

# Processing and Storage of Samples for Metabolomics Assays

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## Outline

- Briefly discuss sample collection since it is the first step of sample processing
- Highlight key points and considerations for sample storage
- Use the example of whole blood extraction to illustrate sample processing for untargeted metabolomics by NMR
- Discuss the importance of biobanking/biorepositories for metabolomics research

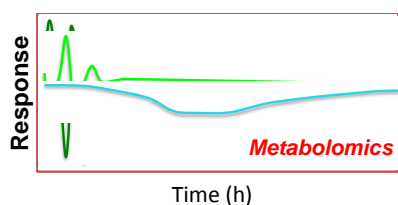
## Sample Collection

- The first step in sample processing
  - depends on the type of sample
  - depends on the source of the sample
    - clinical vs. experimental
- Consistency is key
  - uniformity of supplies
  - standard operating procedures (SOP)
  - prospective collection vs. samples of convenience
- Universal “standards” do not yet exist but will be driven by the advancement of metabolomics technology



image from  
[www.usada.org](http://www.usada.org)

## Sample Collection



- Variables to consider:
  - time of day and circadian variation
  - gender and age of subjects (mammalian)
  - diet, hydration, fasting state, exercise/activity
- Collection vessel
  - glass vs. plastic
  - laboratory vs. clinic
  - presently there are no “metabolomics tubes”

image adapted in part from D. Wishart, Bioinformatics.ca; June 13, 2011 under a creative commons license

Slupsky, CM., et al. Anal Chem 2007;79:6995-7004  
Park, Y., et al. Am J Physiol Regul Integr Comp Physiol 2009;297:R202-9

## Urine Sample Collection



urine collection vessel of metabolic cage (mice)

- Urine
  - for human samples collect a midstream, “clean catch” specimen
  - for animals (e.g., 3-4 mice/metabolic cage) a 12-24h is needed for sufficient volume
    - timeframe from experiment to experiment should be consistent
  - combat bacterial contamination with sodium azide (10%)

image from <http://www.tecniplast.it/usa/>

## Urine Sample Processing

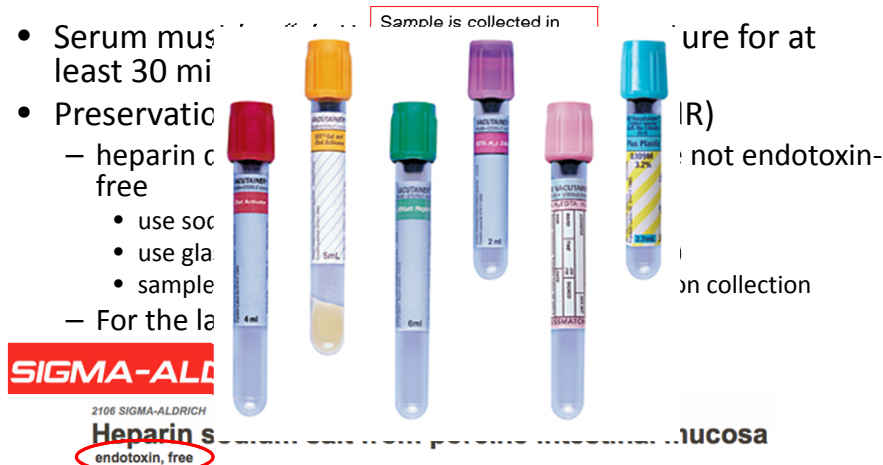
- Following collection, measure volume (can store sample at 4°C for up to 3h)
- Centrifuge (4°C, 2000 x *g*, 10 min) to precipitate sediment.
- Transfer the supernatant into a sterile tube taking care not to disturb the bottom of the tube whether there is a visible pellet or not. Discard the pellet and ~50µL (mouse) or ~500 µl (human) of sample above it.
- Keep samples and tubes on ice during processing

## Urine Sample Processing

- Transfer 500 $\mu$ L-1mL of urine to a clean microcentrifuge tube (1.5mL) and store (-80°C). This is a sample that can be reserved for LC-MS analysis.
- Accurately measure the remaining total sample in the tube. Record the volume. At this point, sample can be aliquoted and frozen.
- Add a volume of buffered internal standard (IS) equivalent to 10% of the final sample volume
  - Na or K phosphate buffer (pH 7.4) containing 10mM DSS
  - Chenomx IS-1 (sample pH'd to 7.0 before addition) Chenomx software is "pH sensitive."
- Following the addition of IS, cap the tube and invert 4X to thoroughly mix.
- Generate technical replicates (500 $\mu$ L) and freeze (-80°C).

