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

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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

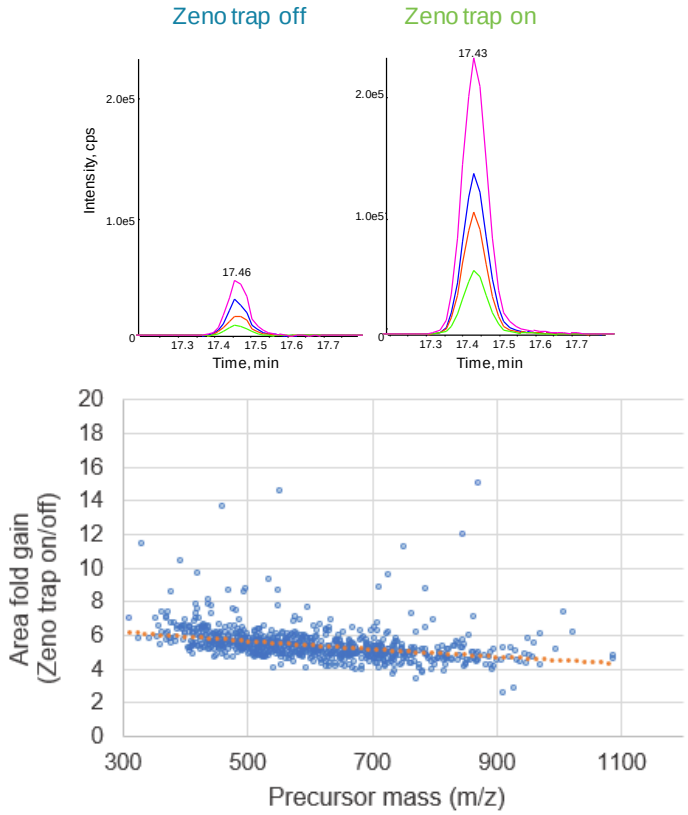

Figure 1. Significant gains in peptide area with Zeno trap activated. (Top) Example data of the sensitivity increases observed when the Zeno trap is activated are shown for ANT3.PFLVFIR, with a ~6x gain in peak area. (Bottom) A summary of the observed sensitivity gains for all 804 peptides is shown,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

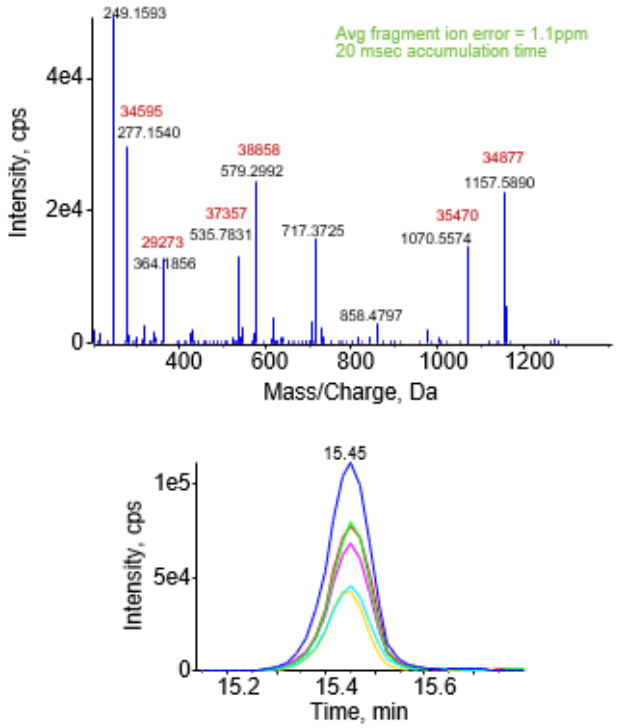

Figure 2. The MRM^{HR} workflow. In this QTOF workflow, the acquisition is set on the Q1 of a target peptide m/z, the peptide is fragmented in the collision cell and the full scan TOF MS/MS is acquired (top). This is repeated each cycle for the duration of the elution of the peptide from the column. 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.

