University of Glasgow | College of Medical, Veterinary & Life Sciences

Glasgow Polyomics

Metabolomics Service Protocol
Sample Preparation for Cells

1 Aim

In all cases described below, one should aim to consistently obtain a final cell or tissue pellet of **approximately 5µL**. The metabolite concentration of a sample depends on the total volume of the material, rather than on cell number as increasing cytosol volumes give increasing metabolite amounts.

A 5µL pellet of cells would typically give us enough material to detect several hundred metabolites. It is possible to work with (much) less, but the sensitivity will decrease in concert with the decrease in metabolite concentration.

To prepare the sample:

- For ***eukaryotic cells***: many eukaryotic cells produce a 5µL pellet with around $8x10^7$ cells for each sample. It's worth pelleting from a range of flask sizes initially to optimise the number of cells required for a 5µL pellet. Consistent volumes are more important than obtaining 5µL exactly. Bear in mind that drug treatments, mutations or other conditions may affect cell size, and therefore the number of cells required for consistent sample extraction.
- For ***bacterial cells***: if you are growing them in large numbers and have a pellet of more than ~5µL, simply increase the extraction volume to suit - as long as a 5:200µL dilution factor is maintained, it won't be an issue for the analysis.

2 Quality Control Notes

Important notes to consider before applying this protocol are:

- Make a pooled sample containing approximately 5–10µL for each sample, to be used as a quality control sample in the LC–MS procedure. Do this either by mixing aliquots from the samples (*e.g.* cell suspension) prior to extaction, or by taking an aliquot from each sample following the extraction. This sample is also useful for metabolite identification. Provide sufficient sample for multiple analyses: > 500µL is preferred. **Supplying a pooled standard is mandatory.**
- Cell density and growth conditions have significant effects on metabolism. For best comparison, grow all replicate cell cultures under identical conditions (*i.e.* seed the same *C* cells/mL in the same *M* mL of medium for the same *D* days before performing the sample preparation). For rigorous biological replicates, use a different passage of cells for each replicate.
- Due to the nature of mass spectrometry, it is not accurate to normalize data *post hoc*. Therefore please ensure that all samples have the same biomass (*e.g.* by cell count), and adjust if necessary during sample preparation by appropriate dilution.

2 Quality Control Notes (Cont'd)

- Always take additional samples of the extraction solvent to allow removal of contaminants at the data-analysis stage. One blank sample only is needed per batch, but please ensure there is a substantial amount (> 200µL) of solvent.
- If you are performing analysis of spent medium, take additional samples of fresh growth medium (5µL) and extract with solvent (see protocol steps 5–9), to allow comparison of medium components/contaminants at the data-analysis stage.
- Biological replicates are essential for data analysis (a minimum of 3 replicates, but a recommendation of 6 replicates, per biological group for cultured cells or organisms).

3 Materials

In addition to standard laboratory materials, this protocol requires:

- Temperature-controlled centrifuge.
- Temperature-controlled mixer such as a Thermomixer®.
- Dry Ice/Ethanol bath.
- Chloroform/Methanol/Water (1:3:1 ratio) mix.
- Screw-capped vials and Dry Ice, for transportation of samples.

4 Method

1. **Set both the centrifuge and the mixer to 4°C.**

   If you don't have a Thermomixer®, you can tape a tube-rack to a vortex mixer and set it going in the cool room.

2. **Quench by rapidly cooling cells to 4°C by submersion of tube in a Dry Ice/Ethanol bath.**

   Mix vigorously during cooling to avoid freezing and possible cell lysis.

3. **Centrifuge for 10 minutes at 1000g at 4°C and remove supernatant to approximately 1mL.**

   Keep 5µL supernatant if analysis of the spent medium is required.

4. **Re-suspend pellet and transfer to 1.5mL Eppendorf® tube.**

5. **Centrifuge for 5 minutes at 2500g at 4°C and remove supernatant completely.**

6. **Suspend pellet in 200µL of Chloroform/Methanol/Water (1:3:1 ratio) at 4°C.**

7. **For solvent lysable eukaryotic cells, mix vigorously with pipette to break up the pellet, followed by rocking for 1 hour at 4°C. If cells are tough or resistant to lysis (such as yeast or bacteria), disrupt the tissue to lyse the cells by sonication, grinding with etched glass beads, homogenisation or use of a French press, then vortex on cooled (4°C) mixer for 1 hour.**

8. **Centrifuge for 3 minutes at 13,000g at 4°C.**

9. **Take supernatant (180µL) and store at -80°C until analysis by LC–MS.**

   ⚠ If you are interested in oxidative effects, it is recommended to store the sample under argon, although this is usually not sufficient for true quantitation of cellular oxidation state.

10. **If shipping is required, please place samples in screw-capped vials and use dry ice for transport.**

END OF METHOD

5 Variations

Method variations that may be applicable depending on the specific needs of your project include:

5.1 Cold Quenching

Rationale

It is generally expected that you should rapidly cool cells to 4°C (by submersion of tube in a Dry Ice/Ethanol bath) before centrifuging. However, the advantage of slowing metabolism during processing may be somewhat offset by the extra stress and extra time required for handling.

Variation relative to the standard method

1.	As previously defined
2.	*[Step omitted]*
3-10.	As previously defined

5.2 Washing

Washing steps (with CBSS) may be added if you are looking at metabolites that are present in your growth medium, however generally this means more handling (therefore more unwanted changes in metabolome) and may also wash out some metabolites of interest.

5.3 Cell Concentration and Re-suspension

After Step 3 you could re-suspend the cell pellet in a small volume of growth medium before the extraction step (4). This is required for short sampling time-frames (seconds-minutes), and may improve reproducibility of extraction (hypothesis not tested), but trypanosomes at this high cell density are highly stressed and the metabolome will change rapidly. Furthermore, this will add more salts and medium components to your final sample.

5.4 Hot Ethanol Extraction Method

Rationale

This appears to extract some metabolites that at not extracted using cold chloroform/methanol, however degradation of other metabolites (esp. phosphates) is a problem. Currently recommended only if the chloroform/methanol method is not giving suitable results.

Variation relative to the standard method

1-5.	As previously defined
6.	Re-suspend pellet in 200uL hot (80^{o}C) 80% ethanol in water and leave on heat for 2 minutes.
7.	Rapidly cool tubes on ice for 5 minutes, then centrifuge for 2 minutes at 13,000g.
8-10.	As previously defined

6 Troubleshooting

If you are having difficulty, or anticipate that you are likely to have difficulty, with obtaining the appropriate sized pellet, please get in touch with our Metabolomics Service Manager by email to gp-metabolomics@glasgow.ac.uk, and we can discuss some modifications to the extraction that might help.