High-throughput proteomics

Efficient tandem capillary flow LC-MS with short µPAC columns and a single ionization source

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Abstract

Purpose: Demonstration of productivity, performance and robustness of high-throughput tandem capillary flow LC-MS setup using improved Thermo Scientific™ µPAC™ Neo High-Throughput Plus columns.

Methods: HeLa cell digests were resuspended in 0.1% TFA and 1% ACN to obtain a 200 ng/µL stock solution, sonicated, diluted, and vortexed before use. A Thermo Scientific™ Vanquish™ Neo UHPLC instrument was configured in tandem direct injection mode with an additional pump module and a 10 µL injection loop for maximum throughput. Two µPAC Neo High-Throughput Plus columns were positioned between low-dispersion 6-port switching valves in the column compartment. A 20 µm ID Thermo Scientific™ nanoViper™ line connected the alternating eluents to an ESI emitter with an integrated liquid junction, positioned in a Thermo Scientific™ EASY-Spray™ source coupled to a Thermo Scientific™ Orbitrap Exploris™ 240 Mass Spectrometer.

Results: Compared to a single-column workflow, instrument productivity increased by over 30% for the highest throughput methods and up to 17% for the 100 SPD methods. Injecting 200 ng HeLa digest at 200 SPD with 2.5 µL/min identified up to 3663 protein groups. With optimized methods and lowering throughput to 180 SPD and 100 SPD, identifications up to 4731 and 5692 protein groups, respectively. Median CVs at the protein group level ranged between 5% and 7% across 2x3 replicates for each method, incorporating inter-column variability.

Introduction

Nano-flow tandem direct injection (TDI) workflows increase sample throughput and instrument productivity with flow rates below 1 µL/min but need dual-column ESI interfacing to maintain chromatographic performance. At flow rates above 1 µL/min with capillary flow columns, post-column dispersion impacts performance less, allowing low-dispersion switching valves in the fluidic path. We demonstrate the Vanquish Neo UHPLC system TDI workflow for capillary flow LC with a high throughput short pillar array column. This dual-column, single-ESI emitter setup reduces variability, extends emitter life through efficient salt management, and ensures high reproducibility with dedicated gradient pumps and intelligent method design.

Materials and methods

Sample preparation

HeLa cell digests were resuspended in 0.1% TFA and 1% ACN to obtain a 200 ng/µL stock solution, sonicated, diluted, and vortexed before use.

LC-MS configuration

Samples were analyzed using a Vanquish Neo UHPLC instrument that was configured in tandem direct injection mode with an additional pump module and a 10 µL injection loop for maximum throughput. Two µPAC Neo High-Throughput Plus columns were positioned between low-dispersion 6-port switching valves in the column compartment. A 20 µm ID nanoViper line connected the alternating eluents to an ESI emitter with an integrated liquid junction, positioned in an EASY-Spray source coupled to a Orbitrap Exploris 240 mass spectrometer.

Data independent acquisition (DIA) parameters for the *method evaluations* were as follows: MS resolution = 60k, MS^2 resolution = 15k, MS AGC = 300%, MS^2 AGC = 800%, precursor mass range = 525-825 Th, MS1/MS^2 maxIT = auto. Isolation widths were varied based on gradient length: 200SPD = 15Th, 180SPD = 10Th, 100SPD = 8Th. DIA parameters for the dilution series and reproducibility studies were as follows: MS resolution = 120k, MS^2 resolution = 30k, MS AGC = 300%, MS^2 AGC = 800%, precursor mass range = 525-825 Th, MS1/MS^2 maxIT = auto. Isolation widths were varied based on gradient length: 180 SPD = 15Th, 100 SPD = 8th.

Data analysis

LC-MS data were analyzed using Spectronaut® 19, if not indicated differently. Results shown have been filtered to a 1% FDR.

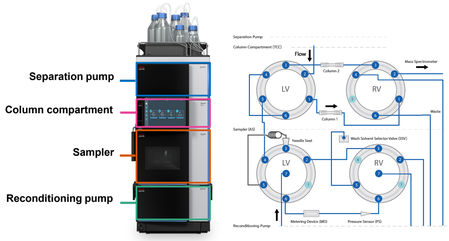

Figure 1. Left: Tandem Vanquish Neo with labelled modules. Right: Flow scheme of the Tandem capillary flow setup.

Tandem Direct Injection (TDI) workflow with 2x µPAC™ Neo High-Throughput Plus

- Two µPAC Neo HT Plus columns were installed between two 6-port valves in the column compartment of the Tandem Vanquish Neo
- The new columns are completely bidirectional, so the attached capillaries have the same length on both sides.
- µPAC Neo Plus configuration minimizes pre and post column dispersion
- A 10 uL sample loop was installed to reduce the overhead time to approx. 1 minute
- Additional grounding of the µPAC Neo HT Plus columns in TDI is not necessary --> this is achieved via the column compartment valves

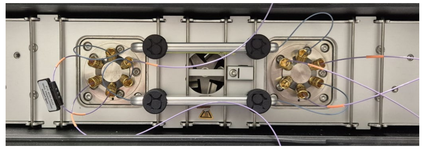

Figure 2. Picture of installed µPAC™ Neo HT Plus columns in the column compartment of the Tandem Vanquish Neo system.

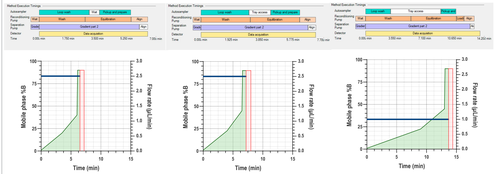

Figure 3. LC gradient profiles and tandem workflow execution timings for 200, 180 and 100 SPD throughput methods.

Results

Productivity gain using µPAC Neo HT Plus in Tandem Direct Injection workflow

- Compared to Direct Injection workflows, the productivity gain for the three optimized TDI-based gradient lengths range from **16 - 33%**
- Compared Trap-and-Elute workflows, the productivity gain for the three optimized TDI-based gradient lengths range from **9 - 17%**

200 Samples per day	Active Elution (min)	Overhead (min)	Total (min)	Productivity
Direct Injection	3.8	3.4	7.2	53%
Trap and Elute	5	2.4	7.2	69%
Tandem Direct Injection	6.2	1	7.2	86%

180 Samples per day	Active Elution (min)	Overhead (min)	Total (min)	Productivity
Direct Injection	4.6	3.4	8	58%
Trap and Elute	5.6	2.4	8	70%
Tandem Direct Injection	7	1	8	88%

100 Samples per day	Active Elution (min)	Overhead (min)	Total (min)	Productivity
Direct Injection	11	3.4	14.4	76%
Trap and Elute	12	2.4	14.4	83%
Tandem Direct Injection	13.25	1.15	14.4	92%

LC method optimization: Flow rate optimization

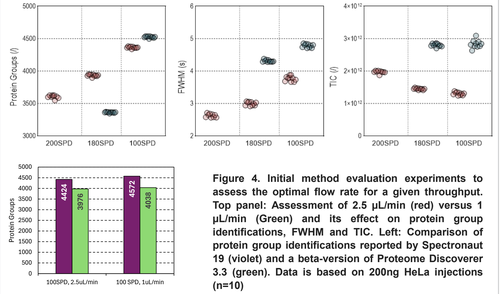

Figure 4. Initial method evaluation experiments to assess the optimal flow rate for a given throughput. Top panel: Assessment of 2.5 µL/min (red) versus 1 µL/min (Green) and its effect on protein group identifications, FWHM and TIC. Left: Comparison of protein group identifications reported by Spectronaut 19 (violet) and a beta-version of Proteome Discoverer 3.3 (green). Data is based on 200ng HeLa injections (n=10)

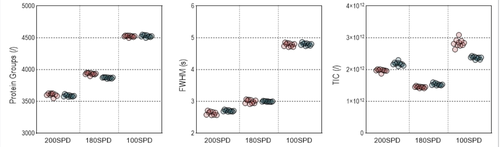

Figure 5. Comparison of different chosen emitter and their effect on protein group identifications (left), FWHM (middle) and TIC (right). Bullet emitter with 15um I.D. fused silica emitter (red) and 30 um I.D. steel emitter (green). Data based on 200 ng HeLa injections (n=10)

HeLa dilution series for two throughputs and different columns

- For the 180 SPD and 100 SPD throughput methods, a HeLa dilution series ranging from **50 ng – 1000 ng** was measured using optimized method parameters to achieve both, a high ID rate and good reproducibility.
- For both gradients, peptides (data not shown) + protein group IDs consistently increased with increasing peptide loads on column
- Using the 180 SPD gradient, IDs ranged from **3687** to **4713** protein groups
- Reducing throughput to 100 SPD, IDs yielded between **4621** and **5692** protein groups

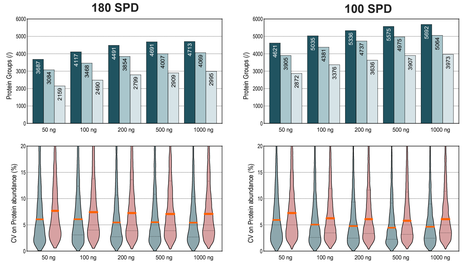

Figure 6. HeLa dilution series for 180 and 100 SPD. Top: Protein group identifications. Each group is presented by the total Ds (dark teal), IDs with CVs lower than 20% (teal) and IDs with CVs lower than 10% (light teal), n=6. Bottom: Violin plots of CVs on protein group level for single column (teal, n=3) versus dual column (red, n=6). Red mark indicates median CV.

Column reproducibility across production batches

- Twelve columns from 4 different production batches were analyzed using the 100 SPD method and PRTC peptides spiked into a HeLa background.
- Assessing PRTC retention time across the columns shows very high reproducibility with the CVs ranging from 1.4 – 3.3% (figure 7)
- Overall chromatogram profile looks very similar from column to column (figure 8)
- The coefficient of variation of protein group IDs across the twelve columns is 1.4%

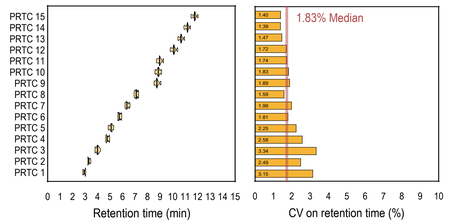

Figure 7. Distribution and variation of PRTC peptide retention times across 12 columns from three different production batches. Violin plots and the coefficient of variation were generated using six replicates from each column. The PRTC peptides were spiked at 25 fmol/µL into a 500 ng/µL HeLa digest background.

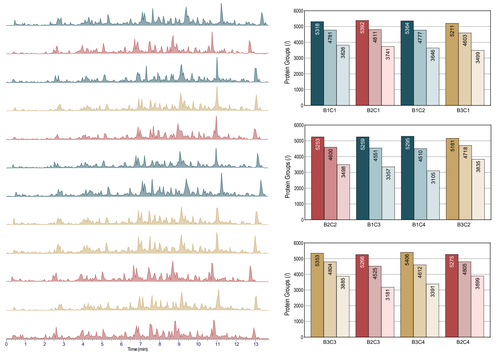

Figure 8. Column reproducibility within 3 production batches (n=4, per batch, total 12 columns, 6 replicates, 200ng HeLa). Left: Chromatograms of each column over a gradient of 13.7 minutes. Right: Protein group IDs per production batch. Each group is presented by the total IDs (dark color), IDs with CVs lower than 20% (color) and IDs with CVs lower than 10% (light),

Conclusions

- Using optimized uPAC Neo HT Plus columns in combination with the Tandem Vanquish Neo UHPLC system heightened the productivity of bottom-up proteomics measurements to 86 - 92% (gradient-dependent)
- At these throughputs, the chosen emitter only has a minor effect on identifications, FWHM and TIC. Decreasing the flow rate to 1 µL/min causes peak broadening and increases ionization efficiency which is reflected in higher TICs. In this study, reducing the flow rate only showed for the longest gradient (100 SPD) a positive effect on protein IDs.
- With the optimized methods, identifications up to 4713 protein groups for the 180SPD and 5692 protein groups for the 100 SPD were achieved. The CVs using the same column were between 5-6% and between two columns at around 7-8% (n=6)
- Column reproducibility was showcased for 12 columns across four different production batches. Overall, the ID rate variation between the columns were 1.4%. The median coefficient of variation of the retention times of spiked-in PRTC peptides across the tested 12 columns was 1.8%

References

R Zheng, M Rendl, AC Valenta, C Pynn, Y Lin, M Daniliuk, E Aydin, R van Ling, L Taujenis, W Decrop, M Samonig, A Morgenstern; *A dual-column, single-spray configuration for capillary and micro-flow LC-MS applications*, TN003314, Thermo Fisher Scientific

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