

PMI Intact Analysis

(Intact Mass™)

User's Manual

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Intact Analysis User's Manual

Overview

Intact Analysis (Intact Mass™) software by Protein Metrics analyzes mass spectra of intact (undigested) proteins. Features include:

- Support for all major mass spectrometry instruments and vendors
- Automatic or manual chromatographic time windows
- Deconvolution of charge states to transform m/z spectra to neutral mass spectra
- Automatic mass peak picking and intensity calculations
- Side-by-side comparison of multiple samples
- Reporting of summary data and figures

Introduction

An intact mass spectrum of a protein sample provides a direct test of protein sequence, truncation, extension, glycosylation, and purity. For example, Figure 2 is a deconvolved mass spectrum from the m/z plots shown in Figure 1. The neutral mass peaks in a deconvolved mass spectrum represent protein forms ("`proteoforms`") in the sample.

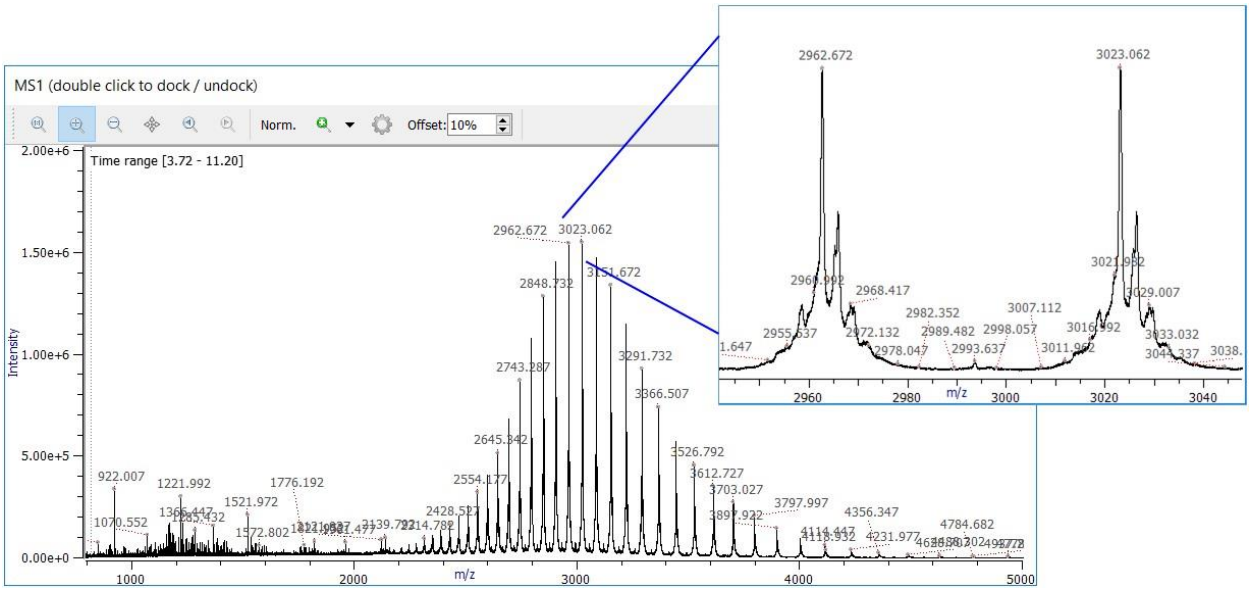


Figure 1: **Summed MS1 scans of Adalimumab**. The peak cluster around *m/z* 2963 shows the protein with charge z=50+ and the cluster around *m/z* 3023 shows charge z=49+.

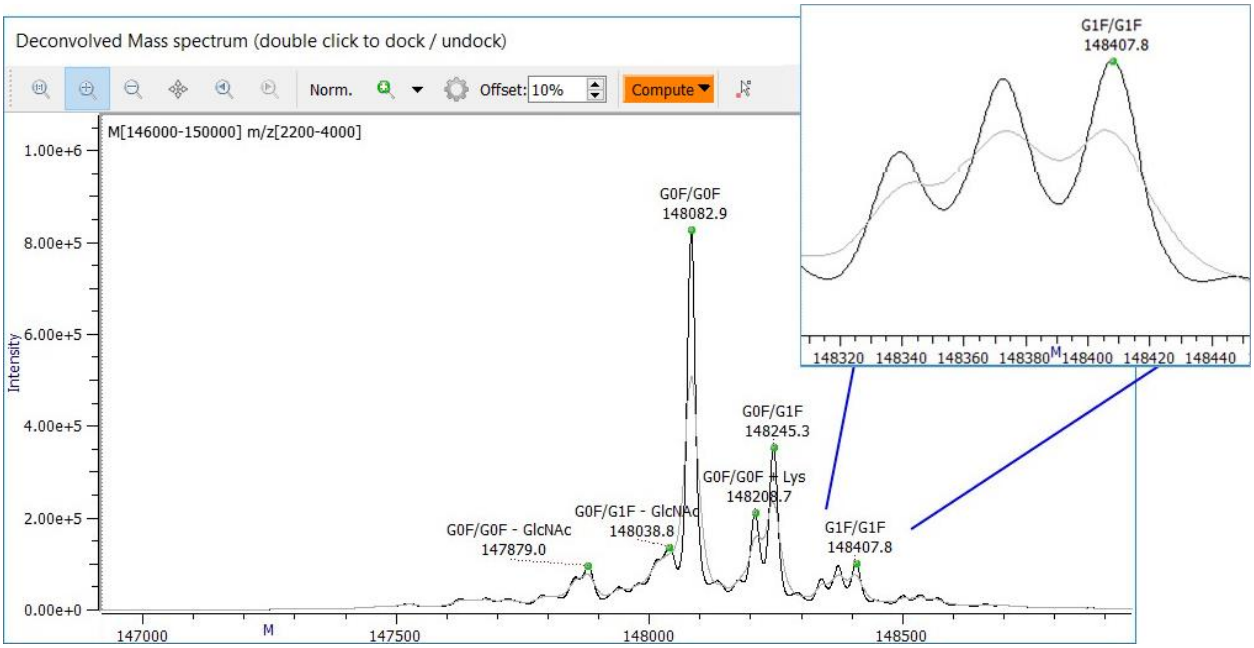


Figure 2: Intact mass spectrum of Adalimumab. The neutral mass spectrum is computed by "deconvolving" the charge states from the summed m/z spectrum in Figure 1. Each peak in an intact mass spectrum represents a "proteoform". For example, the largest peak at 148,083 is Adalimumab with two G0F glycans, and has calculated average isotopic mass of 148,084 Da. The small peak at 146,637 represents the mAb missing one glycan. The inset shows the neutral mass spectrum before (gray) and after (black) peak sharpening.

Intact Analysis typically employs a short (10 to 20-minute) liquid chromatography (LC) gradient. LC helps separate proteins (for example, the heavy and light chains of a reduced mAb), and even in the case that the sample contains only a single protein, LC gives cleaner spectra than does direct infusion.

Protein Metrics Intact Analysis software first divides the LC profile into trace peaks. After reviewing the summed *m/z* spectra, the user can adjust the trace peak chromatogram to add, remove, split, or merge trace peaks. The *m/z* range and output mass range can be set for each trace peak. Alternatively, the software's default settings can be applied over the full range of *m/z* from 600 to 9000 and all masses between 10,000 to 160,000 Da. The peak-picking algorithm finds the most intense mass peaks in the deconvolved mass spectrum and reports these in the Masses table. Mass peaks can be added, removed, and annotated. For example, in the spectrum in Figure 2, the mass peak 148,039 Da may be considered indistinct and then manually removed. Similarly, the small peak at 148,373 Da (middle peak in the inset) may be added and annotated as "`G0F/G1F + Lys`". A mass peak can be evaluated by selecting the mass peak and inspecting the corresponding *m/z* peaks, as shown in Figure 3.

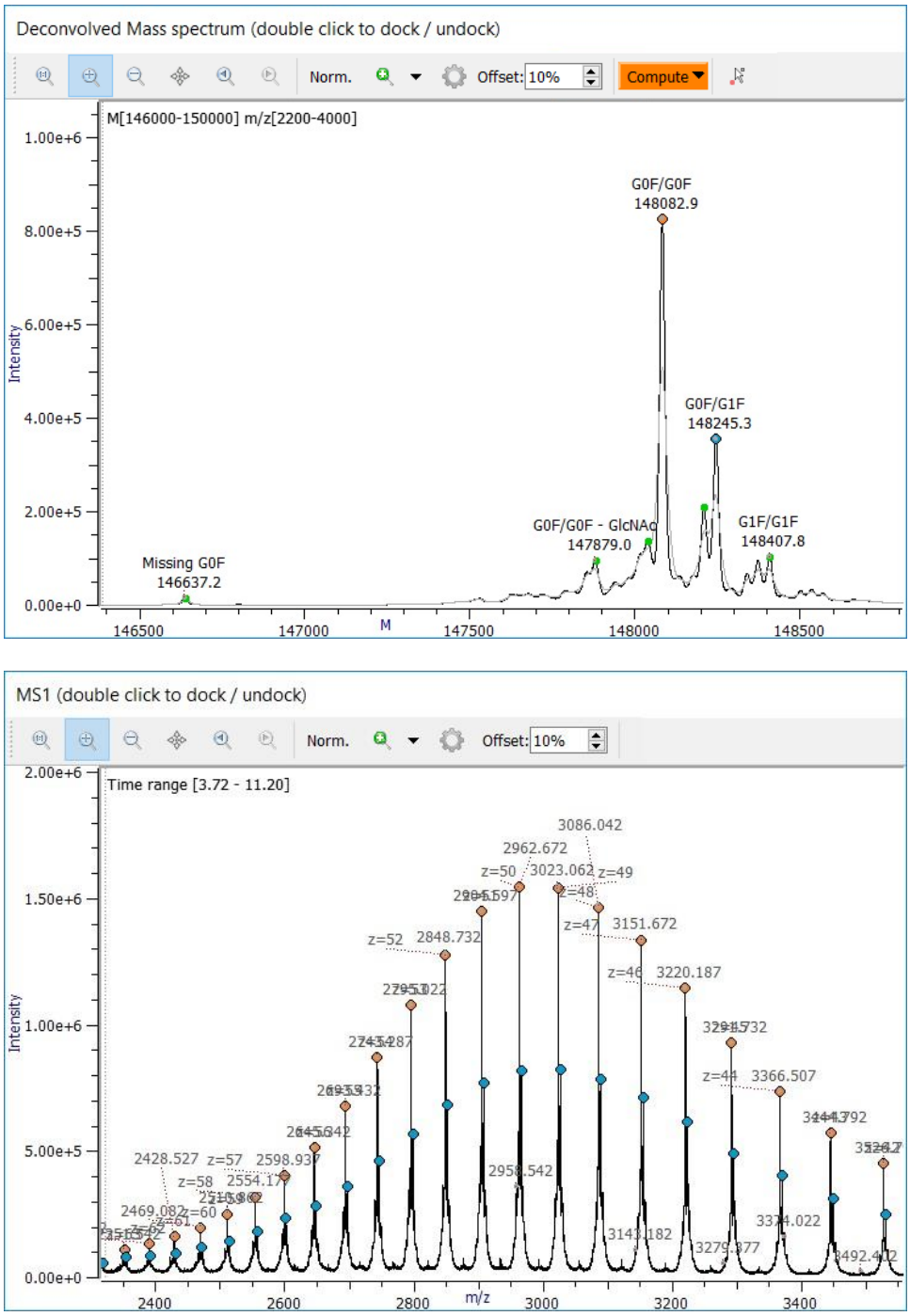


Figure 3: Dots connect neutral masses to m/z peaks. The Intact Analysis software provides interactive views of the data; selecting a mass peak in the spectrum or Masses table colors the m/z bins that combine to give that mass peak. A smooth sequence of m/z dots is a sign of correctness, while an up and down pattern is a sign of an artifact.

Protein Metrics' Intact Analysis software relies on a new "parsimonious" charge deconvolution algorithm ; this algorithm makes no assumptions about peak shapes and requires separate evidence for each mass peak, thereby reducing "harmonic" and "off-by-one" artifacts, which result from assigning a number of charges to the *m/z* peak. The standard MaxEnt algorithm uses peak shape models, which can be helpful in the common cases but detrimental in the less common cases and tends to split

peaks in order to increase the entropy of the deconvolved spectrum. With its default settings, Intact Analysis's deconvolved mass spectra closely reproduce the peak shapes of the *m/z* spectrum. As seen in Figure 2, Intact Analysis also allows peak sharpening or super-resolution of mass peaks by deconvolving a parametrically defined point spread function.

Tour of Intact Analysis Views

The Intact Analysis software user interface (UI) shown in Figure 4 has seven views:

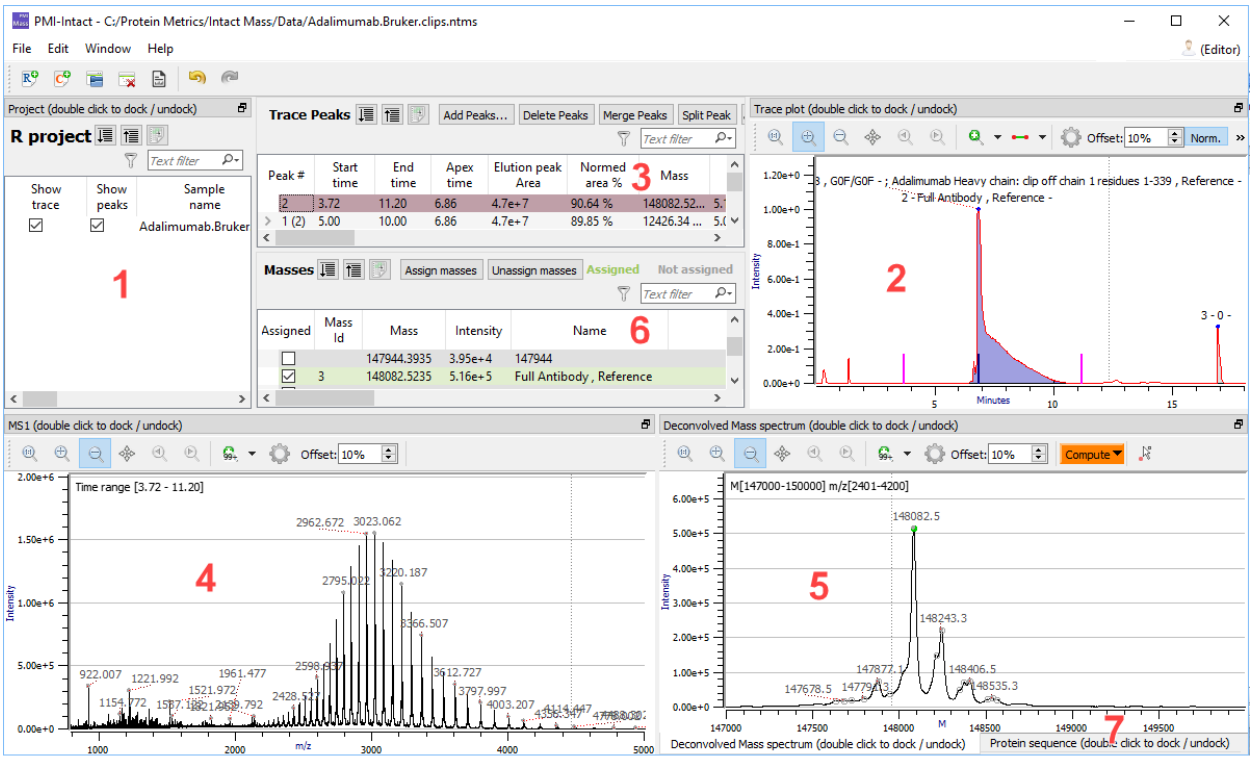


Figure 4: **Seven Intact Analysis views**: (1) Project table with input MS files, (2) Trace plot chromatogram with selected peak, (3) Trace Peaks table, (4) MS1 spectrum (summed spectra), (5) Deconvolved mass spectrum, (6) Masses table, (7) Protein sequence table as a tab hidden behind the Deconvolved mass spectrum. Views can be rearranged, resized, docked, and undocked.

1. The **Project** view lists the data files in use. **R project** is a reference project, **M project** is a multi-sample project and **C project** is a comparison project. The **Show trace** and **Show peaks** check boxes can be used to turn on or off Trace plot and Trace Peak table records in Views 2 and 3.
2. The **Trace plot** shows the total ion chromatogram (TIC), base peak intensity (BPI), ultraviolet (UV) trace, or electropherogram (iCIEF). Electropherograms are read when using **the iCIEF-MS with Blaze from Intabio** workflow. When an electropherogram is loaded, radio buttons appear to enable a switch of the X-axis units from **Minutes** to **Isoelectric point** (pI). The chromatogram of a purified protein typically has a small number of trace peaks. In this example, there are two trace peaks, only one of which, the highlighted peak in the center, contains a strong signal for the intact biotherapeutic protein Adalimumab. The leading and trailing edges of a trace peak may contain different mixtures of "proteoforms".
3. The **Trace Peaks** table summarizes the Trace plot chromatogram or electropherogram. Selecting a row in the table selects a peak in View 2. Trace peaks can be merged, split, added, or deleted using the buttons at the top of View 2. Time limits can be adjusted by editing text in View 3 or dragging the magenta bars shown in View 2.

4. **MS1** shows the summed m/z plot for the chromatogram or electropherogram trace peak selected in Views 2 and 3. Except for summing individual scans, this spectrum is unprocessed.
5. The **Deconvolved Mass spectrum** is the primary output of the software. Neutral masses are plotted for the summed m/z plot (View 4) associated with the trace peak selected in Views 2 and 3. Hollow dots indicate picked peaks, the “candidate” masses that can be assigned for reporting. Once assigned, the dots become solid green and peaks become persistent. Outlined and colored dots show interactively selected peaks, connecting to the corresponding colored dots in the m/z plot.
6. The **Masses** table includes one row for each picked peak from View 5. The user can add, delete, or assign rows in the table. The name of a mass peak can be either its mass or an interpretation.
7. **Protein sequence** shows amino acid sequences optionally input into the software for automatic mass matching as shown in Figure 5. (Intact Analysis also accepts numerical average-isotope masses and includes customizable tables of likely mass deltas.)

ff

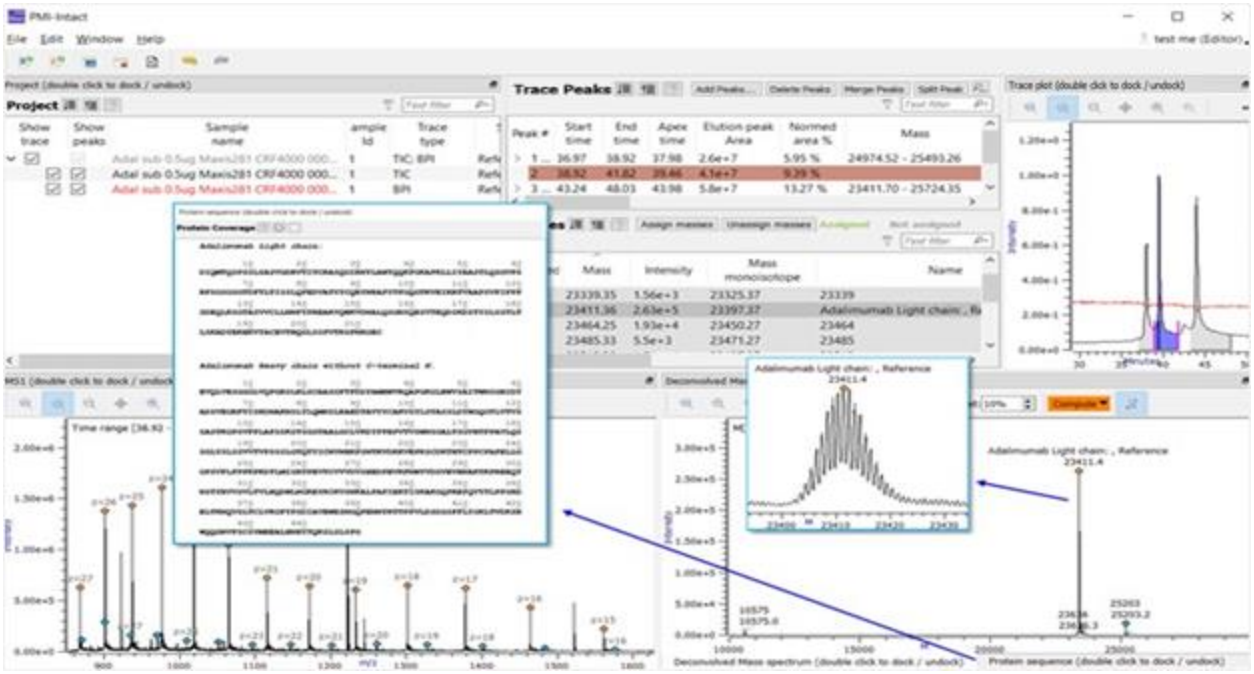


Figure 5: Protein sequence input. Intact Analysis accepts FASTA file input (inset), which it uses to compute average-isotope masses for automatic mass matching, such as the Adalimumab light chain shown here. The Trace plot view shows both BPI (red) and TIC (black). Here the BPI is flat due to the large peak at m/z 1222 from Agilent tuning mix. For isotope-resolved ions like the mAb subunits shown here, Intact Analysis can now show precise monoisotopic masses.

Creating a Reference Project

Intact Analysis can be used to analyze a single sample or to compare a number of related samples. Comparison of related samples starts with the analysis of a sample chosen to be the reference sample. To create a reference sample:

1. Open Intact Analysis.
2. Under the File menu, click “New Reference Project…”
3. Intact Analysis will open the New Reference project dialog.

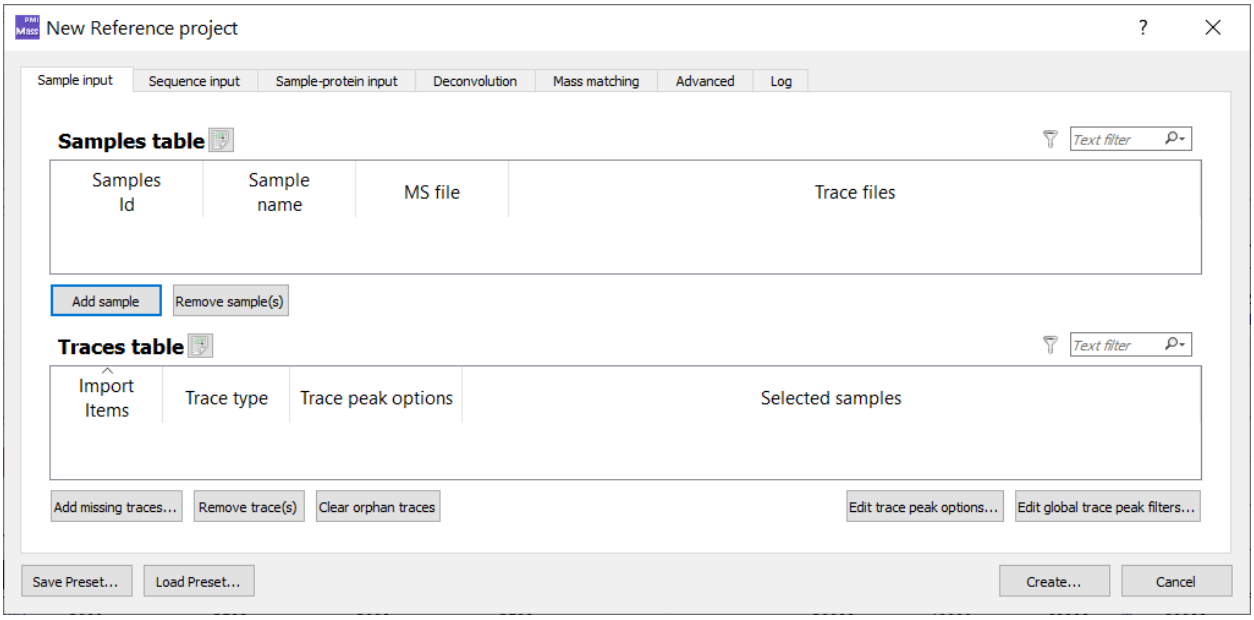


Figure 6: New Reference project

Sample input Tab

MS sample files are added in the **Sample** input tab. Intact Analysis accepts a variety of reference sample files (Bruker: *.d, Thermo: *.raw, Waters: *.raw, Sciex: *.wiff, Agilent: *.d). To enter a file, drag and drop an MS file into the project window. Alternatively, click **Add sample**, double-click in the **MS file** column, click ··· and browse to the sample file. Click **Open**.

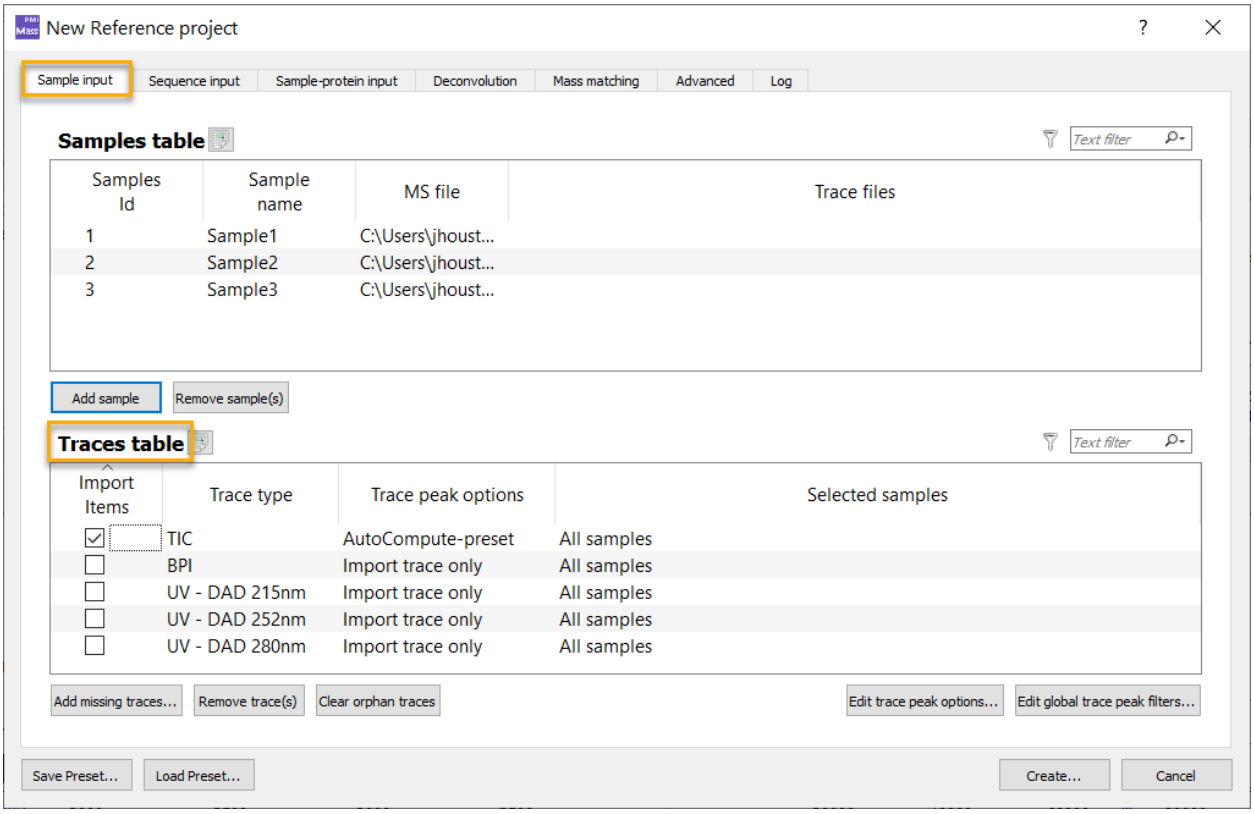


Figure 7: Adding samples and their traces in the Sample input tab

The sample is named for the file name; to change the sample name, click the default name twice and enter a new sample name. Click **Add sample** at left to add a new row to the **Samples table**. Then add the desired files as above. To remove an unwanted sample file row, select the row and click **Remove sample(s)**.

