An Integrated Workflow for Peptide Mapping of Monoclonal Antibodies

Application Note

Biotherapeutics and Biosimilars

Authors

David L. Wong

Agilent Technologies, Inc.

Santa Clara, CA, USA

Jing Chen

Agilent Technologies, Inc.

Madison, WI, USA

Introduction

Monoclonal antibodies (mAbs) represent one of the fastest growing classes of drugs in the pharmaceutical industry. As a protein drug, the manufacturing of an mAb from initial expression to commercialization is a highly complex process, requiring careful characterization of the mAb in each step. Peptide mapping, among the many analytical methods used for mAb characterization, is an essential technique that provides primary sequence confirmation of the mAb as well as the identification and quantitation of post-translational modifications (PTMs) such as deamidation, oxidation, and glycosylation[1].

Peptide mapping of an mAb using an LC/MS/MS approach involves the enzymatic cleavage of a purified mAb into peptides, tandem mass spectrometry analysis, and data interpretation. Sample preparation typically involves multiple steps including denaturation, reduction, alkylation, and enzymatic digestion. Manual sample preparation for peptide mapping can be labor-intensive, and it is susceptible to limited scalability and reproducibility.

This application note presents a high-throughput workflow that enables simultaneous, highly reproducible sample digestion and sample cleanup. This was accomplished using an Agilent AssayMAP Bravo liquid-handling robot, sensitive and accurate spectrum acquisition with an Agilent 1290 Infinity II LC system coupled to an Agilent 6550 Q-TOF, and automated data analysis using Agilent MassHunter BioConfirm software.

Agilent Technologies

Experimental

Materials

Monoclonal antibody standard RM 8671, was purchased from National Institute of Standard & Technology (NIST). Mass spec grade Trypsin/Lys-C enzyme mix was obtained from Promega. All other chemicals were purchased from Sigma-Aldrich. AssayMAP C18 cartridges were from Agilent Technologies.

Sample preparation

NIST mAb standard RM 8671, was denatured, reduced, alkylated, digested, and desalted using the AssayMAP Bravo. After diluting samples to 10, 5, 1, and 0.5 µg/µL in DI water (8 replicates for each concentration), 10 µL of each sample was transferred to a 96-well, opaque, U-bottom plate. Sample preparation was orchestrated by Protein Sample Prep Workbench software. The In-Solution Digestion module was used to perform sample digestion: 30 µL of denaturing and reducing reagent (8 M guanidine, 13.3 mM TCEP, pH 7.5) was added to each sample. The plate was sealed and incubated off deck at 37 °C for 1 hour.

After unsealing the plate, the In-Solution digestion application was used to transfer 10 µL of alkylating agent (132 mM iodoacetamide) from a lidded opaque 96-well U-bottom plate to each sample. The samples were mixed. The sample plate was lidded, then incubated in the dark at room temperature for 30 minutes.

Then, 210 µL of diluent (50 mM Tris, pH 7.5) was added to the samples, followed by 5 µL of Typsin/LysC Combo of various concentrations (1:40 enzyme to protein) diluted in 50 mM acetic acid.

The samples were sealed and incubated at 37 °C for 2 hours off deck. An additional 5 µL of Typsin/LysC Combo was added to the samples, and the samples were incubated off deck at 37 °C for an additional 14 hours. The next day, 30 µL of 10 % TFA was transferred to the sample plate to acidify the samples using the AssayMAP reagent transfer utility. The Peptide Cleanup application was used to desalt the samples using C18 cartridges. The cartridges were primed with 100 µL of 60 % ACN, 0.1 % TFA, equilibrated with 50 µL of 0.1 % TFA, loaded with digested samples at a flow rate of 5 µL/min, washed with 50 µL of 0.1 % TFA, and eluted with 15 µL of 60 % ACN, 0.1 % TFA at a flow rate of 3 µL/min into 165 µL of 0.1 % FA to reduce the ACN concentration to 5 %. Then, 0.5 µg of each sample were analyzed by LC/MS.

LC/MS Analysis

Instrumentation

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

MS system

Agilent 6550 iFunnel Q-TOF LC/MS System with Dual Agilent JetStream

LC/MS/MS and LC/MS analyses were conducted on an Agilent 1290 Infinity II LC system coupled to an Agilent 6550 iFunnel Q-TOF LC/MS system equipped with a Dual JetStream ESI source. LC separation was obtained with an Agilent AdvanceBio Peptide Mapping column (2.1 × 150 mm, 2.7 µm). Tables 1 and 2 summarize the LC/MS/MS parameters used. For LC/MS analyses, the same LC gradient and source parameters were used. MS data were acquired at five spectra per second.

