

The *Scheduled* MRM™ Algorithm Enables Intelligent Use of Retention Time During Multiple Reaction Monitoring

Delivering up to 2500 MRM Transitions per LC Run

Christie Hunter¹, Brigitte Simons²

¹AB SCIEX, USA, ²AB SCIEX, Canada

The utility of Multiple Reaction Monitoring (MRM) for targeted protein quantification and biomarker verification/validation studies on triple quadrupole based MS systems is an active research area¹, driven by the well known sensitivity and selectivity attributes of MRM. As more extensive protein panels need to be monitored in a targeted way across multiple samples, higher MRM multiplexing is becoming essential for throughput. Recent examples in the characterization of cell signaling pathways² or verification of putative biomarker panels³ highlight the need for MRM assays robust enough to quantitatively profile more peptides and proteins in every LC run.

With the *Scheduled* MRM™ Algorithm (Analyst® Software 1.5 or higher), many more MRM transitions can be monitored in a single acquisition while maintaining the quantitative reproducibility that is essential for targeted protein quantification assays. By monitoring each peptide MRM transition only around its expected elution time (Figure 1), many more peptides can be monitored. This MRM scheduling decreases the number of concurrent MRMs monitored at any point in time, allowing both the cycle time and dwell time to remain optimal at higher levels

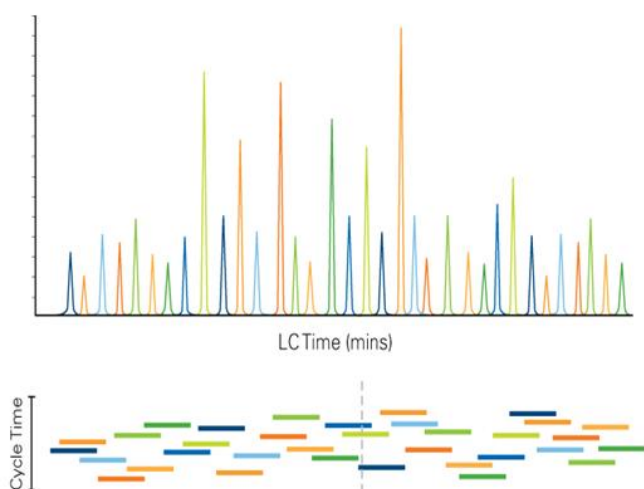


Figure 1. *Scheduled* MRM™ Algorithm. Using knowledge of the elution time of each peptide, each MRM transition is monitored only during a short retention time window. This allows many more MRM transitions to be monitored in a single LC run, while still maintaining maximized dwell times and optimized cycle times.



of MRM multiplexing. MRM assays consisting of 1000 MRM transitions have been run, facilitated by intelligent use of retention time windows during an LC analysis, while maintaining an optimum number of data points for improved precision of peak area and quantitative measurement.

Key Features of *Scheduled* MRM™ Algorithm

- Analyst® Software automates MRM scheduling with the intelligent *Scheduled* MRM™ Algorithm
- The *Scheduled* MRM™ Algorithm automatically associates retention times with MRM transitions and creates an optimized acquisition method based on a few key parameters provided by the user
- MRM scheduling allows many more analytes to be analyzed in a single run (Figure 1)
- The *Scheduled* MRM™ Algorithm enables optimized cycle time and maximized dwell times to be used during acquisition to provide higher multiplexing with good analytical precision
- *Scheduled* MRM™ Algorithm transitions can be used as survey scans in MIDAS™ workflow acquisition methods, to obtain high sensitivity MS/MS spectra for ID confirmation on detected peptides
- Up to 2500 MRMs can be scheduled in a single run on the QTRAP® and Triple Quad™ 5500 Systems, and up to 1000 for the 4000 QTRAP® and API 5000 and 4000 systems
- **Optimized precision, maximized capacity, one click**

Key Parameters of Multiple Reaction Monitoring (MRM)

Dwell Time: Dwell time is the time spent acquiring the specific MRM transition during each cycle. Because of the LINAC[®] Collision Cell in AB SCIEX QTRAP[®] systems and triple quadrupole instruments, very short dwell times can be used (5ms or less)⁴. However, higher dwell times are always desirable for better signal/noise and sensitivity, especially for lower abundant peptides / proteins.

