# Metabolomics Studies Paradigm Shift with Quanfirmation: Integrating Untargeted Profiling, Targeted, and Pseudo-Targeted Analysis on One Platform

Poster Note 64399

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## Overview

**Purpose:** To demonstrate a comprehensive metabolomics research strategy using a high-resolution MS platform with quanfirmation capabilities.

**Methods:** Untargeted profiling, targeted and pseudo-targeted analyses were integrated for an exemplified cellular metabolomic study using quadrupole-Orbitrap MS.

**Results:** Quanfirmation capacity enables a metabolomics studies paradigm shift to integrate three mainstream metabolomics practice styles on one MS platform.

## Introduction

MS-based metabolomics investigates metabolic phenotype variations delineated with qual/quan information of small molecule patterns in biosystems. It can be roughly classified into two subtypes: discovery-driven untargeted profiling analysis mainly using HRAM MS and hypothesis-driven metabolite targeted analysis using tandem triple quadrupole MS. Generally, method transformation between instruments is inevitable and often tedious from differential discovery to quantitative validation/verification experiments in a comprehensive metabolomic workflow. Herein, we proposed a metabolomics research paradigm shift with a hybrid quadrupole-Orbitrap mass spectrometer through its HRAM qualitative power and quantitative ability comparable to triple quadrupoles. We conducted a cellular metabolomic study using hydrophilic interaction chromatography (HILIC) separation, integrated differential metabolites discovery with untargeted profiling, acylcarnitines targeted analysis, and pseudo-targeted analysis with a local metabolome database into one platform, as shown in Figure 1.

## Methods

### Sample Preparation

Intracellular metabolome of G1 high-expressing and normal human pancreatic cancer cell line *Panc-1* were investigated in this study. Cell harvesting and metabolite extraction were implemented using protocols established before.[1] Briefly, flash quenching with liquid nitrogen and extraction with methanol solvent were used.

### Liquid Chromatography / Mass Spectrometry

HILIC separation was carried out on a Thermo Scientific™ Dionex™ UltiMate™ 3000 UHPLC system by using an underivatized bare silica gel HILIC column at a 300 µL/min flow rate and column temperature of 40 °C. Binary mobile phases were (A) 5% water in acetonitrile adjusted with 10 mM ammonium formate and 0.1% formic acid, and (B) 50% water in acetonitrile using the same buffers. A linear gradient was implemented as follows: 0–1.0 min holding at 100% A, linearly increasing to 100% B at 20 min, then washing column for the next 4.9 min, and equilibrating until 30 min. A 5 µL aliquot of extracted sample was injected for detection with the Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer equipped with a heated ESI source. Untargeted profiling and pseudo-targeted analyses used the same raw data, which was acquired with full-scan (80-900 *m/z*) at 70,000 (FWHM at *m/z* 200) resolution followed by Top-10 data-dependent MS/MS at 17,500 (FWHM at *m/z* 200) resolution under positive ionization mode. The main parameters for MS/MS included: AGC target - 2e5, maximum IT - 60 ms, isolation window - 2.0 *m/z*, normalized collision energy - 30% ± 50%, apex trigger - 6–12 s, dynamic exclusion - 6s. Ionization conditions were optimized and finally operated at spray voltage - 3.5 kV, heater and capillary temperature 350 °C and 275 °C, respectively. For targeted analysis, multiplexed tSIM, PRM, and full scan within a scheduled time window were employed for routine quantitation of acylcarnitines (Table 1).

**FIGURE 1. Overview of Q Exactive MS based metabolomics workflow adopted in the study using cell metabolome as research model.**