Starting with Intact Analysis v. 3.8, the MS file populates both the **Samples table** and the new **Traces table** at the bottom. Selected (not checked) traces can be removed by clicking **Remove trace(s)**. Removed traces can be restored by clicking **Add missing traces**, check the trace to restore and click **OK**. To associate specific traces to the samples, double-click the text in the Selected samples column, then click the ... button to open the **Edit samples selection** dialog:

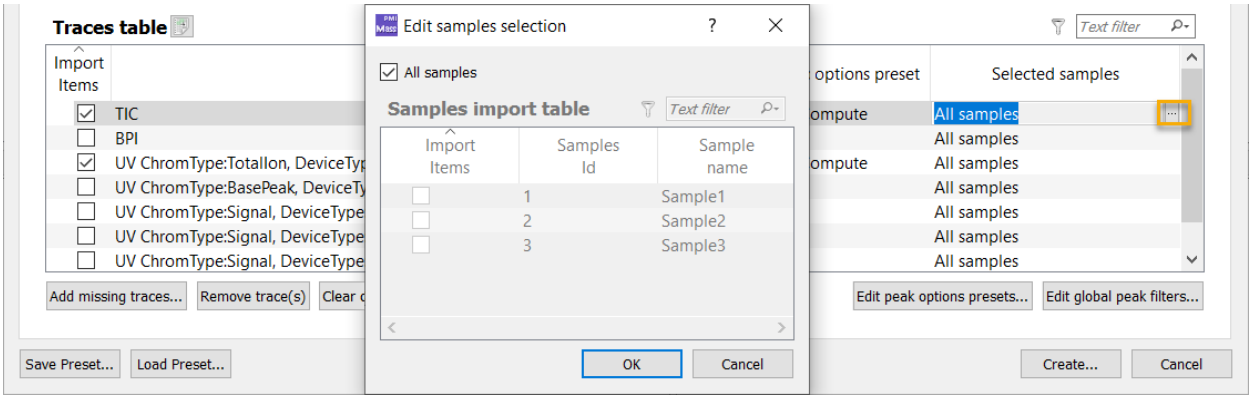


Figure 8: Edit samples selection dialog to associate traces with sample names

To associate the selected trace with an individual sample, uncheck **All samples**, check a single sample, and click **OK**. Note that multiple checked samples are not supported for traces.

Individual traces can be imported from sample file types supported in the MS file cell, as well as *.csv, *.txt files, and Intabio *.blaze. To import a trace from a file, drag the file into the **Traces files** column in the Samples table. Alternatively, double-click in the Samples table row under the Traces file column, click ... and browse to the sample file. Click **Open**. The trace file is added to the sample name and the trace is added to the Traces table:

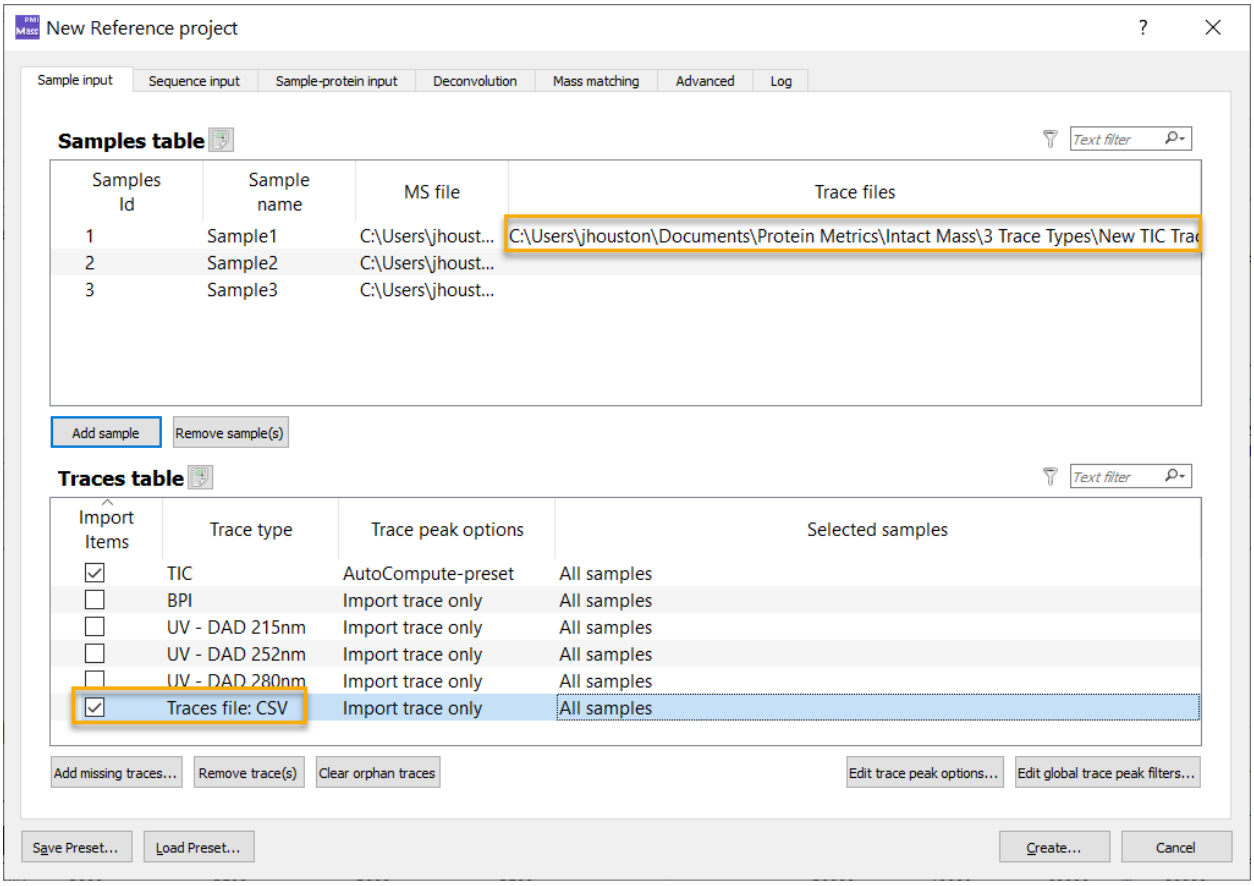


Figure 9: Traces imported from a *.csv file

Multiple traces can be added to a sample. To add additional trace files, click in the **Traces file** cell, click the ✚ icon and navigate to the new trace file, as before:

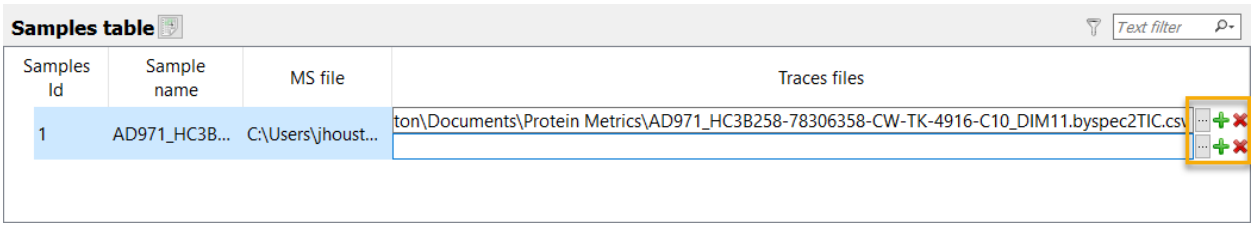


Figure 10: Adding multiple trace files to a sample

To remove a trace file, click in the **Traces file** cell to be removed and click the ✖ icon.

Trace peak options

To control how the trace peaks are processed, click **Edit peak options** at the bottom:

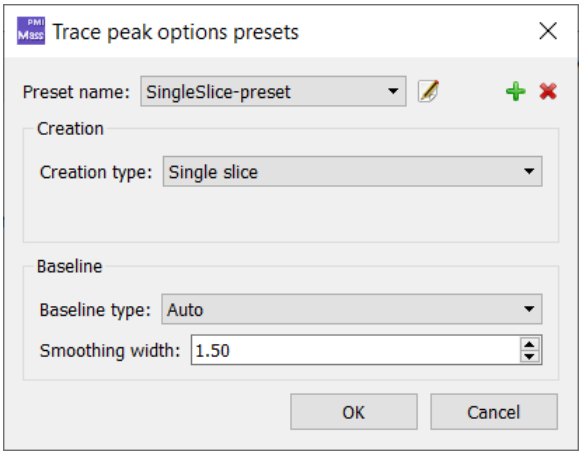


Figure 11: Trace peak options presets

At the bottom of the Trace peak options presets dialog are the Baseline parameters. The baseline type can be set to **Auto** or **Flat**. Baseline smoothing width is also set here. Larger smoothing factors will merge trace peaks while smaller values will split peaks.

The Trace peak options presets dialog contains five default peak processing presets:

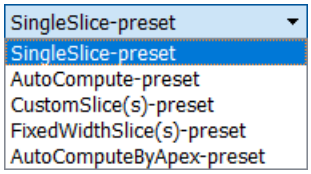


Figure 12: Default trace peak presets

Select a processing preset and edit the peak parameters. To create a custom preset, click the ✚ button, add a preset name, and click **OK**. Next, select a creation type and edit those peak parameters. The new preset is added to the list. Click the ✎ button to edit a selected preset name. Click the ✖ button to delete a selected preset.

The default and custom peak processing presets can use one of four **Creation types**:

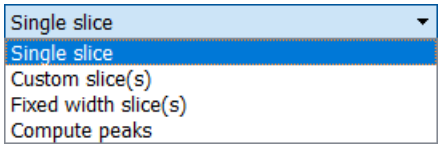


Figure 13: Trace peak Creation types

These Creation types use the following peak processing methods:

- **Single Slice** processes the entire trace as single peak.
- **Custom slice(s)** divides trace peaks by user defined start and end points:

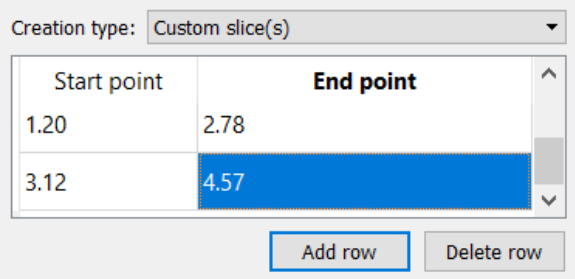


Figure 14: Trace peak Custom slice option

Click **Add row** and enter start and end points for each slice. To remove a slice, select it and click **Delete row**.

- **Fixed width slice(s)** divides trace peaks by regular intervals defined by the user:

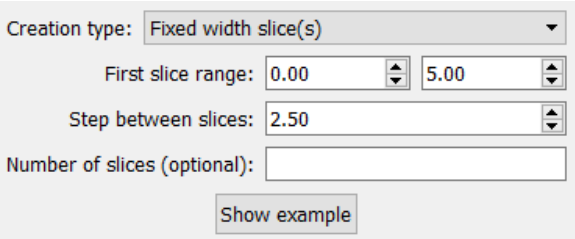
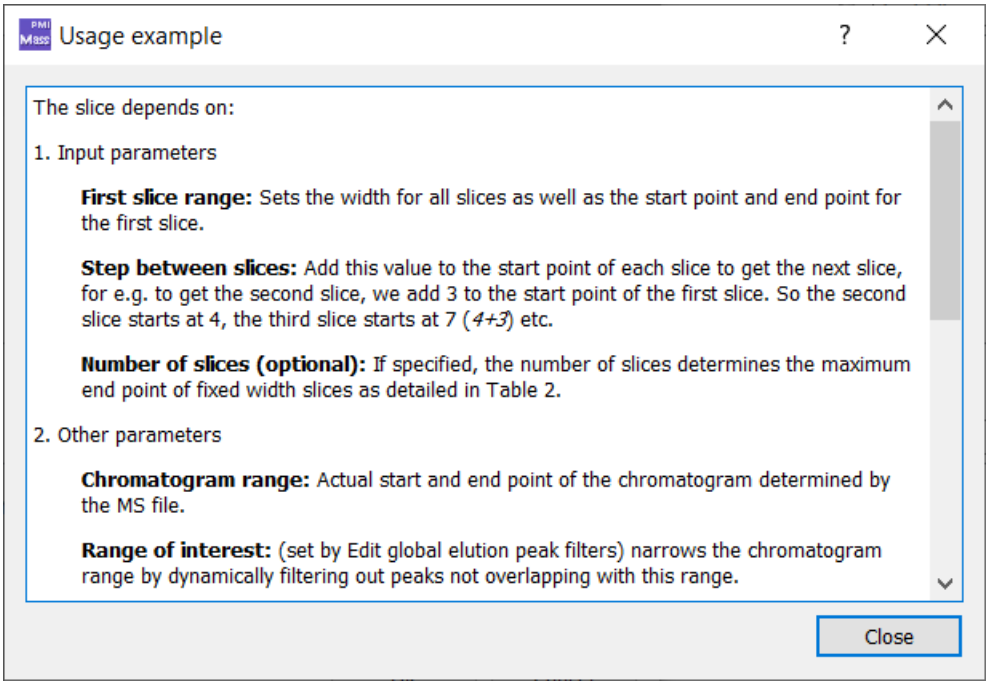


Figure 15: Trace peak Fixed width slice(s) option

- **First slice range** sets the start and end points for the first slice. This also sets the width for all slices.
- **Step between slices** is the delta that sets the start of subsequent fixed width slices by adding the step value to the start of the preceding slices. For example, the values above define the first slice as 0 - 5, the second slice as 2.5 – 7.5, etc., through the end of the trace.
- **Number of slices** sets the maximum number of defined slices. If blank, the last slice is determined by the end of the trace x-axis range.
- **Show Example** displays an example fixed width slice configuration with definitions and explanations:



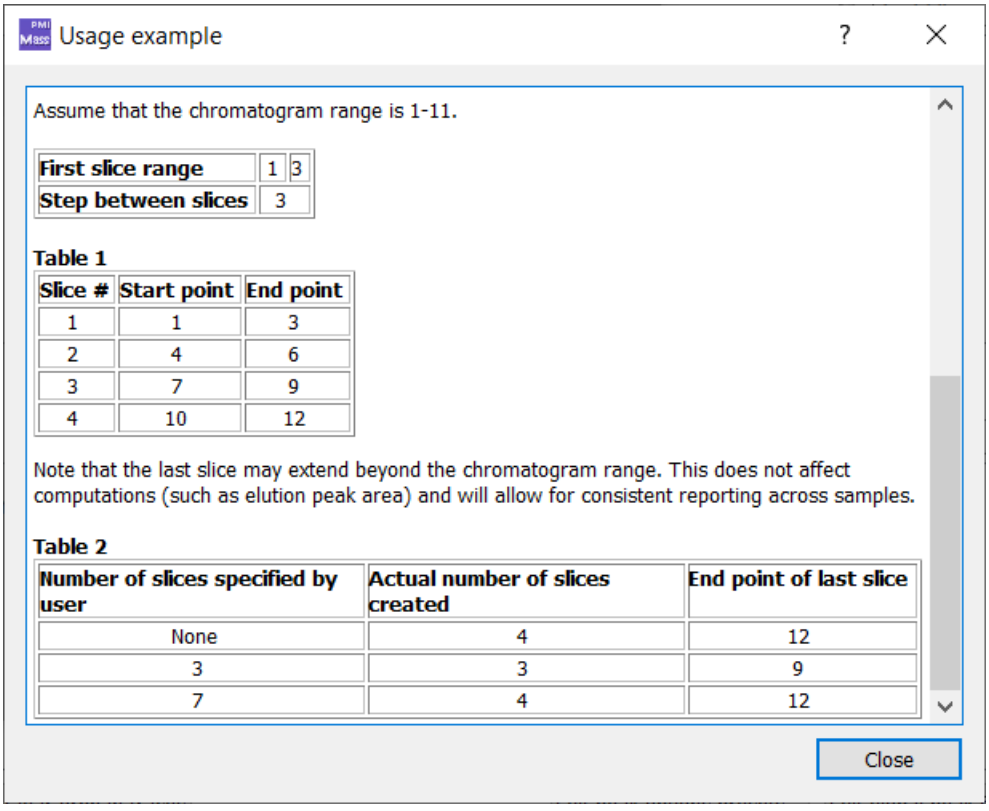


Figure 16: Fixed width slice Usage example

- **Compute peaks** automatically determines the trace peaks based on their minimum width, after smoothing:

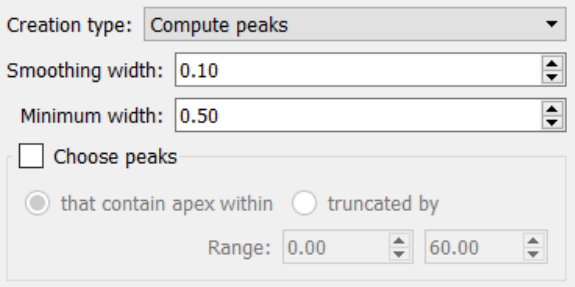


Figure 17: Trace peak Compute peaks option

Choose peaks, when checked, limits the detected peaks to the specified x-axis range. The option **that contain apex within** selects the whole peak of each apex within the specified range. The option **truncated by** trims the first and last peak to fit within the specified range.

Starting with Intact Analysis v. 3.8, individual trace filters are replaced with a single peak filter across all traces. To set peak property filters for all the traces, click **Edit Global trace peak filters**:

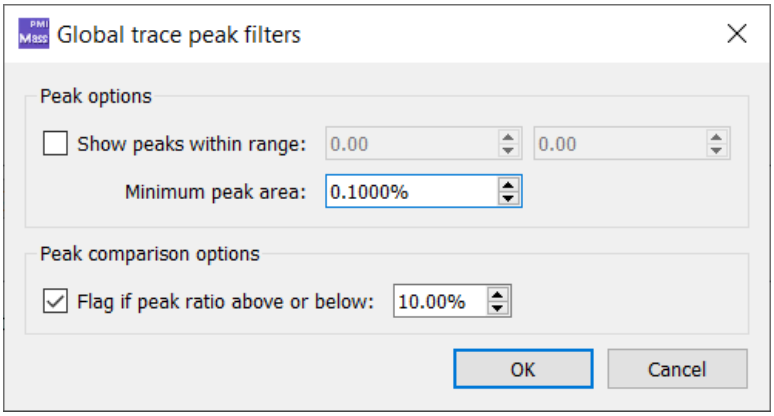


Figure 18: Global trace peak filters

Check **Show peaks within range** to manually set start and end points. **Minimum peak area** and a peak ratio threshold flag can be set in this dialog.

The Traces table and trace peak settings are backwards-compatible. Traces associated with samples in projects from software versions before 3.8 will correctly populate the Traces table in the current version. Trace peak settings and presets from versions before 3.8 will correctly migrate into the current version. However, Intact Analysis and Chromatogram Analysis projects created in versions 3.8 and above will not correctly open in older versions of these applications.

Sequence input tab

Next in the New Reference project menu is the **Sequence input** tab, which is now divided between two sub-tabs: **Sequences** and **Building blocks**. The **Sequences** sub-tab is further divided into three sections: **Chains**, **Sequence combinations**, and a section for clipped species and Mass computation options.

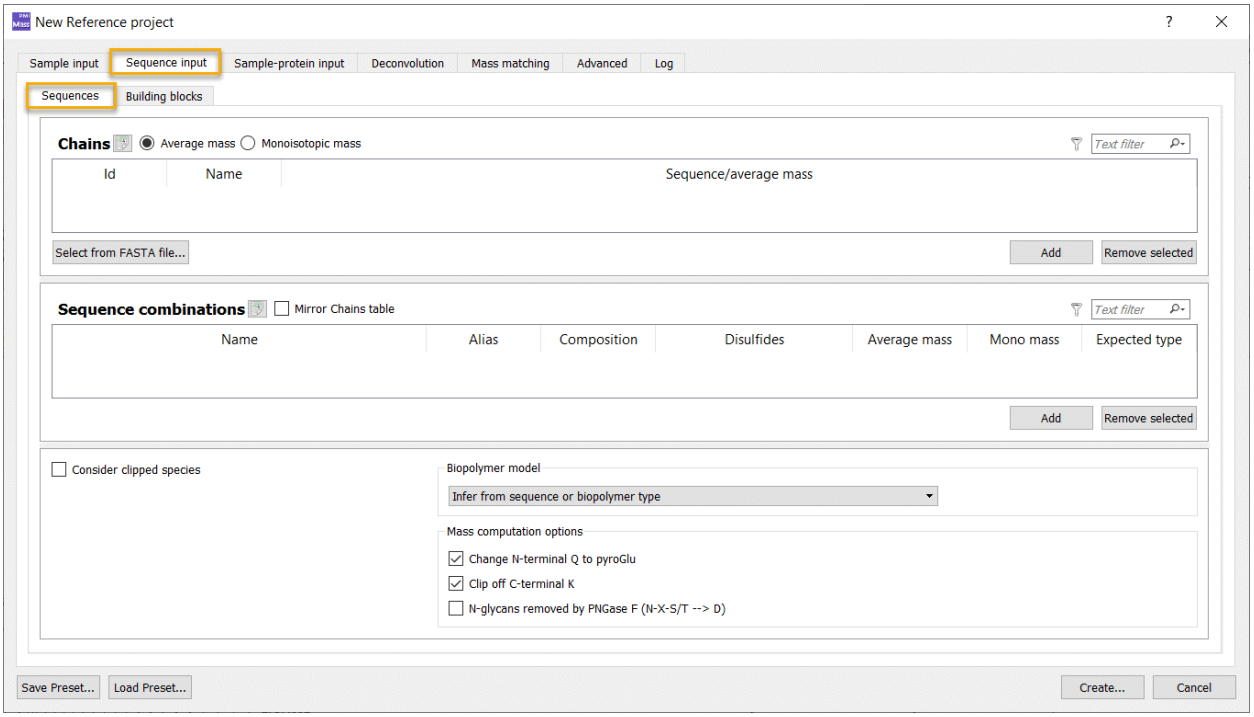


Figure 19: The Sequences sub-tab of the Sequence input tab

Users can add chains to the **Chains** table and then use these chains to add rows to the Sequence combinations table.

FASTA Files

To enter a protein sequence, drag and drop a FASTA file into the **Chains** table. Alternatively, click **Select from FASTA file**, navigate to the file of interest, and click **Open**. The **Select from FASTA** dialog opens (see Figure 20). Protein sequences also can be typed or pasted in manually. To manually enter protein sequences, click **Add** in the Chains section, and click in the **Name** and **Sequence/average mass** cells to enter text in those columns.

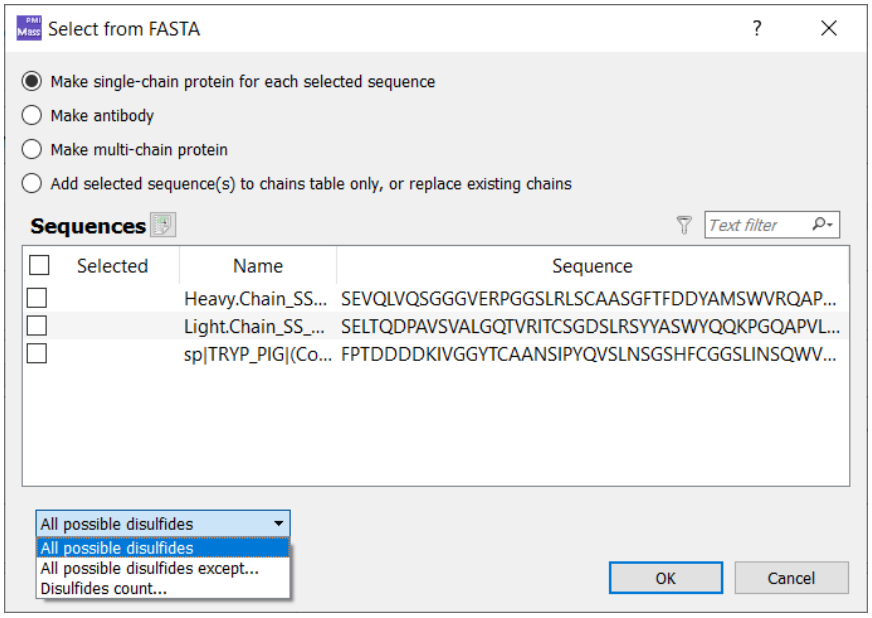


Figure 20: Select from FASTA

Select the disulfide option from the drop-down menu at bottom left.