Duty Cycle: Duty cycle is effectively the amount of time spent monitoring an analyte or peptide, therefore the higher the duty cycle the better the data quality. Duty cycle is inversely proportional to the number of concurrent MRMs monitored. Therefore, an increase in multiplexing resulting from more concurrent MRM transitions can decrease the analytical reproducibility.

Cycle Time: The ideal cycle time for an MRM assay is a chromatographic consideration. A cycle time which provides an LC peak sampling rate such that 6-8 points are obtained across the peak at half height is optimal for LC peak integration. Better peak integration will provide better accuracy, especially for the lower abundant peptides.

Scheduled MRM™ Algorithm - The Ideal Way to Maximize Multiplexing

An advantage to using MRM assays for quantitative analysis is the apparent ease of multiplexing (measuring higher numbers of MRMs per method). However as shown in Figure 2, the key parameters of MRM analysis must be considered. As illustrated in Figure 1, knowledge of the elution time of each peptide allows the *Scheduled MRM™* Algorithm to create an acquisition method where each MRM transition is monitored only across a short time window. At any one point in time, the number of concurrent MRM transitions is significantly reduced resulting in much higher duty cycles for each peptide. The software computes maximal dwell times for the co-eluting species while maintaining the desired cycle time. A maximized dwell time, an optimal cycle time, and the highest possible duty cycle for each MRM ensure that the analytical precision is maintained at higher multiplexing.

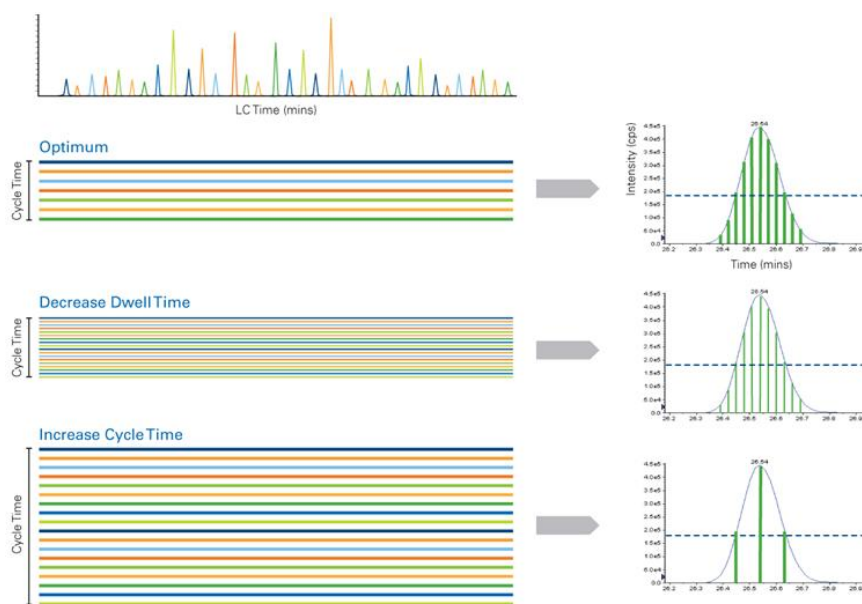


Figure 2. Key Considerations for Maximizing MRM Multiplexing. A) Traditionally MRMs are monitored across the whole LC run with fixed dwell times and cycle times. This produces good sampling of the LC peak (right), where the width of the bars indicates the dwell time and the spaces between the bars indicate the cycle time. B) Tripling the multiplexing by decreasing the dwell time results in good peak sampling, but the dwell time and duty cycle are impacted (reduced line widths). C) Tripling the multiplexing by extending cycle time results in very poor peak sampling (large spaces between lines).

