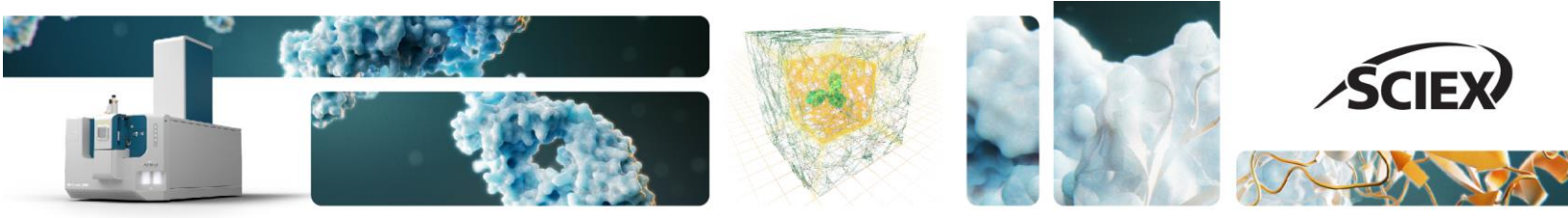

Improved proteomics performance at high throughput using ZT Scan DIA on the ZenoTOF 7600+ system

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This technical note describes improvements in the identification and quantitation of proteins and peptides with high throughput liquid chromatography-mass spectrometry (LC-MS) analyses when using ZT Scan data-independent acquisition (DIA) on the ZenoTOF 7600+ system. ZT Scan DIA enhances proteomics performance compared to traditional discrete-window DIA methods such as Zeno SWATH DIA, particularly as sample loading and complexity increase. Using ZT Scan DIA, up to 70% gains were achieved in the identification and quantitation of proteins and precursors in highly complex lysate digest samples.

Key features of high throughput proteomics with ZT Scan DIA on the ZenoTOF 7600+ system

- **Go deeper:** Identify and quantify up to 70% more proteins and peptide precursors with ZT Scan DIA
- **Go faster:** The benefits of ZT Scan DIA increase as sample throughput increases, allowing confident detections and quantitation at throughputs as high as 500 SPD
- **Get specific:** Performance gains with fast LC gradients using ZT Scan DIA increase as sample loading and complexity increase due to the added specificity of the scanning quadrupole dimension

