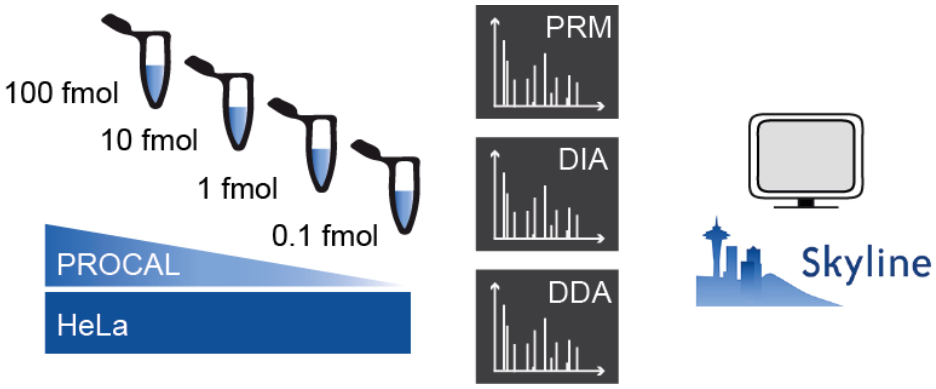
Tutorial: Examination of PRM, DIA and DDA data in Skyline

In this tutorial, we will perform a targeted analysis with Skyline[1] (Step 1 – Step 3) with PRM, DIA and DDA measurement data. The Skyline software is free-of-charge, open-source and can be run on a standard PC or laptop.

The four samples used for this tutorial were generated by mixing HeLa protein digest with PROCAL peptide standard[2] at defined concentrations. Each sample was measured with three technical replicate injections in PRM, DIA and DDA mode on a Quadrupole-Orbitrap-Iontrap (Tribrid) mass spectrometer (Eclipse, Thermo).



Overview

Step 1: Skyline – Libraries and PRM
Step 2: Skyline – DIA data
Step 3: Skyline – DDA data
Appendix: Background information

Credits

The samples for this tutorial have been measured by the BayBioMS@MRI at TU Munich. The Skyline part of this tutorial is based on a tutorial available on the Skyline website (Skyline Targeted Method Editing https://skyline.ms/wiki/home/software/Skyline/page.view?name=tutorial_method_edit) and has been further updated and edited by Julia Mergner and Christina Ludwig.

[1] MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics. 2010 Apr 1;26(7):966-968

[2] Zolg DP, Wilhelm M, Yu P, Knaute T, Zerweck J, Wenschuh H, Reimer U, Schnatbaum K, Kuster B. PROCAL: A set of 40 peptide standards for retention time indexing, column performance monitoring, and collision energy calibration. Proteomics. 2017 Nov; 17(21)

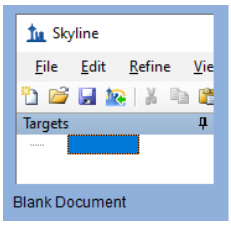
Getting Started

To start the tutorial, copy the complete tutorial folder (Tutorial_Skyline) onto your local PC/laptop.

The **Installers** subfolder from your flash drive "**Skyline (24.1)**" contains an installer for the unplugged 64-bit Skyline 24.1 version that you can use to install Skyline locally on your computer. This version will not receive any updates! (To install Skyline 24.1 from the internet, go to the Skyline installer page, register as a user, and install from there. Skyline will then automatically inform you when an update is available.)

If you have been using Skyline prior to starting this tutorial, it is a good idea to revert Skyline to its default settings. To do so:

- Start Skyline (v.24.1)
- On the **Start Page**, click **Blank Document**



- On the **Settings** menu, click **Default**
- Click **No** on the form asking if you want to save the current settings

The document settings in this instance of Skyline have now been reset to the default. Choose the proteomics interface by doing the following:

- Click the **user interface** control in the upper right-handed corner of the Skyline window, and click Proteomics interface 

1. Skyline – Loading Libraries

Loading an experimental Spectral Library file into Skyline

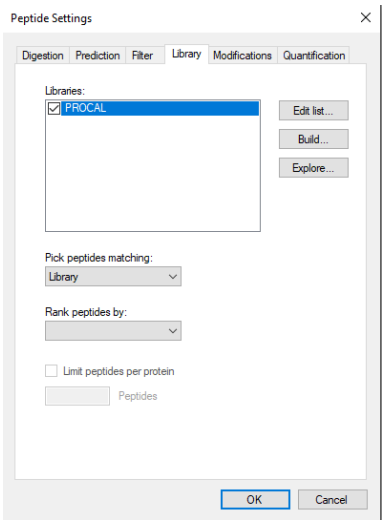
To start this tutorial, you will first of all load a previously generated experimental spectral library called PROCAL.blib. This library file entails experimental spectra for all the 41 PROCAL peptides (proteome tools project, Zolg et al., 2017), which are the target peptides of this tutorial. The PROCAL.blib file was generated with Skyline by loading a DDA measurement file of the PROCAL peptides together with the MaxQuant search result file (msms.txt)
To load this experimental spectra library file

- On the **Settings** menu, click **Peptide Settings**
- Click on the **Library** tab
- Click on the **Edit list…** button
- In the **Edit Libraries** form click on the **Add…** button
- In the **Name** field enter "**PROCAL**"
- Click the **Browse** button and navigate to the **Tutorial\02_PRM** subfolder
- Open the "**PROCAL.blib**" file
- Click **Ok** in the **Edit Library** and **Edit Libraries** form

Skyline has now added the "**PROCAL**" to the Libraries list in the Library tab of the Peptide settings form.

- Check the box ☐ for "**PROCAL-Library**" to tell Skyline to use this library in picking peptides and transitions

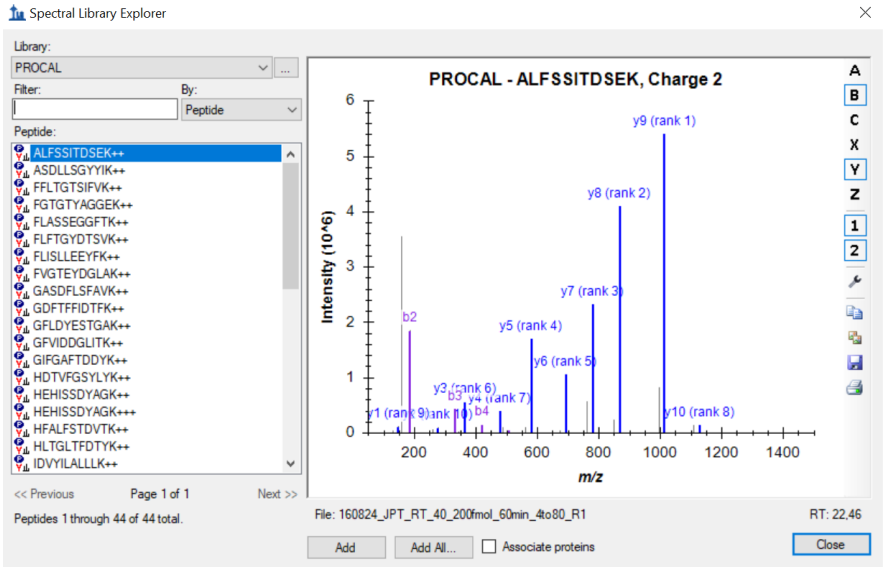
The **Peptide Settings-Library** tab should now look like this:



- Click **Ok**

You can explore the loaded PROCAL library by selecting on the **View** menu **Spectral Libraries.**

A **Spectral Library Explorer** window will open, which will show you all peptides entailed in the loaded library file (for the PROCAL library it is 44 peptides in total), their corresponding spectra as well as raw file and retention time information:

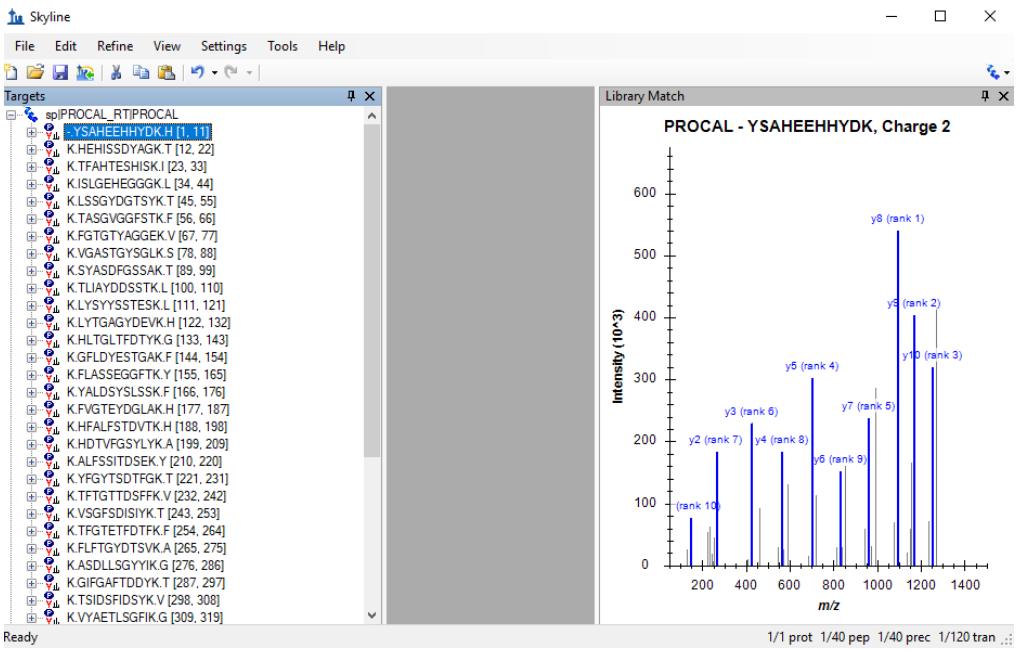


Close again the **Spectral Library Explorer** window by clicking the "x" in the upper right corner.

Inserting FASTA Sequences

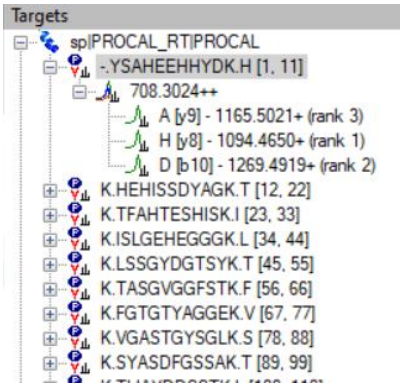
To specify your proteins of interest you can import a fasta file entailing exclusively your target proteins via the **Import** function.

- On the **File** menu, select **Import>** and click **FASTA…**
- Navigate to the **Tutorial\02_PRM** subfolder and select the "**PROCAL.fasta**" file



- In case no Library Match window with spectra information is visible in your document, go to the **View** menu and select **Library Match.**
- Right-click into the **Library Match** window. Make sure that the **Ion Types Y and B are activated.**

Skyline now highlights the y-ions in blue and the b-ions in purple in the spectrum graph.
By default, Skyline chooses the **3 most intense singly-charged product y-ions** as the transitions it will target for doubly charged precursors. This selection can be seen when opening the peptide tree in the Targets window by clicking on the boxes with "+" in front of each peptide sequence:

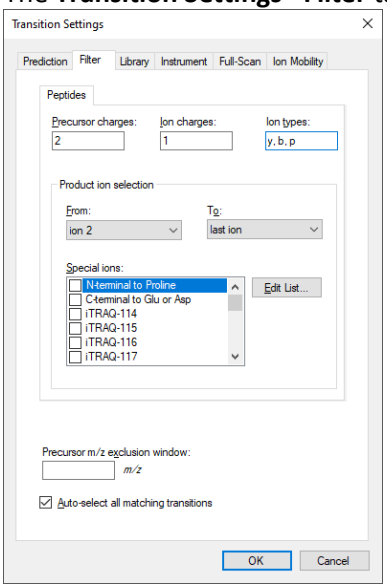


To change the product ion selection setting from the default values, perform the following steps:

- On the **Settings** menu, click on **Transition Settings**
- Select the **Filter** tab
- In the **Precursor charges** and **Ion charges** field confirm the values are "**2**" and "**1**", respectively
- In the **Ion types** field, change the "**y**" to "**p,y,b**" (p- precursor, y- y-ions, b- b-ions)
- In the **Product ion selection** field change **From** to "**ion 2**" and **To** to "**last ion**"

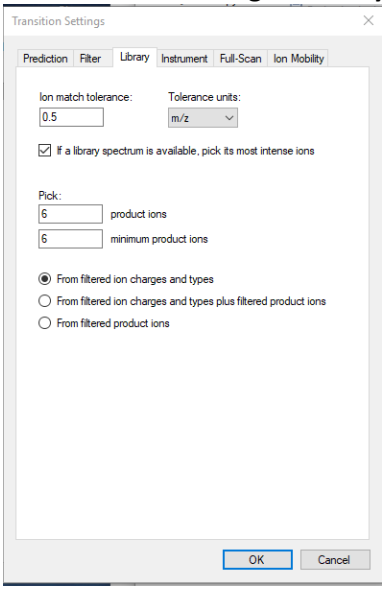
- Uncheck “**N-terminal to Proline**”

The **Transition Settings –Filter** tab should look like this:



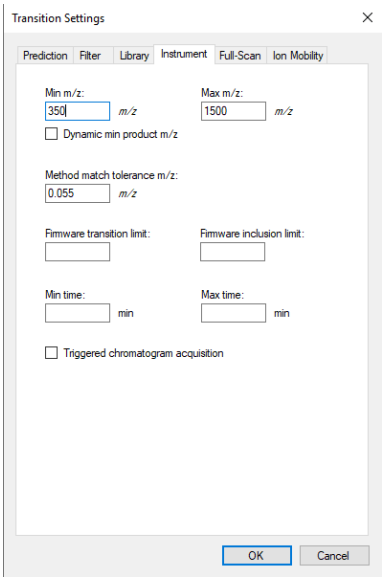
- Select the **Library** tab
- Check that the **Ion match tolerance** is set to the default “**0.5**
- In the **Pick** field, enter “**6**” for **product ions** and **minimum product ions**

The **Transition Settings – Library** tab should look like this:



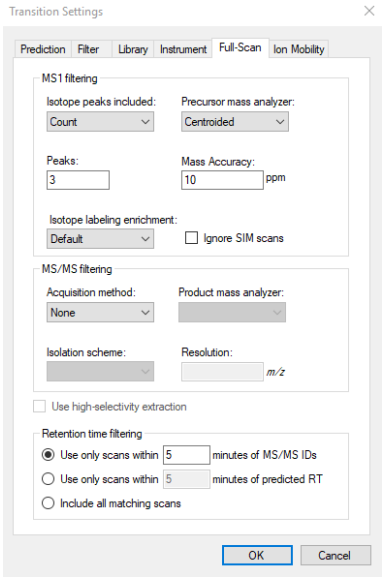
- Select the **Instrument** tab
- Change **Min m/z** to “**350**”

The **Transition Settings – Instrument** tab should look like this:



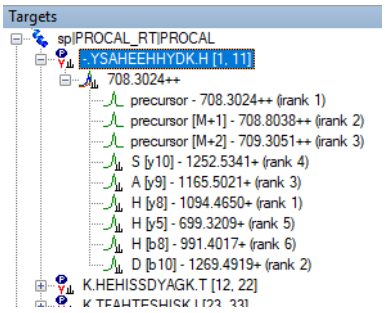
- Select the **Full-Scan** tab
- Change the **Isotope peaks included** field to "**Count**" and the **Precursor mass analyzer** field to "**Centroided**". The **Mass Accuracy** field is by default set to 10 ppm.

The **Transition Settings – Full-Scan** tab should look like this:



- Click Ok

The Skyline document tree should now look like the following:

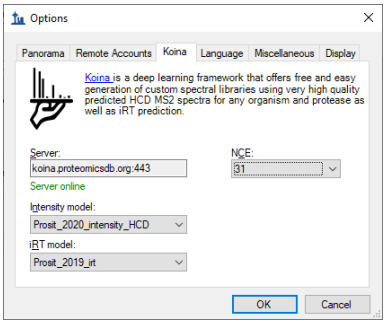


Skyline has selected the top 6 ranked product ions for each peptide precursor, including b-ions, as well as 3 precursor isotopes (irank 1,2,3) for each peptide, based on the spectral library file ("**PROCAL.blib**") your provided in the first step of this tutorial.

