ExDViewer User Guide

Table of Contents

Introduction

Using This Guide

The purpose of this guide is to provide users of ExDViewer a comprehensive instructional manual for ExDViewer software. New users of ExDViewer software should start with the Quick Start Guide. For more detailed information, users can look at the applicable sections in this user guide.

What Is the ExD Solution?

The ExD solution enables comprehensive characterization of proteins and peptides. An ExD cell upgrade with hardware and software lets labs use Electron Capture Dissociation (ECD) to:

- Fragment larger protein ions.
- Characterize labile post-translational modifications (PTMs), e.g., phosphorylation, glycosylation.
- Differentiate isobaric residues, e.g., aspartate/isoaspartate, leucine/isoleucine.
- Obtain complementary sequence information to CID b/y-ions.
- Learn more about disulfide-bonded peptides and proteins.

What Is ExDViewer Software?

ExDViewer rapidly processes mass spectrometry fragmentation data and then displays them in an easy-to-understand format with annotated spectra, sequences, and tables. ExDViewer annotates all protein fragment ion types — b, y, c, z, a, x, including side chain fragmentation which distinguishes isobaric amino acids such as leucine and isoleucine.

Using ExDViewer's unique parameter-free deconvolution algorithm, proteins and peptides can be rapidly sequenced using evidence from raw mass spectra. In addition, unknown amino acids matching an Averagine model are highlighted in the mass spectrum. Results can be shared with colleagues using a file or with a Uniform Resource Locator (URL) that can be opened in a web browser.

ExDViewer Setup

Minimum Configuration

Hardware

- Windows-type PC
- Intel Core i3 CPU or equivalent
- 4 GB Random Access Memory (RAM)
- 50 GB hard disk space (more if many large data files will be processed)
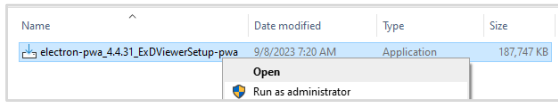
Software

- Microsoft Windows 10 or higher operating system
- For the web browser-based version, Google Chrome version 113 (or higher) or equivalent

Installing ExDViewer

1. Download the ExDViewer executable file.

2. Right-click on the file and select **Run as administrator.**

3. Follow the prompts to select where the application will be installed and whether to create a Windows desktop icon.

Uninstalling ExDViewer

1. Click on the Windows Start icon in the lower left corner.
2. Select the **Settings** item.
3. In the Settings dialog box choose **Apps**
4. In the **Apps & features** dialog box, click on the ExDViewer
5. Click on the **Uninstall** button and follow the instructions:
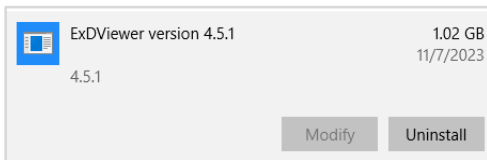
How to Respond to Update Messages

Sometimes when you open ExDViewer you may receive an update message in the lower right corner. This might notify you that:

- A new version of ExDViewer is available.
- The prerelease version that you are using will soon no longer be operational and that you should upgrade to continue using ExDViewer.

- A specific issue exists that you should be aware of.

Follow the instructions then dismiss the update message.

Starting ExDViewer

Right-click on the ExDViewer icon on the Windows desktop and select the Open menu item:
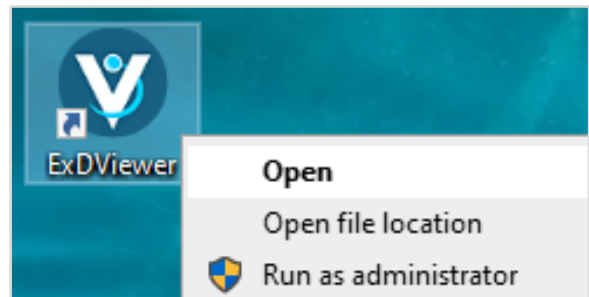
The ExDViewer software program will open:

Example Files Available with ExDViewer

Downloading the Sample Data Files

1. Select the **File > Download Sample Data** menu item. A file will begin to download.

4

2. Go to the Downloads folder in the Windows Explorer and extract the zipped file which contains Agilent MassHunter *.d LC/Q-TOF data files:

3. Move the data files to the normal place for LC/Q-TOF data files on the PC, e.g. \MassHunter\Data.

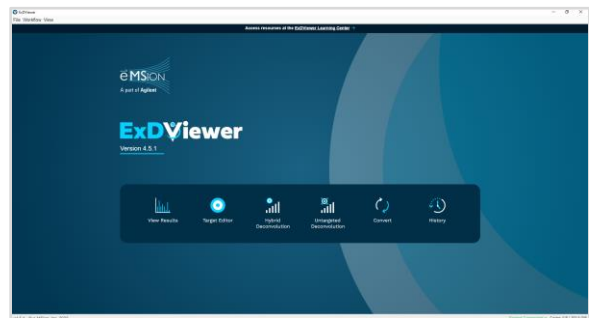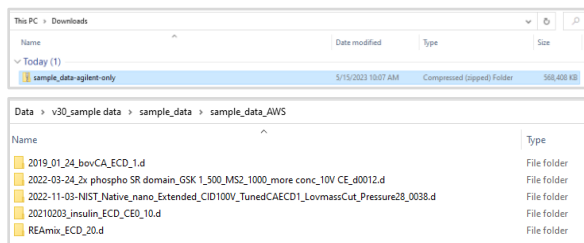4. Example data files can be analyzed using the Hybrid Deconvolution workflow described later in this guide. Example protein sequences are pre-loaded in the Target Editor for use with example files.

Loading Example exdview Files

exdview files are result files created by ExDViewer. They contain annotated mass spectra, sequence information and coverage (including ion types and PTMs assigned to positions), tables of results, and window layouts. ExDViewer lets you load several example exdview files which illustrate many of the features of the ExD solution.

To load an example exdview file, either select **View Results > Browse Examples** in the Navigation UI or go to the **File > Load Example Run** menu item.
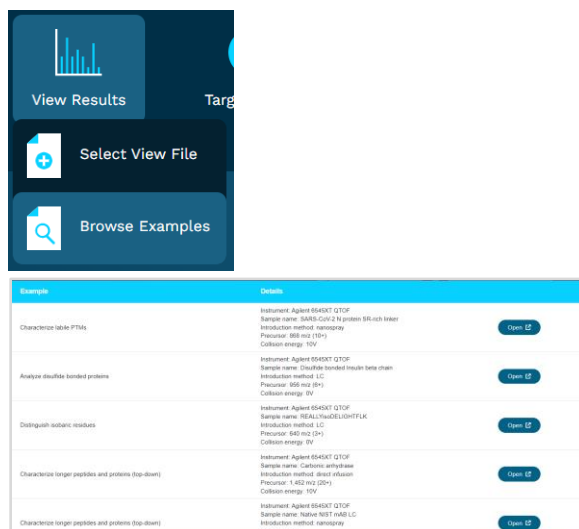
Choose one of the examples, e.g., "Distinguish isobaric residues", and click the **Open** button. The example exdview file will begin to load; if an exdview file has a custom window layout saved with it you will be prompted whether to load it:
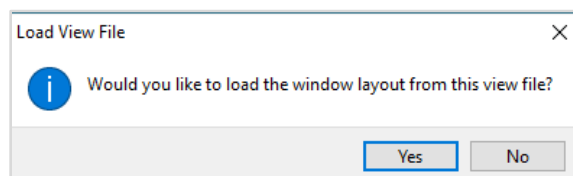
Click "Yes".

The results will load into ExDViewer:

Processing Data Files Using the Hybrid Deconvolution Workflow

What is the Hybrid Deconvolution Workflow?

The Hybrid Deconvolution workflow answers the question "Does this data confirm my hypothesized protein or peptide?" It clearly shows the evidence in the source raw data by highlighting the specific types of ions found. These could be generated by ECD (c and z ions), EID (d and w ions), and CID (b and y ions). In addition, a ions can be generated by both ECD and CID. Finally, ExDViewer will annotate internal ions (which are fragmented on both the amino and carboxyl end) as well as unknown ions that match an Averagine model for amino acids[1].

The Signal Processing Algorithm

ExDViewer uses a signal processing algorithm to measure how well each putative ion in a mass spectrum fits a statistical model. The model has been validated against results from human curators[2]. The fitting from the model calculates an **Ion Score** which assigns a value between 0 and 15.

$$Ion\ Score = (1.0 - chi - squared\ p - value) \times Spearman\ correlation \times log((1.0 - Pearson\ p - value)/noise\ probability)$$

where:

*chi-squared p-value* is based on centroid *m/z* and intensities.
Spearman correlation is based on profile raw signal.

Pearson p-value is based on centroid intensities.
noise probability—assuming independence of centroids, what is the probability of observing peaks with higher intensities? This parameter captures peak density as a function of intensity rank in the wider spectrum.

An Ion Score is classified as:

- Restrictive if ≥ 3.0
- Default if ≥ 1.5 (recommended for most analyses)
- Permissive if ≥ 0.5
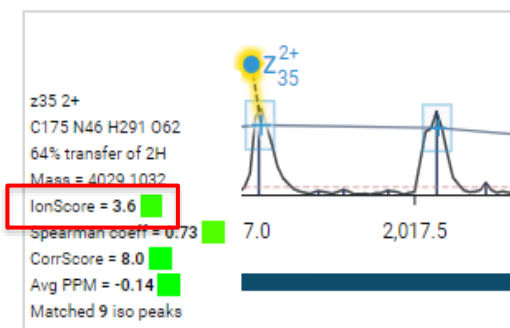
"Parameter-free" Deconvolution

Deconvolution converts complex signals in mass spectra into a simpler form that is easier to understand. In ExDViewer, a unique deconvolution algorithm is used to effectively parse isotope clusters in raw mass spectra. Traditionally, deconvolution algorithms only fit an Averagine isotope distribution to each putative isotopic cluster, allowing for fragment peak charge determination in an un-targeted manner. This is done in ExDViewer, but when matching to a known peptide or protein sequence, ExDViewer will first generate and match an exact isotopic distribution for each putative ion accounting for the specific

residues, post-translational modifications, chemical losses/additions as a result of the ion type (i.e., a/b/c/d/y/w/z), and hydrogen transfer re-arrangements. This helps determine the correct theoretical isotope distribution before determining a quality score of each putative ion match. ExDViewer is unique in its ability to render these deconvolution results at the level of individual charge states in the interactive profile spectrum view, which layers the identified ions on top of the profile spectrum, centroid spectrum, and the computed noise threshold.

