

# Skyline Collision Energy Optimization

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Skyline supports a rich user interface and fully automated pipeline for predicting and optimizing SRM instrument parameters like collision energy (CE) and declustering potential (DP). This tutorial focuses on CE optimization, but the same principles apply to DP optimization. In 2016, concept was expanded to include compensation voltage (CoV) on the SCIEX SelexION and more recently Thermo FAIMS instruments. It could eventually apply to other parameters, such as cone voltage and RF lens. This functionality described here has been tested and in use for over a decade on instruments from Agilent, SCIEX, Shimadzu, Thermo, and Waters.

In most cases, the default method in Skyline of assigning CE values to transitions sacrifices very little peak area to full, empirical optimization of each transition separately<sup>1</sup>, but Skyline provides ample support for performing per-transition CE optimization when you feel the need. The default method in Skyline for calculating CE values is to use a linear equation of the form:

$$\text{CE} = \text{slope} * (\text{precursor } m/z) + \text{intercept}$$

Each charge state is allowed to have a separate equation.

The default equations provided with a new Skyline installation generally cover charges 2 and 3 for charge 1 fragments of peptides. If you are working with higher charge state precursors or fragments, then individual fragment optimization is likely to provide greater benefit<sup>2</sup>. If you are working with molecules other than peptides, you should consult the "[Small Molecule Method Development and CE Optimization](#)" tutorial.

This tutorial will cover how to use Skyline both to derive your own linear equations for CE and to perform empirical, per-transition optimization.

## Getting Started

To start this tutorial, download the following ZIP file:

<https://skyline.ms/tutorials/OptimizeCE.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\OptimizeCE

It will contain all the files necessary for this tutorial. Open the file CE\_Vantage\_15mTorr.sky in this folder, either by double-clicking on it in Windows Explorer, or by clicking **Open** in the **File** menu in Skyline.

## Deriving a New Linear Equation

In most cases, you will be able to use an existing linear equation for calculating the CE of your SRM transitions for peptides. If you have used Skyline to run any experiments before, then you have probably already done this. Skyline also makes it easy to derive your own linear equation, or just to check that your system produces similar results to the linear equation you intend to use. This tutorial will walk you through how we did this originally for our Thermo TSQ Vantage.

The file CE\_Vantage\_15mTorr.sky, which you have opened, contains 20 charge 2 precursors and 10 charge 3 precursors, which we measured successfully in the Michrom bovine protein mix. After determining the CE values that produce the maximum peak area for each of these precursors Skyline can perform a simple linear regression to derive the equation we seek. Skyline can also generate the methods containing the measurements it needs to determine those optimal CE values.

The methods Skyline creates will contain multiple transitions for each product ion to be measured over a range of CE values centered at the CE predicted by an existing linear equation.

To look at the linear equation settings used in this experiment do the following:

- On the **Settings** menu, click **Transition Settings**.
- Click the **Prediction** tab.
- From the **Collision energy** drop-list, Choose **<Edit list...>**.
- In the **Collision Energy Regression** list, select "Thermo".
- Click the **Edit** button.

You should be presented with a form that looks like this:

	Charge	Slope	Intercept
▶	2	0.034	3.314
	3	0.044	3.314
*			

In it you can see the slope and y-intercept values used for both charge 2 and charge 3 precursors. Any precursor charges that are not covered will use the linear equation for the closest charge. At the bottom of the form, you can also see the fields **Step count** and **Step size**. These tell Skyline how many transitions to measure for each product ion, at what voltage interval.

For this experiment, we used 5 steps on either side of the equation predicted value, for a total of 11 transitions per product ion, each 1 volt apart. In your own experiments you may choose to change these values to better suit your instrument and your confidence in the original linear equation.

For this tutorial, simply cancel out of the forms you have opened, and we will turn to method creation.

### Measuring Retention Times for Method Scheduling

The optimization method for this tutorial will contain 11 transitions for every product ion or 1320 transitions total. Initial unscheduled measurement of all 1320 transitions required 22 sample injections. By using the Skyline support for scheduled methods, we were able to decrease this number to 5 and even 4 sample injections.

