# 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. Thereby 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 can so far not be 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 *TarProCourse_Tut3_MtbProtoemeLib_hDP.sky* in folder *Tutorial-3_Library* and save it as *TarProCourse_Tut5_SSRCalc.sky* in folder *Tutorial-5_Scheduling*. The file should contain 444 transitions.

- Go to "Settings" → "Peptide Settings" → "Prediction" → "Retention time predictor" Select from the drop-down list "Add…".
    - Set a retention time predictor name: "SSRCalc_TarProCourse"
    - Click the button "Calculate >>" in the lower right corner.
    - Open in Excel the file *SSRcalc_calibration_peptides.xlsx* (in folder *Tutorial-5_Scheduling)* and copy-paste the peptide sequences and retention times into the table "Measured peptides".
      **Note!** 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 will be used for the main SRM measurements later on. Accurate retention time information for a small number of random peptides (ideally spanning the whole retention time range) 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. Ideally, these peptides span the entire retention time range.

      **Tip!** In case you have imported the data file of your calibration run into the 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.

o Skyline will now generate a calibration curve based on the 12 peptides you pasted. The Slope (0.825) and Intercept (-0.234) of the calibration curve are filled in automatically. The peptide number (12) and the R value (0.9653) are shown right above the table.

o Set the "Time window" to 4 minutes

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



o Visualise the calibration curve by clicking on "Show graph", close it afterwards.
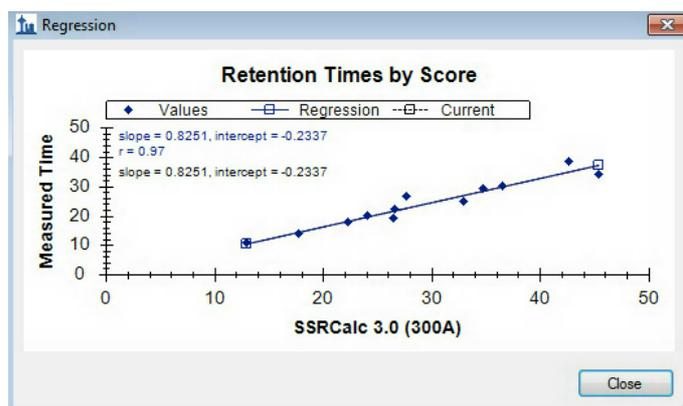


o Accept the retention time predictor with "OK".

o In "Peptide Settings" → "Prediction": Make sure that the above defined retention time predictor "SSRCalc_TarProCourse" is activated. "Use measured retention times when present" should be activated (default) and the time window should be set to "4" minutes.

- o Accept the "Peptide Settings" with "OK"
- o Save the Skyline file (under the name which has already been defined above).

- Now that you have activated a retention time predictor, Skyline can assign expected retention times to all target peptides defined in your document. Skyline provides a graphical view showing the number of transitions, which will be concurrently measured depending on the selected retention time window size. To show this graph go to:
  - o "View" → "Retention times" → "Scheduling"
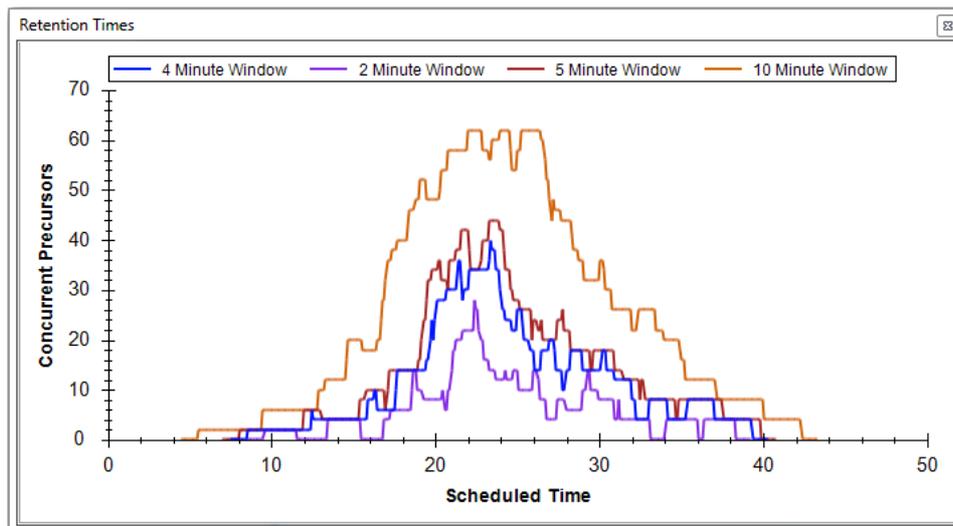


- o Right-click on the graph and select "Properties…" to add more time windows, for example: "1, 2, 3, 4, 5, 10, 20" minutes. Explore the effect of the window size onto the number of concurrent transitions.
- o Close this "Retention Times" window before continuing.