For example, the zoomed-in spectrum below of Bovine Carbonic Anhydrase's c37 4+ ion shows six peaks that make up its annotated isotopic cluster. The profile spectrum is outlined along the x axis while each peak has a vertical line for its centroid location. Boxes around the profile and centroid apexes correspond to match PPM and intensity thresholds. An overarching horizontal curved line links the theoretical values for each peak, visually demonstrating any difference from the observed values:
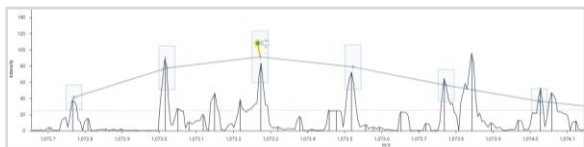

No parameters are required to be adjusted and the signal processing works with default settings on most types of data. A test evaluating over 10,000 putative ions assigned by ExDViewer achieved greater than 99% accuracy compared to assignments made by experts in benchmarking studies evaluating a range of protein fragmentation data.[2]

High-quality fragment ion matches can be viewed in de-charged or de-isotoped spectra which can greatly simplify analysis.

De-charging takes all charge states for an identified ion and collapses it down to one isotope distribution. For example, the c27 ion in thymosin has ions in the +2, +3, and +4 charge states that during de-charging can be collapsed into a molecule with a monoisotopic mass of 3205.6409 Da. This process converts the x axis from m/z to Mass. When switching into de-charged mode, profile data is omitted and all unmatched centroids are assumed to be charge 1.
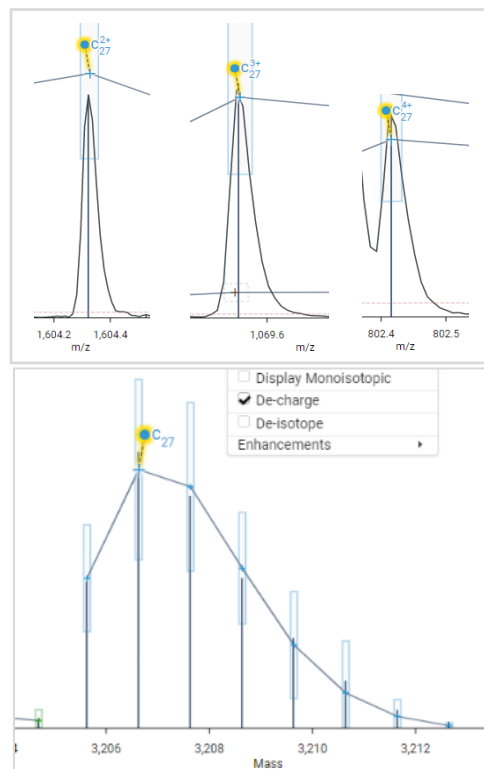

De-isotoping combines the signal intensity from all detected isotopes into a single monoisotopic peak. Measuring distance between deisotoped peaks is useful for sequence confirmation and identifying unknown peaks.
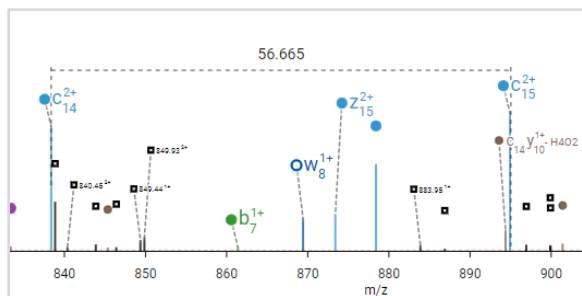
Treatment of Hydrogen Transfer

Transfer of hydrogen is often seen in ECD[3] as well as occasionally in CID. As a result, the mass of a molecule can be off by 1 or 2 Daltons. For specified ion types, ExDViewer will use an algorithm to fit all combinations of the -2H, -H, neutral, +H, and +2H isotope distributions.

The algorithm allows each ion match to use any combination of +/-1 or +/-2 hydrogen transfers. There are three possibilities involving no transfer dist(0), one hydrogen transfer dist(H), and two hydrogen transfers dist(2H). For the first two possibilities, we know that a + b + c = 1, thus we can use linear algebra to determine the coefficients that minimize error between observed and expected centroid values. Applying a single hydrogen transfer event (i.e., dist(+/-H)) to a peak distribution simply increments or decrements each m/z value by a mass(H+)/charge. A hydrogen transfer arrangement is only accepted if it yields a score that is at least 20% better than the neutral species.
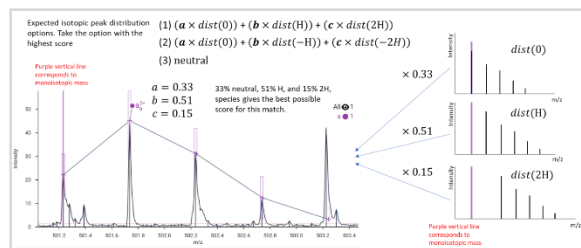
The ion types can be specified in the Hybrid Deconvolution Settings dialog box in the Hybrid Deconvolution workflow:
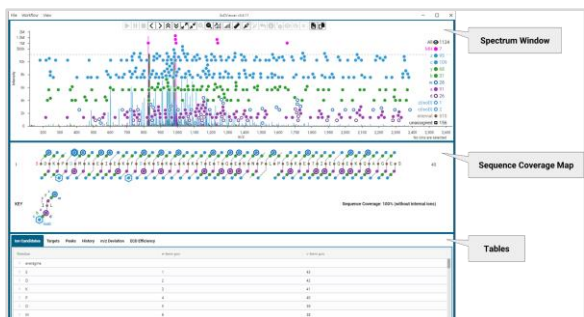

Placing the mouse cursor over a labeled ion will show its degree of hydrogen transfer. The information is also available in the Ion Candidates, Ions, and Isotopic Peaks tables.
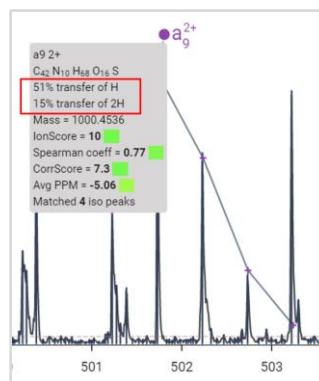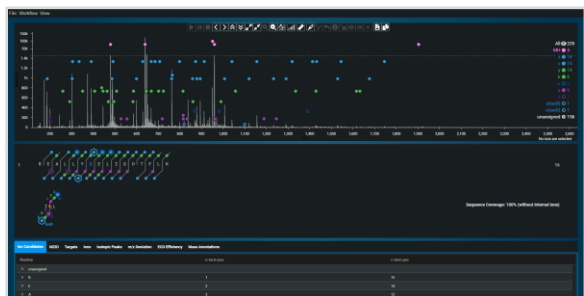

Reviewing ExDViewer Results

Windows

ExDViewer has three main windows: the Spectrum, the Sequence Coverage Map, and Tables.

Spectrum Window

Sequence Coverage Map

Tables

The default mode when loading ExDViewer is called Light Mode. It is possible to switch to Dark Mode using the **View > Dark Mode** menu item:

Windows in ExDViewer can be rearranged by clicking on them and moving them around the program area. They can also be resized individually by using a double-sided arrow that appears in the lower right corner when the mouse is placed on top of them:


For example, this layout can be created which allows clicking on an ion in the Sequence Coverage Map and immediately seeing its spectrum and ion candidate information:
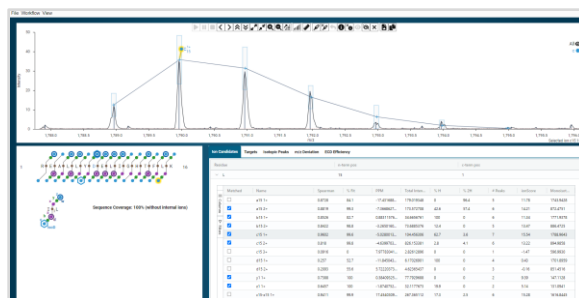
The original sizes and positions can be restored using the **View > Reset Preferences** menu item.

In addition, it is possible to zoom in and out the windows using the **View > Zoom In** and **Zoom Out** menu items. The default zoom level can be restored using the **View > Reset Zoom** menu item.

Using **View > Layout Settings** it is possible to configure which windows and tables are visible. Clicking the New Window button will create an additional window where you can specify which view or table it contains.
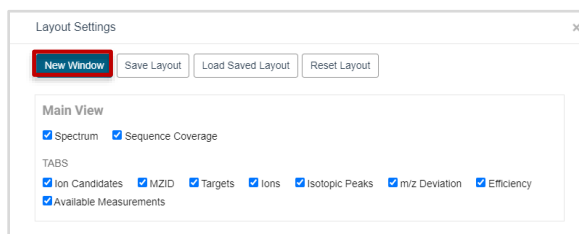
A custom layout can be saved with the exdview file using the **View > Save Layout** menu item. Later, it can be loaded using **View > Load Saved Layout**. These functions are also available as command buttons in the Layout Settings dialog box.

Each window has three horizontal lines in the top left corner. These can be used to open in a new window (e.g., place the Sequence Coverage Map in its own window), move it

back to the Main View, or to close it completely:
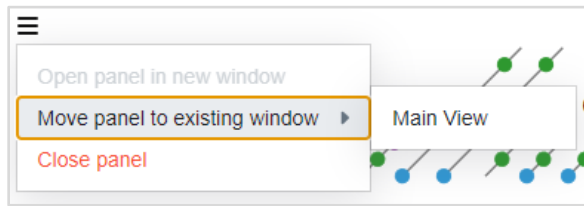
The default set of windows, tables, sizes, and positions can be reloaded using the **View > Reset Layout** menu item or by clicking the **Reset Layout** button in the Layout Settings dialog box.

