Application Note

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Peptide Mapping of Trastuzumab Tryptic Digests on an Agilent 6545XT AdvanceBio LC/Q-TOF

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Abstract

This application note describes a peptide mapping strategy to analyze tryptic digests of trastuzumab on an Agilent 6545XT AdvanceBio LC/Q-TOF. Detailed sample preparation instructions are provided for users that are new to peptide mapping to enable their own biopharmaceutical analysis. For trastuzumab, sequence coverages of 98, 99.7, and 98% were observed across three replicates. In addition, excellent sequence ladders for peptides with MS1 signals in the low ~ 1e4 range are presented, highlighting the excellent dynamic range of the 6545XT AdvanceBio LC/Q-TOF. MS/MS information is also presented for the deamidated peptide FTISADTSKNTAYLQM**N**SLR, which unambiguously assigns the site of deamidation at position 17, despite multiple potential sites. The combination of excellent sequence coverages and dynamic ranges highlights the performance of the 6545XT AdvanceBio LC/Q-TOF for peptide mapping applications.

Introduction

There are several LC/MS techniques that can report on protein sequence and posttranslational modifications (PTMs) present for monoclonal antibodies (mAbs). For example, intact protein mass spectrometry will use MS1 information and deconvolution tools to report on the mass correlation to the original and potentially modified sequence of the mAb, as well as the relative abundances of glycosylation present in the sequence. Since PTMs can play a role in the safety, efficacy, and binding of a therapeutic to its target[1], the ability to digest an intact mAb into smaller peptides and identify the location of unknown PTMs is an important technique in the biotherapeutic process. Peptide mapping uses a combination of MS1 and MS2 information to confidently assess the primary sequence of an mAb, as well as identify the location of PTMs and sequence variants.[2] A 6545XT AdvanceBio LC/Q-TOF was used in combination with Agilent MassHunter BioConfirm software, version 12.1 to characterize trastuzumab for sequence confirmation, glycosylation heterogeneity, and unknown PTMs.

Experimental

Chemicals

The following chemicals were used for the digestion reaction:

- 8 M Guanidine hydrochloride, pH 8.5 (Sigma-Aldrich, part number G7294, 100 mL)
- 0.2 M DL-Dithiothreitol (DTT, Sigma-Aldrich, part number D9779, 1 g)
- 0.4 M Iodoacetamide (IAA, Sigma-Aldrich, part number I1149, 5 g)
- 1 M Tris buffer, pH 8.0 (Sigma-Aldrich, part number 648314, 100 mL)
- Trypsin/LysC Mix, Mass Spectrometry Grade, 5 × 20 µg (Promega, part number V5073)
- 10% Trifluoroacetic acid (TFA, Sigma-Aldrich, part number T6508)

Preparation of solutions

1. **6.4 M Guanidine-HCl, 200 mM Tris-HCl buffer, pH 8.1:** Add 80 mL of 8.0 M guanidine HCl solution to 20 mL of 1 M Tris-HCl buffer in a 100 mL volumetric flask. Mix thoroughly by inversion, and measure the pH using a pipette and pH indicator strip and record. If the pH falls between 7.2 and 8.5, this will be sufficient to proceed with the reducing step.
2. **0.2 M DTT:** Measure 31 mg of DTT using an analytical balance and add 1 mL of 50 mM Tris-HCl solution to dissolve. Vortex this solution prior to the reducing step.
3. **0.4 M IAA:** Measure 74 mg of IAA using an analytical balance and add 1 mL of 50 mM Tris-HCl solution to dissolve. Make immediately before use. Vortex this solution and store in the dark, covered with aluminum foil.
4. **1 µg/µL Trypsin/LysC mix:** Add 20 µL of water to 20 µg of Trypsin/LysC mix to dissolve. **Note:** Trypsin/LysC is enzymatically active at pH 7. Prepare this solution right before overnight digestion.

Standards and sample preparation

The mAb trastuzumab was acquired from Genentech at a concentration of 22 mg/mL. This solution was used directly without any changes.

