

Skyline Targeted Method Editing

This tutorial will cover many of the features available in the Skyline Targeted Mass Spec Environment for creating new target lists for targeted analysis and instrument methods for Selected Reaction Monitoring (SRM also referred to as Multiple Reaction Monitoring - MRM) mass spectrometers. The Skyline support for creating documents from existing transition lists is covered in a separate tutorial.

In developing Skyline we aim to create a vendor-neutral platform for targeted mass spectrometry investigation. From all Skyline documents, you will be able to export transition lists for SRM instruments from Agilent, SCIEX, Shimadzu, Thermo-Scientific, and Waters. Skyline can also export native methods for these instruments as well as methods and isolation lists for Parallel Reaction Monitoring (PRM) which are not covered here.

One of the great benefits of exporting your methods from a Skyline document is that you are assured the instrument output will import seamlessly for use in data analysis with Skyline, though this also is covered in other tutorials.

Understanding how to create a targeted proteomics method in Skyline is a critical starting point, however, a good reason to explore this tutorial.

Getting Started

To start this tutorial, download the following ZIP file:

<https://skyline.ms/tutorials/MethodEdit.zip>

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

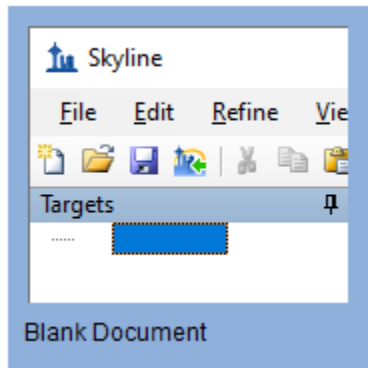
C:\Users\brendanx\Documents

This will create a new folder:

C:\Users\brendanx\Documents\MethodEdit

If you have been using Skyline prior to starting this tutorial, it is 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:

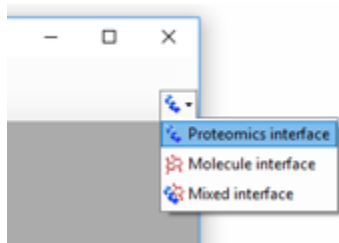



- On the **Settings** menu, click **Default**.
- Click **No** on the form asking if you want to save the current settings.

The document settings in this instance of Skyline have now been reset to the default.

Since this tutorial covers a proteomics topic, you can choose the proteomics interface by doing the following:

- Click the user interface control in the upper right-hand corner of the Skyline window, and click **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 the Skyline window.

You could start editing this blank document in a number of ways, but first you can give Skyline more information about the proteins you will be working with. By giving Skyline this background information, you allow Skyline to help you create information rich methods more quickly.

Creating a MS/MS Spectral Library

Since the method you will create in this tutorial will be designed to measure yeast proteins, you will first create a MS/MS spectral library from part of a yeast data set downloaded from the Peptide Atlas on-line data repository. You can do the same for any data set from Peptide Atlas, or you can just use the full SpectraST libraries supplied by Peptide Atlas. There are 3 large public sources of spectral libraries all supported by Skyline:

- Peptide Atlas (<http://www.peptideatlas.org/speclib/>)
- National Institute of Standards and Technology (NIST) (<http://peptide.nist.gov/>)

- The Global Proteome Machine (GPM) (<ftp://ftp.thegpm.org/projects/xhunter/libs/>)

You can also create new spectral libraries in Skyline using other publicly available data, or peptide search results from your laboratory experiments. Skyline currently supports building libraries from the following search result formats:

- ByOnic (mzIdentML, MGF)
- Comet (sqt / ms2, pepXML / mzXML)
- Mascot (DAT files)
- MaxQuant Andromeda (msms.txt and modifications.xml)
- Morpheus (pepXML/mzML or mzXML)
- MSFragger (pepXML and mzML)
- MSGF+ (mzIdentML, mzML or pepXML and mzXML)
- Myrimatch/IDPicker (ipdXML and mzXML)
- OMSSA (pepXML and mzXML)
- PEAKS (pepXML and mzXML)
- PeptideShaker (mzIdentML)
- PRIDE XML
- Protein Pilot (group or group.xml files)
- Protein Prospector (pepXML and mzXML)
- Proteome Discoverer (MSF and pdResult)
- Scaffold (exported mzIdentML and MGF)
- Spectrum Mill (exported pepXML and mzXML)
- Trans Proteomic Pipeline (pepXML and mzXML)
- Waters PLGS MS^e (final_fragment CSV)
- X! Tandem (BioML XML)
- Custom (SSL) (<https://skyline.ms/blib-formats.url>)

To start this tutorial, you can build your first BiblioSpec spectral library with Skyline by performing the following steps:

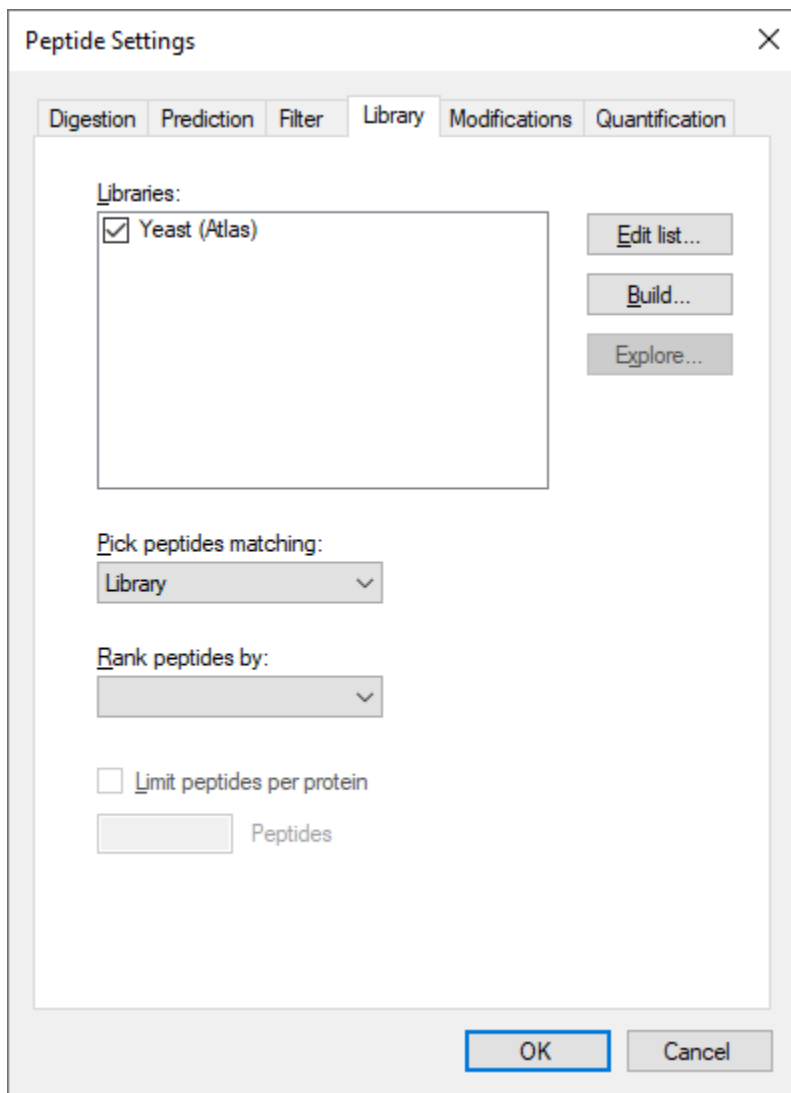
- On the **Settings** menu, click **Peptide Settings**.
- Click on the **Library** tab.
- Click on the **Build** button.
- In the **Name** field of the **Build Library** form, enter “Yeast (Atlas)”.
- Click the **Browse** button.
- Navigate to the Library subfolder under the MethodEdit folder created earlier.
- Click the **Save** button.
- Click the **Next** button.
- Click the **Add Files** button.
- Navigate to the “Yeast_atlas” subfolder under the MethodEdit folder.
- Double-click the interact-prob.pep.xml file in this folder.

