

## Tutorial for the Cross-link Transition Calculator Skyline Plugin

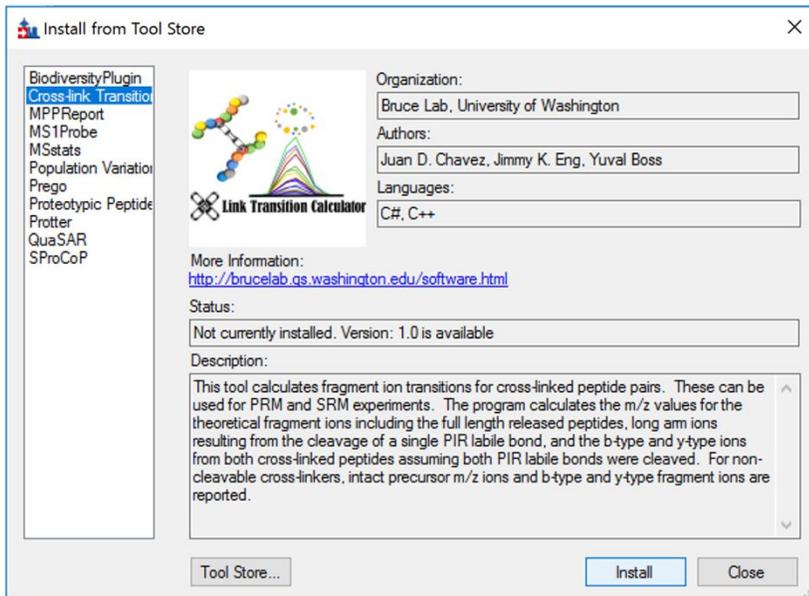
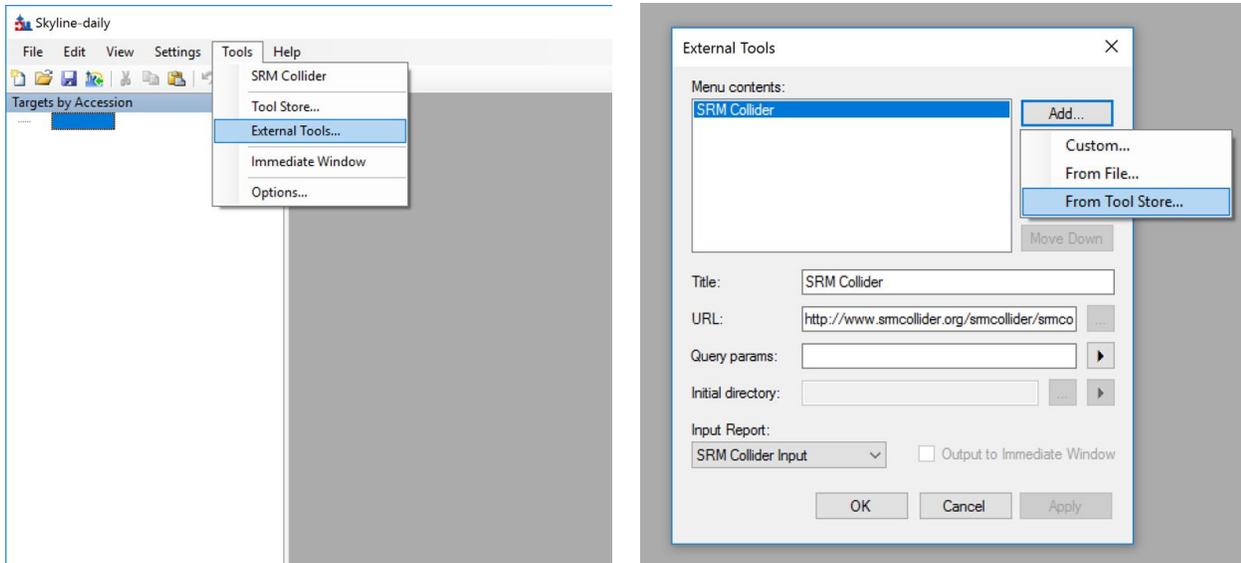
Developed by Juan D. Chavez, Jimmy K. Eng, Devin K. Schweppe, and James E. Bruce.  
Implemented as a Skyline plugin by Yuval Boss.

### **Program Goal:**

The Cross-link (XLink) Transition Calculator generates transitions for cross-linked peptides suitable for use in parallel reaction monitoring (PRM) and selected reaction monitoring (SRM) experiments. The calculator is compatible with both cleavable and non-cleavable cross-linkers.

## Installation:

The XLink Transition Calculator plugin for Skyline can be installed through the Skyline tool store interface. Select Tools→Tool Store→Cross-link Transition Calculator and hit “Install” to install the plugin.



