HPLC columns

Advancing nanoLC-MS sensitivity for single cell proteomics using solid silicon micro-pillar array column technology

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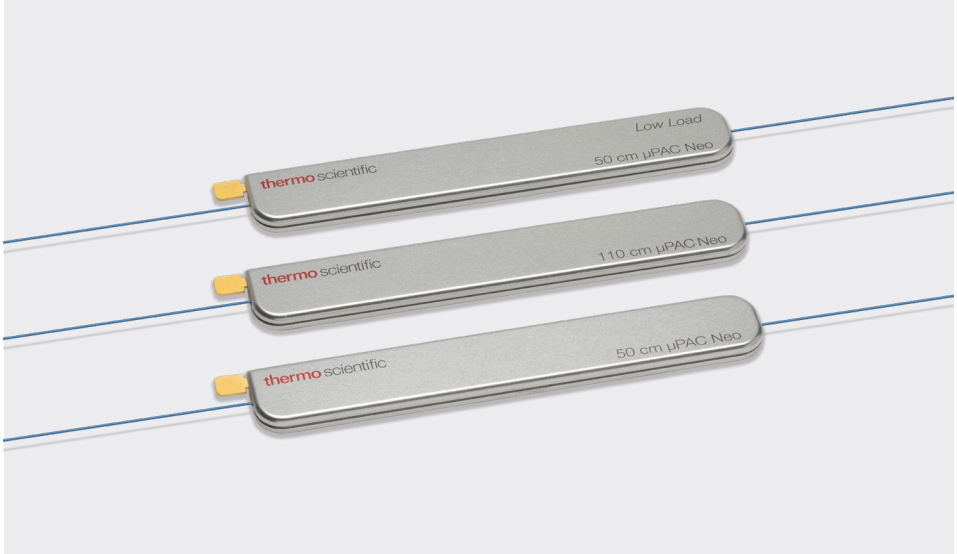


Abstract

Purpose

Sub-nanogram sample load proteome coverage and analysis throughput were increased using a novel non-porous micro pillar array based on Thermo Scientific™ 50 cm µPAC™ Neo low-load HPLC columns.

Methods

LC flow rate ramping methods were used to increase sample throughput for low flow (250, 125 and 65 nL/min) nanoLC methods. Effect on proteome coverage was assessed using standard LC-MS equipment and protein digest standards.

Results

Increases in ionization efficiency at 65 nL/min led to an increase in proteome coverage of up to 16% compared to 250 nL/min. 2862 protein groups could be consistently identified from 1 ng of protein digest sample.

Introduction

The practice of ultrasensitive MS-based proteomics has seen remarkable breakthroughs in the last few years. Improvements at different stages involved in the proteomics workflow have pushed sensitivity to a level which nowadays allows scientists to successfully identify and quantify more than 1000 proteins from a single mammalian cell. Optimized sample handling, maximum separation efficiency at low nanoLC flow rates and high resolution mass spectrometry are key technologies to achieve maximum sensitivity. In this contribution, we will describe a proof-of-concept study where LC separation conditions for a non-porous microfabricated pillar array column (Thermo Scientific µPAC Neo 50 cm HPLC column) type were optimized to get maximum proteome coverage from sub nanogram protein digest samples.

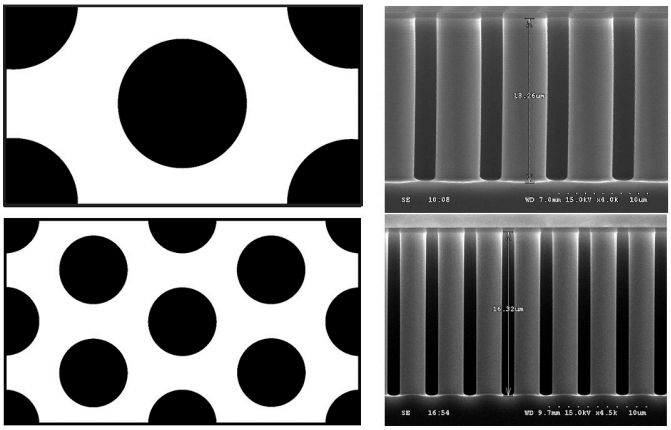


Figure 1. Left: Schematic overview of the unit cells used to design pillar array column chromatographic beds (Thermo Scientific™ µPAC™ GEN1 HPLC column versus 50 cm µPAC Neo low loads HPLC column). Right: Transverse SEM images of the respective Thermo Scientific™ µPAC™ chromatographic beds

