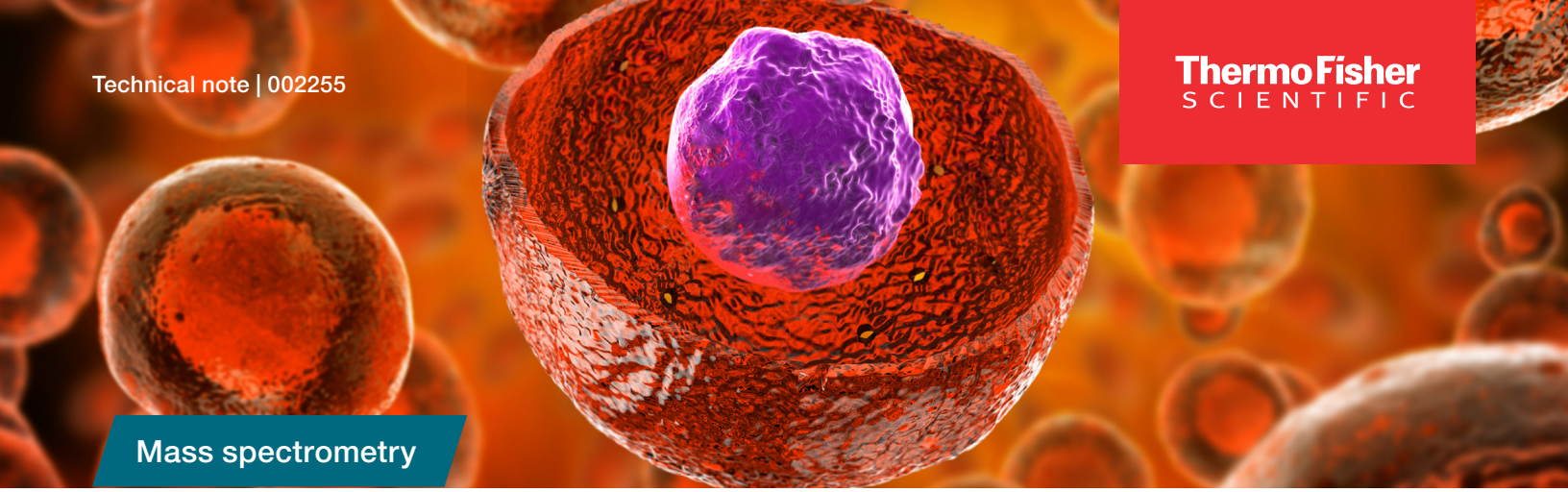

Technical note | 002255

Mass spectrometry

Deeper proteome coverage and faster throughput for low input samples on the Thermo Scientific Orbitrap Astral mass spectrometer

Authors

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Keywords

Orbitrap Astral mass spectrometer, Vanquish Neo UHPLC, µPAC low-load column, FAIMS Pro interface, data-independent acquisition, proteomics

Goal

To assess proteome coverage and sample throughput performance for low input samples using a library-free and library-based data-independent acquisition (DIA) method on the new Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer

Introduction

In recent years there has been a shift to analyzing smaller sample quantities, driven by the interest in profiling the proteome of individual cells. This increased focus on LC-MS analysis of limited sample amounts (e.g., single-cells) requires the highest possible sensitivity. At the same time, throughput and chromatographic performance must be preserved to generate sufficiently high-quality data from large data sets to draw meaningful conclusions. This trend toward analyzing smaller sample quantities reflects the need to understand the heterogenous nature of biology through the dissection of complex systems into their individual parts or specifically individual cells. Traditional proteomics approaches read out the average of a sample, while single-cell proteomics provides insights into the exact nature of each of the components in such samples.

The Orbitrap Astral mass spectrometer delivers a new standard for low-input samples with both deeper proteome coverage and faster throughput due to its high sensitivity. The Orbitrap Astral mass spectrometer enables this deeper coverage and high throughput through the synchronized acquisition of high resolution and high dynamic range full scan spectra with the Thermo Scientific™ Orbitrap™ mass analyzer and high speed and sensitivity MS2 spectra with the Thermo Scientific™ Astral™ mass analyzer. The Thermo Scientific™ Vanquish™ Neo UHPLC system delivers maximum performance in terms of precise low flow rates and gradient formation as well as low injection volumes, an important benefit for single cell proteomics and limited sample amounts. The Thermo Scientific™ µPAC™ Neo Low Load columns with novel micropillar-array deliver highly reproducible chromatographic separation, increased sensitivity with maximum resolution. The Thermo Scientific™ FAIMS Pro™ interface enhances sensitivity by removing background signals.

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In this technical note, we present a low-input proteomics sample workflow (Figure 1) using the Thermo Scientific™ Pierce™ HeLa protein digest standard in nanogram and picogram loads on the Orbitrap Astral mass spectrometer to demonstrate new standards in sensitivity and depth of proteome coverage using a library-free and library-based DIA approach.

