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High-throughput proteomics using narrow window DIA on the Orbitrap Astral Zoom mass spectrometer

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Keywords

Orbitrap Astral Zoom MS, Orbitrap Astral MS, high-throughput proteomics, narrow-window data-independent acquisition, nDIA, three-proteome mixture, coefficient of variation, CV, quantitation, precision, accuracy, data points per peak, DPPP

Goal

Assess the performance of the Thermo Scientific™ Orbitrap™ Astral™ Zoom mass spectrometer for high-throughput proteomics using narrow-window data-independent acquisition (nDIA) methods.

Introduction

High-throughput proteomics is an important tool in chemoproteomic drug discovery and development, translational biology, and clinical research, enabling the comprehensive analysis of complex proteomes within a short timeframe. This approach is crucial for large-scale studies, such as those involving clinical cohorts, where the need for deep proteome coverage and accurate and precise quantitation is paramount. However, achieving this depth and confident quantitation while maintaining high throughput poses significant challenges, such as the need for robust and sensitive instrumentation capable of handling high sample complexity and fast data acquisition.

For many applications, especially single-shot label-free analyses utilizing short gradients, data-independent acquisition (DIA) has emerged as the preferred method for proteome profiling due to its superior reproducibility, coverage, and quantitative performance compared to data-dependent acquisition (DDA). DIA enables the comprehensive and unbiased coverage of peptides within a sample. Despite its advantages, traditional DIA methods often struggle with insufficient precursor selectivity and ambiguous precursor to fragment assignments due to the use of wide isolation windows of 8–25 Th, especially when dealing with complex samples eluting over very short gradients.[1]

nDIA addresses these limitations by using smaller isolation windows, similar to those used in DDA, to improve specificity and reduce chimeric spectra that result from many co-eluting peptides within an isolation window. This approach allows for more precise peptide identification and quantification, even in high-throughput scenarios with short chromatographic gradients. Moreover, nDIA aquisition schemes have advantages for post-translational modifications (PTMs) detection and site localization analyses.[2-3]

The feasibility of measuring such narrow, DDA-like isolation windows of 2 *m/z*, sampling peptides across an appropriate mass range and on a chromatographic timeframe, is restricted by the acquisition speed of most MS instruments, resulting in unrealistic cycle times and suboptimal quantitation.

The Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer uniquely meets the needs of high acquisition speed, sensitivity, and dynamic range to employ nDIA and has thereby significantly advanced the field of proteomics.[2-5]

Building on this innovation, the Orbitrap Astral Zoom MS further pushes the boundaries of high-throughput proteomics and provides even more flexibility with faster scan rates and duty cycle improvements through pre-accumulation of ions. Faster acquisition speeds up to 270 Hz (35% faster) are enabled by improved ion optics settling times and faster ion transfers; higher sensitivity is achieved by bent-trap pre-accumulation (up to 45% more ions per Thermo Scientific™ Astral™ analyzer MS^2 scan); and enhanced spectral processing capabilities provide improved deconvolution of complex MS^2 spectra to further advance nDIA performance.

The Orbitrap Astral Zoom MS, in combination with the Thermo Scientific™ Vanquish™ Neo UHPLC system, offers an outstanding, robust, and high-throughput solution for deep proteome interrogations. This setup is ideal for large-cohort clinical studies or high-throughput screening methods, providing comprehensive proteome profiling with confident quantitation reliably and reproducibly.

Here, we demonstrate the performance of the Orbitrap Astral Zoom MS using nDIA to achieve high-throughput proteome profiling. We highlight the ability to handle throughputs ranging from 30 to 300 samples per day (SPD), showcasing significant improvements in unique peptide and protein group identifications and even deeper coverage while maintaining accurate and precise quantitation. This next-generation instrument represents a further significant advancement in quantitative proteomics, making high-throughput, high-sensitivity, and high-resolution analyses more accessible and practical for a wide range of applications.

Experimental

Recommended consumables

- Fisher Chemical™ Optima™ LC-MS grade water with 0.1% formic acid (FA), (Cat. No. LS118-500)
- Fisher Chemical™ Optima™ LC-MS 80% acetonitrile (ACN) with 0.1% formic acid (Cat. No. LS122500)
- Fisher Chemical ™ Optima™ LC-MS grade formic acid (Cat. No. A117-50)
- Fisher Chemical™ Optima™ LC-MS grade water (Cat. No. 10505904)
- Fisher Chemical™ Optima™ LC-MS grade acetonitrile (Cat. No. A955-1)
- Fisher Chemical™ Optima™ LC-MS grade isopropanol (Cat. No. A461-212)
- Thermo Scientific™ Pierce™ Trifluoroacetic acid (TFA), sequencing grade (Cat. No. 28904)
- Eppendorf™ twin.tec™ 96 Well LoBind PCR Plates, Skirted (Cat. No. E0030129512)
- Axygen™ AxyMats™ 96 Round Well Sealing Mat for PCR Microplates (Cat. No. AM-96-PCR-RD)

Samples

- Thermo Scientific™ Pierce™ HeLa Protein Digest Standard (Cat. No. 88328)
- Promega™ MS-Compatible Yeast Protein Extract, Digest, 100 µg (Cat. No. V7461)
- Waters™ MassPREP™ *E. coli* Digest Standard (Cat. No. 186003196)

LC columns

- Thermo Scientific™ EASY-Spray™ HPLC column, 2 µm C18, 150 µm × 150 mm (Cat. No. ES906)
- Thermo Scientific™ PepMap™ Neo HPLC column, 5 µm C18, 300 µm × 5 mm (Cat. No. 174500)
- IonOpticks™ Aurora Ultimate™ 25 cm x 75 µm ID, 1.7 µm C18 (Cat. No. AUR3-25075C18-XT)
- Column heater for IonOpticks column (Cat. No. COLHTR01)

HPLC system

- Vanquish Neo UHPLC system including Vanquish Neo Pump/ Autosampler (Cat. No. VN-S10-A-01)

Mass spectrometer

- Orbitrap Astral Zoom mass spectrometer
- Thermo Scientific™ EASY-Spray™ ion source

Data analysis software

- Spectronaut™ 19.5 software (Biognosys AG)

HeLa digest standard and HYE (*Homo sapiens*, yeast, *E. coli*) sample preparation

Lyophilized HeLa Digest Standard (20 µg/vial) was reconstituted in H_2O + 0.1% FA by sonication at room temperature for 3 min to reach a final peptide concentration of 100 or 200 ng/µL, and after a short spin-down, was transferred to 96-well plates for LC-MS measurement.

Three-proteome mix samples of human, yeast, and *E. coli* protein digests were prepared from lyophilized peptides from Pierce HeLa Protein Digest Standard, Mass Spec-Compatible Yeast Digest (Promega), and MassPREP *E. coli* Digest Standard (Waters). The lyophilized digests were defrosted and re-suspended in aqueous 0.1% FA with 0.015% DDM to obtain 500 ng/µL stock solutions, which were then used to prepare two mixtures, A and B. Mix A contained 65% of HeLa digest, 15% of yeast digest, and 20% of *E. coli* digest. Mix B contained 65% of HeLa digest, 30% of yeast digest, and 5% of *E. coli* digest.

LC conditions

For the throughput of 30 SPD, the samples were separated on an IonOpticks Aurora Ultimate XT 75 µm × 25 cm C18 column using a Vanquish Neo UHPLC system operated in NanoCap Direct Injection configuration. For the throughputs of 60, 100, 180, and 300 SPD, samples were separated on a 150 µm × 150 mm C18 EASY-Spray HPLC column using a Vanquish Neo UHPLC system operated in NanoCap Trap and Elute configuration with the PepMap Neo HPLC column, 5 µm C18 300 µm × 5 mm cartridge, as a trap column. Mobile phases are shown in Table 1. Flow rates and gradients varied depending on the throughput and are shown in Table 2. The run-to-run throughputs (SPD) are defined for 1 µL injections; column re-equilibration was performed in parallel to sample loading. The columns were connected online via an EASY-Spray source to the mass spectrometer.