Good Chromatography is Key for Highest Order Multiplexing

The key to highest order multiplexing is high quality, highly reproducible chromatography. One of the user inputs to the software for automatically creating the *Scheduled MRM™* Algorithm methods is the MRM Detection Window. This is an estimate of the reproducibility expected in the chromatography of the experiment, and should reflect the width of the peaks at base plus some accounting for any shifts in chromatography. The more narrow the peak widths and the more reproducible the elution times, the more narrow this MRM detection window can be. Shown in Figure 3 is a simulation of the MRM concurrency for 1000 MRM transitions scheduled over a 30 minute gradient (concurrency being the number of MRMs to be monitored at that point in time). The effect of using a more narrow retention time window is clear. With more narrow retention time windows, the number of concurrent MRMs is reduced (Figure 3, top). Reduced concurrency means that higher dwell times can be used for each MRM transition (Figure 3, bottom), improving the quality of the data especially on the low abundant precursors.

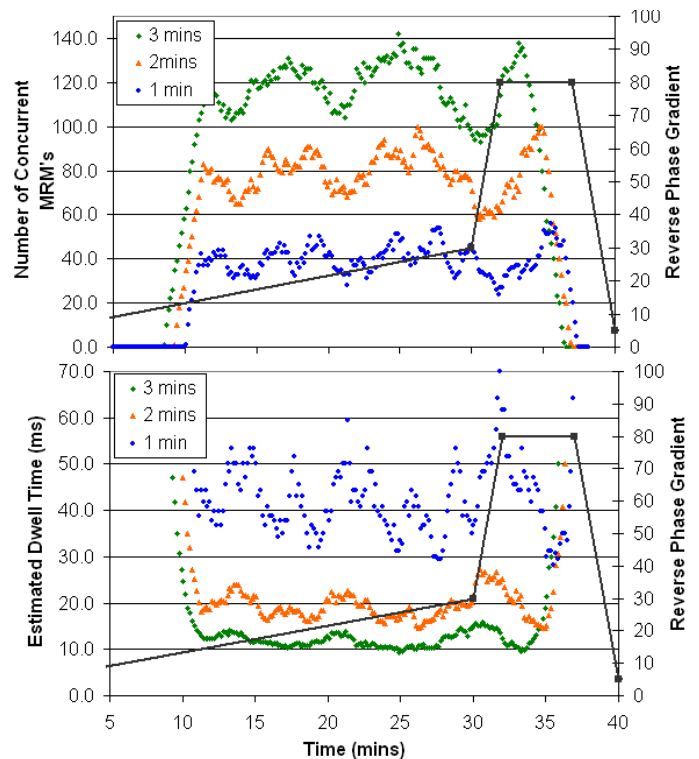


Figure 3. The Effects of MRM Detection Windows on *Scheduled MRM™* Algorithm Acquisition Methods. This simulation of 1000 MRM transitions scheduled over a standard 30 min LC gradient illustrates how narrower MRM detection windows (from 3 mins down to 1 min) can reduce MRM concurrency (top graph). Reduced concurrency means a higher dwell time per MRM is automatically assigned resulting in better data quality (bottom graph).

Easy Method Creation

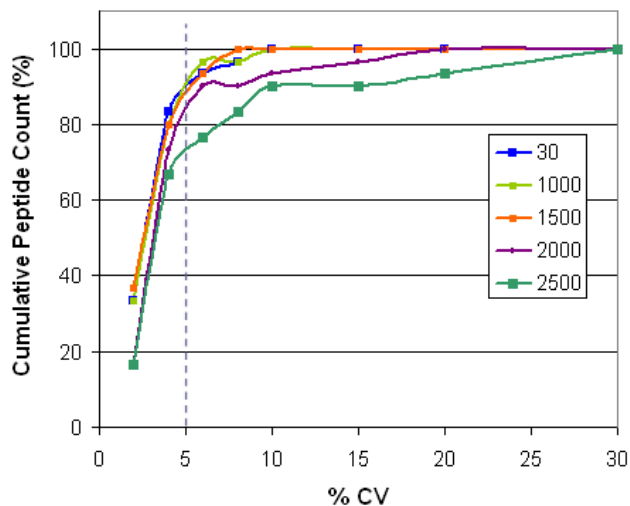
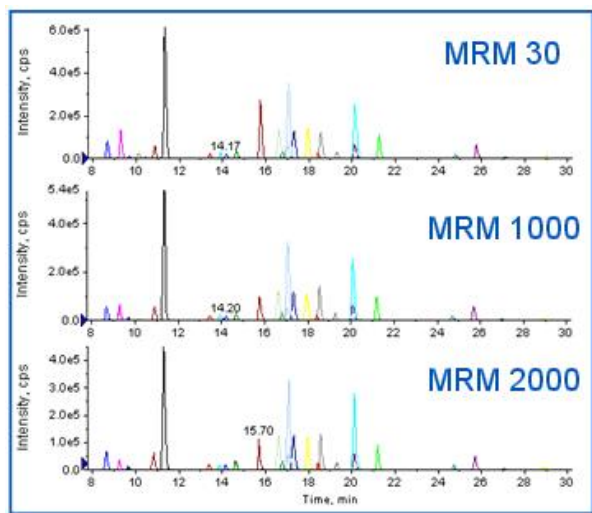
Another key advantage of the *Scheduled MRM™* algorithm is the ease at which powerful quantitative MRM acquisition methods can be created. The user is required to specify a few key parameters:

- MRM Transition – the Q1 and Q3 m/z and any compound dependent parameters
- Expected RT – the retention time for each MRM transition
- MRM Detection Window – a wide enough window is provided such that the MRM peak stays entirely within the window across all the injections
- Target Scan Time – this is effectively the cycle time, how often the chromatographic peak should be sampled

Within seconds, the software algorithm then automatically builds an acquisition method that schedules the appropriate MRM transitions to be monitored only around their expected elution time. No tedious manual scheduling is required.

	Q1 Mass	Q3 Mass	Time	ID	CE (vc)
1	518.0	414.2	21.5	APOB.SVSLPSLDPASAK.3b3.light	43.9
2	518.0	445.3	21.5	APOB.SVSLPSLDPASAK.3y3.light	43.9
3	518.0	613.4	21.5	APOB.SVSLPSLDPASAK.3y5.light	43.9
4	518.0	527.3	21.5	APOB.SVSLPSLDPASAK.3b4.light	43.9
5	520.6	418.2	21.5	APOB.SVSLPSLDPASAK.3b3.heav	44.0
6	520.6	449.3	21.5	APOB.SVSLPSLDPASAK.3y3.heav	44.0
7	520.6	617.4	21.5	APOB.SVSLPSLDPASAK.3y5.heav	44.0
8	520.6	531.3	21.5	APOB.SVSLPSLDPASAK.3b4.heav	44.0
9	666.8	588.3	15.9	APOC1.EFGNTLEDK.2b4.light	51.3
10	666.8	531.3	15.9	APOC1.EFGNTLEDK.2y3.light	51.3
11	666.8	474.2	15.8	APOC1.EFGNTLEDK.2b3.light	51.3

Figure 4. Acquisition Method User Interface for the *Scheduled MRM™* Algorithm. In addition to the traditional MRM parameters, the user provides a peptide elution time, an MRM detection window and a Target Scan Time. The software then automatically designs and optimizes the *Scheduled MRM* Algorithm acquisition method.



	30	500	1000	1500	2000	2500
Area Avg %CV	3.01	2.60	2.94	2.94	3.94	5.28
RT Std Dev	0.03	0.03	0.01	0.01	0.01	0.02

Figure 5. Assessing the Quantitative Accuracy of Higher Multiplexing using Scheduled MRM™ Algorithm. The effect of higher numbers of MRM transitions on the reproducibility of 30 peptide MRMs across 5 replicate injections on the QTRAP® 5500 system was assessed. Because the MRM acquisition methods were created with the Scheduled MRM™ Algorithm, the reproducibility of up to 2500 MRMs showed very similar quantitative accuracy (top right reproducibility plot), with average MRM peak area reproducibility of 3.6% (bottom).

Effect of Scheduling on Quantitative Reproducibility

An experiment was designed to assess the effects of higher MRM multiplexing on analytical reproducibility (Figure 5). Thirty real MRM transitions were designed to E. coli beta-Galactosidase and Bovine Serum Albumin protein digests and these MRMs were used to create the first acquisition method – MRM 30. Random MRM transitions and retention times were added to the 30 real MRM transitions to create 500, 1000, 1500, 2000 and 2500 MRM / retention time pairs. The MRM / retention time pairs were provided to the Scheduled MRM™ Algorithm to build the final MRM acquisition methods. Each acquisition method was run on the same sample (Beta Gal and BSA protein digest) in 5 replicates by high flow chromatography (300 µL/min). The reproducibility of the 30 real MRMs from each method across the replicate injections was used as a measure of the analytical reproducibility of each acquisition method.

