APPLICATION NOTE 65952

A robust mass spectrometer for precision medicine – the Orbitrap Exploris 240 mass spectrometer for large-scale plasma protein profiling

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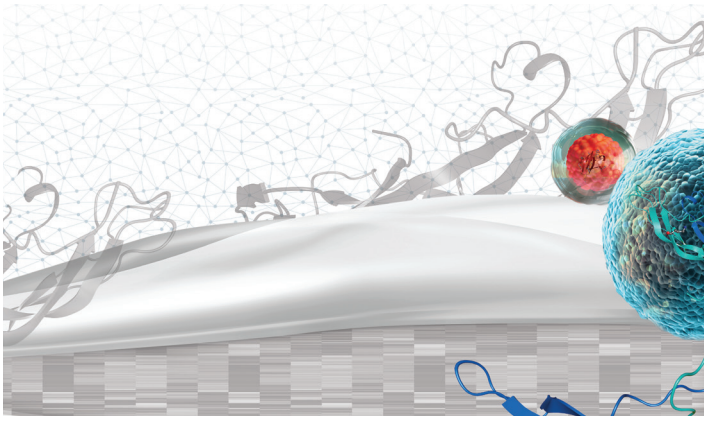


Goal

To demonstrate the reliability and robustness of the Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer for large-scale, untargeted plasma protein profiling.

Introduction

Biomarkers are greatly needed for managing patients with diseases and improving efficacy of biotherapeutics. Blood is an ideal biological material for biomarker discovery because its collection can be done routinely and repeatedly in the clinic. With emerging efforts of establishing large biorepositories, research laboratories race to assemble robust and reproducible mass spectrometry-based workflows that can be scaled up to detect disease relevant plasma protein biomarkers, and can be implemented in the clinic to benefit the affected population.

Previously, we described an automated sample preparation solution that can be utilized to reduce analytical variability by using a liquid handling robotic platform (AN-65727). In this application note, we focus on describing a LC-MS/MS platform that can be easily implemented to conduct large-scale, human cohort plasma protein profiling studies. An easy to operate Evosep One LC was coupled to an Orbitrap Exploris 240 mass spectrometer using the Thermo Scientific™ EASY-Spray™ ionization source and a Thermo Scientific™ EASY-Spray™ HPLC column. We optimized the MS acquisition parameters for short LC gradients (60 SPD), and the resulting MS method is included as

a pre-loaded method template (plasma profiling) in the Method Editor for the Orbitrap Exploris 240 MS. Using the preset method (LC and MS), we performed analyses of over 200 digested pooled human serum injections five weeks apart to examine reproducibility and robustness of the LC-MS/MS workflow.

Experimental

Source of chemicals and reagents

Fisher Chemical™ Optima™ LC/MS grade (A955-500 and W6500) acetonitrile and water were used in these experiments. The Thermo Scientific™ Pierce™ Peptide Retention Time Calibration Mixture (PRTC, P/N 99321), Thermo Scientific™ Pierce™ HeLa Protein Digest Standard (P/N 88328), Thermo Scientific™ EasyPep™ Mini MS Sample Preparation Kit (P/N A40006), and EASY-spray HPLC columns (ES903 and ES906) were obtained from Thermo Fisher Scientific. The Evosep One LC, EvoTips, and the Evosep column (EV-1064) used were from Evosep.

Sample preparation

Pooled human non-small cell lung cancer adenocarcinoma serum was purchased from BioIVT. Serum samples were depleted using the Thermo Scientific™ High Select™ Top 14 Abundant Protein Depletion Mini Spin Columns (A36369). Depleted serum samples were reduced, alkylated, trypsin digested, and purified using the EasyPep MS Sample Prep Kit. Concentrations of trypsin digested peptides purified from the EasyPep sample prep column were measured by a Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay (P/N 23290). PRTC peptides were spiked in biological samples to monitor column stability, peptide retention time, peak width, and mass spectrometer mass accuracy.

