MultiQuant™ 3.0.3 Software

Peptide Quantitation Tutorial

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Contents

Peptide Quantitation 1

Objectives

This tutorial describes how to use the MultiQuant™ software to quantitatively process MRM data acquired on any QTRAP® system or triple quadrupole system.

The MultiQuant™ software can be used to process data from many different types of quantitative MS-based experiments. While the software is very flexible and allows for many different types of experiments to be processed, an overview of two main workflows is provided in this tutorial. In particular, only MRM workflows are discussed although the program can also be used to process scan mode data.

For more information about the software features, refer to the *Reference Guide* that is installed with the MultiQuant™ software. This guide provides a description of the functionality available in the MultiQuant™ software.

The two main types of quantitative analysis that are covered in this tutorial are relative quantitation and accurate quantitation.

To learn about relative quantitation, go to:

- Relative Quantitation Using the MultiQuant™ Software on page 8
- Process Data Acquired Using the *Scheduled* MRM™ Algorithm on page 25

To learn about accurate quantitation, go to:

- Accurate Quantitation Using Calibration Curves on page 37

Related Documentation

The MultiQuant™ software documentation can be found under the MultiQuant™ software Help menu. The Analyst® software documentation can be found under the **Start** menu: (On Windows 7 operating system) **All Programs > SCIEX > Analyst** or (On Windows 10 operating system) **All apps > SCIEX Analyst > Analyst Documentation**.

- *Reference Guide* for the MultiQuant™ software
- *Laboratory Director's Guide* for the Analyst® software
- *Standard Quantitation Tutorial* for the Analyst® software
- *Getting Started Guide* for the Analyst® software

- Instrument operator's manual for your instrument (if you are using a pre 1.5 version of the Analyst® software)

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Technical Support

SCIEX and its representatives maintain a staff of fully-trained service and technical specialists located throughout the world. They can answer questions about the system or any technical issues that might arise. For more information, visit the website at sciex.com.

Example Files

Data files used in this tutorial are provided with the software and can be found in the following folder:

- For Windows 7 and Windows 10: C:\ProgramData\AB SCIEX\MultiQuant\Example Data
  - Tutorial Dataset Heavy_Light.wiff is the file for the relative quantitation experiment
  - mTRAQ HumPlas Apo sMRM 1.wiff is the file acquired using the *Scheduled* MRM™ algorithm
  - Pep Quant Curve 1.wiff and Pep Quant Samples A.wiff are the two files for the accurate quantitation experiment

Copy the files to the <drive>:\Analyst Data\Projects\Example\Data\Example Data folder.

About Calibration Curves

A calibration curve (also known as a standard concentration curve) is a method for determining the concentration of a substance in an unknown sample by comparing the unknown sample to a set of standard samples of known concentration. The calibration curve is a plot of how the instrument responds (the analytical signal) to changes to the concentration of the analyte (the substance to be measured). The user prepares a series of standard samples across a range of concentrations near the expected concentration of the analyte in the unknown sample.

About the MultiQuant™ Software

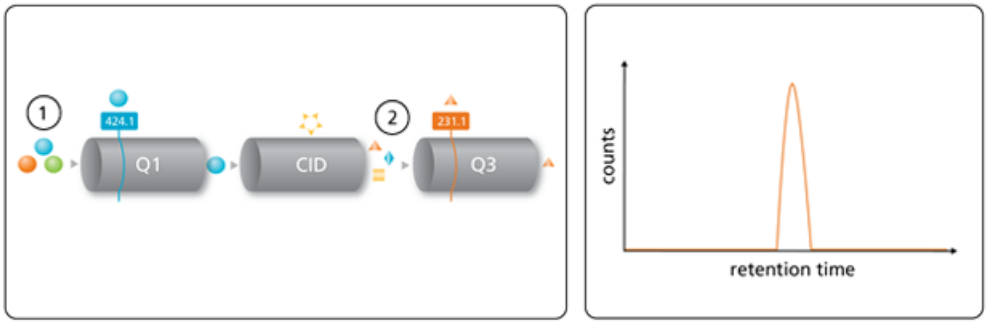
MultiQuant™ software has been specifically developed for users that have large numbers of MRM transitions or samples in their acquisition methods, although it can also be used effectively with acquisition methods that have smaller numbers of MRM transitions. The user interface has been designed to simplify processing of these data files. This software is also compatible with the MIDAS™ Workflow. Although the examples presented in this document all use proteomics data, the software is also useful for processing small molecule data.

MultiQuant™ software has the added ability to deal with stable isotope labeled components in a seamless manner. This type of work is done routinely in protein/peptide quantitation but also applies to other applications.

What is Multiple Reaction Monitoring?

In Multiple Reaction Monitoring (MRM), Q1 is set to transmit only the precursor m/z of the peptide or other molecule, 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. Because of the short dwell times required (5 ms to 50 ms) and the ability to change rapidly between MRM transitions, many components (transitions) in a mixture can be monitored simultaneously in a single LC-MS/MS run.

Figure 1-1 Multiple Reaction Monitoring

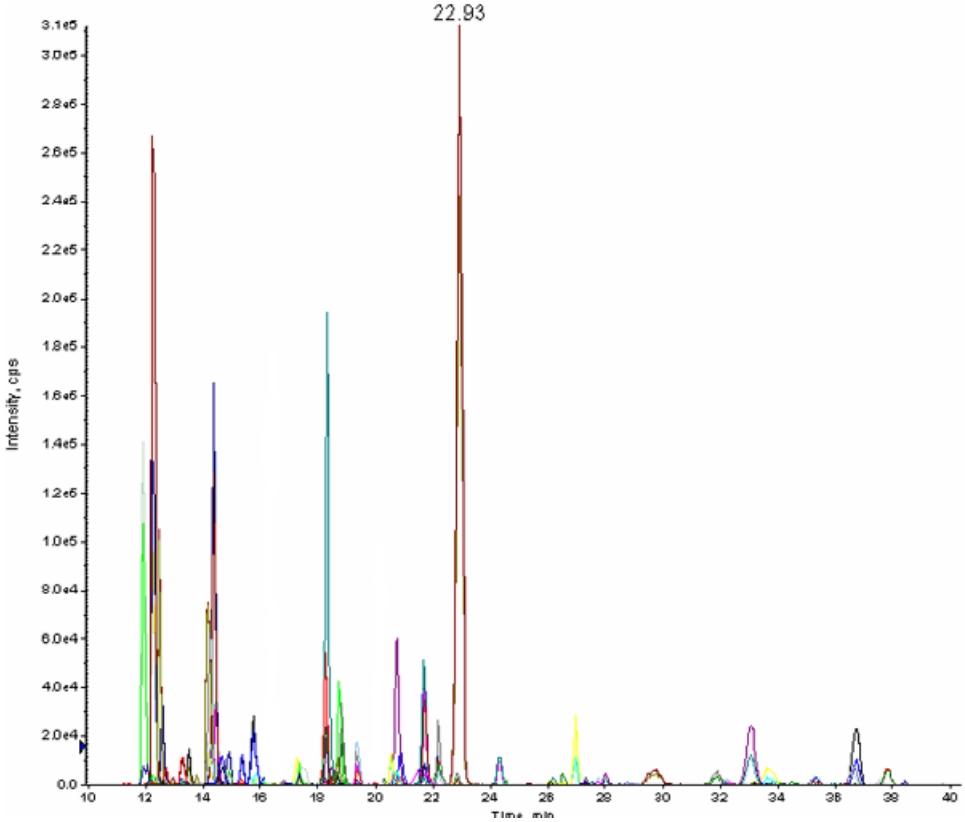


Item	Description
1	Molecule
2	Fragment

Relative Quantitation

In relative quantitation experiments, the MRM peak areas from multiple MRMs can be determined across multiple samples and used to compare the relative amounts of analyte present in each sample. Refer to Figure 1-2. This can be done using raw MRM peak areas or by including isotope-coded internal standards and determining MRM peak area ratios. The example in this tutorial covers the case where there is an isotope-coded internal standard for each analyte of interest and the ratios between the analyte and the internal standard for each MRM are determined and compared between three samples.

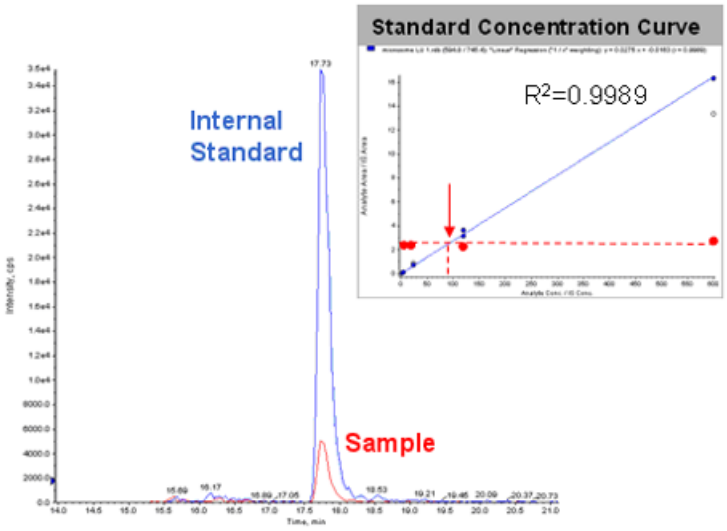
Figure 1-2 Example of Relative Quantitation Experiments



Absolute Quantitation

In absolute quantitation experiments, the analyte of interest is used to generate a calibration curve to characterize the MS response of each analyte. Refer to Figure 1-3. This can be done with the analyte alone or with an additional isotope-coded version of the analyte that has an identical MS response. A calibration curve is generated and the amount of analyte present in each sample is determined by comparing to the curve.

Figure 1-3 Example of an Absolute Quantitation Experiment



Relative Quantitation Using the MultiQuant™ Software

In this section, you will learn how to process the MRM data from a relative quantitation experiment in which there is a heavy labeled peptide internal standard for every peptide of interest. First, the peak integration parameters are set before quantitative processing to avoid any reprocessing of data.

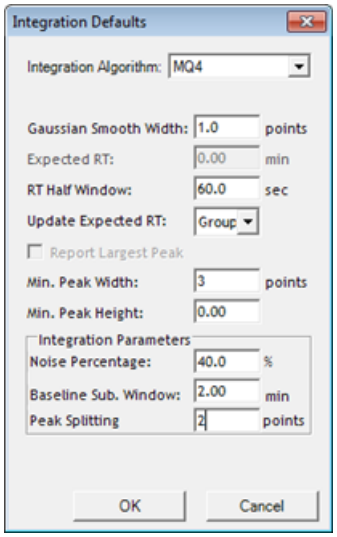
Before you begin

- Make sure you are in the Example project.
- Make sure the Tutorial Dataset Heavy_Light.wiff file is in the <drive>:\Analyst Data\Projects\Example\Data\Example Data folder.

Set the Peak Integration Parameters

1. In the Analyst® software, on the **Navigation** bar, under **Companion Software**, double-click **MultiQuant 3.0.3**.
2. Click **Edit > Project Integration Defaults**.
3. In the Integration Defaults dialog, select **MQ4** from the **Integration Algorithm** list. Good peak integration parameters for this dataset are shown in Figure 1-4.

Figure 1-4 Integration Defaults Dialog



In many cases a smaller smoothing width (for example, 1.0) might be more appropriate. Also note that the peak review process is eased by specifying a non-zero Min. Peak Height, typically corresponding to at least one or two counts. Because the entered threshold should be in counts per second (cps) the actual value required depends on the dwell time. For example, for a dwell time of 100 ms, two counts corresponds to a setting of 20 cps.

When analyzing compounds for which there are multiple MRM transitions and which have been assigned to groups, the Group option of the Updated Expected RT parameter is generally the best choice. In this case, the expected retention time (RT) is updated using the position of maximum overlap of the individual chromatograms for the group (for each sample separately) within the RT window.

4. Adjust the values and then click **OK**.

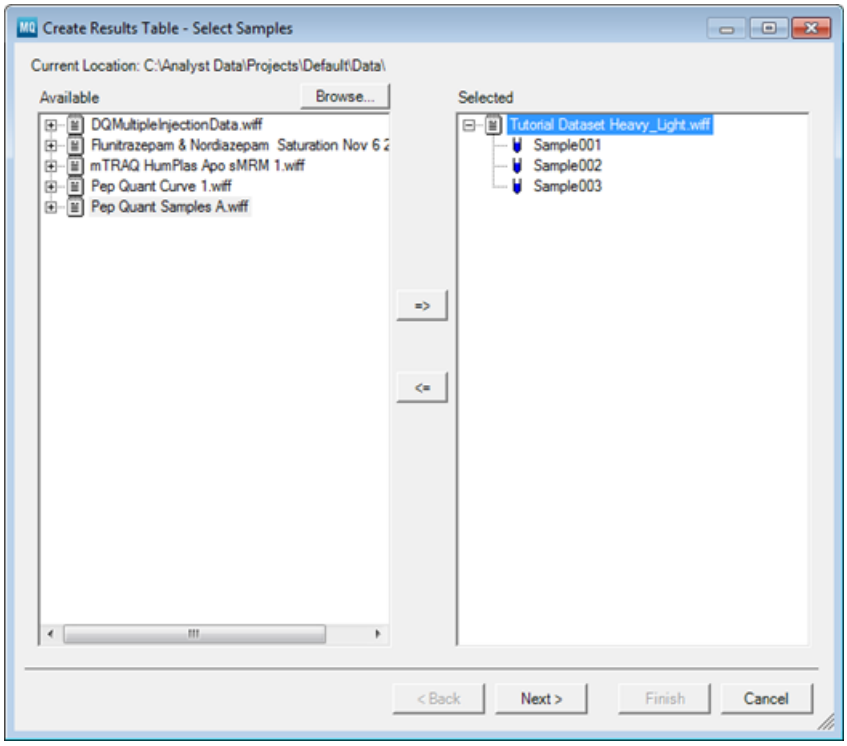
Create a Results Table

Quantitation methods include a set of instructions on how to quantitate the peaks selected for integration. In this tutorial, you will create a quantitation method at the same time that you create a Results Table. Use the following procedures to create a Results Table.

Note: If you are not using the Audit Trail with Security Features edition, then you can launch the MultiQuant™ software directly from the desktop icon.

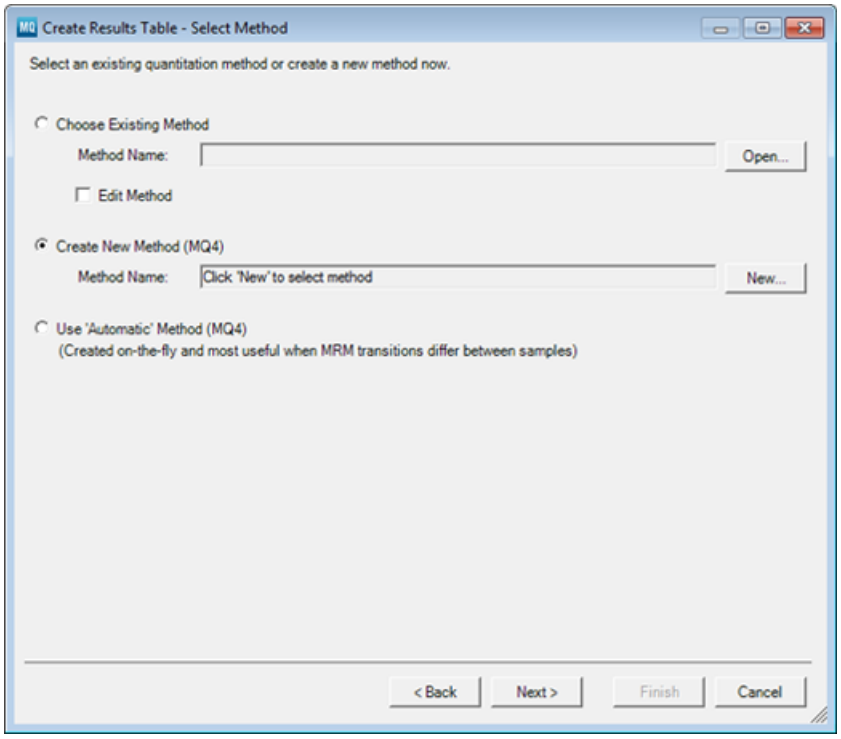
1. Click **File > New Results Table**.
2. On the Create Results Table - Select Samples page, drag the Tutorial Dataset Heavy_Light.wiff file into the **Selected** pane and then click **Next**.

Figure 1-5 Create Results Table - Select Samples Page



3. On the Create Results Table - Select Method page, click the **Create New Method (MQ4)** option and then click **New**.

Figure 1-6 Create Results Table - Select Method Page



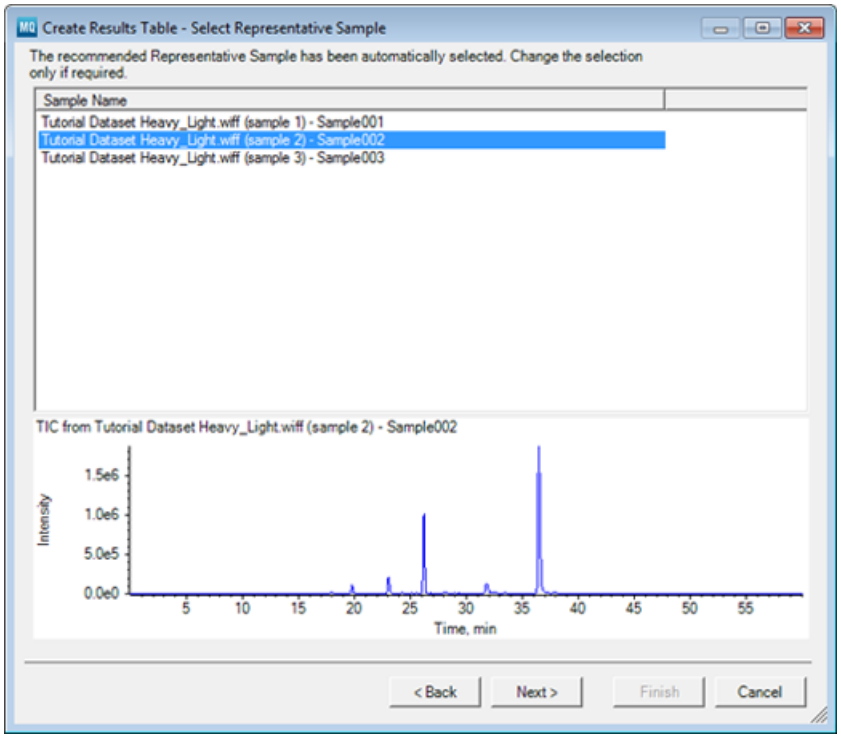
4. Type a name for the method, click **Save**, and then click **Next**.

   In this tutorial, you will create a method. Creating methods gives you an opportunity to review and apply different parameters for the integration of your data.

5. On the Create Results Table - Select Representative Sample page, a representative sample has been recommended and is selected. Click **Next**.

   The software recommends a representative sample based on selecting a chromatogram that provides the best opportunity to select integration parameters that fit the entire batch.

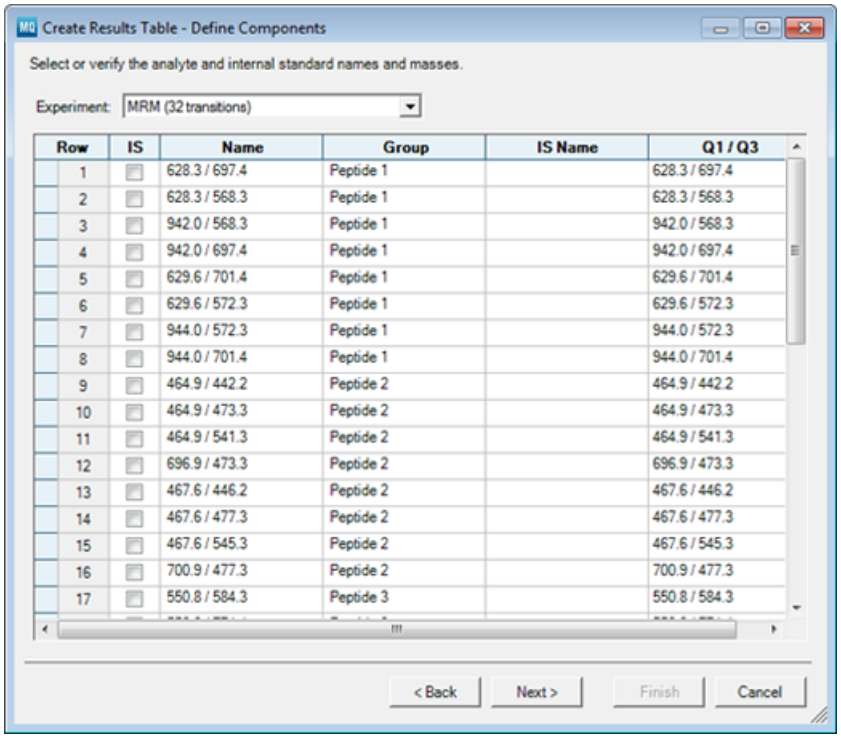
Figure 1-7 Create Results Table - Select Representative Sample Page



On the Create Results Table - Define Components page, the details of the various analytes and internal standards to be processed must be defined. All the MRM transitions from the data file are automatically loaded into the window.

