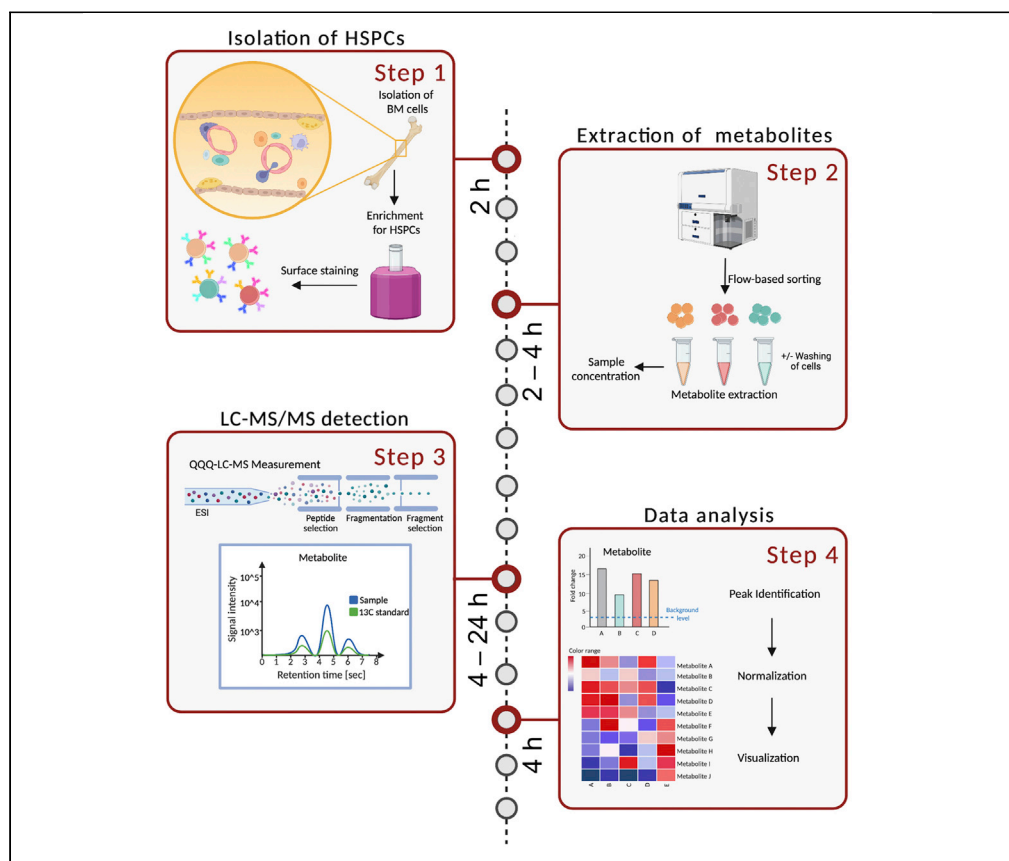


## Protocol

# Targeted LC-MS/MS-based metabolomics and lipidomics on limited hematopoietic stem cell numbers



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**Highlights**  
Isolation of rare  
hematopoietic cell  
types from the murine  
bone marrow

Metabolite extraction of  
limited sample  
amounts after FACS  
purification

Detection of  
signaling lipids,  
retinoids, and polar  
metabolites

Metabolism is important for the regulation of hematopoietic stem cells (HSCs) and drives cellular fate. Due to the scarcity of HSCs, it has been technically challenging to perform metabolome analyses gaining insight into HSC metabolic regulatory networks. Here, we present two targeted liquid chromatography–mass spectrometry approaches that enable the detection of metabolites after fluorescence-activated cell sorting when sample amounts are limited. One protocol covers signaling lipids and retinoids, while the second detects tricarboxylic acid cycle metabolites and amino acids.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Protocol

## Targeted LC-MS/MS-based metabolomics and lipidomics on limited hematopoietic stem cell numbers

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## SUMMARY

Metabolism is important for the regulation of hematopoietic stem cells (HSCs) and drives cellular fate. Due to the scarcity of HSCs, it has been technically challenging to perform metabolome analyses gaining insight into HSC metabolic regulatory networks. Here, we present two targeted liquid chromatography–mass spectrometry approaches that enable the detection of metabolites after fluorescence-activated cell sorting when sample amounts are limited. One protocol covers signaling lipids and retinoids, while the second detects tricarboxylic acid cycle metabolites and amino acids.

For complete details on the use and execution of this protocol, please refer to Schönberger et al. (2022).

## BEFORE YOU BEGIN

The protocol below describes the specific steps for the extraction of metabolites in hematopoietic stem and progenitor populations after fluorescence-activated cell sorting (FACS). However, this protocol is also applicable to other rare cell types that require FACS purification for isolation.

Note that it is critical to perform all steps as quickly and as cold as possible. Before you begin, pre-cool all reagents and centrifuges used in steps 1–9 to 4°C, and after metabolite extraction pre-cool centrifuges to –9°C. It is important to highlight that some of the reagents used are hazardous. When applicable, we have added P Codes and H Codes of Globally Harmonized System (GHS) Precautionary Statements. Moreover, all types of materials used throughout the protocol can influence metabolite recovery. It is highly recommended to use the same materials for all repeating experiments (for recommended materials, see [key resources table](#)).

The establishment of the method, analysis, and interpretation of results for this liquid chromatography–mass spectrometry (LC-MS/MS) protocol require a skilled mass spectrometrist.



### Institutional permissions

All mice were bred in-house in the animal facility at the MP-IE in individually ventilated cages (IVCs). Mice were euthanized by cervical dislocation according to German guidelines. Animal procedures were performed according to protocols approved by the German authorities and the Regierungspräsidentium Freiburg (the sacrificing of animals for scientific purposes according to §4 (3) of the German Animal Protection Act). Please remember that permissions from your local authorities will be required to conduct animal experiments.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
CD8a-PE/Cy7 (1:1000)	BioLegend	Cat#100722; RRID: AB_312761
CD11b-PE/Cy7 (1:1000)	BioLegend	Cat#101216; RRID: AB_312799
Gr1-PE/Cy7 (1:1000)	BioLegend	Cat#108416; RRID: AB_313381
TER119-PE/Cy7 (1:500)	BioLegend	Cat#116221; RRID: AB_2137789
B220-PE/Cy7 (1:1000)	BioLegend	Cat#103222; RRID: AB_313005
CD4-PE/Cy7 (1:1000)	BioLegend	Cat#100422; RRID: AB_2660860
cKit-PE (1:1000)	BioLegend	Cat#105808; RRID: AB_313217
Sca1-APC/Cy7 (1:500)	BioLegend	Cat#108126; RRID: AB_10645327
CD150-BV605 (1:300)	BioLegend	Cat#115927; RRID: AB_11204248
CD48-BV421 (1:1000)	BioLegend	Cat#103428; RRID: AB_2650894
CD8a-Biotin (1:500)	BioLegend	Cat#100704; RRID: AB_312743
CD11b- Biotin (1:500)	BioLegend	Cat#101204; RRID: AB_312787
Gr1- Biotin (1:500)	BioLegend	Cat#108404; RRID: AB_313369
TER119- Biotin (1:500)	BioLegend	Cat#116204; RRID: AB_313705
B220- Biotin (1:500)	BioLegend	Cat#103204; RRID: AB_312989
CD4- Biotin (1:500)	BioLegend	Cat#100404; RRID: AB_312689
<b>Chemicals, peptides, and recombinant proteins</b>		
PBS	Sigma	Cat#D8537
Methanol LC-MS/MS grade	Carl Roth	Cat#HN41.2
Acetonitrile LC-MS/MS grade	VWR Chemicals	Cat#83640.320
2-Propanol LC-MS/MS grade	Carl Roth	Cat#AE73.1
Water (for preparing extraction solutions and LC buffers)	Milli-Q	n/a
<sup>13</sup> C yeast standard	ISOtopic solutions	Cat#ISO-1
Dynabeads Untouched Mouse CD4 Kit	Life Technologies	Cat#11415D
OneComp eBeads	eBioscience	Cat#01-1111-41
ACK Lysing Buffer	Lonza	Cat#10-548E
Ammonium Carbonate	Fisher Chemical	Cat#A/3686/50
Ammonium Hydroxide 25% Solution	Millipore	Cat#30501-1L-M
Ammonium Formate	Sigma-Aldrich	Cat#516961-100G
Glycerol	Carl Roth	Cat#3783.1
<b>Experimental models: Organisms/strains</b>		
C57BL/6J (CD45.2), females, 8–24 weeks old	MPI-IE	RRID: IMSR_JAX:002014
<b>Software and algorithms</b>		
FACSDiva	BD	RRID: SCR_001456
MassHunter8	Agilent	
<b>Other</b>		
Biosphere <sup>®</sup> SafeSeal Tube 1.5 mL	Sarstedt	Cat#72.706.200
Filter-cap FACS tube	Corning	Cat#352235
Cell strainer 40 μm Nylon	Corning	Cat#352340
twintec PCRplate 96LoBind skirted	Eppendorf	Cat#0030129512
Hot seal foil "Seal&Pierce"	neoLab	Cat#7-5218