The sample preparation procedure used for reduction, alkylation, and digestion was performed manually. A description of this procedure is as follows:

1. Add 4.6 µL of trastuzumab stock solution (102 µg) to 27.4 µL of 6.4 M guanidine chloride, 200 mM Tris-HCl.
2. Add 7 µL of 0.2 M DTT in 50 mM Tris-HCl to the denatured trastuzumab solution. Mix gently and spin down, then incubate at 37 °C for 30 minutes.
3. Add 7 µL of 0.4 M iodoacetamide in 50 mM Tris-HCl. Keep in the dark at room temperature for 30 minutes.
4. Add 3.5 µL of 0.2 M DTT to quench any remaining iodoacetamide.
5. Add 130.5 µL of 50 mM Tris-HCl buffer to dilute the guanidine-HCl concentration to 1 M.
6. Add 20 µL of water to the lyophilized Trypsin/LysC vial to bring the concentration to 1 µg/µL. Add 4 µL of Trypsin/LysC to the trastuzumab solution to yield a protein-to-enzyme ratio of 25:1. Incubate overnight at 37 °C.
7. Following overnight digestion, add 10 µL of 10% trifluoroacetic acid to quench the trypsin reaction.

The concentration of digested mAb in each of the samples was ~ 0.5 µg/µL. Using an injection volume of 4.5 µL gives an on-column amount of ~ 2.25 µg for each LC/MS/MS analysis.

LC/MS analysis

LC/MS analysis was performed on an Agilent 1290 Infinity II Bio LC system coupled to a 6545XT AdvanceBio LC/Q-TOF system (Figure 1). To prepare the 6545XT AdvanceBio LC/Q-TOF system for acquisition, a TOF transmission tune was performed with the Fragile Ions checkbox selected. An Agilent AdvanceBio Peptide Mapping column (2.1 × 150 mm, 2.7 µm) was used for chromatographic separation. LC and MS parameters used are listed in Tables 1 and 2.

Data processing

Raw data from LC/MS/MS analysis was processed using MassHunter BioConfirm software, version 12.1. Trastuzumab, Trypsin, and LysC sequences were entered into BioConfirm biopharmaceutical software using the Sequence Manager tool. For trastuzumab, cysteines involved in disulfide bonds were modified directly in the sequence to incorporate iodoacetamide alkylation as a fixed modification. The peptide mapping search includes several filters to restrict the appearance of low-quality MS/MS mass spectra that can artificially inflate the sequence coverage calculation. For example, mass matching used thresholds of 5 ppm for MS1 and 20 ppm for MS2. In addition, a minimum BioScore of 5 for the sequence coverage calculation was set to restrict the inclusion of false positive peptide identifications. The BioScore was calculated as the $-\log_{10}$ of the spectral E-value score, which is a metric that evaluates the statistical significance of individual peptide-spectrum matches relative to a decoy database. Practically speaking, an E-value of 0.00001 (corresponding to a BioScore of 5) means that there is a very low chance of finding a match by random chance within the searched database. More details on the calculation of the spectral E-value score can be found in reference 3.[3] The processing parameters used for the peptide mapping search are listed in Table 3.

Figure 1. Agilent 6545XT AdvanceBio LC/Q-TOF and Agilent 1290 Infinity II Bio LC system.

Liquid chromatography

Table 1. Agilent 1290 Infinity II LC method.

Agilent 1290 Infinity II LC System	
Column	Agilent AdvanceBio Peptide Mapping, 2.1 × 150 mm, 2.7 µm (p/n 653750-902)
Sampler Temperature	5 °C
Mobile Phase A	Water with 0.1% formic acid
Mobile Phase B	Acetonitrile with 0.1% formic acid
Flow Rate	0.4 mL/min
Injection Volume	4.5 µL
Column Temperature	50 °C
Gradient Program	Time (min) %B 0 2 3 2 70 40 70.5 80 74 80 74.50 2 80 40 80.20 80 83.20 80 83.50 2 87 2

Mass spectrometry

Table 2. MS parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF	
Ion Source	Agilent Dual Jet Stream ESI source
Polarity	Positive
Divert Valve	Enabled; LC flow to waste from 0 to 3 minutes, LC flow to MS from 3 to 87 minutes
Data Storage	Both (centroid and profile)
Gas Temperature	250 °C
Drying Gas Flow	10 L/min
Nebulizer	25 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	0 V
Fragmentor	95 V
Skimmer	50 V
Reference Mass	322.0481, 922.0098
MS1 Range	*m/z* 200 to 3,000
Acquisition Rate	3 spectra/second
MS/MS Range	*m/z* 100 to 3,000
Acquisition Rate	3 spectra/second
Isolation Width	Medium (*m/z* ~ 4)
Precursors/Cycle	Top 5
Threshold for MS/MS	6,000 counts and 0.001%
Precursor Charge	2+, 3+, > 3+
Target	50,000 counts/spectrum

Processing in BioConfirm 12.1

Table 3. Agilent MassHunter BioConfirm software, version 12.1 processing parameters.

