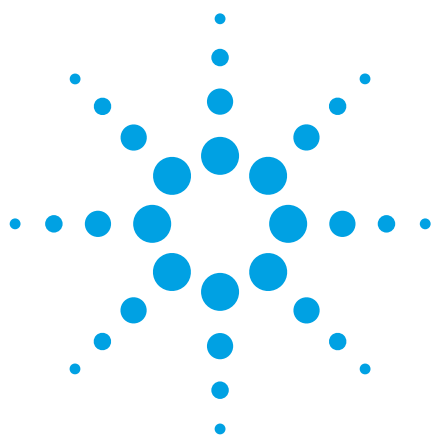




Multi-Omics Compatible Protocols for Preparation and Extraction of Biological Samples for Wide Coverage in Untargeted Metabolomics Experiments

Technical Overview

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Metabolomics, the global study of an organism's metabolites, is a comparative science and relies heavily on statistical comparisons. Thus, successful metabolomics studies require strict consistency from sample collection, to preparation, to analysis. Without this consistency, experimental error will be large and results unreliable. For example, circadian rhythm can affect the contents of urine, plasma, and saliva [1], thus samples are best collected at the same time of day for greatest consistency and highest chance of success. Furthermore, the metabolome changes rapidly *in vitro* and *in vivo*, much faster than the genome or proteome, necessitating prompt and quick sample treatment to quench metabolism [2].

The approach to metabolite extraction presented here is based upon methods developed by Folch [3] and Bligh-Dyer [4] in the 1950s, and extends the work of Sana and Fischer [5] to a variety of biological sample types. The result of this protocol is a water-methanol fraction containing water-soluble metabolites, a protein disc, and a chloroform fraction containing primarily lipids. The aqueous and chloroform fractions may be used for metabolomics and lipidomics studies, respectively. If desired, the protein disc may be resuspended and digested for bottom-up proteomics experiments, making this method a suitable choice to prepare samples for an integrated biology workflow.

Depending on the type of biological sample, tissue, serum, or cells, different preliminary sample preparation is needed, as described below. Certain sample types, particularly those with rigid cell walls or membranes, require more involved preparation, and in some cases, specialized equipment.

Liquid biological samples (serum, CSF, urine, and so forth): These biological fluids are presumed to be majority aqueous, and are used as the 0.8 parts water in the modified Bligh-Dyer extraction described later.

Serum and plasma: Serum and plasma are both complicated matrixes containing multiple classes of biomolecules spanning many orders of magnitude [2]. A Bligh-Dyer extraction is necessary to separate proteins and lipids from water-soluble metabolites.

CSF: While cerebrospinal fluid is predominantly aqueous [2], lipid content can change significantly as a function of many disease states [6,7]. Therefore, a Bligh-Dyer extraction provides the benefit of isolating proteins and a separate lipid fraction for analysis.



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Saliva: The glycoprotein content of saliva can lead to foaming of the sample during preparation. This is addressed simply with a 5x dilution with water prior to extraction. Saliva is not a rich source of lipids; however, they have been reported at a level of 1.3 mg/dL [8].

Urine: Urine has very little lipid content [2]. Thus the benefits of a Bligh-Dyer extraction are principally for co-purification of proteins and metabolites. Since standard protein quantitation methods do not work due to interfering substances, measuring osmolality is recommended, in addition to precipitation-based protein quantitation strategies (described below) [9]. For osmolality measures, immediately after collection, urine should be centrifuged for 30 seconds to sediment debris. Following the osmolality measurement, water should be added to normalize the samples, followed by the coldest possible methanol in a ratio of 2 parts:0.8 parts MeOH:H₂O to quench metabolism.

Tissue: Tissues should be perfused with ice-cold saline or PBS. Samples are snapfrozen in Eppendorf tubes in liquid nitrogen, and stored at -80 °C until processing. A 3-mm punch biopsy is recommended for tissue samples. For processing, frozen samples are weighed, then pulverized to a fine powder (freeze fracturing) with a mortar and pestle in liquid nitrogen. Following pulverization, ice-cold MeOH:H₂O (2 parts:0.8 parts, 1 mL total) is added to quench metabolism, and for samples with high connective tissue content, samples should be further ground using a Duall tissue grinder and liquid nitrogen. The sample is transferred to an Eppendorf tube, vortexed to mix, and pulse sonicated (3–5 pulses, 1 second/pulse each) on wet ice to prevent heat generation. A modified 3 pH Bligh-Dyer extraction is then performed as described below and illustrated on page 6.

