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A Hydrogen-Deuterium Exchange Mass Spectrometry (HDX-MS) Platform for Investigating Peptide Biosynthetic Enzymes

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TITLE:

A Hydrogen-Deuterium Exchange Mass Spectrometry (HDX-MS) Platform for Investigating Peptide Biosynthetic Enzymes

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SUMMARY:

Lanthipeptide synthetases catalyze multistep reactions during the biosynthesis of peptide natural products. Here, we describe a continuous, bottom-up, hydrogen-deuterium exchange mass spectrometry (HDX-MS) workflow that can be employed to study the conformational dynamics of lanthipeptide synthetases, as well as other similar enzymes involved in peptide natural product biosynthesis.

ABSTRACT:

Hydrogen-deuterium exchange mass spectrometry (HDX-MS) is a powerful method for the biophysical characterization of enzyme conformational changes and enzyme-substrate interactions. Among its many benefits, HDX-MS consumes only small amounts of material, can be performed under near native conditions without the need for enzyme/substrate labeling, and can provide spatially resolved information on enzyme conformational dynamics−even for large enzymes and multiprotein complexes. The method is initiated by the dilution of the enzyme of interest into buffer prepared in D_2O. This triggers the exchange of protium in peptide bond amides (N-H) with deuterium (N-D). At the desired exchange time points, reaction aliquots are quenched, the enzyme is proteolyzed into peptides, the peptides are separated by ultra-performance liquid chromatography (UPLC), and the change in mass of each peptide (due to the exchange of hydrogen for deuterium) is recorded by MS. The amount of deuterium uptake by each peptide is strongly dependent on the local hydrogen bonding environment of that peptide. Peptides present in very dynamic regions of the enzyme exchange deuterium very rapidly, whereas peptides derived from well-ordered regions undergo exchange much more slowly. In this manner, the HDX rate reports on local enzyme conformational dynamics. Perturbations to deuterium uptake levels in the presence of different ligands can then be used to map ligand binding sites, identify allosteric networks, and to understand the role of conformational dynamics

in enzyme function. Here, we illustrate how we have used HDX-MS to better understand the biosynthesis of a type of peptide natural products called lanthipeptides. Lanthipeptides are genetically encoded peptides that are post-translationally modified by large, multifunctional, conformationally dynamic enzymes that are difficult to study with traditional structural biology approaches. HDX-MS provides an ideal and adaptable platform for investigating the mechanistic properties of these types of enzymes.

INTRODUCTION:

Proteins are structurally dynamic molecules that sample different conformations on time scales ranging from femtosecond-scale bond vibration to rearrangements of entire protein domains which can occur over many seconds[1]. These conformational fluctuations are often critical aspects of enzyme/protein function. For example, conformational changes induced by ligand binding are often critically important for modulating enzyme function, either by organizing active site residues needed for catalysis, defining substrate binding sites in sequential kinetic mechanisms, shielding reactive intermediates from the environment, or by modulating enzyme function via allosteric networks. Recent studies have also shown that conformational dynamics can be conserved throughout evolution and that perturbations to conserved molecular motions can be correlated with changes in substrate specificity and the emergence of new enzyme functions[2-3].

In recent years, hydrogen-deuterium exchange mass spectrometry (HDX-MS) has rapidly emerged as a powerful technique to probe how protein conformational landscapes respond to perturbations such as ligand binding or mutagenesis[4-7]. In a typical HDX-MS experiment (**Figure 1**), a protein of interest is placed into buffer prepared in D_2O, which triggers replacement of solvent-exchangeable protons with deuteria. The rate of exchange of the amide moiety of the peptide bonds depends strongly on the pH, the local amino acid sequence, and on the local structural environment of the amide[8]. Amides that are engaged in hydrogen bonding interactions (such as those present in α-helices and β-sheets) exchange more slowly than amides in unstructured regions of the protein that are exposed to bulk solvent. Thus, the extent of deuterium uptake is a reflection of the structure of the enzyme. Enzymes that are conformationally dynamic, or that undergo structural transitions upon ligand binding, would be expected to yield a measurable HDX response.

The mechanistic basis for the slow exchange rate of a structured amide is shown in **Figure 2**[5,8-9]. In order to undergo HDX, the structured region must first transiently sample an unfolded conformation, such that the solvent molecules that catalyze HDX exchange via a specific acid/base chemical mechanism, have access to the exchangeable amide. Ultimately, the relative magnitudes of the chemical exchange rate (k_{chem}) and the folding and refolding rates (k_{open} and k_{close}) determine the HDX rate measured in the experiment[5,8]. From this simple kinetic model, it is clear that extent of deuterium uptake will reflect the underlying conformational dynamics (as defined by k_{open} and k_{close}). Most HDX-MS experiments are performed in a bottom-up workflow where, following the exchange reaction, the protein of interest is digested into peptides and the deuterium uptake by each peptide is measured as an increase in mass[7]. In this way, HDX-MS allows perturbations to enzyme conformational dynamics to be mapped on the local spatial scale of peptides, allowing the researcher to assess how the perturbation alters

dynamics in different regions of the enzyme of interest.

The advantages of the HDX-MS approach for elucidating protein structural dynamics are numerous. First, the method can be performed with small quantities of native protein or on protein complexes in systems with quaternary structure[10]. It is not even necessary for the enzyme preparation used in the assay to be highly purified[11-12], as long as the bottom-up HDX-MS workflow provides a sufficient number of confidently identified peptides that cover the protein sequence of interest. Moreover, HDX-MS can provide information on conformational dynamics under near native conditions without the need for site-specific protein labeling as would be used in single molecule fluorescence studies[13], and there is no size limit to the protein or protein complex that can be investigated (which makes approaches such as nuclear magnetic resonance [NMR] spectroscopy challenging)[7,14]. Finally, time-resolved HDX-MS methods can be employed to study intrinsically disordered proteins, which are difficult to study with X-ray crystallography[15-18]. The main limitation of HDX-MS is that the data is of low structural resolution. HDX-MS data are useful for pointing to where conformational dynamics are changing and for revealing coupled conformational changes, but they do not often provide much insight into the precise molecular mechanism driving the observed change. Recent advances in the combination of electron capture dissociation methods with protein HDX-MS data have shown promise for mapping exchange sites to single amino acid residues[19], but follow up biochemical and structural studies are still often needed to provide clarity to structural models forwarded by HDX-MS data.

Below, a detailed protocol for the development of an HDX-MS assay is presented[20]. The sample preparation protocols presented below should be generally applicable to any protein that exhibits good solubility in aqueous buffers. More specialized sample preparation methods and HDX-MS workflows are available for proteins than need to be assayed in the presence of detergent or phospholipids[21-24]. Instrumental settings for HDX-MS data collection are described for a high-resolution quadrupole time-of-flight mass spectrometer coupled to liquid chromatography system. Data of similar complexity and resolution could be collected on any one of a number of commercially available liquid chromatography-mass spectrometry (LC-MS) systems. Key aspects of the data processing using a commercially available software package are also provided. We also present guidelines for data collection and analysis that are consistent with recommendations made by the broader HDX-MS community[12]. The described protocol is used to study the dynamic structural properties of HalM2, a lanthipeptide synthetase that catalyzes the multistep maturation of an antimicrobial peptide natural product[20]. We illustrate how HDX-MS can be used to reveal substrate binding sites and allosteric properties that have eluded previous characterization. Several other protocols on protein HDX-MS have been published in recent years[25-26]. Together with the present work, these earlier contributions should provide the reader some flexibility in experimental design.

PROTOCOL:

1. Preparation of deuterated reagents and enzyme stock solutions

1.1. Prepare reagents needed for the HDX reactions (including any buffers, salts, substrates,

ligands, etc.) as 100−200x concentrated stock solutions in D_2O (99.9% atom fraction D). Prepare at least 50 mL of buffer stock solution.

NOTE: For characterization of HalM2, the following solutions were prepared: 500 mM $MgCl_2$, 100 mM tris(2-carboxyethyl)phosphine (TCEP), 750 mM ATP (in HEPES buffer), 800 mM HEPES pD 7.1, 500 µM HalA2, and 500 mM AMPPNP.

1.2. Freeze and lyophilize the stock solutions to dryness.

1.3. Re-dissolve in D_2O, and repeat lyophilization cycle at least one additional time to replace as many of the exchangeable protons with deuterons as possible.

1.4. Adjust the pD of the deuterated HEPES buffer stock to the desired value with concentrated NaOD/DCl, keeping in mind the following relationship[27]:

$$pD = pH\ meter\ reading + 0.4$$

NOTE: The amide HDX rate is strongly dependent on the pL of the solution (pL = pH or pD)[5]. Different batches of buffer stock solutions need to be prepared, stored, and used in an identical manner to avoid slight pL drift between experiments.

1.5. Calculate the quantity of each reagent needed for a 300 µL HDX assay and store as single use aliquots at -80 °C.

1.6. Prepare a concentrated enzyme stock solution (~100−200 µM) in protiated enzyme storage buffer using a centrifugal filter (**Table of Materials**) or equivalent device.

NOTE: The exact buffer and centrifugal filter molecular weight cutoff will depend on the protein/enzyme of interest. HalM2 is stored in 50 mM HEPES, pH 7.5, 100 mM KCl, and 10% glycerol. 10 kDa filters were used to prepare the concentrated enzyme.

1.7. Aliquot the enzyme into single use portions and store at -80 °C.

NOTE: This can be a stopping point. All stock solutions described in section 1 can be prepared in advance of the HDX reactions. If stored at -80 °C, most enzymes/deuterated stock solutions will be stable for many months.

2. Calibration of the HDX quench volume

2.1. Prepare a 300 µL HDX reaction in D_2O using the deuterated reagents and concentrated enzyme stocks prepared in section 1.

2.1.1. Use a final enzyme concentration of 1−5 µM.

2.1.2. Use a final deuterated HEPES buffer concentration of at least 50−100 mM.

2.1.3. Ensure the concentrations of other components are sufficient to maintain the desired enzyme activity/function.

2.2. Prepare 1 L of HDX quench solution (100 mM phosphate, 0.8 M guanidine-HCl, pH 1.9). Freeze and store in both 50 mL portions (for long term stock) and 1 mL portions (for single use aliquots).

NOTE: The exact composition of the quench buffer will depend on the enzyme that is used in the proteolysis step of the bottom-up HDX-MS workflow (step 3.3.3). The quench buffer given here is compatible with pepsin, the most commonly used protease for HDX-MS. If a different protease is used, check with the protease supplier to ensure buffer compatibility.

2.3. Calibrate the volume of quench buffer needed to adjust the final pL of the quenched HDX reaction mixture to a pH meter reading value of 2.3.

NOTE: The solvent H/D exchange rate of the peptide amide N-H bond is a pH-dependent process that is subject to both acid- and base-catalysis. The minimum exchange rate occurs at a value of pH 2.5 (pH meter reading = 2.3 for a 50:50 H_2O:D_2O mixture). Thus, a final pL value near 2.5 will minimize hydrogen back exchange that occurs during the bottom-up LC-MS analysis, thereby preserving the deuterium label in the peptides.

2.3.1. Mix 50 µL of the HDX reaction mixture from step 2.1 with 50 µL of quench buffer and measure the pL of the quenched mixture with a microtip electrode.

2.3.2. Increase the volume of quench solution as needed to adjust the final pH meter reading to a value of 2.3.

2.3.3. Once the appropriate quench volume has been determined, repeat the quenching process several times using fresh 50 µL aliquots from the HDX reaction (step 2.1) to ensure that a consistent final pL is achieved upon addition of a fixed quantity of quench buffer.

3. Preparation of reference samples and optimization of the bottom-up LC-MS workflow

3.1. Prepare undeuterated reference samples for the protein of interest in triplicate in 0.5 mL tubes. Ensure that the final reaction mixture conditions are identical to those used in the authentic HDX reactions (step 2.1), except that the reactions are prepared in H_2O using reagent stock solutions also prepared in H_2O.

3.2. Quench the samples as in step 2.3 by adding the appropriate volume of quench buffer to adjust the final pH to 2.5. Flash freeze the samples in liquid nitrogen and store at -80 °C until ready for analysis.

3.3. Analyze the protiated enzyme reference samples using a bottom-up LC-MS workflow.

NOTE: Prior to executing these steps, the LC-MS system to be used for data acquisition should be properly calibrated and ready for use. The timing and temperature of all steps in the bottom-up LC-MS workflow must be rigorously controlled in order to minimize differences in back exchange between samples. With the MS instrumentation used in this protocol (**Table of Materials** and **Supporting Information**), most of the steps can be controlled through the instrument software. To ensure the collection of precise replicates, it is recommended to automate as many of the steps in the workflow as possible.

3.3.1. Remove an individual enzyme reference sample (prepared as in step 3.2) from the freezer and thaw at 37 °C for 1 min in a water bath.

3.3.2. At precisely 2 min after removing the sample from the freezer and thawing, inject a 40 µL portion of the quenched reference sample into an ultra-performance liquid chromatography (UPLC) column (2.1 x 30 mm, 300 Å, 5 µM) containing a stationary phase functionalized with pepsin (an acid-stable protease).

3.3.3. Digest the sample at a flow rate of 100 µL/min for 3 min at 15 °C using 0.1% formic acid in H_2O (pH = 2.5) as the solvent.

3.3.4. Collect the peptic peptides as they elute from the pepsin column onto a C18 trap column held at 0.4 °C to minimize back exchange.

