Extraction of polar metabolites from mammalian suspension cells using filtration for cell harvesting

Only use this protocol if specifically interested in metabolites with high turnover rates.

1. Grow enough suspensions cells for three biological replicates, using 0.5e6 – 2.0e6 cells per replicate (depending on the size of the cells). For very small cells (i.e., T-cells), use 5e6 cells per replicate.
2. Do a cell count for each culture/condition on the day of extraction.
3. Items needed:
   a. the extraction solution, either
      - **80% MeOH**:20% H2O; *pre-cooled to -80 °C*
      - OR, **only** if particularly interested in the levels of ATP/ADP or NAD(P) redox factors: **80% MeOH with 0.5% formic acid solution**. Also prepare 15% (w/v) ammonium bicarbonate (ABC) to neutralize the extract. *See reference: Lu et al, Antioxidants & redox signaling 28 (3), 2018.*

   b. **Ice-cold 150 mM ammonium acetate solution, pH 7.4**[(1)].

   c. **Dry ice**.

   d. Optional: Precool a **metal pan** to -80 °C for a flat work surface.

   e. Vacuum filter holder (25 mm inner diameter) https://www.fishersci.com/shop/products/fisherbrand-vacuum-filter-holders-borosilicate-glass-bases-7/0975314?keyword=true

   f. Nylon filter membranes (0.45 um, 25 mm, hydrophilic; Millipore, HNWP02500) https://www.fishersci.com/shop/products/emd-millipore-nylon-hydrophilic-membrane-filters-4/hnwp02500?keyword=true [100/pack $86]

   g. Metal tweezers, cleaned with 70% ethanol.

   h. 6-well plates; one well for each sample.

   i. 60 mm dish for separating the nylon membranes.

4. Set up a vacuum system as shown.

5. Place as many membranes as needed – plus some extra ones – in a 60-100 mm plate (using the tweezers and removing the separation circles).

6. Clean the top of the vacuum filter holder with 70% ethanol.
7. Turn the vacuum on and test the vacuum system by placing a membrane on the vacuum filter holder.
8. Place the pre-cooled metal pan upside down over dry ice to create a cold working surface (or level the crushed dry ice to an even surface).
9. Place a 6-well plate on the metal pan. Add a proper volume of extraction solution in each well (i.e. 0.5 – 1.0 ml). *Use a separate tip for each aliquot in order to get more equal volumes of the cold extraction solution per well.*
10. Place a membrane on the filter holder.
11. Load the appropriate volume of suspension cells containing 0.5–2e6 cells onto the filter. ***Steps 10-12 have to be performed very quickly, within 10 seconds.***
12. Wash the cells on the membrane with 1 ml of the ammonium acetate washing buffer.
13. Take the membrane with the cells from the filter holder and put it **upside down** into a well with extraction solution.
    a. If using **acidic** extraction buffer: ***after 3 min,*** add 8.7 ul 15% (w/v) ABC *per 100 ul extraction solution* and swirl to neutralize the extract.
14. Repeat steps 9-12 for all the samples in the 6-well plate.
    Clean the vacuum filter holder and the forceps with 70% ethanol between every sample, or at least between the samples of different conditions.
15. Incubate the 6-well plate(s) for 20-30 minutes in a -80 °C freezer.
16. Place the 6-well plate back on the cold (-80 °C) metal pan.
17. Transfer the suspensions to an Eppendorf® microcentrifuge tube and place on dry ice.
    a. Flip the membrane to cells up. Pipet up and down 10-15 times to remove the cell debris from the membrane, rotating the filter.
    b. Transfer the suspension to an Eppendorf® microcentrifuge tube. Lift the filter to be able to remove ALL the liquid.
    c. *Clean the tweezers between samples.*
18. Centrifuge at max speed (16,000 x g) for 10 min at 4 °C.
19. Transfer the supernatant to a glass vial (or new Eppendorf tube if using a speed vac later).
20. Dry the samples in an evaporator without heat or at 30 °C (Genevac or speedvac).
21. Store the dried extracts at -80 °C.

Useful reference:

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

(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).

Other supplies and reagents needed for this protocol

H2O:	W5-1	Fisher Scientific	Water; 1L (HPLC)
MeOH:	A456-1	Fisher Scientific	Methanol; 1L (Optima LC/MS)
ACN:	A955-1	Fisher Scientific	Acetonitrile; 1L (Optima LC/MS)

glass vials:	13-622-351 2SVWGK	FisherScientific	Chromacol™ GOLD-Grade Inert Glass Vials; Thermo
caps:	03-379-123 (PTFE,Silicone)	FisherScientific	9 mm autosampler vial screw thread caps