OptiSpray technology

Thermo Scientific™ OptiSpray™ Technology is an intelligent electrospray ionization (ESI) interface designed to simplify the acquisition of high-quality, reproducible nano- and capillary-flow LC-MS data. By combining the Thermo Scientific™ OptiSpray™ Ion Source with Thermo Scientific™ OptiSpray™ Cartridge Columns, OptiSpray technology enables:

- **Simplicity** for multiple users
- **Performance** for deep, reproducible coverage
- **Reliability** across columns, users, and LC-MS systems

OptiSpray ion source features

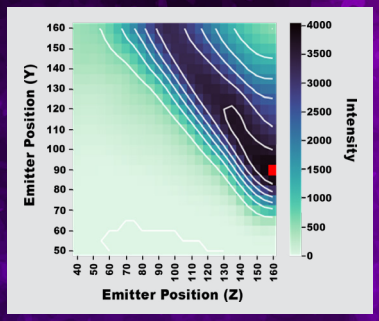
Easy connection to low-flow UHPLC systems and fluidics for optimum separation performance.

Two cameras for spray visualization.

All-in-one ion source for MS calibration, compound optimization, and acquisition.

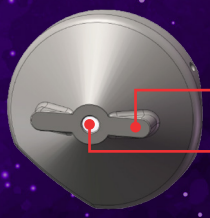
Simple, plug-and-play installation of cartridge nano and capillary columns with a flip of the handle.

Reproducible, high-quality results with automated 3D emitter position optimization.



The Quick Optimization Routine scans the 3D space adjacent to the ion transfer tube or FAIMS device to determine the location that provides the highest signal intensity.

Sweep cone



Inactive spray position

Active spray position

Inactive spray position between injections prevents washing of impurities from the column into the MS.

thermo scientific

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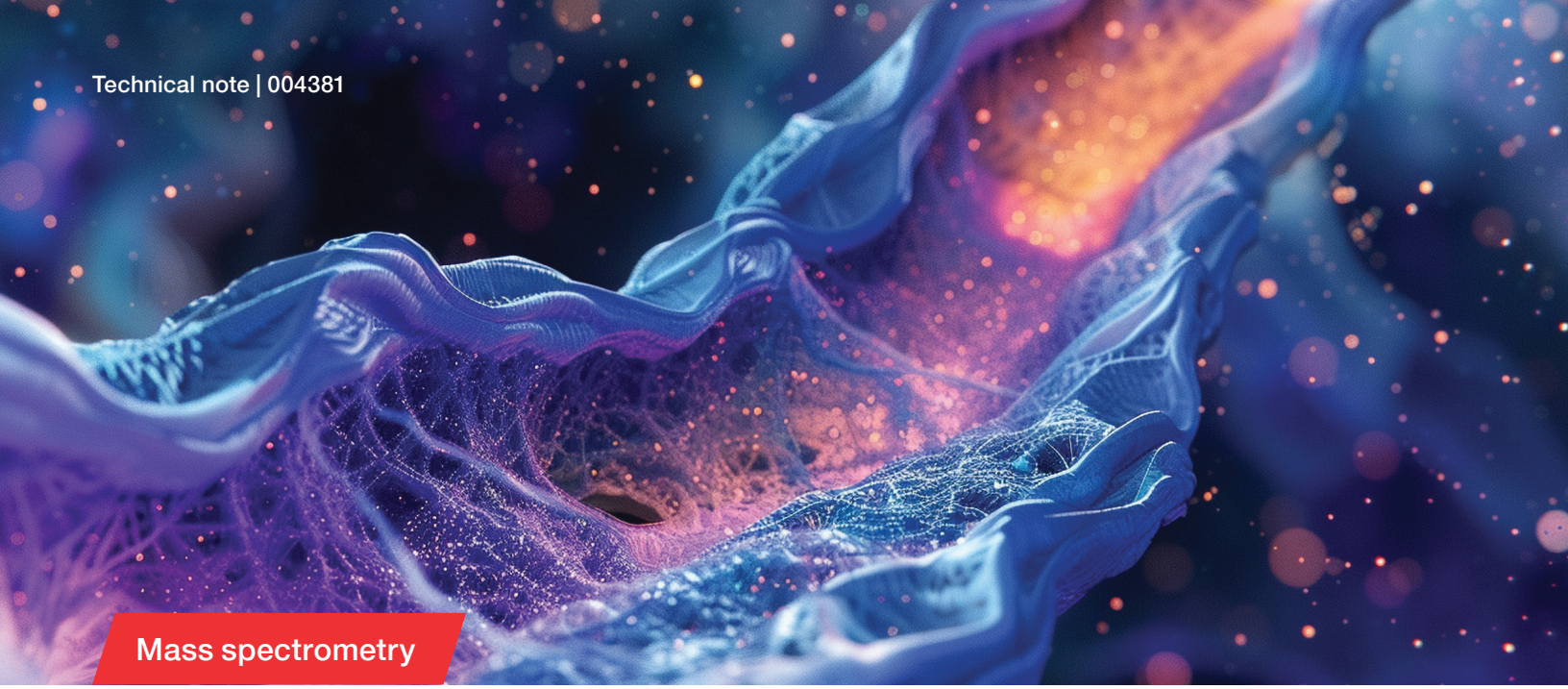

Mass spectrometry

Unlocking the archived proteome: High-throughput, deep FFPE proteome profiling using the Orbitrap Astral mass spectrometer

Authors

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Keywords

Orbitrap Astral, OptiSpray ion source, formalin-fixed, paraffin-embedded, FFPE, cancer, lung tumor

Goal

To demonstrate a streamlined, rapid formalin-fixed, paraffin-embedded (FFPE) proteomics workflow using the Thermo Scientific™ Orbitrap™ Astral™ Mass Spectrometer and multiple liquid chromatography (LC) systems, enabling robust and sensitive protein quantitation and scalable elucidation of biological differences with minimal analysis time.

Introduction

FFPE tissue specimens are foundational to clinical pathology and translational cancer research. FFPE preservation enables long-term storage of patient samples while maintaining tissue morphology required for routine histological evaluation and molecular testing. As a result, FFPE tissues represent a uniquely valuable resource for retrospective studies, biomarker discovery, and precision oncology, particularly when paired with rich clinical annotations such as diagnosis, treatment history, and patient outcomes. Compared to freshly collected tissues which are often difficult to obtain, costly to process, and rarely available at scale, FFPE samples are widely accessible through biobanks and archives, offering a cost-effective and clinically relevant foundation for proteomic analyses aligned with real-world oncology workflows.[1]

Despite their clinical importance, proteomic analysis of FFPE tissues presents methodological challenges that can impact reproducibility and sensitivity if not systematically addressed. Formaldehyde fixation introduces protein crosslinking and chemical modifications that complicate protein extraction and enzymatic digestion, while essential preparatory steps such as paraffin removal and crosslink reversal add processing complexity. Traditional FFPE workflows often involve multiple manual steps and extended processing times, increasing variability in peptide recovery and limiting throughput. Recent advances in sample preparation have created new opportunities to overcome these limitations.[2] However, even well-processed FFPE samples can contain residual contaminants that accumulate over time and require frequent instrument cleaning, limiting robustness and scalability.

Recent advances in FFPE-compatible sample preparation and mass spectrometry technologies are helping to overcome these barriers. Here, we implemented a streamlined FFPE proteomics workflow that simplifies tissue processing, protein recovery, and digestion, reducing sample handling while improving peptide yield and reproducibility. Using FFPE lung tissue sections as a representative cancer model, we leveraged the advanced Thermo Scientific™ OptiSpray™ Ion Source with the Orbitrap Astral mass spectrometer with two different LC platforms to enable confident detection of low-abundance peptides across a wide input range (20–200 ng). When combined with fast and robust LC methods and high acquisition speeds of 60, 180, and 500 samples per day (SPD), this integrated workflow enables deep, scalable proteomic profiling of FFPE cancer tissues. In addition, the automated OptiSpray ion source helps keep the mass spectrometer cleaner, enabling reliable, deep, and scalable proteomic analysis of archived clinical tissues. Together, these advances facilitate and maintain robust detection of biologically meaningful differences across large sample cohorts, supporting biomarker discovery and translational cancer research at scale.

Experimental

Consumables

- Fisher Chemical™ Optima™ LC/MS Grade Water with 0.1% Formic Acid (v/v) (Part No. LS118-500)
- Fisher Chemical™ Optima™ LC/MS 80% Acetonitrile (ACN), 20% Water with 0.1% Formic Acid (Part No. LS122500)
- Fisher Chemical™ Optima™ LC/MS Grade Formic Acid, 99.0+% (Part No. A117-50)
- Thermo Scientific™ EasyPep™ Mini MS Sample Prep Kit
- Invitrogen™ DDM (n-dodecyl β-D-maltoside) (Part No. BN2005)
- Thermo Scientific™ OptiSpray™ µPAC™ Neo 50 cm Nano Cartridge (Part No. OS-UPAC050NAN)
- Thermo Scientific™ OptiSpray™ µPAC™ Neo High Throughput Cartridge (Part No. OS-UPAC005CAP)
- Evosep™ EV1182 Performance Column
- Evosep™ EV2018 Evotip Pure™ Tip Column

UHPLC system

- Thermo Scientific™ Vanquish™ Neo UHPLC System
- Evosep™ Eno

Mass spectrometer

- OptiSpray ion source (Part No. B51004132)
- Orbitrap Astral mass spectrometer

Software

- Biognosys™ Spectronaut™ software

Methodology

Lung tumor and lung normal samples were de-paraffinized using xylene and sequential ethanol washes. The optimized protein extraction protocol and EasyPep Mini MS sample prep kit were used to prepare the digest samples from FFPE sections of normal and tumor lung samples. Protein concentration was measured using the Thermo Scientific™ Pierce™ Rapid Gold BCA kit. The peptides were quantified using the Thermo Scientific™ Pierce™ fluorometric peptide assay kit before LC-MS/MS analysis.

LC-MS analysis

Samples were injected onto an OptiSpray µPAC Neo 50 cm nano cartridge and OptiSpray µPAC Neo high throughput cartridge and separated using 20 min (60 SPD) and 6.8 min (180 SPD) gradients, respectively, in direct injection mode at 55 °C on a Vanquish Neo UHPLC system. For 500 SPD (2.3 min) separation, samples were processed on the Evosep Eno using an Evosep EV1182 Performance Column at 40 °C. Detailed LC and MS parameters are provided in Tables 1 and 2.

Table 1. Vanquish Neo UHPLC system conditions for direct injection mode.

60 SPD (20 min, OptiSpray µPAC Neo 50 cm cartridge, Part No. OS-UPAC050NAN)			
	Time	%B	Flow (µL/min)
Gradient	0	4	0.75
	0.3	8	0.75
	14.1	30	0.4
	17.5	50	0.4
	18	99	0.75
	20	99	0.75
LC parameters	Column temperature	55 °C	
	Fast loading/equilibration	Pressure control	
	Pressure for loading	450 bar	
	Equilibration factor	3	
	Sampler temperature	7 °C	
180 SPD (6.8 min, OptiSpray µPAC Neo high throughput cartridge, Part No. OS-UPAC005CAP)			
	Time	%B	Flow (µL/min)
Gradient	0	4	2.5
	0.1	8	2.5
	0.9	12.5	2.5
	1	12.5	1.4
	4.5	28.5	1.4
	5.8	50	1.4
	6.2	99	2.5
	6.8	99	2.5
LC parameters	Column temperature	55 °C	
	Fast loading/equilibration	Pressure Control	
	Pressure for loading	450 bar	
	Equilibration factor	3	
	Sampler temperature	7 °C	

Table 2. Mass spectrometer parameters.

MS parameters					
	Vanquish Neo UHPLC system (60 & 180 SPD)			Evosep Eno (500 SPD)	
	Sample amount (ng)	**200**	**20**	**200**	**20**
MS^1	Resolution	240,000	240,000	240,000	240,000
	Scan range	380–980	380–920	400–800	400–800
	AGC	500	800	500	500
	Max-IT	5	100	3	3
MS^2	Scan range	150–2,000	150–2,000	150–2,000	150–2,000
	Isolation window	2	5	4	4
	AGC	500	800	500	500
	Max-IT	3	10	3	3
	HCD	25	25	25	25

Data analysis

The raw DIA files from both the labeled and unlabeled samples were analyzed together using Spectronaut software in directDIA™ mode. Protein identification was conducted using the Pulsar search engine, and the reference database employed was UniProtKB/Swiss-Prot, specifically the human proteome database (Homo sapiens: 20,423 entries) with default search settings. All results were processed and filtered with a 1% precursor and 1% protein group false discovery rate (FDR). Exported output files were imported to RStudio (2023.09.0 Build 463) with R (v4.3.1) for downstream data analysis and visualization.

Results and discussion

Fast and scalable proteomics for clinical FFPE specimens

A streamlined workflow was established enabling deep proteomic profiling of FFPE tissues in less than 1 day (Figure 1). The protocol integrates rapid deparaffinization and protein extraction (~3 h), efficient peptide digestion and cleanup using the EasyPep Mini MS sample prep kit (3–4 h, dry time ~4 h), fast LC–MS analysis (<25 min per sample), and automated data processing (~20 min). By minimizing sample handling and total processing time, the workflow addresses key bottlenecks traditionally associated with FFPE proteomics.

This workflow was successfully applied to FFPE lung tissues, including both normal lung and lung cancer samples (Figure 2A). FFPE tissue sections were stained with hematoxylin and eosin (H&E) to verify tumor and normal status (Figure 2B), followed by preparation of the tissue for mass spectrometry-based proteomic analysis. Efficient deparaffinization and lysis enabled effective reversal of formalin-induced crosslinks and robust protein recovery (Figure 2C), which is critical for downstream enzymatic digestion. The resulting peptides were readily compatible with rapid LC-MS analysis, demonstrating that extensive fractionation or long chromatographic gradients are not required to achieve comprehensive proteome coverage from FFPE material.

Notably, the use of short LC gradients combined with high-resolution mass spectrometry supports high-throughput analysis while maintaining sensitivity and reproducibility. This is particularly advantageous for studies involving large FFPE cohorts or comparative analyses between normal and tumor tissues, where throughput and consistency are essential.

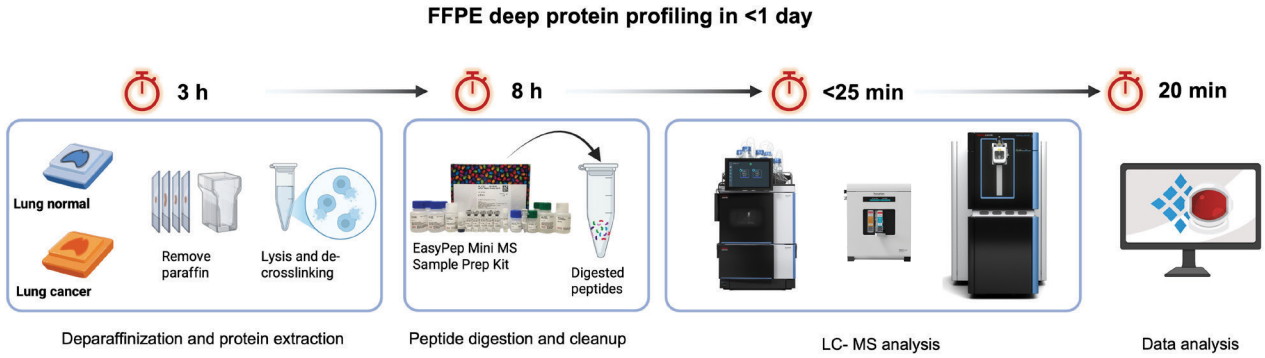

Figure 1. Workflow for FFPE sample preparation and mass spectrometry data acquisition to enable deep protein profiling.

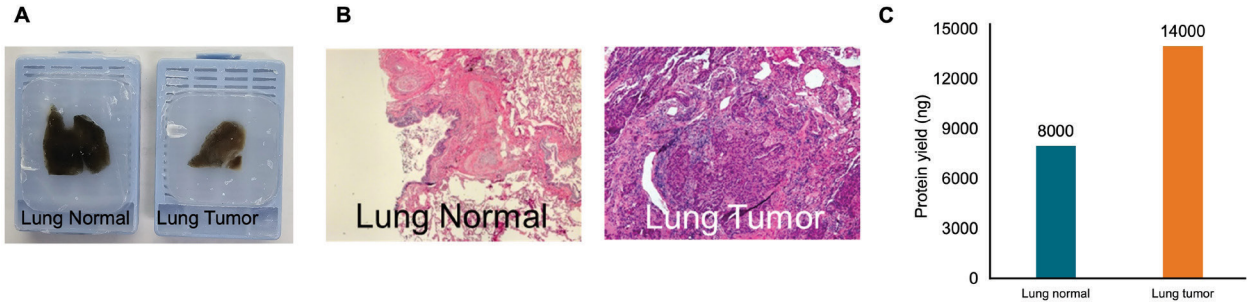

Figure 2. FFPE lung tissue and protein yield. (A) Lung normal and lung tumor tissue in FFPE blocks. (B) H&E staining of lung normal and lung tumor tissue to confirm normal and tumor status. (C) Protein recovery after deparaffinization and lysis.

Protein and peptide identifications across acquisition speeds and input amounts

Across normal and tumor FFPE lung tissues, high proteome coverage was maintained across the tested acquisition speeds, with only gradual reductions in protein group and peptide identifications as throughput increased (Figure 3). At the deepest protein profiling setting (60 SPD; ~20 min gradient), up to ~8,600 protein groups and ~105,000 peptides were identified from 200 ng input, representing the maximum proteome depth achieved in this study.

Increasing throughput from 60 to 180 SPD (~3× higher SPD; ~7 min gradient), at 200 ng load, ~6,100 protein groups and ~62,000 peptides were identified, corresponding to ~70% of protein groups and ~60% of peptides relative to 60 SPD. Notably, even at 20 ng input, ~5,400 protein groups and ~57,000 peptides were identified at 180 SPD, demonstrating robust performance at low sample amounts.

Further increasing throughput to 500 SPD (~8× higher SPD relative to 60 SPD; ~2 min gradient) still enabled identification of ~3,700–3,900 protein groups and ~25,000–27,000 peptides at 200 ng load. This corresponds to ~45% of protein groups and ~25% of peptides compared to the deepest acquisition, despite more than an 8-order-of-magnitude increase in sample throughput. Similar proportional trends were observed at 20 ng input, indicating that depth retention with increasing SPD is largely independent of sample load.

Importantly, normal and tumor FFPE lung tissues exhibited comparable scaling behavior across all SPDs and input amounts, indicating consistent proteome sampling and workflow robustness. Collectively, these results demonstrate that while deeper coverage is achieved at lower SPDs, high-throughput acquisition preserves a large fraction of proteome information. This favorable depth–throughput trade-off enables flexible experimental design, allowing users to prioritize either maximal proteome depth or rapid, large-scale FFPE cohort analysis without compromising data quality.

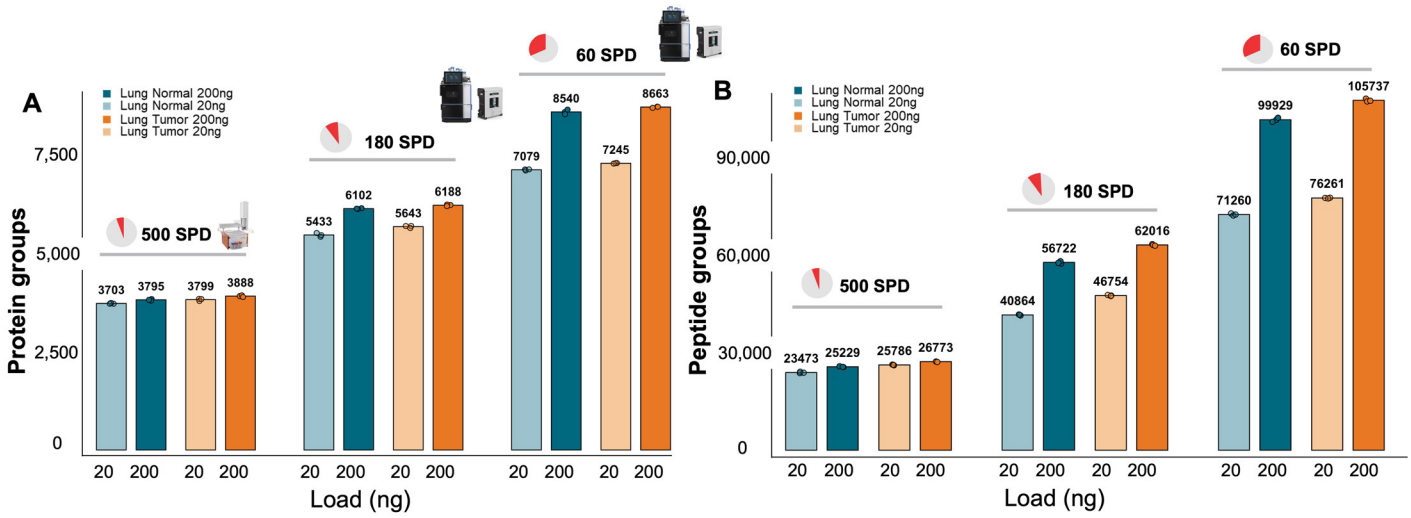

Figure 3. (A) Unique number of identified proteins and (B) peptides from three replicates of FFPE tissue across on-column load (20, 200ng) and gradient length (500, 180, and 60 SPD). Bar plots demonstrate unique identifications for each replicate in the given condition (color and shade identifiers in figure legend). 500 SPD is equivalent to a 2-min gradient, 180 SPD is equivalent to a 7-min gradient, and 60 SPD is equivalent to a 20-min gradient.

High quantitative reproducibility maintained across acquisition speeds

Quantitative reproducibility was consistently high across the tested acquisition speeds, sample types, and input amounts (Figure 4). Median protein abundance coefficients of variations (CVs) across triplicate injections remained low for the tested SPDs, ranging from ~7–10% at 500 SPD and 180 SPD to ~5–6% at 60 SPD (Figure 4A). Importantly, even at the highest throughput (500 SPD), CVs remained within 15% for quantitative proteomics, indicating that accelerated gradients do not substantially compromise measurement precision. Comparable CV distributions were observed for both normal and tumor FFPE lung tissues and across 20 ng and 200 ng loads, demonstrating robustness across biologically and analytically distinct conditions.

Overlap analysis further showed strong consistency in protein identifications across gradient lengths (Figure 4B). A large proportion of proteins identified at 500 SPD were also detected at 180 and 60 SPD, with the highest-throughput method retaining 45% of the core proteome observed at 60 SPD. This indicates that increased throughput primarily affects lower-abundance, gradient-dependent identifications while preserving reproducible detection of the main proteome.

Finally, fold-change measurements between lung tumor and normal tissues were highly concordant across SPDs (Figure 4C). Fold changes quantified at 180 SPD and 500 SPD showed strong correlation with those obtained at 60 SPD (Pearson r = 0.93 and 0.87, respectively), demonstrating that biological trends are consistently captured even under highly accelerated acquisition conditions using two different LC conditions. Further, it demonstrates that high-throughput acquisition preserves biologically relevant proteome observations.

Collectively, these results show that increasing throughput by up to ~8-fold has minimal impact on quantitative reproducibility and biological consistency. The workflow therefore supports reliable, high-throughput FFPE proteomics without sacrificing quantitative accuracy, enabling scalable analysis of large clinical cohorts.

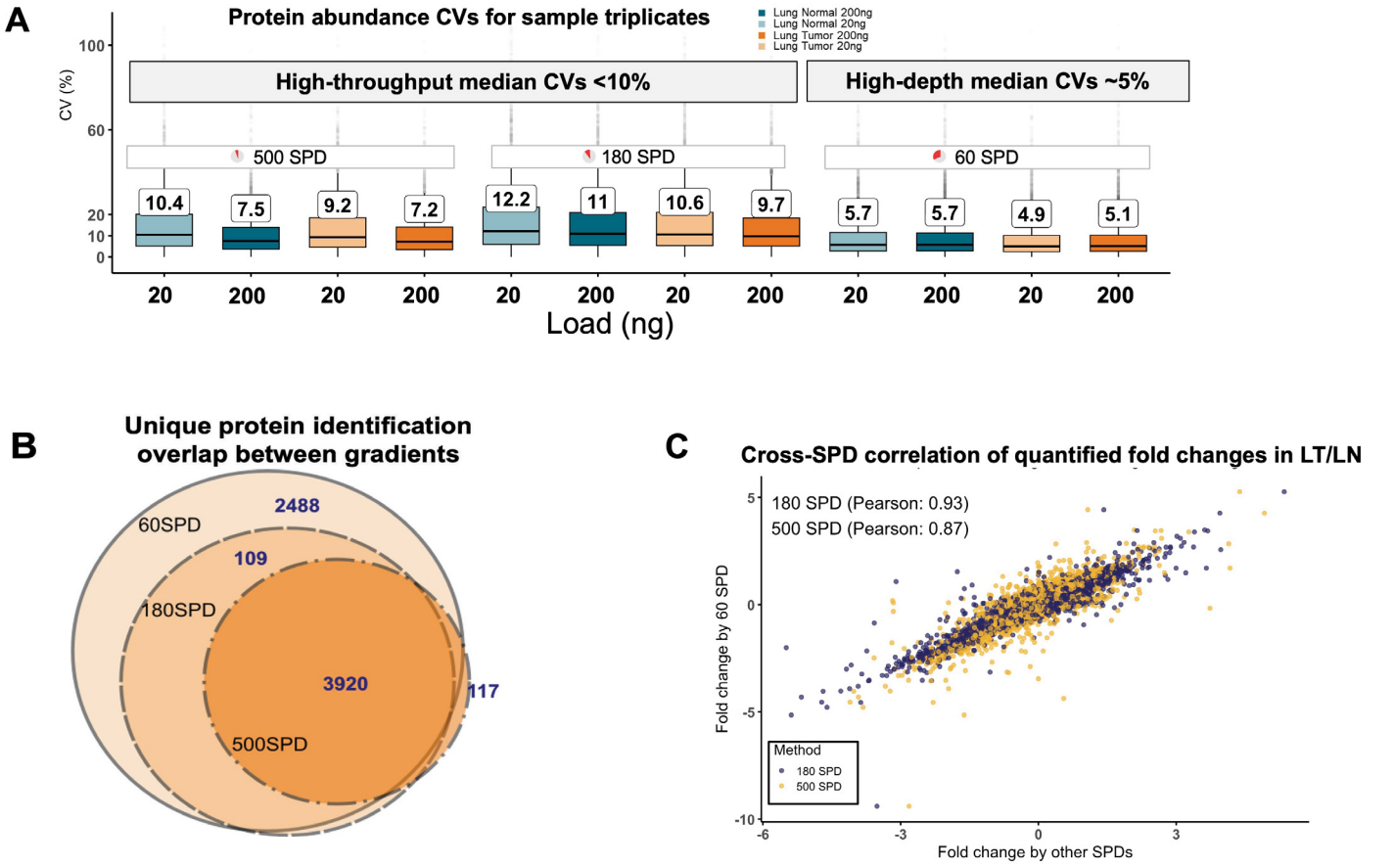

Figure 4. Reproducibility assessment across sample type, on-column amount, and gradient lengths. (A) CVs of peptide abundances for triplicate sample replicates across all FFPE on-column loads and gradients. Median CV marked with black line and labeled in the figure. (B) Unique protein identifications from 200 ng sample load, overlap analysis between gradient lengths. Circles labeled according to gradient length. (C) Fold change calculations between lung normal and lung tissue samples, then correlation between fold changes were plotted between 180 SPD vs. 60 SPD (blue, r = 0.93) and 500 SPD vs. 60 SPD (yellow, r = 0.87).

Differential protein expression and pathway alterations between lung tumor and normal FFPE tissues

Differential expression analysis of 200 ng of sample loads at 60 SPD revealed widespread proteomic alterations between lung tumor and matched normal FFPE tissues (Figure 5 left). About 1,500 protein groups were significantly differentially expressed ($|\log_2 FC| \geq 1$, $p \leq 0.05$), indicating robust detection of tumor-associated proteomic remodeling. The clear separation between significantly regulated and non-significant proteins highlights the sensitivity of the workflow for identifying biologically meaningful differences from FFPE sections.

Ranking proteins by median intensity showed that differentially upregulated proteins spanned a wide dynamic range and were not limited to only the highest-abundance species (Figure 5 center). While many high-intensity proteins exhibited tumor-associated upregulation, a substantial number of regulated proteins were detected at mid- to low-abundance levels, demonstrating that the workflow captures both dominant and subtler proteomic changes. This depth is essential for uncovering signaling and metabolic pathways that may be masked in more shallow analyses.

Pathway enrichment analysis of upregulated proteins identified key biological processes associated with tumor progression and the tumor microenvironment (Figure 5 right). Enriched pathways include neutrophil extracellular trap formation, transcriptional dysregulation in cancer, glycolysis/gluconeogenesis, HIF-1 signaling, ECM–receptor interaction, and central carbon metabolism in cancer. The prominence of metabolic reprogramming, hypoxia signaling, and extracellular matrix remodeling is consistent with known hallmarks of lung cancer biology, 3,4 while enrichment of immune- and phagosome-related pathways suggests contributions from tumor–immune interactions captured within FFPE tissue sections.

Together, these results demonstrate that the rapid FFPE proteomics workflow not only provides deep and reproducible proteome coverage but also enables robust detection of biologically relevant differential expression and pathway-level alterations. This supports its utility for translational studies aimed at interrogating disease mechanisms directly from archived clinical specimens.

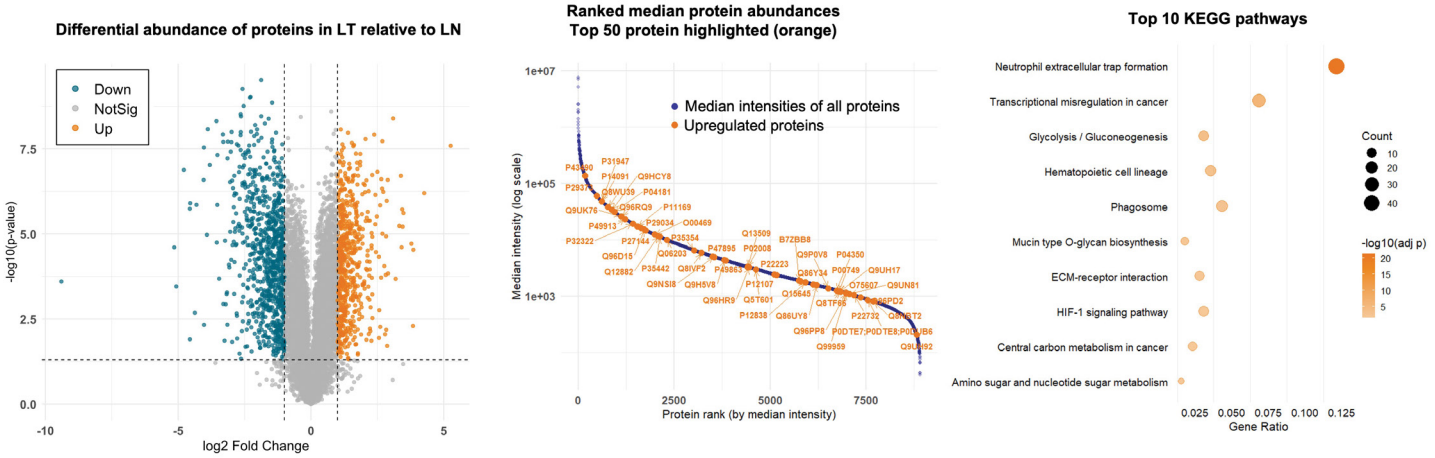

Figure 5. Biological insights for FFPE lung tissue, 200 ng, 60 SPD gradient. (A) Volcano plot: Proteins significantly enriched in lung tumor compared to lung normals samples highlighted in orange, and proteins significantly enriched in lung normal compared to lung tumor highlighted in blue. (B) Rank order plot of median intensities for all proteins, with proteins significantly enriched in lung tumor highlighted in orange. (C) Top 10 KEGG pathways identified from proteins found to be significantly enriched in lung tumor samples. Bubble size indicates numbers of proteins found in the pathway, shade indicates significance.

