

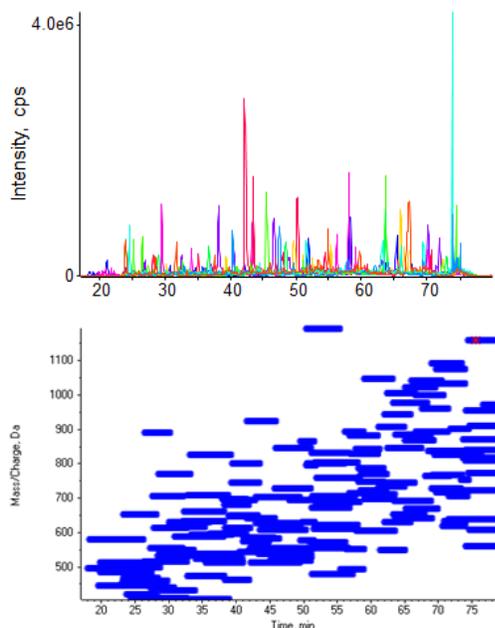
# Increasing the Multiplexing of High Resolution Targeted Peptide Quantification Assays

## *Scheduled MRM<sup>HR</sup> Workflow on the TripleTOF<sup>®</sup> Systems*

Jenny Albanese, Christie Hunter  
SCIEX, USA

Targeted quantitative proteomics has become a key tool in proteomics research and is driven by the well-established sensitivity and selectivity attributes of multiple reaction monitoring (MRM).<sup>1</sup> As more extensive protein panels need to be monitored in a targeted way across multiple samples, higher MRM multiplexing is becoming essential for throughput. With the *Scheduled MRM<sup>TM</sup>* Algorithm (introduced with Analyst<sup>®</sup> Software 1.5), significantly more MRM transitions can be monitored in a single acquisition while maintaining the quantitative reproducibility by time scheduling the acquisition of MRM transitions.<sup>2</sup>

The speed and sensitivity of MS/MS acquisition on the TripleTOF<sup>®</sup> systems has enabled a high resolution targeted quantification strategy. This strategy (termed MRM<sup>HR</sup> workflow) enables a degree of selectivity using high resolution that cannot be reached using standard triple quadrupole based instruments.<sup>3</sup>



**Figure 1. Scheduled MRM<sup>HR</sup> Workflow.** Using knowledge of the elution time of each peptide, each precursor mass is monitored only during a short retention time window and the full scan high resolution MS/MS is acquired. This allows many more peptides to be monitored in a single LC run with higher accumulation time and an optimized cycle time for the highest quantitation quality.

In this technical note, the *Scheduled MRM<sup>HR</sup>* workflow (Figure 1) that is now available in Analyst<sup>®</sup> TF Software 1.6 will be assessed for the degree of multiplexing possible and its impact on quantitative reproducibility. Scheduling the acquisition of high resolution MS/MS on the target peptide in a short time window around its elution time allows more accumulation time to be used per peptide while still maintaining an optimized cycle time. In this way more peptides can be monitored in a single LC-MS run with highest quantitation quality.

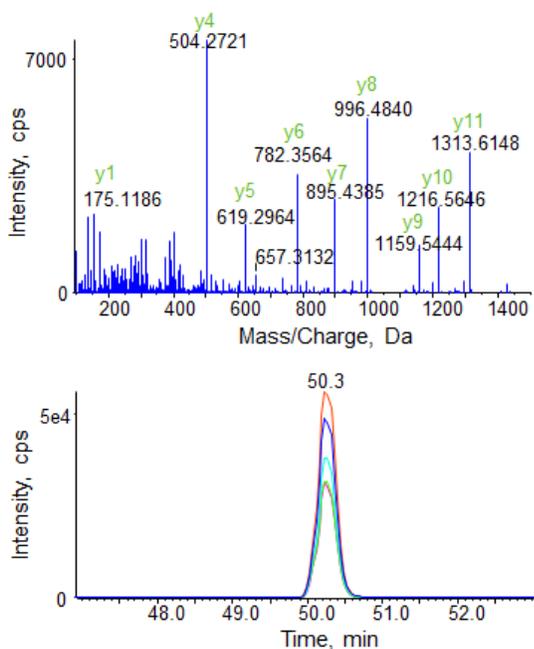
### Key Features of *Scheduled MRM<sup>HR</sup>* Workflow on TripleTOF Systems

