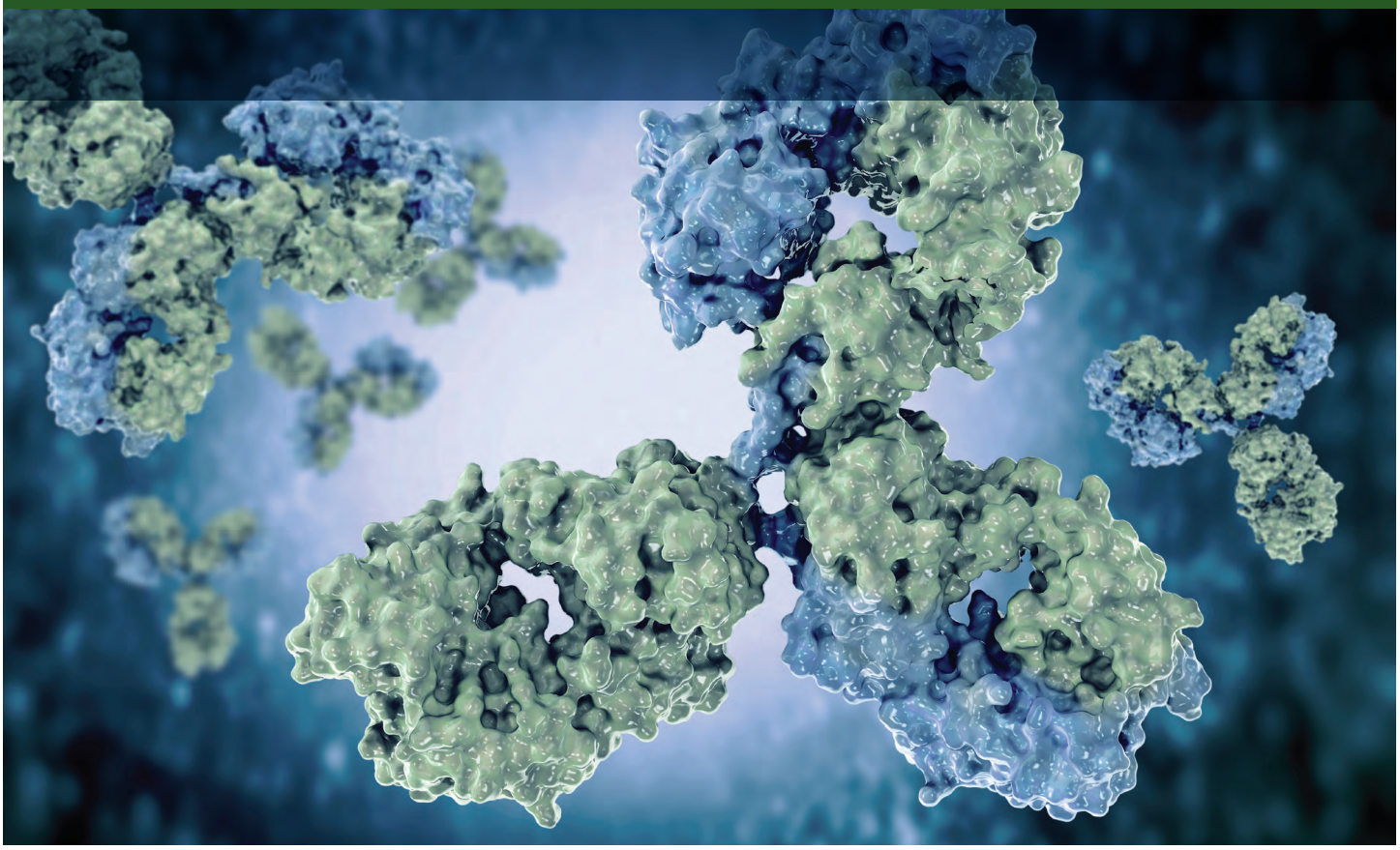
Agilent
Trusted Answers

Agilent-NISTmAb

Peptide Mapping

Agilent BioHPLC Columns
Application Compendium



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Agilent-NISTmAb Standard (P/N 5191-5744; 5191-5745) was aliquoted from NISTmAb RM 8671 batch. Quality control (QC) testing is performed using Agilent LC-MS system. QC batch release test includes aggregate profile, charge variants and intact mass information. A certificate of analysis (CoA) can be found in each product shipment with test results.

Please note that authors used various monoclonal antibodies including Agilent-NISTmAb Standard and NISTmAb RM 8671 to demonstrate critical quality attribute workflows.

Peptide Mapping

Introduction

Peptide mapping – an invaluable tool for biopharmaceuticals – is a very powerful method and the most widely used identity test for proteins, particularly those produced by recombinant means. Peptide mapping can be used to comprehensively identify a protein primary structure. It is also possible to distinguish the exact position of a variant within the protein. It most commonly involves enzymatic digestion (usually using trypsin) of a protein to produce peptide fragments, followed by separation and identification of the fragments in a reproducible manner, allowing the detection and monitoring of single amino acid changes, oxidation, deamidation, and other degradation products. It also enables the direct detection of common monoclonal antibody variants such as N-terminal cyclization, C-terminal lysine processing, and N-glycosylation, as well as other post-translational modifications.

Using this approach, mAb sample will be broken into fragments and a high resolution reversed-phase separation should be able to separate these out into a classic “fingerprint” chromatogram. Combining the separation with mass spectrometry detection should make it possible to correlate the actual peaks observed in the peptide mapping chromatogram with the expected fragments predicted by the analysis software. Different proteins will give different peptide “fingerprints”, and these will include a wide range of sizes (from individual amino acids and dipeptides up to much larger polypeptides), with varying degrees of hydrophobicity. The recommended column for this type of separation is therefore a C18 reversed phase in either superficially porous or totally porous particles.

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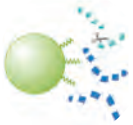

Peptide Mapping

Reversed-phase chromatography (<150 Å)

Reliably characterizes primary sequence and detects PTMs

AdvanceBio Peptide Mapping

Protein identification and PTM analysis

Attribute	Advantage
Endcapped C18 bonded phase	Good retention of hydrophilic peptides
Superficially porous particles	UHPLC-like efficiency at modest back pressure

A peptide map is a fingerprint of a protein and the end product of several processes that provide a comprehensive understanding of the protein being analyzed. It involves four major steps: isolation and purification of the protein; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and validated analysis of the peptides.

Peptide mapping is considered a comparative procedure that confirms the primary structure of the protein and detects alterations in structure. Additionally, it demonstrates process consistency and genetic stability. A peptide map should include positive identification of the protein, maximize coverage of the complete peptide sequence, and provide additional information and sequence identification beyond that obtained at the non-digested protein level.

The selection of a chromatographic technique to separate peptides and generate peptide maps depends on the protein, experimental objectives, and anticipated outcome. However, the excellent resolving power of reversed-phase chromatography (RPC) makes this technique the predominant HPLC technique for peptide mapping separations. It is also ideal for both analytical and preparative separations because of the availability of volatile mobile phase eluents. It is important to note that the preferred columns for peptide mapping separations are similar to those used for small molecules, but because most peptide mapping separations are performed at low pH and elevated temperature, columns with excellent pH stability and minimal silanol effects are routinely used.

Careful inspection of the complete characterization strategy is required to generate successful peptide maps. A profile may consist of over 100 peaks representing individual peptides and their derivatives, so it requires knowledge of sample preparation methods, powerful separation techniques and validated protocols. Having the skill and information to develop a successful peptide map will help you achieve the best possible separation of your proteolytic digests and deliver a successful and reliable peptide characterization outcome. Optimum peak shape is obtained using trifluoroacetic acid as ion pair reagent and for this separation the AdvanceBio Peptide Mapping column is the preferred choice.

This column contains a 120 Å pore size Poroshell particle and provides excellent resolution and peak capacity without the need for UHPLC instrumentation. For extremely hydrophilic, small peptides AdvanceBio Peptide Mapping is recommended for best retention. For applications where MS detection will be used, it is often preferable to use formic acid as ion pairing reagent.

The featured application note in this chapter highlight high-throughput workflow that uses the Agilent AssayMAP Bravo liquid-handling robot, the Agilent 1290 Infinity II UHPLC system coupled to Agilent 6545XT AdvanceBio LC/Q-TOF, and automatic data analysis using Agilent BioConfirm software for complete NISTmAb sequence mapping analysis.

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Quick Start Guide:

Reduce Peptide Mapping time without losing resolution

Biopharmaceutical discovery and development require you to characterize a target molecule's primary sequence to confirm its identity, and/or determine amino acid substitutions or modifications that may occur during manufacturing. It is currently necessary to use high-resolution techniques to resolve individual peptides for identification and quantitation. Accordingly, conventional peptide mapping with fully porous HPLC columns can take 60 minutes or longer to complete.

Agilent AdvanceBio Peptide Mapping columns let you quickly resolve and identify amino acid modifications in primary structure.

These advanced biocolumns feature a 120Å pore size with superficially porous 2.7 µm particles. They are specially tested with a challenging peptides mix to ensure reliable peptide mapping performance. In addition, AdvanceBio Peptide Mapping columns deliver exceptional resolution and speed for UHPLC, and excellent results for conventional HPLC too.

- Greater analytical confidence: Each batch of AdvanceBio Peptide Mapping media is tested with a rigorous peptide mix to ensure suitability and reproducibility, and to enable the identification of key peptides in complex peptide maps.
- Save time: 2 to 3 times faster than fully porous HPLC columns.
- Every instrument works harder: 4.6, 3.0, and 2.1 mm id columns are stable to 600 bar, enabling you to get the most from your UHPLC instruments. They can also deliver excellent performance for your legacy 400 bar instruments, too.
- Increased flexibility: Achieve increased MS sensitivity with formic acid mobile phases on any HPLC.

AdvanceBio columns are rigorously tested to ensure reproducibility and confidence in your results. They are also backed by Agilent's 60-day full satisfaction warranty.

Agilent AdvanceBio Peptide Mapping BioHPLC Columns.

With their 2.7 µm particles and C18 functionality, Agilent AdvanceBio Peptide Mapping columns provide excellent retention, resolution, and peak shape for basic hydrophobic peptides.

