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Omics

Enhancing sample throughput and proteome coverage with a novel tandem-LC MS/MS approach

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Keywords

Tandem direct injection workflow, Evotec, bottom-up proteomics, protein degradation, nano-LC-MS, pulled-tip columns, multiplexing, high-throughput, carryover, Vanquish Neo UHPLC system, Orbitrap Astral MS

Application benefits

- The tandem LC workflow increases the available gradient time during methods, improving separation power, and increases the depth of proteome quantification.
- Quantitative precision is preserved across two columns, allowing small proteome differences to be resolved.
- The tandem direct injection workflow provides additional column washing and equilibration capabilities, which are valuable for high-sensitivity applications.
- Further increases in throughput can be obtained through multiplexed measurements, doubling or tripling the samples/day.

Goal

Evaluate the tandem direct injection workflow for the Thermo Scientific™ Vanquish™ Neo UHPLC system to improve sample throughput and proteome coverage of the Evotec™ ScreenPep™ workflow.

Introduction

Nano-flow (<1 µL/min) liquid chromatography has become an indispensable tool for liquid chromatography-mass spectrometry (LC-MS/MS) based bottom-up proteomics because of its high sensitivity and compatibility with small sample volumes. While providing higher sensitivity, direct injection (DI) nano-flow LC workflows suffer from reduced sample throughput and long overhead times (low mass spectrometer utilization) compared with traditional analytical LC-MS/MS flow rates (e.g., 300 µL/min). For screening pipelines

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used for analyzing thousands of samples, long injection cycles and low MS utilization can become costly in terms of both instrument time and result turnaround time. Method overhead can be partially mitigated by using online trap-and-elute workflows, but this comes at the cost of lower proteome coverage. For such screening pipelines, it is crucial to balance throughput, depth of coverage, and quantitative accuracy and precision to generate meaningful biological data in a timely manner.

Evotec is a leading biotechnology R&D company that aims to advance drug discovery and development through co-creation partnerships and CRO/CDMO services with pharmaceutical and biotechnology companies, foundations, and government agencies. Evotec has worked to address the challenge of deep proteomics coverage at an industrial scale by developing the ScreenPep workflow. The workflow utilizes the Vanquish Neo UHPLC system and the Thermo Scientific™ Orbitrap™ Astral™ Mass Spectrometer, along with automated sample preparation and a dedicated bioinformatics pipeline. To maximize both the sample throughput and the proteome coverage per sample, they adapted a novel Tandem Direct Injection (TDI) workflow on the Vanquish Neo UHPLC system that eliminates method overhead from LC-MS method cycle time. By maximizing the MS utilization during each injection cycle, the TDI workflow provides the sensitivity of direct injection workflows without suffering from significant method overhead time.

Vanquish Neo tandem system installation with the Sonation double barrel nESI source

The Sonation double barrel oven (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). To control the oven temperature independently, the dedicated software (COControl 3.4.8.1) was downloaded from the Sonation GmBH website and installed on the MS control PC. The modified NanoSpray Flex ion source was then installed on the Orbitrap Astral mass spectrometer. Two nanoViper capillaries

Experimental

Table 1. Consumables.

Consumable name	Cat. No.
Thermo Scientific™ Pierce™ HeLa Protein Digest Standard	88329
Promega™ MS-Compatible Yeast Protein Extract, Digest	V746A
Waters™ MassPREP™ *E. coli* Digest Standard	186003196

Table 2. Tandem nano- and capillary-LC hardware and fluidic configurations.

Category	Item name	Cat. No.	Quantity
Vanquish Neo tandem system	Vanquish Neo UHPLC system	VN-S10-A-01	1
	Thermo Scientific™ Vanquish™ User Interface, Vanquish Display	6036.1180	1
	Thermo Scientific™ Vanquish™ Column Compartment N	VN-C10-A-01	1
	Thermo Scientific™ Vanquish™ Switching Valve, 2p–6p low-dispersion	6250.1520	2
	Thermo Scientific™ Vanquish™ Binary Pump N	VN-P10-A-01	1
	Thermo Scientific™ nanoViper™ Capillary Kits for Low-Flow UHPLC Systems, Tandem Workflow Kit for Vanquish™ Neo UHPLC Systems	6250.1030	1
	Vanquish Neo system driver version 1.5 or higher		1
Dual-spray ion source*	Thermo Scientific™ Nanospray Flex™ Ion Source	ES071	1
	Sonation tandem source kit	B51004433	1
	Sonation double barrel oven with mounting kit NG	B51003991	1
Liquid junctions** (for home-made pulled-tip columns)	Upchurch Scientific™ VHP Micro Adapter Tee for 1/16” to 360 µm OD	UH-753	2
	IDEX Health & Science™ VHP MicroFerrule PK 360, 360 µm	PK-152	2
	Thermo Scientific™ HV Cable Assy, liquid junction	G20-2087	2
	Sonation UHP T-pieces with HV connector	004.800.02	2

*Only available from Thermo Fisher Scientific
**Sonation GmbH offers the bundle kit (Cat. No. BB0071) including all required parts for homemade, pulled-tip columns.

(20 µm × 550 mm, Cat. No. 6250.5260, included in the Tandem Workflow Kit (Cat. No. 6250.1030)) were connected from the switching valve located in the LC column compartment to two VHP Micro adapter tees (1/16" to 360 µm OD, IDEX Cat. No. UH-753) placed in T-piece holders (Sonation Cat. No. 004.800.02, Figure 1A).

Two self-packed 75 µm I.D. × 20 cm (360 µm OD, 1,200 bar, C18, 1.5 µm d.p.) C18 columns with pulled-tip emitters were connected using 360 µm UHP Microferrules and placed in the oven. Following this, the high voltage cable assemblies were connected to the third port of the tees from the DBO HV-Switch (Figure 1B). The emitters were positioned at a 45-degree angle to each other, adjacent to the MS ion transfer tube (Figure 1C).

