Label-free quantification

Improved accuracy and identification in label-free quantitative proteomics with the OptiSpray Ion Source and separation cartridge with integrated replaceable emitters

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Abstract

Purpose: Demonstrate the improvement in measurement accuracy and protein identification capability in label-free quantitative proteomics with a novel, automated ion source and column assembly with integrated replaceable emitters for proteomics data acquisition.

Methods: Data was acquired using a Thermo Scientific™ Vanquish™ Neo UHPLC System connected to a Thermo Scientific™ Orbitrap Exploris™ 480 Mass Spectrometer with Thermo Scientific™ FAIMS Pro Duo interface and the novel, automated Thermo Scientific™ OptiSpray™ Ion Source. A cartridge with an integrated Thermo Scientific™ µPAC™ Neo 50 cm Column and a replaceable 15 µm tapered tip emitter assembly was used to analyze three-proteome mix of HeLa, *E. coli*, and Yeast digest, and two-proteome mix of HeLa and *E. coli* digest.

Results: The OptiSpray ion source with a cartridge column assembly, standalone or when combined with the FAIMS interface, enhances spray stability, improving precision by lowering peptide CVs (%) at all levels of abundance, which contributes to improved protein identification and ratio accuracy.

Introduction

Mass spectrometry-based label-free quantitation (LFQ) workflows are widely used in bottom-up proteomics for protein expression studies, enabling insights into biological and disease mechanisms. Recent advances in software have made data-independent and library-free approaches cost-effective, while nano LC-MS remains the gold standard for its sensitivity and low sample consumption. However, technical complexity and usability challenges often hinder adoption. High measurement accuracy is critical for meaningful results with robust statistical power. To this end, we demonstrate improved quantitative performance using a novel ion source designed to enhance data quality and simplify setup. Central to this approach is a cartridge that integrates a column, liquid junction, and replaceable emitter assembly, paired with an automated ion source for consistent and improved performance in LFQ workflow.

Materials and methods

Sample preparation

The following samples were prepared in 1% acetonitrile (aqueous) with 0.1% trifluoroacetic acid from Thermo Scientific™ Pierce™ HeLa Protein Digest Standard, MassPREP™ *E. coli* Digest Standard from Waters™, and Mass Spec-Compatible Yeast Digest from Promega™:

HeLa digest

- **HeLa only:** 200 ng/µL

3-proteome mix

- **A:** HeLa 325 ng/µL, *E. coli* 100 ng/µL, Yeast 75 ng/µL
- **B:** HeLa 325 ng/µL, *E. coli* 25 ng/µL, Yeast 150 ng/µL

2-proteome mix

- **1:** HeLa 400 ng/µL, *E. coli* 12.5 ng/µL
- **2:** HeLa 400 ng/µL, *E. coli* 25 ng/µL
- **3:** HeLa 400 ng/µL, *E. coli* 50 ng/µL
- **4:** HeLa 400 ng/µL, *E. coli* 100 ng/µL

Figure 1. OptiSpray Ion Source

LC-MS methods

After a µPAC Neo 50 cm column cartridge with a 15 µm tapered tip was inserted into the OptiSpray Ion Source (Figure 1) installed on a Orbitrap Exploris 480 mass spectrometer with FAIMS Pro Duo interface, an automated routine was used to move the emitter to the optimal position in front of the FAIMS or ion transfer tube orifice. For taper tip emitters, sheath gas of 0.2-0.6 L/min can be applied. The sheath gas provides nebulization and ensures optimum peak area reproducibility for taper tip emitters.

The samples were analyzed injecting 1 µL in triplicate on a single µPAC Neo 50 cm cartridge, with 3 different DIA methods at sample elution duration of 9 min, 30 min, and 60 min (see Table 1 and 2). 200 ng of HeLa digest was analyzed throughout at regular intervals to demonstrate stability and reproducibility (QC).

Table 1. LC gradients and parameters

9 min active gradient		
Time, min	Flow, µL/min	%B
0	0.7	3
0.1	0.7	6
8.8	0.7	22.5
12	0.7	45
12.1	0.7	99
13	0.7	99
13.6	1	99
15	1	99

30 min active gradient		
Time, min	Flow, µL/min	%B
0	0.5	2
0.1	0.5	4
23.6	0.5	22.5
32.1	0.5	45
32.4	0.5	99
34.7	0.5	99
36.3	1	99
40	1	99

60 min active gradient		
Time, min	Flow, µL/min	%B
0	0.3	1
0.1	0.3	2
44.1	0.3	22.5
60.1	0.3	45
60.6	0.3	99
65	0.3	99
68	1	99
75	1	99

