



HDX User Guide

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Introduction

The HDX module analyzes mass spectrometry data acquired from hydrogen-deuterium exchange experiments. HDX is available as a standard workflow in Byos®.

General Notes

- MS data should be located on the local computer (not on a network drive) and should not be in read-only folders, because Byos writes cache files during project creation.
- Projects created with beta versions of the HDX software may not open properly. Beta version projects should be re-created.
- Processing Waters .raw data (including data collected with ion mobility) is now fully supported.
- If the *.blgc project is copied to another computer, it is best to also copy the data files/folders and cache files to the new computer.

Creating a Project

To create a new project, go to the Byos Workflows tab and click the HDX workflow icon:

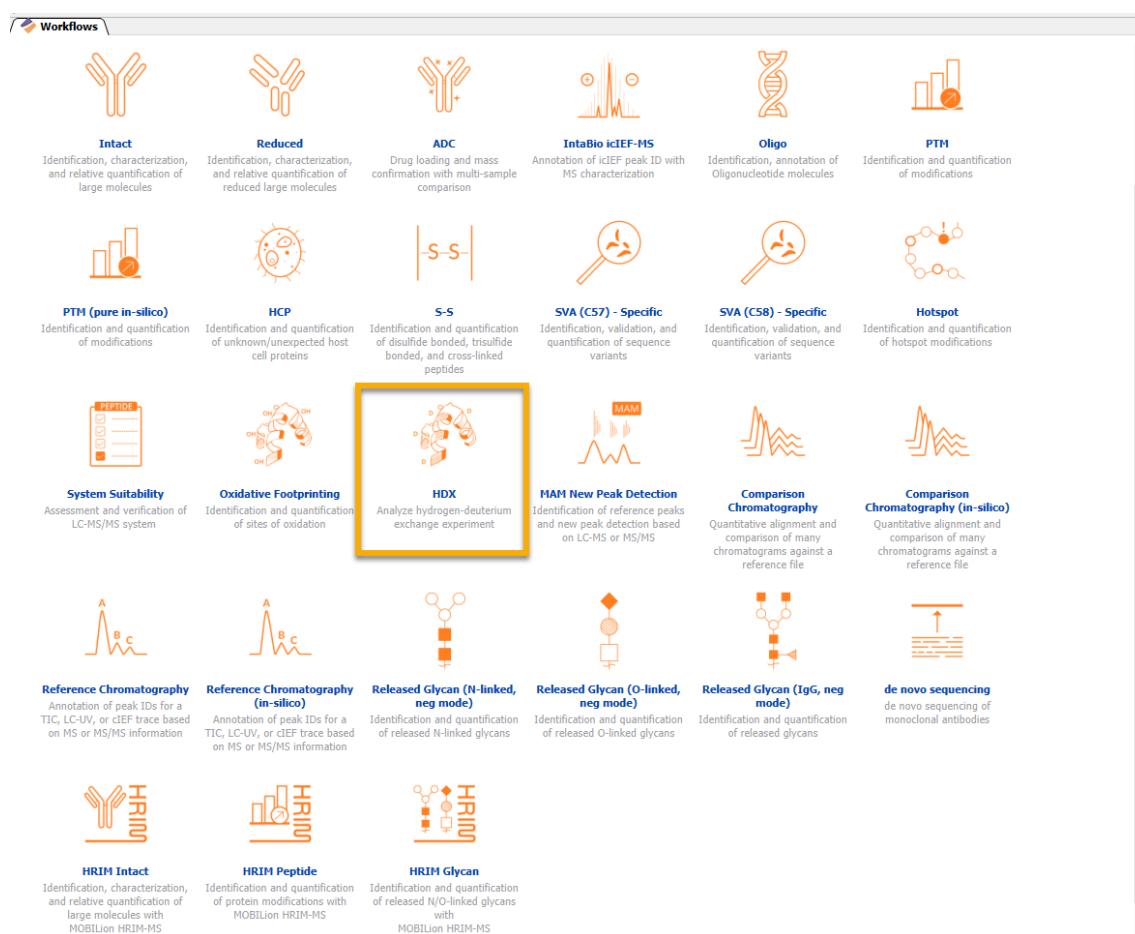


Figure 1: The Byos workflows with the HDX icon highlighted

A multi-tab workflow (e.g., **Samples**, **Sequences** and **Processing nodes** tabs) window allows the analyst to designate one or multiple mass spectrometer files to be analyzed, each with one or multiple set of MS/MS search results and other parameters, as well as a corresponding set of protein sequences.

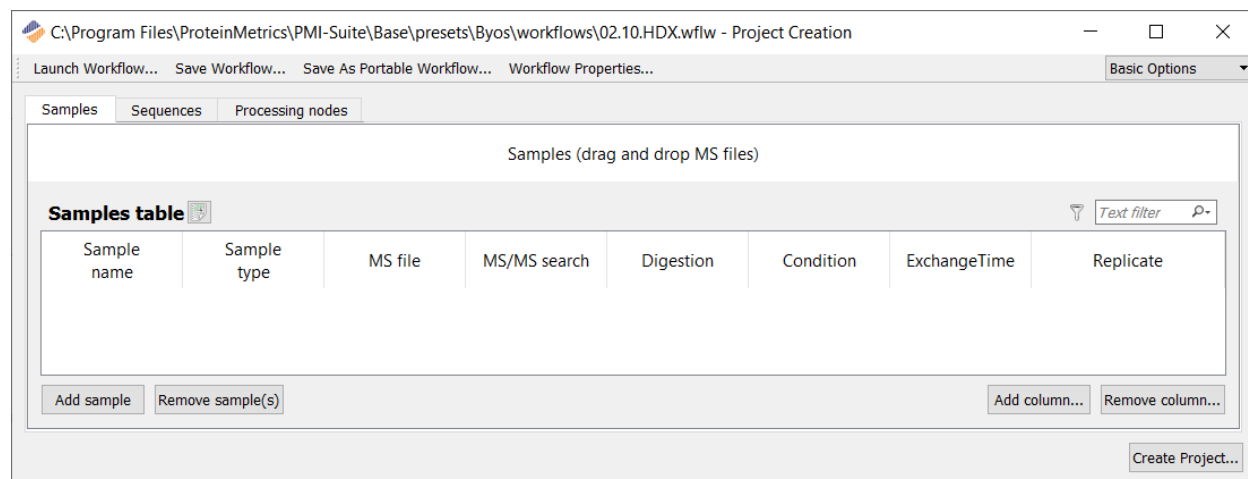


Figure 2: HDX workflow showing Samples tab

Samples Tab

The **Samples** tab allows the user to load raw LC-MS sample files into the project. Drag-and-drop MS data onto the Samples table. **The order of files is important.** Data for the reference condition should be added **first**. (Note that the Sample type, which can be “Reference” or “NonReference” is *ignored*).

Tip — If dragging-and-dropping multiple MS data files/folders simultaneously, it is usually best to place the mouse pointer at the **top** of your list of MS data files/folders. For example, during drag-and-drop of these four files (e.g., file1.raw, file2.raw, file3.raw and file4.raw), if the mouse pointer is over the top file i.e., file1.raw, then the Samples table will also have file1.raw at the top:

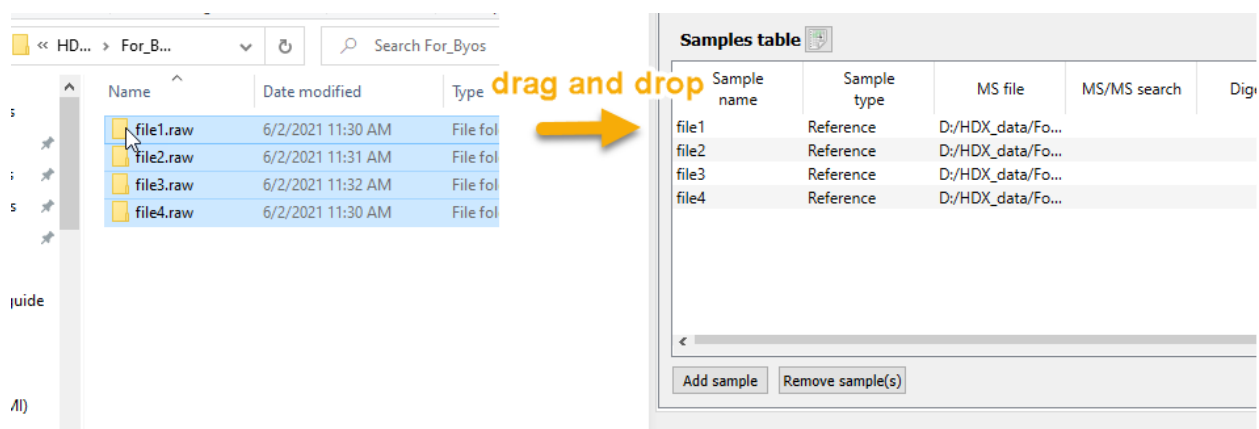




Figure 3: Drag-and-drop from the top file to maintain sample order


If the mouse pointer is **not** over the top file, then the file that the mouse is over will be on top. For example, if it is over file3.raw — then the Samples table will have file3.raw at the top.

To load files by browsing for them, click the **Add sample** button, double-click on the new row under the **MS file** header, click  and browse to the file. Click **Open**.

Sample table Columns

Make sure to fill in the **Condition** and **Exchange Time** columns. The others are auto-filled or optional.

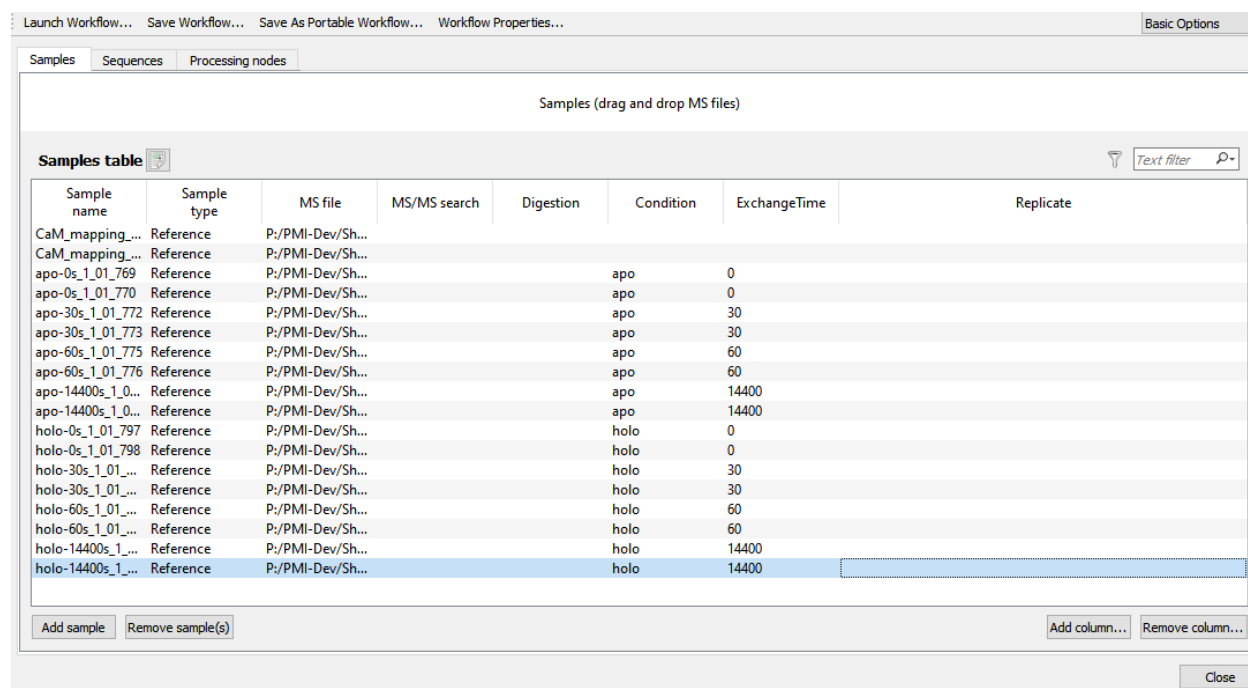
- **Sample name:** defaults to the file name. To change the Sample name, double-click the name and edit the text. The name can also be updated later in the Project table.
- **MS/MS Search:** To add an incremental MS/MS search result to a sample, double-click in that sample row under the **MS/MS Search** header, click  and browse to the MS/MS search result (*.byrslt or *.dat) file. Click **Open**. To attach additional Ms2 search result to that sample, click  to add a new MS/MS Search row and repeat the addition steps.

