Guide to cell extraction, sample normalisation and sample submission for metabolomics

Overview
The following document provides a protocol for metabolite extraction from cells grown under tissue culture conditions. The sample submission form in Part II at the end of this document should be completed and emailed to Elisabete Pires (elisabete.pires@chem.ox.ac.uk) or John Walsby-Tickle (john.walsby-tickle@chem.ox.ac.uk) when submitting samples for analysis.

Analytical methods
There are three separate LC-MS methods available for metabolite. Method 1 and 2 are run as standard, method 3 is optional at present.

LC/MS method 1: 'IC-MS method': Anionic and highly polar compounds (anion-exchange chromatography coupled to mass spectrometry). Metabolic pathways covered include glycolysis, TCA cycle, Pentose phosphate pathway, other carbohydrates, nucleic acids and a wide range of organic acids.

LC/MS method 2: 'C18 method': Hydrophobic low and medium polar compounds (Reversed phase chromatography coupled to mass spectrometry).

LC/MS method 3: 'Derivatised C18 method': Amino acids, primary and secondary amines. (Reversed phase chromatography coupled to mass spectrometry).

Metabolite coverage: Please email to check with us if you want to know whether a particular metabolite of interest is covered by our methods.

Cost of analysis
The cost for collaborative metabolomics analysis is £83 per sample. Currently we focus on the metabolome represented by polar and ionic metabolites. A lipidomics method is being established but currently not available). Targeted methods (i.e. amino acid analysis or TCA cycle metabolite analysis are available at a cost of £48 per sample). All methods provide relative quantitation as standard; absolute quantification is also possible but requires discussion in advance.

Data analysis and results
The time from sample submission to analysis is approximately 3-4 weeks and we aim to get initial result back to you within 1 week after analysis. We will schedule samples for analysis once received along with a complete sample submission form (end of this protocol). If the project leads to a publication we will work closely to tailor the results and provide appropriate figures, graphs and tables for publication. Technical difficulties are not uncommon for hyphenated, high-resolution mass spectrometry and although we are not unfamiliar with these issues, it can lead to delays in sample analysis and project turnaround time. It is worth enquiring if you would like an update on the progress of a particular project.

Metabolite extraction protocols
The extraction protocol below provides high metabolite extraction coverage with bias towards more polar molecules. For targeting more lipophilic compounds specifically please contact us directly as a different extraction procedure may be more suitable.

Growing cells
For the analysis of cell extracts from tissue culture we recommend a minimum of 5 parallel biological replicates per experimental condition. For example in an experiment to compare the effect of hypoxia and normoxia on cell metabolism we would recommend growing 5 (or more) parallel flasks of the same cells in normoxia and 5 parallel flasks in hypoxia. We will run fewer replicates only by prior arrangement so please contact us in advance or discuss with us when planning experiments. Where the analysis is targeted to a particular metabolite and the magnitude of the change in abundance is expected to be significant, then a single replicate can be run as a pilot (please discuss this in advance).

How many cells required?
For cell samples we recommend a minimum of **1 million cells** (roughly a confluent 6cm dish, a T25 cell culture flask can also be used but dishes are easier to scrape, see protocol below) however, cell size and metabolite abundance can differ across cell types and larger dishes/flasks can be used to increase compound coverage. (We use confluent 6 or 10cm dishes and 9 replicates per experimental group for most internal metabolomics experiments for example.)

Normalisation
It is important to normalise samples. This can be done as part of the sample preparation process or after analysis of the samples, each approach has pros and cons). Recording and normalising to the same number of cells is the most common approach however this is not possible using our extraction method (which has other benefits). We suggest recording total DNA content for which we have a separate protocol using a nanodrop™ system or equivalent (please ask if you would like the protocol). Currently we are asking for the total DNA value to be recorded per sample which we will use to normalise the data once samples are analysed.

Media
Certain metabolites can be excreted from cells and it can be useful to identify changes in media metabolites levels at the same time as measuring metabolite levels from cell extracts. We recommend considering the submission of media taken from cell samples in addition to cell extracts. The media can be submitted without any dilution. Make sure you also submit an example of your fresh media for comparison.