For automated spray voltage switching, a contact closure cable (Cat. No. 6000.1004, included in the double barrel oven mounting kit (Cat. No. B51003991)) was connected to Relay Port 1 of the Vanquish Neo column compartment from the Sonation oven regulator. The LC workflow automatically helps ensure that the valve positions and high voltage are synchronized to acquire MS data consecutively on each column. Voltage is only applied to the active column and emitter for data acquisition while effluent from the inactive column drips from the inactive emitter.

Sample preparation

Tryptic digested samples from *Homo sapiens*, Yeast, and *E. coli* were dissolved in H_2O with 0.1% FA individually at a concentration of 1 µg/µL. Then the digests were mixed in the following (v/v) ratios: Mix A (50 % *Homo sapiens*, 45% Yeast, 5% *E. coli*), Mix B (50% *Homo sapiens*, 40% Yeast, 10% *E. coli*), Mix C (50% *Homo sapiens*, 5% Yeast, 45% *E. coli*). 0.8 µL (800 ng) was injected to evaluate the LC-MS quantification accuracy.

For targeted protein degradation (TPD) experiments, cells were treated with either DMSO as vehicle control or commercially available BRD4 degrader MZ1 (5 µM) for 6 h (Figure 2). After automated sample preparation, including cell lysis, tryptic digest, and peptide desalting by SPE (Phenomenex™ Strata™-X-C 33 µm Polymeric Strong Cation, Cat. No. 8B-S029-EBJ), samples were

Table 3. LC solvents.

Module	Solvent	Composition
Vanquish 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
Vanquish 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
Vanquish 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
Vanquish split sampler NT wash port	Weak wash liquid	H_2O with 0.1% FA
	Strong wash liquid	80/20 (v/v) ACN / H_2O with 0.1% FA

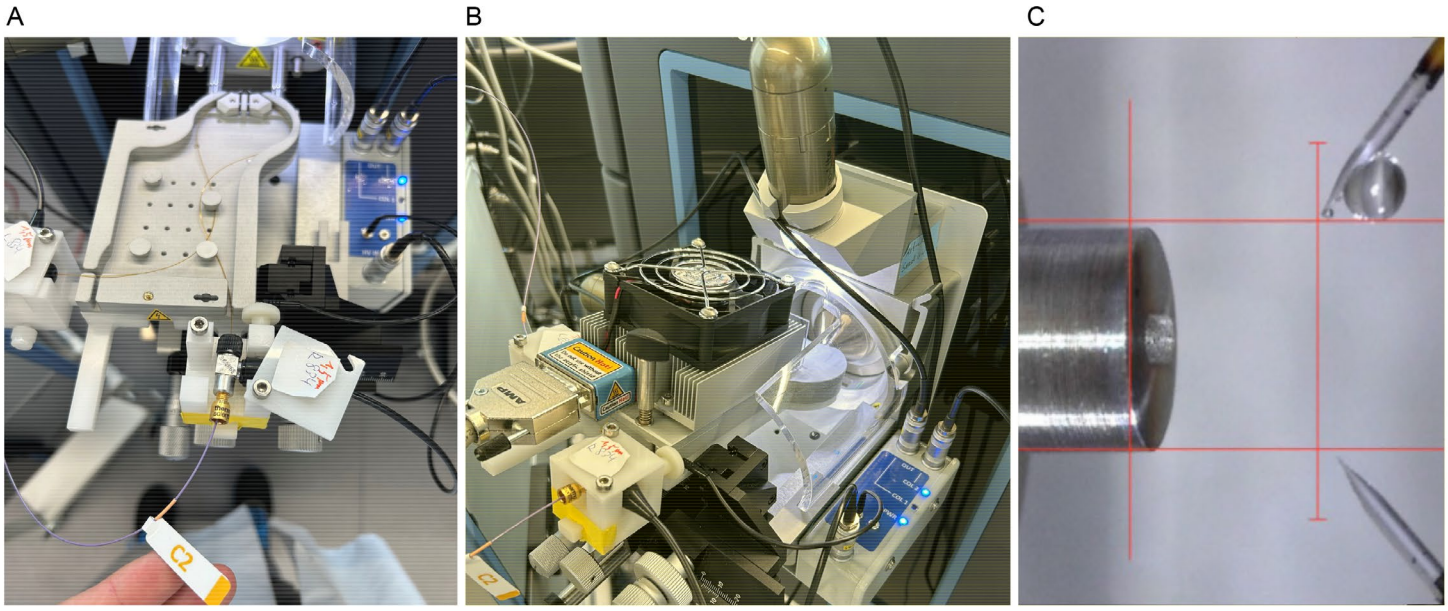


Figure 1. The tandem DI workflow configuration. (A) The liquid junction used for self-pack columns; (B) Double barrel oven mounted onto the ion source; (C) The emitters are positioned 45° to each other in front of the MS inlet, where the spray voltage is applied to the top/right emitter while the bottom/left, inactive emitter drips (automatically controlled by LC software).

adjusted to a concentration of 400 ng/µL. A volume of 2 µL was injected per run for LC-MS analysis resulting in a load of 800 ng.

LC-MS acquisition

For LC-MS measurements, we used in-house-packed columns cut to 20 cm length. Fused silica capillaries with 75 µm inner diameter (360 µm O.D.) were pulled using optimized conditions with a Sutter laser puller. Pulled silica emitter tips were packed using 1.5 µm C18 beads. Columns were maintained at a constant temperature of 60°C using the Sonation DBO. LC settings for each throughput from 30 to 96 samples/day (SPD) method are given in Table 4.

MS data were recorded with an Orbitrap Astral MS in DIA mode. The MS method parameters are given in Table 5. For 3-proteome HYE (human, yeast, *E. coli*) experiments, four replicates of each ratio mix (Mix A, B, C) were injected per column, resulting in 24 injections (2 columns × 4 replicates × 3 mixes) per method. For label-free and SILAC TPD experiments, four replicates of MZ1-treated and DMSO-treated samples were measured per column, resulting in 16 injections (2 columns × 4 replicates × 2 conditions) per method.