Data analysis

Raw data acquired from LC/MS/MS were processed using Agilent MassHunter BioConfirm B.08 software. In brief, raw data were first processed using the Molecular Feature Extractor (MFE). The Find Peptide parameters were set to default, except that the *m/z* range was set to *not restricted*, and for peak filters, *more than 500 counts* was set for peak heights. The results were searched against the NIST mAb sequence with fixed modifications for alkylation (C) and variable modifications for oxidation (M), deamidation (NQ), pyroglutamate (Q), and various common forms of glycosylation (N). The MS matching tolerance was set to ±10 ppm, and the MS/MS matching tolerance was set to ±20 ppm. Two enzymatic missed cleavages were allowed. All identified peptides contained at least 1 MS/MS spectrum. Peptides with low BioScores were manually inspected for validity.

Table 1. Liquid chromatography parameters.

Parameter	Value
Column	Agilent AdvanceBio Peptide Mapping, 2.1 × 150 mm, 2.7 µm
Thermostat	4 °C
Solvent A	0.1 % Formic acid in water
Solvent B	0.1% Formic acid in acetonitrile
Gradient	0–15 minutes, 5–40 %B 15–18 minutes, 40–90 %B 18–20 minutes, 90 %B
Column temperature	60 °C
Flow rate	0.4 mL/min

Table 2. Mass spectrometer parameters.

Parameter	Value
Gas temperature	290 °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	2,000 V
Fragmentor	175 V
Quad AMU	100
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
Minimum MS/MS acquisition rate	3 spectra/sec
Isolation width	Medium (~ 4 *m/z*)
Precursors/cycle	Top 10
Collision energy	3.6*(*m/z*)/100 – 4.8
Threshold for MS/MS	1,000 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

Integrated workflow using Agilent AssayMAP Bravo, Agilent 6550 Q-TOF LC/MS/MS, and Agilent BioConfirm B.08

Although peptide mapping is routinely performed for biopharmaceutical mAb characterization, the sample preparation is complex and essential to the quality of results. Analytical variance may result from subtle differences among techniques, technicians, or different partner labs. This variation can be especially challenging when it is necessary to compare different batches across months or years. In addition, manual sample preparation can be difficult to scale up for high-throughput applications such as clone selection.

The Agilent AssayMAP Bravo automates the physical work of sample preparation for peptide mapping, it accelerates the process, and improves the reproducibility. The AssayMAP software provides easy-to-use applications specifically designed to address various types of protein sample prep workflows, including In-Solution Digestion and Peptide Cleanup. The software directs the robot to execute essential steps such as sample denaturation, reduction, alkylation, enzymatic digestion, and sample cleanup for LC/MS analysis. This open access automation solution is specifically designed for protein sample preparation, and the automation of these mechanical steps (Figures 1 and 2) allows the analyst to walk away to perform other tasks while the samples are being prepared. Sample prep methods are also transferable among AssayMAP systems in different labs to extend reproducibility benefits across an organization.

Figure 1. Integrated workflow for peptide mapping mAbs, from automated sample preparation on the Agilent AssayMAP Bravo Platform to Agilent 6550 Q-TOF LC/MS/MS analysis, and Agilent BioConfirm B.08.

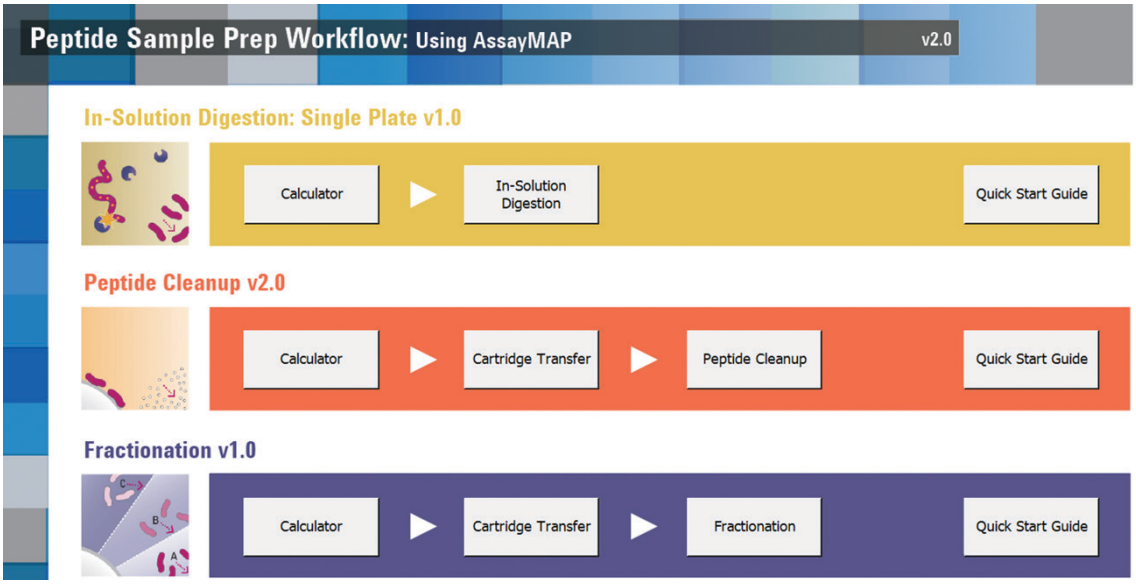

Figure 2. The Agilent AssayMAP Peptide Sample Prep Workflow digests, cleans, and optionally fractionates samples for peptide mapping and other workflows.

