Journal of the American Society for Mass Spectrometry

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Article

Systematic Evaluation of the Impact of Quench Conditions on Deuterium Back-Exchange in HDX-MS

Published as part of Journal of the American Society for Mass Spectrometry special issue "Emerging Investigators 2026".

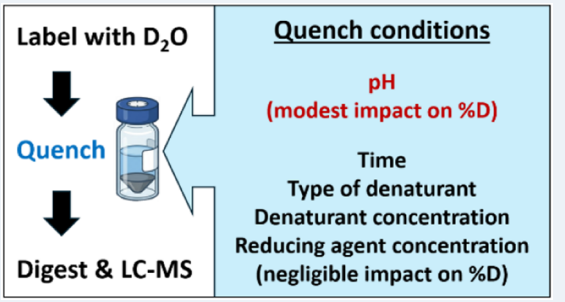
Sandeep Kumar and Kyle W. Anderson*

Cite This: https://doi.org/10.1021/jasms.6c00274

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ABSTRACT: Hydrogen–deuterium exchange mass spectrometry (HDX-MS) is widely used to characterize protein structure and dynamics, but accurate measurements depend on minimizing deuterium back-exchange during sample preparation. Although quench buffers typically contain denaturants and reducing agents under low pH conditions, the effects of quench solution composition and quench duration on deuterium preservation have not been systematically investigated quantitatively. Here, we quantitatively evaluated the influence of denaturant concentration, reducing agent concentration, quench pH, and quench duration on deuterium back-exchange using deuterated phosphorylase b protein and bovine serum albumin (BSA) digest peptides. Variations in denaturant and reducing agent concentrations produced only negligible changes in deuterium retention, whereas increasing the quench pH from 2.5 to 4.0 resulted in an approximately 4 percentage point decrease in average percent deuterium, identifying high quench pH as the condition exerting the greatest influence on deuterium back-exchange among the conditions investigated. Increasing quench duration from 1 to 10 min reduced average deuterium content by approximately 6 percentage points for BSA digest but only 1.6 percentage points for phosphorylase b, highlighting the protective effect of residual protein structure during the quench step. These findings provide practical guidance for selecting HDX-MS quench conditions that balance deuterium preservation with efficient protein processing while improving the accuracy and reproducibility of HDX-MS measurements.



INTRODUCTION

Hydrogen–Deuterium Exchange Mass Spectrometry (HDX-MS) is a powerful analytical technique for studying protein structure, conformational dynamics, and protein–ligand interactions in solutions.[1] This technique relies on the exchange of backbone amide hydrogens with deuterium, allowing the structural and dynamic information to be inferred from changes in protein mass.[1] The accuracy and reproducibility of HDX-MS data depend critically on the preservation of incorporated deuterium throughout sample processing and analysis.[2−4]

Despite its versatility, a limitation of HDX-MS is deuterium back-exchange, the undesired replacement of incorporated deuterium atoms with hydrogen after labeling and prior to mass analysis.[3−5] Back-exchange can occur during quenching, proteolytic digestion, chromatographic separation, and subsequent sample handling, leading to an underestimation of deuterium uptake and reduced experimental accuracy.[3−5] To minimize this effect, HDX-MS workflows typically employ rapid quenching under low pH (≈2.5) and low temperature (0 to 4 °C) conditions, with quench durations typically limited to 2 to 3 min to substantially slow hydrogen–deuterium exchange kinetics.[3,5]

In addition to reducing hydrogen–deuterium exchange rates, quench buffers commonly contain denaturants such as guanidine hydrochloride (GdnHCl) or urea to unfold proteins and improve the efficiency of subsequent proteolytic digestion.[3,5] Reducing agents, such as tris(2-carboxyethyl)-phosphine hydrochloride (TCEP·HCl), are also frequently included to disrupt disulfide bonds, thereby further improving digestion efficiency and peptide recovery.[3,5] Although these additives facilitate HDX-MS sample preparation, variation in their concentrations, quench-solution pH, and exposure times may also influence deuterium back-exchange by altering the extent of back-exchange.[2,3,5]

