Skyline Small Molecule Quantification

The Skyline Targeted Mass Spectrometry Environment provides informative visual displays of the raw mass spectrometer data you import into your Skyline documents. Originally developed for proteomics use, Skyline has been extended to work with generalized molecules. This tutorial explores a relatively straightforward example of using Skyline for targeted quantification of a single small molecule using an external calibration curve and stable-isotope labeled internal standard.

In this tutorial, you will learn about Targeted Quantification based on TQ-MS (in this example, out of crashed plasma) starting from a method you may already be running (e.g. a pharmacokinetic assay). In the analysis of this dataset you will learn:

* Insertion of simple set of known transitions
* Data Analysis and peak integration for non-proteomic molecules
* Small Molecule Quantification workflow in Skyline

You may also wish to view the second half of [Skyline Tutorial Webinar 16](https://skyline.ms/webinar16.url), on which this tutorial is based.

Skyline aims to provide a vendor-neutral platform for targeted quantitative mass spectrometry research. It can import raw data from the instrument vendors Agilent, SCIEX, Bruker, Shimadzu, Thermo-Scientific, and Waters. The ability to import data across various instrument platforms facilitates cross-instrument comparisons and multi-site studies. This remains equally true in using it to target small molecules, as it has been for years in the field of proteomics.

If you have not already looked at the “[Skyline Small Molecule Targets](https://skyline.ms/tutorial_small_molecule.url)” tutorial you should do so now, in order to pick up a few basics about how Skyline works with small molecule descriptions including chemical formulas and adducts.

# Getting Started

To start this tutorial, download the following ZIP file:

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

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

C:\Users\bspratt\Documents

This will create a new folder:

C:\Users\bspratt\Documents\SmallMoleculeQuant

It will contain all the files necessary for this tutorial.

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 small molecule topic, you can choose the molecule interface by doing the following:

* Click the user interface control in the upper right-hand corner of the Skyline window, and click **Molecule interface** which looks like this:



Skyline is operating in molecule mode which is displayed by the molecule icon  in the upper right-hand corner of the Skyline window. Its original proteomics menus and controls are now hidden, allowing you to focus on small molecule analysis.

# Experimental Layout

This experiment was designed according to the FDA Guidance on Bioanalytical Method Validation, and as such contains much more than just the study samples. A full description of the plate layout and run order typically utilized for such a study has been published (<https://www.ncbi.nlm.nih.gov/pubmed/29039849>). Briefly, the samples for this dataset were laid out in a 96 well plate as follows:



Blanks, or “zero” standards, contain only the internal standard, double blanks contain no standard at all.

Calibration curve samples are a dilution series for calibration.

QC samples are “known unknowns”. These are quality control samples which are treated as unknowns in this study. In actuality, it is known what the results should be and thus they can be used to check the accuracy of measurements.

Serum SPQC is a serum pooled QC, a pooling of all study samples which is run at several points at the start, middle and end of the experiment to verify that quantitative reproducibility is constant across the study.

The NIST SRM 1950 is a pooled plasma standard from the National Institute for Standards and Technology, which is available to all researchers as a reference standard for “normal” plasma metabolite measurements. It serves as a reference between studies in different laboratories.

Injections were performed in this order:



In all, 113 injections were used in collecting the mass spec data for these samples.

# Internal Standards

For this study there are just two targets: a molecule and an internal standard, which is an isotopically labeled variant of the molecule and thus co-elutes. It is also possible to establish a relationship between unrelated molecules by declaring one of the molecules as a surrogate standard. The surrogate standard method is covered in the “[Skyline High Resolution Metabolomics](https://skyline.ms/tutorial_hi_res_metabolomics.url)” tutorial.

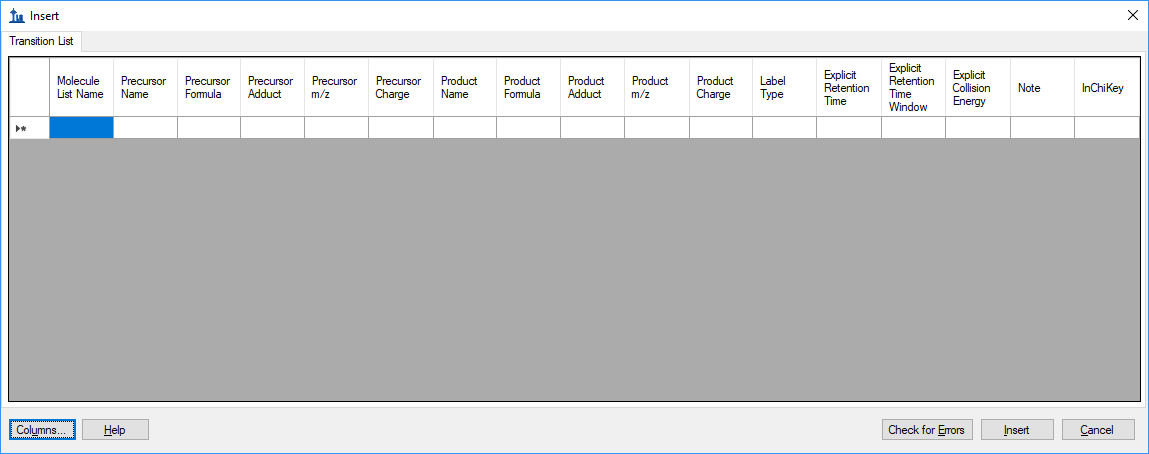
# Importing a Small Molecule Transition List into a Skyline Document

The easiest way to get a small molecule transition list into a Skyline document is to start with an empty document and use the **Edit > Insert > Transition List** menu item.

To begin, do the following:

* On the Skyline **Edit** menu, choose **Insert** and click **Transition List**.