Experimental

Recommended consumables

- Fisher Scientific™ LC-MS grade water with 0.1% formic acid (FA),(P/N LS118-500)
- Fisher Scientific™ LC-MS grade 80% acetonitrile (ACN) with 0.1% formic acid (P/N LS122500)
- Fisher Scientific™ LC-MS grade formic acid (P/N A117-50)
- Fisher Scientific™ Optima™ LC-MS grade water (P/N 10505904)
- Fisher Scientific™ Optima™ LC-MS acetonitrile (P/N A955-1)
- Fisher Scientific™ Optima™ LC-MS isopropanol (P/N A461-212)
- Thermo Scientific™ Pierce™ Trifluoroacetic acid (TFA), sequencing grade (P/N 28904)

Samples

- Thermo Scientific Pierce HeLa protein digest standard (P/N 88328)

LC columns

- Thermo Scientific™ µPAC™ Neo Low Load HPLC 50 cm column (P/N COL-NANO110NEOB)
- Fused silica emitters, 10 µm i.d. (EvoSep Biosystems, P/N EV1111)

HPLC system

- Thermo Scientific™ Vanquish™ Neo UHPLC system including:
  - Thermo Scientific™ Vanquish Neo Pump/Autosampler (P/N VN-S10-A-01)
  - Column Compartment (P/N VN-C10-A-01)

Mass spectrometer

- Thermo Scientific Orbitrap Astral mass spectrometer
- Thermo Scientific FAIMS Pro interface
- Thermo Scientific™ EasySpray™ ion source

Data analysis software

- Spectronaut™ 18 software (Biognosys AG)

HeLa digest standard

All proteomics experiments were performed using the Pierce HeLa protein digest standard. 100 µL of 10% acetonitrile (ACN) in 0.1% TFA was added to the vial containing 20 µg of protein digest. The vial was then sonicated at room temperature for 5 minutes, followed by adding 100 µL of 0.1% TFA to make a final concentration of 100 ng/µL. To the autosampler vial, 95 µL of 0.1% TFA and 5 µL of 100 ng/µL HeLa digest was added to make the final concentration to 5 ng/µL. This solution was pipetted up and down several times to mix and centrifuged at 8,000 × g for 2 minutes.

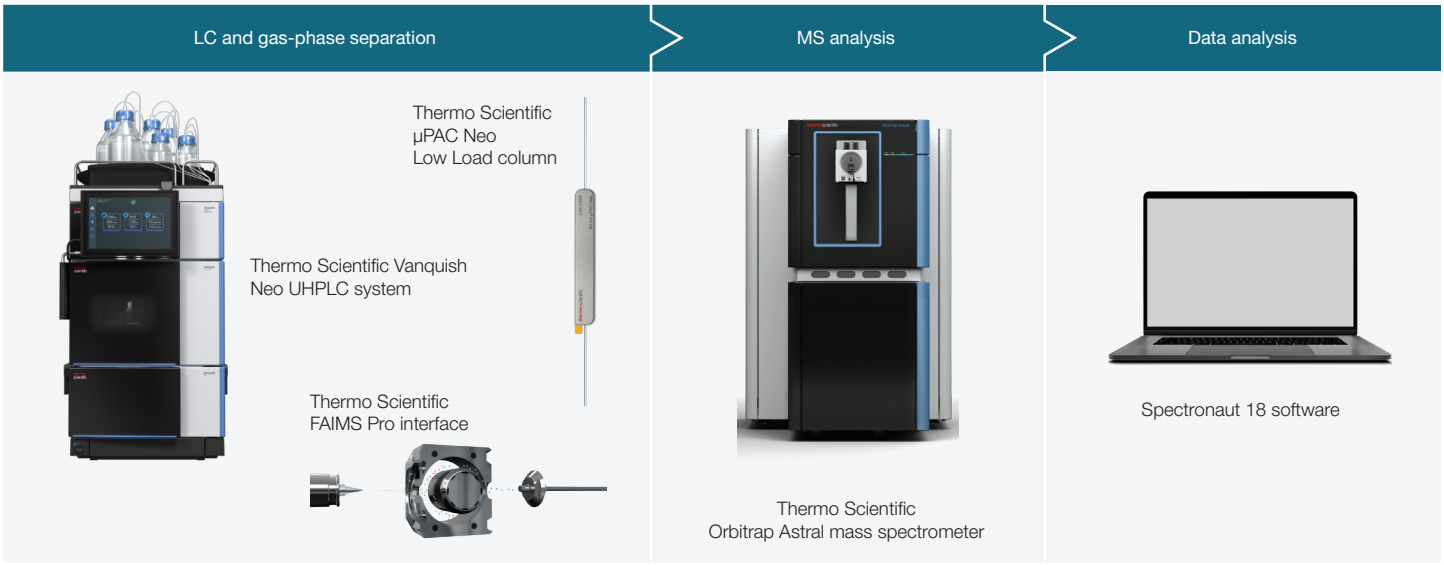

Figure 1. Low-input proteomics workflow with the Orbitrap Astral mass spectrometer for new standards in sensitivity and depth of coverage for single-cell like samples

LC conditions

To evaluate the performance of our workflow, an 80 sample per day (SPD) method was employed consisting of an 8-minute gradient and 10-minute washing, equilibration, and injection steps for a total injection-to-injection cycle time of 18 minutes.

