

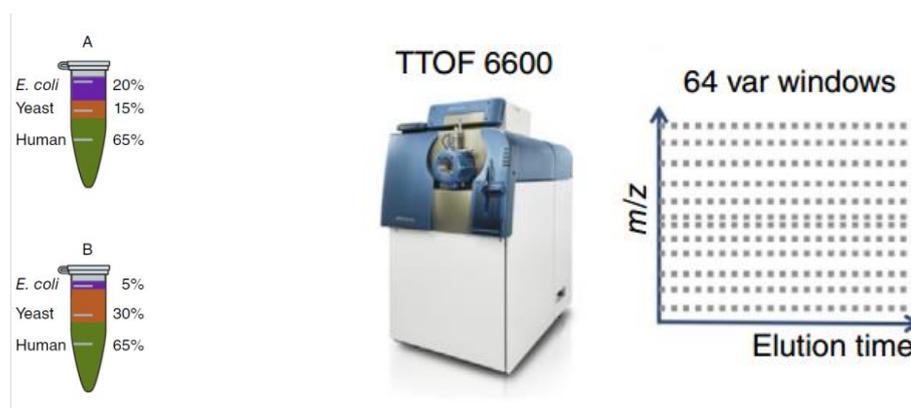
# Analysis of DIA/SWATH data in Skyline

Note: This tutorial uses data from a Sciex TripleTOF 6600 instrument. If you prefer to use the data from a Thermo Q-Exactive Plus, see the version of this tutorial entitled Skyline DIA QE.

In this tutorial you will learn how to use Skyline to perform targeted post-acquisition analysis for peptide and inferred protein detection and quantification using a SWATH-MS dataset acquired on a QqTOF instrument (6600 TripleTOF, Sciex) using an 64 variable width window precursor isolation scheme and a 1 hour gradient.

The data are from samples replicating the [LFQBench study](#) where quantitative benchmarking samples were created by mixing proteomes of 3 organisms in defined ratios (figure).

Initially, you will set all the parameters in the Skyline session required to work with data-independent datasets and then you will proceed to extract the quantification information from the raw data files. You will tell Skyline to deconvolute the DIA spectra to pseudo-DDA spectra using the DIA-Umpire algorithm, then search those spectra with MS Amanda to create a spectral library in order to generate peptide query parameters to analyze the DIA data.



[figure adapted from Navarro, P. et al. A multicenter study benchmarks software tools for label-free proteome quantification. *Nature Biotech* 34, 1130–1136 (2016)]

## Getting Started

To start this tutorial, download the following ZIP file:

<https://skyline.ms/tutorials/DIA-TTOF.zip>

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

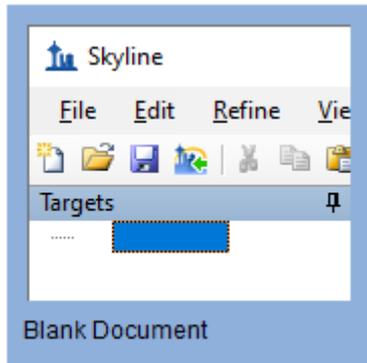
C:\Users\brendanx\Documents

This will create a new folder:

C:\Users\brendanx\Documents\DIA-TTOF

If you have been using Skyline prior to starting this tutorial, it's a good idea to revert Skyline to its default settings. To do so:

- Start Skyline.
- On the **Start Page**, click **Blank Document** which looks like this:

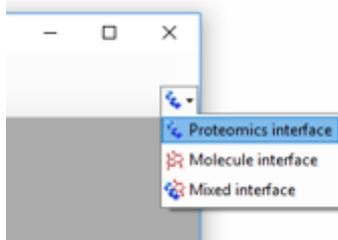


- On the **Settings** menu, click **Default**.
- Click **No** on the form asking to save the current settings.

The settings in your current instance of Skyline have now been reset to the default.

Since this tutorial covers a proteomics topic, ensure that the user interface is set to the “Proteomics interface”

- Click the user interface button in the upper right hand corner on the Skyline toolbar and select **Proteomics Interface** which looks like this:



Skyline is operating in Proteomics mode which is displayed by the protein icon  in the upper right-hand corner of Skyline.

For DIA analysis, you will want to force Skyline to integrate peak areas for all extracted quantitative transitions. To do that:

- On the **Settings** menu, click **Integrate all**.

## Using the Import Peptide Search wizard

If you know where to look, you could perform all of the following steps through various menu options on Skyline. To simplify the normal workflow for DIA, you will instead use a “wizard” which will step you through the critical choices required for Skyline to run the analysis. You will start by telling Skyline to deconvolute the DIA spectra to pseudo-DDA spectra using the DIA-Umpire algorithm, specifying various settings and the FASTA targets which you are interested in querying,

searching the DDA spectra with MS Amanda, and finally building a spectral library from the DDA search results. Skyline will then automatically extract chromatograms from the original DIA files.

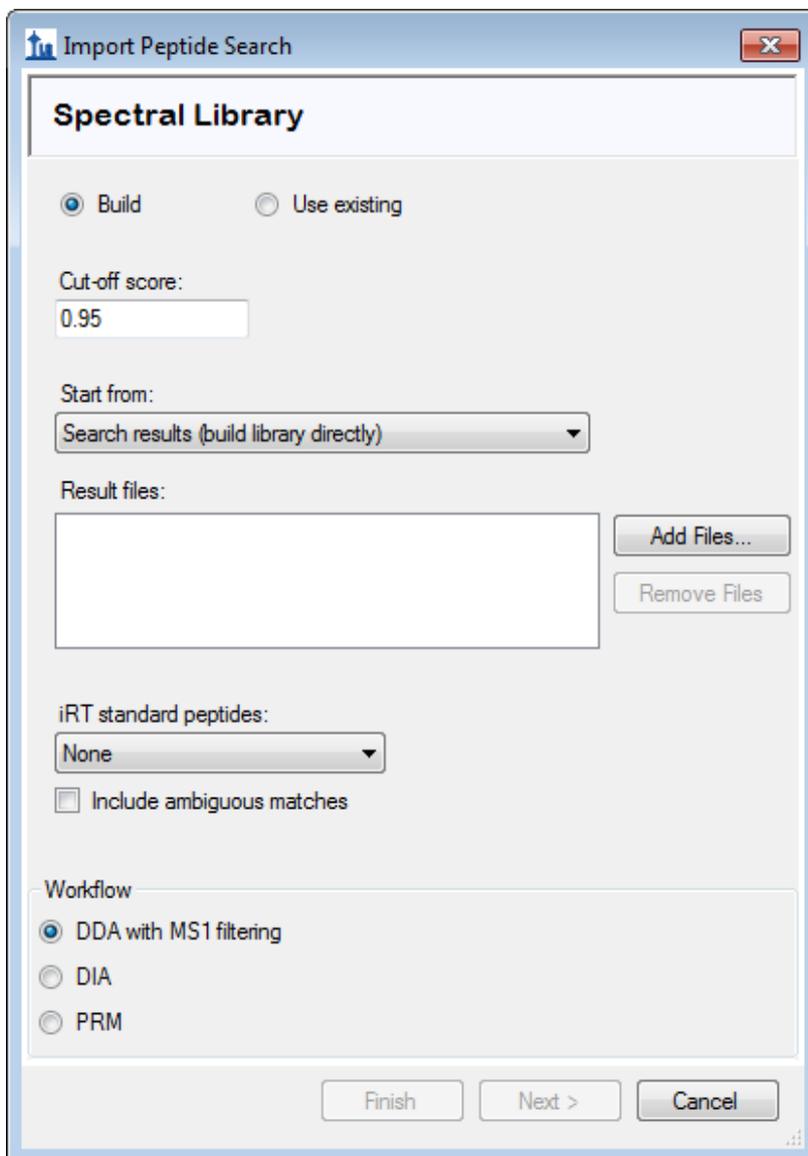
To get started do the following:

- On the **File** menu, choose **Import**, and click **Peptide Search**.

Skyline tells you that you need to save the current document so that it will know where you want to perform the analysis on your computer disk drive.

- Click the **OK** button.
- Navigate to the DIA-TTOF folder created earlier.
- In the **File name** field, type "DIA-TTOF-tutorial".
- Click the **Save** button.

The **Import Peptide Search** wizard should appear looking like this:



Note: 0.95 is the threshold applied to the MS Amanda probability computed for every peptide spectrum match in the DDA database search – in this particular data set this corresponds to a PSM

false discovery rate of 0.2% but this will differ among data sets so a score threshold to achieve the FDR you want to use should be entered here.

- Click the drop down list that says “Search results” and change it to “DIA raw”.
- Click the **Add Files** button.
- Navigate to the DIA-TTOF folder created earlier and into its DIA subfolder.
- Drag a selection box around the first 2 mzML files, “...001\_SW-A” and “...002\_SW-B”.
- In the **iRT standard peptides** dropdown list, click “Biognosys-11 (iRT-C18)”.
- In the **Workflow** box, click the **DIA** option.

