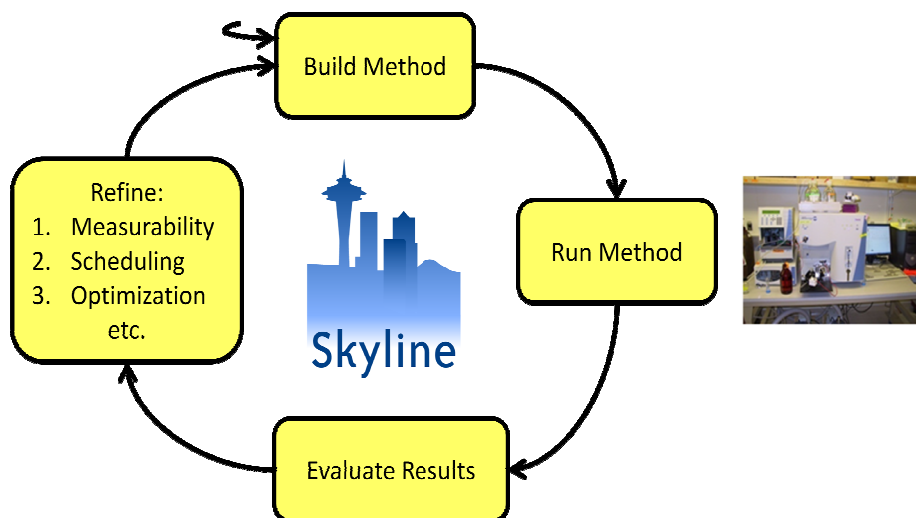


Skyline Targeted Method Refinement

This tutorial will introduce the features available in the Skyline Targeted Proteomics Environment for refining instrument methods for Selected Reaction Monitoring (SRM, also referred to as Multiple Reaction Monitoring, or MRM) mass spectrometry experiments.

When an ideal set of target peptides for a given protein is unavailable or unknown, Skyline makes it easy to create methods that can measure a broad range of peptides in search of the most measurable peptides in a sample matrix. Results from these initial measurements can then be imported into Skyline, and Skyline can help refine your method to improve your next iteration of measurements.

We call this the Targeted Method Refinement Cycle and frequently present the idea as shown below:



By iterating over this cycle, you can start with a broad hypothesis including greater than 100 proteins you are interested in monitoring, and quickly narrow your list down to the best peptides, product ions and instrument settings for achieving your experimental goals.

This tutorial will step you through two-and-one-half iterations of this refinement cycle and leave you with ideas for further iterations that may help in creating a fully refined quantitative assay.

Getting Started

To start this tutorial, download the following ZIP file:

<https://brendanx-uw1.gs.washington.edu/tutorials/MethodRefine.zip>

Extract the files in it to a folder on your computer, like:

C:\Users\brendanx\Documents

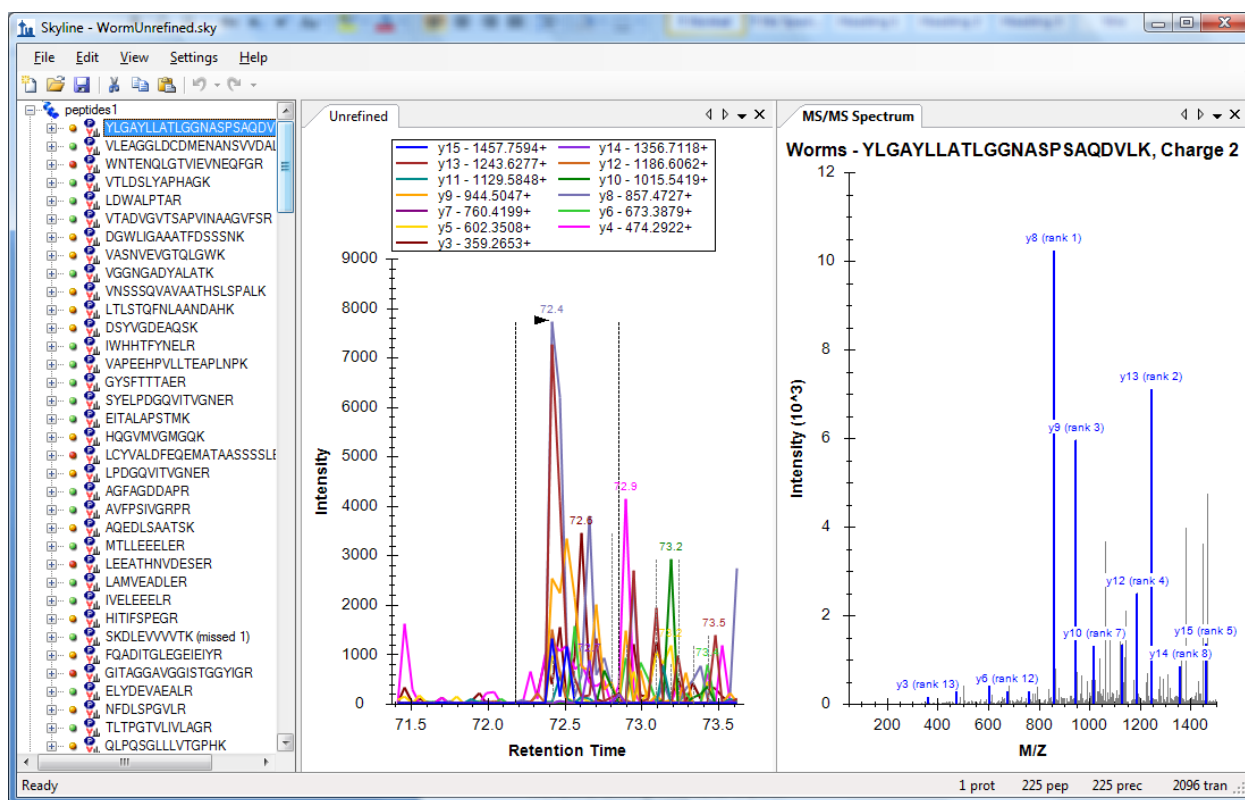
This will create a new folder:

C:\Users\brendanx\Documents\MethodRefine

Now open the WormUnrefined.sky file in this new folder by double-clicking, or by using **Open** on the **File** menu in a running instance of Skyline.

Results Data

Select the first peptide (YLGAYLLATLGGNASPSAQDVLK) in the document. Skyline should present graphs showing both a corresponding MS/MS spectrum from a library and time-intensity chromatogram data measured for product ions y3 – y15 of this peptide on an instrument in the MacCoss lab:



Note that the MS/MS library spectrum associated with each peptide is typically derived from experiments conducted on an ion trap mass spectrometer.

In the peptide view on the left, Skyline displays green, yellow and red dots left of the peptide sequences. There are peak quality icons, and they indicate:

- Green – All transitions contribute a co-eluting peak to the peak Skyline picked as the best.
- Yellow – At least half of the transitions contribute a co-eluting peak.
- Red – Less than half of the transitions contribute a co-eluting peak.

The chromatogram data was originally imported into this document from 39 Thermo RAW files. To understand why 39 separate sample injections were required for a single measurement of the peptides in this document, first note the numbers in the lower right corner of the Skyline window. You can see that the document contains 225 peptides and 2096 transitions, which cover $y_3 - y_{(n-1)}$ for each peptide (where n is the number of amino acids in the full peptide sequence).

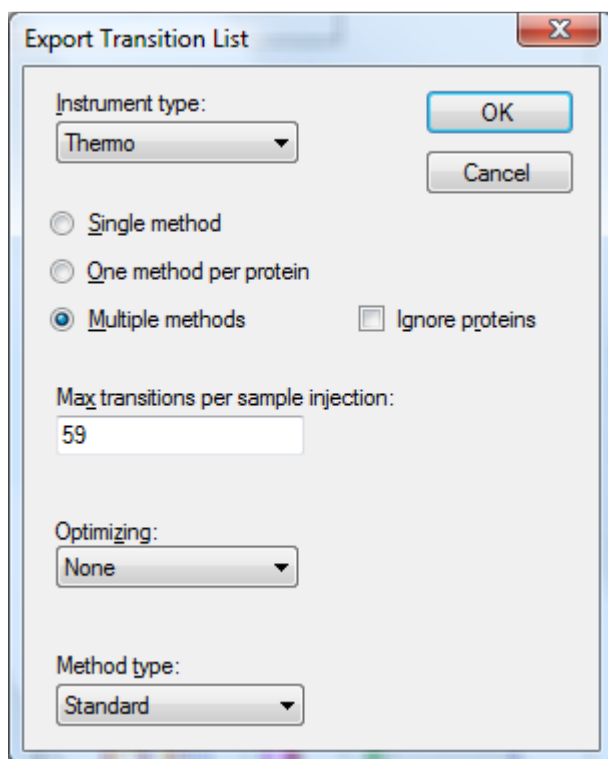
The goal of the Skyline document you are viewing is to help determine which peptides can be measured in a specific target matrix, and which are the best transitions for the measurable peptides. Larger numbers of transitions per peptide allow us to gain confidence that a given peak actually measures the peptide of interest. This confidence is measured by calculating a dot-product correlation between the peak intensities of the transitions for the peptide of interest and the library spectrum^{1,2} for the same peptide.

