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目錄

Sensitive signature peptide quantification in a complex matrix using accurate mass spectrometry

Featuring the ZenoTOF 7600 system and SCIEX OS software

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This technical note describes the achievement of low amol/µL level of quantification for signature peptides in rat plasma, with improvements in MS/MS sampling efficiency on an accurate mass spectrometer. Additionally, with the availability of TOF MS/MS data, further enhancement in sensitivity was achieved by the summation of several highly abundant fragment ions for quantification. As a result, a 2-fold improvement in LLOQ and an overall linear dynamic range (LDR) of greater than 4.3 orders of magnitude (Table 1) was achieved.

Traditional workflows for quantitative bioanalysis of peptides and proteins, such as immunological assays, have been displaced by LC-MS/MS analysis using triple quadrupole mass spectrometers. While the triple quadrupole platform has been the gold standard for most bioanalytical workflows, offering great sensitivity and quantitative performance, accurate mass spectrometry has been increasingly suggested for quantitative bioanalysis.[1,2] However, accurate mass spectrometry platforms, such as traditional time-of-flight (TOF) systems, often lack sensitivity due to limited duty cycle in between TOF pulses.

With the introduction of the ZenoTOF 7600 system, the improvement in MS/MS sampling efficiency offers a robust and sensitive platform to support routine peptide and protein quantification. Here, the Zeno trap controls the ion beam from the collision cell which facilitates greater ion transmission to the TOF accelerator, improving the duty cycle to ≥90% (classical TOF is below 30%). As a result, this enhances the overall MS/MS sampling efficiency enabling the ZenoTOF 7600 system to be highly advantageous for quantitative bioanalysis workflows that can benefit from the accessibility of the full product ion profile and the improvement in sensitivity using the Zeno trap.[3,4]

In this technical note, 3 peptides were selected as model analytes to evaluate the quantitative performance of signature peptides on the ZenoTOF 7600 system. Ultra-low LLOQs, ranging from 0.025 fmol/µL to 0.05 fmol/µL were achieved with an LDR greater than 4.3 orders of magnitude. The measured upper limit of quantification (ULOQ) ranged from 500 fmol/µL to 2000 fmol/µL. Overall, the assay demonstrated outstanding accuracy, precision and linearity, highlighting the robustness and performance of the developed method for the quantification of signature peptides.

Key features of the signature peptide quantification workflow using the ZenoTOF 7600 system and SCIEX OS software

- Achieve low-amol/µL level quantification of signature peptides in complex matrices using the Zeno MRM^{HR} workflow
- Reach enhanced sensitivity by summation of multiple highly abundant fragment ions with the availability of TOF MS/MS data and improvements in MS/MS sampling efficiency using the Zeno trap
- Confirm GLP-level accuracy and precision for quantitative workflows with greater than 4.3 orders of magnitude in LDR using the ZenoTOF 7600 system
- Easily acquire, process and manage data on a single platform using the SCIEX OS software

Table 1. A summary of the quantitative performance of the signature peptide assay on the ZenoTOF 7600 system.

Peptide Sequence	Charge state	Fragment ions used for quantification	LLOQ (fmol/µL)	ULOQ (fmol/ µL)	LDR (orders)
FNWYVDGVEVHNAK	*3+*	*b2, y6 and y4*	*0.05*	*2000*	*4.6*
*AGLIVAEGVTK**	*2+*	*y6*	*0.025*	*500*	*4.3*
*LGLDFDSFR**	*2+*	*y5, y6 and y8*	*0.025*	*500*	*4.3*

Methods

Sample preparation: Plasma proteins were precipitated with cold methanol. After centrifugation, the supernatant was discarded while the pellet was solubilized in 200 mM ammonium bicarbonate in 10:90 (v/v) methanol/water. Digestion was performed using trypsin. After 1 hour at 60°C, the solution was acidified by adding formic acid.[5] The digested plasma was diluted by 200x using 5:1:94 (v/v/v) acetonitrile/formic acid/water. Synthesized peptides (Table 2) were spiked into the digested plasma solution and followed by serial dilution in the matrix. The final injection volume was 10 µL.

Table 2. List of peptide targets.

Peptide Sequence	Description
FNWYVDGVEVHNAK	*Conserved sequence in human immunoglobulin G (IgG)*
*AGLIVAEGVTK**	*Synthetic peptide with C terminal K heavy isotope labeled ($C^{13}N^{15}$)*
*LGLDFDSFR**	*Synthetic peptide with C terminal R heavy isotope labeled ($C^{13}N^{15}$)*

Chromatography: The separation was performed at a flow rate of 0.4 mL/min using an ExionLC system. A Phenomenex bioZen Peptide XB-C18 column (2.1 x 50 mm, 2.6 µm, 100 Å) was used for separation. The column oven temperature was set to 40°C. The mobile phase A consisted of 0.1% formic acid in water, while the mobile phase B was composed of 0.1% formic acid in acetonitrile. Gradient conditions are summarized in Table 3. A volume of 10 µL was injected for analysis. All samples were analyzed in triplicate.

Table 3. LC gradient conditions.

Time (min)	Mobile phase B (%)	Mobile phase A (%)
0	*97*	*3*
1	*97*	*3*
4	*60*	*40*
4.1	*10*	*90*
5.1	*10*	*90*
6.1	*97*	*3*
8	*97*	*3*

Mass spectrometry: Data were acquired in positive mode using Zeno MRM^{HR} on a ZenoTOF 7600 system. Collision energy (CE), source and MS parameters were optimized for all the signature peptides. A summary of the source and MS parameters and the Zeno trap settings is displayed in Table 4. Additionally, the MRM^{HR} parameters and fragments used for quantification for each of the signature peptides are summarized in Table 5. Unit Q1 resolution was used for the analysis.

Table 4. Source and MS conditions.

Parameter	Value	Parameter	Value
Curtain gas	*30 psi*	*Source temp.*	*600˚C*
Ion source gas1	*50 psi*	*Ion source gas 2*	*60 psi*
CAD gas	*7*	*Ion spray voltage*	*5500 V*
MS accumulation time	*80 ms*	*MS/MS accumulation time*	*10 ms*
TOF MS start mass (m/z)	*400*	*TOF MS stop mass (m/z)*	*800*
TOF MS/MS start mass (m/z)	*100*	*TOF MS/MS stop mass (m/z)*	*1200*
Zeno threshold	*20,000 cps*		

Table 5. MRM^{HR} parameters and fragments used for quantification.

Peptide	Q1 mass (*m/z*)	Fragment mass (*m/z*)	DP (V)	CE (V)
FNWYVDGVEVHNAK (b2)	*560.27*	*262.118*	*80*	*30*
FNWYVDGVEVHNAK (y4)	*560.27*	*469.252*	*80*	*30*
FNWYVDGVEVHNAK (y6)	*560.27*	*697.363*	*80*	*30*
AGLIVAEGVTK (y6)*	*533.32*	*612.344*	*80*	*32*
LGLDFDSFR (y5)*	*540.27*	*681.322*	*80*	*34*
LGLDFDSFR (y6)*	*540.27*	*796.351*	*80*	*34*
LGLDFDSFR (y8)*	*540.27*	*966.457*	*80*	*34*

Data processing: Data were processed using the Analytics module in SCIEX OS software with the MQ4 integration algorithm. A $1/x^2$ weighting was used for quantification.

Zeno trap provides greater sensitivity

In traditional TOF MS/MS acquisition, ions are lost between TOF MS spectra acquisitions, resulting in a much lower MS/MS sampling efficiency and sensitivity than MRM on a triple quadrupole mass spectrometer. The Zeno trap increases ion transmission by providing control of the ion beam from the collision cell into the TOF accelerator (Figure 1).

Ions are gated then released based on potential energy. Generally, higher *m/z* ions are released first then followed by lower *m/z* ions. A wide range of ions now arrive in the accelerator to be pushed during the same pulse resulting in a 10-fold increase in MS/MS sampling efficiency.[6]

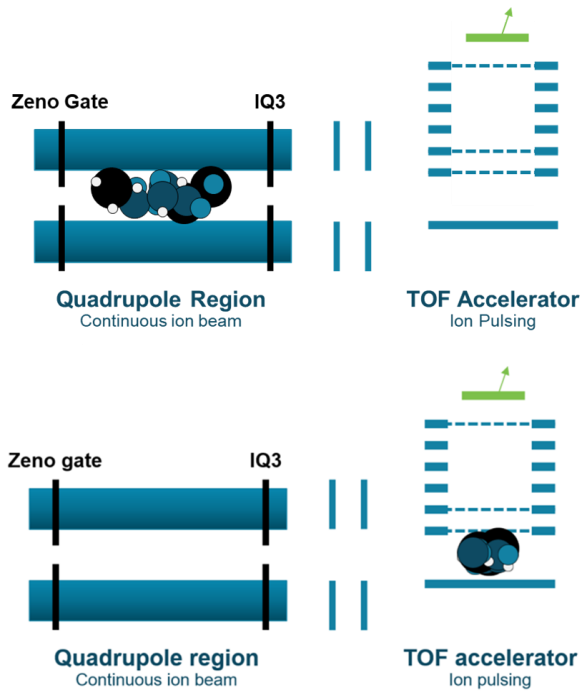

Figure 1. Zeno trap enables ion beam control from the collision cell prior to entrance into the TOF accelerator. Gains in ion transmission improve overall MS/MS sampling efficiency.

Overview of the signature peptide workflow

FNWYVDGVEVHNAK, AGLIVAEGVTK* and LGLDFDSFR* were used as model peptides to evaluate the quantification of signature peptides on the ZenoTOF 7600 system. The peptides were spiked into processed rat plasma at concentrations ranging from 0.025 fmol/µL to 2000 fmol/µL.

Quantification was performed using the following strategies: 1) using the most sensitive fragment ion and 2) using the sum of multiple highly abundant fragment ions. For the former method, fragment ions y6, y8 and y6 were used for the quantification of peptides AGLIVAEGVTK*, LGLDFDSFR* and FNWYVDGVEVHNAK, respectively. For the latter strategy, the XIC responses from fragment ions y5, y6 and y8 were added for the quantification of peptide LGLDFDSFR*. For the quantification of peptide FNWYVDGVEVHNAK, the XIC responses from fragment ions b2, y4 and y6 were added.

All calibration points were measured in triplicate. For the determination of the LLOQ, a %CV value of less than 20% and accuracy between 80% and 120% was required. For all other concentrations, a %CV value of less than 15% and accuracy between 85% and 115% of the nominal concentration was required. This criteria was applied to both quantification strategies.

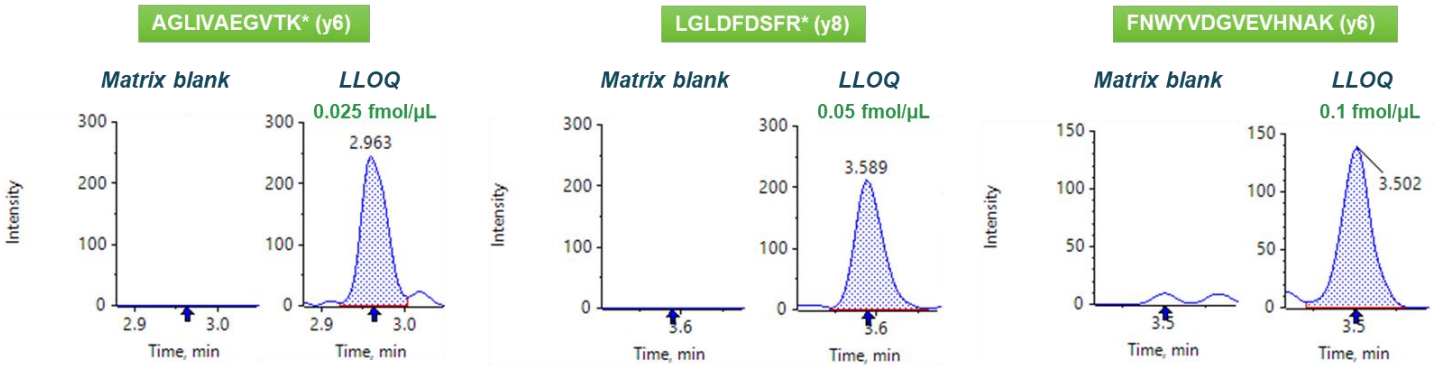

Figure 2. Extracted ion chromatograms (XICs) of the matrix blank and LLOQ using Zeno MRM^{HR}. Using the single fragment ion approach, an LLOQ of 0.025 fmol/µL, 0.05 fmol/µL and 0.1 fmol/µL was achieved for peptides AGLIVAEGVTK*, LGLDFDSFR* and FNWYVDGVEVHNAK, respectively. No matrix interferences were observed in the blank.

Quantification using a single fragment ion

In this workflow, quantification of signature peptides was performed using the Zeno MRM^{HR} workflow. For the following discussion, the most abundant fragment ion was used for the quantification of the signature peptides.

LLOQs of 0.025 fmol/µL, 0.05 fmol/µL and 0.1 fmol/µL were achieved for peptides AGLIVAEGVTK*, LGLDFDSFR* and FNWYVDGVEVHNAK, respectively (Figure 2). No interferences were observed in the matrix blank.

The linear range for the single fragment ion approach covered concentrations from 0.025 fmol/µL to 2000 fmol/µL (Figure 3). An LDR greater than 4 orders of magnitude was observed for each of the calibration curves.

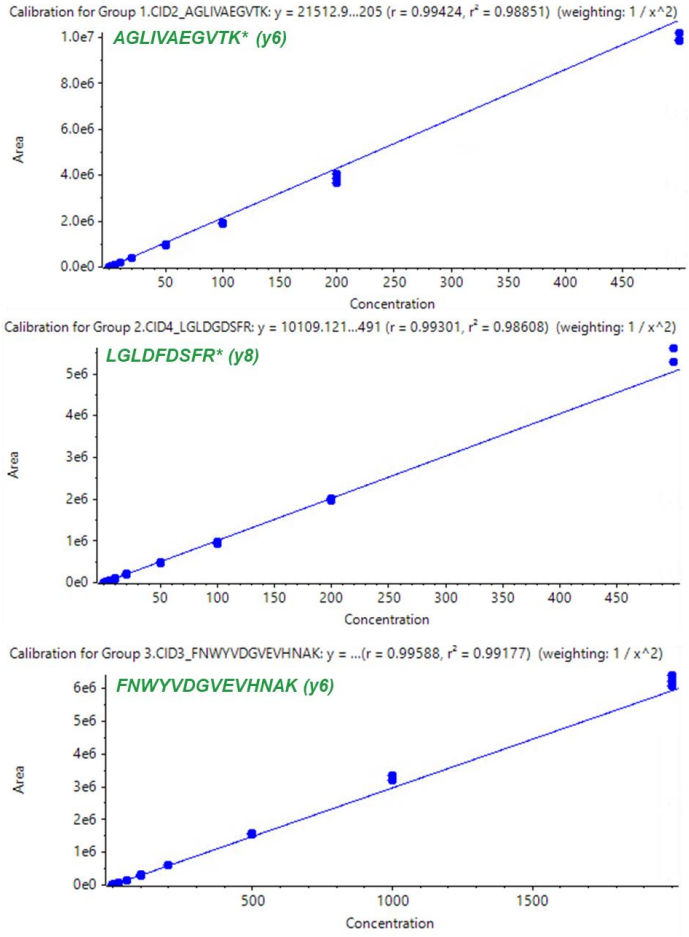

Figure 3. Calibration curves for the signature peptides using single fragment ion quantification. The linear range covered 0.025 fmol/µL to 2000 fmol/µL with an overall LDR of greater than 4 orders of magnitude.

Calculated concentrations for each calibration point were within ±15% of the nominal value (Table 6). The overall accuracy of the LLOQ was within 8% of the nominal concentration, indicating a highly accurate quantification platform for peptides at low concentrations. As shown in Table 6, the %CV value was less than 15%, demonstrating high reproducibility.

