# Flexibility, speed, and throughput for high proteome coverage using Zeno SWATH data-independent acquisition (DIA) coupled with the Evosep One system

***Robust identification and quantification of proteins and peptides using the ZenoTOF 7600 system***

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This work demonstrates the use of Zeno SWATH DIA on the ZenoTOF 7600 system coupled with the Evosep One system to achieve quantitative proteomics on both HeLa cell and non-depleted human plasma digests. The sensitivity of the ZenoTOF 7600 system is demonstrated at different throughput levels. At 30 samples-per-day (SPD), >8,000 protein groups were identified from 200 ng of HeLa digest, while at 500 SPD, >270 protein groups could be identified from 50 ng of non-depleted human plasma.

Scientists performing proteomics analyses are often challenged by issues of sensitivity, reproducibility and experimental design. High LC-MS sensitivity is needed to detect and quantify peptides and proteins that are present at low concentrations in samples or difficult to detect in a complex matrix. Proteomic components can also be present across a wide dynamic range in certain extracts. High system robustness and reproducibility are necessary to achieve quantitative fidelity for large sample cohorts. To navigate these complicating factors, method flexibility can help users dynamically adjust the balance between chromatographic separation and sample turnover/throughput.

The ZenoTOF 7600 system can be used to meet these proteomic analytical requirements. Zeno SWATH DIA is a powerful tool for the sensitive detection and quantification of proteins at low levels using fast acquisition[1], allowing it to be

