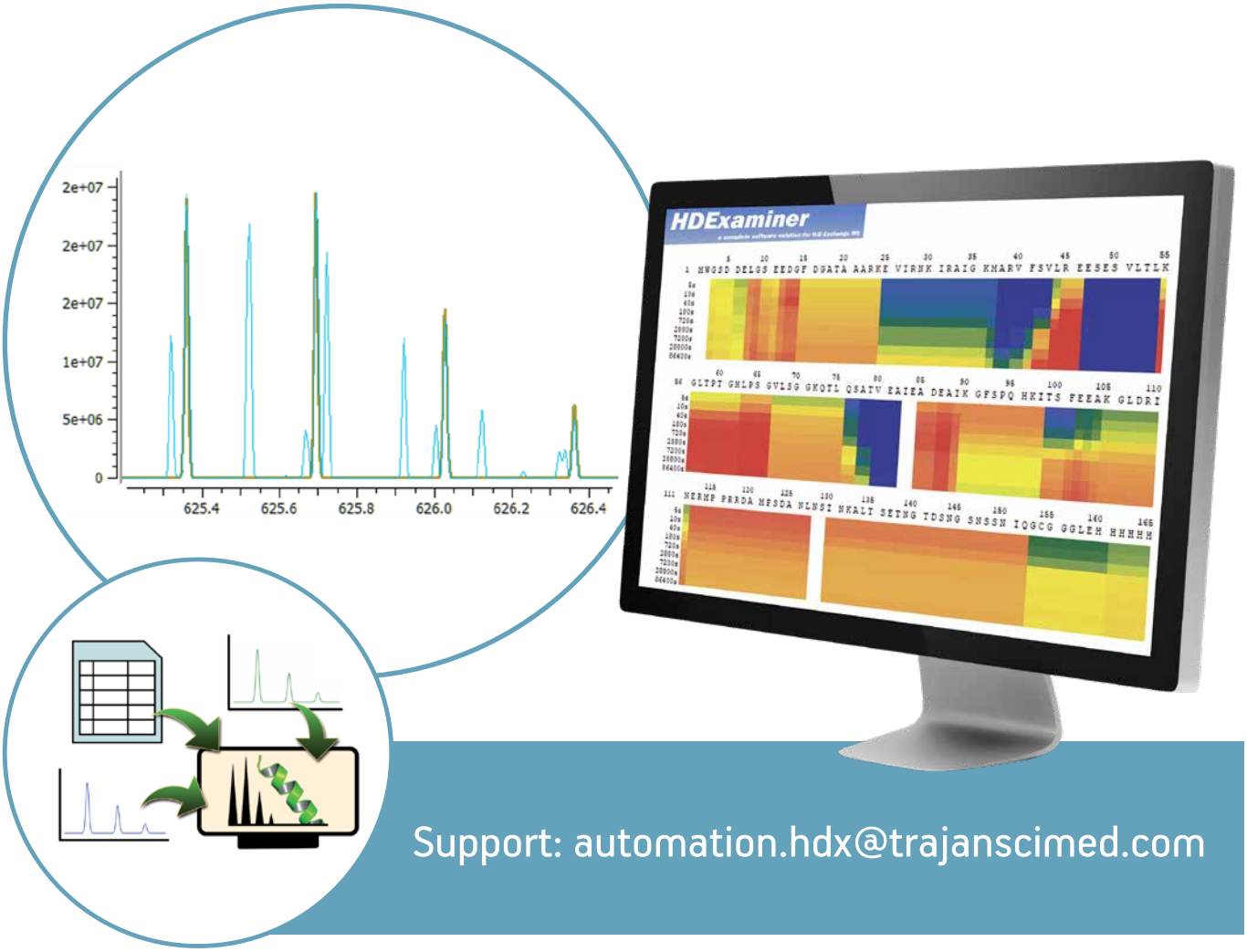
LEAP™

# HDExaminer

## Version 3.4 User Manual




Support: automation.hdx@trajanscimed.com

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# Contents

# Installation and Licensing

## Prerequisites

HDExaminer requires a 64-bit computer running Windows 7, Windows 8 or Windows 10 with at least 8 GB of RAM and at least 2 GB of free hard drive space. It is recommended that the target machine have at least an 8-core processor, 32 GB of RAM and a solid-state hard drive.

## MS Data System Compatibility

HDExaminer can read MS data in the following formats:

- AB Sciex Analyst
- Agilent LC/MSD ChemStation
- Agilent MassHunter
- Bruker Daltonics Compass
- NHFML/Florida State University MIDAS
- Thermo Xcalibur
- Waters MassLynx
- mzXML and mzData
- ASCII (m/z, intensity) peak lists

## Download and Installation

1. Download the installer zipfile from the instructions you received from Trajan.
2. After downloading, uncompress the zipfile.
3. Run HDExaminerDistribution-x64.exe and follow the instructions. During installation you will be asked to choose which data formats you want to install—we recommend only installing support for the formats you need.

## Licensing

HDExaminer uses software license keys that prevent unauthorized use. You must have a valid license to use the software. HDExaminer offers two license types, Fixed and Floating.

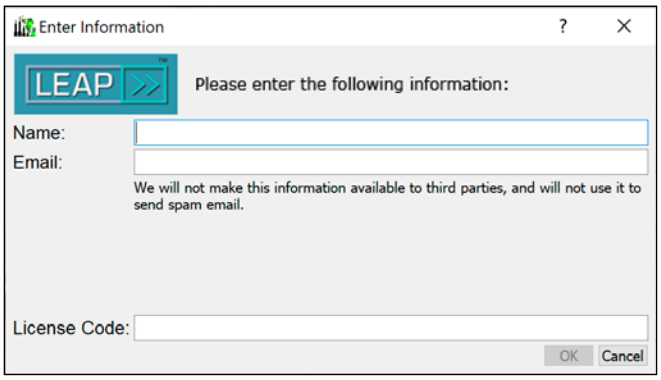
**Fixed:** The software is installed and licensed on a single computer.

**Floating:** The software is installed and licensed on up to four computers, but a specific USB device of your choice (usually a thumb drive) must be plugged into whichever computer is currently using the software. This solution is useful if you want to install the software on, for example, a desktop machine, a lab machine and a laptop, but you only need to use the software on one of those machines at a time.

You will be asked to choose a license type when you first license HDExaminer. Once a license is generated, its type cannot be changed.

### If you are installing HDExaminer onto a machine that has an active, direct Internet connection, follow these steps to license the software:

1. The first time you run HDExaminer, you will see the following window.




2. Enter your name, email address and license code.
3. In the next window, select whether you would like a Fixed or Floating license.
4. If you chose “Floating,” select a USB device to which the Floating license should be attached. Choosing an easily-portable device such as a thumb drive is strongly recommended. If you plug in a new USB device, click the “Refresh List” button to repopulate the list of devices. After you choose the USB device you want, click “OK”.
5. HDExaminer should launch. If it does not, contact automation.hdx@trajanscimed.com. If a file called HDExaminerLicensingDiag.txt appears on your desktop, include this file in your email.

**If you are installing HDExaminer onto a machine that does NOT have an active Internet connection, OR that accesses the Internet through a proxy server, follow these steps to license the software:**

1. First, HDExaminer will ask whether you use a proxy server. If you do, click “Yes” and enter your proxy details (you may need to contact your IT department for these). **After establishing a connection through your proxy server, follow the instructions above.**
2. If you do not use a proxy server, HDExaminer will ask you whether you have an offline key file that you received from Trajan. Say “No”.
3. In the next window, select whether you would like a Fixed or Floating license.
4. If you chose “Floating,” select a USB device to which the Floating license should be attached. Choosing an easily-portable device such as a thumb drive is strongly recommended. If you plug in a new USB device, click the “Refresh List” button to repopulate the list of devices. After you choose the USB device you want, click “OK”.
5. Enter your name and email address.
6. You will then be prompted to save a text file. Save it to a convenient location such as your desktop.
7. Send an email to automation.hdx@trajanscimed.com with the file you just saved as an attachment. Mention in the body of the email that you are requesting an offline HDExaminer license.
8. You will receive a license key file from Trajan via email. Save it in a convenient location such as your desktop.
9. Launch HDExaminer again. You will again be asked whether you use a proxy server. Say “No” again.
10. 1When asked whether you have an offline key file from Trajan, say “Yes”.
11. Browse to the license key file you just saved. (Be sure to browse to the license key file you saved in step 8 and not the original license request file you saved in step 6!)
12. HDExaminer should launch. If it does not, contact automation.hdx@trajanscimed.com. If a file called HDExaminerLicensingDiag.txt appears on your desktop, include this file in your email.
13. After you have successfully licensed the application, it is safe to delete both the License Request File you saved in step 6 and the License Key File you saved in step 8. You may also safely delete the file HDExaminerLicensingDiag.txt, if such a file exists on your desktop.

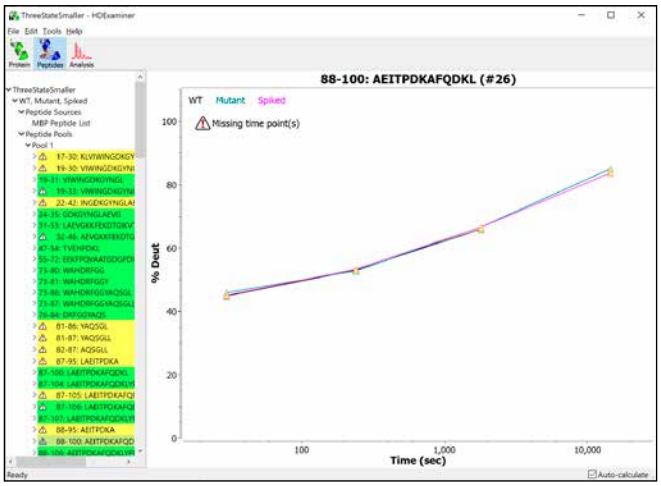
*Note: if you choose the Floating license option, you will need to follow the above licensing instructions for each computer you wish to license. License key files generated for one computer will not work on any other computer, even if those computers are sharing a Floating license.*

HDExaminer version 3.4 includes the following new features:

## Peptide Warnings

Peptide warnings offer a highly-customizable way to tell HDExaminer to highlight any of a number of problems with your peptides and their deuteration data, including:

- Missing data in one or more experiments, time points, replicates, etc
- Impossibly low or high measured %D
- Excessive measured back exchange
- High disagreement between replicates at a time point
- Very different isotope cluster average intensities
- Peptide mass very similar to another peptide in your project
- Peptide signal detected in a blank run
- Peptide found with problematically high MS raw intensity




Under Tools – Options – Peptide Warnings, you can turn warnings on and off globally, and you can select exactly which warnings you wish to see. Whatever warnings you might have will appear in the Peptides View, as warning icons both on the peptide tree view on the left and each individual uptake plot.

## Blank Run Processing

If you run blank LC-MS runs during your HDX experiments, you may now give those MS files to HDExaminer for processing, setting their type to “Blank”. A blank run is calculated similarly to a partially-deuterated file that is ignored by every part of the software except for the peptide warning system. If the software finds a peptide in one of your blank runs and the maximum single-scan intensity of that peptide is at least X% as high as it is in one of your deuterated runs, the software will turn on a warning for that peptide (for whatever X you specify in that warning’s settings).

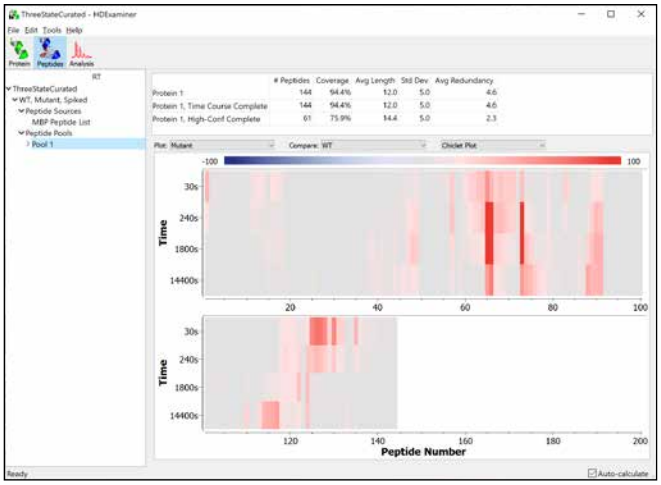
## Missing Data File Alerts

The Analysis View will now put a small alert icon next to any MS data file that cannot be found in its expected location. The right-click menu for such a file now includes “Locate Experiment Folder…”, which allows you to navigate to the folder containing the expected data file.

Related to this, the right-click menu for an MS file that can be found now includes “Show MS File in Explorer”, which will open the file’s containing folder in Windows.

## Chiclet Plot

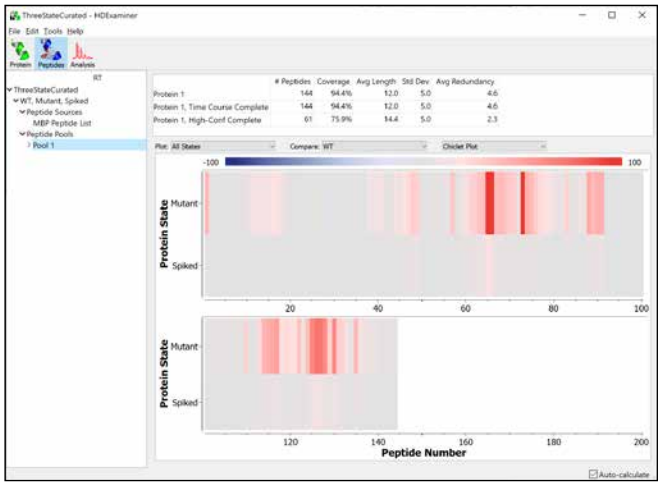
When you select a Peptide Pool in the Peptides View, a new plot type is available: the Chiclet Plot.




In a chiclet plot, the x-axis is peptide number and the y-axis is deuteration time. Each rectangle (“chiclet”) on the plot is colored according to the deuteration level of that peptide at that time

point. By selecting different protein states in the “Plot” and “Compare” dropdown menus, you can show a single state or a comparison between two states.

By selecting “All States” in the “Plot” menu, you can change the y-axis from time to protein state:




This view collapses all of your time points into a single maximum difference between states. This allows you to quickly compare many different protein states (for example, different ligands) against a control state to see which show differences of interest.

(Chiclet plots are further described in Naifu Zhang and others, Bioinformatics, Volume 37, Issue 13, July 2021, Pages 1926–1927, https://doi.org/10.1093/bioinformatics/btaa892)

HDExaminer version 3.3 introduced the following new features:

## Bruker timsTOF Support

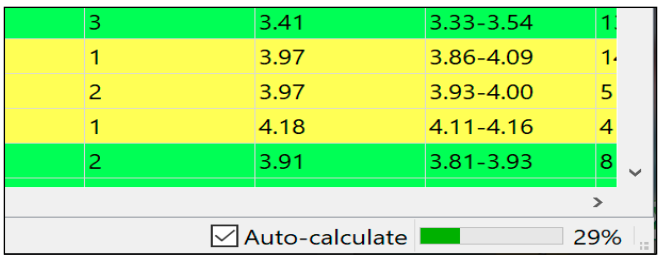
HDExaminer can now read ion mobility generated by Bruker timsTOF instruments. This new data type is treated the same as any other ion mobility data. When looking at an individual result, you can inspect and, if necessary, adjust that result's retention time or ion mobility ranges.

## Centroid MS Data Support

HDExaminer can now import centroid MS data (i.e. "stick data"). Profile-mode data is no longer required, although it is still strongly recommended. If you import data from an MS data file that includes both profile and centroid data, HDExaminer will allow you to choose between the two.

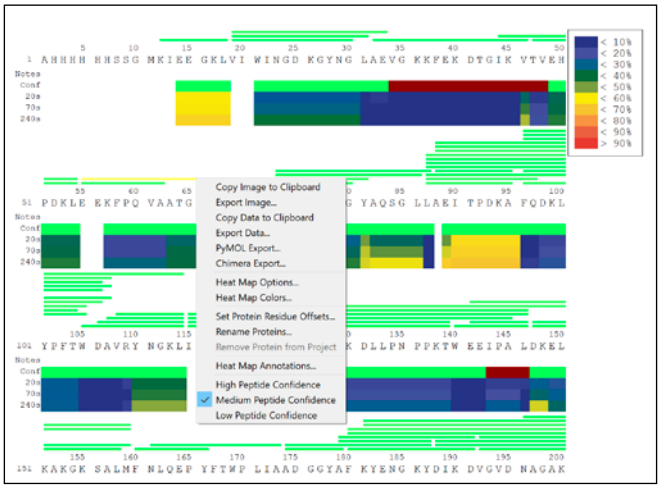
## Calculation Progress Bar

During calculations, the progress indicator of the bottom-right of the HDExaminer window will now (after an initial "spinning" period) show a percentage complete, based on the total number of results in the project.




## Peptide Confidence Overrides in Protein View

You may now override peptide confidence directly in the Protein View by right-clicking on a peptide bar and selecting "High/Medium/Low Peptide Confidence". This changes the peptide-level confidence, but does not alter the confidences for that peptide's individual results.




## Peptide Coverage Statistics Per Protein

When you click on a Peptide Pool in the Peptides View, the Peptide coverage statistics you see are now displayed for each protein individually in a table with much more information than before.

| | # Peptides | Coverage | Avg Length | Std Dev | Avg Redundancy |
|---|---|---|---|---|---|
| Protein 1 | 28 | 98.3% | 12.7 | 5.9 | 5.9 |
| Protein 1, Time Course Complete | 1 | 16.7% | 10.0 | 0.0 | 0.2 |
| Protein 1, High-Conf Complete | 0 | 0.0% | 0.0 | 0.0 | 0.0 |
| Protein 2 | 32 | 100.0% | 13.2 | 6.4 | 5.9 |
| Protein 2, Time Course Complete | 10 | 100.0% | 13.8 | 5.5 | 1.9 |
| Protein 2, High-Conf Complete | 0 | 0.0% | 0.0 | 0.0 | 0.0 |
| Overall | 60 | 99.2% | 13.0 | 6.2 | 5.9 |
| Overall, Time Course Complete | 11 | 61.8% | 13.5 | 5.4 | 1.1 |
| Overall, High-Conf Complete | 0 | 0.0% | 0.0 | 0.0 | 0.0 |

Each protein (plus the "Overall" sequence representing the union of all of your proteins) has three lines in the table. The first line shows coverage for all of the peptides in your pool, ignoring any calculated results or their confidence levels. The second line, "Time Course Complete", counts only peptides that have at least one medium- or high-confidence deuteration result at each time point. The third line, "High-Conf Complete", counts only peptides that have at least one high-confidence deuteration result at each time point.

## Peptide Import Warnings

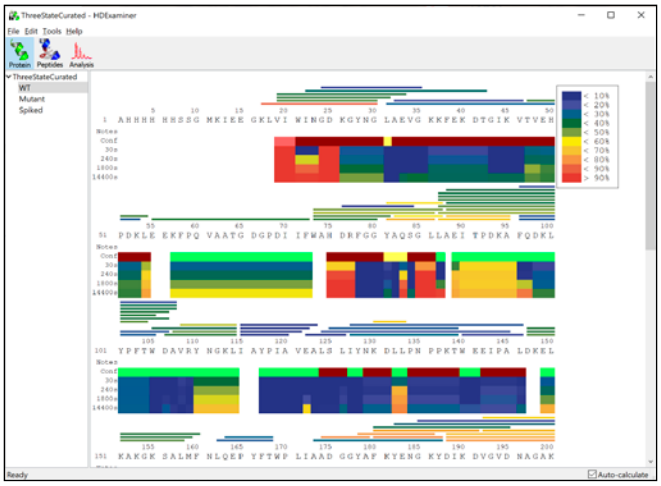
If you import a Peptide Source and there are problems with the import, the software will now give a much more informative warning that includes the number of peptides that could not be imported, along with reasons for the problem. (Remember that HDExaminer cannot import a peptide unless that peptide sequence exists ***exactly once*** across all of the proteins in your project.)

## Set Charge State to Low Confidence

Version 3.3 changes the behavior of the Peptides View context menu item “Set Charge State to Low Confidence”. Previous versions of the software would set the undeuterated results to low confidence for the selected peptide. Starting with version 3.3, it sets the deuterated results to low confidence instead. This is to prevent issues when working with projects that include multiple protein states that share undeuterated MS data files.

HDExaminer version 3.2 introduced the following features:

## Heat Map Confidence

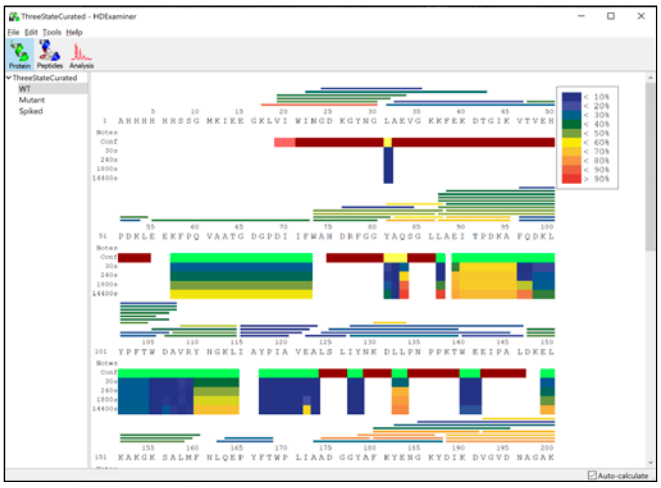


The heat map can now display per-residue confidence ratings, either per time point or as a unified confidence row at the top of the heat map. To turn on this feature, go to Tools – Options – Protein View and select “Show heat map confidence”. If you select “Per time point”, then each time point will have a confidence row above it. If you select “Unified”, then a single row will appear at the top, where the confidence at each residue is the worst confidence from among all of the time points for that residue.

When generating a heat map, HDExaminer performs calculations to find the deuterium occupancy at each residue that minimizes the least-squares error with each of the measured peptides. There are two main problems that can lead to the least-squares model giving an inaccurate or untrustworthy picture of a protein’s deuterium uptake. First, certain peptides can be outliers that have a measured deuteration level that substantially differs from what the model says that that peptide “should” have. Second, certain sections of the protein may have peptide coverage that causes the underlying least squares representation of the system to be underdetermined, which results in those sections having an infinite number of equally good error-minimizing solutions. Both of these issues are addressed by the new confidence assignment algorithms in HDExaminer.

As with the rest of HDExaminer, red means low confidence, yellow means medium confidence, and green means high confidence. You may notice in the screenshot above that there are two different shades of red. The lighter red means that that section of your protein has significant disagreement between the model and the peptides covering that area. The darker red means that that section of your protein has an infinite number of error-minimizing solutions.

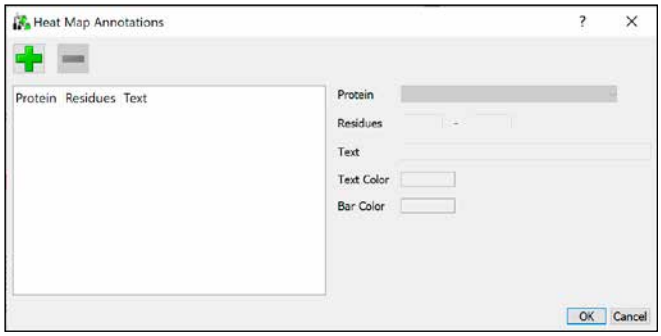
Since low-confidence sections of your protein could give you an inaccurate (or non-unique) picture of your protein’s deuteration behavior, you can also choose whether or not to show the heat map for low-confidence residues. This setting is under Tools – Options – Protein View: “Color low-confidence residues”. This option is off by default, and we recommend that all users leave it off. Note that the above screenshot has this option on. Here is the same screenshot with the option off:



While this screenshot has much less data, we can have much higher confidence in the residues that are colored here.

## Heat Map Annotations

You can now add colored text annotations to the heat map, attached to any residue. To do this, right-click on the heat map and select “Heat Map Annotations…” The following window will appear:




To add an annotation, click on the "+" button, then edit the fields on the right. Select which protein you wish to attach to, the residue range you wish to use, the text of your annotation, the text color and the bar color. Your annotation will be drawn in a new "Notes" row on the heat map.

You may toggle the Notes row on and off by going to Tools – Options – Protein View and toggling the "Show heat map annotations" checkbox. If heat map annotations are off and you add a new annotation, your program settings will automatically change to show them.

## Heat Map Smoothing Deprecated

The smoothing option under Tools – Options – Protein View is now deprecated and will warn the user whenever it is set to a value other than "None". This option will still work as before, but it may be removed in a future version of HDExaminer.

We have found that heat map smoothing generally causes more harm than good, often leading users to inaccurate ideas about the overall deuteration behavior of their proteins. We believe that the new heat map confidence algorithm, without smoothing, will lead to much higher-quality, more trustworthy results.

## Project Notes

In addition to heat map annotations, you may now also add simple text notes to your project, or to any protein state, peptide or experiment in your project. To do this, right-click on the tree view on the left hand side of the HDExaminer window and select the appropriate notes menu item. This menu item changes depending on the item currently selected, but notes can be attached to your project, any protein state, any peptide, or any experiment.

Notes are solely for the user's benefit – they have no effect on calculated results or data exports.

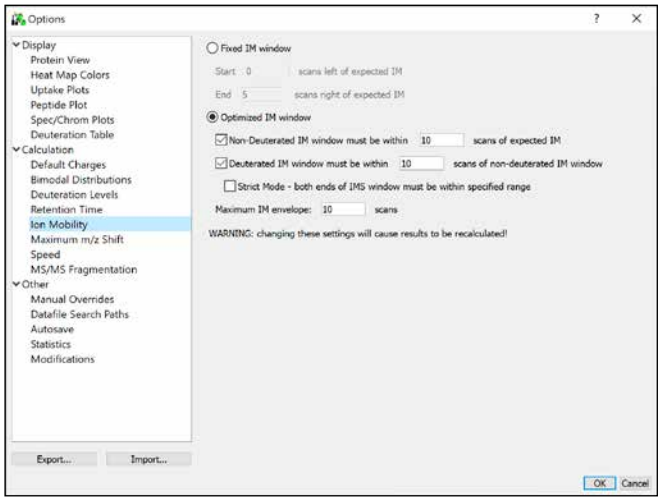
## Changes in Statistical Calculations

Starting with HDExaminer 3.2, different charge states are no longer treated as "pseudo-replicates". In previous versions of HDExaminer, measurements from a peptide at multiple charge states were treated as extra data points for the purpose of statistics, leading to artificially small confidence intervals for some uptake plots. After discussion with several HDX researchers and statisticians, we have determined that such measurements are not sufficiently independent to be usable when testing for statistical significance. The software now uses the single "best" charge state when determining confidence intervals, where "best" is defined as the charge state with the most high-quality data points over the highest number of time points.

