Extraction of metabolites from prokaryotic cells

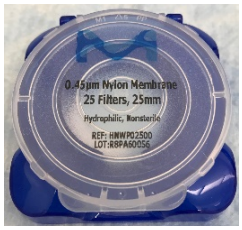
1. Determine cell numbers and culture volumes needed to be able to detect the metabolites by LC-MS (i.e., 1e8 – 1e9 cells)
2. Items needed:
   a. extraction solution **40:40:20 (MeOH:ACN:H2O)** by volumes; *pre-cooled to -20C*.
   (If particularly interested in ATP/ADP or NAD(P) redox factors, add 0.5% formic acid to the 40:40:20 solution. Also prepare 15% (w/v) ammonium bicarbonate (ABC) to neutralize the extract. *Ref: Lu et al, Antioxidants & redox signaling 28 (3), 2018*).

   b. **150 mM ammonium acetate solution, pH 7.3**[(1)] (room temperature) for washing

   c. Precool a **metal pan** *to -20C.*

   d. Have **cold packs** ready at -20C.

   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

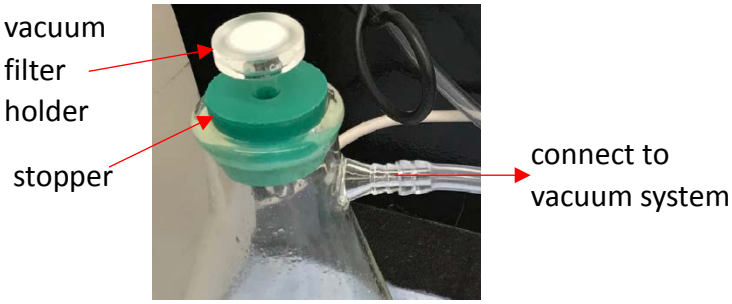
   

   f. Nylon filter membranes (0.45 um, 25 mm, hydrophilic; Millipore, HNWP02500)
   https://www.fishersci.com/shop/products/emd-millipore-nylon-hydrophilic-membrane-filters-4/hnwp02500?keyword=true [100/pack $86]

   g. Metal forceps

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

   i. 60 mm dish for separating the nylon membranes.
3. Set up a vacuum system as shown in the picture below.



4. Place as many membranes as needed in a 60 mm plate (removing the separation circles).
5. Clean the vacuum filter holder with 70% ethanol.
6. Turn the vacuum on and test the vacuum system by putting a membrane on the vacuum filter holder.
7. Place the pre-cooled metal pan upside down over the -20C cold packs to create a cold working surface.
8. Clean the vacuum filter holder with 70% ethanol.

9. Place a 6-well plate on the metal pan. Add a proper volume of extraction solution to cover each well (i.e. 0.4 – 1.0 ml). Use a separate tip for each aliquot (for more accurate measurements of the cold extraction solution).
10. Place a membrane on the filter holder.
11. Load an appropriate volume of cell culture. **Steps 10-12 have to be performed very quickly, within 10 seconds.**
12. Wash the cells on the membrane with 1 ml of the 150 mM NH4OAc solution (at room temperature).
13. Take the membrane with the cells and put it **upside down** into the well with extraction solution.

    ! If using acidic extraction buffer: *after 3 min,* add 8.7 ul 15% (w/v) ABC *per 100 ul 40:40:20:0.5* and swirl to neutralize the extract.
14. Incubate the 6-well plate(s) at -20 °C for (at least) 20 minutes.
15. Repeat steps 10-13 for all the samples (work in batches).
    Clean the vacuum filter holder and forceps with 70% ethanol between samples.
16. Place the 6-well plate back on the cold (-20 °C) metal pan. Flip the membranes to cells up. Remove the cell debris from the membrane by pipetting up and down 10-15 times, rotating the filter.
17. Transfer the entire content to an Eppendorf® microcentrifuge tube on ice. Lift the membrane in order to remove ALL the liquid.
18. Centrifuge at max speed (16,000 x g) for 10 mins at 4 °C.
19. Transfer the supernatant to a glass vial if drying the extracts in a Genevac or to a new Eppendorf® microcentrifuge tube if drying in a speed vac.
20. Dry the extracts down*.
21. Store at -80 °C until ready for LC-MS analysis.

*As an alternative to drying, if the metabolite concentration is high enough:

21-b. Transfer 50-100 ul to a glass HPLC vial for LC-MS analysis.

22-b. Store at -20 °C if analyzed by LC-MS within a few days. Store at -80 °C if longer storage is required.

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:

H2O:	W5-1	FisherScientific	Water; 1L (HPLC)
MeOH:	A456-1	FisherScientific	Methanol; 1L (Optima LC/MS)
ACN:	A955-1	FisherScientific	Acetonitrile; 1L (Optima LC/MS)
glass vials:	13-622-351	FisherScientific	1.8 mL Volume; Clear Glass, 12x32 mm, 9 mm thread
caps:	03-379-123	FisherScientific	Rubber/Silicone Septa