An Ultra High-throughput Plasma Protein Profiling (uHTPPP) Workflow Using a Modified Quadrupole-Orbitrap Mass Spectrometer

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ABSTRACT

Purpose: An automated plasma protein profiling workflow on the new Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer can deliver the consistency, sensitivity, and versatility required for translational research.

Methods: Human serum was processed for LC-MS/MS analysis using the EasyPep™ 96 well kits on a Hamilton STARLet liquid handling robotic system. The resulting peptides were separated on a high throughput EvoSep One LC and analyzed on the new Orbitrap Exploris 240 mass spectrometer.

Results: With a standardized workflow and a robust mass spectrometer (Orbitrap Exploris 240), we observed excellent day-to-day consistency of protein identification and quantitation with less than 5%CV and detected 500 plasma proteins from Top14 depleted lung cancer serum with reproducible quantitation of >60% of proteins with <20% CV.

INTRODUCTION

Using biomarkers from easily assessable biofluids such as blood and urine is highly beneficial for early detection of diseases as well as monitoring therapeutic response from patients. However, proteomics analysis of blood remains challenging because of the vast dynamic range of the plasma proteome and heterogeneity among individuals. The conventional proteomics workflow based on manual manipulation and nanoflow excels in sensitivity but lacks the necessary throughput to analyze population heterogeneity with statistical rigor. In this study, we developed a standardized plasma protein profiling workflow solution (uHTPPP) that consists of an automated sample preparation protocol, a high throughput LC, and the new Orbitrap Exploris 240 mass spectrometer (MS). The uHTPPP workflow enables rapid sample turnover using an automated sample preparation protocol with low variability. The Evosep One LC allows for short LC gradients and standardized methods to maximize the number of samples analyzed per day for large scale studies. In addition, we introduced the utility of a Thermo Scientific™ EASY-Spray™ ES806A column, with integrated emitter and column heater, for the analysis of digested peptides from human serum. Finally, the Orbitrap Exploris 240 MS showed robust performance and demonstrated the feasibility to support biomarker discovery for translational research.

MATERIALS AND METHODS

Material

Reagents and HPLC grade buffers used for proteomics analyses are from Thermo Fisher Scientific. Pierce™ Peptide Retention Time Standards (PRTC) Kit, HeLa protein digest standard, and EasyPep™ MS sample preparation kit are from Thermo Fisher Scientific (Rockford, IL).

Sample Preparation

Commercial human lung cancer serum samples from several individuals were purchased from BioreclamationIVT. Aliquots of lung cancer serum samples were pooled and depleted of abundant proteins using the Thermo Scientific™ High Select™ Top14 Abundant Protein Depletion Midi Columns (A36369). Undepleted plasma or depleted serum samples were processed by automating the Thermo Scientific™ EasyPep™ 96 well MS Sample Prep Kit on a Hamilton STARLet liquid handling system with a high-throughput filtration method [MPE]2 — Monitored Multi-flow, Positive Pressure Evaporative Extraction module. Digested peptides (500ng or 200ng) were loaded onto EvoTip disposable trap columns manually or by automation with a Hamilton liquid handling workstation.

LC-MS Analysis

Peptides from digested samples (HeLa digest standard, serum, and plasma) were separated using a Thermo Scientific™ EASY-Spray™ column ES806A (150 µm x 15 cm) and an EASY-Spray ion source equipped Orbitrap Exploris 240. The integrated column heater setup allows for reproducible peptide separation and stable chromatographic retention time. The mobile phase A is composed of 0.1% formic acid in water (HPLC grade), and the mobile phase B is composed of 0.1% FA in acetonitrile. Peptides were loaded on Evotips based on the manufacturer's protocol. Data dependent acquisition (DDA) and Evosep One optimized gradient methods were used to acquire LC-MS data.

System suitability runs were performed using 1 µg of HeLa digest loaded onto a Thermo Scientific™ ES803A EASY-Spray column (75 µm x 50 cm), separated on a 60 min gradient delivered by a Thermo Scientific™ Ultimate 3000 RSLC.

Data Analysis

Thermo Scientific™ Proteome Discoverer™ 2.5 software was used for multiple precursor spectral library search, AI-driven post-acquisition confidence validation, and quantification. Feature mapping was used to increase both identified and quantified proteins based on MS[1] retention time alignment with MS[2] evidence in each experiment. GraphPad Prism was used for large dataset figure generation and correlation analysis.

Results

Figure 1. Hardware innovations of the new Orbitrap Exploris 240.