Received: July 10, 2026
Revised: September 23, 2026
Accepted: October 26, 2026

ACS Publications

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Considerable efforts have been made to minimize back-exchange during chromatographic separation.[6,7] In particular, the development of subzero temperature liquid chromatography has significantly reduced the deuterium loss, with studies reporting up to a 30% reduction in back-exchange when both trapping and analytical columns are maintained at approximately −30 °C.[7,8] As a result, chromatographic back-exchange can now be effectively minimized through advances in instrumentation and separation methodology. In contrast, comparatively little attention has been directed toward quantifying deuterium loss from sample processing conditions during the quench step. Although current HDX-MS workflows recommend limiting quench times to approximately 2 to 3 min to minimize back-exchange,[3,5] little systematic experimental evidence exists to quantify the effect of quench duration on deuterium back-exchange. Likewise, while denaturants, reducing agents, and low pH quench conditions are routinely incorporated into HDX-MS workflows, the effects of denaturant concentration, reducing agent concentration, quench solution pH, and quench duration on deuterium back-exchange have not been systematically evaluated quantitatively.

Therefore, a systematic quantitative assessment of these parameters is needed to establish evidence-based guidelines for HDX-MS workflows. In this study, we evaluated the effects of denaturant concentration, reducing-agent concentration, quench time, and quench solution pH on deuterium back-exchange in both deuterated bovine serum albumin (BSA) tryptic peptides and phosphorylase b. By quantifying back-exchange under controlled quench conditions, we aim to identify parameters that strongly influence deuterium back-exchange, thereby providing practical guidance for improving the accuracy, reproducibility, and standardization of HDX-MS measurements.

EXPERIMENTAL SECTION

Reagents and Materials

Phosphorylase b from rabbit muscle (P/N P6635) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A tryptic digest of fully reduced and iodoacetamide alkylated bovine serum albumin (BSA) (Thermo Scientific Pierce BSA Protein Digest Standard, LC−MS grade; Catalog No. 88341) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Deuterium oxide (D_2O, 99.96 mol % D) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). An immobilized pepsin column (P/N PDX.PP01-F32) was obtained from Trajan Scientific Americas Inc. (Austin, TX, USA). LC−MS-grade water and acetonitrile, both containing 0.1% (v/v) formic acid, and LC−MS-grade formic acid were purchased from Fisher Scientific (Waltham, MA, USA). HPLC-grade water was obtained from Sigma-Aldrich (St. Louis, MO, USA). FOS-choline-12 (P/N F308S) was purchased from Anatrace (Maumee, OH, USA). Urea (PlusOne) was obtained from Cytiva. Guanidinium hydrochloride (GdnHCl; P/N 24110) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl; P/N 20491) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Sample Preparation

Undeuterated and deuterated stock solutions were prepared by reconstituting lyophilized BSA digest peptides and phosphorylase b in H_2O and D_2O (99.96 mol % D), respectively. Samples prepared in D_2O were incubated at 4 °C for 24 h to maximize deuterium exchange. Aliquots of each stock were stored at −80 °C until use. Prior to analysis, stock solutions were diluted in either H_2O or D_2O to yield 4 pmol of BSA digest peptides or 41 pmol of phosphorylase b per injection. All quench solutions (C1−C8; Table 1) were prepared using LC−MS grade water. Quench solution conditions selected for evaluation were based on a survey of the range of conditions used in the HDX-MS literature.

Table 1. Quench Solution Conditions Evaluated

Condition	Quench solution composition
C1	8 mol/L urea, 1 mol/L TCEP·HCl, pH 2.5
C2	8 mol/L urea, 0.1 mol/L TCEP·HCl, pH 2.5
C3	0.5 mol/L urea, 0.1 mol/L TCEP·HCl, pH 2.5
C4	4 mol/L urea, 0.1 mol/L TCEP·HCl, pH 2.5
C5	6 mol/L urea, 0.1 mol/L TCEP·HCl, pH 2.5
C6	0.5 mol/L GdnHCl, 0.1 mol/L TCEP·HCl, pH 2.5
C7	4 mol/L GdnHCl, 0.1 mol/L TCEP·HCl, pH 2.5
C8	8 mol/L urea, 1 mol/L TCEP·HCl, pH 4.0

Peptide Identification

Peptic peptides of phosphorylase b were generated by passing 41 pmol of protein through an immobilized pepsin digestion column (2.1 mm × 30 mm, 20 μm bead; Trajan Scientific Americas Inc., Morrisville, NC) and analyzed by LC−MS/MS using a Thermo Ascend Tribrid Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). For the BSA digest standard, 4 pmol of the predigested tryptic peptide mixture was analyzed by replacing the immobilized pepsin column with a PEEK union. The same MS acquisition parameters were used for both phosphorylase b and BSA analyses. Data were acquired using one full MS scan followed by data-dependent MS/MS scans of the most abundant precursor ions using the collision-induced dissociation (CID) as the fragmentation method. Peptide identification was performed using the Mascot search engine (v3.1, Matrix Science). For the BSA digest, the search parameters included trypsin as the enzyme specificity, carbamidomethylation of cysteine as a fixed modification, an MS mass tolerance of ±10 ppm, and an MS/MS tolerance of ±0.5 Da. For phosphorylase b peptic peptides, identical search parameters were used except that enzyme specificity was set to none and no modifications were specified.