- In the **Score Threshold** field, enter “0.95” as the minimum PeptideProphet score.
- Click the **Finish** button.

You should see that Skyline has added the newly created “Yeast (Atlas)” library to the **Libraries** list in the **Library** tab of the **Peptide Settings** form. The library build is performed as a background task, freeing you to continue working. If you look at the Skyline status bar, you can see progress status for the library build. This is a small data set, however, and the build will happen quickly. It may already have completed by the time you read this. When it does, you will see a notification of completion slide up from the lower left corner of the Skyline window.

- In the **Peptide Settings** form, check the checkbox for “Yeast (Atlas)” to tell Skyline to use this library in picking peptides and transitions.

The Library tab should now look like this:



Creating a Background Proteome File:

You can also use FASTA sequence files to inform Skyline of the background matrix in which your experiment will take place. In Skyline, this is called the Background Proteome. It can be as broad or as narrow as you like, e.g. the entire organism FASTA for one or multiple organisms to 18 specific proteins you plan to spike into an otherwise blank matrix, or anywhere in between.

For this tutorial, you will use the complete FASTA file for yeast, which you can do by performing the following steps before clicking the **OK** button in the **Peptide Settings** form:

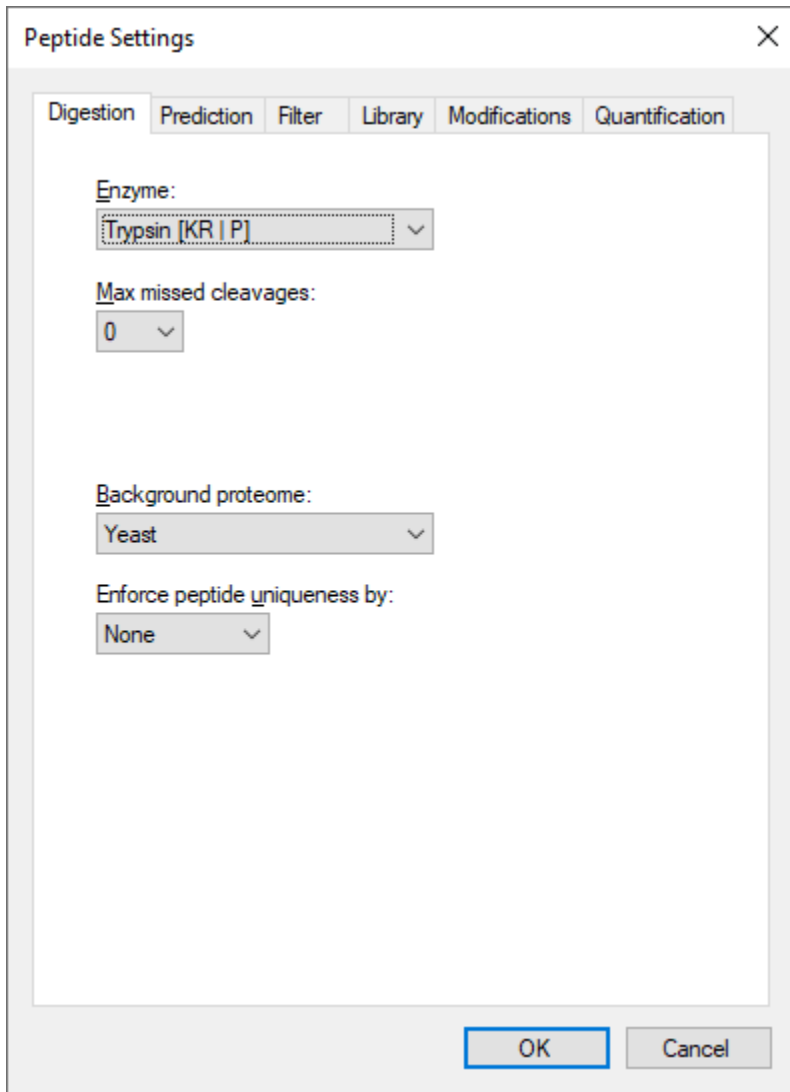
- In the **Peptide Settings** form, click the **Digestion** tab.
- From the **Background proteome** drop-list, choose **<Add...>**.
- Click the **Create** button in the **Edit Background Proteome** form.
- Navigate to the FASTA subfolder of the MethodEdit folder.
- In the **File name** field, enter "Yeast".
- Click the **Save** button.
- Click the **Add File** button.
- Navigate to the FASTA subfolder of the MethodEdit folder.
- Double-click the `sgd_yeast.fasta` file.

Skyline will scan the 5801 protein sequences in this file, create an index for peptide sequence lookup, and save this to the file you created. The Background Proteome form should look like this:

The screenshot shows the 'Edit Background Proteome' dialog box. The 'Name' field is set to 'Yeast'. The 'Proteome file' field is set to 'Skyline Tester Results\MethodEdit\MethodEdit\FASTA\Yeast.protdb'. The 'FASTA files' list contains 'sgd_yeast.fasta'. The status bar indicates 'The proteome file contains 5801 proteins.' The 'OK' button is highlighted with a blue border.

- Click the **OK** button.

This takes you back to the **Digestion** tab of the **Peptide Settings** form, which should now look like this:



The image shows a screenshot of the 'Peptide Settings' dialog box, specifically the 'Digestion' tab. The dialog has a title bar with 'Peptide Settings' and a close button (X). Below the title bar are five tabs: 'Digestion', 'Prediction', 'Filter', 'Library', 'Modifications', and 'Quantification'. The 'Digestion' tab is active. It contains four settings, each with a dropdown menu:

- Enzyme:** The dropdown menu is set to 'Trypsin [KR | P]'.
- Max missed cleavages:** The dropdown menu is set to '0'.
- Background proteome:** The dropdown menu is set to 'Yeast'.
- Enforce peptide uniqueness by:** The dropdown menu is set to 'None'.

At the bottom of the dialog, there are two buttons: 'OK' and 'Cancel'.

Skyline provides many editable lists like the two you just modified. The Enzyme list at the top of the **Digestion** tab is another, and there are others on every tab of the Peptide Settings forms, which you can explore later.

- For now, click the **OK** button.

This commits these changes and returns you to the document in the main Skyline window.

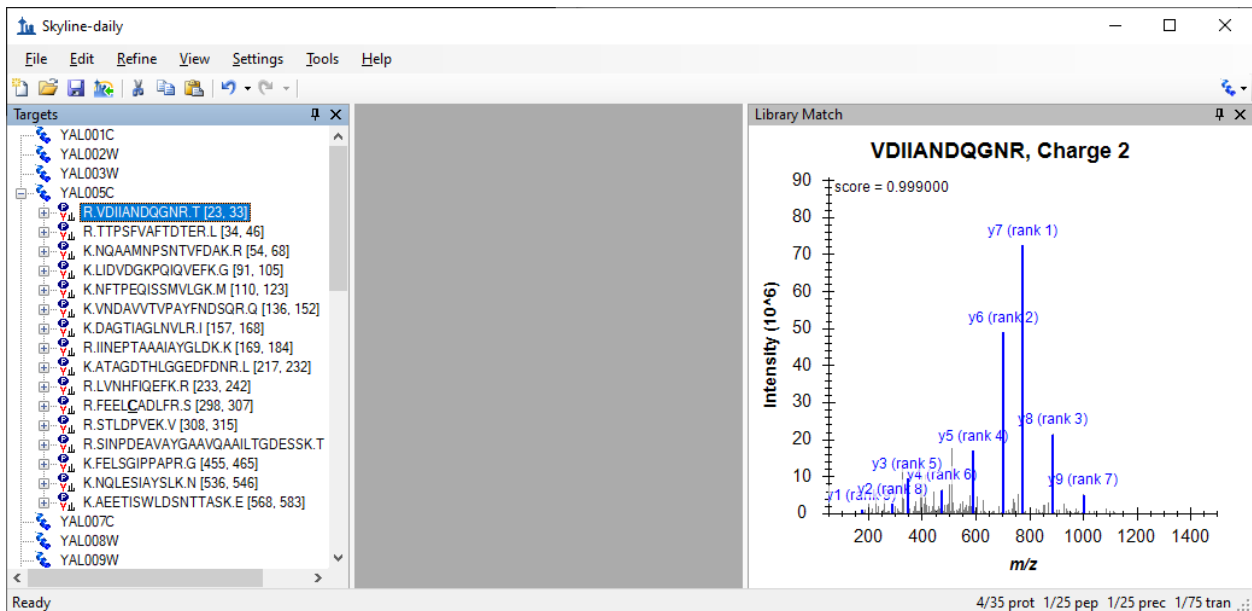
Next you can begin adding proteins to the document to better understand how the new spectral library impacts the peptide and transition choices Skyline makes.