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sample vials with micro glass insert	Carl Roth	Cat#TY82.1
Snap caps for sample vials	Fisherbrand	Cat#11864910
Luna propylamine column (50 × 2 mm, 3 μm)	Phenomenex	Cat#00B-4377-B0
microLC column 100 × 0,3 mm packed with Zorbax Eclipse Plus RP C18 1.8 μm	Dr. Maisch	Cat#x.s100.3
trap column 10 × 1 mm packed with Zorbax Eclipse Plus RP C18 1.8 μm	Dr. Maisch	Cat#x.t0101
MicroLC Pump Zirconium Ultra	Prolab	410F
MicroLC ESI Source	Prolab	AX150
UHPLC Pump	Agilent	G7120A
UHPLC Autosampler	Agilent	G7157B
UHPLC Sample Thermostat	Agilent	G4761A
UHPLC Column Thermostat	Agilent	G7116B
Triple Quadrupole Mass Spectrometer	Agilent	6495B
JetStream ESI Source	Agilent	G1958B
Speedvac EZ-2 Elite	SP Scientific	EZ3T-23050-HN0
Sample Holder for 1.5 mL tubs in Speedvac	SP Scientific	10-5043
Table Top Centrifuge	Eppendorf	5417R

## MATERIALS AND EQUIPMENT

### 50% glycerol (v/v)

Glycerol	25 mL
milliQ H <sub>2</sub> O	25 mL
Total	50 mL

**Note:** Glycerol is highly viscous. Pour the respective amounts of glycerol and water into a graduated cylinder and mix. This mix can be kept at 4°C for several years.

### <sup>13</sup>C internal standard stock solution

milliQ H <sub>2</sub> O	7.5 mL
Methanol	2.5 mL
<sup>13</sup> C labeled extract of 2 billion yeast cells	Dry pellet
Total	10 mL

**Note:** It is helpful to aliquot this solution in smaller quantities to avoid repeated thawing/freezing. This solution can be stored at –80°C for up to 1 month.

△ **CRITICAL:** Methanol is volatile, flammable, and toxic (H255, H301, H331, H311, H370). Keep away from heat, hot surfaces, sparks, open flames, and other ignition sources. Wear protective gloves and protective clothing (P210, P280, P301, P302, P304, P310, P311, P312, P330, P340, P352). Use under a fume hood.

### Wash buffer

50% glycerol	5.6 mL
milliQ H <sub>2</sub> O	94.4 mL
Total	100 mL

**Note:** This wash buffer can be stored for several months at 4°C when handled under sterile conditions.

**80% methanol extraction solution**

methanol	79.75 mL
milliQ H <sub>2</sub> O	24.25 mL
<sup>13</sup> C internal standard stock solution	1 mL
Total	100 mL

**Note:** This extraction solution should be pre-cooled to –20°C before use and can be stored at –20°C for up to 2 weeks.

**Buffer HILIC A**

Ammonium hydroxide 25% solution	0.75 mL
milliQ H <sub>2</sub> O	999.25 mL
Total	1 L

**Note:** Buffer A can be used for up to 2 days at 20°C.

△ **CRITICAL:** Ammonium hydroxide is toxic, can cause severe skin and eye irritation, and poses an aquatic hazard (H314, H318, H335, H400, H410, H411). Wear protective gloves and protective clothing (P261, P271, P273, P280, P303, P305, P338, P351, P353, P361). Use under a fume hood.

**Buffer HILIC B**

Ammonium carbonate	480 mg
milliQ H <sub>2</sub> O	100 mL
Acetonitrile	900 mL
Total	1 L

**Note:** First dissolve ammonium carbonate in water, then add acetonitrile. Buffer B can be kept up to 1 week at 20°C.

△ **CRITICAL:** Ammonium carbonate is harmful when swallowed (H302). Wear protective gloves and protective clothing (H301, P312, P330). Keep refrigerated to minimize unpleasant smell.

△ **CRITICAL:** Acetonitrile is volatile, flammable, toxic, and can lead to severe eye irritation (H225, H302, H312, H319, H332). Wear protective gloves and protective clothing (P210, P280, P301, P303, P304, P305, P312, P338, P351, P353, P361). Use under a fume hood.

**Buffer microLC A1**

Ammonium formate	630 mg
milliQ H <sub>2</sub> O	900 mL
Acetonitrile	100 mL
Total	1 L

△ **CRITICAL:** Ammonium formate can lead to severe eye irritation (H319). Wear protective gloves and protective clothing (P264, P280, P305, P313, P337, P338, P351).

**Note:** The buffer can be kept up to 1 week at 20°C.

### Buffer microLC A2

Ammonium formate	630 mg
milliQ H <sub>2</sub> O	900 mL
Methanol	100 mL
Total	1 L

**Note:** The buffer can be kept up to 1 week at 20°C.

### Buffer microLC B1

Ammonium formate	630 mg
2-Propanol	900 mL
Acetonitrile	100 mL
Total	1 L

**Note:** Stir or sonicate buffer microLC B1 for several hours in a closed bottle to dissolve ammonium formate. The buffer can be kept up to 1 month at 20°C.

**△ CRITICAL:** 2-propanol is volatile, flammable, toxic, and can lead to severe eye irritation (H225, H319, H336). Wear protective gloves and protective clothing (P210, P233, P240, P241, P242, P305, P338, P351). Use under a fume hood.

**Alternatives:** The [key resources table](#) provides information on material and equipment that we have validated for this protocol. In some cases, alternative products may be used; although we have not tested them for this protocol:

- Reagents:
  - Organic solvents: LC-MS/MS grade solvents from other suppliers.
  - Ammonium salts and ammonium hydroxide: p.a. quality or better from other suppliers.
  - Glycerol: > 99% purity from other suppliers.
- Materials:
  - Sample vials/caps: vials with micro glass insert with matching caps from other suppliers.
  - Twintec PCR plate: other clear PCR plate that the LC autosampler will accept.
- Equipment:
  - Speedvac: rotary evaporator that can tolerate organic solvents.
  - Table top centrifuge: refrigerated centrifuge that can be used with 1.5 mL tubes.
  - UHPLC: HPLC or UHPLC that allows for at least 400 bar back pressure, sample cooling and column heating from another supplier.
  - Mass spectrometer: triple quadrupole mass spectrometer with heated ESI source that is sufficiently sensitive.
- Analysis tools:
  - MassHunter8: for data pre-processing, raw data in .d format can be converted to open .mzML format and subsequently be processed in free software such as skyline or R.

## STEP-BY-STEP METHOD DETAILS

### Isolation of murine bone marrow cells

⌚ Timing: 30–45 min

Hematopoietic stem and progenitor cells reside in the bone marrow (BM) niche. In order to recover the maximum cellular output per mouse, pool the BM within mouse femurs, tibias, ilia, and vertebrae.