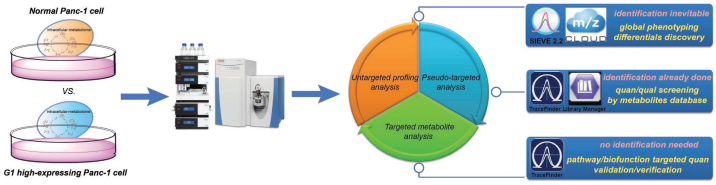


**TABLE 1. Quantitative methods used for acylcarnitines targeted analysis after untargeted profiling analysis revealing lipids and energy metabolism globally changed.**

| Acylcarnitines | Exact *m/z* | Rt /min | Quan mode | Time segment |
|---|---|---|---|---|
| Carnitine | 162.11247 | 12.31 | FS@70K FWHM | 8.5-20 min |
| C2-carnitine | 204.12303 | 11.93 | FS@70K FWHM | 8.5-20 min |
| C3-carnitine | 218.13868 | 11.31 | FS@70K FWHM | 8.5-20 min |
| C4-carnitine | 232.15433 | 10.71 | FS@70K FWHM | 8.5-20 min |
| C6-carnitine | 260.18563 | 8.82 | PRM@35K FWHM | 8.5-20 min |
| C8-carnitine | 288.21693 | 8.27 | 6-msx-tSIM@70K FWHM | 0-8.5 min |
| C10-carnitine | 316.24824 | 8.00 | 6-msx-tSIM@70K FWHM | 0-8.5 min |
| C14-carnitine | 372.31084 | 7.75 | 6-msx-tSIM@70K FWHM | 0-8.5 min |
| C16-carnitine | 400.34214 | 7.69 | 6-msx-tSIM@70K FWHM | 0-8.5 min |
| C18-carnitine | 428.37344 | 7.59 | 6-msx-tSIM@70K FWHM | 0-8.5 min |
| C20-carnitine | 456.40474 | 7.54 | 6-msx-tSIM@70K FWHM | 0-8.5 min |

### Data Analysis

For untargeted profiling, data mining was performed with Thermo Scientific™ SIEVE™ 2.2 label-free differential analysis bioinformatics software. Putative structural annotation was fulfilled through searching against HMDB, KEGG, and METLIN metabolome databases with full-scan exact mass and against mzCloud and METLIN spectral databases for MS/MS confirmation. Thermo Scientific™ TraceFinder™ software was employed for routine quantitation in targeted metabolite analysis. It was also used for the qual/quan data process of pseudo-targeted analysis through matching a local metabolome in-house database, where peak area of XIC in full scan was used for quantitation, and exact *m/z*, isotopic distribution pattern, characteristic fragments, and MS/MS spectrum library were for metabolite qualitative confirmation. Heatmap plots were depicted using free software Cluster 3.0 and TreeView.

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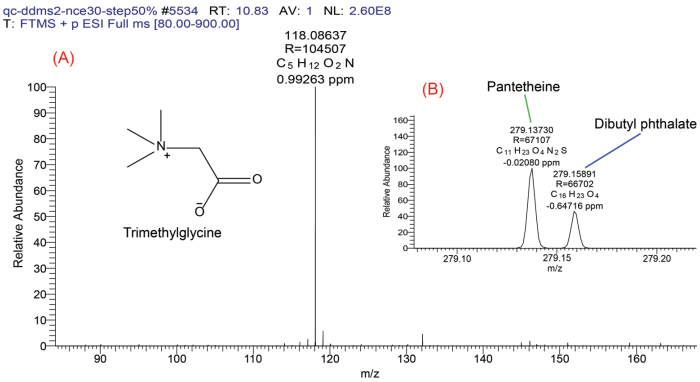
# Results

### Data Quality Evolution

The high data quality of the untargeted profiling analysis was attributed to superior HRAM capacity and quanfirmation (quan/qual) power of the Q Exactive platform, which provide the structural annotation with high confidence and convenience, and the quantitation of metabolites with high specificity and sensitivity in a complex biological matrix.

**FIGURE 2. (A) Q Exactive MS producing more than 100K FWHM resolution for small metabolite trimethylglycine, (B) High resolving power distinguishing pantetheine with ubiquitous plasticizer interference and guaranteeing sub-1 ppm mass accuracy of metabolites.**




### Untargeted Profiling Analysis

Metabolic phenotypes in different cells had been comprehensively changed. By using volcano plot, it's found 430 metabolic components were differentially expressed with significance level p<0.05. It revealed several endogenous metabolism patterns of modified base/base, nucleotide, amino acid/AA derivate, phospholipid/glycerolipid, carnitine shuttle, peptides, cofactors, and vitamins were profoundly transformed.