Summary

- End-to-end FFPE proteomics is completed in under 1 day, integrating rapid sample preparation, fast LC–MS, and streamlined data analysis
- Deep proteome coverage is achieved from FFPE lung tissue, with up to ~8,600 protein groups and >100,000 peptides identified from 200 ng input in 20 min
- Increasing throughput by up to ~8× (60 to 500 SPD) with moderate loss in depth, retaining ~70% of proteins at 180 SPD and ~45% at 500 SPD
- Robust quantitative performance is maintained at low sample input (20 ng) and across acquisition speeds, with median CVs ≤10%
- The workflow enables reliable differential expression and pathway analysis at scale, demonstrating its utility for biological insight into lung tumor and normal tissues

Learn more at thermofisher.com/astral

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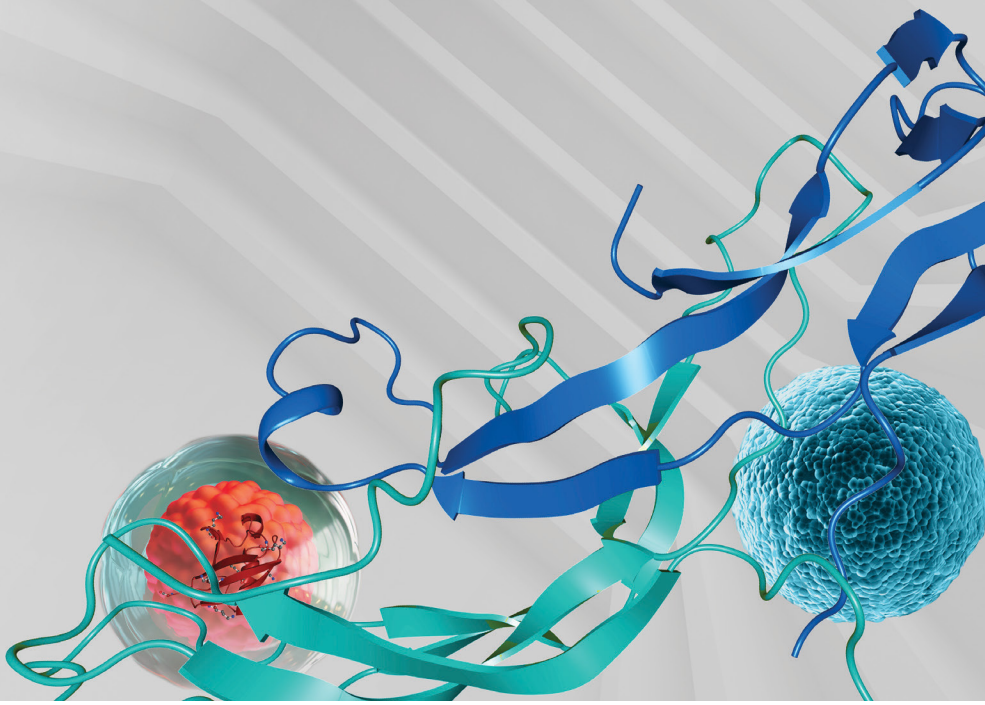

Proteomics

OptiSpray technology for improved sensitivity and precision for low-input samples with the Orbitrap Ascend MultiOmics Tribrid mass spectrometer

Authors

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Keywords

OptiSpray ion source, OptiSpray µPAC Neo cartridges, Orbitrap Ascend Tribrid mass spectrometer, Vanquish Neo UHPLC, FAIMS Pro Duo interface, data-independent acquisition, DIA, proteomics, low load, single-cell, Proteome Discoverer software, CHIMERYS, Spectronaut software, DIA-NN software

Goal

To demonstrate qualitative and quantitative performance of peptide and protein identification in low-input samples with data-independent acquisition (DIA) methods on the Thermo Scientific™ Orbitrap™ Ascend MultiOmics Tribrid™ mass spectrometer and Thermo Scientific™ Vanquish™ Neo UHPLC System using the Thermo Scientific™ OptiSpray™ Ion Source and Thermo Scientific™ OptiSpray™ µPAC™ Neo 50 cm Cartridge Column.

Introduction

Low-input proteomic analysis demands high sensitivity and reliable data quality, and the same requirements apply to single-cell proteomics. Unlike bulk approaches that average signals across many cells, single-cell proteomics captures the molecular diversity of individual cells or small tissue regions. This makes it possible to detect rare cell types or transient states that are often hidden in population-level analyses.

Increased focus on liquid chromatography-mass spectrometry (LC-MS) analysis of limited sample amounts requires the high sensitivity. At the same time, throughput, chromatographic performance, and quantitative precision and accuracy must be preserved to generate sufficiently high-quality data from large sample sets to draw

meaningful biological insights. To this end, we present a sensitive and robust workflow for low-input samples using the Thermo Scientific™ OptiSpray™ Technology on the Orbitrap Ascend MultiOmics Tribrid mass spectrometer.

Advances in LC-MS have pushed the sensitivity limits for low-input analyses, and when operating at flow rates between 0.1–5 µL/min, ionization efficiency is greatly increased, enabling detection of low-abundance peptides with quantitative accuracy. However, fluctuations in electrospray or column performance can severely impact data reproducibility. To maintain stable electrospray ionization and detection of low-abundance peptides, a user may regularly position an emitter at an arbitrary fixed distance from the inlet based on visual alignment, which is prone to error and poor reproducibility. Furthermore, the optimal position is challenging to determine and often differs between individual users. The OptiSpray ion source features cartridge-based columns mounted on an XYZ motorized stage that enables automated optimization routines in the instrument control software, which minimize ambiguity by positioning the emitter based on the mass spectrometer signal detected for solvent or analytical ions, as defined by the user.

Another key challenge in low-flow LC-MS workflows is an increasing column and emitter backpressure and subsequent clogging or degradation of spray quality. With the OptiSpray ion source, the cartridges feature an integrated replaceable emitter, which extends cartridge lifetime.

The OptiSpray ion source and separation cartridges, designed for low-flow applications, further increase capabilities of the Thermo Scientific™ Orbitrap™ Mass Spectrometers, and together with Thermo Scientific™ FAIMS Pro Duo interface, which helps reduce background ions with ease, enable detection of low-abundance features in low-input samples with improved data quality while simplifying the setup.[1]

Our work demonstrates high sensitivity and quantitative performance of low-input samples using the OptiSpray ion source designed to improve data quality and simplify nano-flow LC-MS operation.

Experimental

Recommended consumables

- Fisher Chemical™ Optima™ LC-MS Grade Water with 0.1% Formic Acid (P/N **LS118-500**)
- Fisher Chemical™ LC-MS Grade 80% Acetonitrile (ACN) with 0.1% Formic Acid (P/N **LS122-500**)
- Fisher Chemical™ LC-MS Grade Formic Acid (P/N **A117-50**)
- Fisher Chemical™ Optima™ LC-MS Grade Water (P/N **10505904**)
- Fisher Chemical™ Optima™ LC-MS Acetonitrile (P/N **A955-1**)
- Fisher Chemical™ Optima™ LC-MS Isopropanol (P/N **A461-212**)
- Thermo Scientific™ SureSTART™ Microvial (P/N **60180-1655**)
- Thermo Scientific™ SureSTART™ Screw Caps (P/N **6PSC9STB1**)
- Thermo Scientific™ n-dodecyl-β-D-maltoside (DDM) (P/N **89902**)

Sample

- Thermo Scientific™ Pierce™ HeLa Protein Digest Standard (P/N **88329**)

OptiSpray cartridge

- OptiSpray µPAC Neo 50 cm cartridge (P/N **OS-UPAC050NAN**)

OptiSpray emitter replacement options

- Thermo Scientific™ OptiSpray™ Pulled Emitter, 8 µm (P/N **OSE0835P**) 5 pcs/box

HPLC system

- Vanquish Neo UHPLC system (P/N **VN-S10-A-01**)
- Thermo Scientific™ Vanquish™ User Interface (P/N **6036.1180**)

Mass spectrometer

- Orbitrap Ascend MultiOmics mass spectrometer
- FAIMS Pro Duo interface
- OptiSpray ion source (P/N **B51004132**)

Data analysis software

- Thermo Scientific™ Proteome Discoverer™ 3.2 Software with CHIMERYS™ Intelligent Search Algorithm by MSAID
- Spectronaut™ 19.9 Software by Biognosys AG
- DIA-NN 2.0 Software by Aptila Biotech GmbH

Sample preparation

20 µg of Pierce HeLa protein digest standard were reconstituted in 200 µL of 0.015% DDM dissolved in 0.1% formic acid (reconstitution solution). The solution was sonicated at room temperature for 5 min, making a final concentration of 100 ng/µL. HeLa solutions were prepared at the concentration of 2.5 ng/µL and 5 ng/µL, to cover the range of load with appropriate injection volumes. For example, to achieve a final concentration of 5 ng/µL in an autosampler vial, 10 µL of the reconstituted HeLa digest were mixed with 190 µL of the reconstitution solution.

Vanquish Neo UHPLC system

Orbitrap Ascend MultiOmics mass spectrometer with FAIMS Pro Duo interface and OptiSpray ion source and µPAC Neo 50 cm cartridge

Proteomics software of choice

Figure 1. Overview of DIA workflow for low-input sample analysis

LC-MS method

Methods at 40 and 20 samples per day (SPD) were tested (Tables 1–3). Three replicates were analyzed per level (25 pg–10 ng).

Table 1. LC method parameters

Separation column specifications (set in the Vanquish Neo system)	
Inner diameter	75 µm
Length	50 cm
Maximum pressure and change rate	450 bar, 1,000 bar/min
Maximum flow	1 µL/min
Maximum temperature	60 °C

LC method parameters	
Injection workflow	Direct injection
Fast loading/equilibration pressure	400 bar
Equilibration factor	3.0
Sampler temperature	7 °C

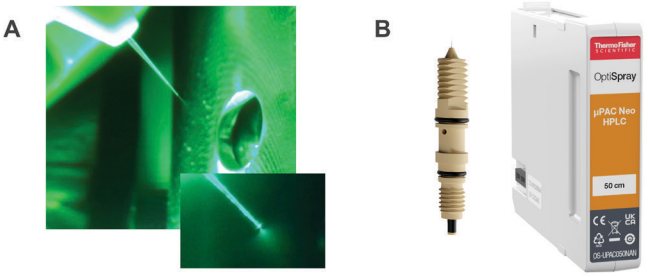

Figure 2. (A) Pulled emitter with enhanced nebulization by cone jet spray as seen in camera view in the instrument control software. (B) OptiSpray µPAC Neo 50 cm cartridge column and a replaceable emitter assembly. The 8 µm OptiSpray pulled emitter is recommended for analysis of single-cell and low-input samples.

Table 2. MS method parameters

MS method global parameters	
Cartridge column temperature	45 °C
Application mode	Peptide
Spray voltage*	+2,200 V
Ion transfer tube temperature	200 °C
FAIMS carrier gas flow / mode	3.5 L/min / standard resolution
EASY-IC	RunStart
Advanced peak determination	On

MS and DIA method parameters	
Scan range (*m/z*) MS^1	400–800
Scan range (*m/z*) MS^2	145–1450
Normalized AGC target	MS^1: 300%, MS^2: 2,000%
Maximum injection time mode	Auto (MS^1 and MS^2)
Window placement optimization	On
Window overlap	1 *m/z*
RF lens	40%
Normalized HCD Collision Energy	25%
FAIMS voltage	–50
Gradient–25 min MS method duration–25 min 40 SPD	Resolution MS^1/MS^2–120,000/60,000 Isolation window–50 *m/z*, 40 *m/z* (>1 ng sample load)
Gradient–50 min MS method duration–50 min 20 SPD	Resolution MS^1/MS^2–240,000/120,000 Isolation window–50 *m/z*, 40 *m/z* (>1 ng sample load)

*Spray voltage is increased due to FAIMS entrance plate offset voltage.

FAIMS voltage(s) and HCD energy may require optimization depending on average peptide length and post-translational modifications (PTMs).

The number of samples per day is determined by the analysis cycle time, including equilibration and sample loading

Table 3. LC gradient method parameters

LC gradients			
	Time (min)	Flow (µL/min)	%B
Gradient–25 min 40 SPD	0	0.75	4
	0.1	0.75	8
	1.9	0.75	12
	2	0.3	12.1
	12	0.3	22.5
	19.5	0.3	40
	22	0.3	99
	25	0.3	99
	Time (min)	Flow (µL/min)	%B
Gradient–50 min 20 SPD	0	0.4	4
	0.2	0.4	8
	3.8	0.4	12
	4	0.15	12.1
	24	0.15	22.5
	39	0.15	40
	44	0.15	99
	50	0.15	99

A = Water with 0.1% formic acid (FA), B = 80% acetonitrile with 0.1% FA

After inserting the µPAC Neo 50 cm HPLC column cartridge into the OptiSpray ion source and setting the column specifications (Table 1), the system was equilibrated at 90% B using the Vanquish Neo column equilibration script B02. The flow rate was then adjusted to match the application conditions (40 SPD: 0.3 µL/min; 20 SPD: 0.15 µL/min) at 20% B in the active spray position. Once pressure and spray stabilized, the automatic nanoflow quick optimization routine was performed in the mass spectrometer tune application (Figure 3). For comparison, the 40 SPD method (Table 3) was modified with flow rates of 0.45 µL/min at 1% B (0 min), 4% B at 0.1 min, 0.2 µL/min at 2 min, and 0.3 µL/min at 22 min, to accommodate a commercially available 75 µm I.D. × 25 cm C18 column with sub-2 µm particles, featuring an integrated pulled emitter. The gradient conditions used with the commercially available column are available in Thermo Fisher Scientific Technical Note 002879.[2] Methods are available for download from the **Thermo Scientific™ AppsLab™ Library of Analytical Applications.**

Data processing and interpretation

Data acquired from three technical replicates at each load level were processed using Spectronaut™ 19.9 software directDIA™ workflow, Proteome Discoverer 3.2 software with CHIMERYS intelligent search algorithm, and DIA-NN 2.0 software. False-discovery rate (FDR) threshold of 1% was applied.

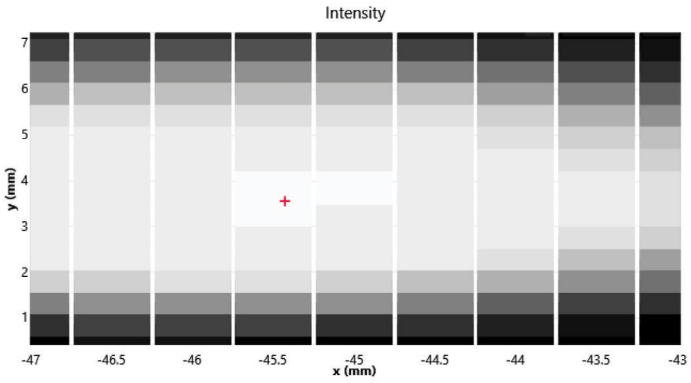

Figure 3. Spatial heatmap from a successful OptiSpray quick optimization report in the instrument control software, showing intensity as a function of x and y coordinates (in mm). Shading represents the relative intensity of solvent ions used to optimize position. Heatmap irregularities, artifacts, or streaking may indicate unstable flow, FAIMS electrode contamination, or other ionization issues. Optimization is cartridge- and flow rate-specific.

Results and discussion

Effect of DDM on peptide recovery

n-Dodecyl-β-D-maltoside (DDM) is a water-soluble, non-ionic detergent widely used for membrane protein solubilization, and to minimize sample loss due to surface adsorption. In its absence, we observed a slightly less than 5% loss in the total number of peptides identified over 12 hours of storage in the autosampler, relative to samples containing DDM. The detergent is compatible with mass spectrometry and typically elutes shortly after most peptides. In positive electrospray mode, it produces a prominent singly charged carbohydrate cluster dimer $[2M + H]^+$ with an accurate *m/z* of 1021.6153. Such dimers are typical of glycosylated compounds at high concentrations in the electrospray.

Improved technical precision with OptiSpray technology

When compared with the commercially available third-party pulled emitter column (75 µm I.D. x 25 cm, sub-2 µm particles), the OptiSpray µPAC 50 cm cartridge showed significantly improved technical precision both at a lower flow rate of 0.15 µL/min and a higher flow rate of 0.3 µL/min. Furthermore, the improvement in median coefficient of variation of proteins remained consistent across the selected levels of 50, 500, and 2,500 picogram load (Figure 4).

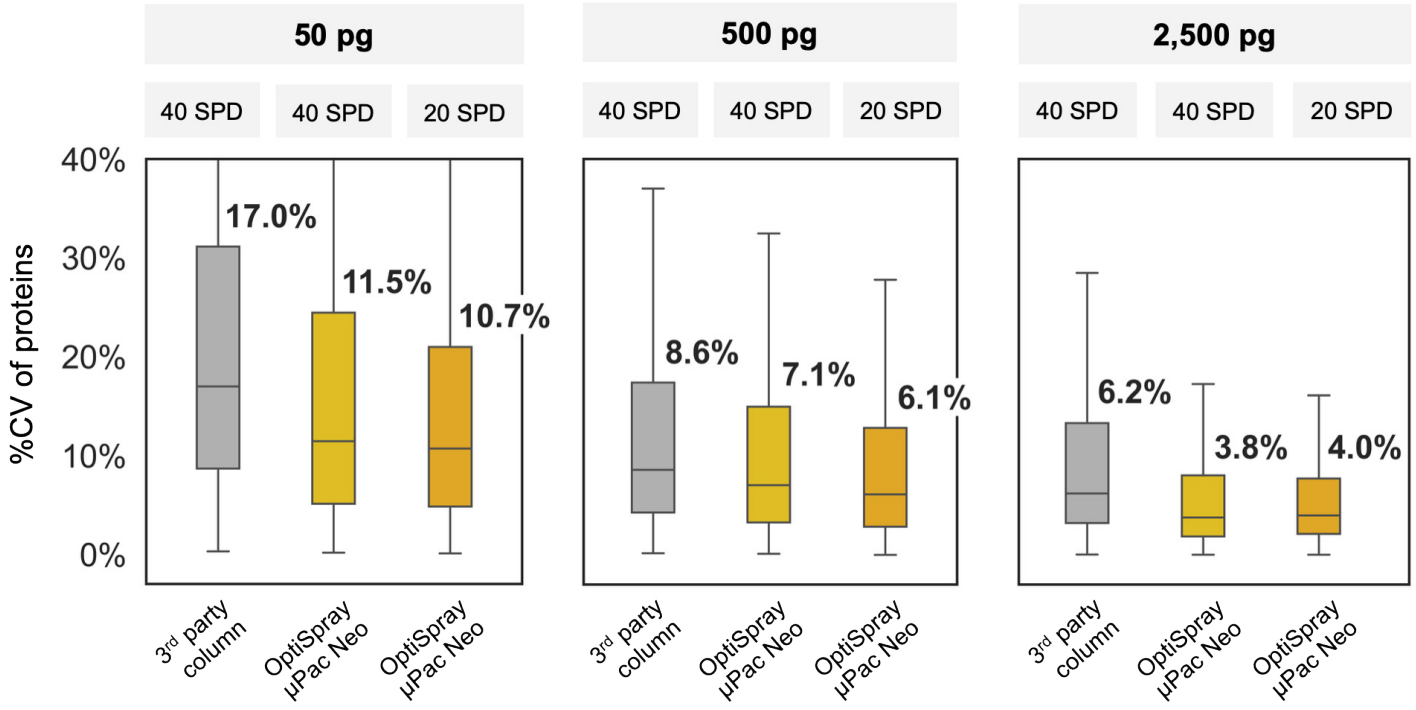

Figure 4. Boxplots of coefficients of variation from HeLa digest analysis at 50, 500, and 2,500 picogram loads using a third-party column (75 µm I.D. × 25 cm, sub-2 µm particles, integrated pulled emitter) compared with the OptiSpray µPAC Neo 50 cm cartridge featuring a pulled emitter. Data labels represent the median %CV of protein quantitation with Spectronaut software (n = 3).

Improved peptide identification and robustness

Acquisition of the dilution series from 250 pg to 5 ng was repeated on fresh samples multiple times over a span of 64 days. On each day, either the column for Thermo Scientific™ EASY-Spray™ Ion Source was manually re-positioned or the OptiSpray µPAC 50 cm cartridge was re-inserted, followed by a quick optimization procedure before running the sequence. Between the days, the instrument remained idle, while the OptiSpray µPAC 50 cm cartridge was stored at room temperature. The number of peptides identified using a single OptiSpray µPAC 50 cm cartridge consistently exceeded that of the third-party column by 17% on average. Across different days, the results with the OptiSpray cartridge varied by less than 5% between any two days, demonstrating higher sensitivity and excellent reproducibility (Figure 5). This increased sensitivity with the OptiSpray µPAC 50 cm cartridge translated into enhanced proteome coverage, and increased peptide identifications by 17% and protein identifications by 5% on average. Figures 6 and 7 illustrate the number of peptides and protein groups identified using Spectronaut software, DIA-NN software, and Proteome Discoverer software.

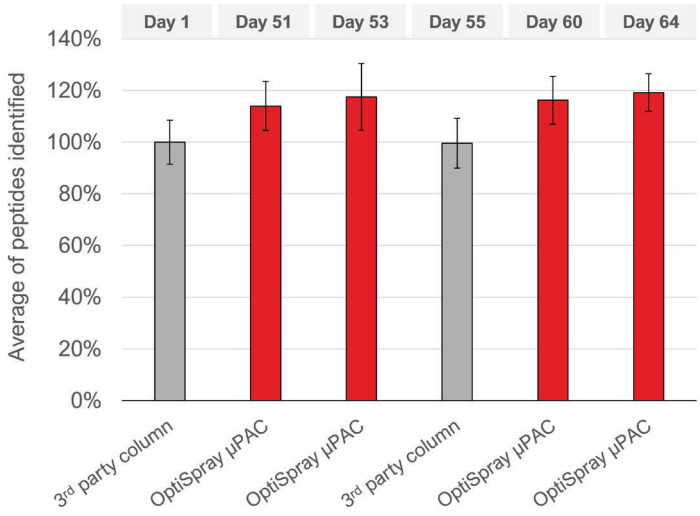

Figure 5. Average of peptides identified in triplicates of 250–5,000 pg dilution series, repeated over 64 days at 40 SPD, using the same OptiSpray µPAC 50 cm cartridge and the same third-party column. Each level was processed separately with Spectronaut software. The OptiSpray µPAC 50 cm cartridge increased peptide identifications by 17% on average. The observed increase, as well as variability, was uniform across levels.

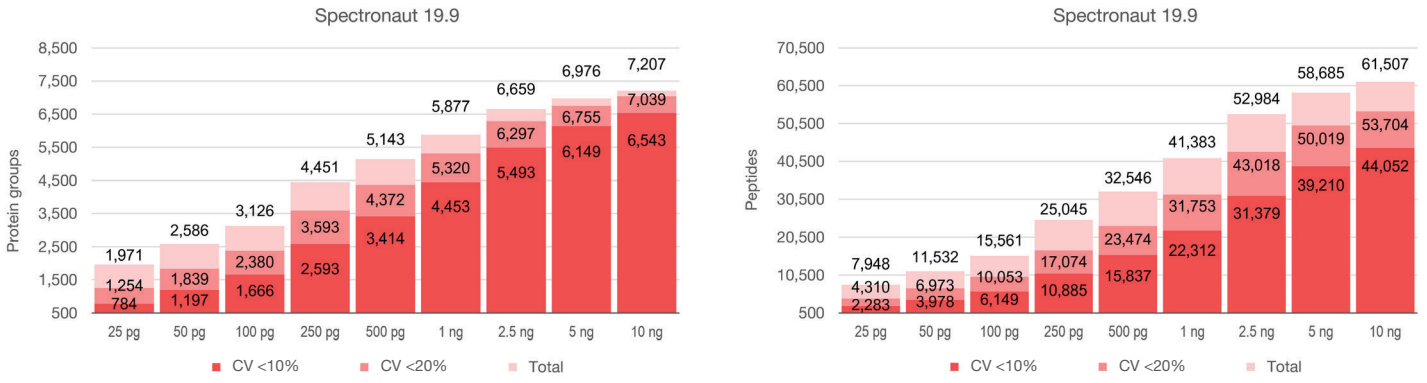

Figure 6. Number of protein groups (A) and peptides (B) identified using Spectronaut 19.9 software in HeLa digest (25 pg–10 ng) acquired at 20 SPD with the OptiSpray µPAC 50 cm cartridge and shown subdivided by thresholds CV<10% and CV<20%

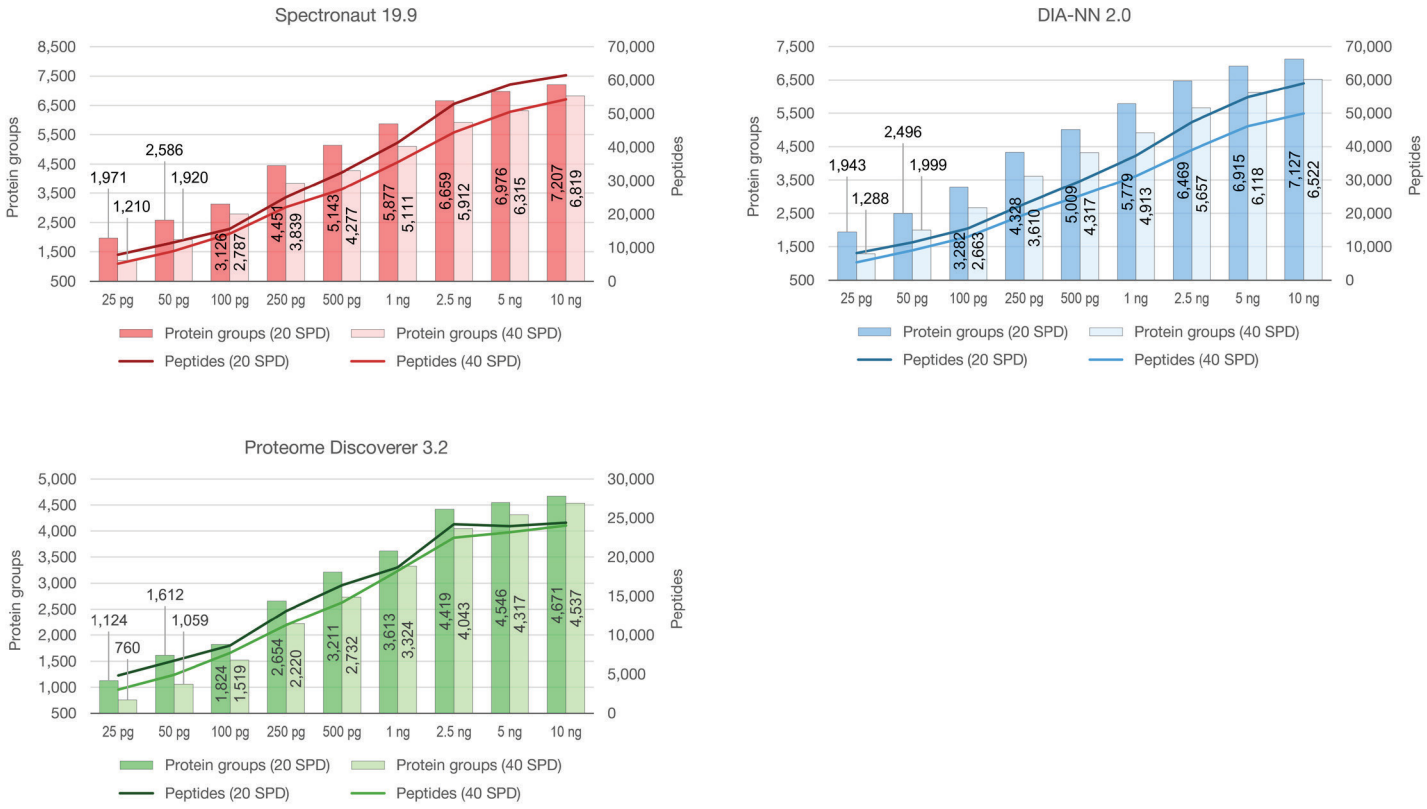

Figure 7. Number of peptides and protein groups identified in HeLa digest (25 pg–10 ng) using Spectronaut 19.9 software (A), DIA-NN 2.0 (B), and Proteome Discoverer 3.2 (C), acquired at 20 SPD and 40 SPD with the OptiSpray µPAC 50 cm cartridge

Leveraging Orbitrap MS high-resolution detection

Orbitrap mass spectrometers offer an excellent linear dynamic range, which supports accurate quantification of individual peptides across a wide range of concentrations. Furthermore, as shown in Table 2, the resolution can be increased for methods with longer gradients. This usually results in an increased number of identifications and increased sensitivity of low-abundance features (Figures 7, 9, and 10).

To demonstrate linearity and the quality of chromatographic features, we selected two putative biomarkers with significance in cancer and examined select peptides with respect to their linearity and data quality.

To demonstrate linearity across a wide concentration range, we selected a tryptic peptide from Importin α2 (UniProt™ recommended name: Importin subunit alpha-1 [UniProt P52292]). Importin α2 (karyopherin alpha-2), encoded by the KPNA2 human gene, has been implicated in mediating the transport of the Human papillomavirus (HPV) L2 structural protein towards the chromosomes during cell division, which enables establishing an HPV infection.[3,4]

In transient expression assays, where HPV oncoproteins were introduced into cells, researchers have shown that the E6 protein binds to several host proteins, including proteins of the minichromosome maintenance (MCM) complex, which is required for DNA replication.[5,6] Elevated expression of MCM proteins has been observed in abnormal HPV-infected cervical cells.[7]

We selected a tryptic peptide from MCM7 protein to demonstrate the quality of chromatographic features at low abundance, and how Orbitrap high-resolution detection can be leveraged to improve sensitivity and data quality of low-abundance features.

Figure 9 shows the excellent linearity of the selected doubly charged tryptic peptide LLGASELPIVTPALR from Importin α2, in HeLa standard digest. Using the 20 SPD method with 240,000 MS^1 and 120,000 MS^2 resolution, both linearity and the limit of detection were improved compared to the 40 SPD method.

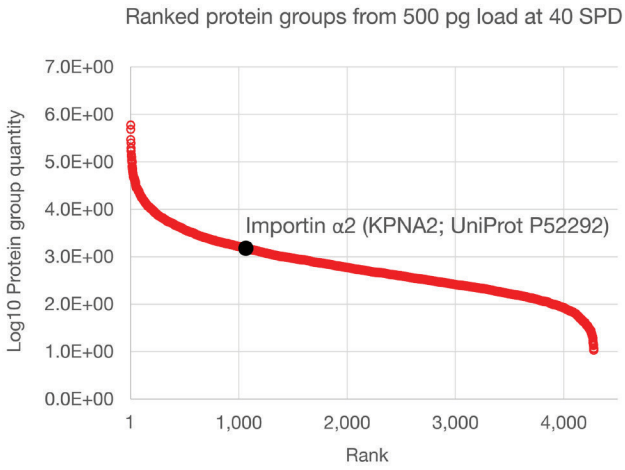

Figure 8. Ranked abundance distribution of identified protein groups from a 500 pg sample analyzed at 40 SPD. Each red circle represents a quantified protein group, plotted by rank versus Log10 protein quantity. The black dot highlights Importin α2 (KPNA2; UniProt P52292), indicating its relative abundance among all detected proteins.

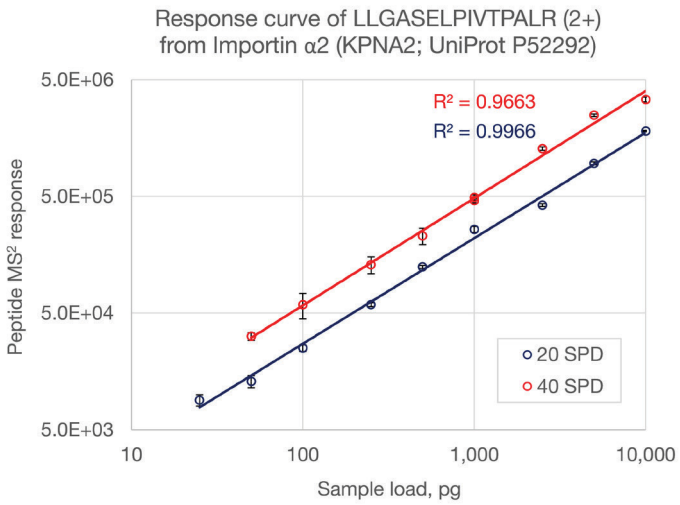

Figure 9. MS^2 response curve of doubly charged LLGASELPIVTPALR peptide from Importin α2 in HeLa standard digest at 25, 50, 100, 250, 500, 1,000, 2,500, 5,000, and 10,000 pg. Triplicates independently processed at each level with DIA-NN software. The signal response curves are evaluated in log–log scale.

Figure 10 shows the MS^1 and MS^2 extracted ion chromatograms of the precursor and fragments of the doubly charged tryptic peptide C(Carbamidomethyl)SILAAANPAYGR from MCM7, in HeLa standard digest at 50 pg load, as visualized in Thermo Scientific™ FreeStyle™ software and Spectronaut software. The 20 SPD method, with 240,000 MS^1 and 120,000 MS^2 resolution, improved detection quality and increased peptide and protein identification rates (see also Figure 7).

With both methods the C(Carbamidomethyl)SILAAANPAYGR peptide was detected with a CV of 20% at 25 pg load, a CV of 10% at 100 pg load, and <5% CV at 1 ng load. From 25 pg to 10 ng, the response curve had an R^2 of 0.9986 with the 40 SPD method, and an R^2 of 0.9977 with the 20 SPD method. Furthermore, with the 40 SPD method, quality of peaks obtained at 100 pg load using the OptiSpray µPAC 50 cm cartridge was consistent between repetitions, while comparable quality was observed with the third-party, EASY-Spray-compatible column only starting at >250 pg load (Figure 11).