Workflow and Sequences		
Workflow	Protein digest	
Conditions	Reduced	
Sequences	Trastuzumab (directly alkylated), Trypsin, LysC	
Variable Modifications	Deamidation, G0, G0F, G0F-GlcNAc, G1, G1F, G1F-GlcNAc, G2, G2F, H5N3F1-1, methylation, oxidation (M, W), pyroGlu (E)	
Enzymes	Trypsin, LysC	
Find Peptides		
Biomolecule Filters	Display Biomolecules Containing MS/MS Scans	Enabled
	Display Biomolecules Containing MS-Only and MS/MS Scans	Disabled
Extraction	Restrict RT to	0 to 65 min
	Use Peaks with Height	≥ 600 counts
Ion Species	Positive Ions	H^+, Na^+, K^+, NH_4^+
Charge State	Isotope Model	Peptides
	Limit Assigned Charge States to a Range of	Allow all charge states
Peak Filters (MS/MS)	Height Filters, Absolute Height Enabled	≥ 50 counts
Match Tolerances		
Mass Matching	± 5 ppm MS1, ± 20 ppm MS2	–
Scoring	Warn if Score is	< 7.00
	Do Not Match if Score is	< 5.00 (Sequence coverage) < 3.00 (Glycopeptide ID)
Matching Rules	MS/MS Fragmentation Type	CID
	Number of Missed Cleavages	3
	Max Number of Matches per Biomolecule	3
	Peptide length range	3 to 70
	Allow Only Either N-Term or C-Term Truncation	Selected
	Maximum Number of Mods	2

Results and discussion

Total ion chromatogram for trastuzumab digest

The total ion chromatogram for trastuzumab digested overnight with Trypsin/LysC at a protein-to-enzyme ratio of 25:1 is illustrated in Figure 2. As indicated in the sample preparation protocol, no desalting was performed to remove guanidine or Tris-HCl. Since the injection volume of the mAb digest was small relative to the flow rate used, the first 3 minutes of the LC/MS method diverted LC flow to waste, to reduce potential ion suppression due to guanidine or Tris-HCl. From 3 to 70 minutes, the diverter valve was switched to direct flow to the MS, where peptide signal was recorded. The most intense peptide signals were observed from approximately 20 to 55 minutes, consistent with peptide lengths that were longer and allow sufficient overlap for high sequence coverage. Following elution of the peptide signals, a series of wash steps were used to flush and re-equilibrate the peptide mapping column for subsequent injections.

