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LC-MS

# Ultra-robust micro-flow LC-MS/MS for targeted high-throughput peptide quantification using the Vanquish Neo UHPLC system


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## Keywords

micro-flow, cycle time, proteomics, screening, peptide quantification, direct injection, LC-MS, UHPLC, high-throughput


## Goal

Demonstrate the robust performance of the Thermo Scientific™ Vanquish™ Neo UHPLC system for micro-flow LC-MS/MS based peptide quantification

## Introduction

Peptide analysis with nano-flow gradient separations on long (≥25 cm) analytical columns coupled with HRAM (high-resolution accurate-mass) mass spectrometry is the standard for discovery-based "bottom-up" proteomics research. While delivering unsurpassed sensitivity and depth of analysis, nano-flow setups have yet to achieve the throughput and robustness required for large sample cohort analysis. Therefore, nano-flow LC-MS has been of limited use for translational proteomics, in particular for validation studies.

Increasing flow rates in combination with shorter gradients and shorter nano-flow columns (75 µm ID × 15 cm length) can increase sample throughput.[1] However, higher flow rates result in reduced electrospray ionization efficiency,[2] which are difficult to compensate for by larger injection amounts due to the limited sample loading capacity of nano-flow columns. Hence, the uptake of such methods for validation studies is limited.

Micro-flow LC can now overcome this limitation. Its inception dates back to the mid-1970s, but its potential is only now being fully realized. Modern mass spectrometers deliver high detection sensitivity at micro-flow flow rates; improved quality of UHPLC separation columns and packing materials results in better run-to-run and column-to-column reproducibility; and the latest generation of low-flow UHPLC systems, such as the Vanquish Neo UHPLC system, deliver outstanding injection and retention time reproducibility at micro-flow rates and system pressures above 1000 bar. The combination of these technological advances in mass spectrometry and liquid chromatography enables high-throughput proteomic screening of large sample cohorts.

In this Technical Note, a previously published high-throughput micro-flow method[3,4] has been adapted to the new capabilities of the Vanquish Neo UHPLC system and rigorously tested for reproducibility and robustness. Fast sample loading and accelerated column washing and equilibration result in shorter cycle times without reduced performance or method robustness. With a flow rate of 50 µL/min during the gradient phase, the method achieves sufficient detection sensitivity and sample throughput while the cycle time is <15 minutes.

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## Materials and methods

LC-MS grade solvents were used (Table 1). HeLa protein digest spiked with PRTC (Table 1) was diluted to 50 ng/µL and 25 fmol/µL with 200 µL water, 0.1% formic acid.

**Table 1. Solvents and additives**

| Reagent | Grade | Supplier | Part number |
|---|---|---|---|
| HeLa Digest spiked with PRTC, 10 µg and 5 pmol | N/A | Thermo Scientific™ Pierce™ | A47996 |
| Acetonitrile with 0.1% formic acid | Optima™ LC-MS | Fisher Chemical | LS120-212 |
| Isopropanol | Optima™ LC-MS | Fisher Chemical | A461-212 |
| Formic acid | Optima™ LC-MS | Fisher Chemical | A117-50 |
| Water with 0.1% formic acid | Optima™ LC-MS | Fisher Chemical | LS118-212 |
| Water | Ultra-Pure, 18.2 MΩ at 25 °C | Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System | |

LC-MS analyses were performed on a Vanquish Neo UHPLC system (Table 2) connected to a Thermo Scientific™ TSQ Altis™ triple quadrupole mass spectrometer. For electrospray ionization a Thermo Scientific™ OptaMax™ NG source in HESI configuration was fitted with a 50 µm ID low-flow HESI needle (PN OPTON-30139). For reduced post-column volume the PEEK capillary connecting the grounding union to the HESI needle was replaced by a 50 µm × 150 mm Thermo Scientific™ nanoViper™ capillary (PN 6041.5124).

**Table 2. Vanquish Neo UHPLC system with thermostatted column compartment**

| Module | Part number |
|---|---|
| Vanquish Neo UHPLC system (comprising Binary Pump N, Split Sampler NT, Solvent Rack, System base with drawer, Ship kit) | VN-S10-A-01 |
| Vanquish Display (required) | 6036.1180 |
| Column Compartment N | VN-C10-A-01 |

The Vanquish Neo UHPLC system was set up in micro-flow direct injection mode with 50 µm ID nanoViper capillaries using the solvents listed in Table 3. A Thermo Scientific™ Acclaim™ PepMap™ 1.0 mm ID × 15 cm column (PN 164711) was placed in the column compartment. LC method details are described in Table 4 and Table 5. MS method details and SRM transitions are described in Table 6 and Table 7 and can be downloaded from Thermo Scientific™ AppsLab Library of Analytical Applications.

