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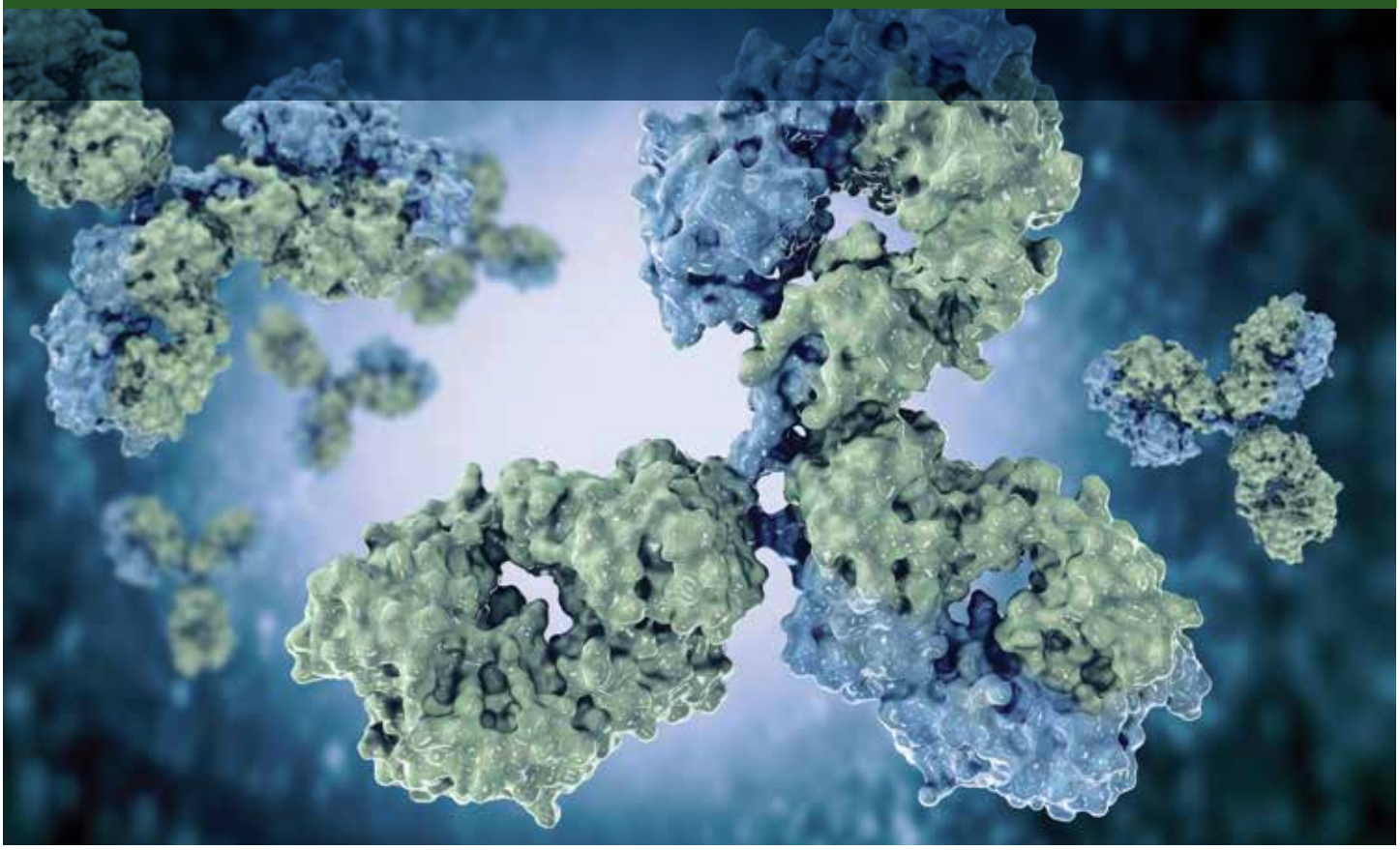
Agilent BioHPLC Columns

Characterization of NIST Monoclonal Antibody Critical Quality Attributes

Application Compendium



Contents

Agilent-NISTmAb Standard (P/N 5191-5744; 5191-5745) was aliquoted from NISTmAb RM 8671 batch. Quality control (QC) testing is performed using Agilent LC-MS system. QC batch release test includes aggregate profile, charge variants and intact mass information. A certificate of analysis (CoA) can be found in each product shipment with test results.

Please note that authors used various monoclonal antibodies including Agilent-NISTmAb Standard and NISTmAb RM 8671 to demonstrate critical quality attribute workflows.

Introduction

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Massachusetts Institute of Technology

Large-molecule therapeutics such as monoclonal antibody (mAb) are typically manufactured through a fermentation process, which intrinsically generates a high level of heterogeneity in the physicochemical and biological attributes of the drug molecule. Among those attributes, variation in the critical quality attributes (CQAs) beyond acceptable ranges compromises both the efficacy and the safety of the drug, and needs to be avoided at all cost. Thus, it's imperative for the developers of large-molecule therapeutics to deploy measurement technologies that both accurately and reliably characterize the CQAs of their drug candidates. Historically, due to the proprietary nature of drug development, there was not a model large-molecule therapeutic agent for which the true or accepted values of CQA measurements are disclosed in the public domain, making it challenging to benchmark different CQA measurement technologies. This changed in mid-2016, when National Institute of Standards and Technology (NIST) released NISTmAb (NIST Monoclonal Antibody Reference Material 8671). NISTmAb is a recombinant humanized IgG1κ expressed in murine suspension culture; it undergoes an industry-standard purification process and is packaged in a formulation appropriate for long-term storage at -80 °C, making it representative of a mAb drug[1]. Accompanying the release of NISTmAb, American Chemical Society published a 3-volume book titled "State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization"[1,2,3]. In Volume 2 of the book[1], CQA measurements for NISTmAb, were independently performed in the labs of multiple pharmaceutical companies using a wide array of technologies, are published in great detail. For the first time, a CQA measurement technology could be evaluated unbiasedly by comparing its results using NISTmAb against the accepted values published in the book.

To further corroborate data reported in the book, NIST in 2018 published a series of papers describing the development of methods by NIST scientists for the measurement of NISTmAb size heterogeneity[4], charge heterogeneity[5], and peptide mapping[6].

As a world leader in analytical instruments, Agilent Technologies offers comprehensive solutions to the measurement of biotherapeutic CQAs, including size variant analysis, charge variant analysis, glycan analysis, intact and subunit analysis, peptide mapping, titer determination, amino acid and cell culture analysis. In this compendium, the Agilent solution suitable for the measurement of each CQA is discussed in the context of characterizing NISTmAb. This compendium also aims to provide guidance for the appropriate liquid chromatography columns for the different types of detection that may be required, and to provide a valuable reference for future consideration.

References

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6. Mouchahoir T, Schiel JE. Development of an LC-MS/MS peptide mapping protocol for the NISTmAb. Anal Bioanal Chem. ***Analytical and Bioanalytical Chemistry;*** **2018**; *410*(8):2111–26.

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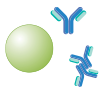


Aggregate/ Fragment Analysis

Size exclusion chromatography

Accurate, precise quantitation for a broad range of biomolecule separations

Charge Variant Analysis

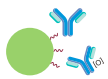
Ion exchange chromatography

Enhances the accuracy and speed of biomolecule characterization

Intact and Subunit Purity

Large molecule chromatography (>150 Å)

Selectivity options for every separation need



Intact Native Protein Analysis

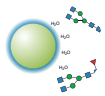
Hydrophobic interaction chromatography

Resolves various protein variants (PTMs) including oxidation in mAbs and drug-antibody species observed in ADCs

Peptide Mapping

Reversed-phase chromatography (<150 Å)

Reliably characterizes primary sequence and detects PTMs



Glycan Analysis

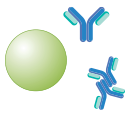
Hydrophilic interaction chromatography

Fast, high-resolution, reproducible glycan separation

Aggregate and Fragment Analysis- Size Exclusion Chromatography

Introduction

Proteins are often susceptible to aggregation, forming dimers and higher order aggregates because of exposure to stress conditions. This is particularly problematical in biotherapeutic protein manufacture since the target protein will be subjected to a wide variety of conditions that may induce aggregation. Changes in temperature and concentration during fermentation, changes in pH and concentration during downstream processing. Even shear forces (from impeller blades, stirrers and other engineering plant equipment) can result in stress related aggregation. The presence of aggregates, particularly very large multimers up to sub-visible particles, are potentially harmful to health. It is therefore a prerequisite that the level of aggregation is quantified and determined, and limits are put into place. Size exclusion chromatography is one of the popular techniques that is particularly suited to the separation of monomer peaks from higher order aggregates and lower molecular fragments, in combination with a suitable concentration detector such as UV or DAD, quantification as relatively straightforward. More complex macromolecules such as monoclonal antibodies (mAbs) may be more challenging due to their larger structure (150kDa) and hydrophobicity that can result in unwanted interactions with many size exclusion chromatography columns. To address this issue, Agilent has developed a new stationary phase that demonstrates greatly reduced risk of secondary interactions. Agilent AdvanceBio SEC columns are packed with highly uniform particles with newly featured 1.9 µm, containing a low-binding, polymer-coated, silica stationary phase that provides efficient separations and minimal non-specific interactions. The AdvanceBio SEC columns are therefore ideally suited for rapid separation and quantification of aggregates and low molecular fragments.



Aggregate/ Fragment Analysis

Size exclusion chromatography

Accurate, precise quantitation for a broad range of biomolecule separations

AdvanceBio SEC

Versatile performance for routine and challenging applications

Attribute	Advantage
Hydrophilic polymer coating	Avoid secondary interactions
Increased analytical speed	Meet vital deadlines
Higher reproducibility	Reduce rework
Greater sensitivity	Quantitate aggregates, even at low levels

Bio SEC-3 and Bio SEC-5

Extra wide pore and scale-up options

Attribute	Advantage
Compatibility with most aqueous buffers	Method flexibility
Wide range of pore size options, including 1000 Å and 2000 Å	Options for everything from peptides to VLPs
Analytical and semi-prep dimensions	Easy scale up or down

Size exclusion chromatography (SEC) is a chromatographic method where molecules in solution are separated by size or, more specifically, by hydrodynamic radius. When applied to large molecules such as mAbs, the larger components of the sample mixture, like aggregates, are excluded from the pores in the chromatographic media and elute first, whereas the smaller components (e.g., heavy chain, fragments, free light chain, clips) diffuse further into the pores and elute later in subsequent peaks according to their size.

Currently, SEC separations are performed in HPLC or UHPLC mode (i.e., SEC-HPLC or SEC-UHPLC) under native or denaturing conditions using specialized, high-performance systems. SEC-UHPLC mode, which operates under the same principles as HPLC, is a step improvement in liquid chromatography based on advances in column particle chemistry performance, system optimization, detector design, and data processing. For mAbs, an SEC chromatogram with good separation will feature an antibody monomer elution peak with a symmetrical shape and allow optimal resolution of higher molecular weight forms (i.e., aggregates) to resolve as distinct earlier peaks relative to the main monomer peak.

Since protein aggregation can occur as a result of external factors, one of the most important steps in aggregate analysis is sample preparation. It is necessary to ensure the proteins are fully dissolved in the mobile phase but not subjected to factors that may alter the level of aggregation, such as sonication, temperature, pH and excessive concentration.

Size exclusion chromatography is a relatively straightforward technique relying on isocratic elution. The mobile phase conditions should ensure there is no secondary interactions between the protein and the column stationary phase. Typically, aqueous buffers such as sodium phosphate (100-200 mM) or PBS (phosphate buffered saline) at neutral pH are used. It is important to use freshly prepared buffer solutions and to filter it through 0.2 µm filter. Under no circumstance should unused buffer be left on the instrument for a prolonged period since these eluents provide ideal conditions for bacterial growth. SEC separations are generally monitored by UV detection at 280 nm, low wavelengths (210 – 220 nm) can greatly improve the sensitivity of the technique.

The resolved molecular species can also be characterized by on-line light scattering techniques such as MALS or may be collected for off-line analysis. With use of volatile buffers such as ammonium acetate it is possible to perform native SEC-MS and this technique is being widely explored for online SEC monitoring with direct mass spec characterization. Preservatives, such as 20% ethanol, are recommended for long term column storage, however care must be taken since the viscosity of the mobile phase containing organic modifiers is often significantly higher and column damage could ensue when performed at higher operating flowrates.

The featured Application note (5994-0876EN) demonstrates method development with an Agilent AdvanceBio SEC 200 Å 1.9 µm column for SEC analysis of the NISTmAb.

Size Exclusion Chromatography (SEC)

Application	Agilent Columns	Notes	USP Designation
Peptides, proteins, aggregate analysis	AdvanceBio SEC	Robust hydrophilic polymer coating yielding minimal secondary interactions; 2.7 µm particles with 130 Å or 300 Å, or 1.9 µm particles with 120 Å or 200 Å pore size for highest resolution.	L59
	Bio SEC-3	Higher resolution and faster separation from 3 µm particles, with 100 Å, 150 Å, and 300 Å pore sizes	L59
Large biomolecules and samples with multiple molecular weight components	Bio SEC-5	More pore size options (100 Å, 150 Å, 300 Å, 500 Å, 1000 Å, and 2000 Å) to cover a wider range of analytes.	L59
Globular proteins, antibodies	ProSEC 300S	Single column option for protein analysis in high salt conditions	L33
Proteins, globular proteins	ZORBAX GF-250/450	Legacy products that should be employed where protocols still require use of USP designation L35.	L35

General recommendations to consider in SEC separation

Column selection

- To ensure sample integrity, SEC is carried out slowly on long columns
- Column lengths are typically 250 or 300 mm
- Normal flow rate is 1.0 mL/min on a 7.5 or 7.8 mm id column and 0.35 mL/min on a 4.6 mm id column
- Columns are often run in series to increase resolution in biopolymer applications
- Smaller particle sizes are used to increase resolution in protein applications
- Separations done on 150 mm columns with smaller particle sizes can reduce analytical time

Column media choice

- There should be no non specific interactions between analytes and column media
- Silica-based sorbents are used for analyzing peptides and proteins
- Polymer-based sorbents are for analyzing biopolymers

Column parameters

- **Pore size**–depends on the molecular weight range of the sample to avoid exclusion of sample components and maximize volume in the required separation region
- **Particle size**–use smaller particles for higher resolution (but higher backpressure)
- **Column length**–compromise between resolution and analysis time
- **Column id**–use smaller columns for reduced solvent consumption and smaller injection volume

Sample preparation

- Ideally, samples should be dissolved in the mobile phase
- If the sample is cloudy, it may be necessary to change the mobile phase conditions
- Filtration or centrifugation can be used to clarify samples, but these processes could alter the molecular weight composition of the sample
- To dissolve a sample, gentle heating, vortexing, or sonication is sometimes used, but should be applied with caution because this can alter the molecular weight composition
- Care should also be taken to ensure the sample does not change during storage
- Samples should be made up fresh and analyzed as soon as possible
- Bacterial growth can develop quickly in buffer solutions
- Samples made up at high concentration can also change over time, leading to aggregation or even precipitation

Mobile phase

- Mobile phase should contain buffer/salt to overcome ionic interactions, but too much may cause hydrophobic interactions
- Do not alter the analyte to avoid degradation/ aggregation, etc
- Make up fresh mobile phase and use promptly, as bacterial growth is rapid in dilute buffer stored at room temperature
- Buffer shelf life is less than 7 days unless refrigerated
- Filter before use to remove particulates in water (less likely) or in buffer salts (more likely)
- High pH phosphate buffers (particularly at elevated temperature) can significantly reduce column lifetime when using silica columns

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Quick Reference Guide

Size Exclusion Chromatography UV/DAD Workflow

Agilent AdvanceBio SEC 2.7 µm Columns

In this document Agilent applications chemists share their recommendations for an optimum LC system and its confi guration for characterizing biomolecules. They also offer guidance on a generic method to get you started, and how this method can be further optimized to meet your specifi c separation goals. Additional application information is available at **www.agilent.com/chem/advancebio**.

Guidelines

- Protein aggregation is impacted by various environmental factors, including pH, ionic strength, and temperature. To quantify levels of aggregation use a mobile phase that does not affect the sample. Typically, start with 150 mM phosphate buffer, pH 7.0.
- For routine SEC use a 300 mm column.
- To increase sample throughput use a 150 mm column and increase the flow rate.
- To increase resolution use two columns in series.
- SEC is a non-interactive LC technique and so small injection volumes must be used to achieve efficient separations. Sample size should be ≤5% of total column volume.
- AdvanceBio 2.7 µm SEC columns are recommended for SEC/DAD, SEC/UV, and SEC/LS applications. For SEC/MS methods Agilent recommends Bio SEC-3 columns.
- To maximize column lifetime Agilent recommends using a guard column and not to exceed 200 bar operating pressure when using a single column or 400 bar for two columns in series.

Agilent 1260 Infinity Bio-Inert LC System

Mobile phases

Isocratic elution with freshly prepared aqueous or aqueous/ organic buffers. Ensure all components of the mobile phase are soluble and filter, 0.2 µm, before use.

Pump (G5611A)

0.1 to 2.0 mL/min for 7.8 mm id
0.1 to 0.7 mL/min for 4.6 mm id.

Sample injection (G5667A)

5-10 µL injection for samples contain 1-5 mg/mL of protein.

Column compartment (G1316C)

20-30 °C is typical temperature used for SEC of biologically active proteins.

Detection (G1315D)

DAD with a bio-inert standard flow cell, 10 mm.

Analytical columns

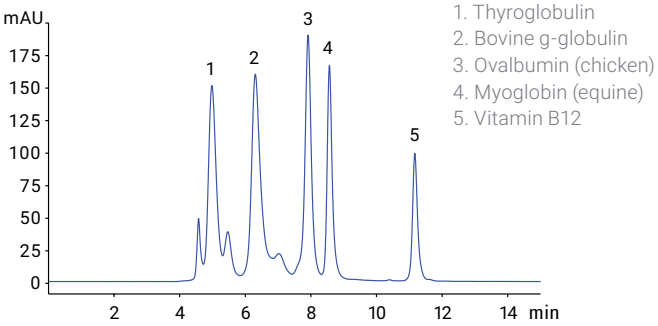
Description	130Å	300Å
4.6 x 300 mm, 2.7 µm	PL1580-5350	PL1580-5301
4.6 x 150 mm, 2.7 µm	PL1580-3350	PL1580-3301
7.8 x 300 mm, 2.7 µm	PL1180-5350	PL1180-5301
7.8 x 150 mm, 2.7 µm	PL1180-3350	PL1180-3301

Analytical guards

Description	130Å	300Å
4.6 x 50 mm, 2.7 µm	PL1580-1350	PL1580-1301
7.8 x 50 mm, 2.7 µm	PL1180-1350	PL1180-1301

AdvanceBio SEC column choice

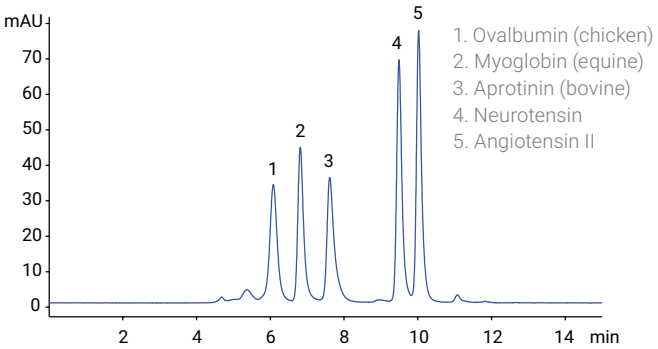
AdvanceBio SEC 300Å, protein resolving range 5 – 1,200 KD



Conditions

Parameter	Value
Column:	AdvanceBio SEC 300Å, 7.8 x 300 mm
	BioRad Gel Filtration Standard #1511901
Mobile phase:	150 mM sodium phosphate, pH 7.0

AdvanceBio SEC 130Å, protein resolving range 0.1 – 100 KD

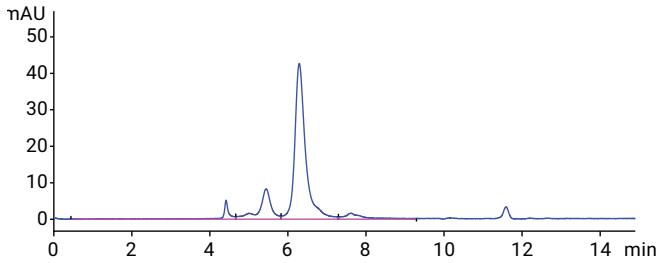


Conditions

Parameter	Value
Column:	AdvanceBio SEC 130Å, 7.8 x 300 mm
Mobile phase:	150 mM sodium phosphate, pH 7.0

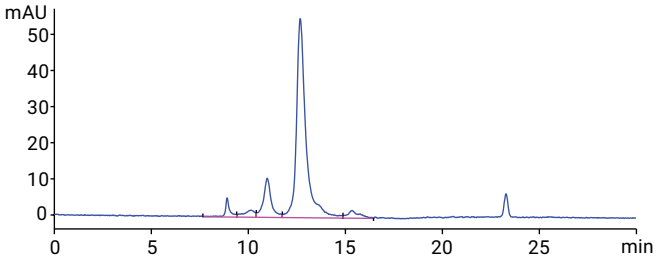
Increasing resolution

Two columns can be run in series to increase resolution.



Conditions

Parameter	Value
Column:	1 x AdvanceBio SEC 300Å, 7.8 x 300 mm)
Run time:	15 min Rs (monomer/dimer) = 2.06
Mobile phase:	150 mM sodium phosphate, pH 7.0
Sample:	IgG

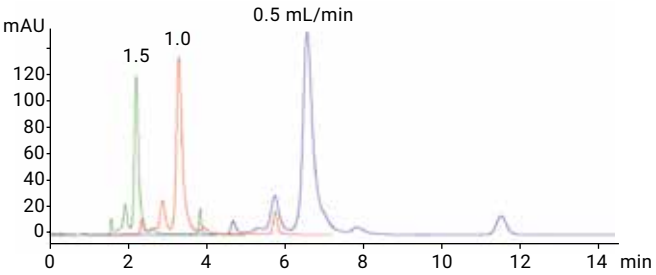


Conditions

Parameter	Value
Column:	2 x AdvanceBio SEC 300Å, 7.8 x 300 mm)
Run time:	30 min Rs (monomer/dimer) = 2.59
Mobile phase:	150 mM sodium phosphate, pH 7.0
Sample:	IgG

Reducing analysis time

Use a shorter column, 150 mm, and for further savings run at higher flow rate.



Conditions

Parameter	Value
Column:	1 x AdvanceBio SEC 300Å, 7.8 x 150 mm
Mobile phase:	150 mM sodium phosphate, pH 7.0
Sample:	IgG

Conditions

Flow rate	Rs (monomer/dimer)	Run time
0.5 mL/min	1.94	15 min
1.0 mL/min	1.63	8 min
1.5 mL/min	1.46	4 min

Application Note

Biologics

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Size Exclusion Chromatography Method Development of NISTmAb Using an Agilent AdvanceBio SEC 200 Å 1.9 µm Column

Author

Veronica Qin
Agilent Technologies, Inc.