Increasing sensitivity using the summation of multiple fragment ions

The accessibility of TOF MS/MS data can be highly advantageous, as fragment ions can be selected based on overall selectivity and sensitivity for quantification. When multiple highly abundant fragment ions are summed from the target peptide, the assay sensitivity can be further enhanced.[3]

The linear range for the multiple fragment ion approach covered concentrations from 0.025 fmol/µL to 2000 fmol/µL (Figure 4). Strong linearity was observed for each of the calibration curves with an LDR of greater than 4.3 orders of magnitude.

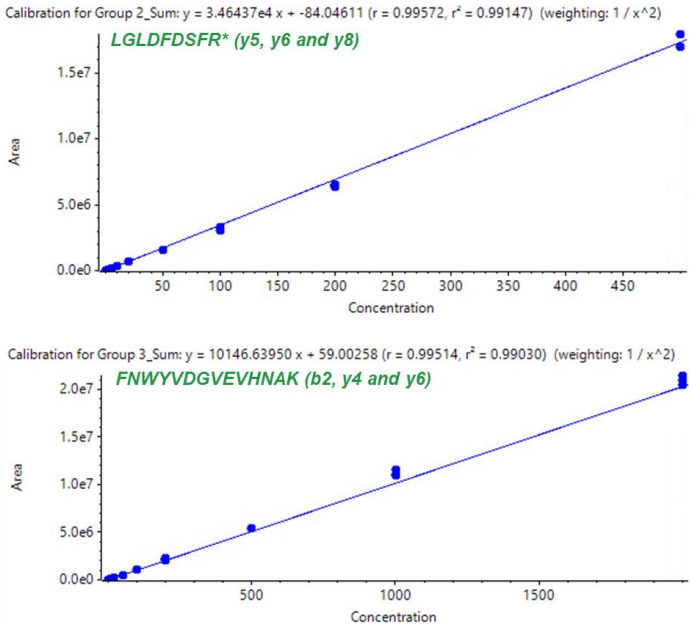

Figure 4. Calibration curves for the signature peptides using multiple fragment ion quantification. The linear range covered 0.025 fmol/µL to 2000 fmol/µL with an overall LDR of greater than 4.3 orders of magnitude.

Table 6. Calculated concentration, precision and accuracy for quantification using a single fragment ion.

	AGLIVAEGVTK*		LGLDGDSFR*		FNWYVDGVEVHNAK	
Concentration (fmol/µL)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
0.025	101.81	13.12	NA	NA	NA	NA
0.05	98.26	14.60	107.89	10.46	NA	NA
0.1	95.82	5.26	114.93	8.42	103.95	13.79
0.2	98.11	7.86	100.17	6.97	NA	NA
0.5	105.30	9.69	100.96	10.42	84.57	12.94
1	99.68	2.91	95.81	1.40	96.83	10.51
2	101.97	3.42	101.73	4.07	91.76	8.17
5	109.58	0.72	99.81	1.44	92.60	4.49
10	104.35	2.45	96.09	7.08	99.28	1.90
20	103.73	4.36	97.55	1.58	102.25	2.49
50	96.32	4.19	92.26	1.74	101.98	1.44
100	94.53	0.81	92.91	4.23	103.62	3.11
200	93.93	4.38	95.87	1.20	103.57	1.77
500	96.61	2.16	104.02	3.42	104.99	0.79
1000	NA	NA	NA	NA	109.50	2.75
2000	NA	NA	NA	NA	105.10	2.56

A 2-fold improvement in LLOQ was achieved when multiple highly abundant fragment ions were summed for quantification (Figure 5). LLOQs of 0.025 fmol/µL and 0.05 fmol/µL were achieved for peptides LGLDFDSFR* and FNWYVDGVEVHNAK, respectively. No interferences were observed in the matrix blank.

Calculated concentrations for each calibration point were within ±15% of the nominal value (Table 7). The overall accuracy was within 8% of the nominal concentration at the level of the LLOQ, indicating a highly accurate platform for low-level quantification. As shown in Table 7, the overall %CV value for the acquired data, including the LLOQ, was less than 20%.

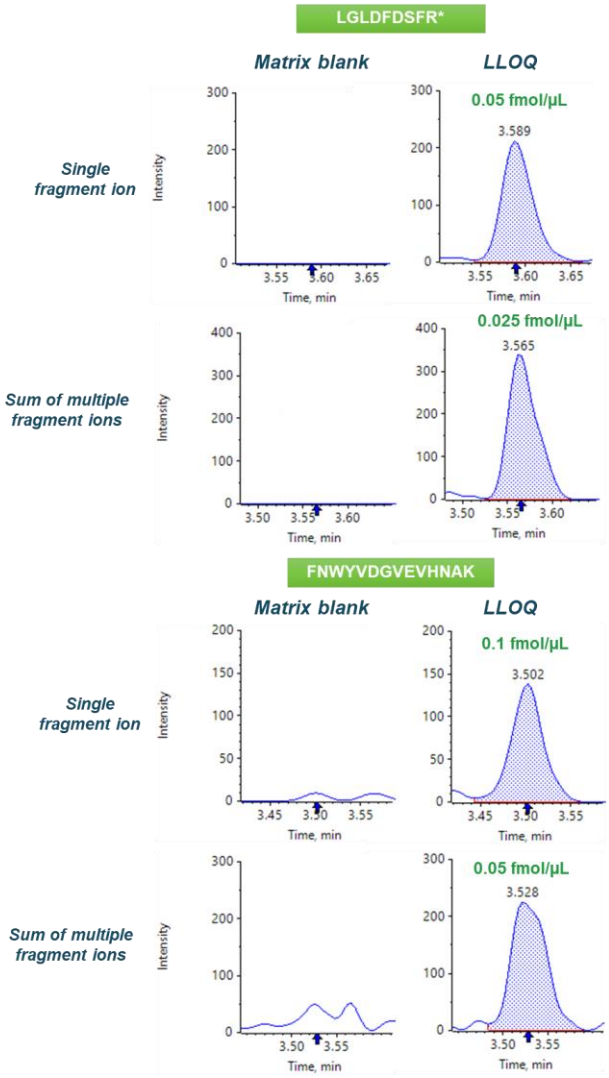

Figure 5. XICs of the matrix blanks and spiked peptides at the LLOQ using summation of multiple fragment ions. A 2-fold improvement in sensitivity was observed when multiple highly abundant fragment ions were summed for quantification. LLOQs of 0.025 fmol/µL and 0.05 fmol/µL were reached for the peptides LGLDFDSFR* and FNWYVDGVEVHNAK, respectively. No matrix interferences were observed in the blank.

Table 7. Calculated concentration, precision and accuracy for quantification using summation of multiple fragment ions.

	LGLDGDSFR*		FNWYVDGVEVHNAK	
Concentration (fmol/µL)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
0.025	93.84	19.71	NA	NA
0.05	107.80	8.65	107.08	4.45
0.1	106.56	2.66	90.44	9.57
0.2	100.82	5.95	NA	NA
0.5	106.00	8.53	85.74	3.14
1	103.68	1.75	88.83	6.67
2	111.02	4.43	89.43	4.66
5	100.69	2.56	90.38	3.45
10	95.64	5.60	103.43	2.71
20	97.52	1.32	107.77	2.87
50	91.46	1.23	104.44	1.23
100	91.87	4.85	105.68	1.50
200	93.23	1.35	106.39	4.00
500	99.85	3.24	106.89	0.80
1000	NA	NA	110.21	2.56
2000	NA	NA	103.28	2.33

Conclusions

- A highly sensitive signature peptide quantification workflow was developed using the ZenoTOF 7600 system
- Low-amol/µL levels of quantification for signature peptides were reached using a Zeno MRM^{HR} workflow
- Summation of multiple fragment ions enhances the LLOQ up to 2-fold with the availability of TOF MS/MS data and improvements in MS/MS sensitivity using the Zeno trap
- GLP-level accuracy and precision for signature peptide quantification was achieved with greater than 4.3 orders of magnitude in LDR

Acknowledgment

We would like to thank Fang Xie and Zhe Wang from Amgen for their consultation and guidance throughout the development of the assay.

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2. Yuan-Qing Xia, Jim Lau, Timothy Olah, Mohammed Jemal (2011). Targeted quantitative bioanalysis in plasma using liquid chromatography/high-resolution accurate mass spectrometry: an evaluation of global selectivity as a function of mass resolving power and extraction window, with comparison of centroid and profile modes. *Rapid Communications in Mass Spectrometry* **25**(19):2863-2878.
3. Enhanced sensitivity for peptide quantification in a complex matrix using high-resolution LC-MS/MS. SCIEX technical note, RUO-MKT-02-13324-A.
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6. Qualitative flexibility combined with quantitative power. SCIEX technical note, RUO-MKT-02-13053-A.

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Enhanced sensitivity for peptide quantification in a complex matrix using high-resolution LC-MS/MS

Featuring the ZenoTOF 7600 LC-MS/MS system

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A significant 5-fold improvement in LLOQ for peptide quantification was achieved using the ZenoTOF 7600 system featuring the Zeno trap. Compared with traditional time-of-flight systems, the Zeno trap enables greater MS/MS sensitivity by enhancing the duty cycle. In addition, the versatility of TOF MS/MS data allows for the capability of post-acquisition decisions for the selection of fragment ion(s) for MRM^{HR}. For cases where multiple dominant fragment ions are generated from the target peptide, the sum of XICs enabled greater sensitivity. A 3-fold improvement in LLOQ was observed for peptides that leveraged the summing of multiple dominant fragment ions when MS/MS ion current was dispersed.

Traditional workflows for quantitative bioanalyses, such as immunological assays, have been displaced by LC-MS/MS analysis on triple quadrupole mass spectrometers. Immunoassays often lack selectivity and specificity, and have a limited linear dynamic range. While the triple quadrupole platform has been a key driver for most bioanalytical workflows, offering great sensitivity and quantitative performance, high-resolution accurate mass spectrometry (HRAMS) has increasingly been adopted for quantitative bioanalysis.[1,2] With the inherent advantage of greater selectivity with improved mass resolution, as well as the flexibility of TOF MS/MS data, the ZenoTOF 7600 system provides excellent quantitative performance in multiple dimensions.

High-resolution platforms, such as traditional time-of-flight systems, often lack sensitivity due to loss of ion transmission in between TOF pulses. The Zeno trap controls the ion beam from the collision cell which facilitates greater ion transmission to the TOF accelerator. Therefore, the duty cycle is improved to ≥90 %, which enhances overall MS/MS sensitivity.

The ZenoTOF 7600 system offers an exceptional combination of mass resolution, sensitivity, and acquisition speed for quantitative analysis. It also aids in the potential for: less ion path tuning, increased sensitivity with the Zeno trap, ability to change measured fragments post-acquisition and improved reproducibility and accuracy.

Key features of the ZenoTOF 7600 system for highly sensitive peptide quantification

- Demonstration of a 5-fold improvement in LLOQ for peptide quantification using the Zeno trap to accumulate ions during each TOF pulse for enhanced duty cycle
- Reach enhanced sensitivity by summing of multiple highly abundant fragment ions with availability of TOF MS/MS data
- Ensure exceptional accuracy and precision for quantitative workflows using the ZenoTOF 7600 system
- Easily acquire, process, and manage data on a single platform using the SCIEX OS Software

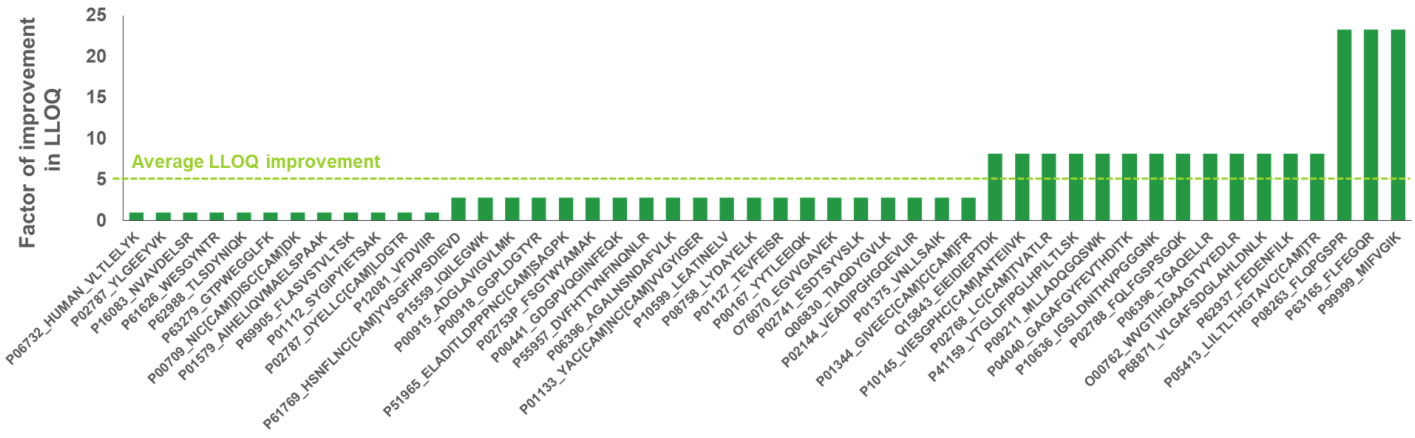

Figure 1. Factor of improvement in LLOQ for peptide quantification with Zeno MRM^{HR} compared to MRM^{HR}. On average, a 5-fold improvement in LLOQ for peptide quantification was observed with Zeno MRM^{HR} in comparison to standard MRM^{HR}.

Methods

Samples and reagents: Universal Proteomics Standard (UPS) was purchased from Sigma-Aldrich. Rat plasma (Sprague Dawley, K2 EDTA) was purchased from BioIVT.

Sample preparation: The calibration curve was prepared by spiking digested UPS into rat plasma digest followed by serial dilution.

Samples were denatured by incubating with N-octyl-glucoside (OGS), followed by reduction with dithiothreitol (DTT) and alkylation with iodoacetamide (IAM). A trypsin/Lys-C digestion was performed at 37 ºC overnight, with an enzyme-protein ratio of 1:25. Formic acid was spiked into the samples to abort digestion. The samples were centrifuged at a speed of 12,000 g and the supernatant was injected for LC-MS analysis.

As a note, proteins used for this study had limited starting concentrations. Therefore, the final LDRs were narrow for the peptides analyzed.

Chromatography: An ExionLC system was used for analyte separation. A volume of 20 µL was injected for analysis. Mobile phase A consisted of water with 0.1% FA in water, while organic phase B was composed of 0.1% FA in acetonitrile. For analyte separation, the operating flow rate was set to 0.5 mL/min using a Phenomenex Kinetex C18 column (3 x 50 mm, 2.6 µm, 100 Å). The column oven temperature was set to 40 ºC. Chromatographic conditions for analyte separation are shown in Table 1.

Table 1. Chromatographic conditions for analyte separation.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.50	*98.0*	*2.0*
1.00	*88.0*	*12.0*
6.00	*68.0*	*32.0*
6.10	*10.0*	*90.0*
7.00	*10.0*	*90.0*
7.10	*98.0*	*2.0*
8.00	*98.0*	*2.0*

Mass Spectrometry: Samples were analyzed in triplicate. Method details such as source and gas parameters and MS conditions are summarized in Table 2. Sample analysis was performed using scheduled Zeno MRM^{HR} on the ZenoTOF 7600 system. The ZenoTOF 7600 system provides a scan speed of 133 Hz.

Data processing: MRM data were processed using SCIEX OS 2.0 software. Integration was performed using the MQ4 algorithm. Linear regression with 1/x weighting was used for quantification of all peptides. The XIC peak width was set to 0.05 Da.

Table 2. Source and MS conditions.

Parameter	Value	Parameter	Value
Curtain gas	*30 psi*	*Source temperature*	*550 °C*
Ion source gas1	*65 psi*	*Ion source gas 2*	*65 psi*
CAD gas	*12*	*Ion spray voltage*	*5500 V*
MS accumulation time	*40 ms*	*MS/MS accumulation time*	*20 ms*
TOF MS start mass	*350 Da*	*TOF MS stop mass*	*1500 Da*
TOF MS/MS start mass	*300 Da*	*TOF MS/MS stop mass*	*≥1000* Da*
ZOD threshold	*20,000 cps*		

**TOF MS/MS stop mass depends on peptide analyzed*

Greater sensitivity with Zeno trap

With traditional time-of-flight MS/MS, fragment ions arriving from the collision cell are often lost in transmission between TOF pulses due to differences in velocity. As a result, for standard time-of-flight MS/MS, duty cycle range is approximately between 5-25%. A decrease in sensitivity occurs as a consequence of loss in ion transmission. The Zeno trap ensures greater ion transmission by controlling the ion beam from the collision cell into the TOF accelerator (Figure 2). Ions exit the Zeno trap based on potential energy.