The first step in creating these scheduled methods is to acquire unscheduled SRM for the peptides in the document using the default equation CE values. The unscheduled data will be used to record the

peptide retention time ranges for building a scheduled SRM method for the actual CE optimization. To create an unscheduled transition list do the following:

- On the **File** menu, choose **Export**, and then click **Transition List**.

Make sure the form looks like this:

The screenshot shows the 'Export Transition List' dialog box with the following settings:

- Instrument type: Thermo
- Single method (selected)
- One method per protein (unselected)
- Multiple methods (unselected)
- Order by m/z (unselected)
- Ignore proteins (unselected)
- Max transitions per sample injection: (empty field)
- Methods: 1
- Write S-Lens values (unselected)
- Optimizing: None
- Method type: Standard

- Click the **OK** button.
- Specify your OptimizeCE folder as the location to save.
- Name the file CE\_Vantage\_15mTorr\_unscheduled.csv
- Click the **Save** button.

When you open the resulting CSV file in Excel, you will find it is an unscheduled Skyline transition list for a Thermo Scientific SRM instrument (pre-Quantiva), as shown below, with 6 columns in the order precursor m/z, product m/z, CE, peptide sequence, protein name, fragment ion:

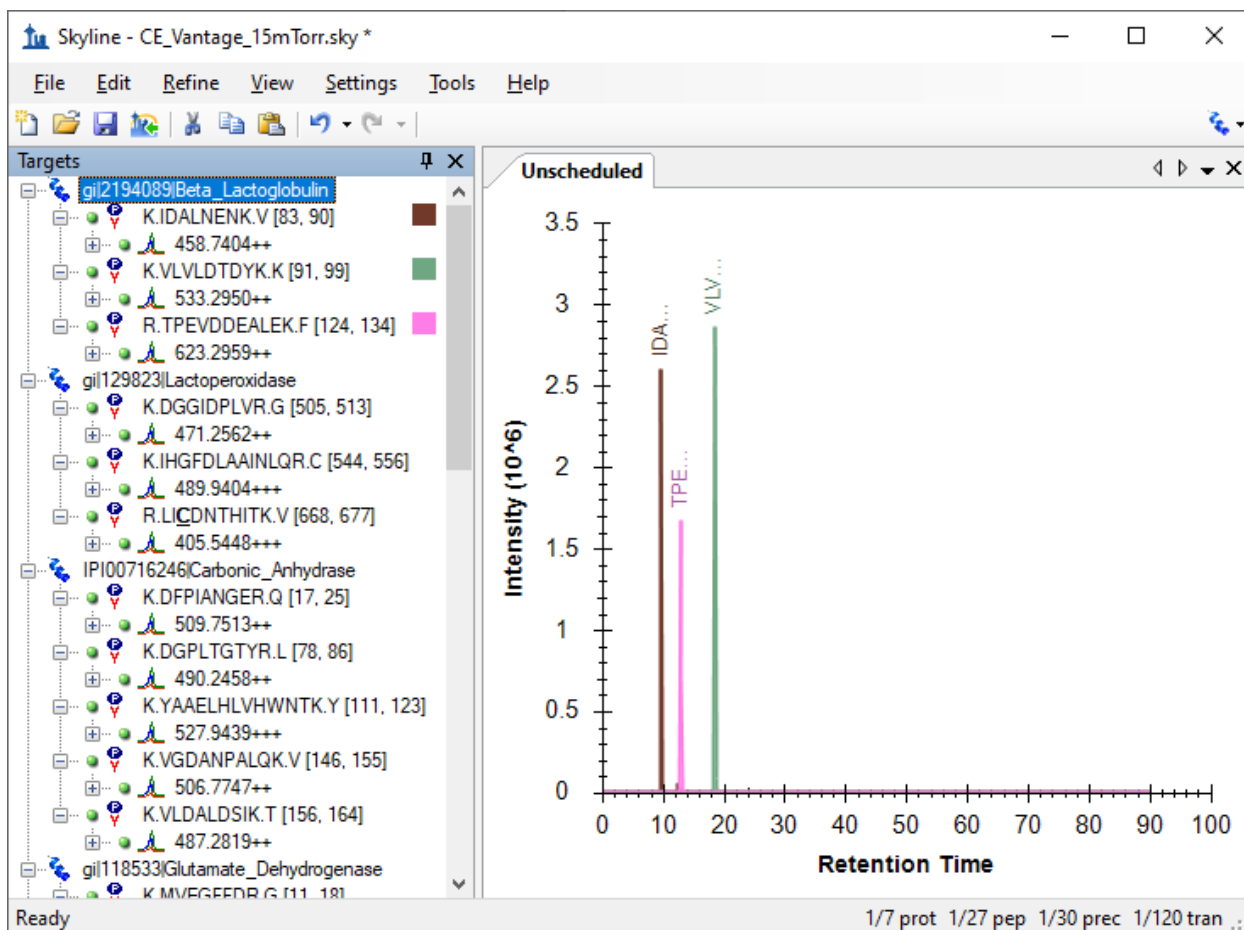
458.7404	688.3624	18.9	IDALNENK	gi 2194089 Beta_Lactoglobulin	y6
458.7404	617.3253	18.9	IDALNENK	gi 2194089 Beta_Lactoglobulin	y5
458.7404	504.2413	18.9	IDALNENK	gi 2194089 Beta_Lactoglobulin	y4
458.7404	390.1983	18.9	IDALNENK	gi 2194089 Beta_Lactoglobulin	y3
533.295	853.4302	21.4	VLVLDTDYK	gi 2194089 Beta_Lactoglobulin	y7
533.295	754.3618	21.4	VLVLDTDYK	gi 2194089 Beta_Lactoglobulin	y6
533.295	641.2777	21.4	VLVLDTDYK	gi 2194089 Beta_Lactoglobulin	y5