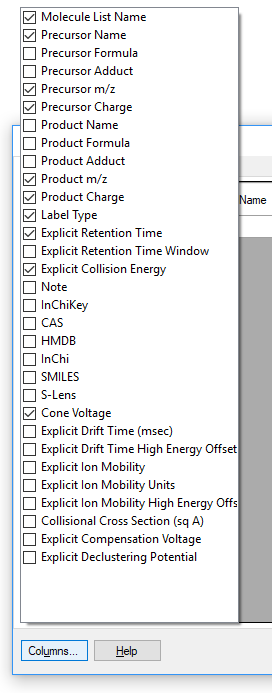
Skyline will show the **Insert** form:



Normally, you would copy and paste a transition list from Excel or some other external source, but in this case, the transition list is small enough it can entered by hand.

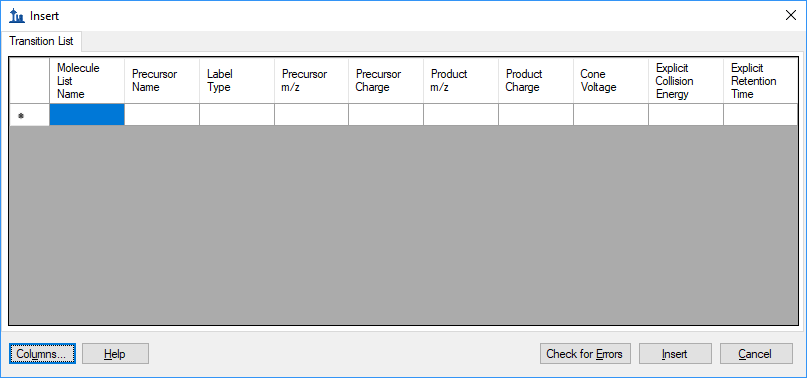
You can see that there are currently a lot of columns in the **Insert** form, and this tutorial will also benefit from a different column order. Both issues are easy to correct:

* Click the **Columns** button and click the check boxes in the popup list to achieve the state below.



Next do the following to reorder the columns in the **Insert** form:

* Click and drag each column header you want to move to the order shown below.



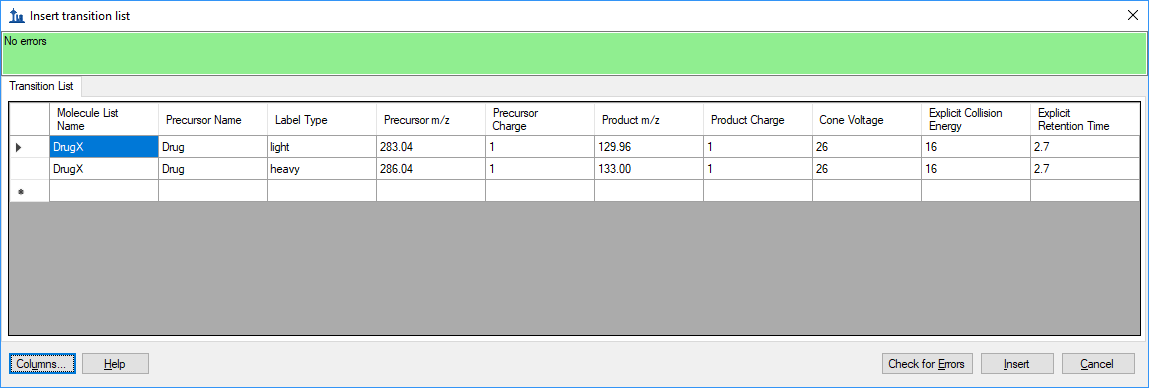
Enter the following values in the **Insert** form (or better yet, copy and paste from this PDF):

* Select the two rows below by dragging over them and then **Copy** (Ctrl-C).

DrugX,Drug,light,283.04,1,129.96,1,26,16,2.7  
DrugX,Drug,heavy,286.04,1,133.00,1,26,16,2.7

* Make sure the selected cell in the **Insert** form appears the same as above (all blue and not with a blinking cursor) and **Paste** (Ctrl-V).

If you accidentally got the column order wrong, then you will see an error at this point. Otherwise, the **Insert** form should look like this:



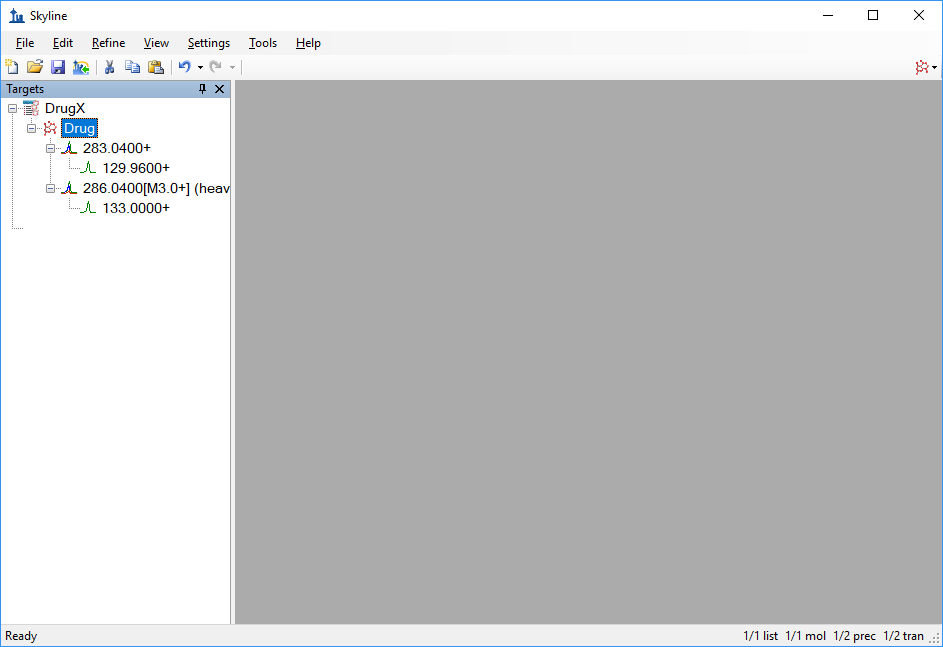
* Click the **Insert** button.

|  |
| --- |
| NOTE: In this tutorial, you have provided only *m/z* and charge values for these targets. Skyline can accept higher level descriptions including chemical formulas and heavy isotope labels etc. Having the chemical formula is especially useful when working with full scan, high resolution data as it allows Skyline to calculate isotopic distributions, but for SRM data such as this, using *m/z* and charge is adequate. |

To see the newly imported targets in full detail:

* On the **Edit** menu, choose **Expand All** and click **Precursors**.