1. Preparation of mouse femurs, tibias, ilia and vertebrae.
  - a. Mice must be handled and euthanized according to the guidelines and protocols approved by the country's authorities.
  - b. Place the mouse on its belly, if needed, secure animal onto dissecting tray by pinning front palms so that they are raised diagonally from its body, and disinfect with 70% ethanol.
  - c. Isolation of mouse femurs, tibias, ilia, and vertebrae can be achieved by competent dissection of the mouse legs and spine using forceps and scissors. Isolated tissue should be kept in PBS-filled 6-well plates on ice until all bones are collected.
  - d. Remove the surrounding tissue from the bones and the spinal cord from vertebrae using a scalpel. This step will avoid possible contamination as well as potential blockage during the filtration step.
2. Preparation of a BM single-cell suspension.
  - a. Use a mortar and pestle to gently crush the bones with 5 mL ice-cold PBS.
  - b. Filter cell suspension through a 40  $\mu$ m sterile filter into a 50 mL falcon tube. Keep the tubes on ice.
  - c. Repeat steps 2a and 2b until the bones appear to be completely white.
3. Lysis of erythrocytes.
  - a. Centrifuge the cell suspension at 400  $\times$  g for 5 min at 4°C and remove supernatant.
  - b. Re-suspend in 2 mL ice-cold ACK lysis buffer and incubate on ice for 5 min. Increase incubation time to up to 10 min dependent on the size of the pellet.
  - c. Stop the reaction by adding 1 mL ice-cold PBS.
  - d. Centrifuge the cell suspension at 400  $\times$  g for 5 min at 4°C and remove supernatant.

**Note:** For further details on how to isolate BM cells, see protocol by [\(Zhang and Cabezas-Wallscheid, 2019\)](#).

**△ CRITICAL:** Cells must be kept on ice from the moment the bones are isolated from the mouse. It is recommended to perform the entire preparation process on ice or in a cold room at 4°C.

### Enrichment for hematopoietic stem and progenitor cells

⌚ **Timing:** 75 min

To enrich for lineage negative (Lin<sup>-</sup>) cells, we use the Dynabeads Untouched Mouse CD4 Cells Kit (Invitrogen). Note that this kit does not contain CD4 and thus does not deplete CD4 positive cells. A biotinylated CD4 antibody can be added if desired or, alternatively, a home-made lineage cocktail of biotinylated lineage antibodies can be used.

4. Incubation of cells using the lineage cocktail.
  - a. Re-suspend cells in 500  $\mu$ L lineage cocktail (100  $\mu$ L of the cocktail provided in the kit added to 400  $\mu$ L PBS per mouse BM) and transfer the supernatant into a 2 mL tube.
  - b. Incubate 35 min on a rotating wheel at 4°C.

**△ CRITICAL:** The incubation time of cells with the lineage cocktail should not exceed 45 min since this will cause lower cell recovery.

**Optional:** Instead of using the lineage cocktail included in the Dynabeads Untouched Mouse CD4 Cells Kit (Invitrogen), a home-made lineage cocktail can be used ([CD4/CD8a/CD11b/GR-1/B220/Ter-119]-all biotinylated). To obtain a good depletion efficiency, however, the incubation time should be adjusted to at least 45 min.

- c. Meanwhile prepare the Dynabeads®.
    - i. Vortex the beads for 30 s to make sure that they are well re-suspended.
    - ii. Add 400  $\mu$ L of the Dynabeads® into a 2 mL tube and incubate for 30 s on a depletion magnet until the solution clears.
    - iii. Remove the supernatant without disturbing the beads and wash with 1 mL ice-cold PBS.
    - iv. Repeat step iii for a total of two washing steps.
    - v. Re-suspend in 500  $\mu$ L PBS and keep on ice until use.
  - d. Wash the cells with 12 mL ice-cold PBS in a 15 mL falcon tube.
  - e. Centrifuge the cell suspension at 400  $\times$  g for 5 min at 4°C and remove supernatant.
5. Depletion of lineage positive cells.
- a. Re-suspend cell pellet in 1 mL ice-cold PBS and transfer them to the tube containing the Dynabeads®.
  - b. Incubate for 20 min on a rotating wheel at 4°C.

**△ CRITICAL:** The incubation time of cells with the Dynabeads® should not exceed 25 min since this will lead to reduced cell recovery.

- c. Incubate cells for 5 min on ice on the depletion magnet until the solution clears.
- d. Transfer the entire supernatant into a new FACS tube and keep cells on ice.
- e. Add 1 mL ice-cold PBS and repeat steps 5c and 5d, then combine the two supernatants.

**△ CRITICAL:** Shorter incubation time will reduce efficiency of the depletion process.

6. Surface staining of lineage depleted cells for sorting.
- a. Centrifuge the cell suspension at 400  $\times$  g for 5 min at 4°C and remove supernatant.
  - b. Re-suspend in ice-cold PBS containing the surface antibody cocktail ([CD4/CD8a/CD11b/GR-1/B220/Ter-119]-all PeCy7, c-Kit/CD117-PE, Sca-1-APC-Cy7, CD150-BV605, CD48-BV421) and incubate for 30 min at 4°C in the dark.

**Note:** Other suitable combinations of fluorescent dyes can be used and will not influence the results.

### Fluorescence-activated cell sorting of purified hematopoietic stem cells

⌚ Timing: 1–3 h

**Note:** Depending on the abundance of the desired cell population, the number of mice, and the event rate of the sorting process, the time required for this step may differ. However, it is recommended to not exceed a sorting time of more than 4 h since this will negatively impact the metabolome and lead to a starvation phenotype of the cells.

7. Preparation of cells for FACS.
- a. Add 1 mL ice-cold PBS.
  - b. Centrifuge the cell suspension at 400  $\times$  g for 5 min at 4°C and remove supernatant.
  - c. Re-suspend pellet in an appropriate volume of PBS dependent on the size of the pellet (usually between 500  $\mu$ L and 2 mL).
  - d. Filter cells through a filter-cap FACS tube.



- e. Prepare 1.5 mL-RNase/DNase/ATP-free tubes containing A) 100  $\mu$ L StemPro®-34 SFM (Life Technologies) without cytokines for measuring TCA-cycle metabolites and amino acids or B) 100  $\mu$ L 100% acetonitrile for measuring signaling lipids and retinoids.
8. Sort A) 10,000 HSCs (Lineage<sup>-</sup> cKit<sup>+</sup> Sca1<sup>+</sup> CD150<sup>+</sup> CD48<sup>-</sup>) using the 100  $\mu$ m nozzle of a FACSAria II, FACSAria III, or FACSymphony (Becton Dickinson) or B) 40,000 HSCs using the 70  $\mu$ m nozzle while constantly cooling the samples.

⚠ **CRITICAL:** Cells must not hit the side of the tube but only the surface of the liquid. Also, prepare at least two tubes as negative controls. It is crucial to treat these samples identically to those containing cells.

⚠ **CRITICAL:** Retinoids are light-sensitive metabolites. It is therefore essential to keep the samples for B) in the dark during and after the sorting process.

**Note:** FACS is a very stressful process for most cells.

Depending on the cell type you are interested in, a short recovery step of 20 min in the incubator (37°C; 5% CO<sub>2</sub>) might increase the number and amount of detected metabolites. However, even a short incubation period will lead to activation of metabolic pathways and, in the case of quiescent populations (such as HSCs), may not reflect the actual *in vivo* phenotype. Thus, it is highly recommended to adjust the experimental setup according to the biological question being asked.

### Targeted polar metabolomics: A) TCA cycle metabolites and amino acids

⌚ **Timing:** 45 min–1 h

9. Wash the cells.
  - a. Add 1 mL 2.8% glycerol solution per 1.5 mL tube.
  - b. Centrifuge the cell suspension at 400  $\times$  g for 5 min at 4°C and remove supernatant.