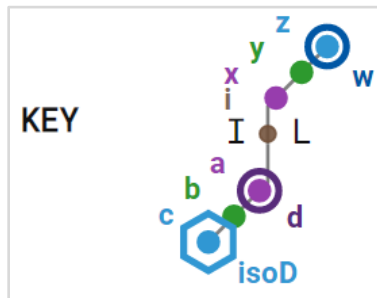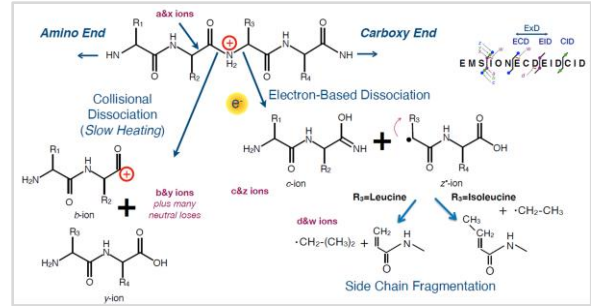## Sequence Coverage Map

The Sequence Coverage Map shows the amino acid residues in the target sequence as well as fragment ion evidence that was matched.

Reviewing the **KEY** which shows the representation of the different types of ions in the Sequence Coverage Map:

These ion types are generated by ECD, EID and CID fragmentation mechanisms. ECD and EID are collectively referred to as ExD:

Most residues in the Sequence Coverage Map are in black text; however, those that have a PTM such as glycosylation or phosphorylation are in red. Isoaspartate (i.e., "D") is in blue text.

Peptide Example The following content provides example results from a 16 residue synthetic peptide with the sequence REALLYisoDELIGHTFLK. These results can also be viewed interactively by loading the example file in ExDViewer.

Clicking on a particular ion in the Sequence Coverage Map will zoom into it in the Spectrum view:
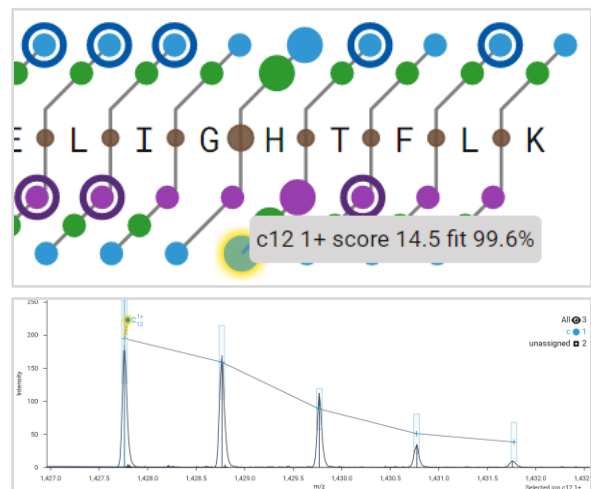
If there are multiple instances of an ion detected (i.e., different charge states or neutral losses), when you click on an ion in the Sequence Coverage Map it will give you a

menu of which (or all) to select:
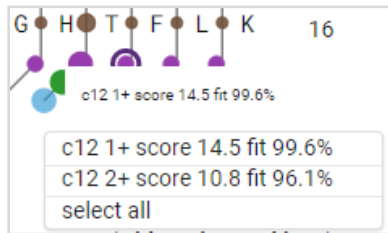
Turning on de-charge in the Spectrum window will collapse all charge states down to one selection.

REALLYisoDELIGHTFLK contains an isoaspartate (the blue D) in position 7 as well as four leucines (L) and one isoleucine (I). One of the challenges of top-down analysis is differentiating isobaric residues such as aspartate/isoaspartate and leucine/isoleucine.

In ExDViewer, leucine and isoleucine can be differentiated by identifying side chain fragmentation w ions. Isoaspartate is indicated by an isotopic cluster 57 Daltons greater than the c ion:
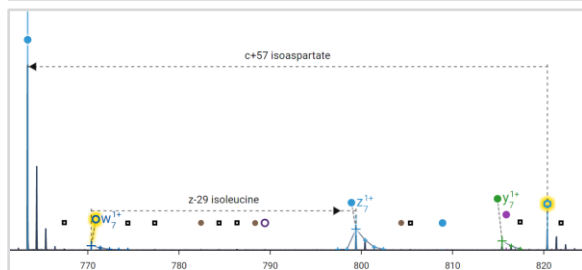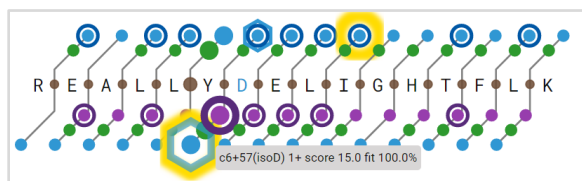

In the lower right corner, the Sequence Coverage Map displays the percentage of the total sequence that is found and annotated:

Internal ions are fragmented on both the amino and carboxyl end. ExDViewer detects and annotates them as brown symbols next to the amino acid letters but does not include them in the total sequence coverage. Detection of internal ions can be configured in the Hybrid Deconvolution dialog box during the Hybrid Deconvolution workflow. Hovering over any ion will bring up a description as well as its Ion Score and Fit percentage (a measure of how well the expected ion mass peaks match the observed isotopic ratios. It is calculated as $((1 - \text{chi-squared p-value}) \times 100)$.
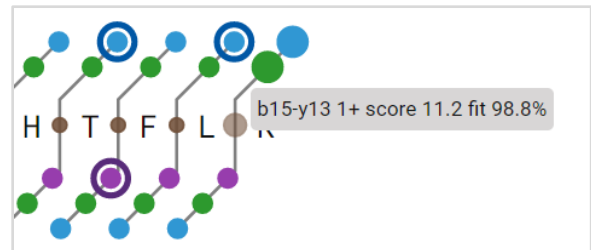
Right-clicking anywhere in the window will bring up a menu to change the format of the Sequence Coverage Map or save an image as a publication-quality graphic:
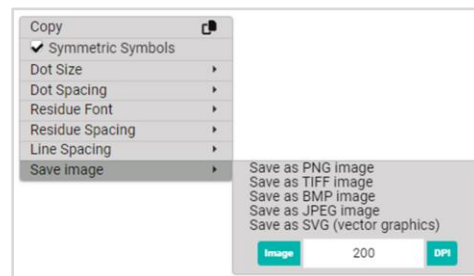
Spectrum Window

The Spectrum window shows the raw mass peaks in the processed spectrum annotated with matched ions and their isotopic clusters.

It has several user interface (UI) elements for transforming or filtering. The Toolbar at the top has command buttons which enable zooming, panning as well as de-charging and de-isotoping the spectrum:

Resume/Pause/Terminate are used while streaming data from an instrument (see "Streaming Spectra Directly From an Instrument.") They allow you to control the flow of data into the ExDViewer software program. When not streaming, they are gray and inactive:

Pan-left and **Pan-right** move the x-axis in either direction to allow you to browse through the spectrum:

Increase y-axis scale and **Decrease y-axis scale** move the maximum value of the Intensity either upward or downward:

Step zoom-in and **Step zoom-out** either reduce or increase the range of the x-axis and y- axis. Each time they are clicked there will be an incremental change to the ranges.

Zoom to selected ion(s) allows you to select one or more ions in the spectrum and then get a zoomed in view of its isotopic peaks. If more than one ion is selected the zoomed in view will have axis ranges to include all of them.

Reset zoom restores the original x and y axis ranges and displays all the ions, whether selected or not.

Toggle ion density view allows the ion symbols to either be lined up neatly (Always On) or shown with all ions in their exact place (Always Off). Dynamic Mode determines the best positioning for the ion symbols:

Dynamic Mode
> Always On
Always Off

Toggle view of peak data has several options.

- Selecting the **Display Monoisotopic** check box will draw a solid vertical line from the monoisotopic mass of each isotopic cluster in the spectrum. The line will be the same color as the type of ion, e.g., blue for c ions, green for b ions.
- Selecting **De-charge** applies an algorithm that rapidly consolidates information from different charge states of the same ion together and transforms the x axis of the spectrum from *m/z* to Mass. This greatly simplifies the mass spectrum and aids in interpretation.
- Selecting **De-isotope** collapses each isotopic cluster into a single mass peak.

Measure distance between any two masses provides a means of displaying the difference on the x-axis between two points.

If the user chooses "Measure centroids" it will on the first mouse-click snap to the closest ion with an annotated centroid. The second mouse-click will draw a dashed horizontal line to the closest centroid and put the difference above. If the difference is approximately the same as a molecular component such as a hydrogen atom, it will also note that:
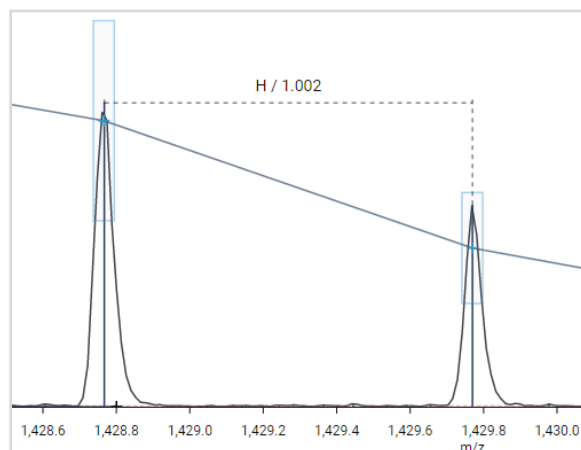
The Available Measurements table contains a set of mass offsets that can be displayed above the dotted line, allowing multiple labels at the same time using the Combinatoric depth feature. Choosing "Measure cursor" will draw a dashed horizontal line between two mouse-clicks regardless if any centroids or ions are nearby.