Your Skyline window should now look like this:

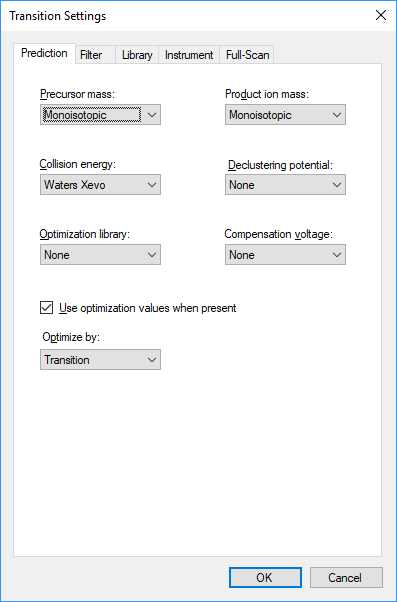


# Transition Settings

The next step is to make sure the transition settings are correct for importing the experimental mass spectrometer results. To do this, perform the following steps:

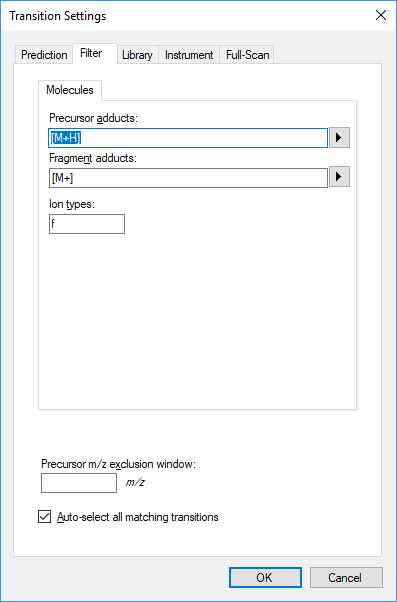
* On the **Settings** menu, click **Transition Settings**.
* On the **Prediction** tab, in the **Collision energy** dropdown list, choose “Waters Xevo”.
* Check **Use optimization values when present**.
* In the **Optimize by** dropdown list which appears when you do this, choose **Transitions**.

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



* Click the **Filter** tab.
* In the **Precursor adduct** field, change the text to “[M+H]”.
* In the **Fragment adduct** field, change the text to “[M+]”.

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



In the **Ion types** field, the value “f” indicates only fragment ion transitions will be measured. If you wanted to measure precursor ions as well, you would use “f, p”.

In the **Instrument** tab, the default values will work for this experiment. In your own work, however, make sure that the minimum and maximum *m/z* values make sense for your actual instrument. The purpose of these settings is to keep you from adding target transitions that your mass spectrometer is not actually able to measure.

Method match tolerance is another important setting in the **Instrument** tab – it determines how closely a measured *m/z* value must match the transition value to be considered a match.

* Click the **OK** button.

The next step is to import the experimental mass spectrometer results.

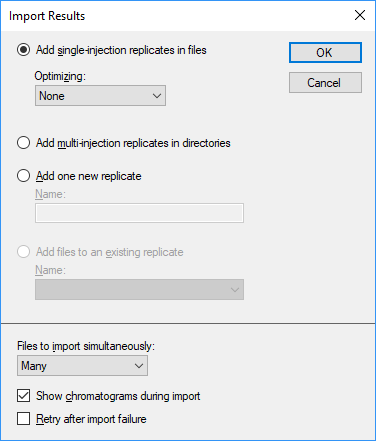
# Importing Mass Spectrometer Runs

This experiment has 113 mass spec result files associated with it. In cases like this, it can be useful to initially import just a handful of the unknowns along with all of the Calibration Curve and Quality Control (QC) files. However, start out with a less complicated document and verify chromatography and calibration curves, etc.

Perform the following steps:

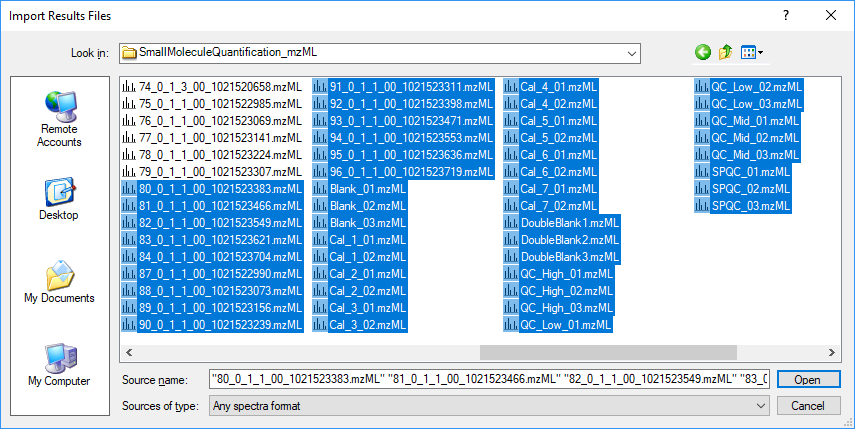
* On the **File** menu, click **Save**. (Ctrl-S)
* Save this document as “SMQuant\_v1.sky” in this tutorial’s folder.
* On the **File** menu, select **Import** and click **Results**.
* In the **Import Results** form, choose **Add single-injection replicates in files**. In the **Files to import simultaneously** dropdown list at the bottom of the form, click **Many** which will provide the best import performance.

