APPLICATION NOTE

A Novel 75 cm Column Size Gives Increased Resolution and Better Sequence Coverage

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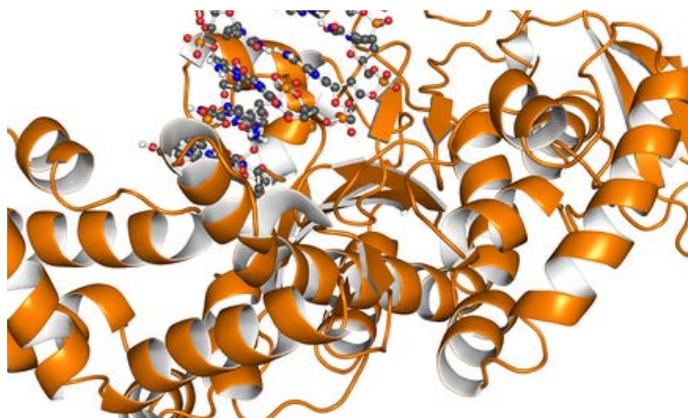
Key Words

EASY-Spray, ES805, HeLa, EASY-nLC 1200, PRTC, Q Exactive, bottom-up proteomics, peptides, sequence coverage, UHPLC, mass spectrometry, nanoLC, nanoLC-MS, RSLC nano

Goal

To demonstrate the improved capabilities of the Thermo Scientific™ EASY-Spray™ Acclaim™ PepMap™ C18 75 cm column and emitter by comparing their performance against the similar format 50 cm column. The increased peak capacity of the 75 cm column was demonstrated by using an extended gradient.

Introduction

The separation of complex peptide mixtures and their subsequent identification is crucial in the field of bottom-up proteomics.[1,2,3] The quality of the separation and the ability to maximize peak resolution, over a large range of peptides with different lengths and properties, is dependent on the separation efficiency of the columns and the choice of gradient elution profiles. Appropriate gradient choice on a high-quality column allows greater peak capacity and separation of more peptides. The use of longer gradient times with both the 50 cm and 75 cm columns has been shown to increase peak capacity.[4] Additionally, higher peak efficiency produces greater sensitivity with higher signal response, which allows detection of more peaks with greater confidence.

The following experiments demonstrate how the use of a novel 75 cm column gives:

• Higher peptide identification rates when same injection volumes are injected on 50 cm and 75 cm columns.

• Improved peak capacity and higher sensitivity allowing better sequence coverage, which is critical for better protein identification.

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A HeLa digest with a spiked peptide retention time calibration (PRTC) mixture was used for determination of peak capacity and efficiency, using an extended gradient.

Thermo Scientific™ Pierce™ HeLa digest provides a reproducible, complex quality-controlled standard for mass spectrometry. Spiking PRTC into this sample adds an additional 15 isotopically labeled peptides to assess optimal equipment and chromatographic performance.

Samples were introduced onto an EASY-Spray Acclaim PepMap C18 column by a Thermo Scientific™ EASY-nLC™ 1200 system. The EASY-nLC 1200 splitless, nanoflow UHPLC system is capable of operating at the higher backpressures that can arise from using longer columns with smaller (2 micron) packing materials.

Nanoflow UHPLC separations and nano-electrospray ionization can maximize LC-MS sensitivity, but are often reliant on numerous critical and error-prone connections to achieve optimum results. The connection of the EASY-Spray columns to the EASY-nLC 1200 was made with Thermo Scientific™ nanoViper™ fittings, which allowed for full use of the high pressure capability while minimizing dead volume. The EASY-Spray Acclaim PepMap C18 LC column integrated column/emitter design eliminates dead volumes and is temperature-controlled for maximum reliability and performance. Rigorously tested to ensure excellent spray stability, these columns are compatible for use up to 1200 bar*.

The Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer provides high resolution and mass accuracy of intact peptides and generates mass accurate MS-MS data using the HCD cell with detection by the Orbitrap™ mass spectrometer. It was set up in data-dependent mode for HeLa/PRTC samples.