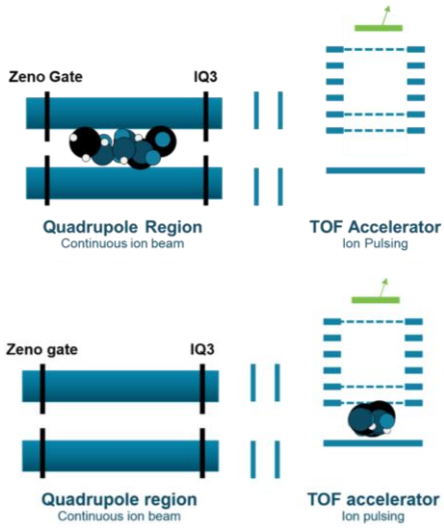

Figure 2. Zeno trap enables ion beam control from the collision cell before entering into the TOF accelerator. Gains in ion transmission improve overall MS/MS sensitivity.

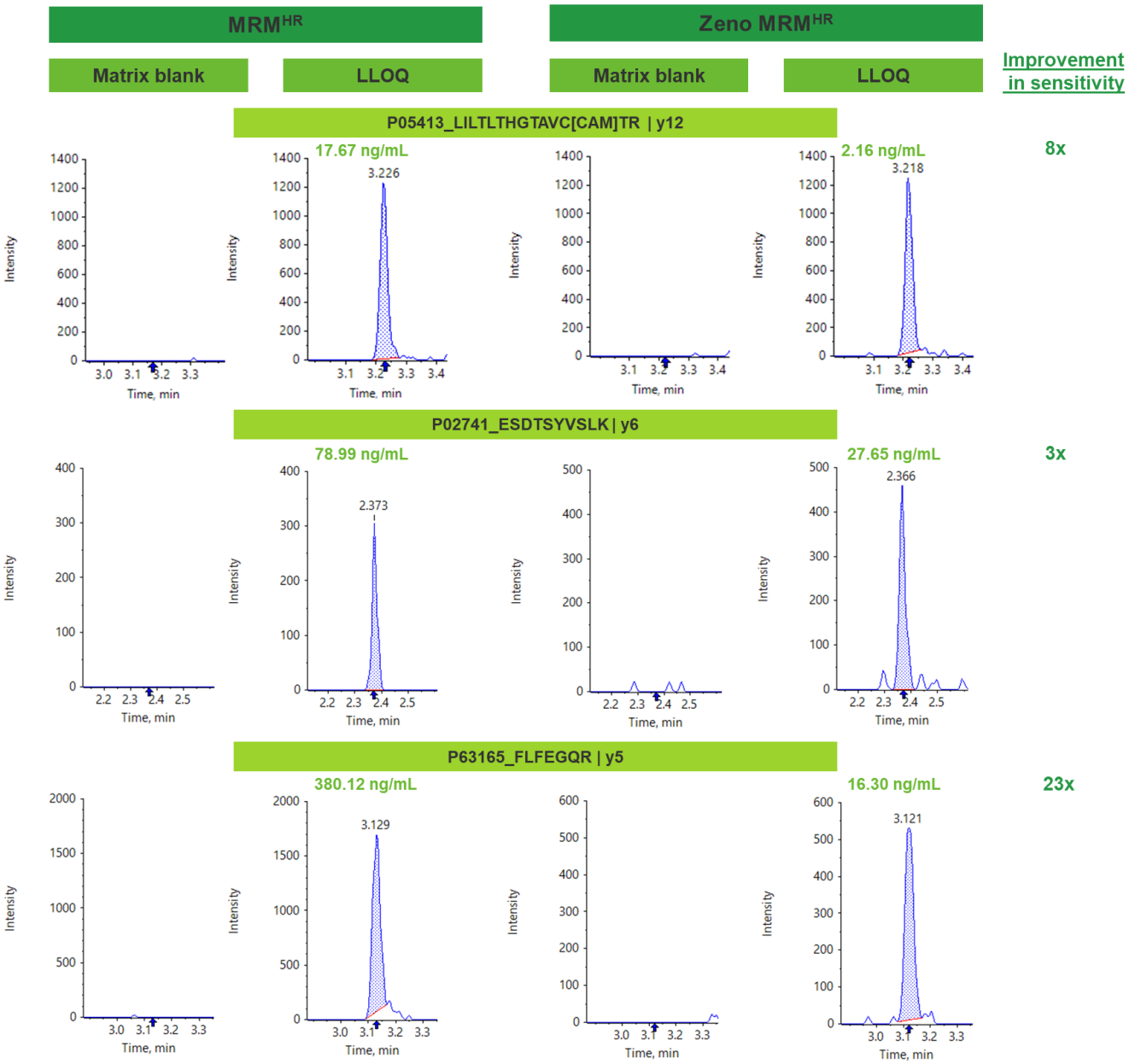

Figure 3. Greater sensitivity for peptide quantification was observed with Zeno MRM^{HR}. Significantly lower LLOQs were achieved using Zeno trap.

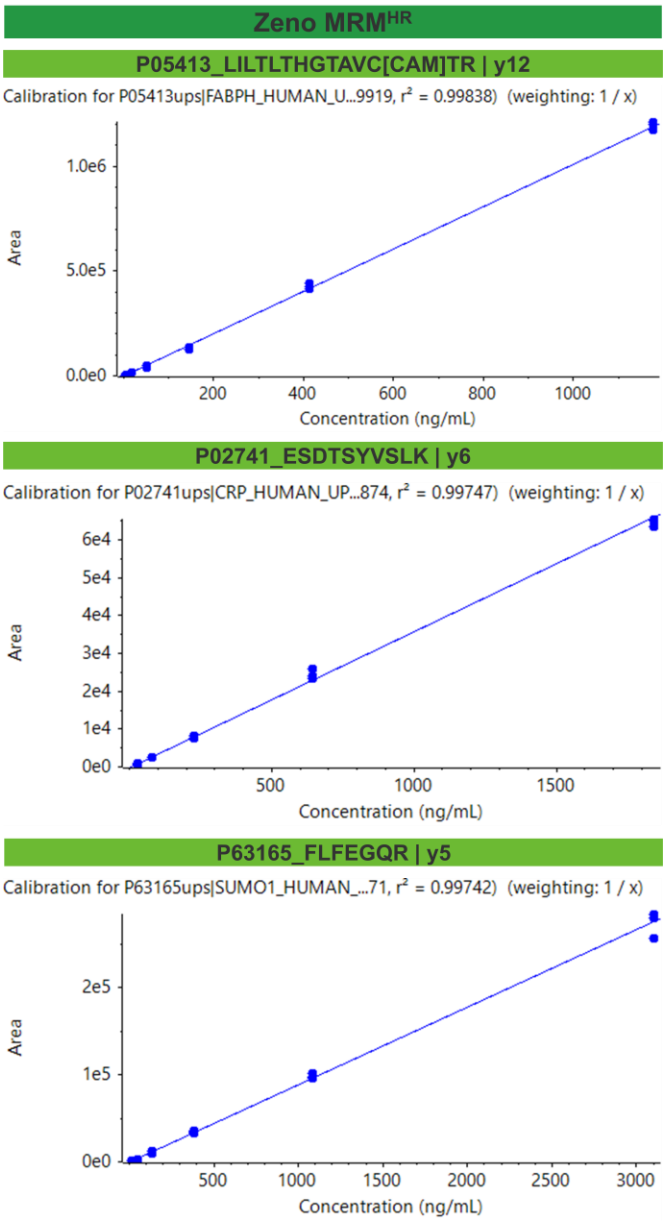

Figure 4. Strong linearity was achieved with Zeno MRM^{HR}.

The Zeno trap enabled significant improvements in MS/MS sensitivity. On average, 5-fold improvement in LLOQ was achieved using Zeno MRM^{HR} in comparison with standard MRM^{HR} (Figure 1). Out of 48 peptides measured, 75% of peptides showed ≥3-fold improvement in LLOQ.

Overall, XICs comparing LLOQs with MRM^{HR} and Zeno MRM^{HR} show significantly lower LLOQs were achieved with the Zeno trap (Figure 3). Strong linearity was achieved for all peptides analyzed (Figure 4). Accuracy at the LLOQ was within 80%-120%, while for all other non-zero calibrators, accuracy was within 85%-115% of the nominal concentration. Overall, precision was <15%, demonstrating high reproducibility (Table 3).

Summation of multiple fragment ions enhances sensitivity

The accessibility of TOF MS/MS data can be advantageous as post-acquisition data decisions can be made on which measured fragments can be utilized for MRM^{HR}. For MRM^{HR}, quantification can be performed using single fragment ion or by summing multiple dominant fragment ions. When multiple, high-abundant, fragment ions are generated from the target peptide, the sum of XICs can further enhance the assay sensitivity.

As shown in Figure 5, summing of multiple dominant fragment ions can achieve up to a 3-fold improvement in LLOQ. Strong linearity was achieved for quantification with single fragment ions and summed multiple fragment ions (Figure 6). Accuracy at the LLOQ was within 80%-120%, while for all other non-zero calibrators, accuracy was within 85%-115% of the nominal concentration. Overall, precision was <14%, demonstrating high reproducibility (Table 4).

As discussed earlier, the Zeno trap provides added sensitivity enhancement through improvements in duty cycle. The cumulative gain from the use of the Zeno trap and summation of highly abundant fragment ions enhances overall assay sensitivity (Figure 7).

Table 3. Accuracy and precision values per concentration level for Zeno MRM^{HR}. Excellent accuracy and precision was achieved using the Zeno trap.

P05413_LILTLTHGTAVC[CAM]TR y12			P02741_ESDTSYVSLK y6			P63165_FLFEGQR y5		
Concentration (ng/mL)	%CV	Accuracy (%)	Concentration (ng/mL)	%CV	Accuracy (%)	Concentration (ng/mL)	%CV	Accuracy (%)
1177.33	1.78	100.43	*1842.46*	*1.66*	*97.90*	*3103.01*	*5.27*	*99.24*
412.06	3.20	*102.60*	*644.86*	*5.54*	*106.65*	*1086.05*	*2.87*	*101.85*
144.22	*5.22*	*91.90*	*225.70*	*3.61*	*99.43*	*380.12*	*3.60*	*104.36*
50.48	*11.05*	*91.71*	*78.99*	*3.92*	*96.48*	*133.04*	*12.48*	*92.80*
17.67	*0.70*	*98.30*	*27.65*	*8.66*	*99.54*	*46.56*	*8.27*	*87.47*
6.18	*9.38*	*102.62*	*N/A*	*N/A*	*N/A*	*16.30*	*14.75*	*114.28*
2.16	*13.00*	*112.44*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*

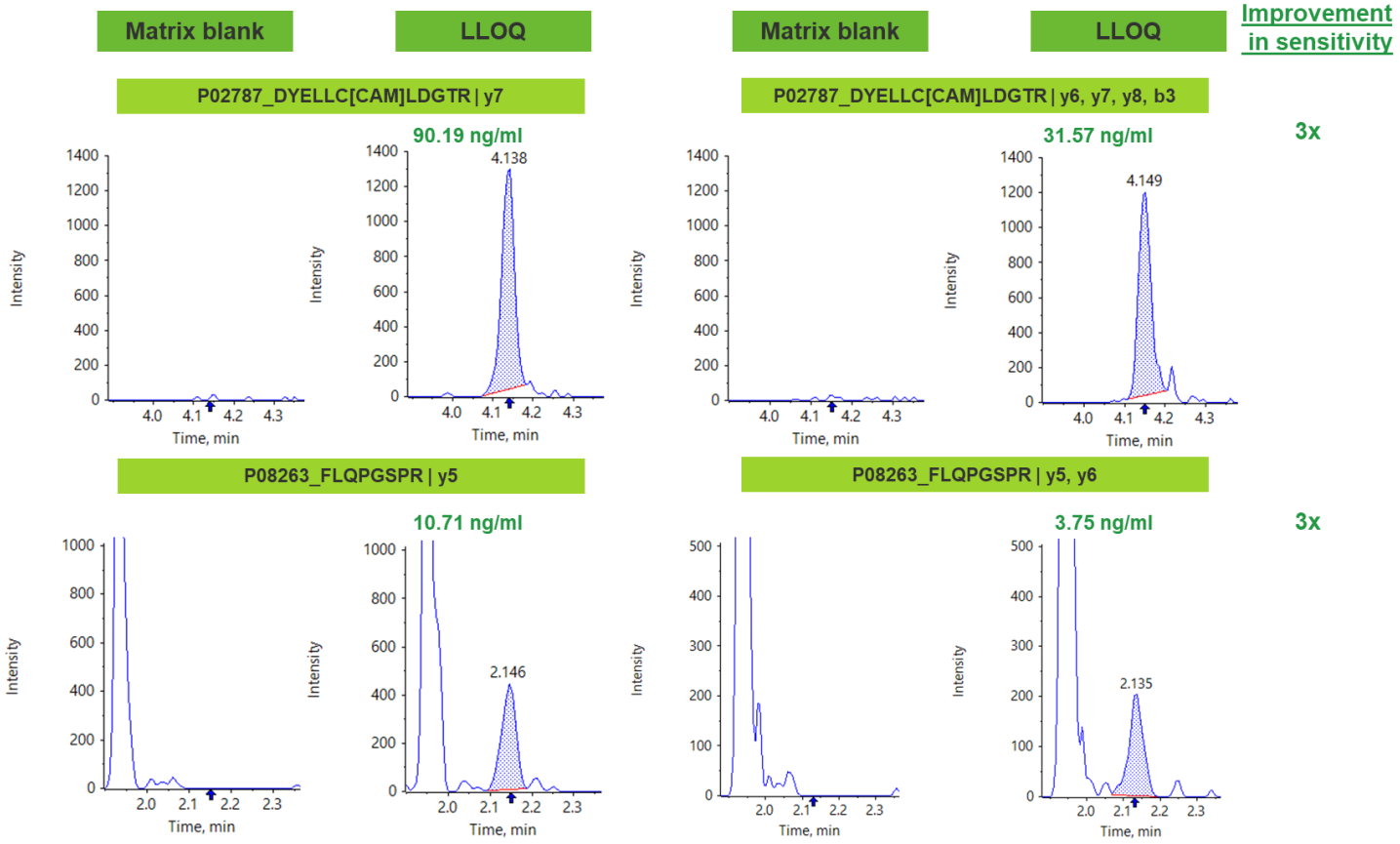

Figure 5. Summation of multiple fragment ions enhances assay sensitivity. A 3-fold improvement in LLOQ was achieved with quantification using summation of multiple dominant fragment ions.