Materials and methods

Sample preparation

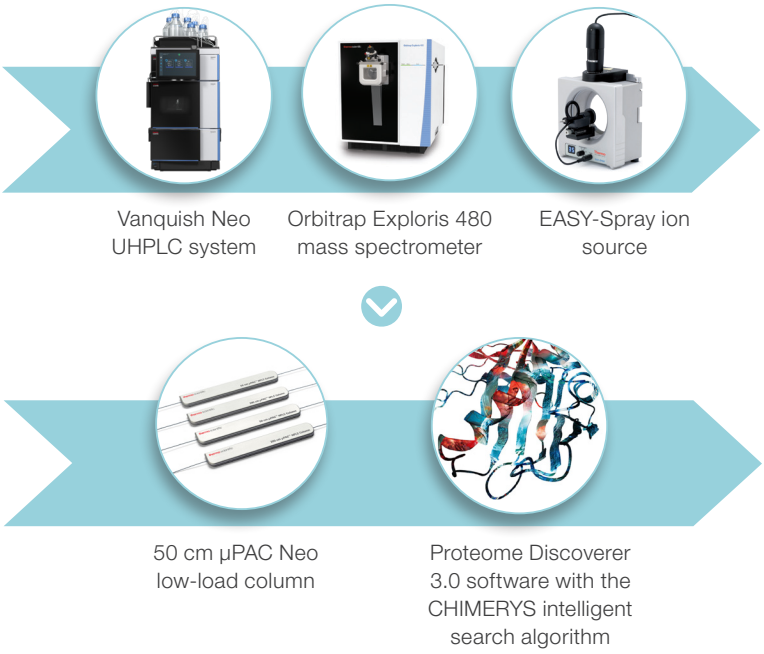
Thermo Scientific™ Pierce™ HeLa Protein Digest Standard was dissolved in 1% acetonitrile with 0.1% TFA to a concentration of 50 ng/µL.

Experimental set-up

All experiments were performed using a Thermo Scientific™ Vanquish™ Neo HPLC system coupled to a Thermo Scientific™ Orbitrap™ Exploris™ 240 mass spectrometer instrument. The Thermo Scientific 50 cm µPAC Neo low-load column was placed in the LC column compartment where it was maintained at a temperature of 40C. The Thermo Scientific™ NanoViper™ columns outlet was directly coupled to a Thermo Scientific™ EASY-Spray™ bullet emitter 10 µm ID (PN **ES993**) that is compatible with Thermo Scientific™ EASY-Spray™ electrospray ion sources.

LC-MS settings

LC separation was performed using a direct injection workflow in nano/cap mode. Inner diameter and column length specifications were set to 75 µm and 50 cm respectively, yielding a column volume of 1.48 µL. Maximum pressure was set at 400 bar, maximum flow rate was set at 0.8 µL/min. Sample loading was performed in CombinedControl mode at a pressure of 400 bar and with a fixed loading volume of 1 µL. Fast equilibration with an equilibration factor of 1.5 and at a pressure of 400 bar was enabled in CombinedControl mode. Injection volumes were varied between 10 and 200 nL. Electrospray ionization voltage was set at 1,9 kV. MS data were collected in data-dependent acquisition mode (Top10) with full scan data collected at 120,000 resolution and fragmentation data collected at 60,000 resolution. Quadrupole isolation width for MS2 acquisition was set at 4 Th. Maximum injection time (MaxIT) was set at 118 ms. The scan range used was 375-1500 *m/z*. Dynamic exclusion was set to 10 ppm with a 20 second duration. Fragmentation was performed using HCD with a fixed collision energy of 30.



Data analysis

The acquired raw data files were processed with Thermo Scientific™ Proteome Discoverer 3.0 software using the default Sequest HT_Percolator, INFERYS_Rescoring_SequestHT_ Percolator, and CHIMERYS_ Percolator workflows paired with a standard consensus workflow. Abundance of identified peptides was determined with a standard label free quantification (LFQ) consensus workflow and chromatgraphic metrics were determined using IMP-apQuant without match beween run mode (MBR).

