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Application News

Biotech-2202

Liquid Chromatography Mass Spectrometry

High Throughput and High Sequence Coverage Peptide Mapping of a Monoclonal Antibody Using an Integrated Protein Digestion LCMS Platform (Perfinity-QTOF LCMS-9030)

■ Summary

This application news demonstrates the advantage of high-throughput and high sequence coverage peptide mapping for the NIST monoclonal antibody (NISTmAb) by coupling an integrated online protein digestion platform, Perfinity Workstation, with a Shimadzu Q-TOF LCMS-9030.

■ Background

Over the past three decades, more than 100 monoclonal antibodies (mAbs) have been successfully used as therapeutics for the treatment of various human diseases[1]. As the understanding of targeted therapies grew and recombinant mAb technology matured, pharmaceutical companies have been discovering and developing mAb therapeutics at an unprecedented pace. This growth has generated a challenging demand for the characterization of therapeutic mAbs, which is essential for patent protection, regulatory approval, and quality control. Peptide mapping is the gold standard approach to establishing protein identity, identifying post-translational modifications (PTMs), and analyzing potential impurities.

A bottom-up strategy using enzymes to digest a therapeutic protein into its constituent peptides is usually used to generate a peptide map. Typical peptide-mapping procedures are tedious, especially for protein digestion, and vary due to the differences among protocols and/or operators. As such, they are susceptible to changes that affect reproducibility, reduce assay sensitivity, and significantly increase analysis times. Protein digestion protocols may also influence correct evaluation of PTM values due to possible introduction of process-induced modifications as a result of the excessive sample handling involved. Overcoming these drawbacks calls for simple, efficient, and reliable alternatives. We demonstrate herein a high-throughput and high sequence coverage approach based on an automated on-column protein digestion platform directly coupled to a Shimadzu Q-TOF mass spectrometer, LCMS-9030. The Perfinity Workstation is a multi-column instrument that automates protein digestion and mass spec sample preparation. The LCMS-9030 is a powerful instrument that integrates highly sensitive quadrupole technology with TOF capabilities for accurate mass measurement.

■ Materials and Methods

NISTmAb Humanized IgG1κ monoclonal antibody reference material 8671 was purchased from the National Institute of Standards & Technology (NIST). LC-MS-grade solvents (formic acid, acetonitrile, water) were sourced from Honeywell. Urea, Tris-HCl, dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from Sigma-Aldrich. The optimized wash solution, digest buffer, and re-equilibrate buffer for the online protein digestion were obtained from Perfinity.

A Perfinity Workstation was used to facilitate rapid online digestion and reversed-phase separation of the NISTmAb and the proteolytic peptides. Trypsin columns, NoRA trypsin columns, and Lys-C columns were used for protein digestion. The operating temperatures for the columns were set based on manufacturers' recommendations. A C18 desalt column was used to clean up the samples after digestion and a Restek Ultra C18 (150mm x 2.1mm, 2.7µm) column was used for reserved-phase separation. All data were acquired on the Q-TOF LCMS-9030 under data dependent acquisition (DDA) mode with three events monitored. Protein metrics PTM workflow was used to obtain sequence coverage map and identify the post-translational modifications (PTMs).

The effect of pre-reduction/alkylation during sample preparation was assessed. In the first set of experiments, NISTmAb was diluted to 1.0 mg/mL with water then directly injected into Perfintiy for a 6-minute on-column protein digestion. Both the trypsin column and NoRA trypsin column were tested at different temperatures. In the second set of experiments, NISTmAb was first reduced with 50 mM DTT in 8 M urea and 50 mM tris-HCl (pH 8.0), then incubated at 37 °C for 30 minutes, and finally alkylated with 500 mM IAA in the dark at room temperature for another 30 minutes prior to injection into Perfinity. The final concentration of NISTmAb after reduction and alkylation was 1.0 mg/mL. All three types of enzymatic columns were tested in each set of the experiments. The analytical conditions for Perfinity Workstation and mass spectrometry are listed in Table 1.