The **Spectral Library** page of the wizard should now look like this:

**Import Peptide Search**

### Spectral Library

Build  Use existing

Cut-off score:  
0.95

Start from:  
DIA raw (deconvolute, search, and build library)

Files to search:  
collinsb\_I180316\_001\_SW-A.mzML  
collinsb\_I180316\_002\_SW-B.mzML

Add Files...  
Remove Files

iRT standard peptides:  
Biognosys-11 (iRT-C18)

Include ambiguous matches

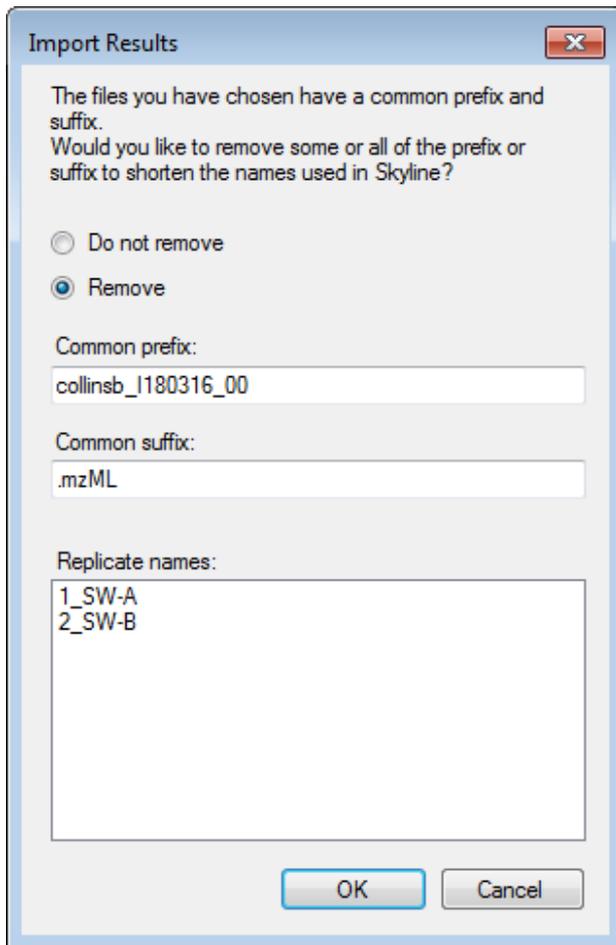
Workflow

DDA with MS1 filtering  
 DIA  
 PRM

Finish Next > Cancel

- Click the **Next** button.

The **Import Results** form should appear asking you how to handle the prefix and suffix shared by the two mzML files:

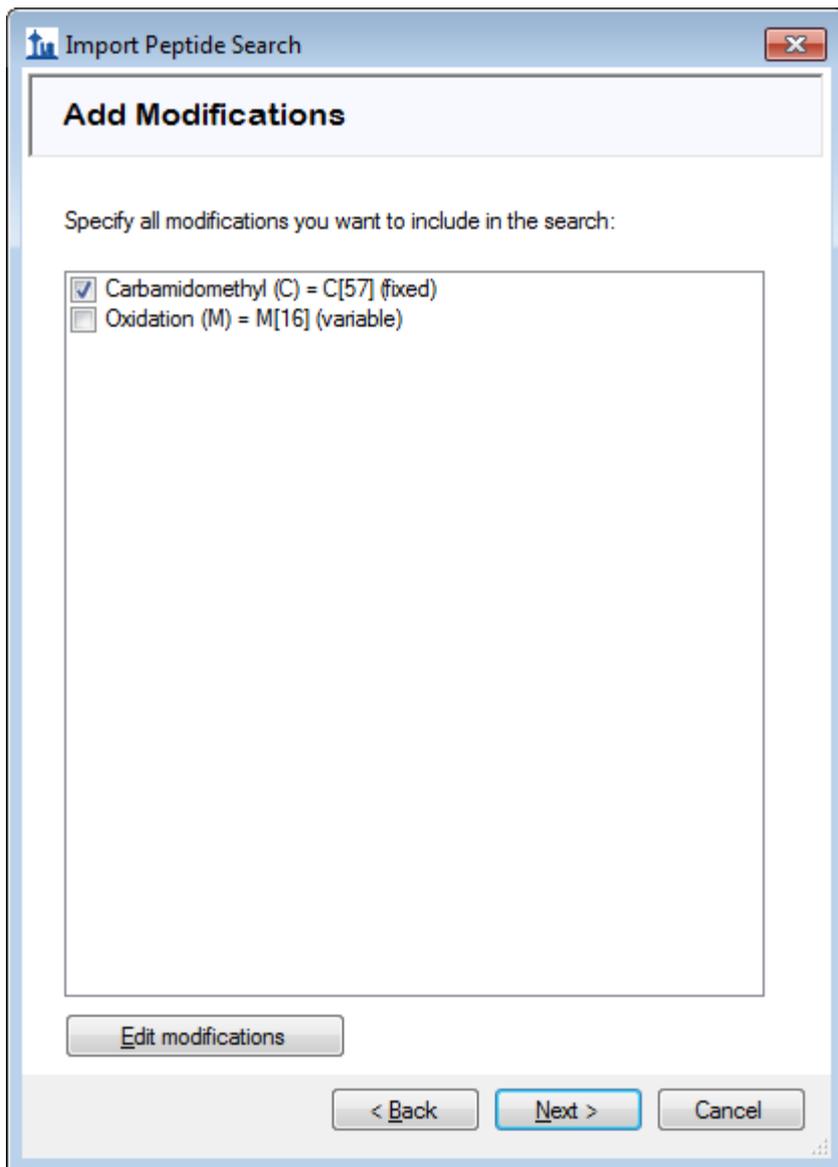


The screenshot shows a dialog box titled "Import Results" with a close button (X) in the top right corner. The text inside reads: "The files you have chosen have a common prefix and suffix. Would you like to remove some or all of the prefix or suffix to shorten the names used in Skyline?". There are two radio buttons: "Do not remove" (unselected) and "Remove" (selected). Below this, there are two text input fields: "Common prefix:" containing "collinsb\_1180316\_00" and "Common suffix:" containing ".mzML". At the bottom, there is a section labeled "Replicate names:" with a list box containing "1\_SW-A" and "2\_SW-B". At the very bottom of the dialog are "OK" and "Cancel" buttons.

- Click the **OK** button.

The wizard will advance to the **Add Modifications** page, where it lists all of the amino acid modifications in the document that you might want to include in the DDA search. It is important to distinguish here between fixed and variable modifications: fixed (sometimes called static) modifications are always applied to the specified amino acids. For example, Carbamidomethyl C is usually treated as a fixed modification because all cysteines in the data are expected to be alkylated. Oxidation M is almost always treated as a variable modification because oxidation is hit-or-miss depending on sample handling. Skyline's search always treats isotopic labels as variable, but you can change whether other modifications are treated as fixed or variable by clicking on the **Edit modifications** button.

You can also add modifications to the document from this page. Since this Skyline document was reset to defaults, the list starts with only Carbamidomethyl (C) and Oxidation (M):



- Make sure the checkboxes for both modifications are checked.
- Click the **Next** button.

The wizard will advance In the **Configure Transition Settings** page. Make the following changes from the default values:

- For **Precursor charges**, enter "2, 3, 4".
- For **Ion types**, enter "y, b".

NOTE: You could leave the ion type "p" which stands for precursor, and Skyline would extract chromatograms covering the precursor isotope distribution from the MS1 spectra in the DIA data files. However, to simplify this tutorial and reduce processing time and output file size, the description below deals only with fragment ion chromatograms extracted from the DIA MS/MS spectra.

The **Import Peptide Search** form should look like:

The screenshot shows a software window titled "Import Peptide Search" with a sub-header "Configure Transition Settings". The window contains several input fields and a checkbox:

- Precursor charges:** Input field containing "2, 3, 4".
- Ion charges:** Input field containing "1, 2".
- Ion types:** Input field containing "y, b".
- Product ions from:** Dropdown menu showing "ion 3".
- Product ions to:** Dropdown menu showing "last ion".
- Min m/z:** Input field containing "50" with "m/z" label.
- Max m/z:** Input field containing "2000" with "m/z" label.
- Use DIA precursor window for exclusion
- Ion match tolerance:** Input field containing "0.05" with "m/z" label.
- Pick:** Two input fields, both containing "6". The first is labeled "product ions" and the second is labeled "min product ions".

At the bottom of the window are three buttons: "< Back", "Next >", and "Cancel".

- Click the **Next** button.

Skyline presents the **Configure Full-Scan Settings** page.

**Note:** These extraction settings depend on the type and settings of the instrument used for data acquisition. The optimal extraction settings may be slightly different for each dataset. In this analysis you will use centroided data to save space. So you should select 'Centroided' and specify a '20' ppm mass accuracy for extraction. With profile mode data the resolving power of the instrument can be specified.

Make the following changes to the default values:

- In the **Mass accuracy** field, use "20" ppm.

Now you need to define a new isolation scheme according to the parameters defined on the instrument for data-independent acquisition.

**Note:** In this example, we used 64 variable width windows that covered the range from 400 to 1200  $m/z$  with 1  $m/z$  overlap to support 0.5  $m/z$  excluded margins at the edges of the quadrupole range.

- In the **Isolation scheme** dropdown list, click “<Add...>”.

This brings up the **Edit Isolation Scheme** form in which you should do the following.

- In the **Name** field, enter “ETH TTOF (64 variable)”.
- Click the **Prespecified isolation windows** option.
- Click the **Import** button.
- Navigate to the DIA-TTOF folder created earlier and into its DIA subfolder.
- Double-click the file “collinsb\_I180316\_001\_SW-A.mzML”.

The **Edit Isolation Scheme** form should now look like this:

**Edit Isolation Scheme**

Name: ETH TTOF (64 variable) [OK] [Cancel]

Use results data isolation targets

Isolation width: [ ] Deconvolution: [ ]

Prespecified isolation windows [Calculate...] [Import...] [Graph...]