⚠ **CRITICAL:** PBS contamination of the flow cytometry sorting process will interfere with the measurement and thus reduce metabolite recovery. Cells must therefore be washed with a glycerol solution. It is important to remove as much supernatant as possible without losing cells. Removing too little supernatant will lead to high background levels for all metabolites contained in the media. To estimate the background levels of the measured metabolites, the two negative controls should be processed in the exact same way (including washing process).
10. Metabolite extraction.
  - a. Add 100  $\mu$ L of the pre-cooled 80% MeOH extraction buffer containing 1  $\mu$ L <sup>13</sup>C yeast extract to the washed cell pellet.
  - b. Ensure complete re-suspension of the cell pellet by repeated pipetting.
  - c. Centrifuge 3 min at 20,000  $\times$  g and 4°C to pellet cell debris.
  - d. Transfer 95  $\mu$ L of clear supernatant to a fresh Eppendorf tube.
11. Vacuum concentration of the samples.
  - a. Vacuum concentrate (EZ2 elite, Genevac) the samples for 35 min using the aqueous program, lamp off.

⚠ **CRITICAL:** Do not over-dry the samples, as this will lead to decreased metabolite recovery.

⏸ **Pause point:** Samples can be stored at this point for up to 2 weeks at –80°C.

### LC-MS/MS analysis of TCA cycle metabolites and amino acids

Ⓞ Timing: 2 h plus 10 min for every sample

12. Calibrate the mass spectrometer following the manufacturer's recommendations. Ensure that the check tune is passed.
13. Install fresh mobile phase buffers HILIC A and HILIC B in sufficient amounts for the expected number of samples.
14. Purge liquid chromatography (LC) system with mobile phase. Purge 5 min with 3 mL/min with a 50:50 mix of both mobile phase buffers.
15. Install the Luna aminopropyl chromatography column.
16. Equilibrate the LC system with mobile phase buffers in starting conditions.
17. Check LC performance by running a blank sample. Ensure that the backpressure is below 170 bar under starting conditions and remains below 300 bar throughout the gradient run. If backpressure is too high, check for restrictions in the mobile phase flow path.
18. Equilibrate the column by running 4 blank samples.
19. It is highly recommended to use quality control samples such as pool samples, mixtures of standards, or reference material to check if the LC-MS/MS performs as expected.

#### HILIC LC gradient Profile

Time (min)	% B	Flow rate (μL/min)
Initial	100	1000
0.5	100	1000
4.7	30	750
5.1	10	750
7.5	10	750
7.8	100	750
8.4	100	1000
9.5	100	1000

#### LC parameter settings

Injection volume	3 μL
Column temperature	30°C
Autosampler temperature	5°C
Max pressure limit	400 bar

**Note:** An Agilent 6495 Triple Quadrupole mass spectrometer coupled to an Agilent 1290 Infinity II ultra-high-performance liquid chromatography (UHPLC) system is used for quantification of metabolites in this protocol. The MS parameters are detailed in [Table 1](#). Other LC-MS/MS systems with similar capabilities can be used. For other LC-MS/MS systems, LC and MS parameters may have to be adapted.

**Note:** Compound-specific MS settings were optimized separately for all compound using pure standards. These settings are machine-specific and optimization must be repeated on a different machine. Settings used for this protocol are listed in [Table 2](#).

⚠ **CRITICAL:** LC systems operate under high pressure. Refer to manufacturer's instructions to avoid leakage of mobile phase buffers.

⚠ **CRITICAL:** Mass spectrometers apply high temperatures to evaporate the stream of mobile phase buffer coming from the LC. Refer to manufacturer's instructions and do not

**Table 1. MS parameter settings for analysis of polar metabolites**

ESI source	JetStream
Gas temperature	200°C
Gas flow	17 L/min N <sub>2</sub>
Nebulizer pressure	60 psi N <sub>2</sub>
Sheath gas temperature	350°C
Sheath gas flow	11 L/min N <sub>2</sub>
Capillary voltage (both polarities)	1,800 V
Nozzle voltage (both polarities)	800 V
iFunnel high pressure RF positive	110 V
iFunnel high pressure RF negative	90 V
iFunnel low pressure RF positive	80 V
iFunnel low pressure RF negative	60 V
MS1 resolution	unit
MS2 resolution	unit
Fragmentor	380
Cell accelerator voltage	4 V
Dwell time	5 ms

touch hot surfaces to avoid burn wounds. Ensure sufficient ventilation to avoid accumulation of harmful or dangerous vapors. Mass spectrometers apply high voltages to ionize metabolites. Refer to manufacturer's instructions to avoid electric shock.

### Targeted metabolomics: B) Signaling lipids and retinoids

⌚ **Timing: 40 min**

⚠ **CRITICAL:** Remember that retinoids are light-sensitive metabolites and it is essential to keep the samples protected from light during processing.

**Note:** Metabolites are already extracted when reaching the extraction solution. The contaminant fluid due to the droplets is roughly 1 nL/droplet when using the 70 μm nozzle and will lead to a final ACN concentration of 60%–80%, depending on the number of cells and the FACS setup.

Of note, steps 1–8 are shared for both protocols (A and B). After step 8 of protocol A, you should continue with this step (20) if assessing protocol B:

20. Vacuum concentration of the samples.

- a. Vacuum concentrate (EZ2 elite, Genevac) the samples for 35 min using the aqueous program, lamp off.

⚠ **CRITICAL:** Do not over-dry the samples, as this will lead to decreased metabolite recovery.

⏸ **Pause point:** Samples can be stored at this point for up to 2 weeks at –80°C.

### MicroLC-MS/MS analysis of signaling lipids and retinoids

⌚ **Timing: 3 h plus 1 h for every sample**

21. Calibrate the mass spectrometer following the manufacturer's recommendations. Ensure that the check tune is passed.
22. Install fresh mobile phase buffer microLC A2 and microLC B1 in sufficient amounts for the expected number of samples on UHPLC System (see [Figure 1](#)).

**Table 2. Compound-specific settings for analysis of polar metabolites**

Compound name	Precursor ion	Product ion	Collision energy	Polarity	Expected RT (min)
4-OH-Proline quantifier	132.1	86	12	+	1.7
4-OH-Proline qualifier	132.1	68	26	+	
4-OH-Proline qualifier	132.1	58	30	+	
Acetyl-CoA quantifier	810.1	303.1	38	+	4.2
Acetyl-CoA qualifier	810.1	136	78	+	
Acetyl-CoA_13C qualifier	833.1	316.1	38	+	
Aconitic acid quantifier	173	129	10	–	3.9
Aconitic acid qualifier	173	85	25	–	
Aconitic acid_13C qualifier	179	89	25	–	
Adenosine quantifier	268.1	136	22	+	0.5
Adenosine qualifier	268.1	119	62	+	
Adenosine_13C qualifier	278.1	141	22	+	
ADP quantifier	426	328	22	–	4.1
ADP qualifier	426	79	66	–	
ADP_13C qualifier	436	338	22	–	
AMP quantifier	348.1	136	22	+	3.0
AMP qualifier	348.1	119	74	+	
AMP_13C qualifier	358.1	141	22	+	
Arginine quantifier	175.1	116	18	+	2.8
Arginine qualifier	175.1	60	14	+	
Arginine_13C qualifier	181.1	61	18	+	
Asparagine quantifier	131	113	8	–	1.8
Asparagine qualifier	131	42	22	–	
Asparagine_13C qualifier	135	43	8	–	
Aspartic acid quantifier	132	115	10	–	2.3
Aspartic acid qualifier	132	88	14	–	
Aspartic acid_13C qualifier	136	91	14	–	
ATP quantifier	506	408	22	–	5.2
ATP qualifier	506	159	46	–	
ATP_13C qualifier	516	418	22	–	
cAMP quantifier	328	134	24	–	1.7
cAMP qualifier	328	107	64	–	
cAMP_13C qualifier	338	139	24	–	
Citric acid quantifier	191	111	10	–	3.6
Citric acid qualifier	191	87	18	–	
Citric acid_13C qualifier	197	90	18	–	
Cystine quantifier	241.03	74	34	+	2.7
Cystine qualifier	239.01	120	10	–	
Cystine_13C qualifier	245.01	123	10	–	
Fumaric Acid quantifier	115	71	6	–	2.4
Fumaric_13C qualifier	119	74	6	–	
Glutamic acid quantifier	146	128	6	–	2.3
Glutamic acid qualifier	146	102	14	–	
Glutamic acid_13C qualifier	151	106	14	–	
Glutamine quantifier	145.1	127	10	–	1.8
Glutamine qualifier	145.1	109	10	–	
Glutamine_13C	150.1	132	10	–	
GSH quantifier	306.1	272.1	10	–	2.4
GSH qualifier	306.1	143	22	–	
GSH_13C qualifier	316.1	282.1	10	–	
Histidine quantifier	156.1	110	14	+	2.0