Protocol for the extraction of metabolites from cells grown in tissue culture[1]

1. Prepare the required volume of ice cold methanol (MS grade if possible, otherwise HPLC grade) solution with milli-Q water.

2. Remove cells from the incubator and pour off the media from a confluent 6cm dish or T25 flask or similar of cells. Label and keep 1mL of media from each dish in a sealed tube if analysis of media is required.

3. Quickly add 5mL of PBS buffer at 37°C, swirl and pour off to waste to remove excess media. Repeat once more.

4. Snap freeze cells by adding ~ 10 mL of liquid nitrogen, enough to cover all cells (note this is an optional step – it is the quickest and most effective way to arrest metabolism, however, it is also common to simply add the ice cold MeOH directly (step 5) to the cells without the prior addition of liquid nitrogen).

5. Add 500uL of methanol to cover the cells in the dish (500uL per 10cm dish or less volume can be used with a smaller dish). Do this before all the liquid nitrogen has boiled away if using the liquid nitrogen (step 4) (probably less than 2 mins). Swirl gently making sure all the cells are covered by the methanol, do this for at least 3 minutes.

6. Note: from here on the process is less time critical as metabolism has been arrested, metabolites have been extracted into the methanol solution and enzymes largely denatured.

7. Use an appropriate cell scraper to remove adhered cells material into suspension. Make sure this is done thoroughly as no trypsin step is used so adherent cells need to be mechanically displaced. The aim is to remove >90% of cells material and take into methanol suspension.

8. On ice: Pipette the resulting lysate solution including cell debris into a 1.5mL Eppendorf tube (or equivalent for centrifugation). It is useful to draw the solvent in the cell dish up and down several times to ensure cells are suspended in solution and no left behind.

9. Check that all cells have been removed from the dish using a microscope. If >10% of cells are still adhered, pipette the methanol suspension back into the dish and re-scrape. It's important to get at least 90% of cells into suspension in the Eppendorf tube to ensure accurate results.

10. Keep the Eppendorf tube ice cold and spin down the mixture in a centrifuge at max rpm (>13,000 rpm) for 30mins (ideally a cooled centrifuge but this is not essential).

11. **Measure the DNA context of the supernatant:** Use our separate **DNA normalisation protocol** to perform this step. This can be found on page 6 (see below).

12. If there is a >20% variability in the DNA context across the samples the next step is to normalise each sample by adding a volume of MeOH to each sample so that the total volume of each sample is proportional to the amount of DNA present (total protein is sometimes used for this step and is optional but be have found DNA to be more accurate) (If there is <20% variability this step can be ignored and move on to step 13 below).

    How to normalise samples: For example if you have 3 samples and you measure the total DNA content for each as 100ng/µL, 75ng/µL and 50ng/µL respectively. You should normalise to the 50 ng/µL sample by diluting the others with MeOH to bring them to the same concentration (I.e. double the volume of the first sample (100ng/50ng/uL) and add a third of the volume of the 75ng/µL sample)**.

    Note: The DNA content for each sample is expected to be in the region of 50-100 ng/uL (from the extraction of a T25 flask or 10cm tissue culture dish of cells (~1-2 million cells). If you are getting DNA values <30 ng/uL the cell extraction efficiency or original starting number of cells is too low and metabolite coverage will be poor. In this case you might want to consider repeating the experiment.

    *Note: **Don't normalise to samples with DNA content <30ng/uL.** If the sample with the lowest total DNA content is <**30ng/uL** or you have a few outliers near this value with the lowest DNA content – ignore these samples and normalise to the next highest total DNA (or lowest in the more representative group in case of clear outliers. **Please report the total DNA content (or protein content or other normalisation measure used) for each sample on the sample submission form**.*

13. 500uL of the normalised sample should next be filtered using a 10 kD molecular weight cut-off filter* (10 kD MWCO). This removes soluble protein from the metabolite solution (Amicon Ultra -0.5mL centrifugal filters UFC501024 recommended). Make sure you follow filter preparation instructions to remove glycerol (washing with milliQ water prior to use for at least 30mins in centrifuge).

14. Transfer the filtered and normalised sample extract into a *Waters Total Recovery autosampler vial**** with pre-slit ptfe cap. **Please make sure this vial type is used**.

