LC-MS

Fast, sensitive, and reproducible nano- and capillary-flow LCMS methods for high–throughput proteome profiling using the Vanquish Neo UHPLC system hyphenated with the Orbitrap Exploris 480 MS

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Keywords

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Goal

Demonstrate the performance of the Thermo Scientific™ Vanquish™ Neo UHPLC System, the next–generation nano-, capillary- and micro-flow LC, coupled to a Thermo Scientific™ Orbitrap Exploris™ 480 Mass Spectrometer for high-throughput bottom–up proteome profiling using a 75 µm I.D. × 15 cm Thermo Scientific™ EASY-Spray™ PepMap™ Neo Column.

Introduction

Historically, discovery proteomics laboratories have achieved deep proteome profiling by using long columns (≥25 cm length) with 75 µm I.D. operated at nano-flow rates (approx. 300 nL/min). Such separation conditions provide high chromatographic resolution, but result in long runtimes and limit LC-MS throughput to several samples per day. While this remains the best route for maximizing protein coverage, there are two main disadvantages: (i) analyzing a sample cohort of sufficient size to generate impactful data is very time-consuming; (ii) actual mass spectrometer utilization is very low in traditional nano-flow applications because of the time required for sample loading, column washing and column equilibration at nano-flow rates.

Throughput limitations often preclude the adoption of nano-LC methods for translational proteomics applications such as biomarker validation and precision medicine study because a statistically significant number of samples must be analyzed to compare the biological variation and cases related variation. Although long capillary columns yield improved peak capacities, the pressure limitations of most commercial low-flow UHPLC systems lead to impractical total cycle times when using columns beyond 15 cm length.[1] Moreover, method overhead times can account for up to 50% of the total analysis time, leaving the MS idle for all but a short elution window.

Recently, high-throughput capillary- and micro-flow LC-MS methods have gained traction for fast proteome profiling due to improved sample throughput and method robustness over traditional nano-flow LCMS approaches.[2,3] The benefits of operating nanoLC columns packed with 2 µm particles at higher flow rates have been, until now, unavailable due to the pressure and workflow limitations of existing low-flow UHPLC systems. Another aspect of developing high-performance, high-throughput LC-MS methods is MS utilization. The impact of sample pickup and loading, column washing, and column equilibration on the cycle times can be reduced by utilizing ultra-high-pressure capabilities and intelligent method execution.

Here, we present the Vanquish Neo UHPLC system for high-throughput, bottom-up proteome profiling using elevated flow rates on 75 µm I.D. columns. The 1500 bar pressure limit for the UHPLC system and 75 µm I.D. × 15 cm, 2 µm EASY-Spray PepMap Neo columns provides the flexibility to run methods with throughputs from 24 to 180 samples per day without any changes to fluidics or hardware. Optimized sample pickup, sample loading, column washing, and column equilibration at increased flow rates enable up to 95% MS utilization. The described methods also provide an excellent level of proteome coverage, robustness, and reproducibility between multiple laboratories.

Experimental materials and methods

Sample preparation

Thermo Scientific™ Pierce™ HeLa Digest/PRTC Standard (A47996, 10 µg/vial) was reconstituted by adding 50 µL of 0.1% formic acid (FA) in water. The vial was subsequently sonicated for 2 min, followed by multiple sample aspiration and release cycles with a pipette to dissolve it completely. The final sample concentration was 200 ng/µL HeLa digest with 100 fmol/µL PRTC.

Consumables

- Fisher Scientific™ LC-MS grade water with 0.1% Formic Acid (P/N LS118500)
- Fisher Scientific™ LC-MS grade 80% acetonitrile with 0.1% Formic Acid (P/N LS122500)
- Fisher Scientific™ LC-MS grade Formic Acid (P/N A117-50)
- Fisher Scientific™ LC-MS grade Isopropanol (P/N A461-212)
- Fluidics and consumables used to set up Vanquish Neo UHPLC system for trap-and-elute injections are given in Table 1

Table 1. Vanquish Neo UHPLC system, fluidics and accessories for trap-and-elute workflow

Part number	Description	#
VN-S10-A-01	Vanquish Neo UHPLC System Binary Pump N, Split Sampler NT, Solvent Rack, Vanquish System Controller, and System base with drawer	1
6036.1180	Vanquish display	1
6PK1655	Vial and septa kit, 100/pack of • Vial 0.2 ml amber TPX screw 9 mm short thread conical glass insert • Cap screw 9 mm black PP white silicone/red PTFE septa bonded 1.0 mm	1
ES75150PN	EASY–Spray PepMap Neo column 75 µm × 150 mm, 2 µm, 1500 bar	1
174500	PepMap Neo trap column 300 µm × 5 mm, 5 µm, 1500 bar	1
174502	Trap column holder + Thermo Scientific™ nanoViper™ Fitting System, 1500 bar	1

LC solvents and system temperature settings

The required solvents to run high-throughput applications are described in Table 2.