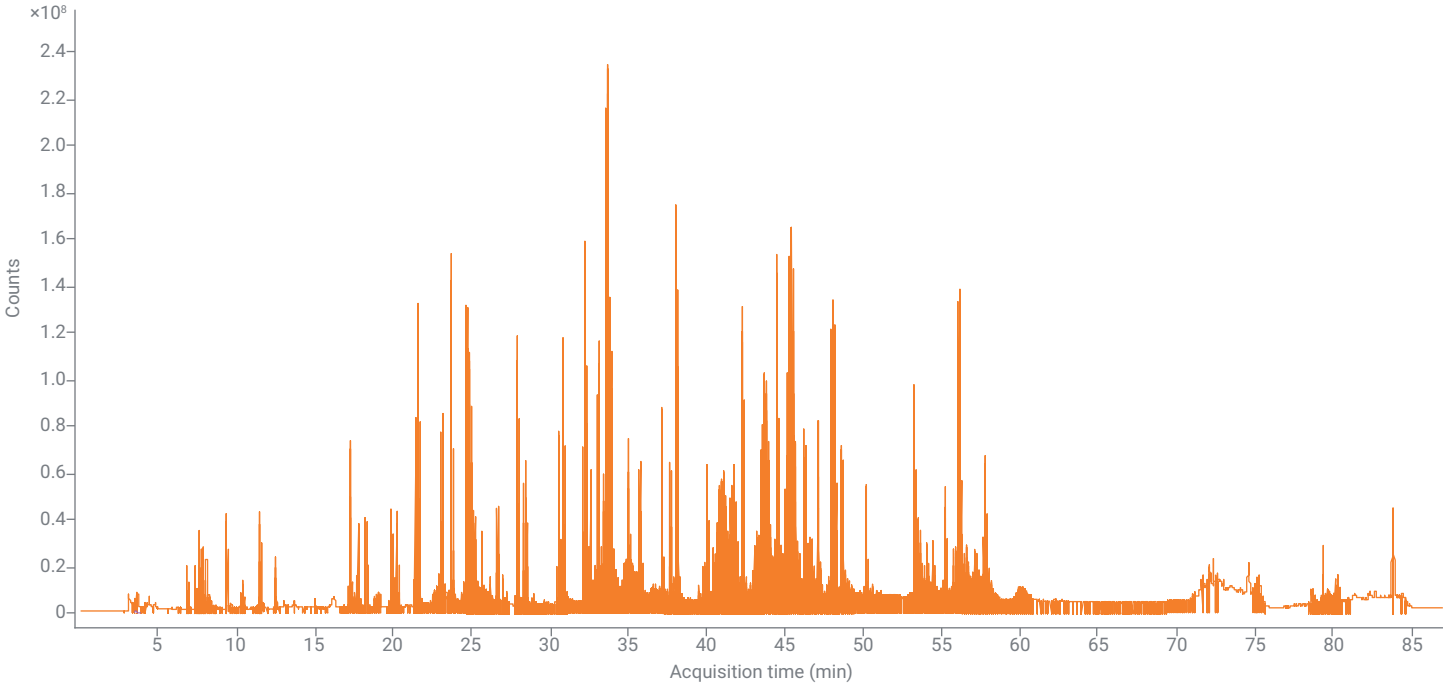


Figure 2. Total ion chromatogram for trastuzumab digested overnight with Trypsin/LysC at a protein-to-enzyme ratio of 25:1. LC/MS parameters are listed in Tables 1 and 2.

Sequence coverage for trastuzumab and trypsin autolysis

Sequence coverage is a commonly reported metric, used to confirm the correct sequence and identify relevant PTMs in a typical peptide mapping experiment. Sequence coverage for trastuzumab and trypsin are reported in Figure 3. The reported sequence coverage for the three injections was 98, 99.7, and 98%, respectively. The stretch of sequence that was not identified corresponds to the glycan-containing asparagine residue at N300. The extent of trypsin autolysis is also presented in Figure 3 and was found to be 54.3% (same value across replicates observed). No peptides arising from the LysC sequence were detected. While the primary goal of a peptide mapping experiment is to report high sequence coverage for the mAb of interest, trypsin autolysis is also an important metric to report. This is because peptides from trypsin autolysis will also be detected by the mass spectrometer and can impact the number of MS/MS spectra matching the mAb of interest due to suppression by intense tryptic peptides. It is therefore important to include the sequences of the digestion enzymes used (Trypsin and LysC) to monitor the extent of autolysis. Furthermore, the extent of autolysis for digestion enzymes can aid in the development of an appropriate sample preparation protocol.

A **Sequence Coverage Map:** Herceptin_Trastuzumab_HC_LC_alkylated (Protein Digest) (98.04%)