The Agilent 6550 iFunnel Q-TOF LC/MS/MS system has been proven to deliver excellent mass accuracy and highly sensitive MS results for protein identification and detailed peptide mapping[2]. The BioConfirm software can provide automatic data processing in the areas of MFE, peptide sequence matching, and protein sequence-coverage mapping. Typically, an LC/MS/MS run of a digested mAb sample contains numerous peptides, of which many may have various PTMs, so samples are complex. Once the MS data are acquired, the BioConfirm peptide mapping workflow can be used to perform the MFE processing, and generate a list of compounds (that is, peptides) from the highly complex data. The list of compounds is then matched against the user-specified mAb sequence, and a sequence coverage map is generated. Figure 3 illustrates the extracted compound chromatogram (ECC) of the NIST mAb, which has been digested with Trypsin/Lys-C mix. All identified peptides are labeled with their corresponding sequence numbers in NIST mAb light chain and heavy chain.

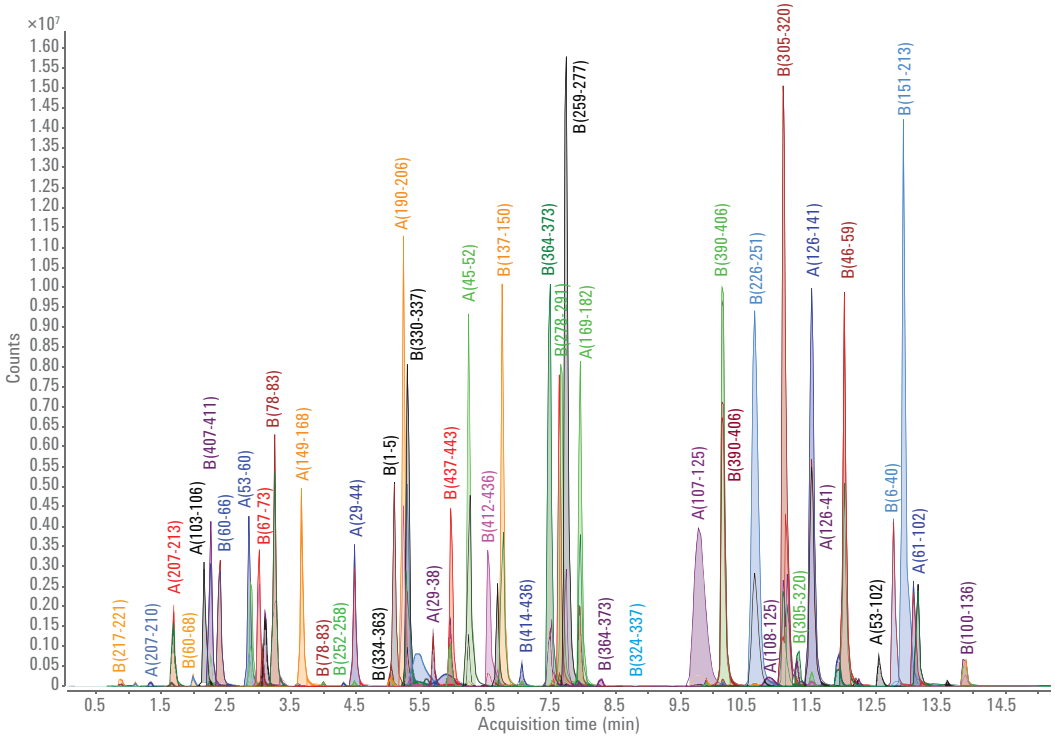

Figure 3. ECC of peptides from Trypsin/Lys-C digested NIST mAb standard RM 8671, separated by Agilent AdvanceBio Peptide Mapping column. Light- and heavy-chain peptides are marked *A* and *B*, respectively.

To identify peptides with high confidence, all matched peptides were required to have <10 ppm MS mass error, and have at least one confirmatory MS/MS spectrum. Figure 4 shows examples of the MS/MS spectra for two selected peptide fragment ions. The BioConfirm scoring algorithm considers factors such as:

- The presence of b and y fragment ions
- Immonium ions
- Mass accuracy
- MS/MS peak intensity
- Other parameters

Integrating high-quality MS and MS/MS data improves the reliability of peptide matching, and provides a wealth of information about peptide modifications (Figures 5 and 6).