Results

LC method optimization

As a result of the inherently low operating pressures associated with pillar array column types, flow rates can be varied over a wide range (50–750 nL/min). This gives the opportunity to operate relatively long columns (50 cm) at higher flow rates than typically used in nanoLC-MS analyses. Even though sensitivity is affected by operating at higher flow rates, column void times can be reduced significantly, which is highly desirable for high throughput analyses. This is clearly illustrated in Figure 2, where 2 ng of tryptic HeLa peptides were separated using different flow rates. At a flow rate of 750 nL/min, peptide elution starts at approximately 2 min (1.5 µL column volume). By reducing the flow rate by a factor of 3 (250 nL/min), column void time is increased to 6 min but a remarkable increase in signal intensity is observed. In order to combine the best of both methods, we have developed flow rate ramping methods that combine early elution at high flow rates with increased ionization efficiency at low flow rates.

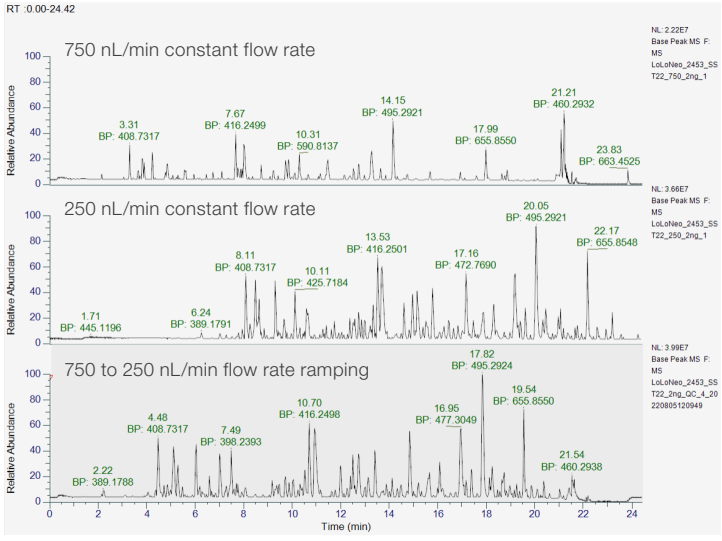


Figure 2A: Base peak chromatograms obtained for the separation of 2 ng of HeLa digest sample using constant flow rate or optimized flow rate ramping methods

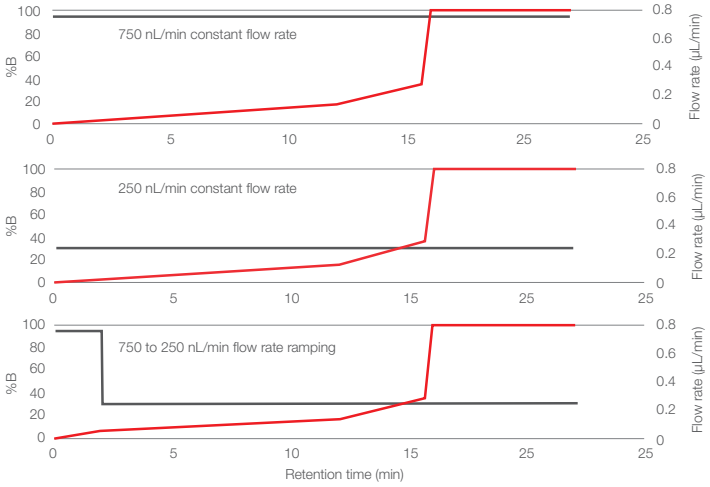


Figure 2B: Solvent gradient and flow rate profiles used for the comparison of constant flow rate to flow rate ramping

With an optimized flow rate ramping method, we could achieve similar sensitivity as obtained with the 250 nL/min constant flow method but with significantly reduced overhead time. The key to the development of these methods is proper determination of column volumes and combination with an LC instrument that can handle fast flow rate ramping (> 5 µL/min^2). Flow rate is reduced at the point where peptides start eluting, but the percentage organic modifier at which the ramping should occur also has to be defined properly. An example is given in Figure 3. In order to have consistent elution of peptides, the evolution of solvent gradient composition as a function of total solvent pumped should remain linear (in the case of a linear gradient segment). For the gradient profile shown in Figure 3, where a higher flow rate (750 nL/min) is used up to a time of 2 min, this implies that a steeper gradient should be used in the first segment in order to maintain linear volumetric gradient formation.