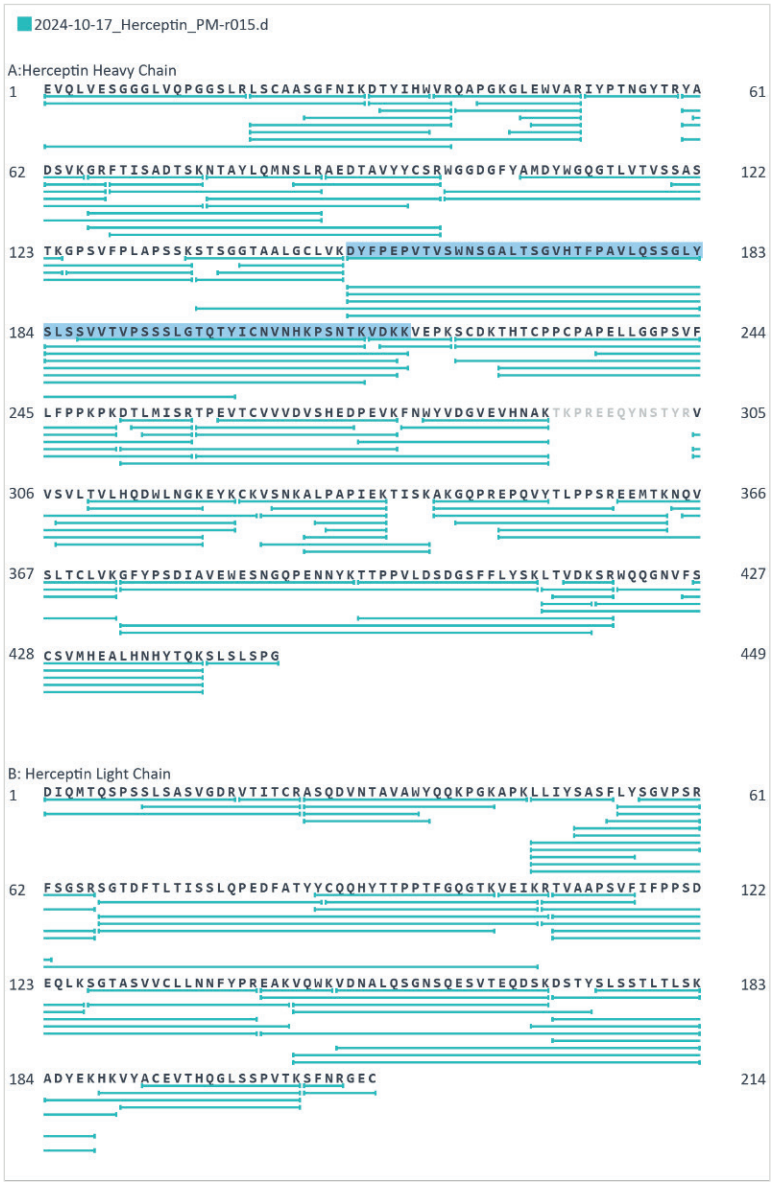


Figure 3A. Sequence coverage map for trastuzumab at a protein-to-enzyme ratio of 25:1 after overnight digestion.

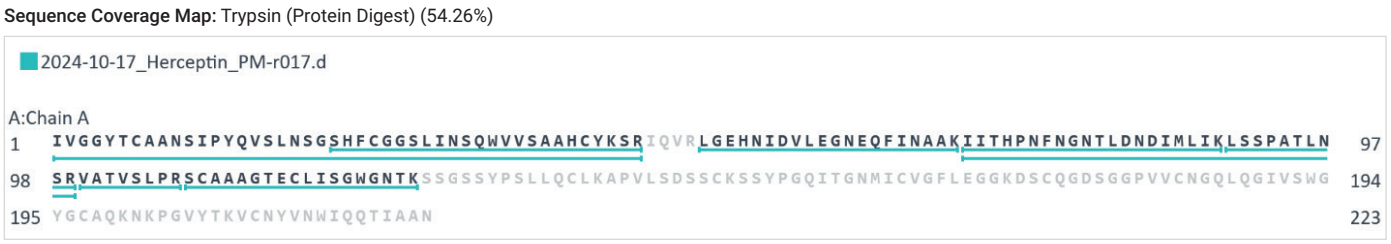


Figure 3B. Sequence coverage map for Trypsin at a protein-to-enzyme ratio of 25:1 after overnight digestion.

The effect of fragmentor voltage on peptide fragmentation

The fragmentor voltage controls the speed at which peptides transmit through the capillary of the 6545XT AdvanceBio LC/Q-TOF. While high fragmentor voltages can boost peptide signal, they can also cause peptide fragmentation in the source. The sequence coverage map in Figure 3 was generated with a fragmentor voltage of 95 V. While higher fragmentor voltages (170 and 125 V) did result in higher MS1 signals overall, they also resulted in the appearance of peptide fragments that are not reflective of the digestion protocol used in this study. Therefore, a fragmentor voltage of 95 V was used since this minimized peptide fragmentation while maintaining good signal intensity.

MS/MS spectra for low abundance peptides

Figure 4 shows representative CID spectra for the following peptides: (A) SASFLYSGVPSR and (B) SGGTAALGCLVK. The MS1 signals for these two peptides are 1.1e4 and 1.7e4, respectively, which is only marginally higher than the MS1 threshold that will trigger MS/MS measurement (6e3, Table 2). Excellent fragment ladders that illustrate near-complete sequence coverage were obtained for these peptides. This highlights that the extended dynamic range capabilities of the 6545XT AdvanceBio LC/Q-TOF system is sufficient to obtain excellent sequence coverage in the MS/MS spectrum.

