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Preparing for a metabolomics experiment using tissues

- *General considerations for stable isotope (e.g. C-13, N-15, H-2 (D)) labeling:*
  The minimum labeling duration depends on the metabolic pathway of interest. For instance, metabolites in the glycolysis pathway only take minutes to reach isotopic steady-state for many cell types, while other metabolic pathways (e.g. lipids) might take days
- you can label animals through i.p. or i.v. injection (bolus) or over time through a catheter into the tail vein, for instance
- optional: use a positive/input control, i.e. blood plasma, to verify that the tracer has gone into the blood stream

Metabolite extraction from tissue

1. Cut off 10-20 mg tissue on ice, rinse if necessary to remove blood, and place in a 2 ml round-bottom cryovial. Tissue can be frozen at -80 °C at this point.
2. One by one, homogenize* the tissue samples in very cold 1 ml 80% MeOH **(-80C/dry ice)**: use 2-3 bursts of +/- 10 sec, *making sure that the samples stay cold* by keeping the samples on dry ice (avoid prolonged homogenization or gripping the tube at the bottom while homogenizing to prevent substantial heating).

   Clean the probe homogenizer between samples by running it in large volumes of water followed by running in some methanol.
3. Transfer the homogenate to an Eppendorf tube and **keep on dry ice while processing the remaining samples.**
4. Incubate the samples for a minimum of 20 min on dry ice. Extraction may benefit from several freeze-thaw cycles using liquid N2 if the homogenization was not 100% efficient.
5. Vortex the samples for 10 sec.
6. Spin down at top speed for 10 min @ 4 °C.
7. Transfer the supernatant to a new Eppendorf tube on ice.
8. Optional: Reextract the pellet with another 0.2 ml cold 80% methanol; add the supernatant to first extract.
9. Resuspend the remaining cellular pellet in 100 ul protein lysis buffer and determine the protein concentration. Then calculate the total protein content per sample.
10. Transfer equivalent amounts of the extracts – usually the equivalent of 10-20 μg protein – into glass vials.
    - i.e., total protein amount sample 1 = 60 ug. If using 15 ug protein equivalent, take 15/60 x 1.2 ml (total volume of extract) = 300 ul of extract.
11. Optional: add 5 nmol norvaline to each sample as an internal standard.
    From a 10 mM stock solution in methanol (stored at -20 °C), make a fresh 1:10 dilution in water. Add 5 ul of the 1 mM solution.
12. Dry the samples using the EZ-2Elite evaporator at 30C using program 3 (aqueous).
13. Store dried samples at -80 °C.

**These instructions are for using a small diameter probe homogenizer. You can also use a bead beating method using beads and a bead beater. Alternatively, pulverize the tissue in liquid nitrogen.*

Useful reference:

Lu et al, 2018: Metabolite Measurement: Pitfalls to Avoid and Practices to Follow
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5734093/

Equipment, materials and reagents needed for this protocol

- round bottom cryovials, 2 ml:
  03-374-22 Fisher Corning Internally Threaded Cryogenic Vials
- Eppendorf centrifuge tubes
- dry ice and ice
- MeOH: A456-1 Fisher Fisher Methanol (Optima* LC/MS)
- H2O: W5-1 Fisher Water, Glass Bottle; 1L
- Norvaline: N7502-25G Sigma DL-Norvaline
  Used as an internal standard: prepare a 100 mM solution in H2O. Make a 10 mM working stock in MeOH. Store both at -20C.

- glass vials:	03-410-151	Fisher	1.8 mL Volume; Clear Glass, 12x32 mm, 9 mm thread
- caps:	03-379-123	Thermo Scientific	Rubber/Silicone Septa
Alternatively:	American Chromatography Supplies		
- glass vials:	VT009M-1232	ACS	1.8 mL Volume; Clear Glass, 12x32 mm, 9 mm thread
- caps:	C395E-09SB	ACS	Bonded PTFE/Silicone Septa
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C-13- and N-15-labeled metabolites (from Cambridge Isotope Laboratories if not otherwise stated)

- U13C Glucose: CLM-1396-1 1 g