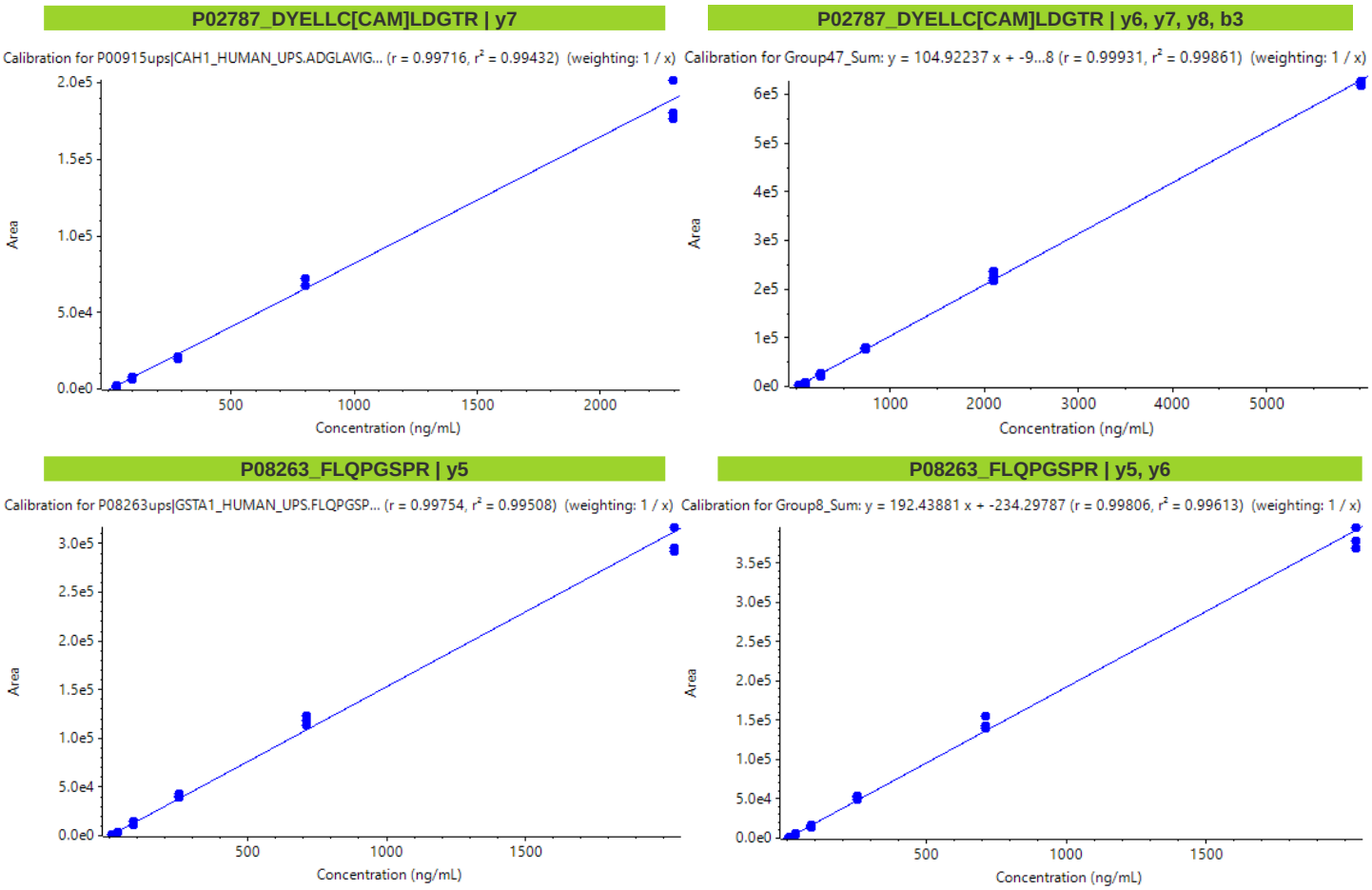

Figure 6. Strong linearity was achieved for all quantification using single fragment ion and summation of multiple fragment ions.

Table 4. Accuracy and precision values per concentration level. Excellent accuracy and precision was achieved for quantification using single fragment ion and summation of multiple fragment ions on the ZenoTOF 7600 system.

P02787_DYELLC[CAM]LDGTR y7			P02787_DYELLC[CAM]LDGTR y6, y7 , y8, b3		P08263_FLQPGSPR y5			P08263_FLQPGSPR y5, y6	
Concentration (ng/mL)	%CV	Accuracy (%)	%CV	Accuracy (%)	Concentration (ng/mL)	%CV	Accuracy (%)	%CV	Accuracy (%)
6010.25	1.19	99.07	*0.68*	*99.07*	*2038.56*	*1.75*	*95.78*	*3.44*	*97.21*
2103.59	3.50	*102.49*	*4.20*	*102.80*	*713.50*	*2.62*	*109.18*	*5.27*	*106.37*
736.25	*4.89*	*101.16*	*1.01*	*103.45*	*249.72*	*3.60*	*108.86*	*5.37*	*108.19*
257.69	*5.40*	*98.28*	*6.90*	*91.37*	*87.40*	*3.01*	*100.79*	*13.87*	*91.93*
90.19	*11.31*	*98.99*	*13.70*	*87.29*	*30.59*	*5.04*	*96.47*	*8.03*	*97.24*
31.57	*N/A*	*N/A*	*8.14*	*116.02*	*10.71*	*4.51*	*88.92*	*4.77*	*85.54*
N/A	*N/A*	*N/A*	*N/A*	*N/A*	*3.75*	*N/A*	*N/A*	*4.37*	*113.53*

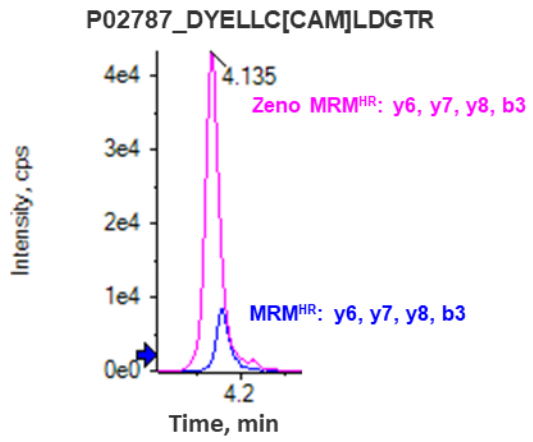

Figure 7. Cumulative gain in sensitivity from Zeno trap and summation of multiple abundant fragment ions. Summed XICs of multiple fragment ions for both Zeno MRM^{HR} and MRM^{HR} are displayed.

Conclusions

- An average of 5-fold in LLOQ improvement was achieved for peptide quantification in this sample set using the Zeno trap, which enhances the duty cycle through the accumulation of ions during each TOF pulse
- Improved LLOQs were reached by summing of multiple highly abundant fragment ions along with the availability of TOF MS/MS data, resulting in a 3-fold enhancement in LLOQ
- A highly accurate and reproducible quantitative workflow for peptides was demonstrated using the ZenoTOF 7600 system

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Large-scale, targeted, peptide quantification of 804 peptides with high reproducibility, using Zeno MS/MS

Using a 20-minute microflow gradient with a Scheduled MRM^{HR} workflow on the ZenoTOF 7600 system

Christie Hunter
SCIEX, USA

There are many powerful workflows available for proteomics research on today's mass spectrometry systems, depending on project goals. They cover a wide range that includes fully untargeted data dependent acquisition approaches for protein identification, comprehensive data independent acquisition strategies for large scale quantification, and also fully targeted quantitative assays for the highest specificity and sensitivity. This last class of assay has been typically performed on triple quadrupole or QTRAP systems because of their very high sensitivity and speed.

The ZenoTOF 7600 system is a QTOF system that can collect high-resolution, high mass accuracy, full-scan MS and MS/MS data. With Zeno trap technology, the system also demonstrates

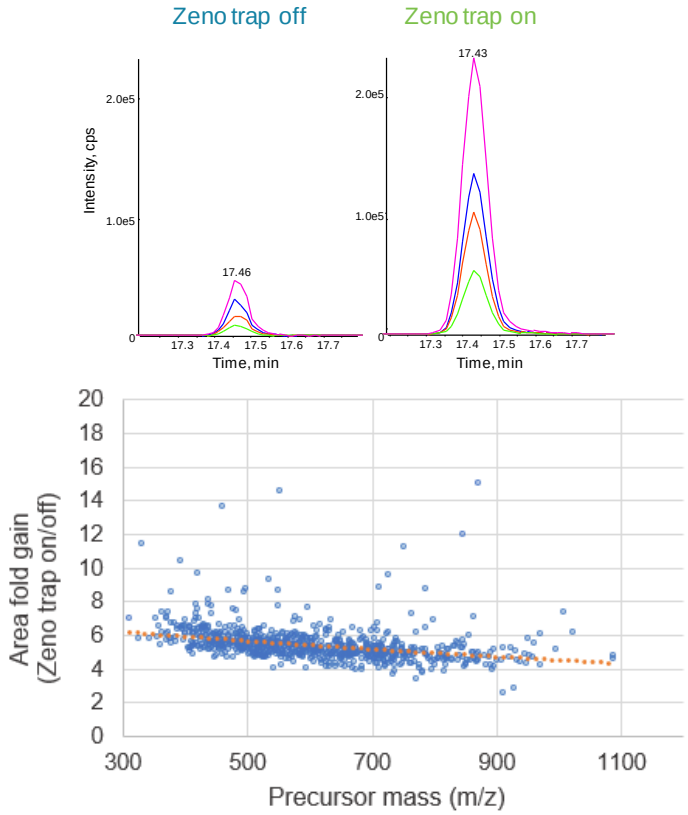

Figure 1. Significant gains in peptide area with Zeno trap activated. (Top) Example data of the sensitivity increases observed when the Zeno trap is activated are shown for ANT3.PFLVFIR, with a ~6x gain in peak area. (Bottom) A summary of the observed sensitivity gains for all 804 peptides is shown, plotted according to precursor mass. The average gain is 5.6-fold.

very high sensitivity MS/MS data. The Zeno trap provides significant increases in MS/MS signal: ~5-fold increase for the higher m/z fragment ions that are typically monitored for peptides (Figure 1). Here, a large-scale targeted assay for peptides in human plasma was developed to explore the quantitative capability of Zeno MS/MS on the ZenoTOF 7600 system. Using the PQ500 kit (Biognosys), an MRM^{HR} assay for 804 peptides was run in human plasma, and the reproducibility and sensitivity of the assay were characterized.

Key features of the ZenoTOF 7600 system for protein ID

- The ZenoTOF 7600 system delivers a 4 to 25-fold gain in MS/MS sensitivity across the entire m/z range, using the Zeno trap technology[1]
  - Zeno MS/MS acquired at 10 msec accumulation time with ≥30000 resolution and high mass accuracy
- For peptide quantification, peak area gains of ~5.6-fold are typical using Zeno MS/MS
- Excellent quantitative reproducibility and sensitivity was observed for 804 peptides in human plasma, in a single acquisition method, with a 20 min microflow gradient
- Microflow chromatography and the OptiFlow ion source enable fast gradients with excellent retention time reproducibility for large-scale, time-scheduled, targeted assays

Methods

Sample preparation: The PQ500 kit (Biognosys), containing 804 heavy labeled tryptic peptides for human plasma proteins, was used to develop a targeted peptide quantification assay. Assay development was performed with ~40 fmol on column peptide concentration. The peptides were then diluted into 500 ng digested human plasma across a concentration range of 2 amol/µL – 40 fmol/µL. 1 µL injections were performed.

Chromatography: A Waters ACQUITY UPLC M-class system was used in a trap-elute workflow. A Phenomenex Kinetex 2.6 µm XB-C18 100A, 150 x 0.3 mm LC column was used (P/N 00F-4496-AC) with a Phenomenex micro trap (P/N 05N-4252-AC). The column temperature was 30 ºC. Mobile phase A was water with 0.1% formic acid. Mobile phase B was acetonitrile with 0.1% formic acid. Samples were trapped at 10 µL/min for 3 min at 100% mobile phase A. A 20 minute linear gradient was used from 3-32% B using a flow rate of 5 µL/min.

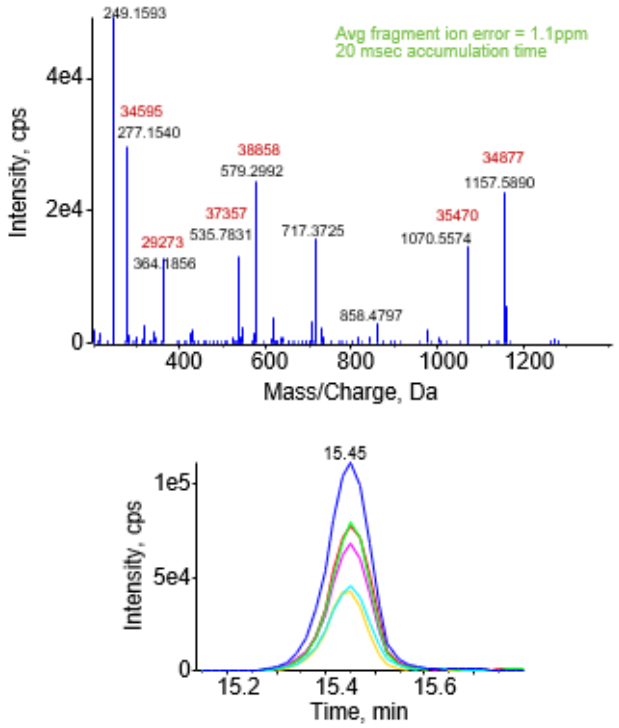

Figure 2. The MRM^{HR} workflow. In this QTOF workflow, the acquisition is set on the Q1 of a target peptide m/z, the peptide is fragmented in the collision cell and the full scan TOF MS/MS is acquired (top). This is repeated each cycle for the duration of the elution of the peptide from the column. After data acquisition, extracted ion chromatograms (XICs) on multiple sequence specific fragment ions are generated using the data processing software. Now, processing for quantification is very similar to how one would handle MRM data acquired on a triple quadrupole or QTRAP System. Multiple target peptides can be monitored, and these can be scheduled in time to allow a larger number of peptides to be analyzed in a single acquisition. With the ZenoTOF 7600 system, the MS/MS is acquired with ≥30000 resolution for the larger fragment ions and with very good mass accuracy.

Mass spectrometry: A SCIEX ZenoTOF 7600 system equipped with the OptiFlow source was used with both the microflow probe and ESI calibration probe.[4] Source conditions were as follows: CUR: 30 psi, GS1: 10 psi, GS2: 25 psi, IS: 5000 V, TEM: 200° C. MRM^{HR} methods were constructed with TOF MS accumulation time of 100 msec. After optimization of charge states, fragment ions, and determination of the retention times for all peptides, they were analyzed in a single method using retention time scheduling. The final method was tested with both the Zeno trap on and the Zeno trap off, and the final concentration curve data was generated with the Zeno trap activated. A minimum accumulation time of 10 msec and a target cycle time of 1 sec was set. A retention time window of 40 sec was used.

Data processing: Method optimization data was processed in Skyline (daily version). Peptide information was then exported and imported into SCIEX OS software in order to process the calibration curves in Analytics. Multiple fragment ions were summed for each peptide during computation of the calibration curves. Lower limit of detection (LLOD) was determined to be the peak detected above the blank (because of the very low noise with this approach, it is difficult to compute a signal/noise ratio). The lower limit of quantification (LLOQ) was determined using standard bioanalytical guidelines, requiring a %CV < 20% and an accuracy of ±20%.

Zeno MS/MS provides significant gains in peptide area

The Zeno trap technology on the ZenoTOF 7600 system provides significant gains in peptide fragment signal when activated, as it increases the duty cycle to ≥90% across the entire mass range in the orthogonal pulsing region of the instrument. This results in gains of 4-25 fold in MS/MS sensitivity.[1] These duty cycle enhancements are accomplished by trapping ions in the Zeno trap region of the collision cell, then releasing them such that all ions arrive as a condensed packet at the same time in the TOF accelerator region.

An example of the gain in peptide signal when Zeno trap is activated is shown in Figure 1. Typically, when building targeted peptide assays, larger m/z fragment ions are used for specificity. With Zeno MS/MS, theoretical sensitivity gains for m/z ions above mass 300 are in the 4-6 x range.[1] A back-to-back experiment was performed using the final assay on the 804 peptides with Zeno trap off vs. Zeno trap on and the gain in MS/MS sensitivity was measured (Figure 1, bottom). The sensitivity gains across the peptides monitored were as expected, with an average peak area gain of ~5-fold.

