

PMI Peptide Analysis (Byologic®) User's Manual

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Introduction

Peptide Analysis (Byologic®) is a unique application developed in partnership with biopharmaceutical scientists for detailed, peptide-centric analysis of protein samples. It is used from late discovery through clinical development to define design space and product profile and supports Multi-Attribute Method (MAM) reporting.

Peptide Analysis enables researchers to confidently characterize their product down to trace components and reduces turn-around time by a factor of weeks. Flexible summary tables can quickly generate reports for non-mass spectroscopy stakeholders for internal review or for application filings.

Peptide Analysis software considers LC-MS and LC-MS/MS results and enables label-free quantification, easy inspection of results, and reporting on summary and detail information for a variety of workflows that rely on peptide-level analysis. The raw data (MS1 recorded in profile or centroid mode) currently can originate from any major mass spectrometry vendor (Thermo Fisher, Waters, Sciex, Bruker, Shimadzu, or Agilent).

Peptide Analysis is a general tool that can be applied to a wide range of degradants, variants, and post-translation modifications. Thus, it can be used in workflows such as PTM analysis, peptide mapping, hotspot analysis, stress studies, MAM profiling, host cell protein analysis glyco analysis, disulfide bond profiling, comparability, oxidative footprinting, and more. It offers a powerful platform for comparing and reporting across samples. More than one type of digestion enzyme may be examined at once, and each may include multiple LC-MS data acquisitions and multiple MS2 searches from any data acquisition. The fragmentation method may be any combination of low-energy CID, HCD/Q-TOF, and/or ETD. The quantification of a modification or variant relative to wildtype is performed by label-free quantification with extracted ion chromatograms (XICs) which have editable limits of integration.

Peptide Analysis enables an analyst to determine if a putative identification is true or false (validation), by making available all relevant information interactively in a single screen. The user may make this decision, as opposed to a black-box software algorithm, and may also add comments. The application also makes room for one or more reviewers to enter their responses and comments. Comprehensive reports of results (tables and figures) may be exported for report generation to share with colleagues.

Peptide Analysis may also be deployed into a fully-automated workflow to process large numbers of samples with consistent analysis and reporting.

Workflow

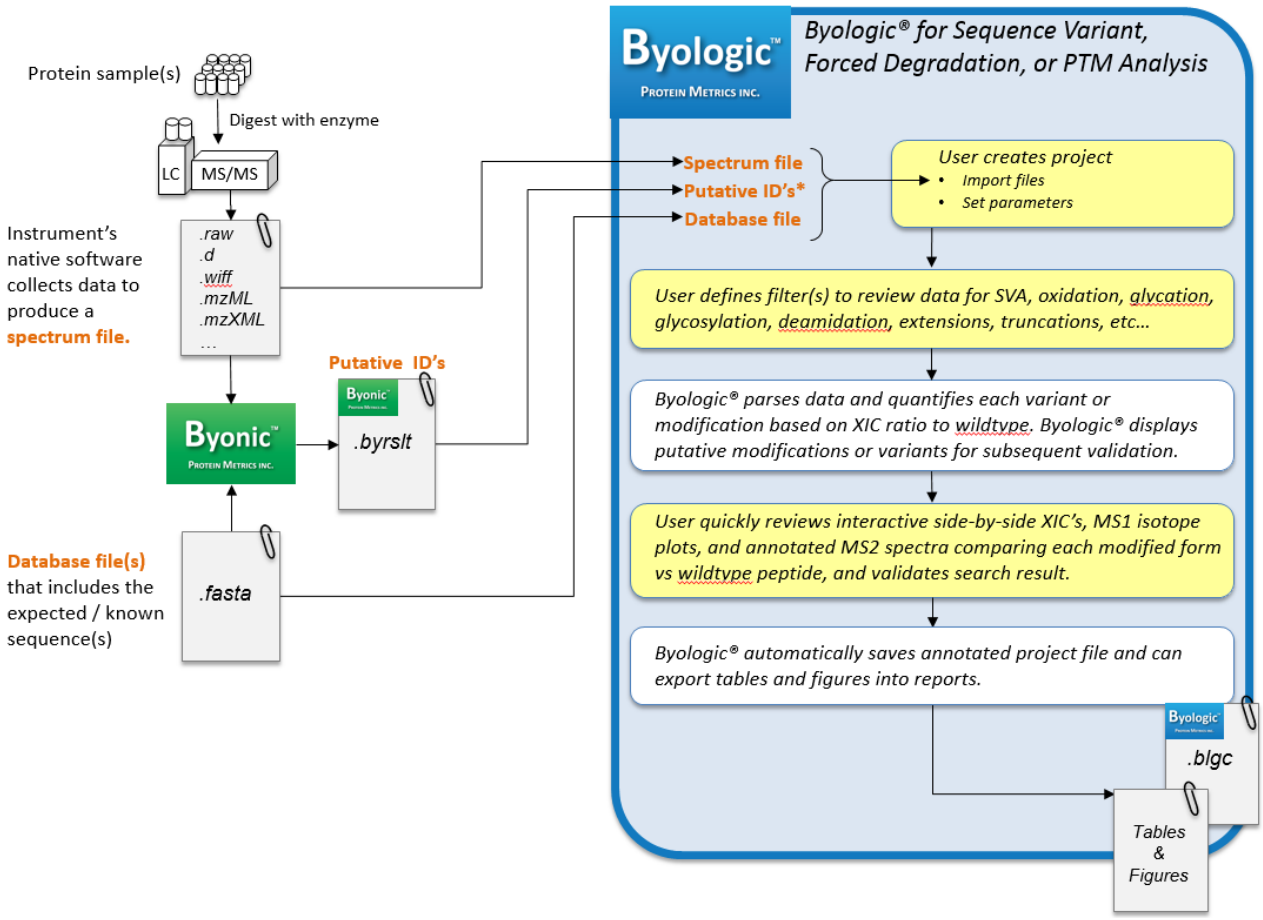


Figure 1: Peptide Analysis workflow

Tour of Peptide Analysis Dashboard Views

The Peptide Analysis user interface (UI), or dashboard, shown in Figure 2 has seven highly interactive plot and table views:

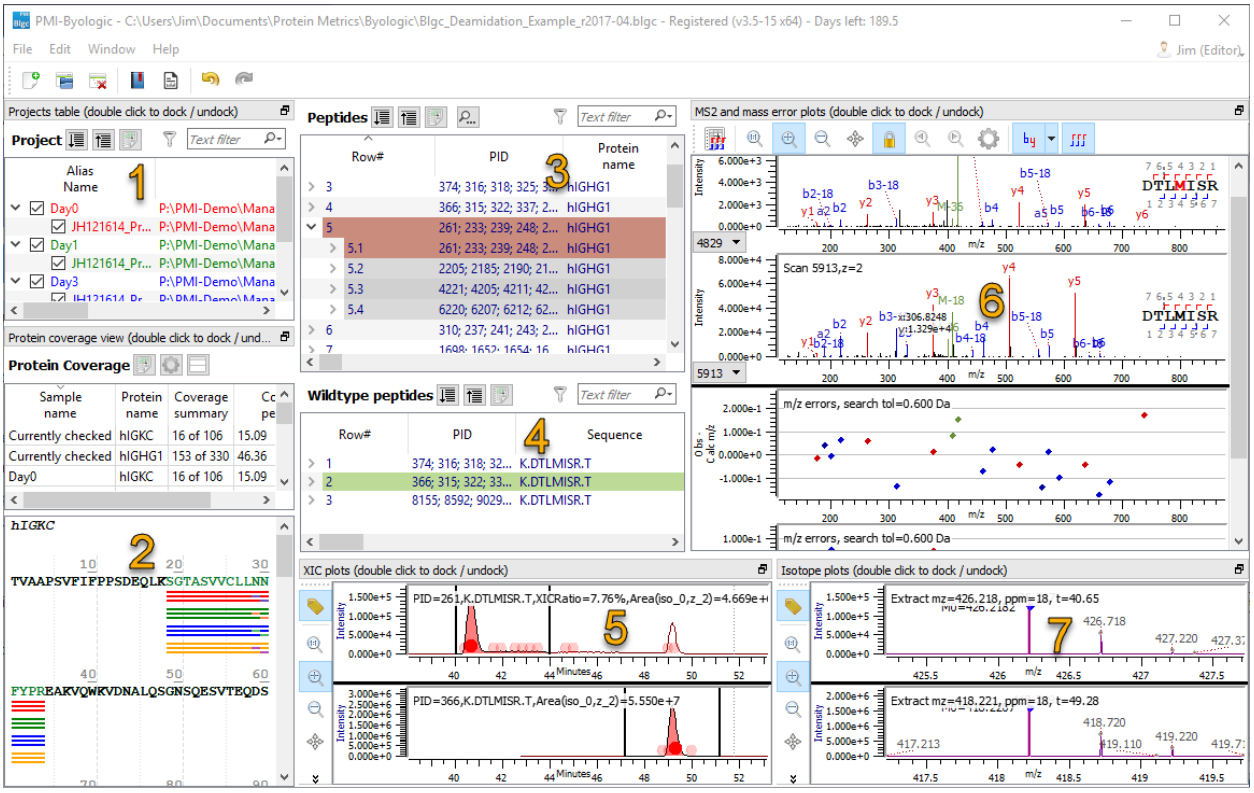


Figure 2: **Seven Peptide Analysis views**: (1) Project table, (2) Protein coverage table, (3) Peptides table, (4) Wildtype peptides table, (5) Ion chromatogram (XIC) plots, (6) MS2 and mass error plots, and (7) Isotope plots. Views can be rearranged, resized, docked, and undocked. Customized layouts can be saved and shared.

The **Project** view lists the data files in use. The paired check boxes for MS files and result files can be used to turn on or off the records displayed in the other views. The colors of the files correspond to colors used in the other views.

1. The **Protein Coverage** views display table data and identify sequences for peptides selected and filtered in the Peptides table in View 3. Bars below the sequences represent MS/MS scans. The bars and sequences share the colors of the corresponding MS files in the Project table in View 1.
2. The **Peptides** table lists all identified and filtered peptides. Selecting a peptide row in the table displays the corresponding wildtype peptides, XIC chromatograms, MS2 and mass error plots, and isotope plots in Views 4 through 7.
3. The **Wildtype peptides** table lists wildtype peptides corresponding to the modified peptides selected in the Peptides table in View 3. Records are shaded with the colors of the source MS files in the Project table in View 1.
4. The **XIC plots** show the ion chromatograms for the peptide and corresponding wildtype peptides, if present, selected in the Peptides table in View 3. The plots are shaded with the colors of the source MS files in the Project table in View 1.

5. The **MS2 and mass error plots** show the MS2 spectrum of the peptide and corresponding wildtype peptides, if present, selected in the Peptides table in View 3. The peptide and wildtype peptide spectra share the same m/z scaling for easy comparison.
6. The **Isotopes plots** show the MS1 isotope spread of the peptide and corresponding wildtype peptides, if present, selected in the Peptides table in View 3.

Creating a Project

To create a new project, go to **File > New Project**, or click on the icon directly below **File** on the toolbar. Byologic will open the **New project** dialog:

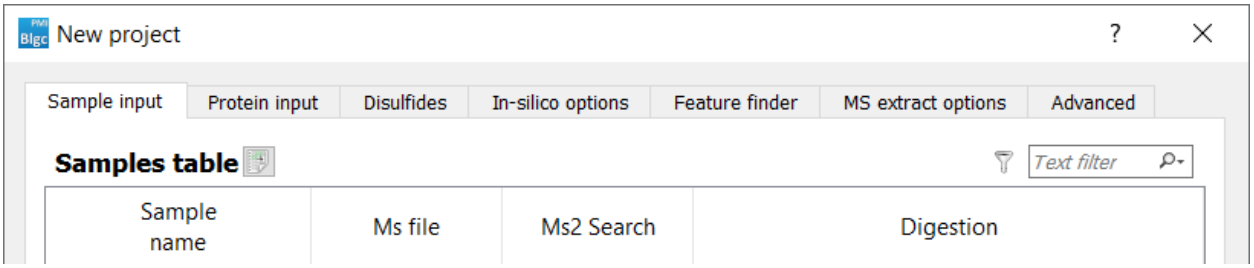


Figure 3: New project dialog headers

This multi-tab window allows the analyst to designate one or multiple mass spectrometer files to be analyzed, each with one or more set of MS/MS search results, as well as a corresponding set of protein sequences.

Sample input tab

The **Sample input** tab allows the user to load raw LC-MS sample files into the project. Byologic accepts a variety of sample file types (Bruker: *.d, Agilent: *.d, Thermo: *.raw, Waters: *.raw, Sciex: *.wiff, Shimadzu *.lcd Alternatively, a project can be opened from a *.byrslt file generated from a previous BYonicTM search. To load a file, drag and drop one or more MS files into the project window. Alternatively, click the **Add sample** button, double-click on the new row under the **MS file** header, click ... and browse to the file. Click **Open**.

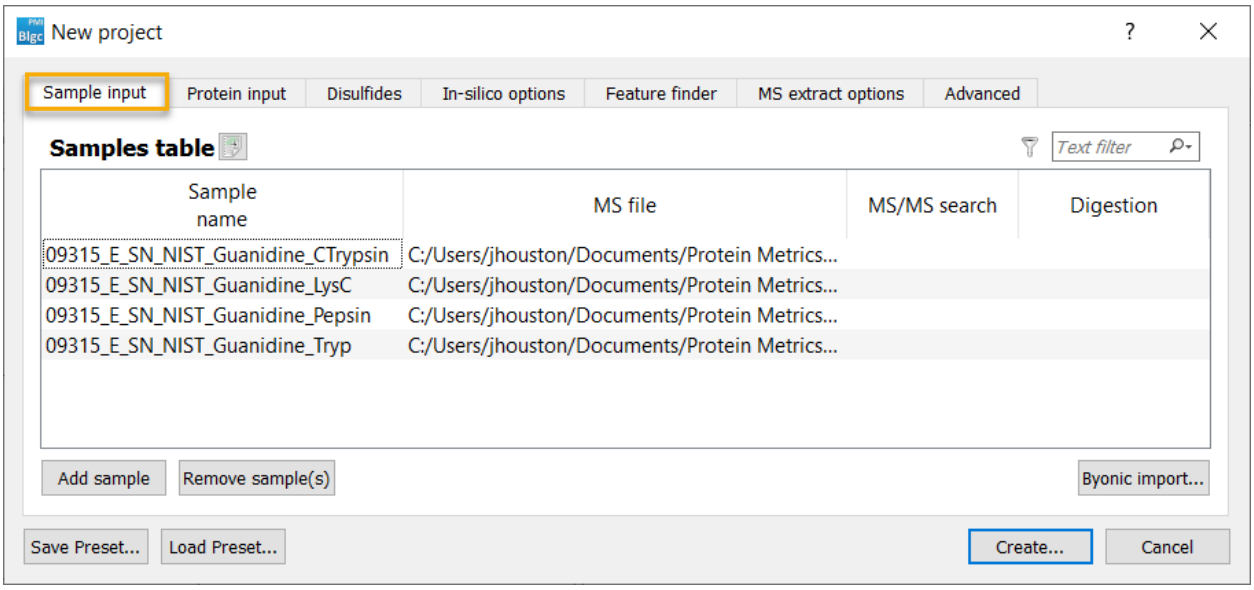


Figure 4: Sample input tab

The Sample name defaults to the file name. To change the Sample name, double-click the name and edit the text. The name can also be updated later in the Project table.

To designate the enzyme used by a sample, double-click the sample row under Digestion and select the enzyme name. This enables Byologic to determine the expected cleavage sites.

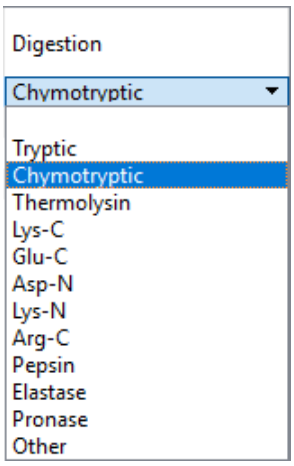


Figure 5: Digestion enzyme selection

To add an incremental MS/MS search result to a sample, double-click in that sample row under the **MS/MS Search** header, click ... and browse to the MS/MS search result (*.byrslt or *.dat) file. Click **Open**. To attach additional Ms2 search result to that sample, click + to add a new MS/MS Search row and repeat the addition steps.

To remove an MS/MS search file, double-click the file name and click ✖,

To import the parameters used in an associated Byonic® search result, select the search result and click the **Byonic Import** button. Check to include protein sequences, lock mass, ppm precursor tolerance, and then click **Import**.

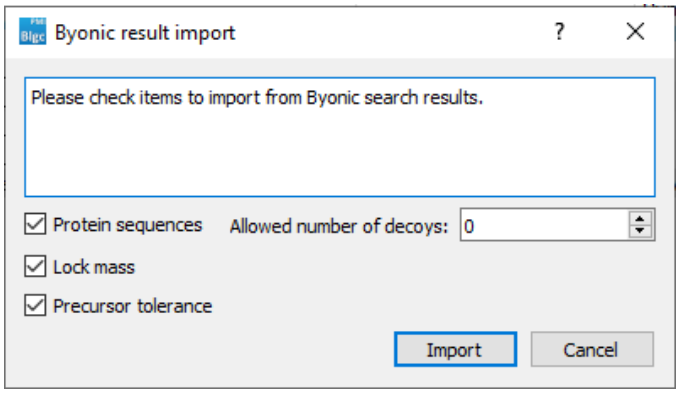


Figure 6: Importing MS/MS search result

The Byonic result import dialog includes the field **Allowed number of decoys**, accepting integers from 0 to 100. The default value is 0. Decoy numbers allows the user to input how many decoys to read up to in the protein list. For example, a value of “3” would allow the reading of the protein list past the third decoy. It would stop reading proteins at the fourth decoy. This option provides enhanced support for HCP analysis.

Project input tab

The **Protein input** tab allows the user to enter a protein sequence either by adding a FASTA file or by typing it in directly.

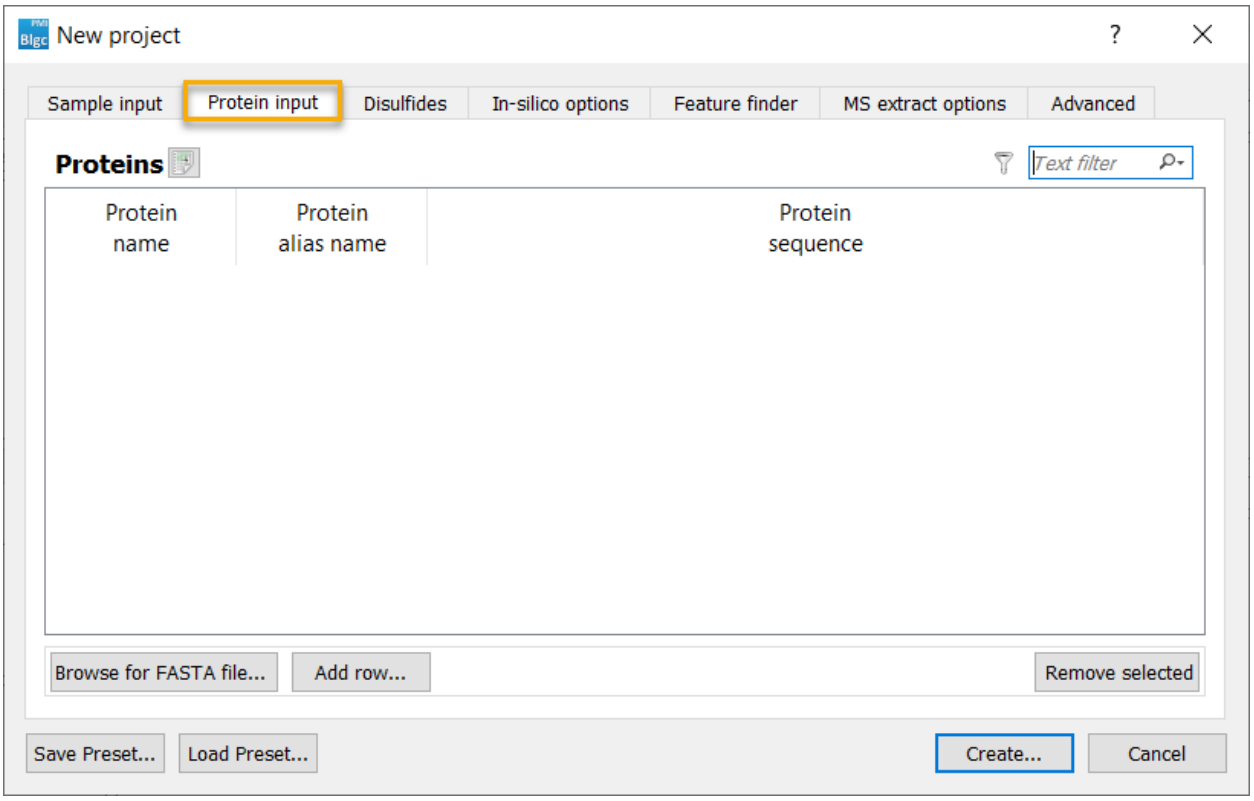


Figure 7: Protein input tab

To add a *.fasta or *.fa file, click **Browse for FASTA file**, locate the file to be added and click **Open**. FASTA files also can be dragged into this dialog directly.