The LC-MS system was operated with Thermo Scientific™ Chromeleon™ 7.2.10 MUd. Alternatively, Thermo Scientific™ Xcalibur™ with Thermo Scientific™ SII for Xcalibur™ 1.5.1 software can be used.

**Table 3. Solvents of LC-MS method**

| Solvent | Composition |
|---|---|
| Eluent A | 100% water, 0.1% formic acid |
| Eluent B | 80% acetonitrile, 20% water (v/v), 0.1% formic acid |
| Weak Wash Liquid of Metering Device | 100% water, 0.1% formic acid |
| Strong Wash Liquid of Metering Device | 80% acetonitrile, 20% water (v/v), 0.1% formic acid |
| Weak (Outer) Needle Wash Liquid | 100% water, 0.1% formic acid |
| Strong (Outer) Needle Wash Liquid | 80% acetonitrile, 20% water (v/v), 0.1% formic acid |

**Table 4. Gradient of LC-MS method**

| Time (min) | Duration (min) | Flow rate (µL/min) | %B |
|---|---|---|---|
| Gradient separation phase | | | |
| 0.0 | 0 | 100 | 1 |
| 0.2 | 0.2 | 100 | 8 |
| 2.2 | 2.0 | 50 | 13 |
| 10.7 | 8.5 | 50 | 35 |
| Column wash phase | | | |
| 10.9 | 0.2 | 50 | 99 |
| 11.2 | 0.3 | 50 | 99 |
| 11.4 | 0.2 | 100 | 99 |
| 12.4 | 1.0 | 100 | 99 |

Table 5. LC method parameters of LC-MS method

| Parameter | Value |
|---|---|
| **Pick-up and loading settings** | |
| Keep Loop Inline | Disabled |
| Fast Loading | Disabled |
| Loading Volume | Automatic |
| 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 settings** | |
| Fast Equilibration | Disabled |
| Equilibration Factor | 1.0 |
| **Temperature settings** | |
| Column Compartment Temperature | 50 °C |
| Autosampler Temperature | 4 °C |

Table 6. MS method parameters of LC-MS method

| Parameter | Value |
|---|---|
| **Source parameters** | |
| Spray Voltage (static, positive) | 3500 V |
| Sheath Gas | 25 psig |
| Auxiliary Gas | 5 psig |
| Sweep Gas | 0 psig |
| Ion Transfer Tube Temperature | 325 °C |
| Vaporizer Temperature | 75 °C |
| **Scan parameters** | |
| Acquisition Mode | SRM (selected reaction monitoring) |
| Polarity | Positive |
| Cycle Time | 0.5 s |
| Use Calibrated RF Lens | Enabled |
| Q1 Resolution (FWHM) | 0.7 amu |
| Q3 Resolution (FWHM) | 1.2 amu |
| CID Gas | 1.5 mTorr |
| Source Fragmentation | 0 V |
| Chromatographic Peak Width | 6 s |
| Use Chrome Filter | Enabled |

Table 7. Selected reaction monitoring (SRM) transitions of LC-MS method. Transition used for quantitation marked in bold. (Note: PRTC peptides are isotopically labeled. PRTC peptides marked with * were not used for method evaluation in this work due to their low signal response).

