High-resolution mass spectrometry peptide quantitation for biomarker verification using the ZenoTOF 7600 system

Using MRM^{HR} and Zeno SWATH data-independent acquisition (DIA) for peptide quantitation on the ZenoTOF 7600 system

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This technical note demonstrates the performance of targeted high-resolution MS/MS (MRM^{HR}) and Zeno SWATH DIA for the quantitation of peptides in a complex matrix using microflow liquid chromatography (LC). A lower limit of quantitation (LLOQ) of 0.02 fmol/µL (0.1 fmol on column) was demonstrated for alcohol dehydrogenase (ADH1) protein digest from yeast spiked into a background of K562 cell lysate tryptic digest. Excellent linearity was observed for both the MRM^{HR} and Zeno SWATH DIA methods, with a linear dynamic range of 3.0 and 2.5 orders of magnitude, respectively. Scheduled MRM^{HR} (sMRM^{HR}) improved the LLOQ by a factor of 3 on average relative to Zeno SWATH DIA. These results highlight the versatility and quantitative capabilities of the ZenoTOF 7600 system, which make it ideal for the identification and robust quantitative verification of protein biomarkers.

Key features of using the ZenoTOF 7600 system for quantitation in proteomics

- The versatility of the ZenoTOF 7600 system is appropriate for high-resolution quantitative analysis using either Zeno SWATH DIA or MRM^{HR}
- LLOQs as low as 0.02 fmol/µL were observed for peptides in a background of 400 ng cell lysate digest (on column) using microflow LC
- Linear dynamic ranges spanning 3 and 2.5 orders of magnitude were achieved using MRM^{HR} and Zeno SWATH DIA, respectively
- On average, a 3-fold increase in sensitivity was achieved with MRM^{HR} using 40 ms MS/MS accumulation times compared to Zeno SWATH DIA with 13 ms MS/MS accumulation times
- sMRM^{HR} and Zeno SWATH DIA methods were highly robust, with peak area relative standard deviations of 5.52% and 6.13%, respectively, over 45 consecutive injections

Table 1. Accuracy and precision for the quantitation of the ADH1 tryptic peptide ANELLINVK using summed MS/MS fragments. Quantitation was linear over 3 orders of dynamic range with sMRM^{HR,} and the LLOQ was 0.02 fmol/µL. With Zeno SWATH DIA, the LLOQ was 0.06 fmol/µL, and the linear dynamic range was 2.5 orders of magnitude.

	Zeno SWATH DIA			sMRM^{HR}		
Actual concentration (fmol/µL)	Mean concentration (fmol/µL)	CV (%)	Accuracy (%)	Mean concentration (fmol/µL)	CV (%)	Accuracy (%)
0.02	N/A	N/A	N/A	0.0196	19.5	97.9
0.06	0.0607	19.7	101	0.0638	6.9	106
0.2	0.186	10.9	93.1	0.195	8.6	97.7
0.6	0.657	5.9	109	0.640	1.5	107
2	1.97	2.2	98.4	2.02	3.7	101
6	6.16	6.6	102	5.88	2.7	98
20	19.0	4.2	95.2	18.5	3.2	92.4

Introduction

DIA has emerged as the preferred analysis method for many proteomics researchers, as it provides an unbiased, global view of the proteome with all peptides within a defined mass-to-charge (m/z) window subject to fragmentation. With the introduction of Zeno SWATH DIA on the ZenoTOF 7600 system, researchers now have access to unparalleled speed, sensitivity and accuracy for the identification and quantitation of proteins, particularly when quantitation is performed on MS/MS fragments. It has been previously shown that high numbers of proteins can be identified and quantified from cell lysate digests with Zeno SWATH DIA using microflow LC and the ZenoTOF 7600 system.[1] After the identification and relative quantitation of proteins between samples from different cell states or patients, proteins identified as potential biomarkers are typically further quantified in larger sample groups as part of the verification process. As this technical note shows, the ZenoTOF 7600 system is the ideal platform for the quantitative verification of identified biomarkers. In addition to biomarker discovery, early validation of potential biomarkers can be performed using the same Zeno SWATH DIA methods, minimizing the need for additional method development. Further improvements in sensitivity and quantitative dynamic range can be achieved using a targeted approach such as sMRM^{HR}.