Adherent cells (see specific sample prep protocol): Plates/wells are placed on wet ice to slow metabolism. Media is removed (can be saved for potential analysis), and cells are washed twice in ice-cold DPBS to remove excess media. Cells in dishes are placed on dry ice to freeze completely. Addition of the coldest possible MeOH:H₂O (2 parts:0.8 parts, typically 1 mL) further quenches metabolism and disrupts cells. The MeOH:H₂O should be more than ice cold. Ideally it should be -80 °C, or at the minimum -40 °C, with the use of a recirculating bath or dry ice slurry. Cells are scraped off the plate/well, and collected with the MeOH:H₂O into Eppendorf tubes for storage at -80 °C. Subsequently, samples are pulse sonicated, and subjected to a modified 3 pH Bligh-Dyer extraction, described below and illustrated on page 6.

Modified 3 pH Bligh-Dyer extraction (BDE): Sana and colleagues showed that the traditional BDE method could be significantly improved by facilitating solvent exchange of metabolites by adjusting the pH of the aqueous phase for extraction, thus performing three sequential extractions (water ~pH 7, water with 1% formic acid, and water with 2% ammonium hydroxide) [10]. However, this method obtains poor phase separation due to the relatively low ratios of chloroform:methanol:water in this method as compared to a traditional BDE (3:4:2 versus 1:2:0.8 for BDE) and thus requires a long chilling step and acetonitrile precipitation of proteins. A modified version of this protocol is presented here, so that initially metabolites are quenched in methanol:water (2:0.8 v/v) and subsequently 1 part chloroform is added, resulting in a ratio of 1:2:0.8 chloroform:methanol:water. An additional 1 part of chloroform and 1 part of water is added for a final ratio of chloroform:methanol:water of 2:2:1.8 to be consistent with a traditional BDE.

The organic phase is dried down and reextracted with the acidic solvent (stage 2 in flow chart) and finally with the basic solvent (stage 3 in flow chart). All aqueous phases are then dried down and re-suspended together in the LC/MS buffer A for analysis. While larger volumes require more time to dry down, the total volume used is not important. The ratios between solvents is critical to this protocol.

Normalization methods: Normalization by protein quantification is the most straight forward approach. Protein may be quantified either by setting aside an aliquot of sample before any chloroform is added or by using the protein disc after the modified Bligh-Dyer extraction if not used for proteomics experiments. Consider typical protein concentration ranges for your biological material when selecting a protein quantification method. We have used a 2D Quant Kit from GE Life Sciences. Other colorimetric assays are available. Amino acid hydrolysis is the most accurate method, but also the most costly.

One must also be aware of circumstances in which protein quantification does not provide good normalization. Urine is one such case [5]. Another example is an experiment in which cell growth is inhibited possibly leading to atypical protein quantities that do not reflect relative abundances of cellular metabolites. In these scenarios, cell counting or DNA quantification may serve as alternative approaches to normalization.

Internal standards: Internal standards serve as a quality check rather than as a means of absolute quantification. Internal standards should be included in the 2:0.8 MeOH/H₂O used at the beginning of the extraction procedure, whether MeOH is added to an aqueous fluid sample, or the MeOH/H₂O mixture is added to tissue or cell culture samples. It is recommended to use a few isotopically labeled standards, such as ²H or ¹³C, that represent a variety of metabolites, such as a free fatty acid, an amino acid, or a nucleotide. Verifying the presence of each of these during data analysis ensures that all of these metabolite classes were successfully extracted and detected.

Cultured Cell Samples

Like the tissues and biological fluids, consistency is equally important for cultured cell samples. For example, one must control cell-cycle phase when planning metabolomics experiments. Another consideration is the number of cells necessary. Analytically, metabolites from as few as 10⁶ cells can be measured, but at the analytical limit, one must consider whether the biology of interest is still being represented.

