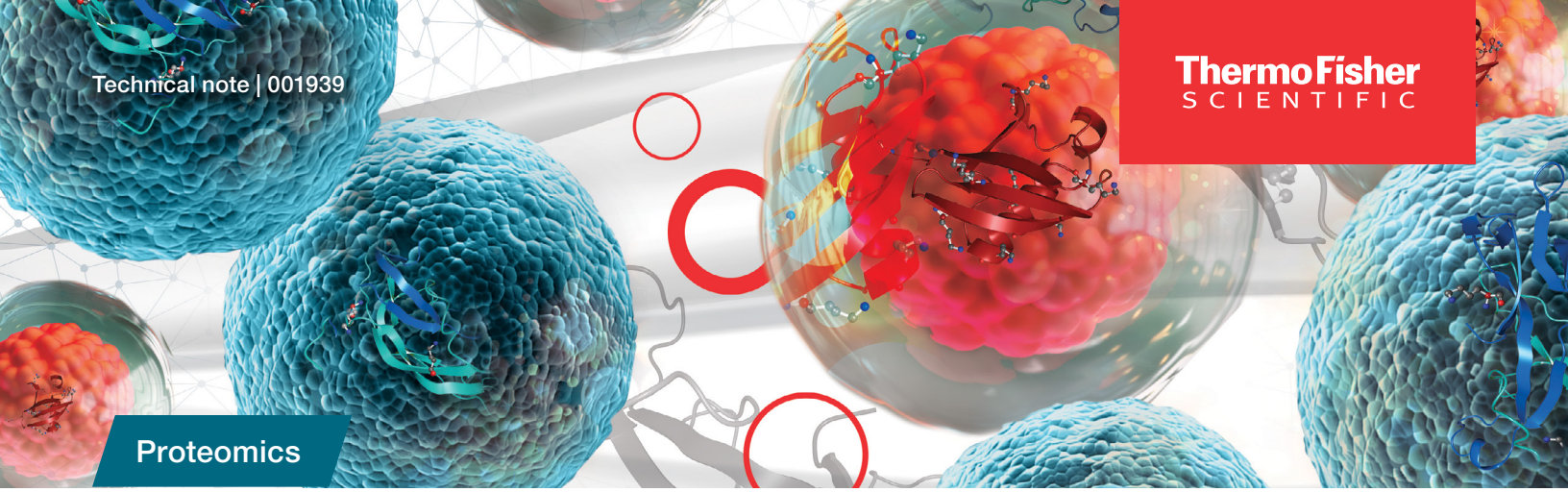

High-sensitivity low-nano flow LC-MS methods for high-throughput sample-limited proteomics

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Keywords

Bottom-up proteomics, single-cell proteomics (SCP), sample-limited, nano-LC-MS, direct injection, Vanquish Neo UHPLC system, Orbitrap Exploris 480 mass spectrometer, HeLa protein digest, Acclaim PepMap 100 C18 column, label-free quantification (LFQ), data-dependent acquisition (DDA), data-independent acquisition (DIA), wide window acquisition (WWA)

Goal

Demonstrate high-throughput and high-sensitivity nano-flow LC-MS methods using a 50 µm i.d. column operated at 100 nL/min for sample-limited proteomics analysis.

Introduction

State-of-the-art liquid chromatography-mass spectrometry (LC-MS) technologies can provide the sensitivity and throughput necessary to support the growing need for analyzing samples with limited amounts, including single-cell proteomics (SCP). In particular, the capacity to generate precise low nano-flow liquid chromatography (LC) gradient separations, highly reproducible low internal diameter separation columns, and leak-free connections are essential for consistent data quality with sufficient sample throughput.

