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

Application Note

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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 locations[1-3]. 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 NIST mAb 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 NIST mAb sequence.

Table 1. Liquid Chromatography parameters.

Agilent 1290 Infinity II UHPLC System	
Column	Agilent AdvanceBio Peptide Mapping, 2.1 × 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

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

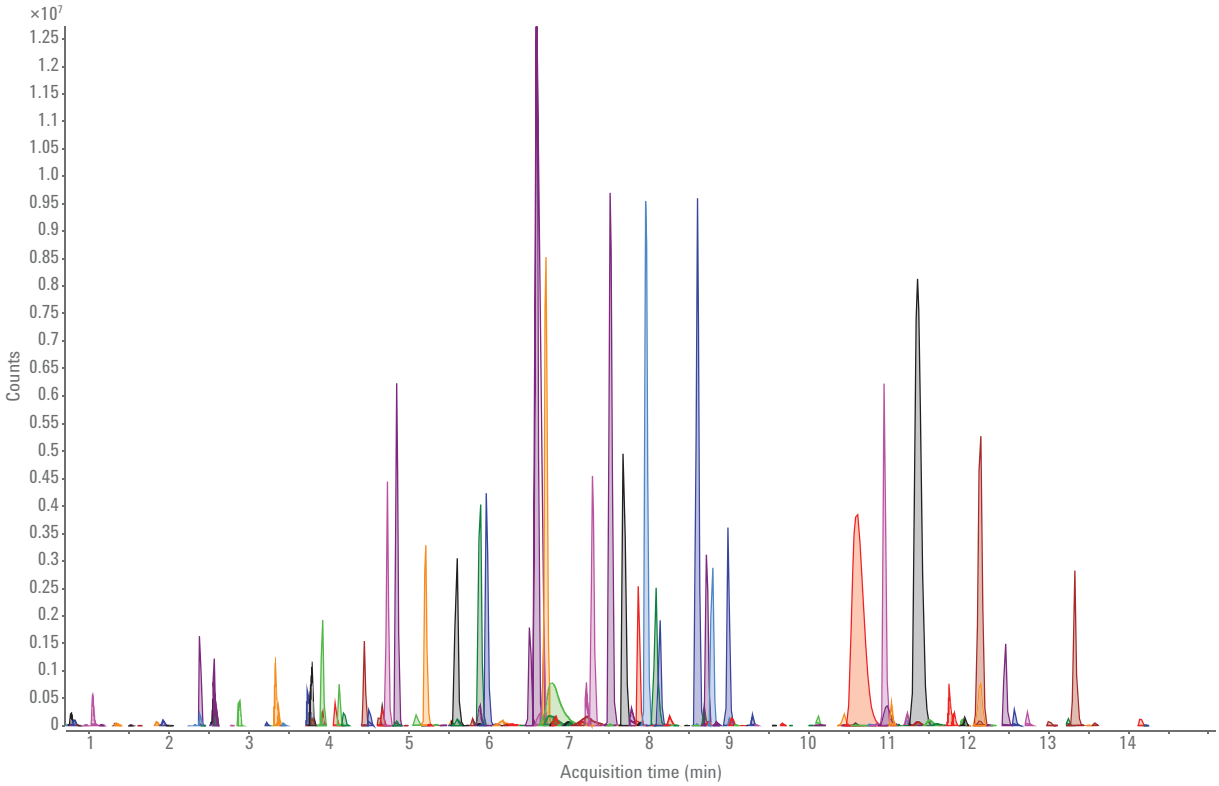

Figure 2. ECC of peptides from Trypsin/Lys-C digested NIST mAb standard RM 8671, separated using an Agilent AdvanceBio Peptide Mapping column.

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 NIST mAb, 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 NIST mAb digest. The majority of identified peptides posted less than 1 ppm of mass accuracy (red box).