The **Import Results** form now looks like this:



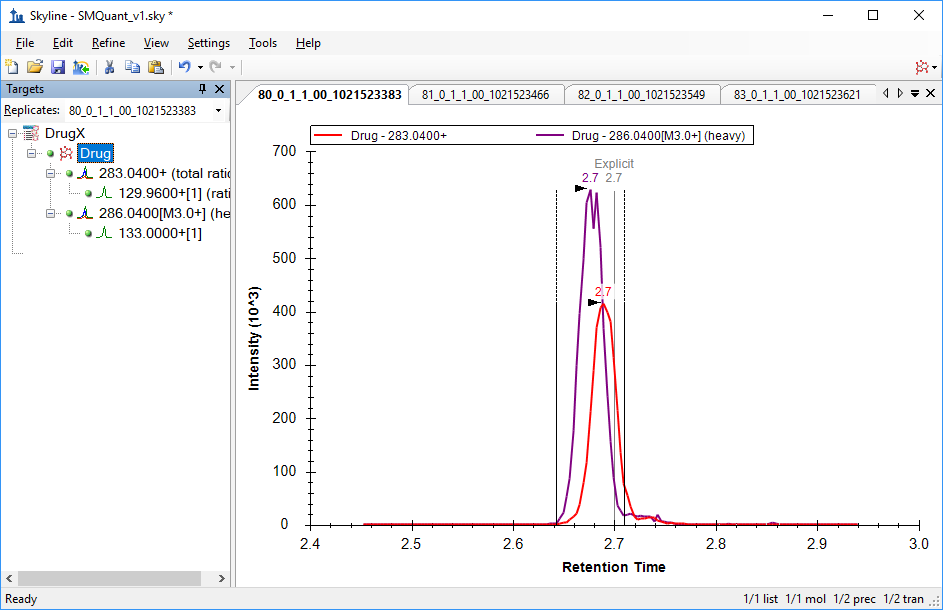
* Click the **OK** button.
* In the **Import Results Files** form that appears, select the last 16 unknowns samples and all the QC samples by clicking the “80\_0\_1\_1\_00\_1021523383.raw” file and then holding down the shift key and clicking the last file in the list.

The **Import Results Files** form should look like:



* Click the **Open** button.

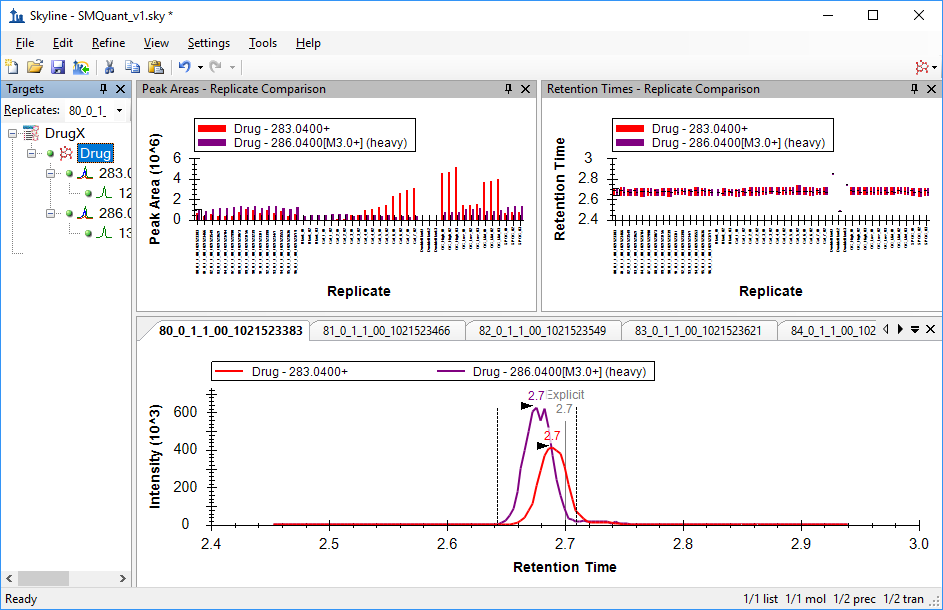
The files should import within 30 seconds or so, leaving your Skyline window looking like this:



To take advantage of the Skyline summary graphs for viewing individual targets, do the following:

* On the **View** menu, choose **Peak Areas** and click **Replicate Comparison**.
* On the View menu, choose **Retention Times** and click **Replicate Comparison**.
* Click and drag these views to the arrows to dock them above the chromatogram graphs.
* Select the first target “Drug” in the **Targets** view.

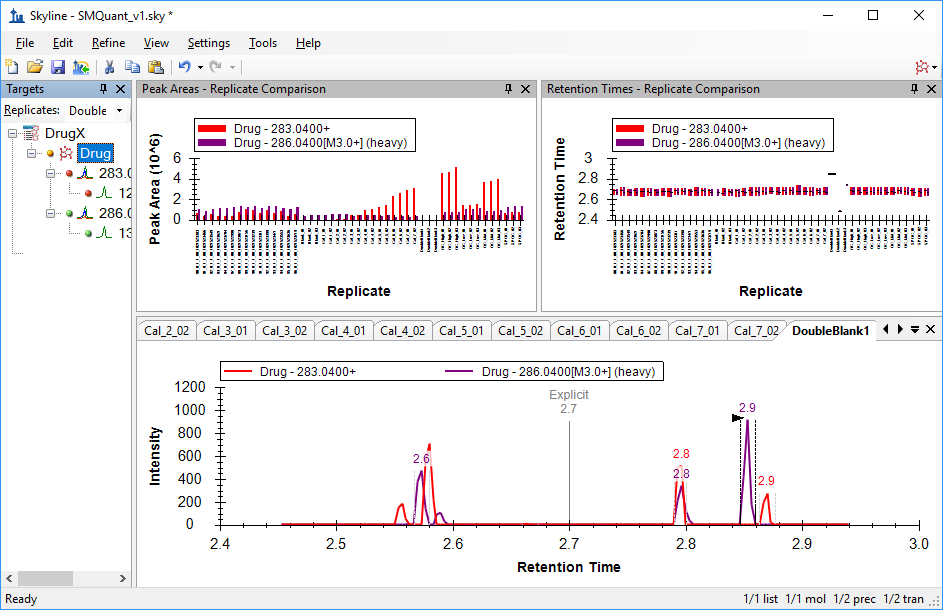
The Skyline window should now look like this:



# Checking Peak Integration

Looking at the **Retention Times – Replicate Comparison** window, you can see by the outliers in a few replicates that Skyline has not chosen peaks at the retention times you might have expected.