| PRTC peptide | Start time (min) | End time (min) | Precursor (*m/z*) | Product (*m/z*) | Collision energy (V) |
|---|---|---|---|---|---|
| SSAAPPPPPR | 0 | 12.4 | 493.768 | 476.286 | 18.9 |
| | | | | 573.338 | 18.9 |
| | | | | **670.391** | **18.9** |
| | | | | 741.428 | 18.9 |
| | | | | 812.465 | 18.9 |
| GISNEGQNASIK | 0 | 12.4 | 613.316 | 540.323 | 23 |
| | | | | **725.403** | **23** |
| | | | | 854.446 | 23 |
| | | | | 968.489 | 23 |
| | | | | 1055.521 | 23 |
| *HVLTSIGEK | 0 | 12.4 | 496.286 | 454.275 | 19 |
| | | | | 541.307 | 19 |
| | | | | 642.355 | 19 |
| | | | | 755.439 | 19 |
| | | | | 854.507 | 19 |
| *DIPVPKPK | 0 | 12.4 | 451.283 | 380.275 | 17.5 |
| | | | | 477.327 | 17.5 |
| | | | | 576.396 | 17.5 |
| | | | | 673.449 | 17.5 |
| | | | | 786.533 | 17.5 |
| IGDYAGIK | 0 | 12.4 | 422.736 | 366.194 | 16.6 |
| | | | | 396.270 | 16.6 |
| | | | | 559.333 | 16.6 |
| | | | | 674.360 | 16.6 |
| | | | | **731.381** | **16.6** |
| *TASEFDSAIAQDK | 0 | 12.4 | 695.832 | 582.334 | 25.8 |
| | | | | 740.403 | 25.8 |
| | | | | 855.430 | 25.8 |
| | | | | 1002.498 | 25.8 |
| | | | | 1218.573 | 25.8 |
| SAAGAFGPELSR | 0 | 12.4 | 586.800 | 611.339 | 22.1 |
| | | | | **668.360** | **22.1** |
| | | | | 815.429 | 22.1 |
| | | | | 886.466 | 22.1 |
| | | | | 943.487 | 22.1 |
| ELGQSGVDTYLQTK | 0 | 12.4 | 773.895 | 660.381 | 28.5 |
| | | | | 761.428 | 28.5 |
| | | | | 876.455 | 28.5 |
| | | | | 1032.545 | 28.5 |
| | | | | **1119.577** | **28.5** |

Table 7. (continued)

| PRTC peptide | Start time (min) | End time (min) | Precursor (*m/z*) | Product (*m/z*) | Collision energy (V) |
|---|---|---|---|---|---|
| GLILVGGYGTR | 0 | 12.4 | 558.325 | 473.273 | 21.1 |
| | | | | 506.260 | 21.1 |
| | | | | **620.303** | **21.1** |
| | | | | 719.371 | 21.1 |
| | | | | 832.455 | 21.1 |
| SFANQPLEVVYSK | 0 | 12.4 | 745.392 | 603.359 | 27.5 |
| | | | | **942.539** | **27.5** |
| | | | | 1070.597 | 27.5 |
| | | | | 1184.640 | 27.5 |
| | | | | 1255.677 | 27.5 |
| GILFVGSGVSGGEEGAR | 0 | 12.4 | 801.411 | 685.314 | 29.4 |
| | | | | 772.346 | 29.4 |
| | | | | 928.436 | 29.4 |
| | | | | **1072.489** | **29.4** |
| | | | | 1171.558 | 29.4 |
| LTILEELR | 0 | 12.4 | 498.801 | 427.254 | 19.1 |
| | | | | 556.296 | 19.1 |
| | | | | **669.381** | **19.1** |
| | | | | 782.465 | 19.1 |
| | | | | 883.512 | 19.1 |
| NGFILDGFPR | 0 | 12.4 | 573.302 | 486.270 | 21.6 |
| | | | | 601.297 | 21.6 |
| | | | | **714.381** | **21.6** |
| | | | | 827.465 | 21.6 |
| | | | | 974.533 | 21.6 |
| ELASGLSFPVGFK | 0 | 12.4 | 680.373 | **555.338** | **25.3** |
| | | | | 702.406 | 25.3 |
| | | | | 789.439 | 25.3 |
| | | | | 959.544 | 25.3 |
| | | | | 1046.576 | 25.3 |
| LSSEAPALFQFDLK | 0 | 12.4 | 787.421 | 658.365 | 28.9 |
| | | | | 805.433 | 28.9 |
| | | | | 918.517 | 28.9 |
| | | | | **1086.607** | **28.9** |
| | | | | 1157.644 | 28.9 |

