Application Note

Biotherapeutics and Biosimilars

In-depth Peptide Mapping with Iterative MS/MS Acquisition on the Agilent 6545XT AdvanceBio LC/Q-TOF

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Introduction

Therapeutic monoclonal antibodies (mAbs) represent one of the fastest growing classes of protein-based drugs in the biopharmaceutical industry. Due to the heterogenous nature of protein drugs, comprehensive analytical characterization is required. Peptide mapping using liquid chromatography and electrospray mass spectrometry (LC/MS) is an essential technique for the characterization of a protein drug. An extensive characterization of a protein drug provides primary sequence confirmation as well as the identification and quantification of post-translational modifications (PTMs) such as deamidation, oxidation, and glycosylation.

This Application Note presents an integrated workflow for peptide mapping of a mAb from automated sample preparation to data analysis, including an Agilent AssayMAP Bravo liquid-handling robot, the Agilent 1290 Infinity II LC system, the Agilent 6545XT AdvanceBio Q-TOF, and the Agilent MassHunter BioConfirm B.09 software. A new data acquisition method, Iterative MS/MS, provided by the Agilent 6545XT AdvanceBio LC/Q-TOF is demonstrated for in-depth peptide mapping to improve sequence coverage and PTM analysis.

Experimental

Materials

CHO-cultured human IgG1 mAb was expressed and purified from the Agilent R&D lab. Monoclonal antibody was reduced, alkylated, and trypsin-digested followed by desalting using the Agilent AssayMAP Bravo liquid-handling robot. The digested sample was subjected to LC/MS/MS analysis with either 0.6 µg or 2 µg loading amount per analysis, as indicated in the content.

Instrumentation

Sample Preparation Workstation

Agilent AssayMAP Bravo system

LC System

Agilent 1290 Infinity II LC system including:

- Agilent 1290 Infinity II High Speed Pump G7120A
- Agilent 1290 Infinity II Multisampler G7167B
- Agilent 1290 Infinity II Thermostatted Column Compartment G7116B

Column

Agilent AdvanceBio Peptide Mapping column (2.1 × 150 mm, 2.7 µm)

MS System

An Agilent 6545XT AdvanceBio LC/Q-TOF with an Agilent Dual Jet Stream ESI source was used.

LC/MS Analysis

LC separation was performed on an Agilent AdvanceBio Peptide Mapping column (2.1 × 150 mm, 2.7 µm) using either 15-minute or 30-minute gradient, as indicated. The data were acquired either using a conventional Auto MS/MS method or an Iterative MS/MS method, as indicated. Tables 1 and 2 list the LC/MS parameters.

Data Processing

Data acquired from LC/MS/MS analysis were processed using Agilent MassHunter BioConfirm B.09.00 software. Searching parameters were set up as trypsin digest allowing semitryptic peptides and a maximum two missed cleavages, variable modifications containing Cysteine (C) alkylation, Asparagine (N) or Glutamine (Q) deamidation, Methionine (M) oxidation and N-terminal pyroGlu (E). Mass tolerance allowed: 10 ppm for MS1 and 30 ppm for MS2. Peptide length was limited to 5 to 60 amino acids (AAs). Peptide-spectrum matches required MS/MS features, and were filtered by a 0.1 % false discovery rate (FDR).

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC system		
Analytical column	Agilent AdvanceBio Peptide Mapping, 2.1 × 150 mm, 2.7 µm (p/n 653750-902)	
Column temperature	60 °C	
Autosampler temperature	4 °C	
Solvent A	0.1 % formic acid in water	
Solvent B	0.1 % formic acid in 90 % acetonitrile	
Gradient	**15-minute gradient** 0–15 minutes, 3–40 % B 15–18 minutes, 40–90 % B 18–20 minutes, 90 % B 20–22 minutes, 90–3 % B	**30-minute gradient** 0–30 minutes, 3–40 % B 30–33 minutes, 40–90 % B 33–35 minutes, 90 % B 35–37 minutes, 90–3 % B
Flow rate	0.4 mL/min	

Table 2. MS parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF	
Gas temperature	325 °C
Drying gas flow	13 L/min
Nebulizer	35 psig
Sheath gas temperature	275 °C
Sheath gas flow	12 L/min
VCap	4,000 V
Nozzle voltage	0 V
Fragmentor	175 V
Skimmer	65
Reference mass	121.050873, 922.009798
Acquisition mode	Extended Dynamic Range (2 GHz)
Mass range	110–1,700 *m/z*
Acquisition rate	8 spectra/sec
Auto MS/MS range	50–1,700 *m/z*
Min MS/MS acquisition rate	3 spectra/sec
Isolation width	Narrow (~ 1.3 *m/z*)
Precursors/cycle	Top 10
Collision energy	3.1*(*m/z*)/100+1 for charge 2; 3.6*(*m/z*)/100–4.8 for charge 3 or greater than charge 3
Threshold for MS/MS	3,000 counts and 0.001 %
Dynamic exclusion On	One repeat, then exclude for 0.1 or 0.2 minutes
Precursor abundance based scan speed	Yes
Target	25,000 counts/spectrum
Use MS/MS accumulation time limit	Yes
Purity	100 % stringency, 30 % cutoff
Isotope model	Peptides
Sort precursors	By charge state then abundance; +2, +3, >+3