Methods

Sample preparation: Human K562 cell lysate tryptic digest was acquired from Promega, and an 80 ng/µL dilution was prepared in water with 0.1% formic acid. A mixture of 20 isotopically labeled synthetic tryptic peptides (PepCalMix, MS Synthetic Peptide Calibration kit, SCIEX P/N 5045759) was added at a final concentration of 1 fmol/µL for each. Standard curves of a mixture of tryptic peptides from 4 proteins, yeast enolase (ENO1), rabbit phosphorylase b (PYGM), yeast alcohol dehydrogenase (ADH1) and bovine serum albumin (ALBU) (MassPREP Digestion Standard Mix 1, Waters) were prepared in the 80 ng/µL K562 dilution spiked with 1 fmol/µL PepCalMix).

Chromatography: A trap-and-elute microflow LC workflow was set up on a SCIEX M5 MicroLC TE ultralow flow system. A Phenomenex trap column (MicroTrap C18, 10 x 0.5 mm, P/N: 05N-4252-AF) and analytical column (Kinetex XB-C18 (150 x 0.3 mm, P/N: 00F-4496-AC) were used with a flow rate of 5 µL/min. Mobile phases A and B were water and acetonitrile, respectively, each with 0.1% formic acid. Trapping and desalting were performed for 1 minute at 50 µL/min with mobile phase A.

A 20-minute gradient from 3% B to 35% B was used, followed by a 1-minute ramp to 80% B, a 2-minute wash at 80% B, a ramp down to 3% B over 1 minute, and a 6-minute re-equilibration. The analytical column was held at 40°C. A 50 µL loop was used with 5 µL injections. Five replicate injections were run for each sample.

Mass spectrometry: The ZenoTOF 7600 system was operated in positive mode using the OptiFlow Turbo V ion source with a microflow probe and 1-10 µL/min electrode. The source conditions used included gas 1 set to 60 psi, gas 2 set to 20 psi, curtain gas set to 35 psi, and source temperature set to 225°C. The electrospray voltage was 4,500 V. The Zeno SWATH DIA and sMRM^{HR} parameters used are listed in Table 2. The declustering potential was 80 V, CAD gas was 7, and time bins to sum was set to 8. The collision energy for TOF MS was 10 V. For MS/MS, the dynamic collision energy calculation for charge state 2 was used.

Data processing: Zeno SWATH DIA and sMRM^{HR} data were processed using the Analytics module of the SCIEX OS software, version 3.1. Precursors and transitions were selected using Skyline software.[2] The extraction width was set to 0.05 Da and used for the extracted ion chromatograms (XICs). LLOQs and the linear dynamic ranges were determined based on precision CV<20% at the LLOQ and <15% at higher concentrations and accuracy between 80% and 120% at the LLOQ and between 85% and 115% at higher concentrations. The level of smoothing was set based on the cycle time, with 1 point used for the sMRM^{HR} data and 0.8 points used for the Zeno SWATH DIA data.

Table 2. MS parameters for the different gradient methods.

Parameter	Zeno SWATH DIA	sMRM^{HR}
TOF MS range (m/z)	400-1,500	400-1,500
TOF accumulation (s)	0.1	0.2
MS/MS range (m/z)	140-1,750	140-1,750
Variable windows (#) *	65	N/A
MRM^{HR} precursors (#) **	N/A	51 (scheduled)
Retention time tolerance (s)	N/A	+/- 60
Q1 resolution	N/A	Low
MS/MS accumulation (s)	0.013	0.04
Zeno threshold (cps)	N/A	20,000
Total scan time (s)	1.28	1.0

** The Zeno SWATH DIA windows used are available on request.*
*** The precursors used and their retention times are available on request.*

