

## Tutorial 1: Setting up your Skyline document

**Caution!** For using Skyline the number formats of your computer have to be set to English (United States). Open the Control Panel → Clock, Language, and Region → Change Location → Formats → English (United States).

Open the **Peptide Settings** (under the menu “Settings”) and go through all tabs to adjust these settings to your current project. **Caution!** Make sure that you understand every single setting that you set here.

### **Digestion:**

- **Enzyme:** Select the proteolytic specificity of the enzyme that was used to digest the proteins in your sample.  
→ We usually use trypsin, which cleaves C-terminally of lysine and arginine, but not if these are followed by proline: select “Trypsin [KR|P]”
- **Max missed cleavages:** Select the number of missed cleavages that you would like to tolerate.  
→ We usually work with fully tryptic peptides only: select “0”
- **Background proteome:** Here you can build a background proteome from a protein fasta file using the digestion settings defined above. Alternatively, you can directly add an already in-silico digested proteome file (format.protdb).  
→ To generate a background proteome according to the digestion settings above click “Add...” → Click on the “Build >>” button. → In the “Name” field, enter “TubercuList\_v2-6”. → Click on “Browse...”. → Define the path (“Skyline” folder on USB key) of your new background proteome and enter the file name “TubercuList\_v2-6” and click “Save” in the lower right corner of the window. → “Add File...” → Select the *TubercuList\_v2-6.fasta* file provided in the folder *Tutorial-1\_Settings*. The generated proteome file should contain 3982 proteins. → Confirm by clicking “OK”, then close the Peptide Settings by again clicking “OK”. Now the background proteome is being generated. You can follow the progress in the bottom lane of the Skyline interface. Depending on the size of the file this may take a few seconds up to several minutes.  
**Tip!** We suggest to work with a general folder called “Skyline” where you save all project-independent spectral libraries, background proteomes, iRT databases etc. that you create within Skyline. This helps to find these important files later on when you have many different Skyline projects.

### **Prediction:**

- Re-open the Peptide Settings and select the “Prediction” tab.
- **Retention time predictor:** Select a retention time predictor and use it to create scheduled SRM methods and to support data analysis.  
→ We typically work with the SSRCalc predictor or with a custom iRT predictor. We will create a scheduled SRM method in a later tutorial, but for now no retention time predictor needs to be activated (“None”).
- **Use measured retention times when present:** This option allows working with actually measured retention times (instead of predicted) for those peptides for which this information is included in the Skyline file.  
→ For non-scheduled SRM this tab is not functional and we leave the default (activated).
- **Time window:** Enter the time window, which you would like to use for your scheduled measurements.  
→ For non-scheduled SRM this tab is not functional and we leave the default (2 minutes).

**Filter:**

- Change to the “Filter” tab.
- Enter the peptide amino acid length that you would like to work with.
  - We work with:
    - Min length: 7**
    - Max length: 25**
- **Exclude N-terminal AAs:** The N-terminus of a protein might be posttranslationally processed (modified and/or cleaved) and might thus not be suited for protein quantification.
  - When working with a synthetic peptide library we typically do not exclude N-terminal amino acids and enter “0”.
- **Exclude potentially ragged ends:** Ragged ends are peptides with KK, RR, RK or KR sequences at one or both ends. Such peptides might not be fully cleaved and hence may not be suited for quantification. However, if no alternative peptides are available one might rather quantify with a ragged end peptide than not at all.
  - When working with a synthetic peptide library we typically do not select this option.
- **Exclude peptides containing:**
  - Cys, Met, His:** These residues are prone to modifications, such as oxidation.
  - NXT/NXS:** This is a glycosylation motif.
  - RP/KP:** Lys or Arg followed by Pro are sometimes cleaved by trypsin.
  - When working with a synthetic peptide library we typically do not select these options.
- **Auto-select all matching peptides:** Needs to be activated if peptides should be automatically selected from a background proteome or a spectral library file.
  - Activate.