There are four options for FASTA sequences that result in different kinds of sequence combinations

- **Make single-chain protein for each selected sequence**

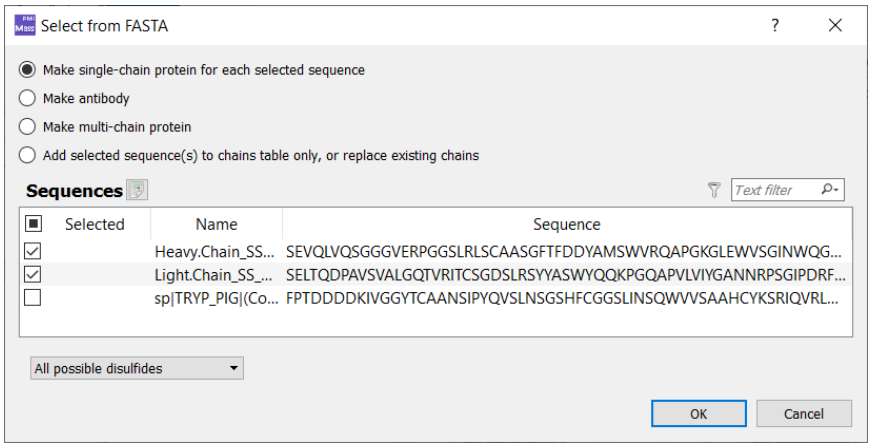


Figure 21: FASTA - Make single-chain protein

This option generates a protein in the Sequence combinations table for each of the checked sequences:

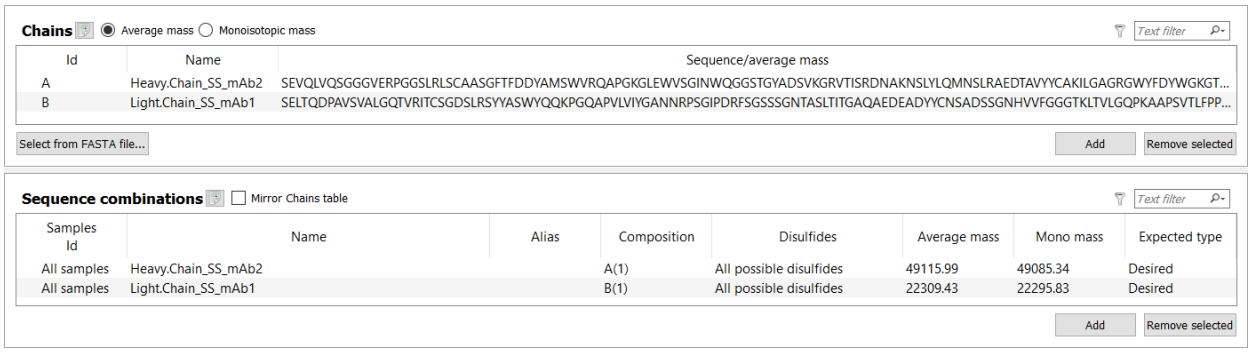


Figure 22: A single protein is generated per chain

- **Make antibody**

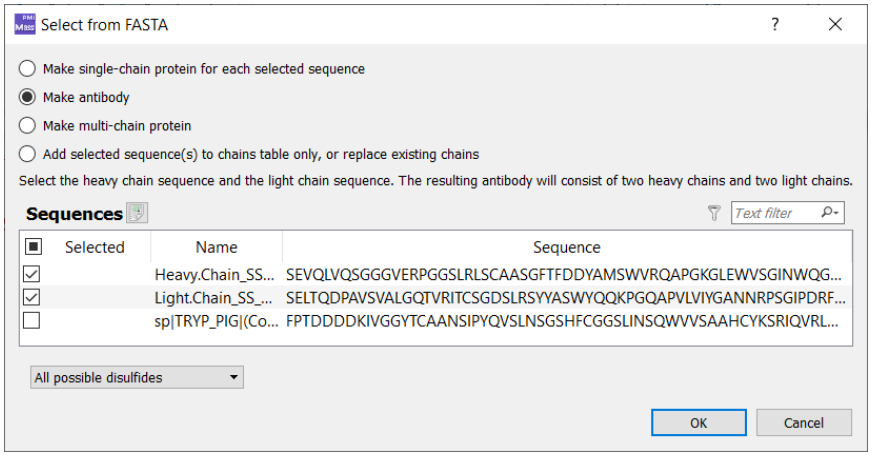


Figure 23: FASTA - Make antibody

This option generates an antibody from two copies of a checked heavy chain sequence and two copies of a checked light chain sequence:



Figure 24: An antibody is generated from two heavy and light chains

- **Make multi-chain protein**

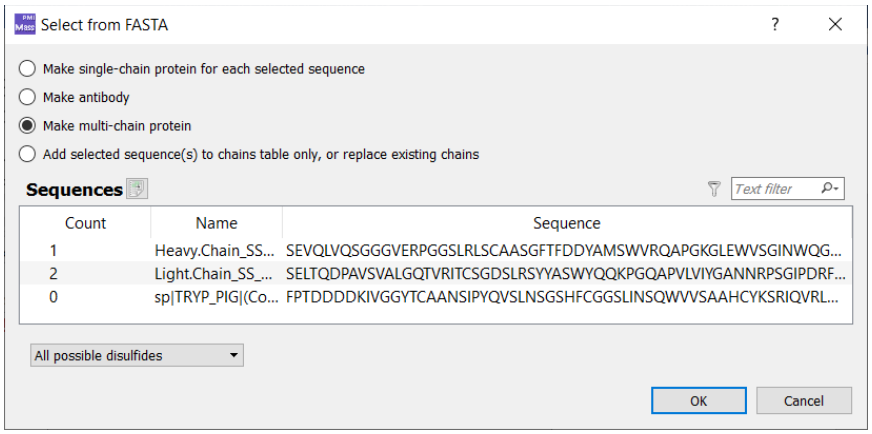


Figure 25: FASTA - Make multi-chain protein

This option generates a protein from the designated counts of the checked sequences, in the case above, one heavy chain and two light chains:

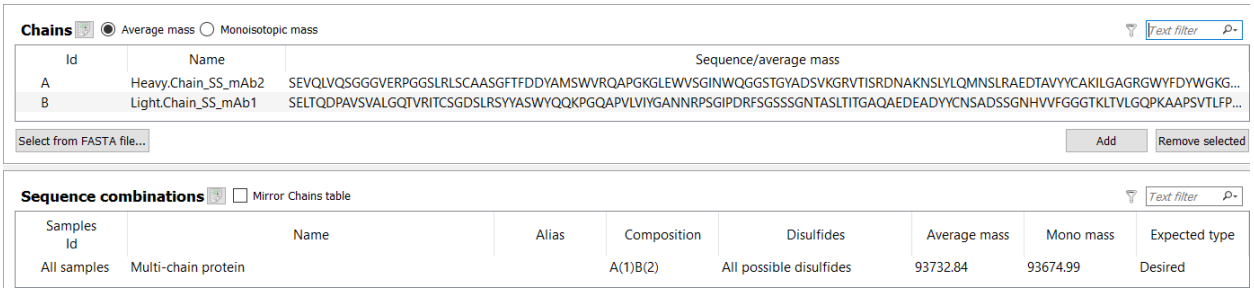


Figure 26: A protein is generated from the counts of checked sequences

- **Add selected sequence(s) to chains table only, or replace existing chains**

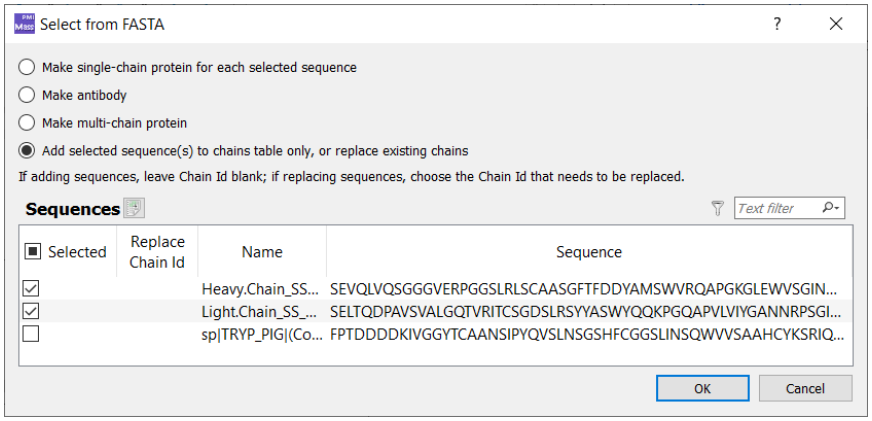


Figure 27: FASTA - Add selected sequences only

This option performs two functions: first, when importing into an empty Chains table, the option loads the Chains table with the checked sequences without generating any sequence combinations:



Figure 28: No Sequence combinations are generated for this option

This option can also be used to replace existing sequences added using any of the four entry options. To replace existing sequences, click **Select from FASTA file** again and select the original or a new FASTA file. Select the **Add selected sequence(s) to chains table only, or replace existing chains** radio option. Choose the rows containing the new sequences by checking the boxes in the **Selected** column. Use the dropdowns in the **Replace Chain Id** column to select the Ids of the sequences to replace (by default, capital letters):

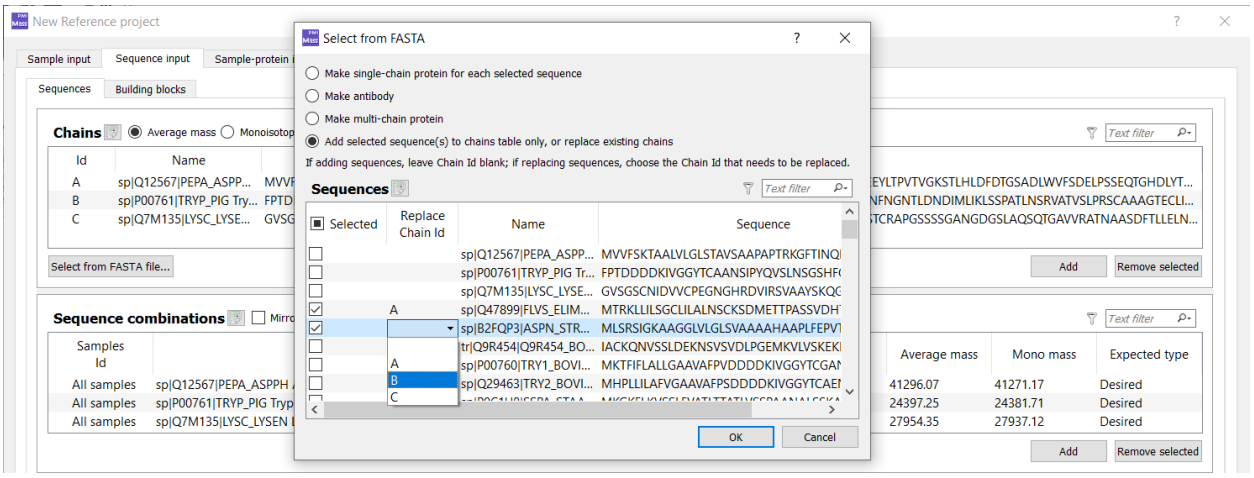


Figure 29: Replacing existing sequences from the original or a new FASTA file

Click **OK** and the **Name** and **Sequence** entries in the Chains table are updated. Likewise, the Sequence combinations table records containing those updated sequences are also updated.

Chains and Sequence Combinations

Users can designate peptide chain masses without sequences as average mass or monoisotopic mass. In the Chains section, a radio button selects between the two types of masses and the mass header updates accordingly:

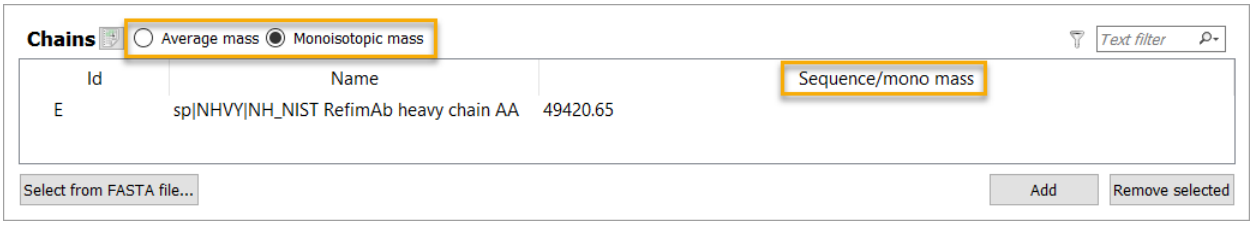


Figure 30: Average mass and Monoisotopic mass options

The software automatically computes the average and monoisotope masses for each selected FASTA entry, and these masses are then available for automatic peak assignment. Alternatively, a known average or mono mass can be added manually by clicking **Add** and clicking in the **Name** and **Sequence** cells to type or paste in names and masses. The default computation assumes that cysteine residues are disulfide bonded, but this default can be changed by the user. Protein sequences can be arbitrary if the user inputs the average or monoisotopic mass, overriding the computed masses. Average-isotope masses give the software reference masses to assign mass peaks automatically based on mass deltas. For example, in Figure 5 above, the peak at 23,411 Da was assigned the name “Adalimumab Light chain, Reference” automatically. “Adalimumab Light chain” came from the name of the entry in the FASTA file, and “Reference” came from the mass delta table in the Mass matching tab (see below). A mass of 23,563 would be matched to “Adalimumab Light chain, Glycation” if the mass delta table included an enabled entry with mass 162 Da and name “Glycation”.

The **Mirror Chains table** check box populates the content from the Chains table into the **Sequence combinations** table. For each row in the Chains table, a corresponding row is added to the Sequence combinations table (with single-chain composition and all possible disulfides).

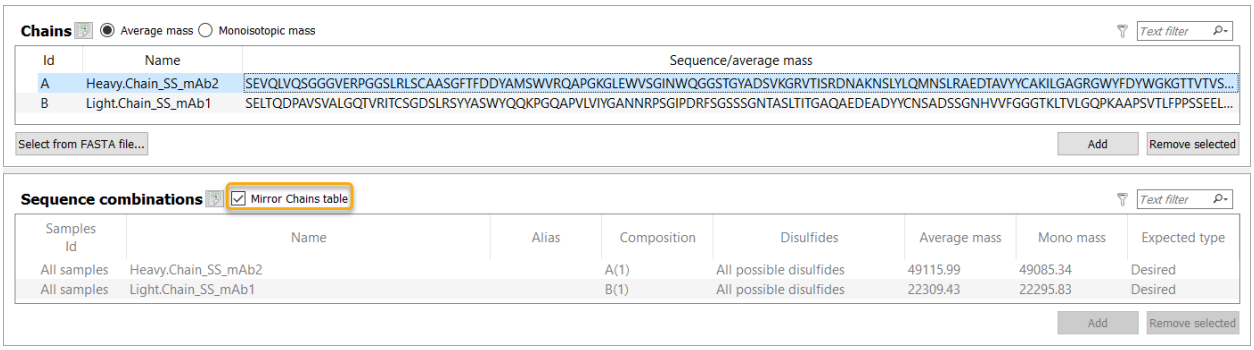


Figure 31: Importing protein sequences from chains using the Mirror Chains table feature

Sequence combination are associated with all samples, by default. However, they can be associated with an individual sample. Click ⋯ after the **Sample Id** for the desired sequence combination to open the **Edit samples selection** dialog:

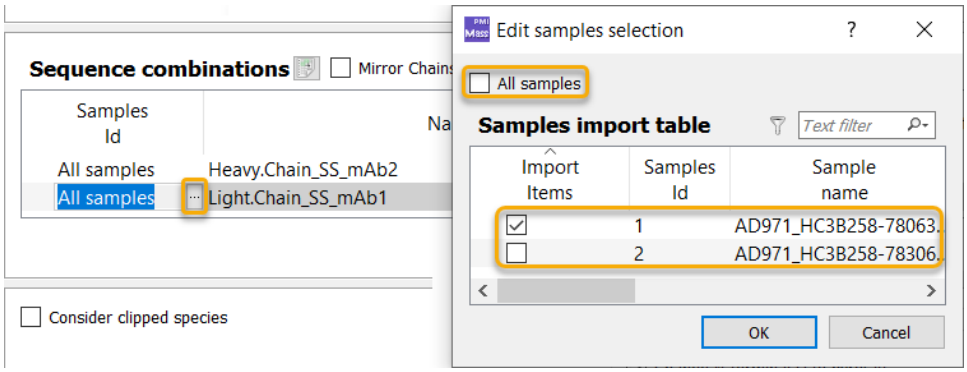


Figure 32: Edit sample selection for sequence combinations

Check either **All samples** or one of the sample rows and click **OK**. More than one checked sample for a sequence combination is not supported.

The user can also set the number of disulfides within the Sequence combinations table. The “Disulfides” window is activated by double clicking within the box within the table.

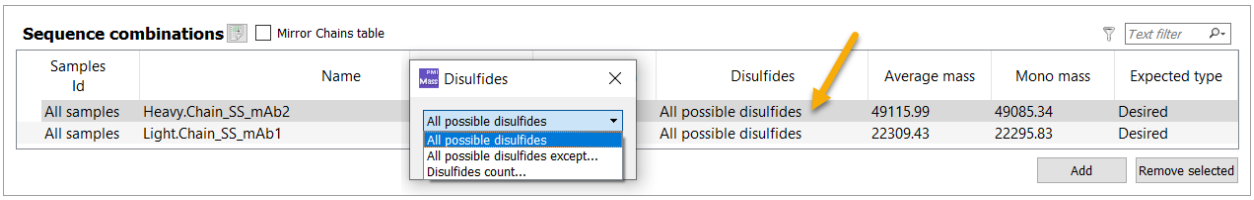


Figure 33: All possible disulfides

To set a specific number, select “Disulfides count…” and enter a value

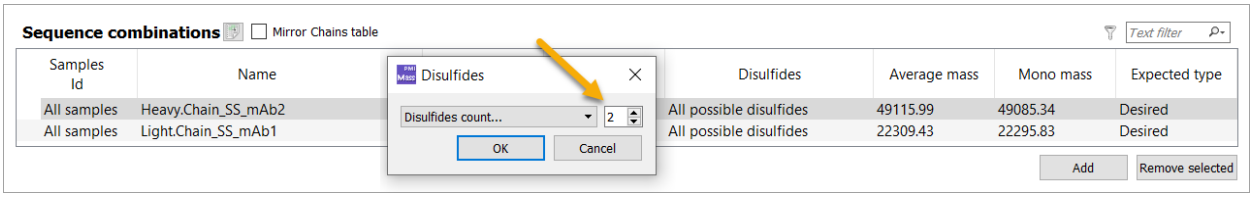


Figure 34: Disulfides by count

Sequences can be designated as **Desired** or **Undesired** in the **Expected type** column:

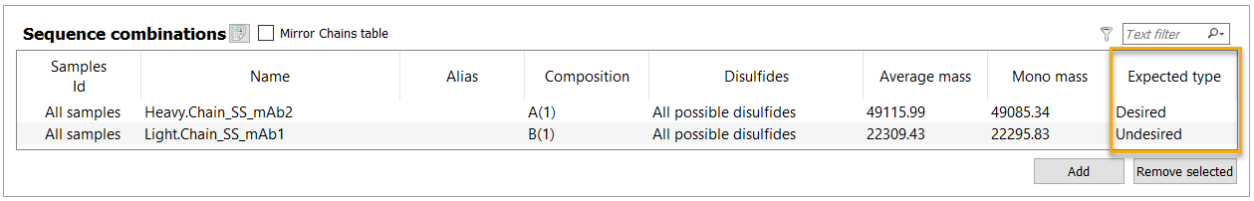


Figure 35: Expected type settings for sequences

The software can be enabled to account for clipping. To enable this, check the “Consider clipped species box”. To specify where the clipping occurs, choose the “Clip at specific sites” radio button, click **Add** and enter specific residues before and/or after the clip site. Alternatively, choose “Clip everywhere”.

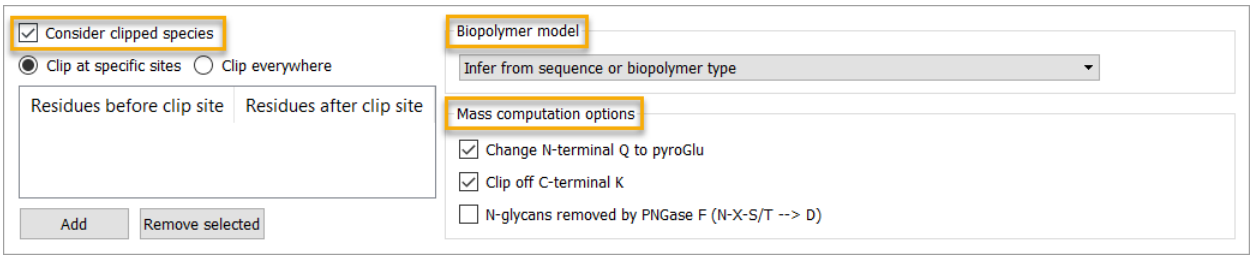


Figure 36: Clipped species, Biopolymer model and Mass computation options

Clipped species are then counted for each sample in the **Project** table in the generated project, and the **Trace peaks** and **Masses** tables will label affected records as **Clipped** in the **Expected type** column.

Users can select the kind of biopolymer model used to convert average mass to monoisotopic mass and monoisotopic mass to average mass:

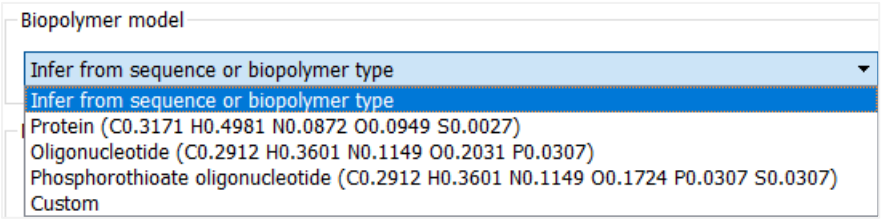


Figure 37: Biopolymer model choices

Biopolymer model options include:

- **Infer from sequence or biopolymer type** is the default selection, which uses the standard model that Intact Analysis has used to calculate mass.
- **Protein** (C0.3171 H0.4981 N0.0872 O0.0949 S0.0027) uses the displayed averagine formula to calculate mass.
- **Oligonucleotide** (C0.2912 H0.3601 N0.1149 O0.2031 P0.0307) uses the displayed formula to calculate mass.
- **Phosphorothioate oligonucleotide** (C0.2912 H0.3601 N0.1149 O0.1724 P0.0307 S0.0307) uses the displayed formula to calculate mass.
- **Custom** opens a cell to the right of the selection to add a custom formula to calculate mass.

Note that the formula values refer to atom counts (molar ratios), not mass ratios.

There are also some Mass computation options available, as shown in Figure 36.

Building blocks

Within the **Sequence Input** tab, is also the **Building blocks sub-**tab.

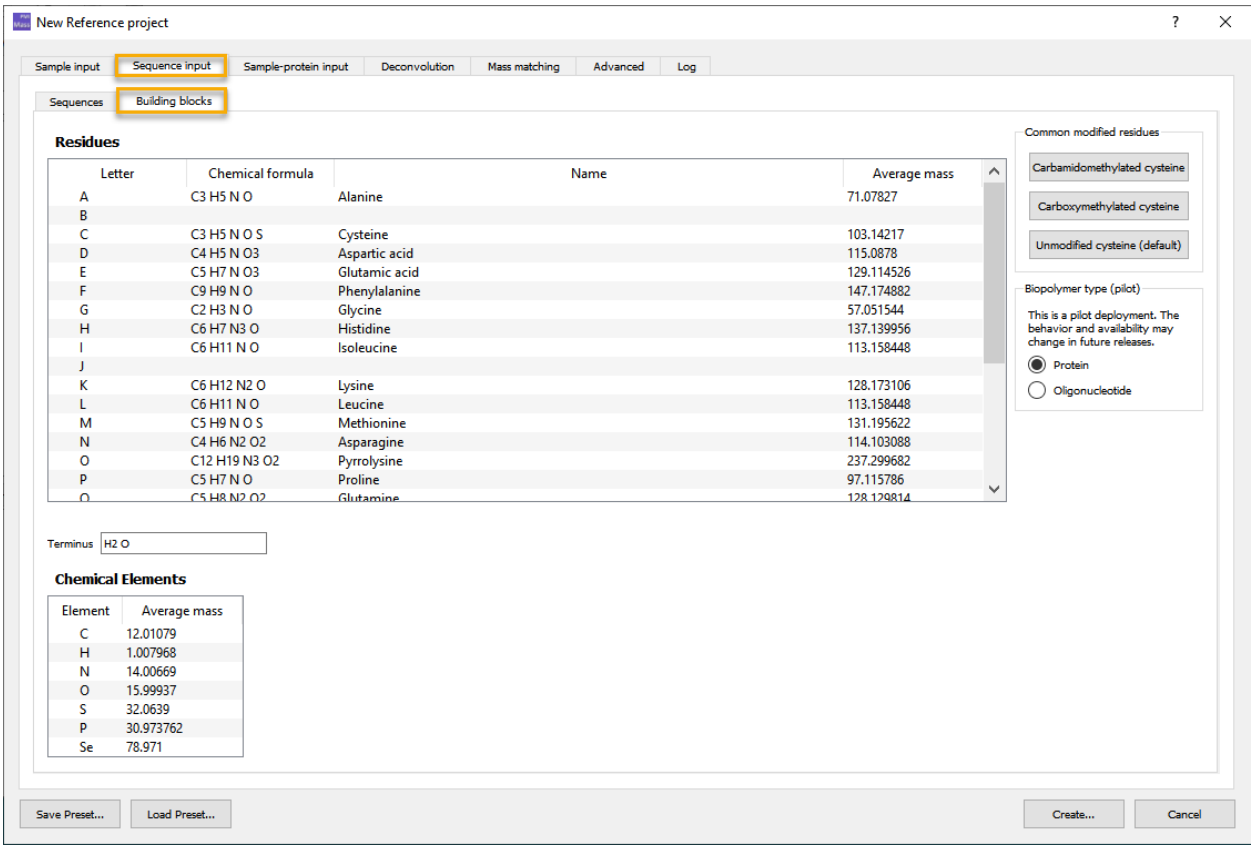


Figure 38: Building blocks tab

The Building blocks tab contains several useful parameters. Cysteine modification can be clarified by selecting amongst the three named options. The chemical formula will be adjusted accordingly.

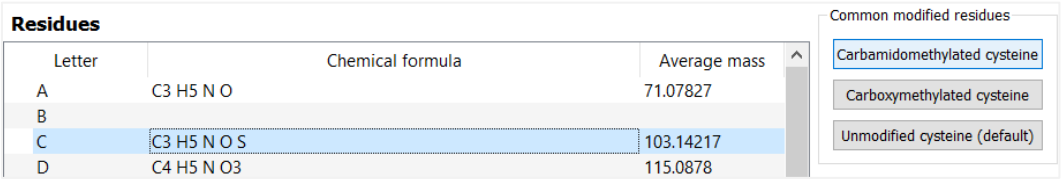


Figure 39: Carbamidomethylated cysteine

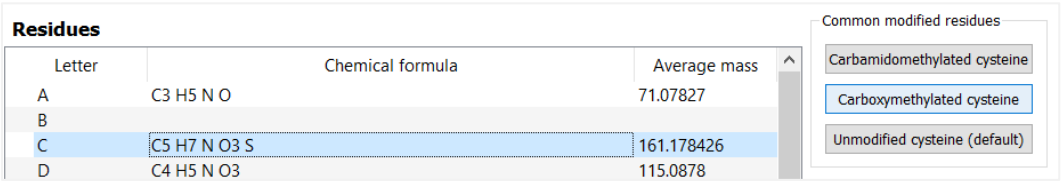


Figure 40: Carboxymethylated cysteine

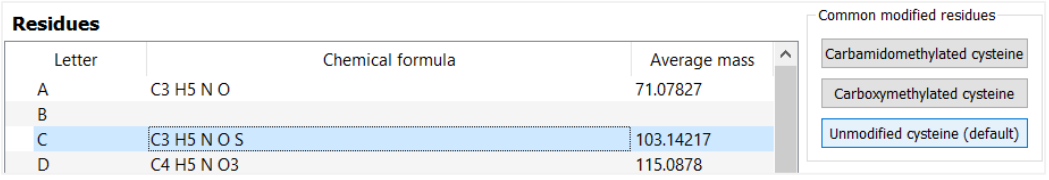


Figure 41: Unmodified cysteine (default)

For Biopolymer type, the application supports oligonucleotides, in addition to the default Protein type (please note this is a pilot deployment). The contents of the table update for the selected type.

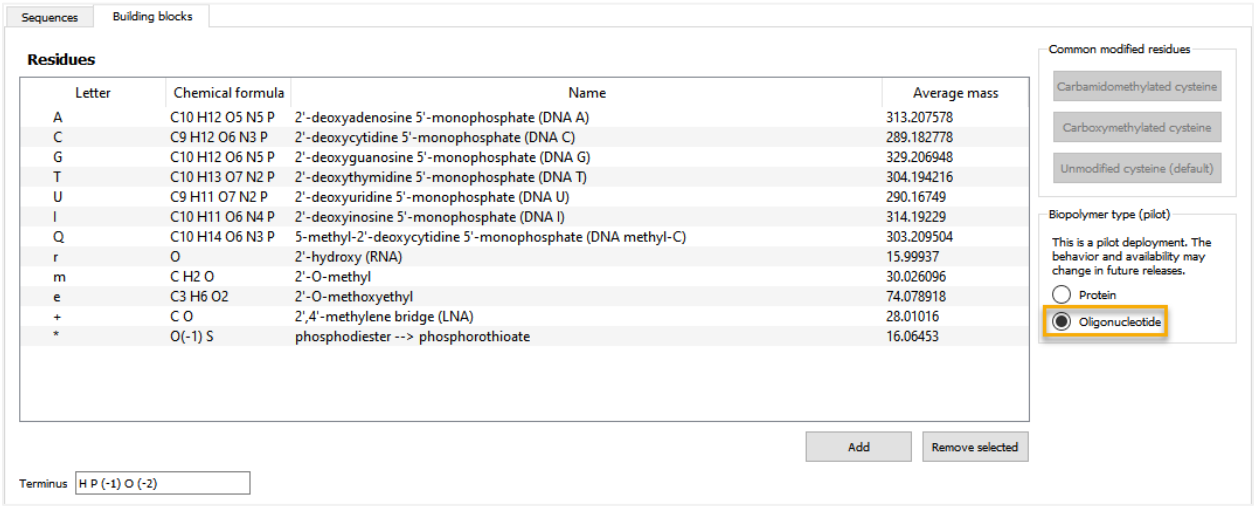


Figure 42: Biopolymer type Oligonucleotide

When Oligonucleotide is selected as Biopolymer type, the following changes appear in the Building blocks sub-tab:

- The Letter column in the Residues table is editable
- When editing the Letter table, the string in the Letter column must have exactly 1 character and must be unique. The following characters are forbidden for the "Letter" column:

  0 1 2 3 4 5 6 7 8 9 - ( )
- Add and Remove selected buttons appear below the Residues table
- The Common modified residues buttons are disabled
- The Terminus field is set to "H P (-1) O (-2)" by default

The following changes appear in the Sequences sub-tab:

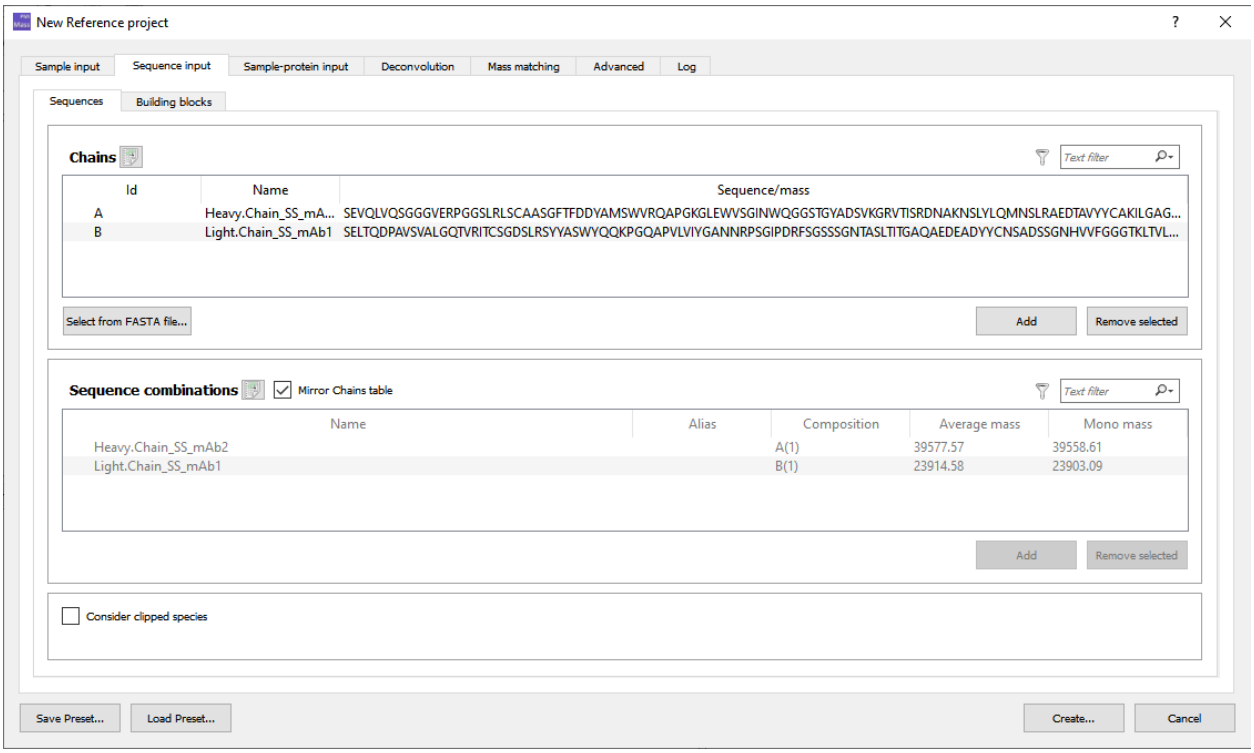


Figure 43: Sequences sub-tab when Oligonucleotides is selected in the Building blocks sub-tab

- The Mirror Chains table mode is enabled by default
- The Mass computation options are hidden
- The Disulfides column in the Sequence combinations table is hidden
- In the Chains table, manually added sequences are not automatically converted to capital letters

Sequences can be manually entered using the following rules (please note “X” identifies a base location):

- Standard and Mixed Bases must be expressed in UPPERCASE
- RNA must be expressed as “rX” (for example rA, rU)
- 2' O-methyl RNA bases are entered as “mX”
- Locked nucleic acid bases (LNA) are entered as “+X”
- Phosphorothioated DNA bases are entered as “X*”
- Phosphorothioated RNA bases are entered as “rX*”
- Phosphorothioated 2'-O-methyl RNA bases are entered as “mX*”
- Phosphorothioated LNA bases are entered as “+X*”
- The user can redefine letters and symbols as desired, as long as they are defined within the Building blocks tab. For example, see Q defined as 5-methyl-Cytosine in the above screen capture.

