TECHNICAL NOTE 000309

Achieving next-level performance for HDX-MS with the Orbitrap Exploris 240 mass spectrometer

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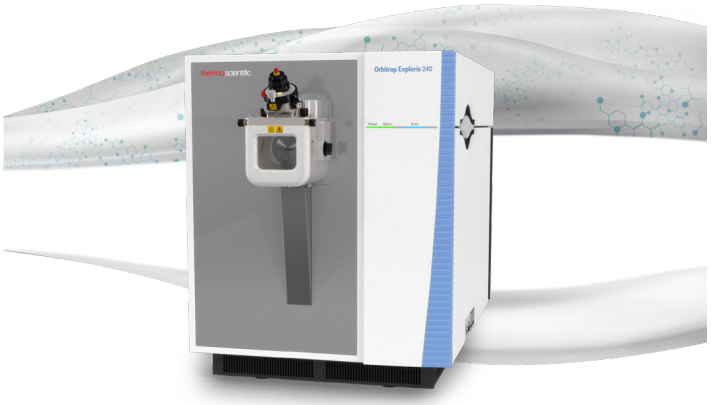
Goal

Benchmark performance of the new generation of the Trajan LEAP™ HDX extended parallel system integrated with the Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer for HDX-MS workflow.

Introduction

There are many analytical tools, such as X-ray crystallography, cryo-electron microscopy (cryo-EM), and nuclear magnetic resonance (NMR) spectroscopy, to study protein conformation, conformation dynamics, and protein-protein interactions. Each method faces different challenges and limitations depending on the protein being studied. Hydrogen deuterium exchange mass spectrometry (HDX-MS) has emerged as a powerful tool to fill the analytical gap.[1,2] In this technical note, we describe and benchmark a fully automated HDX-MS workflow solution interfacing the new Trajan LEAP extended parallel system with the Orbitrap Exploris 240 mass spectrometer.



Experimental

Methods and materials

Protein	Phosphorylase b, Sigma (P6635) Trastuzumab provided by a pharmaceutical company
Columns	
Analytical column	Thermo Scientific™ Hypersil GOLD™, 1.9 µm, 1 mm x 50 mm, (P/N 25002-051030)
Trapping column	Thermo Scientific™ Acclaim™ PepMap™ 300 C18, 5 µm, 0.5 mm x 15 mm (P/N 163593), holder (P/N 164650)
Digestion column	Immobilized protease III/pepsin, NovaBioAssays (NSA2014002)
Labeling buffer	PBS in D_2O
Quenching buffer	2 M GuHCL + 100 mM citric acid, pH 2.1 for phosphorylase b
Quenching buffer	4 M GuHCL + 0.2 M TCEP, pH 2.2 for trastuzumab
Digestion	20 °C, 5 min
Labeling	4 °C
Separation	0.5 °C

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Mobile phase A	0.1% formic acid in water
Mobile phase B	0.1% formaic acid in acetonitrile
Mass spectrometer	Orbitrap Exploris 240 mass spectrometer
Liquid chromatography	Thermo Scientific™ UltiMate™ NCS-3500RS, microflow configuration
Data analysis	Thermo Scientific™ BioPharma Finder™ 4.1 software HDExaminer™ 3.2 software (Sierra Analytics)

Loading conditions:

Retention [min]	Flow [µL/min]	%B
1.0	35	0
3.0	35	0
3.1	300	0
4.9	300	0
5.0	100	0
6.0	100	4
10.0	100	4
11.0	50	0
22.0	50	0
23.0	35	0

Separation conditions:

Retention [min]		Flow [µL/min]	%B
7-minute gradient	3-minute gradient		
4.9	4.9	40.00	3.0
5.0	5.0	40.00	10.0
12.0	8.0	40.00	35.0
13.0	9.0	40.00	35.0
13.9	10.0	40.00	98.0
14.0	11.0	40.00	98.0
15.0	12.0	40.00	3.0
16.0	13.0	40.00	98.0
17.0	14.0	40.00	98.0
18.0	15.0	40.00	3.0

Mass spectrometry
ESI source conditions

Parameter	MS
Spray voltage (V)	3,500
Sheath gas	20
Aux gas	2
Capillary temperature (°C)	220
Vaporizer temperature (°C)	50

