

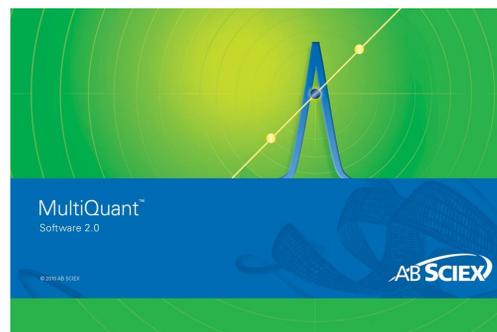
# MultiQuant™ Software 2.0 for Targeted Protein / Peptide Quantification

## Gold Standard for Quantitative Data Processing

Because of the sensitivity, selectivity, speed and throughput at which MRM assays can be performed, they have emerged as the definitive analytical tool for drug quantification in discovery, pre-clinical and clinical studies. For proteomics studies, MRM assays are now the method of choice for monitoring proteins and peptides in biological samples.

Multiple Reaction Monitoring (MRM) is a mass spectrometry experiment that allows very sensitive and specific quantification of targeted analytes. MRM experiments require triple quadrupole systems as described in Figure 1. The QTRAP® system, which is a hybrid triple quadrupole linear ion trap instrument, can also perform these experiments because the Q3 can function as a linear ion trap or as a standard quadrupole. Because there are no compromises in performance in either the linear ion trap mode or the standard quadrupole mode for Q3, the QTRAP system is capable of very sensitive and selective quantitative MRM assays as well as very sensitive qualitative studies.

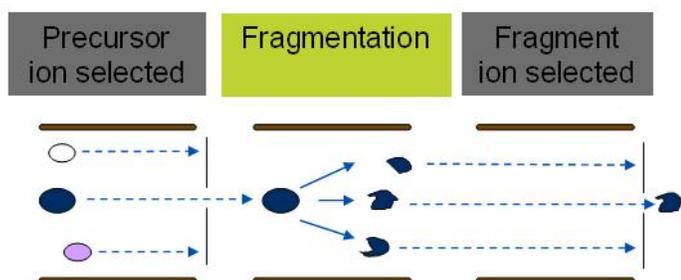
An MRM experiment can be performed on a very fast time scale. Because of the LINAC® collision cell unique to AB SCIEX instruments, very short dwell times can be used for each transition, as low as 2-5 ms, with good sensitivity. Often many MRM transitions are monitored simultaneously in a single LC/MS/MS run, representing the analysis of many different components in a mixture. For assays that require monitoring of very large numbers of transitions (1000-2500 MRMs), acquisition methods can be created using the *Scheduled* MRM™ Algorithm<sup>4</sup> where experiments can be performed which allow retention time acquisition windows to be associated with each MRM transition.



A key component of the MRM assay workflow is the software for processing MRM data and providing quantitative information. MultiQuant™ Software is a powerful quantification package that enables many analytes and samples to be processed all at the same time with additional support for protein / peptide quantitative workflows.

## Key Features of MultiQuant™ Software

- Supports relative and absolute quantification of peptides for biomarker verification / peptide quantification workflows.
- Supports use of both unlabeled or stable isotope labeled peptide internal standards for relative MRM quantification.
- Handles large data sets consisting of both large numbers of MRM transitions, including *Scheduled*™ MRM Algorithm datasets and large numbers of biological samples.
- Features a comprehensive user interface for superior data visualization
- Parsing of MRM ID information from Analyst® Software to automatically create quantification methods.
- Innovative data integration algorithms (MQ4 and SignalFinder™ Algorithm<sup>6</sup>) for robust automated peak review and data queries to reduce and simplify manual review.
- Powerful regulatory compliance and audit trail features if required<sup>5</sup>.
- Report generation and exporting results to other software packages (e.g. LIMS and Microsoft Excel).