Unrefined Methods

To see how we generated the transition lists required for measuring the peptides in this document, perform the following steps:

- On the **File** menu, choose **Export**, and click **Transition List**.
- Choose **Multiple methods**.
- Enter '59' in the **Max transitions per sample injection** field.

The **Export Transition List** form should look like:



The screenshot shows the 'Export Transition List' dialog box. It has a title bar with a close button (X). Inside, there are several controls: a dropdown for 'Instrument type' set to 'Thermo'; two buttons, 'OK' and 'Cancel'; three radio buttons for 'Single method', 'One method per protein', and 'Multiple methods' (which is selected); a checkbox for 'Ignore proteins' which is unchecked; a text input field for 'Max transitions per sample injection' containing the value '59'; a dropdown for 'Optimizing' set to 'None'; and a dropdown for 'Method type' set to 'Standard'.

- Click the **OK** button.
- Navigate to the MethodRefine folder in the next form.
- Enter 'worm' in the **File name** field.
- Click the **Save** button.

If you use Windows Explorer to view the contents of the MethodRefine folder, you will see that it now contains 39 new CSV files (worm_0001.csv – worm_0039.csv). Each is about 4K in size and contains a list of no more than 59 transitions ready for import into an unscheduled Thermo TSQ method.

The number 59 may seem like a strange choice, but it was necessary to get transition lists that match the original experiment. For the original experiment, the number 60 was used. Unfortunately, Skyline had a bug (now fixed) that only allowed transition counts less than the maximum.

Importing Multiple Injection Data

If you would like to learn how to import the initial set of instrument output files for this experiment, you will have to download another, supplementary ZIP file (36 Meg). This ZIP file contains the 39 Thermo RAW files (161 Meg uncompressed) collected at the MacCoss lab to measure the transition lists you just exported in the above section.

The original MethodRefine.zip file you downloaded contained a high-performance data cache file WormUnrefined.skyd, which already has all the data Skyline requires from these files. If you prefer to continue using the existing data cache file, you may skip to the next section.

To re-import the data yourself, download the ZIP file:

<https://brendanx-uw1.gs.washington.edu/tutorials/MethodRefineSupplement.zip>

Extract the files into the folder you used before. This will create a new folder like:

C:\Users\brendanx\Documents\MethodRefineSupplement

In Skyline, perform the following steps to remove the previously cached data:

- On the **Edit** menu, click **Manage Results**.
- Click the **Remove** button.
- Click the **OK** button.

Both the chromatogram chart and the peak quality icons have been removed from the Skyline interface.

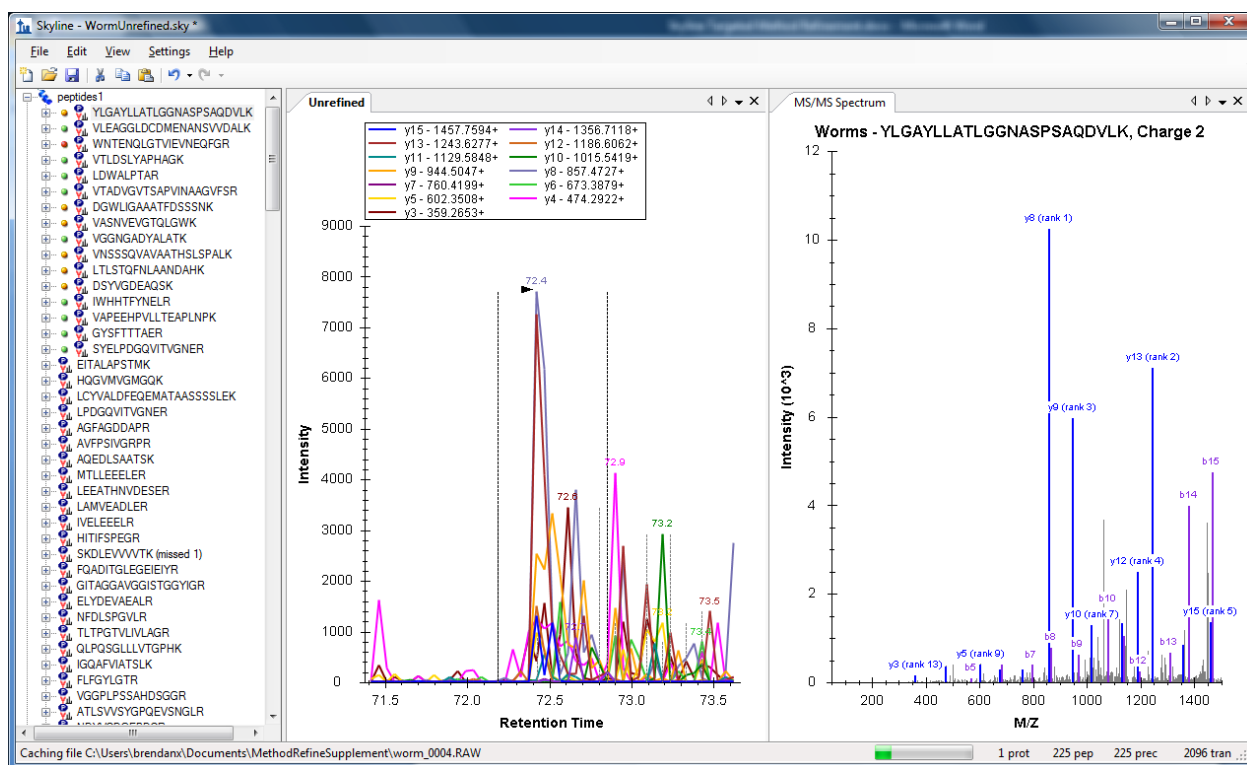
- On the **File** menu, click **Save** (Ctrl-S).

Now you are ready to import the original data yourself. You don't have to import it all at once. This can be useful for checking your data before acquisition is completed for all of the transition lists exported from these large, unrefined documents. In this tutorial, you will import the data in two batches.

First, perform the following steps:

- On the **File** menu, choose **Import**, and click **Results**.
- Choose **Add one new replicate**.
- Enter 'Unrefined' in the **Name** field.
- Click the **OK** button.
- Navigate to the MethodRefineSupplement folder.
- Click on the 'worm_0001.RAW' file.
- Shift-click on the 'worm_0015.RAW' file, to select the first 15 files.
- Click the **Open** button.

Skyline will begin importing those 15 files, and you will see its progress indicated in the status bar at the bottom of the Skyline window, and as the peak quality icons return to the peptides in the peptide view, as shown below:



While Skyline caches this data into its high-performance data file, you are free to continue reviewing the results. You could even begin refining the document, but for this tutorial, you should finish importing all 39 results files by doing the following:

- On the **File** menu, choose **Import**, and click **Results**.
- Choose **Add files to an existing replicate**.
- Click the **OK** button.
- Navigate to the MethodRefineSupplement folder.

- Click on the 'worm_0016.RAW' file.
- Shift-click on the 'worm_0039.RAW' file, to select all remaining files.
- Click the **Open** button.

Once Skyline completes the import, you are ready to start the next section, with a data cache file that matches the one this tutorial came with originally.

Simple Manual Refinement

One way to begin refining a document is by visually reviewing each peptide and deciding what to keep and what to discard based on the rich information Skyline provides. This is how the Skyline document in this tutorial was originally refined for our ASMS 2009 poster. It took less than one hour to review these peptides, and choose the best three transitions for the ones with well defined peaks closely matching the library spectra.