MS conditions

Parameter	MS	MS/MS
MS full resolution (at *m/z* 200)	120K	60k
AGC	200%	200%
MS mass range, *m/z*	300–1300	Start 200
RF lens	40	-
Isolation	-	2
DDA	-	Top 15
Collision energy (%)	-	30
Charge state	2–6	2–6
Intensity threshold	-	5e4
Max injection time (ms)	200	200

HDX workstation

Figure 1 shows the HDX workstation consisting of the Trajan LEAP HDX extended parallel system integrated with the Orbitrap Exploris 240 MS. The new LEAP HDX extended parallel system can accurately schedule fast deuterium exchange time points (approximately 15 s) to probe protein conformation dynamics. There are three valves inside the cooling chamber (Figure 2); the first and the third valves are six-port valves and the second valve is a ten-port valve. The first six-port valve connects the sample loop and the loading pump. The loading pump drives the flow either from the sample loop or bypasses the sample loop entirely depending on the experimental stage. The second ten-port valve connects the enzymatic and trapping columns. The third six-port valve connects the analytical column driving the binary high pressure gradient pump to deliver the gradient flow to the mass spectrometer for data acquisition. This newly designed three-valve system can set up the back-flash function to efficiently reduce the carryover and clean up the buildup on top of the column, which results in highly reproducible chromatography.

Figure 1. HDX-MS workstation

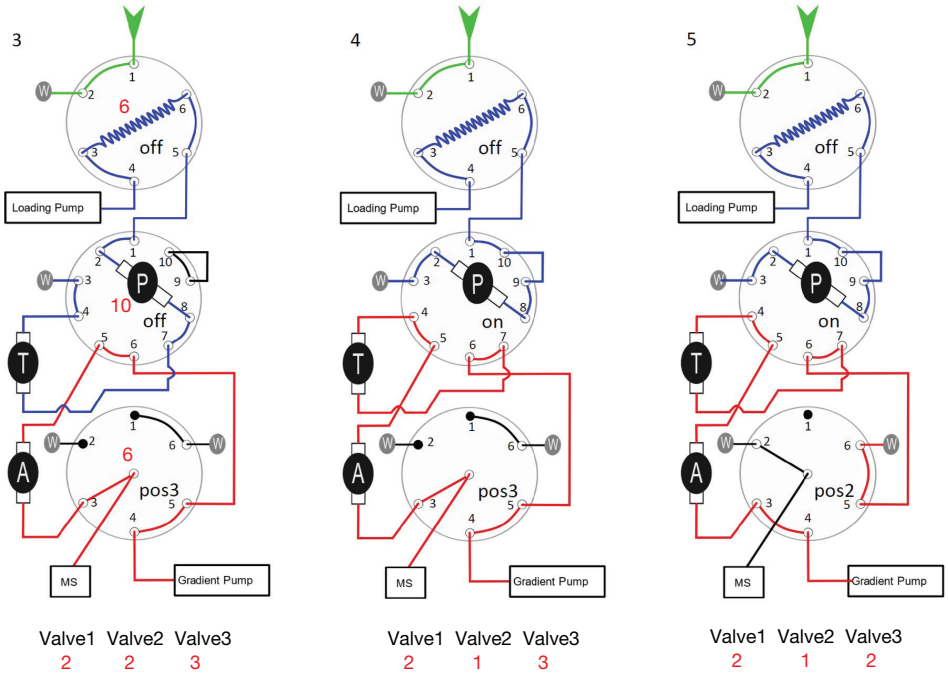

Figure 2. Inside the cooling chamber. The flexible three valve configurations for carrying out different experimental tasks simultaneously are shown.

Experimental method

Phosphorylase b or trastuzumab samples were diluted (1 to 9 ratio) with labeling buffer and incubated in D_2O for five time points (15, 30, 300, 1,800, and 3,600 s) at 4 °C. The samples were then quenched and subjected to online enzymatic digest (NovaBioassay Immobilized protease III/ pepsin, 2.1 x 3 mm). The digested peptides were injected onto an Acclaim PepMap trapping column and eluted onto a Hypersil GOLD C18 reversed phase column. The separated peptides were analyzed with an Orbitrap Exploris 240 mass spectrometer. In the first step of the MS analysis, data dependent MS/MS HCD spectra were collected from the undeuterated protein digest. Using BioPharma Finder software all detected peptides were identified. In the second step, MS full scan spectra were collected for the HDX-MS experiment in triplicate. Figure 3 shows the base peak chromatogram for two different gradient lengths. It is very important to have highly reproducible chromatography separation for deuterium uptake calculation.