HPLC conditions are described in Table 1, with the 80 SPD method gradient details in Table 2.

Table 1. HPLC 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	0.2 µL/min
Column	µPAC Neo Low Load column, nonporous C_{18} silicon chip packing material, bed length 50 cm, pillar length 30 µm, interpillar distance 1.25 µm
Colum temperature	40°C
Autosampler temperature	7°C
Injection wash solvents	Strong wash: 0.1% FA in 80% ACN Weak wash: 0.1% FA in water
Needle wash	Enabled after-draw

Table 2. 80 SPD method

	Time, min	Duration, min	%B	Flow rate, µL/min
	Run			
	0.0	0.0	4.0	0.75
Active gradient	0.1	0.2	4.0	0.75
	1.9	1.8	12.0	0.75
	2.0	0.1	—	0.2
	5.0	3.0	22.5	0.2
	7.5	2.5	40.0	0.18
	Column wash			
	7.9	0.4	99.0	0.18
	13.9	6.0	99.0	0.18
	Stop run			
	Column equilibration			

MS parameters

The Orbitrap Astral mass spectrometer was operated with the parameters shown in Table 3.

Table 3. MS parameters

Source parameters	
Spray voltage	1.9 kV
Capillary temperature	270°C
FAIMS CV	–50
Orbitrap MS full scan parameters	
Resolution	240 k
Normalized AGC target	500%
Maximum IT	100 ms
RF lens	40%
Scan range	400–800 *m/z*
Astral DIA MS^2 scan parameters	
Precursor mass range	400–800 *m/z*
DIA Window type	Auto
Window placement optimization	On
DIA window mode	*m/z* Range
HCD normalized collision energy	25%
Scan range	150–2,000 *m/z*
RF lens	40%
Normalized AGC target	800%
Loop control time	0.6 Sec

The DIA isolation window and maximum injection time used in the experiments were varied based on the sample load as shown in Table 4.

Table 4. DIA isolation windows and maximum injection times

Sample amount	≤250 pg	250 pg–1 ng	1 ng –5 ng	5 ng–10 ng
DIA isolation window (*m/z*)	20	10	8	5
Maximum injection time (ms)	40	20	14	10

Data processing parameters

The HeLa protein digest standard dilution data containing triplicates of 50 pg, 100 pg, 250 pg, 500 pg, 1 ng, 2.5 ng, 5 ng, and 10 ng DIA runs were processed using individual triplicate runs with Spectronaut 18 software using the directDIA+™ workflow with the Spectronaut™ Pulsar search engine against the Human UniProt protein database (20,607 FASTA entries). False-discovery rate (FDR) of 1% was applied at the precursor, peptide, and protein level. Library-based searches were processed with Spectronaut 18 in Bremen, Germany. Spectral libraries were generated using DIA data with Pulsar search engine in Spectronaut 18 against the Human UniProt Protein database.

Results and discussion

Optimization of MS parameters

Several MS parameters were evaluated for optimum protein and peptide identifications using the 250 pg HeLa protein digest standard and analyzed using library-free search.

Effect of DIA isolation windows

The performance of the various DIA isolation windows was evaluated with the 80 SPD method. A 20 *m/z* isolation window provided the optimal window width, identifying >4,300 protein groups. A linear decrease in protein groups and peptide identifications were observed with increasing isolation windows. The decrease in identification can be attributed to the increase in complexity of the DIA MS2 spectra as more precursors are fragmented with wider isolation windows (Figure 2).

Effect of Astral mass analyzer DIA scan injection times

The best results for the analysis of concentrated sample (200 ng HeLa digest) using DIA on the Orbitrap Astral have been achieved with injections times of 3-5 ms and isolation width of 2-4 Th. (data not shown). However, in the case of limited sample amounts, such as 250 pg HeLa protein digest, the Orbitrap Astral mass analyzer DIA scan injection times of 40–50 ms and isolation width of 20 Th were found to be ideal. Since the signal of peptides is lower with small sample amounts, larger injection times significantly boost the protein/peptide identifications. Figure 3 shows the increase in protein and peptide identifications from 5 ms to 60 ms injection times using a 250 pg amount of HeLa protein digest. Furthermore, we optimized DIA isolation windows and injection times for different low sample loads, as shown in Table 4.