We realize that this change in behavior may be an inconvenience for some HDExaminer users. We apologize for this, but we believe that the software's new method for calculating statistical significance is an improvement that will increase the trust users can have in their calculated results.

## Ion Mobility Improvements

Under Tools – Options – Ion Mobility, you can now select "Fixed IM window" or "Optimized IM window".




The optimized algorithm works just like previous versions of HDExaminer, where the software finds a candidate mobility range, then iteratively adjusts that range to try to find a better result. If you select "Fixed IM window", you can specify some number of scans on either side of the expected IM (the peptide's search mobility). In the

fixed IM case, HDExaminer will use the specified range for all peptides and will not attempt any kind of range optimization to get a better result. This algorithm can give slightly less precise results, but can also be significantly faster.

In addition to this new setting, the “ion mobility chromatogram” for each result now shows a range around the relevant mobility value rather than showing the entire mobility range. This makes calculations faster for ion mobility data.

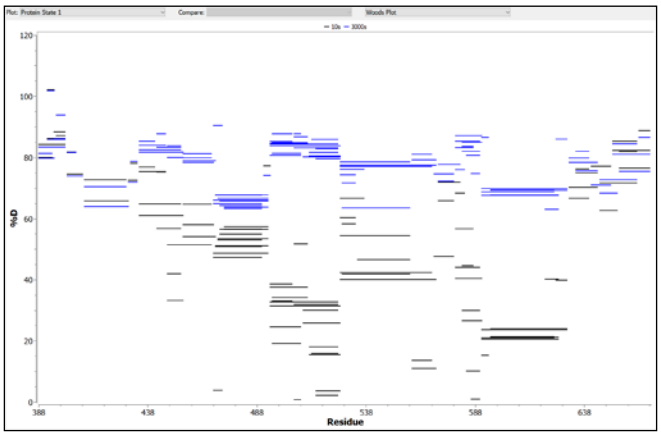
## Direct Interpolation in Peptide Plot

Previously, the butterfly plot and residual plot used a spline-based interpolation algorithm. While this looked nicer, it could lead to some highly misleading plots. The plots now use a simpler direct interpolation algorithm to prevent this.

HDExaminer version 3.1 introduced the following features:

## Woods Plot

HDExaminer's Peptide Pool View now allows a new plot type in addition to the Butterfly Plot, Residual Plot and Volcano Plot. The Woods Plot shows a line for each peptide, with residue range as its x-axis and deuteration level as its y-axis. The peptide lines are color-coded by deuteration time.




Mousing over a peptide line will show you details about that peptide. Clicking on a peptide line will take you to the uptake plot for that peptide. Note that you can zoom this view by clicking and dragging a zoom range.

Note that the Woods Plot is generally more appropriate for projects with a relatively small number of peptides. It is useful for "protein at a glance" information, but can get very crowded in a larger project.

## Other Peptide Pool View Features

Other improvements to the Peptide Pool View have been added. For example, if your project includes multiple protein sequences, then the Butterfly Plot, Residual Plot and Woods Plot will have vertical lines demarcating protein breaks. In addition, you may now export the Volcano Plot as a data table rather than only as an image.

## Batch Retention Time Overrides from a Table

You can now import a spreadsheet of retention time overrides for your entire project all at once. This can be useful if you have done automated analysis of retention time windows in a tool other than HDExaminer. For information about this feature, see "Batch Retention Time Overrides from a Table" on page 27.

## Clickable Data Points in Uptake Plots

You can now click on a data point in the uptake plot to view that point's result in the Analysis View. Note that you may need to zoom your uptake plot first to make sure that you are clicking on a single, non-overlapping data point!

HDExaminer version 3.0 was a major release with many improvements over previous versions. If you have upgraded from version 2.X, read this section to learn about the new features.

## Improved Algorithms

HDExaminer 3.0 includes a significant rewrite of the calculation engine, designed to find better results and dramatically reduce the need for manual overrides.

## Multiple Protein Sequences

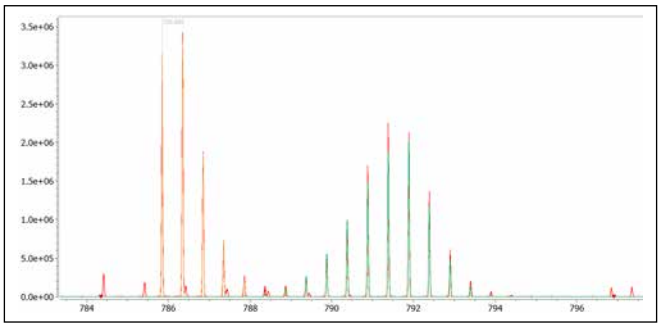
An HDExaminer project can now contain multiple protein sequences rather than just one. So, for example, you can enter your heavy chain and light chain separately, either by importing separate FASTA files or by importing a single FASTA file with multiple sequences in it.

In the Protein View, you can right-click on the protein sequence view to rename proteins, remove any unwanted proteins from your project, or set the sequence numbering offsets for each protein in your project.

When analyzing a project with multiple protein sequences, it is strongly recommended that you give each protein a unique name and turn on the “Protein Name” column in the Analysis View tables by going to Tools – Options – Deuteration Table.

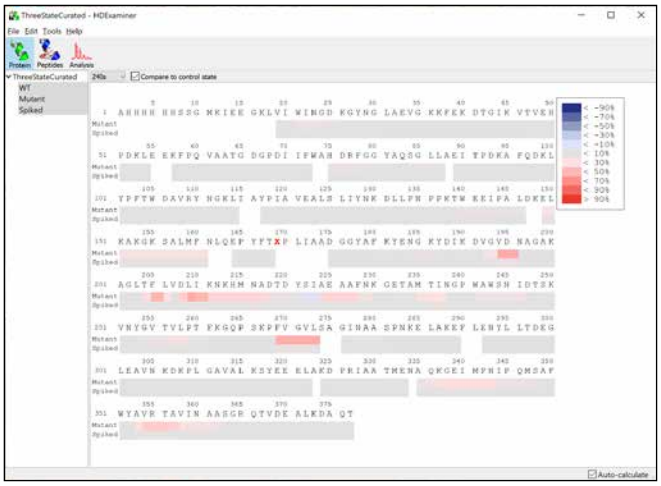
## Bimodal Distributions

HDExaminer now includes comprehensive support for bimodal distributions. See “Analyzing Bimodal Distributions” on page 39 for more information.




## Multistate Heat Map

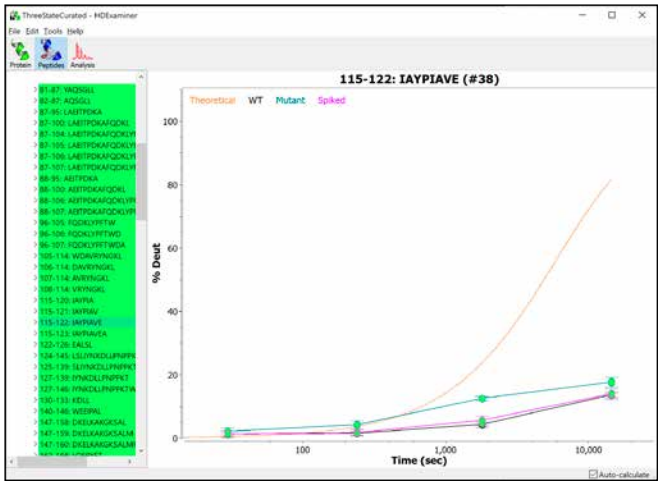
You can now view a single time point at multiple protein states in the Protein View. You can also compare multiple protein states to your control state to generate a view like this:



To access this view, simply select the top level item in the Protein View tree or ctrl-click to select 3 or more protein states, then select the time point you wish to view in the menu that appears to the upper left of the heat map. See “Multistate Heat Maps” on page 32 for more information.

## Theoretical Uptake Curves

An uptake plot can now include the theoretical uptake, calculated using the intrinsic hydrogen exchange rates described in *Nguyen et al., J. Am. Soc. Mass Spectrom. (2018) 29: 1936*. To turn this feature on, go to Tools – Options – Uptake Plots. Check the box that says “Show theoretical uptake curve” and enter the pDcorr and Temp in C values that match your experimental conditions. The theoretical uptake curve will appear on each uptake plot in the color specified in the settings (orange by default).




## Community Paper Exports

*In Masson et al., Nat Methods* ***16,*** *595–602 (2019),* the HDX community codified many recommendations for the design, analysis and reporting of HDX experiments. HDExaminer's data export features now adhere to the recommendations set forth in that paper.

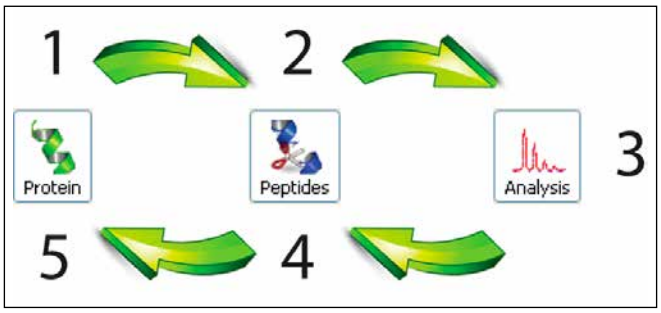
⚠ **Note**

Note that Recommendation 3.1 of that paper states that reported #D results should not be back-exchange corrected. In light of this recommendation, the #D results reported in HDExaminer's Uptake Summary Table are not corrected for back exchange, regardless of your HDExaminer settings. ***This is a change in behavior from previous versions of HDExaminer.***

## High DPI Support

HDExaminer now looks good at Windows display magnifications between 100 and 200%, so if you have a high-DPI monitor, you can now take advantage of that without having to change any compatibility settings within Windows.

The basic workflow for analyzing bottom-up HD Exchange data using HDExaminer is illustrated in the diagram below:




1. Create a new project, import your protein sequence, set up protein comparison states.
2. Import peptide search results and create a peptide pool.
3. Import MS data files and analyze calculated results.
4. Analyze peptide deuteration behavior via comparison view and uptake plots.
5. Analyze overall protein deuteration behavior via heat map and difference map.

The remainder of this section will discuss a simple workflow example.

## Creating a New Project

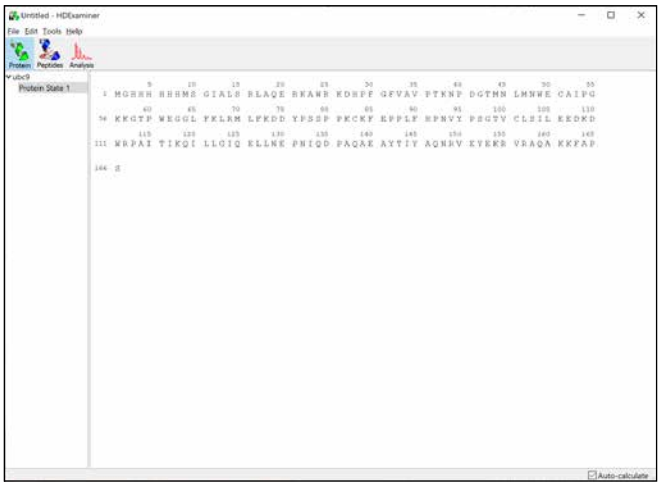
Your “document” in HDExaminer is called a **project.** A project contains an analysis of one or more related protein sequences. Projects may be created, opened and saved using the commands under the File menu. A project file is saved with a file extension of .hdx.

**To create a new project,** select “New…” under the File menu. Browse to the FASTA file containing the protein sequence(s) you wish to use. The protein sequence(s) will be shown in the Protein View.

You may also create a new project by dragging and dropping a FASTA file directly onto your HDExaminer window, or by copying and pasting a protein sequence.

You may also create a new project by importing a protein sequence from the Uniprot database. Select “New from Uniprot…” under the File menu, then type in the 6-character Uniprot code for the protein you want. Click on the “Save downloaded FASTA file locally” checkbox if you wish to have HDExaminer save a copy of the sequence of a FASTA file to your hard drive for future use.

In the Protein View, you may create and edit multiple **protein states** for comparison purposes. Protein states are discussed in more detail on page 34.



## Importing Peptides

Once you have entered a protein sequence, the next step is to import one or more **Peptide Sources** and use them to create one or more **Peptide Pools.**

A **Peptide Source** is any externally-generated list of peptides which you expect to find in your experiments. A **Peptide Pool** is a curated list of peptides generated by filtering and combining one or more Peptide Sources. Peptide Sources are used to create Peptide Pools; the Peptide Pools contain the peptides that HDExaminer will use for its deuteration calculations.

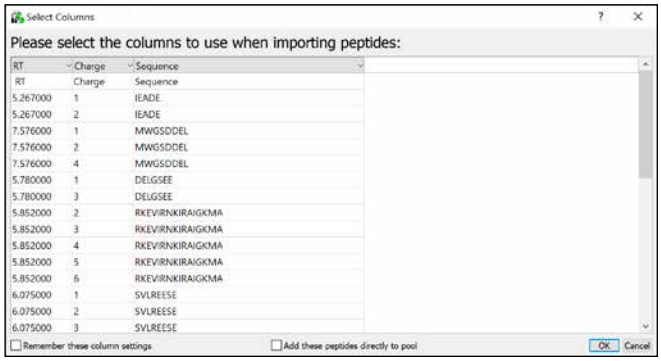
A project will often contain multiple Peptide Sources corresponding to multiple LC-MS/MS peptide search runs. A project may contain multiple Peptide Pools, but it is generally more convenient to combine all Peptide Sources into a single Peptide Pool.

**HDExaminer can read peptides from the following file formats:**

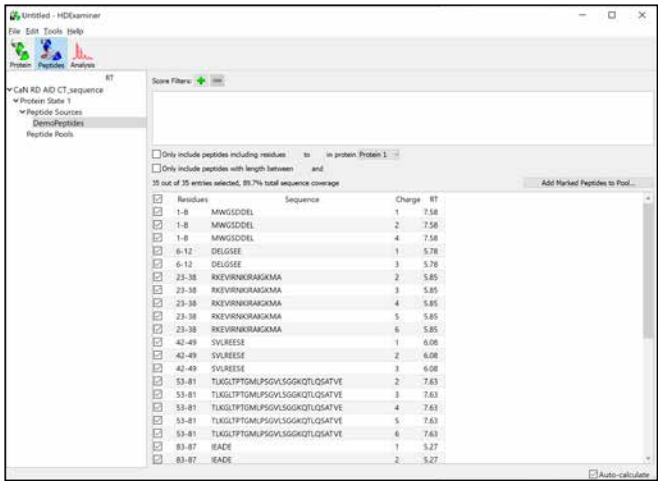
- SEQUEST HTML
- Mascot XML
- pepXML
- Proteome Discoverer xls or xlsx
- PLGS csv
- Generic tabular data in txt, csv, xls or xlsx formats (this format will be used in this example)

### To create a Peptide Source:

1. Switch to the Peptides View by clicking on the “Peptides” button in the upper left of the window.
2. In the tree view on the left side of the window, right-click on “Protein State 1” and select “Add Peptide Source…” You may also add a Peptide Source by dragging the file directly onto your HDExaminer window.
3. Browse to your Peptide Source file. If you are importing tabular data, you will see the following window:




4. Using the popup menus above each column, specify which column corresponds to peptide sequence, charge state and retention time. You may also specify that one or more columns should be treated as score columns.
5. Click “OK” when you are finished. Your Peptide Source will be added under “Peptide Sources” in the tree view:



6. Repeat these steps for each Peptide Source file you wish to import.

Each Peptide Source entry includes a peptide sequence, an optional charge state, a retention time, a mobility value (in the case of ion mobility data), and zero or more peptide quality scores. Peptide Sources may be filtered based on quality scores. See “Working with Peptides: Advanced Features” on page 19 for more information.

In order to begin calculating deuteration levels, you will need to create one or more Peptide Pools from your Peptide Sources.

### To create a Peptide Pool:

1. In the Peptides View, select a Peptide Source in the tree view on the left side of the window.
2. Click on the button “Add Marked Peptides to Pool…” toward the right side of the window.
3. By default, HDExaminer will add all peptides to a single pool called “Pool 1”. You may rename the pool if you wish.
4. Click on “OK”.

You will see a new entry in the tree view called “Pool 1.” It will contain the peptides you just imported.

If you imported a Peptide Source from tabular data and you do not wish to do any filtering on that source, you may check the “Add these peptides directly to pool” to skip the above steps and create a pool directly from your Peptide Source.

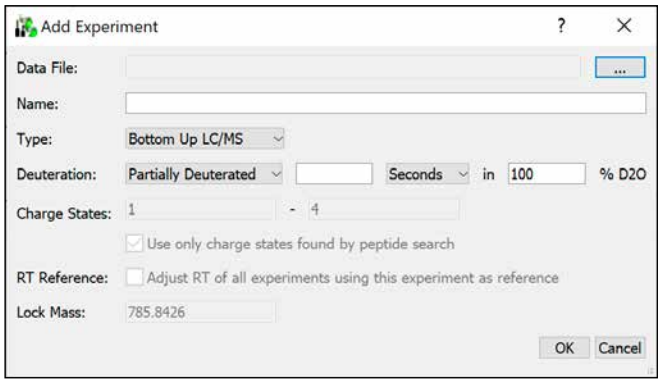
*Note: for simplicity, this example has skipped over several advanced peptide import features, including removing poor-quality peptides via score filters. For more information on this and other topics, please see “Working with Peptides: Advanced Features” on page 19.*

## Importing MS Data

Once you have one or more Peptide Pools, you will need to import your MS experiments. For a list of the MS data formats HDExaminer can import, see “MS Data System Compatibility” on page 1.

### To import an MS experiment:

1. Switch to the Analysis View by clicking on the “Analysis” button in the upper left of the window.
2. In the tree view on the left side of the window, right-click on “Protein State 1” and select “Add Experiment…” You will see the Add Experiment dialog box:

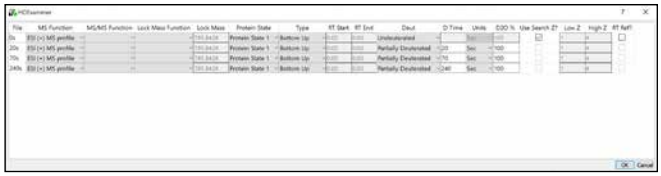



3. Click on the “…” button in the upper right.
4. Browse to your MS data file. Ensure that the data types that show in the dropdown menus in the upper right correspond to the data types you wish to import. (Note that HDExaminer can only import MS data acquired in positive-ion mode. If you wish to import both MS and MS/MS data, see “Analyzing Middle Down ETD/ECD Data” on page 43.)
5. Click “OK”.
6. If you wish to edit the experiment’s name inside of your project, you may do so by altering it in the “Name” field.
7. Leave the type as “Bottom Up LC/MS”. (If you are instead doing a Top Down HD Exchange experiment, see “Analyzing Top Down ETD/ECD Data” on page 42. If you are doing Middle Down HD Exchange, see “Analyzing Middle Down ETD/ECD Data” on page 43.)
8. Select whether the experiment is Undeuterated, Partially Deuterated, Fully Deuterated, or a Blank run.
9. If the experiment is Partially Deuterated, enter the experiment’s deuteration time.
10. If your D2O buffer concentration for this experiment was lower than 100%, enter the actual percentage in the text box labeled “in ___ % D2O”.
11. If the experiment is Undeuterated, select the charge state(s) that you wish HDExaminer to consider when looking for peptides. By default, HDExaminer will search only for the charge states that were imported from your Peptide Sources. You may override this behavior by unchecking “Use only charge states found by peptide search” and entering a charge state range manually.
12. If the experiment is Undeuterated, you may also select it as a reference dataset to use for retention time adjustment by checking the “Adjust RT of all experiments using this experiment as reference” checkbox. If you check this box, then HDExaminer will attempt to perform retention time adjustment on all experiments in that protein state using an algorithm similar to the one described in *Venable et al., J. Am. Soc. Mass Spectrom. (2013) 24:642-645.* (Note that this algorithm is very time-consuming, so you should avoid using it unless you are confident that your data requires retention time adjustment in order for HDExaminer to give acceptable results.)
13. If you imported a lock mass function in step 4, the Lock Mass field will be enabled. Enter the expected m/z value of the reference peak of the lock mass function.
14. When finished, click “OK”.

Once a project contains at least one peptide pool and an undeuterated MS experiment, calculations will begin automatically. (Note that you may override this behavior by unchecking the “Auto-calculate” checkbox in the bottom right of the HDExaminer window.)

If you have replicate experiments, you may enter them here. Simply enter each experiment’s data file separately using the instructions above and HDExaminer will calculate overall deuteration levels based on all of the experiments.

Alternatively, you may provide MS data files to HDExaminer by dragging those files directly onto your HDExaminer window. Doing so will show a window like this:



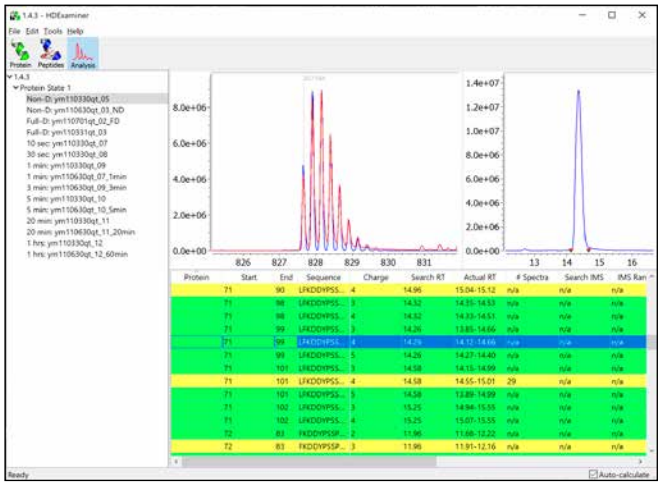
The columns in this table correspond to the fields in the “Add Experiment” dialog described above.

- **File:** this column displays the name of the MS data file.
- **MS Function:** if your MS data file contains multiple functions, select the correct one here. If your data file contains both profile data and centroid (or “stick”) data, you can choose between them here. Using profile data is strongly recommended.
- **MS/MS Function:** if you wish to import an MS/MS function for top-down or middle-down analysis, select it here.
- **Lock Mass Function:** if your data includes a lock mass function, select it here.
- **Lock Mass:** if you selected a lock mass function, enter the expected m/z value of the reference peak.
- **Protein State:** for each imported file, select the protein state that the file should be assigned to.
- **Type:** select whether each file is a Bottom Up LC/MS run, a Middle Down ETD or ECD run, or a Top Down ETD or ECD run.
- **RT Start/End:** if the reported data file is a Top Down ETD or ECD run, specify the retention time range of that run that HDExaminer should use.
- **Deut:** select whether each file is Undeuterated, Partially Deuterated, Fully Deuterated or a Blank run.
- **D Time/Units:** if the file is Partially Deuterated, enter the deuteration time here.
- **D2O %:** if you used a D2O buffer concentration lower than 100%, enter it here.
- **Use Search Z?:** for each Undeuterated file, select whether HDExaminer should limit its search to the charge states specified by your imported peptide search results.
- **Low Z/High Z:** if a file is Undeuterated and “Use Search Z?” is unchecked, you may specify a charge state range that HDExaminer will search for.
- **RT Ref?:** if a file is Undeuterated, you may specify that HDExaminer should use it to do retention time alignment on the other MS runs, as described above.

Once you have imported MS data, HDExaminer uses an isotope cluster modeling algorithm to find each peptide. It then computes the geometric centroid of each peptide’s isotope cluster. A peptide’s deuteration level at a given time point is computed by comparing the centroid of the deuterated isotope cluster with its undeuterated counterpart.

## Analyzing Results

Clicking on any calculation result in the Analysis View table will show a view like this:



Here are a few important features of this view:

**Retention time envelope:** HDExaminer begins looking for a peptide by taking the expected retention time from its Peptide Pool (possibly adjusted based on the experiment you selected as a reference in step 10 above) and summing spectra over a window around that retention time. HDExaminer will then, in many cases, adjust this retention time range in order to find a better fit between the actual and theoretical data. The final retention time envelope is displayed in the table (Actual RT) and in the XIC view (the red triangles).

**Actual and theoretical isotope clusters:** the spectrum view (top center) shows the theoretical isotope cluster in blue and the actual data in red. The actual data represents the sum of the spectra over the calculated retention time envelope.

**XIC view:** the view in the upper right of the window is an extracted ion chromatogram of the theoretical isotope cluster. In general, seeing a single well-defined peak in this view is strong evidence of a good match. If your data includes ion mobility separation, this view will include a toggle switch in the bottom left. Clicking on the switch allows you to toggle back and forth between the extracted ion chromatogram in the retention time domain and the similar chromatogram for the ion mobility separation domain.

**Score:** each entry in the table has a score. This is a measure of how well the theoretical and actual isotope clusters match. In general, a score of 0.8 or higher represents a decent match, while a score of 0.9 or higher represents a good match.

**Color-coded results:** each result in the table is color-coded green (high confidence), yellow (medium confidence) or red (low confidence). The confidence level of a result is calculated using a number of factors, including score, signal to noise and how well the theoretical isotope cluster matches the actual data. Low-confidence results are not considered in any downstream calculations. For example, if all undeuterated results for a peptide have low confidence, that peptide/charge state combination is not considered in any uptake plots or in the protein's heat map. If you disagree with the confidence level that HDExaminer assigns to a result, you may change it by right-clicking on the result in the table and assigning it a new confidence level or by selecting the result and pressing Ctrl-up-arrow or Ctrl-down-arrow.