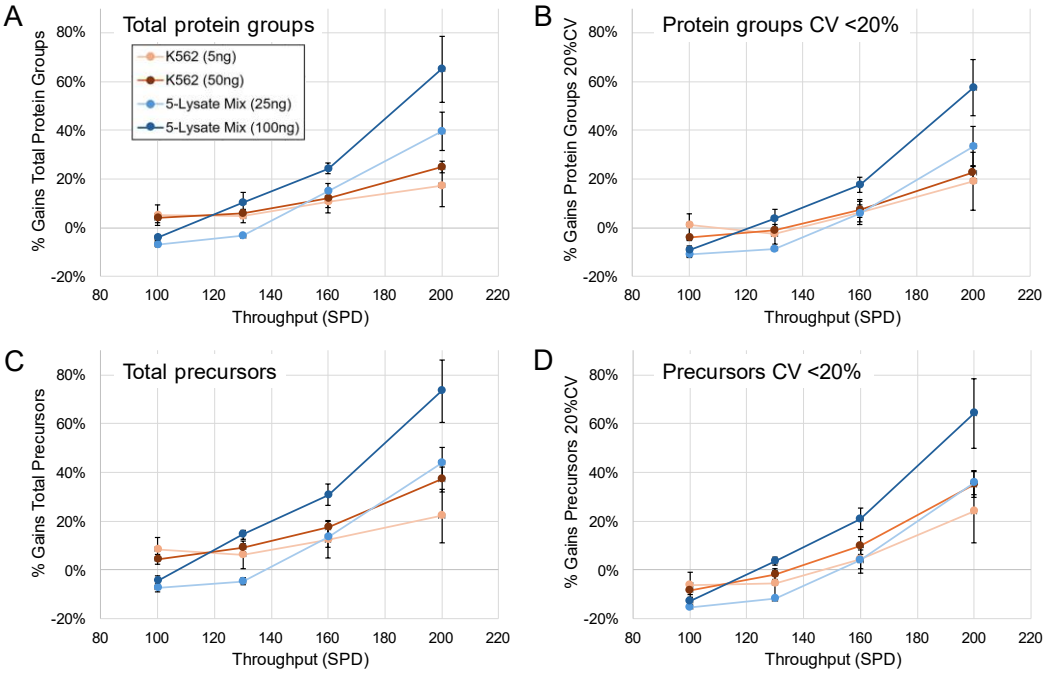

Figure 1. Performance gains with ZT Scan DIA versus Zeno SWATH DIA using a Phenomenex XB-C18 column (15 cm x 300 µm). K562 digest (5 ng or 50 ng on-column loading) and a 5-lysate digest mixture (25 ng and 100 ng total protein on-column loading) were analyzed using either ZT Scan DIA or Zeno SWATH DIA, using 1-min (200 SPD), 3-min (160 SPD), 5-min (130 SPD), and 10-min (100 SPD) active gradients. Percent gains in total protein groups (A) and protein groups identified with CV <20% (B) are shown, along with percent gains in total precursors (C) and precursors with CV <20% (D). Results are averages from data acquired across three ZenoTOF 7600+ systems, with error bars representing the standard deviation. As sample throughput and sample complexity/on-column loading increase, the gains with ZT Scan DIA over Zeno SWATH DIA increase.

