Reproducible targeted peptide profiling using highly multiplexed MRM assays

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

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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.

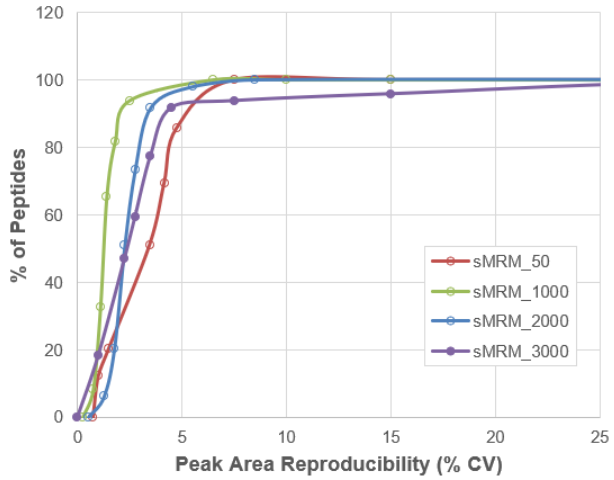

Figure 1. Excellent quantitative accuracy of higher multiplexing in a 5 minute gradient. The effect of higher numbers of MRM transitions on the reproducibility of 50 peptide MRMs from 3 protein digests spiked in complex matrix (1 µg on column) across 10 replicate injections on the SCIEX 7500 System was 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

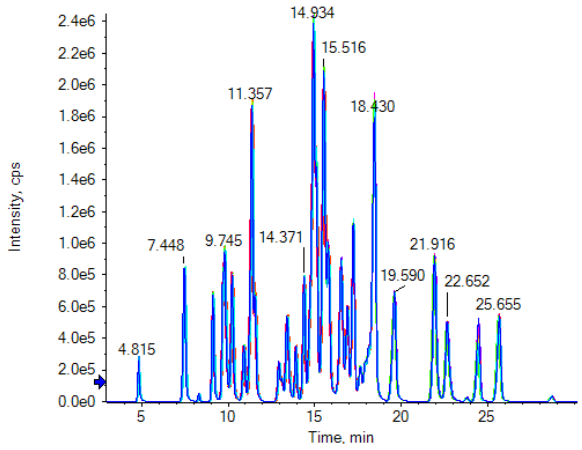

Figure 2. Chromatographic reproducibility for 30 min gradient runs. Using a 3 protein digest in buffer, very good reproducibility was achieved across the replicate analysis (n=15), both in peak area and in retention time stability.

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.

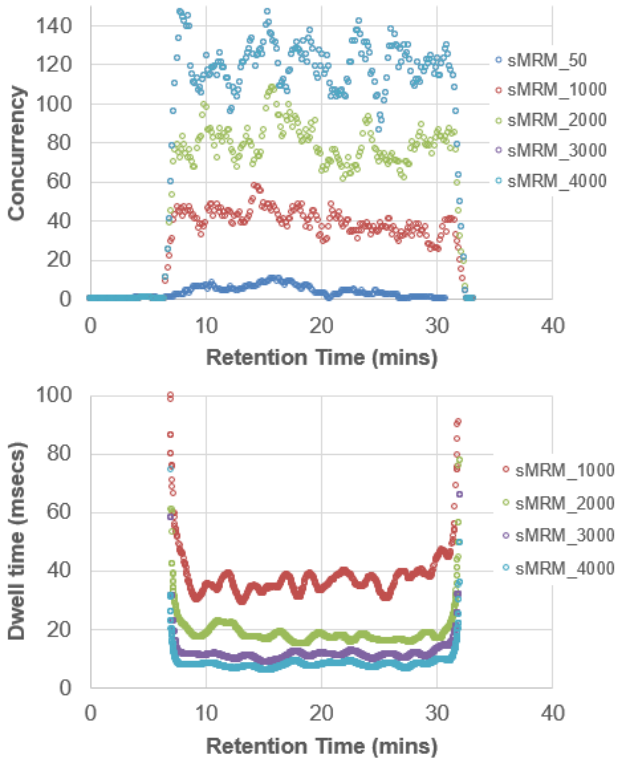

Figure 3. The Effects of MRM detection windows on Scheduled MRM Algorithm acquisition methods. For the 30 min LC gradient, increasing numbers of MRM transitions were added to the method and the MRM concurrency (top) and dwell time used (bottom) are plotted vs retention time. Because of the narrow retention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

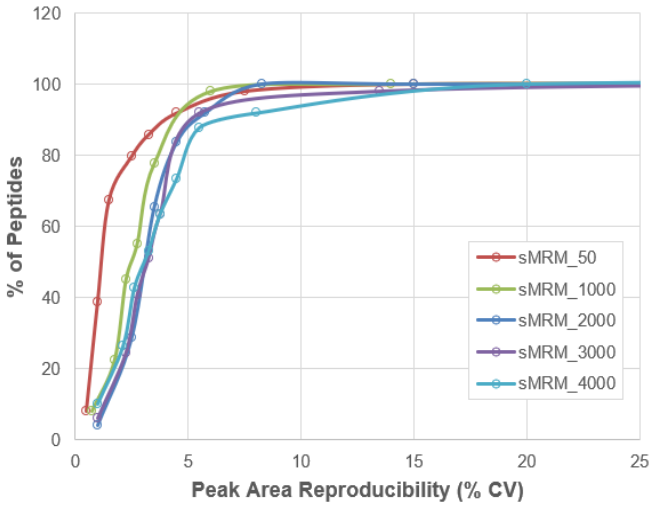

Figure 4. Assessing the quantitative accuracy of higher multiplexing using Scheduled MRM Algorithm. The effect of higher numbers of MRM transitions on the reproducibility of 50 peptide MRMs (3 protein digest in buffer) across 15 replicate injections on the SCIEX 7500 System was assessed using microflow 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

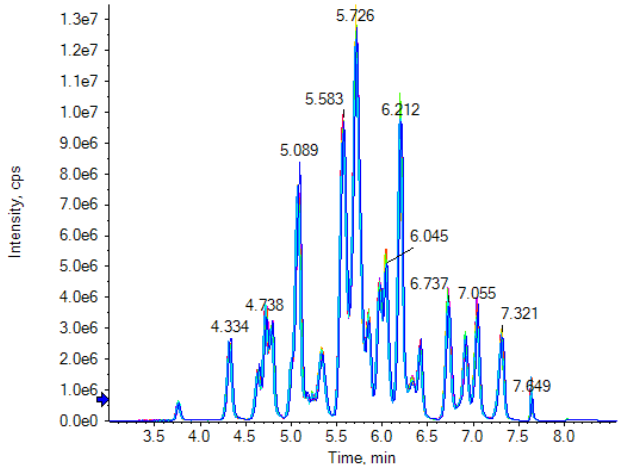

Figure 5. Chromatographic reproducibility for 5 min gradient runs. Again, very good reproducibility was achieved across the replicate analysis (n=10) for the 3 protein digest in complex matrix, both in peak area and in retention time stability.

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.

References

1. Enabling new levels of quantification. SCIEX technical note RUO-MKT-02-11886-A.
2. Using Scheduled MRM™ Algorithm in SCIEX OS Software. SCIEX community post RUO-MKT-18-11941-A.
3. Controlling SCIEX microflow LC Systems using contact closure in SCIEX OS Software. SCIEX community post RUO-MKT-18-11908-A.
4. High Sensitivity MRM workflow for signature peptide quantification. SCIEX technical note RUO-MKT-02-11882-A.
5. AutoPeak Integration Algorithm. SCIEX community post RUO-MKT-18-10329-A.

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