- Using the precursor mass and retention time of each peptide, large numbers of peptides can be analyzed with high resolution and high quality data in a single run
- *Scheduled MRM<sup>HR</sup>* workflow enables longer accumulation times to be used during acquisition to provide good analytical precision at high multiplexing
- Full scan, high resolution data from *Scheduled MRM<sup>HR</sup>* workflow can be searched using ProteinPilot<sup>™</sup> Software for confirmation of detected peptides
- *Scheduled MRM<sup>HR</sup>* Workflow is available on all TripleTOF Systems using Analyst Software 1.6 or higher.

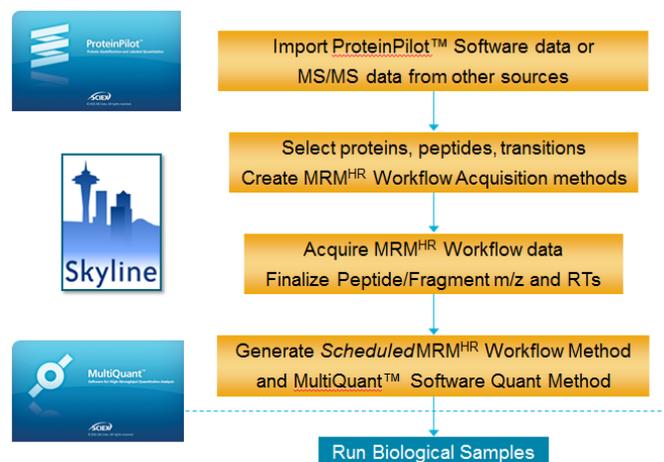
## Methods

**Sample Preparation:** Protein digests of Bovine Serum Albumin (Michrom BioResources), Myoglobin, (Michrom BioResources) and Beta-Galactosidase (SCIEX) were mixed together to create a test sample and loaded at 10 fmol on column. *E.coli* protein digest (Waters) was analyzed at 250 ng on column.

**Chromatography:** Separation of protein digests was performed on an NanoLC™ Ultra 2D System and cHiPLC® system (SCIEX, USA) in serial column mode. For each injection, the sample was desalted on a 75 µm x 15 mm analytical column, then eluted onto a second analytical column to create a 30cm column length for separation<sup>5</sup>. Both column chips were filled with ChromXP™ C18-CL 3µm 120Å phase. Peptides were separated using a linear gradient formed by A (2% ACN, 0.1% FA) and B (98% ACN, 0.1% FA), from 12–32% of B over 60 minutes at a flow rate of 250 nL/min. Each injection was performed using a full loop injection with a 1 µL sample loop.



**Figure 3. High Resolution XIC Generation from a Scheduled MRM<sup>HR</sup> Workflow Demonstrates Sufficient Points Across an LC Peak.** To quantify the peptide DGEDPGYTYLDSER ( $m/z$  577.2566) from Beta-glucuronate decarboxylase in *E.coli* cell lysate, a looped MS/MS spectrum is acquired across the LC peak at high resolution (>15,000) (top). High resolution XICs are generated for the peptide post-acquisition (bottom). Scheduling the acquisition of the MS/MS spectra around the elution time for the target peptide enables higher multiplexing while maintaining data quality.



**Figure 2. Assay Development Process for Scheduled MRM<sup>HR</sup> Workflow.** Experimental MS/MS data from IDA acquisition on TripleTOF Systems or MS/MS data from other sources can be imported for initial assay development. MRM<sup>HR</sup> workflow methods can be built for target proteins and peptides and used for assay refinement and retention time determination. Final Scheduled MRM<sup>HR</sup> workflow methods are built and used to run biological study. At the same time, a MultiQuant™ Software quantitation method is exported for use in final data processing.