The user can customize the average mass for each chemical element.

Chemical Elements

Element	Average mass
C	12.01079
H	1.007968
N	14.00669
O	15.99937
S	32.0639
P	30.973762
Se	78.971
F	18.998403163

Figure 44: Chemical Elements

Sample-protein input tab

The **Sample-protein input** tab allows for association of protein sequences with the samples that contain them:

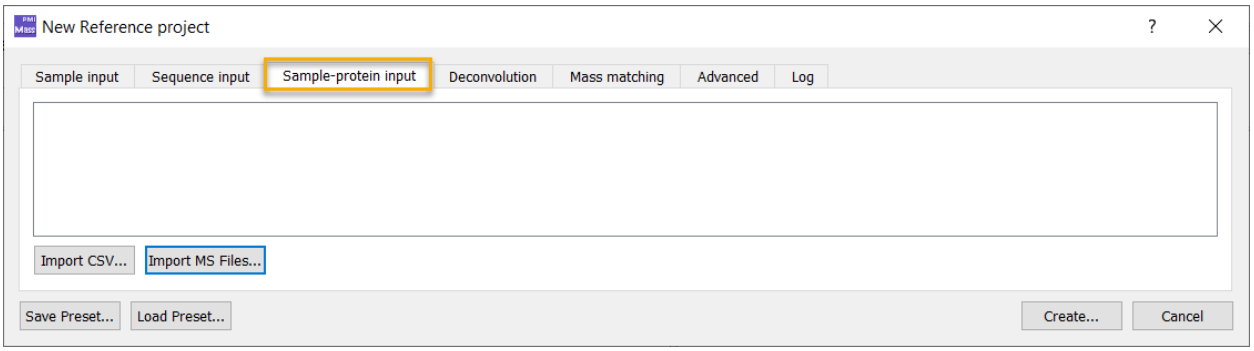


Figure 45: Sample-protein input tab

Sample-protein associations can be imported from *.csv files and from MS files. To create csv files from other projects, use **File > Export > Generate MS path template CSV**. This capability is useful for making a single Intact Analysis project with many different samples.

Deconvolution tab

The **Deconvolution** tab includes two sub-tabs, **Basic** and **Advanced**. The **Basic** tab (Figure 46) contains the primary settings used in computing deconvolved masses. **Mass range** and **m/z range** set ranges for neutral masses and m/z, respectively. Mass range defines the range of neutral masses displayed in the Deconvolved mass spectrum. m/z range defines the segment of the MS1 spectrum used to compute neutral masses. Within the Auto mass peak picking parameters, **Min difference between mass peaks** and **Max number of mass peaks** control peak picking. Min difference between mass peaks prevents the peak picker from picking multiple points on top of a ragged or isotope-resolved mass peak. Max number of mass peaks sets a limit on the number of picked peaks.

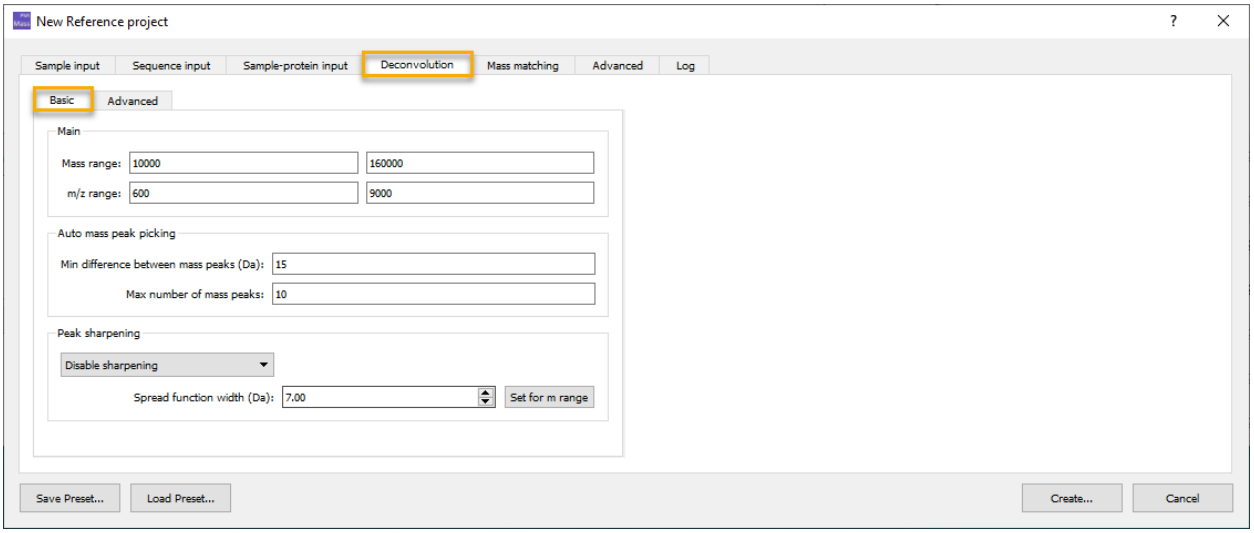


Figure 46: Deconvolution Basic parameters

The **Peak sharpening** dropdown enables or disables an optional "super-resolution step" that sharpens peaks beyond what is seen in the data. It deconvolves a "point spread function" to give super-resolved mass peaks and turn shoulders into separate peaks. The **Spread function width** sets the expected width of a peak when peak sharpening is enabled. A reasonable width is the square root of the peak mass in kilodaltons, for example, 12 for a spectrum from 140,000 to 150,000 Da. A too-narrow width will not sharpen much, and a too-wide width might split peaks. Peak sharpening on mass spectra that span a range of more than about 20,000 Da is not recommended, because no single width will be optimal for the entire range. Peak sharpening should also be avoided on isotope-resolved mass spectra.

These options can be applied to trace peaks one at a time using the orange **Compute** button above the neutral mass spectrum in Figure 4 and Figure 5. See the **Deconvolved Mass Spectrum Menu** section for more information about these parameters.

The **Advanced** tab (Figure 47) mainly concern resolution. These are only needed if the aim is to produce isotopically resolved neutral mass spectra. **Charge vectors spacing** assigns charge probabilities for each small interval of m/z points. Deconvolutions of most MS1 spectra will be almost exactly the same with any charge vectors spacing from 0.2 to 1 m/z units (Thomsons), but a narrow spacing of 0.1 may give better results on isotope-resolved MS1 spectra with interleaved signals, and a wide spacing of 2 may give better results for native MS with broad m/z peaks.

Intact Analysis removes an m/z baseline before deconvolution; this step and removal of charge one or stop-list peaks are the only steps in the algorithm that do not conserve ions. **Baseline radius** controls the stiffness of the baseline. A baseline radius of 8 gives a flexible baseline that will cut into m/z peaks broader than 8 Thomsons; this will often give better visual separation of neutral mass peaks, but may distort peak areas. A baseline radius of 30 will give a stiffer baseline that cannot cut into m/z peaks narrower than 30 Thomsons. An even larger value, 100 or more, may be needed for native MS. The default value of 15 is a compromise.

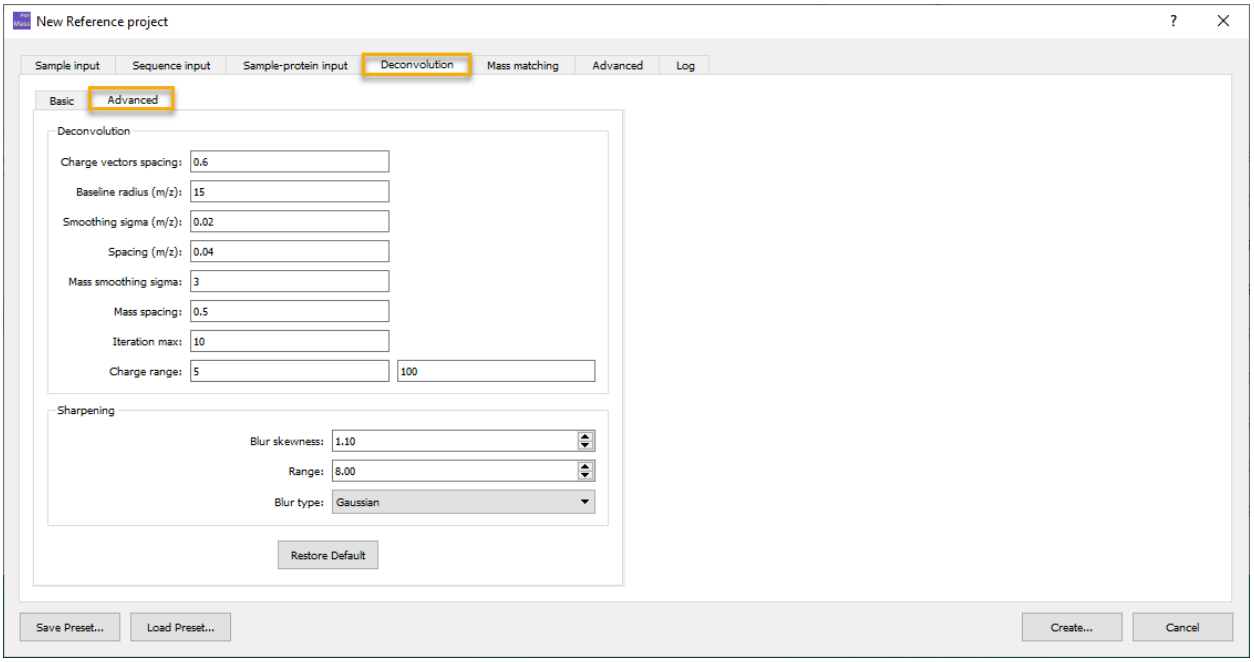


Figure 47: Deconvolution Advanced parameters

Spacing (m/z) controls the spacing of sample points in the m/z spectrum. The raw MS1 data is represented as a continuous piecewise-linear function that can support any spacing of sample points. However, m/z spacing finer than the finest spacing in the original data will slow the computation without adding resolution. Reasonable values for Spacing (m/z) are in the range 0.005 – 0.05 for QTOF instruments, which have almost the same resolution at all m/z; the low setting of 0.005 would be appropriate for Bruker maXis and the higher setting of 0.05 for older instruments with lower resolution. For Orbitrap, the resolution depends upon m/z; the setting of 0.005 shown above is for isotope-resolved 25 kDa masses (antibody subunits). For native MS on Exactive EMR with m/z's in the 5000 – 10,000 range, a spacing of 0.1 is fine enough. **Smoothing sigma** is typically set to the same value as Spacing (m/z), but a larger value can be helpful for producing an appropriately smoothed neutral mass spectrum with less smoothing at lower mass and more smoothing at higher mass.

Mass spacing controls the spacing of points in the neutral mass spectrum. To preserve isotopic resolution, spacing should be set to 0.1 or even 0.05. If the MS1 spectrum does not have isotopic resolution, or isotopic resolution is not needed for analysis, mass spacing in the range 0.2 to 1 is best for target molecules below 200 kDa. Spacing of 10 Da or more is best for targets above 300 kDa. For mass spectra without isotopic resolution, **Mass smoothing sigma** in the range of 2 – 5 will smooth jittery peaks in the range 20 – 200 kDa; larger values will be needed for larger masses. For mass spectra with isotopic resolution 0.1 will work.

Iteration max set to 10 will work for most purposes. A larger value, for example 20 or 30, can be helpful for lower signal-to-noise spectra that take longer to converge.

Charge range is best set to a wide range, in which case the charge range will be implied by the mass and m/z ranges. The default range of 5 – 100 covers most applications, but 5 will need to be reduced for deconvolutions with mass range starting below 10 kDa, and 100 increased for targets that may have charges above 100.

Sharpening uses the **Spread function width** set in the Basic tab to deconvolve the data. **Blur skewness** controls the asymmetry of the point spread function; the default value of 1.1 means that the right tail has sigma (standard deviation) 10% bigger than the left tail. 1.2 gives even more tailing; 1.0 gives a symmetric point spread function. **Range** sets the length of the tails in standard deviations; a small value of 5 or 6 may work better in the case of Lorentzian point spread. **Blur type** has two choices: Gaussian (skinny tails) and Lorentzian (fat tails). Lorentzian should use a slightly smaller sigma than Gaussian.

Mass matching tab

The **Mass matching** tab (Figure 48) includes parameters that control automatic mass peak assignment. The **Delta masses** section of the tab gives a table of likely mass differences between observed peaks and the reference mass input by the user or computed from an amino acid sequence. For example, if "`+ Hex`" (for hexose) is checked, then a peak 162.2 Da heavier than the reference mass (+/- the **Match tolerance**, which is 6 Da in picture below) will be automatically assigned.

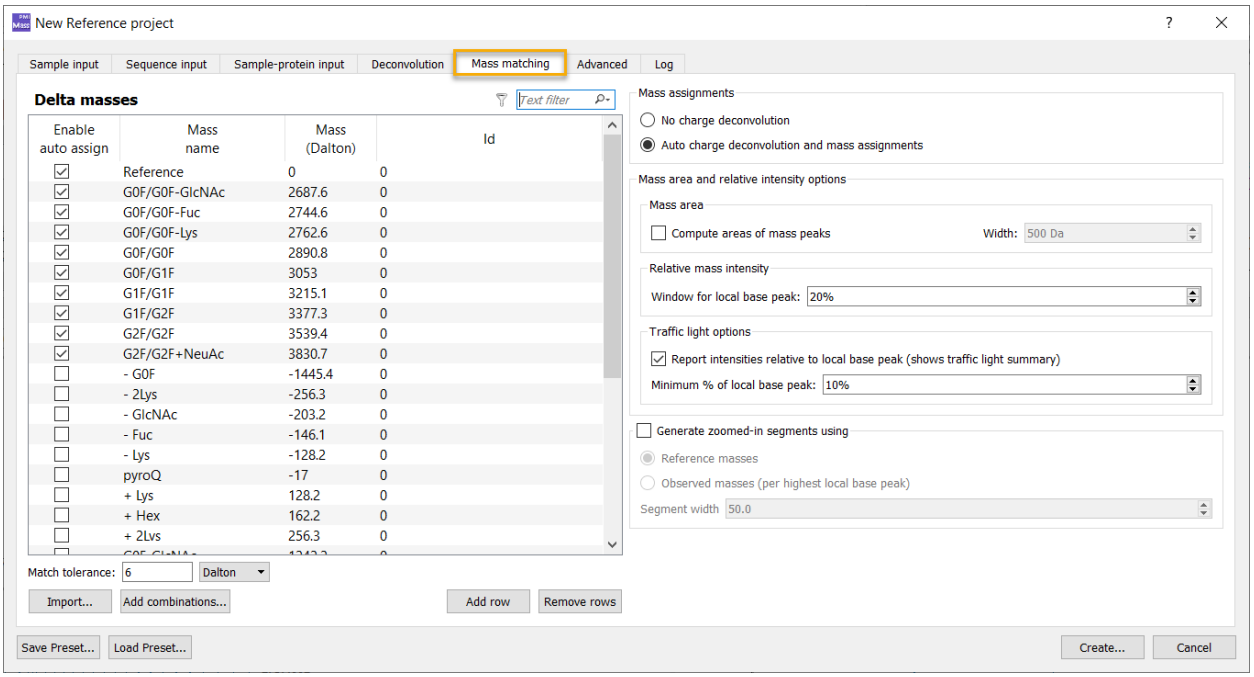


Figure 48: Mass matching tab

Import allows the user to import a *.csv table of custom delta masses. **Add row** and **Remove rows** allow the user to enter individual delta masses. **Add Combinations** opens a dialog that allows the user to assign match tolerance modifications to a delta mass.

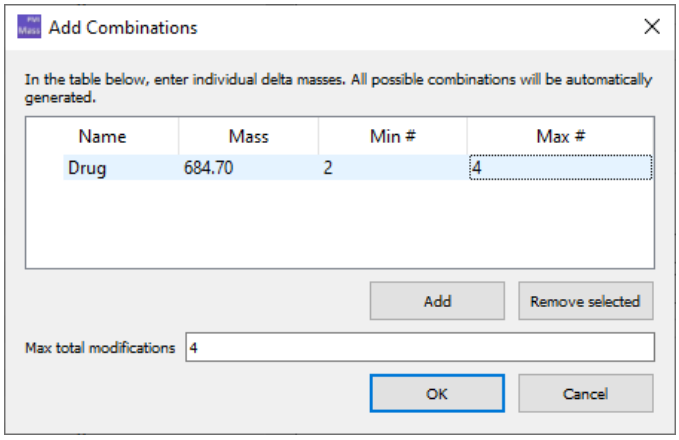


Figure 49: Add Combinations to delta mass values

The **Mass assignments** section of the tab allows the user to turn on/off charge deconvolution and automatic mass assignment. For new projects, especially those with longer chromatography times, significant computation time may be saved by creating the project with the setting **No charge**

deconvolution selected. The summed *m/z* spectra can then be viewed before deciding which trace peaks warrant deconvolution. Similarly, charge deconvolution without mass assignments will let the neutral mass spectra be viewed before mass peak assignment.

By default, PMI Intact Analysis uses peak intensity for quantitation, but will use area instead if **Compute areas of mass peaks** is checked; area is commonly used for antibody-drug conjugates. **Width** defines the width in daltons across which a peak is integrated to determine its area. **Report intensities relative to local base peak** tells the software to define local peak groups, so that proteoforms of similar mass are compared to each other and not to proteoforms of very different mass. **Window for local base peak** sets the percentage range of the major mass peak that defines a local peak group, for example, a 20% window would compare 20 – 30 kDa forms to a locally tallest (base) peak at 25 kDa and 40 – 60 kDa forms to a base peak at 50 kDa. **Minimum % of local base peak** sets the intensity threshold below which a peak is considered too small to report.

The **Generate zoomed-in segments** checkbox will automatically generate segments around either referenced or observed masses with a specified segment width for **MS1** or **Deconvolved Mass** plots.

Advanced and Log tabs

The **Advanced** tab includes several specialized features.

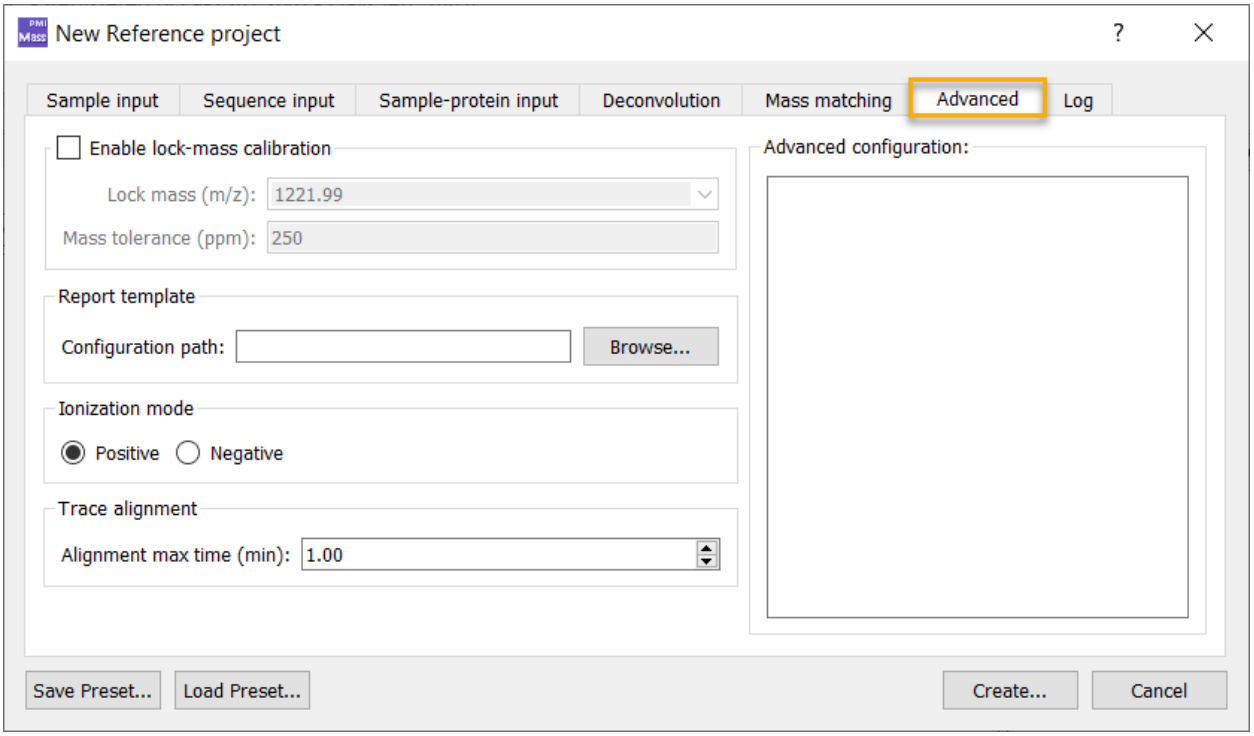


Figure 50: Advanced tab

The **Enable lock-mass calibration** checkbox shifts *m/z* measurements based on an internal calibrant. For example, the tallest peak in the *m/z* spectrum in Figure 5 is at 1221.9771. Shifting the *m/z* measurements so that this peak aligns to the correct value of 1221.9906 reduces the measurement error of the Adalimumab light chain monoisotopic mass from about 10 ppm to 1 ppm.

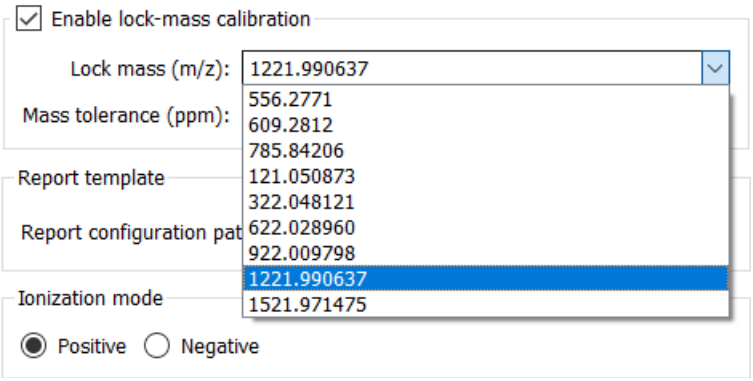


Figure 51: Lock-mass calibration

Report Template allows the user to preload a report configuration to the project.

Ionization Mode has been moved from the advanced command area to this tab. The user can select between Positive and Negative values.

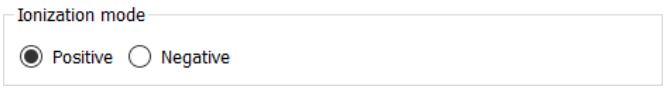


Figure 52: Ionization mode

Trace alignment sets the maximum time alignment in minutes for the traces.

The **Advanced configuration** box is used to enter text commands for less frequently used or newly developed options: monoisotopic mass, removing charge-one peaks (such as the 1222 peak in Figure 5), time-sliced trace peaks, use of isotope spacing to deduce charge, and many more advanced features. See the **Appendix** for more information.

Finally, the **Log** tab reports recent activity, and is useful for troubleshooting problems.

New reference project inputs can be saved to a file with the extension *.ntmsnpo using the **Save Preset** button at lower left. A saved file can then be reloaded with the **Load Preset** button. PMI's Batcher program can be used to run a number of data files with the same *.ntmsnpo parameters; this is useful for automated workflows.

After all values have been entered, click the **Create** button to create the project. The **Save File** dialog will open to enter a folder location and file name for saving the project. Project creation takes less than a minute for a typical spectrum file of size 10–100 MBytes.

The New Reference project dialog does not close. It opens to the **Log** tab and displays the progress of the projection creation:

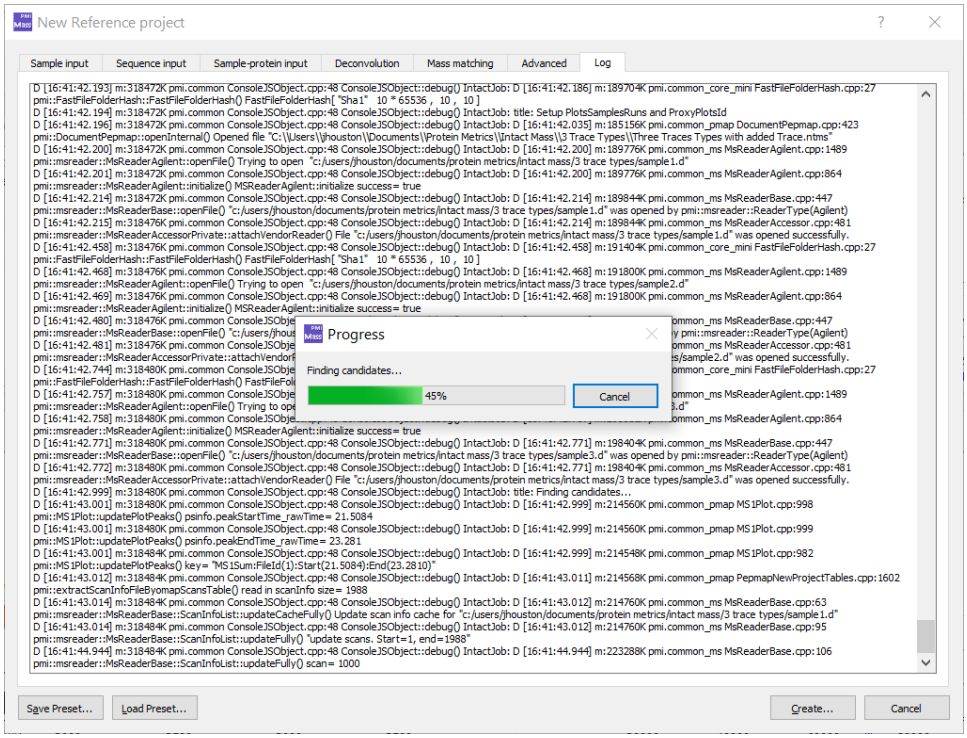


Figure 53: Log file generated by the project creation

Peak Annotation and Quantification

After project creation, a typical workflow filters trace peaks, computes further deconvolutions, and annotates neutral mass peaks. These operations are highly automatable for most samples such as purified mAbs, without sacrificing manual inspection and validation.

