

Tutorial 4: Parameter optimization

The aim of this tutorial is to provide you with a “feeling” of how a few of the parameters that can be set on a QQQ instrument affect SRM results. Therefore, we will ask you several times along the tutorial to browse through all the peptides and observe the effects of a certain parameter.

Information about the sample used: in this tutorial we used the same mixture of 30 crude and heavy synthetic peptides that was used as a reference in the previous tutorials. Such a synthetic peptide mixture allows optimization of all instrument parameters from a low-complexity sample leading to optimal results in terms of sensitivity and data quality. Further, the sample amount of synthetic peptide mixture is typically very high, which for parameter optimization is important, as this requires multiple injections.

A. Collision Energy Optimization (CEO)

Note! On the Skyline website you can find a very detailed tutorial on CEO. The following tutorial has been adapted to our case study.

CEO can be performed for several purposes such as to optimize the general CE equation for your instrument, to further optimize CEs for selected precursors or to even optimize CEs on transition level.

In the following we performed a CEO experiment for the 30 heavy synthetic peptides used already previously throughout our case study.

Transition Settings for CEO

In order to prepare transition lists for CEO, you must choose the optimization parameters in the “Collision Energy” drop-down menu: by how much to increase the CE (step size), and how many steps would you like to include (step count). Note that the final number of steps is defined as $(\text{step count} * 2 + 1)$, as CE steps will be added above and below the default chosen value, which is also included.

- Open the file *TarProCourse_Tut1_Settings.sky*, that was created yesterday.
- Peptide Settings:

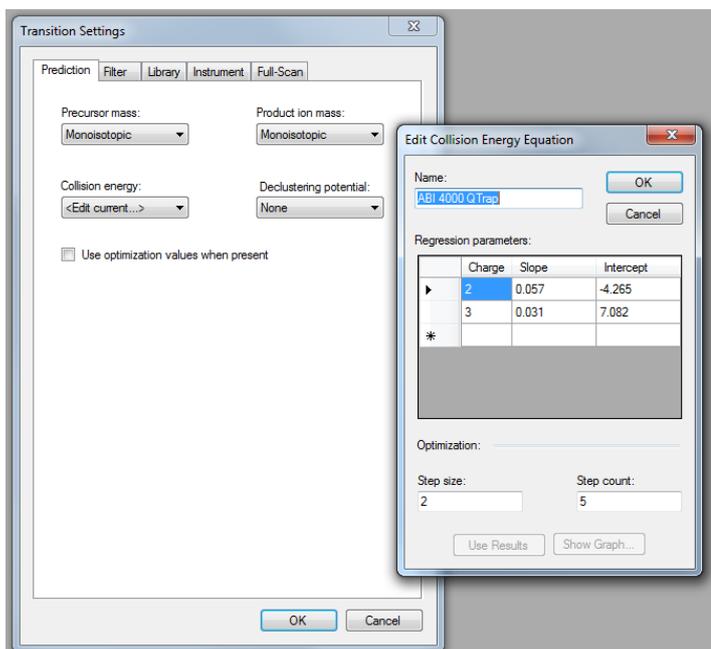
→ Apply peptide settings as described in *Tutorial 1 - Settings*

- Transition Settings:

→ Apply transition settings as described in *Tutorial 1 - Settings*, except for the “Prediction” tab. Here select from the “Collision Energy” drop-down menu:

Edit list → select “ABI 4000 QTrap” → then click “Edit current...”.

An “Edit Collision Energy Equation” window will open. In the lower part of this window you can define the CEO step size (→ select 2), and step count (→ select 5). Click “OK” to close these windows.



Insert a transition list into your Skyline document

Next, we need to insert the list of transitions we wish to optimize. Each transition is then measured in every CE.