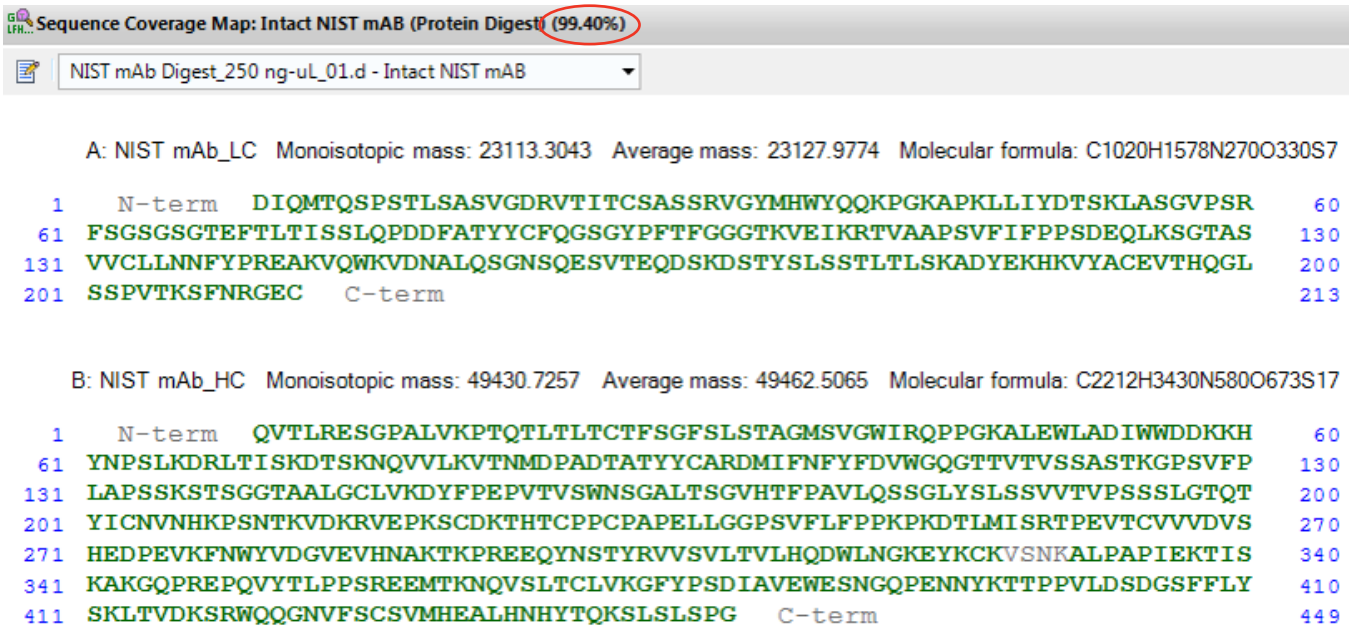

Figure 3B. Summary of sequence coverage of NIST mAbs.

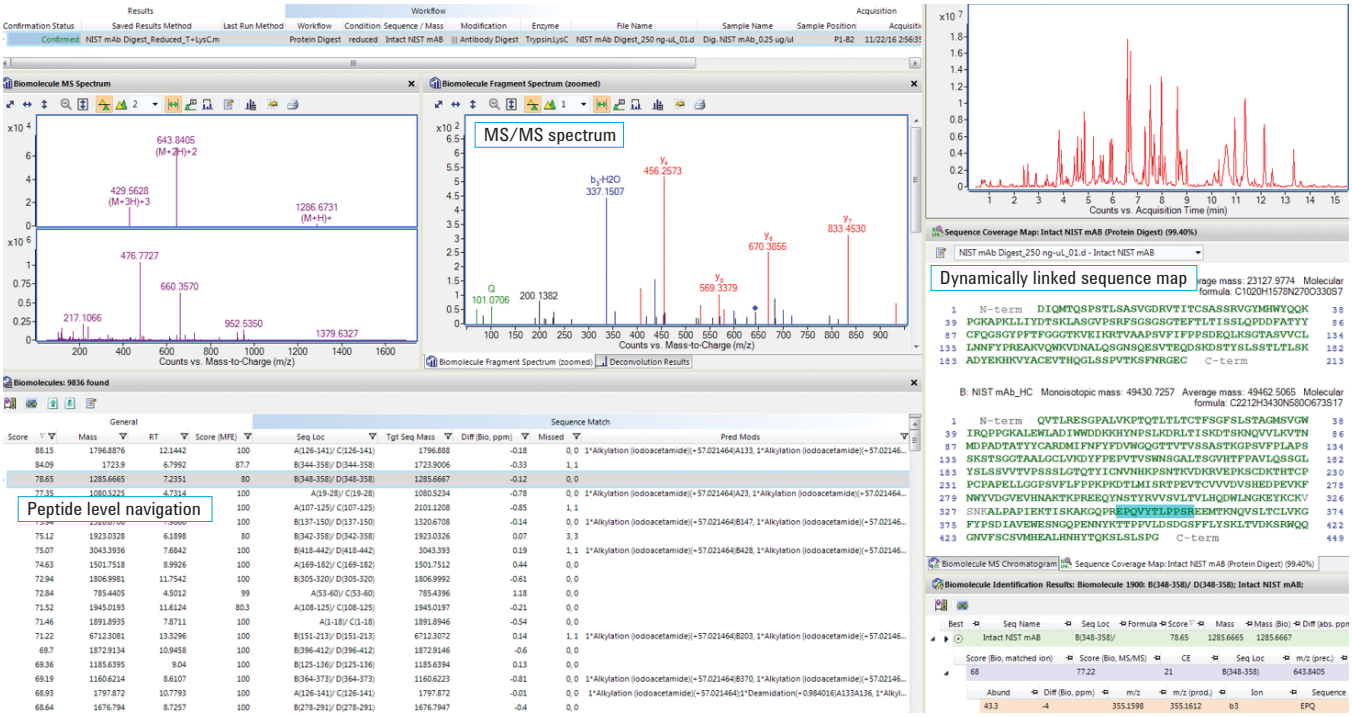

Figure 4. Screenshot of Agilent MassHunter BioConfirm B.08 software with representative peptide mapping results and protein sequence coverage.