Looking at the Skyline document for this tutorial, one question you might ask about the first peptide is whether Skyline missed a peak better than the one it is currently zoomed in on. To answer this question, you can do the following to zoom out:

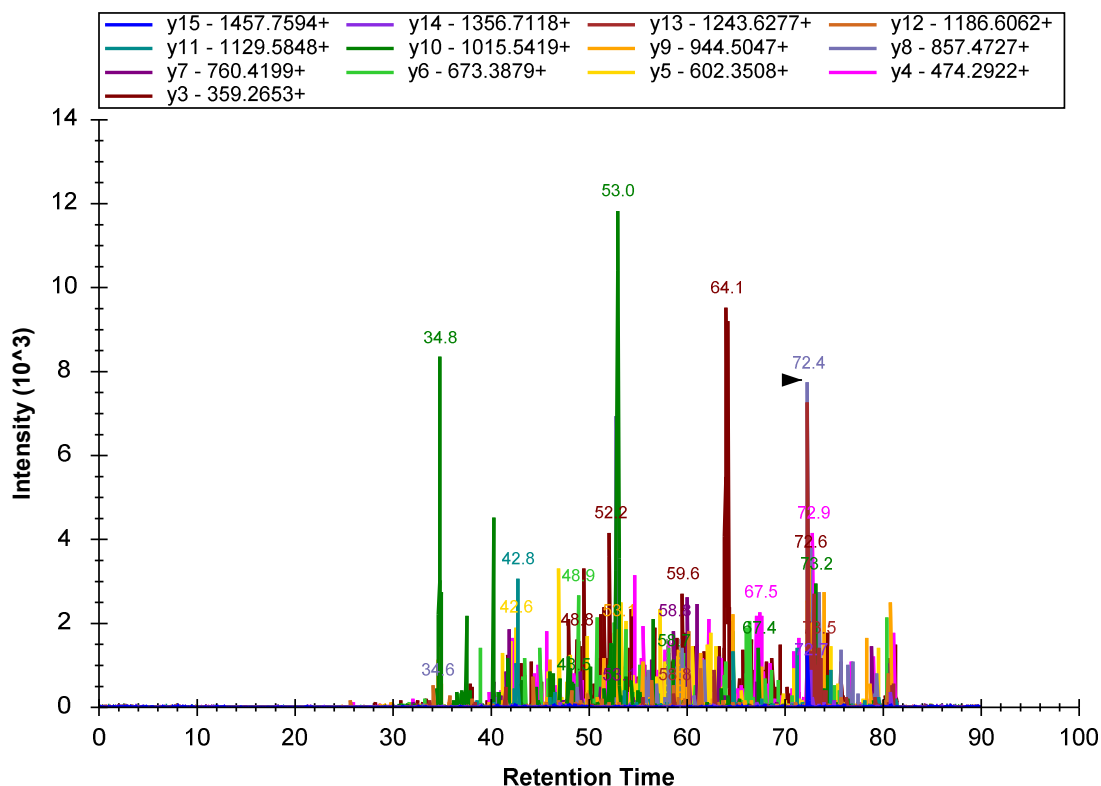
- On the **View** menu, choose **Auto-Zoom**, and click **None** (Shift-F11).

Here you should pause and take a moment to memorize the keyboard short-cuts for

- **View / Auto-Zoom / Best Peak** – F11
- **View / Auto-Zoom / None** – Shift-F11

These will allow you to switch quickly between a close-up view of the currently selected peak, and the entire range of time for which the instrument measured the transitions you are inspecting.

For the first peptide in the document, that full range looks like:



This looks like pretty noisy data at first glance, but if you would like to look deeper, you can click and drag a box around any of the large peaks labeled with their retention times.

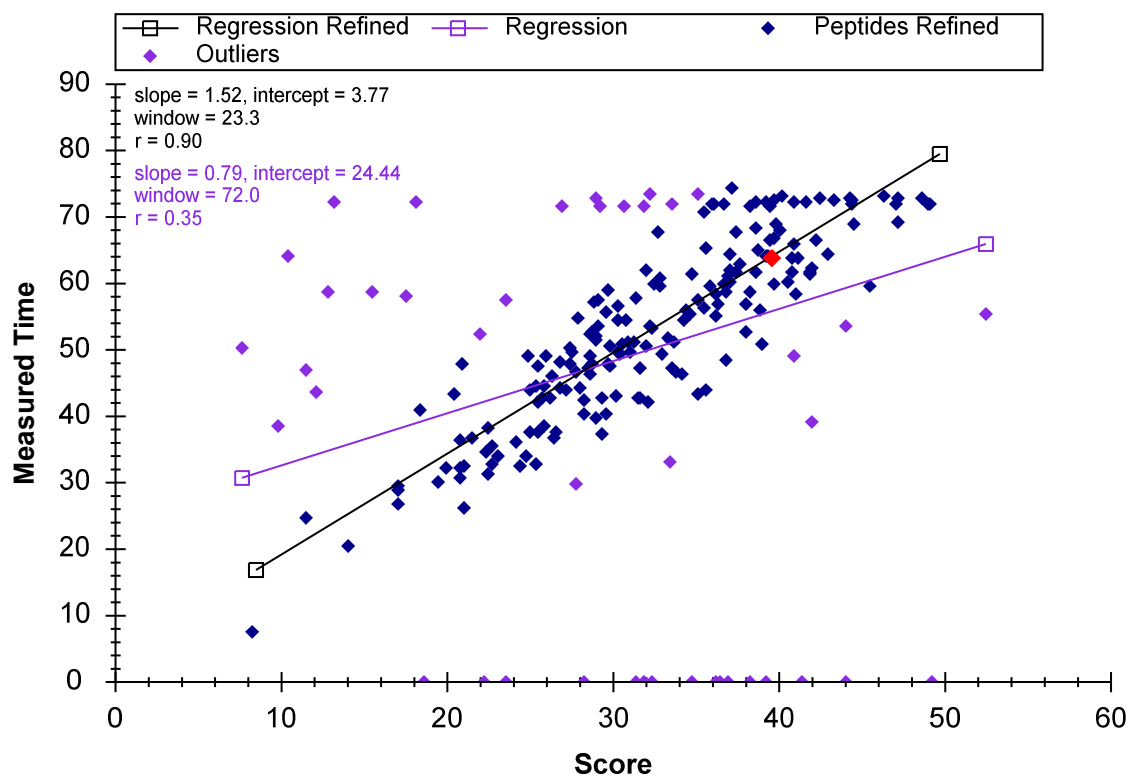
Once you are convinced that none of these contain a genuine measurement of this peptide, you can delete the peptide from the document by pressing the delete key.

Retention Time Prediction

It can also be useful when inspecting chromatogram peaks to have some idea of the expected retention time for a peptide. The Sequence-Specific Retention Calculator (SSRCalc) 3.0³ has been integrated into Skyline to make this possible. To see a linear regression graph of the relationship between SSRCalc score and measured peptide retention time, do the following:

- On the **View** menu, choose **Retention Times**, and click **Linear Regression** (Shift-F8).

Skyline will display a graph like this:

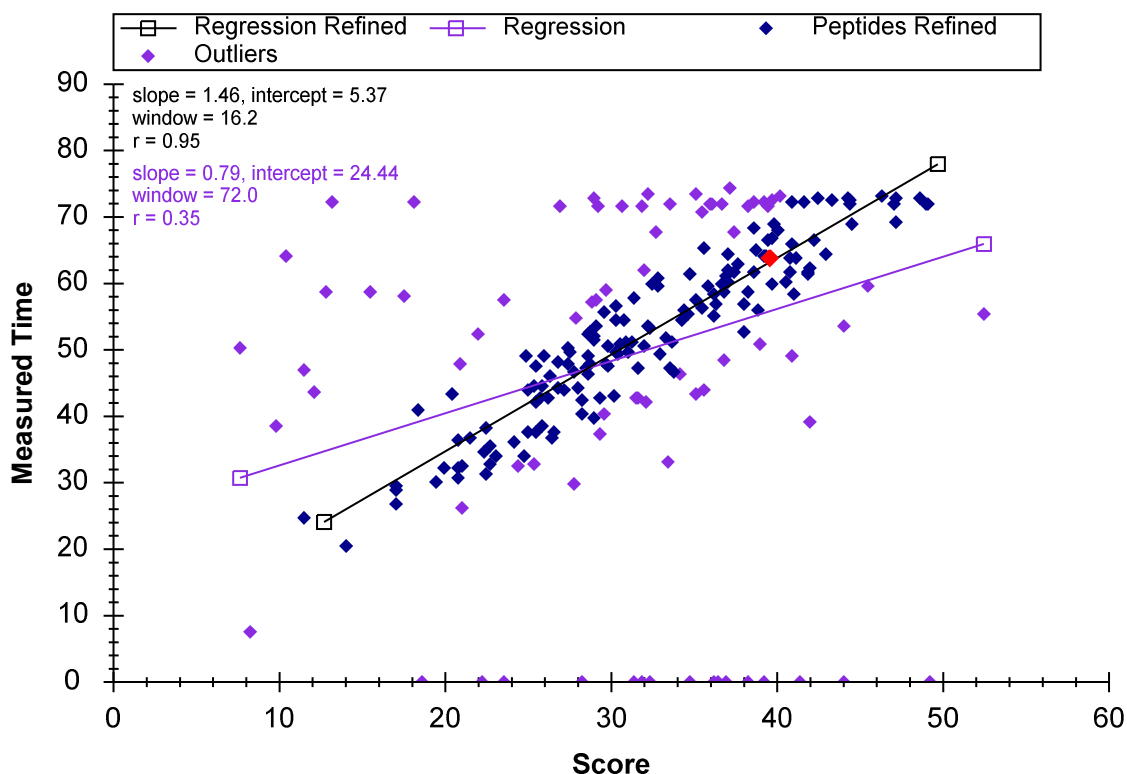


Notice the red point positioned on the current refined regression line. This point shows the SSRCalc score and measured time for the currently selected peptide. The highlighted point will change as you select different peptides in the Skyline document tree.

By default the graph uses a threshold of $r = 0.9$ for the residuals of the refined regression, removing points from the regression and labeling them outliers until the threshold is reached. You can adjust this threshold by doing the following:

- Right-click on the graph, and click **Set Threshold**.
- Enter '0.95' in the **Threshold** field.
- Click the **OK** button.

Skyline recalculates the regression, marking more peptides as outliers to change the graph to:



You can create a new linear equation for retention time prediction by doing the following:

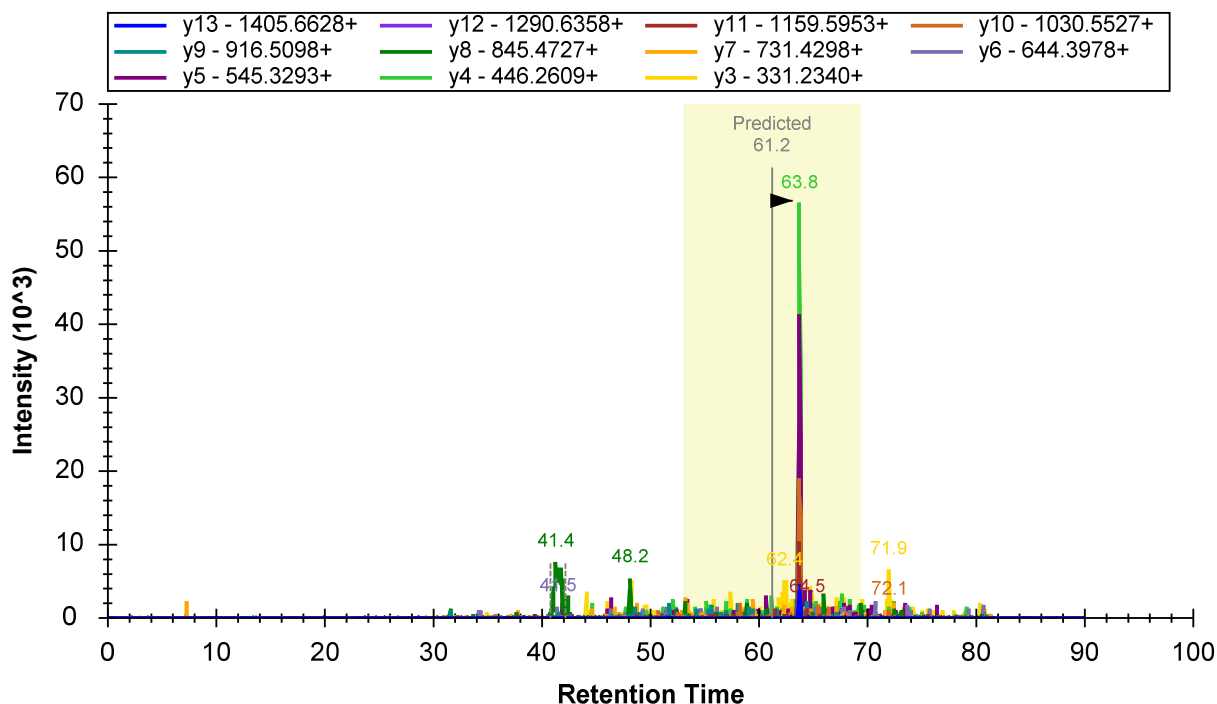
- Right-click on the graph, and click **Create Regression**.

Skyline presents the **Edit Retention Time Regression** form pre-populated with information from the retention time regression graph, including only the refined regression data (145 peptides), and the same slope, intercept and time window. The time window Skyline suggests is 4 standard deviations obtained from the residuals of the regression, which should contain about 95% of the 145 peptides.

Skyline also picks the calculator that yields the best fit (r closest to 1.0) for the data. Currently the choice is just SSRCalc 3.0 trained on columns using reverse-phase packing material with either 100 or 300 Angstrom pore size. In the MacCoss lab, we use packing material with 90 Angstrom pore size, and SSRCalc 3.0 (100Å) usually provides the best fit.

To simply accept the values suggested by Skyline, click the **OK** button.

Skyline will add an indicator of the predicted retention time for the selected peptide to its chromatogram graph, as shown below (you may need to move the regression graph out of the way to see this):



The shaded rectangle around this indicator shows the window (16.2 minutes) you chose in the **Edit Retention Time Regression** form. Anything outside the shaded rectangle is over 2 standard deviations from the predicted value.

Missing Data

Before leaving the retention time regression graph to return to manual refinement of this document, notice that many of the outlier points lie on the x-axis. This means Skyline found no peak at all for the peptide in question. To understand why, move the mouse cursor over the left-most point until the cursor changes to a hand, and then click the left mouse button.

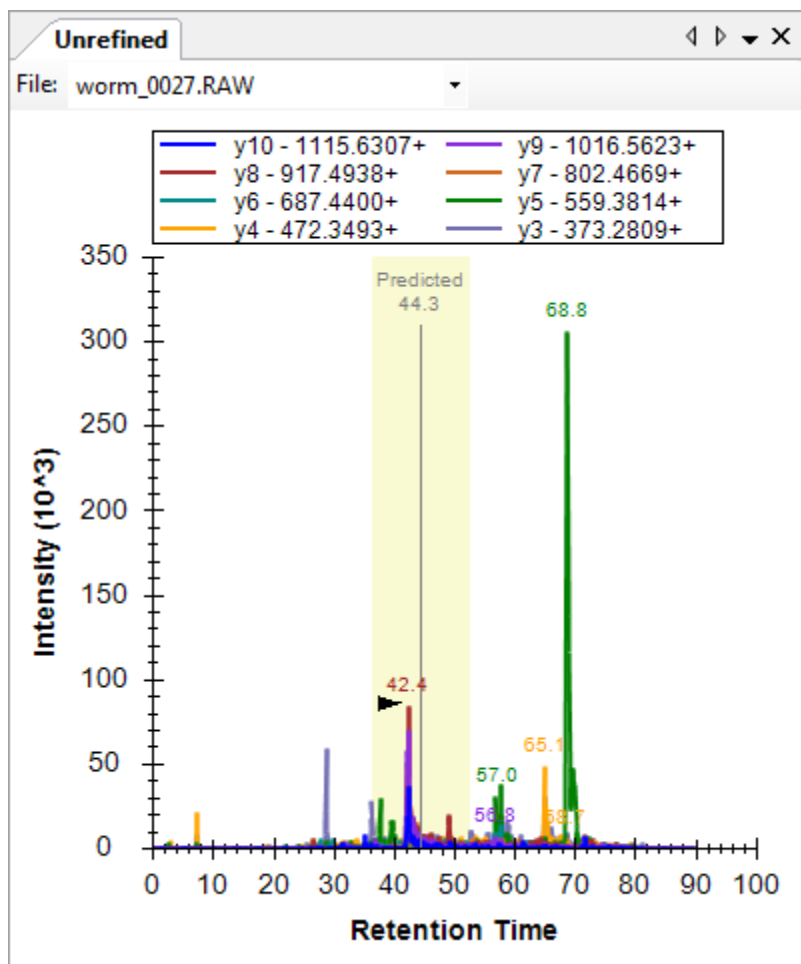
Skyline will highlight the point in red and scroll the peptide view to show the newly selected peptide (YLAEVASEDR). Press the Escape key to return to the peptide view, which should now look something like:

+	+	LIESNDTVWFPEGVTFTVK
+	+	SVYAHFPINVTLQDGNR
+	+	FLEQQNKVLETK (missed 1)
+	+	FSIQYGSNGYLGK
+	+	VTWDDQSVILK
+	+	LEYVGEGETQDTFFVVGK
+	+	LTMLDPLTLAK
+	+	TVQEAEVASLNR
+	+	EAQLLAEEADR
+	+	VKVEQELNDICQDVLK (missed 1)
+	+	YLAEVASEDR
+	+	GPILIAQLVAR
+	+	ARPEYMLPVNFYVK
+	+	YEELQITAGR
+	+	SQVTIEYESR

The absence of even a red peak quality icon indicates that no measurements were present for these 7 peptides in the RAW files imported into this document. That was certainly a surprise when we first imported the RAW files into this document. There are 39 transition lists and 39 RAW files. What happened?