We use a transition list similar to the list you generated in *Tutorial 2 - TransitionList*. The complete list is provided in file *transition_list_for_CEO.xlsx* in folder *Tutorial-4_Parameters*. (reminder for inserting transitions: Edit → Insert → Transition list → copy-paste from Excel)

- You should end up with a Skyline document containing **10 proteins, 30 peptides, 30 precursors and 143 transitions**.
- Save your Skyline document as *TarProCourse_Tut4_Parameters.sky* in the folder *Tutorial-4_Parameters*.

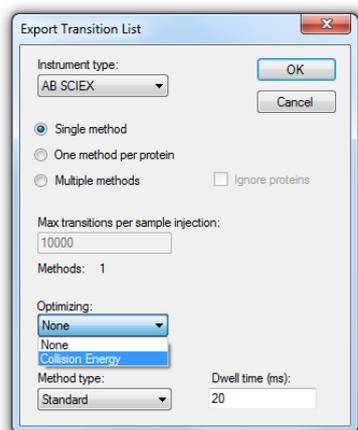
Transition list export for CEO

To perform CEO measurements, Skyline can automatically export a transition list including each transition several times associated with a different CE. To allow the same transition to be concurrently measured and analyzed, the Q3 value for each CE step needs to be slightly changed.

- To export a transition list automatically including all CEO parameters go to: File → Export → Transition List → select: “AB SCIEX”, “Single method”, Optimizing = “Collision Energy”, Method type = “Standard”, Dwell time (ms) = “20”. Click “OK”. You are then asked if to use default settings. Click “No” (meaning that in this case we do not want to use the default).
- Save this transition list as *TarProCourse_Tut4_Parameters_CEO.csv*. Open the file in Excel. You will note that there are more than 1500 transitions generated (143*11). As these are too many transitions to be measured in one

method, we had split this transition list and measured it in several injections. You do not need to do it now, but you can try: export again a transition list, select “multiple methods”. You now need to fill in a number in the window “Max transitions per sample injection”. Skyline will then tell you how many methods will be generated. Try filling in the numbers 100, 300, 500 transitions, and observe the differences.

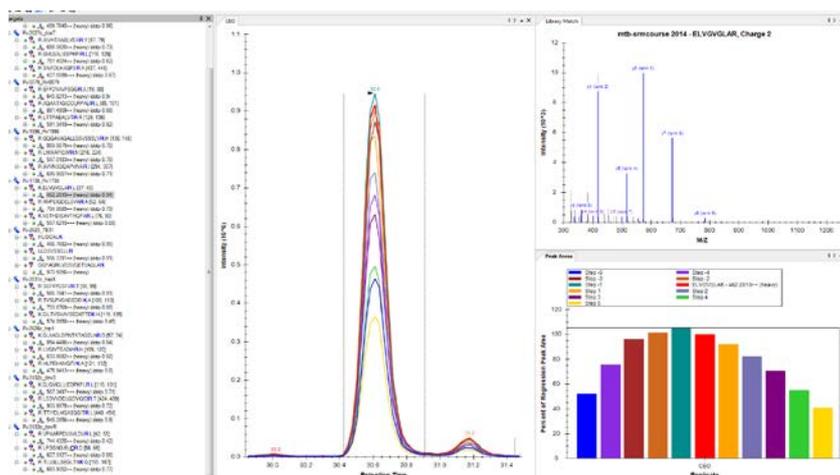
- **Note!** In future, if you perform CEO, you should aim to measure all the CEO transitions of a precursor in the same run. Therefore, in case you split your transition list into several methods, account for the step number in your calculation of transition number per injection, which should be $\#transitions * \#steps$, to ensure that CEO transitions for a precursor are measured concurrently.



Import raw files into your CEO-Skyline document

- Import four measurement files into the Skyline document:
File → Import → Results → Add one new replicate → name it “CEO” → from the Optimizing drop-down menu choose “Collision Energy” → click “OK” → select the 4 measurement files called:
CEO_1.wiff, CEO_2.wiff, CEO_3.wiff, CEO_4.wiff
Note! These files were measured in scheduled SRM mode, a topic which will be covered later in the course in *Tutorial 5 - Scheduling*. →Click “Open”
- Force Skyline to integrate all transitions by selecting: Settings → Integrate all
- Adjust your view settings:
View → Transitions → Single;
View → Peak Areas → Replicate comparison (make sure this is clicked by you);
View → Auto-zoom → Best peak;
(Optional: View → Library Match (to view the library)).