Table 1: Analytical conditions

Perfinity Conditions	[Perfinity Workstation]
Mobile Phase A	: Water containing 0.1% formic acid
Mobile Phase B	: Acetonitrile containing 0.1% formic acid
Gradient Program	: 5% B (0 - 1 min) -> 30% B (30 min) -> 50% B (36 min) -> 95% B (40 - 50 min) -> 5% B (50 - 52 min)
Column	: Restek Ultra C18 (150mm x 2.1mm, 2.7µm)
Flow Rate	: 0.2 mL/min
Column Oven Temperature	: 50 ℃ (trypsin and Lys-C columns); 70 ℃ (NoRA trypsin column)
Injection Volume	: 10 uL
Digest Time	: 6 min
MS Conditions	[LCMS-9030)
Mode	: DDA (3 events)
TOF Start *m/z*	: 200
TOF End *m/z*	: 1500
DDA Start *m/z*	: 200
DDA End *m/z*	: 1500
CE	: 35
CE Spread	: 17
Event Time (s)	: 0.100
Pulser Inj. Times	: 194
Nebulizing Gas Flow	: 2.0 L/min
Heating Gas Flow	: 10.0 L/min
Interface Temperature	: 300 ℃
Drying Gas Flow	: 10.0 L/min

■ Results and Discussion

Figure 1 illustrates two examples of extracted ion chromatograms (XIC) of the NISTmAb proteolytic peptides which were generated on the trypsin column for six minutes at 50 °C. The top chromatogram was obtained for samples with no reduction/alkylation, while the bottom chromatogram included reduction/alkylation. Each identified peptide is labeled with the corresponding sequence number and chromatographic retention time in Protein Metrics software.

Visual comparison of the two XICs revealed significant differences in terms of the number of detected peptide peaks. 91 peaks were detected in the top chromatogram, whereas more than 200 detected peaks were detected in the bottom one, which indicates that the digestion of the NISTmAb protein was more efficient when including a reduction-alkylation step.

Similar results were observed for samples digested on the NoRA trypsin column. Although the NoRA trypsin column increased the number of peptides detected in the digested sample, the reduction/alkylation step was still necessary to achieve a complete digestion.

In the PTM workflow of Protein Metrics software, all matched peptides were set to have < 6 ppm mass error and at least one confirmatory MS/MS spectrum. Missed cleavage was set as 2. Carbamidomethyl was set as the fixed modification. Variable modifications included oxidation, deamidation, pyro-Glu formation, and ammonia loss. The majority of identified peptides showed excellent mass accuracy, with errors less than 1 ppm. After the identification of the peptides, a sequence coverage map for both heavy and light chain was reported.