Results and Discussion

Peptide Mapping with Iterative MS/MS Acquisition

Peptide mapping of a protein drug product often requires reproducible sample preparation, in-depth peptide identification, and comprehensive data analysis of batch data files. We performed peptide mapping experiments on a CHO-cultured human IgG1 mAb using an integrated peptide mapping workflow, including an AssayMAP Bravo liquid handling robot, a 6545XT AdvanceBio LC/Q-TOF system, and MassHunter BioConfirm B.09 software (Figure 1). Automation of sample preparation using the AssayMAP Bravo platform has been demonstrated extensively in other Application Notes[1-3]. Therefore, this Application Note mainly focuses on data acquisition and processing.

Figure 2 illustrates the extracted compound chromatograms (ECCs) of peptides from trypsin-digested mAb with a 15-minute gradient. With a 0.6 µg injection and a 0.1 % FDR filter, 96.24 % sequence coverage of the heavy chain and 98.63 % of the light chain were achieved. For the light chain, the uncovered sequence is a short peptide EAK (Seq Loc 148-150). The missed-cleavage peptide TASVVCLLNNFYPREAK (Seq Loc 134-150) was identified with a 1 % FDR, bringing the total coverage to 100 %. For the heavy chain, the difficulty is in the short sequences TISK (Seq Loc 340-343) and the peptide with an N-glycosylation site TKPREEQYNSTYR (Seq Loc 294-306). Since the glycan of a glycopeptide tends to fragment first leading to poor peptide backbone fragmentation, the resulting identification scores are much lower than for an unmodified one, while the unmodified form exists at a very low level in the sample.

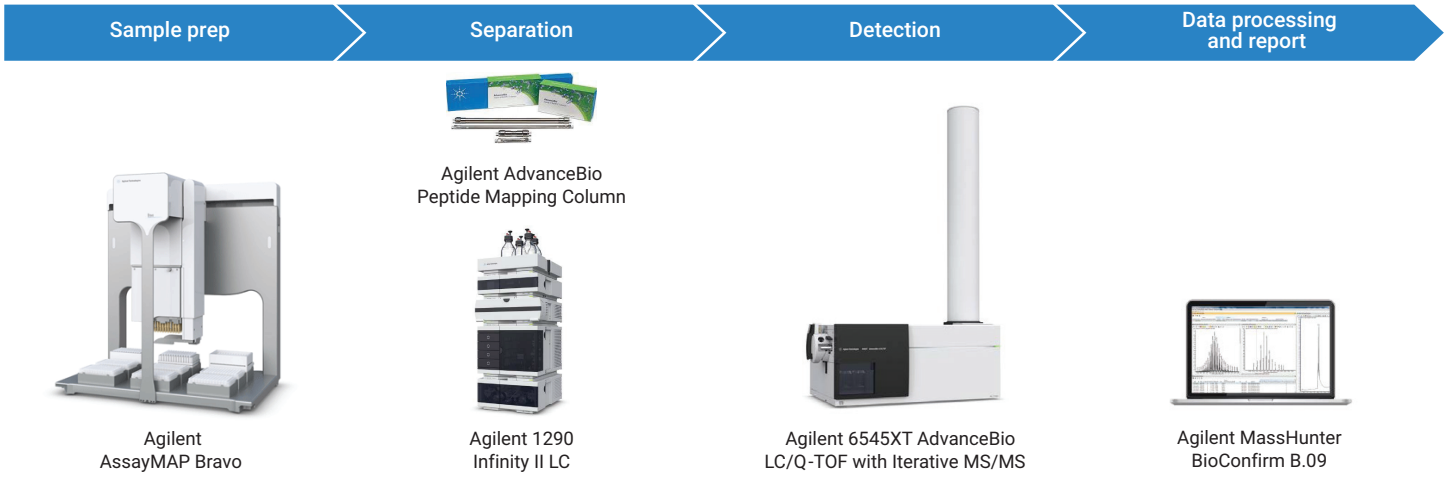


Figure 1. Integrated workflow for peptide mapping.

