GUIDELINES FOR METABOLOMICS
SAMPLE PREPARATION

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**METABOLIC WORKFLOW**

- **Matrix**
- **Sample processing**
- **Instrumental Analysis**
- **Processing**
- **Metabolites**

**Metabolite Extraction**

- Fresh tissue
- Biofluids
- Cells
- Cell culture media (CCM)
- Organelles
- Isolated nucleic acids

**Instrumental Analysis**

- Chromatographic separation coupled with mass spectrometry (LC/MS)
- Run time depends on sample size.

**Processing**

- Internal library
- External software

Analysis will take 3-6 weeks, depending on the sample size.

**Metabolites**

- Amino acids
- Acylcarnitines
- Amines
- Nucleotides & nucleosides
- Glycolysis
- TCA cycle metabolites
- Urea cycle metabolites
1. EXPERIMENTAL DESIGN

Experimental design should be discussed with the mass spec team prior to conducting the metabolomics experiment. Sufficient biological sampling and appropriate controls must be included to meaningfully interpret the data and avoid unnecessary repeats of the experiment.

- How many replicates do I need?
  It is recommended to have at least five replicates (technical replicates) per experimental condition due to the nature of mass spectrometry analysis. For in vitro experiments, experiments should ideally be repeated three times on different days (biological replicates), using different passages of the same cell line, to provide an adequate number of both technical and biological replicates. When seeding cells for metabolite extraction, please always set aside extra wells for cell counting and/or protein assay as pre-acquisition normalization measures. For in vivo experiments, it is recommended to have at least three animals per biological condition and five technical replicates per animal. Exceptions can be made in the case of in vivo experiments when such number of replicates is not permitted by the Animals (Scientific Procedures) Act 1986, and for pilot experiments for method optimization.

- How much sample do I need?
  For primary and cultured cell lines, we recommend having 0.3-1 X10⁶ cells per technical replicate, depending on the cell type. It is recommended to use 70-80% confluent cells for metabolite extraction and have approximately similar number of cells for all experimental conditions. Additionally, the wells used for cell counting or protein assay must be treated exactly as the experimental wells used for metabolite extraction. For tissue samples, we recommend using 10-20mg of tissue in each sample. Where sampling is extremely difficult, a minimal of 2mg of tissue should be provided to guarantee robust results. For blood specimen, we recommend a minimum of 40ul of plasma, with up to 100ul plasma for the detection of lower abundant metabolites, if sample amount is not limiting.

2. STABLE ISOTOPE LABELING

Before any labelled experiments can be conducted, we generally ask for an unlabelled pilot experiment first, which provides a snapshot of the steady state metabolic profile as well as quantitative information for the total amount of each metabolite. The pilot experiment can also be used to determine the most optimal ratio of extraction buffer to be used, that is specific to your sample.
For labelling experiment using stable isotope tracers (13C, 15N, 2H (D)), the labelling duration depends on the metabolic pathway of interest. For instance, labelled isotopes in glycolysis may only take minutes to reach steady state for many cell types, TCA cycle labelling might take up to a few hours, while lipid metabolism may take days. The concentration of the labelled tracer in the culture media should ideally be the same as that used in your normal experimental conditions (e.g. you would use glucose-free media and add back the same amount of labelled glucose). We typically recommend using dialyzed serum for labelling experiments, to minimize the presence of unlabelled metabolites in the serum diluting the tracer. However, this should be discussed beforehand as the dialyzed serum may affect the biological question being addressed. Please always keep back some culture media sample as evidence for the label being added.

For in vivo labelling experiment, animals can be labelled through IP or IV injection, either as a bolus or through a catheter into the tail vein. In this case, blood samples can be taken as a control to determine if the tracer has gone into circulation. Note: this procedure needs specific permission in project licence and training. Check this before planning the experiments.

3. SAMPLE PREPARATION

Metabolite extraction is a crucial step in obtaining good quality metabolomics data. Please refer to the protocols for metabolite extraction from different biological matrices appended at the end of this document.

Samples should be prepared in dedicated mass spec autosampler vials (Merck Life Science, 29659-U) with a minimum final volume of 80ul. Sample coding should include both the researcher’s initials and the sample number, written legibly on a cryo-label (Merck Life Science, Z366218-1PAK), exactly as specified on the sample submission form. As an example, for a researcher named Andrew Brown, the samples should be labelled as AB001…AB060…. AB099…AB120. Please do not write directly on the glass vial, as the markings will come off. The mass spec vials should be stored in a cryobox labelled with the date, name of researcher/lab, and experiment title or number. After analyses, the samples should be collected/shipped back to the owner, or disposed of following acceptance of a manuscript, with the permission from the owner. Note: we will not accept samples that are labelled incorrectly.

