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Maximizing sample throughput and sensitivity in nano- and capillary-LC-MS

Harnessing a tandem direct injection workflow and a dual spray ion source

Authors

Runsheng Zheng[1], Martin Rendl[1], Alec C. Valenta[2], Christopher Pynn[1], Till Reinhardt[1], Felix Josef[3], Maksim Daniliuk[4], Amarjeet Flora[2], Thomas Lanzinner[3], Patrick Allihn[3], Giada Marino[3], Nagarjuna Nagaraj[3], Ece Aydin[1], Tabiwang Arrey[1], Rosa Viner[2], Robert van Ling[5], Lukas Taujenis[4], Bhavin Patel[2], Ryan Bomgarden[2], Eugen Damoc[1], Wim Decrop[1], Andreas Tebbe[3], Christof Völkle[6], Wolfgang Schrader[6], Martin Samonig[7], Anne Morgenstern[1]

[1]Thermo Fisher Scientific, Germany
[2]Thermo Fisher Scientific, USA
[3]Evotec International GmbH, Germany
[4]Thermo Fisher Scientific, Lithuania
[5]Thermo Fisher Scientific, Netherlands
[6]Sonation GmbH, Germany
[7]Thermo Fisher Scientific, Austria

Keywords

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Goal

Exploring the potential of a novel, intelligent tandem LC-MS workflow with near 100% MS utilization from deep-dive and high-throughput proteomics.

Introduction

Nano- and capillary-LC-MS (0.1–5 µL/min) is widely recognized as the gold standard for deep-dive proteomics workflows due to its exceptional sensitivity and separation capacity.[1,2] However, these advantages come at the cost of reduced sample throughput resulting from slow sample loading, long migration times of analytes, and lengthy washing and equilibration steps. Such factors limit the time available for data acquisition and, ultimately, decrease mass spectrometer utilization.

To compensate, the Thermo Scientific™ Vanquish™ Neo UHPLC system expedites each overhead step through fast loading and equilibration, thereby increasing productivity for both deep-dive and high-throughput applications.[1] However, these overhead steps are never entirely eliminated, becoming particularly time consuming in the direct injection (DI) workflow configurations, particularly when using high flow resistance (e.g., long, narrow I.D.) low-flow columns. Here, we present a novel tandem workflow utilizing a second analytical column and second 1,500 bar binary pump. By offsetting the gradient runs on the two separation columns, it becomes possible to achieve nearly continuous sample introduction into the mass spectrometer, eliminating most MS idle time.

We investigated the capabilities of this robust and easy-to-use tandem direct injection (TDI) workflow for both tandem mass tag (TMT) and label-free quantification (LFQ) in both data-dependent and data-independent acquisition modes (DDA and DIA, respectively). The workflow utilizes the Vanquish Neo UHPLC system in a tandem nano-/capillary-flow configuration in combination with a Thermo Scientific™ Nanospray Flex™ ion source and the new double-barrel oven (DBO) from Sonation Lab Solutions. Together, this setup streamlines low-flow LC-MS methods, offering the following

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advantages: negligible post-column dispersion, high inter-column chromatographic reproducibility through dedicated gradient and reconditioning pumps, and an intelligent and easy-to-use LC method editor that automatically populates parameters to align column and pump pressures and flow rates, switching valves, as well as the timing of voltage switches between the two spray emitters. This new TDI workflow combines increased sample throughput and mass spectrometer utilization with the operational simplicity of a single column configuration setup.

Experimental

Sample preparation

Thermo Scientific™ Pierce™ HeLa Digest/PRTC Standard, (10 μg/vial) was reconstituted by adding 50 μL of 0.1% formic acid (FA) in water with 2% acetonitrile (ACN) or 0.015% *n*-dodecyl-D-maltoside (DDM). The vial was subsequently sonicated for 5 min, followed by aspirating 10 times with a pipette to fully reconstitute the sample. Thermo Scientific™ TMTpro™ 18-plex labeled HeLa samples were prepared in-house by evenly mixing each channel, followed by dissolution in 2% ACN / H_2O, 0.1% FA prior to analysis.

Consumables

- Water with 0.1% formic acid (v/v), Optima™ LC/MS grade, Thermo Scientific™ (P/N LS118-500)

- 80% ACN with 0.1% FA, Optima™ LC/MS grade, Fisher Chemical™ (P/N LS122500)

- Formic acid (FA), Optima™ LC/MS grade, Fisher Chemical™ (P/N 10596814)

- Isopropanol (IPA), Optima™ LC/MS grade, Fisher Chemical™ (P/N 10684355)

- TMTpro 18-plex label reagent (P/N A52047)

- Pierce HeLa Digest/PRTC Standard (P/N A47996)

- Fluidics and columns for tandem direct injection workflow are given in Table 1, Figure 1, and Figure 2.

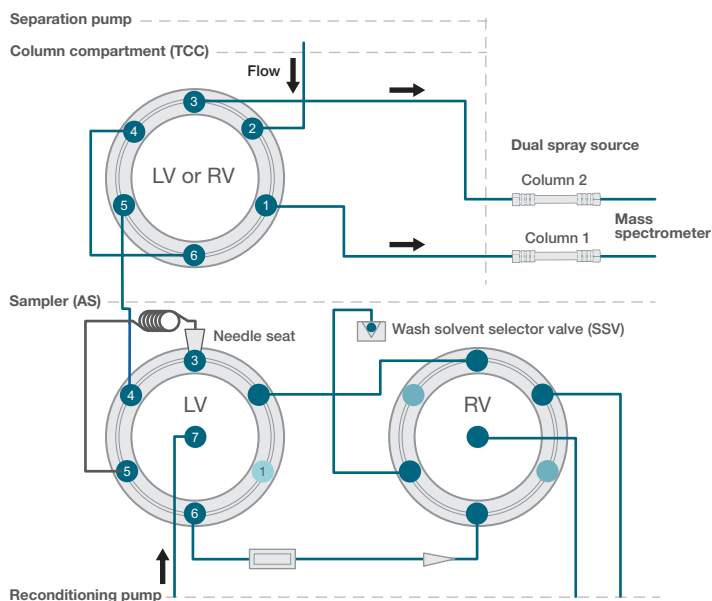**Tandem direct injection, nano/capillary flow (<5 μL/min)**

