Agilent Triple Quadrupole LC/MS
Peptide Quantitation with Skyline
Workflow Guide

A. Peptide Optimization
   - Create method in Skyline
   - Edit Skyline settings
   - Import peptides into Skyline
   - Export method
   - Review results and run method in Data Acquisition
     - Review acquisition method
     - Run method
     - Load results into Skyline
     - Review results in Skyline
     - Export final method
   - Review final method in Data Acquisition
     - Review final acquisition method
     - Save method

B. Review Results in Skyline
   - Review results
   - Export and modify reports

C. Automation
   - Setup Skyline
   - Setup and run Agilent Automation
Notices

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## Contents

1 Before You Begin 5  
Introduction 6  
Overview of the workflow 7  
Required items 9

2 Peptide Optimization 11  
Create method in Skyline 12  
Create and run the method in Data Acquisition 22  
Review results and export final method from Skyline 27  
Review final method in Data Acquisition 34

3 Review Results in Skyline 39  
Review results 40  
Modify and export reports 46

4 Run Skyline Automation 51  
Setup Skyline 52  
Setup and run Agilent Automation 59

5 Reference Information 63  
Installing Skyline 64  
Downloading a library 65  
References 66
Before You Begin

Make sure you read and understand the information in this chapter and have the necessary instrumentation, software, solvents, and lab supplies before you start the analysis.

Introduction  6
Overview of the workflow  7
Required items  9
The MacCoss Lab at the University of Washington has developed the Skyline software package which provides an intuitive set of peptide-centric tools. Among its many features, Skyline can create Agilent TQ acquisition methods to run targeted proteomics experiments. Skyline can also create transition lists. This software is open source and is free for you to install on your computer.

This manual outlines how to use the Skyline software package to do the following tasks:

• Optimization of collision energies for peptides
• Review results
• Modify and export a report
• Use Agilent Automation to automatically optimize collision energies
This guide describes the workflow to use the LC/MS Data Acquisition software with the Skyline software from the University of Washington to optimize the collision energy. This guide also describes how to use Skyline to analyze peptides and create reports that contain quantitative information. In the Automation chapter, you learn how to use the Agilent Automation process to automatically optimize collision energies.

More information

If you need a general introduction to triple quadrupole (TQ) mass spectrometry before you begin, see the Agilent 6400 Series Triple Quadrupole LC/MS System Concepts Guide. The following sections are especially useful:

• “How a triple quadrupole mass spectrometer works”
• “How Dynamic MRM works”

You can also view an online video that describes how a triple quadrupole works.

For more information about Dynamic MRM, refer to the Triple Quadrupole LC/MS System Concepts Guide or the online Help for the MassHunter Data Acquisition software for the Triple Quadrupole instrument.

If you need a general introduction to Skyline software from the University of Washington’s MacCoss lab, you can watch the videos that are available online on the Skyline software website.
Advantages of this workflow

The Skyline program is organized by proteins and then peptides and then transitions. The organization of this program is very intuitive to people working in proteomics.

What you cannot do with the workflow

The Skyline program does not have all of the quantitative analysis functionality that is available in the Agilent Quantitative Analysis program.

Safety Notes

**WARNING**
When you disconnect LC columns or fittings, solvents may leak. Use appropriate safety procedures (for example, goggles, safety gloves and protective clothing), especially when you use toxic or hazardous solvents. Read the material data safety sheets supplied by the solvent vendors.

**CAUTION**
Read, understand, and meet conditions of all cautions in the Safety Guide that you received with your Triple Quadrupole instrument.
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 6400 Series Triple Quadrupole Version B.08.00 or later
• Skyline software version 3.5 or greater from the MacCoss Lab at the University of Washington

The exercises in the next two chapters assume that:
• All instruments have already been installed and are working to specifications.
• You have been trained on the instrumentation and software. For example, you have taken an operator course at an Agilent training center or you have been trained on-site by an Agilent instructor (Application Engineer or consultant).
• That Skyline version 3.5 or later is installed.

Figure 1  The workflow requires an Agilent LC and an Agilent 6400 Series Triple Quadrupole LC/MS System.
These exercises show you how to optimize the collision energy for the transitions that you are acquiring.
Create method in Skyline

In this exercise, you first enter your proteins or peptides 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.

Edit Skyline settings

1. Start the Skyline program.
2. Edit Peptide Settings.

Before you enter your protein or peptide information, you need to verify that the settings in Skyline are correct for your proteins or peptides. For more information on installing the software, see “Installing Skyline” on page 64.

- From the Start button, click All Programs > Skyline > Skyline.
- Click File > New to make sure that you are not starting from a previous project.

a Click Settings > Peptide Settings.

b On the Digestion tab, verify that the Enzyme is set correctly. In this example, the Enzyme is Trypsin [KR | P]. See Figure 2.
c Select 1 for **Max missed cleavages**. See Figure 2.

d Select **None** for **Background proteome**. This setting is useful if you are trying to have unique peptides associated with only one protein of a given organism.

