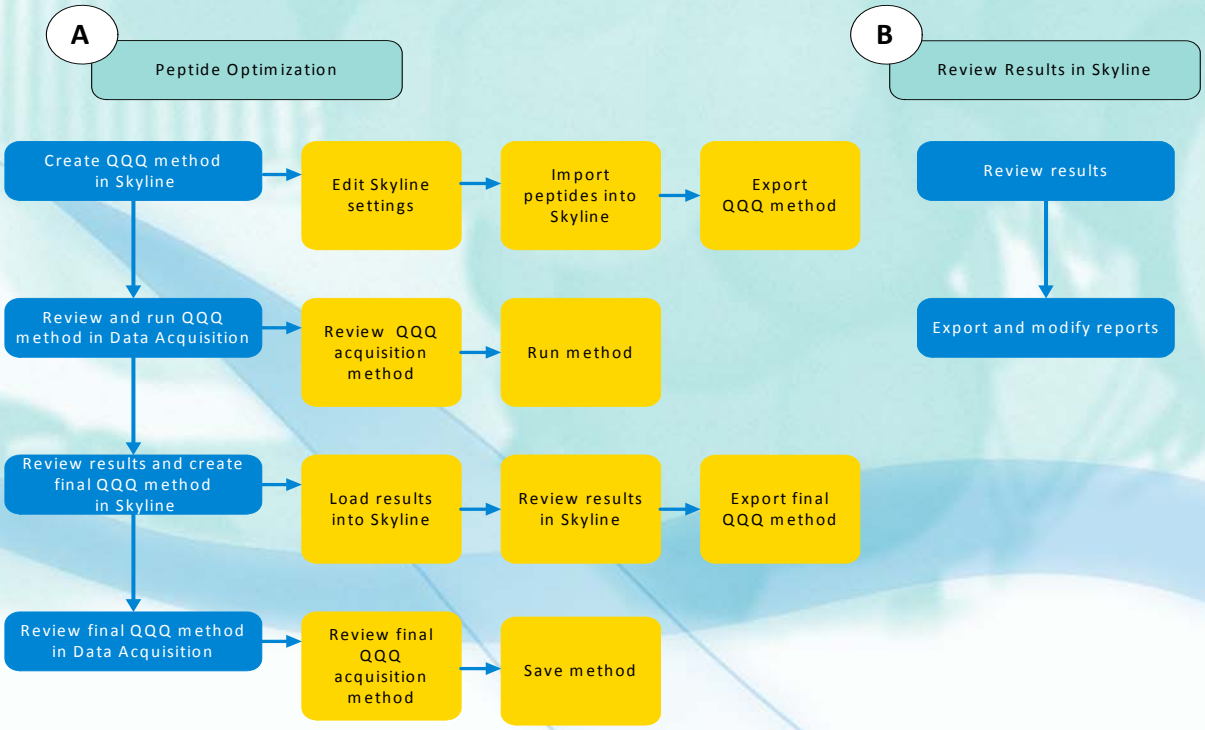
Agilent Triple Quadrupole LC/MS Peptide Quantitation with Skyline

Workflow Overview



Agilent Technologies

Required items

Required hardware and software

Figure 1 *The workflow requires an Agilent LC and an Agilent 6400 Series Triple Quadrupole LC/MS System.*

To do this workflow, you need:

- One of the following LCs:
  - Agilent 1220 Infinity LC
  - Agilent 1260 Infinity LC
  - Agilent 1290 Infinity LC
  - Agilent 1200 Series LC system
  - Agilent 1200 Series Rapid Resolution LC system
- Agilent 6400 Series Triple Quadrupole LC/MS System
- Agilent MassHunter Data Acquisition for QQQ version B.05.00
- Skyline software version 1.1 or greater from the MacCoss Lab at the University of Washington

Peptide Optimization

This section show you how to optimize the collision energy for the transitions that you are acquiring.

Create QQQ method in Skyline

In this exercise, you first enter your peptide into Skyline. Then, Skyline creates a list of precursor ions based on your settings and predicts the product ions. From this list, you can select which precursor ions and product ions to include. Then, you can create a QQQ Data Acquisition method. The LC parameters and the other MS parameters are copied from the template method that you select.

You can also export a transition list that you can use to optimize the collision energy for each transition. This transition list can be pasted into an existing QQQ Data Acquisition method in the Data Acquisition program.