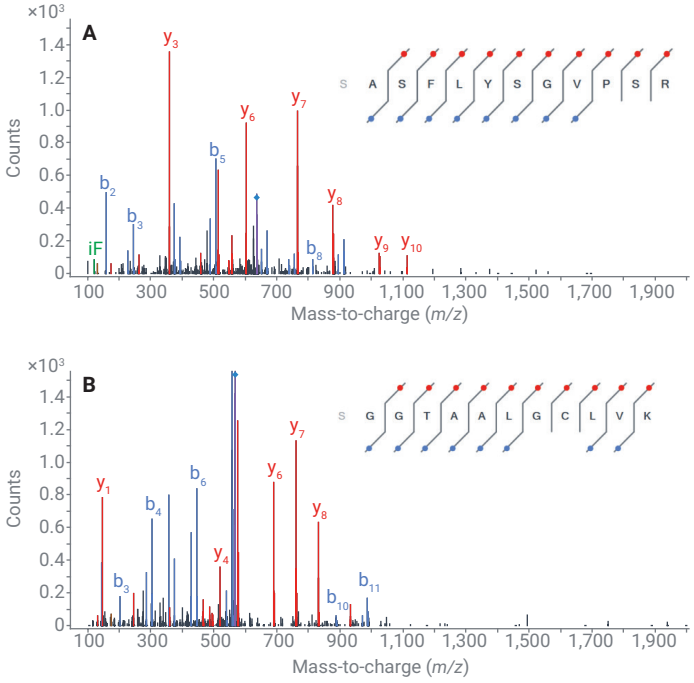


Figure 4. MS/MS spectra and fragment coverage map for the peptides (A) SASFLYSGVPSR and (B) SGGTAALGCLVK. These peptides were highlighted since they contribute signals in the ~ 1e4 range of the MS1 spectrum, yet have excellent fragment ladders.

MS/MS spectra for peptides with deamidation

Asn and Gln deamidations are common PTMs that are routinely observed in mAbs. Combining the small mass shift of 0.98 Da and relatively low signal intensity of the deamidated peptide, it is important to use representative b- and y-ions from the MS/MS spectrum to localize the site of deamidation. Figure 5 shows the CID spectra for the FTISADTSKNTAYLQMNSLR peptide in its (A) native and (B) deamidated form. Here, the MS/MS information can be used to assign the deamidation at position 17 using fragment information. For example, the y_4^{1+} ion in the unmodified form can be observed at *m/z* 489.2779, whereas the y_4^{1+} ion in the deamidated form is seen at *m/z* 490.2621, corresponding to a mass difference consistent with deamidation. Additional MS/MS information illustrates that there is a single site of deamidation for this peptide, despite multiple potential sites. For example, the y_6^{1+} ion is observed at *m/z* 748.3770 and 749.3690 in the unmodified and deamidated forms, respectively. If the glutamine (Q) at position 15 were also subject to deamidation, an additional mass shift would be observed. The fact that the mass shift is the same moving toward the N-terminus illustrates that there is a single site of deamidation for this peptide.