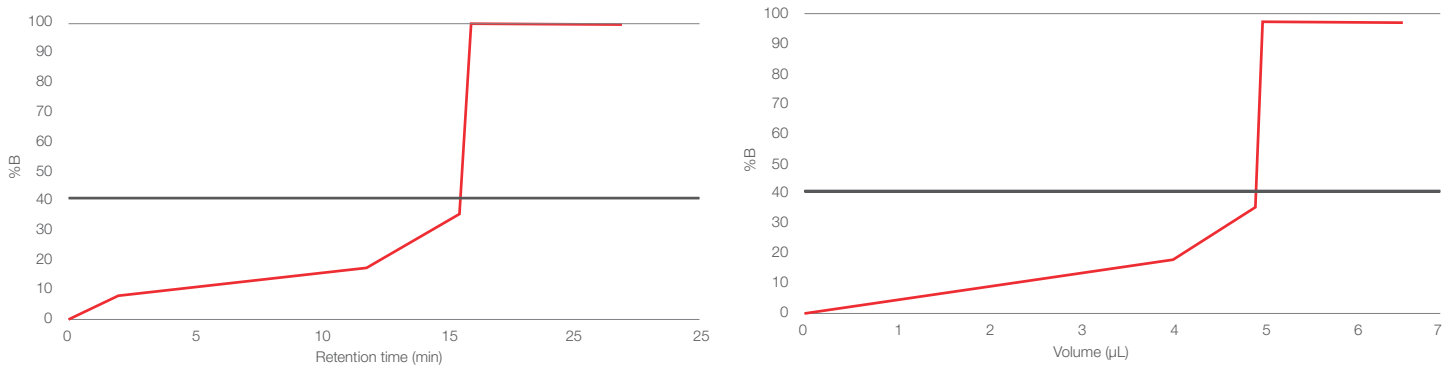
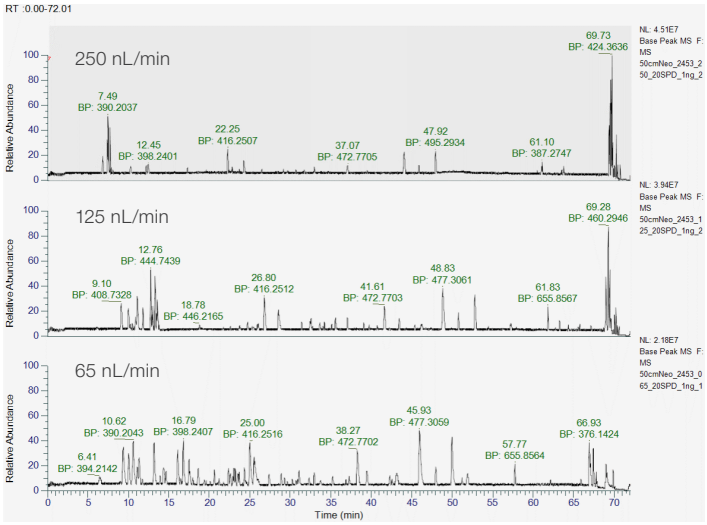


Figure 3. Solvent gradient profile used with flow rate ramping from 750 to 250 nL/min