Table 2. Solvents and temperature 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/ Isopropanol with 0.1% FA

*FA = Formic acid, ACN = Acetonitrile

Vanquish Neo UHPLC system method parameters

The generic parameters for sample aspiration, loading, and column equilibration are shown in Table 3. The separation gradients for five LC methods are described in Table 4. These five standard methods permit the sample throughput of 24, 48, 60, 100, and 180 per day, and available for download from Thermo Scientific™ AppsLab Library of Analytical Applications (AppsLab).

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

	Parameter	Value
Sample loading	Mode	FlowControl
	Flow	60 µL/min
	Loading volume*	Automatic
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
Trap column	Fast wash and equilibration	Enabled
	Wash factor	50
	Equilibration factor*	Automatic (*i.e.*, 2)
	Mode	CombinedControl
	Flow	200 µL/min
	Pressure	800 bar
	Trap flush direction	Backward
Temperature	EASY–Spray column temperature	50 °C
	Autosampler temperature*	7 °C
	Trap cartridge	Room temperature (ca. 23 °C)
Advanced sampler setting**	Neo.SamplerModule. Sampler.OuterNeedle WashCycleTime	Fast

*System default values

**Advanced setting available in the Instrument Setup section of the Instrument Method Script Editor

Table 4. Gradients for five high-throughput LC-MS methods optimized for 75 µm I.D. × 150 mm column in trap-and-elute workflow (excluding ca. 1.4-min duration of sample pickup and sample loading)

8–min method

Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0	0	1.3	4
3.7	3.7	1.3	20
5.5	1.8	1.3	35
Column wash phase			
5.9	0.4	1.3	99
6.6	0.7	1.3	99

14.4–min method

Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0	0	1.3	4
0.7	0.7	1.3	4.5
1	0.3	1.0	5
7.6	6.6	1.0	20
11.3	3.7	1.0	35
11.8	0.5	1.3	55
Column wash phase			
12.3	0.5	1.3	99
13	0.7	1.3	99

24–min method

Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0	0	1.3	4
0.7	0.7	1.3	4.5
1	0.3	0.8	5
14	13	0.8	20
20.9	6.9	0.8	35
21.4	0.5	1.3	55
Column wash phase			
21.9	0.5	1.3	99
22.6	0.7	1.3	99

48–min method

Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0	0	1.3	4
0.7	0.7	1.3	4.5
1	0.3	0.4	5
30	29	0.4	20
44.9	14.9	0.4	35
45.4	0.5	1.3	55
Column wash phase			
45.9	0.5	1.3	99
46.6	0.7	1.3	99

60–min method

Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0	0	1.3	4
0.7	0.7	1.3	4.5
1	0.3	0.3	5
38	37	0.3	20
56.9	18.9	0.3	35
57.4	0.5	1.3	55
Column wash phase			
57.9	0.5	1.3	99
58.6	0.7	1.3	99

MS acquisition parameters

MS data were acquired with an Orbitrap Exploris 480 mass spectrometer in data-dependent acquisition (DDA) mode with MS parameters designated for each LC method (Table 5 and 6). All LC-MS methods are available for download in AppsLab.