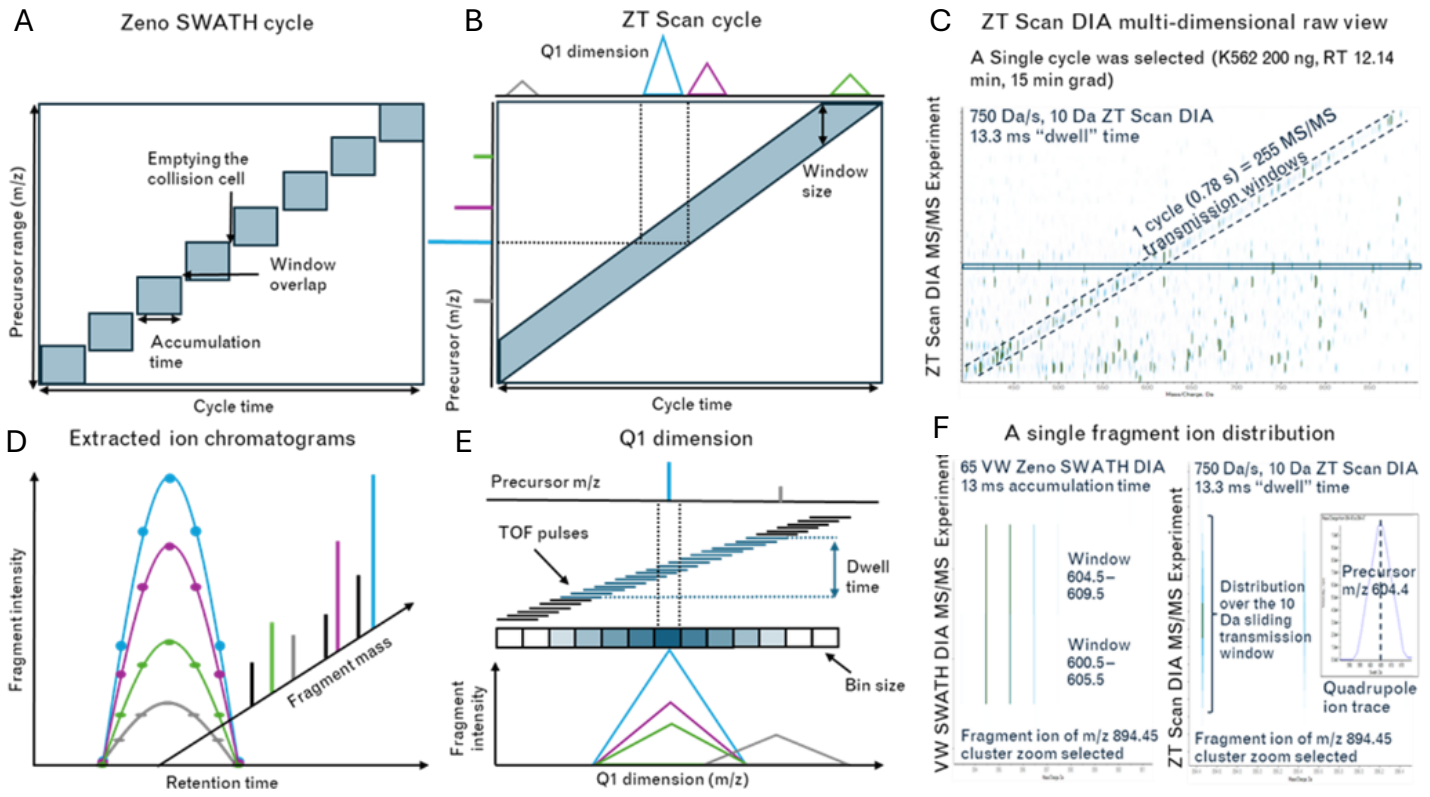

Figure 2. Overview of ZT Scan DIA and the associated Q1 dimension. Visualization of (A) a conventional Zeno SWATH DIA cycle showing data is collected in a stepped manner, and (B) a ZT Scan DIA cycle where the Q1 mass range is scanned, adding this dimension to the data. (C) Raw data showing a single ZT Scan DIA cycle selected (K562 200 ng, RT 12.14 min, 15 min gradient). (D) Extracted ion chromatogram generated for each fragment according to retention time. (E) MS/MS data collected from TOF pulses are binned according to the precursor m/z. Fragments can be distinguished from chimeric MS/MS spectra and aligned to their precursor ions using the Q1 dimension. (F) Fragment ion distribution from a Zeno SWATH DIA experiment and the ZT Scan DIA shown in (C) above.

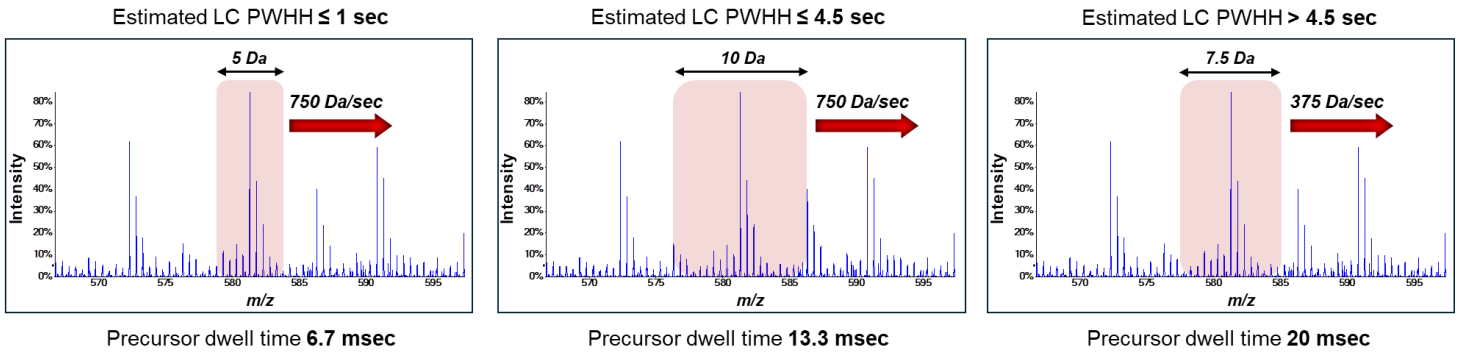

Figure 3. Summary of ZT Scan DIA methodology. The available ZT Scan DIA methods are pre-set based on the user-defined estimated peak width at half height (PWHH), which is dependent on the desired LC regime. PWHH ≤ 1 sec sets a 5 Da-wide Q1 isolation window, scanning at 750 Da/sec. PWHH ≤ 4.5 sec sets a 10 Da-wide Q1 isolation window, scanning at 750 Da/sec. PWHH > 4.5 sec sets a 7.5 Da-wide Q1 isolation window, scanning at 375 Da/sec. TOF MS mass ranges (400-900 Da) and MS/MS mass ranges (140-1750 Da) are fixed for all methods. Apart from the definition of the estimated PWHH, users need only input MS source parameters, making ZT Scan DIA methods quick and easy to set up.

Introduction

DIA has become the cornerstone of MS-based proteomics analysis, enabling the identification and quantification of large numbers of proteins and peptides to enhance the understanding of the complex biological mechanisms of various diseases. A critical aspect of disease research is analyzing large sample cohorts to increase the statistical significance of observed trends and paint a broader picture of the disease mechanisms involved. As such, the ability to analyze large numbers of samples faster, with easy-to-implement methods and improved data quality, is of great value to researchers. The power of Zeno SWATH DIA for fast, high-quality quantitative proteomics has been repeatedly demonstrated[1-3]. ZT Scan is presented as the next step in the evolution of DIA[4]. Using a scanning quadrupole coupled to fast, sensitive, time-of-flight (TOF) analysis improves specificity over existing discrete-window DIA methods for accurately and precisely identifying and quantifying analytes across a given mass range.

This technical note highlights the benefits of ZT Scan DIA for identifying and quantitating proteins and peptides using high throughput LC-MS regimes. Using pre-defined methods requiring minimal optimization and parameter setup, performance improvements over Zeno SWATH DIA methods increase as sampling throughput increases and for samples of higher complexity and loadings. These results indicate that ZT Scan DIA is an ideal method for the high throughput analysis of high-complexity samples, ideally suited for large cohort biomarker research.