Data analysis

A 3-stage data processing workflow in Thermo Scientific™ Proteome Discoverer™ 2.4 software was created to take advantage of the combination of spectral library searching, database searching, and additional modifications commonly found in biological samples all without impacting the false discovery rate (FDR) calculations. The human protein database (SwissProt, December 2018) was used for peptide identification. The search parameter settings for peptide identification are as follows: 10 parts per million (ppm) precursor mass accuracy, 0.02 Dalton (Da) fragment mass accuracy, static modification of carbamidomethyl +57.021 Da (C), and dynamic modification of oxidation +15.995 Da (M). 1% FDR was set as the filtering threshold for protein and peptide identification. In the first processing stage, the MSPep search engine was used to search the acquired data against a Prosit generated spectral library based on the Human SwissProt FASTA file with alkylated cysteine (+57.021 Da) as the only static modification. In the second processing stage, all medium and low confidence PSMs and all unidentified spectra were re-searched using SEQUEST HT against the Human SwissProt FASTA file with oxidation of methionine (+15.995 Da), protein N-terminal acetylation (+42.011 Da), protein N-terminal loss of methionine (-131.040 Da), and protein N-terminal loss of methionine with acetylation of the new N-terminus (-89.030 Da) as the dynamic modification, and alkylation of cysteine (+57.021 Da) as the only static modification. Finally, in the third processing stage, all medium and low confidence PSMs and all unidentified spectra were re-searched using SEQUEST against the Human SwissProt FASTA file with asparagine/glutamine deamidation (+0.984 Da), peptide N-terminal glutamine to pyro-glutamic acid (-17.027 Da), and peptide N-terminal carbamylation (+43.006 Da) as dynamic modifications, in addition to all of the protein N-terminal and static modifications listed above. A 1% peptide FDR was set for all search nodes.

Label-free quantification (LFQ) comparison of detected proteins was processed and visualized in Proteome Discoverer software, which applies normalization of the total abundance values for each run across all files. After aggregating all the normalized abundance values, Proteome Discoverer software scales the abundance values of each sample so that the average of all samples is 100.

Skyline software was used to monitor the retention time, peak area, full width half maximum (FWHM), and mass accuracy of the spiked PRTC peptides during the full acquisition run-time. Prism 8.4 software was used to perform statistical analysis.

Results and discussion

The Orbitrap Exploris 240 mass spectrometer showed excellent inter-laboratory reproducibility

With its small footprint, the Orbitrap Exploris 240 mass spectrometer introduces different hardware such as the S-lens interface to balance the robustness and sensitivity for translational research with the mass accuracy expected of Thermo Scientific™ Orbitrap™ mass spectrometers (Fig. 1A). In addition to its small footprint, high instrument-to-instrument reproducibility allows researchers to increase

throughput of data acquisition for large-scale studies. To examine the inter-laboratory reproducibility of the Orbitrap Exploris 240 MS, we ran the same system suitability method on four different Orbitrap Exploris 240 MS instruments at three different sites. As shown in the Figure 1B, we found similar performance of these four Orbitrap Exploris 240 mass spectrometers at both protein identification (2.58% CV) and peptide identification (5.89% CV)

Plasma profiling method template is pre-loaded on the Orbitrap Exploris 240 MS to harmonize data acquisition parameters and benchmark instrument performance

We optimized the MS parameters to acquire data from digested plasma/serum samples using Evosep LC gradients and the Orbitrap Exploris 240 MS. The optimized MS method is included as a pre-loaded method template (plasma profiling) in the Method Editor of the Orbitrap Exploris 240 MS (Fig. 2).