- Now export a single scheduled transition list. For this go to:
  - o "File" → "Export" → "Transition list" → Select instrument type "AB SCIEX", "Single method", Optimizing = "None", Method type = "Scheduled".
  - o Now click "OK". Skyline will show the following message:
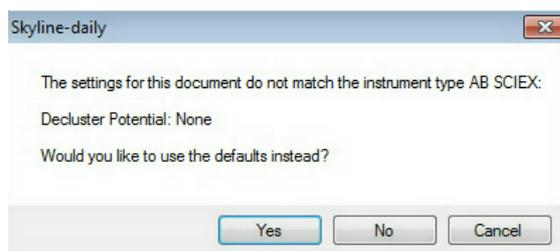


Select "No" to continue.

- Save the transition list as *TarProCourse_Tut5_SSRCalc.*csv in folder *Tutorial-5_Scheduling*
- Inspect the transition list in Excel. The third column now contains the predicted retention time for the current LC setup and no longer the dwell time.

**Tip!:** The scheduled window size around the predicted retention time must be defined for ABSciex instruments directly in the method file on the instrument itself. However, for other instrument vendors, the scheduled window size can be defined within the transition list output (contains two columns, one with start and one with stop window time, e.g. for Thermo).

## 2. Empirical retention time prediction using iRT

- Start again with the Skyline file *TarProCourse_Tut3_MtbProtoemeLib_hDP.sky* from folder *Tutorial-3_Library.*

- Insert iRT standard peptides into your Skyline document (the ones we are using here are commercially available from Biognosys).
    - Open in Excel the file "*Transition_list_iRT_peptides.xlsx*" (in folder *Tutorial-5_Scheduling*) and copy all four columns (peptide sequence, Q1, Q3, protein) without header. Go to "Edit" → "Insert" → "Transition list" → paste the four columns. Make sure the "Peptides" button is selected and click "Insert"
    - Now your Skyline document should contain **466 transitions**.

- Save the Skyline file in folder *Tutorial-5_Scheduling* as *TarProCourse_Tut5_iRT.sky.*

- 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* from folder *Tutorial-5_Scheduling*.
      To generate this file, a pool of the 30 heavy reference peptides of our case study was 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 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 so 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 - Manual Analysis*. 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.
    - Two windows (Peak Areas and Retention Times) might open, which you can close again. We will discuss these in *Tutorial 6 - Manual Analysis*.

### 2.1    Manually calculate iRT values for all target peptides

The file *arielb_Q130619_017.wiff* contains the retention time of all target peptides and the retention time of the iRT standard peptides. With this information we can convert empirical retention times into unit-free iRT values.

- Export the empirical retention time of all targets and iRT standard peptides by selecting: "File" → "Export" → "Report" → select the default export called "Peptide RT Results" → save as *TarProCourse_Tut5_iRT_report.csv* in folder *Tutorial-5_Scheduling*.
- Open the .csv file in Excel.
- Remove the columns with the header "ReplicateName", "PredictedRetentionTime" and "PeptidePeakFoundRatio". Only "PeptideSequence", "ProteinName" and "PeptideRetentionTime" are of importance for us.
- Add two new columns called "iRT standard peptides" and "iRT calculated".
- Into the column "iRT standard peptides" paste the 11 iRT values for the standard peptides given in the Excel file *iRT_calibration_peptides.xlsx*, which can be found in the folder *Tutorial-5_Scheduling*.
  **Tip!** The Excel function "vlookup" can be used to add information from one Excel table to another one.