Abstract

This Application Note demonstrates method development with an Agilent AdvanceBio SEC 200 Å 1.9 µm column for size exclusion chromatography (SEC) analysis of the NIST monoclonal antibody (mAb). A wide range of mobile phase combinations can easily be screened with the bio-inert quaternary pump of the Agilent 1260 Infinity II Bio-inert LC system and Agilent Buffer Advisor software.

Introduction

SEC is a commonly used technique to characterize and quantify size variants from biotherapeutic proteins. A variety of different mobile phase conditions often need to be evaluated to improve peak shape and resolution for a protein of interest. The AdvanceBio SEC 200 Å 1.9 µm column with its unique bonding chemistry offers reduced secondary interactions under different buffer conditions. SEC method development can be time-consuming, with the requirement of screening a number of different buffer compositions and pH combinations. However, Buffer Advisor software, combined with a bio-inert quaternary LC pump provides a simple way of online mobile phase optimization for SEC analysis. This Application Note presents SEC method development for characterizing the NISTmAb.

Experimental

Materials

NISTmAb (RM 8671) (10 mg/mL) was purchased from NIST SRM standards. Monobasic and dibasic sodium hydrogen phosphate (NaH_2PO_4/ Na_2HPO_4) and sodium chloride (NaCl) were purchased from MilliporeSigma. All chemicals used were ≥99.5 % pure. Water was purified from a Milli-Q A10 water purification system (Millipore). Mobile phases were prepared fresh daily and filtered through a 0.22 µm membrane filter prior to use.

Instrumentation

An Agilent 1260 Infinity II Bio-inert LC system with the following configuration was used:

- Agilent 1260 Infinity II Bio-inert quaternary pump (G5654A)
- Agilent 1260 Infinity II Bio-inert multisampler (G5668A) with sample cooler (option #100)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A) with bio-inert heat exchanger (option #019)
- Agilent 1260 Infinity II variable wavelength detector (G7114A)

Column

Agilent AdvanceBio SEC 200 Å 1.9 µm, 4.6 × 300 mm (p/n PL1580-5201)

Software

- Agilent OpenLab CDS 2.2 software
- Agilent Buffer Advisor software

Results and discussion

Conditions

Parameter	Settings
Column Temperature:	25 °C
Mobile phase:	A) Water B) 1 M NaCl C) 245 mM NaH_2PO_4 D) 420 mM Na_2HPO_4
Flow Rate:	0.35 mL/min
Injection Volume:	3 µL
Detection:	UV at 280 nm

To screen a variety of mobile phase conditions for NISTmAb SEC analysis, the following stock solutions were prepared:

- 1 M NaCl
- 245 mM NaH_2PO_4
- 420 mM Na_2HPO_4

Using these stock solutions, it was possible to create sodium phosphate buffer concentrations from 150 to 350 mM without the addition of NaCl, using Buffer Advisor software. In addition, combinations of sodium phosphate buffer with NaCl present at varying concentration were also evaluated. Mobile phase pH was tested at four different values: pH 6.6, 6.8, 7.0, and 7.4. In Buffer Advisor software, we can enter different method development screening conditions including buffer concentration, salt concentration, and pH. The software can then automatically calculate the correct percentage of each stock solution needed to achieve the desired mobile phase conditions. Table 1 shows several selected screening conditions and mobile phase compositions calculated by Buffer Advisor software.

Table 1. Selected screening conditions and mobile phase compositions calculated by Buffer Advisor software.

Buffer (mM)	NaCl (mM)	pH	Software-Calculated Mobile Phase Compositions			
			A%	B%	C%	D%
150	0	6.6	49.9	0.0	34.5	15.6
150	0	6.8	52.6	0.0	27.9	19.5
150	0	7	55.3	0.0	21.5	23.2
150	0	7.4	59.6	0.0	11.3	29.1
50	250	6.6	58.8	25.0	10.2	6.0
50	250	6.8	59.8	25.0	7.9	7.3
50	200	7.4	66.8	20.0	3.1	10.1
25	250	7.0	67.8	25.0	3.0	4.2

A: Water
B: 1 M NaCl
C: 245 mM NaH_2PO_4
D: 420 mM Na_2HPO_4

Figure 1 shows SEC chromatograms of NISTmAb under 150 mM sodium phosphate without the addition of any NaCl at pH ranging from 6.6 to 7.4. As shown in Table 2, at this buffer concentration, a higher pH at 7.4 results in better peak shape and dimer/monomer resolution. In addition, the peak area percentage of high molecular weight species (HMW%) increases gradually from pH 6.6 to 7.4 (Figure 2), and at pH 7.4 the value is close to 3 % as reported by NIST's evaluation of RM 8671[1].If we set pH at 7.4, and increase the buffer concentration from 150 mM up to 350 mM, peaks have less tailing at 300 and 350 mM, but dimer/monomer resolution continues to drop with increased concentration (Table 3). Under mobile phase compositions without NaCl, 150 mM of sodium phosphate at pH 7.4 gives the best result, with a balance of peak shape and resolution and more accurate HMW%.

In addition, mobile phases consisting of different concentrations (25 to 100 mM) of sodium phosphate and 250 mM NaCl at the four pH values were evaluated by comparing peak symmetry and dimer/monomer resolution (results shown in Table 4). It was shown that 50 mM sodium phosphate and 250 mM NaCl at pH 6.8 gave the best result, with a balance of peak shape and resolution. Using 25 mM phosphate, and 250 mM NaCl, pH at 6.8 or above, gave more accurate HMW% results (Figure 3).

Table 2. Effect of pH on peak symmetry and dimer/monomer resolution.

Buffer (mM)	NaCl (mM)	pH	Asymmetry (As)	Resolution (Dimer/Monomer)
150	0	6.6	1.49	2.33
150	0	6.8	1.43	2.35
150	0	7	1.42	2.67
150	0	7.4	1.41	2.78

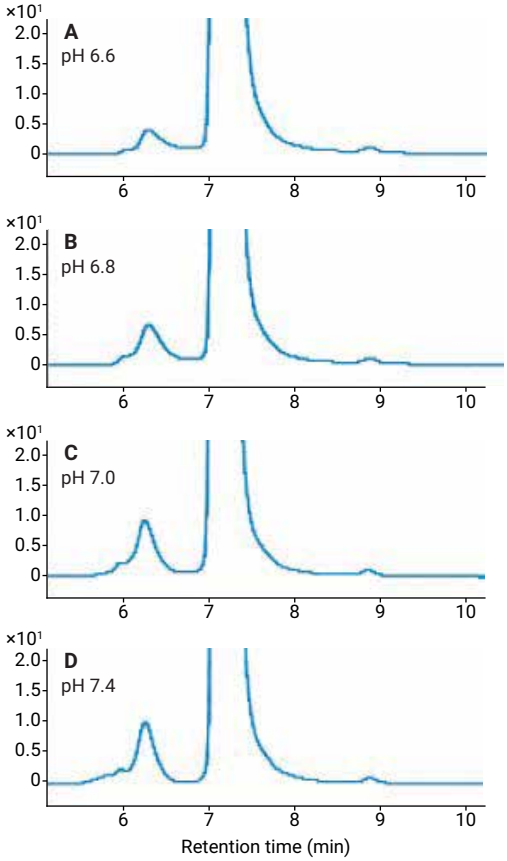


Figure 1. Size exclusion chromatograms of NISTmAb using 4.6 × 300 mm SEC columns running at 0.35 mL/min under 150 mM sodium phosphate at A) pH 6.6, B) pH 6.8, C) pH 7.0, and D) pH 7.4.

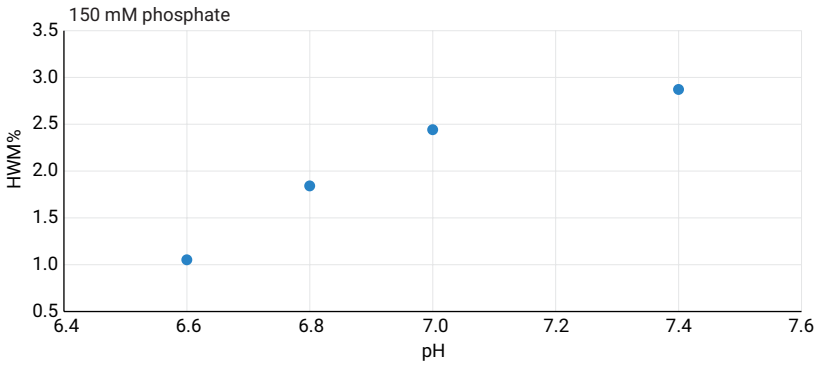


Figure 2. Peak area percentage of HMW of NISTmAb under 150 mM phosphate at different pH values.

Table 3. Effect of buffer concentration (without NaCl) on peak symmetry and dimer/monomer resolution.

Buffer (mM)	NaCl (mM)	pH	Asymmetry (As)	Resolution (Dimer/Monomer)
150	0	7.4	1.41	2.78
200	0	7.4	1.45	2.60
250	0	7.4	1.42	2.57
300	0	7.4	1.40	2.45
350	0	7.4	1.38	2.33

Table 4. Effect of buffer concentration (with NaCl) and pH on peak symmetry and dimer/monomer resolution.

Buffer (mM)	NaCl (mM)	pH	As	Rs (Dimer/Monomer)
25	250	6.6	1.36	2.73
25	250	6.8	1.36	2.86
25	250	7	1.35	2.83
25	250	7.4	1.37	2.86
50	250	6.6	1.35	2.87
50	250	6.8	1.33	2.86
50	250	7	1.36	2.85
50	250	7.4	1.36	2.84
100	250	6.6	1.36	2.87
100	250	6.8	1.36	2.89
100	250	7	1.35	2.83
100	250	7.4	1.37	2.80

Using 50 mM phosphate and 250 mM NaCl, or 100 mM phosphate and 250 mM NaCl, we obtained accurate HMW% at the four pH values were evaluated by comparing peak symmetry and dimer/monomer resolution (results shown in Table 4). It was shown that 50 mM sodium phosphate and 250 mM NaCl at pH 6.8 gave the best result, with a balance of peak shape and resolution. Using 25 mM phosphate, and 250 mM NaCl, pH at 6.8 or above, gave more accurate HMW% results (Figure 3).

Using 50 mM phosphate and 250 mM NaCl, or 100 mM phosphate and 250 mM NaCl, we obtained accurate HMW% at any of the four pH values. Therefore, with these two mobile phase compositions, pH had negligible effect on HMW% results (Figure 4). Table 5 compares the effect of salt concentration and pH on peak symmetry and dimer/monomer resolution when setting buffer concentration at 50 mM. Among these conditions, 50 mM sodium phosphate with 250 mM NaCl at pH 6.8 gave the best result, with a balance of peak shape and resolution. Using 50 mM phosphate, and 200 mM NaCl, similar to 25 mM phosphate with 250 mM NaCl, pH at 6.8 or above, gave more accurate HMW% results (Figure 5). Overall, considering peak symmetry, dimer/monomer resolution, and HMW% accuracy, the optimum mobile phase composition is 50 mM sodium phosphate, 250 mM NaCl at pH 6.8 (chromatogram shown in Figure 6).

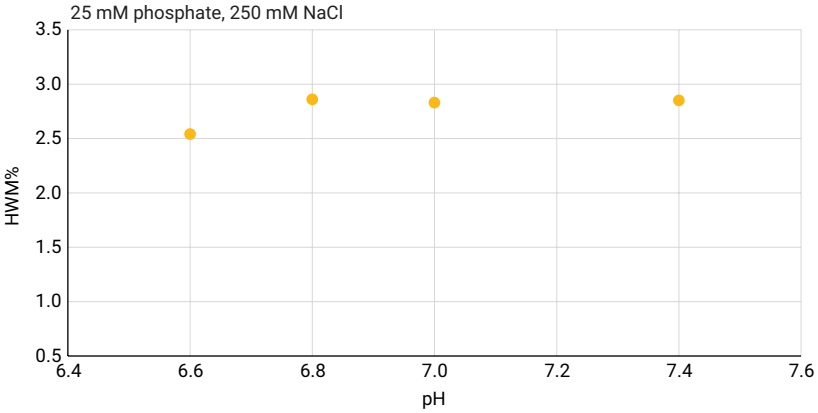


Figure 3. Peak area percentage of HMW of NISTmAb under 25 mM phosphate, 250 mM NaCl at different pH values.

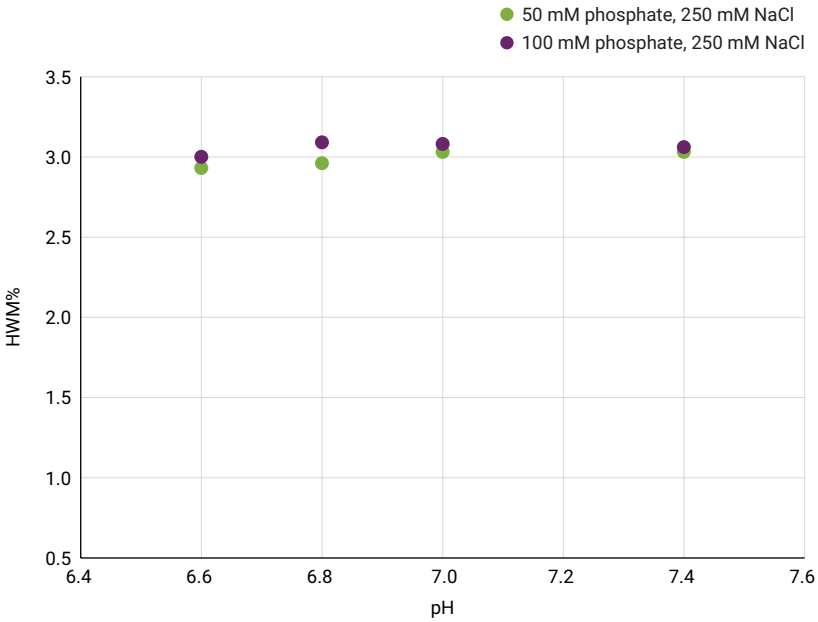


Figure 4. Peak area percentage of HMW of NISTmAb under 50 mM phosphate, 250 mM NaCl or 100 mM phosphate, 250 mM NaCl at different pH values.

Table 5. Effect of salt concentration and pH on peak symmetry and dimer/monomer resolution.

Buffer (mM)	NaCl (mM)	pH	As	Rs (Dimer/Monomer)
50	250	6.6	1.35	2.87
50	250	6.8	1.33	2.86
50	250	7	1.36	2.85
50	250	7.4	1.36	2.84
50	200	6.6	1.36	2.68
50	200	6.8	1.35	2.83
50	200	7	1.36	2.87
50	200	7.4	1.36	2.88

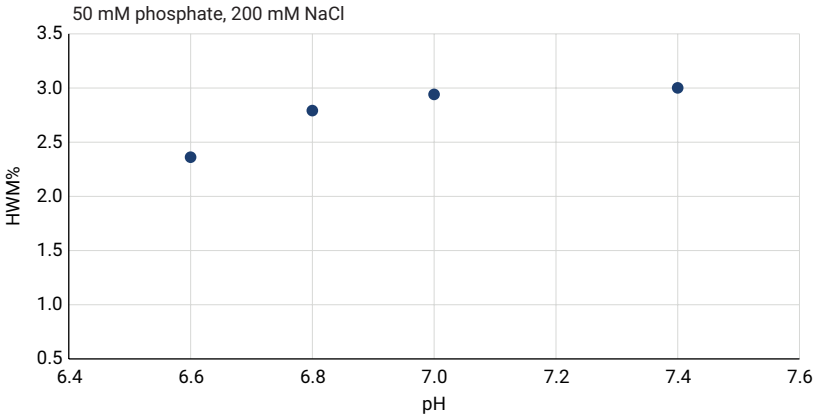


Figure 5. Peak area percentage of HMW of NISTmAb under 50 mM phosphate, 200 mM NaCl at different pH values.

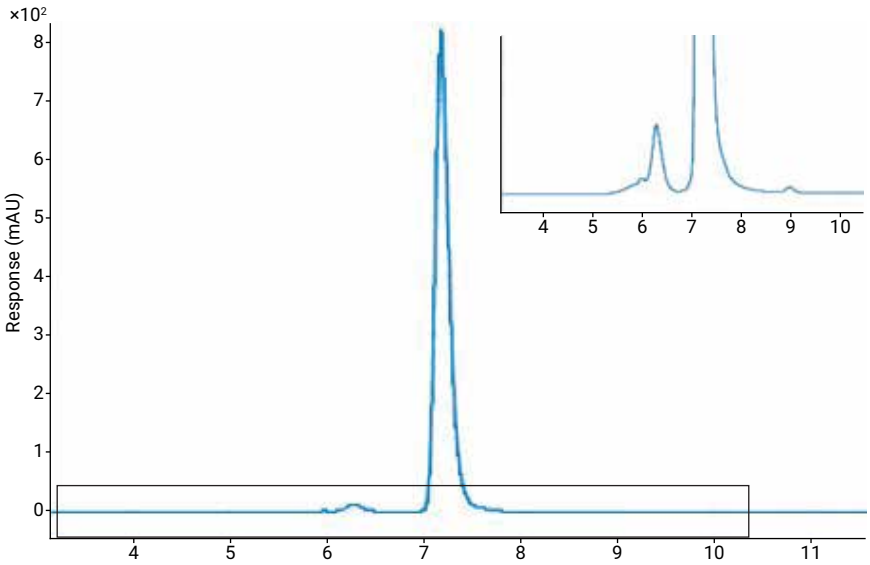


Figure 6. Size exclusion chromatogram of NISTmAb using 4.6 × 300 mm SEC columns running at 0.35 mL/min under 50 mM sodium phosphate, 250 mM NaCl, pH 6.8.

Conclusion

This study demonstrates a simplified approach to mobile phase optimization for SEC analysis of NIST mAb (RM 8671) with the use of an AdvanceBio SEC 200 Å 1.9 µm column together with the bio-inert quaternary pump of the 1260 Infinity II Bio-inert LC system, and Buffer Advisor software. Optimized mobile phase combination and pH is selected considering peak symmetry, dimer/monomer resolution, and quantitation accuracy.

Reference

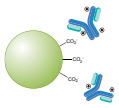
1. Schiel, J. E.; *et al.* The NISTmAb Reference Material 8671 value assignment, homogeneity, and stability, *Anal. Bioanal. Chem.* **2018**, *410*, 2127–2139.

Charge Variant Analysis

Introduction

The presence of positively charged and negatively charged amino acids and negatively charged glycans (sialic acids) means that large proteins exist as multiply charged species and there are several side reactions that can result in a change in the net charge. Understanding which amino acids or glycans are involved and their specific location within a large biotherapeutic protein is of paramount importance. Ion exchange chromatography can enable the separation of some charge variants, particularly those positioned on the surface of the protein. Charge heterogeneity of mAbs comes from various modifications such as oxidation, deamidation, aspartic acid isomerization, glycan modifications, lysine truncation, and other post-translational modifications (PTMs). Variants within the antigen binding region of an antibody are likely to have a more profound effect on function. It is essential to characterize these mAb variants to understand the purity of mAbs for development and administration of a therapeutic agent.

Since most proteins contain more basic amino acids than acidic amino acids, most charge variant separations will require cation exchange. However, every protein is different and finding the conditions to deliver the best resolution will likely require considerable method optimization. Strong cation exchange columns are often easier to work with, however for monoclonal antibodies a weak cation exchange column may be an optimal choice to achieve the desired resolution. Nonetheless, separating a mAb molecule with a net charge of +50 from a variant that is +49 or +51 is still a considerable challenge. Elimination of pore structure and therefore pore diffusion by using nonporous particles goes some way to improving peak shape and gaining resolution. It is often necessary to revert to weak cation exchange columns and to perform extensive method optimization to determine the most appropriate conditions for a protein molecule. Agilent Bio Mab HPLC columns features rigid non-porous particles with hydrophilic, polymeric coating where a highly uniform densely packed, weak cation-exchange layer is chemically bonded designed ideally for mAb charge variant analysis. These columns offer superior performance promoting higher resolution and faster separations eliminating most non-specific interactions.



Charge Variant Analysis

Ion exchange chromatography

Enhances the accuracy and speed of biomolecule characterization

Bio MAb

Ideal for monoclonal antibodies

Attribute	Advantage
Rigid, non-porous particles	High-efficiency separations
Hydrophilic, polymeric layer	Eliminates non-specific binding
High density WCX chemistry	High ion exchange capacity ideal for MAbs

Bio IEX

Ideal for proteins and peptides

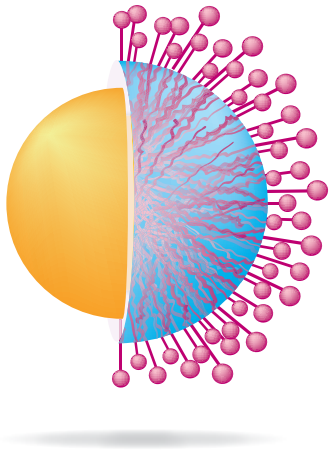
Attribute	Advantage
Rigid particles with hydrophilic coating	Eliminates non-specific binding
Strong/weak anion, cation chemistries	A column for every separation

Ion-exchange chromatography is a widely used method for separating biomolecules based on differences in ionic charge. It is a mild, non-denaturing technique that does not require organic solvents and is therefore frequently used for characterization of proteins in their native or active form, and for purification. Acidic groups include C-terminal carboxylic acids, acidic side chains of aspartic and glutamic acid, and acidic groups arising from sialic acid in glycosylated proteins; basic groups include N-terminal amines and basic side chains of arginine, lysine, and histidine. The overall charge of the molecule is therefore dependent on the pH of the surrounding solution and this in turn will affect the ion-exchange method that can be used. The isoelectric point, pI, is the pH at which the net charge of the protein is neutral (the number of positive charges is equal to the number of negative charges). If the pH is below this value, the protein will possess an overall positive charge and can be retained on a negatively charged cation-exchange sorbent; if the pH is above the pI, the protein will be negatively charged overall and can be retained on an anion-exchange sorbent.

Before beginning method development, it is crucial to determine the isoelectric point, or pI, of the target protein. If the pH of initial mobile phase conditions is too close to the pI of the protein, the protein will not be retained on the column. Depending on how widely the pI of the charge variants differs, the pH may need to be a minimum of 0.5 to 2 pH units away from the isoelectric point of the main species. Proteins may be eluted by either a salt gradient (using high ionic strength to disrupt protein adsorption to the column) or a pH gradient (proteins elute when the pH equals the pI).