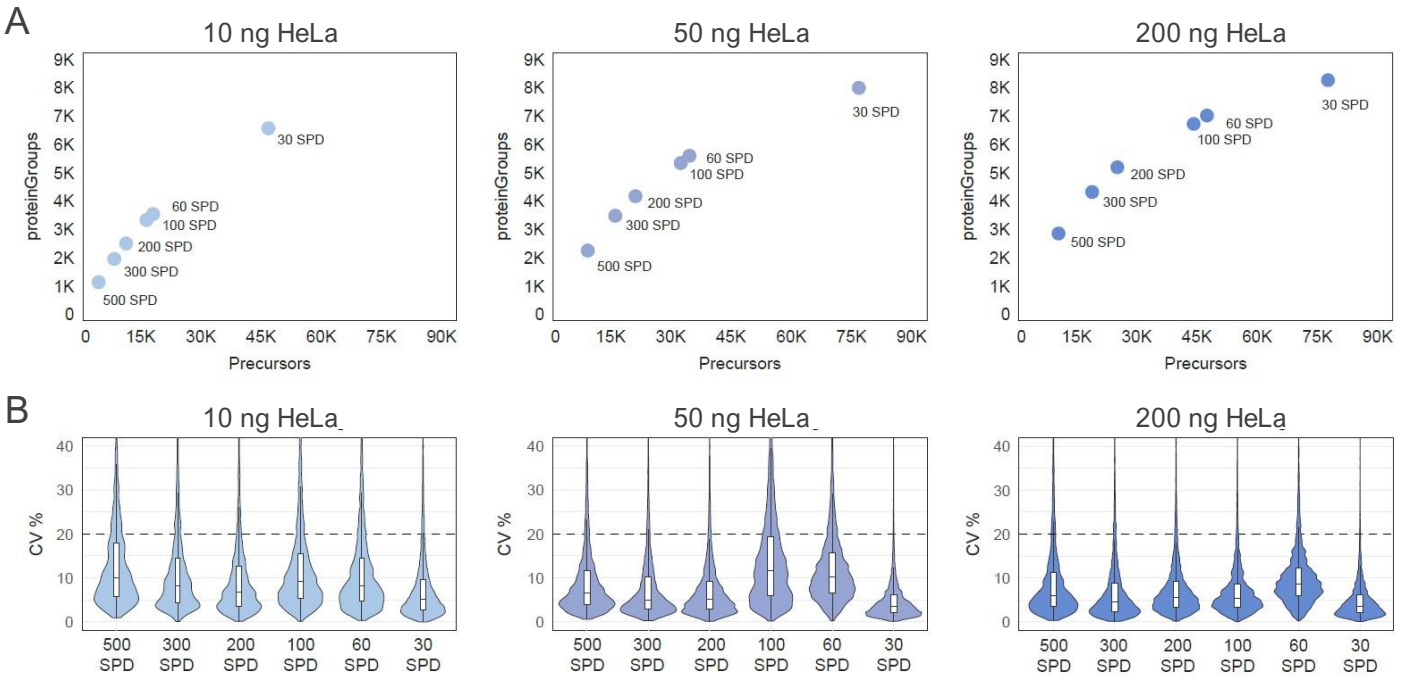


**Figure 1. Quantitative proteomics results with HeLa cell digest at various samples-per-day (SPD) throughput methods using Zeno SWATH DIA.** (A) Numbers of protein groups and precursors quantified from different on-column loadings of HeLa cell digest using different SPD methods. Data were obtained from library-free searches of Zeno SWATH DIA triplicate injections for each experiment using DIA-NN software. The resulting "pg.matrix.tsv" and "pr.matrix.tsv" files were used to report the numbers of protein groups and precursors, respectively. (B) Violin plots showing %CV distributions for precursors from the different on-column HeLa cell loadings using the different SPD methods.

coupled with either ultra-fast or long gradient separations. The Evosep One system simplifies chromatography by utilizing pre-defined methods at user-defined throughput levels for robust and efficient peptide separation. These capabilities of the Evosep One system are synergistic with the capabilities of the ZenoTOF 7600 system.[2] Here, we describe the power of Zeno SWATH DIA for quantitative proteomics using throughput capabilities of the Evosep One system.

## Key features of quantitative proteomics using Zeno SWATH DIA and the Evosep One system

- >7,800 protein groups (>73,000 precursors) were identified from 50 ng HeLa cell digest, and >8,000 protein groups (>83,000 precursors) were identified from 200 ng HeLa cell digest using the 30 SPD method on the Evosep One system.
- > 270 protein groups were identified from 50 ng of non-depleted human plasma digest using the 500 SPD method on the Evosep One system.
- Zeno trapping provided MS/MS with industry-leading sensitivity for qualitative and quantitative protein characterization using simple Zeno SWATH DIA methods.
- The Evosep One system allowed for robust and sensitive chromatographic separation of peptides using standardized methods ranging in throughput from 500 SPD (2.9 minutes) to 30 SPD (48 minutes).

## Methods

***Sample preparation:*** Commercial HeLa human cell line tryptic digest was purchased from Thermo Fisher Scientific and reconstituted to the appropriate concentrations in a buffer containing water with 0.1% formic acid. Non-depleted human plasma was purchased from BioIVT (UK), prepared using an established protocol[3] and resuspended to the desired concentration using the same buffer as above.

***Chromatography:*** Chromatographic separation was carried out using the Evosep One system (Evosep, Denmark) using preset methods with the flow rate, gradient length, overall throughput conditions, and Evosep columns specified in Table 1. Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid.

***Mass spectrometry:*** Zeno SWATH DIA analysis was performed on the ZenoTOF 7600 system using the OptiFlow Turbo V ion source with the 1-10 µL/min microflow electrode in the vertical probe position.[4] Evosep columns were connected to the microflow probe using a PEEK 1/16" high-pressure union (IDEX P/N P-779). The parameters used for Zeno SWATH DIA with the various Evosep methods are described in detail in Table 2.

***Data processing:*** All Zeno SWATH DIA data were processed using DIA-NN software version 1.8.1 using a library-free approach against the human FASTA sequences downloaded from UniProt.[5, 6] Protein groups and precursors detected and quantified were reported in the outputs from DIA-NN software in the pg.matrix.tsv and pr.matrix.tsv files, respectively.