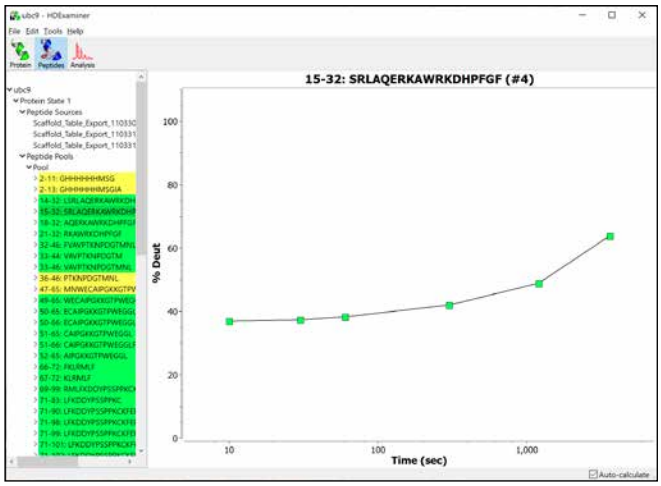
## Analyzing Peptide Deuteration Behavior

Clicking on any calculation result in the Analysis View table will show a view like this:

The Peptides View provides convenient ways to view and analyze each peptide's deuteration behavior over time. This allows you to spot "problem peptides" quickly and easily.

### To view a peptide's uptake plot:

1. Switch to the Peptides View by clicking on the "Peptides" button in the upper left of the window.
2. In the tree view on the left, open "Peptide Pools", then your Peptide Pool.
3. Click on a peptide. You will see a view like this:

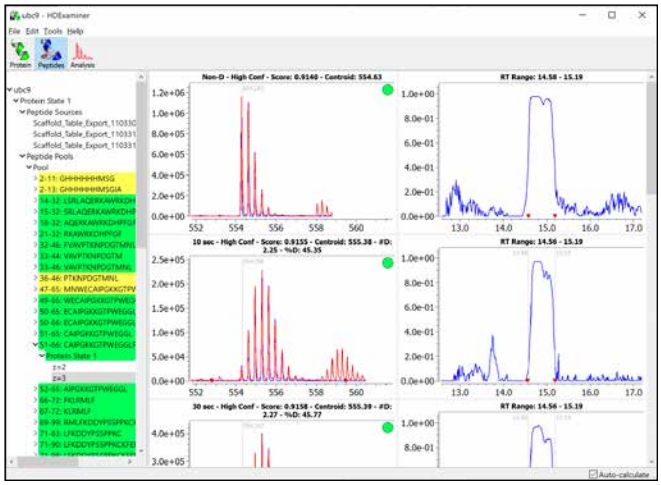



The uptake plot shows deuteration over time. The x-axis shows deuteration time. The y-axis shows the peptide's deuteration percentage by default, but can also show absolute deuteration. Right-click on the plot's y-axis to switch between the two.

Each point on the uptake plot represents a single calculated deuteration result. Each point is color-coded by confidence level (green for high, yellow for medium) and shape-coded by charge state (circle = 1, diamond = 2, triangle = 3, square = 4, pentagon = 5, hexagon = 6+).

Each peptide on the left-side view is color-coded by confidence, as well. Unlike calculation results, a peptide's color isn't determined by the spectra or XIC. Instead, these colors are determined by the shape of the peptide's uptake curve. A sensible curve that increases over time will have a higher confidence than one that wavers, has missing data points or that has replicates that disagree with each other. As with individual calculation results, you may right-click on a peptide to change its confidence. Low confidence results are ignored in all downstream calculations.

Finer-grained analysis for a particular peptide can be achieved by expanding that peptide's entry in the tree view, then expanding a protein state and selecting one of the charge states shown under it.



This view shows the spectrum and XIC views for the selected charge state. The top view(s) correspond to the undeuterated peptide(s), while the subsequent views show the later time points. The statistics at the top of each view give useful information about that result. On deuterated results, the RT range shown by the grey lines indicate the undeuterated RT range.

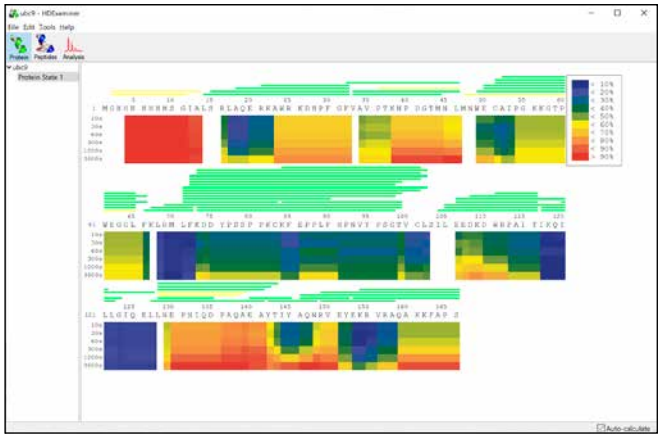
### If you see a result which makes no sense, you have several options:

1. You can try to improve the fit by right-dragging on either the spectrum or XIC views. (See “The Analysis View: Manual Overrides” on page 26 for more information on this.)
2. You can set the problematic result to low confidence by right-clicking on the result and selecting “Low Confidence”.
3. You can set the entire charge state to low confidence, either in the current protein state or across all protein states. To do this, right-click on the charge state entry in the tree view on the left. In the popup menu that appears, select “Set Charge State to Low Confidence (This Protein State)” or “Set Charge State to Low Confidence (All Protein States)”. This is equivalent to setting the deuterated result(s) to low confidence which, in turn, causes that charge state to be ignored in all downstream calculations.
4. You can set the entire peptide to low confidence. To do this, right-click on the peptide entry in the tree view on the left. In the popup menu that appears, select “Low Confidence”.
5. You can delete the peptide from your project completely. Select the peptide entry in the tree view on the left, then right-click on it and select “Delete Peptide”. Note that this will delete that peptide across all protein states and charge states!

Once you believe that your peptide results exhibit sensible deuteration behavior, proceed back to the Protein View to analyze the protein’s heat map.

## Analyzing Protein Deuteration Behavior

After deuteration results have been calculated, the Protein View displays a heat map showing uptake behavior for the entire protein.



This view shows the protein sequence, as well as the peptide coverage map above the sequence and the heat map below.

**Coverage map:** each bar in the coverage map corresponds to a peptide for which an undeuterated result was found. By default, these bars are green for high-confidence peptides and yellow for medium-confidence ones. Low-confidence results, if they are displayed, are displayed in red. If you wish, you can tell HDExaminer to color the bars by average deuteration level instead. See “HDExaminer Options: Protein View” on page 46 for more information. Clicking on a peptide bar in the coverage map takes you to the uptake plot for that peptide.

**Heat map:** each row of the heat map represents one of your partially deuterated time points. Its color corresponds to the computed deuteration percentage of that area of your protein (see the color key in the upper right of the window).

For further information about the heat map see “The Heat Map” on page 30.

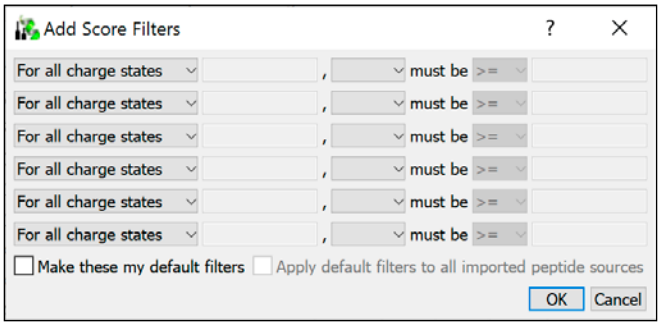
This section explains the peptide import process in more detail. For the basics, please refer to “Getting Started with HDExaminer: Importing Peptides” on page 13.

## Score Filters

After you have imported a Peptide Source, you may improve the quality of your imported peptides by applying any number of score filters to that Peptide Source. The filters you specify will determine which peptides are transferred to a Peptide Pool.

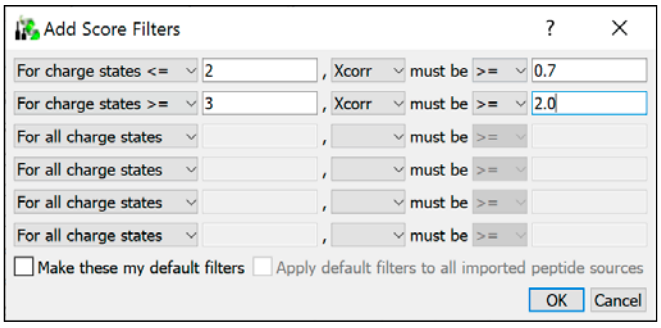
### To add a score filter:

1. Select a Peptide Source in the tree view on the left.
2. Click the add button near the top of the window: 
3. You will see the Add Score Filters dialog box:




Here you may specify up to six score filters. A peptide will be included if it passes all applicable filters.

You may specify different score filters for different charge states. Consider the following example:




This tells HDExaminer that Xcorr must be at least 0.7 for peptides at charge states 1 and 2, but that Xcorr must be at least 2.0 for peptides at charge states 3 or higher.

The Add Score Filters dialog has two checkboxes:

**Make these my default filters:** checking this box tells HDExaminer to auto-populate the Add Score Filters dialog with your current filters every time you bring up the dialog.

**Apply default filters to all imported peptide sources:** checking this box tells HDExaminer that you want the current filters applied to every new Peptide Source you import. In this case, HDExaminer will automatically apply the specified filters—you will not need to bring up the Add Score Filters dialog at all.

After applying score filters, the checkboxes to the left of each peptide in the table will be checked or unchecked depending on whether or not that peptide passed all the filters. In addition, HDExaminer will report the total number of marked peptides and the percentage of the protein covered by the marked peptides.

Note that you may manually mark or unmark any entry in the peptide list.

### To delete a score filter:

1. Select a Peptide Source in the tree view on the left.
2. Select one of its score filters in the list near the top of the window.
3. Click the minus button near the top of the window: 

## Residue Filters

You can also filter peptides based on a residue number range. This is useful for situations where you only wish to analyze the deuteration behavior of a particular section of your protein.

### To create a residue filter:

1. Select a Peptide Source in the tree view on the left.
2. Check the box labeled “Only include peptides including residues…”
3. Enter the residue range and protein you wish to use.

As with score filters, the checkboxes to the left of each peptide in the table will become marked or unmarked as appropriate. If you select a protein but leave the residue range blank, the filter will simply select all peptides from the selected protein. This can be useful for filtering in a project with multiple protein sequences.

To remove your residue filter, simply uncheck the "Only include peptides…" checkbox.

## Length Filters

You can also filter peptides based on length.

### To create a length filter:

1. Select a Peptide Source in the tree view on the left.
2. Check the box labeled "Only include peptides with length between…"
3. Enter the length range you wish to use.

As with other filters, the checkboxes to the left of each peptide in the table will become marked or unmarked as appropriate.

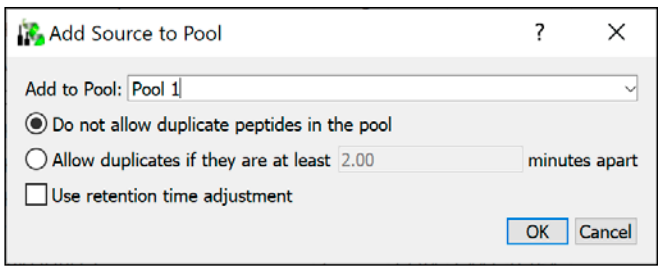
To remove your length filter, simply uncheck the "Only include peptides…" checkbox.

## Working with Peptide Pools

Peptide Pools are created by importing peptides from one or more Peptide Sources. HDExaminer supports multiple Peptide Pools in a project, but putting all peptides into a single Peptide Pool is recommended.

### To create or add to an existing Peptide Pool:

1. Select a Peptide Source in the tree view on the left.
2. Click the button on the right labeled "Add Marked Peptides to Pool…" You will see the following dialog box:




3. Select (or type) the name of the pool to which you wish to add the marked peptides. If a pool by that name does not exist, it will be created.
4. Select other import options (described below).
5. Click "OK".

### Duplicate peptide entries:

Most peptide search engines will report each peptide multiple times—once for each MS/MS spectrum which suggests the presence of that peptide. As a result, a Peptide Source may have multiple entries corresponding to a single peptide. The Add Source to Pool dialog shown above allows you to select whether or not these duplicate peptide entries are allowed in the target Peptide Pool.

The default setting is "Do not allow duplicate peptides in the pool". This means a peptide may only appear in a Peptide Pool once, regardless of the various retention times at which it was found by your peptide search engine. With this setting, an existing peptide in the pool takes precedence over one being imported. If no such peptide already exists and multiple duplicate peptides are being imported from a Peptide Source, the entry with the highest score is imported. If the entry has multiple scores (such as Xcorr and DelCn), the one with the highest first score is imported.

If you wish to allow duplicate peptides, select "Allow duplicates…" and enter the minimum retention time gap between the duplicates that HDExaminer should allow. In this case, HDExaminer will first import the highest-scoring entry that is the required retention time gap away from any instance of the same peptide already in the pool. It will then repeat this process until it cannot add any more entries.

#### ⚠ Warning

**Warning: it is generally considered to be physically impossible for a single peptide to elute at two different retention times!** If you have duplicate peptides in your Peptide Pool, it is most likely because one of them was misidentified. Using such misidentified peptides in HDExaminer is likely to give you an incorrect picture of the overall deuteration behavior of your protein. If you decide to allow duplicates, you must disambiguate all peptides by deleting all but one instance of each before analyzing your heat map.

**It is strongly recommended that you use the default selection: "Do not allow duplicate peptides in the pool".**

## Peptide Pool Retention Time Adjustment

In some cases, the retention times specified in your Peptide Source may not correspond to the retention times at which you expect to find each peptide in your MS runs, because you have chosen to run your MS/MS experiments under different chromatographic conditions from your MS time point experiments. In such a case, you will need to check “Use retention time adjustment” when creating your Peptide Pool and enter one or more translation entries in the table that appears.

*Note: HDExaminer can also do automatic retention time adjustment via a feature alignment algorithm similar to the one described in Venable et al., J. Am. Soc. Mass Spectrom. (2013) 24:642-645. If your experiments were all run under the same chromatographic conditions, but have some unusual retention time drift, you should use that feature (introduced on page 15) rather than the one described here.*

A translation entry consists of a Peptide Source retention time and the corresponding Peptide Pool retention time to which that value should be translated. Retention times will be translated as follows:

- In between the points specified by your translation entries, retention time will be calculated using linear interpolation.
- Outside of the retention time range covered by your translation entries, linear extrapolation will be used.
- The entry (0, 0) is implied unless you enter a translation entry that contains a zero value.

In general, retention time translation will work as expected as long as you can find 2 or 3 analogous retention times between your MS/MS run and your non-deuterated MS run. This is best accomplished by looking at individual spectra in each run and choosing a few which clearly contain similar signals.

HDExaminer’s retention time adjustment behavior is best illustrated with examples:

**Example 1:** your peptide search MS/MS run is 30 minutes long, but your MS time point experiments are 10 minutes long. When you look at the individual spectra, you notice that the MS/MS spectra at 5.2, 10.3, and 22.1 minutes

| Search RT | Pool RT |
|---|---|
| 5.2 | 1.7 |
| 10.3 | 3.5 |
| 22.1 | 8.1 |

HDExaminer will translate all peptide retention times by using linear interpolation and extrapolation from these points. Note that translated retention times do not need to be exact, since HDExaminer searches for each peptide within a retention time window rather than at a precise retention time.

**Example 2:** your peptide search MS/MS run is 60 minutes long, but your MS time point experiments are 30 minutes long (but otherwise use similar chromatographic conditions). You therefore want to tell HDExaminer to map the higher retention times from your Peptide Source to lower retention times at which to look for the peptide in the MS runs. To do this, you enter “60” as the first Search RT and “30” as the corresponding Pool RT. HDExaminer then divides each peptide’s retention time by 2 on import. (Note that entering “2” and “1” would work just as well as entering “60” and “30” here.)

**Example 3:** due to problems you are having with overlapping peptides, you decide to try MS time point experiments which are 40 minutes long--10 minutes longer than your 30-minute peptide MS/MS search runs. Suppose that you decide to slow down your elution partway through your MS runs, so that minutes 20-22 of your MS/MS search run correspond to minutes 20-32 of your MS time point runs. You set the following table entries:

| Search RT | Pool RT |
|---|---|
| 20 | 20 |
| 22 | 32 |
| 30 | 40 |

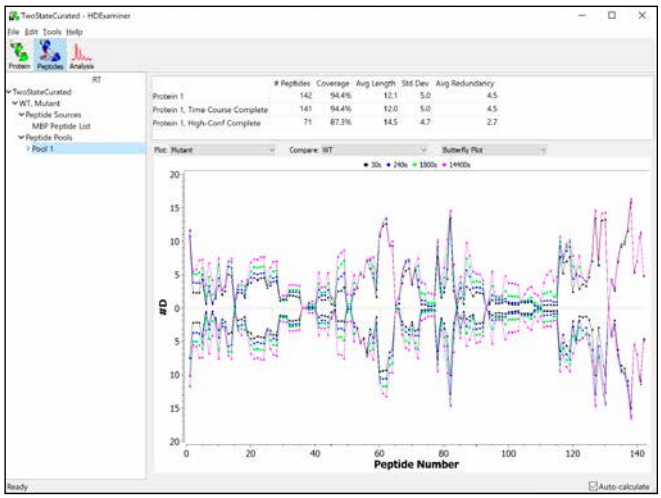
The first line (along with the implied (0, 0) entry) tells HDExaminer that the retention times for the first 20 minutes of the run should be unaltered. The second line tells HDExaminer to stretch minutes 20-22 in the Peptide Source to minutes 20-32 in the Peptide Pool. The final line tells HDExaminer that peptides found at 30 minutes in the MS/MS search run will be found at 40 minutes in the MS time point runs.

## Importing Thermo-Generated Peptide Sources

Some SEQUEST, Mascot and pepXML files generated by older Thermo software refer to peptides by scan number rather than by retention time. If this is the case, then after browsing to a Peptide Source file, HDExaminer will ask you to browse to the corresponding MS/MS dataset that was used to generate the Peptide Source. HDExaminer will use this dataset to convert scan numbers into retention times.

## The Peptide Pool View

If you select a Peptide Pool in the tree view, you will see a view like the following:




This view shows the deuteration level as a function of peptide index, as well as other useful information about how well the Peptide Pool covers your protein. Each colored trace on this graph represents a single time point experiment. By selecting protein states in the “Plot” and “Compare” dropdown menus, you can generate plots similar to the butterfly plots, residual plots, Woods plots, chiclet plots and volcano plots sometimes seen in the HD Exchange literature. (For more information on comparing protein states, see “Comparing Protein States” on page 34.)

You may zoom this graph by left-dragging a rectangle anywhere on it, and unzoom by clicking anywhere that isn’t a data point. Clicking on a data point will take you to the uptake plot for the clicked peptide (see “Analyzing Peptide Deuteration Behavior” on page 17).

## Context Menus

The following commands are available through right-click context menus in the Peptides View.

### Tree View:

**Peptide Notes…:** this allows you to attach a text note to the selected peptide. Notes are for your own benefit and have no bearing on HDExaminer’s calculations or reports.

**Add Peptide Source…:** this allows you to browse to a Peptide Source file. If the import is successful, HDExaminer will create a new Peptide Source based on the file you import.

**Delete Peptide Source:** removes the selected Peptide Source from your project.

**Add Peptide Pool:** adds an empty Peptide Pool to your project.

**Delete Peptide Pool:** removes the selected Peptide Pool from your project. Note that deuteration calculations will be discarded for any peptide which is no longer in your project.

**Delete Peptide:** removes the selected peptide and all of its deuteration calculations (for all protein states and charge states) from your project.

**Delete Peptides with No Uptake Plot…:** deletes any peptide that has no uptake plot, either because the necessary data points do not exist or because they are all low confidence.

**Delete Low Confidence Peptides…:** deletes any peptide that is marked as low confidence (red) in the Peptides View.

**High/Medium/Low Confidence:** sets the selected peptide to the specified confidence.

**Remove Confidence Override:** if you override a peptide’s confidence level, this allows you to remove that override and reset its confidence to the level originally calculated by HDExaminer.

**Recalculate and Force Bimodal:** this tells HDExaminer to recalculate the entire peptide, and to force all of the deuterated results to be matched with a bimodal distribution rather than a unimodal one.

**Recalculate and Force Unimodal:** this tells HDExaminer to recalculate the entire peptide, and to force all of the deuterated results to be matched with a unimodal distribution rather than a bimodal one.

**Set Charge State to Low Confidence (This Protein State):** this sets the selected charge state's deuterated results to low confidence for the current protein state. This removes that charge state from consideration in all downstream calculations.

**Set Charge State to Low Confidence (All Protein States):** this sets the selected charge state's deuterated results to low confidence across all protein states in your project. This removes that charge state from consideration in all downstream calculations.

**Copy Peptide Pool Results to Clipboard:** copies a complete, per-peptide deuteration summary table for the selected Peptide Pool to the clipboard. This table may then be pasted to a spreadsheet program such as Microsoft Excel.

**Export Peptide Pool Results…:** you will be asked to specify a .csv file to which HDExaminer should save. It will then export a complete, per-peptide deuteration summary table for the selected Peptide Pool.

**Copy Uptake Summary Table to Clipboard:** copies a deuteration summary table to the clipboard. This table differs from the table described above in that there is one row in this table per peptide/time point combination. Note that this table adheres to all requirements in Recommendation 3.1 of *Masson et al., Nat Methods* **16***, 595–602 (2019).*

**Export Uptake Summary Table…:** you will be asked to specify a .csv file to which HDExaminer should save. It will then export the deuteration summary table described under "Copy Uptake Summary Table to Clipboard". As above, this table adheres to all requirements in Recommendation 3.1 of *Masson et al., Nat Methods* ***16****, 595–602 (2019).*

**Export Uptake Plots to PDF…:** use this feature to export all of your uptake plots to a tabular PDF file. Specify the number of rows and columns you would like per page, and whether you would like HDExaminer to also export Enhanced Metafiles (EMF) or JPEG image files for each uptake plot. If you are analyzing middle-down data, you may tell HDExaminer to include uptake plots of MS/MS fragments. Click OK, then specify the name of the PDF file you would like to save and HDExaminer will create it. If you chose to generate EMF or JPEG files, HDExaminer will save those in a directory named [file].images, where [file] is the name of the PDF file you saved, without the ".pdf" extension.

**Export Peptide Spectra…:** you will be asked to select a folder. Into that folder, HDExaminer will save the raw spectrum data for each result corresponding to the selected peptide. The exported data will be saved to a subfolder called SpecExport. The individual spectra will be saved in csv format.

**Export Pool Spectra…:** this is the same as "Export Peptide Spectra" described above, except that spectra will be exported for every peptide in the current Peptide Pool rather than just for the selected peptide.

**Export Peptide Pool…:** you will be asked to specify a .csv file to which HDExaminer should save. It will then export your peptide pool in a format that you may import into another HDExaminer project.

### Uptake Plot:

**Copy Uptake Plot to Clipboard:** copies a scalable image of the uptake plot to the clipboard. This image may then be pasted into programs such as Microsoft Word, Microsoft PowerPoint, or Adobe Illustrator.

**Export Uptake Plot…:** you will be asked to specify a .emf (Windows Enhanced Metafile) file to which HDExaminer should save. It will then export a scalable image of the uptake plot.

**Uptake Plot Options…:** this brings up the Uptake Plots page of the HDExaminer program options. For more information, see "HDExaminer Options: Uptake Plots" on page 47.

### Deuteration Comparison View:

**View in Analysis View:** switches to the selected result in the Analysis View.

**High Confidence:** sets the selected result to high confidence.

**Medium Confidence:** sets the selected result to medium confidence.

**Low Confidence:** sets the selected result to low confidence. Remember, low-confidence results are not used in any downstream calculations.

**Remove RT Override:** removes the retention time manual override from the current result and recalculates. See "The Analysis View: Manual Overrides" on page 26 for more information.

**Remove m/z Override:** removes the m/z manual override from the current result and recalculates.

**Remove Drift Override:** removes the ion mobility drift time override from the current result and recalculates.

**Force Unimodal/Bimodal:** recalculates the selected result by matching a unimodal or bimodal distribution instead of the one calculated by default.

**Copy Plots to Clipboard:** copies a scalable image of the stacked spectrum and chromatogram plots to the clipboard. This image may then be pasted into programs such as Microsoft Word, Microsoft PowerPoint, or Adobe Illustrator.

**Export Plots…:** you will be asked to specify a .emf (Windows Enhanced Metafile) file to which HDExaminer should save. It will then export a scalable image of the stacked spectrum and chromatogram plots.

### Peptide Pool View:

**Copy Peptide Plot to Clipboard:** copies a scalable image of the current plot to the clipboard. This image may then be pasted into programs such as Microsoft Word, Microsoft PowerPoint, or Adobe Illustrator.

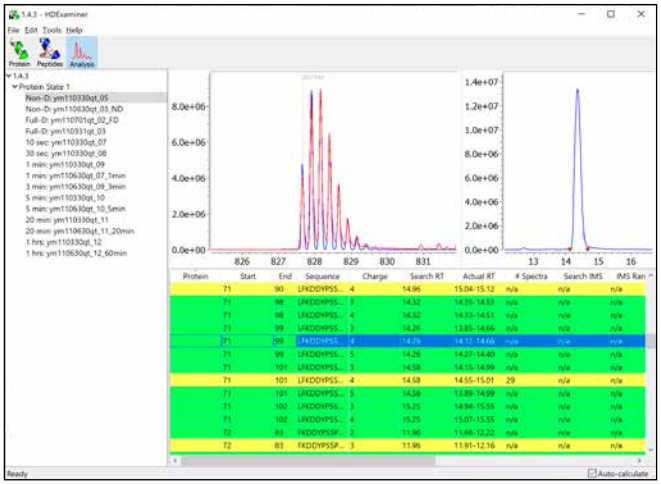
**Export Peptide Plot…:** you will be asked to specify a .emf (Windows Enhanced Metafile) file to which HDExaminer should save. It will then export a scalable image of the peptide plot.

**Copy Peptide Plot Data to Clipboard:** copies a table to the clipboard corresponding to the peptide plot. Each row of this table includes a single peptide and the deuteration data for that peptide for all protein states and time points.

**Export Peptide Plot Data…:** you will be asked to specify a .csv file to which HDExaminer should save. It will then export a table corresponding to the peptide plot, as described above.

**Peptide Plot Options...:** this brings up the Peptide Plot page of the HDExaminer program options. For more information, see “HDExaminer Options: Peptide Plot” on page 50.

This section explains the functionality available in the Analysis View.



## Viewing Results

To select an MS experiment, click on that experiment in the tree view on the left. To view a result, click on its entry in the results table. You will see its theoretical (blue) and actual (red) isotope clusters, as well as its extracted ion chromatogram.

You may zoom the spectrum or chromatogram view by left-dragging over a range. Left-click on the view to return it to its previous zoom level.

If you are analyzing data with ion mobility separation, the chromatogram view will include a switch in the bottom left allowing you to toggle between XIC and IMS. Switching it to IMS will show the extracted ion chromatogram over all ion mobility drift times rather than over retention time.

## The Results Table

The results table contains information about calculated results. If a result shows a score of 0, it is either because the peptide could not be found or because the result has not yet been calculated. You may sort this table by clicking on a column header.

**Protein:** the name of the protein that the current peptide belongs to. In a project with only one protein sequence, this column could be blank.

**Start and End:** the start and end residues of the peptide

**Sequence:** the sequence of the peptide

**Adduct:** the MS adduct used to calculate the result (usually H)

**Charge:** the charge state of the result

**Search RT:** the retention time at which HDExaminer expected to find the peptide, as specified in the Peptide Pool

**Actual RT:** the retention time range for which HDExaminer found the best result

**# Spectra:** the number of spectra (in the RT domain) that were summed to generate the result

**Search IMS:** the ion mobility drift time at which HDExaminer expected to find the peptide, as specified by your imported peptide search results

**IMS Range:** the ion mobility range that was summed to generate the result

**Peak Width:** the peak width in Daltons that resulted in the best fit between the theoretical and actual isotope clusters

**m/z Shift:** the m/z shift that resulted in the best fit between the theoretical and actual isotope clusters

**Max Inty:** the maximum intensity of the isotope cluster summed over all scans in the RT and IMS ranges

**Avg Inty:** the Max Inty field divided by the # Spectra field

**Max Scan Inty:** the maximum intensity of the isotope cluster found in any single MS scan

**Exp Cent:** the centroid of the actual isotope cluster in the experimental data. If the result is bimodal, the centroid of the left distribution.

**Exp Cent 2 (partially-deuterated experiments only):** if the result is bimodal, the centroid of the right distribution

**L/R Ratio (partially-deuterated experiments only):** if the result is bimodal, the ratio of the total intensity of the left distribution to the total intensity of the right distribution

**Left % (partially-deuterated experiments only):** if the result is bimodal, the percentage of total intensity contributed by the left distribution

**Right % (partially-deuterated experiments only):** if the result is bimodal, the percentage of total intensity contributed by the right distribution

**Theor Cent:** the centroid of the theoretical isotope cluster. If the result is bimodal, the centroid of the left distribution.

**Theor Cent 2 (partially-deuterated experiments only):** if the result is bimodal, the centroid of the right distribution

**Theor L/R (partially-deuterated experiments only):** if the result is bimodal, the ratio of the total intensity of the left distribution to the total intensity of the right distribution

**Theor Left % (partially-deuterated experiments only):** if the result is bimodal, the percentage of total intensity contributed by the left distribution

**Theor Right % (partially-deuterated experiments only):** if the result is bimodal, the percentage of total intensity contributed by the right distribution

**Score:** a score (on a 0-to-1 scale) representing how well the theoretical and actual isotope clusters match

**Cent Diff (deuterated experiments only):** the difference between the deuterated and undeuterated centroid for this result. If the result is bimodal, the difference between the left distribution's deuterated centroid and the undeuterated centroid.

**# Deut (deuterated experiments only):** the number of deuterons corresponding to the difference between the deuterated and undeuterated centroids. If the result is bimodal, this is the calculated result for the left distribution.

**% Max D (fully-deuterated experiments only):** the result's percentage of the theoretical maximum deuteration

**Deut % (partially-deuterated experiments only):** the result's percentage of the deuteration level of your fully-deuterated experiment. If the result is bimodal, this is the calculated result for the left distribution.

**Cent Diff 2 (partially-deuterated experiments only):** if the result is bimodal, the difference between the right distribution's deuterated centroid and the undeuterated centroid

**# Deut 2 (partially-deuterated experiments only):** if the result is bimodal, the number of deuterons corresponding to the difference between the right distribution's centroid and the undeuterated centroids

**Deut % 2 (partially-deuterated experiments only):** if the result is bimodal, the right distribution's percentage of the deuteration level of your fully-deuterated experiment

**Mark:** this column contains a checkbox that you may use to "bookmark" certain results. For example, you could use this to mark where you left off in manual verification of results. Whether or not a result is marked has no impact on HDExaminer's deuteration calculations.

## Manual Overrides

In some cases, you may wish to adjust a result's retention time range, ion mobility range or m/z range in an attempt to improve that result. By default, HDExaminer will automatically recalculate any result based on your manual adjustments. For retention time and ion mobility manual overrides, you may change this behavior by going to the Tools menu, selecting Options…, then Manual Overrides, and checking the box marked "Show spectrum before calculating RT/drift override."

### To adjust the retention time envelope or ion mobility range of a result:

1. Select the result in the results table.
2. If you are analyzing ion mobility data, make sure that the chromatogram switch is set to "XIC" if you want to override retention time or "IMS" if you want to override ion mobility range.
3. Right-drag a range anywhere on the chromatogram view to set the retention time range or ion mobility range directly, **OR** left-drag one or both of the red triangles in the chromatogram view to the positions you desire, **OR** left-drag both triangles simultaneously by holding down the Ctrl key.
4. If the Auto-calculate checkbox in the bottom right is unchecked, check it. Depending on your settings, HDExaminer will either recalculate the result automatically or show you the raw spectrum (in black) corresponding to your new range. You will then be able to decide whether to recalculate based on the new spectrum or reject the new spectrum and keep your existing result.

Note that retention time and ion mobility manual overrides can also be specified in the deuteration comparison view inside the Peptides View. When viewing multiple results in the deuteration comparison view, you may shift-right-drag a range on a chromatogram view to set that range for **all** results for the selected peptide/charge state combination. You may also ctrl-shift-right-drag a range to set that range for **all results across all protein states in the project.**

### To adjust the m/z range of a result:

1. Select the result in the results table.
2. Right-drag a range anywhere on the spectrum view to set the m/z range directly, **OR** left-drag one or both of the red triangles in the spectrum view to the positions you desire, **OR** left-drag both triangles simultaneously by holding down the Ctrl key.
3. If the Auto-calculate checkbox in the bottom right is unchecked, check it. HDExaminer will recalculate the result automatically.

Note that m/z manual overrides can also be specified in the deuteration comparison view inside the Peptides View.

Also note that m/z manual overrides are only allowed for deuterated results. Undeuterated results have a fixed isotope cluster which may not be altered.

In the absence of an m/z manual override, HDExaminer will use a Gaussian distribution of deuteration levels to calculate a theoretical isotope cluster. However, when you provide HDExaminer with an m/z manual override, it will instead compute the *arbitrary* distribution of deuteration levels that produces the best match between the theoretical and actual isotope clusters. As a result of this, bimodal distributions are never reported on results with m/z overrides.

### To remove a manual override:

1. Select the result in the results table.
2. Right-click on the result and choose “Remove RT Override”, “Remove m/z Override” or “Remove Drift Override”, as appropriate.
3. If the Auto-calculate checkbox in the bottom right is unchecked, check it. HDExaminer will recalculate the result automatically.

## Batch Retention Time Overrides from a Table

HDExaminer allows batch retention time overrides from a spreadsheet. This can be useful if you have done automated analysis of retention time windows in a tool other than HDExaminer and wish to override your entire project all at once. To use this feature, create a spreadsheet in csv, txt, xls or xlsx format with the following columns:

1. **MS Filename:** this column contains the filenames of the MS raw files in your HDExaminer project whose retention times you wish to override. This can be a full path or just the filename. The filename must include the file extension, such as .raw or .d.
2. **Peptide Sequence:** this column contains the sequence of the peptide whose retention time you wish to override. Any characters in this column that do not correspond to amino acids will be ignored.
3. **Charge:** this column contains the charge of the result you wish to override.
4. **Desired Start Retention Time:** this column contains the start retention time (in minutes) you wish to use for the given file/sequence/charge combination.
5. **Desired End Retention Time:** this column contains the end retention time you wish to use for the given file/sequence/charge combination.

The imported spreadsheet should **not** include a header row. Each row in the file corresponds to a single retention time override. When you import this file, HDExaminer will calculate all of the specified retention time overrides as though you had manually entered each of the overrides in the imported file.

Here is an example of a valid override file:

| | | | | |
|---|---|---|---|---|
| 0s_20200326_235540.raw | HKTGPNLHGL | 2 | 3.42 | 3.56 |
| 0s_20200326_235540.raw | HKTGPNLHGL | 3 | 3.45 | 3.6 |
| 0s_20200326_235540.raw | GLFGRKTGQ | 2 | 4 | 4.3 |
| 0s_20200326_235540.raw | ANKNKGITWKEETL | 3 | 5.8 | 6.1 |
| 0s_20200326_235540.raw | ANKNKGITWKEETL | 4 | 5.75 | 6.08 |
| 30s_20200327_003227.raw | HKTGPNLHGL | 3 | 3.55 | 3.8 |
| 30s_20200327_003227.raw | GLFGRKTGQ | 2 | 4.2 | 4.4 |
| 30s_20200327_003227.raw | ANKNKGITWKEETL | 3 | 5.9 | 6.3 |
| 30s_20200327_003227.raw | ANKNKGITWKEETL | 4 | 5.95 | 6.34 |
| 100s_20200327_061745.raw | HKTGPNLHGL | 3 | 3.52 | 3.76 |
| 100s_20200327_061745.raw | GLFGRKTGQ | 2 | 4.2 | 4.4 |
| 100s_20200327_061745.raw | ANKNKGITWKEETL | 3 | 5.8 | 6.1 |
| 300s_20200327_065934.raw | HKTGPNLHGL | 3 | 3.55 | 3.8 |
| 300s_20200327_065934.raw | GLFGRKTGQ | 2 | 4.2 | 4.4 |
| 300s_20200327_065934.raw | ANKNKGITWKEETL | 3 | 5.9 | 6.3 |
| 300s_20200327_065934.raw | ANKNKGITWKEETL | 4 | 5.95 | 6.34 |

To import an override file, right-click on any result in the Analysis View table and select “RT Overrides from Table…” Select the override file and HDExaminer will override the results and begin recalculating.

Note that it does not matter which experiment or result you have selected in the Analysis View when you select this menu option. The results specified in your imported data file are the ones that will be overridden, regardless of which results are currently showing in the Analysis View.

## Filtering Results

In certain situations, you may wish to change the confidence level of results automatically based on criteria such as score, m/z shift, or maximum peak intensity. You can do this by right-clicking on the tree view on the left or the table view and selecting “Filter Results...” You will see a window like this:


The first menu lets you specify whether you want to apply the new confidence level to matching results in this experiment, this protein state, or your entire project. The second menu lets you specify the parameter you’d like to filter on, such as score. The third menu lets you specify whether you want to change the confidence of results above or below the specified value. The final menu lets you specify the new confidence level that you want to be assigned to all results that meet the condition you specify.

## Context Menus

The following commands are available through right-click context menus in the Analysis View.

### Tree View:

Add Experiment…: this brings up the MS data browser to allow you to select an MS time point experiment to add to your project.

**Edit Experiment…:** this allows you to change the parameters associated with the selected experiment, including experiment name, deuteration time, D2O buffer %, and, in the case of an undeuterated experiment, the charge states to use when searching.

**Remove Experiment:** this removes the selected experiment and all of its calculated results from the project.

**Show MS File in Explorer:** shows the selected MS experiment file in Windows. If HDExaminer can’t locate the MS file, this menu item is “Locate Experiment Folder…” instead, and selecting it allows you to browse to a folder where the MS experiment file is located.

**Experiment Notes…:** this allows you to add a text note to the selected experiment. Notes are for your own benefit and have no bearing on HDExaminer’s calculations or reports.

**Filter Results...:** this allows you to set the confidence level of all results above or below a certain threshold for various parameters, such as score. See “Filtering Results” above for more information.

**Use RT Adjustment:** if you chose an undeuterated experiment to use as a reference dataset for retention time adjustment, then by default HDExaminer will adjust all other experiments. You may override this behavior for a single experiment by unchecking this menu item. If you do this, HDExaminer will use the raw, unadjusted retention times for that experiment.

### Results Table:

**High Confidence:** sets the selected result to high confidence.

**Medium Confidence:** sets the selected result to medium confidence.

**Low Confidence:** sets the selected result to low confidence. Remember, low-confidence results are not used in any downstream calculations.

**Force Unimodal/Bimodal:** if HDExaminer found a bimodal/unimodal result, this tells HDExaminer to try recalculating the result the other way.

**Recalculate:** the recalculate menu allows you to recalculate some or all of the results in your project. Note that the recalculation feature is mainly useful for recalculating results after upgrading to a new version of HDExaminer.

- **Result:** recalculates the selected result.
- **Peptide:** recalculates the selected peptide.
- **Experiment:** recalculates all results for the selected experiment.
- **Protein State:** recalculates all results for the selected protein state.
- **Project:** recalculates all results for the entire project.

**Try Other Charge States:** this selection allows you to look for this peptide at different charge states in this experiment, even if those charge states are not listed in your imported peptide search results.

**RT Overrides from Table…:** allows you to import a retention time override table as described on page 27, above.

**Remove RT Override:** removes the retention time manual override from the selected result.

**Remove m/z Override:** removes the m/z manual override from the selected result.

**Remove Drift Override:** removes the ion mobility range override from the selected result.

**View in Peptides View:** switches to the Peptides View and shows the selected result's peptide and charge state. You will see the deuteration comparison view for the selected result across all MS time point experiments for its protein state. Note that double-clicking on a result in the results table is equivalent to selecting this context menu item.

**Filter Results...:** this allows you to set the confidence level of all results above or below a certain threshold for various parameters, such as score. See "Filtering Results" above for more information.

**Copy This Table to Clipboard:** copies the results table to the clipboard. It may then be pasted to a spreadsheet program such as Microsoft Excel.

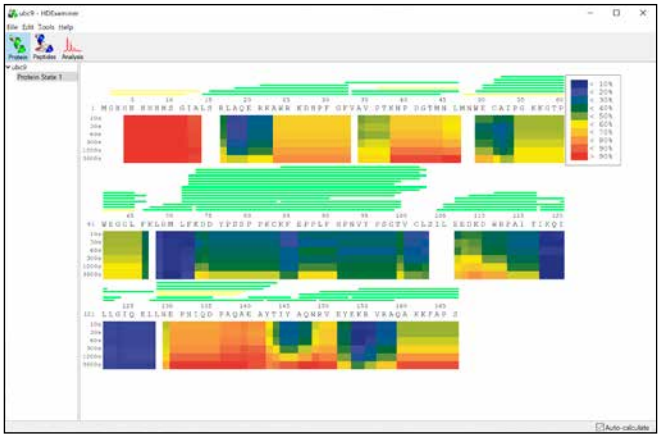
**Export This Table…:** you will be asked to specify a .csv file to which HDExaminer should save. It will then export the results table to that file.

**Copy All Tables to Clipboard:** this is similar to "Copy Table Data to Clipboard," except that instead of copying only the currently-displayed table, it creates a single table with all of your results across all experiments in the project.

**Export All Tables…:** this is similar to "Export Table Data," except that instead of saving only the currently-displayed table, it saves a single .csv with all of your results across all experiments in the project.

**Table Display Options…:** this brings up the Deuteration Table page of the HDExaminer program options. For more information, see "HDExaminer Options: Deuteration Table" on page 51.

This section explains the heat map: how it works, how to use it, and why it is useful.



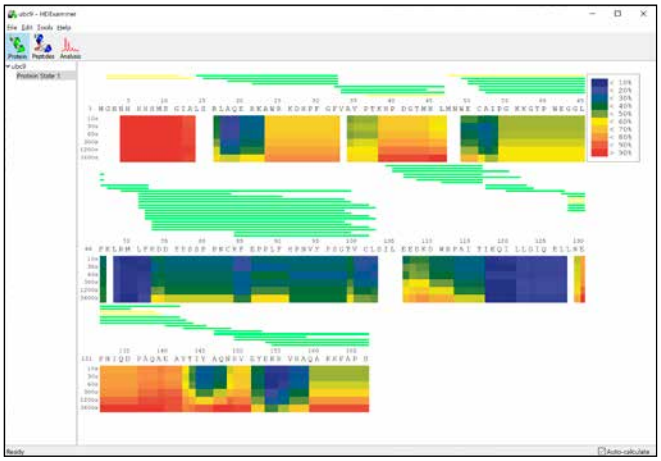
## Coverage Bars

The bars above the sequence are the coverage bars. These show the peptides that HDExaminer was able to find in your experiments. Clicking on any of the coverage bars will take you to the uptake plot for that peptide.

By default, the coverage bars are color coded by peptide confidence. You may change this by right-clicking anywhere on the heat map, selecting “Heat Map Options…”, then selecting “Average deuteration level of peptide” or “Result confidence” under “Coverage map bar color corresponds to:” and clicking OK.

You may turn off the coverage bars by right-clicking anywhere in the heat map, selecting “Heat Map Options…”, then unchecking “Show coverage map” and clicking OK.

By default, the coverage bars will arrange themselves in the compact representation shown above. If you would rather arrange the coverage bars in ascending residue order, right-click on the heat map and select “Heat Map Options…”, then uncheck “Conserve vertical space when drawing coverage map” and click OK. Your coverage bars will then be arranged similarly to the bars in this example:



## Heat Bars

The bars below the sequence are the heat bars. These show, for each time point, the computed deuteration level at each residue that is the best least-squares match to your individual peptide measurements. (See the color key in the upper-right for each color’s meaning.) You may turn off the heat bars by right-clicking anywhere in the heat map, selecting “Heat Map Options…”, then unchecking “Show heat map” and clicking OK.

*Note: if you have chosen to show only the coverage map, then the coverage bars will appear below the protein sequence rather than above.*

The heat bars may be used as a tool to help you analyze the quality of your data. In particular, if your experiments contain internal inconsistencies (such as two overlapping peptides with very different deuteration levels), the heat bars can alert you to that fact.

### How is the heat map computed?

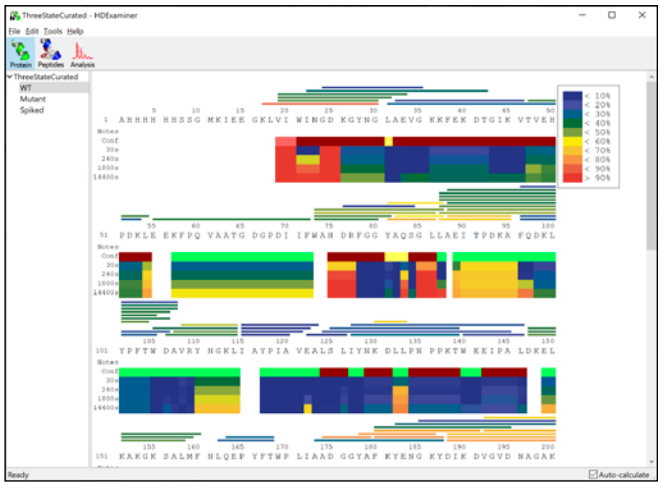
First, HDExaminer ignores the first two residues of each peptide, since these are widely considered to exchange too rapidly to be useful. (Note: this number can be overridden in HDExaminer’s settings. See “HDExaminer Options: Deuteration Levels” on page 51.)
The software then divides the protein into non-overlapping “atomic ranges”. These atomic ranges are formed by dividing the protein everywhere an observed peptide starts or ends. Each observed peptide’s deuteration level can then be expressed as a sum of deuteration levels

for one or more atomic ranges. HDExaminer computes a deuteration level for each atomic range that minimizes the least squares error with the set of observed peptides.

Like many least squares algorithms, this algorithm can be somewhat sensitive to noise. If you see color bars that don't make much sense, it may be an indication that one or more of your peptides has an incorrect or nonsensical result. In this case, you may wish to return to the Peptides View and further analyze your results.

## Heat map confidence

The heat map can also show confidence ratings for each residue:



To turn on this feature, go to Tools – Options – Protein View and select "Show heat map confidence". If you select "Per time point", then each time point will have a confidence row above it. If you select "Unified", then a single row will appear at the top, as in the example above. In the Unified representation, the confidence at each residue is the worst confidence from among all of the time points for that residue.

As described above, HDExaminer performs calculations to find the deuterium occupancy at each residue that minimizes the least-squares error with each of the measured peptides. There are two main problems that can lead to the least-squares model giving an inaccurate or untrustworthy picture of a protein's deuterium uptake. First, certain peptides can be outliers that have a measured deuteration level that substantially differs from what the model says that that peptide "should" have. Second, certain sections of the protein may have peptide coverage that causes the underlying least squares representation of the system to be underdetermined, which results in those sections having an infinite number of equally good error-minimizing solutions. Both of these issues are addressed by confidence assignment.

As with the rest of HDExaminer, red means low confidence, yellow means medium confidence, and green means high confidence. You may notice in the screenshot above that there are two different shades of red (one on residues 19-20 and one on residue 21 and many others). The lighter red means that that section of your protein has significant disagreement between the model and the peptides covering that area. The darker red means that that section of your protein has an infinite number of error-minimizing solutions.

Note also that the above screenshot shows all sections of the heat map, even the ones that are low confidence. *This is not recommended, since low-confidence sections of the heat map are unlikely to be correct and uniquely-determined.* To turn this behavior off, go to Tools – Options – Protein View and uncheck the "Color low-confidence residues" checkbox.

## Redrawing the heat map

When changes to your project require the heat map to be updated, the top of the heat map view will contain the text "The heat map needs to be recalculated. Click here to refresh the heat map." Clicking anywhere on the heat map while this text is showing will cause the heat map to be recalculated and redrawn.

## Heat map smoothing

You may wish to tell HDExaminer to alter the least squares calculation slightly to ensure that calculated deuteration levels don't vary wildly from one residue to the next. To do this, right-click on the heat map, select "Heat Map Options...", then adjust the "Heat map smoothing" setting to your choice of "None", "Very Light", "Light", "Moderate", "Heavy", or "Very Heavy".

### ⚠ Warning

**The heat map smoothing feature can cause misleading results, and may be removed in a future version of HDExaminer. It is strongly recommended that all users leave this setting on "None".**

## Multistate Heat Maps

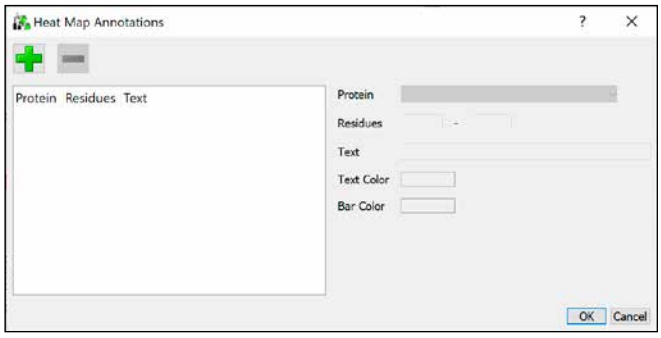
By default, the heat map shows all time points for the selected protein state. However, you may also view a heat map of multiple protein states at a selected time point. To do this, select the top level item in the tree view or individually select 3 or more protein states by ctrl-clicking.

This view shows the heat map for each selected state at the time point selected in the menu just to the upper left of the heat map itself (240s in the above example).

You can also get a multistate comparison heat map by clicking the checkbox marked “Compare to control state”. See page 37 for more information.

## Heat Map Annotations

You may add colored text annotations to the heat map, attached to any residue. To do this, right-click on the heat map and select “Heat Map Annotations…” The following window will appear:




To add an annotation, click on the “+” button, then edit the fields on the right. Select which protein you wish to attach to, the residue range you wish to use, the text of your annotation, the text color and the bar color. Your annotation will be drawn in a new “Notes” row on the heat map.

You may toggle the Notes row on and off by going to Tools – Options – Protein View and toggling the “Show heat map annotations” checkbox. If heat map annotations are off and you add a new annotation, your program settings will automatically change to show them.

## Context Menu

The following commands are available through right-click context menus in the Protein View.

### Tree View:

**Add Protein State…:** allows you to add a new protein state to your project.

**Edit Protein State…:** allows you to edit an existing protein state.

**Remove Protein State:** removes a protein state and all of its calculated results from your project.

**Protein State Notes…:** allows you to add a text note to the selected protein state. Notes are for your own benefit and have no bearing on HDExaminer’s calculations or reports.

**Set As Control State:** by default, HDExaminer treats the first protein state in a project as the control, and will use the control state when doing peptide-level comparisons (such as the butterfly plot or the volcano plot). Selecting this menu item for a different protein state tells HDExaminer to treat that state as the control instead.

For more information about protein states, see “Comparing Protein States” on page 34.

### Heat Map:

**Copy Image to Clipboard:** copies a scalable image of the heat map to the clipboard. This image may then be pasted into programs such as Microsoft Word, Microsoft PowerPoint, or Adobe Illustrator.

**Export Image…:** you will be asked to specify an .emf (Windows Enhanced Metafile) file to which HDExaminer should save. It will then export a scalable image of the heat map.

**Copy Data to Clipboard:** copies a data table to the clipboard. This table may be pasted to a spreadsheet program such as Microsoft Excel. The data table contains a numeric representation of the heat map. For each residue, and each time point, the table contains a number representing

the computed deuteration percentage of that residue, on a zero-to-one scale. The table also includes "spread" columns that indicate how much variance there is between the individual peptide measurements and the heat map data displayed. In general, lower spread values indicate better agreement among all of your peptides and the heat map. Finally, the data table includes confidence columns telling you whether that residue/time point combination is high, medium or low confidence.

**Export Data…:** you will be asked to specify a .csv file to which HDExaminer should save. It will then export the data table described above to that file.

**PyMOL Export…:** this allows you to export the heat bars to a format that can be imported by PyMOL. For more information, see "Exporting Data: Exporting to PyMOL or Chimera" on page 57.

**Chimera Export…:** this allows you to export the heat bars to a format that can be imported by Chimera. For more information, see "Exporting Data: Exporting to PyMOL or Chimera" on page 57.

**Heat Map Options…:** this brings up the Protein View page of the HDExaminer program options. For more information, see "HDExaminer Options: Protein View" on page 46.

**Heat Map Colors…:** this brings up the Heat Map Colors page of the HDExaminer program options. For more information, see "HDExaminer Options: Heat Map Colors" on page 47.

**Set Protein Residue Offsets...:** if you are analyzing a smaller section of a larger protein, it may be helpful to tell HDExaminer to label the first residue of each imported protein with a residue number other than 1. You may do so by selecting this menu item and entering the appropriate label for the first residue in each imported protein sequence. Note that the numbers you enter here can be negative.

**Rename Proteins…:** this allows you to change the name of each protein sequence as displayed on the heat map.

**Remove Protein from Project:** this allows you to remove a protein sequence from your project. The protein currently under the mouse cursor is the one that is removed.

**Heat Map Annotations…:** this allows you to add residue-specific annotations to the heat map, as described above.

**High/Medium/Low Confidence:** if you have right-clicked on a peptide in the coverage bars, then you may use these menu items to override that peptide's confidence to the level you select.

**Many HD Exchange experiments are intended to demonstrate a difference between two or more protein states, for example:**

- Ligand-bound vs. unbound
- Modified vs. unmodified
- Stressed vs. unstressed
- Mutant vs. wild-type

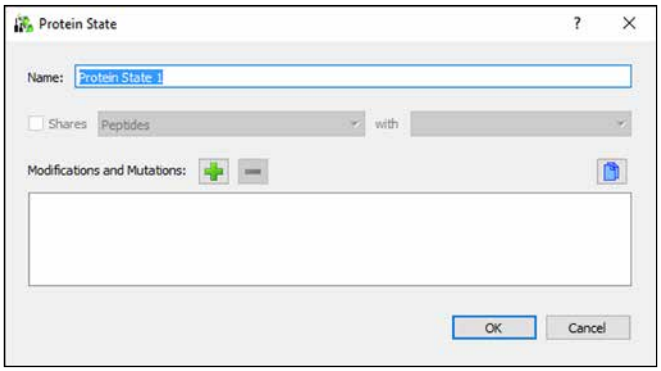
HDExaminer allows you to conveniently view the data in comparison experiments by using **protein states**. If you set up a protein state for each state for which you have experimental data, HDExaminer will allow you to compare uptake plots and heat maps for those states.

## Working with Protein States

By default, a project contains a single protein state called "Protein State 1" with no modifications or mutations.

### To add or edit a protein state:

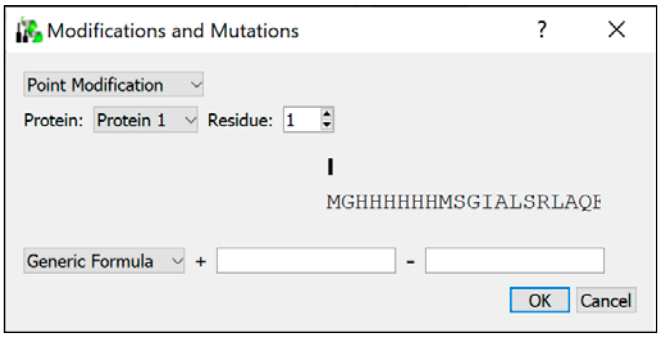
1. In the Protein View, right-click on an entry in the tree view on the left. (If you wish to edit, right-click on the entry you wish to edit.)
2. Select "Add Protein State…" or "Edit Protein State…" as appropriate. You will see the Add Protein State dialog box:




3. If you wish, you may give your protein state a more descriptive name, like "Control" or "Mutant."
4. If your protein state shares peptide lists or other data with another protein state, specify that here. **If you wish to compare peptide uptake plots directly, your protein states must share peptides.**
5. If your protein state contains modifications or mutations, enter them as specified below. You may also copy all modifications and mutations from a previous protein state by clicking on the button.
6. When finished, click "OK".

### To add modifications or mutations to a protein state:

1. In the Protein State dialog box, click on the add button:
2. You will see the Modifications and Mutations dialog box:




3. **If you wish to add a modification to a single residue:**
   a. Select "Point Modification" in the dropdown menu in the upper left.
   b. Select the protein and residue to which the modification should be attached.
   c. In the dropdown menu in the lower left, select your modification. If the modification you need is not in the menu, select "Generic Formula" and enter the chemical formula of the modification in the "+" text field. If the modification involves removing any atoms from the residue, enter the chemical formula that is removed in the "-" text field.
4. **If you wish to add a modification to *all instances* of a particular amino acid:**
   a. Select "Global Modification" in the dropdown menu in the upper left.
   b. Select the amino acid which should be globally modified.
   c. Select your modification, as described in 3c above.

5. **If you wish to add a mutation:**
   a. Select “Point Mutation” in the dropdown menu in the upper left.
   b. Select the protein and residue which should change.
   c. In the dropdown menu in the lower left, select the amino acid which should replace the selected residue.
6. When finished, click “OK”. If you added a “Generic Formula” modification in step 3, HDExaminer will ask if you wish to add that formula to your library of modifications for future use.

**To remove a modification or mutation,** select it in the Protein State dialog box and click the minus button: 

## Importing Data

The process for importing peptides and MS data files is similar whether your project contains one protein state or several.

**To import peptides,** you may add Peptide Sources to individual protein states by right-clicking on a protein state in the Peptides View and selecting “Add Peptide Source…” as normal. Note that protein states are grouped together in the Peptides View when they share peptides.

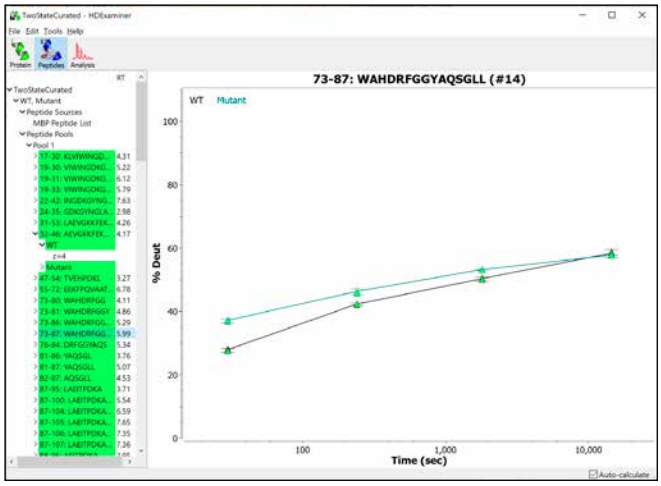
**To import MS time point experiments,** go to the Analysis View, then right-click on the protein state to which you want to add experiments. Select “Add Experiment…” as normal. You may also drag MS data files onto an individual protein state, as described on page 16.

## Analyzing Results

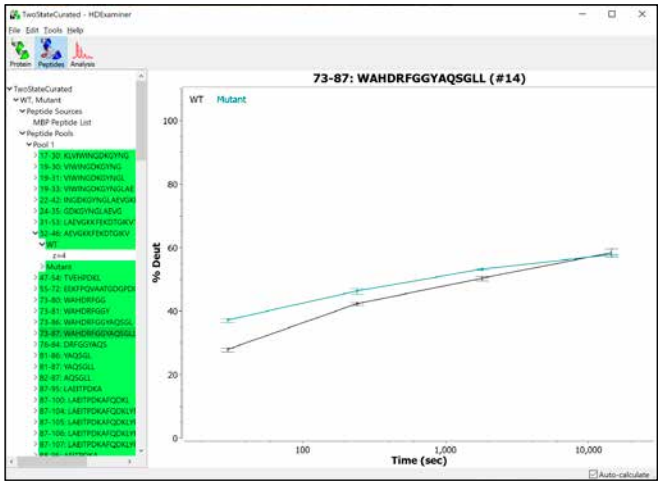
After results have been calculated for a project with multiple protein states, you may compare the results across two or more of those protein states.

### To view comparison uptake plots:

1. Make sure that your protein states share peptides. (See “Working with Protein States” above.) HDExaminer cannot display comparison uptake plots unless the protein states share peptides.
2. In the Peptides View, expand your Peptide Pool and select any peptide. You will see a view like the following:




If your experiments include multiple replicates for a given peptide at a given time point, HDExaminer will draw bars to show you the calculated significance intervals. If your significance intervals are very small (as in the above screenshot), they could be obscured by the shapes showing the individual results. To hide those shapes, right-click on the uptake plot and select “Uptake Plot Options…” In the options window that appears, uncheck “Show all data points”, then click OK. Without data points, the above screenshot looks like this instead:



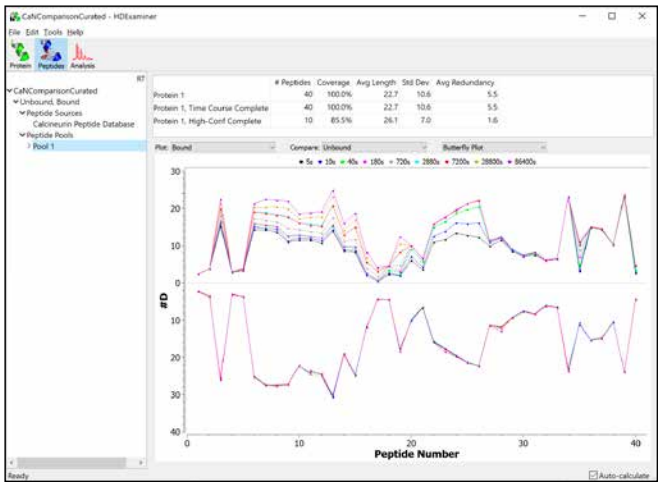

Each confidence interval is drawn by calculating a Welch’s unequal variances t-test using all of the replicates available for the “best” charge state for that protein state, which is defined as the one with the most high-confidence data and the most time points. If a peptide’s confidence interval bars do not overlap for a given peptide, you can be fairly confident that that peptide displays a statistically significant difference in deuteration behavior from one protein state to the other. You may also tell HDExaminer to mark significant

differences with an asterisk by going to the Tools menu, then “Options…”, then selecting “Uptake Plots” and checking the box “Mark significant differences”.

If a significance interval bar is missing, it means that HDExaminer could not find sufficient data points. A significance interval bar cannot be drawn without at least two good-quality replicates. Remember, low-confidence results are ignored.

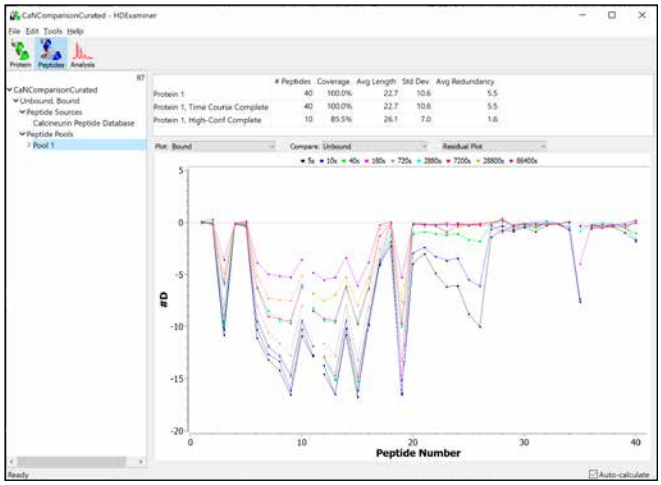
### To view comparison peptide plots:

You may use HDExaminer to create “butterfly plots” similar to those seen in the HD Exchange literature. To do this, go to the Peptides View and click on your Peptide Pool. You will see a view like this one:

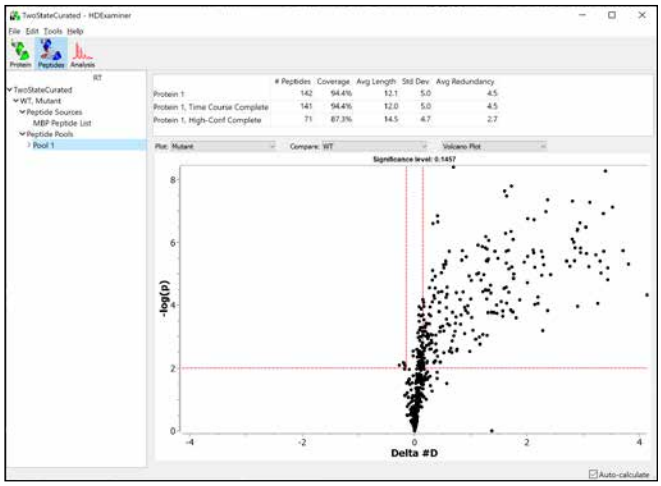



Near the top of this view are two popup menus. You may use these to determine which protein states are plotted.

You may also select “Residual Plot” in the third menu to show the difference between the two protein states you’ve selected:




If your project contains multiple replicates at each time point, then you may select “Volcano Plot” in the third menu to look for peptide/time point combinations that show significant differences between the two selected protein states.




In this view, each point represents a single peptide at a single time point. Mousing over a point will show you information about that point.

The x-axis of the volcano plot shows the change in the measured number of deuterons between the two selected protein states (Mutant and WT in the above image). The red vertical lines on either side of 0 indicate a significance level calculated by taking into account the variance in the replicate experiments provided. Measurements in between these two vertical red lines are not significant, because the measured difference between the two protein states does not exceed the replicate variance.

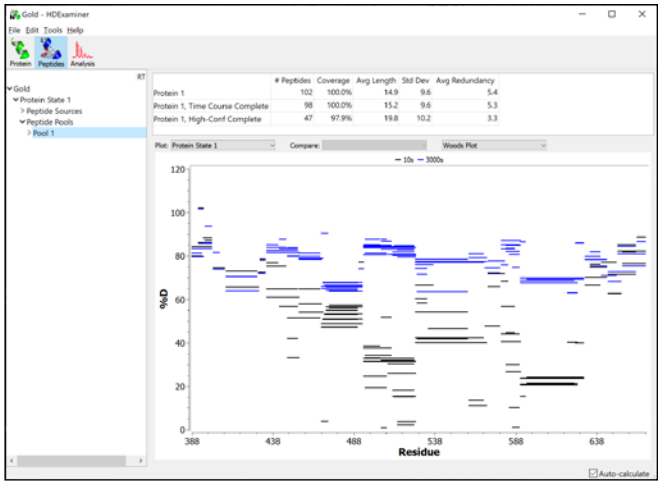
The y-axis of the volcano plot is the negative log of the p-value for that measurement (so a point high on the y-axis has a very low – and therefore significant – p-value). The horizontal red line at y=2 represents a 99% confidence interval (since 99% confidence requires p <= 0.01, or 10-2). Measurements below the horizontal red line are not significant, because their p-value is too high.

Measurements in the upper-left and upper-right areas of the volcano plot are the ones that pass both significance tests.

Another plot you can view when a Peptide Pool is selected is the Woods Plot. In this plot, the x axis is protein residue number and the y axis is either #D or %D, based on program settings. Each peptide/time point combination is represented by a single horizontal bar whose x-position and length represent the position of the peptide in the

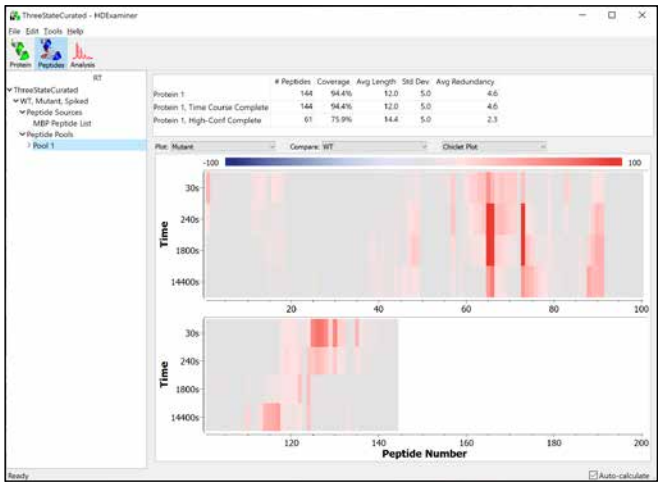
protein and whose y-position is the deuteration level.

If two protein states are selected, then the y-position will represent the difference in deuteration levels for the two protein states.



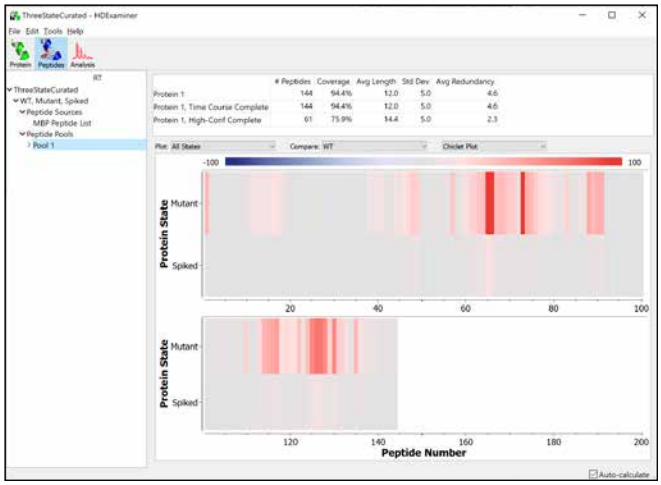

Note that the Woods Plot is best suited for projects with a relatively small number of peptides.

Another way to compare peptides in the Peptides View is with the Chiclet Plot.




In a chiclet plot, the x-axis is peptide number and the y-axis is deuteration time. Each rectangle (“chiclet”) on the plot is colored according to the deuteration level of that peptide at that time point. By selecting different protein states in the “Plot” and “Compare” dropdown menus, you can show a single state or a comparison between two states.

By selecting “All States” in the “Plot” menu, you can change the y-axis from time to protein state:

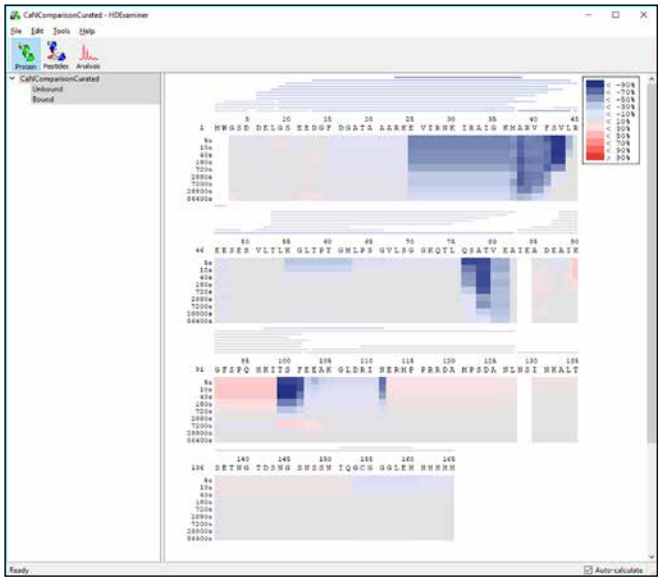



This view collapses all of your time points into a single maximum difference between states. This allows you to quickly compare many different protein states (for example, different ligands) against a control state to see which show differences of interest. In the example above, the chiclet plot clearly shows that the Mutant protein state differs substantially from the wild type control, while the Spiked protein state does not.

Chiclet plots are further described in *Naifu Zhang and others, Bioinformatics, Volume 37, Issue 13, July 2021, Pages 1926–1927,* https://doi.org/10.1093/bioinformatics/btaa892.

### To view comparison heat maps:

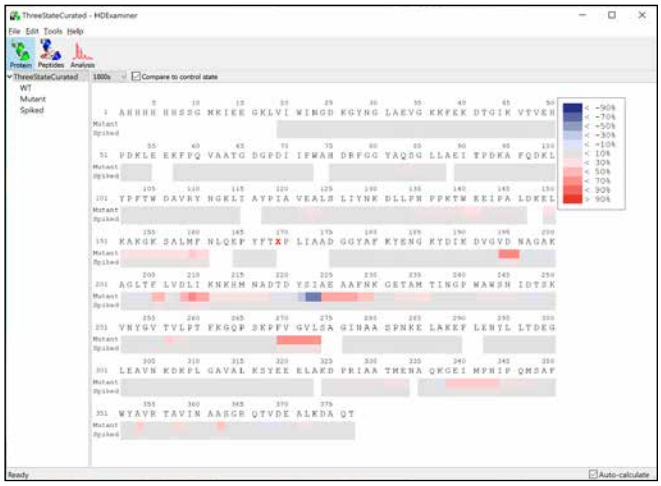
Another way to compare protein states is to compare their heat maps. In the Protein View, clicking on a protein state’s name will show you the heat map for that protein state. If one protein state is selected, you may Ctrl-click on a second protein state to get a difference heat map, such as the one below:



This view shows the areas of your protein which display increased or decreased deuterium uptake between the first and second selected protein states.

### To compare multiple protein states to a control state:

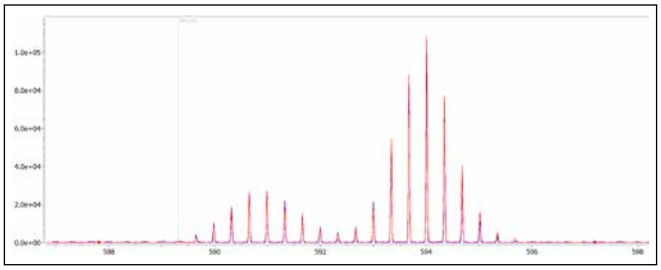
The Protein View also allows you to compare multiple protein states to your control state. To do this, either select the top item (the project name) in the Protein View tree or ctrl-click to select multiple protein states. The multistate heat map (described on page 32) will appear. To see a comparison heat map, check the box near the top of the window labeled “Compare to control state” and select the deuteration time you wish to view in the nearby menu. You will see a heat map like this:



This heat map shows the difference between each selected state and the control state at the selected deuteration time. This view allows you to quickly see which protein states show the largest differences.

Remember, by default the first protein state in your project is marked as the control state, but you may choose any protein state in your project to be the control state by right-clicking on that state in the Protein View and selecting “Set As Control State”.

It is possible for HD Exchange MS data to have two distinct distributions for a given peptide at a certain deuteration time. Causes for this include protein folding dynamics, multiple protein folding conformations, sample contamination and LC column carryover. A typical HDX bimodal distribution looks like this:



HDExaminer can identify such bimodal distributions and display relevant information about them.

## Getting Started

By default, bimodal processing is turned off in HDExaminer, and all result table columns pertaining to bimodal results are hidden. To change these settings, go to Tools – Options and follow these instructions:

**To turn on bimodal processing,** select "Bimodal Distributions" and change the settings from "only when user forces bimodal" to "whenever a unimodal result score is less than". We recommend values of 0.9 and 0.0 respectively in the two numeric field that activate. Note that if you have a project loaded when you change these values, deuterated results in your project will be recalculated.

**To show the individual distributions in the spectrum view,** select "Spec/Chrom Plots" in the and check the box "show individual distributions when bimodal". By default HDExaminer will show the actual MS data in red and the theoretical isotope cluster in blue. For bimodal results, the blue theoretical cluster is the sum of the two underlying distributions that best matches the actual data. If you check this box, HDExaminer will also show those two underlying distributions in the colors specified (orange and green by default).

**To turn on the bimodal fields in the Analysis View results table,** select "Deuteration Table" and select the fields you wish to see in the table:

- Experimental Centroid and Exp. Centroid 2: for bimodal results, Experimental Centroid reports the centroid of the left distribution, and Exp. Centroid 2 reports the centroid of the right distribution.
- Bimodal Dist. L/R ratio: the ratio of the total intensity of the left distribution to the total intensity of the right one.
- Bimodal Dist. Left and Right Percent: these fields report the total intensity of the left and right distributions, but expressed as a percentage of the total. These two fields will always sum to 100%. Note also that these fields express the same information as the L/R ratio field, but in a different way.
- Theor. Centroid 2, Theor. L/R Ratio, Theor. Bimodal Left/Right Percent: these fields are identical to the corresponding Experimental fields described above, but they are calculated based on the theoretical isotope clusters rather than the actual MS data.
- Centroid Difference, Centroid Difference 2: for bimodal results, Centroid Difference reports the centroid difference of the left distribution and Centroid Difference 2 reports the difference of the right distribution.
- # Deuterons, # Deuterons 2, Deuteration %, Deuteration % 2: as above, these fields report data for the left and right distributions, respectively.

## Calculating Bimodals

If bimodal processing is enabled, then for each deuterated result, the software checks to see if its fit quality score is less than the value specified under Tools – Options – Bimodal Distributions. If so, the software will try finding a bimodal distribution that fits the data better than the unimodal distribution did.

By default, the software will accept any bimodal result that gives a better score. However, you can specify a stricter cutoff in the second numeric field under Tools – Options – Bimodal Distributions. By entering a positive number, you can tell the software to only accept a bimodal

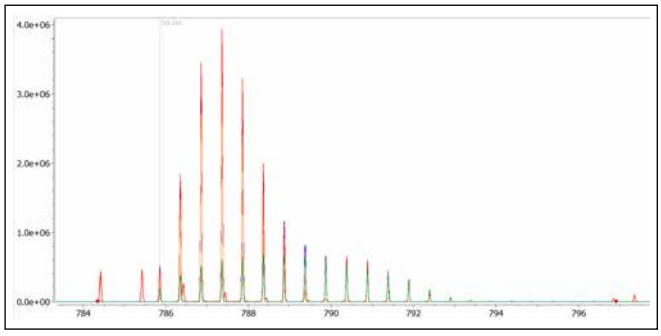
distribution if its score is a certain amount better than the unimodal score. Conversely, you can enter a negative number here to tell HDExaminer to accept a bimodal distribution even if that result's score is slightly worse than the unimodal one.

Any calculated result can be forced to be unimodal or bimodal, even if the software's automated algorithms found something different. To force a result to be unimodal or bimodal, select that result in the Analysis View table, right-click and select "Force unimodal" or "Force bimodal" as appropriate.

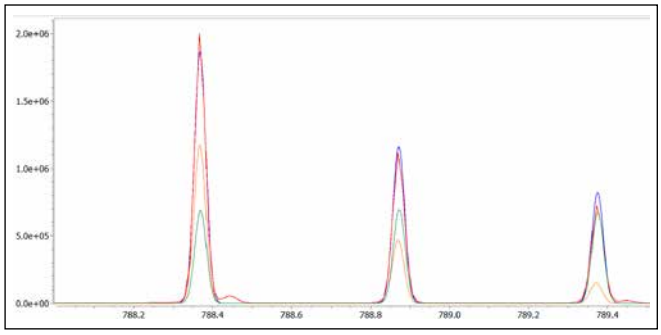
## Bimodals in the Analysis View

When browsing results in the Analysis View, bimodal results will include values for the bimodal columns in the results table. For an explanation of each of these bimodal columns, see "Getting Started" above.

If you have selected "show individual distributions when bimodal" under Tools – Options – Spec/Chrom Plots, then the spectrum view will show both distributions, like this:

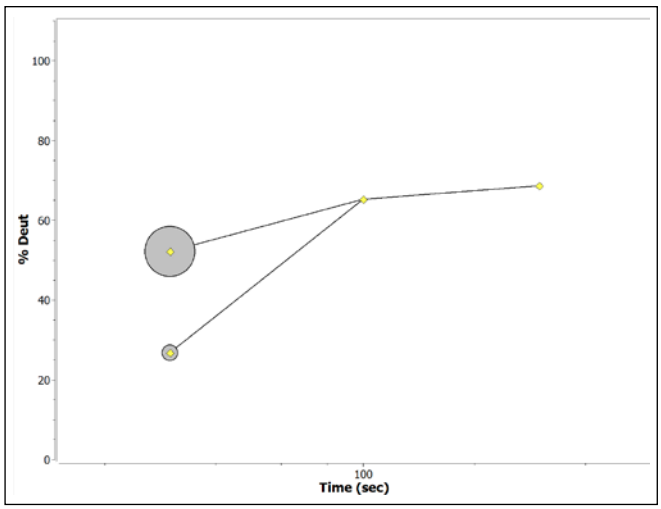



Note the presence of orange and green traces showing the two separate distributions. For high-resolution data, it is useful to zoom in by left-dragging over an m/z range to see the separate red, blue, orange and green traces:




## Bimodal Uptake Plots

At the peptide level, HDExaminer shows bimodal results by drawing "doubled" uptake plots, as often seen in the HDX literature. For example:

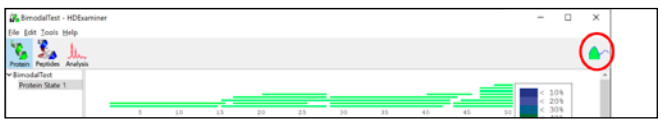



The yellow diamonds show the individual %D results, as usual. The gray circles show the relative intensities of the left (lower) and right (higher) distributions in the MS data. So, this uptake plot indicates that the selected peptide has a bimodal distribution at the first time point, with a higher-mass distribution that is quite a bit larger than the lower-mass one. It also shows that the peptide's results converge to a unimodal distribution for the second and third time points.

## Bimodal Results in Protein-Level Plots

Visualizations such as the butterfly plot, volcano plot and heat map do not readily lend themselves to concise and intuitive representations of bimodal data. For this reason, HDExaminer simply focuses on one distribution at a time (left or right) and draws protein-level plots as though only one of the two distributions exists.

When the currently-open project includes bimodal results, the bimodal toggle switch will appear in the upper right of the HDExaminer window:



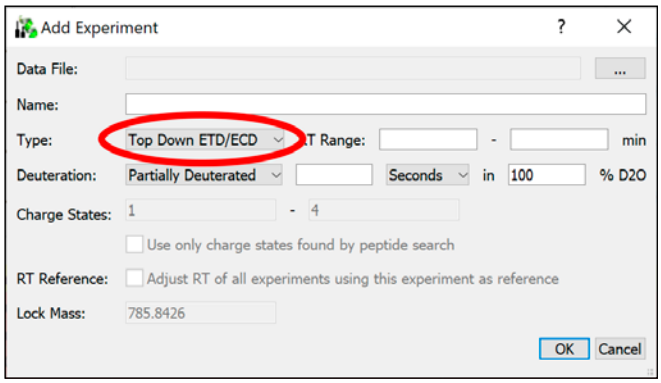
To control which distribution is shown in protein-level views, simply toggle the switch back and forth between left and right. The peak highlighted in green in the toggle switch is the one currently being shown.

Note that the toggle switch affects the heat map, the butterfly plot, the residual plot, the Woods plot, the chiclet plot and the volcano plot. Other views are completely unaffected by the state of the toggle switch. When exporting data or images from HDExaminer, the data exported will be based on the current state of the toggle switch.

In a Top Down HD Exchange experiment, your sample is deuterated, then intact protein is injected into your mass spectrometer, where it is fragmented via ETD or ECD. With proper low-energy settings, this technique can fragment the protein and avoid “scrambling” of deuterium.

To analyze Top Down data in HDExaminer, you will need your protein sequence, an undeuterated control and a time course of MS/MS data files for varying deuteration times. HDExaminer can read either LC-MS/MS data or spectra from a direct infusion. To analyze your Top Down data, follow these steps:

1. Create a new project in HDExaminer and import your protein sequence(s) as normal.
2. Skip the Peptides View and go straight to the Analysis View.
3. Import your undeuterated and deuterated experiment files by right-clicking on the tree view and selecting “Add Experiment...”
4. When selecting an MS/MS data file, ensure that the MS menu in the upper right of the browse dialog is blank and the MS/MS menu contains the MS/MS data you wish to import.
5. Ensure that you specify that each of your MS/MS data files is Top Down by selecting “Top Down ETD/ECD” in the “Type” popup menu:




6. In the “RT Range” fields, enter the retention time range that corresponds to the range of your MS/MS data containing the spectra of interest. By default, the range is the entire retention time range of the data file you are loading.
7. Select whether the sample is Undeuterated, Partially Deuterated, Fully Deuterated or Blank, as usual.
8. If the file you are loading is an undeuterated sample, enter the change state range that HDExaminer should consider for each protein fragment.

The calculations HDExaminer uses when analyzing Top Down data are very similar to those used to analyze Bottom Up data. The main difference is that instead of searching for the y-ions corresponding to the peptides you specified in your Peptide Pool, it searches for all possible c-ions and z-ions for your protein whose theoretical isotope clusters fall within the mass range of your imported MS/MS data. As with Bottom Up data, HDExaminer uses an isotope cluster modeling algorithm and finds the best match it can between theoretical and actual isotope clusters.

For both Top Down and Middle Down data, HDExaminer takes into account that M-1 and M+1 ions can be present. If they are, then the software assumes that the relative abundances of M-1, M and M+1 ions remains constant between all undeuterated and deuterated experiments for a given peptide.

After HDExaminer has finished calculating results, you can browse them similarly to Bottom Up results. If you go to the Peptides View, you will see that instead of “Pool 1”, your Peptide Pool will be called “Top Down Fragments”. This “virtual peptide pool” is automatically generated and contains all c-ion and z-ion fragments of your protein that could potentially be found in the m/z range of your data. You may analyze any of these fragments as though they were peptides in a Bottom Up HDExaminer project. Uptake Plots, the Peptide Plot and the Heat Map all work the same way, but with these fragment ions taking the place of digested peptides.

In a Middle Down HD Exchange experiment, you do ETD or ECD fragmentation on every peptide in your LC/MS run. This could be a small list of large peptides, or even every peptide in a full peptide digest.

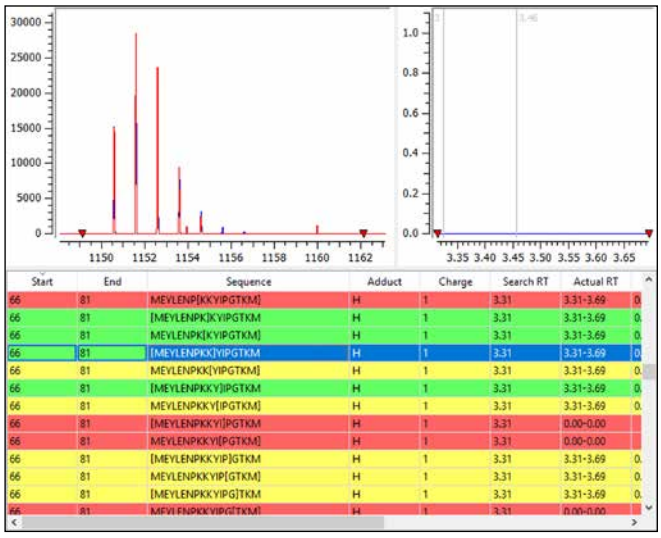
⚠ **Warning**

Analyzing Middle Down data for a project with a large peptide pool takes **substantially longer** than analyzing Bottom Up data for that same peptide pool!

## To analyze your Middle Down LC-MS/MS data, do the following:

1. Import your protein sequences and peptide sources, then create a peptide pool as normal.
2. Import LC-MS/MS data files that contain both MS data and data-directed MS/MS data. Be sure to select “Middle Down LC/MS” as the data type when importing your MS files.
3. HDExaminer will look for each peptide in the MS functions and will also look for every possible fragment of those peptides in the MS/MS functions.

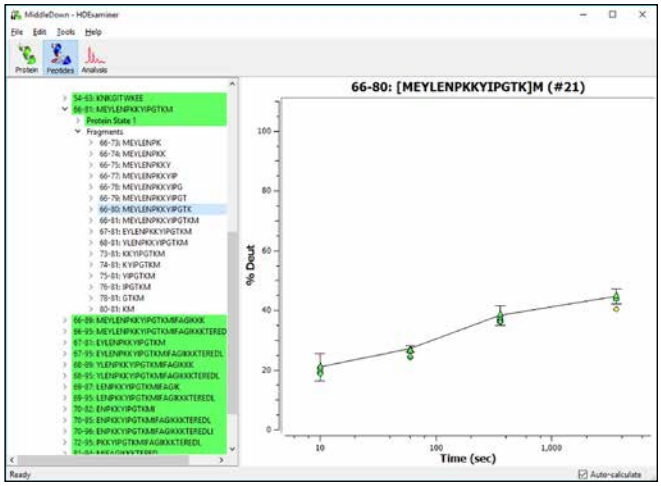
Fragment results look like this in the Analysis View:



| Start | End | Sequence | Adduct | Charge | Search RT | Actual RT | |
|---|---|---|---|---|---|---|---|
| 66 | 81 | MEYLENP[KKYIPGTKM] | H | 1 | 3.31 | 3.31-3.69 | 0. |
| 66 | 81 | [MEYLENPK]KYIPGTKM | H | 1 | 3.31 | 3.31-3.69 | 0. |
| 66 | 81 | MEYLENPK[KYIPGTKM] | H | 1 | 3.31 | 3.31-3.69 | 0. |
| 66 | 81 | [MEYLENPKK]YIPGTKM | H | 1 | 3.31 | 3.31-3.69 | 0. |
| 66 | 81 | MEYLENPKK[YIPGTKM] | H | 1 | 3.31 | 3.31-3.69 | 0. |
| 66 | 81 | [MEYLENPKKY]IPGTKM | H | 1 | 3.31 | 3.31-3.69 | 0. |
| 66 | 81 | MEYLENPKKY[IPGTKM] | H | 1 | 3.31 | 3.31-3.69 | 0. |
| 66 | 81 | [MEYLENPKKYI]PGTKM | H | 1 | 3.31 | 0.00-0.00 | |
| 66 | 81 | MEYLENPKKYI[PGTKM] | H | 1 | 3.31 | 0.00-0.00 | |
| 66 | 81 | [MEYLENPKKYIP]GTKM | H | 1 | 3.31 | 3.31-3.69 | 0. |
| 66 | 81 | MEYLENPKKYIP[GTKM] | H | 1 | 3.31 | 3.31-3.69 | 0. |
| 66 | 81 | [MEYLENPKKYIPG]TKM | H | 1 | 3.31 | 3.31-3.69 | 0. |
| 66 | 81 | MEYLENPKKYIPG[TKM] | H | 1 | 3.31 | 0.00-0.00 | |

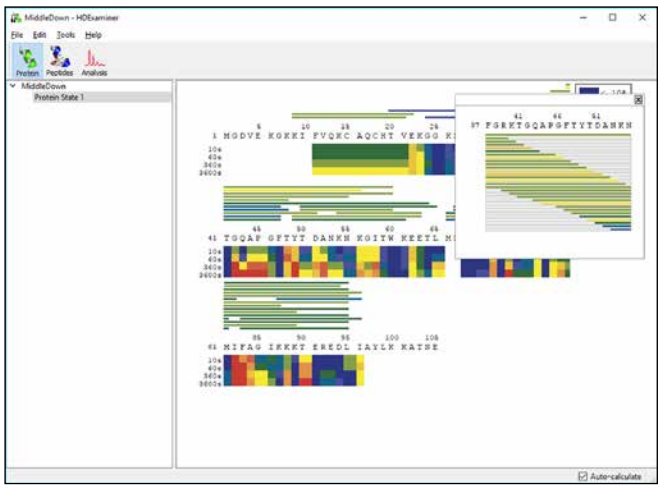
In the results table, the sequence column shows the sequence of the parent peptide. The portion of that sequence inside square brackets shows the fragment of that peptide found in the MS/MS data. Selecting a result shows the isotope cluster as normal. The XIC view remains blank, since an XIC of an isotope cluster is impossible to calculate properly from a data-directed MS/MS function.

In the Peptides View, fragment results appear under their parent peptides:




Individual results appear under fragments and can be explored as usual. Note that peptide pool plots such as the butterfly plot, residual plot, Woods plot, chiclet plot and volcano plot only take intact peptides into account, not peptide fragments.

In the heat map, all peptides and fragments are taken into account. Clicking on a peptide coverage bar shows all of that peptide’s fragments:



Clicking on a fragment takes you to that fragment’s uptake plot in the Peptides View.

When creating an HDExaminer project with multiple protein states, multiple peptide sources and many MS data files, it can be useful to automate the process. HDExaminer allows you to import text files in a special format for this purpose. The software can also be launched from a command line for automation convenience.

## Automation File Format

An HDExaminer automation file is a text file with an .hda extension. When you drag such a file onto HDExaminer, the software will automatically create and populate a new project with the data that you specify. Here is an example of an .hda file:

```
FastaFile: C:\MyData\WT.txt
PeptideFile: C:\MyData\Peptide List.csv
ProteinState: Control
0 Seconds: C:\MS Files\CONTROL 0 sec.d
30 Seconds: C:\MS Files\1 CONTROL 30s.d
30 Seconds: C:\MS Files\2 CONTROL 30s.d
30 Seconds: C:\MS Files\3 CONTROL 30s.d
240 Seconds: C:\MS Files\1 CONTROL 240 sec.d
240 Seconds: C:\MS Files\2 CONTROL 240 sec.d
240 Seconds: C:\MS Files\3 CONTROL 240 sec.d
1800 Seconds: C:\MS Files\1 CONTROL 1800s.d
1800 Seconds: C:\MS Files\2 CONTROL 1800 sec.d
1800 Seconds: C:\MS Files\3 CONTROL 1800 sec.d
14400 Seconds: C:\MS Files\1 CONTROL 14400 sec.d
14400 Seconds: C:\MS Files\2 CONTROL 14400 sec.d
14400 Seconds: C:\MS Files\3 CONTROL 14400 sec.d
ProteinState: Bound
30 Seconds: C:\MS Files\1 Bound 30 sec.d
30 Seconds: C:\MS Files\2 Bound 30 sec.d
30 Seconds: C:\MS Files\3 Bound 30 sec.d
240 Seconds: C:\MS Files\1 Bound 240 sec.d
240 Seconds: C:\MS Files\2 Bound 240 sec.d
240 Seconds: C:\MS Files\3 Bound 240 sec.d
1800 Seconds: C:\MS Files\1 Bound 1800 sec.d
1800 Seconds: C:\MS Files\2 Bound 1800 sec.d
1800 Seconds: C:\MS Files\3 Bound 1800 sec.d
14400 Seconds: C:\MS Files\1 Bound 14400 sec.d
14400 Seconds: C:\MS Files\2 Bound 14400 sec.d
14400 Seconds: C:\MS Files\3 Bound 14400 sec.d
```

An automation file consists of carriage-return-separated lines, each of which begins with a tag followed by a colon. When file names are specified in an automation file, those file names must be complete path names.

Several tags are recognized in the .hda format:

**FastaFile:** this tag specifies the location of the FASTA file to be imported. If multiple FASTA files are listed in an .hda file, all but the last one will be ignored.

**PeptideFile:** this tag specifies the location of a Peptide Source file to import. If an .hda file contains multiple PeptideFile tags, all of the files will be imported. If all of the imported Peptide Source files contain columns labeled “Sequence”, “Charge” and “RT”, then those Peptide Sources will automatically be imported into a single Peptide Pool, so that calculations will begin as soon as the .hda file is imported.

**ProteinState:** this tag tells HDExaminer to create a protein state with the specified name. Subsequent MS data file import tags will be imported to the most recently-specified protein state.

**Experiment descriptor:** any line that begins with a time followed by a colon is treated as an MS data file to be imported with the specified deuteration time. The time syntax is a number followed by a specifier such as “Seconds”, “sec”, “Minutes”, “min” or “Hours”. Any time string specifying no deuteration time, such as “0 sec”, indicates an undeuterated experiment. The special time string of “FD” (without a number) may be used to specify a fully-deuterated control experiment file. The special time string “Blank” may be used to specify a blank experiment file.

An experiment descriptor can also include other information after the time string but before the colon. These fields must be separated by commas and may include % D2O buffer and lock mass m/z, as in the following example:

```
30 Seconds, 50% D2O, Lock Mass 785.8426: C:\MS Files\1 Bound 30 sec.d
```

The experiment descriptor field is not case-sensitive. Uppercase and lowercase letters may be used interchangeably.

## Importing from a Chronos Log File

The Chronos automation software by Trajan Automation allows you to set up an HDX-MS run with a Trajan Automation autosampler. At the end of such a run, you may import the log file generated by Chronos to import the acquired MS files directly into HDExaminer. To do this, first launch HDExaminer and set up your protein sequence and peptide pool normally. After this, simply drag your Chronos log file

onto HDExaminer. The acquired MS files will be imported automatically.

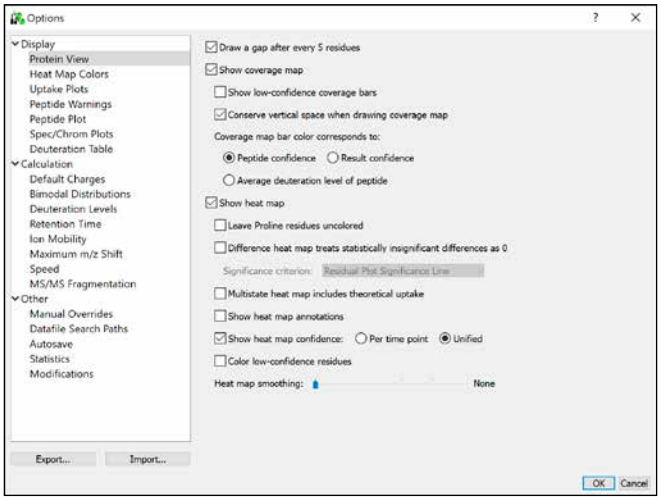
## Launching HDExaminer from a Command Line

You can launch HDExaminer from a Windows command line. When you do this, you may specify any number of files as arguments to the HDExaminer.exe command. HDExaminer will launch and will behave just as it would have if you had launched HDExaminer, then dragged all of the specified files onto your HDExaminer window.

This section explains the various user-definable settings that affect HDExaminer's behavior. The options dialog is available by selecting "Options…" under the Tools menu on the main menu bar.

In the bottom left of the options dialog are "Export…" and "Import…" buttons. These buttons may be used to export your HDExaminer settings to a file for easy transfer to another computer.

## Protein View




**Draw a gap after every 5 residues:** by default, HDExaminer will draw your protein sequence in groups of 5 residues, with a gap after each group. If you would like to eliminate these gaps, uncheck this box.

**Show coverage map:** if checked, HDExaminer will show peptide coverage bars on the Protein View.

**Show low-confidence coverage bars:** if checked, HDExaminer will show coverage bars for low-confidence peptides on the Protein View.

**Conserve vertical space when drawing coverage map:** if checked, HDExaminer will fit all coverage bars into as little vertical space as it can. If unchecked, HDExaminer will draw the coverage bars in a more traditional format, sorted by start and end residues.

**Coverage map bar color corresponds to:** By default, HDExaminer will color coverage bars by confidence; green for high, yellow for medium, and red for low. If you select "Average deuteration level of peptide", coverage bars will instead be colored by average deuteration level, using the same color key as the heat map. If you select "Result confidence", coverage bars will be colored based on their undeuterated result confidence (this corresponds to the algorithm used in HDExaminer 2.0 and before).

**Show heat map:** if checked, HDExaminer will show heat bars on the Protein View.

**Leave Proline residues uncolored:** by default, HDExaminer will color proline residues based on the colors of their neighbors. If you would like HDExaminer to leave all proline residues blank, check this box.

**Difference heat map treats statistically significant differences as 0:** if this is checked, then any peptide that does not have a statistically significant difference between the two protein states being compared will be treated in the heat map calculation as though it had a difference of 0 between the two states. The significance criterion can be the confidence intervals shown in the peptide's uptake plot, the significance line used in the residual plot, or the significance lines used in the volcano plot. See "Comparing Protein States – Analyzing Results" on page 35 for more information.

**Multistate heat map includes theoretical uptake:** if this is checked, then when you display a multistate heat map for a given time point, that heat map will include a row for the theoretical uptake at each residue, calculated using the intrinsic hydrogen exchange rates described in Nguyen et al., J. Am. Soc. Mass Spectrom. (2018) 29: 1936. The temperature and pDcorr used in the calculation are the ones specified in the Uptake Plot options (see page 47).

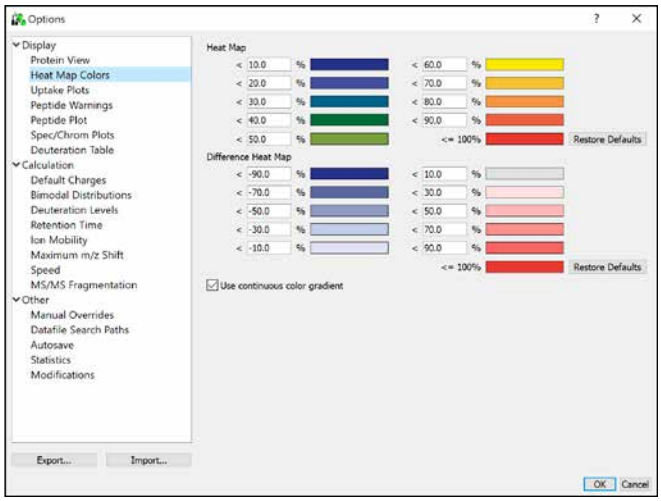
**Show heat map annotations:** if this is checked, then you may annotation your protein sequences with colored text attached to individual residues. For more information, see "Heat Map Annotations" on page 32 for more information.

**Show heat map confidence:** if this is checked, then HDExaminer will calculate a confidence for each residue of your proteins and display that as either a row above each time point row (“per time point”) or as a single row at the top of the heat map (“unified”). For more information, see “Heat Map Confidence” on page 31 for more information.

**Color low-confidence residues:** if this is unchecked, then low-confidence residues will be omitted from the heat map. Including low-confidence residues can give you a misleading picture of your protein’s deuteration behavior, so it is highly recommended that you leave this box unchecked.

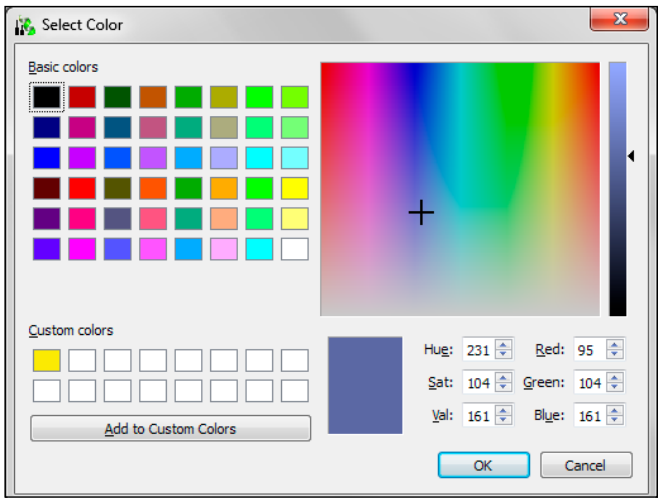
**Heat map smoothing:** this option ranges from “None” to “Very Heavy” and determines the extent to which HDExaminer will alter its heat map calculation algorithm to ensure that neighboring residues are colored similarly. Smoothing can give you a misleading picture of your protein’s deuteration behavior, so it is highly recommended that you leave this set to “None”.

## Heat Map Colors




This view allows you to override the colors that HDExaminer uses to draw heat maps and difference heat maps. You can specify the deuteration percentage used for each range by adjusting the numbers.

If you click on any of the colors, you will see the following:

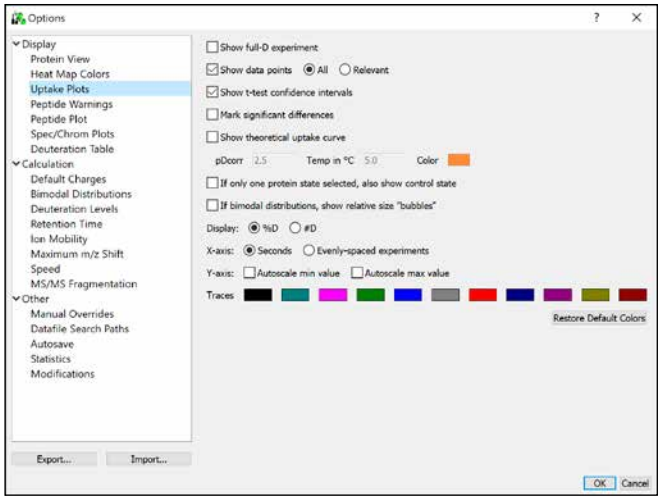



In this window, you may select a color, or enter HSV or RGB values to specify a color.

You may also return HDExaminer to default colors and ranges by pressing the “Restore Defaults” button.

**Use continuous color gradient:** if this is checked, then the heat map will smoothly interpolate between the colors specified here rather than using only the specified colors.

## Uptake Plots




**Show full-D experiment:** if this box is checked, then the deuteration level of the fully-deuterated experiment will be included when drawing uptake plots. If it is not checked, then only the partially-deuterated experiments will be shown.

**Show data points:** if this box is checked, then data points will be shown on uptake plots, color-coded by confidence and shape-coded by charge state. If it is unchecked, the individual data points will be hidden. The “All” and “Relevant” buttons determine which data points will be drawn. If “All” is selected, then all medium- and high-confidence results will be drawn. If “Relevant” is selected, then only the results which contributed to the uptake plot will be drawn. Whether or not a result contributes to the uptake plot is determined by the settings under “Deuteration Levels”, described on page 51.

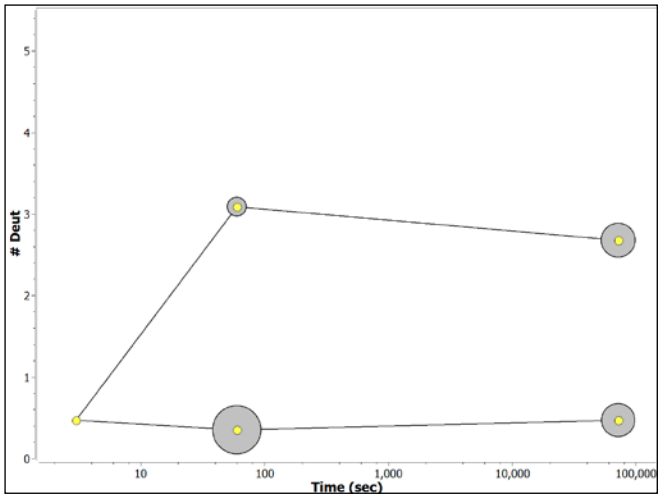
**Show t-test confidence intervals:** if this box is checked, then confidence interval bars will be drawn on uptake plots wherever two or more data points are available. The confidence interval bars are drawn by calculating a Student’s t-distribution from replicates for the best charge state. If this box is unchecked, confidence interval bars will not be drawn.

**Mark significant differences:** if this box is checked, then when an uptake plot is displaying exactly two protein states and the results at a particular time point show a statistically significant difference, those results will be marked with an asterisk. Moving your mouse cursor over the asterisk will show you the corresponding p-value.

**Show theoretical uptake curve:** if this box is checked, then all uptake plots will include the theoretical uptake curve for the current peptide or fragment, using the intrinsic hydrogen exchange rates described in *Nguyen et al., J. Am. Soc. Mass Spectrom. (2018) 29: 1936.* The pDcorr and temperature parameters are required for calculating these theoretical curves, so you must be sure to set these to properly match your experimental conditions.

**If only one protein state selected, also show control state:** if this box is checked and you select a single protein state to view its uptake plot, then HDExaminer will also draw the uptake plot of the control protein state for comparison purposes.

**If bimodal distributions, show relative size “bubbles”:** if the current peptide or fragment includes results with bimodal distributions, then a dual uptake curve will be drawn, like this one:




If you select this checkbox to show the bimodal “bubbles”, then the gray circles shown above will be displayed to show the relative intensity between the lower and higher distributions at each deuteration time.

**Display:** this setting determines whether uptake plots will show a peptide’s deuteration level as a percentage (%D) or as an absolute number of deuterons (#D).

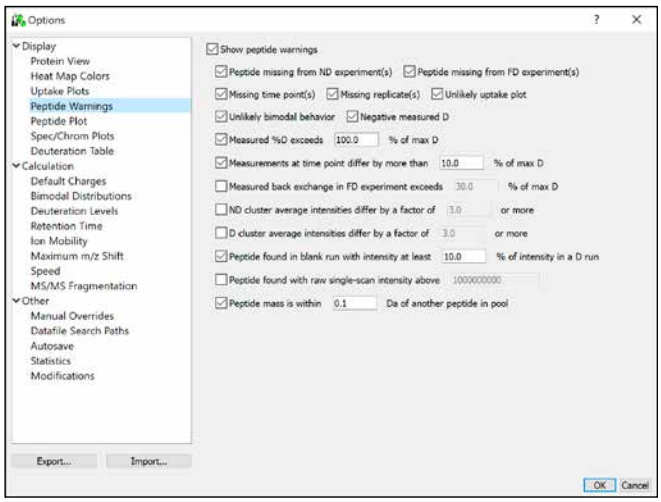
**X-axis:** if set to “seconds”, the uptake plot’s x-axis will be deuteration time, in seconds. If set to “evenly-spaced experiments”, your experimental time points will be evenly spaced along the x-axis.

**Y-axis, Autoscale min value:** if this box is checked, then the minimum y-axis value for uptake plots will be based on the lowest value to be plotted. If it is unchecked, then the minimum y-axis value will be 0, or the minimum calculated y value, whichever is lower.

**Y-axis, Autoscale max value:** if this box is checked, then the maximum y-axis value for uptake plots will be based on the highest value to be plotted. If it is unchecked, then the maximum y-axis value will be either 110% (when plotting %D) or 1.1 times the theoretical maximum deuteration level (when plotting #D).

**Traces:** your protein states’ uptake plots will be drawn in the specified colors. Colors are used from left to right, so your first protein state will use the leftmost color. Clicking on any color button allows you to change that color. Clicking on the “Restore Default Colors” button will restore all of the color buttons to their default values.

## Peptide Warnings




**Show peptide warnings:** if this box is checked, then the selected peptide warnings will be shown in the Peptides View. If it is unchecked, no warnings will be shown.

**Peptide missing from ND experiment(s):** if this box is checked, then a warning will be displayed for any peptide whose isotope cluster could not be found in one or more of your project's undeuterated experiments.

**Peptide missing from FD experiment(s):** if this box is checked, then a warning will be displayed for any peptide whose isotope cluster could not be found in one or more of your project's fully deuterated experiments.

**Missing time point(s):** if this box is checked, then a warning will be displayed for any peptide whose isotope cluster could not be found in any deuterated experiments for some time point.

**Missing replicate(s):** if this box is checked, then a warning will be displayed for any peptide whose isotope cluster could not be found in one or more deuterated experiments for some time point.

**Unlikely uptake plot:** if this box is checked, then a warning will be displayed for any peptide whose uptake plot includes a drop of more than 10% of max D from one time point to the next.

**Unlikely bimodal behavior:** if this box is checked, then a warning will be displayed for any peptide whose uptake plot appears to be bimodal, then unimodal, then bimodal again over the time course.

**Negative measured D:** if this box is checked, then a warning will be displayed for any peptide whose deuteration level drops below 0 at any time point.

**Measured %D exceeds X% of max D:** if this box is checked, then a warning will be displayed for any peptide whose deuteration exceeds the given percentage of max D.

**Measurements at time point differ by more than X% of max D:** if this box is checked, then a warning will be displayed for any peptide whose measurements at some time point differ from each other by more than the given percentage of max D.

**Measured back exchange in FD experiment exceeds X% of max D:** if this box is checked and your project includes one or more fully-deuterated experiments, then a warning will be displayed for any peptide that shows a back exchange percentage greater than the given percentage.

**ND cluster average intensities differ by a factor of X or more:** if this box is checked and your project includes more than one undeuterated experiment, then a warning will be displayed for any peptide for which the maximum raw MS intensity of the isotope cluster, averaged over the scans summed for that result, differs by more than the given factor between any two of your ND replicates.

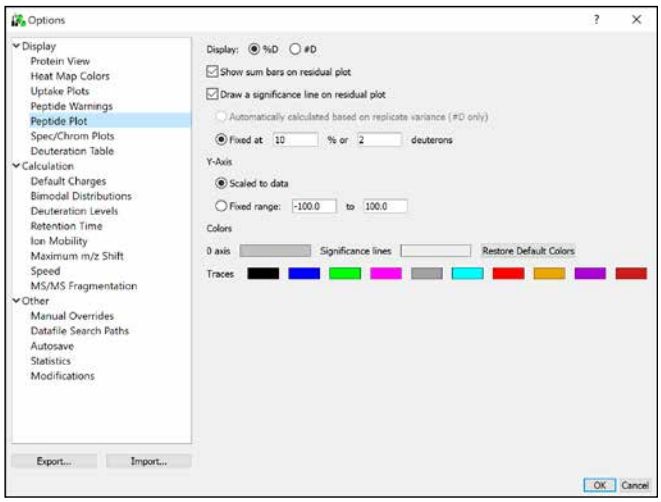
**D cluster average intensities differ by a factor of X or more:** if this box is checked, then a warning will be displayed for any peptide for which the maximum raw MS intensity of the isotope cluster, averaged over the scans summed for that result, differs by more than the given factor between any two time points or between any two replicates within a single time point.

**Peptide found in blank run with intensity at least X% of intensity in a D run:** if this box is checked and your project contains blank experiments, then a warning will be displayed for any peptide whose single-scan maximum raw MS intensity in any blank experiment exceeds the given percentage of the corresponding single-scan maximum raw MS intensity in any time point experiment.

**Peptide found with raw single-scan intensity above X:** if this box is checked, then a warning will be displayed for any peptide whose single-scan maximum raw MS intensity exceeds the given value in any experiment.

**Peptide mass is within X Da of another peptide in pool:** if this box is checked, then a warning will be displayed for any peptide with a mass within the given tolerance of some other peptide in the currently-selected peptide pool.

## Peptide Plot




**Display:** this selection determines whether the butterfly plot and residual plot use deuteration percentage (%D) or absolute number of deuterons (#D) as their y-axis.

**Show sum bars on residual plot:** if checked, the residual plot will show a vertical bar at each peptide indicating the total difference between the selected protein states measured for that peptide at all time points.

**Draw a significance line...:** if checked, a residual plot will include a faint horizontal line showing a significance level.

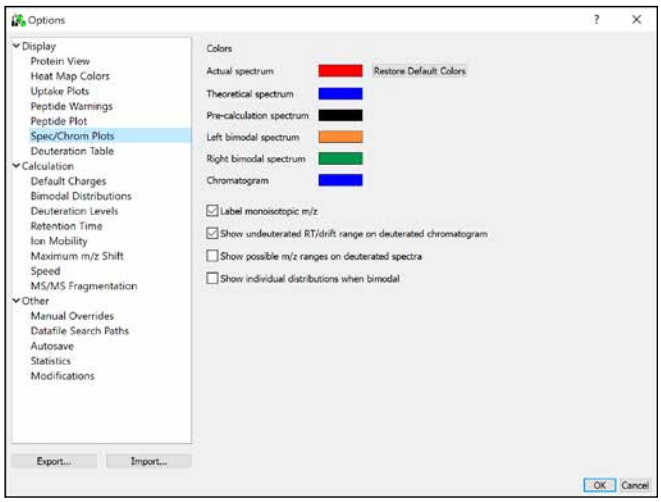
**Automatically calculated based on replicate variance:** this setting tells HDExaminer to automatically calculate a significance level based on replicate variance across your entire project. Note that this option is disabled if your display setting is %D.

**Fixed at:** this tells HDExaminer to draw a fixed significance line at the level you specify. If the Display setting is %D, then the significance line will be drawn at the given percentage. If the Display setting is #D, then the significance line will be at the specified deuteration level instead.

**Y-Axis:** by default, the peptide plot's y-axis is scaled to the data being plotted. You may override this behavior by selecting "Fixed range" and manually entering the range to be used for the y-axis for all peptide plots.

**Colors:** these buttons allow you to change the colors used to draw the Peptide Plot. Clicking on any color button allows you to change that color. Clicking the "Restore Default Colors" button returns all colors to default values.

## Spec/Chrom Plots




**Colors:** these buttons allow you to change the colors used to draw traces on spectrum and chromatogram plots throughout the software. The "Restore Default Colors" button returns all colors to default values.

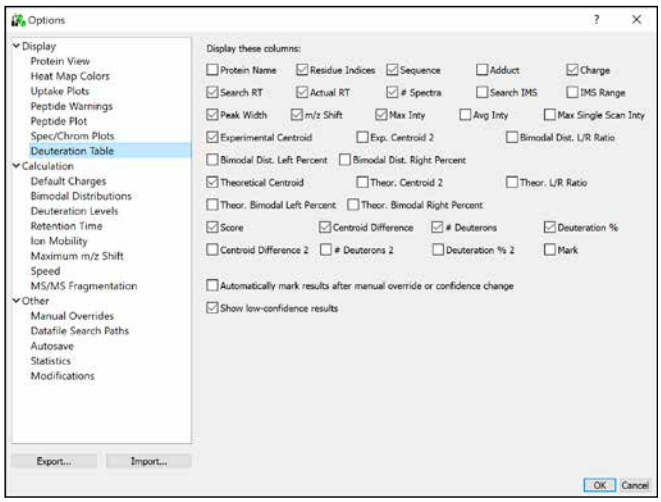
**Label monoisotopic m/z:** if checked, the monoisotopic m/z value will be labeled in grey on all spectrum plots.

**Show undeuterated RT/drift range on deuterated chromatogram:** if checked, then the current peptide's RT/drift range on the undeuterated chromatogram will be indicated by grey lines on all corresponding deuterated chromatogram plots.

**Show possible m/z ranges on deuterated spectra:** if checked, then deuterated spectrum plots will show a faint grey bar at each m/z range that could potentially contain an isotopic peak.

**Show individual distributions when bimodal:** if checked, then a spectrum view for a bimodal distribution will show the actual spectrum, the two individual theoretical distributions, and the combined theoretical distribution.

## Deuteration Table

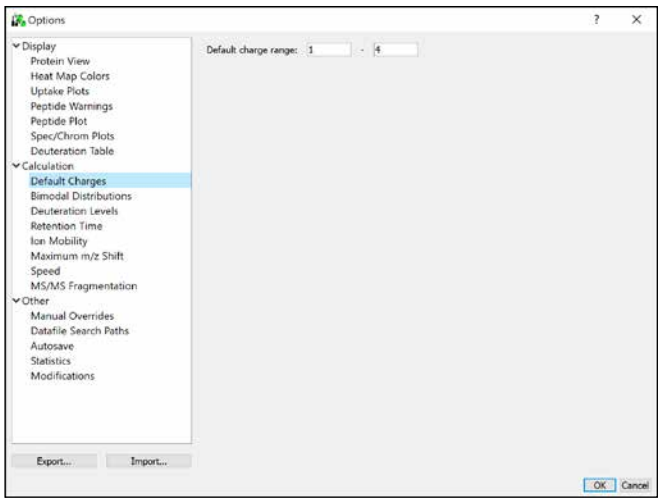



**Display these columns:** these checkboxes determine which columns are shown in the deuteration results table in the Analysis View.

**Automatically mark results after manual override or confidence change:** if checked, HDExaminer will automatically set the "Mark" checkbox on a result after you perform any manual override on that result. See "The Results Table" on page 25 for more information.

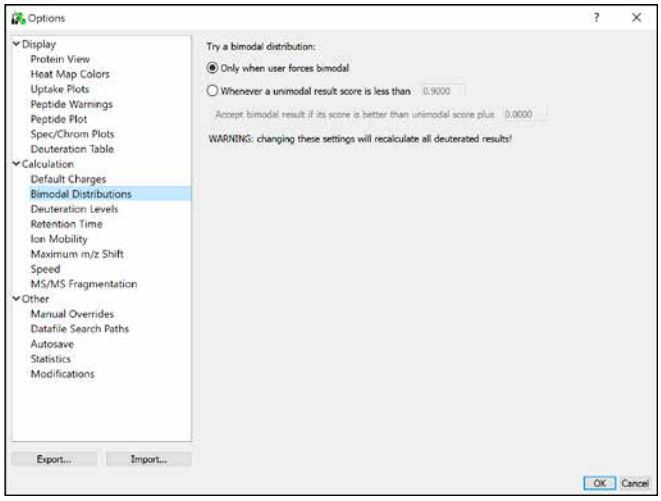
**Show low-confidence results:** if checked, low-confidence results will be shown in the deuteration results table.

## Default Charges




These fields determine the charge state range that HDExaminer will search for by default. Note that selecting "Use only charge states found by peptide search" when importing your undeuterated MS experiment will override these settings.

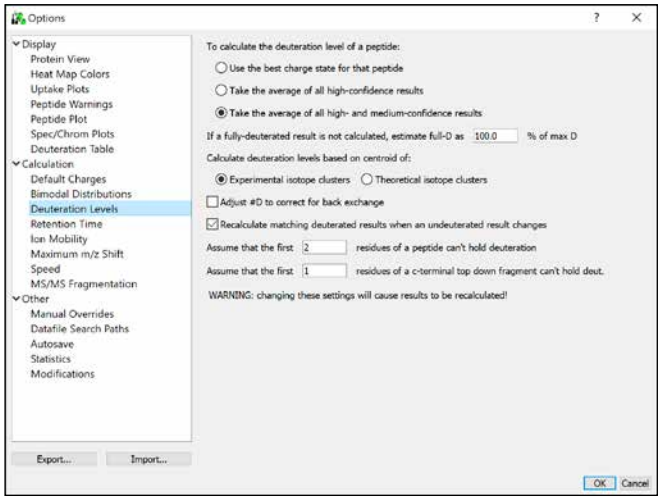
## Bimodal Distributions




**Try a bimodal distribution:** there are two possible selections. "Only when user forces bimodal" tells the software to only use unimodal distributions when matching peptides. "Whenever a unimodal result score…" tells the software to first try a unimodal distribution, but to attempt a bimodal distribution if the unimodal result's score is less than the specified number.

**Accept bimodal result…:** HDExaminer will report a bimodal result only if its score is at least the given amount *better than* the corresponding unimodal result. Note: you may enter a negative value in this box, in which case HDExaminer will report the bimodal result even if it is *slightly worse* than the corresponding unimodal result.

## Deuteration Levels




**To calculate the deuteration level of a peptide:** this setting determines how HDExaminer

aggregates multiple charge states and replicates for a peptide into a single deuteration level at each time point.

- **Use the best charge state for that peptide:** if this is selected, HDExaminer finds the charge state for each peptide that has the highest-quality results over the most time points and averages the results for only that charge state when calculating the peptide's deuteration level.
- **Take the average of all high-confidence results:** if this is selected, HDExaminer takes the average of the deuteration levels determined by high-confidence results.
- **Take the average of all high- and medium-confidence results:** if this is selected, HDExaminer takes the average of the deuteration levels determined by high- and medium-confidence results.

**If a fully-deuterated experiment is not calculated…:** when computing deuteration percentages for uptake plots and the heat map, HDExaminer needs a deuteration level that it can define as "one hundred percent". If your project contains a fully-deuterated experiment, then each result's corresponding fully-deuterated result defines this 100% level. If HDExaminer cannot find a fully-deuterated result for a certain peptide/charge state combination, then the 100% level for that peptide and charge is defined to be the given percentage of that peptide's theoretical maximum deuteration level.

**Calculate deuteration levels based on centroid of…:** if this is set to "Experimental isotope clusters," HDExaminer will calculate the centroid of an isotope cluster based on the experimental data. If it is set to "Theoretical isotope clusters" instead, HDExaminer will calculate the centroid of the theoretical isotope cluster that best matches the experimental data.

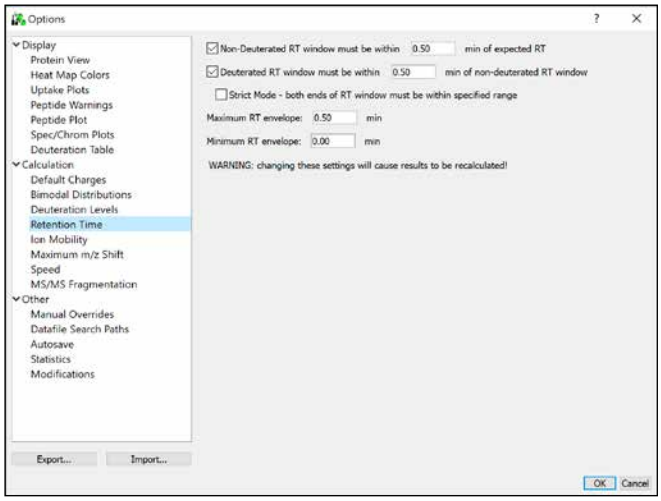
**Adjust #D to correct for back exchange:** by default, HDExaminer corrects for back exchange when reporting a deuteration percentage, but uses the raw centroid difference (without correction) when reporting the number of deuterons on a peptide. If you would like HDExaminer to correct for back exchange on the reported #D value as it does on the %D value, check this box.

**Recalculate matching deuterated results when an undeuterated result changes:** if checked, a change to an undeuterated result will automatically cause all corresponding deuterated results to be recalculated.

**Assume that the first X residues of a peptide…:** HDExaminer defines the maximum deuteration level for a peptide by removing the first X residues (as specified here), then counting the remaining non-proline residues. By default, HDExaminer sets this value to 2.

**Assume that the first X residues of a c-terminal top down fragment…:** This is similar to previous setting, but this setting affects top down c-terminal fragments instead of bottom up digested peptides. By default, HDExaminer sets this value to 1.

## Retention Time




To find a peptide in your undeuterated, bottom-up MS experiment, HDExaminer will start with a small window centered at the retention time specified in your Peptide Pool. Depending on your settings under Speed (see page 54), it may then "wander" away from that initial retention time window if doing so results in a higher-confidence fit. These settings determine the limits HDExaminer enforces when wandering in this way.

In general, you should use the smallest values you can here, taking into account your chromatographic quality, reproducibility and peak width.

**Non-Deuterated RT window…:** if this box is checked, then when calculating non-deuterated results, HDExaminer will never wander more than the specified number of minutes away from the expected retention time specified in your Peptide Pool.

**Deuterated RT window…:** if this box is checked, then when calculating deuterated results,

HDExaminer will never wander more than the specified number of minutes away from the retention time window at which it found the corresponding non-deuterated result. If you are using automatic retention time adjustment, the retention time window used will be based on the *adjusted* non-deuterated retention time.

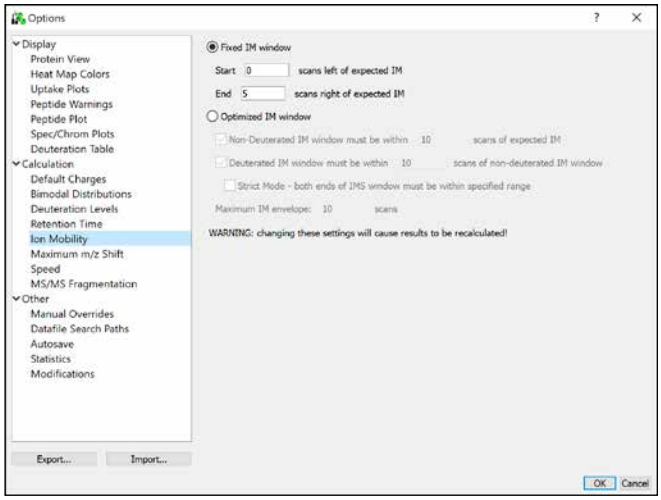
**Strict Mode:** by default, HDExaminer will allow a deuterated retention time window to be the specified distance away from the undeuterated window, even if the endpoint of the undeuterated window is that distance away from the starting point of the deuterated window (or vice versa). If this checkbox is set, then the *starting point* of the undeuterated window must be within the specified distance of the starting point of the deuterated window, and the endpoint of the undeuterated window must be within the specified distance of the *endpoint* of the deuterated window.

**Maximum RT envelope:** HDExaminer will never allow a result's retention time window to be wider than the number of minutes specified here.

**Minimum RT envelope:** HDExaminer will never allow a result's retention time window to be narrower than the number of minutes specified here.

*Note: specifying a retention time manual override for a result will cause HDExaminer to ignore these settings for that result.*

## Ion Mobility




These settings are very similar to the Retention Time settings described above, but allow you to specify the maximum number of scans away that HDExaminer is allowed to "wander" when looking for your peptide in ion mobility drift space. These settings allow you to also tell HDExaminer to not wander at all, but rather to use a fixed mobility range based on the mobility value imported from your peptide search results for each peptide.

**Fixed IM window:** if this is selected, HDExaminer will not attempt to optimize the ion mobility range for each result. Instead, it will calculate a mobility range by taking the specified number of scans to the left and right of the expected mobility value from your peptide search results. This option can give slightly less precise results, but can speed up calculation time considerably.

**Optimized IM window:** if this is selected, HDExaminer will start with an expected mobility range, but will then try other ranges to get the best possible result. (This was the default behavior in HDExaminer versions prior to 3.2.)

**Non-Deuterated IM window…:** if this box is checked, then when calculating non-deuterated results, HDExaminer will never wander more than the specified number of scans away from the expected mobility value specified in your Peptide Pool.

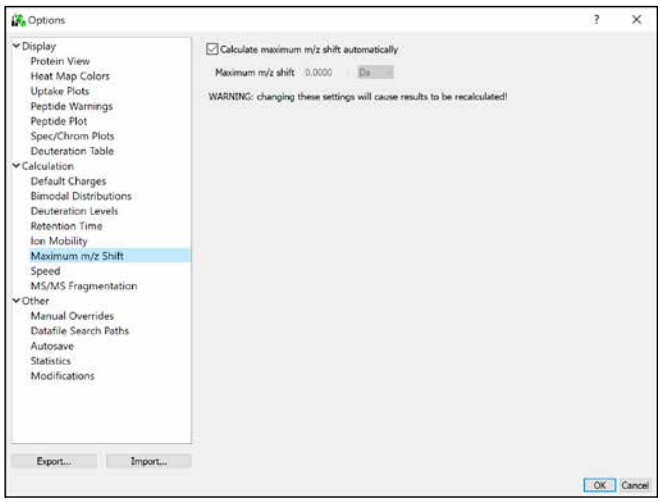
**Deuterated IM window…:** if this box is checked, then when calculating deuterated results, HDExaminer will never wander more than the specified number of scans away from the ion mobility window at which it found the corresponding non-deuterated result.

**Strict Mode:** by default, HDExaminer will allow a deuterated drift time window to be the specified distance away from the undeuterated window, even if the endpoint of the undeuterated window is that distance away from the starting point of the deuterated window (or vice versa). If this checkbox is set, then the starting point of the undeuterated window must be within the specified distance of the *starting point* of the deuterated window, and the endpoint of the undeuterated window must be within the specified distance of the *endpoint* of the deuterated window.

**Maximum IM envelope:** HDExaminer will never allow a result's ion mobility window to be wider than the number of scans specified here.

*Note: specifying an ion mobility manual override for a result will cause HDExaminer to ignore these settings for that result.*

## Maximum m/z Shift

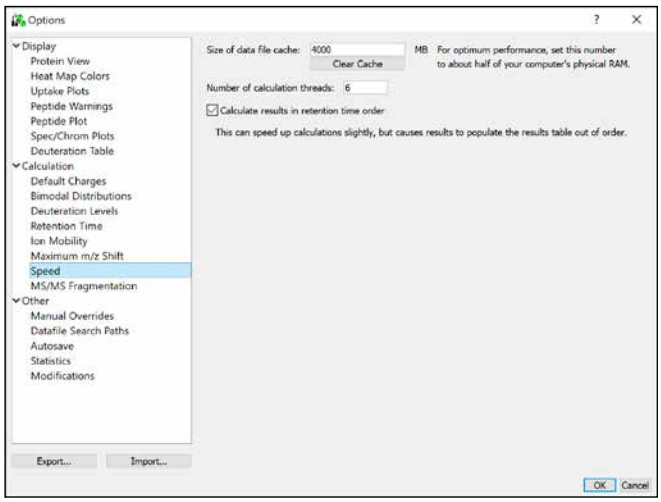



When computing theoretical isotope clusters, HDExaminer will allow the clusters to shift slightly to the left or right to compensate for imperfectly-calibrated data. You may wish to override the default settings if you believe that HDExaminer is shifting isotope clusters far enough for there to be a danger of peptide misidentification.

**Calculate maximum m/z shift automatically:** if this box is checked, HDExaminer will use its default algorithm, which estimates a maximum m/z shift based on the resolution of the data (higher resolution equals a smaller maximum m/z shift). If this box is unchecked, you may specify a maximum m/z shift yourself.

**Maximum m/z shift:** HDExaminer will never shift a theoretical isotope cluster by more than this amount to the left or right. You may specify the amount in either Daltons or PPM. Note that this field is disabled if the above checkbox is checked.

## Speed

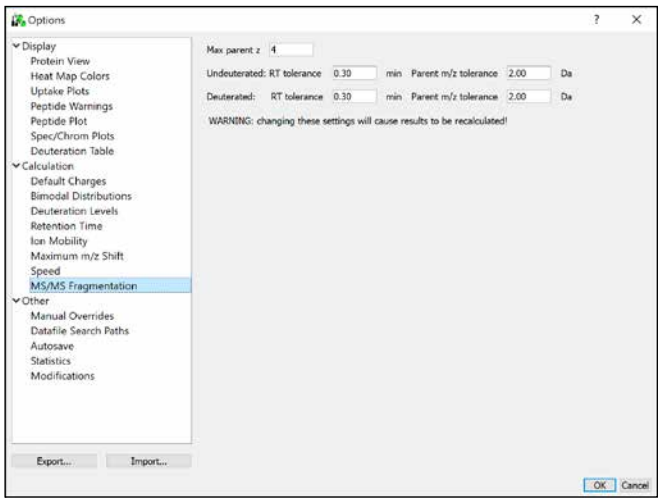



**Size of data file cache:** When HDExaminer does its calculations, it will cache spectra from the MS data file as it calculates. This can allow later calculations to be completed more quickly. This setting determines the amount of memory that HDExaminer will set aside for this spectrum cache. You may wish to experiment with various values to find the setting that maximizes performance. We recommend that you set this number to about half of your computer's physical RAM. HDExaminer will automatically reduce the size of the spectrum cache anytime it needs to do so to prevent low-memory conditions. If you would like to have HDExaminer clear the spectrum cache immediately, click the "Clear Cache" button. Clicking this button during calculations will not change any of your results, although it may make the calculations take longer to complete.

**Number of calculation threads:** HDExaminer creates multiple calculation threads to take advantage of multi-core systems. By default, it creates a number of threads equal to the number of cores on your system minus two. You may override that number here. You should lower this number if you will need to run other programs on your computer while HDExaminer is calculating results.

**Calculate results in retention time order:** by default, HDExaminer calculates results in retention time order. If you would like the software to calculate in protein sequence order instead, uncheck this box.

## MS/MS Fragmentation




These settings all pertain to Middle Down ETD analysis. See “Analyzing Middle Down ETD/ECD Data” on page 43 for more information.

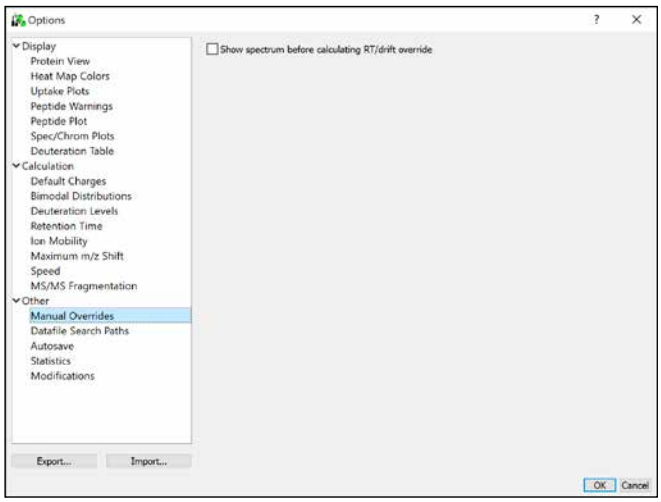
**Max parent z:** HDExaminer will search for appropriate parent scans at charge states up to the value specified here.

**RT tolerance:** a fragment scan in the MS/MS function must have a retention time within this many minutes of the retention time range of its parent peptide.

**Parent m/z tolerance:** a fragment scan in the MS/MS function must have a parent m/z that is within this many Daltons of the expected parent ion m/z.

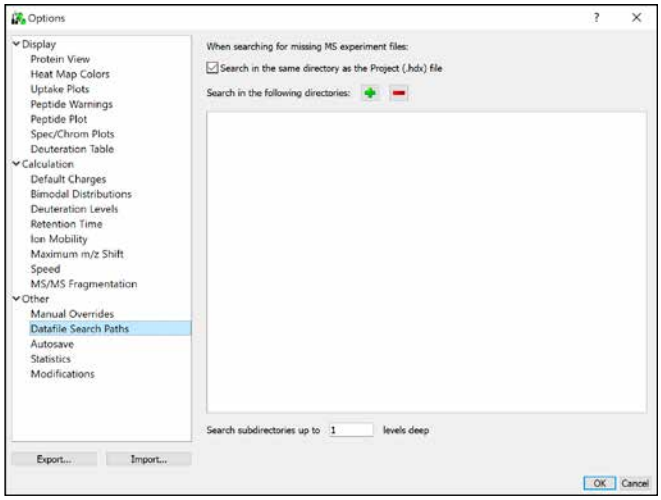
*Note that these last two settings may be set independently for both undeuterated and deuterated results.*

## Manual Overrides




**Show spectrum before calculating RT/drift override:** by default, specifying a retention time or ion mobility drift manual override will cause HDExaminer to automatically recalculate that result. If you check this box, then HDExaminer will instead sum up the spectra in the specified range and display the resulting spectrum in black. You may then decide whether to continue with calculations based on the new spectrum or whether to reject the new spectrum and keep your old result.

## Datafile Search Paths




These settings are useful if you are in an environment where multiple HDExaminer users are sharing project files. An HDExaminer project file includes references to the MS data files that were imported as time point experiments. When HDExaminer opens a project file, it will look for those data files in the expected locations.

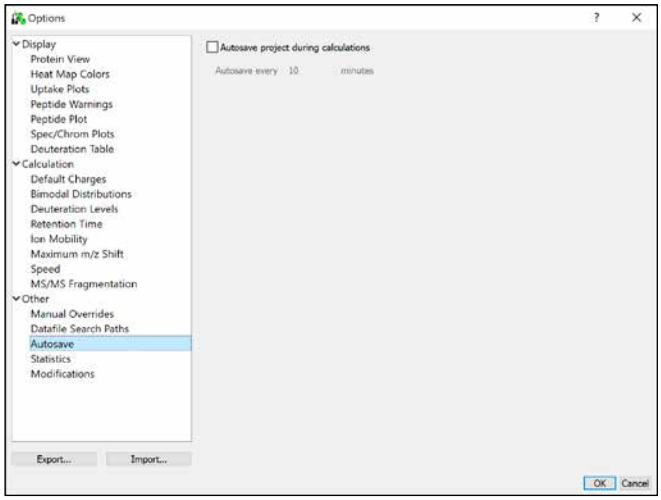
If it cannot find them (because they are on some other user's computer, for example), then these settings determine other locations where HDExaminer will look. If HDExaminer cannot find a required data file, then you will be able to open and view the project, but you will not be able to recalculate results.

**Search in the same directory…:** if this box is checked, HDExaminer will search in the project file's own directory for missing MS data files.

**Search in the following directories:** when searching for missing data files, HDExaminer will search the specified directories in order. To add a new directory, click the Add button . To remove a directory, select it and click the Remove button .

**Search subdirectories…:** when searching for missing data files, HDExaminer will search subdirectories of the specified directories, up to the specified search depth.

## Autosave

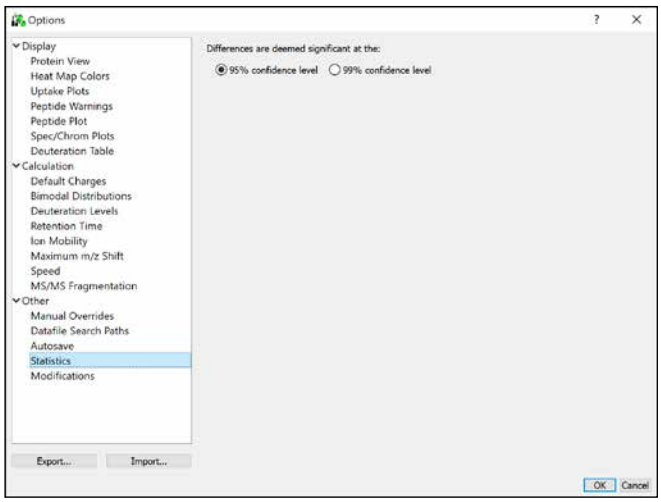



If you check the **"Autosave project during calculations"** checkbox, then while HDExaminer is calculating results, it will save a copy of the project file every X minutes, where X is the number specified here. An autosave file will overwrite the previous autosaved file, but is always separate from the last project file that you saved manually.

The autosave location is the same directory in which you have saved the regular project file. If the project file hasn't been saved yet, then the autosave location is the same directory as the project's first undeuterated dataset.

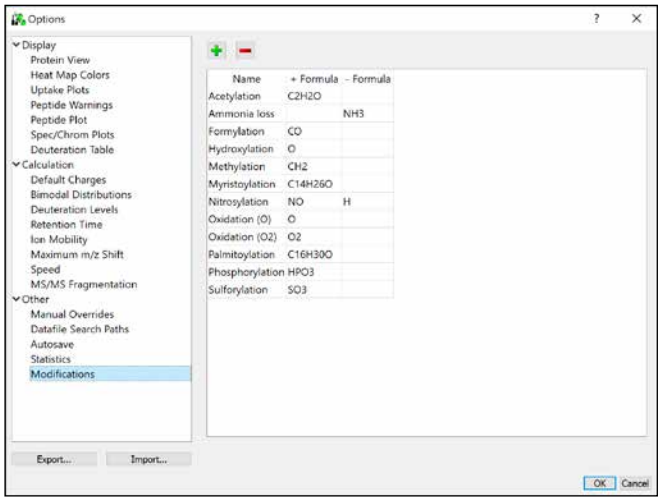
If you open a project file and the software notices a newer autosave file in one of the expected locations, then it will ask you if you want to open the autosave file instead.

## Statistics




This page allows you to set the desired confidence limit for the various statistical algorithms that HDExaminer uses: 95% or 99%.

## Modifications Library




When you add a modification to a protein state, you can choose from a list of built-in modifications. You may edit that list of modifications here. To add a new modification, click the Add button , then specify the name of the modification, the formula that it adds to a residue, and, if applicable, the formula it removes from that residue. To remove a modification from the library, select it and click the Remove button .

HDExaminer's export functionality is available in several places: under "Copy" in the Edit menu, under "Export" in the Tools menu, and in several right-click context menus throughout the software. The Copy functionality places data onto the Windows clipboard for pasting into other applications. Most Export functions behave similarly, but save the data to a specified file rather than to the clipboard.

Some exports use the .emf (Enhanced Metafile) format. This is a scalable vector graphics format suitable for import into many different software packages. If you import an .emf file into a vector-graphics-aware program such as Inkscape or PowerPoint, you will be able to scale the image without seeing scaling artifacts such as jagged lines.

## Project Summary

HDExaminer can export a project summary table in the format specified in Recommendation 2.3 and Supplementary Table 1 of *Masson et al., Nat Methods **16**, 595–602 (2019).* To export this table, go to either Edit – Copy – Project Summary (to copy the table to the clipboard) or Tools – Export – Project Summary… (for a .csv export).

Note that the "HDX reaction details" cells in the exported table will always be left blank, since HDExaminer does not store or track this information.

## Protein View

From the Protein View, you may export the heat map, either as an image or as a data table. The data table contains a numeric representation of the heat map. For each residue and each time point, the table contains a number representing the computed deuteration percentage of that atomic peptide, on a zero-to-one scale. The table also includes "spread" columns that indicate how much variance there is between the individual peptide measurements and the heat map data displayed. In general, lower spread values indicate better agreement among all of your peptides and the heat map. Finally, the table includes columns for the confidence level at each residue.

**To copy the heat map image to the clipboard,** first switch to the Protein View, then select "Edit", then "Copy", then "Heat Map Image" **or** right-click on the heat map and select "Copy Image to Clipboard".

**To export the heat map image to an .emf file,** first switch to the Protein View, then select "Tools", then "Export", then "Heat Map Image…" **or** right-click on the heat map and select "Export Image…". Specify a filename. HDExaminer will save the image to that file.

**To copy the heat map data table to the clipboard,** first switch to the Protein View, then select "Edit", then "Copy", then "Heat Map Data" **or** right-click on the heat map and select "Copy Data to Clipboard".

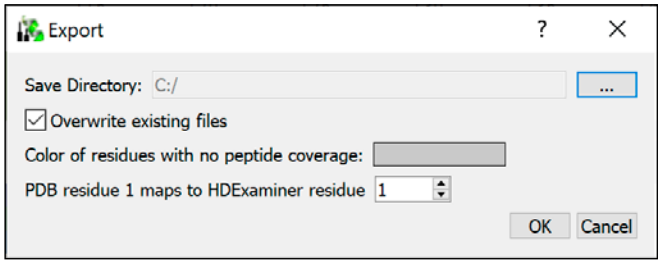
**To export the heat map data table to a .csv file,** first switch to the Protein View, then select "Tools", then "Export", then "Heat Map Data…" **or** right-click on the heat map and select "Export Data…". Specify a filename. HDExaminer will save the table to that file.

## Exporting to PyMOL or Chimera

PyMOL is a molecular modeling software tool produced by Schrödinger, LLC, available at http://pymol.org. Chimera is a similar tool produced by the University of California, San Francisco, available at https://www.cgl.ucsf.edu/chimera/. These software programs are widely used to generate images of protein structures.

Data from an HDExaminer heat map may be exported to a form suitable for import into PyMOL or Chimera. To do this, you will need a structure file for your protein that is compatible with PyMOL or Chimera. (HDExaminer will color an existing protein structure for you, but it cannot generate a protein structure file.)

To export to PyMOL or Chimera, first switch to the Protein View and view the heat map or difference map you wish to export. Select “Tools”, then “Export”, then “PyMOL…” or “Chimera…” or right-click on the heat map and select “PyMOL Export…” **or** “Chimera Export…”. You will see the export window:




Click on the browse button “…” to select a directory to save to (HDExaminer will save one file for each heat map row currently showing in the Protein View).

If you want HDExaminer to create new export files rather than overwriting old ones, uncheck the “Overwrite existing files” checkbox.

By default, HDExaminer will color all residues with no peptide coverage a neutral gray in PyMOL or Chimera. You may override that color by clicking on the gray box marked “Color of residues with no peptide coverage”.

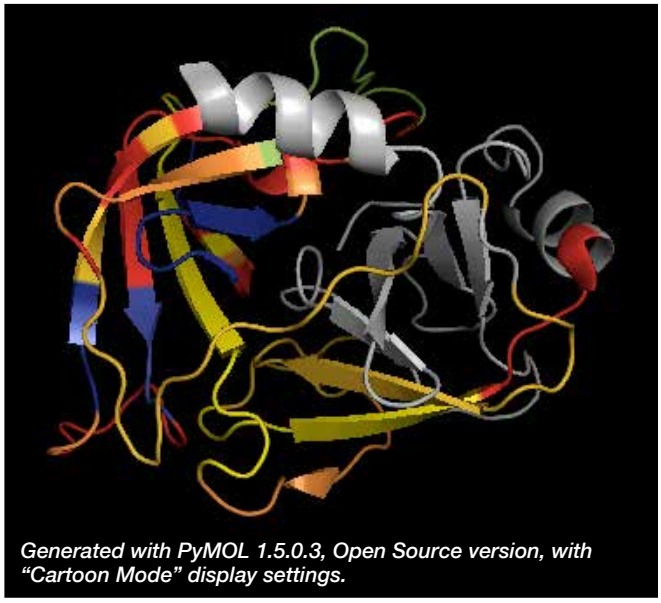
If your protein structure file sequence is offset from the sequence you imported into HDExaminer (for example, because your HDExaminer sequence is a subset of your complete protein), you may enter either a positive or negative offset here to ensure that the correct residues receive the correct coloration.

When you click “OK”, HDExaminer will generate one .pml (for PyMOL) or .com (for Chimera) file for each time point shown on your heat map. Each of these files is a script.

To color your protein in PyMOL, first launch PyMOL, then load your protein’s PDB structure file, then go to “File”, then “Run…”. In the file browse window that appears, browse to the HDExaminer-generated script you wish to use.

To color your protein in Chimera, first launch Chimera, then load your protein’s PDB structure file, then go to “File”, then “Open...”. Open any of the .com files generated by HDExaminer.

Using this functionality, you can generate pictures such as this one:



*Generated with PyMOL 1.5.0.3, Open Source version, with “Cartoon Mode” display settings.*

## Peptides View

From the Peptides View, you may export an image of any uptake plot, or the image or data corresponding to a peptide plot. You may also export data tables showing deuteration levels for every peptide in your Peptide Pool. You may also export the raw spectra used for each calculated result. You may also export a PDF file showing all of your uptake plots. You may export an image of the stacked spectrum/chromatogram plots. Finally, you may export your Peptide Pool so that you can import it into another HDExaminer project.

**To copy an uptake plot to the clipboard,** switch to the Peptides View, then select a peptide. Select “Edit”, then “Copy”, then “Uptake Plot” **or** right-click on the uptake plot and select “Copy Uptake Plot to Clipboard”.

**To export an uptake plot to an .emf file,** switch to the Peptides View, then select a peptide. Select “Tools”, then “Export”, then “Uptake Plot…” **or** right-click on the uptake plot and select “Export Uptake Plot…”. Specify a filename. HDExaminer will save the uptake plot to that file.

**To copy a peptide plot image to the clipboard,** switch to the Peptides View, then select a Peptide Pool. Select “Edit”, then “Copy”, then “Peptide Plot Image” **or** right-click on the peptide plot and select “Copy Peptide Plot to Clipboard”.

**To export a peptide plot image to an .emf file,** switch to the Peptides View, then select a Peptide Pool. Select “Tools”, then “Export”, then “Peptide Plot Image…” **or** right-click on the peptide plot and select “Export Peptide Plot…”. Specify a filename. HDExaminer will save the peptide plot to that file.

**To copy the peptide plot data to the clipboard,** switch to the Peptides View, then select a Peptide Pool. Select “Edit”, then “Copy”, then “Peptide Plot Data” **or** right-click on the peptide plot and select “Copy Peptide Plot Data to Clipboard”. Note that this option is only available for the butterfly plot.

**To export the peptide plot data to a .csv file,** switch to the Peptides View, then select a Peptide Pool. Select “Tools”, then “Export”, then “Peptide Plot Data…” **or** right-click on the peptide plot and select “Export Peptide Plot Data…”. Specify a filename. HDExaminer will save the peptide plot data to that file. Note that this option is only available for the butterfly plot.

**To copy the deuteration level table to the clipboard,** switch to the Peptides View, then select a Peptide Pool or any peptide in that pool. Select “Edit”, then “Copy”, then “Peptide Pool Results” or “Uptake Summary Table” **or** right-click on any Peptide Pool or peptide and select “Copy Peptide Pool Results to Clipboard” or “Copy Uptake Summary Table to Clipboard”. The two tables contain the same data, but are formatted differently (one peptide per line versus one result per line).

**To export the deuteration level table to a .csv file,** switch to the Peptides View, then select a Peptide Pool or any peptide in that pool. Select “Tools”, then “Export”, then “Peptide Pool Results…” or “Uptake Summary Table…” **or** right-click on any Peptide Pool or peptide and select “Export Peptide Pool Results…” or “Export Uptake Summary Table…”. Specify a filename. HDExaminer will save the table to that file. The two tables contain the same data, but are formatted differently (one peptide per line versus one result per line).

⚠ **Note**

Note that the Uptake Summary Table adheres to all requirements in Recommendation 3.1 of *Masson et al., Nat Methods* ***16****, 595–602 (2019).* ***Importantly, the #D results reported in this table are not corrected for back exchange, regardless of your HDExaminer settings!***

**To export all uptake plots to a PDF file:** switch to the Peptides View, then select a Peptide Pool or any peptide in that pool. Select “Tools”, then “Export”, then “PDF Uptake Plots…” **or** right-click on any Peptide Pool or peptide and select “Export Uptake Plots to PDF…”. Specify the number of rows and columns you would like per page, whether you would like HDExaminer to also export Enhanced Metafiles (EMF) or JPEG image files for each uptake plot, and whether fragment uptake plots should be included (in the case of middle down data). Specify the name of the PDF file you would like to save and HDExaminer will create it. If you chose to generate EMF or JPEG files, HDExaminer will save those in a directory named [file].images, where [file] is the name of the PDF file you saved, without the “.pdf” extension.

**To export the raw spectra used for all of a peptide’s calculated results:** switch to the Peptides View, then select a peptide. Select “Tools”, then “Export”, then “Peptide Spectra…” **or** right-click on the selected peptide and select “Export Peptide Spectra…”. Select a folder. HDExaminer will save the raw spectrum data for each result corresponding to the selected peptide. The exported data will be saved to a subfolder called SpecExport, with another subfolder called Fragments in the case of middle down data. The individual spectra will be saved in csv format.

**To export the raw spectra used for all calculated results in a Peptide Pool:** switch to the Peptides View, then select a Peptide Pool or any peptide in that pool. Select “Tools”, then “Export”, then “Pool Spectra…” **or** right-click on any Peptide Pool or peptide and select “Export Pool Spectra…”. Select a folder. HDExaminer will save the raw spectrum data for each result corresponding to the selected Peptide Pool. The exported data will be saved to a subfolder called SpecExport, with another subfolder called Fragments in the case of middle down data. The individual spectra will be saved in csv format.

**To copy the spectrum and XIC plots for a particular charge state of a peptide to the clipboard:** switch to the Peptides View, then select a particular charge state under a peptide so that you see the deuteration comparison view of stacked spectrum and chromatogram plots. Select “Edit”, then “Copy”, then “Deuteration Comparison Plots” **or** right-click on any plot and select “Copy Plots to Clipboard”.

**To export the spectrum and XIC plots for a particular charge state of a peptide:** switch to the Peptides View, then select a particular charge state under a peptide so that you see the deuteration comparison view of stacked spectrum and chromatogram plots. Select "Tools", then "Export", then "Deuteration Comparison Plots…" **or** right-click on any plot and select "Export Plots…". Specify a filename. HDExaminer will save the plots to that file in EMF format.

**To export a peptide pool for use in a different HDExaminer project:** switch to the Peptides View, then select a peptide pool or any item in a pool. Select "Tools", then "Export", then "Peptide Pool…" **or** right-click on the selected item and select "Export Peptide Pool…" Specify a filename. HDExaminer will save a csv file containing your peptides. This file may be used as a peptide source in another HDExaminer project.

## Analysis View

From the Analysis View, you may export the deuteration results table for any experiment in your project.

**To copy the table to the clipboard,** switch to the Analysis View, then select any experiment. Select "Edit", then "Copy", then "Current Results Table" **or** right-click on the results table and select "Copy This Table to Clipboard".

**To export the table to a .csv file,** switch to the Analysis View, then select any experiment. Select "Tools", then "Export", then "Results Table Data…" **or** right-click on the results table and select "Current Results Table…". Specify a filename. HDExaminer will save the table to that file.

**To copy all tables to the clipboard,** switch to the Analysis View, then select any experiment. Select "Edit", then "Copy", then "All Results Tables" **or** right-click on the results table and select "Copy All Tables to Clipboard".

**To export all tables to a .csv file,** switch to the Analysis View, then select any experiment. Select "Tools", then "Export", then "All Results Tables…" **or** right-click on the results table and select "Export All Tables…". Specify a filename. HDExaminer will save the combined tables to that file.

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