

# Accelerating Throughput for Targeted Quantitation of Proteins/Peptides in Biological Samples

## Biomarker Verification Studies using the QTRAP® 5500 Systems and the mTRAQ® Reagents Triplex

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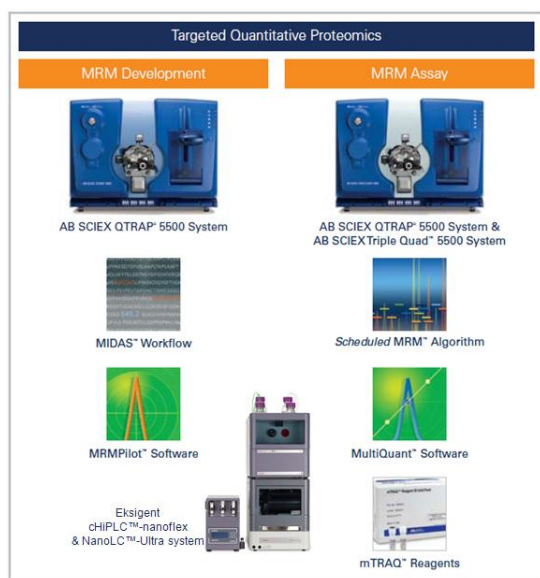
There are many powerful global discovery techniques that have been used to generate panels of candidate protein biomarkers. The big challenge of proteomics today is to move these putative markers forward in the research pipeline, to validate the performance of the markers across larger biological sample sets. Much work has been done over the last few years to develop Multiple Reaction Monitoring (MRM) based strategies to enable and accelerate the transition from discovery to targeted quantitative MS verification assays.

We have been focused on developing tools that rapidly enable the transition from discovery to verification assays, relieving the burden of time and related costs. With innovative hardware of the QTRAP® system, enabling software and multiplexed reagents, significant progress has been made towards rapidly developing MRM assays and running these assays on many biological samples.



## Addressing the Challenges of Biomarker Verification

- Rapid assay development is enabled by both hardware and software innovations
- MIDAS™ Workflow to detect and identify peptides to target proteins that will be good peptides for MRM analysis
- MRMPilot™ Software provides a user friendly workspace that enables the iterative assay development using the MIDAS™ Workflow (Table 1). No synthetic peptides or proteins are required for assay development, reducing time and cost
- mTRAQ® reagents triplex provide a cost effective strategy for developing internal standards for biomarker verification studies<sup>1</sup>
- Global internal standard workflow (Figure 2) enables creation of internal standard peptides for every protein / peptide of interest
- Triplex reagent enables two samples per injection for double the sample throughput
- Scheduled MRMT™ Algorithm<sup>2</sup> enables highest level of MRM multiplexing per injection to monitor more peptides per protein and more proteins in biomarker verification
- Multiplex nanoLC enabled by the Eksigent cHiPLC®-nanoflex (Eksigent Technologies) provides up to a 2x increase in LC throughput in an easy to use set-up (Figure 5)
- Combining all above technologies provides a significant improvement in efficiency of the biomarker verification workflow (Figure 6)