**FIGURE 3. (A) Mirror plot demonstrating clear disparity between comprehensive metabolic profiles of G1 high-expressing and normal *Panc-1*cells. (B) Heatmap of untargeted profiling data with hundreds of metabolic components extracted by SIEVE 2.2 software.**

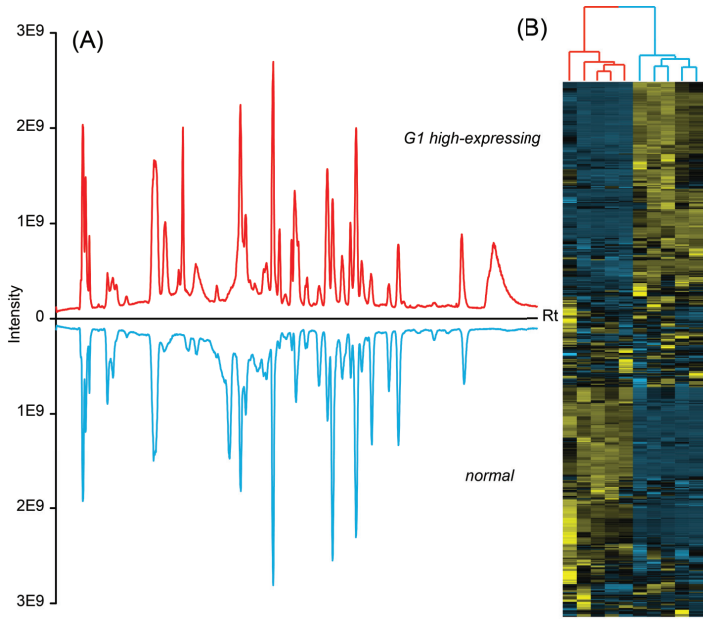
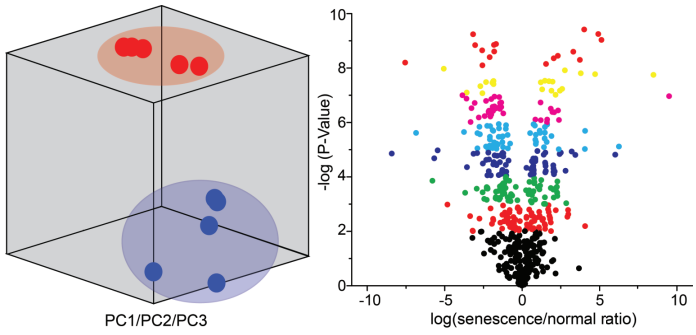


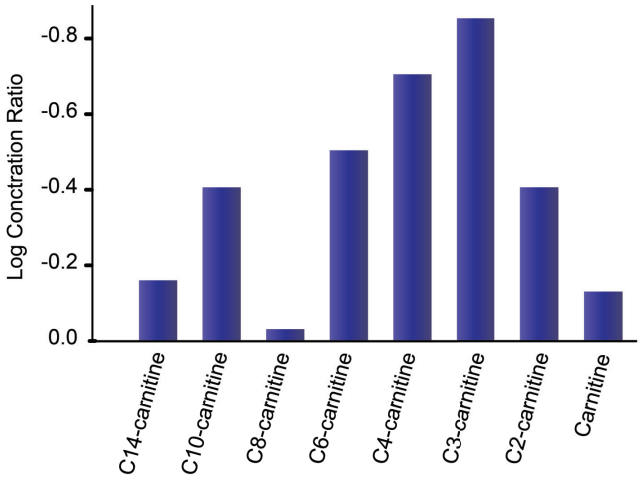

**FIGURE 4. Left: 3D score scatter plot of PCA with untargeted profiling data processed with SIEVE 2.2 software, revealing significant meta-phenotype disparity of G1 high-expressing and normal *Panc-1* cells. Right: Volcano plot of metabolic components deconvoluted by SIEVE2.2 software showing many changed significantly.**



### Targeted Metabolite Analysis

After bio-functional annotation of the differential metabolites discovered in the untargeted profiling analysis, free carnitine and acylcarnitine, an important shuttle molecule in energy metabolism, were submitted to targeted analysis to verify the findings deduced from untargeted profiling analysis and meanwhile to extend metabolome coverage. Based on the quantitative performance with good linearity, high sensitivity, and specificity of Q Exactive MS, as well as high usability of multiple quantitation methods, discovery-driven untargeted profiling research could be easily evolved into validation/verification studies.