A typical chromatogram from each of the three acquisition methods is shown in Figure 5 (left), illustrating the quality and stability of the chromatography achieved in this experiment using a standard high flow LC system. The total number of MRM transitions (in %) at a specific % coefficient of variation (%CV) was computed and plotted in Figure 5 (top right). This cumulative reproducibility plot shows the majority of MRM transitions

achieved %CV of less than 10%, even at the highest MRM multiplexing in this experiment. The plots for the 5 methods are very similar, showing reproducibility across a wide range of multiplexing. Finally, the average %CV of the raw MRM peak areas for the 5 different methods across the 5 replicates (Figure 5, table) were very similar. This experiment clearly illustrates the power of scheduling the MRM transitions for maximizing multiplexing while maintaining reproducibility. A similar experiment was performed on the 4000 QTRAP® system, also showing very high reproducibility up to 1000 MRM transitions in a single 30 min run (Table 1). This experiment was done using nanoflow chromatography.

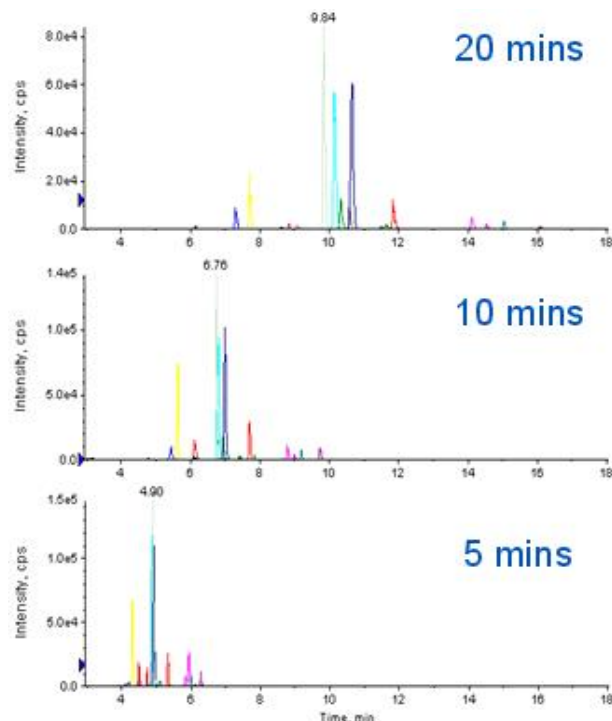
Table 1. Quantitative Accuracy of Higher Multiplexing using Scheduled MRM™ Algorithm – 4000 QTRAP® System. Using a similar experimental strategy discussed (Figure 5), very high reproducibility is obtained with a 1000 MRM method created with the Scheduled MRM™ Algorithm on the 4000 QTRAP® system.

	MRM30	sMRM750	sMRM1000
Avg. Area %CV	5.9	5.1	6.8
RT Std Dev	0.11	0.04	0.09

Increase Throughput with *Scheduled MRM™* Algorithm

In addition to using the *Scheduled MRM™* Algorithm to increase the multiplexing, it can also be used to increase the speed at which experiments are performed. An additional experiment was performed using 1000 MRM transitions, and the length of the high flow LC gradient was progressively decreased from 20 to 5 mins (Figure 6). Good reproducibility was obtained with 1000 MRM transitions in a 5 minute gradient, because with narrow MRM detection windows, dwell times of 30 ms can still be achieved.

As even larger panels of MRM transitions are developed and run on biological samples, it will become increasingly important to have MS/MS confirmation data for every peptide monitored. Using survey scans created by the *Scheduled MRM™* Algorithm, high sensitivity ion trap MS/MS scans can be triggered for many peptides automatically (Figure 7).