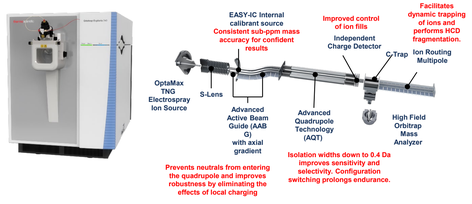


Figure 2. Schematic illustration of the uHTPPP workflow. It is a high throughput and robust workflow solution that can be easily scaled to analyze samples from large human cohorts for biomarker discovery.

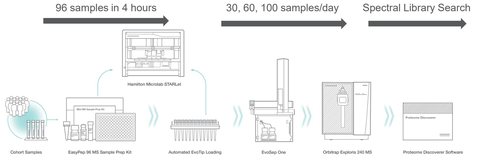


Table 1. Automated sample preparation increases recovery while reducing both the variability and processing time for each sample, compared to manual preparation. A. 20 µg of digested peptides were loaded, washed, and eluted from both manual spin columns and the 96-well plate. Triplicate analyses were carried out per method. **B**. 45 µg of undepleted serum proteins were processed through the entire EasyPep workflow, either manually or with the automated scripts. **C**. The automation workflow has been optimized for both serum and plasma samples, with comparable results, despite differences in sample viscosity.

A	Peptide clean-up only		
	Input	% Recovery	%CV
Manual spin column	20 µg	68.5%	5.7%
Automated 96-well plate on Hamilton	20 ug	72.1%	3.7%

B	Full Protein workflow		
	Input	% Recovery	%CV
Manual spin column	45 µg	77.9%	10.7%
Automated 96-well plate on Hamilton	45 ug	80.7%	7.2%

C	Processing time (per sample)	Serum		Plasma	
		% Recovery	% CV	% Recovery	% CV
Manual spin column	20 mins	77.9%	10.7%	60.4%	6.2%
Automated 96-well plate on Hamilton	2.5 mins	80.7%	7.2%	81.0%	8.9%

Figure 3. Reproducibility of plate-to-plate recovery using the automated sample preparation protocol. We used undepleted pooled serum or lung cancer plasma to examine the performance of the uHTPPP workflow on the new Orbitrap Exploris 240. We used peptide recovery efficiency to demonstrate plate-to-plate consistency for sample preparation. Using the EasyPep 96 well kit and automated sample prep script on the Hamilton robotic liquid handling system, the peptide recovery efficiency from each well and each plate were determined and close to 90% of peptides were recovered consistently from three 96 well plates or 288 serum samples. Less than 10% CV were observed for plate-to-plate variability.

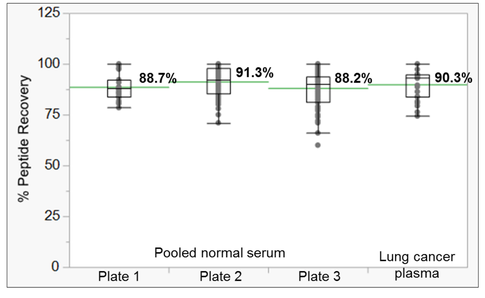


Figure 4. uHTPPP workflow results in consistent protein and peptide identifications over 5 days of consecutive analysis. 20 EvoTips containing 200 ng of Top14 depleted human pooled serum were analyzed each day for 5 consecutive days in a mock-workflow format using the uHTPPP workflow on the new Orbitrap Exploris 240. 100ng of HeLa digested lysate was used to monitor the instrument performance in the workflow. Over 5 days of analysis, we observed consistent performance on LC, column, and instrument, resulting in reproducible protein and peptide identifications.

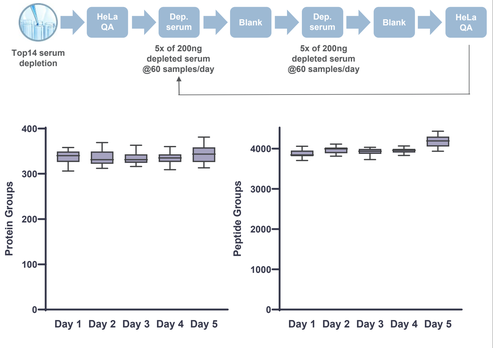


Figure 5. uHTPPP workflow has robust mass accuracy and retention time stability over 5 days of continuous analysis. The EvoSep One LC coupled to an EASY-Spray column allows for excellent run-to-run retention time stability, with the use of the integrated column heater for additional performance improvements. The PRTC peptides spiked into each sample showed < 6% CV for retention time. The new Orbitrap Exploris 240 shows tight mass accuracy more than 5 days out from calibration, with less than 5 ppm of mass accuracy drift. Together, these two parameters allow for more reliable identifications and feature mapping with little manipulation of the raw data in post-acquisition processing.