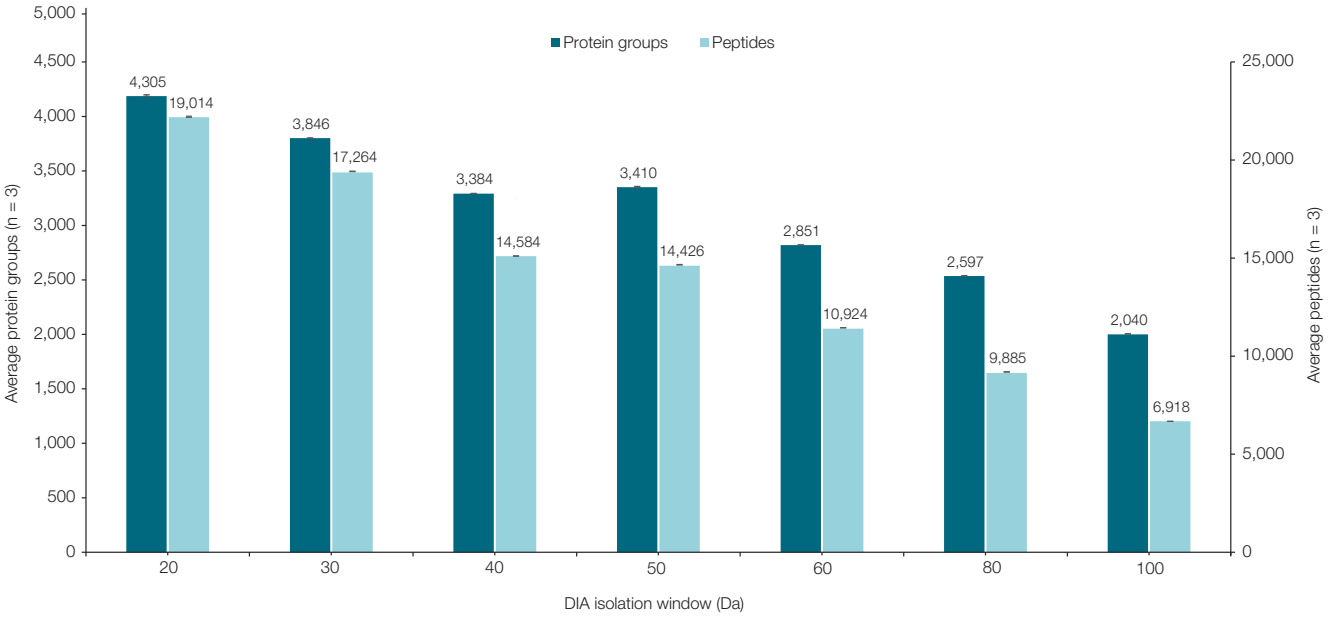

Figure 2. Effect of DIA isolation windows on protein identification numbers from 250 pg HeLa protein digest standard

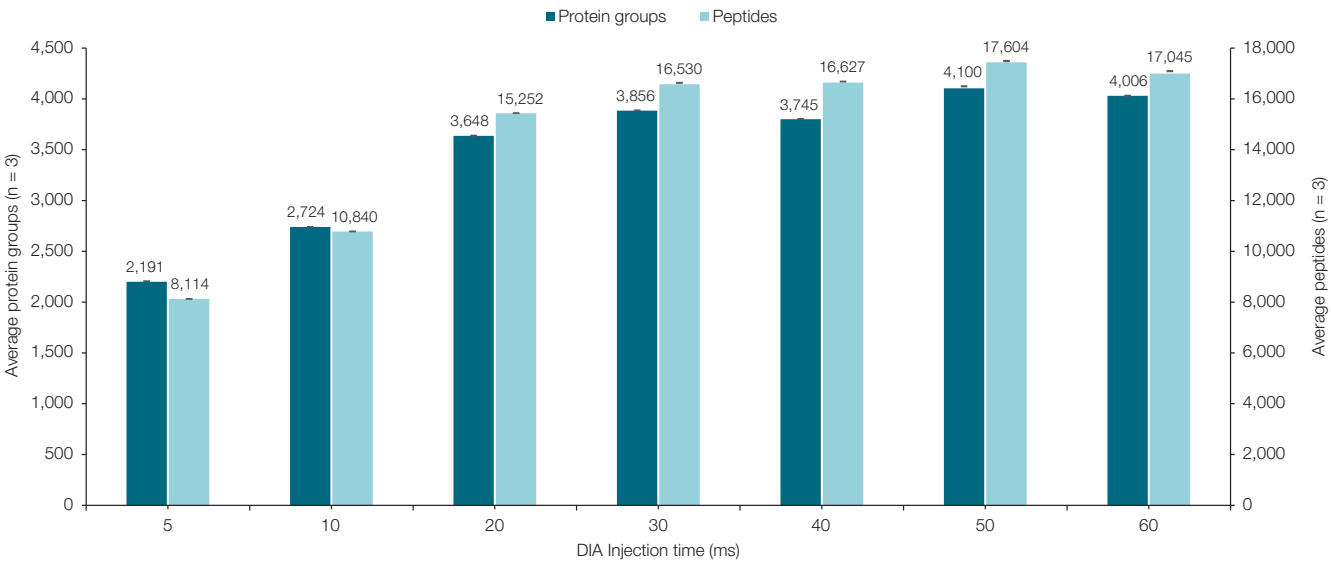

Figure 3. Effect of Orbitrap Astral DIA (DIA MS2) injection times (ms) on protein identification numbers from 250 pg HeLa protein digest standard

High-throughput and high protein coverage from low sample amounts

We created a dilution series from 50 pg - 10 ng HeLa digest and analyzed them using the optimized methods (see Table 4). We identified >2,600 protein groups and >11,600 peptides from 50 pg HeLa digest and >4,000 protein groups and 20,000 peptides from 250 pg HeLa digest in a library-free workflow. Increasing the sample amount to 10 ng, over 6,700 protein groups and 46,000 peptides were identified (see figure 4). Recent studies show increased protein and peptide identifications for DIA data using library-free approaches, incorporating machine learning-based in-silico library predictions from a protein database. For low sample amounts, it was observed that a project-specific library generated from limited sample amounts yielded better results compared to library-free approach. This could be due to the reduced spectral complexity in low sample amounts that negatively impacts the library-free data analysis approach.

