Processing of Cell Culture Samples for Metabolomics Analyses
By Felix Grun, PhD
Adapted by Emi Embler, PhD

There are three basic goals in preparation of samples for metabolomics analyses:

1) Stop metabolism
2) Deproteinate/desalt samples
3) Selectively enrich analytes

Stopping metabolism can be accomplished through several methods, e.g heating (>90 oC; denaturation of enzymes), low pH (addition of acid; pH 2-4) or removal of enzymes (e.g. swing extractions into another solvent).

Deproteination can be accomplished by precipitation of intact proteins with organic solvents, e.g. 5 volumes methanol (MeOH) or acetonitrile (MeCN) followed by centrifugation. This normally results in > 95% removal. Alternatively centrifugal filtration through MW cutoff spin filters (MW3000 or 10000) will remove large proteins but retains metabolites and smaller peptides. Lastly, samples could also be diluted to reduce the amount of protein in the sample.

Non-volatile salts (NaCl or PBS) are not helpful. They will contaminate the inlet source and cause ion suppression reducing sensitivity. The goal is to reduce salts to levels (<10 mM) that are more compatible with MS. Serum, tissue culture media contains >100 mM NaCl. Can remove salts by swing extractions into non-miscible organic solvents (no salts transferred) or dilution with water (10-50 fold).

To process and prep tissue culture cells for MS analyses you can use the following two approaches.

Protocol 1: For adherent cells (e.g. cells grown on 10 cm plates)

The key to obtaining good MS data is to **work QUICKLY and consistently** so that the time taken to get samples prepped is kept to an ABSOLUTE minimum. (Metabolite levels can change on the order of seconds to minutes after disrupting the cells or taken cells out of the incubator/medium !!)

1. Prepare 3 replicate plates for each condition you are testing. I.e. positive control x 3, neg control x 3 and your experiment x 3. Each plate should end up with about $3x10^6$ cells.
2. Treat the SW480 cells with 5mM ibuprofen for 24 hours.
3. Vacuum aspirate (remove the media) from the plates.
4. Wash once with 10 mL PBS to remove serum (proteins). Tilt the plate and aspirate as you are GENTLTY rinsing from the top down.
5. Wash once with 10 mL d.H_2O to remove PBS (salt). You must be quick (5 secs max) so again tilt the plate, rinse from the top and aspirate at the same time.

6. Add 1 ml of ice cold 80 % methanol:20 % dH_20 + internal standard (IS) to each plate.
7. Evenly coat and agitate for 2 mins. Scrape the cells off of the plates.
8. Transfer 1 ml sample + cells into microfuge tubes.
9. Add 300 µL chloroform to the cell suspension. Vortex for 30 secs vigorously.
10. Centrifuge at 14000 rpm for 2 mins at 4 ^{o}C to separate phases. The bottom phase will be chloroform:methanol layer (lipids); the top phase will be water:methanol (polar metabolites); proteins/DNA will form the precipitate located in the middle.
11. Carefully and accurately transfer 300 µl of bottom layer (chloroform:methanol) to a new microfuge tube. These will be your non-polar (NP) metabolite fractions. (Note: You will need to pipette some clean chloroform:methanol several times first to equilibrate the vapor pressure in the pipette tip, otherwise the sample will drip and you will not transfer an accurate amount). **It is critical that you transfer a consistent volume for all your samples!**
12. Transfer 300 µL of the top aqueous layer (polar metabolites) to another new microfuge tube. These will be your polar (P) fractions. Be careful not to transfer any of the gunky precipitate to the new tube. **It is critical that you transfer a consistent volume for all your samples!**
13. Place sample tubes in a Speedvac to evaporate the samples to dryness.
14. Resuspend NP fractions with 200 µL 100 % MeOH by pipetting up/down multiple times to dissolve any pellet.
15. Resuspend P fractions with 200 µL 20 % MeOH:80 % d.H2O by pipetting up/down multiple times to dissolve any pellet.
16. Centrifuge tubes for 2 mins at 14,000 rpm.
17. Transfer samples to MS low volume insert vials for MS analysis. Analyse on Waters Quattro Premier triplequad for targeted quantitation of analytes or Waters Xevo QTOF MS instrument for untargeted metabolomics.
18. Data are processed using MassLynx/QuanLynx software for targeted analysis and Progenesis QI/MetaboAnalyst software for untargeted analysis.

Do a literature search looking for quantitative methods for your specific analytes (use LC-MSMS as a search term). Copy down all the method details but specifically parent ion m/z, daughter ion m/z and cone voltage (CV) and collision energy (CE) parameters.