Scope:

- This SOP applies to sample protein precipitation for global metabolomics analysis by reverse phase or HILIC- HPLC-MS.
- Samples include but are not limited to tissue, cells, plasma, serum, and stool.
- Refer to Appendix A: Standard preparation for global metabolomics assays for preparation of internal standards and injection standards.

Materials:

- Chemicals and Reagents for Tissue, Plasma, Serum, and Stool
  - 5mM Ammonium Acetate in water for homogenizing
  - Precipitate solution, 8:1:1 Acetonitrile: Methanol: Acetone
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  - Mobile Phases: 0.1% Formic Acid in Water, LC/MS grade, and Acetonitrile, LC/MS grade
  - Internal Standard Solution (prepare according to Appendix A)
  - Reconstitution Solution (prepare according to Appendix A)
- Chemicals and Reagents for Cells
  - 5mM Ammonium Acetate in water for homogenizing
  - 40 mM ammonium formate in water for pellet washing (3 mL per sample)
  - Precipitation solution, Ice-cold 80% methanol
  - Mobile phases, H_20 with 0.1% Formic Acid LC/MS grade and Acetonitrile LC/MS grade
  - Internal Standard Solution (prepare according to Appendix A)
  - Reconstitution Solution (prepare according to Appendix A)
- Labeled 1.5 mL or 2 mL Eppendorf tubes
- Appropriate pipette tips (see Table 1)
- Labeled LC vials

Table 1. Precise Micropipette Volume and Transfer capabilities

Pipette Type	Volumes (µL)	Tip color
P10	0.5 – 10	white
P20	2 – 20	yellow
P200	20 – 200	yellow
P1000	200 – 1000	blue
repeater	0.1 µL-10ml	Use appropriate tip for the volume needed to dispense.

Personal Protective Equipment:

- At a minimum, use nitrile gloves, lab goggles and lab coat when performing this SOP.

Equipment:

- Calibrated Repeater Pipette
- Calibrated Micropipettes (See Table 1)
- Refrigerator
- Vortex mixer
- Refrigerated Centrifuge (Eppendorf- 5417R or equivalent)
  Open by pressing blue "open" button on bottom left of display. Check to be sure loading dock is cool. If not cool, close, press fast cool and wait until temperature is <10°C. When temperature is <10°C, press stop, wait for centrifuge to stop spinning, and open. Load samples making sure samples and/or weights are evenly distributed among the wheel.
- N_2 Dryer (Organomation Asscociates, Inc- OA-HEAT or equivalent)
  Flip green power switch to "on" (located on bottom right of box). Of the three black switches, set the start/reset switch to neutral. Set the heat switch to neutral. Set the gas switch to Manual. To obtain gas flow, turn the gas nozzle on right side of hood. Turn the Harris valve in hood to open position. Adjust LPM air to no more than 15. Place samples in drying tray. Open/close N_2 flow lines depending on where samples are placed. Lower N_2 lines to enable drying.

Extraction Procedure: Use the appropriate sample extraction procedure.

1. Tissue

1.1. If frozen, thaw sample on ice or in 4°C refrigerator.

1.2. **Note:** If the tissue has been lyophilized, no beads are necessary for homogenization. Perform one round on the BeadBeater at 1800 rpm for 30 sec followed by a 30 min incubation period in 4°C. Go to Step 1.8 afterwards.

1.3. Add three 3 mm borosilicate glass beads to the tube containing the tissue sample with sterilized foreceps.

1.4. **Note:** For dense and intact tissue, add 1 squirt of 0.7 mm zirconia beads.

1.5. Add 1 mL of 5 mM Ammonium Acetate in water for every 50 mg of tissue.

1.6. Homogenize on the bead beater 2 times for 30 s at 1800 rpm and incubate for 30 minutes in 4°C between each round.

1.7. Homogenize on the bead beater one time for 30 s at 1800 rpm and incubate for 10 minutes in 4°C afterward.

1.8. Centrifuge at 20,000 xg at 4°C for 10 minutes to pellet the tissue debris.

1.9. Transfer the supernatant into a clean Eppendorf tube.

1.10. Determine protein concentration. If necessary, calculate normalization of samples using the lowest determined concentration.

1.10 **Note:** The following procedure is appropriate for 100 µL sample. Adjust to equivalent volumes in the succeeding steps if extracting a different volume of sample.

1.11. Add 100 µL of sample to a labeled, clean Eppendorf tube. If normalizing to a specific and lower protein concentration, add the amount of sample needed and dilute with LC/MS grade water.

1.12. Add 20 µL of Internal Standard Solution using a repeater pipet

1.13. Add 800 µL of precipitate solution with repeater pipette to make a solution of 1:8 (sample: solvent) ratio. Vortex sample to ensure mixing.