The media in which cells are grown can also be a rich source of metabolomic information. If one desires to study the exometabolome as well, a media blank must be set aside and saved prior to the addition of cells.

One of the most critical aspects of sample collection and preparation is preservation. Metabolism does occur during freeze-thaw cycles, and disproportionately affects different metabolic processes [2]. Freezing should be avoided until after metabolism has been quenched (by addition of organic solvent) or protein has been removed. One option is to quench by adding methanol, freeze the samples at -80 °C as methanol/water pellets, and then conduct all further sample preparation at -40 °C or lower. Samples should never approach room temperature, and tubes should be frozen in Eppendorf tubes wrapped with parafilm.

Cells-in-Suspension Sample Preparation

1. Aim for sufficient number of cells for each sample.
 - a. Cells equivalent to at least 1 mg of protein per sample are recommended.
 - b. Successful examples from adherent cell cultures:
 - i. 15×10^6 cells/sample at plating of primary hypothalamic neurons (100-mm dish), at minimum estimated 12×10^6 cells/sample at the time of harvest 10 days later (approximately 1 mg protein/sample).
 - ii. 14×10^6 cells/sample at plating of MCF-7 cancer cells (150-mm dish), up to 20×10^6 cells/sample at the time of harvest the next day (approximately 2 mg protein/sample).

2. At the time of collection/extraction, place flasks on wet ice to slow metabolism.

Maintain consistent timing of treatment and collection from sample to sample. This may require limiting the number of flasks sampled each time to the amount that can be centrifuged at one time.

3. Immediately cold-centrifuge cells (16,000 rpm, 5 minutes or longer as needed, 4 °C). Remove media.

The media can be saved for exometabolomics. Remember to also save a media blank that has never had cells in it. Cold-centrifuge media (16,000 g, 30 minutes or longer as needed, 4 °C), and collect debris-free supernatant. The media serves as the 0.8 parts water; add 2 parts MeOH to quench (2:0.8 MeOH:H₂O total), snap-freeze supernatant in tubes in liquid nitrogen, and store at -80 °C until ready to complete the Bligh-Dyer extraction.

4. Snap-freeze the cell pellet (and media, separately, if desired) in a tube in liquid nitrogen. Store at -80 °C.

For subsequent extraction, refer to the appropriate Comprehensive Metabolomics Extraction flow chart.

Adherent Cells Sample Preparation

1. Aim for sufficient number of cells for each sample.
 - a. Cells equivalent to at least 1 mg of protein per sample are recommended.
 - b. Successful examples:
 - i. 15×10^6 cells/sample at plating of primary hypothalamic neurons (100-mm dish), at minimum estimated 12×10^6 cells/sample at time of harvest 10 days later (approximately 1 mg protein/sample).
 - ii. 14×10^6 cells/sample at plating of MCF-7 cancer cells (150-mm dish), up to 20×10^6 cells/sample at time of harvest the next day (approximately 2 mg protein/sample).

2. At time of collection/extraction, place cells/dishes on wet ice to slow metabolism.

Maintain consistent timing of treatment and collection from sample to sample. This will likely require limiting the number of dishes sampled each time to that which can be done quickly. (Example: 2 dishes at a time, one from each of two treatment groups).