533.295	526.2508	21.4	VLVLDTDYK	gi 2194089 Beta_Lactoglobulin	y4
623.2959	1047.484	24.5	TPEVDDEALEK	gi 2194089 Beta_Lactoglobulin	y9

We used this method to acquire SRM data for the 120 transitions with the default, predicted CE. You can import the resulting instrument output file by doing the following:

- On the **File** menu, choose **Import**, and then click **Results**.
- Select **Add one new replicate**.
- In the **Name** field, enter 'Unscheduled'.
- Click the **OK** button.
- Select the file CE\_Vantage\_15mTorr\_unscheduled.raw
- Click the **Open** button.

After the import is completed, Skyline should look like this:

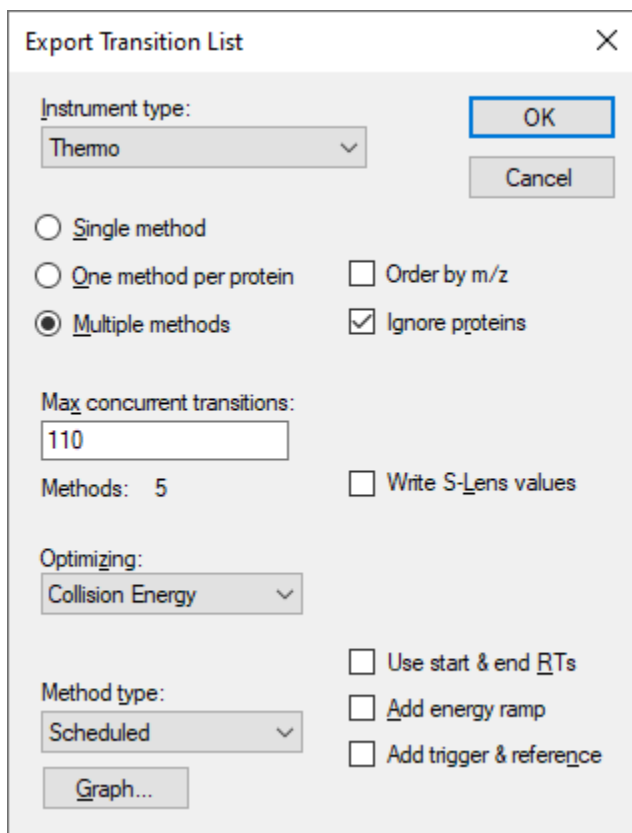


You can select a few of the peptides in the tree-view to see their chromatograms in the chart on the right.