Creating a predicted Spectral Library using PROSIT

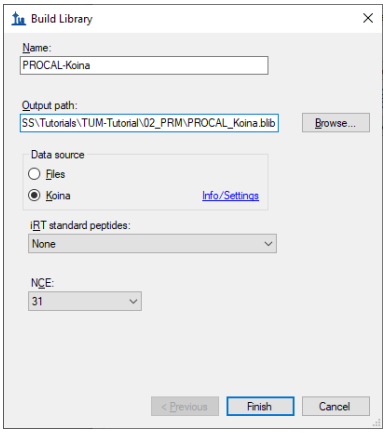
Skyline is not limited to using just a single spectral library. You can for example use the Koina prediction framework to predict spectra for **unmodified** peptides directly in Skyline.

- On the **Settings** menu, click **Peptide Settings**
- Select the **Library** tab and click the **Build…** button
- In the **Name** field of the **Build Library** form, enter "**PROCAL-Koina**"
- Click the **Browse** button and make sure you save the new library to the Tutorial\02_PRM subfolder. Click **Save**
- In the **Data source** field select **Koina**
- In the Pop-up window click **Yes** to confirm that you want to change the Koina settings now. If there is no Pop-up window click on Koina - **Info/Settings**
- The **Options** form opens. In the **Intensity model** field select "**Prosit_2020_intensity_HCD**" and "**31**" in the **NCE** field



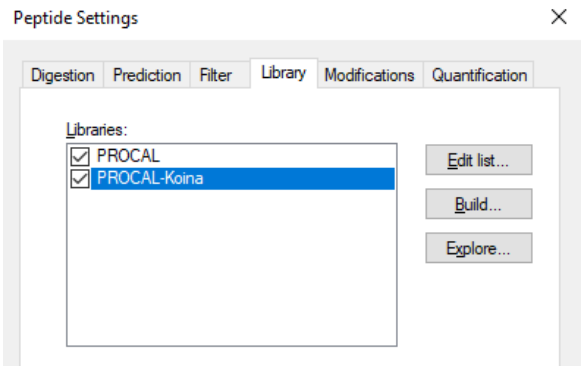
In case you cannot get an online connection to the Koina prediction server please import the PROCAL_Koina.blib spectral library file from the **Tutorial\05_backup** subfolder. Just follow the same steps as for the PROCAL library. You can still perform the Mirror spectrum comparison of both libraries. Only the online Koina prediction that we use later on will not work.

- Click **Ok**
- Select "**31**" in the **NCE** field of the **Build Library** form

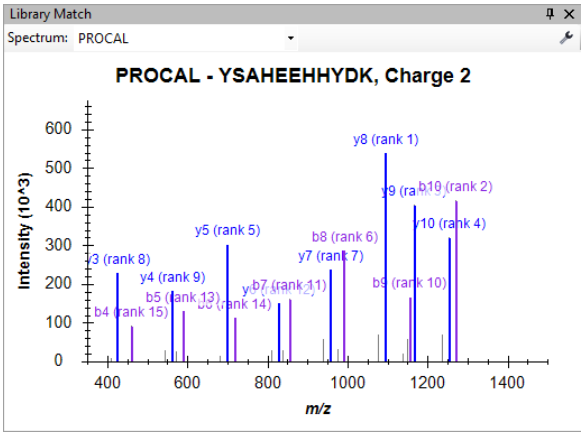


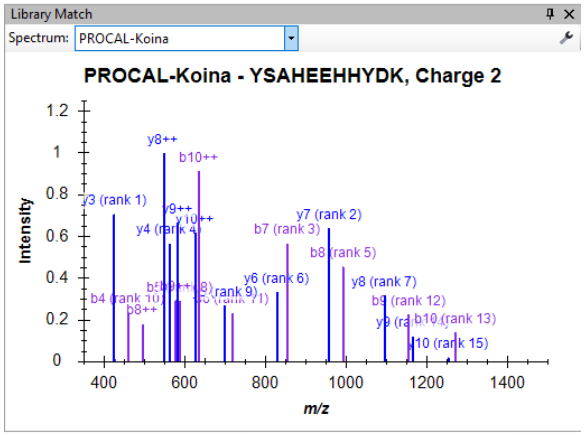
- Click **Finish**

- Make sure both Libraries are ticked in the **Peptide Settings –Library** tab and click **Ok**. The PROCAL_Koina library is loading in the background.



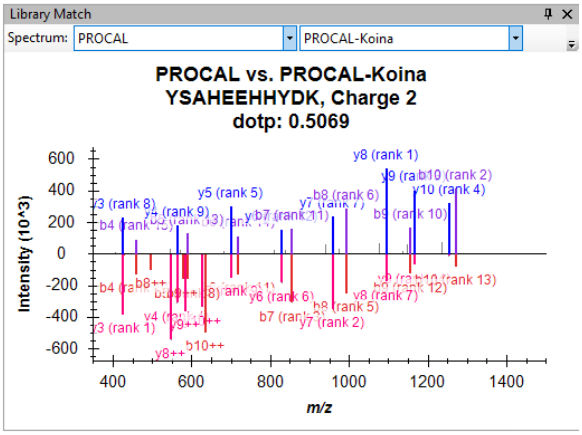
- In the **Library Match** window **Spectrum** field, you can now select one of the two Libraries that are active in the file. Which library spectrum is currently shown is always printed on top of the spectrum together with the spectrum sequence and charge state.





Skyline can only base product-ion selection on a single spectrum from one library. Skyline will search the libraries in the order they appear in the list and use the first spectrum match it finds. With our current settings the PROCAL library is always selected first.

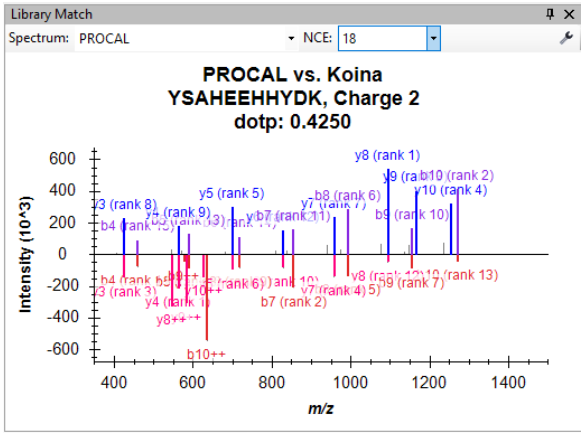
- To compare the libraries in a mirror plot, right click into the **Library Match** window and select **Mirror**
- In the **Spectrum** field select "**PROCAL**" and "**PROCAL_Koina**" in the **Mirror** field

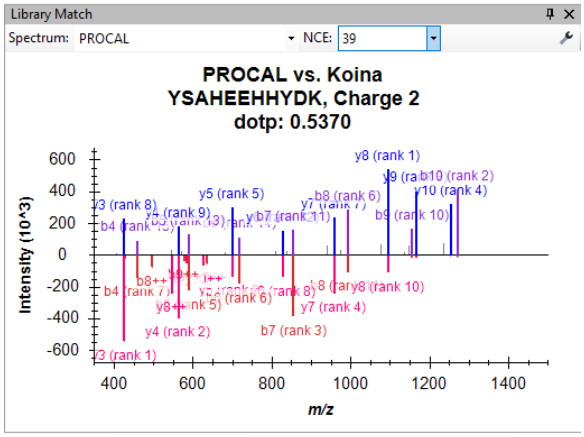


➢ Compare the dotp match factor for different peptides.

- Right click into the **Library Match** window again and select **Koina**. Now you get the same mirror plot with a live Koina prediction for CE 31

➢ Change the CE setting to "**18**" and "**39**" and observe how the fragmentation pattern for peptide changes.
➢ Find for several peptides the optimal CE setting, i.e. the setting where experimental and predicted peptides are as similar as possible. Can the initial setting of "NCE = 31" be further optimized?





- Save the Skyline document as **"Tutorial_Libraries"** in the tutorial folder.

Skyline – PRM data analysis

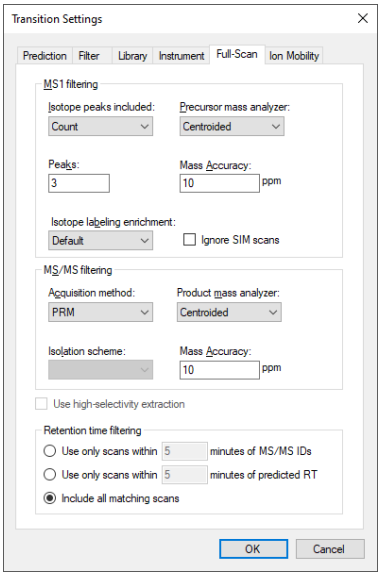
We have created our spectral libraries and selected our target peptide list. In the next step we want to extract the chromatogram information for the precursor and transition ions in our list from Thermo mzML files recorded with a PRM method (for settings see *Appendix: Background information*).