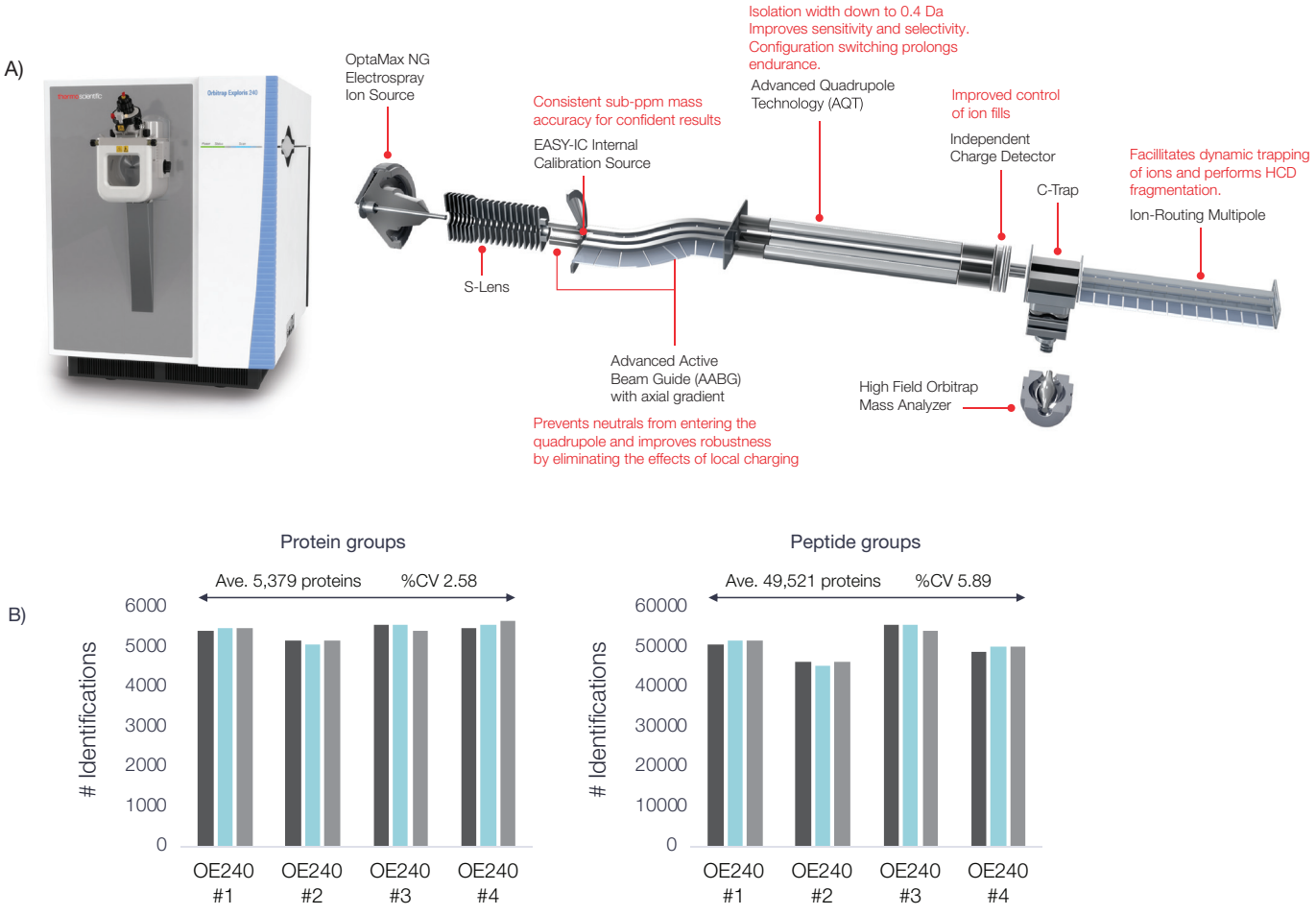
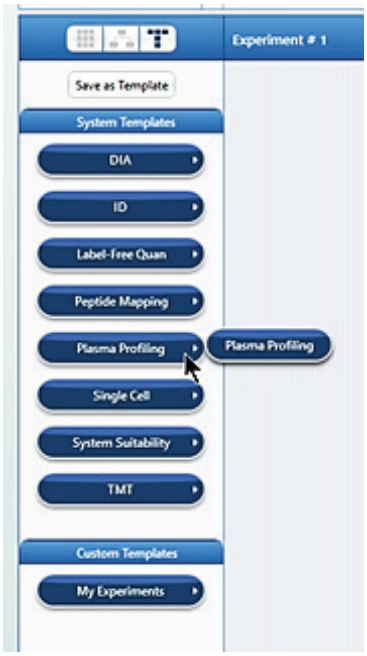


Figure 1. Instrument schematic and inter-lab performance of the Orbitrap Exploris 240 mass spectrometer. A) Schematic of the ion path of the Orbitrap Exploris 240 MS highlighting important features of the system for improved performance and reliability. B) Inter-laboratory study of the Orbitrap Exploris 240 MS – 1 µg of HeLa digest separated on an EASY-Spray HPLC column (ES903) (75 um x 50 cm) coupled with a Thermo Scientific™ EASY-nLC™ 1200 system over a 60 min LC gradient at 300 nL/min flow rate.