6. On the Create Results Table - Define Components page, define all Group members by giving them the same name. In this example, there are four groups, each consisting of eight MRMs. The group names can be typed into the Group column or copied in from an Excel spreadsheet. Refer to Figure 1-8.
7. Type the peptide name in the first row only of each group (row 1, 9, 17, 25), and then right-click in the table and click **Groups > By Filling Down Existing Groups**. In this example, the first eight MRMs are named Peptide 1, the second eight rows are named Peptide 2, the third eight are named Peptide 3, and the final eight rows are named Peptide 4.

Figure 1-8 Create Results Table - Define Components Page



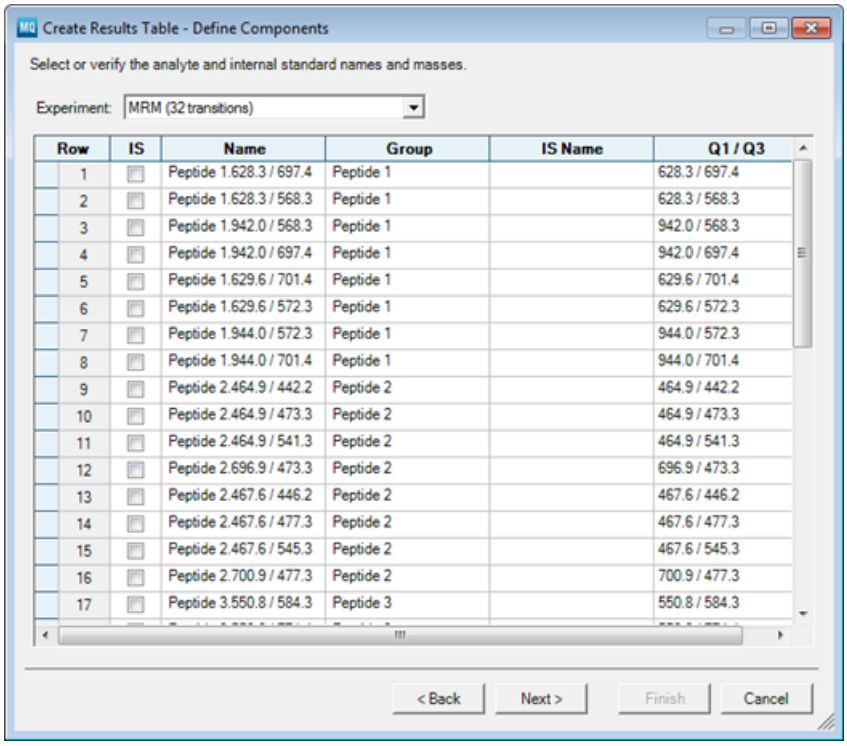
The Groups can be named in many ways, depending on the compounds. Examples of group nomenclature are: using the peptide sequence as the group name or using the Protein name_Peptide sequence.

Note: When you group MRM transitions together, they should correspond to the same compound, including the internal standard version of that compound.

8. Copy the Group column into the Name column. Right-click in the table and then click **Groups > Add Group to Start of Component Name**.

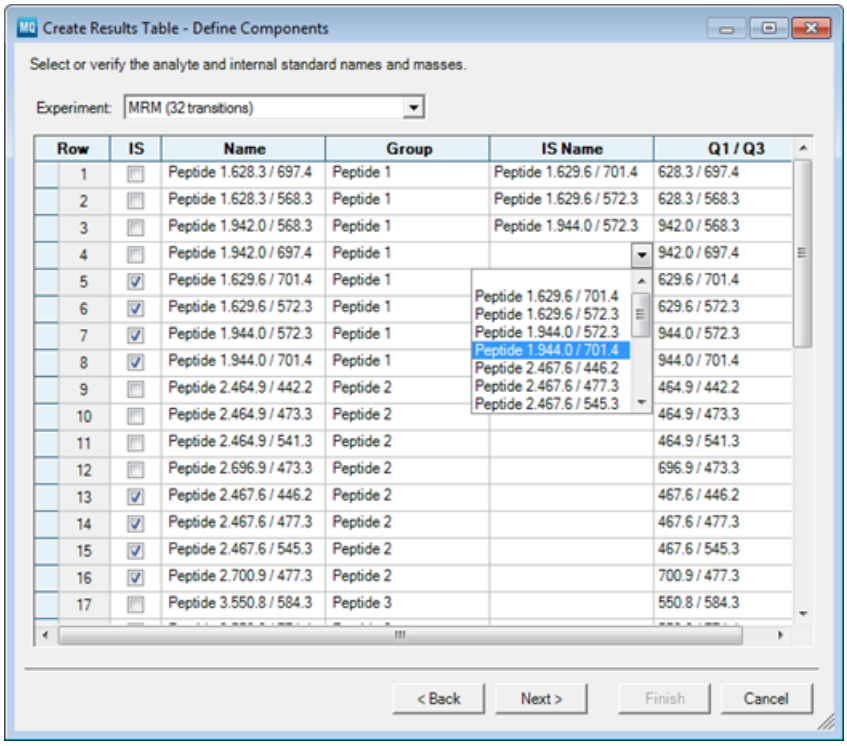
   The Name and Group columns are shown in Figure 1-9.

Figure 1-9 Name and Group Columns



9. Define the MRM transitions to be used as the internal standards for Peptide 1 only by selecting the check boxes in the **IS** column as shown in Figure 1-10. In this example, the last four transitions in each group are the internal standards for the first four transitions in the group.
10. After an MRM transition has been defined as an internal standard, it can be associated with the correct analyte in the **IS Name** column. Click the right side of each row within this column and then select the internal standard from the list that is shown in Figure 1-10.
11. Select the appropriate internal standard from the list for each of the first four MRM transitions for Peptide 1. Within the group, the fifth MRM is the IS for the first row, the sixth MRM is the IS for the second row, and so on.

Figure 1-10 Associating Internal Standards

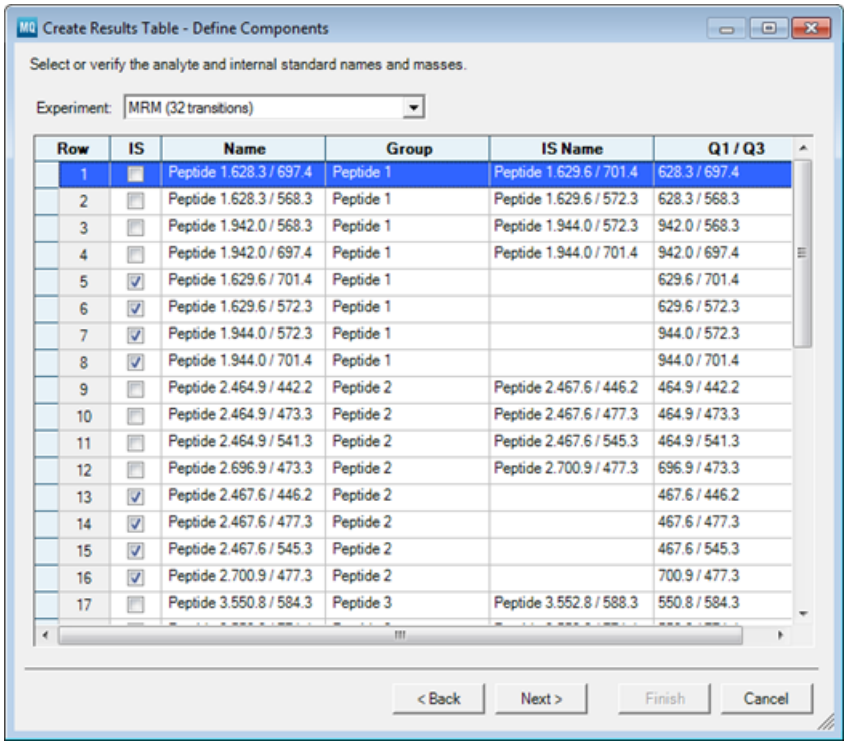


12. To propagate this format to all the groups in the data file, select the first row for Peptide 1, right-click and then click **Internal Standards > Set for all Groups as for Selected Group**.

Note: The pattern of sample and IS MRMs must be constant throughout the data file to be able to propagate the set pattern. This reduces the manual interaction required when setting up the processing method.

The structure of the Peptide 1 group is applied to Peptide 2, 3 and 4 groups as shown in Figure 1-11.

Figure 1-11 Set for all Groups as for Selected Group Option



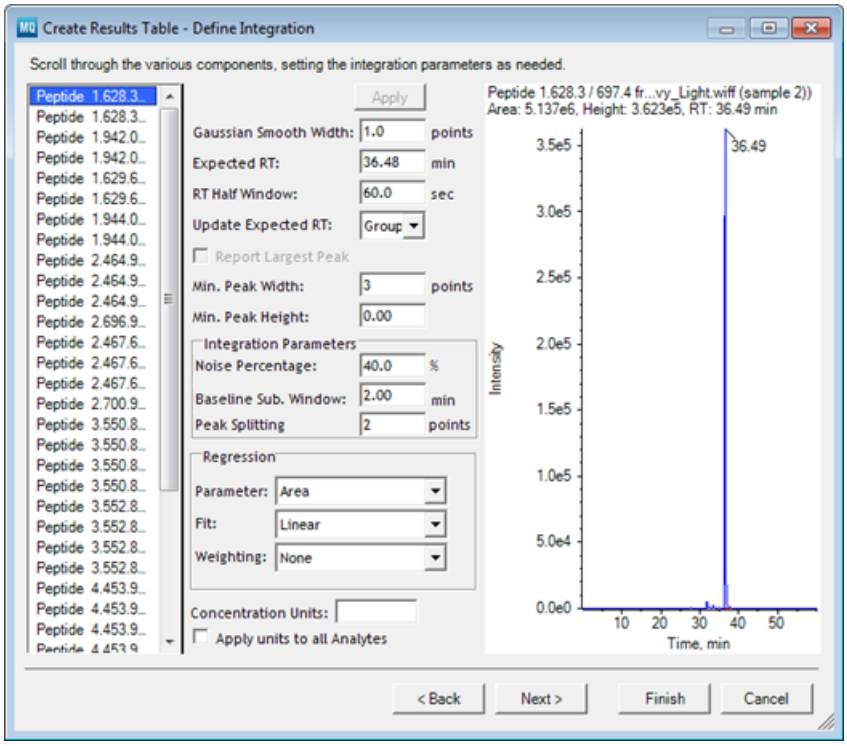
Tip! This highlights one option for structuring the use of internal standards. For more information on how to use the internal standards, refer to the *Reference Guide* that is installed with the MultiQuant™ software.

Note: If a multi-period experiment was used, the previous steps must be repeated for each period. To switch between periods, select the period from the Experiment list and then repeat the process of setting up the processing method as described.

13. Click **Next**.

    On the Create Results Table - Define Integration page, the appropriate peak integration parameters are defined for each individual MRM. Each MRM peak has been automatically integrated, but you have the option to adjust the peak integration (that is, retention time of integrated peak) for each MRM.

Figure 1-12 Create Results Table - Define Integration Page

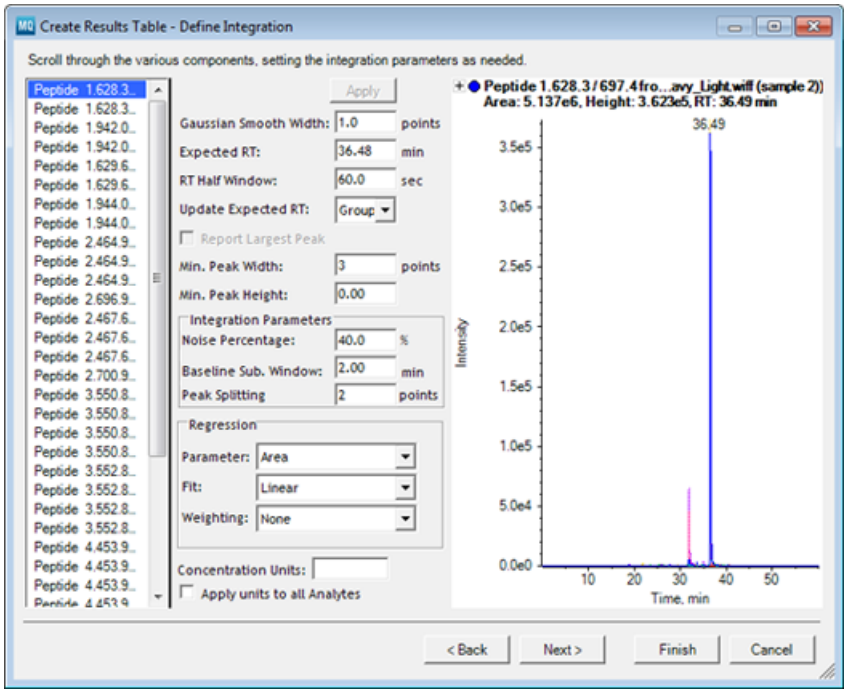


14. Right-click anywhere on the pane and then click **Overlay Other Components for Group**. When there are multiple MRM transitions within a Group, it is convenient to overlay the MRM peaks within a group during integration to be sure that the correct peak is integrated in each case. MRM transitions within the same group should all have the same retention time.

    All the MRM transitions within the selected group are overlaid with different colors specifying each MRM. The specific selected MRM within the group is shown in blue as shown in the Figure 1-13.

Note: When the Update Expected RT is set to Group, the retention time selected for the peak integration of all peaks within the group is based on the position of maximum overlap of the individual chromatograms.

Figure 1-13 Overlay Other Components for Group Option

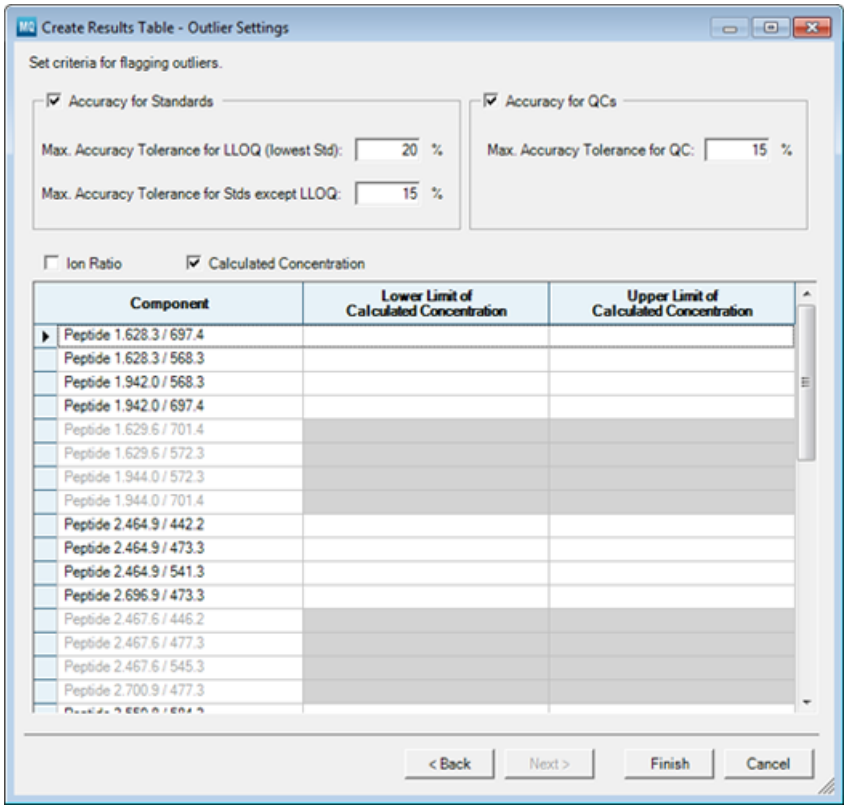


15. Each MRM transition can be viewed by clicking the specific MRM in the list, or by using the Up and Down arrows to automatically advance through the list. When an individual MRM transition has not been integrated at the right retention time, the correct peak can be selected by dragging across it on the pane . The peak is automatically reintegrated. In some cases it might be necessary to adjust the peak-finding parameters.

    If multiple periods are present in the method, all MRM transitions within all periods are integrated in this pane as a single list.

16. After all the MRM transitions have been reviewed and the correct retention times defined for each, click **Next**.

Figure 1-14 Outlier Settings



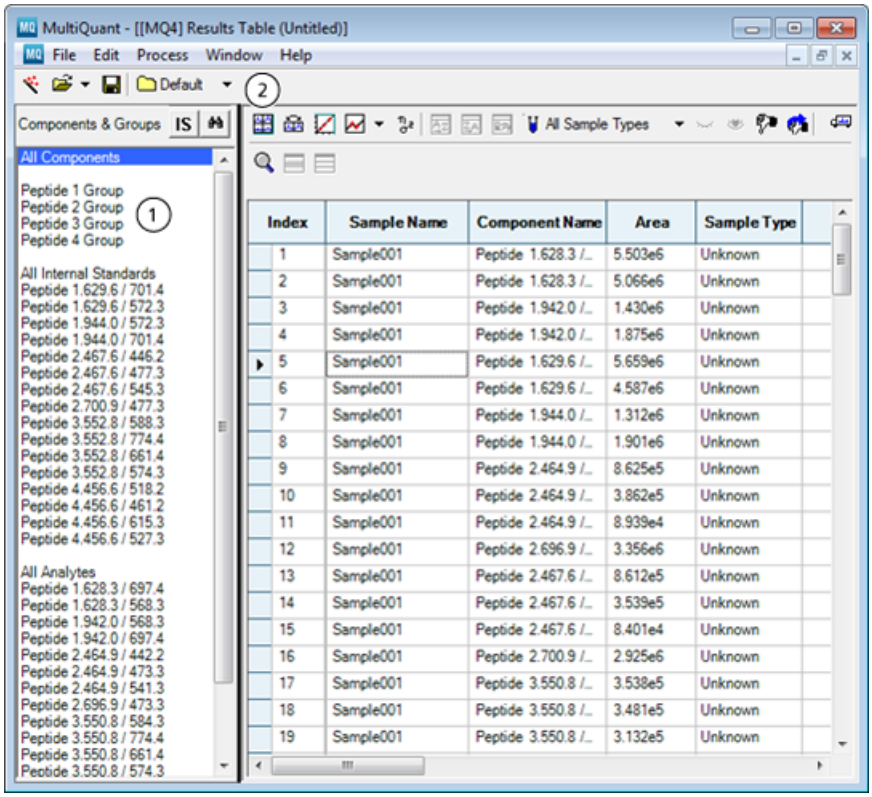
17. Specify the fields as required to automatically flag samples for review in the Results Table. For more information about the Outlier Settings parameters, refer to the *Reference Guide* that is installed with the MultiQuant™ software.
18. Click **Finish**.

Modify the Results Table

All of the MRM transitions in each of the samples are integrated with the specified parameters as shown in Figure 1-15.

On the left side, the information is organized in a number of ways. If All Components is selected, all integrated MRMs for all samples are listed in the order that the MRMs appear in the data file, one sample at a time. If an individual group is selected (Peptide 1 group) then only the MRM transitions within that group for all the samples are shown. The data for all internal standards or all analytes can be visualized by selecting either All Internal Standards or All Analytes. The data for any one IS or analyte can be viewed by selecting its name in the list.

