APPLICATION NOTE

No. 21550

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

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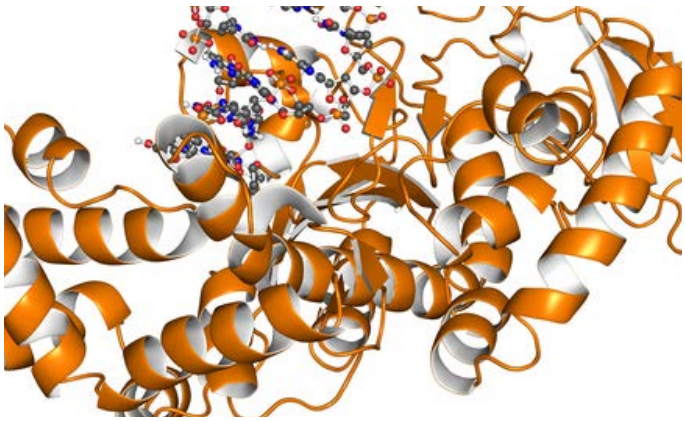

**Key Words**
**EASY-Spray, ES805, HeLa, EASY-nLC 1200, PRTC, Q Exactive, bottom-up proteomics, peptides, sequence coverage, UHPLC, mass spectrometry, nanoLC, nanoLC-MS, RSLC nano**