A)



B)

Global parmeters	
Source	EasySpray
Ion transfer tube temp	280 °C
Spray voltage	1.8 kV
MS parameters	
Orbitrap resolution	60,000 at *m/z* 200
RF lens	80%
AGC target	300%
Max inject time	Auto
MS/MS parameters	
Intensity threshold	1 x 10^5
Resolution	15,000 at *m/z* 200
AGC target	Standard (100%)
Maximum IT	Auto
Top N	20
Isolation window	1.6 *m/z*
Normalized collision energy	30%
Dynamic exclusion	25s

Figure 2. The plasma protein profiling MS method using short gradients (60 SPD and 30 SPD) from Evosep LC is pre-loaded as a method template in the method editor of the Orbitrap Exploris 240 MS. A) A screenshot of the plasma profiling method template in the method editor. B) Detailed MS parameters of the plasma profiling method template.

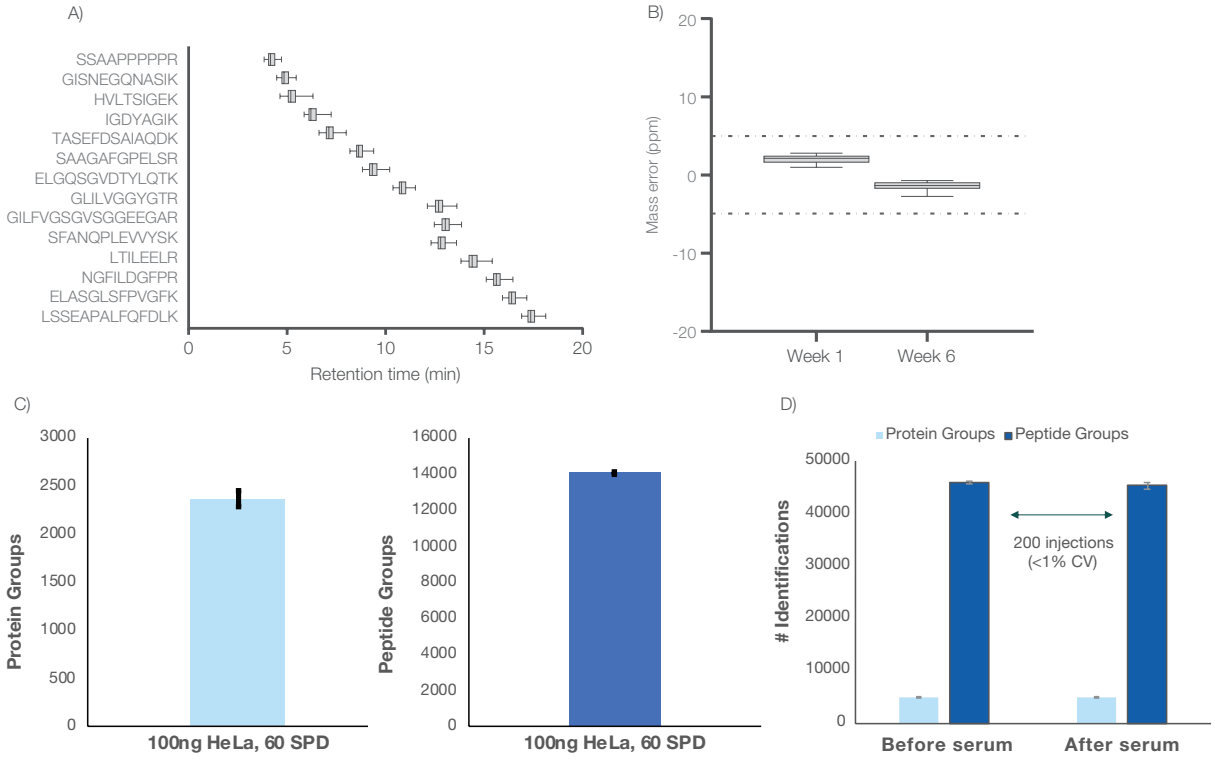


Figure 3. Analytical performance of the EvoSep One LC coupled with the Orbitrap Exploris 240 MS. A) Retention time stability of the PRTC peptides (50 fmol) over 200 runs. B) Mass stability of the PRTC peptides over 200 runs in two non-consecutive weeks (over 100 injections summarized in each time point). C) Protein and peptide identifications at 1 % FDR from 100 ng HeLa digest. D) Results from system suitability tests before and after 200 consecutive injection of human serum.