Agilent Bio MAb HPLC columns: superior performance from the inside out

- Particles, coating, and bonding are resistant to high pressures, promoting higher resolution and faster separations
- Hydrophilic coating eliminates most nonspecific interactions
- A highly uniform, densely packed, weak cation-exchange (WCX) layer chemically bonded to the hydrophilic, polymeric coating



It is worthwhile considering an instrument that allows screening of several different columns during method development. It is difficult to predict the outcome of small changes to method conditions such as ionic strength and pH; both factors will influence the net charge on the protein and, in the case of weak ion exchange columns, the net charge on the column too. A rigorous "Quality by Design" approach is recommended. Software to develop a matrix or systematic design of experiments is advisable. Agilent Buffer advisor software that can utilize the quaternary HPLC pump capabilities of an Agilent 1260 Infinity II Bio-inert LC can save considerable method development time. The featured SOP manual in this section illustrates how to use buffer advisor software to test a range of mobile phase conditions and optimized method development parameters for charge variant analysis of NISTmAb (RM 8671). When the optimum conditions for separation require very low ionic strength buffers and pH levels at the extreme limits of the buffering range then PEEK columns may also be advisable. Like size exclusion chromatography, ion exchange conditions are typically nondenaturing; the separation is conducted on the intact, native proteins. This means that the method is not MS compatible unless combined as the first dimension in a 2D-LC setup. However, quantification can be achieved by UV or fluorescence detection. In recent trend, with the use of volatile salt buffers or dual volatile pH gradients, both chromatographic separation of charge variants species and direct native mass spec detection are possible.

The technique of ion-exchange is therefore suitable for separating proteins with differing isoelectric points, but it is equally valuable in separating charged isoforms of a single protein. In the increasingly important field of biopharmaceuticals, where proteins are manufactured through bioengineering and isolated from fermentation reactions, it is important to identify charged isoforms as these indicate a difference in primary structure of the protein. A difference in primary structure could indicate a change in glycosylation, or degradation pathways such as loss of C-terminal residues or amidation/ deamidation. These protein modifications can likely result in loss in stability or activity and could potentially lead to immunologically adverse reactions. Ion-exchange is used to separate and quantify charge variants during the development process and for quality control and quality assurance during manufacture of biotherapeutics. With large molecules such as monoclonal antibodies (mAbs) it is also important to consider the size and structure of the molecule, particularly as the chromatographic interactions will only occur with surface charges.

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Quick Reference Guide

Cation Exchange Chromatography Workflow

Agilent Bio IEX HPLC Columns
Agilent Bio MAB HPLC Columns

In this document Agilent applications chemists share their recommendations for an optimum LC system and its confi guration for characterizing biomolecules. They also offer guidance on a generic method to get you started, and how this method can be further optimized to meet your specifi c separation goals. Additional application information is available at **www.agilent.com/chem/advancebio**.

Guidelines

- Basic proteins: SCX or WCX
- Consider the isoelectric point (pI) of your protein when choosing the pH of the mobile phase. If pH<pI, your protein will have a net positive charge.
- The pH of the starting buffer should be 0.5 to 1 pH unit from the pI (below pI for cation-exchange)
- If pI is unknown, start with pH 6 for cation-exchange
- Start with SCX columns, which have the widest operating range, WCX can be used to provide a difference in selectivity.
- Buffers for cation-exchange (pH 4 to7 include formate, acetate, MES, phosphate, HEPS

Column Selection

Bonded Phase
SCX (strong cation-exchange) – SO_3H
WCX (weak cation-exchange) – COOH

Samples	Column
Monoclonal antibody	Bio MAb
Peptides and proteins	Bio SCX and WCX
Globular proteins and peptides	PL-SCX 1000Å
Very large biomolecules/ high speed	PL-SCX 4000Å
Proteins, antibodies	Bio-Monolith SO3

Note: For Bio IEX and Bio MAb stainless steel HPLC columns part number, see Agilent BioHPLC Column Selection Guide, 5994-0974EN

Agilent 1260 Infinity Bio-Inert LC System

Mobile phases

Mobile phase should contain buffer to maintain the desired operating pH, typically 20 mM.
Elution salt is typically 400 to 500 mM.

Agilent Buffer Advisor is used to develop the necessary gradient profile by mixing different proportions from the four stock solutions

Sample injection

1 to 10 µL injection for maximum resolution.
Sample must be soluble in the mobile phase.

Flow rate

Typical flow rate for 4.6 mm id columns is 0.5 to 1.0 mL/min..

Column temperature

Maximum limit 80 °C. Column lifetime is optimized when used between 10 to 50 °C.

Detection

UV, G1315D with a 10 mm bio-inert standard flow cell.

Column Selection

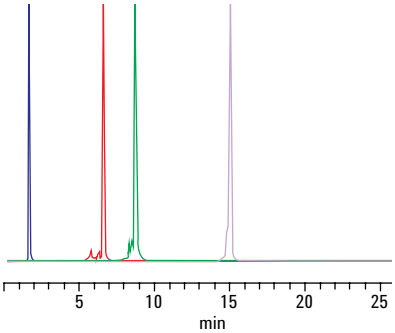
Description	Bio IEX HPLC Columns, PEEK Bio SCX	Bio WCX	Bio MAb HPLC Columns, PEEK
	Part No.	Part No.	Part No.
4.6 x 250 mm, 10 µm	5190-2435	5190-2455	5190-2415
4.6 x 50 mm, 10 µm	5190-2436	5190-2456	5190-2416
4.6 x 250 mm, 5 µm	5190-2427	5190-2447	5190-2407
4.6 x 50 mm, 5 µm	5190-2428	5190-2448	5190-2408
2.1 x 250 mm, 10 µm	5190-2439	5190-2459	5190-2419
2.1 x 50 mm, 10 µm	5190-2440	5190-2460	5190-2420
2.1 x 250 mm, 5 µm	5190-2431	5190-2451	5190-2411
2.1 x 50 mm, 5 µm	5190-2432	5190-2452	5190-2412

Recommended initial conditions

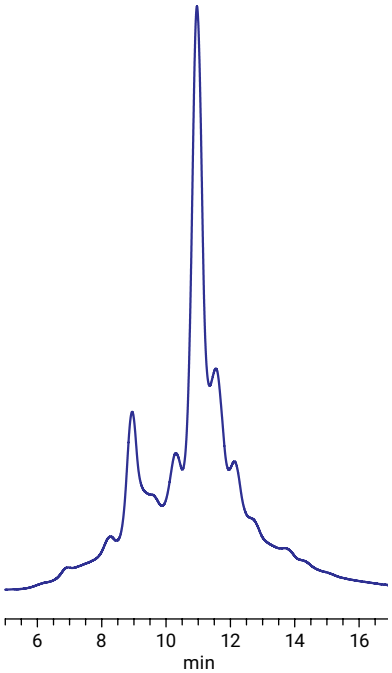
	Monoclonal antibodies		Monoclonal antibodies, Proteins and peptides
	Salt Gradient	**pH Gradient**	**Salt Gradient**
Columns	Bio WCX, 4.6 x 250 mm, 10 µm Bio WCX, 4.6 x 250 mm, 5 µm	Bio MAb, 4.6 x 250 mm, 5 µm	Bio SCX, 4.6 x 50 mm, 3 µm WCX, 4.6 x 50 mm, 3 µm Bio MAb, 4.6 x 50 mm, 3 µm
Mobile Phase	A: Water B: 1.6 M NaCl C: 40.0 mM NaH_2PO_4 D: 40.0 mM Na_2HPO_4 By combining predetermined proportions of C and D, 20 mM buffer solutions at the desired pH range are produced.	A: Water B: 1.6 M NaCl C: 40.0 mM NaH_2PO_4 D: 40.0 mM Na_2HPO_4 By combining predetermined proportions of C and D, buffer solutions at the desired pH range are produced at the selected buffer strengths.	A: 20 mM sodium phosphate, pH 5.0 for WCX or pH 6.0 for SCX B: Buffer A + 1 mM NaCl
Gradient	0 to 50% B, 0 to 20 min (constant pH, for example, pH 6.0) 50% B, 20 to 25 min 0% B, 25 to 35 min	pH 6.0 to 8.0, 0 to 20 min 0 to 800 mM NaCl, 20 to 25 min 800 mM NaCl, 25 to 30 min	1 to 100% B in 30 min for 50 mm columns, 60 min for 250 mm columns
Flow rate	1 mL/min	1 mL/min	0.5 mL/min
Temperature	Ambient	Ambient	Ambient
Injection	10 µL	10 µL	10 µL
Sample	2 mg/mL (in 20 mM sodium phosphate buffer, pH 6.0)	2 mg/mL (in 20 mM sodium phosphate buffer, pH 6.0)	
Detection	UV, 220 nm	UV, 220 nm	UV, 220 nm

Separation of protein standards at pH 7.0 using an Agilent Bio WCX, 4.6 × 250 mm, 10 µm column.

Ovalbumin (pI 4.5)
Ribonuclease (pI 9.4)
Cytochrome C (pI 9.8)
Lysozyme (pI 11)

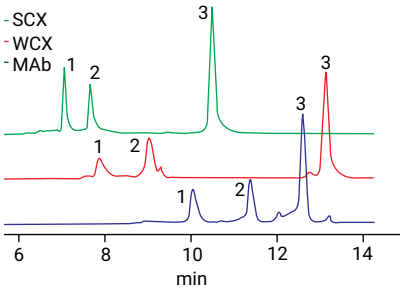


Analysis of a IgG monoclonal antibody using a pH gradient of 6.5 to 7.5 (0-20 min), 50 mM, Agilent Bio MAb, 4.6 x 50 mm, 5 µm



Separation of protein standards on Agilent 3 µm ion-exchange columns by cation-exchange chromatography

Ribonuclease (pI 9.4)
Cytochrome C (pI 9.8)
Lysozyme (pI 11)



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Charge Variant analysis manual for NISTmAb

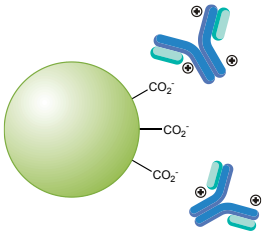
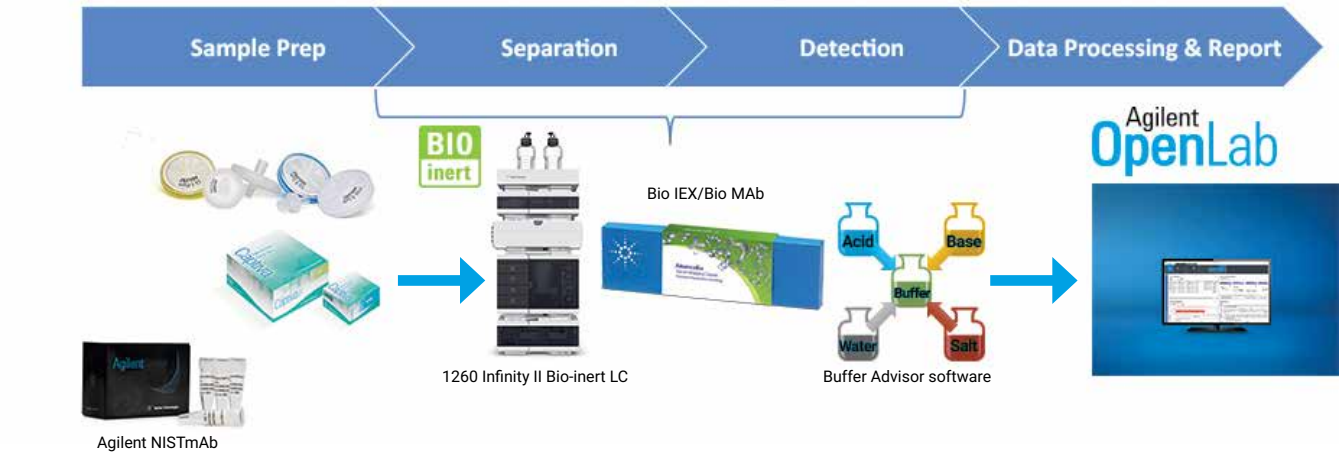


Table of Contents

Charge Variant Analysis - Solution Description



Solution details	
Technology	Liquid Chromatography
Chromatography	Ion exchange chromatography (IEX)
Sample	Agilent-NISTmAb Standard (p/n 5191-5744)
LC	Agilent 1260 Infinity II Bio-inert LC system
Column	Agilent Bio MAb, nonporous, 2.1 × 250 mm, 5 µm, PEEK (p/n 5190-2411)
Detector:	DAD WR with Bio-inert standard flow cell
Software:	Agilent OpenLAB CDS 2.3; Buffer Advisor A.01.01 (009)

Scope

- Operationalize the charge variant analysis of NISTmAb using Ion – Exchange Chromatography using this SOP.

Background

- Charge Variants heterogeneity is considered a Critical Quality Attribute. It can impact drug stability, activity, and efficacy. Charge Variants profiles are used for regulatory drug submissions.
- Charge heterogeneity during production and purification caused by amino acid substitutions, glycosylation, phosphorylation and other post-translational or chemical modifications.
- Monitoring charge variants is a critical part of quality control and quality assurance process during the manufacturing of biotherapeutics.

Checklist:

Experimental checklist		
1260 Infinity II Bio-inert	Quaternary Pump (G5654A)	❑
	Multisampler with sample cooler (G5668A)	❑
	Multicolumn Thermostat with Bio-inert heat exchangers (G7116A)	❑
	DAD WR with bio-inert standard flow cell (G7115A)	❑
Column	Agilent Bio MAb, nonporous, 2.1 × 250 mm, 5 µm, PEEK (p/n 5190- 2411)	❑
Software	OpenLAB CDS 2.3, Buffer Advisor A.01.01 [009]	❑
Chemical	NISTmAb RM8671	❑
	Sodium hydrogen phosphate monobasic (Sigma S8282, BioXtra)	❑
	Sodium hydrogen phosphate dibasic (Sigma S9390, ACS reagent)	❑
	Sodium chloride (Sigma S7653)	❑
Additional equipment	pH meter	❑
	Milli-Q Integral system	❑
	Vacuum filtration unit with 0.2 µm	❑

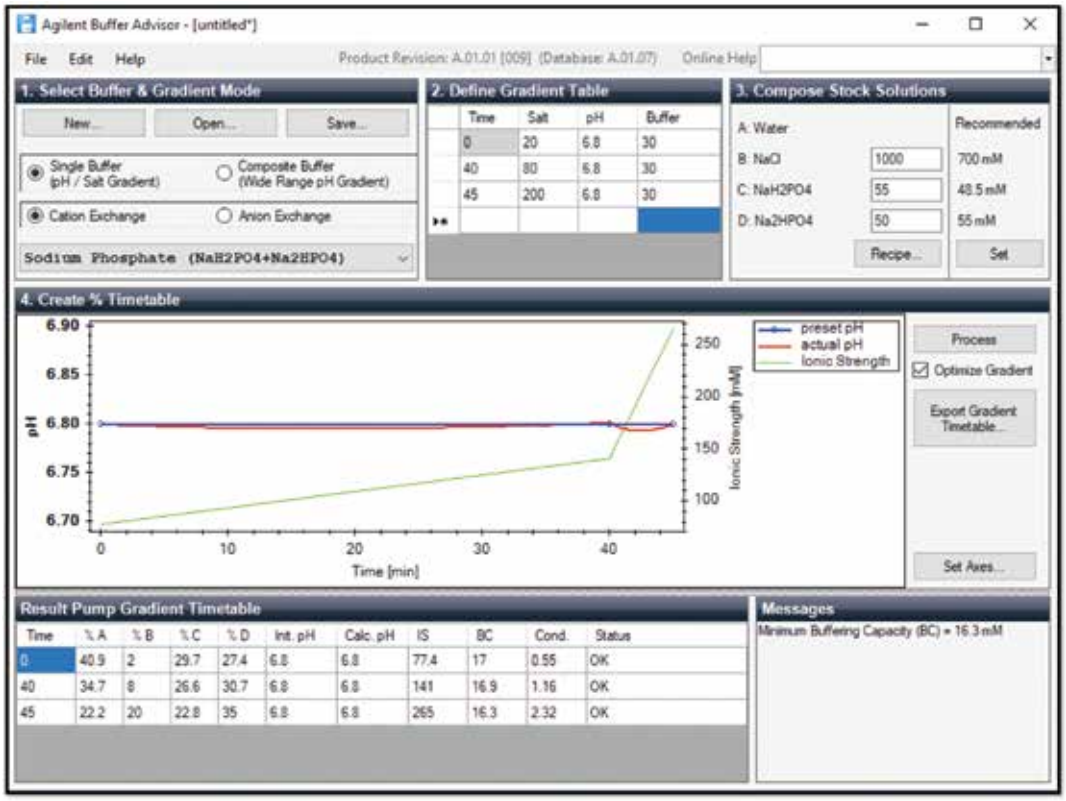
Note:

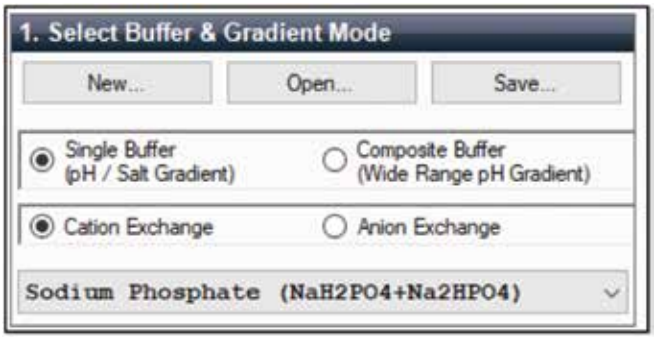
- The expected results may slightly vary due to system-to-system setup. The two LC method conditions and steps (25 mins and 45 mins) are described in this protocol. The methods were optimized and best suited for the NISTmAb sample (RM 8671) IEX analysis.

Agilent Buffer Advisor Software

Buffer Advisor Software facilitates dynamic mixing of solvents from only four stock solutions, simplifying the bioanalysis workflow and significantly reducing the time required for buffer preparation. The Buffer Advisor Software was designed to generate pH gradients for IEX separation. Below discussion illustrates buffer preparation steps for IEX separation of NISTmAb with salt gradient elution method.

Agilent Buffer Advisor software layout





⊖ **Buffer type:** select sodium phosphate buffer system (NaH_2PO_4+Na_2HPO_4)

⊜ **Define Gradient:** Enter the required buffer condition (shown is for Speed and Resolution)

2. Define Gradient Table

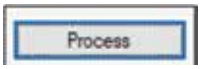
	Time	Salt	pH	Buffer
	0	20	6.8	30
▶	20	80	6.8	30
	25	200	6.8	30
✳				

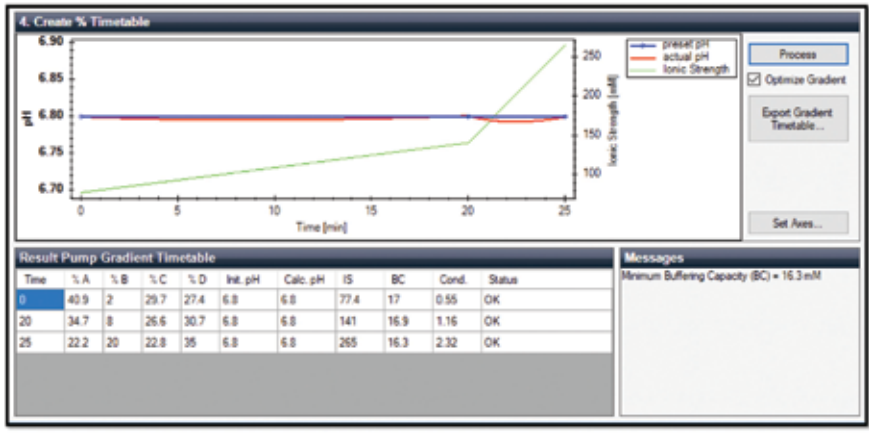
2. Define Gradient Table

	Time	Salt	pH	Buffer
	0	20	6.8	30
	40	80	6.8	30
	45	200	6.8	30
▶✳				

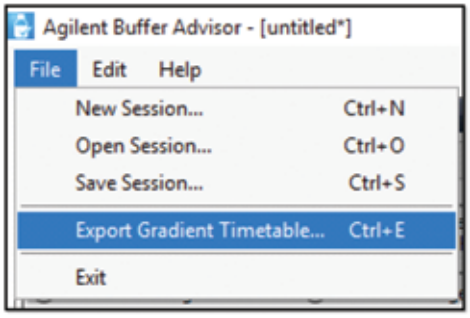
⊛ **Compose Stock Solution:** Enter the required concentrations in stock solution section

④ **Generate Gradient Timetable:** Click the "Process" tab to generate pump gradient timetable. The timetable displays mobile phase percentages to achieve set buffer condition.





Save the timetable using Buffer Advisor “File” tab which generates “xml” file. Import this “xml” file using “Import Timetable” tab in OpenLAB CDS method editor window.



To prepare Stock Solutions: Click “Recipe” tab and it will display absolute amount of chemicals needed for 1 L buffer stock solution preparation

Sample and Buffer Preparation

mAb sample preparation

- Agilent-NISTmAb (p/n 5191-5744) sample contains 25 µL of 10 mg/mL IgG1κ monoclonal antibody in 12.5 mmol/L L-histidine, 12.5 mmol/L L-histidine HCl (pH 6.0).
- Aliquot 25 µL of NISTmAb RM 8671 sample into LC injection vial (p/n 5188-6591) and place it in LC multisampler.

Software

- Prepare following four stock solutions in 18 megOhm-cm water (see Buffer Advisor section)

  Bottle A: Water
  Bottle B: 1000mM NaCl
  Bottle C: 55mM NaH_2PO_4
  Bottle D: 50mM Na_2HPO_4 7 H_2O

Stock Solution Recipes

- NaCl: Sodium chloride
  Recipe Bottle B: Weigh 58.44 g and fill up to 1 L .
- NaH_2PO_4: Monosodium phosphate
  Recipe Bottle C: Weigh 6.5989 g and fill up to 1 L.
- Na_2HPO_4: Sodium phosphate dibasic heptahydrate
  Recipe Bottle D: Weigh 13.404 g and fill up to 1 L.
- Filter the solution through 0.22 µm membrane filter (hydrophilic PTFE) and use immediately. Always prepare mobile phase freshly.