## Blood Collection

- Serum must be collected in a tube that is suitable for at least 30 minutes of storage.
- Preservation
  - heparin coated tubes (HCT)
    - use sodium heparin
    - use glass tubes
    - sample must be kept at room temperature
  - For the laboratory, use heparin sodium (Heparin sodium, endotoxin free) endotoxin, free



Newhall, KJ., et al. Clin Chem 2010;56:1483-91

## Sample Storage

- Upon collection and in advance of assay
  - most biofluids should be stored at  $-80^{\circ}\text{C}$  (urine  $-40^{\circ}\text{C}$ )
  - samples (e.g., urine) that require pre-processing before storage should be kept cold ( $4^{\circ}\text{C}$ )
  - tissue, cell and other types of samples (e.g., sputum) processing is unique
- Few data are available about specific metabolites
  - most have been done in urine

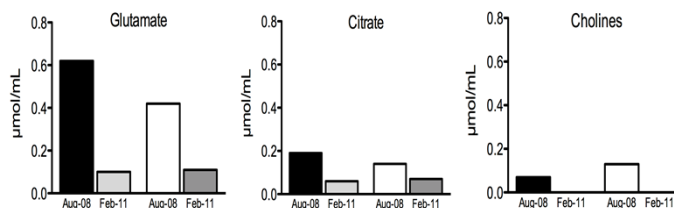


image from [commtechlab.msu.edu](http://commtechlab.msu.edu)

Bernini, P., et al. J Biomol NMR 2011;49:231-43  
 Yuille, M., et al. Biopreserv Biobank 2010;8:65-9  
 Beckonert, O., et al. Nature Protocols 2007;2:2692-2703

## Sample Stability

- Freeze-thaw
  - aliquot technical replicates to avoid freeze-thaw
- Storage duration: Some metabolites may be more vulnerable than others and this may vary based on sample type



- Metabolite stability in complex biological samples
  - should be part of quality control measurements in conjunction with analytical variability

## NMR Assay of Un-extracted Plasma and Serum Samples

- Best approach for the analysis of very low volume (<200  $\mu$ L) samples
- For water-soluble profiling only (using a unique water-suppression and macro-molecule suppression techniques)
- The limitations of this approach:
  - protein and lipoprotein signals obstruct regions of the spectrum and the detection of low-abundant aqueous metabolites
  - no specific lipid information
  - quantification of individual metabolites is difficult

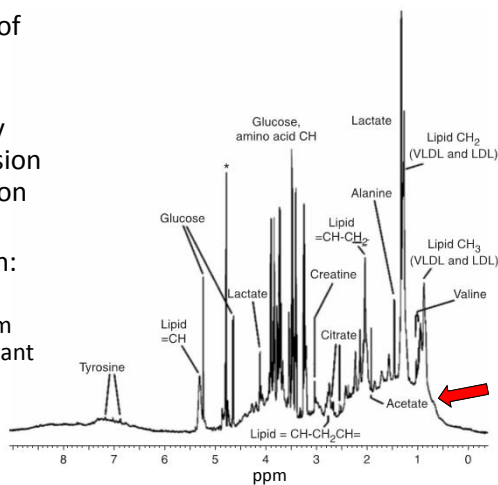
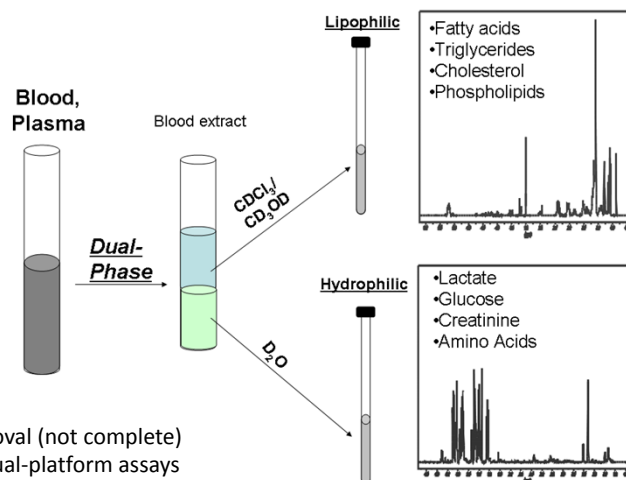