To learn more, visit **agilent.com/chem/AdvanceBio**

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Quickly confirm the identity of target proteins and peptides

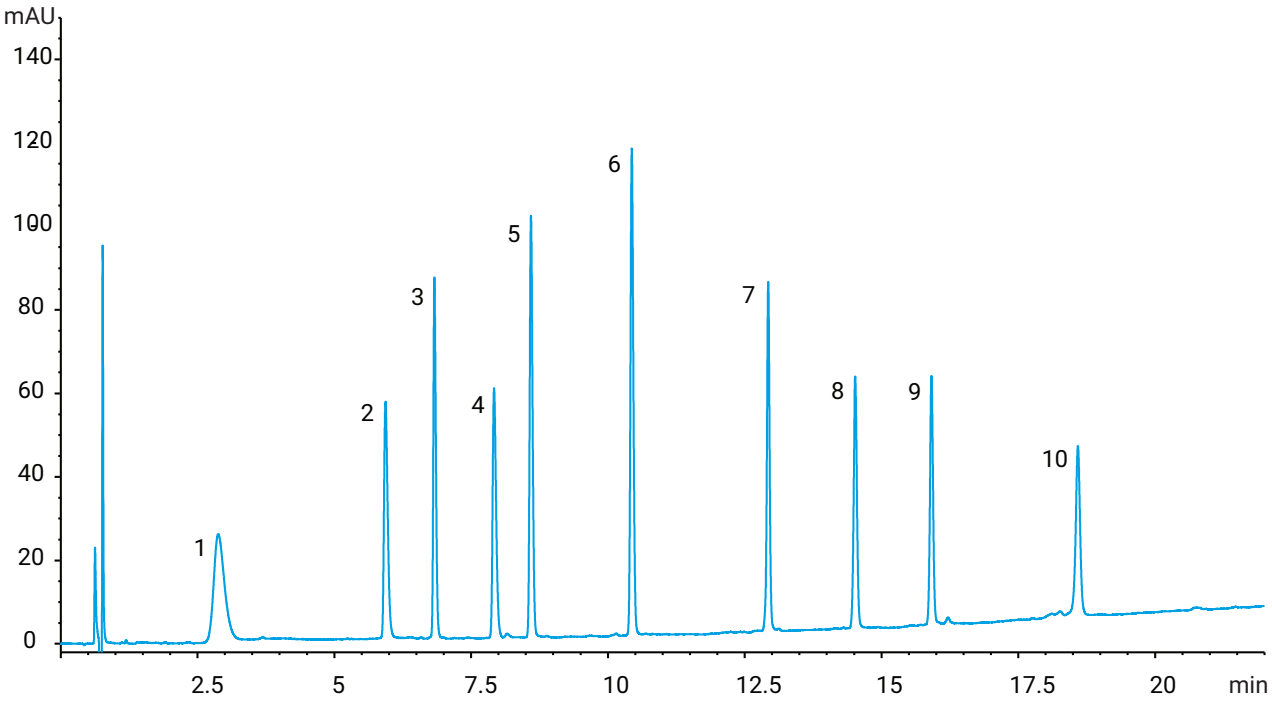
Quality Assurance Testing with Agilent Peptide Mix

Test mix used for every batch of AdvanceBio Peptide Mapping media. The mixture contains 10 hydrophilic, hydrophobic, and basic peptides, ranging in molecular weight from 757 to 2845 Da. Every column is also tested with a small-molecule probe to ensure efficiency.

Conditions

Parameter	Value
Column:	AdvanceBio Peptide Mapping, 2.1 x 150 mm, 2.7 µm, p/n 653750-902
Flow rate:	0.5 mL/min
Injection:	3 µL
Temp:	55 °C
Detection:	220 nm
Gradient:	A, water (0.1% TFA), B, ACN (0.1% TFA), 0-25 min,15-65% B; 25-26 min, 65-95% B
Sample:	Agilent Peptide Mapping Standards Mix (0.5-1.0 µg/µL per peptide) p/n 5190-0583

Pk no.	Peptide
1	Bradykin frag (1-7)
2	Bradykin Acetate
3	Angiotensin II
4	Neurotensin
5	Angiotensin I
6	Renin
7	[Ace-F-3,-2 H-1] Angiotensinogen (1-14)
8	Ser/Thr Protein Phosphotase (15-31)
9	[F14] Ser/Thr Protein Phosphotase (15-31)
10	Mellitin (Honey bee venom)



Separation of Agilent Peptide Mix standard on AdvanceBio Peptide Mapping column

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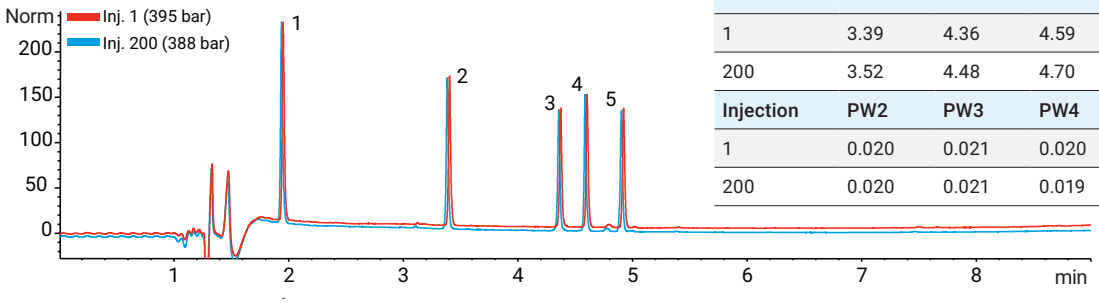
Lot-to-Lot Reproducibility after 200 Injections

Superior reproducibility, lot-to-lot and run-to-run. A 2.1 x 250 mm AdvanceBio Peptide Mapping column was used for maximum resolution.

Conditions

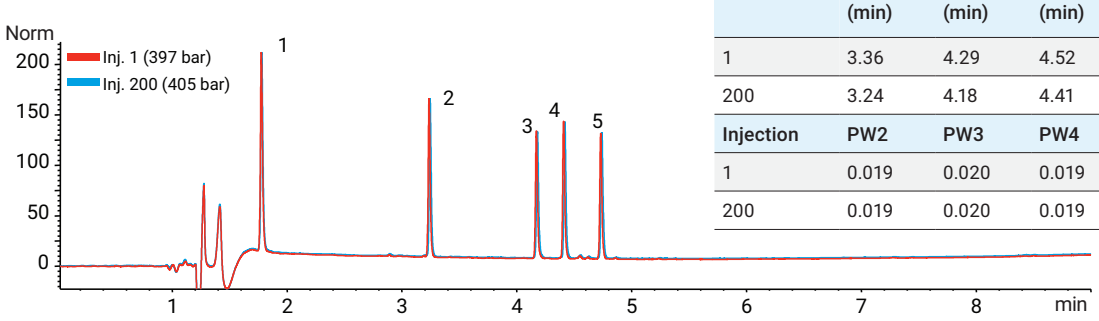
Parameter	Value
Column:	AdvanceBio Peptide Mapping, 2.1 x 250 mm, 2.7 µm, p/n 651750-902
Flow rate:	0.5 mL/min
Injection:	1 µL
Temp:	55 °C
Detection:	220 nm
Gradient:	A, water (0.1% TFA), B, ACN (0.08% TFA), 0-8 min, 10-60% B; 8.1-9 min, hold 95% B
Sample:	Sigma HPLC peptide standards: 1-Gly-Tyr, 2-Val-Tyr-Val, 3-Met Enk, 4-Angio II, 5-Leu Enk

Silica lot PEP1227229



Injection	RT2 (min)	RT3 (min)	RT4 (min)	RT5 (min)
1	3.39	4.36	4.59	4.90
200	3.52	4.48	4.70	5.02
Injection	**PW2**	**PW3**	**PW4**	**PW5**
1	0.020	0.021	0.020	0.022
200	0.020	0.021	0.019	0.021

Silica lot B12169



Injection	RT2 (min)	RT3 (min)	RT4 (min)	RT5 (min)
1	3.36	4.29	4.52	4.85
200	3.24	4.18	4.41	4.74
Injection	**PW2**	**PW3**	**PW4**	**PW5**
1	0.019	0.020	0.019	0.020
200	0.019	0.020	0.019	0.020

Chromatograms represent superior reproducibility on multiple batch lots of AdvanceBio Peptide Mapping column

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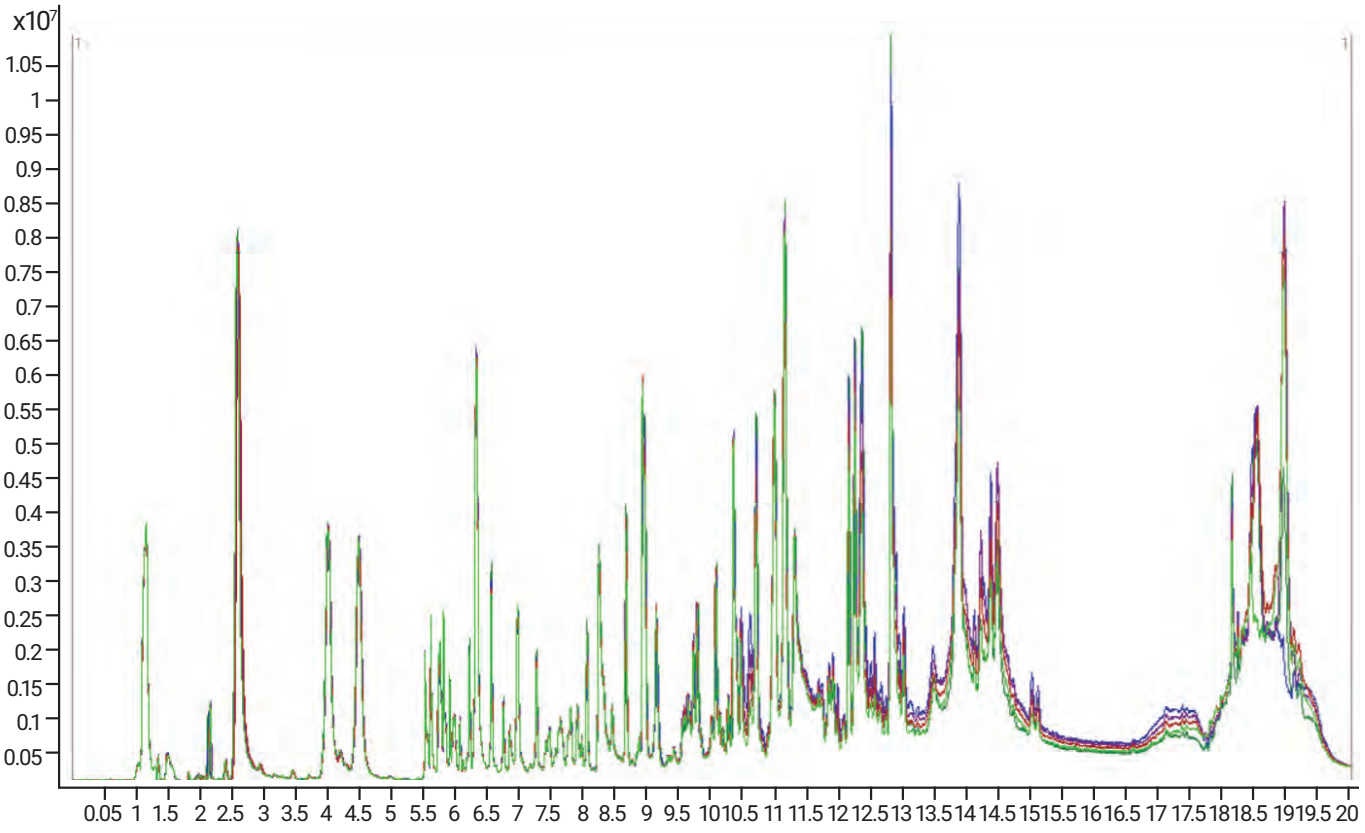
LC /MS Reproducibility

Excellent reproducibility of peak heights and retention times for more accurate identification of target peptides. The entire IgG1 tryptic peptide map was completed in just 20 minutes (n=5).