- **Save** the document again as "**Tutorial_PRM**"

Before importing data go again to the **Settings** menu and click **Transition Settings**

- Select the **Full-Scan** tab and specify the settings for **MS/MS filtering**
- In the **Acquisition method** field select "**PRM**"
- In the **Product mass analyzer** field select "**Centroided**"
- In the **Mass Accuracy** field set "**10**" ppm
- **IMPORTANT**: select the tick box "**Include all matching scans**". You need to change this from the standard setting if the retention times recorded in the library do not match the retention times in the file you are importing. Consider this whenever you are combining libraries and files from different LCs, different method settings, different columns etc.

The **Transition Settings – Full-Scan** tab should look like this:

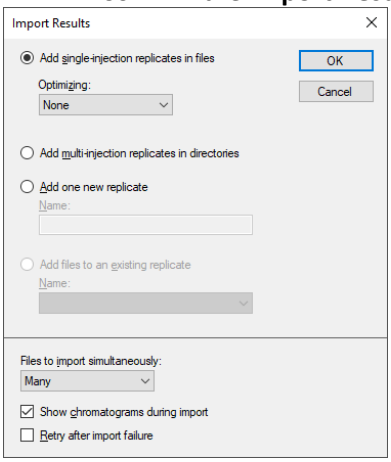


- Click **Ok**

mzML data.
All measurements in this tutorial were recorded as Thermo .raw files. To decrease file size .raw files were converted to .mzML files using MSConvert.

Kessner et al., 2008. ProteoWizard: Open Source Software for Rapid Proteomics Tools Development. Bioinformatics; doi: 10.1093/bioinformatics/btn323).
Martens et al., 2011. mzML – a community standard for mass spectrometry data. MCP; https://doi.org/10.1074/mcp.R110.000133

- To import the mzML data go to the **File** menu, select **Import** and click on **Results**
- Confirm the **Import Results** form settings and click **Ok**



- Navigate to the **Tutorial\02_PRM** subfolder and open the four **PRM mzML files**
- Remove the common prefix "**01_**" (delete "**PRM_**" suggested by Skyline)
- Click **Ok**

The Chromatogram information is extracted from the mzML files. With four or more cores, all files will be processed in parallel. On most laptops with two cores, the import will process two files at a time.

Once the import is finished, we want to adjust the Skyline window view. You can drag and dock any window in Skyline by left clicking on the window's top border, holding the left mouse button down,

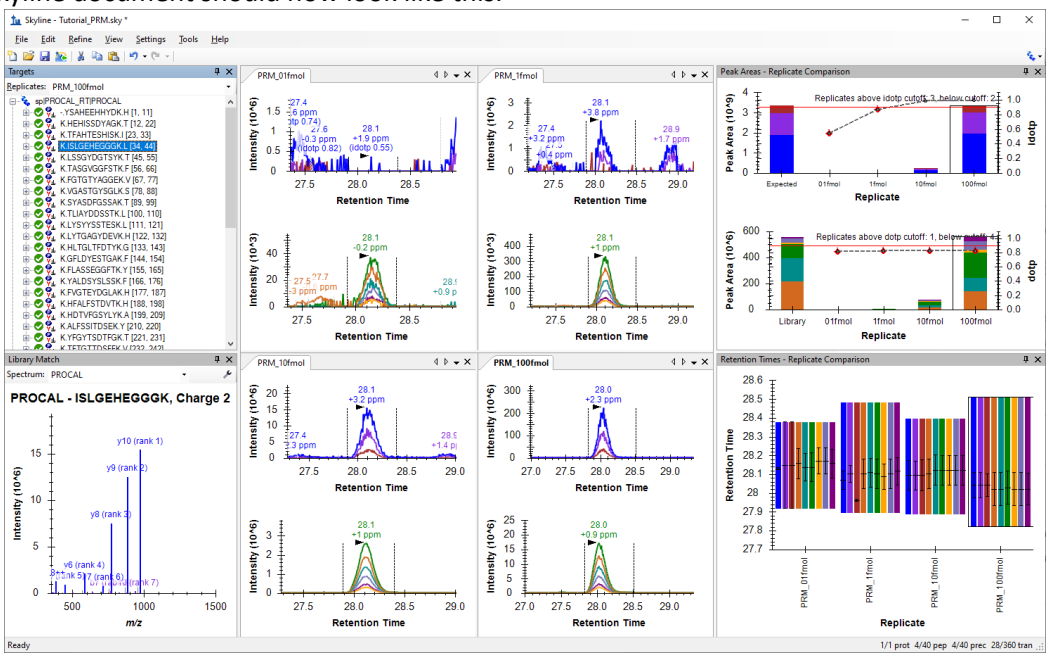
and dragging this window to a new position. Hover over one of the arrow symbols and release the window.

- Drag and drop the "**Library match**" window below the "**Targets**" window
- On the **View** menu go to **Arrange Graphs** and select **Tiled**
- On the **View** menu go to **Retention Times** and select **Replicate Comparison (F8)**
- On the **View** menu go to **Peak Areas** and select **Replicate Comparison (F7)**

If the **Replicate Comparison** windows are floating on your Skyline document, drag and dock the **Peak Areas** - **Replicate Comparison** and **Retention Times** - **Replicate comparison** to a position on the right of the chromatogram windows so that all information is easily visible.

- On the **View** menu go to **Auto-Zoom** and select **Best peak (F11)**
- On the **View** menu go to **Transitions** and select **Split Graph**
- On the **View** menu go to **Transform** and select **None**
- On a small screen the legends take up too much space. Right-click into one **Chromatogram** window and deselect **Legend**. Do the same for the **Replicate Comparison** windows
- Right-click into one **Chromatogram window** and select **Synchronize Zooming**

Your Skyline document should now look like this:



Investigate the different peptide chromatograms.

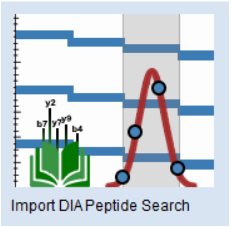
- *Compare the MS1 and MS2 chromatograms for the four PROCAL dilutions*
- *Which peptides are not well detected at which concentration?*
- *Can you find three peptides for which at low concentrations the MS1 signal (precursor ion) has already vanished in the noise, while MS2 signal (product ions) still provide decent chromatogram quality?*
- *Can you find a peptide that behaves the other way around, i.e. for which MS2 signal is of lower quality than MS1?*
- *Can you find a peptide with a strong interference in MS1?*

- **Save** the document

2. Skyline – Read in DIA data

Now that you are already familiar with some Skyline functions, we want to explore how the PROCAL peptide dilution chromatograms look when measured with DIA (for settings see *Appendix: Background information*).
For this we need to open a new Skyline document and adjust the setting for DIA.

- Click the "New Document" button on the toolbar.
- On the **File** menu go **Start**.
- Select the option "**Import DIA Peptide Search**"



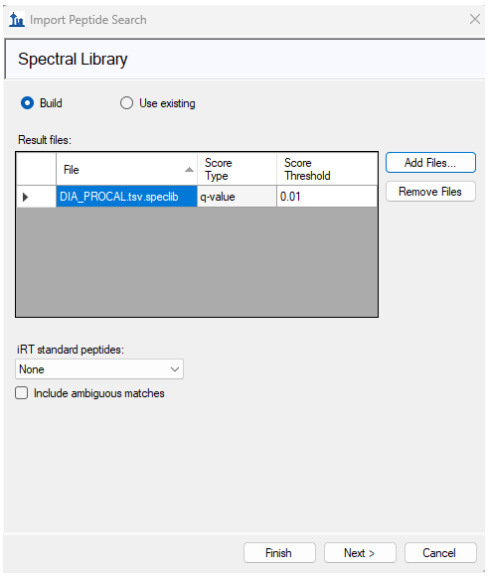
- **Save** the document under "**Tutorial_DIA**"

The **Import Peptide Search** wizard opens. You are presented with the **Spectral Library** page, which allows you to build a project-specific spectral library. Here we use the database search results and library prediction performed in DIA-NN[3] (For settings see *Appendix: Background information*).