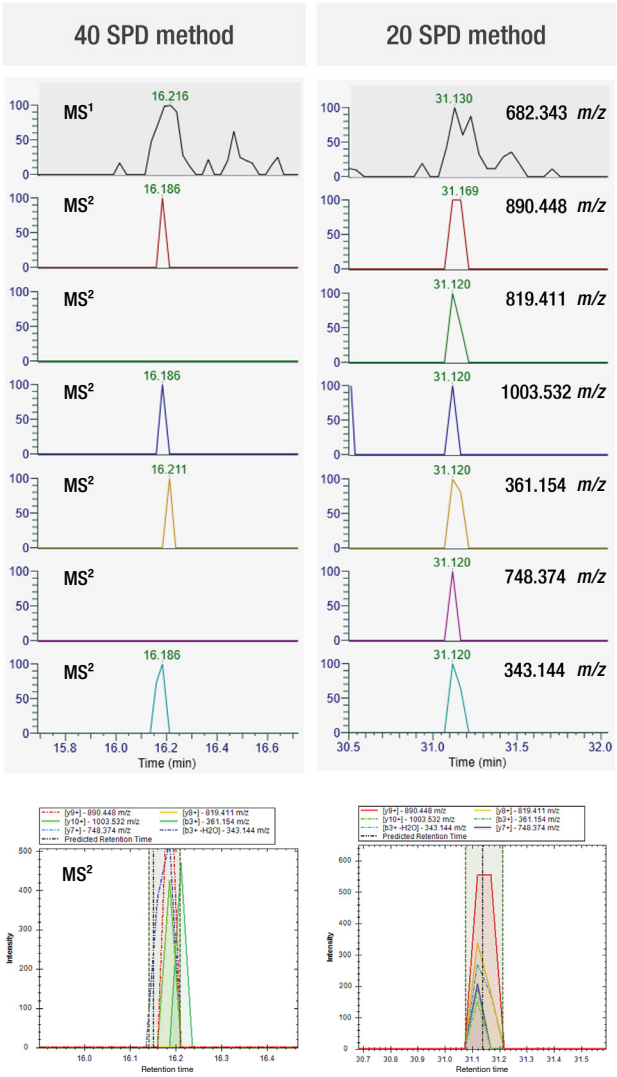

Figure 10. Extracted ion chromatograms of the tryptic peptide C(Carbamidomethyl)SILAAANPAYGR from MCM7 protein in HeLa digest acquired with the OptiSpray µPAC 50 cm cartridge at 50 pg. Data collected using 40 SPD and 20 SPD methods and visualized in FreeStyle software (top) and Spectronaut software (bottom). Quantification was based on MS^2, with resolution of 60,000 (40 SPD) and 120,000 (20 SPD). The higher resolution in the 20 SPD method improved fragment ion peak detection and limits of detection.

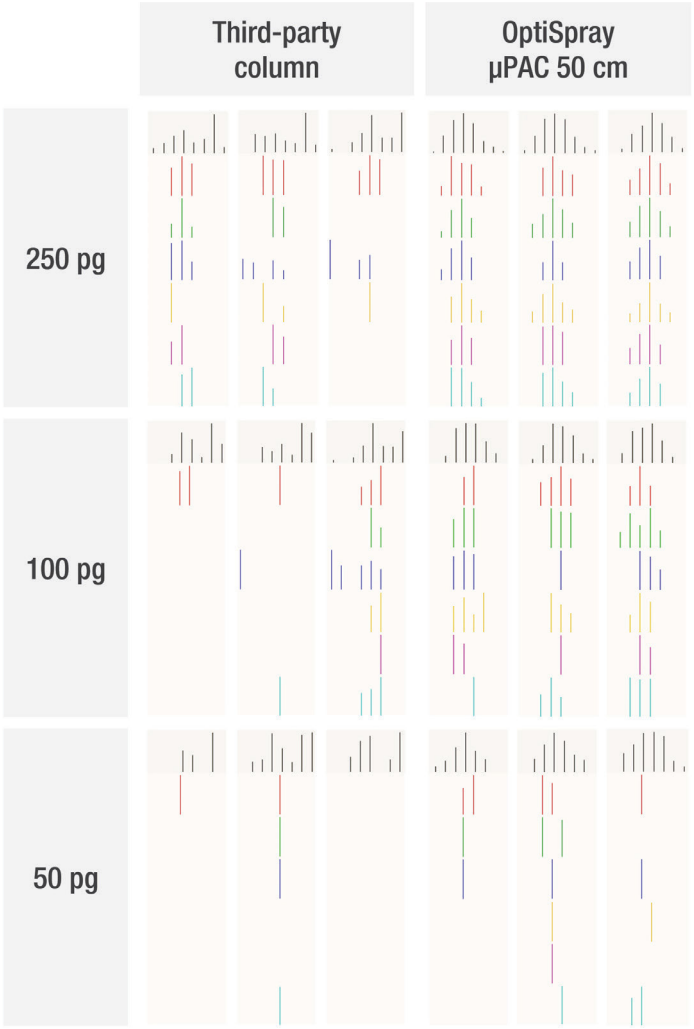

Figure 11. Extracted ion chromatograms of the tryptic peptide C(Carbamidomethyl)SILAAANPAYGR from MCM7 protein in a HeLa digest, acquired using an OptiSpray µPAC 50 cm cartridge and a third-party column (75 µm I.D. × 25 cm, sub-2 µm particles, integrated pulled emitter) with the 40 SPD method. Data in FreeStyle software are shown as individual scans (stick plot). Chromatogram rows match Figure 10—precursor ion (top rows) and fragment ions (rows below top row), and each column belongs to a technical replicate run. OptiSpray technology showed more consistent scans and improved sensitivity at lower loads.

Conclusion

- The OptiSpray ion source and Orbitrap Ascend MultiOmics Tribrid mass spectrometer, combined with a FAIMS Pro Duo interface and a µPAC Neo 50 cm cartridge, offer excellent sensitivity for peptide and protein identification in low-load samples, identifying 1,971 protein groups from 25 pg HeLa standard digest.
- The OptiSpray µPAC Neo cartridge enables the identification of >5,100 protein groups at 20 SPD and >4,200 protein groups at 40 SPD in 500 pg of HeLa standard digest with the Orbitrap Ascend MultiOmics Tribrid mass spectrometer and FAIMS Pro Duo interface.
- The OptiSpray µPAC Neo 50 cm cartridge demonstrated superior technical precision over a third-party 25 cm packed bed column, with consistent improvements across various sample loads. In comparison, the median coefficient of variation was reduced by a third on average, likely due to automated emitter positioning and the novel column assembly providing superior spray stability and reproducibility.
- The 240,000 resolution Orbitrap detection improves data quality of low-abundance features, enabling more accurate identification thanks to an increased number of fragment ions detection.
- The Orbitrap Ascend MultiOmics Tribrid MS offers robust chromatographic feature detection over a wide range, from 25 pg to 10 ng, with excellent linearity.

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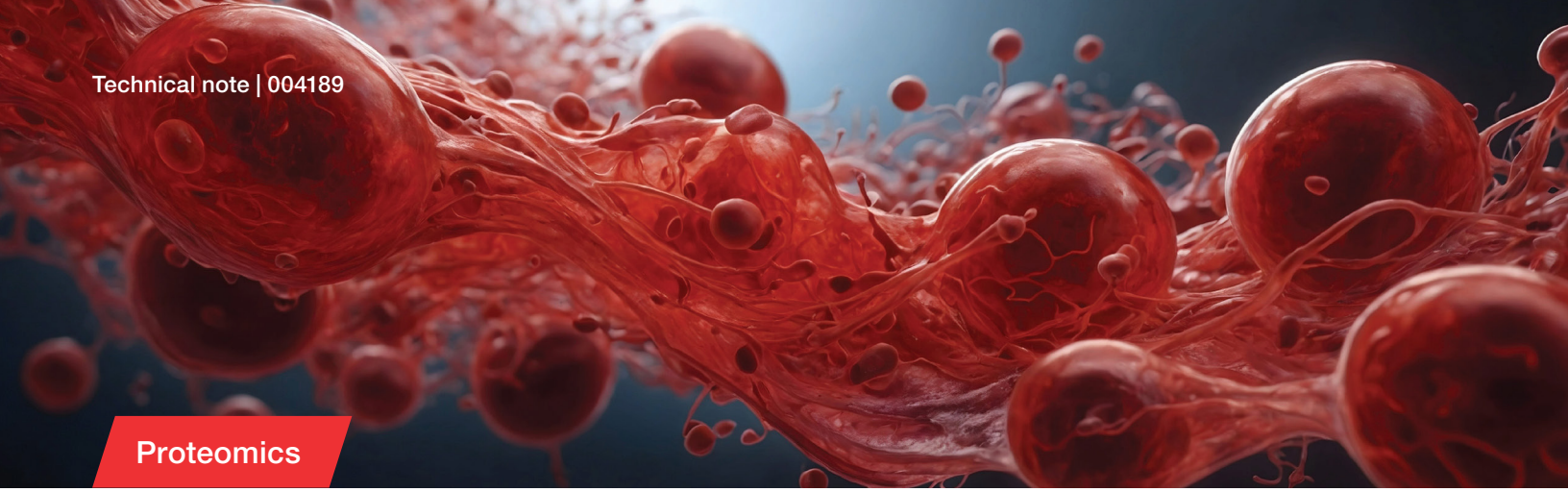

Proteomics

Scalable and deep plasma proteomics on the Orbitrap Excedion Pro mass spectrometer

Authors

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Keywords

Plasma proteomics, Orbitrap Excedion Pro, OptiSpray, translational research

Goal

Demonstrate the capabilities of the Thermo Scientific™ OptiSpray™ Ion Source coupled with the Thermo Scientific™ Orbitrap™ Excedion™ Pro Hybrid Mass Spectrometer for plasma proteomics workflows. Showcase the throughput versatility, peptide and protein group identifications, and quantitative precision with the Orbitrap Excedion Pro MS across multiple plasma sample preparation strategies.

Introduction

Plasma proteomics, the study of proteins in blood plasma, provides a powerful view into human biology and a practical route to novel biomarker discovery. By capturing protein changes linked to complex biological processes, it supports insights into immune responses, signaling pathways, and disease mechanisms, making it an important tool for advancing diagnostics, health monitoring, and personalized medicine.[1]

However, plasma's complexity and dynamic range pose significant analytical challenges. Protein concentrations can vary by up to 12 orders of magnitude,[2] making it difficult to detect low-abundance species. In addition, preanalytical, analytical, and biological factors can impact measurement reproducibility and the reliability of candidate biomarkers, reinforcing the importance of optimized collection, preparation, and analytical strategies.[3]

Liquid chromatography-mass spectrometry (LC-MS) approaches address many challenges by combining a broad analytical dynamic range with the ability to directly measure peptides and post-translational modifications (PTMs). Ongoing advances in sample preparation, separation, MS hardware, and data analysis continue to improve robustness and quantitative reproducibility. The Orbitrap Excedion Pro MS extends these capabilities, enabling flexible method design, consistent quantitative precision, and compatibility with diverse plasma preparation strategies. This versatility makes the Orbitrap Excedion Pro MS well-suited for scalable biomarker discovery studies, where precision, robustness, and throughput are essential for translating proteomic findings into real-world impact.

Combined with the OptiSpray ion source and cartridge columns, researchers can reduce the operational complexity of low-flow separations with automated emitter positioning, exchangeable emitters, and plug-and-play cartridge installation. Additionally, for higher throughput methods with capillary flow, researchers can use sheath gas to increase ionization efficiency, enhance spray stability, and reduce contamination. Together, the Orbitrap Excedion Pro MS equipped with the OptiSpray ion source simplifies data acquisition, enhances scalability and reproducibility, and supports robustness, while producing high-quality LC-MS data for translational research.

Here, we demonstrate the performance of the Orbitrap Excedion Pro MS equipped with the OptiSpray ion source using both high-throughput (HT) and comprehensive profiling (Max ID) workflows applied to neat and immuno-depleted plasma. With both methods, researchers can balance depth of proteome coverage, robust quantitative reproducibility, and throughput, enabling scalable biomarker discovery and reliable analysis across diverse plasma proteomics applications.

Plasma proteomics sample preparation—flexible workflows designed to meet your research needs

- Neat plasma
  - Streamlined, minimal handling preserves the complete plasma protein profile
  - Cost-effective and highly reproducible, ideal for high-throughput studies
  - Kit-based end-to-end sample preparation workflow
  - Provides an unbiased starting point for broad proteome profiling
  - Abundant proteins may mask low-abundance targets but simplify workflow and reduce sample loss
- Immuno-depleted plasma
  - Removes high-abundance proteins to enhance detection of low-abundance species
  - Extends dynamic range and improves sensitivity and quantitative precision
  - Automated, plate-based workflows enable scalability and reduce technical variability
  - Well suited for large-scale biomarker discovery requiring consistent, high-quality data

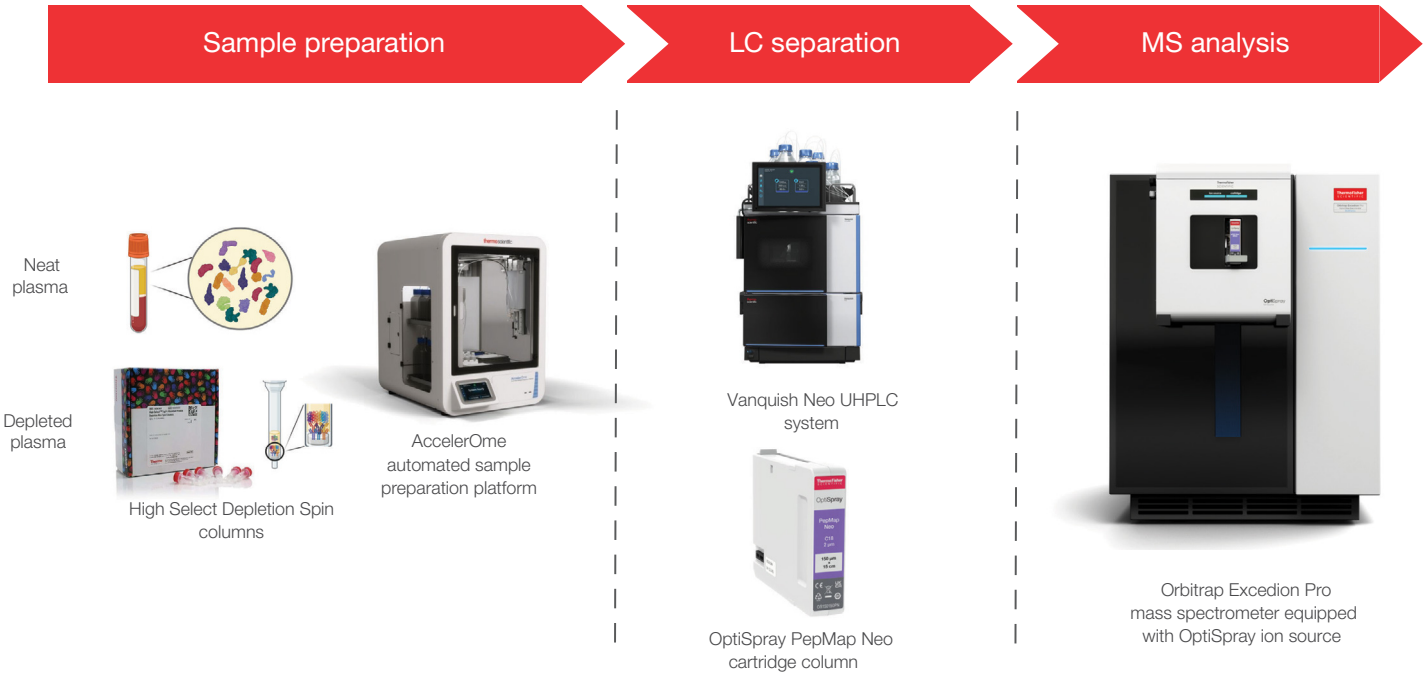

Figure 1. Scalable plasma proteomics workflow using the Orbitrap Excedion Pro mass spectrometer.

Experimental

Common consumables

- Fisher Chemical™ Water with 0.1% formic acid (FA) (v/v), Optima™ LC-MS grade (Cat. No. LS118-500)
- Fisher Chemical™ 80% Acetonitrile (ACN), 20% water with 0.1% formic acid, Optima™ LC-MS (Cat. No. LS122500)
- Fisher Chemical™ Formic acid, 99.0+%, Optima™ LC-MS grade (Cat. No. A117-50)
- Thermo Scientific™ High Select™ Depletion Spin Columns (Cat. No. A36370)
- Thermo Scientific™ AccelerOme™ Label-Free MS Sample Prep Kit (Cat. No. A50945)
- Thermo Scientific™ Pierce™ Quantitative Fluorometric Peptide Assay Kit (Cat. No. 23290)

Analytical columns

- Thermo Scientific™ OptiSpray™ PepMap™ Neo Cartridge
  - 2 µm, C18, 150 µm × 15 cm (Cat. No. OS150150PN)
  - 2 µm, C18, 75 µm × 50 cm (Cat. No. OS75500PN)
- Thermo Scientific™ PepMap™ Neo Trap Cartridge, 5 µm, C18, 300 µm × 5 mm (Cat. No. 174500)

Instrumentation

- Thermo Scientific™ Savant™ SpeedVac™ Concentrator
- Thermo Scientific™ AccelerOme™ Automated Sample Preparation Platform
- Thermo Scientific™ Vanquish™ Neo UHPLC System (Cat. No. VN-S10-A-01)
- Orbitrap Excedion Pro mass spectrometer (Cat. No. BRE725572)
- OptiSpray ion source (Cat. No. B51004132)
- Eppendorf™ ThermoMixer™ C temperature-controlled plate shaker

Sample preparation

Neat and immuno-depleted plasma peptides were prepared from a matched K_2EDTA pooled human plasma sample across both sample preparation methodologies. Neat plasma peptides were prepared using the AccelerOme Label-Free MS Sample Prep Kit on the AccelerOme automated sample preparation platform. Specifically, 1.5 µL of plasma was mixed with 48.5 µL of LYSE solution and samples were loaded onto the AccelerOme automated sample preparation platform for protein preparation, digestion with Trypsin/Lys-C, and peptide clean-up. Peptides were then dried down with a Savant SpeedVac concentrator and reconstituted in water with 0.1% formic acid and 2% acetonitrile to a final concentration of 100 ng/µL. Peptide concentrations were quantified using the Pierce Quantitative Fluorometric Peptide Assay Kit.

Immuno-depleted plasma peptides were prepared using the High Select Depletion Spin columns. Specifically, 10 µL of plasma was used per depletion with the columns, following the manufacturer's protocol. Depleted protein samples were concentrated using the Savant SpeedVac concentrator and reconstituted in 50 µL of LYSE solution from the AccelerOme Label-Free MS Sample Prep Kit. Samples were loaded directly into the AccelerOme automated sample preparation platform for protein preparation, digestion with Trypsin/Lys-C, and peptide clean-up. Peptides were then dried down with a Savant SpeedVac concentrator and reconstituted in water with 0.1% formic acid and 2% acetonitrile to a final concentration of 100 ng/µL. Peptide concentrations were quantified using the Pierce Quantitative Fluorometric Peptide Assay Kit. Depending on the LC-MS throughput, 200 ng, 500 ng, or 1,250 ng of peptide mass was loaded on the column for LC-MS analysis.

LC-MS analysis

All LC-MS runs for neat and depleted plasma matched peptides were analyzed using a Vanquish Neo UHPLC system in trap-and-elute configuration, paired with an Orbitrap Excedion Pro mass spectrometer. Peptides were separated on the Vanquish Neo UHPLC system using OptiSpray PepMap Neo cartridge columns, and chromatographic gradients were formed using 0.1% formic acid in water for mobile phase A and 0.1% formic acid in 80% acetonitrile for mobile phase B. Liquid chromatography parameters and gradient settings can be found in Tables 1 and 2. Mass spectrometer source parameters and scan parameters can be found in Tables 3 and 4. Gas phase fractionation MS1 and MS2 parameters can be found in Tables 5 and 6. All source parameters used for either HT (Narrow-Range, N-HT or Standard-Range, S-HT) or Max ID MS methods were conserved for HT and Max ID gas-phase fractionation (GPF) runs. GPF was performed to generate sample-specific chromatogram libraries to enhance the depth of proteome coverage. By dividing the mass range into smaller segments across six injections, GPF allows for more comprehensive analysis, which is used for spectral library generation to search DIA data.

Table 1. High-throughput HPLC parameters.

High-throughput HPLC parameters			
	Time (min)	% Mobile phase B	Flow (µL/min)
Gradient	0	4	2.0
	0.2	8	2.0
	0.4	8	1.5
	14.7	20.0	1.5
	21.4	35.0	1.5
	21.6	55.0	1.5
	21.65	99.0	2.0
	23.35	99.0	2.0
LC parameters	LC configuration	Trap and elute	
	Fast loading/Equilibration mode	Pressure control	
	Loading/Equilibration/Wash pressure	800/1,000/800 bar	
	Equilibration factor	2	
	Sampler temperature	7°C	
	Mobile phase A / Weak wash	0.1% Formic acid in water	
	Mobile phase B / Strong wash	0.1% Formic acid in 80% acetonitrile	
	Zebra wash	Disabled	
	Analytical column (cartridge) temperature	50°C	
Column specifications	Analytical column	OptiSpray PepMap Neo cartridge; 2 µm, C18, 150 µm × 15 cm (Cat. No. OS150150PN)	
	Trap column	PepMap Neo trap cartridge, 5 µm C18 300 µm x 5 mm (Cat. No. 174500)	

Table 2. Max identification HPLC parameters.

Max identification HPLC parameters			
	Time (min)	% Mobile phase B	Flow (µL/min)
Gradient	0	4	0.5
	0.4	8	0.5
	0.5	8	0.25
	65.1	20.0	0.25
	90.1	35.0	0.25
	90.2	99.0	0.25
	90.3	99.0	0.5
	100.0	99.0	0.5
LC parameters	LC configuration	Trap and elute	
	Fast loading/Equilibration mode	Pressure control/Combined control	
	Loading/Equilibration/Wash pressure	800/1,000/800 bar	
	Equilibration max flow	1.5 µL/min	
	Equilibration factor	2	
	Sampler temperature	7°C	
	Mobile phase A / Weak wash	0.1% Formic acid in water	
	Mobile phase B / Strong wash	0.1% Formic acid in 80% acetonitrile	
	Zebra wash	Disabled	
	Analytical column (cartridge) temperature	50°C	
Column specifications	Analytical column	OptiSpray PepMap Neo cartridge; 2 µm, C18, 75 µm × 50 cm (Cat. No. OS75500PN)	
	Trap column	PepMap Neo trap cartridge, 5 µm C18 300 µm x 5 mm (Cat. No. 174500)	

Table 3. Orbitrap Excedion Pro MS HT method parameters.
(A) Global source and mass spectrometer parameters; (B) MS1 full scan experiment parameters; (C) MS2 DIA scan experiment parameters.

A

Global parameters (Source and MS)	
Positive ion voltage	1,900 V
Ion transfer tube temperature	290°C
Gas mode	Static
Sheath gas (Arb)	10
Sweep gas (Arb)	0
Expected peak width	6 s
Advanced peak determination	True
Pre-accumulation for MS2 scans	True
Default charge state	2
Lock mass correction	Off

B

MS1 Full Scan experiment parameters	
Orbitrap resolution	60K
N-HT / S-HT Method scan range (*m/z*)	380–780 / 380–980
RF lens (%)	40
Normalized AGC target (%) / Absolute AGC value	300% (3.00e6)
Maximum injection time	Auto
Microscans	1

C

MS2 DIA Scan experiment parameters	
Orbitrap resolution	15K
N-HT / S-HT Method scan range (*m/z*)	380–780 / 380–980
Isolation window (*m/z*)	8
Window placement optimization	On
AGC target	Custom
Normalized AGC target (%) / Absolute AGC value	800% (8.00e5)
Maximum injection time	Auto
DIA scan range (*m/z*)	145–2,000
HCD collision energy (%)	25
RF lens (%)	40
Loop control	All

Table 4. Orbitrap Excedion Pro MS Max ID method parameters.
(A) Global source and mass spectrometer parameters; (B) MS1 full scan experiment parameters; (C) MS2 DIA scan experiment parameters.

A

Global parameters (Source and MS)	
Positive ion voltage	1,900 V
Ion transfer tube temperature	290°C
Gas mode	Static
Sheath gas (Arb)	0
Sweep gas (Arb)	0
Expected peak width	6 s
Advanced peak determination	True
Pre-accumulation for MS2 scans	True
Default charge state	2
Lock mass correction	Off

B

MS1 Full Scan experiment parameters	
Orbitrap resolution	60K
Scan range (*m/z*)	380–980
RF lens (%)	40
Normalized AGC target (%) / Absolute AGC value	300% (3.00e6)
Maximum injection time	Auto
Microscans	1

C

MS2 DIA Scan experiment parameters	
Orbitrap resolution	15K
Precursor mass range (*m/z*)	380–980
Isolation window (*m/z*)	4
Window placement optimization	On
AGC target	Custom
Normalized AGC target (%) / Absolute AGC value	1,000% (1.00e6)
Maximum injection time	Auto
DIA scan range (*m/z*)	145–2,000
HCD collision energy (%)	25
RF lens (%)	40
Loop control	All

Table 5. Orbitrap Excedion Pro MS GPF HT method scan parameters. (A). MS full scan GPF full scan parameters; (B) MS2 DIA GPF experiment parameters.

A

MS1 Full Scan experiment parameters	
Orbitrap resolution	60K
Scan range (*m/z*)	380–480; 480–580; 580–680; 680–780; 780–880; 880–980
RF lens (%)	40
Normalized AGC target (%) / Absolute AGC value	300% (3.00e6)
Maximum injection time	Auto
Microscans	1

B

MS2 DIA GPF experiment parameters	
Orbitrap resolution	15K
Precursor mass range (*m/z*)	380–480; 480–580; 580–680; 680–780; 780–880; 880–980
Isolation window (*m/z*)	4
Window placement optimization	On
AGC target	Custom
Normalized AGC target (%) / Absolute AGC value	800% (8.00e5)
Maximum injection time	Auto
DIA scan range (*m/z*)	145–2,000
HCD collision energy (%)	25
RF lens (%)	40
Pre-accumulation	On
Loop control	All

Table 6. Orbitrap Excedion Pro MS GPF Max ID method scan parameters. (A). MS full scan GPF full scan parameters; (B) MS2 DIA GPF experiment parameters.

A

MS1 Full Scan experiment parameters	
Orbitrap resolution	60K
Scan range (*m/z*)	380–480; 480–580; 580–680; 680–780; 780–880; 880–980
RF lens (%)	40
Normalized AGC target (%) / Absolute AGC value	300% (3.00e6)
Maximum injection time	Auto
Microscans	1

B

MS2 DIA GPF experiment parameters	
Orbitrap resolution	15K
Precursor mass range (*m/z*)	380–480; 480–580; 580–680; 680–780; 780–880; 880–980
Isolation window (*m/z*)	2
Window placement optimization	On
AGC target	Custom
Normalized AGC target (%) / Absolute AGC value	1,000% (1.00e6)
Maximum injection time	Auto
DIA scan range (*m/z*)	145–2,000
HCD collision energy (%)	25
RF lens (%)	40
Pre-accumulation	On
Loop control	All

LC-MS data processing and analysis

The acquired LC-MS data was processed using Spectronaut® (v20) software (Biognosys, AG) with default settings. All sample preparation methods were searched separately to minimize any signal inferences made across different sample preparation workflows. A human Uniprot database downloaded on 2024-07-29 containing verified sequences without isoforms (20,435 entries) was used for the analyses. The results were processed and filtered with a 1% precursor and 1% protein group false discovery rate (FDR). Exported output files were imported to Rstudio (2025.05.1 Build 513) with R (v4.5.1) for downstream data analysis and visualization.

Results

Matched neat and depleted plasma pool samples were run with two HT (Standard/Narrow-Range) methods along with a Max ID method to assess neat and immuno-depleted plasma proteome depth of coverage and quantitative precision. Technical triplicate injections across various throughputs and injection amounts yielded the results shown in Table 7 and Figures 2–6.

Table 7. Neat and immuno-depleted plasma proteome depth of coverage and quantitative precision.

Identifications and analytical precision	
Neat plasma	
Narrow-Range High-Throughput (N-HT)	• 200 ng input: Median of 2,746 peptides and 277 protein groups, with 3.8% peptide CV and 1.9% protein group CV • 500 ng input: Median of 2,991 peptides and 347 protein groups, with 3.9% peptide CV and 2.1% protein group CV
Standard-Range High-Throughput (S-HT)	• 200 ng input: Median of 3,187 peptides and 349 protein groups, with 4.8% peptide CV and 3.2% protein group CV • 500 ng input: Median of 3,334 peptides and 343 protein groups, with 4.1% peptide CV and 2.3% protein group CV
Max ID	• 500 ng input: Median of 4,356 peptides and 471 protein groups, with 6.5% peptide CV and 3.9% protein group CV • 1,250 ng input: Median of 4,496 peptides and 485 protein groups, with 5.5% peptide CV and 2.7% protein group CV
Data points per peak	• S-HT: Median of 3 data points per peak • N-HT and Max-ID: Median of 4 data points per peak
Top 14 protein depletion	
Narrow-Range High-Throughput (N-HT)	• 200 ng input: Median of 4,531 peptides and 468 protein groups, with 5.5% peptide CV and 3.1% protein group CV • 500 ng input: Median of 4,966 peptides and 535 protein groups, with 5.3% peptide CV and 2.7% protein group CV
Standard-Range High-Throughput (S-HT)	• 200 ng input: Median of 5,081 peptides and 493 protein groups, with 6.4% peptide CV and 3.7% protein group CV • 500 ng input: Median of 5,367 peptides and 535 protein groups, with 5.3% peptide CV and 3.1% protein group CV
Max ID	• 500 ng input: Median of 7,947 peptides and 894 protein groups, with 5.5% peptide CV and 3.4% protein group CV • 1,250 ng input: Median of 8,598 peptides and 974 protein groups, with 5.3% peptide CV and 2.9% protein group CV
Data points per peak	• S-HT: Median of 3 data points per peak • N-HT and Max-ID: Median of 4 data points per peak

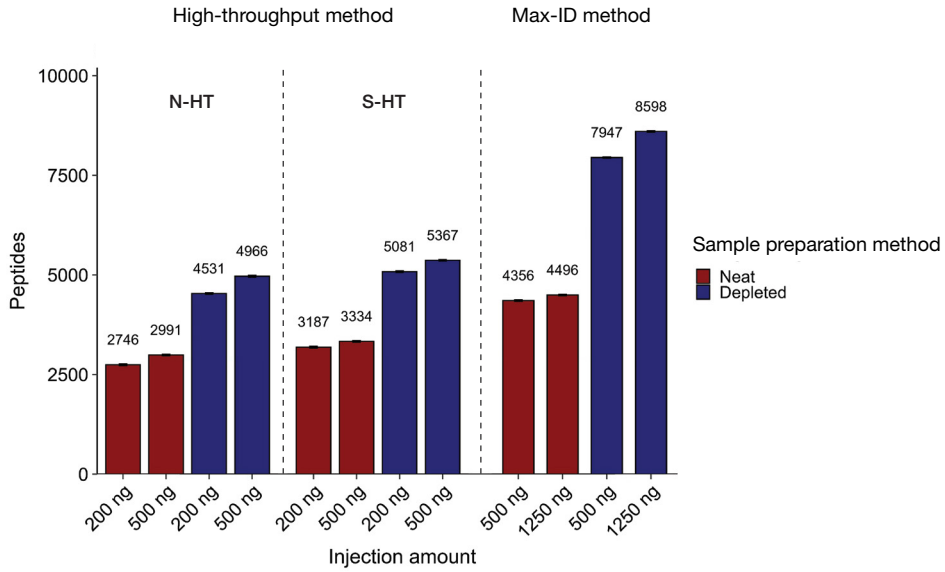

Figure 2. Peptide identification performance across HT and Max ID methods. Triplicate technical injections were performed sequentially from a healthy donor peptide pool across different sample preparation methods, injection amounts, instrument methods, and throughputs. Both HT methods (N-HT; 380–780 *m/z* precursor & DIA scan range and S-HT; 380–980 *m/z* precursor & DIA scan range) are represented above. Average peptide identification numbers are listed above each condition. Error bars represent standard deviation of identifications across technical replicates.

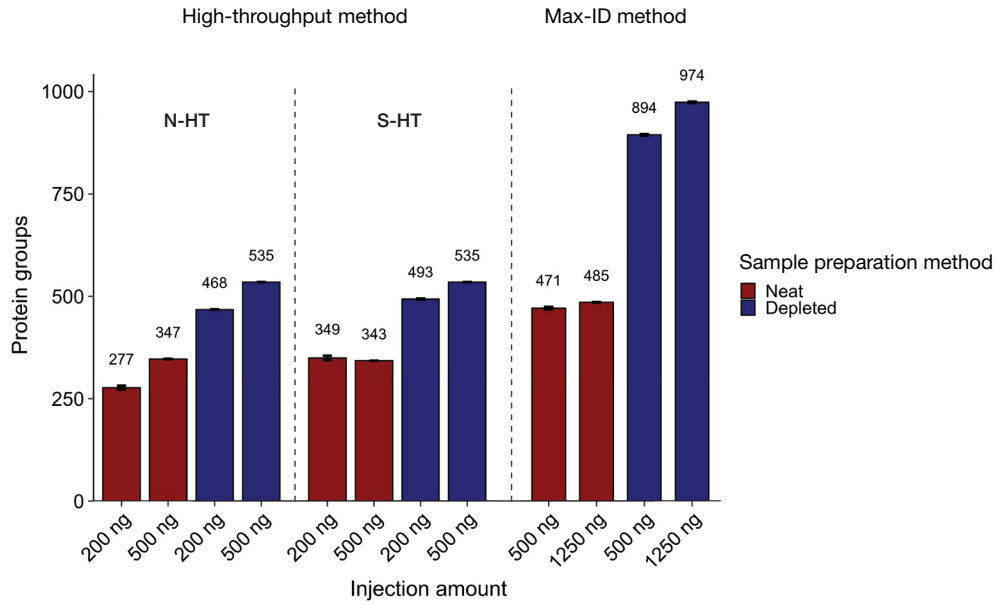

Figure 3. Protein group identification performance across HT and Max-ID methods. Triplicate technical injections were performed sequentially from a healthy donor peptide pool across different sample preparation methods, injection amounts, instrument methods, and throughputs. Both HT methods (N-HT; 380–780 *m/z* precursor & DIA scan range and S-HT; 380–980 *m/z* precursor & DIA scan range) are represented above. Average protein group identification numbers are listed above each condition. Error bars represent standard deviation of identifications across technical replicates.