Measurement [ ]

Start	End	Margin
399.5000	408.2000	0.5
407.2000	415.8000	0.5
414.8000	422.7000	0.5
421.7000	429.7000	0.5
428.7000	437.3000	0.5
436.3000	444.8000	0.5
443.8000	451.7000	0.5

Deconvolution: [None] [Specify Margin] [Specify CE Range]

Windows per scan: [ ]

Skyline has worked out the **Start** and **End**  $m/z$  values for the DIA isolation ranges measured on the mass spectrometer. You can see that they overlap by 1.0  $m/z$  to compensate for the overlap, Skyline has added a 0.5  $m/z$  margin to each side of the isolation windows, which it will exclude from consideration during chromatogram extraction. To see this in action:

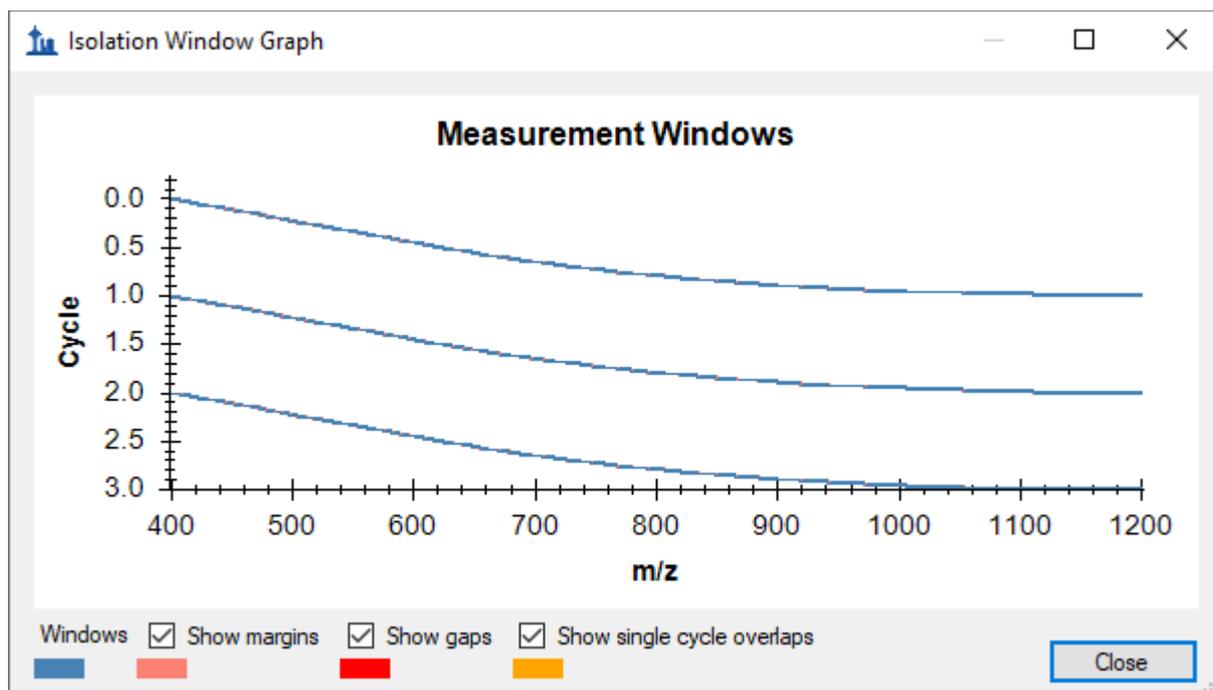
- In the dropdown list beneath **Prespecified isolation window**, click “Extraction”.

You will see the **Start** and **End** values shift inward by 0.5  $m/z$  and the ranges will no longer overlap. These are the ranges that Skyline will use when matching peptide precursor  $m/z$  values to acquired spectra for fragment ion chromatogram extraction.

To visualize the isolation scheme:

- Click the **Graph** button.

You will see a form like the one below:



You can click-and-drag a rectangle to zoom in or use the mouse scroll-wheel, and see the margins. If you uncheck and re-check **Show margins**, the graph title will switch between **Measurement Windows** and **Extraction Windows**, and the pink margin bars will disappear and re-appear. There is no red for **Show gaps** or yellow for **Show single cycle overlaps**, which are both errors DIA isolation scheme design.

- Click the **Close** button.
- Click the **OK** button in the **Edit Isolation Scheme** form.

The **Import Peptide Search** wizard should now look like this:

The screenshot shows a software window titled "Import Peptide Search" with a close button in the top right corner. The main content area is titled "Configure Full-Scan Settings" and is divided into three sections:

- MS1 filtering:** Contains four controls: "Isotope peaks included:" with a dropdown menu set to "None"; "Precursor mass analyzer:" with an empty dropdown menu; "Peaks:" with an empty text input field; and "Resolution:" with an empty text input field.
- MS/MS filtering:** Contains four controls: "Acquisition method:" with a dropdown menu set to "DIA"; "Product mass analyzer:" with a dropdown menu set to "Centroided"; "Isolation scheme:" with a dropdown menu set to "ETH TTOF (64 var"; and "Mass Accuracy:" with a text input field set to "20" followed by "ppm".
- Retention time filtering:** Contains three radio button options: "Use only scans within 5 minutes of MS/MS IDs" (which is selected), "Use only scans within 5 minutes of predicted RT", and "Include all matching scans".

At the bottom of the window, there are three buttons: "< Back", "Next >" (which is highlighted with a blue border), and "Cancel".

- Click the **Next** button.

You will see the **Import FASTA** page, which should look like:

**Import Peptide Search**

**Import FASTA (required)**

Enzyme: Trypsin [KR | P] Max missed cleavages: 0

FASTA records begin with '>' and have the protein name followed by the optional protein description.

Browse... Clear

Import target proteins from a separate FASTA

Decoy generation method: Decoys per target: 1

Automatically train mProphet model

< Back Next > Cancel

- Click the **Browse** button.
- Navigate to the DIA-TTOF folder created earlier and into its DDA\_search subfolder.
- Double-click on the “nodecoys\_3mixed\_human\_yeast\_ecoli\_20140403\_iRT.fasta” file.

The **Import target proteins from a separate FASTA** option tells Skyline that you want to import a (usually smaller) set of proteins as targets but run the MS Amanda search on a different FASTA (usually a full proteome). This is useful because doing a DDA search on a small FASTA introduces problems with estimating FDR.

NOTE: At this point, you could choose to perform a proteome-wide analysis by instead navigating to the DDA\_search subfolder and double-clicking the full FASTA sequence file “nodecoys\_3mixed\_human\_yeast\_ecoli\_20140403\_iRT.fasta” that will be used in the peptide search. This would produce quite a lot more targets and take more time to process, but is still feasible on most modern laptops.

- Check **Import target proteins from a separate FASTA**.
- Navigate to the DIA-TTOF folder created earlier and into its DIA subfolder.
- Double-click on the “target\_protein\_sequences.fasta” file.
- In the **Decoy generation method** dropdown list, click “Shuffle Sequence”.
- Check **Automatically train mProphet model**.

After setting these options, the page should look like:

**Import Peptide Search**

**Import FASTA (required)**

Enzyme: Trypsin [KR|P] Max missed cleavages: 0

FASTA records begin with '>' and have the protein name followed by the optional protein description.

\\napedro\_3mixed\_human\_yeast\_ecoli\_20140403\_iRT.fasta Browse...

Import target proteins from a separate FASTA

\\DIA-TTOF\DIA-TTOF\DIA\target\_protein\_sequences.fasta Browse...

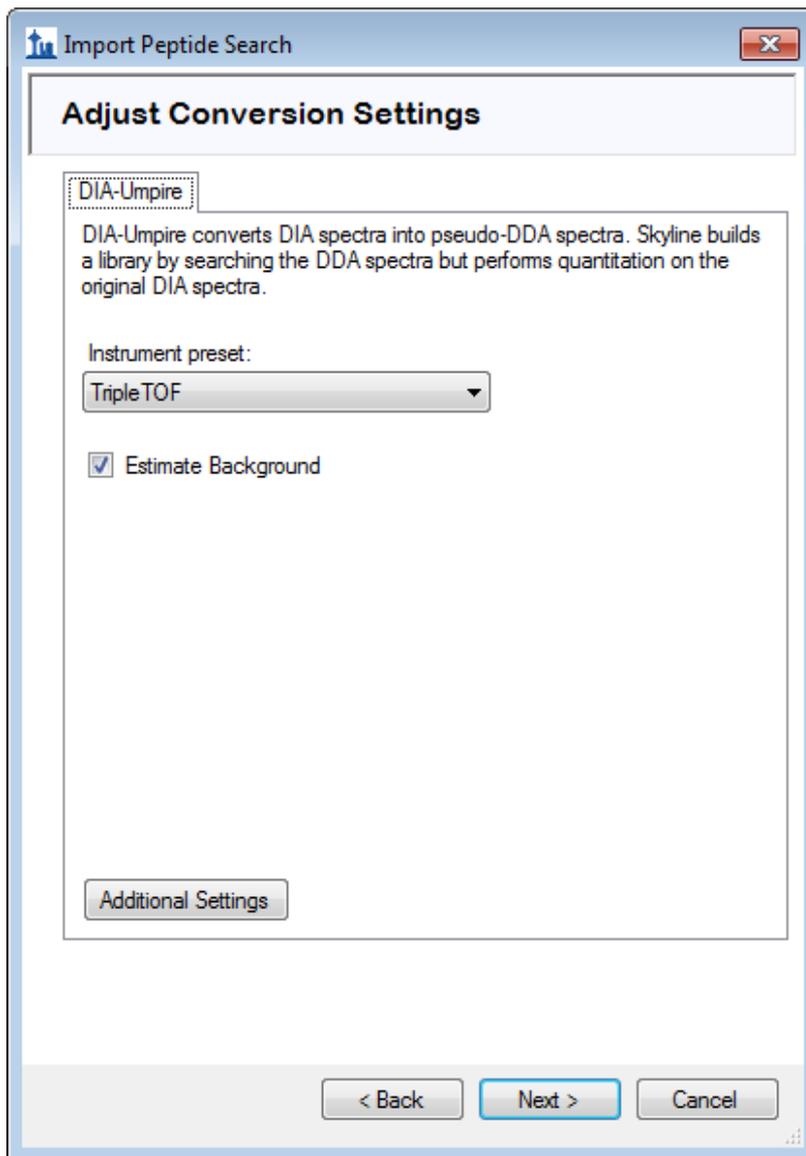
Decoy generation method: Shuffle Sequence Decoys per target: 1

Automatically train mProphet model

< Back Next > Cancel

- Click the **Next** button.

The wizard will advance to the **Adjust Conversion Settings** page. For this tutorial, this page will show DIA-Umpire settings:



The main setting here is “Instrument preset” which sets some established defaults for DIA-Umpire parameters according to what kind of instrument collected the data. All DIA-Umpire parameters can be viewed and changed by clicking on the “Additional Settings” button. The [DIA-Umpire manual](#) should help to understand what these settings do.

- Click **Estimate Background**, which will sacrifice a small number of peptides for a significant improvement in speed.
- Click the **Next** button.

The wizard will advance to the **Adjust Search Settings** page. Here you can set the most important parameters for the DDA search. For this tutorial, do the following:

- In the **MS1 tolerance** field, enter “5”. (Note that when you leave the text box the form will assume you mean ppm and set the unit box accordingly.)
- In the **MS2 tolerance** field, enter “10”.