4. POOLED SAMPLE FOR QUALITY CONTROL
We use a pooled quality control (QC) approach to monitor instrument performance during a long acquisition. Please make a total pool sample by taking an aliquot of all samples after your sample preparation. The pooled sample is run interspersed throughout the sample sequence to monitor stability of chromatography separations and any deviations in mass accuracy. All samples are randomized during instrument analysis to minimize bias, unless otherwise requested by the researcher.

5. SAMPLE SUBMISSION FORM (SSF)

The sample submission form (SSF) must be submitted before any samples can be analysed. Please fill the SSF to the best of your knowledge, stating clearly the type of biological matrices used (e.g. cells, tissues, biofluids, media and etc), genotypes or experimental subgroups, sample numbers, cell counts/tissue weights, volume of extraction buffer used, whether absolute quantitation of specific metabolites are required, and any other information that is relevant. The SSF should be emailed to us before we receive the samples. Please ship all samples on dry ice and enclose a hard copy of SSF when shipping the samples.

The address for shipping is,

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6. DATA ANALYSIS

- TARGETED METABOLOMIC ANALYSIS
  We have two high-resolution Q-Exactive Orbitrap mass spectrometers that are coupled to either a high performance liquid chromatography (HPLC) system for the detection of polar and hydrophilic compounds using a SeQuant ZIC-pHILIC column, or a high pressure ion chromatography (HPIC) system with an IonPac anion exchange column for the detection of organic acids and other anionic compounds. Additionally, we have several other hydrophilic or reverse phase analytical columns for detecting certain metabolites.
Therefore, it is essential that researchers communicate with us the metabolic pathways they are interested in well before sample submission. A list of all metabolites that we can currently detect on our platforms can be provided. If new metabolites are required, we can develop specific methods that suit your needs, provided the compound standards can be obtained.

- **ABSOLUTE QUANTIFICATION AND NORMALIZATION METHODS**

Please state clearly in your sample submission form whether the absolute quantification of any metabolites is required. Importantly, to calculate the intracellular concentration of a metabolite, please provide us with the cell volume. This can be measured using a CASY counter.

- **NORMALIZATION**

Normalization of metabolomics data can be performed using either pre-acquisition strategies (e.g. to cell number, protein content or tissue weight), and/or using total ion count as a post-acquisition method. When designing an *in vitro* experiment, please always set up extra wells for cell counting and/or protein assay for normalization purposes. Prior to metabolite extraction, make sure to count your cells and adjust the amount of extraction buffer used, based on the cell count. Additionally, for experiments looking at the consumption and release (CoRe) of metabolites in the media, please set up extra wells to count cells at BOTH the start and finish of the CoRe experiment, and plate cell-free media into empty wells to extract metabolites as controls for looking at nutrient change in the media over time.

Raw data obtained for each metabolite are generally normalized to the total ion count (sum of all metabolites detected in a targeted analysis) unless otherwise requested. If there is anything else that needs to be considered for normalization, please discuss with us beforehand.

### 7. DELIVERY OF RESULTS

We will provide an excel file containing all the raw and normalized data, bar charts for each metabolite and a metabolite heatmap. Desirably, a follow-up meeting will take place to discuss major findings and further experiments. Spectra or chromatography traces can be supplied for thesis, reports, or manuscripts. Data acquired may be retrieved at any time and will be submitted to Metabolight (mandatory for some journals).
PROTOCOLS and MATERIALS

Metabolite extraction protocol for cell culture media

Extraction protocol for intracellular metabolites from Adherent cells

Extraction protocol for intracellular metabolites from non-adherent cells

Metabolite extraction protocol for blood specimen

Metabolite extraction protocol for mammalian tissues
Preparation of Metabolite Extraction Solution

❖ Prepare the extraction solution in advance and store in a glass bottle at -20°C at least 1 hour prior to extraction. Make sure the glass bottle is clean and free of detergent.

❖ Pay special attention to the metabolite extraction solution homogeneity. Prolonged exposure of the extraction solution to low temperatures may lead to phase separation. Agitate thoroughly before use.