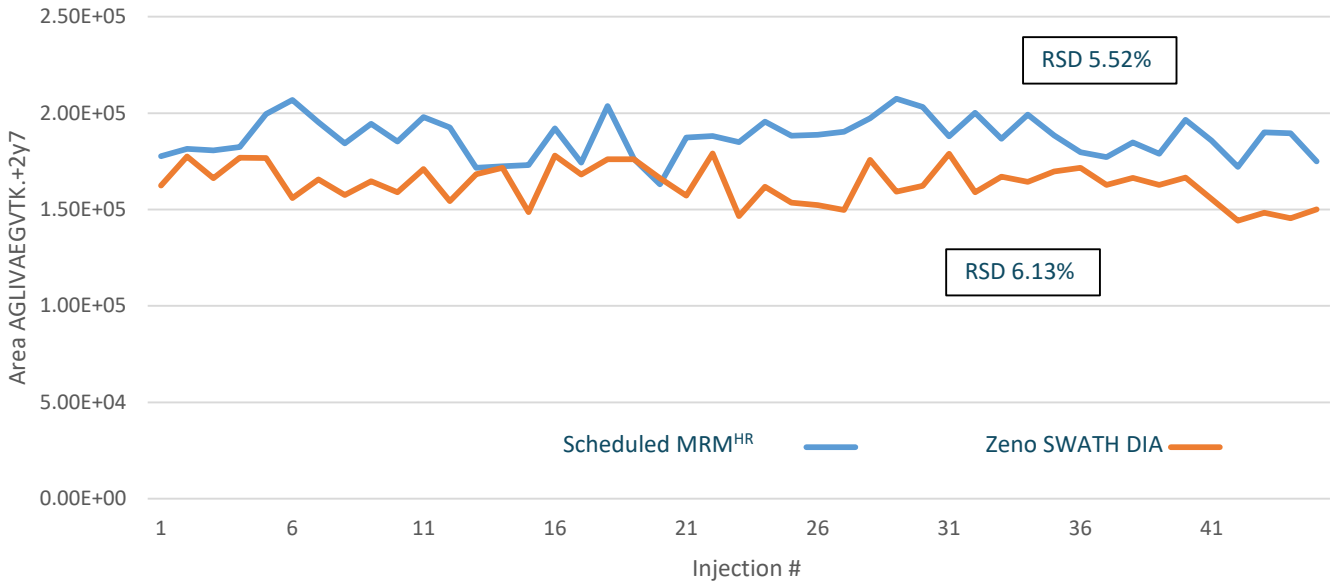

Figure 1. Peak area reproducibility of the +2y7 fragment of the AGLIVAEGVTK PepCalMix peptide. Excellent stability was observed for both the sMRM^{HR} and Zeno SWATH DIA experiments for the 45 consecutive injections of the standard curves.

Stability

The PepCalMix peptides spiked in at a constant concentration of 1 fmol/µL were used to check the run-to-run stability for both the MRM^{HR} and Zeno SWATH DIA experiments. Figure 1 shows the excellent peak area reproducibility of the +2y7 fragment of the AGLIVAEGVTK PepCalMix peptide for the 45 consecutive injections of the standard curves. The relative standard deviation was 5.52% for the sMRM^{HR} experiments and 6.13% for the Zeno SWATH DIA experiments.

Sensitivity comparison

Using Skyline software, data from the Zeno SWATH DIA experiments were used to determine 6-8 of the most intense precursors for each of the spiked-in proteins. These precursors, together with 20 precursors for the PepCalMix peptides, were used to build a sMRM^{HR} method for 51 precursors. Scheduling allowed the MS/MS accumulation times for the sMRM^{HR} method to be increased to 40 ms, compared to 13 ms for the Zeno SWATH DIA method. Using a longer MS/MS accumulation for the more targeted approach of the sMRM^{HR} method was expected to improve signal-to-noise (S/N) ratio for the XICs for the peptide fragments used for quantitation. Figure 2 shows the comparison of Zeno SWATH DIA against sMRM^{HR} for the tryptic peptide AVDDFLISLDGTANK from ENO1. Note that for more precursors and/or faster LC runs, shorter MS/MS accumulation

