# Long-term stability and reproducibility of nano-, capillary- and micro-flow LC-MS separations: the impact of hardware and separation column

Christopher Pynn[1], Runsheng Zheng[1]; Tabiwang Arrey[2]; Amirmansoor Hakimi[3]; Alec Valenta[1]; Susanne Moyer[1]
[1]Thermo Fisher Scientific, Germering, Germany; [2]Thermo Fisher Scientific, Bremen, Germany; [3]Thermo Fisher Scientific, San Jose, United States

## ABSTRACT

**Purpose:** Demonstrate the long-term robustness and consistent chromatographic performance of the next generation Vanquish Neo UHPLC system under nanoLC and microLC conditions for bottom-up proteome profiling on PepMap Neo columns as well as system-to-system reproducibility based on peak properties and proteomic data metrics

**Methods:** The Thermo Scientific™ Vanquish™ Neo UHPLC system, Thermo Scientific™ PepMap™ Neo columns, and Thermo Scientific™ Orbitrap Exploris™ 480 and Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer. Direct injection workflows in nano/cap or micro configurations were used to evaluate long-term stability and reproducibility of low-flow LCMS analysis with 75 µm ID columns and 1.0 mm ID columns.

**Results:** Vanquish Neo UHPLC system and the PepMap Neo columns deliver levels of chromatographic robustness and reproducibility required for long-term trouble-free nano- and micro-flow LC operation under maximum performance and pressure conditions. In addition, the results are reproducible across systems and sites that will help foster the adoption of low-flow LC-MS for large sample cohorts analysis

## INTRODUCTION

NanoLC-MS analysis employing long, narrow-bore columns and long gradients is well established as the gold standard for bottom-up discovery proteomics applications. Aside from the technical challenges associated with nanoLC, concerns remain around the day-to-day and system-to-system reproducibility of results. High system-to-system reproducibility is essential to generate global data sets that can be jointly processed and to gain statistically significant insights for biomarker discovery and validation, characterization of cell lines, and drug development. The latest low-flow UHPLC systems and columns have the potential to overcome many of these challenges and to usher in a new era of robustness and standardization in the field of low-flow LC-MS based analytics.

We tested the Vanquish Neo UHPLC system for robust and consistent long-term LC separation performance under conditions typically adopted for deep dive proteomics experiments using nanoLC or microLC separations.

**Figure 1. Nano/cap LC-MS setup with Vanquish Neo UHPLC system and Orbitrap Exploris 480 mass-spectrometer coupled via Thermo Scientific™ EASY-Spray™ interface**

**Figure 2. Micro LC-MS setup with Vanquish Neo UHPLC system and Thermo Scientific™ TSQ Altis™ triple quadrupole mass-spectrometer coupled via Thermo Scientific™ OptaMax™ NG Ion Source**

## 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. Thermo Scientific™ Cytochrome C protein digest standard (1.6 nmol/vial, PN 161089) was reconstituted by adding 200 µL of 5% Acetonitrile/95% Water (v/v) with 0.1% formic acid (FA) in water. CAM-modified trypsin-digested BSA MS Standard (500 pmol/vial, New England Biolabs, PN P8108S) was reconstituted by adding 500 µL of 0.1% formic acid (FA) in water. The vial was subsequently sonicated for 2 min, followed by mixing with a pipette to fully reconstitute the sample. The final sample concentration was 1 pmol/µL BSA protein digest.

### Instrumentation

All experiments were performed using Vanquish Neo UHPLC systems interfaced to an Orbitrap Exploris 480 mass spectrometer operated in data-dependent acquisition (DDA) mode or TSQ Altis mass-spectrometer operated in MRM mode. Direct injection methods for nanoLCMS analysis utilized Thermo Scientific™ EASY-Spray™ PepMap™ Neo columns and Thermo Scientific™ Acclaim™ PepMap™ columns for microLC-MS analysis. The long-term robustness was tested using the Vanquish Neo UHPLC system including thermostatted column compartment and UV detector. Thermo Scientific™ Double nanoViper™ PepMap™ Neo Column 75 µm × 500 mm, 2 µm was used for nanoLC-UV robustness testing.