Toggle selection of visible ions will alternate between the currently selected ion and all other ions. **De-select all ions** will remove all selection of ions

Occasionally it is useful to move the ion annotations around in the spectrum, for example in setting up graphics for publication. Clicking on the ion name, e.g., c21 2+, will allow this. **Revert to default positioning of selected ions** will move all annotations back to their original position.

Label selected ions will add annotations to any ions in the spectrum that are currently selected (as below). **Un-label selected ions** will remove those labels.
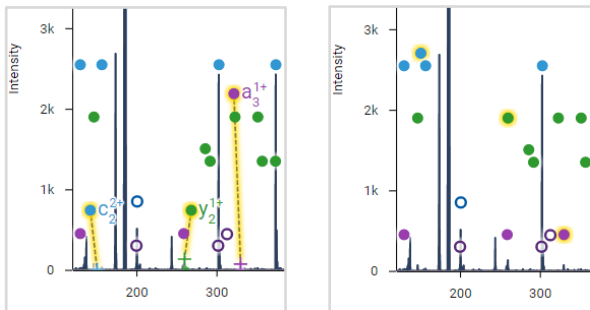
Show all hidden ion(s) will display any ions that have been hidden using the **Hide selected ion(s)** command button.

Hide selected ion(s) will make the currently selected ions disappear.

Unmatch selected ion(s) will remove the match information for those ions that are currently selected.

Save image as file allows you to create a copy in five different formats.

Copy image to clipboard makes a copy available in the system's clipboard.

| Save as PNG image |
| Save as TIFF image |
| Save as BMP image |
| Save as JPEG image |
| Save as SVG (vector graphics) |

The Legend on the right side has a summary of all ions found including unassigned (those matching an Averagine model). Selecting an ion (e.g., the blue z ion symbol) will highlight all ions of that type in the Spectrum and Sequence Coverage Map.
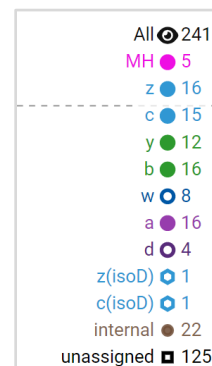
While moving the cursor around the Spectrum window the values of the x and y axis (*m/z* and Intensity) are displayed above the Legend:

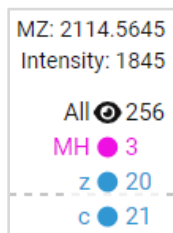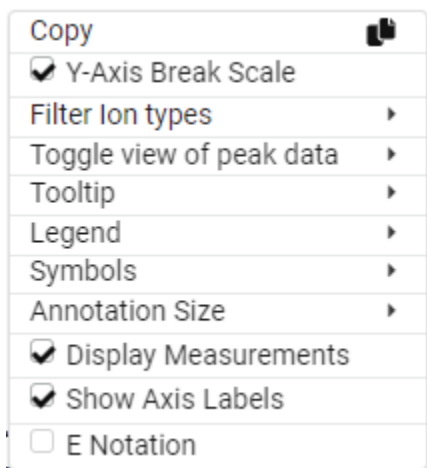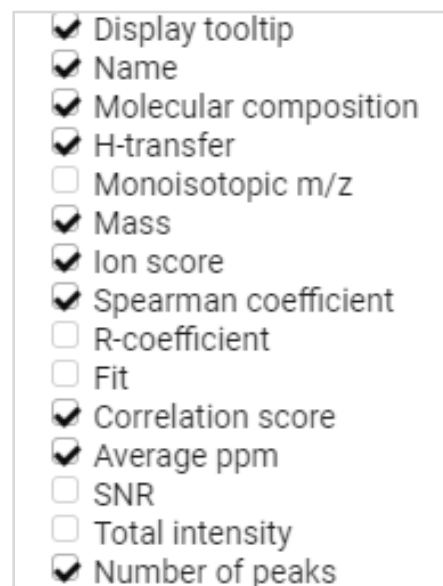MZ: 2114.5645
Intensity: 1845

All 👁 256
MH ● 3
z ● 20
c ● 21

Right-clicking in the Spectrum window brings up a menu:

Copy
✔ Y-Axis Break Scale
Filter Ion types ▶
Toggle view of peak data ▶
Tooltip ▶
Legend ▶
Symbols ▶
Annotation Size ▶
✔ Display Measurements
✔ Show Axis Labels
☐ E Notation

- **Copy** makes a copy available in the system's clipboard.
- The **Y-Axis Break Scale** check box provides a dashed horizontal line in the Spectrum window. Below it is a scale that allows lower intensity ions to appear (e.g., 0 to 2.5K). Above it high intensity ions are displayed (e.g. 2.5K to 300K). Clearing the check box changes the y-axis to one consistent scale (e.g., 0 to 300K).
- **Filter ion types** provides a check box for each type of ion (e.g., a, b, y, z). Clearing the check box for an ion

type removes it from the Spectrum window.
- **Toggle view of peak data** provides all the same options as the **Toggle view of peak data** command button in the Spectrum window's Toolbar.
- **Tooltip** allows the user to configure if the tooltip comes up next to each ion symbol and which information is displayed:

✔ Display tooltip
✔ Name
✔ Molecular composition
✔ H-transfer
☐ Monoisotopic m/z
✔ Mass
✔ Ion score
✔ Spearman coefficient
☐ R-coefficient
☐ Fit
✔ Correlation score
✔ Average ppm
☐ SNR
☐ Total intensity
✔ Number of peaks

- **Legend** toggles the display of the Legend, whether Averagine (i.e., unassigned) ions show up in the Legend, and the font size and radius of symbol in the Legend.
- **Symbols** toggles the display of the ion symbols and whether they are symmetric (i.e., round) or non-symmetric and point in the direction of the N-terminal (a, b, c, d ions) or C-terminal (y, w, z ions).
- **Annotation Size** controls the size of the font of the labels on ion symbols (e.g., z25 2+). It is necessary to zoom into a spectrum to see these

labels since they do not show up on a fully zoomed-out Spectrum window.

- **Display Measurements** toggles display of both system and user generated annotations across the x-axis. For example, when the check box is cleared a system generated annotation such as a dashed horizontal line with "z−43 leucine" will disappear as well as any annotations generated using the **Measure distance between any two masses** command button in the Toolbar.
- **Show Axis Labels** toggles display of the x-axis (m/z) and y-axis (Intensity) labels. The values for these axes continue to be displayed in either setting.
- **E Notation** changes the y-axis (Intensity) from standard notation (e.g., 200) to exponential form (e.g., 2.0e+2).

There are three ways to zoom into the Spectrum window:

- Click the **Step zoom-in** command button in the Toolbar (described above).
- Click on a location in the Spectrum window and drag the mouse cursor to another location. When the mouse button is lifted, the Spectrum will zoom to between those two locations.
- Rotate the mouse scroll wheel (if available) to either zoom in or out of the currently displayed spectrum area.

It is always possible to return to the fully zoomed-out Spectrum window by clicking

the **Reset zoom** command button in the Toolbar.

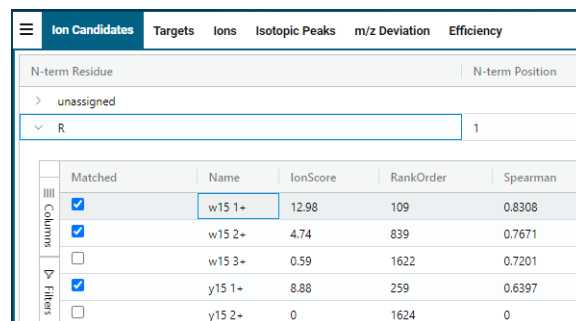There are two ways to pan through the Spectrum window:

- Click the **Pan-left** and **Pan-right** command buttons in the Toolbar.
- Click on the m/z label in the x-axis and move either to the left or right.

Tables

The Tables provide different views on the data including matched and unmatched ions, targets used, raw mass peaks, m/z deviation across the mass range, and fragmentation efficiency.

Ion Candidates Table

The highest level in the Ion Candidates table is a list of the residues in the target sequence. Clicking on a **>** opens up the section to see rows containing detected ions. The first column lists the matched and unmatched ions for each residue and allows you to select or clear their **Matched** check box:


In the default configuration, columns to the right of the **Matched** check box contain

information for each ion including its:

- **Name** is the designation of the ion using ion type, position, and charge e.g., w15 1+.
- The **Ion Score** as calculated in the section [The Signal Processing Algorithm](#).
- **RankOrder** is the rank applied to this ion after sorting by IonScore. If two ions have IonScores within 35%, then the tie is resolved using the ion_types_priority setting from the Hybrid Deconvolution Settings dialog box.
- **Spearman** correlation, a component of the Ion Score.
- **% Fit** calculated as ((1 −chi-squared p-value) x 100), also a component of the Ion Score.
- **PPM**, the weighted average deviation from the theoretical *m/z* for all peaks in the isotopic cluster expressed in parts per million.
- **Total Intensity** is the sum of the intensities for all peaks in the isotopic cluster.
- **% H**, the percentage of single hydrogen transfer.
- **% 2H**, the percentage of double hydrogen transfer.
- **# Peaks**, the number of isotopic peaks in the cluster.
- The **Monoisotopic** mass of the ion, i.e., the mass based on the most abundant stable isotopes of the constituent elements.