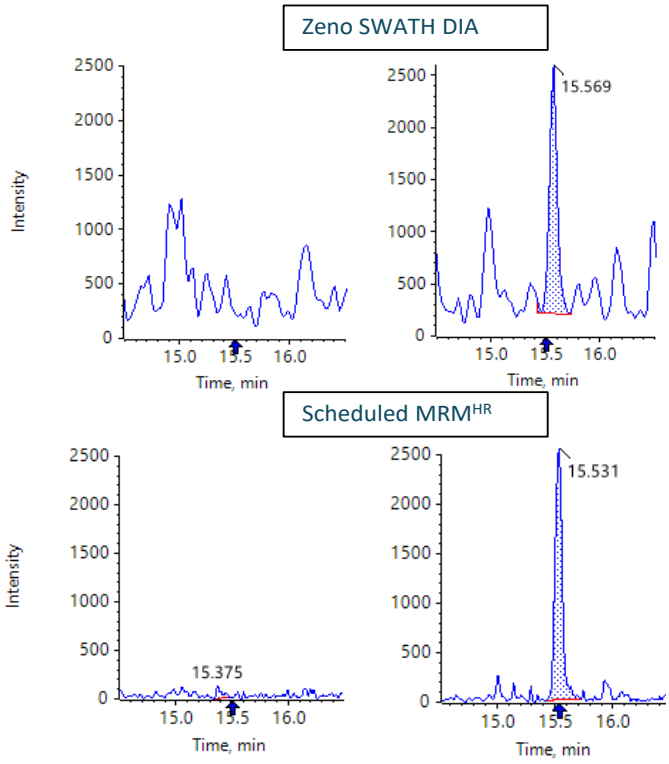

Figure 2. S/N comparison for the +2y8 fragment of the AVDDFLISLDGTANK tryptic peptide of ENO1. The XICs on the left are blanks, and the XICs on the right are at 0.6 fmol/µL. A significant increase in S/N can be seen using the sMRM^{HR} method.

times must be used for sMRM^{HR}, likely resulting in reduced gains in S/N ratio compared to Zeno SWATH DIA. Comparison of 15 of the most intense fragments for selected precursors revealed that the S/N ratio for the sMRM^{HR} experiment improved by 1-7x (average of 3x) relative to the Zeno SWATH DIA experiment. Only fragments from ADH1, PYGM, and ENO1 were used for this comparison, as there was a significant amount of background in the blanks for the ALBU fragments, possibly from the cell culture medium used to grow the K562 cells. In addition to the increased signal averaging with the longer accumulation time, the narrower precursor selection window with MRM^{HR} can minimize interferences attributed to the K562 background.

Table 3 shows the S/N ratio improvements observed for the 15 XICs and Figure 2 shows a representative example of these results.

Table 3. S/N ratio improvement with sMRM^{HR} for 15 fragments from protein tryptic peptides.

Tryptic peptide fragment	Zeno SWATH DIA	sMRM^{HR}	S/N ratio gain with sMRM^{HR}
ADH1_YEAST.ANELLINVK.+2y7	7.9	13.8	1.7
ADH1_YEAST.IGDYAGIK.+2y7	12.0	19.0	1.6
ADH1_YEAST.DIVGAVLK.+2y6	9.4	42.4	4.5
ADH1_YEAST.SISIVGSYVGNR.+2y10	6.5	21.0	3.2
ADH1_YEAST.SISIVGSYVGNR.+2y9	6.5	15.7	2.4
ADH1_YEAST.EALDFFAR.+2y5	22.7	50.3	2.2
ADH1_YEAST.VVGLSTLPEIYEK.+2y9	9.6	26.3	2.7
PYGM_RABIT.APNDFNLK.+2y7	23.8	42.1	1.8
PYGM_RABIT.NLAENISR.+2y5	14.6	26.3	1.8
PYGM_RABIT.FAAYLER.+2y4	23.6	93.5	4.0
ENO1_YEAST.NVNDVIAPAFVK.+2y8	14.5	31.4	2.2
ENO1_YEAST.AVDDFLISLDGTANK.+2y8	2.3	9.2	4.0
ENO1_YEAST.TFAEALR.+2y4	3.3	23.5	7.1
ENO1_YEAST.AADALLLK.+2y7	40.7	107.7	2.6
ENO1_YEAST.VNQIGTLSESIK.+2y9	12.9	17.0	1.3
Average			2.9
Median			2.4

LLOQ and linear dynamic range for ADH1

The LLOQ and linear dynamic range for the quantitation of ADH1 were determined for both acquisition methods for the ANELLINVK tryptic peptide. The precision at lower concentrations improved by summing the 2 most intense fragments (+2y6 and +2y5). Table 1 shows the accuracy and precision across a standard curve from 0.02 to 20 fmol/µL of ADH1 digest in a K562 cell lysate digest. Using sMRM^{HR}, quantitation was linear over 3 orders of dynamic range, and the LLOQ was 0.02 fmol/µL. With Zeno SWATH DIA, the LLOQ was 0.06 fmol/µL, and the linear dynamic range spanned 2.5 orders of magnitude. Figure 3 shows the XICs of the blanks and at the LLOQs. Figure 4 shows the linearity for both acquisition methods.