![Peptide Settings dialog box](image)

**Figure 2**  *The Digestion and Modification tabs in the Peptide Settings dialog box*

e Click the **Modifications** tab.

f On the Modifications tab, mark any **Structural modifications**. In this example, the **Carbamidomethyl Cysteine** check box is marked. See Figure 2 on page 13.

g Click the **Prediction** tab. See Figure 3 on page 14. In order to do retention time scheduling, Skyline needs to have a predictor in; the predictor is overwritten by the MRM results, so it does not need to be perfect. It is important that Skyline believes that it can make the prediction.

h Select **Add** for the **Retention time predictor**. The **Edit Retention Time Predictor** dialog box opens.

i Type a **Name**. In this example, the **Name** is **Test**.

j Type the **Slope** and **Intercept**. In this example, the **Slope** is 1; the **Intercept** is 0.

k Type the **Time window**. In this example, the **Time window** is 2.0.

l Select the **Calculator**. For this example, the **SSRCalc 3.0 (100A)** calculator is selected.
3. **Edit the collision energy settings in the Transition Settings dialog box.**

   Figure 3 The Prediction tab and the Edit Retention Time Predictor dialog box

   m Mark the **Use measured retention times when present** check box.

   n Click the **OK** Button.

   a Click **Settings > Transition Settings**.

   b On the Prediction tab, select `<Edit list...>` in the **Collision energy** box. The **Edit Collision Energy Regressions** dialog box is opened.

   c Select **Agilent QQQ** in the **Edit Collision Energy Regressions** dialog box and click the **Edit** button. If **Agilent QQQ** is not an option, then click **Add**.

   The **Edit Collision Energy Equation** dialog box is opened.

   d **Type Agilent QQQ** for the **Name**.

   e **Type 2** in the **Charge** column.

   f **Type 0.031** in the **Slope** column.

   g **Type 1** in the **Intercept** column.

   h In the next row, type 3 in the **Charge** column.

   i **Type 0.036** in the **Slope** column.

   j **Type -4.8** in the **Intercept** column.

   k **Type 3** for the **Step size**. This step size is the number of volts to change the collision energy for each step. For Agilent Triple Quadrupole, a value of 3 or 4 is fine because Agilent TQ have a relatively wide collision energy optimum.

   l **Type 3** for the **Step count**. A step size of 3 means that when you are optimizing, the collision energy is checked for 3 steps above and 3 steps below the specified collision energy. In this example, the step size is 3, so the collision energy is checked at +9, +6, +3, 0, -3, -6, and -9. This is a reasonable starting point.

   A set of standard peptides was reviewed. Given that the response tends to be 90% of maximum over 5 to 10 V, a step size of 3 V can quickly determine the optimal value.
4. Edit the filter settings in the Transition Settings dialog box.

- a If necessary, click Settings > Transition Settings.
- b Click the Filter tab.
- c Review the parameters. For this example, make the following changes:
  - Type 2, 3 for the Precursor charges.
  - Type 1 for the Ion charges.
  - Type y for the Ion Types.
  - Select (m/z > precursor) - 2 under Product ions in the From list.
  - Select 6 ions under Product ions in the To list.

Figure 4  Edit Collision Energy Equation dialog box

m Click the OK button. The Edit Collision Energy Equation dialog box closes.

n Click the OK button. The Edit Collision Energy Regressions dialog box closes.

o Mark the Use optimization values when present check box.

p Select Agilent QQQ for the Collision energy.

q Select either Precursor or Transition for the Optimize by value.

Figure 5  Two tabs of the Transition Settings dialog box
5. Edit the instrument settings in the Transition Settings dialog box.

   a. If necessary, click **Settings > Transition Settings**.
   b. Click the **Instrument** tab.
   c. Enter a value in the **Max m/z** box that is not greater than the maximum m/z value for the Agilent Triple Quadrupole model that you own. You can find the maximum m/z for your instrument in the Data Acquisition program in the Acquisition > QQQ tab in the Method Editor window. If you right-click a Precursor Ion or Mass value in the Scan segments table, the **Maximum value** is displayed in the shortcut menu. For a 6490, set this value to **1400**.
   d. Click the **OK** button.

6. Save the settings for future use.

   a. Click **Settings > Save Current**.
   b. Type a **Name** and click the **OK** button.

---

**Import peptides into Skyline**

1. Copy your peptide to the Clipboard.

2. Paste the peptide from the Clipboard into Skyline.

   a. Click **Edit > Insert > Peptides**.
   b. Click the first cell in the Peptide Sequence column.
   c. Press **Ctrl** and **V** to paste the peptide into this cell.
   d. Type the **Protein Name**. In this example, the protein name is **beta casein**.

   ![Insert dialog box](image.png)

   **Figure 6** Insert dialog box

   e. Click the **Insert** button.

      • You can also directly paste the peptide into the **Targets** list. Right-click the “...” in the list and click **Paste**.
3. Make modifications to the Peptide.

a. Right-click the peptide and click Modify.

In this example, it is a phosphopeptide, so you have to modify the serine.

b. Select Edit list from the serine (S) list. The Edit Structural Modifications dialog box opens.

c. In the Edit Structural Modifications dialog box, select Phospho (ST) in the Name list. Click the Edit button. If Phospho (ST) is not available, click the Add button.

d. Enter HO3P for the Chemical formula.

e. Type S, T in the Amino acid box.

f. Click the Loss >> button.

 g. Click the button next to the Neutral losses list.

h. Type H3O4P in the Neutral loss chemical formula box.

i. Click the OK button in the Edit Neutral Loss dialog box.

j. Click the OK button in the Edit Structural Modification dialog box.

---

Figure 7  Main window of the Skyline program before modifications

Figure 8  Edit Structural Modification and Edit Neutral Loss dialog boxes
k Select **Phospho (ST)** for the **S** modification.

l Click the **OK** button in the **Edit Modifications** dialog box.

![Edit Modifications dialog box](image)

**Figure 9**  *Edit Modifications dialog box*

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.

![Main window of the Skyline program](image)

**Figure 10**  *Main window of the Skyline program*

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 Click **File > Export > Method**. The Export Method dialog box opens.

b Select **Agilent 6400 Series** as the **Instrument type**.
Click the **Single method** button.

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

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

Click the **Browse** button. The **Browse For Folder** dialog box is opened.

