

Protocol and Methods for Non-targeted Metabolomics Analysis of Cell Samples

6.1.1 Sample preparation and extraction

6.1.1.1 Unconventional samples

Take all samples for freeze-drying. A 500 μ L solution (Methanol : Water = 4:1, V/V) containing internal standard was added into the sample and vortexed for 3 min. The sample was placed in liquid nitrogen for 5 min and on the dry ice for 5 min, and then thawed on ice and vortexed for 2 min. This freeze-thaw circle was repeated three times in total. The sample was centrifuged at 12000 rpm for 10 min (4 °C). All of the supernatant was transferred and concentrated. A 100 μ L solution (Methanol : Water = 7:3, V/V) was used to reconstitute the sample. Then the sample was vortexed for 3 minutes, and sonicate for 10 minutes in an ice bath. The sample was then centrifuged at 12000 rpm for 3 min (4 °C). A 80 μ L aliquots of supernatant were transferred for LC-MS analysis.

6.1.2 HPLC Conditions

All samples were for two LC/MS methods. One aliquot was analyzed using positive ion conditions and was eluted from T3 column (Waters ACQUITY Premier HSS T3 Column 1.8 μ m, 2.1 mm * 100 mm) using 0.1 % formic acid in water as solvent A and 0.1 % formic acid in acetonitrile as solvent B in the following gradient: 5 to 20 % in 2 min, increased to 60 % in the following 3 mins, increased to 99 % in 1 min and held for 1.5 min, then come back to 5 % mobile phase B within 0.1 min, held for 2.4 min. The analytical conditions were as follows, column temperature, 40 °C; flow rate, 0.4 mL/min; injection volume, 4 μ L; Another aliquot was using negative ion conditions and was the same as the elution gradient of positive mode.

6.1.3 MS Conditions (QE)

All the methods alternated between full scan MS and data dependent MS_n scans using dynamic exclusion. MS analyses were carried out using electrospray ionization in the positive ion mode and negative ion mode using full scan analysis over m/z 75-1000 at 35000 resolution. Additional MS settings are: ion spray voltage, 3.5 KV or 3.2 KV in positive or negative modes, respectively; Sheath gas (Arb), 30; Aux gas, 5; Ion transfer tube temperature, 320 °C; Vaporizer temperature, 300 °C; Collision energy, 30,40,50 V; Signal Intensity Threshold, 1*e6 cps; Top N vs Top speed, 10; Exclusion duration, 3s.

6.2.1 PCA

Unsupervised PCA (principal component analysis) was performed by statistics function prcomp within R (www.r-project.org). The data was unit variance scaled before unsupervised PCA.

6.2.2 Hierarchical Cluster Analysis and Pearson Correlation Coefficients

The HCA (hierarchical cluster analysis) results of samples and metabolites were presented as heatmaps with dendrograms, while pearson correlation coefficients (PCC) between samples were calculated by the cor function in R and presented as only heatmaps. Both HCA and PCC were carried out by R package ComplexHeatmap. For HCA, normalized signal intensities of metabolites (unit variance scaling) are visualized as a color spectrum.

6.2.3 Differential metabolites selected

For two-group analysis, differential metabolites were determined by VIP (VIP > 1) and P-value (P-value < 0.05, Student's t test). VIP values were extracted from OPLS-DA result, which also contain score plots and permutation plots, was generated using R package MetaboAnalystR. The data was log transform (log₂) and mean centering before OPLS-DA. In

order to avoid overfitting, a permutation test (200 permutations) was performed.

6.2.4 KEGG annotation and enrichment analysis

Identified metabolites were annotated using KEGG Compound database (<http://www.kegg.jp/kegg/compound/>), annotated metabolites were then mapped to KEGG Pathway database (<http://www.kegg.jp/kegg/pathway.html>).

并使用 DataTrack ID **6594** 和 mwTab 文件名 **Zzm_20251024_193733** 输入研究的元数据和结果