

Methanol Chloroform Extraction of Bovine Plasma and CCMBWB Healthy Human Whole Blood for NMR Analysis

Biomaterials Required:

~1.0 mL Whole Blood (per sample) collected with Heparin***

Samples:

Bovine Plasma (BP): non-sterile, Rockland, Cat #D400-06-0100, Lot #28662, one freeze-thaw cycle

Human whole blood healthy control, no freeze-thaw cycles

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 (-20°C); store mixture (-20°C) so it is ice cold when ready for use
- Ice cold DI water

Other needed materials/equipment:

- A. Parafilm
- B. Pasteur pipets (9" borosilicate glass; Fisher brand; 22-183-632 or 13-678-20D)
- C. Borosilicate glass serological filter-plugged 1mL pipets (VWR 93000-692)
- D. Refrigerated centrifuge
- E. Lyophilizer tubes (Fisher cat# 14-962-26D) – **Use for all areas where "culture tube" is indicated as well. Centrifuge rotor can only fit these tubes.**
- F. Lyophilizer/freezer-dryer- Stringer Lab (LabConco 1L benchtop)
- G. Speedvac (LabConco Centrивap Micro IR)- Stringer lab
- H. "Industrial" Sharpie pen for labeling tubes
- I. Disposable glass vials (Fisher brand; 03-339-25C borosilicate glass) for speedvac
- J. Generic round bottom 15mL flasks (bulbs)

** Blood must be stored on ice immediately after collection and frozen at -80°C as soon as possible (<1 hour).

***The only acceptable anti-coagulant for use with sample collection or storage for this protocol is heparin. EDTA will render samples unusable. Sodium or lithium heparin work equally well.

Use glass graduated cylinders and Pasteur pipets and the glass serological pipets for Chloroform/Methanol measurements.

All samples and solutions must be kept on ice or in the refrigerator at all times!
Only use glass pasteur pipettes for transferring!

Prechill all tubes/reagents in ice or in -20 before use!

1. Thaw samples in the fridge (4°C) and mix needed reagents/solutions.
2. Label two culture tubes for each sample; put the second set of tubes in the fridge (4°C).
3. **For Free and Total Hemoglobin Assay Test:** Gently invert sample tubes (DO NOT VORTEX) and remove one **50uL** aliquot (store at 4C) and one **5uL** aliquot (store at -80C) to labeled 0.7mL microcentrifuge tubes.
4. Add remaining volume (µL) of sample to each culture 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.
5. Add **2mL** 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 culture 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 (figure 1). Pellets from whole blood samples will be larger and harder than those of serum or plasma.
8. Resuspend the pellet of each sample in cold methanol:chloroform (**1mL**). 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.
10. Add **1mL** of ice-cold DI water to each of the fluid tubes. Vortex well.
11. Cool the fluid for **17 minutes** at -20°C. ALWAYS cool for exactly 17 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 **1mL** of ice-cold DI water to the tubes containing pellet. Suspend the pellet by vigorously vortexing or use a spatula as needed.

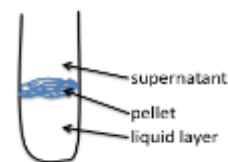


Figure 1: Representative depiction of blood or serum sample following centrifugation in step 5.

14. After the 20min freezer incubation, the fluid samples will have separated into a supernatant, aqueous top layer and a protein layer (see figure 2). 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).

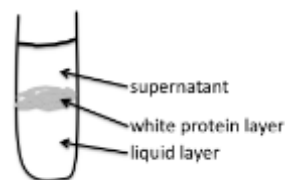


Figure 2: Representative depiction of blood or serum sample following freezer incubation in step 10.

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 **1mL** 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.
19. Allow the water soluble fractions to sit in the lyophilizer bulbs for 5 minutes. 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 glass serological pipet!
NOTE: Add 2mL of distilled water to each bulb to dilute out remaining methanol before freezing.
20. Freeze the lyophilizer glasses in -80°C freezer while tilting the glasses if possible (do this by placing the bulbs in the lyophilizer flask and covering each with kimwipes and placing in -80 overnight). Lyophilize for at least 24 h to ensure that methanol is removed. Do not exceed 48 h.
21. Transfer the lipid fractions to the 3.7mL borosilicate vials (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 tubes¹, wrap in parafilm and store at -20°C (can be stored for months).
22. When the water soluble samples are dry, they can be sealed with parafilm and stored (4°C) for up to a week. At the time of assay or for long-term storage, add 0.5mL of D₂O (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).

¹ solvent vapors can cause degradation and failure of plastic caps