Select the method to use as a template in the **Template file** box. 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. The Time segments table is taken from the template method’s first Time segment row.

Click **OK** in the **Browse For Folder** dialog box.

Click **OK** in the **Export Method** dialog box.

Click the **OK** button in the **Export Agilent 6400 Series Method** dialog box.

Type a **File name** for the new method and click **Save**.

---

**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.

Click **File > Export > Transition List**. The **Export Transition List** dialog box is opened.

Select **Agilent** as the **Instrument type**.

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

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

Click the **OK** button. The “Export Transition List” dialog box opens.

Navigate to the folder where you want to save the list.

Type a name for the transition list.

Click the **Save** button.
Save Skyline project

You can save this project; then, you can open this project at a future time.

a Click File > Save or File > Save As.

b Type a name in the Save As dialog box.

c Click the Save button.

Figure 12  Export Transition List dialog box

i Select Transition List (*.csv) for the Save as type.

j Enter a File name. You can create a folder in MassHunter to store your CSV files.

Figure 13  Export Transition List dialog box

k Click the Save button.
The format of the CSV file that contains the Transition List is:

Figure 14  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. Your file will contain \( (2 \times \text{Step Count} + 1) \) rows for each transition.
- The collision energy increases by the step size that you set in “Edit the collision energy settings in the Transition Settings dialog box.” on page 14.
- 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.
Create and run the method in Data Acquisition

In this exercise, you load the method created by Skyline and run it to optimize the collision energy. You could also import the transition list from Skyline and run 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.

Review acquisition method

1. Start the MassHunter Data Acquisition program.

2. Load the acquisition method from Skyline.

• Double-click the MassHunter Data Acquisition icon.

If you need help, see Step 1 in the “Getting Started” section of the Agilent 6400 Series Triple Quadrupole LC/MS System Quick Start Guide.

a Click Method > Open. The “Open Method” dialog box is opened.

b Navigate to the folder where you saved the method in Skyline.
3. (optional) Prepare the LC modules.

- Select the method that you created in Skyline.
- Click the Open button.

4. Prepare the Agilent 6400 Series Triple Quadrupole LC/MS System.

- Switch the LC stream to waste (or disconnect it from the MS).
- Purge the LC pump.
- Install the column and condition it as described in the column instructions included in the column package.
- Set up to view real-time parameter values (actuals).
- Set up to display real-time plots.
- Set the LC parameter values in the Method Editor window.

If you need help, see Step 2 in the “Getting Started” section of the Agilent 6400 Series Triple Quadrupole LC/MS System Quick Start Guide.

It is very important to purge solvent channels A and B because trapped air causes irreproducible retention times for analytes.

5. Review values for all the LC modules.

- In the MassHunter Data Acquisition program, review the parameters on each LC tab.

If you need help, see Step 4 in the “Getting Started” section of the Agilent 6400 Series Triple Quadrupole LC/MS System Quick Start Guide.

6. Review parameters on the QQQ tabs.

- Review parameters on the Acquisition tab.
  - 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 “Edit the collision energy settings in the Transition Settings dialog box.” on page 14.
  - The Product Ion also changes slightly. The Skyline program uses those slight changes to keep track of which step it is on.

- Review parameters on the Source tab.

- Review parameters on the Chromatogram tab. You can specify which chromatograms to show in the Chromatogram Plot window during a run.
7. Review the parameters on the DA tab.

8. Save the method.

Import transition list

1. Open the CSV file in Excel.

2. Copy the transition list to the Clipboard.

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 22 and then follow these steps to modify the Scan segments table.

   a. Open the Excel program.

   b. Click the Microsoft Office button and then click Open.

   c. Navigate to the folder containing the CSV file and click Open. See Figure 14 on page 21.

   a. Select all of the cells in the transition list including the header.

   b. Click Edit > Copy. You can also press Ctrl and C.
3. Import the transition list.

- In the Data Acquisition program, click the QQQ tab in the Method Editor window.
- Make sure that you have saved your method before changing the Scan Type. The Scan segments table is reset to one, default line when the Scan Type is changed.
- Select MRM for the Scan Type in the first row of the Time segments table.
- Right-click the Scan segments table and click Paste from Clipboard.
- Select the first row in the Scan segments table. This row is the default row in the Scan segments table.
- Right-click the Scan segments table and click Delete Row.

4. Save the method.

- Click Method > Save.

Or, to save with a different name:

- Click Method > Save As.
- Enter a name for the method and click OK.
Run the method

1. Start the MassHunter Data Acquisition program, if it is not currently running.
   • Double-click the MassHunter Data Acquisition icon.
   If you need help, see Step 1 in the “Getting Started” section of the Agilent 6400 Series Triple Quadrupole LC/MS System Quick Start Guide.

2. Load your method.

3. Edit the information in the Sample Run window.
   a Type a Name for the Sample.
   b Type a Comment for the Sample.
   c Type a Name for the Data File.

4. Run the method.
   • Click Sample > Run.
   • Click the ➤ icon in the toolbar in the Sample Run window.
   • Click the icon in the main toolbar.

Figure 17 The Sample Run window
This section shows you how to create the final optimized method. You first need to import the results from the data file you created in “Create and run the method in Data Acquisition” on page 22. Then, you can graphically review the results and then export a Dynamic MRM method.

**Load results into Skyline**

1. Start Skyline program.  
   - From the Start button in the All Programs list, click Skyline > Skyline.

2. Open the Skyline project.  
   - Load the settings that you saved in “Save the settings for future use.” on page 16. A menu item with the name you used to save the settings is added to the Settings menu when you saved the settings.
   - Click File > Open.
   - In the Open dialog box, select the project you saved in “Export method” on page 18.

