

Tutorial 3: Building a spectral library in Skyline

Spectral libraries for SRM method design and for data analysis can be either directly added to a Skyline document (in case a suitable library in the file formats .sptxt, .blib, .hlf or .msp is available) or a custom library can be built within Skyline. For the latter, data-dependent acquisition (shotgun) as well as SRM-triggered MS2 measurements searched with one of the common search engines can be used (currently 15 search engines are supported).

In this tutorial we describe how you can build a spectral library for 30 synthetic peptides that have been measured in SRM-triggered MS2 mode on a QTrap instrument.

1. Short introduction into SRM-triggered MS2

SRM assays can be generated based on fragmentation information of MS2 spectra derived from any shotgun analysis. However, shotgun analyses are typically carried out on ion trap mass spectrometers which show, due to different collision cell architecture, slightly different peptide fragmentation patterns compared to triple quadrupole instruments. One way to generate an MS2 spectrum directly on a triple quadrupole, and hence ensure an optimal fragmentation pattern for SRM assay design, is to perform a so-called SRM-triggered MS2 experiment directly on the triple quadrupole.

In SRM-triggered MS2 target peptides of interest are measured by SRM using a small number of theoretically generated transitions (typically 1 to 2 y-ions with m/z above the precursor mass). Whenever one of these transitions exceeds a defined intensity threshold (typically 1000 counts), the third quadrupole switches from filter mode to trap and scanning mode and records a full MS2 spectrum, which can then be searched with any of the common search algorithms. From the assigned MS2 spectra, the identity of the peptide can be proven and optimal SRM transitions can be identified.

In our case study we would like to generate SRM assays for 30 target peptides representing our 10 proteins of interest. For each of the target peptides we had a synthetic, heavy-labelled reference peptide available, which allowed us to acquire high-quality SRM-triggered MS2 measurements on a QTrap instrument (in 2 injections):

- *olgas_Q130517_hDP_1*
- *olgas_Q130517_hDP_2*

These two files were subsequently converted into mzXML format and searched with the search engine Sequest followed by a statistical analysis with PeptideProphet as part of the trans-proteomic pipeline (TPP). The following file contains for every MS2 spectrum the matched peptide sequence and the corresponding PeptideProphet quality score:

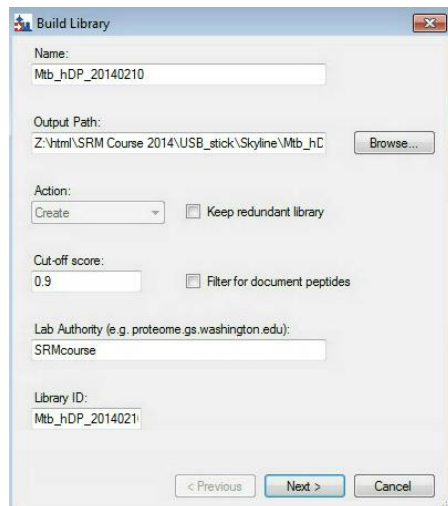
- *interact_hDP_P0-9.pep.xml*

The data files (here in mzXML format) and the search engine output file (here in pep.xml format) are the required input to generate a spectral library in Skyline.

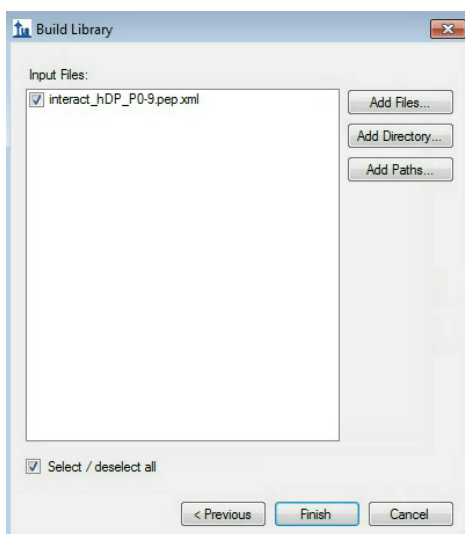
2. Building a spectral library in Skyline

- Open the Skyline document *TarProCourse_Tut2_MtbProteomeLib.sky* from *Tutorial 2 - Transition List*.
- Go to “Settings” → “Peptide Settings” → “Library” → “Build...”
- Give your library a name: *Mtb_hDP_TarProCourse* (hDP = heavy dirty peptides).
- Specify the output path where your library should be saved. We recommend placing it into the project-independent “Skyline” folder on your USB key (see *Tutorial 1 - Settings*).