Using the pre-loaded method (LC and MS), we analyzed commercially available HeLa digest standard to validate the the performance of the Evosep LC and Orbitrap Exploris 240 MS (quality assurance or QA) and to quality control results from using the workflow over time (QC). We also spiked the PRTC peptide standard in human serum to quickly monitor the column integrity and MS stability during the experimental period. Using the 60 SPD LC method and EASY-Spray HPLC column (ES906), we observed minimal retention time shift (<5% CV) (Fig. 3A) and mass accuracy of no more than +/- 5ppm over 200 injections that were five weeks apart (Fig. 3B). Using the same LC-MS method, we detected close to 2,500 proteins and over 14,000 peptides from 100 ng of HeLa digest standard (Fig. 3C). Lastly, to demonstrate the robustness of the Orbitrap Exploris 240 MS for plasma proteome biofluid analysis, we compared results from system suitability tests (method described in Figure 1) before and after 200 consecutive injections of human serum (200 ng per injection) and found good repeatibility (<1% CV difference) without recalibration of the mass spectrometer (Fig. 3D).

The workflow solution of coupling the Evosep LC to the Orbitrap Exploris 240 MS showed robust and reproducible profiling of plasma proteins over hundreds of injections

In addition to robust analytical performance shown in Figure 3, we observed excellent reproducibility of protein and peptide identifications over 100 injections of human serum performed five weeks apart using this workflow at the 60 SPD throughput (Fig. 4). On average, 338 proteins (3977 peptides) were identified in week 1 from 100 injections of 200 ng Top14 depleted serum with less than 5% CV (Fig. 4A & 4B). 337 proteins (4097 peptides)