3. Import results.  
   - Click File > Import > Results.
   - Click the Add one new replicate button.
c Type a **Name** for the import. In this example, **Name** is set to FQS opt.

d Select **Collision Energy** from the **Optimizing** list.

e Click the **OK** button.

![Image](image1.png)

**Figure 18** The Import Results dialog box

f In the **Import Results Files** dialog box, select the data file you created in “Create and run the method in Data Acquisition” on page 22.

g Select **Agilent Data** for the **Sources of type** box.

h Click the **Open** button.

![Image](image2.png)

**Figure 19** The Import Results Files dialog box

---

**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. You can also use the commands in the View menu.

• 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.

---

1. Select the transitions that were found.

   a. Click the down arrow next to the precursor ion. This arrow is visible when you move the mouse over the precursor in the list.

   b. In the list that is displayed, mark the transitions that have a green dot next to them. The transitions with a green dot next to them are found. Clear the check box for each transition that does not have a green dot next to it.

---

![Figure 20](image1)

**Figure 20**  *Skyline program after importing results file*

![Figure 21](image2)

**Figure 21**  *Marking the transitions that were found*

   c. Click the green check mark icon to save the changes.

**NOTE:** In this example, only the transitions that were found are marked.
2. Examine each transition to see which collision energy created the greatest peak area.

   a. Right-click the Peak Areas graph and click **Selection** if it does not have a check next to it. When **Selection** is marked, then a line is drawn from the maximum collision energy to the y-axis.

   b. Click a transition in the list.

   c. Right-click the Peak Areas graph and click **Transitions > Single**.

   d. Examine the Peak Areas graph to determine the collision energy that produces the greatest peak area.

   e. Examine the FQS opt tab to see the overlay of the peaks. This graph is also color coded. You can customize this graph when you right-click and click **Properties**.

![Figure 22](image)

*Figure 22  Comparing collision energies for the same transition*

3. (optional) Edit the regression parameters to change the initial equation.

   a. Click **Settings > Transition Settings**.

   b. Click the **Prediction** tab.

   c. Select **Transition** for **Optimize by**.

   d. Select **Edit list** from the Collision energy list.

   e. Click **Agilent QQQ** in the Edit Collision Energy Regressions dialog box, and then click the **Edit** button.

   f. In the Edit Collision Energy Equation dialog box, click the **Show Graph** button.

   g. Examine the different graphs. The slope and the intercept are shown for each different charge state. Click **Close** to close the **Regression** dialog box.

   h. If you want to use the new information, click the **Use Results** option. Otherwise, click **Cancel** to close the **Edit Collision Energy Equation** dialog box. Then, click **Cancel** to close the **Edit Collision Energy Regressions** dialog box. Finally, click **Cancel** in the **Transition Settings** dialog box.
You do the following steps to use the optimal collision energies for a peptide or set of peptides to create a final method. You can also export a final transition list. You can use this transition list to create a method in the Agilent Data Acquisition program.

- Click File > Export > Method.
- In the Export Method dialog box, select Agilent 6400 Series as the Instrument type.
- Click the Single method button.
- Select None for Optimizing.
- Select Scheduled as the Method type.
- Click the Browse button. The Browse For Folder dialog box is opened.
- Select the method to use as a template in the Template file box. 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 Time segments table and the Scan segments table. The Time segments table and the Scan segments table are created from the information in Skyline.
- Click OK in the Browse For Folder dialog box.
- Click the OK button in the Export Method dialog box.

If you do not have a method to use as the Template file, you can export a transition list instead of a method. You can paste this transition list into the Scan segments table in the MassHunter Data Acquisition program.

- Click File > Export > Transition List.
- In the Export Transition List dialog box, select Method File (*.m) as the Save As type.
- Enter a name for the method and click the Save button.
c Select **Scheduled** as the Method type.

d Click the **Single method** button.

e Select **None** for Optimizing.

f Click the **OK** button.

![Image of Export Transition List dialog box]

**Figure 24**  *Export Transition List - page 1 and page 2*

g In the next **Export Transition List** dialog box, select **Transition List (*.csv)** as the **Save as type**.

h Enter a name for the transition list and click the **Save** button.
The final Transition List contains only one line for each transition including the optimized collision energy:

![CSV file for the Final Transition List](image)

**Figure 25** CSV file for the Final Transition List
This exercise shows you reviewing the final method. You can also review importing the final transition list.

1. Start the MassHunter Data Acquisition program.
   - Double-click the MassHunter Data Acquisition icon. If you need help, see Step 1 in the “Getting Started” section of the Agilent 6400 Series Triple Quadrupole LC/MS System Quick Start Guide.

2. Load the acquisition method from Skyline.
   - Click Method > Open. The Open Method dialog box is opened.
   - Navigate to the folder where you saved the final method in Skyline.
   - Select the final method that you created in Skyline.
   - Click the Open button.
3. Review values for all the LC modules.

4. Review parameters on the QQQ tabs.
   a. Review parameters on the Acquisition tab.
   b. Review parameters on the Source tab.
   c. Review parameters on the Chromatogram tab. You can specify which chromatograms to show in the Chromatogram Plot window during a run.
   d. Review parameters on the Instrument tab. You can specify which instrument curves to store on this tab.

5. Review the parameters on the DA tab.
   a. Click the DA tab.
   b. Review the parameters on the Qual tab.
   c. Click the Quant tab.
   d. Review the parameters on the Quant tab.

Import final transition list

1. Open the method.

2. Open CSV file in Excel.

3. Copy the transition list to the Clipboard.

If you need help, see Step 4 in the “Getting Started” section of the Agilent 6400 Series Triple Quadrupole LC/MS System Quick Start Guide.

**If you created a final transition list, then you follow these instructions to create a final method.**

- Click Method > Open or click the Open Method icon on the main toolbar. Open the method to which you want to add the Scan segments.

  a. Open the Excel program.
  b. Click the Microsoft Office button and then click Open.
  c. Navigate to the folder containing the CSV file and click Open. See Figure 25 on page 33.

  a. Select all of the cells in the transition list including the header.
4. Import the transition list.

a. In the Data Acquisition program, click the QQQ tab in the Method Editor window.

b. Make sure that you have saved your method before changing the Scan Type. The Scan segments table is reset to one, default line when the Scan Type is changed.

c. Select Dynamic MRM for the Scan Type in the first row of the Time segments table.

d. Right-click the Scan segments table and click Paste from Clipboard.

e. Select the first row in the Scan segments table. This row is the default row in the Scan segments table.

f. Right-click the Scan segments table and click Delete row.

5. Review parameters on the other MS tabs.

a. Review parameters on the Source tab.

b. Review parameters on the Chromatogram tab. You can specify which chromatograms to show in the Chromatogram Plot window during a run.

c. Review parameters on the Instrument tab. You can specify which instrument curves to store on this tab.
a  Click Method > Save.

Or, to save with a different name:

a  Click Method > Save As.

b  Enter a name for the method and click OK.
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

In this exercise, you learn several different techniques for reviewing and refining your data in Skyline. When you refine your data, you change the proteins, peptides and transitions that are included. Your data files and knowledge of your experiment help you make changes to the proteins, peptides and transitions.

B

Review Results in Skyline

Import results

Refine list of peptides, precursors and transitions

Modify report

Import results

Review results

Modify report and export reports

Review Results in Skyline

Import results

Refine list of peptides, precursors and transitions

Modify report

Import results

Review results

Modify report and export reports

In this exercise, you learn several different techniques for reviewing and refining your data in Skyline. When you refine your data, you change the proteins, peptides and transitions that are included. Your data files and knowledge of your experiment help you make changes to the proteins, peptides and transitions.

Import results

1. Start the Skyline program.
2. Select previously saved settings.
3. Paste list of proteins or peptides.

- From the Start button in the All Programs list, click Skyline > Skyline.
- In the Settings menu, click the name of the settings that you saved in “Save the settings for future use.” on page 16.

In this example, human serum albumin is loaded by opening an existing Skyline project.

a Click File > Open.

b Select hsa-pep-demo.sky.

c Click the OK button.

It is possible to import proteins and peptides in several different ways:

- 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.
4. Modify the peptides. If necessary, you can modify the peptides after loading the list.
   a. Right-click the name of the peptide and then click **Modify**.
   b. Click the arrow next to the amino acid that you want to modify. NOTE: If the amino acid appears multiple times in the peptide, you need to apply the modification to each amino acid.
   c. Click the modification if it is available. If not, do the following:
      - Click **Add**.
      - Enter the information for your modification.
      - Click the **OK** button.

   ![Image](Add or Edit Structural Modifications dialog box)

5. (optional) Select a library.
   a. Click **Settings > Peptide Settings**. The **Peptide Settings** dialog box opens.
   b. Click the **Library** tab.
   c. Mark the library to use. If no libraries are listed, you can either click **Cancel** or see “Downloading a library” on page 65 for information on getting a library.
   d. Review the other parameters on this tab.
   e. Click **OK**.

![Image](Edit Modifications dialog box)

Each amino acid in the peptide is listed in order, so you can modify any of the amino acids in the peptide. In this example, the first two amino acids are both alanine, and the last amino acid is lysine. This peptide contains two cysteines which are both modified.

6. Save the project.
   - Save the document. Click **File > Save** or **File > Save As** to save the current proteins/peptides in a Skyline project. Type a **File name** and click the **Save** button.
To start reviewing the list of predicted transitions, you first load a data file. After doing an initial review, you can import multiple replicates (see "Import replicate data files." on page 44).

7. Import one data file.

a  Click File > Import > Results.

b  Click the Add one new replicate button.

c  Select None from the Optimizing list. See “Review results and export final method from Skyline” on page 27 for more information on using Skyline to optimize the collision energy.

d  Click the OK button.

e  Select Agilent Data from the Sources of type list.

f  Navigate to your data file. Select one of the data files.

g  Click the Open button.

Skyline has many tools to allow you 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.

1. **Remove peptides that were not present in the data file.**

   a. Select a precursor in the tree view.
   
   b. If you see a green dot next to the peptide, then the specified transitions were found in the data file. You probably will keep this peptide in the list. You can manually review the transitions to make a final decision.
   
   c. Expand the precursor to show the predicted transitions.
   
   d. If no dot is shown next to the precursor, then none of the selected transitions were found. Click the arrow next to the precursor and examine the list of transitions.
   
   e. 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.

   ![Figure 32](image)
   
   *Figure 32  No transitions were found for this precursor*

   f. If no precursors exist for a peptide, then delete the peptide. If you cannot expand the peptide, then no precursors exist.

   ![Figure 33](image)
   
   *Figure 33  No precursors were found for this peptide*

2. **Change selected transitions for a peptide.**

   a. Select a precursor for a peptide in the tree view.
3. Import replicate data files.

If you have multiple replicates available, it may be useful to load the replicates before deleting more peptides. The deleted peptides may be available in one of the replicates.

a. Click **File > Import > Results**.

b. Click the **Add files to an existing replicate** button.

c. Select the **Name** to which to add the replicates.

d. Click the **OK** button.

e. Select the replicate data files that you acquired. You can select a range of data files by pressing the **Ctrl** key while selecting a file. To select a range of data files, you select the first data file in the range. Then, you press the **Shift** key while selecting the last data file in the range.