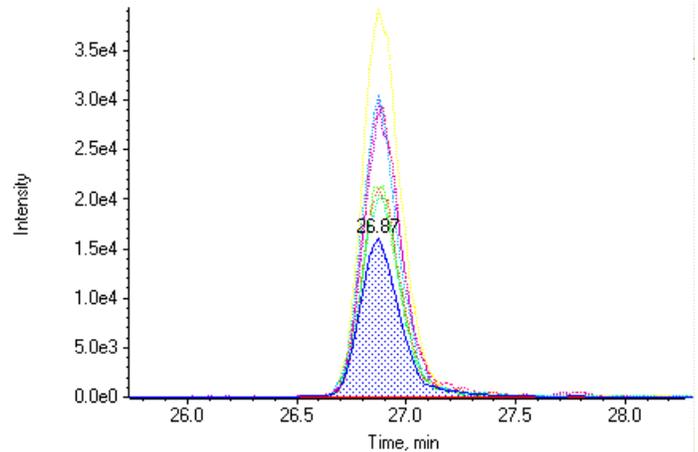
**Figure 1. MRM Experiment.** Q1 is set to transmit only the parent  $m/z$  of the peptide, the collision energy is optimized to produce a diagnostic charged fragment of this peptide in Q2, and Q3 is set to transmit this diagnostic fragment only.

## Intuitive User Interface for Efficient Data Review

Significant advancements in the use of MRM assays for quantitative proteomics have driven the need for intuitive, easy-to-use software, powerful enough to handle the large amounts of proteomics data. Major advancements in the processing and review of MRM data have been made in MultiQuant™ Software.

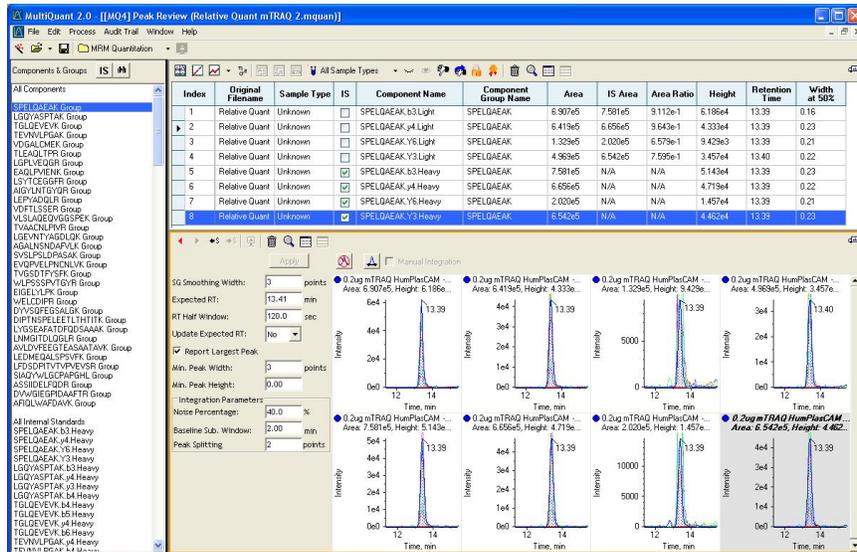
The interface is organized such that important information is at the user's finger tips (Figure 2). The navigation window on the left makes it easy to move from one Peptide Group to the next, display results from internal standards or from analytes only, or review specific transitions. Panes are linked so selecting on one pane will automatically update information on all open panes. Complete flexibility of the workspace allows the user to fine tune the display to the specific data processing required.

While the automated MRM peak integration is based on the industry leading MQ4 algorithm or the novel new SignalFinder Algorithm<sup>6</sup>, many additional tools are available to the user for manual data review if required. Sorting MRM data by the Quality or Signal/Noise columns enables focused review on only the lowest scoring MRM transitions to speed data review. Additional queries are also available to interrogate the data for peak quality, peak ratios, etc, again to minimize peak review. Overlay view of multiple MRM transitions for the same species (Figure 3) allows



**Figure 3. Flexible Peak Review Tools.** Peak overlay to improve manual data review.

quick assessment of peak shape to scan for possible peak interferences in selected channels. Many other ease-of-use features have been implemented with the main goal of minimizing mouse clicks and reducing the data review bottleneck in extracting the quantitative information from every sample set.

