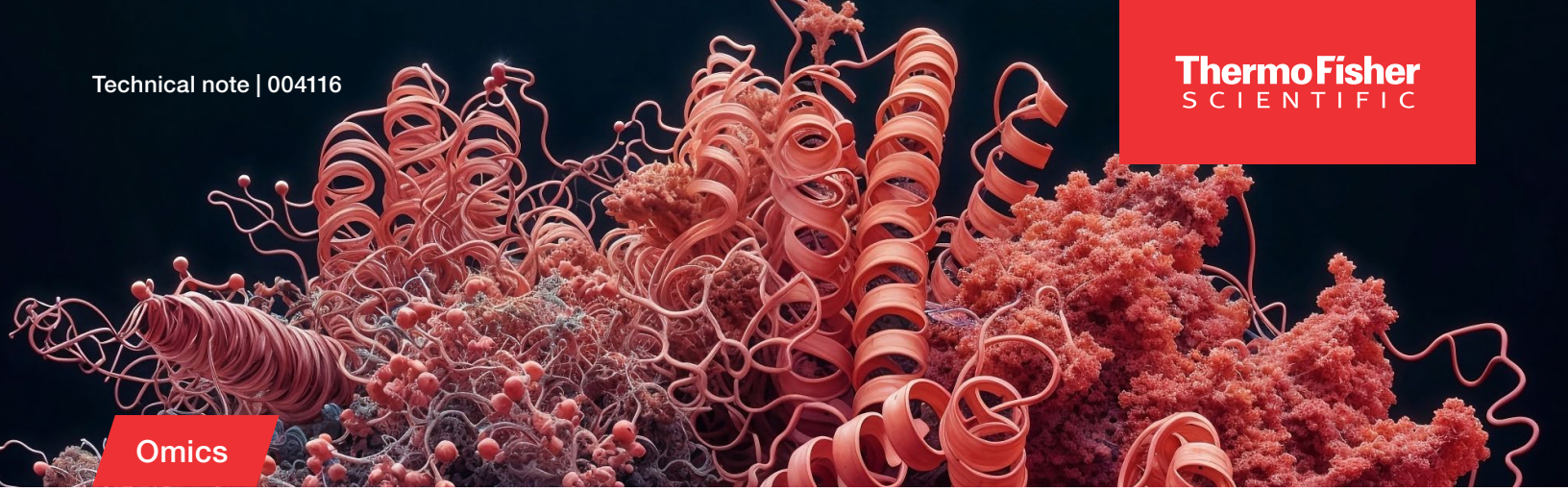

Technical note | 004116

Omics

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

Authors

Patience Sanderson, Katherine Walker, Josh Silveira, Katie Peterson, Amirmansoor Hakimi, Tonya Pekar Hart, Ellen Casavant; Thermo Fisher Scientific

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 essential 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

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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 eliminates the fundamental compromises between comprehensive and targeted analysis. By seamlessly 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.

