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Hydrogen-Deuterium Exchange Mass Spectrometry (HDX-MS) Centroid Data Measured between 3.6 ºC and 25.4 ºC for the Fab Fragment of NISTmAb

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Key words: centroid; hydrogen-deuterium exchange; interlaboratory comparison; mass spectrometry; peptide; precision; proteomics; reference material; repeatability; reproducibility.

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1. Summary

The spreadsheet file reported herein provides centroid data, descriptive of deuterium uptake, for the Fab Fragment of NISTmAb (PDB: 5K8A) reference material, as measured by the bottom-up hydrogen-deuterium exchange mass spectrometry (HDX-MS) method. The protein sample was incubated in deuterium-rich solutions under uniform pH and salt concentrations between 3.6 °C and 25.4 °C for seven intervals ranging over (0 to 14,400) s plus a ∞_{pseudo} s control. The deuterium content of peptic peptide fragments were measured by mass spectrometry. [1-3] These data were reported by fifteen laboratories, which conducted the measurements using orbitrap and Q-TOF mass spectrometers. The cohort reported ≈ 78,900 centroids for 430 proteolytic peptide sequences of the heavy and light chains of NISTmAb, providing nearly 100 % coverage. In addition, some groups reported ≈ 10,900 centroid measurements for 77 peptide sequences of the Fc fragment. The instrumentation and physical and chemical conditions under which these data were acquired are documented.

2. Data Specifications

NIST Operating Unit(s)	Material Measurement Laboratory
Format	xlsx
Instrument	Orbitrap and Q-TOF mass spectrometers
Spatial or Temporal Elements	N/A
Data Dictionary	If applicable, provide URL of data dictionary
Accessibility	All datasets submitted to *Journal of Research of NIST* are publicly available.
License	https://www.nist.gov/open/license

3. Methods

3.1 Bottom-up HDX-MS measurements

These centroid data were obtained using the "bottom-up" HDX-MS experiment (Fig. 1). The first step of the HDX-MS measurement process involves immersion of the protein sample into buffered solution environments (deuterium fraction, $F^{\mathrm{D_2O}}$= 0.8 to 0.96) at temperature T_{HDX} = (3.6 to 25.4) °C and pD 7.48, which induces D for H exchange (Fig. 1A). After the period t_{HDX} the solution is transferred into a cold acidic aqueous solution at T_{quench} ≈ 0 °C and pH ≈ 2.5 (Fig. 1B). This quench solution contains a chaotrope and reducing agent [4] that denatures the protein and reduces disulfide bonds. In this solution environment ($F^{\mathrm{D_2O}}$= 0.18 to 0.48), amide sites of the denatured protein exchange H for D near their minimum chemical rates [5]. NIST supplied the HDX-MS kit that provided buffers and reagents used during the first two steps (ref. yellow/gray box of Fig. 1). The kit assured uniform pH, salt

concentration, and reducing power during these steps. However, due to the individual requirements of each laboratory apparatus, the incubation solution (Fig. 1A) differs in both protein concentration and F^{D_2O}.

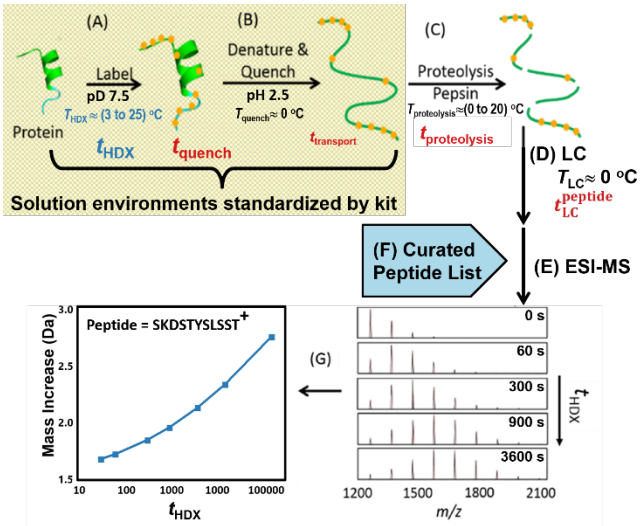

Fig. 1. The "bottom-up" HDX-MS experiment broken into steps controlled by the NIST HDX-MS reagent kit (steps A and B in the yellow/gray box) and by each participating laboratory (steps C through G). H for D back-exchange occurs during time intervals listed in red.