HDX

For HDX-MS experiments, the phosphorylase b protein and BSA digest peptide stock solutions were diluted to final concentrations of 10 μmol/L and 1 μmol/L, respectively, in H_2O or D_2O and equilibrated at 4 °C. Sample preparation was performed using an HDX PAL3 extended rail robotics system (Trajan Scientific Americas Inc., Morrisville, NC) coupled to the NIST cold box apparatus.[8] The preparation and analytical chamber of the NIST cold box were maintained at 0 °C, while the immobilized pepsin column was maintained at 4 °C.

Triplicate measurements were acquired for each experiment. For each experiment, 5 μL of phosphorylase b solution or BSA digest peptide solution was mixed with 25 μL H_2O or D_2O, respectively, for undeuterated or predeuterated samples, at 20 °C. Next, the sample was quenched by adding 26 μL of one of the quench solutions (C1−C8; Table 1) maintained at 4 °C. The 50 μL of quenched sample was injected into the NIST cold box using a 50 μL sample loop. A loading pump operating at 100 μL/min flow rate transferred the sample through the immobilized pepsin column for 3 min. For BSA digest peptides, the enzyme column was replaced with a PEEK union. For comparison among the different quench conditions (C1−C8), a quench time of 2 min was used. To evaluate the effect of quench duration, quench condition C1 was applied for 1, 2, 3, 4, 5, and 10 min.

LC−MS Analysis

Following online digestion, peptides were trapped on a C18 column (Phenomenex, Inc.; Torrance, CA; Model Kinetex EVO C18, 100 Å pore, 2.6 μm particle size, 20 mm × 2.1 mm) and separated on an analytical column (Thermo Fisher Scientific; Waltham, MA; Accucore C18, 80 Å pore, 2.6 μm particle size, 30 mm × 2.1 mm, P/N 17126-

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032130). The analytical pump (Vanquish H, Thermo Fisher Scientific) used 0.1% formic acid by volume in water for solvent A and 0.1% formic acid by volume in acetonitrile for solvent B. Peptides were separated at a solvent A and solvent B combined flow rate of 50 μL/min using the following gradient reported by volume percent: 2% to 50% B for 8 min, 50% to 95% B for 1 min, 95% B for 4 min, 95% to 10% B for 0.5 min, 10% to 95% B for 1.5 min, 95% B for 3 min, and 95% to 4% B for 1.5 min.

After completion of online digestion, switching valves directed the trapped peptides onto the analytical column while simultaneously placing the immobilized pepsin column in line with a dedicated wash pump. The pepsin column was backflushed with 0.1% (w/v) Fos-choline-12 at 200 μL/min for 5 min to minimize the peptide carryover, reducing the number of blank injections required after each HDX sample to one.[9] The injection port was washed twice with acetonitrile, followed by two washes with 0.1% formic acid in water using 50 μL injections.

Mass spectra were measured by a Thermo Ascend Tribrid Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The instrument settings were as follows: spray voltage of 3800 V; sheath gas flow rate of 25 arbitrary units; and ion transfer tube temperature of 275 °C. Full scan MS1 (m/z 300 to 2000) in the Orbitrap was acquired with the resolution set at 90000 for all the HDX-MS samples. For peptide identification and sequence coverage analysis, data-dependent acquisition (DDA) was performed. The acquisition mode was set to Cycle Time with a time between master scans of 2 s. Full MS (MS1) spectra were acquired over an m/z range of 300 to 2000 at a resolution of 90,000. The AGC target was set to Standard, the RF lens to 60%, the maximum injection time mode to Auto, and one microscan was acquired per spectrum. The isolation window was centered on the most abundant precursor ion. Dynamic exclusion was enabled with the following settings: exclusion after one occurrence, exclusion duration of 30 s, mass tolerance of 10 ppm, isotope exclusion enabled, exclusion within cycle enabled, and dependent scans were not restricted to a single charge state per precursor. Precursor ions with charge states of 1 to 6 were isolated using the quadrupole mass filter and fragmented by collision-induced dissociation (CID). The MS/MS acquisition parameters were as follows: isolation mode, ion trap; isolation window, 3 m/z; isolation offset, off; activation type, CID; CID collision energy, 33%; assisted collision energy, disabled; CID activation time, 10 ms; activation Q, 0.25; multistage activation, disabled; detector type, ion trap; ion trap scan rate, normal; mass range, normal; scan range mode, auto; AGC target, custom (normalized AGC target, 200%); maximum injection time mode, auto; and one microscan per spectrum.

