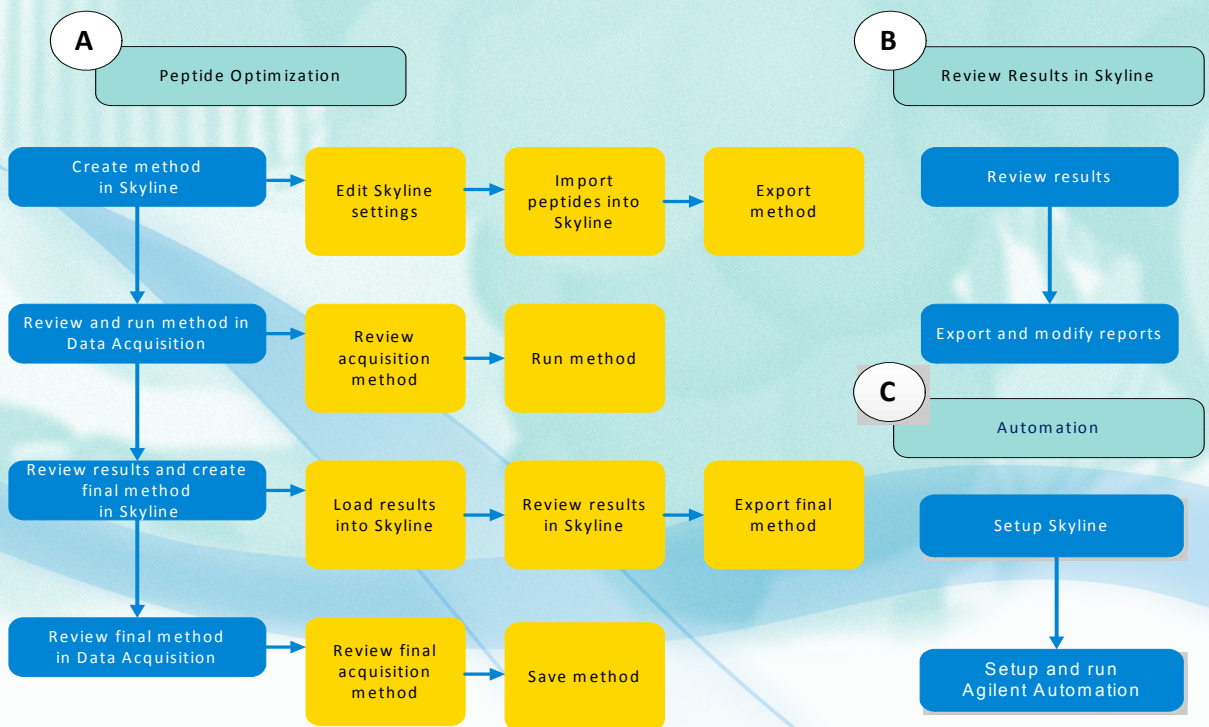


# Agilent Triple Quadrupole LC/MS Peptide Quantitation with Skyline

## Workflow Overview



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**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 1260 Infinity II 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 Triple Quadrupole Version B.08.00 or later
- Skyline software version 3.5 or greater from the MacCoss Lab at the University of Washington

## Peptide Optimization

### Create method in Skyline

#### 1. Edit Skyline settings.

#### 2. Import peptides into Skyline.

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

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 Data Acquisition method. The LC parameters and the other MS parameters are copied from the template method that you select.

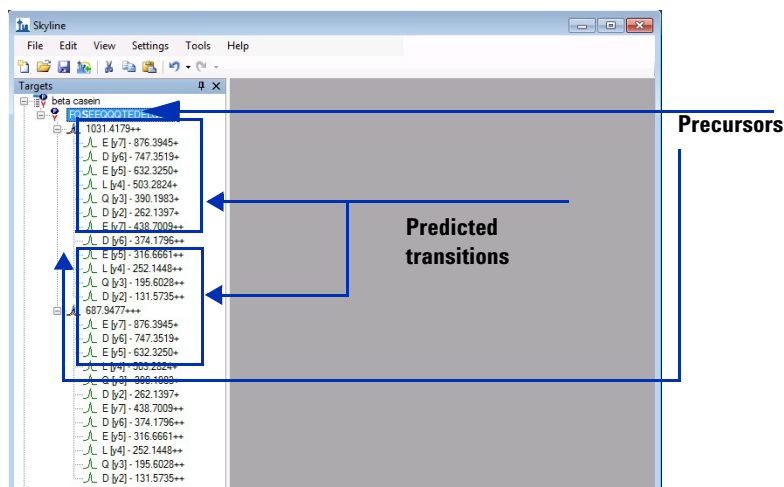
You can also export a transition list that you can use to optimize the collision energy for each transition. This transition list can be pasted into an existing Data Acquisition method in the Data Acquisition program.