The resulting data was interrogated using Thermo Scientific™ Proteome Discoverer™ 2.1 software, where the SEQUEST® HT algorithm was used to provide peptide identifications. Proteome Discoverer software simplifies a wide range of proteomics workflows, from protein and peptide identification to PTM analysis to isobaric mass tagging for quantitation.

Experimental
Consumables
- Fisher Scientific™ Optima™ UHPLC-MS grade water (P/N W8-1)

- Deionized water, 18.2 MΩ/cm resistivity

- Optima UHPLC-MS grade acetonitrile (P/N A956-1)

- Fisher Scientific analytical grade formic acid (P/N F/1900/PB08)

- Fisher Scientific 80% acetonitrile, 20% water, 0.1% formic acid (P/N LS122-500)

- Pierce HeLa Protein Digest Standard (P/N 88329)

- Pierce Peptide Retention Time Calibration Mixture (P/N TS-88320)

Sample Handling
- Thermo Scientific™ 11 mm blue Snap-It cap 6 mm hole, red PTFE/white silicone (P/N C4011-54B)

- Thermo Scientific 11 mm Crimp/Snap 250 µL MicroVial (P/N C4011-13)

* Dependent on column type

Sample Pretreatment

HeLa standard digest with PRTC peptide standards

The solution of HeLa was prepared using a vial of lyophilized standard containing 20 µg of the peptide mix. First, 0.4 µL of acetonitrile (ACN) was added to a conical polypropylene autosampler vial, of volume 300 µL. To this vial was added 4 µL of 0.5 pmol/µL PRTC solution standard. The HeLa digest was then suspended in solution by adding 15.6 µL of water + 0.1% formic acid (FA) to the HeLa standard vial. This solution was pipetted up and down several times to mix and then added to the conical polypropylene autosampler vial. The resulting HeLa concentration was 1 µg/µL and 100 fmol/µL in 98:2 water/acetonitrile with 0.08% formic acid. A 1 µL injection was carried out, loading 1 µg HeLa digest and 100 fmol PRTC on column.

Separation Conditions

Instrumentation

NanoLC-MS analysis was performed using the following:
- EASY-nLC 1200 System (P/N LC140)
- EASY-Spray Source (P/N ES081)
- Q Exactive Hybrid Quadrupole-Orbitrap Mass
  Spectrometer

Columns

EASY-Spray, Acclaim PepMap C18, 2 µm, 100 Å, 75 µm i.d. × 75 cm long column and emitter (P/N ES805)

EASY-Spray, Acclaim PepMap C18, 2 µm, 100 Å, 75 µm i.d. × 50 cm long column and emitter (P/N ES803)

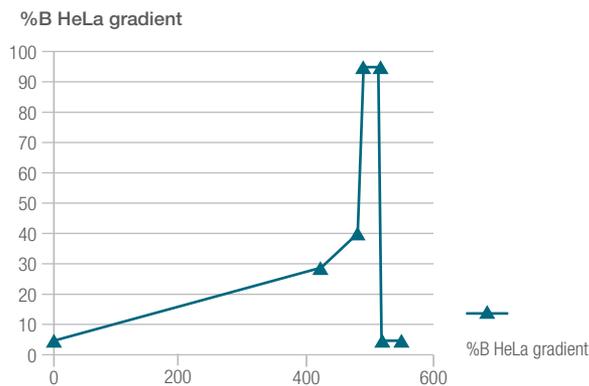|	
Mobile Phase A	100% water with 0.1% formic acid
Mobile Phase B	80:20 acetonitrile/water with 0.1% formic acid
Gradients	See Figure 1
Flow Rate	See Table 1
Column Temperature	35 °C

%B HeLa gradient

Figure 1. Gradient profile of the method used for HeLa + PRTC - 50 cm and 75 cm column.

Table 1. Gradient method (including flowrate), HeLa + PRTC - 50 cm and 75 cm column.