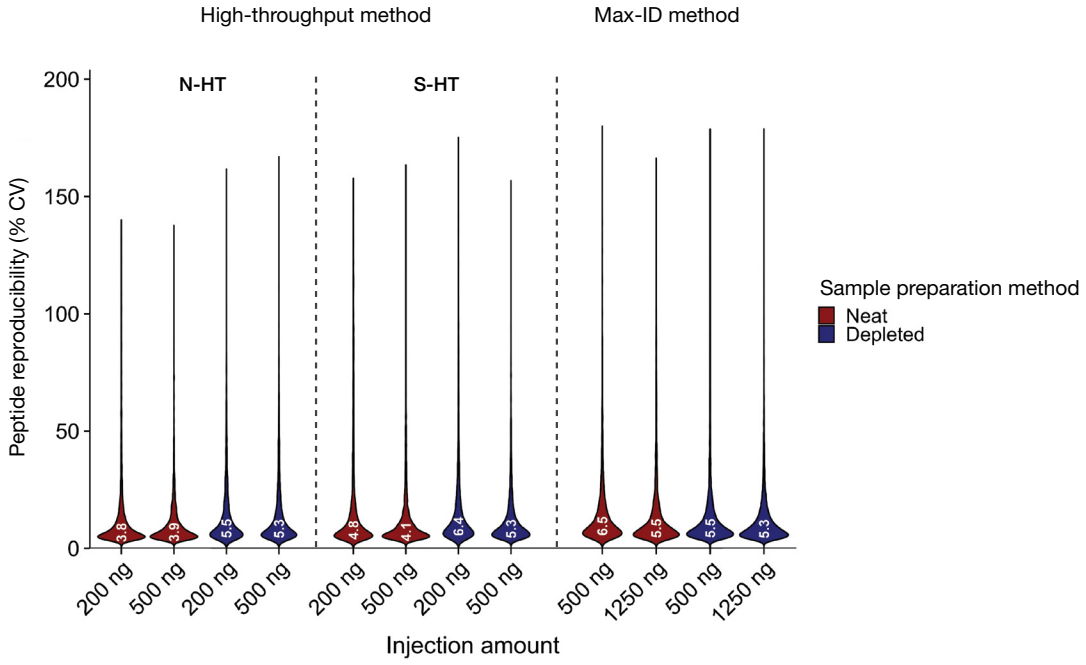

Figure 4. Peptide analytical precision performance across HT and Max ID methods. Triplicate technical injections were performed sequentially from a healthy donor peptide pool across different sample preparation methods, injection amounts, instrument methods, and throughputs. Both HT methods (N-HT; 380–780 *m/z* precursor & DIA scan range and S-HT; 380–980 *m/z* precursor & DIA scan range) are represented above. Median peptide coefficient of variation (% CV) are listed in each violin plot per condition.

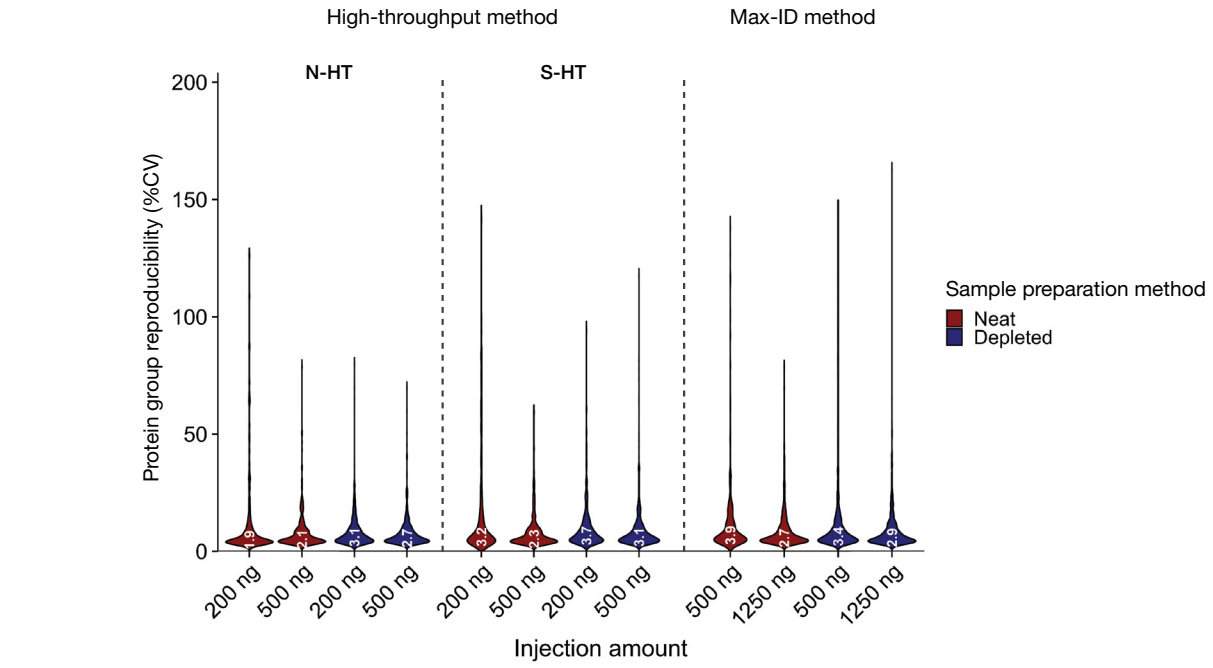

Figure 5. Protein group analytical precision performance across HT and Max ID methods. Triplicate technical injections were performed sequentially from a healthy donor peptide pool across different sample preparation methods, injection amounts, instrument methods, and throughputs. Both HT methods (N-HT; 380–780 *m/z* precursor & DIA scan range and S-HT; 380–980 *m/z* precursor & DIA scan range) are represented above. Median protein group coefficient of variation (% CV) are listed in each violin plot per condition.

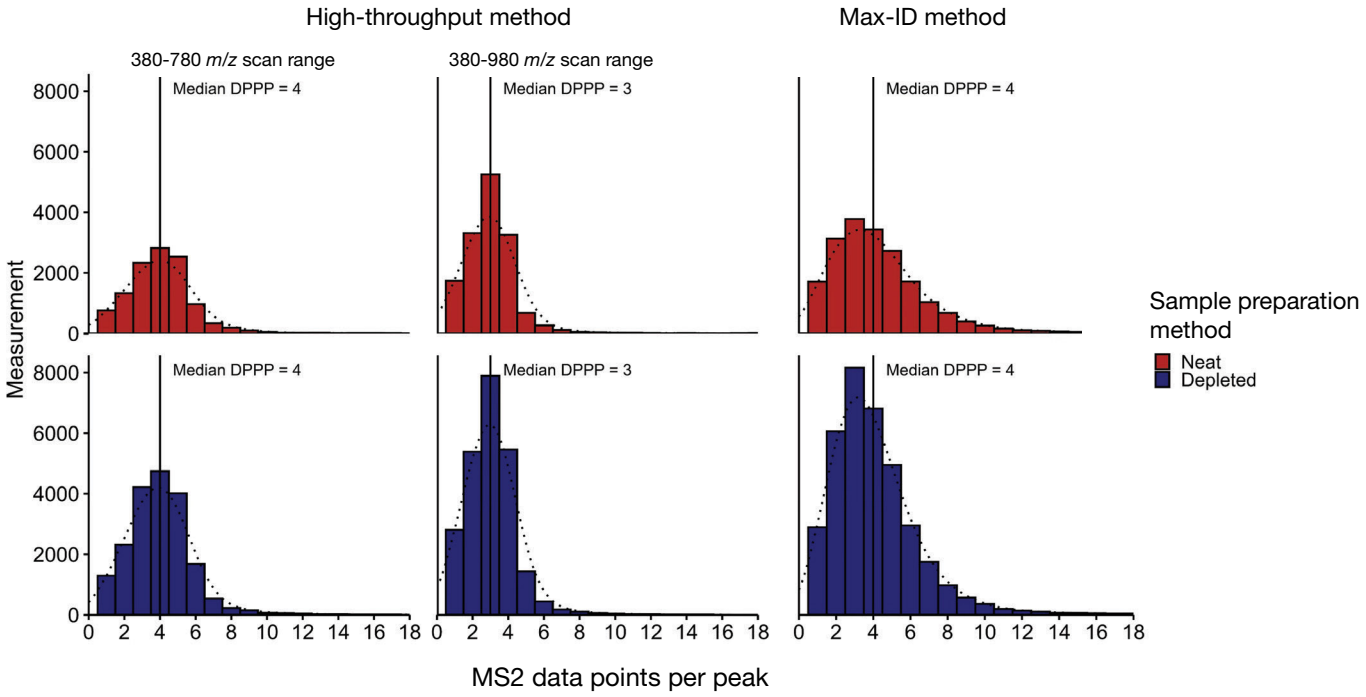

Figure 6. MS2 data points per peak across HT and Max ID methods. Median data points per peak (DPPP) per each method are represented by median lines. Both HT methods (N-HT; 380–780 *m/z* precursor & DIA scan range and S-HT; 380–980 *m/z* precursor & DIA scan range) are represented above. Data points per peak were calculated by counting the number of scans that were reported across the MS2 XIC for each identified precursor. Data visualized is represented from higher injection amounts.

Gas phase fractionation spectral library performance and utility

By using GPF, the number of peptides and protein groups in the spectral library increased by 28%–40% and 46%–66%, respectively, depending on the sample preparation type, injection amount, and instrument method (Figure 7). When using the concatenated directDIA™ search archive + gas phase fractionation spectral libraries in their respective sample preparation specific DIA searches, 15%–27% increases in peptide identifications were observed, corresponding to 23%–33% protein group increases. Overall, sample-specific gas phase fractionation proves beneficial by expanding the spectral library with more peptides and protein groups, enhancing identifications at both the peptide and protein levels, and increasing the depth of proteomic coverage. This further highlights the utility of chromatogram libraries, exemplifies findings from the literature,[4] and supports the use of GPF across pools from biological samples in large-scale cohort analyses for more comprehensive proteomics depth of coverage.

Neat and depletion protein group overlaps and correlation

Between neat and depleted plasma sample preparation methods from the same pooled plasma sample, immuno-depletion gives ~1.55x more depth with the S-HT method using library-free approaches and ~1.65x more depth with the concatenated GPF library (Figure 7). For the Max ID method, immuno-depletion gives ~2x more depth with the S-HT method using library-free approaches and ~1.92x more depth with the concatenated GPF library (Figure 8). When comparing protein group overlaps between both S-HT and Max ID methods across neat and depleted plasma, ~77% and ~83% of neat plasma protein groups are identified in respective throughput using depletion (Figure 9). To evaluate the additional proteins detected from immuno-depletion, protein group identifications were ranked by the average protein group quantity across sample preparation methods using the Max ID method (Figure 10). While the dynamic range is similar for both neat and depleted (~6 orders of magnitude), depletion-based methods identified 704 unique proteins that have lower protein abundances, demonstrating the utility of using depletion to access more lower abundant proteins.

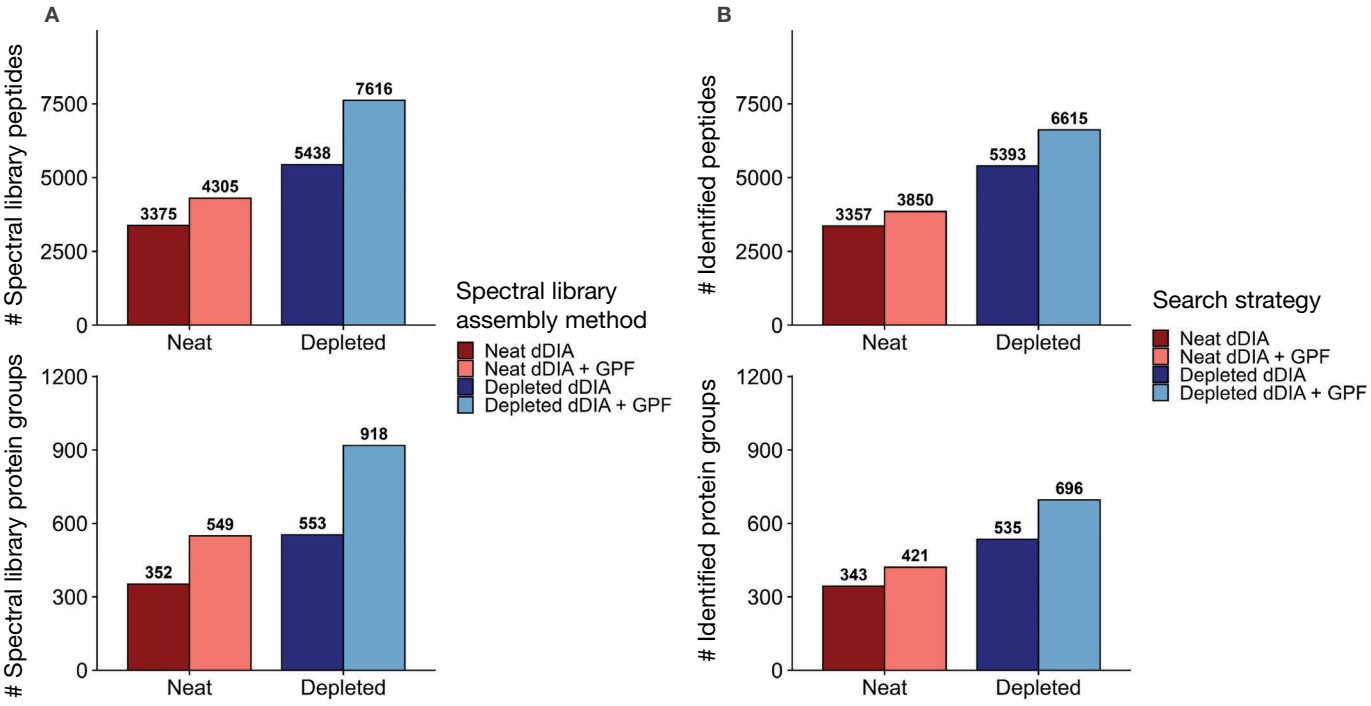

Figure 7. Identification increases in GPF spectral libraries and search implementation for HT method. (A) Peptides and protein groups in spectral library using library-free and GPF concatenated libraries across sample preparation methods. (B) Peptide and protein group identifications across the experiment using library-free and GPF concatenated libraries across sample preparation methods.

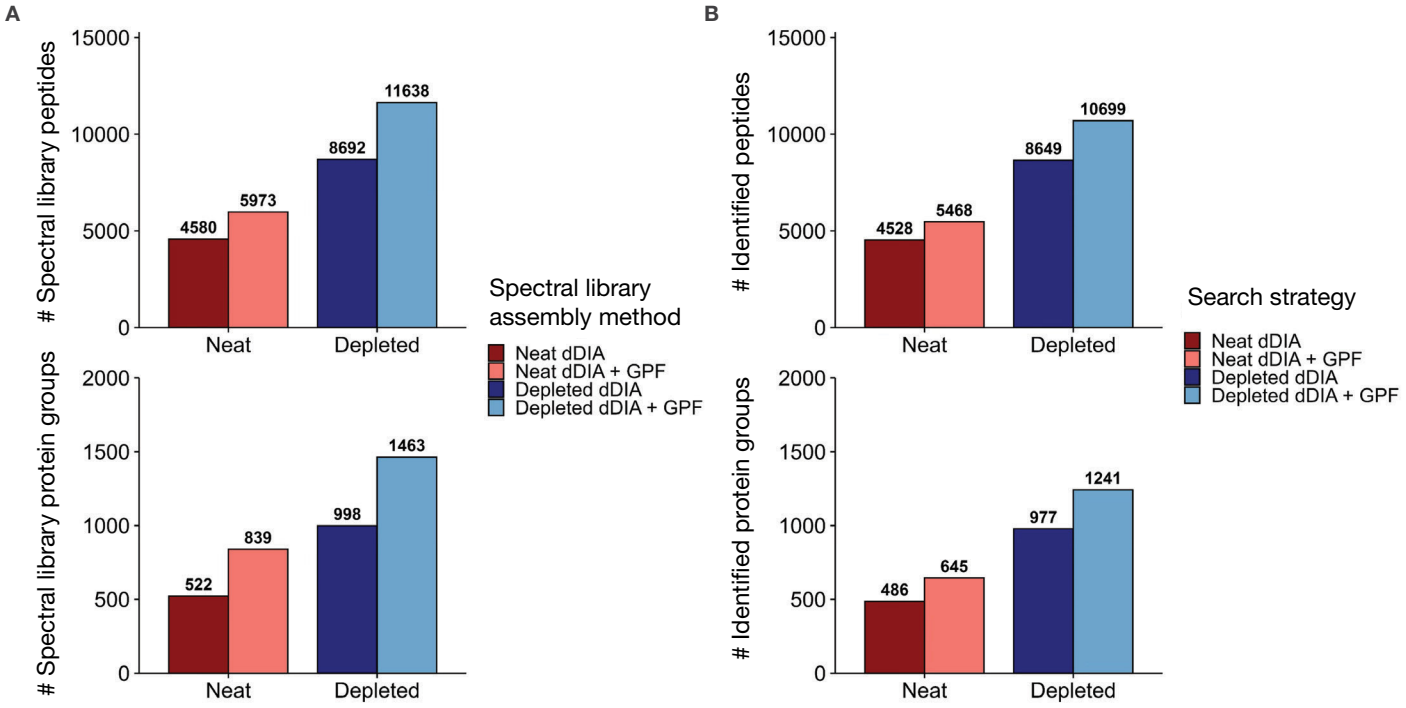

Figure 8. Identification increases in GPF spectral libraries and search implementation for MaxID method. (A) Peptides and protein groups in spectral library using library-free and GPF concatenated libraries across sample preparation methods. (B) Peptide and protein group identifications across the experiment using library-free and GPF concatenated libraries across sample preparation methods.

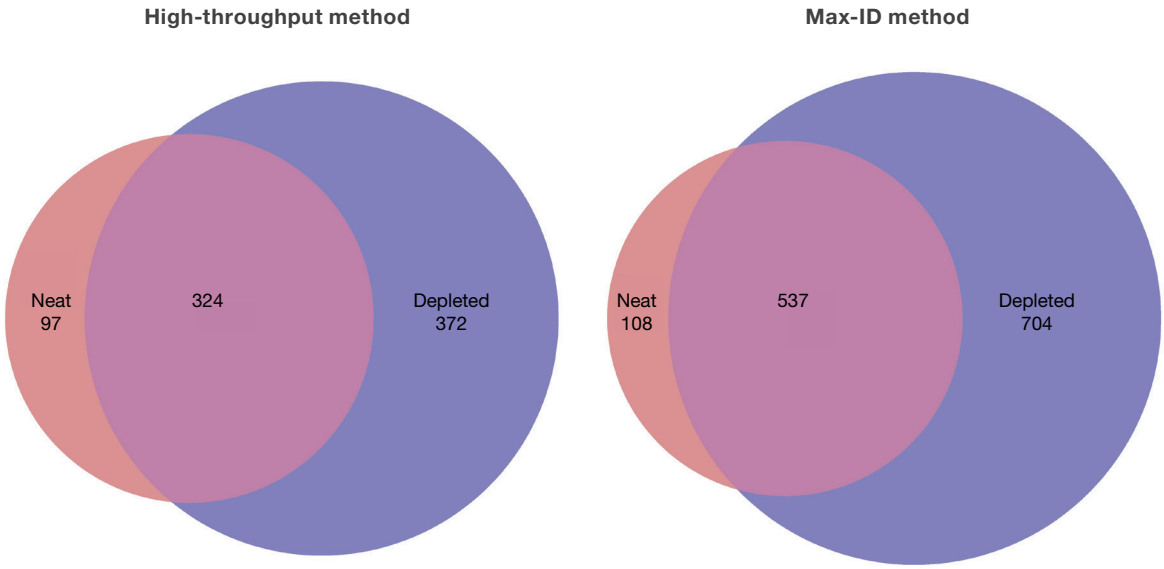

Figure 9. Protein group overlaps and complementarity across identified protein groups identified using each sample preparation method in HT and Max ID methods. The plotted data was derived from GPF concatenated search results, including protein groups that were identified at least once within the respective search.

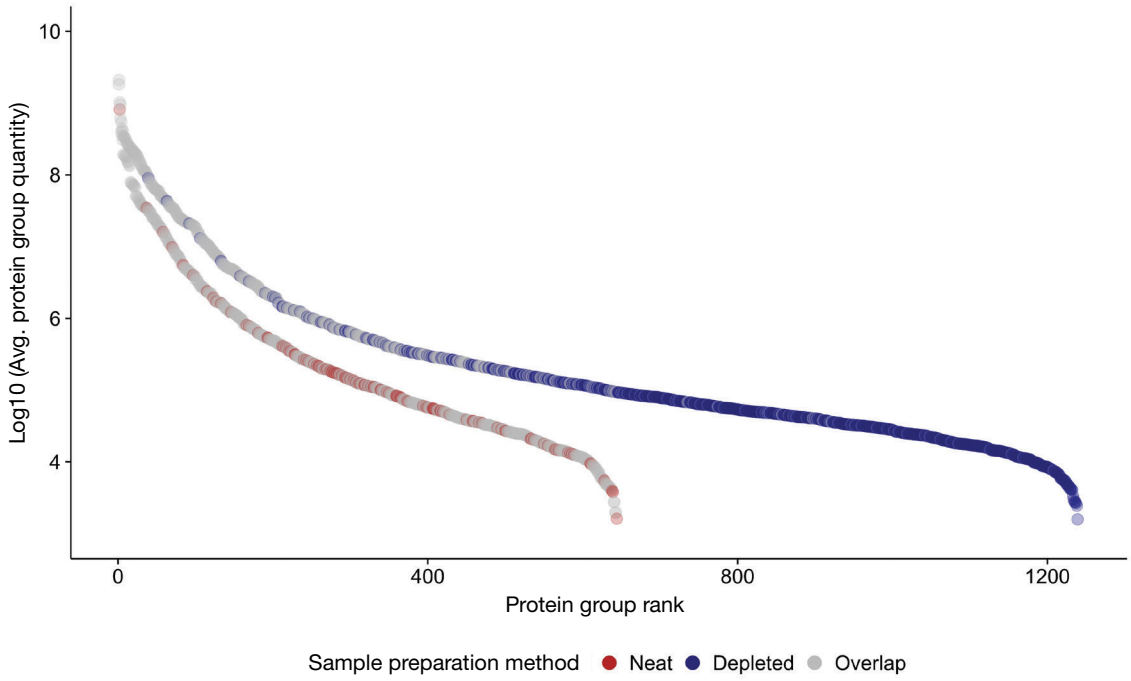

Figure 10. Protein group rank plots across sample preparation methods. Shared protein group identifications are denoted in gray, whereas unique protein groups found in only neat or only depleted are denoted in red or blue, respectively. The plotted data was derived from GPF concatenated search results, including protein groups that were identified at least once within the respective search.

When comparing overlapping protein group correlations across the tested sample preparation and instrument methods (S-HT & Max ID), Pearson correlations greater than 0.97 are observed between S-HT and Max ID methods within each sample preparation strategy. This demonstrates that both instrument methods yield similar protein group quantities, likely due to similar precursor intensity measurements across different throughput methods. This also indicates that scalable studies can be performed with shorter methods, such as the S-HT method, without sacrificing protein quantitation representation relative to longer methods used for more comprehensive plasma proteomic depth of coverage.

When comparing sample preparation strategies, correlations of 0.84–0.85 are observed. This suggests that depletion introduces minimal bias and preserves the protein group quantities observed in neat plasma. As neat plasma best represents the biological amount of protein groups in the sample due to the absence of additional sample handling steps, this highlights the strength of depletion as it maintains accurate biological quantities of proteins in the sample, while identifying up to 1.65x and 2x more protein groups across S-HT and Max ID methods, respectively.

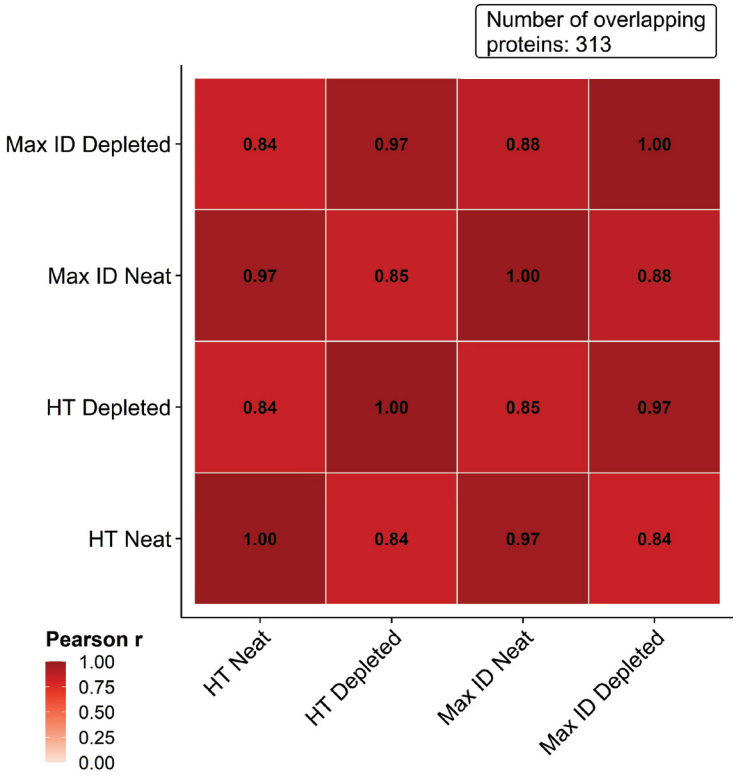

Figure 11. Protein group sample correlation matrix across sample preparation and instrument throughputs. Protein group correlations were calculated by comparing averaged protein group quantity across triplicate injections for overlapping proteins found in all sample preparation and throughput combinations. Protein group data plotted was used from GPF concatenated search results.

Conclusion

- **Plasma proteome depth of coverage:** Identified 421–645 protein groups in neat plasma and 696–1,241 protein groups in depleted plasma, using HT and Max ID methods, respectively.
- **High analytical precision:** Low variability in measurements across HT and Max-ID methods, with peptide CVs ranging from 3.8% to 6.4% for HT and 5.5% to 6.5% for Max-ID. Protein group CVs ranged from 1.9% to 3.7% for HT and 2.7% to 3.9% for Max-ID methods, indicating high precision. Peptide and protein group CVs for the N-HT method were the lowest, indicating an increase in quantitative precision using a more quantitatively focused method.
- **Enhanced depth from GPF:** Increased peptide identifications by up to 24% and protein group identifications by up to 33%, showcasing the utility of building sample-specific chromatogram libraries.
- **Scalable studies with shorter methods:** The S-HT method, which operates 4x faster, provides consistent and highly correlated measurements with the Max ID method, enabling scalable studies without sacrificing protein quantitation representation.
- **Minimal bias from depletion:** Correlations of 0.84–0.85 between neat and depleted plasma suggest minimal bias introduced by depletion, preserving accurate biological quantities of proteins while identifying up to 1.65x and 2x more protein groups across S-HT and Max ID methods, respectively.
- **Utility of depletion for lower abundant proteins:** Depletion-based methods identified 704 unique proteins with lower abundances, demonstrating the effectiveness of depletion in accessing lower abundant proteins.

Overall, the Orbitrap Excedion Pro mass spectrometer, combined with the OptiSpray ion source and cartridge-based columns, provides flexibility in throughput, maximizes plasma protein group and peptide identification, and ensures quantitative precision in both neat and depleted plasma samples. With the Orbitrap Excedion Pro mass spectrometer, researchers can unlock new possibilities in translational research. Its exceptional flexibility, high throughput, and precise quantitative capabilities support reliable and comprehensive proteomic analysis, making it an important tool for biomarker discovery and clinical research.

Learn more at thermofisher.com/orbitrapexcedionpro

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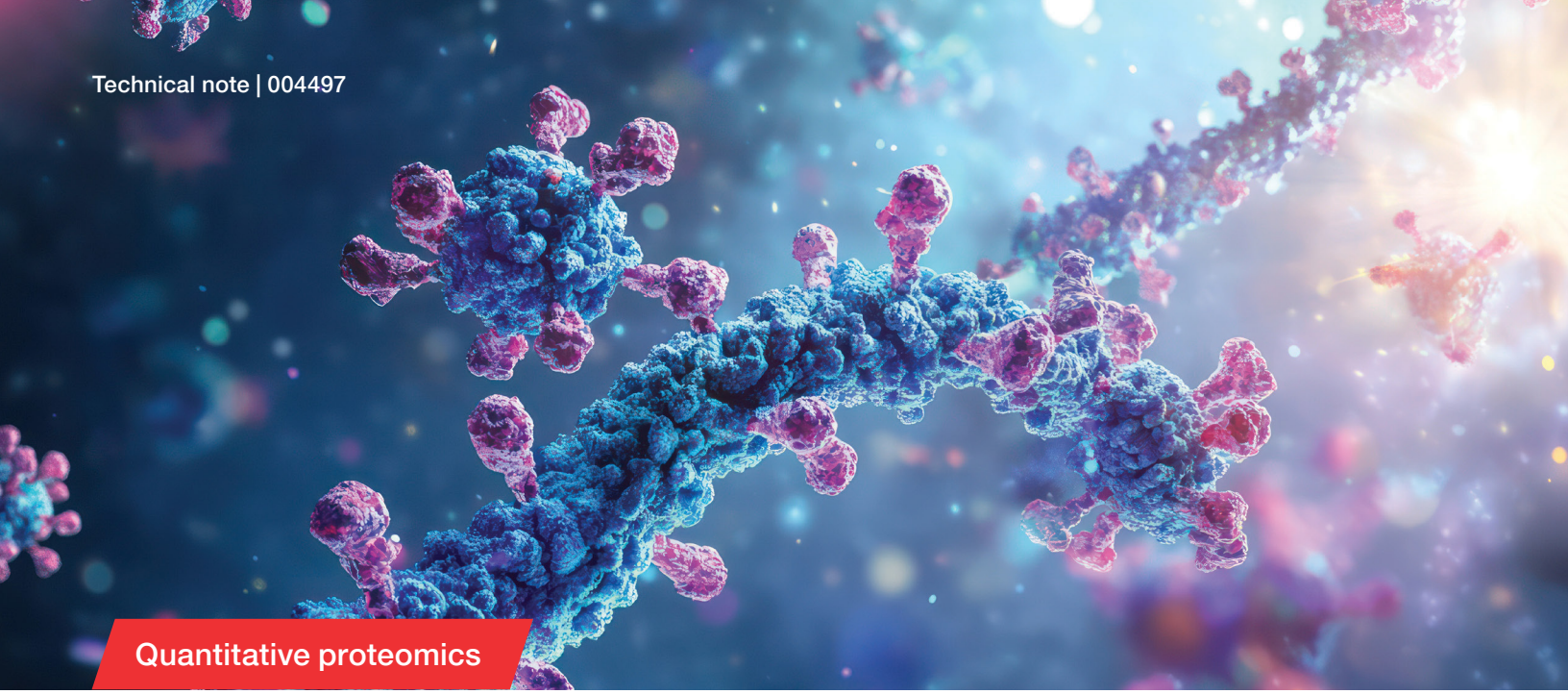

Technical note | 004497

Quantitative proteomics

High coverage and accuracy in quantitative proteomics using the OptiSpray ion source on the Orbitrap Excedion Pro mass spectrometer

Authors

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Keywords

OptiSpray ion source, OptiSpray µPAC Neo cartridges, Orbitrap Excedion Pro, Vanquish Neo UHPLC, FAIMS Pro Duo interface, µPAC Neo Plus HPLC column, data-independent acquisition, DIA, label-free quantitation, LFQ, Proteome Discoverer, CHIMERYS, Spectronaut, DIA-NN

Goal

To showcase the qualitative and quantitative proteome coverage achieved with label-free quantitation (LFQ) using data-independent acquisition (DIA) on the Thermo Scientific™ Orbitrap™ Excedion™ Pro Hybrid Mass Spectrometer with Thermo Scientific™ OptiSpray™ technology.

Introduction

LFQ workflows based on mass spectrometry (MS) are extensively used in proteomics to investigate protein expression and gain crucial insights into biological and disease mechanisms. Recent advances in DIA have significantly improved proteome coverage and quantification accuracy, overcoming the limitations of traditional data-dependent acquisition (DDA). Additionally, advanced software algorithms enable the extraction of LC-MS peaks from highly chimeric raw data supporting identification of peptides across runs through spectral and retention-time alignment. When integrated with nanoflow or microflow chromatography, these workflows maximize identification and quantitation, while minimizing sample consumption.

The Orbitrap Excedion Pro mass spectrometer provides high versatility, accuracy, and robustness. Reduced unintended fragmentation ion source, improved ion processing, and extended fragmentation capabilities, as well as triplicates with intelligent features for discovery and validation in proteomics, position the Orbitrap Excedion Pro mass spectrometer as an excellent choice for quantitative proteomics and biomolecule characterization. The improved design of components results in capabilities for analysis with higher sensitivity and a wider dynamic range, making this instrument a sensitive and versatile tool for the proteomics field.