MS acquisition parameters

Samples were measured on the Orbitrap Astral Zoom MS in DIA mode. Method parameters are shown in Table 3. Comparison data were measured on an Orbitrap Astral MS using the same experimental setup and the same method parameters. For the Orbitrap Astral Zoom mass spectrometer, the novel pre-accumulation feature was additionally enabled.

For throughputs of 60, 100, 180, and 300 SPD, the DIA isolation window was set to 2 Th for all loads. Data were acquired in triplicate for the MS^2 Astral maximum injection time of 3 ms for both instruments and additionally with 2 ms for the Orbitrap Astral Zoom MS. As discussed in this technical note and exemplarily shown for 300 SPD, the scan range/precursor mass range, the DIA isolation window width, and MS^2 maximum injection times can be easily optimized to focus on MS^2-based quantitation. For 30 SPD, the isolation window width and Astral analyzer maximum injection time were adjusted depending on the sample load; details are given in Table 4 for HeLa and in Table 5 for the three-proteome ratio (HYE) samples. For the HYE samples, the Astral analyzer AGC target was set to 100%.

Data processing parameters

The HeLa protein digest standard dilution data was analyzed in triplicate for each injection load and throughput. The triplicates of each load and SPD were analyzed together (but separate from the other loads, 3 files per analysis). The data was searched against the *Homo sapiens* SwissProt database (TaxID = 9606: 42,252 sequences) and contaminants with Spectronaut 19.5 software. The directDIA™ workflow was used with default settings. For three-proteome mix samples, six files (three replicates per mix) were processed together for each load and searched against UniProt FASTAs of human, yeast, and *E. coli* (all without isoforms) using Spectronaut 19.5 directDIA with default settings. Normalization by human FASTA was used. In all cases, the reported peptide numbers correspond to unique (stripped) peptides.

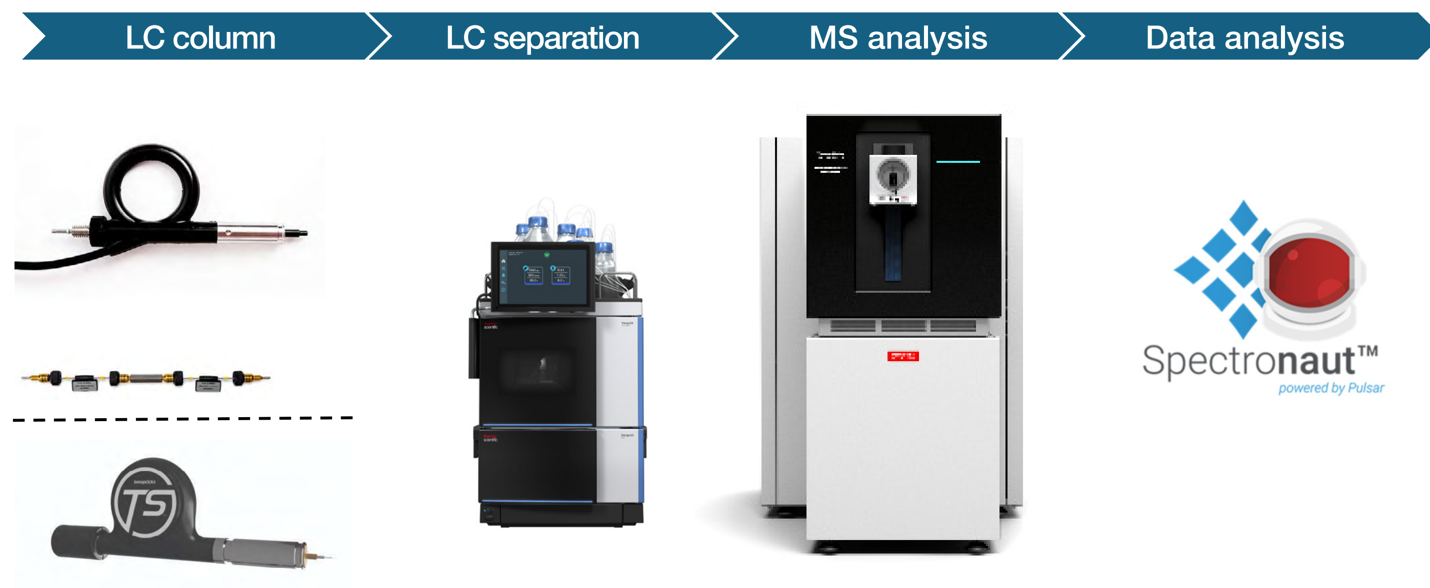


Figure 1. High-throughput nDIA workflow using the Orbitrap Astral Zoom MS.

Table 1. LC conditions.

HPLC method parameters	
Mobile phase A	0.1% formic acid (FA) in water
Mobile phase B	0.1% FA in 80% acetonitrile (ACN)
Flow rate	See Table 2
Column	Thermo Scientific EASY-Spray 2 µm C18, 150 µm × 150 mm for 60–300 SPD IonOpticks Aurora Ultimate 25 cm x 75 µm ID, 1.7 µm C18 for 30 SPD
Column temperature	55 °C
Autosampler temperature	7 °C
Injection wash solvents	Strong wash: 0.1% FA in 80% ACN Weak wash: 0.1% TFA in water

Table 2. LC gradients.

30 SPD - Direct Injection			
Time, min	Duration, min	Flow, µL/min	% B
0.0	0	0.3	4
0.4	0.4	0.3	6
0.9	0.5	0.3	8
31.8	30.9	0.3	28
37.8	6	0.3	45
Column wash			
39.0	1.2	0.3	95
42.0	3.0	0.3	95
Stop run			
Column equilibration			

60 SPD - Trap and Elute			
Time, min	Duration, min	Flow, µL/min	% B
0.0	0	0.8	3
11.0	11.0	0.8	21
16.5	5.5	0.8	31
19.5	3.0	0.8	41
Column wash			
20.0	0.5	3.0	99
22.0	2.0	3.0	99
Stop run			
Column equilibration			

Table 2. LC gradients (cont'd).

100 SPD - Trap and Elute			
Time, min	Duration, min	Flow, µL/min	% B
0.0	0.0	1.8	3
0.5	0.5	1.8	6
6.6	6.1	1.8	21
10.6	4.0	1.8	35
11.4	0.8	2.5	55
Column wash			
11.9	0.5	2.5	99
12.8	0.9	2.5	99
Stop run			
Column equilibration			

180 SPD - Trap and Elute			
Time, min	Duration, min	Flow, µL/min	% B
0.0	0.0	2.5	4
0.2	0.2	2.5	8
3.8	3.6	2.5	20
5.6	1.8	2.5	35
Column wash			
6.0	0.4	3.0	99
6.7	0.7	3.0	99
Stop run			
Column equilibration			

300 SPD - Trap and Elute			
Time, min	Duration, min	Flow, µL/min	% B
0.0	0.0	3.8	8
0.1	0.1	3.8	10
0.15	0.05	3.5	10.5
2.15	2.0	3.5	28
3.0	0.85	3.5	50
Column wash			
3.1	0.1	3.8	99
3.7	0.6	3.8	99
Stop run			
Column equilibration			

Table 3. MS method parameters. Parameters marked with an asterisk can be optimized depending on column, sample load, and desired focus on MS^1-/MS^2-based quantitation.

