Extraction of metabolites from adherent tissue culture cells

Preparing cells for a metabolomics experiment

- Plate 2e5-5e5 cells per well in 6-well plates, or up to 1e6 cells in a 60 mm dish. *Target confluency at the time of extraction is typically 70-80%*. Aim for a similar cell number (or similar confluency) per well/dish ***at the time of extraction***!
- Prepare at a minimum 3 wells/plates per condition for LC-MS analysis.
- Prepare additional wells/plates per condition for doing cell counts at the time of harvest, if desired; AND/OR determine the protein/DNA content of the sample pellets after extraction. The cell count or protein/DNA content can be used for slight normalization of the input amounts.
- Incubate o/n.

Preparing for a metabolomics experiment using isotope tracing

General considerations for stable isotope (e.g. C-13, N-15, H-2 (D)) labeling:

- For the labeling medium, obtain medium that does not contain the nutrient that is used as a tracer (i.e. glucose) and add the isotope tracer at the same concentration as in the regular medium (i.e., 5 mM U13C glucose instead regular glucose).
- The minimum labeling duration depends on the metabolic pathway of interest. For instance, metabolites in the glycolysis pathway only take 30 minutes to reach ***isotopic steady-state*** for many cell types, while other metabolic pathways (e.g. lipids) might take days. An 18-24 h labeling time is sufficient for steady state labeling of most central carbon metabolites.
- Ideally, use dialyzed FBS from which small molecules (metabolites) have been removed. Establish growth curves for the different cell lines using this dialyzed FBS, so that you can aim to have similar numbers of cells at the day of harvesting between conditions with different growth rates.

  Alternatively, you can use regular FBS, but maximum percent labeling will be lower since the FBS contains unlabeled tracer counterparts (i.e., glucose), effectively lowering the labeling percentage of the tracer.
- Plate the required number of cells in regular medium containing dialyzed FBS (if used) and incubate overnight.
- Next day, 3-24 h before extraction (depending on your desired labeling time), switch to medium containing the isotope tracer (and dialyzed FBS if used).

  A volume of 1.5-2 ml is sufficient to cover the cells in a 6-well plate for 24 h, 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 6 hours or so before harvesting.

Polar metabolites extraction using 80% MeOH (adherent cells)

Keep plates in incubator until ready for extraction. Work in smaller batches as needed and is manageable (but prevent creating batch effects).

1. Place the plate(s) on ice. Aspirate the medium.
2. Rinse the cells quickly with ice-cold 150 mM NH4AcO, pH 7.3[(1)] and aspirate.
3. Immediately add 1 ml [80% MeOH/20% water] **precooled in a -80 °C freezer** to each well.
   It is a good idea to take along a 'processing blank', by adding 1 ml 80% MeOH to an empty well.

   Optional: include 1 µM norvaline in the MeOH solution as an internal standard.
4. Incubate the plate(s) in the -80 °C freezer for 30 min to aid proper quenching and protein precipitation.
5. Prepare Eppendorf tubes and place on ice.
6. Place the plate(s) back on ice. Scrape the cells from each well and transfer the content into the prepared Eppendorf tubes.
7. Vortex each sample for 20 seconds.
8. Centrifuge at top speed (16,000 g) for 10 min at 4 °C.
9. Transfer supernatant into a glass vial if using a Genevac EZ-2 Elite evaporator (or into centrifuge tubes if using a speed vac).
10. Dry the metabolite extracts down in an evaporator, i.e., a Genevac EZ-2 Elite, or a speed vac without applying heat . Choose an appropriate drying time for your sample volume and remove the samples promptly when the program is finished.
11. Store the dried extracts at -80 °C.
12. Determine protein content of the pellets:
    a) Briefly airdry the tubes to remove any remaining 80% MeOH from the tubes.
    b) Resuspend the pellets in relatively large volumes (i.e. 20 pellet volumes; i.e. 200 µl) of **0.2 M NaOH**
    c) Heat at 95 °C for 20 min. Flick the tubes to make sure the pellets are completely resuspended.
    d) Cool to RT. Spin the tubes for 15 min.
    e) Collect the supernatant and determine the protein content by **BCA method** using an i.e., 10-fold dilution.

    Of note, instead of protein content, the DNA content of the pellet can be determined using an appropriate resuspension solution

(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 (or something of similar quality/make):

- Ammonium acetate	A1542-500G	FisherScientific	molecular biology grade, ≥98%
- MeOH:	A456-1	FisherScientific	Methanol (Optima* LC/MS)
- H2O:	W5-1	FisherScientific	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 or 1 mM working stock in MeOH. Store at -20C. Dilute the 1 mM norvaline stock solution 1:1000 in 80% MeOH for a 1 µM concentration.

- glass vials:	13-622-351	Fisher Scientific	Thermo 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
	03-379-123	Fisher Scientific	9 mm autosampler vial screw thread caps (PTFE/Silicone septum**)**; Thermo Scientific C500054A

Stable isotope-labeled tracers

Choose C-13- and/or N-15-labeled metabolites of high purity (≥ 98 % labeled)

- Cambridge Isotope Laboratories
  i.e., D-Glucose (U-13C6, 99%) CLM-1396-1 1 g
- Sigma
  i.e., D-Glucose-13C6 389374-1g 1 g