The capabilities of a Thermo Scientific™ Orbitrap™ mass spectrometer can further be enhanced by using the Thermo Scientific™ FAIMS Pro Duo interface, which removes background ions with ease, and the Thermo Scientific™ OptiSpray™ Ion Source simplifies use of nanoflow and capillary flow cartridge columns for expanded functionality, robustness, and reproducibility.

LFQ-DIA is capable of both deep proteome profiling and quantitative analysis in one workflow, which is well-suited for studies requiring analysis of many hundreds of samples, such as large cohort studies or clinical and biomarker discovery. Compared to DDA, methods based on DIA offer improved reproducibility and lower missing data values. DIA can also offer better performance with low-abundance proteins.

Large-scale studies place significant demands on analysts, requiring high sensitivity, quantitative accuracy, reproducibility, and robustness. Advances in technology that deliver strong performance across these areas can help address these challenges. The Thermo Scientific™ Vanquish™ Neo UHPLC System in combination with the Thermo Scientific™ µPAC™ Neo Plus HPLC Column supports high performance for reproducible and versatile LC-MS experiments. The simultaneous detection of ions with the Thermo Scientific™ Orbitrap™ analyzer offers confident measurements by delivering both highly accurate mass and high resolution, providing analytical sensitivity and specificity.

The Orbitrap Excedion Pro mass spectrometer is an excellent choice for applications based on DIA methods, including hybrid DIA,[1] as the improved next-generation Thermo Scientifiic™ Orbitrap hybrid architecture with enhanced sensitivity, dynamic range, and additional features, such as improved intact protein capability and alternative fragmentation with electron transfer dissociation (EASY-ETD),[2] enables analytical flexibility for biomolecule analysis and interdisciplinary proteomics discovery. The analytical challenges can be further addressed by optimized methods that deliver extended proteome coverage with high accuracy.

Here, we present a DIA workflow for LFQ proteomics that delivers high accuracy and broad proteome coverage, demonstrated using the Thermo Scientific™ Pierce™ HeLa Protein Digest Standard and mixtures with yeast and *E. coli* digests. Samples were analyzed on either a Thermo Scientific™ OptiSpray™ µPAC™ Neo 50 cm cartridge or a Thermo Scientific™ µPAC™ Neo Plus HPLC column with the Vanquish Neo UHPLC system coupled to the Orbitrap Excedion Pro mass spectrometer.

Experiment

Recommended consumables

- Fisher Chemical™ Optima™ LC-MS Grade Water with 0.1% Formic Acid (Part No. LS118-500)
- Fisher Chemical™ LC-MS Grade 80% Acetonitrile (ACN) with 0.1% Formic Acid (Part No. LS122-500)
- Fisher Chemical™ Optima™ LC-MS Grade Formic Acid (Part No. A117-50)
- Fisher Chemical™ Optima™ LC-MS Grade Water (Part No. 10505904)
- Fisher Chemical™ Optima™ LC-MS Grade Acetonitrile (Part No. A955-1)
- Fisher Chemical™ Optima™ LC-MS Grade Isopropanol (Part No. A461-212)
- Thermo Scientific™ SureSTART™ Microvial (Part No. 60180-1655)
- Thermo Scientific™ SureSTART™ Screw Cap (Part No. 6PSC9STB1)

Samples

- HeLa protein digest standard (Part No. 88329)
- ProTec Diagnostics HYE Mix A and B
- OptiSpray cartridge and emitter
- OptiSpray µPAC Neo 50 cm cartridge (Part No. OS-UPAC050NAN)
- Thermo Scientific™ OptiSpray™ Pulled Emitter, 8 µm, 5 pcs/box (Part No. OSE0835P)

LC columns

- µPAC Neo 50 cm HPLC column (Part No. COL-NANO050NEOB)
- µPAC Neo Plus 50 cm HPLC column (Part No. COL-UPAC050NAN)
- Thermo Scientific™ EASY-Spray™ Emitter, 10 µm ID (Part No. ES993)
- Thermo Scientific™ µPAC™ Neo Plus ESI-MS Voltage Spacer (Part No. ACC-VSP1006)

HPLC system

- Vanquish Neo UHPLC system (Part No. VN-S10-A-01)
- Thermo Scientific™ Vanquish™ User Interface (Part No. 6036.1180)

Mass spectrometer

- Orbitrap Excedion Pro mass spectrometer
- FAIMS Pro Duo interface (Part No. FMS03-10001)
- Thermo Scientific™ EASY-Spray™ Source (Part No. ES081)
- Sonation lab solutions column oven for µPAC Neo columns (Sonation lab solutions Part No. PRSO-V2-PF)
- OptiSpray ion source (Part No. B51004132)

Data analysis software

- Thermo Scientific™ Proteome Discoverer™ 3.2 Software with MSAID™ CHIMERYS™ intelligent search algorithm
- Biognosys™ Spectronaut™ 19.9 software
- Aptila Biotech DIA-NN 2.2.0 software

Sample preparation

The HeLa protein digest standard was reconstituted to a concentration of 100 ng/µL in 0.1% formic acid. The three-proteome mixtures containing HeLa, *E. coli*, and yeast digest standards were prepared by reconstituting the HYE Mix A and B in 100 µL of aqueous 0.1% formic acid to a total concentration of 200 ng/µL.

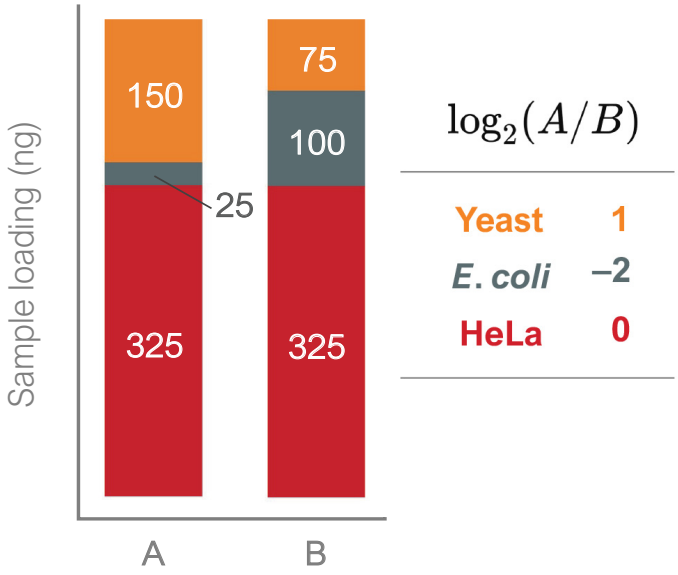

Figure 1. Bar chart of the per-injection amounts of the three proteomes from samples A and B, and the theoretical log2 ratios.

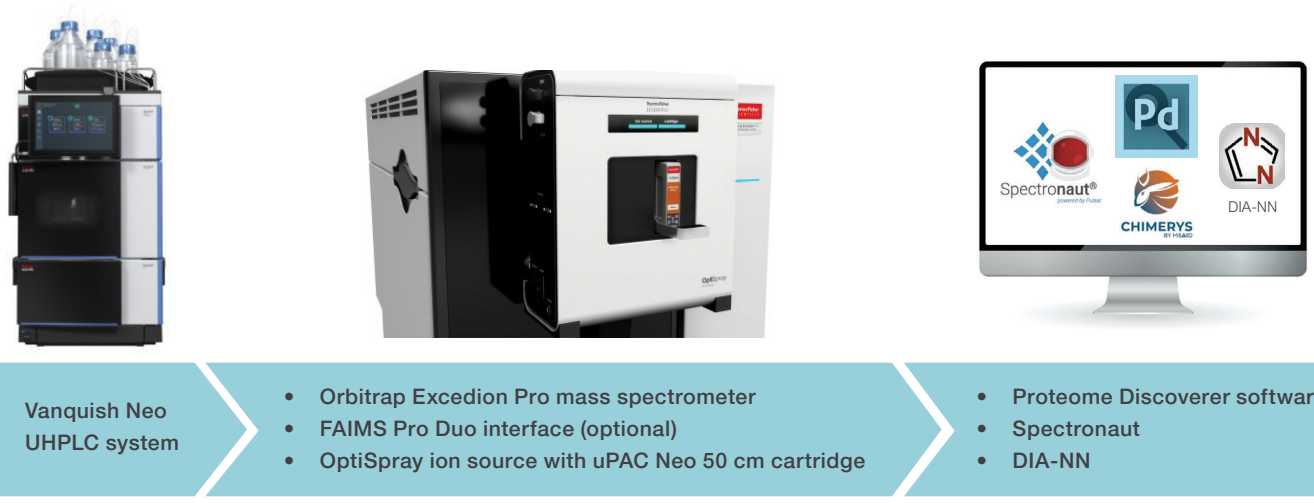

Figure 2. Overview of DIA workflow for LFQ.

LC-MS method

HeLa standard digest was analyzed using 9 min, 30 min, 60 min, 90 min, and 180 min active gradient methods (Table 1) on the Vanquish Neo UHPLC system by injecting 200 ng on-column in triplicate, and carrying out separation on an OptiSpray µPAC Neo 50 cm cartridge featuring a replaceable 8 µm pulled emitter.

To demonstrate protein identification and LFQ with the Orbitrap Excedion Pro mass spectrometer, we evaluated configurations both with and without a FAIMS Pro Duo interface.

Three-proteome mixtures were analyzed in triplicate at 500 ng on-column using all gradient methods with and without FAIMS.

Methods are available for download from the Thermo Scientific™ AppsLab™ Library of Analytical Applications.

For performance comparison with the OptiSpray µPAC Neo 50 cm cartridge, analysis was also performed with a previous-generation µPAC Neo 50 cm HPLC column, and with the latest generation µPAC Neo Plus 50 cm HPLC column, coupled to an EASY-Spray ion source and an EASY-Spray nanoflow emitter (10 µm ID).

The µPAC Neo Plus 50 cm HPLC column was installed on a Sonation lab solutions column oven with the EASY-Spray ion source mounting kit, and a dedicated low-flow voltage spacer with a grounding cable was installed between the column and emitter.

Table 1. LC method parameters.

LC method parameters	
Injection workflow	Direct injection
Sampler temperature	7 °C
Cartridge temperature	50 °C

Separation column specifications	
Inner diameter, length	75 µm, 50 cm
Maximum pressure	450 bar, 1,000 bar/min
Maximum flow	1.0 µL/min
Maximum temperature	60 °C

Separation column specifications	
Mode	Pressure control
Load/equilibrate pressure	400 bar
Loading volume	1 µL
Equilibration factor	2.0

9 min active gradient		
Time, min	Flow, µL/min	%B
0	0.7	3
0.1	0.7	6
9	0.7	22.5
12	0.7	90
12.1	0.75	99
14	0.75	99

30 min active gradient		
Time, min	Flow, µL/min	%B
0	0.5	3
0.5	0.5	6
30	0.5	22.5
36	0.5	90
36.1	0.75	99
38	0.75	99

9 min active gradient		
Time, min	Flow, µL/min	%B
0	0.5	3
1	0.3	6
60	0.3	30
69	0.3	90
69.1	0.75	99
71	0.75	99

9 min active gradient		
Time, min	Flow, µL/min	%B
0	0.5	3
1.5	0.2	6
90	0.2	30
105	0.2	90
105.1	0.75	99
107	0.75	99

9 min active gradient		
Time, min	Flow, µL/min	%B
0	0.5	3
3	0.2	4
150	0.2	28
180	0.2	90
180.1	0.75	99
182	0.75	99

Table 2. MS method parameters.

MS method global parameters	
Application mode	Peptide
Spray voltage	2,200 V
Ion transfer tube temperature	275 °C
FAIMS carrier gas flow	3.5 L/min
FAIMS mode	Standard resolution
EASY-IC	RunStart
Advanced peak determination	On
Default charge state	2

MS and DIA method parameters	
Pre-accumulation for MS^2 scans	Enabled
Scan range (*m/z*) MS^1	400–800
Scan range (*m/z*) MS^2	145–1,450
Normalized AGC target	MS^1: 300%, MS^2: 1,000%
Maximum injection time mode	Auto (MS^1 and MS^2)
Window overlap	1 *m/z*
RF lens	40%
Normalized HCD collision energy	25%

	Without FAIMS	With FAIMS Pro Duo interface
High accuracy and extended coverage		
Gradient: 180 min 7 samples per day (SPD)*	Resolution MS^1/MS^2: 120,000/60,000 Isolation window: 10 *m/z*	Resolution MS^1/MS^2: 60,000/30,000 Isolation window: 13 *m/z* 3 FAIMS voltages: –40, –55, and –70
Gradient: 90 min 13 SPD	Resolution MS^1/MS^2: 120,000/45,000 Isolation window: 10 *m/z*	Resolution MS^1/MS^2: 60,000/15,000 Isolation window: 13 *m/z* 3 FAIMS voltages: –40, –55, and –70
High accuracy		
Gradient: 60 min 18 SPD	Resolution MS^1/MS^2: 60,000/30,000 Isolation window: 8 m/z	Resolution MS^1/MS^2:60,000/15,000 Isolation window: 8 *m/z* 2 FAIMS voltages: –45 and –60
Gradient: 30 min 30 SPD	Resolution MS^1/MS^2: 60,000/15,000 Isolation window: 8 m/z, MS2 AGC 250%	Resolution MS^1/MS^2: 60,000/15,000 Isolation window: 8 m/z, MS2 AGC 500% 1 FAIMS voltage: –50
High throughput		
Gradient: 9 min 70 SPD	Resolution MS^1/MS^2: 45,000/22,500 Isolation window: 18 m/z, MS2 AGC 100%	Resolution MS^1/MS^2: 45,000/22,500 Isolation window: 18 m/z, MS2 AGC 250% 1 FAIMS voltage: –50

*The number of samples per day is determined by the analysis cycle time, including column equilibration and sample loading.

Data processing and interpretation

The data were acquired in triplicate, and for each experiment the three runs were processed together using Proteome Discoverer 3.2 software with CHIMERYS intelligent search algorithm, Spectronaut 19.9 software, and DIA-NN 2.2.0 software. Peptides and protein groups were identified using a 1% false discovery rate (FDR) threshold.

For three proteome mix samples, the FASTA files with canonical sequences were used: *Homo sapiens* (20,428 entries), *Escherichia coli* K-1 (4,530 entries), *Saccharomyces cerevisiae* (6,727 entries), and a common contaminants FASTA file.[1] For HeLa sample only, *Homo sapiens* and the contaminant FASTA were used.

Relative protein abundances were evaluated as ratios of sample A to B using Spectronaut software with cross-run normalization enabled, but without normalization to human proteome. The amounts of the three proteomes in samples A and B, and the theoretical $\log_2$ ratios are shown in Figure 1.

Results and discussion

Improved identifications with µPAC Neo Plus HPLC column or OptiSpray ion source

We compared the OptiSpray µPAC Neo 50 cm cartridge with the latest generation µPAC Neo Plus 50 cm HPLC column and a previous-generation µPAC Neo 50 cm HPLC column.

Across the tested methods (Tables 1 and 2), the OptiSpray µPAC Neo 50 cm cartridge and µPAC Neo Plus 50 cm HPLC column showed similar performance as they contain identical separation columns, with the number of protein identifications from 200 ng of HeLa digest 10% higher without FAIMS and 7% higher with FAIMS compared with the previous-generation µPAC HPLC column. Identification of >100,000 peptides and >8,000 protein groups was achieved from 200 ng of HeLa digest with an OptiSpray µPAC Neo 50 cm cartridge using at least a 60 min active gradient method (Figures 3 and 4).

Compared with a previous-generation µPAC 50 cm HPLC column, the OptiSpray µPAC Neo 50 cm cartridge without FAIMS showed the largest average increase of 10.4% in identified proteins (Figure 4), thanks to improvements in microfluidics technology and the automated emitter position optimization routine, which positions the emitter in the optimal position in front of the ion transfer tube or the FAIMS orifice to maximize ion signal and reproducibility.

Without FAIMS, the OptiSpray µPAC Neo 50 cm cartridge format significantly reduced median protein CVs (%) compared with µPAC Plus Neo 50 cm HPLC column, indicating improved quantitative precision, whereas with FAIMS, consistency was improved by reduced variability from experiment to experiment (Figure 5). While CVs (%) were not found to reduce with FAIMS, typically FAIMS reduces chemical noise, resulting in cleaner spectra, more reliable identifications, and more accurate protein ratios.

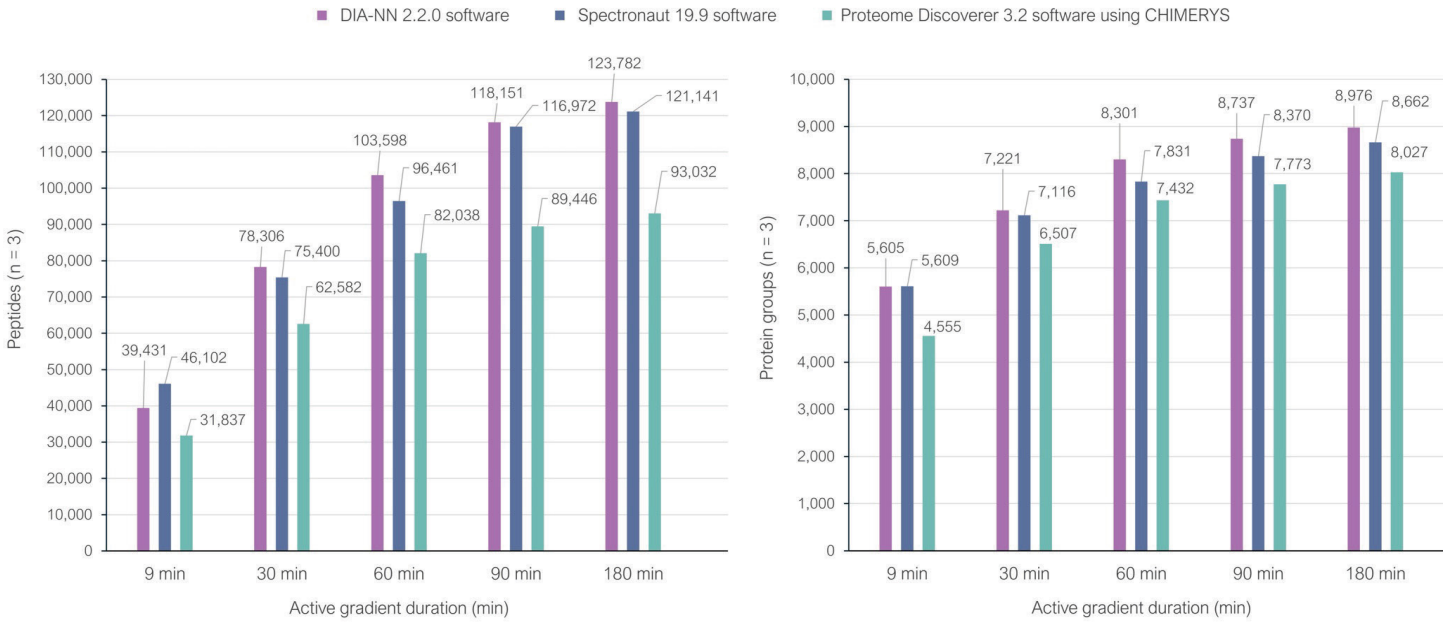

Figure 3. Peptides and protein groups identified in triplicates of 200 ng HeLa protein digest standard using Orbitrap Excedion Pro mass spectrometer without FAIMS equipped with OptiSpray ion source and an OptiSpray µPAC 50 cm cartridge. All data acquired in triplicate, and for each experiment the three runs were processed together using Proteome Discoverer 3.2 software, Spectronaut 19.9 software, and DIA-NN 2.2.0 software. Peptides and protein groups were identified using a 1% FDR threshold.

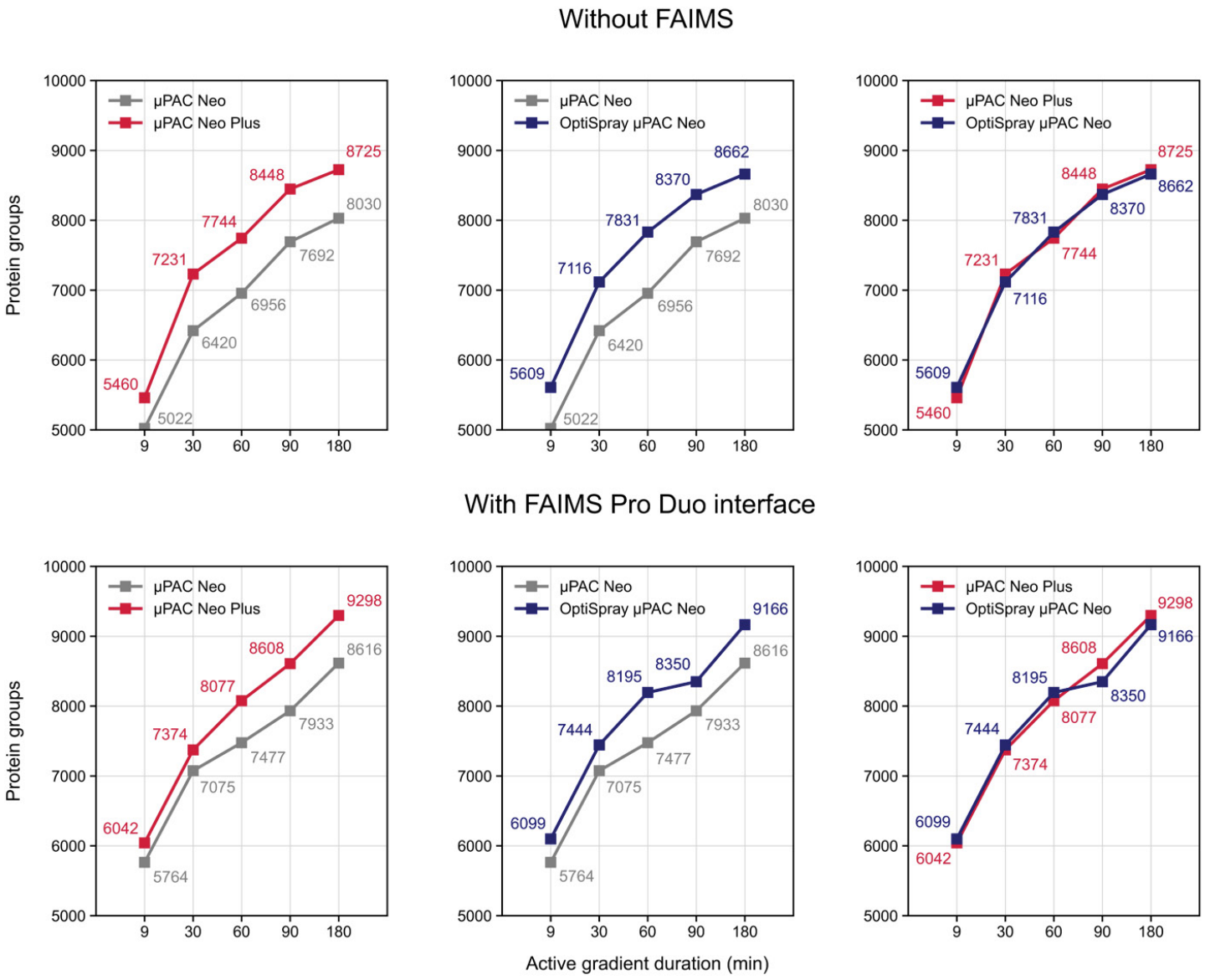

Figure 4. Protein groups and peptides identified in triplicates of 200 ng HeLa digest using the three different column configurations using Orbitrap Excedion Pro mass spectrometer with µPAC Neo 50 cm HPLC column, µPAC Neo Plus 50 cm HPLC column, or OptiSpray µPAC Neo 50 cm cartridge, without and with FAIMS. Use of FAIMS increased protein identifications by 5% on average, while compared with a previous-generation µPAC Neo configuration, protein identifications increased by 7% with FAIMS and by 10% without FAIMS. No significant differences were observed between performance of µPAC Neo Plus 50 cm HPLC column and OptiSpray µPAC Neo 50 cm cartridge in terms of identification counts.

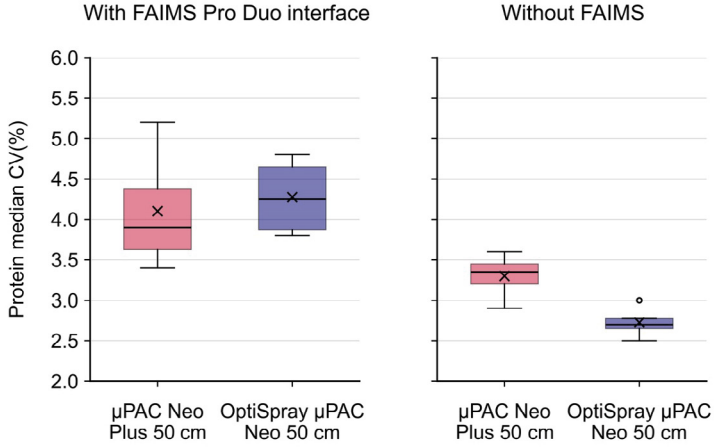

Figure 5. Boxplots of the experiment-level median protein quantity CVs (%) using µPAC Neo Plus 50 cm HPLC column or OptiSpray µPAC Neo 50 cm cartridge, with or without FAIMS across methods with active gradient duration from 30 to 180 minutes. OptiSpray significantly improved protein abundance CVs (%) without FAIMS.

Deeper coverage with FAIMS Pro Duo interface

By using FAIMS, protein coverage can be increased substantially, and additional proteins can be identified, thanks to efficient removal of singly charged background ions and gas-phase fractionation of the sample, which together enhance signal-to-noise ratios and reduce spectral complexity.

Each individual FAIMS compensation voltage selectively transmits a population of ions to enhance dynamic range. For a HeLa protein digest standard, most peptide ions are observed within a compensation voltage range of approximately –80 to –30 V. Employing multiple FAIMS fractions across this range therefore improves overall ion transmission and proteome coverage.

For example, three compensation voltages at –40, –55, and –70 span this region efficiently and sample the ion population comprehensively. This maximizes detection of peptides with the strongest signals and increases the number of high-quality spectra containing meaningful information, ultimately improving protein identification and quantitative performance (Figure 6).

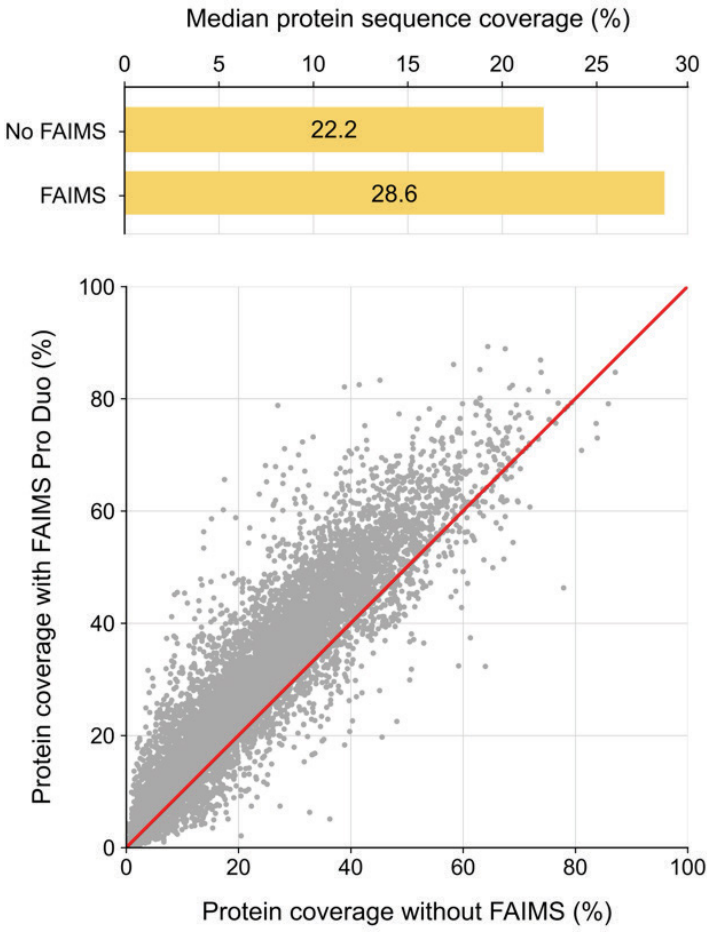

Figure 6. Coverage comparison of shared protein groups identified with and without FAIMS using the µPAC Neo Plus 50 cm HPLC column and a 180 min active gradient. Three FAIMS voltages (–40, –55, and –70) were used to sample the gas-phase fractions comprehensively. Protein coverage can be increased with FAIMS when gas-phase fractions are sampled comprehensively.

High accuracy in quantitative proteomics

Mass accuracy, and especially accuracy of peptide abundance in protein ratio measurements, is greatly affected by the number of ions injected into the ion trap. Injecting too few ions will result in underutilized capacity and decreased number of identified and quantified proteins, while injecting too many ions will result in undesirable ion-to-ion interactions in the ion trap that will negatively affect mass and abundance accuracy. While even with slight trap overloading, mass accuracy is typically very good with Orbitrap analyzer detection, the sensitive and accurate measurement of peptide ratios between samples requires the ion trap to be filled with an appropriate number of ions for every scan. To achieve this, the Automatic Gain Control (AGC) technology monitors the ion input and controls ion injection into the Orbitrap analyzer to support acquisition of high-quality spectra that provide meaningful data.

The optimal AGC target may depend on sample load and electrospray conditions; therefore, it is recommended to optimize it using a three-proteome mixture analysis or a similar experiment at the relevant protein load and ion source parameters. Because FAIMS compensation voltage(s) and HCD collision energy affect ion flux throughout the run—and may themselves require optimization depending on the average peptide length and presence of post-translational modifications (PTMs)—it is recommended to perform survey runs tailored to optimize for the specific samples.

An AGC target of 100% should support acceptable accuracy for many applications; however, in practice, when the Orbitrap analyzer resolution is increased together with increasing gradient duration (Table 2), as the ion flux changes less rapidly from scan to scan, the increased resolution offers improved detection statistics. As a result, thanks to the more uniform ion flux, reduced spectral interference, and improved signal-to-noise, with longer gradients and higher resolution, the AGC target can be relaxed increasingly (Table 2 and Figure 8), especially if using normalization to a reference proteome, which to a certain degree helps to correct for sample loading, instrument response drift, and ratio compression.

The optimized methods in Tables 1 and 2 aim to support a median $\log_2$ ratio error ≤0.1 in a three-proteome mixture of yeast, HeLa, and *E. coli* digests (Figure 7A), with theoretical protein log ratios spanning up to fourfold change. When a higher degree of accuracy is desired, the AGC target can be reduced further, the duration of the active gradient can be increased (Figures 7B, 8, and 9), or both. The number of peptide and protein identifications is maximized when the number of data points per peak is 3.0 (median). A lower number of data points will rapidly decrease the number of peptides and proteins with acceptable CV (%). A higher number of data points may further increase quantitation accuracy; however, this comes at the expense of identifications. With Orbitrap analyzer detection, an increase in resolution may offer a greater increase in quantitation accuracy than an increase in the number of data points per peak. Furthermore, if the complexity of the sample, in combination with the selected parameters, does not afford acceptable quantitation accuracy, it is worth investigating whether the samples have been prepared appropriately, whether the spray is stable, or whether a lower sample load or a narrower mass-to-charge ratio range improves accuracy.

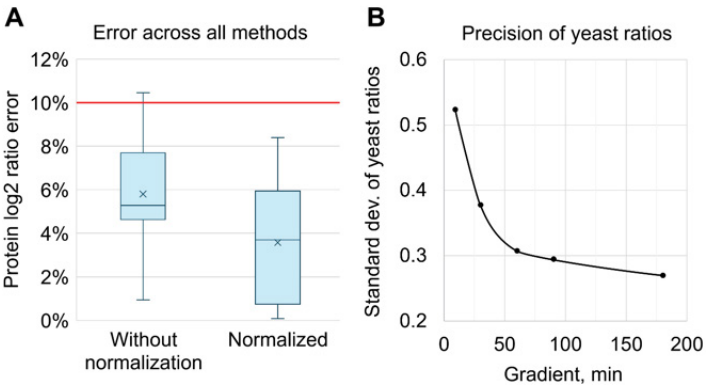

Figure 7. Measurement bias and precision of the LFQ workflow. (A) Boxplots of protein ratio errors of the tested proteomes across the tested methods with gradient duration from 9 to 180 min and the respective gradient-specific MS parameters. (B) Precision of yeast ratios expressed as the standard deviation of measured yeast protein ratios across the methods with different gradient duration.