Clicking on the first outlier in the **Retention Times – Replicate Comparison** window changes the selected replicate to DoubleBlank1, where you would not actually expect Skyline to find a good peak for either the light or heavy form of the drug.



Clicking on the other two outliers reveals that DoubleBlank2 and DoubleBlank3 are the other replicates with apparently poor chromatography. But, of course, as these are double blanks, you don’t expect any actual peaks in these replicates, so you will manually adjust the integration for each of the double blank replicates to center on the low signal area at 2.7 minutes.

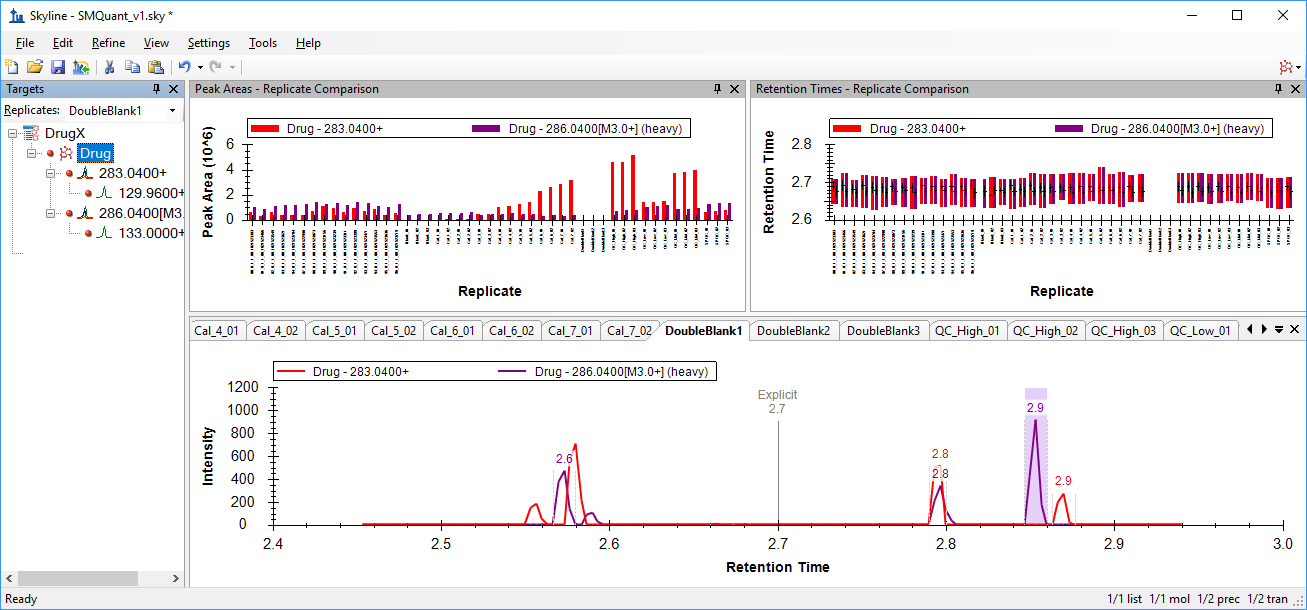
# Adjusting Peak Integration

To adjust peak integration, follow these steps:

* Select the “DoubleBlank1” replicate.
* Position the mouse cursor below the **Retention Time** axis (the cursor will change its shape to this:).
* Click below the **Retention Time** axis at about 2.65 minutes and drag to about 2.75 minutes.

The peak boundaries will change to these new values, and the original boundaries will be marked by a shaded area.

Repeat the steps above for the other two “DoubleBlank” replicates. This should leave Skyline looking like this:

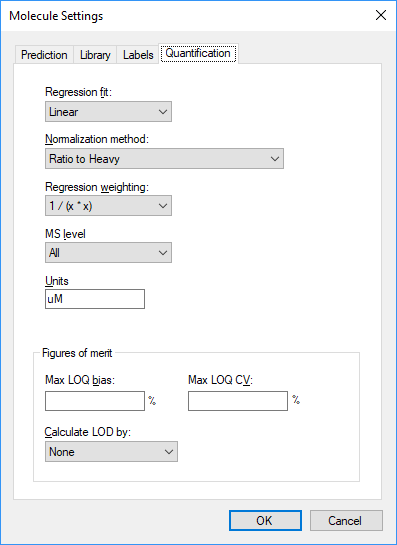


# Preparing for Quantitation

The next step is to move to quantitation. To do this, perform the following steps:

* On the **Settings** menu, choose **Molecule Settings** and then click the **Quantification** tab.
* On the **Regression fit** dropdown list choose “Linear”.
* On the **Normalization method** dropdown list, choose “Ratio to Heavy”.
* On the **Regression weighting** dropdown list, chose “1 / (x\*x)”
* Make sure the **MS level** dropdown list remains showing “All”.
* In the **Units** field, enter “uM”.

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



This experiment uses a linear regression fit, normalizing to the heavy labeled drug. Skyline offers options for weighting across the curve as a function of x: None, 1/x, and 1/(x\*x). This tutorial uses a regression weighting of “1 / (x\*x)”. The **Units** field is for display purposes, and can be set to any value that makes sense for your experiment. The data experiment was calibrated in micromolar, so the **Units** field is set to “uM”.

* Click the **OK** button.