## Creating Optimization Methods

Skyline now has the information it needs to create scheduled optimization methods for the 1320 transitions required. To create these methods:

- On the **File** menu, choose **Export**, and then click **Transition List**.
- Edit the form to look like this:

















The screenshot shows the 'Export Transition List' dialog box with the following settings:

- Instrument type: Thermo
- Max concurrent transitions: 110
- Methods: 5
- Optimizing: Collision Energy
- Method type: Scheduled
- Ignore proteins:
- Order by m/z:
- Write S-Lens values:
- Use start & end RTs:
- Add energy ramp:
- Add trigger & reference:

NOTE: We eventually realized that 132 was a better value for **Max concurrent transitions**, because it allows 3 precursors \* 4 transitions \* 11 CE values to be measured concurrently. The number 110 used in this tutorial is a vestige of initial measurements made with 5 transitions. We encourage you to consider your transitions per precursor \* CE values carefully in choosing this value to maximize your measurements per method.

- Click the **OK** button.
- Specify your OptimizeCE folder as the location to save.
- Name the file CE\_Vantage\_15mTorr.csv
- Click the **Save** button.

These actions should cause Skyline to create 5 new transition lists of similar size, and Windows Explorer should show something like the following for your OptimizeCE folder:

Name	Date modified	Type	Size
 CE_Vantage_15mTorr.sky	3/16/2010 10:28 AM	SKY File	46 KB
 CE_Vantage_15mTorr.skyd	3/16/2010 12:00 PM	Skyline SRM Data	1,317 KB
 CE_Vantage_15mTorr_0001.csv	3/16/2010 12:56 PM	Microsoft Office E...	20 KB
 CE_Vantage_15mTorr_0001.raw	12/13/2009 4:44 PM	Xcalibur Raw File	1,889 KB
 CE_Vantage_15mTorr_0002.csv	3/16/2010 12:56 PM	Microsoft Office E...	24 KB
 CE_Vantage_15mTorr_0002.raw	12/13/2009 4:44 PM	Xcalibur Raw File	1,874 KB
 CE_Vantage_15mTorr_0003.csv	3/16/2010 12:56 PM	Microsoft Office E...	24 KB
 CE_Vantage_15mTorr_0003.raw	12/13/2009 4:44 PM	Xcalibur Raw File	2,302 KB
 CE_Vantage_15mTorr_0004.csv	3/16/2010 12:56 PM	Microsoft Office E...	24 KB
 CE_Vantage_15mTorr_0004.raw	12/13/2009 4:44 PM	Xcalibur Raw File	2,550 KB
 CE_Vantage_15mTorr_0005.csv	3/16/2010 12:56 PM	Microsoft Office E...	27 KB
 CE_Vantage_15mTorr_0005.raw	12/13/2009 4:44 PM	Xcalibur Raw File	2,631 KB
 CE_Vantage_15mTorr_unscheduled.csv	3/16/2010 11:50 AM	Microsoft Office E...	9 KB
 CE_Vantage_15mTorr_unscheduled.raw	12/13/2009 4:44 PM	Xcalibur Raw File	20,022 KB

If you open one of the CSV files in Excel, it should contain a transition list like the one below, with 9 columns in the order precursor m/z, product m/z, CE, start time, stop time, polarity, peptide sequence, protein name, fragment ion:

458.7404	688.3124	13.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y6
458.7404	688.3224	14.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y6
458.7404	688.3324	15.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y6
458.7404	688.3424	16.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y6
458.7404	688.3524	17.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y6
458.7404	688.3624	18.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y6
458.7404	688.3724	19.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y6
458.7404	688.3824	20.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y6
458.7404	688.3924	21.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y6
458.7404	688.4024	22.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y6
458.7404	688.4124	23.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y6
458.7404	617.2753	13.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y5
458.7404	617.2853	14.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y5
458.7404	617.2953	15.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y5
458.7404	617.3053	16.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y5
458.7404	617.3153	17.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y5
458.7404	617.3253	18.9	7.81	11.81	1	IDALNENK	gi 2194089 Beta_Lactoglobulin	y5

There are 11 CE values for each product ion. The product m/z value is incremented slightly for each value as first described by Sherwood et al., 2009<sup>3</sup>. This provides a platform independent means for Skyline to recognize the CE values when the measured data is imported.