1260 Infinity II Bio-Inert LC Installation and Method Setup

- Follow the manual and quick guide for 1260 Infinity II Bio-inert LC installation and configuration settings (https://www.agilent.com/cs/library/usermanuals/public/G5654System.pdf)

Column

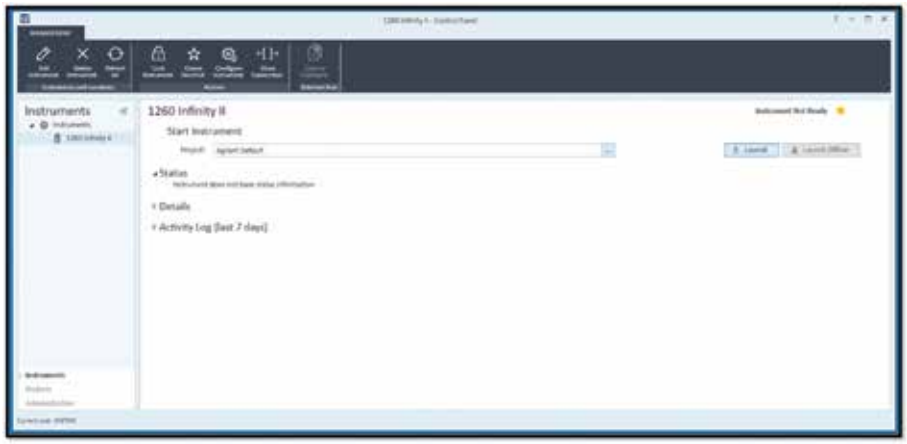
- Remove both end plugs and ensure that your system's flow direction matches the arrow on the column. Do not use the column with the flow in the reverse direction.
- Prior to applying flow over the column make tight ferrule connections.
- The columns are shipped in a 20 mM phosphate buffer, pH 6.0. Prior to first injection of the sample, purge the column with 20 column volumes of mobile phase buffer at 0.1 mL/min (starting condition) and gradually increase the flow rate (0.250 mL/min) and allow until the baseline to flatten.
- Whenever a column is not installed on the LC tightly, seal both ends of the column with the removable end plugs supplied with the column. For short term storage of less than one week, store the column in the mobile phase. For extended storage of longer than one week, flush the column with 20 mM phosphate buffer, pH 6.0 containing 0.1 % NaN_3 (sodium azide). Recommended storage temperature is 4 to 35°C.
- Further details on column maintenance can be found in the data sheet of the column (https://www.agilent.com/cs/library/datasheets/public/5973-1745.pdf)

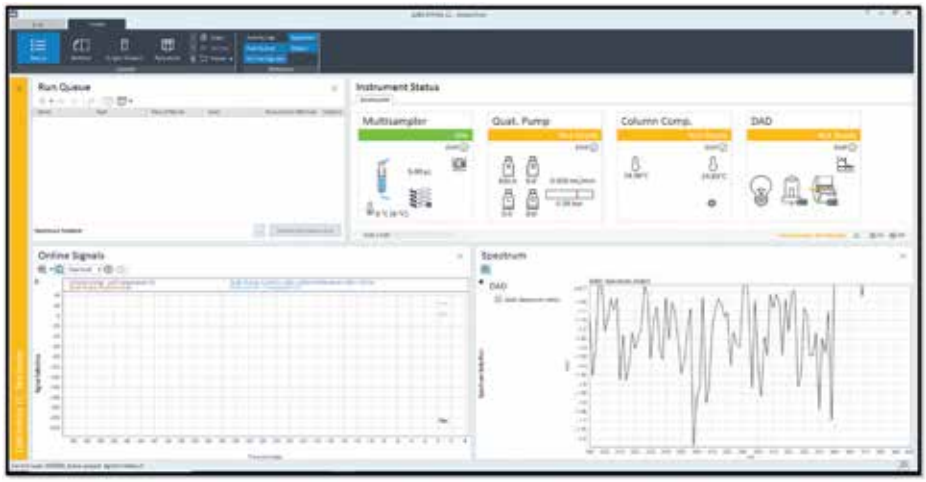
Prepare for run and method setup

Turn on the modules of the instrument. Launch the OpenLab Control panel software from the desktop.

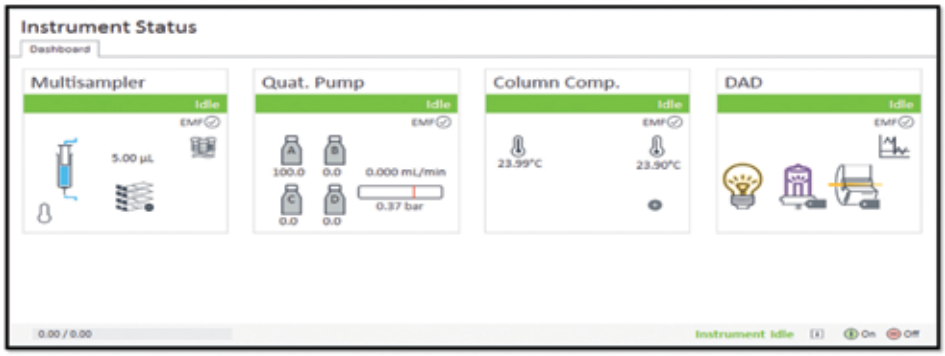
Click on the icon to launch the OpenLab Control panel.

In the opened OpenLab Control panel, click launch button to bring up the Acquisition panel.

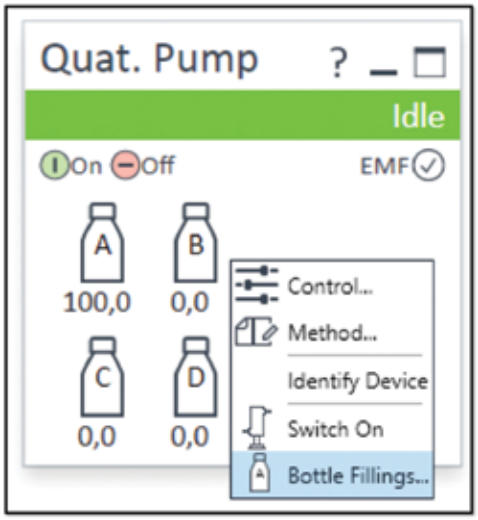


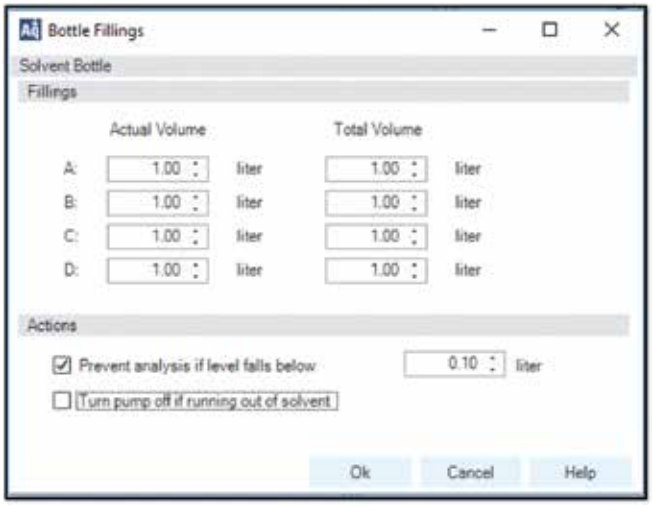


Switch on the instrument modules from the Acquisition panel



- Fill the solvent bottles with adequate buffer solutions and place it into the solvent cabinetVariation of buffer pH (± 0.2)
- Open solvent bottle filling dialog and fill in the volume of the solvents in the bottle
- Purge the pump by opening purge valve





– Enter column information using column assignment tab

Method setup

Load the default method → Change the method for NISTmAb charge variant analysis → Save as a new method

The following screenshots shows the parameters settings for each module

Multisampler (G5668A)

Quat. Pump (G5654A)

Please note the post time of 30 mins which is required to re-equilibrate the column to its initial condition. This is very important step to be included into your method. Failing to do the re-equilibration of the column leads to protein (in this case mAb) not binding to the column.

Column Comp. (G7116A)

Column Comp. (G7115A)

LC method condition summary table:

Conditions

Parameter	Settings
Column	Agilent Bio mAb, nonporous, 2.1 × 250 mm, 5 µm, PEEK (p/n 5190-2411)
Mobile phase	A: Water B: 1000mM NaCl C: 55 mM NaH_2PO_4 D: 50 mM Na_2HPO_4
TCC Temperature	25 °C
Gradient:	Fast gradient or high-resolution gradient
Run time	25 and 45 mins
Sample	NISTmAb
Injection volume	2 µL
Flow rate	0.25 mL/min
DAD	220 and 280 nm

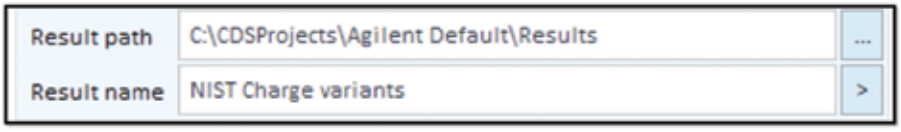
Sequence setup and sample run

– To create a sequence, navigate to sequence layout.

– In the sequence table, add lines and enter the runs as shown below and save the sequence.

		Action	Vial	Sample type	Inj/Vial	Volume	Acq. method	Injection sour	Sample name	Data file
1	☑	Inject	D1F-A1	Blank	1	Use Method	Char_VER_6.8_salt gradient 3_30mm_25...	HipAls	Blank	blank
2	☑	Inject	D1F-A1	Sample	10	Use Method	Char_VER_6.8_salt gradient 3_30mm_25...	HipAls	NISTmAb	charge variant
3	☑	Inject	D1F-D1	Blank	1	Use Method	Char_VER_6.8_salt gradient 3_30mm_25...	HipAls	Blank	blank

– Verify the sequence result path and enter a name for results file

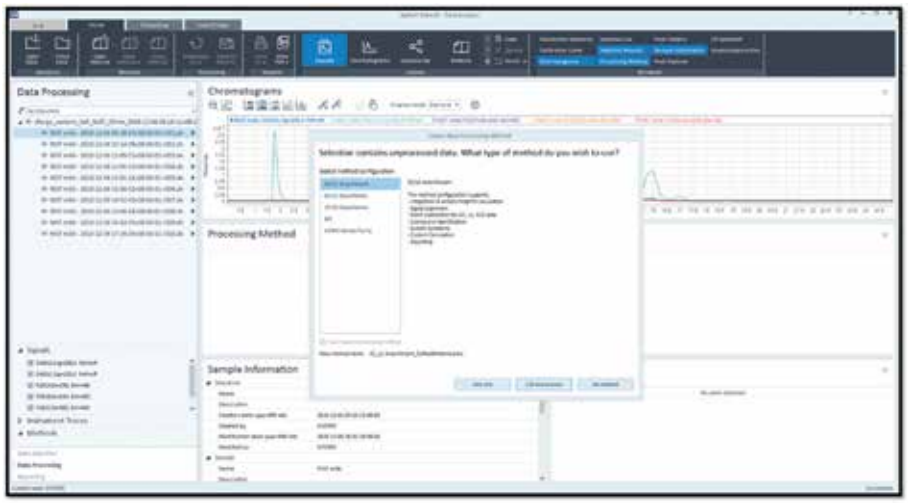


– Click run button to run the sequence

– Once the sequence is submitted, it will automatically show up in Run queue window.

Sequence setup and sample run

- In the Data Selection view, navigate to the required result set and double-click on result set.
- Navigate to the folder containing the corresponding data files, and select the required injections in the Injection List window.
- The selected result sets or injections are loaded, and the application switches to the Data Processing view.
- The workspace will be as in the following Figure:

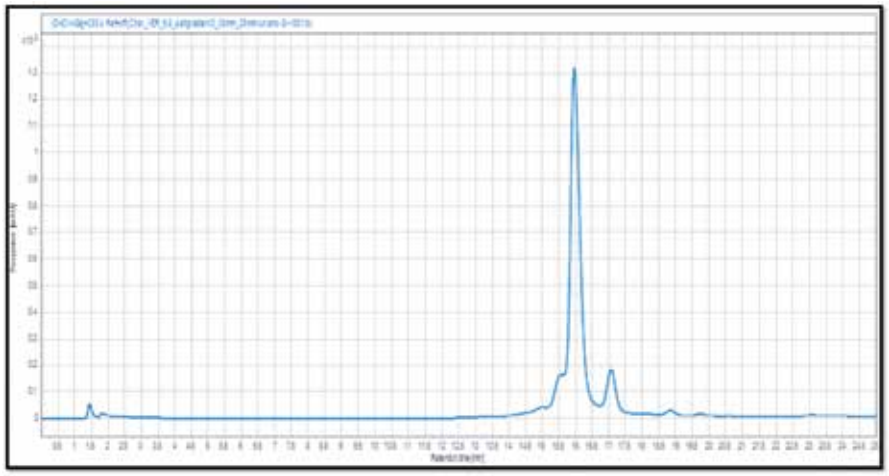


- Use the default GC-LC processing method and link and process all the data files from the sequence. Make sure to delete the integration peaks from the injection peak manually using this button
- Export all the method attributes (retention time, area, resolution) to MS Excel and calculate the % relative standard deviations (%RSD)

Expected results:

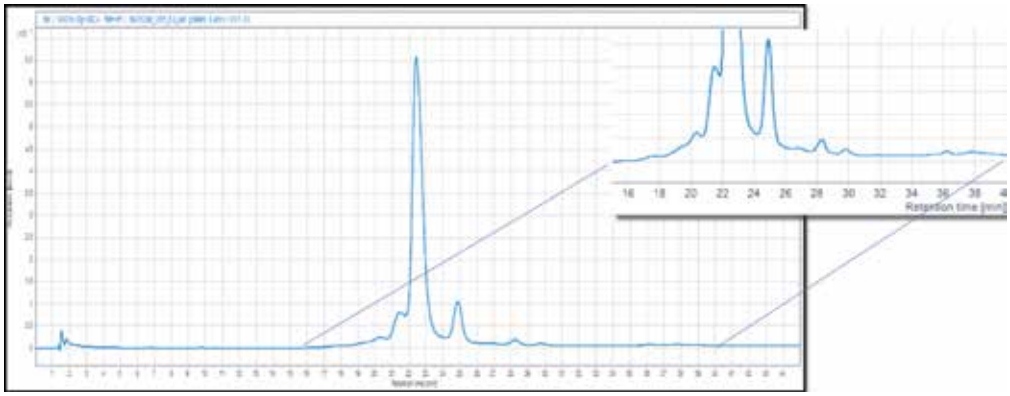
– NISTmAb RM 8671

Faster analysis



Peak id	RT (min)	Area %	Resol. USP
Acidic variants	12.09-15.65L	13.03	
Main peak	15.65-16.71	73.66	0.24 (main peak/acidic)
Basic variants	16.71-19.98	13.31	2.14 (main peak/basic)

High resolution analysis



Peak id	RT (min)	Area %	Resol. USP
Acidic variants	16.27-21.66	13.00	
Main peak	21.66-23.95	73.83	0.59 (main peak/acidic)
Basic variants	23.95-30.1	13.17	2.50 (main peak/basic)

Troubleshooting:

Condition	Reason	Fix
Poor resolution of peak of interests	Suboptimal elution conditions	Change elution conditions: use shallower gradient or reduce flow rate
	Sample is viscous	Dilute the sample with buffer to reduce viscosity of the sample
	Column overloaded	Decrease sample load
	Microbial contamination in the column	Follow cleaning procedures as recommended
Proteins does not bind to the column	Some particulates in sample	Filter the sample and re-run the experiment
	Sample condition changed during storage	Prepare fresh samples
	Column equilibration incompletes	Increase the equilibration time for the column
	Incorrect buffer pH and/or ionic strength	Prepare new solutions
Protein elutes late or does not elute from the column	Incorrect buffer pH	Prepare new solutions
	Ionic strength too low	Increase salt concentration in elution buffer
	Ionic strength of sample or buffer is too high	Decrease ionic strength of sample or buffer
Protein elutes earlier	Column equilibration incomplete	Increase the equilibration time for the column

Application Note

Biopharmaceuticals, Biotherapeutics

Agilent

Trusted Answers

High-resolution Analysis of Charge Heterogeneity in Monoclonal Antibodies Using pH-gradient Cation Exchange Chromatography

Agilent 1260 Infinity Bio-inert Quaternary LC System with Agilent Bio Columns

Authors

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Agilent Technologies, Inc.
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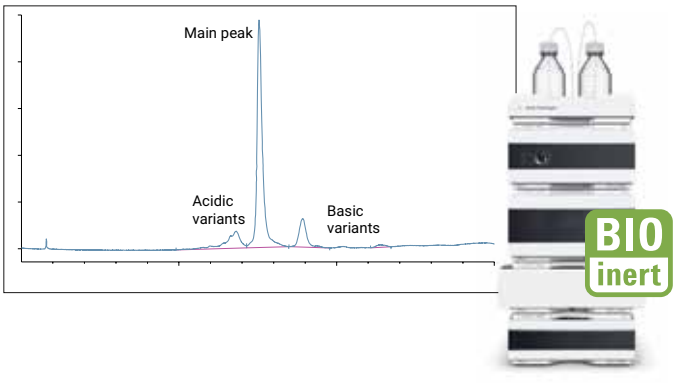
Abstract

Antibody charge variants have gained considerable attention in the biotechnology industry due to their potential influence on stability and biological activity. Subtle differences in the relative proportions of charge variants are often observed during routine manufacture or process changes and pose a challenge when demonstrating product comparability. These changes include differences in glycosylation, deamidation, oxidation, isomerization, incomplete C-terminal processing, and other post-transitional modifications that modify the isoelectric pH (pI) values. In the biotechnology industry, ion-exchange chromatography is widely used for profiling the charge heterogeneity of proteins, including monoclonal antibodies. This Application Note describes a high-resolution, pH-based separation of acidic and basic charge variants for monoclonal antibodies using the Agilent 1260 Infinity Bio-inert Quaternary LC System and an Agilent BiomAb PEEK 4.6 × 250 mm, 5 µm ion exchange column that features a unique resin specifically designed for the charge-based separation of mAbs. The robustness of the method for routine analysis was established by validation studies.

Introduction

Monoclonal antibodies (mAb) are glycoproteins of the immunoglobulin (Ig) family. MAbs have become the most rapidly growing class of biotherapeutics in the development for many different disease conditions. Novel mAb molecules are entering clinical studies at a rate of almost 40 per year, and the research pipeline includes approximately 250 therapeutic mAbs in clinical studies. There is steadily increasing need for an analytical method that can be used for high-throughput analysis of a large number of samples to support bioprocesses and formulation development. Biotherapeutics, such as mAbs, are complex molecules, and a variety of methods is required to monitor the heterogeneities associated with the mAb to ensure product quality and consistency.[1] Cation exchange chromatography is the gold standard for charge-sensitive antibody analysis. In cation exchange chromatography, method parameters often need to be optimized for each individual protein as ion exchange is dependent on the reversible adsorption of the charged protein molecules to immobilized ion exchange groups. Several authors have made significant progress in demonstrating practical separations using pH changes in the mobile phase to elute the proteins.[2] The Agilent ion exchange column family offers strong cation exchange (SCX), weak cation exchange (WCX), strong anion exchange (SAX) and weak anion exchange (WAX). The Agilent Bio MAb NP 5 (nonporous, 5 µm) PEEK, 4.6 × 250 mm, column is specifically designed to characterize the charge heterogeneity of monoclonal antibodies, including C-terminal lysine variance. The column offers even higher resolution, enabling better peak identification and accurate quantification. This Application Note describes a pH gradient based method for separating the charge variants of IgG1 using a 1260 Infinity Bio-inert Quaternary LC System and a Bio MAb NP 5 PEEK, 4.6 × 250 mm, ion exchange column. Method validation and robustness of an optimized ion exchange method are described.

Equipment



Instrumentation

A completely biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC System operating up to a maximum pressure of 600 bar was used for the experiments (Table 1). The entire sample flow path is free of any metal components so that the sample does not come in contact with metal surfaces. Solvent delivery is free of any stainless steel or iron components.

Table 1. Configuration of the Agilent 1260 Infinity Bio-inert Quaternary LC System.

Description	Model number
Agilent 1260 Infinity Bio-inert Quaternary Pump	G5611A
Agilent 1260 Infinity Bio-inert High Performance Autosampler	G5667A
Agilent 1290 Infinity Thermostat (for autosampler)	G1330B
Agilent 1260 Infinity Thermostatted Column Compartment with bio-inert click-in heating elements (option 019)	G1316C
Agilent 1260 Infinity Diode Array Detector with 60-mm Max-Light high sensitivity flow cell (option 033)	G4212B

Software

Agilent OpenLAB CDS ChemStation Edition, revision C.01.04.

Ion Exchange Chromatography Parameters

Table 2 shows the Chromatographic parameters for Ion Exchange Chromatography using Agilent 1260 Infinity Bio-inert LC System.

Reagents, samples and materials

Human monoclonal antibody IgG1 was a proprietary pharmaceutical molecule. Sodium phosphate dibasic dihydrate, sodium phosphate monobasic dihydrate, sodium chloride, sodium bicarbonate hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from Sigma Aldrich. All the chemicals and solvents used were HPLC grade and high purity water from Milli Q water purification system (Millipore Elix 10 model, USA) was used.

Procedures

Mobile phase A was 10 mM sodium phosphate, pH 6.0 and mobile phase B was 10 mM sodium phosphate, pH 9.5. Monoclonal antibodies were diluted to approximately 2 mg/mL in mobile phase A and elution was monitored at 220 nm and 280 nm. Area and retention time (RT) were used to calculate standard deviation (SD) and relative standard deviation (%RSD). For each elution, the column was pre-equilibrated with at least three column volumes of mobile phase A prior to sample injection. After the injection of the monoclonal antibody sample onto the column, a linear increase in the percentage of mobile phase B was delivered. The linear gradients were run from 0 to 100% B in 27 minutes at 1 mL/min flow rate. After the gradient, the mobile phase was pumped at 100% B until at least one column volume passed before the composition was returned to 100% A in preparation for the subsequent analysis. Relative percent area was used to quantify the charge variants of monoclonal antibodies.

Robustness Study

The four critical method parameters listed below were varied to validate the IEX procedure.

- Variation of injection volume (± 10%)
- Variation of buffer pH (± 0.2)
- Variation of flow rate (± 2%)
- Variation of column temperature (± 5%)

For each robustness parameter, 10 µL of IgG1 was injected six times to calculate average area and RT. The percentage deviation (% accuracy) of area and retention time (RT) was calculated from the optimized method.

Table 2. Chromatographic parameters used for IEX chromatography.

Conditions

Parameter	Settings	
Column:	Agilent Bio MAb PEEK, 4.6 × 250 mm, 5 µm column (p/n 5190-2407)	
Mobile phase A:	10 mM sodium phosphate buffer, pH 6.0	
Mobile phase B:	10 mM sodium bicarbonate buffer, pH 9.5	
Gradient:	Time (min) 0 minutes 25 minutes 27 minutes 30 minutes	Mobile phase (% B) 0% B 100% B 100% B 0% B
Injection Volume:	10 µL (needle with wash, flush port active for 7 seconds)	
Flow Rate:	1.0 mL/min	
Data acquisition:	214 and 280 nm	
Acquisition rate:	20 Hz	
Flow cell:	60-mm path	
Column Temperature:	30 °C	
Sample Throughput:	5 °C	
Post time:	5 minutes	

Results and Discussion

Separation and detection

The Agilent Bio MAb columns are highly uniform, densely packed, weak cation exchange resin. This Application Note used the 5-µm column that features a unique resin specifically designed for high-resolution chargebased separations of monoclonal antibodies. The peaks of the ion exchange profiles were typically denoted into three distinct components[3]. Early and late-eluting peaks were called acidic and basic variants, respectively. The most abundant peak was designated as the main peak. Figure 1 shows the optimized cation exchange elution profile of IgG1 on Bio MAb PEEK column demonstrating excellent separation of IgG1 in 30 minutes into three distinct peaks: basic variants, main peak, and acidic variants. The overlay of six replicates of IgG1 shows excellent separation reproducibility (Figure 2). The high resolution separation of IgG1 facilitated the quantification of charge variants using peak areas (Figure 3). The relative peak areas for the charge variants of IgG1 are shown in Table 3. The IgG1 possessed approximately 9.97% of acidic variants, 76.92% main peak and 13.21% basic variants of the total peak area, respectively. The reproducibility of analysis was tested with six replicates.

