**Protocol for Liquid NMR Studies of Tissues/Cells Extractions**

This Protocol is based on metabolomics studies from Jeremy K Nicholson, *et al.* Nature Protocols, 2007, 2(11):2692-2703 and Mark R. Viant, *et al*. Metabolomics, 2007,3(1):55-67.

**1. Collection and Storage.**

All samples were stored at -80 °C until used randomly.

**2. Ground/Homogenized**

Tissues could be ground by mortar/pestle manually or disrupted by homogenizer in solvents. It was found that the homogenization method (Sonicator or mechanical homogenizer) gave less inter-sample variability than mortar/pestle ground method. So the homogenization method was adopted.

**3. Extraction Procedures (MeOH-CHCl3-H2O)**

The extraction procedure with ice-cold MeOH-CHCl3-H2O had the best overall results than other solvents.

**3.1** Prepare ice-cold solvents (MeOH, CHCl3 and H2O).

**3.2** Weigh the frozen tissue and transfer into a glass vial.

**3.3** Homogenize after adding 0.5 ml MeOH and 106 μl deionized water for each sample in ice bath. Vortex the mixture and then add 0.25 ml chloroform. Vortex again. (Appendix for solvent to tissue ratio is attached)

**3.4** Add 0.25 ml chloroform and 0.25 ml deionized water in the mixture and vortex again. At this stage, the mixture will become white cloudy suspension.

**3.5** Let the mixture sit in ice bath or refrigerator for 15 min, centrifuge at 7200 rpm for 18 minutes at 4 °C. The upper layer is MeOH/H2O phase with polar metabolites; the lower layer is CHCl3 phase with the non-polar metabolites. Protein and cellular debris are between those two layers. If the separation is not good, centrifuge the mixture again.

**3.6** Transfer the different layers into different glass vials separately with syringe (1000 µl, hamilton) and label them clearly. Remove the solvents with speedvac or lyophilizer (MeOH/H2O layer).

**3.7** The metabolites will be frozen at -80 °C after solvents removal.

**4. NMR Spectra**

Suspend the tissue extracts with 600 μl D2O (Containing 0.05mM DSS, internal reference), vortex samples and centrifuge at 5000 rpm for 2 min, transfer about 550 μl D2O solution into a 5 mm NMR tube for measurement (4 °C).

0.05 mM DSS D2O solution was prepared as following: 98.2mg DSS was dissolved into 100ml D2O, and 1ml of this solution was diluted to 100 ml with D2O in 100 ml volumetric flask. Shake well and seal with parafilm.

**5. Data Analysis from NMR Spectra**

The intergral of peaks was normalized by internal reference and mass and compared between the control and the treatment groups. PCA and OPLS were used to explore the biomarkers metabolomics.

\*Glass vials are needed for all steps when using chloroform. Tests have showed polypropylene tube is not good for this protocol.

Table 1. Solvents-Material ratio for Extraction (0 °C)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Material | MeOH | H2O | CHCl3 | CHCl3 | H2O |
| (g) | (ml) | (μl) | (ml) | (ml) | (ml) |
| 1 g | 4 | 850 | 2 | 2 | 2 |
| 0.5 g | 2 | 425 | 1 | 1 | 1 |
| 0.25 g | 1 | 213 | 0.5 | 0.5 | 0.5 |
| 125 mg | 0.5 | 106 | 0.25 | 0.25 | 0.25 |
| 62.5 mg | 0.25 | 53 | 0.125 | 0.125 | 0.125 |
| 31.3 mg | 0.25 | 53 | 0.125 | 0.125 | 0.125 |
| 10.0 mg | 0.25 | 53 | 0.125 | 0.125 | 0.125 |