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

- **Digestion:** This column is not used for HDX projects.
- **Condition:** Different conditions in the HDX experiment should be assigned different labels (for example, “Apo,” “Holo,” etc.). In the plots (XIC plots, Isotope plots, Uptake plot), each condition is assigned a unique color. The reference condition should be listed **first**. Note that the reference condition cannot be changed after project creation.
- **ExchangeTime:** Fill in the exchange time as a number (for example, “30,” not “30 sec”). ExchangeTime for all samples should have same units (i.e., “3600”, not “1 hr”, if rest of samples are in seconds).
- **Replicate:** Optional. Must be an integer number. Can be useful for filtering the data when inspecting the project. For example, this can reduce clutter on the screen by restricting the results to a single replicate.

Tip — To fill in multiple cells simultaneously: Select multiple rows using shift-left-mouse-click or control-left-mouse-click. In the column of interest, right-mouse-click and select **Edit selected row(s)**, select or enter the value, and click **OK**.

An example of a completed Samples tab is shown below:



Sample name	Sample type	MS file	MS/MS search	Digestion	Condition	ExchangeTime	Replicate
CaM_mapping_...	Reference	P:/PMI-Dev/Sh...					
CaM_mapping_...	Reference	P:/PMI-Dev/Sh...					
apo-0s_1_01_769	Reference	P:/PMI-Dev/Sh...			apo	0	
apo-0s_1_01_770	Reference	P:/PMI-Dev/Sh...			apo	0	
apo-30s_1_01_772	Reference	P:/PMI-Dev/Sh...			apo	30	
apo-30s_1_01_773	Reference	P:/PMI-Dev/Sh...			apo	30	
apo-60s_1_01_775	Reference	P:/PMI-Dev/Sh...			apo	60	
apo-60s_1_01_776	Reference	P:/PMI-Dev/Sh...			apo	60	
apo-14400s_1_0...	Reference	P:/PMI-Dev/Sh...			apo	14400	
apo-14400s_1_0...	Reference	P:/PMI-Dev/Sh...			apo	14400	
holo-0s_1_01_797	Reference	P:/PMI-Dev/Sh...			holo	0	
holo-0s_1_01_798	Reference	P:/PMI-Dev/Sh...			holo	0	
holo-30s_1_01_...	Reference	P:/PMI-Dev/Sh...			holo	30	
holo-30s_1_01_...	Reference	P:/PMI-Dev/Sh...			holo	30	
holo-60s_1_01_...	Reference	P:/PMI-Dev/Sh...			holo	60	
holo-60s_1_01_...	Reference	P:/PMI-Dev/Sh...			holo	60	
holo-14400s_1_...	Reference	P:/PMI-Dev/Sh...			holo	14400	
holo-14400s_1_...	Reference	P:/PMI-Dev/Sh...			holo	14400	

Figure 4: Completed Samples tab

Sequences Tab

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

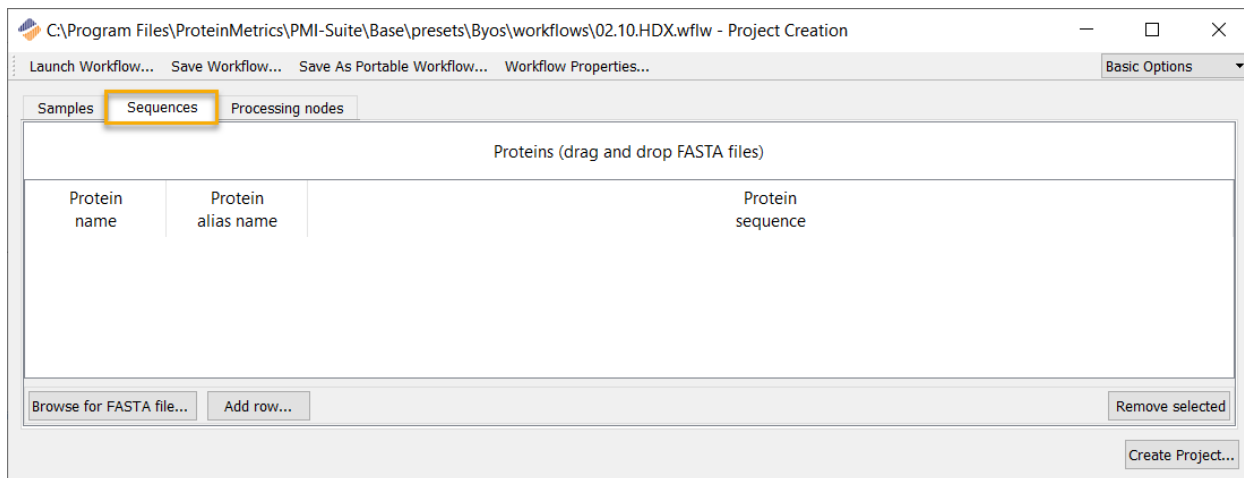


Figure 5: Sequences tab

To add a *.fasta or *.fa file, click **Browse for FASTA file**, locate the file to be added and click **Open**. FASTA files also can be dragged into this dialog directly. An example of a sequence added from a FASTA file is shown below:

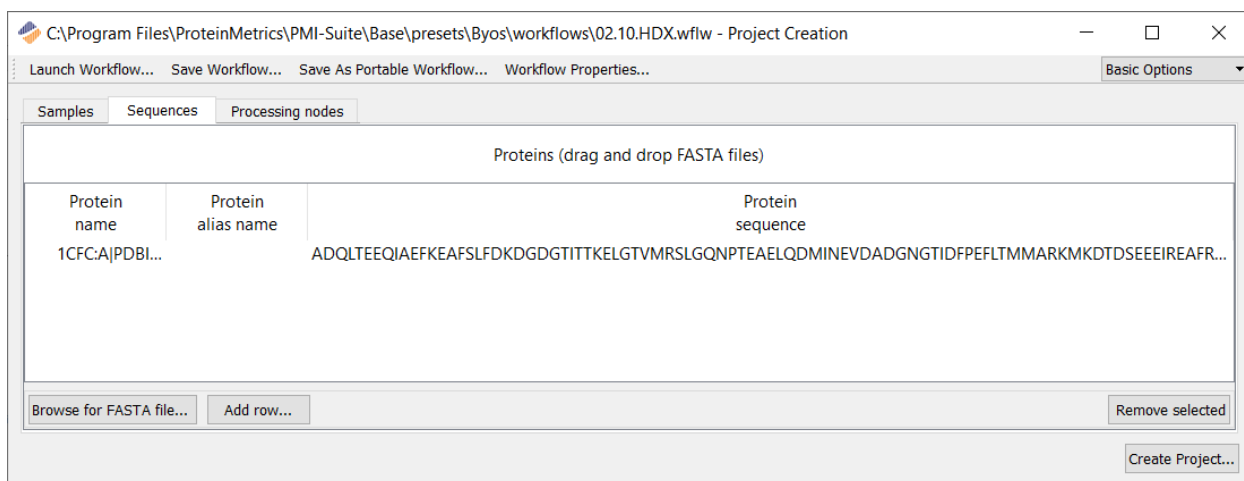


Figure 6: Sequences tab with protein added from a FASTA file

Processing nodes Tab

The **Processing nodes** tab is where the user sets experimental parameters.

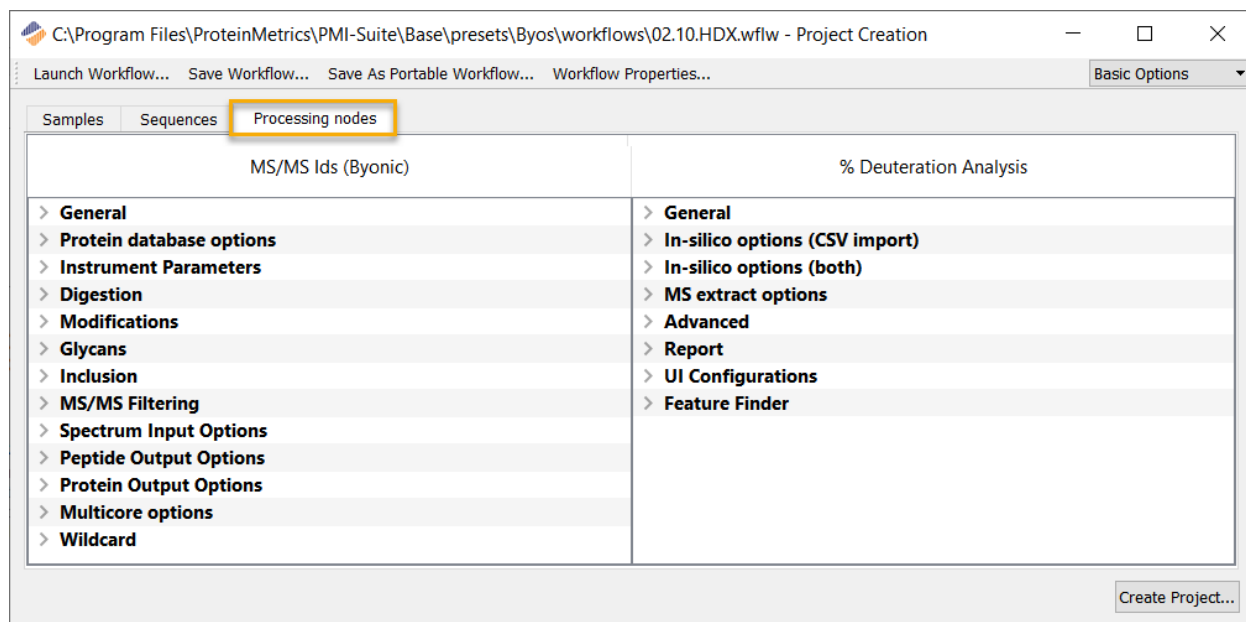


Figure 7: Processing nodes tab

The **left side** of the Processing nodes tab holds parameters for the MS/MS database search for peptide identification. The **right side** holds parameters for the % deuteration analysis of the MS¹ data. Most of these parameters are described in **02 PMI Byos Manual.pdf** in the **PTM > Processing nodes** section. A few parameters are critical to HDX.

