

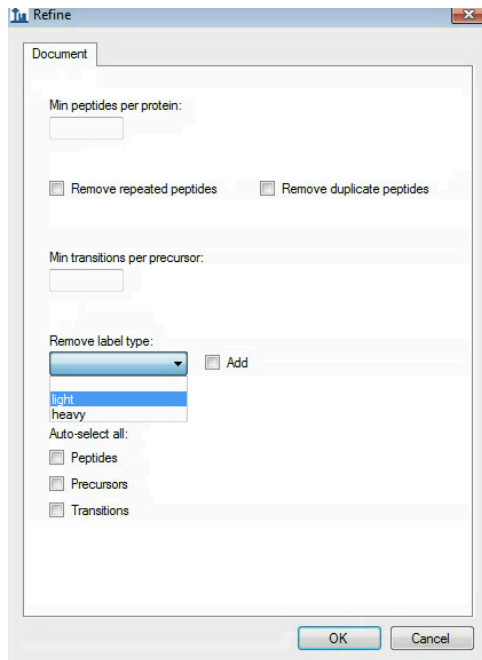
Tutorial 3: Building a spectral library in Skyline

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. SRM method preparation for SRM-triggered MS2 measurements

- Start with an empty Skyline document where the settings are already correctly set: Open *SRMcourse_20130715_Settings.sky* from the folder *Tutorial-1_Settings* and save it as *SRMcourse_20130715_SRM-MS2.sky* in folder *Tutorial-3_Library*.
- Adapt the settings as follows:
 - Peptide Settings:
 - Keep settings as described in *Tutorial 1 – Settings*, except for the Library tab:
 - Library: → No library should be activated.
 - Transition Settings
 - Keep settings as described in *Tutorial 1 - Settings*, except for the Filter and Library tabs:
 - Filter:
 - Precursor charges: 2,3
 - Product ion charges: 1
 - Product ion types: y
 - Product ions from: “m/z > precursor”, To: “2 ions”
 - Always add “N-terminal to Pro” and “C-terminal to Glu or Asp”.
 - Precursor exclusion window: 5 Th
 - Activate “Auto-select all matching transitions”
 - Library: → Deactivate “If a library spectrum is available, pick its most intense ions”
- Insert the list of peptides that were synthesised and for which you would like to select transitions to trigger an MS2 spectrum:
 - Open *target_peptides.xls* in folder *Tutorial-2_TransitionList*, copy the peptide sequences and paste them into Skyline: Edit → Insert → Peptides. Skyline automatically inserts all peptides with the correct protein names and selects the 5 best transitions for each precursor (light and heavy each).
- If all settings are setup correctly, Skyline will automatically insert the 30 target peptides under the correct protein name with selected transitions according to the filter definition.
In total you should end up with a document containing:
408 transitions, 120 precursors, 30 peptides and 10 proteins (see lower right corner).
- As we want to trigger MS2 spectra only from heavy isotope-labelled peptides, all light transitions need to be removed:
Edit → Refine → Advanced... → Remove label type → select “light”
203 heavy transitions will remain.