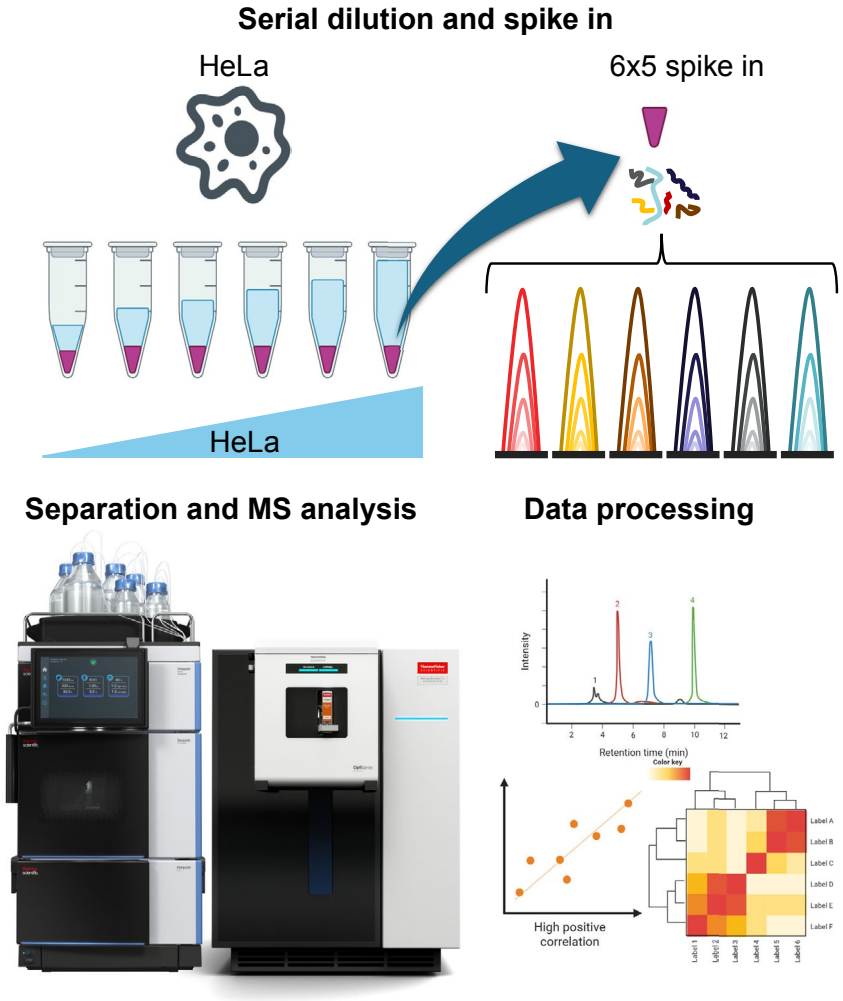


Figure 1. Experimental outline for hybrid DIA on the Orbitrap Excedion Pro MS.

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

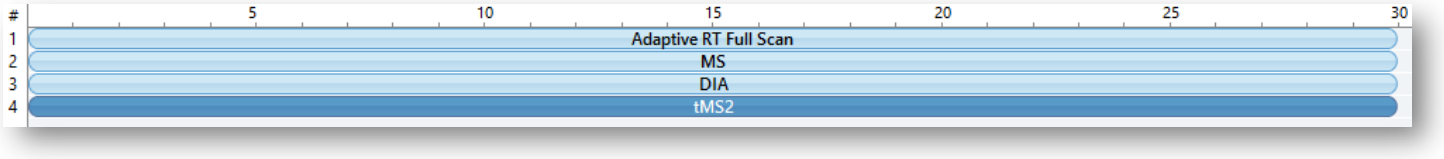


Figure 2. Hybrid DIA experiment setup.

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, ensuring that all 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%.

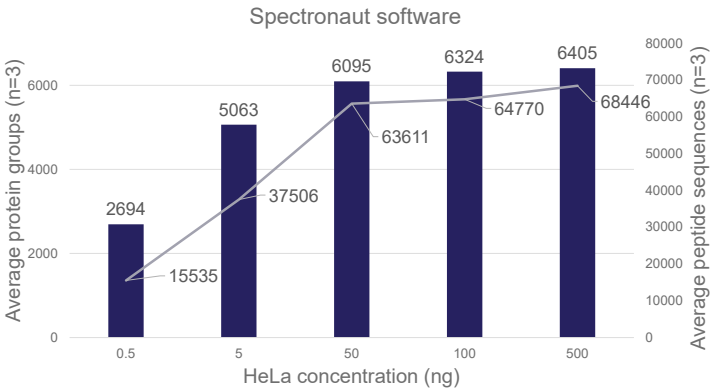


Figure 3. Comparison of protein and peptide identifications at various HeLa concentrations (0.5–500 ng) for hybrid DIA.