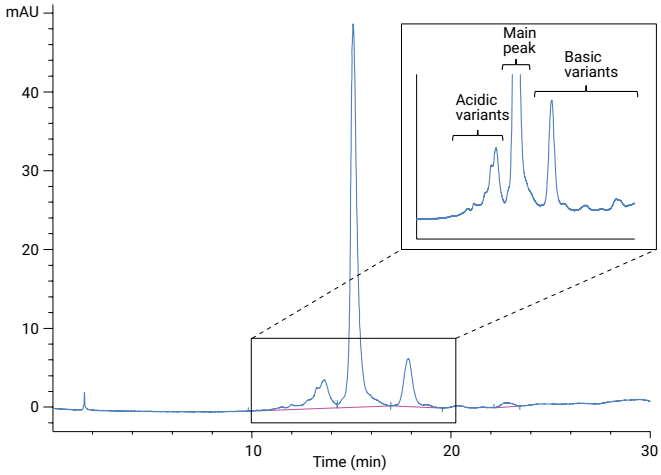


Figure 1. Elution profile of pH based separation of charge variants of IgG1 on an Agilent Bio MAb PEEK, 4.6 × 250 mm, 5-µm column. The acidic, main peak and basic variants are enlarged in the magnified view.

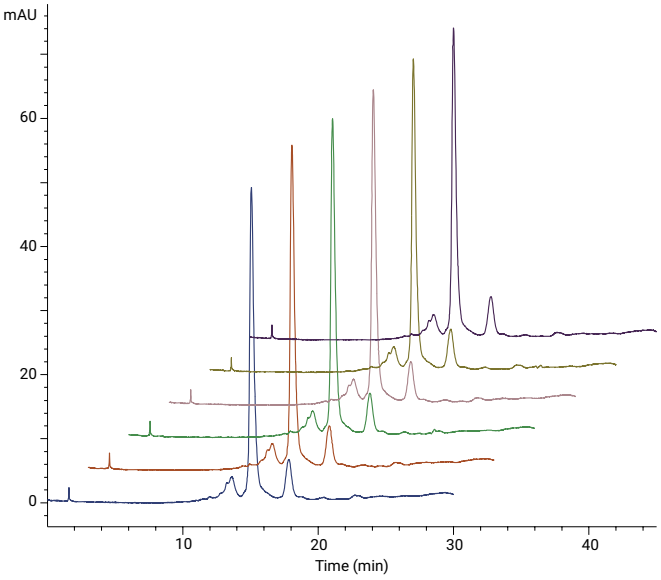


Figure 2. Overlay of six replicates of IgG1 on an Agilent 1260 Infinity Bio-inert Quaternary LC System using an Agilent Bio MAb PEEK, 4.6 × 250 mm, 5 µm column.

Precision of Retention Time and Area

The precision of a procedure expresses the closeness of agreement between a series of measurements obtained from multiple analyses of the homogeneous sample under the prescribed conditions and often expressed as relative standard deviation (RSD). Table 4 shows the average retention times and area RSDs from six replicates of an IgG1 injection. The retention time and peak area RSDs for the main peak were 0.106% and 1.60% respectively which demonstrates excellent reproducibility of the method and thus the precision of the system.

Table 3. Charge variants quantification by area %, n = 6.

	RT (min)	Area %
Acidic variants	13.28 13.61	9.87
Main peak	15.058	76.92
Basic variants	17.82 22.69	13.21

Table 4. Retention time and area RSD (%), n = 6 for main peak.

	RT (min)	Area %
Mean (min)	15.058	1172
RSD	0.106	1.60

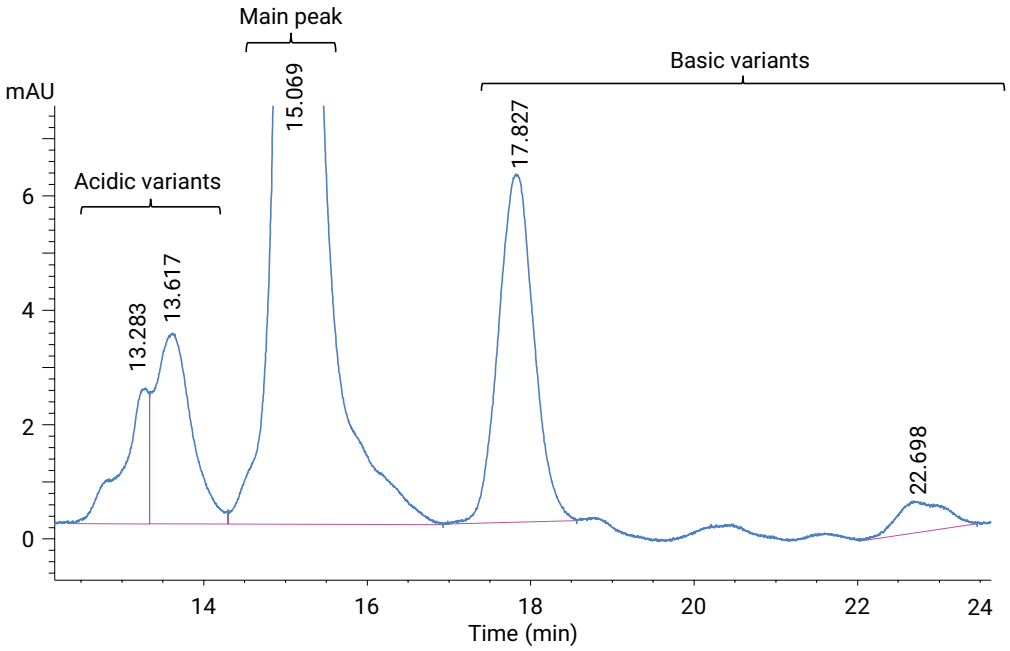


Figure 3. Expanded scale chromatogram of pH gradient-based cation exchange chormatogram of IgG1 separation using an Agilent Bio MAb PEEK, 4.6 × 250 mm, 5 µm column.

Precision of Retention Time and Area

The The robustness of an analytical procedure is the persistence of a method under perturbations or conditions of uncertainty and provides an indication of its consistency during routine use. To evaluate the robustness of the method, four critical parameters of the optimized method were varied (Table 5). Allowed deviations for RT and area RSD were set to ± 3.0% and ± 5% respectively. The red numbers indicate where the result exceeded the allowed deviation. The impact of injection volume, column temperature, buffer pH and flow rate on RT and area RSD was within the acceptable limits.

A variation in injection volume by ± 10% compared to the actual method caused the area RSD to deviate significantly; however, this deviation is an expected due to the load on the ion exchange column. There were no further significant changes in the chromatographic pattern when deliberate variations were made in experimental conditions, thus showing the method is robust. Our results show that the method is reliable for routine QA/QC application for manufacturing and storage consistency. However, some parameters such as injection volume are critical and must be carefully controlled.

Table 5. Robustness (RT and Area % RSD) n = 6.

		RT deviation (limit: ± 3.0 %)	Area deviation (limit: ± 5.0 %)
Parameters	**Variations**	**Main peak**	
Variation in injection volume (10 µL ± 10%)	− 1 µL + 1 µL	-0.19 0	10.49 -9.89
Variation in column temperature (30 °C ± 5%)	− 5% + 5%	-1.19 0.66	2.73 2.13
Variation in buffer pH (6.0 ± 0.2)	− 0.2 + 0.2	0.199 0.99	-0.68 -0.08
Variation of flow rate (1.0 ± 2%)	− 2% + 2%	0.66 0	2.73 -1.10

Conclusion

Cation exchange chromatography has been widely used for separating charge heterogeneity of monoclonal antibodies. This Application Note shows how the Agilent 1260 Infinity Bio-Inert LC System and an Agilent Bio MAb PEEK, 4.6 × 250 mm, 5 µm column were used to perform reproducible and high resolution analysis of charge variants in monoclonal antibodies for biopharmaceutical process development and process monitoring. A simple pH gradient-based cation exchange method for separation and quantification of charge variants was developed. Area, RT precision, and robustness of the method were excellent and show the reliability of the method. There were no significant changes in the chromatographic profile when the modifications were made in experimental conditions, thus showing the method to be robust. The bio-inertness and corrosion resistance of the instrument coupled with simple and reproducible method make this solution particularly suitable for the QA/QC analysis of monoclonal antibodies in the biopharmaceutical industry.

Reference

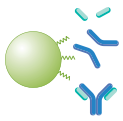
1. Yan He *et al., J. Sep. Sci.,* **2011**, 34, 548–555
2. Dell Farnan, *Anal. Chem.,* **2009**, 81, 8846–8857
3. Jennifer C. Rea, *Journal of Pharmaceutical and Biomedical Analysis,* 54 (**2011**) 317–323

Intact and Subunit Analysis- Reversed Phase Chromatography

Introduction

Reversed-phase chromatography remains one of the most valuable tools in the chromatographer's armory. In reversed-phase chromatography, proteins are separated based on relative hydrophobicity under denaturing conditions by adsorption on a hydrophobic column followed by a gradient elution with an increasing concentration of an organic solvent. For intact proteins, the technique uses gradients of organic solvents as mobile phase, typically with an ion pair reagent. Under these conditions, the molecule is likely to become denatured. It is a sensitive technique as the sample is concentrated as it is retained by the column and it is monitored with mass spectrometry. It is therefore suitable for determining the accurate mass of an intact protein. For large proteins, such as monoclonal antibodies, and even for smaller fragments such as heavy and light chains or Fab and Fc regions of an IgG molecule, wide pore columns are recommended. Agilent offers 300 and 450 Å products in many configurations including fully porous, Poroshell technology featuring superficially porous particles, and a selection of alkyl bonded phases. The bonded phase is typically shorter chain length (C8 or C4/C3) or more unique ligands such as diphenyl that may offer different selectivity.

Although the intact reversed-phase analysis of the mAb standard was able to provide a relatively detailed assessment of product quality, for mAb fragments and structural variants, Reversed-phase analysis under reduced conditions provides a more detailed view of post-translational modifications on the individual light and heavy chains. Reversed-phase chromatography it is possible to use intact and fragment analysis to accurately compare biosimilars with originator biotherapeutics. However, it is always necessary to perform orthogonal tests to identify the specific location of the different variants that may be detected.



Intact and Subunit Purity

Large molecule chromatography (>150 Å)

Selectivity options for every separation need

AdvanceBio RP-mAb

Ideal for monoclonal antibodies

Attribute	Advantage
450 Å pore, superficially porous particles	Optimum design for high-resolution mAb separations
Extended column lifetime	Lower operating costs

ZORBAX RRHD 300 Å 1.8 µm

UHPLC separations

Attribute	Advantage
1200 bar maximum pressure	UHPLC-compatible
1.8 µm particles	Maximum resolution

PLRP-S

Ideal formic acid performance for MS detection

Attribute	Advantage
Polymeric particle with no silanol interactions	Better peak shape, better recovery, and lower carryover
Durable, resilient particles	Reproducible results over longer lifetimes

Introduction

The successful development of a reversed-phase mAb method is often considered a challenging task due to the molecule's size, the structural complexity inherent to mAbs and numerous post-translational modifications. Selecting a reversed-phase column for intact protein analysis requires consideration of several interrelated factors: Sample molecular weight and the best suited particle pore size, column chemistry, the instrumentation to be used particularly the type of detector, mobile phase conditions, and speed or throughput requirements to name a few. For reversed-phase columns, a general guideline for choosing a column chemistry, is the higher the molecular weight, the shorter the alkyl chain should be. Hence, C18 columns are commonly used for peptides while C8, C4, and C3 columns are commonly used for intact protein separations. In addition to linear alkyl chains, diphenyl phases are available for the AdvanceBio RP mAb, ZORBAX RRHD 300, and ZORBAX 300SB columns. Sometimes, the alternate selectivity of the diphenyl phase can provide the separation needed. PLRP-S is a polymeric particle rather than a silica-based particle. It gives a typical reversed-phase separation, although with somewhat different selectivity and the advantage of wide pH tolerance. Larger analytes require larger pore sizes. With some exceptions, pore sizes for intact protein analysis are typically 300–500 Å. As a rule of thumb, the pore size should be at least three times the hydrodynamic radius of the protein. The AdvanceBio RP mAb column has 450 Å pores, ZORBAX RRHD 300 Å, ZORBAX 300SB, and Poroshell 300 all have 300 Å pores, and PLRP-S is available in many pore sizes. While substantially larger than is commonly used for intact proteins, the 1000 Å, 5 µm PLRP-S columns give excellent results for intact protein and protein fragment analysis.

The instrumentation available determines what maximum pressure can be achieved. One can certainly use a column with a 600 bar pressure maximum on a UHPLC capable of 1200 bar. But care should be taken not to over pressure the column, which can lead to premature column failure. Within Agilent's reversed-phase portfolio, the ZORBAX RRHD column has a maximum backpressure of 1200 bar, and can thus be used for high speed, high-pressure separations. When considering instrumentation and backpressure capabilities, it is worth considering whether the method under development will ever need to be transferred to another LC system with a different maximum backpressure. If so, it would be cost- and time-effective to develop a method that can be run on all platforms. Detector selection and mobile phase conditions are often related.

For protein separations, this is commonly a decision between using UV detection or mass spectrometry (MS). Traditionally trifluoroacetic acid (TFA) has been used as an ion pairing agent for separations with UV detection, while formic acid is preferred for MS detection. TFA is typically used for UV detection as it gives excellent peak shape on silica-based columns, however it leads to ion suppression in mass spectrometry. Formic acid preserves MS sensitivity, but gives less than ideal peak shape on silica-based columns, therefore polymeric PLRP-S column is recommended for formic acid mobile phases. With an understanding of the trade-offs, one can use formic acid mobile phase with silica-based columns, or TFA with mass spectrometry. There's also no disadvantage to using formic acid or PLRP-S with UV detection.

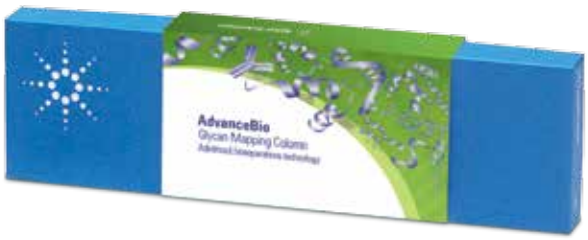
Water/acetonitrile gradients are commonly used for reversed-phase separations of intact proteins and monoclonal antibody fragments and are generally suitable for Agilent reversed-phase columns. A different organic solvent, such as methanol or isopropanol may produce a helpful change in selectivity in the case of some separations. The AdvanceBio RP mAb columns give their best results with an organic mobile phase containing isopropanol, acetonitrile, and water. In addition, low pH (~2) ion pair reagent and high column temperature have been shown to be critical parameters for protein recovery and peak shape. In fact, column temperatures of at least 65 to 70 °C should be used to provide optimal protein recovery and resolution.

Featured application notes in this section describes a seamless Integrated workflow using the Agilent 1290 Infinity II UHPLC system, 6545XT AdvanceBio LC/Q-TOF, and automatic data processing with Agilent MassHunter BioConfirm software to analyze a variety of Intact mAb products and subunits including NISTmAb standard.

Agilent
Trusted Answers

Quick Reference Guide

Reversed-Phase LC Primary Structure Characterization Workflow



Agilent AdvanceBio RP-mAb Columns

In this document Agilent applications chemists share their recommendations for an optimum LC system and its configuration for characterizing biomolecules. They also offer guidance on a generic method to get you started, and how this method can be further optimized to meet your specific separation goals.
Additional application information is available at **www.agilent.com/chem/advancebio**

Guidelines

- **Bonded phase:** C4 and SB-C8 are routinely used. For alternative selectivity use the Diphenyl column.
- **Gradient:** IPA:ACN:water with 0.1% TFA or 0.1% FA to elute all components of interest.
- **Sample solubility:** Mix with starting mobile phase.
- **Temperature:** Higher column temperature can dramatically improve resolution and recovery of proteins.
- **Resolution/selectivity**: A blend of IPA:ACN provides better resolution. Other organic solvent substitutions can be used for different selectivity. Use of IPA results in increased pressure, which can be managed by column temperature and flow rate.

LCMS

- Desalt protein samples before injection.
- Small id columns (e.g. 2.1 mm) are often the best choice with a 0.5 to 1.0 mL/min flow rate.
- FA provides better MS signals. Use less TFA in the eluent to enhance MS signals, or use AcOH.
- IPA with ACN in the mobile phase provides sharper TIC peaks.

Agilent 1260 Infinity Bio-Inert LC System

Mobile phases

Eluent A: 0.1% TFA

Eluent B: IPA, ACN, and water with 0.09% TFA

Pump (G5611A)

Typical flow rate for 2.1 mm id columns is 1.0 mL/min

Sample injection (G5667A)

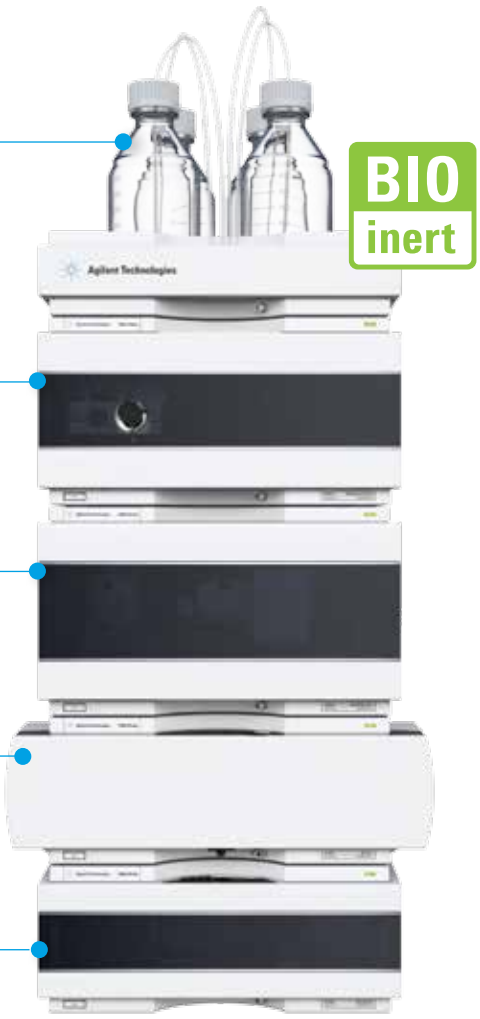
1 to 5 µL injection for samples containing 1 to 5 mg/mL of mAb.
Samples can be dissolved in water or eluent A.

Column compartment (G1316C))

60 to 90 °C is a typical temperature for good separation

Detection (G1315D)

UV (210, 280 nm) with a 10 mm bio-inert standard flow cell

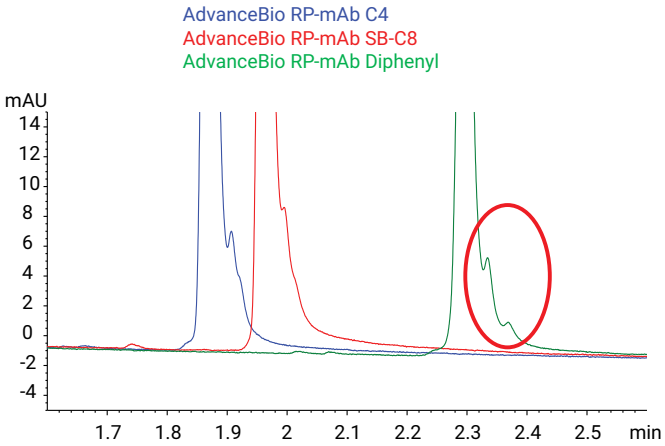


Suggested gradient for resolution

Column	C4	SB-C8	Diphenyl
2.1 x 50 mm, 3.5 µm	799775-904	789775-906	799775-944
2.1 x 75 mm, 3.5 µm	797775-904	787775-906	797775-944
2.1 x 100 mm, 3.5 µm	795775-904	785775-906	795775-944
2.1 x 150 mm, 3.5 µm	793775-904	783775-906	793775-944
4.6 x 50 mm, 3.5 µm	799975-904	789975-906	799975-944
4.6 x 100 mm, 3.5 µm	795975-904	785975-906	795975-944
4.6 x 150 mm, 3.5 µm	793975-904	783975-906	793975-944

Intact mAb Analysis

Fast and high-resolution separation

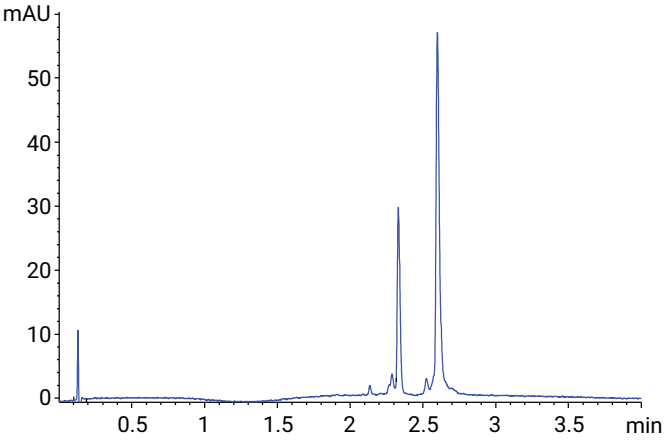


Conditions

Parameter	Value
Columns:	2.1 x 100 mm, 3.5 µm
Eluent A:	0.1% TFA in water:IPA (98:2)
Eluent B:	IPA:ACN:Eluent A (70:20:10)
Flow rate:	1.0 mL/min
Gradient:	10-58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B
Injection:	5 µL (1 mg/mL)
Sample:	Herceptin IgG1 variant
Temperature:	80 °C
Detection:	UV, 254 nm

mAb Fragment Analysis

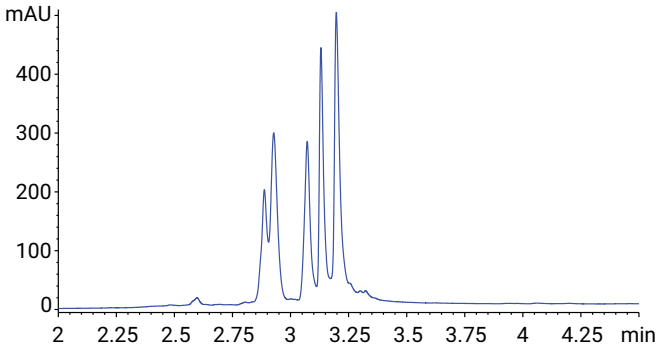
Chemical digestion – heavy chain/light chain