Pasting FASTA Sequences

The first method added to Skyline for specifying proteins of interest was the ability to paste full FASTA sequence text directly into the document. To use this method, perform the following steps:

- Use the Windows **Notepad** application to open the “Fasta.txt” file in the FASTA subfolder of the MethodEdit folder.
- On the **Edit** menu in Notepad, click **Select All** (ctrl-A).
- On the **Edit** menu in Notepad, click **Copy** (ctrl-C).
- Switch back to Skyline.
- On the **Edit** menu, click **Paste** (ctrl-V).
- Press the down arrow key until the first pasted peptide is selected.

This should leave Skyline looking something like this:

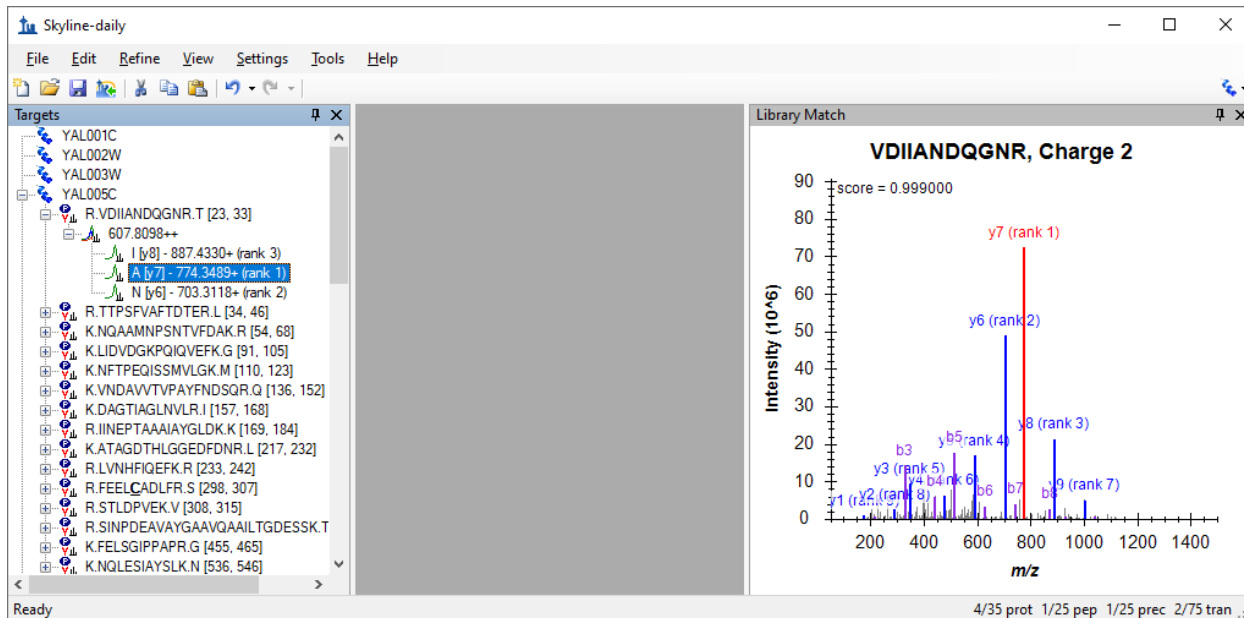


- On the **View** menu, choose **Ion Types**, and then click **B**.

This makes Skyline highlight the b-ions for this peptide in purple in the spectrum graph. Here are the steps to show precursor m/z and product m/z transitions Skyline has picked to monitor for this peptide:

- Click on the **+** to the left of the peptide sequence displayed.
- Press the **down-arrow key** to move the selection to the transition assigned “(rank 1)”.

Skyline highlights ions in the graph as you select the corresponding transitions to present the following view:



By default, Skyline has only chosen the 3 most intense charge 1 product y-ions as the transitions it will measure for the charge 2 precursor. All this may be adjusted, however. To change the settings from their default values, perform the following steps:

- On the **Settings** menu, click **Transition Settings**.
- Click the **Filter** tab.
- In the **Precursor charges** field, change the “2” to “2, 3”.
- In the **Ion charges** field, confirm the value is “1”.
- In the **Ion types** field, change the “y” to “y, b”.

The **Transition Settings** form should look like this:

Transition Settings

Prediction Filter **Library** Instrument Full-Scan

Peptides

Precursor charges: 2, 3 Ion charges: 1 Ion types: y, b

Product ion selection

From: m/z > precursor To: 3 ions

Special ions:

- N-terminal to Proline
- C-terminal to Glu or Asp
- iTRAQ-114
- iTRAQ-115
- iTRAQ-116
- iTRAQ-117

Edit List...

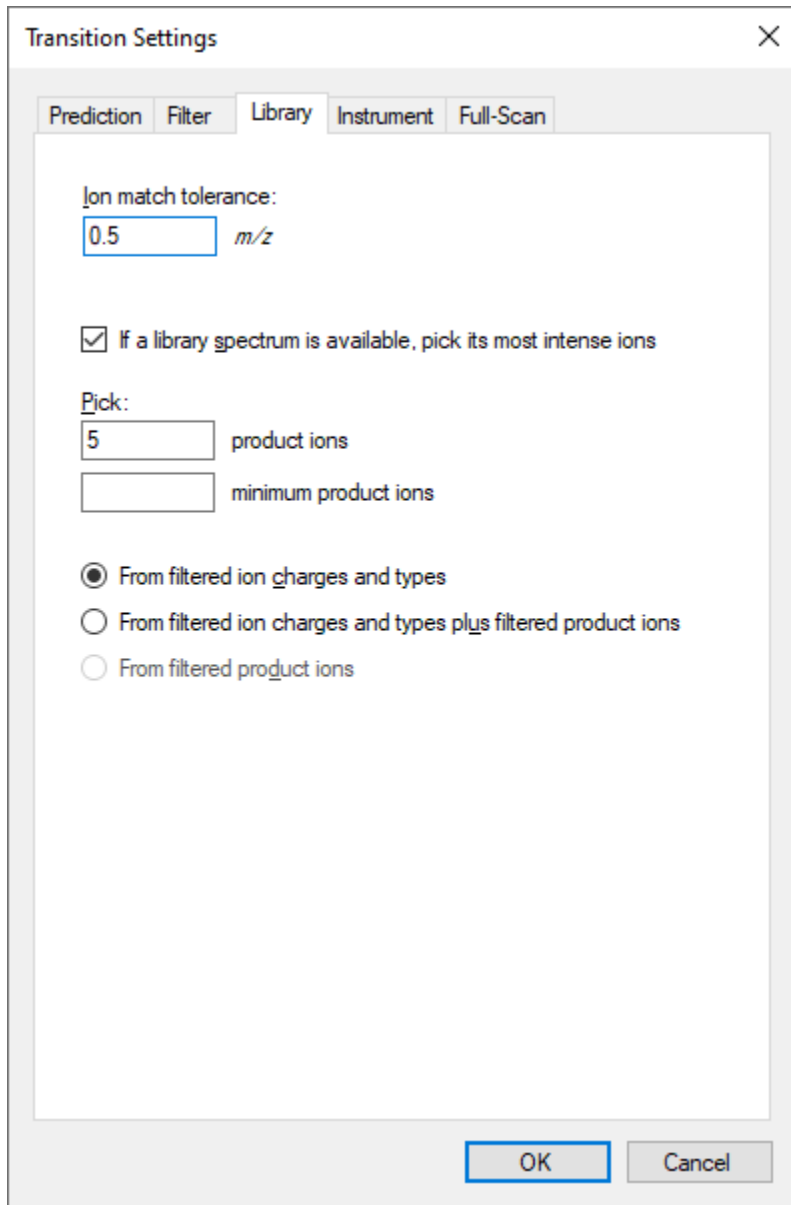
Precursor m/z exclusion window: m/z

Auto-select all matching transitions

OK Cancel

- Click the **Library** tab.
- In the **Pick** field, change “3” to “5” **product ions**.