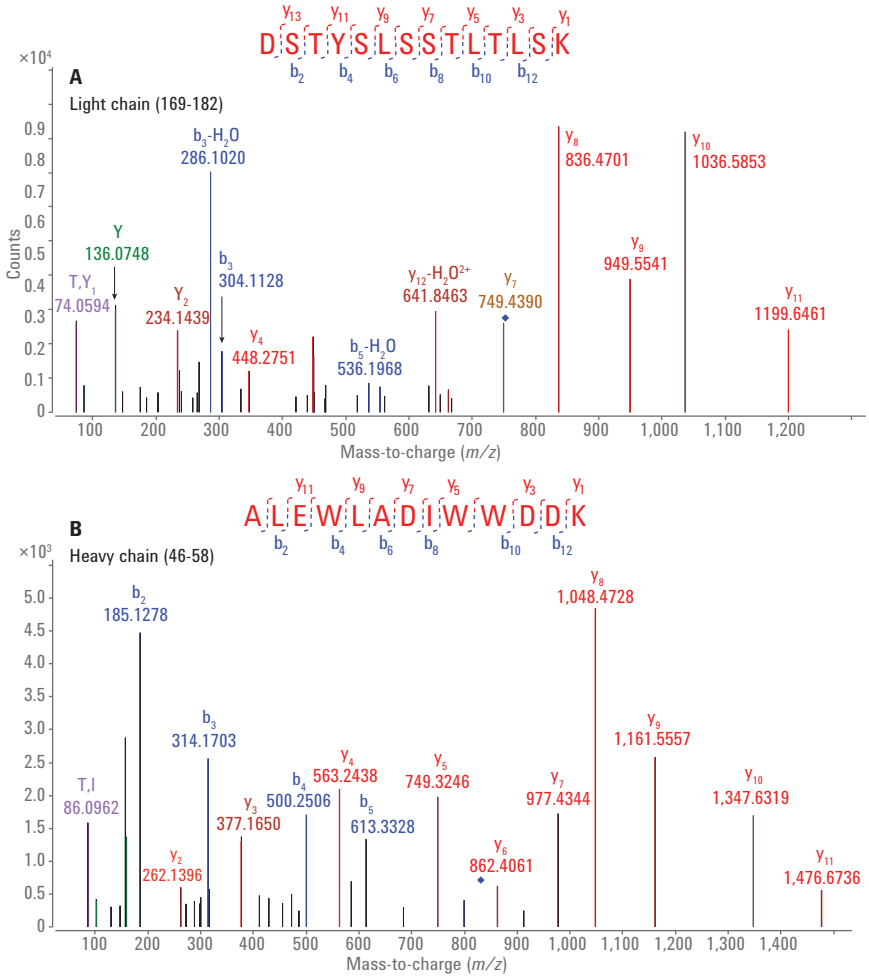

Figure 4. Representative MS/MS spectra of peptides from digested NIST mAb. A) Light chain peptide. B) Heavy chain peptide.