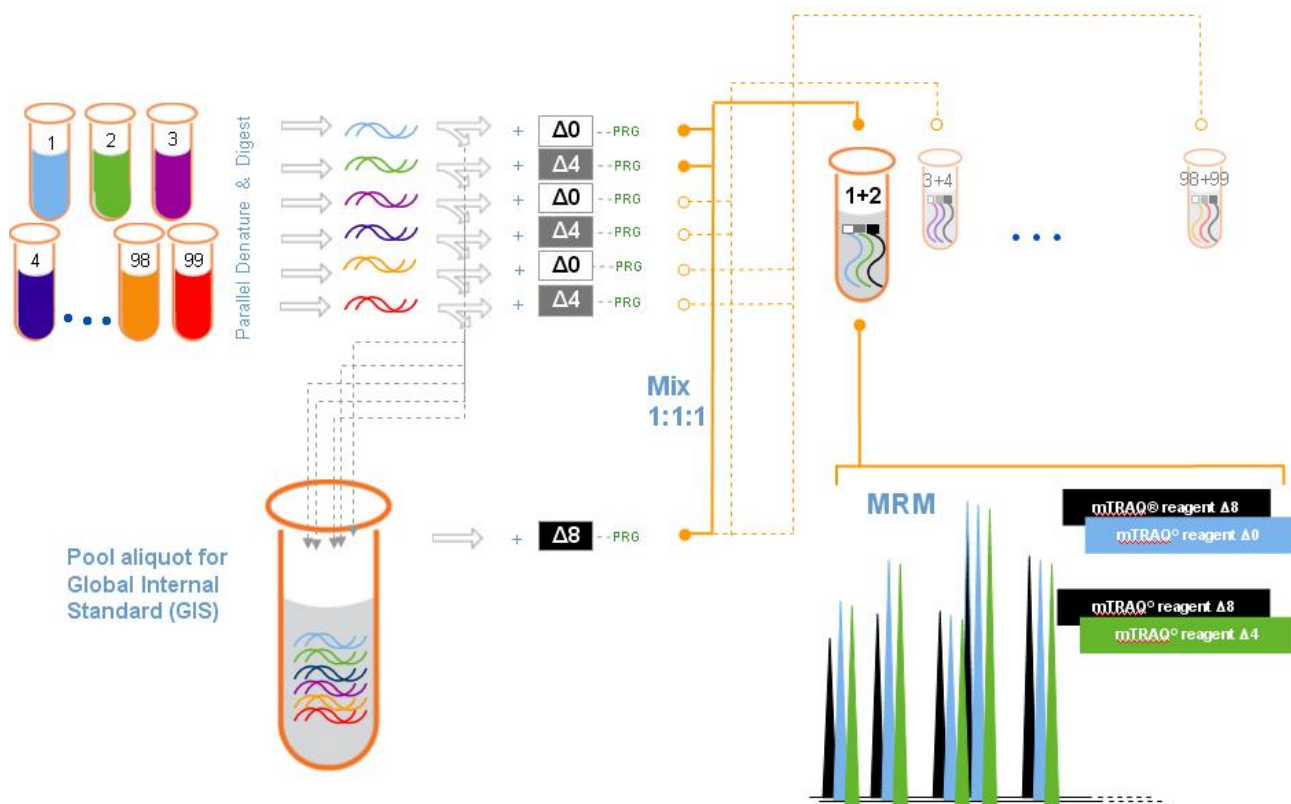
**Figure 1. Whole System Solution for Biomarker Verification Studies:** Putting together the highest efficiency workflow for developing and running MRM assays for proteins / peptides requires an integrated system of hardware, software and reagents.

## Materials and Methods

**Sample Preparation:** 24 different *E. coli* samples were grown under various conditions. Each *E. coli* sample was reduced with TCEP, alkylated with MMTS and digested overnight with trypsin at 37 °C. An aliquot was removed from each individual sample and pooled to create a global internal standard (GIS). The GIS was labeled with the mTRAQ reagent  $\Delta 8$  and each of the individual samples was labeled with either the  $\Delta 0$  or  $\Delta 4$  reagent. The mTRAQ reagent selectively labels the N-terminus and  $\epsilon$ -lysine function of each peptide. Triplex samples were prepared by mixing 1:1:1 an aliquot of the GIS ( $\Delta 8$ ) with two different individual biological samples ( $\Delta 0$  and  $\Delta 4$ ), creating 12 triplex samples. Each sample was cleaned up using a single SCX elution against 500mM ammonium formate.

**Liquid Chromatography:** Peptide separations were performed using the Two-Column Switching with Off-line Column Regeneration on the Eksigent cHiPLC<sup>®</sup>-nanoflex and the Eksigent nanoLC-Ultra<sup>®</sup> system.

**Mass Spectrometry:** Mass spectrometric analysis was performed using the NanoSpray<sup>®</sup> II source interfaced to QTRAP<sup>®</sup> 5500 system (AB SCIEX). MIDAS<sup>™</sup> Workflow acquisition strategy was employed using MRMPilot<sup>™</sup> Software to generate and refine MRM transitions to the proteins of interest. All steps of the workflow were utilized, including optional steps such as collision energy optimization, and time of data acquisition and processing recorded. Final acquisition method was generated using the *Scheduled MRM*<sup>™</sup> Algorithm. Quantitative data processing was performed using MultiQuant<sup>™</sup> software. mTRAQ reagent labeled *E. coli* samples were run in triplicate.



**Figure 2. Global Internal Standard (GIS) Workflow:** All biological samples are prepared in parallel and digested. Then, an aliquot of each sample is taken and mixed together to create the Global Internal Standard. The individual samples are labeled with mTRAQ<sup>®</sup> Reagent  $\Delta 0$  and  $\Delta 4$ , the GIS sample is labeled with the mTRAQ<sup>®</sup> Reagent  $\Delta 8$  and will act as the internal standard. An aliquot of the  $\Delta 8$  labeled GIS is then mixed with a  $\Delta 0$  and  $\Delta 4$  labeled sample at a 1:1:1 ratio. Finally, MRM transitions to all versions of the peptides are monitored and the ratio to the GIS  $\Delta 8$  is computed for each MRM pair. Because of the internal standard, the ratios for all MRM transitions and peptides can be compared between all samples.

