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

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Yeganeh Habibi**: This protocol can provide spatially resolved information about protein conformational dynamics, which can help to identify functionally important regions of proteins in interest **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Yeganeh Habibi**: This technique probes protein conformational dynamics under near native conditions without the need for protein labeling using nanomolar amounts of material for each reaction **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. Bottom-Up Liquid Chromatography-Mass Spectrometry (LC-MS) Reference Sample Workflow Preparation

2.1. Begin by preparing undeuterated reference samples for the protein of interest in triplicate in 500-microliter tubes **[1-TXT]**.

2.1.1. WIDE: Talent adding sample(s) to tube(s), with sample container(s) visible in frame **TEXT: See text for sample preparation details**

2.2. Quench the samples with the appropriate volume of quench buffer per tube **[1-TXT]** to achieve a pH meter reading of 2.5 **[2]**.

2.2.1. Talent adding buffer to tube(s) **TEXT: See text for all buffer and reagent preparation details**
2.2.2. Talent adding pH meter to tube(s), with buffer container visible in frame

2.3. Flash freeze the samples in liquid nitrogen **[1]** for storage at minus 80 degrees Celsius until their analysis **[2]**.

2.3.1. Talent freezing sample(s) in LN2
2.3.2. Talent placing sample(s) at -80 °C

2.4. Then analyze the protiated enzyme reference samples using a bottom-up liquid chromatography-mass spectrometry workflow according to standard protocols **[1]**.

2.4.1. Talent loading sample onto LC-MS instrument, using a syringe.

3. Reference Data Processing and Peptide List Definition

3.1. To analyze the raw mass spectrometry data, in the appropriate proteomics software program **[1]**, navigate to **Libraries** and **Protein Sequence Databanks** to import the amino acid sequence of the protein of interest **[2]**.

3.1.1. WIDE: Talent opening software, with monitor visible in frame
3.1.2. SCREEN: screenshot_1: 00:03-00:07

3.2. Enter a name for the protein sequence of interest and import the protein sequence in FAST-A (fast-A) format **[1]**.

3.2.1. SCREEN: screenshot_1: 00:07-00:30 *Video Editor: please speed up*

3.3. To define the processing parameters, under the **Library** menu, select **Electrospray MS^E** (M-S-E) as the data acquisition type **[1]**.

3.3.1. SCREEN: screenshot_1: 00:33-00:41

3.4. In the **Lock Mass for Charge 2** field, enter 785.8426 for the mass to charge ratio for the 2-plus ion of glucan-1-fibrinopeptide B and click **Finish [1]**.

3.4.1. SCREEN: screenshot_1: 00:54-01:03

3.5. To define the **Workflow** parameters, select **Electrospray MS^E** for the search type **[1]**.

3.5.1. SCREEN: screenshot_1: 01:07-01:19 *Video Editor: please speed up*

3.6. Under the **Workflow** and **Database Search Query** heading, select the database protein created in the **Databank** field and change the **Primary Digest Reagent** to nonspecific **[1]**.

3.6.1. SCREEN: screenshot_1: 01:19-1:50 *Video Editor: please speed up*

3.7. To clear the **Fixed Modifier Reagent** field, hold the **control** button while clicking **Carbamidomethyl C**. To specify the output directory, select **Options**, **Automation setup**, and **Identify E [1]**.

3.7.1. SCREEN: screenshot_1: 01:50-02:05

3.8. Then check the **Apex 3D and Peptide 3D Output** and **Ion Accounting Output** boxes and specify the desired directory **[1]**.

3.8.1. SCREEN: screenshot_1: 02:05-02:12

3.9. To process the reference sample data, right click on **Microtiter plate** to create a new plate, left click in one well, and drag to three other wells to highlight one well for each reference sample **[1]**.

3.9.1. SCREEN: screenshot_2: 00:03-00:21

3.10. Next, right click and select **add raw data**. In the window, navigate to the directory containing the three reference files and select all three files at the same time **[1]**.

3.10.1. SCREEN: screenshot_2: 00:22-00:42

3.11. Click **Next** and select the defined processing parameters defined. Click **Next** again and select the defined workflow parameters. Then click **Finish [1]**.

3.11.1. SCREEN: screenshot_2: 00:42-00:58

3.12. Once the raw data, processing parameters, and workflow parameters have been assigned to each well on the plate, the wells will turn blue **[1]**.

3.12.1. SCREEN: screenshot_2: 00:58-01:03

3.13. Select the wells, right click, and select **process latest raw data [1]**.

3.13.1. SCREEN: screenshot_2: 01:05-01:14

3.14. To track the processing of the data, click the right bottom corner of the window. When **No job to run** appears, the processing is complete **[1]** and the wells in the microtiter plate will turn green **[2]**.

3.14.1. SCREEN: screenshot_2: 01:14-01:23
3.14.2. SCREEN: screenshot_3: 00:06-00:09

3.15. Right click on the wells and select **view workflow results**. A separate window will open for each reference data file **[1]**.