The addition of a FASTA file opens the **Protein candidates** dialog:

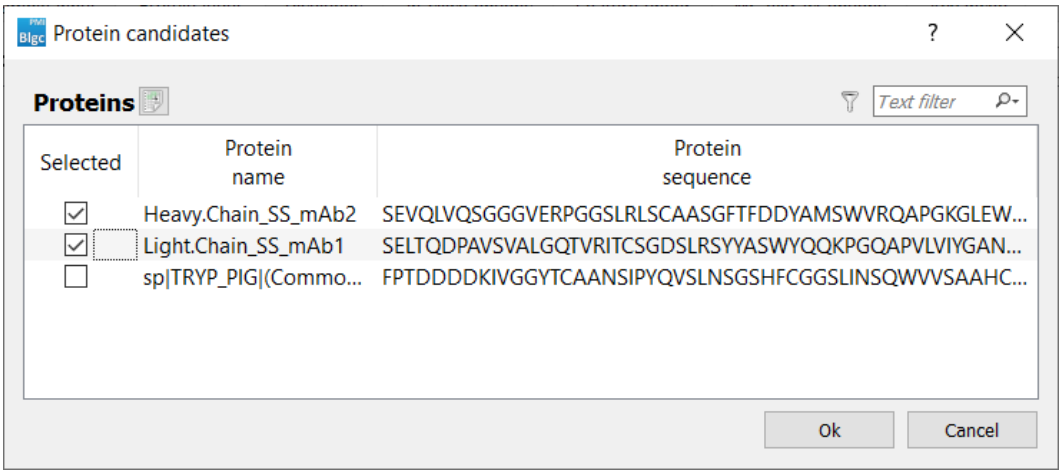


Figure 8: Adding protein candidates from a FASTA file

Check the box for each protein to include in the project. To edit **Protein name**, click on the name and enter a new one. When finished, click **Ok**. The Protein input tab is updated with the proteins and sequences for the project. To add a **Protein alias name**, click that field, and enter a new value.

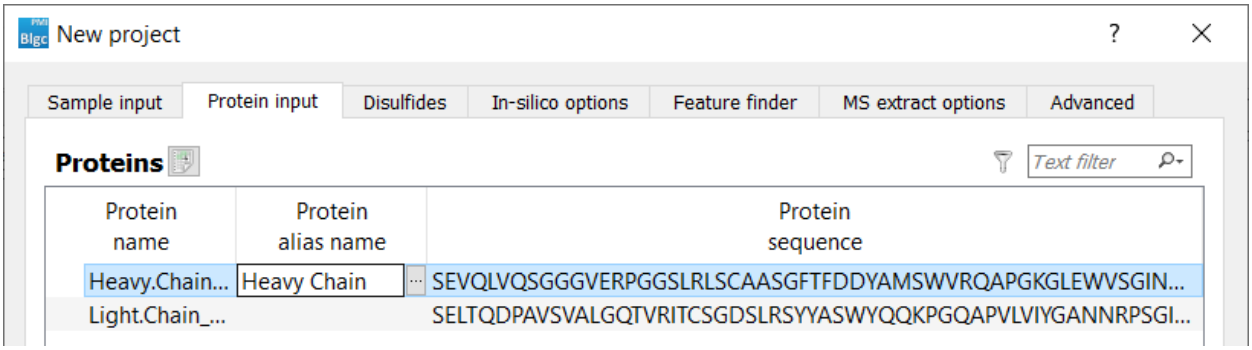


Figure 9: Editing proteins

To add protein data from a URL or to enter it manually, click **Add row** (see Figure 7). This opens the **Add Protein** dialog.

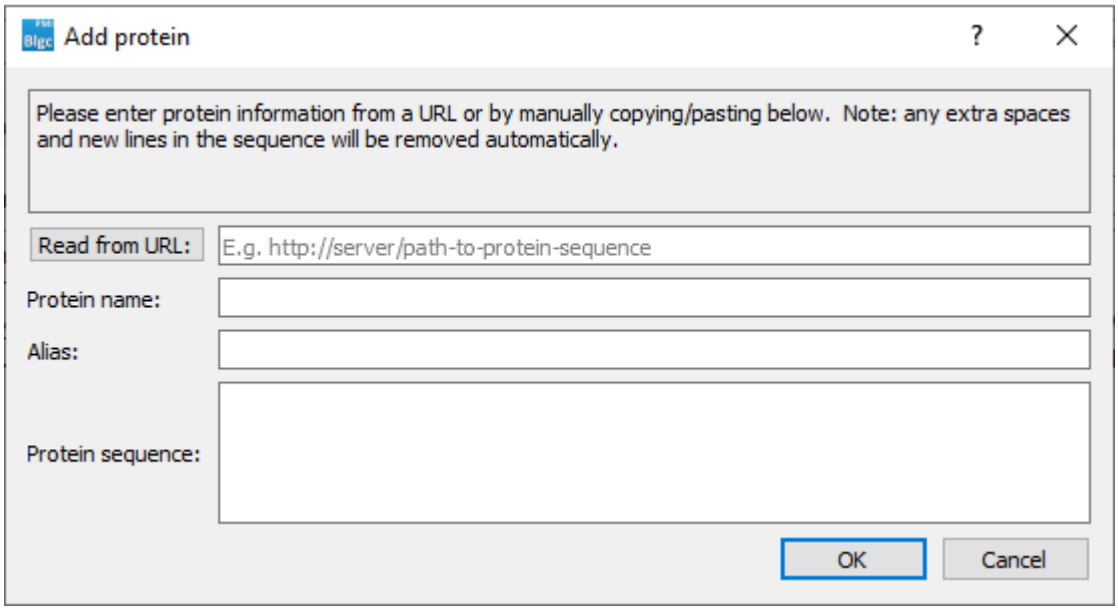


Figure 10: Adding a protein from a URL or as text entry

To add a protein from a URL address, enter the address and click **Read from URL**. To enter the protein data manually, enter or paste in the Protein name, Alias and Protein sequence. Click **OK** to add the protein as a row in the Protein input tab. To remove an added protein from the Protein input table, select that protein and click **Remove all selection**.

Disulfides tab

The **Disulfides** tab enables manual assignment or automatic inference of expected disulfide bond locations. Identified disulfide bonds are marked as **expected** or **shuffled** in the **Peptides** table of the result screen. Disulfides are applied to IgG by default. To assign disulfide bond locations to chains, click the drop-down arrow for the heavy or light chains and select the corresponding input protein. Byologic will populate the **Disulfide links** table to show expected peptide pairs. To add a disulfide link, click **Add row**, scroll to

the bottom of the list and enter the new disulfide link. To remove a disulfide link, select the link and click **Remove rows**.

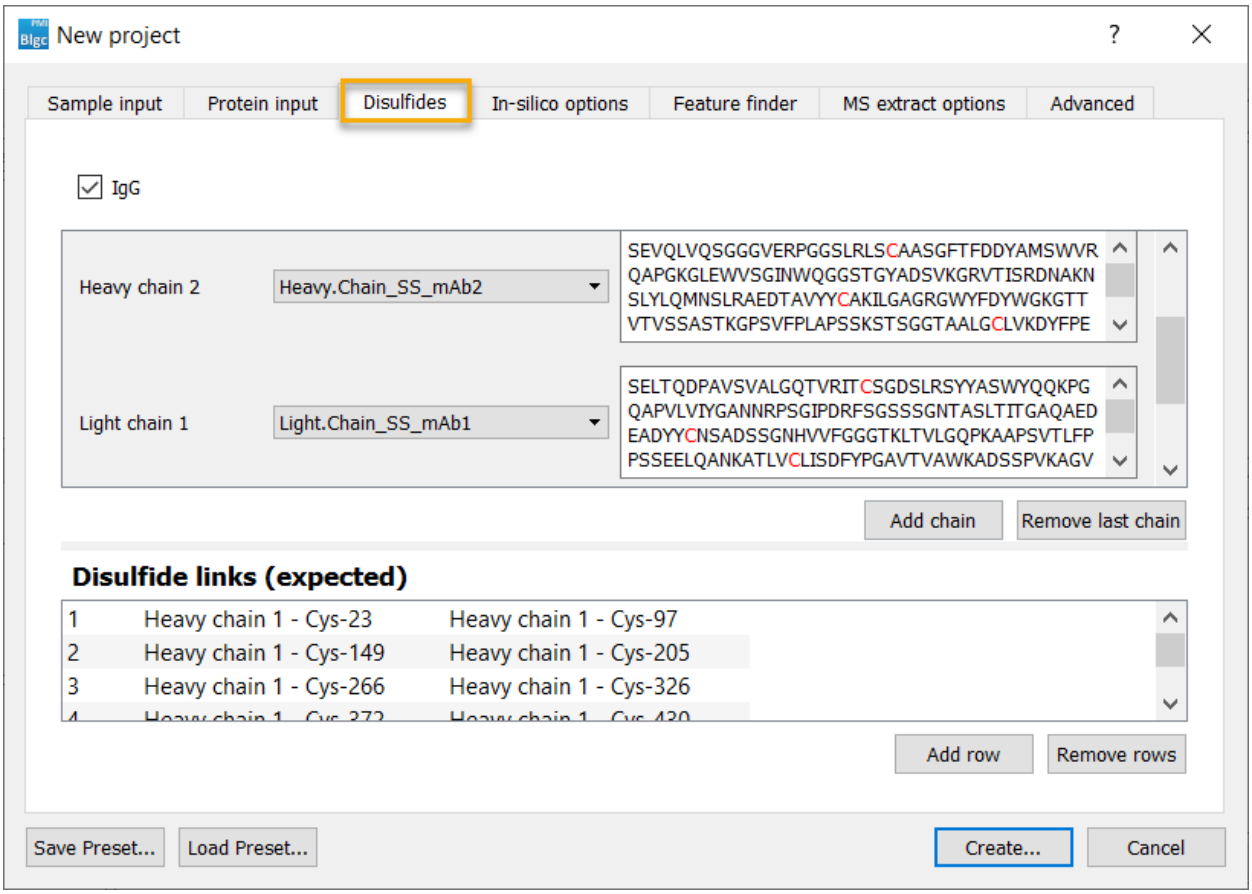


Figure 11: Disulfide tab

In-silico options tab

The **In-silico options** tab allows the user to include in-silico information for a digested protein or glycan mixture into the analysis. Check the **Enable in-silico** checkbox to enable this feature. There are two in-silico options: **Digest proteins** and **Use table**. The **Digest proteins** dialog box is similar to the Byonic™ user interface for users familiar with that software. As the name indicates, it is used to perform an in-silico digestion of the input proteins using specified digestion parameters and modifications. For further information, please see the Byonic software manual.

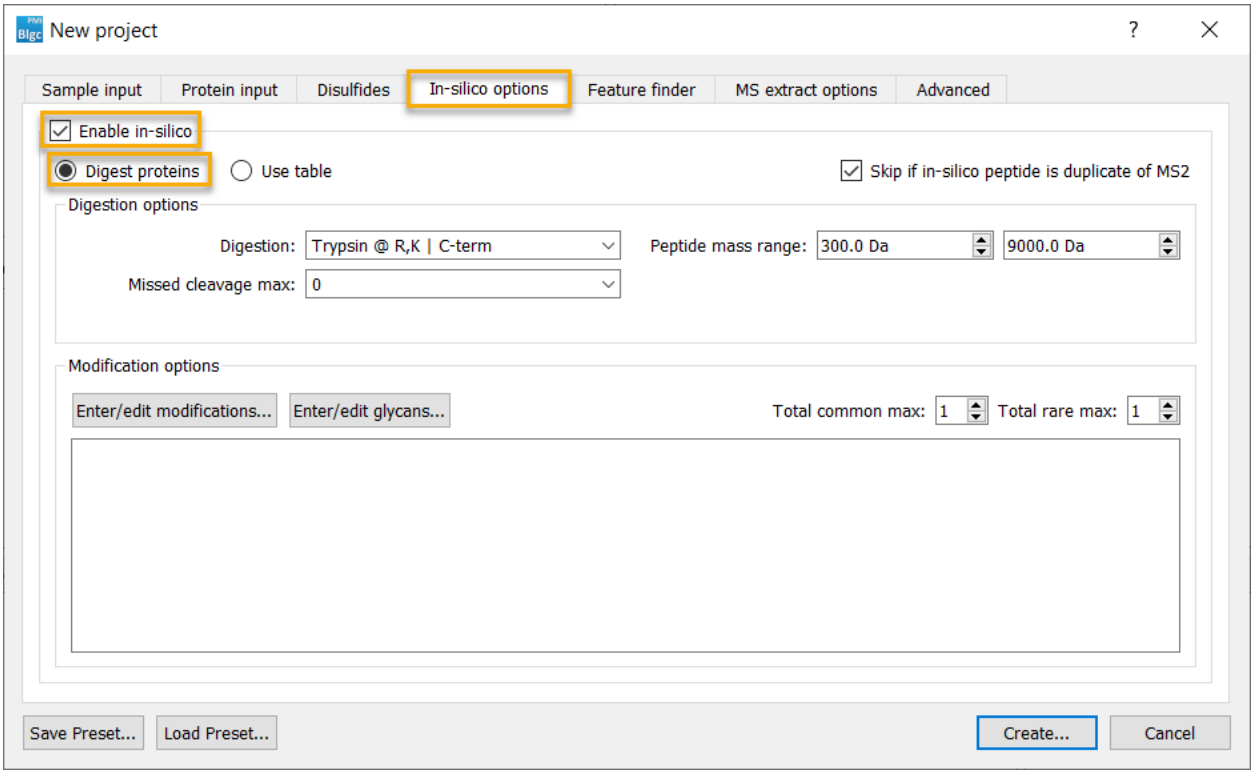


Figure 12: In-silico options tab: Digest proteins

The **Use table** dialog box allows the user to include in the analysis a table of any relevant molecular forms of peptides or glycans.

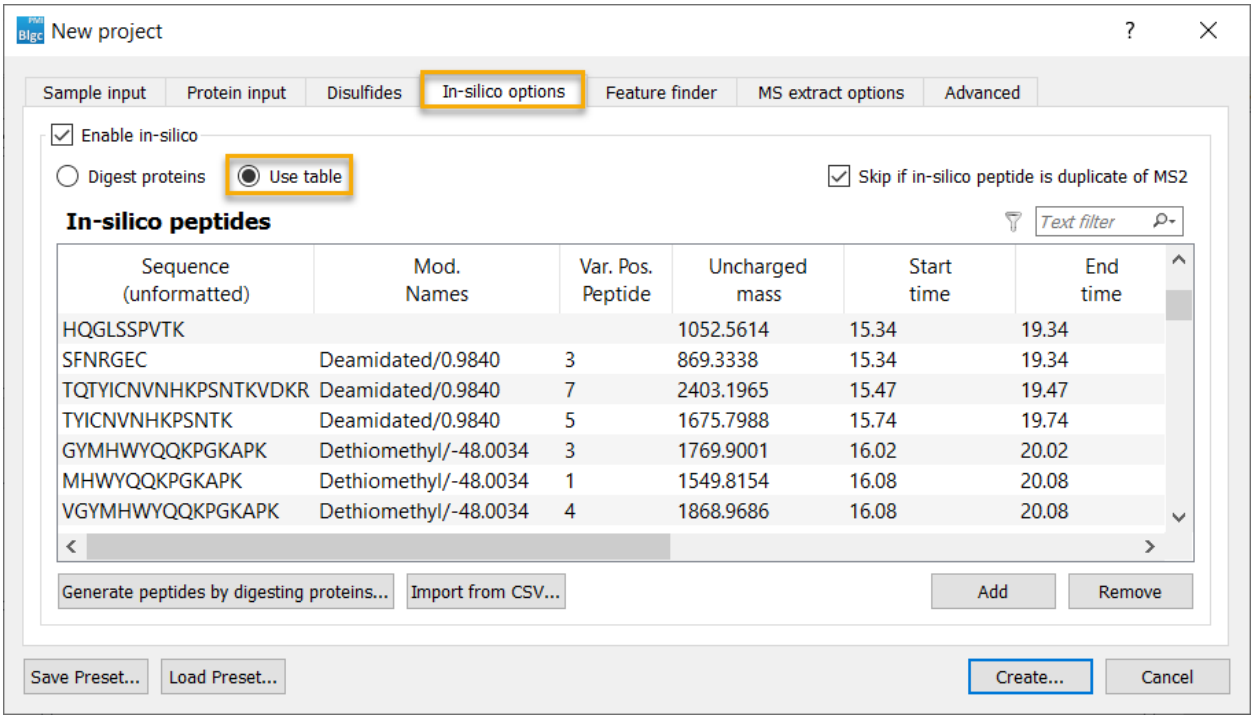


Figure 13: In-silico options tab: Use table

The **Generate peptides by digesting proteins** button at the lower left will bring up essentially the same dialog as for the **Digest proteins** dialog, but the results of the digestion will be displayed in this table.

Click **Import from CSV** at the lower left and browse to an in-silico protein input file. Upon loading the file, the table is populated with the peptides and/or glycans. The *.CSV format required for data entry can be seen in the file **ImportExample.Byologic.csv** located in **Program Files > ProteinMetrics > PMI-Suite > ExampleData**. This *.csv file is for a protein digest, but the same format applies to a glycan library, where the glycan name is placed in the first column, currently named **Sequence**. Certain special characters, such as parentheses, are currently not allowed for glycan names. Only **Sequence**, **Uncharged mass**, **Start times**, and **End times** are required fields. The Start and End times sometimes define narrow time windows, and sometimes define large time windows when detailed elution is not known.

Click **Add** at the lower right to enter in-silico peptides manually. Click the fields in the table and enter or paste in the required information. To delete in-silico peptide rows, select them, and click **Remove**.

Feature finder tab

Feature Finder is an algorithm that allows the user to scan the MS1 domain for all existent peptides in a sample. In a typical data dependent acquisition assay, peptides are identified only if an MS2 scan is triggered from a precursor ion in an MS1 scan. Therefore, if a peptide ion does not trigger an MS2 scan, or its score excludes it from identification, it will not be detected. Feature Finder ensures that a peak will not go undetected by identifying all possible isotopic distributions in a sample that could potentially be a peptide.

Feature Finder also allows the user to match these features to the current identifications and to add a specified number of the unidentified features as unknowns to the project. Doing so will generate XICs for the unknowns and allow reporting on features such as retention time, mass, intensity, and fold change across samples.