❖ Valine-d8 (internal standard for LC-MS) can be prepared as a 10mM stock solution and 1mM working solution in water.

❖ Hippuric acid-d5 (internal standard for IC-MS) can be prepared as a 10mM stock solution and 1mM working solution in water.

❖ Metabolite extraction solution for LC-MS

✓ 50% LC-MS grade Methanol (Fisher Scientific, 10284580)
✓ 30% LC-MS grade Acetonitrile (Fisher Scientific, 10001334)
✓ 20% ultrapure water
✓ Valine-d8 final concentration 5 µM (CK isotopes, DLM-488)

❖ Metabolite extraction solution for IC-MS

▪ 50% LC-MS grade Methanol (Fisher Scientific, 10284580)
▪ 30% LC-MS grade Acetonitrile (Fisher Scientific, 10001334)
▪ 20% ultrapure water
▪ Hippuric acid-d5 final concentration 5 µM (CK isotopes, DLM-7703)
Metabolite extraction protocol for cell culture media (CCM)

Materials
- Metabolite extraction solution for LC-MS
- Eppendorf tubes
- Cell scrapers
- Autosampler vials (MERCK LIFE SCIENCE, 29659-U)
- Cryo labels (MERCK LIFE SCIENCE, Z366218-1PAK)
- Dry ice
- Temperature controlled centrifuge
- Eppendorf Thermomixer (placed in the cold room)

Metabolite extraction from media

- Take 200 µL of CCM and centrifuge for 5 minutes (4°C) at maximum speed to eliminate dead cells and debris.
- Take 80 µL of the supernatant and add it to pre-labelled Eppendorf tube containing 400 µL of extraction solution on dry ice.
- Vortex for 20 sec and mix for 15 minutes in a Thermomixer at 4°C at maximum speed.
- The samples can be stored at -80 overnight at this step if needed.
- Vortex briefly and centrifuge for 20 minutes (4°C) at maximum speed (13000 rpm or higher).
- Carefully transfer the top 80% of the supernatant into autosampler vials, taking care not to disturb any insoluble debris. If any solid residual is aspirated, please centrifuge the samples again, otherwise the precipitates may block the needle or column during mass spec runs.
- Prepare a pooled sample by mixing equal volumes of each sample extract in a single vial. This pool will be analyzed repeatedly throughout the run to monitor instrument stability and assess matrix-associated sample variability.
- Store all samples at -80 °C until analysis.
Extraction for intracellular metabolites from Adherent cells

**Materials**
- Metabolite extraction solution for either LC-MS or IC-MS
- Eppendorf tubes
- Cell scrapers
- Autosampler vials (MERCK LIFE SCIENCE, 29659-U)
- Cryo labels (MERCK LIFE SCIENCE, Z366218-1PAK)
- PBS
- Dry ice
- Temperature controlled centrifuge
- Eppendorf Thermomixer (placed in the cold room)
- For IC-MS only: Amicon Ultra-0.5 centrifugal filter unit (Merk Life Sciences, UFC501096)

**Metabolite extraction from adherent cells**

1. Count the cells in your counting plate (plated and treated in the same way as the cells that will be used for extraction) to calculate how much extraction solution to use. We recommend using 500 µl of extraction buffer per $10^6$ cells as a starting point, although further optimization may be required for individual cell types. If you are using 6 wells plates it can be helpful to use 500 µL of trypsin and then inactivate it with 500 µL medium (for cell counting?).
2. Collect cell culture medium from experimental plates for LC-MS analysis if needed.

*Steps 3-5 should be performed as swiftly as possible.*

3. Remove the medium from wells and wash quickly with PBS 2X at room temperature. Remove PBS by inverting the plate over a piece of tissue paper.
4. After the last wash, aspirate each well quickly to remove ALL residual PBS (it will interfere with downstream analysis).
5. Place the plate on dry ice and add 500ul of extraction buffer per million cells. Prolonged exposure of the extraction buffer on dry ice will result in phase separation. Take care to homogenize the extraction buffer before adding to the wells.

6. Gently swirl the plate making sure all cells are covered by the extraction buffer. Incubate for 5 min on a dry ice-methanol bath to break cell membranes.

7. The plate can be stored at -80 overnight at this step or until ready to be processed further.

8. The day after, scrape the cells off the plate and transfer the entire suspension into pre-chilled Eppendorf tubes. Keep everything on dry ice during this step.