The form should now look like this:

Import Peptide Search

### Adjust Search Settings

MS Amanda search settings:

MS<sub>1</sub> tolerance:  Unit:

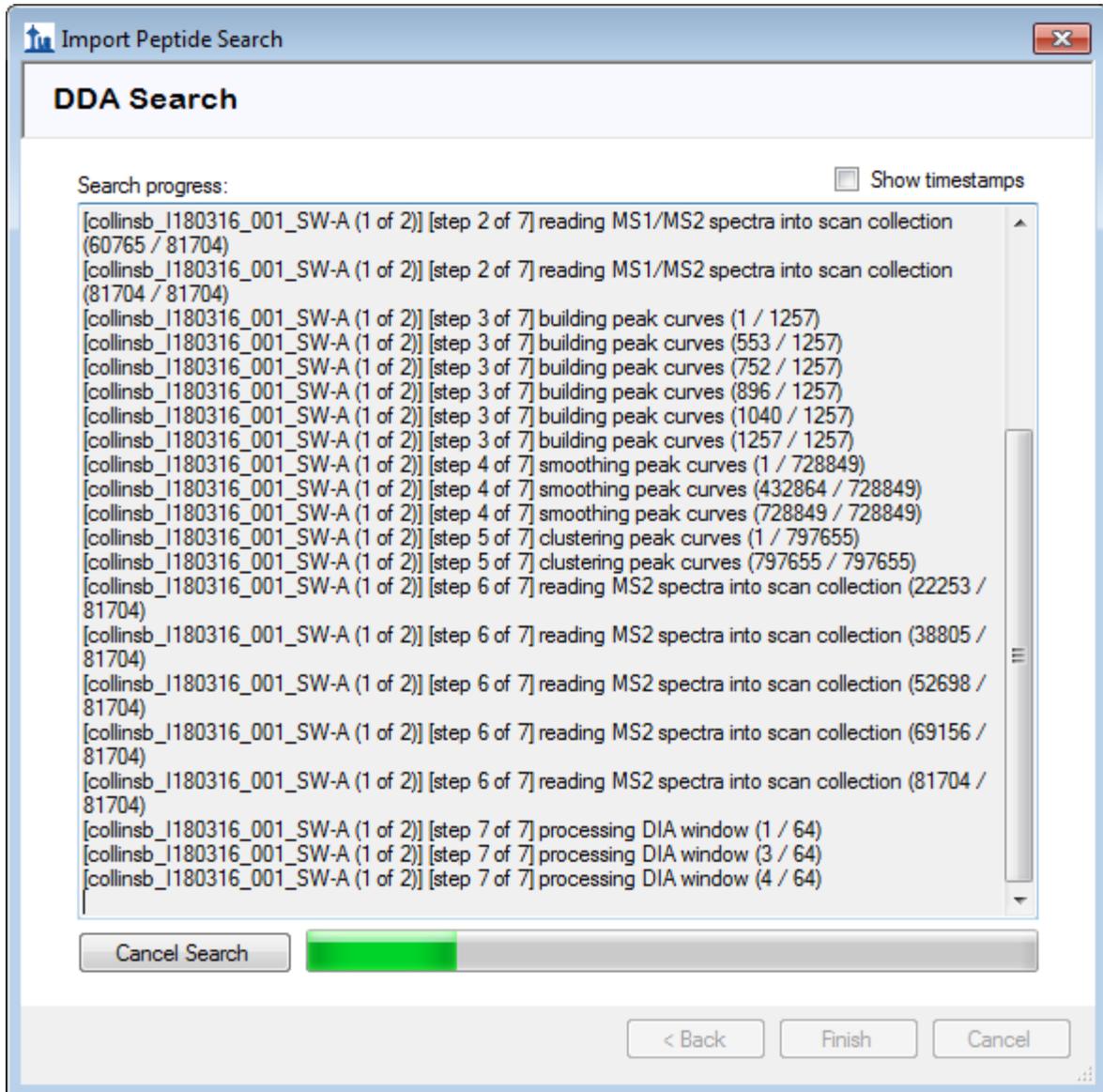
MS<sub>2</sub> tolerance:  Unit:

Fragment ions:

Max variable mods:

- Click the **Next** button to start the search.

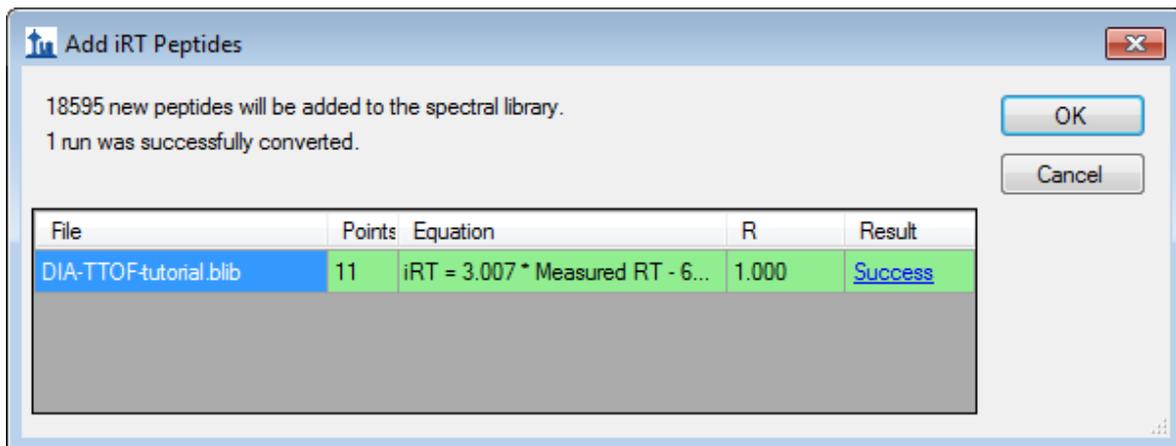
The **DDA Search** page will show you the progress of the DIA-Umpire deconvolution and MS Amanda search. You may also cancel the process here.



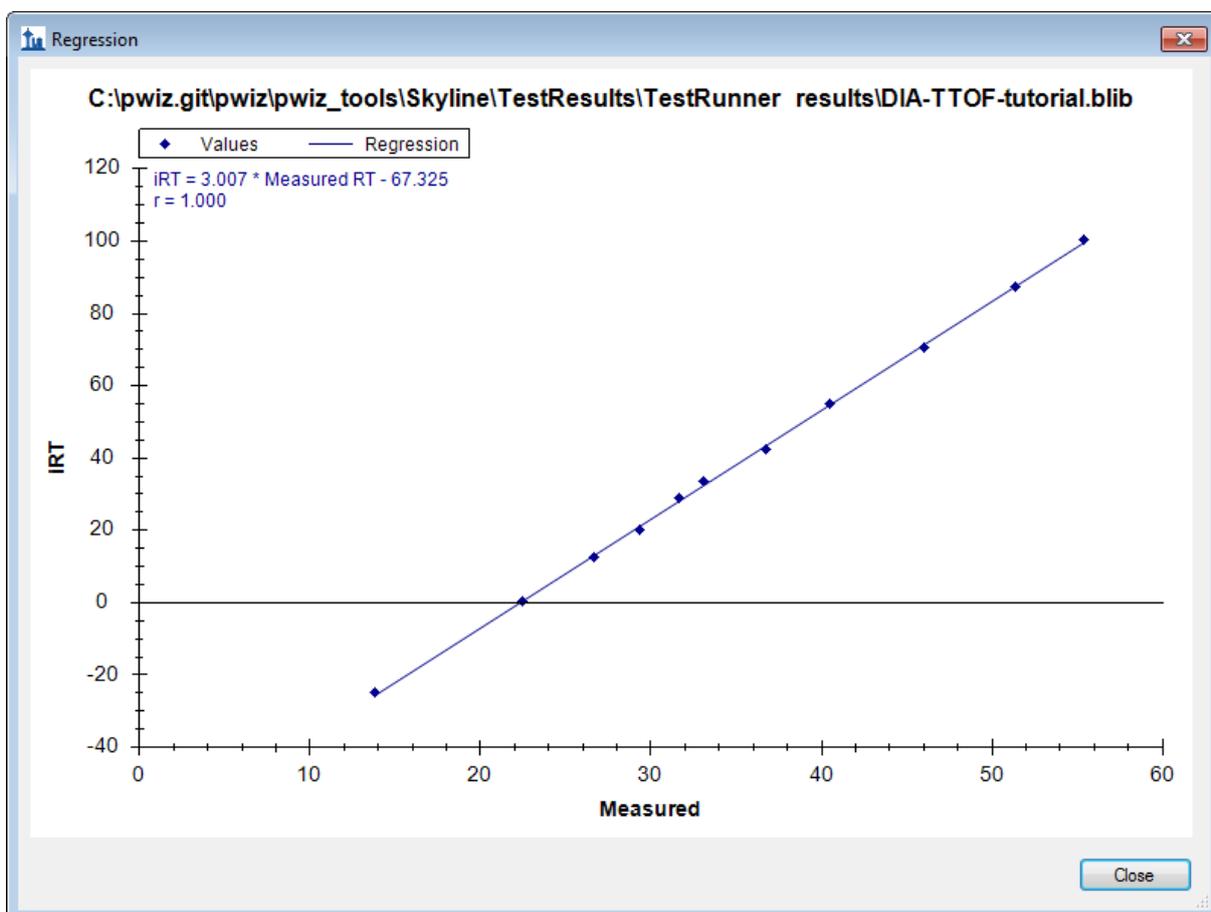
After the search is finished:

- Click the **Finish** button.

Skyline should begin building the library, showing progress in a form titled **Build Peptide Search Library**. When the library build is complete will attempt to calibrate iRT values from the peptide search results based on the Biognosys iRT standards which were spiked into these samples. This should succeed and present a summary form like the one below:



- Click the **Success** link (highlighted in blue) to see the linear regression.



- Click the **Close** button.
- Click the **OK** button.