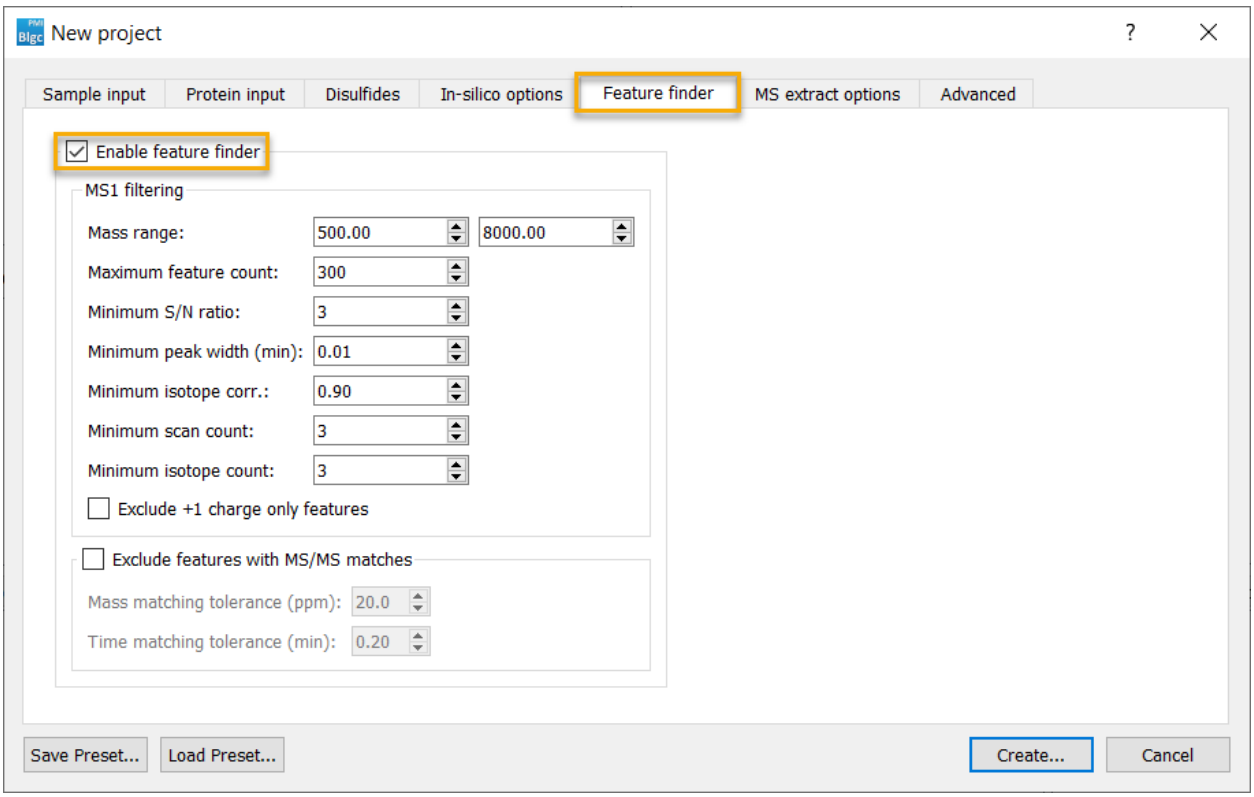


Figure 14: Feature Finder tab

Feature finder is not enabled by default; to enable it, check the **Enable Feature finder** checkbox as shown in Figure 14. **Mass range** sets the minimum and maximum mass for a feature to be considered. To include only the most intense features set a **Maximum features count**. **Minimum S/N Ratio** limits features to small charge ratios. **Minimum Peak Width (min)** limits features to the given peak width. **Minimum Isotope Corr.** sets the minimum cutoff to match the found isotopic distribution to the theoretical distribution using Pearson's correlation. If a feature has an isotopic distribution that is vastly different from its theoretical, this can be used to exclude it. **Minimum Scan Count** limits features to peaks with a minimum number of scans. **Minimum Isotope Count** sets the minimum number of isotope peaks required for a feature to be considered. This applies only if its charge is greater than 2. Check **Exclude +1 charge only features** to exclude those peaks. Check **Exclude features with MS/MS matches** to exclude those features using the specified mass matching and apex time matching tolerances.

MS extract options tab

The **MS extract options** tab sets parameters for creating XIC plots:

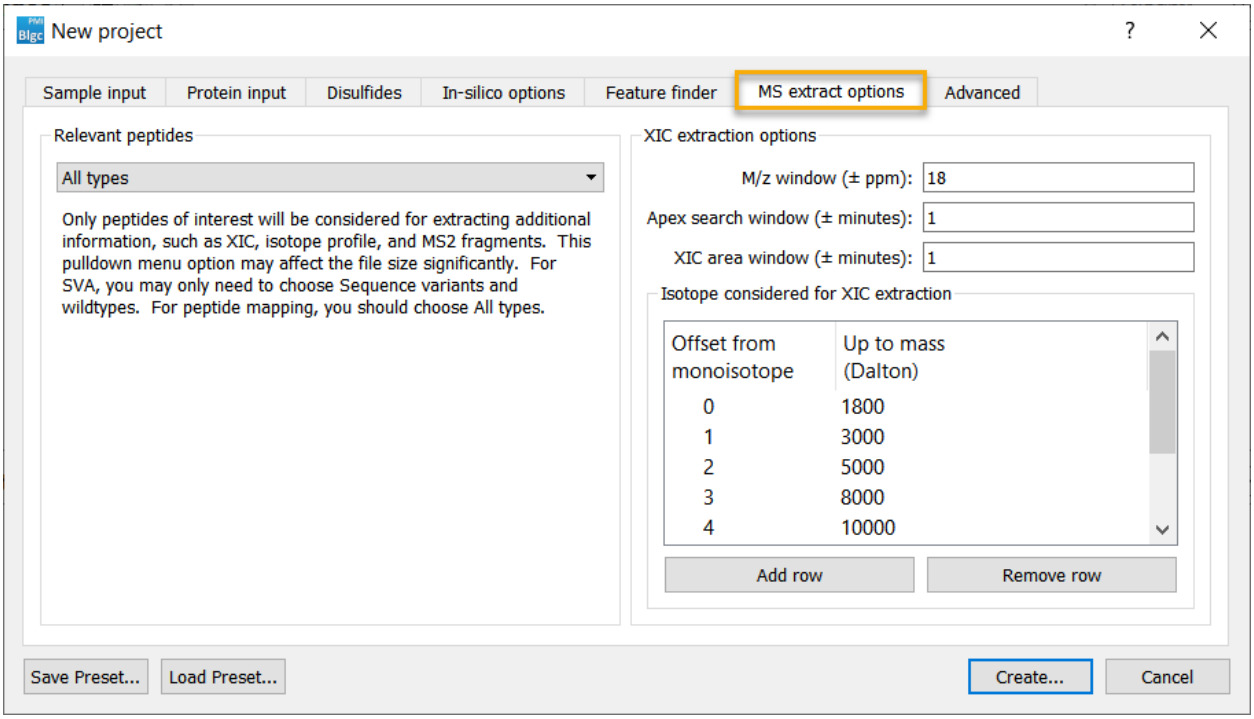


Figure 15: MS extract options tab

The **Relevant peptides** drop-down selects the peptide types to process, including sequence varieties, wildtypes, oxidations and deamidations. Under **XIC extraction options**, the user can adjust the M/z integration range, the search time range, area range and the offset allowed for monoisotopes by total mass.

Advanced tab

The **Advanced** tab contains less-commonly used features.

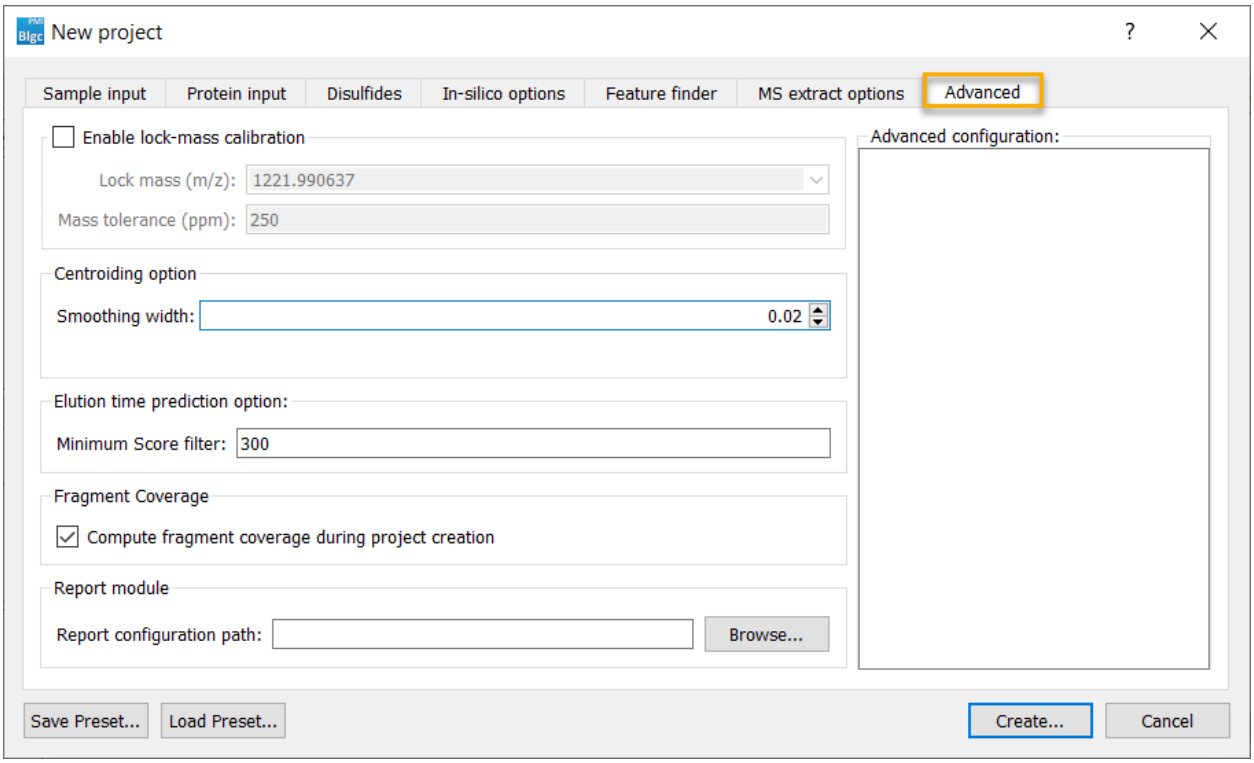


Figure 16: Advanced tab

The tab includes a checkbox labeled **Enable lock-mass calibration**. This option shifts *m/z* measurements based on an internal calibrant. Click the Enable lock-mass calibration drop-down arrow to show a list of common lock masses; however, the user may type in any *m/z* value.

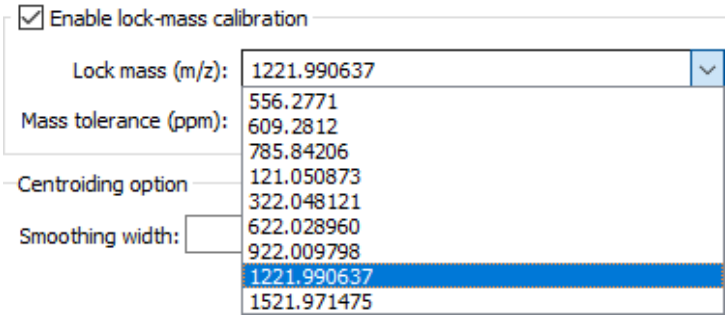


Figure 17: Lock mass dropdown

The **Centroiding option** sets the smoothing width. The **Elution time prediction option** sets the minimum time after which a peak is processed. The **Fragment Coverage** checkbox computes the identities of fragment ions.

The **Report module** setting **Report configuration path l**oads a previously saved report configuration file with the file type *.rptc or *.lvcfg (see **File > Export > Generate multi-document report**). To load a report configuration, click **Browse**, locate the file to be added and click **Open**.

After all project parameters have been entered, click the **Create** button to create the project. The **Save File** dialog will open to enter a file name and directory to save the project.

Main Menu Bar

The topmost menu bar includes four items: **File**, **Edit**, **Window**, and **Help**.

File Menu

The **File** pull-down menus manage project files and export reports and data:

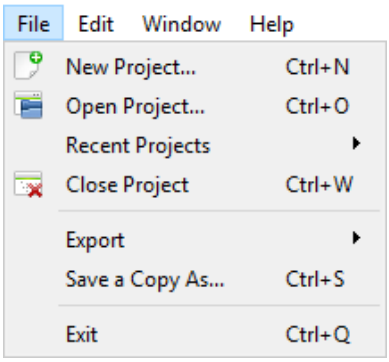
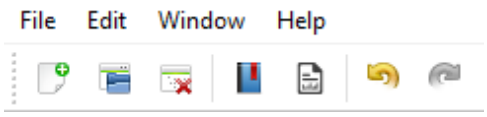


Figure 18: File menu

The File menus includes items to create new projects, open previously created projects from saved files, save a copy of an open project, close a project (yet leave the application open), and exit Byologic. Mouse over the **Recent Projects** menu to open a side window with a list of recently visited projects that can be reopened.

The File menu functions **New Project**, **Open Project**, **Close Project**, **Label Manager** and **Report**, respectively, are also available as icon buttons below the topmost menu bar:



File > Export Menu

There are also three File sub-menu items to export reports.

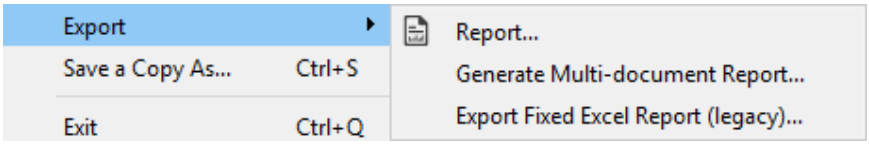


Figure 19: File > Export menu

File > Export > Report generates a report that includes a summary of the project files, one or more pivot tables (that can be visualized as bar charts, etc.), along with plot images. There are other valuable tools for making customizable reports and regulatory filing documents. When a report is perfected, a copy of the report configuration can be saved to use with other reports. If no report configuration is attached to the project, a default report configuration is used to generate the report.

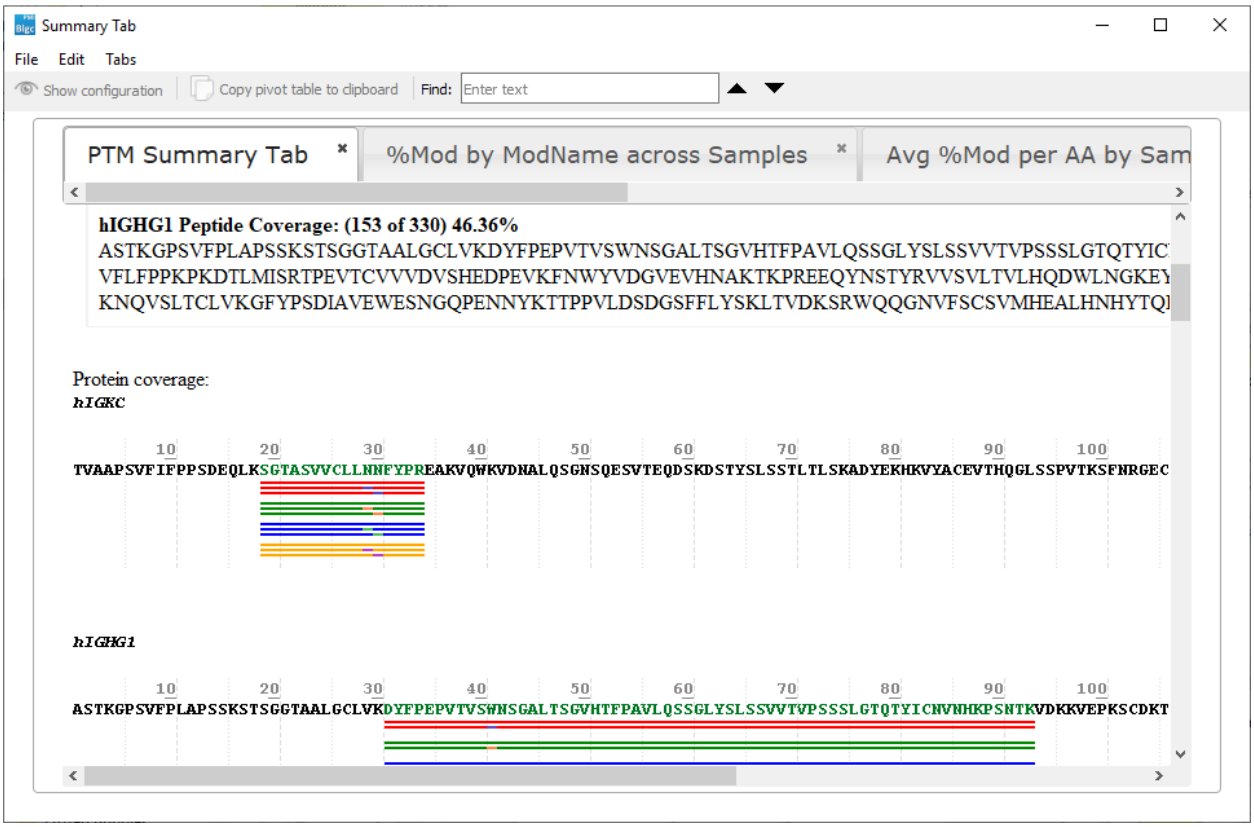


Figure 20: Default report format

File > Export > Generate Multi-document report opens a dialog into which the user drags and drops multiple Byologic project *.blgc files:

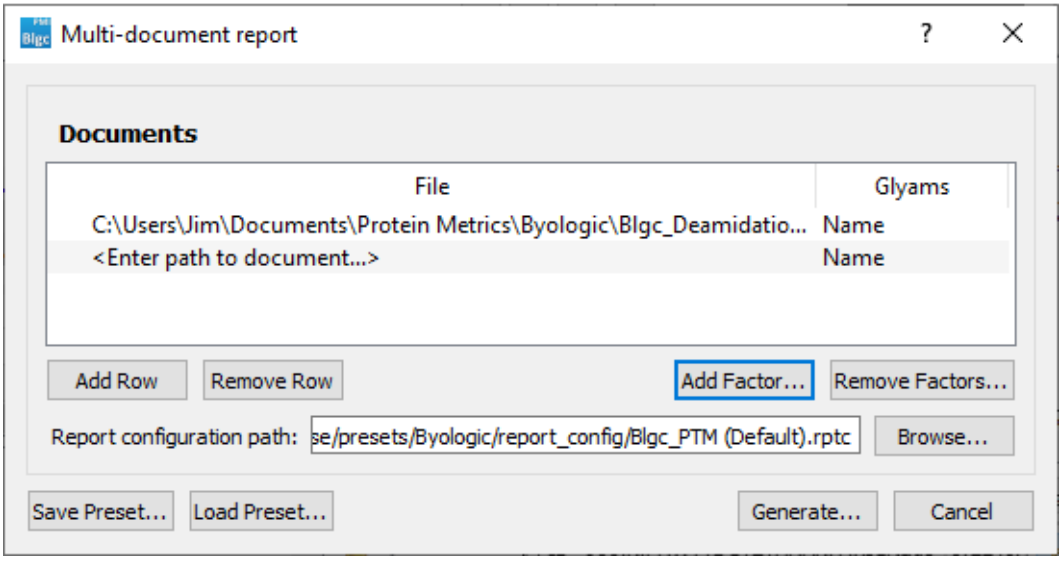


Figure 21: Multi-document report export

File > Export > Fixed Excel report (legacy) exports the Byologic project to an Excel file in the same way that **File > Export > Export to Excel workbook** exports from a standard report.

To learn about reports in detail see the document **PMI Reporting Manual.pdf**. Contact support@proteinmetrics.com for information on a variety of existing and new report templates.

Edit Menu

The **Edit** pull-down menu manages program settings.

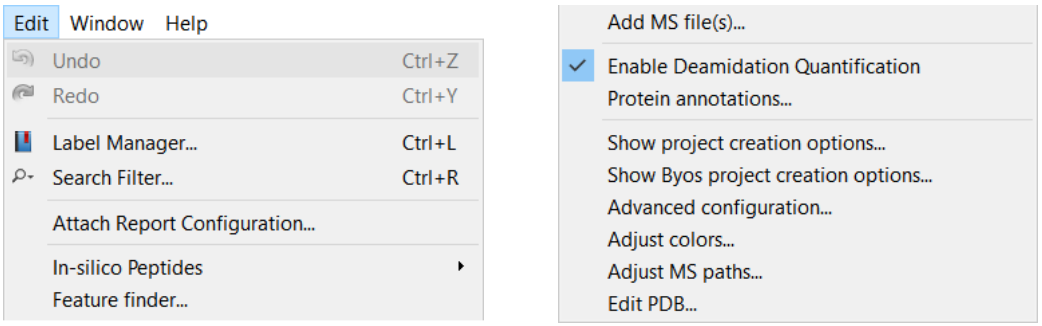


Figure 22: Edit menu

Undo and **Redo** allow the user to undo or redo changes. These Edit menu functions are also available as the arrow toolbar buttons below the menu bar to the right of the **Help** menu.