Data processing

Acquired .raw files were processed with Biognosys™ Spectronaut™ 18 software (directDIA+, Biognosys AG). SILAC data were analyzed using Spectronaut 19 software (directDIA+, Biognosys AG). For 3-proteome HYE analyses, a mixed FASTA of *E. coli* UniProt, *S. cerevisiae* UniProt and *H. sapiens* Swiss-Prot sequences (total of 53747 entries, version: April 2021) was used. For label-free and SILAC TPD samples a *H. sapiens* Swiss-Prot FASTA (42397 entries) was used. All files of one throughput experiment (24 files for HYE, 16 files for label-free and SILAC) were searched together using default directDIA+ settings. False discovery rate (FDR) was set below 1% at both the precursor and protein levels.

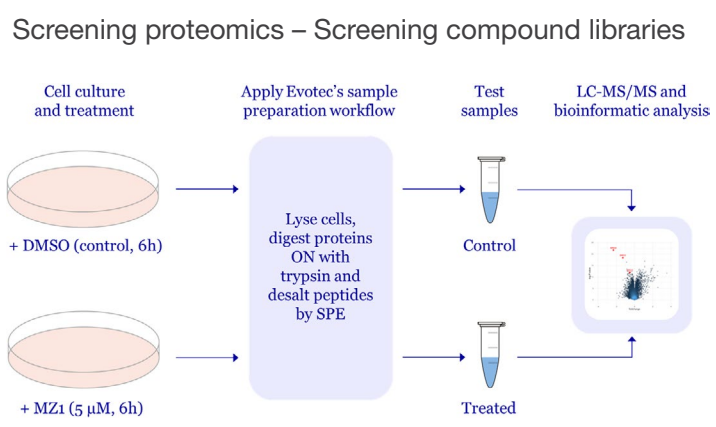
Results and discussion

Increased effective gradient time using the TDI workflow results in more proteins quantified

The Vanquish Neo tandem direct injection workflow has the unique capability of preparing the second column while the first one is being actively used for data acquisition. During this preparation step, the column is extensively washed and equilibrated, followed by sample loading. Since this preparation takes place in parallel to data acquisition on the active column, the fastest loading settings are not required to maintain a certain throughput. Conversely, for a single column setup (i.e., DI workflow) to keep up with throughput requirements, the effective gradient time must be shortened to accommodate washing, equilibration, and loading steps. For example, to achieve 30 SPD, the total runtime including the preparation steps for each sample is 48 minutes. Using the TDI workflow results in 47 minutes of total gradient time, whereas in a single column setup with fast loading, only 38 minutes are available for the gradient (Figure 3). The effects are more pronounced at faster throughputs like 96 SPD, where tandem operation enables up to 14 minutes of effective gradient time compared to just under 5 minutes with a single column configuration.

To evaluate the tandem workflow, we first constructed a triple species sample of human, yeast, and *E. coli* peptides in three different mixing ratios termed Mix A, Mix B, and Mix C, respectively. The mixed peptides were measured at 30, 48, 60, and 96 SPD both in DI and TDI configurations. Because of the longer effective gradient time in the TDI configuration compared to DI, the number of proteins identified was systematically higher at increased sample throughput (e.g., 96 SPD in the TDI workflow delivers an equal performance as 60 SPD in the DI workflow) (Figure 4A). The MS method used for each workflow was identical; therefore, the improvement resulted from the performance boost achieved using TDI. To verify this,

A



B

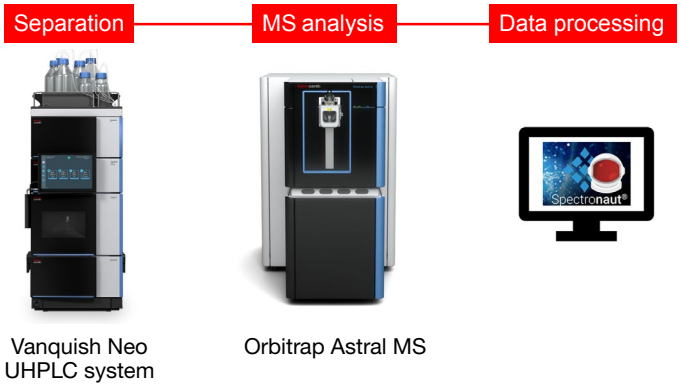


Figure 2. Evotec screening proteomics pipeline for targeted protein degradation analysis (A) and the LC-MS/MS workflow comprising a Vanquish Neo UHPLC system using the tandem direct injection workflow and Orbitrap Astral mass spectrometer (B).

Table 4. Basic TDI method settings.

Parameter	30 SPD	48 SPD	60 SPD	96 SPD
Flow rate (µL/min)	0.400	0.400	0.400	0.700
Duration (min)	47.0	29.0	23.0	14.4
Gradient duration (min) (3–41 %B)	44.5	27.5	22.0	13.1
Delay time (min)	Automatic	Automatic	Automatic	0.7
Injection volume (µL)	2.0			
Injection volume limit (µL)	5.0	5.0	5.0	2.0
Draw speed (µL/s)	0.2	0.2	0.2	0.2
Loading volume (µL)	Automatic	Automatic	Automatic	4.0
Wash factor	10.0	18.0	10.0	8.0
Equilibration factor	5.0	2.0	6.0	2.0

Table 5. MS method parameter settings for standard DI and TDI methods.