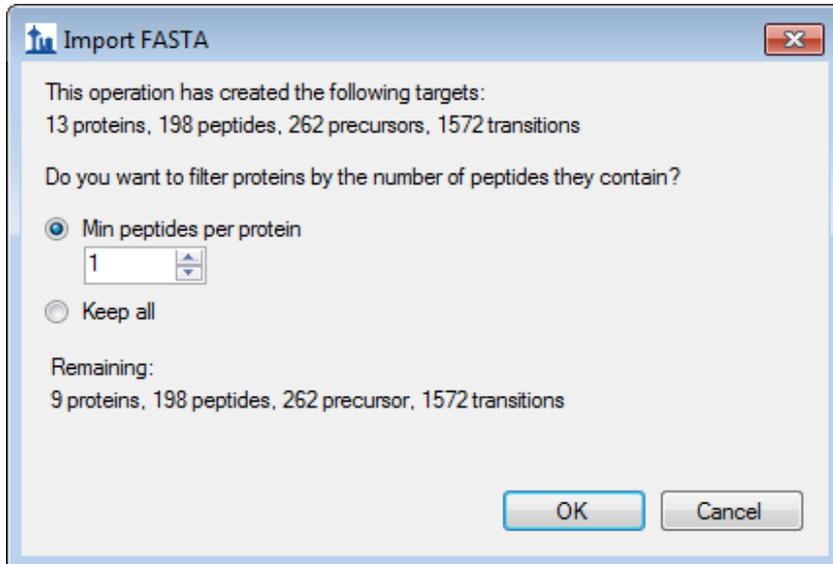
Skyline will ask if you want to recalibrate the iRT standard values onto the regression line calculated and shown in the graph above.

- Click the **No** button.

Skyline presents a long list of peptides with ambiguous matches. In other words, the only evidence for these peptides came from spectra which were matched to multiple peptides, making those spectra and matching peptides not ideal for subsequent targeted analysis.

- Click the **OK** button.

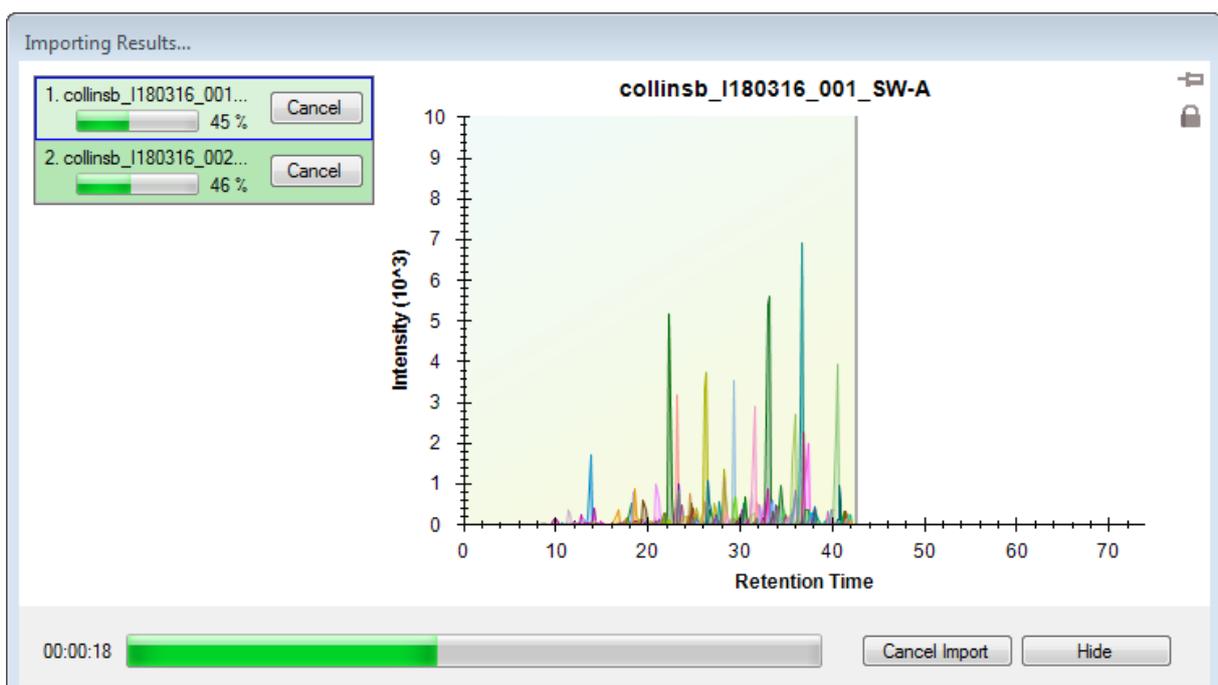
You should be presented with a form describing the targets calculated based on your settings and the FASTA sequence text provided that looks like this:



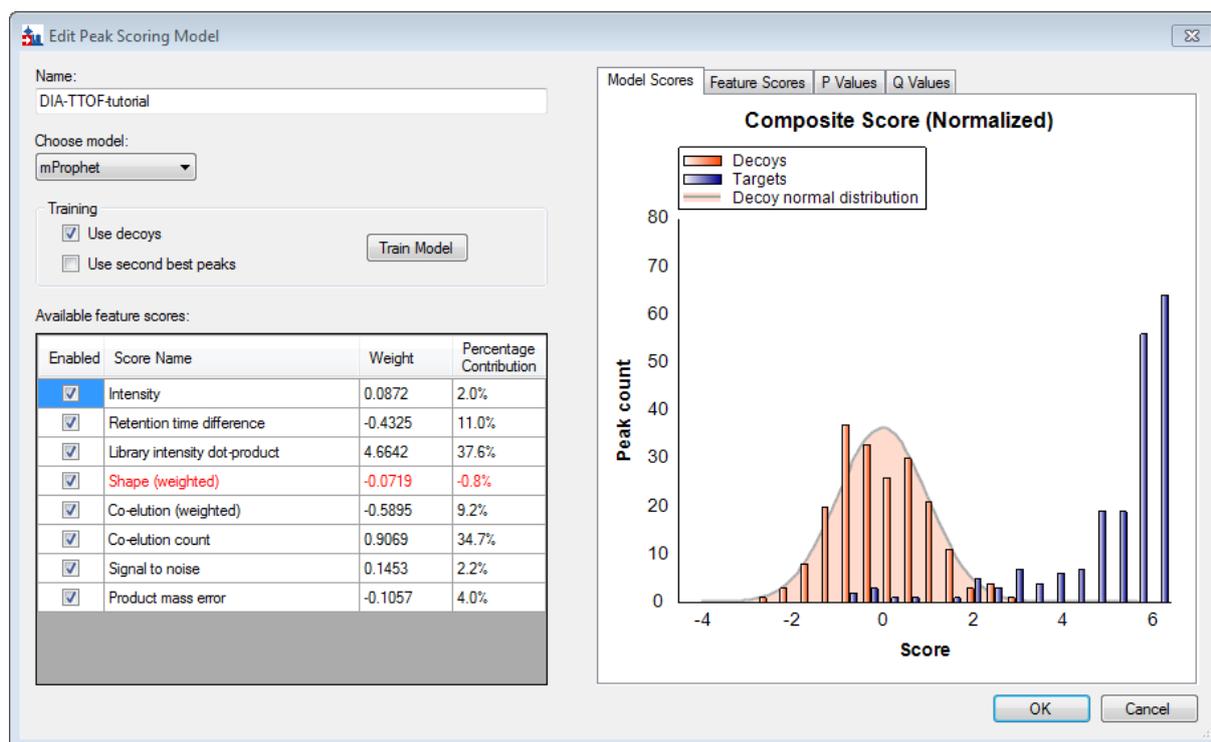
Notice that with a **Min peptides per protein** filter of “1” the 13 proteins found in the FASTA file produce 9 **Remaining** proteins. This is because four proteins have no peptides which are found in the spectral library. If you click **Keep all**, you will see a warning “4 empty proteins will be added”, but switch back before continuing to avoid adding the empty proteins to your targets list.

- Click the **OK** button.

Skyline begins extracting chromatograms:



After the import is completed, Skyline will show you the mProphet model you requested on the **Import FASTA** page of the **Import Peptide Search** wizard. It should look like this:



It has already been used to re-score the 10 best peaks Skyline found in each set of chromatograms, using its default score, and re-choose the peaks to be the ones with the best mProphet scores. These mProphet scores (given the name **Detection Z Score** in Skyline) are scaled such that 1.0 is 1 standard deviation from the mean mProphet score for the distribution of similar best peaks for the sequence shuffled decoy peptides you requested. Each has also been assigned a q value (given the name **Detection Q Value** in Skyline).

## Inspect the data manually

Next you will manually inspect some of the chromatography and underlying spectra. To do that, you will want the **Retention Times** and **Peak Areas** views are showing:

- On the **View** menu, choose **Retention Times**, and click **Replicate Comparison (F8)**.
- On the **View** menu, choose **Peak Areas**, and click **Replicate Comparison (F7)**.