Your Skyline document should now look like this:

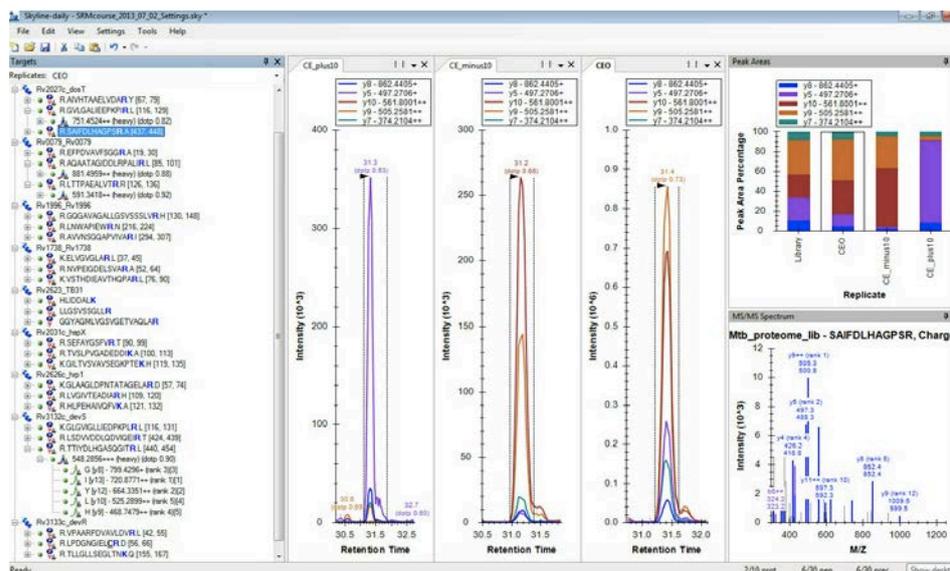


- In the CEO window, when clicking on a precursor, each trace represents the sum of transition intensities measured in a single CE-step, and the matching peak area is presented in the Peak Areas window. These are often centered around the default value (in red).
- Browse through all precursors and check out the effect of CEs onto summed intensity.
- Different transitions of the same precursor might be optimized at different CEs: Browse through the transitions of several peptides and inspect the effect CEO has on the transition level (good example: TTIYDLHGASQGITR). **Note!** CEO will affect the intensity of each transition in a different manner!
- Save this Skyline document as *TarProCourse_Tut4_Parameters_CEO.sky*.
- **Tip!** Following a very similar workflow, you can also perform optimization for declustering potential (DP). It is not covered in this tutorial.

Effect of CEO onto the relative transition intensities per peptide

Since CEO affects transition intensities differently it can also implicate relative intensities, which consequently must be taken into account when comparing SRM traces to a library MS2 spectrum. To see this, carry out the following steps:

- Import two new results as single replicates: CE_plus10.wiff and CE_minus10.wiff. As the names suggest, these files were acquired with a difference of ± 10 V. Arrange the windows so that you can see them all side by side. As these two files start with the letters "CE", Skyline might ask you if to remove "CE" from the name. Select "No".
- Change your view to see all transitions (View → Transitions → All).
- Change the y-axis in the peak area window by normalizing all transition intensities to the total intensity: right click on the window → Normalized To → Total.
- Now browse through all peptides and examine the impact of CE on relative intensities: while for some peptides we saw that CEO improved signal intensities, it can also result in modified relative intensities. To further exemplify this, we can look at the change in the dot product (dotp). To compare the dot product over different samples, right click on the window Peak Areas → Normalized To → None. Inspect the change in dotp between the three runs with different CEs.