Conditions

Parameter	Value
Column:	AdvanceBio Peptide Mapping, 3.0 x 150 mm, 2.7 µm, p/n 653950-302
	LC /MS (Agilent 6520 Q-TO F) P arameters: Dry gas: 10 L/min, Vcap: 4000 V, fragmentor: 150 V
Flow rate:	0.3 mL/min
Injection:	1 µL
Temp:	40 °C
Detection:	220 nm
Gradient:	A, water (0.1% FA), B, ACN (0.10% FA), 0-3 min, 2% B; 3-13 min, 2-45% B; 13-15 min, 45-65% B; 15.1-17 min., hold 90% B
Sample:	Stratagene mAb, in-house tryptic digestion



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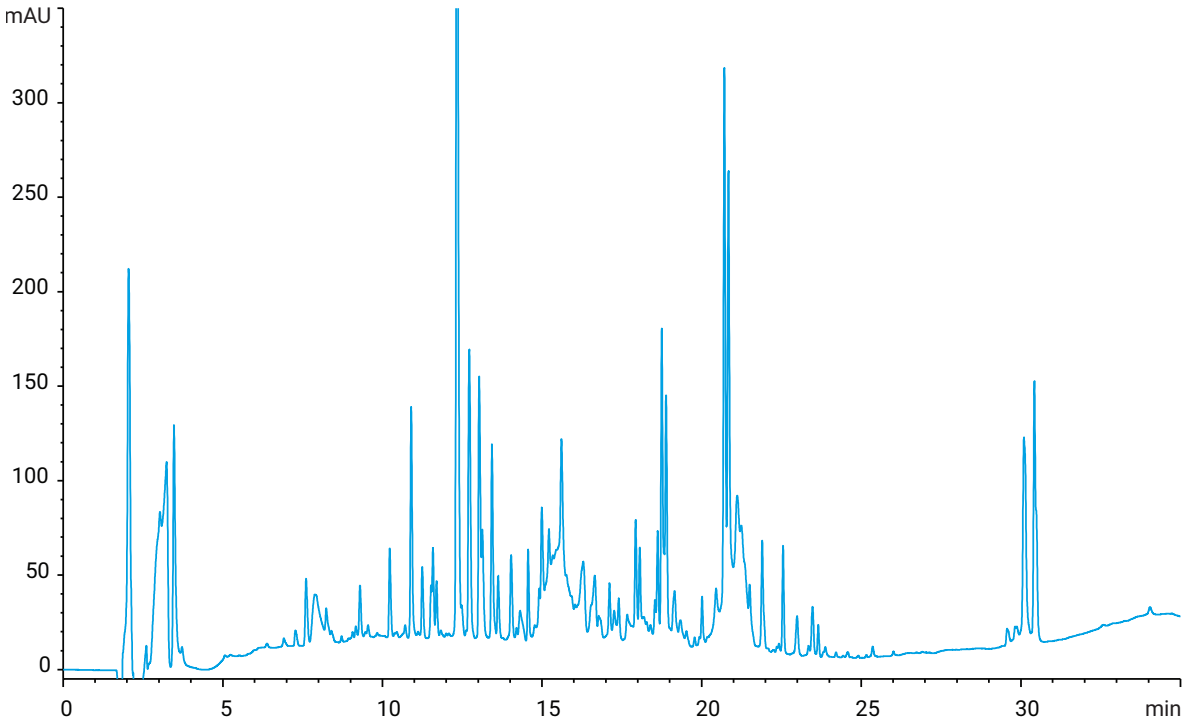
Peptide Map of a Biosimilar EPO

The top chromatogram shows a peptide map of a highly glycosylated EPO from a biosimilar. Note the excellent resolution achieved for small peptide fragments using UV detection. The bottom chromatogram shows the same separation using mass spectroscopy to determine the sequence coverage (100%). UV detection is used for comparing peptide maps, while MS is ideal for identifying amino acid substitutions and modifications.

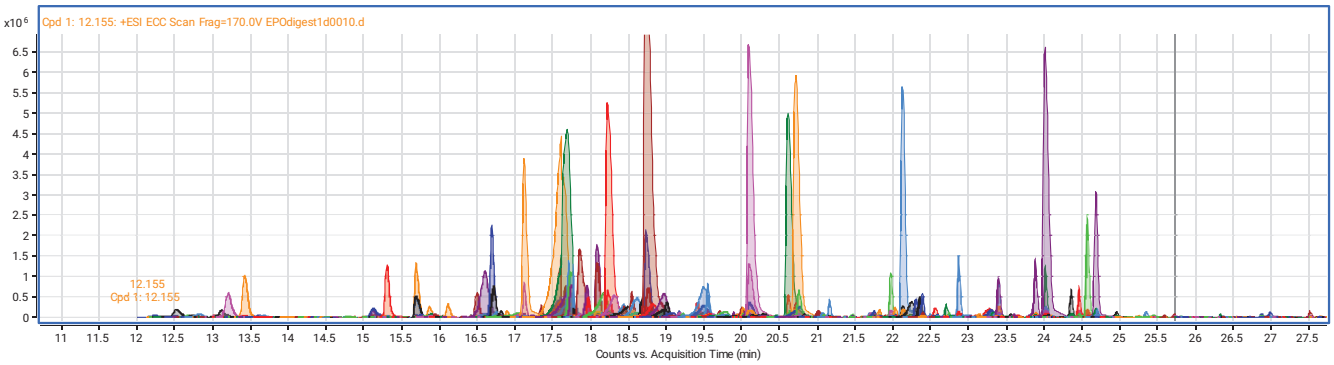
So, you can easily confirm protein identity, and identify any post-translational modifications, using the AdvanceBio Peptide Mapping column.

Conditions

Parameter	Value
Column:	AdvanceBio Peptide Mapping, 2.1 x 250 mm, 2.7 µm, p/n 651750-902
Flow rate:	0.4 mL/min
Injection:	5 µL (2.0 mg/mL)
Temp:	55 °C
Detection:	220 nm
Gradient:	A, water (0.1% FA); B, ACN (0.1% FA), 0-28 min, 3-45% B; 28-33 min, 45-60% B; 33-34 min, 60-95% B



EPO digest, Peptide sequence coverage achieved using UV Detection



EPO digest, LC/MS TOF 100% sequence coverage achieved using MassHunter Workstation software

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Agilent AdvanceBio columns:

For faster, more consistent biopharmaceutical analysis

AdvanceBio Peptide Mapping columns are part of Agilent's growing state-of-the-art family of biocolumns. They are designed to deliver consistent, exceptional performance for the separation and characterization of peptides and proteins, antibodies, conjugates, new biological entities, and biopharmaceuticals. The science behind AdvanceBio columns helps to advance accuracy and productivity that support faster analysis and efficiency in your lab.

Agilent AdvanceBio Peptide Mapping Columns

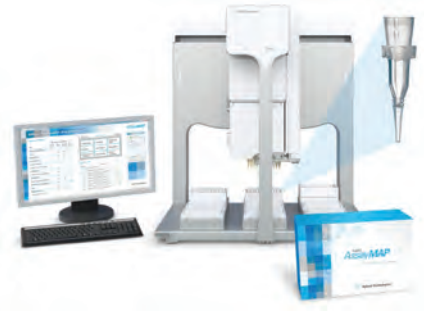
Description	Part Number
4.6 x 150 mm, 2.7 µm	653950-902
3.0 x 150 mm, 2.7 µm	653950-302
2.1 x 250 mm, 2.7 µm	651750-902
2.1 x 150 mm, 2.7 µm	653750-902
2.1 x 100 mm, 2.7 µm	655750-902
4.6 mm Fast Guard*	850750-911
3.0 mm Fast Guard*	853750-911
2.1 mm Fast Guard*	851725-911

* Fast Guards extend column lifetime without slowing down the separation or affecting resolution.

Peptide sample preparation for mass spec analysis, intelligently automated

Manual sample preparation of peptides is a time-consuming process. If you are doing peptide mapping applications on MS, you are likely looking for increased throughput. And you are going to be reliant on a highly reproducible end-to-end workflow to ensure your results are consistent. AssayMAP transforms digestion, cleanup, and fractionation workflows to enable previously unachievable precision and throughput:

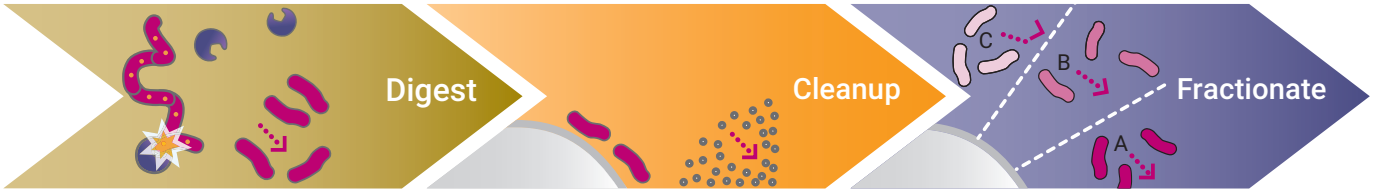
- Improved reproducibility, due to decreased human error – <5 % CVs
- Increased throughput – up to 384 samples each day
- Significantly reduces hands-on time – freeing up scientists to do analytical work
- Faster method development – the automated platform enables you to quickly optimize methods



AssayMAP Peptide Sample Prep Solution is based on the powerful combination of miniaturized, packed bed chromatography, the state-of-the-art Bravo Liquid Handling Platform and a simple, applications-based user interface that creates an open access environment for both novices and experienced users and simplifies the most challenging sample preparation workflows.

AssayMAP peptide sample prep solution

For Mass Spec Analysis



Digestion:

- In-solution digestion with user-supplied reagents
- Parallel process up to 4x96-well plates
- 1 manual pipetting step

Benefits:

- Reduce user variability
- Improve throughput and reproducibility

Cleanup:

- Quantitative separation method using reversed-phase cartridges
- Parallel process 1x96-well plate

Benefits:

- 10 µL elution equals short dry down times or "dilute and shoot" method
- Process control – every sample is treated identically

Fractionation:

- Strong cation exchange (SCX) cartridges generate up to 6 fractions to simplify the sample using step-wise elution with pH or salt
- Parallel process 1x96-well plate

Benefits:

- Increases LC/MS throughput by taking fractionation offline, reducing long LC gradient times
- Powerful enrichment tool for simplifying samples and isolating target peptides prior to analysistreated identically

Total workflow benefit:

- User interfaces for workflows are standardized for ease-of-use and linked for workflow integration.
- AssayMAP reduces the need for sample replicates and requires fewer repeated samples.