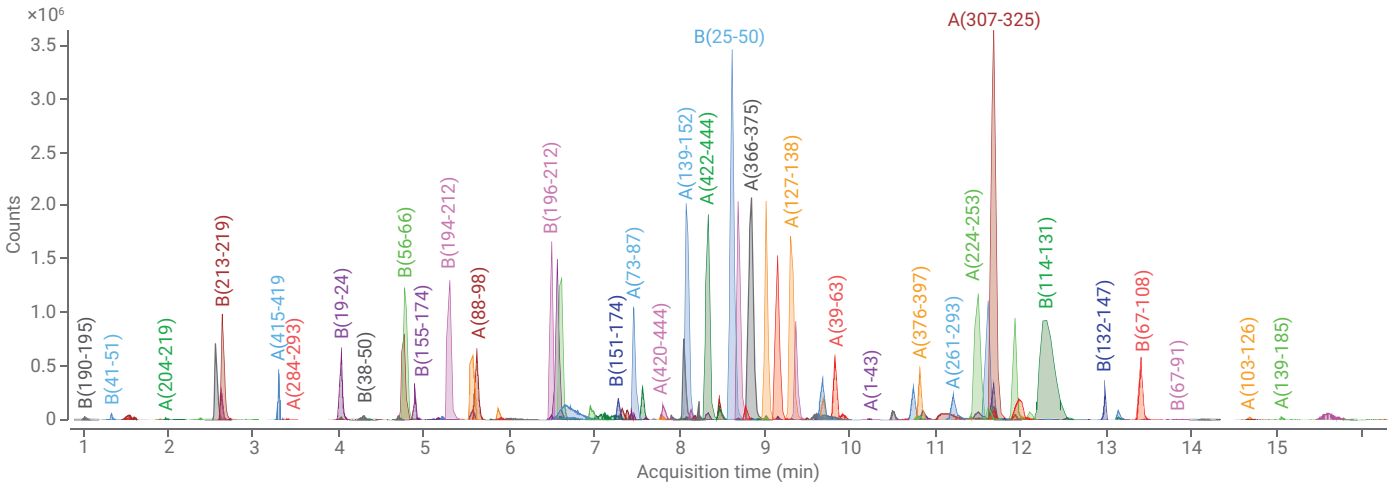


Figure 2. ECCs of peptides from trypsin-digested mAb, separated using an Agilent AdvanceBio Peptide Mapping column. Heavy- and light-chain peptides are marked as A and B, respectively.

The 6545XT AdvanceBio Q-TOF system provides an Iterative MS/MS data acquisition method, which improves identification of low-abundant precursors (Figure 3). In this acquisition method, protein digest sample was subjected to multiple LC/MS/MS analyses as needed. The first analysis was performed as a conventional Auto MS/MS; in the following consecutive LC/MS/MS analyses, precursors previously selected for MS/MS fragmentation were excluded on a rolling basis with customizable mass error tolerance and retention time exclusion tolerance. Figure 4 shows overlaid sequence coverage on the difficulty region of mAb heavy chain with eight LC/MS/MS analyses including six Auto MS/MS analyses (three replicates with 15-minute gradient and three replicates with 30-minute gradient) and two Iterative MS/MS analyses with 15-minute gradient. Each LC/MS/MS analysis was carried out with 0.6 µg sample injection, and filtered by 0.1 % FDR. The unmodified TKPREEQYNSTYR peptide was not identifiable in any of the replicate Auto MS/MS analyses or the first injection of Iterative MS/MS analysis, and only identified in the second injection of Iterative MS/MS analysis, increasing the sequence coverage of the heavy chain to 99.12%. This demonstrates the advantage of Iterative MS/MS acquisition on in-depth peptide mapping, providing an alternative approach to identify low-abundant peptides.

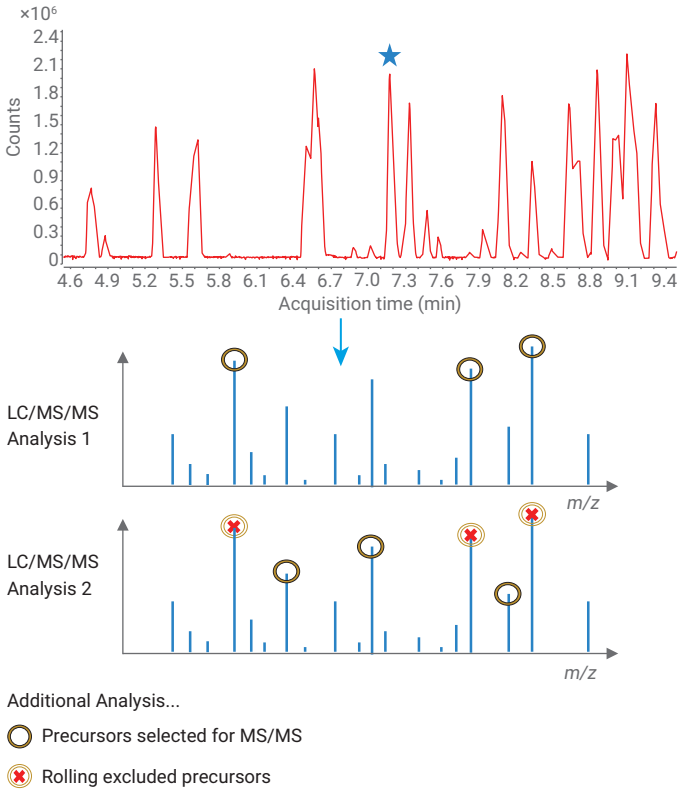


Figure 3. A diagram of an automated Iterative MS/MS acquisition method.

