EMBL

EMBL Metabolomics Core Facility
(Heidelberg, Germany)

Protocol/MCF/SamplePrep/06: Simultaneous metabolite and lipid (biphasic) extraction from biological samples/ adherent mammalian cells

Aim: Biphasic Metabolite (small, polar molecules) and Lipid extraction from **biological (cells & microbial) samples** for LC-MS analysis

Sample requirements:
For cultured cells, use the equivalent of 1–3 million cells. For other sample types a minimum mass of 5-25 mg is required.

Materials:
High purity (MS-grade) methanol (MeOH), methyl tertiary-butyl ether (MTBE), water (H_2O), Internal standards (consult MCF staff)

Note: Keep diluents in chilled condition (0-4°C)

Procedure for metabolite extraction

- As far as possible, all preparation steps should be done on ice.
- Consider addition of antioxidants or usage of UV-absorbing tubes (consult MCF staff)
- This protocol is designed for an exemplary initial extraction volume (75% MeOH) total of **1000 µL** but can be upscaled if necessary (consult MCF staff)
- Use tube size that is sufficient for your volume. For the exemplary initial/quenching volume of 1000 µL, 5 mL tubes are suggested (final volume of biphasic system: 4.125 mL).
- Blank control: prepare processed blank samples using the same procedure but without biological samples (use water instead).
- Internal standards (IS): Please add to the methanolic quenching solution (step 4) if you are aiming for a 2-step transfer of extraction solvent (step 6). If not, please add internal standards at step 7. You should receive information about instructions how and how much to add from MCF staff.

1. Take 250 µL spent growth medium and place it in Eppendorf tubes which already contain 750 µL very cold (- 80°C) MeOH. (Keep it in case you wish to analyze extracellular metabolites). [optional]
2. Aspirate the medium completely.
3. Pour 10mL (adjust volume as per the size of dish/ plate) of 10 mM Ammonium acetate or compatible washing buffer (e.g. PBS) all over the petri dish, wash cells carefully and gently and then discard the washing solution.
4. Prepare a 75% MeOH stock (according to sample number +5-10% overage)
   Put the plates on dry ice and add 1 mL (or less if you are planning to do an additional extraction/solvent transfer → step 6) of the 75% (vol/vol) methanol + IS stock (cooled to - 20/-80°C or on dry ice or liquid nitrogen) or MeOH:ACN:H_2O; 4:4:2 (on ice or

-20°C). Adjust volume as per the size of dish/plate. Incubate the plate at -20/-80 °C for 20 min.

5. Scrape the plates on dry ice with a cell scraper.
6. Transfer the cell lysate/methanol mixture to a 5 mL tube. Try to do the transfer as complete as possible. If you notice residual cells on the plate, introduce an additional extraction/transfer step (e.g. with another 500 µL 75% MeOH aliquot without internal standards) to increase the yield and reduce inter-sample variability. Adjust/rescale the volumes for MTBE and H_2O in the subsequent steps.
7. (Alternative option for Internal Standards - see “Procedure for metabolite extraction” : Add appropriate internal standards (if you didn't already add them at step 4))
8. Vortex for 5 min at maximum speed and make sure that pellet disintegrates and mixed thoroughly with extraction solvent.
9. Sample lysis and homogenization (discuss details and alternatives with MCF members). Simplest procedure: place the samples in an ultrasonication bath and sonicate the sample tube for 5 min (Note: Put some ice in sonicator water bath to avoid heating of sample during sonication; rotate/change position of samples during sonication as the energy is not homogeneously distributed in most ultrasonication baths) and vortex briefly after sonication.
10. Add 2500 µL of cold MTBE (2.5x the amount of initial extraction/quenching volume)
11. Vortex and mix thoroughly
12. Incubate on ice or at -20°C for 20 min
13. Add 625 µL of cold H_2O (0.625x the amount of initial extraction/quenching volume)
14. Vortex and mix thoroughly
15. Incubate on ice or at -20°C for 20 min
16. Centrifuge at 14.000 × g for 10 - 15 min at 4°C
17. You should have a biphasic solvent system now, the ***upper phase*** is the ***organic phase (for lipidomics)***, the **lower phase** is the **aqueous phase (for metabolomics)**.
18. Transfer the upper phase to a labelled tube (lipidomics/organic etc.). Choose a uniform transfer volume per sample if possible or transfer as much volume of this phase as possible and avoid mixing with the lower phase or interphase (middle-phase). This phase can be tricky to pipette and may require pre-equilibration of the pipette tip. 1.5 mL tubes are preferred for transfering if possible.
19. Transfer the lower phase to a labelled tube (metabolomics/aqueous etc.). Choose a uniform transfer volume per sample if possible or transfer as much volume of this phase as possible and avoid mixing with the upper phase, interphase (middle-phase) or bottom phase/pellet (precipitated proteins / residuals). When poking through the upper/interphase, make sure to press a little air through your pipette to remove residuals of these phases in the pipette tip before aspirating the lower phase. 1.5 mL tubes are preferred for transfering if possible.
20. Dry the separated phases under a stream of nitrogen. The aqueous phase will take significantly longer to dry. When using SpeedVacs, please pretest the feasibility of programs as the organic phase is prone to bumping/boiling.
21. Reconstitute the upper phase (lipidomics) in 100 µL IPA:H2O (90:10, v:v)

22. Leave the dried lower phase in dried state without reconstitution
23. Ship samples on dry ice for analysis.

Ref:
https://doi.org/10.1194/jlr.D700041-JLR200
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