HDX-MS Data Processing

HDX-MS data were processed using HDX Workbench.[10] A peptide library for phosphorylase b and BSA digest peptides was generated from LC−MS/MS peptide identification experiments using the Mascot search engine (v3.1, Matrix Science). This peptide library was imported into HDX Workbench and used for peptide assignment during HDX-MS data analysis. The centroid mass of each deuterated peptide isotopic envelope was determined, and relative deuterium uptake was calculated for each peptide. Only peptides that were consistently identified under all quench conditions were included in the deuterium uptake analysis.

RESULTS AND DISCUSSION

Effect of Quench Solution Composition on Deuterium Back-Exchange

To evaluate the influence of quench solution composition on deuterium back-exchange, eight quench solutions (C1−C8; Table 1) differing in denaturant concentration, reducing agent concentration, and pH were examined using deuterated phosphorylase b protein and BSA digest peptides. Figure 1 summarizes the average deuterium uptake of 39 peptic peptides from phosphorylase b and 60 tryptic peptides from

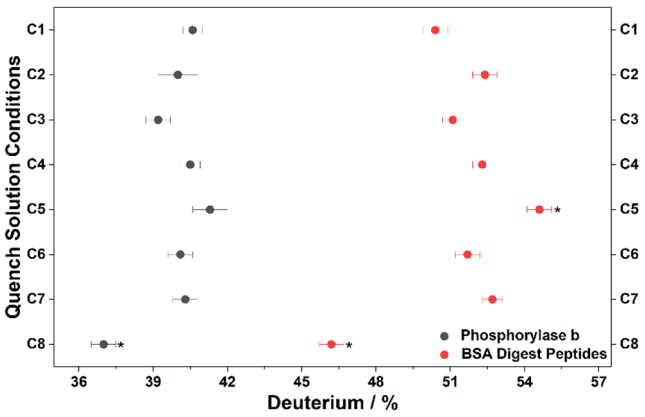


Figure 1. Effect of different quench conditions on the deuterium retention of phosphorylase b and BSA digest peptides. Values are presented as mean ± pooled standard deviation of all individual peptide means from triplicate measurements. * denotes p-value ≤ 0.02 calculated using Welch's t-test by comparing the condition's mean ± pooled standard deviation with the grand mean ± pooled standard deviation across all conditions.

the BSA digest that were consistently identified under all quench conditions. Individual peptide-level percent deuterium values are provided in Tables S1 and S2 of the Supporting Information in addition to p-values for each condition calculated using Welch's t-test by comparing the condition's mean ± pooled standard deviation with the grand mean ± pooled standard deviation across all conditions.

As shown in Figure 1, only modest differences in average deuterium uptake were observed among low pH quench conditions (C1−C7). The effect was negligible for phosphorylase b but somewhat greater for the BSA digest peptides, which likely reflects the distinct structural states of the two analytes. BSA was digested with trypsin prior to deuteration and quenching; therefore, BSA peptides lacking higher-order structure remained fully solvent accessible throughout the quench step, making them more responsive to subtle differences in quench solution composition. In contrast, phosphorylase b protein was deuterated and entered the quench solution as an intact, folded protein. Although denaturants in the quench solution facilitate unfolding, this process is not instantaneous and is unlikely to reach completion, protecting a fraction of exchangeable sites from solvent.[11] Additionally, the variation among the pH 2.5 conditions remained considerably smaller than the decrease in deuterium uptake observed for the pH 4.0 quench condition (C8). These results indicate that among the conditions investigated, quench pH 4.0 exerted the greatest influence on deuterium back-exchange.