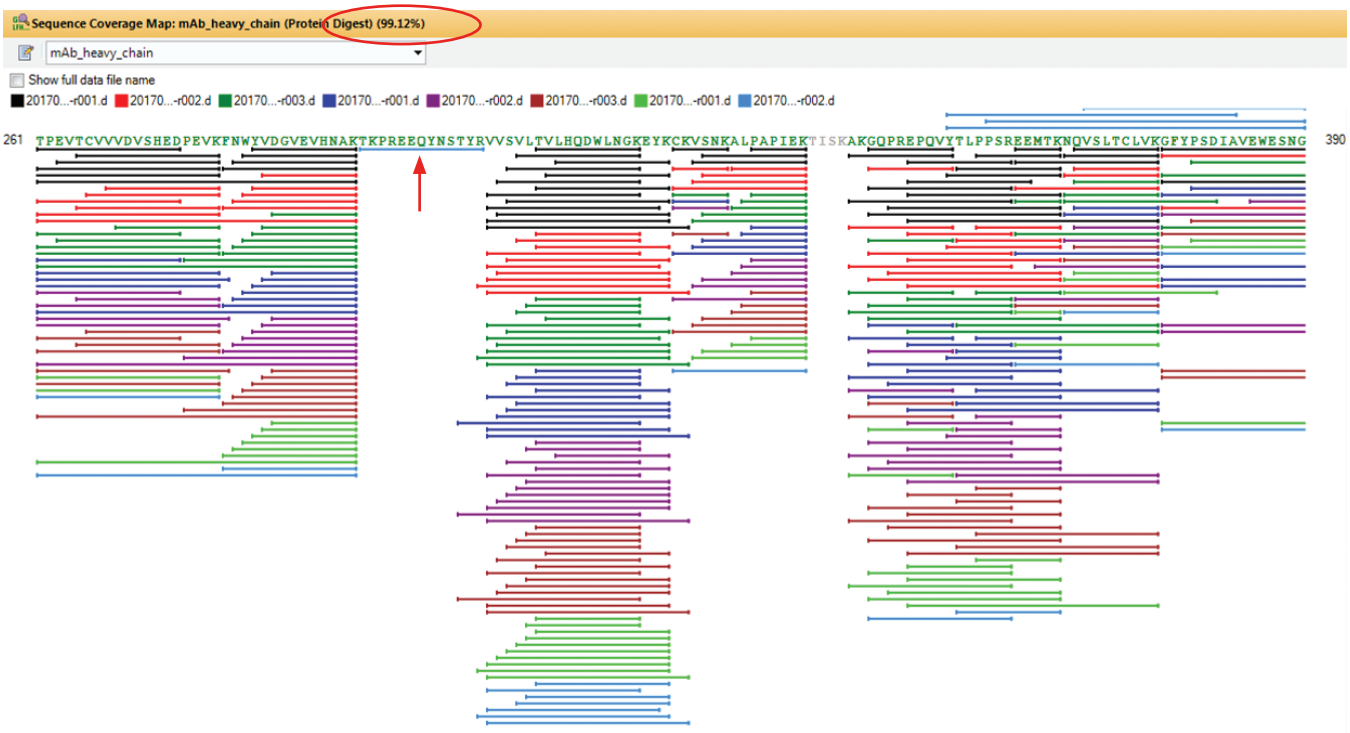
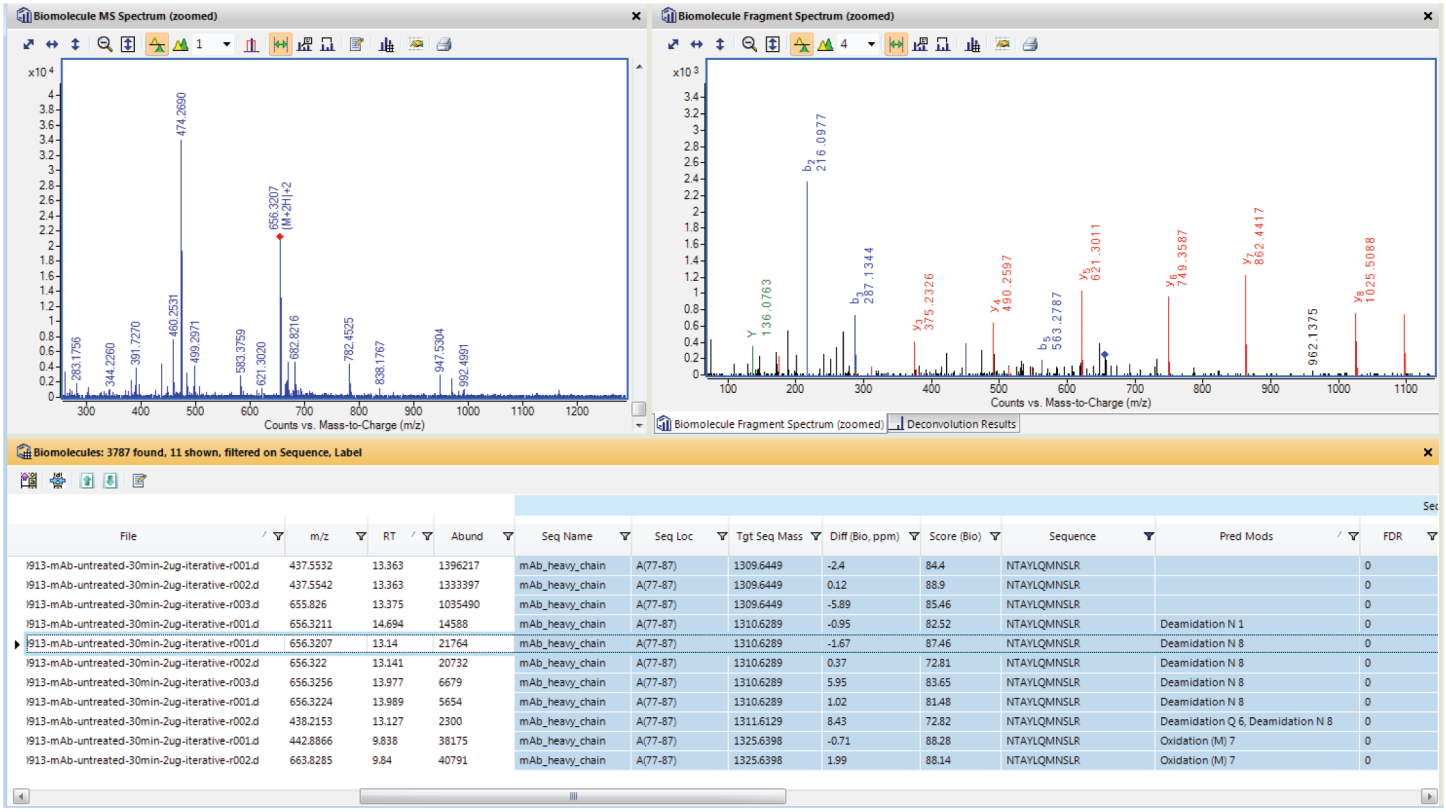