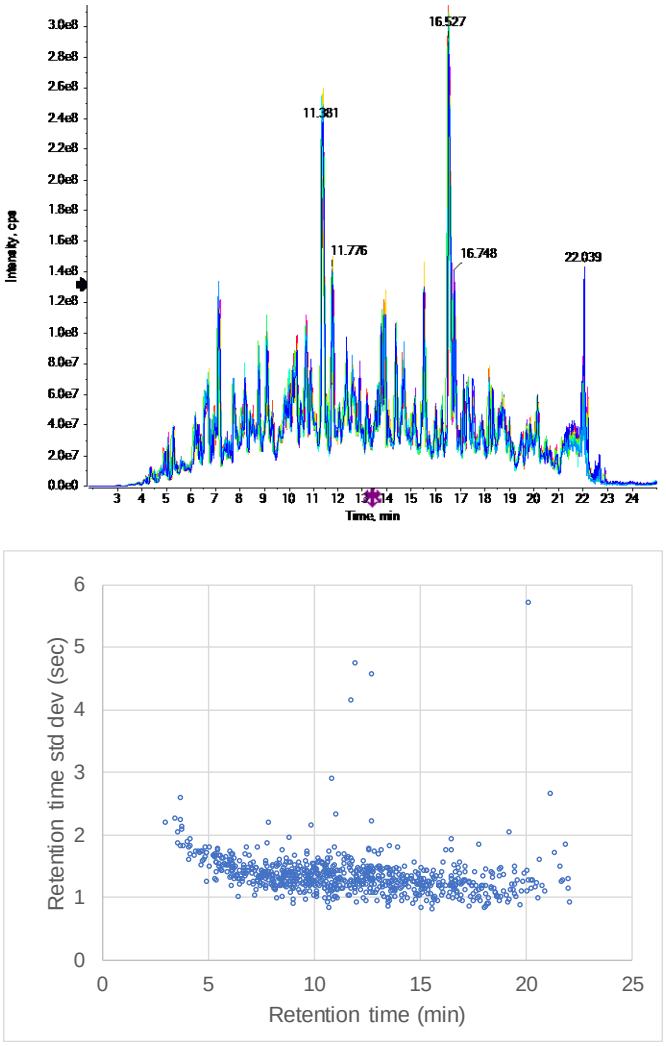

Figure 3. Chromatographic reproducibility across 10 replicates in plasma. (Top) Very good chromatographic reproducibility was observed throughout the method development and final data generation, with an average %RSD of 0.23 across the 804 peptides and all 10 replicates. (Bottom) Plot of observed retention 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).

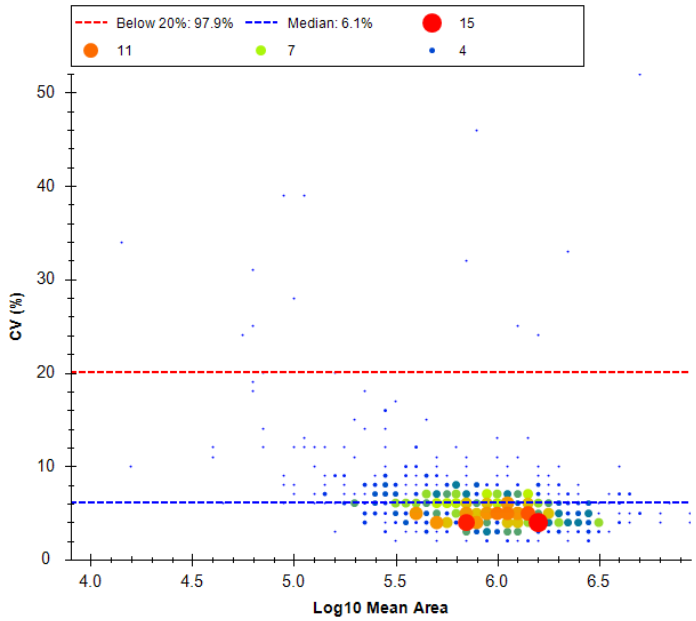

Figure 4. High reproducibility of targeted peptide quantification. Ten replicate injections of the PQ500 peptide (40 fmol on column) dosed into plasma (500 ng on column) were performed and the peptide peak area reproducibility was determined. The size and color of the dot indicate a larger number of 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*

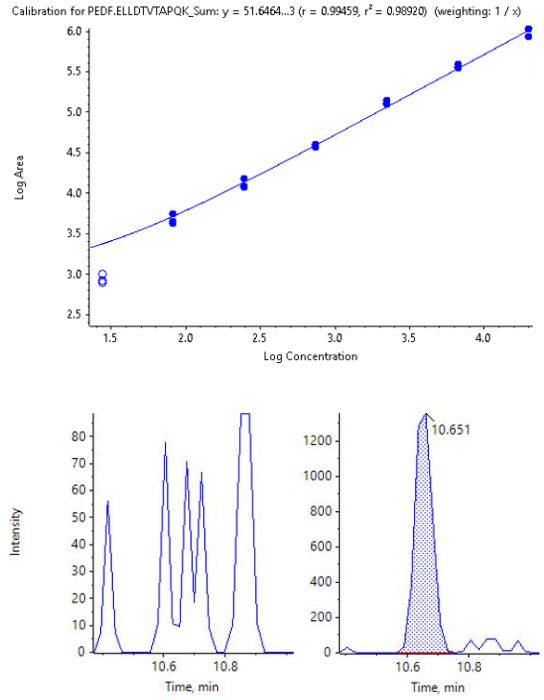

Figure 5. Concentration curve for PEDF.ELLDTVTAPQK. Concentration curve for the ELLDTVTAPQK in 500 ng plasma showed good reproducibility and linearity from 82.2 amol on column to the highest measured point at 19.9 fmol on column (r^2 = 0.995). The peptide signal (sum of 4 fragment ions) for the lower 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.

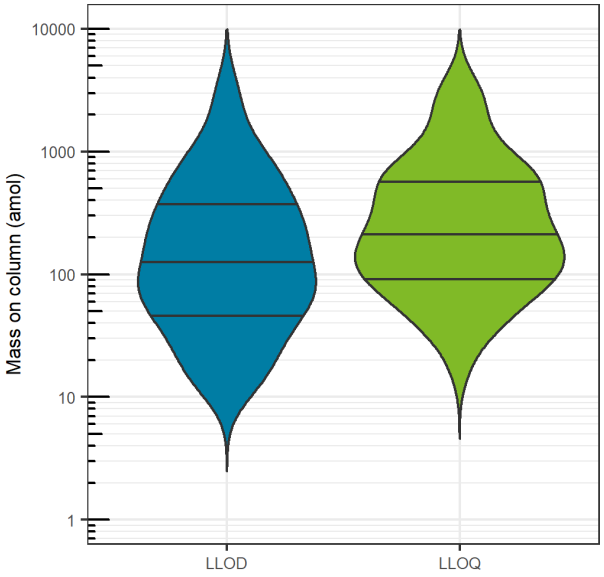

Figure 6. Summary of observed quantification results. The LLOQ and LLOD values were determined for the heavy labeled peptides in plasma. The median LLOQ was found to be 193 amol on column (green) and the median LLOD was 114 amol on column (blue).

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.

References

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