[3] Demichev V, Messner CB, Vernardis SI, Lilley KS, Ralser M. DIA-NN: neural networks and interference correction enable deep proteome coverage in high throughput. Nature Methods 17, 41-44 (2020)

- In the **Spectral Library** form, select **Add Files...** and navigate to the **Tutorial\03_DIA subfolder**
- Open the "DIA_**PROCAL.tsv.speclib**" file

The DIA_PROCAL.tsv.speclib-file was predicted with DIA-NN based on the PROCAL.fasta (see *Appendix: Background information*). To read in the spectra information from the measurement files requires in addition the report.tsv result file from the DIA-NN database search. Both files, the .speclib and report.tsv file need to have the same naming. The measurement files do not need to be located in the same folder.

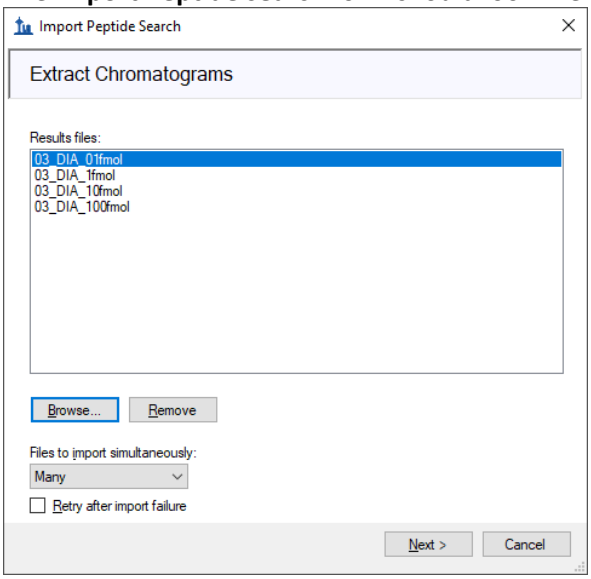


- Click **Next**

You are presented with the **Extract Chromatograms** form, in which you can navigate Skyline to the path for the DIA data files it will use for chromatogram extraction, peak detection, and peak area calculation.

- Click **Browse** and navigate to the **Tutorial\03_DIA subfolder**
- Open the four **DIA mzML files**

The **Import Peptide search** form should look like this:

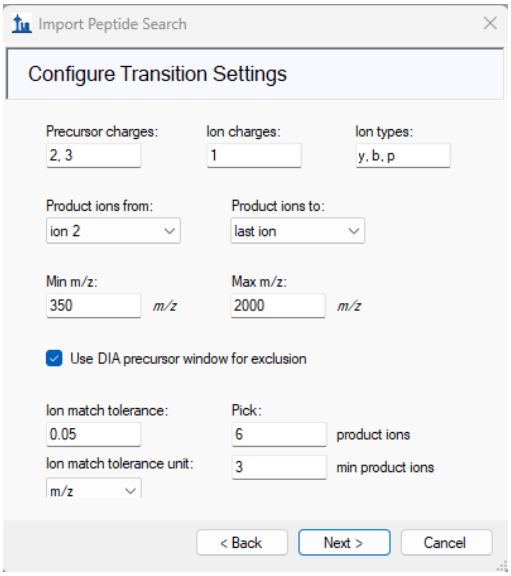


- Click **Next**

- Remove the common prefix "**03_**" (delete "**DIA_**" suggested by Skyline) and click **Ok**
- The Import wizard asks if you want to add modifications. Leave the field empty and click **Next**

In the **Configure Transition Settings** form select:

- **Precursor charges** field "**2,3**"
- **Ion charges** field "**1**"
- **Ion types** "**y, b, p**"
- **Product ions** from "**ion 2**" to **"last ion"**
- **Min m/z** "**350**"
- Tick the box for "**Use DIA precursor isolation window for exclusion**"
- Check the **Ion match tolerance** is set to the default "**0.05**"
- Set Pick: to "6" product ions and "3" min product ions

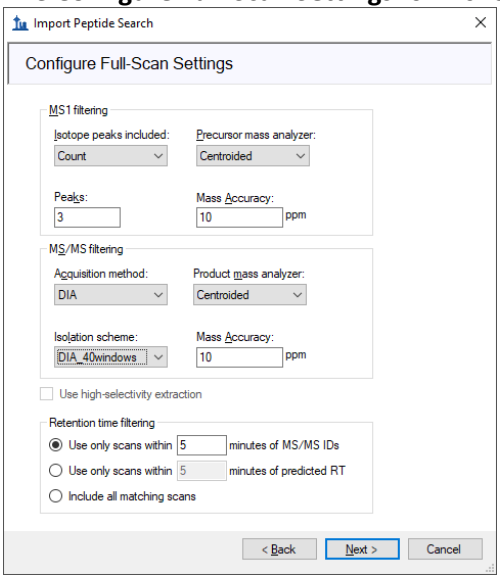


- Click **Next**

In the **Configure Full-Scan Settings** form select:

- MS1 filtering **Precursor mass analyzer** "**Centroided**"
- **Mass Accuracy** "**10 ppm**"
- MS/MS filtering. In the **Isolation scheme** field select **<Add>**. Type in the name "**DIA_40windows**"
- Change to the option "**Prespecified isolation windows**"
- Click on **Import**, navigate to the **Tutorial\03_DIA** subfolder and open the first DIA mzML file. Skyline automatically reads in the DIA windows settings from the mzML file.
- Click **Graph** to have a look at the isolation scheme and then **Close**.
- Click **Ok**

The **Configure Full-Scan Settings** form should look like this:



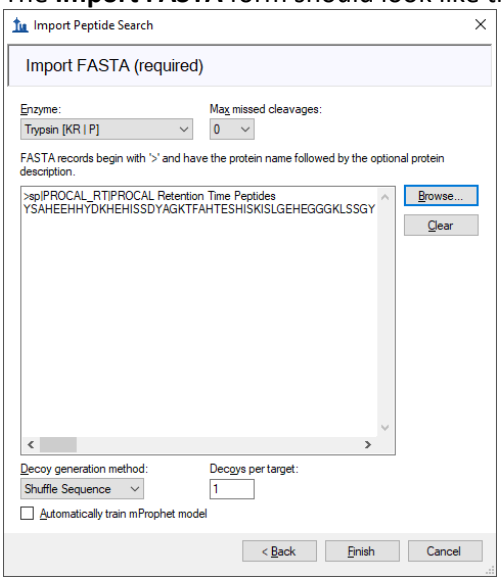
- Click **Next**

In the **Import FASTA (requires)** form go to:

- **Browse** and navigate to the **Tutorial\02_PRM** subfolder. Open the "**PROCAL.fasta**" file

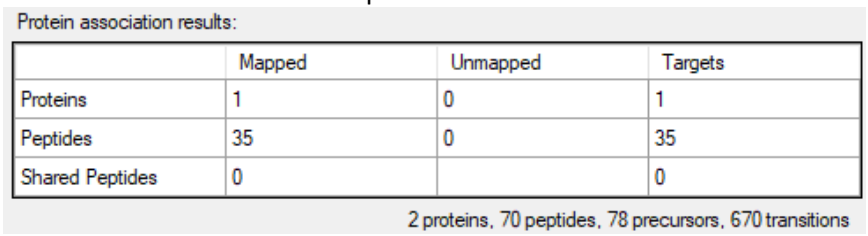
- In the **Decoy generation method** field select "**Reverse Sequence**"

The **Import FASTA** form should look like this:



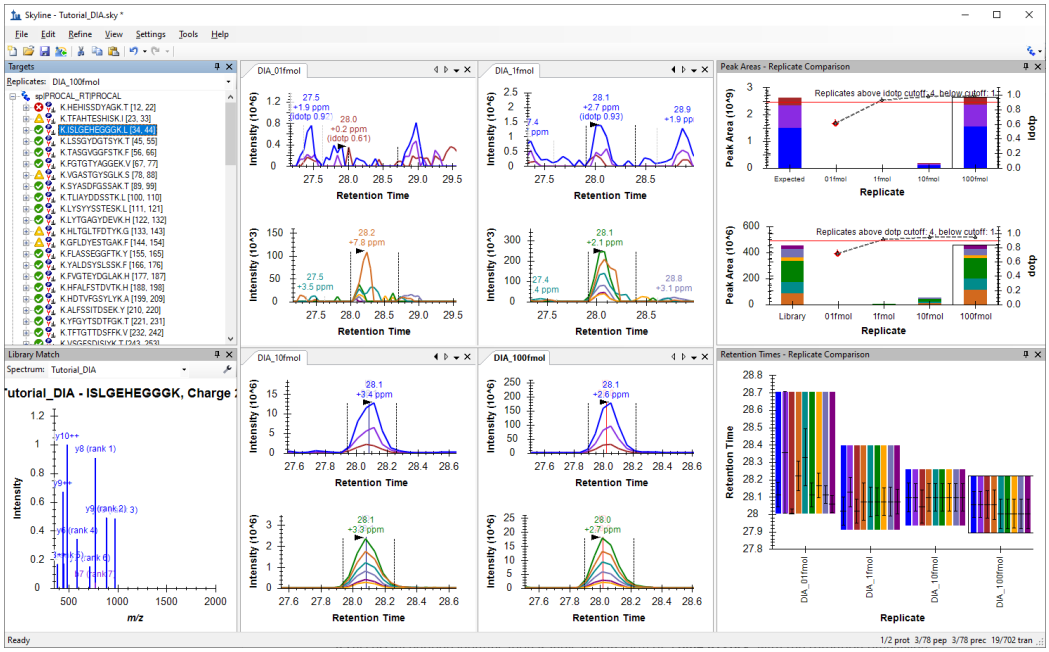
- Click **Finish**

The **Associate Proteins** form opens:



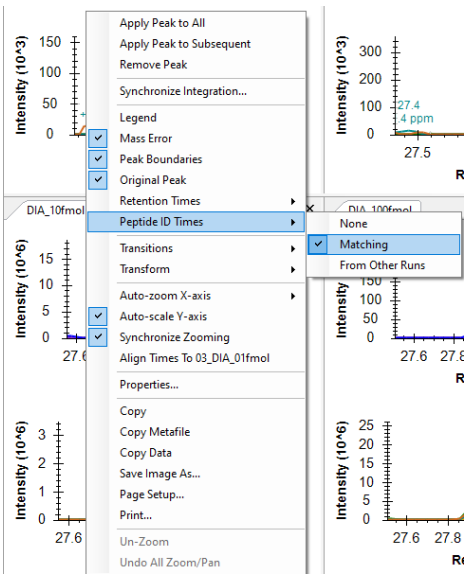
- Leave everything as default and click **Ok**

Skyline begins extracting chromatograms from the DIA mzML files. During this time you can already organize the Skyline windows as before. You should only need View > Arrange Graphs > Tiled.



As you have loaded at the beginning of the Import DIA Peptide Search procedure the results file from a DIA-NN analysis (the PROCAL-tsv.speclib and the PROCAL-report.tsv present in the same folder), Skyline is able to show you at which retention time DIA-NN has successfully identified each peptide in each raw file.

A successful peptide identification is indicated in form of a **blue ID stick**, with the retention time given on top. You can add or remove this peptide identification information by right-clicking onto any chromatogram window, selecting "**Peptide ID Times"** and activating or de-activating **"Matching".**



Now quantitatively investigate the different peptide chromatograms.

- *Compare the MS1 and MS2 chromatograms for the four PROCAL dilutions*
- *Which peptides are not well detected? At which concentrations?*
- *Look up the peptides you investigated in detail in the PRM data before and compare those in the DIA data.*

- *Compare the retention time in the 0.1fmol sample for "TASGVGGFSTK". Compare the mass errors for the selected peaks of this peptide over all concentration. Manually correct the peak area integration in the 0.1fmol sample.*
- *Check the peptide "HDTVFGSYLYK". What is the interfered product ion here? Remove it from the document.*
- *Manually correct peak area integration and remove interfered product ions for the whole document.*
- *Have a look at the decoy peptides "GAYDSSIHEHK", "LGSYGTSAGVK" or "TILGDDIVFGK". What is the dotp score for the 0.1fmol sample?*

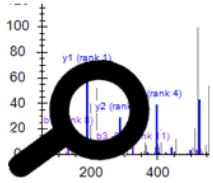
- **Save** the document.

3. Skyline – Read in DDA data

Now that you are already familiar with some Skyline functions, we want to explore how the PROCAL peptide dilution chromatograms look when measured with DDA (for settings see *Appendix: Background information*).

For this we need to open a new Skyline document and adjust the setting for DDA.

- Click the "New Document" button on the toolbar.
- On the **File** menu go **Start**.
- Select the option "**Import DDA Peptide Search**"



Import DDA Peptide Search

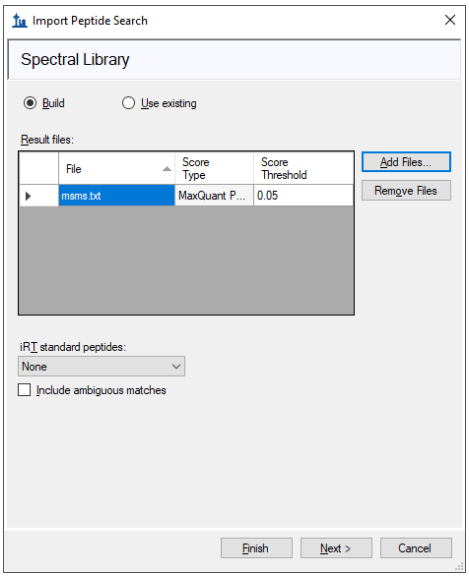
- **Save** the document under "**Tutorial_DDA**"

The **Import Peptide Search** wizard opens. You are presented with the **Spectral Library** page, which allows you to build a project-specific spectral library. Here we use the database search results from **MaxQuant**[4] (For settings see *Appendix: Background information*)

Reading the spectra information form the measurement files requires msms.txt result file and mqpar file from the MaxQuant database search. The measurement files need to be located in the same folder/ or subfolder.

[4] Cox J, & Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nature Biotechnology 26, 1367-1372 (2008)

- In the **Spectral Library** form, select **Add Files...** and navigate to the **Tutorial\04_DDA** subfolder
- Open the "msms.txt" file



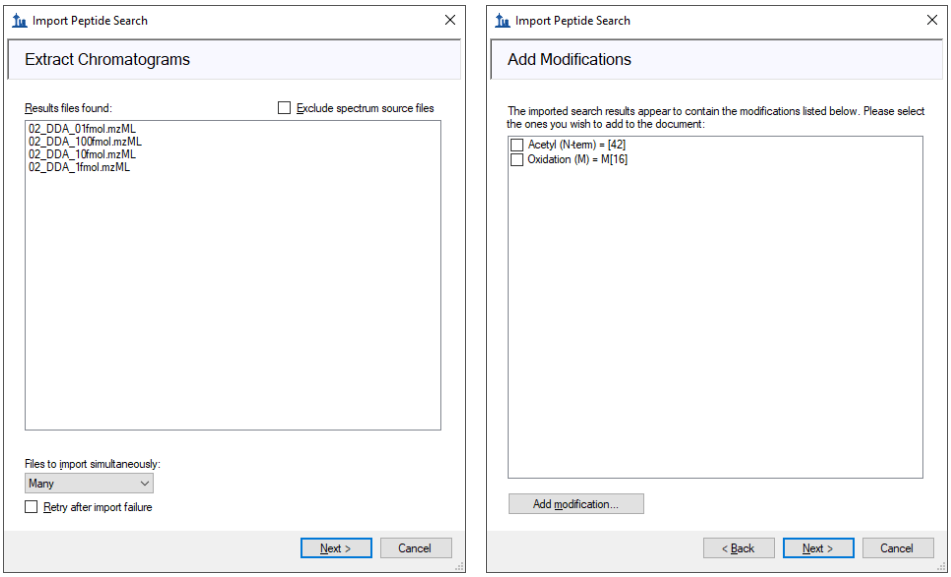
- Click **Next**

Skyline reads in the identified spectra from the DDA database search and creates a new Spectral library file.

You are presented with the **Extract Chromatograms** form where the mzML file(s) used in the database search is usually automatically selected if it is located in the same folder as the txt folder. If not navigate to the **Tutorial\04_DDA** subfolder and select the four DDA mzML files.

