Metabolite extraction from tissues using LN2 pulverization

Use at a minimum 5 samples/replicates per condition.

Important: *When dissecting the tissues, work quickly and on ice! Try to obtain very similar-sized tissue pieces for each sample in the range of 20 – 40 mg.*

1. Prepare 1.5 ml microcentrifuge tubes and place on ice.
2. Dissect the tissue(s) on ice. Rinse the tissue in ice-cold 150 mM NH4OAc pH 7.4[(1)] to remove any blood, if desired, and blot dry.
3. Cut the tissue in smaller pieces (see above), transfer the pieces to a cold microcentrifuge tube, and *quickly snap-freeze the sample in liquid nitrogen (LN2) for 5 min*.
4. Store the tissues in a -80 ˚C freezer until ready for further processing.

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5. Homogenize the tissues by pulverization in liquid nitrogen (WEAR PROPER PPE!)

   Practice this first on test tissues, and make adaptations as needed!

   There are several ways of doing this:

   i. Using mortar and pestle

   - Precool a mortar, pestle, and spatula in a (Styrofoam) container with LN2 in it.
   - Remove the mortar and pestle from the container and place on the bench.
   - Pour a small volume of clean LN2 from a dewar into the mortar. Place the frozen tissue piece in the LN2 in the mortar, and let cool for 1 min to get brittle. Then carefully pound and grind the tissue into a fine powder.
   - With a precooled spatula, scrape the powder into a heap and transfer to an Eppendorf tube sitting on dry ice. Work quickly so that the mortar, pestle, and spatula do not get the chance to warm up.
   - Clean the mortar, pestle, and spatula in between samples by rinsing with 100% methanol and wiping them clean with large Kimwipes.
   - Precool the mortar, pestle, and spatula again in LN2 before proceeding with the next sample.

   ii. Using heavy-duty aluminum foil, a cold metal surface and a hammer

   - Precool two pieces of thick metal on dry ice.
   - Place the frozen tissue piece on a small piece of heavy-duty aluminum foil (pre-cooled) and wrap it closed.
   - Place this in a dewar with LN2 for a minute or two.

- Remove from LN2 and place on one of the metal surfaces on dry ice. Overlay with the other cold metal piece and use the hammer to pulverize. Check and repeat if needed.
- Put the wrapped pulverized tissue back in LN2 for 1 minute
- Transfer the ground tissue into a new microcentrifuge tube sitting on dry ice.

iii. **Using a device like the Cellcrusher**: https://cellcrusher.com/

6. To the Eppendorf tubes with the pulverized tissues, add 1 ml **80% MeOH (at -80°C/dry ice temperature)** and vortex vigorously for 30 sec.

   It is a good idea to take along a 'processing blank'; process without any tissue present.

7. Place the samples for a 30-60 min at -80°C to aid proper quenching, extraction, and protein precipitation.

   Optional: The extraction may benefit from one or more freeze-thaw cycles if the homogenization was not 100% efficient. If using LN2 to do this, then you will need to use cryovials!

8. Allow the samples to warm up a bit on ice.
9. Vortex the samples again for 30 s.
10. Spin the samples at top speed (16,000 g) for 15 min @ 4 °C.
11. Transfer the entire supernatant to a new Eppendorf tube (or glass vial) and place on ice. Keep the tubes with the remaining pellets on ice as well.

    Optional: Re-extract the pellet with another 0.2 ml cold 80% methanol (-80 °C); spin 15 min at 4°C, and add the supernatant to the first extraction volume.

12. Determine the protein content of the pellets.
    a) Decant any remaining MeOH by placing the tubes upside down on a Kimwipe tissue to drain.
    b) Resuspend the sample pellets in **0.2 M NaOH** (~ 600 ul per 20 mg tissue).
    c) Heat the pellet samples for 20 min at 95°C to resuspend completely (flick to check).
    d) Cool the samples to RT and vortex briefly to mix.
    e) Spin 15 minutes at max speed in microcentrifuge to pellet the insolubles.
    f) Remove the supernatant and determine the protein concentration by the **BCA method** using an i.e. 1:10 dilution.
    g) Then calculate the total protein content for each sample.
13. Transfer equivalent amounts of the extracts – i.e. 500 µg protein equivalent or an amount roughly equal to 5 mg tissue equivalent – into either flat bottom glass vials (if using a Genevac) or new Eppendorf tubes (if using speed vac):
    - I.e., the total protein amount of sample #1 pellet = 2200 µg.

      For 500 µg protein equivalent, use 500/2200 x 1.0 ml (the total volume of added 80% methanol) = 227 µl of extract #1.

For the blank, transfer an aliquot similar to the largest volume dispensed for the tissue samples.

14. Make all the sample volumes the same by adding fresh 80% methanol to those samples with a smaller sample volume.
15. Dry the samples using the Genevac EZ-2Elite evaporator or a speed vac without applying heat. Do not dry the samples longer than needed, and remove promptly from the evaporator when the cycle is finished.
16. Store the dried samples at -80 °C until ready for LC-MS analysis.

(1) 150 mM ammonium acetate solution, pH 7.4:

Dissolve 1.1562 g ammonium acetate (Molecular Biology grade) per 100 ml of Millipore Milli-Q water (18 uΩ) to make a 150 mM solution.

Adjust the pH to 7.4 using a few drops of 1 M NH4OH (i.e, a 1:11 dilution of a 21% NH4OH stock solution).

If stored at room temperature, sterilize the solution using a Stericup Vacuum Filter Cup (i.e. Millipore Corp).

Materials and reagents needed for this protocol (or similar):

- Optional: round bottom cryovials, 2 ml:
  03-374-22 Fisher Scientific Corning Internally Threaded Cryogenic Vials
- Snap-Cap Safe-Lock Eppendorf™ microcentrifuge tubes, 1.5 ml (#22363204)
  05-402-25 Fisher Scientific
- Liquid nitrogen
- Dry ice
- Ice
- MeOH: A456-1 Fisher Scientific Fisher Methanol (Optima* LC/MS)
- H2O: W5-1 Fisher Scientific Water, Glass Bottle; 1L
- Glass vials: 13-622-351 Fisher Scientific Chromacol™ GOLD-Grade Inert Glass Vials; Thermo 2SVWGK
- Caps 03-452-327 Fisher Scientific 9 mm Screw Caps, SureSTART™ Level 2 (Silicone/PTFE septum); Thermo Scientific 6ASC9STB1
  or 03-379-123 Fisher Scientific 9 mm autosampler vial screw thread caps (PTFE/silicone septum); Thermo Scientific C500054A
- 0.2 M NaOH in water
- BCA assay kit