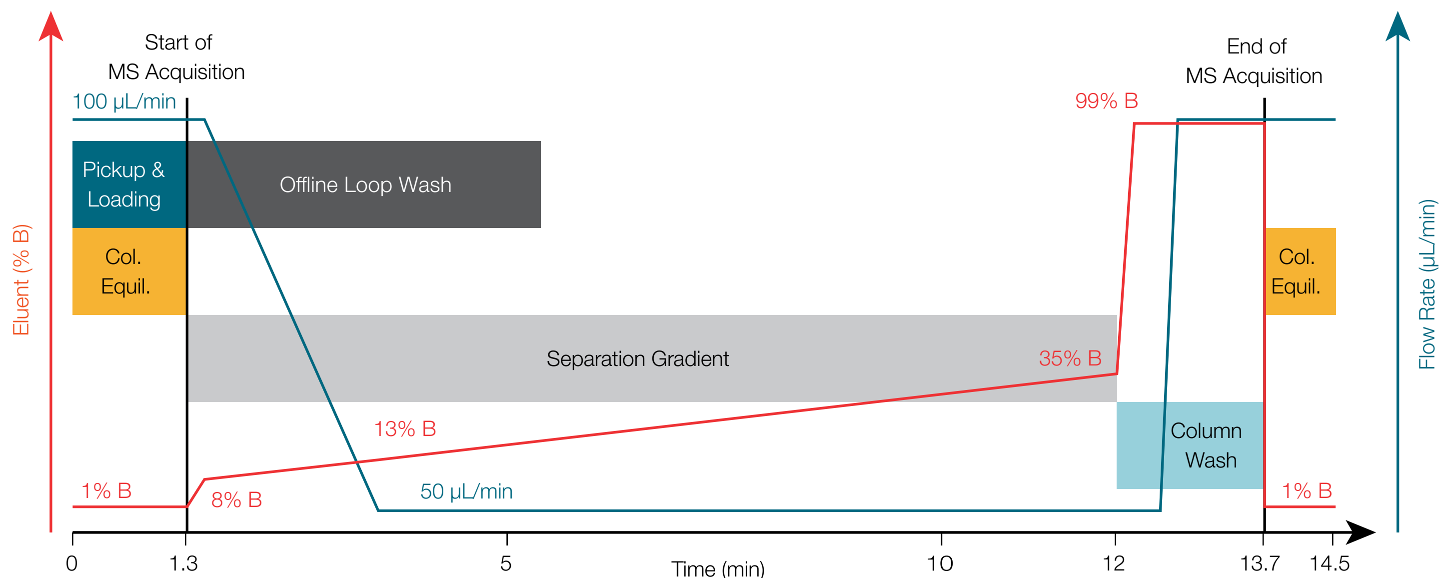


**Figure 1. Scheme of the LC method. Both the gradient phase and additional method overhead including sample pickup, loading and column equilibration are depicted. Colored squares indicate the different phases of the run: the injection phase (includes sample aspiration and its loading onto the column), the sample loop wash phase, the column equilibration phase, the column wash phase, and the gradient separation phase. The red trace shows the percentage of eluent B being delivered by the analytical pump. Lowest value is 1% B, highest value is 99% B (Table 4). The blue trace shows the flow rate delivered by the analytical pump. Lowest value is 50 µL/min, highest value is 100 µL/min (Table 4).**

## Results and discussion

A high-throughput micro-flow LC-MS method was developed based on previously published work.[3,4] The goal was to create a highly robust, short method (ca. 14.4 min cycle time enabling 100 samples per day) which delivers good chromatographic separation and sufficient detection sensitivity for proteomic analysis of large sample cohorts. The gradient flow rate of 50 µL/min was determined as the optimum to deliver both high throughput and high detection sensitivity. For a 1 mm ID column, larger injection amounts are sufficient to compensate for loss in ionization efficiency at micro-flow rate (50 µL/min). Sample loading onto the column, column washing, and column equilibration were performed at 100 µL/min (Table 4 and 5, Figure 1). After sample loading the sample loop was switched offline to minimize the gradient delay volume. In addition, the flow rate at the start of the gradient was 100 µL/min and then ramped down to 50 µL/min within the first 2.2 minutes. This reduces the time until the first peptides are eluted and maximizes the elution window. The total cycle time is ca. 14.4 minutes. The duration of separation gradient phase is 10.7 minutes, or 74% of the cycle time.

To ensure minimal carry-over the sample loop was washed offline using the metering device with both high and low organic containing wash liquids.

The sensitivity of the method was assessed with 1 µL injections corresponding to 50 ng HeLa digest and 25 fmol of each PRTC peptide on column. LC-MS analysis with a TSQ Altis applying default source settings (Table 6) and generic SRM transitions for the PRTC peptides (Table 7) resulted in 12 quantifiable PRTC peptides (Figure 2). The other three PRTC peptides showed low signal intensity and were, therefore, omitted from further analyses.