Several aspects of the analysis of limited samples must be considered in the creation of robust and reproducible methods for this type of application. First, flow rate must be optimized for both sensitivity and throughput. Second, the LC platform must permit efficient sample analysis without wasting valuable mass spectrometry (MS) acquisition time. Third, MS acquisition parameters must be optimized for the relatively low signal intensity observed from small sample quantities. Here, we introduce five optimized UHPLC-MS methods for use with a 50 µm i.d. column operated at 100 nL/min in the direct injection workflow on the Thermo Scientific™ Vanquish™ Neo UHPLC system coupled to a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer to achieve maximum sensitivity, throughput, and MS utilization.

Experimental materials and methods

Sample preparation

The Thermo Scientific™ Pierce™ HeLa Digest/PRTC Standard (A47996, 10 µg/vial) was reconstituted by adding 100 µL of 0.1% formic acid (FA) in water with 10% acetonitrile (ACN, stock solution). The vial was subsequently sonicated for 5 min, followed by dilution of 10 µL sample in 990 µL water (0.1% FA, v/v) to 1 ng/µL HeLa digest and homogenized with 20 min sonication.

Consumables

- Water with 0.1% formic acid (FA) (v/v), Optima™ LC/MS grade, Thermo Scientific™ (P/N LS118500)
- 80% acetonitrile (ACN), 20% water with 0.1% formic acid (FA), Optima™ LC/MS, Fisher Chemical™ (P/N LS122500)
- Formic acid (FA), 99.0+%, Optima™ LC/MS grade, Fisher Chemical™ (P/N 10596814)
- Isopropanol (IPA), Optima™ LC/MS grade, Fisher Chemical™ (P/N 10684355)
- Fluidics and consumables used to set up the Vanquish Neo UHPLC system for direct injection are given in Table 1.

Table 1. Vanquish Neo system and Orbitrap Exploris 480 MS, fluidics, and accessories for direct injection workflow

Category	Description	#	Part number
UHPLC components	Vanquish Neo UHPLC system consisting of Binary Pump N, Split Sampler NT, Solvent Rack, Vanquish System Controller, and System Base with drawer	1	VN–S10–A–01
	Vanquish display	1	6036.1180
	Thermo Scientific™ nanoViper™ Capillary, i.d. × L 10 µm × 350 mm (connect the column to the sampler left valve port 4)*	1	6250.5135
Mass spectrometer	Thermo Scientific™ Nanospray Flex™ ion sources	1	ES072
	Thermo Scientific™ FAIMS Pro™ interface	1	FMS02-10001
	Orbitrap Exploris 480 mass spectrometer	1	BRE725539
Consumables	Total recovery vial 1.5 mL	1	6PSV9-TR1
	Talcum-free cap screw 9 mm	1	6PSC9STB1
Column and emitter	Thermo Scientific™ Acclaim™ PepMap™ 100 C_{18} column 50 µm × 150 mm, 2 µm size, 280 µm o.d.	1	164943
	10 µm i.d. × 360 µm o.d., 5 cm emitter**	1	—
	MicroTight™ Union Assembly 1/32†	1	G10-0035
	Sleeves 1/32 for 280 µm o.d. capillary	1	SC903
	Sleeves 1/16 for 360 µm o.d. capillary	1	SC603
Liquid junction unit	Sonation™ holder for liquid junction unit	1	004.800.01
	Low dispersion Y-piece, 50 µm bore with insert	1	6250.1009
	Thermo Scientific™ Viper™ plug titanium	1	6040.2303

*Due to its narrower i.d., some scripts (e.g., D01 Test System Back Pressure) will deliver a warning message.

**Primary experiment was performed with emitters from Fossiliontech

†This union and the following sleeves could be replaced with PTFE tubing (P/N 160489, 250-350 µm i.d.) when using 10 µm emitters with constant i.d.

LC solvents and system temperature settings

The recommended solvents to run high-sensitivity and high-throughput applications are described in Table 2.