## High Throughput Assay Development

Here, a study was undertaken to establish a metric for the efficiency of MRM assay development using MRMPilot™ Software, MIDAS™ Workflow and the mTRAQ® Reagents. Using an *E.coli* sample set, the total time taken to develop 1500 MRM transitions to 125 different peptides (4 MRMs per peptide) was monitored.

The initial input included both previously detected proteins / peptides (ProteinPilot™ Software search results) and a list of proteins of interest (in silico input). Additional peptides to previously detected proteins were also added to increase the number of peptides per protein. Peptides of interest were detected using the MIDAS™ workflow, with MRMs driving the acquisition of full scan MS/MS to confirm detection of desired peptide. From these confirmed peptide detections, an initial set of quantitative MRMs was designed (6 MRMs per mTRAQ reagent labeled peptide) and evaluated for good LC and MS properties. Peptides and MRMs which are interference free and have the best sensitivity were chosen. Collision Energy (CE) optimization was performed on just the peptides with lower signal

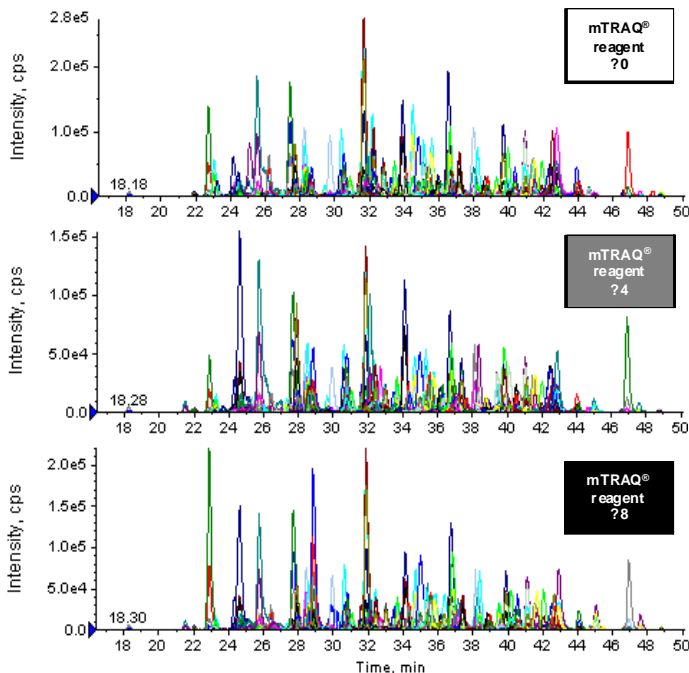
**Table 1. Efficiency of Assay Development:** Shown is the time spent for each step of the assay development. The time includes mass spectrometric sample analysis and data processing using MRMPilot™ Software and MultiQuant™ Software. A total of 62 hrs (2.6 days) was required for developing the final assay consisting of 1500 MRMs, corresponding to a total of 125 peptides. Assay development using this workflow is calculated to be ~48 peptides / day.

Analysis Phase	# of MRM Transitions	Analysis + Data Processing Time (Hrs)
Protein ID + DB Search		3
MIDAS™ Workflow Detection	440	4
MIDAS™ Workflow Verification	1992	20
CE Optimization	1134	15
mTRAQ® Reagent Isotope Conversion	856 (x3 for triplex)	11
Final Assay	1500 (125 peptides)	9
Total Assay Time		62
<b>Speed</b>	<b>48 peptides / day</b>	

intensity (~30) to try to enhance signal and improve data quality (Figure 4 shows the effects of signal intensity on data reproducibility).

This set of good MRM transitions was then converted to MRMs for the three different labeled forms of each mTRAQ® reagent labeled peptide. These MRMs were tested to ensure each labeled form was free of interference and had good reproducibility. The final 4 best MRM transitions to each peptide were chosen for the best 125 peptides and used to build a final *Scheduled MRM™* algorithm acquisition method.

The time required to acquire and process the data was tallied for this assay development process, totaling ~62 hours. This corresponds to peptide MRM assay development throughput (including internal standard MRMs) of about 50 peptides per day. The combination of the MIDAS™ Workflow capabilities, *Scheduled MRM™* Algorithm and the easy-to-use MRMPilot™ Software enable highly multiplexed assay development.