Subsequently, the protein solution passes into the fluidic system comprising a column containing an immobilized protease (usually pepsin) and reverse-phase chromatography components (Fig. 1C). The solution undergoes proteolysis for an interval, $t_{\text{proteolysis}}$ = (4 to 240) s at a chosen temperature, $T_{\text{proteolysis}}$ = (0 to 20) °C, and the resulting effluent passes through a guard column at $T_{\text{LC}} \approx 0$ °C that traps proteolytic peptides (Fig. 1D). To optimize the mass spectrometer sensitivity, many laboratories wash out salts by flowing additional loading solution through the guard column for t_{wash} = (30 to 180) s. Reverse-phase chromatography, conducted at $T_{\text{LC}} \approx 0$ °C, releases and separates peptides, which elute from the analytical column at $t_{\text{LC}}^{\text{peptide}}$ (Fig. 1D). Electrospray ionization mass spectrometry (ESI-MS) detects the peptide ions (Fig. 1E).

Some laboratories use a variation of this procedure, where after D_2O incubation has completed, the protein sample is quenched and flash frozen at −196 °C. Subsequently, the sample is thawed and analyzed using a workflow similar to that depicted in Figs. 1C through 1G.

The average mass change of each selected peptide (Fig. 1F) is calculated and plotted as a function of t_{HDX} (Fig. 1G). Since side chains generally have more rapid exchange rates than backbone amides at pH 2.5 and ≈ 0 °C, [5, 6] the deuterium labels at these positions are equilibrated back to the natural protic isotope abundance during digestion and separation. This simplifies data analyses since each amino acid (except proline) can have a maximum of one deuterium label.

Separate experiments, equivalent to the HDX-MS experiment for t_{HDX} = 0 s, determine the initial curated list of peptides (Fig. 1F) that associates each chromatographic peak with mass spectra. To improve the veracity of peptide identifications, the operator observes MS/MS data for the eluting peptides. With reference to the known sequence of fab fragment of NISTmAb [2] peptide ion identification software analyzes these data and proposes an amino acid sequence, charge state (z), and confidence rank for each peptide ion. The list of retention times and sequences becomes the filter through which peptide ions are selected for HDX-MS analyses. However, the curation process continues throughout the data analysis. Curation of the list assures that the kinetics behavior of sequences adheres to EX2 behavior, that the D-uptake kinetics match the sequence assignments, that the LC retention times remain stable, and that the peptides exhibit adequate intensity to support reliable centroid determinations and remain free of interfering ion signals. These requirements may disqualify many identified peptides from use in the HDX-MS

analysis. Thus, the peptides listed on each spreadsheet may represent only a fraction of the peptide sets found by investigators during the proteomics discovery process. The discarded peptides are not reported.

Each laboratory conducted proteomics studies on the Fab fragment of NISTmAb (PDB: 5K8A) [1-3] and performed three HDX–MS runs. Here, the term "run" is defined by the procedures and capabilities of the laboratory equipment. Practically speaking, the term denotes approximately one day of HDX-MS measurements. Each "run" may include several replicant measurements, termed "reps". Across the participating laboratories, each run comprised between one and five HDX-MS reps at each time point, t_{HDX}. The database contains anonymized copies of the data spreadsheets and auxiliary information.

3.2 Reagents and Materials[1]

All reagents used to prepare samples and supplied with the HDX-MS kits originated from the same chemical lots. D_2O (99.96 mole % D) was acquired from Cambridge Isotopes (Andover, MA). Sodium phosphate dihydrate, sodium phosphate monohydrate, and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 98 % guanidine hydrochloride (GdmCl) were acquired from Thermo Scientific (Thermo Fisher Scientific, Pittsburgh, PA).

The test protein for the HDX-MS interlaboratory comparison was the Fab fragment that is enzymatically cleaved from Candidate RM 8670 (Lot #5F1b), which has the same structure as the Fab of the NIST IgG1κ reference material, NISTmAb RM8671. [1-3] Material used for this study contained a small fraction of free Fc fragment. Triple-angle light scattering data indicated that the Fab fragment of NISTmAb sample was in a monomeric state [2, 3]. Testing showed that the protein is unaltered by the mechanical shocks associated with shipping [2].