The **Label Manager** is used to add, remove and edit label values. These labels can then be assigned to records in the Peptides table. To add a new label, click the **Add** button, enter the label text, and click **OK**. To add or edit a comment to a new or existing label, select the label in the Label Manager view, and edit the comment in the **Edit comment** view. To edit a label, select it, click **Edit**, edit the label text, and click **OK**. To remove a label, select it, click **Remove**. Click **OK** in the main window to save all changes.

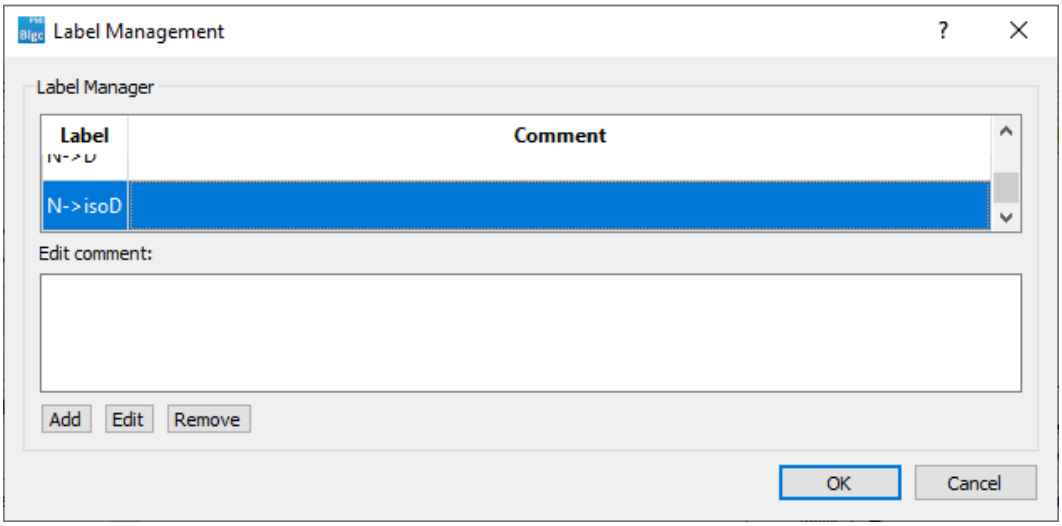


Figure 23: Label manager

To add or remove labels to a record in the Peptides table, click the peptide row, scroll to the Labels column, click the cell in this column to open a dialog. Check or uncheck desired labels and click **OK**. The Label Manager can be opened from this dialog by a click on the **Manager** button.

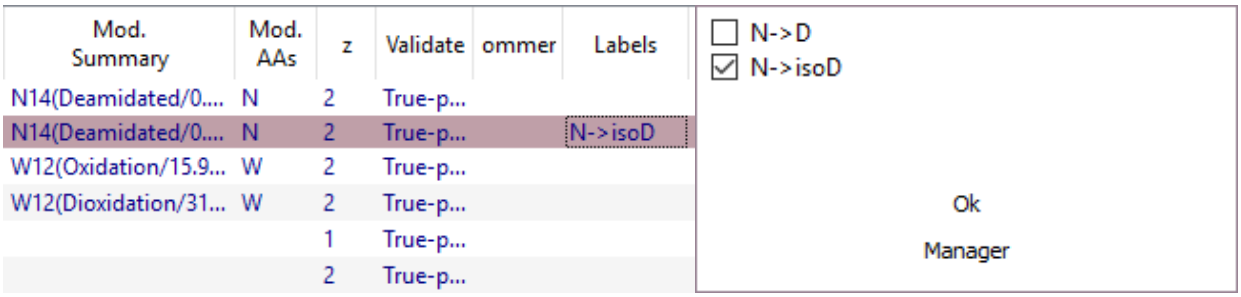


Figure 24: Adding labels in the Peptides table

The **Search Filter** filters records displayed in the Peptides table according to a variety of properties and categories. Edit values for properties to tighten or loosen restrictions. Check additional **Show** categories to include peptides that are not true positives. Click the **Peptide grouping method** drop-down arrow to combine peptides that differ only by their charge states.

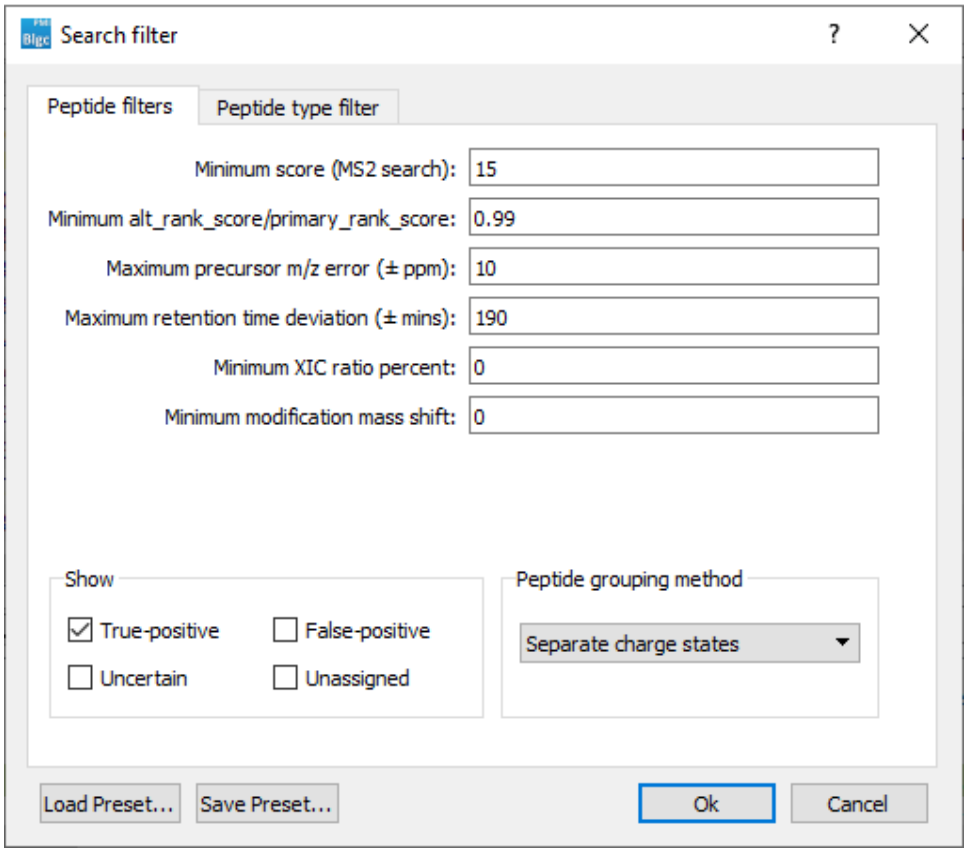


Figure 25: Peptide search filter

Attach report configuration attaches a report configuration file to the project, to be used instead of the default report configuration. Report configuration files (graphical layouts) are generated from **File > Export > Report** and have the extension *.rptc. This menu can also be used to remove an attached report configuration file.

The **Peptide type filter** tab allows the inclusion or exclusion of peptide modification types. Check modifications to include and uncheck modifications to exclude. Byologic defaults to all peptide types:

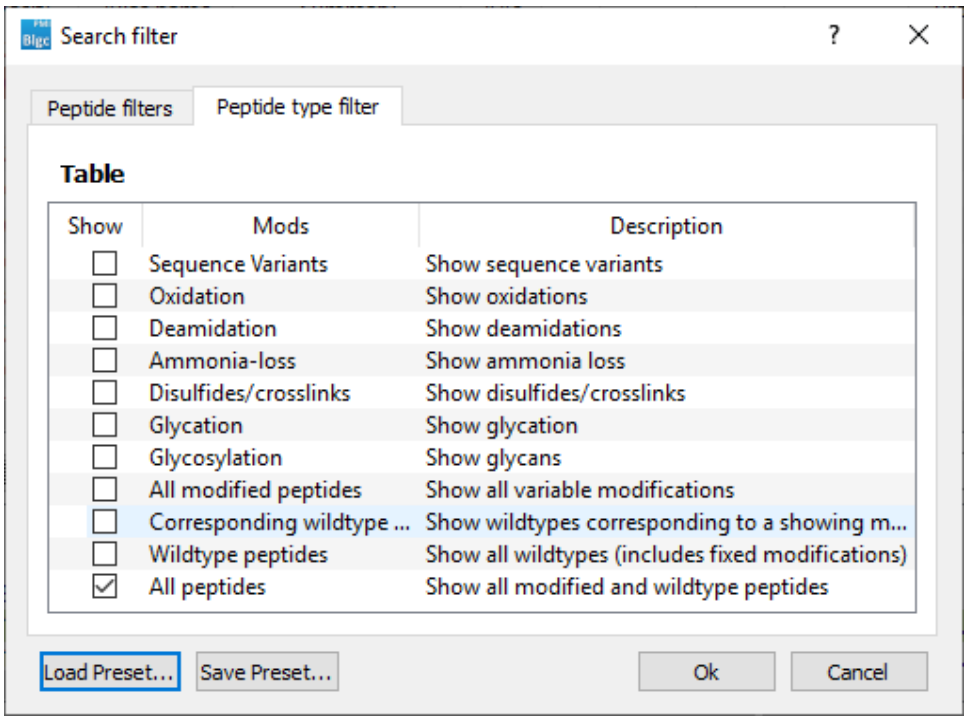


Figure 26: Filtering by peptide type

To save the edited settings from the two tabs to a configuration file, click the **Save Preset** button, enter a file name, navigate to a directory, and click **Save**. To load settings from a previously-saved configuration file, click the **Load Preset** button, navigate to the desired directory, select a *.blgcsfson or *.blgcsf file, and click **Open**.

Attach report configuration attaches a report configuration file to the project, to be used in place of the default report configuration. Report configuration files (graphical layouts) are generated from the project report opened using **File > Export > Report,** and then saved using **File > Save to file**. Configuration have the extensions *.rptc, *.tabc, *.lvcfg, or *.pvtcfg. This menu can also be used to remove an attached report configuration file.

In-silico Peptides contains four sub-menus to import or export in-silico peptide candidates before or after project creation:



Figure 27: Edit > In-silico Peptides menu

- **Add in-silico Peptides From CSV File** opens a browser to load a *.csv file of in-silico candidates. Use of the correct format for the *.csv file is required. For an example of the correct *.csv format, see the file **ImportExample.Byologic.csv** in **Program Files > ProteinMetrics > PMI-Suite > ExampleData**.
- **Add Missing Via Existing Peptides** identifies missing peptide sequences and fills in those sequences from equivalent peptides in the sample files.

- **Export in-silico to CSV** exports the in-silico peptides to a *.csv file. This peptide file is then available for import into a different project. Enter a file name, navigate to a directory, and click **Save**.
- **Peptide Manager** combines all of the In-silico functionalities from **Edit > In-silico peptides** with a color-coded preview of the project changes and enhanced control over which rows and columns are affected.

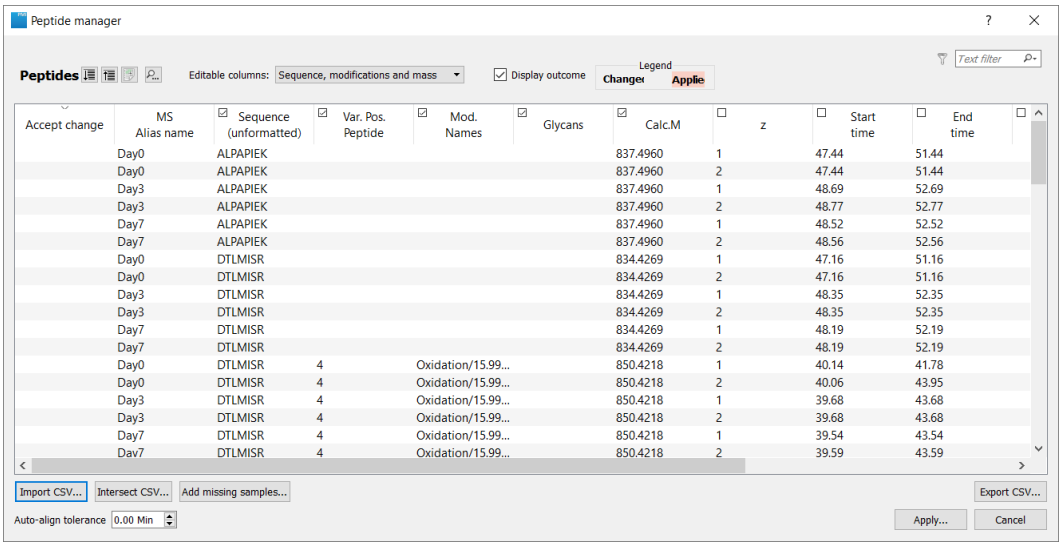


Figure 28: Peptide manager

The user can set which columns are editable using the drop-down menu.

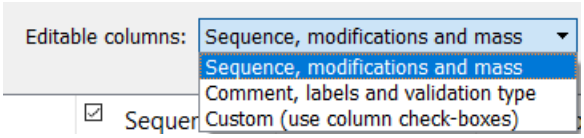


Figure 29: Editable columns in Peptide manager

- Click Export CSV is the same as Edit > In-Silico > Export CSV.

  Start and End times may differ from the previous CSV export. This is because Peptide Manager exports the most centered XIC start/end, while the old method exported the first available start/end range. The difference is considered an improvement.
- Import CSV is the same as in Edit > Import in silico from CSV
- **Intersect CSV** replaces the old menu item **Edit > In-Silico > Intersect with CSV library**. This menu item has been removed and can only be accessed from Peptide Manager.

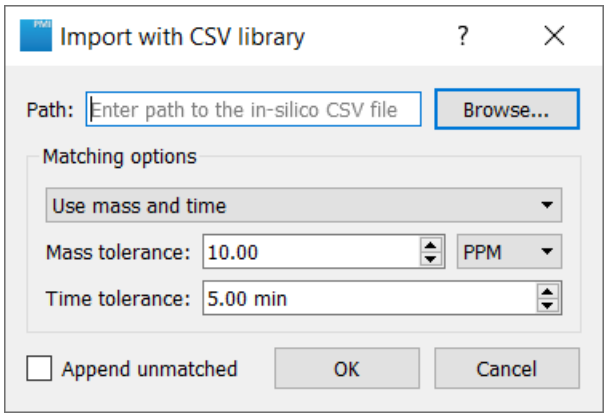


Figure 30: Intersect CSV: import with CSV library dialog

Select a CSV file using the **Browse** button. **Matching options** include **Use mass and time** and **Use sequence and modifications**. Set mass and time tolerances. Check **Append unmatched** to include non-intersected peptides. Click **OK** to return to Peptide Manager and select columns to review. New values will be show in red.

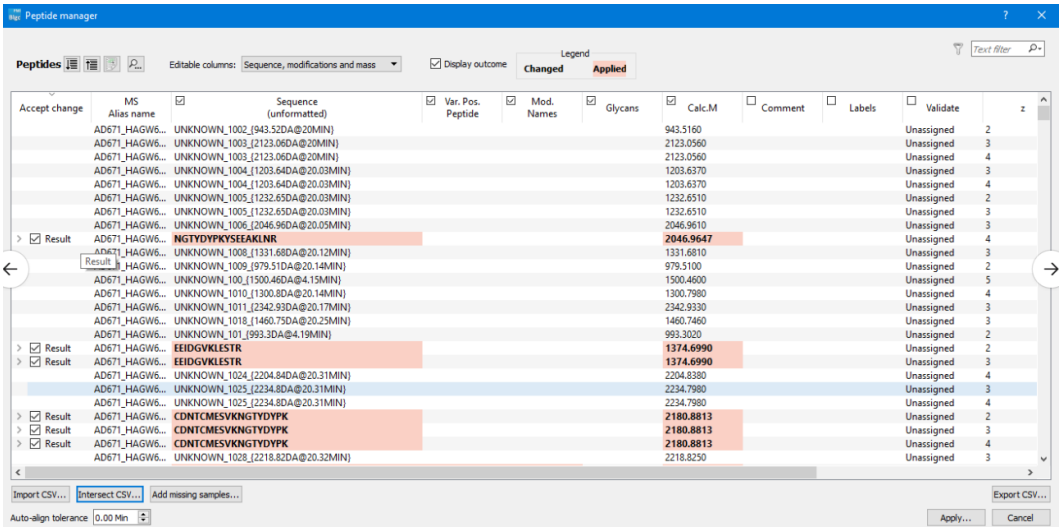


Figure 31: Peptides imported from CSV library are marked in red

Expanded rows to showcase additional content:

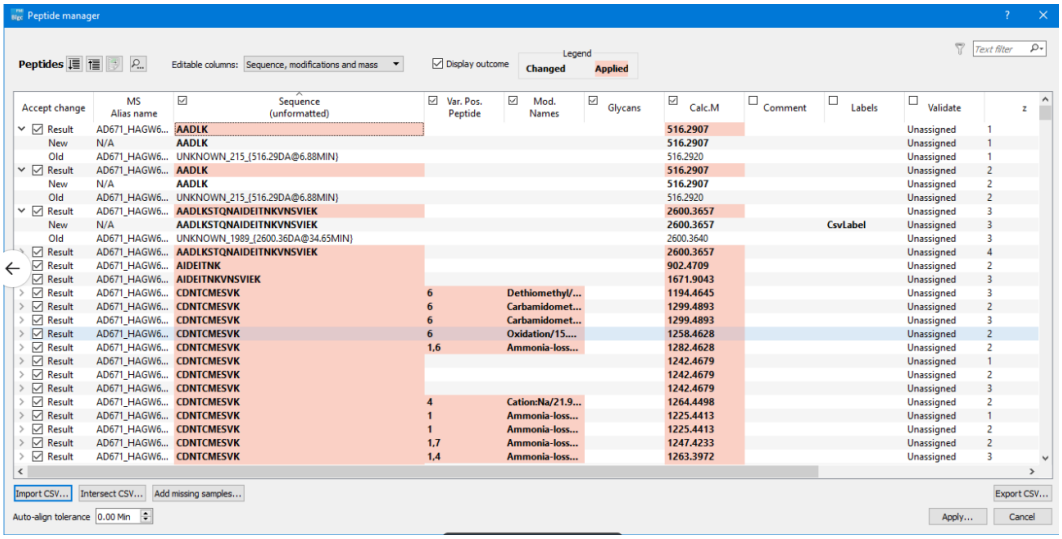


Figure 32: Imported peptides, expanded

- Add missing samples is the same as in Edit > In-Silico > Add missing.

  Click the button and then Apply to update the project with new in-silico peptides. These will be visible at the end of the list.