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 b_4–b_7 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 b_2–b_3 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 (y_4–y_8, highlighted in red) remain the same (except the y_{10} ion) as in the native form (top panel), it is clear that the deamidation occurred at the heavy chain Asn-364 position.

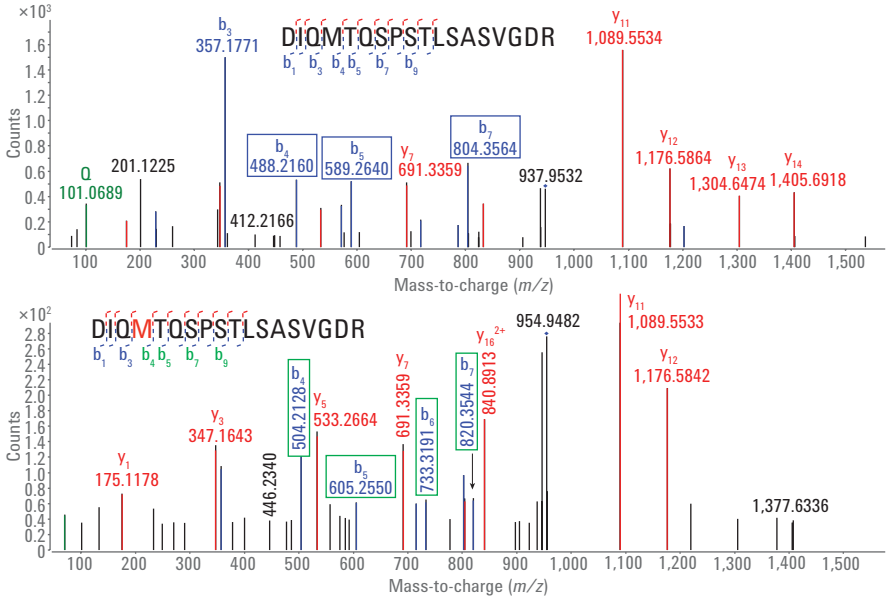

Figure 5. Post-translational modification (methionine oxidation) analysis. MS/MS spectrum of native and Met-oxidized peptides (light chain peptide 1-18). Top: native peptide, Bottom: oxidation at Met 4 (confirmed fragment ions in green boxes).

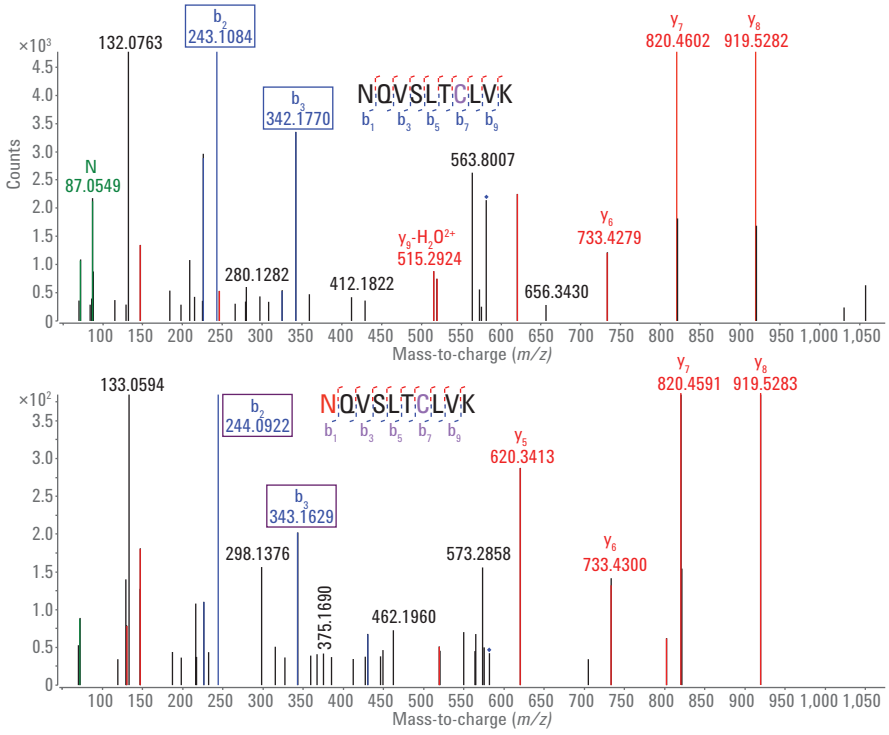

Figure 6. Post-translational modification (deamidation) analysis. MS/MS spectrum of native and deamidated peptides (heavy chain: 364-373). Top: native peptide, Bottom: deamidation at Asn 364.

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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