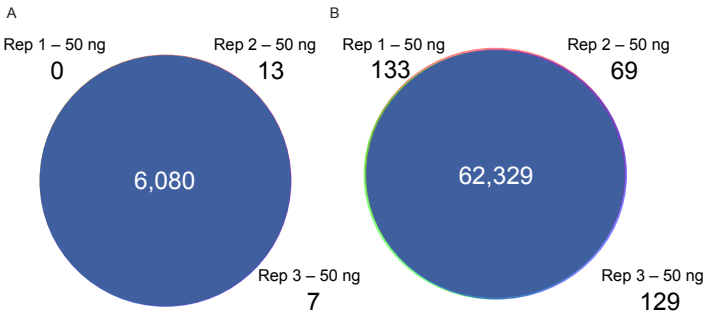


Figure 4. Reproducibility of identified proteins (A) and peptides (B) from three replicates of 50 ng HeLa. Venn diagram demonstrates overlap (middle of circle) and proteins unique to each sample (found under the Replicate label).

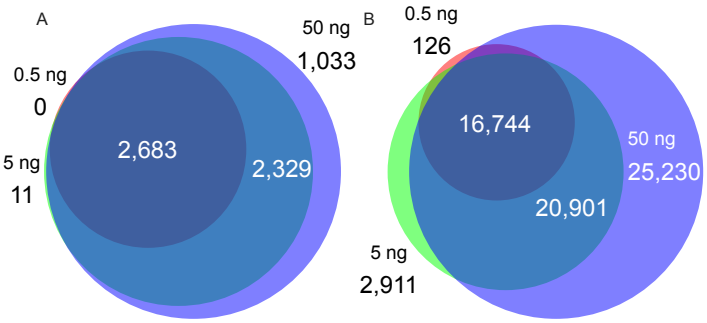


Figure 5. Comparison of identified proteins (A) and peptides (B) of 0.5, 5, and 50 ng HeLa.

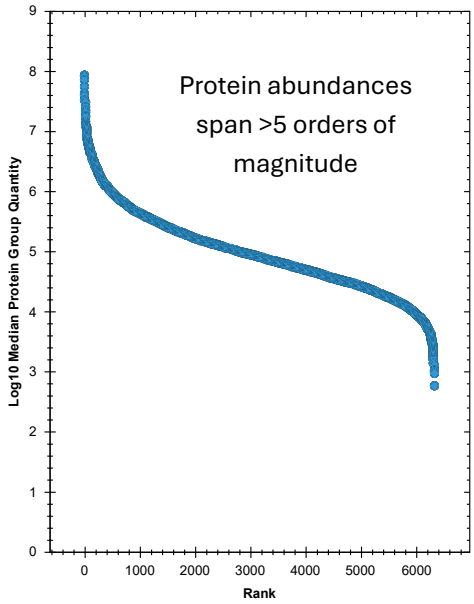


Figure 6. Dynamic range of global proteome coverage using hybrid DIA, represented by 50 ng HeLa. Protein abundances span over five orders of magnitude.

