General Sample Collection Recommendations for Metabolomic Studies: Quantity, Preparation and Handling

Guidelines for all samples:

- Please notify the Metabolomics Core of any preservatives or additives that have been applied during the collection process.
- Use permanent markers to directly write on tubes or plates. Labels with adhesive often fall off when frozen.
- Submission of an *electronic* document containing sample ID information is required. The Sample Submission Form can be found on the Project Requests page of the MRC2 website: http://mrc2.umich.edu/project-requests
- A service request must be initiated in the MiCores website: https://my.ilabsolutions.com/account/login.
- The completed Sample Submission Form must be uploaded to the MiCores website before samples are submitted for analysis.
- Ship samples to:

  Attn: Kari Bonds
  University of Michigan
  Metabolomics Core
  1000 Wall Street
  5305 Brehm Tower
  Ann Arbor, MI 48105-5714

Consistency in sample collection and handling is the most important aspect of sample preparation for metabolomics studies.

Blood Plasma and Serum

- Plasma**:** Collect whole blood in tubes containing EDTA (K2, K3, or Na) as anti-coagulant (lavender colored tops). Follow collection tube manufacturer recommendations or your internal protocol for proper consistent collection. Transfer supernatant or plasma to appropriately labeled tubes and freeze at -80°C immediately.
- Serum: Collect whole blood in serum separator tubes (SST) and follow tube manufacturer's processing instructions. Extract serum, transfer to appropriately labeled tubes and store at -80°C immediately.
- Volume requirements: generally 50 - 150 µL per assay is required; please check specific requirements in the assay descriptions available on the Services & Fees page of the MRC2 website. If multiple assays are requested and there is limited volume, please contact the Core to discuss options.
- Samples should be shipped frozen, in a secondary container, on dry ice. Tubes should be clearly labeled with sample identifiers (described in the Sample Submission Form) and include a hard copy of the Sample Submission Form with the shipment.

Urine

- Accurate 24-hour whole urine collections kept at 4°C during the duration are highly recommended, especially for steroid measurements. Aliquot a minimum of 5 mL after thorough mixing of all timepoint collections. If 24-hour collections are not practical or indicated, then first catch is preferred.
- Post collection place sample in -80°C.
- Samples should be shipped frozen, in a secondary container, on dry ice. Tubes should be clearly labeled with sample identifiers (described in the Sample Submission Form) and include a hard copy of the Sample Submission Form with the shipment.

Saliva

- Collect saliva samples in a non-stimulated fashion. Dietary restrictions are not necessary, but food intake should be documented.
- Instruct research subjects to rinse their mouths vigorously three times with at least 30 mL of water before saliva collection, without brushing their teeth or using any mouthwashes. Five minutes after the water rinse, collect about 3 ml of saliva in a clean plastic container by expectorate sputum.
- Freeze samples as soon as possible and maintain at −80°C. If saliva collection tubes were purchased from a manufacturer containing preservative, please send an unused container to determine any additive/preservative interaction with the analysis.

Tissues

- For solid tissues (e.g., non-plasma/serum/urine/CSF), the amount of tissue/sample can vary depending upon study objectives and tissue type—typically, a 100 mg (wet tissue weight) sample is required; please check specific requirements in the assay descriptions available on the Services & Fees page of the MRC2 website. Please note there will be a difference in the sample weight recorded before and after the -80°C storage due to “freeze drying.”
- Flash-freeze sample immediately after sectioning and store at -80°C.
- Samples should be shipped frozen, in a secondary container, on dry ice. Tubes should be clearly labeled with sample identifiers (described in Sample Submission Form) and include a hard copy of the Sample Submission Form with the shipment.

Fecal/Large Intestinal Samples

- Collect and weigh 50-100mg of fecal, cecal, or chyme samples in an appropriate tube with sample identification information.
- Immediately store samples frozen at -80°C.
- Samples should be shipped frozen, in a secondary container, on dry ice. Tubes should be clearly labeled with sample identifiers (described in Sample Submission Form) and include a hard copy of the Sample Submission Form with the shipment.

Plated Cultured Cells Procedure

1. Cultures should be prepared with cells grown as defined per your individual study design.
2. If your study is a time course, stagger the beginning of the treatment to account for sample processing time at the end.
3. Each sample should optimally contain 1 x 10^7 cells, preferably 3-5 x 10^6 cells, and at minimum 1 x 10^6 cells. Quantitative analysis will be performed on protein content on each sample extracted by a colorimetric assay based spectrometer analysis.
4. You can use individual 10 cm or 6 cm plates. Multi-well plates can be used as long as the required cell population is met. For robust statistical analysis, a minimum of 3 replicate plates or wells per condition is required; 5 or more is recommended.
5. To process samples for Metabolomics analysis:
    a. Remove any solutions (media/ PBS) adjacent to the sample.
    b. Wash with 150 mM ammonium acetate and remove quickly (<30 sec.)(We use Sigma Aldrich A7330-100g.)
    c. Quench samples by pouring liquid nitrogen on the plate (enough to cover the bottom generously); allow evaporation to occur until most of the liquid nitrogen is gone; place on dry ice until all samples are processed.
    d. Wrap plates in aluminum foil (with labeling) and store at -80°C.
    e. Ship samples frozen and on dry ice. Plates should be clearly labeled with sample identifiers (described in Sample Submission Form) on both the plate lid and the outer aluminum foil using a permanent maker and a hard copy of the Sample Submission Form should be included with the shipment.