With a little more exploration, Skyline makes the issue clear. Click on the peptide VTVVDDQSVILK above the peptides with missing data.

The chromatogram graph should now look something like:



Notice the toolbar that has been added at the top, with the **File** choice list. If you click on this list, it will show that both worm_0027.RAW and worm_0028.RAW contained measurements for this peptide.

Although there may be reasons in the future to measure a peptide twice in a single sample injection, at present a chromatogram graph showing the **File** list is a good indication that a mistake has been made somewhere. Either you have imported files measured as separate replicates into the same logical replicate in Skyline, or, as in this case, the sample list repeated a transition list for two output files and accidentally omitted another transition list. If you scroll the peptide view up, you can see that this happened another time for worm_0015.RAW and worm_0016.RAW.

You could delete these peptides without data now, but you can do this as part of a single refinement operation later in this tutorial. Now, press the Home key and close the retention time regression graph.

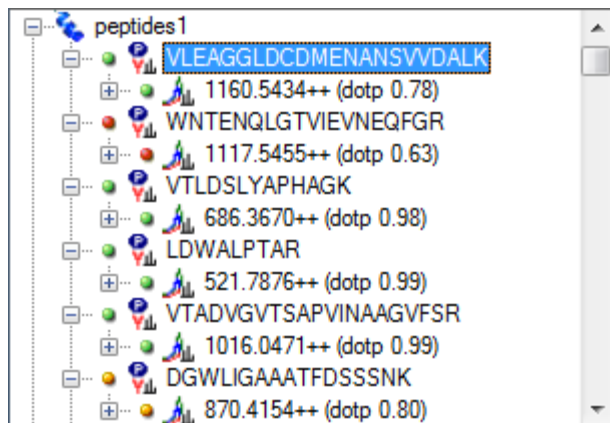
Picking Measurable Peptides and Transitions

Even if you may eventually use more powerful operations available in Skyline to refine your document, it is a good idea to understand how you might choose peptides and transitions individually, using the information Skyline provides. To prepare for initial manual review of this document, do the following:

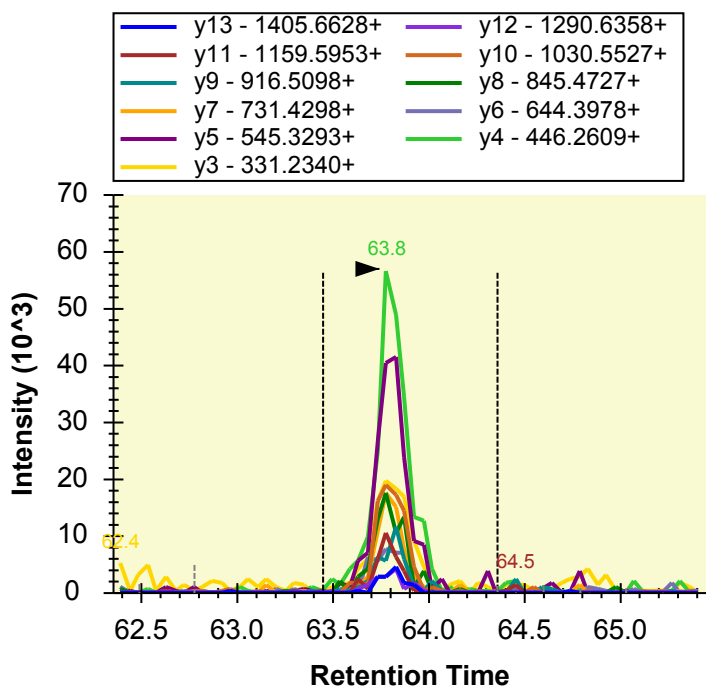
- Select the first peptide in the document.
- Press F11 to zoom in on the best peak in the chromatogram view.
- On the **Edit** menu, choose **Expand All**, and click **Peptides**.

This last operation exposes the 'dotp' value in the peptide view, which is a dot-product similarity metric^{4,5} between the measured SRM peak areas and the MS/MS library peak intensities. The closer this value is to 1.0, the better the match.

The peptide view should now look like this:

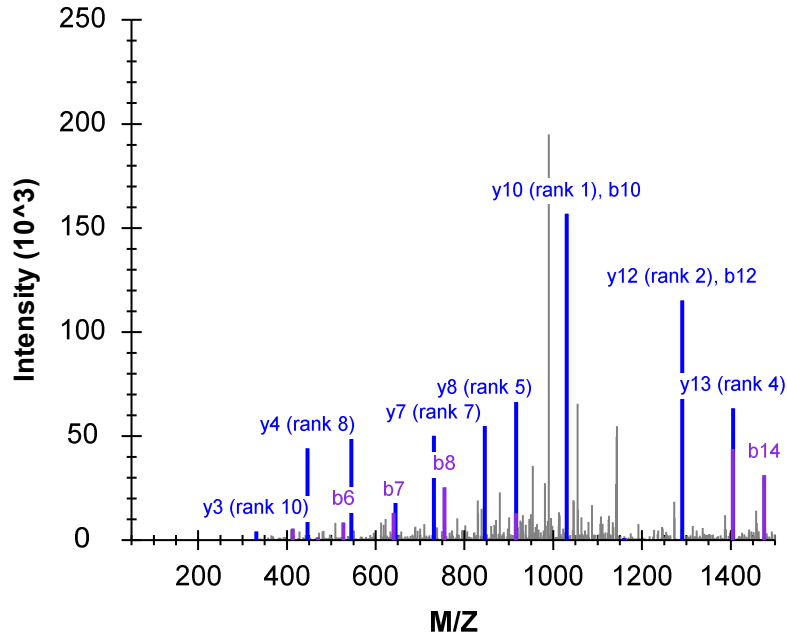


The dot-product (0.78) of the selected peptide is not that good, but all 11 y-ions measured have nicely co-eluting peaks:



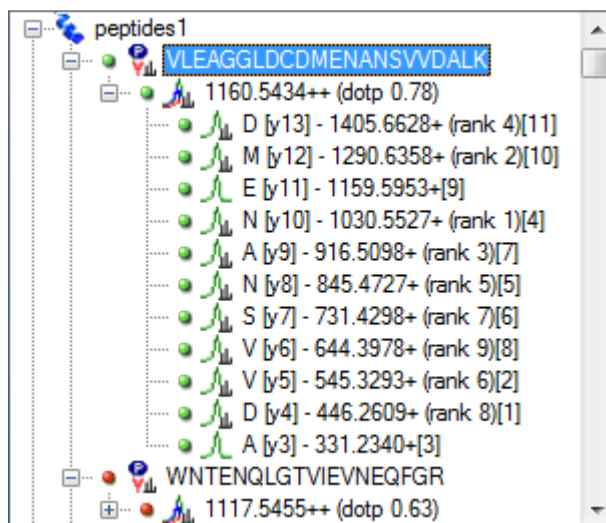
If you look at the MS/MS library spectrum graph, you can see an issue that might be causing the poor dot-product:

Worms - VLEAGGLDCDMENANSVVDALK, Charge 2



Notice that the two most intense peaks in the MS/MS library spectrum are annotated with both y- and b-ions (y10, b10 and y12, b12). The Thermo TSQ instrument, used for the SRM measurements, is known not to preserve b-ions well, which means the b10 and b12 components of these peaks will be absent in the SRM measurements.