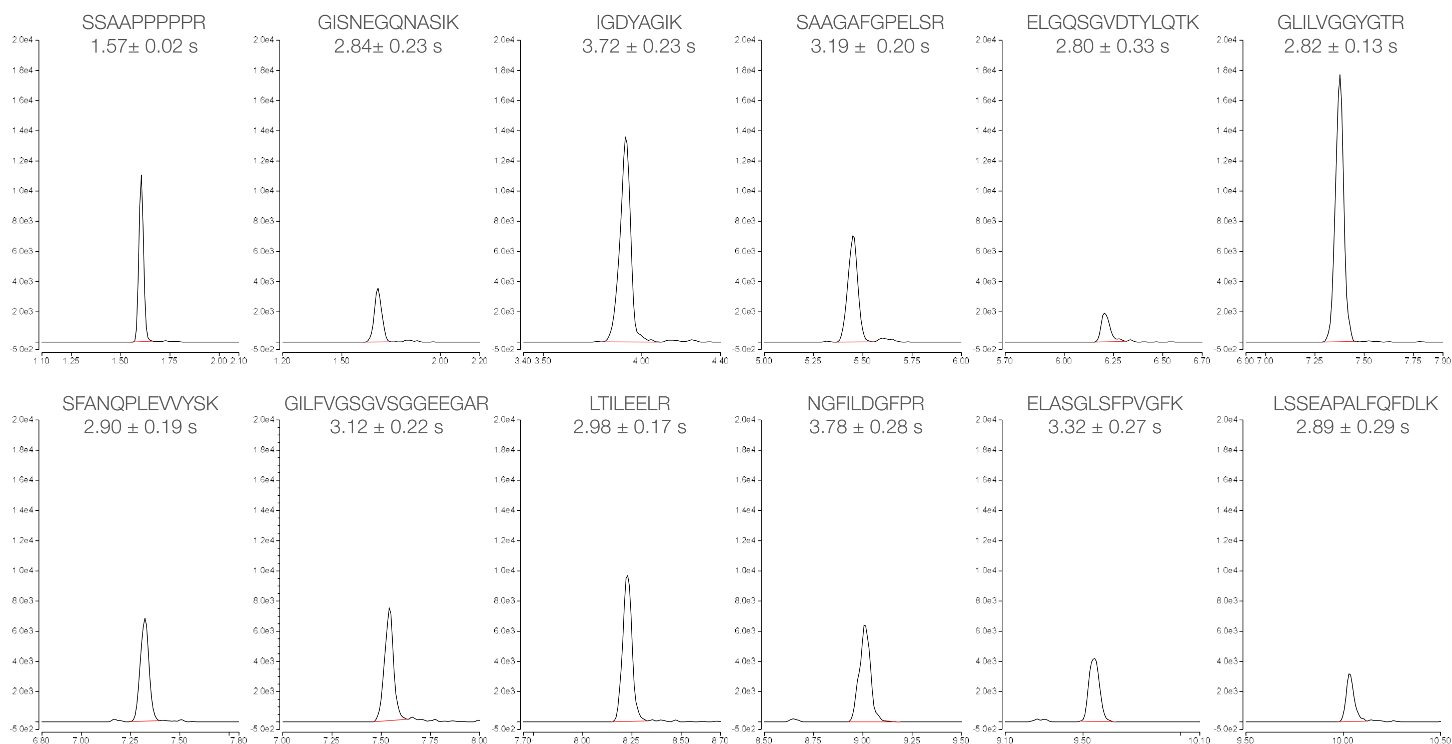


**Figure 2: Representative chromatograms of 12 PRTC peptides used for method assessment. The peptide sequence is stated above each chromatogram. The average full width at half maximum (FWHM) and standard deviation over the test sequence is given below.**

Since these 12 peptides were detected well above the limit of quantitation (signal-to-noise ratio = 10) they were used for method robustness evaluation. A sequence of 760 injections was run over a period of 7.5 days. The sequence was a repetition of the following injections: 3 sample injections, 3 blank injections, 3 matrix injections resulting in a total of 254 HeLa digest/PRTC injections. After the first 452 injections, sample vials containing fresh matrix and sample were exchanged because the 200 µL (10 µg) of matrix and sample were nearly depleted.