Figure 1. Vanquish Neo tandem configuration for direct injection workflow

Table 1. Fluidic connections, accessories, and columns for tandem nano- and capillary-flow LC-MS configurations

Flow regime	Separation column	Emitter	Column-emitter connector
Binary Pump N (Upper pump / separation pump)	Thermo Scientific™ Single nanoViper™ PepMap™ Neo column, 75 μm I.D. × 75 cm (1,500 bar, P/N SNV75750PN)	FOSSILIONTECH 10 μm I.D. × 5 cm (LOTUS)	MicroTight™ union (P/N 13040420)
	Thermo Scientific™ Acclaim™ PepMap™ column, 75 μm I.D. × 15 cm (1,200 bar, P/N 164940)	FOSSILIONTECH 10 μm I.D. × 5 cm (LOTUS)/ CoAnn 30 μm I.D. × 5 cm	MicroTight™ union (P/N 13040420)
	Thermo Scientific™ Acclaim™ PepMap™ column, 50 μm I.D. × 15 cm (1,200 bar, P/N 164943)	FOSSILIONTECH 10 μm I.D. × 5 cm (LOTUS)	MicroTight™ union (P/N 13040420)
	Self-packed 75 μm I.D. × 20 cm (1,200 bar)	Pulled-tip	–

Tandem nano- and capillary-LC hardware and fluidic configurations

- Vanquish Neo UHPLC System (P/N VN-S10-A + Vanquish Display P/N 6036.1180)

- Vanquish Column Compartment N (P/N VN-C10-A) with two 2p-6p low-dispersion switching valves (P/N 6250.1520)

- Vanquish Binary Pump N (P/N VN-P10-A-01)

- Thermo Scientific™ Nanospray Flex™ Ion Source (P/N ES072)

- Vanquish Neo system driver 1.5 for tandem workflow execution

- Tandem Workflow Kit, Vanquish Neo (P/N 6250.1030)

- Tandem Source Kit (P/N B51004433)

- Sonation Double Barrel Oven with Mounting Kit NG (P/N B51003991) (Figure 2)

This nano- and capillary-flow configuration was plumbed with 20 μm I.D. Thermo Scientific™ nanoViper™ Fingertight Fittings.

LC solvents

The recommended solvents are listed in Table 2.

Installing and connecting the Sonation double barrel ESI source

The Sonation DBO was installed onto the NanoSpray Flex ion source using the corresponding source mounting kit as per the instructions provided by the manufacturer (Sonation GmbH)[3]. To control the oven temperature independently, the dedicated software (COControl 3.4.8.1) was installed on the MS control PC. Two single-nanoViper (SNV) PepMap columns with identical dimensions were coiled within the oven, with the outlets connected to the emitters via two MicroTight Unions P/N 13040420). The emitters were positioned at a 45-degree angle to each other, interfacing with the MS ion transfer tube. The connections between the LC and column inlets were made using two Y-pieces (P/N ES269, served as liquid junction units) for nanoViper-to-nanoViper connections.
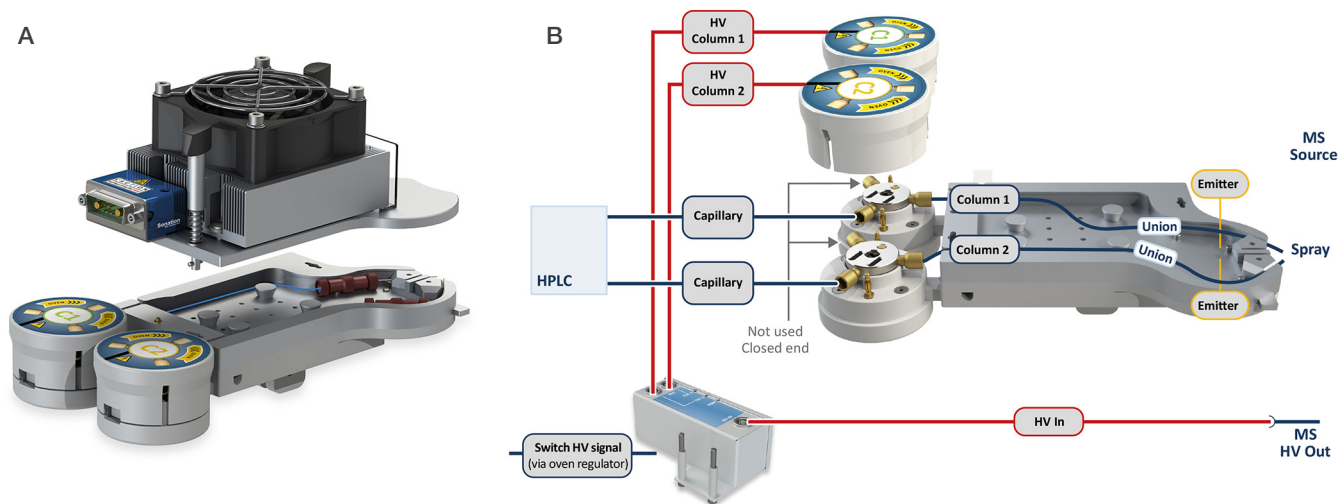

Figure 2. Sonation DBO for tandem nano-flow direct injection application, (A) Oven overview, (B) Fluidic connections inside the oven

Table 2. Solvents used for instrument operation

	Solvent	Composition
Binary Pump N (Upper pump / separation pump)	Mobile phase A	H_2O with 0.1% FA
	Mobile phase B	80/20 (v/v) ACN / H_2O with 0.1% FA
Binary Pump N (Lower pump / reconditioning pump)	Mobile phase A	H_2O with 0.1% FA
	Mobile phase B	80/20 (v/v) ACN / H_2O with 0.1% FA
Split Sampler NT Metering Device	Weak wash liquid	H_2O with 0.1% FA
	Strong wash liquid	80/20 (v/v) ACN / H_2O with 0.1% FA
Split Sampler NT Wash Port	Weak wash liquid	H_2O with 0.1% FA
	Strong wash liquid	ACN with 0.1% FA
Binary Pump N and Split Sampler NT	Rear seal wash buffer	25/75 (v/v) H_2O / IPA with 0.1% FA

The modified NanoSpray Flex ion source was then installed on a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer or Thermo Scientific™ Orbitrap Astral™ mass spectrometer. For automated spray voltage switching, a contact closure cable (P/N 6000.1004) was connected to Relay Port 1 of the Vanquish Neo Column Compartment from the oven regulator. The intelligent LC workflow ensures that the valve positions and high voltage are synchronized to consecutively acquire data in the MS. Voltage is only applied to the active column and emitter while the second column and emitter is dripping.