To select a trace peak, either click the peak row in the Trace Peaks table (View 3), or Alt-click the peak in the Trace plot view (View 2) (see Figure 4). The pink vertical lines represent the trace integration start and end times. To change the time limits of the peak, hover over a pink line until the arrow icon appears, then click and drag to the desired integration limit.

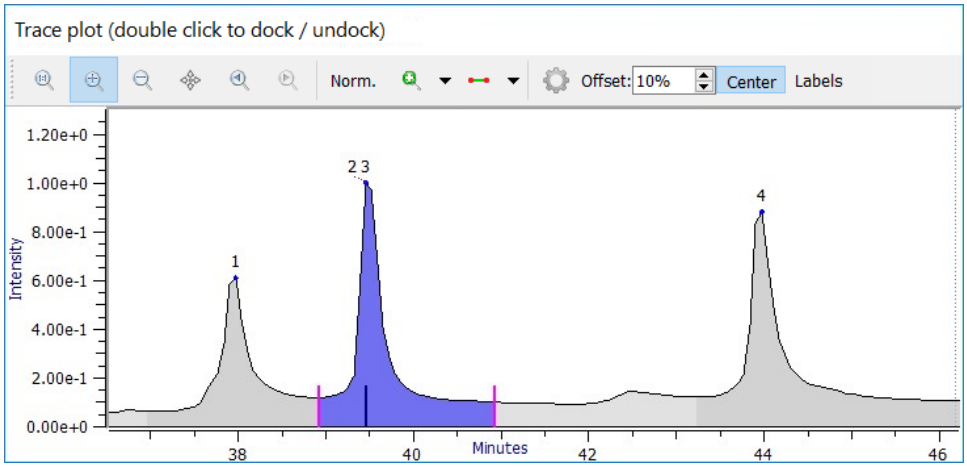


Figure 54: Modifying peak integrations

The MS1 plot recomputes the summed *m/z* spectrum automatically. Click the orange **Compute** button above the deconvolved mass spectrum and apply desired parameters to produce a new neutral mass spectrum. A variety of time windows can be used to obtain the clearest set of deconvolved mass spectra. A single trace peak can be split into finer time slices to distinguish early versus late eluters in a chromatographic profile. Trace peaks can overlap arbitrarily; trace peaks 2 and 3 above share the same apex, but peak 3 covers 36 to 48 min, that is, all three of the peaks we see by eye. The **Labels** button lets the user change the way trace peaks are labeled.

After deconvolution, Intact Analysis chooses a number of peaks (the default is 10) in the deconvolved spectrum and labels them with their masses. Peak picking is controlled by two parameters in the **Compute** menu: the minimum difference between peaks and the maximum number of picked peaks. Other controls are available through Advanced commands; see the Appendix. Picked peaks can be **assigned** either automatically or manually to make them persistent and available for reporting. Mass assignments remain when the maximum number of picked peaks is reduced, but are discarded when the deconvolution is recomputed, because the mass peaks may change.

The picked peaks can be labeled through the Masses table. When the software assigns masses, `"Reference"` labels are automatically derived from the input protein sequences, and labels such as `"G0F/G0F"` derived from the enabled mass deltas (differences from reference mass). Names and other fields can be edited manually. In the Masses table, click the Name field in a mass that is checked as assigned, click the name, and then either click the drop-down arrow and select a value or enter free text.

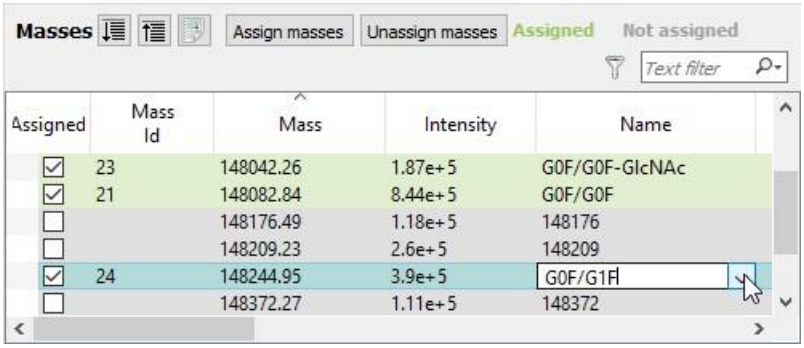


Figure 55: Editing assigned masses in the Masses table

Assigned masses in the Masses table appear as sub-rows in the Trace Peaks table. The trace peak header row inherits values from the mass sub-rows.

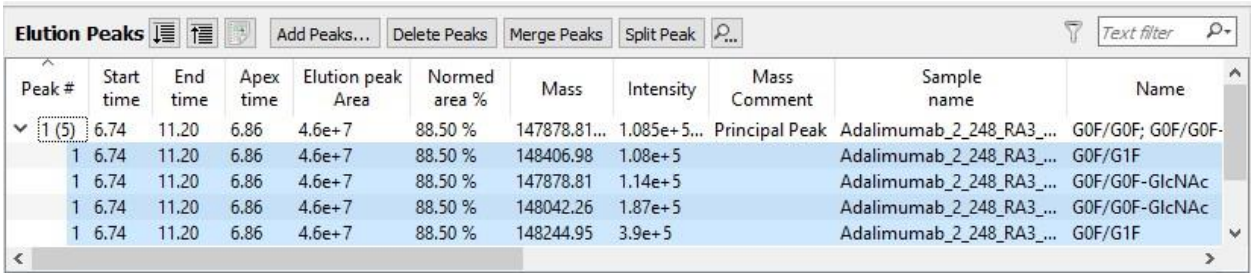


Figure 56: Mass information in the Trace Peaks table is inherited from assigned masses in the Masses table

By default, candidate annotations display values from **Protein name** and **Delta name** in the Deconvolved Mass spectrum. To display mass names in this view, do the following:

1. In the Deconvolved Mass spectrum view, click the gear icon.
2. Click Edit annotations. Click the Short Codes icon.

3. Select the **[Sequence]** short code and click **Insert**.
4. In the Template Editor, click **OK**. In the Render and zoom options dialog, click **OK**. This setting will be saved with the project.
5. The assigned mass name for a peak will now be displayed above its neutral mass in the Deconvolved Mass spectrum view. Horizontal grid lines can be turned on/off and font sizes can be adjusted by right-clicking on the plot.

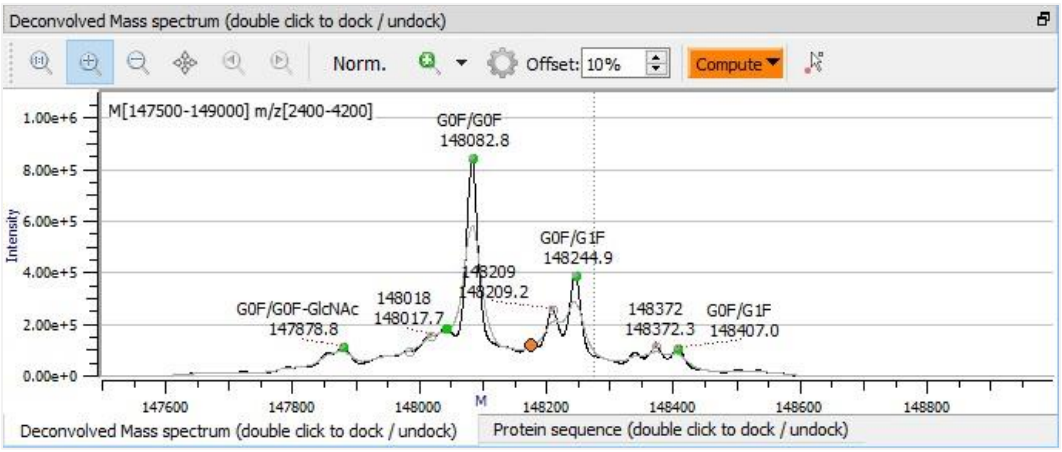


Figure 57: Displaying mass names in the Deconvolved Mass spectrum

Creating a Comparison Project

Intact Analysis supports comparison of multiple intact mass experiments, for such cases as lot-to-lot comparability, biosimilarity, and degradation studies.

To create a new comparison project, select **File > New Comparison Project**.

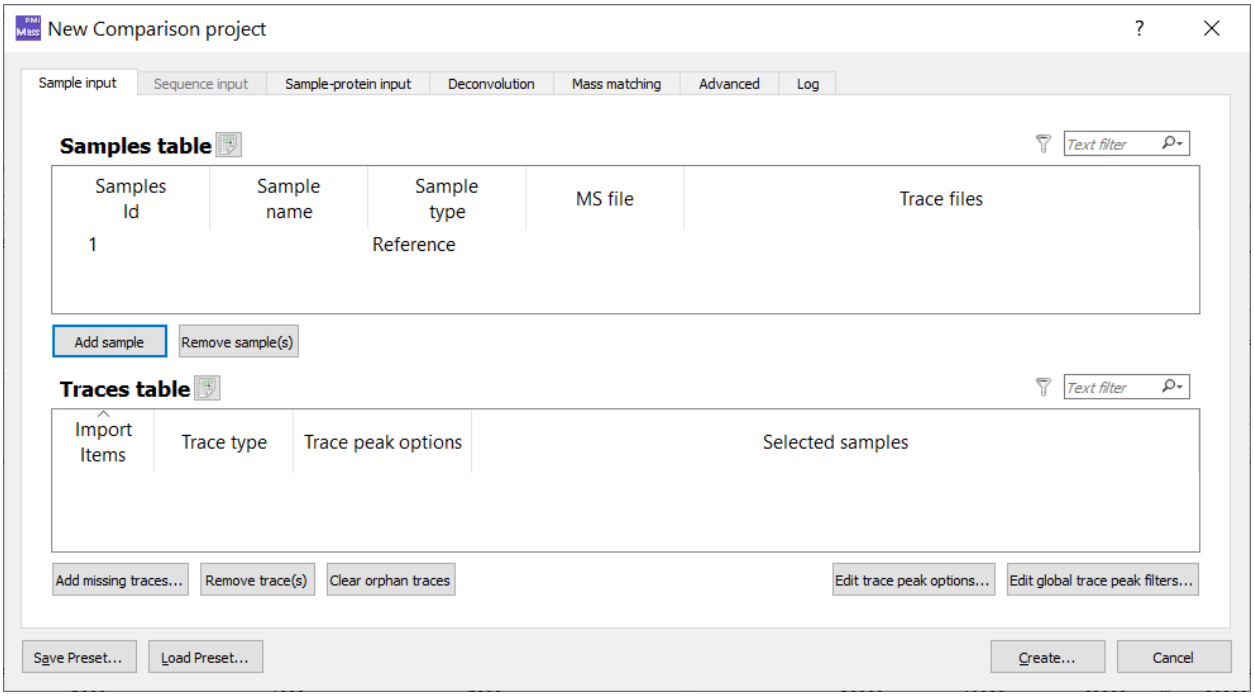


Figure 58: New Comparison project dialog

In the New Comparison dialog, drag in or navigate to a previously saved reference project. Next, drag in or navigate to the files to be compared to the reference. Enter the initial settings as with a new reference project and click **Create**. Enter a comparison project file name and location, and then create the new project.

In a comparison project (Figure 59), the Project window lists all raw files included. The Trace plot window shows an overlay or stacked display of all TIC traces. For each peak identified in the reference project, the program attempts to identify the "same" peak in each comparison file. Mismatched peaks, which should be rare in most cases, can be corrected manually. The Trace Peaks table contains a series of expandable rows ("groups") for each identified trace peak; groups are organized hierarchically by trace peak, by MS file, and finally by identified peak masses.

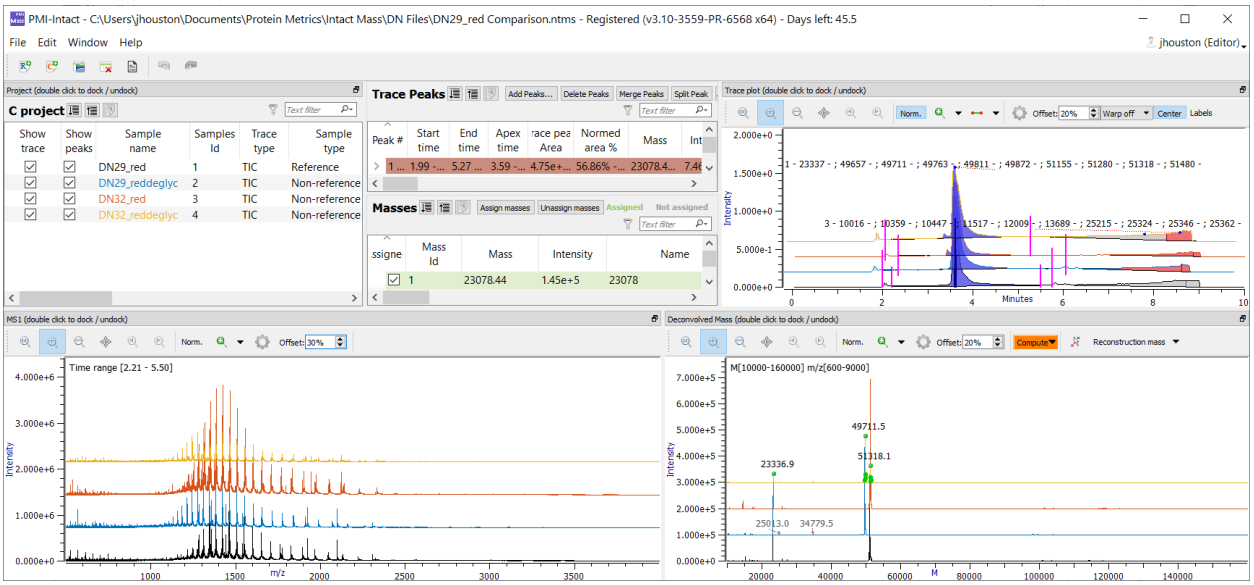


Figure 59: Comparison Project. Trace plot, MS1, and Deconvolved Mass spectrum show stacked views with the reference sample in black at the bottom. Offset controls the spacing of the plots. This project shows two different reduced mAbs with and without deglycosylation.

A good way to compare samples is to reuse deconvolution parameters (time, *m/z*, and mass windows) from the reference sample for all the other samples. This approach is especially convenient when there are multiple trace peaks with different mass options. The next section (under **File Menus**) explains how to produce comparability reports.

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. The **File** menus includes items to create new reference or comparison projects, to open previously created projects, to save a copy of an open project, to close a project, and to exit the program. There are also three menu items to export reports and data.

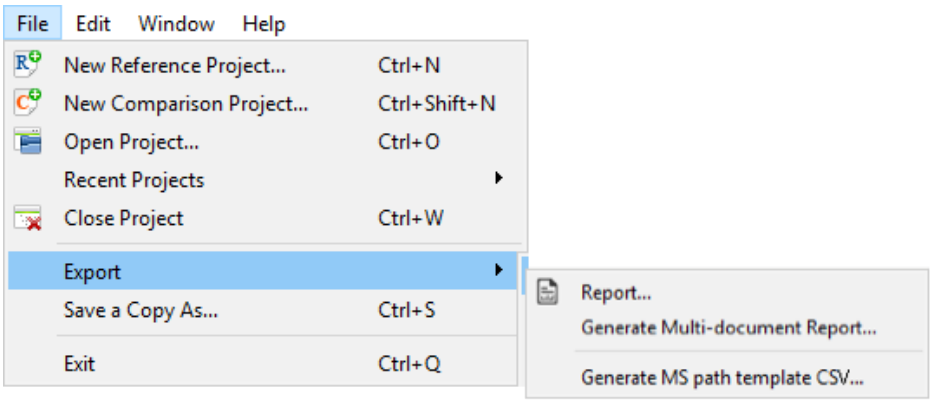


Figure 60: File menu with Export sub-menu

File > Export > Report generates a report that includes a summary of the project files, one or more pivot tables (that can be visualized as heatmaps, bar charts, etc.), along with plot images.

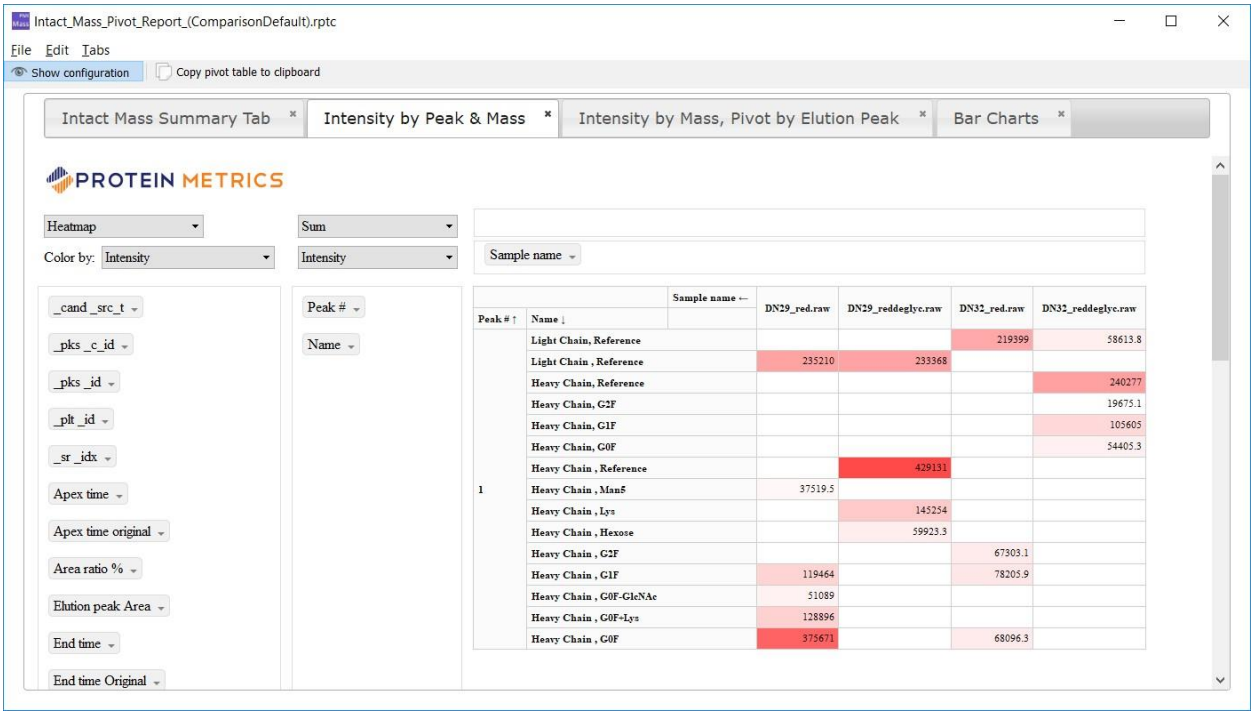


Figure 61: Default project report generated from **File > Export > Report**. The heatmap view of a pivot table gives the intensity information from Figure 59. Highest intensity overall is from the deglycosylated DN29 heavy chain.

To replace the report with another report template, choose in the report window **File > Presets > Report presets > <template name>**:

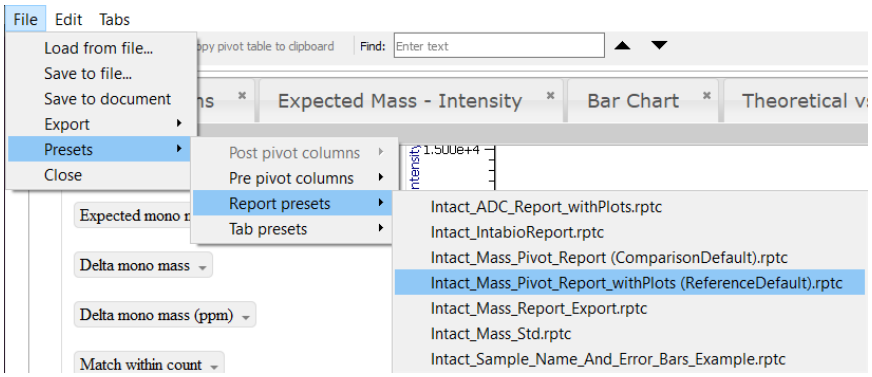


Figure 62: Changing the report template in a created report

The template **intact_IntabioReport.rptc** supports electropherograms from Blaze trace files; all the others support chromatograms. Choose **File > Save to document** to persist the new report to the project.

The **File > Export** sub-menu includes the item Generate Multi-document Report. A multi-document report creates report tabs derived from the table and graphical data from a series of reports.

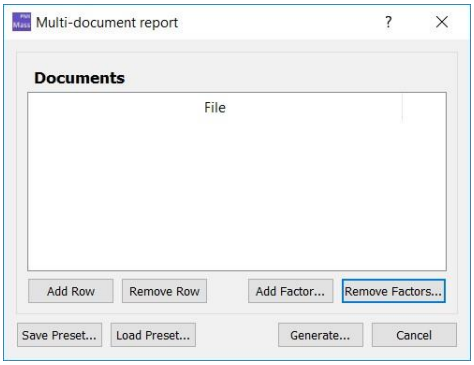


Figure 63: Multi-document report

To learn about reports in detail see the document **PMI Reporting Manual.pdf**.

Edit Menu

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

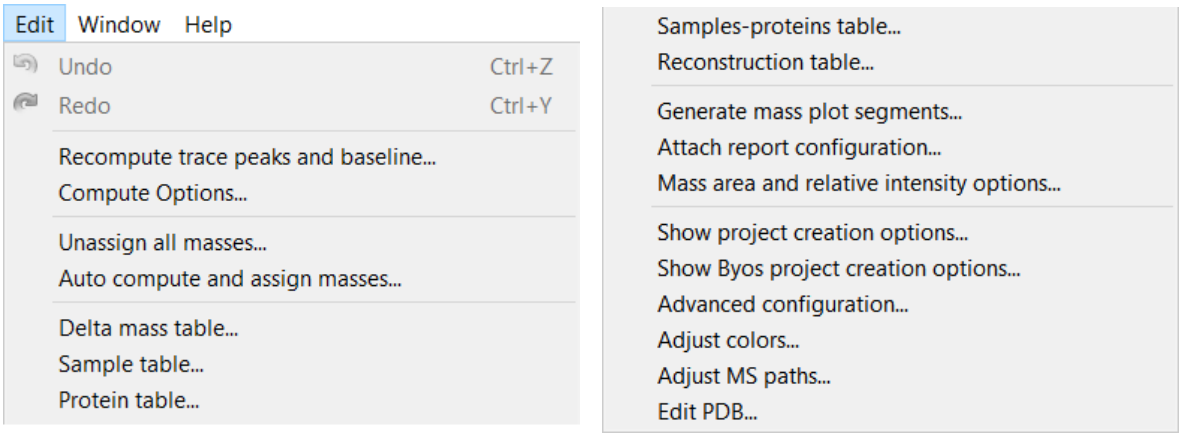
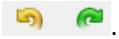


Figure 64: Edit menu

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

Recompute trace peaks and baseline opens the Trace peak options dialog that includes settings to modify and recompute the trace peaks.

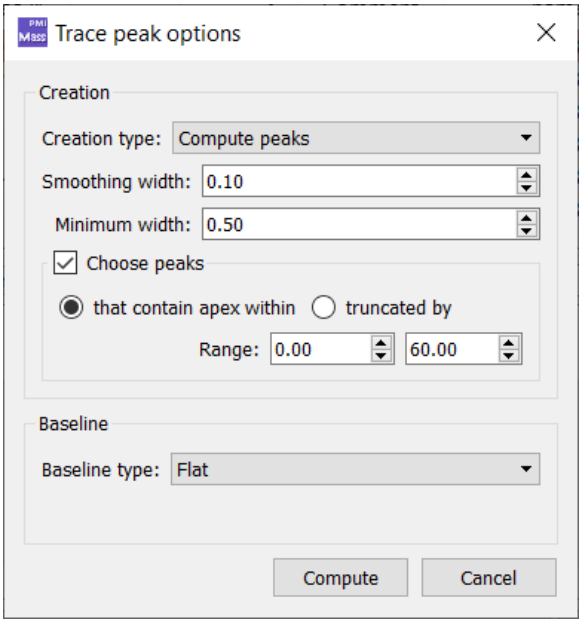


Figure 65: Trace peak options using Edit > Recompute trace peaks and baseline

This is equivalent to the dialog opened by the **Edit trace peak options** button in the **Sample Input** tab when creating a new project. Thus, the user has the option to alter the trace processing before and/or after project creation. For details, see the **Trace peak options** section.

Compute Options opens the **Trace peaks compute options** dialog, which includes a list of trace peaks, the samples that contain these peaks, and the peak start and end points. To change the deconvolution parameters (*m/z* range, m range, etc.) for a specific trace peak, click **Edit** for that peak. This opens the **Intact options** dialog:

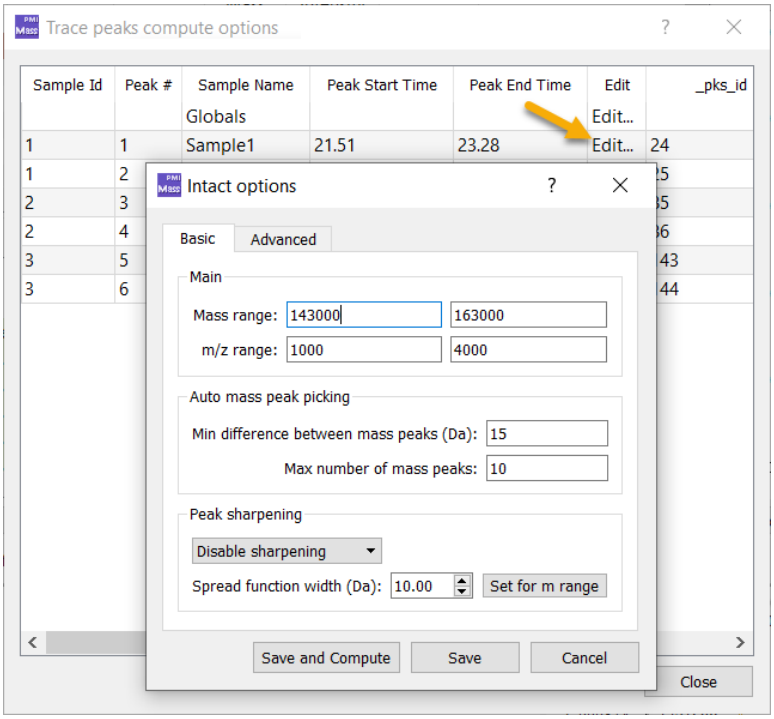


Figure 66: Compute options

The **Basic** tab contains the primary settings used in computing deconvolved masses. **Mass range** defines the range of neutral masses displayed in the Deconvolved mass spectrum. ***m/z* range** defines the segment of the MS1 spectrum used to compute neutral masses. **Min difference between mass peaks** prevents the peak picker from picking multiple points on top of a ragged or isotope-resolved mass peak. **Max number of mass peaks** sets a limit on the number of picked peaks. The **Peak sharpening** dropdown enables a `"super-resolution step"` that sharpens peaks beyond what is seen in the data. **Spread function width** sets the expected width of a peak when peak sharpening is enabled. A reasonable width is the square root of the peak mass in kilodaltons, for example, 12 for a spectrum from 140,000 to 150,000 Da. A too-narrow width will not sharpen much, and a too-wide width might split peaks. Peak sharpening on mass spectra that span a range of more than about 20,000 Da is not recommended, because no single width will be optimal for the entire range. Peak sharpening should also be avoided on isotope-resolved mass spectra. See the **Deconvolved Mass Spectrum Menu** section for more information about the basic and advanced Compute options.

After the settings are modified, click **Save and Compute**. The program computes an MS1 summed *m/z* spectrum, a deconvolved (neutral) mass spectrum, and assigns a peak list in the Masses table for each trace peak in the Trace Peaks table.