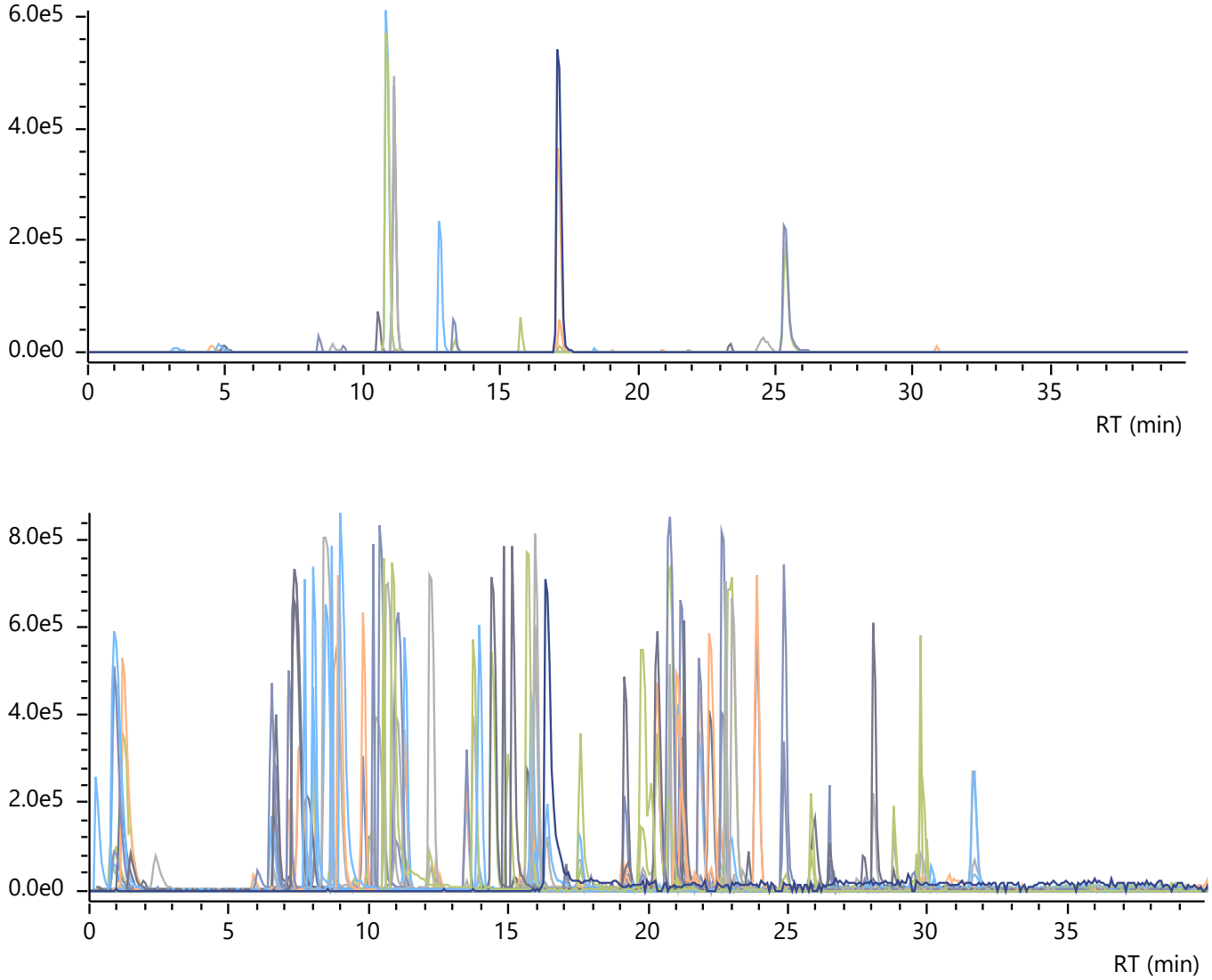

Figure 1: Extracted ion chromatogram (XIC) of peptide fragments from trypsin digested NISTmAb. Top: trypsin column without reduction/alkylation (RA). Bottom: trypsin column with RA. NISTmAb was digested on column for 6 minutes at 50 °C in both cases.

The sequence coverage maps of NISTmAb digestion on different types of enzymatic columns are shown in Figure 2 and the results are summarized in Table 2. For samples injected directly into Perfinity without reduction/alkylation, 30.2% sequence coverage of the heavy chain and 44.1% sequence coverage of the light chain were obtained with the trypsin column. When using the NoRA column, 37.3% sequence coverage of the heavy chain and 50.2% sequence coverage of the light chain were obtained.

For samples with reduction/alkylation steps, 98.7% sequence coverage of the heavy chain and 100% sequence coverage of the light chain were obtained with the trypsin column. A 100% sequence coverage for both the heavy and light chains was obtained when using the NoRA trypsin column. The coverage drops to 94.4% sequence of the heavy chain and 95.3% of the light chain when using the Lys-C column.

Figure 2: NISTmAb peptide coverage on Perfinity-QTOF system. Black: trypsin column without reduction/alkylation (RA). Blue: NoRA trypsin column without RA. Red: trypsin column with RA. Green: NoRA trypsin column with RA. Magenta: Lys-C column with RA.

Table 2: Summary of sequence coverage

NISTmAb	Sequence coverage (%)				
	Trypsin column w/o RA, 50 °C	NoRA trypsin column w/o RA, 70 °C	Trypsin column with RA, 50 °C	NoRA trypsin column with RA, 70 °C	Lys-C column with RA, 50 °C
Heavy chain	30.21	37.33	98.67	100	94.42
Light chain	44.13	50.23	100	100	95.31

Figure 3 illustrates the MS/MS spectra comparison of the native (precursor at *m/z* = 418.2207, +2) and Met-oxidized peptides (precursor at *m/z* = 426.2182, +2) in the heavy chain 252-258. The differences in *m/z* (+15.99 Da in the oxidized form) for the y4–y5 fragment ions (green boxes) clearly distinguish the modified forms from the native species, and the *m/z* of y1-y3 ions remain constant, indicating that the Met-4 in the peptide is the location of oxidation.

Similarly, Figure 4 shows the MS/MS spectra of the native and the deamidated peptides of 305-323 in the heavy chain, where the y6-y8 fragment ions (green boxes) all show the signature mass shift of 0.98 Da, and y14++ - y17++ ions have the *m/z* difference of 0.49, indicating the presence of deamidation. Moreover, as the *m/z* of y3-y5 ions remain the same with the native form, the deamidation occurs at the Asn-14 position.