MS acquisition

MS data were recorded with an Orbitrap Exploris 480 MS or Orbitrap Astral MS in DDA or DIA modes. All method templates are available for download in the Thermo Scientific™ AppsLab Library of Analytical Applications.

Data acquisition and processing

Acquired HeLa digest .raw files were processed with Thermo Scientific™ Proteome Discoverer™ 3.1 software using a 2-step SEQUEST™ HT search algorithm and the INFERYS™ rescoring node or CHIMERYS™ DDA node (DDA data), CHIMERYS DIA node (MSAID), or Spectronaut™ 19 software (DIA data, Biognosys AG). The false discovery rate (FDR) was set below 1% at both the peptide and protein levels.

Results and discussion

Tandem methods overview

The Vanquish Neo tandem direct injection workflow distinguishes itself from the conventional method creation process for tandem workflows in the legacy low-flow LC systems[4,5] by providing a user-friendly experience empowered by system intelligence. Users create methods from scratch by defining standard method parameters including flow rate, duration, and injection volume. Based on the input, the method editor automatically schedules the execution steps for each module in the workflow (Figure 3). This process is similar to the standard method creation in the Vanquish Neo system, ensuring familiarity and ease of use.

The Vanquish Neo system must first be configured to run the workflow on the Vanquish System Controller (VSC) via the Vanquish User Interface (VUI) using the A00 script using the settings below (Figure 4). Care should be taken to ensure the pumps are correctly assigned—lower (reconditioning pump) vs. upper (separation) pump. The corresponding serial number of each pump can be found on both the front and the back of the module.

Note that for the example described here (Figure 4), the "switching valve connected to detector" setting is set to "right". In this case the post-column capillary is connected between the right valve of the column compartment and the MS ion source and should be applied when the LC system is positioned on the left-hand side of the MS ion source to allow for the shortest possible post-column connection. If the LC system is positioned on the right-hand side of the source, the user should select "left" from the "switching valve connected to detector" drop down menu.
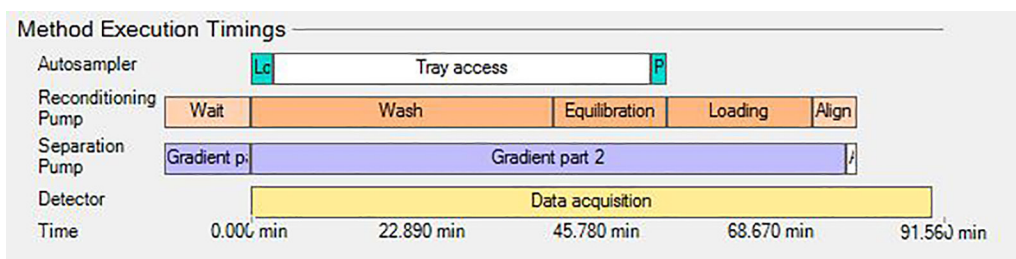

Figure 3. Automatic workflow parameter population in the instrument method editor

Figure 4. Configuring tandem nano- and capillary-flow LC on the Vanquish User Interface

The script guides the user through the entire fluidic configuration process, finishing with a summary page detailing which scripts should be run next including setting solvent types (assuming they differ from those previously selected), running the auto start up script, and setting the separation column specifications. The final script in the list is B06 – condition columns.

The column resistances are determined as part of the B06 script. Determination of the column resistance is essential for proper tandem operation as it is used to define the maximum flow rate available for column washing, equilibration, and sample loading.

The user is required to define three parameters before starting the script (Figure 5). In particular, the column temperature must be set to the temperature that will be used to run the application. It is essential that the column oven temperature should also be set to the correct temperature for the DBO using the COControl software on the LC-MS control PC (see the section on installing and connecting the Sonation double barrel oven). Note that if the columns have already been in use and column reconditioning

is not required, setting the redetermine column resistance only toggle to "on" will skip the column conditioning part of the script, speeding up the script execution time.

The system uses the column resistance values to determine specific method boundary conditions which are automatically uploaded into the instrument method editor, such as the minimum possible cycle time.

Once the instrument has been configured, the instrument method editor is used to program parameters such as flow rate, gradient length, and gradient composition. Here the column wash type and number of wash cycles are also defined. The instrument driver then auto-optimizes the method accordingly.

Details on understanding and optimizing the tandem workflow using the instrument method editor can be found in the Vanquish Neo User Guide version 2.0 or above.[6] For convenience, all method templates presented in this document have been uploaded to the AppsLab Library. The corresponding method overview is in Table 4.

Parameters

Final Pump Flow:	0.250	µL/min	Final Pump %B:	1.0	%
Enter Column Temperature:	50.00	°C	Redetermine Column Resistance Only:	✕	

Figure 5. Measuring column resistance values to determine optimal flow rates for applications

Table 4. Key attributes of the twelve high-throughput and high-sensitivity tandem nano- and capillary-flow methods (flow rate range 0.1–1 µL/min). The 10 µL sample loop (P/N 6252.1960) is required for methods with throughputs of 72 and 180 samples/day.

Separation column	Flow rate (µL/min)	Throughput (samples/day)	Cycle time (min)	Elution window (min)	MS utilization (%)
PepMap Neo 75 µm I.D. x 75 cm	0.25	18.0	91.6	86.7	95
	0.25	7.9	181.6	176.5	97
	0.25	5.3	271.6	267.0	98
	0.25	4.0	361.6	357	99
Acclaim PepMap 75 µm I.D. x 15 cm	1	180	8	7	88
	0.8	120	12	10.8	90
	0.5	100	14.4	13	90
Acclaim PepMap 50 µm I.D. x 15 cm	0.1	72	20	14.8	74
	0.1	48	30	24.5	82
	0.1	36	40	34.5	86
	0.1	28.8	50	44.5	89
	0.1	24	60	54.5	91

Achieving consistent nano-LC-MS performance for LFQ and TMTpro 18-plex samples

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 using a 75 µm × 75 cm column and 90-min gradient (86.7-min elution window) at 250 nL/min, permitting 12 samples per day throughput.[1] Using the TDI workflow, MS utilization was increased to 93%. This improvement raised sample throughput to 18 samples/day (50% increase) while affording a similar performance in protein identification and quantification with 1 µg HeLa peptides in DDA mode (Figure 6A). Although column to column variation was observed, this could be accounted for by normalizing the data using the total peptide amount, a common practice in proteomics data analysis. The same practice is applied for more accurate and reliable protein abundance level comparisons where different columns were used for large-cohort studies (Figure 6B).