Conditions

Parameter	Value
Column:	AdvanceBio RP-mAb Diphenyl, 2.1 x 50 mm, 3.5 µm
Eluent A:	0.1% TFA in water
Eluent B:	IPA:ACN:water (70:20:10) + 0.09 % TFA
Flow rate:	1 mL/min
Gradient:	0 min, 15% B; 0.5 min, 25% B; 1.5 min, 35% B; 1.51 min, 35% B; 3 min, 60% B; 4 min, 60% B
Injection:	1 µL (1 mg/mL) (TECP reduction)
Sample:	Rituximab innovator
Temperature:	80 °C
Detection:	UV, 220 nm

Enzymatic digestion – Fab/Fc regions

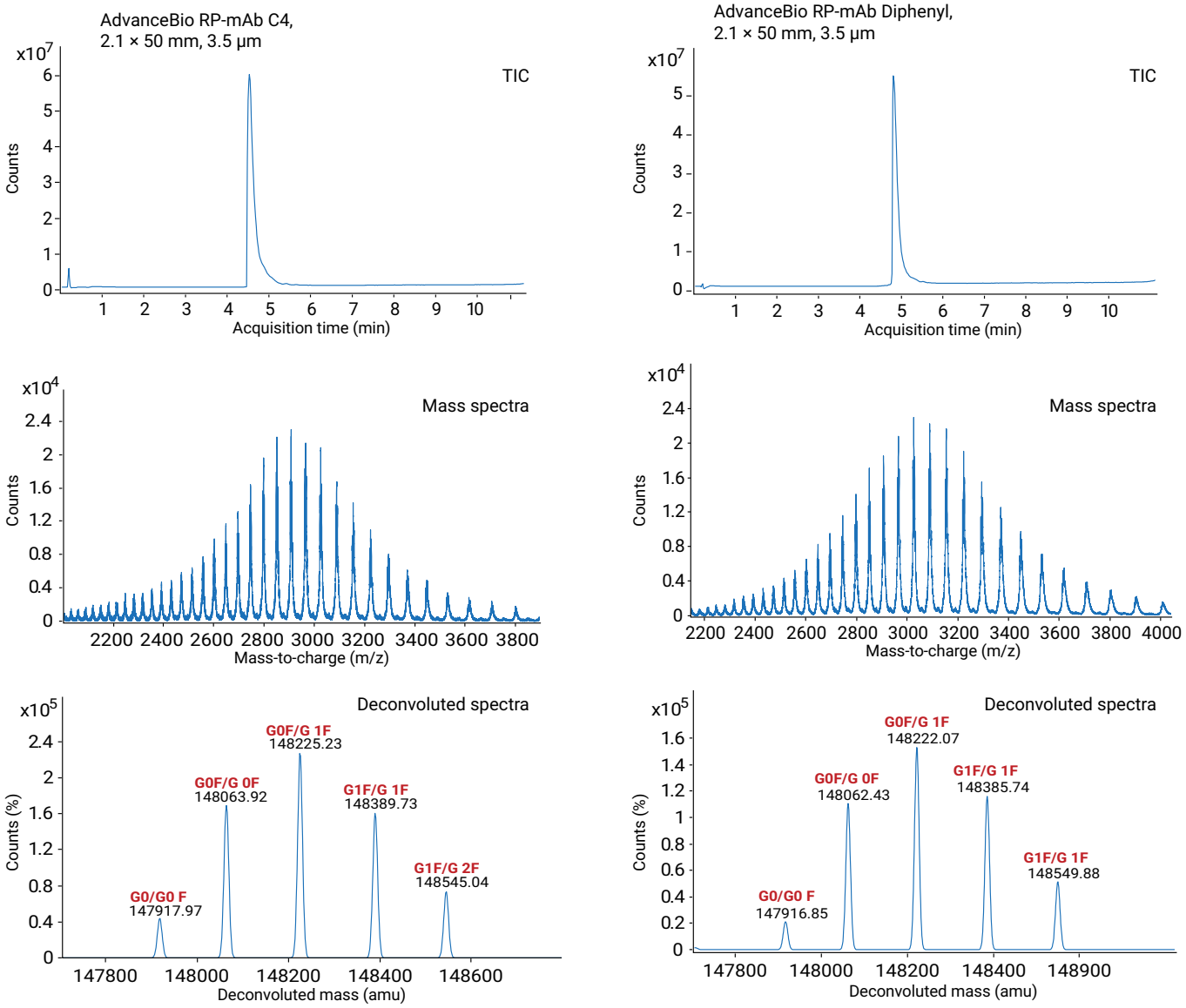


Conditions

Parameter	Value
Column:	AdvanceBio RP-mAb C4, 2.1 x 100 mm, 3.5 µm
Eluent A:	0.1% TFA in water
Eluent B:	n-Propanol:ACN:eluent A (80:10:10)
Flow rate:	0.8 mL/min
Gradient:	5-40% B in 5 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B
Injection:	1 µL (2 mg/mL)
Sample:	Herceptin IgG1 variant – papain digested
Temperature:	60 °C
Detection:	UV, 220 nm

LC/MS Analysis of Intact mAbs

Fast chromatography with excellent peak shape and MS data using formic acid mobile phase



Conditions

Parameter	Value
Eluent A:	0.1% FA in water
Eluent B:	IPA:ACN:water (80:10:9.9) + 0.1% FA
Gradient:	0 min, 20% B; 4 min, 20% B; 5 min, 40% B; 10 min, 70% B; 11 min, 90% B; 11.1 min, 20% B
Flow rate:	0.6 mL/min

Parameter	Value
Temperature:	80 °C
Injection:	1 µL (1 µg/µL)
Sample:	Innovator Herceptin
Detection:	Agilent 6530 Accurate-Mass Q-TOF LC/MS

Application Note

Biotherapeutics and Biosimilars

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Precise Characterization of Intact Monoclonal Antibodies

Using the Agilent 6545XT AdvanceBio LC/Q-TOF

Authors

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Introduction

Monoclonal antibodies (mAbs) are a very important class of biopharmaceutical molecules. As a protein drug, thorough characterization of the mAb is required in each of the manufacturing steps. Intact mAb analysis offers rapid assessment on determining the accurate molecular weight of an mAb product and its degree of heterogeneity, such as post-translational modifications (PTMs), antibody-drug conjugate (ADC), mAb sequence variations, or degradation products. Quadrupole Time-of-flight (Q-TOF) LC/MS systems are often used to analyze intact proteins or antibodies due to excellent resolution at the high mass range[1-3]. The Agilent 6545XT AdvanceBio LC/Q-TOF system includes hardware and software features to significantly improve the measurement of biomolecules up to 30,000 m/z. This Application Note describes a seamless workflow using the Agilent 1290 Infinity II UHPLC system, 6545XT AdvanceBio LC/Q-TOF, and automatic data processing with Agilent MassHunter BioConfirm software to analyze a variety of mAb products.

Figure 1. Agilent 6545XT AdvanceBio LC/Q-TOF system.

Experimental

Materials and methods

Monoclonal antibody standard RM 8671 was purchased from National Institute of Standards & Technology (NIST). The formulated Herceptin (Trastuzumab) and formulated ADC (T-DM1) were from Genentech (So. San Francisco, California, USA). All mAb samples were diluted with DI water to 1.0 µg/µL.

LC/MS analysis

LC/MS analyses were conducted on an Agilent 1290 Infinity II UHPLC system coupled with an Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with a Dual Agilent JetStream source. LC separation was obtained with an Agilent PLRP-S 1000 Å column (2.1 × 50 mm, 5 µm). Table 1 and Table 2 list the LC/MS parameters used. Approximately 0.5 µg of mAb sample was injected for each analysis.

Data processing

All MS data of the mAbs were analyzed using the Protein Deconvolution feature of MassHunter BioConfirm B.08.00 software that uses the Maximum Entropy algorithm for accurate molecular mass calculation. Averaging spectra across the top 25 % of peak height over the mass range of 2,000 to 5,000 m/z was used for mass deconvolution. The deconvoluted mass range was set at 140,000 to 160,000 Daltons.

Results and Discussion

While multiple mAbs were analyzed, a common methodology led to excellent data quality for all samples examined. Figure 2 demonstrates the intact protein analysis on the NISTmAb standard. Approximately 0.5 µg of was injected (without sample desalting preparation) onto an Agilent PLRP-S column using a 4-minute gradient with a flow rate of 0.5 mL/min. High-quality MS spectra with multiply-charged ion envelopes range of intact mAb were obtained over the mass range of 2,000 to 5,000 m/z. The zoom-in spectrum (Figure 2 inset) of each charge state clearly shows the six major glycoforms of the NISTmAb.

The BioConfirm B.08 Protein Deconvolution feature provides not only automatic mass range detection, but also accurate determination of zero-charge state spectra, resulting in excellent mass accuracy. Figure 3 illustrates the MS deconvolution result of the intact NISTmAb. Low ppm errors (average: <0.5 ppm) and superior MS resolution were achieved for all six major glycoforms of the NISTmAb. Beyond the major features of the mAb, other minor heterogeneities of glycosylation such as the loss of GlcNAc residues were easily identified. The raw data gathered by the LC/Q-TOF and the minimal processing of the Maximum Entropy deconvolution algorithm allow the user to detect and preserve fine details about the intact protein composition. This is important, especially when comparing more aggressive analysis techniques that employ high levels of data processing and manipulation that can obscure minor structures.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II UHPLC Systemm	
Column	Agilent PLRP-S, 1000 Å, 2.1 × 50 mm, 5 µm (p/n PL1912-1502)
Thermostat	4 °C
Solvent A	0.1 % Formic acid in DI water
Solvent B	0.1 % Formic acid in 100 % acetonitrile
Gradient	0–1 minutes, 0–20 % B 1–3 minutes, 20–50 % B 3–4 minutes, 50–70 % B
Column temperature	60 °C
Flow rate	0.5 mL/min
Injection volume	0.5 µL

Table 2. MS Acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System	
Source	Dual Agilent Jet Stream
Gas temperature	350 °C
Gas Flow	12 L/min
Nebulizer	60 psig
Sheath gas temperature	400 °C
Sheath gas flow	11 L/min
Capillary voltage	5,500 V
Nozzle voltage	2,000 V
Fragmentor	380 V
Skimmer	140 V
Quad AMU	500 *m/z*
Mass range	100–10,000 *m/z*
Acquisition rate	1.0 spectra/s
Reference mass	922.0098
Acquisition mode	Positive, Extended (10,000 *m/z*) Mass Range

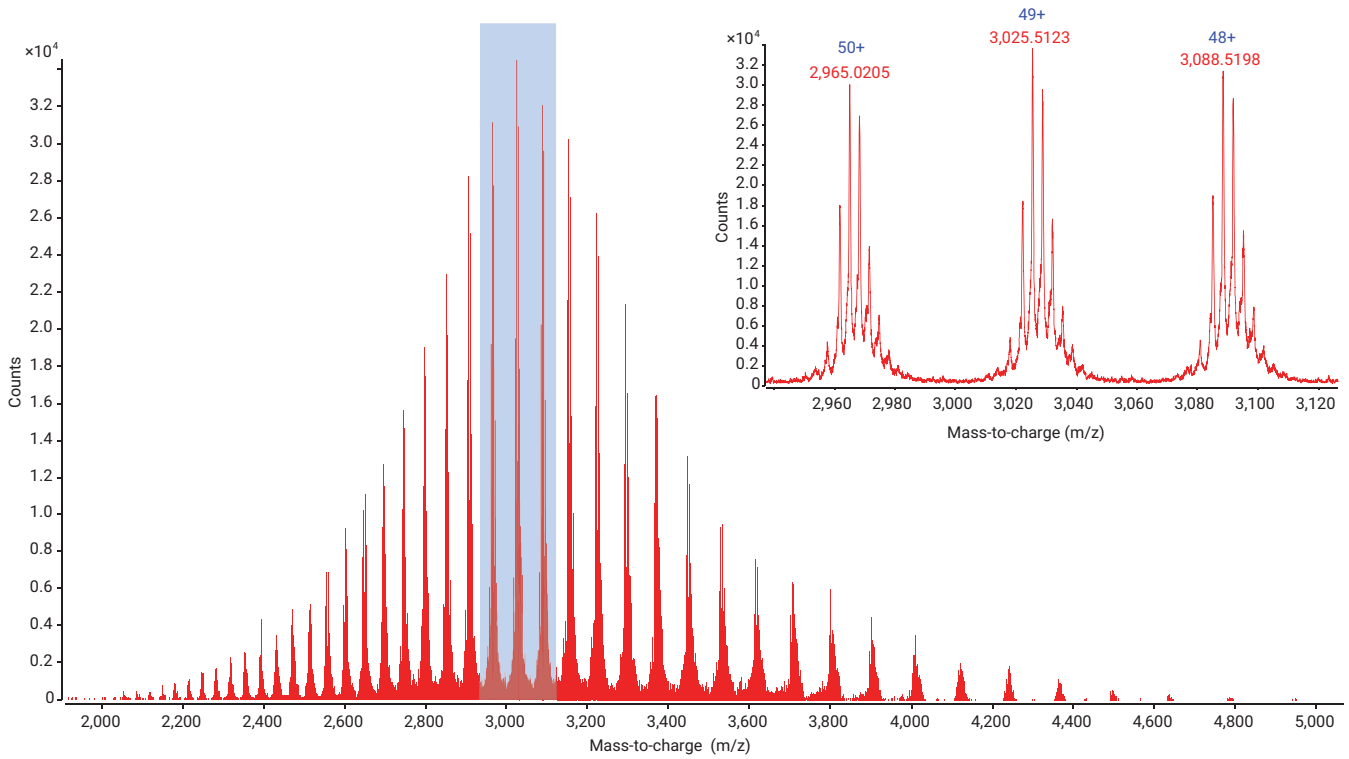

Figure 2. Intact NISTmAb analysis (0.5 µg injection).

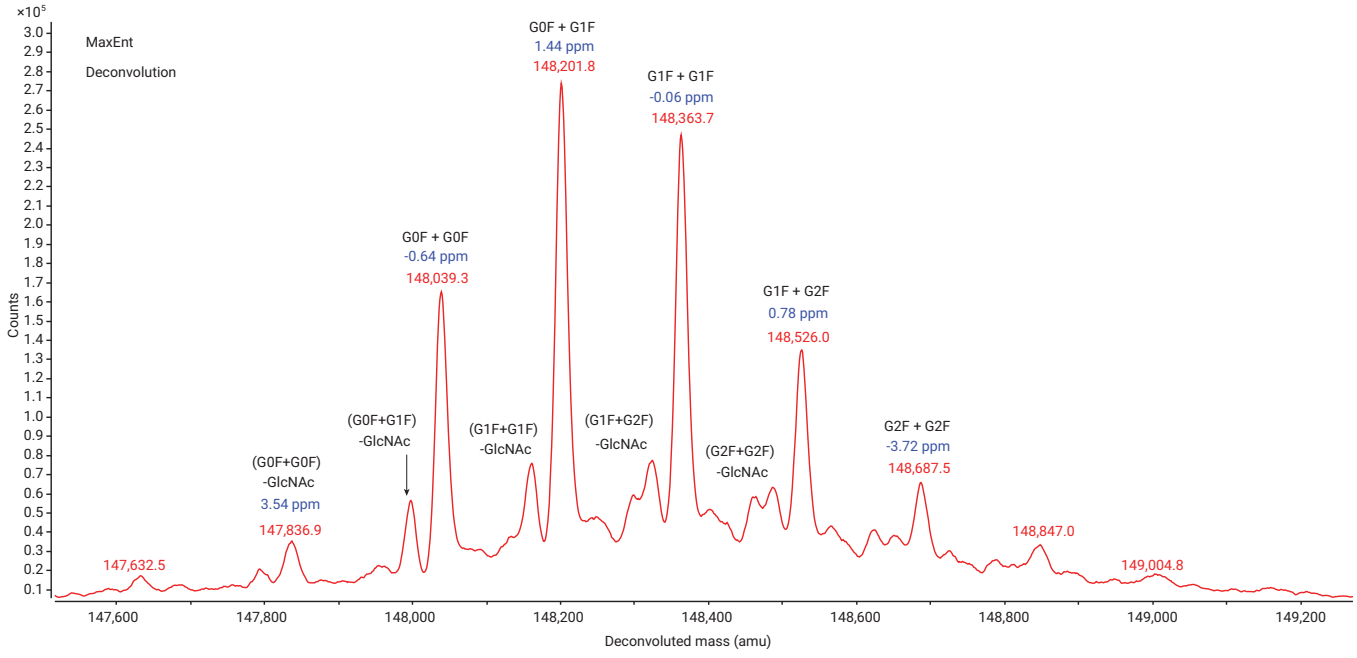

Figure 3. MS Deconvolution of intact NISTmAb (0.5 µg injection).

To confirm the reproducibility of mass accuracy of the 6545XT system on mAb analysis, several mAbs were also analyzed with the same amount of sample injection and HPLC conditions. Table 3 shows the deconvoluted mass results of the top six glycoforms of NISTmAb and Herceptin. Impressive low-ppm mass accuracy and clear representation of all glycoforms was consistently shown.

ADCs represent a new generation of effective biotherapeutics that are target-specific. It is crucial to obtain the accurate drug-to-antibody ratio (DAR) to optimize the efficacy, and minimize the toxicity of the ADC. They present an added challenge due to the increased complexity presented by variable levels of drug conjugation. Figure 4 shows the deconvoluted spectra of an intact glycosylated ADC. Nine mass clusters were observed with masses matching D0–D8 of ADC. The three major peaks in each cluster group correspond with the G0F/G0F, G0F/G1F, and G1F/G1F glycoforms. Most importantly, the average DAR value calculated using the BioConfirm DAR calculator was 3.5, which is consistent with the DAR values of the intact ADC reported previously using data from other analytical methods.

Table 3. Summary of intact mAbs analysis..

	NISTmAb		Herceptin	
mAb	Cal. MW (Da)	Mass error (ppm)	Cal. MW (Da)	Mass error (ppm)
G0 + G0F			147,912.6887	0.76
(G0F + G0F) - GlcNAc	147,836.3503	3.54		
G0F + G0F	148,039.4297	-0.64	148,058.8326	4.76
G0F + G1F	148,201.5729	1.44	148,220.9758	0.16
G1F + G1F	148,363.7162	-0.06	148,383.1191	-5.07
G1F + G2F	148,525.8595	0.78	148,545.2623	-6.74
G2F + G2F	148,688.0027	-3.72	148,707.4056	-13.23

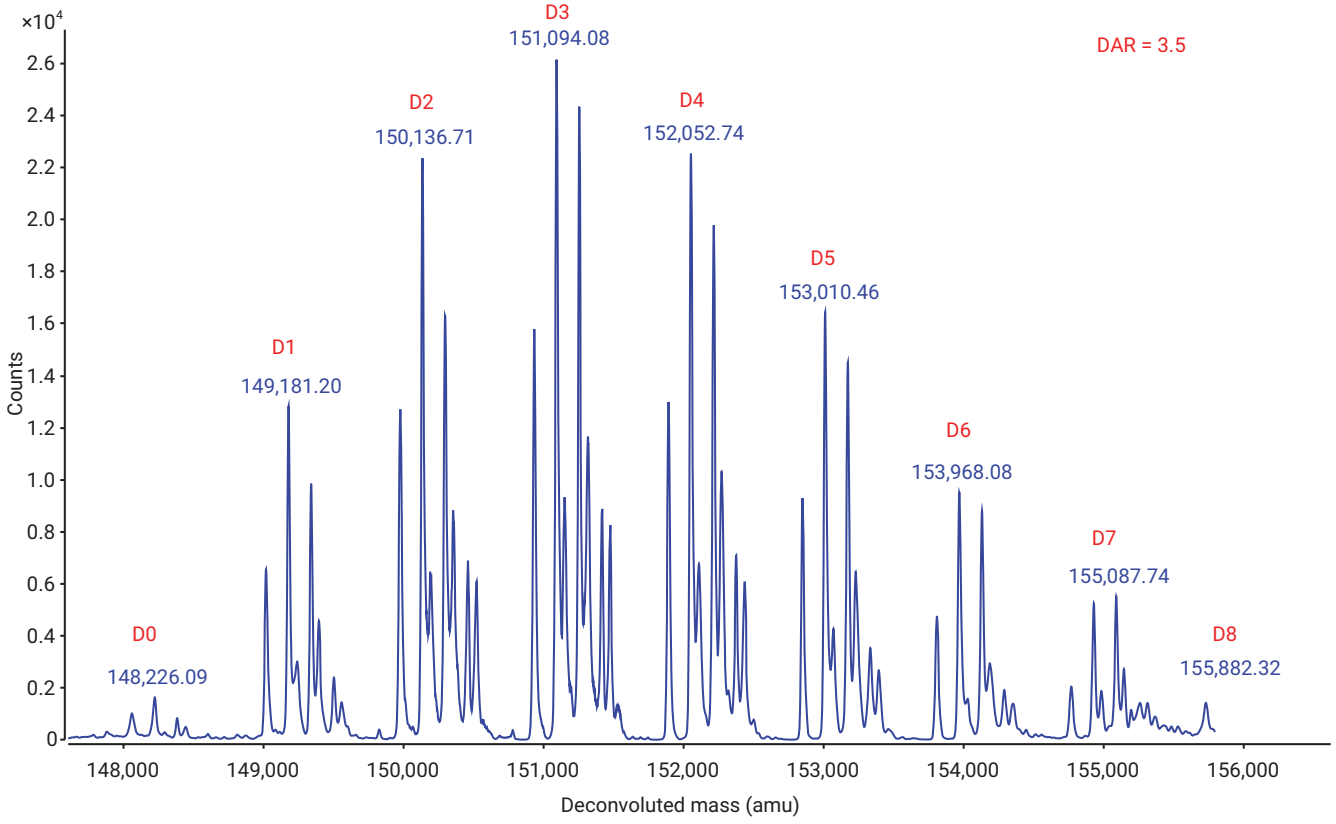

Figure 4. Intact T-DM1 analysis (0.5 µg injection).

The high level of detail provided by the 6545XT AdvanceBio LC/Q-TOF has a direct benefit when developing and analyzing biosimilar therapeutics. BioConfirm permits a direct mirror-plot comparison of deconvoluted spectra (Figure 5) to allow quick visualization of differences between protein samples. In this example, the two samples appear to be very similar in nature. Differences that could alter the quality of the drug product, such as protein sequence (mutation), glycosylation, or protein truncation could quickly be observed.