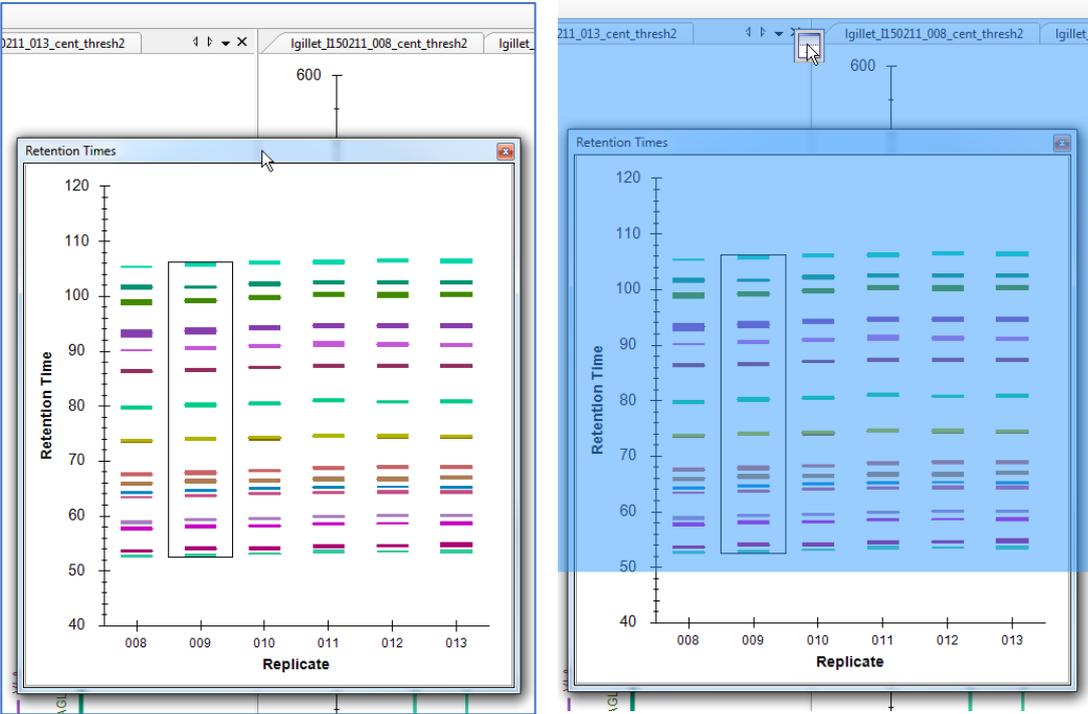
Now both views should be visible. You can configure them optimally on the screen by doing the following:

- If these new windows are floating you can dock them to the main window by clicking on the top border of the floating window, holding the left mouse button down, and dragging this window.

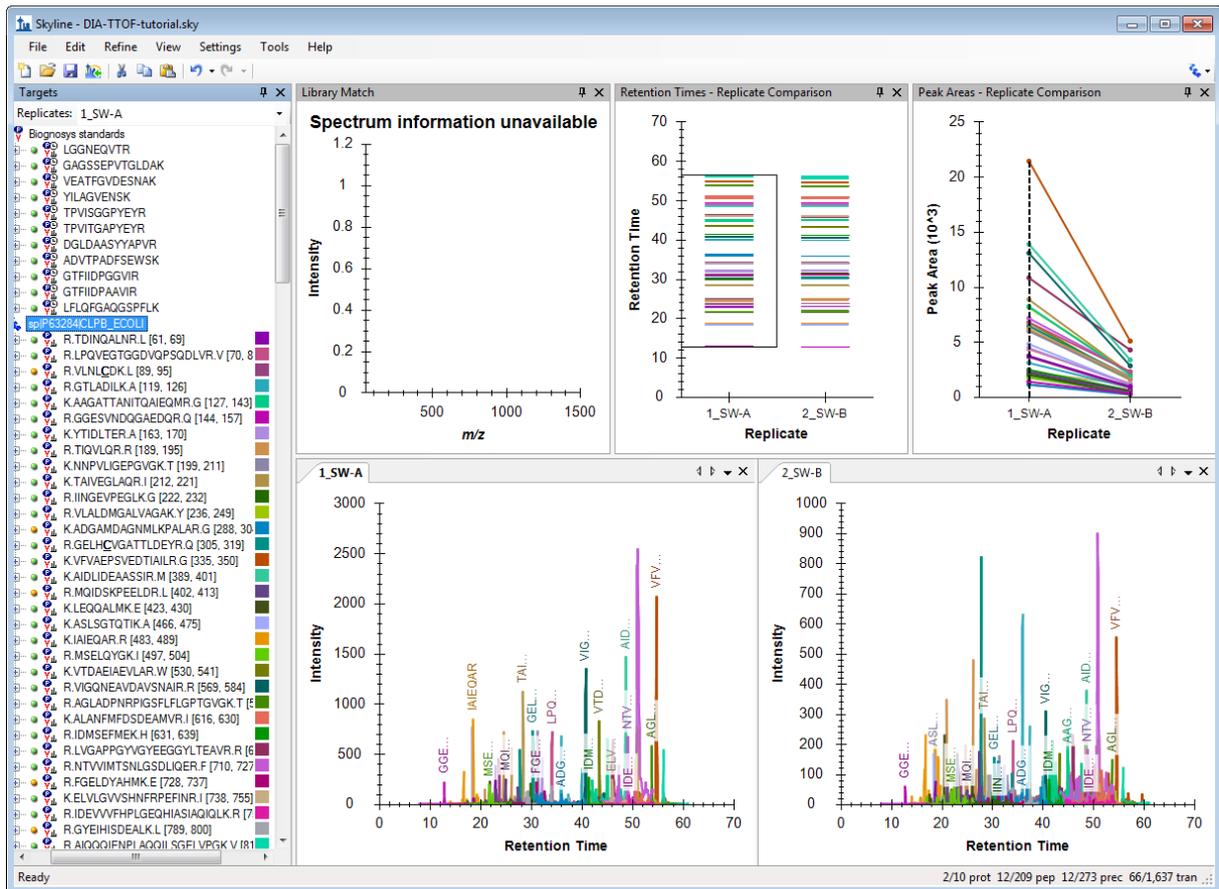
You can perform that same operations shown below with the **Library Match** window.

Note: In order to dock a window like this, the mouse arrow cursor must be placed inside one of the docking icons, in this case, at the top. Once you begin dragging with the left-mouse button held

down, Skyline will show the docking icons and a transparent blue rectangle where the window will be placed if you were to release the left-mouse button at that moment.



- Similarly, dock the **Peak Area: Replicate Comparison** and **Library Match** windows so that all information is easily viewable, as below:



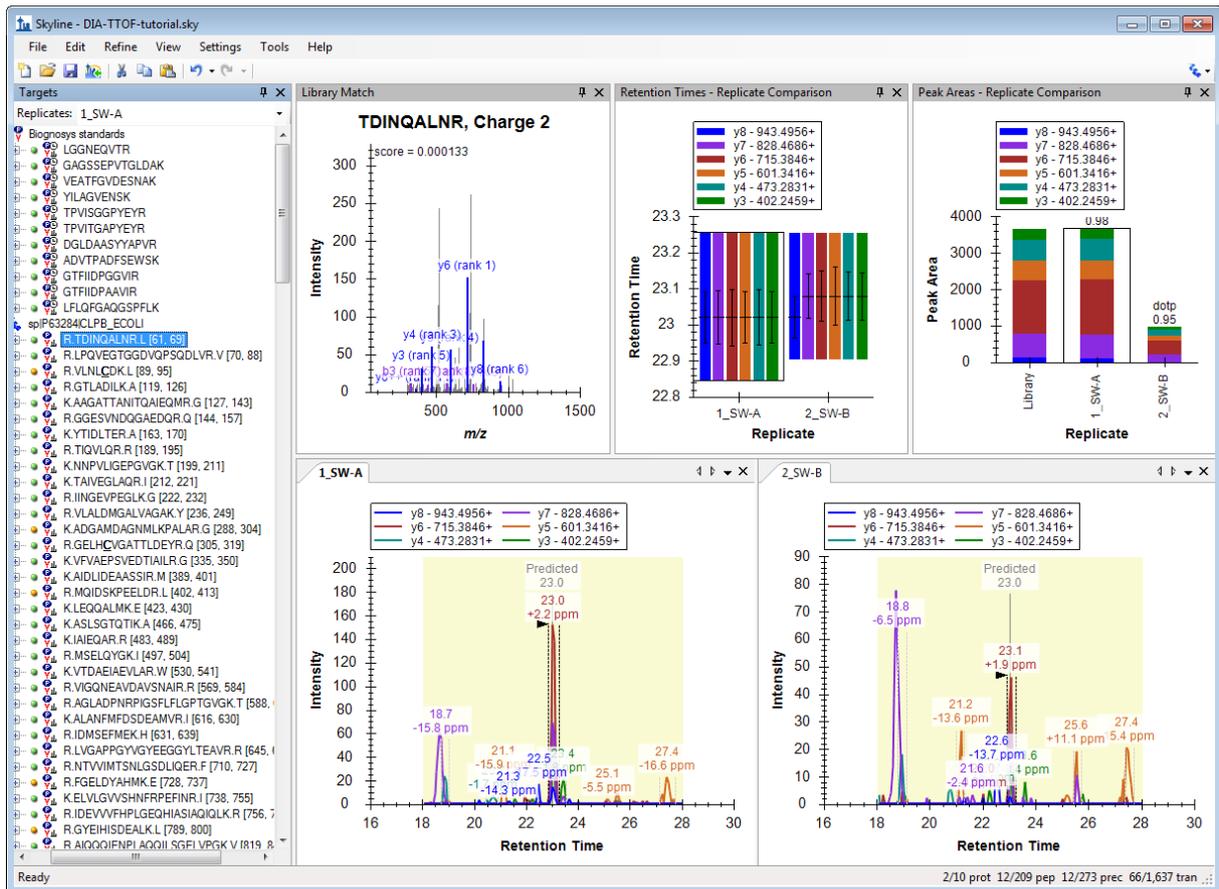
- Click on the first E. coli protein: “sp|P63284|CLPB\_ECOLI”.

You should see all of the peptides for this protein shown on the various plots (chromatogram, peak areas, and retention time replicate graphs). The screenshot above is an example of when one protein is selected and all of the peptides for this protein are summarized in each of these views (except library match window where nothing is shown).

Based on what you see in the **Peak Area - Replicate Comparison** plot, does this protein appear to be differentially regulated? Recall that the expected fold-change ratio between A:B is 4:1 for E. coli.

- Select the peptide TDINQALNR in this protein.

You get specific information for this peptide in all of the views, as shown below:



Examine the peak area patterns for the rest of the peptides belonging to this protein. Is the quantitative pattern for the peptides from this protein consistent with the expected differential regulation pattern (4:1)?