Table 2. Solvents used for instrument operation

	Solvent	Composition
Binary Pump N	Mobile phase A	H_2O with 0.1% FA
	Mobile phase B	80/20 (v/v) ACN/H_2O with 0.1% FA
Split Sampler NT Metering Device	Weak wash liquid	H_2O with 0.1% FA
	Strong wash liquid	80/20 (v/v) ACN/H_2O with 0.1% FA
Split Sampler NT Wash Port	Weak wash liquid	H_2O with 0.1% FA
	Strong wash liquid	80/20 (v/v) ACN/H_2O with 0.1% FA
Binary Pump N and Split Sampler NT	Rear seal wash buffer	25/75 (v/v) H_2O/IPA with 0.1% FA

Vanquish Neo UHPLC system method parameters

The generic parameters for sample aspiration, loading, and column equilibration are shown in Table 3. An example method is described in Table 4, and all other optimized methods are available for download from Thermo Scientific™ AppsLab Library of Analytical Applications.

Table 3. Generic LC parameters for five high-throughput LC methods

	Parameter	Value
Sample loading	Mode	PressureControl
	Pressure	1,500 bar
	Loading volume	2 µL
Sample pick–up	Outer needle wash mode	After draw
	Outer needle wash time (strong)	3.0 s
	Outer needle wash speed (strong)	80.0 µL/s
	Outer needle wash time (weak)	5.0 s
	Outer needle wash speed (weak)	80.0 µL/s
	Draw speed	0.2 µL/s
	Draw delay	2.0 s
	Dispense speed	5.0 µL/s
	Vial bottom detection	Enabled
Column equilibration	Fast equilibration	Disabled
	Mode	–
	Pressure	–
	Equilibration factor	0.1
Temperature	Column temperature*	50°C
	Autosampler temperature	7°C

*Controlled by external heating sleeve/oven

Table 4. High-throughput 10-min gradient method in the direct injection workflow

Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0	0	0.5	1
0.5	0.5	0.1	1
1	0.5	0.1	8
8	7	0.1	20
10	2	0.1	35
Column wash phase			
10.1	0.1	0.1	99
14	3.9	0.1	99

MS acquisition parameters

MS data were acquired with an Orbitrap Exploris 480 mass spectrometer in data-dependent acquisition (DDA) and data-independent acquisition (DIA) modes with a FAIMS Pro interface installed. The MS parameters designated for the 10-min gradient method are described in Table 5, and all the other methods are available for download in the AppsLab Library.

Table 5. MS acquisition parameters for 10-min gradient method

Category	Property	DDA	DIA
Method settings	Application mode	Peptide	
	Method duration	14 min	
Ion source	Positive ion	1,600–2,200* V	
	Ion transfer tube temp.	275°C	
	FAIMS mode	Standard resolution	
	Total carrier gas flow	Static	
	Total carrier gas flow	3.5 L/min	
MS global settings	Infusion mode	Liquid chromatography	
	Expected LC peak width	15 s	
	Advanced peak determination	True	
	Default charge state	2	
	Internal mass calibration	Off	
Full scan	Resolution	120,000	
	Scan range	375–1,200 *m/z*	375–1,200 *m/z*
	FAIMS voltage	On	
	FAIMS CV	-50 V	
	RF lens	45%	
	AGC target	300	
	Maximum injection time mode	Auto	
	Data type	Profile	
	Polarity	Positive	
	Source fragmentation	Disable	
MIPS	Monoisotopic peak determination	Peptide	–
	Relax restrictions when too few precursors are found	True	–