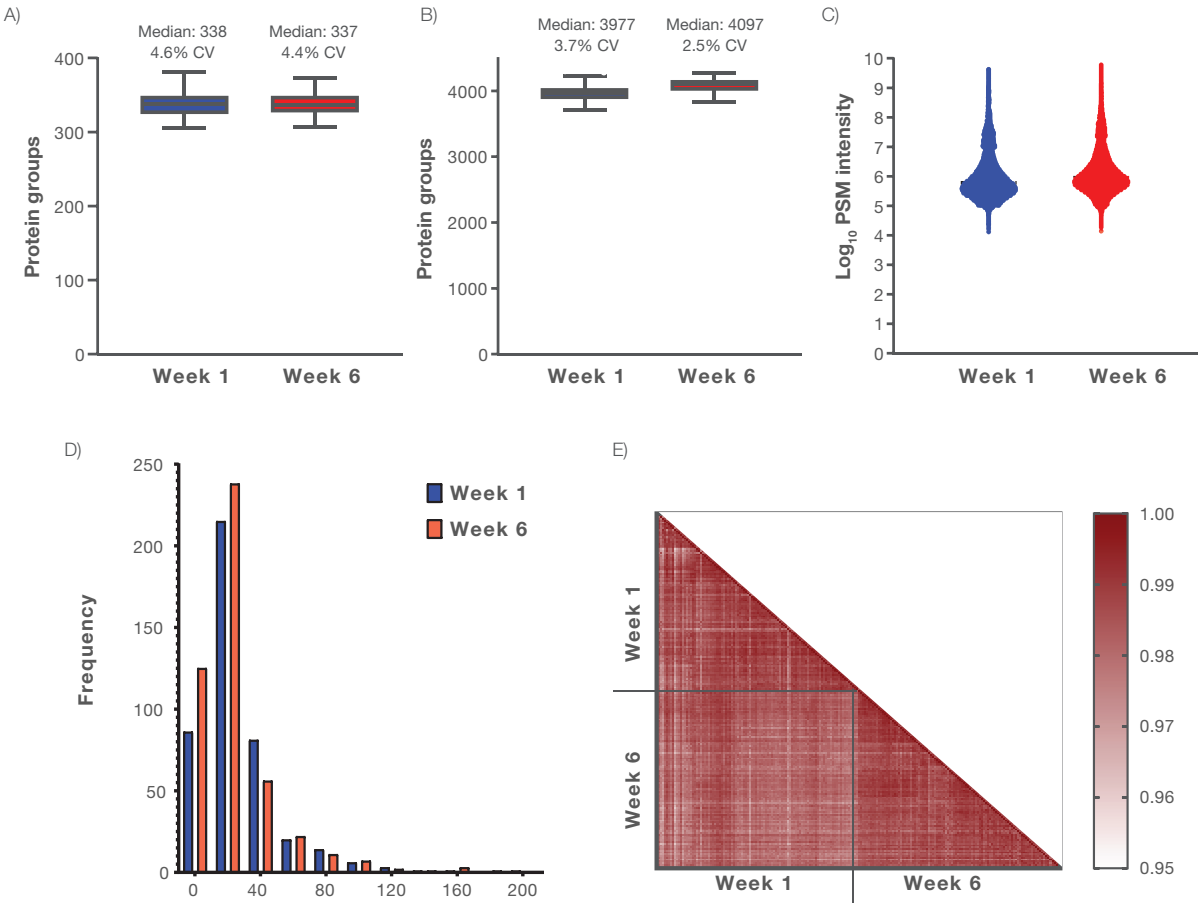


Figure 4 – Robust and reproducible analytical performance of the plasma protein profiling workflow. A) Box-and-whisker plots show median and maximum 1.5 interquartile range (IQR) of protein (1% FDR) from 100 consecutive runs of 200 ng Top14 depleted serum at two time points (200 injections total). B) Peptide identification (1% FDR) from 100 consecutive runs of 200 ng Top14 depleted serum at two time points (200 injections total). C) Distribution of annotated PSMs intensities at each time point. D) A histogram of % CVs from LFQ of 100 Top14 depleted serum injections at each time point. E) Correlation of 200 runs from two different time points based on LFQ.

were identified in week 6 also with less than 5% CV from another batch of 100 injections of 200 ng Top14 depleted serum (Fig. 4A & B). Moreover, over 5 orders of magnitude of dynamic range of PSMs was detected from over 200 injections of Top14 depleted human serum (Fig. 4C).

While the depth of proteome coverage is important, robust quantification is equally important to identify actionable biomarkers. To evaluate the quantification consistency, we combined replicate runs from each time period using a feature mapping consensus workflow and looked at the distribution of the cumulative CV of LFQ. We found more than 70% of proteins were quantified with less than 20% CV from 100 replicate injections at each time point (Fig. 4D) and LFQ values were highly correlative between week 1 and week 6 runs (Fig. 4E). Our results showed good repeatibility (runs within the week) and reproducibility (runs between two weeks) of ths workflow for large-scale studies.

Varying loading amounts and gradient lengths allowed reproducible characterization of 500 plasma proteins

To determine if one can increase the depth of plasma proteome coverage by increasing the loading amount and gradient length, we tested two different loading amounts (200 ng and 500 ng) and two different gradient lengths (21 minutes and 44 minutes). We found that 500 plasma proteins could be detected by loading 500 ng of Top 14 depleted human serum with the 44-minute gradient (30 SPD) method and results showed consistent quantitative with a consistent quantitative performance (Fig. 7A & B). Among these 500 plasma proteins, many of them are known FDA approved biomarkers. We highlighted some of these FDA-approved biomarkers across different concentrations (Fig. 5). Ultimately, our results demonstrated that the workflow solution can be scaled based on the user's need for analytical throughput, sample availability, and biological/disease relevance for large-scale studies.