Table 5. MS acquisition parameters for five high-throughput methods

Category	Property	Common setting
Method Settings	Application Mode	Peptide
Ion Source	Positive Ion (V)	1900
	Ion Transfer Tube Temp (°C)	275
MS Global Settings	Infusion Mode	Liquid Chromatography
	Expected LC Peak Width (s)	15
	Advanced Peak Determination	TRUE
	Default Charge State	2
	Internal Mass Calibration	Off
Full Scan	Scan Range *(m/z)*	375–1200
	AGC Target	250
	RF Lense (%)	45
	Data Type	Profile
	Polarity	Positive
	Source Fragmentation	Disable
MIPS	Monoisotopic peak determination	Peptide
	Relax restrictions when too few precursors are found	TRUE
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
ddMS^2	Multiplex Ions	FALSE
	Isolation window *(m/z)*	2
	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

Table 6. Specific MS method parameters optimized for each method length

Category	Property	8–min method	14.4–min method	24–min method	48–min method	60–min method
Method Settings	Method Duration (min)	6.6	13	22.6	46.6	58.6
Full Scan	Orbitrap Resolution	45,000	60,000	60,000	60,000	60,000
	Maximum Injection Time (ms)	20	20	20	20	20
Intensity	Intensity Threshold	5.0E+03	8.0E+03	8.0E+03	8.0E+03	8.0E+03
Dynamic Exclusion	Exclusion duration (s)	25	25	30	45	50
Data Dependent	Number of Dependent Scans	40	40	40	30	30
ddMS^2	Orbitrap Resolution	7,500	7,500	7,500	15,000	15,000
	Maximum Injection Time (ms)	12	12	12	Auto	Auto

Data processing and analysis

Acquired data files were processed with Thermo Scientific™ Proteome Discoverer™ Software (version 2.5) using a 2-step Sequest™ HT search algorithm and INFERYS rescoring node. The false discovery rate (FDR) was set below 1% at the peptide and the protein level, respectively. The data processing templates are also available for download in AppsLab. Further data analysis and plotting were performed with R script.[3] The Skyline (version 21.1.1.160)[4] software package was used to process extracted ion chromatograms of PRTC peptides.

Results and discussion

Maximizing MS utilization for high throughput proteomics

Five LC-MS methods were developed to demonstrate the versatility of the Vanquish Neo UHPLC system and PepMap Neo column to perform across a wide range of sample throughputs. All five LC-MS methods utilize the 75 µm I.D. × 150 mm separation column and 0.3 mm I.D. × 5 mm trap column because they provide the flexibility to perform each method without the need to make any changes to the system or consumable setup. By operating the Vanquish Neo UHPLC system at different flow rates, MS utilization (calculated as the ratio of peptide elution window to cycle time) ranged from 68% to 95% with a throughput of up to 180 samples per 24 h (Table 7 and Figure 1). The obtained results show that the full width at half-maximum (FWHM) is reaching <3 sec for the shortest method and gradually increases with the increased gradient duration and the corresponding reduction of the flow rate (Figure 1A). The peptide and proteins identifications appear to reach the plateau when employing the 48-min method (Table 8) so we did not test methods with total cycle times above 60 min using a 150 mm long column. The longer columns should be utilized to achieve deeper proteome coverage for longer gradients.

Table 7. Overview of main characteristics for five high-throughput methods

Flow rate (µ/min)	Sample throughput/day	Cycle time (min)	Method duration (min)	Elution window (min)	MS utilization (%)
1.3	180	8	6.6	5.4	68
1.0	100	14.4	13.0	11.8	82
0.8	60	24	22.6	21.0	88
0.4	30	48	46.6	45.1	94
0.3	24	60	58.6	57.0	95