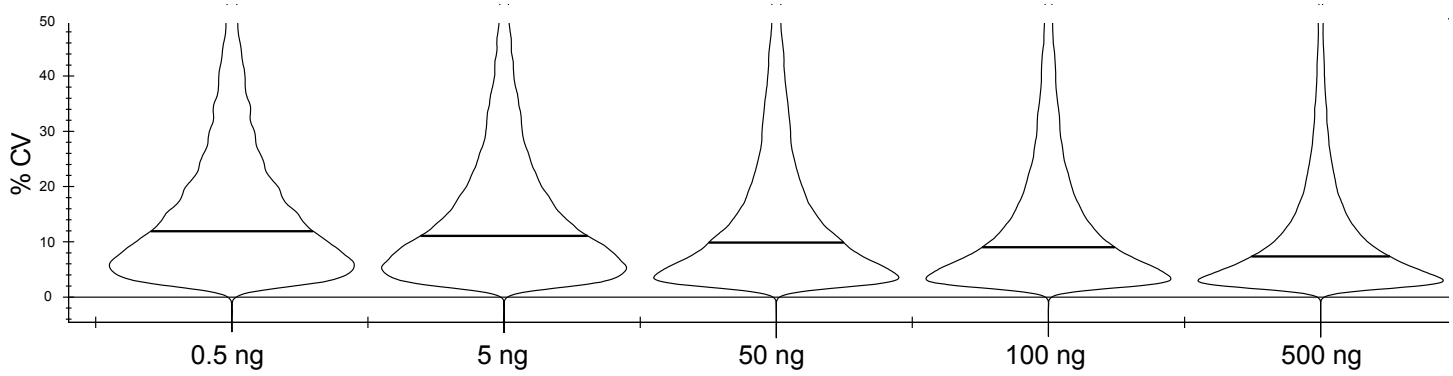


Figure 7. Coefficients of variation of peptide abundances across all HeLa concentrations. Median CV marked with black line.

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 every protein observed in a concentration, then a linear model was fit to every 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.

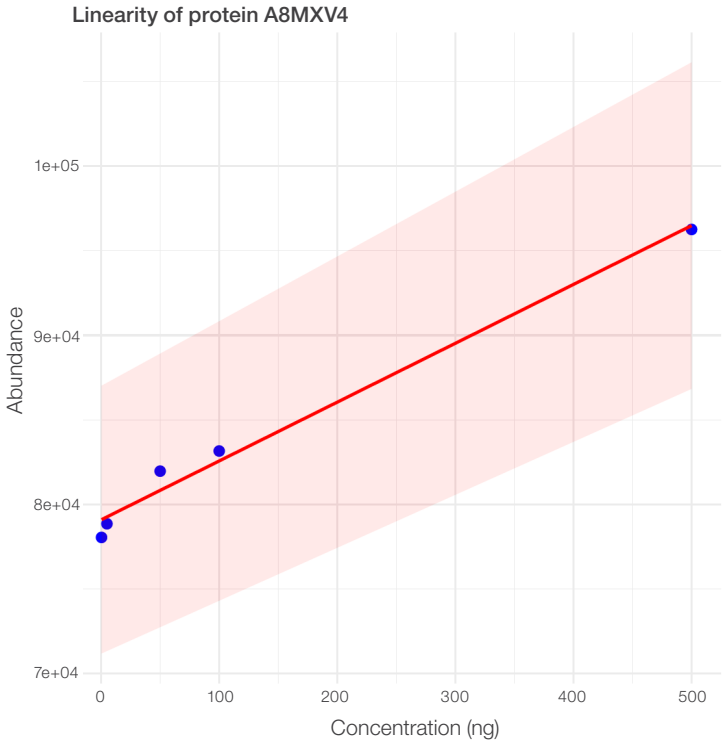


Figure 8. Example of linearity for identified protein at concentrations from 0.5 ng to 500 ng using DIA quantitation (R^2=0.98). Red shading represents a 10% confidence interval.