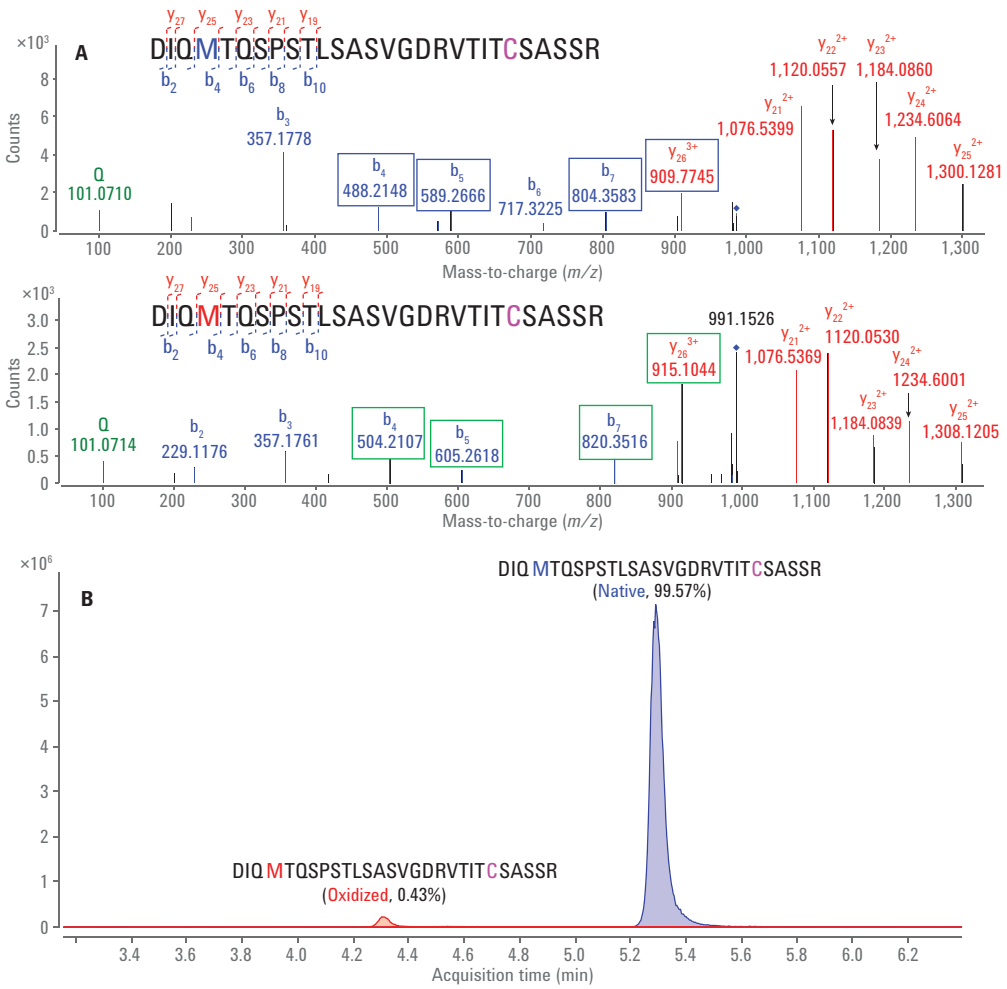

Figure 5. A) Post-translational modification (methionine oxidation) analysis. MS/MS spectra of native and Met-oxidized peptides (Light chain peptide 1-28). Top: native peptide, Bottom: oxidation at Met 4 (green boxes). B) Overlaid ECCs and relative quantitation analysis of native (blue) and oxidized (red) peptides (light chain peptide 1-28).