Parameter	30 SPD	48 SPD	60 SPD	96 SPD
Global parameters				
Spray voltage	2.2 kV			
Ion transfer tube temp. (°C)	290			
Orbitrap full scan properties				
Orbitrap resolution	240,000			
Scan range *(m/z)*	380–980	380–980	400–800	400–800
RF lens (%)	40			
Normalized AGC target (%)	500			
Max. injection time (ms)	5			
Data-independent acquisition properties				
Precursor mass range *(m/z)*	380–980	380–980	400–800	400–800
Isolation width (Th)	2	2	4	4
Window placement optimization	On			
NCE (%)	25			
Scan range *(m/z)*	150–2,000			
Max. injection time (ms)	3.5	3.5	3.5	3
AGC target (%)	500			
Loop control	Time			
Time (s)	0.6			

we compared LC performance metrics for data acquired in TDI and DI column workflows and found that the peak capacity is significantly higher when using the tandem workflow, resulting in higher protein identification.

In addition to identifications, protein quantification precision with sufficient points-per-peak is crucial for acquiring high-quality data. Because of the increased effective gradient time when using TDI, there is a marginal increase in the number of proteins identified but a notably significant increase in the number of proteins quantified with CV less than 10%. While the 30 SPD method showed little differentiation in quantified proteins at CV <10%, faster throughputs yielded increased gains using TDI. At 96 SPD, the number of proteins quantified with less than 10% CV is nearly doubled compared with DI (Figure 4B).

Quantitative accuracy is preserved across two columns in tandem operation

The above data demonstrates that the tandem workflow significantly increases the quantitative proteome depth in very

short gradients compared to single column injection. However, it is important to note that the quantification of proteins is performed across two columns in the tandem workflow and to evaluate the variations that could arise from the two columns used in the measurement. To test this, we used the same three species mixed proteome of HeLa, yeast, and *E. coli* peptides at three different mixing ratios designated as Mix A, Mix B, and Mix C samples. These samples were analyzed using 30, 48, 60, and 96 SPD methods in quadruplicates on each column in a tandem setup, resulting in eight total replicates for each mix at each throughput. All samples were processed in Spectronaut software using the MaxLFQ algorithm for label-free quantification.

We investigated if the quantitative comparison is different depending on whether the replicates are measured within a single column or across two different columns in the tandem workflow. First, in a scatterplot of the log 2 median intensity versus log 2 fold change for Mix A versus Mix C, the overall distribution of the plot looks nearly identical for the replicates that are selected from a single column or across the two columns (data not shown). Furthermore, if all eight replicates from the three mixed samples for each throughput are visualized in a dimensionality reduction plot like PCA analysis, the three different samples separate very clearly from each other without any major contribution resulting from the column (C1 or C2) in which the samples were measured (Figures 5A and 5C). Not surprisingly, in a dedicated PCA plot with only the samples from a single mix, the replicates could be seen separating according to the column in which they were measured (Figures 5B and 5D). Such differences coming from the columns (Figures 5B and 5D), however, are negligible compared to the differences between Mix A, Mix B, and Mix C samples (Figures 5A and 5C). This indicates that the quantitative variations arising from two different columns were much smaller than the difference coming from the samples under study. Therefore, samples could be analyzed in a high-throughput manner across two columns in tandem mode without any compromise in quantitative accuracy.

Effective washing in tandem system minimizes separation column carryover

In the tandem workflow, when one column is used for separation, the other column is washed and equilibrated. The amount of time available for this preparation is proportional to the gradient time allocated for sample acquisition. In general, the time available for column preparation in TDI is significantly higher than in the single column DI workflow at the same throughput. The extent of washing varies also with the column dimensions. A longer column or a narrow ID column would result in higher back pressure, which subsequently leads to fewer wash cycles and equilibration in a given time compared to a shorter or wider column that results in a lower back pressure.

In this study, we used a 20 cm long, 75 µm ID column, which represents the shortest possible length compatible with the column oven geometry. Given the extensive washing capabilities when using TDI compared to single column operation in DI, the amount of carryover from one run to the next run could also be significantly reduced in large-scale proteomics operations. To test this, we injected different amounts of peptide loads ranging from 200 ng to 800 ng on single and tandem columns followed directly by blank injections to examine the amount of carryover. As expected, given the more extensive washing (18 column volumes with trapezoidal washing pattern) in TDI for the 48 SPD method, the amount of carryover in terms of quantified peptide peak area is less than half of what is observed in DI. At faster throughputs, like 96 SPD, the effect is even more drastic with four-fold less carryover with the TDI configuration (Figure 6).

The extensive washing capabilities would be beneficial in samples with hydrophobic contaminants that could be more efficiently removed from the column between sample injections. In turn, this would further increase the lifetime of the column when analyzing large sample cohorts. Moreover, the extensive washing capabilities may also improve the sensitivity and robustness of the mass spectrometer with fewer cleaning cycles, as the washed contaminants are not sprayed directly into the instrument.

Targeted protein degradation assay in tandem mode of operation

The results from HYE mixed sample measurement indicate that the TDI workflow yields robust quantitative proteome analysis across two columns. We next wanted to investigate whether this platform could be used in one of our routine screening proteomics platforms to assay thousands of compounds to find potential neo substrates for E3 ligases. Molecular glues are small molecules that bind to E3 ligases, changing the way they interact with proteins and sequestering the interacting protein for ubiquitin-proteasomal pathway for degradation. This proves to be an effective way to degrade specific proteins using small molecules as potential drugs with many advantages compared to proteolysis-targeting chimera (PROTAC) based drugs. To identify novel molecular glues that bind E3 ligases to target compounds, typically tens of thousands of compounds are screened in cell model systems by interrogating the proteome for degradation. High-throughput proteomics plays a central role in such screening studies and increased throughput capabilities provided by TDI might prove highly beneficial in such studies to further increase the throughput and scale of operations.