1.14. Cool samples in the 4°C refrigerator for 30 minutes to further precipitate proteins.

1.15. Centrifuge at 20,000 xg for 10 mins at <10° C to create a pellet of proteins.

1.16. Transfer 750 µL of supernatant to a new, labeled tube, making sure to leave behind protein pellet.

1.17. Dry liquid sample using Nitrogen gas.

1.18. Add 100 µL of Reconstitution Solution. Vortex to ensure mixing. Note that a lower reconstitution volume can be used if concentration of the sample is warranted.

1.19. If analyzing in HILIC mode, reconstitute samples in 90: 10 Acetonitrile: 10mM Ammonium Acetate

1.20. Place on an ice bath or in a 4°C refrigerator for 10-15 minutes. Centrifuge again with the above settings.

1.21. Carefully transfer entire supernatant to a labeled, glass LC vial with a fused glass insert.

1.22. **Note:** 2 µL of aliquots of each sample within a group/treatment are pooled into a clean vial. This pooled sample will be analyzed by MS/MS data dependent scan. Label the vial correctly.

1.23. Prepare a Blank of 200 µL of 0.1% Formic Acid in Water and a Neat QC of 1: 1: 3 Internal Standard Solution: 20 µL/mL Amino Acid Standards: 0.1% Formic Acid in Water (1 µL per sample)

1.24. After all samples have been transferred, ensure vials are free of air bubbles.

1.25. Store in 4°C refrigerator (or -80C for longer storage) until ready for analysis.

2. Cells

2.1. If frozen, thaw sample on ice or in a 4°C refrigerator.

2.2. If already pelleted, proceed to 2.3. If not yet pelleted, centrifuge cell suspension at 2000 rpm for 5 minutes at 5°C to pellet the cells. Discard the supernatant.

2.3. Wash pellet by adding 1 mL of 40 mM ammonium formate in water. Vortex and centrifuge at 2000 rpm for 5 min at 5C. Discard supernatant.

2.4. Complete step 2.3 two more times for a total of 3 washings.

2.5. Add 50 µL of 5mM Ammonium Acetate in water to pellet.

2.6. Homogenize on the Bead Beater for 30 sec at 1800 rpm with a 30 min incubation in 4°C afterward.

2.7. Determine protein concentration. If necessary, normalize samples using the lowest determined concentration in a clean 2 mL Eppendorf tube using LC/MS water to dilute.

2.8. Add 2 µL of the internal standard solution with repeater pipette.

2.9. Add 1 mL ice-cold 80% methanol in water with repeater pipette.

2.10. Homogenize on the Bead Beater for 30 s at 1800 rpm.

2.11. Incubate on ice or in a 4°C refrigerator for 10 min to further precipitate proteins.

2.12. Centrifuge at 2000 rpmfor 5 min at 5°C to pellet the proteins.

2.13. If cell count is less than 3×10^6, do not split the supernatant. Otherwise, transfer 500 µL of the supernatant into a clean Eppendorf tube making sure to not disturb the pellet. Transfer the remaining 500 µL into another clean, labeled tube and store in -80°C for further testing.

2.14. Dry the liquid sample using Nitrogen gas.

2.15. Add 30 µL of Reconstitution Solution (refer Appendix A). Vortex to ensure mixing.

2.16. Place on an ice bath or in the 4°C refrigerator for 10-15 minutes. Centrifuge again with the above settings.

2.17. **Note:** If sample volume allows, 2 µL of sample from each group are pooled into a clean vial. This sample will be analyzed by MS/MS data dependent scan. Label the vial correctly.

2.18. Carefully transfer entire supernatant to a labeled, glass LC vial with a fused glass insert.

2.19. Prepare a Blank of 200 µL of 0.1% Formic Acid in Water and a Neat QC of 1: 1: 3 Internal Standard Solution: 20 µL/mL Amino Acid Standards: 0.1% Formic Acid in Water (1 µL per sample)

2.20. After all samples have been transferred, ensure vials are free of air bubbles.

2.21. Store in a 4°C refrigerator (or -80°C for longer storage) until ready for analysis.

3. Plasma or Serum

3.1. If frozen, thaw sample on ice or in a 4°C refrigerator.

3.2 **Note:** The following procedure is appropriate for 100 µL sample. Adjust to equivalent volumes in the succeeding steps if extracting different volume of sample.

3.3. Add 100 µL of sample to a labeled, clean Eppendorf tube.
3.4. Add 20 µL of Internal Standard Solution using a repeater pipette
3.5. Add 800 µL of precipitate solution with repeater pipette to make a solution of 1:8 (sample: solvent) ratio. Vortex sample to ensure mixing.
3.6. Cool sample in the 4°C refrigerator for 30 min to further precipitate proteins.
3.7. Spin at 20,000xg for 10 mins at <10° C to create a pellet of proteins.
3.8. Transfer 750 µL of supernatant to new, labeled tube, making sure to leave behind protein pellet.
3.9. Dry liquid sample using Nitrogen gas.
3.10. Add 100 µL of Reconstitution Solution. Vortex to ensure mixing.
3.11. Place on an ice bath or in the 4°C refrigerator for 10-15 minutes. Centrifuge again with the above settings.
3.12. Carefully transfer entire supernatant to labeled, glass LC vial with a fused glass insert.
3.13. **Note:** 2 µL of aliquots of each sample within a group/treatment are pooled into a clean vial. This pooled sample will be analyzed by MS/MS data dependent scan. Label the vial correctly.
3.14. Prepare a Blank of 200 µL of 0.1% Formic Acid in Water and a Neat QC of 1: 1: 3 Internal Standard Solution: 20 µL/mL Amino Acid Standards: 0.1% Formic Acid in Water (1 µL per sample)
3.15. After all samples have been transferred, ensure vials are free of air bubbles.
3.16. Store in 4°C refrigerator (or -80°C for longer storage) until ready for analysis.