3.3.5. Pass the desalted peptic peptides from the trap column to a C18 analytical column (1 mm x 100 mm, 1.7 µM, 130 Å) held and operated at 0.4 °C for separation of the peptic peptides.

NOTE: Steps 3.3.3−3.3.5 can be automated by certain LC-MS systems used for HDX-MS data acquisition. Alternatively, these steps can be performed independently, keeping in mind that the timing and temperature of each step needs to be carefully controlled to achieve a consistently low back exchange.

3.3.6. Elute the C18 column with an acetonitrile/water/0.1% formic acid solvent system. Optimize the LC gradient for the protein of interest in order to maximize the separation of and to preserve the deuterium label in the peptic peptides.

NOTE: Gradient elution details are provided in the **Supporting Information**.

3.3.7. Subject the peptic digest to electrospray ionization (ESI) mass spectrometry.

NOTE: The source conditions provided in the **Supporting Information** will provide sufficient ionization for most peptic peptides.

3.3.7.1. Once ionized in the MS instrument, perform a gas phase ion mobility separation using nitrogen as the buffer gas to enhance the peak capacity of the method.

3.3.7.2. Following the ion mobility separation, subject the peptic peptide precursor ions to an MS^E workflow involving alternating cycles of low collision energy (4 V) and high collision energy (21−40 V).

NOTE: The alternating low and high collision energy regimes allow for the collection of MS data (low collision energy) simultaneously with MSMS data (high collision energy). This, in turn, allows for the time-correlation of precursor ions with their respective fragment ions. This correlation is essential for confident peptide identification described in section 4.

3.3.7.3. Detect the peptide precursor and fragment ions using a mass analyzer with a resolving power of at least 20,000.

3.3.7.4. Simultaneous with the data acquisition, acquire MS data for a [Glu-1]-fibrinopeptide B (GluFib) external standard.

NOTE: The MS workflow described in step 3.3.7 is referred to as an MS^E protocol. Complete instrumental settings for an MS^E protocol that is suitable for bottom-up HDX-MS are provided in the **Supporting Information**.

3.3.8. Assess the quality of the LC-MS data.

NOTE: Using the protocol described above and the instrumental settings provided in the **Supporting Information**, the reference samples should produce a total ion chromatogram with a maximum signal intensity of approximately 1 x 10^8. There should be many peptic peptides eluting between 3−9 min (**Figure 3A–C**).

3.3.9. Inject 40 µL of blank samples (0.1% formic acid in water) to clean the pepsin and analytical C18 columns.

NOTE: In general, 2−3 blanks should be sufficient.

3.3.10. Repeat steps 3.3.1−3.3.9 for each of the triplicate reference protein samples.

4. Processing the reference data and defining a peptide list

4.1. Analyze the raw MS^E data (step 3.3.7) using proteomics software (**Table of Materials**). Using the proteomics software, navigate to **Libraries** | **Protein Sequence Databanks** to define the protein database by importing the amino acid sequence of the protein of interest.

NOTE: The goal of this step is to search the reference MS data for peptic peptides derived from the protein of interest, and to use the MSMS data (acquired simultaneously with the MS data) to validate any putative peptide identifications.

4.2. Give a name to the protein sequence of interest. Import the protein sequence (in FASTA format). The software will perform an in silico digestion of the database protein to generate a list of peptides that will be used to search the LC-MS data.

4.3. Define the processing parameters (located under the **Library** menu). Select **Electrospray MS^E** as the data acquisition type. In the **Lock Mass for Charge 2** field, enter **785.8426** for the m/z for the 2^+ ion of [Glu-1]-fibrinopeptide B (GluFib) and click **finish**.

4.4. Define the **Workflow** parameters (located under the **Library** menu).

4.4.1. Select **Electrospray MS^E** for the search type. Under the **Workflow** | **Database Search Query** heading, select the database protein created in step 4.2 in the **Databank** field.

4.4.2. Change **Primary Digest Reagent** to nonspecific, and clear the **Fixed Modifier Reagent** field by holding the **Ctrl** button while clicking on **Carbamidomethyl C**.

4.5. Specify the output directory by navigating to **options** | **automation setup** | **Identity E**. Check the boxes for **Apex 3D and Peptide 3D Output** and **Ion Accounting Output** and specify the desired directory.

4.6. Process the reference sample data.

4.6.1. On the left tool bar of the proteomics platform workspace, create a new plate by right clicking on **Microtiter Plate**. Highlight three wells in the microtiter plate (one for each reference sample collected in section 3). Left click in one well, hold and drag to three wells.

4.6.2. Right click and select **add raw data**. In the window that appears, navigate to the directory containing the three reference files from section 3 and select them at the same time.

4.6.3. Click on **next** and choose the processing parameters defined in step 4.3. Click on **next** and select the workflow parameters defined in step 4.4. Then click on **finish**.

4.7. Once the raw data, processing parameters, and workflow parameters have been assigned to each well on the plate, the wells will appear blue. Select the wells, right click and select **process latest raw data**. Click on the right bottom corner of the window to track the processing of the data. Once the message **No job to run** appears, the processing is fully done.

4.8. After data processing is complete, the wells in the plate will turn green. Right click on the wells and select **view workflow results**. A separate window will open for each reference data file.

4.9. Inspect the data to ensure that the majority of MS signals in the reference sample data were successfully mapped to peptides predicted from the in-silico digestion of the protein of interest. Matched peptides will be colored blue in the output spectrum (**Figure 4**). Double-click on the **OK filter** and check that the percent coverage is greater than 99%.

NOTE: Upon processing, data output will be automatically saved with the file extension (*raw_data_file_name*_IA_final_peptide) in the directory specified in step 4.5.

4.10. Import the proteomics software output into the HDX-processing software (**Table of Materials**) for additional thresholding.

4.10.1. Click on **Data** in the left corner of the HDX-processing software window. Click on **import PLGS results** and click on the add icon. Choose the processed data files from step 4.9 by navigating to the appropriate directory.

4.10.2. Click on **Next** and specify the following parameters: minimum consecutive ions ≥ 2, mass error = 5 ppm, and file threshold = 3. Click on **finish**.

4.11. Once satisfied with the thresholding parameters, save the HDX project. All HDX data will be imported into this project for analysis and display.

NOTE: The deuterium exchanged samples described in the next section will need to be processed with an identical LC-MS workflow. Therefore, before proceeding with HDX assays (section 5), ensure that the sample preparation (section 2), bottom-up LC-MS workflow (section 3) and data processing workflows (section 4) are providing the desired reproducibility and sequence coverage of the target protein. If any of these processes need to be changed to improve coverage, it is advised to return to step 2.1, prepare fresh reference samples in triplicate, and to repeat sections 2−4 (while making the necessary adjustments to the protocol) to ensure that each peptide can be reproducibly generated and detected.

5. Conducting HDX reactions

5.1. Prepare workspace for HDX reactions.

5.1.1. Pre-aliquot quench buffer into properly labeled 0.5 mL tubes. Prepare a different tube for every time point, every replicate, and every biochemical state to be analyzed. Use the appropriate volume of quench buffer from step 2.2 needed to adjust the final pH meter reading of a 50 µL portion of the HDX reaction to a value of 2.3.

5.1.2. Briefly centrifuge the 0.5 mL tubs to transfer all of the quench buffer to the bottom of the tube. Place the tubes on ice.

5.1.3. Fill a small Dewar with liquid nitrogen and keep adjacent to workspace.

5.2. Prepare the HDX reactions. Ensure that there is sufficient reaction volume to collect the desired number of exchange time points (one 50 µL aliquot for each desired time point). Collect at least 4−5 time points over 3−4 orders of magnitude in time scale (e.g., quench times of 15 s, 60 s, 300 s [5 min], 1,800 s [30 min], and 14,400 s [4 h] provide adequate coverage of exchange dynamics for most enzymes).

5.2.1. Pre-mix all of the deuterated components (minus enzyme) from step 1.1 in D_2O.

5.2.2. For each biochemical state to be examined (free enzyme, enzyme + ligand, enzyme + inhibitor, etc.), prepare HDX reactions in at least triplicate.

5.2.3. Incubate the reaction mixtures in a temperature-controlled water bath at 25 °C for 10 min prior to addition of the enzyme.

NOTE: The enzyme should be prepared as a concentrated stock solution (~100−200 µM, step 1.6) so as to minimize the addition of protium into the HDX assay.

5.2.4. Upon adding the enzyme to a final concentration of 1−5 µM, start the timer. Carefully and quickly mix the solution using a 200 µL pipette to ensure that the enzyme is evenly distributed in the sample.

5.2.5. At the desired exchange time points, remove 50 µL aliquots from the HDX reaction and mix quickly and evenly with the pre-aliquoted, ice cold quench buffer in a 0.5 mL tube.

NOTE: The mixing volumes and mixing procedure must be as precise and reproducible as possible to ensure that the desired final quench pL of 2.3 is achieved rapidly in all samples. Keeping the quench buffer ice cold will help to minimize back exchange upon denaturation of enzyme.

5.2.6. Immediately after quenching the HDX sample, cap the tube and flash freeze in liquid nitrogen.

5.2.7. Continue collecting time points until all assays are complete, then transfer samples to the -80 °C freezer for storage.

NOTE: This can be a stopping point. After collecting all HDX time points, the samples can be stored at -80 °C until ready for LC-MS analysis. Ideally, triplicate HDX reactions should be performed for all biochemical states of interest on the same day. At a minimum, all replicate HDX reactions for a given biochemical state should be run in parallel on the same day.

5.3. After collecting all of the quenched HDX time points, subject the samples to the optimized bottom-up LC-MS workflow developed as described in step 3.3. Inject HDX samples in a randomized order with an appropriate number of blanks between samples to ensure that any peptide carry over is minimal.

NOTE: HDX data do not need to be collected in MS^E mode. Thus, the high collision energy segment (step 3.3.7.2) should be removed from the MS duty cycle. This should be the only alteration made to the workflow described in step 3.3.

5.4. Assess the quality of the HDX data as it is being collected.

5.4.1. Ensure that chromatographic peaks present in the total ion chromatogram of the undeuterated reference samples appear at the same retention time in the deuterated samples (as in **Figure 3D–F**).

5.4.2. Ensure that mass spectra summed over specific time intervals from reference and deuterated samples show evidence for deuteration (i.e., a shift in the isotopic envelope of individual peptides to higher m/z values in the deuterated samples [**Figure 6**]).

6. Processing HDX data

6.1. Import the HDX data into the HDX project created in step 4.11 by clicking on **Data** | **MS Files** in the top tool bar.

6.1.1. Click on **New State** and **New Exposure** as needed to define the biochemical states (e.g., free enzyme, enzyme + ligand, etc.) and deuterium exposure times, respectively, that are pertinent to the analysis.

6.1.2. Click on **New Raw** to select the HDX data files to be analyzed. Assign the appropriate exchange times and biochemical state to each raw data file that is imported.

NOTE: The data can be imported and processed in batches, or all at once. Adding data to the project will not undo any analysis that has been previously performed within that project.

6.2. Once the data files have been added, click **finish** to begin the data processing. After a short delay, the software will ask if the user wants to save the data before continuing. Click **yes**.

NOTE: The initial processing can take up to several hours depending on how many samples are being analyzed, how many peptides are in the final peptide list (step 4.10), the size of the chromatographic window, and the frequency of spectral acquisition.

6.3. If desired, alter the processing parameters in the **Configuration** menu to change the ion search parameters. Make sure to employ the same ion search parameters for all data in a given project.

7. Analysis and visualization of the HDX data

NOTE: Once the initial processing of the raw data has been completed (step 6.2), the HDX-processing software will have located peptides from the peptide list (generated in step 4.10) in

each of the raw data files that were analyzed. Once the isotope distribution for a peptide in the list is located in a raw data file, the HDX-processing software represents each isotope with a "stick" (as in **Figure 6C–E**). The relative intensities of the sticks for a given peptide are then used to calculate the deuterium uptake relative to the reference spectra. While the HDX-processing software does an admirable job of properly assigning "sticks" to most peptides, significant manual curation of the deuterium uptake values will still be required.

7.1. Analyzing peptide deuterium uptake values

7.1.1. Select the first peptide in the peptide list and open the stacked spectral plot from the **Views** menu. Scroll up and down in the stacked spectra plot window to see the mass spectra for the selected peptide as function of deuterium exchange time (**Figure 7D,E**).

7.1.2. Assign and unassign sticks as necessary using mouse clicks to ensure that the proper isotope distribution has been located in the data and that each isotope peaks has been assigned (assigned sticks will appear blue). Clicking on any of the spectra in the stacked spectral plot (**Figure 7D,E**) will allow the user to assign/unassign sticks in the active data viewer window (**Figure 7C**).

7.1.3. Check the stick assignments for each charge state by toggling the charge state at the top of the stacked spectral plot window.

7.1.4. Repeat steps 7.1.1−7.1.3 for each biochemical state of interest. The biochemical state can also be toggled at the top of the stacked spectral plot window. For the most accurate deuterium uptake difference measurements, ensure that sticks are assigned for the same set of charge states for each biochemical state.

7.1.5. Repeat steps 7.1.1−7.1.4 for each peptide in the peptide list.

7.1.6. Check the standard deviation of peptide deuterium uptake values using the coverage map.

7.1.6.1. Access the coverage map from the **Views** menu, which displays each peptide in the peptide list mapped along the amino acid sequence of the protein of interest (**Figure 8C**). Color the peptides according to relative standard deviation (units of Da).