(Continued on next page)

**Table 2. Continued**

Compound name	Precursor ion	Product ion	Collision energy	Polarity	Expected RT (min)
Histidine qualifier	156.1	83	30	+	
Histidine_13C qualifier	162.1	115	14	+	
IMP quantifier	349	137	22	+	3.0
IMP qualifier	347	79	74	–	
IMP_13C qualifier	359	142	22	+	
Isocitric acid quantifier	191	73	25	–	3.6
Isocitric acid_13C qualifier	197	75	25	–	
Isoleucine quantifier	132.1	86	10	+	1.0
Isoleucine qualifier	132.1	69	18	+	
Isoleucine_13C qualifier	138.1	74	18	+	
Itaconic acid quantifier	129	85	8	–	2.7
Itaconic acid qualifier	129	41	12	–	
Itaconic acid_13C qualifier	134	89	8	–	
Lactic Acid quantifier	89	45	10	–	1.2
Lactic Acid qualifier	89	43	10	–	
Leucine quantifier	132.1	86	10	+	0.9
Leucine qualifier	132.1	43	26	+	
Leucine_13C qualifier	138.1	46	26	+	
Lysine quantifier	147.1	130	6	+	2.8
Lysine qualifier	147.1	84	18	+	
Lysine_13C qualifier	153.1	89	18	+	
Malic acid quantifier	133	115	14	–	2.7
Malic acid qualifier	133	71	10	–	
Malic acid_13C qualifier	137	119	10	–	
Methionine quantifier	150.1	104	10	+	1.1
Methionine qualifier	150.1	56	14	+	
Methionine_13C qualifier	155.1	108	10	+	
N-Acetylaspartic acid quantifier	174	130	14	–	2.5
N-Acetylaspartic acid qualifier	174	88	18	–	
Niacinamide quantifier	123.1	80	24	+	0.3
Niacinamide qualifier	123.1	53	40	+	
Niacinamide_13C qualifier	129	85	24	+	
Phenol Red quantifier	353	273.1	28	–	2.0
Phenol Red qualifier	353	195.1	48	–	
Phenylalanine quantifier	166.1	120	10	+	1.1
Phenylalanine qualifier	166.1	103	30	+	
Phenylalanine_13C qualifier	175.1	128	10	+	
Proline quantifier	116.1	43	30	+	1.5
Proline qualifier	116.1	30	30	+	
Proline_13C qualifier	121.1	45	30	+	
Pyruvic acid quantifier	87	43	36	–	1.0
Pyruvic acid qualifier	87	41	4	–	
Pyruvic acid_13C qualifier	90	45	4	–	
Riboflavin quantifier	377.2	243	28	+	0.4
Riboflavin qualifier	377.2	172	40	+	
Riboflavin_13C qualifier	394.2	184	28	+	
Serine quantifier	104	104	10	–	1.9
Serine qualifier	104	74	10	–	
Serine_13C qualifier	107	76	10	–	
Succinic Acid quantifier	117	73	10	–	2.7

(Continued on next page)

**Table 2. Continued**

Compound name	Precursor ion	Product ion	Collision energy	Polarity	Expected RT (min)
Succinic Acid_13C qualifier	121	76	10	–	
Taurine quantifier	124	80	22	–	1.3
Taurine qualifier	124	64	66	–	
Thiamine quantifier qualifier	266.1	123	14	+	1.5
Thiamine qualifier	266.1	122	18	+	
Tyrosine quantifier qualifier	180.1	163	18	–	1.5
Tyrosine qualifier	180.1	119	14	–	
Tyrosine_13C qualifier	189.1	172.1	18	–	
Valine quantifier qualifier	118.1	72	10	+	1.2
Valine qualifier	118.1	55	22	+	
Valine_13C qualifier	123.1	76	10	+	

23. Purge UHPLC system with mobile phase. Purge 5 min with 3 mL/min with a 50:50 mix of both mobile phase buffers.
24. Equilibrate the UHPLC system with mobile phase buffers in starting conditions.

**Valve switching time table**

Time (min)	Valve state
initial	load (dashed lines)
2	elute (dotted lines)

**UHPLC gradient Profile**

Time (min)	% B	Flow rate (μL/min)
initial	0	100
5	0	100
10	100	100
25	100	100
60	0	100

**UHPLC parameter settings**

Injection volume	8 μL
Autosampler temperature	5°C

25. Install fresh mobile phase buffer microLC A1 and microLC B1 in sufficient amounts for the expected number of samples on microLC pump.
26. Purge microLC system with mobile phase with 6 full strokes on each channel.

**microLC gradient Profile**

Time (s)	% B	Flow rate (μL/min)
initial	0	5
180	0	5
190	25	5
1800	100	5
2700	100	5

#### microLC parameter settings

Continuous Flow Mode	on
CL Flow Control	on
B Start Delay	10 s
Compartment Temp	30°C
Equilibration Time	720 s
Max. Pressure Limit	1,000 bar

27. Equilibrate complete system in starting conditions.
28. Check system performance by running a blank sample. Ensure that the UHPLC backpressure is below 120 bar under starting conditions and remains below 250 bar throughout the gradient run. Ensure that the microLC backpressure remains below 900 bar throughout the gradient run. If backpressure is too high, check for restrictions in the mobile phase flow path.

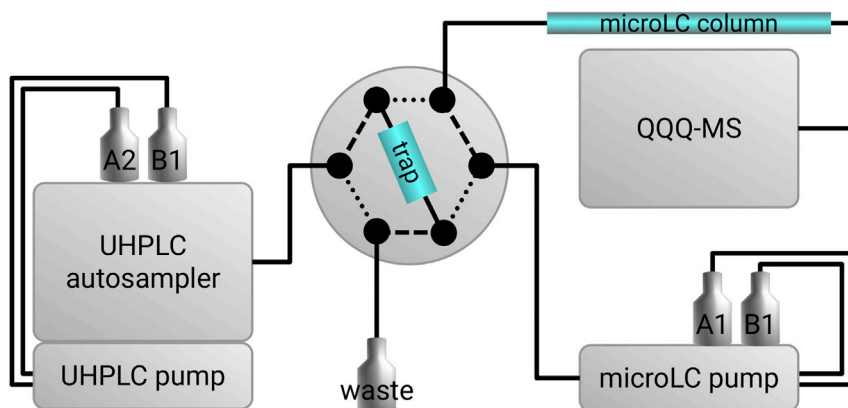
**Note:** An Agilent 6495 QQQ mass spectrometer coupled to an Agilent 1290 Infinity II UHPLC system and a ProLab Zirconium Ultra is used for quantification of metabolites in the is protocol. Other LC-MS/MS systems with similar capabilities can be used. In any case, the MS parameters detailed in [Table 3](#) may need to be adapted.

**Note:** Compound-specific MS settings were optimized separately for all compounds using pure standards. These settings are machine-specific and optimization must be repeated on a different machine. Settings used for this protocol are listed in [Table 4](#).