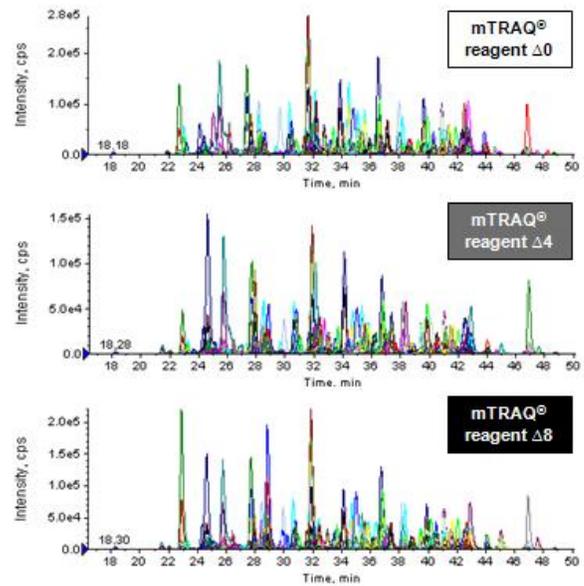


**Figure 2. Intuitive User Interface.** The Peak Review window in MultiQuant Software provides an intuitive, customizable user interface for data review. In this figure, the first peptide group is selected: the important information for that peptide is displayed in tabular form above and the raw MRM peaks are displayed in the bottom pane.

## Relative Quantitation using MultiQuant™ Software

Often it is desirable to understand to what extent a set of specific analytes are changing across different samples. In relative quantification experiments, the MRM peak areas from multiple analytes can be determined across multiple samples and used to compare the relative amounts of analyte present in each sample. This can be done using raw MRM peak areas or by using labeled internal standards, such as non-isobaric reagent labeled samples (Figure 4), and assessing MRM peak area ratios for more accurate calculations. MultiQuant Software is capable of processing large relative quantification proteomics datasets.

For the quantitative MRM analysis of peptides, multiple MRM transitions per peptide are typically monitored to improve the quantitative robustness of the assay. MultiQuant Software provides the ability to process MRM transitions as part of a Peptide Group, consisting of multiple transitions for a peptide as well as the corresponding internal standard transitions, automatically computing all the relevant peptide ratios. Selecting the Group from the navigation bar makes it very easy to immediately see all of the data for one peptide at the same time. Figure 2 shows the selection of a peptide group; all ratios are calculated automatically but the user has the option of manually adjusting integration parameters for specific peaks if desired.



**Figure 4. Relative Quantitation using Non-Isobaric Triplex Amine Labeling Reagents.** In relative quantitation experiments, MRM peak areas for specific proteins/peptides are monitored across many samples and the relative peak areas or ratios to an internal standard are compared. In this example, 1500 MRM transitions to proteins in *E. coli* are monitored.

## Automatic Creation of Quantitation Methods

In the Analyst Software 1.5 and later, there is an ID column in the acquisition method where individual MRM transitions can be defined (i.e. Protein.Peptide.Fragment.IS Indicator). This information is used by MultiQuant Software to automatically create quantitation methods. Peptide groups are automatically configured and peak elution time is automatically determined from the co-elution of the multiple MRMs per peptide.