- 
- 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 3 or 4 and a **Step count** of 3.
  - 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.

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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 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.

- Open the Export Method dialog box.
- Select **Agilent 6400 Series** as the **Instrument type**.
- Select **Collision Energy** for **Optimizing**.
- Select **Standard** for **Method type**.
- Select the method to use as a template.
  - This method is an existing Data Acquisition method. All of the parameters in the existing 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.
- 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:

## Review and run method in Data Acquisition

### 1. Review acquisition method.

	A	B	C	D	E	F	G	H	I	J	K	L
1	Compound	Compound Name	ISTD?	Precursor	MS1 Res	Product Ion	MS2 Res	Dwell	Fragment	Collision Energy	Cell Acceleration	Ion
2	beta casein	FQS[+80.0]EEQQQTEDELQDK.light	FALSE	1031.418	Unit	876.3745	Unit	20	130	24.3	4	y7
3	beta casein	FQS[+80.0]EEQQQTEDELQDK.light	FALSE	1031.418	Unit	876.3845	Unit	20	130	28.3	4	y7
4	beta casein	FQS[+80.0]EEQQQTEDELQDK.light	FALSE	1031.418	Unit	876.3945	Unit	20	130	32.3	4	y7
5	beta casein	FQS[+80.0]EEQQQTEDELQDK.light	FALSE	1031.418	Unit	876.4045	Unit	20	130	36.3	4	y7
6	beta casein	FQS[+80.0]EEQQQTEDELQDK.light	FALSE	1031.418	Unit	876.4145	Unit	20	130	40.3	4	y7
7	beta casein	FQS[+80.0]EEQQQTEDELQDK.light	FALSE	1031.418	Unit	747.3319	Unit	20	130	24.3	4	y6
8	beta casein	FQS[+80.0]EEQQQTEDELQDK.light	FALSE	1031.418	Unit	747.3419	Unit	20	130	28.3	4	y6
9	beta casein	FQS[+80.0]EEQQQTEDELQDK.light	FALSE	1031.418	Unit	747.3519	Unit	20	130	32.3	4	y6

**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.

After you create the 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*.

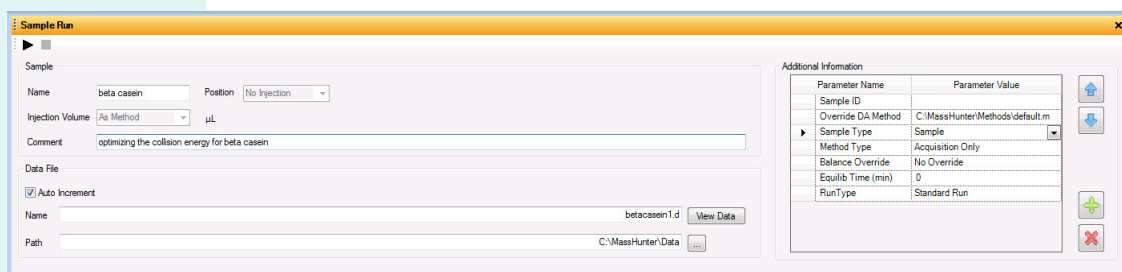
- Start the MassHunter Data Acquisition program.
- Load the acquisition method from Skyline.
- Review values for all of the LC modules.
- Review parameters on the QQQ tabs.
  - Each transition has (2\*Step Count + 1) rows in the Scan segments table. In this example, three steps are below the original collision energy and three steps are above the original collision energy plus 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.
- Review parameters in the DA tab. Clear the **Qual Automation** check box and the **Quant Automation** check box.
- Save the method.

## Import a transition list

### 2. Run the method.

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 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.
  - 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 method in Skyline

### 1. Load results into Skyline.

This section shows you how to create the final optimized 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.

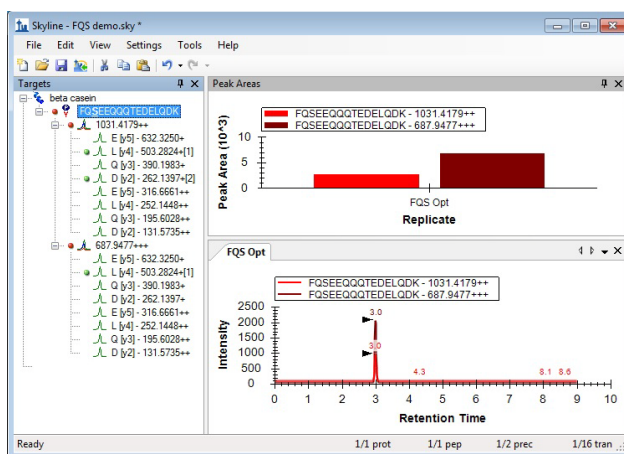
- a Load the settings that you created in “[Save the settings.](#)” on page 3.
- b Open the Skyline project you saved in “[Export method](#)” on page 4.
- c Import the results.
  - Click the **Add one new replicate** button in the **Import Results** dialog box.
  - Select **Collision Energy** from the **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

- Select the transitions that were found.
- 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 determine a new equation only 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.