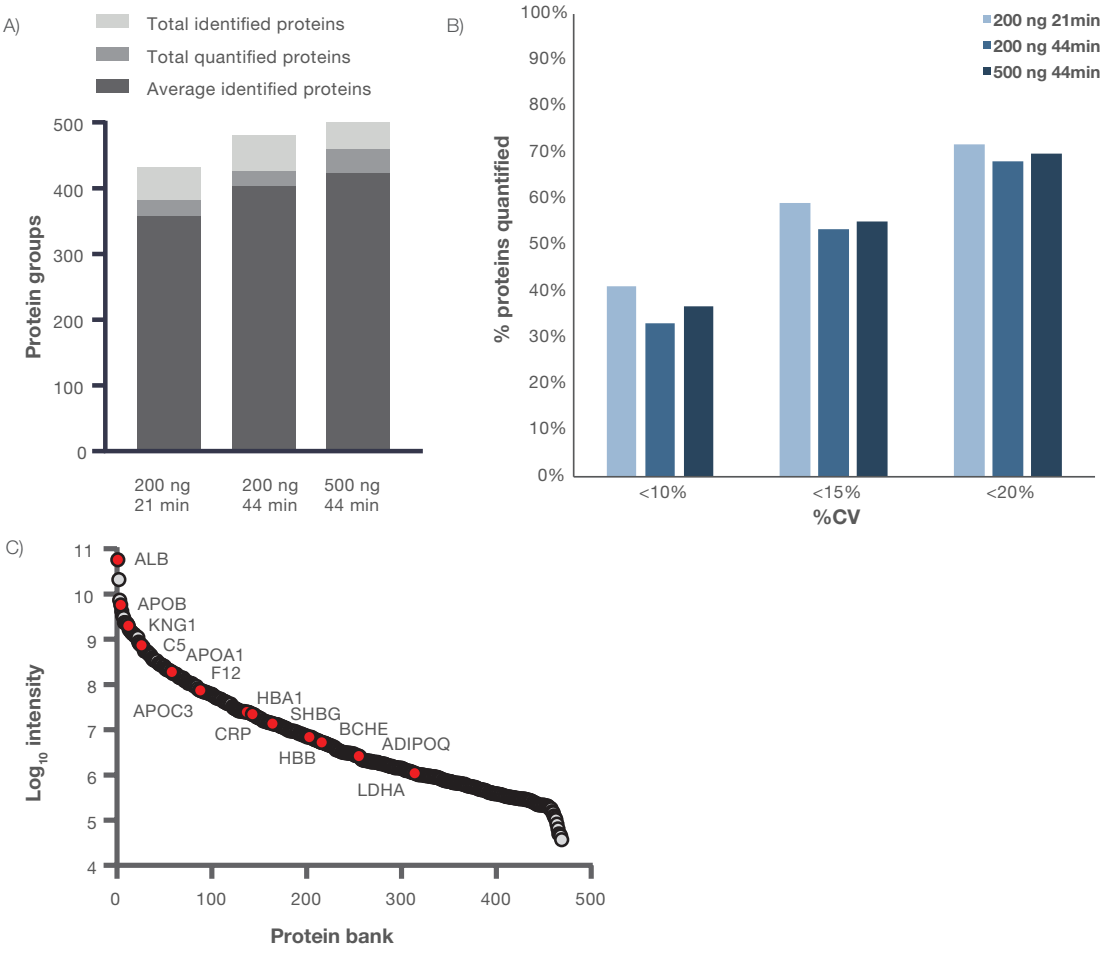


Figure 5. Reproducible detection of 500 plasma proteins from Top14 depleted human serum by varying loading amounts and gradient lengths. A) 200 or 500 ng of Top14 depleted human serum were loaded on Evotips for LC MS/MS analysis using the Orbitrap Exploris 240 MS and the 60 SPD (21 minute) or 30 SPD (44 minutes) Evosep LC methods. Three types of numbers were reported: averaged identified proteins (per run), total quantifiable proteins (n=5), and total identified proteins (n=5). Feature mapping was applied to repeated injections (n=5) to determine the total number of proteins identified and quantified. B) LFQ analysis of plasma proteins from replicate injections (n=5) of different loading amounts in combination with the gradient length. Percentages of protein quantified with less than 10, 15, and 20% CV were shown. C) Known FDA approved biomarkers across a range of concentrations were highlighted among 500 plasma proteins identified.

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Summary

In summary, we presented a robust, reproducible, and high-throughput workflow solution for plasma protein profiling, which can be integrated with our automated sample preparation solution to support biomarker discovery for large-scale human cohort studies. This analytical solution includes a robust and easy to use Orbitrap Exploris 240 mass spectrometer for qualitative and quantitative characterization of human plasma proteins from healthy and diseased individuals. Our results demonstrated:

- The Orbitrap Exploris 240 mass spectrometer showed excellent inter-laboratory reproducibility.
- The plasma profiling method template, which is pre-loaded on the Orbitrap Exploris 240 MS, could be used to harmonize data acquisition parameters and benchmark the instrument performance.
- The workflow solution of coupling the Evosep LC with the Orbitrap Exploris 240 MS showed robust and reproducible profiling of plasma proteins over hundreds of injections.
- The Orbitrap Exploris 240 MS is robust and easy to maintain, which is ideal for routine plasma protein profiling analysis.
- The workflow solution can be scaled based on the user's need for analytical throughput, sample availability, and biological/disease relevance for large-scale studies.

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