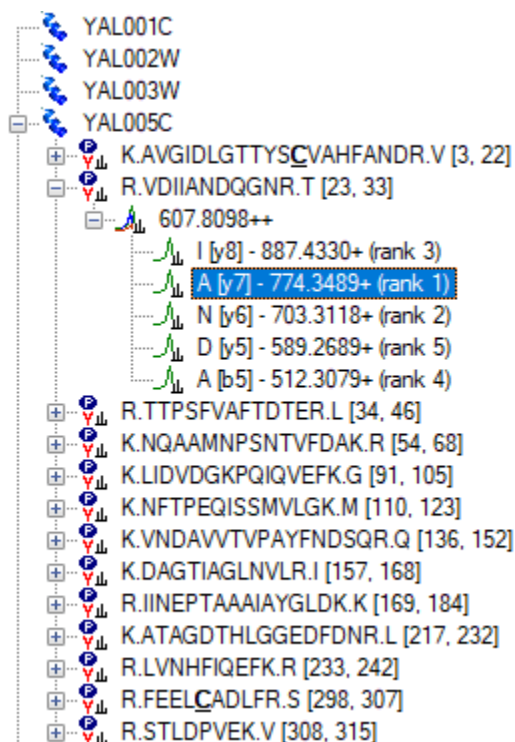
The **Transition Settings** form should look like:



The image shows a dialog box titled "Transition Settings" with a close button (X) in the top right corner. The dialog has four tabs: "Prediction", "Filter", "Library" (which is selected), and "Full-Scan". Inside the "Library" tab, there is a section labeled "Ion match tolerance:" with a text input field containing "0.5" and the unit "m/z" to its right. Below this is a checked checkbox with the text "If a library spectrum is available, pick its most intense ions". Underneath is a section labeled "Pick:" with two text input fields. The first field contains "5" and is followed by the text "product ions". The second field is empty and is followed by the text "minimum product ions". At the bottom of the dialog, there are three radio button options: the first is selected and labeled "From filtered ion charges and types"; the second is labeled "From filtered ion charges and types plus filtered product ions"; and the third is labeled "From filtered product ions". At the very bottom of the dialog box, there are two buttons: "OK" and "Cancel".

- Click the **OK** button.

The Skyline document tree should update to look like the following:



Skyline has added the rank 4 and rank 5 ions for the VDIIANDQGNR peptide, including its b5 ion. A new peptide has been added as the first peptide in the YAL005C protein. By expanding it, you can see that it is a peptide for which the new spectral library contains only a charge 3 peptide-spectrum match, while the library clearly does not contain a charge 3 spectrum for the VDIIANDQGNR peptide. The library peptide settings still direct Skyline to choose only precursors for which the spectral library contains a matching spectrum. You can expand the LIDVDGKPKIQVEFK peptide to see an example of a peptide where both charge 2 and charge 3 spectra were found in the spectral library.

Using a Public Spectral Library

The new spectral library contains a lot of matches for the YAL005C protein, but because it was built from such a small data set many proteins show no peptides at all. Skyline is not, however, limited to using just a single spectral library. You can, for example, add the publicly available library for Yeast from the GPM. The yeast library from NIST is more extensive, but the smaller GPM library was chosen for this tutorial. To add the GPM library included in the tutorial ZIP file, perform the following steps:

- On the **Settings** menu, click **Peptide Settings**.
- Click the **Library** tab.
- Click the **Edit List** button.
- Click the **Add** button in the **Edit Libraries** form.
- In the **Name** field of the **Edit Library** form, enter “Yeast (GPM)”.
- Click the **Browse** button.

- Navigate to the Library subfolder of the MethodEdit folder.
- Double-click the “yeast_cmp_20.hlf” file.
- Click the **OK** button in the **Edit Library** form.
- Click the **OK** button in the **Edit Libraries** form.
- In the **Libraries** list, check the newly created “Yeast (GPM)” item.

The Peptide Settings form should now look like the following:

The screenshot shows the 'Peptide Settings' dialog box with the 'Library' tab selected. The 'Libraries' list contains two entries: 'Yeast (Atlas)' and 'Yeast (GPM)', both with checked checkboxes. To the right of the list are three buttons: 'Edit list...', 'Build...', and 'Explore...'. Below the list, the 'Pick peptides matching:' dropdown menu is set to 'Library'. The 'Rank peptides by:' dropdown menu is currently blank. There is an unchecked checkbox for 'Limit peptides per protein' and a corresponding input field labeled 'Peptides'. At the bottom of the dialog, the 'OK' button is highlighted with a blue border, and the 'Cancel' button is also visible.

Since Skyline can only choose a single spectrum to inform its product-ion decisions, and the value by which to rank these matches remains blank in the Rank peptides by field, the libraries will be searched in the order they appear in the list. Skyline will use the first spectrum match it finds. To see this in action:

- Click the **OK** button.

Once the library is loaded, Skyline updates the document to include a lot of new peptides. If you select peptides and peptide precursors that were already present before the GPM library was added, you can see that the spectrum chart title still attributes the spectra to the “Yeast (Atlas)” library. The spectrum chart for new peptides and peptide precursors, however, displays “Yeast (GPM)” in the title.

NOTE: The GPM libraries unlike all other formats, store only the 20 most intense MS/MS peaks. You can make your own judgment of how well you think the stored spectra actually match the spectra they claim to, but you should do so with full knowledge of why so few peaks are displayed, compared with the unfiltered spectra in other libraries.

Limiting Peptides per Protein

Some of the peptides now have quite a lot of peptide precursors to measure. You might really want to measure them all before you start refining your document, a process covered in another tutorial. In this tutorial, suppose you want to somehow limit the number of peptides you measure for each protein.

In the future, we hope to add predictive algorithms to Skyline that will allow this type of peptide ranking without experimental data. At the moment, however, the only way to limit peptides per protein without resorting to manually picking the peptides yourself is to use a ranking value supplied by a spectral library. Unfortunately, the BiblioSpec library built in this tutorial and the public library from the GPM do not share a comparable score. This means you will also have to uncheck one of the libraries to use a ranking score.

Perform the following steps to limit the peptides per protein in the current document:

- On the **Settings** menu, click **Peptide Settings**.
- Uncheck the “Yeast (Atlas)” library. (The **Library** tab should still be active.)
- From the **Rank peptides by** drop-list, choose **Expect**.
- Check the **Limit peptides per protein** checkbox.
- In the **Peptides** field, enter the number “3”.
- Click the **OK** button.

You should have many fewer peptides now. You can now also get rid of the empty proteins:

- On the **Refine**, click **Remove Empty Proteins**.

Now the tutorial returns to the background proteome file you created, and how it also can be used to add information to the peptides and proteins you are working with as you edit your document.

Inserting a Protein List

Suppose you are working with a list of proteins. You may have the IDs used to identify the proteins in your FASTA file, but you do not want to go through the FASTA file pasting each sequence one by one.

Since you have set up a background proteome, all Skyline needs is a line-separated list of protein IDs from the FASTA file you used to create the background proteome.

To add a list of new proteins to the current document perform the following steps:

- Use the Windows **Notepad** application to open the “Protein List.txt” file in the FASTA subfolder of the MethodEdit folder.
- On the **Edit** menu in Notepad, click **Select All** (ctrl-A).
- On the **Edit** menu in Notepad, click **Copy** (ctrl-C).
- Switch back to Skyline.
- Select the blank element at the end of the document.
- On the **Edit** menu, choose **Insert**, and then click **Proteins**.
- Press ctrl-V to paste from the clipboard.