Method robustness was evaluated according to the consistency of the pressure traces as well as the retention time stability and peak area reproducibility of the 12 PRTC peptides.

The pressure traces were highly reproducible (Figure 3) with a run-to-run pressure variation of less than 3 bar observed throughout the sequence. It is important to note that the pressure consistency was not only present during the separation gradient phase. The pressure traces were also fully reproducible during the initial flow rate ramp down (0.2 to 2.2 min) and during the column wash phase where the flow rate is ramped up (Figure 3).

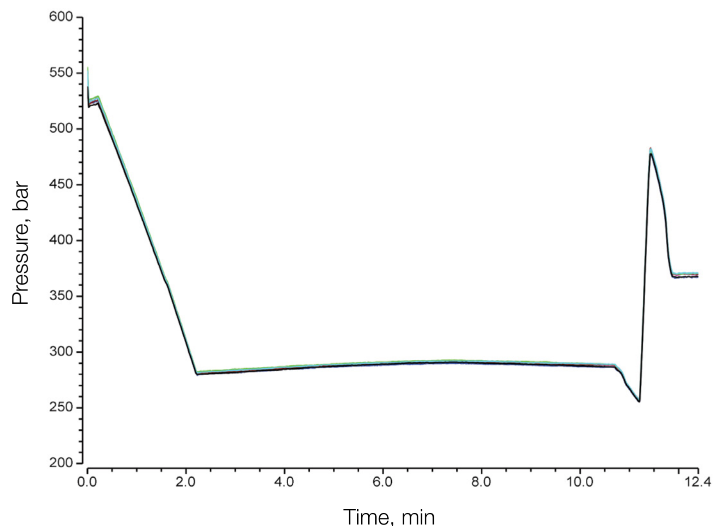


**Figure 3. Pressure traces of early (black and purple), intermediate (blue and dark red) and late (green and light blue) runs of the sequence. The pressure traces are highly consistent with a minimal increase of less than 3 bar observed over the entire sequence. Data acquisition starts when the sample loop is switched offline after the sample is loaded onto the column. Therefore, the time scale between Figure 1 where the complete method is outlined and this figure where only the data acquisition during Separation Gradient and Column Wash phases is shown is shifted by 1.3 minutes.**

The high congruency of pressure traces was also reflected in the observed retention time stability (Figure 4 and Table 8). The retention times were stable for any of the 12 peptides. Relative standard deviation was well below 0.5% for all peptides during seven days of system operation. Ten peptides had an RSD smaller than 0.1%, one had 0.14% RSD, and one had 0.31% RSD.

There was a minimal retention time reduction (≤3 s or ≤1%) for the first 20 injections (Figure 3). The initial retention time shift is most likely due to stationary phase conditioning. Afterwards, no further retention time changes were observed.

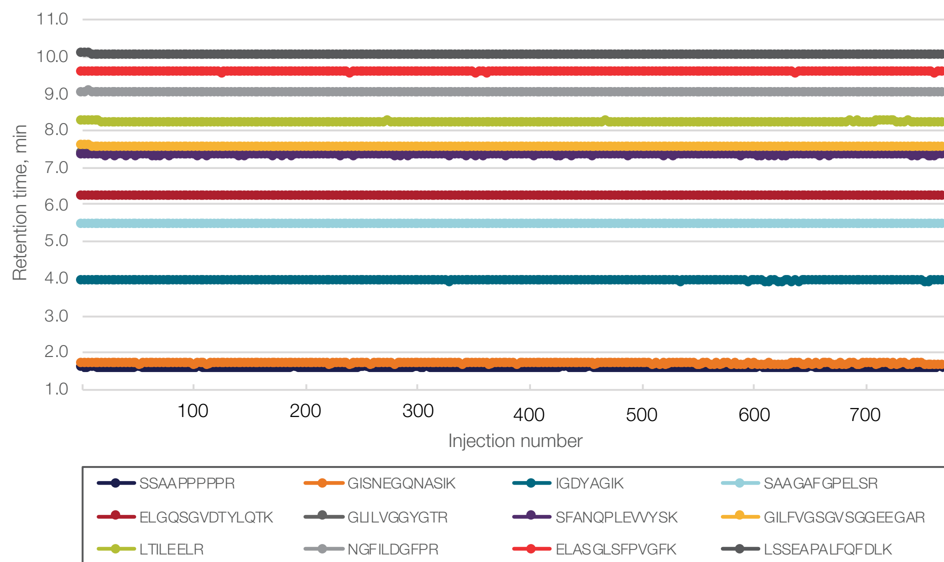


**Figure 4. Retention time stability of 12 PRTC peptides for all sample injections of the sequence.**

**Table 8. Average retention time (RT), standard deviation (SD) and relative standard deviation (RSD, %) for 12 PRTC peptides**