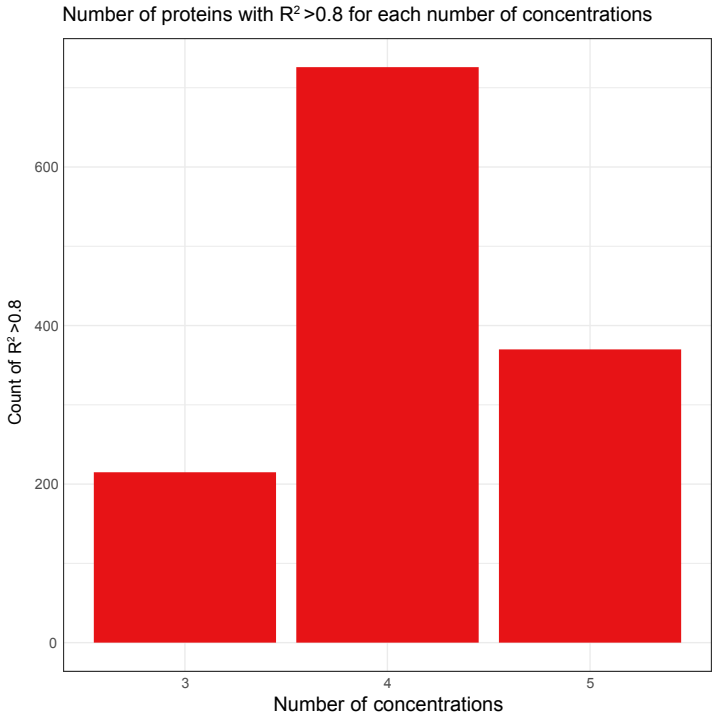


Figure 9. The histogram shows the number of protein abundances with linear behavior across indicated numbers of concentrations. In this experiment, five HeLa concentrations were tested (Table 5). The number of protein abundances displaying linearity of R^2 >0.8 at the indicated number of concentrations 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 all targeted peptides, (R^2 >0.9) (Figure 10). All 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 all peptides considered (6) and across all concentrations (5), 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.

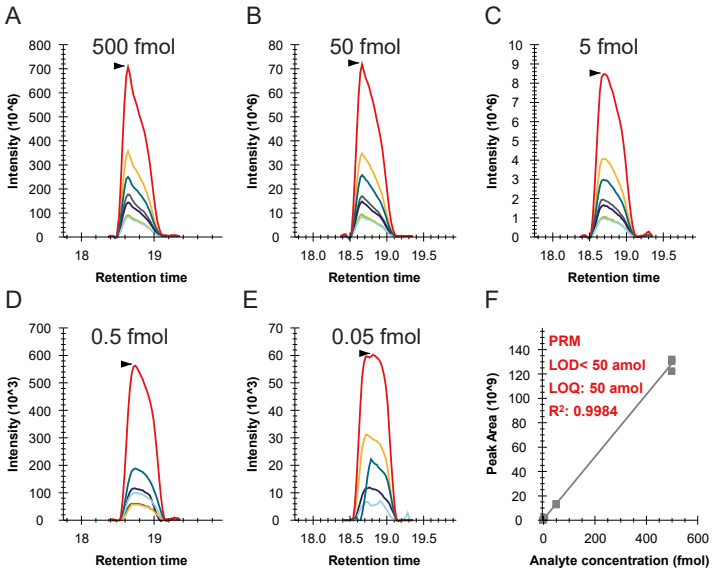


Figure 10. Example dilution curve of 6x5 spike-in standard. PRM chromatograms for the YVYVADVAAK peptide are shown at 500, 50, 5, 0.5, and 0.05 fmol (A, B, C, D, and E, respectively) and the resulting calibration curve (F).