|    | Q1 Mass | Q3 Mass | Time | ID                            | CE (volts) |
|----|---------|---------|------|-------------------------------|------------|
| 9  | 771.9   | 758.4   | 21.8 | CFAH.DGWSAQPTCIK.2y5.light    | 44         |
| 10 | 771.9   | 499.2   | 21.8 | CFAH.DGWSAQPTCIK.2b3.light    | 44         |
| 11 | 771.9   | 560.3   | 21.8 | CFAH.DGWSAQPTCIK.2y3.light    | 44         |
| 12 | 771.9   | 586.3   | 21.8 | CFAH.DGWSAQPTCIK.2b4.light    | 44         |
| 13 | 775.9   | 762.4   | 21.8 | CFAH.DGWSAQPTCIK.2y5.heavy    | 44         |
| 14 | 775.9   | 503.2   | 21.8 | CFAH.DGWSAQPTCIK.2b3.heavy    | 44         |
| 15 | 775.9   | 564.3   | 21.8 | CFAH.DGWSAQPTCIK.2y3.heavy    | 44         |
| 16 | 775.9   | 590.3   | 21.8 | CFAH.DGWSAQPTCIK.2b4.heavy    | 44         |
| 17 | 597.0   | 456.3   | 22.1 | CFAB.VSEADSSNADWVTK.3b3.light | 35         |
| 18 | 597.0   | 642.3   | 22.1 | CFAB.VSEADSSNADWVTK.3b5.light | 35         |
| 19 | 597.0   | 527.3   | 22.1 | CFAB.VSEADSSNADWVTK.3b4.light | 35         |
| 20 | 597.0   | 729.3   | 22.1 | CFAB.VSEADSSNADWVTK.3b6.light | 35         |
| 16 |         |         |      | CFAH.DGWSAQPTCIK.2b4.heavy    | 44         |
| 17 |         |         |      | CFAB.VSEADSSNADWVTK.3b3.light | 35         |
| 18 |         |         |      | CFAB.VSEADSSNADWVTK.3b5.light | 35         |
| 19 |         |         |      | CFAB.VSEADSSNADWVTK.3b4.light | 35         |
| 20 |         |         |      | CFAB.VSEADSSNADWVTK.3b6.light | 35         |
| 21 |         |         |      | CFAB.VSEADSSNADWVTK.3b3.heavy | 35         |
| 22 |         |         |      | CFAB.VSEADSSNADWVTK.3b5.heavy | 35         |

| Name                          | Group               | IS Name                       | Q1 / Q3       | RT (min) |
|-------------------------------|---------------------|-------------------------------|---------------|----------|
| fCIK.2y5.light                | CFAH.DGWSAQPTCIK    | CFAH.DGWSAQPTCIK.2y5.heavy    | 771.9 / 758.4 | 22.20    |
| fCIK.2b3.light                | CFAH.DGWSAQPTCIK    | CFAH.DGWSAQPTCIK.2b3.heavy    | 771.9 / 499.2 | 22.20    |
| fCIK.2y3.light                | CFAH.DGWSAQPTCIK    | CFAH.DGWSAQPTCIK.2y3.heavy    | 771.9 / 560.3 | 22.20    |
| fCIK.2b4.light                | CFAH.DGWSAQPTCIK    | CFAH.DGWSAQPTCIK.2b4.heavy    | 771.9 / 586.3 | 22.20    |
| fCIK.2y5.heavy                | CFAH.DGWSAQPTCIK    |                               | 775.9 / 762.4 | 22.20    |
| fCIK.2b3.heavy                | CFAH.DGWSAQPTCIK    |                               | 775.9 / 503.2 | 22.20    |
| fCIK.2y3.heavy                | CFAH.DGWSAQPTCIK    |                               | 775.9 / 564.3 | 22.20    |
| fCIK.2b4.heavy                | CFAH.DGWSAQPTCIK    |                               | 775.9 / 590.3 | 22.20    |
| CFAB.VSEADSSNADWVTK.3b3.light | CFAB.VSEADSSNADWVTK | CFAB.VSEADSSNADWVTK.3b3.heavy | 597.0 / 456.3 | 22.70    |
| CFAB.VSEADSSNADWVTK.3b5.light | CFAB.VSEADSSNADWVTK | CFAB.VSEADSSNADWVTK.3b5.heavy | 597.0 / 642.3 | 22.70    |
| CFAB.VSEADSSNADWVTK.3b4.light | CFAB.VSEADSSNADWVTK | CFAB.VSEADSSNADWVTK.3b4.heavy | 597.0 / 527.3 | 22.70    |
| CFAB.VSEADSSNADWVTK.3b6.light | CFAB.VSEADSSNADWVTK | CFAB.VSEADSSNADWVTK.3b6.heavy | 597.0 / 729.3 | 22.70    |
| CFAB.VSEADSSNADWVTK.3b3.heavy | CFAB.VSEADSSNADWVTK |                               | 599.6 / 460.3 | 22.70    |
| CFAB.VSEADSSNADWVTK.3b5.heavy | CFAB.VSEADSSNADWVTK |                               | 599.6 / 646.3 | 22.70    |

