

## Supplementary Note 1: Analyzing pulseSILAC-DIA data with Prosit+EncyclopeDIA and Skyline

This tutorial is a practical guide for how to use the Encyclopedia software suite to build. . In this tutorial, we will detail how we analyzed the pulse SILAC-DIA data using EncyclopeDIA to first build a library of detected endogenous peptides, then using Skyline to pair the endogenous precursors to their “heavy” SILAC counterpart and extracting their chromatograms for quantification.

### SUMMARY: Four steps for pulseSILAC-DIA analysis

1. Convert .raw files to .mzML using MSConvert

*\*Not covered here, please see Pino et al 2020 Supplementary Note 1 (<https://doi.org/10.1074/mcp.P119.001913>) for a detailed tutorial.*

2. Build time point zero library using Prosit and EncyclopeDIA

3. Search pulsed data with library from step 2 using EncyclopeDIA

4. Extract light/heavy chromatograms using Skyline

### Citations

MSconvert (<https://www.nature.com/articles/nbt.2377>)

A cross-platform toolkit for mass spectrometry and proteomics. Chambers MC et al. *Nat Biotech* 30, 918-920 (2012). doi.org/10.1038/nbt.2377

Encyclopedia (<https://www.nature.com/articles/s41467-018-07454-w>)

Chromatogram libraries improve peptide detection and quantification by data independent acquisition mass spectrometry. Searle BC et al. *Nat Comm* 9, 5128 (2018). doi.org/10.1038/s41467-018-07454-w

Prosit+Encyclopedia (<https://www.nature.com/articles/s41467-020-15346-1>)

Generating high quality libraries for DIA MS with empirically corrected peptide predictions. Searle BC et al. *Nat Commun* 11, 1548 (2020). <https://doi.org/10.1038/s41467-020-15346-1>

You will need:

- MSConvert from Proteowizard: *Windows only!*
  - <http://proteowizard.sourceforge.net/download.html>
- EncyclopeDIA suite (\*.jar file): *command line and cross-platform GUI*
  - <https://bitbucket.org/searleb/encyclopedia/wiki/Home>
- Skyline: *Windows only!*
  - <https://skyline.ms/project/home/software/Skyline/begin.view>

To exactly replicate the results here, you will also need:

- RAW DIA data files from the tutorial bortezomib dataset (PXD022659)
- Ready-made predicted spectral library in \*.dlib format and accompanying FASTA of the Uniprot human reference proteome (reviewed; 20,350 entries) available on the Prosit Libraries website

## BUILD THE POOL OF PEPTIDES PRESENT AT TIME ZERO

1. Launch EncyclopeDIA. Under “Parameters:” click the “Library:” Edit button. Navigate to and select your Prosit predicted library (in DLIB format). Then, click the “Background:” Edit button to navigate to and select the corresponding FASTA for that Prosit library.

**! NOTE:** for a detailed tutorial on generating custom Prosit libraries, please see the *Supplementary Info for Searle et al 2020*

**! NOTE:** Prosit libraries for common organisms (human and yeast) can also be found on the [Prosit website here](#).

EncyclopeDIA: Peptide Searching for DIA

File View Convert Help

EncyclopeDIA Thesaurus Walnut

### EncyclopeDIA: Library Searching Directly from Data-Independent Acquisition (DIA) MS/MS Data

EncyclopeDIA extracts peptide fragmentation chromatograms from MZML files, matches them to spectra in libraries, and calculates various scoring features. These features are interpreted by Percolator to identify peptides.

**Parameters:**

Library:  Edit

Background:  Edit

Target/Decoy Approach: Normal Target/Decoy

Data Acquisition Type: Non-Overlapping DIA

Enzyme: Trypsin

Fragmentation: HCD (Y-Only)

Precursor Mass Tolerance: 10.0 PPM

Fragment Mass Tolerance: 10.0 PPM

Library Mass Tolerance: 10.0 PPM

Percolator Version: v3-01

Number of Quantitative Ions: 5

Minimum Number of Quantitative Ions: 3

Number of Cores: 8

Additional Command Line Options:

**Console:**  
EncyclopeDIA Graphical Interface (version 0.9.5)

52 of 9102 MB used

**Jobs:** Add MZML Save Chromatogram Library Save Quant Reports Save BLIB

File Progress

2. At the top next to “Jobs:”, click “Add MZML” to select the narrow window, gas phase fractionated (GPF) MZML files for your experiment.

**! NOTE:** The GPF library should be composed of the “time zero” sample from your pulse SILAC experiment. In other words, this pooled library sample should be composed entirely of endogenous/light peptides prior to incorporation of heavy SILAC amino acids.

The screenshot displays the EncyclopeDIA software interface. The main window title is "EncyclopeDIA: Peptide Searching for DIA". The interface includes a menu bar (File, View, Convert, Help) and a toolbar with buttons for "Add MZML", "Save Chromatogram Library", "Save Quant Reports", and "Save BLIB".

The central panel features a header "EncyclopeDIA: Library Searching Directly from Data-Independent Acquisition (DIA) MS/MS Data" with a sub-image of a book. Below this, a description states: "EncyclopeDIA extracts peptide fragmentation chromatograms from MZML files, matches them to spectra in libraries, and calculates various scoring features. These features are interpreted by Percolator to identify peptides."

The "Parameters:" section contains the following settings:

- Library: `uniprot_human_25Apr2019.fasta.z2_nce33.dlib` (Edit)
- Background: `uniprot_human_25Apr2019.fasta` (Edit)
- Target/Decoy Approach: Normal Target/Decoy
- Data Acquisition Type: Non-Overlapping DIA
- Enzyme: Trypsin
- Fragmentation: HCD (Y-Only)
- Precursor Mass Tolerance: 10.0 PPM
- Fragment Mass Tolerance: 10.0 PPM
- Library Mass Tolerance: 10.0 PPM
- Percolator Version: v3-01
- Number of Quantitative Ions: 5
- Minimum Number of Quantitative Ions: 3
- Number of Cores: 8
- Additional Command Line Options: (empty)

At the bottom left, a scrollable list shows "Parsed" percentages from 49% to 65%. At the bottom right, a "Jobs:" table lists several reads with their corresponding progress status:

File	Progress
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_L1B1_400-500...	Converting files...
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_L1B2_500-600...	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_L1B3_600-700...	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_L1B4_700-800...	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_L1B5_800-900...	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_L1B6_900-100...	


At the bottom of the interface, it indicates "166 of 9102 MB used".