**Figure 34**  This precursor is missing some of the selected transitions.

b. Mark the check boxes next to the transitions that have a green dot next to them.

c. Clear the check boxes next to the transitions that do not have a green dot next to them.

**Figure 35**  Only the transitions that were found are marked.

d. Click the green check mark to keep changes in the **Transitions** list.

- A green dot is placed next to the precursor because all of the transitions are found. If all of the precursors have a green dot next to them, then the peptide also has a green dot next to it.
- The number in brackets at the end of the list of transitions shows the peak rank by area of the coeluting peaks for a precursor. In this example, the three most abundant peaks by peak area are found.
- If you selected a library in the Peptide Settings dialog box, then the rank of that peak in the library spectrum is also shown. The number in parentheses with the word “rank” shows you how large that peak is in the library spectrum. If the rank of the peak in the library spectrum is approximately the same as the peak rank of the spectrum, that is an indication that the peptide is correctly identified.
4. Display the retention time graph.

5. Remove additional peptides and transitions.

**Figure 36** Import replicates by clicking *Add files to an existing replicate*

- Click the *Open* button.

  - **Click View > Retention Times > Replicate Comparison.**
  - You can rearrange the windows to different positions if the window has a tab. You drag the window by the tab to reposition it.

**Figure 37** The Library Match, Chromatograms and Retention Times windows

- In this example, these data files only contain one peptide, so all of the other peptides can be removed.
Modify and export reports

In this exercise, you learn how to modify and export a report. A report in Skyline is a CSV file. It 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 many 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.

1. Display the Export Report dialog box.
2. Export a report.
   - Click **File > Export > Report**.
     a. Select the report that you want to export.
     b. (optional) Click **Preview**. The Preview Report dialog box is opened, and you can review the report on the screen. You use this feature to make sure that the correct columns are included in the report and that all of the information is being displayed properly. Click the red X to close the Preview Report dialog box.
     c. Click the **Export** button. The **Save As** dialog box opens.
Open CSV file in Excel.

Modify and share reports

1. Modify a report.

   a Click File > Export > Report.
   b Click the Edit list button.
   c Select a report that closely matches the report that you want to create.
   d Click the Copy button. The Edit Report dialog box is opened.
   e Type a name for the View Name.
   f Remove any columns that you do not want to include in your report. Click the item in the right column and then click the button.
   g Add values to the report. Select the value from the left column that you want in your report. Click the Add > button. You can only add a value to the report one time.
   h Arrange the items in the report in the order that you want them to appear in the report. You click the button to move an item up in the list, and you click the button to move an item down in the list.

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.
The first item in the list is the first item in the exported table.

![Edit Report dialog box](image1)

**Figure 39** Edit Report dialog box

- **i** Click the **Preview** button. Examine the report to see if all columns are included.

  - Click the **Close** button to close the Preview Report dialog box.

![Preview Report dialog box](image2)

**Figure 40** Preview Report dialog box

- **j** Click the **OK** button in the Edit Report dialog box.

A report with the name you entered is added to the list of possible reports in the Edit Reports dialog box.

![Edit Reports dialog box](image3)

**Figure 41** Edit Reports dialog box

- **k** In the **Edit Reports** dialog box, click the **OK** button.
2. Save the report definitions (share a report).

   
   b. In the Export Report dialog box, click the Share button.
   
   c. In the Save Report Definitions dialog box, mark the check box next to the Report Definitions that you want to save. Then, click the OK button.

   ![Save Report Definitions dialog box](image)

   **Figure 42**  *Save Report Definitions dialog box*

   d. In the Save As dialog box, type the File name that you want to use to save the report definitions. Reports have the extension “.skyr”.

   e. Click the Save button.

   ![Save As dialog box](image)

   **Figure 43**  *Save As dialog box*
3. Import a report.

You can add a report to the list that you created and shared. Thus, you can move a report from one computer to another computer in your lab.


b. Click the Import button. The Open dialog box is opened.

c. Select the report that you want to be able to use in this program. Reports have the extension “skyr”.

![Open dialog box](image)

**Figure 44 Open dialog box**

d. Click the Open button.

The name of the report is added to the list of reports in the Export Report dialog box.

![Export Report dialog box](image)

**Figure 45 Export Report dialog box**

e. Click Cancel if you do not want to print a report at this time.
Run Skyline Automation

In this exercise, you learn 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.
In this exercise, you learn how to activate the Agilent Automation tool, modify the Skyline settings, and save a method in Skyline.

Run Skyline Automation Enabler

1. Open the Acq Tools folder.
   - Double-click the Agilent MassHunter Workstation icon.

2. Run the Skyline Automation Enabler.
   a. Double-click the Acq Tools folder.
   b. Double-click the Skyline Automation Enabler tool.

      You can instead click All Programs > Agilent > MassHunter Workstation > Acq Tools > Skyline Automation Enabler command.

      The program runs automatically. A message appears when it is enabled.

Edit Skyline settings

1. Start the Skyline program.
   - From the Start button, click All Programs > Skyline > Skyline.
   - Click File > New to make sure that you are not starting from a previous project.

Before you enter your protein or peptide information, you need to verify that the settings in Skyline are correct for your proteins or peptides. For more information on installing the software, see “Installing Skyline” on page 64.
2. Edit Peptide Settings.

a. Click **Settings > Peptide Settings**.

b. On the Digestion tab, verify that the **Enzyme** is set correctly. In this example, the Enzyme is **Trypsin [KR | P]**. See Figure 46.

c. Select **1** for **Max missed cleavages**. See Figure 46. For other target peptides, you may need to enter a higher value.

d. Select **None** for **Background proteome**. This setting is useful if you are trying to have unique peptides associated with only one protein of a given organism.