1. Edit Skyline settings.

a Start the Skyline program.

b Edit Peptide Settings.
- On the Digestion tab, set the Enzyme correctly.
- Make any Structural modifications.

c Edit collision energy settings in the Transition Settings dialog box.
- After reviewing a standard set of peptides, Agilent recommends a **Step size** of 4 and a **Step count** of 2.

d Edit filter settings in the Transition Settings dialog box.
- On the Filter tab, specify the Precursor charges and the **Ion charges**.

e Set the maximum *m/z* in the Instrument tab. This value cannot be larger than the value entered in the Data Acquisition program in the QQQ tab.

f Save the settings.

2. Import peptides into Skyline.

The protein name and the peptide are shown in the Skyline program. The Skyline program uses the Peptide Sequence and the values in the Filter tab of the Transition Settings to determine the precursors. Then, the Skyline program predicts the transitions.

a Copy your peptide to the Clipboard.

b Paste the peptide in the **Peptide Sequence** column in the Insert dialog box.

c Make any necessary modifications to the peptide.

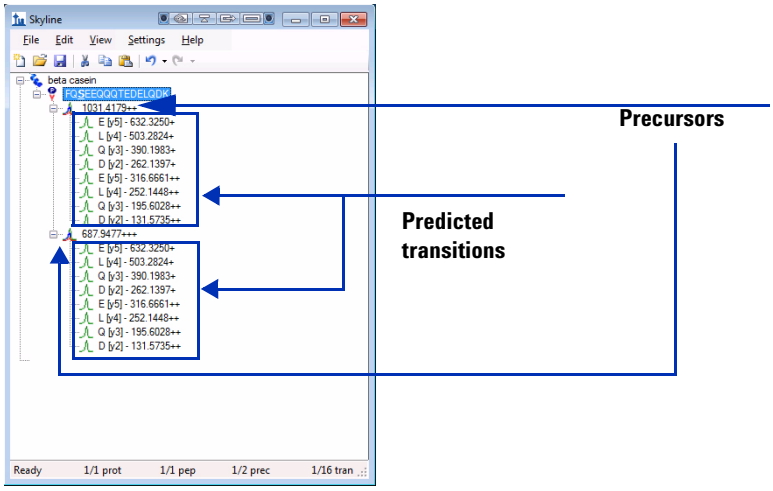


Figure 2 *Main window of the Skyline program*

3. Export QQQ method

You can either export a method directly from Skyline or you can export a transition list that you can import into the MassHunter Data Acquisition program. Exporting a method is the recommended option, but both options are documented here.

a Open the Export Method dialog box.

b Select **Agilent 6400 Series** as the **Instrument type**.

c Select **Collision Energy** for **Optimizing**.

d Select **Standard** for **Method type**.

e Select the method to use as a template.
- This method is an existing QQQ Data Acquisition method. All of the parameters in the existing QQQ Data Acquisition method are copied to the new method except for the Scan segments table. The Scan segments table is created from the information in Skyline. Also, only the first segment in the Time segments table in the template method is used. All other time segments are deleted.

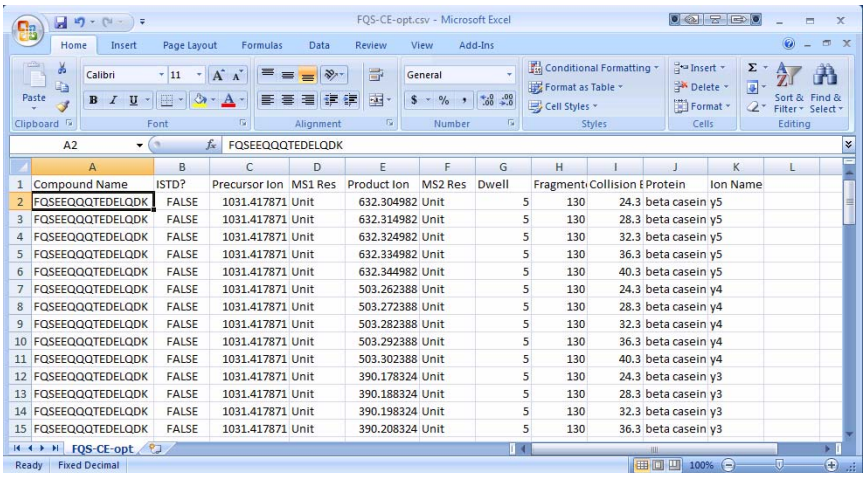
f Save the Skyline project.