Figure 4. Screenshot of Agilent MassHunter BioConfirm B.09 software with overlaid sequence coverage on a region of mAb heavy chain from multiple data files. Peptides identified from different files were color coded. The TKPREEQYNSTYR peptide, highlighted by a red arrow, was only identified in the second run of Iterative MS/MS analysis with a short gradient.

Post-translational Modification Analysis

Characterization of peptide PTMs is critical during the development and manufacture of protein therapeutics in the biopharma industry. MassHunter BioConfirm B.09 software provides automatic data batch processing, allowing users to view multiple peptide attributes, such as a Biomolecules Table, Sequence Coverage Map, ECC, MS spectrum, fragment spectrum, peptide abundance, and so forth. Figure 5 shows a screenshot of a MassHunter BioConfirm B.09 software Biomolecules Table and spectra for a representative peptide NTAYLQMNSLR (heavy chain 77-87) and all the identified PTM forms. Table 3 shows the identification comparison of this native peptide and its PTM forms using different methods, including two LC gradients (15 minutes versus 30 minutes), two loading amounts (0.6 µg versus 2 µg), and two acquisition methods (Auto MS/MS versus Iterative MS/MS). Positive identification of each peptide form is labeled with a green check mark ✓. The native, the M7-oxidized, and the N1-deamidated peptides were identified under all of the conditions. With a 30-minute gradient and 0.6 µg sample loading, another deamidated form, N8-deamidated peptide, was identified at a retention time (RT) of 13.14 minutes, which eluted close to the native form.



File	m/z	RT	Abund	Seq Name	Seq Loc	Tgt Seq Mass	Diff (Bio, ppm)	Score (Bio)	Sequence	Pred Mods	FDR
I913-mAb-untreated-30min-2ug-iterative-r001.d	437.5532	13.363	1396217	mAb_heavy_chain	A(77-87)	1309.6449	-2.4	84.4	NTAYLQMNSLR		0
I913-mAb-untreated-30min-2ug-iterative-r002.d	437.5542	13.363	1333397	mAb_heavy_chain	A(77-87)	1309.6449	0.12	88.9	NTAYLQMNSLR		0
I913-mAb-untreated-30min-2ug-iterative-r003.d	655.826	13.375	1035490	mAb_heavy_chain	A(77-87)	1309.6449	-5.89	85.46	NTAYLQMNSLR		0
I913-mAb-untreated-30min-2ug-iterative-r001.d	656.3211	14.694	14588	mAb_heavy_chain	A(77-87)	1310.6289	-0.95	82.52	NTAYLQMNSLR	Deamidation N 1	0
I913-mAb-untreated-30min-2ug-iterative-r001.d	656.3207	13.14	21764	mAb_heavy_chain	A(77-87)	1310.6289	-1.67	87.46	NTAYLQMNSLR	Deamidation N 8	0
I913-mAb-untreated-30min-2ug-iterative-r002.d	656.322	13.141	20732	mAb_heavy_chain	A(77-87)	1310.6289	0.37	72.81	NTAYLQMNSLR	Deamidation N 8	0
I913-mAb-untreated-30min-2ug-iterative-r003.d	656.3256	13.977	6679	mAb_heavy_chain	A(77-87)	1310.6289	5.95	83.65	NTAYLQMNSLR	Deamidation N 8	0
I913-mAb-untreated-30min-2ug-iterative-r001.d	656.3224	13.989	5654	mAb_heavy_chain	A(77-87)	1310.6289	1.02	81.48	NTAYLQMNSLR	Deamidation N 8	0
I913-mAb-untreated-30min-2ug-iterative-r002.d	438.2153	13.127	2300	mAb_heavy_chain	A(77-87)	1311.6129	8.43	72.82	NTAYLQMNSLR	Deamidation Q 6, Deamidation N 8	0
I913-mAb-untreated-30min-2ug-iterative-r001.d	442.8866	9.838	38175	mAb_heavy_chain	A(77-87)	1325.6398	-0.71	88.28	NTAYLQMNSLR	Oxidation (M) 7	0
I913-mAb-untreated-30min-2ug-iterative-r002.d	663.8285	9.84	40791	mAb_heavy_chain	A(77-87)	1325.6398	1.99	88.14	NTAYLQMNSLR	Oxidation (M) 7	0

Figure 5. Screenshot of Agilent MassHunter BioConfirm B.09 software Biomolecules Table and spectra for representative peptides.