Table 1. LC methods used with respective sample throughput

	100 SPD			80 SPD			60 SPD			40 SPD			20 SPD		
	Time (min)	Flow rate (µL/min)	%B	Time (min)	Flow rate (µL/min)	%B	Time (min)	Flow rate (µL/min)	%B	Time (min)	Flow rate (µL/min)	%B	Time (min)	Flow rate (µL/min)	%B
250 nL/min	0	750	1	0	750	1	0	750	1	0	750	1	0	750	1
	2	750	12	2	750	9	2	750	7	2	750	4.5	2	750	3
	2.1	250	12.1	2.1	250	9.1	2.1	250	7.1	2.1	250	4.6	2.1	250	3.1
	4.9	250	17.5	7	250	17.5	12.1	250	17.5	21.5	250	17.5	50	250	17.5
	5.4	250	35	8.9	250	35	15.6	250	35	27.7	250	35	63.7	250	35
	5.8	250	99	9.3	250	99	16	250	99	28.1	250	99	64.1	250	99
	11.8	250	99	15.3	250	99	22	250	99	34.1	250	99	70.1	250	99
125 nL/min				0	750	1	0	750	1	0	750	1	0	750	1
				2	750	13	2	750	11	2	750	9	2	750	4.5
				2.1	125	13.1	2.1	125	11.1	2.1	125	9.1	2.1	125	4.6
				3.2	125	17.5	6.7	125	17.5	16	125	17.5	44.1	125	17.5
				3.9	125	35	8.8	125	35	21	125	35	57.2	125	35
				4.7	125	99	9.6	125	99	21.8	125	99	58	125	99
				16.7	125	99	21.6	125	99	33.8	125	99	70	125	99
65 nL/min										0	750	1	0	750	1
										2	750	15	2	750	10
										2.1	65	15.1	2.1	65	10.1
										5.6	65	17.5	27.1	65	17.5
										9.1	65	35	44.1	65	35
										10.7	65	99	45.7	65	99
										34.7	65	99	69.7	65	99

The effect of flow rate ramping becomes even more pronounced when elution at ultra low flow (ULF) rates is desired. For eluting flow rates of 125 and 65 nL/min, flow rate ramping allows reducing column void time from 12 to 2 and from 24 to 2 min respectively. Parameters used for a range of flow rate ramping methods (100 to 20 samples per day and eluting flow rates of 250, 125 and 65 nL/min have been listed in (Table 1).



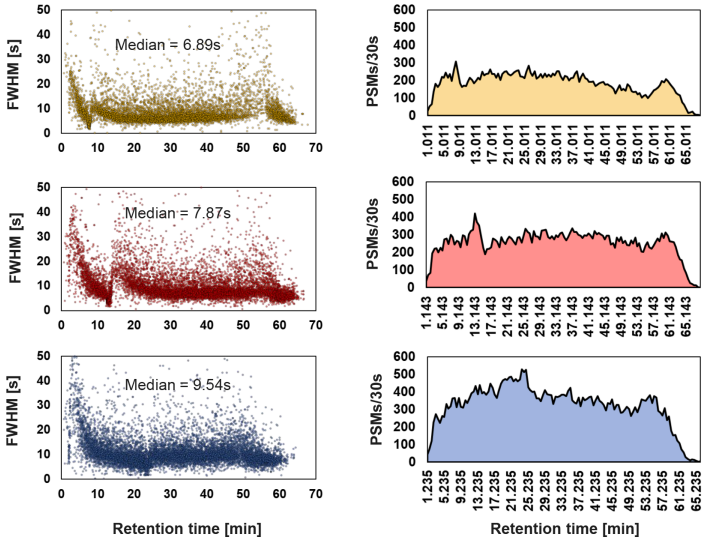


Figure 4. Results obtained at eluting flow rates of 250, 125 and 65 nL/min are compared at a sample throughput rate of 20 samples per day. Top: base peak chromatograms obtained for the separation of 1 ng of Hela cell digest. Bottom left: FWHM distribution. Bottom right: PSM identification rate

Proteome coverage

Using a complex protein digest standard (Hela cell lysate digest), we then evaluated the impact of increased ionization efficiency on proteome coverage that could be achieved for low input data dependent proteomics experiments. In agreement with earlier reports on the use of the CHIMERYS intelligent search algorithm, significantly more features could be identified compared to processing workflows using Sequest HT or Sequest HT combined with INFERYS rescoring. For sample loads of 1 ng and at a flow rate of 250 nL/min, the increase was most pronounced at the highest sample turnover rate (100 SPD), resulting in a 3-fold increase in protein group identifications. At 20 SPD, an increase of about 70% could be achieved yielding a total of close to 2500 protein groups.