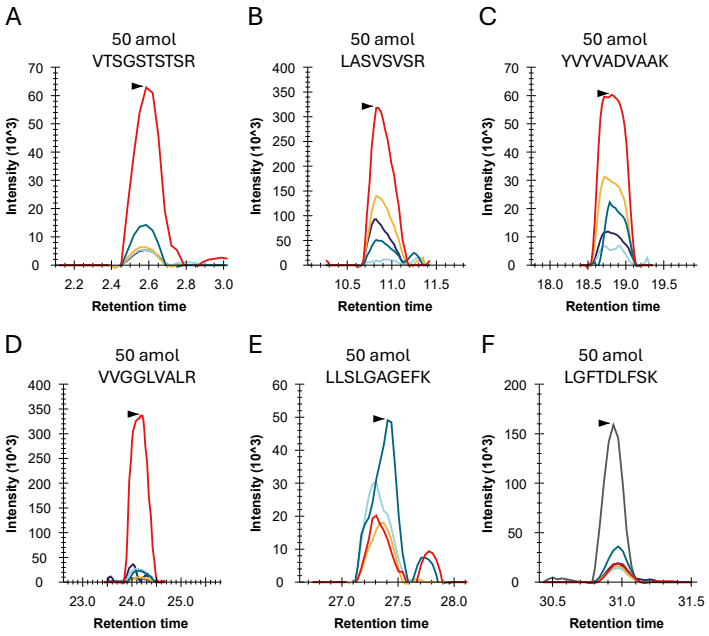


Figure 11. PRM chromatograms for 6x5 peptides are shown at 50 amol (A: VTSGSTSTSR, B: LASVSVSR, C: YVYVADVAAK, D: VVGGLVALR, and E: LLSLGAGEFK, and F: LGFTDLFSK).

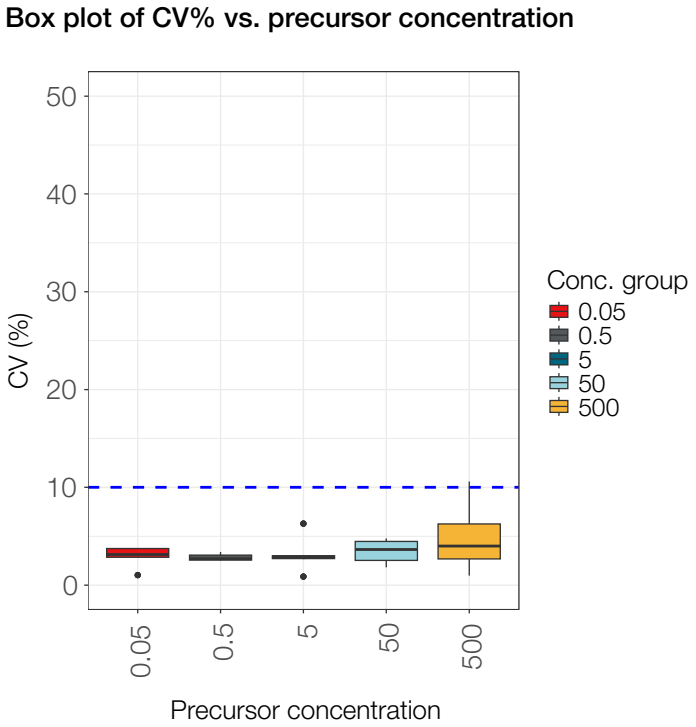


Figure 12. Average CV for peptide-concentration pair replicates across all concentrations of HeLa. The blue dashed line represents 10% CV.

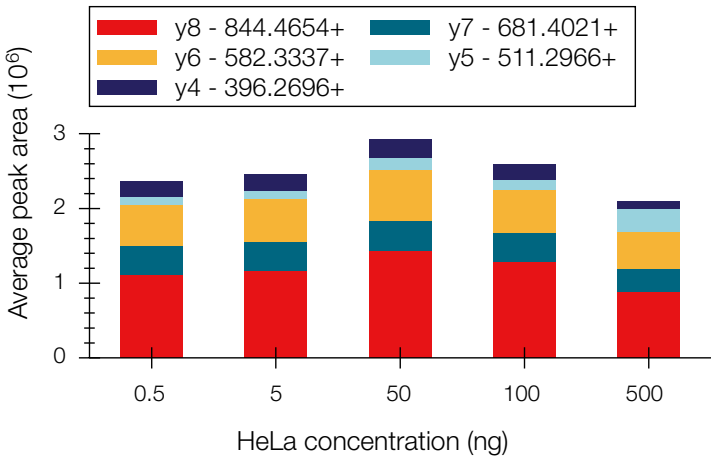


Figure 13. Fragment ion areas for peptide YVYVADVAAK (50 amol) across each HeLa concentration.