*Depending on the emitter type

Table 5. MS acquisition parameters for 10-min gradient method *(continued)*

Category	Property	DDA	DIA
Intensity	Intensity threshold	5.0E+03	–
Charge state	Include charge state(s)	2–5	–
	Include undetermined charge states	False	–
Dynamic exclusion	Perform dependent scan on single charge state per precursor only	True	–
	Exclusion duration	30 s	–
ddMS^2	Multiplex Ions	False	–
	Isolation window	2 *m/z*	–
	Collision energy type	Normalized	–
	HCD collision energy	26%	–
	Scan range mode	Define first mass	–
	First mass	*m/z* 120	–
	Normalized AGC target	50%	–
	Data type	Centroid	–
	Number of dependent scans	10	–
	Orbitrap resolution	60,000	–
	Maximum injection time	118 ms	–
DIA	Precursor mass range	–	400–800 *m/z*
	DIA window type	–	Auto
	Isolation window	–	40 *m/z*
	Window placement optimization	–	On
	Number of scan events	–	9
	Collision energy type	–	Normalized
	HCD collision energies	–	28%
	Orbitrap resolution	–	60,000
	Scan range mode	–	Defined first mass
	First mass	–	120 *m/z*
	AGC target	–	Custom
	Normalized AGC target	–	1,000%
	Maximum injection time	–	118 ms
	Loop control	–	All

Data processing and analysis

The DDA dataset was processed with Thermo Scientific™ Proteome Discoverer™ 3.0 software using a 2-step SEQUEST™ HT search algorithm and INFERYS™ rescoring node. The chimeric spectra in the DDA dataset from a wide window acquisition approach were searched with the CHIMERYS™ algorithm, and DIA files were submitted to Spectronaut™ 17 (SN17) for peptide and protein ID and quantification. The false discovery rates (FDR) were set below 1% at both the peptide and the protein levels. Further data analysis and plotting were performed with R script.[1]

Results and discussion

Column and liquid junction configuration for high-sensitivity, high-throughput analysis

Considering multiple factors of balancing the sensitivity and sample throughput in low-flow LC-MS separations, we established a high-performance configuration running gradients at 100 nL/min using a liquid junction on the column inlet (Figure 1).

Taking full advantage of the ultra-low gradient delay volume (GDV) in the LC fluidic connections (<300 nL), we developed five high-throughput methods (Figures 2 and 3) that enable fast column washing and equilibration while maintaining the high sensitivity 100 nL/min gradient separations.

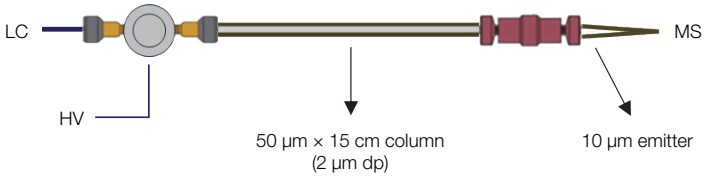


Figure 1. Direct injection workflow using 1,500 bar for sample loading to increase sample throughput

Sample throughput/day	Cycle time (min)	Gradient length (min)	Elution window (min)	Sample injection & loading (min)*	MS utilization (%)
72	20	10	11	5.4	55
60	24	14	14	5.4	62.5
48	30	20	21	5.4	70
36	40	30	31	5.4	77.5
24	60	50	51	5.4	85