**Library:**

- Change to the “Library” tab.
- **Libraries:** Here you can insert or build spectral libraries containing MS2 spectra. Spectral libraries can be downloaded from public sources or built within Skyline from your own data. Several libraries can be selected at the same time. Be aware that the order in the list matters: the higher up in the list, the higher the priority in case there is an MS2 spectrum for the same peptide in more than one library. For a more detailed library generation protocol please refer to *Tutorial 3 - Library*.
  - For now we will work with a comprehensive public Mtb library (<http://www.srmatlas.org/mtb/>), which was generated on a QTrap instrument using synthetic peptides (for more information see O. T. Schubert et al., Cell Host Microbe, 2013, 13, 602). This library can be found on your USB key in the project-independent *Skyline* folder and is called *Mtb\_DirtyPeptides\_QT\_filtered\_cons.sptxt*.
  - Click “Edit list” → “Add...” → Name: “Mtb\_proteome\_library” → “Browse...”
  - Select the sptxt file in the folder called *Skyline* → Click “OK” twice. → Activate the new “Mtb\_proteome\_library” by checking the box. → Confirm by clicking “OK”. Skyline will now read this library, which takes a few seconds. Again you can follow the progress in the bottom lane of the Skyline interface.
- Re-open the Peptide Settings and select again the “Library” tab.
- **Pick peptide matching:** Select if peptides should be automatically selected according to the filter settings (defined in the Filter tab before) or according to the library settings defined below.
  - For this study we pre-selected the target peptides and we do not need this setting. We keep the default setting (“Library”).
- **Rank peptides by:** Here you can define a ranking of all peptides available for a given protein in the library. Depending on which tool you used to create your library, you can select between:
  - Picked intensity (sum of selected peak intensities in the spectrum for a given peptide)
  - Spectrum count (sum of all spectra for a given peptide)
  - TFRatio (SpectraST score for spectrum quality)
  - Total intensity (sum of all peaks in the MS2 spectrum)

→ For this study we do not need this setting.

- **Limit peptides per protein:** Limits the number of automatically selected peptides per protein from the library.

→ For this study we do not need this setting.

#### Modifications:

- Change to the “Modifications” tab.
- **Structural modifications:** Structural modifications concern chemical modifications of peptides. They can either be static (always present) or variable (sometimes present, sometimes not).
  - By default “Carbamidomethyl (C)” is activated, which comes from the reduction and alkylation step during sample preparation to avoid formation of disulphide bonds between cysteine residues. Keep this modification.
- **Max variable mods** and **Max neutral losses:** Select the maximal number of variable modifications and neutral losses according to your project.
  - Leave the default setting (3 variable modifications and 1 neutral loss).
- **Isotope label type:** Here you can define the isotope label type you plan to work with.
  - Leave the default “heavy” as label type.
- **Isotope modifications:** Here you can define the chemical composition of your isotopic modification.
  - For our case study we will work with stable isotope labelled synthetic reference peptides of which the C-terminal Arg or Lys are heavy labelled. Activate this modification by going to “Edit list” → “Add...” → Name: select the following two modifications from the drop-down menu one after another:
    - Label:13C(6)15N(2) (C-term K)
    - Label:13C(6)15N(4) (C-term R)

After they have been added, activate both label types by checking the boxes in the “Isotope modifications” window.

- **Internal standard type:** Define which labelling state should be your internal standard
  - Our internal standard will be the spiked-in heavy reference peptides and we thus keep the default setting “heavy”.