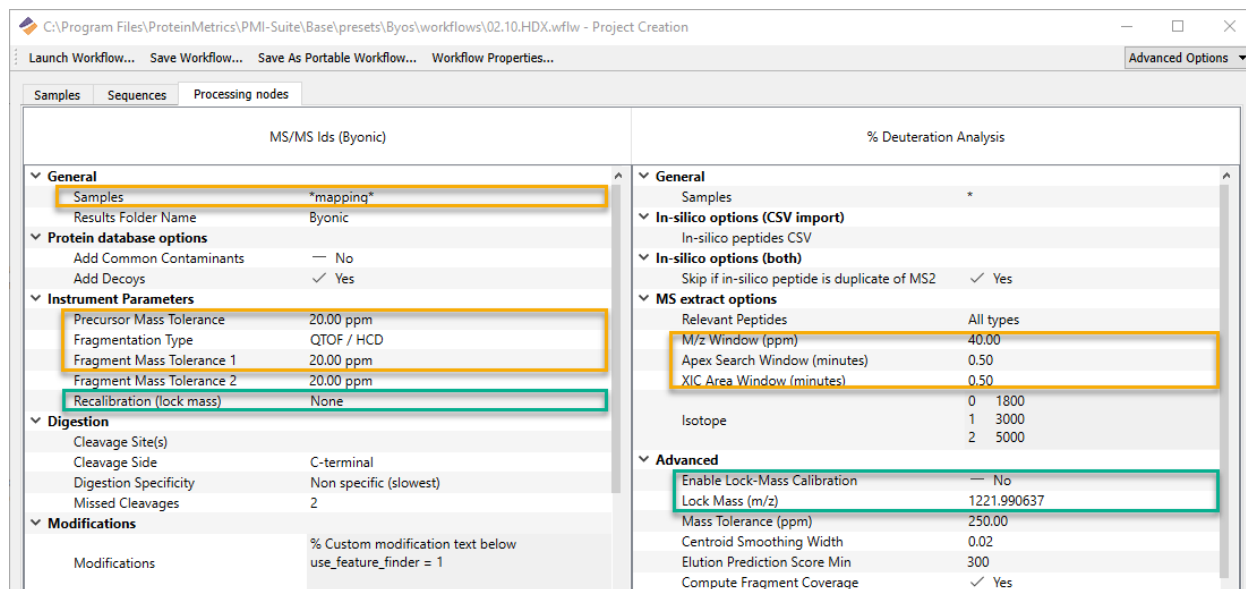


Figure 8: Critical HDX parameters marked in gold, instrument-specific parameters marked in green

Critical Parameters

The parameters that are required for HDX workflows are highlighted in gold in the figure above:

- **Samples:** Specify a text pattern that matches the undeuterated MS/MS data to be used for database search but **does not match the MS¹ data**. For example, "****mapping***" can be used if all

of the undeuterated MS/MS data contain “mapping” in their file names but none of the MS¹ data of the deuterated samples contain “mapping” in their file names. (*Note: Edit the sample file names to fit to this criterion before running the HDX workflow*)

- **Mass tolerances and fragmentation type:** Choose appropriate settings for the data.
- **Time tolerances:** If the run-to-run chromatography is highly reproducible, the retention time tolerance can be decreased.

Parameters Specific to Instruments

The following three lock mass parameters (highlighted in green in the figure above) should be set for specific instruments (e.g. Waters):

- **Recalibration (lock mass)**
- **Enable Lock-Mass Calibration**
- **Lock Mass (m/z)**

Predefined Peptides Without Byonic Database Search

For predefined peptides (i.e., in-silico peptide list), the left side of the **Processing nodes** tab should be deleted (i.e., the Byonic search), and an in-silico peptides CSV should be specified. For an example file showing the format, see `C:\Program Files\ProteinMetrics\PMI-Suite\ExampleData\ImportExample.Byologic.csv`.

To remove the Byonic search from the Processing nodes tab, right-click in the **MS/MS Ids (Byonic)** header and click **Remove**:

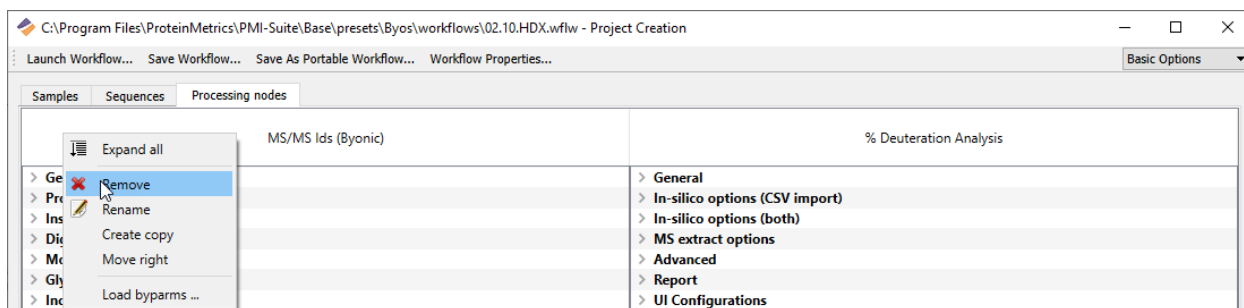


Figure 9: Removing the Byonic Processing nodes

To add an in-silico peptides CSV file, expand **In-silico options (CSV Import)**, click in the **In-silico peptides CSV** cell, click the “...” button, navigate to and select the CSV file, and click **Open**.

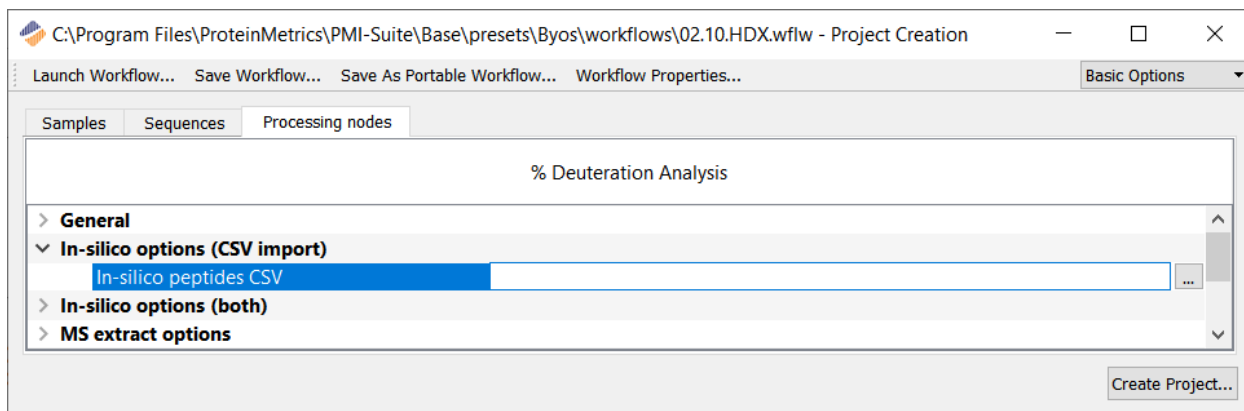


Figure 10: In-silico CSV file selection

Save Workflow and Create Project

The customized workflow can be used as a template for future HDX runs. To save the workflow, click the **Save Workflow** button, assign a custom workflow name, and click **Save**.

To generate the project, click **Create Project**, assign a project name, and click **Save**.

Project Inspection

An example of a completed project is shown below:

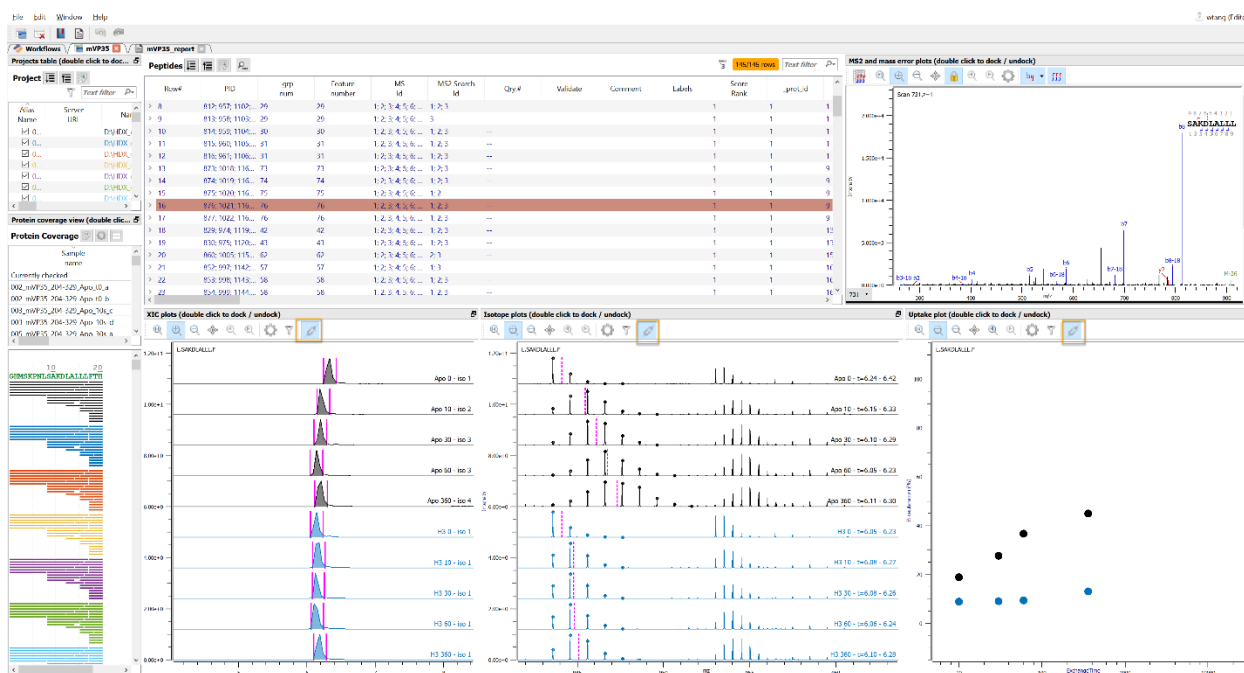


Figure 11: HDX project



A good column sort can greatly facilitate inspection. Click on the column headers of the **Peptides** table in the reverse order from the field sort priorities. For example, to generate the following sort:

- Start AA

- End AA
- Condition
- ExchangeTime

then click on **ExchangeTime**, **Condition**, **End AA**, and **Start AA**, in that order.

Other columns that may be helpful to sort/filter are XIC area summed and PEP 2D.