Table 3. Identification comparison of peptide NTAYLQMNSLR native and modified forms. Positive identification in Auto MS/MS analysis or the first run of Iterative MS/MS analysis are marked as ✓. A **+** mark indicates the peptide only identified in the second run of Iterative MS/MS analysis.

Peptides	Modifications	Retention time with 30-minute gradient (min)	Relative abundance	15 minute, 0.6 µg		30 minute, 0.6 µg		30 minute, 2 µg	
				Auto MS/MS	Iterative MS/MS	Auto MS/MS	Iterative MS/MS	Auto MS/MS	Iterative MS/MS
NTAYLQMNSLR	none	13.37	94.09 %	✓	✓	✓	✓	✓	✓
NTAYLQMNSLR	Oxidation M7	9.84	2.79 %	✓	✓	✓	✓	✓	✓
NTAYLQMNSLR	Deamidation N8	13.14	1.50 %	–	–	✓	✓	✓	✓
NTAYLQMNSLR	Deamidation N8	13.98	0.44 %	–	–	–	–	✓	✓
NTAYLQMNSLR	Deamidation N1	14.69	1.03 %	✓	✓	✓	✓	✓	✓
NTAYLQMNSLR	Deamidation Q6N8	13.13	0.16 %	–	–	–	–	–	+

By further increasing the loading amount to 2 µg, the N8-deamidated form was also identified at a different RT (13.98 minutes), which could be showing the presence of aspartate isomerization[4]. With a combination of Iterative MS/MS, 30-minute gradient, and 2 µg sample loading, a double-deamidated species, Q6N8-deamidated peptide, was identified in the second injection of Iterative MS/MS, but not in replicate Auto MS/MS analyses, marked as **+**. This Q6N8-deamidated peptide coeluted with N8-deamidated peptide. Figure 6 illustrates MS/MS spectra comparison of native and all PTM forms of peptide NTAYLQMNSLR. The fragment ions highlighted in the blue box are representative ions, which clearly distinguish the native and various PTM forms from each other, confirming the high quality of peptide identification.