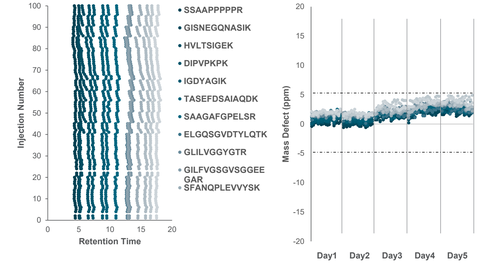


Figure 6. The new Exploris 240 showed consistent and reliable instrument performance. System suitability runs were performed to collect data from 4 different Exploris 240 at three different sites. Less than 5%CV was observed among these four Exploris 240 demonstrating exceptional reproducibility of the analytical performance.

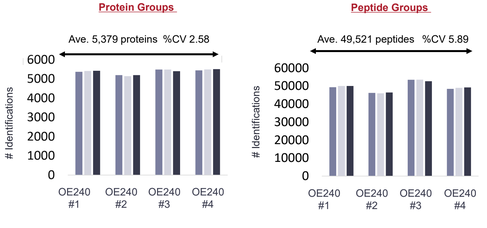


Figure 7. Consistent dynamic range detection on the Orbitrap Exploris 240 allows for excellent Label-Free Quantitation for biomarker discovery across 5 days of analysis. 5 days of analysis of Top14 depleted pooled serum shows excellent PSM dynamic range reproducibility. The result is consistent quantitation for 5 straight days, which is essential for biomarker discovery, and shows the robustness of the Orbitrap Exploris 240 in the analysis of large cohorts of biofluids.

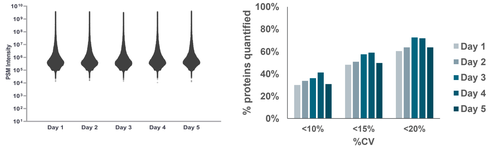


Figure 8. Varying loading amounts and gradient lengths allow reproducible detection of 500 plasma proteins from depleted lung cancer serum. 200 or 500 ng of depleted serum were loaded for LC MS/MS analysis on the new Orbitrap Exploris 240 MS using the 60 sample per day (21 minute) or 30 sample per day (44 min) Evosep One LC methods to demonstrate the sensitivity and robustness of the instrument performance. Feature mapping was applied to the dataset to determine the total identified proteins.

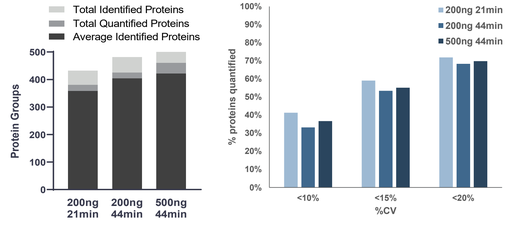
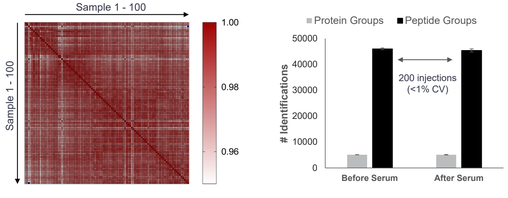


Figure 9. Data generated from the uHTPPP workflow on the new Orbitrap Exploris 240 were consistent across hundreds of injections, with a minimal impact on overall system performance. Over 100 sequential injections, LFQ values of plasma proteins detected on the Orbitrap Exploris 240 showed high correlation (spearman's correlation >0.95) for protein quantitation among replicates. Additionally, the Orbitrap Exploris 240 showed no deterioration in performance after over 200 injections of biological samples.



CONCLUSIONS

- The uHTPPP workflow on the new Orbitrap Exploris 240 allows for robust analysis of human plasma and serum with maximum instrument uptime, high throughput sample preparation, and excellent quantitation reproducibility for translational research. Less than 10% CV was observed from sample preparation variability and less than 5% CV was observed from protein detection.
- The Evosep One LC, combined with the EASY-Spray ES806A column with an integrated emitter and column heater on the EASY-Spray source, demonstrated excellent run-to-run reproducibility of retention time, total protein and peptide identification, and label free quantitation across multiple days of runtime and hundreds of injections. Additionally, ES806A column (150µm x 15 cm) has a higher peptide loading capacity compared to the Evosep column solution (100µm x 8 cm).
- An optimized method for plasma protein profiling is included as a method template on the Exploris 240 to assist researchers to generate high quality data with precise mass accuracy from precious human samples and serve as a reliable workhorse for proteomics analysis.

TRADEMARKS/LICENSING

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