The XIC plots, Isotope plots and Uptake plot all contain the **Sync filtering and sorting** icon . When the icon is active in the plots, the plots apply the same filtering and sort orders set in the **Peptides** table for the selected record by clicking the  filter icon:

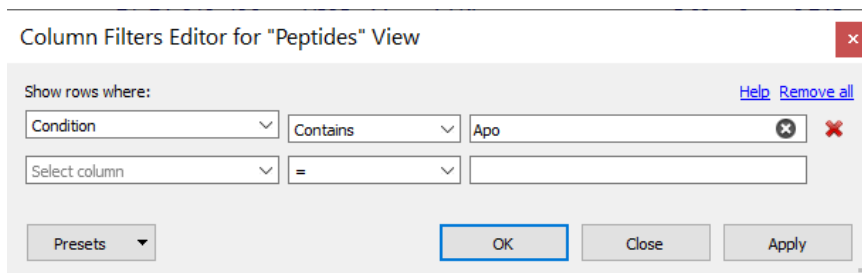


Figure 12: Example filter applied to Peptides table records

By default, the Peptides table filtering is synced to all the plots:

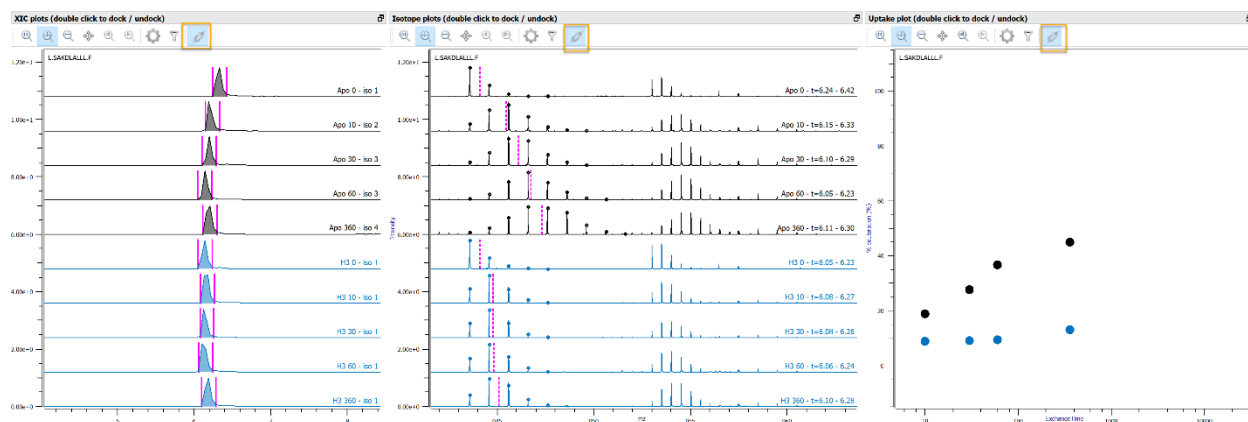


Figure 13: Synced filtered plots

To un-sync the filtered plots, de-select the sync button (shown in the orange boxes above).

Uptake Plot

Uptake plots estimate the percent deuterium uptake of each selected peptide:

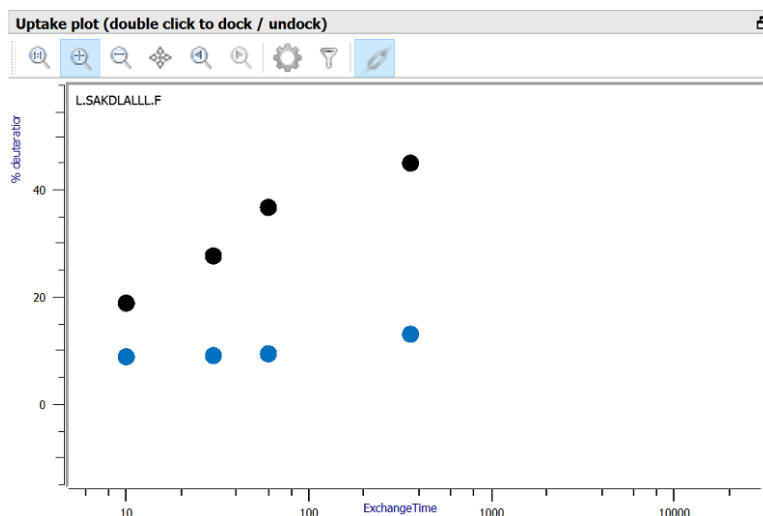


Figure 14: Uptake plot

“100% Deuteration” is defined by the number of amino acids in the peptide, minus the number of proline residues, minus 2 (for fast back-exchanging N-term residues).

When a project is first opened, the Uptake plot’s default y-axis range is -15% – 115%. After performing any zoom operations on the Uptake plot (e.g., zoom in, zoom out, pan, reset zoom), the default zoom can be restored using advanced commands, as described below.

The behavior of the Reset zoom button (1:1 button) can be altered with an advanced command that fixes the y-axis range. For example, if you use the following advanced command:

```
[XY]
YRange = 0, 50
```

and then you press the Reset zoom button (1:1 button), the plot’s y-axis range becomes 0 – 50.

To restore the default y-axis range (i.e., the y-axis range when you first opened the project), use the following advanced command:

```
[XY]
YRange = -15, 115
```

and then you press the Reset zoom button (1:1 button). To add an advanced command, choose **Edit > Advanced configuration**:

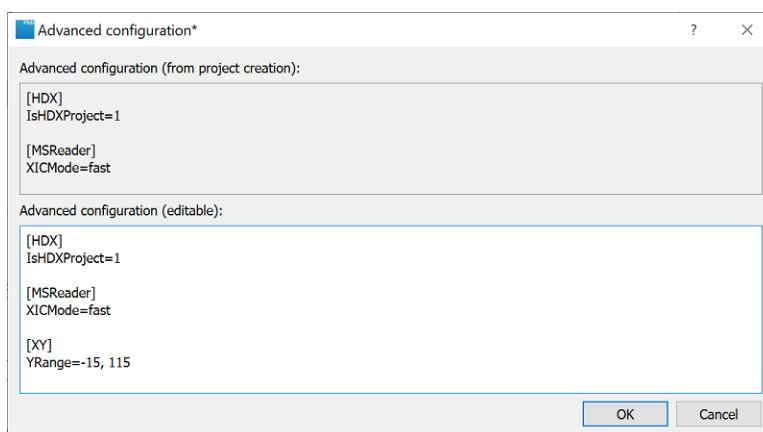



Figure 15: Advanced commands in the Advanced Configuration dialog

The y-axis variable can be changed by clicking on the  icon.

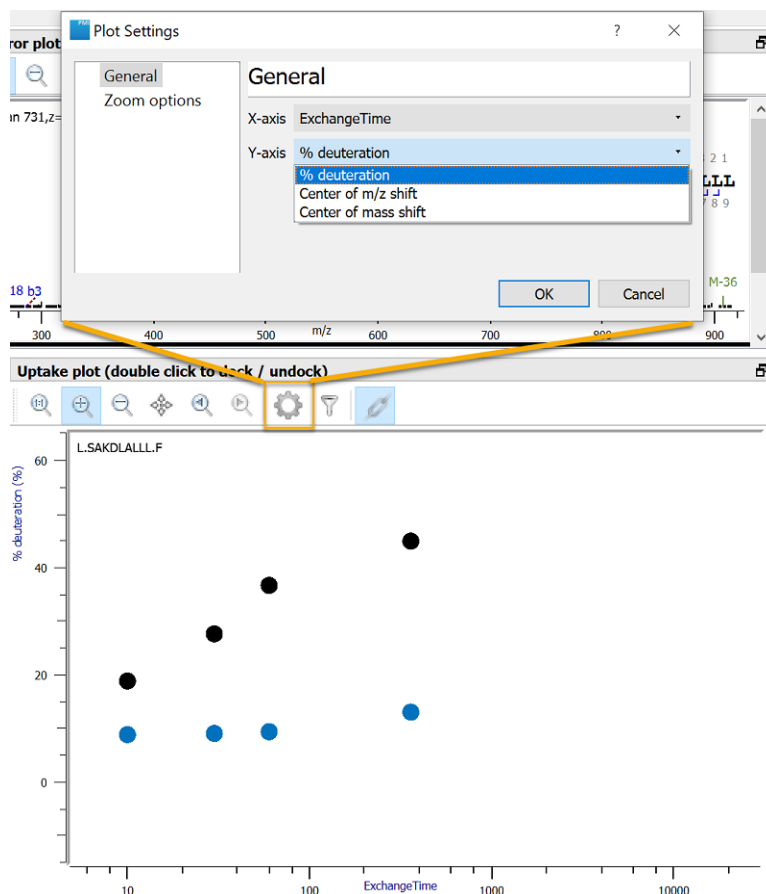


Figure 16: Changing the y-axis variable

The x-axis variable cannot be changed (it is always ExchangeTime). Y-axis options include **% deuteration**, **Center of m/z shift**, or **Center of mass shift**. The **Zoom options** section controls the same zoom and scrolling options available for other plots.

Isotope Plots

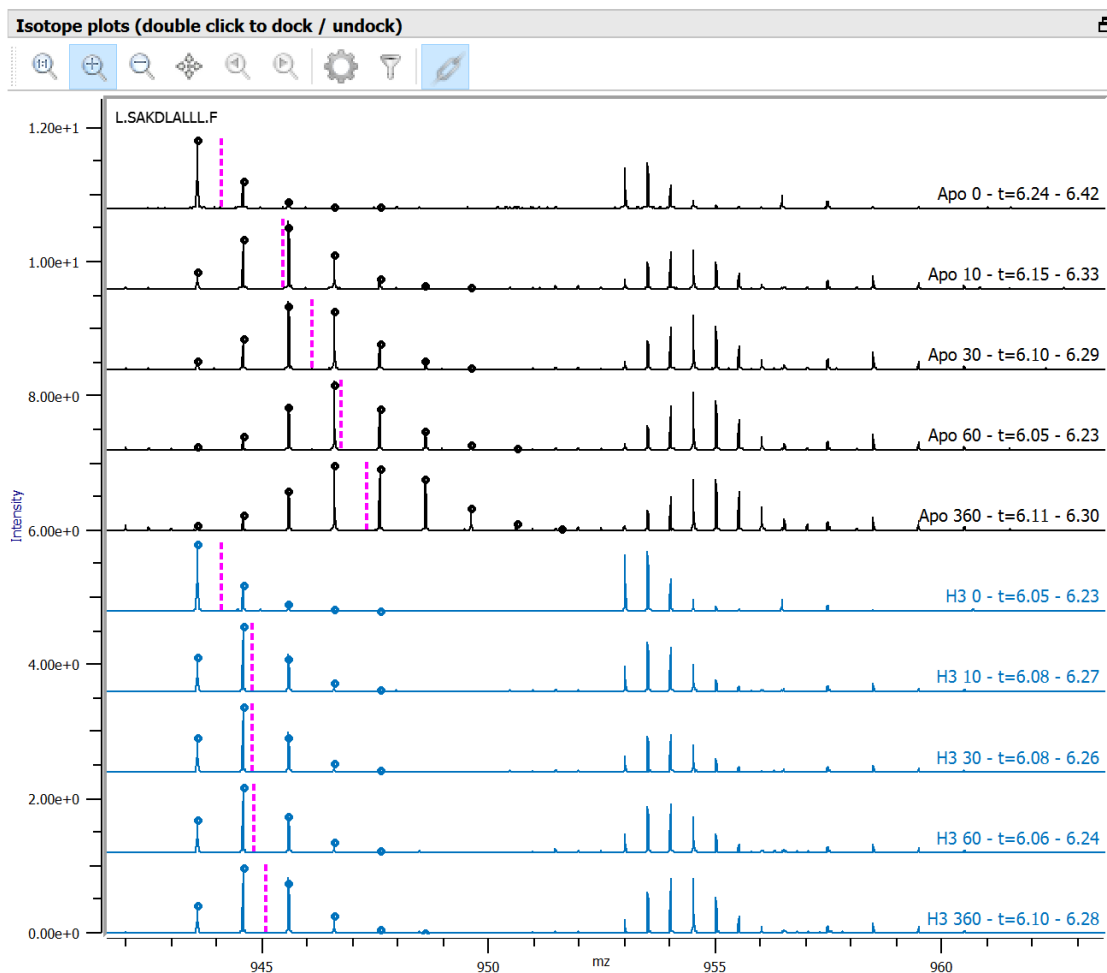



Figure 17: Isotope plots

The magenta dotted lines show the center of m/z, and the dots on the peaks show the isotopes used to calculate the center of m/z. Show/hide of either of the magenta dotted line and/or the dots can be configured by clicking on the  icon.