**FIGURE 5. Log transformed abundance ratio values of acylcarnitines in G1 high-expressing *vs.* normal group.**

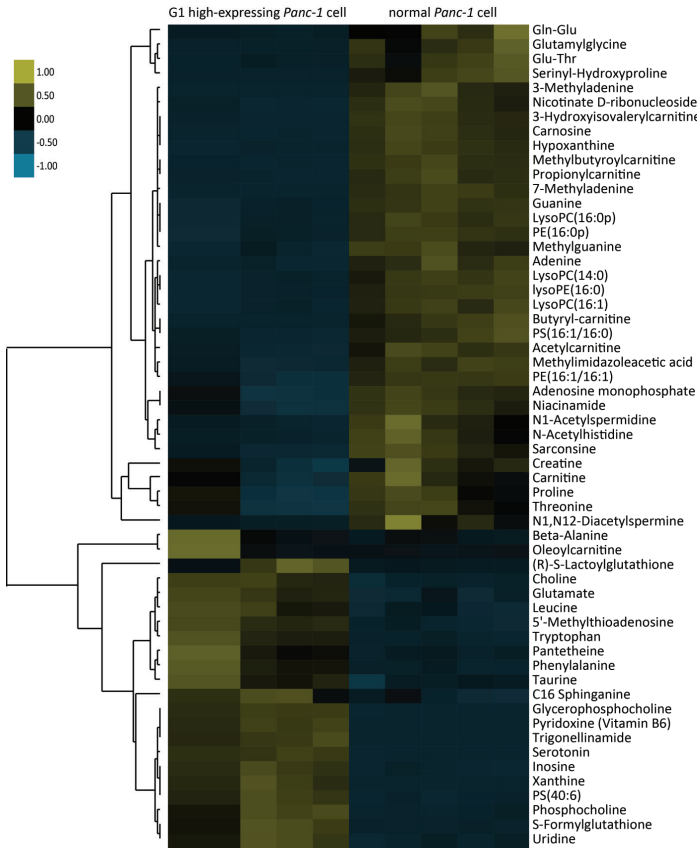



### Pseudo-Targeted Analysis

Chemical identification of differential metabolic components was inevitable in most cases of untargeted profiling analysis. Alternatively, pseudo-targeted analysis, another strategy with high efficiency and throughput of data-processing, was adopted to circumvent tedious identification work one-by-one by using a predefined in-house metabolome database/library to take full use of the qual/quan power of the Q Exactive MS simultaneously.

**FIGURE 6. TraceFinder software squeezes all LC-MS data to automate qual/quan information extracting of the metabolome, i.e. XIC within narrow mass tolerance for relative quan and qual confirmation combining exact *m/z*, fine isotopic pattern, characteristic fragments, reference MS² spectrum as well as retention time.**

**FIGURE 7. Heatmap of pseudo-targeted analysis data processed with TraceFinder software to automatically quantify metabolites and identify them using a predefined metabolome database and MS² spectral library (partial list).**




## Conclusions

Wide popularity of the Q Exactive mass spectrometer with leading HRAM characteristics and quanfirmation capacity enables a metabolomics studies paradigm shift to integrate three mainstream metabolomics practice styles: untargeted metabolic profiling, pseudo-targeted, and targeted metabolite analysis on one MS platform. This will facilitate not only delineating holistic and subtle metabolic patterns but also circumventing method transformation between platforms.

## Reference

1. Bi, H.C.; Krausz K.W., *et al. Anal Bioanal Chem.*, **2013**, *405*(15), 5279-5289.

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