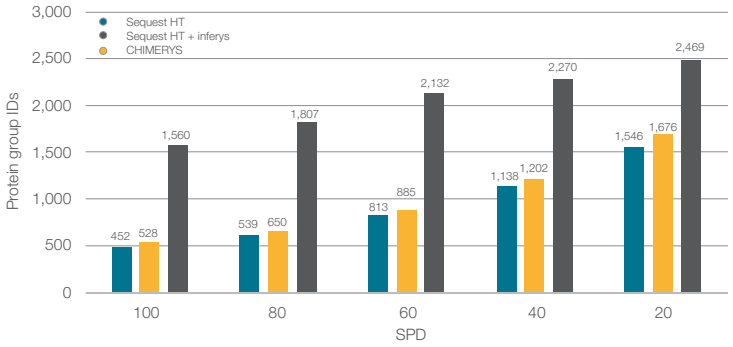


Figure 5. Comparison protein groups identified from 1 ng of HeLa digest sample using either Sequest HT, Sequest HT with INFERYS rescoring and CHIMERYS intelligent search algorithm processing. All processing with Proteome discoverer 3.0 at 1% FDR

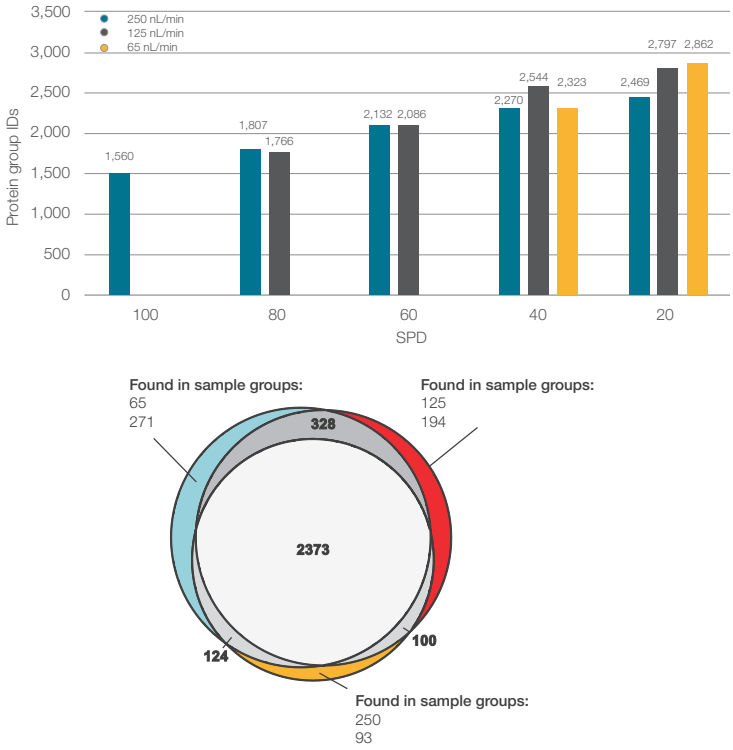


Figure 6. Top: Comparison of protein groups identified from 1 ng of HeLa digest sample using different flow rate ramping methods. Eluting flow rates of 250, 125 and 65 nL/min are compared. All processed with CHIMERYS at 1% FDR. Bottom: Venn diagram showing overlap between protein groups identified at flow rates of respectively 65, 125 and 250 nL/min, 20 samples per day method

Even though lower flow rates clearly give rise to increased ionization efficiency (Figure 4), the lowest flow rate does not consistently produce the most identification hits. For sample throughput rates ≥ 60 SPD, 250 nL/min gives the best results, whereas for 40 and 20 SPD, the 125 and 65 nL/min respectively give the highest proteome coverage.