Time (min)	Duration (min)	Flow (nL/min)	%B HeLa gradient
0	0	200	5
420	420	200	28
480	60	200	40
490	10	200	95
515	25	200	95
520	5	200	5
545	25	200	5

MS Detection Conditions

Instrumentation
Q Exactive mass spectrometer

Source Parameters

Spray Voltage	1.9 kV
Capillary Temperature	320 °C

General Method Parameters

Chrom. Peak Width (FWHM)	10 s

Full MS Parameters for HeLa + PRTC Analysis

Resolution	60,000
AGC Target	3e6
Maximum IT	50 ms
Number of Scan Ranges	1
Scan Range	350–1200 *m/z*

MS/MS Instrument Parameters for HeLa + PRTC Analysis

Resolution	15,000
AGC Target	2e5
Maximum IT	80 ms
Isolation Window	1.4 *m/z*
Peptide Match	Preferred
Exclude Isotopes	On
Dynamic Exclusion	20.0 s

Software
Thermo Scientific™ Xcalibur™ 3.1, Foundation 3.1
Q Exactive – Orbitrap MS 2.7
EASY-nLC 3.0.0
Proteome Discoverer 2.1

To determine the ability of the longer columns to resolve an increased numbers of peptides, which can lead to a larger number of positive identifications by LC-MS/MS, the database conditions were set in Proteome Discoverer 2.1 software using parameters set in Table 2.

Table 2. Database setup.

Proteome Discoverer Software 2.1	Parameter
Min./Max Precursor Mass	400/5000 Da
Database	Human Swissprot (Downloaded 20160213)
Enzyme	Trypsin (Full)
Min./Max Peptide Length	6/144
Precursor/Fragment Mass Tolerance	10 ppm/0.02 Da
Dynamic Modifications	Oxidation (+15.995 Da M)
	Deamidated (+0.984 Da N)
Static Modifications	Carbamidomethyl (+57.021 Da C)
Percolator Data	
Input Data	Max Delta Cn (0.05)
Decoy Database Search	Target FDR (Strict) 0.01
	Target FDR (Relaxed) 0.05
	Variation based on q-Value

Results and Discussion

From a series of injections of the HeLa digest standard with the PRTC peptides added as markers, the 75 cm column gave representative base peak traces as shown in Figure 2. This was compared with a similar series of injections onto a 50 cm ES803 column (Figure 3).

The operating pressure of both the columns at 200 nL/min volumetric flow, under similar gradient conditions, showed pressures of 600–700 bar for the 75 cm column compared to 450–500 bar for the 50 cm column. These are well within the pressure range of the EASY-nLC 1200 system.
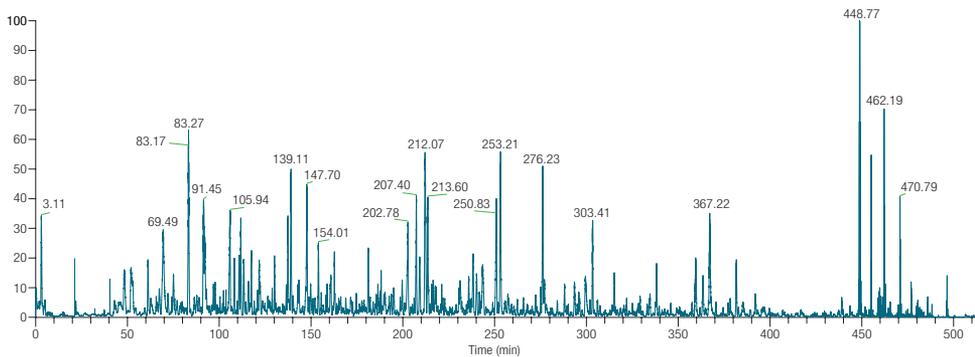
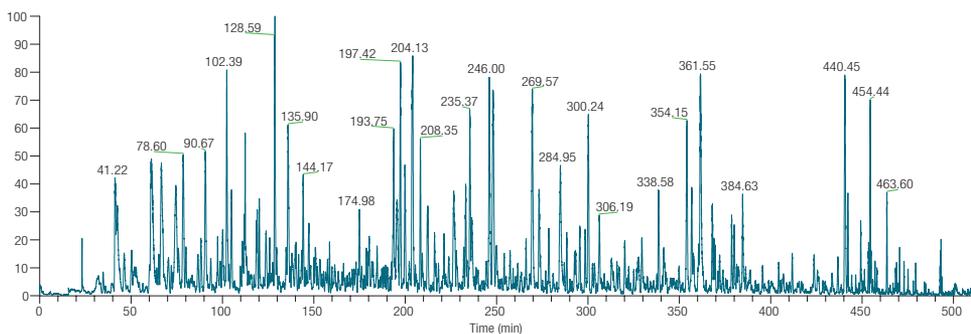Figure 2. 75 cm column HeLa PRTC base peak full scan.