Global parameters (source and MS)	
Positive ion voltage (V)	1,900–2,200*
Ion transfer tube temperature (°C)	280
Expected peak width (s)	6
Default charge state	2
Orbitrap full scan properties	
Orbitrap resolution	240,000
RF lens (%)	40
Scan range (*m/z*)	380–980*
Normalized AGC target (%)	500
Max injection time (ms)	3 (180, 300 SPD) 5 (30, 60, 100 SPD)
Microscans	1
Data-independent acquisition properties	
Precursor mass range (*m/z*)	380–980*
Isolation width	2-5 Th*
Window placement optimization	On
Detector type	Astral
HCD collision energy (%)	25
Scan range (*m/z*)	150–2,000
RF lens (%)	40
Pre-accumulation	On
Max injection time (ms)	2–10*
AGC target (%)	500* 100 (HYE studies)
Loop control	Time
Time (s)	0.6

Table 4. DIA isolation windows and Astral maximum injection times used for 30 SPD HeLa loads.

HeLa load	Isolation window width	Astral max injection time
20–50 ng	5 Th	10 ms
100 ng	3 Th	5 ms
≥200 ng	2 Th	3.5 ms

Table 5. DIA isolation windows and Astral maximum injection times used for 30 SPD HYE loads.

HYE load	Isolation window width	Astral max injection time
20 ng	5 Th	10 ms
50 ng	4 Th	8 ms
100 ng	3 Th	5 ms
≥200 ng	2 Th	3 ms

Results and discussion

To investigate the performance of the Orbitrap Astral Zoom MS for high-throughput proteomics, we used HeLa sample loads ranging from 20 ng to 200 ng on fast methods ranging from 30 to 300 SPD (run-to-run time). For each throughput, datasets were recorded on the Orbitrap Astral Zoom MS and the Orbitrap Astral MS to facilitate a direct comparative analysis.

At full speed (up to a maximum Astral injection time of 2 ms), the Orbitrap Astral Zoom MS operates at up to 270 Hz. At a maximum Astral injection time of 3 ms, the scan rate is 210 Hz. This is significantly faster (35% increase) than the Astral MS (200 Hz at 2.5 ms injection time (IT)). Importantly, with the Astral detector, no spectral averaging of multiple scans is needed, as is often required for classic time-of-flight instruments, because each scan on its own generates a distinct spectrum with a good signal-to-noise ratio. Because of these fast acquisition speeds, both instruments are uniquely suited to perform nDIA across a large precursor selection range.

For maximal identifications, the precursor selection range of 380–980 *m/z*, comprising >90% of the tryptic HeLa precursor density, was covered with a fixed, non-overlapping 2 Th isolation scheme, resulting in 300 DIA windows.

Orbitrap Astral Zoom MS excels at high-throughput DIA

As expected, identification counts increased with sample load and longer gradients (Figure 2). Importantly, for 200 ng HeLa at 300 SPD, close to 8,000 PG (approx. 100,000 unique peptides) were identified with Spectronaut 19.5 software. Longer gradient times of 30 SPD provided a comprehensive and deep coverage of more than 11,000 PGs (>200,000 peptides). Results of the dilution series of 20 ng to 200 ng HeLa for both instruments at 30 SPD are depicted in Figure 2D.

Orbitrap Astral Zoom MS generates a consistent gain of identifications

Comparative analysis of the novel Orbitrap Astral Zoom MS versus the Orbitrap Astral MS revealed higher numbers of identifications due to technological improvements of the instrument design. Exact gains are dependent on throughput and load. However, the Orbitrap Astral Zoom MS consistently identified 10%–15% more peptides and precursors and approximately 5% more protein groups (Figure 2C, Figure 3). Notably, the highest gains were observed at very short gradients with 15%–25% on peptides and precursors and 11%–17% on protein groups (Figure 3).

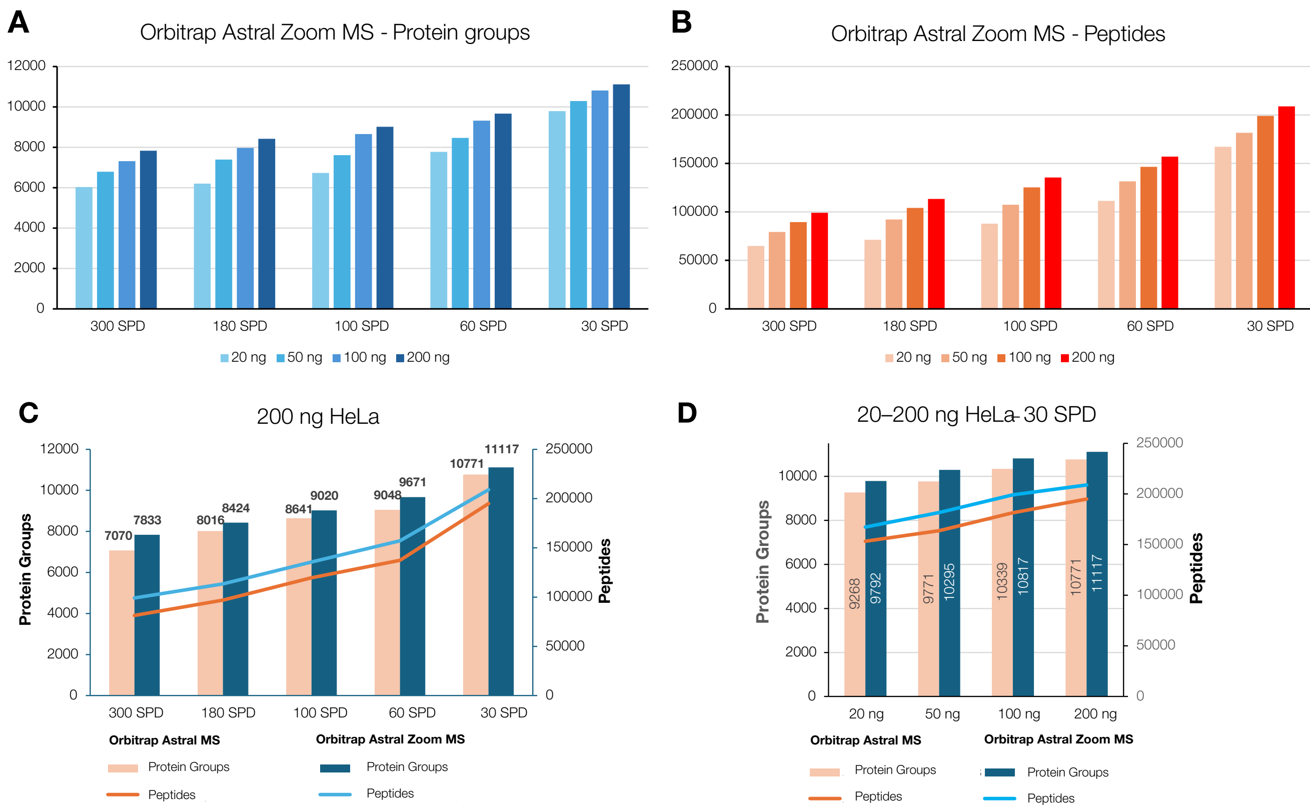
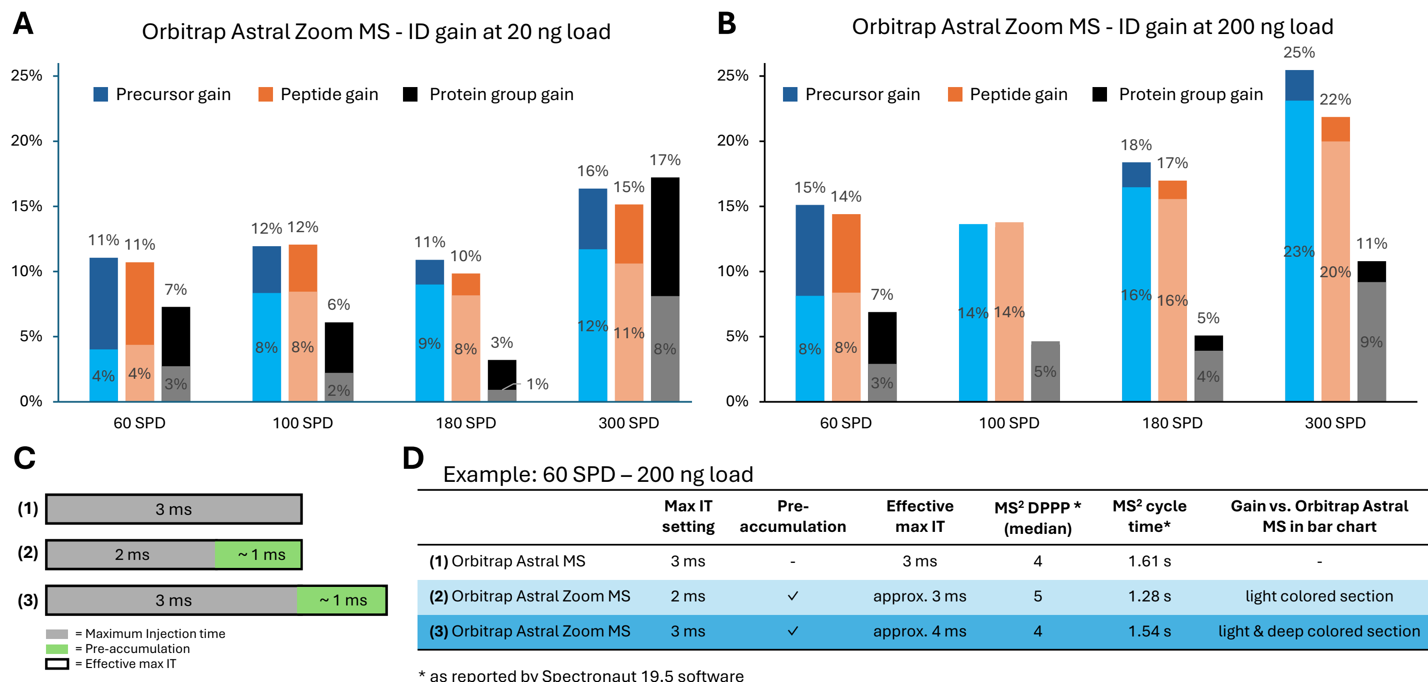