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Achieve total workflow reproducibility with Agilent AssayMAP solution for sample prep before mass spec analysis

The AssayMAP Peptide Sample Prep Solution was used to digest 64 replicates each of two sample types: BSA in urea and guanidine HCL. The samples were cleaned using AssayMAP reversed-phase cartridges and analyzed using an Agilent AdvanceBio Peptide Mapping column, Agilent 1290 Infinity LC, and an Agilent 6550 iFunnel Q-TOF mass spectrometer. The experiment was repeated on day two to examine reproducibility. %CV was determined for 25 peptides within each sample as shown in Table 1. The different %CV bins are shown. Illustrating the contributions of the total average %CV. To further showcase the reproducibility, peak area for representative peptides are shown in Figure 12.

The AssayMAP sample prep took about four hours per day, with only two hand-on hours per day. Manual sample prep for the same workflow would take about eight hours per day, with four hands-on hours each day.

Total workflow CVs were <4%. The full workflow included AssayMAP Peptide Sample Prep system, an Agilent AdvanceBio Peptide Mapping Column, the 1290 Infinity LC System, and an Agilent 6550 iFunnel Q-TOF mass spectrometer.

For more details about this application, see Agilent publication 5991-2474EN.

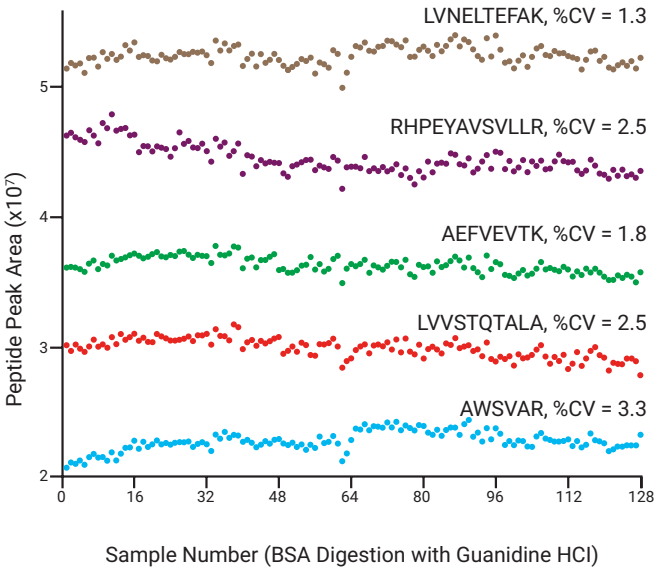

Figure 12. Scatter plots showing peak area of 4 peptides over 2 days.

Table 1. – %CV by day with different %CV bins.

	Urea (n=64, 62)		Guanidine HCl (n=64, 62)	
25 Peptides	**Day 1**	**Day 2**	**Day 1**	**Day 2**
Average Peak Area %CV	3.3	3.7	2.3	2.6
Peptides with %CV<5	23	21	25	23
Peptides with 5>%CV<10	2	3		1
Peptides with %CV>10		1		1

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Application Note

Biotherapeutics and Biosimilars

Making Peptide Mapping Routine with the Agilent 6545XT AdvanceBio LC/Q-TOF

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Introduction

Monoclonal antibodies (mAbs) comprise a rapidly growing group of protein-based biomolecules being researched. Due to the heterogeneous nature of protein drugs, extensive analytical characterization is required.

Peptide mapping by the combination of liquid chromatography and electrospray mass spectrometry (LC/MS) is a well-established technique used by the biopharmaceutical industry for the confirmation of the primary sequence of an mAb. The comprehensive characterization provides not only the complete amino acid sequences of mAbs and their variants, but also the information on post-translational modifications (PTMs) and locations1-3. However, the lack of automatic workflow in the data processing and result interpretation has been the rate-limiting step for most biopharmaceutical analytical or clinical research laboratories.

In this study, we have developed a high-throughput workflow that uses the Agilent AssayMAP Bravo liquid-handling robot, the Agilent 1290 Infinity II UHPLC system, the Agilent 6545XT AdvanceBio LC/Q-TOF, and automatic data analysis using Agilent BioConfirm software for complete sequence mapping analysis.

Figure 1. Agilent 6545XT AdvanceBio LC/Q-TOF system.

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Experimental

Materials and methods

Monoclonal antibody (mAb) standard RM 8671 was purchased from National Institute of Standards and Technology (NIST). DL-Dithiothreitol (DTT), iodoacetamide (IAA) and guanidine-hydrochloride were purchased from Sigma-Aldrich. High quality mass spec grade Trypsin/Lys-C enzyme mix was obtained from Promega. AssayMAP C18 cartridges were from Agilent Technologies.

The Agilent AssayMAP Bravo liquid handling system was used to dilute, digest, and desalt the NISTmAb sample. Samples were then dried down and resuspended with 0.1 % TFA in DI water. Approximately 0.5 µg of mAb digested sample was injected for each LC/MS/MS analysis.

LC/MS analysis

LC/MS analyses were conducted on an Agilent 1290 Infinity II UHPLC system coupled with an Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with an Agilent Dual Jet Stream ESI source. LC separation was obtained with an Agilent AdvanceBio Peptide Mapping column (2.1 × 150 mm, 2.7 µm). Tables 1 and 2 list the LC/MS parameters used.

Data processing

Raw data acquired from LC/MS/MS were processed using Agilent MassHunter BioConfirm B.08.00 software. This powerful algorithm simplifies downstream data analysis, enabling the automatic identification of peptides and PTMs when compared to a theoretically digested NISTmAb sequence.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II UHPLC System	
Column	AdvanceBio Peptide Mapping column, 2.1 x 150 mm, 2.7 µm (p/n 653750-902)
Thermostat	4 °C
Solvent A	0.1 % Formic acid in water
Solvent B	0.1 % Formic acid in 90 % acetonitrile
Gradient	0–15 minutes, 0–40 % B 15–18 minutes, 40–90 % B 18–20 minutes, 90 % B
Column temperature	60 °C
Flow rate	0.4 mL/min
Injection volume	3.0 µL

Table 2. MS Acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System	
Gas temperature	325 °C
Drying gas	13 L/min
Nebulizer	35 psig
Sheath gas temperature	275 °C
Sheath gas flow	12 L/min
VCap	4,000 V
Nozzle voltage	500 V
Fragmentor	175 V
Skimmer	65
Quad AMU	95
Reference mass	121.0509, 922.0098
Acquisition mode	Extended Dynamic Range (2 GHz)
Mass range	*m/z* 100–1,700
Acquisition rate	5 spectra/sec
Auto MS/MS range	*m/z* 50–1,700
Min MS/MS acquisition rate	3 spectra/sec
Isolation width	Narrow (~ 1.3 *m/z*)
Precursors/cycle	Top 10
Collision energy	3.6*(*m/z*)/100–4.8
Threshold for MS/MS	3,000 counts and 0.001 %
Dynamic exclusion	On; 3 repeat then exclude for 0.2 minutes
Precursor abundance based scan speed	Yes
Target	25,000
Use MS/MS accumulation time limit	Yes
Purity	100 % stringency, 30 % cutoff
Isotope model	Peptides
Sort precursors	By abundance only; +2, +3, >+3

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Results and Discussion

A comprehensive peptide mapping of an antibody can be a complex and time-consuming process due to the necessary sample preparation and data analysis for hundreds of peptides with various modifications. We used the high-throughput AssayMAP Bravo liquid handling system, Agilent Infinity II UHPLC, and Agilent accurate-mass AdvanceBio Q-TOF system to overcome these challenges. In addition, the automatic data processing workflow by Agilent MassHunter BioConfirm B.08 software improved the overall data mining and resulting accuracy significantly.

The Figure 2 illustrates the extracted compound chromatogram (ECC) of peptides from Trypsin/Lys-C digested NISTmAb. Excellent chromatographic resolution was achieved with a short 15-minute gradient. Each identified peptide from the NISTmAb light chain and heavy chain are labeled with their corresponding sequence numbers.

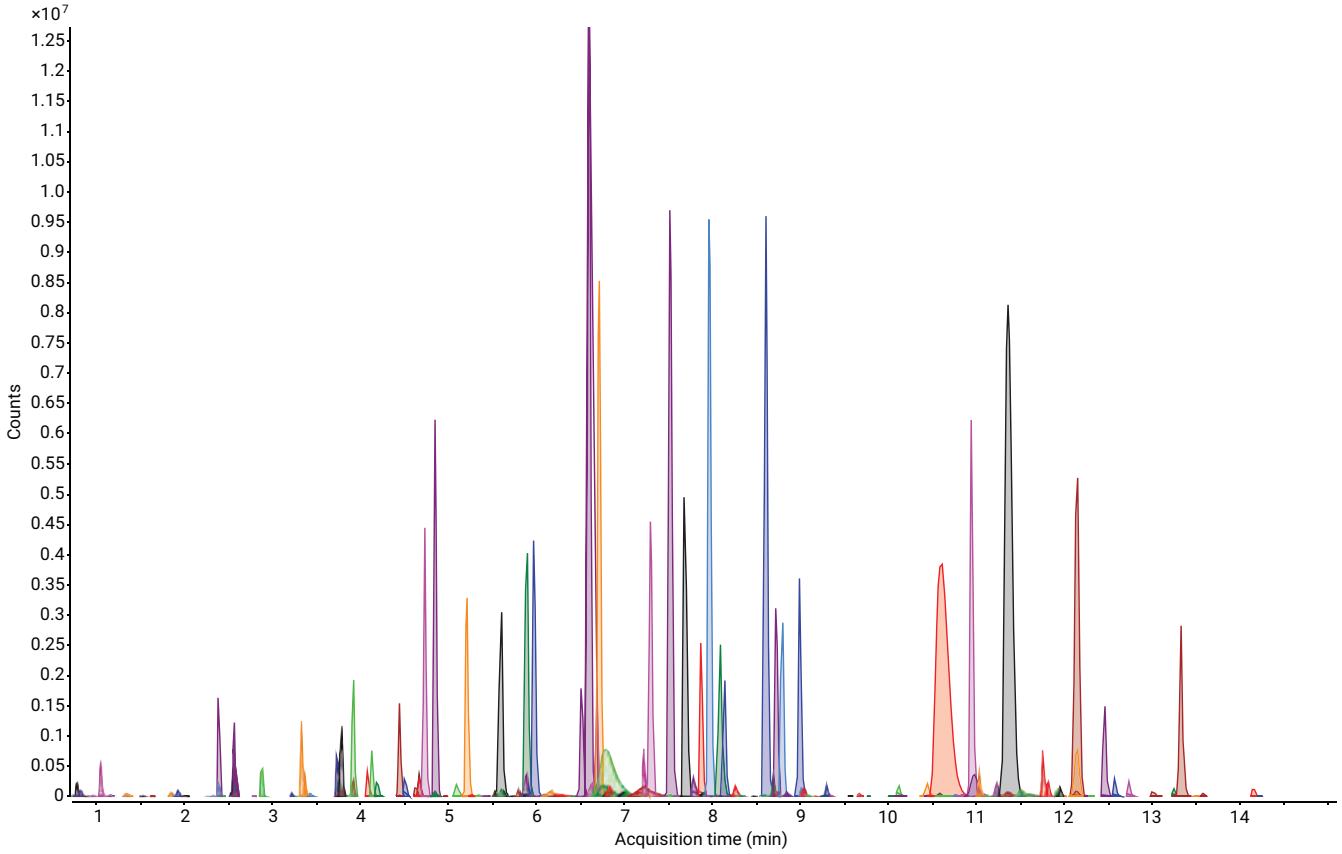

Figure 2. ECC of peptides from Trypsin/Lys-C digested NISTmAb standard RM 8671, separated using an Agilent AdvanceBio Peptide Mapping column.

