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Notices

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Introduction

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 QQQ 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

Overview of the workflow

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.



More information

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

- "How a triple quadruple mass spectrometer works"
- "How Dynamic MRM works"

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

For more information about Dynamic MRM, refer to the *Triple Quadrupole 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 prograr The organization of	n is organized by proteins and then peptides and then transitions. this program is very intuitive to people working in proteomics.
What you cannot do with the workflow	The Skyline program is available in the A	n does not have all of the quantitative analysis functionality that Igilent 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 <i>Maintenance Guide</i> that you received with your Triple Quadrupole instrument.

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

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).



Create QQQ 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 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.



Edit Skyline 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 50.

- 1. Start the Skyline program.
- 2. Edit Peptide Settings.
- From the Start button in the All Programs list, click MacCoss Lab, UW > Skyline.
- 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.

Digestion Prediction Filter Library Modifications Enzyme: Typein [KR P]	Prediction Fitter Library Modifications Structural modifications: If Cabanidomethyl Cysteine Edt liet
Max missed cleavages:	Max variable mods: Max neutral losses:
	Isotope Isolel type: Isotope modifications: Edit Ist
OK Cancel	Internal standard type: heavy OK Cancel

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

- c On the Modifications tab, mark any **Structural modifications**. In this example, the **Carbamidomethyl Cysteine** check box is marked. See Figure 2.
- d 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** 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.

- d Type Agilent for the Name.
- e Type 2 in the **Charge** column.
- f Type 0.036 in the **Slope** column.
- g Type -4.8 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 4 for the **Step size**. This step size is the number of volts to change the collision energy for each step.
- 1 Type 2 for the **Step count**. A step size of 2 means that when you are optimizing, the collision energy is checked for 2 steps above and 2 steps below the specified collision energy. In this example, the step size is 4, so the collision energy is checked at +8, +4, 0, -4 and -8. For a precursor with m/z 1000, the collision energies would be 39.2, 35.2, 31.2, 27.2, and 23.2 V.

3. Edit the collision energy settings in the Transition Settings dialog box. 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 4 V can quickly determine the optimal value.

ame: gilent			OK
egress	charge	slope	Intercept
	2	0.036	-4.8
0	3	0.036	-4.8
./ *	3	0.036	-4.8



m Click the **OK** button.

- n Mark the Use optimization values when present check box.
- o Select Agilent.
- 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, 2 for the lon charges.
 - Select ion 2 under Product ions in the From list.
 - Select 4 ions under Product ions in the To list.

	ary instrument Full-S	can	
Precursor charges:	lon charges:	lon types:	
2,3	1,2	У	
Erom: ion 2	T <u>o</u> : ◀ ions	•	
Verminal to	Proline Glu or Asp	<u>E</u> dit List	
Precursor m/z exc	lusion window: Th		
Auto-select all ma	tching transitions		

insition Settings			
Prediction Filter I	Library Instrume	ent Full-Scan	
Min m/z:		Max m/z:	
50	Th	1500	Th
Dynamic min	product m/z		
Match tolerance	m/z:	Emware trans	tion limit:
0.055	ſh		
Min time:	nin	Ma <u>x</u> time:	min

Figure 4 Two tabs of the Transition Settings dialog box

4. Edit the filter settings in the Transition Settings dialog box.

6. Save the settings for future use.

Import peptides into Skyline

- 1. Copy your peptide to the Clipboard.
- 2. Paste the peptide from the Clipboard into Skyline.

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

- In this example, a peptide from beta casein is typed into Notebook.
- Highlight the entire peptide and press **Ctrl** and **C**. You can also highlight the entire peptide and click **Edit > Copy**.
- The example peptide is FOSEEQQQTEDELQDK.
- 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.

	Peptide Sequence	Protein Name	Protein Description
Ø	FQSEEQQQTEDELQDK	beta casein	
ŧ			
i.			



e Click the Insert button.

Ja Skyline File € ● ⊕ ● ⊕ ●	dit View Se a casen Frss=covorted y S14347- A E b49 - 63 A b49 - 84 A E b49 - 63 A b49 - 85 A b49 - 85 A b49 - 95 A	Image: Constraint of the second sec			Precursors before modifications
Ready	1/1 prot	1/1 рер	1/2 prec	1/16 tran .,;;	

Figure 6 Main window of the Skyline program before modifications

- 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, ${\mathbb T}$ 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.

		ОК
Phospho (ST)	-	
		Cancel
Amino acid: Termin	nus:	
S, T 👻	 Variable 	0
Chemical formula:		
UO3P		
	-	
Monoisotopic mass:	Average mass:	
79.966331	79.979901	Loss <<
Veutral losses:		
Veutral losses: 97.9769 - H3O4P		
Veutral losses: 97.9769 - H3O4P	÷	



Figure 7 Edit Structural Modification and Edit Neutral Loss dialog boxes

- k Select Phospho (ST) for the S modification.
- I Click the **OK** button in the Edit Modifications dialog box.

tu Edit Modifications			•
Structural:		Isotope heavy:	ОК
•	F	•	Cancel
-	Q		Cancer
Phospho (ST) -	<u>s</u>		Create copy
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	Е		
	Q		
	Q		
	Q		
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	Е		
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	0		
	D		
	к		Reset
			,ii

Figure 8 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.



Figure 9 Main window of the Skyline program

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 Click File > Export > Method. The Export Method dialog box opens.
- b Select Agilent 6400 Series as the Instrument type.

- c Click the Single method button.
- d Select Collision Energy for Optimizing.
- e Select Standard for Method type.
- f Click the **Browse** button. The Browse For Folder dialog box is opened.
- g Select the method to use as a template in the **Template file** box. 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. The Time segments table is taken from the template method's first Time segment row.
- h Click OK in the Browse For Folder dialog box.
- i Click the **OK** button in the Export Agilent 6400 Series Method dialog box.

Export Method	—	Export Agilent 6400 Ser	ies Method				
Instrument type:	ОК	() •) • M	assHunter 🕨 Metho	ds ► SkylineMethods	- 47	Search SkylineMethod	15
Agricite 0400 Joines	Cancel	Organize • Ne	w folder				80 • 6
Single method		🔶 Favorites	Name	-	Date modified	Type	Size
One method per protein		C Librariar		No it	ems match your search.		
Multiple methods	Ignore proteins	Contractory of the second seco					
Max transitions per sample injecti	ion:	🔧 Homegroup					
		Computer					
Methods: 1		🗣 Network					
Optimizing:			×				
Collision Energy		File name:	pfh_Skyline_Optimi	izeCE			
Method type:	Dwell time (ms):	Save as type:	Method File (*.m)				
Standard	20	Hide Folders				Save	Cancel
Template file: C:\MassHunter\Methods\defau	t.m Browse						

Figure 10 Export Method and Export Agilent 6400 Series Method dialog boxes

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.

- a Click File > Export > Transition List. The Export Transition List dialog box is opened.
- b Select Agilent as the Instrument type.
- c Select Collision Energy for Optimizing.
- d Select Standard for Method type.
- e Click the **OK** button. The "Export Transition List" dialog box opens.
- f Navigate to the folder where you want to save the method.
- g Type a name for the new method.
- h Click the Save button.

xport Transition List	×
Instrument type: Agilent	OK Cancel
Single method	
One method per protein	
Multiple methods	Ignore proteins
Methods: 1 Optimizing:	
collision Energy	

Figure 11 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 12 Export Transition List dialog box

k 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.

Format of CSV file

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

6					FQS-CE-opt.c	sv - Micros	oft Excel				780		х
C	Home Insert	Page Layo	ut Formulas	Data	Review V	iew Ad	d-Ins					🥑 -	σx
Pi	Calibri Iste V B Z U V	• 11 •			Ge	neral	▼ 00+ 00*	Condition	nal Formattin Table *	g ▼ 🚰 Inse ∰ Del	ert * Σ * ete * 💽 * mat * 2 *	Sort & F Filter * S	nd &
Cit	board () F	ont	6	Alignment		Number		5	tyles	Cei	IS	Editing	
-	AZ 👻		FQSEEQQQ	TEDELQDK		-		1				-	*
	A	B	C	D	E	F	G	н	1	J	K	L	-
1	Compound Name	ISTDP	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Fragment	Collision	Protein	Ion Name		
2	FQSEEQQQTEDELQDK	FALSE	1031.41/8/1	Unit	632.304982	Unit		5 130	24.3	beta casein	γ5		- 1
3	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	632.314982	Unit		5 130	28.3	beta casein	γ5		
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	3	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	1	5 130	36.3	beta casein	y4		
11	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	503.302388	Unit	1	5 130	40.3	beta casein	y4		
12	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	390.178324	Unit	1	5 130	24.3	beta casein	уЗ		
13	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	390.188324	Unit		5 130	28.3	beta casein	у3		
14	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	390.198324	Unit	1	5 130	32.3	beta casein	у3		
15	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	390.208324	Unit	3	5 130	36.3	beta casein	у3		-
14	FQS-CE-opt	2/					14	(- MIL			
Rea	dy Fixed Decimal									100	% Θ		- 🕀 ,;;

Figure 13 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 the collision energy settings in the Transition Settings dialog box." on page 13.
- 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 QQQ acquisition method

- 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 Quad LC/MS System Quick Start Guide.

2.	Load the QQQ acquisition method from Skyline.	 a Click Method > Open. The "Open Method" dialog box is opened. b Navigate to the folder where you saved the method in Skyline. c Select the method that you created in Skyline. d Click the Open button.
3.	(optional) Prepare the LC modules.	 a Switch the LC stream to waste (or disconnect it from the MS). b Purge the LC pump. c Install the column and condition it as described in the column instructions included in the column package. d Set up to view real-time parameter values (actuals). e Set up to display real-time plots. f Set the LC parameter values in the Method Editor window.
4.	Prepare the Agilent 6400 Series Triple Quadrupole LC/MS System.	 If you need help, see Step 2 in the "Getting Started" section of the Agilent 6400 Series Triple Quad 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. a Do a Checktune, or if necessary do an Autotune. b Switch the LC stream to MS. c Start the flow at initial method conditions. d Monitor the MS baseline and spectral displays. If you need help, see Step 3 in the "Getting Started" section of the Agilent 6400 Series Triple Quad LC/MS System Quick Start Guide.
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 Quad LC/MS System Quick Start Guide (Agilent publication G3335-90077, Eighth Edition, January 2010).
6.	Review parameters on the QQQ tabs.	 a 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 13. The Product Ion also changes slightly. The Skyline program uses those slight changes to keep track of which step it is on. b Review parameters on the Source tab.

	c Review parameters on the Chromatogram tab. You can specify which chromato- grams 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.
7. Review the parameters on the DA tab.	You typically do not run Qualitative Analysis or Quantitative Analysis with this data file because the software doesn't know that these are the same compound since the product ions are different. Skyline changes the product ion to a slightly different value for each of the different collision energies as an internal way of keeping track of the collision energy optimization.
	a Click the DA tab.
	b Clear the Qual Automation check box on the Qual tab.
	c Click the Quant tab.
	d Clear the Quant Automation check box on the Quant tab.
8. Save the method.	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 .
Import 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 Clip-board . You can follow the steps in the "Review QQQ acquisition method" on page 21 and then follow these steps to modify the Scan segments table.
1. Open the CSV file in Excel.	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 13 on page 20.
2. Copy the transition list to the Clipboard.	a Select all of the cells in the transition list including the header.