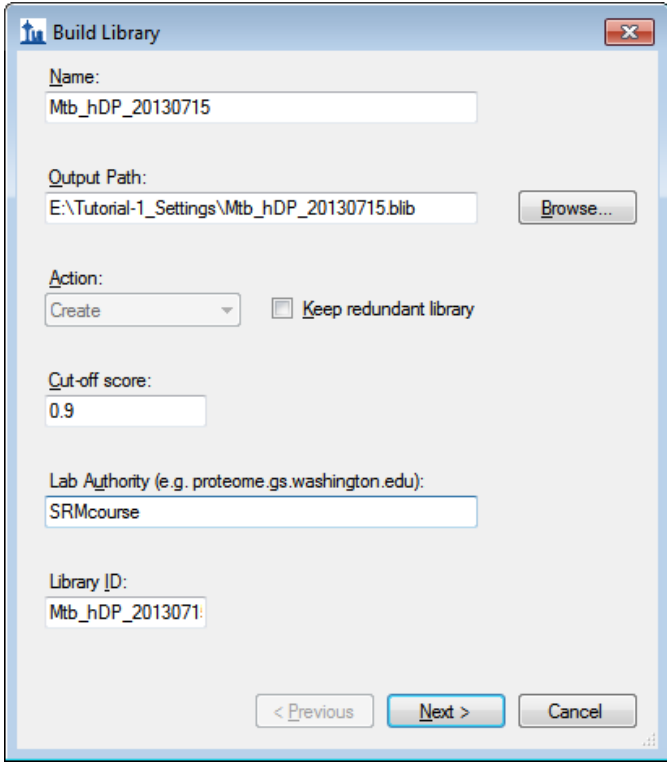
- **Tip!** Do not remove labelled peptides by deactivating the respective modification in the Modifications tab in the Peptide Settings, because then Skyline does not “know” them anymore. (E.g. Skyline can not use a library generated from labelled peptides with a label type that is not entered in the modification tab.)
- Export this transition list split into 2 method files for an AB Sciex instrument type
 - File → Export → Transition list:
 - “AB Sciex”
 - “Multiple methods”
 - “Max transitions per sample injection = 103”
 - “Dwell time = 20 ms”
 - Activate the tab “ignore proteins”, which means that not all peptides for a given protein will be measured in one transition list, but distributed among different lists in case that would help to have less co-eluting transitions.
 - In case, Skyline gives a notification that the settings do not match → “No”.
 - Export transition list as: *SRMcourse_20130715_SRM-MS2.csv* (the extensions 0001 and 0002 will be generated automatically).
- Save the Skyline document.

2. Building a spectral library in Skyline

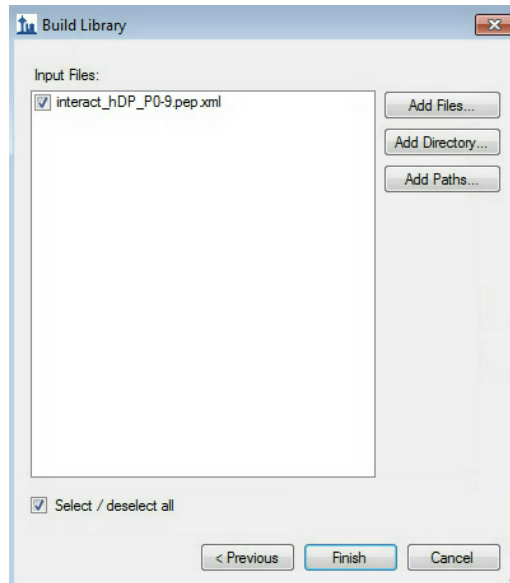
- After these two SRM methods were run in SRM-triggered MS2 mode on a QTrap instrument, the files were searched with Sequest followed by PeptideProphet as part of the trans-proteomic pipeline (TPP).
 - Name of the two SRM-triggered MS2 files:
 - olgas_Q130517_hDP_1.wiff*
 - olgas_Q130517_hDP_2.wiff*
 - Name of the TPP output file:
 - interact_hDP_P0-9.pep.xml*
- Import the .wiff files into to see the SRM traces that were used to trigger the MS2 spectra.
 - File → Import → Results → Add one new replicate → Name: *olgas_Q130517_hDP* → select *olgas_Q130517_hDP_1.wiff* and *olgas_Q130517_hDP_2.wiff*.

Note! These runs can be merged because they are complementary, i.e. no transitions were measured in both injections.

- Build a spectral library from the acquired SRM-triggered MS2 spectra.
 - Settings → Peptide Settings → Library → Build
 - Give your library a name: *Mtb_hDP_20130715* (hDP = heavy Dirty Peptides)
 - Specify the output path where your library should be saved. We recommend placing it into the project-independent “Skyline” folder (see Tutorial 1).
 - Do not activate the “Keep redundant library” option, as we would like to get for each peptide just the single best spectrum, which is automatically selected based on the best identification score (based on 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 “SRMcourse”.
 - The field “Library ID” is optional and by default filled with the library name.



- Click “Next” and “Add directory” to specify 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.
- Your “Build Library” tab should now look like this:



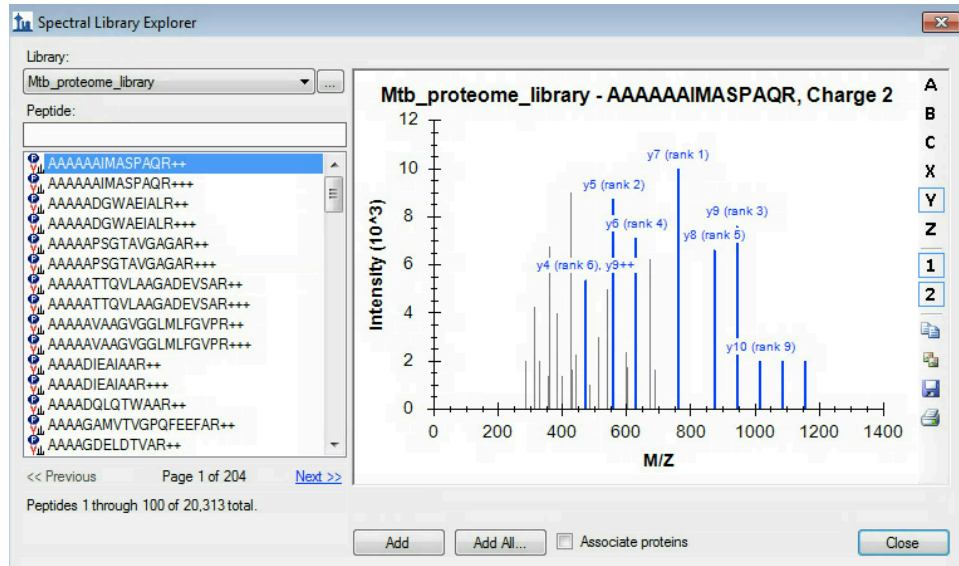
- “Finish” the library build.
- Activate both libraries. Place the Mtb_proteome_library first, such that it has priority over the newly created Mtb_hDP_20130715 library (Edit list → Select “up” or “down”).
- Place the MS/MS spectrum tab next to your SRM traces by drag-and-drop.
- At the top of the MS/MS spectrum tab you can select from the drop-down menu, which library spectrum you would like to see first (in case both libraries contain an MS2 spectrum for the respective precursor). For now, select the Mtb_hDP_20130715 library.
- Go through all 30 target peptides and check the obtained SRM traces as well as the quality of the MS2 spectra.
- **Note!** Some of the spectra in the Mtb_proteome_library contain profile data, and, therefore, do not work so well with Skyline’s ion ranking. There is a 50-peak cut-off imposed for performance reasons and this causes that in some spectra the peak picking is not optimal.

3. Selecting the most intense transitions based on spectral library information

- Remove the SRM-triggered MS2 data from the current document:
 - Edit → manage results → select olgas_Q130517_hPD and “remove”.
- Remove all peptides from the left window so that you end up with a blank document.
 - This is necessary to get rid of those precursor charge states that are not present in the libraries but that were selected with the filter settings used above.
- Change the transition settings as described in *Tutorial 1 – Settings*:
 - 5 most intense y-ion transitions, singly and doubly charged
 - Doubly and triply charged precursor ions.
- Insert again the 30 peptides from file “target_peptides.xls” (in folder *Tutorial-2_TransitionList*): Edit → Insert → Peptides → Paste the peptide list.
 - Skyline automatically inserts all peptides with the correct protein names and selects the 5 best transitions for each precursor (light and heavy each).
 - You will end up with a transition list of 449 transitions, 96 precursors, 30 peptides and 10 proteins.
- Export a transition list based on the new settings:
 - AB Sciex, Multiple methods, Ignore proteins, Max transitions per sample injection: 100, Dwell time: 20 ms, Name: SRMcourse_20130715_MtbProteomeLib_hDP_Qtrap.csv.
→ You will get 5 transition lists.

- Save the Skyline file as *SRMcourse_20130715_MtbProteomeLib_hDP.sky*.

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



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? What could you do to get MS2-information?
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?
5. In file *SRMcourse_20130715_SRM-MS2*, why do some precursors have >2 transitions?
6. For SRM-triggered MS2, why do we select transitions above the precursor and always add transitions N-terminal to Pro and C-terminal to Glu/Asp?

We would like to thank Prime-XS and SystemsX for supporting the SRM Course 2013.