**Finally:** Click “OK” to confirm all peptide settings.

## Overview Peptide Settings:

Peptide Settings

Digestion Prediction Filter Library Modifications

Enzyme:  
 Trypsin [KR | P]

Max missed cleavages:  
 0

Background proteome:  
 TubercuList\_v2-6

OK Cancel

Peptide Settings

Digestion Prediction Filter Library Modifications

Retention time predictor:  
 None

Use measured retention times when present

Time window:  
 2 min

OK Cancel

Peptide Settings

Digestion Prediction Filter Library Modifications

Min length: 7 Max length: 25

Exclude N-terminal AAs:  
 0

Exclude potential ragged ends

Exclude peptides containing:  
 Cys  
 Met  
 His  
 NXT/NXS  
 RP/KP

Auto-select all matching peptides

OK Cancel

Peptide Settings

Digestion Prediction Filter Library Modifications

Libraries:  
 Mtb\_proteome\_library

Pick peptides matching:  
 Library

Rank peptides by:

Limit peptides per protein

Peptides

OK Cancel

Peptide Settings

Digestion Prediction Filter Library Modifications

Structural modifications:  
 Carbamidomethyl (C)

Max variable mods: 3 Max neutral losses: 1

Isotope label type:  
 heavy

Isotope modifications:  
 Label-13C(6)15N(4) (C-term R)  
 Label-13C(6)15N(2) (C-term K)

Internal standard type:  
 heavy

OK Cancel

Now open the **Transition Settings** (under the menu “Settings”) and go through all tabs to adjust the settings to your current project. **Caution!** Make again sure that you understand every single setting that you set here.

#### **Prediction:**

- Open the Transition Settings and go to the “Prediction” tab.
- **Precursor mass** and **Product ion mass**: Here you define the basic MS parameters of your data.
  - For both, precursor and product ion mass, we work with the monoisotopic mass.
- **Collision energy**: Define how the collision energy that you are going to use should be calculated. Ideally, the optimal collision energy equation is determined for each instrument and we will discuss how to do this in *Tutorial 4 - Parameters*. If you do not have any information available, use the recommendations by the vendor or by Skyline.
  - Select the “ABI 5500 QTrap” collision energy. To see the underlying formula of the collision energy calculation select “Edit current”, go back with “Cancel”).
- **Declustering potential**: Define the declustering potential that should be applied to your sample when it is injected into the mass spectrometer. This parameter will as well be discussed in *Tutorial 4 - Parameters*.
  - We do not set a declustering potential.
- **Use optimisation values when present**: If you have carried out a collision energy optimisation experiment within Skyline, you can directly apply the optimised values.
  - We do not activate this option for now.

#### **Filter:**

- Change to the “Filter” tab.
- **Precursor charges**: Define which precursor charge states you would like to consider for your SRM measurements.
  - We usually consider doubly and triply charged peptides, enter “2, 3”.
- **Product ion charges**: Define which product ion charge states you would like to consider for your SRM measurements.
  - We usually consider singly and doubly charged product ions, enter “1, 2”.
- **Ion types**: Define which product ion types you would like to consider for your SRM measurement (you can define a,b,c,x,y,z and p ions, p stands for precursor).
  - In this study we only focus on y-ions, so enter “y”.
  - Note: We do not consider b-ions in this case study, because compared to y-ions they tend to be less intense and less conserved between instruments.
- **Product ions**: In this window you can define a filter to automatically select transitions for all peptides in your Skyline document. For example, to filter for the whole y-ion series, ranging from the first to the last y-ion of a peptide, enter: From: “ion 1” and To: “last ion”. Further criteria to automatically include typically very intense transitions, such as N-terminal to Pro or C-terminal to Glu or Asp, can be selected or custom-defined.
  - In our case study we want to consider all y-ions, hence we select **From:** “ion 1” **To:** “last ion”.
  - Uncheck “N-terminal to Proline”, as we would like to select transitions based on a library spectrum only.
- **Precursor m/z exclusion window**: Here you can exclude a certain mass window around the precursor m/z value for transition selection. In this m/z range transitions are typically very noisy and therefore not suited for identification and quantification.
  - Set the precursor exclusion window to “5” Th (which is +/- 2.5 Th around the precursor).
- **Auto-select all matching transitions**: Needs to be activated if transitions should be automatically selected for all peptides based either on the filter settings or the library.
  - Activate.