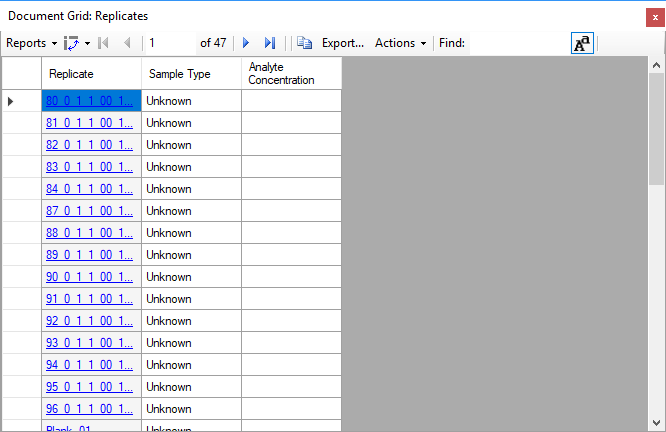
The calibration curve is not quite ready to view yet as it requires declaring the sample types of various replicates for Skyline.

# Declaring Sample Types for Calibration Curve Display

The **Document Grid** will be used to examine and add information about the various replicates. The **Document Grid** is a highly useful tool in Skyline, providing spreadsheet-like views of many document details, many of which can be edited right in the grid. In this case, the focus is in the details of the various replicates, so follow these steps:

* On the **View** menu, click **Document Grid**
* Click on **Reports** in the upper left corner of the grid, and choose **Replicates**

The **Document Grid** should look like this:



* Expand the **Document Grid** if needed so that you can see all the replicates at one.
* If needed, alphabetize the list by clicking on the “Replicate” column header and choose “Sort Ascending”.

The various sample types are displayed. The replicates with the unknowns are ready to go by default.

* Click on the **Sample Type** for “Blank\_01”. Change the sample type from “Unknown” to “Blank”.
* Now shift+click on the **Sample Type** for “Blank\_03” to select all three blank replicates at once.
* Right-click and select **Fill Down**. Everything in the multiple selection now has the same value as the first item in the selection.

Repeat as needed (or, skip ahead to the table below):

* Set all the “Cal\_” replicates with the “Standard” **Sample Type**
* Set the “DoubleBlank\_” replicates as “Double Blank” **Sample Type**
* Set the “QC\_” replicates as **“**Quality Control” **Sample Type**

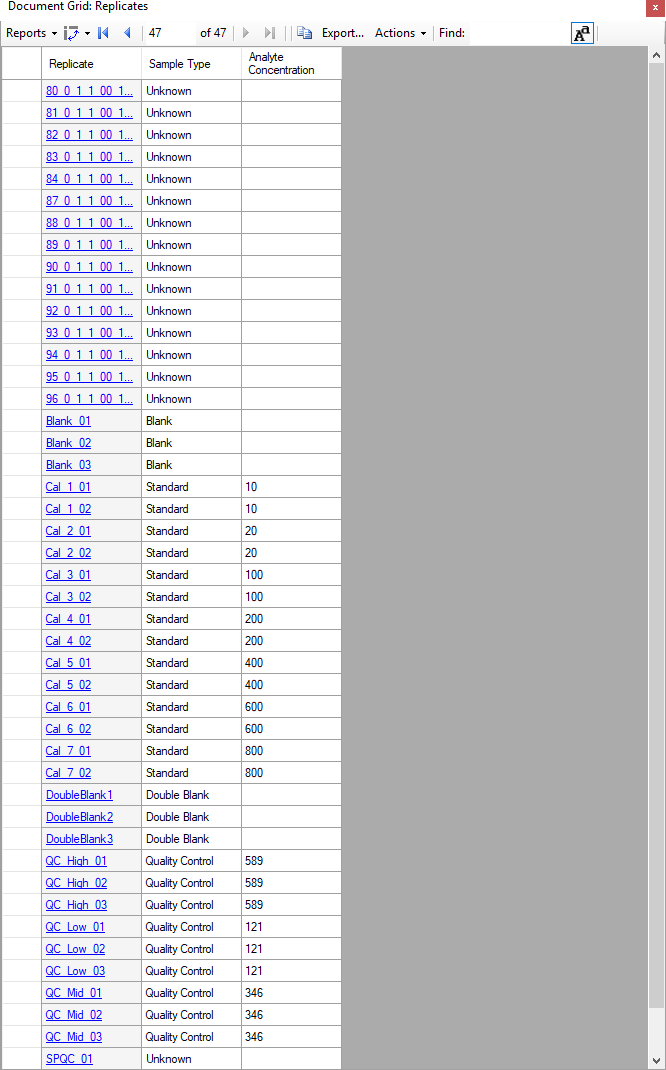
Recall that the “SPCQC\_” replicates are quality control in a different sense (a pooling of all study samples), so those are left as “Unknown”.

Analyte concentrations can be entered by hand, but it is much easier to copy and paste them into the grid.

* Navigate to the “SmallMoleculeQuant” folder and open the “Concentrations.xlsx” file in Excel or any text editor. It should look like this:

|  |  |  |
| --- | --- | --- |
| Blank\_01 | Blank |  |
| Blank\_02 | Blank |  |
| Blank\_03 | Blank |  |
| Cal\_1\_01 | Standard | 10 |
| Cal\_1\_02 | Standard | 10 |
| Cal\_2\_01 | Standard | 20 |
| Cal\_2\_02 | Standard | 20 |
| Cal\_3\_01 | Standard | 100 |
| Cal\_3\_02 | Standard | 100 |
| Cal\_4\_01 | Standard | 200 |
| Cal\_4\_02 | Standard | 200 |
| Cal\_5\_01 | Standard | 400 |
| Cal\_5\_02 | Standard | 400 |
| Cal\_6\_01 | Standard | 600 |
| Cal\_6\_02 | Standard | 600 |
| Cal\_7\_01 | Standard | 800 |
| Cal\_7\_02 | Standard | 800 |
| DoubleBlank1 | Double Blank |  |
| DoubleBlank2 | Double Blank |  |
| DoubleBlank3 | Double Blank |  |
| QC\_High\_01 | Quality Control | 589 |
| QC\_High\_02 | Quality Control | 589 |
| QC\_High\_03 | Quality Control | 589 |
| QC\_Low\_01 | Quality Control | 121 |
| QC\_Low\_02 | Quality Control | 121 |
| QC\_Low\_03 | Quality Control | 121 |
| QC\_Mid\_01 | Quality Control | 346 |
| QC\_Mid\_02 | Quality Control | 346 |
| QC\_Mid\_03 | Quality Control | 346 |
| SPQC\_01 | Unknown |  |
| SPQC\_02 | Unknown |  |
| SPQC\_03 | Unknown |  |