The **Name** column in the **Masses** table is also updated when newly added delta masses or proteins have been matched after recomputing. The new value in the Name column is most often created using the values in the **Protein name** and **Delta name** columns. The report is updated automatically with the new Name values.

Unassign all masses removes all mass peak assignments over all trace peaks.

Auto compute and assign masses computes and optionally assigns convolved masses using the last used set of compute mass settings. Check **Auto assign masses** to have the application assign deconvolved masses. Click **Selected Peaks** to assign masses to selected trace peaks. Click **All identified Peaks** to assign masses to all trace peaks.

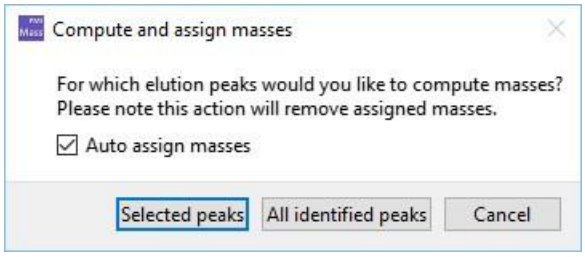


Figure 67: Compute and assign masses

The **Delta masses** table assigns peaks based on mass differences from Reference peaks. Check each group to include for peak assignments. The mass names and Dalton values can be edited by selecting them and typing new values. To add an additional delta mass record, click **Add row** and enter a new mass name and Dalton value to the new record at the bottom of the table. **Import** opens a saved delta mass table in CSV format. The Delta mass table is also useful in automated mass assignments when processing many data files.

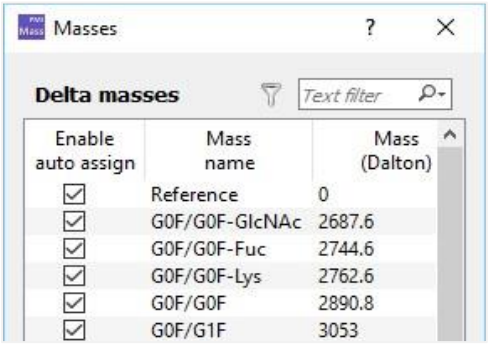


Figure 68: Delta masses

The **Sample table** is used to customize the Project view. To add a comment for a sample, click in the **Comment** cell and enter text. To add a new column, click **Add custom column**, enter the column name and an optional default value and click OK twice. The column will appear after Comment in the Project View. To remove a custom column, click **Remove custom column**, select the column and click **OK**.

The **Protein table** is used to add proteins associated with a sample. Proteins are described by name (Accession name), sequence, and average mass. They are assigned to samples by the Sample ID. A protein defined in this table will automatically be labeled as **Reference** during automated mass assignments. As above, custom columns can be added and removed.

The **Sample-proteins table** combines the Sample and Protein table so that they can be edited simultaneously. The Sample-proteins table is especially convenient for monoclonal antibodies (mAbs). Each row corresponds to one sample (MS spectrum file) with a mAb containing one light and one heavy chains. As with the **Protein table**, custom columns can be added and removed.

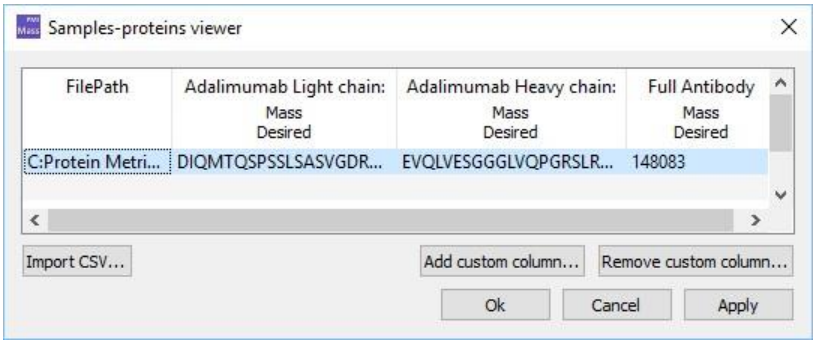


Figure 69: Sample-proteins viewer

Reconstruction table allows the user to reconstruct intact mass spectra of proteins from peptide mapping data Protein degradations at the amino acid level quantified by peptide mapping methods are convolved against each other to reconstruct a theoretical intact mass spectrum. Comparing the theoretically reconstructed intact mass spectrum against the experimental spectrum provides a way to compare peptide mapping data against intact mass spectrometric analysis. The differences between the two spectra can then be used to compare how well each method correlates with the other. This can then be used to adjust acquisition or data analysis settings accordingly. For detailed information, see the PMI Intact Reconstruction Beta Tutorial package available in the Current Release section at http://www.proteinmetrics.com/biopharma-download/.

Generate mass plot segments controls how neutral mass spectra will be plotted in reports. A small **Segment width** will give many plots; a larger width will give fewer plots but less detail.

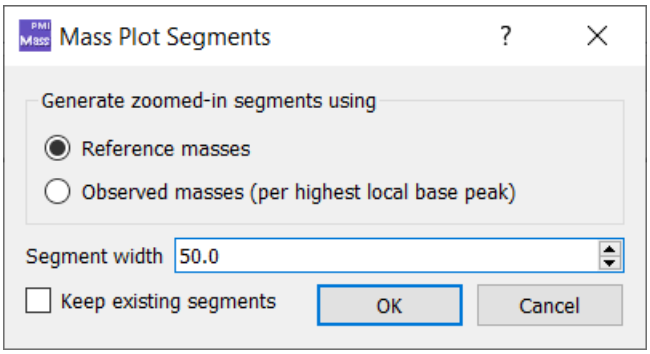


Figure 70: Generate mass plot segments

Plot segments of the size specified will automatically be generated around either referenced or observed masses.

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 extensions *.rptc or *.lvcfg. This menu can also be used to remove an attached report configuration file.

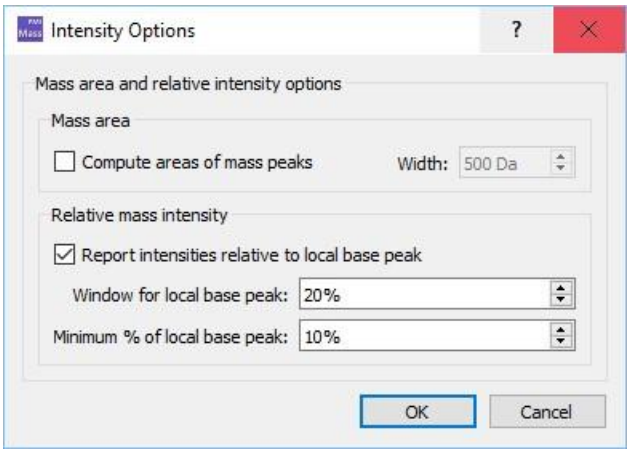


Figure 71: Intensity options

Mass area and relative intensity options control quantification method. The default method uses peak intensities (heights), but **Compute areas of mass peaks** uses area instead, and allows the user to define the width in daltons across which a peak is integrated to determine its area. **Report intensities relative to**

local base peak defines local peak groups. **Window for local base peak** sets the percentage range of the base peak that defines a local peak group. **Minimum % of local base peak** sets the minimum intensity for a reported peak.

Show project creation options displays in a single window the values and inputs used to create the current project. This data is read-only in this window. 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.

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:

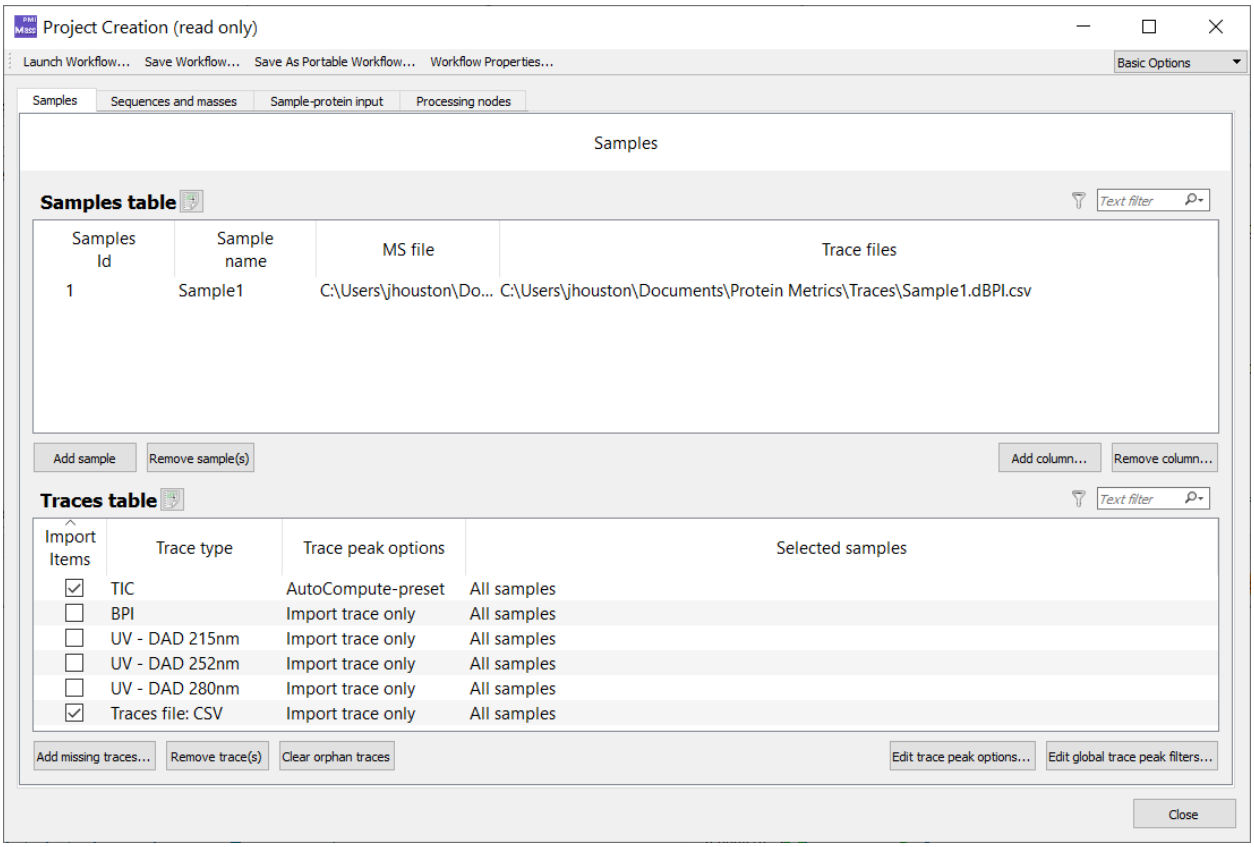


Figure 72: Show Byos project creation options

The **Advanced configuration** dialog is used to enter text commands, which are often new features still in beta testing. Text commands include controls for: use of isotope spacing to infer charge, removal of charge-one *m/z* peaks, expected mass differences between molecules (for example, 162 Da for hexose), and negative mode (for example, deprotonated proteins and DNA).

Adjust colors is used to modify the default colors associated with samples and traces.

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.

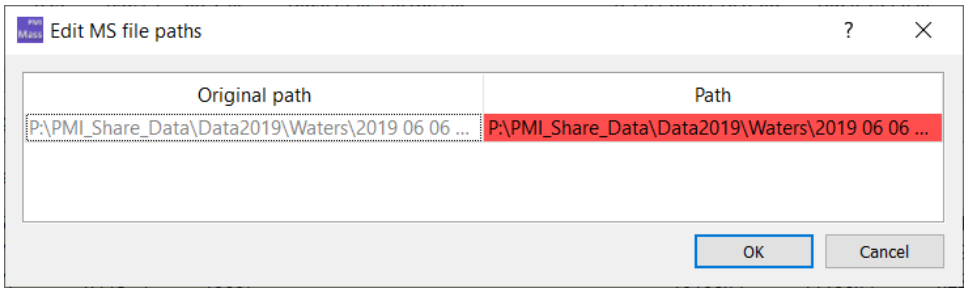


Figure 73: Editing the MS file path

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

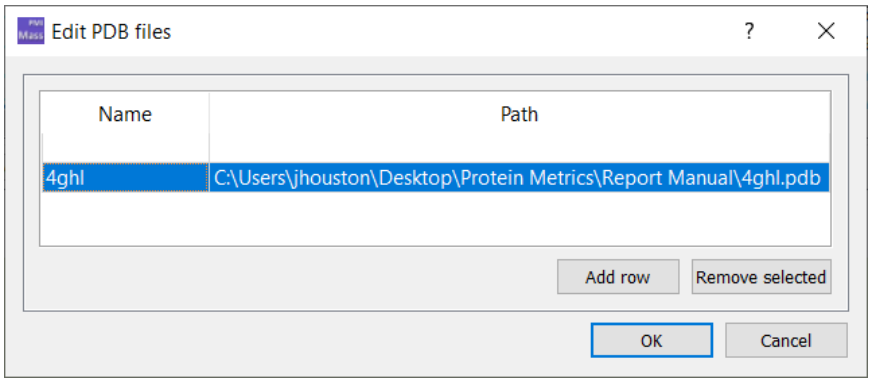


Figure 74: Edit PDB files

Window Menu

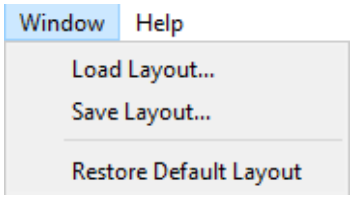


Figure 75: Windows menu

The **Window** pull-down menu manages layouts, the arrangement and sizes of the various table and plot views. **Load Layout** opens a saved layout stored to files with extension .ini. **Save Layout** saves the current layout to an .ini file. **Restore Default Layout** applies the layout used when the application is first opened. The current layout is customized 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.

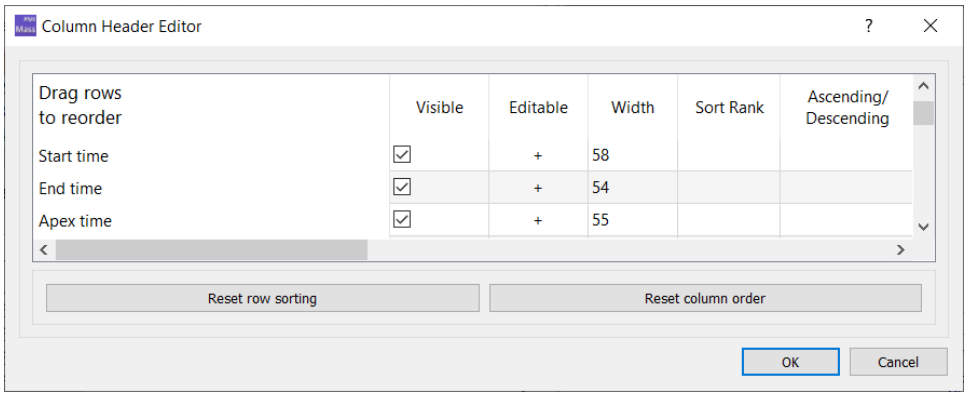


Figure 76: Column Header Editor

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.

Help Menu

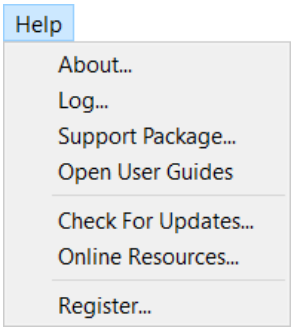


Figure 77: Help menu

The **Help** pull-down menu provides information about the software. 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 staff via support@proteinmetrics.com. **Support Package** collects all relevant information helpful for troubleshooting problems together with Protein Metrics staff via support@proteinmetrics.com. **Open User Guides** opens the Document directory where this and other useful guides can be found, including PMI Intact Analysis Manual.pdf. **Check For Updates** checks whether software updates are available on www.proteinmetrics.com. **Online Resources** opens the www.proteinmetrics.com/secure-resources site which offers specialized workflows and additional information about Protein Metric products. **Register** is used to activate the software upon first use.

Tables and Menus

The menu bars at the top of the three table views, Project, Trace Peaks, and Masses, share icons that manage hierarchical lists and filter 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 Trace Peaks table a row is a trace peak and a sub-row is a mass peak within the deconvolved spectrum.

- The  icon exports the table to a .csv file for opening with Excel.
- The  icon opens a dialog to create custom filters for the data table. 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.

com

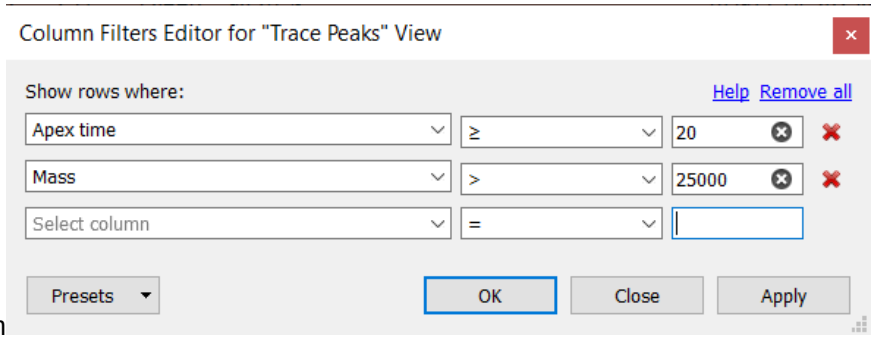


Figure 78: Column Filters Editor

- 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, Trace Peaks, and Masses 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.)

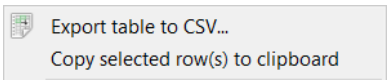


Figure 79: Table right-click menus

- **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.

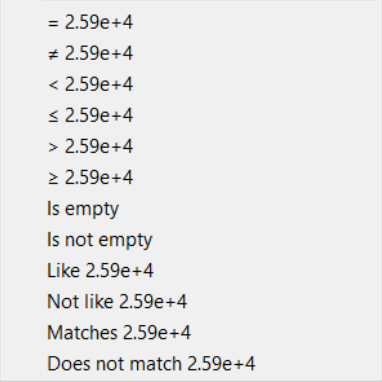


Figure 80: Right-click field searches

A table view's menu bar may also include buttons and icons specialized for that table.

Project Table and Menu

The **Project** table contains information about the MS samples:

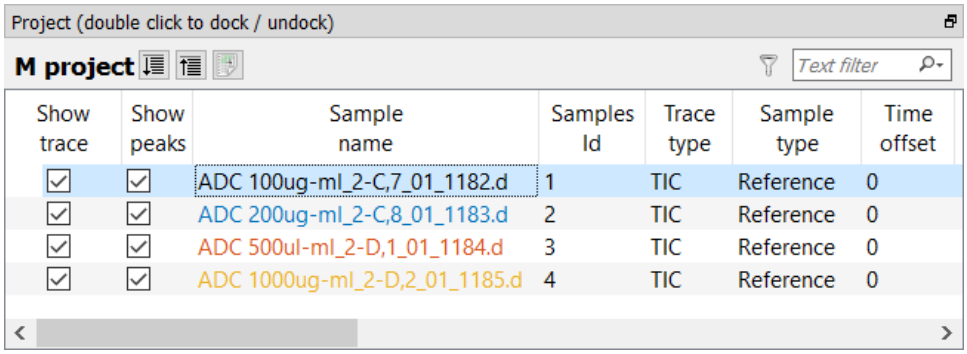


Figure 81: Project Table

Check or uncheck **Show trace** or **Show peaks** to show or hide these features for particular samples. To edit a MS sample, right-click the entry to edit under the **Sample Name** header, click **Edit selected row(s)**, edit the name, and then click **OK**.

Trace Peaks Table Menu

The **Trace Peaks** table contains details about identified peaks, organized by sample:

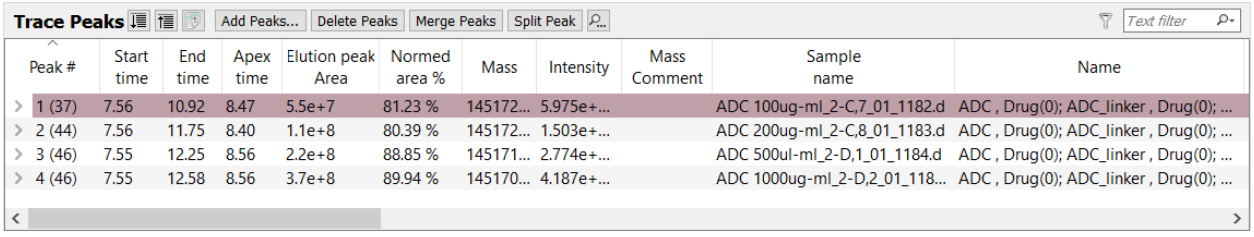


Figure 82: Trace Peaks table

To view specific peak data for a sample, click the > symbol to the right of that sample:

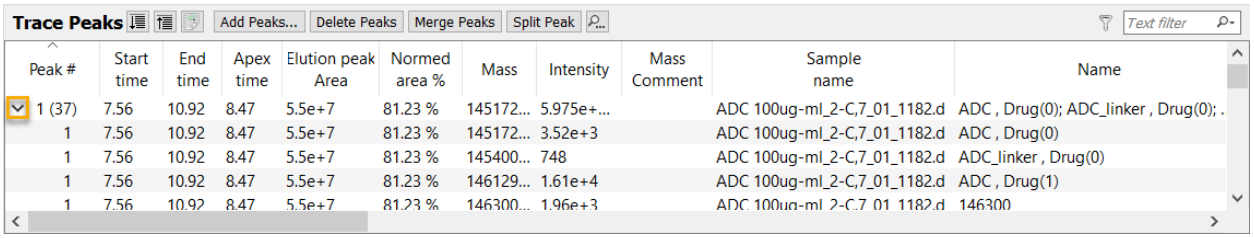


Figure 83: Trace Peaks sub-table

The Trace Peaks table includes the following specialized buttons:

- **Add Peaks** opens a dialog for the input of start and end times for a new peak.
- **Delete Peaks** deletes the selected peak.
- **Merge Peaks** merges a contiguous set of selected peaks into a single peak. Use left-click and shift-click to select a set.
- **Split Peak** splits a peak into two peaks. The time values for the two peaks can then be adjusted separately.
- **Filter options**: The magnifying glass opens a dialog that allows the user to adjust the trace peak filter by overall trace time or minimum peak area (percent of TIC) to determine which trace peaks will be processed.

The Trace Peaks table includes the specialized right-click menu **Edit selected row(s)**. This menu is available from certain numeric fields such as Start time, End time and Apex time, and for annotation fields, such as Mass Comment, Peak Comment and Visible. This allows the user to overwrite the computed values for those fields for the given sample, as well as mark or comment on samples peak records.

Masses Table Menu

The **Masses** table reports the masses of the most intense peaks that are selected in the Trace Peaks table.

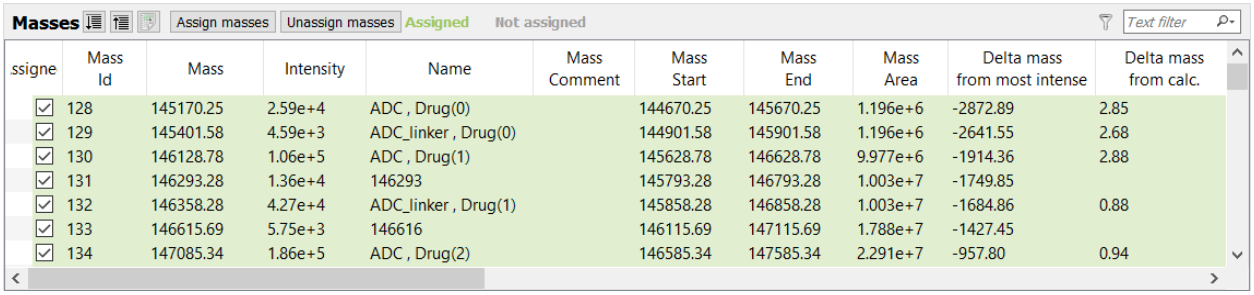


Figure 84: Masses table

The Masses table includes the following specialized buttons:

- **Assign Masses** assigns selected rows, an alternative to checking the Assigned boxes. If there is no selected row, **Assign Masses** opens a dialog to define a new mass by name, neutral mass, start and end times, intensity, and comment.
- **Unassign Masses** can be used to unassign selected rows.

The Masses table includes the specialized right-click menu **Edit selected row(s)**. This menu is available from certain numeric fields such as Mass Start, Mass End and Mass area, and along with other fields, such as Mass Comment and Name. This allows the user to overwrite the computed values for those fields for the given sample, as well as mark or comment on mass records.

Plots and Menus

The menu bars at the top of the three plot views, Trace plot, MS1, and Deconvolved mass spectrum, share icons that that support generic operations on plots. These icons appear in almost all of Protein Metrics' software products.

- The icon resets the plot to default zoom level. Shortcut = double left-click.
- The icon enables zooming in. After clicking, the cursor changes to . 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 described 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 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 Norm. button scales the y-coordinates so that the tallest peak has intensity 1.
- The icon manages plot segments or zoom states. The number displayed in the icon shows how many plot segments are stored (named by the date and time of the save). To save the current zoom state as a plot segment, simply click the icon. To switch to a previously stored plot segment, click the drop-down arrow and select the segment. To add, remove, rename or edit plot segments, click the drop-down arrow and then click **Edit**. To display all the plot segments at once, as seen on the right, check **Show segments** in the Plot segments dialog.

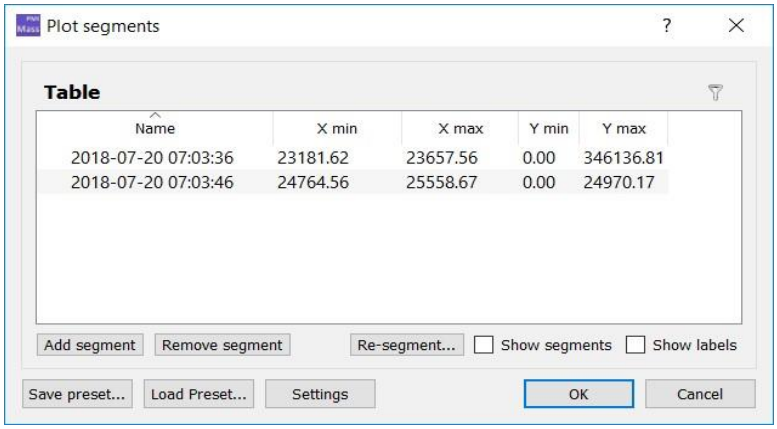


Figure 85: Defining plot segments

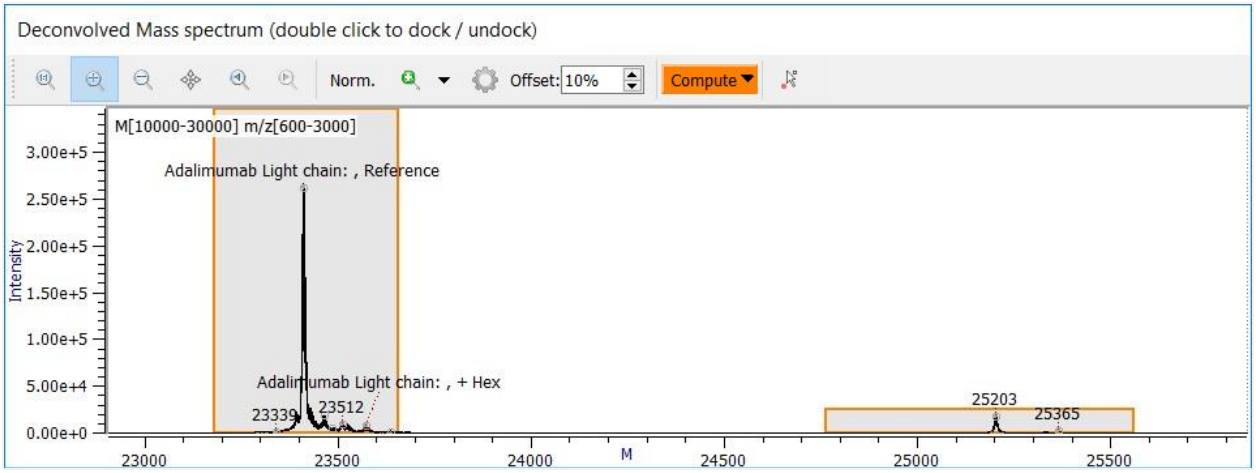


Figure 86: Spectra resulting from defined plot segments

- The icon manages how plots are displayed (render options), as well as zoom modes.