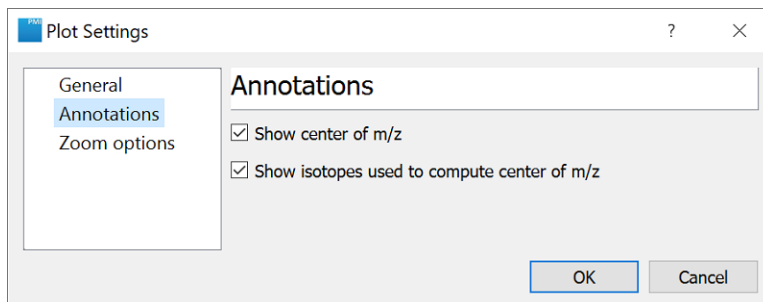


Figure 18: Isotope plot Annotation settings

XIC Plots

The XIC plots show the time range over which the center of m/z analysis was performed:

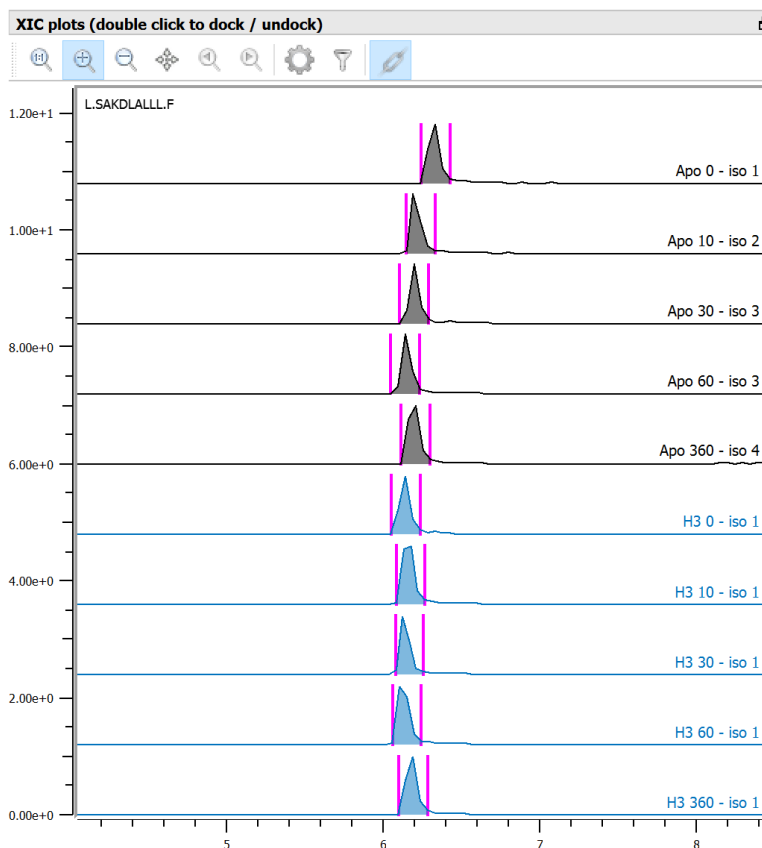


Figure 19: XIC plots

Curating Peptides

Poor peptides can be excluded by marking them as false positives in the Peptide table's **Validate** column (or by using the keyboard shortcut Ctrl+F). Then apply a filter to exclude all false positives.

Row#	PID	_grp num	Feature number	MS Id	MS2 Search Id	Qry.#	Validate	Comment	Labels	Score Rank	_prot_id	S
> 9	122; 127; 300; ...	24	24	1; 2; 3; 4; 5; 6; ...	1	--			1	1	1	
> 10	186; 154; 156; ...	25	25	1; 2; 3; 4; 5; 6; ...	1	--			1	1	1	
> 11	155; 160; 302; ...	25	25	1; 2; 3; 4; 5; 6; ...	1	--			1	1	1	
> 12	6; 349; 474; 59...	60	60	1; 2; 3; 4; 5; 6; ...	1	--			1	1	9	
> 13	91; 94; 350; 47...	61	61	1; 2; 3; 4; 5; 6; ...	1	--			1	1	9	
> 14	138; 351; 476; ...	62	62	1; 2; 3; 4; 5; 6; ...	1	--			1	1	9	
> 15	195; 199; 353; ...	63	63	1; 2; 3; 4; 5; 6; ...	1	--			1	1	9	
> 16	193; 198; 215; ...	63	63	1; 2; 3; 4; 5; 6; ...	1	--			1	1	9	
> 17	201; 204; 312; ...	34	34	1; 2; 3; 4; 5; 6; ...	1	--			1	1	13	
> 18	266; 254; 257; ...	35	35	1; 2; 3; 4; 5; 6; ...	1	--			1	1	13	
> 19	227; 332; 457; ...	47	47	1; 2; 3; 4; 5; 6; ...	1	--			1	1	16	
> 20	197; 200; 333; ...	48	48	1; 2; 3; 4; 5; 6; ...	1	--			1	1	16	
> 21	202; 334; 459; ...	48	48	1; 2; 3; 4; 5; 6; ...	1	--			1	1	16	
> 22	189; 336; 461; ...	49	49	1; 2; 3; 4; 5; 6; ...	1	--			1	1	16	
> 23	187; 194; 335; ...	49	49	1; 2; 3; 4; 5; 6; ...	1	--			1	1	16	
> 24	248; 251; 252; ...	50	50	1; 2; 3; 4; 5; 6; ...	1	--			1	1	16	

Figure 20: Marking a peptide as a false positive

Click the Search filter button and check **False-positive** under **Show**:

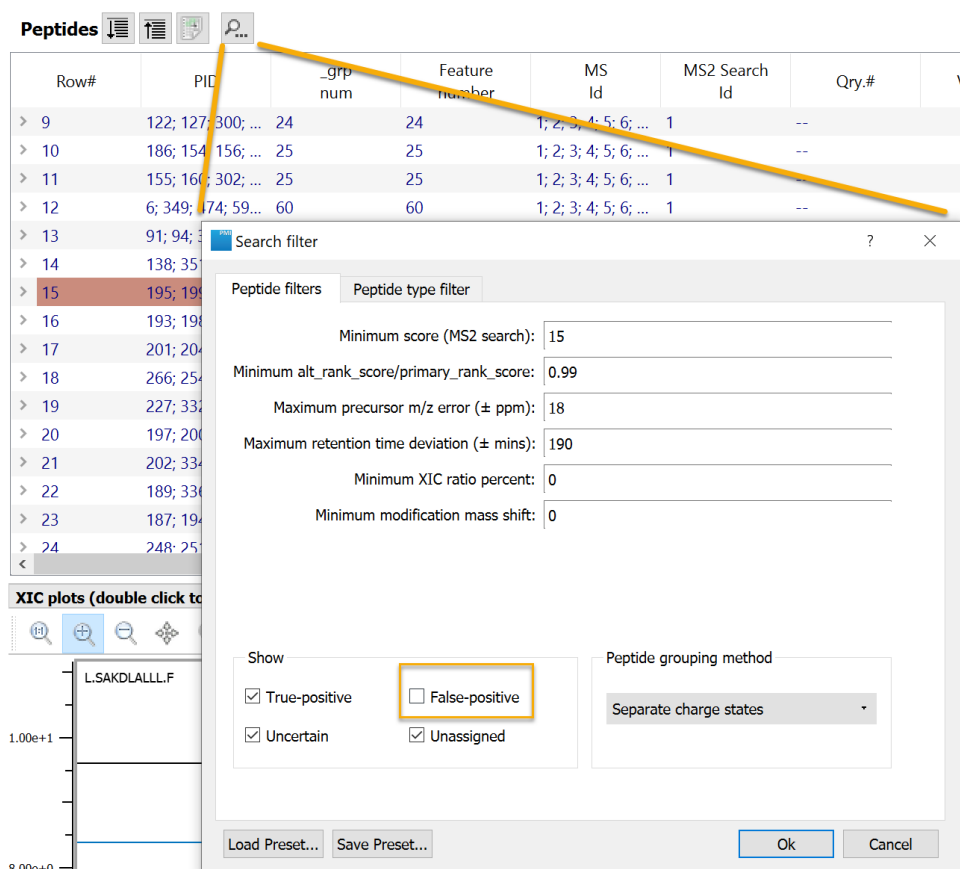


Figure 21: Filtering out false positives

Updating Plots With Custom XIC Time Range

To change an XIC time window for a peptide of interest, select only that peptide row (e.g., peptide of a specific sample). In the Peptides table, expand the tree view and select a single peptide (the XIC plots pane as well as the Isotope plots pane should each display a single trace). Then, in the XIC plots pane, hold down the shift key and with the mouse key depressed, drag across the desired time window. A black horizontal bar will indicate the selected time range.

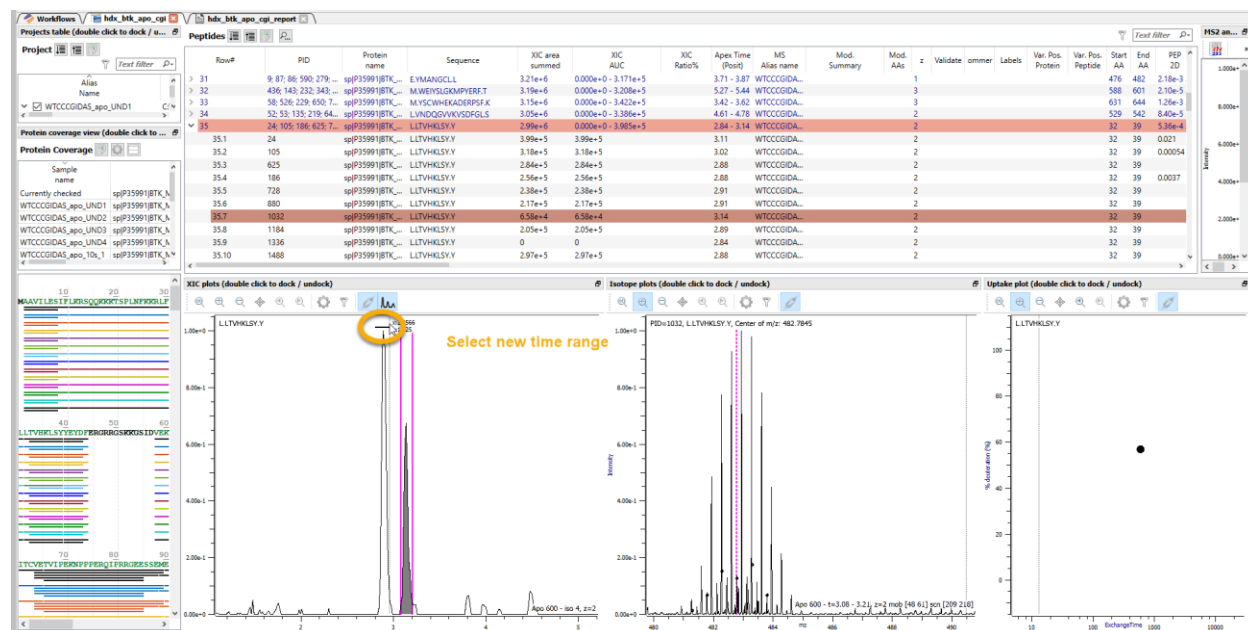



Figure 22: Selecting a new XIC time range

Upon releasing the mouse button, the Isotope plot and the uptake plots will update to reflect the new time range selected in the XIC plot, as shown in the figure below:



Figure 23: Updated plots to reflect new XIC time range

The change is immediately saved to the project. Note that an uptake plot exists for “deuterated samples” only. **NOTE:** Instead of shift/drag option, the same mode can also be enabled by selecting **Inspection mode** icon  (this icon only appears after selecting a single peptide from a single sample from the tree view). When selected, Inspection mode allows the user to mark a range within a XIC plot to inspect the Isotope plots for that range.