9. Shake for 15 minutes at 4 °C in a Thermomixer at maximum speed.

10. Centrifuge for 20 minutes (4°C) at maximum speed. Collect only the top 80% of the supernatant and put into pre-labelled autosampler vials, taking care not to include any solid debris. If any solid residual is aspirated, please centrifuge the samples again, otherwise the precipitates may block the needle or column during mass spec runs.

11. Optional: for samples to be run on the IC, load the cleared supernatant onto an Amicon Ultra-0.5 centrifugal filter unit with 10 KDa MWCO membrane, and spin at full speed for 30 min. Collect the FLOW THROUGH.

12. Prepare a pooled sample by mixing equal volumes of each sample extract in a single vial. This pool will be analyzed repeatedly throughout the run to monitor instrument stability and assess matrix-associated sample variability. Store all samples at -80 until analysis.
Extraction for intracellular metabolites from Non-Adherent cells

**Materials**

- Metabolite extraction solution for either LC-MS or IC-MS
- Eppendorf tubes
- Autosampler vials (MERCK LIFE SCIENCE, 29659-U)
- Cryo labels (MERCK LIFE SCIENCE, Z366218-1PAK)
- PBS
- Dry ice
- Temperature controlled centrifuge
- Eppendorf Thermomixer (placed in the cold room)
- For IC-MS only: Amicon Ultra-0.5 centrifugal filter unit (Merk Life Sciences, UFC501096)

**Metabolite extraction from non-adherent cells**

1. Count the cells in your counting plate (plated and treated in the same way as the cells that will be used for extraction) to calculate how much extraction solution to use. We recommend using 500ul of extraction buffer per $10^6$ cells as a starting point, although further optimization may be required for individual cell types.

2. Quickly and gently collect the contents of each well into an Eppendorf tube, and centrifuge briefly (30 seconds, low speed). Collect cell culture medium for LC-MS analysis if needed. Refer to the cell culture media extracts protocol on Page 9 for media sample preparation.

3. **Steps 3-5 should be performed as swiftly as possible.**

4. Aspirate the medium and replace by roughly the same volume of PBS. Pipette up and down to remove any traces of cell culture medium. Centrifuge and remove the PBS.

5. Repeat the PBS wash. Make sure to remove ALL PBS residue from the cell pellet before adding metabolite extraction buffer. Incomplete removal of PBS will interfere with downstream analysis.

6. Add 500ul of extraction buffer per million cells, vortex for 20 sec, and incubate for 5 min on a dry ice-methanol bath.
6. The tubes can be stored at -80 overnight at this step or until ready to be processed further.

7. The day after, shake the suspension for 15 minutes at 4 °C in a Thermomixer at maximum speed.

8. Centrifuge for 20 minutes (4°C) at maximum speed. Collect only the top 80% of the supernatant and put into pre-labelled autosampler vials, taking care not to include any solid debris. If any solid residual is aspirated, please centrifuge the samples again, otherwise the precipitates may block the needle or column during mass spec runs.

9. Optional: for samples to be run on the IC, load the cleared supernatant onto an Amicon Ultra-0.5 centrifugal filter unit with 10 KDa MWCO membrane, and spin at full speed for 30 min. Collect the FLOW THROUGH.

10. Prepare a pooled sample by mixing equal volumes of each sample extract in a single vial. This pool will be analyzed repeatedly throughout the run to monitor instrument stability and assess matrix-associated sample variability. Store all samples at -80 until analysis.
Metabolite extraction protocol for blood specimen

Materials

- Heparin-containing anticoagulant tubes
- Metabolite extraction solution for either LC-MS or IC-MS
- Eppendorf tubes
- Autosampler vials (MERCK LIFE SCIENCE, 29659-U)
- Cryo labels (MERCK LIFE SCIENCE, Z366218-1PAK)
- Dry ice and ice
- Temperature controlled centrifuge
- Eppendorf Thermomixer (placed in the cold room)
- For IC-MS: Amicon Ultra-0.5 centrifugal filter unit (Merk Life Sciences, UFC501096)