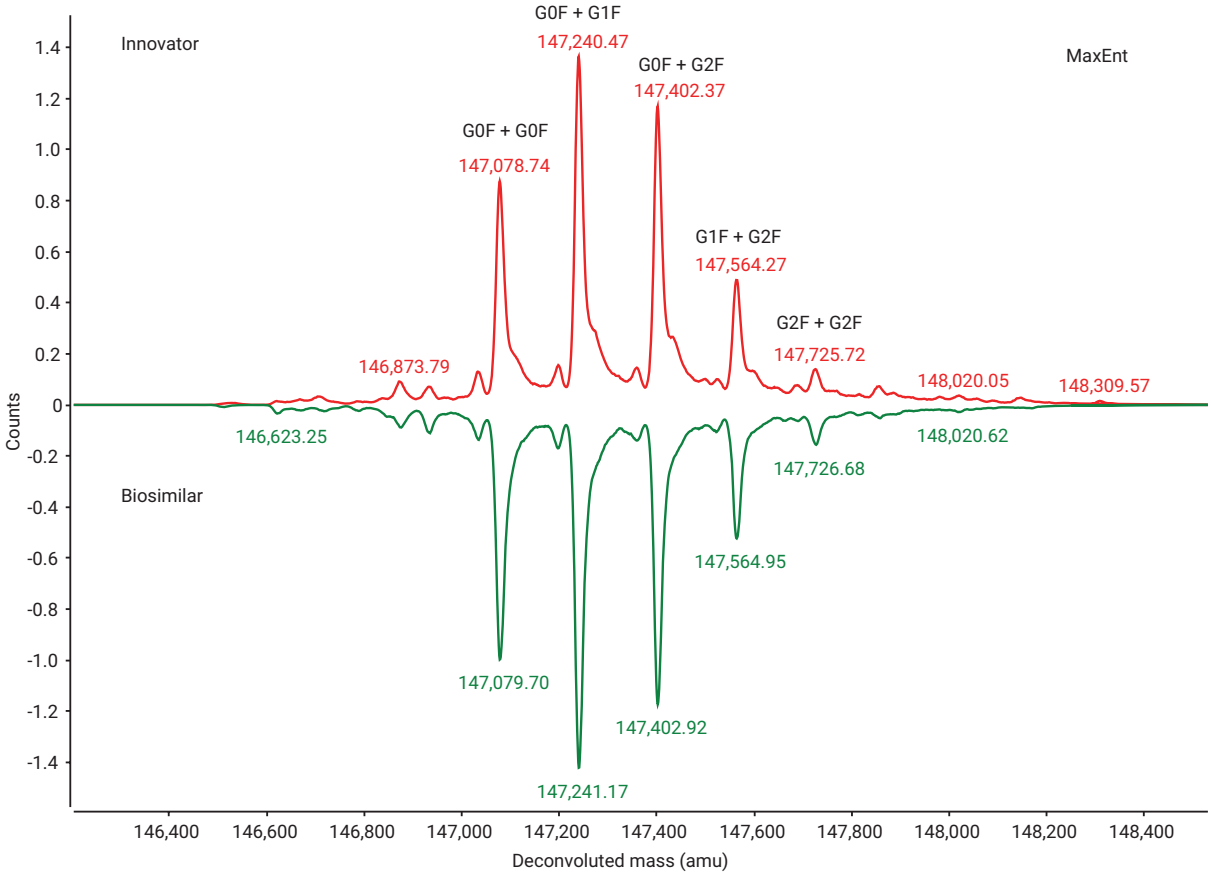

Figure 5. Intact Rituximab analysis (Innovator versus Biosimilar) (0.5 µg).

Conclusion

Monoclonal antibodies have a high level of complexity associated with them, requiring high resolution, precision, and dynamic range to fully characterize them with confidence. This Application Note demonstrates a high-throughput intact mAb analysis workflow solution integrating high-performance chromatography technologies, the Agilent 6545XT AdvanceBio LC/Q-TOF, and Agilent MassHunter BioConfirm software for automatic data processing.

- The workflow permits excellent mass accuracy down to the single ppm level for glycoforms measured during the intact mAb mass analysis.
- Highly detailed information was obtained about the heterogeneous composition of mAb proteins. In addition to major glycoforms, minor components of low intensity and similar molecular weight were clearly resolved. Variations such as all major glycoforms with loss of a GlcNAc sugar moiety and the full length protein sequences with a C-terminus lysine of the heavy chain were easily distinguished.

The total analysis time needed with this method is very short, allowing it to be used for large sample sets. With just a 4-minute LC gradient, and the Agilent BioConfirm automatic data processing, it is possible to run an entire 96-well plate in 8 hours.

References

1. Intact Protein Analysis Using an Agilent 6550 Q-TOF Mass Spectrometer, Agilent Technologies, publication number 5991-2116EN.
2. Analysis of Monoclonal Antibody (mAb) Using Agilent 1290 Infinity LC System Coupled to Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF), Agilent Technologies, publication number 5991-4266EN.
3. An Integrated Workflow for Automated Calculation of Antibody-Drug Conjugate (ADC) Drug-to-Antibody Ratio (DAR), Agilent Technologies, publication number 5991-7366EN.

Application Note

Biotherapeutics and Biosimilars

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An Integrated Workflow for Intact and Subunits of Monoclonal Antibody Accurate Mass Measurements

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Introduction

Monoclonal antibody (mAb) based entities represent a rapidly growing class of biologics that require extensive characterization to obtain approval for clinical trials and subsequent market release. Accurate mass measurement is a challenging step in the analytical characterization of antibodies because of their large size and the presence of post-translational modifications such as glycosylation. These characteristics also make determining the location of modifications more complex.

To overcome the challenges associated with antibody mass measurement, a number of complementary approaches are typically used. Antibodies can be treated with PNGase F to remove the N-Glycans, digested with proteases such as IdeS to generate antibody fragments, or reduced to generate light and heavy chains prior to mass measurement. These techniques can be used in various combinations. Sample preparation can be laborious, time-consuming, and have limited reproducibility. This Application Note demonstrates how these approaches can be streamlined by automation on the Agilent AssayMAP Bravo to reduce the probability of human error, increase reproducibility, and create more walk-away time (Figures 1 and 2).

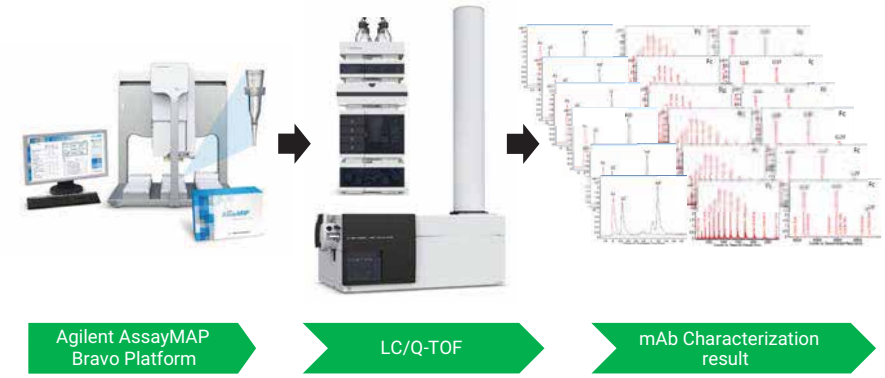

Figure 1. Integrated workflow for automated antibody characterization using Agilent AssayMAP Bravo.

We demonstrate how Agilent provides a complete solution for intact mass analysis from raw sample, through sample preparation, data collection, and data analysis using two well characterized antibodies. Herceptin (Trastuzumab), a monoclonal antibody specific for the HER2 extracellular domain (ECD), and the NIST Monoclonal Antibody Reference Material 8671 were affinity purified with the AssayMAP Bravo from cell culture supernatant using either biotinylated HER2 ECD (Trastuzumab) or biotinylated Protein L (NISTmAb) bound to AssayMAP streptavidin (SA-W) cartridges. Protein L is an affinity reagent for antibody kappa light chains.

Both affinity purified Herceptin and NISTmAb were then either left intact, deglycosylated with PNGase F, or digested with IdeS while still immobilized on the AssayMAP cartridges using the On-Cartridge Reaction application. The soluble reaction products from the PNGase F and IdeS reactions (glycans and Fc/2 heavy chain fragments, respectively) were collected in one plate. One half of the immobilized intact mAb, deglycosylated mAb, and F(ab')2 fragments were eluted from the cartridge into wells containing reducing buffers, while the other half of each of the samples was eluted into nonreducing buffers. All of these steps were automated on the AssayMAP Bravo. To acquire data with high mass accuracy, the proteins resulting from these steps were then analyzed with a UHPLC coupled to a Q-TOF mass spectrometer.

Experimental

Materials and methods

Recombinant human HER2 extracellular domain (ECD) was purchased from ACRO Biosystems (Newark, DE). The EZ-Link Sulfo-NHS-LC biotin kit and Pierce Biotin Quantitation Kit were purchased from Thermo Fisher Scientific (Grand Island, NY). Rapid PNGase F was obtained from New England Biolabs (Ipswich, MA). IdeS protease was purchased from Promega (Madison, WI). The formulated Herceptin (Trastuzumab) was manufactured by Genentech (South San Francisco, CA). Monoclonal Antibody Reference Material 8671 was purchased from the National Institute of Standards & Technology (NIST). The spent CHO cell media was obtained from Aldevron (Madison, WI). AssayMAP Streptavidin cartridges (SA-W) were from Agilent Technologies, Inc. (Santa Clara, CA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Generation of antibody affinity cartridges

Human Epidermal Growth Factor Receptor (HER2) ECD and Protein L were biotinylated using the EZ-Link Sulfo-NHS-LC biotin kit. The molar ratio of biotin to HER2 ECD was determined to be 9.5, the molar ratio of biotin to Protein L was determined to be 5.3. These ratios were determined by following the instructions in the Pierce Biotin Quantitation Kit.

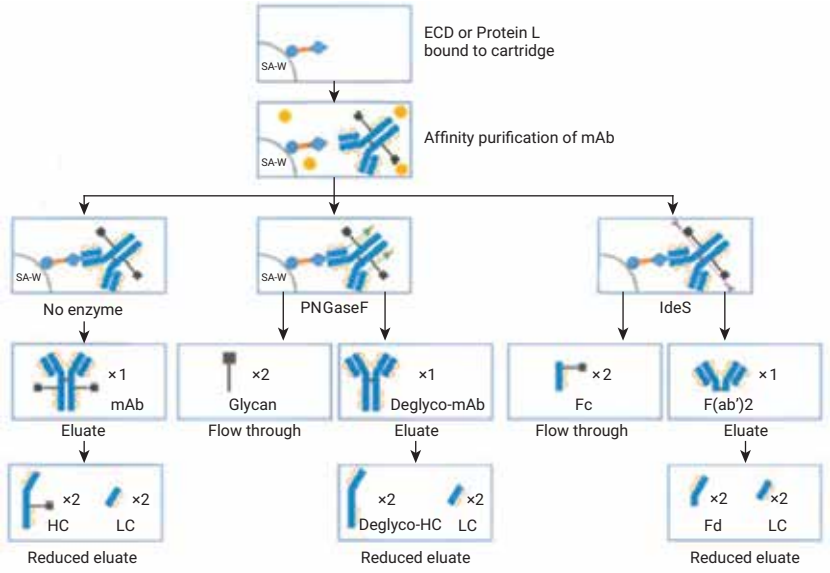

Figure 2. Antibody sample preparation performed by the Agilent AssayMAP Bravo.

The AssayMAP Bravo then was used to immobilize 2 µg of biotinylated HER2 ECD (Column 1 and 2 in Table 1) and 2 µg of biotinylated Protein L (Columns 3 and 4 in Table 1) on each streptavidin (SA-W) cartridge with the Immobilization application on the AssayMAP Bravo (Figure 3). The minimum mass of biotinylated ligand required to efficiently bind the target molecule at a slow flow rate was determined empirically, and found to be approximately a 5:1 molar ratio of biotinylated capture ligand to target. Briefly, SA-W cartridges were primed and equilibrated with 1 % formic acid (deck location 3, Figure 3), then washed with 50 µL HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) (deck location 5, Wash 1) using the **Internal Cartridge Wash 1** step.

Priming and equilibrating with 1 % formic acid purged the entrained air from the cartridges, and acted as a stringent wash to remove streptavidin monomers that dissociated from the solid support in the low pH condition. The cartridges were then re-equilibrated with HEPES buffer. This put the buffer on the cartridge resin bed in preparation for the cartridges to bind the biotinylated antigen in HEPES buffer. Next, the SA-W cartridges were loaded with 2 µg of biotinylated HER2 ECD or Protein L in 100 µL HEPES buffer (deck location 9) at a flow rate of 5 µL/min using the **Load Blocking Reagent** step. The final step in the generation of the affinity cartridges was one wash with 50 µL HEPES buffer using **Internal Cartridge Wash 2**. All other steps in the Immobilization application were turned off and automatically skipped. Figure 3 shows a screenshot of the Immobilization application settings that were used to execute this run.

Table 1. Experimental design and final sample in the elution plate.

		Step 1: ECD-biotin(2 µg/100 µL)	Step 1: Protein L -biotin(2 µg/100 µL)	
		Herceptin (2 µg/100 µL)	NIST mAb (2 µg/100 µL)	
Step 2		Columns 1 and 2	Columns 3 and 4	Step 3
No enzyme	A	Intact Herceptin with glycan	Intact NIST mAb with glycan	
	B	Herceptin LC/HC with glycan	NIST mAb LC/HC with glycan	TCEP
PNGase F	C	Herceptin without glycan	NIST mAb without glycan	
	D	Herceptin LC/HC	NIST mAb LC/HC	TCEP
IdeS	E	Herceptin F(ab')2	NIST F(ab')2	
	F	Herceptin Fd', LC	NIST Fd', LC	TCEP
	G			
	H			

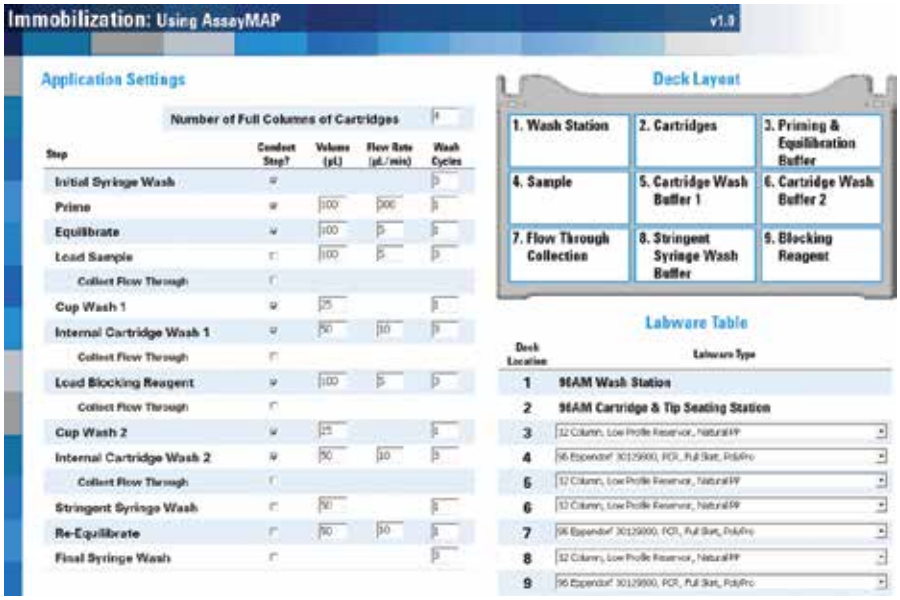

Figure 3. User interface for Agilent AssayMAP Bravo, showing the setup screen for the Immobilization application on AssayMAP Bravo used to generate antibody affinity cartridges.

Antibody purification

Commercially obtained Herceptin (Trastuzumab) and NISTmAb were reconstituted in deionized water to 20 mg/mL, aliquoted, and stored at –80 °C. Just before use, Herceptin and NISTmAb were diluted to 1 µg/µL with water. Both mAbs were then spiked into spent CHO cell supernatant to a concentration of 2 µg/100 µL. The Affinity Purification application on the AssayMAP Bravo was used to purify the antibodies out of the cell culture supernatant (Figure 4). The prime and equilibration steps were turned off because these were done during the Immobilization protocol.

Then, 100 µL of CHO cell supernatant spiked with either Herceptin mAb or NISTmAb was loaded onto each HER2 ECD and Protein L affinity cartridge, respectively, at 3 µL/min, followed by a 150 µL HEPES buffer wash (Internal Cartridge Wash 1) at 10 µL/min. Table 1 (Step 1) shows how the cartridges were used for different samples. Columns 1 and 2 from A to F (12 cartridges) were loaded with biotinylated HER2 ECD. Columns 3 and 4 from A to F (12 cartridges) were loaded with biotinylated Protein L.

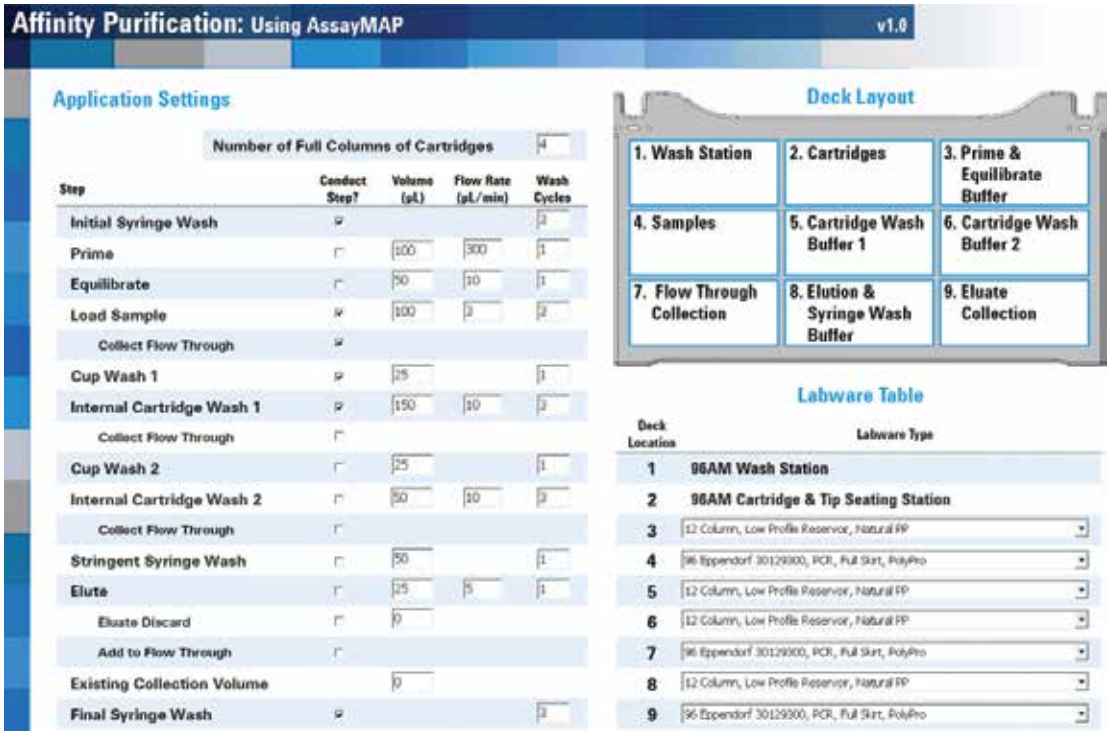

Figure 4. The setup screen for the Affinity Purification application on Agilent AssayMAP Bravo used to purify antibody from cell culture supernatant.

On-cartridge deglycosylation and IdeS digestion

Following capture of the Herceptin and the NISTmAb, the cartridges were equilibrated with 50 µL water control or enzyme buffer at 10 µL/min flow rate to prepare for the On-Cartridge Reaction (Figure 5). Table 1 (Step 2) shows that the:

- Cartridges in rows A and B were equilibrated with water
- Cartridges in rows C and D were equilibrated with deglycosylation buffer (20 mM Tris, pH 8.0)
- Cartridges in rows E and F were equilibrated with IdeS proteolysis buffer (50 mM Tris, 150 mM NaCl, pH 6.6)

The On-Cartridge Reaction (Figure 5) was carried out by aspirating 4 µL of heated water, rapid PNGase F (1:12), or IdeS enzyme (4 U/µL) through the cartridges at 10 µL/min (Figure 5). An additional 2 µL of heated enzyme solution or water was then aspirated through each cartridge over the course of 30 minutes (total volume of each enzyme used was 6 µL).

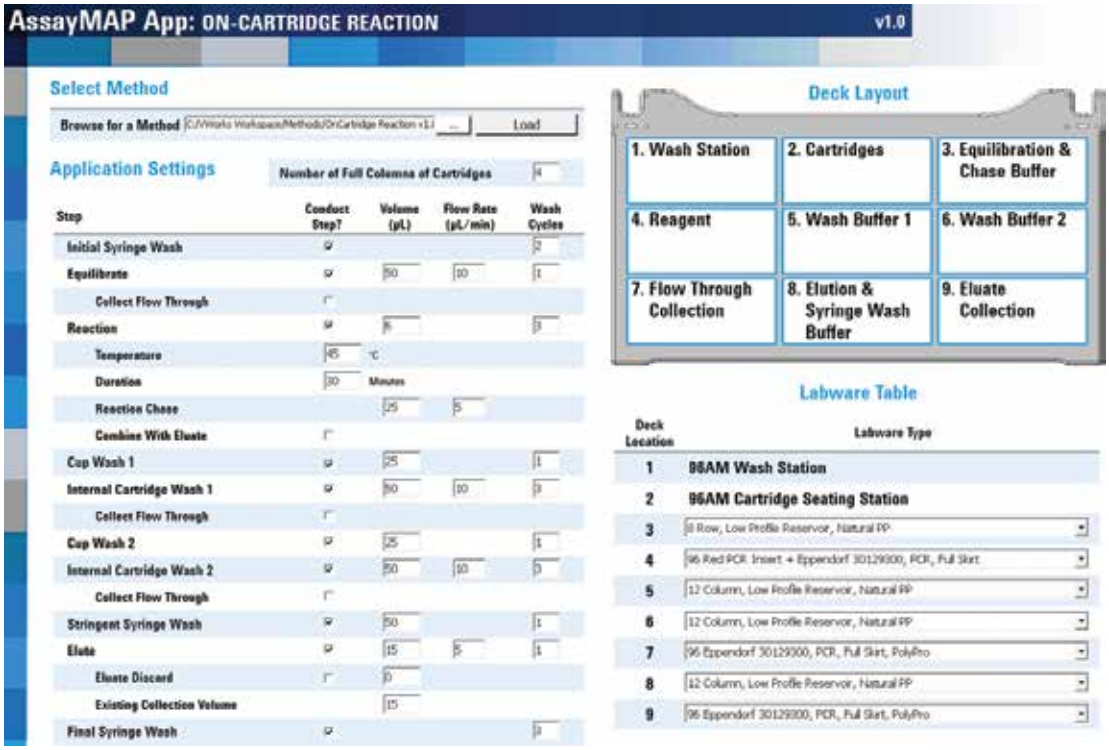

Figure 5. The setup screen for the On-Cartridge Reaction application, used in this case for deglycosylation (PNGase F) and proteolysis (IdeS) of the mAbs.