To revert the change, click the Undo button or select Undo from the Edit menu (see figure below).

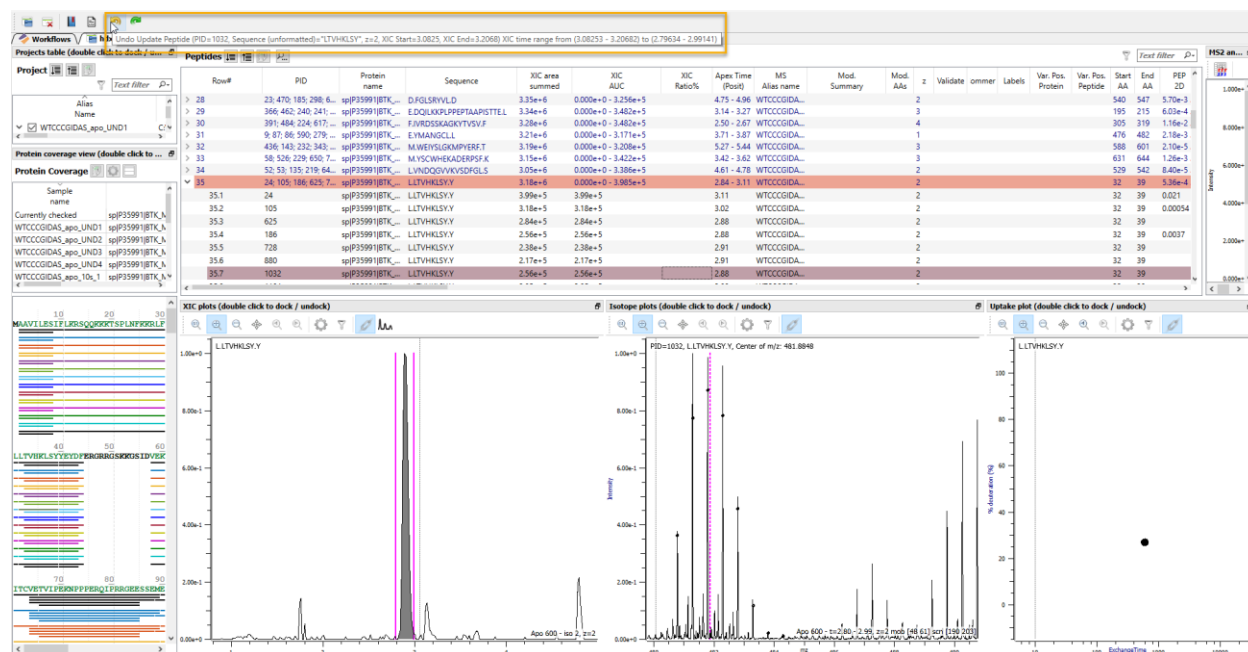


Figure 24: Revert change with Undo button

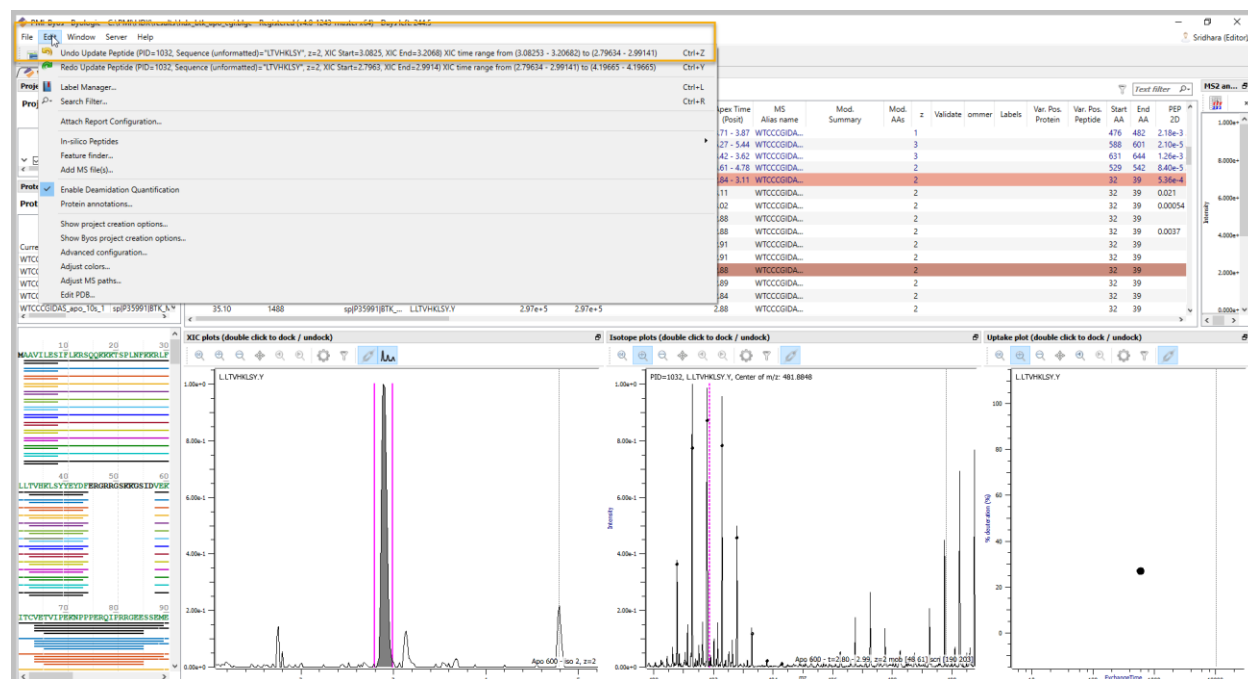


Figure 25: Revert change with Undo from Edit menu

Reporting

While the HDX project inspection view is designed for inspecting peptides in detail one at a time, the Report tab is designed to show information about all peptides in aggregate. The HDX workflow uses a

specialized report template designed for their unique features (the file is found in **File > Presets > Report presets > Blgc_HDX.rptc**). The report tabs include:

- **Protein Map** – shows a peptide-level view
- **Heatmap** – shows a residue-level view
- **Protein 3D** – If the protein's 3D structure is known, the residue-level data can be overlaid onto the 3D structure
- **Uptake plots** – shows all the plots (uptake plot, MS² plot, MS¹ plot, XIC plot) all at once, allowing for convenient export of all plots to PDF

The reference condition is the first non-null condition in the MS data list (as specified during project creation).

Protein Map Tab (Peptide-Level View)

In the Protein map visualization, each horizontal line represents a peptide:

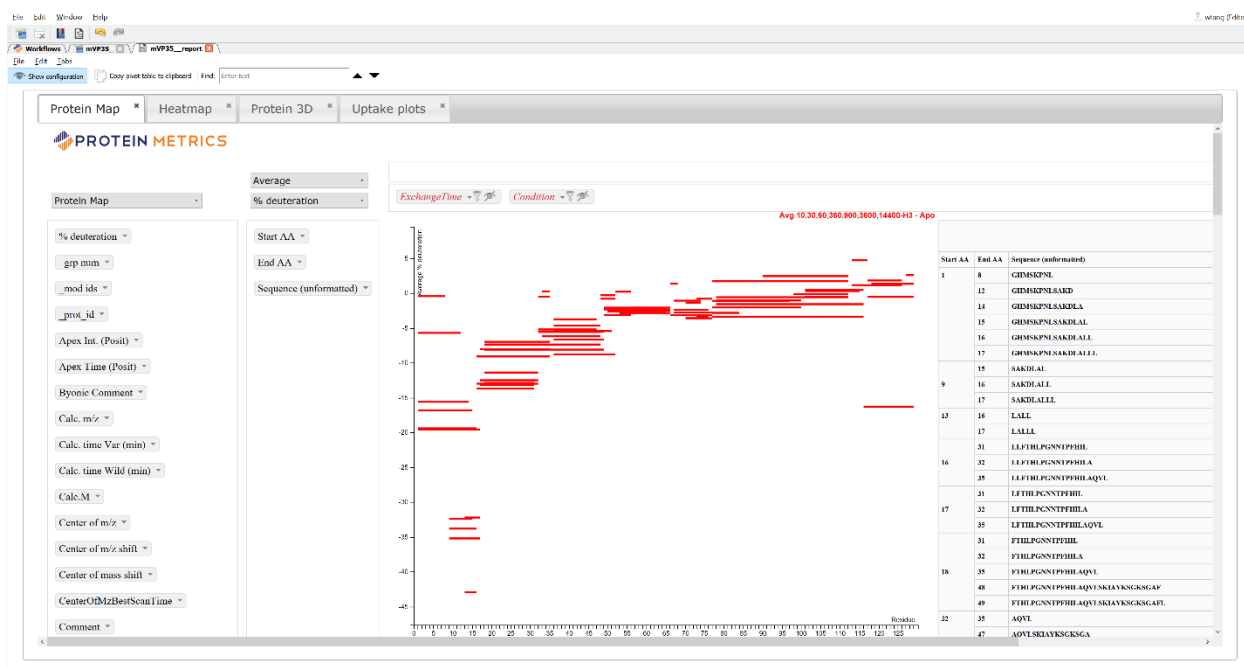


Figure 26: Peptide-level protein map

X-Axis

The protein residue number and sequence are on the x-axis. For long sequences, the window should be made wider.