*MS utilization (%) = Peptide Elution Window (min)/Cycle Time (min) *100%

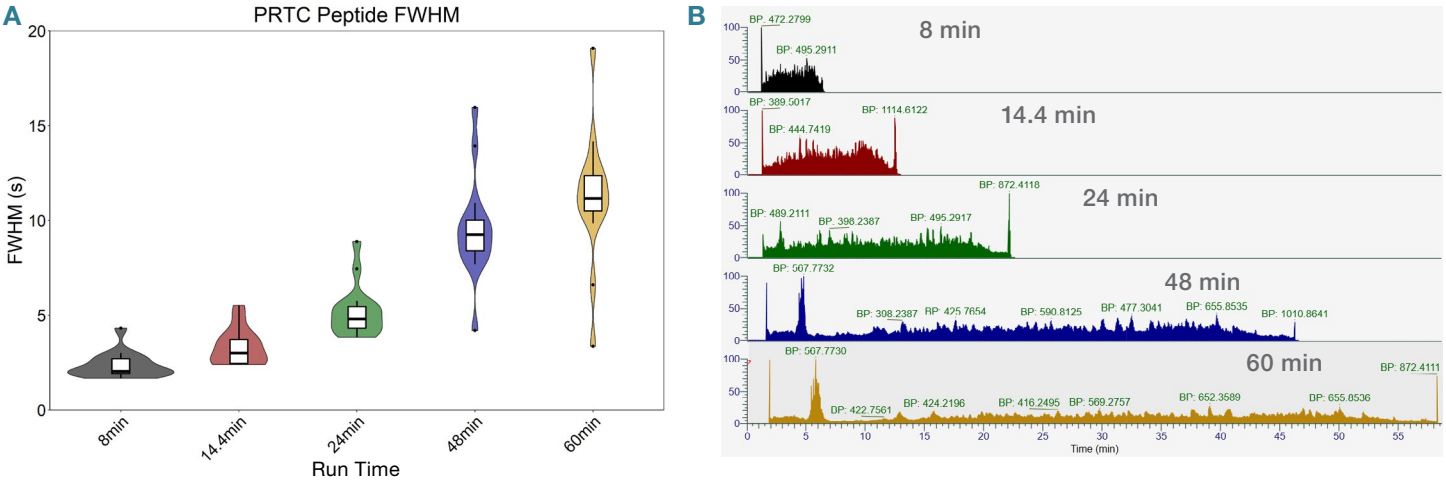
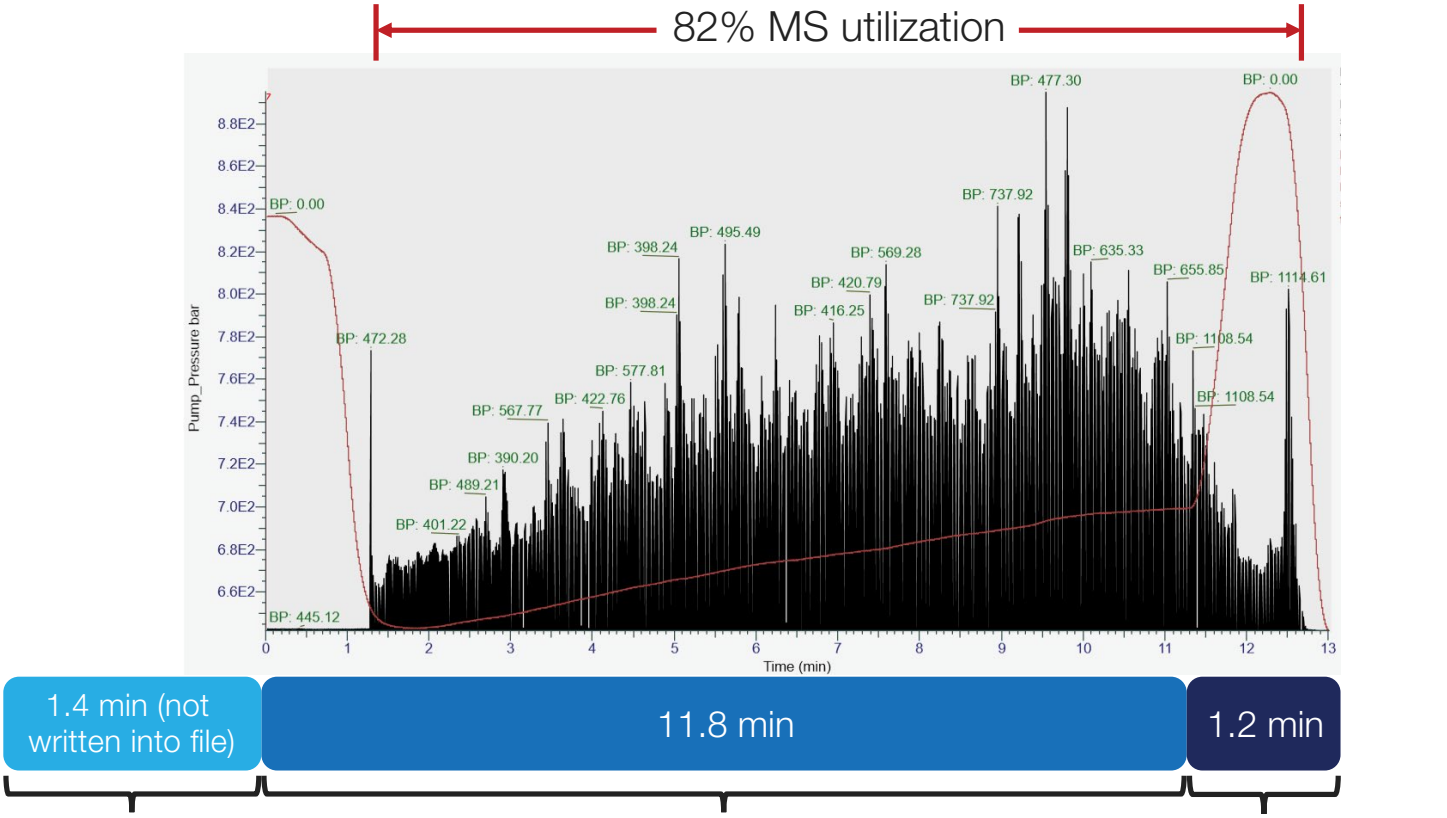