3. After all GPF library files have completed, click “Save Chromatogram Library” to perform the final FDR filtering and save the ELIB.

EncyclopeDIA: Peptide Searching for DIA

File View Convert Help

EncyclopeDIA Thesaurus Walnut



## EncyclopeDIA: Library Searching Directly from Data-Independent Acquisition (DIA) MS/MS Data

EncyclopeDIA extracts peptide fragmentation chromatograms from MZML files, matches them to spectra in libraries, and calculates various scoring features. These features are interpreted by Percolator to identify peptides.

**Parameters:**

Library:  Edit

Background:  Edit

Target/Decoy Approach: Normal Target/Decoy

Data Acquisition Type: Non-Overlapping DIA

Enzyme: Trypsin

Fragmentation: HCD (Y-Only)

Precursor Mass Tolerance: 10.0 PPM

Fragment Mass Tolerance: 10.0 PPM

Library Mass Tolerance: 10.0 PPM

Percolator Version: v3-01

Number of Quantitative Ions: 5

Minimum Number of Quantitative Ions: 3

Number of Cores: 8

Additional Command Line Options:

```

t Extracting 980.7 to 982.7 m/z (26.119476 to 98.64414 min)
t Extracting 982.7 to 984.7 m/z (19.837406 to 99.33775 min)
t Extracting 984.7 to 986.7 m/z (17.656512 to 105.60191 min)
t Extracting 986.7 to 988.7 m/z (23.385439 to 105.625496 min)
t Extracting 988.7 to 990.7 m/z (12.423291 to 107.52876 min)
t Extracting 990.7 to 992.7 m/z (24.696596 to 101.166595 min)
t Extracting 992.7 to 994.7 m/z (19.949072 to 101.021515 min)
t Extracting 994.7 to 996.7 m/z (13.809503 to 101.38755 min)
t Extracting 996.7 to 998.7 m/z (23.661320 to 99.38044 min)
t Extracting 998.7 to 1000.7 m/z (27.054232 to 100.47675 min)
t Extracting 1000.7 to 1002.7 m/z (35.593396 to 99.344025 min)
g EncyclopeDIA ELIB from 20200828_QEHFX_lkp_pSILAC-DIA_btz_LIB8_900-1000MZ_149.mzML (6174 entries)...
g 6174 peptides to entries table...
g 6174 peptides to peptidequants table...
ed writing to EncyclopeDIA ELIB at Tue Sep 22 18:21:17 EDT 2020
g global target/decoy peptides: 62731/653, pi0: 0.926237
g global target/decoy proteins: 9064/90

```

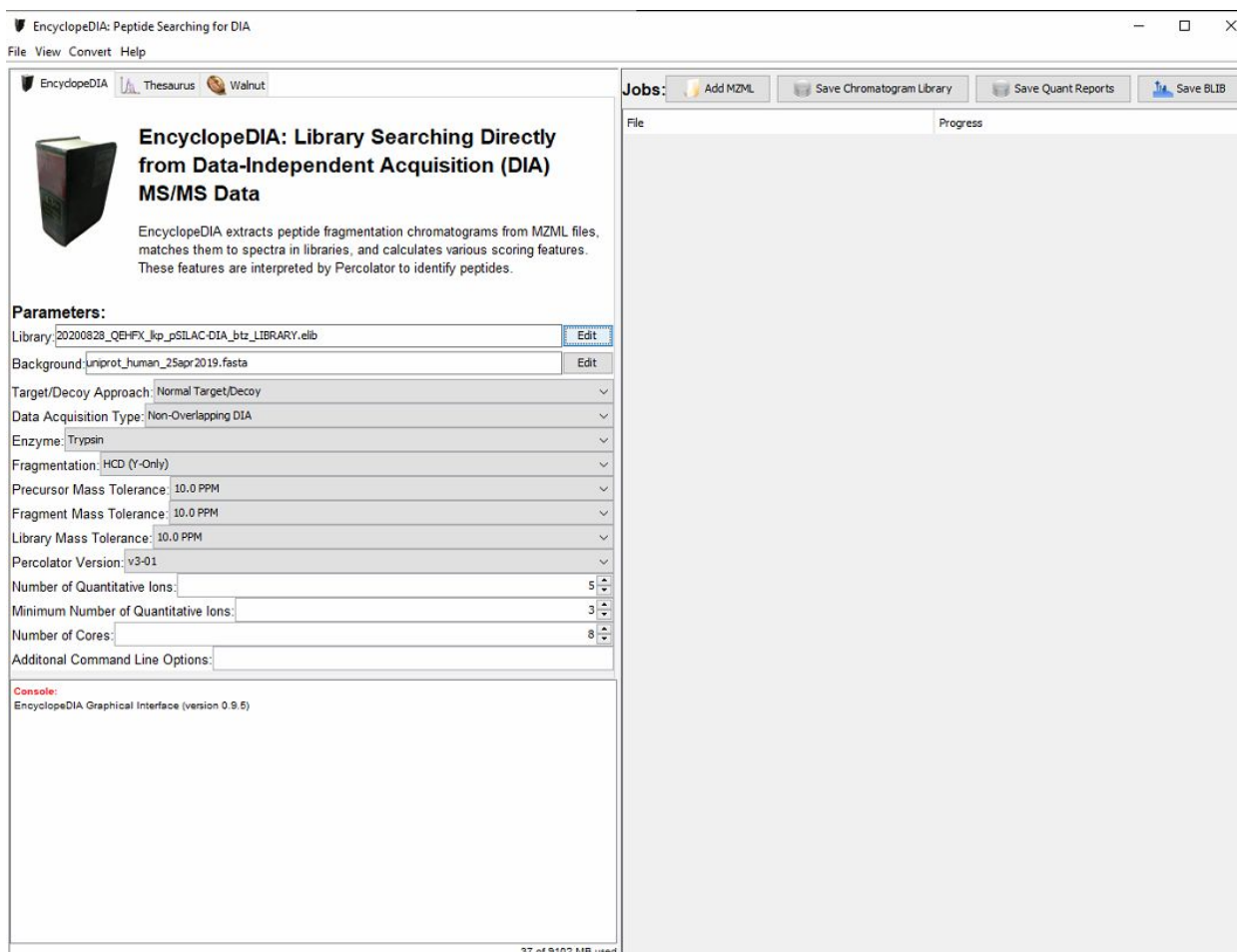
427 of 9102 MB used

**Jobs:** Add MZML Save Chromatogram Library Save Quant Reports Save BLIB

File	Progress
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_LIB1_400-500...	Wrote 10414 peptides identified at 1.0% FDR
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_LIB2_500-600...	Wrote 11321 peptides identified at 1.0% FDR
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_LIB3_600-700...	Wrote 11970 peptides identified at 1.0% FDR
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_LIB4_700-800...	Wrote 12329 peptides identified at 1.0% FDR
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_LIB5_800-900...	Wrote 7117 peptides identified at 1.0% FDR
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_LIB6_900-100...	Wrote 16448 peptides identified at 1.0% FDR
Write Library 20200828_QEHFX_lkp_pSILAC-DIA_btz_LIBRA...	62731 peptides identified at 1.0% FDR

