

Metabolomics Core -BCM

Extraction protocols of metabolites from cells and control liver tissue samples

(to handle 20 exptl and 6 control samples)

1. Preparation of internal standard solution

- i) For example, prepare 1 mL of 10 mM solution for each internal standard and prepare similar solutions for up to 10 internal standards.
- ii) Combine all these 1 mL and 10 mM solutions to get final 10 mL of 1mM internal standard stock solution
- iii) Take two aliquots of 10 ul from the above stock solution in to two vials and dry them completely.
- iv) Reconstitute each vial with 200 ul of methanol:water (1:1), sonicate and combine the solutions of two vials
- v) Again rinse each vial with 100 ul of methanol:water (1:1), sonicate and combine the solutions of two vials
- vi) Combine solutions from v and vi steps to get 600 ul of final internal standard solution.
- vii) Keep the final solution on ice

2. Making of solutions for sample preparation

- i) For example, make 26 mL of methanol:water (4:1) sample solution under ice cold temperature to process 20 experimental samples and 6 liver sample controls

3. Preparation of a master solution spiked with internal standard

- i) Add 600 ul of internal standard solution from step 1 (vi) to 26 mL of methanol:water (4:1) sample solution of step 1 (i) to make final 26.6 mL final master solution
- ii) Vortex to mix the above master solution thoroughly and keep it under ice cold temperature

4. Preparation of experimental and control samples (*always keep samples on ice*)

- i) In case of cell metabolite analysis, freeze and thaw cell pellets in liquid nitrogen and water for three cycles, 30s each time
- ii) In case of control samples, use one 100 ug vial of liver tissue

5. Preparation of Experimental and control sample solutions

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A) Experimental sample solutions

- i) Add 750 ul of ice cold internal standard spiked master solution from step 3 (ii) to each 3 mL size sample vial containing freeze-thawed cell pellet solutions
- ii) Sonicate all the solutions (Settings: Amp 30% , 20-30s, 3 times)
- iii) Wash rotor with 10ml de-ion water in between each group.
- iv) Wash rotor with Methanol and water separately between each sample sonication

B) Control sample solutions

- i) Add 3 mL of ice cold internal standard spiked master solution from step 3 (ii) to 100 ug vial of liver tissue.
 - ii) Homogenize all the solutions (settings: 6, 30 sec and 30 sec)
 - iii) Split the homogenized solution into 4 replicates (each having ~750 μ l, weighing roughly 25mg per replicate.
 - iv) Take **first** three replicates
- C) Vortex both experimental and control samples for 5 min and keep them on ice

6) Extraction of metabolites

A) CHCl₃ extraction (caution: extraction should be performed in glass tubes instead of plastic vials)

- i) Add 450 ul of ice cold CHCl₃ (preserved at -20 for 10 min) to each sample solution in step 5 (C) and vortex for 10 min at speed 8
- ii) Add 150 ul of ice cold water to the above each sample mixture and vortex for 2 min
- iii) Keep the solutions at -20C for 20-30min
- iv) Centrifuge all the sample solutions at 4000 rpm for 10 min @ 4C
- v) Pipette out separate layers (organic and aqueous phase), combine them and use the entire supernatant.
- vi) Dry the supernatant solution @ 37C, 30-45 min.
- vii) Dissolve again in 500 ul of methanol:water (1:1) (vortex - 5min, sonicate - 5min, spin @ 5000rpm-5min) and filter to separate proteins

B) Protein separation by Amicon filters

- i) Precondition the Amicon protein filters by prewashing and centrifuging them w/ 500 μ l 50:50 Methanol: Water (15000 rpm @ 4C -20min) until the entire solution is filtered out
- ii) Discard the tube and keep the filter in a new tube
- iii) Transfer the samples from 6 A (vii) into the pre-washed filters and centrifuge it to collect the filtered samples. Add additional 100 μ l 50:50 Methanol: Water to the filter and centrifuge it to collect the remaining metabolites if any.
- iv) Collect the filtered solution and dry them.

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Metabolomics Sample Extraction Protocols

Tissue Extraction: Weight the tissues and add the extraction buffers according to the weight.

Cell line: 3 - 5 million cells and each five replicates.