* 1 µL sample injection + 2 µL loading

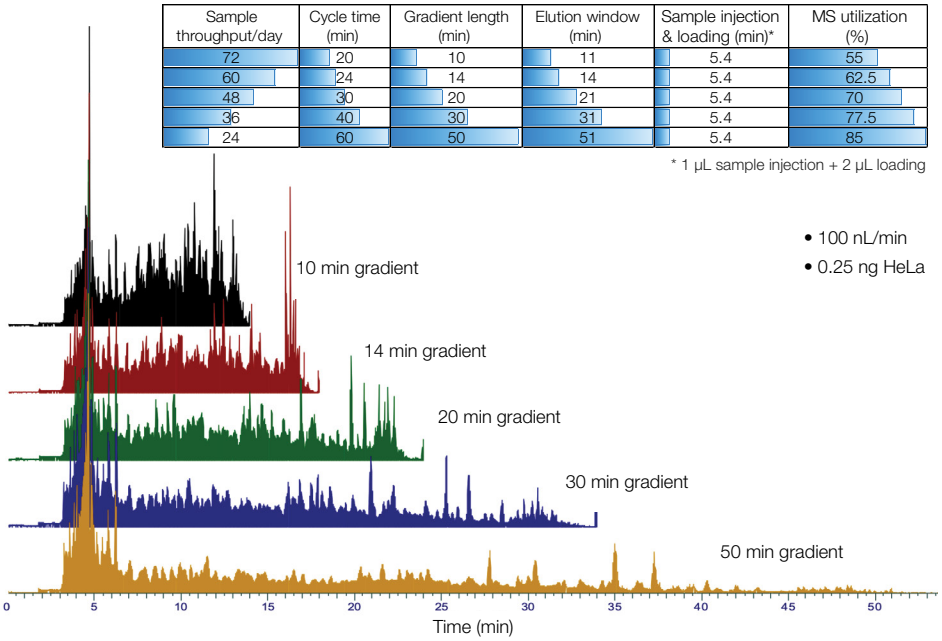


Figure 2. Five optimized LC-MS methods balancing sensitivity and throughput for different proteome coverage needs

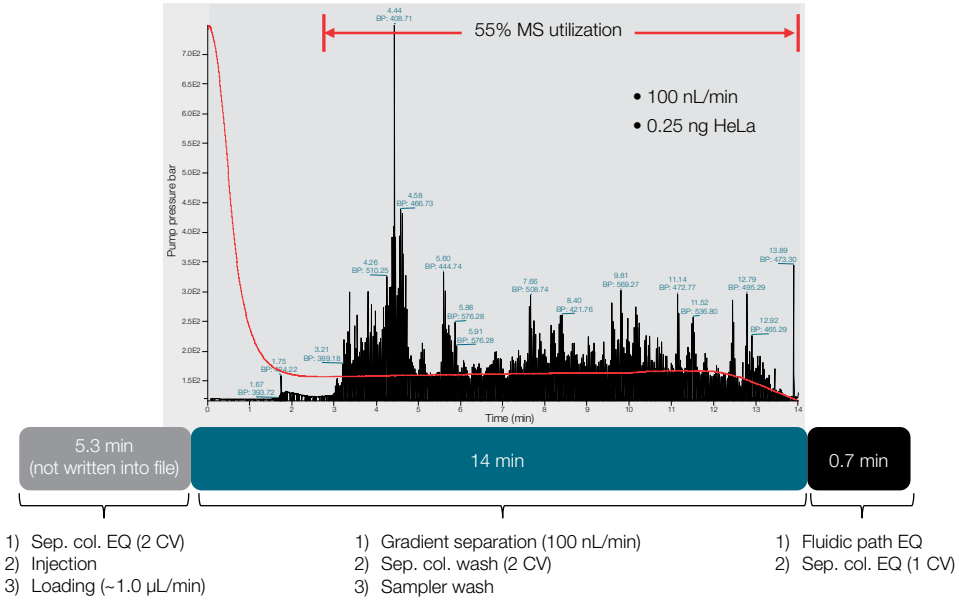


Figure 3. High-throughput method highlight. The 20-min cycle time with 11 min elution window (72 samples/day) enables 55% MS utilization through parallel gradient separation and sampler washing.

Outstanding performance in DDA mode

A linear increase of peptide and protein IDs from 250 pg to 5 ng HeLa digest (Figure 4) suggests excellent method suitability for sample-limited proteomics analysis. We identified ~1,500 protein groups from a 250 pg sample, which—to the authors' knowledge—represents the most comprehensive DDA data to date.[2] Moreover, ~800 protein group IDs in a 10-min gradient demonstrate outstanding potential for the DIA strategy. In addition, the low variation in peptide retention time and protein abundance confirms reproducible LC-MS performance for protein quantification (Figure 5).