image from Beckonert, O., et al. Nature Protocols 2007;2:2692-2703

## Methanol:Chloroform Extraction



### Advantages:

- Protein removal (not complete)
- Allows for dual-platform assays
- Ideal for untargeted quantitative metabolomics of aqueous and lipid metabolites (required for use of Chemomx software)

### Disadvantages:

- Time consuming ( $\geq 24$  h)- not “real time”
- Metabolite recovery

## Methanol:Chloroform Extraction

### Whole Blood Extraction SOP

- Biomaterials Required:

~0.5 to 1.0 mL plasma/serum or whole blood (per sample) collected with heparin\*

Preservative will vary depending on planned analytical platform

- Other reagents and solutions:

Methanol and chloroform (reagent or HPLC grade) mix 1:1 (volume) fresh in a tightly sealed (Corning screw top bottle) that has been pre-cooled (-20°C); store mixture (-20°C) so it is ice cold when ready for use

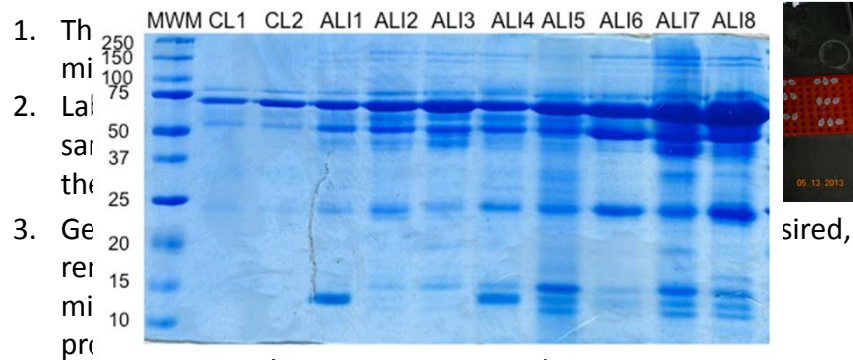
– Ice cold DI water

## Methanol:Chloroform Extraction

### A few things to keep in mind...

- Use glass graduated cylinders and Pasteur pipets and the glass serological pipets for methanol and chloroform measurements.
- All samples and solutions must be kept on ice or in the refrigerator at all times!
- Only use glass pipettes for transferring!
- Pre-chill all tubes/reagents in ice or in -20°C before use!

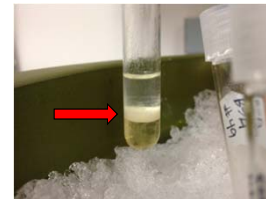
## Methanol:Chloroform Extraction



4. Add 500 $\mu$ L of sample to each glass tube labeled with "A."  
Record the actual volume that goes into each tube. This can vary if a clot has formed in the sample. Keep samples on ice.