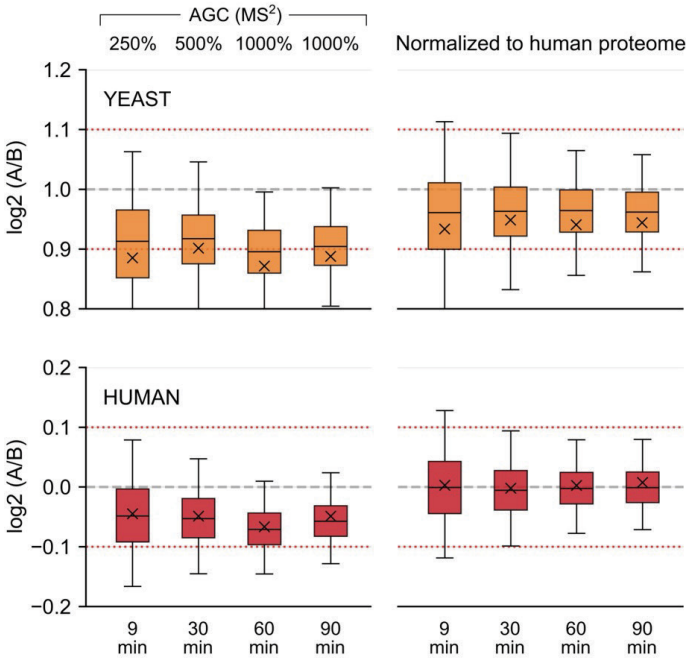

Figure 8. Protein ratios in yeast and human proteomes across active gradient durations and MS² AGC settings measured from three-proteome mixture using an Orbitrap Excedion Pro mass spectrometer equipped with FAIMS Pro Duo interface and an OptiSpray µPAC Neo 50 cm cartridge. Dotted red lines indicate the ±10% error threshold. Boxes represent the interquartile range (IQR; 40th–60th percentiles), with whiskers covering the central 50% of the distribution.

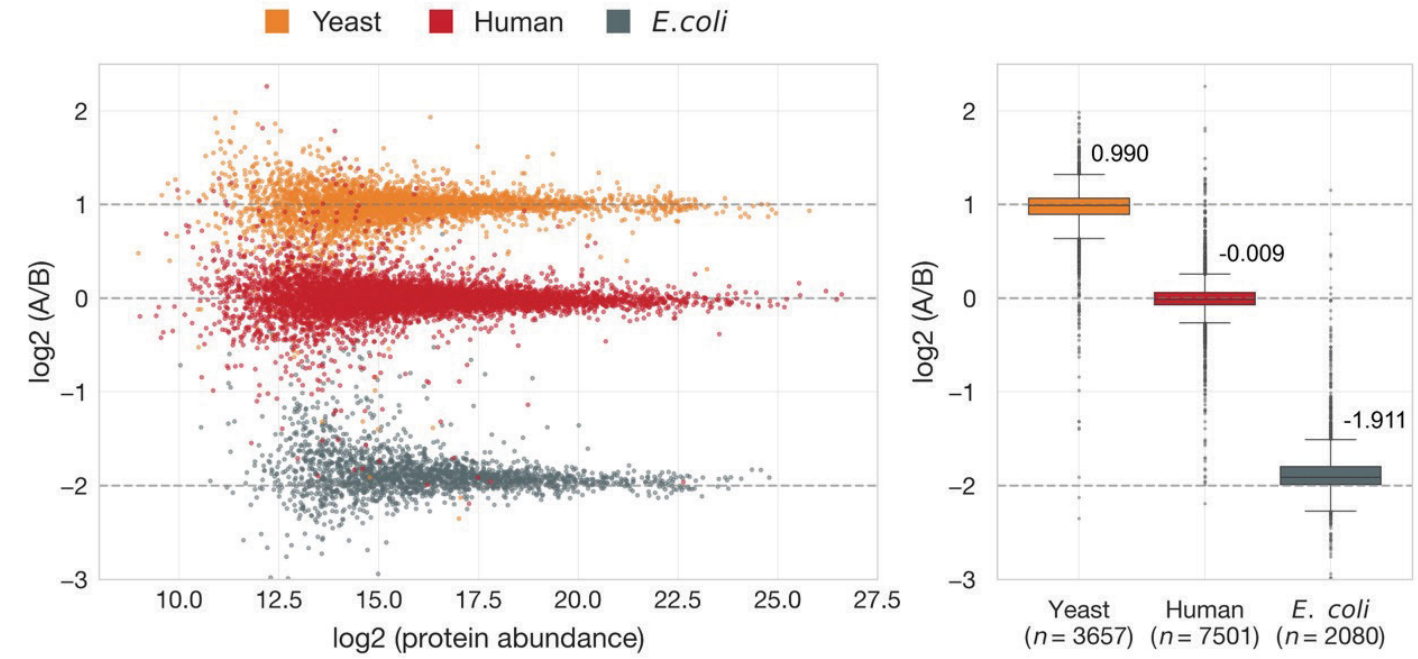

Figure 9. Scatterplot and boxplots of protein ratios from analysis of three-proteome mixture (n = 3) with the Orbitrap Excedion Pro mass spectrometer equipped with an OptiSpray µPAC Neo 50 cm cartridge (without FAIMS) using 180 min active gradient, processed with Spectronaut 19.9 software with normalization to human proteome. A total of 13,591 protein groups were identified using a 1% FDR threshold, of which 13,238 proteins were quantified. The error of median protein $\log_2$ ratio was –0.99% for yeast, 0.94% for human, and 4.47% for *E. coli* proteome.

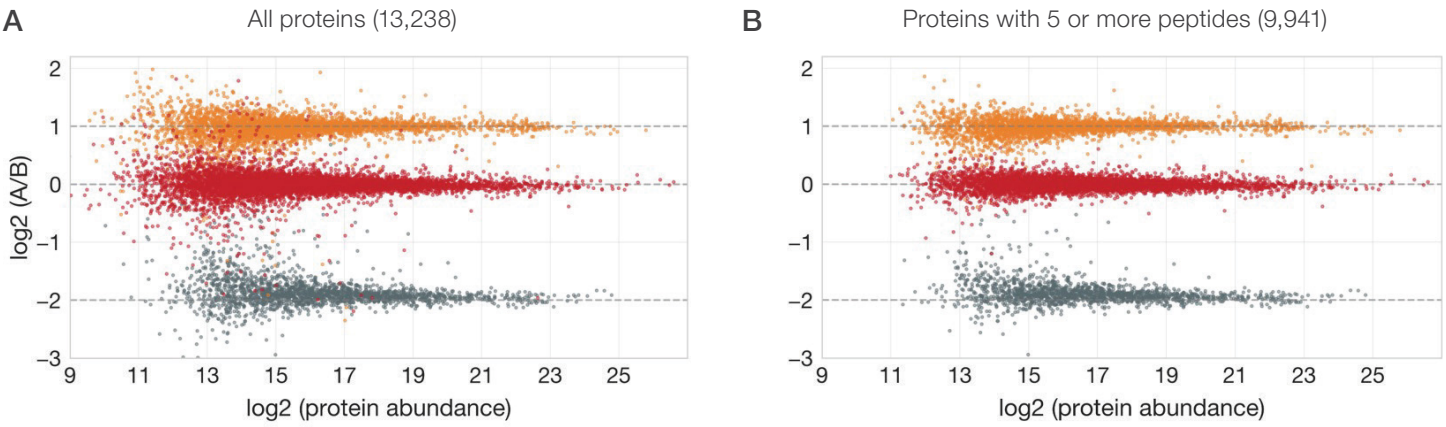

Figure 10. Scatterplots of protein ratios from analysis of three-proteome mixture (n = 3) with the Orbitrap Excedion Pro mass spectrometer equipped with an OptiSpray µPAC Neo 50 cm cartridge (without FAIMS) using 180 min active gradient, processed with Spectronaut 19.9 software with normalization to human proteome. (A) All proteins with at least one peptide identified and quantified. (B) Proteins with at least five or more peptides identified and quantified. Filtering out proteins with fewer than 5 peptides resulted in narrower ratio distributions and lower standard deviations across all three proteomes. The improved precision with increased peptide evidence highlights the value of high proteome coverage for accurate quantitative measurements.

Ease of use with the OptiSpray ion source

To evaluate ease of use and reproducibility under low-input conditions (1 ng HeLa digest analyzed with FAIMS), a comparative experiment was performed, switching between EASY-Spray source configuration using a µPAC Neo Plus 50 cm column and an OptiSpray configuration using a µPAC Neo 50 cm cartridge. The evaluation was performed over three installation cycles to introduce operator-dependent variability.

Sixteen peptides commonly detectable in HeLa digests were checked, and to exclude chemical instability, only six peptides showing stable behavior (≤10% loss) were included in the analysis. Performance was evaluated using two metrics: relative standard deviation (RSD) of peak area (Figure 11A) and relative area deviation from the fitted peak profile (Figure 11B).

Across the three installation cycles, the OptiSpray configuration demonstrated approximately twofold lower RSD of peak area compared to the µPAC Neo Plus setup, indicating improved quantitative reproducibility following source removal and reinstallation. In addition, the OptiSpray configuration showed closer agreement between the observed and idealized peak areas, suggesting more stable spray conditions.

The reduced variability observed with the OptiSpray cartridge indicates that automated spray positioning and cartridge-based integration effectively reduce operator-dependent variability, improving reproducibility under demanding low-load conditions where spray stability is critical.

Importantly, unlike the EASY-Spray source configuration, which required prior operator experience and special care during handling and adjustment, the OptiSpray configuration achieved better reproducibility with a simpler, easier-to-use setup.

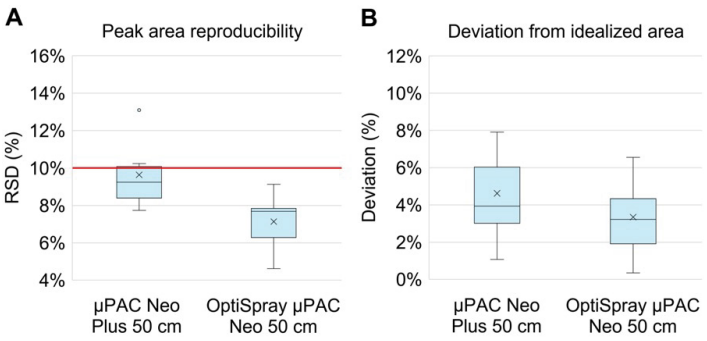

Figure 11. Reproducibility with µPAC Neo Plus 50 cm column and OptiSpray µPAC Neo 50 cm cartridge over three independent installation cycles. 1 ng of HeLa digest was analyzed with FAIMS. The peptides included in the analysis were identified as –MC(Carbamidomethyl) HPSIEGFTPR (3+), GGIVDEGALLR (2+), GIAYIEFK (2+), AITGASLADIMAK (2+), AQFEGIVTDLIR (2+), and ILEFFGLK (2+). (A) RSD of peak area. (B) Relative area deviation from fitted peak profile. This metric expresses a fractional deviation relative to the area under an idealized peak profile and serves as an indicator of spray instability or signal distortion.

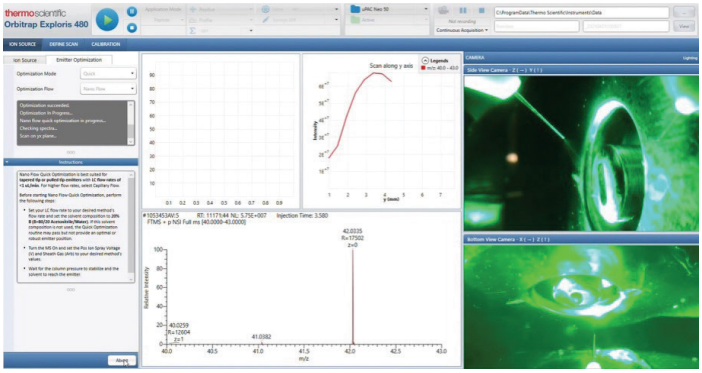

Figure 12. Instrument control software during automated emitter position optimization. The last optimized position is recorded into the cartridge memory, and the source motors returns the emitter to the exact same position when the cartridge is inserted again.

Conclusion

- The Orbitrap Excedion Pro mass spectrometer enables robust DIA-LFQ performance across a wide throughput range, achieving >100,000 peptides and nearly 9,000 protein groups from 200 ng HeLa digest with ≥60-min gradients. In addition, when methods are appropriately optimized, it maintains high quantitative accuracy (log_2 protein ratio error ≤10%) even with short (9–30 min) gradients.
- OptiSpray µPAC Neo 50 cm cartridge DIA-LFQ performance for protein identifications was found equivalent to µPAC Neo Plus 50 cm HPLC columns, offering a 10% increase in proteins identified when compared with a previous-generation µPAC Neo configuration, while providing improved quantitative precision attributable to improved microfluidics and automated emitter positioning.
- Across three independent reinstallation cycles selected peptides from 1 ng HeLa digest analyzed with the OptiSpray µPAC Neo 50 cm cartridge showed approximately twofold lower peak area relative standard deviation compared to the µPAC Neo Plus 50 cm HPLC column, indicating improved installation-to-installation reproducibility under low-input conditions with a simpler, easier-to-use setup.
- FAIMS Pro Duo interface increases proteome coverage and identification robustness when multiple compensation voltages are applied. On average, a 5% increase in protein identifications can be expected with FAIMS when methods are appropriately optimized.
- Quantitative accuracy is controlled by AGC target, gradient length, and Orbitrap resolution, with optimized combinations achieving median protein log_2 ratio errors ≤5% across yeast, human, and *E. coli* proteomes spanning up to fourfold abundance differences.
- Longer gradients and higher Orbitrap analyzer resolution allow relaxation of MS^2 AGC targets without compromising quantitative accuracy, resulting in ultimate identification depth and quantitation precision.

Learn more at thermofisher.com/proteomics

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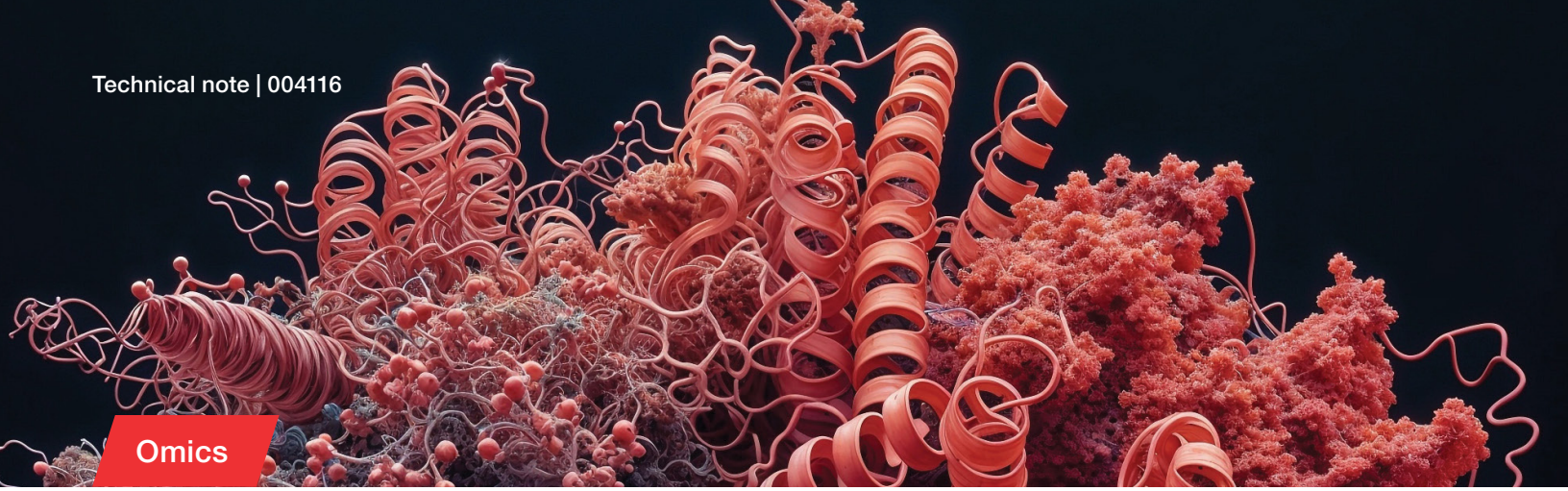

Technical note | 004116

Omics

Hybrid DIA on the Orbitrap Excedion Pro mass spectrometer: Bridging global depth and targeted sensitivity for proteomic analysis

Authors

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Keywords

Hybrid DIA, Orbitrap Excedion Pro, OptiSpray ion source, data-independent acquisition, PRM

Goal

To develop and evaluate the performance of hybrid data-independent acquisition (hybrid DIA) on the Thermo Scientific™ Orbitrap™ Excedion™ Pro Hybrid Mass Spectrometer for proteomic analysis.

Introduction

In discovery proteomics research, scientists face two critical challenges: achieving comprehensive protein identification across a wide dynamic range while simultaneously obtaining precise and accurate quantitation of low-abundance targets. Traditional data-independent acquisition (DIA) provides broad proteome coverage but may lack the sensitivity needed for precise quantitation of low-abundance proteins. Conversely, parallel reaction monitoring (PRM) offers exceptional sensitivity and accurate quantitation but sacrifices the comprehensive view important for discovery proteomics.

This analytical compromise typically forces researchers to run separate experiments for discovery and targeted quantitation, doubling sample requirements and instrument time while potentially introducing variability between runs. This traditional workflow is especially problematic for precious samples or time-sensitive studies, where sample quantity is limited and experimental reproducibility is crucial.

Reliable electrospray ionization performance and consistent chromatography are fundamental requirements for achieving reproducible results in such analyses. Traditional low-flow LC-MS systems often present operational challenges with spray stability and complex column installation procedures that can impact data quality. Thermo Scientific™ OptiSpray™ technology addresses these challenges through its innovative ion source and cartridge design, featuring automated emitter positioning, exchangeable emitters, and plug-and-play cartridge installation. This intelligent electrospray ionization interface simplifies the acquisition of reproductible, high-quality data, enabling researchers to

focus on their analyses rather than system optimization. The Thermo Scientific™ OptiSpray™ uPAC™ Neo Cartridge Column utilizes micro pillar array technology, yielding superior robustness, reproducibility, and chromatographic performance, offering deeper and more reliable coverage of complex biological samples.

Building on this robust analytical foundation, the introduction of hybrid DIA powered by an adaptive retention time (adaptive RT) routine on the Orbitrap Excedion Pro MS minimizes the fundamental compromises between comprehensive and targeted analysis. By efficiently integrating DIA and PRM capabilities within a single experiment, hybrid DIA enables both comprehensive proteome profiling and high-sensitivity quantitation of targeted proteins. While earlier studies with hybrid DIA used different instruments and required stable isotope-labeled (SIL) peptides and an application programming interface (API), the Orbitrap Excedion Pro MS supports both labeled and non-labeled peptides through its standard instrument control software.[1,2] Enhanced by adaptive RT technology, this method ensures reliable quantification with narrower retention time windows even in complex samples, while maintaining the simplicity of traditional DIA workflows.[3]

This technical note demonstrates how the combination of hybrid DIA and the OptiSpray ion source enables both comprehensive and targeted protein analysis with exceptional precision and sensitivity. Using a HeLa digest dilution series (0.5 ng – 500 ng) with a spike-in standard (0.05 fmol – 500 fmol), we showcase deep proteome coverage (>6,000 proteins) with excellent reproducibility (median CVs <15%), while simultaneously achieving robust quantitation of targeted peptides (CV <10%, R^2 >0.9) across varying sample loads in a single 35-minute analytical run. The spike-in demonstrates precise quantitation across four orders of magnitude with LOQ and LOD values down to 50 and 5 amol, respectively.

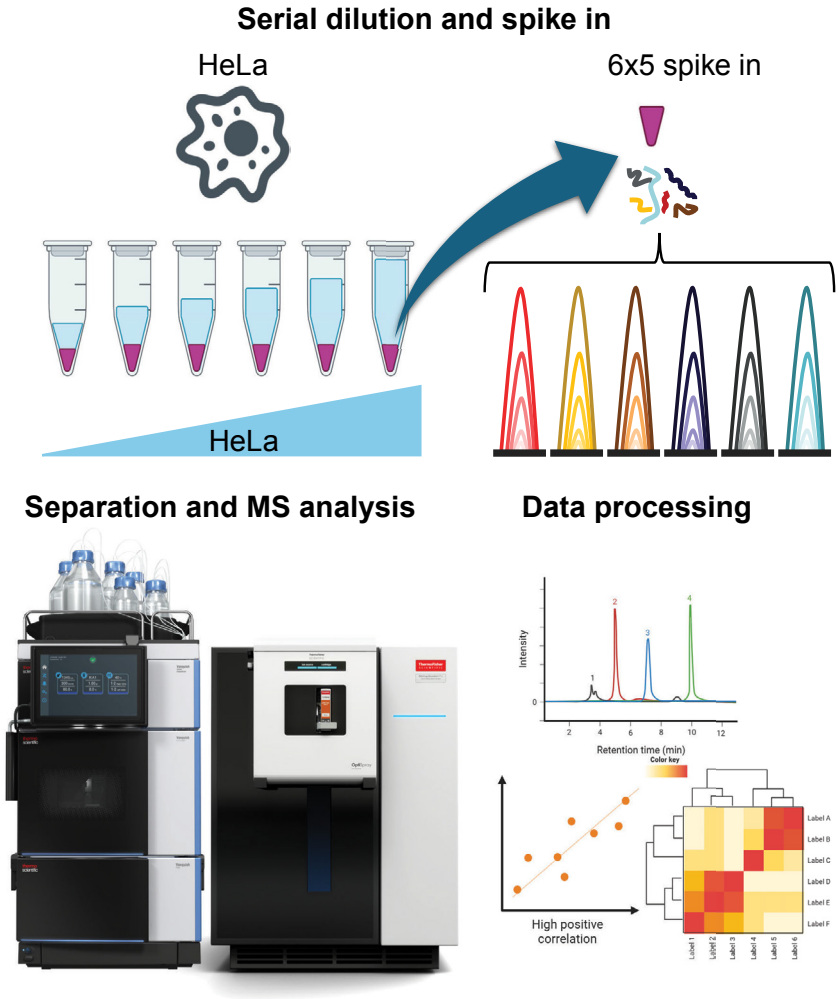


Figure 1. Experimental outline for hybrid DIA on the Orbitrap Excedion Pro MS.

Experimental

Recommended consumables

- Fisher Scientific™ Optima™ LC-MS grade water with 0.1% formic acid (Cat. No. LS118-500)
- Fisher Scientific™ Optima™ LC-MS grade 80% acetonitrile with 0.1% formic acid (Cat. No. LS122-500)
- Fisher Scientific™ Optima™ LC-MS grade 100% acetonitrile with 0.1% formic acid (Cat. No. LS120-212)
- Thermo Scientific™ n-Dodecyl-β-D-maltoside DDM (Cat. No. 89902)

Samples

- Thermo Scientific™ Pierce™ HeLa Protein Digest Standard (Cat. No. 88328)
- Promega™ 6×5 LC-MS/MS Peptide Reference Mix

LC columns

- Thermo Scientific™ OptiSpray™ µPAC™ Neo 50 cm Cartridge (Cat. No. OS-UPAC050NAN)

UHPLC system

- Thermo Scientific™ Vanquish™ Neo UHPLC System (Cat. No. VN-S10-A-01)

Mass spectrometer

- Thermo Scientific™ OptiSpray™ Ion Source (Cat. No. B51004132)
- Orbitrap Excedion Pro MS

Data analysis software

- Biognosys Spectronaut® 20.1 software
- MacCoss Lab Software Skyline-Daily V25.1

Sample preparation

HeLa Protein Digest Standard was reconstituted in 0.02% dodecyl-β-D-maltoside and 0.1% formic acid (FA) with 30 seconds of vortexing. Five concentrations of HeLa were prepared: 0.5, 5, 50, 100, and 500 ng. The same amount of 6x5 LC-MS/MS Peptide Reference mix was added to each HeLa concentration. Peptide concentrations of the 6x5 mixture ranged from 0.05 to 500 fmol.

LC and MS conditions

Samples were injected onto an OptiSpray µPAC Neo 50 cm cartridge and separated using a 35 min gradient in direct injection mode using a Vanquish Neo UHPLC system and the Orbitrap Excedion Pro MS (Figure 1). The details for LC and MS parameters are reported in Tables 1-5.

Table 1. HPLC conditions.

Global LC parameters	
Mobile phase A	0.1 % FA in water
Mobile phase B	0.1% FA in 80% acetonitrile
Separation column specifications	
Inner diameter	75 µm
Length	50 cm
Maximum pressure	450 bar
Maximum flow	100 uL/min
Maximum temperature	60°C
Experimental conditions	
Column temperature	50°C
Fast loading/equilibration	PressureControl
Pressure loading/equilibration	400 bar
Equilibration factor	0.2
Sampler temperature	7°C

Table 2. HPLC gradient.

Time (min)	%B	Flow rate (nL/min)
0	1	750
0.05	4	750
2.75	12	750
2.85	12.1	200
16.35	22.5	200
26.35	45	200
35	99	200

Table 3. Ion source and global MS method parameters.

Parameter	Setting
Spray voltage	1,900 V
Sheath gas	0 arb
Cartridge temperature	50°C
ITT temperature	305°C
Expected peak width	10 s
Advanced peak determination	TRUE
Default charge state	2

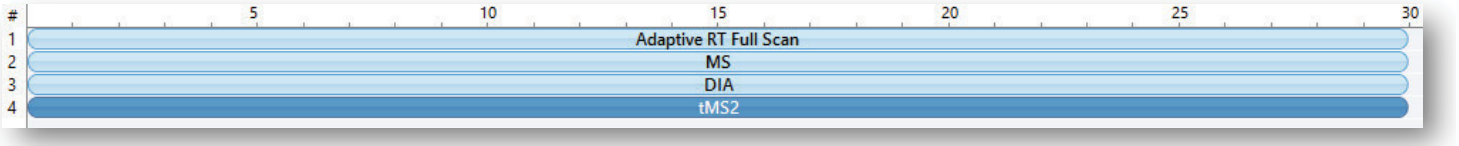


Figure 2. Hybrid DIA experiment setup.

Table 4. DIA MS method parameters.

Full Scan		
Orbitrap resolution	120,000	45,000
Scan range (*m/z*)	400–900	400–900
RF lens	50%	50%
AGC target	300%	300%
Maximum IT	Auto	Auto
DIA		
	0.5-5 ng HeLa	50-500 ng HeLa
Orbitrap resolution	60,000	15,000
Precursor scan range (*m/z*)	400–900	400–900
Isolation width (*m/z*)	50	12
Window placement optimization	On	On
Normalized HCD energy	30%	30%
AGC target	1,000%	1,000%
Maximum IT	Auto	Auto
Loop	2 s	2 s

Table 5. PRM MS method parameters.

Adaptive RT Full Scan	
Orbitrap resolution	7,500
Scan range (*m/z*)	400–1,200
RF lens	50%
AGC target	Standard
Maximum IT	Auto
PRM	
Orbitrap resolution	15,000
Isolation window (*m/z*)	0.7
Normalized HCD energy	30%
AGC target	500%
Maximum IT	200 ms
Time mode	Retention Time Window
Dynamic retention time	Adaptive RT
Reference file	Load appropriate .Rtbin file

The adaptive RT full scan (RT Full Scan) experiment is optional but strongly encouraged with large target of interest lists or narrow retention windows. This creates an .RTbin file that is used to shift retention time windows based on the matrix background.[3] For this experiment, the targeted peptide list consisted of 30 peptides from the 6x5 mixture with their scheduled elution time.

Data processing parameters

Acquired DIA data was searched and processed by Spectronaut 20.1 software using a directDIA™ approach. Default settings and the human UniProt database were used. Peptide and protein identifications were filtered for 1% FDR, and a q-value cutoff of 1% was used for the DIA analysis.

PRM data was analyzed in Skyline-daily V25.1 software. Average fragment areas were calculated from y-ion transitions. For figures of merit calculation, the regression was fit to bilinear turning point for limit of detection (LOD) and max CV <20% for limit of quantitation (LOQ).

Results

Deep proteome profiling with hybrid DIA analysis

To evaluate proteome profiling of hybrid DIA on the Orbitrap Excedion Pro MS, a dilution series using HeLa was performed ranging from 0.5 ng to 500 ng. The 6x5 LC-MS/MS Peptide Reference mix was spiked into each HeLa sample, so that target peptide concentrations from 0.05 to 500 fmol were represented in the HeLa samples. Each concentration was evaluated using three technical replicates.

Table 6. 6x5 standard.

Each peptide group	Amount on column (fmol)
5 heavy	500
4 heavy	50
3 heavy	5
2 heavy	0.5
1 heavy	0.05

Dilution series experiments were performed to represent various concentrations that might be used in scientific research to demonstrate that the hybrid DIA method is appropriate for various experimental concentrations and can handle potential unknown sample loads. Using the hybrid DIA method, average protein and peptide identifications at 0.5 ng to 500 ng HeLa ranged from 2,694 to 6,405 and 15,000+ to 68,000+, respectively (Figure 3).

Although five concentrations were tested, 50 ng HeLa is used as the standard in the following figures and discussion for simplicity. Comparing identified proteins and peptides within replicates for 50 ng HeLa reveals high overlap, with 99% for proteins and 96% for peptides, demonstrating high reproducibility of the method (Figure 4). The three replicates differed by an average of 15 proteins, while over 6,000 proteins were consistently identified in each replicate (Figure 4A). More than 62,000 peptides were identified with 96% overlap and a mean of 1,282 peptides between replicates (Figure 4B). When comparing overlap between concentrations of 0.5, 5, and 50 ng HeLa, we observed an overlap of 2,683 and 16,744 in proteins and peptides, respectively (Figure 5A,B). Higher concentrations do not result in vastly different proteins or sequences but rather expand the number of identifications.

Extensive proteome coverage with precise quantitation

To investigate the range and quantitation from a global proteome perspective using hybrid DIA, protein abundances from DIA data were analyzed. With 50 ng HeLa, protein abundances spanned over five orders of magnitude (Figure 6). The median coefficient of variation (CV) for peptide abundances was <15% for each concentration of HeLa and three technical replicates measured, respectively (Figure 7). For three replicates of 50 ng HeLa, the median CV was 9.9%.

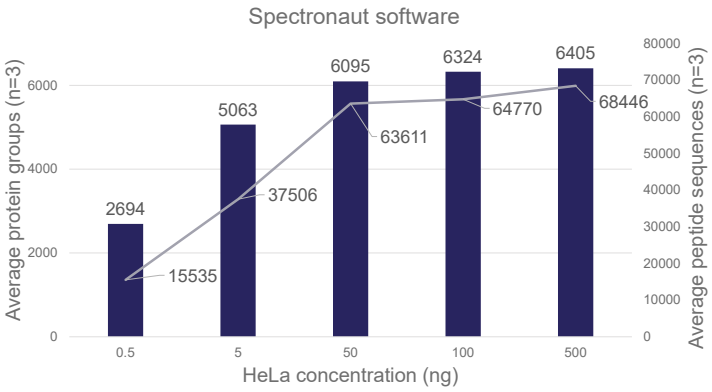


Figure 3. Comparison of protein and peptide identifications at various HeLa concentrations (0.5–500 ng) for hybrid DIA.

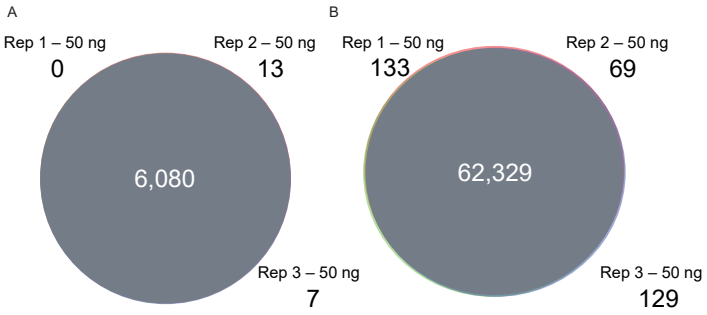


Figure 4. Reproducibility of identified proteins (A) and peptides (B) from three replicates of 50 ng HeLa. Venn diagram demonstrates overlap (middle of circle) and proteins unique to each sample (found under the Replicate label).

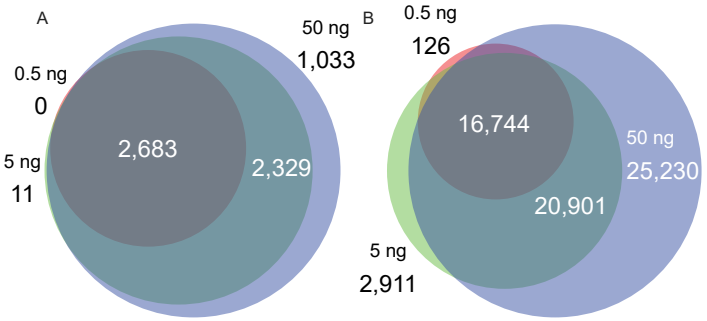


Figure 5. Comparison of identified proteins (A) and peptides (B) of 0.5, 5, and 50 ng HeLa.

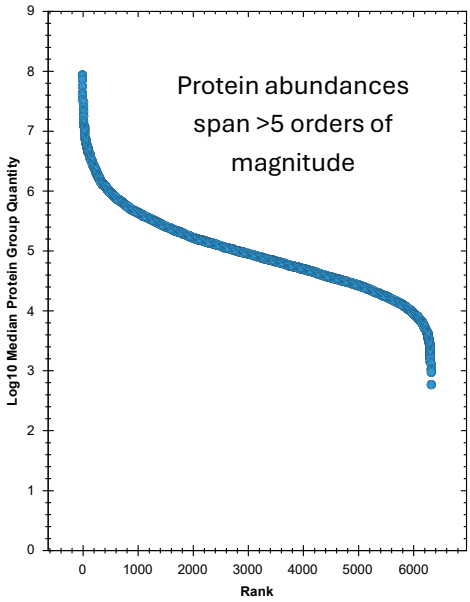


Figure 6. Dynamic range of global proteome coverage using hybrid DIA, represented by 50 ng HeLa. Protein abundances span over five orders of magnitude.

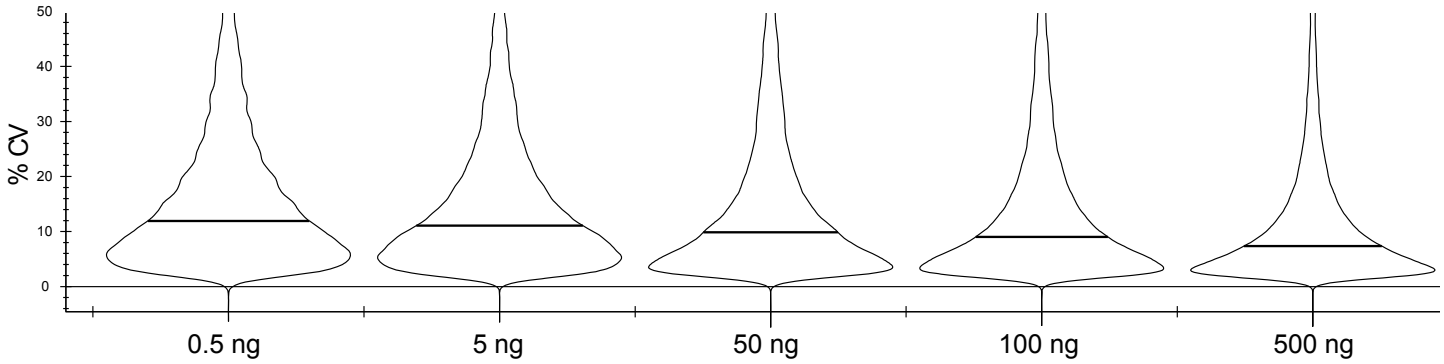


Figure 7. Coefficients of variation of peptide abundances across all HeLa concentrations. Median CV marked with black line.

Further quantitative performance of DIA is exemplified by the linearity of proteins across varying sample load concentrations. For this analysis, mean abundances were taken for each protein observed in a concentration, then a linear model was fit to each protein across the varying concentrations. Proteins were deemed as behaving linearly, and thus having accurate quantitation, if R^2 >0.8 and slope >0. As an example of a protein abundance with linear behavior, the protein A8MXV4, acyl-coenzyme A diphosphatase NUDT19, had an R^2 >0.95 across all five HeLa digest concentrations that were measured (Figure 8). Protein abundances for 370 proteins behaved linearly (R^2 >0.8) across all concentrations, an inter-sample dynamic range of three orders of magnitude (Figure 9). More than 1,300 protein abundances (20% of total unique proteins observed across entire dataset) exhibited linear behavior (R^2 >0.8) in at least three different concentration levels.

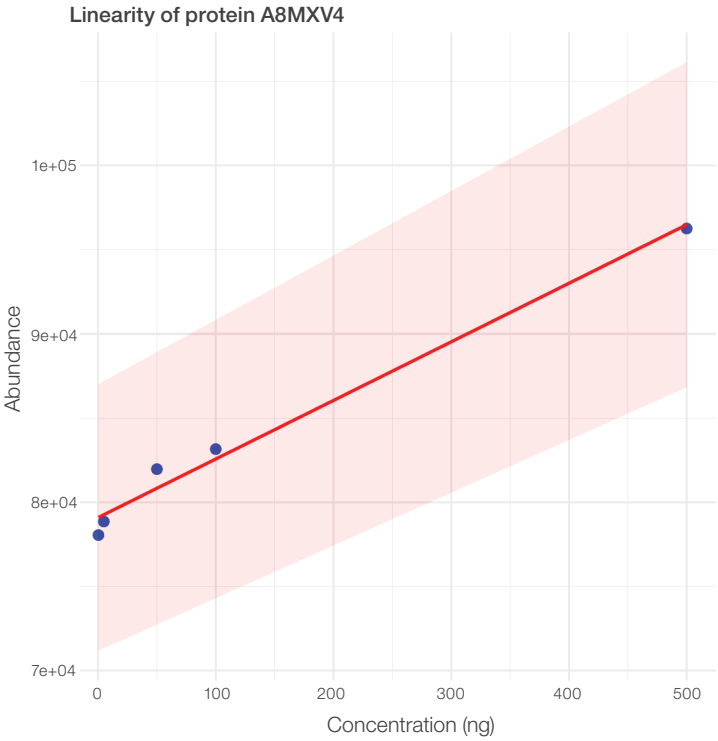


Figure 8. Example of linearity for identified protein at concentrations from 0.5 ng to 500 ng using DIA quantitation (R^2=0.98). Red shading represents a 10% confidence interval.

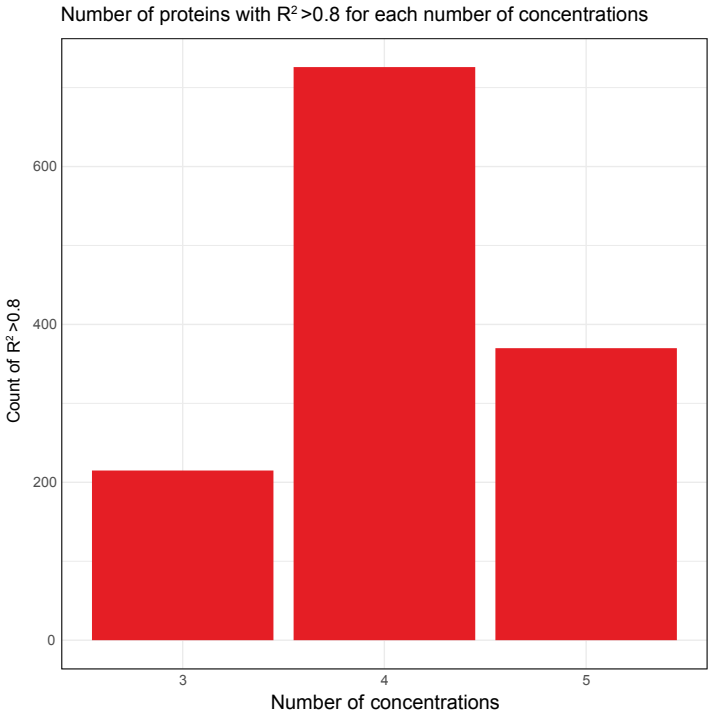


Figure 9. The histogram shows the number of protein abundances with linear behavior across indicated numbers of concentrations. In this experiment, five HeLa concentrations were tested (Table 5). The number of protein abundances displaying linearity of R^2 >0.8 at the indicated number of concentrations are shown.

Robust quantitative performance with spike-in standards

For the hybrid DIA experiments, 30 peptides were analyzed using PRM. There were six peptide groups at five concentration levels (0.05, 0.5, 5, 50, 500 fmol), therefore testing an intra-sample dynamic range of four orders of magnitude. Accurate quantitation was observed for the targeted peptides, (R^2 >0.9) (Figure 10). The peptides were detected at 50 amol (Figure 11). Linearity was observed at the lowest concentration tested, demonstrating an LOQ of 50 amol for this experiment and suggesting that LOD and LOQ for these peptides, except VTSGSTSTSR, could be lower. VTSGSTSTSR, being the first eluting peptide and extremely hydrophilic, exhibited different behavior. For the six peptides considered and across the five concentrations, data demonstrated precise quantitation with median CVs (n=3) <10%. (Figure 12). Consistent ion fragment areas and proportions of the lowest concentration (50 amol) peptide across all HeLa concentrations is highlighted using the average peak area of fragment ions, demonstrating reproducible quantitation (Figure 13). Matrix effects on spike-ins were assessed using the 6x5 peptide standard in different HeLa concentrations.

The wide range of HeLa concentrations provided confidence that the hybrid DIA method works for the typical range used by nanoflow discovery for interrogation of biological samples. Low, high, and potentially unknown concentrations can be used with hybrid DIA.

Comparison of DIA and PRM MS^2 spectra and fragment extracted ion chromatograms demonstrate the increased sensitivity of PRM (Figure 14 A,B). With a narrow isolation window and increased accumulation time, the data shows high sensitivity and specificity for PRM, whereas the high *m/z* isolation window in DIA demonstrates that other precursors eluting at the same retention time dominate the signal intensity.

To further assess the sensitivity of the Orbitrap Excedion Pro MS, data was collected with decreasing volumes of the 6x5 mixture spiked into HeLa. The lowest concentration levels tested were 25, 10, and 5 amol. Figure 15 highlights the sensitivity with peptide YVYVADVAAK, but similar responses were found for all peptides. The lowest concentration tested (5 amol) produces detectable peaks, indicating high peptide sensitivity on the Orbitrap Excedion Pro MS.

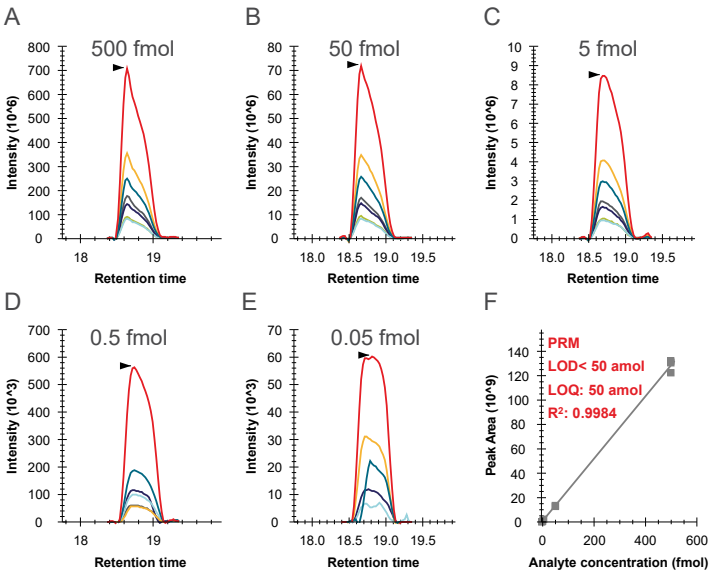


Figure 10. Example dilution curve of 6x5 spike-in standard. PRM chromatograms for the YVYVADVAAK peptide are shown at 500, 50, 5, 0.5, and 0.05 fmol (A, B, C, D, and E, respectively) and the resulting calibration curve (F).

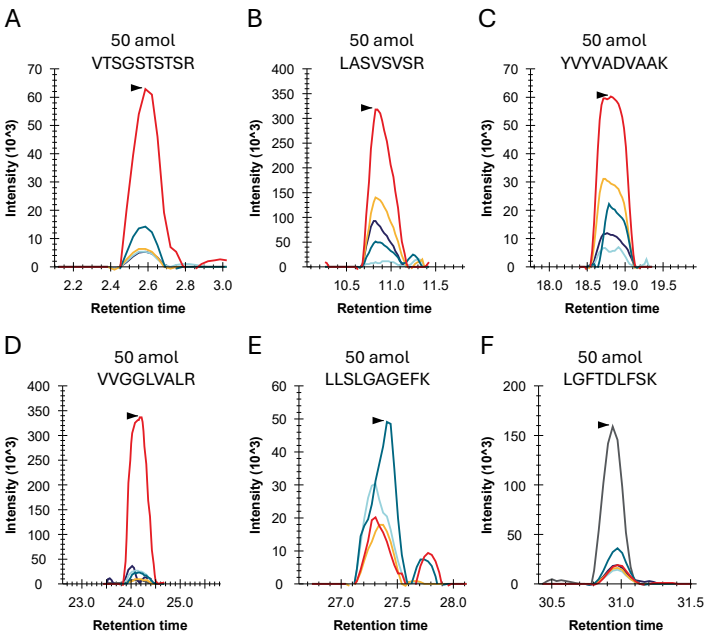


Figure 11. PRM chromatograms for 6x5 peptides are shown at 50 amol (A: VTSGSTSTSR, B: LASVSVSR, C: YVYVADVAAK, D: VVGGLVALR, and E: LLSLGAGEFK, and F: LGFTDLFSK).

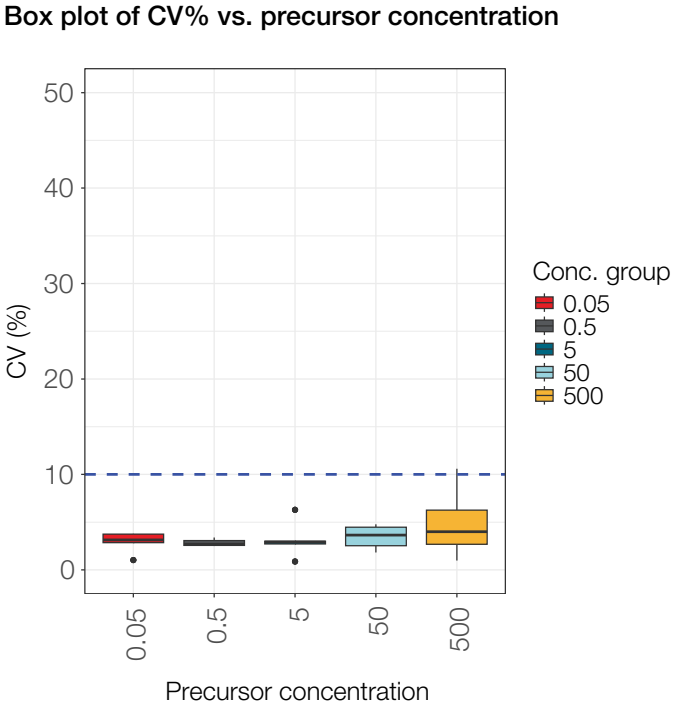


Figure 12. Average CV for peptide-concentration pair replicates across the tested concentrations of HeLa. The blue dashed line represents 10% CV.

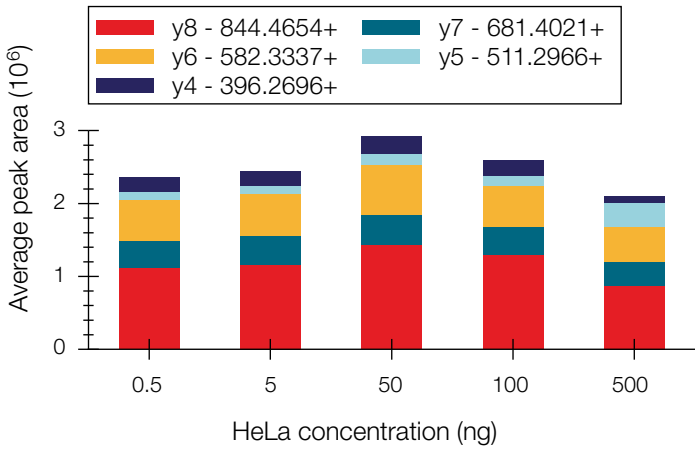


Figure 13. Fragment ion areas for peptide YVYVADVAAK (50 amol) across each HeLa concentration.

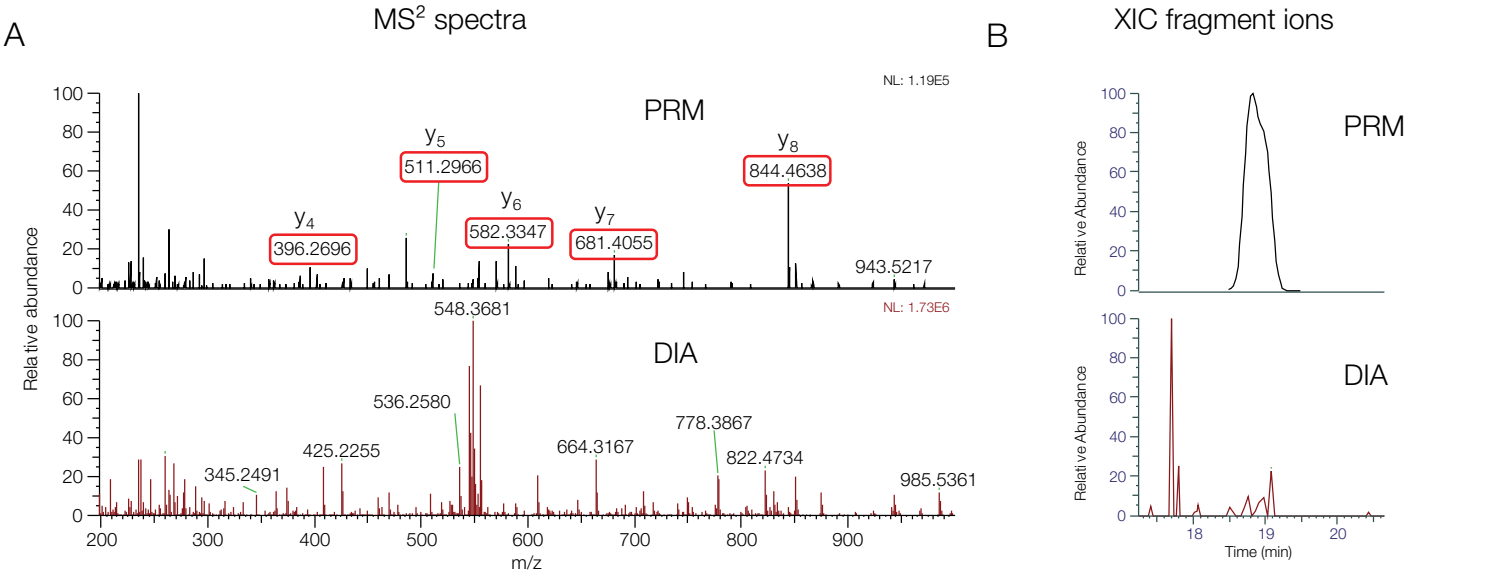


Figure 14. Comparison of PRM and DIA MS² spectra (A) and extracted ion chromatograms using y-ions (B) for peptide YVYVADVAAK at the lowest concentration, 50 amol.

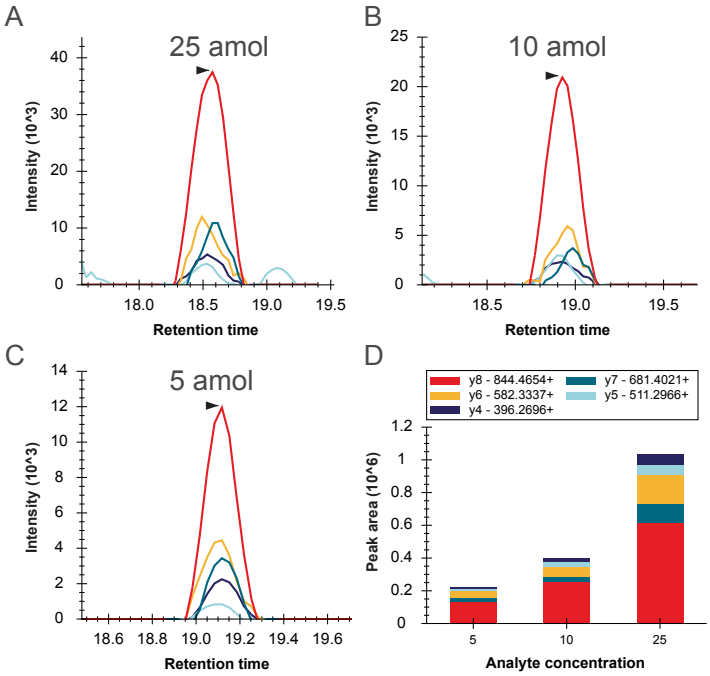
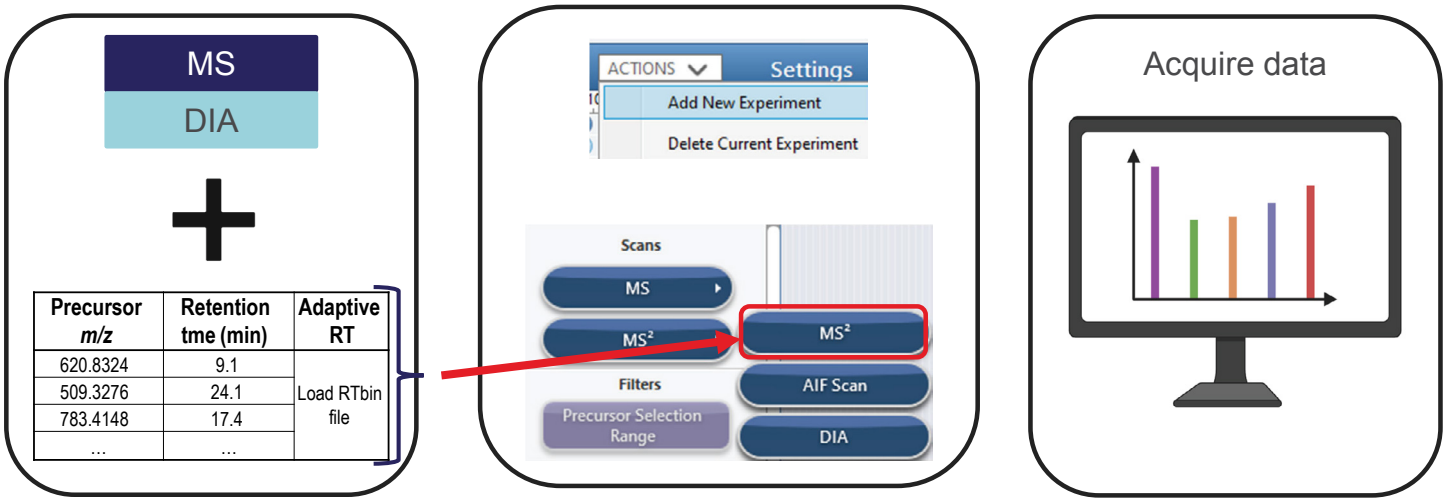


Figure 15. PRM chromatograms for the YVYVADVAAK peptide are shown at 25, 10, and 5 amol (A, B, and C, respectively) and the peak area comparison (D).

Easy implementation of hybrid DIA

Hybrid DIA powered by adaptive RT method creation was straightforward (Figure 16). To implement hybrid DIA, one method and one file is required. Below are the steps for easy creation of hybrid DIA powered by adaptive RT:

1. Open or create a DIA experiment and generate or use a tMS^2 input file. The input file will require precursor mass, RT, and RTbin file.
2. Add the tMS^2 experiment and import precursor list: Turn on adaptive RT dynamic scheduling and load the appropriate .RTbin file.
3. Acquire hybrid DIA data.



1. Start with DIA and tMS^2 precursor list
2. Add tMS^2 experiment and import precursor list
3. Queue injection and acquire nybrid DIA data

Figure 16. Integration of tMS^2 with DIA to create hybrid DIA experimental workflow.

Summary

The hybrid DIA approach combines global proteome coverage and robust quantitative performance with high dynamic range.

- Protein abundances span >5 orders of magnitude with >6,000 protein identifications in 35 minutes for 50 ng HeLa.
- High reproducibility and consistency for discovery proteomics with median CVs <15% across 500 picograms to 500 nanogram.
- Linear quantitation of targets at low levels down to 50 attomole with R^2 >0.9 when leveraging PRM quantitation.
- Easy to implement with three clicks to import tMS^2 experiment and targets into DIA method.
- Addition of adaptive RT minimizes the need for inclusion list adjustments and enables targeting more analytes within narrower retention time windows, which increases the number of DIA scans.
- OptiSpray technology supports plug-and-play simplicity with automated emitter optimization and cartridge-based columns, enabling consistent performance and data acquisition throughout the entire experiment.

Learn more at thermofisher.com/orbitrapexcedionpro

References

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2. Goetze, S., et al. Simultaneous targeted and discovery-driven clinical proteotyping using hybrid-PRM/DIA. *Clin. Proteom.* **2024**, 21, 26. https://doi.org/10.1186/s12014-024-09478-5
3. Remes, P., et al. (2020, September). Highly Multiplex Targeted Proteomics Enabled by Real-Time Chromatographic Alignment. *Anal Chem.* **2020**, 92, 11809. https://doi.org/10.1021/acs.analchem.0c02075

Clinical research

Robust peptide quantitation using the TSQ Certis triple quadrupole mass spectrometer and OptiSpray ion source and cartridges

Authors

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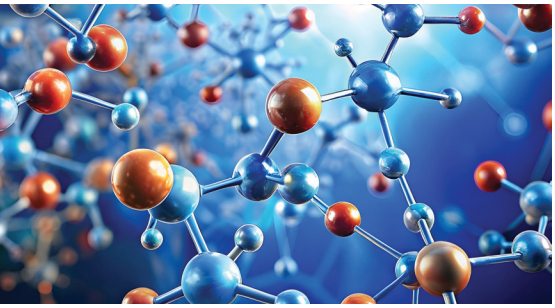
Keywords

TSQ Certis mass spectrometer, Vanquish Neo UHPLC, OptiSpray ion source and cartridge, automation, peptides, biomarkers, human plasma, sensitivity, robustness



Application benefit

The newly introduced Thermo Scientific™ TSQ Certis™ Triple Quadrupole Mass Spectrometer supports exceptional speed, reproducibility, and quantitative precision for targeted peptides and biomarkers. Its enhanced duty cycle and advanced ion optics enable fast acquisition speeds, as well as robust peptide quantitation across in a highly multiplexed assay. The Thermo Scientific™ OptiSpray™ Ion Source and Cartridge Columns improve precision and reproducibility for targeted workflows and make capillary-flow workflows easy to operate with an automated routine for optimized emitter positioning and integrated sheath gas. Additionally, OptiSpray technology used in combination with the TSQ Certis MS improves workflow operability, making it easier to acquire data for routine users and over large cohorts. Together, they are well-suited for translational research and peptide biomarker applications which require confident and consistent results.

Goal

The goal of this work was to demonstrate the quantitative performance of targeted peptide analysis on the next-generation TSQ Certis triple quadrupole mass spectrometer with a focus on operability. The TSQ Certis MS is a high-speed, highly sensitive platform equipped with the OptiSpray ion source and cartridge columns, which provide an intelligent electrospray ionization (ESI) interface optimized for nano- and capillary flow applications (<5 µL/min). The study assessed reproducibility, retention time stability, and sensitivity for a multiplexed assay comprising 82 peptides representing 57 plasma proteins, including >20 recognized biomarkers, to illustrate increased functionality, efficiency, and manageability for users and operators, which is especially needed for large cohorts.

Introduction

Precise and accurate peptide quantitation is critical to advancing proteomics, biomarker discovery, and biotherapeutic development. Triple quadrupole mass spectrometers remain widely-used for targeted peptide quantitation due to their superior sensitivity, selectivity, and quantitative precision. As peptide biomarker workflows increasingly demand higher throughput and greater robustness, innovations in acquisition speed and ion transmission efficiency have become essential. Thus, operability of the workflow becomes central where ease, efficiency, and manageability are key.

The TSQ Certis MS is the next evolution in Thermo Scientific™ triple quadrupole mass spectrometers, designed to deliver faster acquisition with uncompromised performance. The OptiSpray ion source offers simplified operation for both experienced professional and routine users while delivering consistent ionization performance across instruments and analytical runs. This technical note presents a comprehensive evaluation of the TSQ Certis MS equipped with the OptiSpray ion source and column cartridges, highlighting its quantitative performance for targeted peptide analysis.

To evaluate the TSQ Certis MS with the OptiSpray ion source and column cartridges, we adapted a previously described Health Surveillance Panel (HSP) assay[1,2] consisting of 82 peptides from 57 proteins, which was originally developed to quantify biomarkers associated with various diseases such as cardiovascular, inflammatory, and metabolic conditions and includes 24 well-recognized markers in the field. In this work, key performance characteristics—including reproducibility, retention time stability, and sensitivity—were determined using this multiplexed HSP assay.

Together, these results demonstrate the operability of the next-generation TSQ Certis MS with the OptiSpray ion source and cartridge columns to enable faster, more reliable, and higher-throughput peptide quantitation, supporting both research and applied targeted biomarkers laboratories in achieving consistent, high-quality results.

Experimental

Reagents

- Thermo Scientific™ Human plasma digest (Cat. No. NCI4852)
- Stable isotope–labeled (SIL) peptides, New England Peptide (Biosynth Gardner, MA, USA)
- Thermo Scientific™ Water, HPLC–MS grade (Cat. No. W8-1)
- Fisher Chemical™ Formic acid, Optima™ LC-MS grade, 99.0+% (Cat. No. A117-50)
- Thermo Scientific™ Acetonitrile, UHPLC–MS grade (Cat. No. A956-1)
- Thermo Scientific™ Pierce™ Peptide Retention Time Calibration Mixture (PRTC) (Cat. No. 88321)

Calibration standards

Calibration and linearity experiments were conducted at a throughput of 100 samples per day (SPD) to evaluate instrument performance under high-throughput conditions. An 11-point SIL peptide calibration curve was generated, spanning load levels from 0 to 120 fmol on-column (0, 0.0061, 0.0183, 0.0548, 0.16, 0.49, 1.48, 4.4, 13.3, 40, 120 fmol). Each sample was prepared in a constant matrix containing 50 ng of digested control plasma and 50 fmol PRTC to maintain consistent background complexity. For each concentration point, six replicate injections were analyzed to assess reproducibility and quantitative precision.

Liquid chromatography-mass spectrometry

A Thermo Scientific™ Vanquish™ Neo UHPLC System was coupled to the next-generation TSQ Certis MS equipped with the OptiSpray ion source for data acquisition. Ionization was performed using the OptiSpray ion source fitted with a Thermo Scientific™ OptiSpray™ PepMap™ Neo 150 µm × 15 cm Cartridge with a tapered emitter (Cat. No. OS150150PN). Source parameters are summarized in Table 1. SIL-spiked plasma digests were analyzed in a trap-and-elute configuration with a total run time of 13 minutes (100 SPD throughput). The Thermo Scientific™ PepMap™ Neo Trap Cartridge (300 µm × 5 mm, Cat. No. 174500) was used for preconcentration and desalting. Mobile phases consisted of Solvent A: 0.1% formic acid in water; Solvent B: 0.1% formic acid in 80% acetonitrile. The LC gradient and flow rate conditions are detailed in Table 2. Thermo Scientific™ Xcalibur™ Software was used to acquire data on the TSQ Certis MS with SRM transitions of the target peptides listed in Table 3. Raw files and the LC-MS method are available for download from the Thermo Scientific™ AppsLab Library.

Table 1. OptiSpray ion source parameters.

Ion source parameter	Set value
Positive ion voltage (V)	1,800
Sheath gas (arb)	10
Sweep gas (arb)	0
Ion transfer tube temperature (°C)	250
Cartridge temperature (°C)	45

Table 2. LC gradient conditions.

Time (min)	Flow rate (µL/min)	%A	%B
0.0	1.8	99	1
0.7	1.8	96	4
1.0	1.8	92	8
7.7	1.8	77.5	22.5
11.4	1.8	65	35
11.8	2.5	45	55
12.3	2.5	1	99
13.0	2.5	1	99
End	—	—	—

Data analysis

Following SRM data acquisition, the raw files were imported into Skyline-daily software (version 25.1.1.271, MacCoss Lab Software) for automated peak detection, integration, and visualization. The area under the curve (AUC) was determined for each peptide to quantify signal intensity. Limits of detection (LOD) and quantification (LOQ) were established individually for each peptide. In addition, the raw data files were processed using Thermo Scientific™ TraceFinder™ Software (TF, version 5.2 SP3) for automated peak detection, integration, and data review. The AUC was calculated for each peptide transition to quantify signal intensity. Skyline-daily software includes a built-in TF Export tool that enables seamless data export to TraceFinder software.

Results and discussion

The performance was evaluated using an 11-point stable isotope–labeled (SIL) peptide dilution curve spiked into a pooled human plasma digest. Quantitative performance was benchmarked by assessing points per peak, peak area, and relative standard deviation (RSD) for the 82 peptides across six replicate injections (Figure 1). For 1,080 transitions acquired, the number of data points acquired across chromatographic peaks ranged from 8 to 30 [>96% transitions (1,036 transitions) have data points >8 across the peak] with a median of 16, thereby exceeding standard practice for accurate quantification and precision. The average RSD of peak areas in six injections was 3.2%, with values ranging from 0.77% to 12.9%. Notably, >98% peptides (81 of 82) exhibited RSDs below 10%, demonstrating excellent reproducibility of signal intensity and overall method robustness.

Subsequently, the sensitivity, linearity, lower limit of detection (LLOD), and lower limit of quantification (LLOQ) for each of the 82 peptides were determined using a 11-point serial dilution curve with the 100 SPD method (Table 3). The median sensitivity observed across methods was in the sub-femtomole range (LLOD = 0.019 fmol; LLOQ = 0.164 fmol). Evaluation of the quantitative performance demonstrated excellent linearity for >99% of the peptides, exhibiting coefficients of determination (R^2) greater than 0.99 across the dilution series.

Vanquish Neo UHPLC system

TSQ Certis MS with OptiSpray ion source and cartridge

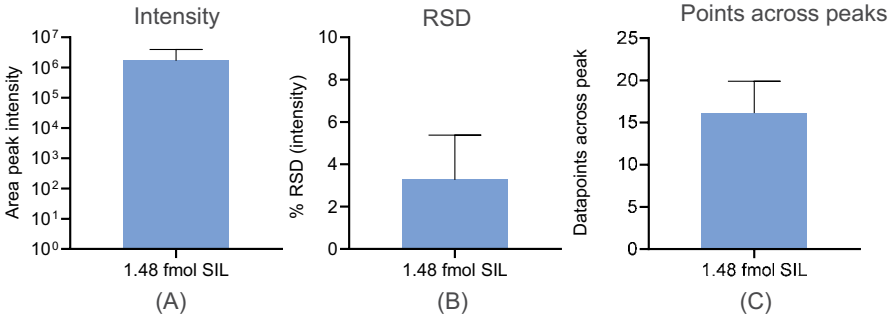


Figure 1. Technical evaluation with repeated injections. The performance of 100 sample per day (SPD) methods was evaluated using 1.48 fmol of the 82 SIL peptides spiked into 50 ng of digested human plasma used as the matrix (n=6 technical) and Skyline software was used to

The scheduled method for the HSP panel includes 1,080 transitions (57 HSP proteins plus the Pierce Peptide Retention Time standard) with a 2-minute retention window (Table 3). Under these conditions, the system consistently maintains short dwell times of approximately 1.3–2 ms and cycle durations of ~0.4–0.6 s for the majority of transitions. This high duty cycle efficiency enables the acquisition of 10–20 data points per chromatographic peak, supporting quantitative robustness even in highly multiplexed targeted workflows. Additionally, the adaptive increase in dwell time as transition density decreases demonstrates the instrument's dynamic and intelligent scheduling capability, supporting optimal sensitivity across the chromatographic run.

Table 3. Summary of LODs, LOQs, R^2 values, and retention times obtained for each peptide. Data was processed by Skyline software.

UniProt ID	Protein name	Peptide sequence	SIL-C-terminal residue modification	Retention time (min)	LOD (fmol on column)	LOQ (fmol on column)	R^2
O14791	APOL1_HUMAN	VAQELEEK	$^{13}C_6,^{15}N_2$	3.15	0.0438	0.0549	0.9972
P00734	THRB_HUMAN	ELLESYIDGR	$^{13}C_6,^{15}N_4$	9.35	0.0250	1.4815	0.9996
P00736	C1R_HUMAN	YTTEIIK	$^{13}C_6,^{15}N_2$	4.92	0.0175	0.0183	0.9971
P00738	HPT_HUMAN	VGYVSGWGR	$^{13}C_6,^{15}N_4$	6.62	0.0238	0.1646	0.9979
P00738	HPT_HUMAN	VTSIQDWVQK	$^{13}C_6,^{15}N_2$	7.99	0.0182	0.4938	0.9989
P00739	HPTR_HUMAN	TEGDGVYTLNDK	$^{13}C_6,^{15}N_2$	5.47	0.0680	0.4938	0.9984
P00742	FA10_HUMAN	MLEVPYVDR	$^{13}C_6,^{15}N_4$	8.84	0.0141	1.4815	0.9988
P00747	PLMN_HUMAN	LFLEPTR	$^{13}C_6,^{15}N_4$	7.16	0.0119	0.1646	0.9965
P00747	PLMN_HUMAN	LSSPAVITDK	$^{13}C_6,^{15}N_2$	5.39	0.0906	0.1646	0.9965
P00748	FA12_HUMAN	VVGGLVALR	$^{13}C_6,^{15}N_4$	7.5	0.0170	0.0549	0.9959
P00748	FA12_HUMAN	EQPPSLTR	$^{13}C_6,^{15}N_4$	3.87	0.0392	0.1646	0.9977
P00751	CFAB_HUMAN	EELLPAQDIK	$^{13}C_6,^{15}N_2$	7.31	0.0434	0.1646	0.9968
P00751	CFAB_HUMAN	ALFVSEEEK	$^{13}C_6,^{15}N_2$	5.91	0.0268	0.0549	0.9969
P01008	ANT3_HUMAN	DDLYVSDAFHK	$^{13}C_6,^{15}N_2$	7.27	0.0934	0.4938	0.9980
P01008	ANT3_HUMAN	FATTFYQHLADSK	$^{13}C_6,^{15}N_2$	7.54	0.0331	4.4444	0.9992
P01009	A1AT_HUMAN	SVLGQLGITK	$^{13}C_6,^{15}N_2$	8.84	0.0169	0.4938	0.9975
P01009	A1AT_HUMAN	QINDYVEK	$^{13}C_6,^{15}N_2$	4.27	0.0374	0.1646	0.9983
P01011	AACT_HUMAN	ADLSGITGAR	$^{13}C_6,^{15}N_4$	5.64	0.0065	0.0183	0.9967
P01011	AACT_HUMAN	EIGELYLPK	$^{13}C_6,^{15}N_2$	8.89	0.0227	0.4938	0.9980
P01023	A2MG_HUMAN	SDIAPVAR	$^{13}C_6,^{15}N_4$	3.77	0.0079	0.0183	0.9973
P01023	A2MG_HUMAN	TEHPFTVEEFVLPK	$^{13}C_6,^{15}N_2$	10.1	0.0406	13.3330	0.9984
P01024	CO3_HUMAN	TGLQEVEVK	$^{13}C_6,^{15}N_2$	5.1	0.0084	0.0183	0.9973
P01024	CO3_HUMAN	ISLPESLK	$^{13}C_6,^{15}N_2$	7.37	0.0266	0.1646	0.9975
P01031	CO5_HUMAN	LQGTLPVEAR	$^{13}C_6,^{15}N_4$	5.87	0.0048	0.0061	0.9961
P01031	CO5_HUMAN	TDAPDLPEENQAR	$^{13}C_6,^{15}N_4$	5.26	0.2558	0.4938	0.9989
P01871	IGHM_HUMAN	GFPSVLR	$^{13}C_6,^{15}N_4$	7.26	0.0164	0.1646	0.9969
P01876	IGHA1_HUMAN	TPLTATLSK	$^{13}C_6,^{15}N_2$	5.34	0.0126	0.0549	0.9961
P01876	IGHA1_HUMAN	DASGVTFTWTPSSGK	$^{13}C_6,^{15}N_2$	9.8	0.0166	1.4815	0.9992
P02647	APOA1_HUMAN	ATEHLSTLSEK	$^{13}C_6,^{15}N_2$	3.19	0.0125	0.1646	0.9931
P02649	APOE_HUMAN	LGPLVEQGR	$^{13}C_6,^{15}N_4$	5.42	0.0069	0.0549	0.9961
P02649	APOE_HUMAN	AATVGSLAGQPLQER	$^{13}C_6,^{15}N_4$	6.77	0.0057	0.1646	0.9991
P02652	APOA2_HUMAN	SPELQAEAK	$^{13}C_6,^{15}N_2$	3.24	0.0199	0.0183	0.9976
P02655	APOC2_HUMAN	TYLPAVDEK	$^{13}C_6,^{15}N_2$	6.03	0.0092	0.0183	0.9968
P02655	APOC2_HUMAN	TAAQNLYEK	$^{13}C_6,^{15}N_2$	3.56	0.0412	0.1646	0.9977
P02656	APOC3_HUMAN	DALSSVQESQVAQQAR	$^{13}C_6,^{15}N_4$	6.89	2.3678	4.4444	0.9987
P02671	FIBA_HUMAN	NSLFEYQK	$^{13}C_6,^{15}N_2$	6.36	0.0057	0.1646	0.9944
P02741	CRP_HUMAN	ESDTSYVSLK	$^{13}C_6,^{15}N_2$	5.61	0.0186	0.1646	0.9966
P02741	CRP_HUMAN	GYSIFSYATK	$^{13}C_6,^{15}N_2$	9.07	0.0329	1.4815	0.9993

UniProt ID	Protein name	Peptide sequence	SIL-C-terminal residue modification	Retention time (min)	LOD (fmol on column)	LOQ (fmol on column)	R^2
P02748	CO9_HUMAN	TSNFNAAISLK	$^{13}C_6,^{15}N_2$	7.24	0.0514	0.1646	0.9986
P02748	CO9_HUMAN	LSPIYNLVPVK	$^{13}C_6,^{15}N_2$	10.48	0.0089	4.4444	0.9990
P02750	A2GL_HUMAN	ALGHLDLSGNR	$^{13}C_6,^{15}N_4$	5.18	0.0392	0.1646	0.9947
P02750	A2GL_HUMAN	DLLLPQPDLR	$^{13}C_6,^{15}N_4$	10.77	0.0250	1.4815	0.9992
P02760	AMBP_HUMAN	ETLLQDFR	$^{13}C_6,^{15}N_4$	8.82	0.0281	0.4938	0.9976
P02763	A1AG1_HUMAN	SDVVYTDWK	$^{13}C_6,^{15}N_2$	7.16	0.0510	0.0549	0.9976
P02765	FETUA_HUMAN	FSVVYAK	$^{13}C_6,^{15}N_2$	5.8	0.0111	0.0183	0.9957
P02768	ALBU_HUMAN	LVNEVTEFAK	$^{13}C_6,^{15}N_2$	7.29	0.0164	0.1646	0.9969
P02768	ALBU_HUMAN	DLGEENFK	$^{13}C_6,^{15}N_2$	5.58	0.1156	0.0549	0.9979
P02774	VTDB_HUMAN	VLEPTLK	$^{13}C_6,^{15}N_2$	5.18	0.0100	0.0549	0.9925
P02787	TRFE_HUMAN	DGAGDVAFVK	$^{13}C_6,^{15}N_2$	6.51	0.0204	0.0183	0.9970
P02787	TRFE_HUMAN	SASDLTWDNLK	$^{13}C_6,^{15}N_2$	8.41	0.0289	0.0549	0.9993
P02790	HEMO_HUMAN	NFPSPVDAAFR	$^{13}C_6,^{15}N_4$	9.44	0.0183	1.4815	0.9993
P03952	KLKB1_HUMAN	YSPGGTPTAIK	$^{13}C_6,^{15}N_2$	4.42	0.0072	0.0549	0.9968
P04003	C4BPA_HUMAN	EDVYVVGTVLR	$^{13}C_6,^{15}N_4$	9.6	0.0281	1.4815	0.9992
P04004	VTNC_HUMAN	FEDGVLDPDYPR	$^{13}C_6,^{15}N_4$	9.01	0.0263	1.4815	0.9996
P04114	APOB_HUMAN	TEVIPPLIENR	$^{13}C_6,^{15}N_4$	9.23	0.0109	1.4815	0.9990
P04217	A1BG_HUMAN	NGVAQEPVHLDSPAIK	$^{13}C_6,^{15}N_2$	6.35	0.0209	0.1646	0.9932
P04217	A1BG_HUMAN	LLELTGPK	$^{13}C_6,^{15}N_2$	6.83	0.0122	0.1646	0.9969
P04275	VWF_HUMAN	ILAGPAGDSNVVK	$^{13}C_6,^{15}N_2$	5.76	0.0071	0.0549	0.9961
P04278	SHBG_HUMAN	VVLSQGSK	$^{13}C_6,^{15}N_2$	3.14	0.0154	0.0549	0.9960
P05155	IC1_HUMAN	FQPTLLTLPR	$^{13}C_6,^{15}N_4$	10.43	0.0115	4.4444	0.9995
P05546	HEP2_HUMAN	TLEAQLTPR	$^{13}C_6,^{15}N_4$	5.82	0.0185	0.0183	0.9971
P06396	GELS_HUMAN	TGAQELLR	$^{13}C_6,^{15}N_4$	4.9	0.0125	0.0549	0.9979
P06396	GELS_HUMAN	AGALNSNDAFVLK	$^{13}C_6,^{15}N_2$	8.17	0.0146	0.1646	0.9982
P06727	APOA4_HUMAN	ISASAEELR	$^{13}C_6,^{15}N_4$	4.4	0.0086	0.0549	0.9961
P08603	CFAH_HUMAN	IDVHLVPDR	$^{13}C_6,^{15}N_4$	6.39	0.0305	0.1646	0.9946
P08603	CFAH_HUMAN	SPDVINGSPISQK	$^{13}C_6,^{15}N_2$	6.02	0.0155	0.1646	0.9978
P08697	A2AP_HUMAN	LGNQEPGGQTALK	$^{13}C_6,^{15}N_2$	3.92	0.0199	0.0549	0.9977
P09871	C1S_HUMAN	TNFDANDIALVR	$^{13}C_6,^{15}N_4$	8.7	0.0378	1.4815	0.9997
P0C0L5	CO4B_HUMAN	DHAVDLIQK	$^{13}C_6,^{15}N_2$	4.53	0.0309	0.0183	0.9975
P0C0L5	CO4B_HUMAN	VGDTLNLNLR	$^{13}C_6,^{15}N_4$	8.11	0.0148	0.4938	0.9984
P10909	CLUS_HUMAN	EIQNAVNGVK	$^{13}C_6,^{15}N_2$	3.93	0.0133	0.1646	0.9974
P10909	CLUS_HUMAN	TLLSNLEEAK	$^{13}C_6,^{15}N_2$	8.27	0.0078	1.4815	0.9982
P19823	ITIH2_HUMAN	SLAPTAAAK	$^{13}C_6,^{15}N_2$	3.19	0.0323	0.1646	0.9956
P19823	ITIH2_HUMAN	IYLQPGR	$^{13}C_6,^{15}N_4$	4.8	0.0106	0.0183	0.9972
P19827	ITIH1_HUMAN	AAISGENAGLVR	$^{13}C_6,^{15}N_4$	5.18	0.0062	0.1646	0.9955
P25311	ZA2G_HUMAN	AGEVQEPELR	$^{13}C_6,^{15}N_4$	4.83	0.0239	0.0183	0.9974
P32119	PRDX2_HUMAN	GLFIIDGK	$^{13}C_6,^{15}N_2$	9.55	0.0201	0.0549	0.9975
P43652	AFAM_HUMAN	FLVNLVK	$^{13}C_6,^{15}N_2$	8.99	0.0243	0.4938	0.9977
P51884	LUM_HUMAN	SLEYLDLSFNQIAR	$^{13}C_6,^{15}N_4$	11.81	6.8849	13.3330	0.9656
P69905	HBA_HUMAN	VGAHAGEYGAEALER	$^{13}C_6,^{15}N_4$	4.85	0.0466	0.1646	0.9982
Q14624	ITIH4_HUMAN	LALDNGGLAR	$^{13}C_6,^{15}N_4$	6.27	0.0599	0.1646	0.9970
Q14624	ITIH4_HUMAN	LGVYELLLK	$^{13}C_6,^{15}N_2$	11.29	0.0213	4.4444	0.9990

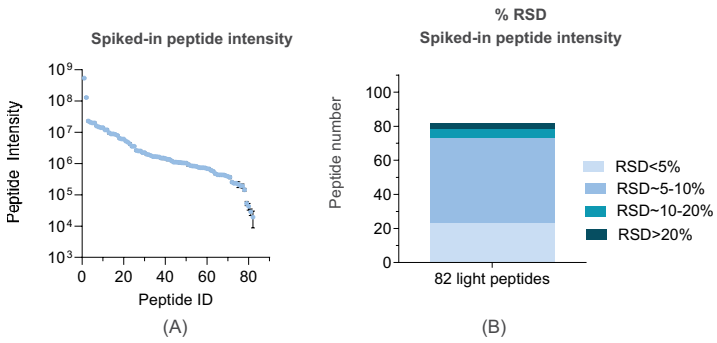


Figure 2. Reproducibility of peptide quantitation using the TSQ Certis mass spectrometer with the OptiSpray ion source and PepMap Neo 150 µm x 15 cm cartridge column across 71 injections of 50 ng plasma digest. Skyline software was used for data processing. (A) Average light peptide intensity with standard deviations. (B) Relative standard deviation (RSD) for all 82 native peptides.

The reproducibility and robustness of the TSQ Certis MS equipped with the OptiSpray ion source and PepMap Neo 150 µm x 15 cm cartridge column were evaluated using a panel of 82 peptides representing 57 HSP proteins. A total of 71 injections of 50 ng plasma digest spiked with internal standards was analyzed. The average and standard deviation of the light peptide signals are shown in Figure 2A, and % RSD values across the 71 injections are summarized in Figure 2B. The results demonstrated excellent reproducibility, with approximately 89% (73) of native peptide signals exhibiting RSDs below 10%, and 95% (78) showing RSDs below 20%. The remaining 5% (4 peptides) with RSDs greater than 20% were attributed to low endogenous peptide concentrations in plasma.

A critical aspect in the development of a SRM targeted peptide assay is the maintenance of consistent retention times, which allows for accurate and efficient scheduling of multiple precursor ions. To evaluate chromatographic reproducibility, retention time variations were analyzed for the SIL peptides. Specifically, the assay includes 24 well-recognized protein markers represented by 35 peptides.

As shown in Figure 3A, the retention times of these 35 peptides ranged from 3.09 to 10.42 minutes, with %RSD between 0.084% and 0.56% across 71 injections, indicating exceptional retention time stability (Figure 3B).

Furthermore, Figure 3C illustrates the reproducibility of peptide peak areas across 71 injections. The % RSD values for the 35 peptides (representing 24 recognized protein markers) ranged from 4.3% to 55.1%, with a median RSD of 3.2%. Notably, 88% of the peptides (31 out of 35) exhibited peak area RSDs below 10% (Figure 3D).

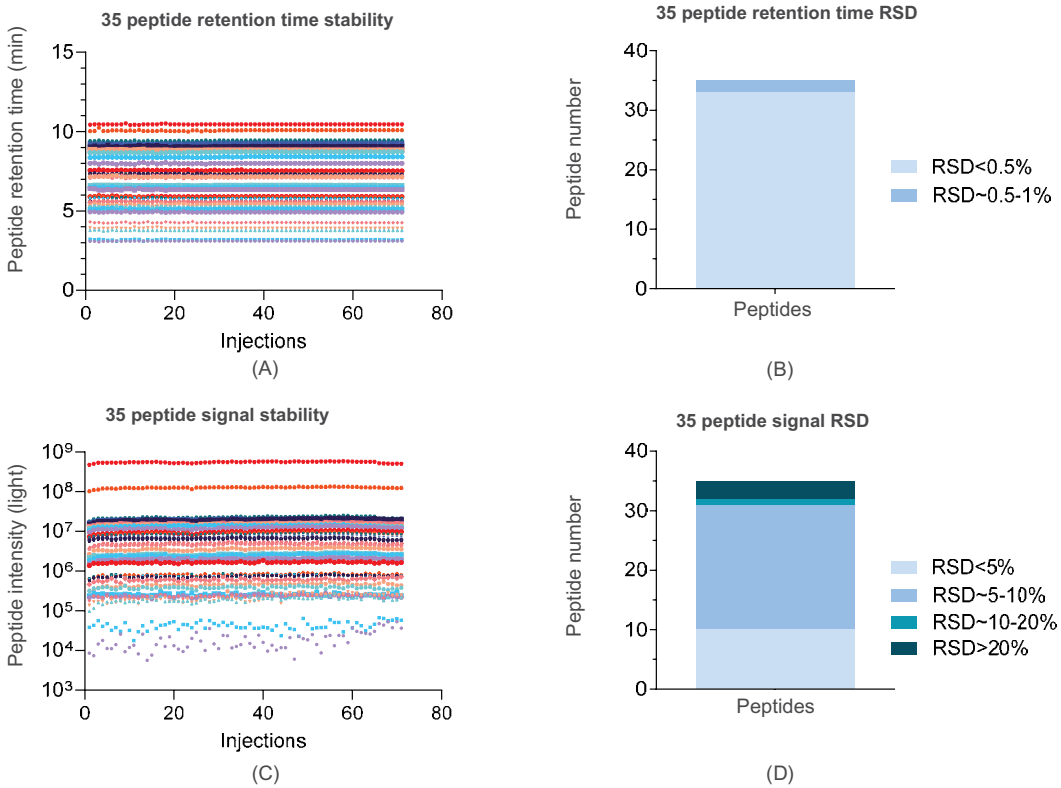


Figure 3. Retention time and peak area reproducibility. Over 71 injections, 35 peptides representing 24 well-recognized protein markers were investigated. Skyline software was used for data processing. (A) Retention times ranged from 3.09 to 10.42 min with RSDs between 0.084% and 0.56%. (B) 33 peptides showed RT RSD <0.5%, and 2 peptides had RT RSDs between 0.5% and 0.56% across 71 injections, indicating excellent chromatographic stability. (C) Peak area reproducibility and (D) variation across 71 injections, with 31 peptides showing RSD <10% and 33 peptides showing RSD <20%.

The remaining 47 peptides also exhibited excellent signal reproducibility (average RSD = 6.9%) and stable retention times (average RSD = 0.3%). The consistent performance highlights their potential analytical robustness and suitability for research or pre-clinical biomarker studies. Overall, the peptide quantitation data acquired on the TSQ Certis MS demonstrates outstanding quantitative performance.

The 11-point serial dilution peptide dataset was also analyzed with TraceFinder software. In Figure 4, extracted data are presented for two well-recognized protein biomarkers: the FSVVYAK peptide from Alpha-2-HS-glycoprotein and the SDIAPVAR peptide from Alpha-2-macroglobulin. Serial dilutions of the heavy peptides were analyzed over a dynamic range of 0–120 fmol on-column (Figure 4A). Both peptides exhibited excellent linearity, with correlation coefficients (R^2 = 0.9981 and R^2 = 0.9974, respectively), confirming accurate quantitative response across the tested range. Insets highlight expanded views of the low-concentration regions (0–2 fmol), demonstrating consistent sensitivity (R^2 > 0.996).

Signal intensity reproducibility for the corresponding light (unlabeled) peptides was evaluated across 71 consecutive injections, yielding RSD of 3.6% for SDIAPVAR and 2.8% for FSVVYAK, indicating high analytical precision. Retention time reproducibility showed minimal variation, with mean retention times of 3.775 min (RSD = 0.195%) for SDIAPVAR and 5.80 min (RSD = 0.0975%) for FSVVYAK, demonstrating excellent chromatographic stability across repeated injections.

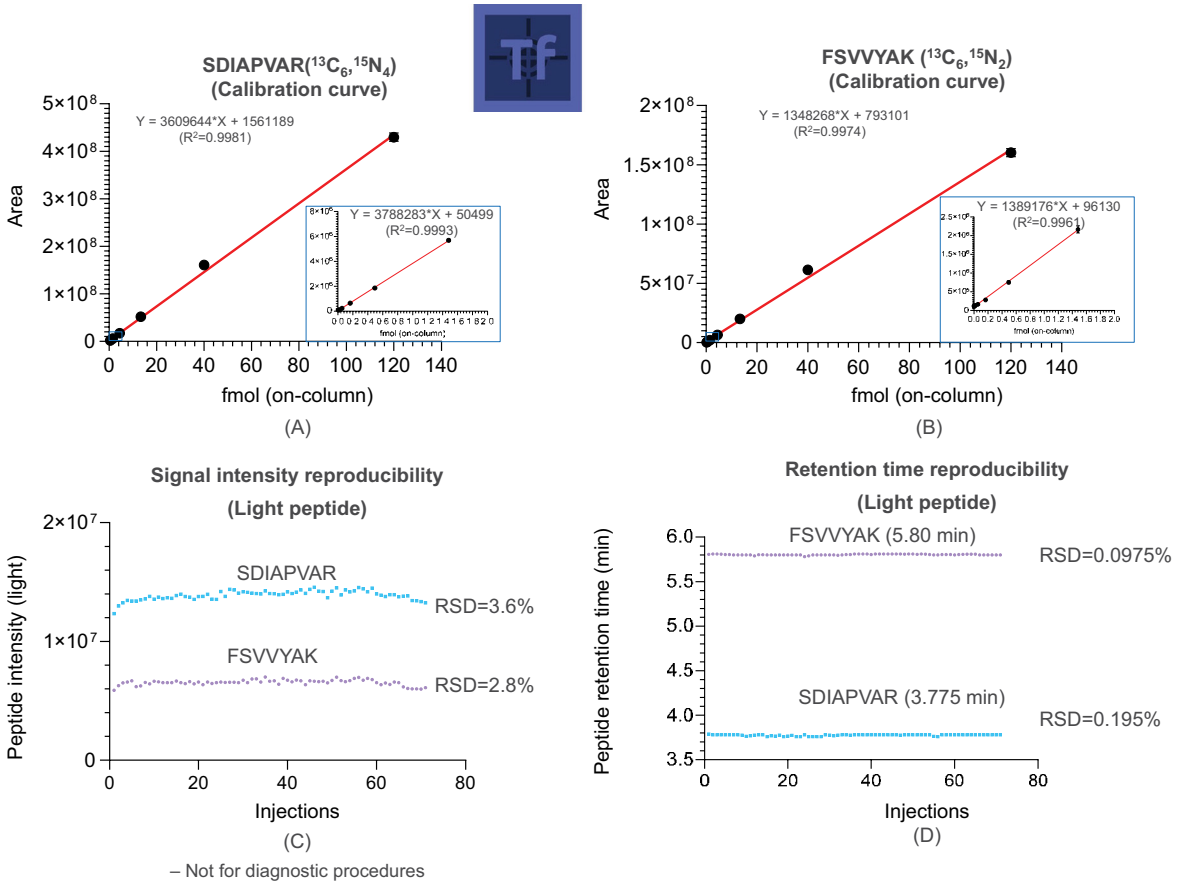


Figure 4. Calibration curves and reproducibility assessment of targeted peptide quantification using TraceFinder software.
(A) and (B) Calibration curves for the stable isotope–labeled internal standard peptides SDIAPVAR($^{13}C_6$,$^{15}N_4$) and FSVVYAK($^{13}C_6$,$^{15}N_2$), respectively. (C) Signal intensity reproducibility of the corresponding light (unlabeled) peptides was assessed across 71 consecutive injections. (D) Retention time reproducibility for the same light peptides. TraceFinder software was utilized for data generation.

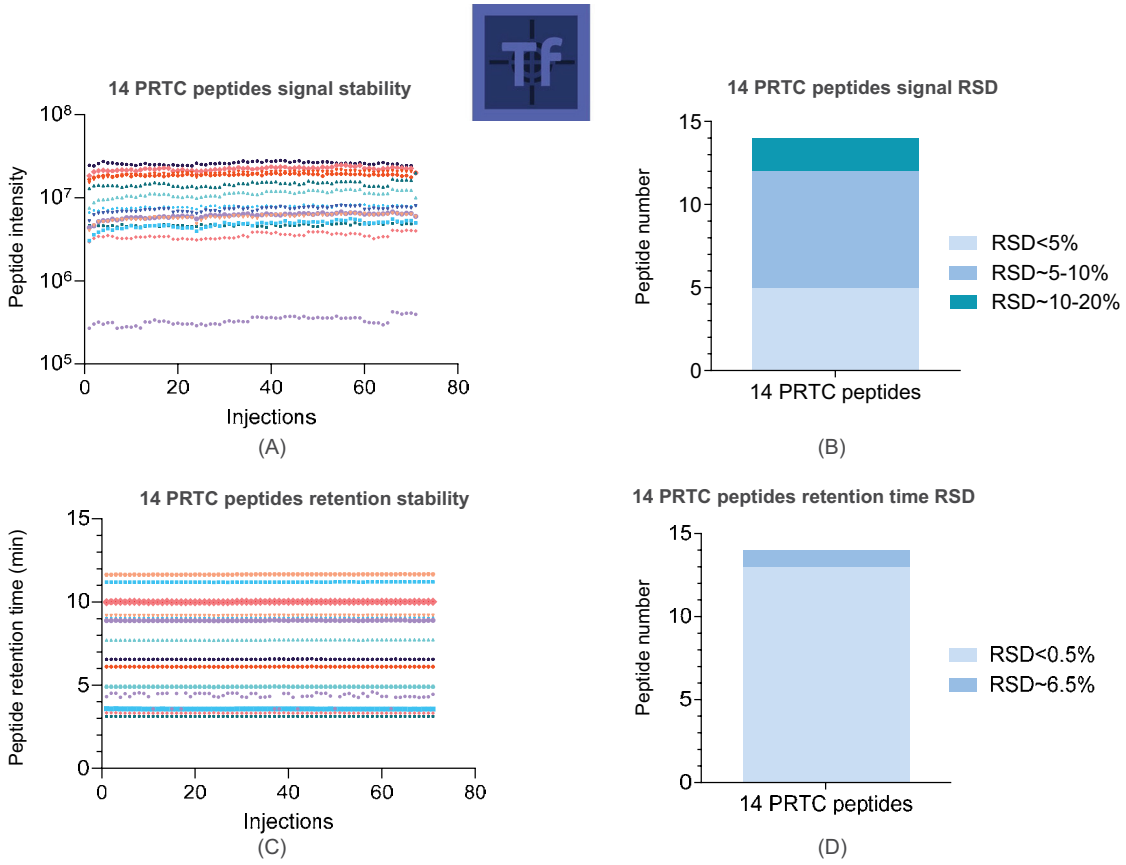


Figure 5. Robust performance of Pierce Peptide Retention Time Calibration Mixture (PRTC) intensity and retention time. (A) Signal stability of 14 PRTC peptides across 71 injections. (B) Signal RSD distribution for the 14 PRTC peptides. (C) Retention time stability of 14 PRTC peptides across 71 injections. (D) Retention-time RSD distribution for the 14 PRTC peptides. TraceFinder software was utilized for data generation.

All samples were spiked with Pierce Peptide Retention Time Calibration Mixture and 14 PRTC peptides were targeted. As expected, PRTC peptides demonstrated excellent LC–MS performance across 71 consecutive injections. As shown in Figure 5A, peptide signal intensities remained highly stable throughout the run. Correspondingly, the signal RSD analysis in Figure 5B revealed that most peptides exhibited RSD values below 10%, and 4 out of 14 were below 5%, indicating strong quantitative reproducibility. Retention time stability (Figure 5C) was also consistently maintained, with the peptides displaying tightly clustered retention times across injections. The retention time RSD distribution (Figure 5D) further confirmed this stability, as nearly the entire peptide set (13 out of 14) showed RSD values below 0.5%, with the remainder still under 10%. Together, these results demonstrate robust instrument stability and reproducible chromatographic performance suitable for high-confidence LC–MS analyses.

Conclusion

The TSQ Certis triple quadrupole mass spectrometer equipped with the OptiSpray source and cartridge columns provides exceptional reproducibility, sensitivity, and stability for multiplexed peptide quantitation. The key outcomes include sub-femtomole sensitivity; >99% linearity for >98% of the peptides, <0.6% retention time variation; and high reproducibility at 100 SPD (13 minutes per run) throughput. These results demonstrate the instrument's suitability for peptide quantitation, pre-clinical biomarker verification, and translational research applications.

The TSQ Certis MS is a next-generation, high-performance triple-quadrupole platform designed for fast, precise quantitative analyses. Its rapid-scanning (900 SRM/second) architecture supports large transition loads without compromising data quality.

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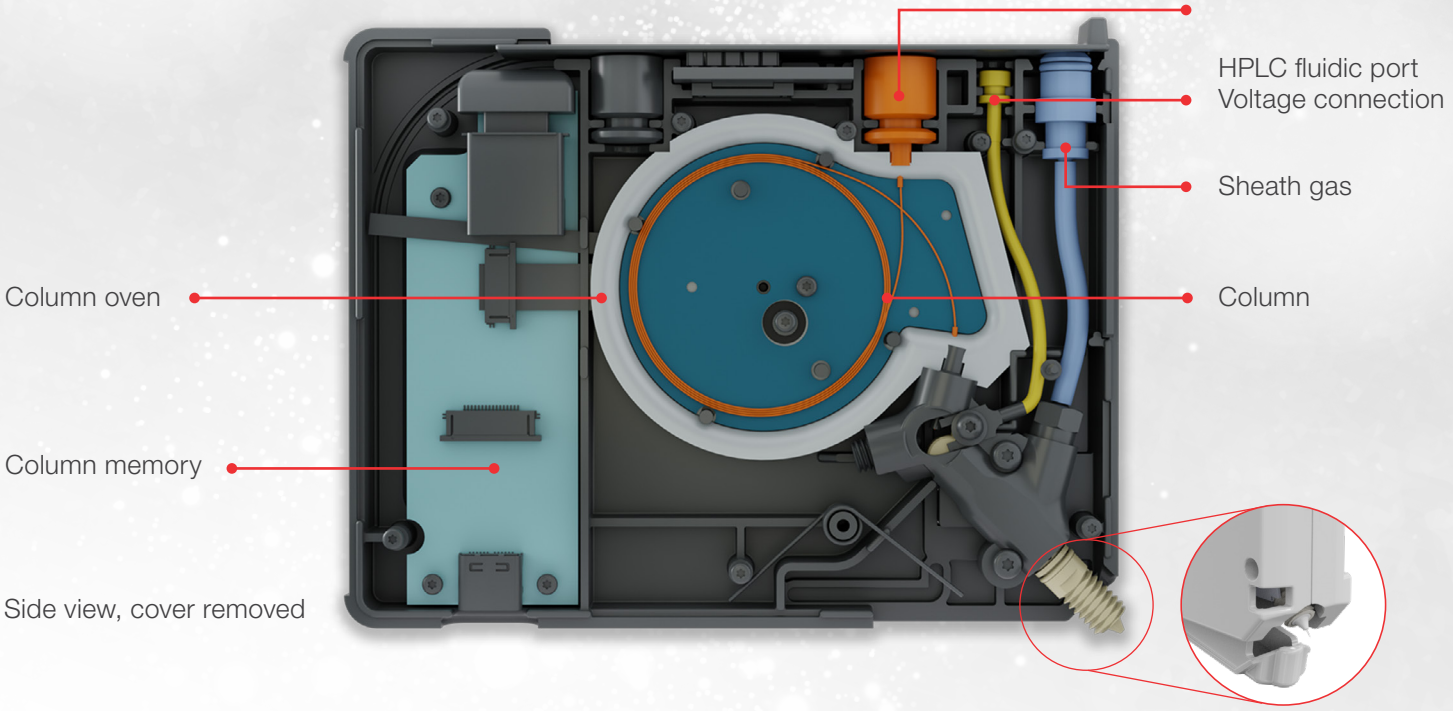

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