Figure 2. Identifications of HeLa dilution series at various throughputs. Protein groups (A) and peptides (B) as identified by Spectronaut 19.5 software with the Orbitrap Astral Zoom MS. (C) Identified protein groups and peptides of 200 ng Pierce HeLa across the various throughputs (SPD, run-to-run times) for Orbitrap Astral Zoom MS and Orbitrap Astral MS. (D) Dilution series of 20 ng to 200 ng HeLa measured at 30 SPD. Three raw files were analyzed together, but separately for each load, using Spectronaut 19.5 software. The Orbitrap Astral Zoom MS consistently generates more identifications.



	Max IT setting	Pre-accumulation	Effective max IT	MS² DPPP * (median)	MS² cycle time*	Gain vs. Orbitrap Astral MS in bar chart
(1) Orbitrap Astral MS	3 ms	-	3 ms	4	1.61 s	-
(2) Orbitrap Astral Zoom MS	2 ms	✓	approx. 3 ms	5	1.28 s	light colored section
(3) Orbitrap Astral Zoom MS	3 ms	✓	approx. 4 ms	4	1.54 s	light & deep colored section

* as reported by Spectronaut 19.5 software

Figure 3. Gain of IDs of Orbitrap Astral Zoom MS vs. Orbitrap Astral MS. Precursor, peptide, and protein group gain of Orbitrap Astral Zoom MS over Orbitrap Astral MS across various throughputs at 20 ng sample load (A) and 200 ng load (B). Identification gains using the same effective MS^2 maximum injection time (max. IT, Orbitrap Astral Zoom MS: 2 ms + approx. 1 ms pre-accumulation, Orbitrap Astral MS: 3 ms) are depicted in light colors. Additional gains using the pre-accumulation time on top of 3 ms max. IT on the Orbitrap Astral Zoom MS are depicted in deep colors. These two strategies and their effects on effective MS^2 maximum injection time, MS^2 data points per peak (DPPP), and cycle time are illustrated in panels (C) and (D).

The observed identification gains are in line with the instrument improvements of the Orbitrap Astral Zoom MS, such as the higher acquisition speed, bent-trap pre-accumulation, and enhanced spectral processing. The ability to pre-accumulate ions in the bent-trap for approximately 1 ms adds flexibility to method design. The feature is automatic-gain-control (AGC)-target controlled and is applied to MS^2 scans only if necessary. Especially when using short maximum MS^2 injection times, the extra in parallel pre-accumulated ions can be highly beneficial and can be used in two ways (Figure 3C-D).

For example, compared to a maximum MS^2 injection time of 3 ms on the Orbitrap Astral MS, the maximum MS^2 injection time on the Orbitrap Astral Zoom MS can be reduced by 1 ms to maintain the same effective maximum injection time (since pre-accumulation adds approximately 1 ms). This strategy results in a slightly faster MS^2 cycle time.

Alternatively, the pre-accumulation can be added on top of the 3 ms maximum injection time, resulting in an effective maximum injection time of about 4 ms. Consequently, the options are either more scans with approximately the same number of ions or more ions at the same scan rate. In terms of identifications, the second strategy can have advantages, especially on slightly lower loads, such as 20 ng. When more MS^2 data-points-per-peak (DPPP) are desired, the first option can be advantageous.

Orbitrap Astral Zoom MS generates better identifications and precise quantitation

The instrument evolution of the Orbitrap Astral Zoom MS is not only generating deeper coverage of the proteome but also identifications of higher quality. For example, the shared proteins identified both by the Orbitrap Astral Zoom MS and the Orbitrap Astral MS are based on additional unique peptides (for example, at 300 SPD 200 ng load: +2 unique peptides in median) and consequently feature higher sequence coverage (Figures 4A and 4B). Furthermore, the investigation of proteins uniquely identified by the Orbitrap Astral Zoom MS at 30 SPD and 200 ng load shows a tendency towards low abundance proteins, showcasing the increased sensitivity of the Orbitrap Astral Zoom MS (Figure 4C).