To exploit the potential of this configuration, we conducted a single-shot DDA experiment in an Orbitrap Exploris 480 MS with 1,000 ng TMTpro 18-plex HeLa digest (with each channel mixed equally) in gradients ranging from 90 to 360 minutes (Table 4, Figure 7). The highest number of quantified proteins were reached using the 360-minute gradient at >5,700 even without pre-fractionation. Accurate MS2 quantification was also obtained in all conditions (Figure 7). Adopting the CHIMERYS node substantially increased the performance by increasing the number of quantified proteins to >6,700 proteins in a 360-min gradient and facilitating 72 proteomes/day throughput in DDA mode.

Enhancing sample throughput using shorter columns

In the field of high-throughput proteome analysis, the inclusion of a trap column has traditionally been employed to increase sample throughput by increasing sample loading speed, which can be a limiting factor in direct injection workflows. In a previous Technical Note,[7] we used a 300 µm I.D. × 0.5 cm trap cartridge to achieve a throughput of 24–180 samples/day from a 75 µm × 15 cm PepMap Neo column at flow rates ranging from 0.4 to 1.3 µL/min. In that study, we observed MS utilization rates ranging from 68% to 95%.

Through the adoption of the TDI workflow, it was possible to maintain sample throughput at reduced flow rates while significantly enhancing sensitivity and MS utilization to over 90% (Table 4). For instance, at 100 samples/day and 1,000 nL/min, we identified 20% more protein groups compared to the equivalent trap-and-elute workflow when measuring 200 ng samples[7] in DDA mode searched by using the SEQUEST HT node (Figure 8A). Using the TDI workflow running a flow rate at 500 nL/min, we observed an approximately 62% increase in MS intensity (Figure 8B) enabling us to identify >4,300 protein groups from 40 ng samples in DIA mode (Figure 8C).

These findings demonstrate some of the advantages of the tandem direct injection workflow, including improved sensitivity, increased MS utilization, and therefore enhanced protein group identification at reduced flow rates. In other words, this allows for efficient high-throughput proteome analysis with enhanced performance compared to traditional trap-and-elute workflows.
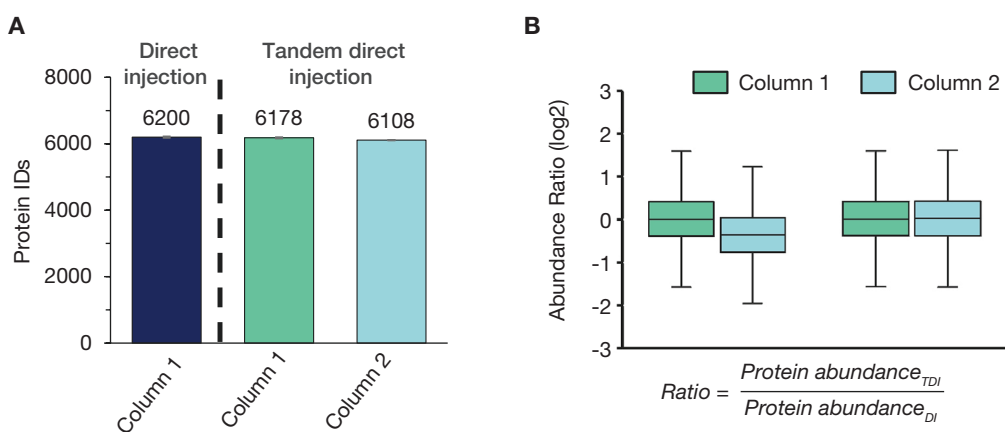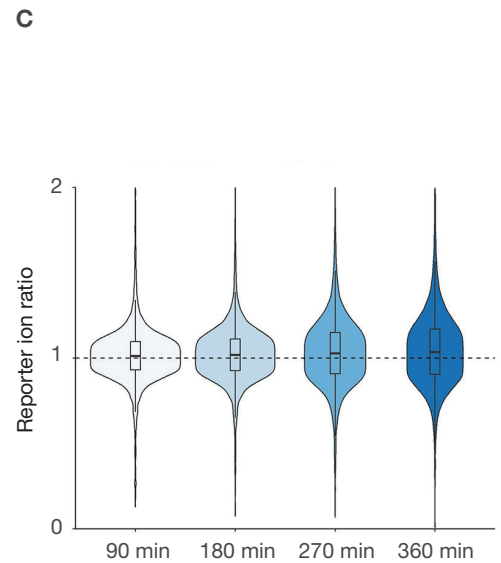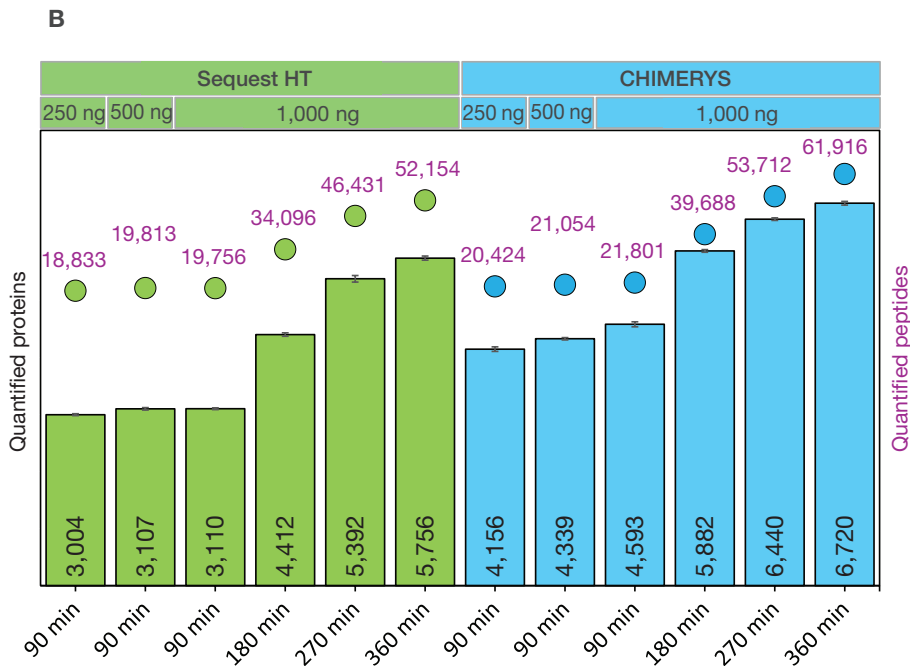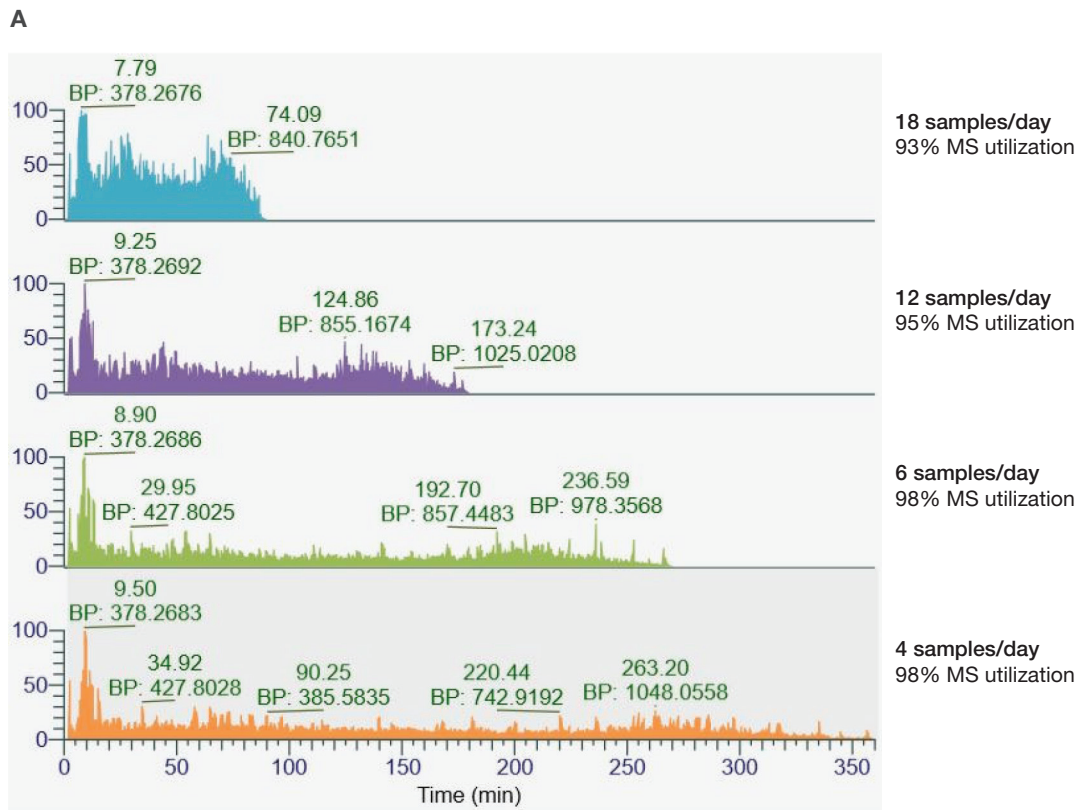