| Peptide | SSAAPPPPPR | GISNEGQNASIK | IGDYAGIK | SAAGAFGPELSR | ELGQSGVDTYLQTK | GLILVGGYGTR | SFANQPLEVVYSK | GILFVGSGVSGGEEGAR | LTILEELR | NGFILDGFPR | ELASGLSFPVGFK | LSSEAPALFQFDLK |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RT (min) | 1.604 | 1.683 | 3.920 | 5.449 | 6.213 | 7.376 | 7.322 | 7.540 | 8.228 | 9.010 | 9.558 | 10.035 |
| SD (min) | 0.000 | 0.005 | 0.006 | 0.004 | 0.005 | 0.005 | 0.006 | 0.006 | 0.005 | 0.007 | 0.006 | 0.006 |
| RSD (%) | 0.01 | 0.31 | 0.14 | 0.08 | 0.08 | 0.07 | 0.08 | 0.08 | 0.06 | 0.08 | 0.06 | 0.06 |

Peak area reproducibility over the injection sequence was also assessed. As previously stated, a fresh vial of HeLa digest/PRTC was used after the first 452 injections due to sample depletion. Since slight differences in signal intensities between the two sample vials were observed, the peak area stability was considered separately for the first 452 injections and the subsequent 308 injections. A peak area reproducibility of <15% RSD is considered good for quantitative MS analyses.[5] The observed peak area reproducibility was below 10% for 10 of the peptides. The two peptides with lowest peak areas showed the peak area variabilities of 11.1 and >15%.

In conclusion, a micro-flow LC-MS method on the Vanquish Neo UHPLC system with a cycle time of ca. 14.4 minutes was developed and evaluated. The method delivered highly robust and reproducible results for a sequence of 760 injections over a period of more than one week and did not reveal any LC, MS or column performance declines. Therefore, this method would be suitable for high-throughput proteomic analyses of large sample cohorts needed for validation studies.

**Table 9. Average peak area, standard deviation (SD) and relative standard deviation (RSD) for 12 PRTC peptides for injections 1–452 and 453–760**

| | Peptide | SSAAPPPPPR | GISNEGQNASIK | IGDYAGIK | SAAGAFGPELSR | ELGQSGVDTYLQTK | GLILVGGYGTR | SFANQPLEVVYSK | GILFVGSGVSGGEEGAR | LTILEELR | NGFILDGFPR | ELASGLSFPVGFK | LSSEAPALFQFDLK |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Injections 1–452 | Peak Area (counts * min) | 346.8 | 174.2 | 925.6 | 459.5 | 101.2 | 881.5 | 340.8 | 381.0 | 478.8 | 393.3 | 245.2 | 136.5 |
| | SD (counts * min) | 27.9 | 15.6 | 65.6 | 31.0 | 11.2 | 53.9 | 19.8 | 27.2 | 36.9 | 28.4 | 22.7 | 21.2 |
| | RSD (%) | **8.0** | **8.9** | **7.1** | **6.7** | **11.1** | **6.1** | **5.8** | **7.1** | **7.7** | **7.2** | **9.2** | **15.5** |
| Injections 453–760 | Peak Area (counts * min) | 412.3 | 204.8 | 1,155.4 | 562.3 | 119.2 | 1,148.2 | 405.0 | 462.6 | 605.5 | 506.5 | 304.9 | 168.3 |
| | SD (counts * min) | 27.9 | 15.6 | 65.6 | 31.0 | 11.2 | 53.9 | 19.8 | 27.2 | 36.9 | 28.4 | 22.7 | 21.2 |
| | RSD (%) | **6.8** | **7.6** | **5.7** | **5.5** | **9.4** | **4.7** | **4.9** | **5.9** | **6.1** | **5.6** | **7.4** | **12.6** |

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