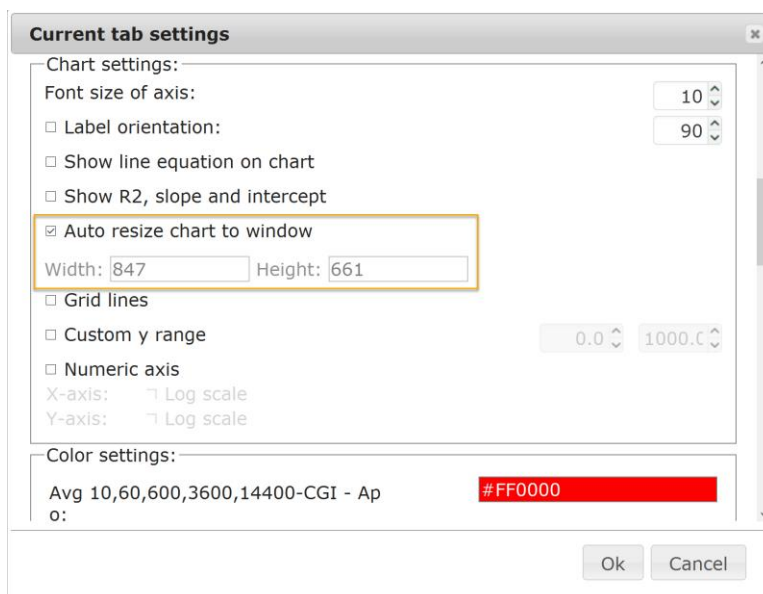


Figure 27: Resetting the chart width for long sequences

Y-Axis

By default, the y-axis shows the **difference** in % deuteration between two conditions averaged over all exchange times. % deuteration alternatives can be center of m/z shift or center of mass shift.

Filtering

The plot legend (or which Exchange time or conditions are visible) can be adjusted using the following user interface controls:

- Exchange time
- Condition

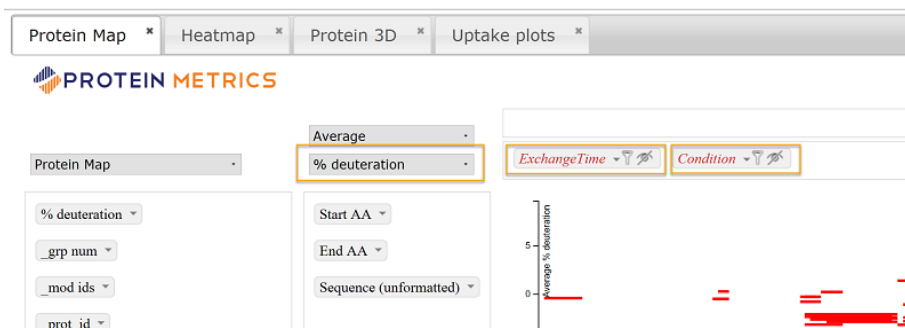


Figure 28: User interface controls for adjusting the y-axis variable

Visualization Types

An alternative visualization to the Protein Map is the **Bar Chart**, where each bar represents a peptide, and the bars are ordered from N-terminus to C-terminus. Note that only the relative position of the bars is meaningful; the distance of the bar along the x-axis is not meaningful in the peptide-level view bar chart.

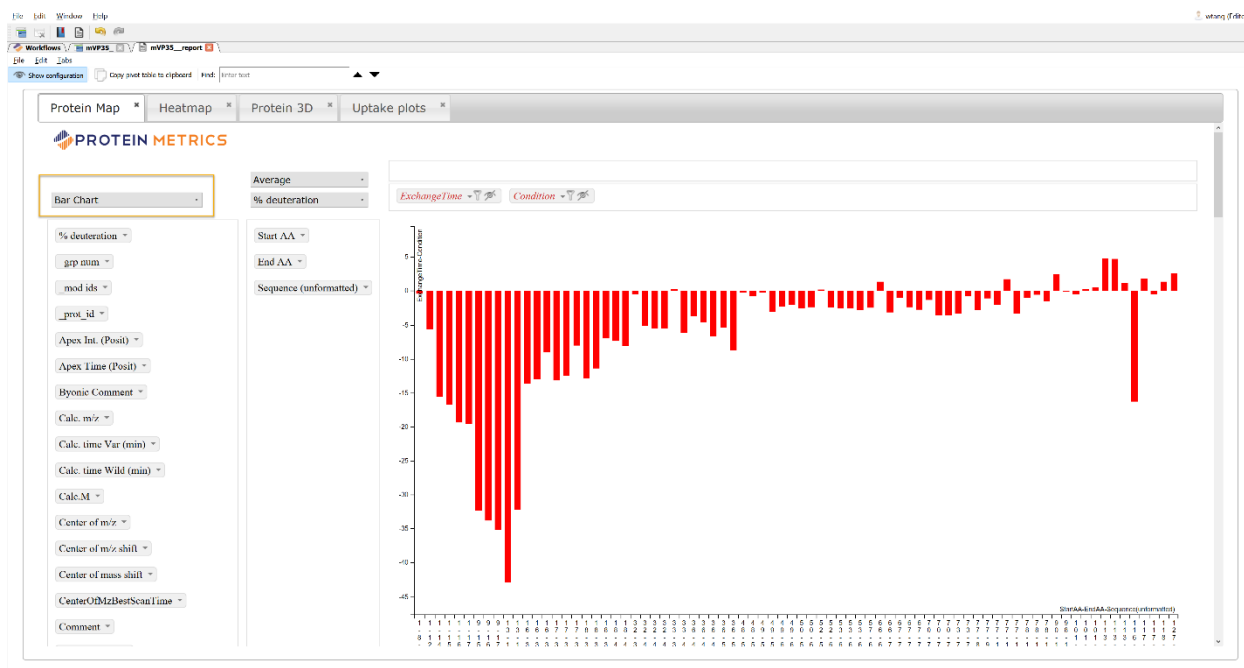


Figure 29: Peptide-level bar chart

Heatmap Tab (Residue-Level View)

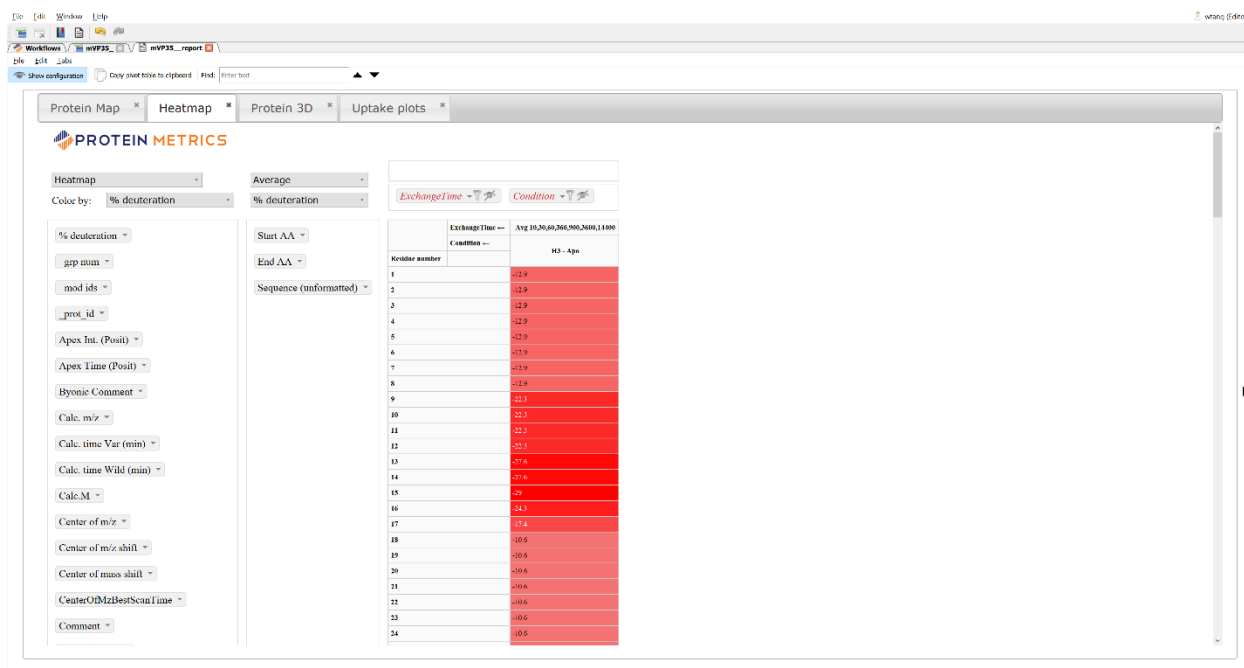


Figure 30: Residue-level heatmap

X-Axis

Same as the peptide-level view.

Same as the peptide-level view, except the calculation is done for each residue by averaging over all the peptides that include that residue.

The default visualization type is the heat map, but interesting alternative visualization types include the Bar Chart and the Line Chart.

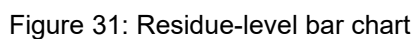


Figure 32: Residue-level line chart

For the residue-level bar chart and line chart, it is best to enable the numeric X-axis. To do so, chose **Edit > Current tab settings**, and check **Numeric X-axis**.

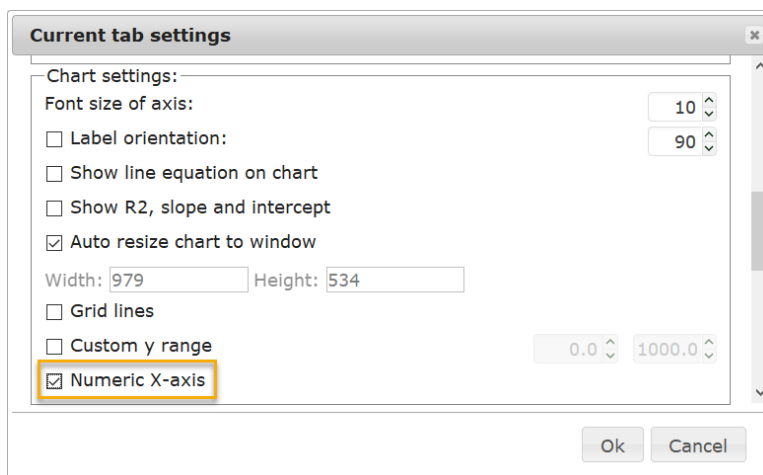


Figure 33: Numeric X-axis

Protein 3D Tab

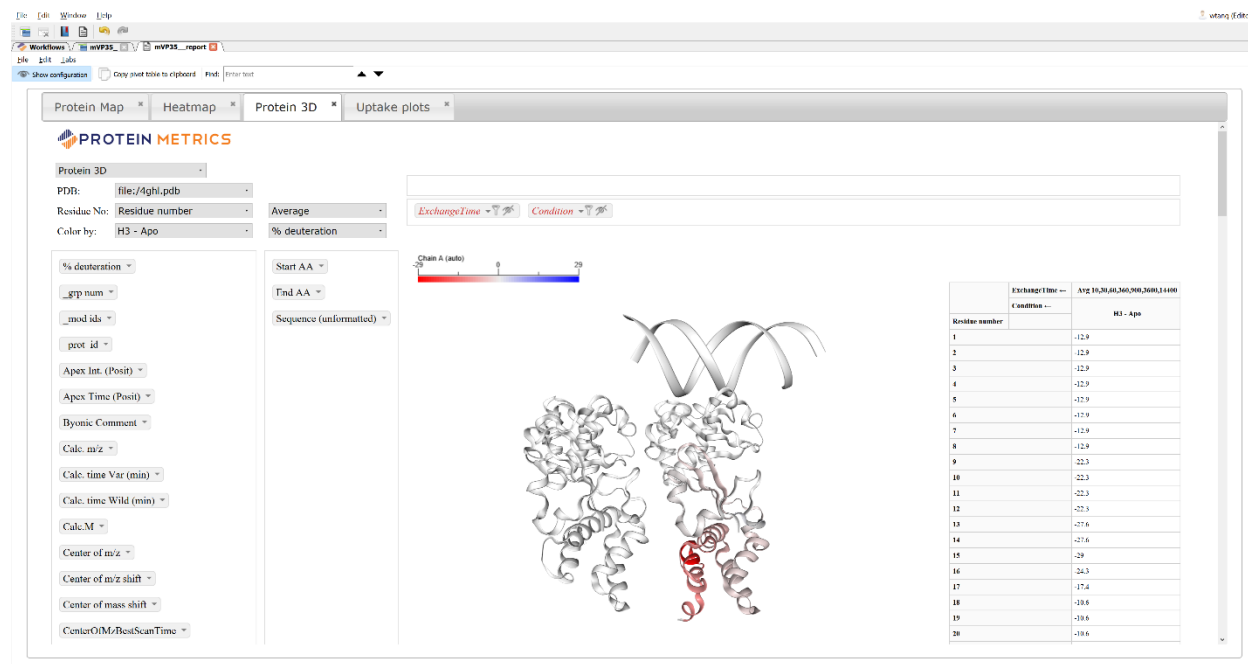


Figure 34: Protein 3D structure (gray regions of 3D plot indicate values near 0, as shown in legend)

The calculations and user interface controls for % deuteration alternatives, exchange time, and condition are identical to the residue-level view.