Methods

Sample preparation: Human K562 and yeast protein tryptic digest standards were purchased from Promega. *Drosophila melanogaster* protein lysate was purchased from GenLysate. Human HEK 293, mouse 3T3, and Chinese hamster ovary (CHO) protein lysates were purchased from Innovative Research. Drosophila, HEK 293, mouse, and CHO protein lysates were digested with trypsin and purified using S-Traps (Protifi) according to the manufacturer's instructions. Drosophila, HEK 293, yeast, mouse, and CHO digests were combined at equivalent nanogram amounts to create the lysate digest mixture. K562 and the lysate mixture were reconstituted to the indicated concentrations in a buffer containing 5% acetonitrile and 0.1% formic acid in water.

Chromatography: High throughput microflow LC separations were performed using either the Waters M-Class UPLC system (in direct-inject LC mode) or the Evosep One system. Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Chromatographic separations with the Waters M-Class system were done with a Phenomenex XB-C18 analytical column (15 cm x 300 µm, 2.6 µm particle size), at a flow rate of 8 µL/min. Throughput methods of 200, 160, 130, and 100 SPD were used, with respective 1-min, 3-min, 5-min, or 10-min active gradients (see Table 1). For chromatographic separations using the Evosep One system, throughput methods of 200, 300, and 500 SPD were used with the EV1107 analytical column (4 cm x 150 µm, 1.9 µm particle size) as previously described[2].

Mass spectrometry: Data-independent acquisition experiments were performed on a ZenoTOF 7600+ system using the OptiFlow Turbo V ion source with the microflow probe (1-10 µL/min electrode). Zeno SWATH DIA experiments used 65 variable-width windows spanning the TOF MS mass range 400-900 Da, MS/MS mass range 140-1750 Da, and Zeno trap pulsing turned on. ZT Scan DIA experiments used an estimated peak width at half-height (PWHH) setting of ≤ 1 sec. Additional information about source parameters and MS/MS accumulation times for the various experiments are described in Table 1. All data was acquired in triplicate for all conditions.

Data processing: All data was processed using DIA-NN software version 1.8.1[5]. Human K562 data was processed against a spectral library previously described[6]. Data acquired on the lysate mixture was processed using a combined predicted spectral library comprising *Homo sapiens*, *Saccharomyces cerevisiae*, *Mus musculus*, *Cricetulus griseus*, and *Drosophila melanogaster* protein FASTA sequences downloaded from www.uniprot.org. Default DIA-NN software search settings were used with the following changes: Precursor m/z range was adjusted

Table 1. LC column, gradient, and mass spectrometry parameters. Experiments using the Phenomenex XB-C18 column were performed using the Waters M-Class LC system, while experiments using the EV1107 column were performed using the Evosep One system.

Column	Phenomenex XB-C18 (15 cm x 300 µm)				EV1107 (4 cm x 150 µm)		
Throughput (SPD)	**100**	**130**	**160**	**200**	**200**	**300**	**500**
Flow rate (µL/min)	8	8	8	8	2	4	4
Active gradient length (min)	10	5	3	1	5.6	3.2	2.2
Total method length (min)	14	11	9	7	7.2	4.8	2.9
Gas 1 (psi)	20						
Gas 2 (psi)	60						
TEM (°C)	225						
Q1 m/z range	400 - 900						
TOF MS/MS m/z range	140 - 1750						
Zeno SWATH DIA - # windows	65						
Zeno SWATH DIA - MS/MS accumulation time (msec)	13	13	10	7	7	7	7
ZT Scan DIA - estimated PWHH (sec)	≤ 1						
ZT Scan DIA - Q1 window width (Da)	5						
ZT Scan DIA - Q1 window scan speed (Da/sec)	750						

to 400-900, Fragment m/z range was adjusted to 140-1750, Mass accuracy was set to 20 ppm, MS1 accuracy set to 0 ppm, Scan window was set to 6, and MBR was checked. The **--scanning-swath** command option was used for ZT Scan DIA data processing. Searches were done individually, processing only triplicate data files from the same loading/gradient/method.

Unlock the power of ZT Scan DIA with easy-to-setup methods

Traditional DIA techniques such as Zeno SWATH DIA divide the precursor mass range into discrete windows, with either fixed or variable widths across the Q1 range. Ideally, DIA window widths are kept as narrow as possible to maximize MS/MS selectivity, which necessitates using more DIA windows spanning the Q1 range. However, with DIA methods having many windows, maintaining a reasonable overall cycle time (based on LC peak width) requires sacrificing MS/MS scan times per window, potentially impacting overall data quality. Conversely, ZT Scan DIA utilizes a sliding quadrupole precursor isolation window scanning across the Q1 range (Figure 2). As precursor ions fall in and out of the sliding isolation window, they are fragmented in Q2, followed by Zeno trap pulsing into the TOF analyzer. MS/MS spectra are collected from TOF pulses and are binned according to the precursor m/z from the Q1 dimension. Fragment ions can be mapped to their precursor ion based on the correlation of the fragment ions' appearance and disappearance to the precursor ion's appearance and disappearance in the Q1 isolation window.