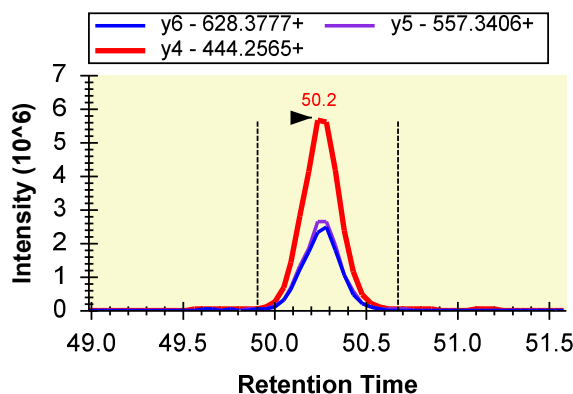
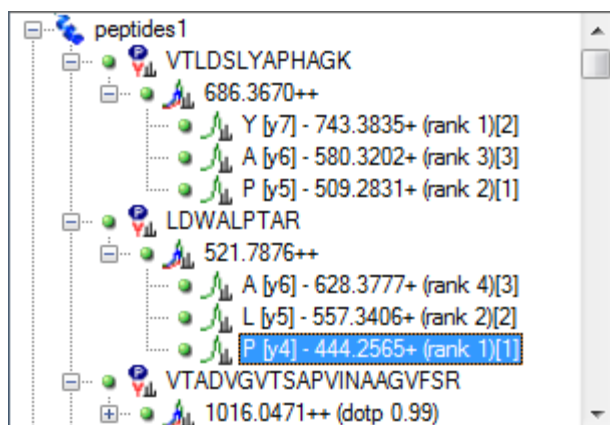
Expand the precursor 1160.5434++ to have a closer look at the transitions, and you will see:



The rank numbers on the left are the MS/MS library spectrum peak ranks, while the bracketed numbers to the far right are the SRM peak ranks. These numbers might help you sort out whether you believe the measurements on these transitions correspond to the appropriate peaks in the MS/MS library peptide identification. In this tutorial, however, just delete this peptide by pressing the Delete key.

The next peptide (WNTENQLGTVIEVNEQFGR) was clearly not measured, since its best peak group contains peaks for less than half of its transitions, producing a 0.63 dot-product. You can delete it also.

The next three peptides are very nice examples of candidates that meet the criteria for this level of refinement. All three peptides have peaks for all transitions, and they have dot-products of 0.98 or better. If you were looking to choose just 3 transitions for each peptide, the first peptide would be easy. Expand it, and delete all but the 3 most intense product ions, on which both the MS/MS library spectrum and SRM measurements agree. In the next peptide, note that the MS/MS library spectrum rank 3 and 4 peaks are nearly identical, and keep the three most intense SRM peaks. This should leave you with:



Looking at the next peptide and its transitions, you will see that y3 had the third largest peak area in SRM, but y13, with the fourth largest peak, was not much smaller. If you delete all but the four most intense peaks, you can press Shift-F11 to zoom out and see that neither the y3 or y13 transition encountered much noise or other features. A y13 ion, however, is generally more selective than a y3 ion (since it contains more of the complete peptide sequence), and in refinement you should be trying to produce the most selective method you possibly can. For this tutorial, keep y14, y13 and y11 for this peptide.

You could continue in this way, deleting the next two peptides and keeping the one after that, keeping the transitions with the highest signal, least noise and best selectivity. Or, you could use the Skyline refinement form to get a jump-start on this work.

Automated Refinement

The Skyline **Refine** form automates the most common refinement operations. The kind of refinement you have been doing manually so far in this tutorial can be performed in a single operation by doing the following:

- On the **Edit** menu, choose **Refine**, and click **Advanced**.
- Click the **Results** tab.
- Enter '3' in the **Max transition peak rank** field.
- Check the **Prefer larger product ions** checkbox.
- Choose **Remove nodes missing results**.
- Enter '0.95' in the **Target r value for linear regression** field.
- Enter '0.95' in the **Min dot-product** field.
- Click the **OK** button.

This will leave you with 72 peptides and 216 transitions, and they should all be pretty high quality. Take a moment to review them in the chromatogram graph by doing the following:

- On the **Edit** menu, choose **Collapse All**, and click **Peptides**.
- Press the Home key.
- Press the Down Arrow key until you have reached the last peptide.

This may be a little aggressive, however. To take a less aggressive approach that combines initial automated refinement with subsequent manual review, you can now perform the following additional steps:

- On the **Edit** menu, click **Undo** (Ctrl-Z).
- On the **Edit** menu, choose **Refine**, and click **Advanced**.
- Click the **Results** tab.
- Enter '6' in the **Max transition peak rank** field.
- Choose **Remove nodes missing results**.
- Enter '0.9' in the **Target r value for linear regression** field.
- Enter '0.9' in the **Min dot-product** field.
- Click the **OK** button.

This reduces the number of peptides to 110 and preserves enough transitions to keep the dot-product numbers useful in distinguishing peak quality. You can make the final refinement pass manually, taking into account factors the **Refine** form may still be missing.

Scheduling for Efficient Acquisition

The Skyline document you are editing was used in real experiments in the MacCoss lab in Spring 2009; you are following the same steps we took then. However, at that time, Skyline did not have the **Refine** form, nor was it capable of calculating dot products. At that time, therefore, we manually reduced the

list to 86 peptides for the next iteration of the refinement cycle. You can follow along with the choices we made in by doing the following:

- On the **Edit** menu, click **Undo** (Ctrl-Z).
- On the **Edit** menu, click **Manage Results**.
- Click the **Remove** button.
- Click the **OK** button.

This should remove all of the unrefined results and the chromatogram graph.

- On the **File** menu, choose **Import**, and click **Results**.
- Choose **Add multi-injection replicates in directories**.
- Click the **OK** button.
- Click the **OK** button in the **Browse For Folder** form.
- Click the **Do not remove** button in the form that appears to ask about removing the common prefix 'Unscheduled0'.

This will start Skyline importing 2 new unscheduled replicates from the 2 folders (Unscheduled01 and Unscheduled02) in the MethodRefine folder. Each folder contains 2 RAW files with the unscheduled chromatograms of 3 transitions for every measurable peptide remaining after the first refinement pass.

The current document still contains a lot of transitions that were not measured in these RAW files. To reduce the document to only the transitions measured, do the following:

- On the **Edit** menu, choose **Refine**, and click **Remove Missing Results**.

This will leave you with 86 peptides and 255 transitions.

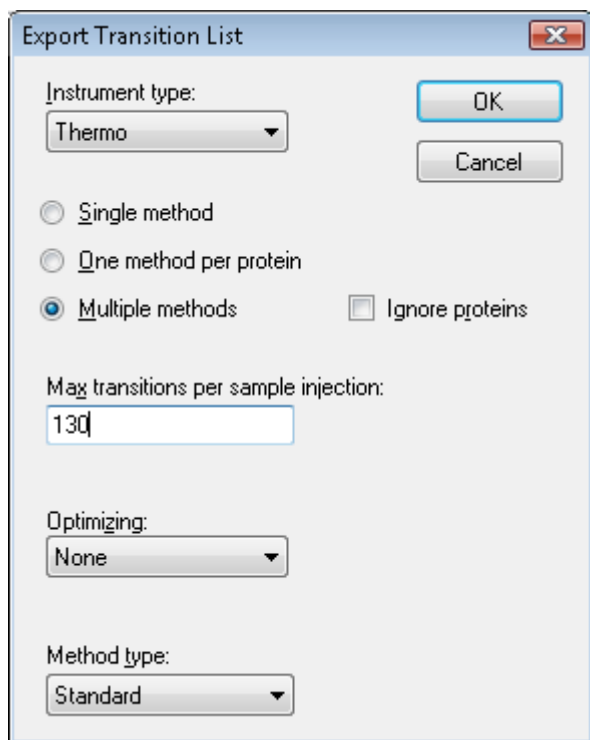
Measuring Retention Times

These peptides might have been better measured in 4 separate injections, rather than just 2. However, as the purpose of this next phase of refinement was to determine expected retention times for scheduling, we decided to allow longer cycle times and fewer points across the elution curves to reduce the number of injections required.

You can create transition lists similar to the ones we used to measure these peptides by doing the following:

- On the **File** menu, choose **Export**, and click **Transition List**.
- Enter '130' in the **Max transitions per sample injection** field.

The **Export Transition List** form should look like this:



- Click the **OK** button.
- Enter 'Unscheduled' in the **File name** field.
- Click the **Save** button.