7.1.6.2. Visually search the map for outlier peptides with high relative standard deviation. Click on the outlier peptides in the coverage map to populate the stacked spectral plots (**Figure 8B**) and data viewer window (**Figure 8A**) with the target peptide.

7.1.6.3. Using the stacked spectral plot, carefully check that all charge states and all time points of the outlier peptide have appropriately assigned sticks.

NOTE: Most often, peptides with large standard deviations (>0.3 Da) have isotopic peaks that were not appropriately assigned sticks by the software (as indicated in **Figure 8B**). Assigning any

missing sticks will generally enable the relative standard deviation of a peptide to be reduced to <0.3 Da.

7.1.6.4. Hide the peptide from the list if the relative standard deviation cannot be reduced to less than 0.3 Da.

7.2. Export HDX difference data between two biochemical states for mapping onto a structural model of the protein of interest.

7.2.1. Display the difference of interest in the coverage map. Right click on the coverage map to export the difference data to a .csv file. Export the state data (in .csv format) by navigating to **Data** | **Export State Data** in the main tool bar.

NOTE: Appropriate formatting of the difference data and state data files is provided in the **Supporting Information**.

7.2.2. Import the difference data, the state data, and the pdb file of the protein of interest into Deuteros[28]. Choose the 99% confidence interval, select **enable sum**, and process the data.

NOTE: MATLAB must be installed on the PC in order to run Deuteros. Deuteros will use the replicate measurements in the data set to calculate the standard deviation of the uptake data for each peptide. This standard deviation will be used to define the confidence interval for significant exchange, which will be displayed on the plots.

7.2.3. Under **PyMOL** Options, select **export uptake** | **export** to generate a Pymol script to map regions of significant exchange difference onto the pdb structure of the protein of interest using PyMOL software.

NOTE: Using the workflow described in this protocol, the 99% confidence interval for significant deuterium uptake difference for a given peptide at a single time point is typically 0.3−0.5 Da. The 99% confidence interval for the difference summed over all exchange time points is typically 0.7−1.0 Da.

REPRESENTATIVE RESULTS:

It is necessary to assess the quality of the proteolytic digestion and the reproducibility of the workflow for each set of sample injections. Thus, prior to performing HDX-MS assays, it is essential to establish effective conditions for the proteolysis of the protein of interest, for the separation of peptides using reverse phase liquid chromatography and gas phase ion mobility, and for the detection of peptides using MS. For this purpose, the reference samples for the protein of interest (collected in the absence of deuterium) should be investigated first (section 3). The chromatographic data in **Figure 3A–C** show the total ion chromatograms (TICs) for three reference samples of the HalM2 lanthipeptide synthetase. The TIC is the time dependent change in the sum of all ion counts at all m/z values included in the mass spectral scan. Closer views of the TICs between 3 and 8 min are shown in **Figure 3D–F**. The mass spectra shown in **Figure 3G–I**

represent a summation of all mass spectra over a small time interval (from 5.0 to 5.1 min) of each TIC. Particular attention should be paid to the following features in **Figure 3**.

First, the large peak near 9.3 min at the end of the gradient represents incompletely digested (and hence, large and more hydrophobic) fragments of HalM2 (**Figure 3A–C**). The digestion could be made more efficient by reducing the HalM2 concentration, but this will reduce peptide signal intensity and signal:noise ratio. The digestion could also be made more efficient by increasing the contact time (from 3 min, protocol step 3.3.3) with the pepsin column. However, increased contact time will result in more back exchange. Ultimately, these parameters need to be balanced for the protein of interest in order to provide the desired sequence coverage, signal intensity, and deuterium retention. Second, the shape and intensity of the TIC profiles should be similar (as in **Figure 3D–F**). This suggests that the proteolytic digestion of HalM2 is reproducible and is of similar efficiency in all three reference samples. The expectation is that a similar digestion of a deuterium-exchanged sample will produce the same set of peptides with similar retention times. Third, the mass spectra over a given time interval should also be similar (**Figure 3G–I**). A quick visual comparison of the summed mass spectra over the 5.0−5.1 min time interval of the chromatographic separation show that mass spectral signals in each sample are indeed very similar, providing confidence that similar peptides are present in each sample and are eluting at similar times from the C18 column. A similar quick visual inspection should be performed over other time intervals across the chromatogram.

After visually verifying the similarity in the LC-MS data of the reference samples, proteomics software is used to search the MS data for peptides derived from the amino acid sequence of the protein of interest. After defining the protein sequence databank (the amino acid sequence of the protein of interest), the processing and the workflow parameters as described in protocol steps 4.2−4.4, each individual reference sample is processed to detect peptic peptides that match predicted peptides derived from the protein of interest. An example of the proteomics software output for an undeuterated HalM2 reference sample is shown in **Figure 4**. Particular attention should be paid to the following features. First, any MS signal that is matched to a specific peptide within the protein of interest according to the values defined in the processing and workflow parameters will be colored blue in the bottom spectrum. If a reference sample is “successfully” processed, the majority of signals will appear blue (as is the case for the data in **Figure 4**). Furthermore, In the top left panel, ensure that that the “OK Filter” is toggled to the green checkmark. When this is done, the statistics in the top bar of the top right panel (highlighted in blue in **Figure 4**) will reflect only those peptides that give high confidence scores. These peptides exhibit low ppm mass accuracy, multiple fragment ions, and good correlation between fragment and parent ion retention times, and are considered to be confident identifications. For the HalM2 reference sample shown, 1,421 peptides were detected with high scores, covering 99.6% of the HalM2 sequence. Near 100% sequence coverage should be attainable for most proteins using the bottom-up LC-MS workflow described in protocol section 3. Moreover, Additional useful statistics for every peptide are tabulated in the top right panel, including the mass error, precursor retention time, the number of fragment (product ions), the complete list of fragment ions by name, and the ion mobility drift time. This information is useful for the interpretation of results, which should always be focused on the most confidently identified peptides.

The final peptide list should be determined by additional thresholding in the HDX-processing software. After analyzing the reference data to generate the peptide list (protocol section 4), the output should be imported into the HDX-processing software for additional thresholding. The output files will be stored in a directory as defined in protocol step 4.5 with the file extension "…_IA_final_peptide.csv." Upon uploading the output into The HDX-processing software , the complete list of peptides will be shown in the "peptide preview" panel (**Figure 5**), and the number of peptides, sequence coverage, and redundancy will be displayed in the bottom right corner of the window. These values will change in real time as additional thresholding is applied. After clicking **next**, thresholding values can be set in the indicated fields. The most critical filters are the "file threshold" which requires all peptides to be located in each of the three reference samples, the "maximum MH+ error (ppm)" which should be set to a value <10 ppm, and "minimum consecutive products," which requires the peptide to generate at least two consecutive fragment ions during the MSMS (i.e., high collision energy) phase of the MS^E workflow (protocol step 3.3.7.2).

The most significant technical limitation of the HDX-MS assay is the back exchange of deuterium for protium that occurs as soon as deuterium-exchanged samples are quenched (with protiated buffer). Back exchange continues during the protease digestion and LC-MS portions of the workflow. Back exchange is unavoidable, but if the pH, temperature, and timing of all post-quench steps are carefully controlled as described in the protocol, 60%−70% of the deuterium label can be maintained. For this reason, it is essential to check during the early stages of assay development that most of the peptides are retaining deuterium. This can be achieved quickly by comparing the raw data of a deuterated sample with that of a reference sample. As was done to ensure reproducibility of the reference samples (**Figure 3**), compare the TICs of the deuterium-exchanged and undeuterated reference samples, ensuring that the shapes of the profiles resemble each other. In addition, sum the mass spectra over the same chromatographic time interval in both samples. Most of the peptides in the deuterium exchanged sample should exhibit obvious shifts in their isotopic distributions towards higher m/z values (as in **Figure 6**). This data indicates that a substantial fraction of the deuterium label is being maintained throughout the course of the acid quench, pepsin digestion, and LC-MS data collection.

After verifying that the workflow is providing sufficient deuterium retention, the deuterium uptake needs to be quantified. Thus, the raw data for deuterium-exchanged samples are imported into the HDX-processing software. Using the reference samples and the finalized peptide list, the HDX-processing software will locate the peptides from the peptide list in each of the raw data files and will assign "sticks" to each of the isotopic peaks. In the representative data for HalM2 shown in **Figure 7**, the successfully assigned sticks are colored blue in all of the spectra. After assigning sticks, the centroid m/z value for the isotopic distribution will be determined by the software and used to calculate deuterium uptake relative to the undeuterated reference sample. The deuterium exchange value for each peptide should be manually checked. In **Figure 7**, the currently selected peptide (HIDKLTVGL, spanning HalM2 residues 110−118) is shown in the left panel of the main data viewer (**Figure 7A**). The other panels show HDX data associated with this peptide. Clicking on any peptide in the peptide list will populate the other panels with HDX

data from that peptide. **Figure 7B** shows the deuterium uptake plots for peptide 110−118. This data set contains four exchange time points: 0.5, 5, 30, and 240 min (collected in triplicate). Exchange data were collected for two biochemical states: free HalM2 enzyme (red) and the HalM2 enzyme complexed to AMPPNP and its substrate peptide HalA2 (blue). Note the significant deuterium uptake difference into peptide 110−118 across the entire time course and the high precision of the replicate measurements (error bars are shown on the plot in **Figure 7B**). In general, similar precision in the uptake measurement will be obtained for most peptides, underscoring the reproducibility of the workflow presented in this protocol. Upon ligand binding (blue curve in **Figure 7B**), the peptide bond amides in the 110−118 region of HalM2 apparently undergo significant protection from deuterium exchange, suggesting that the amino acids in the 110−118 region are becoming more stably structured upon ligand binding. Subsequent mutagenesis of this region indicated a role in binding to the precursor peptide, HalA2[20]. Shown also in **Figure 7** are the stacked spectral plots for the HalM2 state (**Figure 7D**) and the HalM2:AMPPNP:HalA2 state (**Figure 7E**). In these plots, the exchange time increases from the bottom to the top. The mass increase of peptide 110−118 upon deuterium exchange at longer time points is evident from visual inspection. It is also evident that peptide 110−118 uptakes more deuterium in the HalM2 state (**Figure 7D**) than in the fully liganded state (**Figure 7E**). If necessary, clicking on any of the subplots in **Figure 7D** or **Figure 7E**, will allow the user to manually modify the assignment of sticks in the main data viewer (**Figure 7C**). Upon stick assignment/unassignment, the HDX-processing software will recalculate the deuterium uptake values, and all of the plots will be updated in real time. Similarly, clicking on individual data points in panel B will populate the data viewer in panel C for manual stick assignment.

After sticks have been assigned for each peptide and exchange time point for all biochemical states, the standard deviation of the measured uptake values should be checked. This is easiest to perform with the coverage map and stacked spectral plot (**Figure 8**). In the coverage map (**Figure 8C**), select the state and exchange time of interest, then select relative uptake standard deviation. The peptides in the coverage map will be colored according to the standard deviation in the measured deuterium uptake value. In this way, it will be very easy to visually identify peptides that have isotope stick mis-assignments. Clicking on the outlier peptide (with high standard deviation) in the coverage map will populate the main data viewer and the stacked spectra plot with HDX data from the outlier peptide. The stick assignments for each time point and charge state can then be modified as needed to correct the measured uptake value. Again, the HDX-processing software will update all of the data displays in real time as the stick assignments are changed.

After fully curating the HDX data set, the significance of the deuterium uptake difference measurement for each peptide needs to be considered prior to data interpretation. Using an approach described by Engen and co-workers[29], we have estimated an average uptake value of 0.1 ± 0.1 Da for the HalM2 protein using the workflow described in the above protocol[20]. This value is consistent with the errors reported by others for similar bottom-up, continuous exchange HDX workflows[29,30]. Recently, Politis and co-workers developed Deuteros, a useful open source tool (implemented in MATLAB) for rapidly determining significant uptake differences in HDX data sets[28]. Representative input files for Deuteros ("State_Data_for_Deuteros" and

"Difference_Data_for_Deuteros") are included in the **Supporting Information**. If exported directly from the HDX-processing software described in this protocol (**Table of Materials**), the difference and state data files will have the appropriate format for file reading by Deuteros. If HDX data are generated on a different LC-MS system, data files resembling the ones provided in the **Supporting Information** will have to be manually constructed.

The Dueteros workspace is shown in **Figure 9**. Once the data are imported into Deuteros, the user selects the desired confidence limit (>98% recommended) and clicks **Import & Calculate**. For the flattened data map, select **coverage** for data type and **absolute** for color scale. Click **plot**. For the woods plots, select **binary filter** as data type, the desired confidence filter and enable the sum. Upon clicking **plot**, Deuteros will plot all peptides at each exchange time point as a function of their position in the amino acid sequence and their deuterium uptake values. Peptides that exhibit significant exchange will be colored either red or blue, depending on whether the peptide takes up more or less deuterium, respectively, according to the difference being calculated. The significance value reported in each plot (ranging from 0.39 to 0.72 Da in the data plotted in **Figure 9**) is calculated from the standard deviations of the uptake differences measured for every peptide as determined from the replicate measurements present within the data set. The confidence intervals are shown as dashed bars across each individual plot. If enabled, the "Sum" simply adds the uptake difference measured at each time point for every peptide. Finally, for visualization of the uptake data on the three-dimensional structure of the protein of interest, select **export uptake** under PyMOL options, then **export**. Deuteros will generate a PyMOL script that can be dragged and dropped into the PyMOL workspace containing the open pdb file of the protein of interest.

