04/00/2018 – 233.5.1 – Methanol:Chloroform Precipitation of HAPS Human WB (Mike Puskarich)

Biomaterials Required:

Samples:

~0.5 mL HAPS human WB, from patients with and without sepsis, collected in Sodium Heparin tubes

Internal Controls:

Human WB from two individuals (Race: B, H; male; age: 53, 58) pooled together (lot #s: 2400097E, 2400100C)

Details on the pooling/aliquotting of this control in 233.1.2

Other reagents and solutions:

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

Equipment:

A. Vortex (Fisher cat# 12-812 or similar)
B. Micropipetters: 10uL or similar for glucose testing, 20-100uL for 20uL pre-extraction aliquots, 1000uL for water additions
C. Pipet aid to use with serological pipettes
D. Refrigerated microcentrifuge (FisherScientific Accuspin 17R or similar)
   - Rotor: 16x10mL vials (Fisher 10-269-653)
E. Lyophilizer (Labconco 1 L Benchtop 77400 Series)
F. NMR

Disposables/Consumables:

A. Glass bottle for storage of Methanol:chloroform
B. 2-mL microcentrifuge tubes (one per sample)
C. 0.5mL cryo-vials (white top) to store pre-extraction aliquots in (one per sample)
D. Glucose meter (Contour Blood Glucose Monitoring System or similar)
E. Glucose meter test strips (Contour Blood Glucose Test Strips or similar)
F. Disposable glass serological pipettes, 2-mL (1mL: VWR 93000-692; 5mL: 93000-696)
G. Bath Sonicator
H. Pasteur pipets, 9-inch borosilicate glass (Fisher 22-183-632 or 13-678-20D)
I. Kim-wipes
J. Rubber bands
K. Parafilm
L. Sharpie or permanent marker for labeling tubes

Disclaimers:

1. Keep all samples on ice or in the refrigerator at all times!
2. Prechill all tubes and reagents on ice, or at 4°C. Do not use warm or room temperature reagents

Procedure:

I. Preparation of samples and reagents

1. Remove samples from -80°C to thaw in an ice-water bath, remove shells that form around samples to speed up thaw time.
2. Prepare a 1:1 solution of Methanol:Chloroform
   Use the formula: 2n+3=mL of MeOH:$CHCl_3$ to prepare (where n=number of samples). Add half of that volume of methanol and chloroform each to a glass bottle and chill on ice or at 4°C before adding to samples.
3. For *each* sample prepare the following tubes, labeled completely and including a unique sample identifier.
   a. One 2-mL microcentrifuge tube to precipitate sample in and to dry the lipid/protein pellet in
      i. Label as being the lipid/protein fraction even during extractions
      After labeling chill this tube on ice or at 4°C before adding sample.
   b. One 0.5-mL cryovial for pre-precipitation aliquots (this needs to be a **cryovial**, since it will be stored in LN_2)
      Chill before sample addition
   c. One 5-mL centrifuge tubes to dry the sample in
      i. One for aqueous fraction.
4. Add ice to bath sonicator to create an ice-water bath.
5. Gently swirl or invert sample tubes as they thaw in an ice-water bath (**do not vortex**), try to dissolve any clots that are present. **Record any clots or unusual samples**
6. If clots are present in most of the samples, allow the samples to rest at RT for 3 minutes prior to removing any sample from tubes.
7. Measure and Record the glucose concentration of each sample by spotting serum onto a fresh test strip for each sample. Spot 2uL aliquots of each sample onto a piece of saran-wrap and then dip the test strip into the sample.
8. Remove 50uL from sample as pre-extraction aliquot. Store in LN_2.

II. Precipitation

1. Add the complete volume of each sample to the corresponding Eppendorf tube. Use a micropipetter to transfer the complete volume and **record the volume transferred**.
   If there are less than 300uL of any sample do not precipitate the sample. Keep samples in an ice water bath at all times.
2. Add two volumetric equivalents of cold MeOH:$CHCl_3$ (for a sample:MeOH:$CHCl_3$ ratio of 1:1:1) to each sample, cap and vortex thoroughly (~30s).
3. Sonicate tubes for 2 minutes at 4_oC (using ice-water bath in bath sonicator).
4. Incubate tubes at -20_oC for 20 min, chill in a pre-chilled or open-sided rack to insure samples are chilled sufficiently. Samples should be a thick, opaque slurry after the chill step.
5. Centrifuge tubes at 13,400 x *g*, 4°C for 30 min. There should be a pellet with a clear interface, which is typically fairly distinct after centrifugation.
   Record any unusual samples after centrifugation
6. Transfer aqueous fraction to a labeled 5-mL centrifuge tube using a Pasteur pipette.

Measure and **record** the volume of the aqueous fraction in the tube with a 2-mL glass serological pipette.

7. **Record** the volume of lipid remaining in the 2-mL centrifuge tube with a 1-mL glass serological pipet, try to avoid getting pellet in the pipet.
8. Cap centrifuge tubes by covering with a kim-wipe folded in half, and secure with a cap with a hole drilled in the middle.
   Lyophilization chambers can be shared between 3-4 samples (more for lipid fractions).
9. Freeze samples in LN2 for at least 3 minutes before lyophilizing 24-48 hours
10. After lyophilization, resuspend in 500uL of 50mM phosphate buffer.

Sample ID	Glucose conc (mg/dL)	Initial vol (uL)	MeOH:$CHCl_3$ added	Aqueous Pre-lyophilization vol	Lipid pre-lyophilization vol	Time on lyophilizer
HAPS_233_41						
HAPS_233_42						
HAPS_233_43						
HAPS_233_44						
HAPS_233_45						
HAPS_233_46						
HAPS_233_47						
HAPS_233_48						
HAPS_233_49						
HAPS_233_50						
HAPS_233_51						
HAPS_233_52						
HAPS_233_53						
233_C09						
233_C10						
233_C11						
233_C12						

III. Resuspension

1. Once samples have dried, **record time on lyophilizer.**
   Carefully uncap vials by removing kim-wipes, slowly lifting kim-wipe to observe any solids on the paper, **record** if any are present/visible.
   Resuspend samples in 500 uL of 50 mM phosphate buffer (pH 6.8) in D_2O.
2. Cap the tube sample was dried in, invert to dissolve solids, vortex briefly (~10s) to mix.
3. Transfer resuspended sample to a green-cap cryo vial appropriately labeled for an NMR prepped sample, **record this volume.**
4. Add D_2O to bring the volume of each sample up to 500 uL, **record this volume.**
5. Add 50 uL of 4.99 mM DSS-d_6 internal standard to each sample.
   check the actual concentration of DSS added!
   Store samples at -80$_o$C until NMRs are run

Sample ID	Vol transferred (uL)	D_2O added (uL)	DSS added (uL)	Total vol (uL)
HAPS_233_41				
HAPS_233_42				
HAPS_233_43				
HAPS_233_44				
HAPS_233_45				
HAPS_233_46				
HAPS_233_47				
HAPS_233_48				
HAPS_233_49				
HAPS_233_50				
HAPS_233_51				
HAPS_233_52				
HAPS_233_53				
233_C09				
233_C10				
233_C11				
233_C12				