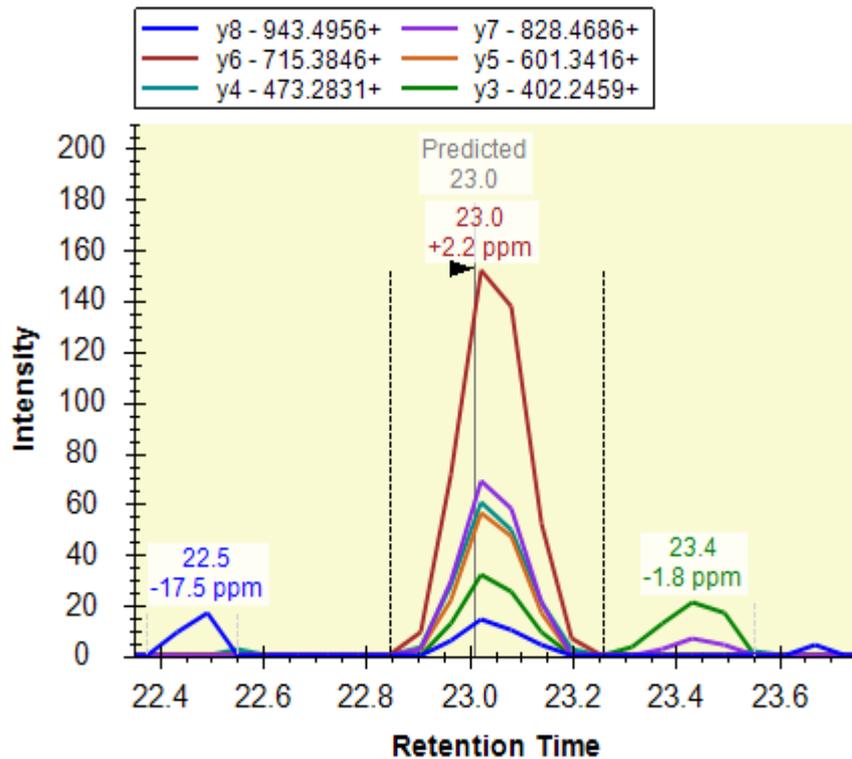
**NOTE:** If there is more than one precursor charge state for a given peptide sequence these are extracted and scored separately. You can look at these by clicking the + next to the peptide sequence and clicking on the individual charge states.

- Click on some of the human proteins in the document.

Examine the replicate peak areas from the protein level view and the peptide level view. Are the peak areas consistent with the expected ratio (1:1)? What about the yeast proteins (1:2)?

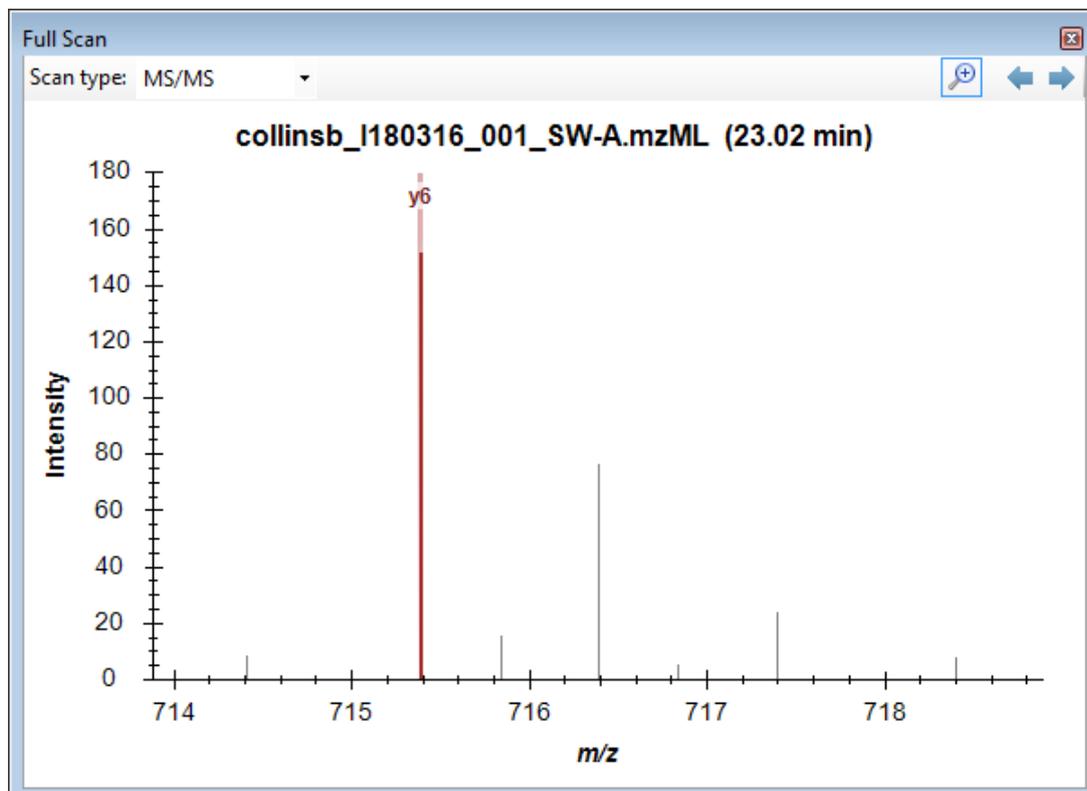
- Click again on the TDINQALNR peptide in the first protein again.
- On the **Views** menu, choose **Auto-Zoom**, and click **Best Peak (F11)**.

Notice that if you hover the cursor over a chromatogram curve a circle appears on the curve with the same color as the curve.

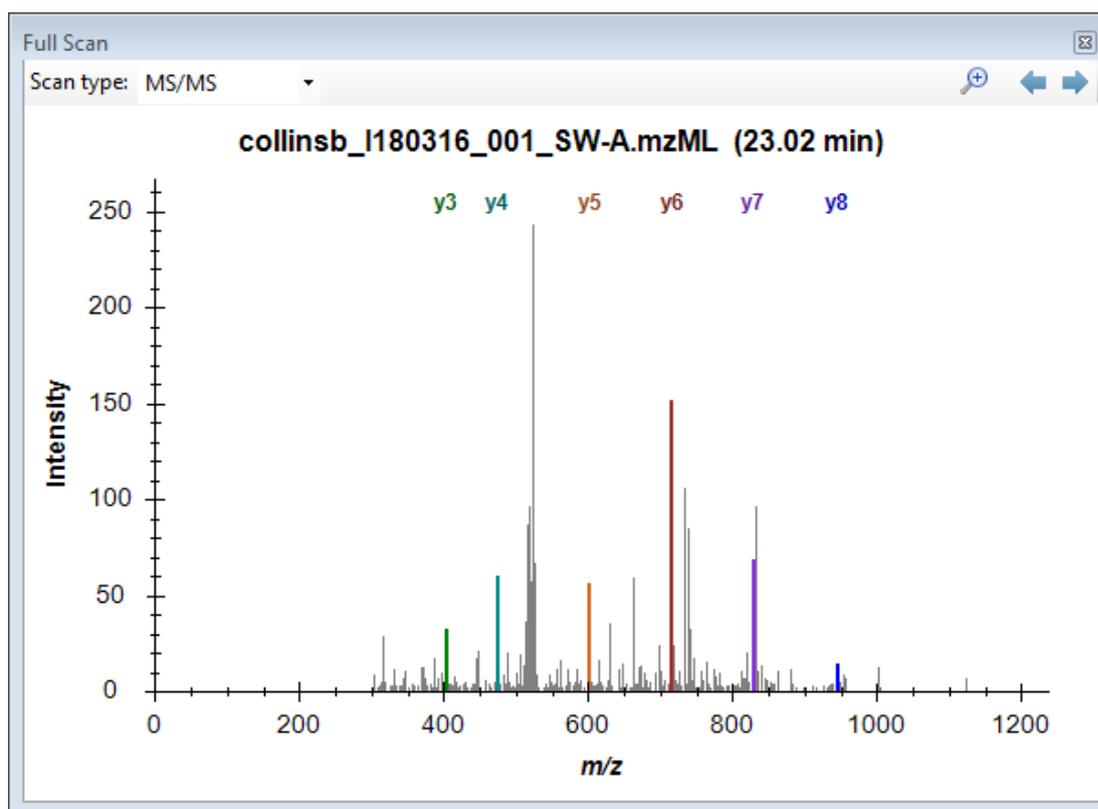


- Click the circle on a chromatogram point.

Skyline will open the **Full-Scan** view on the spectrum from which the chromatogram point was extracted zoomed on the ion that you selected.



- Click the magnifying glass button in the **Full-Scan** toolbar to view the full spectrum.



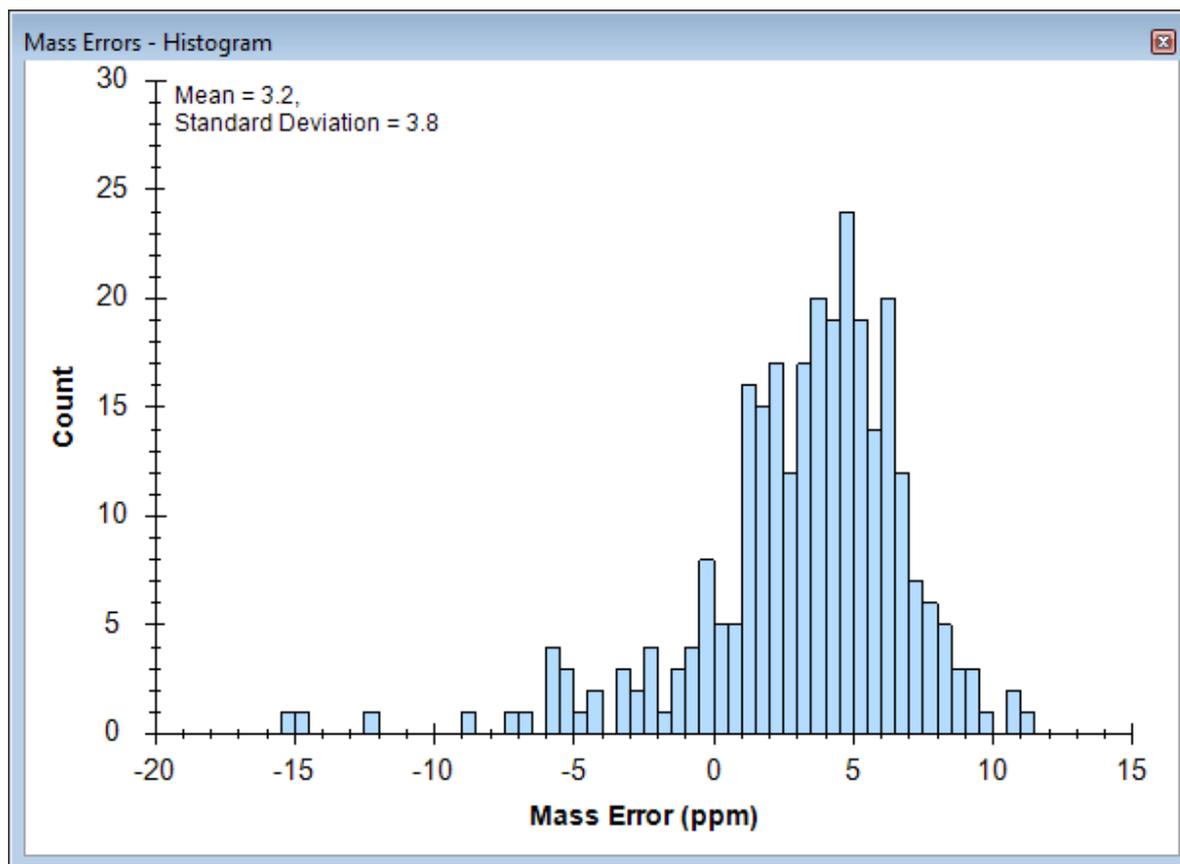
Explore the data further manually (including some decoys).