Skyline adds the list of proteins to the Protein List grid, and assigns the Description and Sequence field values found for the IDs in the background proteome. Because the background proteome lacks accession numbers, preferred names, genes and species information, the columns will be empty. To see more of the protein sequence, do the following:

- Double-click the line between the “Sequence” and “Accession” column headers.
- Click and drag the line between the “Description” and “Sequence” column headers.

So that the form looks like:

	Name	Description	Sequence
▶	YDL227C	HO SGDID:S000002386, Chr IV from 480...	MLSENTTILMANGEIKDIANVTANSYVMCADGSAARVINVTQGYQKIYNIQKTK...
	YDL229W	SSB1 SGDID:S000002388, Chr IV from 4...	MAEGVFQGAIGIDLGTYSVCVATYESSVEIIANEQGNRVTPSFVAFTPEERLIGDA...
	YDL230W	PTP1 SGDID:S000002389, Chr IV from 4...	MAAAPWYIRQRDLDLLGKFKFIQNGEDGRLREATNGTVNSRWVSLGVSIEPRND...
	YDL231C	BRE4 SGDID:S000002390, Chr IV from 4...	MKRYERDRSPTDPDIVKGSYSQTSLSRSLHELNYKNPAGISGLSFAGSPQQSVA...
	YDL233W	YDL233W SGDID:S000002392, Chr IV fr...	MYQGPPQPPPQAVPMPYIVNNNTPPYPNGNINFPPTAQQNIPPTVYPQQVFPFG...
	YDL234C	GYP7 SGDID:S000002393, Chr IV from 3...	MSKILFCKSKVFLHPTSDARDNIAGFLLLTLEANKLSHQAILGYIPESGLSTLEISKL...
	YDL236W	PHO13 SGDID:S000002395, Chr IV from ...	MTAQGGVPIKITNKEIAQEFLDKYDTFLFDCDGLVWLGSQLPYTLEILNLLKQLG...
	YDL237W	YDL237W SGDID:S000002396, Chr IV fr...	MLGLKGCLTILIGYIIVCALFSSRGRNPSLTDWEKLDQKSIDIDNFGLTGQHLL...
	YDL238C	GUD1 SGDID:S000002397, Chr IV from 3...	MTKSDLLFDKFNDKHGKFLVFFGTFVDTPKLGELRIREKTSVGLNLIIRFVNRNS...
	YDL239C	ADY3 SGDID:S000002398, Chr IV from 2...	MNHWLAFLNKPESLKEQNSDCDQGGEMRHVTDGTLTKSPESKPFRRERSQT...
	YDL240W	LRG1 SGDID:S000002399, Chr IV from 2...	MIQNSAGYRSLNTASPMTVQVKNQKICARCNKLVIPDSQRKTTLKALGKYH...
	YDL243C	AAD4 SGDID:S000002402, Chr IV from 1...	MGSMNKEQAFELLDIFYEAGGNICDTANSYQNEESEIWIGEWKSRKLRDQIVI...
	YDL244W	THI13 SGDID:S000002403, Chr IV from 1...	MSTDKITFLLNWQPTPYHIPIFLAQTGKYFKEQGLDIALEPTNPSDVELIGSGKV...
	YDL245C	HXT15 SGDID:S000002404, Chr IV from ...	MASEQSSPEINADNLNSSAADVHVQPPGEKEWSDGFYDKEVINGNTPDAPKRG...
	YDL246C	SOR2 SGDID:S000002405, Chr IV from 9...	MSQNSNPVAVLEKVGDIIEQRPIPTIKDPHYVKLAIKATGICGSDIHYYRSGGIGK...
	YDL247W	MPH2 SGDID:S000002406, Chr IV from 5...	MKNLSFLINRRKENTSDSNVYPGAKASHEPSWIEMDDQTKKDGLDIVHVEFSPD...
	YDL248W	COS7 SGDID:S000002407, Chr IV from 1...	MKENEVKDEKSVDLVLSFKQLESQKIVLPQDLFRSSFTWFCYIYKSLAFRIWMLL...
*			

- Click the **Insert** button.

This will add these proteins to the end of the document. Many of them have no peptides matching spectra in the GPM library, so follow these steps to remove empty proteins:

- On the **Refine** menu, click **Remove Empty Proteins**.

Inserting a Peptide List

There are two ways to insert a list of peptides into a Skyline document, and they have the following results:

1. A list of peptides separate from any protein information
2. Each peptide associated with a specific protein

To achieve the first result, perform the following steps:

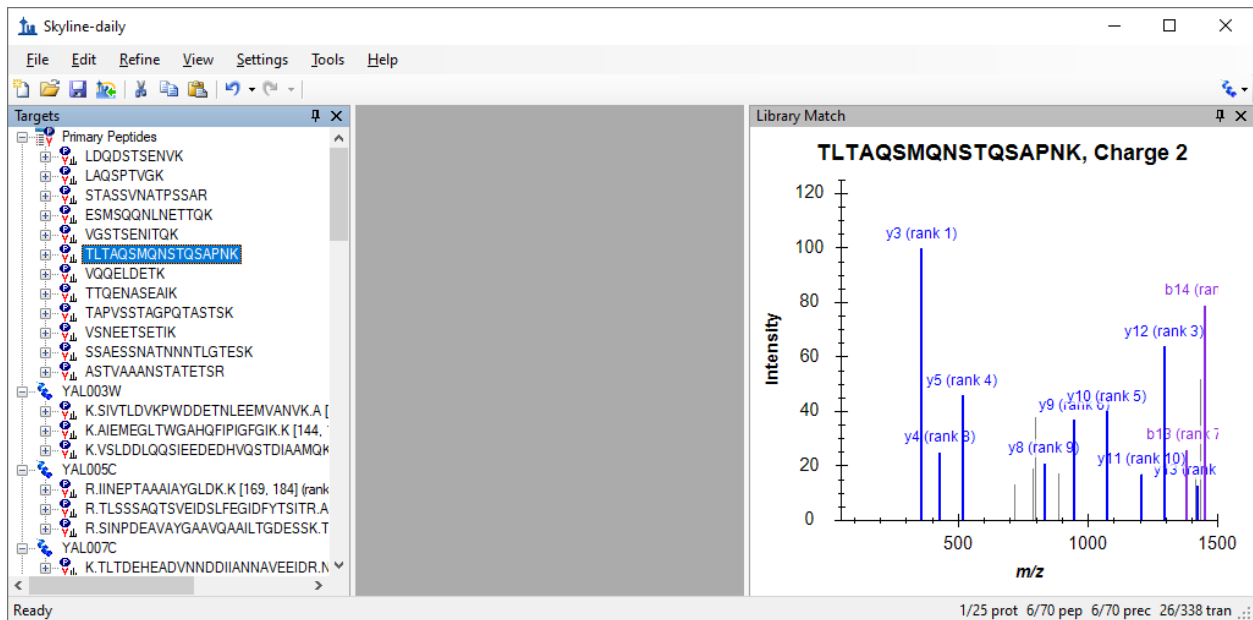
- Use the Windows **Notepad** application to open the “Peptide List.txt” file in the FASTA subfolder of the MethodEdit folder.
- On the **Edit** menu in Notepad, click **Select All** (ctrl-A).

- On the **Edit** menu in Notepad, click **Copy** (ctrl-C).
- Switch back to Skyline.
- Select the first protein in the document.
- On the **Edit** menu, click **Paste** (ctrl-V).

Skyline adds the complete list of peptides to a single new peptide list element in the document, named “peptides1”. You can rename the list now by simply typing the name you prefer.

- Type “Primary Peptides” and press Enter.

The GPM library has spectra for all of the added peptides, which you can review by pressing the down-arrow key to select each of the pasted peptides. The Skyline document should now look something like this:



To insert the same list so that each peptide is associated with its respective protein in the background proteome, you need to use the **Insert Peptide List** form. Perform the following steps to do this now:

- First click the **Undo** button in the toolbar twice (ctrl-Z, ctrl-Z).
- On the **Edit** menu, choose **Insert**, and click **Peptides**.
- Press ctrl-V to paste from the clipboard.