Clicking on the vertical **Columns** command button to the left of the **Matched** column opens an area with check boxes that allow further configuration of the Ion Candidates table.
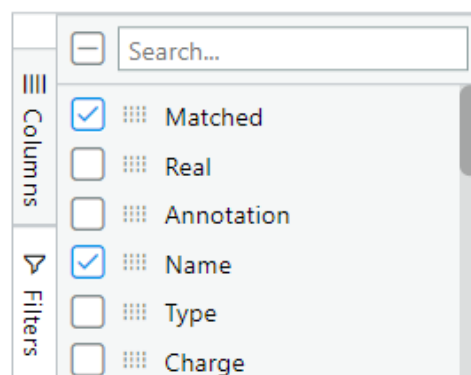
Existing columns can be removed and new ones can be added including:

- **Real** indicates if the ion is present in the spectrum and is a true match. This column can be used for benchmarking and human curation of processed data.
- **Annotation** when double-clicked in the cell, opens a dropdown with explanations about why the ion was not matched or is not real. You can select an explanation and it will be saved with the exdview file.
- **Type** of ion i.e., a, b, c, d, i (internal), w, x, y, z, and # (unassigned).
- **Charge** e.g., +1, +2, +3.
- **Pearson** correlation, a component of the Ion Score.
- **SNR,** the signal-to-noise ratio of the highest theoretical isotope.
- **corrScore**, also a component of the Ion Score.
- **Mass** is the de-charged mass of the ion.
- **Average** is the average mass of the ion, i.e., the average of the isotopic masses weighted by the isotopic abundances.

In addition, it is possible to move columns by clicking and holding the left mouse button on the column name at the top and moving it to the left or right. A cross will appear underneath the columns that shows the column being moved and its new location once the left mouse button is released:


Columns can be sorted by clicking once on the column name. The first click will sort the column by ascending values and a ↑ symbol will appear after the column name; the second click by descending values and a ↓ symbol will appear:

You can remove the sorting from the column by clicking a third time on the column name.

At the top of the Columns area there is a Search field. Begin typing the name of a column to see if it is available:
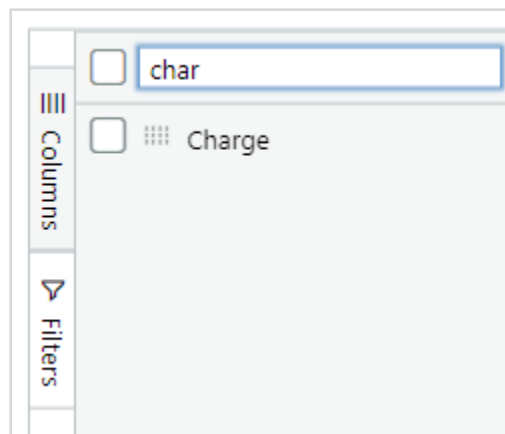

Clicking on the vertical **Filters** command button to the left of the **Matched** column opens an area that allows you to set up filters. For example, you can filter by the parts-per-million deviation from the theoretical *m/z* in the range of -2 to +2 ppm and exclude ions with a higher mass error. Clicking the **Apply** button updates the Ion Candidates table:

The **Clear** command button clears the current values out of the input fields but does not remove the filter from the Ion Candidates table. The **Reset** command button completely removes the values and the filter.

Multiple filters can be applied at the same time by selecting either **AND** or **OR** within the currently chosen filter, or by setting up another filter on a column, e.g., **SNR**. Columns that have a filter applied will have a funnel symbol after the column name:

% Fit	SNR ▽	PPM ▽
100	98.31	-1.7469042539596558
80.1	27.66	-0.8679099678993225
99.7	136.49	0.37330594658851624

And in the Filters area:

> % Fit

> SNR ▽

> PPM ▽

> % H
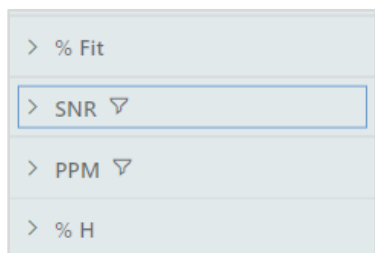
Right-clicking on the Ion Candidates table brings up a menu:
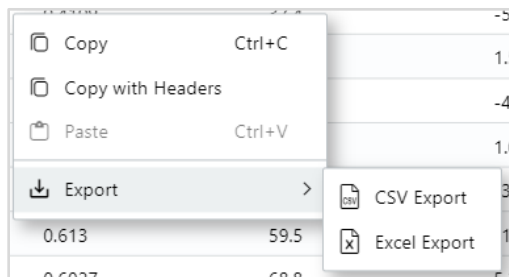
- **Copy** copies the contents of the currently selected cell to the clipboard.
- **Copy with Headers** copies both the contents of the currently selected cell and its column header.
- **Export > CSV Export** brings up a dialog box to save the current

contents of the Ion Candidates table in Comma Separated Value (.csv) format. Note that only columns that are displayed in the table will be exported.
- **Export > Excel Export** brings up a dialog box to save the current contents of the Ion Candidates table in Microsoft Excel worksheet (.xlsx) format. Note that only columns that are displayed in the table will be exported.

MZID Table

The MZID table shows information about target sequences that were used while running the Hybrid Deconvolution workflow from an MZID file (see the section "Running Targets From An MZID File"). The columns contain values taken from the mzIdentML[4] file (aka MZID file) specified in the Input dialog box. Double-clicking on any row in the MZID table will use cached data from the input file to quickly process that target sequence. The MZID table will appear after running an MZID file with the Hybrid Deconvolution workflow or loading an exdview file with MZID results.

Targets Table

The Targets table shows information about the target sequence and spectra analyzed:

The Targets table replicates information from the **Scan Selection** dialog box which is filled out during the setup of the Hybrid Deconvolution workflow. The rows are each segment of the data file that has results. These may be distinct in terms of precursor and time range. For multiple, user specified target sequences in the same data file each row may have a different name under the Target column because they represent a different molecule, e.g., apamin, melittin, neurotensin, etc.

The columns in the Targets table are:

- **Target** is the target peptide or protein used to annotate each spectrum.
- **MS Level** is the MS level of all scans averaged together. "MS2" implies that quadrupole precursor isolation was used. "All" implies that all ions were fragmented without precursor isolation.
- **Collision Energy** is the collision energy of the first scan in this retention time (RT) range.
- **Precursor** is the precursor *m/z* of all scans to extract for averaging (within the tolerance set in the **Input** dialog box for Hybrid Deconvolution).
- **MZ Error** is the difference between the observed precursor and the target's first isotope at maximum charge.
- **RT Begin** is the beginning retention time in minutes of each averaging window.
- **RT End** is the ending retention time in minutes of each averaging window.
- **# Scans** is the number of scans selected in each averaging window.

If Average Spectrum is not selected in the **Input** dialog box this will be 1.
- **Min Charge** is the minimum charge of MS/MS ions to match.
- **Max Charge** is the maximum charge of MS/MS ions to match.

Clicking on the three horizontal lines in a column gives access to the menus:
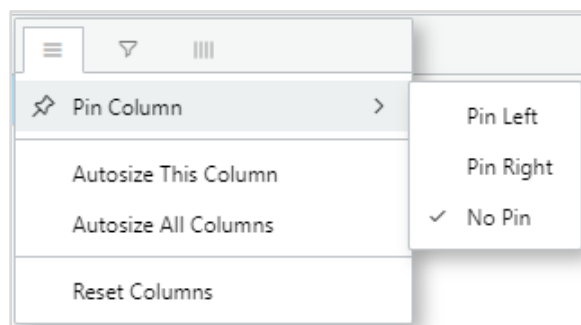

- **Pin Column** places the column in a location from which it will not move despite moving the table around.
  - **Pin Left** places the column on the far left of the table
  - **Pin Right** places the column on the far right of the table
- **Autosize This Column** fits the size of the column so all text is visible.
- **Autosize All Columns** fits the size of all columns in the table so all text is visible.
- **Reset Columns** restores the default columns, positions and sizes.

Clicking the funnel symbol makes it possible to filter on a text string:
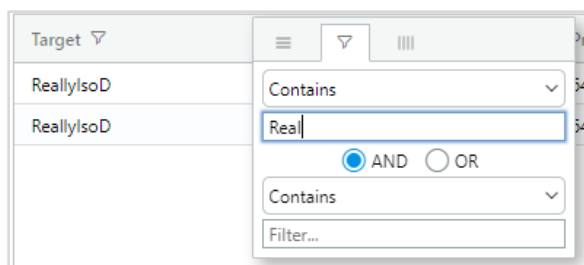
Clicking the four vertical lines brings up a set of check boxes to configure the columns shown in the Targets table:
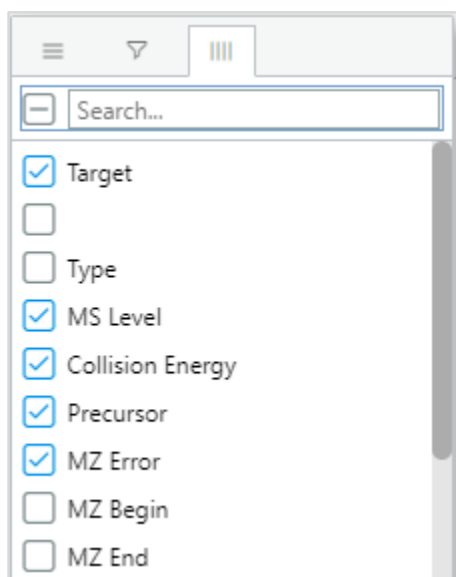
Ions Table

The Ions Table lists all ions found during the Hybrid Deconvolution workflow. Unlike the Ion Candidates table, its highest level is not a putatively matched Residue but individual ions. Although its first column is the **Matched** column, you are also unable to unmatch individual ions in the table. To do that, you should go to the Ion Candidates table and uncheck their check box.

The Ions table has the same default columns displayed as the Ion Candidates table. However, there are additional columns

that are not displayed by default and can be added by clicking on the vertical **Columns** command button to the left of the **Matched** column:

- **Internal Base Type** is the type of C-terminal cleavage for internal ions.
- **Pos** is the numeric position of the residue that the ion may match as measured from the N-terminal.
- **Residue Index** is the index of the residue this ion is associated with.
- **Internal Residue Index** is the index of the internal C-terminal cleavage residue for internal ions.