* Make sure the column order matches the **Document Grid**
* In Excel, **Select All** (Ctrl-A), then **Copy** (Ctrl-C).
* In the **Document Grid,** click on the “Blank\_01” cell and click **Paste** (Ctrl-P)

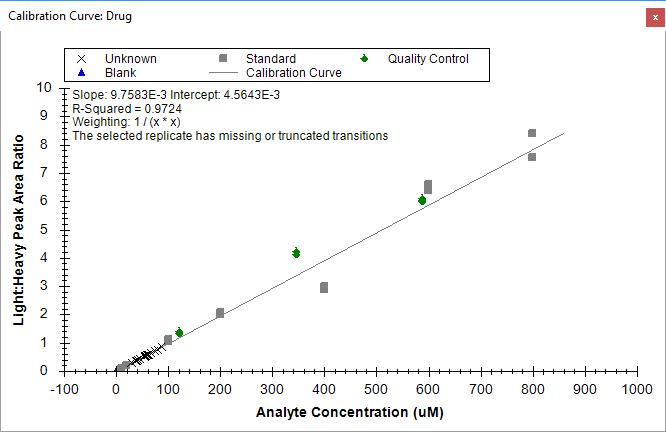
The **Document Grid** should look like this when done: 

# Inspecting the Calibration Curve

It is time to examine the calibration curve.

* Close the **Document Grid**.
* On the **View** menu, click **Calibration Curve**.

The **Calibration Curve** form should appear:

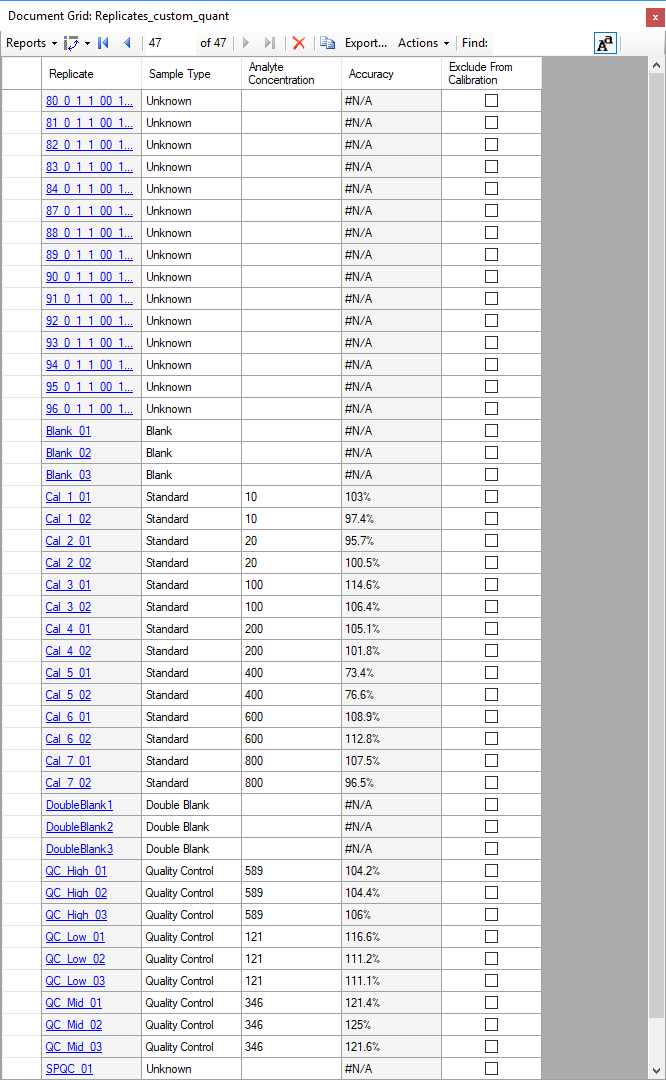


The note about the selected replicate missing transitions is expected, as the currently selected replicate is a double blank.

Looking at the graph, you can see the “Unknowns” are loaded and have relatively low dynamic range.

It is also apparent that some of the calibration samples look like they might be outliers. By using the **Document Grid** to get a qualitative sense of that, you can exclude any samples that are not suitable: To do that, follow these steps:

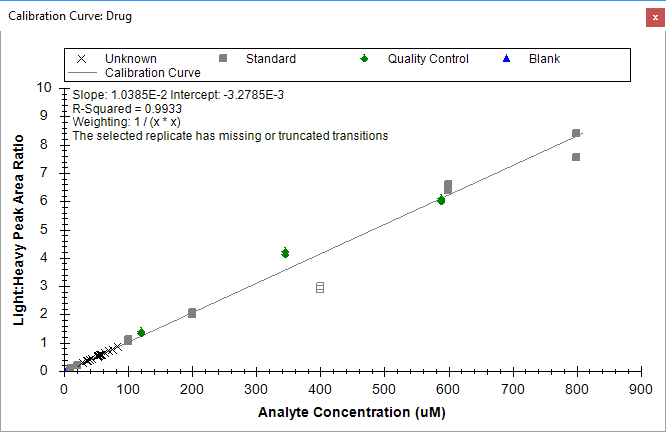
* On the **View** menu, click **Document Grid**
* Click on **Reports** in the upper left corner of the grid, and then click **Replicates**
* Click again on the **Reports** control in the upper left corner of the grid, and select **Customize Report**
* Click on the search button  and enter “accuracy” into the **Find what** field
* Click **Find Next**
* Click the **Close** button on the **Find Column** form.
* In the **Customize Report** form, **Accuracy** should be highlighted under the **Quantification** subcategory.
* Click the **Accuracy** checkbox**.**
* In **Molecule Results** (which is just above **Quantification**), check **Exclude From Calibration**
* In the **View Name** field at the top of the **Customize View** form, enter “Replicates\_custom\_quant”.
* Click the **OK** button.