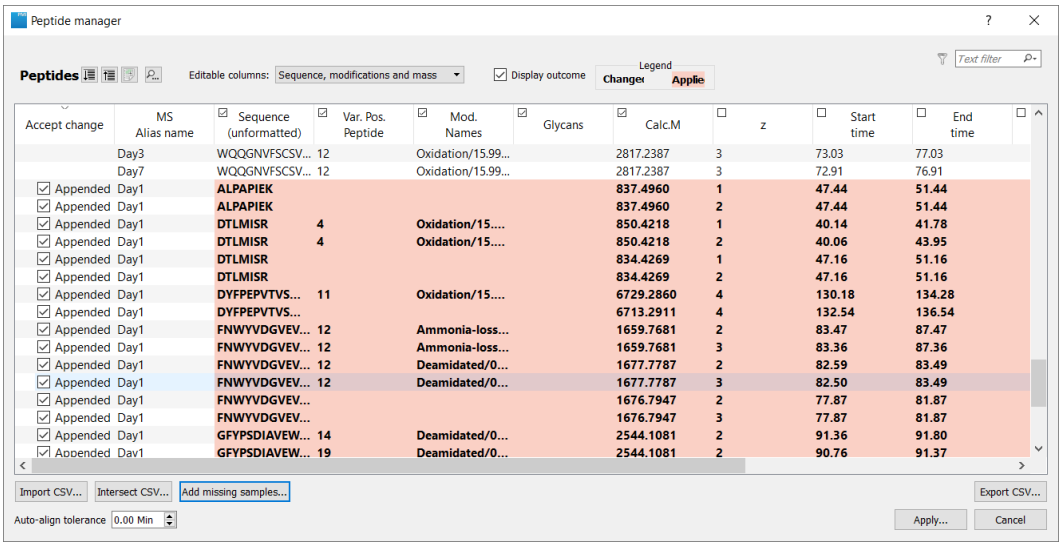
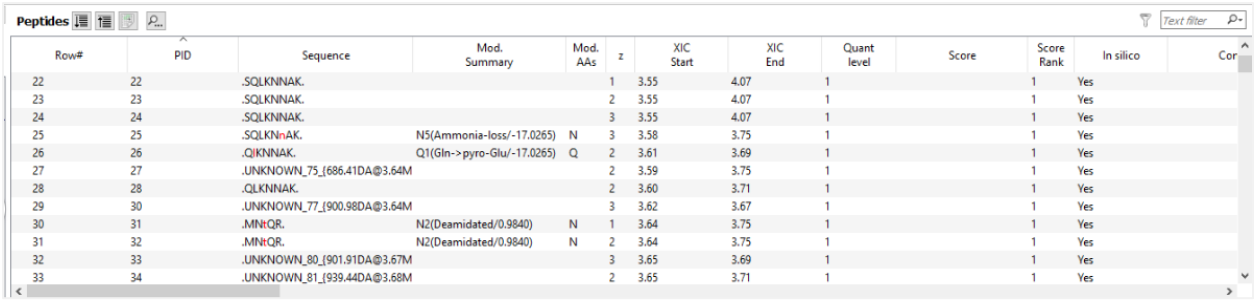


Figure 33: Peptide manager after using Add missing samples

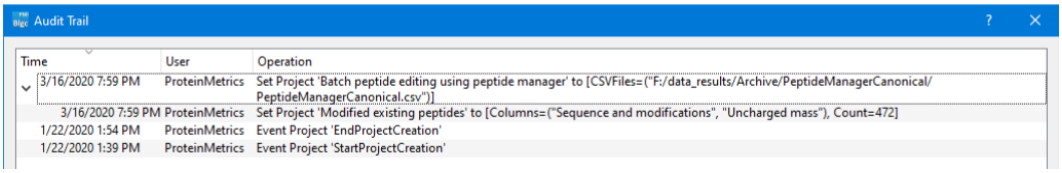
- Click **Apply** to update the project. Figure 34 displays a common use case: Feature Finder unknowns matched with in-silico content.



Row#	PID	Sequence	Mod. Summary	Mod. AAs	z	XIC Start	XIC End	Quant level	Score	Score Rank	In silico	Cor
22	22	.SQLKNNAK.			1	3.55	4.07	1		1	Yes	
23	23	.SQLKNNAK.			2	3.55	4.07	1		1	Yes	
24	24	.SQLKNNAK.			3	3.55	4.07	1		1	Yes	
25	25	.SQLKNnAK.	N5(Ammonia-loss/-17.0265)	N	3	3.58	3.75	1		1	Yes	
26	26	.QIKNNAK.	Q1(Gln->pyro-Glu/-17.0265)	Q	2	3.61	3.69	1		1	Yes	
27	27	.UNKNOWN_75_[686.41DA@3.64M			2	3.59	3.75	1		1	Yes	
28	28	.QLKNNAK.			2	3.60	3.71	1		1	Yes	
29	30	.UNKNOWN_77_[900.98DA@3.64M			3	3.62	3.67	1		1	Yes	
30	31	.MNtQR.	N2(Deamidated/0.9840)	N	1	3.64	3.75	1		1	Yes	
31	32	.MNtQR.	N2(Deamidated/0.9840)	N	2	3.64	3.75	1		1	Yes	
32	33	.UNKNOWN_80_[901.91DA@3.67M			3	3.65	3.69	1		1	Yes	
33	34	.UNKNOWN_81_[939.44DA@3.68M			2	3.65	3.71	1		1	Yes	

Figure 34: Peptides added with Add missing samples

- The Audit Trail documents all change made through the Peptide Manager, including
  - The CSV intersected
  - The columns altered
  - How many peptides were affected



Time	User	Operation
3/16/2020 7:59 PM	ProteinMetrics	Set Project 'Batch peptide editing using peptide manager' to [CSVFiles=("F:/data_results/Archive/PeptideManagerCanonical/PeptideManagerCanonical.csv")]
3/16/2020 7:59 PM	ProteinMetrics	Set Project 'Modified existing peptides' to [Columns=("Sequence and modifications", "Uncharged mass"), Count=472]
1/22/2020 1:54 PM	ProteinMetrics	Event Project 'EndProjectCreation'
1/22/2020 1:39 PM	ProteinMetrics	Event Project 'StartProjectCreation'

Figure 35: Audit Trail after using Peptide manager

Feature finder opens a dialog which includes the settings available in the **Feature Finder** tab during project creation:

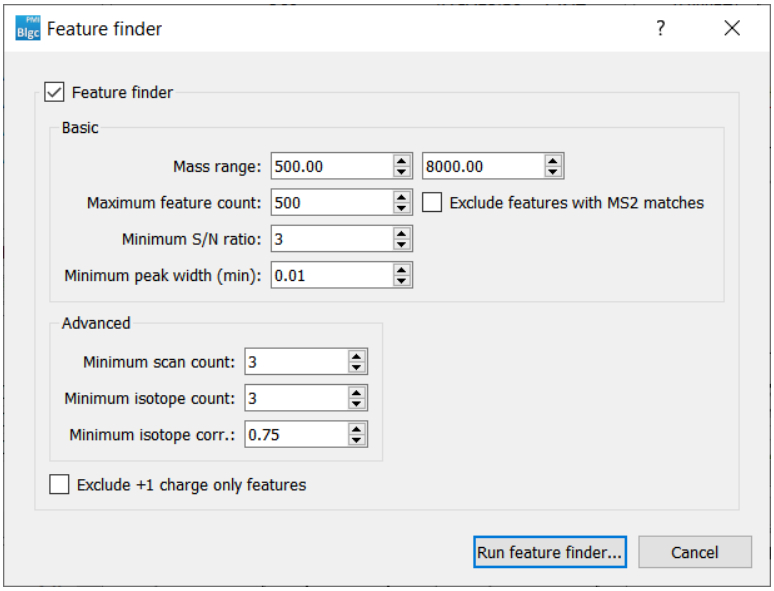


Figure 36: Feature finder dialog

See the **Feature Finder tab** section under **Creating a Project** for more information.

Add MS File(s) imports additional MS files after project creation. The MS files have extensions *.d, *.raw, *.wiff, *.lcd, and *.byspec2. Navigate to the desired file and click **Open**.

Enable Deamidation Quantification recalculates XIC integrations to account for the interference between a deamidated peptide and a wildtype peptide with a similar isotope value. The wildtype integration is subtracted from the total to yield a “pure deamidated” XIC plot.

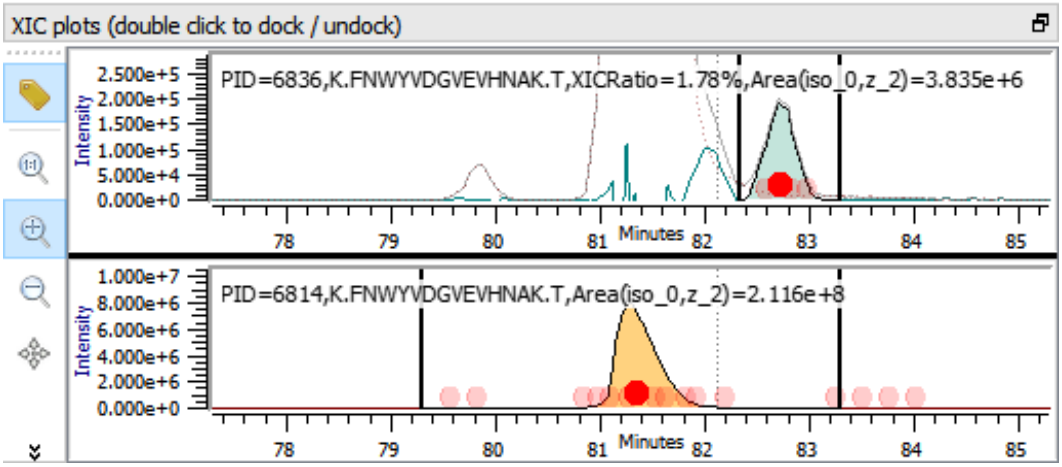


Figure 37: Deamidation quantification subtracts the wildtype integration from the total

Protein Annotations is used to annotate individual proteins for display in the Peptides table:

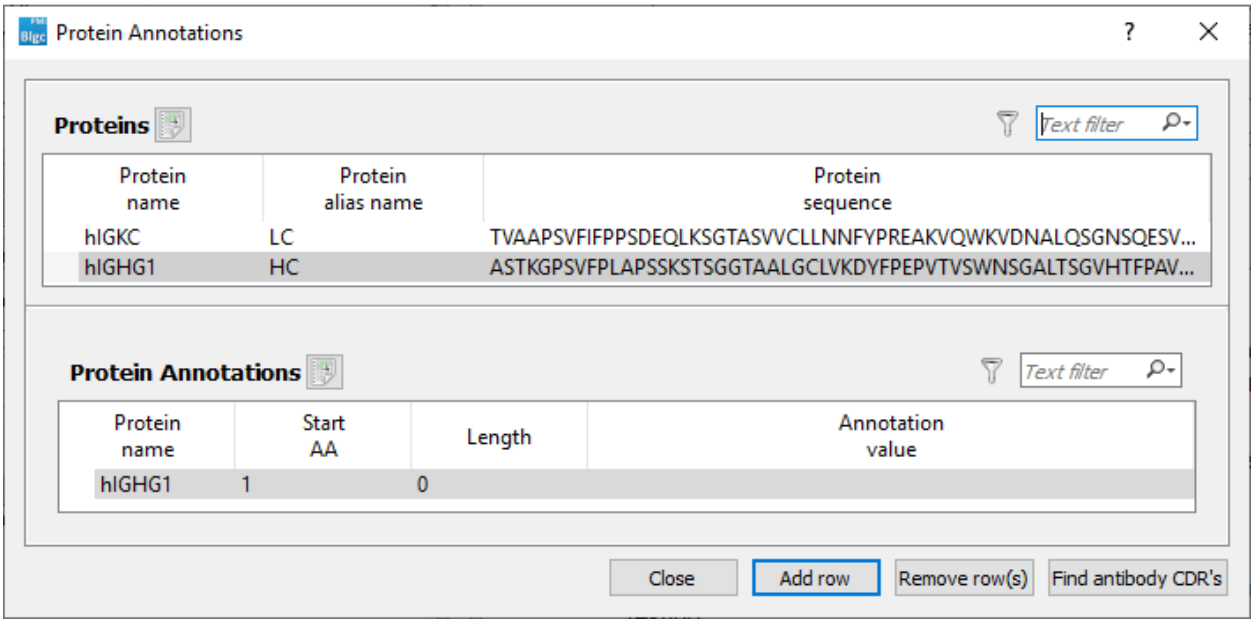


Figure 38: Protein Annotations

To annotate a protein, select it in the Proteins window, click **Add row**, specify the starting amino acid and sequence length, and then enter text under **Annotation value** in the Protein Annotations window. The **Find antibody CDR’s** button finds antibody complementarity determining regions and displays them in the Protein Annotation column of the Peptides table. Click **Close** when finished.

Show Byos project creation options displays the Byos workflow project creation options used to generate the project. This feature is only available for projects created through Byos. A read-only dialog similar to the Byos Project Creation dialog opens:

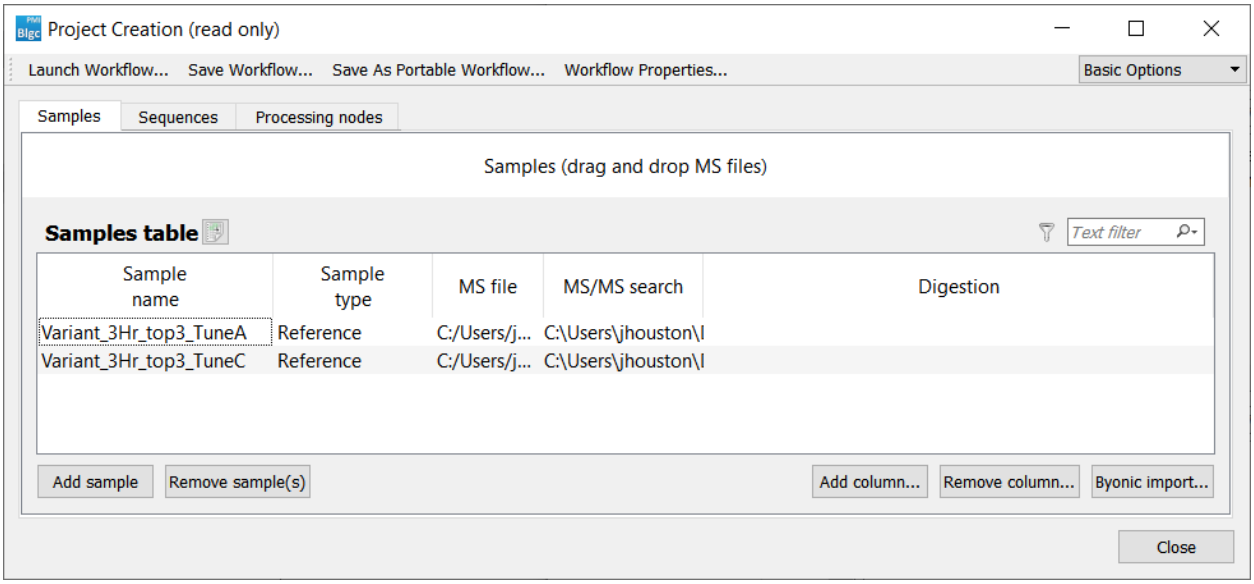


Figure 39: Show Byos project creation options

The **Sequences** and **Processing** nodes tabs open to show all the entries used in those tabs.

Show project creation options displays in a single window the values and inputs used to create the current project. Apart from some import options, the data is generally read-only in these tabs. To edit specific functions and values, use the corresponding Edit menu items described in this section. This feature allows the user to save the current settings used in the project with the **Save Preset** button or load new ones with the **Load Preset** button.

Advanced configuration is used to enter text commands, which are often new features still in beta testing. This feature is generally not used.

Adjust colors is used to modify the default colors associated with MS samples and corresponding plots.

Adjust MS paths is used to modify the path to the source MS file, should the file be moved. Enter or navigate to the new directory containing the MS file.

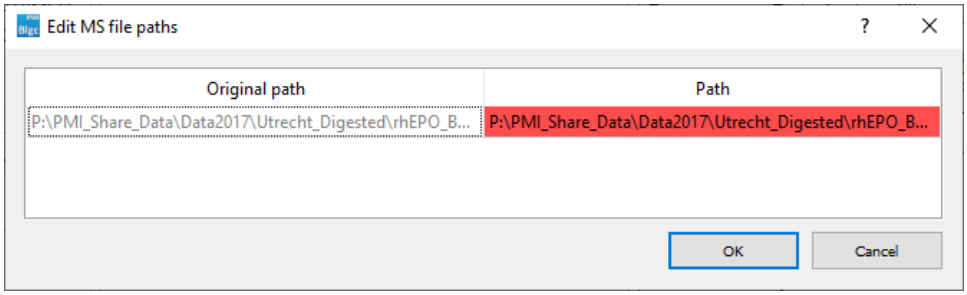


Figure 40: Editing the MS file path

Edit PDB loads protein database files for use by Protein 3D reports.

Window Menu

The **Window** pull-down menu manages the visual layout of the user interface, and the arrangement and sizes of the various table and plot views:

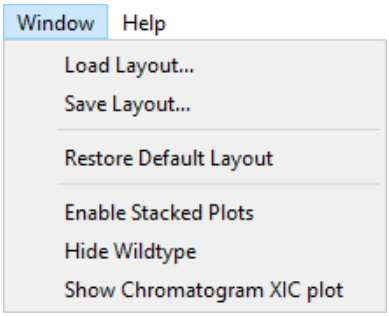


Figure 41: Window menu

Load Layout opens a saved layout stored to files with extension *.ini. **Save Layout** saves the current layout to an *.ini file which can be used in other projects or shared with other users. **Restore Default Layout** applies the layout used when the application is first opened.

The current layout can be customized by moving, resizing or editing the view panes. Many of the views (Project, Protein coverage, XIC, MS2 and mass error, and Isotope plots) can be undocked by double-clicking on the tops of that view to create standalone windows that can be moved to another monitor. These views can be click-dragged to new locations in the layout. The view can also be resized by hovering over the vertical or horizontal edges between table and plot views so that the cursor changes to arrows:  or . Left click and drag the edges up or down or right or left to change the sizes of the views. Columns in the table views can be made larger or smaller in same manner. To reorder columns in a table, left click the name of the column, and drag it to the new location. To do a simple sort, left click the header once to sort ascending and twice to sort descending. Mouse over a column header to see a description of that field.

Column headers can be modified through the **Column Header Editor** dialog, opened by a right-click anywhere on a table column header.

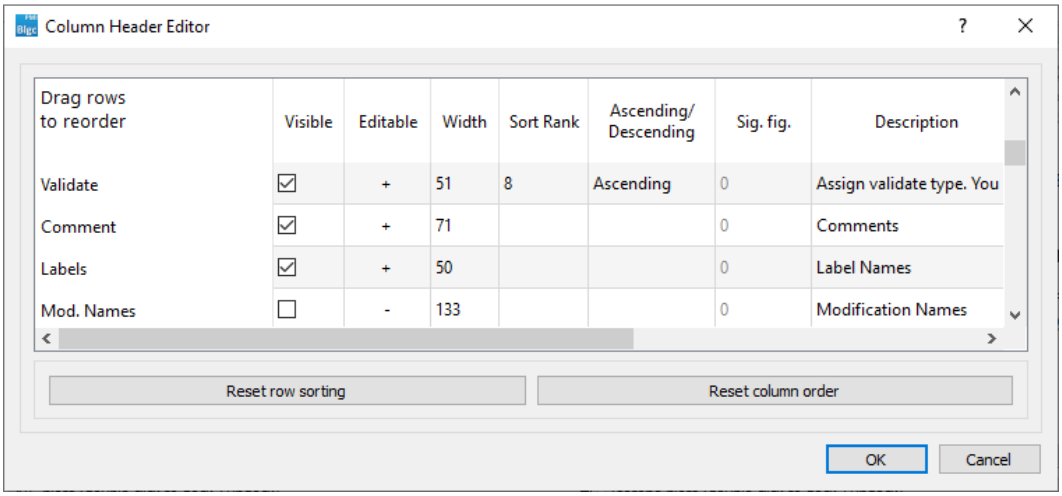


Figure 42: Column Header Editor

Columns can be rearranged by dragging the header names up or down. To hide a column, uncheck the **Visible** box for that column. This dialog can also be used to specify number of significant figures and ranked sorting. The **Window > Save Layout** menu preserves these edits for future use and sharing. The **Reset row sorting** and **Reset column order** buttons restores the default row and column properties.