3. Collect as much media as possible, being consistent from sample to sample.
 - a. Example: Tilt cold dish 30°, collect the majority of media within 20 seconds, wait 30 seconds for additional media to drain to bottom of tilted dish, then collect remaining media.
 - b. The media can be saved for exometabolomics. Remember to also save a media blank that has never had cells in it. Cold-centrifuge media (16,000 rpm, 30 minutes or longer as needed, 4 °C) and collect debris-free supernatant. The media serves as the 0.8 parts water; add 2 parts MeOH to quench (2:0.8 MeOH:H₂O total), snap-freeze supernatant in tubes in liquid nitrogen, and store at –80 °C until ready to complete the Bligh-Dyer extraction.
4. Wash cells twice with ice-cold DPBS (Ca²⁺-free, Mg²⁺-free). Make washes consistent and complete.
5. Freeze cells/dishes on dry ice. Ensure all the cells are frozen.
6. Have available an adequate total volume of coldest possible MeOH:H₂O (2:0.8 mix) per sample.
 - a. Cells should never approach room temperature. MeOH:H₂O should be –80 °C, or at least –40 °C.
 - b. Successful examples (above) should be approximately 1 mL total volume of the MeOH:H₂O mixture, with internal standards, for each sample.
Example: 750 µL MeOH + 300 µL H₂O = 1,050 µL total volume MeOH:H₂O (2:0.8) per sample.
7. Add half of the MeOH:H₂O to a dish. Transfer the dish to wet ice. Scrape the cells off the dish. Collect cells and extract into a 2-mL (or other) Eppendorf tube on wet ice.

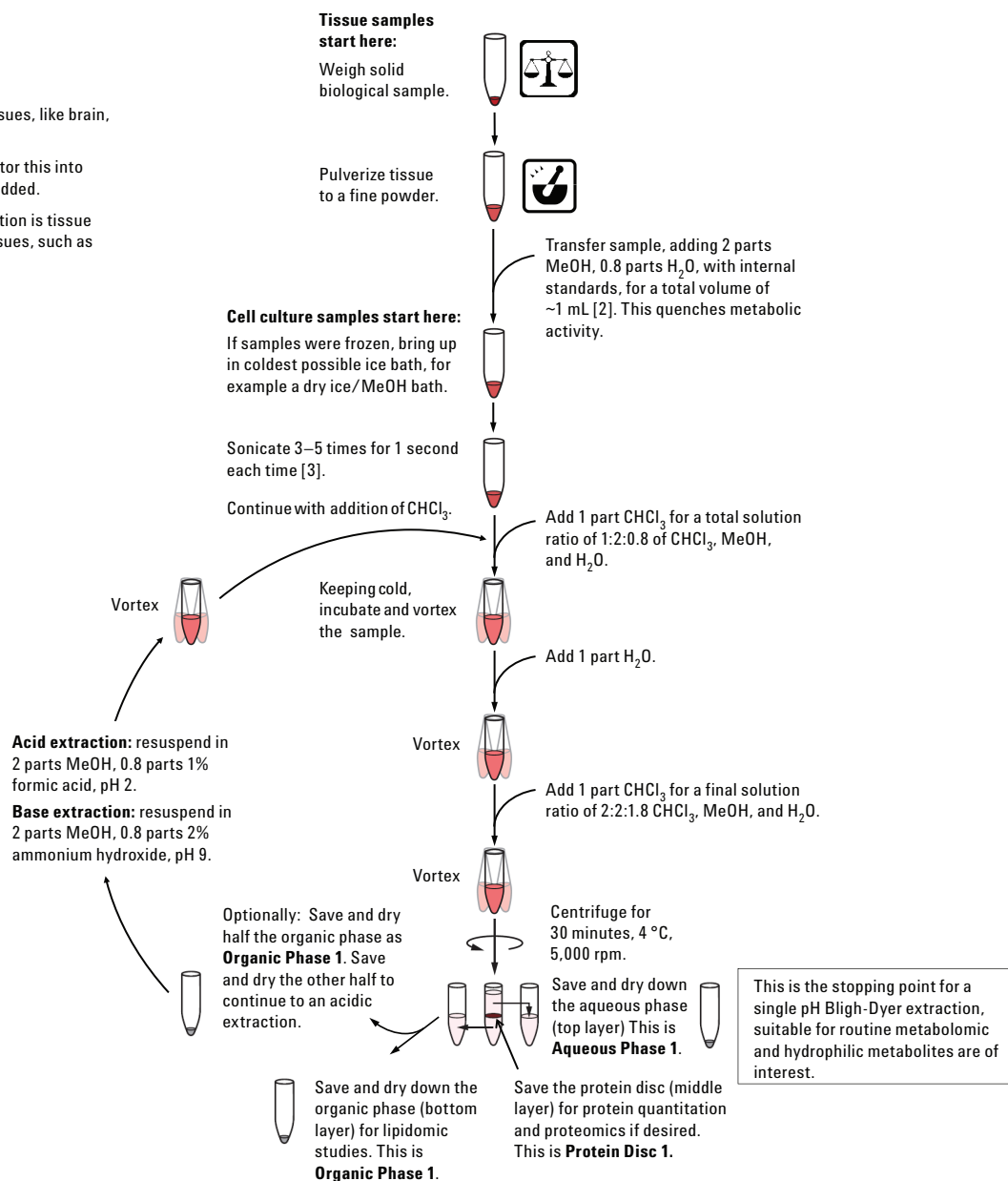
This step completes the freeze-thaw. Cold organic solvent helps to disrupt cells, quench metabolism, and begin the extraction (completed with appropriate Extraction protocol).
8. Repeat Step 7, collecting remaining cells and extract into the Eppendorf tube.
9. If not completely processing immediately, snap-freeze cells and extract in tubes in liquid nitrogen. Store at –80 °C.