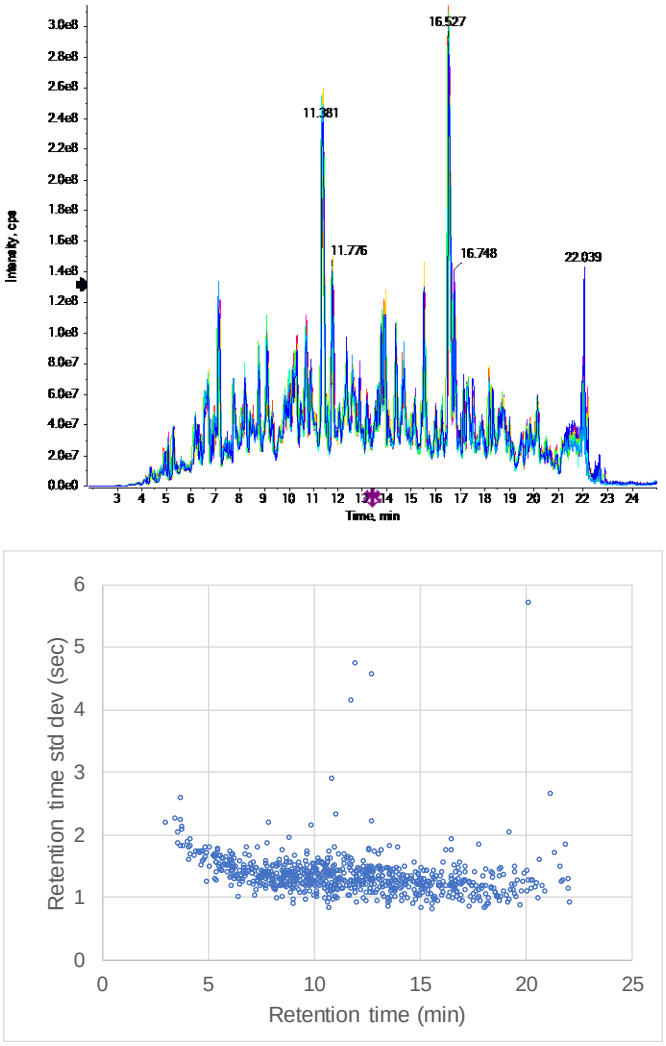

Figure 3. Chromatographic reproducibility across 10 replicates in plasma. (Top) Very good chromatographic reproducibility was observed throughout the method development and final data generation, with an average %RSD of 0.23 across the 804 peptides and all 10 replicates. (Bottom) Plot of observed retention time standard deviation across run vs. the observed retention time showing the majority of peaks shift less than a few seconds across 10 replicates.

Good chromatographic reproducibility

In order to perform such a highly multiplexed assay using a fast gradient of just 20 minutes, it is essential to have highly reproducible chromatography and good peak shape such that very narrow time scheduling windows can be used (Figure 3). Using the 15 cm Kinetex column in trap elute mode, the observed average peak width was 17.7 sec. The retention time standard deviation was mostly <2 sec across the gradient using the Waters ACQUITY UPLC M-class system (Figure 3, bottom).

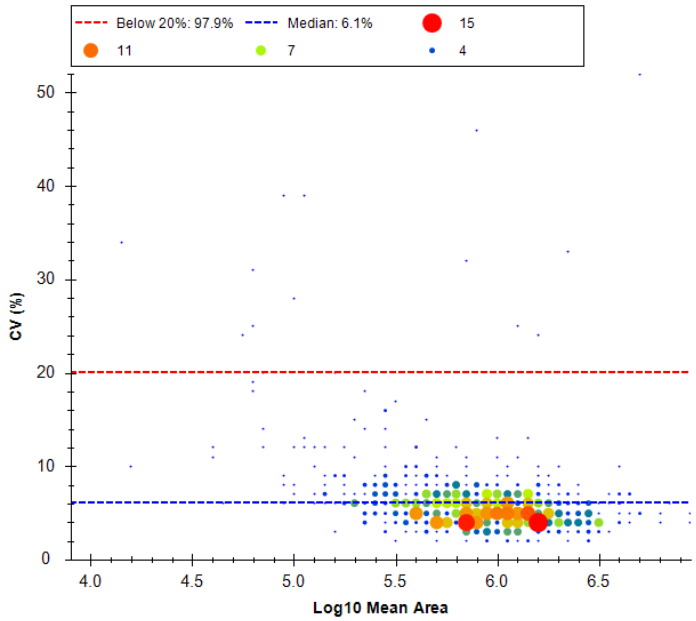

Figure 4. High reproducibility of targeted peptide quantification. Ten replicate injections of the PQ500 peptide (40 fmol on column) dosed into plasma (500 ng on column) were performed and the peptide peak area reproducibility was determined. The size and color of the dot indicate a larger number of peptides at that point in the reproducibility plot, as indicated by the legend at the top. The median CV is 6.1% and 97.9% of the peptides have ≤20% CV highlighting the very good reproducibility of the assay.

This enabled use of 40 sec wide time scheduled retention time windows.

Excellent peak area reproducibility

Once the final time-scheduled MRM^{HR} assay was established for all 804 peptides, the reproducibility of peptide quantification was assessed. From the same ten replicate injections in plasma, the peak areas of the fragment ions were extracted and summed. Figure 4 highlights the reproducibility observed across the 10 injections with the %CV plotted for each peptide for all 804 peptides dosed into digested plasma. All the individual peptides are plotted based on their area on the x-axis and their reproducibility on the y-axis (note dots plotted on matrix). The median %CV was 6.1% indicating extremely high reproducibility of this very highly multiplexed assay of 804 heavy peptides. 97.9% of peptides had %CV less than 20%.

Concentration (amol on column)	% CV	Accuracy (%)	Area 1	Area 2	Area 3
82.2	19.6	90.2	*6.91E+01*	*9.05E+01*	*6.28E+01*
246.6	14.3	*94.8*	*2.10E+02*	*2.20E+02*	*2.72E+02*
739.8	4.5	*98.4*	*7.13E+02*	*7.66E+02*	*7.06E+02*
2219.3	5	*113.6*	*2.42E+03*	*2.66E+03*	*2.49E+03*
6657.8	*5.24*	*106.5*	*7.49E+03*	*7.03E+03*	*6.76E+03*
19973.3	*12.22*	*96.5*	*2.06E+04*	*1.66E+04*	*2.07E+04*

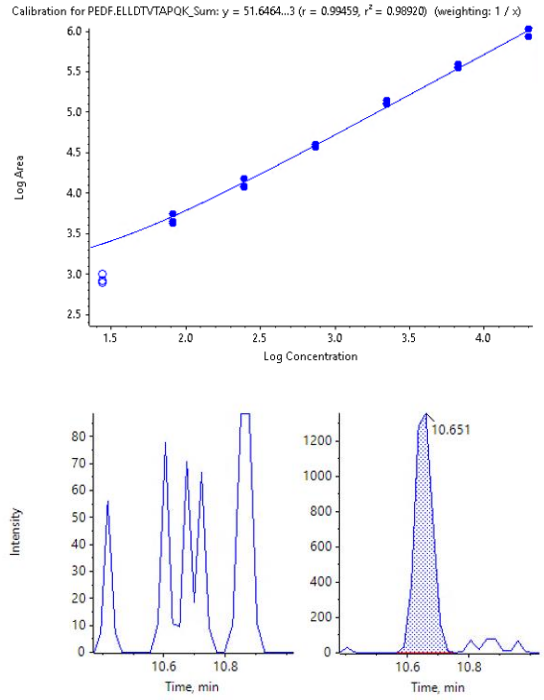

Figure 5. Concentration curve for PEDF.ELLDTVTAPQK. Concentration curve for the ELLDTVTAPQK in 500 ng plasma showed good reproducibility and linearity from 82.2 amol on column to the highest measured point at 19.9 fmol on column (r^2 = 0.995). The peptide signal (sum of 4 fragment ions) for the lower limit of quantification (LLOQ) is shown bottom right and has good S/N and passes bioanalytical critieria for an LLOQ. The signal for the blank injection is on the bottom left. The statistics for the curve are shown in the table on top.

Peptide concentration curves

Finally, concentration curves were generated for the peptide mixture from 2 amol/µL – 40 fmol/µL in 500 ng/ µL of digested plasma and 1 µL injections were performed in triplicate. Because the concentration of the stock solution did not allow exploration of the high end of the system's linear dynamic range, the focus was on sensitivity. All 804 peptides were analyzed in a single acquisition method. XICs for the selected fragment ions were generated and then summed for each peptide using Analytics in SCIEX OS software. The peptide areas were then evaluated across the concentration range for signal/noise, reproducibility and accuracy of the calibration curve.

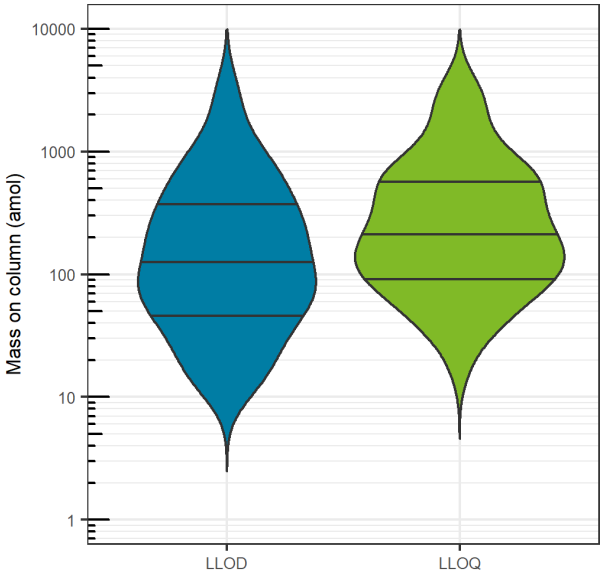

Figure 6. Summary of observed quantification results. The LLOQ and LLOD values were determined for the heavy labeled peptides in plasma. The median LLOQ was found to be 193 amol on column (green) and the median LLOD was 114 amol on column (blue).

Shown in Figure 5 is an example of the data obtained. Very good linearity was observed across the concentration range interrogated. The Zeno trap is activated for the lower part of the concentration curve when the peptide intensity drops below the Zeno trap threshold.[5] Statistics for the calibration curve were evaluated to ensure reproducibility and accuracy across the triplicate injections. To determine the lower limit of quantification (LLOQ), standard bioanalytical rules were used, ensuring the signal had <20% CV and 90 – 120% accuracy. In particular, for the peptide ELLDTVTAPQK from PEDF, an LLOQ of 82.2 amol on column was observed, with 19.2% CV and 90.2% accuracy.

Lower limits of detection (LLOD) were also evaluated. As the noise in the high resolution extracted data is so low, it is difficult to compute an accurate signal/noise value. For determination of the LLOD, the concentration below the LLOQ was evaluated to determine if there was measurable signal above the previous concentration. If there was measurable signal, the LLOD was recorded. If not, the LLOD was set at the LLOQ. The LLOQ and LLOD values for the large set of peptides are summarized in Figure 6.

Conclusions

Here, the quantitative performance of the ZenoTOF 7600 system was evaluated using the Scheduled MRM^{HR} workflow and Zeno MS/MS. The sample tested was the PQ500 sample of 804 heavy synthetic peptides in a digested human plasma matrix.

- 804 heavy labeled peptides were analyzed using a 20 min microflow gradient and a Scheduled MRM^{HR} workflow
  - Full scan MS/MS spectra were acquired with a minimum accumulation time of 10 msec and ≥30000 resolution for higher mass peptide fragments
- Zeno MS/MS provides a 5.6-fold increase in peptide area when comparing the same method acquired with Zeno trap off vs. Zeno trap on
- The quantitative reproducibility of the time-scheduled MRM^{HR} assay was extremely good, with a median %CV of 6.1 across 10 replicates in plasma digest
- Concentration curves were generated to evaluate the sensitivity of the system for this targeted workflow. The median LLOQ for the peptides in plasma was 207 amol on column.

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High sensitivity MRM workflow for signature peptide quantification

Featuring the SCIEX Triple Quad™ 7500 LC-MS/MS System – QTRAP® Ready, powered by SCIEX OS Software

Ian Moore[1], Lei Xiong[2]
[1]*SCIEX, Canada;* [2]*SCIEX, USA*

Quantification of peptide/protein therapeutics in biological matrices is crucial for therapeutic development. Serving as an orthogonal technology to the traditional ligand binding assays (LBAs), LC-MS has been routinely adopted for quantitative measurement of protein levels in bioanalytical laboratories. The capability of a triple quadrupole system to quantify low concentration analytes from small sample volumes, in line with current LBAs, has been a main stay of analytical analysis in this area. However, further sensitivity, improved %CV and linear dynamic range (LDR) operating down to lower concentrations would greatly benefit this analytical methodology.

While different MS approaches have been investigated by researchers, quantification using peptides as surrogates (bottom-up proteomic workflows) using targeted LC-MRM strategies remains the most common. It offers not only high sensitivity, but also a wide linear dynamic range (LDR) combined with high reproducibility to provide reliable quantitative measurements.

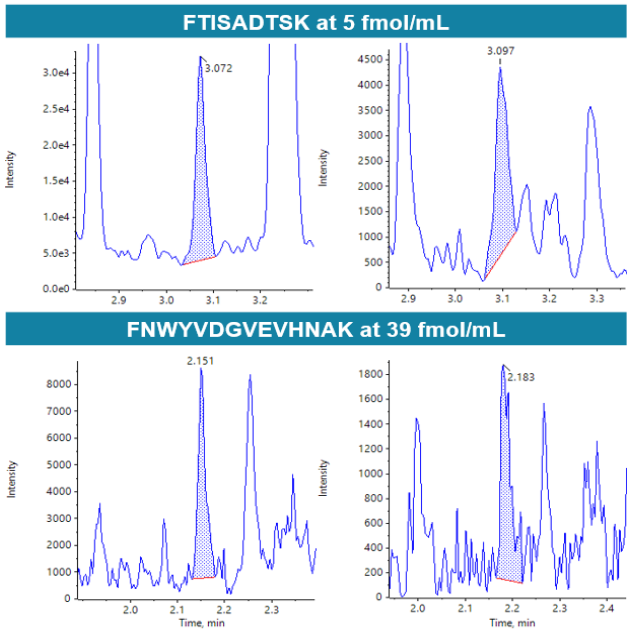

Figure 1. Sensitivity gains for peptide quantification. MRM XIC comparison between SCIEX 7500 System (left) and QTRAP 6500+ LC-MS/MS System (right) for mAb signature peptides at 5 or 39 fmol/mL in rat plasma.

Here, a SCIEX 7500 System was used to quantify a series of surrogate peptides in rat plasma. Multiple hardware improvements on the ion source and the front end of the mass analyzer significantly boost the systems sensitivity and low-level %CV values. Ultra-low LLOQs, ranging from 5 to 39 fmol/mL are achieved. The assay shows high reproducibility, precision, accuracy, and linearity, proving the robustness and performance of the developed method.

Key features of peptide quantification workflows

- Hardware improvements on the SCIEX 7500 System provide significant gains in sensitivity for peptide quantification: the OptiFlow® Pro Ion Source with E Lens™ Technology provides improvements in ion generation and the D Jet™ Ion Guide improves ion sampling[1]
- An average of 3-fold improvement in S/N was observed when analyzing surrogate peptides in biological matrices with high reproducibility, accuracy, and linearity
- SCIEX OS Software—an easy to use, compliance ready and single platform for acquisition, processing and data management

Methods

Sample preparation: Plasma proteins were precipitated with cold methanol. Upon centrifugation, supernatant was discarded. The pellet was solubilized in 200 mM ammonium bicarbonate in 10/90 (v/v) methanol/water. Digestion was performed using trypsin. After one hour at 60°C, the solution was acidified by adding formic acid.[1] The digested plasma was diluted by 200x using 5:1:94 (v/v/v) acetonitrile/formic acid/water. Synthesized peptides (Table 1) were spiked into the digested plasma solution and followed by serial dilution in matrix. Final injection volume was 10 µL.

Table 1. List of peptide targets.

Peptide Sequence	Description
FTISADTSK	*trastuzumab CDR region peptide*
FNWYVDGVEVHNAK	*conserved sequence in human immunoglobulin G (IgG)*
*AGLIVAEGVTK**	*synthetic peptide with C terminal K heavy isotope labeled ($C^{13}N^{15}$)*
*LGLDFDSFR**	*synthetic peptide with C terminal R heavy isotope labeled ($C^{13}N^{15}$)*

LC-MS conditions: Samples were analyzed in triplicate by a SCIEX Triple Quad 7500 LC-MS/MS System – QTRAP Ready, coupled with an ExionLC system. The method details are summarized in Tables 2 and 3. The same sample set was also injected into a SCIEX Triple Quad 6500+ LC-MS/MS System, coupled with the same HPLC system, to characterize the performance difference between the two mass spectrometers. All MRM parameters are optimized on both mass spectrometers for accurate performance comparison

Data processing: Data are processed using Analytics in SCIEX OS Software 2.0.