As proof of principle for such large-scale compound screening proteomics analysis, we used MZ1, a well-known reference compound that is a strong degrader of the protein BRD4. Cell lines in culture were treated with 5 µM MZ1 compound

for 6 hours or with DMSO vehicle control. After the 6-hour incubation, cells were lysed and digested, and peptides were purified according to Evotec's automated sample preparation pipeline. These peptides from the compound-treated and control samples were then injected using TDI at four sample throughputs (30, 48, 60, and 96 SPD). In this case, a comparison to the single column setup was not performed and all the samples were solely measured using the tandem workflow. Like in the previous HYE experiments, this experimental design provides quadruplicates for each sample within a single column and across two columns. In the HYE experiments, a significant portion of proteins changed quantitatively. However, MZ1 is known to specifically degrade a small number of proteins with the vast majority of the proteome unchanged upon 6-hour treatment. We therefore investigated whether such minute changes in the proteome were still larger than intercolumn variation using TDI and whether such changes were robustly quantifiable across the two columns.

The fast scanning speed of the Orbitrap Astral MS together with the increased effective gradient time available via TDI largely preserves the number of proteins identified as the throughput of measurement is increased from 30 to 96 SPD. We identified close to 11,000 protein groups from the cell lines in 30 SPD, and even at 96 SPD we identified close to 10,000 protein groups. The performance remained constant across the two columns for both DMSO- and MZ1-treated samples with no systematic variations. Furthermore, we could still quantify close to 7,500 protein groups with less than 10% CV in 96 SPD. To investigate the quantitative performance, we checked the sample separation in a PCA plot. In all throughputs from 30 to 96 SPD, the samples separated

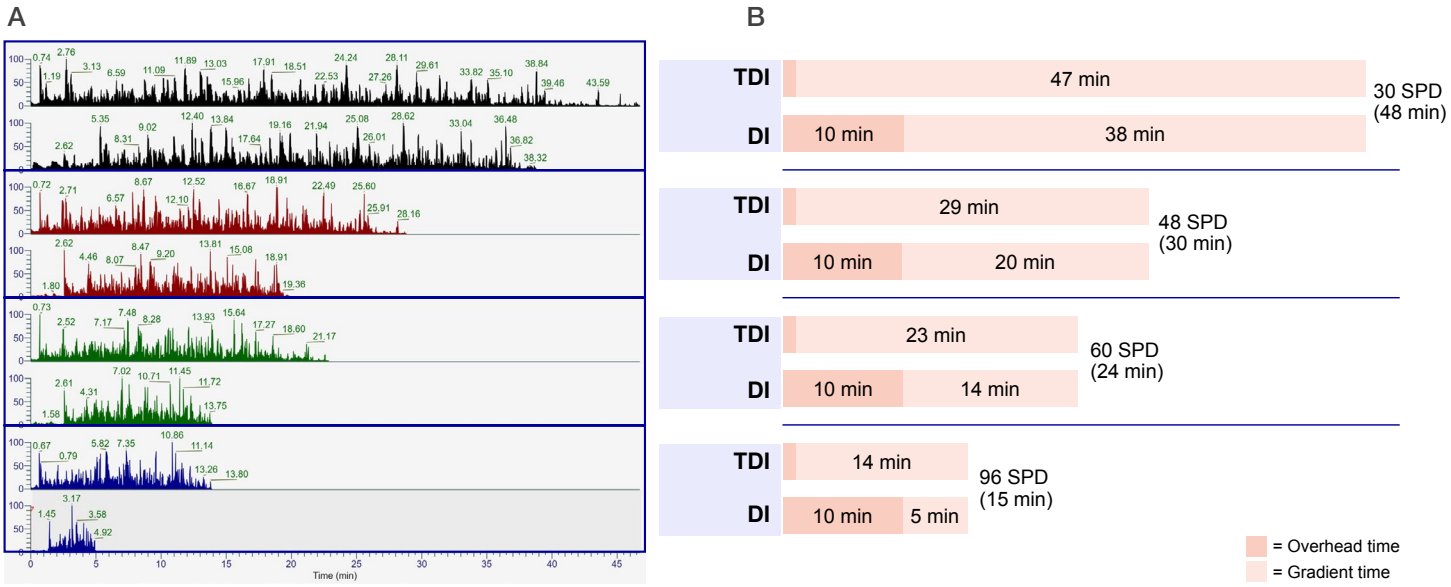


Figure 3. Gradient and throughput comparison of standard DI and Tandem DI workflows. Elution profile of 30–96 SPD methods (A) with corresponding method overhead time and MS utilization comparison (B).

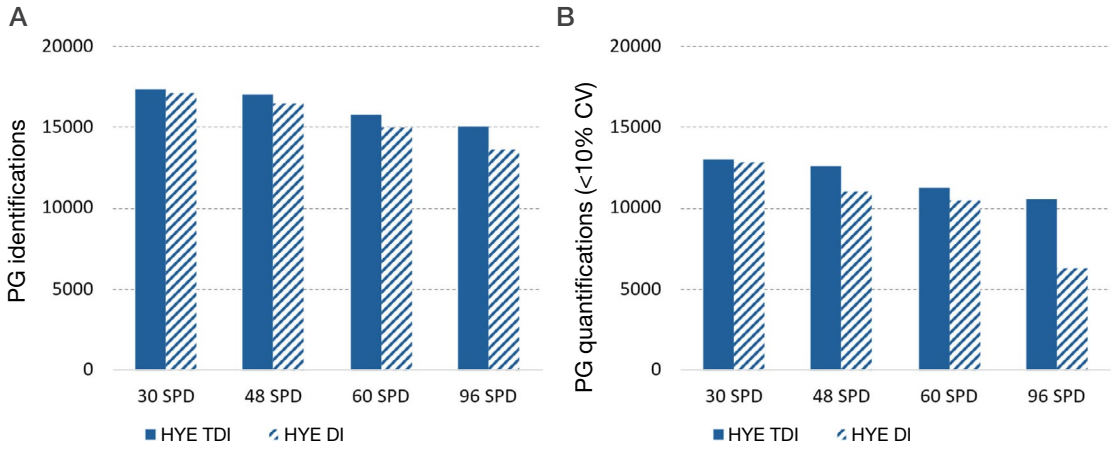


Figure 4. (A) The number of proteins identified in the HYE sample (Mix B shown here) and (B) the number of proteins that are quantified with a CV less than 10%.