## SEARCH PULSED DATA WITH LIBRARY FROM TIME ZERO

4. Close and relaunch EncyclopeDIA to clear the cache. Under “Parameters:” click the “Library:” Edit button. Navigate to and select the chromatogram library you created in Step 3 above (in ELIB format). Then, click the “Background:” Edit button to navigate to and select the corresponding FASTA for the original Prosit library.




The screenshot displays the EncyclopeDIA software interface. The main window title is "EncyclopeDIA: Peptide Searching for DIA". The interface includes a menu bar (File, View, Convert, Help) and a toolbar with buttons for "Add MZML", "Save Chromatogram Library", "Save Quant Reports", and "Save BLIB". The main content area is divided into two panes: "File" and "Progress". The "Parameters:" section is visible, showing various settings for the search process. The "Library:" field is set to "20200828\_QEHFX\_lkp\_pSILAC-DIA\_btz\_LIBRARY.elib" and has an "Edit" button next to it. The "Background:" field is set to "Unprot\_human\_25Apr2019.fasta" and also has an "Edit" button. Other parameters include "Target/Decoy Approach" (Normal Target/Decoy), "Data Acquisition Type" (Non-Overlapping DIA), "Enzyme" (Trypsin), "Fragmentation" (HCD (Y-Only)), "Precursor Mass Tolerance" (10.0 PPM), "Fragment Mass Tolerance" (10.0 PPM), "Library Mass Tolerance" (10.0 PPM), "Percolator Version" (v3-01), "Number of Quantitative Ions" (5), "Minimum Number of Quantitative Ions" (3), and "Number of Cores" (8). The "Additional Command Line Options:" field is empty. A "Console:" window at the bottom left shows the text "EncyclopeDIA Graphical Interface (version 0.9.5)". The status bar at the bottom indicates "37 of 9102 MB used".

5. At the top next to “Jobs:”, click “Add MZML” to select the wide window, single-shot MZML files for your experiment.

EncyclopeDIA: Peptide Searching for DIA

File View Convert Help

EncyclopeDIA Thesaurus Walnut



### EncyclopeDIA: Library Searching Directly from Data-Independent Acquisition (DIA) MS/MS Data

EncyclopeDIA extracts peptide fragmentation chromatograms from MZML files, matches them to spectra in libraries, and calculates various scoring features. These features are interpreted by Percolator to identify peptides.

**Parameters:**

Library: 20200828\_QEHFX\_lkp\_pSILAC-DIA\_btz\_LIBRARY.elib

Background: Uniprot\_human\_25Apr2019.fasta

Target/Decoy Approach: Normal Target/Decoy

Data Acquisition Type: Non-Overlapping DIA

Enzyme: Trypsin

Fragmentation: HCD (Y-Only)

Precursor Mass Tolerance: 10.0 PPM

Fragment Mass Tolerance: 10.0 PPM

Library Mass Tolerance: 10.0 PPM

Percolator Version: Y3-01

Number of Quantitative Ions: 5

Minimum Number of Quantitative Ions: 3

Number of Cores: 8

Additional Command Line Options:

```

Adding mzMML import to queue for
[C:\Users\linds\Desktop\20200828_pSILAC-DIA\btz\20200828_QEHFX_lkp_pSILAC-DIA_btz_38_DIA_130.mzML]
Adding new job to queue: Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_38_DIA_130.mzML
Adding mzMML import to queue for
[C:\Users\linds\Desktop\20200828_pSILAC-DIA\btz\20200828_QEHFX_lkp_pSILAC-DIA_btz_37_DIA_122.mzML]
Adding new job to queue: Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_37_DIA_122.mzML
Adding mzMML import to queue for
[C:\Users\linds\Desktop\20200828_pSILAC-DIA\btz\20200828_QEHFX_lkp_pSILAC-DIA_btz_38_DIA_141.mzML]
Adding new job to queue: Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_38_DIA_141.mzML
Adding mzMML import to queue for
[C:\Users\linds\Desktop\20200828_pSILAC-DIA\btz\20200828_QEHFX_lkp_pSILAC-DIA_btz_39_DIA_118.mzML]
Adding new job to queue: Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_39_DIA_118.mzML
Adding mzMML import to queue for
[C:\Users\linds\Desktop\20200828_pSILAC-DIA\btz\20200828_QEHFX_lkp_pSILAC-DIA_btz_40_DIA_130.mzML]
Adding new job to queue: Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_40_DIA_130.mzML
Adding mzMML import to queue for
[C:\Users\linds\Desktop\20200828_pSILAC-DIA\btz\20200828_QEHFX_lkp_pSILAC-DIA_btz_40_DIA_157.mzML]
Adding new job to queue: Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_40_DIA_157.mzML
Converting 20200828_QEHFX_lkp_pSILAC-DIA_btz_01_DIA_156.mzML ...
  
```


55 of 9102 MB used

**Jobs:**

File	Progress
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_01_DIA_156....	Converting files...
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_02_DIA_129....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_03_DIA_113....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_04_DIA_153....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_05_DIA_109....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_06_DIA_133....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_07_DIA_124....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_08_DIA_130....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_09_DIA_119....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_10_DIA_131....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_32_DIA_126....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_33_DIA_114....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_34_DIA_152....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_35_DIA_110....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_36_DIA_130....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_37_DIA_122....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_38_DIA_141....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_39_DIA_118....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_40_DIA_130....	
Read 20200828_QEHFX_lkp_pSILAC-DIA_btz_40_DIA_157....	

- After all wide window, single-shot files have completed, click "Save Quant Report" to perform the global retention time alignment, fragment ion refinement, and FDR filtering; and save the ELIB.





### EncyclopeDIA: Library Searching Directly from Data-Independent Acquisition (DIA) MS/MS Data

EncyclopeDIA extracts peptide fragmentation chromatograms from MZML files, matches them to spectra in libraries, and calculates various scoring features. These features are interpreted by Percolator to identify peptides.