Table 2. Chromatographic conditions.

Parameter	Value
Column	*Phenomenex bioZen Peptide XB-C18 50×2.1 mm; 2.6 µm*
Mobile Phase A	*Water with 0.1 % formic acid*
Mobile Phase B	*Acetonitrile with 0.1 % formic acid*
Flow Rate	*500 µL/min*
Column Temperature	*40 °C*
Injection Volume	*10 µL*

Table 3. Gradient conditions.

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]
0	*95*	*5*
5.5	*75*	*40*
5.6	*10*	*90*
6.0	*10*	*90*
6.1	*95*	*5*
7.0	*95*	*5*

Table 4. MS parameters on the SCIEX 7500 System.

Parameter	Value	Parameter	Value
Curtain gas	*42 psi*	*Source temperature*	450 °C
Ion source gas 1	*50 psi*	*Ion source gas 2*	70 *psi*
CAD gas	*9 psi*	*Ion spray voltage*	1500 V

Signature peptide quantification

The SCIEX 7500 System integrates innovations that provide improvements in both ion generation and ion sampling. The OptiFlow Pro Ion Source with E Lens Technology provides improvement in ion generation and the D Jet Ion Guide efficiently captures and transmits the ions in the high gas flow behind the orifice plate.

To identify the sensitivity improvements provided by these innovations, the same peptide sample sets were analyzed on both the SCIEX 7500 System and the SCIEX 6500+ System. On average, a 6-fold difference in peak area and a 3-fold difference in S/N were observed (Figure 1).

The peptide serial dilution samples were injected onto a SCIEX 7500 System to evaluate the overall quantification performance. As shown in Figures 2, 3 and 4, the LLOQs of the target peptides range from 5 to 40 fmol/mL, with LDR up to 4 orders of magnitude without internal standard normalization. For all quantified samples, the %CV was within 10% and the accuracy was within 94-106%.

Conclusions

- A highly sensitive peptide quantification workflow using the SCIEX Triple Quad 7500 LC-MS/MS System – QTRAP Ready has been developed
- When combining the OptiFlow Pro Ion Source with E Lens Technology and D Jet Ion Guide, an average 3-fold improvement in sensitivity over the previous generation of instrumentation was observed when quantifying surrogate peptides in biological matrix

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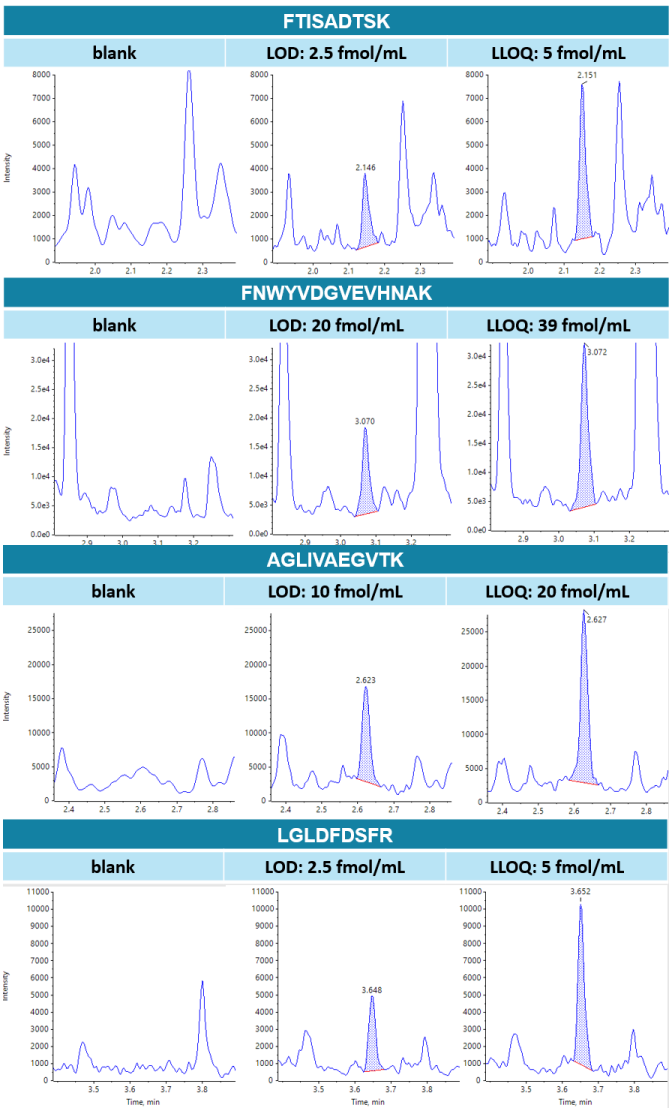

Figure 2. XICs of target peptide quantification in rat plasma. From left to right: in matrix blank, at LODs and LLOQs.

Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
FTISADTSK	5.00	3 of 3	4.868e0	3.894e-1	8.00	97.36
FTISADTSK	10.00	3 of 3	9.419e0	1.550e-1	1.65	94.19
FTISADTSK	20.00	3 of 3	1.951e1	6.531e-1	3.35	97.56
FTISADTSK	39.00	3 of 3	3.967e1	5.048e-1	1.27	101.73
FTISADTSK	78.00	3 of 3	7.901e1	3.394e0	4.30	101.29
FTISADTSK	156.00	3 of 3	1.601e2	3.603e0	2.25	102.66
FTISADTSK	313.00	3 of 3	3.206e2	5.314e0	1.66	102.44
FTISADTSK	625.00	3 of 3	6.381e2	2.658e1	4.17	102.10
FTISADTSK	1250.00	3 of 3	1.272e3	5.123e1	4.03	101.75
FTISADTSK	2500.00	3 of 3	2.497e3	5.252e1	2.10	99.89
FTISADTSK	5000.00	3 of 3	4.930e3	1.015e2	2.06	98.60
FTISADTSK	10000.00	3 of 3	1.016e4	1.551e2	1.53	101.61
FTISADTSK	20000.00	3 of 3	1.967e4	3.129e2	1.59	98.34
FTISADTSK	40000.00	3 of 3	4.020e4	9.680e2	2.41	100.49

Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
FNWYVDGVEVHNAK	39.00	3 of 3	3.943e1	2.157e0	5.47	101.10
FNWYVDGVEVHNAK	78.00	3 of 3	7.691e1	2.047e0	2.66	98.60
FNWYVDGVEVHNAK	156.00	3 of 3	1.536e2	5.674e0	3.69	98.45
FNWYVDGVEVHNAK	313.00	3 of 3	3.104e2	2.079e1	6.70	99.16
FNWYVDGVEVHNAK	625.00	3 of 3	6.216e2	1.164e1	1.87	99.45
FNWYVDGVEVHNAK	1250.00	3 of 3	1.244e3	1.977e1	1.59	99.49
FNWYVDGVEVHNAK	2500.00	3 of 3	2.594e3	3.973e1	1.53	103.76
FNWYVDGVEVHNAK	5000.00	3 of 3	4.949e3	1.497e1	0.30	98.98
FNWYVDGVEVHNAK	10000.00	3 of 3	1.025e4	1.141e2	1.11	102.46
FNWYVDGVEVHNAK	20000.00	3 of 3	1.989e4	2.022e2	1.02	99.45
FNWYVDGVEVHNAK	40000.00	3 of 3	3.943e4	8.714e2	2.21	98.58
FNWYVDGVEVHNAK	80000.00	3 of 3	8.041e4	1.874e3	2.33	100.51

Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
AGLIVAEGVTK	20.00	3 of 3	1.905e1	1.436e0	7.54	95.27
AGLIVAEGVTK	39.00	3 of 3	4.116e1	4.313e-1	1.05	105.53
AGLIVAEGVTK	78.00	3 of 3	8.045e1	1.473e0	1.83	103.15
AGLIVAEGVTK	156.00	3 of 3	1.648e2	6.464e0	3.92	105.62
AGLIVAEGVTK	313.00	3 of 3	3.248e2	1.000e1	3.08	103.78
AGLIVAEGVTK	625.00	3 of 3	6.429e2	3.048e1	4.74	102.86
AGLIVAEGVTK	1250.00	3 of 3	1.276e3	4.018e1	3.15	102.08
AGLIVAEGVTK	2500.00	3 of 3	2.556e3	1.313e2	5.14	102.23
AGLIVAEGVTK	5000.00	3 of 3	4.970e3	5.112e1	1.03	99.40
AGLIVAEGVTK	10000.00	3 of 3	9.883e3	1.213e2	1.23	98.83
AGLIVAEGVTK	20000.00	3 of 3	1.886e4	4.672e2	2.48	94.32
AGLIVAEGVTK	40000.00	3 of 3	3.643e4	1.269e3	3.48	91.07
AGLIVAEGVTK	80000.00	3 of 3	7.669e4	2.622e3	3.42	95.86

Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
LGLDFDSFR	5.00	3 of 3	5.058e0	4.337e-1	8.58	101.16
LGLDFDSFR	10.00	3 of 3	1.010e1	3.713e-1	3.68	100.96
LGLDFDSFR	20.00	3 of 3	1.890e1	9.260e-1	4.90	94.51
LGLDFDSFR	39.00	3 of 3	3.883e1	1.325e0	3.41	99.58
LGLDFDSFR	78.00	3 of 3	7.613e1	7.507e-1	0.99	97.60
LGLDFDSFR	156.00	3 of 3	1.530e2	3.346e0	2.19	98.10
LGLDFDSFR	313.00	3 of 3	3.138e2	9.416e0	3.00	100.25
LGLDFDSFR	625.00	3 of 3	6.195e2	9.281e0	1.50	99.12
LGLDFDSFR	1250.00	3 of 3	1.264e3	4.096e1	3.24	101.09
LGLDFDSFR	2500.00	3 of 3	2.506e3	1.276e2	5.09	100.25
LGLDFDSFR	5000.00	3 of 3	4.960e3	8.268e1	1.67	99.21
LGLDFDSFR	10000.00	3 of 3	1.025e4	3.253e2	3.17	102.53
LGLDFDSFR	20000.00	3 of 3	1.998e4	1.481e2	0.74	99.89
LGLDFDSFR	40000.00	3 of 3	4.230e4	1.816e3	4.29	105.76

Figure 3. Quantification result summaries.

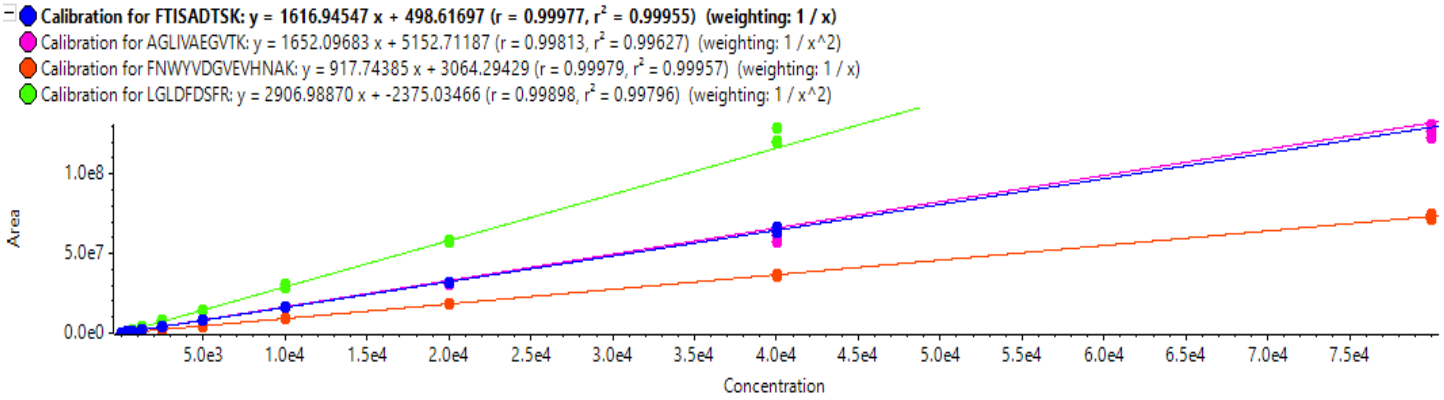

Figure 4. Calibration curves of target peptides with R^2 from 0.996 to 0.999.

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Reproducible targeted peptide profiling using highly multiplexed MRM assays

Using SCIEX Triple Quad™ 7500 LC-MS/MS System – QTRAP® Ready

Christie L Hunter
SCIEX, USA

The utility of Multiple Reaction Monitoring (MRM) for targeted protein quantification and biomarker verification/validation studies on triple quadrupole-based MS systems is driven by the renowned sensitivity and selectivity attributes the technique delivers. As more extensive protein panels need to be monitored in a targeted way across multiple samples, higher multiplexing of MRM transitions is becoming essential for throughput. This growing need to run higher numbers of samples in biomarker research projects is also driving a shift from longer nanoflow gradients to faster microflow chromatography runs.

But quantitative robustness remains key, to ensure both large and small biological changes are accurately measured across large sample cohorts. Biological matrices also have a very wide range of protein abundance, which requires the LC-MS/MS system to have high sensitivity as well as wide linear dynamic range.

Here a simulated study was performed to test the performance of the SCIEX Triple Quad™ 7500 LC-MS/MS System – QTRAP Ready for large scale targeted peptide quantification. Microflow chromatography was used to explore both longer and short gradients. Methods with up to 4000 MRM transitions were run and the quantitative reproducibility was evaluated.

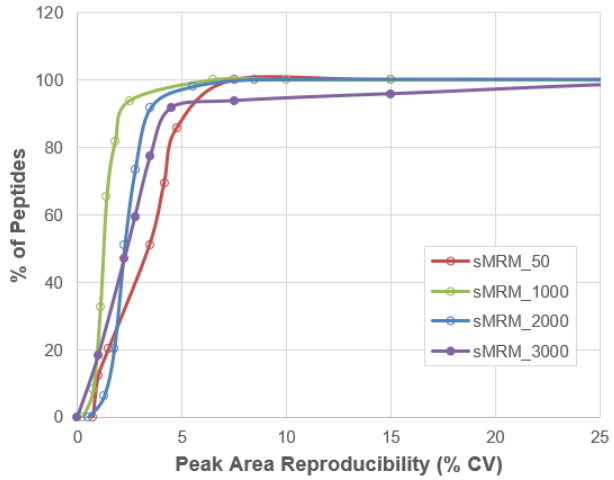

Figure 1. Excellent quantitative accuracy of higher multiplexing in a 5 minute gradient. The effect of higher numbers of MRM transitions on the reproducibility of 50 peptide MRMs from 3 protein digests spiked in complex matrix (1 µg on column) across 10 replicate injections on the SCIEX 7500 System was assessed using microflow chromatography. High reproducibility of up to 3000 MRMs was achieved using Scheduled MRM Algorithm, with 90% of peptides having %CV lower than 4.5%.

Key Features of the SCIEX 7500 System for highly multiplexed peptide MRM assays

- SCIEX Triple Quad™ 7500 LC-MS/MS System – QTRAP Ready provides high sensitivity for peptide quantification, with improvements in ion generation due to the OptiFlow® Pro Ion Source with E Lens™ Technology and in ion sampling due to the D Jet™ Ion Guide[1]
- OptiFlow Pro Ion Source provides flexibility and ease of use, with interchangeable probes and electrodes that require minimal user optimization
- Switch to microflow chromatography in minutes
- SCIEX OS Software 2.0 automates MRM time scheduling with the intelligent Scheduled MRM™ Algorithm, allowing many more analytes to be analyzed in a single run
- The Scheduled MRM Algorithm uses peptide retention times to automatically compute an optimized acquisition method based on a few key parameters provided by the user[2]

Methods

Sample Preparation: A simple mixture of PepCalMix and digested beta-galactosidase (SCIEX), and a digest of bovine serum albumin was prepared at 1 fmol/µL. Samples were prepared both in buffer and spiked into digested human K562 cell line sample.