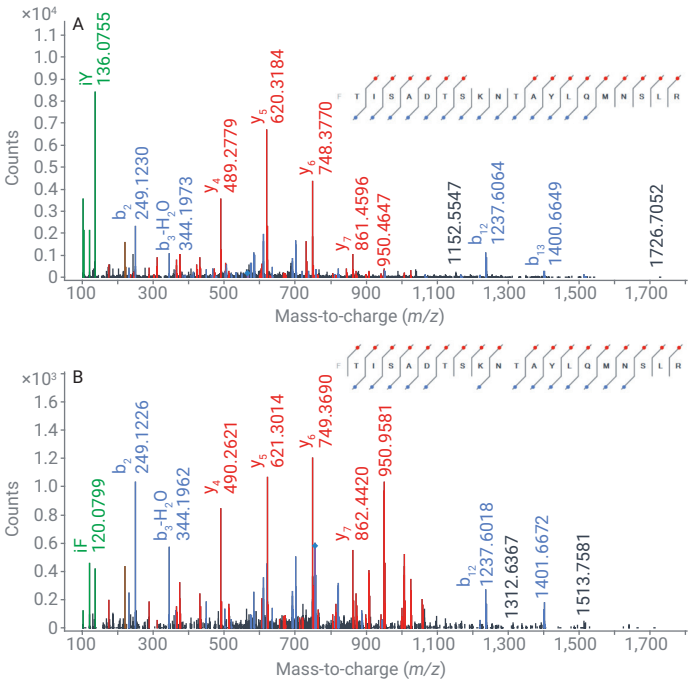


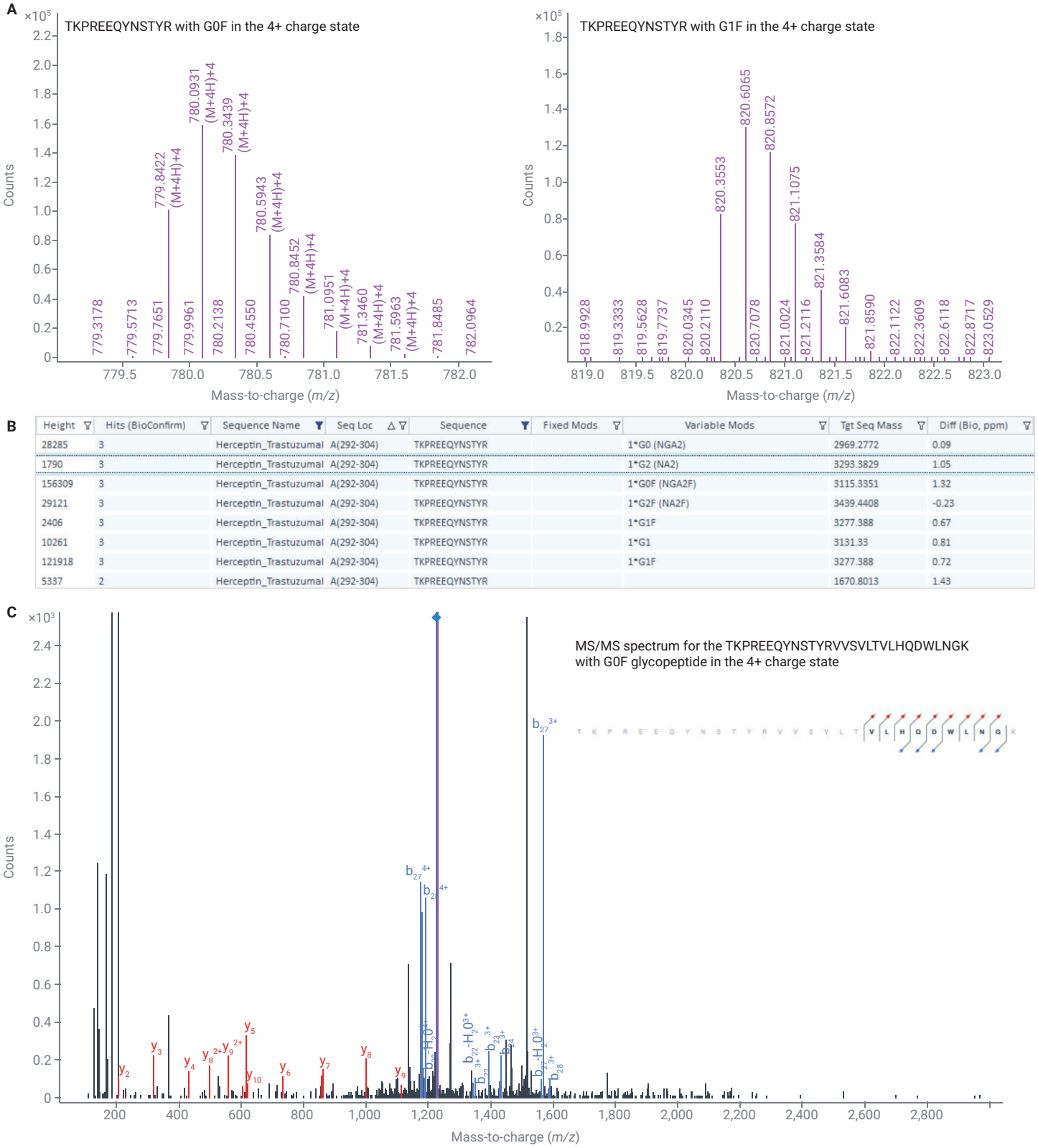
Figure 5. MS/MS spectra and fragment coverage map for peptide FTISADTSKNTAYLQMNSLR in its (A) native and (B) deamidated forms. Fragment ions around the asparagine site at position 17 can be used to unambiguously assign deamidation at that site.