To evaluate the impact of different library sizes, we generated a DIA library from triplicate runs of 500 pg, 1 ng, 2.5 ng, 5 ng, and 10 ng and a combined library using 250 pg to 10 ng HeLa digests. The 250 pg DIA runs were then searched against the respective libraries as shown in Figure 5. DIA library generated from ≥2.5 ng DIA runs yielded >5,500 proteins from 250 pg HeLa digest triplicate runs, The protein and peptide identifications from a library of 5 ng and above plateaued without any further gains in identifications (Figure 5).

Using a spectral library-based approach with a DIA library comprising 3 x 10 ng DIA runs resulted in identification of 4,196 and 5,911 protein groups from 50 pg and 250 pg HeLa protein digests, respectively, and >6,700 proteins were identified from 2.5 ng sample (Figure 6).

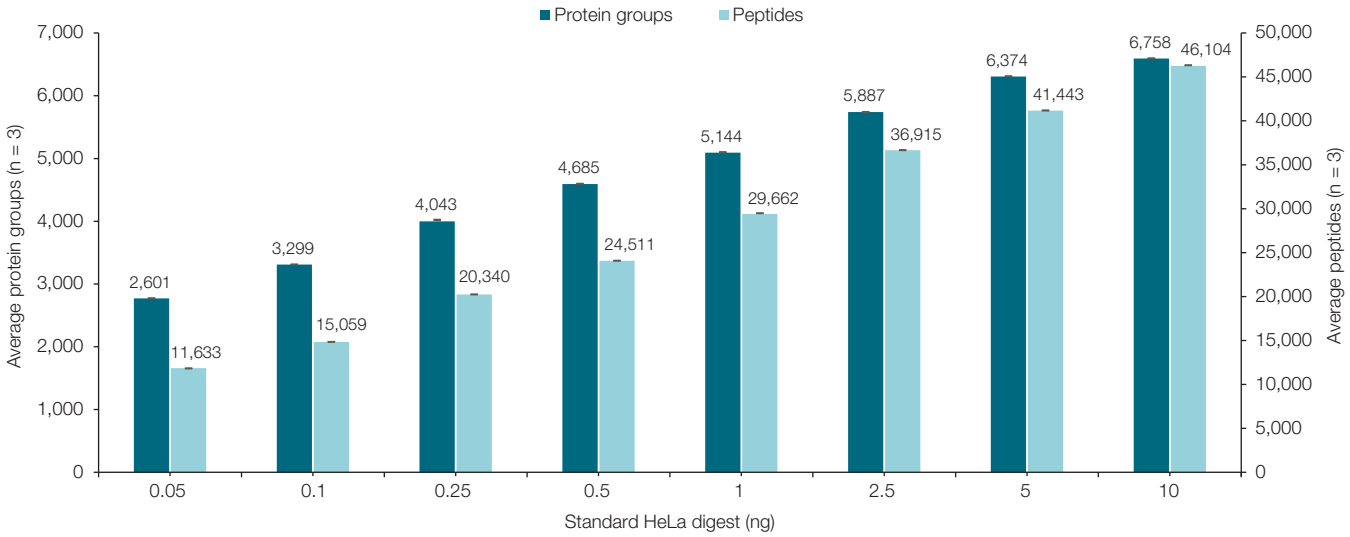

Figure 4. Protein groups and peptides identified using an 80 SPD method with library-free search