Chromatography: Sample separation was performed in trap elute mode using the NanoLC™ 425 System operated at 5 µL/min.[3] A 5 and 30 minute linear gradient was used to separate the peptides, on a Phenomenex C18 column (Luna Omega Polar, 150 x 0.3mm, PHX P/N 00F-4760-AC). Column temperature was maintained at 30 ºC. A 6 µL injection was performed.

Mass Spectrometry: Data was acquired using the SCIEX Triple Quad 7500 LC-MS/MS System – QTRAP Ready, controlled by SCIEX OS Software. The OptiFlow Pro Ion Source was used, using the microflow probe and microflow E Lens Technology. Using the Scheduled MRM Algorithm, a series of methods with increasing numbers of MRMs were tested to assess assay reproducibility with increasing multiplexing.

Data Processing: All data was processed using Analytics in SCIEX OS Software using the AutoPeak Algorithm.

Good Chromatography is key for highest multiplexing

To analyze the increasing numbers of analytes in a single targeted MRM assay, high quality, highly reproducible chromatography is essential. One of the user inputs for the Scheduled MRM Algorithm is Retention Time Tolerance. Here, the peak width at base and the observed retention time variance per peptide was used to compute a retention time tolerance. Of course the tighter both of those LC attributes were per peptide, the more narrow the retention time tolerance was in the method. Using microflow chromatography, very high quality chromatography was achieved (Figure 2 and 5). The average retention time %RSD observed for the 10 to 15 replicates analyzed using each method was 0.13% and 0.2% for both the 5 and 30 min gradients. This means most peaks were shifting by less than 2 seconds across the replicates.

Shown in Figure 3 is a simulation of the MRM concurrency for increasing numbers of MRMs when running a 30 minute gradient (concurrency being the number of MRMs to be monitored at any point in time). Because the chromatography was very good, up to 4000 MRM transitions could be run in the single method and still maintain lower concurrency (top figure) as well as higher dwell times (bottom figure). The dwell times to be used in the

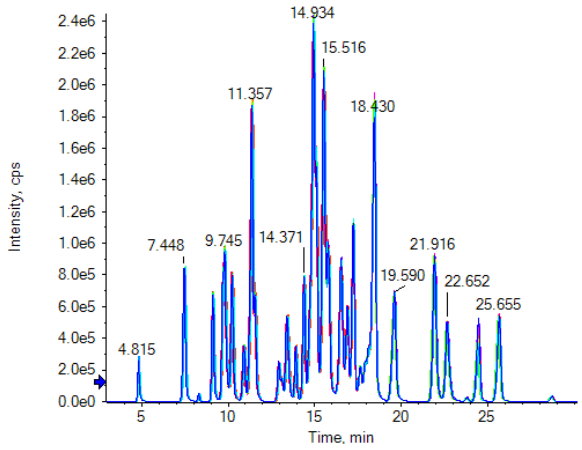

Figure 2. Chromatographic reproducibility for 30 min gradient runs. Using a 3 protein digest in buffer, very good reproducibility was achieved across the replicate analysis (n=15), both in peak area and in retention time stability.

method is computed and displayed in SCIEX OS Software in sMRM Summary to help with method development.[2] Keeping the dwell times above 5 or 10 msec ensures the quantitative data is of high quality especially for the low abundant precursors.

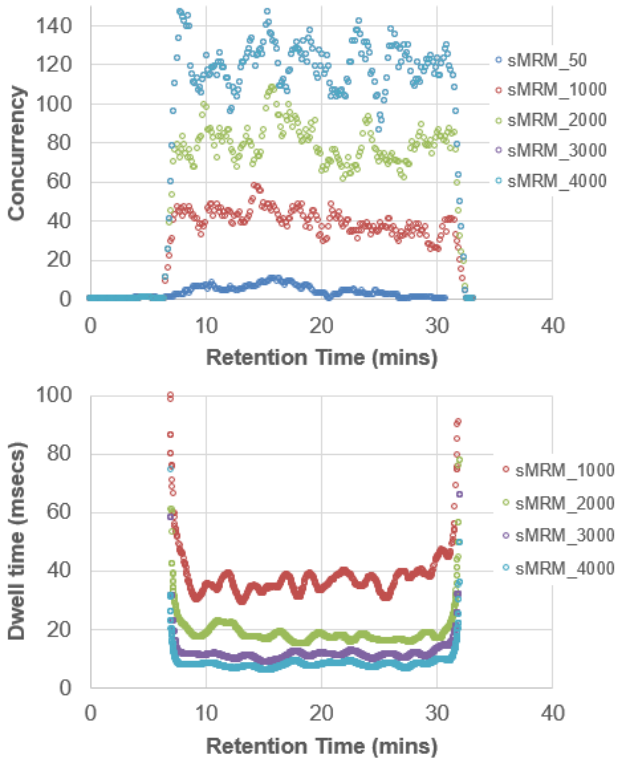

Figure 3. The Effects of MRM detection windows on Scheduled MRM Algorithm acquisition methods. For the 30 min LC gradient, increasing numbers of MRM transitions were added to the method and the MRM concurrency (top) and dwell time used (bottom) are plotted vs retention time. Because of the narrow retention time tolerances possible with the highly reproducible chromatography, sufficient dwell times were maintained for high quality quantification.

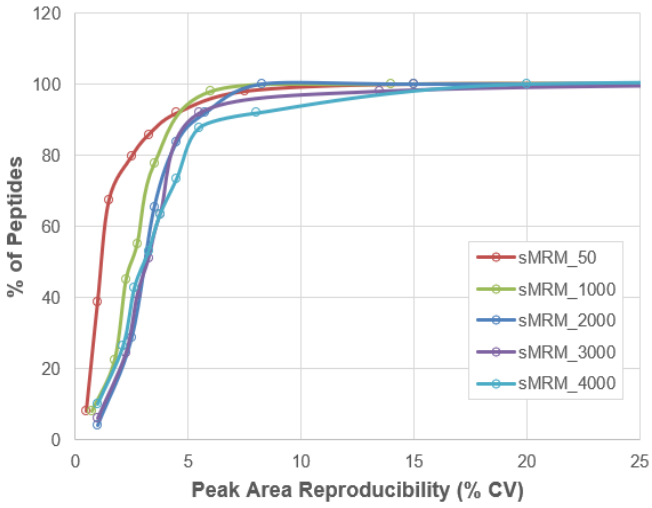

Figure 4. Assessing the quantitative accuracy of higher multiplexing using Scheduled MRM Algorithm. The effect of higher numbers of MRM transitions on the reproducibility of 50 peptide MRMs (3 protein digest in buffer) across 15 replicate injections on the SCIEX 7500 System was assessed using microflow chromatography (30 min gradient). High reproducibility was observed for each of the methods with up to 4000 MRMs per method, with ~85-90% of peptides showing %CV <5%.

Effect of scheduling on quantitative reproducibility

An experiment was designed to assess the effects of higher MRM multiplexing on analytical reproducibility (Figure 4) on the SCIEX Triple Quad 7500 LC-MS/MS System – QTRAP Ready. MRM transitions of 50 tryptic peptides were developed and tested for reproducibility (sMRM_50). Next, random MRM transitions and retention times were computed and added to the 50 real MRM transitions to create assays with 1000, 2000, 3000 and 4000 MRM transitions using the Scheduled MRM Algorithm. The reproducibility of the 50 real MRM transitions from each method across the replicate injections was used as a measure of the analytical reproducibility of each acquisition method, for peak area and retention times.

These assays were tested for reproducibility by measuring the peak areas and retention times for the 50 tryptic peptides across the 15 replicates for each of the assays (Figure 4). Plotting the cumulative %CV curves allows easy visualization of the impact of increasing numbers of MRMs per assay. For the 30 minute gradient across all of the assays, ~85-90% of peptides showed %CV of <5%. Table 1 highlights the average MRM peak area %CVs, the retention time reproducibility as well as the average dwell time per assay.

Table 1. Average reproducibility observed across the experiments.

	Avg peak area %CV	Avg retention time %RSD (min)	Avg dwell time (msec)
	30 minute gradient		
sMRM_50	*2.46*	*0.24*	*250.0*
sMRM_1000	*3.20*	*0.20*	*39.1*
sMRM_2000	*3.81*	*0.19*	*25.6*
sMRM_3000	*4.31*	*0.25*	*15.6*
sMRM_4000	*4.53*	*0.20*	*12.4*
	5 minute gradient		
sMRM_50	*4.01*	*0.11*	*148*
sMRM_1000	*1.94*	*0.13*	*12*
sMRM_2000	*2.72*	*0.10*	*4.4*
sMRM_3000	*3.81*	*0.17*	*3*

Enabling faster chromatography

In addition to using the Scheduled MRM Algorithm to increase multiplexing, it can also be used to increase the gradient speeds used in quantitative experiments. An experiment was performed using a 5 minute gradient, and again increasing numbers of MRM transitions were run in replicate. (Figure 1, Table 1). In this case, the 3 protein digest was spiked into digested human cell lysate to account for any matrix effects. Again, good data reproducibility was obtained with up to 3000 MRM transitions in a 5 minute gradient, with average peak area %CV below 5%. This high quality data was possible because of the sensitivity the

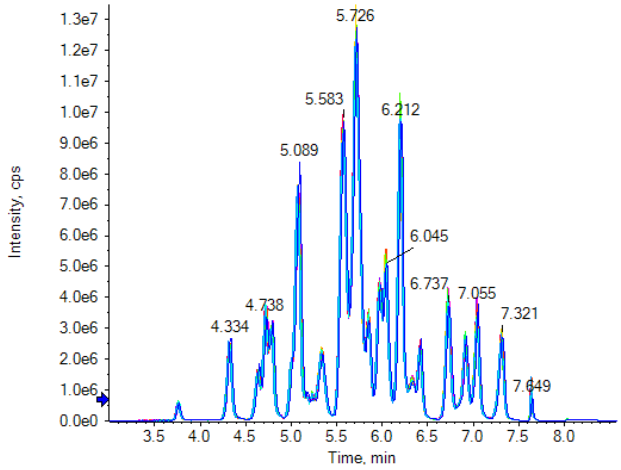

Figure 5. Chromatographic reproducibility for 5 min gradient runs. Again, very good reproducibility was achieved across the replicate analysis (n=10) for the 3 protein digest in complex matrix, both in peak area and in retention time stability.

SCIEX 7500 System and the reproducible chromatography that allowed the use of narrow retention time tolerances (Figure 5).

Powerful software for MRM data processing

SCIEX OS Software provides a comprehensive package for the acquisition and processing of peptide quantification data from large scale MRM experiments. As mentioned above, assay development is streamlined with the ability to view the impact of method settings on the computed dwell times across the method. Using the Analytics module for data processing, reproducible peak integrations of MRM assays using AutoPeak Algorithm is very automated, enabling streamlined and rapid quantitative data processing.[5] Because of its ability to support many samples and highly multiplexed peptide MRM experiments, the software is ideal for biomarker verification assays, tracking changes in post-translational modifications (e.g. phosphorylation) across different samples, biological pathway analysis, and other targeted peptide quantitative assays.

Conclusions

Using the Scheduled MRM Algorithm to build MRM acquisition methods provides tremendous advantages for the generation and use of MRM assays. It allows a higher number of transitions to be monitored concurrently without having to resort to shorter dwell times or longer cycle times. This ensures that the analytical reproducibility of the MRM assays is maintained at even 4000 MRM transitions during a single LC run.

The sensitivity and quantitative reproducibility of the SCIEX Triple Quad 7500 LC-MS/MS System – QTRAP Ready enables very good peak reproducibility to be observed even with very high numbers of MRM transitions in a single assay. Coupled with highly reproducible microflow chromatography, this LC-MS/MS system provides a powerful tool for large scale peptide quantification for targeted biomarker research.

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Targeted assay for quantification of proteins from the SARS-CoV-2 coronavirus

Using the SCIEX Triple Quad™ 5500+ System – QTRAP® Ready

Catherine S. Lane [1], Bart Van Puyvelde[2], Katleen Van Uytfanghe[2], Maarten Dhaenens[2]
[1]SCIEX, UK, [2]Ghent University, Belgium

SARS-CoV-2 is the virus that causes COVID-19 infections in humans and belongs to the family of viruses known as coronaviruses. The coronavirus is an RNA-based virus, and therefore encodes its genome using RNA, similarly to influenza viruses, HIV, and rhinoviruses (common cold). Coronaviruses primarily infect human lung cells through a receptor for an enzyme called angiotensin-converting enzyme 2 (ACE2) through recognition by the spike protein.[1]

Currently, the majority of testing for active infection is carried out using a polymerase chain reaction (PCR)-based test, which can quantify the amount of viral RNA present. However, an active particle does contain a significant amount of protein encapsulating the RNA genome (Figure 1). There are a number of different viral proteins present in a varying number of copies per viral particle. The interesting question being posed by mass spectrometry researchers is whether a protein-based test could be used to detect viral particles at a level of sensitivity that would provide an alternative strategy.

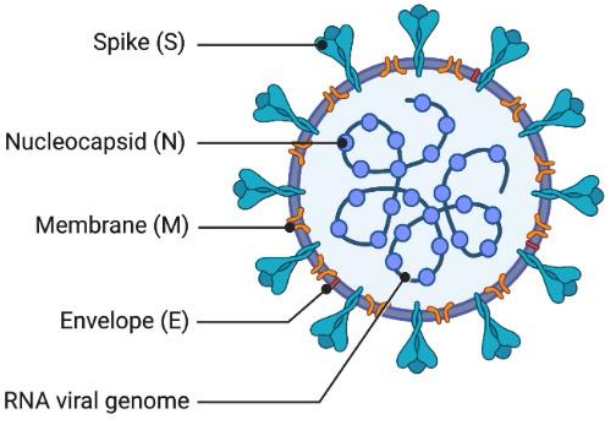

Figure 1. Structure of the SARS-CoV-2 coronavirus.[1] The coronavirus particle is a protective capsid made of nucleocapsid (N) proteins that contain the RNA genome. This is surrounded by a membrane with various viral proteins inserted, the spike (S) protein, the membrane (M) protein and the envelope (E) protein.

Here, the assay development work has been done to build a targeted peptide quantification assay for the detection of two of the viral proteins from SARS-CoV-2. Using recombinant proteins, target peptides were determined, and the MS parameters were optimized. Next, the assay was tested on recombinant proteins spiked into nasopharyngeal swabs from healthy patient samples (stored in UTM, a matrix commonly used for the collection and storage of viral samples). Detection limits were determined for the targeted peptides in this matrix. This information will be useful for researchers who want to explore the use of proteins for viral detection.

Key features of the targeted peptide quantification assay for the detection of coronavirus proteins

- A fast, robust peptide quantification assay has been developed for the detection of two of the viral proteins from the SARS-CoV-2 particle
- MRM transitions and compound dependent parameters have been optimized using the SCIEX 5500+ System
- Detection limits of 0.14–4.4 fmol protein per µL of UTM matrix
- Use of stable, isotope-labeled peptides, matched to the best performing peptides determined here, would provide enhanced quantitative robustness for the assay in the future

Methods

Sample preparation: One µg of spike glycoprotein (P0DTC2, SPIKE_SARS2) and nucleocapsid protein (P0DTC9, NCAP_SARS2) were obtained, digested with Trypsin/LysC as per standard protocols and then lyophilized. These simple digests were used for initial MRM assay development by re-suspending in 0.1% formic acid in 5% acetonitrile.

Dilution series were prepared by spiking the recombinant proteins (0, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 500 ng) into 250 µL of two different pooled nasopharyngeal swabs in UTM from healthy donors. Samples were vortexed with ice-cold acetone (1750 µL), then centrifuged at 16,000g for 10 mins at 0 °C. Acetone was removed and pellets were left to dry.

Dried pellets were re-suspended in 250 µL of 500 mM TEAB, then 50 µL of 0.1µg/µL Trypsin/LysC was added. Digestion was done for 4 hours at 37 °C. 50 µL were removed and dried. For analysis, the samples were re-suspended in 50 µL of 0.1% formic acid in 5% acetonitrile and 5 µL was injected per analysis.