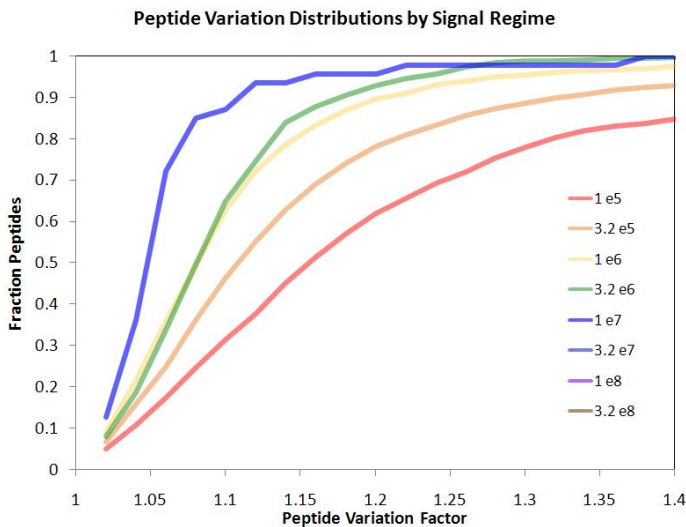


**Figure 3. MRM with the different mTRAQ® reagent labels:** Using an MRM acquisition method created with the *Scheduled MRM™* Algorithm, 1500 MRM transitions to 125 peptides from *E. coli* were monitored across triplicate injections. The peptides were labeled with mTRAQ reagent  $\Delta 0$ ,  $\Delta 4$ , and  $\Delta 8$ , 4 MRM transitions to each reagent version of each peptide were monitored. The  $\Delta 8$  labeled peptides were used as internal standard for normalization purposes.

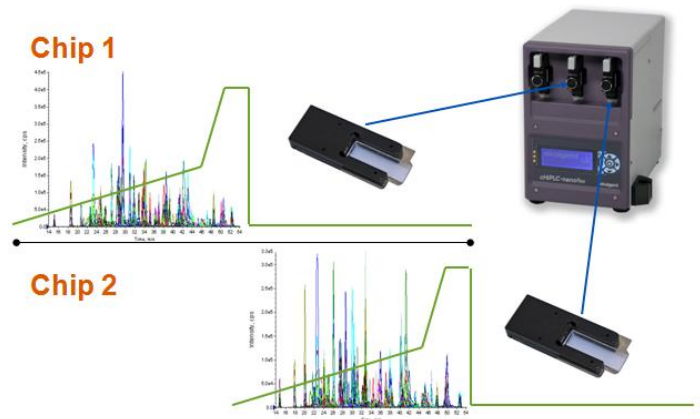
## Reproducibility of MRM Assay

In this study, a total of 1500 MRMs (500 for each mTRAQ reagent) were analyzed per run. Triplicate analysis of 24 biological samples was performed and analyzed. Here, the mTRAQ  $\Delta 8$  reagent labeled peptides act as the global internal standard which provides added robustness to the MRM assay as every peptide now has an internal standard (to correct for analytical variability). This global internal standard strategy provides a cost effective way to generate large numbers of stable isotope labeled peptide standards.<sup>1</sup>

The variation in the data at the peptide level (Figure 4) was determined as a function of MRM peak area and as expected, larger MRM peak areas yield greater reproducibility in the data. For proteins / peptides at lower abundance, the MRM peak areas are reduced and there is more error observed in the peptide level measurements. However, the use of internal standard peptides improves the data quality at the lower signal intensities and allows robust peptide measurements to be obtained on most peptides.



**Figure 4. Cumulative Distributions of Peptide Variation at Different Signal Intensities.** Replicate injections were performed on the same samples to obtain an understanding of the assay reproducibility. The peptide variation factor is the standard deviation of the log peptide ratios across the replicates, transformed into linear space (variation factor =  $10^{(\text{std dev log peptide ratios})}$ ). As MRM peak area increases, the observed variability in the data decreases.



**Figure 5. Two Column Switching with Off-line Regeneration using the Eksigent cHiPLC<sup>®</sup>-nanoflex system:** This easy-to-setup workflow provides up to a 2 fold increase in throughput. One column is used for running the biological sample, while the other column is regenerated offline.