Metabolic extraction from plasma

1. Collect blood into a lithium heparin containing tube. Immediately invert tube 5-6 times to mix with anticoagulant. Avoid using EDTA-containing anticoagulant tubes as it can interfere with mass spec analysis.
2. Centrifuge sample within 30 minutes at 1,000g for 10 minutes at room temperature (or store sample on ice until centrifugation is possible).
   a. Set the centrifuge braking speed on a low setting to avoid disturbing the plasma and buffy coat layers when the centrifuge completes the spin.
   b. Take care to avoid any buffy coat layer in this step.
3. Transfer 200μl of plasma to a labelled Eppendorf tube.
   Recommended: transfer up to 1 mL of plasma so that there is enough sample to prepare backup aliquots.
4. Centrifuge plasma aliquots in a microcentrifuge for 5 minutes at 16,000g at room temperature if processed immediately or at 4 °C if initially stored on wet ice.
5. Take 80 μL of the plasma supernatant and add it to pre-labelled Eppendorf tube containing 400 μL of metabolite extraction solution on dry ice.
6. Vortex for 20 sec and mix for 15 minutes in a Thermomixer at 4°C at maximum speed.

7. The samples can be stored at -80 overnight at this step if needed.

8. Vortex briefly and centrifuge for 20 minutes (4°C) at maximum speed. Carefully collect only the top 80% of the supernatant and put into pre-labelled autosampler vials, taking care not to include any solid debris. If any solid residual is aspirated, please centrifuge the samples again, otherwise the precipitates may block the needle or column during mass spec runs.

9. For samples to be run on the IC, transfer the cleared supernatant onto Amicon Ultra-0.5 centrifugal filter unit with 10 KDa MWCO membrane, and spin at full speed for 30 min. Collect the FLOW THROUGH into pre-labelled mass spec autosampler vials.

10. Prepare a pooled sample by mixing equal volumes of each sample extract in a single vial. This pool will be analyzed repeatedly throughout the run to monitor instrument stability and assess matrix-associated sample variability.

11. Store all samples at -80 °C until analysis.
Metabolite extraction protocol for mammalian tissues

**Materials**
- Metabolite extraction solution for either LC-MS or IC-MS
- Precellys 2ml tissue homogenizing tubes (CKMIX, Stretton Scientific, P000918-LYSK0-A.0)
- Eppendorf tubes
- Autosampler vials (MERCK LIFE SCIENCE, 29659-U)
- Cryo labels (MERCK LIFE SCIENCE, Z366218-1PAK)
- Dry ice
- Temperature controlled centrifuge
- Eppendorf Thermomixer (placed in the cold room)
- For IC-MS: Amicon Ultra-0.5 centrifugal filter unit (Merk Life Sciences, UFC501096)

**Tissue harvesting and storage**
- Carefully pat the organ with lint-free tissue paper.
- Quickly collect enough tissue sample for your experiments.
- Cut into small pieces, and place into pre-labeled tubes.
- Snap-freeze in liquid nitrogen and store at −80°C until extraction.

**Metabolite extraction from mammalian tissues**

1. Label and pre-weigh Precellys tubes. Place the tubes and metabolite extraction solution on dry ice prior to start.
2. Cut out 10-20 mg of tissue and place directly in a pre-chilled Precellys tube on dry-ice. Keep a note of the tissue weight. Please note a minimal of 2mg of tissue is required.
3. Add the correct amount of metabolite extraction solution (25 µL/mg tissue).
4. Lyse the tissue in a homogenizer (4°C) until complete disruption of sample (1 or 2x 30 seconds cycles at 6000 rpm will suffice in most cases).
5. Incubate the samples for a minimum of 20 min on dry ice. Extraction may benefit from several freeze-thaw cycles if the homogenization was not 100% efficient. Samples can be stored at -80 overnight.
6. Vortex the samples for 20 sec. Shake in a Thermomixer for 15 min at 4°C and maximum speed.
7. Centrifuge for 20 minutes (4°C) at maximum speed. Carefully collect only the top 80% of the supernatant and put into pre-labelled autosampler vials, taking care not to include any solid debris.
8. For samples to be run on the IC, transfer the cleared supernatant onto Amicon Ultra-0.5 centrifugal filter units with 10 KDa MWCO membrane, and spin at full speed for 30 min. Collect the FLOW THROUGH into pre-labelled mass spec autosampler vials.
9. Prepare a pooled sample by mixing equal volumes of each sample extract in a single vial. This pool will be analysed repeatedly throughout the run to monitor instrument stability and assess matrix-associated sample variability.
10. Store all samples at -80 °C until analysis.