3.15.1. SCREEN: screenshot_3: 00:09-00:17

3.16. Inspect the data to ensure that the majority of the mass spectrometry signals in the reference sample data were successfully mapped to the peptides predicted from the in silico digestion of the protein of interest. Matched peptides will appear blue in the output spectrum **[1]**.

3.16.1. SCREEN: screenshot_3: 00:17-00:30 *Video Editor: please indicate blue peptides when mentioned*

3.17. Double-click the **OK filter** and check that the percent coverage is greater than 99% **[1]**.

3.17.1. SCREEN: screenshot_3: 00:33-00:39

3.18. Import the proteomics software output into the hydrogen-deuterium exchange-processing software for additional thresholding and click **Data** and **Import PLGS** (P-L-G-S) **results [1]**.

3.18.1. SCREEN: screenshot_3: 00:39-00:55 *Video Editor: please speed up*

3.19. Click the **Add** icon and navigate to the appropriate directory to select the processed data files **[1]**.

3.19.1. SCREEN: screenshot_3: 00:55-01:05

3.20. Click **Next** and set the minimum consecutive ions to greater than or equal to 2, the mass error to 5 parts per million, and the file threshold to 3. Then click **Finish [1]**.

3.20.1. SCREEN: screenshot_3: 01:05-01:32 *Video Editor: please speed up*

4. Hydrogen-Deuterium Exchange (HDX) Reactions

4.1. To conduct hydrogen-deuterium exchange reactions, aliquot quench buffer in 500-microliter tubes **[1]** and briefly centrifuge to transfer all of the quench buffer to the bottom of each tube **[2]**.

4.1.1. WIDE: Talent adding buffer to tube(s)
4.1.2. Talent placing tube(s) into centrifuge

4.2. Place the tubes on ice **[1]** and mix the deuterated reagents rapidly by pipetting up and down. **[2]**.

4.2.1. Talent placing tube(s) on ice
4.2.2. Talent mixing reagents

4.3. Then transfer the reaction mixture to a 25-degree Celsius, temperature-controlled water bath for a 10-minutes incubation **[1]**.

4.3.1. Talent placing tube(s) into water bath

4.4. At the appropriate experimental exchange time points, remove 50-microliter aliquots from each hydrogen-deuterium exchange reaction **[1]** and mix the reactions quickly and evenly with one aliquot of ice-cold quench buffer in individual 500-microliter tubes **[2]**.
Author Note: Here we also had a wide shot to show the overall workflow, since the procedure(4.4.1, 4.4.2, 4.5.1) must be performed very quickly, the close-up shots were not able to track them.

4.4.1. Talent removing aliquot from tube, while the remaining is kept in 25-degree Celsius water bath. *Videographer: Important/difficult step*
4.4.2. Reaction being mixed with quench buffer, with reaction and buffer containers visible in frame *Videographer: Important/difficult step*

4.5. Then immediately cap the tubes for flash freezing in liquid nitrogen. **[1]** After thawing the frozen samples, analyze the deuterated enzyme reference samples using a bottom-up liquid chromatography-mass spectrometry workflow according to standard protocols **[2]**.

4.5.1. Talent capping tube(s) *Videographer: Important step*
4.5.2. *Added shot: Talent freezing the sample in LN2. (this is visible in wide shot)*
4.5.3. Talent loading sample onto LC-MS instrument while holding a timer and keep track of time.

5. HDX Data Processing, Analysis, and Visualization

5.1. For processing of the hydrogen-deuterium exchange data, import the data into the previously created hydrogen-deuterium exchange product **[1]** and click **New State** and **New Exposure** to define the biochemical states and deuterium exposure times, respectively, pertinent to the analysis **[2]**.

5.1.1. WIDE: Talent importing data, with monitor visible in frame
5.1.2. SCREEN: screenshot_4: 00:05-00:31 *Video Editor: please speed up*

5.2. Click **New Raw** to select the hydrogen-deuterium exchange data files to be analyzed and assign the appropriate exchange times and biochemical state to each raw data file that is imported **[1]**.

5.2.1. SCREEN: screenshot_4: 00:31-00:58

5.3. For analysis and visualization of the data, select the first peptide in the peptide list and open the stacked spectral plot from the **Views** menu **[1]**.

5.3.1. SCREEN: screenshot_5: 00:11-00:24

5.4. Click the mouse to assign and unassign sticks as necessary to ensure that the proper isotope distribution has been located in the data and that each isotope peak has been assigned **[1-TXT]**.

5.4.1. SCREEN: screenshot_5: 00:24-00:43 *Video Editor: can speed up* **TEXT: Assigned sticks will appear blue**

5.5. To check the stick assignments for each charge state, toggle the charge state at the top of the stacked spectral plot window **[1]**.