## Methanol:Chloroform Extraction

5. Add 1mL of **cold** methanol:chloroform to each tube, cap and vortex thoroughly. Be careful not to let the methanol:chloroform come in contact with the tube cap. The caps are not solvent-tight.
6. Centrifuge tubes at 1300 x g, **4°C** for 20 min.
7. Following centrifugation, transfer the supernatant and lower fluid layer to the second labeled tube ("L")(keep on ice). The samples may form a pellet and supernatant, or a supernatant, solid layer and lower layer of fluid. Either is acceptable, just be sure to remove all fluids leaving only the pellet (it doesn't matter if you get some of the pellet). Reserve this fluid layer in the refrigerator (4°C) or on ice. **Pellets from whole blood samples will be larger and harder than those of serum or plasma.**

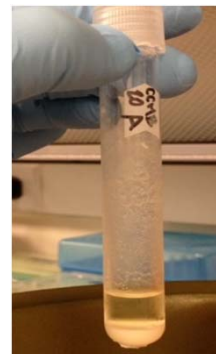


Representative sample at **step 7**.  
Transfer both the top and bottom layers to the "lipid" tube.



## Methanol:Chloroform Extraction

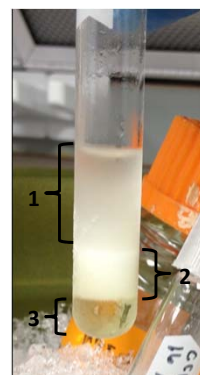
8. Resuspend the pellet of each sample in **cold** methanol:chloroform (0.5mL). Vigorously vortex to break up as much of the pellet as possible. Centrifuge (1300 x *g*, 4°C for 20 min).
9. Collect the supernatant (at this point, there should only be a supernatant and pellet but there may be some “floaters”) and add it to the fluid layer that was reserved in step 6. Reserve pellet and any floaters.



Sample at step 9. Add all supernatant to the fluid layer reserved in step 6.

## Methanol:Chloroform Extraction

10. Add 0.5mL of ice-cold DI water to each of the fluid tubes. Vortex well.
11. Cool the fluid for 20 minutes at -20°C. ALWAYS cool for exactly 20 minutes. If you should run over, remove tubes from the freezer re-vortex and repeat this freezing step (step 10). You don't want the aqueous phase to freeze. Ice crystals will form and precipitate in the organic phase and this will lead to erroneous results. If the sample freezes, thaw it (4°C) and repeat step 10.
12. The tubes containing the pellet can also go in the freezer (-20°C) at this point (step 10).
13. Upon removal of the tubes from the freezer, add 0.5mL of ice-cold DI water to the tubes containing pellet. Suspend the pellet by vigorously vortexing or use a spatula as needed.
14. After the 20min freezer incubation, the fluid samples will have separated into a supernatant, aqueous top layer and a protein layer (see figure 3). Add the top layer to the resuspended pellet (step 12). Do not disrupt or penetrate white protein layer. This remaining protein layer and lower liquid layer is the lipid fraction which can be capped and placed in the fridge (4°C).



**Step 14. Transfer layer 1 to the “aqueous” tube.**

1. Aqueous top layer
2. Protein layer
3. Lipid layer

## Methanol:Chloroform Extraction

15. Vortex the tubes containing the pellet and the newly added aqueous top layer (these are the water soluble samples) and centrifuge (1300 x *g*, 20 min, 4°C).
16. Transfer the supernatant to a lyophilizer bulb/flask. Cover with lid or parafilm.
17. Add 0.5mL ice cold DI water to pellet and resuspend as in step 10 and centrifuge (1300 x *g*, 20 min, 4°C).
18. Transfer the supernatant to the existing samples in the lyophilizer bulbs and discard the pellet.



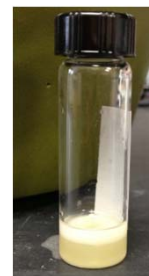
Round-bottom flask (lyophilizer bulb) with aqueous fraction prior to lyophilization. Steps 16 and 18.

## Methanol:Chloroform Extraction

19. Allow the water soluble fractions to sit in the lyophilizer bulbs for 5 min. Carefully observe each glass. If oil droplets have formed at the bottom of the glass, use a Pasteur pipet to remove them and add to the lipid fraction. **Measure and record the volume in the glasses before freezing with pipet! NOTE: Add 1mL of DI water to each remaining methanol before freezing.**

Alternatively, liquid nitrogen can be used; MeOH/dry ice is not a reliable alternative. Sample must be completely frozen.
20. Freeze the lyophilizer bulbs in -80°C freezer while tilting the glasses if possible (do this by placing the bulbs in the lyophilizer flask, covering each bulb with kimwipes and placing in -80°C overnight). Lyophilize for at least 24 h to ensure that methanol is removed. Do not exceed 48 h.
 