	1000 MRM Transitions		
	5 mins	10 mins	20 mins
Avg. Area %CV	9.40	7.30	8.10

Figure 6. Increase Speed of Chromatography with *Scheduled MRM™* Algorithm. Using high flow chromatography, 1000 MRMs were scheduled into increasingly shorter gradients. Reproducibility is maintained because narrow MRM Detection Windows enable good dwell times to be maintained.

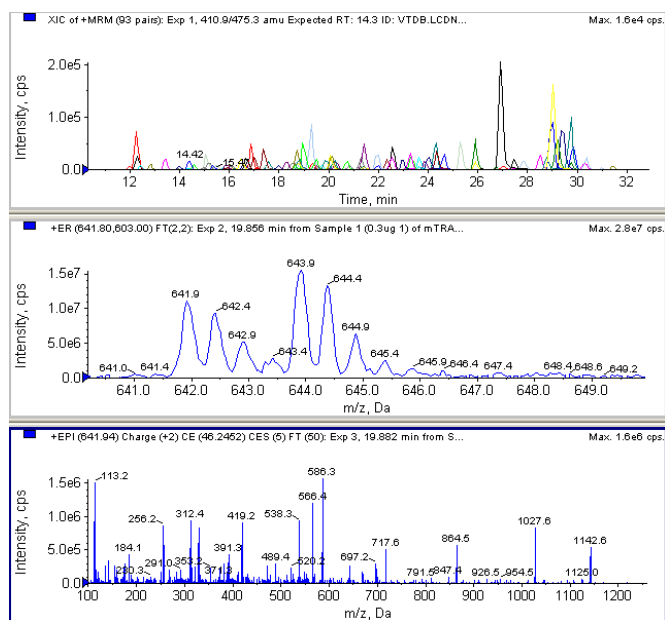


Figure 7. MIDAS™ Workflow Detection using a survey scan created by the *Scheduled MRM™* Algorithm. High sensitivity ion trap MS/MS data is obtained using the MIDAS™ Workflow, enabled on QTRAP® system instruments. The ion trap MS/MS is over 100X more sensitive over standard triple quadrupoles for full scan MS/MS data. These MS/MS spectra help confirm the MRM transition.

Creation of Internal Standards for MRM Assays

The ability to monitor up to 1000 or 2500 MRM transitions in a single acquisition method means the requirement for internal standard peptides is greatly increased. Using the non-isobaric amine labeling reagents to create global internal standards (GIS) provides a cost effective way to create internal standards for all the proteins/peptides/post translational modifications to be monitored in the multiplexed MRM assay generated with the *Scheduled MRM™* Algorithm. It also greatly improves the reproducibility of the data.

Powerful Software for MRM Assay Development and Data Processing

MRMPilot™ Software allows a user to create MRM based experiments to identify or quantify peptides⁴. The selection of MRM is based on either previously acquired MS/MS identifications, or predicted based on peptide sequence leveraging the MIDAS™ Workflow on the QTRAP® systems (Figure 7). MRMPilot™ Software then helps to iteratively optimize MRM transitions by allowing the evaluation of quantitative and qualitative results by calculated metrics as well as intelligent and user-friendly graphs and tables. A final optimized MRM assay is automatically to use in targeted quantitative assays. *Scheduling* MRM transitions using the *Scheduled* MRM™ Algorithm can maximize the throughput during the assay development process and deliver high quality quantitative measurements in the final assay.

MultiQuant™ Software provides a comprehensive package for processing peptide quantification data from MRM experiments⁵. Enabling the MRM peak integration of both MRM assays and MIDAS™ workflow data, workflows both with and without isotopic labeling strategies for high numbers of relative and absolute peptide quantification datasets can be processed. 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 in Analyst® software (build 1.5 or higher) to build MRM acquisition methods provides tremendous advantages for generation and use of MRM assays. It allows higher numbers 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 2500 MRM transitions. The powerful *Scheduled* MRM™ Algorithm makes it extremely easy to create highly multiplexed MRM assays, without the complicated and tedious manual interaction required for creation of time period methods. The input of just a few key parameters allows the algorithm to automatically and intelligently create the optimum assay in seconds.

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Headquarters
110 Marsh Drive | Foster City CA 94404 USA
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