

Tutorial 5: Retention time scheduling

The term “scheduled SRM” refers to measuring SRM transitions not over the whole chromatographic gradient but only for a short time window around a peptide’s expected retention time. Hereby the number of measurable transitions per SRM run can be significantly increased. The more precise retention times of peptides can be predicted, the narrower a retention time window can be defined and the more peptides can be measured in a single run without loss of sensitivity.

Hence, peptide retention time prediction is very important for targeted proteomics. Within Skyline, peptide retention times can for example be predicted using bioinformatic algorithms, such as SSRCalc (Krokhin et al., Analytical Chemistry, 2006, 78, 6265), which estimate retention times from the amino acid composition and sequence of a peptide (modifications were so far not considered). However, the precision of predictions with bioinformatic tools is limited. Improved values can be obtained using empirically determined reference retention times (iRTs, Escher et al., Proteomics, 2012, 12, 1111). In the following tutorial we will show you how to do retention time prediction with SSRCalc and iRTs.

1. Theoretical retention time prediction with SSRCalc

- Open *SRMcourse_20130715_MtbProtoemeLib_hDP.sky* in folder *Tutorial-3_Library* and save it as *SRMcourse_20130716_SSRCalc.sky* in folder *Tutorial-5_Scheduling*. The file should contain 449 transitions.
- Go to Settings → Peptide Settings → Prediction → Retention time predictor: Select from the drop-down list “Add”.
 - Set a retention time predictor name: “SSRCalc_SRMcourse”
 - Click the button “Calculate >>” in the lower right corner.
 - Open in Excel the file *SSRcalc_calibration_peptides.xlsx* and copy-paste the peptide sequences and retention times into the table “Measured peptides”. This Excel file contains 12 of our target peptides whose retention times have been measured in a survey experiment under the same LC conditions which were used for the main SRM measurements later on. The accurate retention time information is necessary to calibrate the SSRCalc-predicted retention times to your actual LC system.

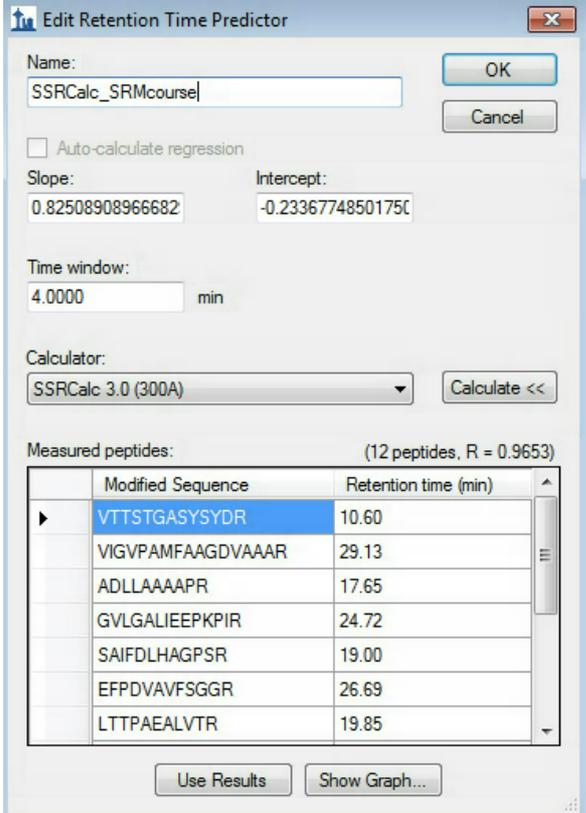
Caution! The measurement from which this retention time information is taken should be carried out immediately before the main SRM measurements in order to avoid retention time shifts due to column aging, column exchange, temperature changes, etc.

Tip! It does not matter which peptides you use for calibration and you can work for example with a subset of your endogenous target peptides (as in our case) or synthetic reference peptides spiked into sample background. For a good calibration curve, however, at least 10 peptides with accurate retention times are required.

Tip! In case you have imported the data file of your calibration run into your Skyline document you can also directly insert retention time results via the “Use results” button (we will use this function for the iRT calculator, see below).

- Select “SSRCalc 3.0 (300A)” from the Calculator drop-down list.
- Skyline will now generate a calibration curve based on the 12 peptides you pasted. The Slope (0.825) and Intercept (-0.233) of the calibration curve are filled in automatically. The peptide number (12) and the R value (0.9653) are shown right above the table.