#### LC-QQQ-MS data pre-processing

Raw liquid chromatography triple quadrupole mass spectrometry (LC-QQQ-MS) data require pre-processing to extract peak area or peak height as alternative measures of signal intensity. Signal intensity can then be used as a proxy for the concentration of metabolites in a sample. Alternative software solutions exist for LC-QQQ-MS data pre-processing, including fully automatic solutions such as MRMprobs ([Tsugawa et al., 2014](#)) and automRm ([Eilertz et al., 2022](#)), as well as solutions that facilitate manual peak review, such as skyline ([MacLean et al., 2010](#)), and vendor-specific solutions such as MassHunter.

Low-input metabolomics experiments often suffer from low signal intensity. In addition, both hydrophilic interaction liquid chromatography (HILIC) and microLC are notorious for suboptimal retention



**Figure 1. Plumbing scheme of microLC setup**

During the loading phase, the dashed connections are used to load the sample onto the trap column and discard excess mobile phase. During the analytical phase, the dotted connections are used. Flow from the microLC pump elutes metabolites from the trap column to enable separation on the microLC column and subsequent detection by QQQ-MS.

**Table 3. MS parameter settings for analysis of polar lipids**

ESI source	ESI
Gas temperature	200°C
Gas flow	16 L/min N <sub>2</sub>
Nebulizer pressure	20 psi N <sub>2</sub>
Capillary voltage positive	3,700 V
Capillary voltage negative	2,800 V
iFunnel high pressure RF positive	110 V
iFunnel high pressure RF negative	90 V
iFunnel low pressure RF positive	80 V
iFunnel low pressure RF negative	60 V
MS1 resolution	unit
MS2 resolution	unit
Fragmentor	380
Cell accelerator voltage	4 V
Dwell time	5 ms

time reproducibility. Consequently, manual peak review is required to maximize the number of quantified metabolites. We opted to use MassHunter8 because it does not require conversion of our original data to an open format. During manual peak review, it is important to follow a set of guidelines to obtain reliable results:

Signal intensity in cell extract must be higher than in the blank sample.

Chromatographic peaks of quantifier and qualifier must align well.

The ratio in signal intensity between quantifier and qualifier must be similar across all samples. This does not apply to <sup>13</sup>C qualifiers.

If shifts in retention time occur, they should typically be in the same direction for all metabolites and might increase in the order of measurement. Moreover, retention times could continue to shift in the same direction from sample to sample.

The location of the start and end of a chromatographic peak relative to the top of the peak should be as similar as possible for all peaks.

The choice of baseline relative to a chromatographic peak and the surrounding background signal should be as similar as possible for all peaks.

If chromatographically separated isotopes are observed, their elution order must always remain the same, even if their retention time shifts.

Signals that do not meet these criteria should be disregarded in subsequent analyses.

Some additional considerations that aid data interpretation:

The signal intensity of phenol red can be used as a proxy for the amount of medium carry over. Lower signal intensity indicates less carry over.

Some peaks can show persistent background signals (e.g., cholic acid in the microLC analysis of polar lipids or citric acid in the analysis of polar metabolites). In these cases, subtraction of background signal intensity from the signal intensities recorded for cell extracts may be advisable.



**Table 4. Compound-specific settings for analysis of polar lipids**

Compound name	Precursor ion	Product ion	Collision energy	Polarity	Expected RT (min)
Cholic acid quantifier	453.3	407.3	16	–	13.1
Cholic acid qualifier	426.3	355.3	20	+	
Muricholic acid quantifier	453.3	407.3	16	–	11.9
Muricholic acid qualifier	426.3	355.3	20	+	
7,25 dihydroxy cholesterol quantifier	383.3	91.1	80	+	18.1
7,25 dihydroxy cholesterol qualifier	383.3	81.1	44	+	
4-oxo-(9-cis,13-cis)Retinoic acid quantifier	315.2	297.1	8	+	23.2
4-oxo-(9-cis,13-cis)Retinoic acid qualifier	313.2	269.3	16	–	
carnitine-C02 quantifier	204.1	85	20	+	9.8
carnitine-C02 qualifier	204.1	43	72	+	
carnitine-C04 quantifier	232.2	85	20	+	10.2
carnitine-C04 qualifier	232.2	43	48	+	
carnitine-C06 quantifier	260.2	85	28	+	11.9
carnitine-C06 qualifier	260.2	43	56	+	
carnitine-C08 quantifier	288.2	85	24	+	13.6
carnitine-C08 qualifier	288.2	57	48	+	
carnitine-C10 quantifier	316.2	85	32	+	14.2
carnitine-C10 qualifier	316.2	43	76	+	
carnitine-C12 quantifier	344.3	85	32	+	15.1
carnitine-C12 qualifier	344.3	43	76	+	
carnitine-C14 quantifier	372.3	85	28	+	17.2
carnitine-C14 qualifier	372.3	57	72	+	
carnitine-C16 quantifier	400.3	85	36	+	19.9
carnitine-C16 qualifier	400.3	57	62	+	
carnitine-C18 quantifier	428.4	85	44	+	22.7
carnitine-C18 qualifier	428.4	57	52	+	
carnitine-C20 quantifier	456.4	85	44	+	24.2
carnitine-C20 qualifier	456.4	57	52	+	
Chenodeoxycholic acid quantifier	437.3	391.2	16	–	16.0
Chenodeoxycholic acid qualifier	357.3	91.1	80	+	
Cholesterol quantifier	369.4	91.1	76	+	32.4
Cholesterol qualifier	369.4	81	56	+	
Glycochenodeoxycholic acid quantifier	450.32	414.2	16	+	12.4
Glycochenodeoxycholic acid qualifier	448.3	74.1	52	–	
Glycocholic acid quantifier	466.4	412.4	16	+	11.1
Glycocholic acid qualifier	466.4	337.3	24	+	
LPC 14-0 quantifier	468.3	184	28	+	18.3
LPC 14-0 qualifier	468.3	104.1	32	+	
LPC 16-0 quantifier	496.3	184	28	+	21.0
LPC 16-0 qualifier	496.3	104.1	32	+	
LPC 18-0 quantifier	524.4	184	28	+	24.0
LPC 18-0 qualifier	524.4	104.1	32	+	
LPC 18-1 quantifier	522.3	184	28	+	21.9
LPC 18-1 qualifier	522.3	104.1	32	+	
LPC 20-4 quantifier	544.4	184	28	+	20.2
LPC 20-4 qualifier	544.4	104.1	32	+	
LPE 16-0 quantifier	452.2	255.1	24	–	21.4
LPE 16-0 qualifier	452.2	195.9	24	–	
LPE 18-0 quantifier	480.2	283	24	–	24.2
LPE 18-0 qualifier	480.2	195.9	24	–	
LPE 18-1 quantifier	478.2	281	24	–	22.0
LPE 18-1 qualifier	478.2	195.9	24	–	
7-hydroxy cholesterol quantifier	367.3	81	52	+	22.2

(Continued on next page)

Table 4. Continued

Compound name	Precursor ion	Product ion	Collision energy	Polarity	Expected RT (min)
7-hydroxy cholesterol qualifier	367.3	55.3	72	+	
25-hydroxy cholesterol quantifier	367.3	81	52	+	27.3
25-hydroxy cholesterol qualifier	367.3	55.3	72	+	
PC 32-0 quantifier	734.5	184.1	32	+	33.9
PC 32-0 qualifier	734.5	86.2	76	+	
PC 34-0 quantifier	762.6	184.1	32	+	34.3
PC 34-0 qualifier	762.6	86.2	76	+	
PC 34-1 quantifier	760.6	184.1	32	+	34.0
PC 34-1 qualifier	760.6	86.2	76	+	
PC 34-2 quantifier	758.6	184.1	32	+	33.3
PC 34-2 qualifier	758.6	86.2	76	+	
PC 36-2 quantifier	786.6	86.2	76	+	34.5
PC 36-2 qualifier	786.6	184.1	32	+	
PC 36-4 quantifier	782.6	184.1	32	+	33.0
PC 36-4 qualifier	782.6	86.2	76	+	
PE 32-0 quantifier	692.5	551.5	24	+	32.4
PE 32-0 qualifier	690.5	195.9	52	–	
PE 34-1 quantifier	718.5	577.4	24	+	33.4
PE 34-1 qualifier	716.5	195.9	52	–	
PE 36-2 quantifier	742.5	281.1	40	–	33.5
PE 36-2 qualifier	742.5	195.9	52	–	
PE 36-4 quantifier	738.5	281.1	40	–	33.0
PE 36-4 qualifier	738.5	195.9	52	–	
Retinal quantifier	285.2	91.2	56	+	22.2
Retinal qualifier	285.2	41.2	64	+	
Retinoic acid quantifier	301.2	41.2	72	+	20.8
Retinoic acid qualifier	299.2	255.2	20	–	
Retinol quantifier	287.2	41.2	76	+	22.3
Retinol qualifier	269.2	93.3	24	+	
Taurochenodeoxycholic acid quantifier	517.3	464.3	20	+	12.4
Taurochenodeoxycholic acid qualifier	498.3	80	80	–	
Taurocholic acid quantifier	533.32	337.2	36	+	11.1
Taurocholic acid qualifier	533.3	462.3	28	+	