## Mass error and retention time deviation

You can examine the mass accuracy and retention time prediction accuracy to determine whether the optimal extraction parameters have been used or whether some adjustment may improve the results.

- On the **View** menu, choose **Mass Errors**, and click **Histogram**.

This will show the distribution of mass errors over the data set. Could the extraction window ( $\pm 20$  ppm) have been further optimized? Generally the mean error  $\pm 3$  standard deviations is sufficient. If the histogram appears cut off, however, with high counts at the extremes then you may want to widen your extraction windows.



Note that the distribution appears to have two peaks, one around 0 and one around 5. To understand this better, it helps to know that these are the mass error values across all 6 runs.

- Right-click the graph, choose **Replicates**, and click **Single**.
- Click in the **Replicates** dropdown list at the top of the **Targets** view.
- Use the up and down arrow keys on your keyboard to review the mass error values in all 6 replicates.

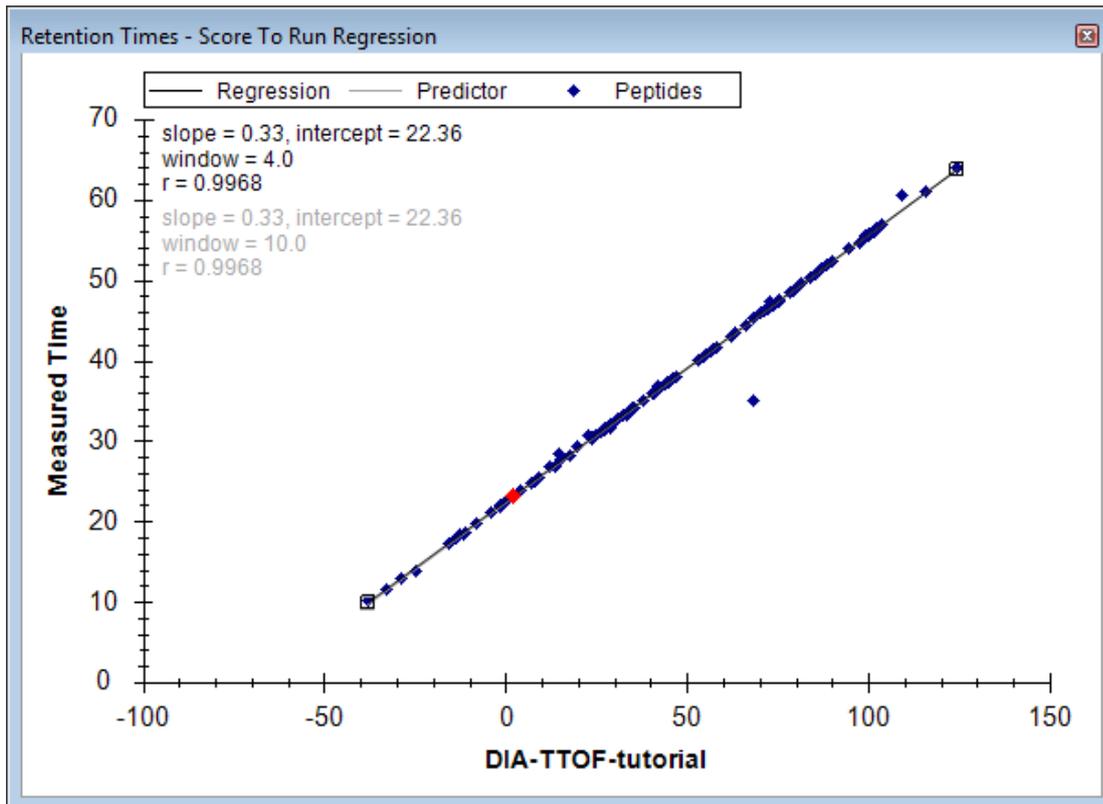
You will see that the mean value ranges from -0.4 to 4.5, while the standard deviation value ranges from 3.7 to 4.5. Using the simple calculation  $\text{Mean} + 3 * \text{SD} = 4.5 + 4.5 * 3 = 18 \text{ PPM}$  at the most extreme, this seems to indicate that a 20 PPM tolerance is a pretty good approximation of what these data require.

To view the mass errors for the decoys also:

- Right-click the graph, choose **Points**, and click **Decoys**.
- Right-click the graph, choose **Replicates**, and click **All**.  
(and then switch back to **Targets**)

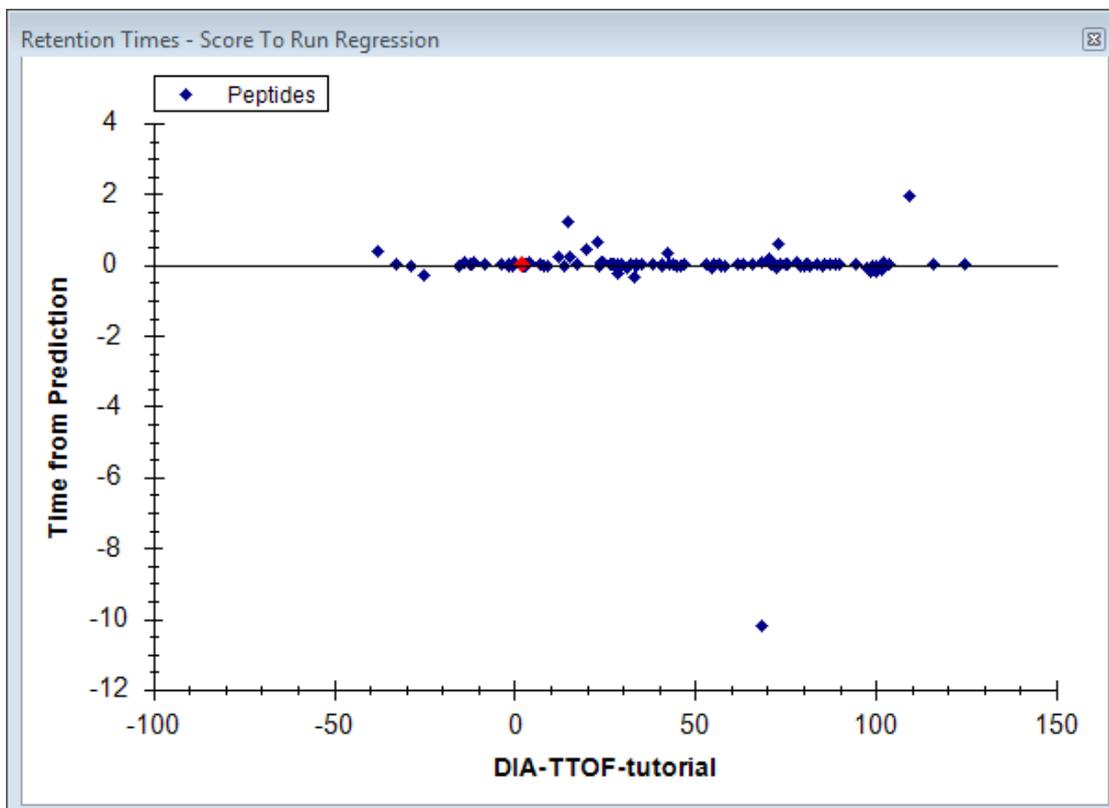
To see the linear regression used to predict the target peptide retention times based on the iRT peptides and library iRT values from the target peptides:

- On the **View** menu, choose **Retention Times**, then **Regression**, and click **Score to Run**.



- Right-click the graph, choose **Plot**, and click **Residuals**.

This will show the deviations from the predicted retention times in this data set. Could the extraction window ( $\pm 5$  minutes) have been further optimized for this analysis? Check the decoys as well.



**Note:** The spectral library for this analysis was constructed from the DIA-Umpire pseudo-DDA spectra. As such, the accuracy of the retention time predictions are very good. Retention times from external spectral libraries acquired on different instruments, at different times, from different samples would lead to larger errors in these predictions.

## Further Reading

At this point, this tutorial may be continued at another tutorial, [Analysis of DIA/SWATH Data](#). If you have not viewed that tutorial before, we recommend that you do it now, starting at the **Sample Annotation** and **Quantitative Comparison** sections, to learn how to perform a quantitative analysis once some DIA data has been imported into Skyline.

## Conclusion

Congratulations! You have completed your first analysis using Skyline's DIA-Umpire implementation. You have learned how to use the **Import Peptide Search** wizard to seamlessly make a spectral library out of DIA data, extract quantitative chromatograms from them, and create and apply an mProphet statistical peak detection model to improve peak selection and assign q values which can be used to control the false discovery rate among the peak areas you used in your group comparison. You learned to first assess the DIA data quality with the mass error and retention time regression plots.

Now consider retrying these steps with the proteome-wide FASTA file, using all 6 DIA files as input, and/or including precursor ions to see how this works with a broader target set and/or another dimension (precursor ions in MS1 spectra). Or move on to trying this with your own data.