### Methods

All experiments were performed using with the following solvents: eluent A – 100% water, 0.1% formic acid; eluent B – 80% acetonitrile/20% water (v/v), 0.1% formic acid. The sample amount on the column was varied by altering the injection volume or sample concentration.

### 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. LC-UV data were acquired and analyzed using the Thermo Scientific™ Chromeleon™ Chromatography Data System (version 7.2.10 MUd). Peak properties (full width at half maximum) FWHM, retention time—RT) were extracted for 8 selected BSA peptides using the Cobra peak detection algorithm.

## RESULTS

### Long-term nanoLC-MS robustness

The Vanquish Neo UHPLC system was continuously run using a single 75 µm × 50 cm PepMap Neo column for a period of 6 months. The BSA protein digest was separated using a classic 90-minute nano-flow gradient (100-minute method including column washing) typical for bottom-up proteomic experiments. The peak resolution of BSA peptides afforded by the separation, permitted the evaluation of chromatographic parameters for 8 selected peaks using UV detection (Figure 3). Peptide retention times were stable across all 1600 injections (Figure 4). The retention time standard deviation was below 0.3 min over the entire 6 months period for each set of 100 injections.

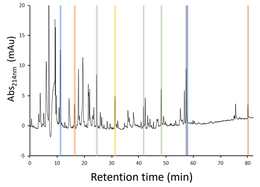


**Figure 3. Representative LC-UV chromatogram for a 1 pmol injection of BSA protein digest onto a 75 µm × 50 cm PepMap Neo column. The 8 peaks selected for evaluation are highlighted.**

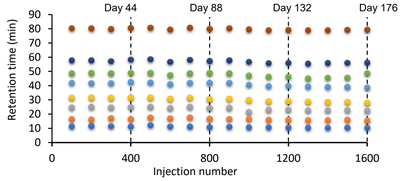


**Figure 4. The retention time for 8 selected peptides from 1,600 injections of BSA protein digest over 176 days (approximately 6 months). Retention time values are the means per set of 100 injections**

FWHM was also assessed for the same set of peptides as a marker of resolution consistency and robustness (Figure 5). Consistent FWHM for the 8 selected peptides was observed throughout the study. There was no trend towards increased peak width associated with any of the peptides studied. In contrast a "random" variation of FWHM can be seen for some of the peptides measured which can be attributed to overlapping peaks which in some instances could not be resolved by UV. The hallmarks of reproducible gradient delivery and separation column robustness observed for the duration of the study can be attributed to various system features designed to prolong column lifetime and maintain optimal separation performance such as the controlled flow ramping employed during fast sample loading and fast column equilibration and Thermo Scientific™ SmartInject functionality that reduces the pressure shock on the column material. Additionally, a filter frit integrated into the needle seat prevents sample debris from accumulating on the head of the column, further improving method robustness and column lifetime. The robustness of the separations is also reflected by the very stable column back pressure which varied by less than 25 bar over the 1600 injection/6 month analysis period (Figure 6).

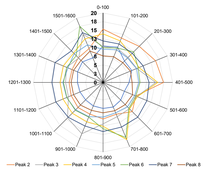


**Figure 5. Mean values for FWHM of 8 selected peptides over 1,600 replicate injections of BSA protein digest. Displayed are mean peak widths (seconds) per 100 injections for each of the selected peptides.**

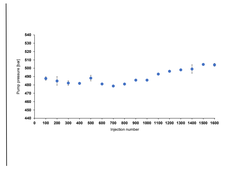


**Figure 6. Column back pressure measured 1 minute after the start of the gradient at a flow rate of 300 nL/min and 50°C column temperature on a 75 µm × 50 cm PepMap Neo column. Results are mean values per 100 injections ± S.D.**

## SYSTEM-TO-SYSTEM REPRODUCIBILITY

### Reproducible nanoLCMS proteomics results

There is an increasing requirement for high retention time reproducibility in nanoLC-MS proteomics workflows. This is particularly true for label-free quantitative LC-MS analysis where peptide retention times can be used to increase identification confidence and to compare results collected using different LC-MS setups. The results for 4 replicate injections on 6 systems showed excellent retention time precision with <0.2% RSD for all peptides selected for data processing. Mean retention times (Figure 7A) and relative standard deviation (RSD), % (Figure 7B) for each peptide across all 6 Vanquish Neo UHPLC systems are given below.