Figure 6. Equivalent performance of direct injection and TDI workflows in protein identification (A) without matching and quantification (B)

Figure 7. High-performance nano-LC-MS for deep-dive proteome profiling from HeLa digest TMTpro 18-plex samples. (A) Elution profiles of 90-, 180-, 270-, and 360-min gradient TDI methods; (B) quantified peptides and proteins (n=4); (C) quantification accuracy (n=4).
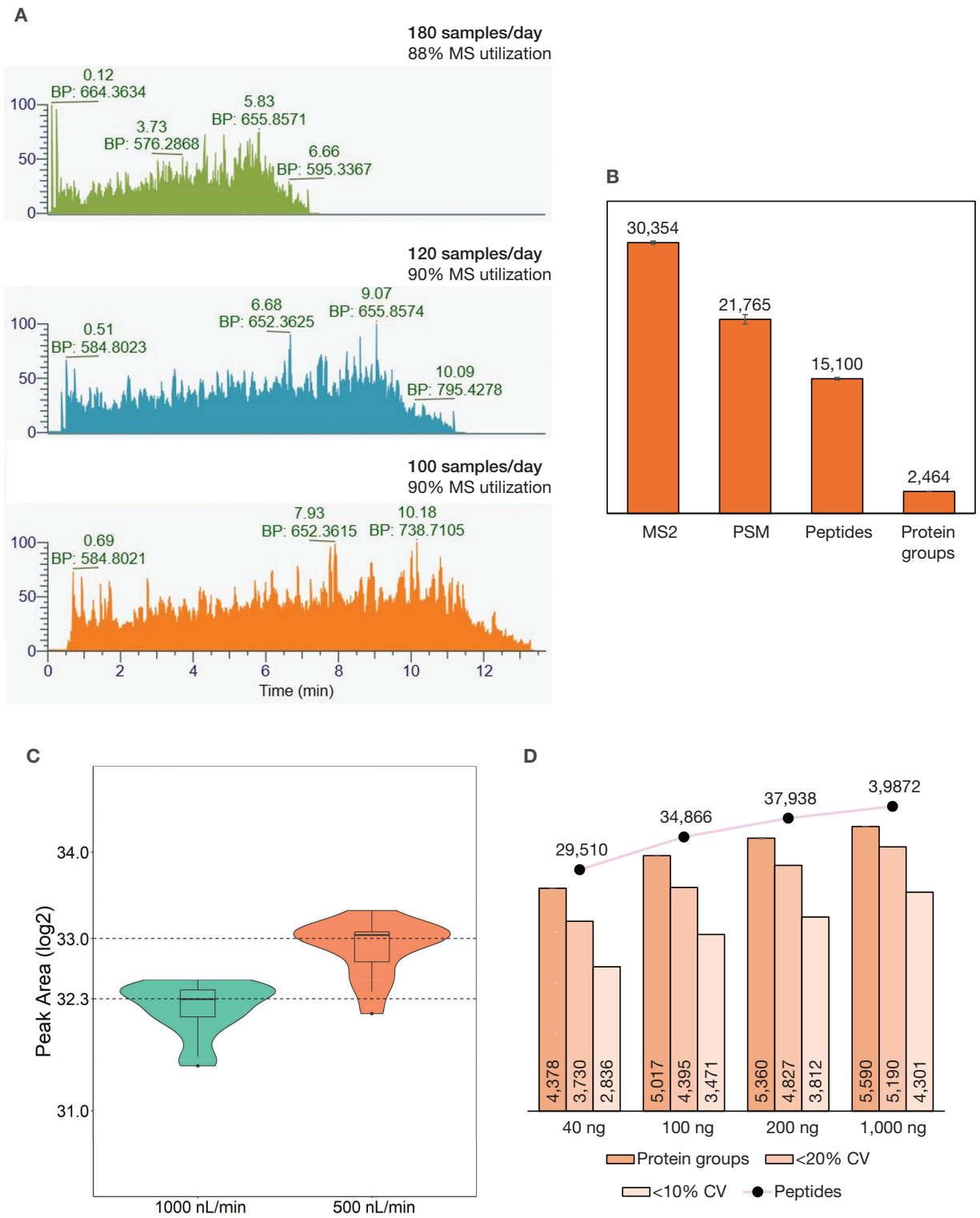
Figure 8. High-performance nano-LC-MS applications with the 75 µm × 15 cm column. (A) 100–180 samples/day at 500–1,000 nL/min; (B) DDA performance at 100 samples/day with 200 ng samples (n=24, w/o matching); (C) Increasing >60% PRTC peptide (50 fmol) intensity at 500 nL/min with 200 ng samples (n=24); (D) Proteome depth and quantitative precision using the 100 samples/day method (n=6) in DIA mode.