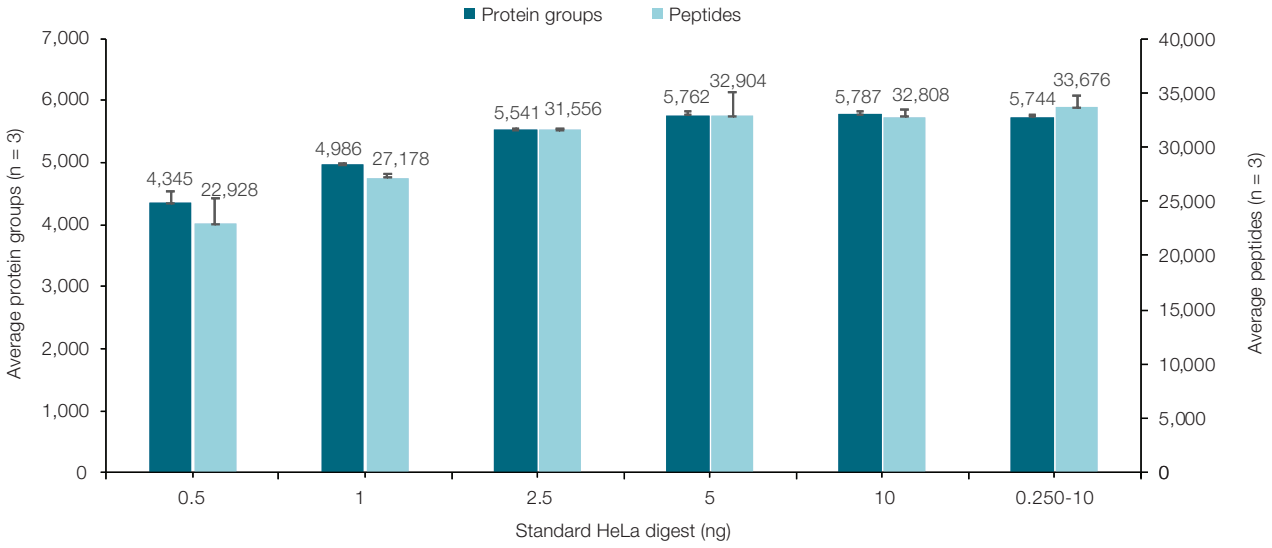

Figure 5. Effect of DIA library size—250 pg HeLa protein digest standard (n = 3) runs were searched against DIA libraries generated with varying amounts of Hela digests

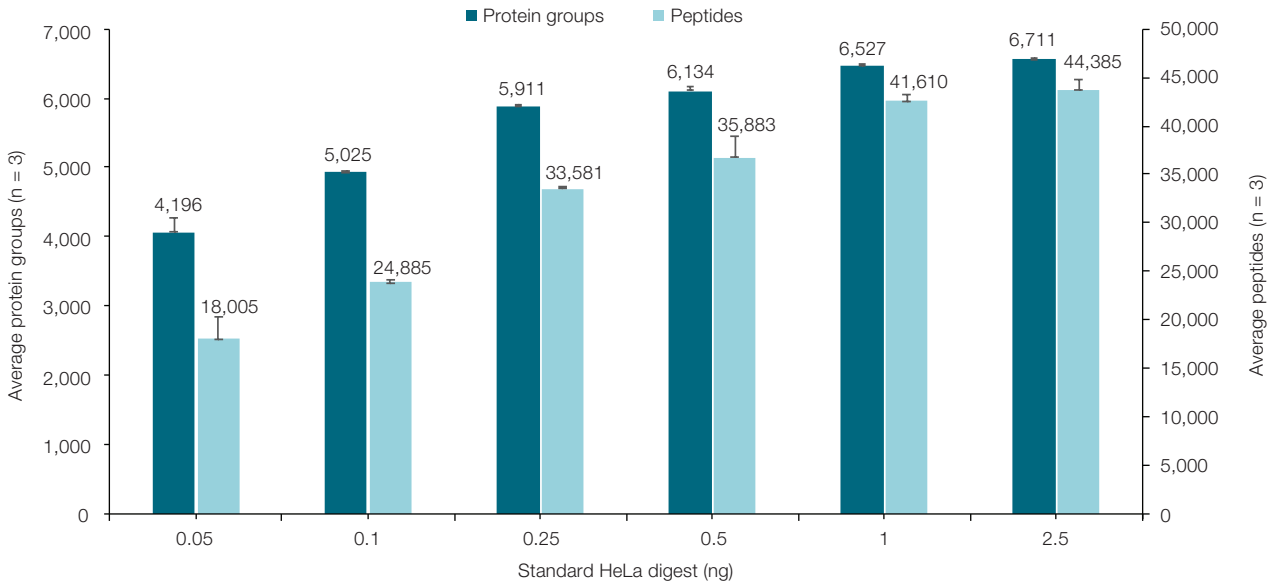

Figure 6. Average number of protein groups and peptides identified using an 80 SPD method with DIA library-based approach

High accuracy and precision of quantitation

In addition to a list of protein identifications, quantitative data is necessary to study biomarkers and get insights into biological pathways. The quantitative data must be highly precise and accurate to reflect subtle changes in biological systems. We observed that ~90% of identified proteins in 250 pg HeLa digest were <20% CV and 71% proteins were <10% CV using library-free approach (Figure 7). Using library-based approach, 75% of identified proteins were <20% CV and 56% proteins were <10% CV for 250 pg HeLa digest (Figure 8).

Median CVs of protein abundances across triplicate runs from 50 pg to 10 ng were <10% and <12% with library-free (Figure 9) and library-based search (Figure 10), respectively. For 250 pg HeLa digest (equivalent to protein amount found in single HeLa cell), the median CV was 5.7% and 8% with library-free and library-based search, respectively. This clearly shows highly reproducible measurements with the new Orbitrap Astral mass spectrometer across low to high sample inputs (Figure 9 and Figure 10).