## Use of the XLink Transition Calculator:

Cross-linked peptide information is input as tab delimited text file. The format of the input files is as follows:

```
short_arm_mass long_arm_mass
peptideA peptideB modificationA modificationB precursor_charge
peptideA peptideB modificationA modificationB precursor_charge
peptideA peptideB modificationA modificationB precursor_charge
```

A PIR (BDP-NHP) cleavable cross-link example:

```
197.032422 948.437502
QKAEADKNDK QKAEADKNDK 197.032422@2 197.032422@2 4
MEESKAK KVEKVTISNR 197.032422@5 197.032422@4 4
MKETQK IMKAQALR 197.032422@2 197.032422@3 4
IMKAQALR KVEKVTISNR 15.9949@2,197.032422@3 197.032422@4 4
```

The first line of the input file specifies the short arm and long arm mass of the cleavable cross-linker. For PIR, this would be “197.032422 948.437502” and for DSSO the masses would be “54.010565 103.99320”. For a non-cleavable cross-linker, the first line should be set to “0.0 0.0”. The format of the modification string is “mass@residue\_position”. The modification mass is the mass added from the modification to the mass of the respective residue. In the above example, the PIR cross-linker attaches to lysine residues and after dissociation leaves a stump mass of 197.032422 Da on the lysine residues. Once loaded into Skyline the modification mass will be displayed as the total mass of the cross-linker modification plus the residue mass 325.13. In this case, mass is the mass of lysine (128.094963 Da) plus the cross-link stump mass (197.032422 Da). The string “325.13@2” specifies the 2<sup>nd</sup> residue has a total modification mass of 325.13 Da. Additionally, the last entry above shows a modification string “15.99@2” which corresponds to oxidation of methionine on the second residue of the first peptide.

Here is an example of an input file for two peptides linked with DSS non-cleavable cross-linker with a linker mass of 138.07 Da and a precursor charge state of 6:

```
0.0 0.0
DFNKVPNFSIR IVQSKSGLNMEMLANHEHLLSPVR 2823.470148@4 1473.761615@5 6
```

The “0.0” on the first line indicates a non-cleavable cross-linker. The modification mass is the new mass of the modified residue. In the case of this cross-link, 2823.470148@4 corresponds to the modification of the 4<sup>th</sup> residue lysine where 2823.470148 is the mass of the peptideB (2685.402068) plus mass of the cross-linker (138.06808). “1473.761615@5” is derived as the mass of peptideA (1335.693535) plus mass of cross-linker (138.06808).

When a non-cleavable cross-linker is used, the masses that are calculated and exported as transitions are:

- Intact precursor m/z for all charge states from 1 to precursor\_charge
- b- and y-ions for both peptides, from charges 1 to 3

For cleavable cross-linkers, the calculated transition masses are:

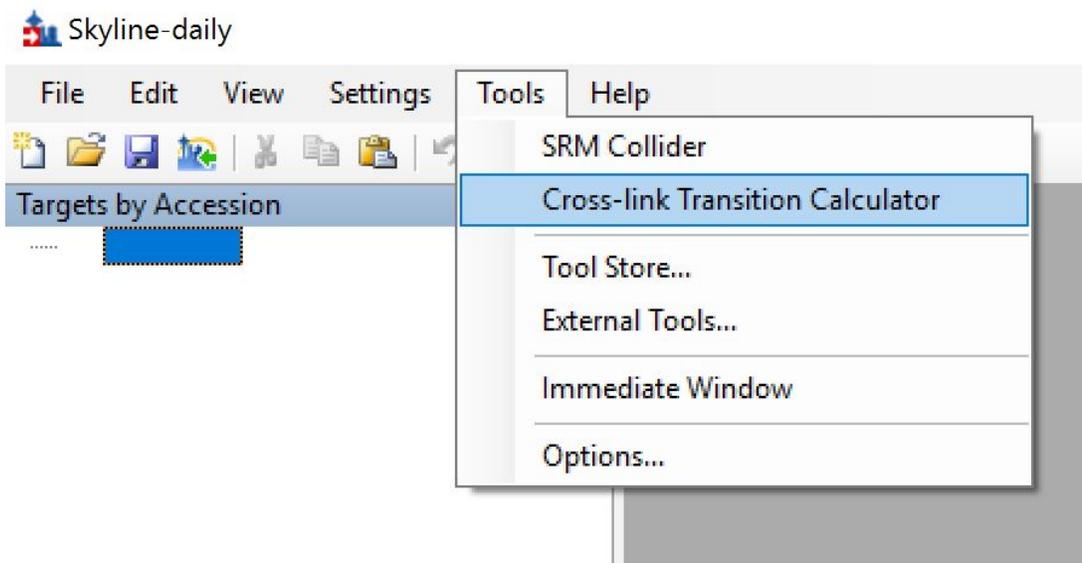
- released precursor m/z for charge states 1 and 2 for each released peptide
- long arm m/z for charge states 1 to 3 for each released peptide. The long arm is an ion resulting from cleavage of a single cleavable bond within the cross-linker resulting in a peptide with the reporter and stump portions of the cross-linker attached.
- b- and y-ions for both peptides of charge 1+

NOTE: The default precursor m/z reported is the most intense theoretical C13 isotope peak. If you would like the monoisotope m/z value reported for the precursor, add "MONO" to the end of the first line after the short arm and long arm masses. For example with the DSSO crosslinker:

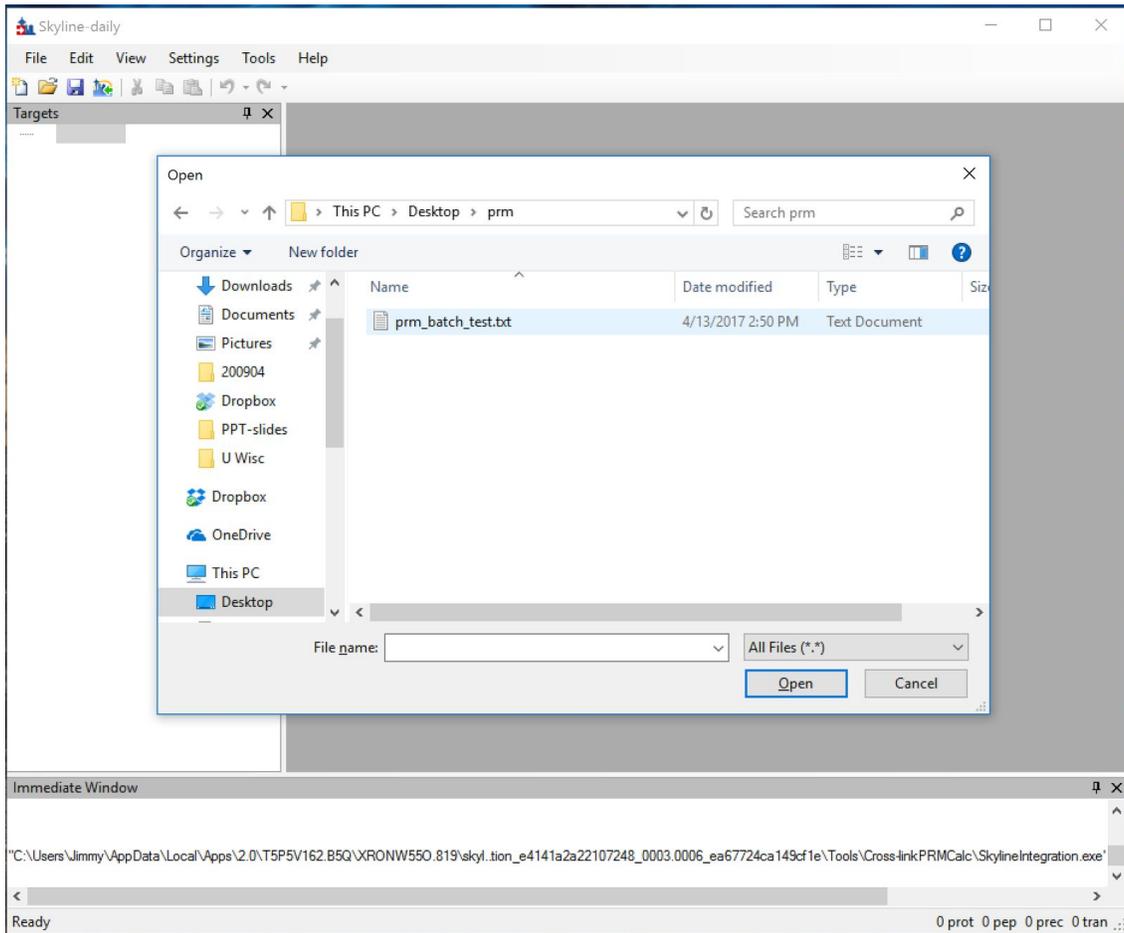
```
54.010565 103.993200          this example reports max isotope m/z  
VEK GK FDNLTKAER 54.010565@3 54.010565@6 3
```

```
54.010565 103.993200 MONO     this example reports monoisotopic m/z  
VEK GK FDNLTKAER 54.010565@3 54.010565@6 3
```

To run this plug-in in Skyline, select the "Cross-link Transition Calculator" menu item from the "Tools" menu.



This immediately brings up a file selection box. Choose the appropriate input file and hit the “Open” button.



If the right input file is selected and the entries in the file are formatted correctly, you will see a list of cross-linked peptides as below. Note: modification masses when loaded into Skyline are displayed in the format of residue mass + modification mass (i.e K[325.13] = 128.094963 Da for Lys plus the cross-link stump mass 197.032422 Da).

The screenshot shows the Skyline software interface. The 'Targets' window on the left lists several cross-linked peptides:

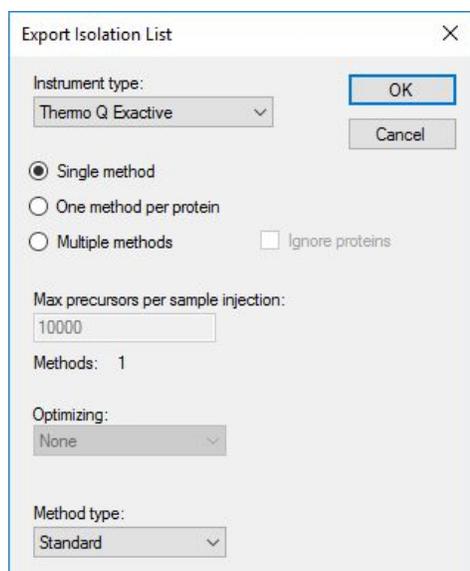
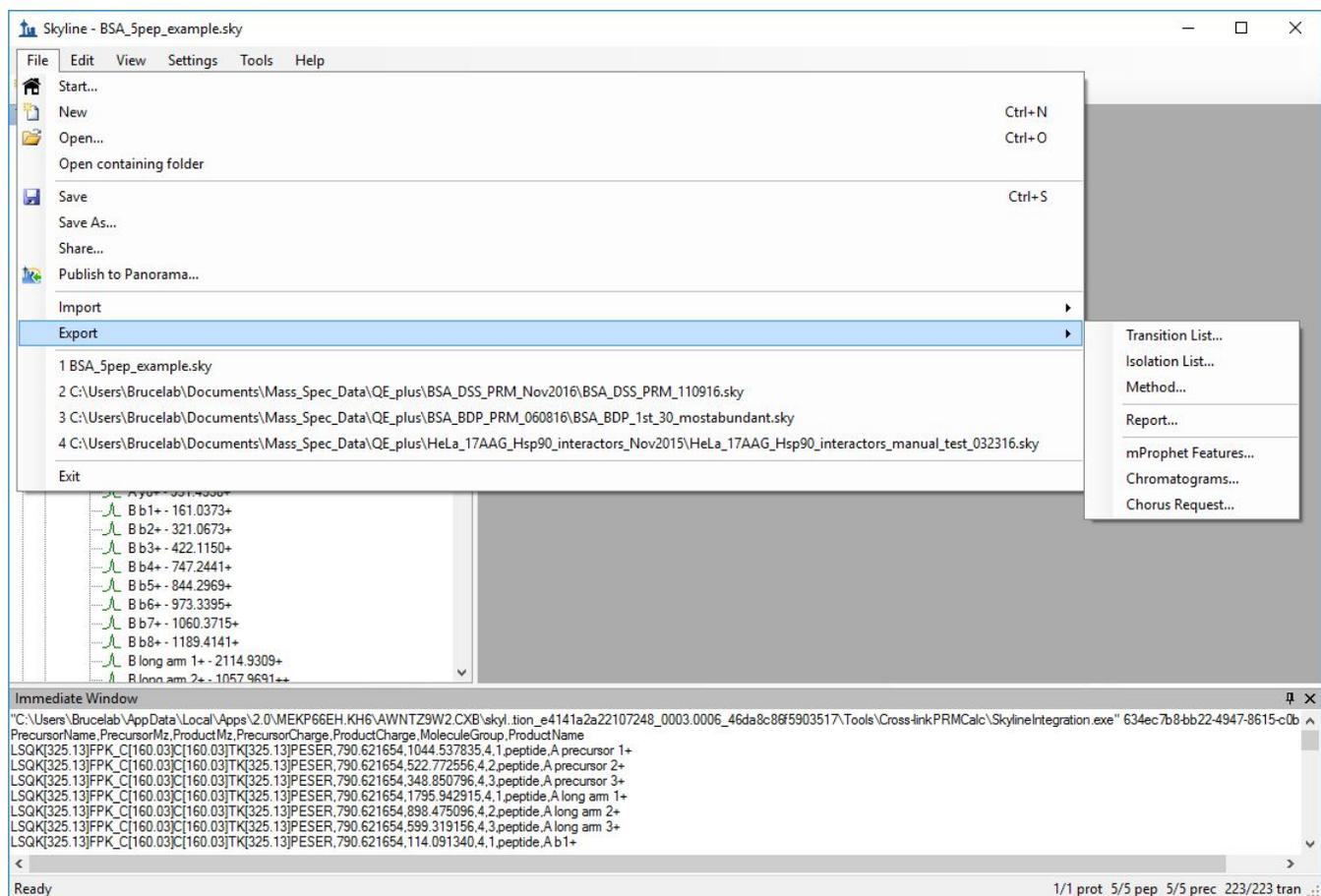
- LSQK[325.13]FFPK\_C[160.03]C[160.03]TK[325.13]PESER
- DTHK[325.13]SEIAHR\_FK[325.13]DLGEEHFK
- ALK[325.13]AWSVAHR\_DTHK[325.13]SEIAHR
- SLGK[325.13]VGTR\_LSQK[325.13]FFPK
- SLGK[325.13]VGTR\_LC[160.03]VLHEK[325.13]TPVSEK

The 'Immediate Window' at the bottom displays the following information:

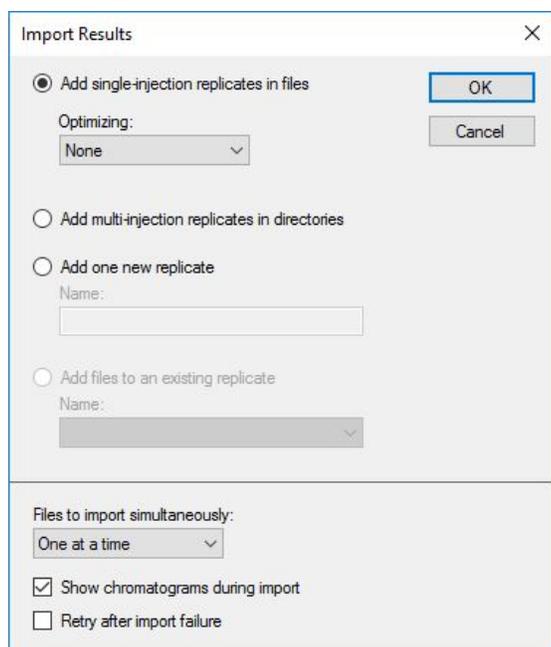
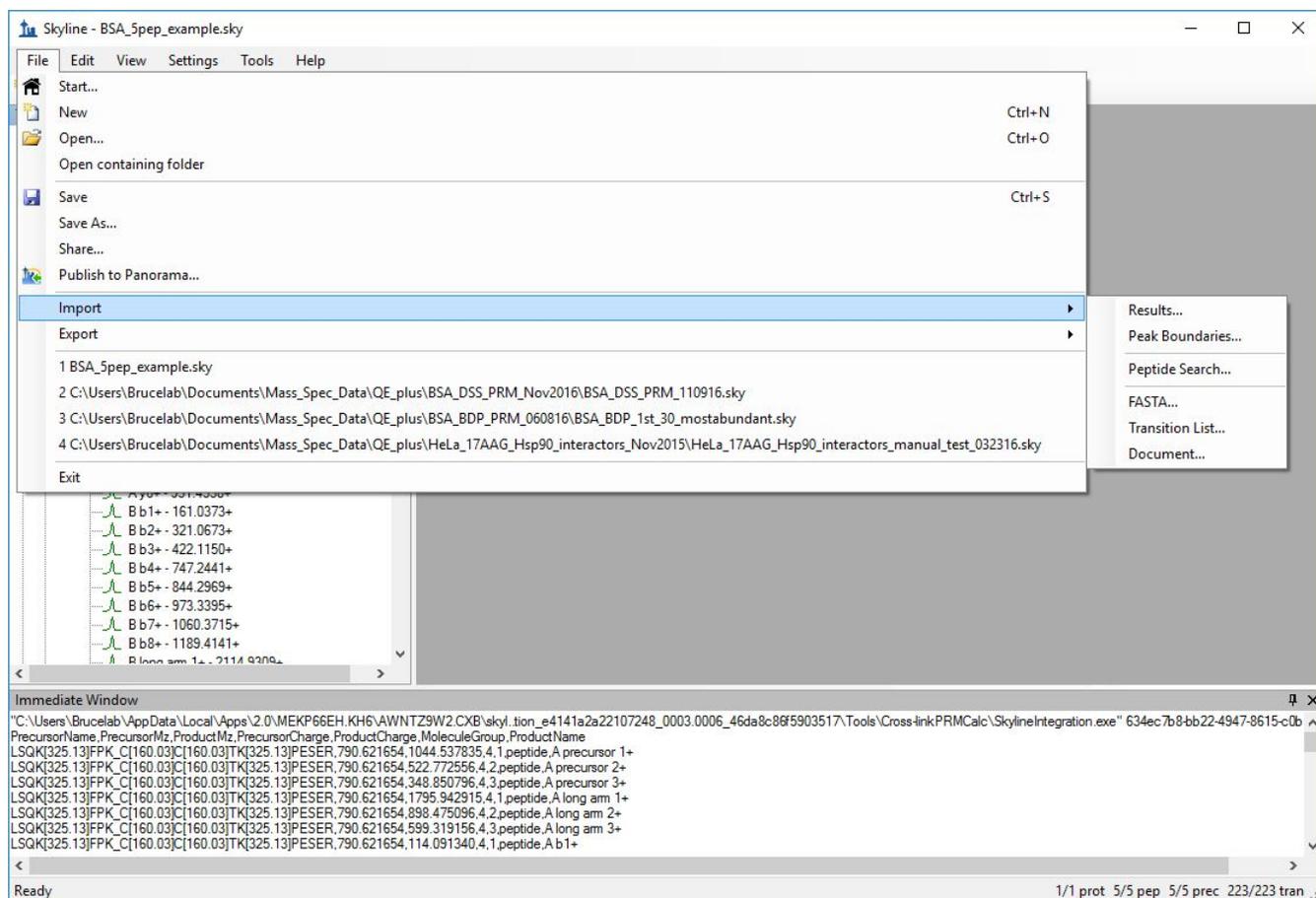
```
"C:\Users\BruceLab\AppData\Local\Apps\2.0\MEKP66EH.KH6\AWNTZ9W2.CXB\skyl.sion_e4141a2a22107248_0003.0006_46da8c86f5903517\Tools\Cross-linkPRMCalc\SkylineIntegration.exe" 634ec7b8-bb22-4947-8615-c0b6f08136cf
PrecursorName, PrecursorMz, ProductMz, PrecursorCharge, ProductCharge, MoleculeGroup, ProductName
LSQK[325.13]FFPK_C[160.03]C[160.03]TK[325.13]PESER, 790.621654, 1044.537039, 4, 1, peptide, A precursor 1+
LSQK[325.13]FFPK_C[160.03]C[160.03]TK[325.13]PESER, 790.621654, 522.772556, 4, 2, peptide, A precursor 2+
LSQK[325.13]FFPK_C[160.03]C[160.03]TK[325.13]PESER, 790.621654, 348.850796, 4, 3, peptide, A precursor 3+
LSQK[325.13]FFPK_C[160.03]C[160.03]TK[325.13]PESER, 790.621654, 1795.942915, 4, 1, peptide, A long arm 1+
LSQK[325.13]FFPK_C[160.03]C[160.03]TK[325.13]PESER, 790.621654, 898.475096, 4, 2, peptide, A long arm 2+
LSQK[325.13]FFPK_C[160.03]C[160.03]TK[325.13]PESER, 790.621654, 599.319156, 4, 3, peptide, A long arm 3+
```

The status bar at the bottom indicates 'Ready' and '1/1 prot 5/5 pep 5/5 prec 223/223 tran ...'.