ZT Scan DIA methods are pre-defined in terms of the Q1 window size and the speed at which they scan across the Q1 mass range, and these have been optimized based on the expected peak widths at half height (PWHH) (Figure 3). Users define their expected PWHH based on their chosen chromatographic regime. An expected PWHH setting of ≤ 1 sec uses a ZT Scan DIA method with a 5 Da Q1 window scanning at 750 Da/sec. This method is ideal for faster gradient separations with correspondingly narrow peaks. The other ZT Scan DIA methods, with settings of PWHH ≤ 4.5 sec (utilizing a 10 Da Q1 window scanning at 750 Da/sec) and PWHH > 4.5 sec (utilizing a 7.5 Da Q1 window scanning at 375 Da/sec), are optimized for LC-MS methods with progressively longer active gradient lengths, with broader LC peaks and slower throughputs. The TOF MS and MS/MS mass ranges are pre-defined for all methods. Users only need to set the estimated LC peak widths when building methods and the source parameters associated with the desired flow rate and LC regime, thereby significantly simplifying overall method development. This work evaluated high throughput methods between 100 and 500 SPD. Henceforth, all data described here utilized the ZT Scan DIA method with PWHH ≤ 1 sec.

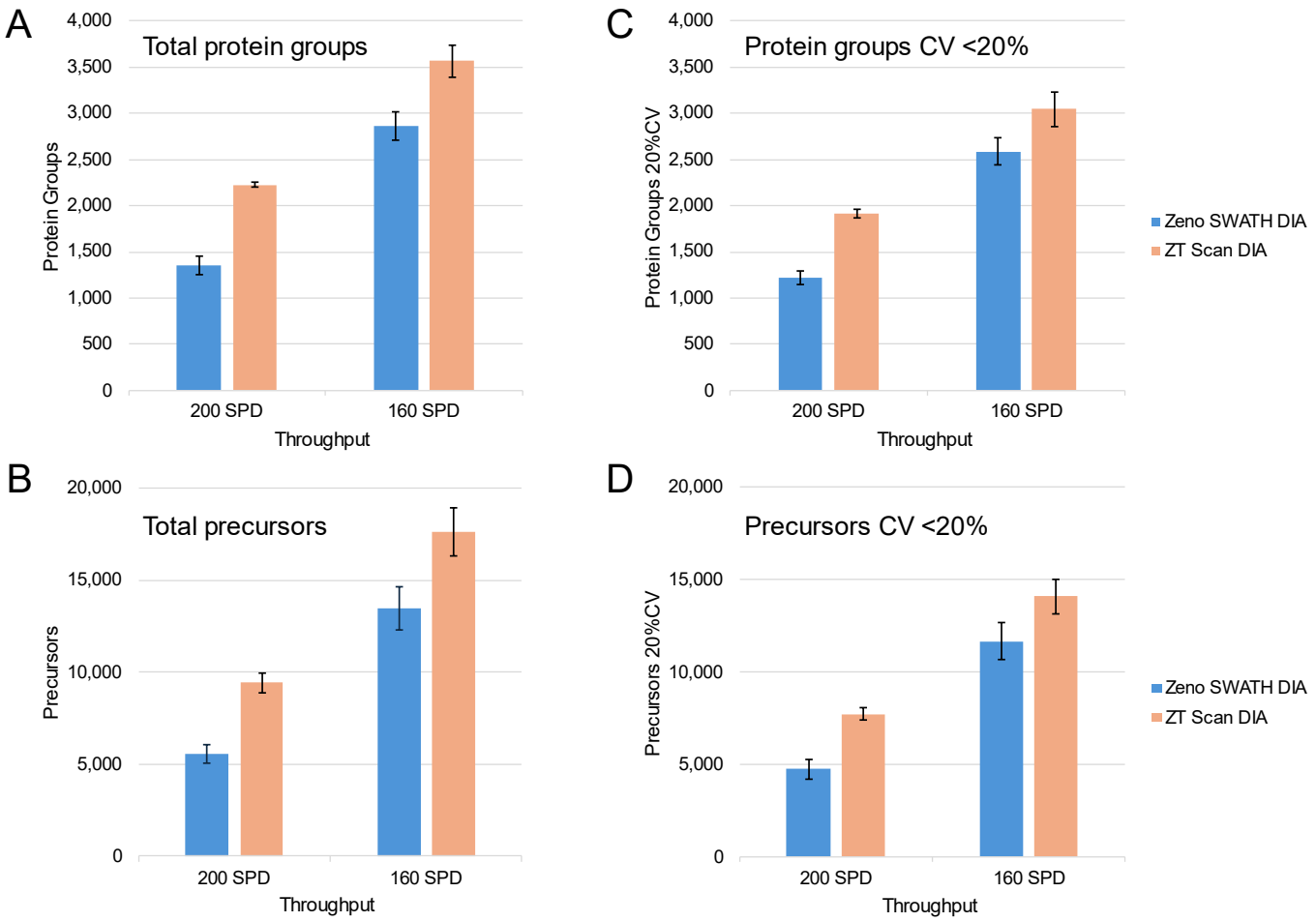

Figure 4. Protein groups and precursors identified and quantified from 100 ng of a 5-lysate mixture using either ZT Scan DIA or Zeno SWATH DIA using the Waters M-Class LC system and Phenomenex XB-C18 column (15 cm x 300 µm). 100 ng on-column loadings of a 5-lysate mixture were analyzed using active gradients of 1 minute (7-min total run time; 200 SPD) and 3 minutes (9-min total run time; 160 SPD). (A) Total protein groups identified. (B) Protein groups identified with CV <20%. (C) Total precursors identified. (D) Precursors identified with CV <20%. Results are averages from data acquired across three ZenoTOF 7600+ systems, with error bars representing the standard deviation. As sample throughput increases, the gains with ZT Scan DIA over Zeno SWATH DIA increase for this sample and loading.

Benefits of ZT Scan DIA with analysis of up to 200 SPD using the Waters M-Class LC system and Phenomenex XB-C18 column (15 cm x 300 µm)

Active gradients of 1 minute (7-min total run time), 3 minutes (9-min total run time), 5 minutes (11-min total run time), and 10 minutes (14-min total run time) were used, equating to throughputs of 200, 160, 130 and 100 SPD, respectively. Two different sets of samples were analyzed, one consisting of commercial human K562 digest (5 ng and 50 ng on-column loadings), the other being a mixture of HEK 293, mouse, CHO, yeast, and Drosophila protein digests combined at equivalent nanogram amounts (on-column loadings of either 25 ng or 100 ng total protein digest). Samples were analyzed using ZT Scan DIA (PWHH ≤ 1 sec) or a 65 variable-window Zeno SWATH DIA method, with triplicate runs for all conditions. Comparisons were made between the ZT Scan DIA and Zeno SWATH DIA methods in terms of the resulting numbers of total protein group and precursor identifications, along with the numbers of protein groups and precursors identified with a coefficient of variation (CV) <20% representing the numbers of quantifiable protein groups and precursors. Figure 1 summarizes the gains in both total protein groups and protein groups with CV <20% using ZT Scan DIA for