**Parameters:**

Library: 20200828\_QEHFX\_kp\_pSILAC-DIA\_btz\_LIBRARY.elib

Background: lniprot\_human\_25Apr2019.fasta

Target/Decoy Approach: Normal Target/Decoy

Data Acquisition Type: Non-Overlapping DIA

Enzyme: Trypsin

Fragmentation: HCD (Y-Only)

Precursor Mass Tolerance: 10.0 PPM

Fragment Mass Tolerance: 10.0 PPM

Library Mass Tolerance: 10.0 PPM

Percolator Version: v3-01

Number of Quantitative Ions: 5

Minimum Number of Quantitative Ions: 3

Number of Cores: 8

Additional Command Line Options:

```

            want Extracting 964.7 to 968.7 m/z (85.45108 to 98.28442 min)
            want Extracting 970.7 to 980.7 m/z (82.15037 to 82.983696 min)
            want Extracting 980.7 to 984.7 m/z (84.38043 to 75.40048 min)
            want Extracting 992.7 to 996.7 m/z (91.428474 to 92.28181 min)
            want Extracting 996.7 to 1000.7 m/z (89.964115 to 75.600695 min)
            fitting Encyclopedia ELIB from 20200828_QEHFX_kp_pSILAC-DIA_btz_04_DIA_153.mzML (16745 entries) ...
            fitting 16745 peptides to entries table ...
            fitting 16745 peptides to peptidequants table ...
            finished writing to Encyclopedia ELIB at Wed Sep 23 20:41:39 EDT 2020
            fitting local target/decoy peptides: 16792/277, pF0. 0.431048
            fitting local target/decoy proteins: 2738/27
            finished analysis! 16792 peptides identified at 1.0% FDR (11.5 minutes)

            error deleting temp file!
            doing ELIB export to queue for
            ?:\Users\lind\Desktop\20200828_pSILAC-DIA\btz\20200828_QEHFX_kp_pSILAC-DIA_btz_QUANT.elib)
            doing new job to queue: Write Library 20200828_QEHFX_kp_pSILAC-DIA_btz_QUANT.elib
        
```

**Jobs:**

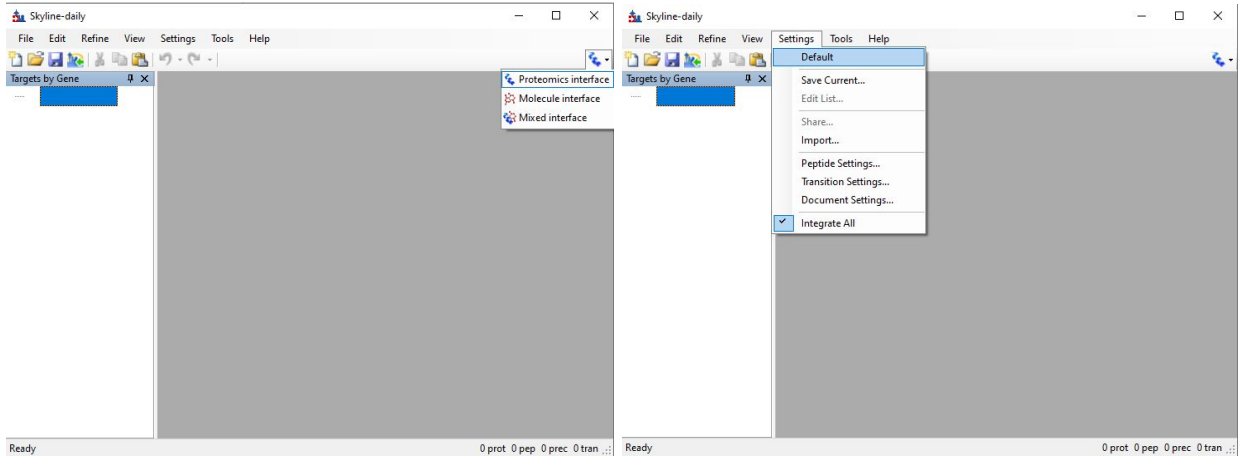
File	Progress
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_01_DIA_156.mzML	Wrote 17574 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_02_DIA_129.mzML	Wrote 9524 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_03_DIA_113.mzML	Wrote 18411 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_05_DIA_109.mzML	Wrote 17816 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_06_DIA_133.mzML	Wrote 15258 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_07_DIA_124.mzML	Wrote 11645 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_08_DIA_130.mzML	Wrote 9673 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_09_DIA_119.mzML	Wrote 3042 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_10_DIA_131.mzML	Wrote 0 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_32_DIA_126.mzML	Wrote 14710 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_33_DIA_114.mzML	Wrote 19094 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_34_DIA_152.mzML	Wrote 18029 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_35_DIA_110.mzML	Wrote 13540 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_36_DIA_130.mzML	Wrote 15994 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_37_DIA_122.mzML	Wrote 15944 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_38_DIA_141.mzML	Wrote 11243 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_39_DIA_118.mzML	Wrote 3785 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_40_DIA_130.mzML	Wrote 0 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_40_DIA_157.mzML	Wrote 17870 peptides identified at 1.0% FDR
Read 20200828_QEHFX_kp_pSILAC-DIA_btz_04_DIA_153.mzML	Wrote 16792 peptides identified at 1.0% FDR
Write Library 20200828_QEHFX_kp_pSILAC-DIA_btz_QUANT.elib	