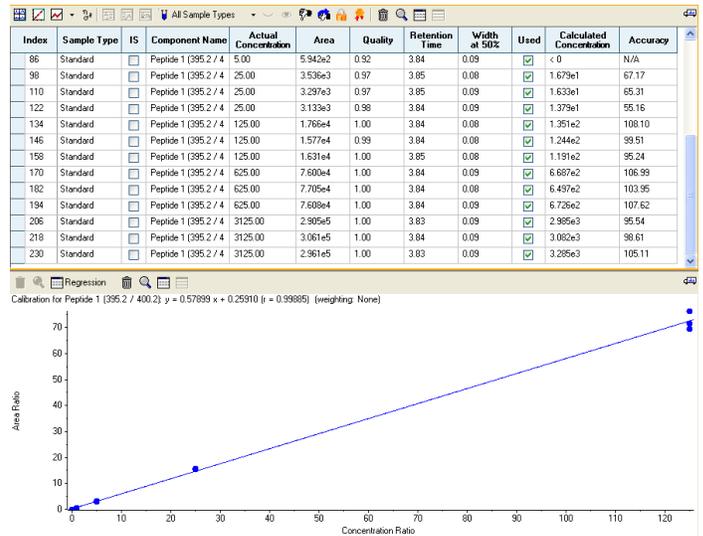
**Figure 5. Automatic Creation of Quantitation Methods.** Using the pre-defined naming convention for proteins and peptides in the Analyst Software MRM method ID column (top) allows MultiQuant Software to automatically build quantitation methods (bottom). This naming also allows multiple MRM transitions per peptide to be considered as part of a single analyte group for automatic retention time determination and interference analysis.

## Absolute Quantitation using MultiQuant™ Software

Absolute quantitation of analytes is also possible using MultiQuant Software. In absolute quantitation experiments, a standard concentration curve is generated using internal standard peptides and the amount of analyte present in each sample is determined by comparing to the curve. Typically stable isotope labeled peptides are created using stable isotope labeled amino acids and used as internal standards.