15. Put the sample in a vial in a -80°C freezer until the day of MS analysis. Ensure the sample vial is stored upright in a suitable tray to avoid loss as samples can remain in liquid state. Avoid freeze-thaw cycles. Transport on dry ice to the MS lab when submitting samples.

16. The sample is ready for LC-MS/MS analysis using method 1 or 2 (see page 1). For method 3 (amino acids) an additional derivatisation step is required which can be performed in the McCullagh lab when you deliver the samples (please arrange this with Elisabete or Kourosh prior to sample delivery).

17. **Finally a quality control (QC)** sample should be made to monitor analytical reproducibility (do this before freezing all the samples for the first time). A QC sample contains an equal volume from each sample combined into a single vial. To make a QC sample add together an equal volume of each sample into a fresh total

recovery vial to make at least 250 uL in total. The volume taken from each sample depends on the number of samples you have but typically this would be 10-20uL per sample. Label this 'QC' and submit along with your other samples.

18. **Optional:** You may wish to also keep the **media** removed from each cell sample prior to metabolite extraction and submit for analysis along aside the cell extracts. This can be interesting as it allows changes in the use or excretion of metabolites to be monitored which can be useful depending on the metabolic system and experimental aims. Comparing fresh and spent media enables a comparison of what compounds the cells have utilised and excreted. All media samples must be normalised by the same ratio as the cells from each cell dish (I.e. according to the step 10) and filtered using a 10 kD MWCO filter as for cells above.

19. **Submitting samples for analysis:** Once you have prepared your samples please contact the researcher in the McCullagh lab who you are working with on the project (usually Elisabete Pires or David Hauton) and arrange to deliver the samples and to make up any additional samples (for example derivatisation for amino acid normalisation) and create the unique sample sequence for your samples. These will all be done in the McCullagh lab. Next please complete the sample submission form below and submit, by email as well as paper copy at the time you bring your samples to the MS lab.

[1]**Note:** For this protocol no cell counting is performed on flask harvested for MS analysis but additional flasks of cells should be gown and harvested *for each experimental condition* in order to determine the number of cells present. We make the fundamental assumption that there are a similar number of cells in the same flasks from the same experimental conditions. We recommend using a nanodrop or similar to determine the amount of DNA in each sample after cell lysis and normalise samples using methanol accordingly. Please record the normalisation factor you calculated and supply this in a separate excel sheet along with the completed sample submission form.

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*MILLIPORE AMICON Ultra molecular weight cut-off filters

Millipore Amicon Ultra -0.5mL centrifugal filters UFC501024 are recommended either 3kD or 10kD cut-off. If you are unable to obtain these they are available from the MS facility at a cost of £2.40 per filter.

** The purpose of the normalisation step is ensure that the abundances measured by mass spectrometry for a particular metabolite relate to the same cell content to ensure any differences observed between samples or experimental group is likely due to differences in metabolism rather than differences in the number of cells present or differential effects resulting from the extraction protocol. We have shown that relying on the same number of cells present in flasks at the start of the extraction protocol is not as accurate as careful normalisation based on DNA content measurements described here. However if this normalisation step described is not possible in your lab or you are not comfortable with this step you can submit sample without normalisation and rely on counting the number of cells in each flask to be roughly the same. In our experience however this can be more variable and lowers the power of your experiments.

***Autosampler vials appropriate for analysis

It is essential that samples are prepared for analysis in the right type of autosampler vial. Waters Total Recovery (part number: 186000385C. If you are unable to obtain these they are available from the MS facility at a cost of £1.50 per vial.

Figure 1: Waters Total Recovery Vial

Cell DNA content analysis for normalisation prior to targeted and untargeted metabolomics.

Overview

The following normalisation protocol is for cells and tissues based on the analysis of DNA content. For any questions about how to perform this protocol please contact Elisabete Pires elisabete.pires@chem.oxa.c.uk or David Hauton david.hauton@chem.ox.ac.uk in the McCullagh lab.

Normalisation

It is important to normalise samples. Recording the same number of cells is the most common approach however this is not easy to do using our optimised metabolite extraction protocol. Instead we recommend evaluation and comparison of total DNA content to determine a normalisation factor which is then applied later in the analysis.