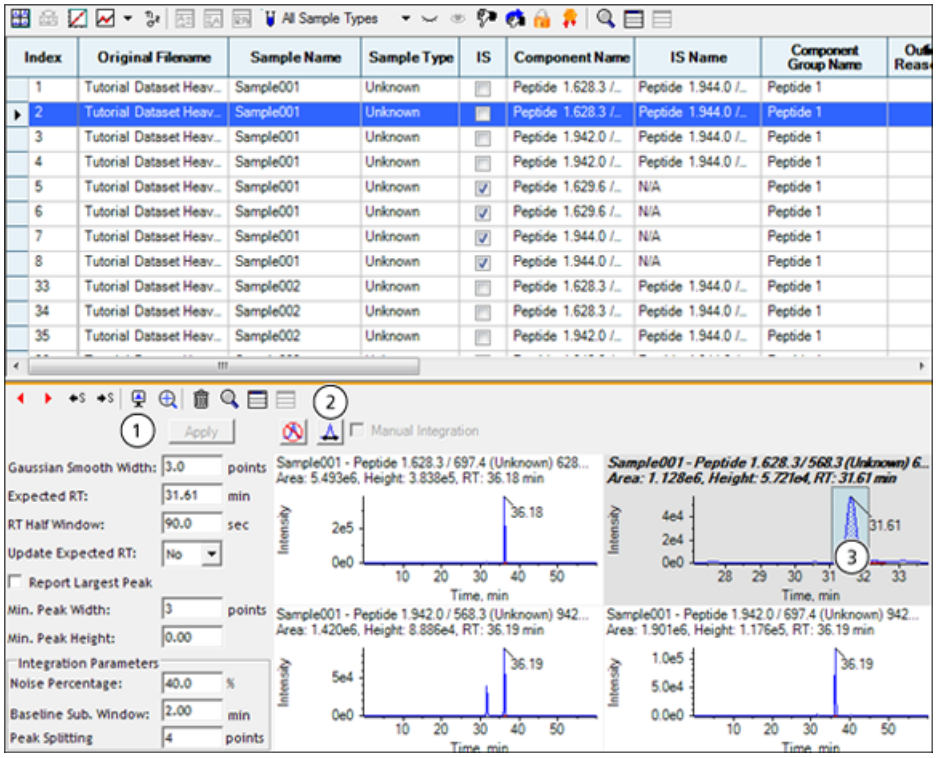
Figure 1-15 Results Table Interface



Item	Description
1	Peptide group
2	Peak Review icon

1. Save the Results Table by clicking **File > Save**.
2. To visualize the MRM transitions for selected group for all samples, select a Peptide group (Figure 1-15 item 1) from the left panel and then click the Peak Review icon (Figure 1-15 item 2). If a row is selected within the table, the selected MRM transition is shown in the pane below with the name highlighted in bold. The up and down arrows or the scroll wheel on the mouse can be used to advance through the rows of the table to change the active pane.
3. If an incorrect peak is integrated, the correct peak can be selected and integrated by dragging the mouse across the peak. If the peak does not integrate, click the Enable Manual Integration Mode icon (Figure 1-16 item 2) to integrate manually. Define the peak area by dragging the mouse across the bottom of the peak area to be integrated (Figure 1-16 item 3). The selected peak integration are shown automatically by the peak filling.

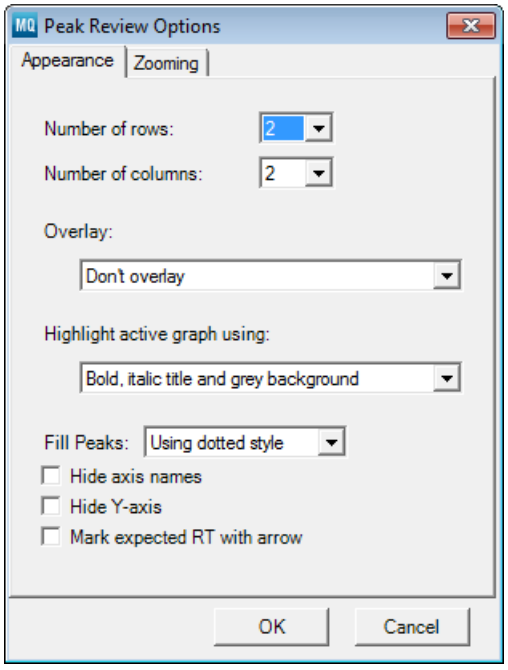
Figure 1-16 Peak Review Interface



Item	Description
1	Slide Show Peak Review icon
2	Enable Manual Integration Mode icon
3	Selecting a peak for integration

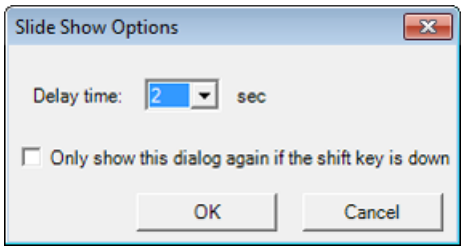
4. Right-click anywhere in the graph pane and then click **Options**. The settings on this dialog can be used to adjust the appearance of the view.

Figure 1-17 Peak Review Options Dialog



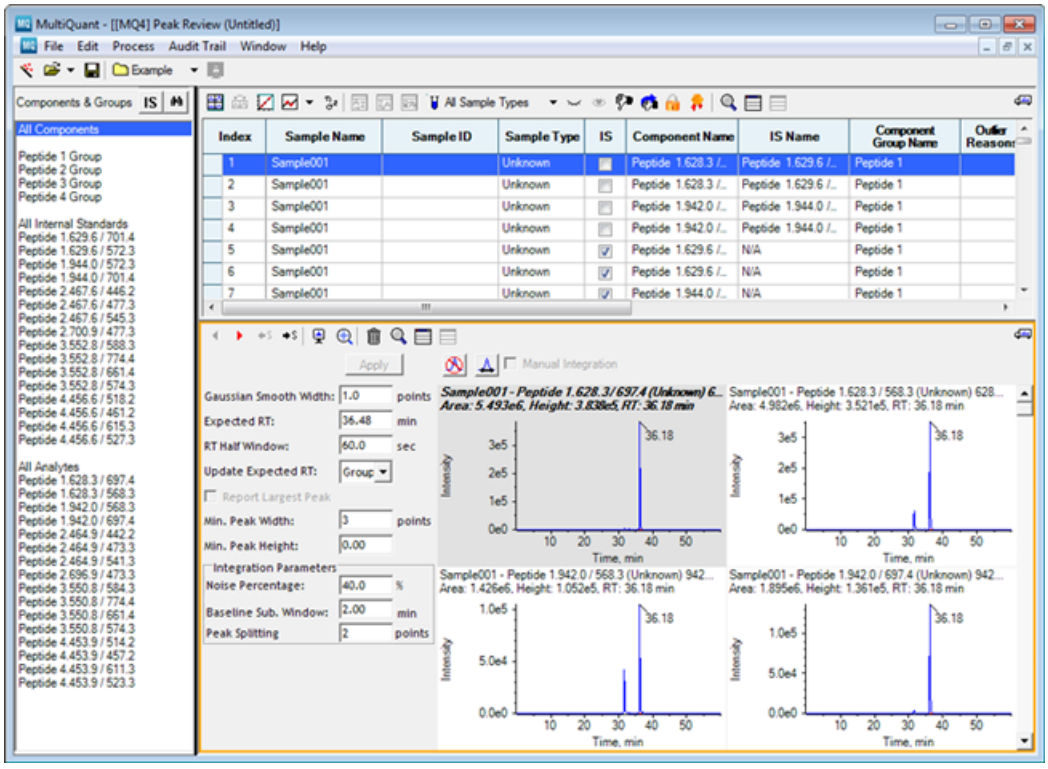
5. From the toolbar, the slide show mode can be selected by clicking the **Slide Show Peak Review** icon (Figure 1-16 item 1). This is a convenient way to review the data.

Figure 1-18 Slide Show Options Dialog



Tip! You can review the data within the Results Table in many ways. For example, the Area column can be selected by clicking on the column header and then selecting the Ascending Sort or Descending Sort (Figure 1-16). This sorts the data within the table.

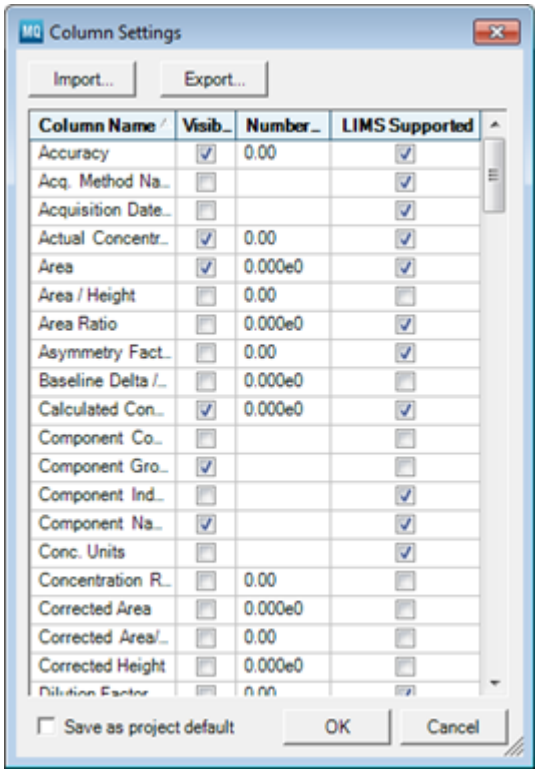
Figure 1-19 Quality Column



6. To edit the structure of the Results Table and make sure that the required columns for determining calibration curves are visible, right-click in the table and then click **Column Settings**. Columns can be added or removed from the Results Table view by selecting or clearing the check boxes in the **Visible** column. For relative quantitation experiments, the common columns required are shown in Figure 1-19.

   It is useful to sort on the Quality column. All the peaks with low quality scores can be viewed together and you can decide if it is necessary to reintegrate some of the peaks. Quality is computed by determining the percentage of the peak area that the integrated peak contributes to the total area under the MRM trace (in a region around the integrated peak, that is, 1.5 times the size of the user specified RT Window). For more information about Results Table columns, refer to the *Reference Guide* that is installed with the MultiQuant™ software.

Figure 1-20 Column Settings Dialog



When you perform relative quantitation experiments, using internal standards provides extra robustness and quantitative accuracy and reproducibility. The Area Ratio column is the peak area of the analyte divided by the peak area of the corresponding internal standard.

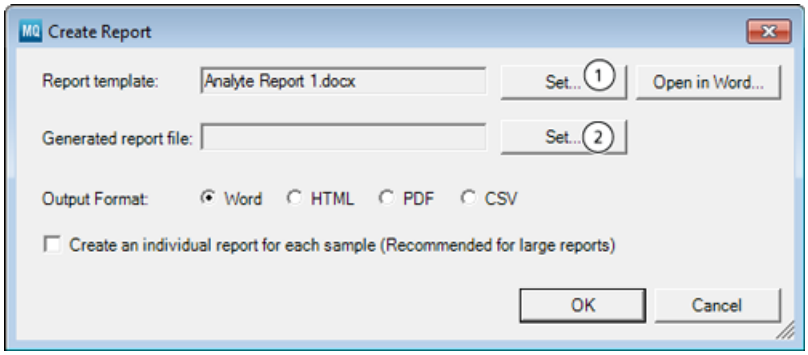
If further manipulation of the data is required, the data can be exported and opened in other programs such as Microsoft Excel. For more information about the Export command, refer to the *Reference Guide* that is installed with the MultiQuant™ software. Refer to Run Queries on page 35.

Create Reports

If you are creating reports using unlocked Results Tables, make sure that you are allowed to create and export unlocked Results Tables. See your administrator if you are unable to create reports.

1. Click **File > Create Report and Save Results Table.**

Figure 1-21 Create Report Dialog



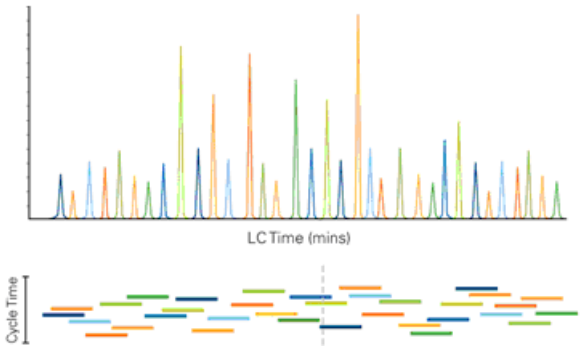
Item	Description
1	Click to select the template.
2	Click to save the report.

2. Click **Set** (Figure 1-21 item 1) to navigate to the Tutorial Dataset Heavy_Light.xml template in the following folder:
   - For Windows 7 and Windows 10: C:\ProgramData\AB SCIEX\MultiQuant\Reporter.
3. Click **Set** (Figure 1-21 item 2) to create the name and location of the report.
4. Click **OK**.

Process Data Acquired Using the *Scheduled* MRM™ Algorithm

The *Scheduled* MRM™ algorithm is a powerful acquisition tool in Analyst® software 1.5 or later versions that enables much higher multiplexing of MRM transitions per acquisition method. From the user-supplied retention time, individual acquisition windows are built for each separate analyte, minimizing the total number of MRM transitions monitored at any single point in time. This improves data quality by enabling the use of higher dwell times for every analyte.

Figure 1-22 Example of *Scheduled* MRM™ Algorithm Data



Another feature in Analyst® Software 1.5 and later is the ID column. Individual MRM transitions can be named in the Analyst® software Acquisition Method Editor. Refer to Figure 1-23. The IDs should be of the in the following formats:

{Root Name}.{Group}.{IS Indicator}

Figure 1-23 Example of the ID Column

	Q1 Mass	Q3 Mass	Time	ID	CE (vc
1	518.000	414.200	21.5	APOB.SVSLPSLDPASAK.3b3.light	43.900
2	518.000	445.300	21.5	APOB.SVSLPSLDPASAK.3y3.light	43.900
3	518.000	613.400	21.5	APOB.SVSLPSLDPASAK.3y5.light	43.900
4	518.000	527.300	21.5	APOB.SVSLPSLDPASAK.3b4.light	43.900
5	520.600	418.200	21.5	APOB.SVSLPSLDPASAK.3b3.heav	44.000
6	520.600	449.300	21.5	APOB.SVSLPSLDPASAK.3y3.heav	44.000
7	520.600	617.400	21.5	APOB.SVSLPSLDPASAK.3y5.heav	44.000
8	520.600	531.300	21.5	APOB.SVSLPSLDPASAK.3b4.heav	44.000
9	666.800	588.300	15.9	APOC1.EFGNTLEDK.2b4.light	51.300
10	666.800	531.300	15.9	APOC1.EFGNTLEDK.2y3.light	51.300
11	666.800	474.200	15.8	APOC1.EFGNTLEDK.2b3.light	51.300
12	666.800	644.400	15.9	APOC1.EFGNTLEDK.2y4.light	51.300

The Group and IS Indicator are both optional. For small molecule applications, the IS Indicator should be absent for analytes and set to IS or internal standard for internal standards. For example if the IDs are:

- Compound1
- Compound1.IS
- Compound2
- Compound2.IS

Then Compound1.IS and Compound2.IS are both automatically set as internal standards. Additionally, Compound1 will be setup to use 'Compound1.IS' as its internal standard and similarly for Compound2.

For proteomics applications, the ID will usually be of the form:

{Peptide Sequence}.{Transition}.{Light or Heavy}

For more information about triplex mTRAQ® reagent support, refer to the *Reference Guide* that is installed with the MultiQuant™ software. The nomenclature is as follows:

- Protein.peptide.transition.M00
- Protein.peptide.transition.M04
- Protein. peptide. transition.M08

Before you begin

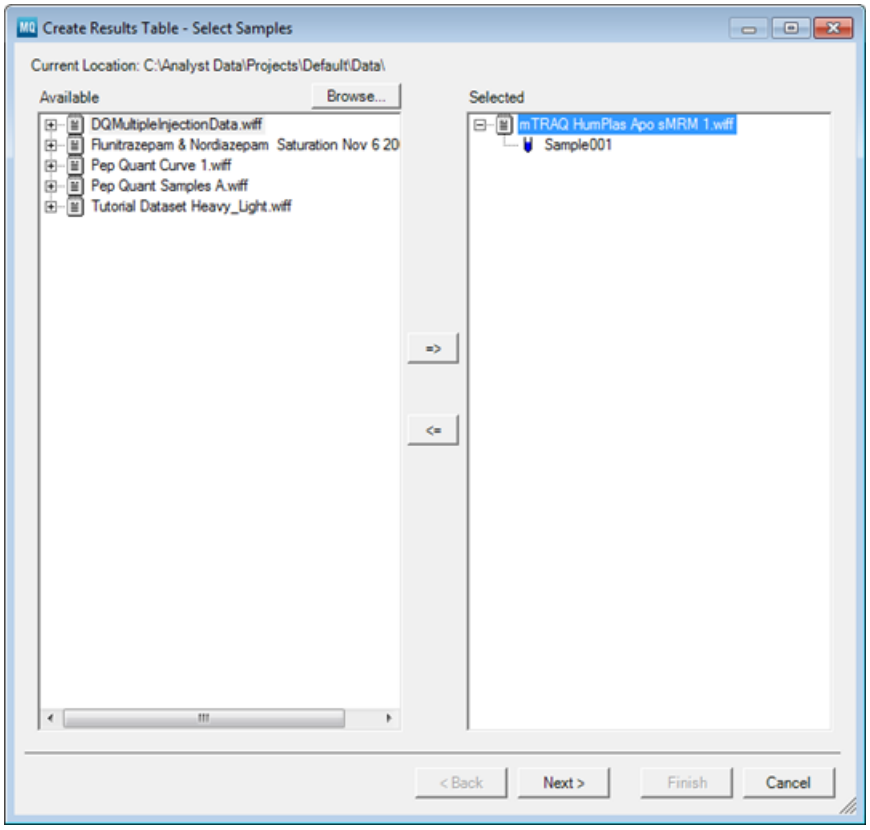
- Set the integration parameter defaults. Refer to Set the Peak Integration Parameters on page 9.
- Make sure you are in the Example project.
- Make sure the mTRAQ HumPlas Apo sMRM 1.wiff file is in the <drive>:\Analyst Data\Projects\Example\Data\Example Data folder.

Create a Results Table

Quantitation methods include a set of instructions on how to quantitate the peaks selected for integration. In this tutorial, you will create a quantitation method at the same time that you create a Results Table.

1. In the Analyst® software, on the Navigation bar, under **Companion Software**, double-click **MultiQuant 3.0.3**.
2. Click **File > New Results Table.**
3. On the Create Results Table - Select Samples page, drag the **mTRAQ HumPlas Apo sMRM 1.wiff** file into the **Selected** pane and then click **Next**.

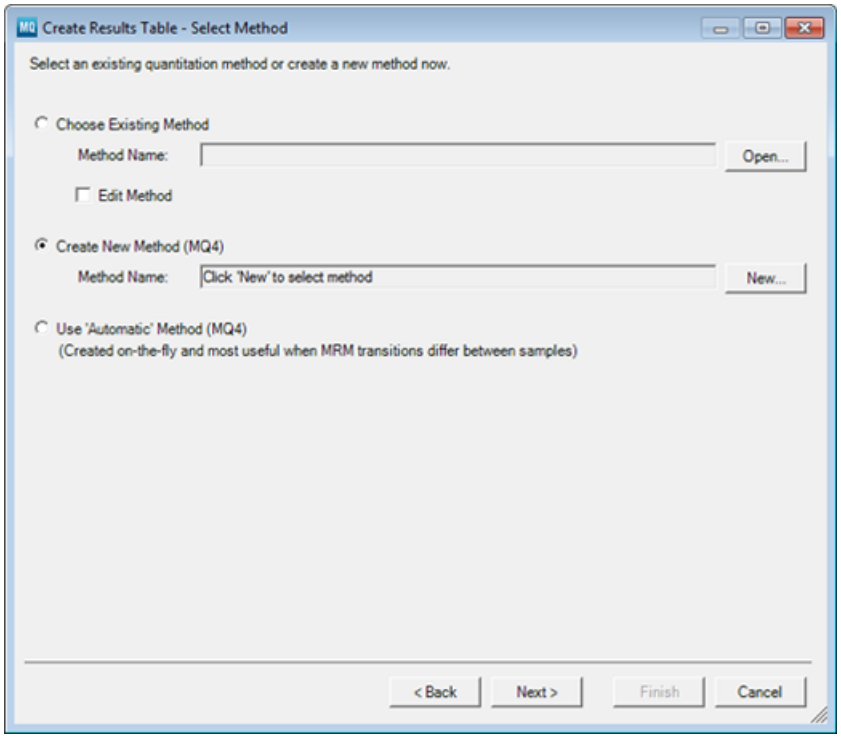
Figure 1-24 Create Results Table - Select Samples Page



4. On the Create Results Table - Select Method page, click the **Create New Method (MQ4)** option and then click **New**.