113 of 9102 MB used

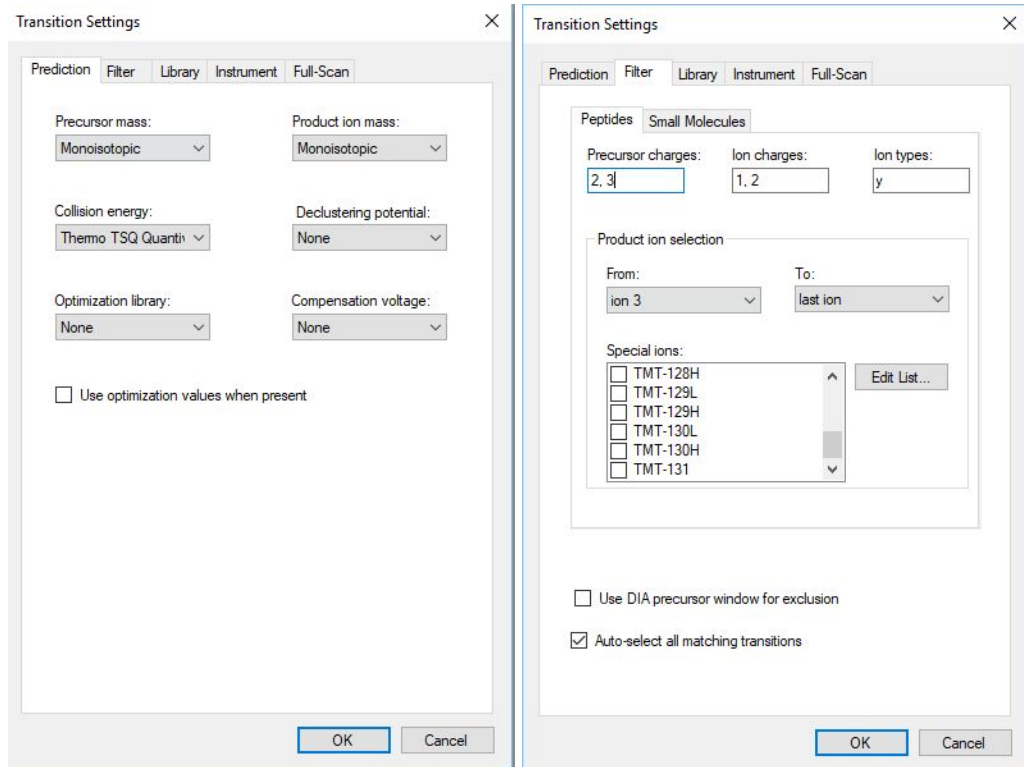


## QUANTIFY SILAC PAIRS USING SKYLINE

7. Launch Skyline and open a blank document. Ensure that you are in protein mode and default settings (Settings > Default).



8. Prepare the Skyline document with the appropriate settings per the EncyclopeDIA search performed above and as dictated by the instrument method settings. For this experiment, match the following parameters in Settings > Transition Settings:



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  
 minimum 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

Transition Settings

Prediction Filter Library Instrument Full-Scan

MS1 filtering

Isotope peaks included:       Precursor mass analyzer:

Peaks:       Resolution:

Isotope labeling enrichment:

MS/MS filtering

Acquisition method:       Product mass analyzer:

Isolation scheme:       Mass Accuracy:  ppm

Use high-selectivity extraction

Retention time filtering

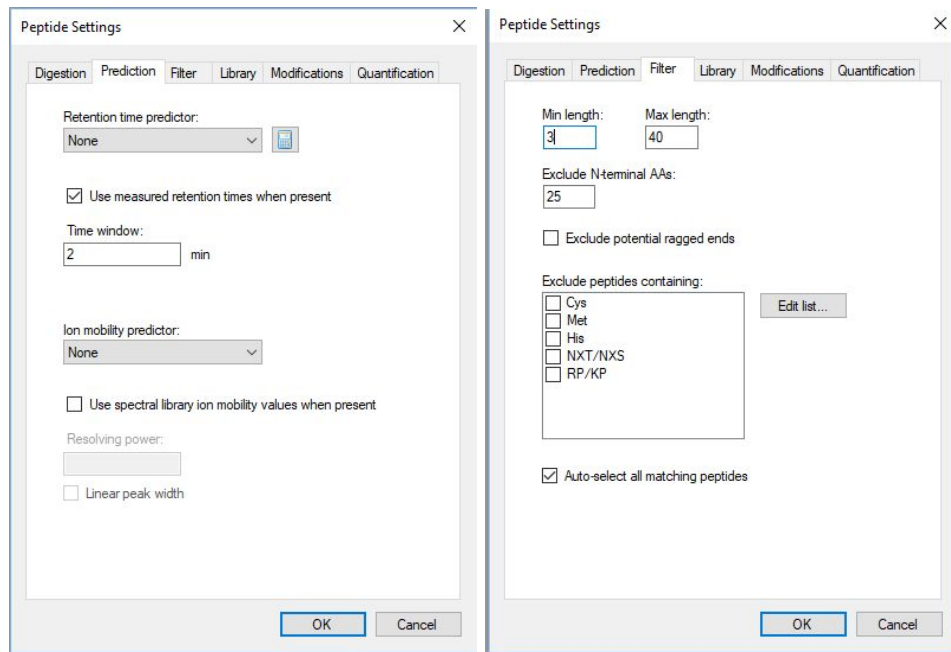
Use only scans within  minutes of MS/MS IDs  
 Use only scans within  minutes of predicted RT  
 Include all matching scans

OK Cancel

- Prediction: Precursor/Product ion mass="Monoisotopic"
- Filter: Ion charges=1,2 Ion types="y"\*, From=Ion 3, To=last ion, no special ions

- Library: Ion match tolerance=0.005 m/z, check “If a library spectrum is available, pick its most intense ions”, pick=5 product ions, select “From filtered product ions”
- Instrument: “Min m/z=50, Max m/z=1500, Method match tolerance m/z=0.005
- Full-Scan (MS1): “Isotope peaks included=None”
- Full-Scan (MS/MS): “Acquisition method=DIA, Product mass analyzer=Centroided, Isolation scheme=Results only, Mass Accuracy=10 ppm, check “Use only scans within “2” minutes of MS/MS IDs