Chromatography: Peptide separation was performed using an ExionLC™ System and a Phenomenex Luna Omega Polar C18 column (2.1 x 100 mm, 3 µm). A flow rate of 0.6 mL/min was used for fast analysis times. The gradient is described in Table 1. The column temperature was 40 °C.

Table 1. Gradient for peptide separation.

Time (min)	% A	% B
0	*97*	*3*
5	*60*	*40*
5.1	*10*	*90*
5.6	*10*	*90*
5.7	*97*	*3*
7	*97*	*3*

Mobile phase A – 0.1% formic acid in water
Mobile phase B – 0.1% formic acid in acetonitrile

Mass spectrometry: MRM analysis of the selected peptides was performed using a SCIEX Triple Quad 5500+ System operating in positive ionization mode. Source conditions were an ISV of 5500 V, GS1 of 60, GS2 of 50, TEM 600 °C and CUR of 30. A Scheduled MRM™ Algorithm was used for optimum detection efficiency, using an MRM detection window of 40 sec and a target scan time of 0.5 sec. Optimized MRM transitions and compound dependent parameters are shown in Table 2.

Data processing: MRM transition optimization was performed using Skyline software. Data was processed using SCIEX OS-Q Software.

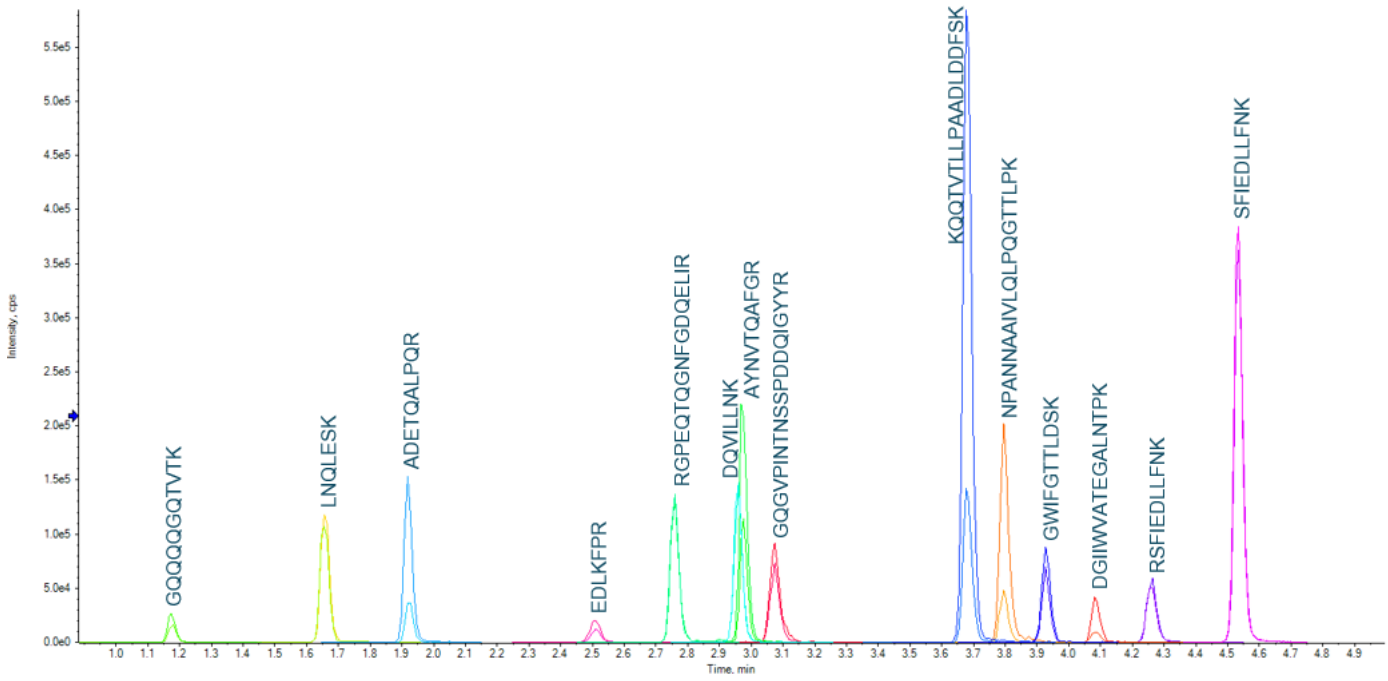

Figure 2. Chromatographic separation of the 14 peptides in the recombinant protein digests. Using high flow rates and fast run times gave sharp peaks for every peptide. Good separation was achieved for all target peptides.

Table 2. Optimized MRM transitions for quantification of key SARS-CoV-2 coronavirus proteins, SPIKE and NCAP.

Q1	Q3	RT (mins)*	ID	DP	CE
612.8	868.4	3.9	sp|P0DTC2|SPIKE_SARS2.GWIFGTTLDSK.+2y8.light	110	32.6
612.8	721.4	3.9	sp|P0DTC2|SPIKE_SARS2.GWIFGTTLDSK.+2y7.light	110	32.6
461.3	504.3	4.3	sp|P0DTC2|SPIKE_SARS2.RSFIEDLLFNK.+3b4.light	65	22.7
461.3	748.4	4.3	sp|P0DTC2|SPIKE_SARS2.RSFIEDLLFNK.+3b6.light	65	26.7
613.3	991.5	4.5	sp|P0DTC2|SPIKE_SARS2.SFIEDLLFNK.+2y8.light	110	28.7
613.3	878.5	4.5	sp|P0DTC2|SPIKE_SARS2.SFIEDLLFNK.+2y7.light	110	32.7
452.7	547.3	2.5	sp|P0DTC9|NCAP_SARS2.EDLKFPR.+2y4.light	50	28.6
452.7	419.2	2.5	sp|P0DTC9|NCAP_SARS2.EDLKFPR.+2y3.light	50	32.6
727.7	920.4	3.1	sp|P0DTC9|NCAP_SARS2.GQGVPINTNSSPDDQIGYYR.+3y16+2.light	80	27.2
727.7	563.8	3.1	sp|P0DTC9|NCAP_SARS2.GQGVPINTNSSPDDQIGYYR.+3y9+2.light	80	31.2
562.3	700.4	4.1	sp|P0DTC9|NCAP_SARS2.DGIIWVATEGALNTPK.+3y7.light	50	23.4
562.3	572.3	4.1	sp|P0DTC9|NCAP_SARS2.DGIIWVATEGALNTPK.+3y5.light	50	23.4
687.4	841.5	3.8	sp|P0DTC9|NCAP_SARS2.NPANNAAIVLQLPQGTTLPK.+3y8.light	50	29.3
687.4	865.5	3.8	sp|P0DTC9|NCAP_SARS2.NPANNAAIVLQLPQGTTLPK.+3b9.light	50	33.3
416.2	718.4	1.7	sp|P0DTC9|NCAP_SARS2.LNQLESK.+2y6.light	50	22.8
416.2	604.3	1.7	sp|P0DTC9|NCAP_SARS2.LNQLESK.+2y5.light	50	22.8
601.8	761.4	1.2	sp|P0DTC9|NCAP_SARS2.GQQQQGQTVTK.+2y7.light	80	32.1
601.8	633.4	1.2	sp|P0DTC9|NCAP_SARS2.GQQQQGQTVTK.+2y6.light	80	36.1
563.8	892.5	3.0	sp|P0DTC9|NCAP_SARS2.AYNVTQAFGR.+2y8.light	65	30.2
563.8	679.4	3.0	sp|P0DTC9|NCAP_SARS2.AYNVTQAFGR.+2y6.light	65	26.2
649.0	830.4	2.7	sp|P0DTC9|NCAP_SARS2.RGPEQTQGNFGDQELIR.+3y7.light	110	31.5
649.0	558.3	2.7	sp|P0DTC9|NCAP_SARS2.RGPEQTQGNFGDQELIR.+3b10+2.light	110	23.5
471.8	699.5	3.0	sp|P0DTC9|NCAP_SARS2.DQVILLNK.+2y6.light	50	25.6
471.8	600.4	3.0	sp|P0DTC9|NCAP_SARS2.DQVILLNK.+2y5.light	50	25.6
564.8	712.4	1.9	sp|P0DTC9|NCAP_SARS2.ADETQALPQR.+2y6.light	95	30.2
564.8	400.2	1.9	sp|P0DTC9|NCAP_SARS2.ADETQALPQR.+2y3.light	95	26.2
664.0	1078.5	3.7	sp|P0DTC9|NCAP_SARS2.KQQTVTLLPAADLDDFSK.+3y10.light	80	28.2
664.0	539.8	3.7	sp|P0DTC9|NCAP_SARS2.KQQTVTLLPAADLDDFSK.+3y10+2.light	80	28.2

* Retention times should be time scheduled based on LC and chromatography used.
EP of 10 and CXP of 20 were used for all MRM transitions.

MRM assay optimization

Using the digested recombinant peptides, MRM transitions were developed to previously selected peptides from the SPIKE and NCAP proteins. MS parameters including declustering potential (DP) and collision energy (CE) were optimized using Skyline software. Final MRM transitions (2 per peptide) were selected from a wider, optimized set of transitions on the basis of signal to noise, intensity and specificity in matrix. Final MRM transitions to the 14 best peptides are shown in Table 2.

A simple linear gradient was used for peptide chromatography, which provided good separation of all targeted peptides (Figure 2).

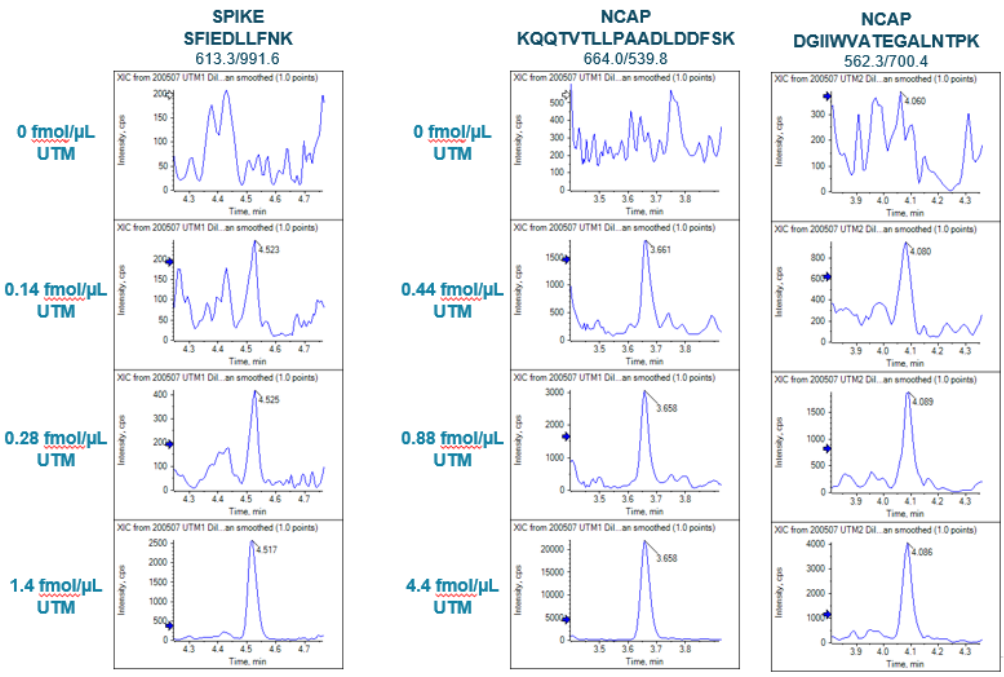

Figure 3. Example data from three peptides. Signals from three of the better performing peptides are shown here for the low end of the dilution series.

Dilution series

Dilution series were prepared by spiking the recombinant proteins into pooled nasopharyngeal swabs in UTM from healthy donors. After addition of the recombinant protein, acetone precipitation was performed, then the protein pellet was re-suspended and digested with Trypsin/LysC. The samples were then analyzed using the optimized MRM assay.

The typical bioanalytical criteria for determination of the lower limits of quantification (LLOQ) are accuracy of ±20% and precision of <20% at LLOQ, and <15% across the calibration curve. A range of LLOQs were found for the 14 peptides and are outlined in Table 3. Examples of the MRM signal observed from three of those peptides in UTM matrix are shown in Figure 3. Example calibration curves are shown in Figure 4.

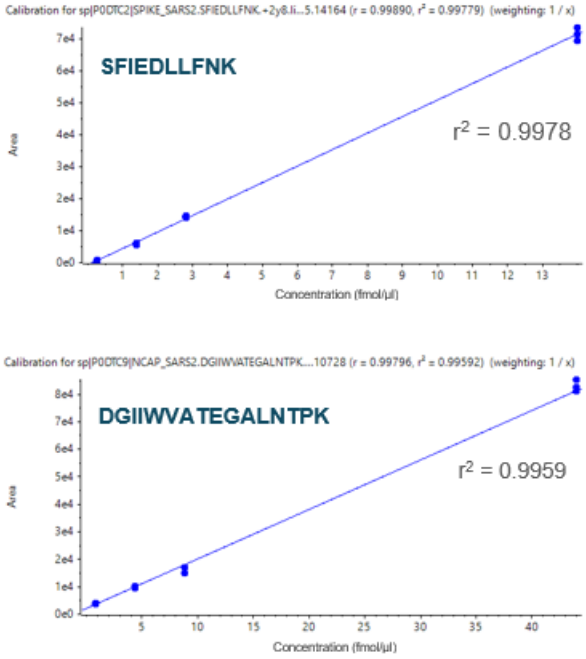

Figure 4. Calibration curve examples. Good linearity was observed across the dilution series for the majority of tested peptides with r^2 values ≥0.99.

Table 3. Lower limits of detection and quantification obtained for the 14 peptides. The LLODs ranged from 0.14–4.4 fmol/µL in pooled nasopharyngeal swabs in UTM. The best responding peptides are in bold.

Peptide	LLOQ in UTM (fmol/µL)	Area %CV for LLOQ	LLOD in UTM (fmol/µL)	LLOD on column (fmol)
SPIKE.GWIFGTTLDSK	*1.4*	*16*	*1.4*	*6*
SPIKE.RSFIEDLLFNK	*0.28*	*16*	*0.28*	*1.2*
SPIKE.SFIEDLLFNK	*0.28*	*19*	*0.14*	*0.6*
NCAP.EDLKFPR	*4.4*	*5.4*	*4.4*	*18*
NCAP.GQGVPINTNSSPDDQIGYYR	*4.4*	*1.3*	*4.4*	*18*
NCAP.DGIIWVATEGALNTPK	*0.88*	*3.9*	*0.44*	*1.8*
NCAP.NPANNAAIVLQLPQGTTLPK	*4.4*	*7.0*	*4.4*	*18*
NCAP.LNQLESK	-	-	*N/A**	-
NCAP.GQQQQGQTVTK	*4.4*	*7.9*	*4.4*	*18*
NCAP.AYNVTQAFGR	*4.4*	*14*	*4.4*	*18*
NCAP.RGPEQTQGNFGDQELIR	*0.88*	*9.8*	*0.88*	*3.7*
NCAP.DQVILLNK	*4.4*	*22*	*4.4*	*18*
NCAP.ADETQALPQR	*4.4*	*17*	*4.4*	*18*
NCAP.KQQTVTLLPAADLDDFSK	*0.44*	*4.0*	*0.44*	*1.8*

**Detection limit obscured by interfering peaks at <4.4 fmol/µL*

Conclusions

Here a targeted peptide quantification assay has been developed and tested on nasopharyngeal swabs in a typical sample collection medium for the detection and quantification of the presence of SARS-CoV-2 coronavirus proteins. The assay has been optimized for sensitivity and for fast, robust chromatography in a high-throughput environment. Next steps are to test this assay on a wider range of matrices to confirm detection limits. In addition, use of stable, isotope-labeled peptides is planned to provide further improvements to the quantitative accuracy of this method.

Note that this assay has not been validated on real test samples and should not be used for patient testing. Much more work would be required to translate this peptide quantification assay into a useful assay for determination of viral particle numbers.

References

1. King J, Kosinski-Collins M, Sundberg E. Coronavirus Structure, Vaccine and Therapy Development.

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