**MPLEx preparation of bacterial samples**

Procedure:

1. Prepare cold chloroform:methanol (CHCl3:MeOH, prepared 2:1 v/v and kept at -20°C). This is added at a 5:1 ratio over sample volume.
2. If the samples are in solution, spin samples for 5 minutes at max speed to pellet cells. Remove and dispose of supernatant, and resuspend each pellet in 200uL HPLC water.
3. Transfer the sample into a 1.5mL Sorenson brand tube (these are the most solvent compatible tubes).
4. Add 1 mL of cold 2:1 CHCl3:MeOH for a 5:1 ratio of (CHCl3:MeOH):sample.
5. Vortex samples for 30 seconds following addition of solvent.
6. Let stand in the refrigerator for 5 minutes.
7. Vortex for 1 minute. Transfer samples to 1.5mL snap cap tubes.
8. Centrifuge the sample at 12,000 rpm, 4°C for 10 minutes.
9. Carefully remove the upper layer (water soluble metabolites) and lower layer (lipid soluble) into fresh 1.5mL Sorenson tubes. Dry down both aqueous and organic layers. Resuspend the lipid samples in 500 μL of 2:1 CHCl3:MeOH for storage (at -70°C).
10. Wash the interphase (protein) pellet with 1 mL of ice-cold MeOH. Vortex for 30 seconds then centrifuge at 12,000 rpm, 4°C for 10 minutes. Remove the MeOH and allow sample to completely dry in the Speed Vac (usually takes about 5 minutes).
11. Resuspend each sample in 100uL of freshly made lysis buffer (6M urea, 14.3mM β-ME in 50mM Tris-HCl, pH8)
    1. Aliquot 0.54g urea to 1.5mL tube.
    2. Add 1mL 50mM Tris-HCl, pH8. Vortex.
    3. Add 1.5uL β-ME.
12. Incubate at 60°C in the Thermomixer for 1 hour with shaking at 500rpm.
13. Add 900uL 50mM ammonium bicarbonate to dilute urea.
14. Add 2uL of trypsin gold. Incubate overnight at 37°C with shaking at 500rpm.
15. Perform C18 SPE clean-up. Pellet any debris in samples prior to applying to column.
    1. Condition columns with 1ml methanol.
    2. Rinse with 1ml 0.1% TFA water
    3. Add samples.
    4. Wash columns with 1 ml 95% 0.1%TFA water/5% ACN.
    5. Place labeled tubes under the columns and elute 1 ml of 80% acetonitrile and 20% 0.1% TFA water.
16. Dry down volume in speed vac to dryness (~3.5 hrs).
17. Resuspend samples in 50 µL 0.1% formic acid in water.
18. Perform BCA analysis of cleaned peptides (results below).
19. Adjust to desired concentration with 0.1% formic acid or other diluent and transfer to glass vials.
20. Store at -20°C until analysis.