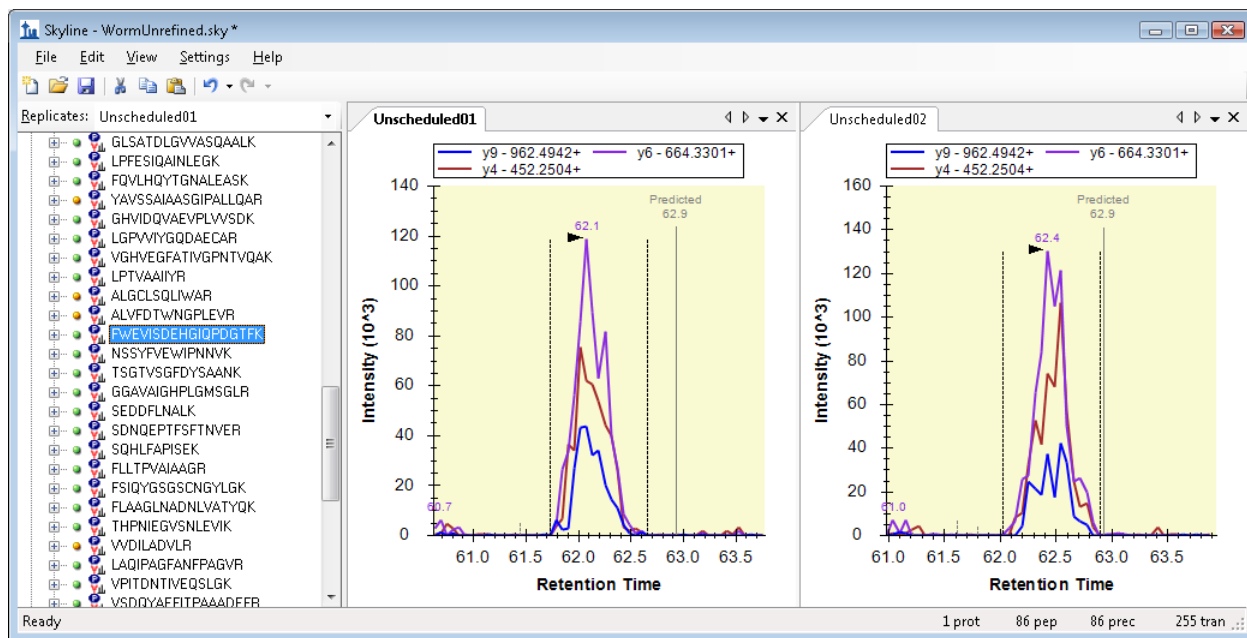
If you look in the MethodRefine folder, you will see two transition list CSV files (Unscheduled_0001.csv and Unscheduled_0002.csv). These could be used to collect new data like the files you just imported.

Reviewing Retention Time Runs

To review the unscheduled retention time runs you imported:

- Close the **MS/MS Spectrum** chart.
- On the **View** menu, choose **Arrange**, and click **Tiled** (Ctrl-T).
- Use the Down Arrow key to select peptides.

Skyline should be showing charts for both of the replicates you created, as shown below:

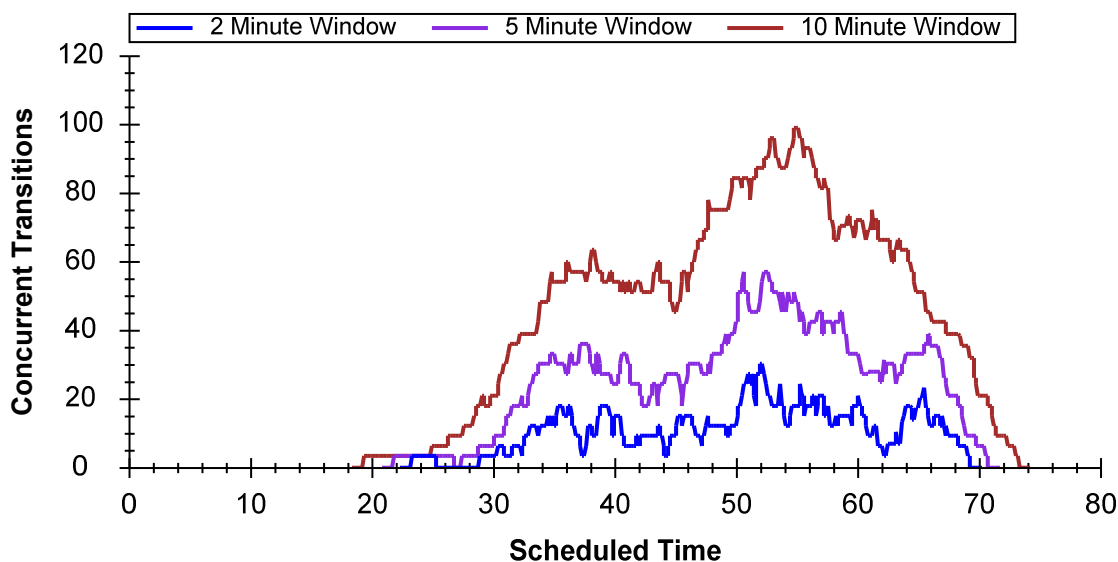


Press Shift-F11 to see the full range of the data acquired, and then press F11 to return to best peak zooming.

Given these measured retention times, you can get an overview of how Skyline might schedule these transitions by doing the following:

- On the **View** menu, choose **Retention Times**, and click **Scheduling**.

Skyline presents a graph like:



From this graph, you can see the number of concurrent transitions the instrument will measure over your full chromatography run using several possible time windows around the previously measured

retention times. The larger the time window, the more concurrent transitions you will see. For this document, a window of 5 minutes causes a maximum number of concurrently measured transitions of about 60. Depending on the speed of your instrument, it should be possible to measure all the remaining peptides in a single injection.

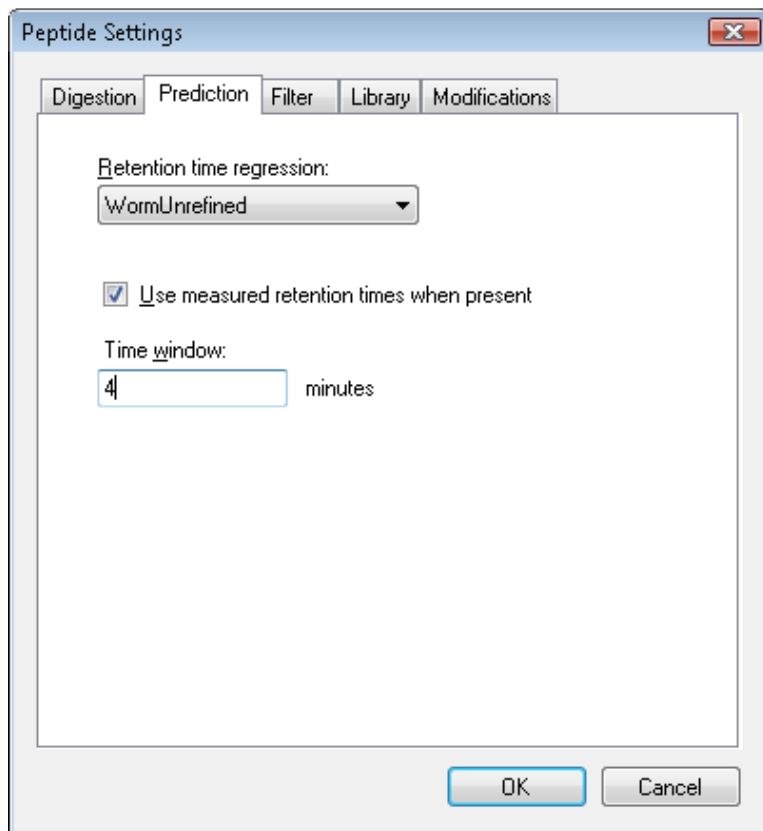
Creating a Scheduled Transition List

Choosing the time window you will actually use for your scheduled transition lists depends on reproducibility of your chromatography. If you schedule too narrow a window to account for the variance in the retention times of your peptides, you will see truncated or missing peaks. Before creating a scheduled transition list, make sure you have a good understanding of the variance in your chromatography.

We used a 4 minute window in this experiment to allow single-injection replicates without negatively impacting the dwell times or cycle time when the maximum number of transitions are measured concurrently. You can do the same by performing the following steps:

- Close the **Retention Times** view.
- On the **Settings** menu, click **Peptide Settings**.
- Click the **Prediction** tab.
- Enter '4' in the **Time window** field.