Serum: 100ul

Non Polar/Polar Metabolites (Methods 1-11)

1. Take experimental samples in tubes (e.g.: total number of experimental samples and liver samples =10 samples).
2. Add 750 μ l chilled Methanol:water (4:1, v/v) to each sample. Make one initial stock solution; e.g.: for 10 samples: $750 \times 10 = \sim 10000 (\mu\text{l})$ or 10ml.
3. Add 200 μ l of 1:1 Methanol:Water to 1 vial of internal standards and then add that into the stock solution.
4. Add additional 200 μ l of 1:1 Methanol: water to the empty IST vial to wash out the remaining solution into the stock solution (8ml.) as well. At that point total volume of the stock solution:
 $(10,000 + 200 + 200) \mu\text{l} = 10,400 \mu\text{l}$.
5. Add 750 μ l of that IST spiked stock solution into each experimental sample.
6. For liver samples: add $\sim 3\text{ml}$ (300 μ l) into single aliquot tube (100mg/ml) and vortex the mixture to make homogenous solution then split that into 4 replicates (each having $\sim 750 \mu\text{l}$, weighing roughly 25mg per replicate. Take three replicates).
7. Homogenize and vortex (~ 10 min.)
8. Add 450 μ l chilled HPLC grade chloroform (CHCl_3) to each sample & vortex (~ 10 min).
9. Add 150 μ l chilled HPLC grade water to each sample and vortex 2 min.
10. Keep the entire mixture in freezer (-20°C , ~ 30 min).
11. Centrifuge the solution (rpm/g-4000rpm, 40°C , ~ 10 min).
12. Pipette out separate layers (organic and aqueous phase). The top layer (aqueous) is used for LC and bottom (organic) layer is used for GC. But if only LC-MS is needed, then combine the two layers and used the entire supernatant.
13. Dry the each separated layers (or the one layer depending on research) in SpeedVac (37°C , until it dries out).
14. Re-suspend each sample in 500 μ l 50:50 Methanol: Water.

15. Protein Separation: Precondition the Amicon protein filters by prewashing and centrifuging them w/ 500 µl 50:50 Methanol: Water until the entire solution is filtered out.
16. Transfer the re-suspended samples into the pre-washed filters and centrifuge it to collect the filtered samples. Add additional 100 µl 50:50 Methanol: Water to the filter and centrifuge it to collect the remaining metabolites if any.
17. Collect the filtered solution and dry them in the SpeedVac. Discard the filters.
18. Re-suspend the dried samples in 0.1% Formic acid w/ 50:50 Methanols:Water (this solution will be different based on mobile phase). Also the initial volume would be adjusted depending on the number of methods. E.g.: for 3 methods 40 µl; for 8 methods 100 µl. Also consider the final volume factoring the sample aliquots which would be stored for later use.
19. Sonicate (5min) and vortex (5min) and centrifuge (5min) this re-suspended mixture. Also split the samples two/three aliquots and store one sample aliquot set at -80°C to re-run the samples if needed later.
20. Next, transfer the remaining sample into an insert placed inside the auto-sampling vial (top-screw glass vials).

Extraction of Steroids/Vitamins (Method 12)

Application/Scope: This SOP is applicable to the extraction and analysis of the following steroids from plasma and serum:

Aldosterone	11-Deoxycortisol
Androsterone	11-Deoxycorticosterone
Androstenedione	Estradiol
Corticosterone	Estrone
Cortisol	17-OH-Progesterone
Cortisone	Progesterone
Dihydrotestosterone	Testosterone
Dehydroepiandrosterone sulfate	

Sample Preparation:

Plasma/serum extraction (w/o DHEAS):

1. Aliquot 100 ul DI H₂O & 100 ul serum/plasma sample to labeled 1.5 ml eppendorf centrifuge tubes.

2. Add 150 ul methanol (MeOH) to each centrifuge tube, then 20 ul of internal standard mixture and 225 ul acetonitrile (ACN).
3. Mix on vortex mixer for 30s; allow to stand for 5 min. and vortex a second time for 30s.
4. Centrifuge @ 15,000 rpm and 4°C for 5 min., transfer supernatant to a labeled 2 mL autosampler vials.
5. Add 1.0 mL MTBE and 300 ul DI H₂O, mix on vortex mixer for 1 min., allow to stand for 5 min. and vortex a second time for 1 min.
6. Allow to stand to facilitate phase separation, transfer MTBE to a new, labeled, 2 mL autosampler vial, dry under UHP N₂ @ 37°C.
7. Reconstitute sample in 200 ul 50:50 (v/v) MeOH/DI H₂O, vortex mix for 15s and transfer to 350 ul autosampler vial insert.