Tip! If the interface has Create New Method (Signalfinder1), then refer to Set the Peak Integration Parameters on page 9 to change the algorithm to MQ4.

Figure 1-25 Create Results Table - Select Method Page



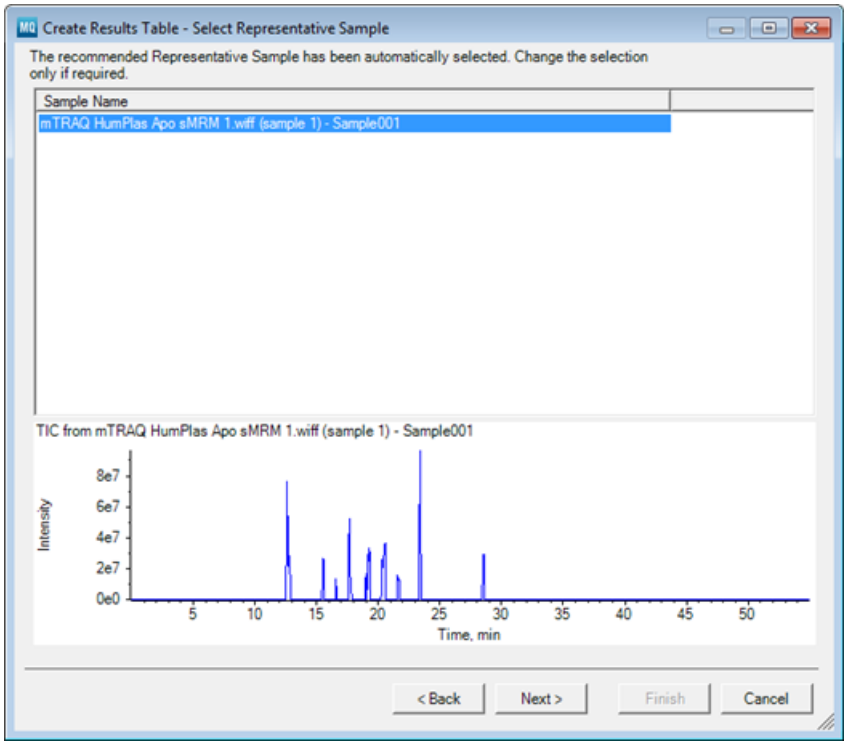
5. Type a name for the method, click **Save**, and then click **Next**.

   In this tutorial, you will create a method. Creating methods gives you an opportunity to review and apply different parameters for the integration of your data.

6. On the Create Results Table - Select Representative Sample page, a representative sample has been recommended and is selected. Click **Next**.

   The software recommends a representative sample based on selecting a chromatogram that provides the best opportunity to select integration parameters that fit the entire batch.

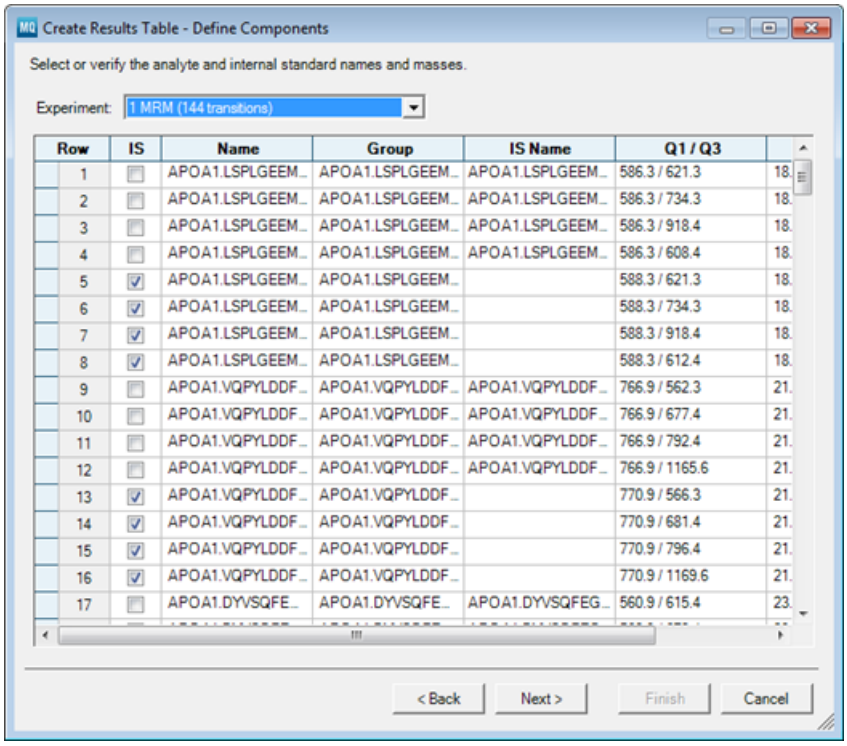
Figure 1-26 Create Results Table - Select Representative Sample Page



On the Create Results Table - Define Components page, the details of the various analytes and internal standards to be processed must be defined. All the MRM transitions from the data file are automatically loaded into the window.

Tip! When the ID column is correctly entered in the Analyst® software MRM acquisition method, the information is used to automatically populate the Group and internal standard information in the following pane.

Figure 1-27 Create Results Table - Define Components Page



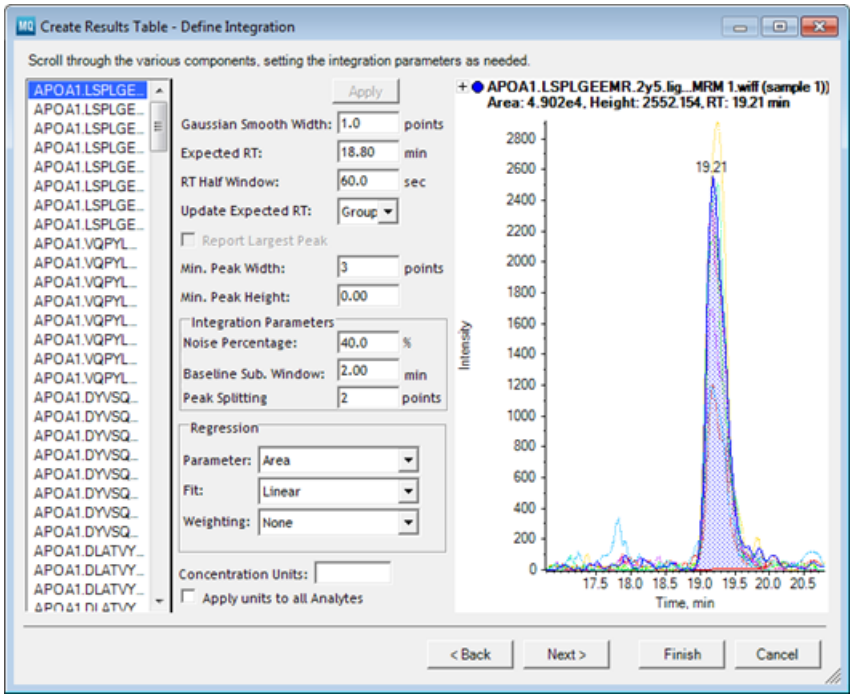
Tip! The MRM transitions marked *.IS or *. heavy are automatically used as internal standards. However, if the light transitions are to be used as the internal standard, this can be easily reversed by right-clicking in the table and then clicking **Internal Standards > Reverse Analyte/IS Assignments.**

7. Click **Next**.

Tip! If you acquire data using a MIDAS™ Workflow, there are additional experiments in the acquisition method because of the MS/MS spectra acquired. If you click Next before reviewing all the experiments, an error message is shown advising you to review all the experiments before proceeding. No review is required, click **OK** to continue.

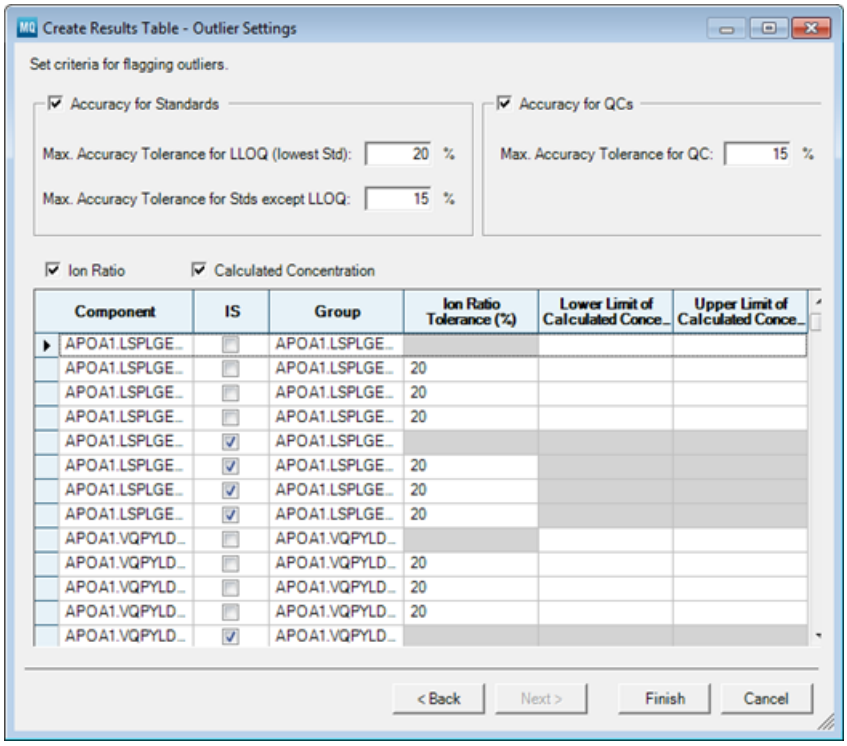
8. On the Create Results Table - Define Integration page, the automatic integrations can be reviewed. Because Update Expected RT is selected, the retention time is automatically determined from all members of each group as explained for the previous example. To see all members of each group, right-click and then click **Overlay Other Components for Group**.

Figure 1-28 Create Results Table - Define Integration Page



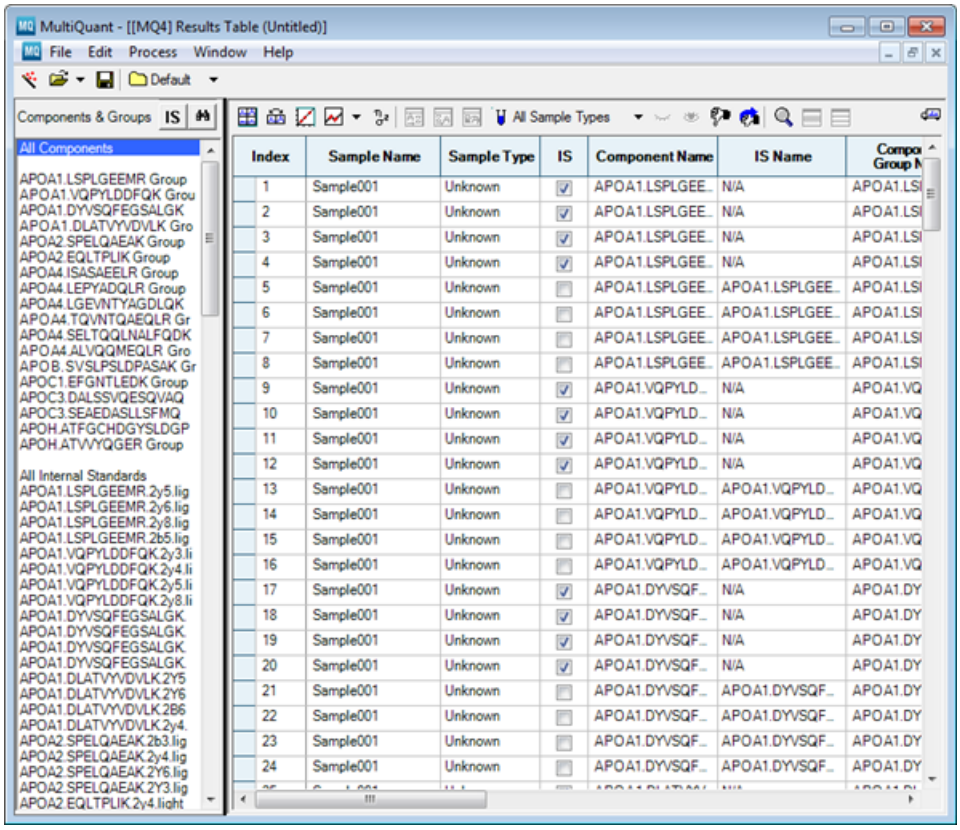
9. Because the MRM data is collected using the *Scheduled* MRM™ algorithm, data is only collected over a short retention time. This is why a narrow time axis is shown for each group. Review each group and then click **Next**.

Figure 1-29 Outlier Settings



10. Specify the fields as required to automatically flag samples for review in the Results Table. For more information about the Outlier Settings parameters, refer to the *Reference Guide* that is installed with the MultiQuant™ software.
11. Click **Finish**.
12. After the Results Table opens, click the **Peak Review** icon.

Figure 1-30 Results Table and Peak Review Pane



13. A useful way to visualize the grouped data is to overlay all members of the same group. Right-click in the Peak Review pane and then click **Options**.
14. In the Peak Review Options dialog, in the **Overlay** field, select **All components for group** and then click **OK**.

Figure 1-31 Peak Review Options Dialog

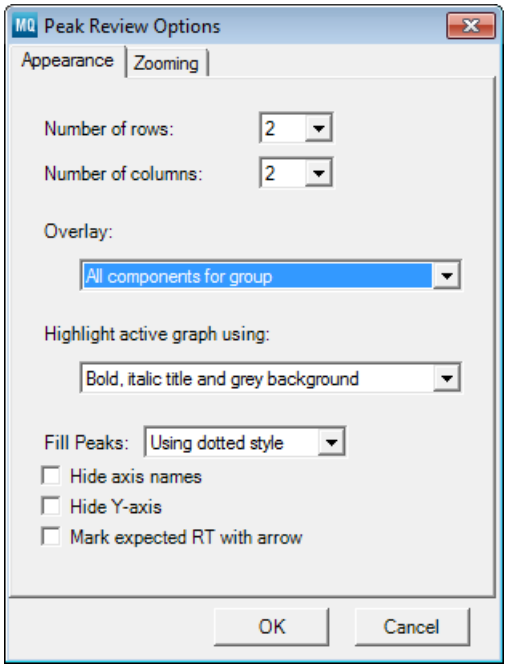
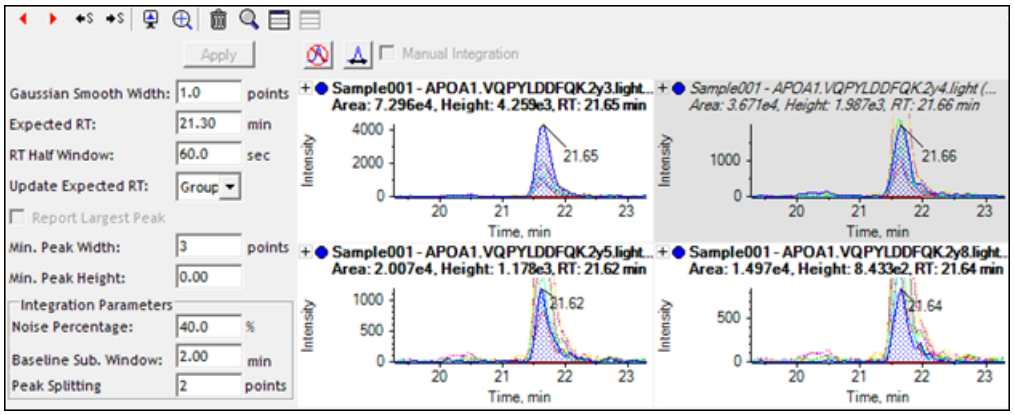


Figure 1-32 Overlay: All components for group



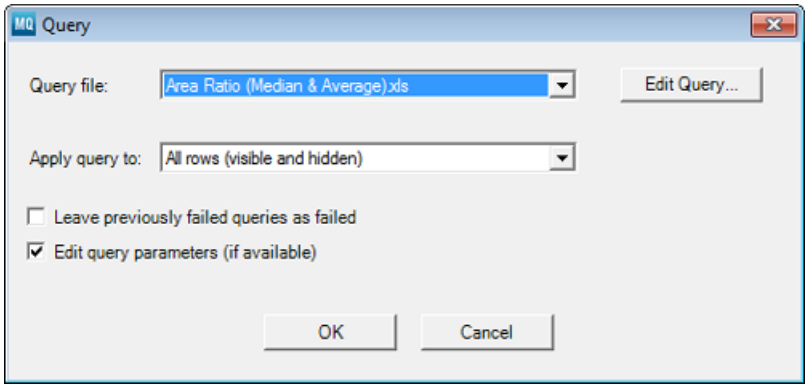
Run Queries

The primary purpose of queries is to allow potentially problem peak integrations to be flagged so that only a subset of the chromatograms needs to be manually reviewed. Queries can also be used to create and populate custom columns. For more information on using and creating queries, refer to the *Reference Guide* that is installed with the MultiQuant™ software.

1. Click **Process > Query.**
2. In the **Query file** list, select **Area Ratio Query** and then click **OK**.

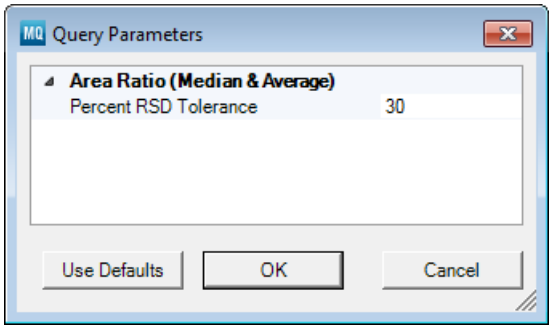
   This query is used to assess the Light/Heavy ratios for each MRM per peptide and flag any cases where there is disagreement between multiple ratios.

Figure 1-33 Query Dialog



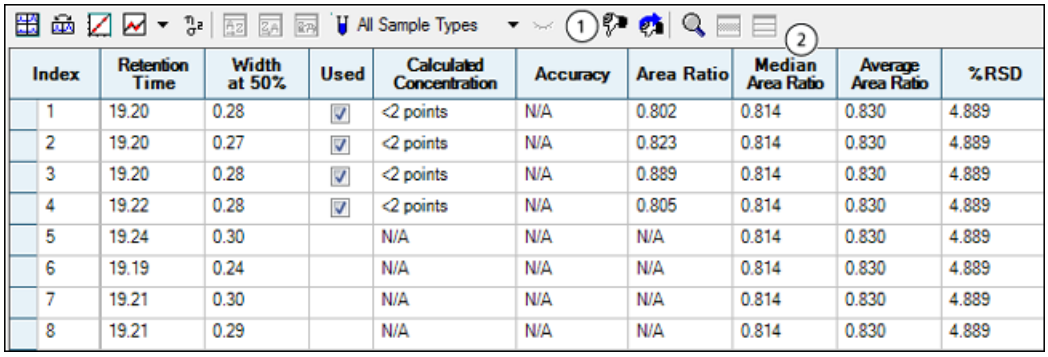
Good default values for this query are shown in Figure 1-34. A peak integration is flagged as Failed if it does not pass the criteria.

Figure 1-34 Query Parameters Dialog



3. Several custom columns are added to the Results Table. In the last column, the %RSD computed for each group is shown. The first peptide group passes the **Query** filter, with a %RSD result of 5.4% (Figure 1-35 item 2). To advance to the first failed peak, click the **Query Failure** icon (Figure 1-35 item 1).