Suspension Cells Procedure

1. Cultures should be prepared with cells grown as defined per your individual study design.
2. If your study is a time course, stagger the beginning of the treatment to account for sample processing time at the end.
3. Each sample should optimally contain 1 x 10^7 cells, preferably 3-5 x 10^6 cells, and at minimum 1 x 10^6 cells. Quantitative analysis will be performed on protein content on each sample extracted by a colorimetric assay based spectrometer analysis.
4. To process samples for Metabolomics analysis:
    a. Centrifuge cells at 750-100 g for 1-3 min. (less than 2500 RPM) to avoid lysing cells.
    b. Wash with 150 mM ammonium acetate and remove quickly (<30 sec.); remove as much liquid as possible; it's best to have a dry pellet for freezing.) (We use Sigma Aldrich A7330-100g.)
    c. Quench samples by pouring liquid nitrogen directly into the conical vial, allow evaporation to occur until most of the liquid nitrogen is gone; alternatively, you can dip the bottom of vial into liquid nitrogen for 10-15 seconds until pellet is frozen.
    d. Place vials on dry ice until all samples are processed.
    e. Ship samples frozen and on dry ice. Tubes should be clearly labeled with sample identifiers (described in Sample Submission Form) and include a hard copy of the Sample Submission Form with the shipment.

Alternatively, pre-extracted samples can be sent. Please contact us for further details on extraction procedure if desired.

References

Lorenz MA, Burant CF, Kennedy RT. Reducing Time and Increasing Sensitivity in Sample Preparation for Adherent Mammalian Cell Metabolomics Anal Chem. 2011 May 1;83(9):3406-14

Strategy for Fluxomics Studies: Parallel Labeling Using Glucose, Glutamine and Oleate

(Note: This is a general protocol. Always consult the Metabolomics Core before preparing to do a fluxomics experiment, to discuss the biological aims and other aspects of the study, such as the time course and any other possible interferents in the media.)

Products you may need for fluxomics studies:

- *The U13C Glucose:: Sigma: 389374 or Cambridge:*CLM-1396-PK
- *Ammonium acetate = Sigma Aldrich A7330-100g*
- *FFA-free BSA = Sigma A7030-50g*
- *Sodium oleate (12C) = Sigma O7501-1g.*
- *Potassium oleate (13C) = Sigma 714313-0.25g Make sure to get a quote first because it will save you half or more.*
- *Glucose/pyruvate/glutamine/phenol red-free RPMI 1640, 1X: custom ordered from Cellgro (cellgro.com). We don't have a part number but they will work with you to make it if you tell them what you need.*

General Procedure Using RPMI Media

Plain RPMI media contains 11.1 mM glucose, 2 mM glutamine. The media is supplemented with 100 µM fatty acid such as Oleic acid. The same media is prepared three times: once with 11.1 mM ^{13}C glucose while maintaining the Oleate and the glutamine concentration. For the second experiment, the media will have 2 mM ^{13}C glutamine, while maintaining the Oleate and the glucose concentration. For the third experiment, the media will contain 100 µM ^{13}C Oleate, while maintaining the glucose and the glutamine concentration. If you would like to use DMEM, please check the concentrations of glucose and glutamine to supply your tracer in the same amount.

For Glucose flux:

1. Get RPMI with 0 mM glucose (or DMEM, depending on what your cells are using).
2. RPMI will contain 2 mM glutamine (some RMPI is 4 mM glutamine – be sure to check this).
3. Prepare stock solution of 1M -U-^{13}C glucose (dissolve 186 mg in 1 mL). Sterile filter it using a syringe filter if you will be incubating your cells for a long period of time.
4. Prepare stock solution of 1 mM Oleate (10X) in 1% Fatty acid free BSA. BSA:Fatty acid concentration should be 1:6 (see Fatty Acid Preparation, below).
5. Spike the media with Fatty acid complexed with BSA to achieve 100 µM in the media.
6. Spike your ^{13}C glucose from stock solution to achieve 11.1 mM glucose (or an appropriate concentration that makes sense for your study).

Fatty Acid Preparation:

1. Dissolve 200 mg of Fatty acid free BSA in 20 mL of RPMI (without glucose) in 20 mL glass scintillation vial. Do not vortex, just keep in water bath for 10 minutes and BSA will dissolve without forming any froth.

2. Add to the BSA solution 6 mg of Sodium Oleate (MW=304). If you think the fatty acid powder is coarse, then it is better to grind it in a mortar for better solubility. Adjust the amount of your fatty acid if it has different molecular weight.
3. Add a small magnetic stirrer and keep it rotating for ~1-2 hours at high speed at room temperature. Clear solution should be obtained.
4. If your experiment will require more than ~6 hours incubation, then you need to sterile filter your fatty acid solution before using it.

For Glutamine flux:
Repeat the experiment from the "Glucose flux," but using Glutamine-free media. Add 2 mM labeled-Glutamine, keeping the concentration of the unlabeled Glucose and unlabeled Fatty Acid consistent with the Glucose flux experiment.

For Oleate flux:
Repeat the experiment from the "Glucose flux," but with 100 µM labeled-Oleate, keeping the concentration of the unlabeled Glucose and unlabeled Glutamine consistent with the Glucose flux experiment.