- Your Edit Retention Time Predictor tab should now look like this:



Name:

Auto-calculate regression

Slope: Intercept:

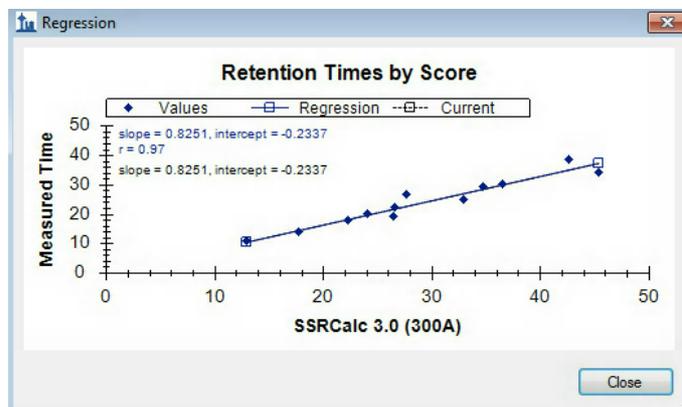
Time window: min

Calculator:

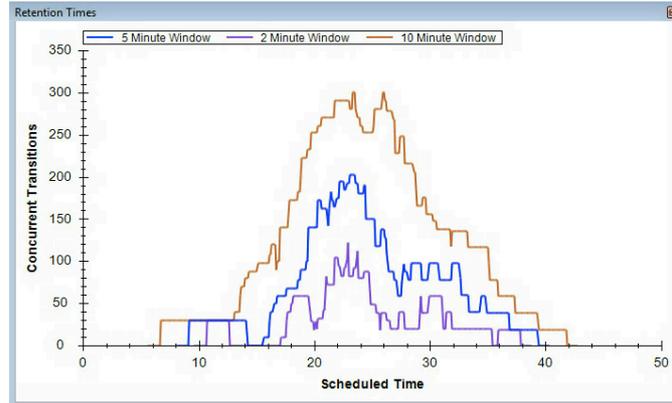
Measured peptides: (12 peptides, R = 0.9653)

	Modified Sequence	Retention time (min)
▶	VTTSTGASYSDR	10.60
	VIGVPAMFAAGDVAAAR	29.13
	ADLLAAAAPR	17.65
	GVLGALIEEPKPIR	24.72
	SAIFDLHAGPSR	19.00
	EFPDVAVFSGGR	26.69
	LTTPAEALVTR	19.85

- Visualise the calibration curve by clicking on “Show graph”:



- Accept the retention time predictor with “OK”.
 - In the Peptide Settings Prediction tab, select from the drop-down menu the “SSRCalc_SRMcourse” as Retention time predictor, activate “Use measured retention times when present”, and set the Time window to 4 minutes.
 - Accept the Peptide Settings with “OK” and save the Skyline file.
- Check the number of transitions which would be concurrently measured depending on the selected retention time window size.
 - View → Retention times → Scheduling



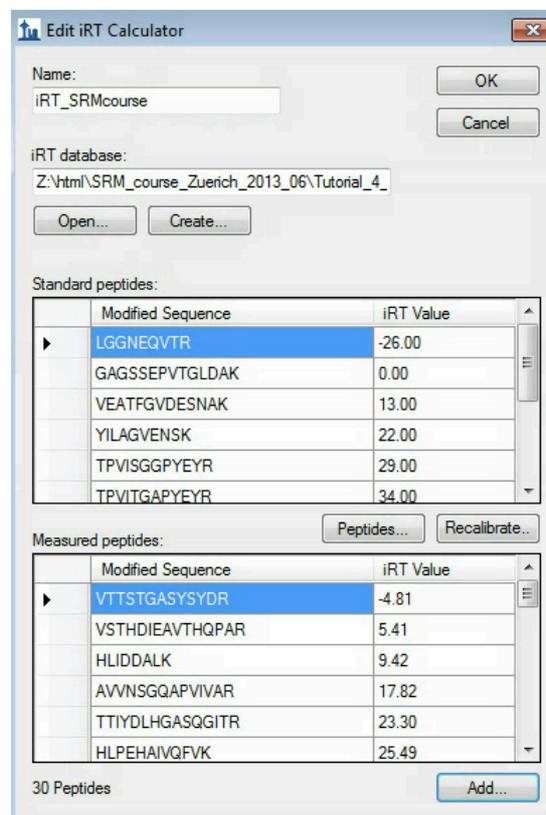
- Right-click on the retention time window → Properties → Add more Time windows: “1, 2, 3, 4, 5, 10, 20” minutes. Explore the effect of the window size onto the number of concurrent transitions.
- Close the “Retention Times” window again before continuing.
- Export a single scheduled transition list for the QTrap 5500 instrument:
 - File → Export → Transition list → Instrument type = “ABSciex”, “single method”, Optimizing = “None”, Method type = “scheduled”.
 - Skyline will show the following message: Select “No” to continue.
- Save the transition list as *SRMcourse_20130716_SSRcalc.csv*.
- Inspect the transition list in Excel. The third column now contains the predicted retention time for the current LC setup.