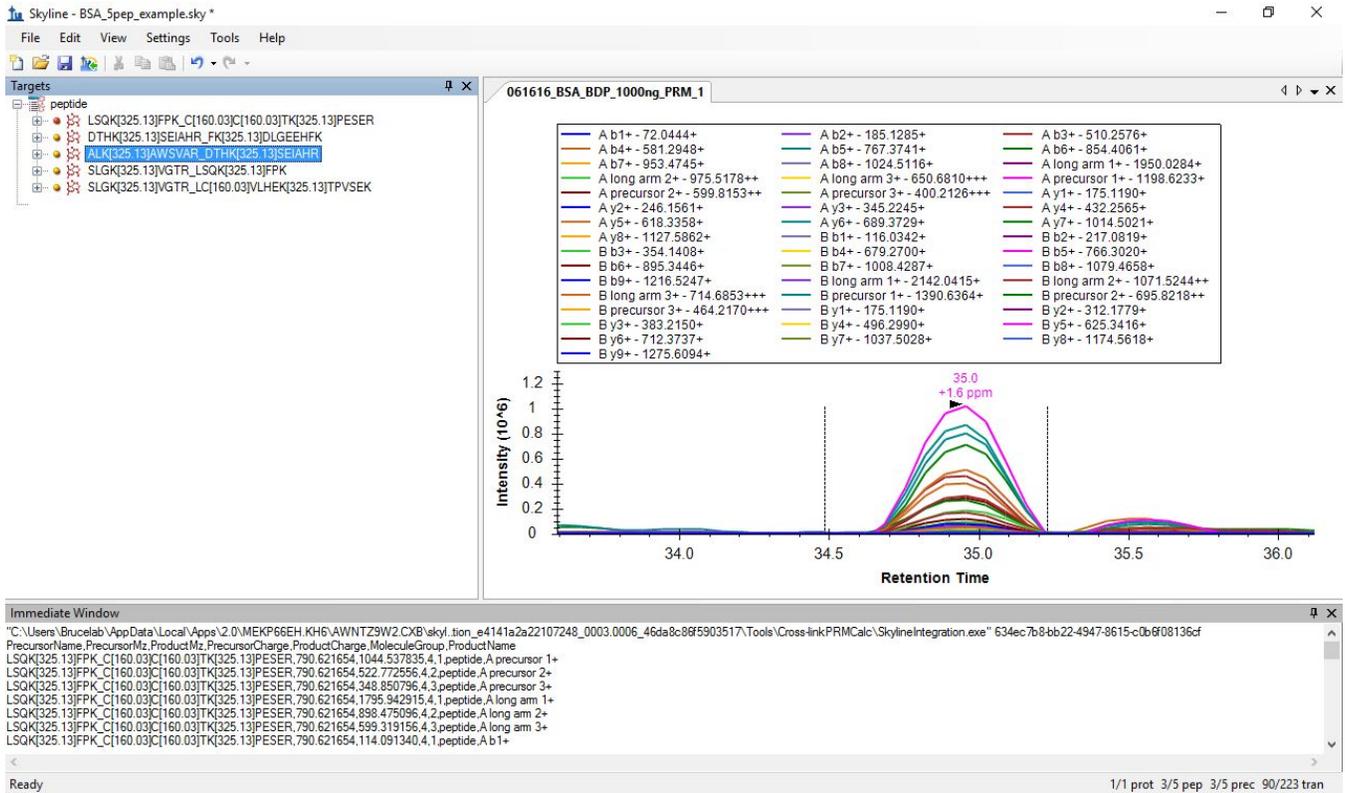
At this point Skyline can be used to export a precursor isolation list for use in generating a PRM method by selecting File→Export→Isolation List then selecting the appropriate instrument type.



Mass spec data can now be loaded by selecting File→Import→Results and selecting appropriate mass spec data file.



Once mass spec data is loaded, transition extracted ion peak areas can be viewed.





Select Peptide Quantification. Note: before exporting the report first click on Edit List→Peptide Quantification and expand the dropdown menu to make sure that the Peptide Results→Quantification→Normalized Area check box is selected.

The screenshot displays the Skyline software interface for peptide quantification. The main window shows a list of peptides and their corresponding mass spectrometry data. The 'Edit Report' dialog box is open, showing the 'Peptide Quantification' view. The 'Columns' list includes 'Normalized Area', 'Calculated Concentration', 'Accuracy', 'RatioLightToHeavy', and 'DilProductLightToHeavy'. The 'Filter' list includes 'Peptides', 'Proteins', 'Normalized Area', 'Peptide Retention Time', 'Precursor Mz', 'Precursor Charge', and 'File Name'. The 'Immediate Window' at the bottom shows a list of peptides and their retention times, including:

Precursor Name	Precursor Mz	Product Mz	Precursor Charge	Product Charge	Molecule Group	Product Name
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER	790.621654	1044.537335	4.1	1	peptide	A precursor 1+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER	790.621654	522.772556	4.2	2	peptide	A precursor 2+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER	790.621654	348.850796	4.3	3	peptide	A precursor 3+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER	790.621654	1795.942915	4.1	1	peptide	A long arm 1+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER	790.621654	898.475636	4.2	2	peptide	A long arm 2+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER	790.621654	599.319156	4.3	3	peptide	A long arm 3+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER	790.621654	114.091340	4.1	1	peptide	A b1+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER	790.621654	201.123369	4.1	1	peptide	A b2+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER	790.621654	329.181946	4.1	1	peptide	A b3+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER	790.621654	654.311130	4.1	1	peptide	A b4+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER	790.621654	801.379543	4.1	1	peptide	A b5+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER	790.621654	898.432307	4.1	1	peptide	A b6+

The chromatogram shows the retention time of the peptides, with peaks labeled from 37.0 to 38.5. The 'Immediate Window' at the bottom shows the list of peptides and their retention times, including:

```

C:\Users\brucelab\AppData\Local\Temp\2\0.MEKP66EH.KH5\AWNTZ9W2.CXB\skyl_ton_e4141a2a22107248_0003.0006_46da8c88f5903517\Tools\CrosslinkPRMCalc\SkylineIntegration.exe" 634ec7b8bb22-4947-8615-cb6f08136cf
PrecursorName: PrecursorMz: ProductMz: PrecursorCharge: ProductCharge: MoleculeGroup: ProductName
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER.790.621654.1044.537335.4.1.peptide.A precursor 1+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER.790.621654.522.772556.4.2.peptide.A precursor 2+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER.790.621654.348.850796.4.3.peptide.A precursor 3+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER.790.621654.1795.942915.4.1.peptide.A long arm 1+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER.790.621654.898.475636.4.2.peptide.A long arm 2+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER.790.621654.599.319156.4.3.peptide.A long arm 3+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER.790.621654.114.091340.4.1.peptide.A b1+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER.790.621654.201.123369.4.1.peptide.A b2+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER.790.621654.329.181946.4.1.peptide.A b3+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER.790.621654.654.311130.4.1.peptide.A b4+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER.790.621654.801.379543.4.1.peptide.A b5+
LSQK[325.13]FPK_C[160.03]C[160.03]TK[325.13]PESER.790.621654.898.432307.4.1.peptide.A b6+

```

## Data export from XLinkDB:

XLinkDB (<http://xlinkdb.gs.washington.edu>) is a data resource for cross-linked peptide and protein analysis developed by the Bruce lab at the University of Washington. In the "PRM" tab of XLinkDB, there's a calculator where you can input two peptide sequences, specify the cross-linker and the residues that are cross-linked. This will generate PRM transitions that you can directly load into Skyline using the File→Import→Transition List option.

**Generate a PRM transition file for analysis of cross-links with Skyline.**

For tutorial more information pertaining to Parallel Reaction Monitoring (PRM) and XL-MS, refer to these papers/resources:

- [Chavez, et al. PLOS ONE](#) A general method for targeted quantitative cross-linking mass spectrometry.
- [Chavez, et al. Cell Chem Biol](#): In Vivo Conformational Dynamics of Hsp90 and Its Interactors.
- [Skyline](#), a targeted mass spec environment.
- [Batch input](#) to generate PRM transitions now available.

2017/03/24 update: To open the created transition file in Skyline: go to File > Import > Transition List, and select the csv file.

**Single PRM Transition Input:**

Peptide A: DIGSKLK

Peptide B: DKCSTR

Peptide A xlinked residue position: 5

Peptide B xlinked residue position: 2

Cross-linker Type:  
 BDP-NHP

DSSO  
 Other Reporter Mass: Stump Mass:

**Precursor Charge State:**  
 4+  
 5+  
 6+

**Modifications:**  
**Peptide A:**  
 None.  
 use carbamidomethyl cysteine (C, monoisotopic) - 57.021464  
 User Defined Modifications:  
#@pos, i.e. "15.9949@2,57.0215@3", space separated for addition to N-term use '[' and for C-term use ']', e.g. "15.9949@[

**Peptide B:**  
 None.  
 use carbamidomethyl cysteine (C, monoisotopic) - 57.021464  
 User Defined Modifications:  
#@pos, i.e. "15.9949@2,57.0215@3", space separated for addition to N-term use '[' and for C-term use ']', e.g. "15.9949@[

**Batch PRM Transition input:**

**Select batch file input:**  
Choose File No file chosen

Generate Batch PRM Transitions

Batch input files should be space or tab-separated (.txt) files of the format:  
neutral\_reporter\_mass  
peptideA peptideB pepmodA pepmodB charge\_state  
peptideA peptideB pepmodA pepmodB charge\_state  
peptideA peptideB pepmodA pepmodB charge\_state

Modification masses should include be the s  
(e.g. BDP-NHP: 197.0324 or DSSO: 54.011  
For non-cleavable cross-linker, the modifica  
mass of the cross-linker plus the mass of the  
Format of modification is: mass@position, l  
"197.0324@2,57.0215@3".  
The charge state is the precursor charge of  
molecule.