The HDX-MS workflow presented in this protocol was used to characterize the biochemical properties of an enzyme (HalM2) that catalyzes a series of post-translational modifications onto a genetically-encoded peptide (HalA2)[20]. In **Figure 10**, representative HDX-MS results are shown for the binding of the HalA2 precursor peptide to the HalM2:AMP-PNP complex. Panel A shows the HalM2 coverage map, where the color indicates the relative uptake difference between the [HalM2:AMPPNP] biochemical state and the [HalM2:AMPPNP:HalA2] state. Deuteros was used to map these uptake differences onto a homology model of the HalM2 enzyme (**Figure 10B,C**). Peptides that are colored red indicate a decrease in deuterium uptake upon HalA2 binding, suggesting that these regions of HalM2 may be involved directly in binding of the precursor peptide. To investigate this hypothesis, regions I-III were mutated and the kinetic properties of the variant enzymes were investigated. Mutations in region I and III both led to significant perturbations in the HalA2 peptide binding affinity, suggesting that these regions of HalM2 are either interacting with the peptide substrate directly, or are required to form structures that enable HalA2 binding. In contrast, mutation of region II had no effect on HalA2 binding affinity, but this mutant was almost devoid of catalytic activity. One explanation for this finding is that the organization of region II observed upon HalA2 binding triggers a conformational change that activates the enzyme. It should be noted, that prior to this study, no information was available on the HalM2-HalA2 binding mode or on the catalytically relevant conformational changes in the system, primarily because the large size and flexible nature of both HalM2 and HalA2 have precluded structural studies. Thus, these representative data on the HalM2 lanthipeptide

synthetase illustrate how HDX-MS can be used to rapidly locate functionally relevant regions of structurally dynamic enzyme systems−even in the absence of high-resolution structural data.

FIGURE LEGENDS:

Figure 1: A continuous exchange, bottom-up HDX-MS workflow. See text for details.

Figure 2: The HDX rate depends on protein conformational dynamics (k_{open} and k_{close}) and the pH-dependent rate of chemical exchange of the N-H bond for an N-D bond (k_{chem}).

Figure 3: Representative LC-MS data for the triplicate HalM2 reference samples. The number in the top right corner of each panel represents the total ion counts. Each row shows data for a different reference sample. The first column (**A–C**) shows the total ion chromatograms (TICs). The large peak at 9.3 min represents large, undigested peptides. The middle column (**D–F**) shows a closer view of the TICs between 3 and 8 min. Note the good agreement of the shapes of the profiles, which indicates a similar underlying mixture of peptide signals in each reference sample across the entire chromatogram. The third column (**G–I**) shows mass spectra for each reference sample generated by summing all of the mass spectra recorded between time point 5.0 and 5.1 min of the chromatographic run. Visual inspection of this data indicates that many of the same peptides are being detected in each sample.

Figure 4: Representative proteomics software output for a HalM2 reference sample. The top left panel shows the complete list of HalM2-derived peptic peptides detected in the MS data. Note that the "OK Filter" is showing the green checkmark. This filters the data according to the confidence score and removes peptide identifications of low confidence. The top bar of the right panel (highlighted in blue) shows cumulative statistics for the set of peptides with high scores. The most important statistics are the number of peptides detected (in this case 1,421) and the sequence coverage (in this case 99.6%). Additional statistics for every peptide are also shown in the top right panel. Each column in this data table is sortable. The bottom panel shows all of the MS signals that were matched to a predicted HalM2-derived peptic peptide. Note that the majority of MS signals were assigned and are colored blue.

Figure 5: Additional thresholding in the HDX-processing software. The proteomics software output for each of the three HalM2 reference samples is imported into the HDX-processing software (left panel). After importing data, additional thresholding is performed (right panel). The "minimum consecutive products" field refers to fragment ions generated by cleavage of adjacent peptide bonds in the peptide. The "minimum MH+ error" parameter allows the user to define the acceptable mass accuracy, and the "file threshold" allows the user to restrict peptides to only those peptides that were detected in all three reference files.

Figure 6: Representative data for a HalM2 reference sample (red) and a HalM2 sample incubated in D_2O buffer for 5 min (green). Both samples were subjected to the bottom-up LC-MS workflow described in protocol section 3. The left column shows mass spectra summed over the 6.0−6.1 min time window. The three right columns show closer views of the same mass

spectra, where the shift to higher m/z values in the deuterated sample (green, top) is obvious for most of the peptide signals.

Figure 7: Screenshot of workspace in the HDX-processing software. (**A**) The list of HalM2-derived peptides obtained from analysis of HalM2 reference samples with the proteomics software (**Figure 3**) and subsequent thresholding in the HDX-processing software (**Figure 4**). The currently selected peptide (HIDKLTVGL, spanning HalM2 residues 110−118) is highlighted in blue. (**B**) The deuterium uptake curves (as a function of exchange time) for the two biochemical states of interest: the free HalM2 enzyme, and the HalM2 enzyme bound to AMPPNP and the precursor lanthipeptide, HalA2. (**C**) The actively selected sample (in this case, one of the undeuterated HalM2 reference samples). The blue sticks in panel C can be manually assigned/unassigned as needed if they were not properly assigned by the HDX-processing software during initial data processing. (**D,E**) Stacked spectral plots for the HalM2 state (**D**) and the HalM2:AMPPNP:HalA2 state (**E**). The time-dependent increase in deuterium uptake is readily visible, as is the uptake difference between the two biochemical states.

Figure 8: Minimizing standard deviation in measured uptake values. The coverage map in the HDX-processing software (panel C) provides a convenient means to rapidly identify peptides with large standard deviations in their measured deuterium uptake values. In this hypothetical case, some of the isotope peaks (blue sticks) for the HalM2 derived peptide spanning residues 110−118 are unassigned for the 5 min exchange time points (the gray sticks in panel B). This is leading to a large standard deviation in the deuterium uptake value measured for the 5 min exchange time point. The large standard deviation is readily obvious from the blue color of the 110−118 peptide in the coverage map (panel C) and from the data point scatter and large error bar in the uptake plot (panel A).

Figure 9: The Deuteros workspace. In this example, the uptake difference values were calculated in the HDX-processing software by subtracting the deuterium uptake of the HalM2:AMPPNP:HalA2 state from that of the HalM2:AMPPNP state. The goal of the comparison was to visualize how peptide (HalA2) binding altered the structural dynamics of the HalM2:AMPPNP complex. The data set contained 4 exchange time points (0.5, 5, 30, and 240 min). The uptake difference for every peptide at each exchange time point is illustrated in the Woods plots. The colored peptides in the Woods plots indicate peptides that exhibit a significant uptake difference, as defined by the standard deviation of the replicate measurements present in the data set and the user-defined confidence limits selected in Deuteros.

Figure 10: HDX-MS data guides functional analysis of peptide binding and allosteric activation in the lanthipeptide synthetase HalM2. The deuterium uptake change upon binding of the HalA2 precursor peptide to the HalM2 lanthipeptide synthetase was investigated. The uptake difference for each peptide after a 5 min exchange reaction is plotted (**A**). This plot was generated in the HDX-processing software. Peptides colored red and blue undergo less and more deuterium uptake, respectively, in the presence of the HalA2 peptide. These HDX “hot spots” are mapped onto a HalM2 homology model (**B,C**). The identification of peptides undergoing significant exchange differences was determined in Deuteros. The PyMOL script used to map the difference

values onto the homology model was generated in Deuteros.

DISCUSSION:

The HDX-MS workflow presented in this protocol provides a remarkably robust platform for mapping the spatial distribution of structurally dynamic elements in proteins and for investigating how these dynamics change in response to perturbation (ligand binding, enzyme mutagenesis, etc.). HDX-MS holds several distinct advantages over other structural biology approaches that are commonly used to investigate conformational dynamics. Most notably, only small quantities of protein are needed. Using the workflow described herein, a 1 mL sample of 1 µM protein provides enough material for triplicate HDX reactions each containing 5 exchange time points. Moreover, there is virtually no size limit on the protein of interest, and protein complexes are equally amenable to the HDX-MS approach. The size limit is only restricted by the extent to which the peptic peptides can be resolved chromatographically, and in the m/z and ion mobility dimensions. Thus, for many proteins/protein complexes, HDX-MS will provide valuable information on protein-protein interaction interfaces, ligand binding sites, conformational dynamics, and allosteric networks. Finally, the HDX reaction is performed under gentle, near-native conditions without the need for site-specific labeling/engineering of the protein, which should help to ensure that the endogenous activity is preserved. The main limitation of the approach (in terms of the mechanistic information it provides on the protein of interest) is that the peptide level HDX data is of inherently low spatial resolution. Thus, the data is sufficient for inferring that a change in secondary structure is occurring, but the mechanistic impacts of the structural perturbation requires higher resolution (e.g., NMR, cryoEM, or X-ray crystal) structures, computation studies, and/or biochemical studies to fully interpret.

In order to ensure the reproducibility and reliability of the results, several critical aspects of the protocol should be kept in mind. First, the extent of deuterium exchange during the HDX reaction and the extent of back exchange during the work-up and analysis are strongly dependent on pH, time, and temperature. Thus, the procedures for the preparation of buffers, HDX reactions, and LC-MS methods must be as systematic as possible to minimize variation in these physical parameters across data sets. Whenever possible, HDX reactions for biochemical states to be compared should be performed by the same researcher on the same day, and the LC-MS data for these samples should be collected over consecutive days with the same batch of solvent. Over time, the efficiency of the pepsin digestion will also decrease, so it is optimal for samples that will be compared to be digested and analyzed within a relatively narrow time window−especially if the pepsin column is being used to digest many other types of samples. It is good practice to periodically check the digestion efficiency of the pepsin column using a standard protein. To achieve this, a dedicated HDX-processing software project can be set up where newly digested samples can be compared with older samples to ensure that the same set of target peptides are being detected with similar intensities.

While the workflow presented in this protocol should provide adequate data for many enzyme/protein systems, there are several potential points of optimization. First, the time scale of the HDX reaction can be modified to capture dynamics that are faster/slower. Most notably, many enzymes will contain highly dynamic elements that become fully exchanged within several

seconds. If these extremely dynamic elements are of interest, methods for pre-steady state, continuous exchange HDX-MS have been reported in the literature[31]. Second, the LC method conditions can be readily changed to alter the digestion efficiency (which ultimately determines sequence coverage) and the deuterium retention (which ultimately determines the sensitivity of the method). Considering the duration and temperature of the pepsin digestion, slower flow rates and higher temperature will favor more thorough digestion of the protein. The protein concentration in the HDX assay can also be increased if the signal intensity is too low, or decreased if the pepsin digestion is too inefficient. Considering the acetonitrile gradient used to separate the peptic peptides on the analytical C18 column, a faster gradient will preserve the deuterium label, but at the expense of the chromatographic resolution of the peptic peptides present in the digested reaction mixture. For smaller proteins (~200 amino acids) where fewer peptic peptides are present in the digest, a faster LC gradient may be easier to implement. For larger proteins such as HalM2 (~1,000 amino acids), a longer gradient is needed to resolve the additional spectral complexity generated by the larger number of peptides in the mixture. In this latter scenario, the inclusion of a gas phase ion mobility separation can help to dramatically improve the peak capacity of the analysis. The inclusion of the ion mobility separation comes at a cost of a slightly reduced signal to noise ratio. Finally, it should be noted that the MS data for the HDX samples do not need to be collected in MS^E mode. The MSMS portion of the MS^E duty cycle (i.e., the high collision energy segment, step 3.3.7.2) is only required for the reference samples in order to define the peptide list (protocol section 4). Thus, HDX samples should be analyzed in MS-only mode to increase the signal:noise ratio.

If the workflow described in this protocol is operating properly, fully exchange peptides should exhibit a relative deuterium uptake value of 60%−70%. If the deuterium uptake is found to be substantially lower than this (for a solvent-exposed, unprotected peptide), the most likely explanation is that the pH of the sample is changing during some portion of the workup/analysis. In this scenario, a microtip electrode should be used to carefully monitor the pH of the HDX assay and of the quenched reaction aliquot. The pH of the LC-MS solvents should also be checked. To minimize the occurrence of this problem, it is strongly recommended to prepare and store concentrated stock solutions of all buffers and reagents needed for the assay (as outlined in protocol section 1).

In recent years, HDX-MS has emerged as a powerful analytical tool to investigate protein structural dynamics. The development of commercially available LC-MS systems designed and optimized for HDX experiments (such as the system used in this study and listed in the materials) coupled with powerful software packages, has extended the HDX-MS approach into many academic and industrial labs, and has transformed what was once a niche technique 15 years ago into a more user-friendly analytical platform. Despite the limitations in spatial resolution, HDX-MS provides highly quantitative and reproducible measurements on protein motions and is ideally suited for studying conformationally dynamic enzymes that are difficult to investigate with other approaches. Because of these characteristics, HDX-MS fills an essential niche in the structural biology of disordered and dynamic protein systems which have relevance in many fundamental areas of biology and medicine. As such, HDX-MS analyses are expected to remain an important tool in the arsenal of the structural biologist for the foreseeable future.

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DISCLOSURES:

We have nothing to disclose.

