Metabolite extraction from mammalian suspension cells using centrifugation for cell harvesting

Preparing cells for a labeled metabolomics experiment

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 need at least 3 samples per condition. Aim for equal cell numbers per sample at the time of harvest!
- plate about 2e5 – 1e6 cells per well in 6-well plates or 60 mm dishes or T25 flasks. Use more cells for very small cell types like T-cells, i.e. 2-5e6 cells per sample.
  Optional: add additional wells/plates for cell counts at the time of harvest (OR determine protein content of the sample pellets left over after extraction)
- incubate o/n
- 3-24 h before extraction, switch to medium with dialyzed FBS and labeled tracer in place of the normal nutrient in the medium (i.e., 13C6 Glucose instead of normal glucose). A volume of 1.5-2 ml is sufficient to cover the cells in a 6-well plate for 24h, but keep in mind that the cells might deplete nutrients within that amount of time! In that case, replace a portion (i.e., 1/3th) of the medium several hours before harvesting.

Polar metabolites extraction with 80% MeOH (suspension cells)

Note: work on ice unless indicated otherwise

1. Remove a small amount of cell suspension for cell count or use another way to estimate cell count per sample/condition.
2. transfer the cells to a polypropylene centrifuge tube and spin at low g to gently pellet the cells
3. place the tubes on ice; aspirate the medium
4. wash the cells briefly by resuspending them in ice-cold 150 mM NH4AcO solution, pH 7.4 (1)
5. spin down gently as short as possible at 4 °C; aspirate the NH4AcO wash buffer
6. on ice, add 1 ml 80% MeOH/20% water pre-cooled in a -80 °C freezer or on dry ice
7. vortex for 10 sec
8. optional: add 1 nmol norvaline to each sample (as a sample preparation internal control) prepare a fresh 1:50 dilution in H2O from the 10 mM stock solution (see below) add 5 ul of the prepared 1 mM solution per sample
9. incubate for 20-30 min at -80 °C or on dry ice for effective protein precipitation
10. vortex the samples for 10 sec
11. centrifuge in a microcentrifuge at top speed (16,000 g) for 10 min at 4 °C
12. place the tubes on ice; transfer the supernatant into a glass vial on ice
13. optional 2nd extraction of the pellet: resuspend the pellet in 50-200 µl cold 80% MeOH and vortex; centrifuge at top speed for 5 min at 4 °C and add the supernatant to the same glass vial as the 1st extraction
14. Keep the centrifuge tubes with the pellets if normalization by protein content is desired. Resuspend the pellets in 10 pellet volumes of 0.1-0.2 M NaOH and determine the protein content (BCA or Bradford methods).

Notes: to aid complete solubilization of the pellet in protein lysis buffers like RIPA, an overnight incubation at 4 °C may be required. 0.1-0.2 M NaOH (and heating for 20 min at 95°C) will solubilize the pellet more effectively. If desired, instead of protein content, the DNA content can be determined.

15. dry the metabolite extracts down at 30 °C (or without heat) in the evaporator – i.e., the Genevac EZ-2 Elite using program 3 (aqueous) – or a speed vac (if using Eppendorf tubes instead)

16. store the dried extracts at -80 °C.

(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).

Supplies needed for this protocol
- Ammonium acetate A1542-500G Fisher for molecular biology, ≥98%
- 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 -20°C.
- 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
- caps: C394-09SB ACS Bonded PTFE/Rubber Septa

C-13- and N-15-labeled metabolites (from Cambridge Isotope Laboratories if not otherwise stated)
- U13C Glucose: CLM-1396-1 1 g