## Analyzing Optimization Data

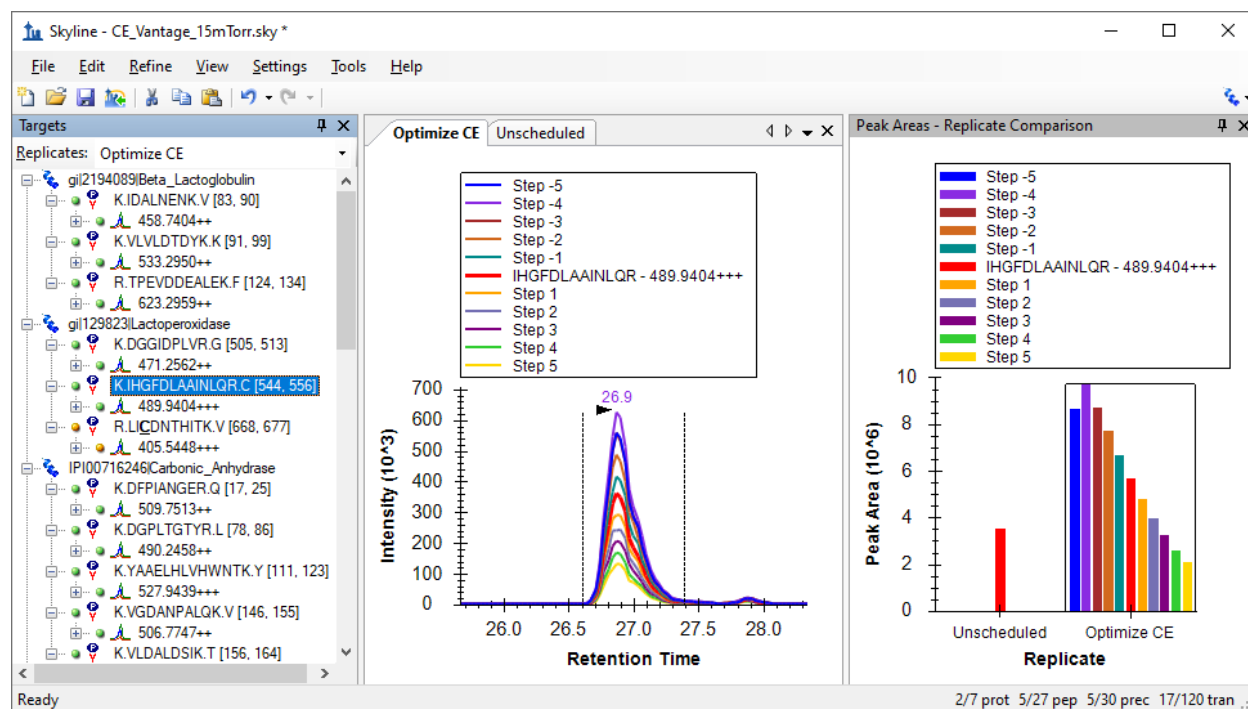
Once data for each of the exported methods is collected, you can import it into Skyline for subsequent analysis. For this tutorial, you will import the instrument output files we have supplied by doing the following:

- On the **File** menu, choose **Import**, and then click **Results**.
- Select **Add one new replicate**.
- In the **Name** field, enter “Optimize CE”.
- From the **Optimizing** drop-list, choose “Collision Energy”.
- Click the **OK** button.
- In the Import Results Files dialog use shift-click to select the 5 raw files CE\_Vantage\_15mTorr\_0001 – 0005.raw
- Click the **Open** button.

While the files are importing, do the following to prepare for viewing the collected data:

- On the **View** menu, choose **Transitions**, and then click **Single** (F10).
- On the **View** menu, choose **Peak Areas**, and then click **Replicate Comparison** (F7).
- On the **View** menu, choose **Auto-Zoom**, and then click **Best Peak** (F11).
- Arrange the graph windows for side-by-side viewing.
- Select a peptide or precursor in the **Targets** view.