Maximizing low-nanoflow LC-MS throughput

Compared to the standard direct injection workflow offering a 72 samples/day throughput with 55% MS utilization (11-min elution window, 50 μm I.D. × 15 cm column),[8] the TDI workflow significantly enhances MS utilization to 74% (14.8-min elution window) at 100 nL/min and the same throughput (Table 6 and Figure 9A). Furthermore, a higher throughput of 80 samples/day is possible with a 12.6-minute elution window (70% MS utilization).
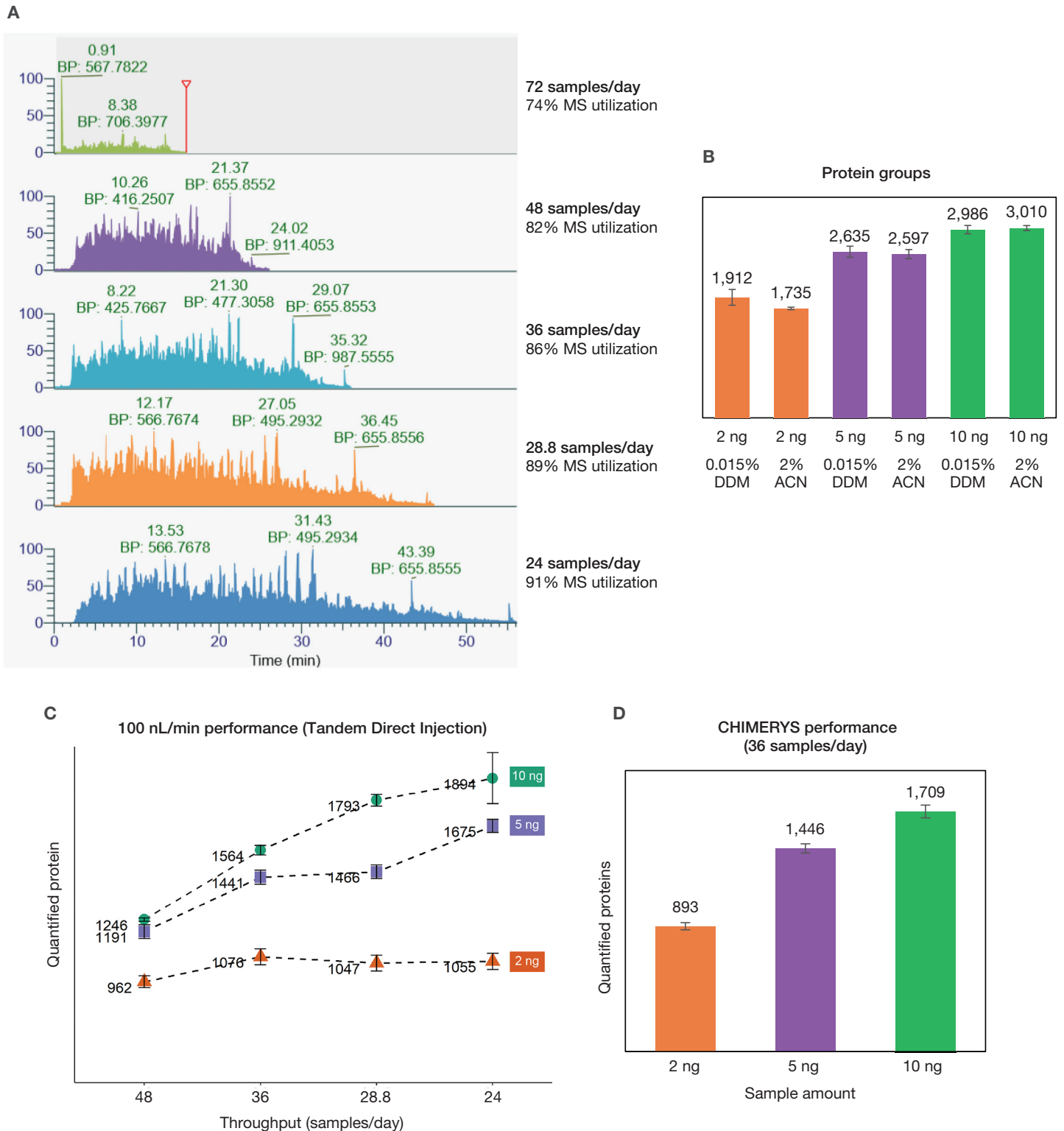

Figure 9. High-sensitivity, low nano-flow LC-MS for sample-limited proteome profiling. (A) 24–72 samples/day at 100 nL/min with 10 ng samples; (B) LFQ-DDA performance at 36 samples/day; (C) Linearity experiments with 2–10 ng TMTpro 18-plex HeLa samples (SEQUEST HT search); (D) CHIMERYS increased the number of quantified proteins at 10 ng sample input in a throughput of 36 samples/day.