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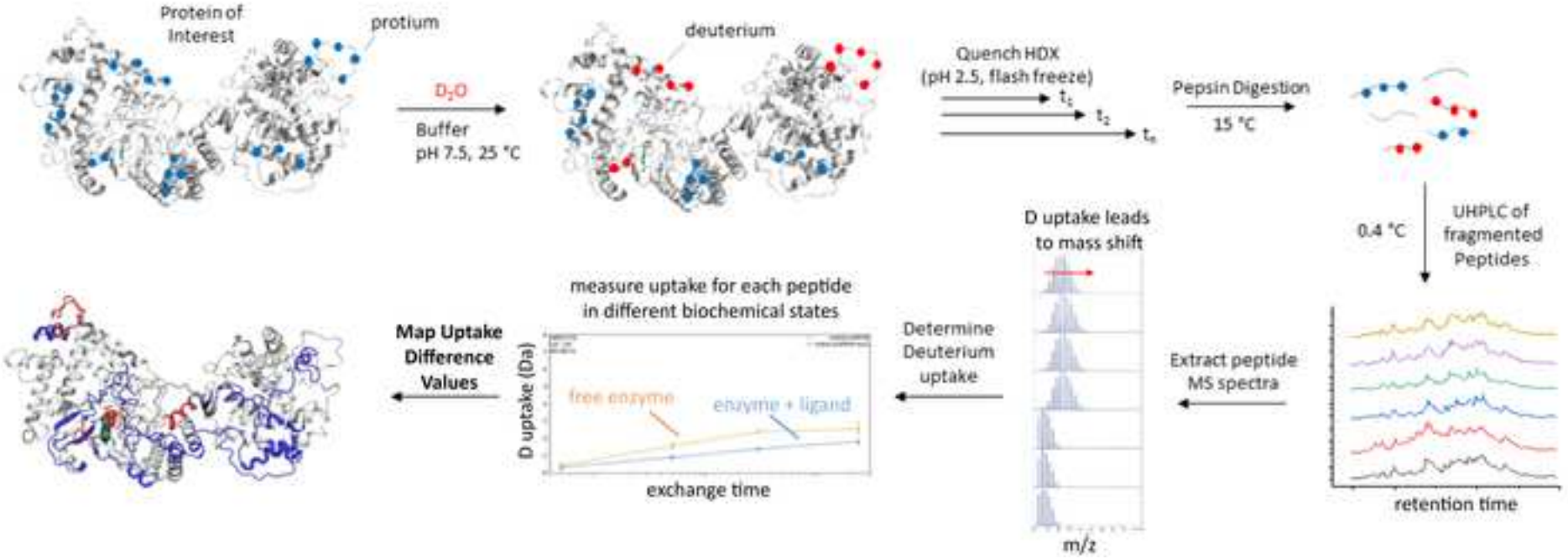
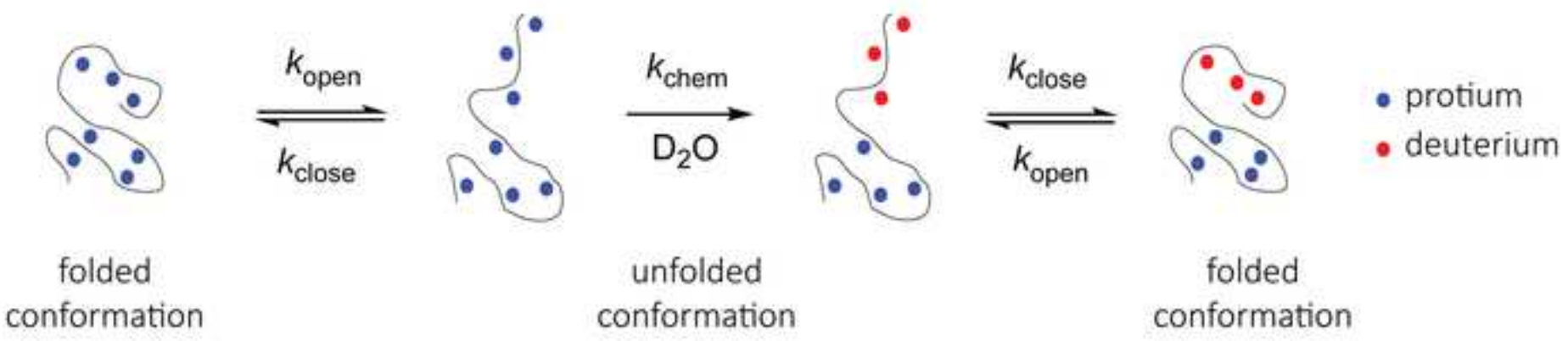
31. Rob, T., Gill, P. K., Golemi-Kotra, D., Wilson, D. J. An electrospray ms-coupled microfluidic device for sub-second hydrogen/deuterium exchange pulse-labelling reveals allosteric effects in enzyme inhibition. *Lab on a Chip*. **13**, 2528-2532 (2013).