A new 3D protein structure (PDB) file can be added by choosing **Edit > Edit PDB** in the main menu (see figure below) and selecting a PDB file from your local device:

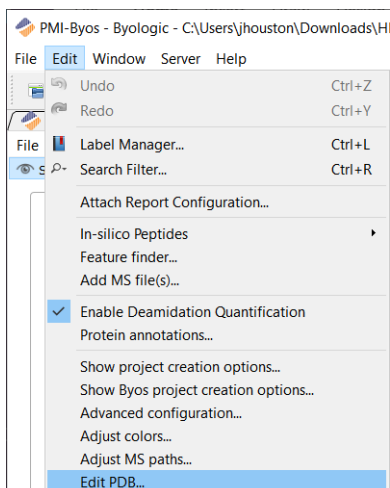


Figure 35: Changing the PDB file

Once a new PDB file has been added, click on **Tab** > **Update tab content** in the Report menu to view the new PDB structure in the drop-down menu.

To adjust the alignment, click **Edit** > **Current tab settings** > **Add chain configuration** (within **Protein 3D settings**) that opens a **Add chain configuration** pop-up dialog where **Position offset** field for the selected chain can be adjusted.

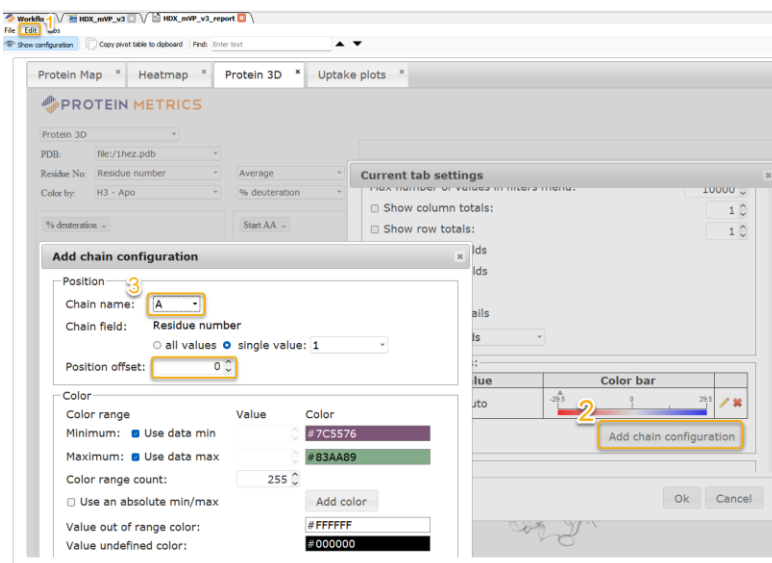


Figure 36: Aligning Sequence to Protein 3D structure

Uptake Plots Tab

Uptake plots estimate the percent deuterium uptake of each peptide. Each colored dot represents a sample. Also provided are the MS², MS¹, and XIC plots. Options for plot rescaling and the order can be set using the **Resize plots** checkbox and **Plot scaling %**.

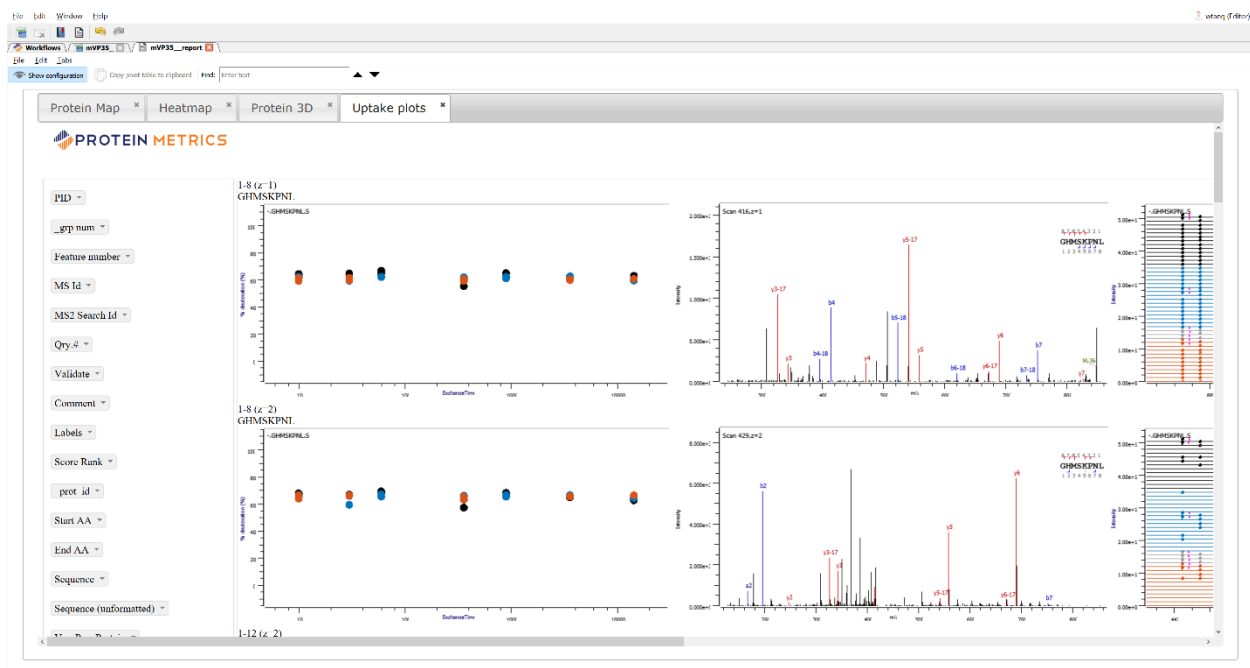
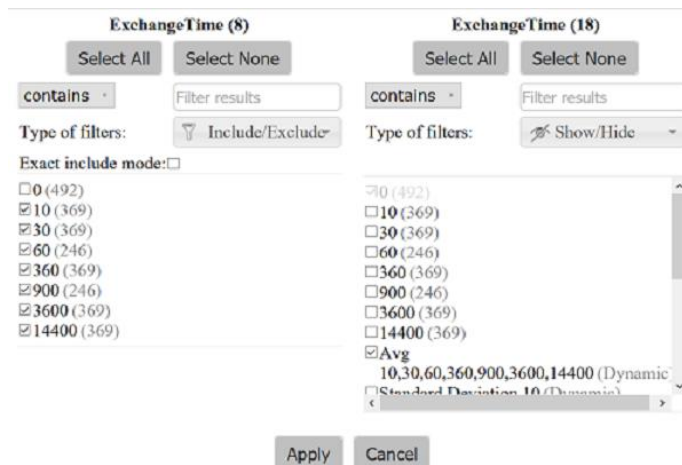


Figure 37: Plots

Managing ExchangeTime and Condition Values

The Include/Exclude checkboxes determine which exchange times or condition values are included (e.g., in the average). The Show/Hide checkboxes determine which columns are shown.



The screenshot shows the 'ExchangeTime' filter configuration dialog. It is divided into two sections: 'ExchangeTime (8)' and 'ExchangeTime (18)'. Each section has 'Select All' and 'Select None' buttons, a 'contains' dropdown, a 'Filter results' input field, and a 'Type of filters' dropdown. The 'Type of filters' dropdown is set to 'Include/Exclude' for the first section and 'Show/Hide' for the second. Below these are checkboxes for 'Exact include mode'. The first section lists exchange times: 0 (492), 10 (369), 30 (369), 60 (246), 360 (369), 900 (246), 3600 (369), and 14400 (369). The second section lists exchange times: 10 (492), 30 (369), 60 (369), 360 (369), 900 (246), 3600 (369), 14400 (369), and 'Avg'. Below the second section is a text input field containing '10,30,60,360,900,3600,14400 (Dynamic)' and a dropdown menu set to 'Standard Deviation 10 (Dynamic)'. At the bottom are 'Apply' and 'Cancel' buttons.

Figure 38: Include/Exclude and Show/Hide for ExchangeTime

Like the exchange times, one can filter on the conditions of interest.

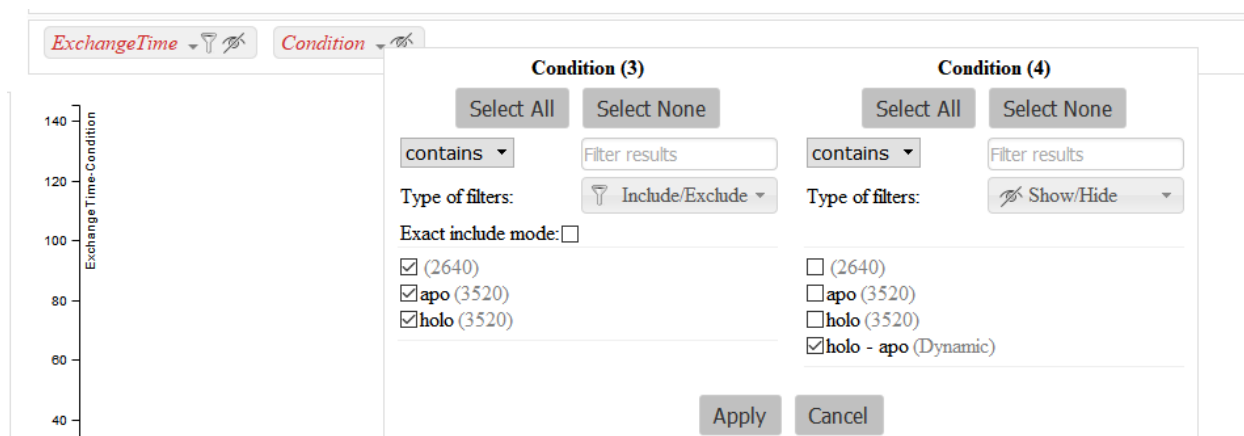


Figure 39: Include/Exclude and Show/Hide for Condition