Many operations in the Ions table are the same as the Ion Candidates table, including:

- Move
- Sort
- Search
- Filter
- Copy
- Export

Also, many operations are the same as the Targets table using the three horizontal lines in a column header, including:

- Pin Column
- Autosize (this column or all columns)
- Reset Columns
- Filter (using the funnel symbol)
- Configure columns (using the four vertical lines)

Many operations are the same as the Isotopic Peaks table (described next) including:

- The command buttons at the top with the exception of not having a **Group by Ion** button (the Ions table is already grouped in that manner).
- The Group by feature (when right-clicking in the table or dragging a column to the Group by area above the **Matched** column).
- The Select Column feature (when right-clicking in the table).
- The ability to create Charts using the Chart Range menu item (available by right-clicking in the table).

Isotopic Peaks Table

The Isotopic Peaks table allows the user to examine the raw ion data. Each row is a mass peak detected in the processed spectrum. In the Ion Candidates and Ions tables those rows are consolidated into ions with charges such as w15 1+, w15 2+, w15 3+. In the Isotopic Peaks table an ion such as w15 1+ will have five rows, one for each peak in its isotopic cluster. Multiple columns are available by default or can be added.

At the top are seven command buttons:

- **Clear All Matches** changes the values in the Matched column to 0.
- **Add to Coverage** marks all ions as matched, changing the values in the Matched column to 1.
- **Subtract** changes the values in the Matched column to 0.

- **Group by Ion** moves the Name column up to the Group By area, thus displaying the Isotopic Peaks table as ion groups. Each distinct ion (e.g., w15) will have a section under the Group column that opens up when you click on the >. You can turn off Group by Ion by clicking on the x next to Name above the Group row.
- **Filter Unmatched** turns on a filter in the Matched column that displays only ions with a value of 1. You can turn off Filter Unmatched by clicking the Remove Filters command button.
- **Filter Unassigned** removes # ions in the Type column. Type # ions are unassigned ions that match an Averagine model for an amino acid residue. Note that the Type column is not displayed by default. You can turn off Filter Unassigned by clicking the Remove Filters command button.
- **Remove Filters** turns off all filters and restores the original ions displayed in the Isotopic Peaks table.

In the default configuration, columns are displayed which contain information for each ion including:

- **Matched** which contains a 0 or 1 depending if it is a matched mass peak.
- **Residue** is the amino acid in the target sequence that the ion associated with that mass peak may match.
- **Name** is the designation of the ion using ion type, position, and charge e.g., w15 1+.

- **Pos** is the numeric position of the residue that the ion may match as measured from the N-terminal.
- **MZ** is the observed *m/z* of the mass peak found in the data file by the Hybrid Deconvolution workflow.
- **Theo MZ** is the theoretical *m/z* of the mass peak.
- **PPM** is the difference between the observed and theoretical *m/z* for the mass peak as expressed in the ratio parts-per-million. PPM = (theoretical *m/z* value - observed *m/z* value / theoretical *m/z* value) * 10^6 .
- **Intensity** is the observed value of the spectrum's y axis for this mass peak.
- **SNR** is the signal-to-noise ratio of the observed intensity value.
- **Abundance** is the theoretical abundance ratio calculated for this isotope.
- **% H** is the percentage of single hydrogen transfer.
- **% 2H** is the percentage of double hydrogen transfer.
- **r Coeff** is the Pearson correlation coefficient, which is a component of the Ion Score.
- **% Fit** is calculated as ((1 −chi-squared p-value) x 100), also a component of the Ion Score.
- **# Peaks** is the number of peaks that match between the observed and theoretical isotopes.
- **ionScore** is the Ion Score as calculated in the section The Signal Processing Algorithm.

Clicking on the vertical **Columns** command button to the left of the **Matched** column opens an area with check boxes that allow

further configuration of the Isotopic Peaks Table:
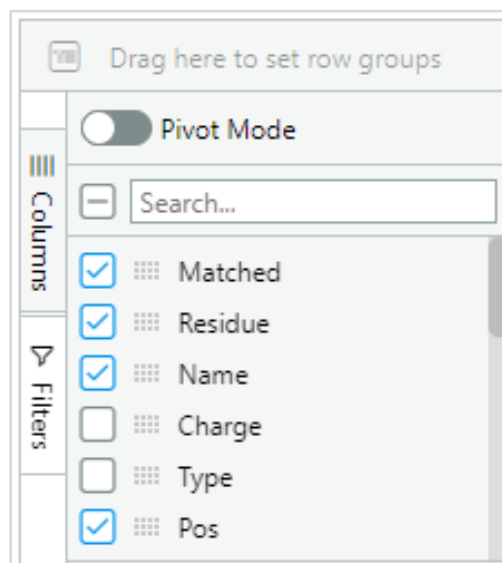
Existing columns can be removed and new ones can be added including:

- **Charge** e.g., +1, +2, +3.
- **Type** of ion i.e., a, b, c, d, i (internal), w, x, y, z, and # (unassigned).
- **N/C Pos** is the position of the fragmentation site relative to the N-terminal as the beginning.
- **Monoisotopic** indicates a Yes or No depending if the mass peak is the monoisotopic mass for the ion, i.e., the mass based on the most abundant stable isotopes of the constituent elements.

Many operations in the Isotopic Peaks table are the same as the Ion Candidates table, including:

- Move
- Sort
- Search
- Filter

- Copy
- Export

Also, many operations are the same as the Targets table using the three horizontal lines in a column header, including:

- Pin Column
- Autosize (this column or all columns)
- Reset Columns
- Filter (using the funnel symbol)
- Configure columns (using the four vertical lines)
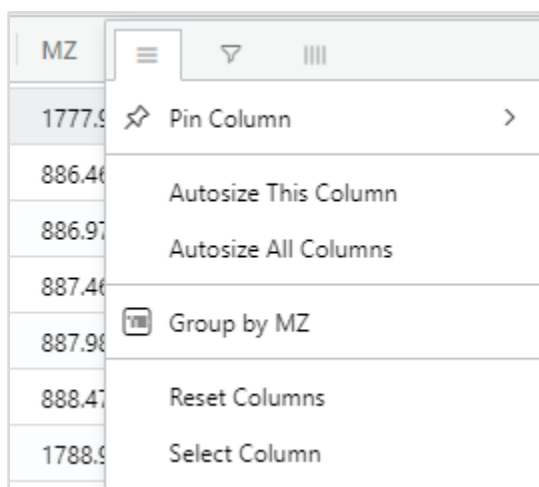
The Isotopic Peaks table has some features that are not available in other ExDViewer tables:

- Group by, which allows transforming the Isotopic Peaks table to look at specific sets of information. For example, selecting Group by Residue puts every mass peak row associated with a particular residue into a group. It is possible to create a group underneath the group, e.g., Group by Name will put each ion under the Residue group:

There are two ways to use Group by: select it as a menu item underneath the three horizontal lines in a column header, or drag the column up to the Group by field underneath the **Clear All Matches** command button. The Group by functionality can be turned off by clicking in the column header and selecting Un-Group by, clicking the x next to the column name in the Group by box at top or by selecting Reset Columns.

- Select Column is also a menu item underneath the three horizontal lines in a column header. It will highlight the current column as well as any other column that has a cell selected. It is possible to multi-select cells by holding down the Ctrl button and clicking with the mouse cursor.

Right-clicking in a cell provides access to the Chart Range menu item. For example, by selecting the PPM and MZ columns (as above) it is possible to create a scatterplot of the mass error in parts-per-million (ppm) across the mass range:
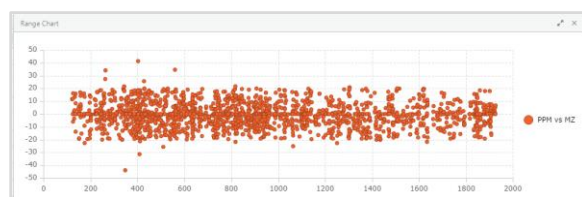
Once the columns have been selected, right-click in a cell and click on **Chart Range > X Y (Scatter) > Scatter:**
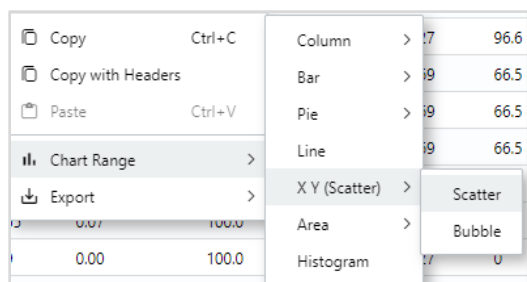
It is possible to create many other types of charts using the data in the Isotopic Peaks table including bar charts, pie charts, and histograms.

m/z Deviation

The *m/z* Deviation tab provides a graph of *m/z* delta (i.e., mass error) vs the Theoretical *m/z*. This is useful in looking at the performance of the instrument that the data were acquired on.
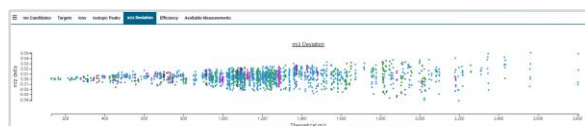
Efficiency

The Efficiency tab displays the intensity of components detected during the analysis including the mass spec precursor, reduced precursor ions, ECD, EID, CID, internal, and unassigned ions. The count of ion clusters by charge state is graphed, which can assist in determining optimal conditions.
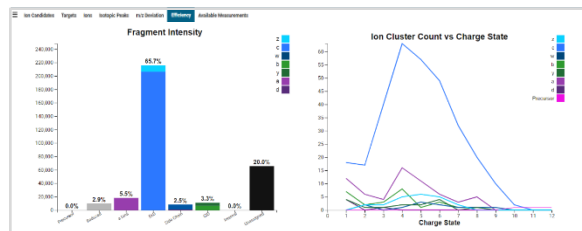
Available Measurements Table

The Available Measurements table allows users to customize the set of known mass offsets that get used when measuring the distance between mass peaks in the Spectrum window. It is preloaded with common amino acids, post translational modifications, and chemical adducts. These measurements are accessed via the measuring tool in the Spectrum window, making it easy to interpret unassigned peaks which may not match known ion types. The Cominatoric depth feature enables multiple combinations of mass offsets, with up to three permutations.