Additional Extraction for DHEAS:

After performing MTBE extraction of serum/plasma sample perform the following extraction on the aqueous phase:

1. Add 300 ul of 1 M Ammonium sulfate;
2. Add 600 ul of a 50:50 (v/v) Chloroform/2-Butanol mixture to the vial containing the aqueous phase, mix on vortex mixer for 1 min. allow to stand for 5 min then vortex a second time for 1 min.;
3. Allow vial to stand to allow for phase separation, then transfer the organic layer to the 2 ml autosampler vial containing the MTBE extract (before drying the extract);
4. Dry under UHP N₂.
5. Reconstitute sample in 50:50 (v/v) MeOH/DI H₂O as in MTBE procedure;
6. Vortex mix for 15s then transfer to 350 ul vial inserts.

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An isotope-labeled chemical derivatization method for the quantitation of Short-chain fatty acids

#	SCFA
1	Acetate
2	Propanate
3	Iso Butyrate
4	Butyrate
5	2-methyl butyrate
6	Isovalerate
7	Valerate
8	3-methyl valerate
9	Isocaproate
10	Caproate

Preparation of standard stock solutions: Prepare 20mM SCFA stock solutions (1-10) in 50% aqueous acetonitrile. Combine each stock solution by 20 ul except for acetate and propionate for which combine 50 ul each. Add additional 110 ul of 50% aqueous acetonitrile to bring total volume 400 ul. Take 40uL of STD mix and 80uL of 50%Acetonitrile (3xdilution) and make up to 10 dilutions. Use 40uL of each dilution for derivatization.

Preparation of light labeled SCFAs: Take 40uL of each dilution (Total 10 dilutions) and add 20 ul of 200 mM $^{12}\text{C}_6\text{-}^{13}\text{NPH_HCl}$ solution in 50% ACN–followed by 20 ul of 120 mM EDC. HCl – 6% pyridine solution in 50% ACN. Mix this solution in 3 mL glass vial and incubate at 40C for 30 min and then cool on ice for 1 min. Dilute this reaction mixture with 1.92 mL of 10% aqueous ACN. Spike 20uL of derivatized $^{13}\text{C}_6\text{-}^{13}\text{NPH_HCl}$ solution in to 20 ul of each derivatized standard dilution and mix well. Finally inject 5uL solution for LC-MS analysis

Preparation of heavy labeled SCFAs: Take 40uL from the above 400 ul of standard stock solution and add 20 ul of 200 mM $^{12}\text{C}_6\text{1}^{13}\text{C}_6\text{-}^{13}\text{NPH_HCl}$ solution in 50% ACN–followed by 20 ul of 120 mM EDC. HCl – 6% pyridine solution in 50% ACN. Mix this solution in 3 mL glass vial and incubate at 40C for 30 min and then cool on ice for 1 min. Dilute this reaction mixture with 1.92 mL of 10% aqueous ACN.

Preparation and extraction of test samples

Test Sample type	Minimum weight/volume	Vol. for derivatization
Feces	50 mg	40uL
Serum	40uL+40 uL 100% ACN	40uL
Tissue	50 mg	40uL

Weigh the samples according to the table and add 0.5 mL of 50% aqueous acetonitrile into each sample (In case of liquid test samples, take the volume directly from sample). Homogenize all samples using probe sonicator. Collect 300 ul of the clear supernatant for all the samples and use 40 ul of each supernatant for derivatization. For this derivatization, follow exactly same procedure as used to derivatize SCFA standards. Spike 20uL of derivatized $^{13}\text{C}_6\text{-}^{13}\text{NPH_HCl}$ solution in to 20 ul of each derivatized test dilution and mix well. Finally inject 5uL solution for LC-MS analysis.