![Peptide Settings dialog box](image)

**Figure 46** The Digestion and Modification tabs in the Peptide Settings dialog box

e. Click the **Modifications** tab.

f. On the Modifications tab, mark any **Structural modifications**. In this example, the **Carbamidomethyl Cysteine** check box is marked. See Figure 46 on page 53.

g. Click the **Prediction** tab. See Figure 47 on page 54. In order to do retention time scheduling, Skyline needs to have a predictor in; the predictor is overwritten by the MRM results, so it does not need to be perfect. It is important that Skyline believes that it can make the prediction.

h. Select **Add** for the **Retention time predictor**. The **Edit Retention Time Predictor** dialog box opens.

i. Type a **Name**. In this example, the **Name** is **Test**.

j. Type the **Slope** and **Intercept**. In this example, the **Slope** is **1**; the **Intercept** is **0**.

k. Type the **Time window**. In this example, the **Time window** is **2.0**.

l. Select the **Calculator**. For this example, the **SSRCalc 3.0 (100A)** calculator is selected.
3. Edit the collision energy settings in the Transition Settings dialog box.

Figure 47  The Prediction tab and the Edit Retention Time Predictor dialog box

m Mark the Use measured retention times when present check box on the Prediction tab.

n Click the OK Button.

a Click Settings > Transition Settings.

b On the Prediction tab, mark the Use optimization values when present check box.

c Select <Edit list...> in the Collision energy box. The Edit Collision Energy Regressions dialog box is opened.

d Select Agilent in the Edit Collision Energy Regressions dialog box and click the Edit button. If Agilent is not an option, then click Add.

The Edit Collision Energy Equation dialog box is opened.

e Type Agilent for the Name.

f Type 2 in the Charge column.

g Type 0.03 in the Slope column.

h Type 1 in the Intercept column.

i In the next row, type 3 in the Charge column.

j Type 0.036 in the Slope column.

k Type -4.8 in the Intercept column.

l Type 2 for the Step size. This step size is the number of volts to change the collision energy for each step. For Agilent, a value of 2, 3, or 4 is fine because Agilent TQ have a relatively wide collision energy optimum.

m Type 4 for the Step count. A step size of 4 means that when you are optimizing, the collision energy is checked for 4 steps above and 4 steps below the specified collision energy. In this example, the step size is 4, so the collision energy is checked at +8, +6, +4, +2, 0, -2, -4, -6, and -8. This is a reasonable starting point.
4. Edit the filter settings in the Transition Settings dialog box.

- If necessary, click **Settings > Transition Settings**.
- Click the **Filter** tab.
- Review the parameters. For this example, make the following changes:
  - Type 2, 3 for the **Precursor charges**.
  - Type 1 for the **Ion charges**.
  - Type y for the **Ion Types**.
  - Select \((m/z > \text{precursor}) - 2\) under Product ions in the **From** list.
  - Select **6 ions** under Product ions in the **To** list.
  - Mark the **N-terminal to Proline** check box.
5. Edit the instrument settings in the Transition Settings dialog box.

6. Save the settings for future use.

**Define peptides and transitions**

1. Copy your peptide to the Clipboard.

**Figure 49** Two tabs of the Transition Settings dialog box

- If necessary, click **Settings > Transition Settings**.
- Click the **Instrument** tab.
- Enter a value in the **Max m/z** box that is not greater than the maximum m/z value for the Agilent Triple Quadrupole model that you own. You can find the maximum m/z for your instrument in the Data Acquisition program in the Acquisition > QQQ tab in the Method Editor window. If you right-click a **Precursor Ion** or **Mass value** in the Scan segments table, the **Maximum value** is displayed in the shortcut menu. For an Agilent 6490, set this value to **1400**.
- Click the **OK** button.

- Click **Settings > Save Current**.
- Type a **Name** and click the **OK** button.

In this example, several peptide sequences are used.

In this example, a peptide from beta casein is typed into Notebook.
- Type the following peptides into Notepad:
  - AAFTECCQAADK
  - YLYEIAR
  - LVNEVTEFAK
  - KVPQVSTPTLVEVSR
  - RPCFSALEVDETYVPK
  - AVMDDFAAVEK
- Highlight all of the peptides and press **Ctrl** and **C**. You can also click **Edit > Copy**.
2. Paste the peptide from the Clipboard into Skyline.

   a. Right-click the gray bar in the **Targets** pane. Click **Paste**.
   
   b. Press **Ctrl** and **D** to expand all of the peptides.

   ![Image of Skyline software with Targets pane expanded]

   c. Remove unwanted precursors. Select the value, and then right-click and click **Delete**. The final precursor list should look like the following for this example.

   ![Image of Skyline software with precursor list]

   d. Press **Ctrl** and **W** to expand transitions. The final list for transitions should look like the following:
e  Save the Skyline document. Click File > Save As.
Setup and run Agilent Automation

In this exercise, you use the Skyline Automation tool to create methods and worklists to optimize collision energy for your peptides.

Setup Step A, Step B, and Step C

1. Start the Skyline Automation program.
   a) If necessary, start Skyline.
   b) Click Tools > Agilent Automation.
      If you see the message “This tool requires a Document Path to run”, save your list of peptides. The Document Path is set when you save the current list of peptides.