Figure 1. Five standard high-throughput LCMS methods provide (A) median value for FWHMs ranging from 2 to 11 sec (calculated for 15 PRTC peptides in 200 ng HeLa/PRTC sample) and deliver (B) excellent separation profiles for methods with 8 to 60 min total cycle time.

Vanquish Neo UHPLC methods are optimized to deliver high-throughput balanced with sensitivity, high MS utilization, low carryover, and high resolution. Here we explain the overall structure of developed methods based on the LC method with 14.4 min total cycle time (Figure 2). During the first phase with 1.4 min duration (Figure 2), the analytical column re-equilibration (EQ) was completed in parallel to sample pick-up (1 µL) and sample loading onto the trap column. Four column volumes were used to re-equilibrate the separation column at an elevated flow rate of 1.3 µL/min delivered by the pump. During the second phase with 11.8 min duration, peptides were separated with a multistep gradient of mobile phase B reaching 55% at the end of the gradient (Table 4). The flow rate was reduced to 1.0 µL/min to increase ESI MS sensitivity during the separation. In parallel to this, the autosampler performed an automated washing routine. During the third phase with 1.2 min duration, the extensive wash of the separation column was performed by increasing the flow rate to 1.3 µL/min and the proportion of mobile phase B to 99%. At the same time, the trap column was automatically switched offline by the LC system and extensively washed with 50 column volumes of strong wash liquid (12 µL 80/20% ACN/Water, 0.1%FA) and re-equilibrated with 100 column volumes of weak wash liquid (24.5 µL water with 0.1%FA) (Figure 2).



1) Separation Col. EQ
2) Injection
3) Loading

1) Gradient separation (1.0 µL/min)
2) Sampler wash

1) Separation col. wash (3 CV)
2) Trap col. wash (50 CV)
3) Trap col. EQ (100 CV)

Figure 2. The typical chromatogram of the 14.4 min method overlaid with the pressure profile (red). A breakdown of the steps demonstrates 82% MS utilization through parallel gradient separation and sampler washing procedures along with fast sample loading and column equilibration.

Column-to-column reproducibility enables reliable and reproducible results

We consistently identified ca. 1,200 proteins with the fast 8-min method and up to ca. 4,000 proteins with the 60-min method (Table 8). The Vanquish Neo UHPLC system allowed execution of all methods without changes to the column or fluidics. Such versatility enables selection of the most suitable cycle time for a particular application or sample complexity. The high-throughput LC-MS methods are usually deployed for the analysis of large sample cohorts. Thus, it is essential to achieve a low level of analytical variability not only for the analysis on the one set of separation and trap columns and one system but also across multiple sets of consumables and across multiple laboratories. Result reproducibility was verified using three sets of trap and separation columns. The Vanquish Neo UHPLC system coupled to an Orbitrap Exploris 480 delivered highly consistent results with below 2% RSD for the number of peptide and protein identifications within each of the five methods using the same set of trap and separation columns (Table 8). We also observed low inter-column variability that resulted in below 6% RSD in the number of proteins and peptides identified while using three different sets of PepMap Neo trap and separation columns on the same LC and MS hardware (Table 8).