Skyline populates the protein fields for each peptide to present the form looking like this:

Peptide Sequence	Protein Name	Protein Description
LDQDSTSENVK	YIL075C	RPN2 SGDID:S000001337, Chr IX from 220697-...
LAQSPTVGK	YGL234W	ADE5,7 SGDID:S000003203, Chr VII from 56482-...
STASSVNATPSSAR	YEL032W	MCM3 SGDID:S000000758, Chr V from 86937-8...
ESMSQQNLNETTQK	YFL038C	YPT1 SGDID:S000001856, Chr VI from 55985-5...
VGSTSENIQK	YOR204W	DED1 SGDID:S000005730, Chr XV from 722911-...
TLTAQSMQNSTQSAPNK	YOR153W	PDR5 SGDID:S000005679, Chr XV from 619840-...
VQQLDETK	YKL196C	YKT6 SGDID:S000001679, Chr XI from 75539-7...
TTQENASEAIK	YAL035W	FUN12 SGDID:S000000033, Chr I from 76428-7...
TAPVSSTAGPQTASTSK	YDR233C	RTN1 SGDID:S000002641, Chr IV from 930351-...
VSNEETSETIK	YCR053W	THR4 SGDID:S000000649, Chr III from 216692-...
SSAESSNATNNNTLGTESK	YBR086C	IST2 SGDID:S000000290, Chr II from 423035-42...
ASTVAAANSTATETSR	YML057W	CMP2 SGDID:S000004521, Chr XIII from 16018-...

- Click the **Insert** button to insert the peptides into the document.

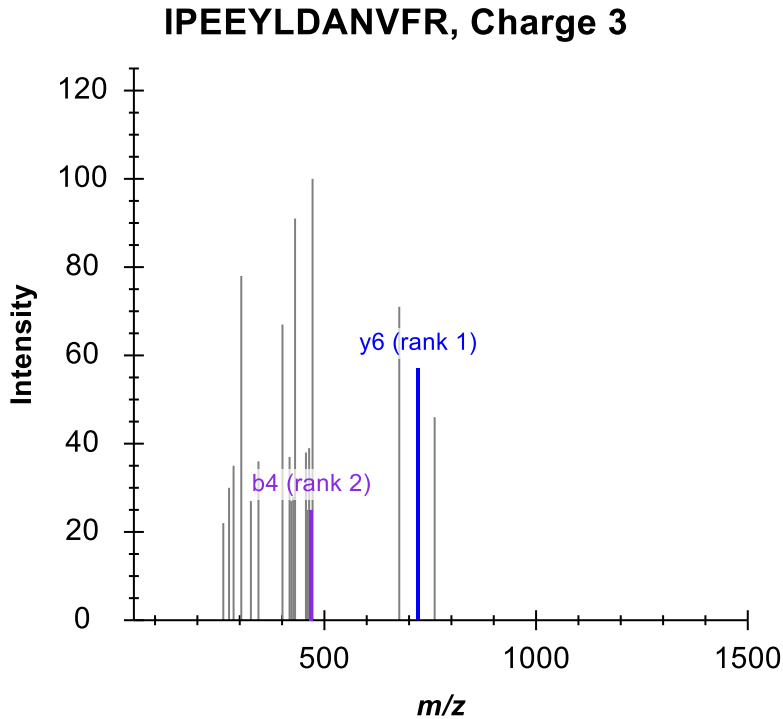
The peptides have been added to the proteins by name instead of a single peptide list, as before.

Simple Refinement

At this point, you have added 70 peptides to the document without doing any confirmation further than that the GPM yeast library contains a spectrum for each. To have a look at a peptide where the library spectrum seems like a particularly poor match for what this document is trying to measure, do the following:

- On the **Edit** menu, click **Find ...** (ctrl-F).
- Enter "IPEE" in the **Find what** field.
- Click the **Find Next** button.

This spectrum shows only 1 matching y-ion and 1 matching b-ion, and looks like:



Measuring these two ions is unlikely to produce useful information about this peptide.

To remove all peptides for which the library spectrum failed to provide the desired 5 product ions, perform the following steps:

- On the **Refine** menu, click **Advanced**.
- In the **Min transitions per precursor** field, enter the number "5".
- Click the **OK** button.

The status bar indicators at the bottom-right corner of the Skyline window show that the number of peptides has been reduced from 70 to 64:

29/35 prot 50/64 pep 50/64 prec 246/320 tran ...

Checking Peptide Uniqueness

Another thing you might want to check is how unique the chosen peptides are to the proteins you are trying to measure. Unfortunately, we have found that FASTA sequence files are frequently redundant enough that it would be unwise to simply remove all peptides which are not unique to a single sequence in the background proteome. Many peptides belong to multiple protein homologues of a single gene model.

Skyline does, however, offer a form for inspecting uniqueness of the peptides for each protein in the document. Perform the following steps to review the last 2 proteins in the document you are editing:

- Select the last protein in the document.
- On the **Edit** menu, click **Unique Peptides**.

Skyline displays the **Unique Peptides** form with the following information:

<input checked="" type="checkbox"/> Included	Peptide	YDR342C	YDR343C	YEL069C	YJR158W	YNR072W
<input checked="" type="checkbox"/>	SASWVPPSR	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>

Protein Name: YDL245C

Details: Searched: Uniprot:SGD_S000002404

Description: HXT15 SGDID:S000002404, Chr IV from 13360-11657, reverse complement, Verified ORF, "Protein of unknown function with

Sequence: MASEQSSPEINADNLNSSAADVHVQPPGEKEWSDGFYDKEVINGNTPDAPKRGFLGYLIYLLCYPVSFSGGFLPGWDSGITAGFI
 NMDNFKMNFSGSYKHSTGEYYLSNVRMGLLVAMFVSGCSIGGVAFARLADTLGRRLAIVVLLVYMVGAIQISSNHKWKYQYFV
 GKIIYGLGAGGCSVLCPLLSEIAPDLRGGLVSLYQLNMTFGIFLGYCSVYGTRKYSNTAQWRIPVGLCFLWALIIIVGMLLVPE
 SPRYLIECERHEEACVSIKINKVSPEDPWVLKQADEINAGVLAQRELGEASWKELFSVKTQVLRITGILVQTFQLTGENYFF

OK Cancel

A close review of these 6 proteins shows that some are similar to each other, but there is certainly enough variation that this one peptide gives little confidence of measuring the protein of interest. You can view the protein sequence for each protein by selecting its column in the grid view.

- Close the **Unique Peptides** form, and press the Delete key to remove this protein from the document.
- Now repeat the same steps for the new last protein.

Skyline shows that the one peptide chosen for this protein also maps to multiple proteins, in this case 4, but they are much more similar. You may want to keep this one.

- Click the **Cancel** button.

Note: Since this tutorial was originally written a setting for enforcing peptide uniqueness has been added to the **Peptide Settings – Digestion** tab, below the **Background proteome** field. It is labeled **Enforce peptide uniqueness by**. You may want to explore how it impacts the peptides chosen in this document.

Direct Document Editing

You have already seen that it is possible to delete items in the document using the delete key, as well as to change the name of a peptide list by typing over the existing name. In this section, you will be

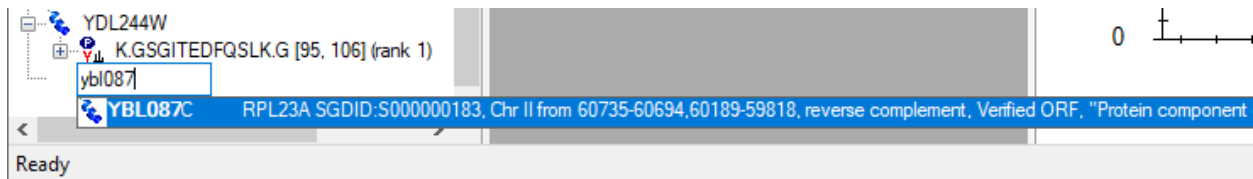
introduced to a few more direct document editing features that allow you to quickly modify the proteins, peptides, precursors and transitions you wish to measure.