Figure 3. 50 cm column HeLa PRTC base peak full scan.

Multiple injections of the HeLa/ PRTC solution (n = 5) were compared for the 50 cm and 75 cm columns. The data was processed and the average number of hits achieved was compared to highlight any differences in sequence coverage as shown in Table 3.

Table 3. Peptide and protein coverage search.

Column	PSM	Peptide Groups	Protein Groups
ES805 Average	170574	40230	5070
ES803 Average	118050	32013	4828
Improvement % of ES805 vs ES803	43.40%	25.67%	5.01%

Three peptides from the PRTC mixture were then monitored at their specific *m/z* values to allow discrimination of the labeled peptides from any native peptides. The peptides added have a heavy stable isotope incorporated to a single amino-acid in its sequence giving a +8 Da (K) or +10 Da (R).

IGDYAGI**K**   422.7363 *m/z*
GLILVGGYGT**R**   558.3259 *m/z*
SFANQPLEVVYS**K**   745.3924 *m/z*

The complete calibration mix composition is shown in Table 4.

Table 4. Peptide sequences, masses, and hydrophobicity factors.

Peptide Number	Peptide Sequence*	Mass	Observed Mass, Charge = +2	Hydrophobicity Factor
1	SSAAPPPPPR	985.522	493.7683	7.56
2	GISNEGQNASIK	1224.6189	613.3167	15.5
3	HVLTSIGEK	990.5589	496.2867	15.52
4	DIPVPKPK	900.5524	451.2834	17.65
5	IGDYAGIK	843.4582	422.7363	19.15
6	TASEFDSAIAQDK	1389.6503	695.8324	25.88
7	SAAGAFGPELSR	1171.5861	586.8003	25.24
8	ELGQSGVDTYLQTK	1545.7766	773.8955	28.37
9	GLILVGGYGTR	1114.6374	558.3259	32.18
10	GILFVGSGVSGGEEGAR	1600.8084	801.4115	34.5
11	SFANQPLEVVYSK	1488.7704	745.3924	34.96
12	LTILEELR	995.589	498.8018	37.3
13	NGFILDGFPR	1144.5905	573.3025	40.42
14	ELASGLSFPVGFK	1358.7326	680.3735	41.18
15	LSSEAPALFQFDLK	1572.8279	787.4212	46.66

*Amino acids in bold are labelled with heavy stable isotopes. Lysine (K) is 8 Da heavier and arginine (R) is 10 Da heavier.

These calibration peptides allowed the retention time and peak width to be directly compared between the 50 cm and 75 cm columns. An assessment was then made on how the resulting differences in chromatographic behavior affected the sequence coverage. The results are summarized in Figure 4 for peak efficiency and Figure 5 to show recorded retention times.

Comparing results from three 75 cm columns with the reference 50 cm column showed that for the three peptides the peak widths at half height were significantly less than the same peptides on the 50 cm column.
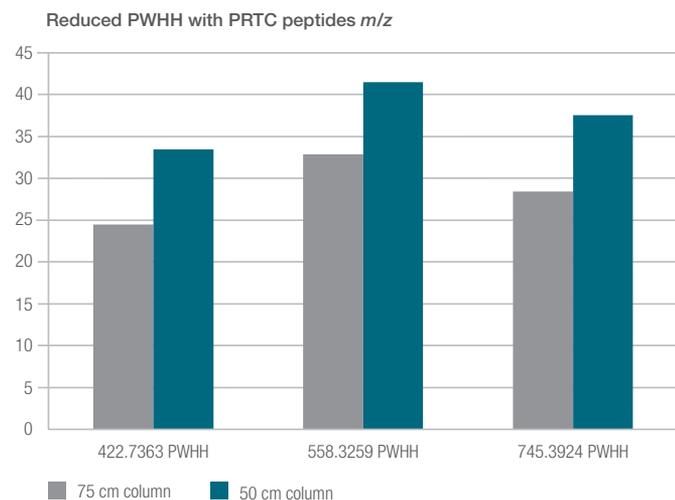