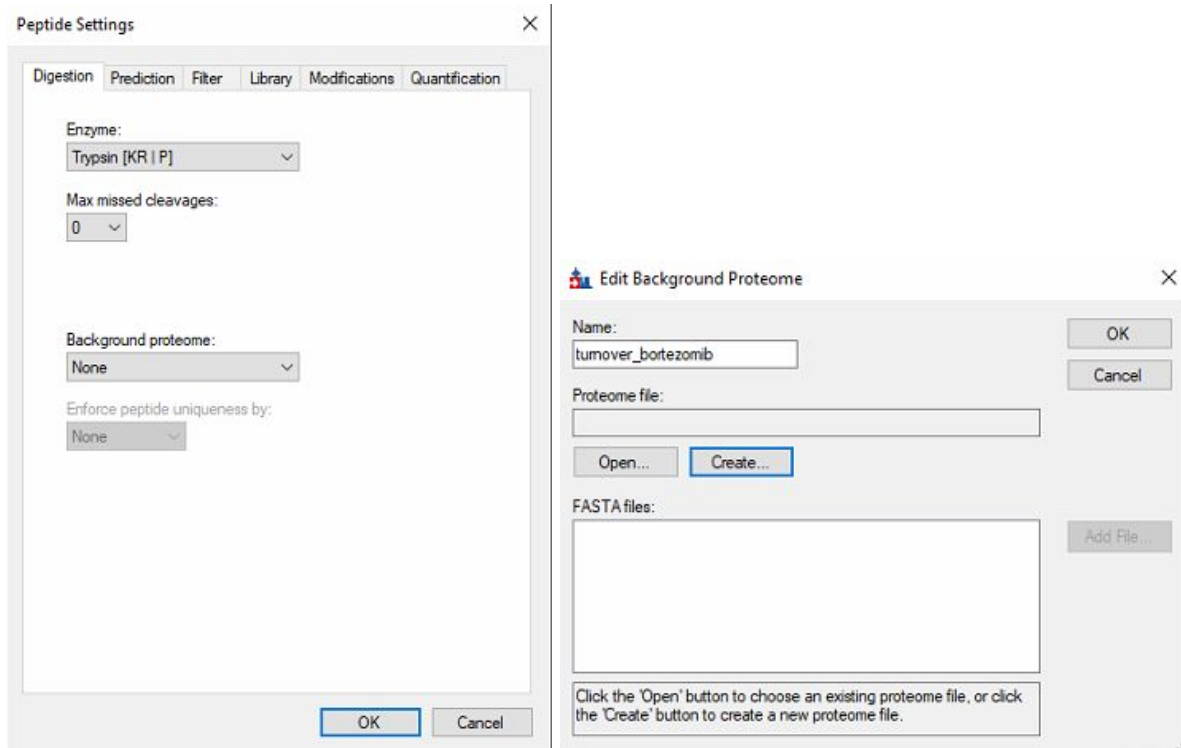
9. Next, set the parameters for the Prediction and Filter tabs of the peptide settings (Settings > Peptide Settings), again per the EncyclopeDIA search performed above and as dictated by the instrument method settings. For this experiment:



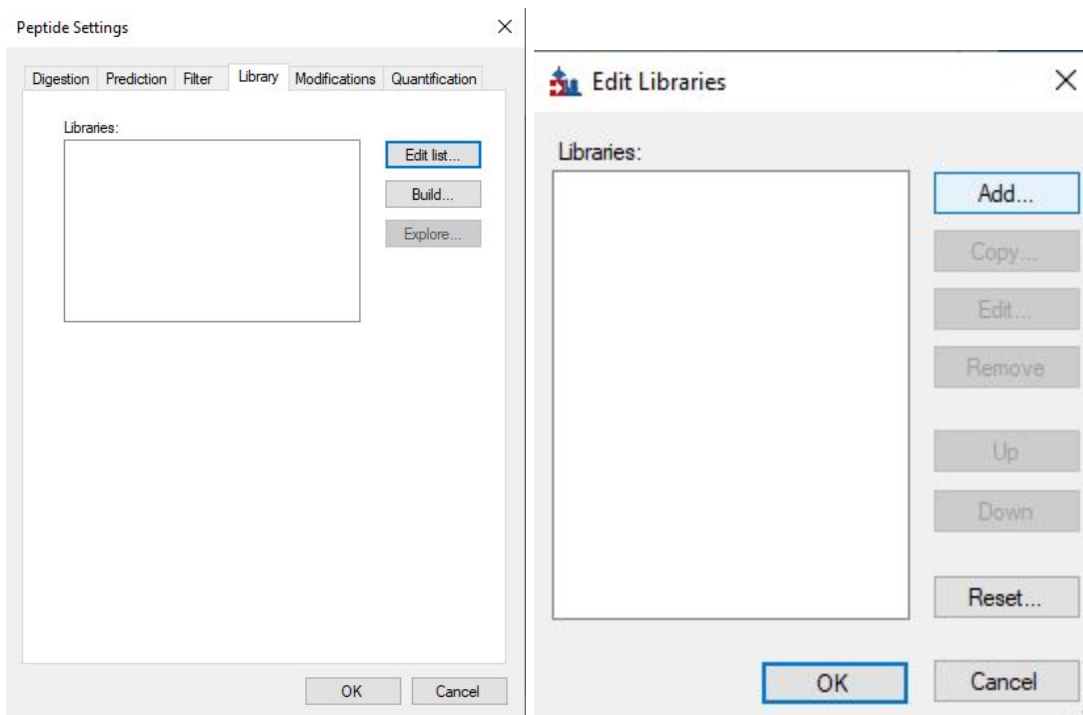
- Prediction: Check “Use measured retention times when present”, Time window=2
- Filter: Min length=3, Max length=40, no excluded amino acids checked

10. In the Digestion tab of the Peptide Settings (Settings > Peptide Settings > Digestion) expand the “Background proteome:” drop down to select <Add...>. Type a working name for this background proteome and then click “Create...” to give the background proteome file a name and filepath location. Click “Add...” and navigate to the same FASTA used in steps 1 and 4 above. Once the background proteome file has been created, press “OK” to return to the Peptide Settings window.