- Plot the empirical retention times and iRT values of the 11 standard peptides using the scatter plot function. Label the axes with "iRT" and "Empirical RT".
- Right click on the data points and add a linear trendline. Display the equation and the R-squared value on the chart (under "Options").
Below is what you should have from excel now:
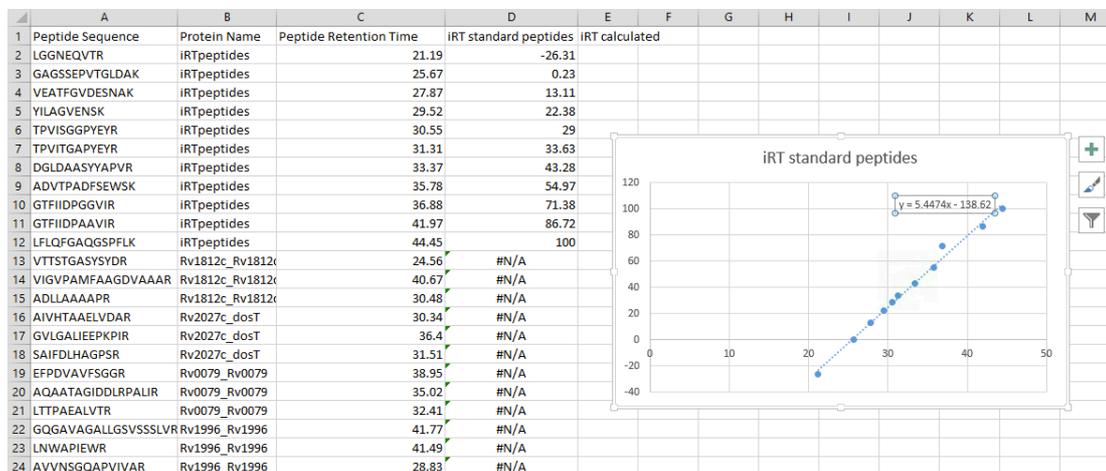


- Use the generated linear equation to calculate iRT values for all your target peptides in the column "iRT calculated". You did it the right way if for the iRT peptides, the calculated iRT values are similar to the given iRT values.
- Save the file as an Excel workbook (not a csv file) with the name *TarProCourse_Tut5_iRT_report.xlsx.*

## 2.2 Automatically generate iRT values within Skyline

- To create an iRT calculator in Skyline, go to "Settings" → "Peptide Settings" → "Prediction".
- Click on the little calculator icon [icon] and click "Add…" to create a new iRT Calculator.
    - o **Name:** "iRT_TarProCourse"
    - o **iRT database:** Click on "Create…" and define the output path of this calculator. Name it "iRT_TarProCourse" and place it into the project-independent "Skyline" folder. It will be saved as an .irtdb file.
    - o **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. 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.
    - o **Measured peptides**: Click "Add…" and select "Add Results…".
      Skyline now converts all retention time results from your current document automatically into iRT values (i.e. what we did manually in Excel before).
      Click "OK" to close the notification about successful calculation.

      **Caution!** It is crucial that the iRT peptides were spiked into the sample, measured, and correctly identified by Skyline.

      **Caution!** If you manually 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].

**Tip!** 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.

o    The "Edit iRT Calculator" window should now look like this:
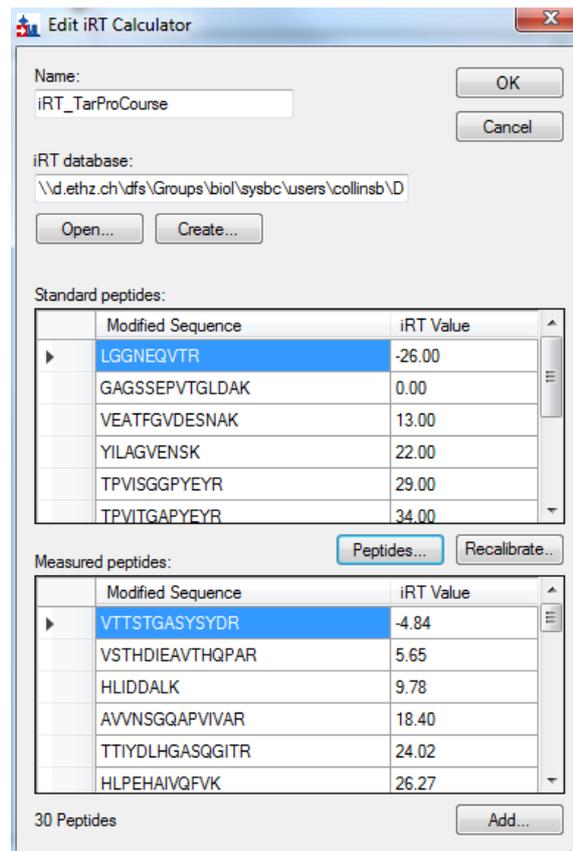


o    Confirm your iRT calculator with "OK".