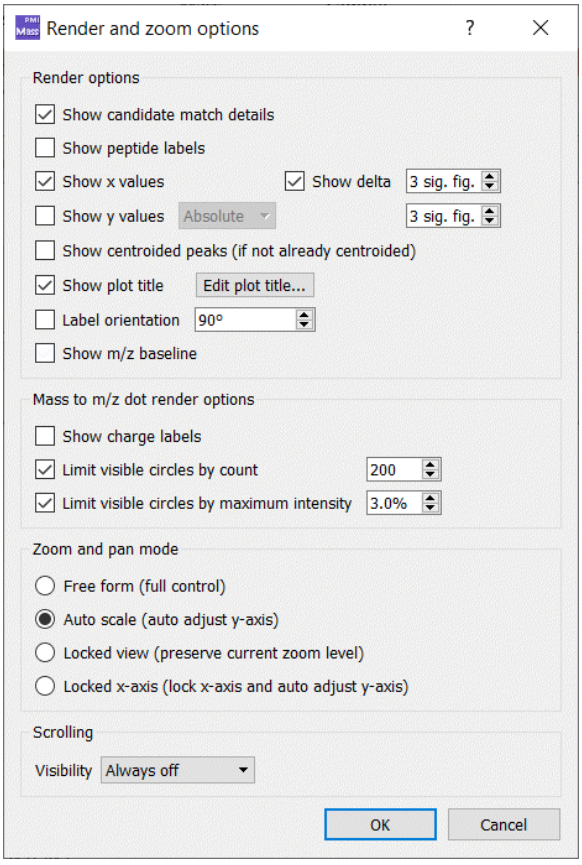


Figure 87: Render and zoom options

- **Show candidate match details** (MS1 only) applies to Chromatogram Analysis only.
- **Show peptide labels** (MS1 only) displays peptide labels in red below the identifying labels for the corresponding peaks.

- **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** displays the x or y differences from a reference peak. To display the deltas, hover the cursor over the reference peak (which displays as 0). The other peaks show the positive or negative differences for that axis.
- **Show centroided peaks** darkens the peak positions that are determined to be centroided.
- **Show plot title** displays the title of the plot, for example, “M[10000-30000] m/z [600-3000]” seen above. The information shown in the plot title can be customized by clicking the Edit plot title button. This opens the Template Editor dialog. The editor accepts free text or programmatic “short codes” that insert data values into the title. For example, “Time range [[StartTime] - [EndTime]]” displays the starting and ending times derived from the trace as part of the title for any of the plots.
- **Show annotations** (Trace plot and Deconvolved mass spectrum) displays text values beside peaks, which can be customized with the Edit annotations button. This opens the **Template Editor** dialog, which displays the default annotations:

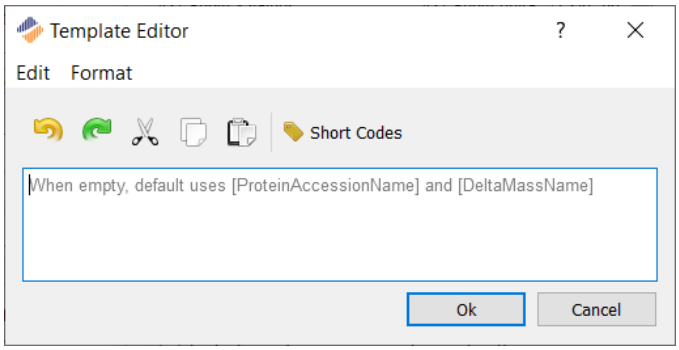


Figure 88: Template Editor for Edit annotations

The editor accepts free text or programmatic “short code” that use data values in the peak annotations. For example, “[ExpectedMass]” displays expected mass labels for individual peaks in the Deconvolved mass spectrum.

- **Label orientation** sets the angle for the label text, where 0º is vertical.
- **Show m/z baseline** (MS1 only) – overlays a smooth m/z baseline.
- **Show selected plots only** (Trace plot only) shows only the selected trace trace(s).
- **Mass to m/z dot render options** (MS1 only) control the display of the colored dots linking neutral mass peaks to the MS1 spectrum. **Show charge labels**, which is on by default, displays the charge assignments next to the colored dots. **Limit visible circles by count** places a limit on the number of dots of each color, and **Limit visible circles by maximum intensity** places a minimum intensity threshold, a percentage of the maximum y-value for that color. Dots are placed at computed m/z values for a selected neutral mass peak, regardless of whether the m/z value is a peak (local maximum); m/z values are adjusted slightly to avoid falling into a gap between isotope-resolved peaks. There is no limit on the number of selected mass peaks, but there is a limit of 10 dot colors.

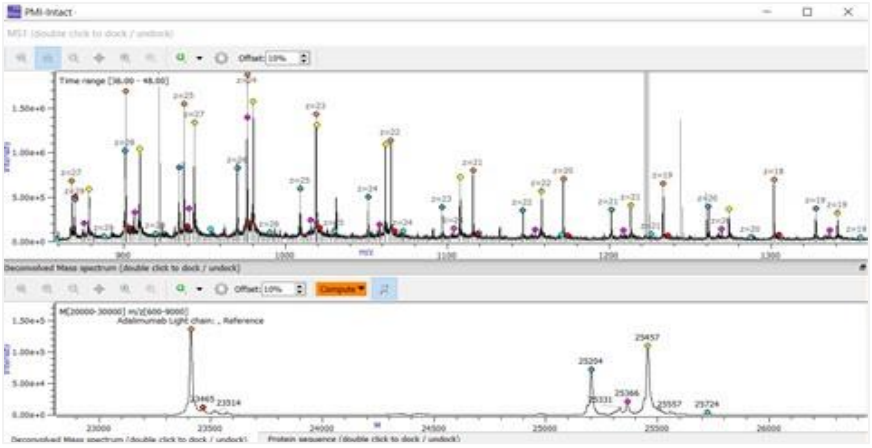


Figure 89: Mass to m/z dot renderings

Zoom and pan mode options include:

- **Free form** mode to select the desired y range as well as x range
- **Auto scale** mode to select only the x range (the y range is then determined by 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 trace peaks
- **Locked x-axis** mode which 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

- The **Offset** entry on a plot menu bar controls the vertical spacing for stacked plots. Offset of 100% means that plots stack top-to-bottom without any overlap. Offset of 70%, as seen to the right, means that plots overlap by 30%, and offset greater than 100% leaves white space between stacked plots.

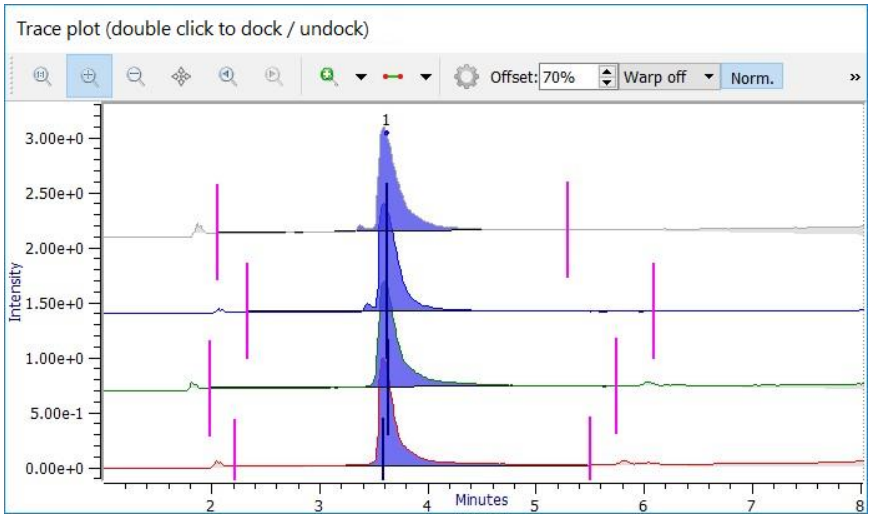


Figure 90: Trace offsets

Plot Right-Click Menus

The Trace plot, MS1, and Deconvolved mass spectrum have a variety of context menus for plot styling and exporting revealed by a right-click on the plots:

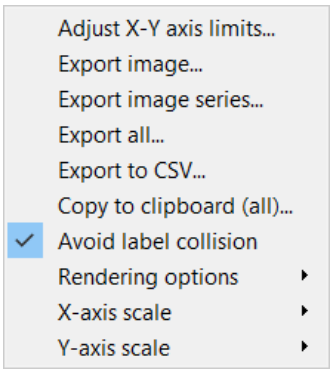


Figure 91: Plot right-click menu

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. 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:

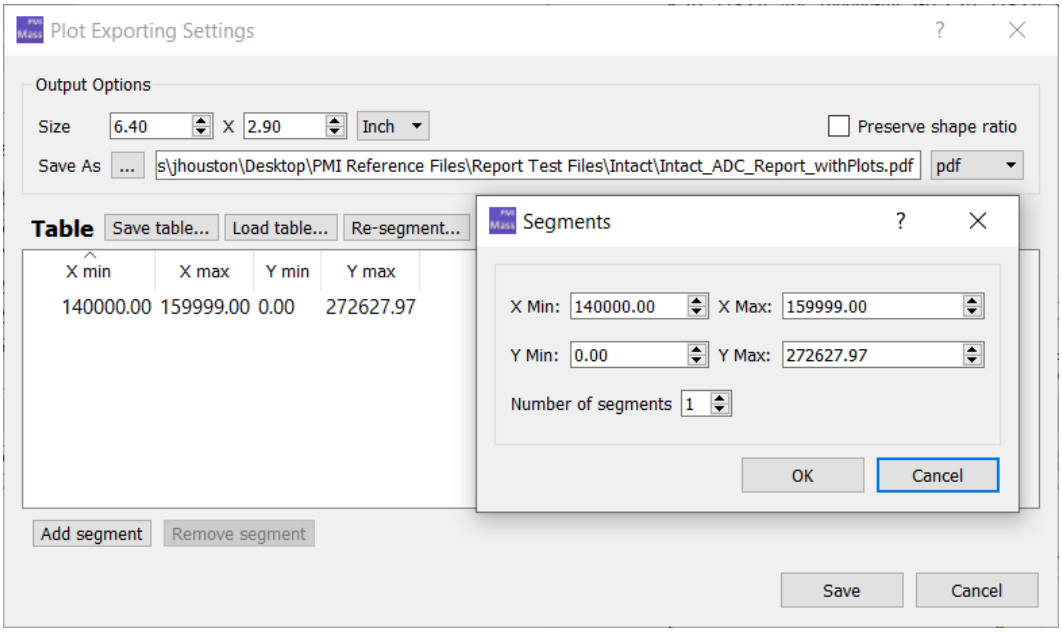


Figure 92: Plot Exporting Settings with the Re-segment option

- **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:

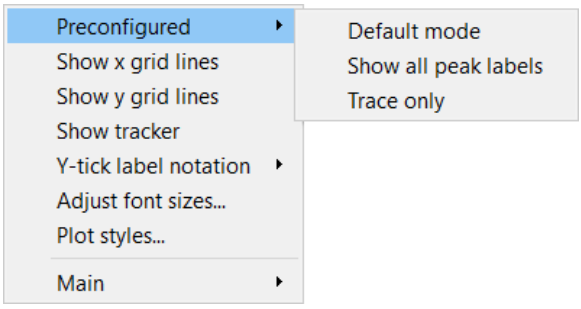


Figure 93: Rendering options sub-menu

  - **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** switches between display of **Absolute** y value, and **Percent** value as a function 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.

- X-axis scale and Y-axis scale toggle between linear and logarithmic scales.

Trace Plot Menu

The Trace plot menu bar includes the following specialized controls:

- The icon shows the options for managing baseline anchors. The Trace plot baseline has no effect on the neutral mass spectrum, only the areas of the trace peaks, so most users can ignore these options:
  - The icon moves the ends of a baseline anchor. Move the cursor to either end of the baseline to see the anchor displayed in green and the cursor changed to an arrow. Click, and drag the end of the baseline to the new location.

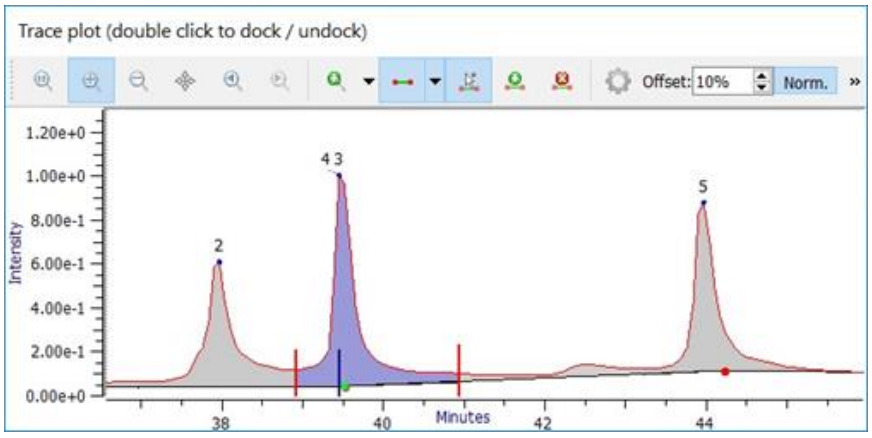


Figure 94: Moving the baseline

  - The icon inserts a new baseline anchor (polygon vertex). Move the cursor to the baseline to see the anchor displayed in green, as shown at the right, and the cursor changed to an arrow. Click anywhere within the baseline anchor to divide it into two parts at that point. The Move baseline anchor mode can be used to move the endpoints of the baseline anchors.
  - The icon deletes a baseline anchor. Move the cursor to the end of the unwanted baseline to see the anchor displayed in green and the cursor changed to an arrow. Click to delete that baseline anchor.
- The **Center** button pans the trace to put a selected peak in the center.
- The **Labels** button opens the Template Editor for peak labels. The editor accepts free text or programmatic `"short codes"` that can place data into peak labels.
- **Minutes/pI** radio buttons appear when an electropherogram is loaded when using the **iCIEF-MS with Blaze from Intabio** workflow. The user can change the X-axis unit label to **Isoelectric point** (pI):

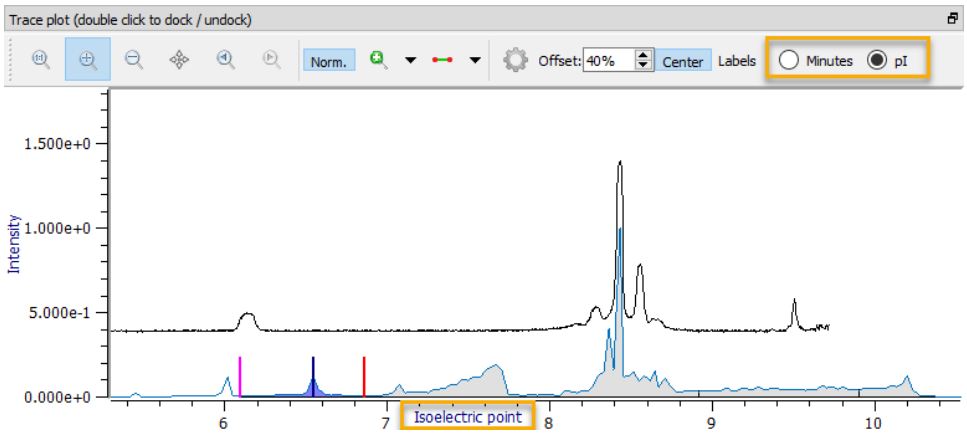


Figure 95: Electropherograms expose radio buttons to choose pI units

The Trace plot includes the following specialized right-click menu:

- **Show/hide sample name** displays or removes the labels from the staggered plots.
- **Sample name position** opens a dialog to set label placement and alignment:

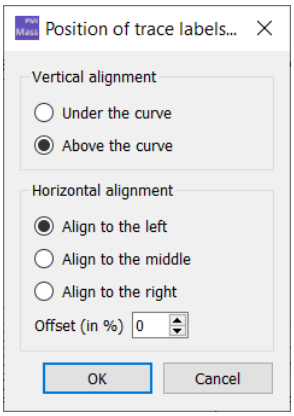


Figure 96: Sample name position

By setting **Vertical alignment** to **Above the curve**, the sample name label for the bottom trace will be displayed correctly:

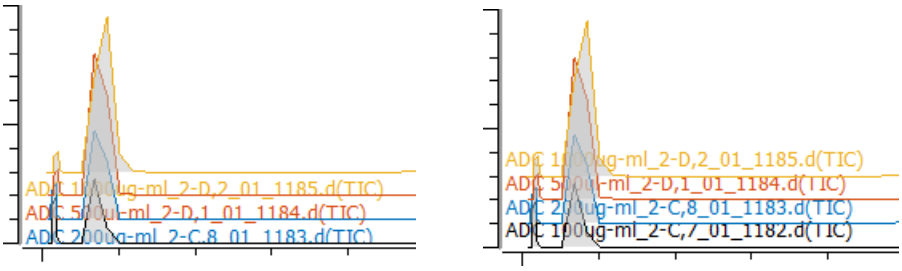


Figure 97: Trace plot labels displayed below (left) and above the curve (right)

- **Labels** opens the Template Editor, allowing the user to define the peak label:

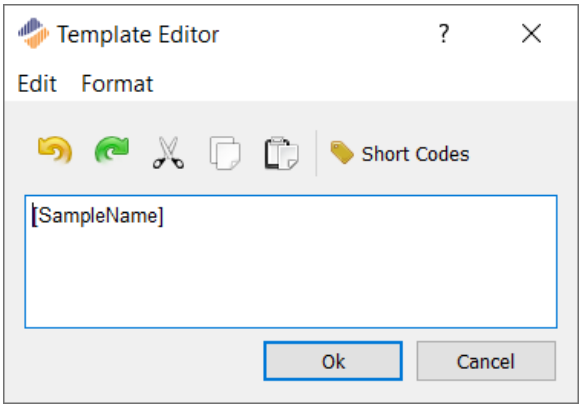


Figure 98: Template Editor for labels

The editor accepts free text or programmatic "`short code`"

Deconvolved Mass Spectrum Menu

The Deconvolved Mass spectrum menu includes the **single most important** specialized control: the orange **Compute** button, which sets the parameters for the deconvolution. A click on this button opens the **Intact options** dialog:

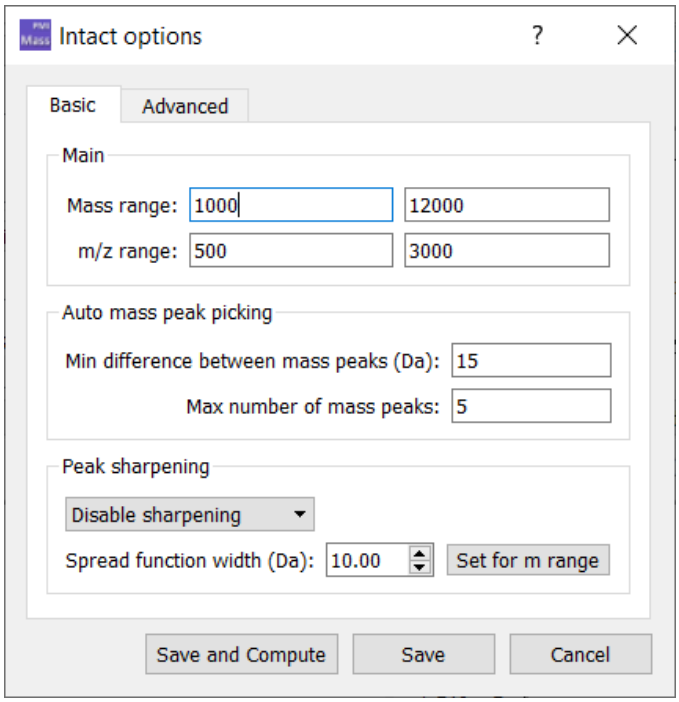


Figure 99: Intact compute options Basic tab

The **Basic** tab contains the primary deconvolution parameters. **Mass range** and **m/z range** set ranges for neutral masses and m/z, respectively. **Min difference between mass peaks** and **Max number of mass peaks** control peak picking. **Peak sharpening** deconvolves a "`point spread function`" to give super-resolved mass peaks and turn shoulders into separate peaks. **Spread function width** gives the halfwidth of the point spread function; a reasonable width is a little less than the square root of the peak mass in kilodaltons, for example a width of 10 – 12 for peaks in the 140 – 160 kDa range. We do not recommend peak sharpening for mass ranges wider than about 20,000 Da or for mass spectra with more

than about 40,000 points. Thus, it is not advisable to use peak sharpening on a spectrum with mass range 140,000 to 160,000 with mass spacing (see below) of 0.2, but it would be okay if mass spacing is changed to 0.5.

The most important parameter is the **Mass range**. The example in Figure 2 and Figure 3 at the beginning of the manual used a mass range of 146,000 – 150,000 Da, wide enough to include the major proteoforms of Adalimumab, but narrow enough to run quickly and achieve maximum sensitivity and resolution. The default mass range is 10,000–160,000 Da, which gives a wide survey of the ions in the *m/z* spectrum. A typical workflow starts with a wide range and then narrows the range to capture all the major proteoforms. Narrowing the range to capture only a single proteoform and exclude all the others is not recommended, unless the *m/z* range can also be narrowed to capture the single proteoform. Forcing essentially all the ions in the *m/z* range to map onto the mass range is a deliberate design choice: this design allows Intact Analysis to deconvolve spectra with low signal-to-noise ratio so long as the user has some prior knowledge of the protein's intact mass.

Noisy MS1 spectra tend to produce "furry" peaks at the low end of the mass range, due to chance ratio relations among noise peaks. Thus extending the **Mass range** to lower masses (for example, using 5 – 30 kDa for mAb subunits rather than 20 – 30 kDa) may actually improve the deconvolution of the masses of interest by moving the noise farther away. The Advanced configuration parameter **MinPeakMass** (see Appendix) can be used to exclude low-mass peaks from peak picking.

The **m/z range** is less crucial than the m range, but best results will be obtained with a range that covers the signal and excludes the noise. The m/z range also constrains the color dots that are rendered on the MS1 plot when the selection marquee  is toggled.

If the MS1 spectrum contains two or more molecules with non-overlapping *m/z* ranges, for example, a full mAb at 150 kDa with signal at *m/z* 2000 – 3000, and a free light chain at 25 kDa with signal at *m/z* 1000 – 2000, then the best detail will be obtained by performing multiple deconvolutions with different *m/z* and mass ranges. An initial survey deconvolution, along with inspection of peaks with colored dot peaks, will guide the mass and *m/z* ranges for the subsequent deconvolutions. PMI Intact Analysis has no set limits on mass, *m/z*, nor charge, and it has been used successfully on masses from 2 to 2000 kDa, *m/z* from 400 to 20,000, and charges from 2 to 200.

The Advanced options mainly concern resolution. You will need to use this tab if your aim is to produce **isotopically resolved neutral mass spectra**.

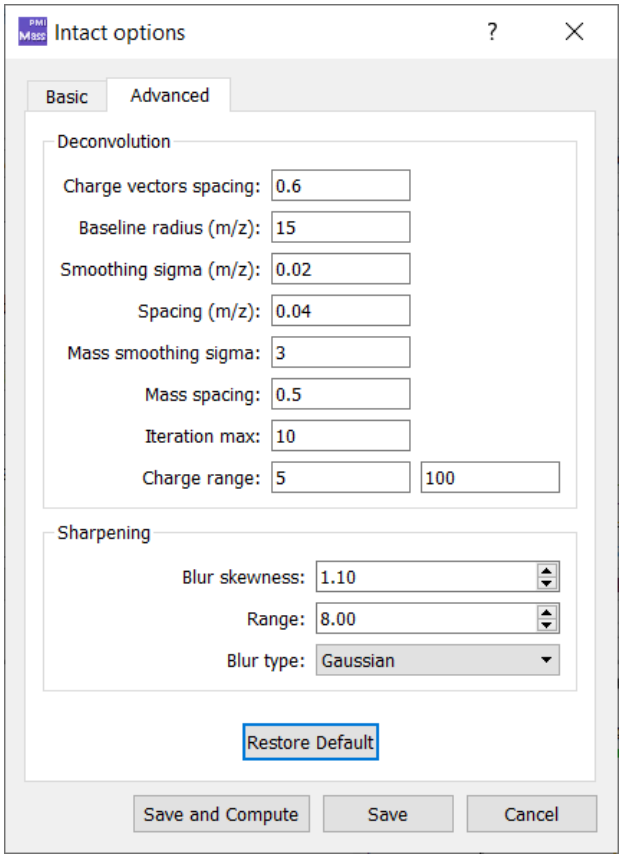


Figure 100: Intact compute option Advanced tab

"Charge vectors" give the charge assignment probabilities for each small interval of *m/z* points. Deconvolutions of most MS1 spectra will be almost exactly the same with any **Charge vectors spacing** from 0.2 to 1 *m/z* units (Thomsons), but a narrow spacing of 0.1 may give better results on isotope-resolved MS1 spectra with interleaved signals, and a wide spacing of 2 may give better results for native MS with broad *m/z* peaks.

Intact Analysis removes an *m/z* baseline before deconvolution; this step and removal of charge-one or stop-list peaks (see Appendix) are the only steps in the algorithm that do not conserve ions. **Baseline radius** controls the stiffness of the baseline. A baseline radius of 8 gives a flexible baseline that will cut into *m/z* peaks broader than 8 Thomsons; this will often give better visual separation of neutral mass peaks but may distort peak areas. A baseline radius of 30 will give a stiffer baseline that cannot cut into *m/z* peaks narrower than 30 Thomsons. An even larger value, 100 or more, may be needed for native MS. The default value of 15 is a compromise.

Spacing (m/z) controls the spacing of sample points in the *m/z* spectrum. The raw MS1 data is represented as a continuous piecewise-linear function that can support any spacing of sample points, but *m/z* spacing finer than the finest spacing in the original data will slow the computation without adding resolution. Reasonable values for **Spacing (m/z)** are in the range 0.005 – 0.05 for QTOF instruments, which have almost the same resolution at all *m/z*; the low setting of 0.005 would be appropriate for Bruker maXis and the higher setting of 0.05 for older instruments with lower resolution. For Orbitrap, the resolution depends upon *m/z*; the setting of 0.005 shown above is for isotope-resolved 25 kDa masses (antibody subunits). For native MS on Exactive EMR with *m/z's* in the 5000 – 10,000 range, a spacing of 0.1 is fine enough. **Smoothing sigma (m/z)** is typically set to the same value as Spacing (*m/z*), but a larger value can be helpful for producing an appropriately smoothed neutral mass spectrum with less smoothing at lower mass and more smoothing at higher mass.

Mass spacing controls the spacing of points in the neutral mass spectrum. To preserve isotopic resolution, spacing should be set to 0.1 or even 0.05. If the MS1 spectrum does not have isotopic resolution, or isotopic resolution is not needed for analysis, mass spacing in the range 0.2 to 1 is best for target molecules below 200 kDa. Spacing of 10 Da or more is best for targets above 300 kDa. For mass spectra without isotopic resolution, **Mass smoothing sigma** in the range of 2 – 5 will smooth jittery peaks in the range 20 – 200 kDa; larger values will be needed for larger masses. For mass spectra with isotopic resolution 0.1 will work.

Iteration max set to 10 will work for most purposes. A larger value, say 20 or 30, can be helpful for lower signal-to-noise spectra that take longer to converge.

Charge range is best set to a wide range, in which case the charge range will be implied by the mass and *m/z* ranges. The example in Figure 2 and Figure 3 has *m/z* range 2200 – 4000 and m range 146,000 – 150,000, so the charge range spans 36 (146,000 / 4000) to 68 (150,000 / 2200). The default range of 5 – 100 covers most applications, but 5 will need to be reduced for deconvolutions with mass range starting below 10 kDa, and 100 increased for targets that may have charges above 100.

Peak sharpening uses the **Spread function width** set in the Basic tab to deconvolve the data. The Advanced tab offers three infrequently used controls for peak sharpening. **Blur skewness** controls the asymmetry of the point spread function; the default value of 1.1 means that the right tail has sigma (standard deviation) 10% bigger than the left tail. 1.2 gives even more tailing; 1.0 gives a symmetric point spread function. **Range** sets the length of the tails in standard deviations; a small value of 5 or 6 may work better in the case of Lorentzian point spread. **Blur type** has two choices: Gaussian (skinny tails) and Lorentzian (fat tails). Lorentzian should use a slightly smaller sigma than Gaussian.