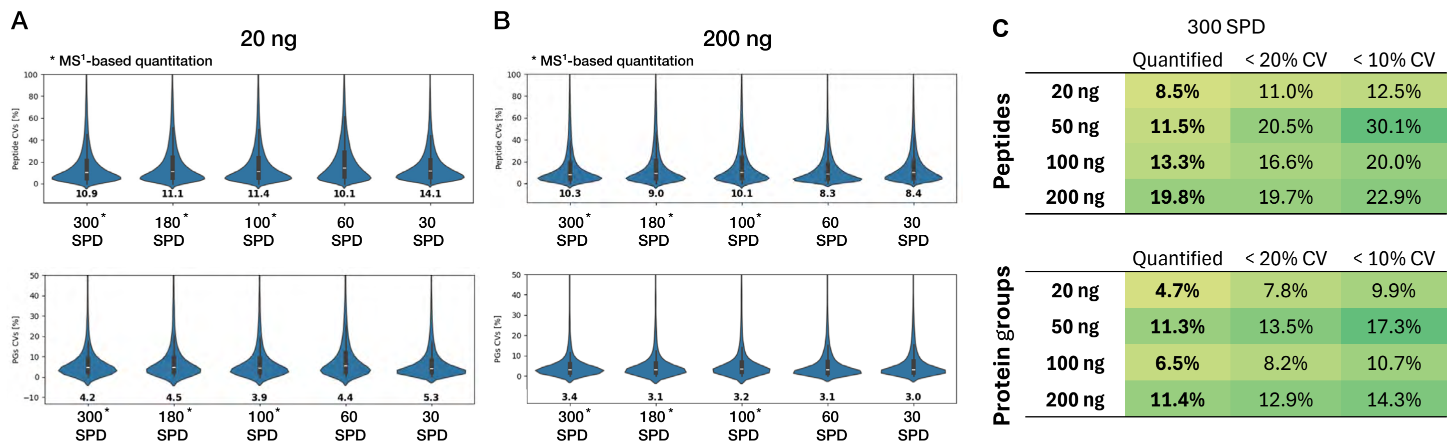
Importantly, the boost in identifications is achieved while maintaining the good quantitative precision of the Astral platform (Figure 5). In fact, the percentage gains on protein groups and peptides below 10% and 20% CV significantly exceed the gain in total quantified protein groups and peptides, as shown exemplarily for the 300 SPD method (Figure 5C).

Optimizing MS parameters of high-throughput methods for MS^2-based quantitation

For high-load HeLa samples (≥20 ng), the standard 2 Th (380–980 *m/z*) DIA isolation window scheme generates the maximum number

Figure 4. Orbitrap Astral Zoom MS generating high quality identifications and precise quantitation. Distributions of unique peptides per protein (A) and protein sequence coverage (B) of identifications generated both by the Orbitrap Astral Zoom MS and the Orbitrap Astral MS. (C) Distribution of proteins uniquely identified by the Orbitrap Astral Zoom MS at 30 SPD with a 200 ng load.

of identifications. Using this in combination with very short gradients of 300, 180, and 100 SPD, MS^1-based quantitation generates good quantitative precision, as the detector parallelization of the Orbitrap Astral Zoom MS generates MS^1 data points at 240k resolution in the Orbitrap analyzer every 0.6 s (Figures 5A and 5B, Figure 6A). However, the platform also offers the full flexibility to balance identification depth with precise MS^2-based quantitation by simply widening the DIA isolation windows slightly to 3–5 Th and potentially limiting the precursor selection range. This further reduces the number of DIA windows and consequently decreases the MS^2 cycle time while increasing the number of MS^2 DPPP. In this context, it should be noted that up to a maximum MS^2 injection time of 2 ms, the Orbitrap Astral Zoom MS is operating at its maximum speed using narrow isolation windows. An exemplary study showcasing this flexibility for 300 SPD is shown in Figure 6, resulting in outstanding precision for this 4.8 min run-to-run method. Based on this data, a good starting point for a MS^2-based quantitation method at 300 SPD would be 4–5 Th DIA windows over a precursor



Peptides	Quantified	< 20% CV	< 10% CV
20 ng	8.5%	11.0%	12.5%
50 ng	11.5%	20.5%	30.1%
100 ng	13.3%	16.6%	20.0%
200 ng	19.8%	19.7%	22.9%

Protein groups	Quantified	< 20% CV	< 10% CV
20 ng	4.7%	7.8%	9.9%
50 ng	11.3%	13.5%	17.3%
100 ng	6.5%	8.2%	10.7%
200 ng	11.4%	12.9%	14.3%

Figure 5. Quantitative precision of the Orbitrap Astral Zoom MS. Peptide (upper pane) and protein group (lower pane) CVs of 20 ng (A) and 200 ng (B) HeLa measured at the different throughputs. (C) Gain of total quantified, quantified <20%, and <10% CV peptides and protein groups at 300 SPD with the Orbitrap Astral Zoom MS.

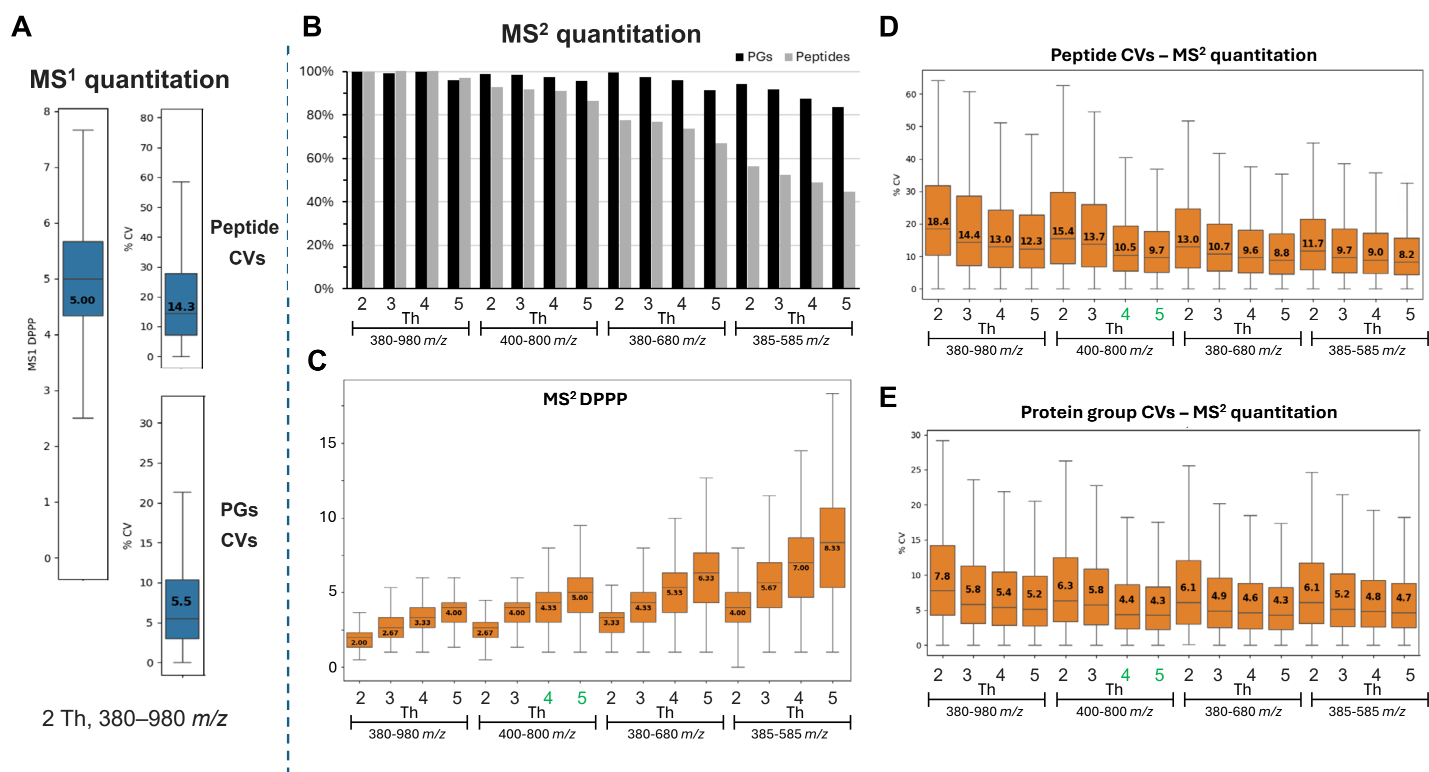


Figure 6. DIA window schemes offer full flexibility to focus on MS^2 quantitation, exemplary study at 300 SPD. (A) MS^1 DPPP, peptide and protein groups CVs of the maximum ID method (2 Th, 380–980 *m/z*) using MS^1 quantitation. (B) Percentage drop in identifications versus the maximum ID method when using incrementally wider isolation windows and limiting the precursor range. (C) MS^2 DPPP. (D) Peptide CVs. (E) Protein group CVs of the various DIA window schemes using MS^2 quantitation. Data extracted from Spectronaut 19.5 software reports.

selection range of 400–800 *m/z*. However, as demonstrated, simple modifications to the method can be made if an even faster MS^2 cycle time and, consequently, even more MS^2 DPPP for quantitation are desired. Importantly, this general strategy can be applied and optimized for all throughputs; the longer the gradient (increased peak width), the smaller the adjustments need to be. A future technical note will explore this strategy in detail.