Figure

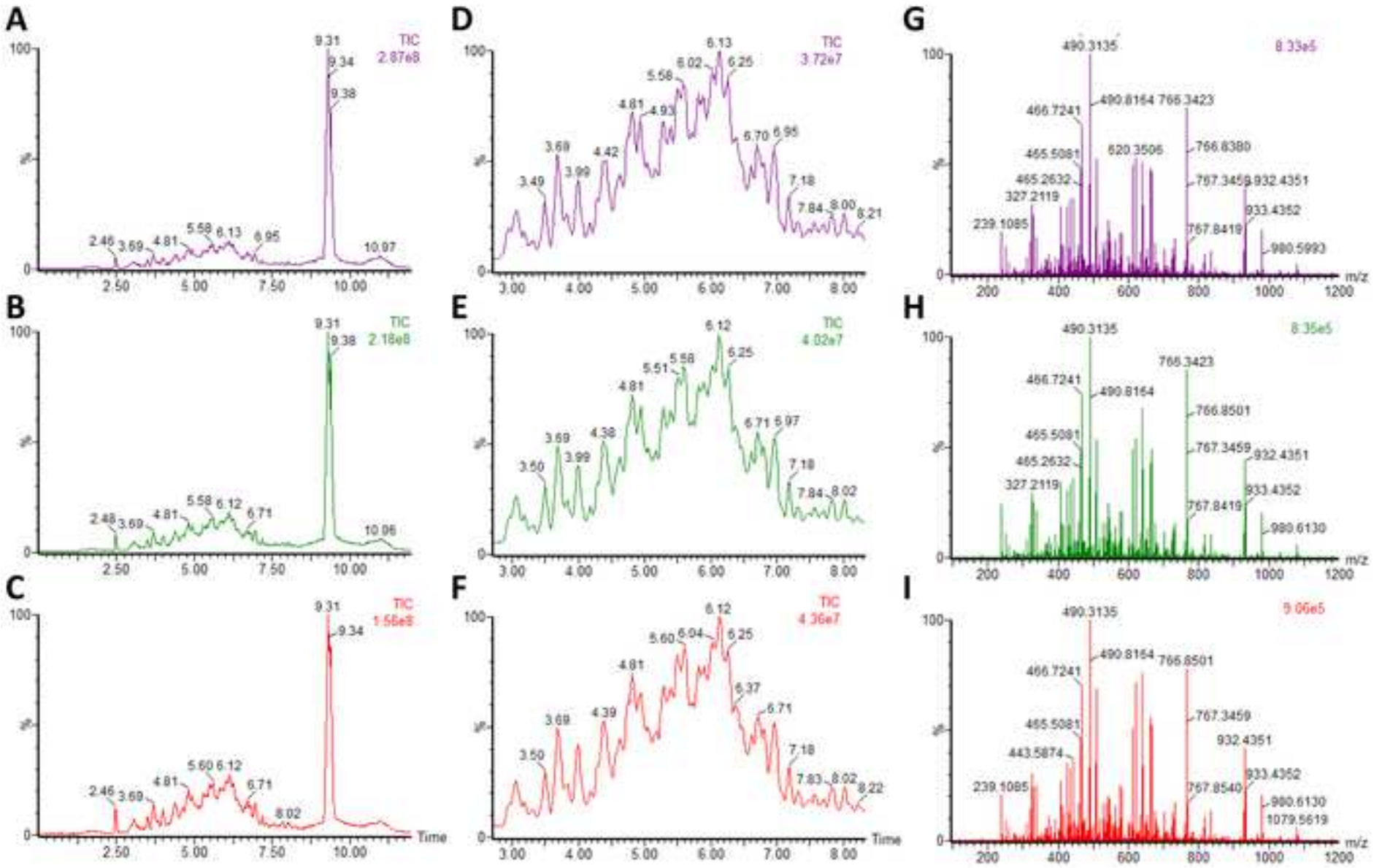


Figure



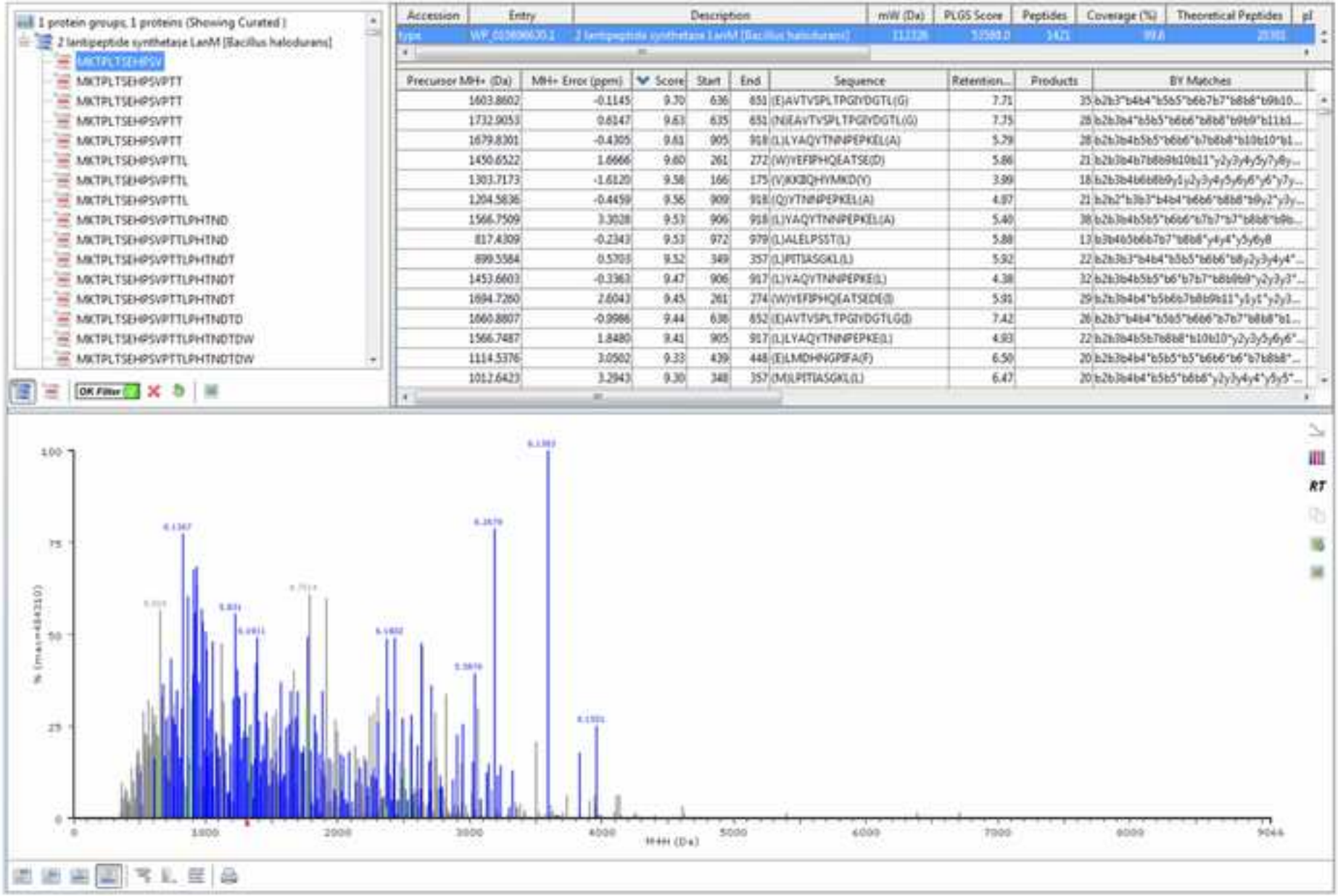
Figure

Click here to access/download;Figure;Fig3-01.tif



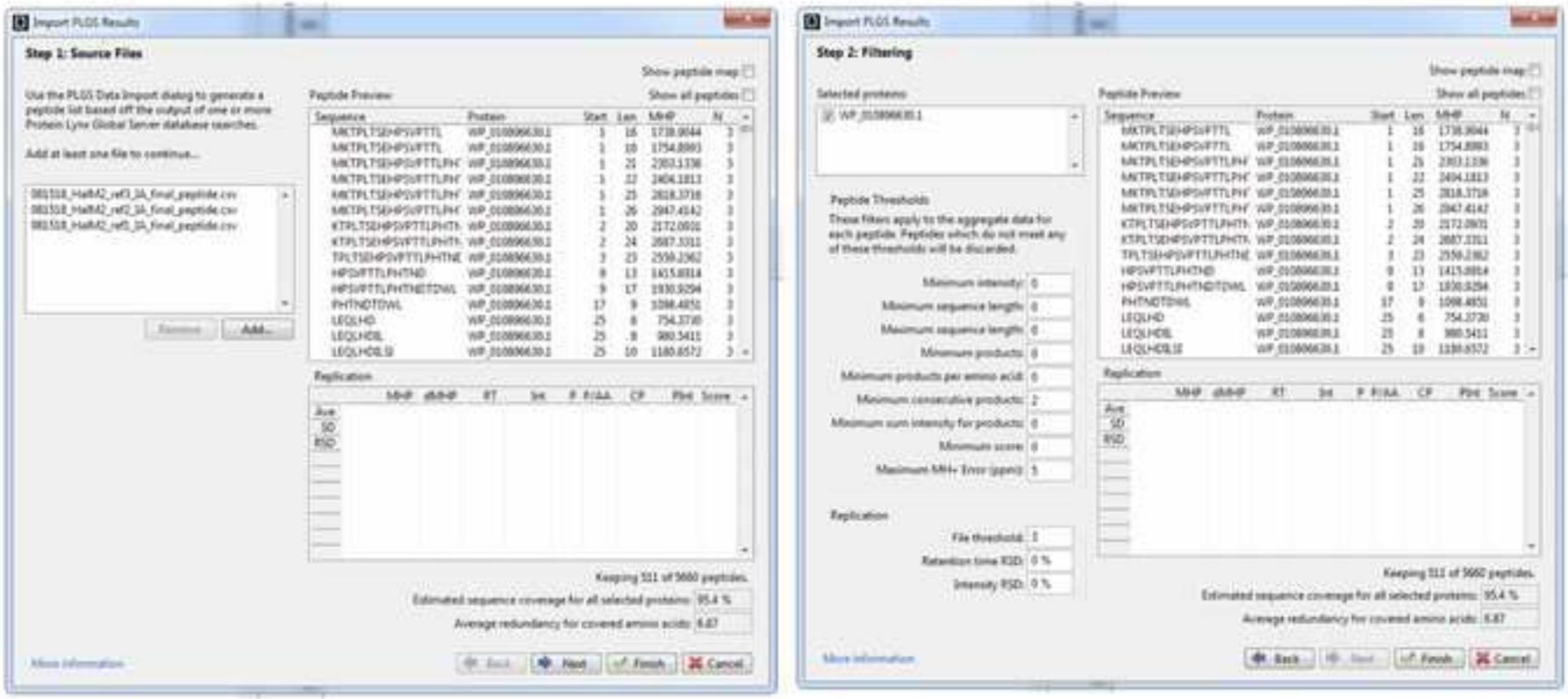
Figure

Click here to access/download;Figure;Fig4-01.tif



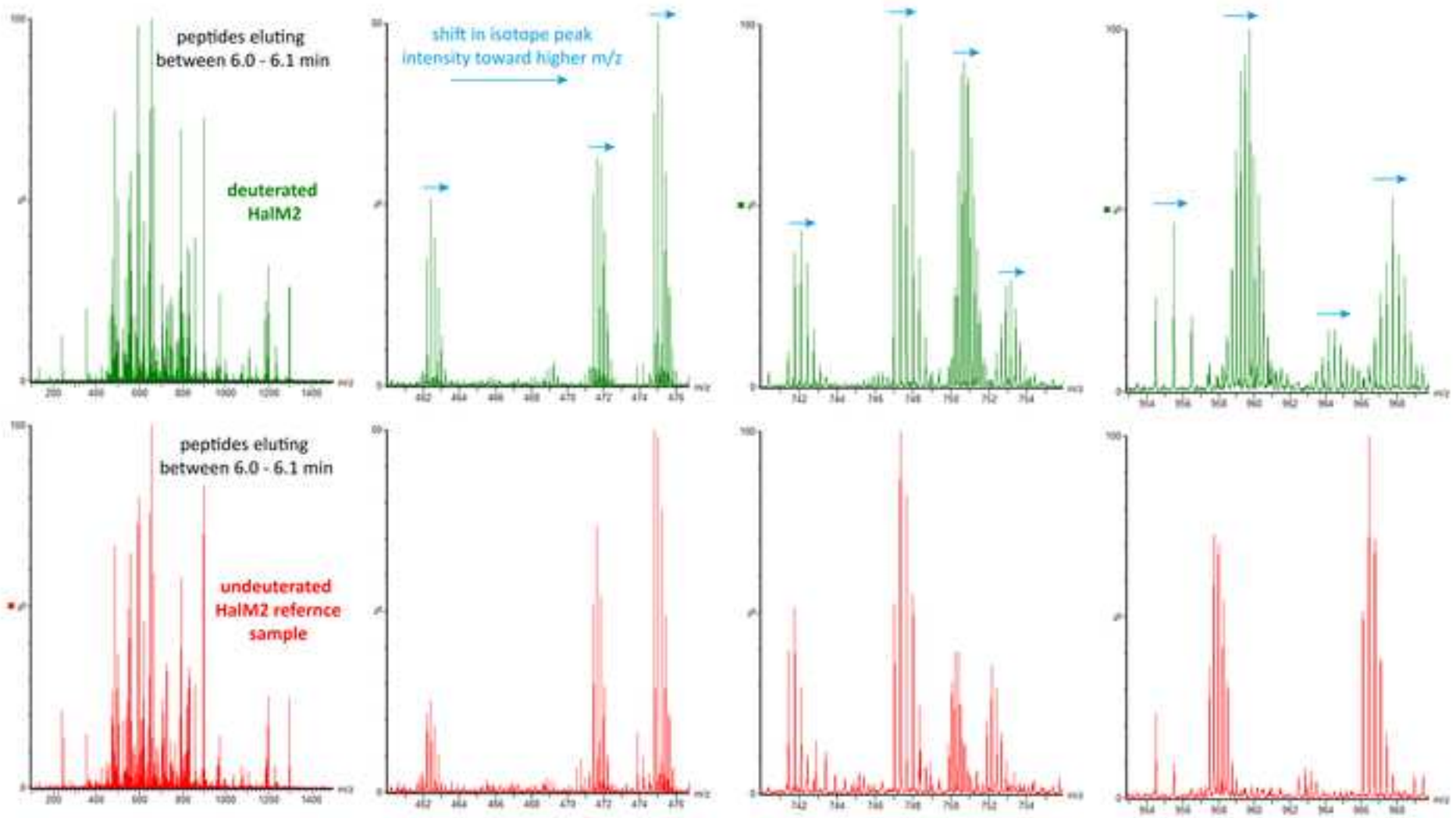
Figure

Click here to access/download;Figure;Fig5-01.tif



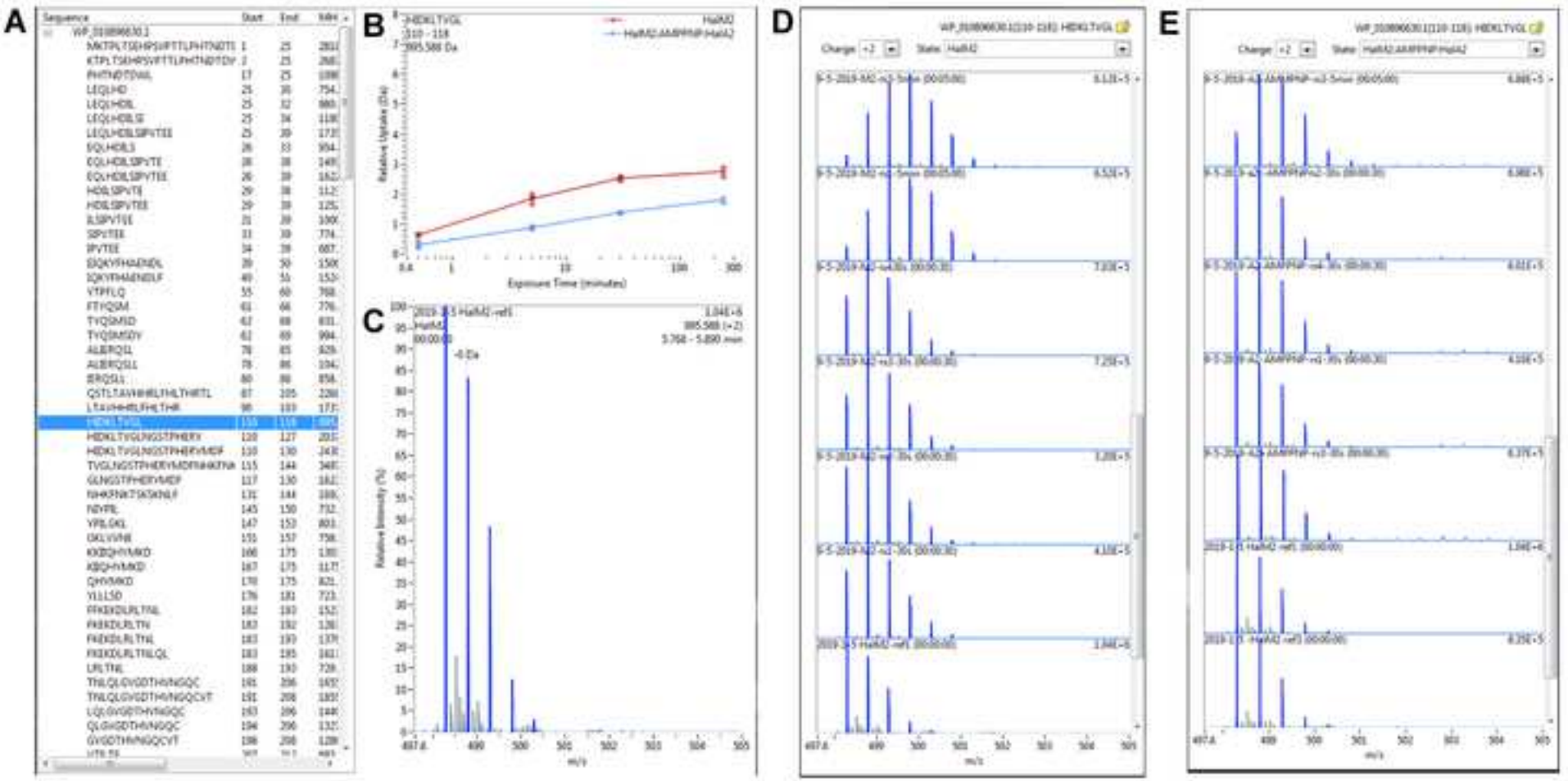
Figure

Click here to access/download;Figure;Fig6-01.tif



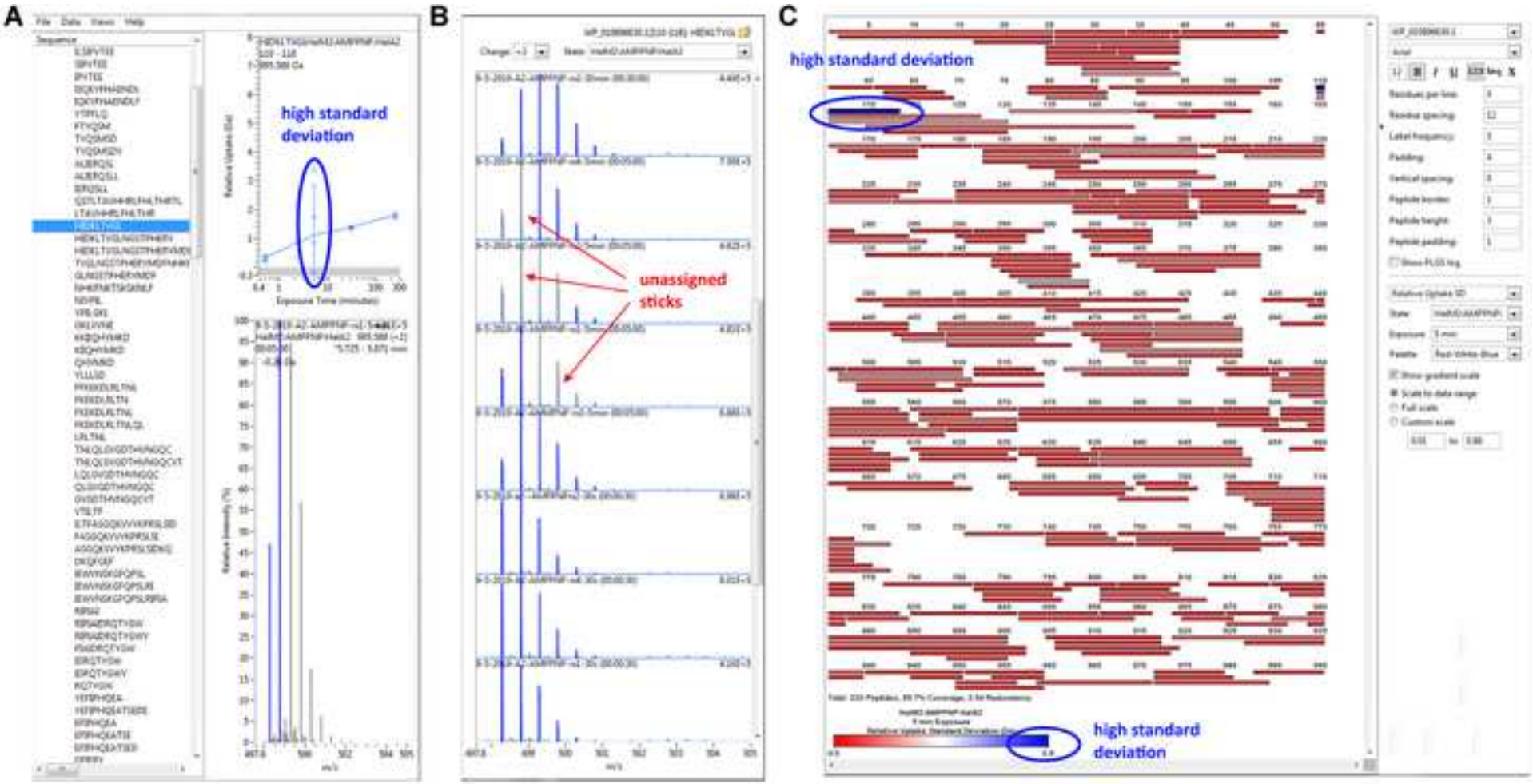
Figure

Click here to access/download;Figure;Fig7-01.tif



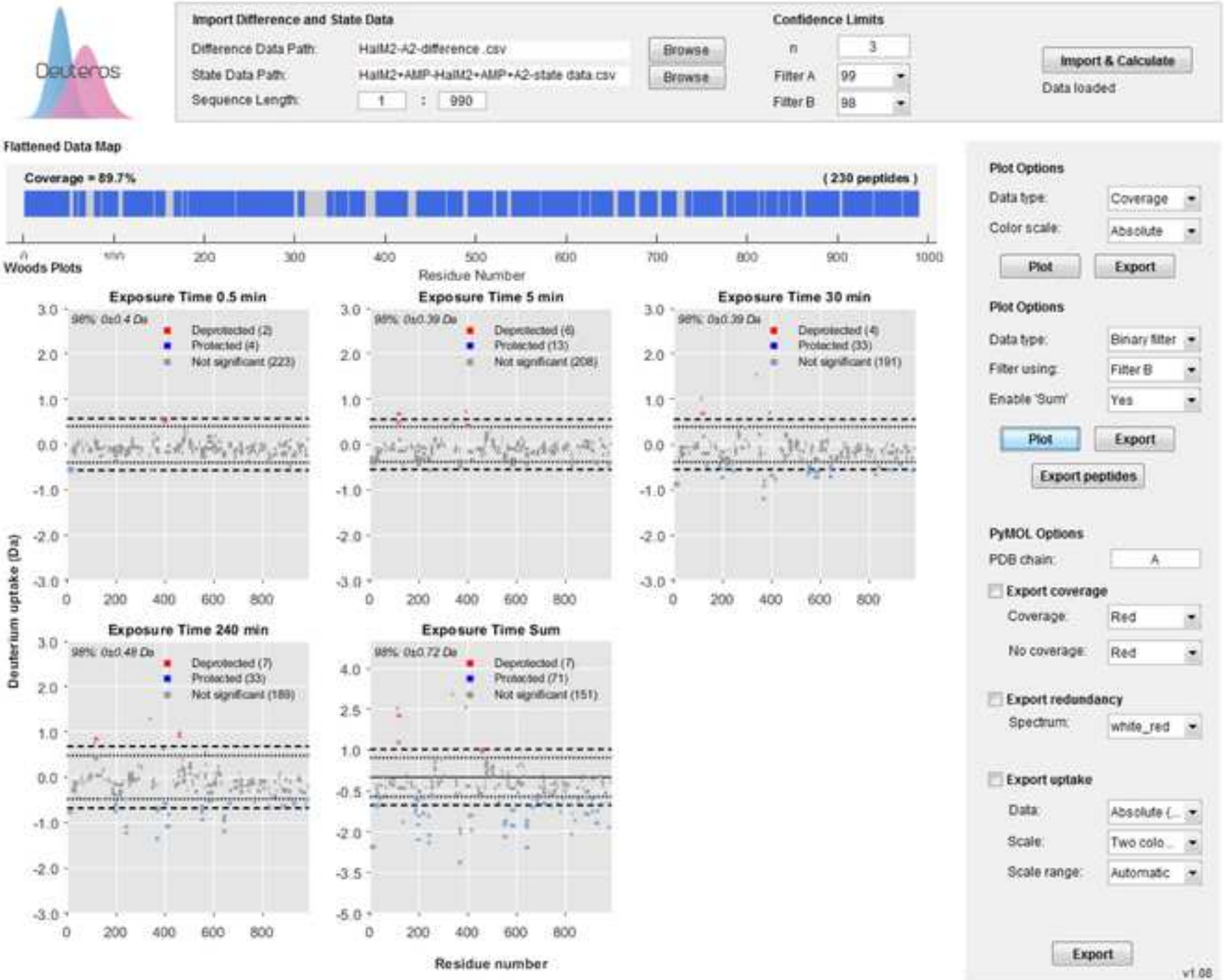
Figure

Click here to access/download;Figure;Fig8-01.tif



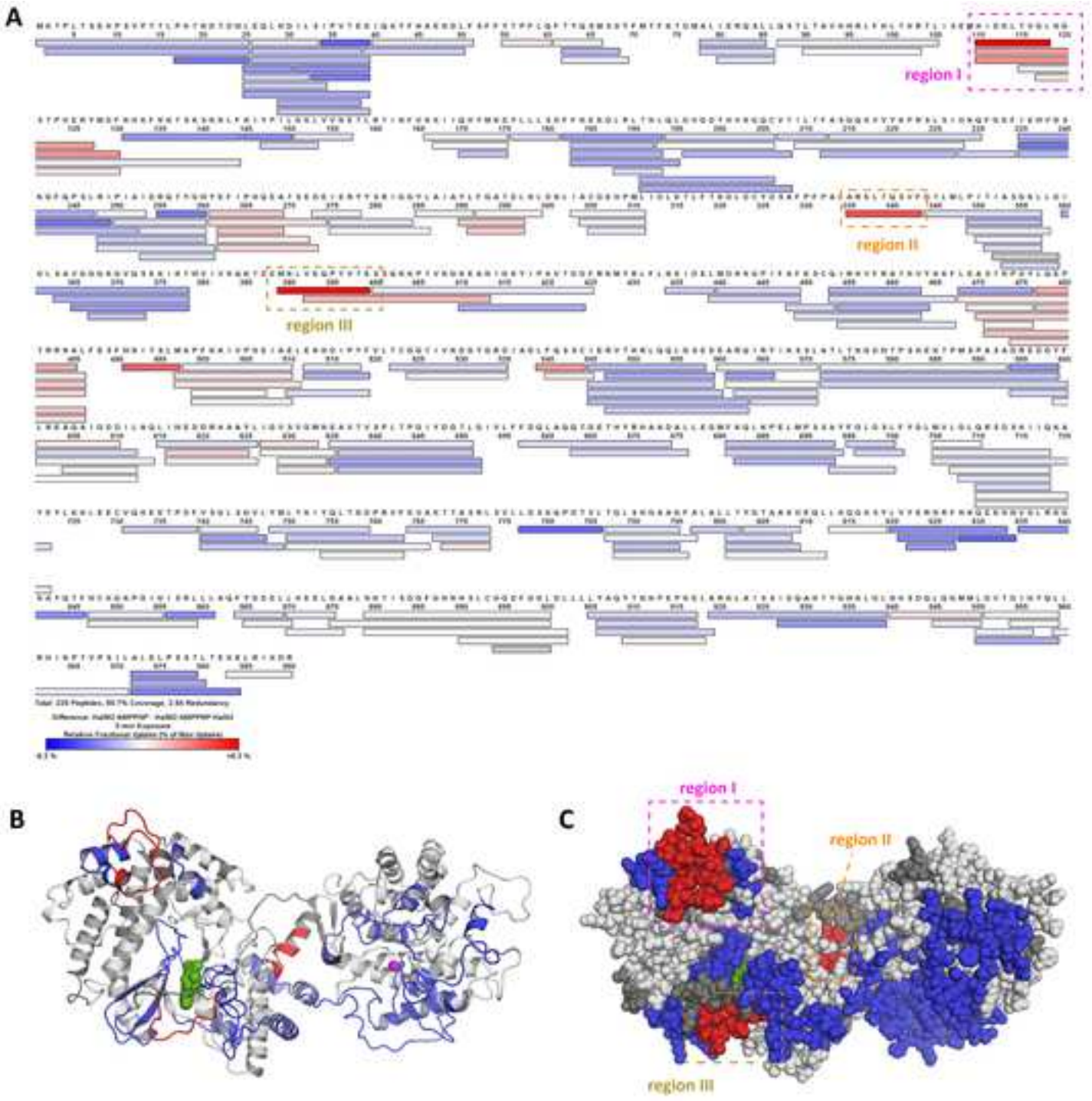
Figure

Click here to access/download;Figure;Fig9-01.tif



Figure

Click here to access/download;Figure;Fig10-01.tif



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Reagents			
[glu-1]-fibrinopeptide B (Glu-Fib)	BioBasic	NA	
0.5 mL Amicon Ultracel 10k centrifugal filtration device (Millipore)	Milipore Sigma	UFC501096	
acetonitrile	Fisher	A955-1	
AMP-PNP	SIGMA	A2647-25MG	
ATP	SIGMA	a2383-5G	
D2O	ALDRICH	435767-100G	
formic acid	Thermo Fisher	28905	
guanidine-HCl	VWR	97063-764	
HEPES	Fisher	BP310-1	
Magnesium chloride	SiGMA-Aldrich	63068-250G	
Potassium chloride	BioBasic	PB0440	
potassium phosphate	BioBasic	PB0445	
TCEP Hydrochloride	TRC Canada	T012500	peptide was synthesized upon request
Name of Material/ Equipment	**Company**	**Catalog Number**	**Comments/Description**
software			
Deuteros	Andy M C Lau, et al	version 1.08	
DynamX	Waters	version 3.0	
MassLynx	Waters	version 4.1	
Protein Lynx Global Server (PLGS)	Waters	version 3.0.3	
PyMOL	Schrödinger	version 2.2.2	
Name of Material/ Equipment	**Company**	**Catalog Number**	**Comments/Description**
Instrument and equipment			
ACQUITY UPLC BEH C18 analytical Column	Waters	186002346	

ACQUITY UPLC BEH C8 VanGuard Pre-column	Waters	186003978
ACQUITY UPLC M-Class HDX System	Waters	
HDX Manager	Waters	
microtip pH electrode	Thermo Fisher	13-620-291
Waters Enzymate BEH column or Pepsin solumn	Waters	186007233
Waters Synapt G2-Si	Waters	

Manuscript changes made in response to editorial comments:

"*Lines 209-213 must be made into a note*."

"*Split long steps (e.g.3.3.7) into 2 or more steps*."

"use notes sparingly"

- We have made numerous changes to the protocol to reflect these editorial comments. Please let us know if additional alterations are needed.
- many of the steps were broken down into sub-steps
- the imperative voice is now used for all steps
- the use of notes was minimized

"*Protocol Detail: … Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps….*"

- We have added more specific details in many parts of the protocol, especially in Steps 4 and 7, which involve many software commands for data processing.
- Highlighted protocol length is 3 pages

"*Results:* *Avoid subheadings and numbered lists in this section*."

- The text has been altered to remove all subheadings and numbered lists.

"*Discussion:* *JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol*."

- The requirements/recommendations for the Discussion section have been followed

"*Figures:* *Scheme 1 should be labeled as figure 2*."

- Scheme 1 has been relabelled as Figure 2 and the numbering for all Figures has been updated throughout the text.

"*Commercial Language:* *JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Waters Synapt G2-Si, DynamX 3.0 software (Waters), etc.*"

- All commercial language has been removed from the manuscript
- Specific details regarding the MS instrument and software are now provided only in the Materials List

Manuscript changes made in response to reviewer comments

Reviewer #1:

"*A number of other JoVE papers exist on this subject (one highly similar, but a bit old), and these *should be cited* (10.3791/55464; 10.3791/50839; Distinguishing Allosteric Effects from Orthosteric Binding in Protein-Ligand Interactions (in production))*."

- Several lines of text have been added to the Introduction (lines 123-126) to cite this earlier literature (references 25 and 26 in the revised manuscript.

"*The citations are rather inadequate. In particular, the paragraph on 'advantages' (lines 91 - 106) needs at least some literature support for the claims made*"

- References 7 and 10-14 and have been added.

"*In the same paragraph, the authors mention intrinsically disordered proteins, which can indeed be a strength of HDX, but requires a different setup than the one described here (10.3791/55464 and/or many publications by the Weis and/or Wilson groups or others*)"

- References 15-18 have been added

Reviewer #2:

"*SI: Cone Gas (L/h) = 0. This setting is not advisable, because the ion optics in the source will get contaminated quickly. A moderate cone gas flow will help keep things clean, thereby ensuring that the instrument operates with high sensitivity over a long period of time*."

- Thanks for noticing this oversight. We have altered the conditions in the SI to read: Cone Gas (L/h) = 50.

"*According to the SI section, the workflow uses MS^E, but section 3.3.7 only mentions MS and MS/MS. The MS^E aspect seems to be lacking in the main text. Please briefly explain the MS^E concept*."

- The MS^E concept is now defined in Protocol Step 3.3.7 and further explained on lines 656-659 of the Results and lines 928-932 of the Discussion
- Protocol step 3.3.7 has also bee broken down into sub-steps to more effectively emphasize the different aspects of MS^E data acquisition.

"*Related to the previous point: MS^E (or MS/MS) are only required for identification of the unlabeled peptides. Here, it appears that the actual HDX data are also acquired in MS^E mode. This is not good, because the high energy channel of the MS^E data provides fragmented peptides that are meaningless for probing peptide deuteration kinetics. In other words, the instrument would spend a lot of time generating useless data. Therefore: the HDX data should be acquired in MS mode, without collision-induced dissociation. Fragmentation is only needed for peptide identification*."

- A note has been added to the protocol (lines 455-457) to emphasize this point.
- The point is also emphasized in the discussion (lines 928-932)

"*The daunting (very long) appendix tables require headers that explain what is being shown here. Just cutting and pasting raw files is not necessarily helpful*."

- The attached .xls files are meant to instruct the reader on how to format their data files for successful processing by Deuteros. A note has been added at the beginning of the S.I. instructing the reader that, if they wish to process the data with Deuteros, then they need to prepare input files in .xls format that look exactly like the sample files shown.
- Additional descriptions of the individual data columns are also now given in the S.I.

"*SI Appendix of identified peptides: Why are most peptides listed numerous times*?"

- This is the format of the "state data" file that is needed as input into Deuteros. The peptides are listed multiple times, because there is a separate entry (row) for each exchange time point (5 time points in our data set – 0, 0.5, 5, 30, and 240 min), and for each biochemical state (two states in our example). We have now clarified this in the S.I.

"*Summary: "structural properties of conformationally dynamic enzymes" change to "structural properties and conformational dynamics of enzymes" (the original wording is confusing because all enzymes exhibit conformational dynamics - some more, some less*)."

- The wording has been altered

Abstract: "in the peptide bond amide" should be "in peptide bond amides".

- Correction made

SUPPORTING INFORMATION

Description of file formats for data processing by Deuteros

Two .xls files are included with this manuscript: "State_Data_for_Deuteros" and "Difference_Data_for_Deuteros." These files show the exact format which is needed for analyzing HDX difference data with Deuteros.[1] If generated directly with the specific HDX-processing software used in this protocol, the files will be correctly read by Deuteros. Otherwise, the user will have to manually generate .xls files using the file structure shown in the supporting files. A description of the required fields for Deuteros input file is provided below.

"Difference_Data_for_Deuteros" file

- Column A: the peptide amino acid sequence
- Columns B-C: the residue numbering for the start and stop of the peptide
- Column D: Any peptide modification that is present (oxidation, etc.)
- Columns E-H: the deuterium exchange time points included in the analysis. For this experiment, time points of 0.5, 5, 30, and 240 min were used. The data in columns E-H represent HDX difference values calculated by subtracting the deuterium uptake of biochemical state 1 from biochemical state 2.

"State_Data_for_Deuteros" file

- Column A: the accession number of the protein (in this case, HalM2)
- Column B-C: the residue numbering for the start and stop of the peptide
- Column D: the amino acid sequence of the peptide
- Column E: lists any peptide modification that might be present
- Column F: The "fragment" column will not be populated with data if the HDX data are collected as described in this protocol. The column should still be included to ensure that the text file is properly read by Deuteros.
- Column G: the maximum number of deuteria that can be taken up by the peptide. This value equals the number of peptide bonds – 1 for each Pro resiude (Pro residues do not have a solvent exchangeable amide).
- Column H: The exact (monoisotopic) mass of the molecular ion.
- Column I: the biochemical state as defined in the HDX processing software (protocol step 6.1.1.). In the sample data show, the biochemical states are the HalM2 enzyme complexed to AMP-PNP, and the HalM2 enzyme complexed to both AMP-PNP and the HalA2 peptide. Note, the peptide deuterium uptake by these two states were subtracted to give the deuterium difference data located in the "Difference_Data_for_Deuteros" file.
- Column J: the deuterium exposure time (in minutes)
- Column K: the centroid mass of the signal, which is calculated as a weighted distribution of the observed isotope peaks.
- Column L: the standard deviation of the centroid mass as determined by the biological replicates included in the data set.
- Column M and N: The deuterium uptake and its standard deviation (in units of Da).
- Column O-P: the peptide chromatographic retention time and standard deviation

Instrumental Settings

The following sections provide settings for HDX-MS data acquisition on a high-resolution electrospray ionization quadrupole time-of-flight mass spectrometer coupled to a liquid chromatography system as described by Thibodeaux and co-workers.[2] There are three portions to the instrumental settings detailed here, the "MS Tune" page settings, the "MS Method" and the "Inlet Method." Together, these parameters establish the ionization conditions in the ESI source, the parameters necessary to effect the gas phase ion mobility separation, the collection of both MS and MSMS data, as well as all chromatographic conditions needed for sample digestion by the pepsin column and analytical separation of the peptic peptides on the analytical C18 column. When used in combination, these instrument and data acquisition settings will provide suitable initial HDX-MS data for most proteins

I. MS Tune

These parameters are defined in the "MS Tune" window of the mass spectrometry data acquisition software. Start by placing the instrument in mobility-TOF mode, positive ion mode, resolution mode, and MS mode. Populate the settings in the various tabs of the MS Tune page with the following values:

- ES+
    - Source
        - Capillary (kV) = 2.8
        - Sampling Cone = 30
        - Source Offset = 30
    - Temperature
        - Source = 80
        - Desolvation = 175
    - Gas Flows
        - Cone Gas (L/h) = 50
        - Desolvation Gas (L/h) = 400
        - Nebulizer (Bar) = 6.5
- Instrument
    - Trap collision energy – off
    - Transfer collision energy – off
    - Detector Control
        - Automatic detector check – not checked
    - Gas Controls
        - Enable manual control – checked
        - Trap = 2.0
        - Helium cell = 180
        - IMS = 90.0
    - Resolving Quadrupole – *not changeable in MS mode*
- TriWave DC
    - Trap DC
        - Enable manual control – checked
        - Entrance= 3
        - Bias=45

        - Trap DC=0
        - Exit= 0
    - IMS DC
        - Enable manual controls- checked
        - Entrance= 20
        - Helium Cell DC= 50
        - Helium Exit= -20
        - Bias= 3.0
        - Exit= 0
    - Transfer DC
        - Enable manual controls- checked
        - Entrance=5.0
        - Exit=15
- TriWave
    - Trap
        - Enable Manual Controls- not checked
    - IMS
        - Enable Manual controls- checked
        - Wave Velocity(m/s) =650
        - Wave Height(v) = 40.0
    - Transfer
        - Enable Manual controls- checked
        - Wave Velocity=175
        - Wave Height= 4.0
- Quad Profile
    - Quadrupole Options= Manual Profile
    - Quadrupole MS Profile
        - 1) Mass = 400, Dwell Time (% Scan Time) = 25, Ramp Time(%Scan Time) = 25
        - 2) Mass = 600, Dwell Time (% Scan Time) = 25, Ramp Time(%Scan Time) = disable
        - 3) Mass = 800
- RF setting
    - StepWave RF Amplitude auto values
        - StepWave=300 volts
        - Ion Guide= 350 volts
    - TriWave RF Amplitude
        - Trap=300 volts
        - IMS(Mobility) 250 volts
        - Transfer= 350 volts

- Step Wave
    - StepWave 1
        - Enable Manual Controls- checked
        - Enable Reverse Operation- not checked
        - Wave velocity(m/s)=300

        - Wave Height(V)= 30.0
    - StepWave 2
        - Enable Manual controls- checked
        - Wave velocity(m/s)=200
        - Wave Height(V)= 30.0
    - StepWave DC
        - Enable Manual controls- checked
        - StepWave 2 offset= 20
        - Diff Aperture 1=0.0
        - Diff Aperture 2= 0.0
    - Source Ion Guide
        - Enable Manual controls- checked
        - Wave Velocity(m/s)=300
        - Wave Height (V)=1.0
    - RF setting
        - Enable Manual controls- checked
        - Step/Wave= 200
        - Ion Guide= 350
- IMS-Config
    - IMS Wave Height
        - Use Variable IMS Wave Height- not checked
    - IMS Wave Velocity
        - Use Variable IMS Wave Velocity-checked
        - Wave Velocity Ramping- checked
        - Start Velocity (m/S)=900
        - End Velocity (m/S)=300
        - Ramp Over Full IMS Cycle-checked
- Trapping
    - Targeted Enhancement
        - Use Targeted Enhancement-not checked
        - Target Enhancement Wave= 556.0
        - No Wideband Enhancement Table File Selected
    - Mobility Trapping
        - Use Manual Release-checked
        - Release Time(µs)= 500
        - Trap Height(V)= 15.0
        - Extract Height(V)=0.0
    - Mobility Delay
        - Trim Drigt Time for wave velocities- not checked
        - Enable Mobility separation delay after Trap release-checked
        - IMS Wave delay(µs)= 450

II. MS Method

These parameters are defined in the "MS Method" window of the mass spectrometry data acquisition software. Start by creating a new HDMS^E function, then update the fields in the various tabs of the window with the values provided below. Note, the HDMS^E protocol given here is only needed during data collection for the reference files. When collecting data for deuterated samples, the "High Energy" function under the "Collision Energy" tab is not needed. For these samples, the user should create an analogous "HDMS" method that lacks high energy peptide fragmentation steps.

- Acquisition
    - Acquisition Times
        - Start Time = 0 min
        - End Time = 12 min
    - Source
        - ES
    - Acquisition Mode
        - Polarity – positive
        - Analyzer mode – resolution
- TOF MS
    - Da Range
        - Acquire MSe data over the range
            - Low Mass – 100 Da
            - High Mass – 2000 Da
    - Scanning Conditions
        - Scan Time – 0.4 sec
        - Data format – continuum
- Mobility
    - Instrument conditions
        - Override IMS wave velocity in tune file – unchecked
        - Override transfer wave velocity in tune file – unchecked
    - Function 1 – Low Energy
        - Apply rule file for charge state/Drift time stripping – unchecked
    - Function 2 – High Energy
        - Apply rule file for charge state/Drift time stripping – unchecked
- Collision Energy
    - Function 1 – Low Energy
        - Trap collision energy – on – 6 V
        - Transfer collision energy – off
    - Function 2 – High Energy
        - Ramp trap collision energy – off
        - Ramp transfer collision energy
            - Use collision energy ramp – 21 to 44 V
- Cone Voltage – all default settings
- Lockmass
    - Collect lockmass – do not apply correction

III. Inlet Method

All of the following settings are defined in the Inlet Method window provided by the mass spectrometry data acquisition software. For HDX data acquisition, a "trapping" method is used. The trapping method has two phases – a 3 min trapping phase where the auxiliary solvent manger (ASM, a binary HPLC pump) is pushing sample through the pepsin column to the trapping column, and a 12 min analytical phase where µBinary Solvent Manager (BSM, a second binary HPLC pump) is eluting the peptic peptides from the trap column onto the C18 analytical column and is separating the peptides with an acetonitrile gradient. In our method, ASM pump A is used to push the sample through the pepsin column with 0.1% formic acid in water. ASM pump B is used to deliver the GluFib lockmass solution to the ESI source. BSM pumps A and B deliver water (0.1% FA) and acetonitrile (0.1% FA), respectively. The following lists the exact settings on all tabs of the Inlet Method window.

- Mode – trapping
- Run time – 12 min
- Loading time – 3 min
- µBinary Solvent Manager (the HPLC pump that runs the analytical column)
    - Trapping Tab
        - Pressure limits
            - Low – 0 psi
            - High – 15000 psi
        - Gradient

Time (min)	Flow (µL/min)	%A	%B	Curve
initial	40.000	95.0	5.0	Initial

    - Analytical Tab
        - Solvents: A1 – water, B1 – acetonitrile
        - Pressure limits: Low – 0 psi, high – 15000 psi
        - Seal wash – 30 min
        - Gradient

Time (min)	Flow (µL/min)	%A	%B	Curve
initial	40.000	95.0	5.0	Initial
7.00	40.000	65.0	35.0	6
8.00	40.000	25.0	75.0	6
10.00	60.000	25.0	75.0	6
11.00	40.000	95.0	5.0	6

    - Data
        - Select data channels to acquire
            - System pressure – checked
            - Measured flow rate A – checked
            - Measured flow rate B – checked
- Auxiliary Solvent Manager

    - Trapping
        - Pump A flow table
            - time – initial, flow = 100 µL
            - pressure limit: low = 0, high = 15000 psi
        - Pump B flow table:
            - time – initial, flow = 10 µL
            - pressure limit: low = 0, high = 15000 psi
    - Analytical
        - Solvent: A1 and B1, water
        - Pump A flow table
            - Time – initial, flow = 25 µL/min
            - pressure limit: low = 0, high = 15000 psi
        - Pump B flow table
            - Time – initial, flow = 10 µL/min
            - pressure limit: low = 0, high = 15000 psi
- Advanced tab
    - Select one or more pumps to enable load ahead
        - Nothing selected
    - Specify a trapping pump to enable multi load
        - Disable multi-load – checked
    - Specify flow rate behavior when a valve change position
        - Desired flowLis rate is applied immediately - checked

References

1 Lau, A. M. C., Ahdash, Z., Martens, C. & Politis, A. Deuteros: software for rapid analysis and visualization of data from differential hydrogen deuterium exchange-mass spectrometry. *Bioinformatics.* **doi: 10.1093/bioinformatics/btz022**, (2019).

2 Habibi, Y., Uggowitzer, K. A., Issak, H. & Thibodeaux, C. J. Insights into the Dynamic Structural Properties of a Lanthipeptide Synthetase using Hydrogen-Deuterium Exchange Mass Spectrometry. *J. Am. Chem. Soc.* **141**, (2019).

State_Data

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Supplemental Coding Files

State_Data_for_Deuteros.xlsx