The **Peptide Settings** form should look like:



The screenshot shows a dialog box titled "Peptide Settings" with a standard Windows-style title bar (minimize, maximize, close buttons). Inside the dialog, there are five tabs: "Digestion", "Prediction", "Filter", "Library", and "Modifications". The "Prediction" tab is currently selected. Below the tabs, there is a section labeled "Retention time regression:" with a dropdown menu showing "WormUnrefined". Below this is a checkbox labeled "Use measured retention times when present" which is checked. Further down is a section labeled "Time window:" with a text input field containing the number "4" and the word "minutes" to its right. At the bottom of the dialog are two buttons: "OK" and "Cancel".

- Click the **OK** button.
- On the **File** menu, choose **Export**, and click **Transition List**.
- Choose **Single method**.
- Select 'Scheduled' in the **Method Type** list.

The **Export Transition List** form should look like:

- Click the **OK** button.
- On the **Scheduling Data** form that appears, choose **Use retention time average**.
- Click the **OK** button.
- Enter 'Scheduled' in the **File name** field.
- Click the **Save** button.

In the MethodRefine folder, you will find a new transition list file (Scheduled.csv) for scheduled SRM of the remaining peptides. If you look at the file in Excel, you can see that start and stop times, 4 minutes apart, have been added in columns D and E:

	A	B	C	D	E	F	G
1	686.367	743.3835	26.7	39.01	43.01	1	VTLDSLYAPHAGK
2	686.367	580.3202	26.7	39.01	43.01	1	VTLDSLYAPHAGK
3	686.367	509.2831	26.7	39.01	43.01	1	VTLDSLYAPHAGK
4	521.7876	628.3777	21.1	54.29	58.29	1	LDWALPTAR
5	521.7876	557.3406	21.1	54.29	58.29	1	LDWALPTAR
6	521.7876	444.2565	21.1	54.29	58.29	1	LDWALPTAR
7	1016.047	1389.748	37.9	62.81	66.81	1	VTADVGVTAPVINAAGVFSR
8	1016.047	1130.632	37.9	62.81	66.81	1	VTADVGVTAPVINAAGVFSR
9	618.8146	909.4676	24.4	29.32	33.32	1	VGGNGADYALATK
10	618.8146	781.409	24.4	29.32	33.32	1	VGGNGADYALATK
11	618.8146	319.1976	24.4	29.32	33.32	1	VGGNGADYALATK

Reviewing Multi-Replicate Data

You are now ready to begin another cycle of refinement of this SRM method. In the experiment you are following, we chose to begin running multiple technical replicates to become more familiar with the peptides we were measuring and any potential issues they might still have, such as:

- Peak area variance
- Retention time variance
- Potential interference

To review the data for the 5 injections we measured for this method, perform the following steps:

- On the **Edit** menu, click **Manage Results**.
- Click the **Remove All** button.
- Click the **OK** button.

This should remove the unscheduled replicates and their chromatogram graphs.

- On the **File** menu, choose **Import**, and click **Results**.
- Choose **Add single-injection replicates in files** (default).
- Click the **OK** button.
- Click on the file 'Scheduled_REP01.RAW'.
- Shift-click on the 'Scheduled_REP05.RAW' file, to select 5 files.
- Click the **Open** button.
- Edit the **Common prefix** field to contain 'Scheduled_' in the form that asks to remove the common prefix.
- Click the **Remove** button.

Skyline creates a tab for each of the 5 replicates and begins importing the data, displaying progress in the status bar at the bottom of the Skyline window.

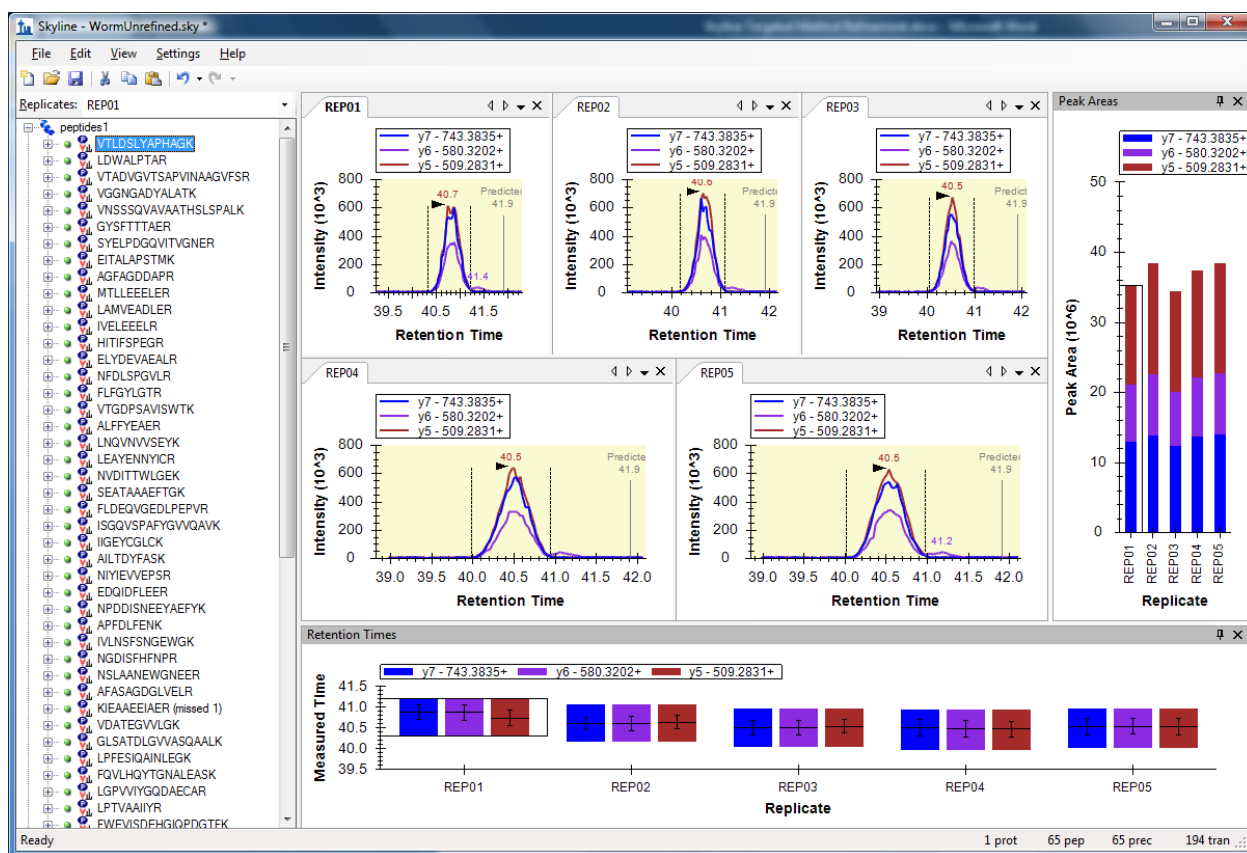
When the import is complete, you can see that some of the peptides measured in the unscheduled runs were removed for these scheduled runs. Again, you can reduce the document to just the peptides that were measured by doing the following:

- On the **Edit** menu, choose **Refine**, and click **Remove Missing Results**.

To prepare for reviewing these data, do the following:

- On the **View** menu, choose **Arrange Graphs**, and click **Tiled** (Ctrl-T).
- On the **View** menu, choose **Retention Times**, and click **Replicate Comparison** (F8).
- Drag the window that appears, position the cursor over the arrow at the bottom edge of the Skyline window, and release.
- On the **View** menu, choose **Peak Areas**, and click **Replicate Comparison** (F7).
- Drag the window that appears, position the cursor over the arrow at the right edge of the Skyline window, and release.

Adjust splitters and window sizing until the Skyline window looks like this:



You can now use the Down-Arrow key to review the data collected for these peptides. Other tutorials give more attention to analysis of multi-replicate refined SRM methods.

Conclusion

In this tutorial, you have been introduced to the powerful tools available in Skyline for creating new targeted proteomics methods and refining them for use in multi-replicate quantitative studies. You have traversed the Targeted Method Refinement Cycle over 2 iterations, and you should be able to use this process to begin exploration of your own proteomics hypotheses. Subsequent refinements might include optimizing instrument parameters like collision energy (CE), introducing synthetic labeled internal standard peptides for improved quantitative accuracy, or running calibration curves. Look for other Skyline tutorials and other Skyline features to help you make the most of your targeted proteomics experiments.

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