Evaluation of quantitative precision and accuracy with mixtures of three proteomes

In MS-based proteomics, both quantitative precision and accuracy are fundamental for reliable and meaningful data generation, which is the foundation of scientific research, drug discovery and development, and clinical diagnostics. Both are critical metrics to evaluate the performance of a mass spectrometer. Quantitative precision ensures the consistency and reproducibility of the measurements, indicating the degree to which the instrument can reliably produce the same results for a given sample, which is crucial for comparing data across different runs or experiments. This metric is determined by the coefficient of variation (CV) between replicate measurements. Quantitative accuracy, on the other hand, defines the instrument's ability to reliably measure the actual concentration of an analyte. High accuracy is vital for quantitative analysis (e.g., ratio determination, regulatory analyses), which is the backbone of any biological question.

While excellent quantitative precision was demonstrated for the Orbitrap Astral Zoom MS with a HeLa digest, a mixture of three proteomes (human, yeast, *E. coli*) poses an even greater challenge due to its complexity, and it allows to demonstrate the quantitation accuracy of the mass spectrometer. To reveal the benefits of using the Orbitrap Astral Zoom MS for analyzing complex proteomic samples, we analyzed a dilution series of three-proteome mixtures with two distinct compositions (Figure 7A) across sample loads from 1 µg to 20 ng. The results were compared with results obtained using the Orbitrap Astral MS. Both precision and accuracy of quantitation were evaluated in this study.

Similar to the results observed for the HeLa high loads, the Orbitrap Astral Zoom MS generated 10%–17% more peptides and 3%–9% more protein group identifications for the three-proteome mixture sample, with >16,000 protein groups identified from the three species at the highest load (Figure 7B).

Table 6 illustrates the precision of quantitation for the three-proteome mixtures at both peptide and protein levels obtained using the Orbitrap Astral MS and Orbitrap Astral Zoom MS. At the protein level, both systems show an average median CV of 4.4% across all loads for mixtures A and B. At the peptide level, the CV increases slightly from 12.4% with the Orbitrap Astral MS to 13.4% with the Orbitrap Astral Zoom MS, likely due to the identification and quantitation of more low abundant peptides with the Orbitrap Astral Zoom MS.

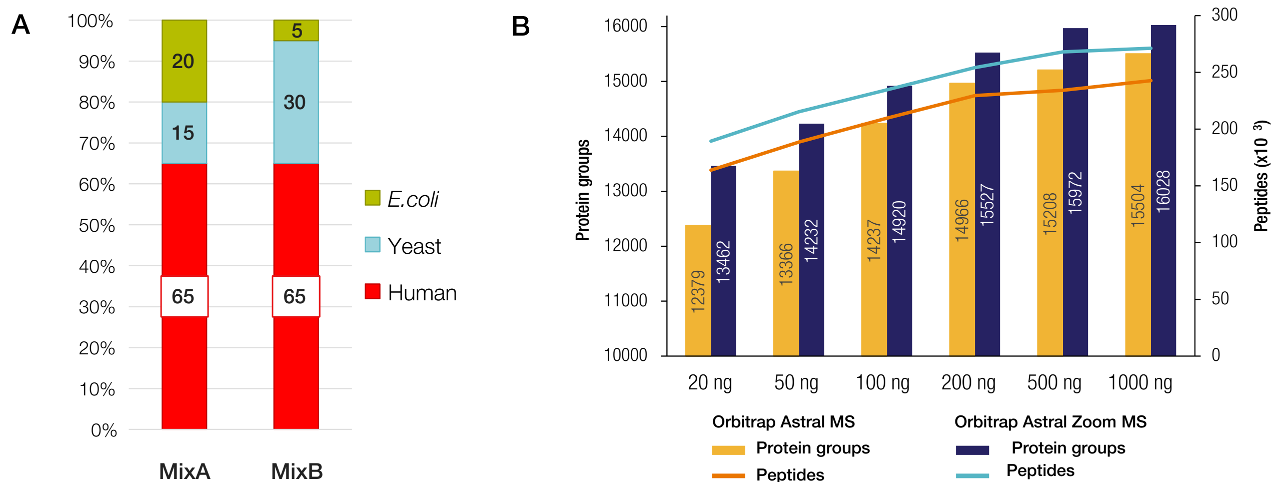


Figure 7. Label-free quantitation with the Orbitrap Astral Zoom MS vs. the Orbitrap Astral MS using three-proteome mixtures. (A) Composition of the three-proteome mixtures. (B) Peptide and protein group identifications at various loads using the 30 SPD throughput. Three replicates of each composition (3×A, 3×B) were processed together with Spectronaut 19.5 software using a library-free directDIA workflow.

The gain in protein IDs from the Orbitrap Astral Zoom MS compared to the Orbitrap Astral MS is more significant for proteins with CV <20% and CV <10% than for the total gain in protein IDs (Table 7 and Figure 8), suggesting that the Orbitrap Astral Zoom MS delivers higher quality data. The highest gain was observed for protein groups identified with CV <10% (8%–13% gain range, averaging 10%), followed by protein groups identified with CV <20% (7%–12% gain range, averaging 8.7%), with the smallest gain for all identified protein groups (4%–9% gain range, averaging 5.8%). This finding is consistent with the data shown earlier for HeLa digest dilution series.

The median log2 ratios for all three species between the two sample compositions are in good agreement with the theoretical values (human: 0, yeast: -1, *E. coli*: 2), with relative errors within 7.5%. This highlights the excellent quantitative accuracy of the Orbitrap Astral Zoom MS (see Figure 9 and Table 8 for more details).

The Orbitrap Astral Zoom MS offers an unprecedented acquisition speed in MS^2, allowing for more DPPP compared to the Orbitrap Astral MS.

To further investigate the influence of DPPP on both quantitation precision and accuracy, a label-free quantitation (LFQ) study

Table 6. Median CV (%) at protein groups and peptide levels obtained with the Orbitrap Astral MS and the Orbitrap Astral Zoom MS (triplicate runs, three-proteome mix, processed with Spectronaut 19.5 software).

Orbitrap Astral MS

	%CV, Protein groups mixA	%CV, Protein groups mixB	Median %CV, Average A,B	Median %CV, Peptides mixA	Median %CV, Peptides mixB	Median %CV, Average A,B
20 ng	5.7	5.4	5.55	14.6	14.2	14.4
50 ng	4.9	4.9	4.9	13.2	13.4	13.3
100 ng	4.4	4.3	4.35	12.2	12.1	12.2
200 ng	4.3	4.2	4.25	12.7	12.9	12.8
500 ng	3.7	3.9	3.8	11.3	11	11.2
1000 ng	3.5	3.6	3.55	10.6	10.6	10.6
Average All			**4.4**			**12.4**

Orbitrap Astral Zoom MS

	%CV, Protein groups mixA	%CV, Protein groups mixB	Median %CV, Average A,B	Median% CV, Peptides mixA	Median %CV, Peptides mixB	Median %CV, Average A,B
20 ng	5.4	5.2	5.3	14.6	14.7	14.7
50 ng	4.6	4.5	4.55	13.5	13.4	13.5
100 ng	4.4	4.3	4.35	13.4	13.2	13.3
200 ng	4.3	4.2	4.25	13.6	13.2	13.4
500 ng	3.9	3.9	3.9	12.3	12.7	12.5
1000 ng	4	4	4	13.1	12.9	13
Average All			**4.4**			**13.4**

Table 7. Protein groups (total, <20% and <10% CV) quantified with the Orbitrap Astral MS and the Orbitrap Astral Zoom MS (triplicate runs, processed by Spectronaut 19.5 software).