Protein Name Auto-Completion

With a background proteome defined, you can get help in adding proteins and peptides simply by typing on the blank element at the end of the document. To add a protein by name, do the following:

- Select the blank element at the end of the document.
- Type “ybl087”

Skyline will offer to complete this by adding a protein as shown below:

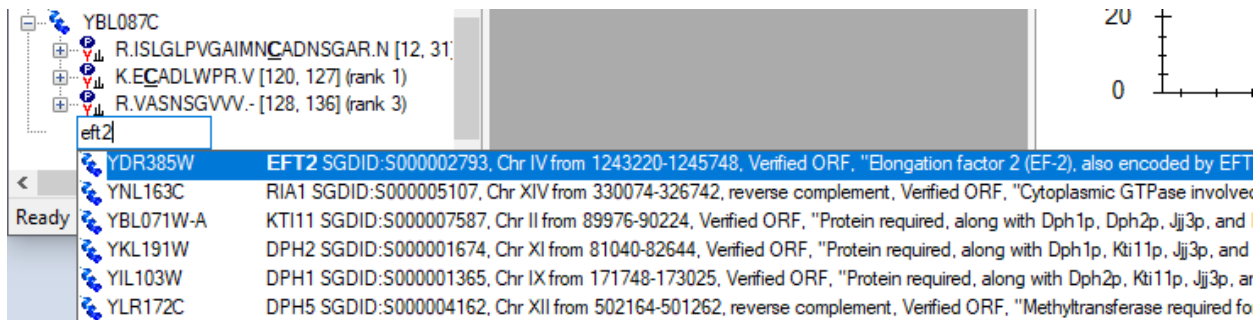


- Press the Enter key to add the protein to your document.

Protein Description Auto-Completion

Skyline will also search the description text for the protein sequences from a FASTA file. To find and add a protein based on its description, do the following:

- Type “eft2”
- Press the down-arrow key once to select the first protein listed, as shown:



- Press the Enter key to add the protein to your document.

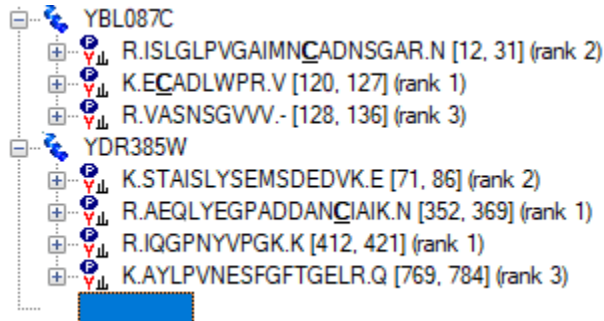
Peptide Sequence Auto-Completion

A third auto-completion option is to begin typing a peptide sequence, and Skyline will help identify the peptide and the protein or proteins that contain it. To find and add a peptide by its sequence, do the following:

- Press Caps-Lock.
- Type “IQGP”.
- When Skyline shows the peptide IQGPNYVPGK, press the down-arrow key to select it.

- Press the Enter key.

The peptide is added to the document, but it is added to the existing protein YDR385W, just above the final blank element. The added proteins should now look like:



Pop-up Pick-Lists

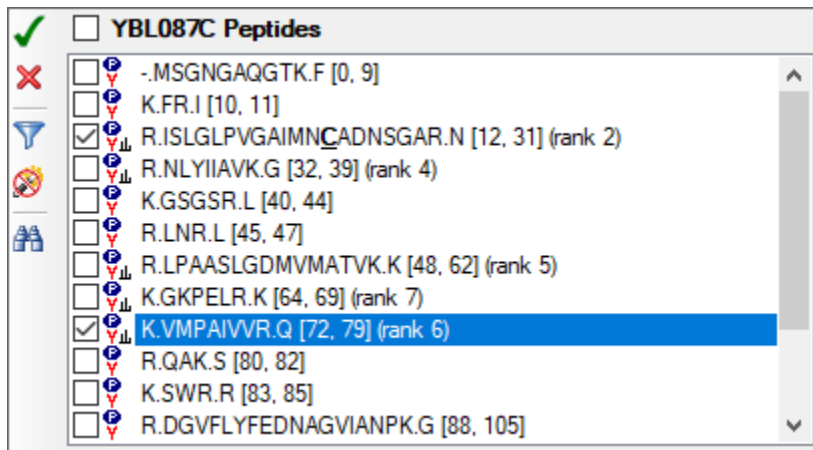
Once you have added proteins to your document, you can also use the Skyline pop-up pick-lists to modify the peptides, precursors and product ions you will target. You can add another peptide to the YBL087C protein by doing the following:

- Move the mouse cursor over the YBL087C protein until a drop-arrow appears beside the name.
- Move the mouse cursor over the drop arrow until the cursor image changes to a hand.
- Click the left mouse button.

Skyline will show a pop-up pick-list that is initially filtered, and shows only the set of peptides it has already added to the document. You could uncheck any of these to have the same effect as using the Delete key to delete peptides from this protein. Instead, you can add a new peptide by doing the following:

- Click the button with the funnel image to get an unfiltered list.
- Check the peptide with the suffix “(rank 6)”.

This should leave the pick-list looking like:



- Press the Enter key or click the button with the green check image to commit the change to your document.

All items in the document that support sub-lists will display the same kind of pick-list. To change the product ion transitions for a peptide precursor, do the following:

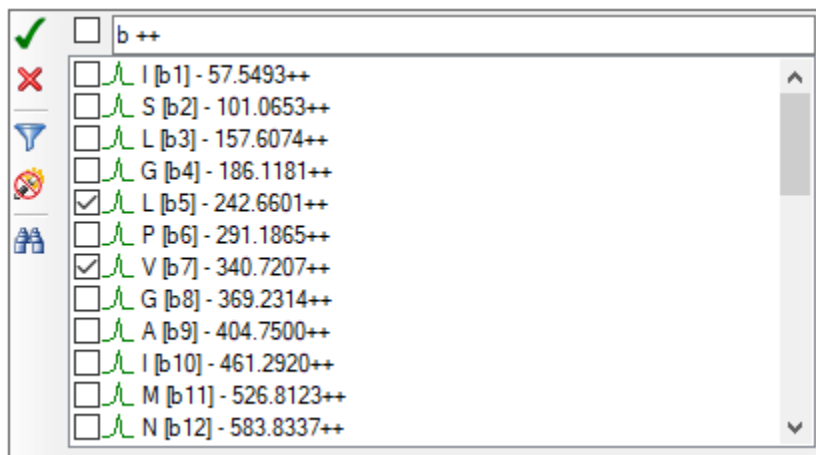
- Expand the first peptide in the YBL087C protein (ISLGLP...) by clicking the + to the left of the peptide sequence.
- Move the mouse cursor over the 672.6716+++ precursor until a drop-arrow appears beside the name.
- Move the mouse cursor over the drop arrow until the cursor image changes to a hand.
- Click the left mouse button.
- Click the funnel icon to get an unfiltered list of possible product ions.

Suppose you had some reason to believe this particular precursor would be better measured with only b-ions, and in fact you want to exchange the current 2 y-ions for 2 doubly charged b-ions. To do this, perform the following steps:

- Uncheck the two y-ions currently in view (y9 and y6).
- Click on the button with the binoculars image to show the find field.
- Type “b ++” (b-space-++) to filter the list to only items containing a b and ++.
- Check the doubly charged b5 and b7 ions.

Note: This is just an example, and these ions were not found in the MS/MS spectrum.

The pick-list should now look like this:



- Press the Enter key or click the button with the green check image to commit the change to your document.

Bigger Picture

Sometimes it helps to be able to see the information in your document in a broader context. You may have noticed this already, as you moved the mouse over the document, but if not, hover the mouse cursor now over some of the elements in the document tree, and Skyline will present data tips like the ones shown below. In these tips, the selected element is highlighted in red, elements in the document in blue, and bold is used for elements which match the filter but are not included in the document.