Among the pH 2.5 quench conditions, C5 produced the highest average deuterium retention for BSA digest peptides, although the improvement relative to the remaining pH 2.5 quench conditions was small. In contrast, C8 consistently resulted in the lowest deuterium retention for both analytes, corresponding to an additional loss of approximately 4 percentage points of deuterium than C1. C8 differed from C1 only in quench pH (approximately pH 4.0 versus pH 2.5), while the denaturant and reducing agent concentrations remained consistent. The increased deuterium loss is attributed to the higher quench pH, which permits faster residual hydrogen−deuterium exchange during the quench step. We did not compare pH 2.5 to pH 3, as these pH values were

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previously reported to produce similar back-exchange.[5] Conditions C4 and C7 showed no difference for either sample type, meaning neither urea nor GdnHCl had a greater impact on back-exchange when used at 4 mol/L concentration.

Although C8 resulted in slightly greater back-exchange, it may still be advantageous for proteins containing multiple disulfide bonds or proteins that undergo inefficient digestion under conventional HDX quench conditions.[5] Pepsin has been shown to have optimal activity between pH 4 and pH 6.5, likely due to an intermediate pepsin conformation that is dominant in this pH range.[12] Similarly, fungal protease type XIII also increases in digestion efficiency as pH increases from pH 2.5 to pH 4.[13] Performing digestions nearer pH 4 would improve digestion efficiency, thereby likely improving sequence coverage. Although this benefit to sequence coverage is protein dependent and could result in overdigestion, therefore, pH impact on sequence coverage should be evaluated by the user for their specific protein. The reducing efficiency of TCEP increases with increasing pH,[14] and the improved reduction may also enhance proteolytic digestion and sequence coverage. In such cases, the modest increase in back-exchange may be offset by the improved structural information obtained.

Overall, these results demonstrate that among the quench parameters investigated, quench pH exerted the greatest influence on deuterium back-exchange, whereas variations in denaturant and reducing agent concentrations had comparatively minor effects over the ranges examined.

Effect of Quench Duration on Deuterium Back-Exchange

Longer quench times are sometimes used because it has been shown that increasing the time for denaturation and disulfide reduction during the quench step improves sequence coverage.[15,16] To evaluate the influence of quench duration on deuterium back-exchange, quench condition C1 was applied for quench times of 1, 2, 3, 4, 5, and 10 min, while all the other experimental parameters were held constant. Figure 2 summarizes the average deuterium uptake of the same 39 phosphorylase b peptic peptides and 60 BSA digest peptides used in Figure 1. Individual peptide-level data are provided in Tables S3 and S4 of the Supporting Information.

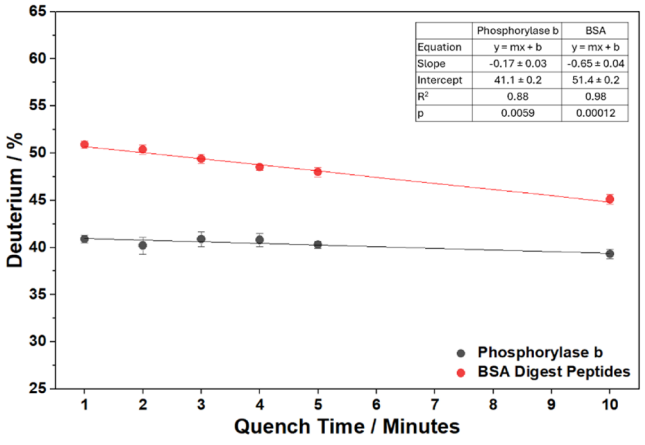


Figure 2. Effect of quench time on the deuterium retention of phosphorylase b and BSA digest peptides using quench condition C1. Values are presented as mean ± pooled standard deviation of all individual peptide means from triplicate measurements. Values from linear regression analysis are provided in the table.

As shown in Figure 2, increasing the quench duration resulted in a gradual decrease in average deuterium uptake for both analytes, confirming that hydrogen−deuterium exchange is substantially slowed but not completely arrested under conventional HDX-MS quench conditions.[3] However, the magnitude of this effect differed markedly between the two analytes, with the rate of back-exchange for BSA digest peptides being 3.8 times greater than for phosphorylase b. Extending the quench duration from 1 to 10 min reduced average deuterium content by approximately 6 percentage points for BSA digest peptides but only 1.6 percentage points for phosphorylase b. The much greater deuterium loss observed for the BSA digest peptides further supports the interpretation that structural protection plays an important role in limiting back-exchange during quenching. Because the digest peptides remain fully accessible throughout the quench period, the residual exchange continues over the entire range of quench intervals. In contrast, the much smaller decrease observed for phosphorylase b indicates that residual higher-order structure continues to protect many backbone amide deuterons even after exposure to the denaturing quench solution. Thus, extending the quench duration has a much greater impact on unstructured peptides than on intact proteins.