## Quantification of protein groups and precursors in HeLa cell digests

The numbers of peptides and protein groups identified and quantified from HeLa cell tryptic digests using Zeno SWATH DIA with the various SPD methods are highlighted in Figure 1. The relationship between protein groups and precursors quantified as a function of on-column loadings was consistent across Evosep One system methods. Using the 30 SPD method, >6,400 protein groups were identified from 10 ng of HeLa cell digest, while >7,800 protein groups and >8,000 protein groups were identified from 50 ng and 200 ng loadings, respectively. Most of the protein groups in HeLa cell digest yielded %CV values below 20% (Figure 1B).

**Table 1. Columns and chromatographic methods**

| Column | Throughput (SPD) | Gradient length (min) | Flow rate (µL/min) | Total cycle time (min) |
|---|---|---|---|---|
| EV1137 (15 cm x 150 µm, 1.5 µm particle size) | 30 | 44.0 | 0.5 | 48.0 |
| EV1109 (8 cm x 150 µm, 1.5 µm particle size) | 60 | 21.0 | 1.0 | 24.0 |
| | 100 | 11.5 | 1.5 | 14.4 |
| EV1107 (4 cm x 150 µm, 1.9 µm particle size) | 200 | 5.6 | 2.0 | 7.2 |
| | 300 | 3.2 | 4.0 | 4.8 |
| | 500 | 2.2 | 4.0 | 2.9 |

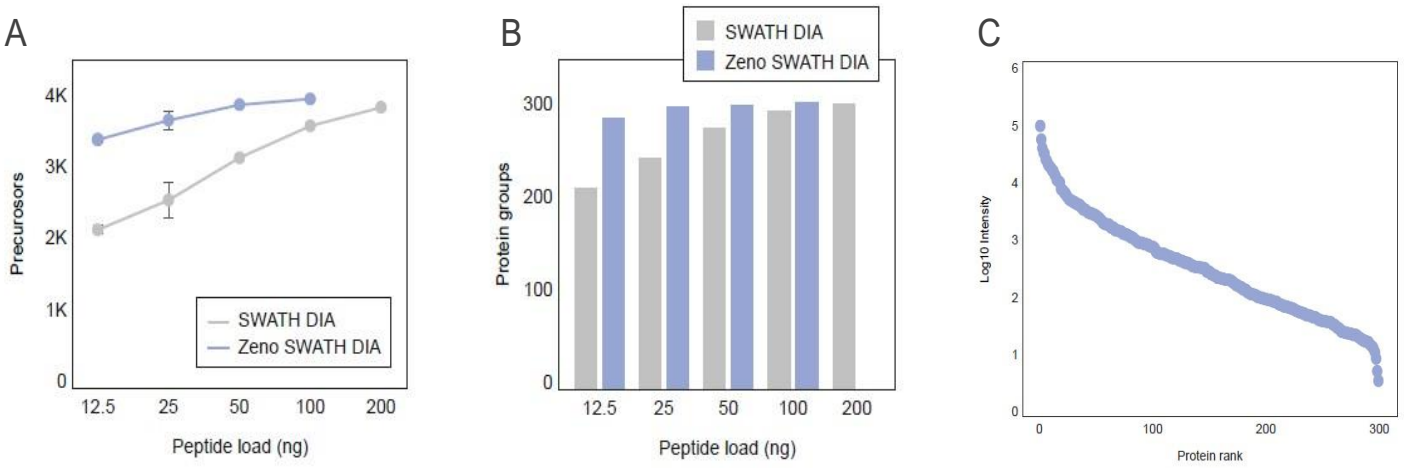


**Figure 2. Zeno SWATH DIA analysis of non-depleted human plasma digest using the 60 SPD method**. Numbers of precursors (A) and protein groups (B) quantified from different on-column loadings of plasma digest. Data were obtained from library-free searches of Zeno SWATH DIA triplicate injections for each experiment using DIA-NN software. The resulting "pg.matrix.tsv" and "pr.matrix.tsv" files were used to report the numbers of protein groups and precursors (respectively). (C) Log10 intensity distribution of protein groups identified and quantified from 50 ng injections of non-depleted human plasma.