- a In the Export Method dialog box, select **Agilent 6400 Series** as instrument type.
- b Click the **Single method** button.
- c Select **None** in the **Optimizing** list.
- d Select **Scheduled** as the **Method type**.
- e Select a Template file which is an existing 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 method in Data Acquisition

- a Start the MassHunter Data Acquisition program.
- b Load the 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.

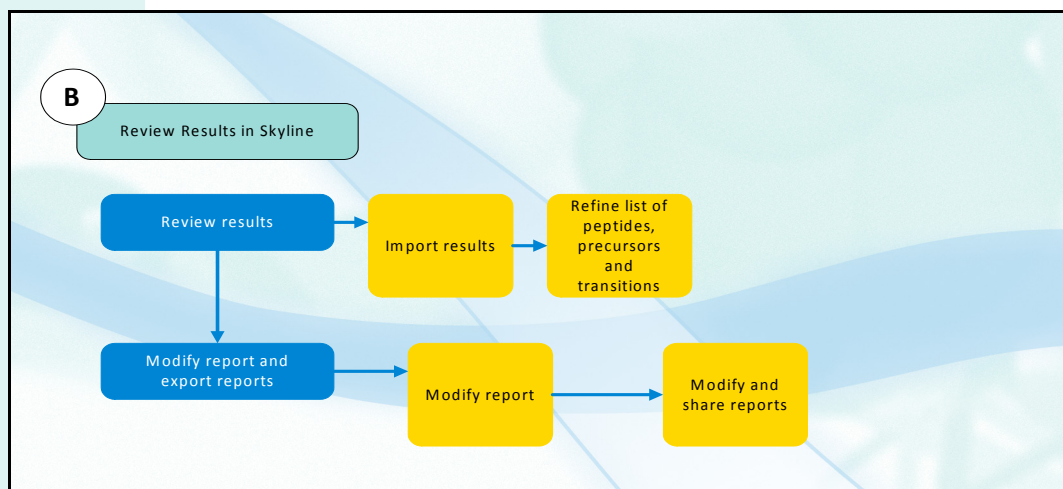


## Review results in Skyline

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.

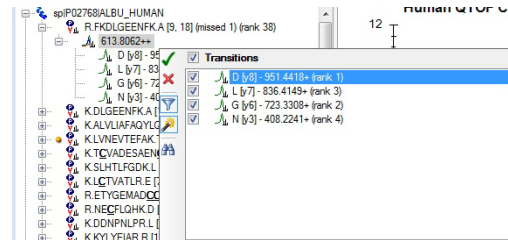
#### 2. Refine list of peptides, precursors and transitions.

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

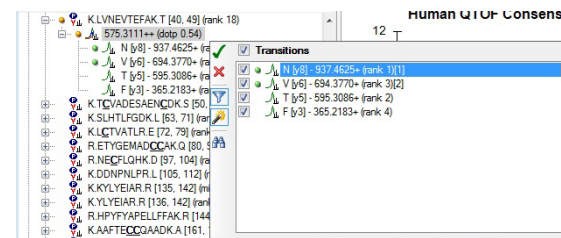
Skyline can automatically refine proteins and peptides when you use the commands in the **Edit > Refine** menu.

- 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.

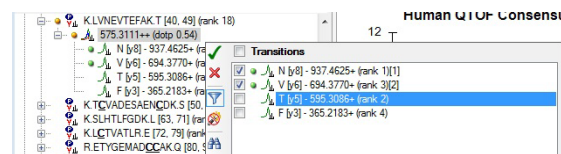


**Figure 7** No transitions were found for this precursor

- 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.

## 1. Export report.

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.

When Skyline is installed, three report definitions are installed:

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

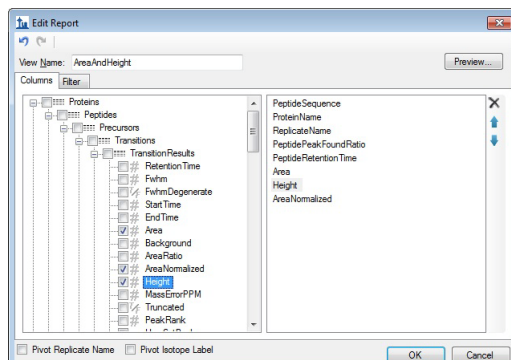
This exercise shows you how to export a report. The next exercise shows you how to modify and save a new report definition. You also learn how to import a report definition from a file.

- 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.