Orbitrap Astral MS

Load per run	All protein groups identified (mix A)	Protein groups with CV<20%	Protein groups with CV<10%	% of CV<20%	% of CV<10%
20 ng	12348	10787	8579	87.4	69.5
50 ng	13349	11882	9761	89.0	73.1
100 ng	14217	12799	10686	90.0	75.2
200 ng	14931	13382	11189	89.6	74.9
500 ng	15193	13897	11959	91.5	78.7
1000 ng	15476	14178	12331	91.6	79.7

Orbitrap Astral Zoom MS

Load per run	All protein groups identified (mix A)	Protein groups with CV<20%	Protein groups with CV<10%	% of CV<20%	% of CV<10%
20 ng	13419	12003	9693	89.4	72.2
50 ng	14192	13056	10895	92.0	76.8
100 ng	14898	13738	11624	92.2	76.0
200 ng	15509	14289	12125	92.1	78.2
500 ng	15950	14960	12961	93.8	81.3
1000 ng	16013	15055	13050	94.0	81.5

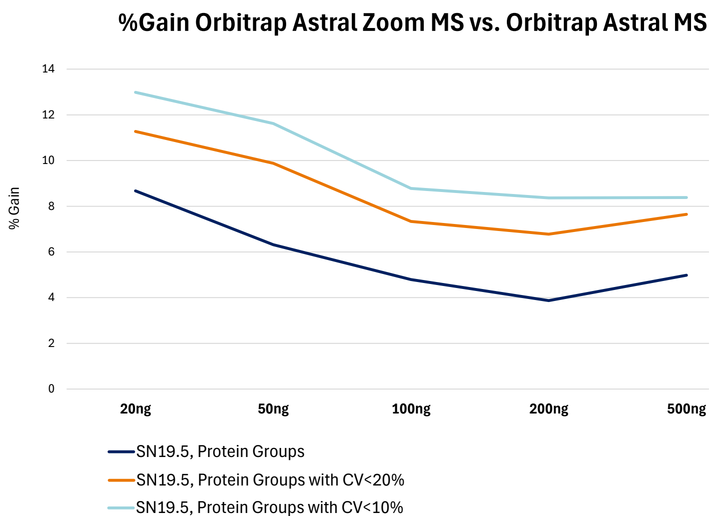
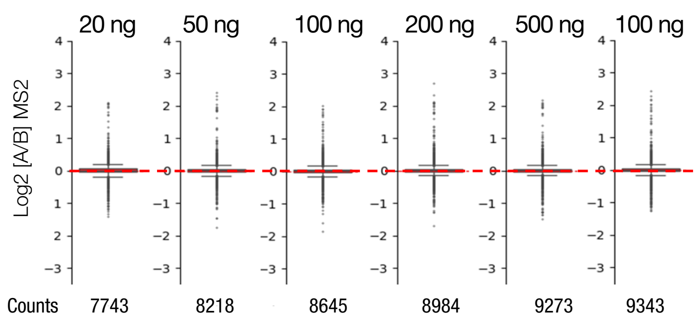
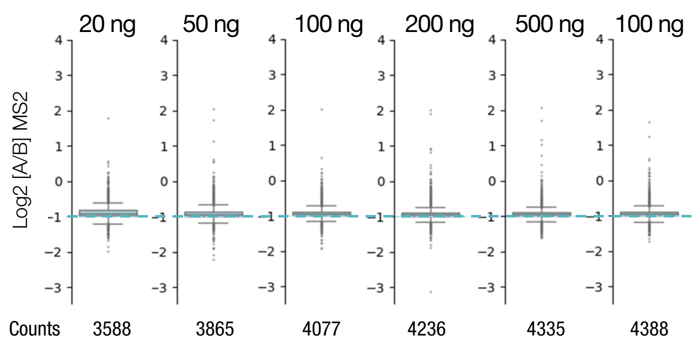


Figure 8. %Gain for the Orbitrap Astral Zoom MS vs. the Orbitrap Astral MS for proteins quantified with CV <20% and <10% (triplicate runs, processed by Spectronaut 19.5 software).

was performed using a medium-length gradient (30 SPD), two mixtures of human, yeast, and *E. coli* proteomes, and three different DIA methods. These methods varied parameters such as isolation window and maximum injection time, leading to different numbers of DPPP. All other LC and MS method parameters remained the same.

The study was performed using the Orbitrap Astral Zoom MS with a 100 ng sample load, and three DIA methods were tested: 2 Th/3 ms, 3 Th/5 ms, and 5 Th/10 ms, resulting in median DPPP of 3, 4, and 5, respectively. The results are presented in Figure 10 and Tables 9-10. For a 100 ng load, the method using 3 Th isolation window and 5 ms was optimal for maximizing identifications and achieving the best coefficients of variation at both peptide and protein group levels. The 5 Th/10 ms method, which produced the highest DPPP, showed some improvement in median CV at the peptide level, but the difference from the 3 Th/5 ms method was minor. At the protein level, the 3 Th/5 ms method provided the highest precision among the three methods.





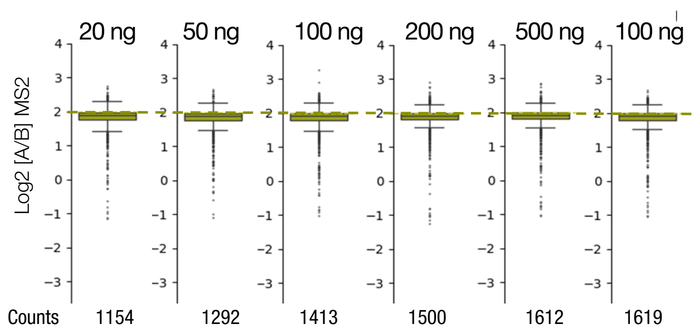


Figure 9. Evaluation of quantitative accuracy of the Orbitrap Astral Zoom MS. Boxplots showing log2 ratios for each of the three proteomes, measured on the Orbitrap Astral Zoom MS at various loads. Counts correspond to the numbers of protein groups quantified, filtered for missing values, and used for median log2 ratio calculations.

Table 8. Relative errors of quantitation calculated based on the experimental data for the two mixtures of three proteomes analyzed with the Orbitrap Astral Zoom MS.

Load	Human	Human	Human	Yeast	Yeast	Yeast	E. coli	E. coli	E. coli	Median CV, %
	Median Log2-ratio	Median ratio	Relative error, %	Median Log2-ratio	Median ratio	Relative error, %	Median Log2-ratio	Median ratio	Relative error, %	
20 ng	0.000	1.000	0.03	-0.920	0.528	5.7	1.895	3.718	7.0	5.2-5.4
50 ng	0.004	1.003	0.28	-0.941	0.521	4.2	1.890	3.706	7.4	4.5-4.6
100 ng	0.003	1.002	0.20	-0.950	0.517	3.5	1.915	3.770	5.8	4.3-4.4
200 ng	0.002	1.002	0.15	-0.955	0.516	3.2	1.918	3.778	5.5	4.2-4.3
500 ng	0.005	1.003	0.32	-0.957	0.515	3.0	1.916	3.775	5.6	3.9-3.9
1000 ng	-0.002	0.999	0.12	-0.955	0.516	3.2	1.904	3.742	6.4	4.0-4.0

The quantitation accuracy results (Figure 10, Table 10) showed that all three methods provided equally accurate proteome ratio estimations, with no significant differences in the obtained ratios.