Evaluation of DNA content and normalisation using a NanoDrop 1000

1. **Operation**

   A 10 ul sample is pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap is controlled to both 1mm and 0.2 mm paths. A pulsed xenon flash lamp provides the light source and a spectrometer utilizing a linear CCD array is used to analyze the light after passing through the sample. The instrument is controlled by PC based software, and the data is logged in an archive file on the PC.

2. **Applications**

   The small sample requirement and ease of use make the NanoDrop 1000 Spectrophotometer ideally suited for measuring:

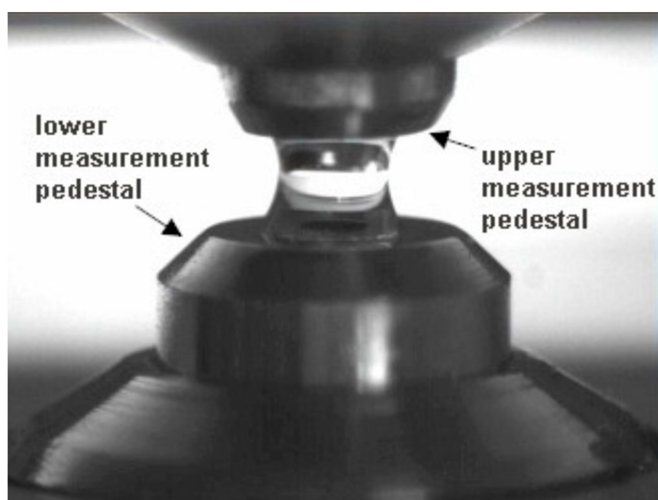
- Nucleic acid concentration and purity of nucleic acid samples up to 3700 ng/ul (dsDNA) without dilution
- Fluorescent dye labelling density of nucleic acid microarray samples
- Purified protein analysis (A280) up to 100 mg/ml (BSA)
- Expanded spectrum measurement and quantitation of fluorescent dye labelled proteins, conjugates, and metalloproteins
- Bradford Assay analysis of protein
- BCA Assay analysis of protein
- Lowry Assay analysis of protein
- Pierce Protein 660 nm Protein Assay

3. General Operation

Basic Use

The main steps for using the sample retention system are listed below:

A. With the sampling arm open, pipette the sample onto the lower measurement pedestal.



B. Close the sampling arm and initiate a spectral measurement using the operating software on the PC. The sample column is automatically drawn between the upper and lower measurement pedestals and the spectral measurement made.

C. When the measurement is complete, open the sampling arm and wipe the sample from both the upper and lower pedestals using a soft laboratory wipe. Simple wiping prevents sample carryover in successive measurements for samples varying by more than 1000 fold in concentration.