After the deconvolution settings are modified, click **Save and Compute**. For difficult data, you may need to explore the space of settings to obtain the best deconvolution. As a general rule, correct deconvolutions are stable under changes of parameters. If a neutral mass peak appears with a specific mass range setting, but disappears when the settings are varied, the peak may be an artifact.

The Deconvolve Mass spectrum view includes another very important specialized menu: the Toggle selection marquee  icon. A click on this icon sets the cursor to selection mode. When a picked peak is clicked in the plot, a colored dot appears over that peak (Figure 101) and the same colored dots appear over the corresponding peaks in the MS1 view (Figure 102). Click on top of another deconvolved peak to assign a different colored dot to that peak and its MS1 peaks. The colored dots in the MS1 spectrum are the best way to tell true mass peaks from artifacts; the MS1 dots for true mass peaks have contiguous charges and tend to hit local maxima for *m/z* intensity.

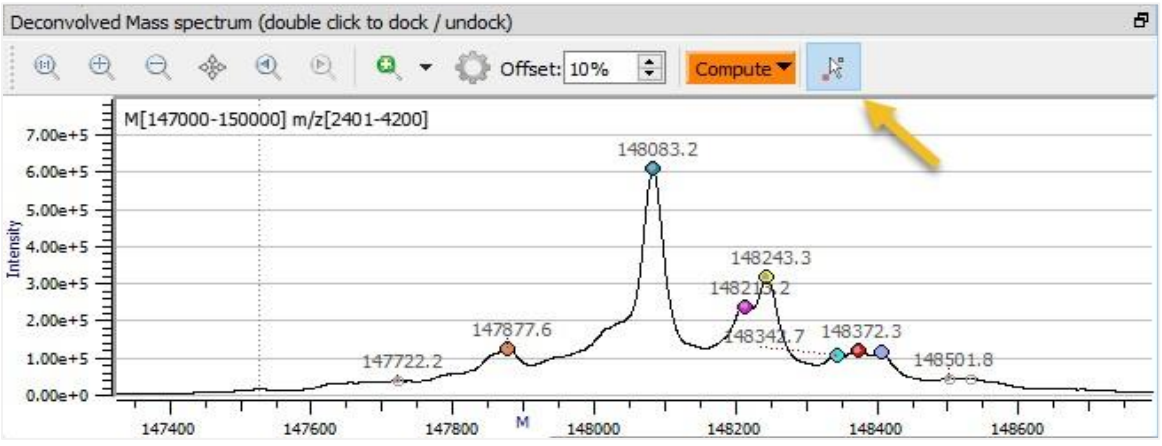


Figure 101: Deconvolved Mass spectrum peaks marked with colored dots in selection marquee mode

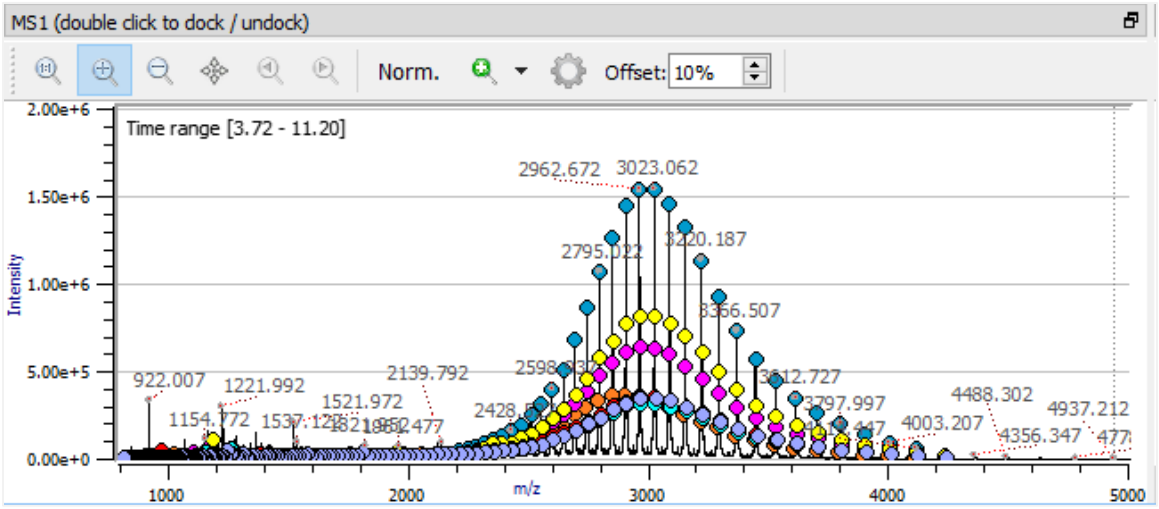


Figure 102: Corresponding peaks in MS1 plot are marked by the same colored dots

The m/z range settings in **Intact options** dialog, opened by a click on the orange **Compute** button will constrain the rendering of the colored dots in the MS1 plot.

Dragging the cursor over a rectangular area of the plot selects all the picked peaks in that area. Dragging the cursor while holding down shift adds more selections without losing current selections. Click outside the peaks to remove all colored dots.

A second click on a selected mass peak will open a dialog box to assign the peak; this allows the user to add a name to the peak and to make the peak persistent.

When the  icon is clicked again to turn off the selection mode, peak selections remain. The cursor can now be used to zoom without affecting the peak selections. Peaks can also be selected by selecting rows in the **Masses** table as explained above. Shift-click and control-click can be used to select multiple rows in the **Masses** table.

Appendix

Intact Analysis: Advanced Commands

Intact Analysis facilitates quick and accurate deconvolution of intact mass spectra and automatic assignment and annotation of the resulting spectra. However, there are many ways to customize the processing to fit specific needs. This section describes several text-format Advanced Commands that will enable finer control over processing. Protein Metrics uses Advanced Commands to test new ideas, beta-test new features, and enable specialized options, without adding complexity to the graphical user interface.

Introduction

Intact Analysis Advanced commands control deconvolution, mass peak selection, mass calculation, annotation and reporting. These commands are preceded by the entry [Intact] to identify them as control commands. Spaces between the command text and values do not affect processing (text=1 or text = 1 are equivalent). Advanced commands may be entered during project creation in the **Advanced configuration** panel of the **Advanced** tab or after project creation by selecting **Edit -> Advanced configuration**.

Peak Detection and Assignment

1. **Automatic Assignment of Reference Masses** assign reference masses from Protein or Sample-protein input even if there are no detectable peaks at the reference masses.

```
[Intact]
ForceShowMassesOfInterest = 1
```

2. **Stop List** removes unwanted signals from the spectra before processing. For example, the user may wish to remove intense calibration ions. The list consists of *m/z* values used to remove signal from the MS1 data before deconvolution. The example below shows removal of *m/z* peaks from Agilent tuning mix.
    a. UseStopList – This enables or disables the use of the Stop List.
    b. StopList – This identifies the *m/z* values of signals to remove.
    c. StopTolerance – This sets the *m/z* interval used for matching peaks on the StopList.

```
[Intact]
UseStopList = 1
StopTolerance = 0.1
StopList = 622.02896, 623.032, 922.009798, 923.012,
1221.990637, 1222.993, 1521.971475, 1522.974,
1821.952313, 1822.955, 2121.933152, 2122.936
```

3. **Remove Singly Charged Peaks** allows the user to remove singly charged peaks with *m/z* (maximum 2500). For example, a value of 0.02 directs the software to remove singly charged peaks down to 2% of base peak.

```
[Intact]
RemoveChargeOne=0.02
```

4. **Isotope Peak Spacing** directs the software to use isotope peak spacing (as well as multiple charge states) to determine charge for ions with charge up to the input number (maximum of 12). Isotope spacing helps the deconvolution whenever peaks are isotope resolved and do not appear in 3 or more charge states.

    IsotopeSpacingThreshold sets an intensity threshold. A value of 0.05 limits isotope spacing to peaks down to 5% of base peak. The default is 10% of base peak.

```
[Intact]
IsotopeSpacing=5
IsotopeSpacingThreshold=0.05
```

5. **Monoisotopic Mass** directs the software to compute monoisotopic mass using a protein averagine model for mass peaks up to the set MaxMonoisotope value. This command should be used in conjunction with convolution parameters that support isotopic resolution (for example, *m/z* spacing of 0.01 Thomsons and mass spacing 0.1 Da, with similarly small smoothing sigmas). However, the software will fit the averagine model whether or not the mass peak has isotopic resolution.

```
[Intact]
MaxMonoisotope=30000
```

6. **Known Mass Deltas** assigns known mass differences. For example, a mass of 162.1 can be assigned to glycosylation or the drug mass for antibody-drug conjugates (ADCs), to help determine charge of *m/z* peaks.

    CombFilter counts the number of KnownMassDelta to the left and the right. A value of 1 is suitable for glycosylation and ADCs. A larger value, for example, CombFilter=3, is appropriate for targets with long chains of equally spaced masses, such as nanodisks or PEGylated proteins. Excessively large values may cause artifacts.

```
[Intact]
KnownMassDelta=162.1
KnownMassDelta2=111.1
```

```
KnownMassDelta3=222.2
CombFilter=1
```

7. **Noise Level** controls the minimum signal-to-noise ratio of a picked peak in the neutral mass spectrum. Use of EnableBaselineRemoval (see below) improves the signal-to-noise ratio, so there may be no need to adjust NoisePercentileFactor.

    a. **NoisePercentileFactor** sets the minimum signal-to-noise ratio (amplitude of mass peak relative to amplitude of background noise peaks – see below) for picked mass peaks. A low value such as 2 will pick peaks barely above the noise; a high value such as 10 will pick only clear peaks.

    b. **NoiseSigmaFraction** is the fraction of mass bins assumed to contain only background noise. For example, a value of 0.25 indicates that at least 25% of the mass bins in the neutral mass spectrum are assumed to be without signal peaks. There should be no need to adjust this parameter unless the mass spectrum is extremely crowded with true masses.

```
[Intact]
NoisePercentileFactor = 4
NoiseSigmaFraction = 0.25
```

8. **Baseline Removal** enables baseline removal in the neutral mass spectrum.

    a. **EnableBaselineRemoval** – Set this to 1 to enable baseline removal from the m spectrum.

    b. **BaselineRemovalFraction** is the fraction of mass bins assumed to contain only background noise. In almost all neutral mass spectra, peaks occupy less than 80% of the mass bins so any value from 0.05 to 0.2 should give similar results.

```
[Intact]
EnableBaselineRemoval = 1
BaselineRemovalFraction = 0.05
```

In the Deconvolved Mass spectrum, baseline removal is controlled by **Baseline radius (m/z)** in the Advanced tab of the Compute button.

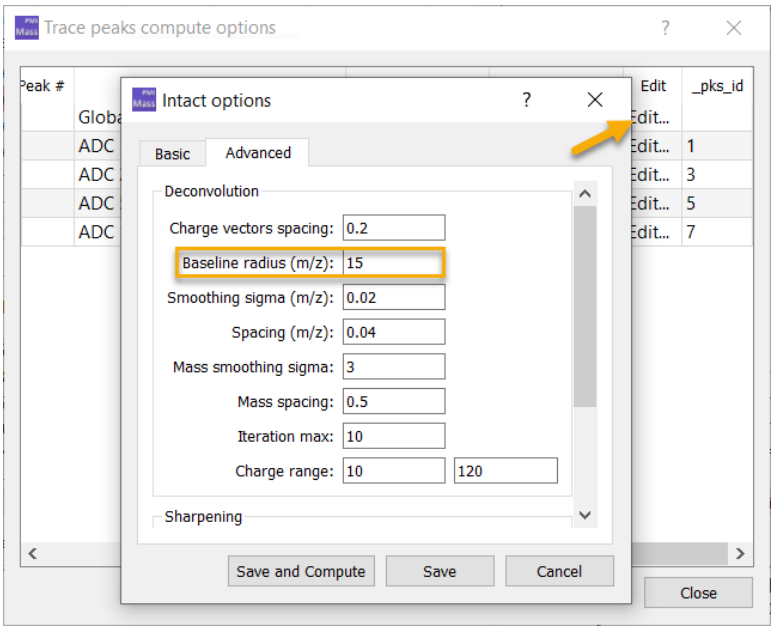


Figure 103: Baseline radius in Compute options Advanced tab

9. **Peak Picking** is a group of commands that allow greater control over which peaks in the neutral mass spectrum are picked for labeling, colored dots, automatic matching, etc.

    a. **PeakDetectorWidth** –sets the peak width (half width half height) used by the Intact Analysis "matched filter" to pick peaks in the mass spectrum. By default, PeakDetectorWidth is one-third of the minimum difference between mass peaks, set on the Basic tab of the deconvolution parameters. A PeakDetectorWidth value of 5, implied by the default value of 15 for "Min difference between mass peaks", will work well for a wide mass range such as 10,000 – 160,000. Too wide a setting will pull the picked peak off the visual apex in the case of asymmetric peaks. Too narrow a setting will pick up jitter on top of jagged peaks. A very large number (for example, 500) can be used to pick just one representative peak for a peak series. This is useful for antibody-drug conjugates with the same number of drugs but varying glycosylation and dead-end linkers. A PeakDetectorWidth of 5 also works well for picking the isotope peak closest to the average-isotope mass for isotopically resolved mass peaks, but this width may sometimes pick a gap between isotopes.

    ```
    [Intact]
    PeakDetectorWidth = 5
    ```

    b. **MinPeakMass** excludes peaks with masses below the set minimum. For example, the user can deconvolve over a mass range of 6000 - 200,000, but only ask for picked peaks with masses > 20,000. Deconvolving over a wider range can clean up noisy spectra, because noise peaks tend to concentrate at the low end of the mass range. Similarly, **MaxPeakMass** excludes peaks with masses above the set maximum. . If not specified, MinPeakMass and MaxPeakMass span the same range as the mass range for deconvolution.

    ```
    [Intact]
    MinPeakMass = 20000
    MaxPeakMass = 150000
    ```

    c. **MinBasePeakRatio** excludes peaks which are less than the set fraction of the intensity of the tallest peak in the range [MinPeakMass, MaxPeakMass]. By default, MinBasePeakRatio=0. For example, a value of 0.05 excludes peaks that are less than 5% of the tallest peak in the range.

    ```
    [Intact]
    MinBasePeakRatio = 0.05
    ```

10. **Antibody Clip Naming** names the antibodies for the clips, by default. Setting this to 0 turns off this feature and also prevents multiple recalculations of the clips.

    ```
    [Intact]
    DoAntibodySpecificClipsAndNames=0
    ```

11. **Insert Anchors** injects manual baseline points when the baseline smoothing parameter alone is inadequate. This will help to keep big dips out of the calculated area if points just outside the time range are included, as depicted below:

    ```
    [Baseline]
    InsertAnchors=19.27,20.12
    ```

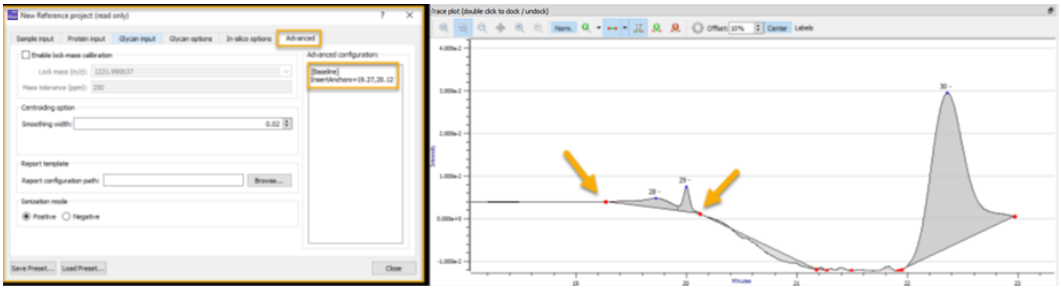


Figure 104: Insert baseline anchors

Note that this function requires the user to have knowledge of where to place the baseline points. The values 19.27 and 20.12 were observed from the chromatogram.

12. **Show Observed Mono** shows orange diamonds at the observed monoisotopic mass on the Deconvolved Mass Spectrum plot

```
[Intact]
ShowObservedMono=true
```

13. **Mass charge vector** improves charge distribution modeling for native MS.
    - This command is especially important for native-MS spectra that contain both monomers and dimers, or large homo-multimers.
    - The command assumes that larger masses appear at higher average m/z, that is, average charge scales less than linearly with mass.
    - "MassCharge=1" is recommended for native MS. This setting assumes that a homodimer will tend to have less than twice the charge of the monomer, so that its charge distribution is centered at higher m/z. This assumption does not always hold for denatured proteins, for example, an antibody heavy chain may hold more than twice the charge of antibody light chain. In this case, "MassCharge=1" will not give an accurate deconvolution.
    - "CheckDoubleMass=1" is another advanced command that will help with masses in 1:2 ratio. This command does not make the assumption stated above, so it may work when "MassCharge=1" does not. It is recommended to use either "CheckDoubleMass" or "MassCharge", but not both at once.

```
[Intact]
MassCharge = 1
```

Rendering

1. **ShowMSLabelDots**, when set to false, hides the dots for the MS1 and deconvolved mass plots. Setting **ShowChromLabelDots** to false hides the dots for the Trace plots:

```
[Rendering]
ShowMSLabelDots=false
ShowChromLabelDots=false
```

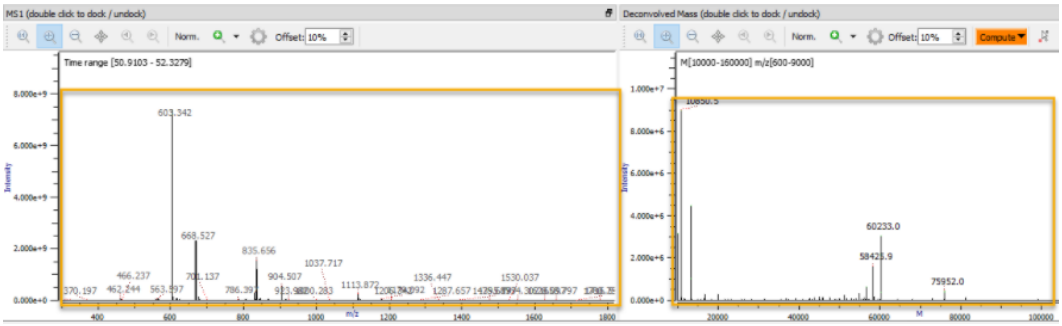


Figure 105: Plots with the dots hidden

These functions are set to true by default.

2. **Label Density in Plots -** adjusts the density/count of labels for plots by the highest peaks. The default value is 1. The minimum value is 0.01; smaller values will be treated as 0.01. The maximum value is 10; larger values will be treated as 10. Note: it is not recommended to set the label density factor below 1.

```
[Intact]
DeconvolutionPlotLabelFactor=1
MS1PlotLabelFactor=0.01
ChromatogramPlotLabelFactor=3
```

Additionally, `LabelFactor` sets the density for all plot types to the same value. Example displays for label densities:

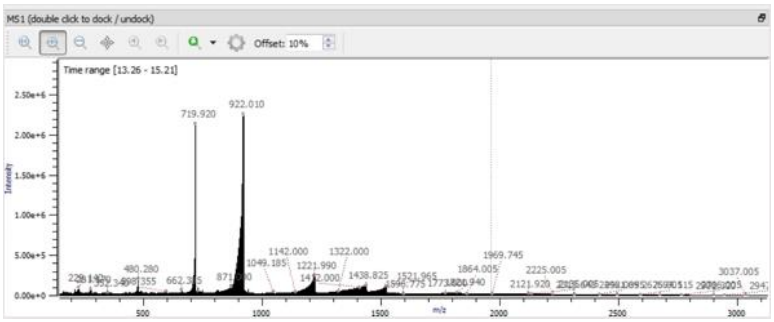


Figure 106: LabelFactor = 1 (default)

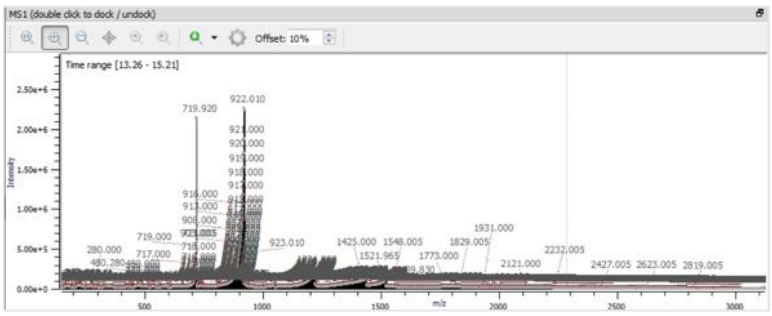


Figure 107: LabelFactor = 0.01 (minimum setting)

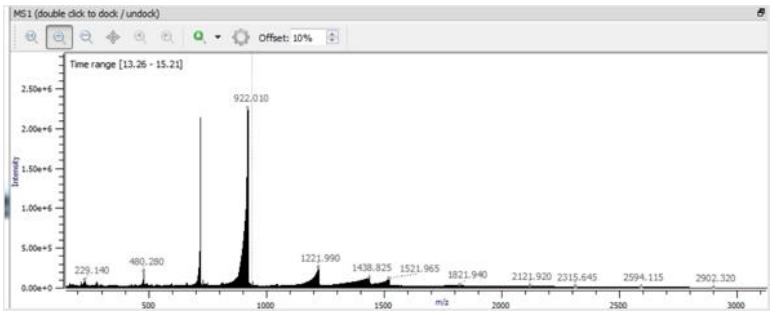


Figure 108: LabelFactor = 3

3. **Trace plot annotations –** can be modified for maximum values in table columns. By default, the Trace plot annotations report the peak number and the name of the most abundant mass. This is represented by the annotation script access by opening **Rendering options** and clicking **Edit annotations**:

```
PeaksDynamicNumber] - <script>max('[ApexPositIntensity]', '[Sequence]')</script>
```

The Trace plot uses this setting to display the most abundant mass name for each peak:

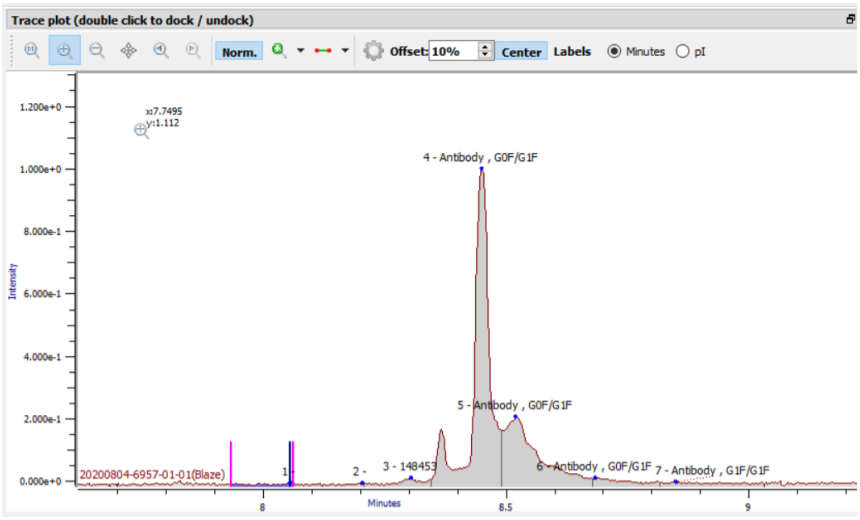


Figure 109: Default Trace plot annotations using most abundant mass

The **max** function can be used for other annotation labels, for example:

- **<script>max('[ApexPositIntensity]')</script>** returns the maximum value of the **Intensity** column
- **<script>max('[ApexPositIntensity]', '[Sequence]')</script>** returns the value of the **Name** column for which Intensity is maximum
- **<script>max('[ApexPositIntensity]', '[Sequence], [ProteinAccessionName]')</script>** returns the value of the **Name** column and **Protein Name** column for which Intensity is maximum

Reporting

1. **MassNameTemplate** customizes the Name column in the Masses table of Intact Analysis to meet user needs. The display may be configured to show any text along with data from any of the following five fields:

a. [ReferenceName] = The reference mass name
b. [DeltaName] = Matching delta mass name
c. [ExpectationType] = Desired, Undesired, Unexpected, Ignored
d. [ExpectedMass] = Sum of reference and delta mass value
e. [ObservedMass] = The deconvoluted mass value For example, the following definition:

```
[Intact]
MassNameTemplate = [ReferenceName], [DeltaName] / [ExpectedMass]
```

Would give the following output mass peak name: HC, G0F / 1234.123

As a second example, assume theMassNameTemplate is defined as follows:

```
[Intact]
MassNameTemplate=rn:[ReferenceName] dn:[DeltaName]
et:[ExpectationType] em:[ExpectedMass] om:[ObservedMass]
```

The matched masses (light and heavy chains of an antibody) receive the names as shown below:

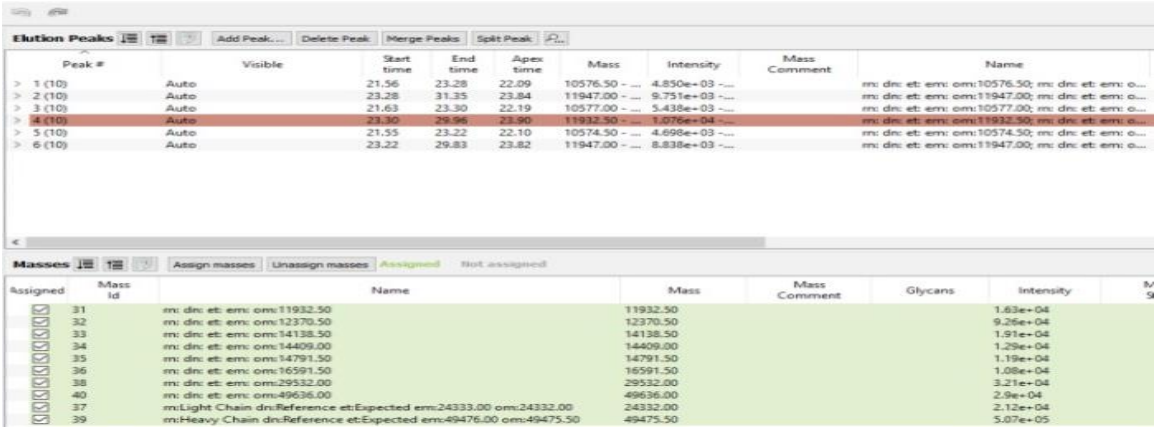


Figure 110: Named masses with the MassNameTemplate

2. **EnableValidateOverride** allows manual control over the Status setting. The Traffic Light view displays the status of Desired, Undesired, and Unexpected counts, but sometimes the user would like to manually change the Pass / Fail value in the Status column based on the value from Validate column. This is possible if the Validate column is not empty and the new Advanced configuration detailed below is enabled:

```
[Intact]
EnableValidateOverride = 1
```

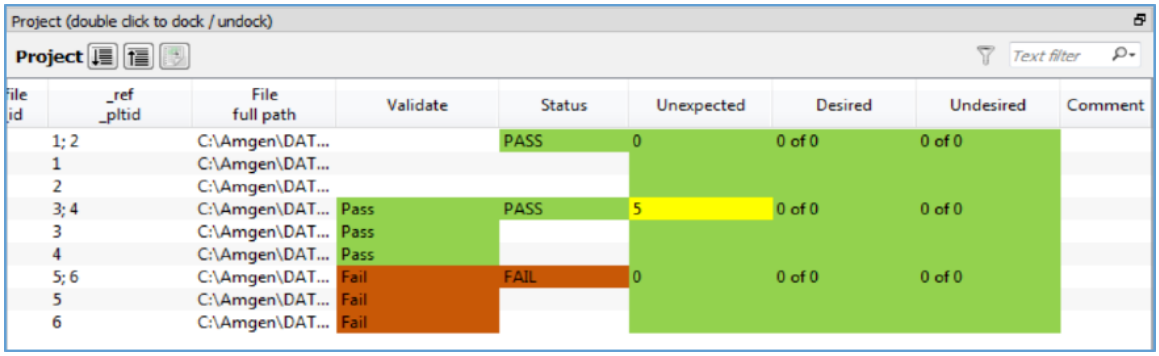


Figure 111: Setting Pass/Fail values in the Project table Status column