- **Create an iRT Retention time predictor.**

- o In the "Prediction" tab of the "Peptide Settings" add a "Retention time predictor" which uses the newly generated iRT calculator. To do so select from the drop-down list of the Retention time predictor "Add…".
- o **Name:** "iRT_TarProCourse"
- o **Calculator:** select from the drop-down list the "iRT_TarProCourse" calculator we just generated.
- o 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. Be aware that this requires that those standard peptides were present and measured in each run and that they were correctly picked by Skyline, otherwise significant errors in retention time prediction might occur.
- o **Time window:** 4 min.
- o Confirm the retention time predictor by clicking "OK".
- Activate the newly generated retention time predictor "iRT_TarProCourse" by choosing it from the drop-down menu in the "Prediction" tab of the "Peptide Settings".
- 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.

## 2.3 Export a scheduled transition list using iRT retention time prediction

- The file *arielb_Q130619_017.wiff* was used to extract retention times of all our target peptides and to store them in the iRT calculator. Now we do not need this file anymore: "Edit" → "Manage Results…" → "Remove". → "OK"
- For every 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. This information can then be used to convert the stored iRT values of all target peptides into expected retention times on the current LC setup.
  **Caution!** For such a calibration run, the standard peptides have to be measured within the same peptide background as the following runs (your samples of interest), because sample complexity can significantly influence peptide retention time.
- Import the iRT 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".
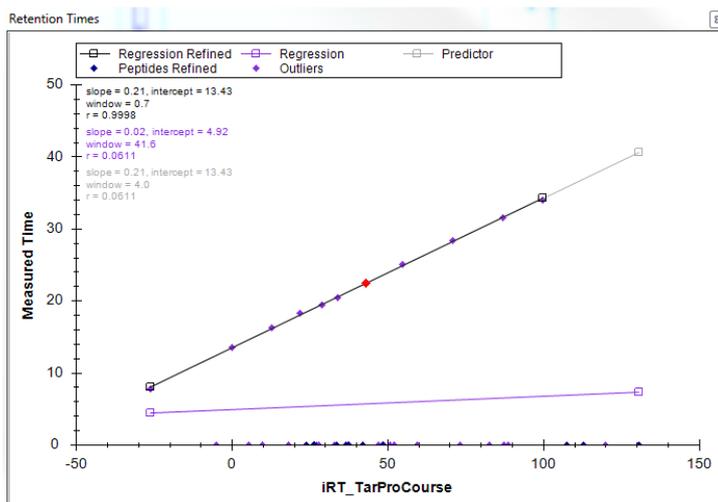
- Right-click onto the linear regression graph to switch between regression curves obtained with various calculators.
- Save the Skyline file in folder *Tutorial-5_Scheduling* as *TarProCourse_Tut5_iRT_scheduled.sky*
- Export a single scheduled transition list for the QTrap instrument by clicking: "File" → "Export" → "Transition list" → Instrument type "AB SCIEX", "Single method", Optimizing "None", Method type "scheduled".
- Save the transition list as *TarProCourse_Tut5_iRT_scheduled.csv* and inspect it in Excel.

## Exercises

1.  Compare the iRT values that you generated manually in Excel with the iRT values generated automatically within Skyline (you can copy-paste the Skyline values out of the "Retention Time Calculator" table).

2.  iRT standard peptide GTFIIDPGGVIR seems to be an outlier in the retention time linear regression viewer of file *arielb_Q130619_017.wiff* (in Skyline file SRMcourse_20140211_iRT.sky). Can you explain what is the issue here?

3.  What is the difference between a "Retention time calculator" and a "Retention time predictor"?

4.  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?

5.  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?

6.  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?

7.  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)

8.  What is the difference between SSRCalc 3.0 (100A) and SSRCalc 3.0 (300A)?

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