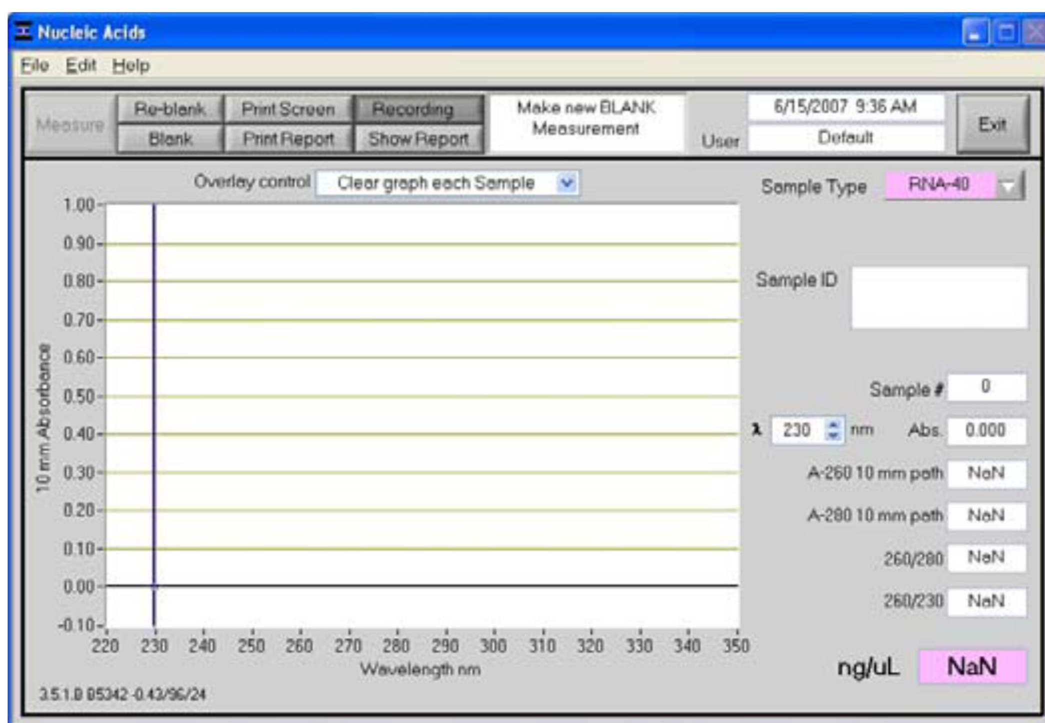
4. Sample measurement

Before making a sample measurement, a blank must be measured and stored (see "Blanking and Absorbance Calculations" in the appendix for more details on absorbance calculations). After making an initial blank measurement, a straight line will appear on the screen; subsequent blanks will clear any sample spectrum and display a straight line as shown in the following image:

Measurement Concentration Range

The NanoDrop 1000 Spectrophotometer will accurately measure dsDNA samples up to 3700 ng/ul without dilution. To do this, the instrument automatically detects the high concentration and utilizes the 0.2mm pathlength to calculate the absorbance.

Detection Limit (ng/ul)	Approx. Upper Limit (ng/ul)	Typical Reproducibility (minimum 5 replicates) (SD= ng/ul; CV= %)
2	3700 ng/ul (dsDNA) 3000 (RNA) 2400 (ssDNA)	sample range 2-100 ng/ul: ± 2 ng/ul sample range >100 ng/ul: ± 2%


Nucleic Acids
File Edit Help
Measure
Re-blank
Blank
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Print Report
Recording
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User
6/15/2007 9:36 AM
Default
Exit
Overlay control
Clear graph each Sample
Sample Type
RNA-40
Sample ID
Sample #
0
230 nm
Abs.
0.000
A-260 10 mm path
NaN
A-280 10 mm path
NaN
260/280
NaN
260/230
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ng/uL
NaN
10 mm Absorbance
Wavelength nm
3.5.1.8 B5342-0.43/96/24

Submission form: Metabolic profiling

(Please complete this submission form electronically and send by email to: Elisabete Pires (elisabete.pires@chem.ox.ac.uk) or John Walsby-Tickle (john.walsby-tickle@chem.ox.ac.uk)

Full name of PI:
Full name of researcher submitting samples:
Date:
Department:
Researcher's Email:
Tel:
Total number of samples submitted:

Two sentence summary of the project:

What you would like to achieve from the metabolomics analysis (e.g. answer a specific hypothesis (targeted analysis), generate new hypotheses (untargeted analysis):

Type of study (e.g. longitudinal, pairwise or case control experimental design)?

Have samples been normalised during preparation (e.g. by weight, cell count, total DNA, total protein, other?) and if so please provide a copy of each sample's normalisation factor measurement in the form below or as a separate document:

<u>Costs:</u> The direct costs of untargeted metabolomics analyses on a collaborative basis is £83 per sample. Cost of quantitative targeted analysis for 10 or more samples and a single target analyte is £48 per sample and £250 per additional targeted analyte.

Please complete the following form and submit with your samples.

Sample number	Up to 6 letter /number unique sample vial identifier clearly labelled on the sample vial.	Experimental group the sample belongs to:	DNA concentration (or cell count or tissue weight protein content).	Confirm sample volume supplied is >300uL and a normalised volume.
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Please expand table for samples and necessary.

*Note the design of successful metabolomics experiments is almost always based on one of two approaches:

1) A binary comparison of a control group versus a treated group. For example: untreated versus drug treated cells or wild type versus mutant cells.

2) Alternatively a time course experiment such as treatment of a cell line with and without a drug over a period of time.