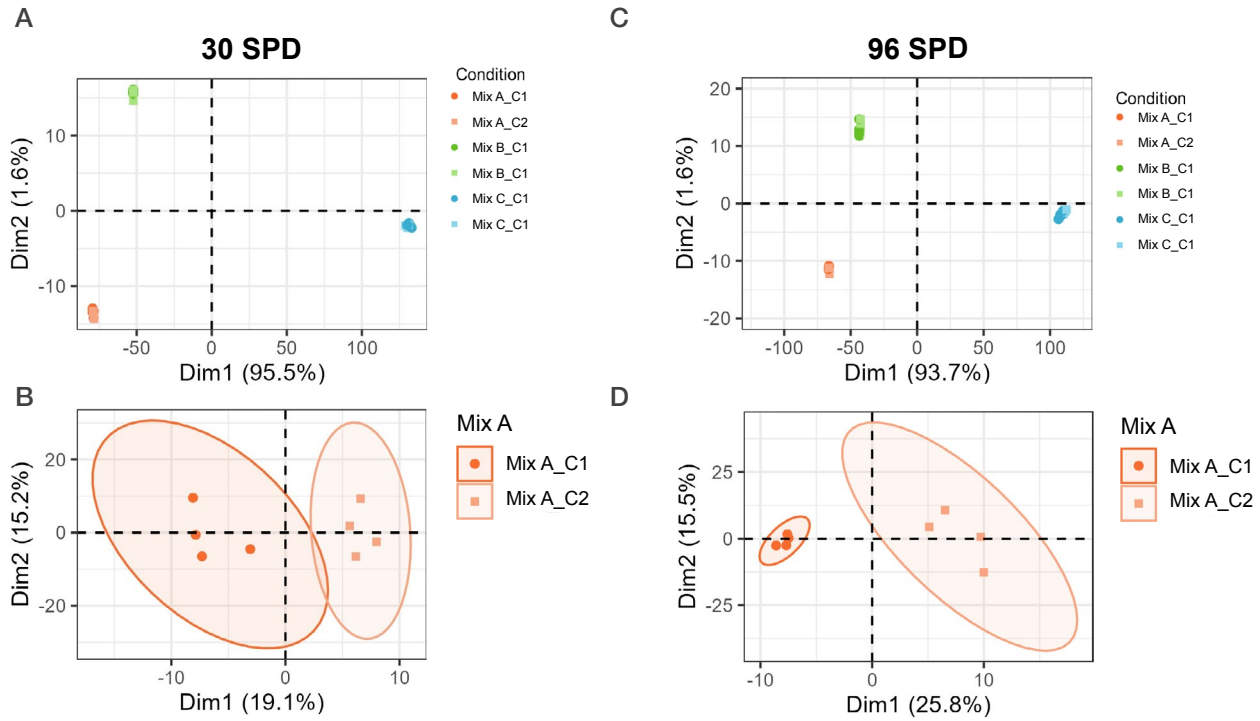


Figure 5. PCA plots. (A) and (C): For the three HYE sample mixes (n = 8 per mix, 4 replicates per column) at 30 and 96 SPD, respectively. Clear separation of sample in all throughputs without any distinction on which column (C1 or C2) the samples were measured. (B) and (D): When plotting for only one sample mix (e.g. Mix A), partial clustering of samples depending on which column they were measured on was observed.

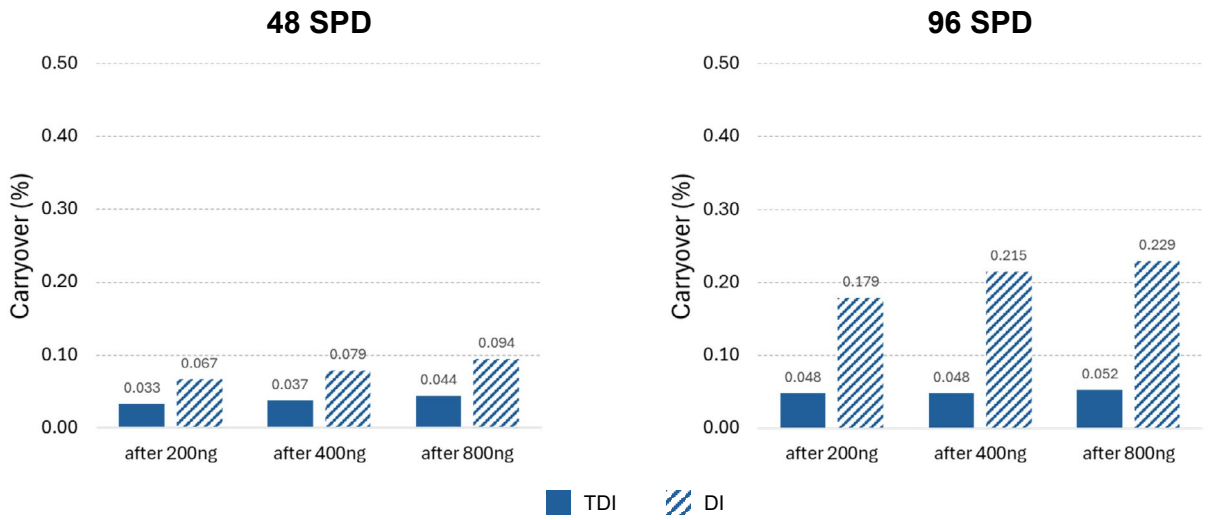


Figure 6. Percentage of column carryover at peptide level (quantified peptide peak area) in TDI and DI workflows at 48 and 96 SPD. Significant reduction in carryover was observed at both throughputs.

according to the compound treatment in the first component, indicating that the proteome differences were clearly visible and, more importantly, larger than the intercolumn variation, despite only few proteins changing upon the compound treatment (Figure 7).