Table 8. Reproducible peptide and protein identification with three columns using five high throughput methods

	Method	Average ID (n=15)	RSD, %, Intra-column			RSD, % Inter-columns (n=15)
			Column #1 (n=5)	Column #2 (n=5)	Column #3 (n=5)	
Peptide group	8 min	5670	0.8	0.7	1.2	5.8
	14.4 min	12056	0.9	0.9	0.4	2.3
	24 min	18469	0.4	1	0.7	2.4
	48 min	27110	0.3	0.4	0.4	4.3
	60 min	29081	0.5	0.3	0.4	5.5
Protein	8 min	1218	0.9	1.2	1.1	3.4
	14.4 min	2063	0.7	0.8	0.7	1.6
	24 min	2881	0.4	1.1	0.6	1.9
	48 min	3810	0.4	0.5	0.6	3.2
	60 min	4051	0.5	0.2	0.2	4.1

Reproducible performance during the longevity study

We tested the reproducibility of LC-MS results over a sequence of 100 injections with 200 ng HeLa protein digest during 24 hours using the 14.4-min method. Stable retention times for PRTC were observed during the analysis (Figure 3 and Table 9). The RSD values for retention times were between 1.1 and 5.5%. The method consistently identified ca. 11,800 peptide groups and >2,000 proteins (1% FDR) in each injection. After the joined processing of all injections we were able to quantify ca. 2,800 proteins when enabling the match between runs functionality (Figure 4A). Furthermore, 67% of all quantified proteins showed less than 25% abundance variation (Figure 4B) for 100 injections.

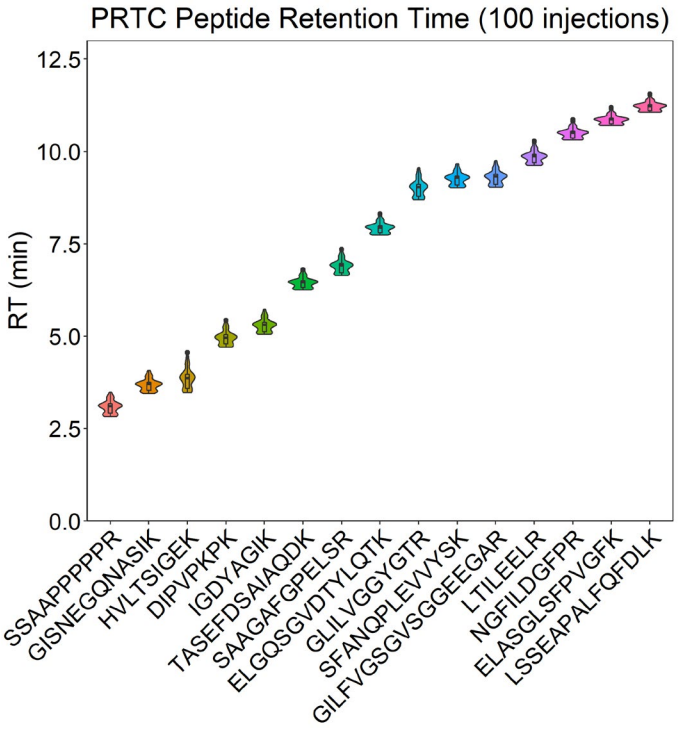


Figure 3. Stable retention times for PRTC peptides over 100 injections in 200 ng HeLa protein digest matrix with the 14.4-min method

Table 9. PRTC peptide retention times median values and variation over 100 injections

Peptide	RT (min)	RSD (%)
SSAAPPPPPR	3.1	5.5
GISNEGQNASIK	3.7	4.1
HVLTSIGEK	3.9	7.0
DIPVPKPK	5.0	3.6
IGDYAGIK	5.3	3.2
TASEFDSAIAQDK	6.4	2.1
SAAGAFGPELSR	6.9	2.6
ELGQSGVDTYLQTK	7.9	1.8
GLILVGGYGTR	9.0	2.5
SFANQPLEVVYSK	9.3	1.8
GILFVGSGVSGGEEGAR	9.3	1.9
LTILEELR	9.9	1.6
NGFILDGFPR	10.5	1.3
ELASGLSFPVGFK	10.9	1.1
LSSEAPALFQFDLK	11.2	1.1