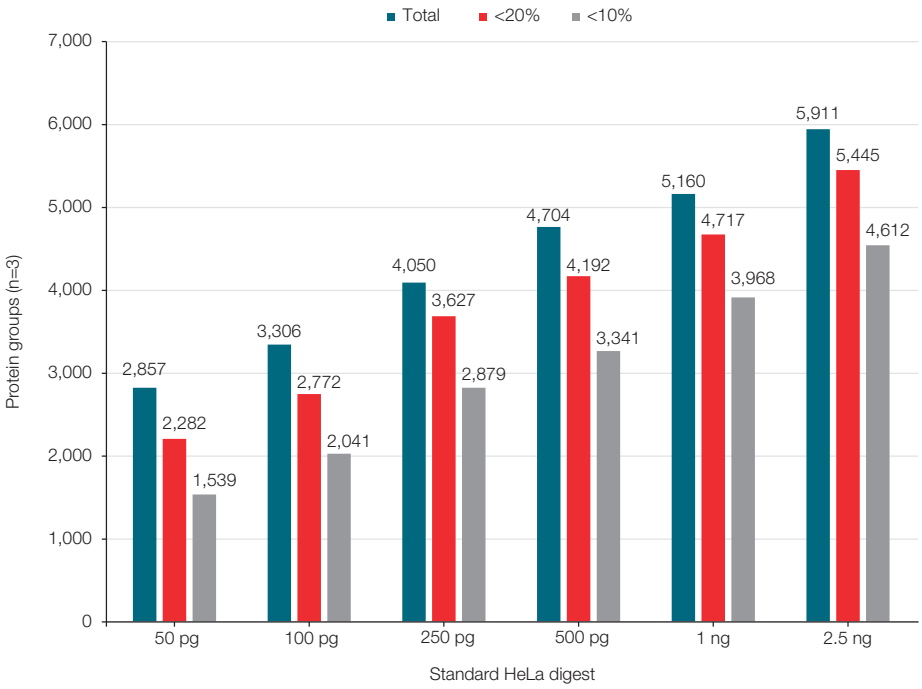

Figure 7. Total protein groups identified in triplicate runs (teal) with protein groups CVs <20% (red) and <10% (gray) analyzed using library-free search

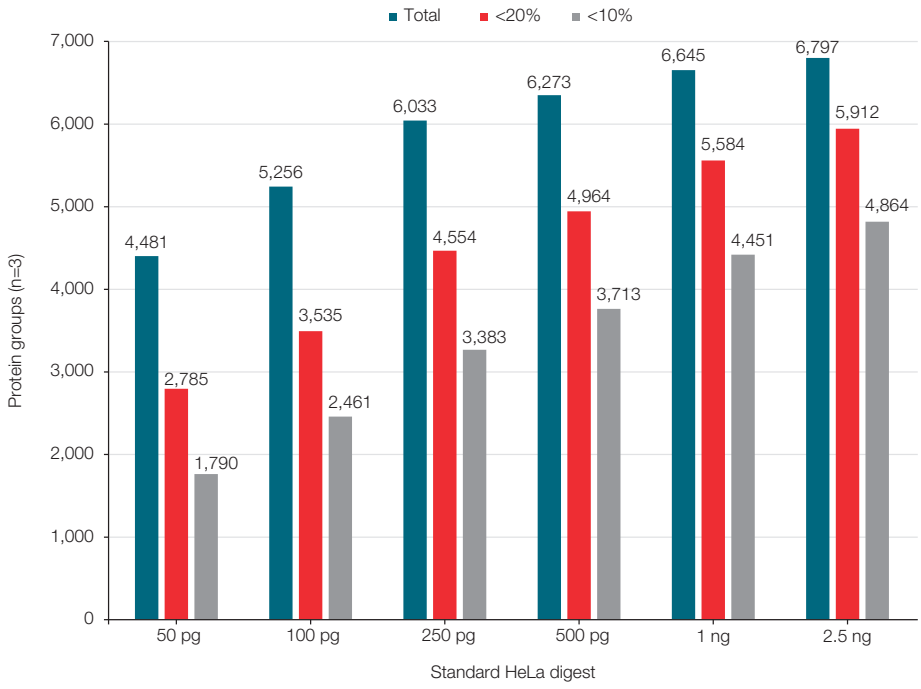

Figure 8. Total protein groups identified in triplicate runs (teal) with protein groups CVs <20% (red) and <10% (gray) analyzed using library-based search