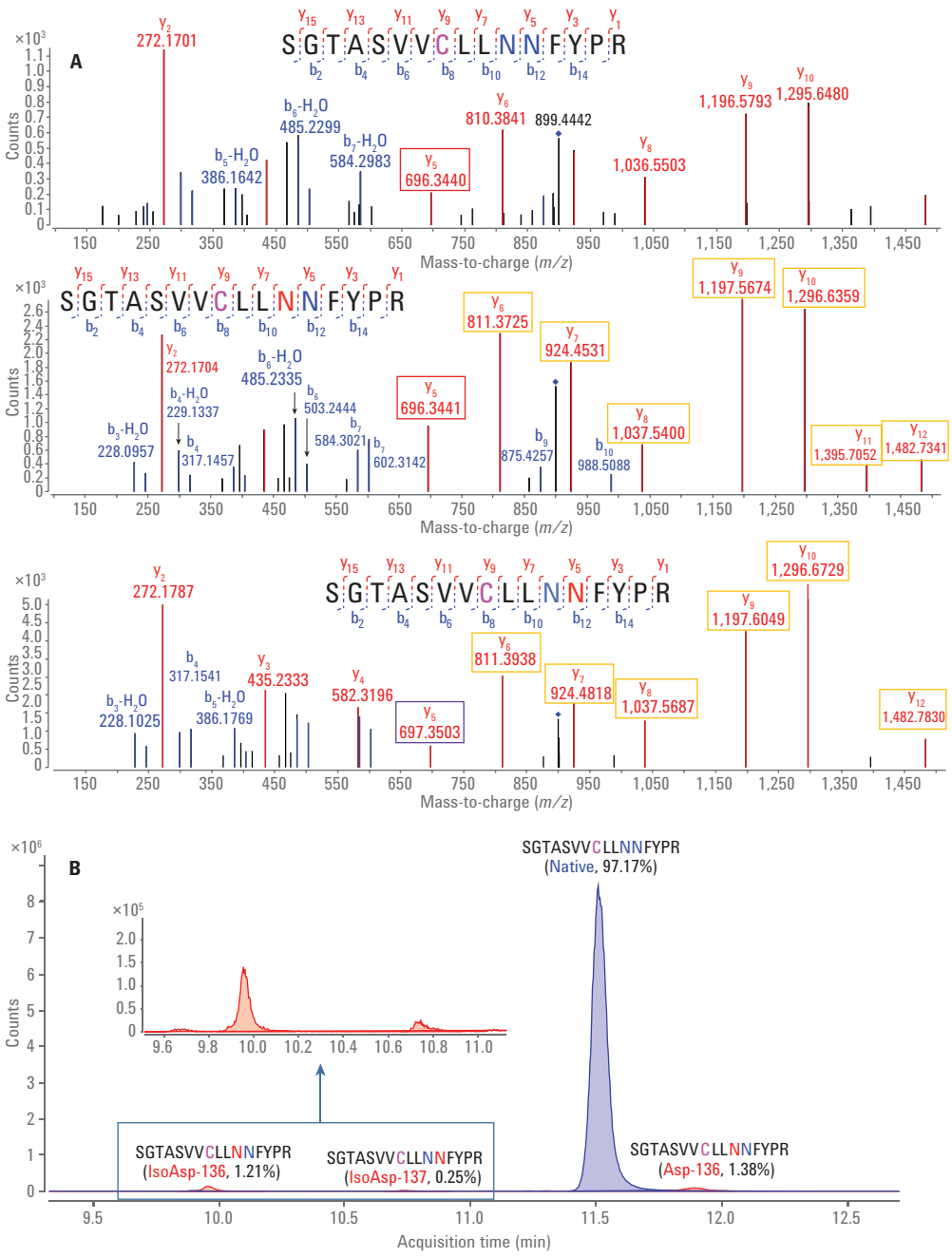

Figure 6. A) Post-Translational Modification (Deamidation) Analysis. MS/MS spectra of native and deamidated peptides (Light chain: 126-141). Top: Native Peptide, Middle: Deamidation at Asn 136, Bottom: Deamidation at Asn 137. B) Overlaid ECCs and relative quantitation analysis of native and deamidated peptides (light chain: 126-141). Inset: zoom in view of IsoAsp-136 and IsoAsp-137.

The relationship between mAb protein sequence coverage and the amount of sample digested was also investigated. Table 3 summarizes the protein sequence coverage for NIST mAb digests prepared at different sample concentrations. On average, greater than 95 % protein sequence coverage was achieved for all of NIST mAb digests analyzed (n = 8) for each protein amount. Figure 7 shows a representative peptide mapping result from BioConfirm B.08 software, with protein sequence coverage.

Table 3. Sequence coverage summary of enzymatically digested NIST mAb at various sample amounts.

Sample digested (µg)	NIST mAb Digest (replicate number)								
	1	2	3	4	5	6	7	8	Average
100	96 %	96 %	96 %	95 %	96 %	96 %	95 %	97 %	96 %
50	96 %	96 %	97 %	96 %	96 %	97 %	96 %	96 %	96 %
10	97 %	97 %	97 %	97 %	97 %	97 %	98 %	98 %	97 %
05	97 %	93 %	97 %	97 %	94 %	94 %	97 %	94 %	95 %

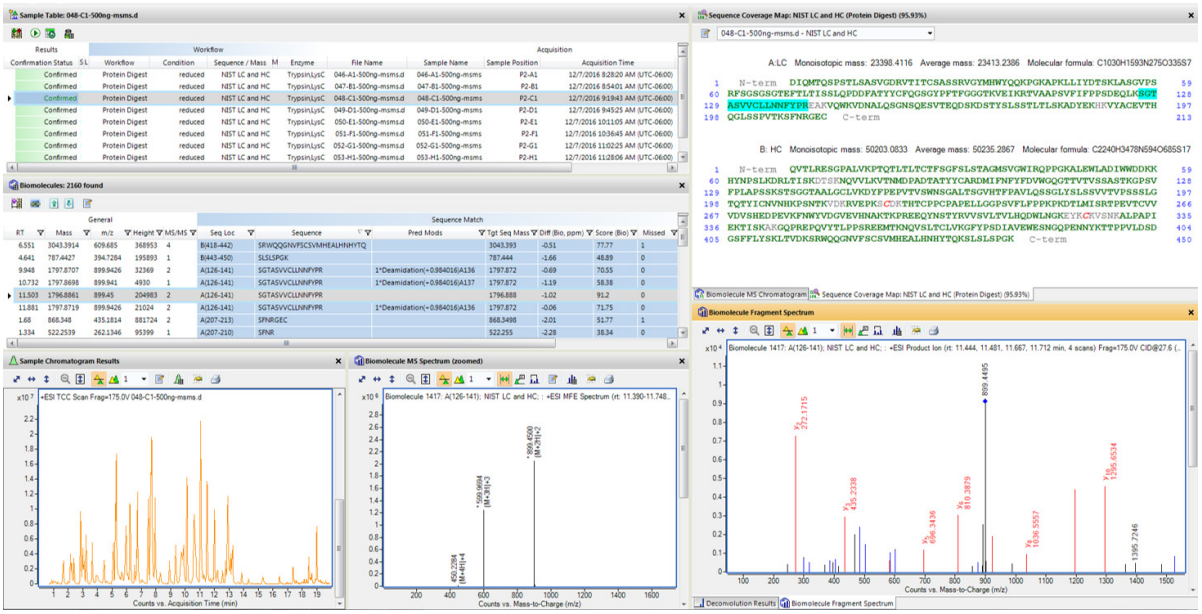

Figure 7. Screenshot of Agilent MassHunter BioConfirm software with representative peptide mapping results and protein sequence coverage.