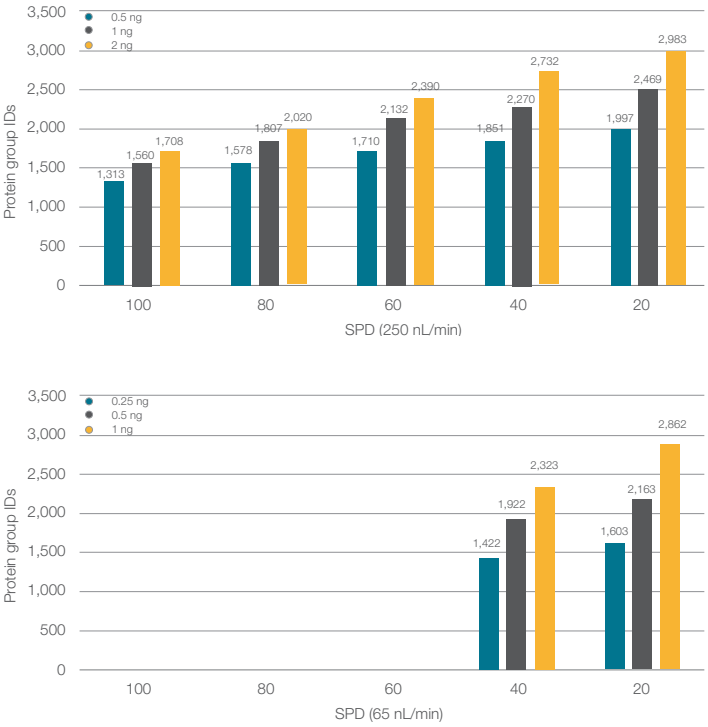


Figure 7. Protein group ID's obtained for the separation of 0.25-2 ng HeLa digest at different sample throuput rates. 250 and 65 nL/min methods shown. Technical injection replicates, n=3

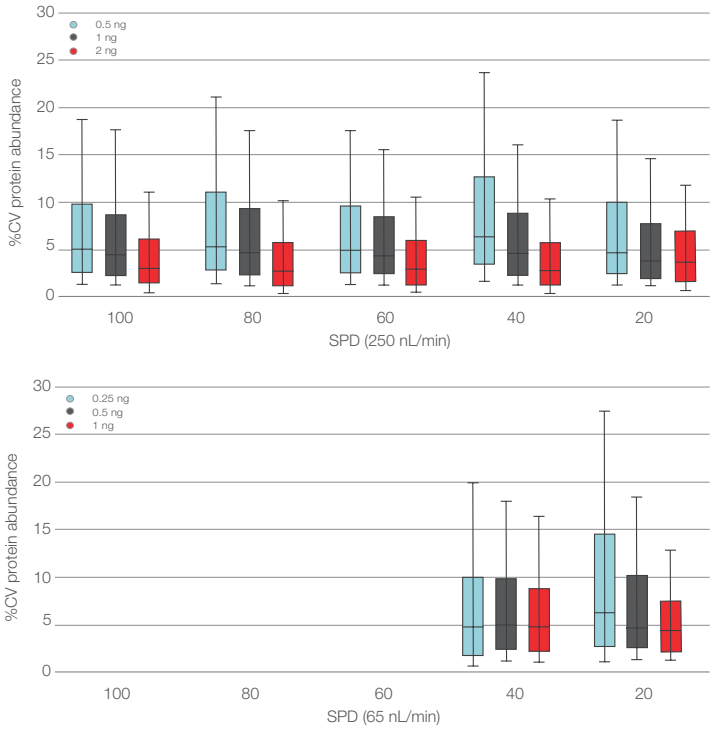


Figure 8. %Coefficient of variation on protein abundance obtained for label free quantitation of 0.25-2 ng HeLa digest samples at different sample throughput rates. 250 and 65 nL/min methods shown. Whiskers from 10-90th percentile. Technical injection replicates, n=3

Using optimized flow rate ramping methods at 250 nL/min, we were able to identify close to 2000 protein groups from as little as 500 pg of protein digest. By reducing the eluting flow rate by a factor of approximately 4 (down to 65 nL/min), proteome coverage could be even further increased, yielding a total of 2163 and 1603 protein groups identified on average from 500 and 250 pg of sample material. When performing label free quantitation using a standard consensus workflow, between 60 and 80% of the identified proteins could be quantified at CVs ≤ 20%. This resulted in consistent quantification of nearly 1900 proteins from 250 pg and over 2500 proteins from 500 pg sample material using the 20 samples per day 65 nL/min method.

Conclusions

With a standard bottom-up proteomics LC-MS set-up equipped with a Thermo Scientific µPAC Neo 50 cm HPLC column, sub nanogram protein digest samples were analyzed at sample throughput rates between 100 and 20 samples per day.

- The low back pressure allows for flow rate ramping with high flow rate at the beginning of the gradient
- Ultra low flow peptide elution can be achieved as from 2 minutes after sample injection
- Consistent identification and quantifaction of more than 2000 proteins from sub nanogram protein samples

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