0) 🖬 🔊 - (°' -) =				FQS-CE-opt.o	sv - Micros	oft Excel				7 0	_	= x
	Home Insert	Page Layo	ut Formulas	Data	Review V	iew Ad	d-Ins					۲	- 🕫 X
Pa	Ste 3 B Z U -	• 11 •		= <mark>=</mark> »-	Ge	eneral	▼ 0.00, 00 0.0	Condition Format as Cell Styles	nal Formatting Table *	 The Inset Deletion Form 	ert * Σ ete * J mat * Z*	Sort &	Find & Select *
Clip	board 🖗 🛛 F	ont	Gr	Alignment	G	Number	G	S	tyles	Cell	s	Editin	9
	A1 - (e ;	fx Compound	Name									×
	А	В	С	D	E	F	G	Н	1	J	К	L	-
1	Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Fragment	Collision EP	rotein	Ion Name		1
2	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	632.304982	Unit	5	5 130	24.3 b	eta casein	y5		-
3	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	632.314982	Unit	5	5 130	28.3 b	eta casein	y5		
4	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	632.324982	Unit	5	5 130	32.3 b	eta casein	y5		
5	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	632.334982	Unit	5	5 130	36.3 b	eta casein	y5		
6	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	632.344982	Unit	5	5 130	40.3 b	eta casein	y5		
7	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	503.262388	Unit		5 130	24.3 b	eta casein	y4		
8	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	503.272388	Unit	5	5 130	28.3 b	eta casein	y4		
9	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	503.282388	Unit		5 130	32.3 b	eta casein	y4		
10	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	503.292388	Unit	5	5 130	36.3 b	eta casein	y4		
11	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	503.302388	Unit	5	5 130	40.3 b	eta casein	y4		
12	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	390.178324	Unit	5	5 130	24.3 b	eta casein	у3		
13	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	390.188324	Unit		5 130	28.3 b	eta casein	y3		
14	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	390.198324	Unit	5	5 130	32.3 b	eta casein	y3		
15	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	390.208324	Unit		5 130	36.3 b	eta casein	y3		
14 4	+ H FQS-CE-opt	2/					14			Ш)
Rea	dy Fixed Decimal				Average: 27	1.2648722	Count: 891	Sum: 1085	05.9489	1009	6 🕞	U	(+) .

Figure 14 After selecting all of the cells in the transition list

- b Click Edit > Copy. You can also press Ctrl and C.
- 3. 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 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.

Tune file Stop time	Ac	quisition Source Chro	matogram	Instrument [Diagnostics							
Browse 6d C 1 min		Compound Name	ISTD?	Precursor Ion 🗸	MS1 Res	Product Ion V	MS2 Res	Dwell	Fragmentor	Collision Energy	Cell Accelerator Voltage	F.
Ion source Time filtering		FQSEEQQQTEDELQDK	Γ	1031.417871	Unit	632.304982	Unit	5	130	24.3	7	Pos
	Bash width 0.07 min	FQSEEQQQTEDELQDK		1031.417871	Unit	632.314982	Unit	5	130	28.3	7	Pos
Peak width U.U7 min		FQSEEQQQTEDELQDK		1031.417871	Unit	632.324982	Unit	5	130	32.3	7	Pos
Time segments		FQSEEQQQTEDELQDK		1031.417871	Unit	632.334982	Unit	5	130	36.3	7	Pos
# Start / Scan Type Div Valve Delta Delta Stored		FQSEEQQQTEDELQDK		1031.417871	Unit	632.344982	Unit	5	130	40.3	7	Pos
		FQSEEQQQTEDELQDK		1031.417871	Unit	503.262388	Unit	5	130	24.3	7	Pos
		FQSEEQQQTEDELQDK		1031.417871	Unit	503.272388	Unit	5	130	28.3	7	Pos
		FQSEEQQQTEDELQDK		1031.417871	Unit	503.282388	Unit	5	130	32.3	7	Pos
		FQSEEQQQTEDELQDK	Γ	1031.417871	Unit	503.292388	Unit	5	130	36.3	7	Pos
		FQSEEQQQTEDELQDK		1031.417871	Unit	503.302388	Unit	5	130	40.3	7	Pos
		FQSEEQQQTEDELQDK		1031.417871	Unit	390.178324	Unit	5	130	24.3	7	Pos 🗸
1.47 cycles/s 680.0 ms/cycle		•										•

Figure 15 The QQQ > Acquisition tab in the Method Editor window

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 Quad 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.

Sample	Addition	nal Information		
New Design (Protection)		Parameter Name	Parameter Value	
Name beta casein Postion No injection *		Sample ID		
injection Volume As Method v µL		Override DA Method	C:\MassHunter\Methods\default.r	n
	•	Sample Type	Sample	-
Comment optimizing the collision energy for beta casein		Method Type	Acquisition Only	
in the second		Balance Override	No Override	
		Equilib Time (min)	0	
V Auto Increment		RunType	Standard Run	
lame betacasein1 d View Data				
Path C:\MassHurter\Data				

Figure 16 The Sample Run window

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.

Review results and export final method from 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 in "Create and run the method in Data Acquisition" on page 21. Then, you can graphically review the results and then export a Dynamic MRM method.