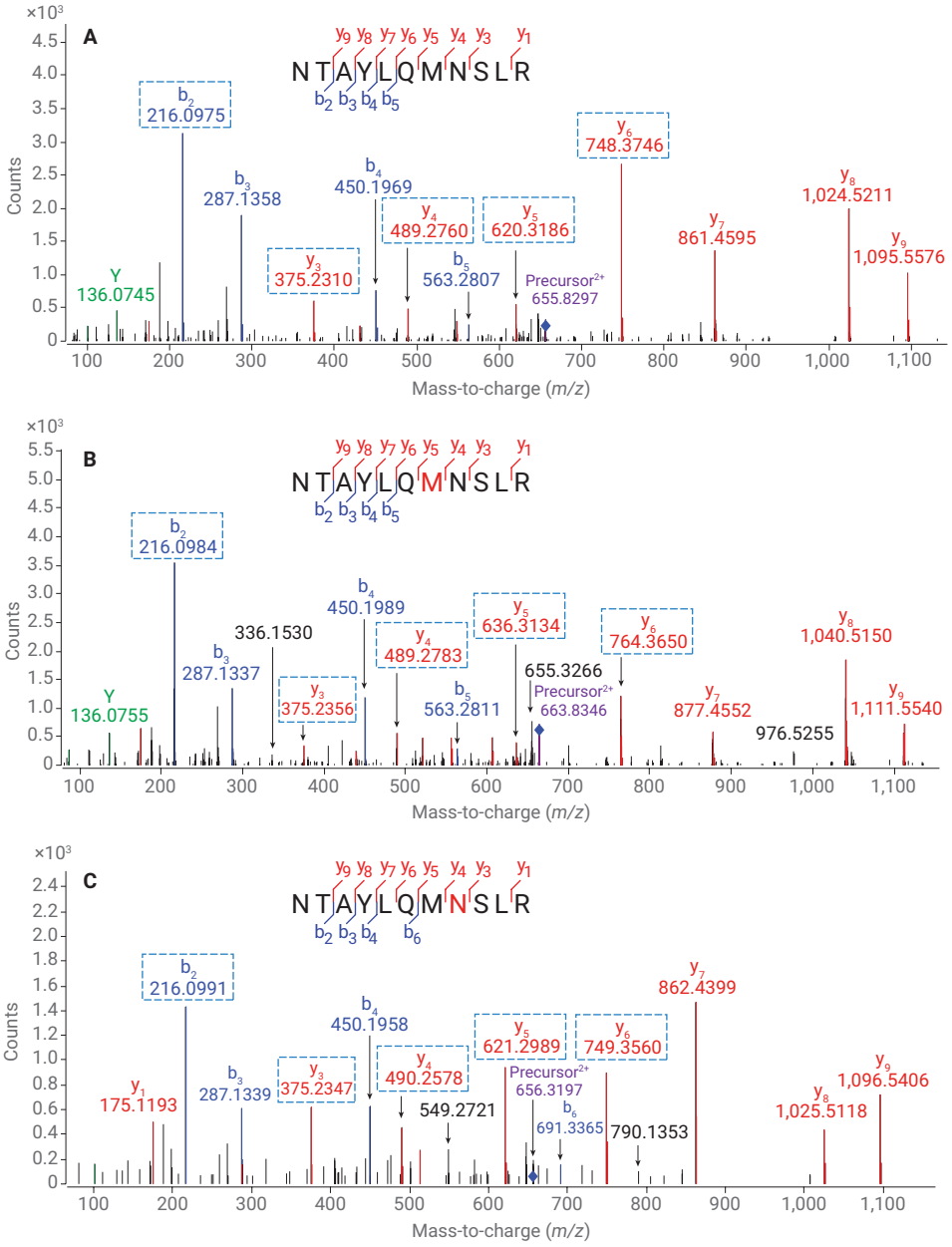


Figure 6. Post-translation modification analysis of peptide NTAYLQMNSLR. MS/MS spectra of native form (A), M7-oxidized form (B), N8-deamidated form, RT = 13.14 minutes (C); On next page: N8-deamidated form, RT = 13.98 minutes (D), N1-deamidated form (E),and Q6N8-deamidated form (F). Modified amino acids in a peptide sequence were in highlighted red. Representative fragment ions that distinguish each species were highlighted in blue box.

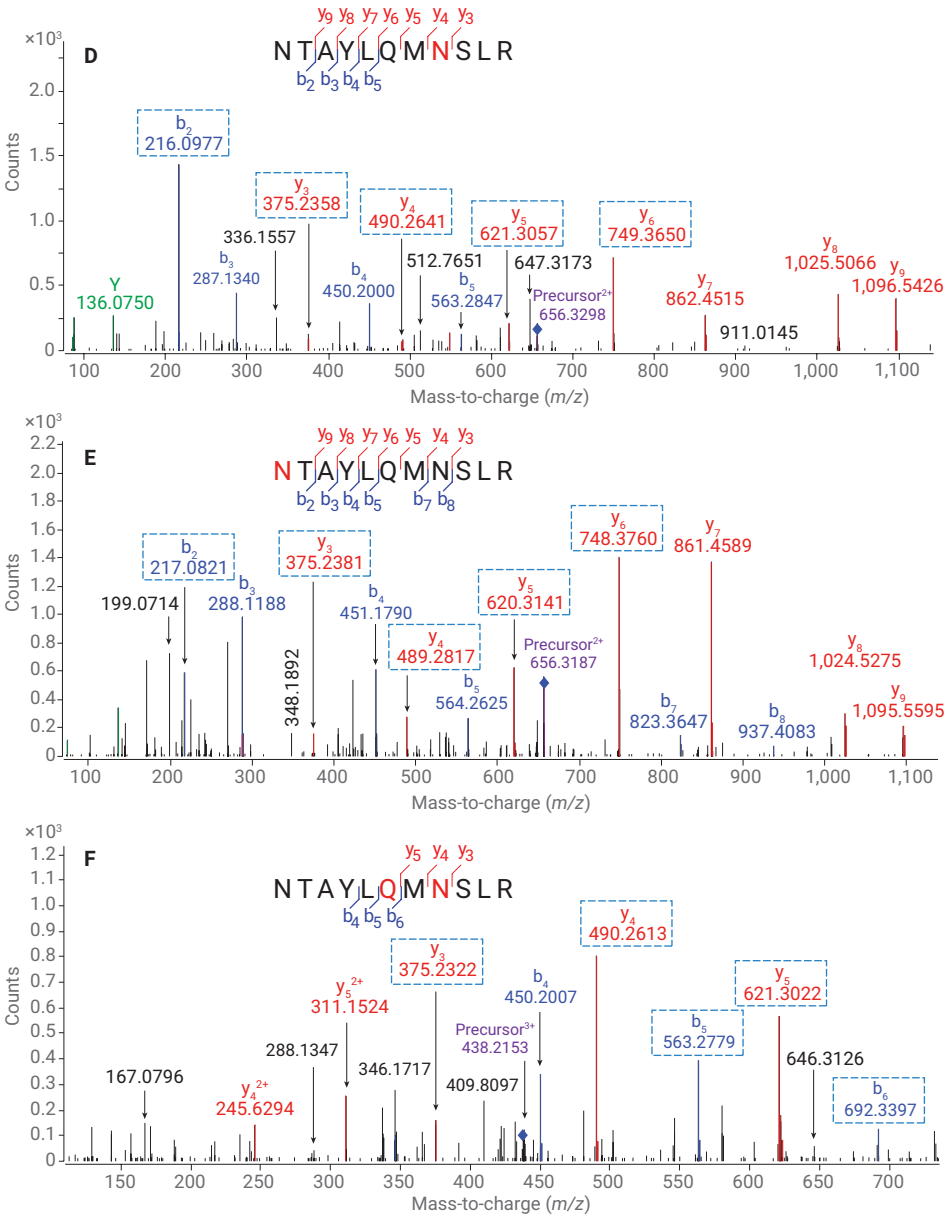


Figure 6. Continued from previous page: post-translation modification analysis of peptide NTAYLQMNSLR. MS/MS spectra of N8-deamidated form, RT = 13.98 minutes (D), N1-deamidated form (E),and Q6N8-deamidated form (F). Modified amino acids in a peptide sequence were in highlighted red. Representative fragment ions that distinguish each species were highlighted in blue box.