Post-translational modification analysis

Protein methionine oxidation and asparagine deamidation are two of the most frequent PTMs that have biological significance. Methionine (Met) is a sulfur-containing amino acid that is particularly susceptible to oxidation by reactive oxygen species. The characterization of Met oxidation is often critical during the development of mAb-based therapeutics.

In our peptide mapping results, a NIST mAb light chain peptide 1-28, which contains Met 4, was identified as partially oxidized. Figure 5A illustrates the MS/MS spectra comparison of the native (precursor at *m/z* 985.8097, +3) and Met-oxidized peptides (precursor at *m/z* 991.1414, +3). The major differences (+16 Da) in the b_4–b_7 fragment ions (green box) clearly distinguished the native species from the modified forms, and indicate the specific location of the modification. Moreover, the MS data indicate the relative amounts of each peptide species, with the oxidized form representing approximately 0.43 %, and the native form representing approximately 99.57 % of the peptides (Figure 5B).

Deamidation is another common PTM. In this case, a spontaneous nonenzymatic reaction modifies an asparagine or a glutamine by removing an amide group, and replacing it with a carboxylate group. Typically, asparagine is converted to aspartic acid (Asp) or isoaspartic acid (Iso Asp). The rate of deamidation depends upon primary sequence, protein structure, and solution properties such as pH, temperature, and ionic strength[3].

In our analyses, three deamidated forms of the NIST mAb light chain peptide 126-141 were found with MS/MS spectra at three distinct retention times. Figure 6A shows the MS/MS spectra of the native and the deamidated peptides, where the y-series fragments are the predominant ions in the larger mass range, and the b-series fragments are the predominant ions in the lower mass range. The y_6–y_{12} fragment ions (orange boxes) all show the signature mass shift of 0.98 Da, clearly indicating the presence of deamidation in this region of the peptides. In addition, as the y_5 ion (red box) remains the same (middle panel) as in the native form (top panel), it is evident that the deamidation for this peptide occurred at the Asn 136, and not at Asn 137. In contrast, the y_5 ion in the bottom panel (purple box) presents 0.98 Da higher than the masses in the native sequence, confirming the deamidation at the Asn 137 position in this population of peptides.

A quantitative analysis of the MS data reveals considerably more information about the deamidation dynamics of this light chain 126-141 peptide. The native, unmodified form of the peptide made up 97.17 % of the sample, and three deamidated variants were observed in different proportions: IsoAsp-136 (1.21 %), IsoAsp-137 (0.25 %), and Asp-136 (1.38 %). Figure 6B summarizes these results. It is noteworthy that this kind of position-specific PTM information, the relative quantitation of each modification, is often critical for therapeutic drug (mAb) development. This workflow makes such information readily accessible. Because peptide-mapping workflows are so complex, with so many sample manipulation steps required to produce a precisely reduced, alkylated, digested, desalted sample for the LC/MS measurement, this type of quantitative analysis benefits greatly from highly reproducible robotic sample processing, which is easily automated by the AssayMAP liquid handling system.

Automated peptide mapping workflow produces highly reproducible results

Since this automated workflow solution makes it easy to scale an experiment up or down to accommodate any number of samples, we ran replicate analyses in this study to evaluate the precision and reproducibility of the system at two levels. At one level, we evaluated the quantitative reproducibility of the LC/MS system by injecting the same digested mAb sample eight times from one well. Secondly, we analyzed eight replicate mAb samples that were denatured, digested, cleaned up in separate wells, then injected separately. For this experiment, all quantitative measurements were made in MS-only mode on the 6550 Q-TOF LC/MS/MS instrument. Twenty-eight peptides with no missed cleavages were quantified by EIC for samples prepared with 5 µg of mAb. Overlaid EICs from two representative peptides are shown for LC/MS injection replicates (Figures 8A and 8B) and sample preparation replicates (Figures 8C and 8D). Of the 28 peptides quantified, 17 had CVs less than 10 % (average 6 %), seven had CVs between 10 % and 20 % (average 15 %), and four had CVs greater than 20 % (Figure 8E). These results are consistent with others that have been reported for AssayMAP peptide mapping studies[4].

Although it was not described in this study, the AssayMAP Affinity Purification application with protein A, protein G, or streptavidin affinity cartridges allows this workflow to be expanded for use with samples suspended in growth medium, plasma, or other complex matrices.

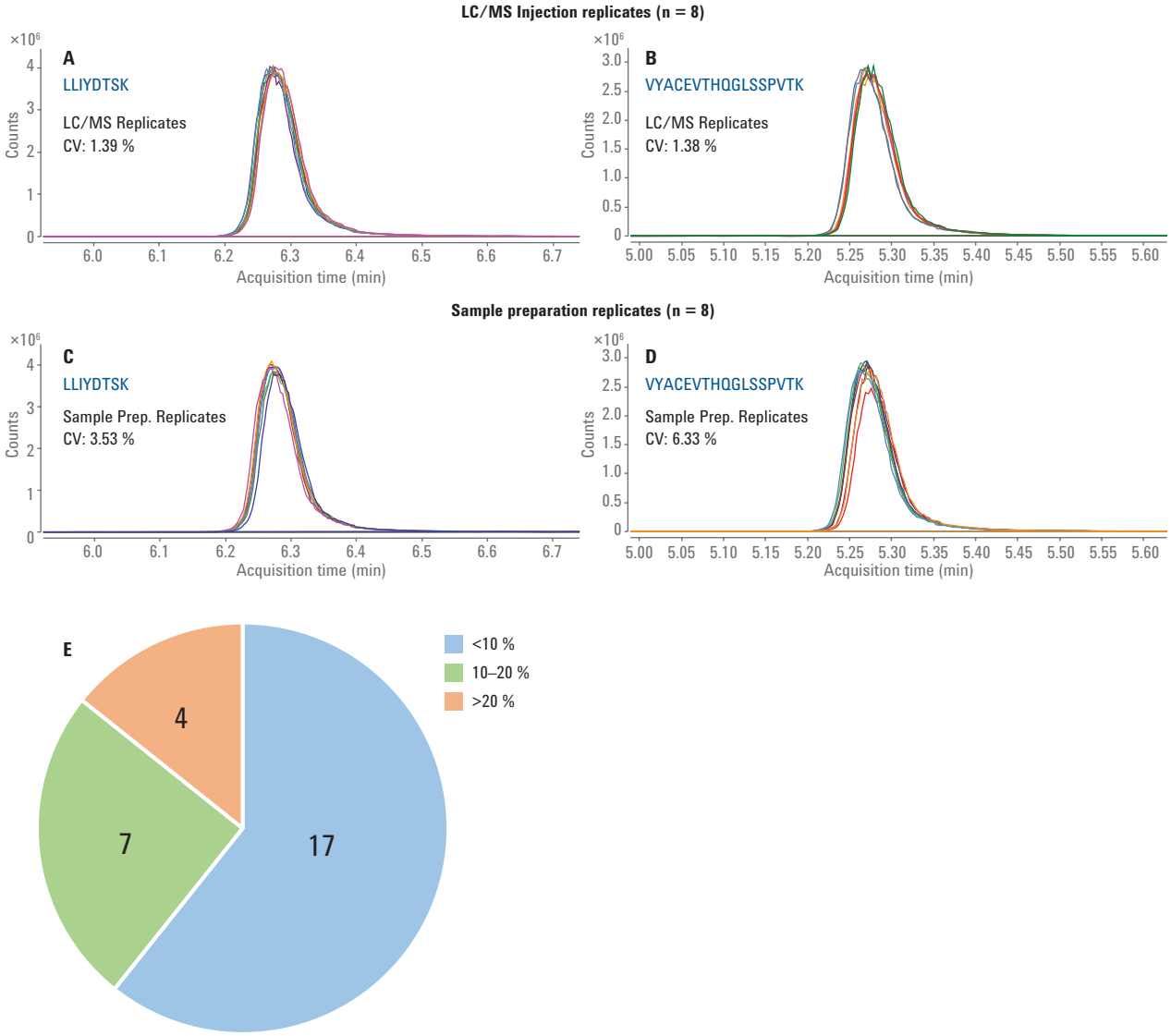

Figure 8. EICs of representative peptides digested from 5 µg NIST mAb. A, B) LC/MS injection replicates of the same sample (n = 8). C, D) Complete-sample-preparation replicates (n = 8). E) Pie chart of the number of peptides with %CV less than 10 %, between 10 % and 20 %, and over 20 %.

Conclusions

The Agilent peptide mapping workflow solution integrates state-of-the-art liquid handling robotics, high-performance chromatography technologies, accurate-mass Q-TOF LC/MS, and powerful software to automate and streamline a complex process that is critical to the development of mAb biologics. This workflow featured:

- An Agilent AssayMAP Bravo system, which enabled automated enzymatic digestion and sample cleanup with excellent reproducibility.
- Superior LC resolution from an Agilent AdvanceBio Peptide Mapping column, which enabled rapid separation for improved analytical laboratory efficiency.
- The highly accurate and sensitive Agilent 6550 Q-TOF LC/MS/MS system, which produced unbiased peptide mapping data to achieve comprehensive sequence coverage of monoclonal antibody proteins.
- Agilent MassHunter BioConfirm data analysis software, which performed automated data extraction, sequence matching, PTM identification, and sequence coverage calculation.

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www.agilent.com/chem/peptide-mapping

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