At 36 samples/day, we identified approximately 2,600 protein groups from a 5 ng standard HeLa digest (Figure 9B) dissolved in 2% ACN or 0.015% DDM in LFQ-DDA (without FAIMS), which is consistent with the benchmark performance[8]. In the next linearity experiment using TMTpro 18-plex HeLa samples, where each channel was mixed equally, we quantified 1070, 1441, 1564 proteins, respectively, from 2, 5, 10 ng samples (equivalent to TMT 10plexed, 18plexed, and 35plexed individual Hela cells at 0.25 ng protein/HeLa cell) in 40 minutes (Figure 9C). Moreover, the performance was boosted by 9.3% for 10 ng samples using the CHIMERYS node, which yields the potential of analyzing up to 1,260 HeLa cells/day when applying the SCoPE-MS approach for TMT 35plex samples.[9] Taken together, these results demonstrate the power of the TDI workflow for enabling high-throughput proteome analysis for limited sample types.

Minimizing column carryover for reliable identification and quantification in TMT-DDA and LFQ-DIA applications

Another facet of the tandem direct injection workflow compared to its single system counterpart is the capacity to carry out more thorough column washing without impacting sample throughput or MS utilization. The Vanquish Neo tandem workflow incorporates four pre-programmed washing patterns (isocratic, trapezoidal, rectangular, and triangular), catering to different applications, sample types, or user preferences (Figure 10A). By using these washing patterns, column carryover can be minimized, extending the column lifetime while maintaining sample throughput.

Here, we show a proof-of-principle study specifically focusing on the effectiveness of the trapezoidal wash pattern for TMT-DDA and LFQ-DIA applications. Both the quantitative (quantified peptide area) and qualitative (number of quantified peptides) carryover for a 90-gradient method using 250, 500, and 1,000 ng TMTpro 18-plex samples were negligible when using a wash factor of 8 (i.e., 8 column volumes). (Figure 10B). Furthermore, by increasing the washing volume 3-fold in a 180-min gradient with 1,000 ng HeLa digest, it was possible to eliminate the carryover, with no identified peptides (i.e., no peptide-spectrum match) detected in consecutive matrix blank runs (Figure 10B). Furthermore, a subsequent experiment utilizing a more sensitive LFQ-DIA mode with a shorter method (14.4 min, 100 samples/day, 75 µm × 15 cm Acclaim PepMap column) demonstrated its efficiency in minimizing carryover to approximately 0.006% peak area and 0.9–1.8% peptide identifications for 200 and 1,000 ng samples (Figure 10C).