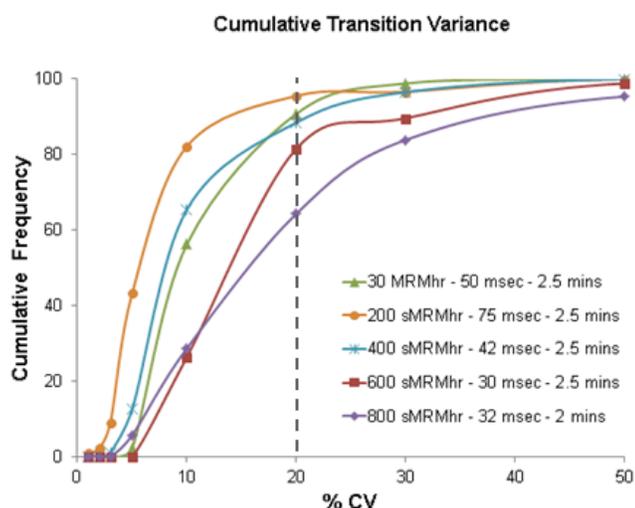
**Mass Spectrometry:** The MS analysis was performed on a TripleTOF® 5600 LC-MS/MS System (SCIEX) using both the MRM<sup>HR</sup> Workflow<sup>3,4</sup> and Scheduled MRM<sup>HR</sup> Workflow. MS spectra were acquired in high resolution mode (>30,000) using 250 ms accumulation time *per* spectrum. Full scan MS/MS was acquired in high sensitivity mode with an accumulation time optimized *per* cycle. Collision energy (CE) was set using rolling collision energy with a collision energy spread (CES) of 5 V. Retention time window used for most datasets was 2.5 minutes, except for the 800 peptide method where a window of 2.0 minutes was used.

**Data Processing:** Peptide identification on the MS/MS collected on the *E.coli* sample was performed using ProteinPilot Software 4.0 and an xml file was created for import into Skyline Software (University of Washington, USA). Proteins and peptides were selected for analysis and MRM<sup>HR</sup> workflow data was acquired and processed to determine peptide retention times. Final Scheduled MRM<sup>HR</sup> workflow acquisition methods and MultiQuant Software quantitation methods were created simultaneously by Skyline. Final data processing was performed using MultiQuant Software.

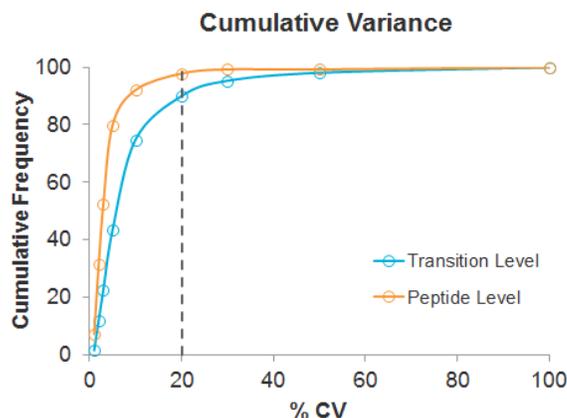
## Assay Development Workflow

Assay development is simplified for MRM<sup>HR</sup> and *Scheduled* MRM<sup>HR</sup> workflow as full scan MS/MS is always acquired and fragment ion selection is done post-acquisition. Only the peptide precursor ion m/z and the collision energy are set up-front. This process of developing *Scheduled* MRM<sup>HR</sup> workflow assays has been implemented within the Skyline Software and consists of four simple steps (Figure 2).

1. Previously acquired MS/MS data from a database search result, such as ProteinPilot Software, can be brought into the Skyline software to provide protein, peptide, and MS/MS information. Assays can also be developed from protein sequence information.
2. Initial MRM<sup>HR</sup> workflow acquisition methods are constructed using the intensity information from MS/MS data
3. These acquisition methods are run against the biological sample of interest and the MRM<sup>HR</sup> data is evaluated. Peptide and fragment ion selections can be refined to develop the highest quality assay (Figure 3). Retention times of each peptide are also determined
4. The final *Scheduled* MRM<sup>HR</sup> workflow acquisition method is built and will be used for data collection on the biological sample of interest. In addition, a MultiQuant Software quantitation method is created to be used for final data processing



**Figure 4. Assessing the Quantitative Accuracy of Higher Multiplexing using *Scheduled* MRM<sup>HR</sup> Workflow.** The effect of higher numbers of peptides on the reproducibility of 30 peptides across 5 or more replicate injections on the TripleTOF 5600 system was assessed. Because the data was acquired using the *Scheduled* MRM<sup>HR</sup> Workflow, the reproducibility of up to 800 peptides showed very good quantitative accuracy.