Figure 1-35 Query Results



Index	Retention Time	Width at 50%	Used	Calculated Concentration	Accuracy	Area Ratio	Median Area Ratio	Average Area Ratio	%RSD
1	19.20	0.28	☑	<2 points	N/A	0.802	0.814	0.830	4.889
2	19.20	0.27	☑	<2 points	N/A	0.823	0.814	0.830	4.889
3	19.20	0.28	☑	<2 points	N/A	0.889	0.814	0.830	4.889
4	19.22	0.28	☑	<2 points	N/A	0.805	0.814	0.830	4.889
5	19.24	0.30		N/A	N/A	N/A	0.814	0.830	4.889
6	19.19	0.24		N/A	N/A	N/A	0.814	0.830	4.889
7	19.21	0.30		N/A	N/A	N/A	0.814	0.830	4.889
8	19.21	0.29		N/A	N/A	N/A	0.814	0.830	4.889

Item	Description
1	Show only query failures
2	Custom columns

Accurate Quantitation Using Calibration Curves

When you perform accurate quantitation of peptides or proteins (using peptides as surrogates), it is possible to create stable isotope labeled peptides to use as concentration standards. These heavy peptides can be created using labeling strategies such as mTRAQ® reagents or by making synthetic peptides using stable isotope-labeled amino acids. By proper use of these peptides, experiments can be designed that leverage the internal standard concept and add robustness to your experiments.

A calibration curve can be generated for the accurate quantitation experiment using increasing concentrations of the "heavy peptide" in the presence of a constant amount of the biological sample of interest (the matrix that contains the light version of the same peptide). In this case, the endogenous light peptide acts as the constant internal standard for the calibration curve.

Note: If you are not using the Audit Trail with Security Features edition, then you can launch the MultiQuant™ software directly from the desktop icon.

Before you begin

- Make sure you are in the Example project.
- Make sure the Pep Quant Curve 1.wiff file and the Pep Quant Samples A.wiff file are in the <drive>:\Analyst Data\Projects\Example\Data\Example Data folder.

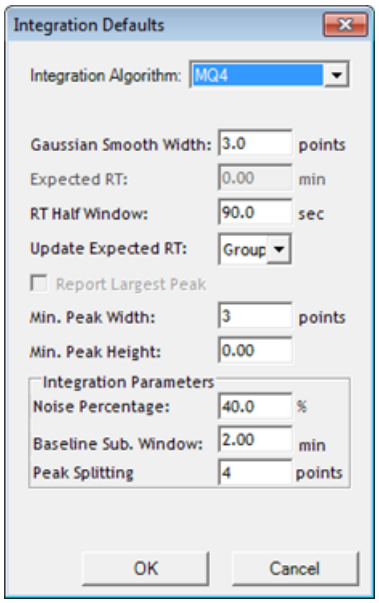
Set the Peak Integration Parameters

1. In the Analyst® software, on the Navigation bar, under **Companion Software**, double-click **MultiQuant 3.0.3**.
2. Click **Edit > Project Integration Defaults.**

> **Note:** If you are not using the Audit Trail and Security Features edition, then click **Edit > User Integration Defaults.**

3. In the Integration Defaults dialog, select **MQ4** from the **Integration Algorithm** list. Good peak integration parameters for this dataset are shown in Figure 1-36.

Figure 1-36 Integration Defaults dialog



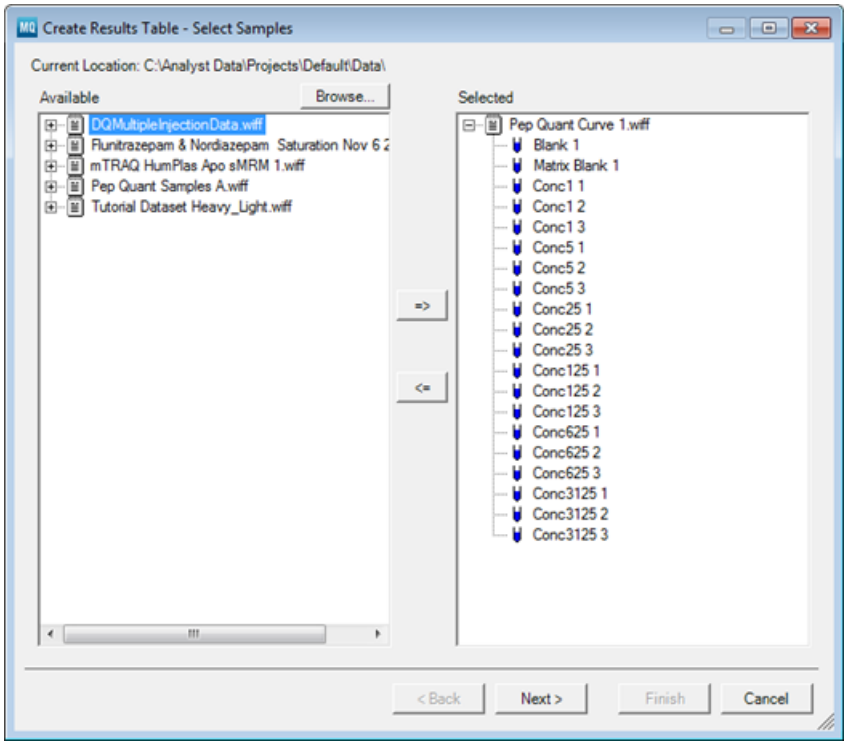
In many cases a smaller smoothing width (for example, 1.0) might be more appropriate. Also note that the peak review process will be eased by specifying a non-zero Min. Peak Height, typically corresponding to at least one or two counts. Because the entered threshold should be in counts per second (cps), the actual value required will depend on the dwell time. For example, for a dwell time of 100 ms, two counts corresponds to a setting of 20 cps.

4. For this dataset, good results are achieved using the parameters shown in the previous figure. Adjust the values and then click **OK**.

Create a Results Table

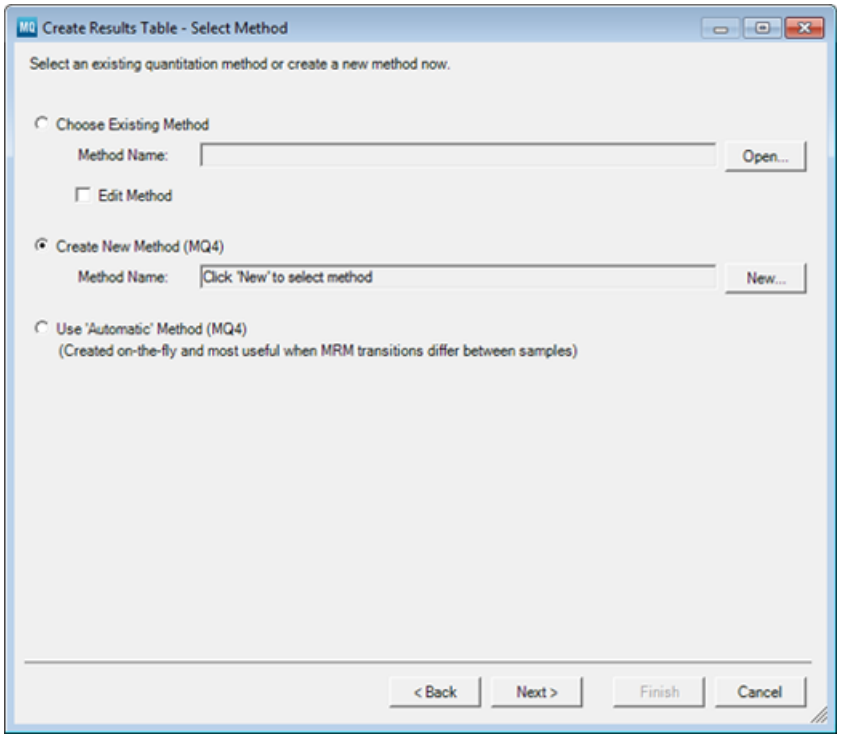
1. Click **File > New Results Table.**
2. On the Create Results Table - Select Samples page, drag the Pep Quant Curve 1.wiff into the Selected pane and then click **Next**.

Figure 1-37 Create Results Table - Select Samples page



3. On the Create Results Table - Select Method page, click the **Create New Method (MQ4)** option and then click **New**.

Figure 1-38 Create Results Table - Select Method page



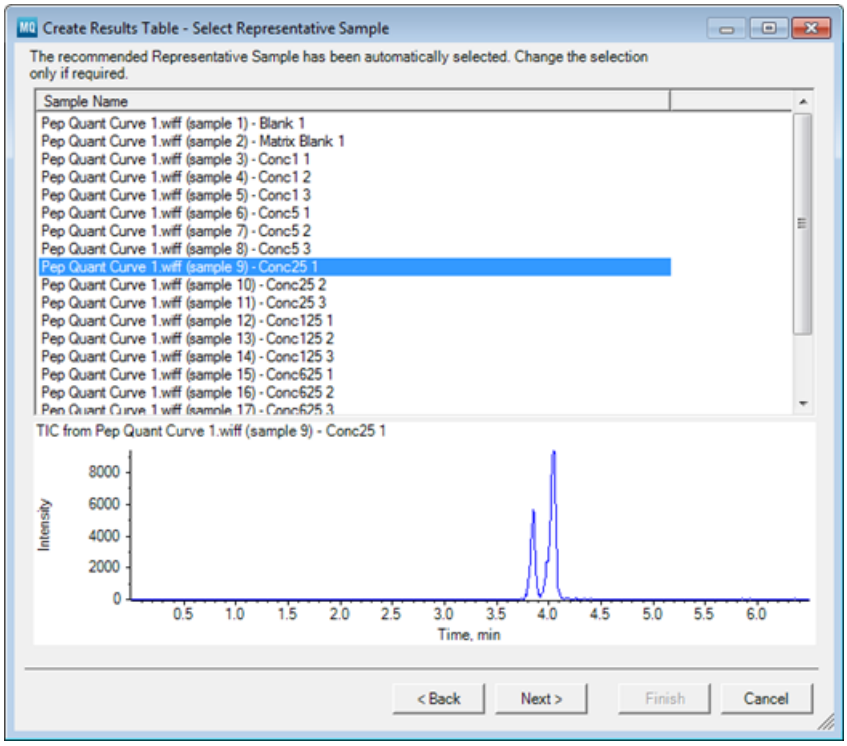
4. Type a name for your method, click **Save**, and then click **Next**.

   In this tutorial, you will create a new method. Creating new methods gives you an opportunity to review and apply different parameters for the integration of your data.

5. On the Create Results Table - Select Representative Sample page, a representative sample has been recommended and is selected. Click **Next**.

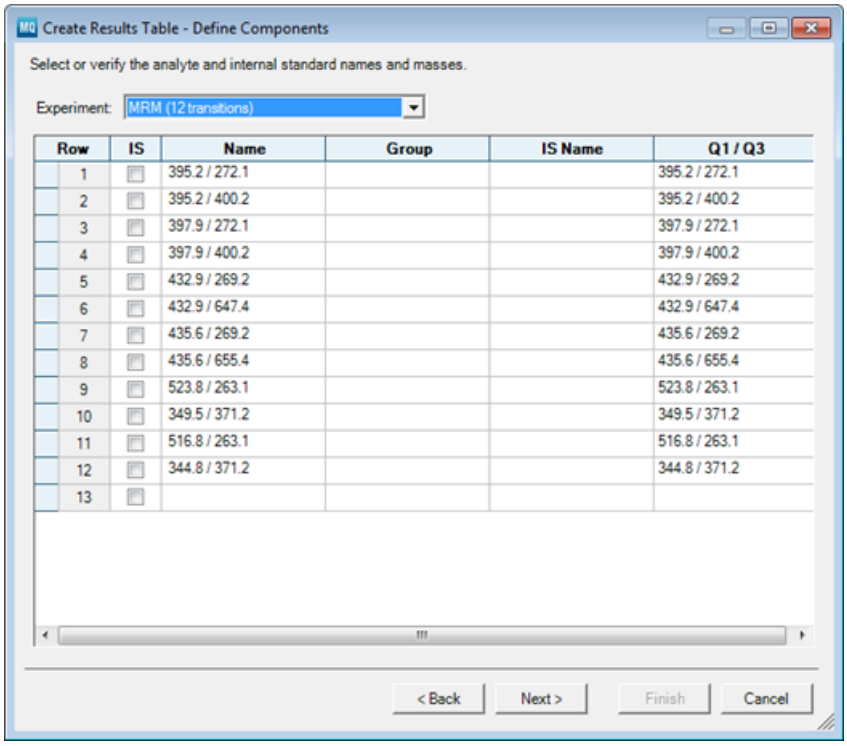
   The software recommends a representative sample based on selecting a chromatogram that provides the best opportunity to select integration parameters that fit the entire batch.

Figure 1-39 Create Results Table - Select Representative Sample page



6. On the Create Results Table - Define Components page, the details of the various analytes and internal standards to be processed must be defined. This step is required because the ID field of the Analyst software Acquisition Method Editor was not completed as discussed in Process Data Acquired Using the *Scheduled* MRM™ Algorithm on page 25. All the MRM transitions from the data file are automatically loaded into the window. First, all the members of a Group must be defined by giving them the same name. In this example, there are three groups, each consisting of four MRMs. The group names can be typed into the Group column or copied from an Excel spreadsheet. The Groups can be named in many ways, depending on the compounds.

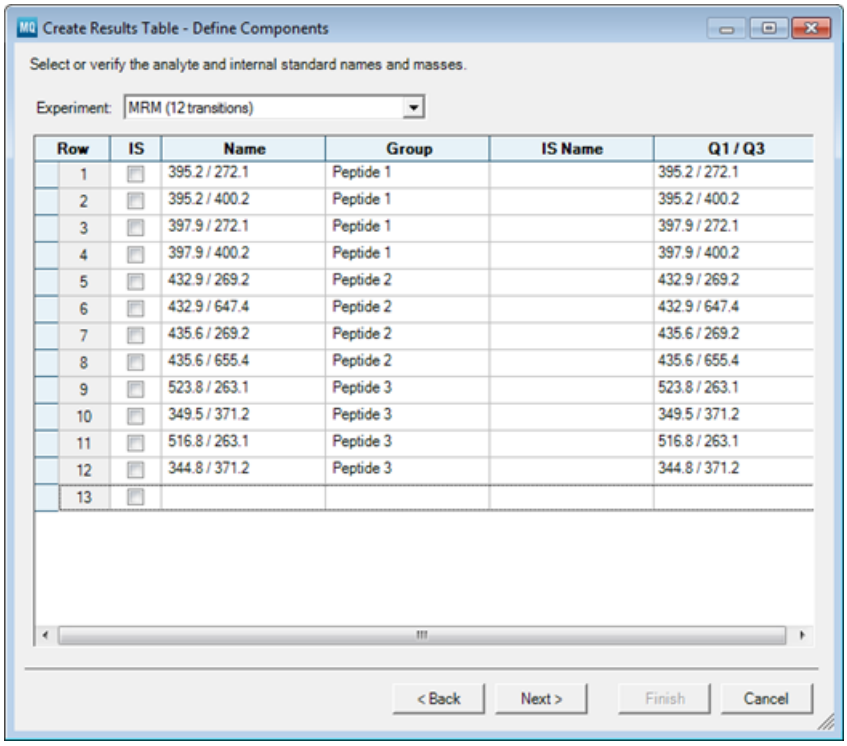
Figure 1-40 Create Results Table - Define Components page



Note: When you group MRM transitions together, they should be to the same compound, including the internal standard version of that compound.

7. In this example, name the first 4 MRMs Peptide 1, the second four rows Peptide 2, and the third four are Peptide 3. Type the peptide name in the first row only of each group (Rows 1, 5, 9), and then right-click in the table and then click **Groups > By Filling Down Existing Groups.**

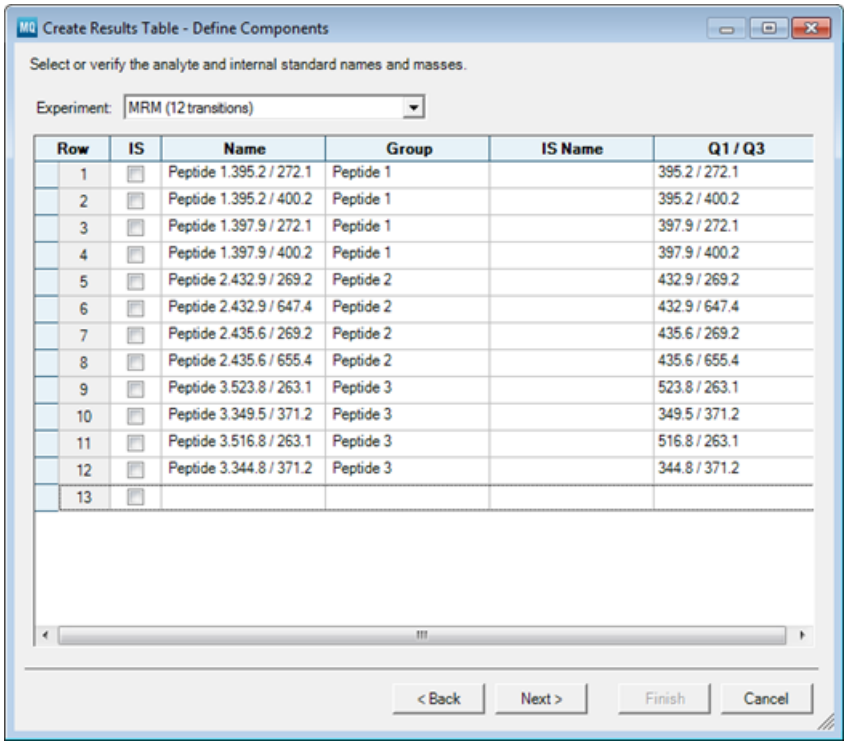
Figure 1-41 Groups > By Filling Down Existing Groups option



Note: Specifying the names and groups here is necessary because this information was not specified in the original data acquisition method. Refer to the Tip on page 30 to learn how to use the ID field of the Acquisition Method Editor to enter this information.

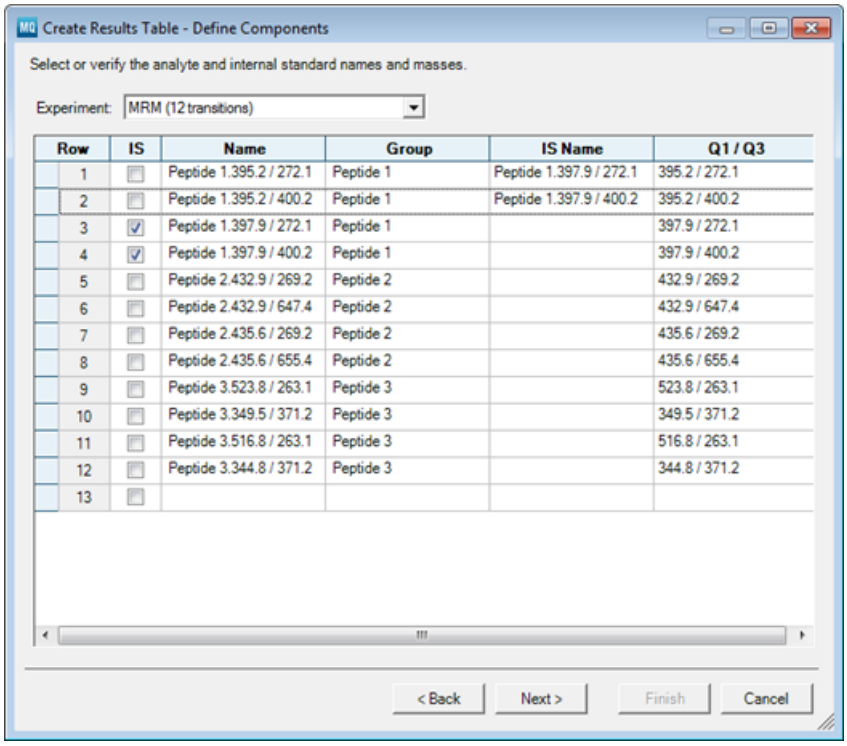
8. Copy the **Group** column into the **Name** column. Right-click in the table and then click **Groups > Add Group to Start of Component Name**.