2. Set the Project settings and the Action selections.
   a) Select the Template method. You can click Browse to change the method.
   b) Select the Study folder. All data files are saved to this folder.
   c) Type the Study name.
   d) Under Action selections, mark Step-A (Update Retention Times).
   e) Mark Step-B (Optimize Collision Energy).
   f) Mark Step-C (Export method, create worklist).
   g) Mark Execute worklist.
3. Set parameters in the **Step-A** tab.

   a. Click **Multiple methods**.
   b. Type 50 for the **Max transitions per sample injection** value.
   c. Select **None** for Optimizing.
   d. Select **Standard** for Method type.
   e. Type 5 for the **Dwell time (ms)** value.

![Figure 50](image)

**Figure 50**  
*Tab A in Skyline Automation*

4. Set parameters in the **Step B** tab.

   a. Click the **Step-B** tab.
   b. Click **Multiple methods**.
   c. Type 70 for **Max concurrent transitions**.
   d. Select **Collision Energy** for Optimizing.
   e. Select **Scheduled** for Method type. See **Figure 51**.

![Figure 51](image)

**Figure 51**  
*Step B and Step C in Skyline Automation*
5. Set parameters in the **Step C** tab.

a. Click the **Step-C** tab.

b. Click **Single method**.

c. Select **None** for **Optimizing**.

d. Select **Scheduled** for **Method type**. See Figure 51 on page 60.

6. Select vial positions for standards.

- Select vial position for Step-A and Step-B (the 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.

---

**Create, submit, and run project**

1. Create the project.

a. Click the **Create Project** button.

b. (optional) Edit the Step-C worklist to add vials and samples. The worklist editor is similar to the Worklist window in the MassHunter Data Acquisition program.

---

2. Submit the project to Study Manager.

- Click **Submit to Study Manager**.

You will get a message indicating that you should not open the Skyline project during the automation process. The Skyline Automation will be importing data into
Skyline and exporting new methods dynamically. After you click OK on this message, Study Manager will automatically launch.

3. Start the MassHunter Study Manager.

   a. Double-click the Agilent MassHunter Workstation folder.
   b. Double-click the Study Manager icon.
      
      You can instead click All Programs > Agilent > MassHunter Workstation > Study Manager.

4. Set options in Study Manager.

   a. Click the Settings tab in the Ribbon.
   b. Mark the Enable standby script execution on idle check box.
   c. Select the Standby Script. For example, you can select SCP_InstrumentStandby.

5. Start running the automation.

   a. Click the Home tab in the Ribbon.
   b. Click the Start icon to start the Automation.
      • During the Automation process, the MassHunter Worklist editor is locked by the Study Manager.
   c. When the automation is complete, click Stop > Stop Immediately. When you click Stop, the Skyline project is released.
Reference Information

This chapter includes the instructions on how to install the MacCoss Skyline software. A list of reference manuals is also included.
Installing Skyline

Skyline software is developed at the University of Washington in the MacCoss lab. You can download it from the internet for free.

1. Find the location of the software online.
   a. Start your internet browser.
   b. Start a search engine, such as Google.
   c. Type Skyline Peptide in the search box.
   d. Find the link to the proteome.gs.washington.edu site.

   ![Figure 53 Searching for Skyline Peptide using Google.]

   Figure 53 Searching for Skyline Peptide using Google.

   e. Connect to that site.

2. Download and install the software.
   a. Find the Download & Install buttons. This button also shows the version number. This guide is written using version 2.5.
   b. Click either the Skyline 3.5 - 32 bit button or the Skyline 3.5 - 64 bit button.
   c. Sign in or register for the Skyline software.
   d. Click the I agree button to agree to the license terms.
      The Name, Version and Publisher information is displayed.
   e. Click the Install Skyline button.
   f. The “Opening setup.exe” message box appears. Click the Save File button.
   g. In the Downloads dialog box, double-click the setup.exe program. Then, follow the instructions to install the program. You may need to click OK in the “Open Executable File?” message box and click Run in the “Open File - Security Warning” message box.

When the installation program finishes installing the software, the Skyline program is started automatically.
You can download a library from the internet. The Skyline web site contains links to allow you to download the following files:
• PeptideAtlas
• NIST - peptide.nist.gov
• GPM

You can use the WinZip program to unzip files with the extension “tar.gz”. You can also build a library using the Skyline software.

You can use the links within the Skyline software to download a library, or you can search for the download page manually.

At the bottom of the Edit Library dialog box, you can click one of the Spectral Library Links to connect to the correct web site to download the library.

a Click Settings > Peptide Settings.
b Click the Library tab.
c Click the Edit list button.
d Click the Add or Edit button. The Edit Library dialog box is opened.

a Start your internet browser.
b Start a search engine, such as Google.
c Type Skyline Peptide begin.view in the search box.
d Find the link to the proteome.gs.washington.edu site.

e Connect to that site.
f Scroll to the bottom of the home page.
g In the Spectral Library Links section in the right column, click one of the links.

• Follow the instructions on the page that is opened to download the library.
The references in this list give valuable information that help you use Skyline software with the Agilent 6400 Series Triple Quadrupole LC/MS System.

**Manuals**

*Agilent 6400 Series Triple Quadrupole LC/MS System Concepts Guide*

*Agilent 6400 Series Triple Quadrupole LC/MS System Quick Start Guide*

*Agilent 6000 Series LC/MS Safety Guide*

*Agilent 6000 Series LC/MS Maintenance Guide (animated)*

*Agilent MassHunter Workstation Software – Data Acquisition for 6400 Series Triple Quadrupole LC/MS Familiarization Guide*

*Agilent TQ Data Acquisition eFamiliarization Guide*

*Note: The MassHunter software includes online Help, in addition to manuals. See the online Help for details about the software.*