Once the data is loaded, Skyline should look something like this:





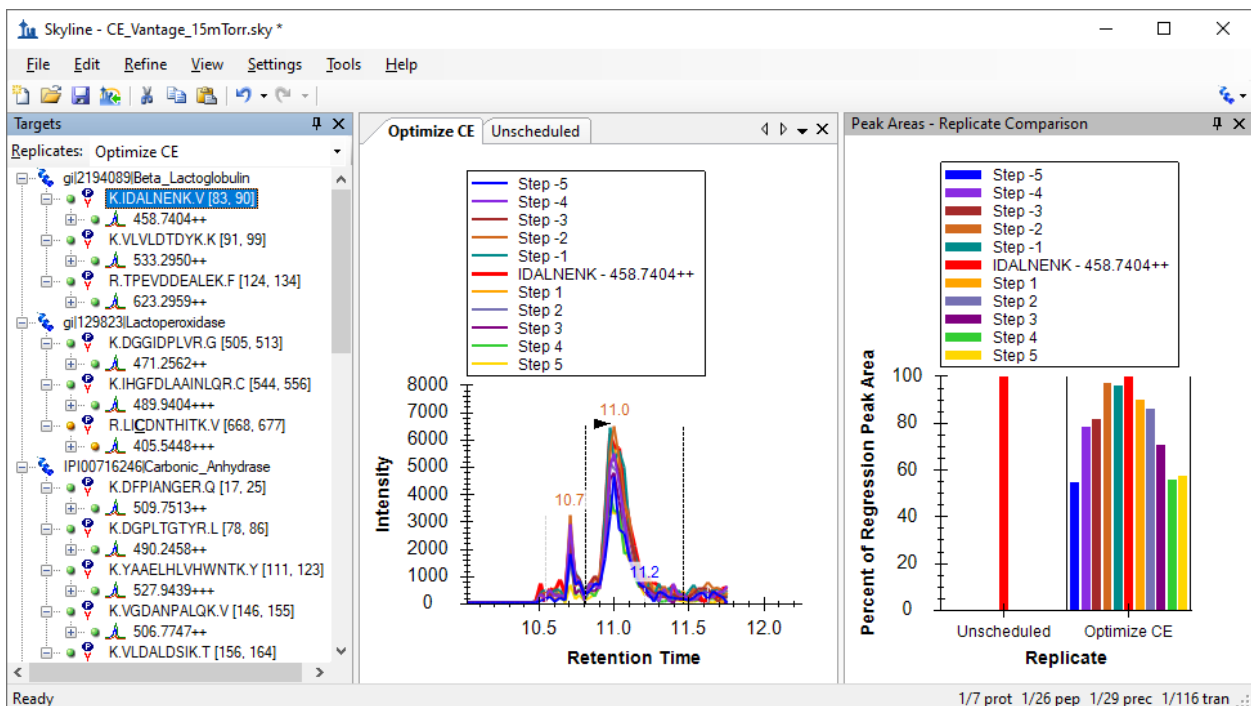
The red bar in the middle of the **Peak Areas** view and the red curve among the chromatograms is the measurement for the transition with the CE calculated by the starting linear equation. In the image above, the maximum peak area was achieved at a CE value 4 volts lower (labeled Step -4 in the legend) than the calculated default CE.

You should now review the peaks for all of the peptide precursors to verify the integration boundaries for each peak. When you get to the peptide EGIHAQQK, you will find that it measured very little signal. Given the quality of the unscheduled peak, this may be due to a retention time shift that caused the peptide to elute outside the scheduling window. Before recalculating the linear equation for CE with these data, you will want to delete this peptide.

The first peptide in the document, IDALNENK, is probably also questionable given its significant drop in intensity and its shift from a retention time of 9.8 minutes to 11.0 minutes. But it is worth looking at from another perspective:

- Select IDALNENK in the **Targets** view.
- Right-click in the peak area chart, choose **Normalize To** and click **Total**.