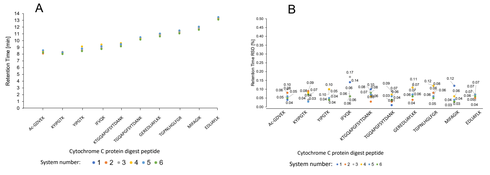


**Figure 7. Mean retention times (A) and RSD, % values (B) for selected Cytochrome C protein digest peptides separated on 6 Vanquish Neo UHPLC systems using a 20 min nanoLC method; 4 replicate injections were carried out for each system.**

High-quality, reproducible nanoLC-MS data generation across systems and sites is crucial to the success of routine quantitative proteomics applications. System-to-system reproducibility according to peptide and protein IDs was evaluated using the 100 min method with 200 ng injections of a HeLa cell protein digest under standardized MS acquisition conditions. On average 33,000 peptides from 4,400 protein groups could be identified with a single-shot nanoLC-MS analysis. As little as 4.1% variation in peptide groups and 2.2% in protein groups was observed for the 6 Vanquish Neo UHPLC systems coupled to an Orbitrap Exploris 240 mass spectrometer.

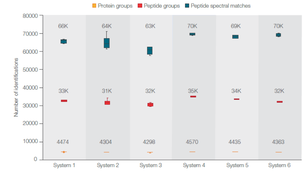


**Figure 8. Comparison of 200 ng HeLa protein digest profiling with 6 different Vanquish Neo UHPLC systems coupled to an Orbitrap Exploris 240 mass spectrometer. For the separation, 75 µm × 50 cm, 2 µm EASY-Spray PepMap Neo columns, a 90 min gradient (100 min method), 300 nL/min flow rate, direct injection, and 1500 bar sample loading and column equilibration were used.**

### Reproducible microLC-MS results

A high-throughput micro-flow LC-MS method was developed based on previously published work[1,2]. We created 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. A sequence of 760 injections was run over a period of 7.5 days to test the results reproducibility with microLC-MS analysis. 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. 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 9) with a run-to-run pressure variation of less than 3 bar observed throughout the sequence. 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.

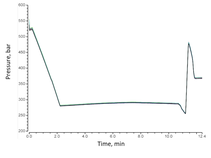


**Figure 9. Pressure traces of early (black and purple), intermediate (blue and dark red) and late (green and light blue) runs of the sequence.**

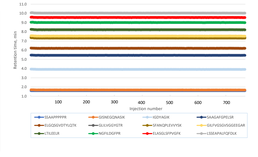


**Figure 10. Retention time stability of 12 PRTC peptides for all sample injections of the sequence**

The high congruency of pressure traces was also reflected in the observed retention time stability (Figure 10). 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.

## CONCLUSIONS

We evaluated the long-term stability and reproducibility of results generated with Vanquish Neo UHPLC system coupled to HRAM or QqQ mass-spectrometers. The comprehensive testing under nano- and micro-flow LC conditions revealed high consistency of results and ruggedness of system operation. Taken together, these data are evidence of the reliability and performance capabilities of modern low-flow UHPLC systems and consumables.

- Vanquish Neo UHPLC system and the PepMap Neo columns deliver levels of chromatographic robustness and reproducibility required for long-term trouble-free nanoLC operation under maximum performance and pressure conditions proven by 6 months of continuous operation
- Nano-flow LCMS proteomics results generated within one system as well as suing 6 Vanquish Neo UHPC systems showed high consistency and reproducibility
- 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

## REFERENCES

1. Bian, Y., Zheng, R., et al. Robust, reproducible and quantitative analysis of thousands of proteomes by micro-flow LC–MS/MS. Nat. Comm. 2020, 11, 157.

2. Bian, Y., Bayer, F. P. et al. Robust Microflow LC-MS/MS for Proteome Analysis: 38000 Runs and Counting. Anal. Chem. 2021, 93, 8, 3686-3690.

## TRADEMARKS/LICENSING



PO66145-EN0422S