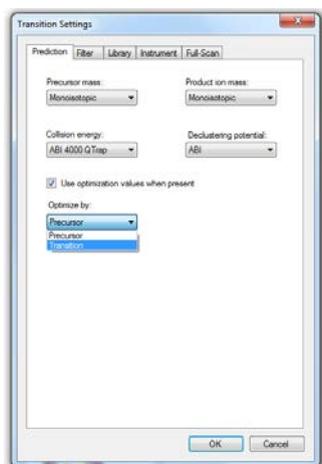


- o In the next few days we will use relative intensities and dotp in choosing the correct peak. Therefore, remember that these are also affected by CE.
- o Save this Skyline document as *TarProCourse_Tut4_Parameters_CE_plusminus10.sky*.
- o Remove the two result files CE_plus10.wiff, CE_minus10.wiff: Edit → Manage results → Select these files → Click “Remove”. Make sure to leave the file “CEO”. In case it disappeared from the view: View → Arrange graphs→ Tiled.

Export a new transition list with optimized CEs

You can export transition lists with optimized CEs either on precursor or transition level by activating the corresponding check box:

Settings → Transition Settings → Prediction → activate “Use optimization values when present” → Optimize by = “Transition” .



Export now a transition list for CE optimization and name it *TarProCourse_Tut4_Parameters_optimizedCE.csv*.

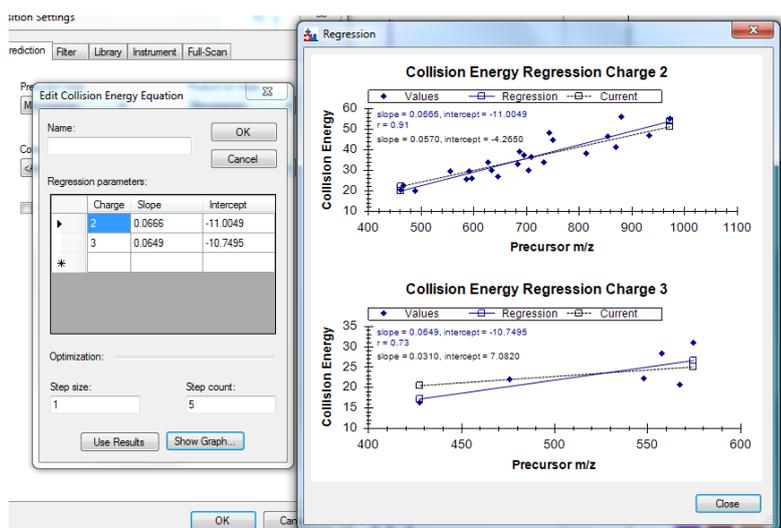
Caution! Optimized values are not stored within Skyline, and therefore you will need to store them elsewhere.

Generate a custom-made CE equation for your own QQQ instrument

Measured CEO values can also be used to extrapolate a new optimal CE equation for your own QQQ instrument. For this select:

→ Transition settings → Prediction tab → Collision energy → select from the drop-list “Add” → Use Results → to visualize the linear regression select “Show Graph” → Name the new equation as “QTrap_TarProCourse” and activate it as the collision energy equation of choice for the document

- How does the new equation compare to the one provided by the manufacturer? (check the r value). You may also receive values that are different than those that appear in the picture below. If so, make sure that “Integrate All” is indeed selected.
- Save this Skyline document as *TarProCourse_Tut4_Parameters_custom-CE-equation.sky*



Caution! Newly generated CE equations can be very sensitive to the peptides chosen for optimization (in this tutorial you may get a different slope. Also, if you wish: unpick several precursors and see what happens to the equation). Hence, to generate a robust and truly representative equation it is advisable to work with a sufficiently large number of peptides, covering the whole range of physicochemical properties (ideally 100s of peptides), at sufficient amounts per injection.