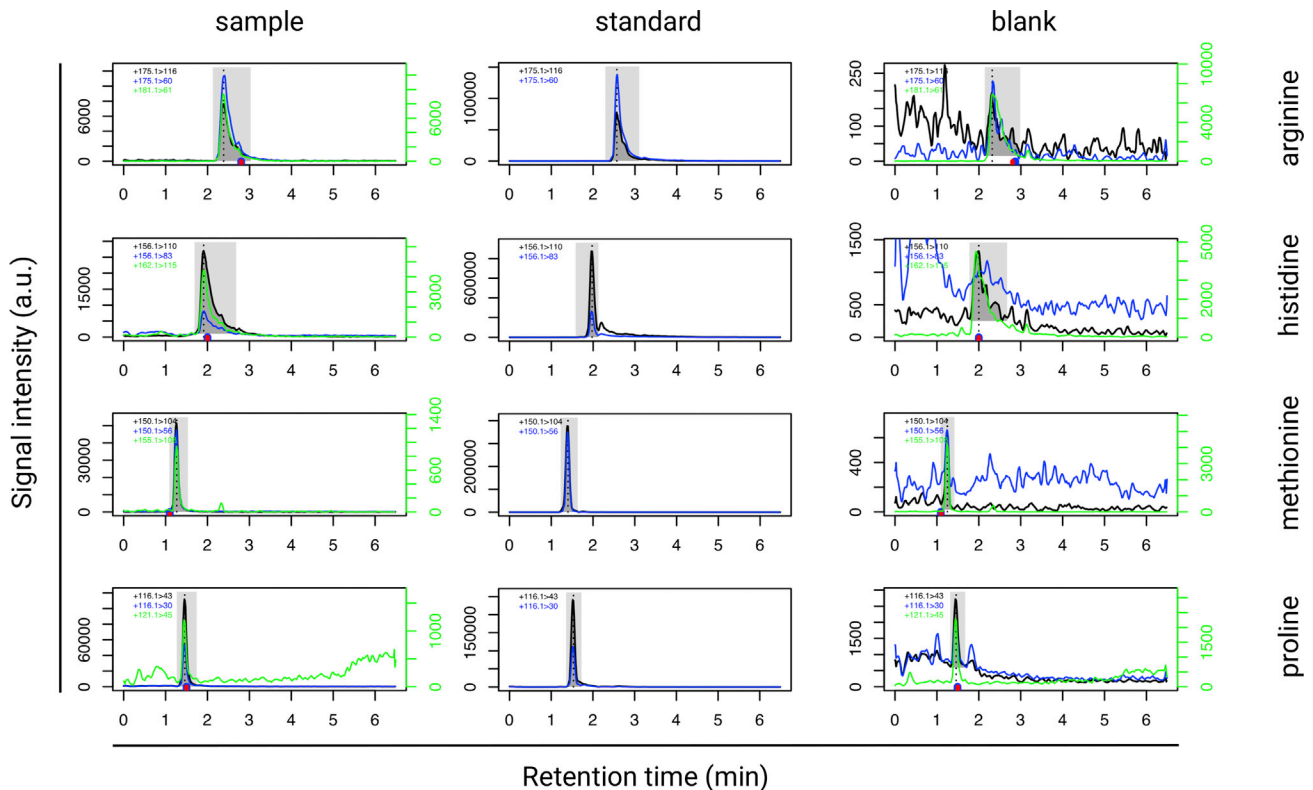
Normalization of signal intensity values can improve the quality of the results:

Normalization to cell number (determined by FACS during sample preparation) can be used to compensate for differences in the amount of input material. Differences greater than 3-fold should not be compensated in this way because non-linear effects can occur.

Normalization to total protein or total DNA in the cell pellet after extraction of metabolites can be used to compensate for differences in the amount of input material. Differences greater than 3-fold should not be compensated in this way because non-linear effects can occur.

Normalization to the signal intensity of a  $^{13}\text{C}$  qualifier can be used to compensate for degradation of compounds prior to analysis and differences in ionization efficiency during MS analysis. This is only reliable if a matching  $^{13}\text{C}$  qualifier has been recorded with sufficient signal intensity. Note that for some polar metabolites no  $^{13}\text{C}$  quantifier was recorded.

We advise against using quantile normalization or normalization to the sum of all metabolite signals for the data generated with the methods described in this protocol because the limited number of metabolites covered by these LC-QQQ-MS methods can introduce a bias in the data.



**Figure 2. Representative chromatograms of polar metabolites in biological samples, standards, and blank samples**

Quantifier (black) and qualifier (blue) are plotted on the left-hand axis,  $^{13}\text{C}$  qualifier (green) is plotted on the right-hand axis. Integrated regions are highlighted in gray and the top of peaks are indicated by vertical dotted lines.

## EXPECTED OUTCOMES

### (A) TCA cycle metabolites and amino acids

Some representative chromatographic peaks of polar metabolites are plotted in Figure 2 and additional examples are given in Data S1. Note that the width of chromatographic peaks can vary between metabolites but is consistent between quantifiers and qualifiers.