Data analysis

Peptide identification, peptide mapping, PTM analysis, and protection factor plots based on the experimental deuterium incorporation calculation were performed within BioPharma Finder 4.1 software. The deuterium uptake calculation was performed using HDExaminer 3.2 software (Sierra Analytics).

Results and discussion

Different concentrations of phosphorylase b sample were used to evaluate the system performance at two gradients, 3 and 7 minutes (Separation table, Experimental section), as shown in Figure 3. Over 1,000 peptides were identified for the three tested protein concentrations. The protein sequence coverage was above 95%. The system was highly sensitive and allowed identification of over 600 peptides with close to 90% sequence coverage for the 3-minute gradient. Peptide mapping results are summarized in Table 1.

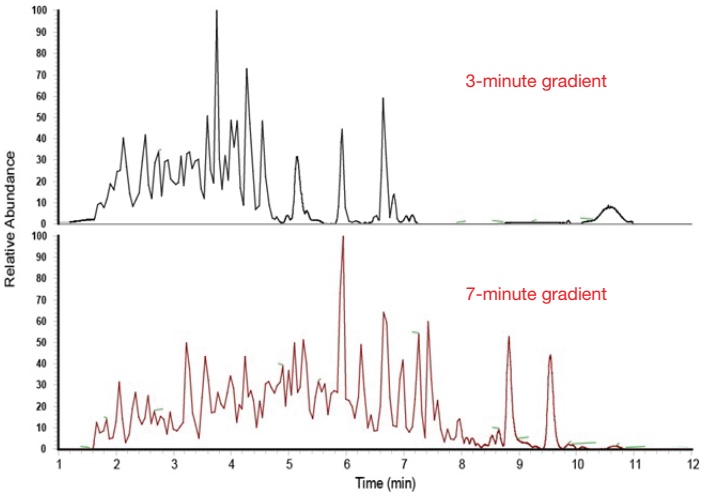

Figure 3. Phosphorylase b (32 µmol) base peak chromatograms at different gradient lengths

Table 1. Phosphorylase b results

Concentration (µm)	7-minute gradient		3-minute gradient	
	Peptides identified	Sequence coverage	Peptides identified	Sequence coverage
8	1054	95.0%	651	89.0%
16	1330	95.8%	713	90.4%
32	1684	96.7%	950	92.2%

The two parallel arms of the LEAP HDX system make it possible to accurately schedule short time exchange experiments. For the two short exchange time experiments (15 and 30 s incubations) at 32 µM concentration, the deuterium uptake calculation standard deviation was in the range of 0 to 0.14 with an error of less than 10% (Figure 4) for the measured 700 data points.

To evaluate the dynamic range of the system, dilution series (Table 2) of trastuzumab samples were analyzed. The quenching buffer of 4 M GuHCL with 0.2 M TCEP was used to break the disulfide bond of the sample. The lowest concentration of 0.33 µM was tested. To maximize peptide identification for the low protein concentration, the mass spectrometry method was optimized by increasing the injection time for the MS^2 acquisition to 400 ms to improve the spectra quality. The results are summarized in Figure 5.

With the optimized method, approximately 200 peptides were identified for the lowest concentration of sample (top panel, Figure 5), with the sequence coverage of 69% and 73% for the light and heavy chains, respectively (low panel, Figure 5).