**Figure 5. Quantitative Reproducibility for Peptides in *E. coli* Cell Lysate Digest.** 139 peptides from proteins across the abundance range in an *E. coli* digest were monitored using the *Scheduled* MRM<sup>HR</sup> workflow. Replicate analysis was performed and the reproducibility at both the XIC and peptide level was assessed. Extremely low variance was found at the transition XIC level (blue trace) across replicates with 90% of data having CVs of less than 20% and over 95% of data at the peptide level (orange trace).

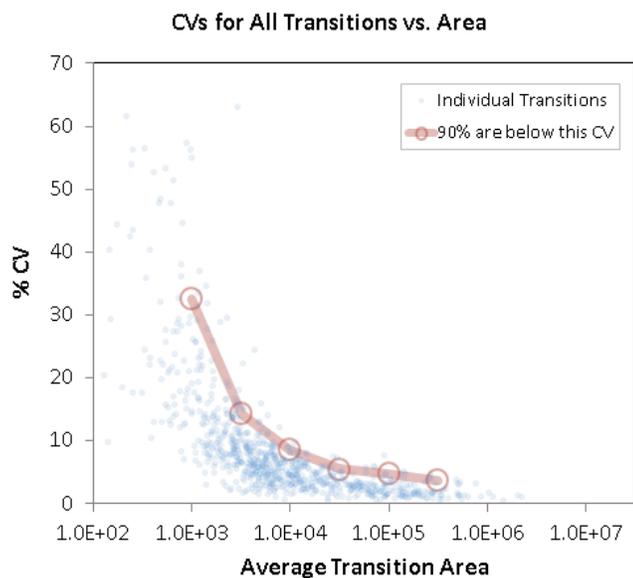
## Assessing Quantitative Reproducibility at High Multiplexing

An experiment was designed to assess the effects of higher multiplexing on analytical reproducibility on the TripleTOF 5600 system. Thirty peptides from Beta-Galactosidase (BG), Horse Myoglobin (Mb) and Bovine Serum Albumin (BSA) protein digests were monitored using the MRM<sup>HR</sup> Workflow with a cycle time of 1.8 seconds. Random generated Q1 m/z values and retention times were then added to the real peptides and these were used to build *Scheduled* MRM<sup>HR</sup> workflow methods with the same cycle time of 1.8 seconds and a retention time window of 2.5 minutes. Each acquisition method was run on the protein digests in 5 or more replicates by NanoLC. The reproducibility of fragment ion XICs for the 30 real peptides from each method across the replicate injections was used as a measure of the analytical reproducibility (Figure 4). The total number of XICs (in %) at a specific % coefficient of variation (%CV) was computed and plotted in Figure 4 (top), the median %CV was also computed (Figure 4, bottom). This cumulative reproducibility plot shows the majority of XICs achieved %CV of less than 20%, even at the highest peptide multiplexing in this experiment. *Scheduled* MRM<sup>HR</sup> workflow for 200 and 400 peptides (orange and blue) showed reproducibility similar or better to the MRM<sup>HR</sup> workflow data (green) on 30 peptides as the MS/MS accumulation time was greater than 40 msec in those methods. For 600 peptides (red line), there was a small drop in data quality as the accumulation time decreased to 30 msec. To run 800 peptides (purple line), the retention time window was reduced from 2.5 minutes to 2 minutes to maintain an accumulation time

of around 30 msec and good quantitative reproducibility was observed.

In addition, the effect of multiplexing on quantitative reproducibility for a set of peptides from proteins across the abundance range in an *E. coli* digest was studied. A total number of 139 peptides from 32 proteins were analyzed in a single LC/MS run using *Scheduled* MRM<sup>HR</sup> workflow and 795 fragment ions were quantified. Replicate analysis was performed and the reproducibility was assessed at both the transition XIC area and peptide level (multiple fragment ion XIC areas were summed to obtain a peptide area). Figure 5 shows extremely low variance at the transition XIC level (blue trace) and across replicates with 90% of data having CVs of less than 20% and over 95% of data at the peptide level (orange trace). The reproducibility of the retention times for the peptides across the replicate injections was an average of 0.2% RSD.