Figure 1-42 Groups > Add Group to Start of Component Name option



9. Define the MRM transitions to be used as the internal standards by selecting the check boxes in the IS column. In this example, the last four transitions in each group are the internal standards for the first four transitions in the group. Select the **IS** boxes as shown in the following figure for Peptide 1.
10. After an MRM transition has been defined as an internal standard, it can be associated with the correct analyte in the **IS Name** column. By clicking on the right side of each row within this column, a list of internal standards is shown and can be selected. Select the appropriate internal standard from the list for each of the first two MRM transitions for Peptide 1. Within the group, the third MRM is the IS for the first row, the forth MRM is the IS for the second row.

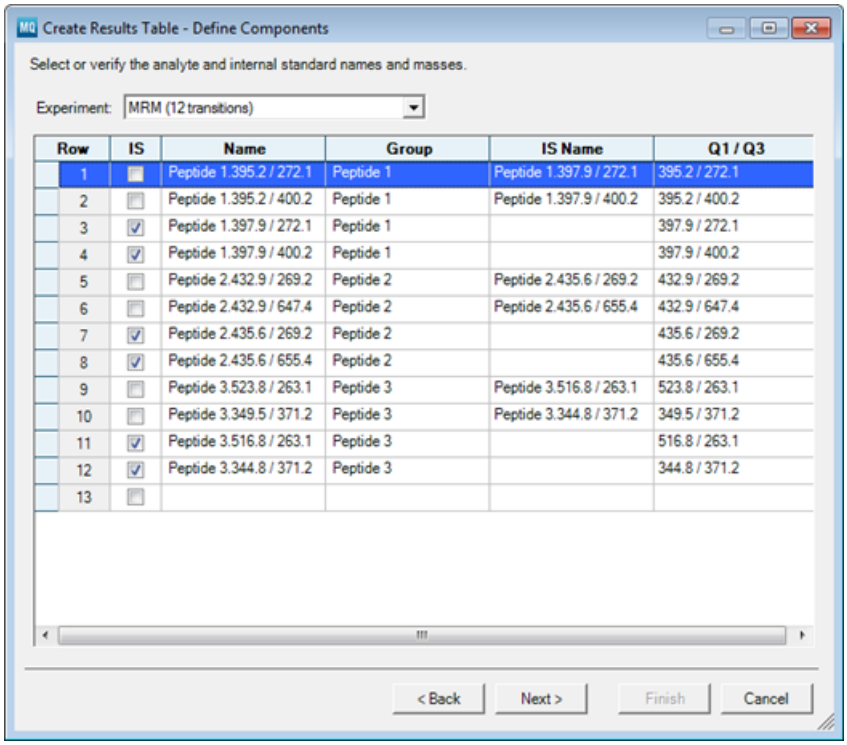
Figure 1-43 Selecting Internal Standards



11. To propagate this format to all the groups in the data file, select the first row for Peptide 1, right-click and then click **Internal Standards > Set for all Groups as for Selected Group.**

Note: The pattern of sample and IS MRM transitions must be constant through the data file to be able to propagate the group pattern. This reduces the manual interaction required when setting up the processing method.

Figure 1-44 Standards > Set for all Groups as for Selected Group Option



Note: If a multi-period experiment was used, the previous steps must be repeated for each period. To switch between periods, select the period from the Experiment list and then repeat the process of setting up the processing method as described. This dataset does not contain multiple periods.

Tip! If required, multiple MRM transitions for the same analyte can be summed during data processing. Right-click in the Define Components table and then click **Sum Multiple Ions**.

12. Click **Next**.
13. On the Create Results Table - Define Integration page, the appropriate peak integration parameters will be defined for each individual MRM. Each MRM peak has been automatically integrated, but you have the option to adjust the peak integration (that is, retention time of integrated peak) for each MRM.
14. Each MRM transition can be viewed by clicking the specific MRM in the left pane list, or by using the Up / Down arrows or mouse scroll wheel to automatically advance through the list. When an individual MRM transition has not been integrated at the right retention time, the correct peak can be selected with the mouse by just dragging across it on the pane and it will be automatically reintegrated. In some cases it might also be necessary to adjust the peak-finding parameters.

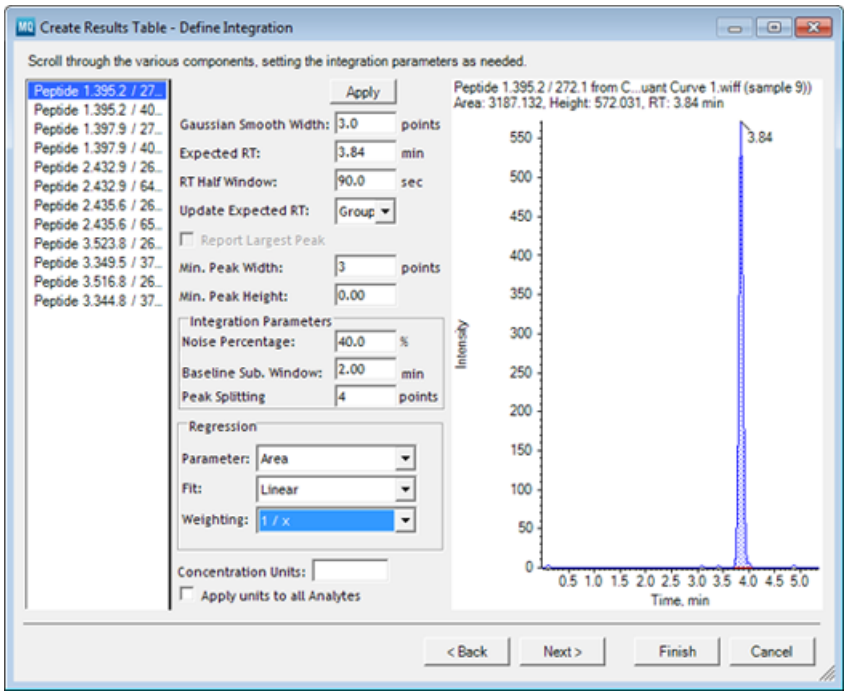
If multiple periods are present in the method, all MRM transitions within all periods are integrated in this pane as a single list.

15. Select the parameters for generating the calibration curve. Typically, the peak area is used in the calculation. In the **Regression** group, select the following and then click **Apply**:

    - **Parameter**: Area
    - **Fit**: Linear
    - **Weighting**: 1/x.

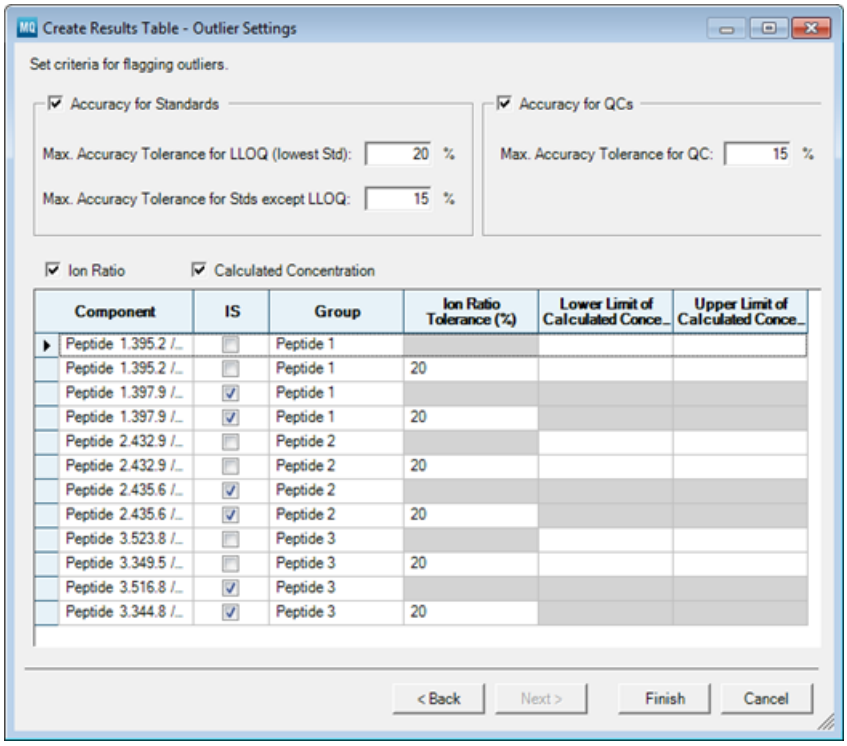
    Typically, the peak area is used in the calculation. A linear calibration curve with a weighting of 1/x is most commonly used for calculating the calibration curve.

Figure 1-45 Create Results Table - Define Integration page



16. Click **Next**.

Figure 1-46 Outlier Settings



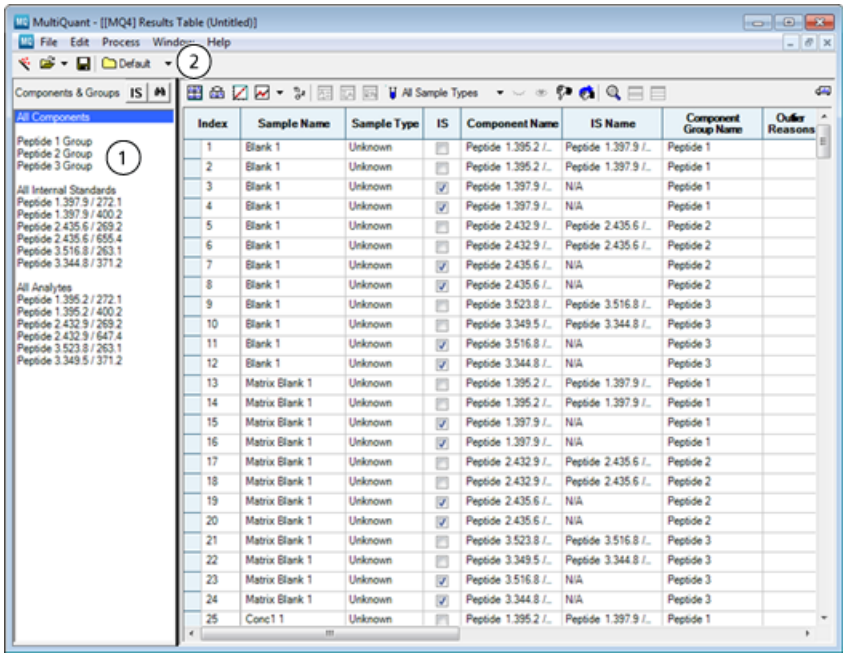
17. Specify the fields as required to automatically flag samples for review in the Results Table. Refer to the *Reference Guide* for more information about the **Outlier Settings** parameters.
18. Click **Finish**.

Modify the Results Table

All of the MRM transitions in each of the samples are integrated with the specified parameters as shown in Figure 1-47.

On the left side, the information is organized in a number of ways. If All Components is selected, all integrated MRMs for all samples are listed in the order that the MRMs appear in the data file, one sample at a time. If an individual group is selected (Peptide 1 Group) then only the MRM transitions within that group for all the samples are shown. The data for all internal standards or all analytes can be visualized by selected either All Internal Standards or All Analytes. Finally, the data for any one IS or analyte can be viewed by selecting its name in the list.

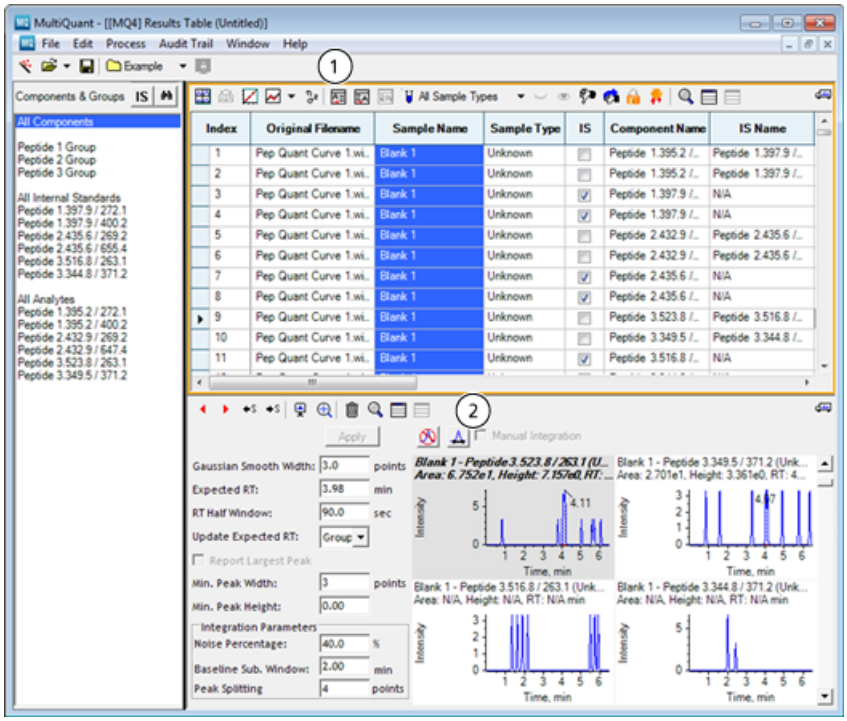
Figure 1-47 Results Table Interface



Item	Description
1	Peptide Group
2	Peak Review icon

1. Save the Results Table by clicking **File > Save.**
2. To visualize the MRM transitions for selected group for all samples, select a **Peptide Group** from the left panel and then click the **Peak Review** icon. If a row is selected within the table, the selected MRM transition is shown in the pane below with the name highlighted in bold. The Up / Down arrows or the scroll wheel on the mouse can be used to advance through the rows of the table to change the active pane.
3. If an incorrect peak is integrated, the correct peak can be selected and integrated by dragging the mouse across the peak. If the peak does not integrate, click the **Enable Manual Integration Mode** icon to integrate manually. Define the peak area by dragging the mouse across the bottom of the peak area to be integrated. The selected peak integration will be shown automatically by the peak filling.

Figure 1-48 Peak Review Interface



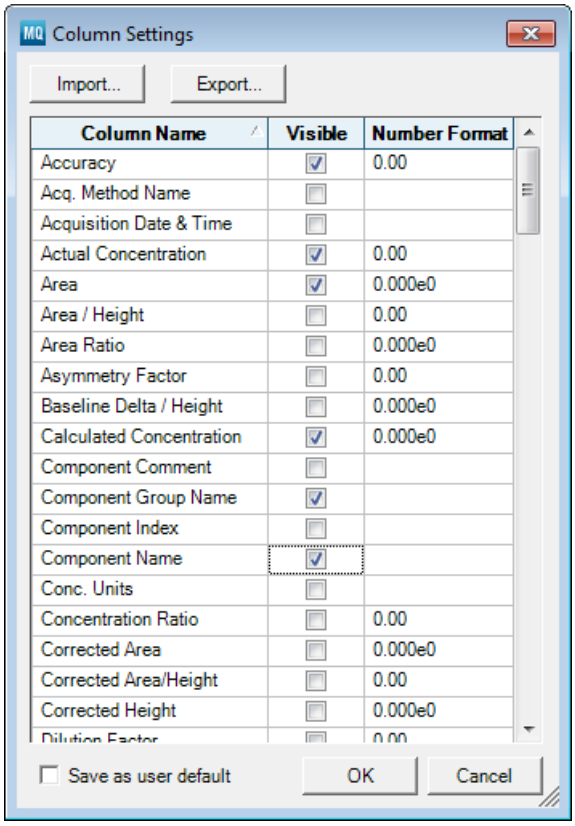
Item	Description
1	Sort buttons
2	Enable Manual Integration Mode icon

Tip! You can review the data within the Results Table in many ways. For example, the Area column can be selected by clicking on the column header and then selecting the **Ascending Sort** or **Descending Sort**. This sorts the data within the table.

4. To edit the structure of the Results Table and ensure that the required columns for determining calibration curves are visible, right-click in the table and then click **Column Settings**. Columns can be added or removed from the Results Table view by selecting or clearing the check boxes in the Visible column.

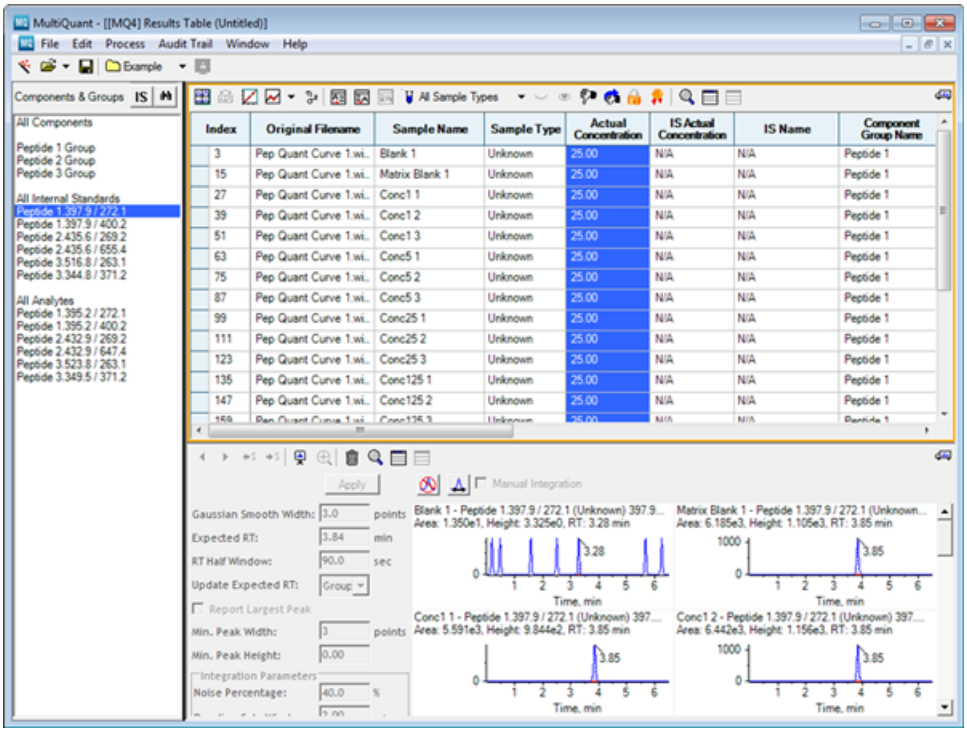
   Recommended columns to use for accurate quantitation include: Sample Index, Sample Name, Sample Type, Actual Concentration, Area, IS Area, S/N, Modified, Used, Calculated Concentration and Accuracy.

Figure 1-49 Column Settings Dialog



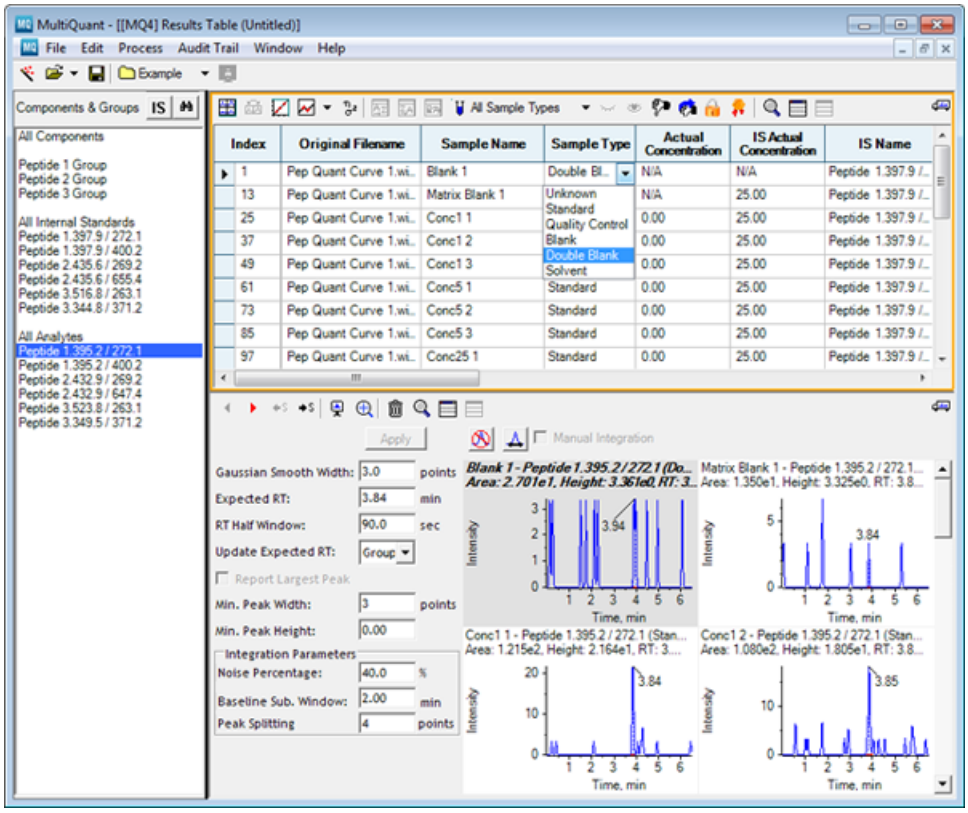
5. The information about the internal standards and samples must be defined. Select one of the peptide MRMs under All Internal Standards and then enter the Actual Concentration (25 in this example) in the correct column for the first sample. Select the column by clicking the column header, and then click **Edit > Fill Down**. The concentration are filled in down the column.