With the available eight replicates for each sample, the MZ1 versus DMSO comparison could be performed either within the single column or across the two columns. To evaluate the effectiveness of quantification across the two columns, we used all replicates coming from the two columns for quantitative analysis of protein regulation upon MZ1 treatment. As visible from the volcano plots, down-regulation of BRD4 and BRD2 is quantifiable across the two columns in the TDI workflow (Figure 8).

Increased measurement throughput by multiplexing samples using SILAC

From the label-free cell line samples, we identified close to 10,000 protein groups and quantified 7,500 protein groups with less than 10% CV at 96 SPD. While this is already a high-throughput approach, large-scale studies that involve thousands of samples would require further increases in throughput. We hypothesize that a further increase in throughput by reducing the gradient length would likely preserve the number of proteins identified, but quantification would deteriorate due to a lack of sufficient data points per peak. An alternative approach would be to multiplex the samples either by SILAC or dimethyl labeling approaches at the same method throughput. The multiplexing is only applied to increase the throughput, but without relative quantification between channels to avoid missing ratios in protein quantification and to avoid including a reference sample. Together with the multiplexing, we introduce here the concept of injections per day (IPD) in contrast to samples per day (SPD). This is because if we multiplexed the samples by a factor of N and measured at the 96 SPD gradient, the actual throughput of measurement would be N times 96 SPD. Thus, with the injections per day concept, the gradient throughput and actual sample throughput are distinguishable. For example, a double SILAC multiplexed sample measured at 60 IPD gradient would have 120 SPD throughput (Table 6).

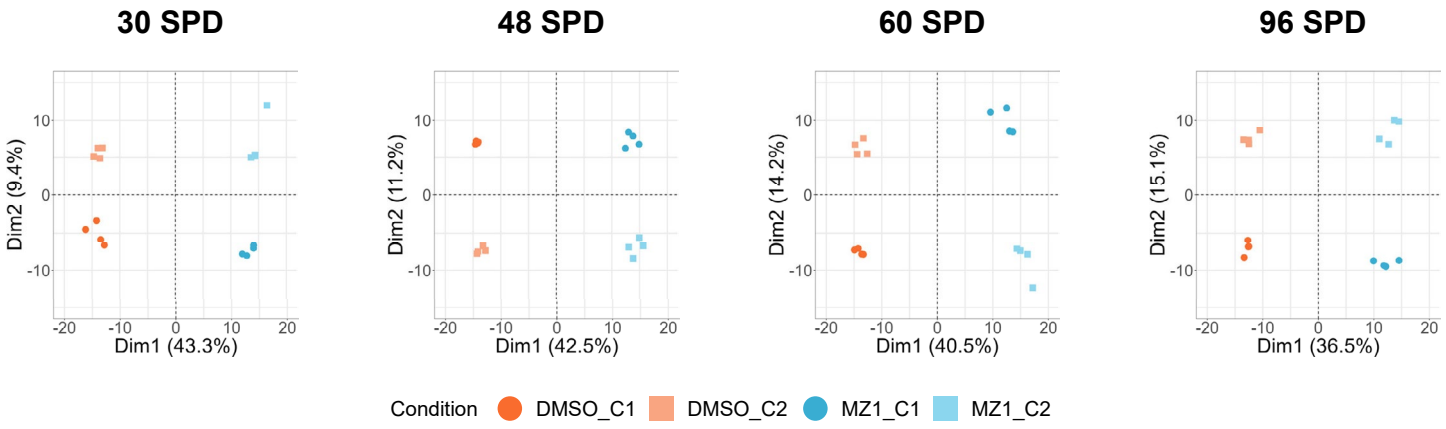


Figure 7. PCA plots show that even in samples with minute proteome variation, separation based on the sample (horizontal axis – PC1) is far larger than the separation based on the analytical column (vertical axis – PC2). Four replicates of each sample condition were analyzed on each column (total of eight per condition).

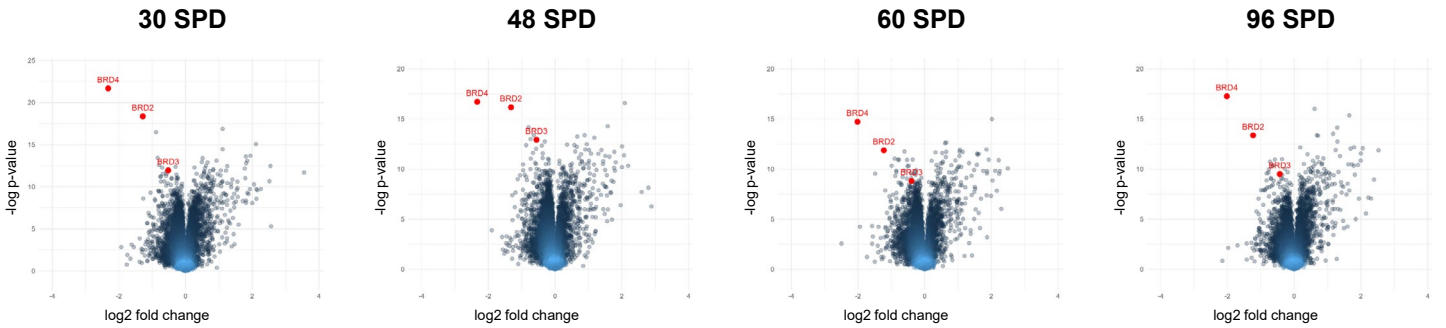


Figure 8. Quantitative analysis using all replicates—four replicates per sample condition per column. At all throughputs down-regulation of BRD4 is clearly observed, demonstrating the utility of the TDI workflow in quantifying candidate substrates. BRD2, BRD3, and BRD4 demonstrate expected behaviors upon MZ1 treatment.