Enable Stacked Plots can be turned on to separate the plot view into a series of normalized traces of all the sample data:

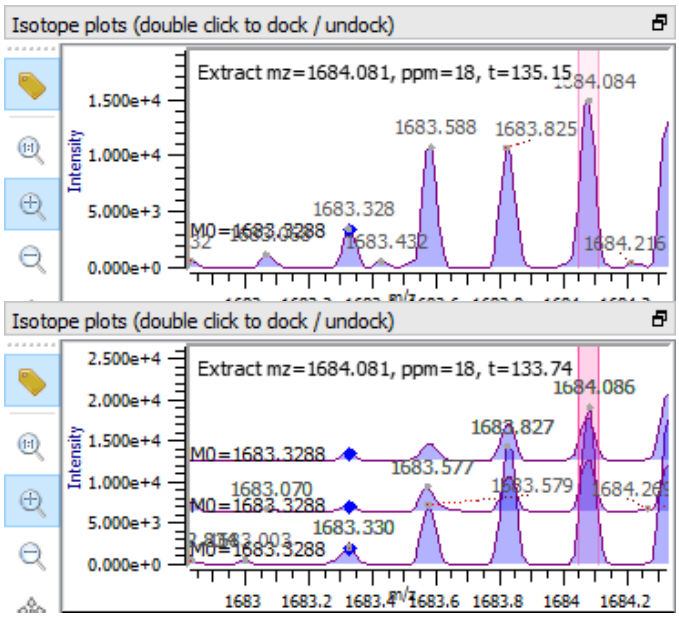


Figure 43: Example of stacked isotope plots

Hide Wildtype removes the wildtype peptide records in the **Wildtype peptides** table and the corresponding wildtype peptide plots in the **XIC plots**, **MS2 and mass error plots** and **Isotope plots**:

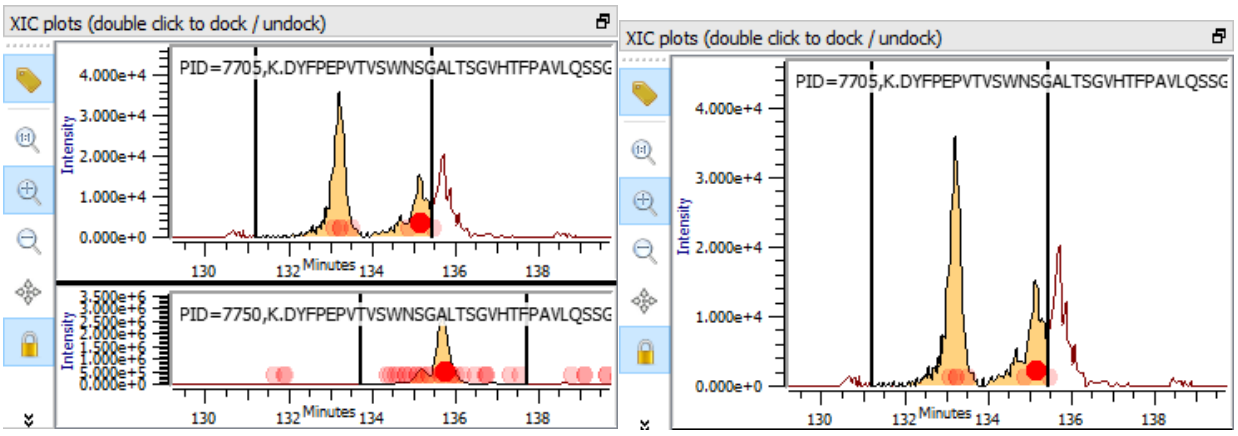


Figure 44: Example of hidden wildtype plot

The **Show Wildtype** menu displays the Wildtype peptide table records and wildtype plots again.

Show Chromatogram XIC Plot opens an undocked window displaying chromatograms for the projects. The traces are displayed in the colors of the samples they represent:

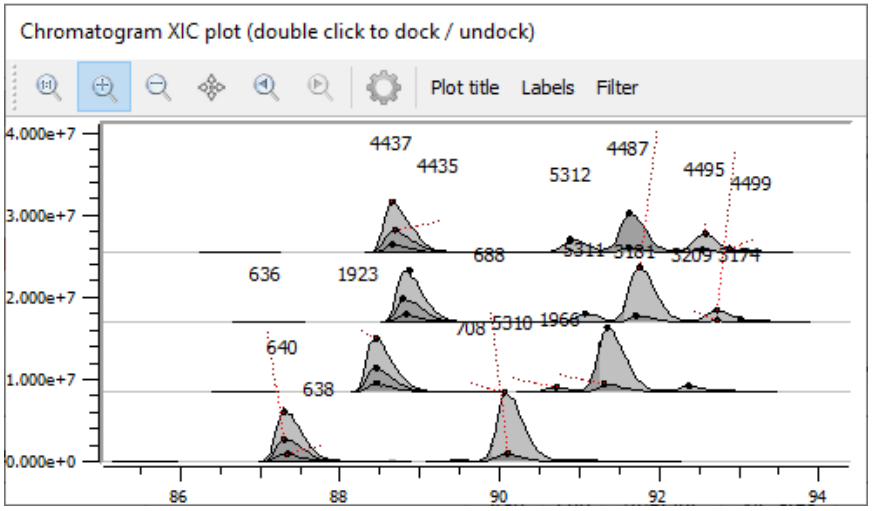


Figure 45: Undocked chromatogram XIC plots

Help Menu

The **Help** pull-down menu provides information about the software:

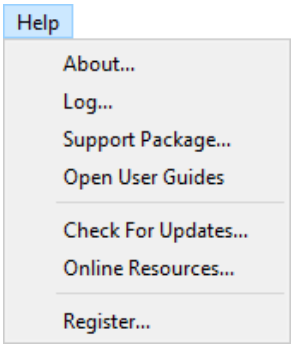


Figure 46: Help menu

The **About** menu shows the software version number, which is needed when reporting issues. The **Log** menu opens a log containing recent activity; this is helpful for troubleshooting problems together with Protein Metrics, Inc. staff via support@proteinmetrics.com. **Support Package** opens a dialog to generate a comprehensive set of files to better enable Protein Metrics to troubleshoot problems. Click **Review & edit the package** to generate a directory of individual files as directed. Click **Compress** to generate a zip file of this support directory. An information box indicates where the zip file can be found and gives a link for support@proteinmetrics.com. **Open User Guides** opens the installed folder with the manuals and quick start guides for Protein Metrics, Inc. software, including Byologic. **Check For Updates** will report if an online update is available for installation. **Online Resources** opens the Protein Metrics, Inc. Secure Resources webpage containing a variety of technical pages and tutorials related to Protein Metrics, Inc. software. **Register** is used to activate the software upon first use.

Table Menus

The menu bars at the top of the three table views, Project, Peaks, and Candidates, share icons that manage hierarchical lists and filter on data.

- The  icon expands rows to show “sub-rows”.

- The  icon collapses rows to hide sub-rows. The use of sub-row depends on the table. For example, in the Peptides table, a row is a peptide record and a sub-row is the data resulting from an individual sample that contained the peptide.
- The  icon exports the table to a .csv file for opening with Excel.
- The  icon opens a dialog to create custom filters for that data table. (A single column filter can also be set by right-clicking on an entry in the column.) Select a column in the first cell, select an operator in the second cell, and enter text in the third cell. A second filter row becomes available for further entry. Click the red X after a filter row to delete it. Custom filters allow masses to be filtered by mass range, annotation, intensity, and so forth. Custom filters can be stored with the project document or exported and imported using the **Presets** dropdown.
- The 

cell filters the entered text across the content of all columns in the protein and peptide tables. The records are filtered automatically as text is entered. Click the icon to search the string as a whole word or as case sensitive.
- To sort the contents of a column by ascending value, click the column header. Click the column header again to sort by descending value.

Table Right-Click Menus

The Project, Protein Coverage, Peptides, and Wildtype peptides tables have context menu items revealed by a right-click on the rows within the tables. (Recall that a right-click on the header of these tables opens the Column Header Editor dialog.)

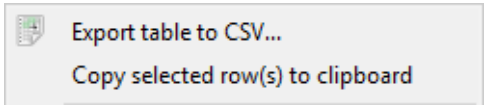


Figure 47: Table right-click menus

- **Export table to CSV** saves the table content and headers to a .csv file using parameters chosen in the **Export data** dialog.
- **Copy selected row(s) to clipboard** copies selected table rows, with their headers to be available for pasting into another application. (This menu is not available in the Protein Coverage table.)
- Table context menus contain a set of query options for searching the clicked text value using any of the available search operators. This is an easy way to filter a table based on a specific field value.

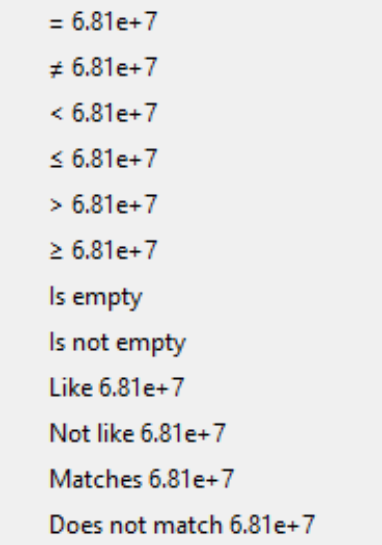


Figure 48: Search table values through a right-click menu

Project Table

The **Project** table contains information about the MS samples and results files:

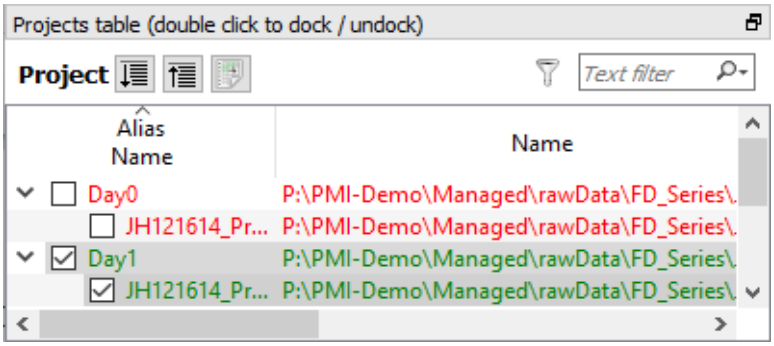


Figure 49: Project table

To display or remove the MS sample and result combination, check or uncheck the box at left of either a sample or result record. To edit a MS sample or result alias name, right-click the entry to edit under the **Alias Name** header, click **Edit selected row(s)**, edit the name, and then click **OK**.

Protein Coverage Table and Menu

The **Protein Coverage** table displays peptide sequences and corresponding protein coverage. The accompanying display maps the protein sequences of detected peptides to colored lines below each sequence representing colors assigned to different MS samples:

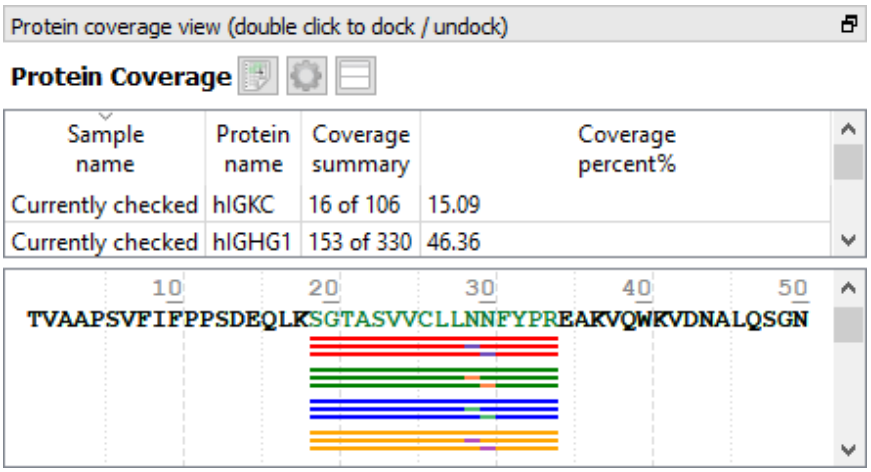


Figure 50: Protein Coverage table

The different colors within the colored bars indicate the presence of peptide modifications at those positions. (The colors used are chosen for contrast and do not reflect the colors assigned to the MS sample files.)

The Protein Coverage table also includes the following specialized menu icon buttons:

- The  icon opens the Protein coverage rendering options dialog:

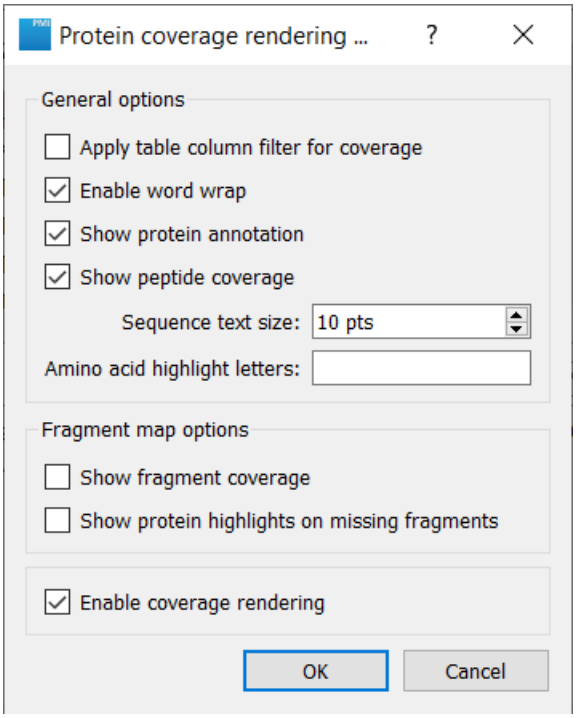


Figure 51: Protein coverage rendering options

- The  icon turns on and off the display of the tabular protein coverage data.

The Protein Coverage view has context menu items revealed by a right-click on the graphical portion of the display:

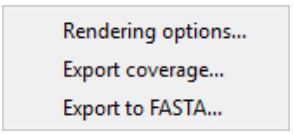


Figure 52: Protein Coverage right-click menu

- Rendering options also opens the Protein coverage rendering options dialog show above.
- **Export coverage** creates a *.png file of the graphical protein coverage display.
- **Export to FASTA** creates a *.fasta file of the proteins found in the Protein Coverage view. The *.fasta file is then available for import into new projects.

Peptides Table and Menu

The **Peptides** table contains detailed information about all identified peptides:

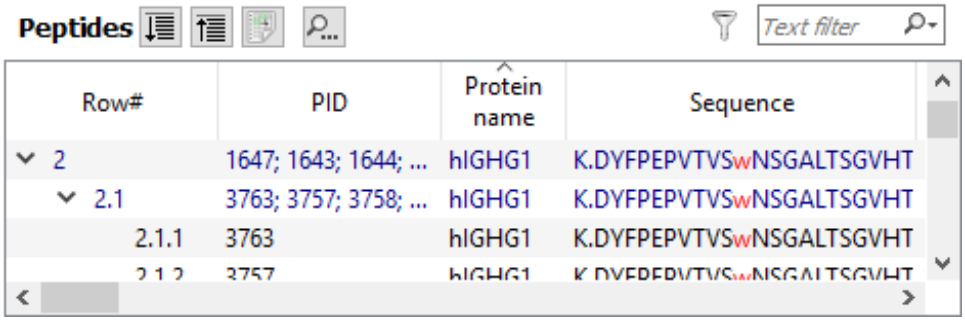


Figure 53: Peptides table

The Peptide table organizes records in the following hierarchy: Peptide > Sample > Charge state (if combined) > MS2 scan. Records in the Peptide table can be modified directly by the user for three fields: Validate, Comment and Labels. In the Validate field, the user selects between “True-positive”, “False-positive” and “Uncertain”. Comments are input directly. Values for Labels are selected from a list. To create new labels to use in the Labels field, see **Edit > Label Manager**.

Note: The MS1 correlation score compares the measured MS1 isotope distribution to the theoretical distribution calculated using cosine similarity. A score of 1 is a perfect match.

In addition to the usual table menu buttons, the Peptides table contains the icon button which opens the same Search Filter dialog that is opened by the **Edit > Search Filter** menu. The “*text filter*“entry box allows the records to be filtered based on the occurrence of the given text string searched across all fields.

The Peptides table has context menu items applicable to specific peptides. The menu is revealed by a right-click on a peptide cell in the table:

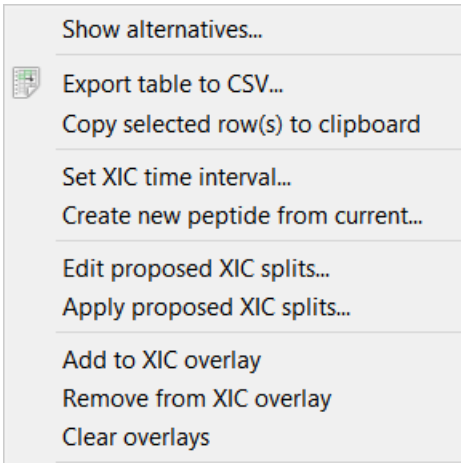


Figure 54: Peptides table right-click menus

- **Show alternatives** displays a table of alternative MS2 identifications (with different sequences and/or modifications) for the selected peptide, along with their scores:

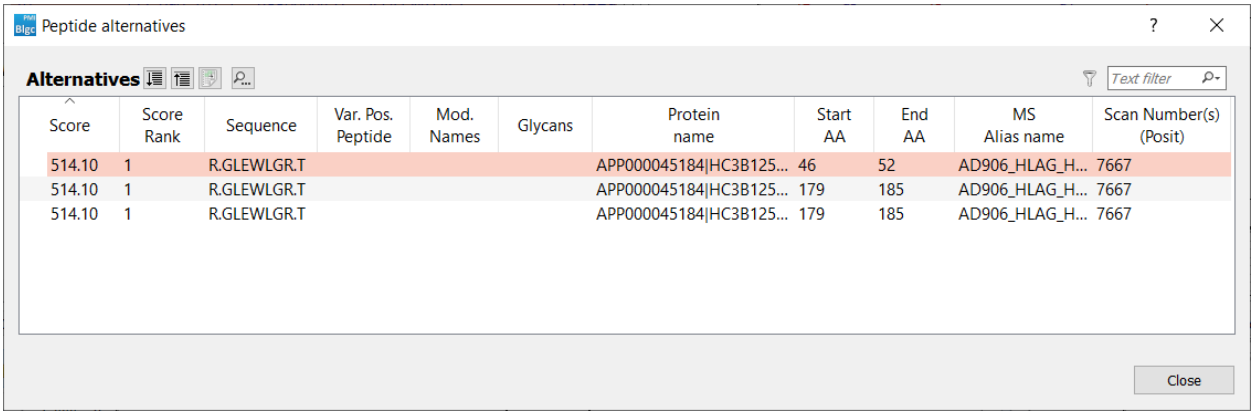


Figure 55: Peptide alternatives table

- **Set XIC time interval** allows the user to edit the XIC start and end times for that peptide:

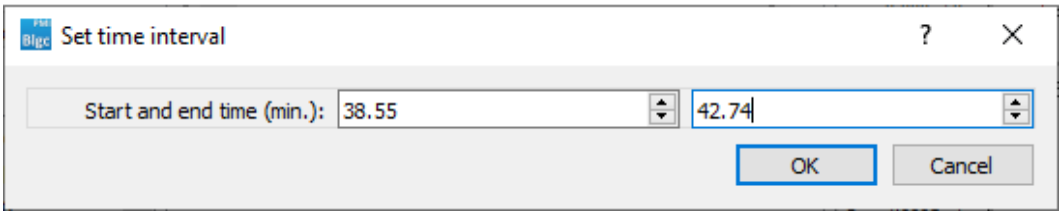


Figure 56: Set XIC time interval

- **Create new peptide from current** makes a copy of that peptide. This allows XIC plots to be split according to separate integrations. The user edits the start and end times and the charge of the peptide before the copy is created:

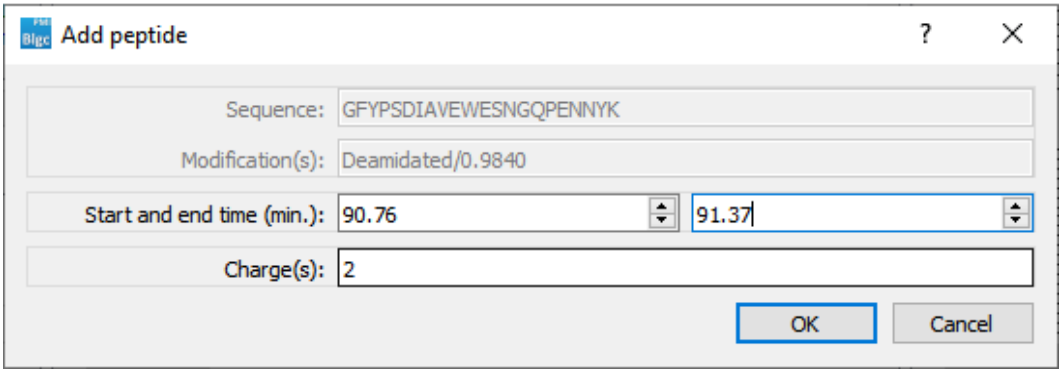


Figure 57: Create new peptide from current

- **Edit proposed XIC splits** opens a dialog to enter pairs of time ranges to split XIC plots for that peptide record into two or more chromatograms:

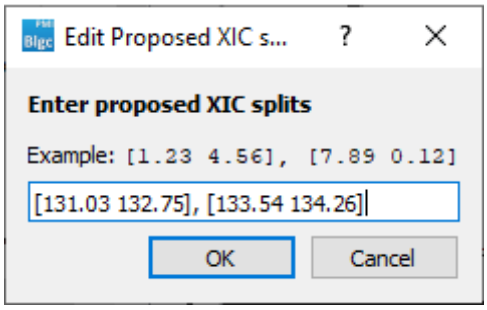


Figure 58: Edit proposed XIC splits

After a click on the **OK** button, the Peptides table record field **XIC proposals** is updated with the time range entries. The split must be applied to take effect.

- **Apply proposed XIC splits** separates a record in the Peptides table into two or more records using the time ranges entered using the Edit proposed XIC splits function.
- **Add to XIC overlay** adds the selected Peptides table record (subject to the right-click) or collection of records as an overlay to the XIC plots view mapped to the same time axis:

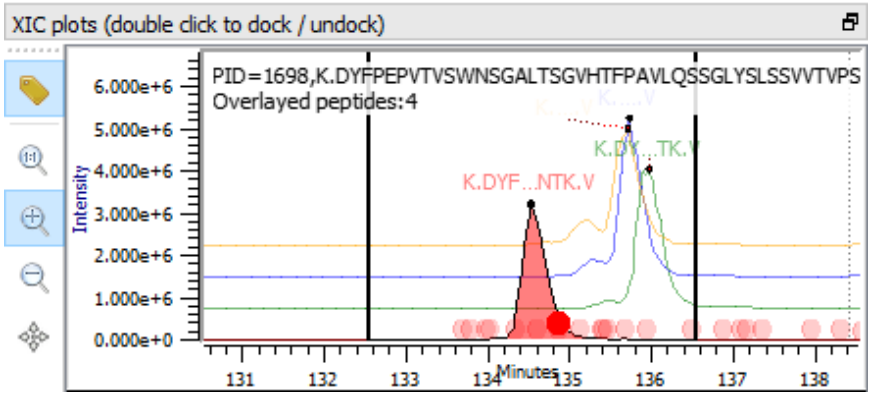


Figure 59: Add to XIC overlay

When this function is applied to a collection of records in the table, all the records below it are added to the XIC plots view.

- **Remove from XIC overlay** removes the overlay from the selected Peptides table record (subject to the right-click) from the XIC plots view.
- **Clear overlays** removes all sample overlays from the XIC plots view.

Mod and SVA Validators

Additional validation is an essential step of degradant and sequence variants analysis. The SVA report, provided as a preset and used as the default report in the SVA workflow, contains a column for auto-validation called “SV auto-comment”. These are now also available in the inspection view. The following two sets of advanced commands enable the user to complete validation through comments automatically populated in the Peptides table, as shown below.

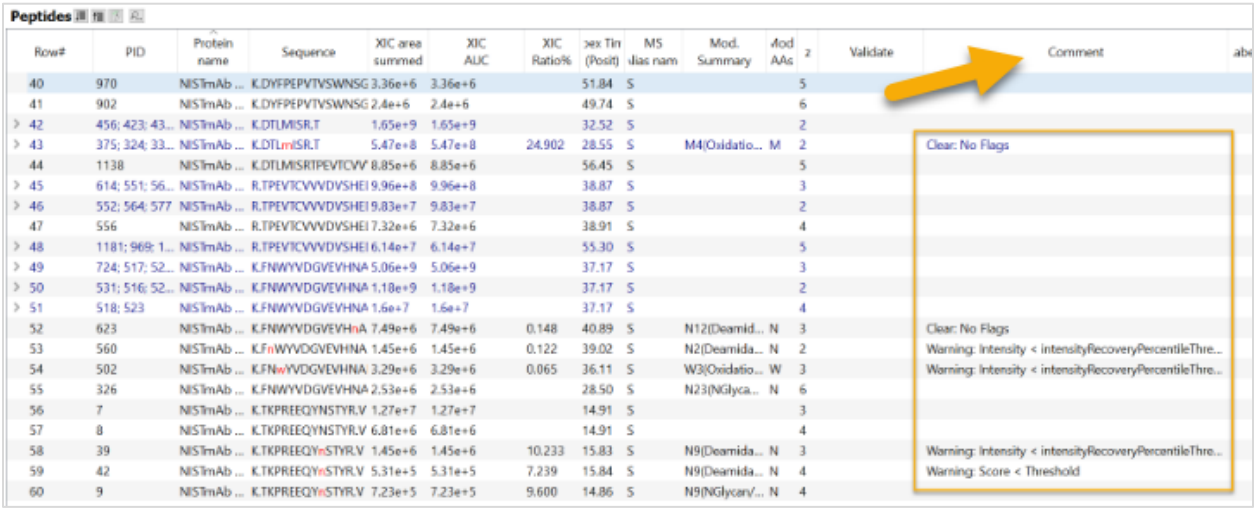


Figure 60: Peptide table Comments apply the Mod and SVA Validators

Mod Validator

Allows a user to assess the validity of a degradation analysis automatically. When this feature is activated, the algorithm will iterate row by row and assess whether the modification meets or fails certain thresholds (as explained below). Please note that this algorithm does not assess sequence variants. The SVA Validator algorithm is specifically designed for sequence variants (detailed directly after this).

The feature can be activated in the advanced commands in Byologic / Byos as follows:

```
[Byologic]
ModValEnabled = true;
```

Threshold parameters that can be modulated are as follows (with default settings):

```
[ModValidatorOptions]
DeltaModScoreThreshold = 10.0;
ScoreThreshold = 225.0;
FwhmThreshold = -1;
IntensityRecoveryPercentileThreshold = 0.05;
```

Mod Validator Option Parameter	Description
DeltaModScoreThreshold	The acceptable threshold of the delta mod score. If a degradation score falls below the set threshold, a warning is added to the Comments field to alert the user.

ScoreThreshold	Same as deltaModScoreThreshold except it will alert if a Score is below the threshold.
FwhmThreshold	The FWHM is used as the minimum retention time difference that categorizes a modification as not co-eluting with its wildtype. A setting of -1 determines the average fwhm of the input files and then automatically calculates the threshold. To set this manually, use any real number greater than 0. For example, if this is set to 0.05, this means that if a degradation has a retention time difference from its wildtype of more than 0.05 minutes, the user will be alerted in the Comments field.
IntensityRecoveryPercentileThreshold	This will alert the user if the apex intensity of the degradation lies within the bottom x percentile. For example, if this is set to 0.05, then whenever the intensity of a peak is in the bottom 5%, a warning will be added to the Comments field.

The Comments Field in the Peptide window will be filled out for various rows. Please note that this field sorts with no comments first, so the user may have to scroll to the end of the peptide window to find the labels.

SVA Validator

Allows a user to assess the validity of sequence variants automatically. When this feature is activated, the algorithm will iterate row by row and assess whether the sequence variant meets or fails certain thresholds as explained below.

Reference: Zhongqi Zhang, et al, *Biochemistry*, 2013, 52, 45, 8165-8176.

The feature can be activated in the advanced commands in Byologic / Byos as follows:

```
[Byologic]
SvaEnabled = true


[SvaOptions]
DeltaModScoreThreshold = 10.0;
PercentXICRatioThreshold = 1.0;
ScoreThreshold = 225.0;
FwhmThreshold = -1;
PredictedTimeDiffThreshold = 5.0;
CommonModLookBothWaysFromModResidue = 3;
```

SVA Validator Option Parameter	Description
DeltaModScoreThreshold	See Mod Validator above
PercentXICRatioThreshold	The acceptable threshold of the XIC Percentage Ratio. If the ratio falls below the set threshold, a warning is added to the Comments field. Depending on how the *.rptc file is configured, the XIC Ratio % value may not match the quantification in the reports since the reports allow multiple kinds of quantifications (one charge state, multiple charge states, etc.).
ScoreThreshold	See Mod Validator above
FwhmThreshold	The FWHM is used as the minimum retention time difference that categorizes a modification as not co-eluting with its

	wildtype. A setting of -1 determines the average fwhm * 2 of the input files and then automatically calculates the threshold. This is the recommended setting for this parameter (feedback is welcomed). However, this can be manually set to any positive value. This is not recommended.
PredictedTimeDiffThreshold	This threshold alerts the user if the predicted retention time in minutes for the sequence variant exceeds the set threshold. A “Critical” label is displayed in the Comments field.
CommonModLookBothWaysFromModResidue	The count of residues on either side of the putative site of a sequence variance to search across for a more common modification that better explains the results. For example, consider the wildtype peptide AAVLAMK, when this parameter is set to 3. Then, a putative sequence variant of AAdLAMK (V->D) is proposed with a delta modification of ~16 Daltons. The program searches the 3 amino acid residues to the left and 3 to the right of this site for a more common modification (such as methionine oxidation) with the +16 Da shift. In this case, there is a methionine residue 3 residues to the right of the putative sequence variant, so a “Warning” label is displayed in the Comments field.

Now the Comments Field in the Peptide window will be filled out for various rows. Please note that this field sorts with no comments first, so the user may have to scroll to the end of the peptide window to find the labels.

- Filtering – “Var. Pos. Protein” and “Var. Pos. Peptide”

  The values in "Var. Pos. Protein" and "Var. Pos. Peptide" columns during filtering are treated as a list of integers. For these columns only the list of integers (integers separated by a comma) is acceptable when entering filter conditions in the dialog.

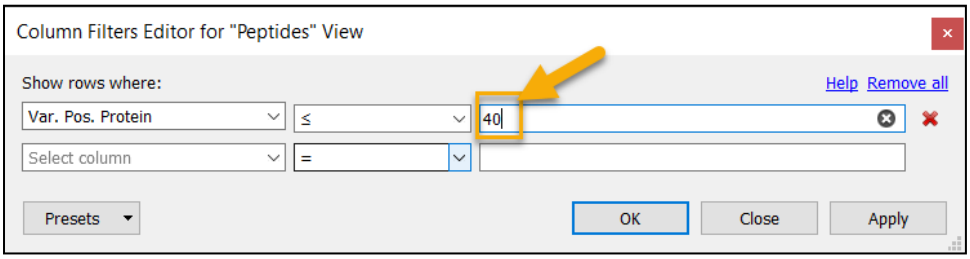


Figure 61: Filter by Var. Pos. Protein and Peptide

If a non-integer value is entered, the user will receive an error message to correct the value entered, as shown below.

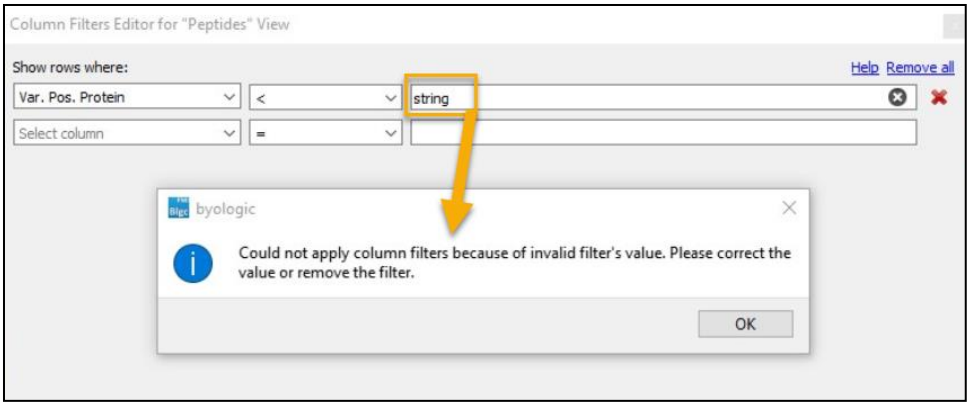


Figure 62: Var. Pos filtering requires integers

Wildtype Peptides Table and Menu

The **Wildtype peptides** table contains detailed information about naturally occurring or wildtype peptides associated with the peptide selected in the Peptides table:

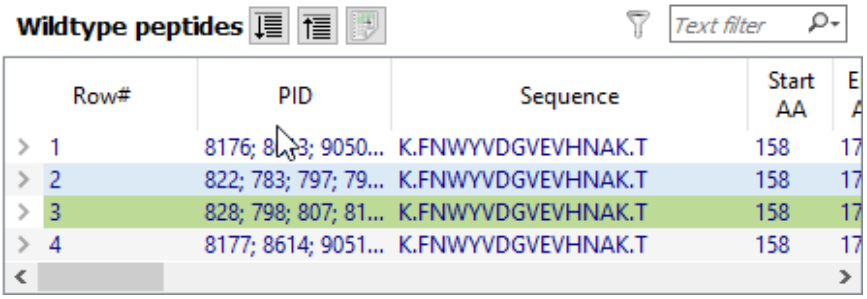


Figure 63: Wildtype Peptides table

The Wildtype peptides table has one context menu item specific to wildtype peptides. The menu is revealed by a right-click on a wildtype peptide cell in the table. **Set primary wildtype** marks that record as the primary wildtype peptide for the selected record in the Peptides table.

Plot Menus

The menu bars at the top or sides of the three plot views, XIC, MS2 and mass error, and Isotope, share icons that support generic operations on plots. These icons appear in almost all Protein Metrics, Inc. software products.

- The icon resets the plot to default zoom level. Shortcut = double left-click.
- The icon enables zooming in. The cursor changes to this icon. Drag across the range of the plot to display to zoom to that x-range. By default, the plot's y-range scales according to the maximum y-value within the x-range, but the software also supports freeform zooming as enabled under the icon.
- The icon enables zooming out. Click anywhere in the plot to zoom out.
- The icon enables moving (panning) across the plot. The cursor changes to this icon. Click the plot and drag up or down, right or left to view a part of the plot that is off-screen.

- The icon locks the x-axis for stacked plots in a plot view to use the same ranges. Unselecting this allows stacked plots to apply independent x-axis ranges.
- The icon performs an undo of the last zoom step. Shortcut = Shift-left arrow key.
- The icon performs a redo of the last zoom step. Shortcut = Shift-right arrow key.
- The icon manages how plots are displayed (render options), as well as zoom modes. The Render and zoom options include:

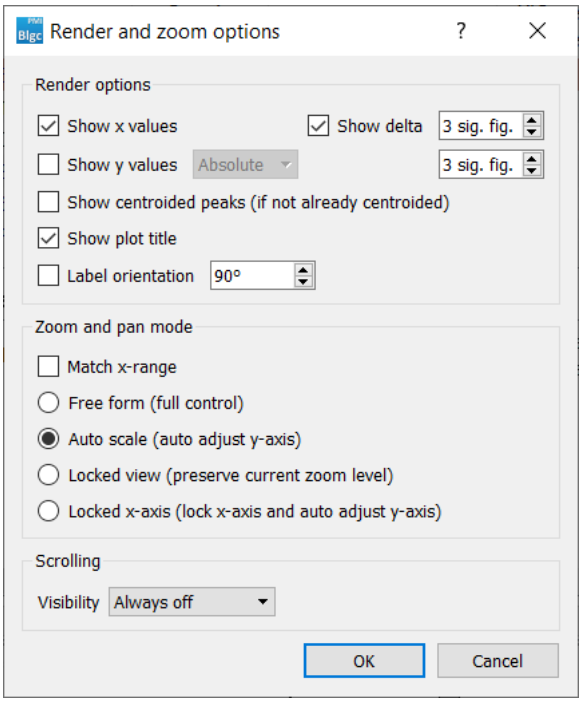


Figure 64: Render and zoom options

  - **Show x values** and **Show y values** display the x- or y-coordinates beside plot peaks to the chosen number of significant figures.
  - **Show delta**: when checked, displays the x or y differences from a reference peak when the cursor hovers over a peak tip.
  - **Show centroided peaks** darkens the peak positions that are determined to be centroided.
  - **Show plot title** displays the title of the plot, as generated from field values.
  - **Label orientation**, when checked, orients labels to some angle other than horizontal.
  - **Show XIC proposals** (XIC only) displays the proposed XIC plot splits generated by the **Edit proposed XIC splits** context menu in the Peptides table. The plot splitting is represented graphically as gray rectangles showing the edited integration limits:

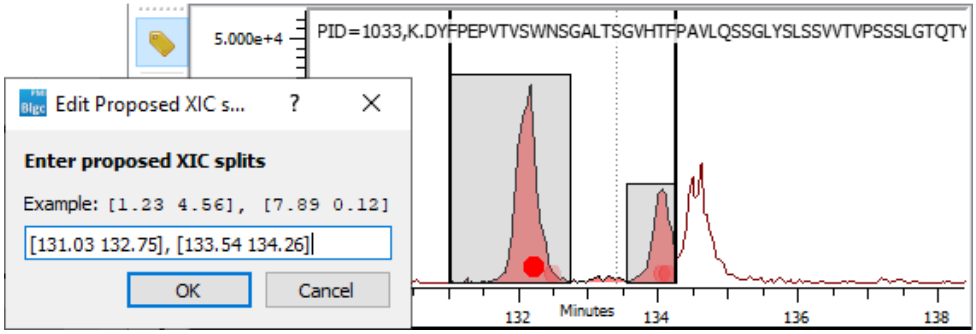


Figure 65: Edit proposed XIC splits

The **Zoom and pan mode** options include:

- **Match x-range**, when checked and followed by reset zoom (click on the icon) the peptide and wildtype peptide plots are redrawn using the same x-axis values.
- **Free form** mode to manually select the desired y range as well as x range
- **Auto scale** mode to select only the x range (the y range is then adjusted to the value of the highest peak)
- **Locked view** mode to keep the current x range (for either *m/z* and/or m) when moving between elution peaks. In Isotope plots
- **Locked x-axis** mode turns off autoscaling for the x-axis (but not the y-axis) and applies the current x-axis scale across all Peptide table selections

Under **Scrolling**, the **Visibility** setting controls display of the scroll bars:

- **Always on** displays scroll bars even when the full plot is shown
- **Always off** turns off display of the scroll bars even when a partial plot is shown
- **Show as needed** displays scroll bars only when a partial plot is shown

Plot Right-Click Menus

The XIC, MS2 and mass error, and Isotope plots have a variety of context menus for plot styling and exporting revealed by a right-click on the plots:

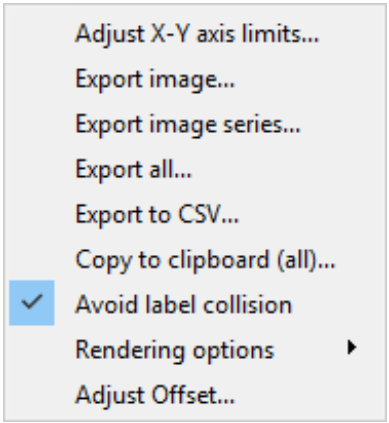


Figure 66: Plot right-click menus

Figures exported can be useful for reports, publications, regulatory filings, or internal communications. There are many options for rendering and exporting so that the user has much freedom to prepare a plot or figure style as needed.

- **Adjust X-Y axis limits** opens a dialog for the user to manually edit the plots x and y maxima and minima. This is a less convenient but more precise (and reproducible) alternative to the  and  icons.
- **Export image** allows the user to save the plot as a *.pdf or *.png file. It opens the **Plot Exporting Settings** dialog, which controls image size, file name and folder, and x and y minima and maxima:

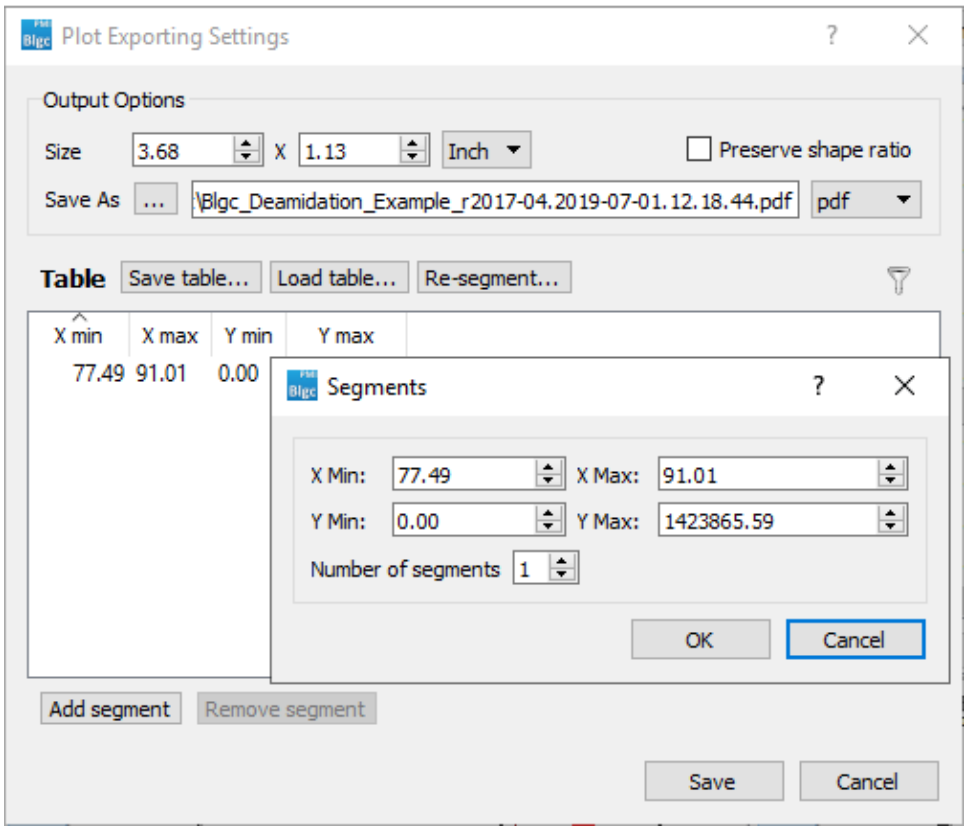


Figure 67: Plot export settings

Sometimes a user wishes to display a wide range across the x-axis and yet retain significant detail. This can be done by breaking up the plot into a series of panels. The **Add segment** button creates segments with user-defined x and y values. A series of these segments eliminates unneeded portions of the plot and increases the effective detail in the image. This is also a useful function for automated reporting. The **Re-segment** button allows edits to the reported x and y minima and maxima for existing segments

- **Export image series** also opens the Plot Exporting Settings dialog, except that the segments are prepopulated with six equal sized segments. This simplifies the edits of the segments. The **Add segment** and **Remove segment** buttons control the number of segments that divide up the plot.
- **Export all** also opens the Plot Exporting Settings dialog, except that the wildtype peptide plots are exported along with the peptide plots. Segment editing functions are disabled for this option.
- **Export to CSV** exports the plot trace x-y points to a *.csv file.

- **Copy to clipboard (all)** enables pasting the plot image into another application.
- **Avoid label collision** staggers the label text to reduce overlap.
- **Rendering options** controls how a plot is displayed in the application. It includes several sub-menus:

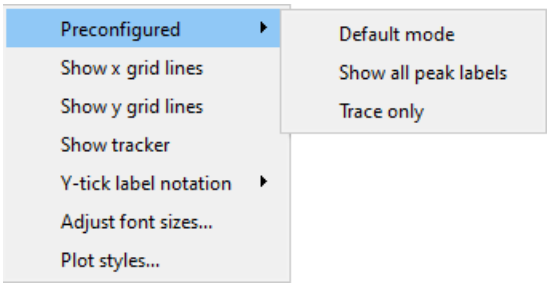


Figure 68: Rendering options menu

  - **Preconfigured** sub-menus control what is displayed in the plots. **Default mode** displays the trace, peak labels and plot title. **Show all peak labels** does exactly that. **Trace only** turns off the peak labels and plot title.
  - **Show x grid lines** turns on and off the x grid lines.
  - **Show y grid lines** turns on and off the y grid lines.
  - **Show tracker** displays a vertical dotted line that follows the cursor when moved in the plot. This allows a more precise determination of the X and Y positions of the cursor.
  - **Y-tick label notation** toggles between y-axis notation options of absolute amounts or as percentages of the highest peak.
  - **Adjust font sizes** controls the font size for each type of text on the plot.
  - **Plot styles** allows changes to the graphic styling of the plot, including trace width, axis width, and total *m/z* dot colors to be used.
  - **First Layer** (XIC and Isotope plots only) reveals sub-menus to turn on and off the display of traces, integrations, and labels in the plots. The show/hide all sub-menu turns all on or off.

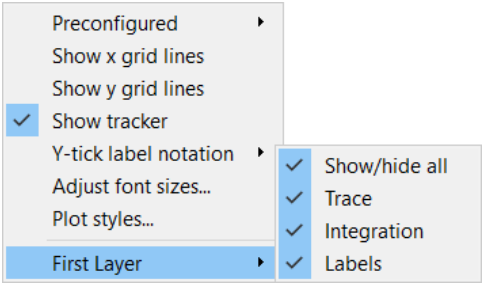


Figure 69: Rendering options > First Layer menu

The **XIC plots** view displays chromatograms for specific regions of the peptides and wildtype peptides associated with the record selected in the Peptides table:

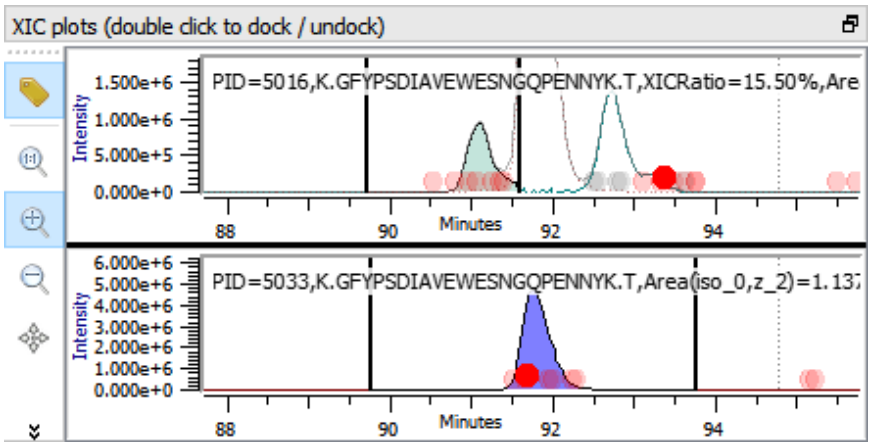


Figure 70: XIC Plots

In addition to the menu icon functions shared by all the plot views, there are some functions specific to XIC plots:

- The icon turns plot annotations on or off. In the XIC plots, annotations are displayed as circles at the base of the chromatograms. Dark red circles represent the position of currently displayed MS2 scans. Light red circles represent the position of other MS2 scans with the same identification. Light gray circles represent the position of MS2 scans with the same precursor mass as the selected peptide, but with different attribution. Hover over the circles and the associated sequence ID and value are displayed. Note that the cursor changes to a hand. Click the circle and the corresponding Peptides table row is selected and the views are updated to display for that record.
- The icon is used to compute the XIC plot integration for a specified time range. A dialog opens to enter start and end times.
- The icon enables MS scan extraction. This displays isotope values derived from the XIC plot. A blue vertical line appears in the XIC plots view. As the line is dragged across the XIC trace, the Isotope plots view is updated to show the MS scan for that XIC time value. Two new icons appear on the Isotope plots menu bar in this mode: The icon clears the extracted MS scan and restores the original isotope plots. The icon saves the extracted MS scan to the project document.

The XIC plots view has one specific context menu item revealed by a right-click in the plot:

- **Adjust Offset** sets the x-axis offset between multiple plots as percentage of the highest peak when the top-level menu Window > Enable Stacked Plots is checked.

MS2 and Mass Error Plots and Menu

The **MS2 and mass error plots** display plots of the MS2 peptide fragments with the corresponding m/z mass error table:

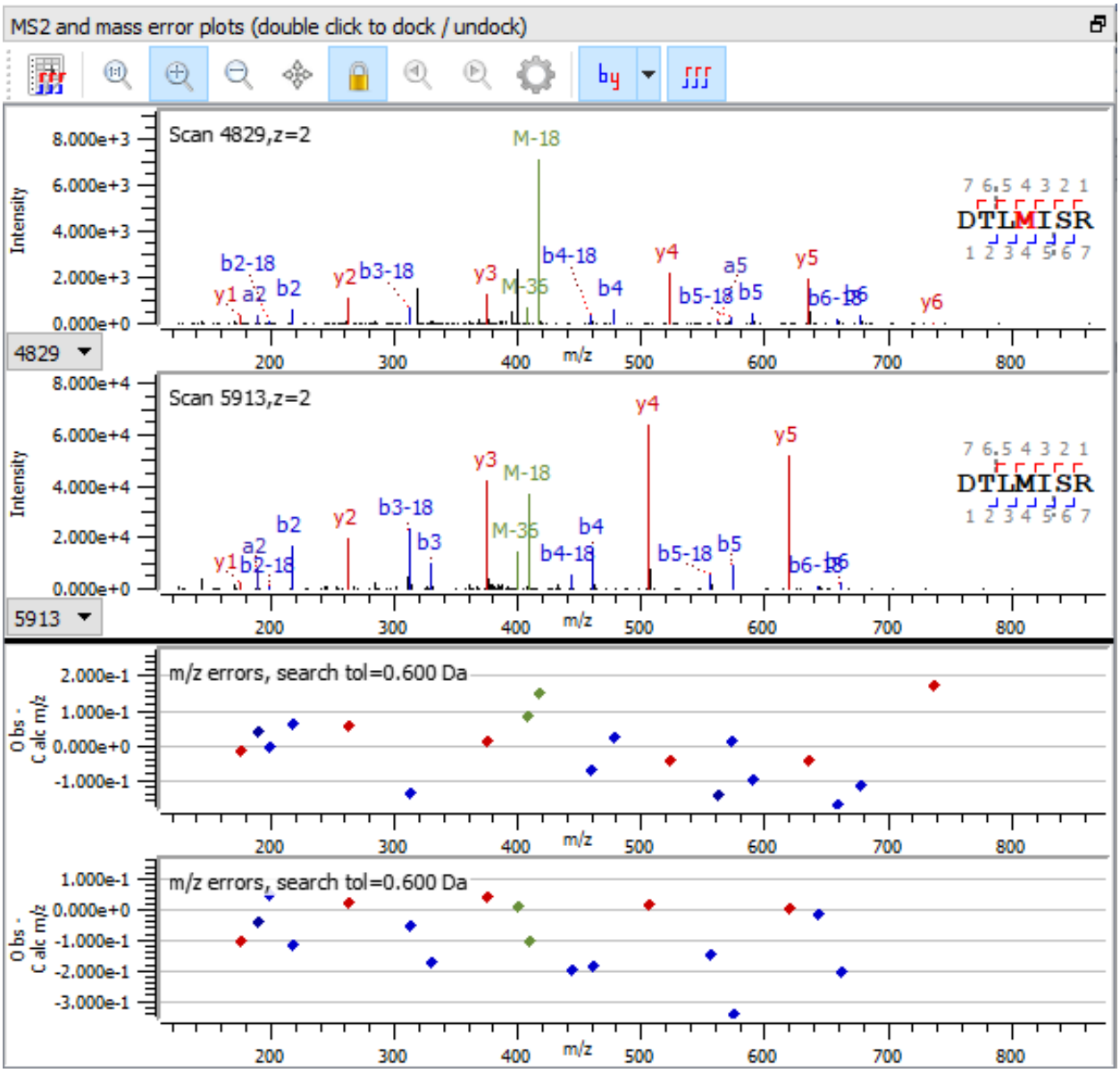
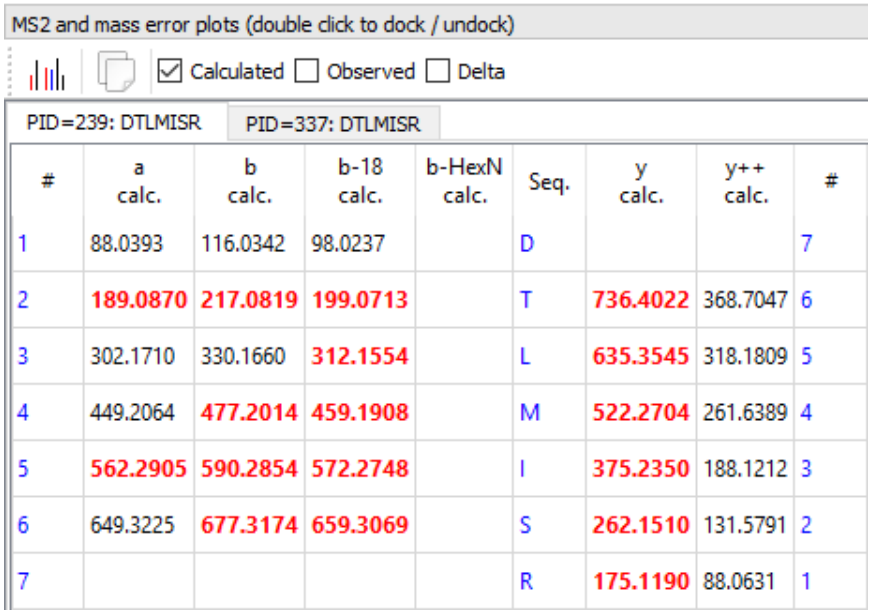


Figure 71: MS2 and mass error plots

The MS2 plots are identified by Scan ID. The plots are colored blue for N-terminus b ions and red for C-terminus y ions. Other fragments, including c and z ions are colored green

In addition to the menu icon functions shared by all the plot views, there are some functions specific to MS2 and mass error plots:

- The  icon switches between the default plot view and the mass table view:



#	a calc.	b calc.	b-18 calc.	b-HexN calc.	Seq.	y calc.	y++ calc.	#
1	88.0393	116.0342	98.0237		D			7
2	**189.0870**	**217.0819**	**199.0713**		T	**736.4022**	368.7047	6
3	302.1710	330.1660	**312.1554**		L	**635.3545**	318.1809	5
4	449.2064	**477.2014**	**459.1908**		M	**522.2704**	261.6389	4
5	**562.2905**	**590.2854**	**572.2748**		I	**375.2350**	188.1212	3
6	649.3225	**677.3174**	**659.3069**		S	**262.1510**	131.5791	2
7					R	**175.1190**	88.0631	1

Figure 72: MS2 and mass error plots: mass table view

The reported fragment data that was confirmed in the analysis is displayed in red bold font. Unconfirmed fragment data is displayed in black font. In this view, the icon switches the view back to MS2 plots. The icon copies the content of this table to the clipboard.

- The icon switches between showing and hiding the b and y ion labels. The icon is accompanied by a drop-down arrow that reveals three sub-menus:
  - **Open annotation options** opens a dialog that allows the user to set options for determining the assignment of the MS2 annotations:

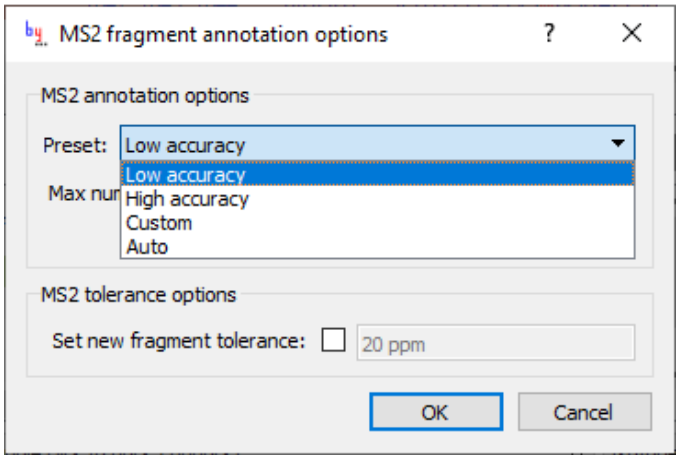


Figure 73: MS2 fragment annotation options

The maximum number of peaks per 100 m/z and MS2 mass-accuracy tolerance options can use presets for Low accuracy, High accuracy, or Custom, in which the user sets these values. The dialog defaults to the Auto preset, in which the software determines the MS2 fragment annotation settings.

  - **Remove fragment coverage cache** clears the existing cache of detected fragments used for the protein fragment coverage rendering.

- **Export fragment to CSV**, exports the fragment sequence to a *.csv file.

- The icon turns on or off the fragment sequences displayed to the right of the plots.

The MS2 and mass error plots view has two specific context menu item revealed by a right-click in the plot:

- **Apply peak filtering** opens a dialog that allows the filtering of peaks by an entered signal-to-noise ratio value.

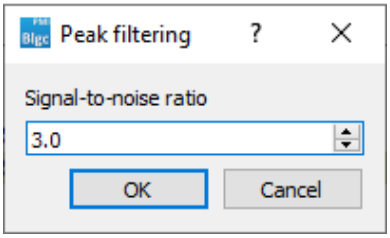


Figure 74: Peak filtering by signal-to-noise ratio

- **Combine MS2 spectra** (MS2 and mass error plots only) sums the MS2 Plots for all the peptides for a selected row in the Peptides table. The title of the plot updates to show the counts of the peptide scans in the combined MS2 plot:

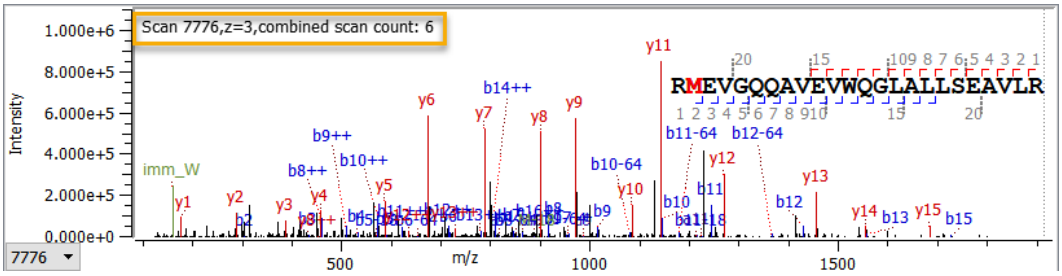


Figure 75: MS2 plots after Combine MS2 spectra is applied

Isotope Plots and Menu

The **Isotopes plots** display the peptide isotope (m/z scan) for the selected Peptides table record:

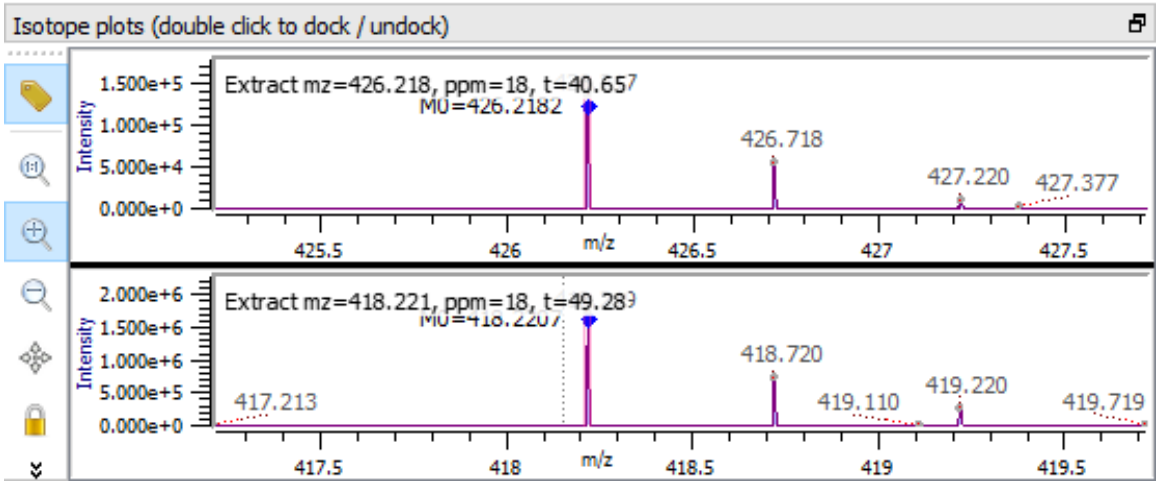


Figure 76: Isotope plots

In addition to the menu icon functions shared by all the plot views, there are some functions specific to XIC plots:

- The  Show/hide annotations icon turns on and off the display of the molecular weight for the primary peptide beside a blue dot that identifies the peak with that calculated value.