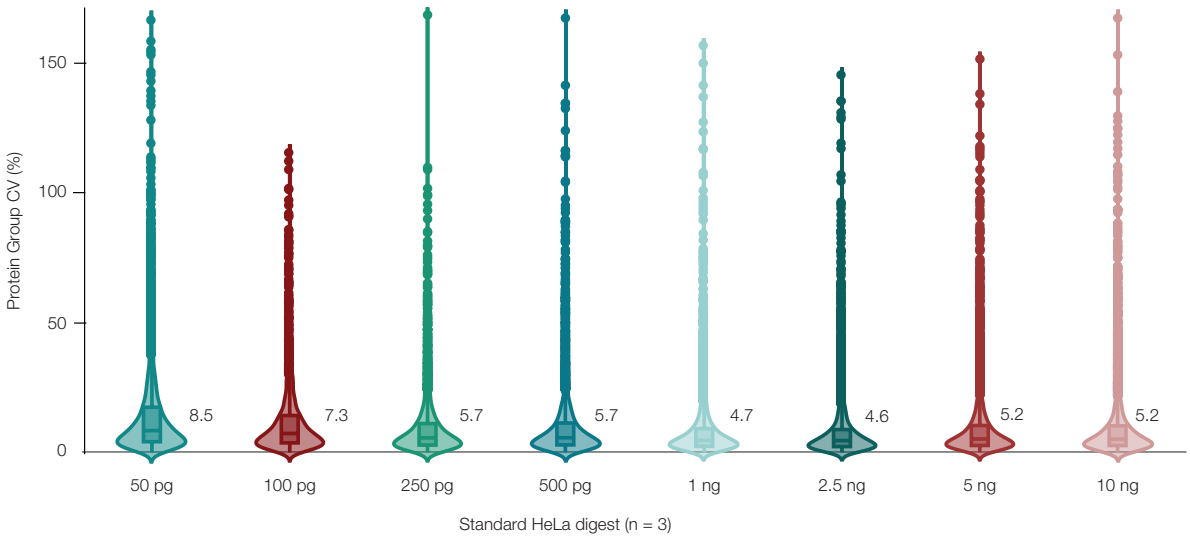

Figure 9. Violin plots indicating %CV of protein groups identified in each triplicate HeLa digest dilution data point with median %CVs labeled in the figure using library-free search

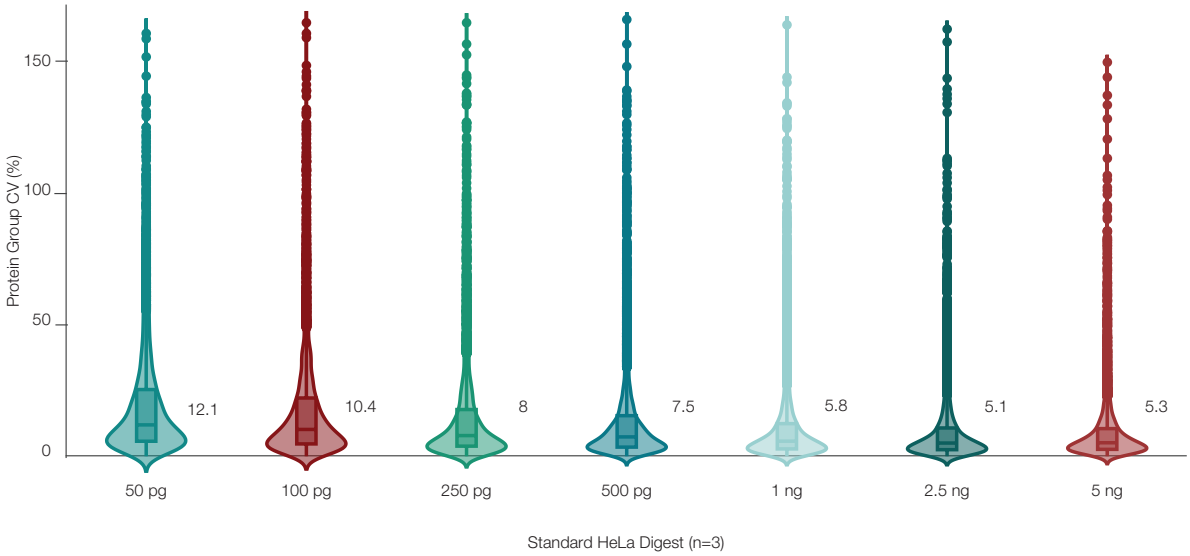

Figure 10. Violin plots indicating %CV of protein groups identified in each triplicate HeLa digest dilution data point with median %CVs labeled in the figure using library-based search

To our knowledge, the identifications reported in this technical note are the highest ever reported on any commercial mass spectrometer for throughput of 80 SPD using a library-free and library-based DIA approach. High quantitative precision and accuracy were achieved with CVs <8.5% in the protein groups analyzed in a library-free search at each dilution point. For 250 pg HeLa digest, CVs were <10% for protein groups quantified using either library-free or spectral library search.

Summary

- The new Orbitrap Astral mass spectrometer combines an Orbitrap mass analyzer and a novel Astral mass analyzer to enable robust, reproducible, rapid (80 SPD), and sensitive deep proteome coverage from low sample amounts using DIA.
- High quality single-cell amount level data showed impressive quantitative accuracy and precision.
- The FAIMS Pro interface improves the signal-to-noise (S/N) ratio with decreased background ions enhancing spectral quality for improved protein and peptide identifications for low sample amounts.
- Reproducible separations were achieved with a next generation 50 cm µPAC Neo Low Load column with consistent performance for 50 pg to 10 ng sample loads.

Learn more at **thermofisher.com/OrbitrapAstral**