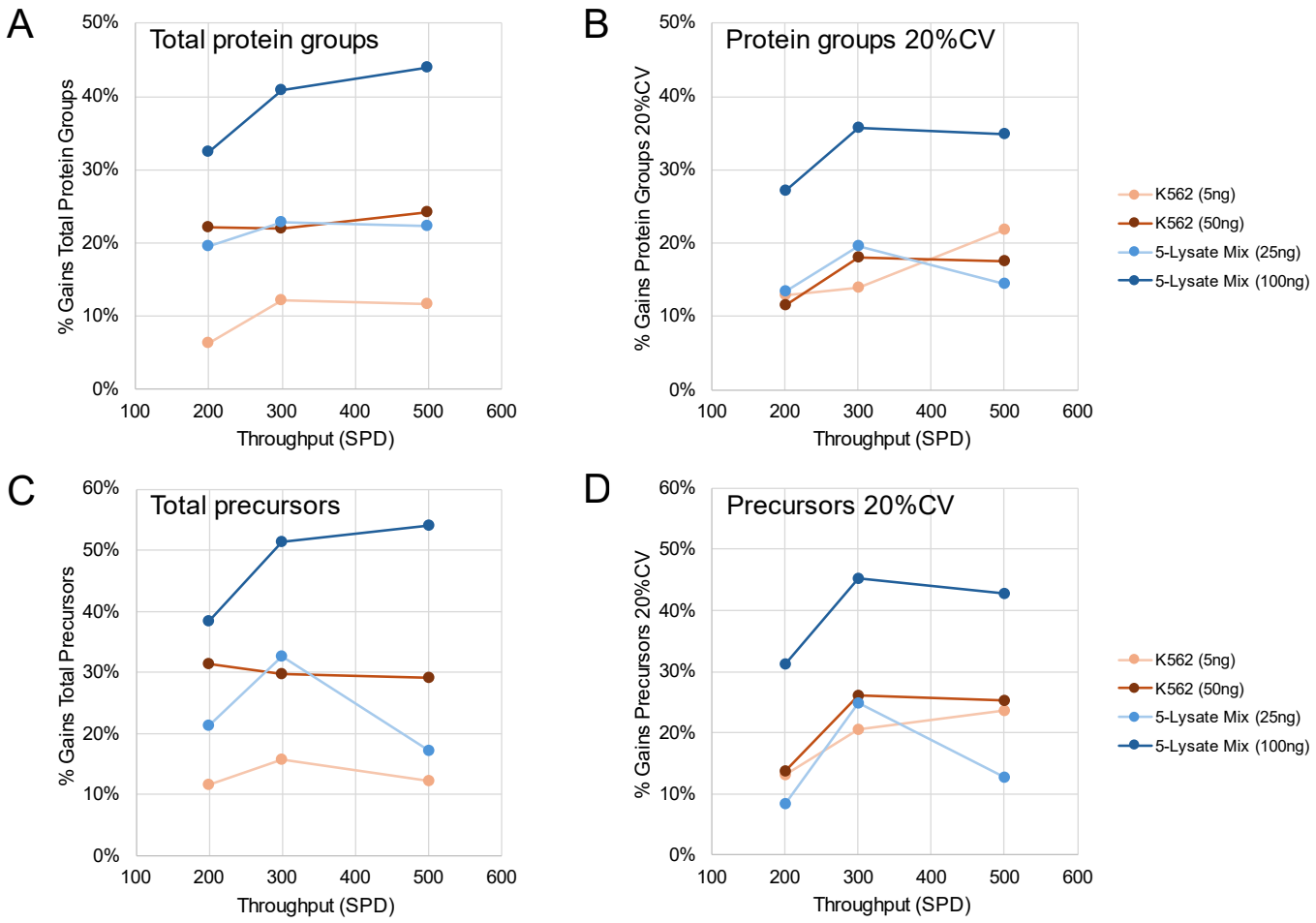

Figure 5. Performance gains with ZT Scan DIA versus Zeno SWATH DIA using the Evosep One system at 200, 300, and 500 SPD. K562 digest (5 ng or 50 ng on-column loading) and a 5-lysate digest mixture (25 ng and 100 ng total protein on-column loading) were analyzed using either ZT Scan DIA or Zeno SWATH DIA, using the indicated throughput levels and the EV1107 column (4 cm x 150 µm). (A) Percent gains in total protein groups identified. (B) Percent gains in protein groups identified with CV <20%. (C) Percent gains in total precursors identified. (D) Percent gains in precursors identified with CV <20%. As sample throughput and sample complexity/on-column loading increases, the gains with ZT Scan DIA over Zeno SWATH DIA increase.

the different samples and on-column loadings tested at throughputs from 100 to 200 SPD. The figure shows that the gains improved as throughput increased, reaching 70% for 200 SPD. This was observed for both the identified and quantified protein groups, and this trend was also evident for the identified and quantified precursors. It was also observed that the gains increased as the sample complexity and loading increased, with the most significant improvements seen for the 100 ng loading of lysate mix, followed by 25 ng lysate mix, 50 ng K562, and 5 ng K562, respectively. This demonstrates that ZT Scan DIA is particularly effective for high-complexity analyte mixtures, especially when the chromatographic space is condensed with shorter gradients. Presumably, this is due to the higher degree of specificity afforded by ZT Scan DIA, in that fragment ions can be effectively mapped to their corresponding precursor ions across the Q1 dimension of the sliding isolation window. The corresponding numbers of protein groups and precursors (total number identified, along with the numbers identified with CV <20%) for the 100 ng lysate mixture at the 160 and 200 SPD throughput levels are shown in Figure 4. Although the absolute number of identifications was higher when using slower throughput LC methods, the highest proportion of gains were observed at 200 SPD, where ZT Scan DIA identified 2224 total protein groups (1919 with CV <20%) and 9415 total precursors (7737 with CV <20%).