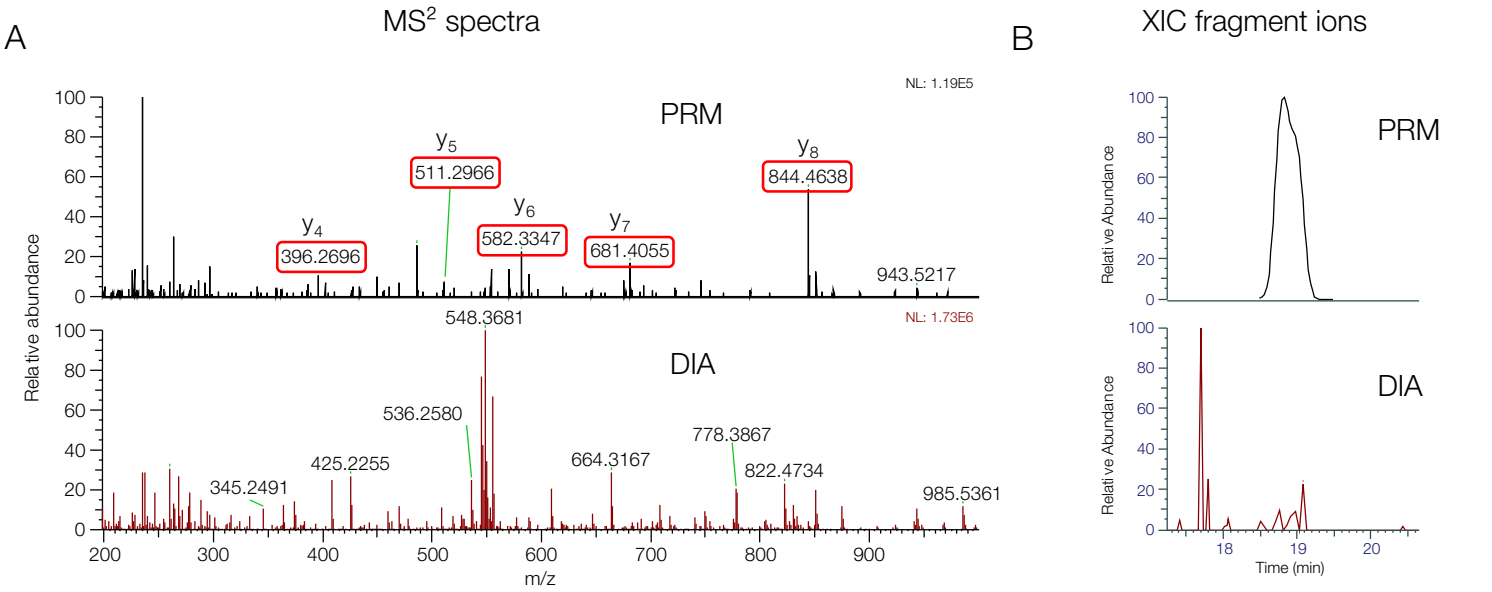


Figure 14. Comparison of PRM and DIA MS² spectra (A) and extracted ion chromatograms using y-ions (B) for peptide YVYVADVAAK at the lowest concentration, 50 amol.