Load results into Skyline

- 1. Start Skyline program.
- 2. Open the Skyline project.
- From the Start button in the All Programs list, click MacCoss Lab, UW > Skyline.

a Load the settings that you saved in "Save the settings for future use." on page 15. A menu item with the name you used to save the settings is added to the Settings menu.

- b Click File > Open.
- c In the Open dialog box, select the project you saved in "Export QQQ method" on page 17.

3. Import results.

- a Click File > Import > Results.
- b 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.

X
ОК
Cancel

Figure 17 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 21.
- g Select Agilent Data for the Sources of type box.
- h Click the **Open** button.

mport Results Files					? 💌
Look in:	🚞 Skyline files		•	G 🤌 🗔-	
My Recent Documents	C gdp hsa hsa-pep NIST MST skyline TestExport1.n	03.d			
My Documents					
My Computer	Source name:	Sigma-FQS+r003.d			Open
	Sources of type:	Agilent Data		•	Cancel

Figure 18 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.
- 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 19 Skyline program after importing results file

- 1. Select the transitions that were found.
- a Click the 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 of the transitions that do not have a green dot next to them.

File Edit View Settings Help	
🖞 🗃 📓 🖄 🖏 🔊 • 🕫 -	-
	Peak Areas
	(√5) - 632 3250+ (√4) - 603 2824+11 (√3) - 903 1983+ (√2) - 825 1187+12 (√5) - 316 6661++ (√4) - 825 1187+12 (√3) - 195 6661++ (√3) - 195 662++ (√2) - 131 5725++

Figure 20 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 Examine the Peak Areas graph to determine the collision energy that produces the greatest peak area. In this example, the peak area is greatest for this transition for Step-1 or -4 volts.
- d Examine the FQS opt tab to see the overlay of the peaks and this graph is also color coded.



Figure 21 Comparing collision energies for the same transition

 (optional) Edit the regression parameters to change the initial equation. Typically, you only want to determine a new equation if you have a much bigger distribution of peptides. However, this example is included for demonstration purposes.

- a Click Settings > Transition Settings.
- b Select Transition for Optimize by.
- c Click the **Prediction** tab.
- d Select Edit list from the Collision energy list.
- e Click **Agilent** in the Edit Collision Energy Regressions dialog box and the 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. If you want to use the new information, click the **Use Results** option.

Export 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 can also export a final transition list. You can use this transition list to create a method in the Agilent Data Acquisition program.

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

Export Method	—	Export Agilent 6400 S	eries Method		
Instrument type:	ОК	C3 (2) ▼ ≪ 1	MassHunter + Skyline files + skyline	ب ب ب	learch styline 🔎
Agilent 6400 Series V	Cancel	Organize 🔻 🛛 N	New folder		ji • 🛛
Single method		⊳ 🚖 Favorites	Name	Date modified	Type Size
 One method per protein Mathematical 		> 🥽 Libraries	🅌 skyline	2/3/2012 12:40 PM	File folder
 Multiple methods 	ignore proteins	🕨 🔧 Homegroup			
Max concurrent transitions:		> (S Computer			
Methods: 1		> 🗣 Network			
Optimizing: None			•		,
		File name	6		-
Method type: Scheduled		Save as type	e Method File (".m)		•
		Hide Folders			Save Cancel
Template file:					<u>اللہ</u>
C:\MassHunter\Methods\defa	ault.m Browse				

Figure 22 Export Method and Export Agilent 6400 Series Method dialog boxes

- j In the Export Agilent 6400 Series Method dialog box, select **Method File (*.m)** as the **Save As type**.
- k Enter a name for the method and click the **Save** button.

Export final transition list

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.

- a Click File > Export > Transition List.
- b In the Export Transition List dialog box, select Agilent as the Instrument type.

- c Select **Scheduled** as the Method type.
- d Click the **Single method** button.
- e Select None for Optimizing.
- f Click the **OK** button.

ort Transition List	•••	Export Transition List	
Instrument type: Agilent	ОК	🚱 🗢 📕 « MassHunter 🕨 Skyline files	Search Skyline file
 Single method 	Cancel	File name: DMRM FQS Save as type: Transition List (*.csv)	
One method per protein		~. <u></u>	
Multiple methods	Ignore proteins	Browse Folders	Save
Max concurrent transitions:			
Methods: 1			
Optimizing:			
None 🔻			
Method type:			
Scheduled •			

Figure 23 Export Transition List - page 1 and page 2

- g In the next Export Transition List dialog box, select **Transition List** as the Save As type.
- h Enter a name for the transition list and click the **Save** button.

Format of the final transition list file

The final Transition List contains only one line for each transition including the optimized collision energy:

E	Home Insert	Page Layo	out Formulas	Data	Review Vie	w Add	-Ins				0 - 5
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	н9 🔫 (۹	<i>f</i> _x 24								
	A	В	С	D	E	F	G	н	1	J	К
1	Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Fragment	Collision	Ret Time (Delta Re	Protein
2	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	1361.617929	Unit	130	28.3	3.01	2	beta casei
3	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	1233.559351	Unit	130	28.3	3.01	2	beta casei
4	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	1105.500774	Unit	130	32.3	3.01	2	beta casei
5	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	977.442196	Unit	130	32.3	3.01	2	beta casei
6	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	503.282388	Unit	130	36.3	3.01	2	beta casei
7	FQSEEQQQTEDELQDK	FALSE	1031.417871	Unit	262.139747	Unit	130	32.3	3.01	2	beta casei
3	FQSEEQQQTEDELQDK	FALSE	687.947673	Unit	977.442196	Unit	130	0.24	3.02	2	beta casei
9	FQSEEQQQTEDELQDK	FALSE	687.947673	Unit	876.394518	Unit	130	24	3.02	2	beta casei
0	FQSEEQQQTEDELQDK	FALSE	687.947673	Unit	747.351925	Unit	130	24	3.02	2	beta casei
1	FQSEEQQQTEDELQDK	FALSE	687.947673	Unit	503.282388	Unit	130	24	3.02	2	beta casei
2											
3											
4											
10											

Figure 24 CSV file for the Final Transition List

Review final QQQ method in Data Acquisition

This exercise shows you reviewing the final method. You can also review importing the final transition list.



Review QQQ acquisition method

- 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 Quad LC/MS System Quick Start Guide.

2. Load the QQQ acquisition method from Skyline.

- a Click **Method > Open**. The "Open Method" dialog box is opened.
- b Navigate to the folder where you saved the final method in Skyline.
- c Select the final method that you created in Skyline.

	d Click the Open button.
3. 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 <i>Agilent 6400 Series Triple Quad LC/MS System Quick Start Guide</i> (Agilent publication G3335-90077, Eighth Edition, January 2010).
4. Review parameters on the	a Review parameters on the Acquisition tab.
QQQ tabs.	b Review parameters on the Source tab.
	c Review parameters on the Chromatogram tab. You can specify which chromato- grams 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	a Click the DA tab.
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	If you created a final transition list, then you follow these instructions to create a final method.
1. Open the method.	 Click Method > Open or click the Open Method icon on the main toolbar.
2. Open CSV file in Excel.	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 24 on page 32.
3. Copy the transition list to the Clipboard.	a Select all of the cells in the transition list including the header.

6						DMRM2 core M	icrosof	t Evcal					- \	~
6) • (= •) •				Dividual Car	icrosol	C EXCEI		1		- 60	-	~
	Hon	Insert	Page Layou	it Formulas	Dat	ta Review	View	Add-In	s			C		×
1	× ×	Calibri	- 11 -	A		General		Conditi	onal Formatt	ing - 🔤 -	Insert *	Σ · A		
-			(· · · · %	, ,	Format	as Table *	3*	Delete *			
Fe	- V	R T R			39	00. 0. 0.€ 00.		📑 Cell Styl	es *		Format *	2 Filter	Select *	
Clip	board 🛱	F	ont	G Aligr	nment	Number	G		Styles		Cells	Editir	g	
	A1 • fr Compound Name *													
		A	В	С	D	E	F	G	н	1	J	К	L	F
1	Compou	nd Name	ISTD?	Precursor Ion	MS1	Product Ion	MS2 I	Fragment	Collision E	Ret Time	Delta Ret	Protein	Ion Nam	
2	FQSEEQ	QUTEDELQDK	FALSE	1031.417871	Unit	1361.617929	Unit	130	28.3	3.01	2	beta casei	y11	
3	FQSEEQ	QQTEDELQDK	FALSE	1031.417871	Unit	1233.559351	Unit	130	28.3	3.01	2	beta casei	y10	
4	FQSEEQ	QUTEDELQDK	FALSE	1031.417871	Unit	1105.500774	Unit	130	32.3	3.01	2	beta casei	y9	
5	FQSEEQ	QUTEDELQDK	FALSE	1031.417871	Unit	977.442196	Unit	130	32.3	3.01	2	beta casei	y8	
б	FQSEEQ	QUTEDELQDK	FALSE	1031.417871	Unit	503.282388	Unit	130	36.3	3.01	2	beta casei	y4	=
7	FQSEEQ	QUTEDELQDK	FALSE	1031.417871	Unit	262.139747	Unit	130	32.3	3.01	2	beta casei	y2	
8	FQSEEQ	QUTEDELQDK	FALSE	687.947673	Unit	977.442196	Unit	130	24	3.02	2	beta casei	y8	
9	FQSEEQ	QUTEDELQDK	FALSE	687.947673	Unit	876.394518	Unit	130	24	3.02	2	beta casei	y7	
10	FQSEEQ	QUTEDELQDK	FALSE	687.947673	Unit	747.351925	Unit	130	24	3.02	2	beta casei	y6	
11	FQSEEQ	QUTEDELQDK	FALSE	687.947673	Unit	503.282388	Unit	130	24	3.02	2	beta casei	y4	
12														
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14 4	FHD	MRM2 / 😏 /	1			210 2225 222	-							
кеа	ay Fixed	Decimal			verage	: 518./3/5222 (ount:	132 Sum: 1	9124.25133		100% (-)		÷	.::

Figure 25 After selecting all of the cells in the final transition list

- b Click Edit > Copy. You can also press Ctrl and C.
- 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.

Method Editor	ethod Editor														
🗅 📝 🖬 📓 🖻 PeptideOptimize.m															
Properties DA QQQ	Yroperties DA QQQ														
Tune file	Stop time	Ac	Acquisition Source Chromatogram Instrument Diagnostics												
atunes.tune.xml	No limit/As Pump	s Pump Scan segments													
Browse 66	C 1 min		Compound Name - 7	ISTD?	Precursor Ion ∇	MS1 Res	Product Ion V	MS2 Res	Fragmentor	Collision Energy	Cell Accelerator Voltage	Ret Time (min)	Delta Ret Time	F	
Ion source	Time filtering	•	FQSEEQQQTEDEL		1031.417871	Unit	1361.617929	Unit	130	28.3	7	3.01	2	205	
FSI	Reak with 0.07 min	FQSEEQQQTEDEL		1031.417871	Unit	1233.559351	Unit	130	28.3	7	3.01	2	Pos		
	Je reak wouri j0,07 min	IV Peak width 0,07 min	FQSEEQQQTEDEL		1031.417871	Unit	1105.500774	Unit	130	32.3	7	3.01	2	20s	
Time segments			FQSEEQQQTEDEL		1031.417871	Unit	977.442196	Unit	130	32.3	7	3.01	2	Pos	
# Start / Scan Type D	iv Valve Delta Delta Stored		FQSEEQQQTEDEL		1031.417871	Unit	503.282388	Unit	130	36.3	7	3.01	2	Pos	
► 1 0 Dynamic MB ▼ To	MS 0 0 V		FQSEEQQQTEDEL		1031.417871	Unit	262.139747	Unit	130	32.3	7	3.01	2	Pos	
			FQSEEQQQTEDEL		687.947673	Unit	977.442196	Unit	130	24	7	3.02	2	Pos	
			FQSEEQQQTEDEL		687.947673	Unit	876.394518	Unit	130	24	7	3.02	2	× 20 ⁶	
		ŀ	•												

Figure 26 The QQQ > Acquisition tab in the Method Editor window

- 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.

Save the method

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 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 MacCoss Lab, UW > Skyline.
- In the **Settings** menu, click the name of the settings that you saved in "Save the settings for future use." on page 15.

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

- a Clip 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.

Edit Structural Modifi	cation	×
Name: Carbamidomethyl Cyst	eine	ОК
Amino acid: Tem C 🔹	variable	Cancel
Chemical formula: C2H3ON		
Manajaatanja masa:	Average mass:	

Figure 27 Add or Edit Structural Modifications dialog box

d Click the **OK** button.

tu Edit Modifications			×
Structural:		Isotope heavy:	ОК
-	Α	-	Cancel
•	Α	•	Cancer
-	F		Create copy
-	т	•	
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Carbamidomethyl Cvi 🔻	с		
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-	D		
•	ĸ	-	Reset

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.

Figure 28 Edit Modifications dialog box

5. (optional) Select a library.

a Click Settings > Peptide Settings.

- 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 51 for information on getting a library.

- 6. Save the project.
- Save the document. Click File > Save to save the current proteins/peptides in a Skyline project. Type a File name and click the Save button.

🕽 🔾 🗢 🚺 « Ma	ssHunter 🕨 Skyline files 🕨 hsa	-pep 🕨	▼ ⁴ → Searci	h hsa-pep	
Organize 👻 Ne	w folder				
🔆 Favorites	Name	Date modified	Туре	Size	
	hsa-seq-PFH2.sky	2/3/2012 9:41 AM	Skyline SRM Docu	11 KB	
ز Libraries	hsa-seq-PFH1.sky	2/2/2012 8:53 AM	Skyline SRM Docu	49 KB	
	hsa-pep-PFH.sky	2/2/2012 7:15 AM	Skyline SRM Docu	86 KB	
😽 Homegroup	hsa-seq-PFH.sky	2/1/2012 2:43 PM	Skyline SRM Docu	12 KB	
Computor	hsa-seq-PFH.skh.sky	2/1/2012 2:42 PM	Skyline SRM Docu	12 KB	
1 Computer	hsa-pep-demo.sky	1/31/2012 11:56 PM	Skyline SRM Docu	248 KB	
💁 Network	hsa-pep-PAH.sky	1/31/2012 10:12 PM	Skyline SRM Docu	45 KB	
-	hsa-pep-20min.sky	10/31/2011 3:10 PM	Skyline SRM Docu	404 KB	
	pfh TextExport BetaCa	2/3/2012 11:02 AM	File folder		
File name:	hsa-pep-demo				
Save as type:	Skyline Documents (*.sky)				

Figure 29 Save As dialog box

Import data file or data files

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 42).

- 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 26 for more information on using Skyline to optimize the collision energy.
- d Click the OK button.



Figure 30 Import Results dialog box

- 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.

Refine list

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.

	18] (missed 1) (rank 33) MS/MS Spectrum human gtof consen:
D [y8] - 95 ✓	Transitions
∫_ G [y6] - 72 ×	
	✓ J _M K (y3) - 10/5/3366+ (ank 5) ✓ J _M D (y8) - 951.4418+ (rank 1)
KALVLIAFAQYLQ	✓ <u>√</u> L [y7] - 836.4149+ (rank 3)
H - • 🕵 KLVNEVTEFAK	∫ G [/6] - 723.3308+ (rank 2) ↓ E [/5] - 666.3093+ (rank 9)
E- KILVADESAEN	☐ ¹ / ₁ E [y4] - 537.2667+ (rank 7)
🗎 🛛 🕵 K.LCTVATLR.E [
R.ETYGEMADCC	K [y1] - 147.1128+ (rank 8)
🗉 🔮 K.DDNPNLPR.L [∫_ K [y9] - 540.2720++ ↓ D [x8] - 476 2245++ (rank 10)
🕀 🗧 🚼 K KYLYEIAR R [1	JD

Figure 31 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.

🖃 🌜 spiP0.	2768JALBU_HUMAN
- 💡	R.FKDLGEENFK.A [9, 18] (missed 1) (rank 33)
😐 – 🂡	K.DLGEENFK.A [11, 18] (rank 29)
😐 – 🍕	K.ALVLIAFAQYLQQCPFEDHVK.L [19, 39] (rank 41)
🔅 - 🖕 🌍	K.LVNEVTEFAK.T [40, 49] (rank 18)
i 🖓 🖓	K.TCVADESAENCDK.S [50, 62] (rank 21)
i 💮 🖓	K.SLHTLFGDK.L [63, 71] (rank 4)
i 💮 🖓	K.LCTVATLR.E [72, 79] (rank 9)
i 💮 🖓	R.ETYGEMADCCAK.Q [80, 91] (rank 1)
i 💮 😲	R.NECFLQHK.D [97, 104] (rank 23)
i 🤨	K.DDNPNLPR.L [105, 112] (rank 19)
÷ 🤨	K.KYLYEIAR.R [135, 142] (missed 1) (rank 7)
6	K VI VEIAR R [126 142] (ook 12)

Figure 32 No precursors were found for this peptide

2. 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.

a Select a precursor for a peptide in the tree view.

spiP02768IALBU_HUMAN	3) MS/MS Spectrum human atof consensus fin
- · · · · · · · · · · · · · · · · ·	↓ pinconcer.070.3111++ ↓ V b/g1.1036.53110+ rank 59 ↓ N b/g3.374625+ rgmk for 1011 ● ↓ k b/g3.74625+ rgmk for 1011 ● ↓ k b/g3.74625+ rgmk for 1011 ● ↓ k b/g4.7470+ rgmk for 1011 ● ↓ k b/g4.740+ rgmk for 1011 ↓ ↓ k b/g4.2609+ rgmk for 1011 ↓ k b/g4.2609+ rgmk for 1011

Figure 33 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.

	8) MS/MS Spectrum human_otof_consensu	s fii
V b61-684.3770-ra V b63-684.3770-ra V b63-685.3086-ra V b63-685.3086-ra	$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$	

Figure 34 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.
- 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 Click the **OK** button.
- d 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.

port Results	×	Import Results Files			8
Add single-injection replicates in files	ОК	Look in:	🚞 hsa	•	G 🤌 💷-
Add multi-injection replicates in directories	Cancel		HSA-ST-10fmol	r001.d ILL HSA-ST-1pmol-r001.d	ILL HSA-ST-50amol-r001.d
Add one new replicate			III HSA-ST-10fmol	r002.d ILL HSA-ST-1pmol-r002.d	ILL HSA-ST-50amol-r002.d
Name		La Devent	ilit HSA-ST-10fmol	r003.d ILL HSA-ST-1pmol-r003.d	ILL HSA-ST-50amol-r003.d
Nono.		Documents	ilil HSA-ST-10fmol	r004.d ILL HSA-ST-1pmol-r004.d	ILL HSA-ST-50amol-r004.d
			ILL HSA-ST-10fmol	r005.d ILL HSA-ST-1pmol-r005.d	ILL HSA-ST-50amol-r005.d
0.000			ILL HSA-ST-10pmo	I-r001.d ILL HSA-ST-20amol-r001.d	ILL HSA-ST-5pmol-r001.d
Optimizing:			ILL HSA-ST-10pmo	I-r002.d ILL HSA-ST-20amol-r002.d	ILL HSA-ST-5pmol-r002.d
· · · · · · · · · · · · · · · · · · ·		Dealstan	ILL HSA-ST-10pmo	I-r003.d ILL HSA-ST-20amol-r003.d	ILL HSA-ST-5pmol-r003.d
		Desktop	ILL HSA-ST-10pmo	I-r004.d ILL HSA-ST-20amol-r004.d	ILL HSA-ST-5pmol-r004.d
Add files to an existing replicate			ILL HSA-ST-10pmo	I-r005.d ILL HSA-ST-20amol-r005.d	ILL HSA-ST-5pmol-r005.d
Name:			ILL HSA-ST-1fmol-r	001.d ILL HSA-ST-500amol-r001.d	
Channel a			ILL HSA-ST-1fmol-r	002.d ILL HSA-ST-500amol-r002.d	
Chromatograms •		My Documents	ILL HSA-ST-1fmol-r	003.d ILL HSA-ST-500amol+r003.d	
			ILL HSA-ST-1fmol-r	004.d ILL HSA-ST-500amol-r004.d	
			ILL HSA-ST-1fmol-r	005.d ILL HSA-ST-500amol-r005.d	
			*	п	
		My Computer	Source name:	"HSA-ST-10fmol+001.d" "HSA-ST-10fm	nol+r002.d" "HSA-ST- Open
		My Computer	c ()	Aniant Data	

Figure 35 Import replicates by clicking Add files to an existing replicate

- e 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 36 The MS/MS Spectrum, Chromatograms and Retention Times windows

• In this example, these data files only contain one peptide, so all of the other peptides can be removed.

4. Display the retention time graph.

5. Remove additional

peptides and transitions.

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.



Export report

- 1. Display the Export Report dialog box.
- 2. Export a 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.

- 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.

- d Type a new **File name**. The default name of the File name is the name of the current project. If you do not type a unique name for the report, you are asked whether or not to replace the file.
- e Click the Save button.

You can open this CSV file in Excel or another program. Then, you can use additional features in Excel to further process the data.

6		u (*) =		hsa-sec	q-PFH2.csv	- Microsoft	Excel					= x
C	Home	Insert Page Lay	out Formu	ulas Data Review Vie	w Ad	d-Ins					۲	- = x
P	Calib	ri • 11 •	A A	Wrap Tes	xt	General	+	Conditional	Format Cell	ar= Insert →	Σ - Α 	7 A
Clin	* 🧭 🗾	Z U T		Alignment	Center •	3 · 70	7 .00 ÷.0	Formatting *	as Table + Styles	- Format -	Q* Filte	er * Select *
City	board (a)	Fonc	0.15	Angriment	100	Num	Der (4)		styles	Cells	, Eu	ung
	Al	- (3	Jx Peptide	eSequence								*
	A	В	С	D		E F		G	Н	1	1	K
1	PeptideSeque	ProteinName	Replicatel	PeptidePeakFoundRatio	Peptide	Retentior R	atioToStand	ard				- II
2	LVNEVTEFAK	peptides1	HSA-ST-10	1		2.82	#N/A					=
3	LVNEVTEFAK	peptides1	HSA-ST-10	1		2.82	#N/A					
4	LVNEVTEFAK	peptides1	HSA-ST-10	1		2.82	#N/A					
5	LVNEVTEFAK	peptides1	HSA-ST-10	1		2.82	#N/A					
6	LVNEVTEFAK	peptides1	HSA-ST-10	1		2.84	#N/A					
7												
8												
9										1		*
14	hsa-se	q-PFH2					1					
Rei	ady Fixed Decin	nal								100% (=)		🕀

Figure 37 Excel Program with the CSV file from a default exported report

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.

1. Modify a report.

Open CSV file in Excel.

- 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 **Report 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 kolumn.
- 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.

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

Report Name: AreaAndHeight			Preview
Peptides Peptides Prevann Transitions Transitions TransitionResults TransitionResults Potential Time Potential StartTime StartTime StartTime Area Atalia AreaNamaized Height Truncated PeakSark Note TransitionResultsSummary	E Add >	PetitoRequence ProteinName RepicateName PetitoRefactiounTatio PetitoRefactiounTatio ProductN2 Area Area Area AreaNomalized	

Figure 38 Edit Report dialog box

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

Click the evice button to close the Preview Report dialog box.

Peptide Sequence	Protein Name	ReplicateName	PeptidePeakFound	Peptide Retention Ti	ProductMz	Area	Height	AreaNormalize
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r001	1	2.82	937.462538	16773411	4166639	73.1996%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r002	1	2.82	937.462538	16905592	4171568	73.0409%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r003	1	2.82	937.462538	16499654	4107749	73.2347%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r004	1	2.82	937.462538	16298528	4033779	73.3593%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r005	1	2.84	937.462538	15829372	3908214	73.3783%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r001	1	2.82	823.41961	2311871	573511	10.0891%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r002	1	2.82	823.41961	2337780	579302	10.1004%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r003	1	2.82	823.41961	2273557	570663	10.0913%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r004	1	2.82	823.41961	2212379	551912	9.9579%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r005	1	2.84	823.41961	2164263	538283	10.0326%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r001	1	2.82	694.377017	3829344	959020	16.7114%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r002	1	2.82	694.377017	3902016	973039	16.8587%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r003	1	2.82	694.377017	3756622	943441	16.674%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r004	1	2.82	694.377017	3706495	929091	16.6828%
LVNEVTEFAK	peptides1	HSA-ST-10pmol-r005	1	2.84	694 377017	3578655	900873	16.5891%

Figure 39 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	l
Report:	
Peptide Ratio Results	Add
Peptide RT Results Transition Results	Copy
	Edit
	Remove
	Up
	Down
	Reset
ОК	Cancel

Figure 40 Edit Reports dialog box

k In the Edit Report dialog box, click the **OK** button.

I In the Export Report dialog box, click the **Cancel** button.

- 2. Save the report definitions (share a report).
- a Click File > Export > 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	×
Select the report definitions you want to save to a file.	
Peptide Ratio Results Peptide RT Results Transition Results Transition Results Tares deviced	OK Cancel
V Meantanagat	
Select / deselect all	

Figure 41 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.



Figure 42 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.

- a Click File > Export > Report.
- 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".

Organize 🔻 New folder			88 -	
Area_Height.skyr				
CopyPeptide Ratio Results.sky	т			

Figure 43 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		×
Report:		
Peptide Ratio Results Peptide RT Results Transition Results		Preview
AreaAndHeight		Edit list
		Share
		Import
	Export	Cancel

Figure 44 Export Report dialog box

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



Installing Skyline

1. Find the location of the software online.

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

- 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 45 Searching for Skyline Peptide using Google.

- e Connect to that site.
- 2. Download and install the software.
- a Find the **Skyline Release** button. This button also shows the version number. This guide is written using version 1.2.
- b Click the Skyline Release button.
- c Click the **I agree** button to agree to the license terms.

The Name, Version and Publisher information is displayed.

- d Click the Install button.
- e The "Opening setup.exe" message box appears. Click the Save File button.
- f 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.

Downloading a library

1. Find the location of the Skyline software online.

You can download a library from the internet. The Skyline web site contains links to allow you to download the following files:

- MacCoss BiblioSpec
- 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.

- 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.

Google	skyline peptide	٩	H
Search	About 486,000 results (0.11 seconds)		
Everything Images Maps	Skyline: /home/software/Skyline proteome.gs.washington.edu/software/skyline/ Aug 8, 2011 – Skyline v1.2 Work Continues! New features include: Integrated display of MS/MS peptide ID spectra in MS1 chromatograms; Peak picking in		Ŧ
•	III		F

Figure 46 Searching for Skyline Peptide using Google.

- e Connect to that site.
- a Scroll to the bottom of the home page.
- b In the Spectral Library Links section, click one of the links.
- c Follow the instructions on the page that is opened to download the library.
- 2. Download a library using the links to the libraries at the bottom of the home page for Skyline.

References	The references in this list give valuable information that help you use Skyline soft- ware with the Agilent 6400 Series Triple Quadrupole LC/MS System.
Manuals	Agilent 6400 Series Triple Quad LC/MS System Concepts Guide
	Agilent 6400 Series Triple Quad LC/MS System Quick Start Guide
	Agilent 6400 Series Triple Quad LC/MS Maintenance Guide
	Agilent MassHunter Workstation Software – Data Acquisition for 6400 Series Triple Quadrupole LC/MS Familiarization Guide
	Note: All MassHunter software includes online Help, in addition to manuals. See the online Help for details about the software.





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