Samples of Fab fragment were used to prepare Fab-H_2O and Fab-D_2O buffered stock solutions. Fab-D_2O solution was prepared by resuspending lyophilized Fab fragment of NISTmAb in buffered, 99.96 % D_2O. Separate HDX-MS experiments revealed negligible differences between freshly prepared Fab-H_2O and Fab solutions prepared by resuspending lyophilized Fab fragment of NISTmAb in buffered H_2O (data not shown). The Fab-H_2O and Fab-D_2O solutions were dispensed through a 0.2 µm filter into 1 mL glass vials.

3.3 HDX-MS Kit

The NIST HDX-MS standard reagent kit comprising a padded box and 26 vials contained all solutions and materials necessary for conducting proteomics studies on Fab samples; for conducting three runs of HDX-MS kinetics studies involving immersion of Fab in buffered D_2O for selected durations, t_{HDX} (Fig. 1A); and for quenching the H/D exchange process, denaturing the protein, and reducing disulfide bonds during the analysis process (Fig. 1B).

Each kit contained a glass vial holding ≈200 µL Fab–H_2O and a glass vial containing ≈200 µL Fab–D_2O, respectively. Each kit contained one 4 mL glass vial and one 2.5 mL plastic vial of H_2O dilution buffer solution, comprising 20 mmol/L sodium phosphate buffer and 150 mmol/L sodium chloride in deionized H_2O (pH 7.50 ± 0.02). Each kit contained three 4 mL glass vials and one 2.5 mL plastic vial of D_2O exchange buffer solution, comprising 20 mmol/L sodium phosphate buffer and 150 mmol/L sodium chloride in 99.96 % D_2O ($pD_{corrected}$ 7.48 ± 0.02).

Each kit contained four 4 mL glass vials of quench buffer solution, comprising 8 mol/L guanidine-HCl and 0.4 mol/L sodium phosphate in H_2O (pH 3.1 ± 0.02). To assure the preparation of solutions with uniform disulfide bond reducing potential, the kits contained four samples of dry ≈0.70 g tris(2-carboxyethyl) phosphine

[1] Certain commercial equipment, instruments, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

hydrochloride (TCEP). Each TCEP sample (Pierce TCEP; Thermo Fisher Scientific, Pittsburgh, PA) was double-sealed within a 1.5 mL Eppendorf tube inside a 5 mL glass vial.

3.4 pH Measurements

During preparation of solutions provided in the HDX-MS kit, pH measurements were conducted with a Thermo Scientific Model Orion 3-Star Benchtop pH meter (Thermo Fisher Scientific, Pittsburgh, PA) coupled to a Fisherbrand double junction refillable glass pH Electrode (Fisher Scientific Catalog# 13-620-223A, Thermo Fisher Scientific, Pittsburgh, PA). The meter was calibrated using a point-by-point method with four point calibration solutions, pH 1.68, 4.01, 7.00, 10.01 (Oakton Instruments, Vernon Hills, IL).

3.5 Intact Mass Analysis of Fab fragment of NISTmAb

Intact mass analyses were conducted by direct infusion of native and perdeuterated Fab fragment of NISTmAb into a Thermo Fisher Scientific LTQ Orbitrap Elite (Thermo Fisher Scientific Inc., San Jose, CA) and an Agilent 6545 Q-TOF (Agilent Technologies, Santa Clara, CA). For these instruments, MagTran 1.03 software (Amgen Inc., Thousand Oaks, CA) [7] and BioConfirm 8.0 (Agilent Technologies, Santa Clara, CA), respectively, deconvoluted the resulting spectra. The 1 221.990 637 *m/z* ion of the HP-1221 calibration standard (Agilent Technologies, Santa Clara, CA) served as the reference mass for Q-TOF measurements. A native Fab control was treated similarly to perdeuterated Fab by incubating at 37 °C for 96 hours in 99.96 % D_2O. Deconvolution of the $[M+nH^+]^{n+}$ mass envelopes of the Fab-H_2O sample yielded a molecular mass of [M] = 47,628 (± 2) g/mol. This mass is in good accord with the theoretical mass of Fab-H_2O, [M] = 47,628 g/mol and a previous measurement of [M] = 47,628 (±5) g/mol with a Q-TOF mass spectrometer. [2] Calculation of the theoretical mass accounts for the N-terminal pyroglutamate residue.