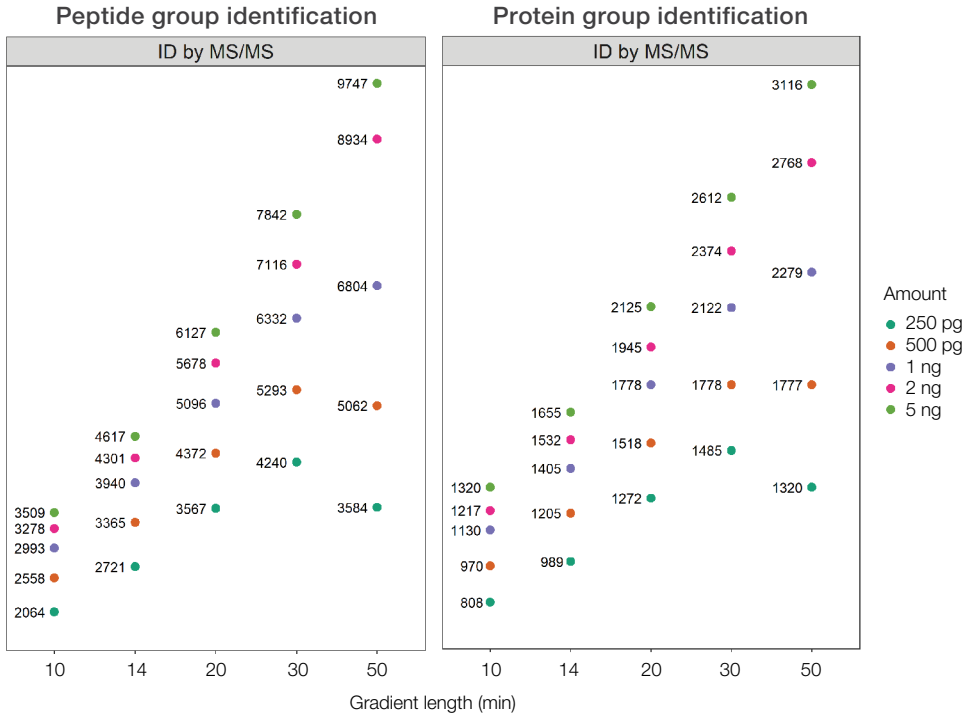


Figure 4. The workflow facilitates state-of-the-art sensitivity with industry-leading throughput.