This time may vary depending on lyophilizer model and relative humidity!
21. Transfer the lipid fractions to the 3.7mL (measure and record volumes using serological glass pipets) and dry the lipid fractions in a speedvac at 50°C on heat + IR setting for 1h or until dry – record time for each. Cap tubes, wrap in parafilm and store at -20°C (can be stored for months).

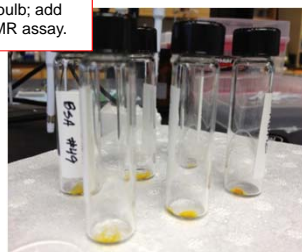


Borosilicate vial with protein and lipid bottom layer from step 21.

## Methanol:Chloroform Extraction

22. When the water soluble samples are dry, they can be sealed with parafilm and stored at 4°C for up to a week. At the time of assay or for long-term storage, add 0.5mL (record volume after resuspending) to each lyophilizer glass to resuspend sample. For assay, transfer each sample to an NMR tube for analysis. For long-term storage (years), transfer sample to a micro-centrifuge tube, seal with parafilm and store (-20°C). Freeze this aliquot at -80°C.
23. At the time of assay of the lipid samples, discard cap, resuspend samples in 0.6mL 1:2 deuterium methanol:deuterium chloroform solution (volume to volume), cap with a new cap and centrifuge (1300 x g, 10 min, 4°C). Transfer supernatants to NMR tubes for assay.

Measure volume removed from lyophilizer bulb; add 10% DSS for NMR assay.



Speedvac-dried lipid fraction

## Equipment and Lab Requirements



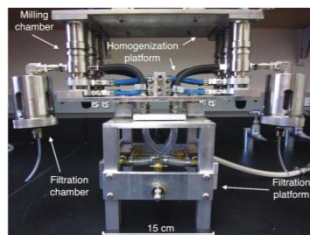
- Extraction of samples for metabolomics assays require:
  - Lyophilizer
  - "Speed vac"
- Sufficient bench and ventilation hood space to accommodate these pieces of equipment



## Technology will Drive the Field of Metabolomics

### Semiautomated Device for Batch Extraction of Metabolites from Tissue Samples

James J. Ellinger,<sup>†,‡</sup> Dan C. Miller,<sup>†</sup> Ian A. Lewis,<sup>§</sup> and John L. Markley<sup>\*,†,‡</sup>



- Automation of extraction process will reduce time of sample processing and improve consistency

Ellinger, JJ., et al. Anal Chem 2012;84:1809-12

## Other Extraction Methods

- Perchloric acid (PCA) extraction for adherent cell culture and mammalian tissues (e.g., lung)
- For targeted metabolomics and lipidomics
  - assay and analytical platform dependent



Agilent 7500A Triple Quadrupole GC/MS



images from Agilent.com

## Biobanking/Biorepositories

- Systematic collection and storage of human samples for “omics” research (and potentially clinical use)
  - samples are collected and processed using SOPs
  - storage conditions are monitored and documented
  - QC processes are integrated from collection through processing, storage and assay
  - samples are labeled (barcoded) and cataloged

## Biobanking/Biorepositories



- Circumvents use of samples of convenience
- Creates an infrastructure for human systems biology research
  - necessary for reliable biomarker discovery and validation studies
- Best option for long-term reliable storage of samples

image from Poste, G. Nature 2011;469:156-7

## Summary

- Consistent, prospective sample collection is the first step of sample processing for the generation of reliable metabolomics data
- Sample stability may vary based on type of biofluid/sample and overall stability of individual metabolites
- Methanol:chloroform extraction of whole blood yields a aqueous and lipid fraction for untargeted metabolomics that can be used for NMR and LC-MS analysis
- A move towards biobanking/biorepositories will improve overall reliability of clinical samples used for metabolomics assays

## Acknowledgements



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