## Analysis of non-depleted human plasma using Zeno SWATH DIA

Zeno trapping on the ZenoTOF 7600 system improved the overall duty cycle and resulted in significant MS/MS sensitivity gains. This was demonstrated with different on-column loadings of non-depleted human plasma digests analyzed with Zeno SWATH DIA using the 60 SPD method (Figure 2). As the method throughput decreased, the gains in protein groups and precursors identified increased, thereby enabling the detection and quantification of lower-abundance analytes.

Additionally, the ZenoTOF 7600 system can perform Zeno SWATH DIA at high speeds. Figure 3 shows the distributions of protein groups identified in non-depleted plasma using either the 60 or 300 SPD method to analyze a selection of 50 FDA-approved biomarkers.[7, 8]

Even at 300 SPD, nearly all biomarkers were quantifiable with %CV values <20%. Using ultra-fast methods (200, 300, and 500 SPD) the numbers of protein groups and precursors quantified in non-depleted human plasma remained consistently high (Figure 4). These results can be attributed to the highly reproducible separation method and the highly precise analyte quantification using Zeno SWATH DIA. Notably, the novel 500 SPD method maintained high precision and accuracy with an injection-to-injection turnaround time of less than 3 minutes (Figure 5). These results suggest that this method could have applications in settings in which high throughput and robustness are of great importance.

**Table 2. Zeno SWATH DIA parameters used on the ZenoTOF 7600 system with the various Evosep One system methods.** For all experiments, TOF MS accumulation times were 50 ms, curtain gas was set to 30 psi, ionization voltage was set to 4500 V, heater gas was set to 60 psi and source temperature was set to 225°C.

| Throughput (SPD) | # SWATH windows | Q1 mass range (amu) | MS/MS accumulation time/window (ms) | Nebulizing gas (psi) |
|---|---|---|---|---|
| 30 | 85 | 400 – 903 | 18 | 12 |
| 60 | 65 | 400 – 903 | 13 | 20 |
| 100 | 56 | 400 – 750 | 11 | 20 |
| 200 | 56 | 400 – 750 | 11 | 20 |
| 300 | 56 | 400 – 750 | 11 | 20 |
| 500 | 56 | 400 – 750 | 11 | 20 |

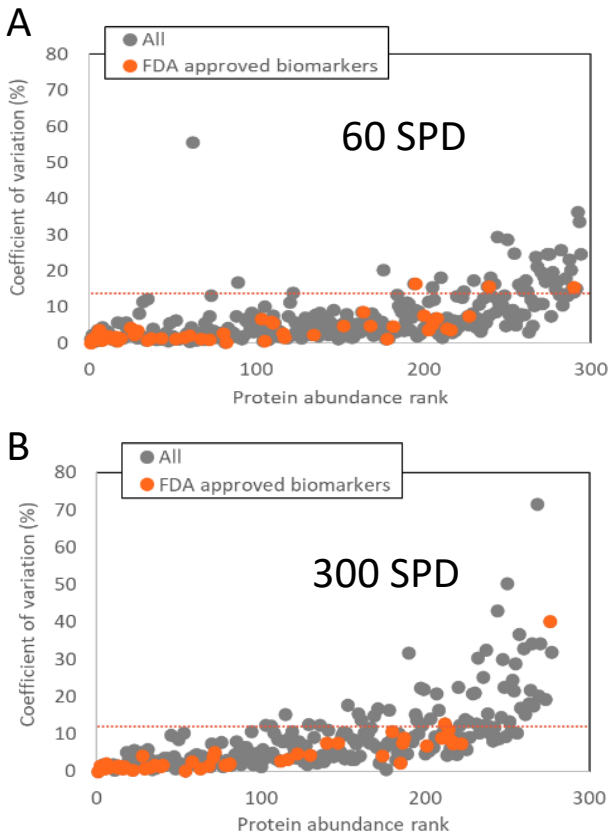


**Figure 3. %CV distributions of protein groups identified from 50 ng of non-depleted human plasma digest analyzed with Zeno SWATH DIA at (A) 60 SPD or (B) 300 SPD.** Protein groups were quantified from triplicate Zeno SWATH DIA injections with the 2 SPD methods using library-free searches with DIA-NN software. FDA-approved biomarkers are highlighted.

