

## Metabolomics Extraction Protocol of Saliva Samples

1. Time of sample collection must be justified to decrease the interference from the food and drink. It is better to fasten the animals for 1-2hr.
  2. Visual inspection must revealed no contaminations, e.g. hair and blood, in the samples.
  3. The samples must be stored in a freezer at  $-80^{\circ}\text{C}$  until extraction.
  4. Equal samples amount must be used during the extraction.
  5. Cold extraction buffer with thawing and freezing technique
- Prepare extraction solvent: formed from methanol: dichloromethane: ethyl acetate 10:2:3.
  - Extraction solvent kept in  $-80^{\circ}\text{C}$  freezer for at least 2hr.
  - 200  $\mu\text{l}$  of the sample mixed with 900  $\mu\text{l}$  of pre-cooled extraction solvent in an Eppendorf tube.
  - Vigorous mixing using vortex for 1 minutes.
  - The tube is transferred into dry ice or freezer ( $-80^{\circ}\text{C}$ ) for 30 min.
  - The sample is thawed in an ice bath then it is sonicated for 5 min at 20-30 kHz. (temperature must be not greater than  $20^{\circ}\text{C}$ )
  - The sample is centrifuged at 10,000 for 10 min  $4^{\circ}\text{C}$ .
  - The supernatant is transferred to a new tube.
  - The sample is centrifuged at 10,000 for 10 min. The supernatant is transferred to a new tube.
  - The supernatant evaporated using vacuum rotary evaporator at  $30^{\circ}\text{C}$  to approximately 25  $\mu\text{l}$  volume.
  - The sample reconstituted in 200 $\mu\text{l}$  solvent (water: methanol: acetonitrile 2:1:1).
  - Centrifuged at 10,000 for 5min. The supernatant is transferred to analysis tube.
  - Analysis using 5-25  $\mu\text{l}$  injection volume (depending on the complicity of the sample) is applied to the LC-MS/MS. This will be used for positive and negative mode.

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- Mobile phase depending on the mode:

Positive mode mobile phase	Negative mode phase
DI-Water contains 0.1%FA	5mM ammonium format containing 1% methanol. Then the pH will be adjusted to 8 using ammonium hydroxide.

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1. Quality control (QC) samples, which are a mixture of equal volume taken from each real sample, also underwent LC-MS/MS analysis for quality assurance of the experiment.
2. It is better to spike the sample with internal standards. These internal standard is better that not included in the endogenetic metabolites. You can also use another one depending on experimental design.

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