For subsequent extraction, refer to the appropriate Comprehensive Metabolomics Extraction flow chart.

Modified 3-ph Bligh-Dyer extraction for comprehensive metabolomics of tissue and cell culture samples

Notes

1. Alternatively, for softer tissues, like brain, use tissue homogenizer.
2. If tissue is very watery, factor this into the total amount of water added.
3. The duration of this sonication is tissue dependent. For tougher tissues, such as muscle, sonicate longer.



Acid extraction:

- Save and dry down the aqueous (top layer). This is **Aqueous Phase 2**. Among other things, it contains the polar head groups of some lipids, creating some artificial metabolites. Keep it separate from **Aqueous Phase 1**.
- Save the protein disc, if it exists, for quantitation. This is **Protein Disc 2**.
- Save and lay down half of the organic phase (bottom layer). This is **Organic Phase 2**. This will contain lipids that have been stripped of their polar head groups.
- Save and dry the other half to continue to a basic extraction.

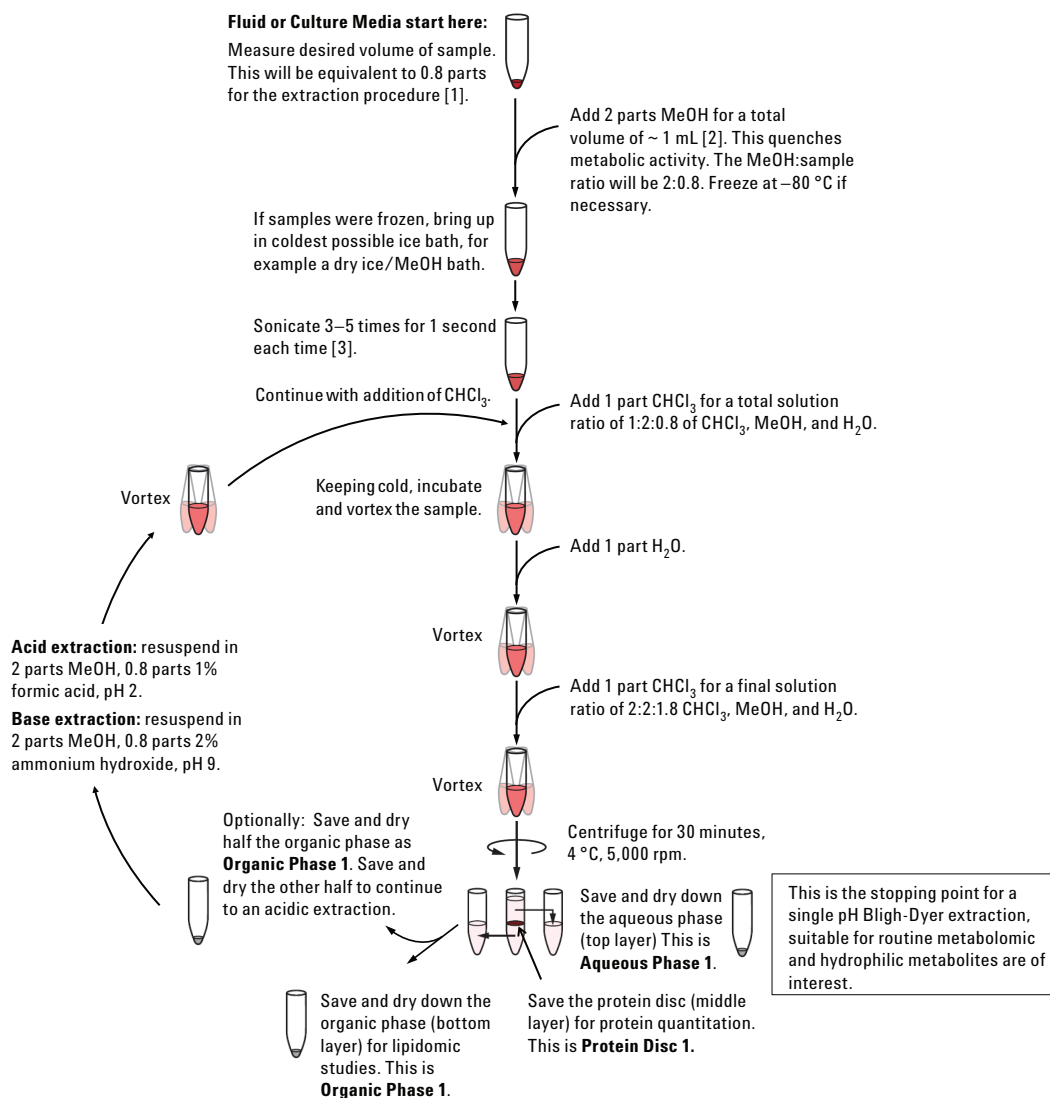
Basic extraction:

- Save and dry down the aqueous (top layer). This is **Aqueous Phase 3**. Keep it separate from **Aqueous Phase 1 and 2**.
- Save the protein disc, if it exists, for quantitation. This is **Protein Disc 3**.
- The bottom layer, **Organic Phase 3**, will be a sludge containing saponified lipids. It will be analytically useless. Discard it.

This completes the modified, 3-ph Bligh-Dyer extraction. When ready for analysis, combine **All Aqueous Phases** and reconstitute in mobile phase A of the intended chromatography (for example, HILIC, mixed-mode, or ANP). Likewise, combine **Organic Phase 1** and **Organic Phase 2** in mobile phase A of the intended chromatography (most likely reverse-phase).

For more explanation of the advantages of the 3-pH Bligh-Dyer extraction, please see Agilent Technologies application note 5989-7407.

Modified 3-ph Bligh-Dyer extraction for comprehensive metabolomics of fluid or media samples



Acid extraction:

- Save and dry down the aqueous (top layer). This is **Aqueous Phase 2**. Among other things, it contains the polar head groups of some lipids, creating some artificial metabolites. Keep it separate from **Aqueous Phase 1**.
- Save the protein disc, if it exists, for quantitation. This is **Protein Disc 2**.
- Save and lay down half of the organic phase (bottom layer). This is **Organic Phase 2**. This will contain lipids that have been stripped of their polar head groups.
- Save and dry the other half to continue to a basic extraction.

Basic extraction:

- Save and dry down the aqueous (top layer). This is **Aqueous Phase 3**. Keep it separate from **Aqueous Phase 1 and 2**.
- Save the protein disc, if it exists, for quantitation. This is **Protein Disc 3**.
- The bottom layer, **Organic Phase 3**, will be a sludge containing saponified lipids. It will be analytically useless. Discard it.

This completes the modified, 3-ph Bligh-Dyer extraction. When ready for analysis, combine **All Aqueous Phases** and reconstitute in mobile phase A of the intended chromatography (for example, HILIC, mixed-mode, or ANP). Likewise, combine **Organic Phase 1** and **Organic Phase 2** in mobile phase A of the intended chromatography (most likely reverse-phase).

For more explanation of the advantages of the 3-pH Bligh-Dyer extraction, please see Agilent Technologies application note 5989-7407.

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