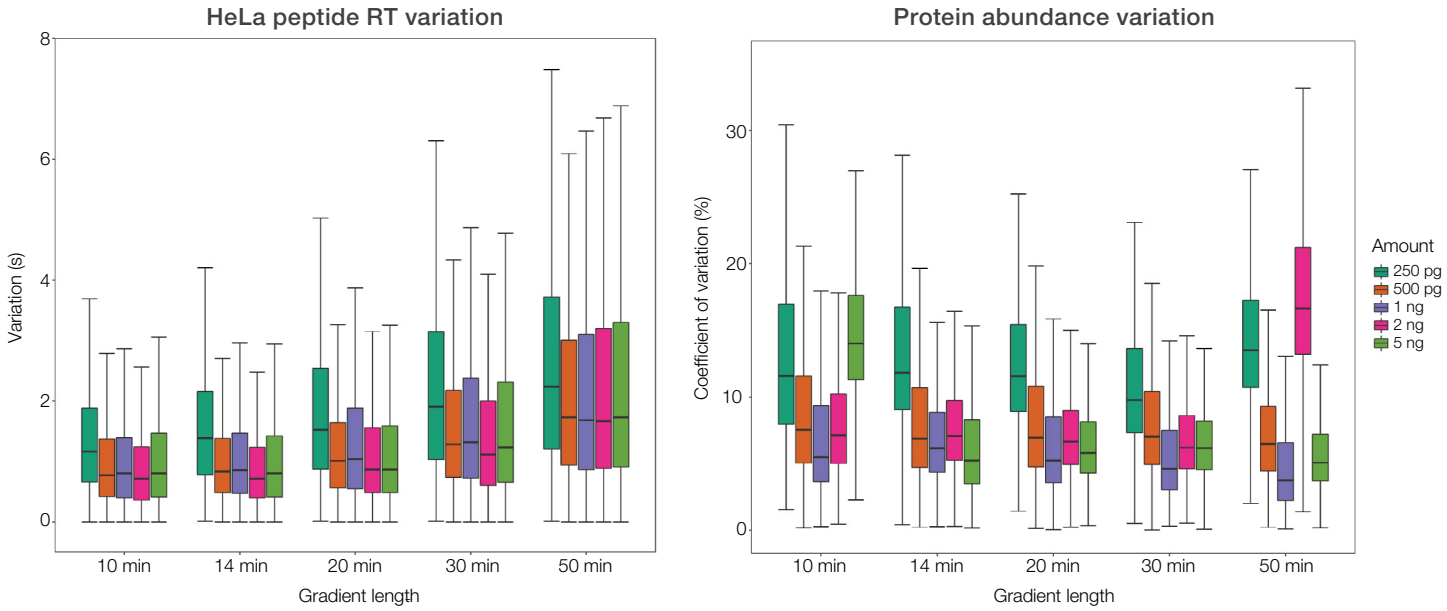


Figure 5. Low retention time variation and high quantitation accuracy of the LC-MS performance

By employing an advanced AI-driven algorithm to deconvolute the MS^2 spectra with a wide window acquisition strategy, we identified ~1,800 protein groups from a 250 pg HeLa digest in a 10-min gradient (Figure 6).

Extraordinary proteome coverage empowered by DIA

A systematic evaluation of the MS^1 open window in DIA indicated >3,000 protein groups from a 250 pg HeLa sample could be identified in a 10-min LC gradient that covers more than four orders of magnitude of the protein abundance dynamic range (Figure 7).