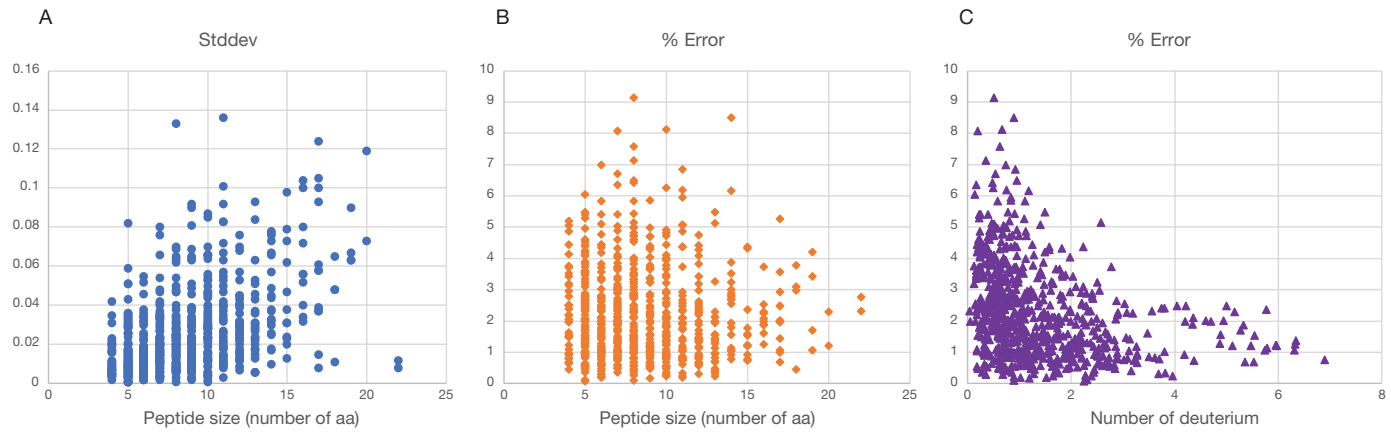

Figure 4. (A) Standard deviation vs. peptide size, (B) percentage error vs. peptide size, (C) percentage error vs. number of deuterium atoms

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Table 2. Dilution series of trastuzumab sample analysis

Sample	Concentration (µM)	On column (pmol)
1	0.33	0.8
2	1	2.3
3	3	6.8
4	9	20
5	13.8	31
6	27	60

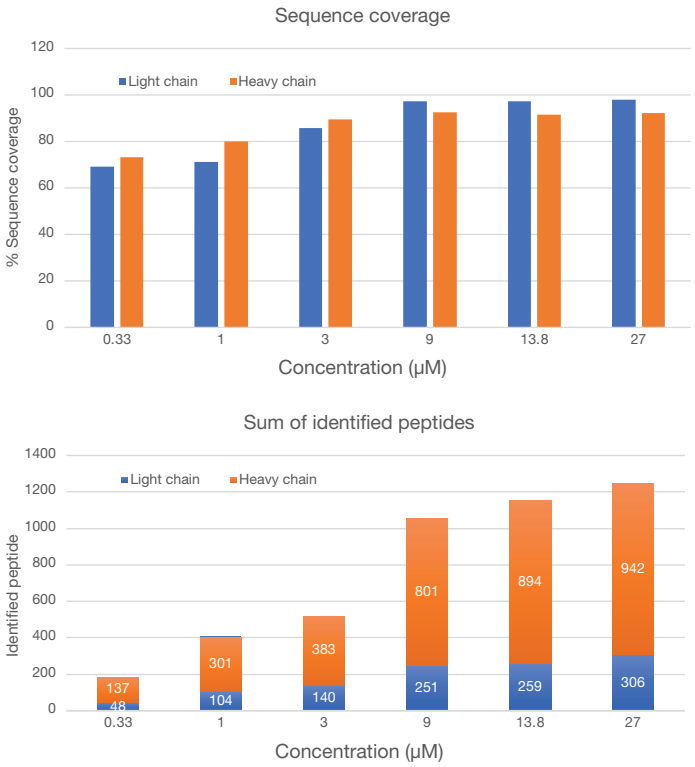

Figure 5. Trastuzumab analysis results

Conclusions

- Performance of the fully automatic, next-generation HDX workstation consisting of the Trajan LEAP HDX extended parallel system and the Orbitrap Exploris 240 mass spectrometer was evaluated for HDX experiment speed, reproducibility, and sensitivity.
- The sensitivity of the system was excellent, attaining close to 90% sequence coverage with more than 600 peptides identified for the short 3-minute gradient method for phosphorylase b protein at 8 µM concentration.
- The system can generate a highly reproducible deuterium uptake measurement for short exchange time HDX experiments with less than 10% error.
- The optimized mass spectrometry method enabled 70% sequence coverage for 1 pmol trastuzumab for both light and heavy chains using a 7-minute gradient.

References

1. Houde, D. et al., Anal. Chem. 2009, 81, 2644-51.
2. Rose, R. J. et al., mAbs 2013, 5, 219-228.

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