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In our peptide mapping workflow, all matched peptides were required to have < 5 ppm MS mass error and have at least one confirmatory MS/MS spectrum. The BioConfirm scoring algorithm considers factors such as: the presence of b and y fragment ions, immonium ions, mass accuracy, MS/MS peak intensity, and other parameters. Figure 3A highlights the detailed example of the identified peptides by the BioConfirm software. The majority of identified peptides displayed excellent mass accuracy, with errors less than 1 ppm. After the peptides were identified, an mAb sequence coverage map was reported automatically. Figure 3B shows a sequence coverage of 99.4 % on the NISTmAb, achieved using the 15-minute UHPLC gradient.

The peptide mapping result summary (Figure 4) in BioConfirm allows quick review of detailed peptide information including mass, retention time, matched peptide sequence, modifications, and matching score. It allows users to review the TIC of the sample as well as the individual peptide MS and MS/MS spectra. In addition, the abundances of the precursor molecule along with its fragment ions are also provided for relative quantitation analysis.

Score	Mass	RT	Score (MFE)	Seq Loc	Tgt Seq Mass	Diff (Bio, ppm)
88.15	1796.8876	12.1442	100	A(126-141)/ C(126-141)	1796.888	-0.18
84.09	1723.9	6.7992	87.7	B(344-358)/ D(344-358)	1723.9006	-0.33
78.65	1285.6665	7.2351	80	B(348-358)/ D(348-358)	1285.6667	-0.12
77.35	1080.5225	4.7314	100	A(19-28)/ C(19-28)	1080.5234	-0.78
77.03	2101.119	10.6051	100	A(107-125)/ C(107-125)	2101.1208	-0.85
75.94	1320.6706	7.9666	100	B(137-150)/ D(137-150)	1320.6708	-0.14
75.12	1923.0328	6.1898	80	B(342-358)/ D(342-358)	1923.0326	0.07
75.07	3043.3936	7.6842	100	B(418-442)/ D(418-442)	3043.393	0.19
74.63	1501.7518	8.9926	100	A(169-182)/ C(169-182)	1501.7512	0.44
72.94	1806.9981	11.7542	100	B(305-320)/ D(305-320)	1806.9992	-0.61
72.84	785.4405	4.5012	99	A(53-60)/ C(53-60)	785.4396	1.18
71.52	1945.0193	11.6124	80.3	A(108-125)/ C(108-125)	1945.0197	-0.21
71.46	1891.8935	7.8711	100	A(1-18)/ C(1-18)	1891.8946	-0.54
71.22	6712.3081	13.3296	100	B(151-213)/ D(151-213)	6712.3072	0.14
69.7	1872.9134	10.9458	100	B(396-412)/ D(396-412)	1872.9146	-0.6
69.36	1185.6395	9.04	100	B(125-136)/ D(125-136)	1185.6394	0.13
69.19	1160.6214	8.6107	100	B(364-373)/ D(364-373)	1160.6223	-0.81
68.93	1797.872	10.7793	100	A(126-141)/ C(126-141)	1797.872	-0.01
68.64	1676.794	8.7257	100	B(278-291)/ D(278-291)	1676.7947	-0.4
68.24	1847.7825	7.2197	100	B(84-99)/ D(84-99)	1847.7818	0.36
68.23	951.5279	7.5208	100	A(45-52)/ C(45-52)	951.5277	0.16
67.57	1787.889	12.5731	100	B(46-59)/ D(46-59)	1787.8883	0.38
67.42	659.3488	7.2981	100	B(443-449)/ D(443-449)	659.349	-0.28
67.13	1923.0327	6.3056	63.1	B(342-358)/ D(342-358)	1923.0326	0.04
66.7	2228.1847	11.2323	100	B(305-323)/ D(305-323)	2228.1841	0.26
65.35	834.4266	6.6893	100	B(252-258)/ D(252-258)	834.4269	-0.42
65.15	2138.0206	8.795	100	B(259-277)/ D(259-277)	2138.0202	0.22
64.64	487.3003	3.7822	100	A(103-106)/ C(103-106)	487.3006	-0.59
64.38	559.3119	4.4421	100	A(145-148)/ C(145-148)	559.3118	0.06
64.1	574.3323	3.9175	100	B(413-417)/ D(413-417)	574.3326	-0.6

Figure 3A. An example of an Agilent MassHunter BioConfirm B.08 peptide mapping results table, summarizing the details of all matched peptides from the NISTmAb digest. The majority of identified peptides posted less than 1 ppm of mass accuracy (red box).

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Figure 3B. Summary of sequence coverage of NISTmAb.

Figure 4. Screenshot of Agilent MassHunter BioConfirm B.08 software with representative peptide mapping results and protein sequence coverage.

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Figure 5 illustrates the MS/MS spectra comparison of the native (precursor at m/z = 631.6385, +3) and Met-oxidized peptides (precursor at m/z = 636.9698, +3). The major differences (+15.99 Da) in the b4–b7 fragment ions (green box) clearly distinguished the native species from the modified forms, and indicate that the Met-4 in light chain is the location of oxidation.

Similarly, Figure 6 shows the MS/MS spectrum of the native and the deamidated peptides, where the b2–b3 fragment ions (purple boxes) all show the signature mass shift of 0.98 Da, clearly indicating the presence of deamidation. Moreover, as most of the y ions (y4–y8, highlighted in red) remain the same (except the y10 ion) as in the native form (top panel), it is clear that the deamidation occurred at the heavy chain Asn-364 position.

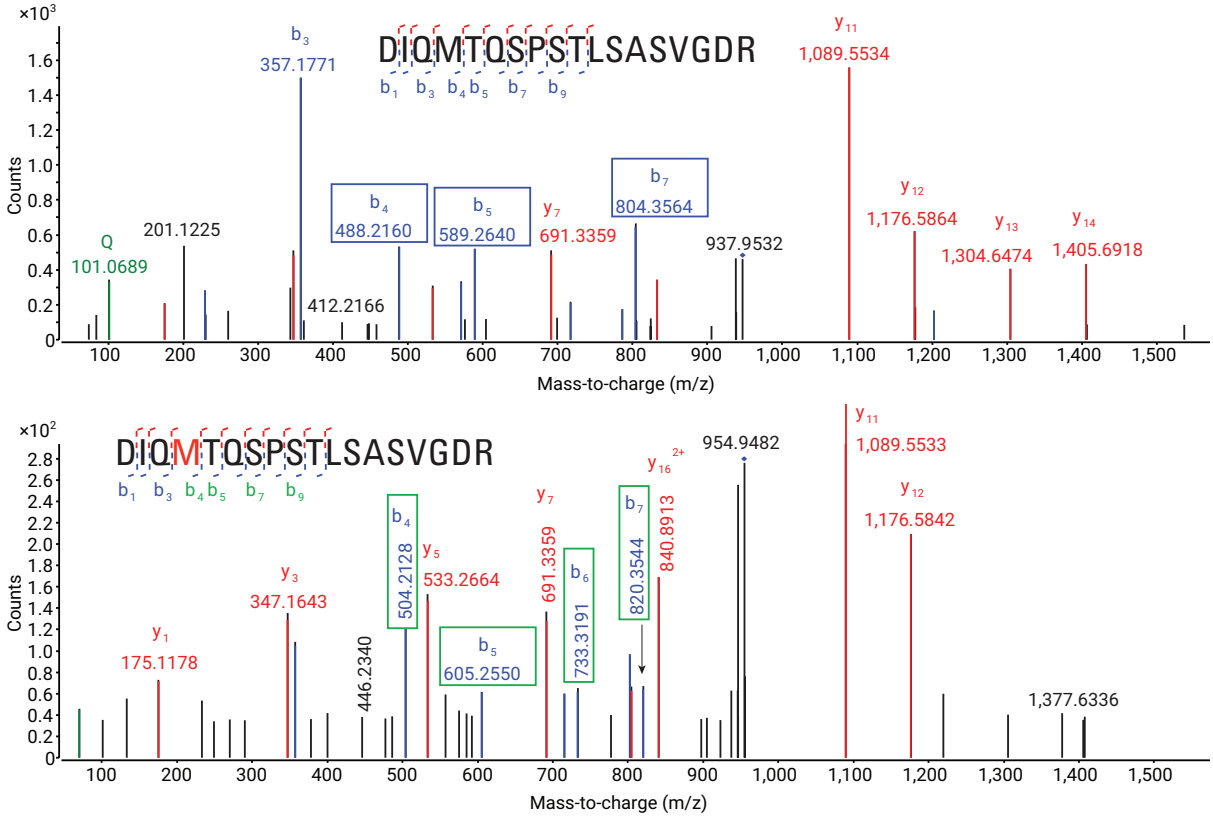

Figure 5. Post-translational modification (methionine oxidation) analysis. MS/MS spectrum of native and Met-oxidized peptides (light chain peptide 1-18). Top: native peptide, Bottom: oxidation at Met 4 (confirmed fragment ions in green boxes).