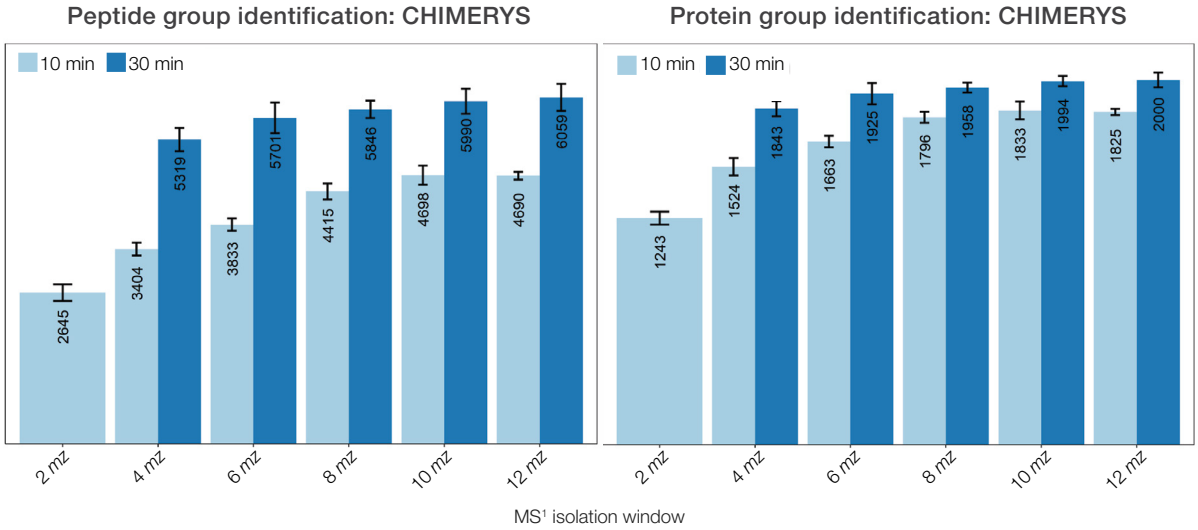
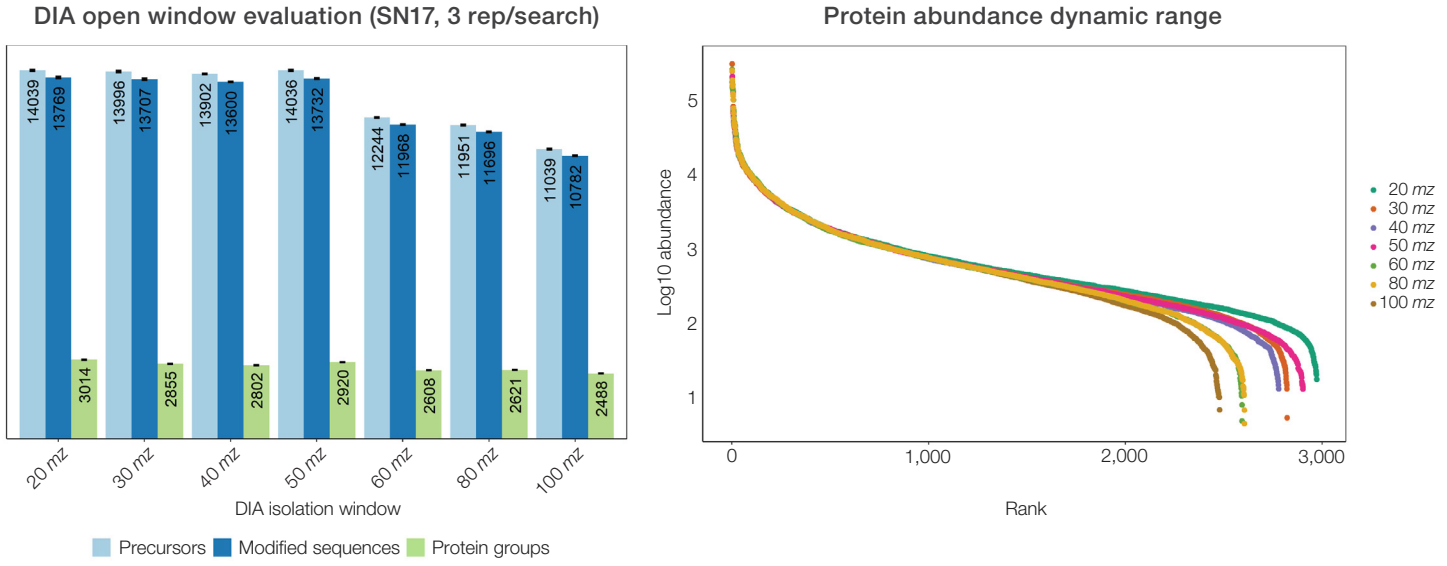


Figure 6. A wide window acquisition strategy from a 250 pg HeLa digest indicates a 10–12 *m/z* isolation window in a 10-min gradient significantly boosts protein group IDs.



Library-free, 250 pg Hela, 10 min gradient

Figure 7. Proteome coverage and dynamic range covered using different DIA isolation windows in LFQ-DIA

Conclusions

We developed five high-sensitivity, high-throughput nano-LC-MS methods that afford industry-leading protein coverage. We were able to identify the following in 250 pg HeLa digest using a 10-min LC gradient:

- ~800 protein groups in DDA (SEQUEST and INFERYS)
- >1,800 protein groups in DDA (CHIMERYS)
- >3,000 protein groups in DIA across >4 orders of magnitude of protein abundance (SN17)

These methods support, for example, SCP analysis with up to 72 cells per day (CPD) in LFQ-DDA and DIA modes, and foreseeably, it would increase sample throughput up to a dozen times when employing a multiplexing strategy (e.g., labeling with TMT reagents, to enable larger-scale sample analysis).

References

1. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, **2020**. https://www.R-project.org/
2. Stejskal, K.; Op de Beeck, J. et al. Ultrasensitive NanoLC-MS of Subnanogram Protein Samples Using Second Generation Micropillar Array LC Technology with Orbitrap Exploris 480 and FAIMS PRO, *Anal. Chem.* **2021**, *93*, 25, 8704–8710.

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