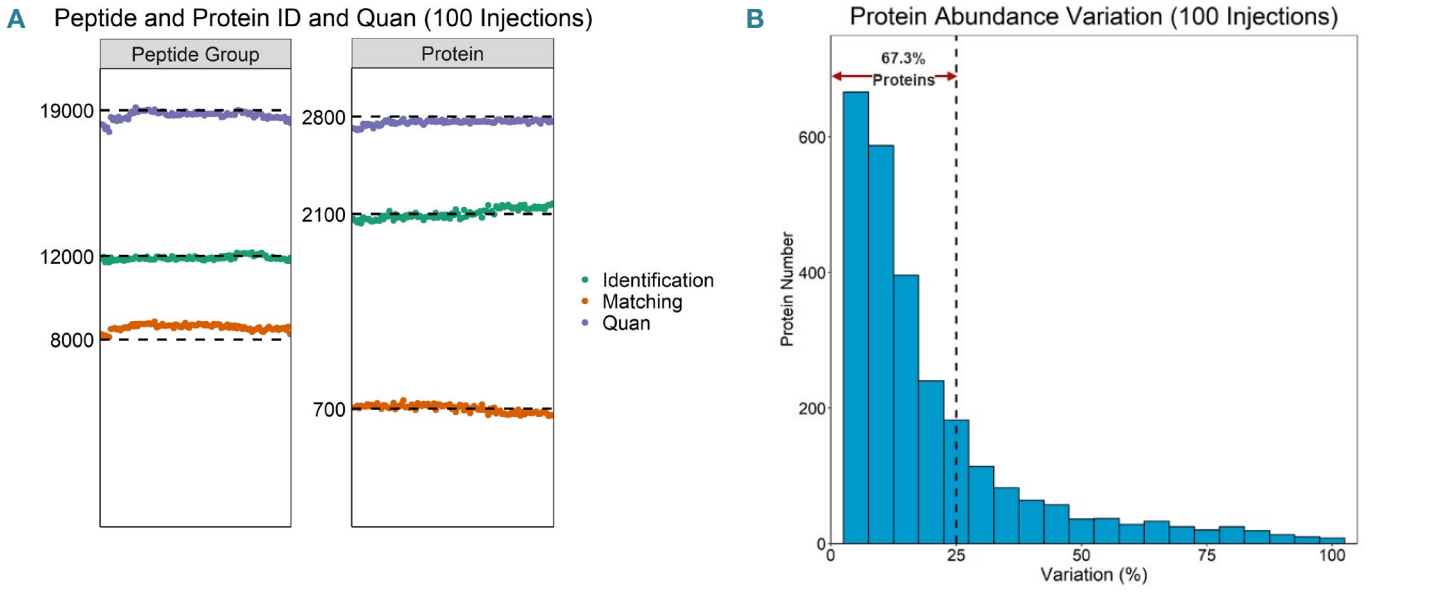


Figure 4. (A) The number of peptides and proteins (1% FDR) identified over 100 injections of 200 ng HeLa sample and (B) protein abundance variation for all quantified proteins.

Inter-laboratory reproducibility of high-throughput LC-MS analysis

Variations in LC, column, MS, sample preparation, solvents, operators, temperature, etc., can significantly impact overall LC-MS performance. In order to test the reproducibility of the presented high-throughput methods the same five methods were used in three different laboratories located in Germering (Germany), Bremen (Germany), and San Jose (USA). 3 to 5 replicates of each method length were performed at each location by different operators. The same data processing pipeline was used. Each individual MS data file was searched independently and all files were combined for global comparison of results across sites. Using optimized parameters from the method templates, highly reproducible performance was achieved both within each site and across sites (Figure 5). Strong reproducibility was observed with 72.5% to 88.5% of proteins commonly identified across all sites (Figure 6).

