**2018/05/16– Experiment #235.01: Methanol Precipitation of Human Sepsis Serum from RACE**

**Biomaterials Required:**

- Human serum (NHS): non-sterile, pooled, off-the-clot, AB type, Innovative research, Cat# IPLA-SERAB-OTC, Lot #20636**,** one freeze-thaw cycle

**Other reagents and solutions:**

- Methanol (reagent or HPLC grade; Fisher brand) in a tightly sealed (Corning screw top) bottle that has been pre-cooled (-20°C); store at -20°C so it is ice cold when ready for use
- Ice cold DI water

**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 for use with serological pipettes
D. Refrigerated Centrifuge (Eppendorf 5810R or similar)
E. Lyophilizer/freeze-dryer capable of removing methanol

**Disposables/Consumables:**

A. Glass bottle for storage of methanol
B. 0.6-mL microcentrifuge tubes (Fisher 2681250) and 2.0-mL microcentrifuge tubes (Fisher 2681258)
C. Glucose meter (Contour Blood Glucose Monitoring System or similar)
D. Glucose meter test strips (Contour Blood Glucose Test Strips or similar)
E. Disposable glass serological pipettes, 1-, 2- and 5-mL (1mL: VWR 93000-692; 5mL: 93000-696)
F. Pasteur pipets, 9-inch borosilicate glass (Fisher 22-183-632 or 13-678-20D)
G. Kim-wipes
H. Rubber bands
I. Sharpie or permanent marker for labeling tubes
J. 50 mM phosphate buffer in $D_2O$, pH approximately 6.8, make sure there is sufficient volume for resuspension before starting precipitations

**Disclaimers:**

1. Keep all samples in an ice-water bath or in the refrigerator at all times!
2. Prechill all tubes and reagents on ice, at 4°C or -20°C. Do not use warm or room temperature reagents

**Procedure:**

**I. Preparation of samples and reagents:**

1. Thaw samples in ice-water bath, to decrease thaw time, remove ice shells that form around samples by removing samples from their floats.
2. Chill enough methanol (MeOH) for all samples at -20°C (2:1 ratio of MeOH:sample)
3. For *each* sample prepare the following tubes, labeled completely with a unique sample identifier.
    a. Two 2.0-mL microcentrifuge tubes, after labeling chill one on ice or at 4°C *before* adding sample
        i. One is used for the precipitation, the other is for the supernatant that is dried and used for NMR.
    b. One 0.6-mL microcentrifuge tube for pre-extraction aliquots
4. Put samples in ice-water bath. Gently swirl or invert sample tubes (**do not vortex**), try to dissolve any clots that are present. Remove one 30uL aliquot from each sample and add to the labeled 0.6mL microcentrifuge tubes. Store in an ice-water bath until all aliquots are collected, store long-term at -80°C.
5. Measure and record glucose level by spotting a few uL of each sample onto saran wrap and dipping test strips into each drop.

**II. Protein Precipitation**

1. Transfer remaining sample to corresponding 2.0-mL microcentrifuge tubes, **record intital volume**
   Do not exceed 600uL of sample volume in each tube, keep samples on ice.
2. Add cold MeOH to each tube to maintain a 2:1 MeOH: sample ratio.
   For samples with volume 440-520 add 1mL MeOH
   For samples w/volume less than 440uL or more than 520uL add the appropriate volume of MeOH to maintain a 2:1 ratio, rounding to the 10s place for the volume of MeOH to add.
   We have found that the best way to do this addition is to sort the samples by volume to add then add the appropriate volume to each sample (so all samples that get 1mL will have 1mL added to them, then all samples that get 880 will have MeOH added, etc).
   Vortex thoroughly (30s)
3. Incubate samples at -20°C for 20 minutes, be careful not to leave samples longer than 20 minutes, as ice crystals may form in the samples. Put samples in a pre-chilled or open sided rack to ensure they are adequately chilled.
4. Centrifuge tubes at 13,400 x *g*, 4°C for 30 min to pellet the proteins.
5. Transfer the supernatant to a clean, labeled 2.0 mL microcentrifuge tube. Use a glass pasteur pipet to transfer supernatant off the pellet, being careful not to disturb the pellet, **measure and record pre-lyophilization volume** with a 2-mL glass serological pipet.
6. Cap each 2.0-mL microcentrifuge tube with a kim-wipe secured with two rubber bands.

Immediately before lyophilizing, submerge each sample in liquid nitrogen for at least 2 minutes. Transfer samples to pre-chilled, insulated chambers and open vacuum, once samples reach low vacuum, the vacuum can be cut off from each chamber while the next sample is added. During lyophilization, chambers can be covered with foil to improve insulation.

7. Remove samples once dry, **record total lyophilization time and final lyophilizer vacuum**.

| Sample ID | Glucose conc (mg/dL) | Initial vol (uL) | MeOH added (uL) | Pre-lyophilization vol (mL) |
|---|---|---|---|---|
| R_001 | | | | |
| R_002 | | | | |
| R_003 | | | | |
| R_004 | | | | |
| R_005 | | | | |
| R_006 | | | | |
| R_007 | | | | |
| R_008 | | | | |
| R_009 | | | | |
| R_010 | | | | |
| R_011 | | | | |
| R_012 | | | | |
| R_013 | | | | |
| R_014 | | | | |
| R_015 | | | | |
| R_016 | | | | |
| R_017 | | | | |
| R_018 | | | | |
| R_019 | | | | |
| R_020 | | | | |
| R_021 | | | | |
| R_022 | | | | |
| R_023 | | | | |
| R_024 | | | | |

**Additional notes:**

**III. Resuspension**

1. Remove rubber bands and kim-wipe from the top of each microcentrifuge tube. If there is additional sample left on a kim wipe, scrape off with a clean spatula. Be careful when opening tubes, as the eppendorfs tend to have static.
2. Resuspend each sample in 500uL of 50mM sodium phosphate buffer in $D_2O$.
   To make 50mM sodium phosphate buffer make 50mM of mono- and di-basic sodium phosphate in $D_2O$, then add both types of buffer to create a solution with pH 6.8 (usually requires more monophosphate buffer than dibasic). Store buffer at 4 C.
3. Invert tubes to dissolve solids, vortex briefly (<10s) to resuspend all solids.
4. Samples can be transferred directly to filters for filtration, or can be
   Stored at -80°C. If samples are transferred to filters record the exact volume transferred (it may be different than 500uL) before starting filtration.