## Multiplexed nanoLC

Most proteomics applications are sample limited and require separations to be carried out at nanoliter flow rates in order to maximize sensitivity during the analysis. The data quality of the LC-MS analysis is greatly dependent on the quality of the peptide separation. As a result, shallow gradients are often necessary to achieve adequate separation of complex peptide mixtures. These gradients are generally, an hour or more and may incorporate column washing and equilibration steps to ensure the lowest carryover. The time spent regenerating the column often significantly adds to the analysis time in serial workflows. If, however, the column regeneration step is performed off-line of the analysis, precious mass spectrometer time can be recouped.

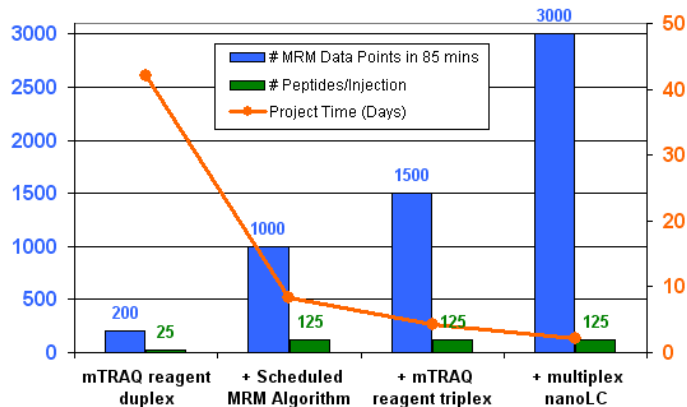
In this study, an Eksigent cHiPLC<sup>®</sup>-nanoflex system is used in a two-column switching mode with the Eksigent nanoLC-Ultra<sup>®</sup> system. The nanoflex is installed with two identical column chips: with the analysis running on the first column, the second column is offline being washed and equilibrated. At the end of the analysis, the nanoflex automatically switches the columns, making the equilibrated column immediately available for the next sample. The column chips have very high chip to chip retention time similarity (<2% RSD) which ensures easy method setup. This LC multiplexing can increase the throughput of MRM analysis by up to 2-fold. The high reproducibility of this multiplexed LC system also allows narrow MRM detection windows to be used in the *Scheduled* MRMTM Algorithm acquisition methods, enabling the creation of very highly multiplexed MRM assays.

## Accelerating MRM Assays

The goal in this test case here was to monitor 125 peptides with 4 fragment ions per peptide plus an internal standard across 24 biological samples, analyzed in triplicate. The acquisition of the final MRM data took 2 days to acquire using the QTRAP® 5500 system with the *Scheduled* MRM™ Algorithm and the Eksigent cHiPLC®-nanoflex / nanoLC-Ultra® system. In contrast, the project would have required much more time using conventional technologies, and the throughput impact of each feature is considered (Figure 6). Using multiplexed LC can increase LCMS throughput from 1.5-2x. Use of the mTRAQ® reagents triplex provides a 2x increase in sample throughput by running two biological samples per injection. Finally, the *Scheduled* MRM™ Algorithm provides a significant increase in throughput enabling all transitions to be run in a single injection. In our efforts to improve the efficiency of biomarker verification assays, we have achieved a 15-20 fold decrease in the time required to complete this biological study.

## Conclusions

- An efficient workflow using both MRMPilot™ and MultiQuant™ Software was demonstrated that decreases the amount of time to develop an MRM assay from a matter of weeks to days, 48 peptides per day. In addition, assay development is performed directly on biological samples, eliminating the need for synthetic peptides or recombinant proteins.
- Acquisition methods created with the *Scheduled* MRM™ algorithm enable much higher MRM multiplexing by intelligent use of peptide elution time, monitoring only peptides that are currently eluting, thus maximizing cycle time.
- The use of one of the triplex mTRAQ® reagents as an internal standard increases the robustness of the MRM assay and doubles project throughput by enabling analysis of two samples per injection.
- Multiplexed LC acquisition with the Eksigent cHiPLC®-nanoflex further doubles throughput by better use of MS time.



**Figure 6. Highly Multiplexed MRM Assays for Highest Throughput:** Running 1500 MRM transitions per acquisition method, the 12 triplex mixed samples (24 biological samples) were run in triplicate on the QTRAP® 5500 system. Using the Eksigent cHiPLC®-nanoflex in two column switching mode, all data were acquired (54000 MRM data points) in ~2 days of acquisition time. Combining the *Scheduled* MRM Algorithm with mTRAQ reagent triplex and multiplex LC provides a 15-20x increase in total project throughput.

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3. MRMPilot™ Software: Developing MRM Assays for Peptide Quantitation. AB SCIEX Technical Note, Publication 0960210-01.
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