Exercises A

Look at the following transition table for CEO:

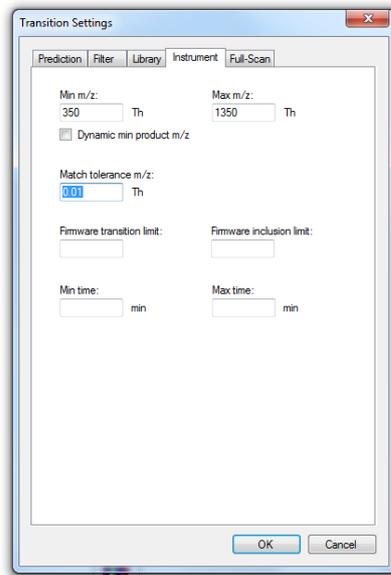
813.43 9	740.342 5	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y8.he avy	31.1
813.43 9	740.352 5	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y8.he avy	33.1
813.43 9	740.362 5	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y8.he avy	35.1
813.43 9	740.372 5	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y8.he avy	37.1
813.43 9	740.382 5	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y8.he avy	39.1
813.43 9	740.392 5	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y8.he avy	41.1
813.43 9	740.402 5	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y8.he avy	43.1
813.43 9	740.412 5	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y8.he avy	45.1
813.43 9	740.422 5	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y8.he avy	47.1
813.43 9	740.432 5	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y8.he avy	49.1
813.43 9	740.442 5	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y8.he avy	51.1
813.43 9	669.305 4	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y7.he avy	31.1
813.43 9	669.315 4	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y7.he avy	33.1
813.43 9	669.325 4	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y7.he avy	35.1
813.43 9	669.335 4	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y7.he avy	37.1
813.43 9	669.345 4	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y7.he avy	39.1
813.43 9	669.355 4	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y7.he avy	41.1
813.43 9	669.365 4	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y7.he avy	43.1
813.43 9	669.375 4	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y7.he avy	45.1
813.43 9	669.385 4	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y7.he avy	47.1
813.43 9	669.395 4	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y7.he avy	49.1
813.43 9	669.405 4	Rv1812c_Rv1812c.VIGVPAMFAAGDVAAAR.+2y7.he avy	51.1

1. How many steps were used ? What is the step count ?
2. What was the step size used ?

3. What is the increment used for Q3 ?

In our tutorial :

4. For which peptide has CEO generated the most significant improvement in signal intensity ?
5. To clarify the use of Q3 increments, set the Match tolerance m/z to 0.01 (Settings → Transition settings → Instrument). How many steps do you see now ? Re-adjust the value to 0.055.