For example, when using the **Measure distance between any two masses** command button in the Spectrum window's Toolbar multiple mass offsets with their *m/z* error can be suggested as the user moves the cursor parallel to the x axis. This can be useful for de novo sequencing when the target sequence is not unambiguously known.
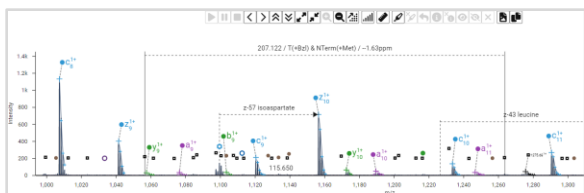
There are five command buttons at the top of the Available Measurements table (four are shown here):


- **Load XML (BioConfirm Format)** opens a dialog box that filters for XML files. Users with Agilent MassHunter BioConfirm installed can provide an updated set of identified mass offsets from the BioConfirm XML file available at D:\MassHunter\ChemicalDataDictionary\preset\10.0\datadictionarypreset_peptide.xml.
- **Add New Annotation** creates a new row at the top of the Available Measurements table. Users can fill in the information about the custom annotation in the columns.
- **Remove Selected** deletes those annotations that have the check box in the first column checked.
- **Regenerate Combinations** quickly calculates the use of multiple mass offsets together, either two or three at a time.
- **Reset to Defaults** changes the table back to an initial state.

The **Combinatoric depth** radio button allows you to select either 2 or 3 annotations applied. Next to the radio buttons it will display the number of measurement labels that are available.

Setting up Target Sequences with Target Editor

The Target Editor enables the user to add, modify and curate target sequences used in the Hybrid Deconvolution workflow. To access it, click the **Target Editor** icon in the home page Navigation UI or the **File > Target Editor** menu item.
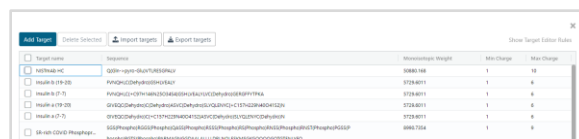
To add a target sequence, click the **Add Target** button below. A new row will be created at the top with the dummy sequence name "target name." Fill this field in with your sequence name. Double-click in the **Sequence** field to enter your new amino acid sequence. The monoisotopic weight will automatically be calculated.
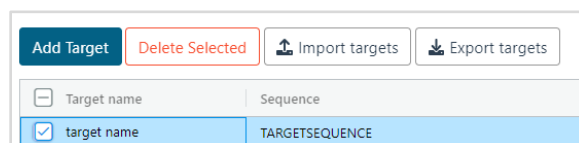
To edit a sequence, double-click in its **Target name** and/or **Sequence** field. It is also possible to edit the **Min Charge** and **Max Charge** fields to values appropriate for the molecule. When you are finished, press the Enter key to save the new information.

To delete a target sequence, select the check box next to its Target name and click the **Delete Selected** button. It is also possible to import and export target sequences as JSON files, a text-based format for exchanging information. This capability can be useful when working with collaborators

and reviewers. Click the **Export targets** button to write out a JSON file to the PC's hard drive. Click the **Import targets** button to bring in target sequences using a JSON file accessible to the PC. If any of the targets already exist in ExDViewer you will be prompted as to whether to keep the existing versions or overwrite with those from the JSON file.

To add PTMs to target sequences, ExDViewer uses a derivative of [Unimod](#) syntax. To access a summary, click the **Show Target Editor Rules** button in the upper right corner. For example, a serine (S) residue that has a phosphorylation PTM will be annotated with (Phospho) as below:

To exit the Target Editor, click the **X** in the upper right corner.

Running the Hybrid Deconvolution Workflow

The Hybrid Deconvolution workflow makes top-down analysis of a protein or peptide fast and easy. It answers the question "Does this spectrum match this protein or peptide?" The default set of preferences can be used to analyze a wide variety of proteins and peptides.

To begin the Hybrid Deconvolution workflow, click the **Hybrid Deconvolution** icon in the home page navigation UI or the **Workflow > Hybrid Deconvolution** menu item.

Hybrid Deconvolution Step 1 – Input Dialog Box

The Input dialog box allows you to select the data file or spectra to analyze as well as its target sequence. In the Input File field you can select the type of file using the buttons. Note that Agilent MassHunter *.d, Thermo, Waters, mzML and MGF formats are supported.
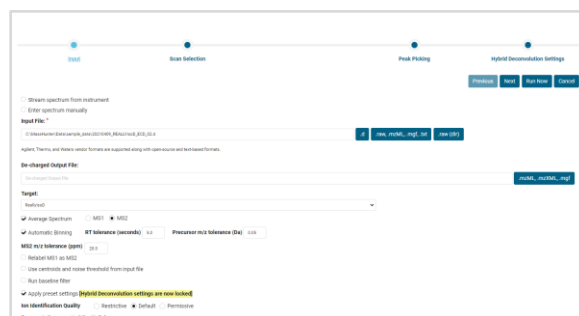
The optional De-charged Output field can be used to write out the deconvolution results. Enter a file name in and append an extension, i.e. .mzML, .mzXML, or .mgf. The type of output file will depend on the extension.

In the Target field use the dropdown to select the target sequence. All target sequences available in the Target Editor will show here.
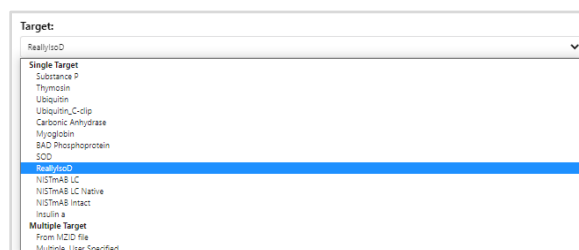
Choose whether the spectra should be Averaged, and with MS1 or MS2 data. Automatic binning also allows you to set an

RT tolerance and Precursor m/z tolerance for the bins.

In addition, select the preferred Ion Identification Quality as Restrictive, Default, or Permissive. It is also possible to generate internal fragments, neutral losses, and common precursor adducts.

Leave the rest of the preferences as their default values. Click the **Next** button.

Hybrid Deconvolution Step 2 – Scan Selection Dialog Box

The Scan Selection dialog box allows you to select which spectra should be processed. The Precursor m/z has been selected based on the target sequence. When the MZ Error is green, it indicates that the precursor MZ error is within specification. If the MZ Error is red, click the Estimate Max Charge check box. This may find a precursor maximum charge that is a better fit for the experimental spectrum. At a later point you can update the Max Charge field for that target sequence in the Target Editor.

Below, the Chromatogram window is interactive; you can zoom in or out and

sliders at each end allow you to select the range of scans for analysis
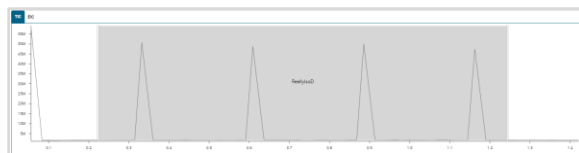
Click the **Next** button.

Hybrid Deconvolution Step 3 – Peak Picking Dialog Box

The Peak Picking dialog box controls the selection of centroid peaks from the profile data.

Most applications can use default values for peak picking. Clicking the Show advanced settings check box greatly expands the number of settings available. After initial analysis, it is possible to return to this dialog box if adjustments are needed for settings such as signal to noise ratio.
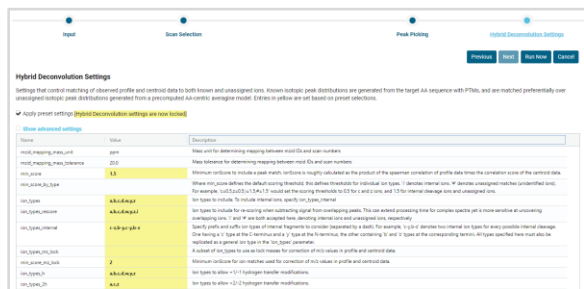

Click the **Next** button.

Hybrid Deconvolution Step 4 – Hybrid Deconvolution Settings Dialog Box

Like the Peak Picking dialog box, most applications can use default match setting values. The values in yellow are locked to preset matching settings. Removing the check mark from the Apply matching presets
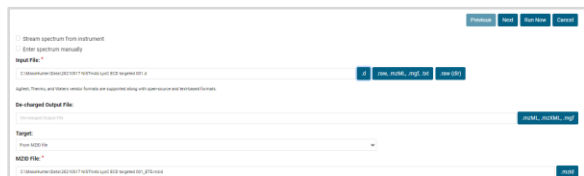
preferences check box will allow them to be modified.

When finished, click the **Run Now** button to begin the analysis.

Running Targets from an MZID File

It is possible to use an mzIdentML file (aka MZID file) as a source of targets. To do this, enter an input file used to create the MZID file and select From MZID file under the Target dropdown in the Input dialog box:

In the Scan Selection dialog box click the check box for the target sequences of interest:
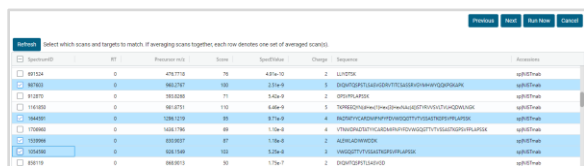
The first target sequence will be processed and appear in the Spectrum, Sequence Coverage Map and Targets table. Double-clicking on any other row in the MZID table will use cached data from the input file to

quickly process the next target sequence:
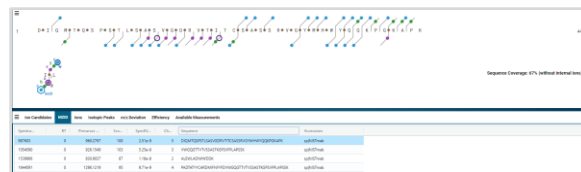
Running Multiple, User Specified Targets

Another option within ExDViewer is to run multiple target sequences at the same time. To do this, enter an input file with a mixture of compounds and select Multiple, User Specified under the Target dropdown in the Input dialog box:

In the Scan Selection dialog box click the check box for the valid Precursor and RT combinations for each target sequence:

The first target sequence will be processed and appear in the Spectrum, Sequence Coverage Map and Targets table. Double-clicking on any other row in the Targets table will use cached data from the input file to quickly process the next target sequence:
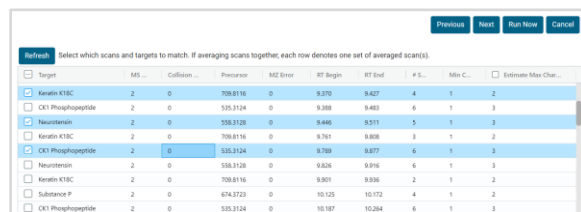
Running the Untargeted Deconvolution Workflow

The Untargeted Deconvolution workflow finds all ion fragments in a spectrum or set of spectra that match an Averagine model for amino acid residues. The ions are labeled as unassigned and annotated in the spectra. The unassigned peptide ions can then be used for de novo sequencing.