Figure 1-50 Edit > Fill Down Option



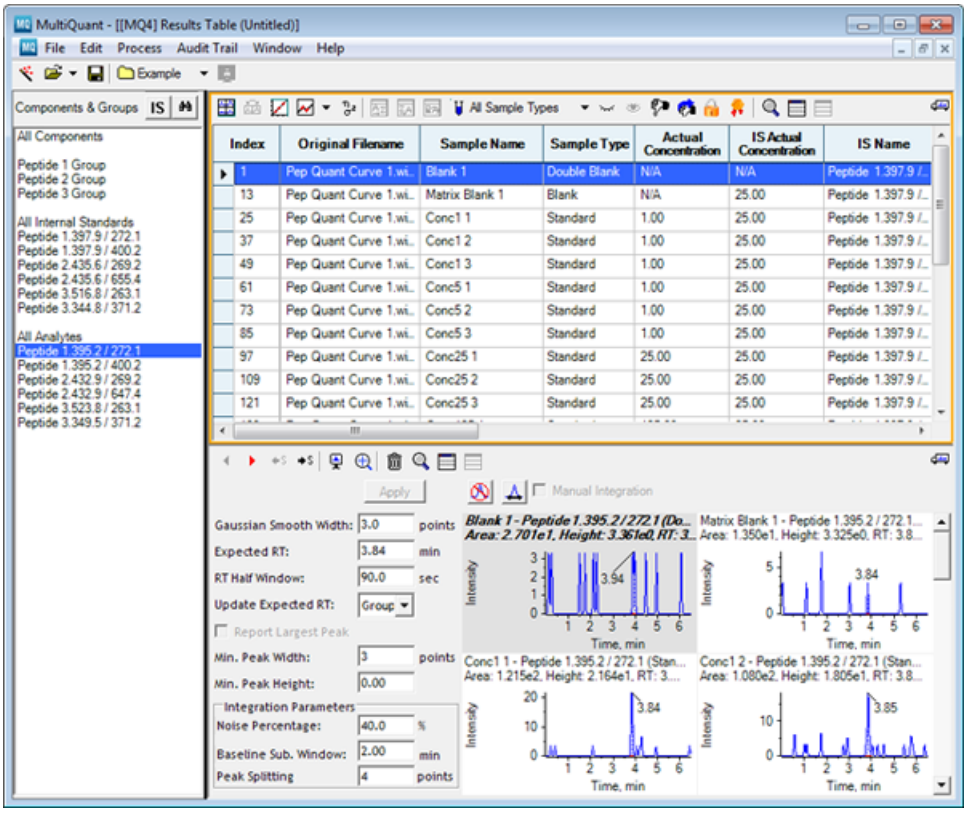
6. To translate this information to all peptide MRMs, right-click anywhere in the Results Table and then click **Apply Current IS's Actual Concentration to All**. Click another peptide under **All Internal Standards** and then make sure that each **Actual Concentration** is 25.
7. The sample types for each sample in the data file must be defined. Select one of the peptide MRMs under **All Analytes**. Select the correct Sample Type for each row by clicking the right corner of the **Sample Type** cell and then selecting the correct sample type. The Blank 1 in row 1 is a double blank as it contains no analyte or no internal standard (note the **IS Actual Concentration** is reset to N/A). The Matrix Blank 1 in row 2 is the blank as it contains only the internal standards. The remaining rows are all Standard samples and will be used for calculating the concentration curve. An alternative approach is to first set all samples to Standard using the **Fill Down** command and then to adjust the type for the first two samples.

Figure 1-51 Selecting Sample Types



8. Enter the concentrations for each sample. The concentrations to be entered are specified in the sample name (Sample Conc1 1 has a concentration of 1, Conc5 1 has a concentration of 5, and so forth).
9. After all the rows have been correctly set, right-click in the table and then click **Apply Current Analyte's Actual Concentrations to All**. This propagates these values to all analytes.

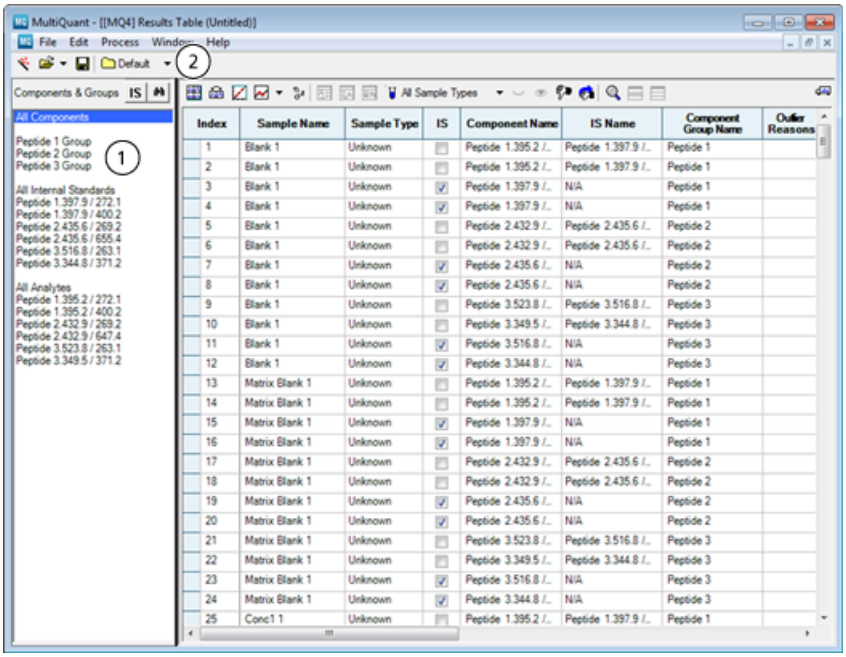
Figure 1-52 Apply Current Analyte's Actual Concentration to All Option



View Calibration Curves

1. To show the calibration curve for an MRM transition for a specific peptide, select the MRM transition from the left pane, and then click the **Show Calibration Curve** icon to view calibration curve. Refer to Figure 1-53.
2. To create more room on the screen, the peak review pane can be removed from view by clicking the **Delete** pane button or the **Hide** pane button. Alternatively, a tabular view can be created by clicking the **Tab mode** button. Each open pane will appear in its own tab.
3. Open the calibration curve pane for the first MRM transition for Peptide 1. The y-axis on the plot is the peak area ratio of the analyte over the internal standard. Normally, the internal standard concentration is held constant over a concentration curve, but in cases where this is not the case, using the concentration ratio for the x-axis automatically compensates for this.

Figure 1-53 Calibration Curve Interface



Item	Description
1	Show Calibration Curve icon
2	Toggles to tab mode
3	Deletes this pane
4	Hide pane

4. From the peak review performed, notice that there was very little peak area for Conc 1 in the dataset. Therefore this concentration can be removed from the calculation of the calibration curve by clearing the **Used** check box in each Conc1 row.

Tip! Remove a data point from the curve by right-clicking on the data point on the calibration curve pane and then clicking **Exclude**.

Notice that the three replicates for the highest concentration show a small amount of variability. Notice also that the data point now seems 'hollow' and the correlation co-efficient (r value) is improved (increased in value). In addition, it is important to observe the accuracy column in the table as this reflects how close the computed concentration at each data point is to the expected concentration. It is desirable for the accuracy of all data points in the calibration curve to lie between 80 to 120%.

The calibration curves for all the analytes can be assessed by removing outlying data points to improve the accuracy of the calibration curve. This is one advantage of performing replicates at each concentration, as the occasional data point can then be removed.

5. Sometimes, a complete concentration point must be removed, at either the low end of the curve (due to low signal for example) or at the high end of the curve (due to saturation or injection issues). Select one of the MRM transitions for Peptide 3 and notice the shape of the calibration. The curve is not very linear but can be greatly improved by removing all the points at the very highest concentration.

Figure 1-54 Before removal of high concentration data points

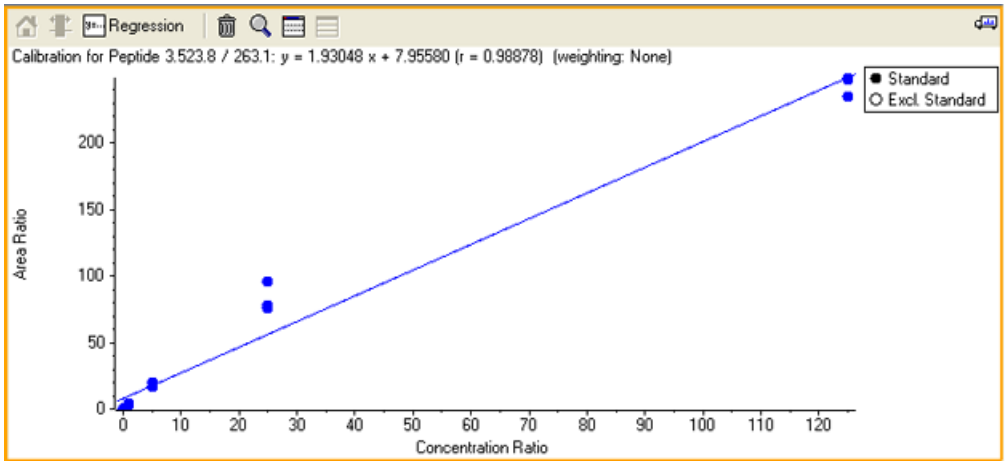
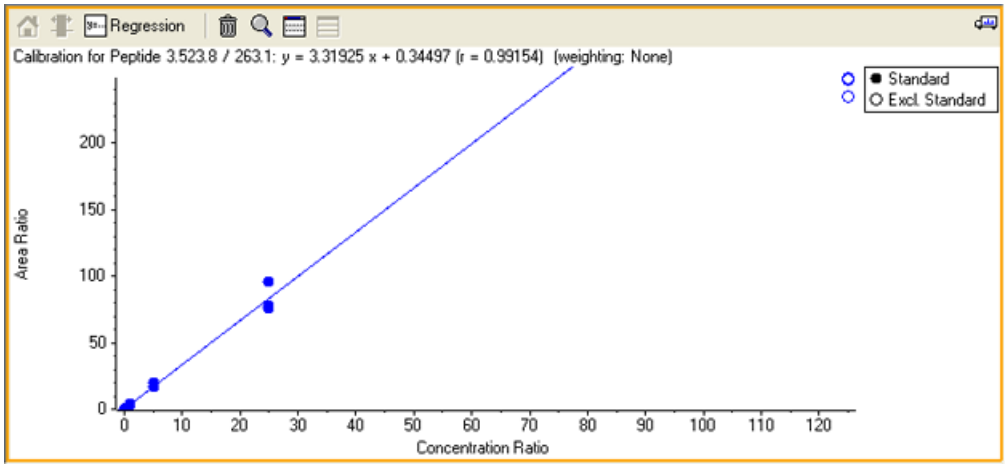


Figure 1-55 After removal of high concentration data points



Note: Any reported concentrations for unknown samples greater than the concentration of the most concentrated standard used for the calibration should be treated with some suspicion. The quality of the calibration curve can be evaluated using the accuracy values calculated in the Results Table. Typically most values across the curves should fall between 80 to 120% accuracy to be included. Points that fall outside the line can be omitted by clearing the check box in the Used column.

6. Before exporting the calibration curve, check the calibration curve statistics. Go to Evaluate the Statistics of the Standard Calibration Curve on page 57.

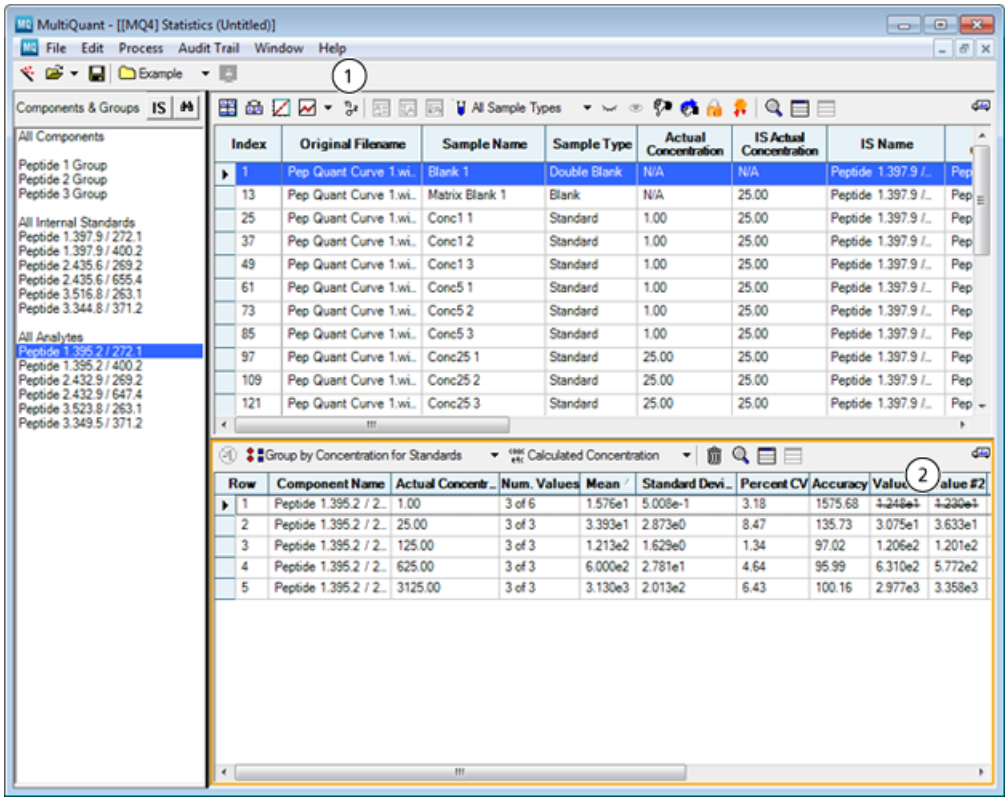
Evaluate the Statistics of the Standard Calibration Curve

The Statistics Table is used to view information related to the reproducibility of an analysis. Each row of the table summarizes information such as the average, standard deviation, and so forth for a group of related peaks, from the same analyte, that would ideally be expected to have the same response.

- Click the active Results Table, and then click the **Statistics** icon to open the Statistics pane.

  Users can assess the reproducibility of the replicates. Notice that when a peak is removed from the quantitation curve by clearing the Used check box, it is not considered in the calculations and is visualized as a struck out value.

Figure 1-56 Statistics Table



Item	Description
1	Statistics icon
2	Peak removed from statistics calculation

Export Calibration Curves

1. After the calibration curves have been optimized for all the MRM transitions for all the peptides, the calibration curve can be saved by clicking **Process > Export Calibration.** This creates a *.mqcal file.

> **Note:** This step is only necessary if you will subsequently apply this external calibration to a new Results Table.

2. For this tutorial, export the calibration noting where you save the file.

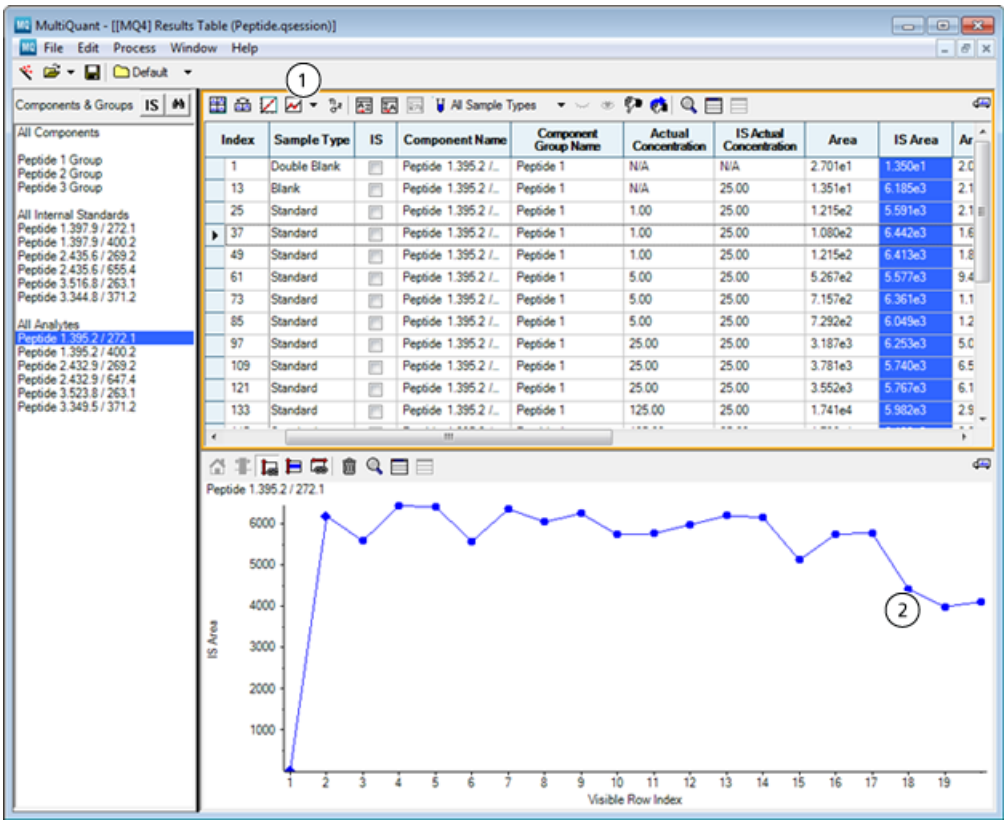
Create a Metric Plot

Metric plots are very useful for quickly assessing data quality or helping to answer specific questions of a dataset. In this example, a metric plot will be generated for the area of the internal standard to enable a quick assessment of the stability of the chromatography.

1. Select Peptide 1 in the **Components and Groups** pane and then select the IS Area column. Click the **Metric Plot** icon.

   A plot of the internal standard area for each injection is generated for that peptide. Notice the slight decrease in signal of the IS in the final three injections. The area for the first sample is very small; a small noise peak was integrated for this double-blank sample.

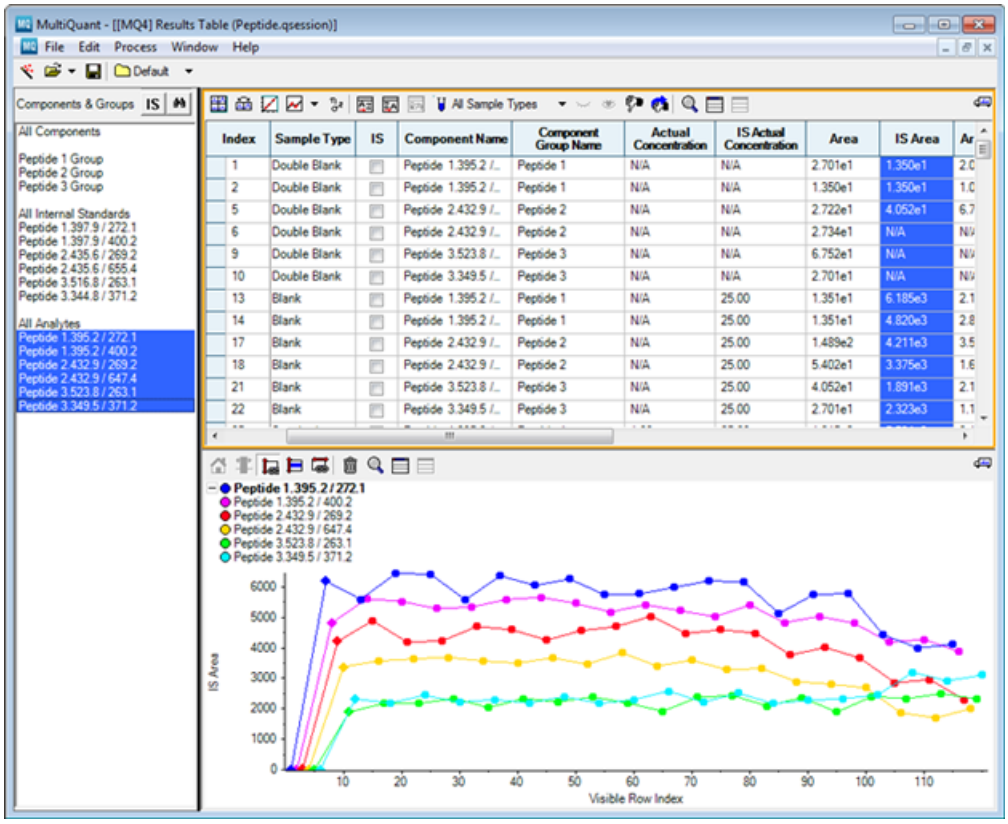
Figure 1-57 Metric Plot Interface



Item	Description
1	Display metric plot
2	Decrease in signal

2. Select a different peptide and then review the metric plot updates. Finally, select all peptides by selecting the first peptide and then holding the **Shift** key while selecting the final peptide. An overlay of all 6 metric plots is generated.

Figure 1-58 Overlaid Metric Plots

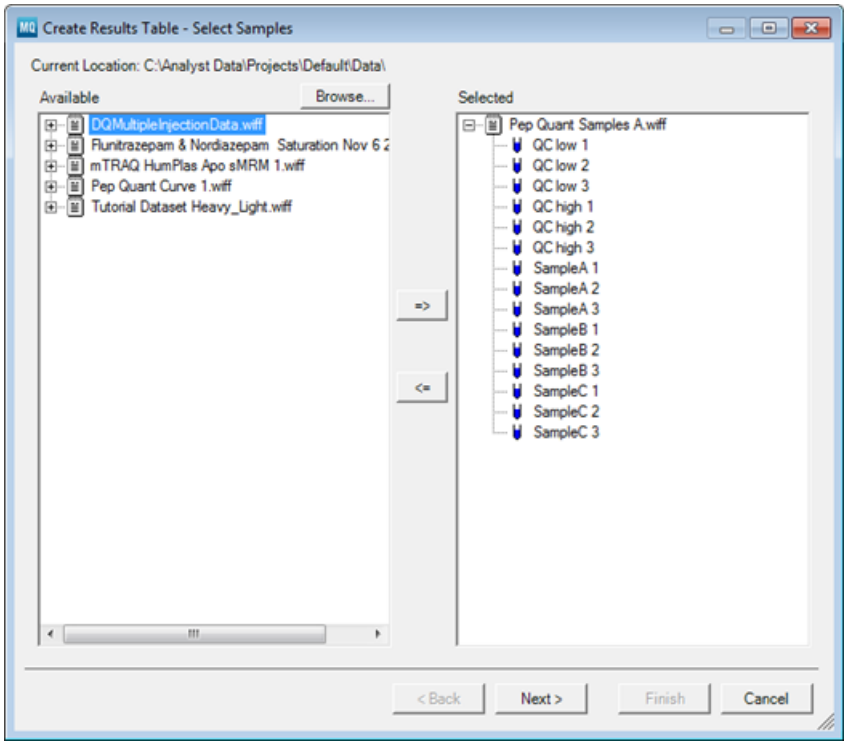


Process Unknown Samples

After the calibration curve is generated, the biological samples (unknowns) can be processed. In these samples, you want to measure the amount of the endogenous light peptide present in each sample. The heavy peptide can be added into each biological sample at a fixed amount. It can then act as the internal standard for these samples to add robustness to your quantitation experiment. Using the calibration curve previously generated, 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).

1. Click **File > New Results Table.**
2. On the Create Results Table - Select Samples page, drag the **Pep Quant Samples A.wiff** file into the Selected pane and then click **Next**.

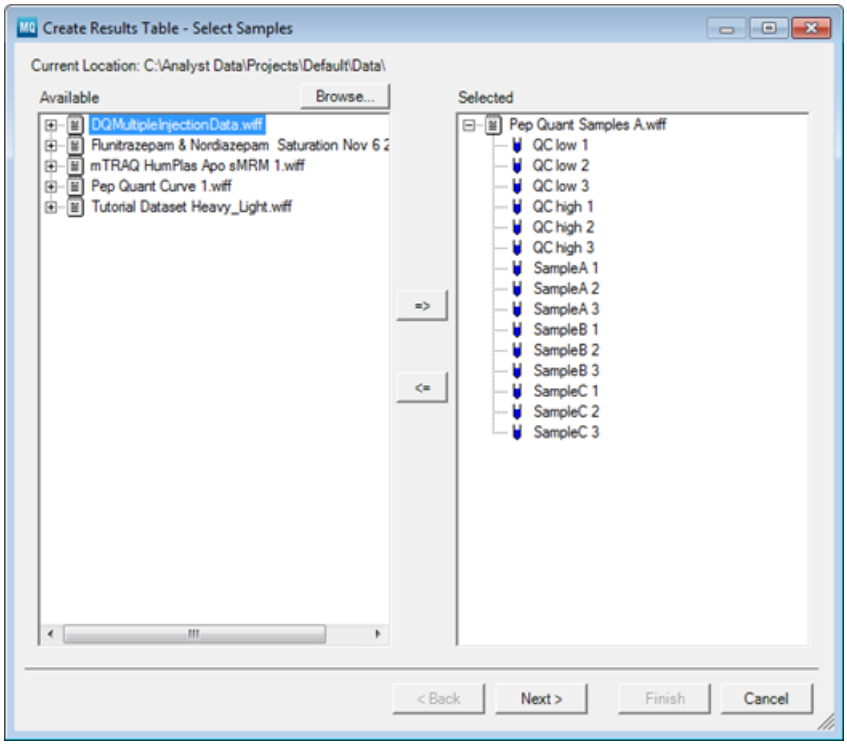
Figure 1-59 Create Results Table - Select Samples Page



3. On the Create Results Table - Select Method page, click the **Choose Existing Method** option and then select the **Edit Method** check box.
4. Click **Open**, select the method created in Accurate Quantitation Using Calibration Curves on page 37, and then click **Next**.

   In a subsequent step we will switch the analyte and internal standard MRMs. The net effect will be to overwrite the method.
5. On the Create Results Table - Select Representative Sample page, a representative sample has been recommended and is selected. Click **Next**.

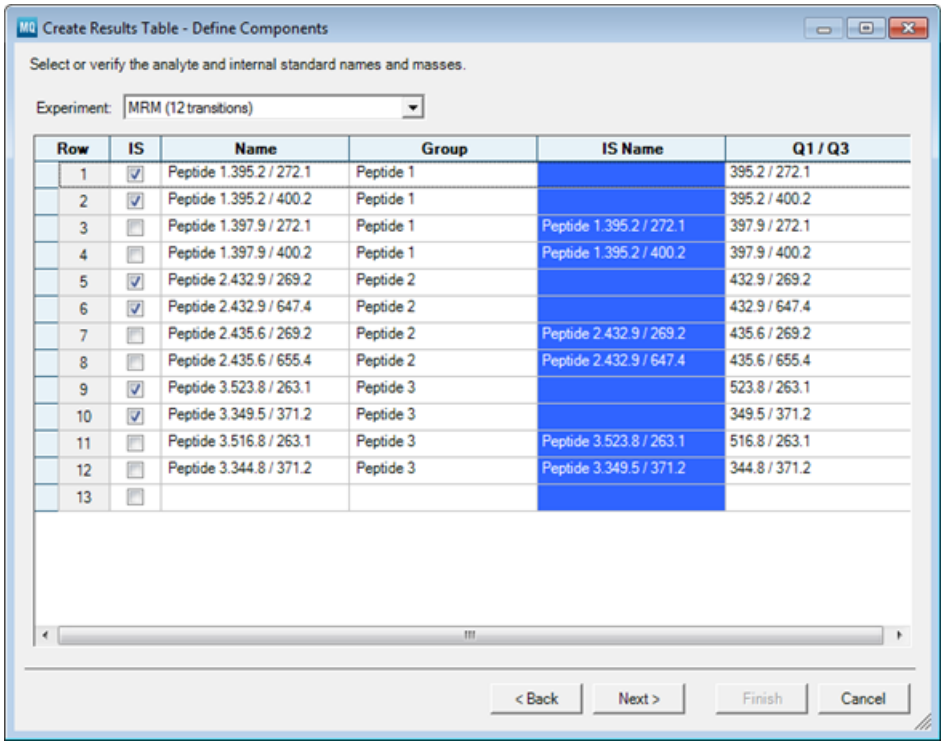
Figure 1-60 Create Results Table - Select Representative Sample Page



6. On the Create Results Table - Define Components page, right-click in the table and then click **Internal Standards > Reverse Analyte/IS Assignments**.

   The analytes and internal standards should interchange so that the method now looks as shown in Figure 1-61.

Figure 1-61 Create Results Table - Define Components Page

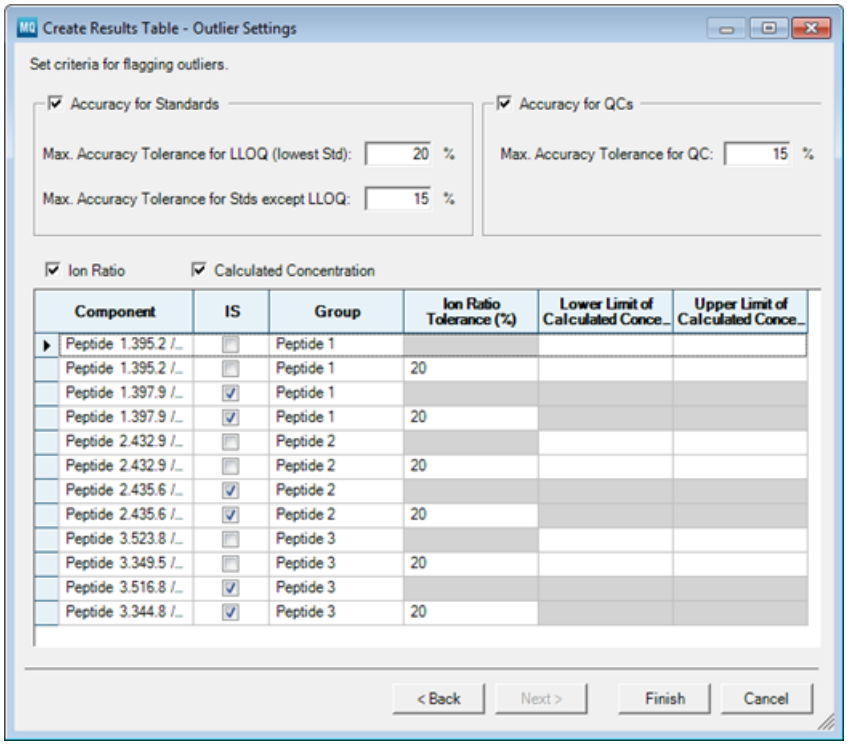


7. Click **Next** to advance to the Create Results Table - Define Integration page.

   Because the integration parameters were previously reviewed when the method was first created, you do not have to make any further changes.

8. Click **Next**.

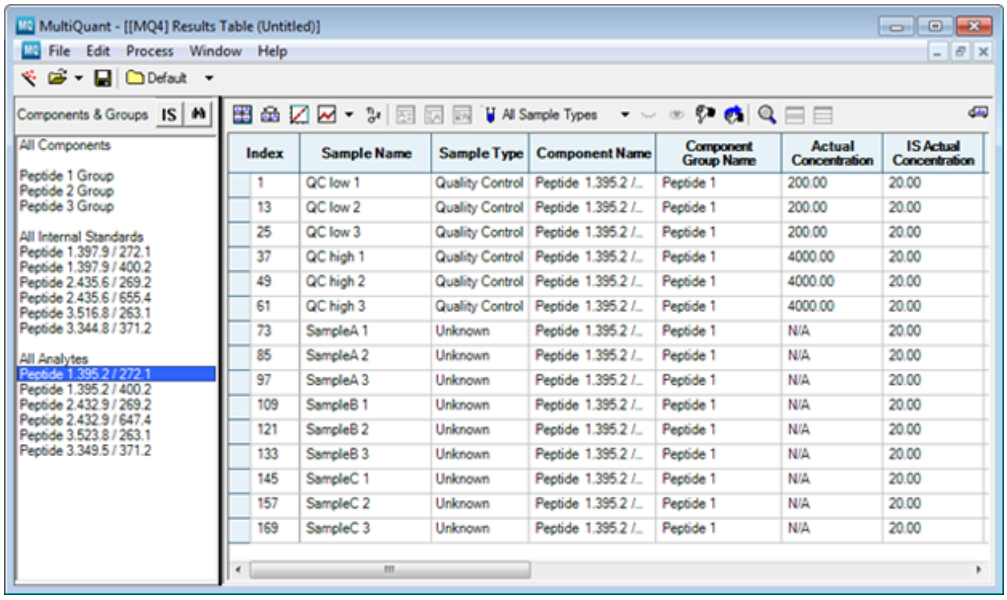
Figure 1-62 Outlier Settings



9. Specify the fields as required to automatically flag samples for review in the Results Table. Refer to the *Reference Guide* for more information about the **Outlier Settings** parameters.
10. Click **Finish**.
11. Define the **Sample Type** for the QC samples as Quality Control and the leave the remaining samples as Unknown.
12. Type an **IS Actual Concentration** of 20 for every sample.
13. Type 200 for the **Actual Concentration** for the QC low samples and 4000 for the QC high samples. The Results Table should now look the following figure.

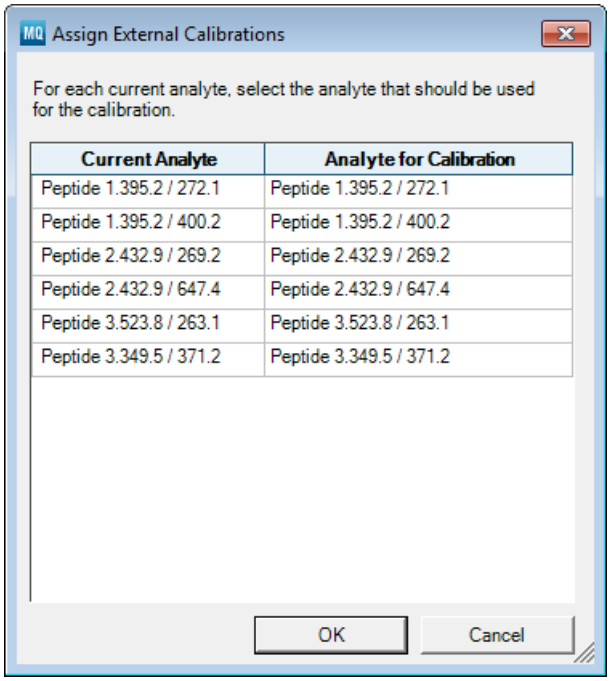
Tip! To fill down a specific section of cells in a table, click the first and last cells in the section while pressing the Shift key. The cells will be selected and you can click **Edit > Fill Down** to populate the cells.

Figure 1-63 Actual Concentrations



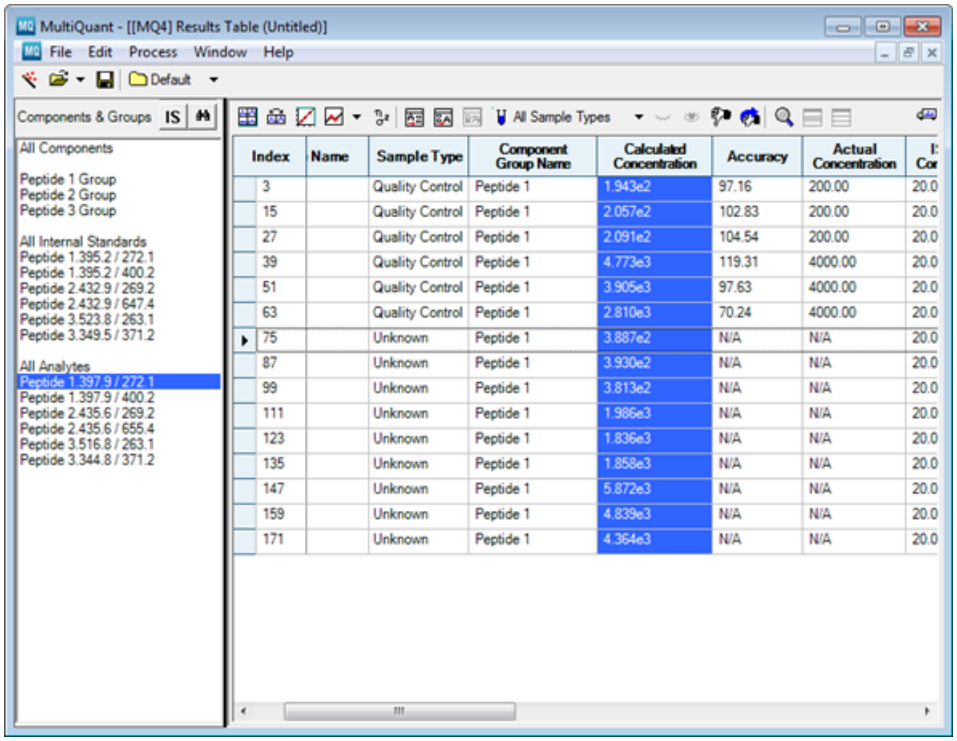
14. Click **Process > Import External Calibration** to import the calibration curve calculated in the previous section into this Results Table. Browse for the *.mqcal file that was previously saved and then click **Open**.
15. In the Assign External Calibrations dialog, select the calibration curve that should be used for each specific analyte in the current data file. Specify the relationships as shown and then click **OK**.

Figure 1-64 Assign External Calibrations dialog



After the calibration file is loaded, the Calculated Concentration and Accuracy columns are automatically calculated. The accuracy obtained for the QC samples is very good, indicating the assay is working as expected. The measured concentrations of the three unknowns in each of the samples A, B, and C can be found in the Calculated Concentration column.

Figure 1-65 Calculated Concentration and Accuracy columns



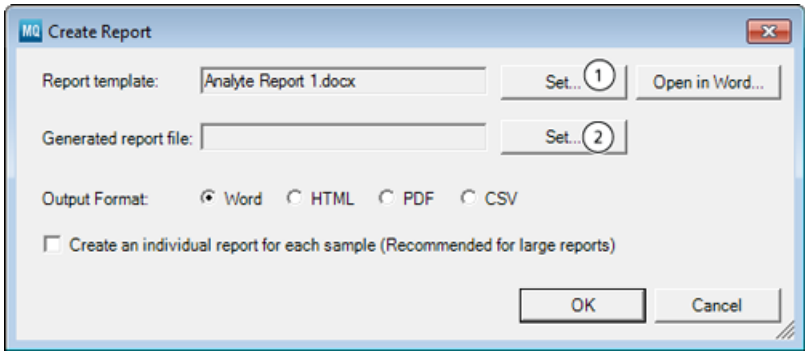
16. Save the Results Table.

Create Reports

If you are creating reports using unlocked Results Tables, make sure that you are allowed to create and export unlocked Results Tables. See your administrator if you are unable to create reports.

1. Click **File > Create Report and Save Results Table.**

Figure 1-66 Create Report Dialog



Item	Description
1	Click to select the template.
2	Click to save the report.

2. Click **Set** (Figure 1-66 item 1) to navigate to the Tutorial Dataset Heavy_Light.xml template in the following folder:

   - For Windows 7 and Windows 10: C:\ProgramData\AB SCIEX\MultiQuant\Reporter.

3. Click **Set** (Figure 1-66 item 2) to create the name and location of the report.
4. Click **OK**.

Revision History 2

Revision	Reason for Change	Date
A	First release of document.	August 2013
B	Rebranded to SCIEX, updated the copyright page, added Windows 10, changed software version to 3.0.3, added Contact Us and Technical Support sections, fixed link in step 5 in the Modify the Results Table section. Added path for Analyst® software documentation on Windows 10. Removed references to Windows XP.	July 2017