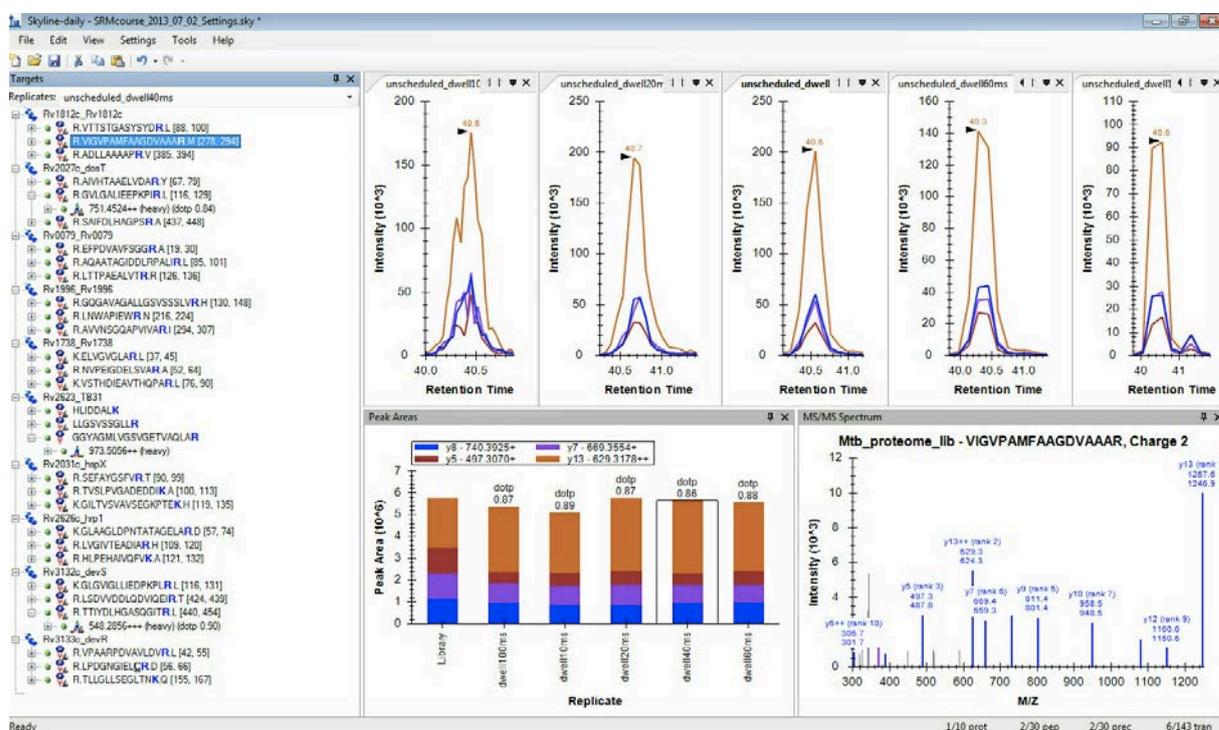
6. Although we have optimized the CE per transition, we will not use it in the next tutorials. Why ?
7. Homework: Further explore CEO by plotting the default CE and the optimized CE per transition in Excel. Approximately how big is the difference ? How could this information affect the choice of step count and step size for future CEO experiments ?

B. Dwell time/ Cycle time

To explore the effects of increasing the cycle time we have measured the same transition list at different dwell times, in an unscheduled mode (reminder: dwell time * #transitions = cycle time). Although we have increased the dwell time, the main take home message of this part relates to increasing cycle time.

1. Import Dwell time results into the Skyline document

- Remove all the results related to CEO via Edit → Manage results → Remove all
- Import as single injection replicates the 5 files from folder *Tutorial-4_Parameters* that are named *unscheduled_dwell*ms.wiff* with * standing for 10, 20, 40, 60 and 100 ms. When asked, do not remove the common prefix.
- Your Skyline document should look like this:



- Browse through the peptides and examine the effect of dwell time onto the peak shape (for example the peptide HLPEHAIVQFVK, or the peptide VIGVPAMFAAGDVAAAR). Note the change in the shape of peaks, resulting from increased cycle time (which means less points per peak).
- Skyline offers several options for smoothing peaks (aiding in visualization and peak picking). In general, smoothing relies on sufficient points of measurement per peak (and this we have here changed by changing the cycle time).
- Have a look at the intensity of a peptide (for example: go to the peptide VIGVPAMFAAGDVAAAR and note down the intensity on the y-axis in each file). Now, perform a visual smoothing of the data by selecting: View → Transform → Savitzky-Golay smoothing. Note that as the cycle time gets longer (in this case because the dwell time gets longer) we have fewer measurement points and the intensity of the peak is changed considerably after smoothing. Important: the areas in the "Peak areas" do not change after smoothing (nor those exported), since smoothing is only affecting visualization.
- Save this Skyline document as *TarProCourse_Tut4_Parameters_cycletime.sky*.

Exercises B

1. Since you know the dwell times we used for each file, how long was the cycle time in each file ? Given the cycle time, estimate how many points are measured on average. (for simplicity, use only the formula given).
2. In this tutorial we changed the dwell time, and thus increased the cycle time. How could be change only the cycle time or only the dwell time ?

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