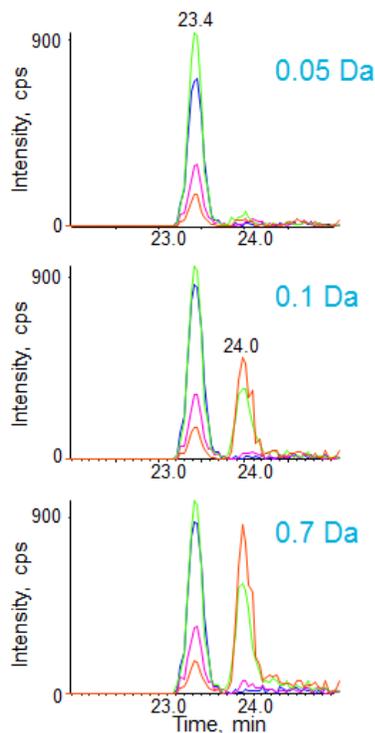
To evaluate the XIC peak area quality and reproducibility further, each individual data point was plotted based on its intensity and %CV (Figure 6). The variance in the data increases at the lower intensity regimes as expected. The red line shows that only at the very lowest intensity bins does the %CV exceed 10%.



**Figure 6. Reproducibility Assessment of Transition Peak Area versus %CV.** The blue dots represent the individual transition peak areas and are plotted based on their intensity and observed variance. The red line describes that 90% out of the total transitions area are below the shown %CV. This demonstrates the very high data reproducibility that was obtained by using the *Scheduled* MRM<sup>HR</sup> workflow on the TripleTOF 5600 system.

## Increased Specificity on a TripleTOF Systems

When targeted quantification is done on a triple quadrupole based instrument, quadrupoles are typically set at unit resolution, transmitting a 0.7 Da wide window in both Q1 and Q3. The difference with the MRM<sup>HR</sup> Workflow is that the TOF analyzer replaces the 'Q3' fragment ion detection with its inherent advantages of detecting all ions, at high resolution and high mass accuracy. To illustrate the advantage of high resolution, multiple fragment ions for the peptide VNAIAAPTR from an uncharacterized protein *yraR* were extracted at variable extraction widths (Figure 7). At the narrow extraction widths possible from the high resolution TOF data, good specificity is observed with minimal interfering peaks (Figure 7, top). When the extraction width is widened to simulate monitoring using a quadrupole, an interference is observed for two of the transitions (Figure 7, bottom).



**Figure 7. Removing Interferences or Background through High Resolution Fragment Ion Extractions.** Multiple fragment ions for the peptide VNAIAAPTR from an uncharacterized protein *yraR* were extracted at variable extraction widths. High resolution extraction of fragment ions can provide higher specificity in complex matrices when specific interferences are present.

## Conclusions

High resolution targeted assays can provide higher specificity for some peptides in complex biological samples and now with the ability to time-scheduled acquisition, high multiplexing can be achieved.

- TripleTOF Systems have the MS/MS sensitivity and speed to perform MRM-like analysis

Post-acquisition extraction of fragment ions from the high resolution TOF MS/MS data allows for high specificity MRM-like data (MRM<sup>HR</sup> Workflow) to be obtained

- *Scheduled* MRM<sup>HR</sup> Workflow extends the multiplexing possible with this high resolution workflow, maintaining quantitative robustness

In the multiplexing test on standard protein digests, up to 800 precursors were analyzed in a single method and high reproducibility was observed

This reproducibility translated into the complex biological sample test, where 130 peptides were monitored in *E. coli* with 90% of the transition peak areas showing reproducibility better than 20%

## References

1. Anderson L and Hunter CL (2006) MCP 5.4, 573-588.
2. The Scheduled MRM™ Algorithm Enables Intelligent Use of Retention Time During Multiple Reaction Monitoring - Delivering up to 4000 MRM Transitions per LC Run. SCIEX Technical Note RUO-MKT-02-8539-A.
3. Impact of High-Resolution MRM Analysis on Complex Proteomes - Targeted Protein Quantification using MRM<sup>HR</sup> Workflow on the TripleTOF® 5600 System. SCIEX Technical Note RUO-MKT-02-5733-A.