For non-cleavable cross-linkers, set the neu  
first line to zero (0.0) and make the encodec  
link site the mass of the other peptide plus r

**Example of input:**  
751.40508  
QKAEADKNDK QKAEADKNDK 197.0324@2 19  
MEESKCK KVEKVTISNR 197.0324@5, 57.02

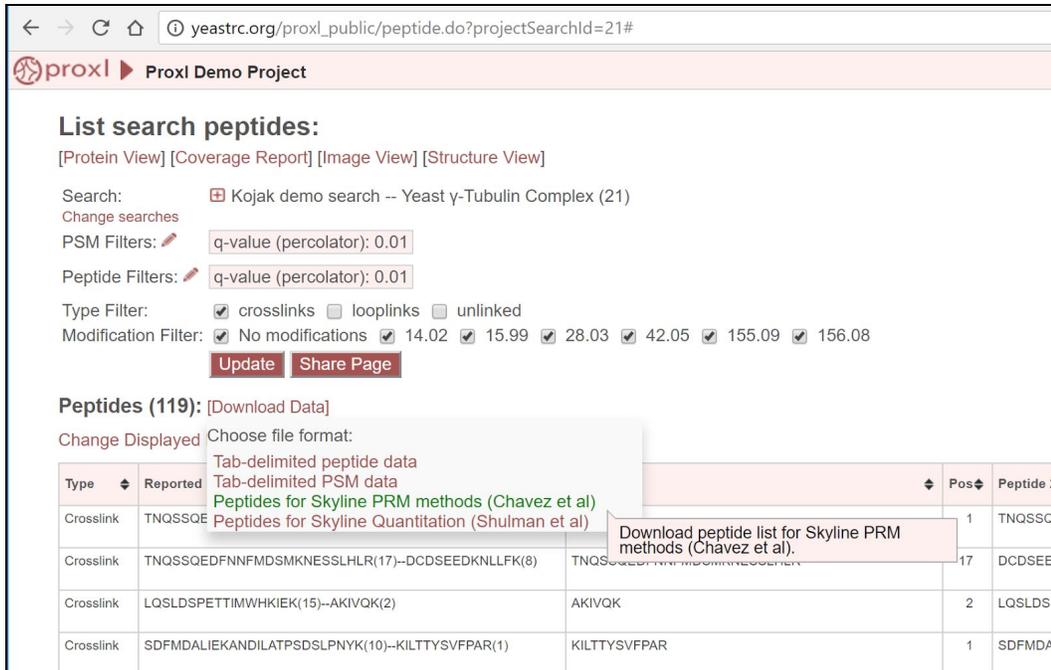
[Download a brief example input file here](#)  
[Download corresponding example tra](#)

Generate Individual PRM Transitions

The batch calculator on the same page replicates the functionality of this plugin. It takes the same input files as this plugin, calculates transitions, and exports them as a tab-delimited text file. This file can be imported into Skyline directly using the same File→Import→Transition List option.

## Data export from ProXL:

ProXL is a cross-linking resource developed by the Yeast Resource Center at the University of Washington. Cross-link peptides in ProXL (proxl.yeastrc.org) can be exported into the right input format for this plugin. Look for the “Download Data” link and select “Peptides for Skyline PRM methods (Chavez et al)” option. This will generate a tab-delimited text file of the selected cross-linked peptides. You can further edit this file by deleting any rows of cross-link pairs that are unnecessary or redundant such as the same cross-linked peptide pairs identified in different precursor charge states. Select the output file from ProXL as the input to this plugin.



The screenshot shows the ProXL website interface. At the top, the URL is `yeastrc.org/proxl_public/peptide.do?projectSearchId=21#`. The page title is "ProXL Demo Project".

**List search peptides:**  
[Protein View] [Coverage Report] [Image View] [Structure View]

Search:   
Change searches

PSM Filters:   
Peptide Filters:

Type Filter:  crosslinks  looplinks  unlinked  
Modification Filter:  No modifications  14.02  15.99  28.03  42.05  155.09  156.08

**Peptides (119):** [Download Data]

Change Displayed Choose file format:  
Tab-delimited peptide data  
Tab-delimited PSM data  
Peptides for Skyline PRM methods (Chavez et al) **(Selected)**  
Peptides for Skyline Quantitation (Shulman et al)

Type	Reported	Pos	Peptide 2
Crosslink	TNQSSQE	1	TNQSSQE
Crosslink	TNQSSQEDFNNFMDSMKNESSLHLR(17)--DCDSEEDKNLLFK(8)	17	DCDSEED
Crosslink	LQSLDSPETTIMWHKIEK(15)--AKIVQK(2)	2	LQSLDSP
Crosslink	SDFMDALIEKANDILATPSDSLPNYK(10)--KILTTYSVFPAR(1)	1	SDFMDAL

## Possible reasons for failure:

If you open an input file and no transitions appear in the Target window, this could happen for various reasons. Unfortunately, the mechanism by which the Cross-link Transition Calculator tool is implemented as a Skyline plugin does not allow the software to report why a failure might have occurred nor can Skyline currently pass information, like transition mass ranges, to the plugin to mitigate possible problems. In testing, we have observed the program to fail for these reasons:

- Incorrect format of the input file. Please confirm your input file is of the right format and has all required fields.
- Precursor m/z of the intact cross-link molecule is larger than the transition settings allow in Skyline. Check your Skyline settings and make sure the precursor m/z range is set to accommodate the data in the input file.