The DPPP study for 100 ng HYE analyzed at 30 SPD using the Orbitrap Astral Zoom MS led to the following conclusions:

- **Quantitation precision:** All three methods produced MS^2 quantities of mixtures A and B with a median CV of less than 5% at the protein level, and the differences between the results were negligible. At the peptide level, the median CV differences between the 3 Th/5 ms and 5 Th/10 ms methods were also insignificant. However, the 5 Th/10 ms method resulted in 500 fewer protein IDs and 20,000 fewer peptide IDs compared to the 3 Th/5 ms method.
- **Quantitation accuracy:** The calculated median log2 values for the ratios of MS^2 quantities of mixtures A and B showed no significant differences between the methods with 3, 4, and 5 DPPP. All methods yielded results within 6.2% of the theoretical ratios, with less than 1% difference between proteome ratios generated by the three methods.
- **Comparing methods** with DIA windows from 2 to 5 Da at a throughput of 30 SPD revealed negligible differences in quantitation precision and accuracy. All methods achieved very high quantitative performance.

Evaluation of instrument-to-instrument variability

To further highlight the excellent instrument-to-instrument repeatability, we acquired data over the course of 4 months on 25 different Orbitrap Astral Zoom MS instruments (Figure 11). The spread of the protein group identifications is within 5% across the instruments.

Table 9. The results of identification and precision of quantitation at peptide and protein levels for three methods yielding different numbers of DPPP.

Astral settings (DIA window, IT)	2 Th, 3 ms	3 Th, 5 ms	5 Th, 10 ms
Precursors	295,157	297,405	273,648
Unique peptides	236,132	234,610	215,806
Protein groups	14,807	14,940	14,424
Median data points per peak, MS^2*	3	4	5
Median %CV (MixA-MixB), peptides*	14.9–14.8	13.4–13.2	13.5–13.2
Median %CV (MixA-MixB), proteins*	4.7–4.8	4.3–4.4	4.5–4.6

*As reported in Spectronaut 19.5 software, post analysis.

Table 10. Relative errors of proteome ratios obtained using the Orbitrap Astral Zoom MS and three DIA methods with different numbers of DPPP.

Load	Human	Human	Human	Yeast	Yeast	Yeast	*E. coli*	*E. coli*	*E. coli*
	Median Log2-ratio	Median ratio	Relative error, %	Median Log2-ratio	Median ratio	Relative error, %	Median Log2-ratio	Median ratio	Relative error, %
2 Th, 3 ms	0.006	1.004	0.44	-0.956	0.515	3.1	1.920	3.784	5.4
3 Th, 5 ms	0.002	1.002	0.15	-0.951	0.517	3.5	1.911	3.761	6.0
5 Th, 10 ms	0.003	1.002	0.19	-0.951	0.517	3.5	1.908	3.753	6.2

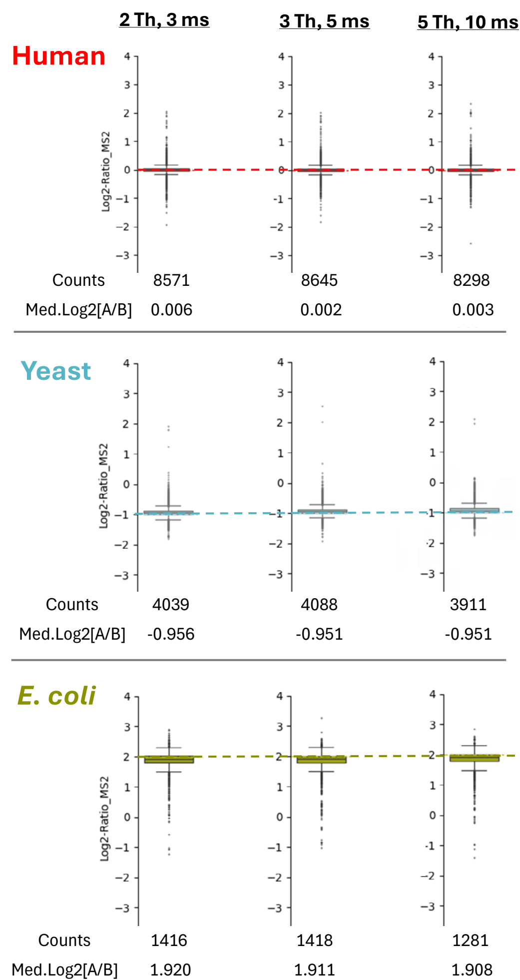


Figure 10. Boxplots showing log2 ratios for each of the three proteomes using the Orbitrap Astral Zoom MS and three DIA methods with different numbers of DPPP. Counts correspond to the numbers of protein groups quantified, filtered for missing values, and used for median Log2 ratio calculations.

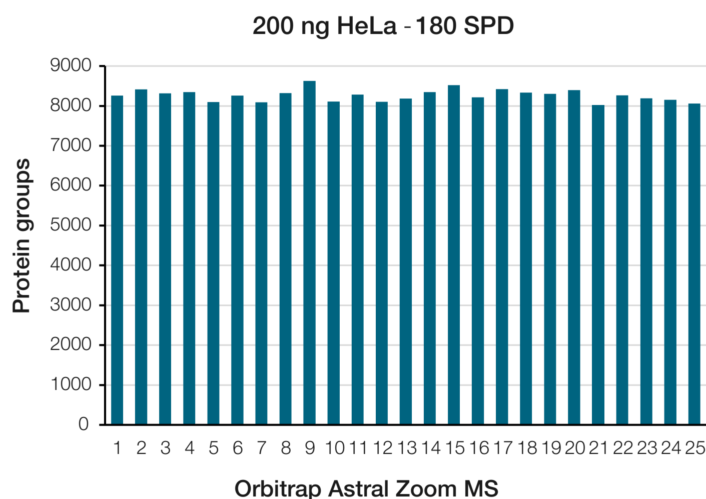


Figure 11. Instrument-to-instrument repeatability. Data acquired using the same setup type by various users over a period of 4 months on 25 different Orbitrap Astral Zoom mass spectrometers, measuring various sample preparations. Three replicate injections per instrument were analyzed together.

Conclusion

The novel Orbitrap Astral Zoom mass spectrometer, with its broad and comprehensive range of applications, is an excellent instrument for high-throughput proteomics, enabling researchers to further streamline their proteomic pipelines. The key points elaborated in this technical note are:

- nDIA improves specificity and enables comprehensive, precise, and accurate investigations of complex proteomes.
- High-throughput nDIA on the Orbitrap Astral Zoom MS generates deep coverage of complex proteomes. At 30 SPD, 11,117 protein groups and >200,000 peptides were identified from 200 ng HeLa.
- The instrument enables precise quantitation with full flexibility to focus on MS^2-based quantitation even for short gradients.
- With the Orbitrap Astral Zoom MS, high quantitative accuracy was achieved, with measured median values of the ratios lying within 7.5% of the expected ratios.
- The Orbitrap Astral Zoom MS enables significant enhancement of throughput. At 300 SPD, results are comparable to data generated at 180 SPD on the Orbitrap Astral MS.
- The novel Orbitrap Astral Zoom MS further pushes the boundaries in terms of quantity and quality of identifications.
- A comparative study involving 25 different Orbitrap Astral Zoom MS instruments demonstrated outstanding instrument-to-instrument repeatability, with protein group identifications varying by less than 5% across the instruments.

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