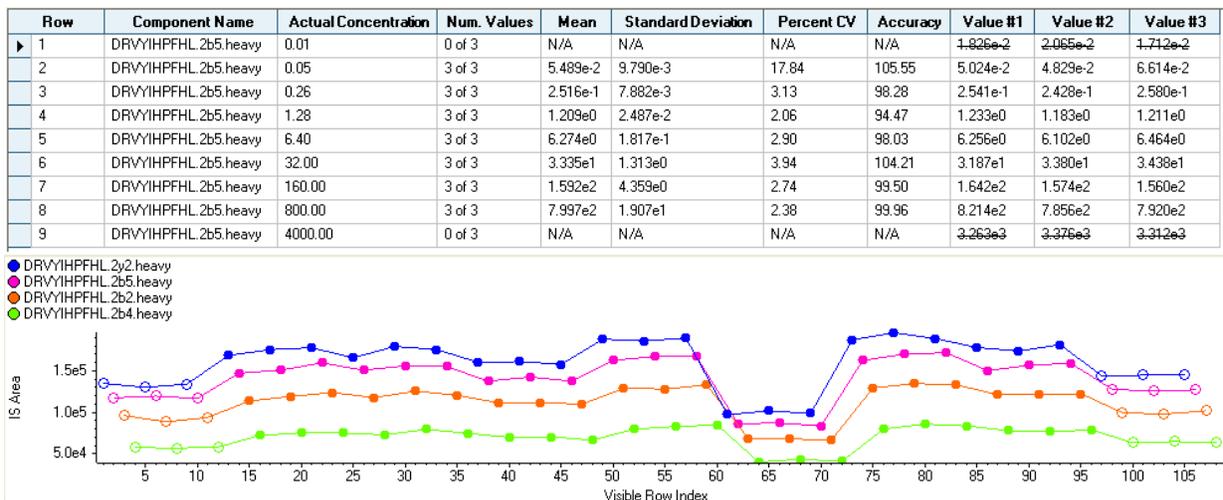
Standard concentration curves are easy to generate in the software (Figure 6). Peaks can be manually added or removed from the calibration curve to determine the correlation coefficient and quantification accuracy. A statistics table is available to quickly assess the quality of the standard concentration curve, where parameters such as the %CV and accuracy are automatically computed. (Figure 7, top).

Once the calibration curve is generated, the biological samples (unknowns) can be processed. In these samples, the amount of the endogenous 'light' peptide present in each sample is measured. To add robustness to the quantification experiment, the 'heavy' peptide can be added into each biological sample at a fixed amount to act as the internal standard for these samples. The standard concentration curve generated to the heavy peptide can be easily linked to the unknown samples. The actual concentration of peptide in the biological sample can be computed from the ratio of the endogenous peptide (light) to the added internal standard (heavy). Once the calibration file is loaded, the Calculated Concentration and Accuracy columns are automatically computed for all the unknown samples.



**Figure 6. Absolute Quantification.** For absolute quantitation, a standard concentration curve is created from the analysis of known concentrations of a labeled version of a peptide of interest. To determine the amount of peptide in a biological sample, the MRM response is compared to the standard concentration curve.

Finally, metric plots can be instantly created from the results table to easily assess data quality. As an example (Figure 7, bottom), the change in area of the internal standard for one peptide is plotted versus injection number. Multiple peptides or multiple MRMs can be plotted by selecting multiple rows from the left hand navigation bar.



**Figure 7. Statistics Table and Metric Plots.** With single button clicks, useful tables of statistics (top) or informative metric plots (bottom) can be quickly generated for easy data assessment.

## Importing and Exporting of Data

In addition to being easy to use, MultiQuant™ Software is extremely flexible for importing and exporting data. Quantification methods can be created when using MRMPilot™ Software<sup>2</sup> to create acquisition methods. Results can be visualized within the software or easily exported to use in other software packages (e.g. Excel). In addition, flexible reporting capabilities are available to create user-specific reports.

## Conclusions

MultiQuant™ Software provides a comprehensive package for quantification of peptides/proteins using MRM assays. It supports the MIDAS™ Workflow, *Scheduled* MRM™ assays, and labeled and unlabeled workflows for relative and absolute peptide quantification. Because of its ability to support many samples, peptides, and MRM transitions, the software is ideal for biomarker verification assays, tracking changes in post-translational modifications (e.g., phosphorylation) across different samples, biological pathway analysis, quantification of biologics / protein therapeutics and other targeted peptide quantitative assays.

## References

1. To download a trial version of MultiQuant Software please visit: [http://www.absciex.com/mk/get/Software\\_downloads](http://www.absciex.com/mk/get/Software_downloads)
2. MRMPilot™ Software: Developing MRM Assays for Peptide Quantitation. AB SCIEX Technical Note, Publication 0960210-01.
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