The cells were treated with either MZ1 or DMSO and mixed after lysis to multiplex the samples. Given that the SILAC multiplexing is performed to only increase the throughput but not to perform SILAC quantification, there is full flexibility of what each channel contains in terms of treatment. The heavy and light channels could correspond to one replicate each of MZ1 and DMSO samples or could contain two replicates of one condition, depending on the experimental design. In this study, four injections of SILAC sample would already encompass the eight replicates that were measured in label-free fashion, demonstrating the throughput gains via multiplexing. In double SILAC measurements, the number of proteins identified and quantified per channel is similar to the number of proteins identified and quantified in the label-free samples without a drop in the number of identifications. In a double SILAC 60 IPD gradient using our proprietary workflows, we quantified more than 10,000 protein groups and close to 7,500 protein groups were quantified with less than 10% CV at an effective sample throughput of 120 SPD. For double SILAC multiplexing at 96 IPD, just under 10,000 protein groups could be identified with close to 7,000 protein groups quantified with less than 10% CV at an effective throughput of 192 SPD. Similarly, in a triple SILAC 60 IPD method, we could quantify more than 10,000 protein groups with a median CV of 5.7% with still close to 7,500 protein groups quantified with less than 10% CV. Based on our triple SILAC and 60 IPD methods, we could observe the down-regulation of BRD4 upon MZ1 treatment. In this experiment, we could quantify proteins within each SILAC channel for samples that were measured across the two analytical columns in the TDI setup (Figure 9). For our experiments, this setup of triple SILAC at 60 IPD delivering a sample throughput of 180 SPD seems to provide the right balance between quantification metrics and throughput of operation in the TDI workflow.

Table 6. SPD vs. IPD for different multiplexing schemes.

Sample	Multiplex factor	Gradient throughput (IPD)	Sample throughput (SPD)
Label free	1	30	30
Label free	1	96	96
Double SILAC	2	30	60
Double SILAC	2	60	120
Triple SILAC	3	30	90
Triple SILAC	3	48	144
Triple SILAC	3	60	180

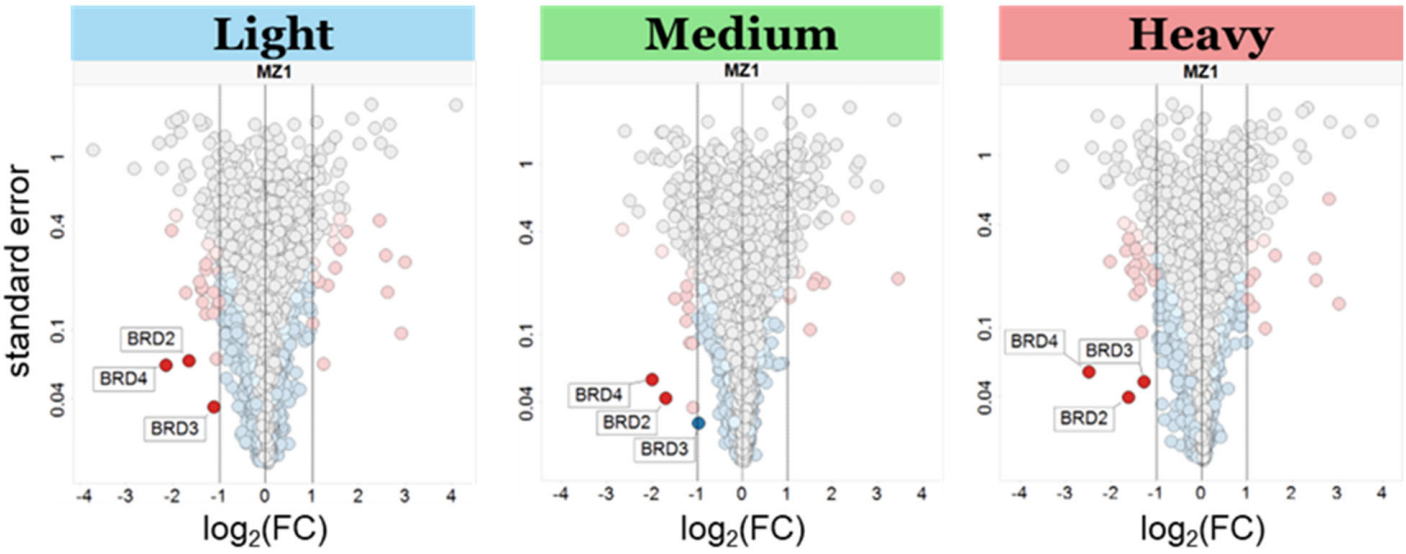


Figure 9. Multiplexing based on triple SILAC for targeted protein degradation studies. Quantification was performed independently on each channel measured across two analytical columns in the TDI workflow consistent with previous results.

Conclusion

The Vanquish Neo tandem direct injection workflow presents a significant advancement in proteomics, offering enhanced sample throughput and quantitative depth. Our comprehensive evaluation demonstrates several key benefits:

- **Increased protein identification and quantification:** The TDI workflow allows for a higher number of proteins to be identified and quantified with a CV <10%. This is particularly evident at higher throughput levels, where the number of proteins quantified at CV <10% is nearly doubled compared to single-column injections.
- **Improved sample throughput:** The workflow efficiently handles increased sample throughput while maintaining high data quality. For example, at 96 SPD, we identified >10K protein groups per sample in a HYE mix. Even higher sample throughputs can be obtained through SILAC multiplexing.
- **Reduced carryover:** Extensive washing capabilities inherent in the TDI workflow significantly reduce sample carryover—up to 4-fold at 96 SPD.
- **Robust quantitative analysis across columns:** The TDI workflow maintains quantitative accuracy across two columns, enabling small proteome differences to be resolved effectively.

In summary, the tandem LC-MS/MS workflow significantly enhances the efficiency and depth of proteome analyses, making it a valuable tool for high-throughput applications. This innovative approach provides a balanced solution between sample throughput and quantitative precision and accuracy, paving the way for more large-scale proteomics studies.

References:

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