2. Empirical retention time prediction with a custom iRT calculator

- Start again with the Skyline file *SRMcourse_20130716_MtbProtoemeLib_hDP.sky* from folder *Tutorial-3_Library* and save it in folder *Tutorial-5_Scheduling* as *SRMcourse_20130716_iRTcalculator.sky*.
- Insert iRT standard peptides into your Skyline document.
 - Open in Excel the file “Transition_list_iRT_peptides.xlsx” and copy the iRT peptides with all four columns without header (peptide sequence, Q1, Q3, protein) into Skyline: Edit → Insert → Transition list → paste.
 - Now your Skyline document should contain 471 transitions.
- Import empirical retention time information for target peptides into Skyline.
 - Import the data file “*arielb_Q130619_017.wiff*” into Skyline: File → Import → Results → Add single injection replicates in file → Select: *arielb_Q130619_017.wiff*
To generate this file, a simple mix of the 30 heavy synthetic peptides of our case study were measured together with spiked-in iRT peptides in non-scheduled mode.
Note! Skyline will indicate a successful data upload for a given peptide/transition by the appearance of a coloured circle in front of the respective peptide/transition. The colour of this dot indicates the quality of the picked chromatographic peak (green = good quality, yellow = medium quality, red = bad quality), but is not reliable and should always be manually confirmed one by one.
 - In a typical SRM experiment we would now have to go through the peptides one by one to make sure that always the correct peak is picked. This process will be described in detail in Tutorial 6 – ManualAnalysis. Because this sample was a pure mix of synthetic peptides, the peaks are all picked correctly by Skyline and we do not need to manually interfere.

- Create an iRT calculator.
 - Settings → Peptide Settings → Prediction.
 - Select the little calculator next to “Retention time predictor”. 
 - Click “Add” to bring up the “Edit iRT Calculator” window.
 - Name: “iRT_SRMcourse”
 - iRT database: click on “create” and define the output path of this calculator. It will be saved as a .irtdb file. Place it into the project-independent “Skyline” folder you created in a previous tutorial.
 - Standard peptides: Copy the peptide sequences and iRTs of the 11 iRT peptides from the file “iRT_calibration_peptides.xlsx” and paste them into the Standard peptides table.

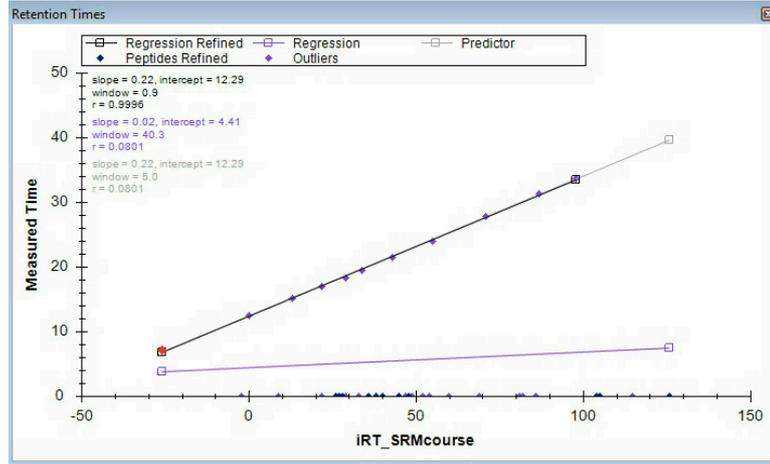
Tip! Here we use the iRT peptides and corresponding iRT values which have been introduced by Biognosys (Escher et al., Proteomics, 2012, 12, 1111). However, you can also use any other standard peptides and fix points for the calibration using the button “Calibrate” right below the Standard peptides table.
 - Measured peptides: Go to “Add...” and select “Add results...”
Skyline takes all retention time results from your current document and converts them into iRT values using information from the measured iRT peptides.
 - Caution!** It is crucial that the iRT peptides were spiked into the sample, measured, and correctly identified by Skyline.
 - Note:** Via the “Add...” button not only iRT values from results, but also from spectral libraries can be added. This is however only possible if the spectral library contains retention time information for target peptides AND standard (iRT) peptides.
 - Caution!** If you insert peptide sequences and iRT values into the Measured peptides table from an external source by copy-paste, make sure that all peptide modifications are indicated correctly. For example carbamidomethylated cysteines need to be written as C[+57.0].
 - Your “Edit iRT Calculator” window should now look like this:



- Confirm your iRT calculator with “OK”.
- Create an iRT Retention time predictor.
 - In the Prediction tab of the Peptide Settings now add an iRT Retention time predictor which uses the newly generated iRT calculator: From the drop-down list in “Retention time predictor” select “Add...”.
 - In the “Edit Retention Time Predictor” window enter:
 - Name: “iRT_SRMcourse”
 - Calculator: select from the drop-down list the “iRT_SRMcourse” calculator we just generated.
 - Activate “Auto-calculate regression”.

Caution! Skyline will now automatically re-calculate, for every data file that you import, the calibration curve based on the iRT peptides, if they were measured in the respective run. Be aware that this requires correctly picked peaks for all iRT peptides in every single SRM run, otherwise significant errors in retention time prediction might occur.
 - Time window: 4 min.
 - Confirm the retention time predictor by clicking “OK”.
 - Select the newly generated retention time predictor in the Prediction tab of the Peptide Settings.
 - Select from the drop-down menu the “iRT_SRMcourse” as Retention time predictor, activate “Use measured retention times when present”, and set the Time window to “4” minutes.
 - Accept the Peptide Settings with “OK” and save the Skyline file.
- Tip!** To show the predicted retention time of each peptide directly within the chromatogram window, right-click on the data/chromatogram window and select “Retention time prediction” (should be shown by default). Note that this is only possible if an iRT value had been stored in the selected iRT calculator.
 - The size of the yellow shaded area can be defined in Peptide Settings → Prediction → Retention time predictor → Drop-down menu: Edit current → Time window.
- Save the Skyline file.
- The file *arielb_Q130619_017.wiff* was used to extract retention times of all our target peptides and we stored them into our iRT calculator. Now we do not need this file anymore: Edit → Manage results → Remove.
- Export a scheduled transition list using iRT retention time prediction
 - Before each scheduled SRM experiment, we need an initial unscheduled calibration run to determine the retention times of the 11 iRT peptides on the current instrument setup (as for SSRCalc prediction). This information can then be used to convert the stored iRT values of all target peptides into expected retention times on the current setup.

Caution! For such a calibration run, the standard peptides have to be measured within the same peptide background as the following runs (i.e. your samples of interest), because sample complexity can significantly influence peptide retention time.
 - Import the calibration run into Skyline:
File → Import → Results → Add single injection replicates in file → Select: *olgas_S130701_001_A1_RTcal.wiff*
 - Click through the chromatograms of the 11 iRT peptides.
 - Inspect the linear regression obtained with the iRT calculator:
View → Retention Times → Linear Regression.
Right-click onto the linear regression graph to switch between regression curves obtained with various calculators.



- Save as *SRMcourse_20130716_iRT.sky*.
- Export a single scheduled transition list for the QTrap instrument by clicking: File → Export → Transition list → Instrument type = “ABSciex”, “single method”, Optimizing = “None”, Method type = “scheduled”.
- Save the transition list as *SRMcourse_20130716_iRT.csv* and inspect it in Excel.

Exercises

1. What is the difference between a “Retention time calculator” and a “Retention time predictor”?
2. Which retention time window size would you apply if you wanted to run a single scheduled method with maximally 150 concurrent transitions? Could you apply this to SSRCalc as well as iRT prediction?
3. To compare the predicted retention times from SSRCalc and iRT calculator, generate a graph showing the correlation between both predictors. Which correlation coefficient (R^2) is achieved?
2. In a scheduled SRM method, not the dwell time but the cycle time is kept constant. This means that the dwell time for a given transition can change over time depending on the number of concurrent transitions. To which minimal dwell time does this approximately lead when using the transition list generated above with a 4 min retention time window size and a fixed cycle time of 2 seconds? At which time of the gradient does this minimal dwell time approximately occur?
3. Why do continuously changing dwell times in scheduled SRM runs not lead to observable differences in signal intensities? (longer dwell times should increase signal intensity, shorter dwell times decrease signal intensity)
4. What is the difference between SSRCalc 3.0 (100A) and SSRCalc 3.0 (300A)?

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