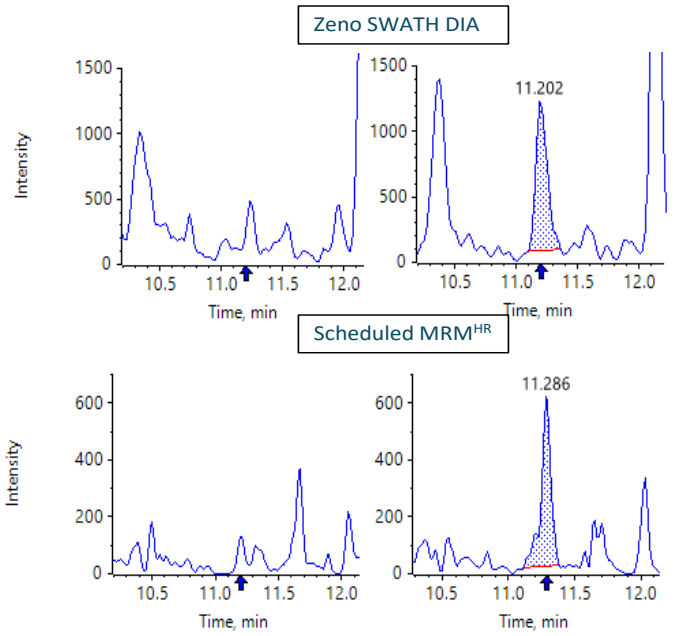

Figure 3. XICs for the summed +2y6 and +2y5 fragments of the ANELLINVK tryptic peptide of ADH1. The top graph shows the XICs of the blank and the LLOQ of 0.06 fmol/µL using Zeno SWATH DIA. The lower graph shows the blank and LLOQ of 0.02 fmol/µL using the sMRM^{HR} method.

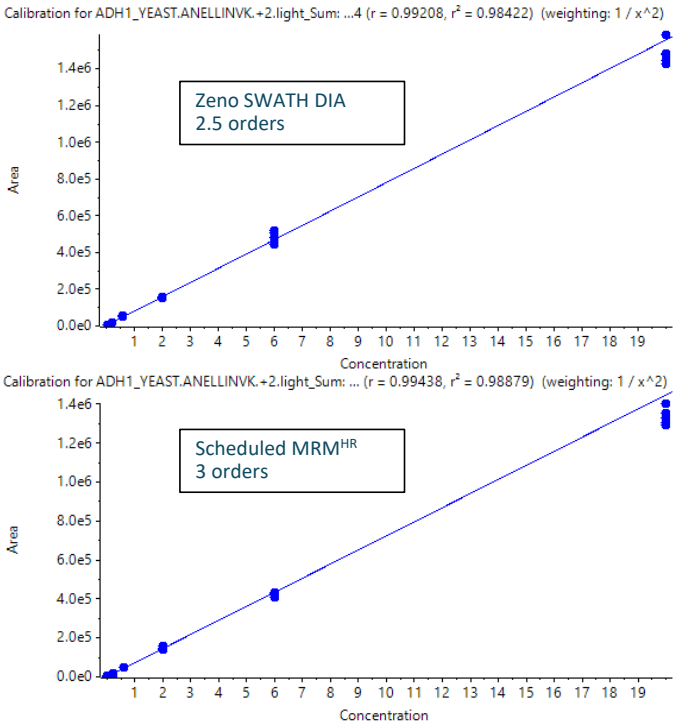

Figure 4. Linearity of the Zeno SWATH DIA and sMRM^{HR} methods for the ANELLINVK tryptic peptide of ADH1. Using sMRM^{HR}, 3 orders of linear dynamic range was achieved, while with Zeno SWATH DIA, 2.5 orders of linear dynamic range was observed.

Conclusions

- Excellent linearity and accuracy were achieved with both MRM^{HR} (3 orders of magnitude) and Zeno SWATH DIA (2.5 orders of magnitude) for high-resolution quantitative analysis
- MRM^{HR} achieved an LLOQ of 0.02 fmol/µL for peptides spiked into a background of 400 ng cell lysate digest
- MRM^{HR}, using MS/MS accumulation times of 40 ms, was approximately 3x more sensitive than the Zeno SWATH DIA method with 13 ms MS/MS accumulation times
- The ZenoTOF 7600 system is a robust platform that offers high-quality reproducibility for the quantitative verification of protein biomarkers

References

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2. Michael J. MacCoss *et al.* (2010) Skyline: an open-source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics. 26(7): 966–968.

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