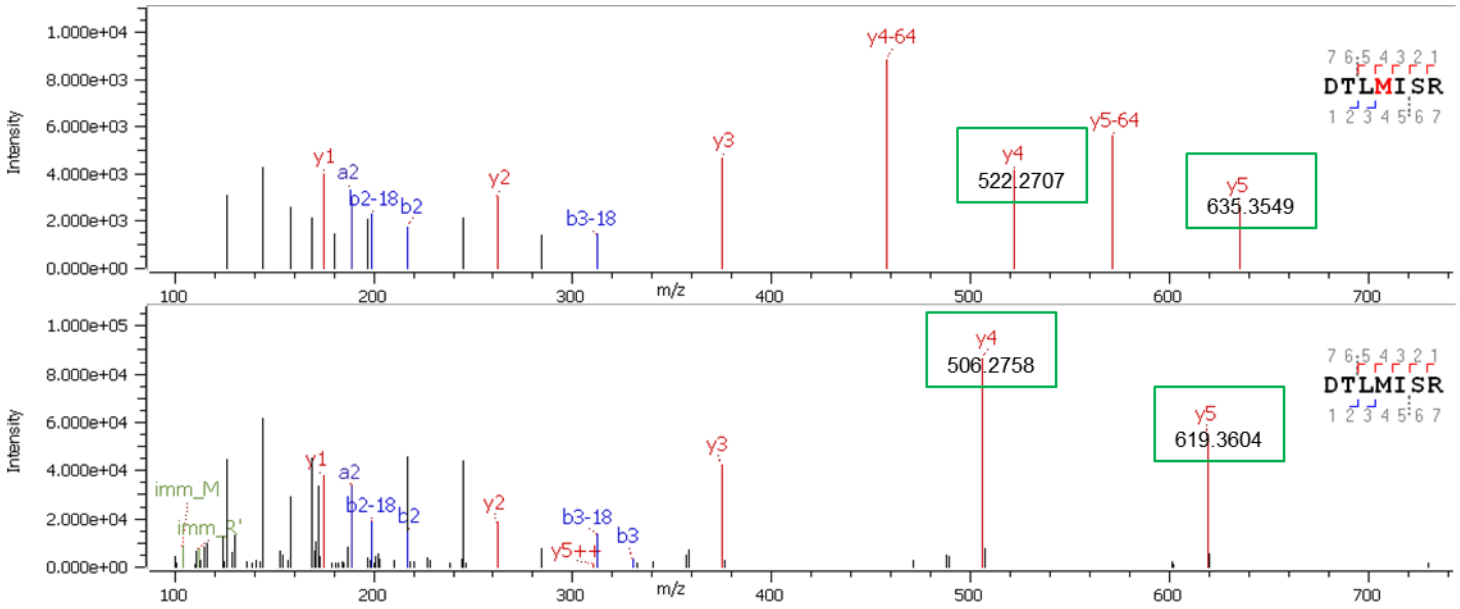

Figure 3: Post translational modification (methionine oxidation) analysis. MS/MS spectra of Met-oxidized and native peptides (heavy chain peptide 252-258). Top: oxidation at Met 255 in heavy chain. Bottom: native peptide. Green boxes show confirmed fragment ions.