4. Stool

4.1 If frozen, thaw sample on ice or 4°C refrigerator.
4.2 **Note:** The following procedure is appropriate for 100 µL sample. Adjust to equivalent volumes in the succeeding steps if extracting different volume of sample.
4.3 Add 1 mL of 5mM Ammonium Acetate in water for every 40 mg of tissue.
4.4 Homogenize on the Bead Beater for 30 s at 1800 rpm with a 10 min incubation in 4°C afterward.
4.5 Determine protein concentration. If necessary, normalize samples using the lowest determined concentration in a clean 2 mL Eppendorf tube using LC/MS water to dilute.
4.6 **Note:** The following procedure is appropriate for 100 µL sample. Adjust to equivalent volumes in the succeeding steps if extracting a different volume of sample.
4.7 Add 100 µL of sample to a labeled, clean Eppendorf tube.
4.8 Add 20 µL of Internal Standard Solution using a repeater pipette.
4.9 Add 800 µL of precipitate solution with repeater pipette to make a solution of 1:8 (sample: solvent) ratio. Vortex sample to ensure mixing.
4.10 Cool sample in the 4°C refrigerator for 30 minutes to further precipitate proteins.

4.11 Spin at 20,000xg for 10 mins at <10° C to create a pellet of proteins.

4.12 Transfer 750 µL of supernatant to new, labeled tube, making sure to leave behind protein pellet.

4.13 Dry liquid sample using Nitrogen gas.

4.14 Add 100 µL of Reconstitution Solution. Vortex to ensure mixing.

4.15 Place on an ice bath or in the 4°C refrigerator for 10-15 minutes. Centrifuge again with the above settings.

4.16 Carefully transfer entire supernatant to labeled, glass LC vial with a fused glass insert.

4.17 **Note:** 2 µL of aliquots of each sample within a group/treatment are pooled into a clean vial. This pooled sample will be analyzed by MS/MS data dependent scan. Label the vial correctly.

4.18 Prepare a Blank of 200 uL of 0.1% Formic Acid in Water and a Neat QC of 1: 1: 3 Internal Standard Solution: 20uL/mL Amino Acid Standards: 0.1% Formic Acid in Water (1 uL per sample)

4.19 After all samples have been transferred, ensure vials are free of air bubbles.

4.20 Store in 4°C refrigerator (or -80°C for longer storage) until ready for analysis.

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Revision Number	Name	Reason for Revision	Effective Date
01	Sandi Batson	Creation of SOP	04/22/14
02	Sandi B. Sternberg	Changed Precipitate solution to 8:1:1 Acetonitrile:MeOH:Acetone. Added new step (9) in procedure. Both changes will give a cleaner sample.	08/01/14
03	Sandi B. Sternberg	Changed Reconstitution amount from 150uL to 100uL	09/18/14
04	Sandi B. Sternberg	Changed amount of Internal Standard to be added from 10uL to 20uL due to change in Internal Standard mix.	10/04/14
05	Sandi B. Sternberg	• Update in Instrumentation to reflect purchase and use of new nitrogen dryer. • Took out calibration info and added reference to calibration SOP. • Added the exact method(s) used with Dionex.	10/29/14

06	Sandi B. Sternberg	• Updated Data collection section to name exact method and tunefile to be used. • Removed Instrument parameters since they are saved in tunefile and method. • Changed gradient information to show increased use of pump B. • Added section for Pre-processing	10/31/14
07	Sandi B. Sternberg	• Added step in Pre-processing to accommodate XReport.	12/5/14
08	Sandi B. Sternberg	• Changed Step 6 in Extraction Procedure from 250µL to 750µL. • Added instrument parameters	12/15/14
09	Sandi B. Sternberg	• Changed method and gradient information to reflect new pump. • Updated the retention times in pre-processing	03/17/15
10	Joy Guingab	• Updated to included protein precipitation extraction procedure for tissue, cell, plasma and serum. • Revised the title to indicate that the SOP applies samples for both reverse phase and HILIC LC-MS analysis • Minor edits to provide clarity to the procedures • Separated the LC-MS procedure	07/13/16
11	Laurel Meke	• Added Stool extraction procedure • Replaced water with 5mM Ammonium Acetate in water as the homogenizing solvent • Added that a Blank and Neat QC should be made for each extraction and how to make each • Minor edits to provide clarity	11/23/2016