## 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 |

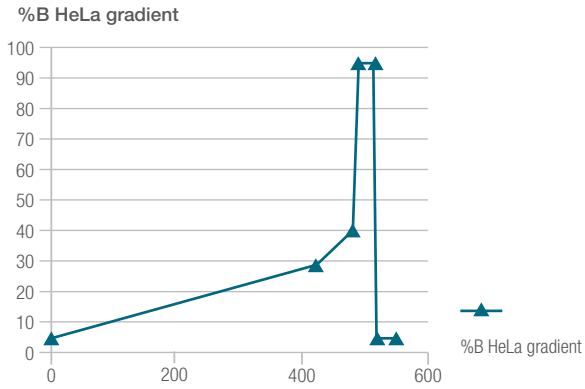


**Figure 1. Gradient profile of the method used for HeLa + PRTC - 50 cm and 75 cm column.**

**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.

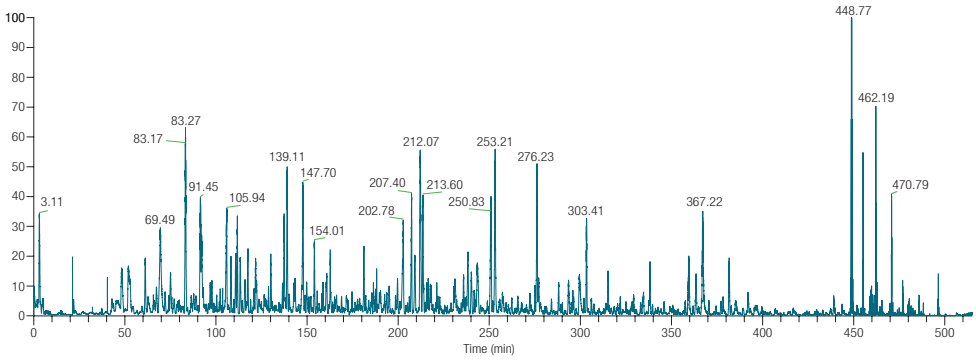


**Figure 2. 75 cm column HeLa PRTC base peak full scan.**

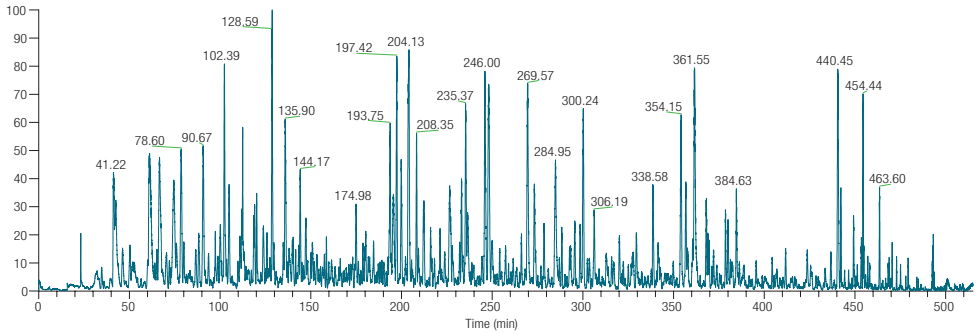


**Figure 3. 50 cm column HeLa PRTC base peak full scan.**

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 | SSAAPPPPP**R** | 985.522 | 493.7683 | 7.56 |
| 2 | GISNEGQNASI**K** | 1224.6189 | 613.3167 | 15.5 |
| 3 | HVLTSIGE**K** | 990.5589 | 496.2867 | 15.52 |
| 4 | DIPVPKP**K** | 900.5524 | 451.2834 | 17.65 |
| 5 | IGDYAGI**K** | 843.4582 | 422.7363 | 19.15 |
| 6 | TASEFDSAIAQD**K** | 1389.6503 | 695.8324 | 25.88 |
| 7 | SAAGAFGPELS**R** | 1171.5861 | 586.8003 | 25.24 |
| 8 | ELGQSGVDTYLQT**K** | 1545.7766 | 773.8955 | 28.37 |
| 9 | GLILVGGYGT**R** | 1114.6374 | 558.3259 | 32.18 |
| 10 | GILFVGSGVSGGEEGA**R** | 1600.8084 | 801.4115 | 34.5 |
| 11 | SFANQPLEVVYS**K** | 1488.7704 | 745.3924 | 34.96 |
| 12 | LTILEEL**R** | 995.589 | 498.8018 | 37.3 |
| 13 | NGFILDGFP**R** | 1144.5905 | 573.3025 | 40.42 |
| 14 | ELASGLSFPVGF**K** | 1358.7326 | 680.3735 | 41.18 |
| 15 | LSSEAPALFQFDL**K** | 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.

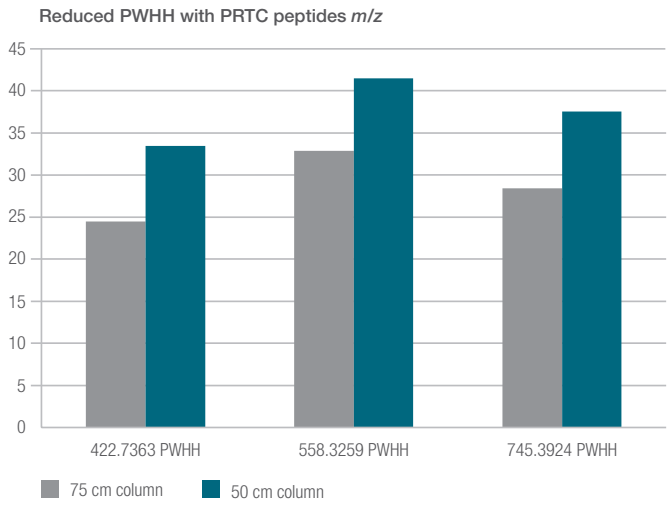


Figure 4. The PWHH values for the three peptides on the two column types.

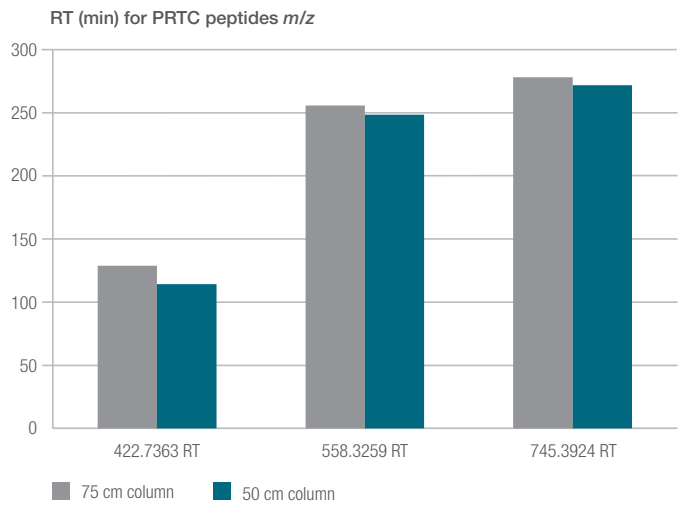


Figure 5. RT (min) for PRTC peptides on the two column types.

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.**

$$C_p = 1 + \frac{T_G}{\frac{1}{n}\sum_1^n W_p}$$

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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## References


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