In this view all peak areas are normalized to the area of the calculated CE value in red. The area of the peak with the calculated CE is given 100%, and the optimization values go from almost invisible beside the unscheduled peak to showing a curve a little less smooth than others, but not that bad.



For this tutorial, however, remove this peptide before calculating the new equation for the Thermo TSQ Vantage.

## Creating a New Equation for CE

To create a newly optimized linear equation for CE using this data, perform the following steps:

- On the **Settings** menu, choose **Transition Settings**.
- From the **Collision energy** drop-list, choose **<Add...>**.
- In the **Name** field, enter 'Thermo Vantage Tutorial'.
- Click the **Use Results** button.

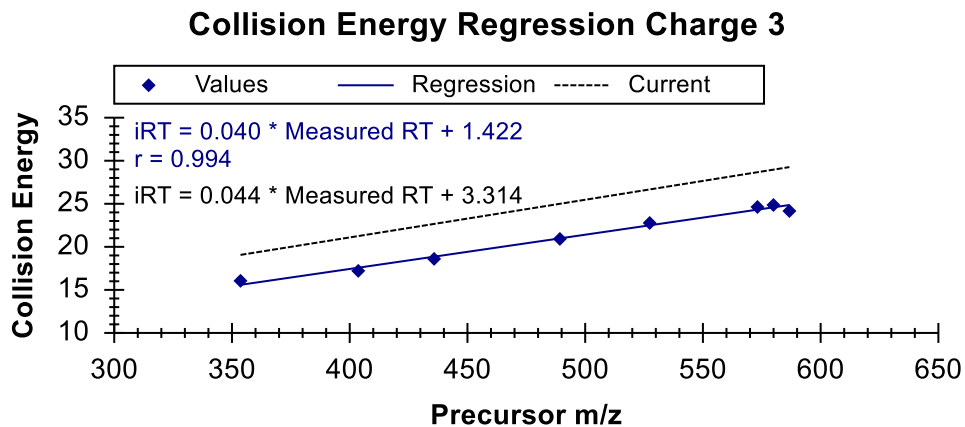
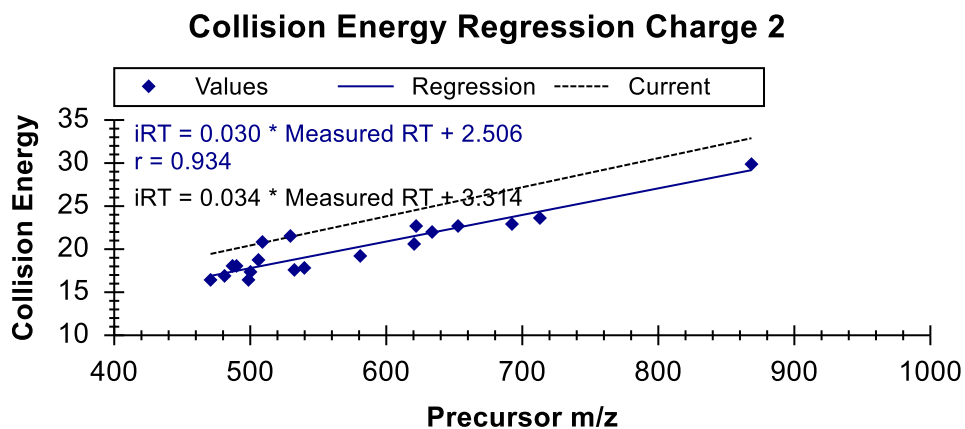
If you deleted the two suggested peptides, Skyline should calculate the equation coefficients as:

Charge	Slope	Intercept
2	0.0305	2.5061
3	0.0397	1.4217

You may wonder how close they are to the original values.

- Click the **Show Graph** button to find out.

Skyline will present the following graphs:



The points correlate very well to the new linear equation, and appear to be on average 3-4 volts below the CE values chosen by the default equations.

- To return to the document, click the **Close** button and two **OK** buttons.

## Optimizing Each Transition

The document settings have been changed to make Skyline use a new linear equation for calculating the CE values in any new method or transition list export. But what if you are planning on moving to measuring large numbers of replicates, and just want to use the CE value which produced the maximum peak area in the optimization data set you imported?