*ND: Not Determined (Sequest HT search)
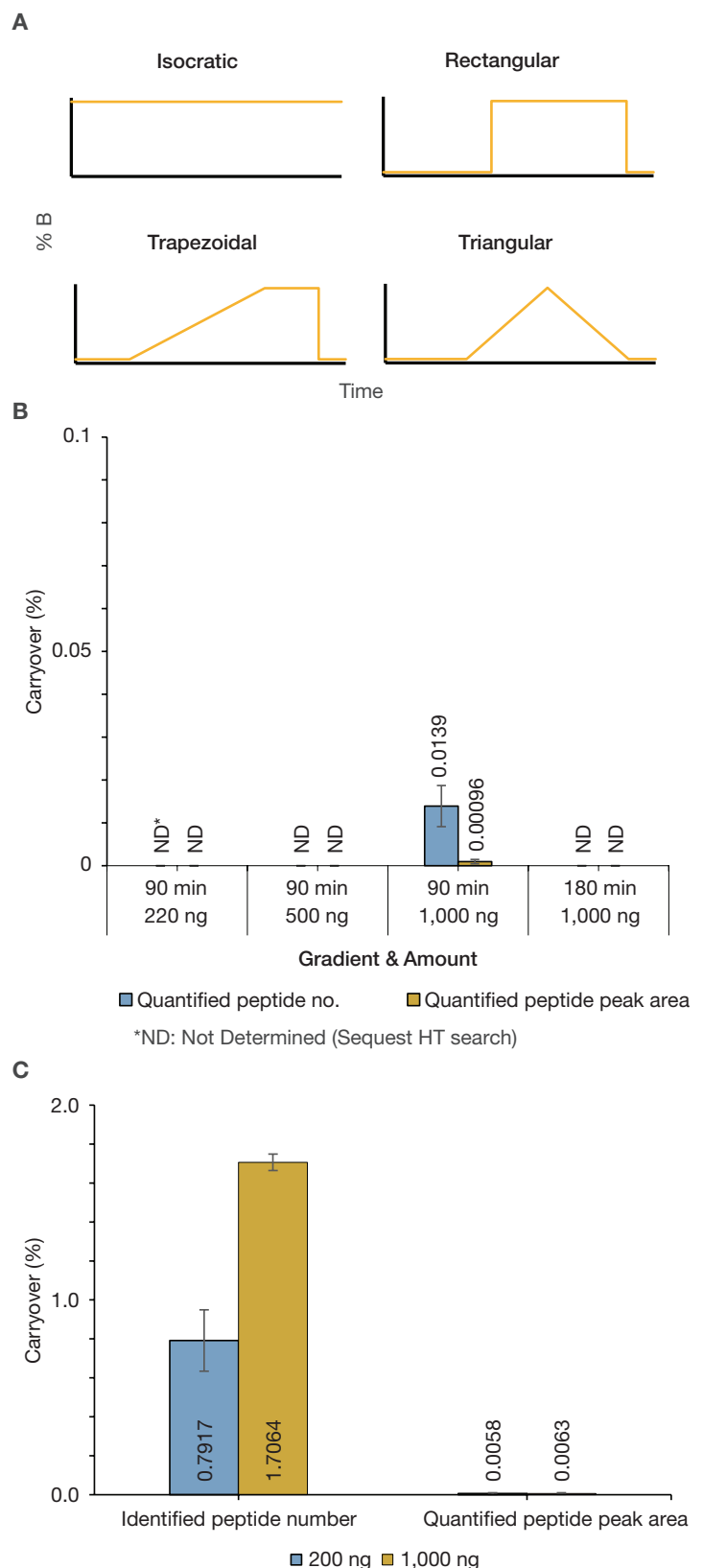
Figure 10. Minimizing carryover for DIA applications at 100 samples/day (n=6). (A) Automatic column washing patterns; (B) negligible qualitative and quantitative carryover at the peptide level (TMTpro 18-plex samples, n=4); (C) low qualitative and quantitative carryover in peptide level (LFQ-DIA samples, n=6).
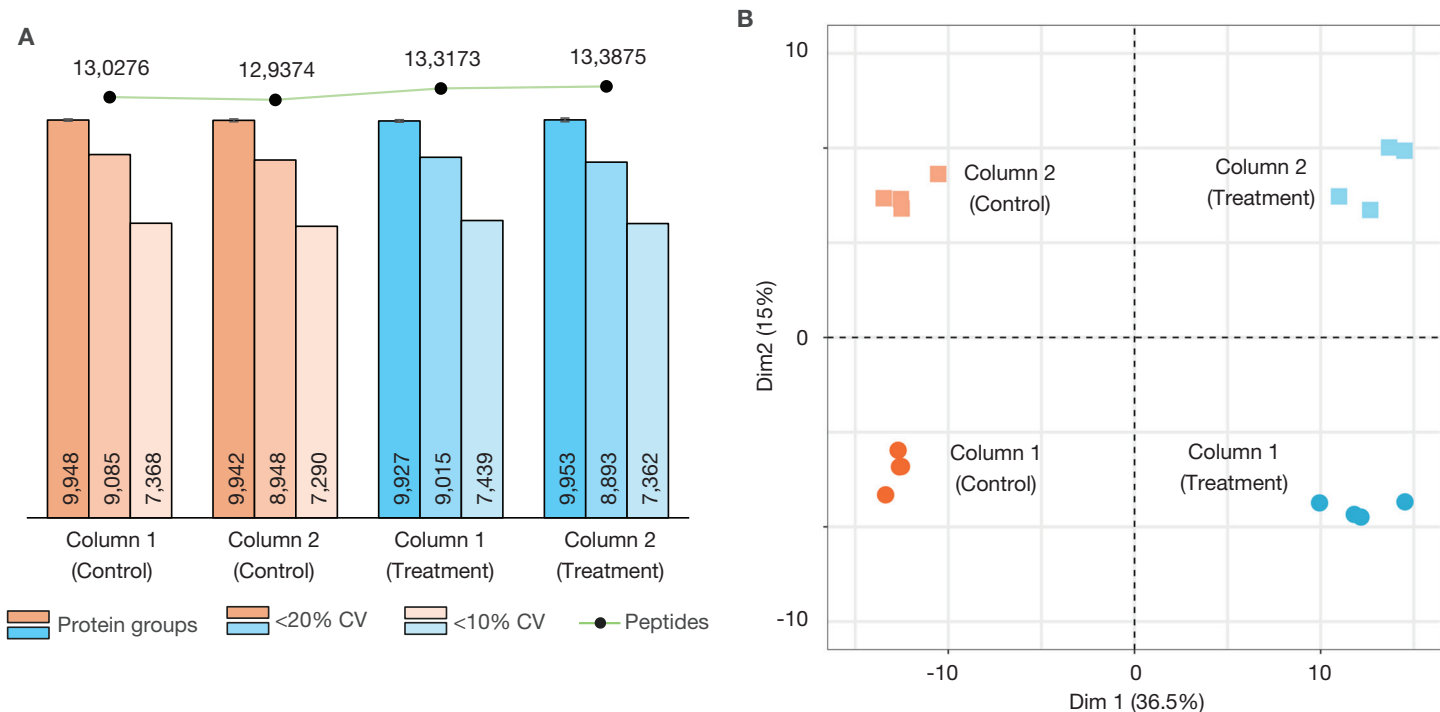
Figure 11. Orbitrap Astral MS DIA performance using the TDI workflow. (A) 96 samples/day performance analyzing cell lysate (n=3); (B) Unbiased differentiation of control (DMSO) and treatment (protein degrader) cell culture groups between columns.

Versatile support of self-packed, pulled-tip nano-LC columns

In addition to commercial SNV PepMap Neo columns, the Sonation DBO also supports self-packed columns ≥20 cm long manufactured from fused silica with 360 μm I.D.

A proof-of-principle experiment was conducted using an Orbitrap Astral MS at 96 samples/day and 700 nL/min using with 75 μm × 20 cm pulled tip columns (1.5 μm particle size / d_p). Approximately 10,000 protein groups were reproducibly quantified from cell lysate in DIA mode (Figure 11A). Principal Component Analysis (PCA) revealed discernable differences in column performance. However, it is important to note that the results also highlighted that the variation in the samples themselves (Control vs. Treatment) had a much more significant influence (Figure 11B). Therefore, these findings demonstrate that the tandem configuration effectively preserves the biological information necessary for large-cohort sample analysis, despite the inter-column variability.

Conclusions

The novel tandem nano- and capillary-LC-MS configuration supporting flow rates of 0.1–5 μL/min with columns from 50–75 μm I.D. described here affords optimal LC-MS performance for columns with I.D. of 50 μm up to 150 μm, maximizing the active data acquisition window for a given

method cycle time. Together with the Nanospray Flex ion source equipped with the novel Sonation DBO, the TDI workflow confers the following key attributes:

- **Ease-of-configuration:** The system is designed with standardized fluidic connections, making it straightforward to set up and configure.

- **Intelligent workflow execution:** The workflow employs smart software tools designed to simplify the process of method creation, enhancing user-friendliness.

- **Maximized MS utilization:** The workflow permits the largest possible data acquisition window for a given method cycle time without sacrificing throughput, ensuring optimal efficiency.

- **Sample throughput**: Improved across all the supported columns, accommodating a wide range of flow rates.

- **Column carryover:** Minimized through extended washing routines without impacting cycle time, enhancing the confidence in identification and quantification.

- **Versatile column compatibility:** Versatility for use with commercial SNV PepMap columns and user generated self-packed (emitter-) columns

Overall, the Vanquish Neo tandem direct injection workflow offers a versatile and efficient solution for a wide range of LC-MS applications, providing ease of use, high MS utilization, robust performance, and reproducible results.

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