Export transition list

If you have not yet developed your template method, you can instead export a transition list. Then, when you create your method in the Data Acquisition program, you can paste this transition list into the Scan segments table.
- Select **Agilent 6400 Series** as the **Instrument type**.
- Select **Collision Energy** for **Optimizing**.
- Select **Standard** for **Method type**.

Format of CSV file

The format of the CSV file that contains the Transition List is:



	A	B	C	D	E	F	G	H	I	J	K	L
1	Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Fragment	Collision E	Protein	Ion Name	
2	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	632.304982	Unit	5	130	24.3	beta casein	y5	
3	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	632.314982	Unit	5	130	28.3	beta casein	y5	
4	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	632.324982	Unit	5	130	32.3	beta casein	y5	
5	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	632.334982	Unit	5	130	36.3	beta casein	y5	
6	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	632.344982	Unit	5	130	40.3	beta casein	y5	
7	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	503.262388	Unit	5	130	24.3	beta casein	y4	
8	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	503.272388	Unit	5	130	28.3	beta casein	y4	
9	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	503.282388	Unit	5	130	32.3	beta casein	y4	
10	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	503.292388	Unit	5	130	36.3	beta casein	y4	
11	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	503.302388	Unit	5	130	40.3	beta casein	y4	
12	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	390.178324	Unit	5	130	24.3	beta casein	y3	
13	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	390.188324	Unit	5	130	28.3	beta casein	y3	
14	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	390.198324	Unit	5	130	32.3	beta casein	y3	
15	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	390.208324	Unit	5	130	36.3	beta casein	y3	

Figure 3 *CSV file for the Transition List*

- Each transition has five rows in the transition list. There are two steps below the original collision energy and two steps above the original collision energy.
- The collision energy increases by the step size that you set in "Edit Skyline settings." on page 3.
- The Product Ion also changes slightly. The Skyline program uses those slight changes to keep track of which step it is on.
- Skyline always exports the transition list using **Unit** for the **MS1** and **MS2** resolution. You can modify the **MS1** and **MS2** resolution before or after you paste the transition list into the **Scan segments** table.

Review and run QQQ method in Data Acquisition

After you create the QQQ method in Skyline, you load the method in the Data Acquisition program and run it to optimize the collision energy. You could also import the transition list from Skyline and create a method that optimizes the collision energy. This task gives you the basic steps to set up an LC/MS method with the MassHunter Data Acquisition program. If you need more details and practice, see the *Agilent MassHunter Workstation Software – Data Acquisition for 6400 Series Triple Quadrupole LC/MS Familiarization Guide*.

1. Review QQQ acquisition method.

a Start the MassHunter Data Acquisition program.

b Load the QQQ acquisition method from Skyline.

c Review values for all of the LC modules.

d Review parameters on the QQQ tabs.
- Each transition has five rows in the Scan segments table. There are two steps below the original collision energy and two steps above the original collision energy.
- The collision energy increases by the step size that you set in the Transition Settings dialog box.
- The Product Ion also changes slightly. The Skyline program uses those slight changes to keep track of which step it is on.

e Review parameters in the DA tab. Clear the **Qual Automation** check box and the **Quant Automation** check box.

f Save the method.

Import a transition list

If you created a transition list, then the contents of the CSV file can be pasted directly into the Scan segments table using the shortcut command, **Paste from Clipboard**. You can follow the steps in the "Review QQQ acquisition method." on page 5 and then follow these steps to modify the Scan segments table.

a Open the Transition List file in Excel. You saved this CSV file in "Export transition list" on page 4.

b Copy the transition list to the Clipboard.

c Import the transition list. Click the **Paste from Clipboard** menu item in the shortcut menu in the Scan segments table to update the Scan segments table.

d Save the method.

2. Run the method.

a Start the MassHunter Data Acquisition program, if it is not currently running.

b Load your method.

c Edit the information in the Sample Run window. Enter the **Name** for the sample, a **Comment**, and a **Name** for the data file.

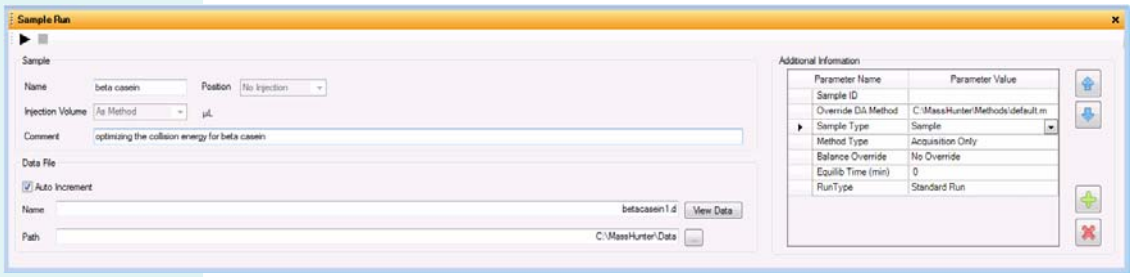


Figure 4 *The Sample Run window*

d Run the method.

Review results and create final QQQ method in Skyline

This section shows you how to create the final optimized QQQ method. You first need to import the results from the data file you created. Then, you can graphically review the results and export a Dynamic MRM method.

1. Load results into Skyline.

a Load the settings that you created in "Save the settings." on page 3.

b Open the Skyline project you saved in "Export QQQ method" on page 4.

c Import the results.
- Click the Add one new replicate button in the Import Results dialog box.
- Select **Collision Energy** from the **Optimizing** list.
- In the "Import Results Files" dialog box, select the data file you created.

d Make modifications to the peptide.

2. Review results in Skyline.

The graphical user interface contains a lot of information.

- A green dot next to a product ion means that the transition was found in the data file.
- The numbers after the peptide represents the relative abundance of that transition compared to the other transitions for that peptide. This value helps when you have optimized more transitions than you will use in the final method which is the typical method development strategy.
- If you place the mouse over the items in the list, a tooltip is displayed which gives you more information.
- You can change the graphs that are displayed using the commands in the View menu.
- You can right-click each graph to change how the graph is displayed. For example, you can right-click the graph and click Transitions > Single to display the transitions as a bar graph or click Transitions > Total to display the transitions stacked together.
- If a green dot is beside a precursor ion, then all of the selected transitions were found.
- If a yellow dot is beside a precursor, then at least one of the selected transitions was not found.
- If a red dot is beside a precursor, that means that over half of the selected transitions were not found.

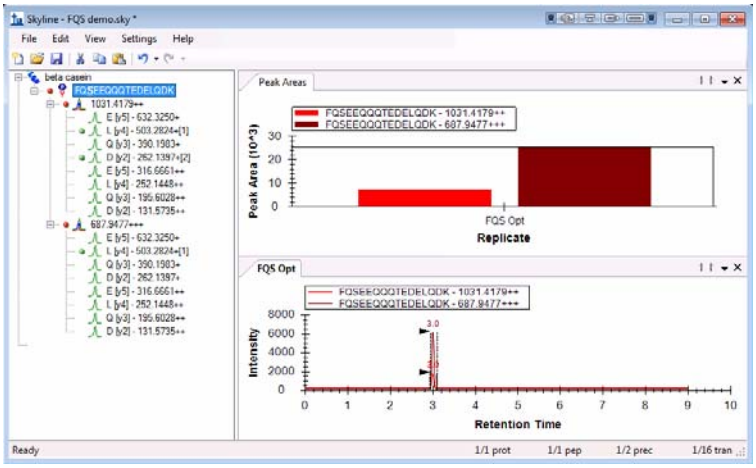


Figure 5 *Skyline program after importing results file*

a Select the transitions that were found.

b Examine each transition to see which collision energy created the greatest peak area. You can examine the Peak Areas graph and the Retention Time graph.

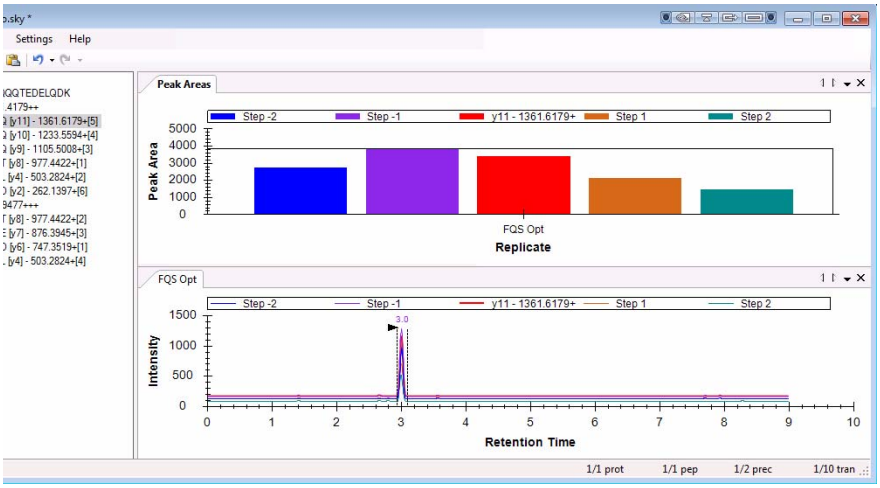


Figure 6 *Comparing collision energies for the same transition*

c (optional) Edit the regression parameters to change the initial equation. Typically, you only want to determine a new equation if you have a large distribution of peptides.

3. Export the final method.

You do the following steps to use the optimal collision energies for a peptide or set of peptides to create a final method. You select a template method to use to create the final method in the Agilent Data Acquisition program.

- In the Export Method dialog box, select **Agilent 6400 Series** as the **Instrument type**.
- Click the **Single method** button.
- Select **None** in the **Optimizing** list.
- Select **Scheduled** as the **Method type**.
- Select a Template file which is an existing QQQ Data Acquisition method. All of the parameters in this method are copied to the new method except for the Scan segments table. This table is created from the information in Skyline. Also, only the first segment in the Time segments table in the template method is used. All other time segments are deleted.

You also can export a final transition list. See "Export transition list" on page 4 for instructions on exporting a transition list. If you are exporting a final transition list, select **None** in the **Optimizing** list.

Review final QQQ method in Data Acquisition

a Start the MassHunter Data Acquisition program.

b Load the QQQ acquisition method from Skyline.

c Review parameters on all of the LC modules.

d Review parameters on all of the QQQ tabs.

e Review parameters on the DA tab.

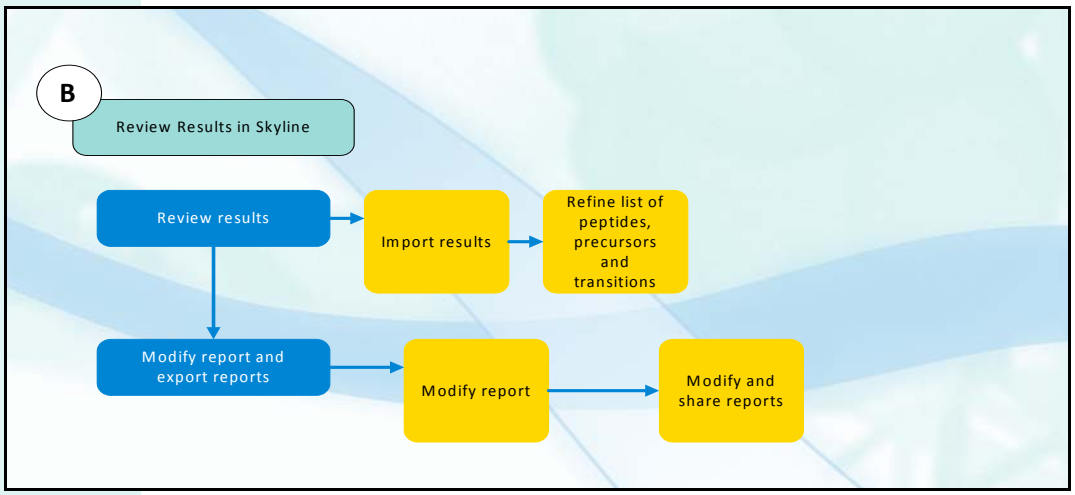
f (optional) Save any changes you made to the method.

Review results in Skyline

These exercises show you how to use Skyline to review the proteins, peptides and transitions. You can remove proteins, peptides, and transitions, and you can also add other proteins, peptides, and transitions.

You can also export reports which are CSV files. The CSV file contains a table with quantitative information for each data file that is opened. You can load this CSV file into Excel or another program to do further analysis.

Review results



1. Import results.

a Start the Skyline program.

b Select previously saved settings.

c Paste list of proteins or peptides.
- Open an existing project.
- Copy the protein or peptides into the Clipboard and paste them into Skyline.
- Click **Edit > Insert > FASTA** and select a FASTA file to import.
- Click **Edit > Insert > Proteins** and specify the proteins to import.
- Click **Edit > Insert > Peptides** and specify the peptides to import.

d Modify the peptides by right-clicking the name of the peptide and then clicking **Modify**.

e (optional) Select a library to use to help verify the results.

f Save the project.

g Import one data file.