To cause Skyline to use optimal measured values in exported methods:

- On the **Settings** menu, click **Transition Settings**.
- Check the **Use optimization values when present** checkbox.
- From the **Optimize by** drop-list, choose **Transition**.
- Click the **OK** button.

And, to export a method with each transition optimized separately:

- On the **File** menu, choose **Export**, and click **Transition List**.
- Select **Single method**.
- Click the **OK** button.
- Name the file `CE_Vantage_15mTorr_optimized.csv`
- Click the **Save** button.

If you open the exported transition list in Excel, you will see the same 9 columns, because this is still a scheduled method. Even when you have enough cycle time to cover all your transitions for the entire gradient of your experiment, the instrument output files will be smaller and import faster, if you use a scheduled method. These are obviously desirable attributes of data for a large enough multi-replicate study to warrant empirical CE optimization of each transition.

The exported transition list should look like:

533.295	853.4302	17.4	16.35	20.35	1	VLVLDTDYK	gi 2194089 Beta_Lactoglobulin	y7
533.295	754.3618	17.4	16.35	20.35	1	VLVLDTDYK	gi 2194089 Beta_Lactoglobulin	y6
533.295	641.2777	18.4	16.35	20.35	1	VLVLDTDYK	gi 2194089 Beta_Lactoglobulin	y5
533.295	526.2508	23.4	16.35	20.35	1	VLVLDTDYK	gi 2194089 Beta_Lactoglobulin	y4
623.2959	1047.484	21.5	10.98	14.98	1	TPEVDDEALEK	gi 2194089 Beta_Lactoglobulin	y9
623.2959	918.4415	21.5	10.98	14.98	1	TPEVDDEALEK	gi 2194089 Beta_Lactoglobulin	y8
623.2959	819.3731	22.5	10.98	14.98	1	TPEVDDEALEK	gi 2194089 Beta_Lactoglobulin	y7
623.2959	460.2766	24.5	10.98	14.98	1	TPEVDDEALEK	gi 2194089 Beta_Lactoglobulin	y4
471.2562	769.4567	16.3	15.03	19.03	1	DGGIDPLVR	gi 129823 Lactoperoxidase	y7

471.2562	712.4352	15.3	15.03	19.03	1	DGGIDPLVR	gi 129823 Lactoperoxidase	y6
471.2562	599.3511	15.3	15.03	19.03	1	DGGIDPLVR	gi 129823 Lactoperoxidase	y5
471.2562	484.3242	20.3	15.03	19.03	1	DGGIDPLVR	gi 129823 Lactoperoxidase	y4

You can see that the CE values in the third column differ among transitions of the same precursor. Skyline has chosen the CE value that produced the maximum measured peak area for each transition.

## Conclusion

There is certainly more to learn about CE optimization. For instance, you can look into storing your optimized transition CE values in an optimization library, also on the **Transition Settings – Prediction** tab. Hopefully this tutorial will be enough to get you started on using Skyline for your CE optimization needs. If your instrument is not now explicitly covered by name in the Transition Settings list of linear equations for CE calculation, you may want to run your own tests to ensure you are using a linear equation that calculates the best CE values as accurately as possible. If you are performing SRM experiments with many peptides in charge states not covered by an existing equation, you probably will want to calculate new equations for those charge states. This tutorial should have provided you with the tools you will need in these cases. We hope you will use them.

## Bibliography

1. MacLean, B. *et al.* Effect of Collision Energy Optimization on the Measurement of Peptides by Selected Reaction Monitoring (SRM) Mass Spectrometry. *Anal. Chem.* **82**, 10116–10124 (2010).
2. Schmidlin, T. *et al.* Diet-induced neuropeptide expression: feasibility of quantifying extended and highly charged endogenous peptide sequences by selected reaction monitoring. *Anal. Chem.* **87**, 9966–9973 (2015).
3. Sherwood, C. A. *et al.* Rapid Optimization of MRM-MS Instrument Parameters by Subtle Alteration of Precursor and Product m/z Targets. *J. Proteome Res.* **8**, 3746–3751 (2009).