The **Document Grid** should now look like this: 

The FDA Guidance on which this assay was based states that calibration points should have bias < 15% (accuracy between 85% and 115%) between the known concentration and the backcalculated concentration from the calibration curve. **Accuracy** column shows that “Cal\_5” does not meet that test. These replicates can be removed from consideration using the checkbox in the **Exclude from Calibration** column in the **Document Grid**, or by right-clicking on the outlier in the **Calibration Curve** form and selecting **Exclude from Calibration.** Follow these steps:

* In the **Document Grid**, click on the checkbox in the **Exclude from Calibration** column for the “Cal5\_01” replicate, then press the down arrow key
* Repeat for “Cal5\_02”

The Calibration Curve should now look like the image below. Note that the R-squared value improves from 0.97 to >0.99 by excluding the outlier “Cal\_5”.



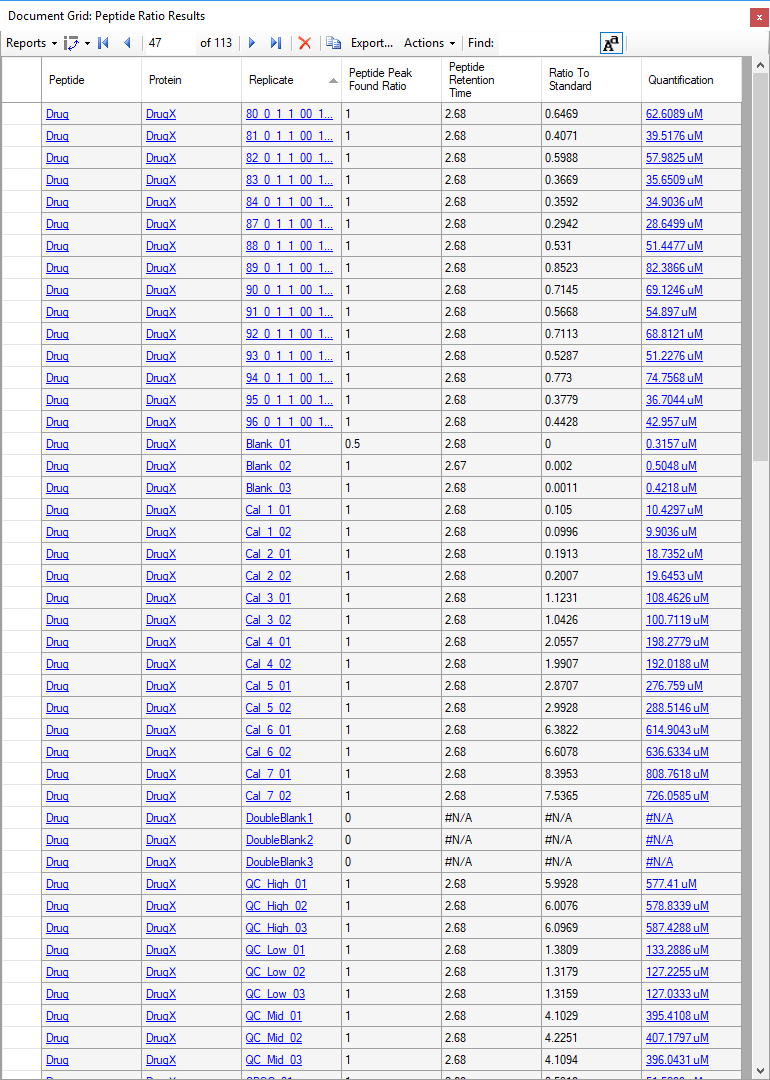
Now it’s time to import the rest of the unknowns:

* On the **File** menu, choose **Import** and then click **Results**.
* In the **Import Results** form, choose **Import single-injection replicates in files**.
* In the **Files to import simultaneously** dropdown list at the bottom of the form, click **Many** which will provide the best import performance.
* Click the **OK** button.
* The **Import Results Files** form appears and displays a collection of raw data files. Select the as-yet-unloaded unknowns
* Click the **OK** button.

A convenient way to view quantitation data is to once again use the **Document Grid**, this time with the **Peptide Ratio Results** view.

* In the **View** menu, click **Document Grid.**
* In the **Reports** dropdown list, click **Peptide Ratio Results**.
* Click the **Replicate** column header and select **Sort Ascending**

The **Document Grid** should look like this:



After removal of the two “Cal\_5” datapoints, further exploration of the data reveals that one of the “Cal\_7” points has accuracy <85% and should likely be removed. This will have little effect on the measurement of the samples, since there are no samples above the level of “Cal\_6”, and only four samples have levels between “Cal 4” and “Cal 6”.

To enable easier visualization of the dynamic range of the samples along the calibration curve:

* Right-Click in the calibration curve window and select **Log Plot**.

The calibration curve should look like this:

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This presents a view which allows the user to easily observe that the samples mostly fall between “Cal\_2” (20 uM) and “Cal\_3” (100 uM), and well within the linear calibration range of the assay. The Quality Control samples (known unknowns, green diamonds above) all have accuracy measured between 85 and 115%, which meets the FDA Guidance criteria.

From here, the next steps would be to export the data for external statistical processing, or establish biological grouping within this document and utilize some of the statistical analysis tools or plugins within skyline. These options are covered in other tutorials.

# Conclusion

In this tutorial, you have learned how to create a Skyline document that targets small molecules specified as precursor ion chemical formulas and adducts, and product ion m/z values. You imported a multi-replicate data set collected using LC-MS/MS on a triple quadrupole, and saw how many existing Skyline features created initially for targeted proteomics use can now be applied to small molecule data. Non-proteomic molecule support is still a relatively new feature area for Skyline. As such, you can expect it to continue improving rapidly.

# Bibliography