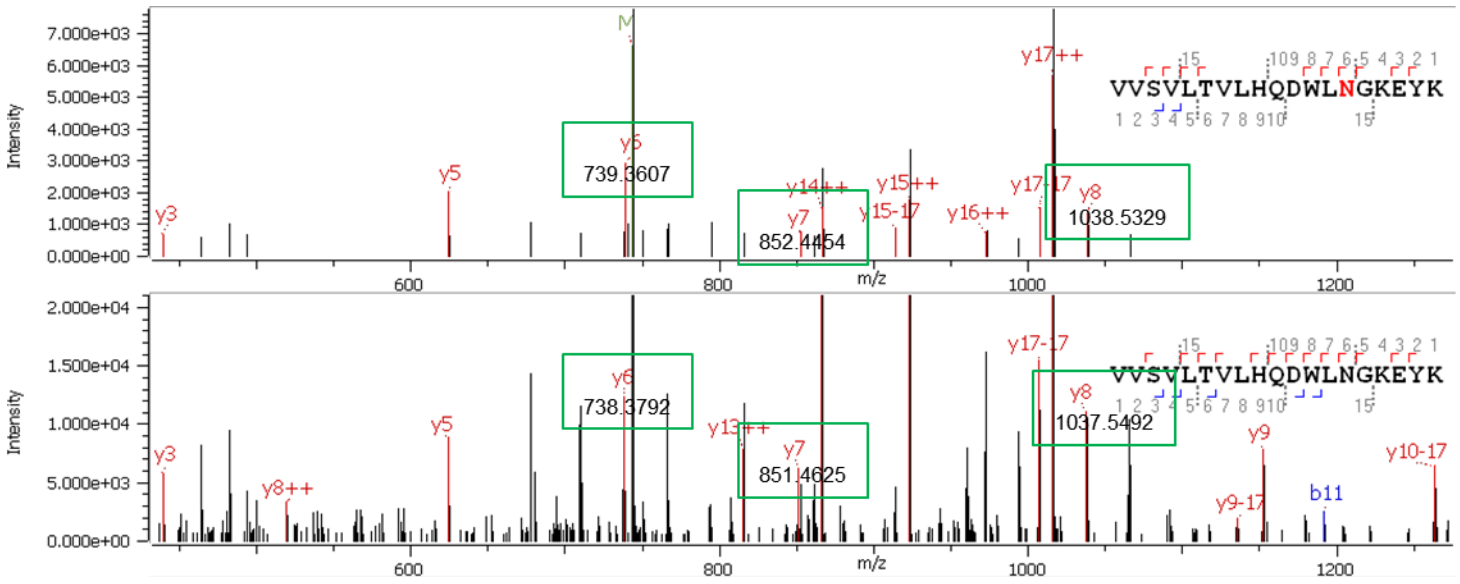

Figure 4: Post translational modification (asparagine deamidation) analysis. MS/MS spectra of Asn-deamidated and native peptides (heavy chain peptide 305-323). Top: deamidation at Asn 318. Bottom: native peptide. Green boxes show confirmed fragment ions.

Table 3: Summary of critical PTMs identified and quantified for NISTmAb with different digestion conditions

Modification	Sequence	Relative abundance (%)				
		Trypsin column w/o RA, 50 °C	NoRA trypsin column w/o RA, 70 °C	Trypsin column with RA, 50 °C	NoRA trypsin column with RA, 70 °C	Lys-C column with RA, 50 °C
HC Q1+Gln -> PyroGlu	QVTLR	100	100	100	100	100
HC M255 + Oxid	DTLMISR	2.39	3.01	3.63	4.97	2.53
HC ~N392/N387 + Deam	GFYPSDIAVEWESNGQPENNYK	1.24	2.89	1.21	1.95	2.54
HC K450 Lys loss	SLSLSPGK	90.55	89.75	87.73	88.79	90.23
HC N300+A2G0F	EEQYNSTYR	NA	NA	40.14	38.36	37.52
	TKPREEQYNSTYR					
HC N300+A2G1F	EEQYNSTYR	NA	NA	36.65	34.11	33.56
	TKPREEQYNSTYR					
HC N300 unglycosylated	EEQYNSTYR	NA	NA	1.53	1.28	1.28
	TKPREEQYNSTYR					

Table 3 summarizes the quantification results of the critical PTMs identified with different digestion conditions. Complete conversion of N-terminal glutamine (Gln) to pyroglutamate (Pyro-Glu) was observed under all conditions. Conversion from N-terminal Gln to pyro-Glu has been reported to be above 95% in mAbs, and it is known that the conversion occurs primarily inside bioreactors with little contribution from downstream purification and analytical processes[2]. The loss of lysine from the C-terminus of the heavy chain, in the range of 87-90%, was comparable at different digestion conditions. C-terminal Lys clipping is another common modification for mAbs and occurs during the manufacturing process[2]. The NISTmAb also has low abundance of PTMs, such as methionine oxidation and asparagine deamidation. Methionine oxidation at heavy chain M255, and asparagine deamidation at heavy chain N387 and N392, are usually monitored for NISTmAb. Similar levels for the two modifications were detected for all digested samples. High abundance of glycosylation at the heavy chain N300 was observed for the reduced and alkylated samples where the main glycans were complex biantennary oligosaccharides containing from 0 or 1 non-reducing galactose with fucose attached to the reducing end of N-acetylglucosamine (A2G0F, A2G1F). The sequence was not covered for the non-reduced/alkylated samples, so the glycosylation modification was not detected. Overall, the reported PTM values are very close among experiments and show low amounts of sample preparation-induced modifications.

■ Conclusion

This application news demonstrates a fast, reproducible peptide mapping workflow using Shimadzu's unique solution: Perfinity-LCMS-9030 system. The combination of automated sample preparation, reversed-phase separation, and accurate detection upgraded the process of peptide mapping from time-consuming and tedious to an easy, reproducible, and automated one. The analysis of NISTmAb RM 8671 protein produced outstanding sequence coverage with high confidence due to the Q-TOF mass accuracy. Reduction and alkylation steps were necessary in order to improve sequence coverage. Even with the added steps, the total analysis time was still less than two hours. The most targeted modifications were successfully identified and quantified relatively. We confirmed that PTMs induced by sample preparation were low and insignificant.

■ References

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2. Li, W.Z.; Kerwin, J.L.; Schiel, J. et al. Structural elucidation of post-translational modifications in monoclonal antibodies. *State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 2. Biopharmaceutical Characterization: The NISTmAb Case Study*, 2015.

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