Loading settings		Wash and equilibration settings	
Fast loading	Yes	Fast equilibration	Yes
Mode	Pressure control	Mode	Pressure control
Loading pressure	400 bar	Equilibration pressure	400 bar
Loading volume	1.0 µL	Equilibration factor	2.0

Table 2. MS methods for the different gradients

MS global parameters			
Spray voltage	+ 2200 V	Cartridge temp.	50 °C
Ion transfer tube temp.	290 °C	Sheath gas	1.0
FAIMS carrier gas flow	3.5 L/min	EASY-IC	RunStart

Expected peak width: 6 s (15 min method), 9 s (40 min method), 15 s (75 min method).
Ion source type: OptiSpray. FAIMS mode: Standard resolution.

MS experiment parameters			
MS experiment duration	15 min	40 min	75 min
Number of MS experiments	2	2	4
MS[1] resolution	45 000	60 000	60 000
FAIMS CV	-50	-50	-45 and -60
MS[1] scan range	400 – 750 m/z	400 – 900 m/z	
AGC target	300 %		
RF lens	70 %		
MS[2] DIA resolution	22 500	30 000	30 000
FAIMS CV	-50	-50	-45 and -60
Precursor range	400 – 750 m/z	400 – 900 m/z	
Isolation window	18 m/z	24 m/z	
Scan range	145 – 1450 m/z		
AGC target	1000 %		
HCD energy	30 %		

Application mode: Peptide. MS[1] data type: Profile. MS[2] data type: Centroid. Maximum injection time mode: Auto. DIA window type: Auto. DIA window optimization: On. DIA window overlap: 1 m/z.

Data analysis

DIA data was processed using Spectronaut® 19.5 from Biognosys AG with default settings, Thermo Scientific™ Proteome Discoverer™ Software 3.2 using CHIMERYS and DIA-NN 2.0 with appropriate settings. PRTC peptides were integrated by targeted processing workflow with Skyline 24.1 from MacCoss Lab.

Protein groups were identified at 1% FDR. The following sample ratios were evaluated: A/B, 4/1, 3/1, and 2/1. FASTA files with canonical sequences were used: Homo sapiens (20428 entries), Escherichia coli K-1 (4530 entries), Saccharomyces cerevisiae (6727 entries), and a common contaminants FASTA file (doi.org/10.1101/2022.04.27.489766).

Peptide and protein identification was performed, log2 ratio accuracy was evaluated. QC data was checked for trends and PRTC peak CVs (%) were evaluated. To demonstrate precision, distributions of coefficients of variation from results obtained with the novel ion source were compared to data from a Thermo Scientific™ Velocity DIA platform workflow (with the µPAC Neo 50 cm column connected to a 10 µm fused silica emitter) acquired on Orbitrap Exploris 480 mass spectrometer with FAIMS interface and Thermo Scientific™ EASY-Spray™ Ion Source.

Results

Distributions of coefficients of variation show that with the novel ion source median CVs decreased in all cases, especially at high throughput (Figure 2) where CVs decreased from 16% to 8% for low-abundance peptides and from 6% to 2% for abundant peptides.

Figure 2. Distribution of all peptide quantity CVs (%) from 200 ng HeLa (*n* = 3)

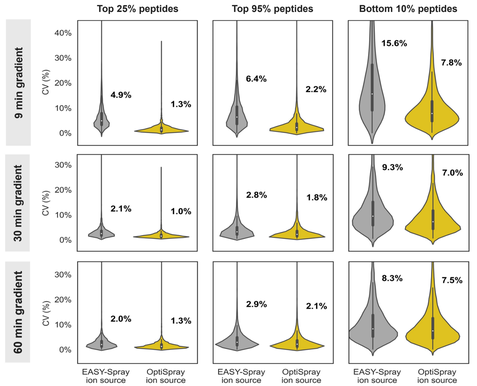
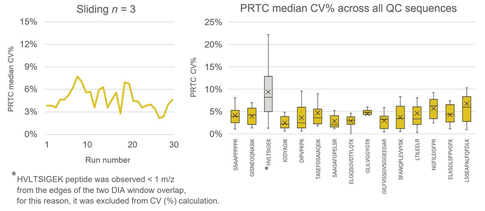


Figure 3. PRTC CVs (%) from 200 ng HeLa analyzed throughout the data set (QC)



*HVLTSIGEK peptide was observed < 1 m/z from the edges of the two DIA window overlap, for this reason, it was excluded from CV (%) calculation.