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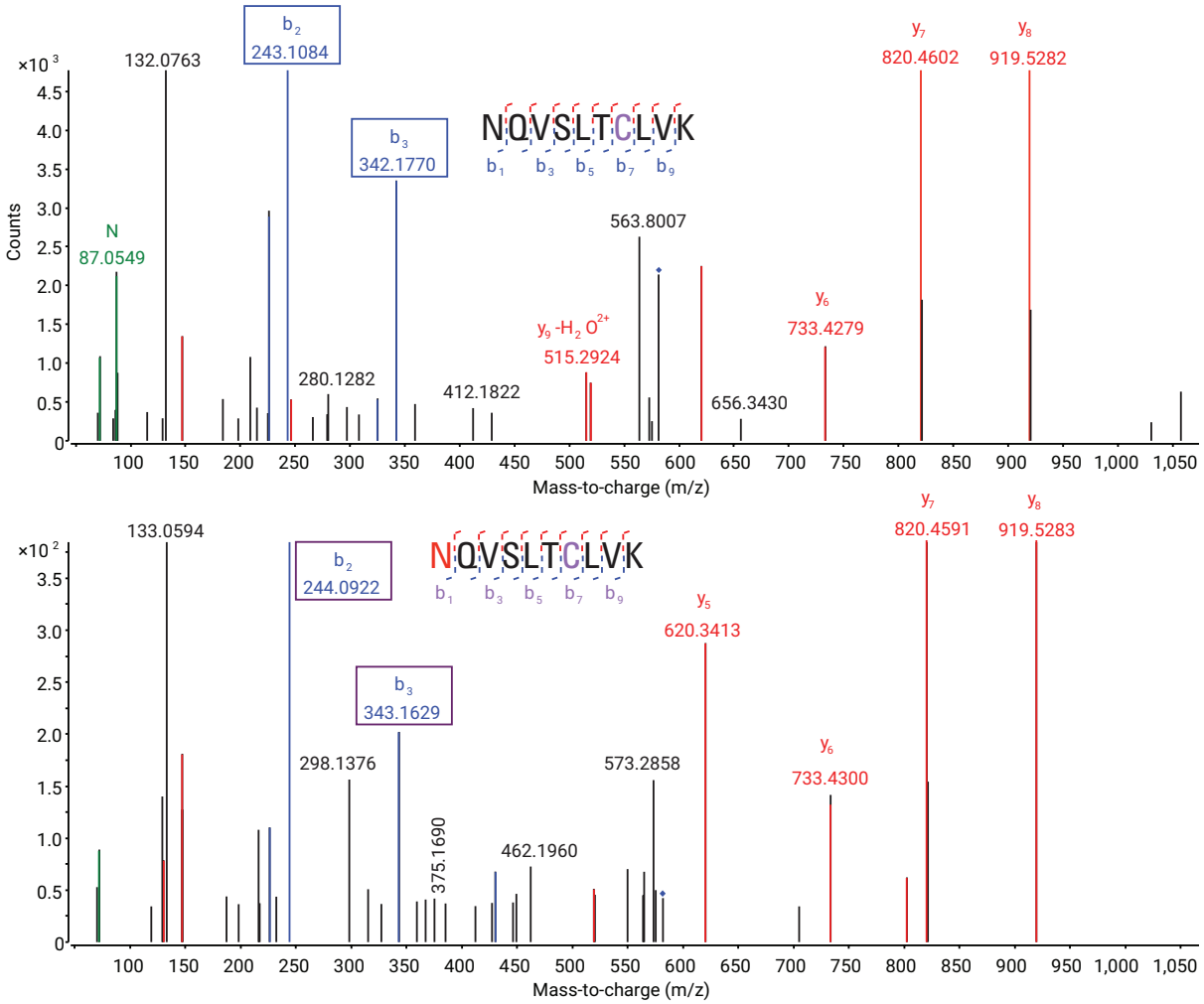

Figure 6. Post-translational modification (deamidation) analysis. MS/MS spectrum of native and deamidated peptides (heavy chain: 364-373). Top: native peptide, Bottom: deamidation at Asn 364.

Conclusion

The combination of automated sample preparation, rapid separation, confident detection, and streamlined processing changes the process of peptide mapping from a time-consuming and tedious effort into a routine workflow. This is possible due to the reliable nature and high performance of each component in this process, starting with Agilent AssayMAP Bravo through the data processing in Agilent MassHunter BioConfirm B.08. Total analysis time is significantly condensed by the separation capabilities of the Agilent 1290 Infinity II UHPLC and the Agilent AdvanceBio Peptide Mapping column as well as the automated processing capability of MassHunter BioConfirm. The combination of accuracy and resolution provided by the Agilent 6545XT AdvanceBio LC/Q-TOF is demonstrated by the uniformly precise results seen when analyzing a complete protein digest.

References

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Application Note

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Monitoring Product Quality Attributes of Biotherapeutics at the Peptide Level Using the Agilent InfinityLab LC/MSD XT System

Authors

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Agilent Technologies, Inc.

Introduction

Single quadrupole (SQ) LC/MS has been adopted in the biopharmaceutical QC labs for its low-cost, robustness, and simple operation. This Application Note describes a simple, generic method for routine biotherapeutic peptide map analysis using the Agilent InfinityLab liquid chromatography/mass selective detector XT (LC/MSD XT), an SQ system with an extended mass range up to m/z 3,000, in combination with an Agilent 1290 Infinity II LC System and Agilent OpenLab ChemStation software. Streamlined data processing and reporting were demonstrated for pre-identified peptides of a recombinant monoclonal antibody (mAb), including complementary-determining regions (CDR) peptides, deamidated peptides, oxidized peptides, and glycopeptides using OpenLab ChemStation. This study serves as a proof of concept for monitoring multiple product quality attributes (PQAs) using an SQ LC/MS system with software that is recommended for laboratories requiring regulatory compliance.

Introduction

In the biotherapeutic industry, optically based chromatographic methods have widely been used for quality control (QC). However, protein-based biotherapeutics are generally very complex, making an orthogonal detection method (for example, mass spectrometry) very attractive or necessary to assess product quality attributes at a molecular level. Therefore, SQ-based LC/MS has been adopted in the QC environment. Due to the product complexity, comprehensive analysis of protein-based therapeutics often requires running a panel of analytical methods. The concept of using a single LC/MS analytical method to monitor multiple PQAs has gained momentum in the biopharmaceutical industry. Therefore, it is valuable to develop an SQ-based LC/MS assay for monitoring multiple PQAs.

In the QC environment, an important need is to support regulatory compliance. OpenLab ChemStation in combination with central data storage (OpenLab ECM or OpenLAB Server) provides functionality that labs need to achieve compliance: controls for managing system access, audit trail, versioning of data, electronic signature, secured records and data archival.1,2

This Application Note develops a simple, untargeted, generic LC/MS method for routine biotherapeutic peptide map analysis using the InfinityLab LC/MSD XT system, coupled with a 1290 Infinity II LC and OpenLab ChemStation software. In a stress study using NIST monoclonal antibody (NISTmAb), we demonstrate that this compliance-ready system allows streamlined data processing and reporting for multiple PQAs in a single analysis, such as product identification confirmation, post translation modification (PTM) analysis, and glycopeptide analysis.

Experimental

Materials

All reagents and solvents were LC/MS grade. The NISTmAb reference material was purchased from National Institute of Standards and Technology.

Sample preparation

To induce asparagine deamidation, NISTmAb was exposed to elevated temperature (37 °C) in a Tris-HCl buffer system at pH 8.7 for six days. To induce methionine oxidation, NISTmAb was incubated in Tris-HCl buffers containing 0.002% (v/v) oxidizing agent H2O2 overnight at room temperature. Both reference and stress-induced NISTmAb were denatured, reduced, alkylated, and trypsin-digested followed by desalting using the Agilent AssayMAP Bravo platform.3 Digested samples were injected at a concentration of approximately 0.5 µg/µL onto the LC/MS system.

LC/MS analysis

LC separation was carried out using an Agilent 1290 Infinity II LC, consisting of an Agilent 1290 Infinity II High-Speed Pump (G7120A), an Agilent 1290 Infinity II Multisampler (G7167B) with sample cooler (option 100), and an Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with an Agilent ZORBAX 300StableBond C18 column (2.1 × 150 mm, 300 Å, 1.8 µm, p/n 863750-902) (Table 1). The MS system used was the Agilent InfinityLab LC/MSD XT system (G6135BA) with the Agilent Jet Stream source (G1958-65138). Agilent OpenLab ChemStation (version C 01.09) was used for data acquisition, processing, and reporting. The data were acquired in positive scan mode ranging from m/z 360 to 1,400 (Table 2).

Table 1. LC conditions.

Agilent LC Parameters		
Column	Agilent ZORBAX RRHD 300Å StableBond C18, 2.1 × 150 mm, 1.8 µm (p/n 863750-902)	
Mobile Phase A	H_2O with 0.1% (v/v) formic acid	
Mobile Phase B	Acetonitrile with 0.1% (v/v) formic acid	
Flow Rate	0.25 mL/min	
Injection volume	5.0 µL	
Gradient	Time (min) 0 5 6 70 72 77 79 81	%B 1 1 10 35 90 90 1 1
Column temperature	50 °C	

Table 2. MS conditions.

Agilent MSD XT Parameters		
Drying Gas Flow	11 L/min	
Drying Gas Temperature	325 °C	
Sheath Gas Flow	10 L/min	
Sheath Gas Temperature	325 °C	
Nebulizer Pressure	35 psi	
Capillary Voltage	4,000 V	
Nozzle Voltage	0 V	
Peak Width	0.07 minutes	
Scan	360 to 1,400 *m/z* in positive mode from 5 to 80 minutes, step size 0.1	
Gradient	Mass 300 2,000	Value 125 V 200 V
Cycle Time	0.62 sec/cycle	

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Results and discussion

Monitoring multiple PQAs in a single analysis

To evaluate the InfinityLab LC/MSD XT system for monitoring multiple attributes of biomolecules, NISTmAb was stressed under two conditions to induce deamidation and oxidation, respectively. The LC/MS method using MS positive scan mode described above was applied to collect the full peptide map for each sample. Figure 1 shows the total ion chromatogram of the peptide map data with 2.5 µg of NISTmAb digest loaded on-column, showing the sample complexity, as well as the high sensitivity and ultrafast scan speeds of the MSD within the InfinityLab LC/MSD XT system. The full scan of the NISTmAb peptide map allows monitoring of multiple attributes of interest using customized data processing methods. The scan also avoids re-acquiring data if additional attributes are of interest in the future.

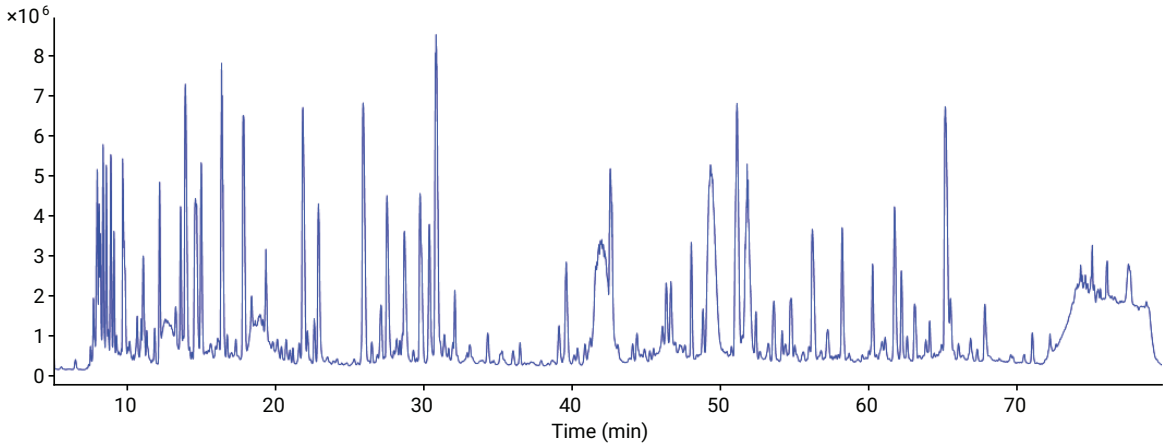

Figure 1. Total ion chromatogram of peptide map detection by Agilent LC/MSD XT with positive scan.

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OpenLab ChemStation software supports automated data processing and reporting. To avoid manual extraction and integration of each peptide, a processing method can be created for extracted ion chromatograms (EICs) of multiple peptides of interest. Figure 2 shows screen captures of the EIC method setup for multiple peptides by the following steps:

1. MS chromatograms for the peptides of interest are defined with targeted m/z, then the targeted MS chromatograms are extracted accordingly (Figure 2A).
2. These targeted EICs are added to the processing method with adjustable retention time windows for automatic signal extraction and loading (Figure 2B).
3. The compound names, associated retention times, and EIC signals are linked through the Calibration Table setup (Figure 2C).