The minimal impact of prolonged quench duration on phosphorylase b suggests that longer quench times may be used, when necessary, to improve protein denaturation, disulfide-bond reduction, and proteolytic digestion without substantially compromising deuterium back-exchange. This may be particularly useful for proteins with extensive disulfide bonds, membrane proteins, and large protein complexes. In contrast, peptide standards represent a worst-case scenario because they lack structural protection during quenching and therefore should not be used to directly estimate quench-induced back-exchange in intact proteins. Intrinsically disordered proteins would likely have responses to quench conditions somewhere between those for phosphorylase b and the peptide standards reported herein.

Collectively, these findings indicate that quench duration is a comparatively minor contributor to deuterium back-exchange for proteins relative to quench pH. Therefore, optimization of HDX-MS quench conditions should prioritize maintaining low pH conditions (pH 2.5 to 3.0) while selecting a quench duration that provides adequate time for protein denaturation and reduction to maximize digestion efficiency and peptide sequence coverage.

CONCLUSION

This study presents a systematic experimental evaluation of the effects of quench solution composition and quench duration on deuterium back-exchange during HDX-MS sample preparation. Among the variables investigated, quench solution pH exerted the greatest influence on deuterium back-exchange. Increasing the quench pH from approximately 2.5 to 4.0 produced an approximately 4 percentage point decrease in average percent deuterium, whereas varying denaturant and reducing agent concentrations over the ranges examined had minimal effects on back-exchange.

The influence of quench duration depends strongly on the structural state of the analytes. Predigested BSA peptides exhibited progressive deuterium loss with increasing quench time because their backbone amide hydrogens remained fully solvent accessible throughout the quench period. In contrast,

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intact phosphorylase b showed only an approximately 1.6 percentage point reduction in average deuterium uptake between 1 and 10 min of quenching, demonstrating that residual higher-order structure retained during the quench step continues to protect many backbone amide hydrogens from solvent-mediated back-exchange. These findings also indicate that peptide standards represent a worst-case scenario for quench-induced back-exchange and should not be used to directly estimate deuterium loss in intact proteins.

From a practical perspective, the results demonstrate that conventional low pH quench conditions remain optimal for minimizing deuterium loss, whereas moderately higher quench pH may provide a useful compromise when improved disulfide-bond reduction, digestion efficiency, or peptide sequence coverage is required. Furthermore, the minimal effect of prolonged quench duration on intact proteins suggests that longer quench times can be employed, when necessary, to improve protein processing without substantially compromising deuterium preservation. Overall, this work establishes practical, evidence-based guidelines for optimizing the HDX-MS quench step and provides a framework for selecting quench conditions that balance deuterium preservation with efficient protein processing, thereby improving the accuracy, reproducibility, and standardization of HDX-MS workflows.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.6c00274.

The percent deuterium for individual peptides for all conditions (Tables S1−S4) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Kyle W. Anderson − *Institute for Bioscience and Biotechnology Research, National Institute of Standards and Technology and the University of Maryland, Rockville, Maryland 20850, United States; National Institute of Standards and Technology, Bioprocess Measurements Group, Biomolecular Measurement Division, Gaithersburg, Maryland 20899, United States;* orcid.org/0000-0002-2808-3026; Email: kyle.anderson@nist.gov

Author

Sandeep Kumar − *Institute for Bioscience and Biotechnology Research, National Institute of Standards and Technology and the University of Maryland, Rockville, Maryland 20850, United States;* orcid.org/0000-0002-3867-5586

Complete contact information is available at: https://pubs.acs.org/10.1021/jasms.6c00274

Author Contributions

The manuscript was written through contributions of all authors. All authors have approved the final version of the manuscript.

Notes

Certain commercial materials and equipment are identified to adequately specify experimental procedures. Such identifications neither imply recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the material or equipment identified is the best available for the purpose.

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

S.K. was funded through the NIST Cooperative Agreement with the University of Maryland, College Park (70NANB24H164). All other funding was provided through the NIST Biomanufacturing Program of the NIST Biomolecular Measurement Division.

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