High-throughput LC-MS

A novel tandem LC workflow for proteome analysis with near 100% MS utilization

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Abstract

Purpose: We present a novel, facile, and robust workflow that eliminates idle MS time by employing a Thermo Scientific™ Vanquish™ Neo UHPLC system with the tandem direct injection workflow compatible with a flow range from 100 nL/min to 100 µL/min for discovery and targeted proteomics analysis in DDA, DIA, and PRM.

Methods: Performance was evaluated using 75 - 1000 µm i.d. columns.

Results: The tandem LC-MS workflow was tested using gradients from 4.4 to 90 min (300 to 18 samples/day) to demonstrate high throughput, run-to-run reproducibility, and close to 100% MS utilization for discovery and targeted proteomics. Specifically, a 65-min gradient with a 75 µm x 75 cm column (2 µm dp) at 300 nL/min enabled the identification of >11,000 protein groups per run in DIA from 1000 ng HeLa digest with > 91% MS utilization using the Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer. While moving to the targeted proteomics analysis using the Thermo Scientific™ TSQ Altis™ Plus Triple Quadrupole mass spectrometer, this new tandem LC workflow supports accurate and reproducible quantification across various columns supporting high-confidence peptide verification.

Introduction

Low-flow UHPLC coupled with mass spectrometry (MS) is the gold standard for deep quantitative analysis of complex proteomes. However, the unmatched sensitivity of low-flow LC-MS typically comes at the cost of low MS utilization and sample throughput. A high proportion of idle MS time results from long sample injection, loading, column washing, and equilibration cycles. Here, we present a novel, facile, and robust workflow that eliminates idle MS time by employing a tandem LC workflow compatible with a flow range from 100 nL/min to 100 µL/min.

Materials and methods

Sample preparation and column-emitter connections

The Thermo Scientific™ Pierce™ HeLa digest were separated using two Thermo Scientific™ PepMap™ Neo UHPLC columns. For applications < 5 µL/min requiring the double barrel column oven, the columns were connected to two pulled fused silica ESI emitters (10 µm I.D. x 5 cm) with a MicroTight® union (IDEX Health and Science LLC). While in capillary- (1 - 5 µL/min) and micro-flow (1 - 5 µL/min) experiments, the EASY-Spray™ capillary emitter (ES994, 15 µm I.D.) and low-flow metal needle insert (50 µm I.D.) were used.

Methods

The innovative workflow incorporates "look-ahead" injections, allowing for sample loading onto the second column while the separation on the first column is still in progress. This is followed by automated column switching. This intelligent system design enhances the user experience of LC-MS sequence programming and execution by providing superior automation and efficiency. (**Table 1 and Figures 1-5**).

Data analysis

The raw files were processed with the Thermo Scientific™ Proteome Discoverer™ 3.1 software for peptide and protein identification and quantification. The false discovery rates (FDR) were all set below 1% at both the peptide and the protein levels.

Results

State-of-art configuration for tandem nano-, capillary-, and micro-flow LC-MS

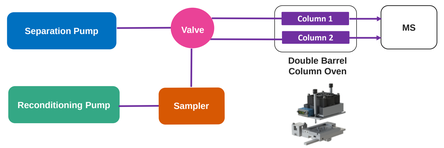
The tandem workflow utilized the following configuration:

1) Vanquish Neo UHPLC System
2) Vanquish Column Compartment N with two 2p-6p low-dispersion switching valves
3) Thermo Scientific™ Vanquish™ Binary Pump N
4) Thermo Scientific™ Orbitrap Exploris™ 480, Orbitrap Astral, or TSQ Altis Plus mass Spectrometer
5) Thermo Scientific™ PepMap™ Neo UHPLC columns
6) Intelligent method for automated column switching and data acquisition.
7) MS ion sources:
    1) Nano-flow (<5 µL/min): Double Barrel Column Oven (Sonation GmbH) installed onto the Thermo Scientific™ Nanospray Flex™ Ion Source (**Figure 2**)
    2) Nano/capillary-flow (1-5 µL/min): Thermo Scientific™ Nanospray Flex™ Ion Source or Easy-Spray Ion Source
    3) Micro-flow (5-100 µL/min): Thermo Scientific™ OptaMax NG™ Ion Source

Figure 1. Vanquish Neo UHPLC system with the Tandem Direct Injection workflow and Orbitrap Astral or Exploris 480 MS

The standardized configuration supports the tandem direct injection workflow using Thermo Scientific™ nanoViper™ Fingertight Fittings for fluidic connections and are optimized for maximum separation performance. For flow rates (< 5 µL/min), the Sonation double barrel column oven and Nanospray Flex ion source are required (**Figure 2**).

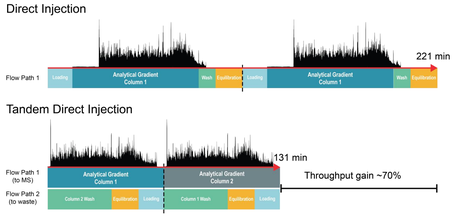
Figure 2. Fluidics connection for tandem direct injection workflow (< 5 µL/min)



Next generation system intelligence for seamless tandem workflow execution

The separation pump continually delivers the analytical gradient for sample elution on column 1 or column 2 while the reconditioning pump and autosampler are used for column washing, equilibration, sample injection and loading (**Figure 3**).

Figure 3. The operation principle of the Tandem Direct Injection workflow.



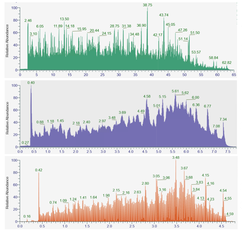
Maximized MS utilization for high-throughput bottom-up proteomics

The tandem direct injection workflow provides >90% MS utilization across the entire flow regime for 24/7 routine operation of profiling complex protein digests (**Table 1 and Figure 4**). Only a single LC-MS/MS method is required to perform peptide separation and MS acquisition on columns 1 and 2.

Table 1. Overview of tandem direct injection LC methods

Column	Emitter I.D. (µm)	Flow Rate (µL/min)	Elution Window (min)	Cycle Time (min)	Throughput (samples/day)	MS utilization (%)
PepMap 75 µm I.D. x 75 cm	20	0.3	61	67	22	91
	10	0.25	86	92	18	93
PepMap 150 µm I.D. x 15 cm	15	1.5	7.5	8	180	90
PepMap 1000 µm I.D. x 5 cm	50	100	4.4	4.8	300	92

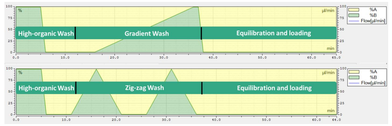
Figure 4: TIC profiles for peptides separation at different flow rates and columns



Prominent reduction of column carryover

The tandem workflow not only enables users to adjust separation conditions based on the application, e.g., phosphoproteome and TMT-labeled peptides, but also provides extensive column washing cycles (**Figure 5**) to reduce column carryover, extend column lifetime, and enhance result reproducibility.

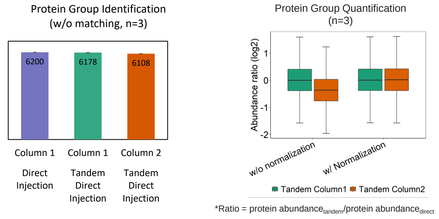
Figure 5. Extended column washing cycles to reduce column carryover



Identical LC-MS performance with increased sample throughput

Leveraging the performance of the Vanquish Neo UHPLC system and Orbitrap Exploris 480 MS, we initially achieved a 70% MS utilization for deep dive proteomics analysis with a 75 µm x 75 cm column and 90-min gradient (86-min elution window) at 250 nL/min, allowing us to process 12 samples per day.[1] Using the tandem workflow, MS utilization was increased to 93%. This improvement raised sample throughput to 18 samples/day (50% increase) while maintaining the similar performance in protein identification and quantification with 1 µg HeLa peptides (**Figure 6**) .

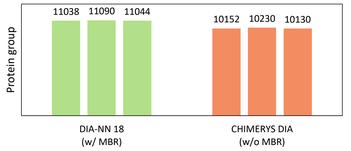
Figure 6. Equivalent performance of direct injection and Tandem direct injection workflows



LFQ-DIA performance

We improved the throughput from 13 to 22 samples/day (61-min elution window) while maintaining performance of deep-dive HeLa proteome (>11,000 protein group identifications) using the Orbitrap Astral MS (**Figure 7**) operated in DIA mode, significantly reducing the overall cost per sample.

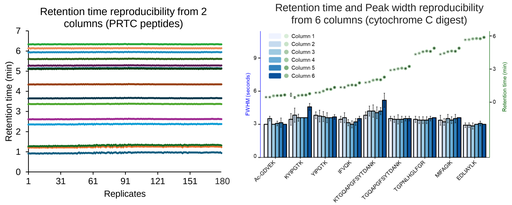
Figure 7. Proteome depth using the Orbitrap Astral mass spectrometer



Reproducible high-throughput target analysis in capillary-flow

The tandem direct injection workflow supports reproducible intra- and inter-column retention times of standard PRTC peptides and Cytochrome C digest in two systems with eight 150 µm x 15 cm DNV PepMap columns operated at 1.5 µL/min for 180 samples/day throughput (**Figure 8**).

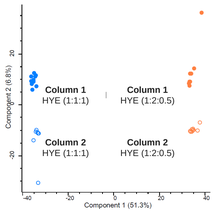
Figure 8. Intra- and inter-column reproducibility



Unbiased differentiation of regulated proteins from multiple columns

We conducted a proof-of-principle experiment to address concerns regarding column-to-column variation and its impact on proteomics conclusions. Two sets of samples (Human, Yeast, and E. coli digests mixed differently) were sequentially injected into two self-packed pulled-tip emitter columns (75 µm x 25 cm) operating at 250 nL/min. Principal Component Analysis (PCA) revealed distinguishable differences in column performance. However, it is important to note that the results also highlighted that the variation in the samples themselves had a more significant influence (**Figure 9A**). Therefore, these findings demonstrate that the tandem configuration effectively preserves the biological information necessary for large-cohort sample analysis, despite the observed column-to-column variations.

Figure 9. Biological variation using tandem direct injection workflow (n=10)



Conclusions

We developed a novel tandem direct injection workflow that maximizes MS utilization for both deep-dive and high-throughput proteome profiling and quantification. It shows high reproducibility in peptide separation and quantification between columns.

We demonstrate how the Vanquish Neo UHPLC system coupled with HRAM MS and double barrel ESI source can be combined to create a new industry standard in the speed and depth of proteome profiling. This configuration seamlessly integrates with all Thermo Scientific mass spectrometers, including the Orbitrap Astral MS.

The Vanquish Neo tandem direct injection workflow represents a promising alternative to conventional nano/capillary LC-MS setups for both shotgun proteomics and targeted analyses in complex matrices.

Reference

[1] Runsheng Zheng, etc. J. Proteome Res. 2022, 21, 10, 2545–2551

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