5.5.1. SCREEN: screenshot_5: 00:43-00:58

5.6. To check the standard deviation of the peptide deuterium uptake values, in the **Views** menu, select the coverage map, which displays each peptide in the peptide list mapped along the amino acid sequence of the protein of interest **[1]**.

5.6.1. SCREEN: screenshot_6: 00:03-00:07

5.7. Color the peptides according to relative standard deviation and visually search the map for outlier peptides with a high relative standard deviation **[1]**.

5.7.1. SCREEN: screenshot_6: 00:07-00:30 *Video Editor: please speed up*

5.8. Click the outlier peptides in the coverage map to populate the stacked spectral plots and the data viewer window with the target peptide **[1]**.

5.8.1. SCREEN: screenshot_6: 00:30-00:45 *Video Editor: please speed up*

5.9. Display the difference of interest in the coverage map and right click on the map to export the difference data to a .csv file **[1]**.

5.9.1. SCREEN: screenshot_7: 00:02-00:30 *Video Editor: please speed up*

5.10. Import the difference and state data and the pdb file of the protein of interest into Deuteros and select the 99% confidence interval and **enable sum** and process the data **[1-TXT]**.

5.10.1. SCREEN: screenshot_8: 00:02-00:21 **TEXT:**
https://github.com/andymlau/Deuteros

5.11. Then, under **PyMOL** (pie-mall), select **export uptake** and **export** to generate a Pymol script to map the regions of significant exchange difference onto the pdb structure of the protein of interest in the PyMOL software **[1]**.

5.11.1. SCREEN: screenshot_8: 00:24-00:28

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
4.4., 4.5.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
4.4. The quenching of the HDX reaction aliquots has to been done in a consistent manner, using a 100 µL pipette for mixing will be.
5.4. During data processing, you must ensure the correct isotope distribution has been assigned for each peptide. You should compare the observed and theoretical monoisotopic mass for each peptide.

Results

6. Results: Representative HDX-MS Peptide Biosynthetic Enzyme Evaluation

6.1. These representative total ion chromatograms for three reference samples of HalM2 (hal-M-two) Lanthipeptide synthetase **[1]** show the time dependent changes in the sum of all of the ion counts at all of the mass to charge ratio values included in the mass spectral scan **[2]**.

6.1.1. LAB MEDIA: Figures 3A-3C

6.1.2. LAB MEDIA: Figures 3A-3C *Video Editor: please sequentially emphasize data line in each graph*

6.2. The shape and intensity of the total ion chromatogram profiles should be similar **[1]**, indicating that the proteolytic digestion of HalM2 is reproducible and is of similar efficiency in all three reference samples **[2]**.

6.2.1. LAB MEDIA: Figures 3D-3F

6.2.2. LAB MEDIA: Figures 3D-3F *Video Editor: please emphasize/trace data lines in all three graphs*

6.3. The mass spectra over a given time interval should also be similar **[1]**, providing confidence that similar peptides are present within each sample and are eluting at similar times from the C18 (C-eighteen) column **[2]**.

6.3.1. LAB MEDIA: Figures 3G-3I *Video Editor: please emphasize/trace data lines in all three graphs*

6.4. Most of the peptides in the deuterium exchanged samples should exhibit obvious shifts in their isotopic distributions toward higher mass to charge ratio values **[1]**.

6.4.1. LAB MEDIA: Figure 6

6.5. For example, these data indicate that a substantial fraction of the deuterium label was being maintained throughout the course of the acid quench, pepsin digestion, and liquid chromatography-mass spectrometry data collection **[1]**.

6.5.1. LAB MEDIA: Figure 6 *Video Editor: please add/emphasize blue arrows and text over green graphs*

6.6. After characterization of the biochemical properties of the enzyme as demonstrated **[1]**, representative hydrogen-deuterium exchange-mass spectrometry results such as these can be generated **[2]** to illustrate the binding of the HalA2 precursor peptide **[2]** to the HalM2-AMP-PNP (A-M-P-P-N-P) complex **[3-TXT]**.

6.6.1. LAB MEDIA: Figure 10A

6.6.2. LAB MEDIA: Figures 10B and 10C

6.6.3. LAB MEDIA: Figures 10B and 10C *Video Editor: please emphasize red and blue regions of Figure 10C*

6.6.4. LAB MEDIA: Figures 10B and 10C *Video Editor: please grey regions in Figure 10C*
TEXT: AMP-PNP: adenylyl imidodiphosphate

Conclusion

7. Conclusion Interview Statements

7.1. **Yeganeh Habibi**: The hydrogen-deuterium exchange reactions must be prepared and quenched in a consistent manner and you must be methodical when determining the deuterium exchange values for the numerous peptides detected **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (4.2., 5.4.)

7.2. **Yeganeh Habibi**: Hydrogen-deuterium exchange-mass spectrometry provides peptide level information on dynamic protein conformational changes. The functional relevance of any hydrogen-deuterium exchange "hot spots" should be validated through mutagenesis and orthogonal activity assays **[1]**.

7.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera