

Automated Workflow for Peptide Mapping of Pembrolizumab on the Agilent 6545XT AdvanceBio LC/Q-TOF

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Abstract

This application note presents an integrated workflow for the peptide mapping of pembrolizumab monoclonal antibody (mAb), from automated sample preparation to data analysis. The workflow includes use of an Agilent AssayMAP Bravo Protein Sample Prep platform, an Agilent 1290 Infinity III bio LC system, an Agilent Altura Ultra Inert Peptide Plus column, an Agilent 6545XT AdvanceBio LC/Q-TOF, and Agilent MassHunter BioConfirm 12.1 software.

Introduction

Therapeutic monoclonal antibodies (mAbs) are structurally complex biopharmaceuticals that require detailed characterization to ensure safety, efficacy, and batch-to-batch consistency. Due to their heterogeneity, arising from post-translational modifications (PTMs) such as glycosylation, oxidation, and deamidation, comprehensive analytical strategies are essential.

By digesting the therapeutic protein into peptides and analyzing them using LC/MS/MS, this workflow delivers comprehensive sequence-level characterization and a high-resolution molecular fingerprint, enabling:

- Confirmation of primary amino acid sequence
- Identification and localization of PTMs
- Monitoring critical quality attributes (CQAs) throughout development and manufacturing

The Agilent AssayMAP Bravo Protein Sample Prep platform automates mAb sample preparation workflows, including digestion and peptide cleanup, to improve reproducibility, reduce hands-on time, and increase throughput. Automated processing ensures consistent peptide mapping results and supports reliable characterization of CQAs in biotherapeutics.

Experimental

Chemicals and reagents

The following chemicals were used for the digestion reaction:

- Urea (U5128-500G, Sigma)
- Ammonium bicarbonate (5330050050, Merck)
- Dithiothreitol (DTT) (D9779-10G, Merck)
- Trypsin (11418475001, Merck)
- Trifluoroacetic acid (28904, ThermoFischer)
- Formic acid (Cat No. 5.33002, Merck)
- Acetonitrile (MS grade, Biosolve, Dieuze, France)
- 1 N HCl (1.09057.1000, Supelco)
- LC/MS-grade water (Milli-Q-IQ 7003 purification system (Merck KGaA, Darmstadt, Germany))

Instruments

- Agilent 1290 Infinity III bio high-speed pump (G7132A)
- Agilent 1290 Infinity III bio multisampler (G7137A) with Agilent InfinityLab sample thermostat (G7167A-60201)
- Agilent 1290 Infinity III multicolumn thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF with Agilent Dual Jet Stream ESI source
- Agilent AssayMAP Bravo system (G5542A) – for reduced and nonreduced analysis

Software

- Agilent MassHunter data acquisition software for LC/TOF and LC/Q-TOF version 11.1
- Agilent MassHunter BioConfirm software version 12.1
- Agilent VWorks version 13.1

Samples

Reduced and nonreduced peptide mapping sample

preparation: Pembrolizumab was provided by Daicel Chiral Technologies India Pvt. Ltd. at a concentration of 25 mg/mL. For the mAb peptide mapping experiments, samples were diluted to 6 mg/mL in solution 1 (20 mM ammonium bicarbonate buffer). To 5 μ L of the 6 mg/mL mAb solution, 10 μ L of solution 2 was added to denature and reduce the sample. For the nonreduced sample, 10 μ L of solution 2 without DTT was added. The solutions were incubated at 70 °C in a dry bath for 5 minutes for heat denaturation.¹ After incubation, 100 μ L of solution 4 (trypsin, 15 ng/ μ L) was added, and the solutions were further incubated at 37 °C in a dry bath for 45 minutes for enzymatic digestion. All of these steps were automated by the Agilent AssayMAP Bravo liquid handling system.^{2,3} The digested samples were then subjected to LC/MS/MS analysis with a column loading amount of 2.5 μ g.

LC/MS analysis

LC/MS analysis was performed on an Agilent 1290 Infinity III bio LC system coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF system (Figure 1). A TOF transmission tune was performed to prepare the 6545XT AdvanceBio LC/Q-TOF system for acquisition. An Agilent Altura Peptide Plus column (2.1 $\times$ 150 mm, 2.7 μ m) was used for chromatographic separation. LC and MS parameters used are listed in Tables 1 and 2.

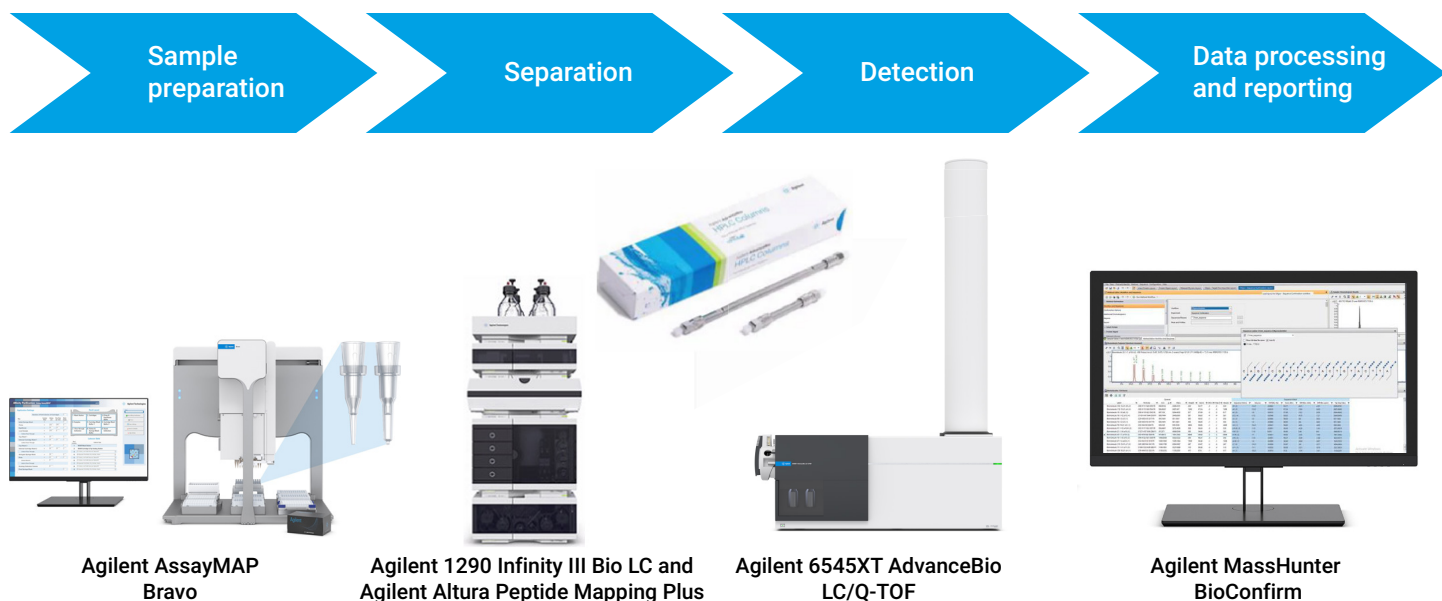


Figure 1. End-to-end Agilent workflow for automated peptide mapping.

Table 1. Liquid chromatography parameters.

Parameter	Value																
Column	Agilent Altura Peptide Plus column, 2.1 × 150 mm, 2.7 μm (p/n 227215-903)																
Thermostat	5 °C																
Solvent A	0.1% FA in Milli-Q water																
Solvent B	0.1% FA in acetonitrile																
Flow Rate	0.4 mL/min																
Injection Volume	10 μL																
Column Temperature	50 °C																
Wavelength	215 nm																
Gradient Program	<table> <tr> <th>Time (min)</th><th>%B</th></tr> <tr><td>0</td><td>2</td></tr> <tr><td>5</td><td>2</td></tr> <tr><td>80</td><td>40</td></tr> <tr><td>82</td><td>80</td></tr> <tr><td>85</td><td>80</td></tr> <tr><td>86</td><td>2</td></tr> <tr><td>100</td><td>2</td></tr> </table>	Time (min)	%B	0	2	5	2	80	40	82	80	85	80	86	2	100	2
Time (min)	%B																
0	2																
5	2																
80	40																
82	80																
85	80																
86	2																
100	2																

Table 2. MS parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF																	
Ion Source	Agilent Dual JetStream ESI source																
Gas Temperature	350 °C																
Drying Gas Flow	11 L/min																
Nebulizer	35 psi																
Sheath Gas Temperature	275 °C																
Sheath Gas Flow	12 L/min																
Capillary Voltage	4,500 V																
Nozzle Voltage	2,000 V																
Fragmentor	175 V																
Skimmer	45 V																
Oct 1 RF Vpp	750 V																
Mass Range	MS1: 200–3,000 m/z, MS2: 50–3,000 m/z																
Scan Rate	MS1: 1 spectra/s, MS2: 0.5 spectra/s																
Isolation Width	Medium (~4 m/z)																
Collision Energy	Use formula: (slope) × (m/z)/100 + offset <table> <tr> <th>Charge</th><th>Slope</th><th>Offset</th></tr> <tr><td>2</td><td>3.1</td><td>1</td></tr> <tr><td>>3</td><td>3.6</td><td>-4.8</td></tr> <tr><td>3</td><td>3.6</td><td>-4.8</td></tr> <tr><td>1</td><td>3.1</td><td>1</td></tr> </table>		Charge	Slope	Offset	2	3.1	1	>3	3.6	-4.8	3	3.6	-4.8	1	3.1	1
Charge	Slope	Offset															
2	3.1	1															
>3	3.6	-4.8															
3	3.6	-4.8															
1	3.1	1															
Precursor Selection I	1 maximum precursor per cycle Precursor threshold: 1,000 counts Relative threshold: 0.1% Active exclusion: unticked																
Precursor Selection II	Isotope model: peptides Precursor charge state selection and preference: 2>3>[>3]>1>Unk Sort precursor by charge state then abundance Purity stringency: 100%, purity cutoff: 30%																
Acquisition Mode	Standard (3,200 m/z) mass range Extended dynamic range (2 GHz)																

Data processing

Raw LC/MS/MS data were processed using Agilent MassHunter BioConfirm software version 12.1. Pembrolizumab⁴ trypsin-digested peptide sequences were entered into the Sequence Manager tool in MassHunter BioConfirm software. For the pembrolizumab disulfide mapping experiment, cysteines involved in disulfide bonds were linked directly in the sequence to incorporate disulfide-linked peptides. The peptide mapping search included several filters to restrict the appearance of low-quality MS/MS mass spectra. For example, mass matching thresholds of 10 ppm for MS1 and 20 ppm for MS2 were used. The processing parameters used for the peptide mapping searches of both reduced and nonreduced samples are listed in Table 3.

Table 3. Agilent MassHunter BioConfirm software data processing parameters.

Agilent MassHunter BioConfirm Workflow Settings		
Workflow	Protein Digest	Protein Digest
Condition	Reduced	Nonreduced
Sequence	Pembrolizumab, Trypsin-digested	Pembrolizumab, Cystine-linked, Trypsin-digested
Variable Modification	Deamidation, G0, G0F, G1, G1F, G1FGlcNAc, G2, G2F, oxidation (M, W), pyroGlu (E)	Deamidation, G0, G0F, G1, G1F, G1FGlcNAc, G2, G2F, oxidation (M, W), pyroGlu (E)
Enzymes	Trypsin	Trypsin
Find Peptides		
Biomolecule Filters	Display biomolecules containing MS/MS scans	Display biomolecules containing MS-only and MS/MS scans
Extraction, Use Peaks with Height	≥ 600 counts	≥ 600 counts
Isotope Model	Peptides	Peptides
Peak Filters (MS/MS)	≥ 50 counts	≥ 50 counts
Match Tolerances		
Mass Matching	± 10 ppm MS1, ± 20 ppm MS2	± 10 ppm MS1, ± 20 ppm MS2
MS/MS Fragmentation Type	CID	CID
Number of Missed Cleavages	2	2
Max Number of Matches per Biomolecule	3	3
Peptide Length Range	3–70	3–70
Allow Only Either N-Term or C-Term Truncation	Selected	Selected
Maximum Number of Modifications	2	2

Results and discussion

Reduced peptide mapping – sequence confirmation

Total ion chromatogram for pembrolizumab digest

The total ion chromatogram (TIC) for trypsin-digested pembrolizumab is shown in Figure 2. As indicated in the sample preparation protocol, no manual desalting was performed. The first three minutes of the LC/MS method diverted the LC flow to waste to reduce potential ion suppression from the denaturing buffers used during sample preparation. From three to 100 minutes, the diverter valve was switched to direct flow to the MS, where peptide signals were recorded. The most intense peptide signals were observed between approximately 30 and 75 minutes, consistent with longer peptide lengths that allow sufficient overlap for high sequence coverage.

Sequence coverage for pembrolizumab

Sequence coverage is a commonly reported metric, used to confirm the correct sequence and identify relevant PTMs in a typical peptide mapping experiment. The list of peptides extracted from raw data using the "find peptide" algorithm is matched against the user-specified mAb sequence, and a sequence coverage map is generated. Sequence coverage for pembrolizumab is reported in Figure 3. The 100% amino acid sequence coverage was reported.

All identified peptides are labeled with their corresponding sequence numbers in pembrolizumab mAb light chain (represented as chain A) and heavy chain (represented as chain B).

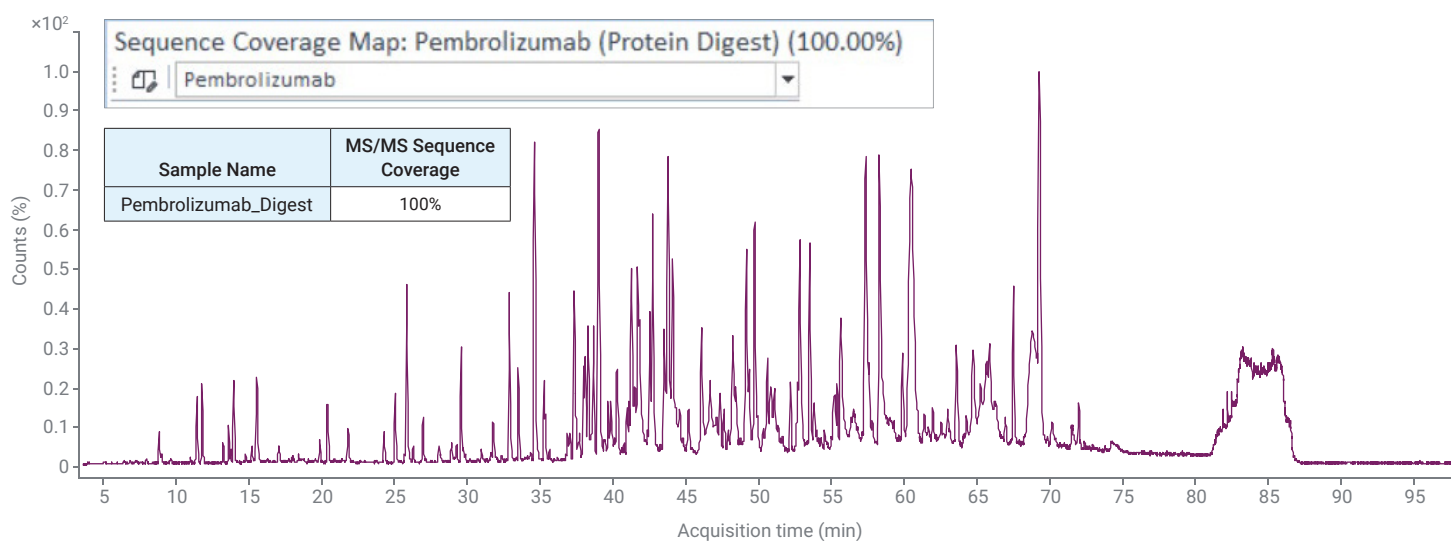


Figure 2. TIC for pembrolizumab digested with trypsin. LC/MS parameters are listed in Tables 1 and 2.

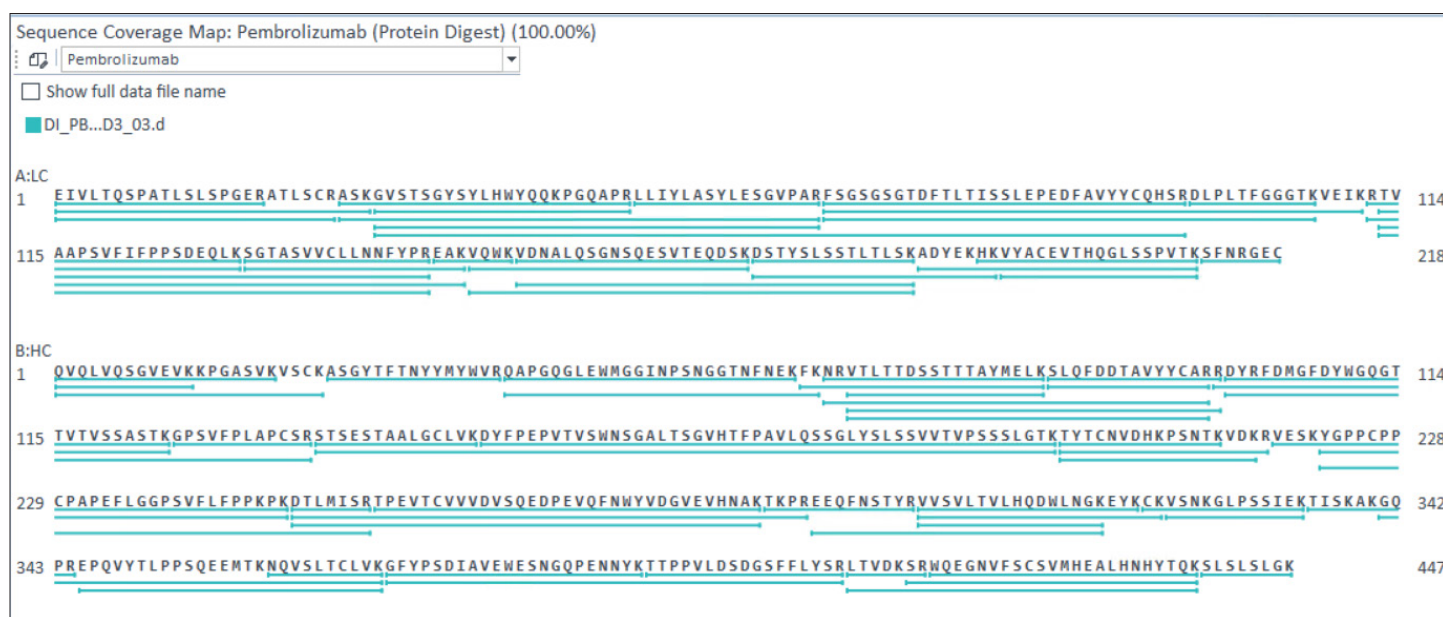


Figure 3. Sequence coverage map for pembrolizumab with trypsin digestion.

N- and C-terminal sequence confirmation

In accordance with ICH Q6B, comprehensive characterization of a biotechnological product must include determination of the complete amino acid sequence, including evaluation of terminal residues and any heterogeneity.⁵

MS/MS fragment spectra confirmed the terminal peptides from both the heavy and light chains, ensuring unambiguous assignment of both the N and C-termini, as represented in Figure 4. The terminal peptides were well resolved on the chromatographic separation, as illustrated in Figure 5.

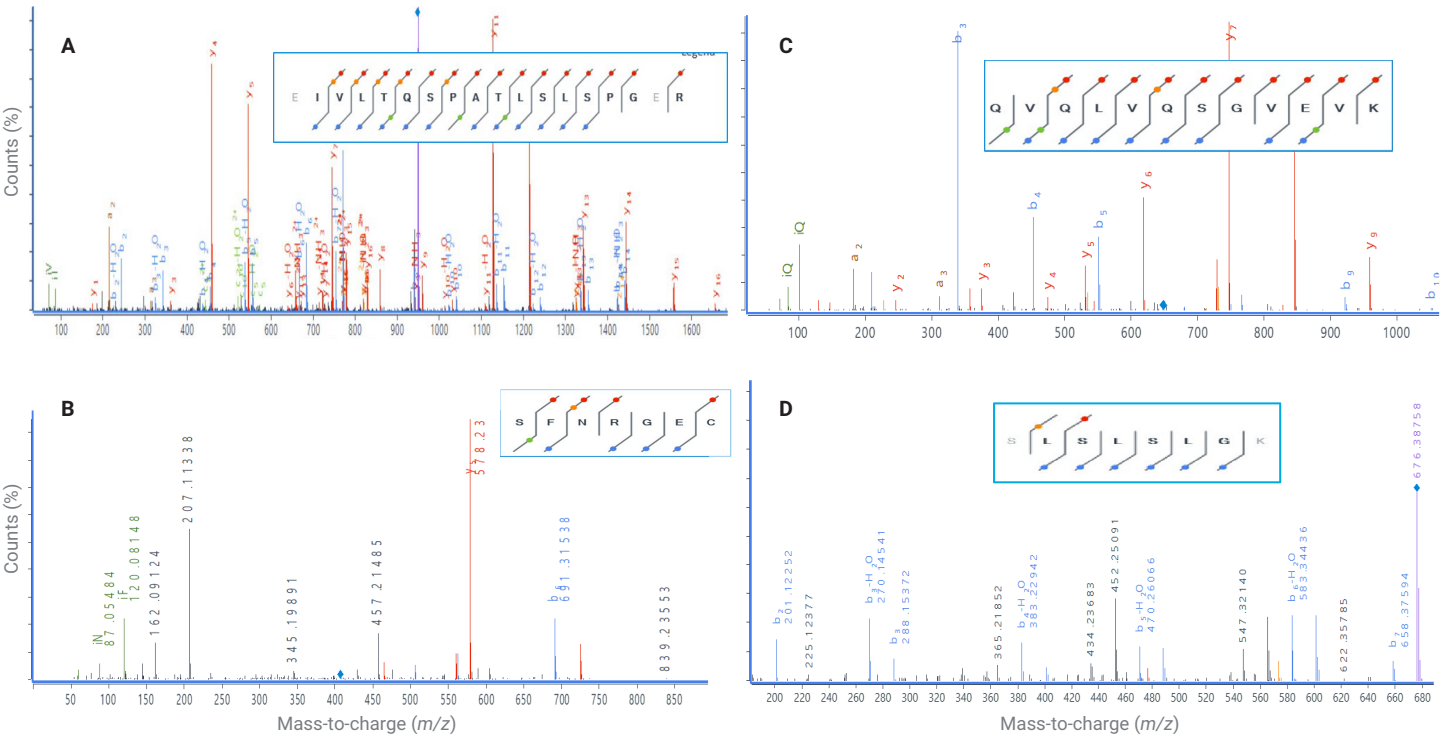


Figure 4. MS/MS sequence confirmation for the terminal amino acids. (A) Light chain N-terminal sequence and (B) C-terminal sequence; (C) Heavy chain N-terminal sequence and (D) C-terminal sequence.

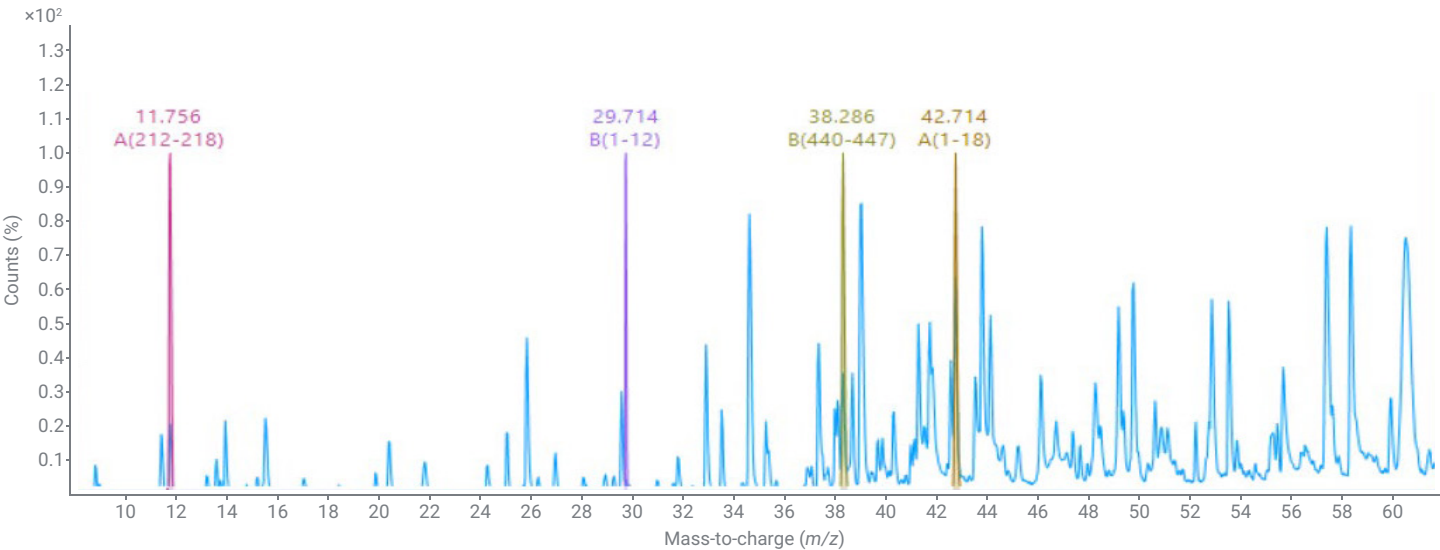


Figure 5. Overlaid extracted biomolecule chromatograms of the terminal peptides on the TIC (blue trace). Light- and heavy-chain peptides are labeled A and B, respectively.

S228P intended point mutation identification and characterization

A key modification in pembrolizumab is the serine-to-proline mutation (S228P) in the Fc hinge region, which prevents Fab-arm exchange, a phenomenon common in IgG4 antibodies that can lead to loss of bivalent binding and reduced efficacy.^{6,7} Identification and characterization of this engineered mutation are required, and LC/MS/MS provides sequence-level confirmation of the amino acids within the peptide containing this modification, as shown in Figure 6. Details for the peptide sequence identification in both oxidized and native form are summarized in Table 4.

Post-translational modification analysis

Among critical PTMs, oxidation is of particular interest because it commonly affects methionine and tryptophan residues and can arise during manufacturing, storage, or sample processing.

For oxidation analysis, MS/MS fragmentation is particularly critical, as it allows site-specific assignment of oxidation (for example, methionine (Met) or tryptophan residues) by pinpointing the exact residue exhibiting the characteristic mass shift (+16 Da). This capability helps ensure differentiation between isobaric or closely eluting modified peptides.

The DTLM₂₅₂ ISR peptide matched to this region (heavy chain sequence location 249–255) contains a single Met residue. This peptide was used to demonstrate the simultaneous identification and quantification of Met oxidation using the Agilent peptide mapping workflow.

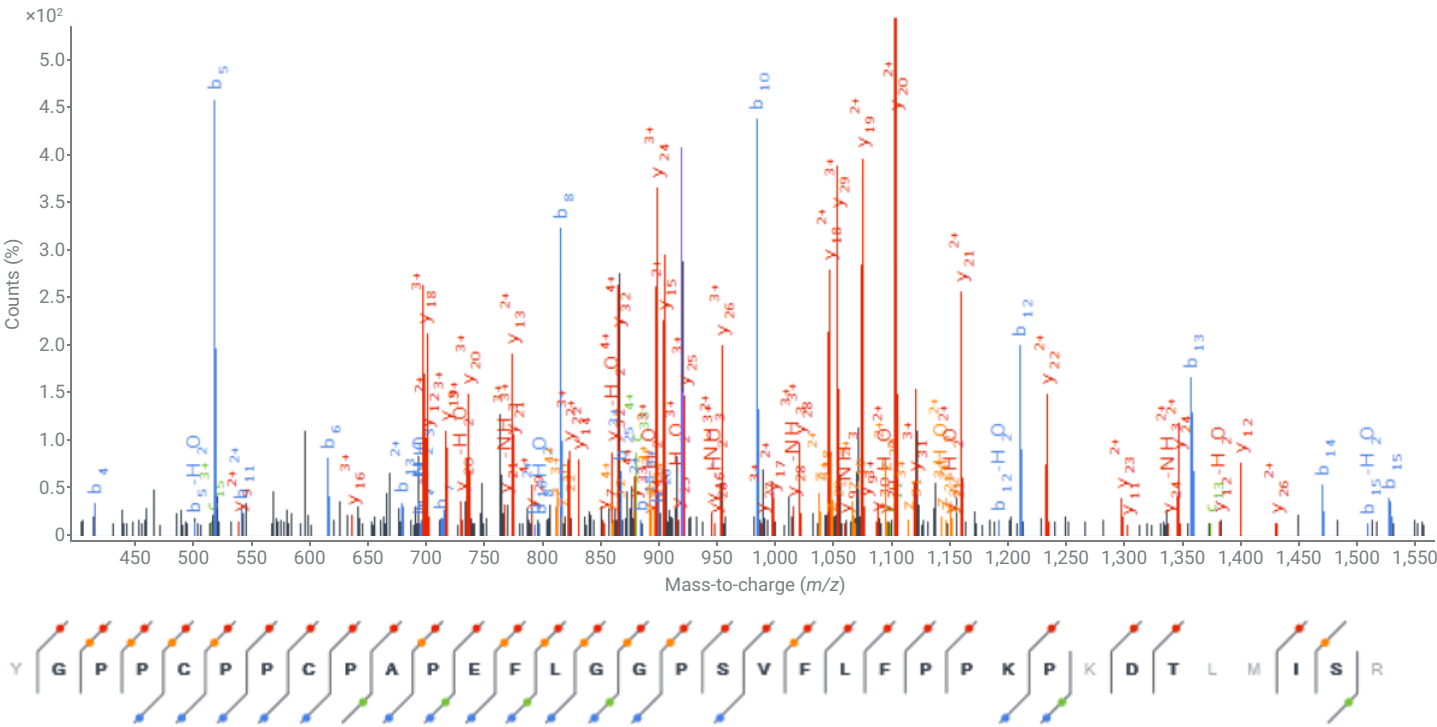


Figure 7A shows the extracted compound chromatograms (ECCs) for the oxidized and native (nonoxidized) forms of the DTLMISR peptide, well-separated and overlaid on the TIC.

Figure 7B shows the CID spectra for the DTLMISR peptide in its native and oxidized form, compared using a mirror plot. Here, the MS/MS information were used to assign oxidation at position 4 using fragment information. For example, the y_4 1+ ion in the unmodified form can be observed at m/z 506.2747, whereas the y_4 1+ ion in the oxidized form is seen at m/z 522.2766, corresponding to a mass

difference consistent with oxidation. Comparison of the y_5 ions from the MS/MS spectrum of the modified peptide to those from the unmodified peptide clearly showed an increase of approximately 16 Da, confirming oxidation at the Met 4 position.

Figure 7C shows the quantitative histograms of Met oxidation at the M_{252} and M_4 residues of pembrolizumab mAb. The low-abundance oxidized form of pembrolizumab was also identified and confirmed by MS/MS fragmentation.

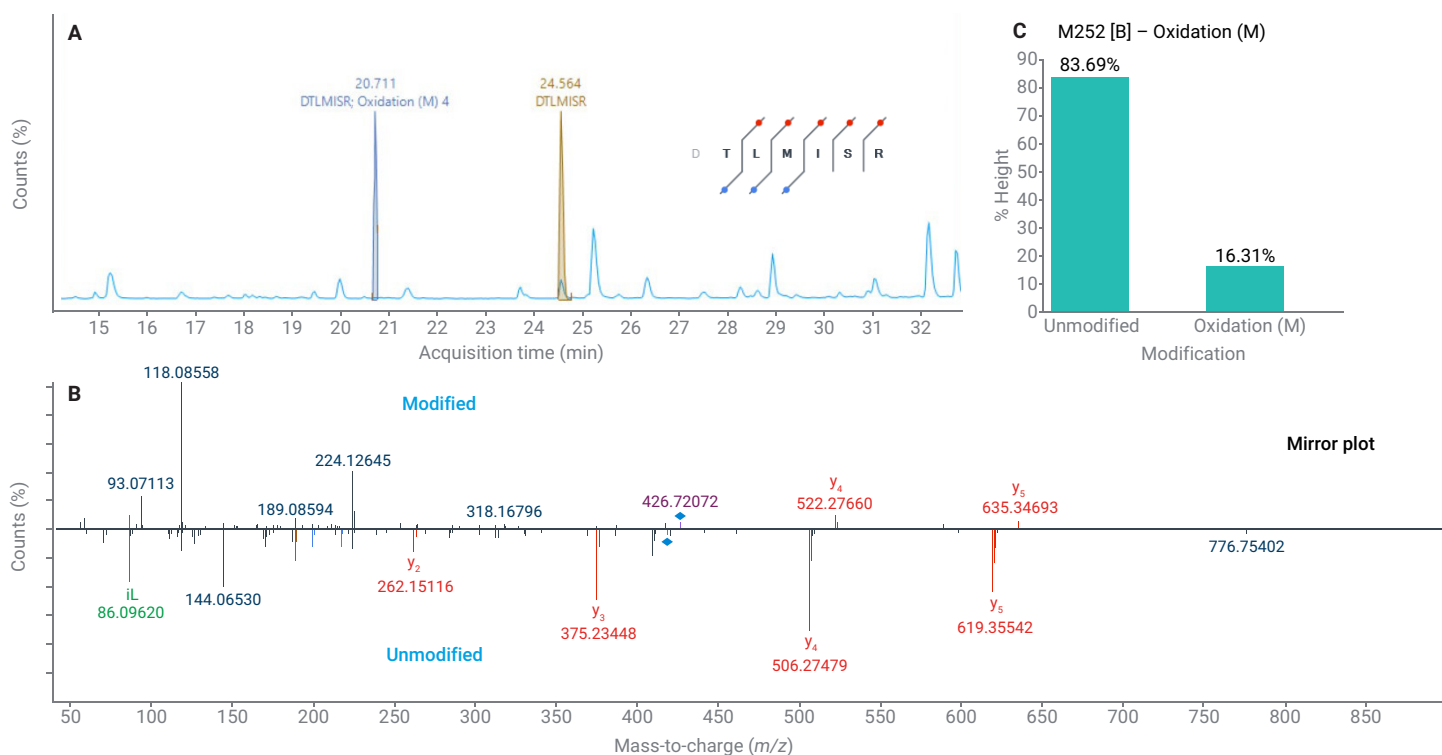


Figure 7. (A) Extracted compound chromatograms of the oxidized and native DTLM₂₅₂ISR peptide, overlaid on the TIC. Well-separated peaks were observed for both the oxidized and native forms. (B) Mirror plot of the MS/MS spectra of the oxidized and native forms of the DTLM₅₂₂ISR peptide. (C) The relative abundance histogram represents the %Quant of the specific modification, here, it is oxidation of the methionine residue at HC₂₅₂.

Disulfide bond mapping of pembrolizumab mAb

The pembrolizumab mAb contains a total of 16 disulfide bonds, 12 of which are intrachain linkages (four in the light chain and eight in the heavy chain). In addition, there are four interchain disulfide bonds: two linking each light chain to a heavy chain and two in the hinge regions linking the two heavy chains.

Reversed-phase LC/MS/MS data were acquired for nonreduced and reduced samples under the LC/MS conditions described in Table 2. In the reduced sample, the disulfide bonds of the mAb were cleaved, whereas the intact disulfide-linked peptides (larger mass) remained intact in the nonreduced sample. The mirror plot in Figure 8 compares the TICs of the two samples and highlights their major differences. Distinct changes in the chromatographic profiles were observed at retention times between 15 and 18 minutes, 26 and 33 minutes, and 57 and 65 minutes.

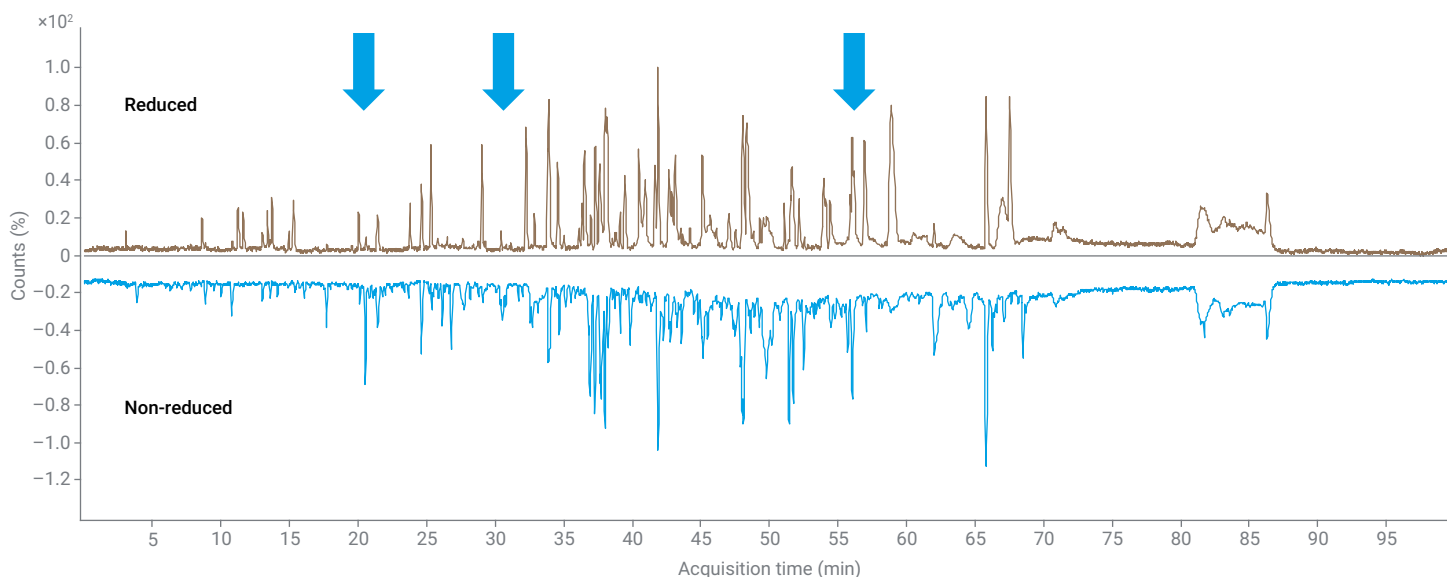


Figure 8. Mirror plot of the reduced and nonreduced TICs for pembrolizumab mAb.

Based on the protein sequence, the BioConfirm software nonreduced protein digest algorithm generates all theoretically possible peptides based on experimental conditions (proteolytic enzymes, alkylating reagents). The algorithm then rapidly searches for these features in peptide

MS data. All 16 pairs of disulfide bonds in the pembrolizumab sample were correctly detected and identified using BioConfirm. The results are summarized in Table 5, and the extracted ion chromatograms (EICs) for all 16 pairs were overlaid on the TIC of the nonreduced sample in Figure 9.

Table 5. Summary of disulfide bonds identified in the pembrolizumab mAb.

Retention Time	Mass	Mass Difference (ppm)	Disulfide Bond Location	Disulfide Links	Sequence	Missed Cleavages
63.8	5,672.8356	2.01	HC–HC	B226–C226/C229–B229	YGPPC(226)PPC(229)PAPEFLGGPSVFLFPPKPK+ YGPPC(226)PPC(229)PAPEFLGGPSVFLFPPKPK	
33.1	2,083.9367	1.13	HC	B22–B96/C22–C96	VSC(22)K+SLQFDDTAVYYC(96)AR	
49.7	3,977.8489	0.93	HC	B261–B321/C261–C321	TPEVTC(261)VVVDVSQEDPEVQFNWYVDGVEVHNAK+C(321)K	
25.8	2,970.4103	1.34	HC	B147–B203/C147–C203	STSESTAALGC(147)LVK+TYTC(203)NVDHKPSNTK	
46.5	3,555.7535	1.25	LC	A138–A198/D138–D198	SGTASVVC(138)LLNNFYPR+VYAC(198)EVTHQGLSSPVTK	
34.99	2,038.9381	1.36	LC–HC	A218–B134/D218–C134	SFNRGEC(218)+GPSVFPLAPC(134)SR	1 + 0
40.7	3,845.811	0.87	HC	B367–B425/C367–C425	NQVSLT(367)LVK+WQEGNVFSC(425)SVMHEALHNYHTQK	
52.6	4,047.8323	1.68	LC	A23–A92/D23–D92	ATLSC(23)R+FSGSGSGTDFTLTISSELEPEDFAVYYC(92)QHSR	

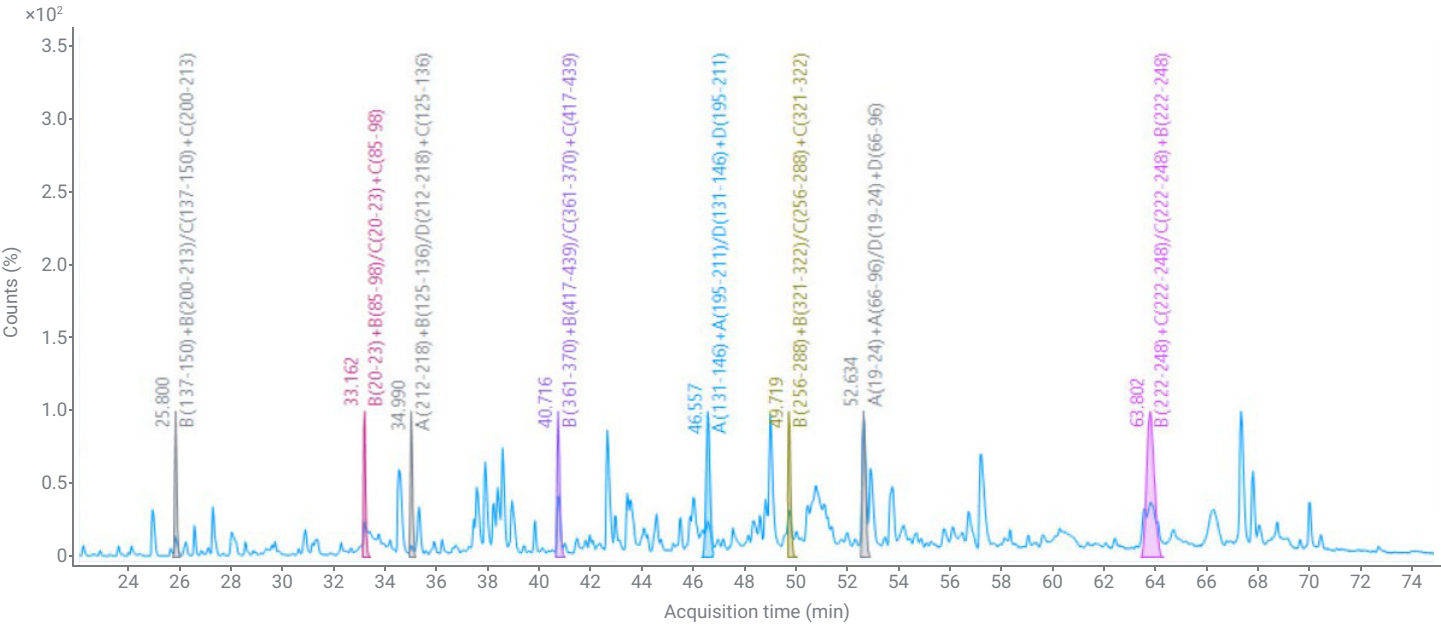


Figure 9. EICs for the 16 disulfide-linked peptide pairs overlaid on the TIC (blue trace), representing chromatographic separation of the disulfide-linked peptides from the LC method.

Conclusion

A comprehensive workflow has been demonstrated, encompassing automated sample preparation on an Agilent AssayMAP Bravo platform, LC separation using an Agilent 1290 Infinity III bio LC system, data acquisition with an Agilent 6545XT AdvanceBio LC/QTOF, and data analysis through Agilent MassHunter BioConfirm software. This approach enables amino acid sequence identification and confirmation, verification of the intended S₂₂₈P mutation, terminal sequence analysis, as well as characterization of PTMs including the low-abundance oxidation form and disulfide linkages in pembrolizumab mAb.

The high-quality MS/MS spectra, combined with extensive coverage of low-abundance peptides, underscore the strong performance of the Agilent 6545XT AdvanceBio LC/QTOF for peptide mapping applications.

References

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