Prior to conducting measurements on Fab-D_2O the mass spectrometer sample handling systems were pre-conditioned with 99.96 % D_2O. Using the same mass spectrometers and infusion procedures, deconvolution of the $[M+nD^+]^{n+}$ data for Fab-D_2O yielded a molecular mass of [M] = 48,206 (± 2) g/mol. The theoretical mass of Fab-D_2O is 48,390 g/mol. [8] The difference indicates that Fab-D_2O control samples contain (184 ± 2) protons. These protons may indicate that certain portions of the 412 amide sites of Fab-D_2O are essentially inert to H/D exchange. Alternately, the protons may reside mainly among the 346 amino acid side-group sites that are available to rapid H/D exchange with H_2O within the MS electrospray source. The intact molecule ESI-MS data do not resolve this question of site-occupancy type. This uncertainty of the amide occupancy type within the finished Fab-D_2O control directs us to assume that $\langle m \quad (\infty_{\text{pseudo}})\rangle^{\text{peptide}} \leq \langle m \quad (\infty)\rangle^{\text{peptide}}$.

3.6 Onsite Sample and Control Preparation

The kit was shipped to participants via an overnight delivery service in an insulated box. Dry gel packets maintained the package contents at +4 °C and had sufficient capacity to accommodate transits of at least 48 h. Upon receipt of a package, each participant ascertained that the internal temperature was near +4 °C. (Replacement kits were issued to participants upon request.) Using H_2O dilution buffer solution or D_2O exchange buffer solution, as appropriate, laboratories promptly diluted the Fab stock solutions to the concentration suitable for use in HDX-MS studies. These vials were stored at −80 °C until needed.

Onsite preparation included additional finishing of the Fab-D_2O control sample to assure that the deuterium content corresponded to $t_{\text{HDX}} \approx \infty_{\text{pseudo}}$ material. At each participating laboratory the Fab-D_2O sample was diluted with 99.96 % D_2O buffered solution to the concentration suitable for the incumbent HDX-MS apparatus. The sample was then incubated at 37 °C for 96 h prior to its centroid measurement. Although this procedure does not assure that the amide sites, $(NH)_k$, contain 99.96 % D, it does assure that all $(NH)_k$ sites contain maximum deuterium, as dictated by structural properties.

3.7 Summary of the Data

The centroid data and additional documentation for each laboratory (numbered #X = 1 to 15) is reported on four distinct Worksheets within the Excel Workbook:

1. **Lab#X A-Centroids.** The A-centroid data sheet from each laboratory lists the centroid mass observed at time t_{HDX} = 0 s, 30 s, 60 s, 300 s, 900 s, 3,600 s, 14,400 s and ∞_{pseudo}. Each centroid is computed by integrating the *m/z* spectrum of the peptide isotopic envelope:

$$\langle m(t_{HDX})\rangle^{peptide} = z \cdot \left[\frac{\sum_{i=1}^{n}\left(m/z_i\right)\cdot I_i}{\sum_{i=1}^{n} I_i} - m_{H^+}\right] \quad (1)$$

   where *z* is the ion charge, *n* is the number of isotopic *m/z* features in the mass spectrum of the ion, $(m/z)_i$ is the measured mass to charge ratio, I_i is the intensity of the *i*th ion feature, and m_{H^+} is the proton mass. [9]
2. **Lab#X B-Questionnaire.** This data sheet details the solution conditions of the proteomics and HDX-MS experiments and a description of the experimental protocol, including a list of protease, guard, analytical columns, chromatography gradients, and ion source conditions.
3. **Lab#X B-Supplemental.** This data sheet reports the temperature of the D_2O incubation solution and the uncertainty or variation of the solution temperature measurement.
4. **Lab#X C-Instruments.** This sheet lists the make and model of chromatograph, mass spectrometer, sample-handling system (i.e. robot), the search engine used to identify peptides, and the software used to conduct the HDX-MS analyses.

4. Impact

These data have use for the development and testing of HDX-MS analysis software. The original use of these spreadsheets was for a determination of the reproducibility of bottom-up HDX-MS. [10, 11] The data comprise the most comprehensive collection of HDX-MS measurements for an antibody fragment reported by a cohort of laboratories. The laboratories have measured the same sample using the same reagents. Protein concentrations and D_2O fraction varied in the incubation solutions, but the salt concentrations were uniform. The study spans multiple laboratories, diverse instrumentation, and different software. These attributes are documented.

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