2. Refine list of peptides, precursors and transitions.

Skyline has many tools to allow you to refine the list of transitions. The following methods are described in this section:
- Remove peptides that are not present in the data file
- Change the transitions that are selected

Skyline can automatically refine proteins and peptides when you use the commands in the **Edit > Refine** menu.

a Remove peptides that were not present in the data file. If none of the transitions have a green dot next to them, then no transitions were found in the data file. Click the green check mark. You may decide to delete this precursor.
   - If no precursors exist for a peptide, then delete the peptide. If you cannot expand the peptide, then no precursors exist.

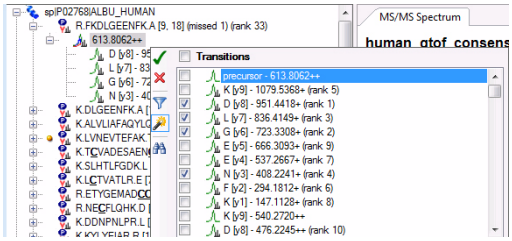


Figure 7 *No transitions were found for this precursor*

b Change selected transitions for a peptide.
   - You can also change the transitions that are selected for a precursor. If the dot before the precursor is orange, then one or more selected transitions were not found.

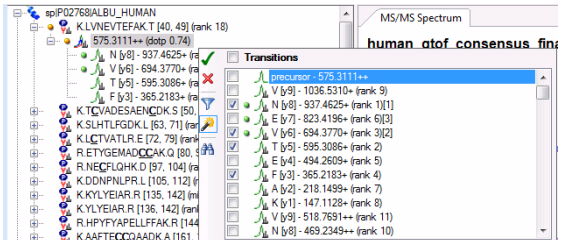


Figure 8 *This precursor is missing some of the selected transitions.*

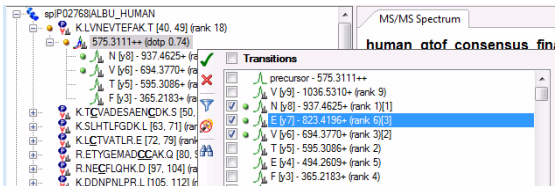


Figure 9 *Only the transitions that were found are marked.*

c Import replicate data files.

Modify and export reports

You can modify and export a report from Skyline. A report in Skyline is a CSV file which contains a single table with many columns of information. Each row in the table is a different sample. You can load the report file into Excel to continue to review the data.

You can use the tools provided by Skyline to create and save new reports. You can add new columns to the existing reports, and you can remove the columns that the report starts with. The output of a report is a CSV (comma separated variables) file.

1. Export report.

When Skyline is installed, three report definitions are installed:

- Peptide Ratio Results definitions
- Peptide RT Results definitions
- Transition Results definitions

This exercise shows you how to export a report. The next exercise shows you how to modify and save a new report definition. You also learn how to import a report definition from a file.

a Display the Export Report dialog box.

b Export a report.

2. Modify and share reports.

Skyline allows you to customize a report. You can remove columns from the report and add other columns. When you have created the report definitions, you can save the report definition to a file and move it to other computers in your lab.

a Modify a report.

- Copy an existing report before modifying the report.
- In the Edit Report dialog box, type a new name for the report.
- Remove any columns that you do not want to include in the report.
- Add any columns that you do want to include in the report.
- Arrange the items in the report in the order that you want them to appear in the report.

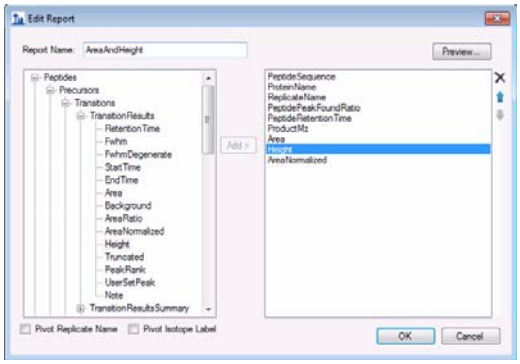


Figure 10 Edit Report dialog box

b Save the report. Click **File > Export > Report**.

- You can copy the report to another system to use. To import the report, you click **File > Export > Report** and then click the **Import** button.

For More Information

For details about these procedures, see the *Agilent Triple Quadrupole LC/MS Peptide Quantitation with Skyline Workflow Guide*.

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