A quantitative analysis of the MS data reveals the relative amounts of each species of peptide NTAYLQMNSLR. Figure 7 shows the overlaid ECCs of the native and all modified forms of this peptide and its relative abundance with a 30-minute LC separation. The native form consists of approximately 94.09 % of the peptides, with the:

- M7-oxidized form comprising 2.79 %
- The two N8-deamidated forms comprising 1.5 % and 0.44 %, respectively
- The N1-deamidated form comprising 1.03 %
- The Q6N8-deamidated form comprising 0.16 %

In summary, two injections with Iterative MS/MS acquisition method enabled identification of all the native and PTM forms, which could be as low as 0.16% for coeluting deamidated peptides. These results demonstrate the power of this integrated workflow with Iterative MS/MS for in-depth peptide mapping.

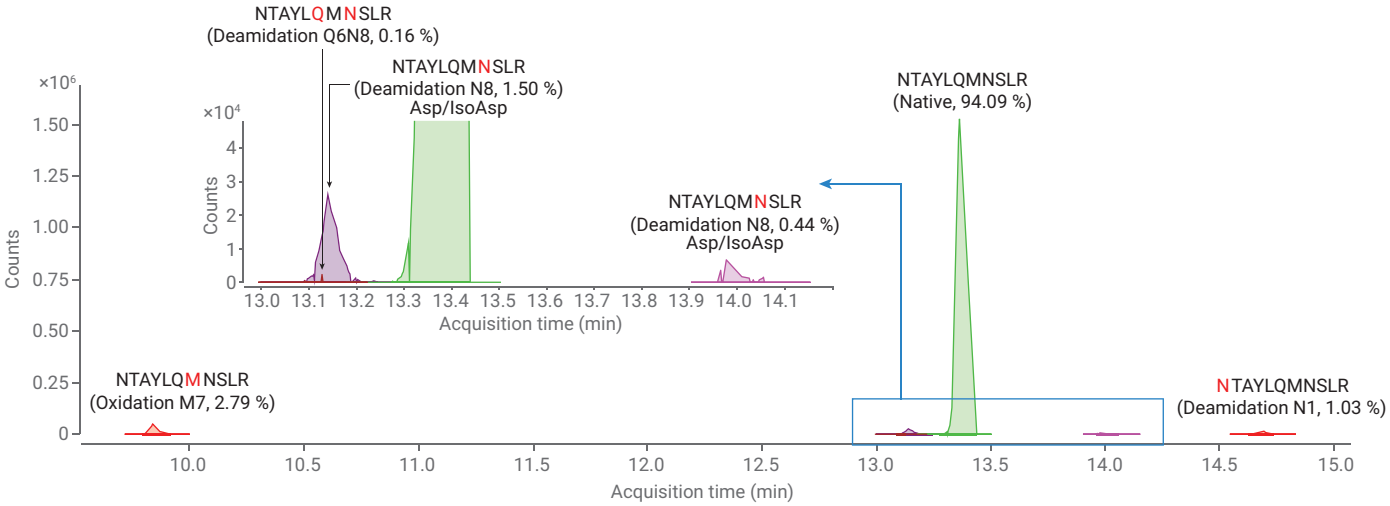


Figure 7. Overlaid ECCs and relative quantitation analysis of peptide NTAYLQMNSLR native and modified forms.

Conclusion

A complete workflow from automated sample preparation using Agilent AssayMAP Bravo for digestion, LC/MS/MS analysis by Agilent 1290 Infinity II LC coupled to an Agilent 6545XT AdvanceBio Q-TOF, to data analysis using Agilent MassHunter BioConfirm B.09 software has been demonstrated for peptide mapping with high sequence coverage and detection of low-abundant PTMs. The acquisition method, Iterative MS/MS, provided by the 6545XT AdvanceBio LC/Q-TOF system greatly improves the identification of low-abundant peptides in a complex protein digest sample. MassHunter BioConfirm B.09 software is capable of automated data batch processing, peptide-spectrum matching with statistical score and FDR to filter the MS/MS spectra quality, sequence coverage overlapping from multiple data files, and linked navigation through the result table to the mass spectra and chromatograms for selected peptides. A combination of these functions provided by the 6545XT AdvanceBio LC/Q-TOF system and MassHunter BioConfirm B.09 software enhances the workflow for in-depth peptide mapping during the development and manufacture of protein biotherapeutic drugs.

References

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Printed in the USA, June 16, 2020
5991-8633EN

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