Name	YBL087C
Searched	Uniprot:SGD:S000000183
Description	RPL23A SGDID:S000000183, Chr II from 60735-60694,60189-59818, reverse complement, Verified ORF, "Protein component of the large (60S) ribosomal subunit, identical to Rpl23Bp and has similarity to E. coli L14 and rat L23 ribosomal proteins"
MSGNGAQGTKFRI ISLGLPVGAIMNCADNSGAR NLYIIAVKGGSGSRLNRLPAASLGDMMATVKKGKPELRKK VMPAIVVRQAKSWRRRDG VFLYFEDNAGVIANPKGEMKGSAITGPVGK ECADLWPRVASNSGVVV	

Modified	ISLGLPVGAIMNC[+57]ADNSGAR					
Precursor charge	3					
Precursor m/z	672.6716					
Precursor m+h	2016.0001					
Expect	2.884318E-11					
Processed intensity	146.0582					
b++	b+	#	AA	#	y+	y++
57.55	114.09	1	I			
101.07	201.12	2	S	19	1902.92	951.96
157.61	314.21	3	L	18	1815.88	908.45
186.12	371.23	4	G	17	1702.80	851.90
242.66	484.31	5	L	16	1645.78	823.39
291.19	581.37	6	P	15	1532.69	766.85
340.72	680.43	7	V	14	1435.64	718.32
369.23	737.46	8	G	13	1336.57	668.79
404.75	808.49	9	A	12	1279.55	640.28
461.29	921.58	10	I	11	1208.51	604.76
526.81	1052.62	11	M	10	1095.43	548.22
583.83	1166.66	12	N	9	964.39	482.70
663.85	1326.69	13	C	8	850.35	425.68
699.37	1397.73	14	A	7	690.32	345.66
756.88	1512.75	15	D	6	619.28	310.14
813.90	1626.80	16	N	5	504.25	252.63
857.42	1713.83	17	S	4	390.21	195.61
885.93	1770.85	18	G	3	303.18	152.09
921.45	1841.89	19	A	2	246.16	123.58
			R	1	175.12	88.06

Drag and Drop

Finally, you can also use drag and drop to reorder certain elements in the document. In the document you have created for this tutorial, only the proteins themselves may be reordered. The other elements have inherent ordering, and cannot be moved. If, however, you create a peptide list with no protein information, as you did above by pasting the list directly into the document, then you could also use drag and drop to reorder the peptides within that list.

- Use drag and drop now to reorder the proteins, and see that it works.

Preparing to Measure

Of course, the end goal of all this document editing is to attempt to measure the peptides in the document on a mass spectrometer. But, first you need to decide which mass spectrometer. Skyline exports transition lists for triple quadrupole instruments from five manufacturers: Agilent, SCIEX, Shimadzu, Thermo Scientific, and Waters. It can also now export native method files for all of these, but some require instrument software and all require a method template to do this.

For this tutorial, you will export only one transition list for a SCIEX Q Trap. Before you do, you should change some settings. To prepare your document for exporting a Q Trap transition list, do the following:

- On the **Settings** menu, click **Transition Settings**.
- Click the **Prediction** tab.
- From the **Collision energy** drop-list, choose “SCIEX”.
- From the **Declustering potential** drop-list, choose “SCIEX”.
- Click the **Instrument** tab.
- Change the **Max m/z** field to “1800”.
- Click the **OK** button.

Before exporting your first transition list, first save your document to the MethodEdit folder by doing the following:

- On the **File** menu, click **Save** (ctrl-S).
- Navigate to the MethodEdit folder.
- In the **File name** field, enter “MethodEditTutorial”.
- Click the **Save** button.

Next, you should note that the document created in this tutorial contains 355 transitions. If you already had accurate measurements of when these peptides elute with the column you will be using, you might be able to schedule them all in a single method, so that each is measured for only a small window of time. Since you do not yet have these measurements, however, you will first need to measure over the entire gradient, dividing the measurements into groups that can be measured throughout the entire gradient for a run. The appropriate number to use will vary depending on the speed and sensitivity of the instrument. The number can be many hundreds with modern triple quadrupole instruments, but this tutorial was originally written for a SCIEX 4000 Q Trap on which 75 was thought to be appropriate.

With this information, you are ready to export a transition list for the SCIEX 4000 Q Trap. To do so, perform the following steps:

- On the **File** menu, choose **Export**, and then click **Transition List**.
- Click the **Multiple methods** radio button.
- Check the **Ignore proteins** checkbox.
- In the **Max transitions per sample injection** field, enter “75”.

This should leave the Export Transition List form looking like:

Export Transition List

Instrument type: SCIEX

OK

Cancel

Single method

One method per protein Order by m/z

Multiple methods Ignore proteins

Max transitions per sample injection: 75

Methods: 5

Optimizing: None

Method type: Standard Dwell time (ms): 20

- Click the **OK** button.
- In the **File name** field, enter “Yeast_list”.
- Click the **Save** button.

Switch to a Windows Explorer window, and navigate to the MethodEdit folder to see the transition list files you just created. The contents of the MethodEdit folder should now look like:

Name	Date modified	Type	Size
FASTA	10/5/2017 4:28 PM	File folder	
Library	10/5/2017 3:18 PM	File folder	
Yeast_atlas	3/26/2010 2:31 PM	File folder	
MethodEditTutorial	10/5/2017 4:34 PM	Skyline SRM Docu...	278 KB
MethodEditTutorial.sky.view	10/5/2017 4:34 PM	VIEW File	4 KB
Yeast_list_0001	10/5/2017 4:35 PM	Microsoft Excel C...	6 KB
Yeast_list_0002	10/5/2017 4:35 PM	Microsoft Excel C...	6 KB
Yeast_list_0003	10/5/2017 4:35 PM	Microsoft Excel C...	6 KB
Yeast_list_0004	10/5/2017 4:35 PM	Microsoft Excel C...	6 KB
Yeast_list_0005	10/5/2017 4:35 PM	Microsoft Excel C...	5 KB

Open the first of the five new files, and you should find a transition list that looks like:

	A	B	C	D	E	F
1	618.2911	879.4054	20	YIL075C.LDQDSTSENVK.+2y8.light	76.2	31
2	618.2911	764.3785	20	YIL075C.LDQDSTSENVK.+2y7.light	76.2	31
3	618.2911	677.3464	20	YIL075C.LDQDSTSENVK.+2y6.light	76.2	31
4	618.2911	576.2988	20	YIL075C.LDQDSTSENVK.+2y5.light	76.2	31
5	618.2911	472.2038	20	YIL075C.LDQDSTSENVK.+2b4.light	76.2	31
6	450.7611	716.3937	20	YGL234W.LAQSPVGVK.+2y7.light	64	21.4
7	450.7611	588.3352	20	YGL234W.LAQSPVGVK.+2y6.light	64	21.4
8	450.7611	501.3031	20	YGL234W.LAQSPVGVK.+2y5.light	64	21.4
9	450.7611	185.1285	20	YGL234W.LAQSPVGVK.+2b2.light	64	21.4
10	450.7611	697.3879	20	YGL234W.LAQSPVGVK.+2b7.light	64	21.4
11	668.3286	803.4006	20	YEL032W.STASSVNATPSSAR.+2y8.light	79.8	33.8
12	668.3286	689.3577	20	YEL032W.STASSVNATPSSAR.+2y7.light	79.8	33.8
13	668.3286	618.3206	20	YEL032W.STASSVNATPSSAR.+2y6.light	79.8	33.8
14	668.3286	517.2729	20	YEL032W.STASSVNATPSSAR.+2y5.light	79.8	33.8
15	668.3286	819.3843	20	YEL032W.STASSVNATPSSAR.+2b9.light	79.8	33.8

The columns are in the order: precursor m/z , product m/z , dwell time, extended peptide, declustering potential, and collision energy. This should be enough to paste into the Analyst software method creation user interface and create a method that you could use to run the instrument on a yeast sample to attempt measuring these peptides.

Conclusion

There is certainly a lot more to learn about using the Skyline application for your targeted proteomics experiments. You should, however, feel confident enough to begin creating your own real Skyline documents. Hopefully these documents will help you get set up to measure peptides that test new hypotheses quicker than you have before. Then you will want to import your instrument output files back into Skyline for peak integration and results analysis. We think you will find that the context you create in a Skyline document greatly simplifies understanding the instrument output. Just how to perform these next steps, however, is covered in other instructional materials you can find on the Skyline website.