- In the **Import Results** form remove the prefix "**02_**" (delete "**DDA_**" suggested by Skyline)
- Click **Ok**

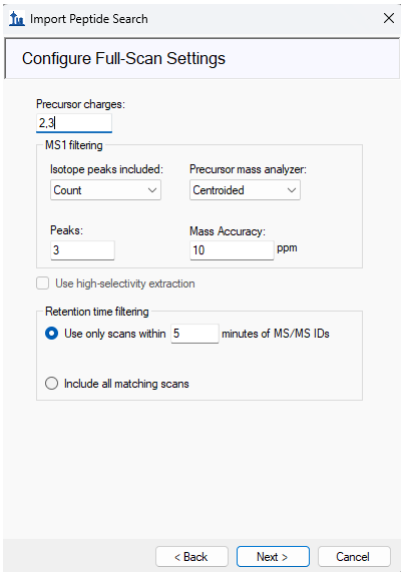
- Click **Next** in the **Add Modifications** tab



In the **Configure Full-Scan Settings** form change Precursor charges to "2,3" and otherwise accept defaults:

- **Precursor mass analyzer** "**Centroided**"
- **Mass Accuracy** "**10 ppm**"

The **Configure Full-Scan Settings** form should look like this:

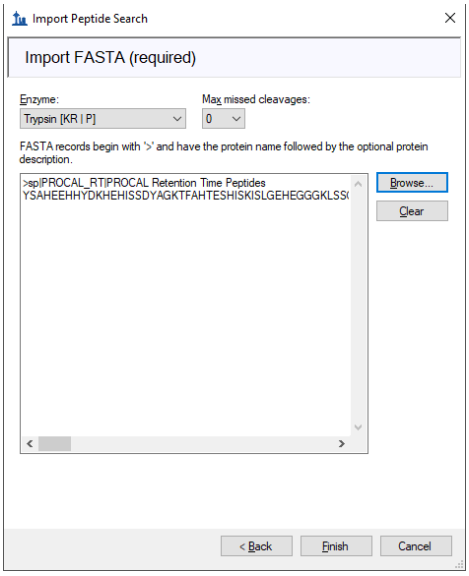


- Click **Next**

In the **Import FASTA (requires)** form go to:

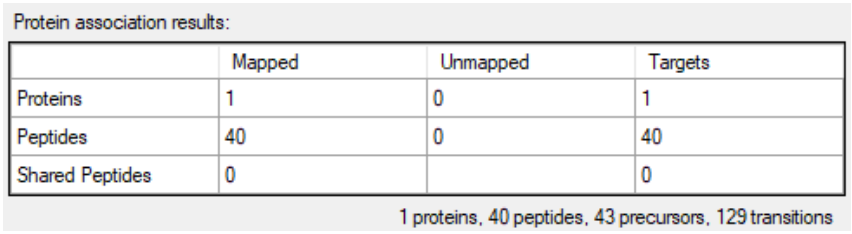
- **Browse** and navigate to the **Tutorial\02_PRM** subfolder. Open the "**PROCAL.fasta**" file

The **Import FASTA (required)** form should look like this:



- Click **Finish**

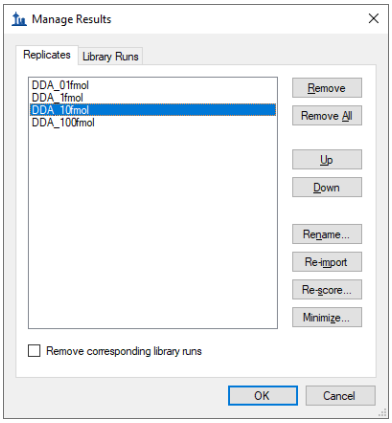
The **Associate Proteins** form opens:



- Leave everything as default and click **Ok**

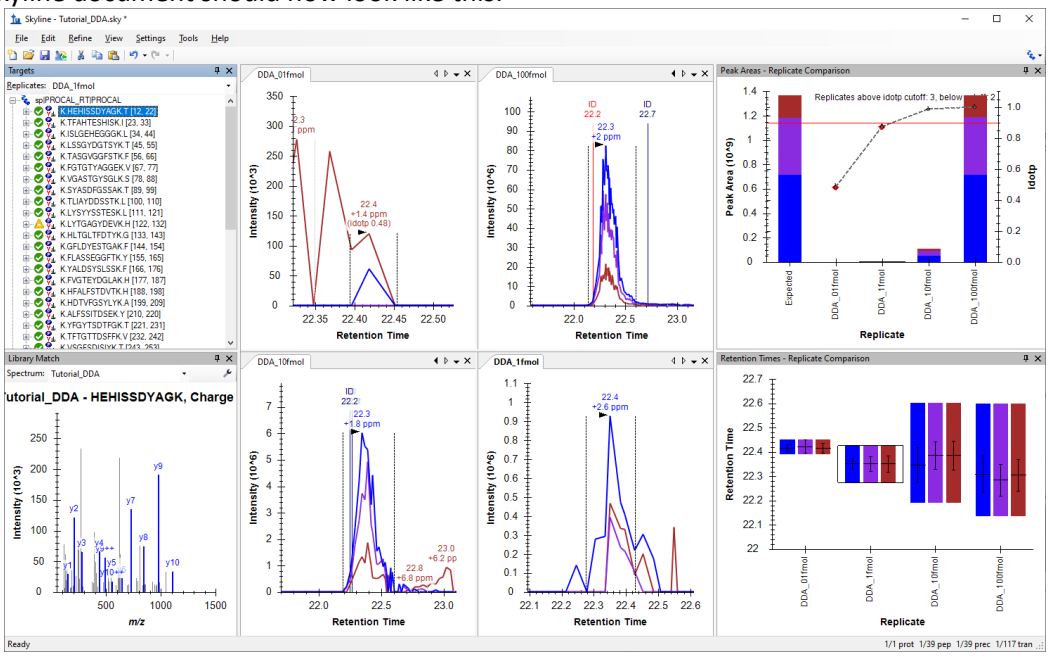
Skyline begins extracting chromatograms.

As the order of the raw files is not according to increased concentration, go to **Edit** menu and select **Manage Results...** Change the order of the Replicates according to increasing concentration using the UP and DOWN buttons.

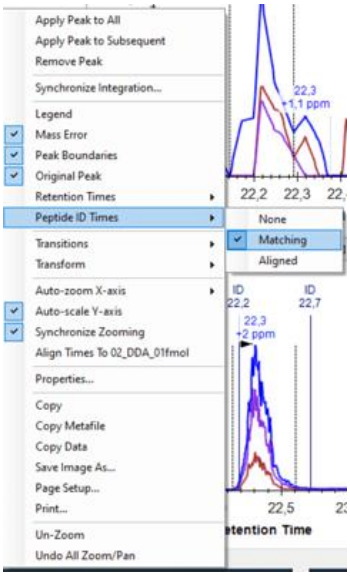


- Select on the **View** menu **Arrange Graphs → Tiled.**

Your Skyline document should now look like this:



As you have loaded DDA data, Skyline will only show you MS1 chromatograms but no product ion (MS2) chromatograms. However, the information if and at which retention time an MS2 spectra was recorded that led to a successful peptide identification with MaxQuant is indicated again in form of a blue ID stick, with the retention time given on top. You can add or remove the MS2 spectra information by right-clicking onto any chromatogram window, selecting "**Peptide ID Times"** and activating or de-activating **"Matching".**



Investigate the identified MS2 spectra for all peptides in your Skyline document.

- *How many identified spectra do you tend to get per precursor in each raw file?*
- *At which concentrations do you tend to get more identifications?*
- *Do you tend to get MS2 identifications rather in the beginning, the end, or in the middle of the peak/chromatogram?*

Now quantitatively investigate the different peptide MS1 chromatograms.

- *Compare the MS1 chromatogram peak areas for the four PROCAL dilutions for all peptides.*
- *Which peptides are not well detected? At which concentration(s)?*
- *Specifically, look up the peptides "TASGVGGFSTK" and "HLTGLTFDTYK". Carefully check retention time, idotp and mass errors and how those values change between the different concentrations. What is the issue with the 0.1 fmol sample?*
- *Manually correct the wrongly integrated peptides in the document.*

- **Save** the document.

Conclusion

Congratulations, you have completed this Skyline tutorial! You have learned to import and build spectral libraries and how to investigate target peptides in PRM, DIA or DDA measurement files. The doors are open to go crazy and explore your data in more detail.