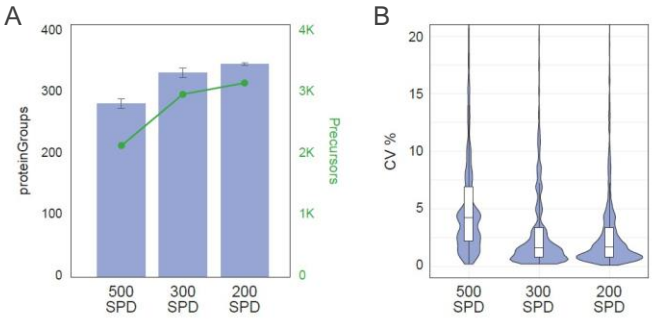


**Figure 4. Quantitative proteomics results for non-depleted human plasma digest for ultra-fast methods using Zeno SWATH DIA.** (A) Numbers of protein groups and precursors quantified from 50 ng on-column loadings of non-depleted human plasma digest with the 200, 300, or 500 SPD methods. Data were obtained from library-free searches of Zeno SWATH DIA triplicate injections for each experiment using DIA-NN software. The resulting "pg.matrix.tsv" and "pr.matrix.tsv" files were used to report the numbers of protein groups and precursors, respectively. (B) Violin plots showing %CV distributions for precursors from the same experiments.

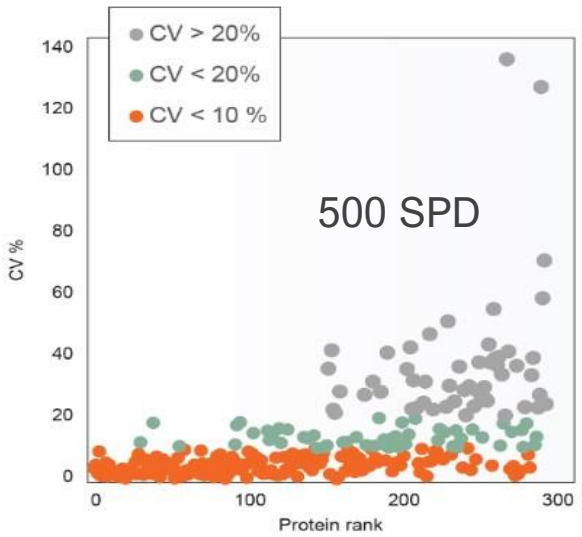


**Figure 5. Zeno SWATH DIA analysis of 50 ng of non-depleted human plasma digest with the 500 SPD method.** Protein groups were quantified from triplicate Zeno SWATH DIA injections using library-free searches with DIA-NN software. Distributions of protein groups at different %CV ranges are shown.

## Conclusions

- Zeno SWATH DIA was used on the ZenoTOF 7600 system to detect and quantify peptides and protein groups from various on-column loadings of HeLa cell and non-depleted human plasma digests, using 30, 60, 100, 200, 300, and 500 SPD Evosep One system methods with high-quality qualitative and quantitative results
- From the analysis of HeLa cell digest at 30 SPD, >8,000 protein groups (>83,000 precursors) could be identified from 200 ng loadings, >7,800 protein groups (>73,000 precursors) could be identified from 50 ng loadings, and >6,400 protein groups (>44,000 precursors) could be identified from 10 ng loadings
- At 500 SPD, 277 protein groups could be identified from 50 ng of non-depleted human plasma
- Using 60 or 300 SPD methods, a selection of 50 FDA-approved biomarkers could be quantified from 50 ng of non-depleted human plasma using Zeno SWATH DIA

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