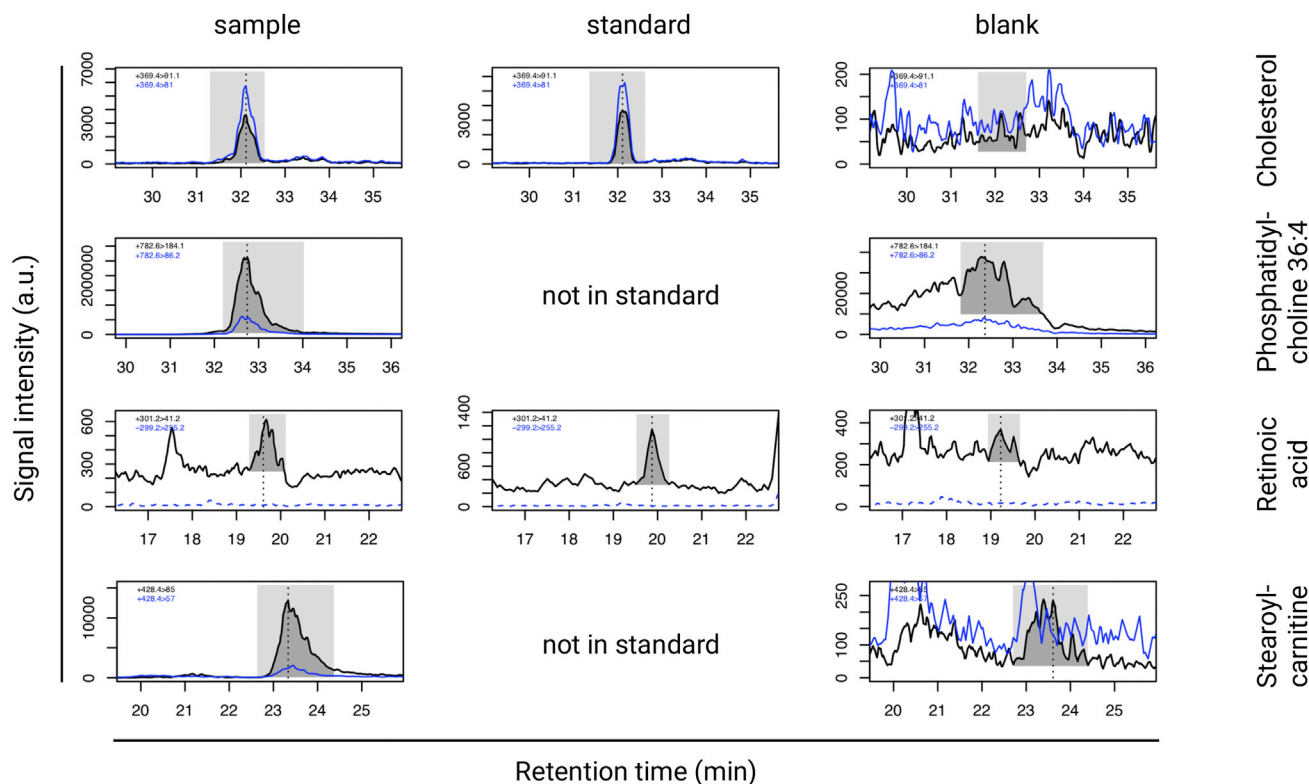
### (B) Signaling lipids and retinoids

Some representative chromatographic peaks of polar metabolites are plotted in Figure 3 and additional examples are given in Data S2. Note that the width of chromatographic peaks can vary between metabolites but is consistent between quantifiers and qualifiers.

## LIMITATIONS

Hematopoietic stem and progenitor cells are small compared to many other cell types and popular cancer cell lines (Shariatmadar et al., 2008). Therefore, an equivalent number of hematopoietic stem and progenitor cells will contain smaller amounts of metabolites. Moreover, hematopoietic stem and progenitor cells are quiescent, which leads to lower amounts of metabolites compared to metabolically active cells. Consequently, metabolomics analysis is limited to those metabolites that exhibit a relatively high intracellular abundance.

The detection of a metabolite in a given sample depends on its concentration in the cell extract. However, additional factors are also important: I) The ionization efficiency during electro spray ionization; II) The formation of adducts or in-source fragments during electro spray ionization; III) The number of fragments formed in the collision cell; IV) The presence of other substances in the sample that have the same retention time, and thus, can cause ion suppression; V) The presence of other



**Figure 3. Representative chromatograms of polar lipids in biological samples, standards, and blank samples**

Black and blue lines represent quantifier and qualifier, respectively. Dashed blue lines indicate that the qualifier only gives a very weak signal and can be disregarded. Integrated regions are highlighted in gray and the top of peaks are indicated by vertical dotted lines.

substances in the sample that can cause background signals. The first three points are likely to differ among different mass spectrometers, whereas the latter two factors depend on the sample composition. Consequently, the suitability of the described workflow has to be tested for every metabolite and every type of sample.

During the isolation of primary cells from tissues and during flow cytometry-based sorting, cells are largely deprived of nutrients and encounter suboptimal conditions with respect to temperature, osmolarity, and oxygen tension. These conditions are known to impact the metabolome (Llufrio et al., 2018; Ryan et al., 2021). However, exposure of hematopoietic stem and progenitor cells to rich culture media and growth stimuli can induce activation and differentiation and thus skew the metabolic composition. To alleviate these issues, we minimize the time from isolation of mouse tissue to extraction of metabolites by reducing the number of samples that are handled in parallel. In addition, the isolated cells are kept as cool as possible to slow down metabolite interconversion.

The use of rich medium as sheath fluid during cell sorting has been described (Ryan et al., 2021), however, this approach is susceptible to contaminations in the cell sorter. Although we have tested an additional short cultivation of the sorted cells prior to metabolite extraction, we have opted to not include this step in the protocol to avoid undesired activation or differentiation of stem cells.

For data interpretation, it has to be taken into account that sample processing is expected to induce a starvation-like phenotype. Of note, we do not draw any conclusions on high-turnover metabolites such as glycolytic intermediates or adenosine triphosphate (ATP).

## TROUBLESHOOTING

### Problem 1

FACS staining pattern looks unusual (step: [fluorescence-activated cell sorting of purified hematopoietic stem cells](#)).

### Potential solution 1

- Fluorescent dyes can degrade, and thus, lead to unusual staining patterns. Especially, coupled dyes (e.g., PeCy7) might degrade into the respective single dyes (PE and Cy7) and cause off-channel signals. Make sure to store the fluorophore-coupled antibodies according to the manufacturer's recommendations and prepare a single staining for each antibody to assure good quality before starting the sort.
- Dead cells can cause auto-fluorescence (specifically in the FITC channel). In some cases, the quality of the metabolomics data might benefit from a live/dead staining during the sort, especially when handling cell types sensitive to tissue processing (e.g., endothelial cells). This can further improve the quantity of metabolites detected and the overall quality of the data.

### Problem 2

Low internal diameters used in microLC are prone to block. This can cause excessive high backpressure in analysis of polar lipids (step: [microLC-MS/MS analysis of signaling lipids and retinoids](#)).

### Potential solution 2

We routinely use in-line filters in the autosampler, at the entry to the trap column, and at the entry to the analytical column to minimize the problem. In addition, rigorous sample cleanup by centrifugation and transfer of clean supernatant is very important.

### Problem 3

In microLC early eluting compounds are missing (step: [microLC-MS/MS analysis of signaling lipids and retinoids](#)).

### Potential solution 3

Possibly, the trap column has not been sufficiently equilibrated. Use the loading pump to equilibrate the loading pump for longer with buffer A2.

### Problem 4

Some organic acids (in particular malic acid and citric acid), as well as some organo-phosphates, are known to interact with metal surfaces such as the capillaries, column housing, and frits that are routinely used in UHPLC systems. This can cause background levels increasing in the sequence of measurement (step: [LC-QQQ-MS data pre-processing](#)).

### Potential solution 4

Modification of mobile phase buffers, changes in LC hardware, and organization of LC-MS/MS measurements can help mitigate this problem:

- Use high-pH mobile phase buffers to reduce the interaction of acids and phosphates with metal surfaces.
- Consider suitable mobile phase additives such as medronic acid ([Hsiao et al., 2018](#)).
- Use fused silica capillaries or PEEK capillaries if possible. Note that there are limitations in the use of PEEK in combination with some organic solvents and the use of fused silica in combination with very high pH.
- Avoid mixing regular samples and low-input samples within one batch.
- Remove residual acids and phosphates by a sequence of blank runs prior to analysis of low-input samples.

### Problem 5

Background metabolite levels within negative controls are as high as within samples (step: [LC-QQQ-MS data pre-processing](#)).

### Potential solution 5

- Make sure flow stream of the fluorescence cell sorter is adjusted properly and cells do not hit the tube wall, causing cell death.
- Media contamination (method A) causes high background levels in the negative control but also the actual samples. Ensure removal of the entire supernatant after washing the cell with wash buffer.

### Problem 6

Retinoids cannot be detected (step: [LC-QQQ-MS data pre-processing](#)).

### Potential solution 6

Light exposure can lead to degradation of retinoids after metabolite extraction. Protect samples from light during further sample preparation and measurement.

## RESOURCE AVAILABILITY

### Lead contact

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### Materials availability

This study did not generate new materials.

### Data and code availability

The published article Schönberger et al. Cell Stem Cell (2022) includes all datasets analyzed during this study. No new datasets were generated.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101408>.

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## AUTHOR CONTRIBUTIONS

Conceptualization: K.S. and J.M.B.; methodology: K.S., M.M., N.C.-W., and J.M.B.; investigation: K.S., M.M., and J.M.B.; writing and editing: K.S. and J.M.B.; supervision: N.C.-W. and J.M.B.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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