Extraction protocols of metabolites from cells and control liver tissue samples
(to handle 20 exptl and 6 control samples)

1. Preparation of internal standard solution
   i) For example, prepare 1 mL of 10 mM solution for each internal standard and prepare similar solutions for up to 10 internal standards.
   ii) Combine all these 1 mL and 10 mM solutions to get final 10 mL of 1mM internal standard stock solution
   iii) Take two aliquots of 10 ul from the above stock solution in to two vials and dry them completely.
   iv) Reconstitute each vial with 200 ul of methanol:water (1:1), sonicate and combine the solutions of two vials
   v) Again rinse each vial with 100 ul of methanol:water (1:1), sonicate and combine the solutions of two vials
   vi) Combine solutions from v and vi steps to get 600 ul of final internal standard solution.
   vii) Keep the final solution on ice

2. Making of solutions for sample preparation
   i) For example, make 26 mL of methanol:water (4:1) sample solution under ice cold temperature to process 20 experimental samples and 6 liver sample controls

3. Preparation of a master solution spiked with internal standard
   i) Add 600 ul of internal standard solution from step 1 (vi) to 26 mL of methanol:water (4:1) sample solution of step 1 (i) to make final 26.6 mL final master solution
   ii) Vortex to mix the above master solution thoroughly and keep it under ice cold temperature

4. Preparation of experimental and control samples (always keep samples on ice)
   i) In case of cell metabolite analysis, freeze and thaw cell pellets in liquid nitrogen and water for three cycles, 30s each time
   ii) In case of control samples, use one 100 ug vial of liver tissue

5. Preparation of Experimental and control sample solutions
A) *Experimental sample solutions*

i) Add 750 ul of ice cold internal standard spiked master solution from step 3 (ii) to each 3 mL size sample vial containing freeze-thawed cell pellet solutions

ii) Sonicate all the solutions (Settings: Amp 30%, 20-30s, 3 times)

iii) Wash rotor with 10ml de-ion water in between each group.

iv) Wash rotor with Methanol and water separately between each sample sonication

B) *Control sample solutions*

i) Add 3 mL of ice cold internal standard spiked master solution from step 3 (ii) to 100 ug vial of liver tissue.

ii) Homogenize all the solutions (settings: 6, 30 sec and 30 sec)

iii) Split the homogenized solution into 4 replicates (each having ~750 µl, weighing roughly 25mg per replicate.

iv) Take first three replicates

C) Vortex both experimental and control samples for 5 min and keep them on ice

6) **Extraction of metabolites**

A) *CHCl₃ extraction (caution: extraction should be performed in glass tubes instead of plastic vials)*

i) Add 450 ul of ice cold CHCl₃ (preserved at −20 for 10 min) to each sample solution in step 5 (C) and vortex for 10 min at speed 8

ii) Add 150 ul of ice cold water to the above each sample mixture and vortex for 2 min

iii) Keep the solutions at −20C for 20-30min

iv) Centrifuge all the sample solutions at 4000 rpm for 10 min @ 4C

v) Pipette out separate layers (organic and aqueous phase), combine them and use the entire supernatant.

vi) Dry the supernatant solution @ 37C, 30-45 min.

vii) Dissolve again in 500 ul of methanol:water (1:1) (vortex − 5min, sonicate − 5min, spin @ 5000rpm−5min) and filter to separate proteins
B) Protein separation by Amicon filters

i) Precondition the Amicon protein filters by prewashing and centrifuging them w/ 500 µl 50:50 Methanol: Water (15000 rpm @ 4°C ~20min) until the entire solution is filtered out

ii) Discard the tube and keep the filter in a new tube

iii) Transfer the samples from 6 A (vii) into the pre-washed filters and centrifuge it to collect the filtered samples. Add additional 100 µl 50:50 Methanol: Water to the filter and centrifuge it to collect the remaining metabolites if any.

iv) Collect the filtered solution and dry them.