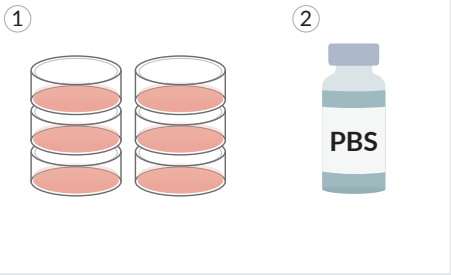
CELL CULTURE

From Sample Collection to LC-MS Analysis of Moderately Polar Intracellular Metabolites

STEPS 1-2

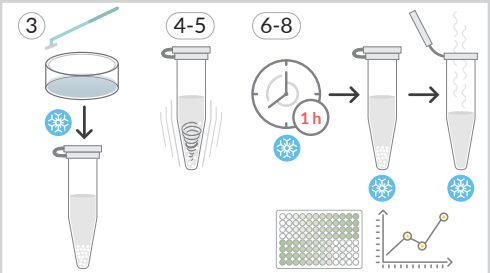


SAMPLE COLLECTION

1. Grow cells with 5-6 replicates/condition to yield ~1 mg protein/sample
2. Wash cells twice with room temperature PBS

Note: *Remove PBS thoroughly as salts are not MS-friendly*

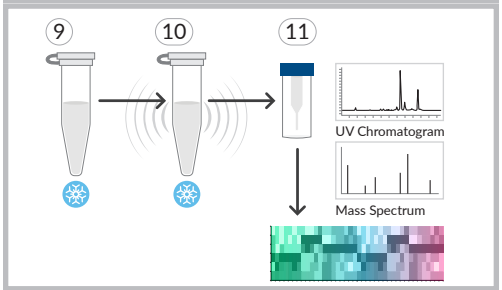
STEPS 3-8



EXTRACTION

3. Place samples on dry ice and add cold MeOH:H_2O (4:1) to wells; scrape cells and transfer to a tube with beads
4. Vortex for 30 s
5. Homogenize
6. Incubate for 1 h at 20°C; centrifuge for 15 min at 4°C
7. Transfer supernatants to a new tube and evaporate solvent
8. Quantify protein concentration of cell pellets

STEPS 9-11



ANALYSIS

9. Reconstitute extracts in MeOH:H_2O (1:1)
10. Sonicate for 1 min in ice-cold bath; centrifuge for 15 min at 4°C
11. Transfer supernatant to UPLC vials and inject for LC-MS analysis

SAMPLE COLLECTION

① Grow cells in individual 100 or 60 mm plates, 5-6 biological replicates per sample group, to have an equivalent of ~1 mg of proteins or 1 x 10^6 cells per sample (*an estimation that depends on the type/size of cells*) → protocol can be adjusted for metabolite profiling from lower amounts of biological material (down to 100 K cells)

② Washing off the spent media: Wash the cells twice with PBS at room temperature (**DO NOT** expose your cells to cold stress using ice-cold solvent!) ~4 ml for 60 mm plates, ~10 ml for 100 mm plates (5-10s), to wash off the spent media x 2. Amino acids (and other metabolites) are usually abundant in the media and can contribute to the measurement of intracellular concentrations. Remove the PBS thoroughly (salts are NOT MS-friendly).

METABOLITE EXTRACTION

③ Place the cell cultures on dry ice and add 1 ml of cold MeOH:H_2O (4:1, v/v) (UPLC-MS grade) to quench the metabolism and burst/release the cell contents. Scrape the cells off the dish with cell scraper and transfer the solution with cells to a lysis tube and add the ceramic beads.

④ Vortex briefly (~30 seconds) and sonicate on ice at least 30 seconds to extract metabolites out of cells. This step is also required if performing freeze-thaw.

⑤ Homogenize the solution in tissue homogenizer (Precellys®) 3 x 20s at 6,000 rpm (with 5s between each homogenization). A cold trap (Cryolys) of the homogenizer should be filled with dry ice to keep the sample cold. ***Remark:*** *If you don't have a tissue homogenizer you can perform 2 x freeze-thaw cycles to extract the cellular content. This is particularly important for bacterial cells.*

⑥ Incubate samples for 1 hour at -20°C, then centrifuge at 16,000 g at 4°C for 15 minutes

⑦ Transfer the supernatants to another 1.5 ml Eppendorf and evaporate the supernatant to dryness in a vacuum concentrator (Labconco™ CentriVap Benchtop) at 10°C (*If the time is limited, the dry extracts can be stored at –80°C at this stage, prior to further processing*). Save cell pellets for step 8.

8. Quantify the total protein content on cell pellets (using BCA or another protein assay)
9. Reconstitute the dry extracts in MeOH/water (1:1, v/v) normalized to protein content
10. Sonicate for 1 minute in an ice-cold bath and centrifuge for 15 minutes at 16,000 g and 4°C to remove debris
11. Transfer supernatants to UPLC vials with inserts and inject them for LC/MS analysis. (The storage at −80°C should be avoided at this stage because it might cause the metabolite precipitation and therefore compromise the coverage)

LIQUID CHROMATOGRAPHY CONDITIONS

· LC System: UPLC
· Column & Guard: WATER ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 100 mm BEH C18, 2.1x 100 mm, 1.7 µm & WATER ACQUITY UPLC BEH C18 VanGuard Pre-column, 130Å, 1.7 µm, 2.1 mm X 5 mm
· Flow rate: 400 ul/min
· Mobile phases:

Time (minutes)	A (H_20 + 0.1% FA)	B (ACN + 0.1% FA)
0	99	1
1	99	1
10	1	99
13	1	99
14	99	1
17	99	1

MASS SPECTROMETRY CONDITIONS

· Ionization modes: ESI (+) and ESI (-)
· Acquisition mode: MS and Auto MS/MS
· *m/z* range: 50-1,000 Da
· Spectra rate: 500 milliseconds per MS spectra, 130 milliseconds per MS/MS spectra (10-50 eV)

MATERIALS

· All solvents are HPLC grade
· UPLC Column: Waters ACQUITY PREMIER BEH C18, 130Å, 1.7 µm VanGuard FIT 2.1 x 100 mm MVK
· Glass vials: Thermo Fisher, Part No. 4000-S1W
· Vial caps: Agilent, Part No. 5182-0717
· Glass inserts: Agilent, Part No. 5181-8872
· 1.5 ml tubes (natural): Eppendorf, Part No. 022363204
· Standards mixture: Cayman, Vascular Eicosanoid Urinary Metabolite LC-MS Mixture, Part No. 19668

REFERENCE

https://www.nature.com/articles/s41592-021-01197-1
https://www.nature.com/articles/s41592-020-0942-5

RESOURCES

METLIN Gen2 (https://massconsortium.com/)
XCMS Online (https://xcmsonline.scripps.edu/)
Cayman Chemical Lipidomic Standards (https://www.caymanchem.com/)

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