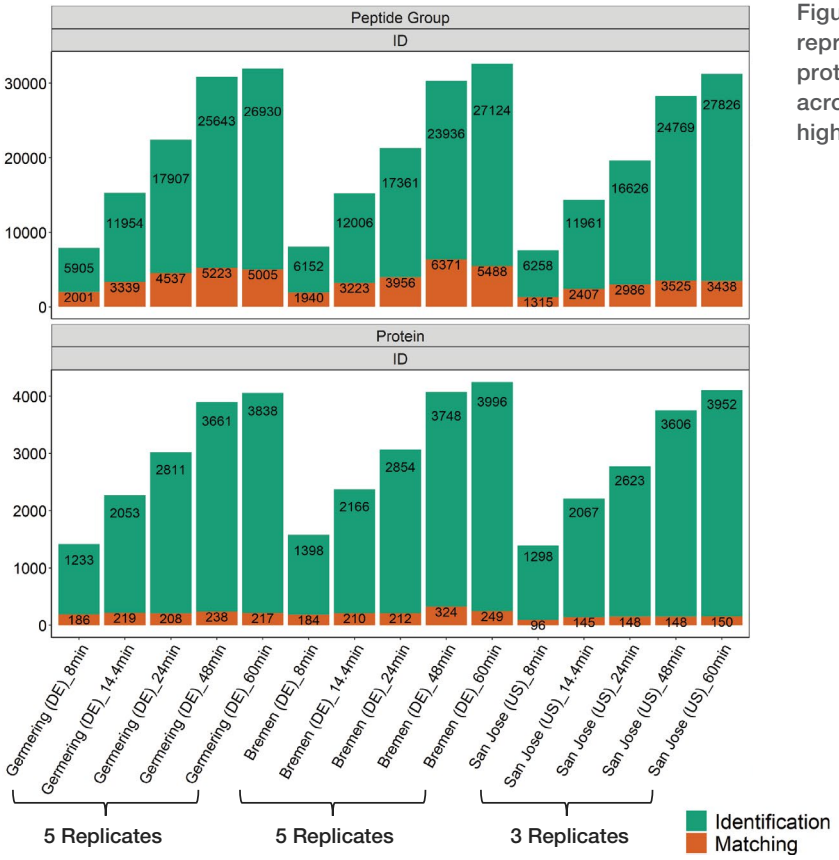


Figure 5. Highly reproducible peptide and protein identifications across three sites with five high–throughput methods

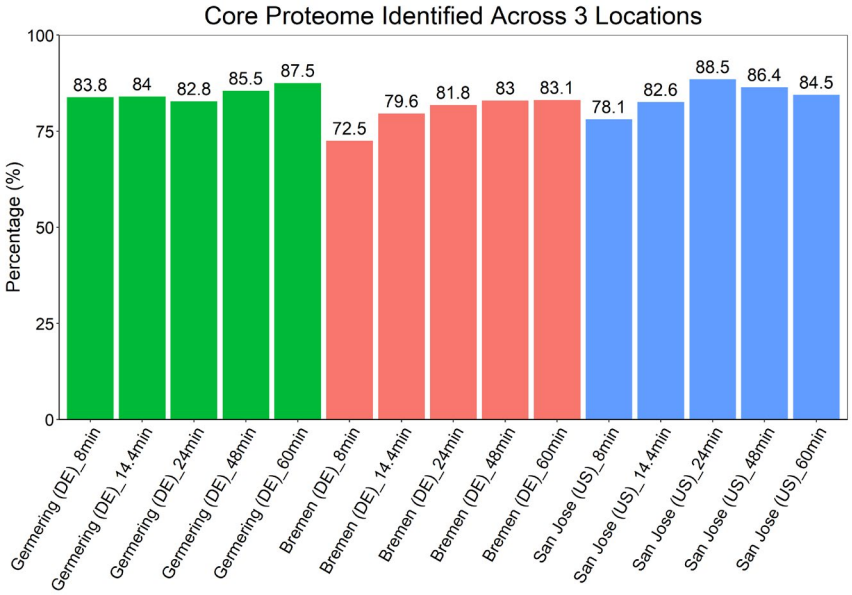


Figure 6. The proportion of proteins that were commonly identified across three laboratories (Germering, Bremen, San Jose) for each method length

Conclusions

We developed five high-throughput methods using the Vanquish Neo UHPLC system and a PepMap Neo column coupled with an Orbitrap Exploris 480 mass spectrometer for fast bottom-up proteome profiling to achieve 24, 30, 60, 100, and 180 samples per 24 hours. The Vanquish Neo UHPLC system enabled up to 95% MS utilization for enhanced peptide and protein identification and quantification when using a 75 µm I.D. × 150 mm column (2 µm particle) in the trap-and-elute workflow. The methods showed excellent reproducibility both intra- and inter-laboratories, regardless of the sample, column, instrument, and operator. Taken together, the Vanquish Neo UHPLC system combined with the latest PepMap Neo columns and Orbitrap HRAM MS is well-suited for sensitive, fast and robust LC-MS analysis of large sample cohorts.

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