Extra task for quick people ☺

- Export precursor and product ion total areas from the Skyline document using **File → Export → Report**
- Visualize those ion intensities using your favourite data visualization tool. Think about optimal ways how to plot the data. For example, plot intensity ratios per peptide (measured peptide intensity at each concentration divided by the peptide intensity at 100 fmol) versus peptide retention time for all three data acquisition methods on MS1 and/or MS2 level.
- What general trends do you observe?

Do you see something that does not fit your expectations?

4. Background information

1) Spectral Libraries

In this tutorial we created spectral libraries for the PROCAL peptide selection. You can do the same for any peptide selection for example using public available spectral library sources like:

- Peptide Atlas (http://www.peptideatlas.org/speclib/)
- National Institute of Standards and Technology (NIST) (https://peptide.nist.gov/)
- The Global Proteome Machine (GPM) (ftp://ftp.thegpm.org/projects/xhunter/libs/)

The Proteome Tools project offers spectral libraries recorded from synthesized peptides at different collision energies and fragmentation settings.

- Proteome Tools (https://www.proteometools.org/index.php?id=53)

You can also create new spectral libraries in Skyline using other publicly available data, or peptide search results from your laboratory experiments. Skyline supports building libraries from the following search result formats:

BlibBuild (https://skyline.ms/wiki/home/software/BiblioSpec/page.view?name=BlibBuild)

Database search	Peptide ID file extension	Spectrum file extension *RAW includes vendor formats like RAW, WIFF, .D, etc.	Score Used	Notes
Generic SSL	.ssl		score column	A generic format for encoding spectrum library entries.
ByOnic	.mzid	.MGF, .mzXML, .mzML	AbsLogProb	
Comet/SEQUEST /Percolator	.perc.xml, .sqt	.cms2, .ms2, .mzXML	q-value	Percolator v1.17 does not include sequence modification information therefore the .sqt file from the SEQUEST search must be present in the same directory, the directory containing the cms2/ms2 spectrum files, or the current working directory.
DIA-NN	.speclib		none	No separate spectrum file. In the current implementation, no score is imported from the library, so all spectra are imported.
IDPicker	.idpXML	.mzXML, .mzML	FDR	The name(s) of the spectrum file(s) are given in the .idpXML file.
MS Amanda	.pep.xml, .pepXML	.mzML, .mzXML, .MGF, RAW*	q-value	
MSFragger	.pep.xml, .pepXML	.mzML, .mzXML, .MGF, RAW*	q-value	
MSGF+	.mzid, .pepXML	.mzML, .mzXML, .MGF, RAW*	expectation value	
Mascot	.dat		expectation value	No separate spectrum file.
MaxQuant Andromeda	msms.txt + evidence.txt + mqpar.xml + modifications.xml	.mzML, .mzXML, .MGF, RAW*	PEP	It is possible to use peaks embedded in the msms.txt, but external spectra files are preferred because the embedded peaks are charge deconvoluted. `mqpar.xml` must be located in the grandparent, parent, or same directory. A custom `modifications.xml`, `modifications.local.xml`, or `modification.xml` can be placed in the same directory as the search results (or specified using the `-x` option).
Morpheus	.pep.xml, .pepXML	.mzXML, .mzML	q-value	The names of the .mzXML files are given in the .pep.xml file and may be in the parent or grandparent directory. Spectra are looked up by index, which is calculated using (scan number - 1).
OMSSA	.pep.xml, .pepXML	.mzXML, .mzML	expectation value	The names of the .mzXML files are given in the .pep.xml file and may be in the parent or grandparent directory.
OpenSWATH	.tsv		m_score column	No separate spectrum file.
PEAKS DB	.pep.xml, .pepXML	.mzXML, .mzML	confidence score	The names of the .mzXML files are given in the .pep.xml file and may be in the parent or grandparent directory.
PLGS MS^E	final_fragment.csv		score column	There need not be a . before 'final_fragment'..
PRIDE	.pride.xml		various	No separate spectrum file.
PeptideProphet/iProphet	.pep.xml, .pepXML	.mzML, .mzXML, .MGF, RAW*	probability score	The names of the .mzXML files are given in the .pep.xml file and may be in the parent or grandparent directory.
PeptideShaker	.mzid	.MGF	confidence score	
Protein Pilot	.group.xml		confidence score	No separate spectrum file.
Protein Prospector	.pep.xml, .pepXML	.mzML, .mzXML, .MGF, RAW*	expectation value	
Proteome Discoverer	.msf, .pdResult		q-value	No separate spectrum file. Libraries cannot be built from databases that do not contain q-values, unless a cutoff score of 0 is explicitly specified.
Proxl XML	.proxl.xml	.mzML, .mzXML, .MGF, RAW*	q-value	
Scaffold	.mzid	.MGF, .mzXML, .mzML	peptide probability	
Spectronaut	.csv		none	Spectronaut Assay Library export. No separate spectrum file.
Spectrum Mill	.pep.xml, .pepXML	.mzXML, .mzML	expectation value	The names of the .mzXML files are given in the .pep.xml file and may be in the parent or grandparent directory.
X! Tandem	.xtan.xml		expectation value	No separate spectrum file.

- Custom (SSL) (https://skyline.ms/blib-formats.url)

2) Eclipse instrument settings

LC-Settings for PRM, DIA & DDA
Ultimate 3000 RSLCnano system

- Trap column (ReproSil-pur C18-AQ, 5 µm, Dr. Maisch, 20 mm × 75 µm, self-packed) at a flow rate of 5 µL/min in HPLC grade water with 0.1% (v/v) formic acid
- Analytical column (ReproSil Gold C18-AQ, 3 µm, Dr. Maisch, 450 mm × 75 µm, self-packed)
- 50 min linear gradient from 4% to 32% of solvent B (0.1% formic acid and 5% (v/v) DMSO in acetonitrile) at 300 nL/min flow rate. nanoLC solvent A was 0.1% (v/v) formic acid, 5% (v/v) DMSO in HPLC grade water

Orbitrap Eclipse

Parameter	PRM	DIA	DDA
MS1			
Orbitrap Resolution	60,000	120,000	60,000
Scan Range (m/z)	360-1300	360-1300	360-1300
Max IT (ms)	50	50	50
Norm.AGC Target	100%	100%	100%
MS2			
Isolation Window	1.3	-	1.3
NCE (%)	30	30	30
Orbitrap Resolution	30,000	30,000	15,000
Scan Range (m/z)	140-2000	200-1800	-
Max IT (ms)	120	54	22
Norm.AGC Target	400%	1000%	200%
Cycle time	-	-	2
Dynamic exclusion	-	-	30s
Windows	-	40 variable	

3) MaxQuant & DIA-NN settings

MaxQant v.2.4.0.0

Parameter	Settings
Type	Standard
Enzyme	Trypsin/P
Missed clevages	1
Modifications	Fixed: Carbamidomethyl (C) Variable: Oxidation (M);Acetyl (Protein N-term)
Label-free quantification	None
Sequences	PROCAL.fasta; human reference UP000005640 Swiss Prot fasta
Match between runs	FALSE
PSM FDR	1%
Protein FDR	1%
Min peptide length	7

DIA-NN v.1.8.1

Parameter	Settings
Spectral library prediction	PROCAL.fasta; human reference UP000005640 Swiss Prot fasta ; contaminants.fastaTrypsin/P; 1 Missed cleavages 0 Maximum number of variable modifications
DIA search	
Spectral library	PROCAL predicted library (.speclib)
Peptide length range	7-30
Precursor m/z range	360-1300
Fragment ion m/z range	200-1800
MBR	TRUE
Protein inference	Genes
Neural network classifier	Single-pass mode
Quantification strategy	Robust LC (high precision)
Cross-run normalization	RT-dependent
Library generation	Smart profiling
Speed and RAM usage	Optimal results

4) File conversion

Thermo .raw files were converted to mzML files using MSConvert (ProteoWizard: Open Source Software for Rapid Proteomics Tools Development. Darren Kessner; Matt Chambers; Robert Burke; David Agus; Parag Mallick. Bioinformatics 2008; doi: 10.1093/bioinformatics/btn323).

Parameter	**Settings**
Output format	mzML
Binary encoding precision	64-bit
Write Index;Use zlib compression;TPP compatibility	TRUE;TRUE;TRUE
Filters	Peak Picking; MS Levels 1-2