Figure 4. The PWHH values for the three peptides on the two column types.
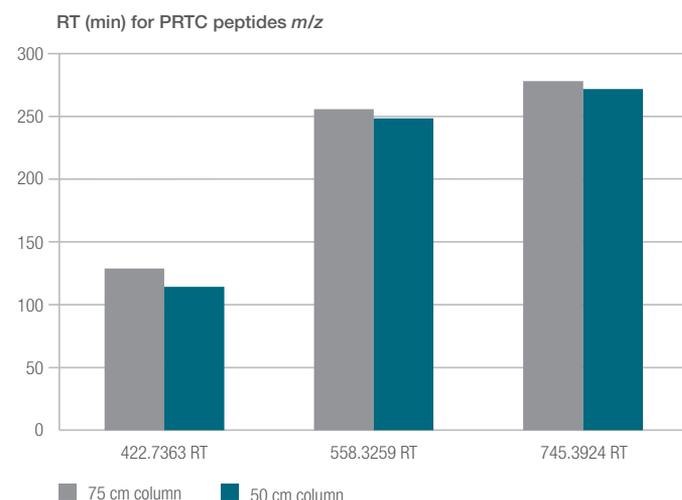

Figure 5. RT (min) for PRTC peptides on the two column types.

The positions of the PRTC peptides are not significantly lengthened by the use of the gradient, so existing sequencing rates will not increase by the use of the longer column and existing methods may be used for the searching of peptides. The gradient times of between 125 minutes and 275 minutes were covered by these three peptides.

Using the peak capacity calculation in Equation 1, peak capacity, C_p, was used to evaluate the performance, where n is the number of peaks used for the calculation, T_G is the gradient length, and W_p is the width at half of the peak height (PWHH). The 75 cm column has been shown to achieve a C_p of over 800 employing a 240-minute gradient.[1] For the two columns using an extended 500-minute gradient, this calculation gives a value of 1050 for the 75 cm column compared to the 800 for the 50 cm column.

The 75 cm column showed an increase in line with previous work carried out with shorter gradient times[1] with a difference in peak capacity of 20% between the two columns. The linear relationship with column length and gradient length would predict that at the same flow and gradient settings the potential increase in C_p would be limited to the effect of the gradient and the peak widths would also show some increase. The increase is approximately 25% from doubling the gradient length while retaining the same volumetric flow.

Equation 1. Calculation of peak capacity.

$$C_p = 1 + \frac{T_G}{\frac{1}{n} \Sigma_1^n W_p}$$

With the gradient time of 490 minutes, the EASY-nLC 1200 system and the 75 cm columns showed an RSD of approximately 0.4% for the above components. This corresponded to less than a 1.5 minute retention time shift over the mid-range of the gradient.

Table 5. Summary of the comparative performance of the columns.

Column	PWHH (s)		
	422.7363 m/z	558.3259 m/z	745.3924 m/z
75 cm column	24.48	32.88	28.44
50 cm column	33.492	41.52	37.56
Retention (min)			
	422.7363 m/z	558.3259 m/z	745.3924 m/z
75 cm column	128.79	255.58	277.88
50 cm column	114.07	248.69	271.87
	Gradient Time (Min)	Average PWHH (s)	C_p (calc)
75 cm column	500	28.6	1050
50 cm column	500	37.5	800

Conclusions

- The 75 cm column with a HeLa digestion method showed that the peptide group differentiation was increased by over 25%, showing greater peptide recognition and increased sequence coverage.

- The chromatographic performance of standard peptides from the PRTC test mix showed that the peak width at half height was reduced from 40 s to between 25 s and 38 s with the 75 cm column, without any significant increase in the retention time of the calibration peaks selected.

- Comparison of the peak capacity for the two column types showed an increase of 20% for the 75 cm column compared to the 50 cm column.

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