**! NOTE:** After selecting the FASTA, this may take some time. Skyline should display a progress bar.

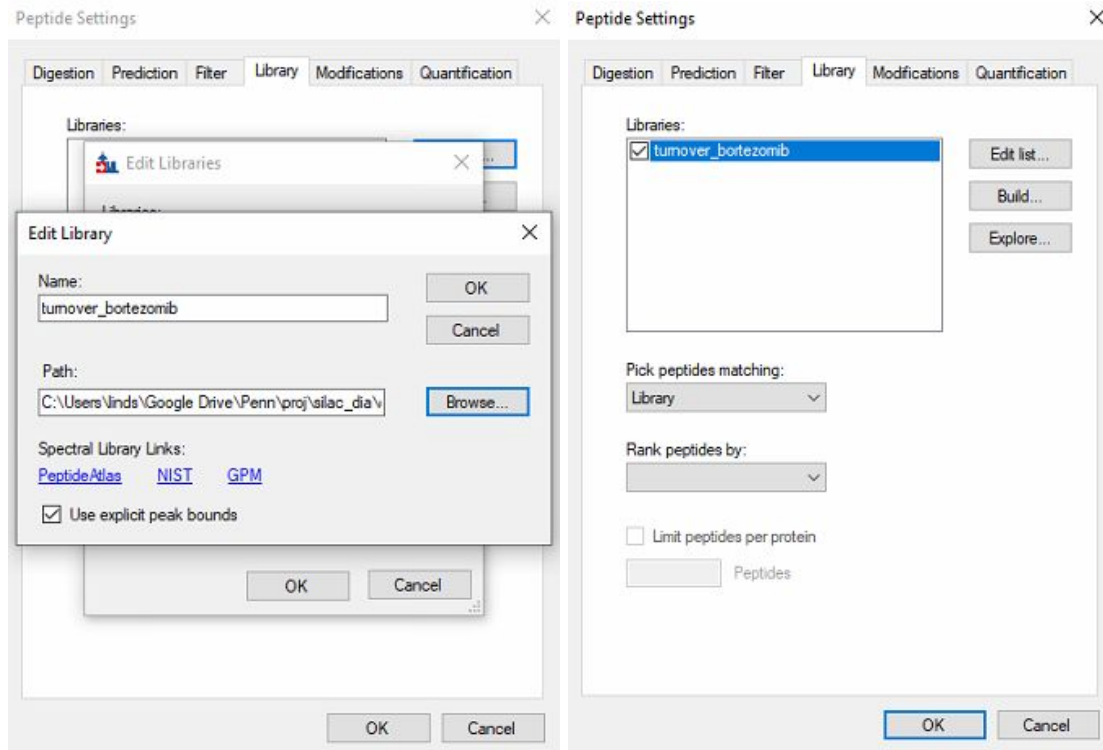


11. In the Library tab of the Peptide Settings window (Settings > Peptide Settings > Library) click “Edit list...” and then “Add...”



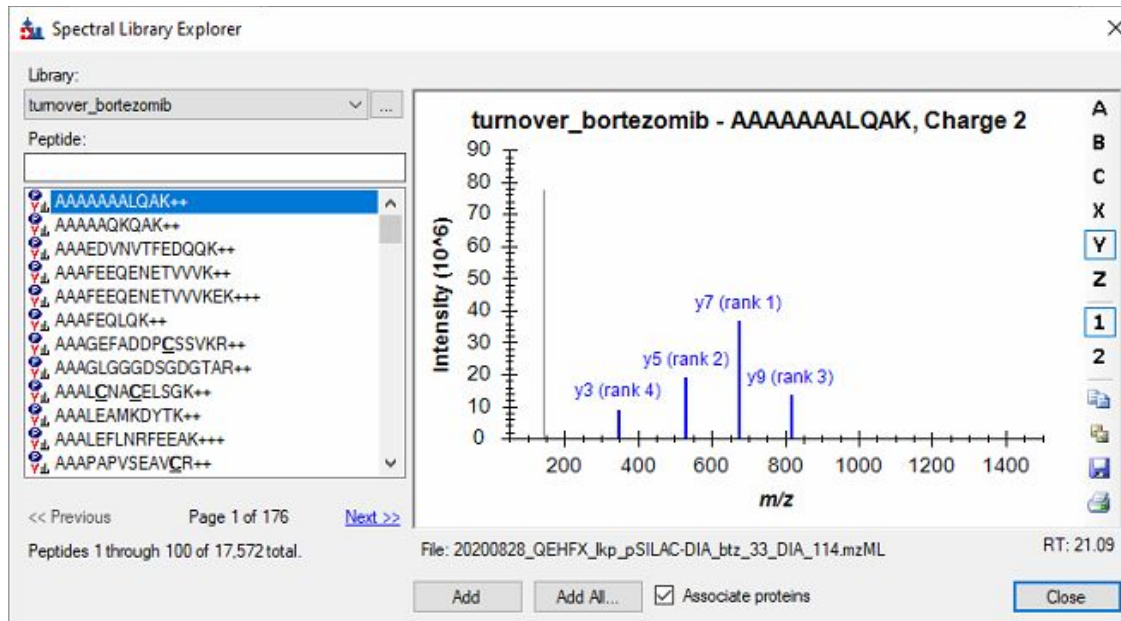
- Fill out a working name for the library, then next to “Path:” click “Browse...” and navigate to the final ELIB that was saved in step 6 above. Select “OK” and then “OK” again to get back to the Peptide Settings window. Check the box next to the library that was just set up, ensure that the dropdown for “Pick peptides matching:” has “Library” selected, and then click “Explore...”

**! NOTE:** After clicking “Explore...” a pop-up may appear notifying that “Peptide settings have been changed. Save changes?” Click YES

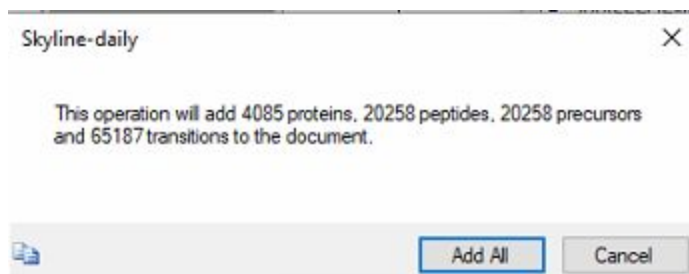


- Once the spectral library explorer window is launched, check the box at the bottom to “Associate proteins” and then click “Add all...”

**! NOTE:** After clicking “Add all...”, a popup progress bar for “Matching peptides to the current document settings” should appear. This may take some time.



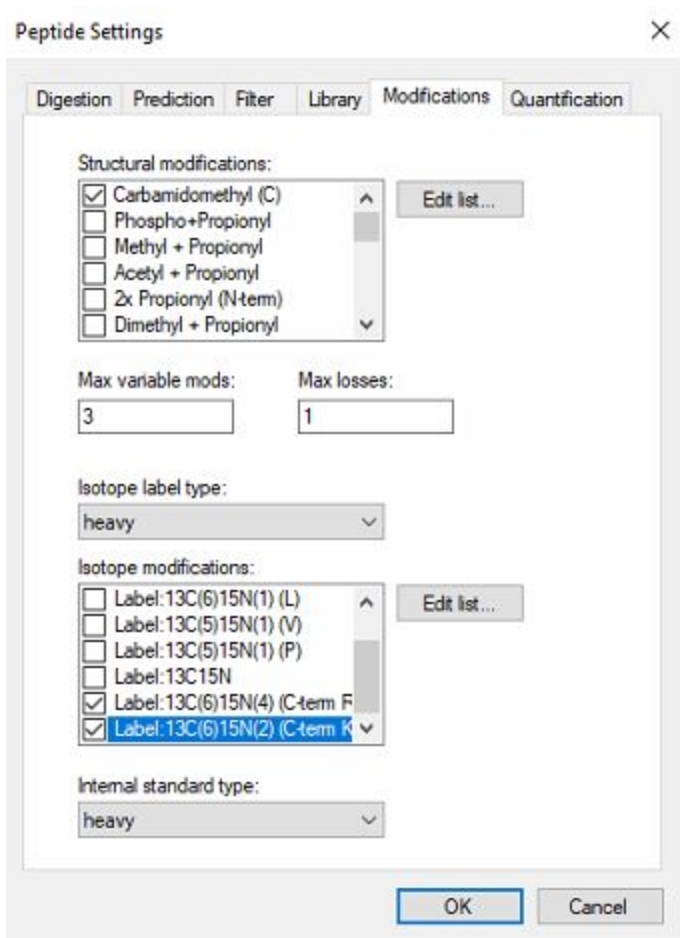
14. When the protein association is complete, a popup window should appear with options for how to handle certain peptide-to-protein mapping situations. Skyline does not use protein groups, so “Add to all matching proteins” is recommended for downstream analysis to ensure that peptide mapping is possible. Click “OK”. After a moment, another popup will appear describing the final results. Select “Add All”. Close out of the spectral library explorer.