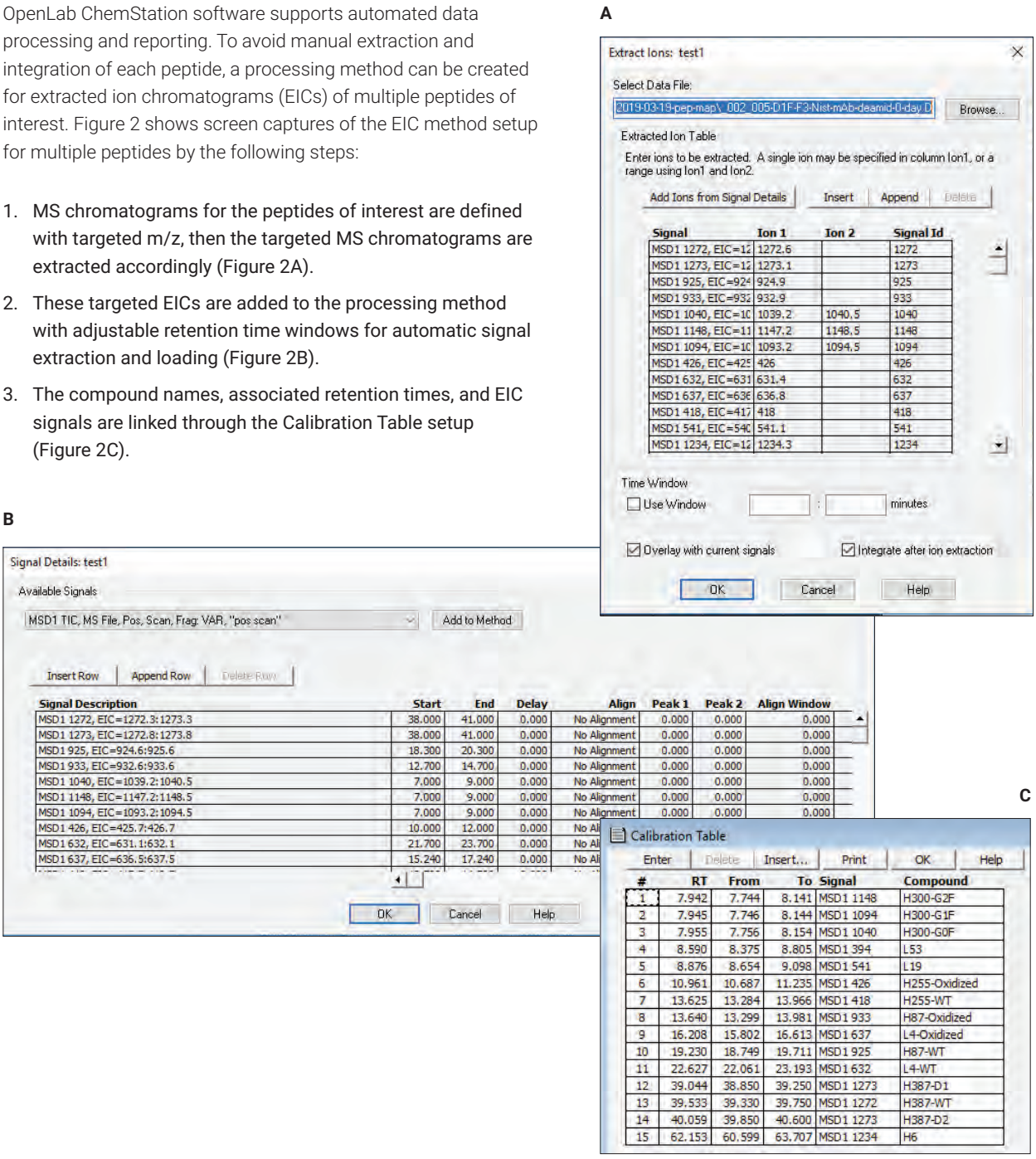

Figure 2. ChemStation screen captures of EIC method setup for multiple peptide attributes.

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EICs for monitoring product attributes

To To evaluate the performance using the InfinityLab LC/MSD XT, 15 precharacterized peptides were selected for identification and quantification analysis for the NISTmAb stress study (Table 3).4,5 The identity and retention time of these peptides was predetermined using a high-resolution LC/Q-TOF system with the same LC gradient as in Table 1. A processing CH3method, including all 15 peptides, was created using the steps described earlier, and a single dominant charge state was used to identify each of the peptides. If desired, the user could sum up additional charge states for each peptide.

The peptides listed in Table 3 can be separated into three categories according to the different monitoring purposes. The first category is the CDR peptides including peptides L4, L19, L53, H6, and H87. During product monitoring, an important need is to confirm the identity of a given biomolecule product. The sequences of CDR peptides are variable among different mAbs and can be used to confirm the product identity. Figure 3 shows the EIC of the CDR peptides that can be used to confirm protein identity.

Table 3. Peptide information for monitored attributes.

Peptide	Peptide sequence	Modification	Calculated *m/z*	Charge state (z)	Expected retention time (min)	mAb region
L4	DIQMTQSPSTLSASVGDR	Oxidation	637.0	3	16.24	CDR
L4	DIQMTQSPSTLSASVGDR	WT	631.6	3	22.68	CDR
L19	VTITCSASSR	WT	541.3	2	8.966	CDR
L53	LASGVPSR	WT	393.7	2	8.353	CDR
H6	ESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIR	WT	1234.3	3	62.105	CDR
H87	VTNMDPADTATYYCAR	WT	924.9	2	13.64	CDR
H87	VTNMDPADTATYYCAR	Oxidation	932.9	2	19.23	CDR
H255	DTLMISR	Oxidation	426.2	2	11.01	CH2
H255	DTLMISR	WT	418.2	2	13.71	CH2
H300	TKPREEQYNSTYR	G0F	1039.5	3	7.97	CH2
H300	TKPREEQYNSTYR	G1F	1093.5	3	7.97	CH2
H300	TKPREEQYNSTYR	G2F	1147.5	3	7.98	CH2
H387	GFYPSDIAVEWESNGQPENNYK	Deamidation	1273.1	2	39.07	CH3
H387	GFYPSDIAVEWESNGQPENNYK	WT	1272.6	2	39.56	CH3
H387	GFYPSDIAVEWESNGQPENNYK	Deamidation	1273.1	2	40.06	CH3

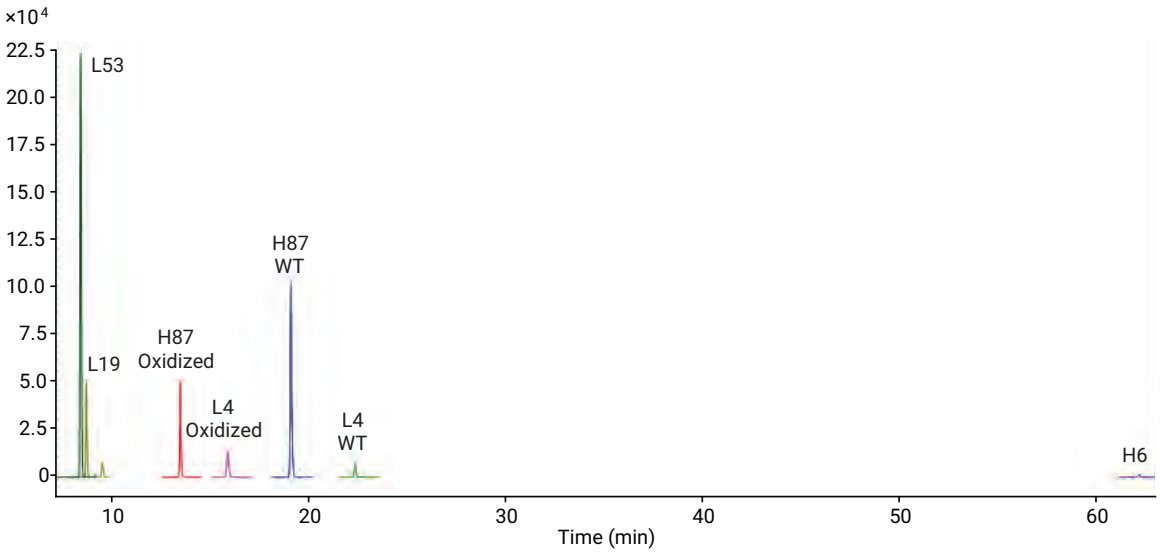

Figure 3. EICs of the CDR peptides.

The second category is peptides with variable modification sites, which are responsive for chemically induced deamidation and oxidation (L4, H87, H255, and H387).6,7 PTMs such as asparagine deamidation, aspartate isomerization, and methionine oxidation lead to degradation products typical for recombinant antibodies. Process changes during manufacturing or storage conditions can affect the rate and extent of these modifications, which could potentially impact the stability and function of the protein drug. Therefore, these PTMs are closely monitored during process development and drug production. Figure 4A shows EICs of the wild type H387 peptide and its deamidation forms, which is also called the PENNY peptide, in the reference and deamidated samples. The deamidated forms of H387 are elevated after deamidation induction. Figure 4B shows the overlaid EICs of the wild type peptide and its oxidized form from peptide H87 in both NISTmAb reference and oxidized samples. As expected, the extent of oxidation of H87 peptide was increased after oxidation induction.

The third category is glycopeptide (H300). Relative abundance of each glycopeptide can provide valuable information about the abundance of protein glycoforms. According to a previous publication on glycoanalysis in the NISTmAb tryptic digest using high-resolution LC/MS/MS, the glycopeptide located at heavy chain 292–304 (TKPREEQYNSTYR) was chosen as the dominant tryptic form5. Figure 5 shows the overlaid EICs of three glycopeptides (G0F, G1F, and G2F) used for determining their relative abundance. This result is consistent with a previous report on the relative abundance of these NISTmAb glycopeptides obtained using high-resolution LC/MS/MS.5

Intelligent reporting

OpenLab ChemStation software enables automated intelligent reporting. Intelligent reporting provides superior flexibility and allows the user to customize their report templates as desired. Figures 6A and 6B show examples of intelligent reports generated for monitoring multiple attributes.