To begin the Untargeted Deconvolution workflow, click the **Untargeted Deconvolution** icon in the Navigation UI or select the **Workflow > Untargeted Deconvolution** menu item. The Input dialog box will appear:

Similar to the Hybrid Deconvolution workflow, choose an input file and settings. It is possible to create a De-charged Output

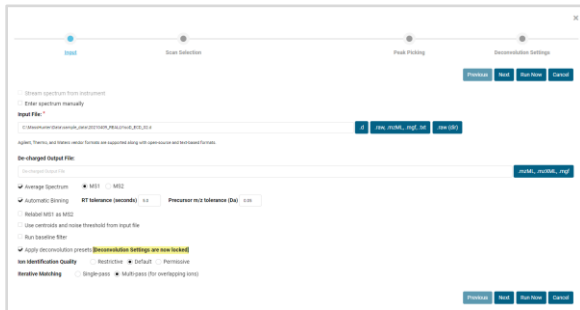file in mzML, mzXML or MGF format for moving results to another software program.

The Untargeted Deconvolution can also be a form of batch deconvolution by processing every spectrum in the data file.
The next three steps after Input are a subset of the Hybrid Deconvolution workflow with the same settings and options.

Reviewing History and Loading Cached Runs

ExDViewer has a history feature which allows you to examine previous runs. To access it, click the **History** icon in the Navigation UI or select the **File > History** menu item. The History dialog will appear:

The processed target sequences are listed with the most recent at top. Each run has cached an associated exdview file. To load a cached run, double-click in its row. Once it has loaded, close the dialog box by clicking the **X** in the upper right corner. It is also possible to remove a processed target sequence by clicking its check box to the left of the Target column and clicking the **Remove Selected** button. You might want to do this to save space on your PC's hard drive.

The Cache limit is displayed at the top as well as the current Cache size. The Cache limit can be adjusted by clicking the **Edit** button.

Saving Runs as exdview Files

ExDViewer allows you to save your processed runs as exdview files. These can then be loaded at a later time or shared with another ExDViewer user.

- To save an exdview file the first time, select the **File > Save Run As** menu item. It is also possible to save the exdview file with a custom layout using the **File > Save Run As With Layout** menu item.
- To update an exdview file which you have loaded, select the **File > Save Run** menu item (this will be unavailable until you have an exdview loaded).
- To load an exdview file, either click the **View Results > Select View File** icon in the Navigation UI or select the **File > Load Saved Run** menu item.
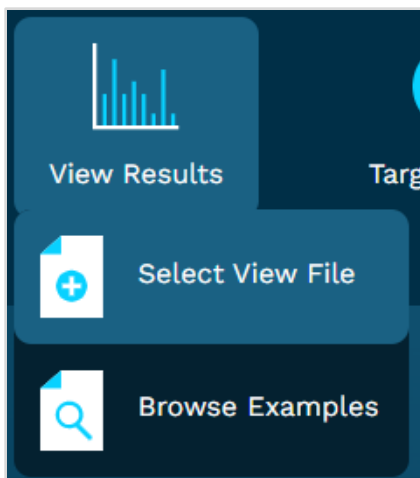
Streaming Spectra Directly From an Instrument

Streaming ExD Cell Spectra From an Agilent LC/Q-TOF

Streaming allows you to collect data in real time and view it in ExDViewer. You can use streaming to optimize your acquisition parameters while watching the changes in the output. To run streaming on an Agilent LC/Q-TOF with an ExD cell installed, follow these steps:

1. Begin acquiring data from a sample on the instrument in MassHunter software's Tune context.
2. Click the **Hybrid Deconvolution** icon in the Navigation UI or the **Workflow > Hybrid Deconvolution** menu item.
3. Click the **Stream spectrum from instrument** check box in the Input dialog box.
4. Enter or confirm the instrument's IP address in the IP Address field as well as the A Value and T0 Value (which are available in the most recent tune report). Alternatively, click the **Auto detect calibration parameters** check box to have ExDViewer obtain them from the MassHunter software.
5. Enter a name for the output file in the **Output File** field. This will be saved to the computer's hard drive as an mzML file.
6. Enter a value in the **Averaging Window** field. This will specify the number of scans averaged before the data is analyzed and displayed. A

lower number allows faster updating but possibly less accuracy.

7. Select a target sequence in the **Target** field.
8. Set up all other preferences in the Input dialog box as you would for processing an acquired data file.
9. In most cases, the settings in the Peak Picking and Hybrid Deconvolution Settings dialog boxes can remain unchanged unless you identify a specific reason to adjust them.
10. Click the **Run Now** button.

You will see the data from the sample appear in the ExDViewer as it is acquired by the instrument.   The spectrum will be updated every few seconds. The time interval depends on the number of scans averaged, how complex the sample is, and the specific preferences that have been set.

The constant updating allows the user to optimize results for specific ion types including all those generated by ExD or CID. In addition, it is possible to adjust fragmentation conditions on the mass spectrometer to achieve optimal sequence coverage. All features in ExDViewer can be used in real time including zooming in the Spectrum window and clicking on ions in the Sequence Coverage Map. The Fragment Intensity graph in the Efficiency tab will

continuously update with percentages of each ion type.

When you want to stop streaming, select the **Workflow > Hybrid Deconvolution** menu item and click the **Terminate** button in the upper right corner.

Converting Spectra to Other Formats

ExDViewer enables you to convert data files and spectra to other industry standard formats. For input, Agilent MassHunter *.d, Thermo, Waters, mzML[5] and MGF[6] formats are supported. They can be converted to mzML, mzXML[7], and MGF files.

To convert data files and spectra:

1. Click the **Convert** icon in the ExDViewer Navigation UI or the **Workflow > Convert Spectrum** menu item.
2. Select a data or text file in the **Input File** field using the buttons next to it. It is also possible to enter a spectrum manually by clicking the **Enter spectrum manually** check box and entering it in a text format. Follow the instructions in the field that appears for the supported formats.
3. Enter a file name in the Output File field and append an extension, i.e.. mzML, .mzXML, or .mgf. The type of output file will depend on the extension.
4. Select the Output data file type, either profile or centroid.

5. Choose whether the spectra should be Averaged, and with MS1 or MS2 data. Automatic binning also allows you to set an RT tolerance and Precursor m/z tolerance for the bins.
6. Set other options in the Input Files dialog box and click the **Next** button.
7. In most cases, the settings in the Convert Options dialog box can remain unchanged unless you identify a specific reason to adjust them.
8. Click the **Run Now** button.

The data file or spectra will be converted to the selected format and placed on the file system in the location in the Output File field.

Running the Browser-based ExDViewer

The browser-based version of ExDViewer is available at the URL https://viewer.e-msion.com/ The browser-based version has most of the same menu items and capabilities as the desktop version.

The browser-based version does not allow you to upload data files at this time. As a result, the following capabilities are not available:

- Running the Hybrid Deconvolution workflow on a data file that is not on the server (i.e., a data file on your hard disk)
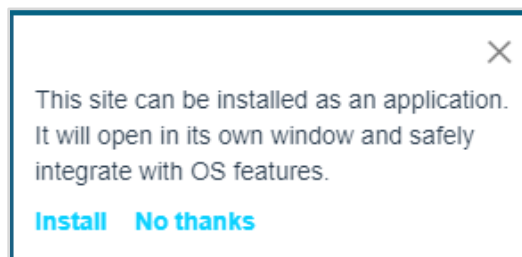- Running the Convert workflow

The browser-based version can be used to share results with users who do not have ExDViewer installed on their PC, as described in the section Sharing Results using a URL. It can also be used to view an exdview file on your hard disk.

The first time you open up the browser-based version it will offer you the option of downloading and installing the program on your hard drive. This hard drive-based version has the same restrictions as the version that runs in a browser.

Sharing and Exporting Results

Sharing Results Using a URL

ExDViewer allows you to share results with users who do not have the software program installed. This allows you to collaborate with others as soon as you have processed your data. To share your results using a Uniform Resource Locator (URL) aka a web address:

1. Run the Hybrid Deconvolution, Untargeted Deconvolution workflow or load a saved run (i.e. an exdview file).
2. Select the **File > Share Run** menu item.
3. A message will appear letting you know that the results will be uploaded to a private server. Click the **Share** button.
4. The results will upload and then a message will appear with a URL. The URL is also copied to your Windows clipboard. Click the **Close** button.
5. Copy the URL into a message that you can send to a collaborator. They will be able to click on the URL and open up your results in the browser-based ExDViewer. They can examine the results including clicking on individual ions to inspect them.

Exporting Results to Excel

There are several ways to export your results and settings to Microsoft Excel.

- The File > Export ion state as CSV menu item creates a Comma Separated Value file with a column for each ion's parameter such as charge or molecular composition. The CSV file will be saved wherever you choose and can be opened in Microsoft Excel.
- The Ion Candidates, Ions, and Isotopic Peaks tables allow you to right-click and select the Export > CSV Export and Excel Export menu items. These files will also be saved wherever you choose. Note that Ion Candidates and Ions will export at the complete ion level and Isotopic Peaks will export all isotopic peaks. Unlike the ion state export, only columns visible in the tables will be exported.
- The Available Measurements table will also allow you to right-click and select the Export > CSV Export and Excel Export menu items.

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