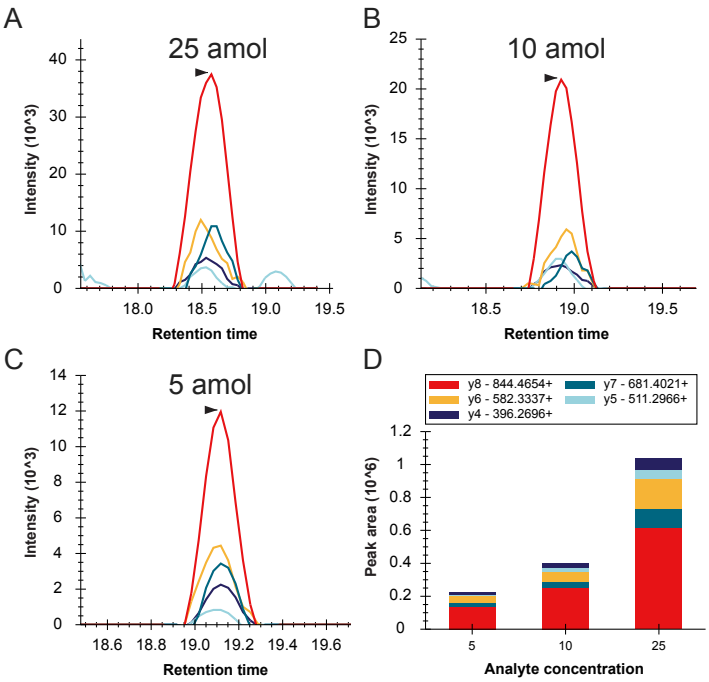
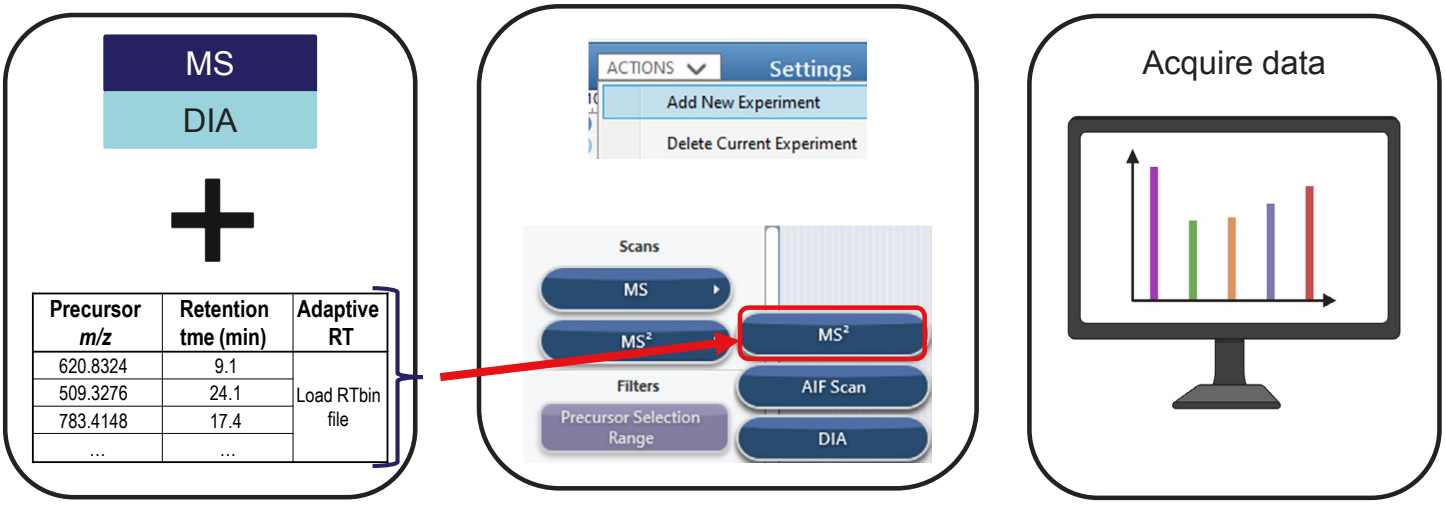


Figure 15. PRM chromatograms for the YVYVADVAAK peptide are shown at 25, 10, and 5 amol (A, B, and C, respectively) and the peak area comparison (D).

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 tMS² input file. The input file will require precursor mass, RT, and RTbin file.
2. Add the tMS² 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 tMS² precursor list
2. Add tMS² experiment and import precursor list
3. Queue injection and acquire nybrid DIA data

Figure 16. Integration of tMS² 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 delivered plug-and-play simplicity with automated emitter optimization and cartridge-based columns, ensuring consistent performance and data acquisition throughout the entire experiment.

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