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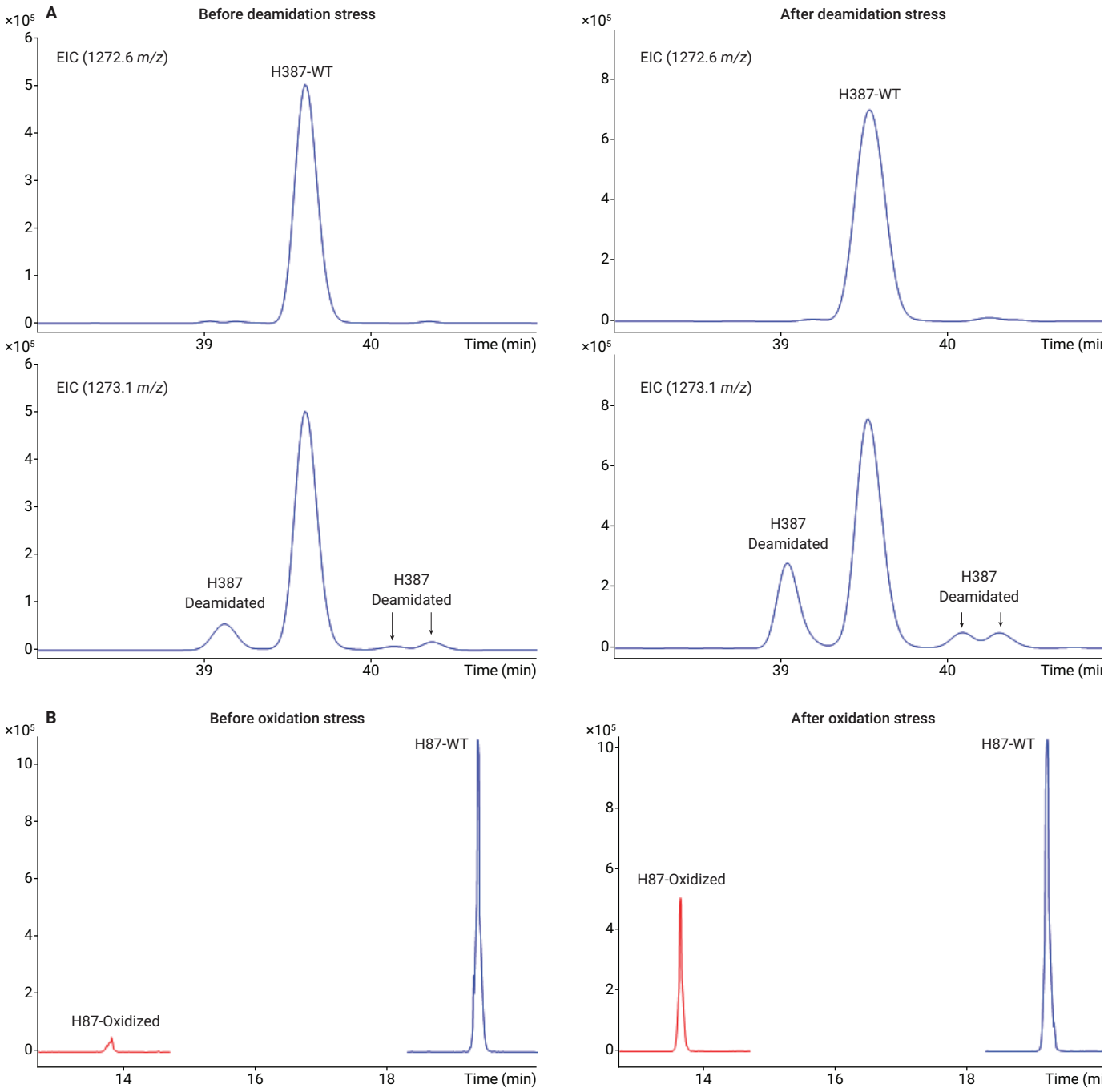

Figure 4. EICs of the peptides with variable PTMs. A) EIC comparison of the WT and deamidated H387 peptides before and after deamidation stress induction. B) EIC comparison of the WT and oxidized H87 peptides before and after oxidation stress induction.

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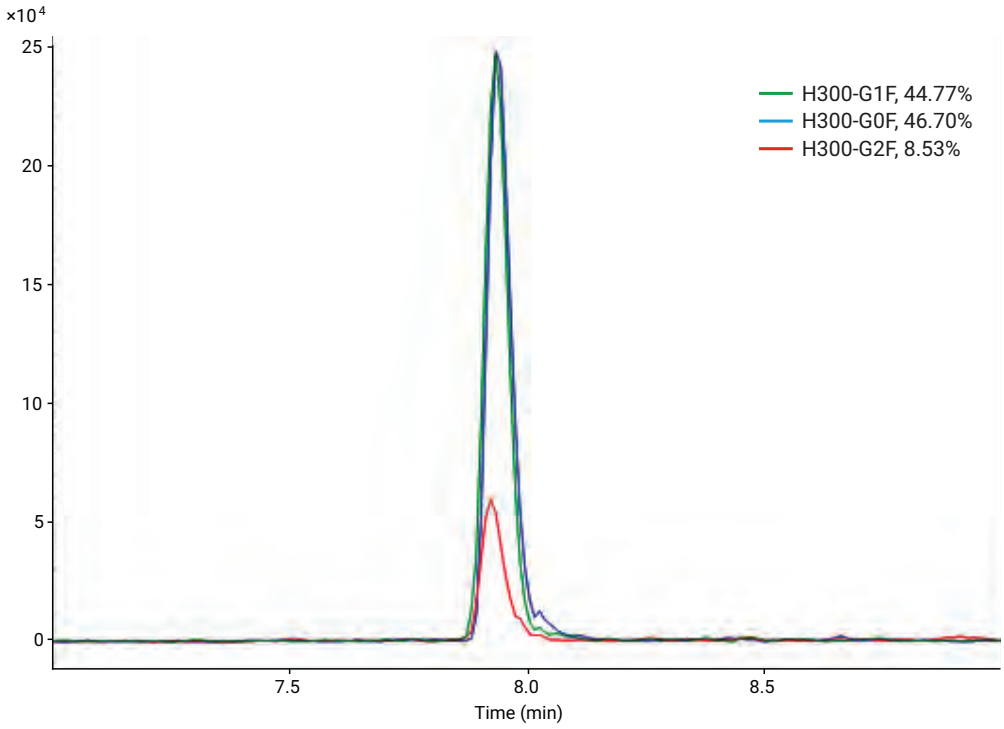

Figure 5. EICs of the three glycopeptides for determining relative abundance.

Single Injection Report

Agilent

Data file:
Sample name:
Description:
Sample amount:

Instrument:
Injection date:
Acq. method:
Analysis method:

Location:
Injection:
Injection volume:
Acq. operator:

Analyst: _____

Date: _____

Pass/Fail: _____

Peak Summary Table: Glycopeptides

Name	RT [min]	Area	Area Percent
H300-G0F	7.955	1207180	46.70%
H300-G1F	7.945	1157171	44.77%
H300-G2F	7.942	220399	8.53%
	AreaSum	2584749	

Peak Summary Table: Deamidation

Name	RT [min]	Area	Area Percent	P/F
H387-D1	39.112	146688	2.86%	Pass
H387-WT	39.592	4914364	95.93%	Pass
H387-D2	40.123	61809	1.21%	Pass
	AreaSum	5122861		

Peak Summary Table: Oxidation

Name	RT [min]	Area	Area Percent	P/F
H87-Oxidized	13.811	113448	3.47%	Pass
H87-WT	19.367	3160505	96.53%	Pass
	AreaSum	3273952		

Name	RT [min]	Area	Area Percent	P/F
L4-Oxidized	16.208	105370	9.00%	Pass
L4-WT	22.627	1065867	91.00%	Pass
	AreaSum	1171237		

Name	RT [min]	Area	Area Percent	P/F
H255-Oxidized	10.961	1967116	16.80%	Fail
H255-WT	13.625	9738910	83.20%	Fail
	AreaSum	11706026		

Figure 6A. Intelligent reporting by Agilent OpenLab ChemStation. Example of a single injection report. Peak tables summarize the relative abundance of WT and PTM forms for each peptide sequence by custom calculation.

Sequence Summary Report

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Sequence name:

Acquisition date:

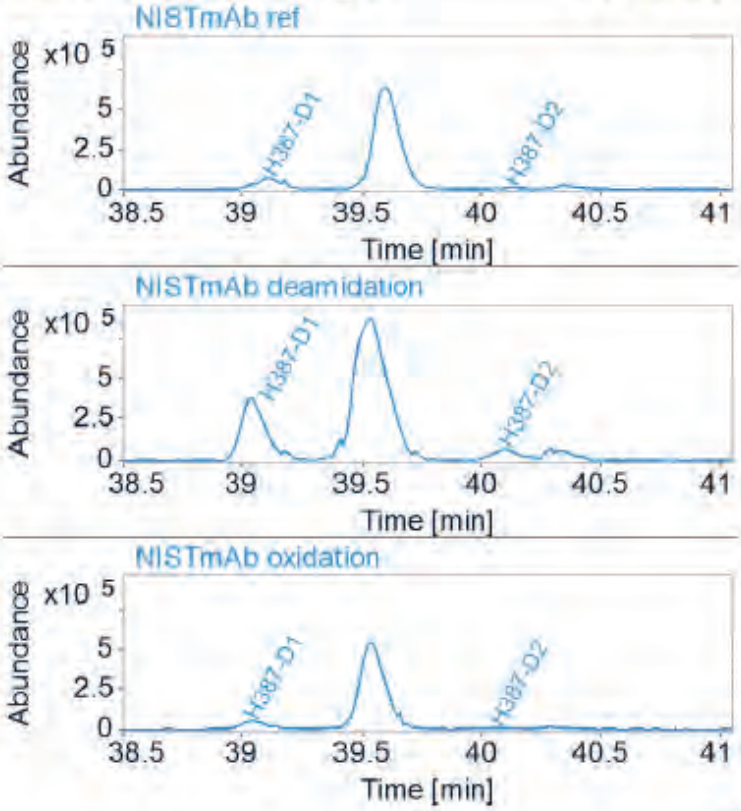
Acquired by:

Approved by: _____

Signature: _____

Date: _____

Extracted Ion Chromatogram **EIC=1272.8 :1273.8**



			H387-D1	H387-WT	H387-D2
			RT (min)	RT (min)	RT (min)
1	NISTmAb ref	_001_005-D1F-F3-Nist-mAb-deamid-0-day.D	39.11	39.59	40.12
2	NISTmAb deamidation	_002_006-D1F-F4-Nist-mAb-deamid-6-day.D	39.04	39.53	40.10
3	NISTmAb oxidation	_003_003-D1F-F2-Nist-mAb-H2O2-002pct.D	39.04	39.53	40.06

Figure 6B. Intelligent reporting by Agilent OpenLab ChemStation. Example of a sequence summary report comparing a NISTmAb reference sample, deamidated sample, and oxidized sample for the EIC and retention time of H387 peptide.

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Conclusion

The Agilent InfinityLab LC/MSD XT system provides a simple and cost-effective solution for monitoring multiple PQAs in a development and quality control environment, assuming those attributes that have been precharacterized using a high-resolution MS instrument. This Application Note demonstrates that the InfinityLab LC/MSD XT system can deliver quantitative analysis for monitoring multiple attributes of biotherapeutics at the peptide level, including CDR peptides, oxidized and deamidated peptides, and glycopeptides in a single analysis. Automated data processing and reporting through Agilent OpenLab ChemStation software avoid manual interrogation and allow high-throughput analysis. OpenLab ChemStation in combination with central data storage provides a compliance solution for chromatography and mass spectrometry data collected in compliant environments.

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RA.1719907407

Published in the USA, June 01, 2020
5994-2073EN

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