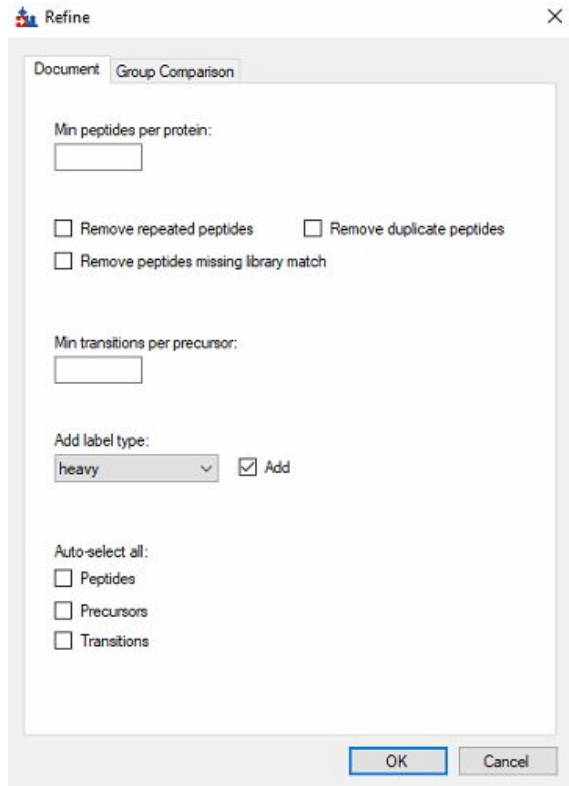
15. The left-hand “Target List” pane should now be populated with proteins, but all these proteins are light/endogenous. Go to Settings > Peptide Settings > Modifications. Under “Isotope modifications”, check the box corresponding to the experiment SILAC labeling scheme, here “heavy” <sup>13</sup>C(6)<sup>15</sup>N(4) arginine and “heavy” <sup>13</sup>C(6)<sup>15</sup>N(20) lysine. Under the “Isotope label type” dropdown, select “heavy”. Click OK to exit out of the Peptide Settings window.

**! NOTE:** Less conventional or custom isotope modifications can be added with Edit list > Add > Edit Isotope Modifications.





16. Navigate to Refine > Advanced and under the “Remove label type” options, check the box next to “Add” and select “heavy” from the dropdown. Click “OK”.



17. Save the Skyline document. Then, choose File > Import > Results to add single-injection replicates and click OK. Navigate to and select all the single-shot, wide-window MZML files used in the EncyclopeDIA analysis above. Choose whether to shorten the file names or not, click “OK”, and the import should begin.

***! NOTE:*** *The chromatogram import graphic should appear and can be used to track progress. This may take time.*

Import Results ✕

Add single-injection replicates in files OK

Optimizing:  
 Cancel

Add multi-injection replicates in directories

Add one new replicate  
 Name:

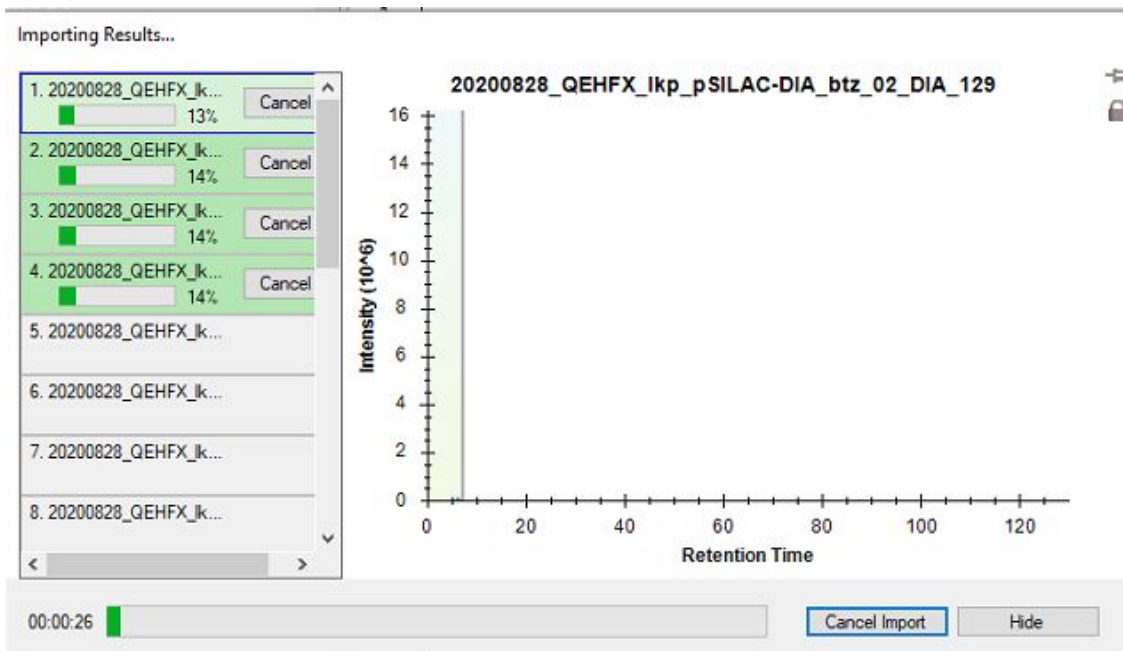
Add files to an existing replicate  
 Name:

---

Files to import simultaneously:

Show chromatograms during import

Retry after import failure



18. Paired SILAC peptides can now be viewed in Skyline. Quantifications can be exported using File > Export > Report...