**Library:**

- Change to the “Library” tab.
- **Ion match tolerance:** Here you can define the mass accuracy you would like to tolerate for the selection of fragment ions from your spectral library. This depends on the instrument type that was used to acquire the library spectra. Lower values help to get a more specific peak assignment of the spectra, but if the instrument did not have this accuracy you will lose your peaks.
  - Our library has been acquired on a QTrap instrument for which we usually use a mass tolerance of 1.0 m/z.
- **If a library spectrum is available, pick its most intense ions:** Here you can specify if the library should be used to guide transition selection and how many transitions per precursor should be considered.
  - Activate and enter “5” product ions
- **From filtered...:** Here you can choose which settings from the “Filter” tab you would like to consider for the automatic transition selection.
  - We select “From filtered product ions”.

**Instrument:**

- Change to the “Instrument” tab.
- Specify the m/z range of your instrument
  - **Min m/z:** 300 m/z to **Max m/z:** 1250 m/z (maximum value for QTrap 5500)
- **Dynamic min product m/z:** This function is only useful for chromatograms from LTQ MS2 data.
  - Do not activate this option.
- **Method match tolerance:** Here you can define the tolerance in mass difference between the theoretical masses calculated by Skyline and the masses given in an imported raw file. If for a given raw file the corresponding method file has been generated with Skyline beforehand, the difference between Skyline and raw file masses will be 0. However, if other mass calculators have been used for method generation and/or due to rounding issues, minor mass differences can occur.
  - We leave the default setting of “0.055 m/z”.
- **Firmware transition limit:** In case your instrument of choice has a maximal transition number limit you can enter this here.
  - We do not enter a limit.
- **Firmware inclusion limit:** In case you use Skyline for inclusion list generation (e.g. for PRM) you can specify the list limit here.
  - We do not enter a limit.
- **Min time, Max time:** Here you can limit the part of the HPLC gradient to extract data from (e.g. in SWATH experiments).
  - We leave these settings undefined.

**Full-Scan:**

- This tab is not needed for SRM measurements, but for MS1 and/or MS2 filtering analysis.
  - For our case study we do not activate this tab

**Finally:** Click “OK” to confirm all transition settings.

Save the Skyline document to folder *Tutorial-1\_Settings* with the name: *SRMcourse\_20140210\_Settings.sky*.

Overview Transition Settings:

Transition Settings

Prediction Filter Library Instrument Full-Scan

Precursor mass:  Product ion mass:

Collision energy:  Declustering potential:

Use optimization values when present

OK Cancel

Transition Settings

Prediction Filter Library Instrument Full-Scan

Precursor charges:  Ion charges:  Ion types:

Product ions

From:  To:

Always add:

N-terminal to Proline  C-terminal to Glu or Asp

Precursor m/z exclusion window:  m/z

Auto-select all matching transitions

OK Cancel

Transition Settings

Prediction Filter Library Instrument Full-Scan

Ion match tolerance:  m/z

If a library spectrum is available, pick its most intense ions

Pick:  product ions

From filtered ion charges and types  
 From filtered ion charges and types plus filtered product ions  
 From filtered product ions

OK Cancel

Transition Settings

Prediction Filter Library Instrument Full-Scan

Min m/z:  m/z Max m/z:  m/z

Dynamic min product m/z

Method match tolerance m/z:  m/z

Firmware transition limit:  Firmware inclusion limit:

Min time:  min Max time:  min

OK Cancel

## Exercises

1. What is the exact monoisotopic mass difference for peptides carrying a  $^{13}\text{C}$  (and NO additional  $^{15}\text{N}$ ) labelled C-terminal lysine residue?
2. What is the chemical formula of the loss occurring in pyro-glutamate formation on N-terminal glutamic acid?
3. How many proteins are in the fasta file and how many are in the Skyline background proteome? What could be the reason for the discrepancy?
4. Chymotrypsin cleaves C-terminal to which amino acids?
5. What is the slope for triply charged precursor ions defined in the collision energy equation of the ABI 5500 QTrap Instrument?
6. Which transition settings do you need to apply to automatically select the complete doubly-charged y-ion series of a peptide between 500 and 1000 Da?

**Caution!** Do not save any of the changes you made for the exercises.

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