PRTC CVs (%) from 200 ng HeLa (Figure 3) analyzed throughout the QC data and calculated in sliding manner of 3 injections, as well as median CVs (%) of all QC sequences (peptide sequences in order of elution) show stable variation. Stability was observed over all 170 hours of acquisition where mixed proteome samples were analyzed with the 9, 30, and 60 min greadient methods between 10 QC sequences of 200 ng HeLa in triplicate with the 60 min gradient. Across QC runs total PRTC peptide area was stable with a CV of 2.4%, peptide and protein identification was stable with CVs of 1.0% and 1.2%, respectively, and the retention times of PRTC peptides were stable with RT deviations from 0.18% to 0.86%. While improved CVs (%) alone usually do not drive increases in protein identification rates, the application of FAIMS or OptiSpray, or combination thereof, show improvement in protein identification compared to EASY-Spray, magnitude depends on instrument methods and software for processing and data interpretation (Figure 4, 5, and 6).

Figure 4. Protein and peptide groups identified at 1% FDR in triplicates of HeLa digest and three-proteome mix A and B using FAIMS and OptiSpray ion source

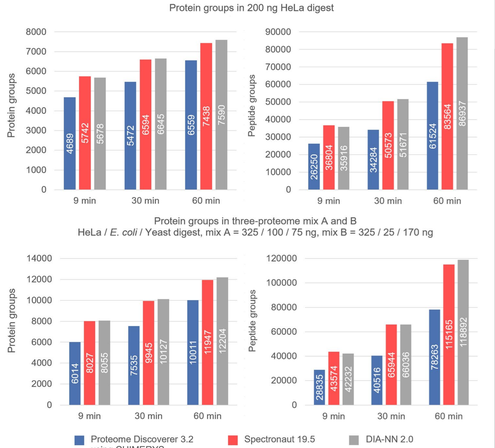


Figure 5. Protein groups in triplicates of 3-proteome mix A and B (30 min gradient) with Spectronaut software

	EASY-Spray	OptiSpray
with FAIMS	9296	9945
without FAIMS	8584	9199

Figure 6. Increase in protein groups with OptiSpray ion source or FAIMS in 3-proteome mix (30 min gradient) with Spectronaut software

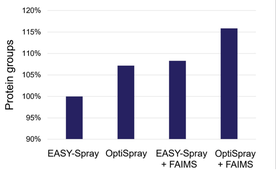
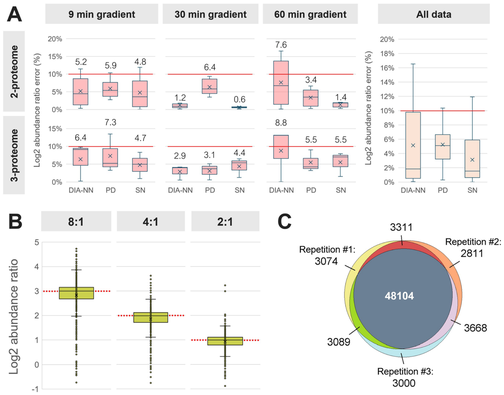


Figure 7. (A) Boxplots of log2 abundance ratio error of proteins across all samples, methods, and software used. (B) Determined log2 abundance ratios for *E. coli* analyzed with 30 min gradient in samples having the true ratios of 8:1, 4:1, and 2:1, processed using Spectronaut. (C) Venn diagram of peptide sequences identified in 3 repetitions of Sample 4 analyzed with 60 min gradient, processed using Proteome Discoverer.



The log2 abundance ratios (Figure 7A) show <10% error across all samples over 170 hours of acquisition, or 4.5% average error. The ratio error can further be reduced by optimizing MS acquisition and processing software parameters. The accurate determination of ratios of *E. coli* proteins is shown in Figure 7B. An overlap of >70% of peptide sequences identified in 3 repetitions of a same sample was observed, example shown in Figure 7C.

Conclusions

- The novel, automated OptiSpray ion source improves precision thanks to improved spray stability, which contributes to improved protein identification and protein ratio accuracy.
- Combination of the novel, automated OptiSpray ion source with gas phase fractionation by FAIMS module enables identification of a broader set of peptides, leading to increased protein identification rates (IDs). While number of peptide IDs may be slightly lower with short runs due to increased demand for scan time, the vastly reduced spectral complexity still leads to improved protein IDs.
- DIA window overlap of 1 m/z may be insufficient as this equals to 0.5 m/z at each window edge. For this reason, the PRTC peptide HVLTSIGEK had increased CV (%). Based on distances from DIA window edges of other PRTC peptides, 3 m/z may be a more suitable value, resulting in at least 1.5 m/z distance from each edge.

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