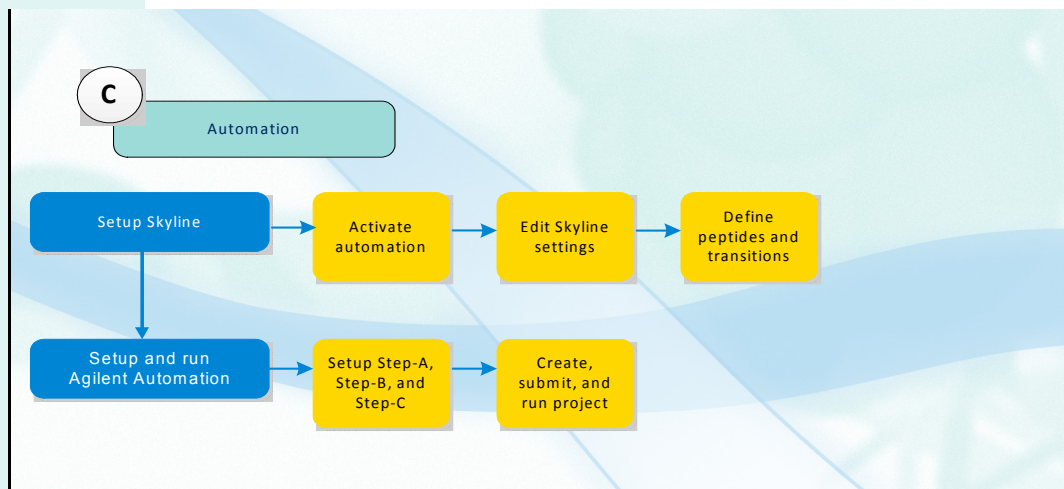
**Figure 10** Edit Report dialog box

- 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.

## Run Skyline Automation

These exercises show you how to activate the Agilent Automation tool modify the Skyline settings, and save a method in Skyline. For Agilent Automation to work, Skyline must be installed on the same computer as the Agilent Data Acquisition software.

### Setup Skyline



1. Activate Automation.

2. Edit Skyline settings.

3. Define peptides and transitions.

- Double-click the **Skyline Automation Enabler** tool in the Act Tools folder.
  - a Start the Skyline program.
  - b Edit the values on the **Peptide Settings** dialog box. Select the **Enzyme**, and enter the **Max missed cleavages**.
  - c Edit the values on the **Transition Settings** dialog box. Edit the **Collision energy** value. Edit the filter settings.
  - d Save your settings to use in the future.

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- a Copy peptides to Skyline from Notepad or some other program.
- b Remove unwanted precursors. Right-click the precursor and click **Delete**.
- c Save the Skyline document.

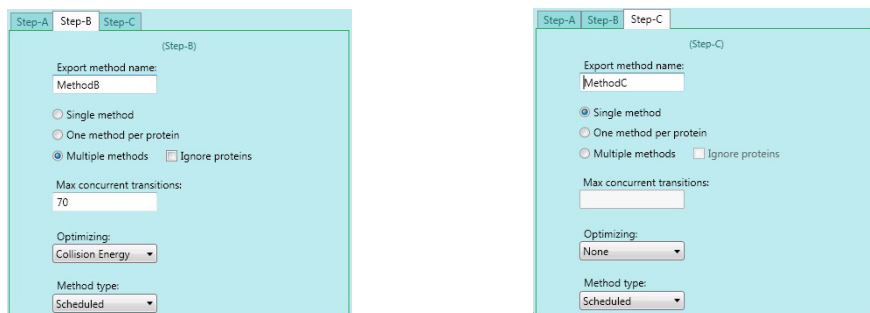
### Setup and run Agilent Automation

1. Setup Step A, Step B, and Step C.

You learn to use the Skyline Automation tool to create methods and worklists to optimize collision energy for your peptides.

- a Click **Tools > Agilent Automation** in the Skyline program. This tool is only available if you used the Skyline Automation Enabler tool.

- b Set the Project settings and the Action selections. Under Action selections, mark each check box.
- c Set parameter in the Step-A tab. Select **None** for Optimizing and **Standard** for the Method Type.
- d Set parameters in the Step-B tab. Click **Multiple methods** and select **Collision Energy** for Optimizing.
- e Set parameters in the Step-C tab. Click **Single method**, and select **None** for Optimizing.



**Figure 11 Step B and Step C in Skyline Automation**

- f Select vial positions for standards.

All peptides in the project must be in a single vial for the standard. If your standard peptides are in more than 1 vial, make a separate Skyline project (document) for each vial. You can submit multiple projects to the Study Manager and queue the analyses. After method development is completed, you can combine documents in Skyline.

## 2. Create, submit, and run project.

- a Click the **Create Project** button to create the project.
- b Click **Submit to Study Manager**.
- c Start the Study Manager program.
- d Set options in Study Manager.
- e Click the Start icon in the Study Manager program to start the queue.

## 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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