Identification of glycosylated peptides

Glycosylation at asparagine consensus sites (NXS/T) represent important PTMs that should be identified in a peptide mapping experiment. One challenge in the identification of these PTMs is that asparagine residues can accommodate many unique glycans that are large and have poor ionization efficiency. Figure 6A shows the zoomed-in region for two glycopeptides, TKPREEQYNSTYR with G0F (left) and TKPREEQYNSTYR with G1F (right) in the 4+ charge state. The measured MS1 signal intensities for these glycopeptides are in the e5 range, indicating that this LC/MS method is sensitive enough for the detection of glycopeptides. Figure 6B shows the complete list of glycans that are identified for the TKPREEQYNSTYR peptide, illustrating the MS1 signal intensity and mass error.

MS/MS spectra for glycosylated peptides

Figure 6C shows the MS/MS spectrum for the TKPREEQYNSTYRVVSVLTVLHQDWLNGK with G0F glycopeptide in the 4+ charge state. While CID tends to fragment the glycosidic bond and generate glycan fragments at *m/z* 204.0865, Figure 6C illustrates that this glycopeptide can pass the BioConfirm 12.1 filtering criteria, and a peptide ladder can be generated. Notably, the most intense b_{27}^{3+} fragment ion at *m/z* 1,568.0915 provides mass confirmation for a large part of the glycopeptide sequence. Together with the MS1 identification criteria, this allows users to perform additional investigations for glycopeptides.



Height	Hits (BioConfirm)	Sequence Name	Seq Loc	Sequence	Fixed Mods	Variable Mods	Tgt Seq Mass	Diff (Bio, ppm)
28285	3	Herceptin_Trastuzumal	A(292-304)	TKPREEQYNSTYR		1*G0 (NGA2)	2969.2772	0.09
1790	3	Herceptin_Trastuzumal	A(292-304)	TKPREEQYNSTYR		1*G2 (NA2)	3293.3829	1.05
156309	3	Herceptin_Trastuzumal	A(292-304)	TKPREEQYNSTYR		1*G0F (NGA2F)	3115.3351	1.32
29121	3	Herceptin_Trastuzumal	A(292-304)	TKPREEQYNSTYR		1*G2F (NA2F)	3439.4408	-0.23
2406	3	Herceptin_Trastuzumal	A(292-304)	TKPREEQYNSTYR		1*G1F	3277.388	0.67
10261	3	Herceptin_Trastuzumal	A(292-304)	TKPREEQYNSTYR		1*G1	3131.33	0.81
121918	3	Herceptin_Trastuzumal	A(292-304)	TKPREEQYNSTYR		1*G1F	3277.388	0.72
5337	2	Herceptin_Trastuzumal	A(292-304)	TKPREEQYNSTYR			1670.8013	1.43

Figure 6. (A) Zoomed-in region of the MS1 spectrum illustrating the appearance of glycopeptides TKPREEQYNSTYR with G0F and G1F glycans in the 4+ charge state. (B) The complete list of glycopeptides identified in Agilent MassHunter BioConfirm software, version 12.1 for the TKPREEQYNSTYR glycopeptide. (C) The MS/MS spectrum for the TKPREEQYNSTYRVVSVLTVLHQDWLNGK with G0F glycan in the 4+ charge state.

Conclusion

Peptide mapping is a routinely used LC/MS/MS technique, employed in the biopharmaceutical industry to confirm mAb sequence and identify PTMs. This application note presents a workflow solution that generates a sequence coverage of 98% for trastuzumab when digested with Trypsin and LysC at a protein-to-enzyme ratio of 25:1. The peptide mapping coverage map was collected with a fragmentor voltage of 95 V, since higher fragmentor voltages (170 and 125 V) resulted in the appearance of peptide fragments that were not reflective of the digestion protocol outlined here. Furthermore, the extended dynamic range capabilities of the Agilent 6545XT AdvanceBio LC/Q-TOF are also highlighted, where two peptides that have MS1 signals in the ~ 1e4 range show near-complete fragment ladders. The native and deamidated forms of the FTISADTSKNTAYLQMNSLR peptide show unique fragment ions that unambiguously identify the site of deamidation. Finally, the TKPREEQYNSTYRVVSVLTVLHQDWLNGK with G0F glycopeptide was identified from the MS/MS spectrum, despite the dominant fragmentation pathway for the glycopeptide generating the glycan fragment at *m/z* 204.0865. The combination of quality MS/MS spectra and depth of coverage for low-abundant peptides highlight the capabilities of the 6545XT AdvanceBio LC/Q-TOF for peptide mapping applications.

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