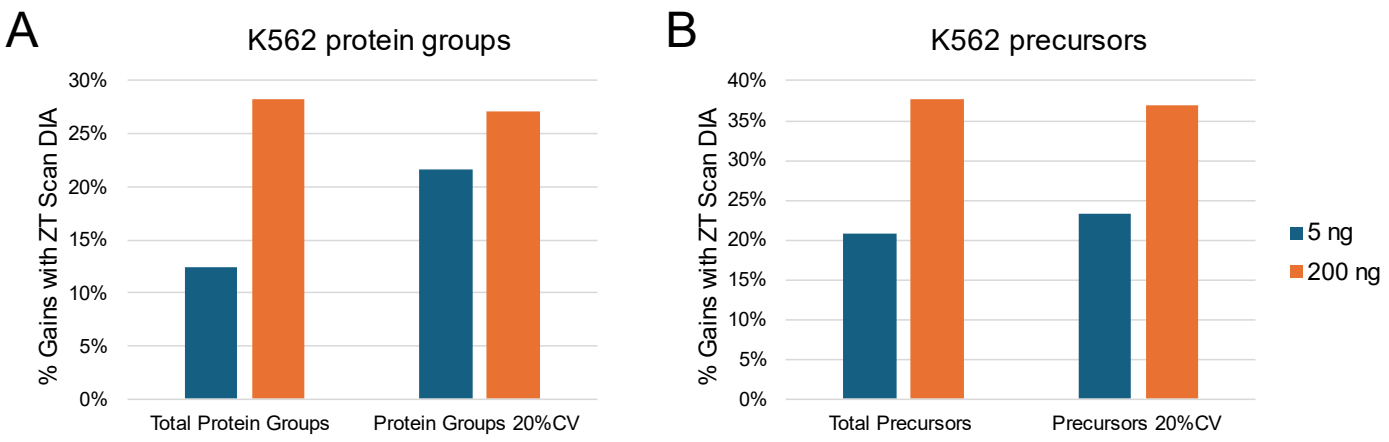

Figure 6. Performance gains with ZT Scan DIA versus Zeno SWATH DIA using the Evosep One system at 500 SPD. 5 ng and 200 ng of K562 digest were analyzed using either ZT Scan DIA or Zeno SWATH DIA at 500 SPD using the EV1107 column (4 cm x 150 µm). (A) Percent gains in total protein groups and protein groups with CV <20%. (B) Percent gains in total precursors and precursors with CV <20%. As sample on-column loading increases, the gains with ZT Scan DIA over Zeno SWATH DIA increase.

Benefits of ZT Scan DIA with analyses up to 500 SPD using the Evosep One system

The same set of samples was analyzed using the Evosep One system, which enables faster LC separations of up to 500 SPD, albeit using the shorter 4 cm x 150 µm EV1107 column (thereby limiting column capacity and chromatographic separation). Throughputs of 200, 300, and 500 SPD were tested with the same on-column loadings of K562 and lysate mixture described above, comparing ZT Scan DIA and the 65 variable-window Zeno SWATH DIA methods.

Figure 5 summarizes the gains achieved with ZT Scan DIA over Zeno SWATH DIA for these samples and throughput levels. As before, the gains for protein groups and precursors identified and quantified were most significant for the 100 ng loading of lysate mixture – up to 44% for identified protein groups, 35% for quantified protein groups, 54% for identified precursors, and 45% for quantifiable precursors.

Using the Evosep One system, the improvements seen with ZT Scan DIA over Zeno SWATH DIA were highest at the 500 SPD level. This was further highlighted in another experiment comparing ZT Scan DIA versus Zeno SWATH DIA for 5 ng and 200 ng loadings of K562. Figure 6 and Table 2 summarize the percentage and absolute gains for K562 protein groups and precursors identified and quantified using ZT Scan DIA at 500 SPD. A total of 896 protein groups (635 with CV <20%) and 3262 precursors (1952 at CV <20%) were identified from 5 ng K562 at 500 SPD using ZT Scan DIA, increasing to 3362 total protein groups (2952 with CV <20%) and 18388 precursors (15610 at CV <20%).

Collectively, these results highlight the effectiveness of ZT Scan DIA for improving the identification and quantitation of proteins and peptides, particularly for highly complex samples and with faster LC regimes. ZT Scan DIA on the ZenoTOF 7600+ system is therefore ideal for high throughput biomarker studies involving large, complex sample cohorts.

Table 2. Performance gains with ZT Scan DIA versus Zeno SWATH DIA using the Evosep One system at 500 SPD for 5 ng or 200 ng loadings of K562.

	5 ng K562		200 ng K562	
	Zeno SWATH DIA	ZT Scan DIA	Zeno SWATH DIA	ZT Scan DIA
Total Protein Groups	797	896	2623	3362
Protein Groups CV<20%	522	635	2323	2952
Total Precursors	2702	3261	13350	18388
Precursors CV<20%	1583	1952	11394	15610

Conclusions

- ZT Scan DIA improved the numbers of protein groups and precursors identified and quantified by up to 70% over Zeno SWATH DIA – these gains were highest as sample throughput increased
- The performance gains with ZT Scan DIA on the ZenoTOF 7600+ system also improved as sample complexity and on-column loading increased due to the added specificity of the Q1 dimension of ZT Scan DIA
- ZT Scan DIA methods are easy to set up, with minimal user optimization required, making the ZenoTOF 7600+ system ideally suited to the analysis of large sample cohorts

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