- Do not activate the “Keep redundant library” option, as we would like to get for each peptide just the single best spectrum (dot product all vs. all).
- Choose a false discovery rate of below 1%, which in our case is represented by a PeptideProphet cut-off score of 0.9.
- A lab authority name needs to be given for each library, say “TarProCourse”.
- The field “Library ID” is optional and by default filled with the library name.



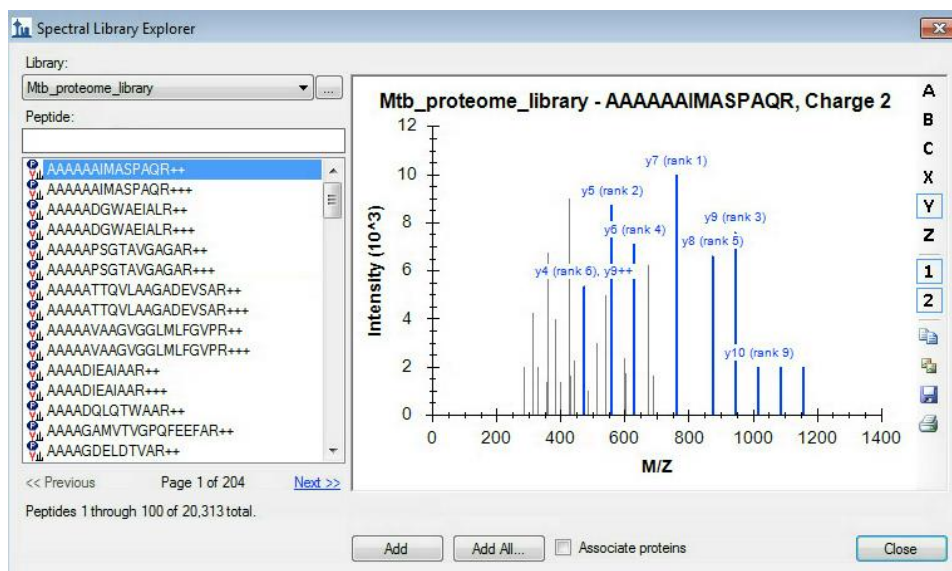
- Click “Next” and “Add directory...” to choose the *Tutorial-3_Library* folder where the search engine output (*interact_hDP_P0-9.pep.xml*) as well as the SRM-triggered MS2 files (converted to mzXML format) are stored. Click “OK”.
- Your “Build Library” tab should now look like this:



- “Finish” the library build.
- Activate (check the box) of both libraries in your “Peptide Settings” → “Library” tab. Leave the *Mtb_proteome_library* first, such that it has priority over the newly created *Mtb_hDP_TarProCourse library*.
- Confirm and apply the changes in the “Library” tab by clicking “OK”
- Skyline will automatically update the transition selection including the new information from the library we just created.
- Your document will now contain **444 transitions, 96 precursor, 30 peptides and 10 proteins**

- **Tip!** In case both libraries contain an MS2 spectrum, at the top of the MS/MS spectrum tab you can select from the drop-down menu, which library spectrum you would like to see first.

Tip! You can visualise and browse all peptides of your library in the spectral library viewer (View → Spectral Libraries):



- Save the Skyline file as *TarProCourse_Tut3_MtbProteomeLib_hDP.sky*.
- Export a transition list based on the new settings. This time however, we do not want to have all transitions exported into one single list, but already split them into several lists, each one containing maximally 150 transitions:
 - Go to “File” → “Export” → “Transition list”
 - Select “AB SCIEX”, “Multiple methods”, “Max transitions per sample injection = 150”, “Dwell time = 20 ms”, activate the tab “ignore proteins” (which means that not all peptides for a given protein have to be measured in one transition list, but that they might be distributed among different lists).
 - Skyline gives a notification that the settings do not match. → Click “No” to ignore it.
 - Save the transition lists as: *TarProCourse_Tut3_MtbProteomeLib_hDP_TransList.csv* (extensions 0001 to 0004 will be generated automatically)
 - Have a look at the generated .csv files in Excel.

Exercises

1. For which peptides did the custom-made SRM-triggered MS2 library not lead to an identified SRM spectrum? What could be the reasons for this?
2. How can you also include b-ions into the automatic transition selection? How many b-ions show up among the best 5 transitions?
3. What are the reasons for the discrepancy in the number of transitions that are picked for the light and heavy peptide versions?
4. Manually curate all peptides with less than 5 transitions. How do you choose which other transitions to add?

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