During both aspiration steps, the three reagents were heated to 37 °C using the peltier device at deck location 4 of the Bravo (Figure 5). The temperature was set to 45 °C in the application form, as this results in a reaction temperature in the cartridge of approximately 37 °C due to losses in heat transfer from the heater through the sample plate and into the cartridge resin bed. The respective reaction buffers or water controls (25 µL) were aspirated through each cartridge at the conclusion of the digestion during the Reaction Chase, combining it with the enzyme solution or control that had passed over the cartridge to collect the released glycans or the Fc. These released reaction products were then collected in the flowthrough collection plate at deck location 7. Each cartridge was washed with 50 µL of 1 M NaCl in HEPES buffer (deck location 5, Wash 1) at 10 µL/min followed by 0.003 % formic acid (deck location 6, Wash 2) at 10 µL/min (Figure 5). The purified mAb, deglycosylated mAb, or F(ab')2 fragments were eluted with 15 µL of 1 % formic acid (deck location 8) per cartridge into an existing volume of 15 µL 0.5 % ammonium hydroxide in the elution plate to neutralize the eluant. TCEP was added to the eluant to a final concentration of 5 mM (Step 3 rows B, D, and F in Table 1), and samples were reduced at room temperature for 30 minutes. All other steps in the On-Cartridge Reaction application were turned off (Figure 5).

Table 2. Liquid chromatography parameters.

	Agilent 1290 Infinity II UHPLC System			
Column	Agilent PLRP-S 1000Å, 2.1 × 50 mm, 8 µm (p/n PL1912-1502)			
Solvent A	0.1 % Formic acid in water			
Solvent B	0.1 % Formic acid in acetonitrile			
Gradient	1. Intact mAb		2. Reduced mAb	
	Time (min)	B(%)	Time (min)	B(%)
	0	5	0	25
	1	20	1	25
	3	50	6.5	60
	4	95	7.5	60
	4.1	5	7.6	25
	5	5	8.5	25
Column Temperature	60 °C for intact and deglycosylated mAbs, and 40 °C for the other samples			
Flow rate	0.5 mL/min for intact mAb, 0.8 mL/min for subunits			
Injection Volume	1 µL			

LC/MS analysis

LC/MS analysis was conducted using an Agilent 1290 Infinity II UHPLC system with a PLRP-S column (PL1912-1502) coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF with a Dual Agilent Jet Stream source. Tables 2 and 3 list the LC/MS parameters used. All the intact mAb and deglycosylated mAb samples were analyzed with a 5-minute gradient. All the IdeS fragments and the reduced light and heavy chains were analyzed with an 8.5-minute gradient.

Table 3. Mass spectrometer parameters.

	Agilent 1290 Infinity II UHPLC System		
Parameter	**Intact antibody**	**F(ab')2**	**Subunits**
Source	Dual Agilent Jet Stream	Dual Agilent Jet Stream	Dual Agilent Jet Stream
Gas Temperature	350 °C	350 °C	350 °C
Gas flow	12 L/min	12 L/min	12 L/min
Nebulizer	60 psi	35 psi	35 psi
Sheath gas Temperature	400 °C	400 °C	400 °C
Sheath gas flow	11 L/min	11 L/min	11 L/min
VCap	5,500 V	4,000 V	4,000 V
Nozzle voltage	2,000 V	2,000 V	2,000 V
Fragmentor	380 V	180 V	180 V
Skimmer	140 V	65 V	65 V
Mass range	800–5,000 *m/z*	800–5,000 *m/z*	400–3,200 *m/z*
Scan rate	1 spectrum/sec	1 spectrum/sec	1 spectrum/sec
Acquisition mode	High (10,000 *m/z*) mass range	High (10,000 *m/z*) mass range	Standard (3,200 *m/z*) mass range
	Extended dynamic range (2 GHz)	Extended dynamic range (2 GHz)	High resolution (4 GHz)
Injection Volume	1 µL		

Data analysis

Spectra were extracted for each total ion current (TIC) peak, and deconvoluted using the Agilent MassHunter BioConfirm Maximum Entropy Algorithm. Table 4 shows the deconvolution parameters.

Table 4. Maximum entropy deconvolution parameters.

	Maximum Entropy Deconvolution Setting			
Parameter	**Intact**	**F(ab')2**	**LCHC**	**Subunits**
Mass range (Da)	140–160 K	90–110 K	20–60 K	21–28 K
Mass step (Da)	1	1	1	1
Use limited *m/z* range	2,000–4,500	1,800–3,000	900–2,600	1,000–2,600
Baseline factor	3.5	6.0	6.0	6.0
Adduct	Proton	Proton	Proton	Proton
Isotope width	Automatic	Automatic	Automatic	Automatic

Results and Discussion

The performance of an integrated antibody accurate mass measurement workflow from raw sample to data analysis is demonstrated for two monoclonal antibodies. The Herceptin example shows how a very specific antibody antigen interaction can be used to purify a specific antibody out of complex matrices for the subsequent characterization of its mass. The NISTmAb illustrates how a more generic affinity ligand (Protein L-affinity for the kappa light chain of antibodies) that binds to a wide range of antibodies can also be used as a purification tool without the need to generate antibody-specific reagents. Herceptin (Trastuzumab) and an NISTmAb standard were affinity purified, deglycosylated, or digested with IdeS, and subsequently reduced using the AssayMAP Bravo platform (Table 1 and Figure 2). They were then subjected to LC/MS analysis. Figures 6 to Figure 11 show the results.

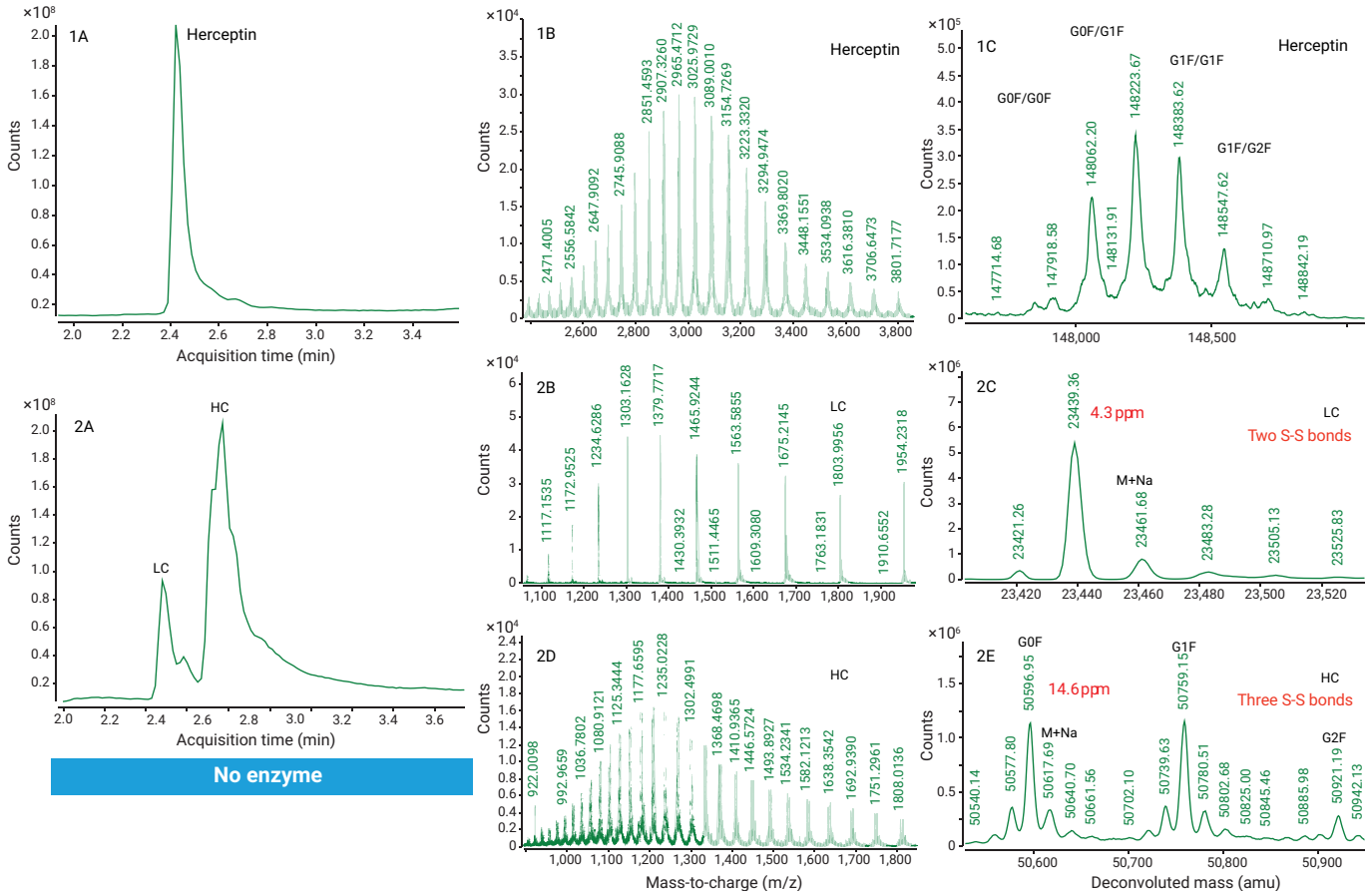

Figure 6. 1A and 2A) TIC of ECD purified intact and reduced Herceptin. 1B, 2B, and 2D) Mass spectra of intact and reduced Herceptin. 1C, 2C, and 2E) Deconvoluted spectra of intact and reduced Herceptin. LC = light chain, HC = heavy chain.

The purified intact Herceptin (Figure 6-1) and NISTmAb (Figure 9-1) were analyzed by LC/Q-TOF, with both showing only one peak after UHPLC separation. This demonstrates that the affinity purification performed on the AssayMAP Bravo results in highly purified mAbs. The deconvoluted Q-TOF MS spectra in Figures 6-1C and 9-1C provided a neutral mass of 148,062.20 for intact Herceptin, and 148,040.02 (4.02 ppm) for intact NISTmAb. The purified Herceptin mass was a few Daltons more than the theoretical value of 148,058.83. This nonspecific adduct could be not completely desolvated in the ion source, and partially resolved on the intact mass level. However, the NISTmAb showed good mass accuracy after purification, proving that the affinity purification application works well.

After reduction of the purified intact mAb with TCEP at room temperature, the light and heavy chains were separated by UHPLC with an 8.5-minute gradient (Gradient 2 reduced mAb in Table 2). Both deconvoluted light chain masses showed good mass accuracy of 4.3 ppm for the Herceptin light chain at 23,439.36 (Figure 6-2C) and 2.39 ppm for the NISTmAb light chain at 23,124.00 (Figure 9-2C).

This result also showed that TCEP only reduced the inter-chain disulfide bond between the light chain and heavy chain, while preserving the two intra-chain disulfide bonds in both light chains. When the heavy chain masses were examined, the heavy chains in both antibodies had one intra-chain disulfide bond reduced with a neutral mass of 50,596.95 (14.6 ppm) for Herceptin (Figure 6-2E) and 50,901.81 (0.18 ppm) for the NISTmAb (Figure 9-2E). The mass difference observed between the theoretical and measured mass for the intact Herceptin was not observed in the light or heavy chains. Instead we can clearly see the sodiated species of Herceptin light chain with neutral mass of 23,461.68 (Figure 6-2C), and the sodiated species of Herceptin heavy chain at 50,617.69 (Figure 6-2E).

On-cartridge deglycosylation was performed on purified intact mAb sample at rows C and D (Table 1), and half of the samples underwent reduction with TCEP (rows B, D, F in Table 1). The deconvoluted spectra for both deglycosylated Herceptin and deglycosylated NISTmAb showed one major peak without the glycans (Figures 7-1C and 10-1C).

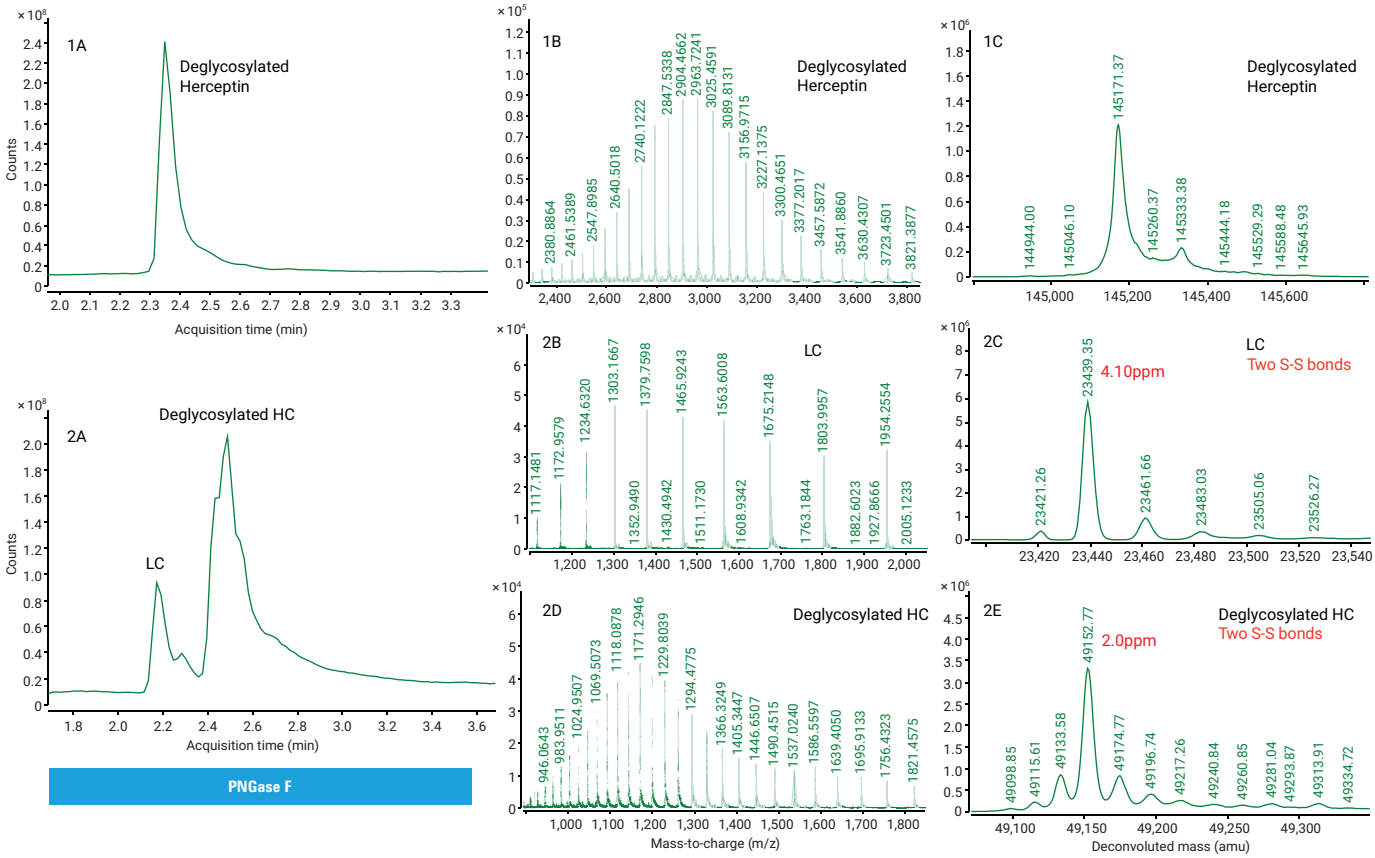

Figure 7. 1A and 2A) TIC of ECD purified intact and reduced Herceptin after on-cartridge deglycosylation with PNGase F. 1B, 2B, and 2D) Mass spectra of deglycosylated Herceptin, LC and HC. 1C, 2C, and 2E) Deconvoluted spectra of deglycosylated Herceptin, LC and HC. LC = light chain, HC = heavy chain.

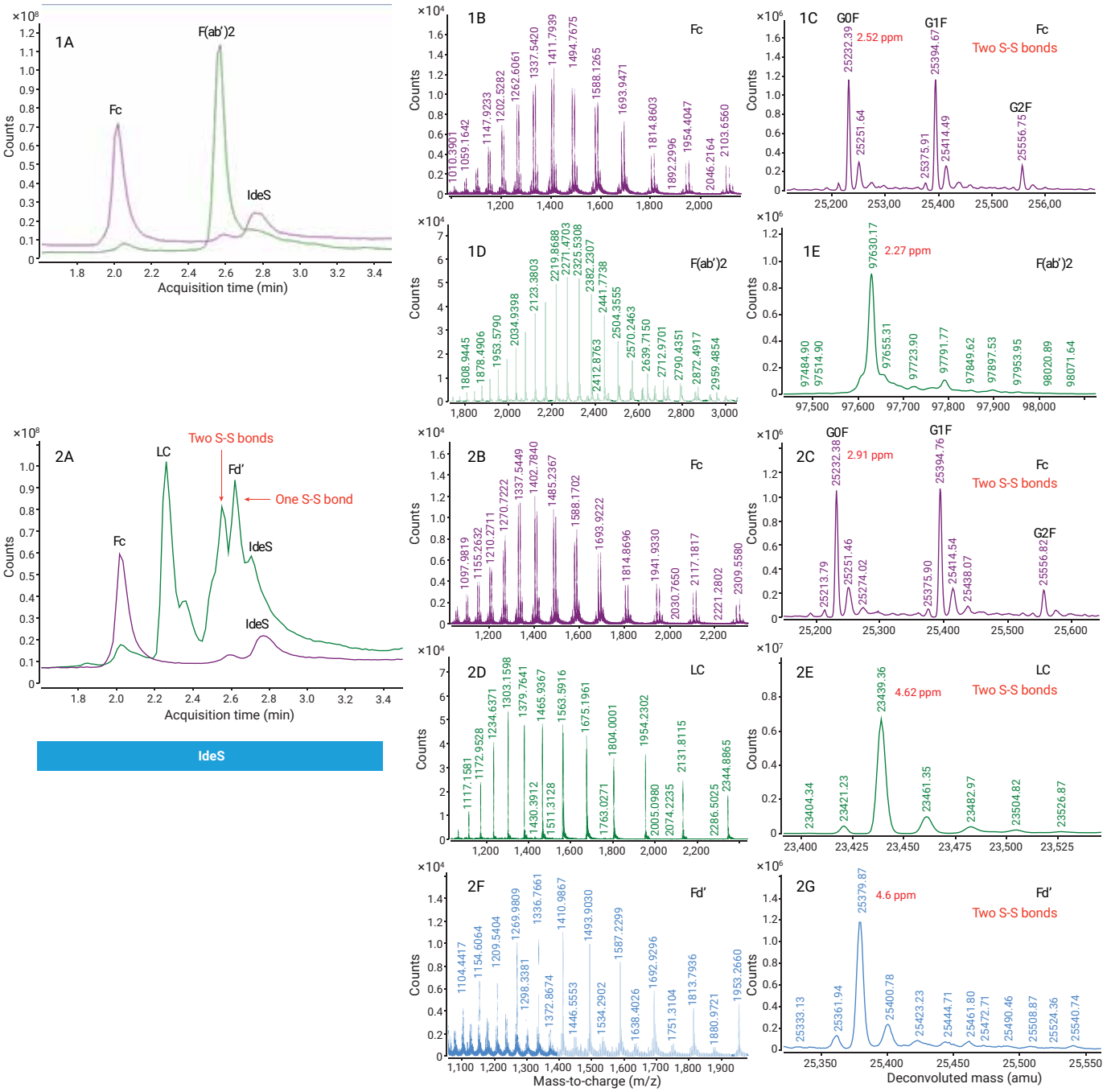

Figure 8. 1A and 2A) TIC of subunits from ECD-purified Herceptin after on-cartridge IdeS reaction and reduction. 1B, 1D, 2B, 2D, and 2F) Mass spectra of Herceptin subunits. 1C, 1E, 2C, 2E, and 2G) Deconvoluted spectra of Herceptin subunits.

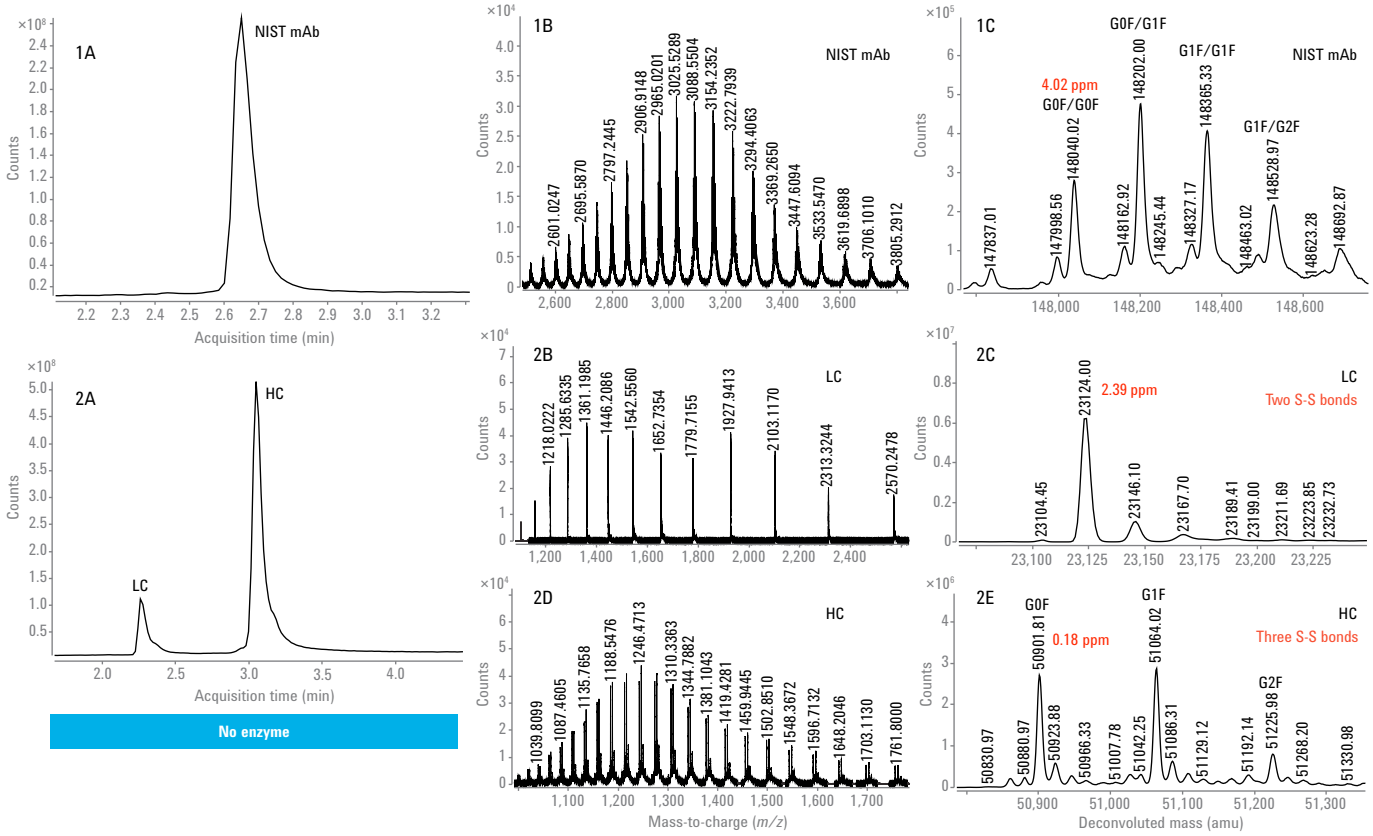

Figure 9. 1A, 2A) TIC of Protein L purified intact and reduced NISTmAb. 1B, 2B, 2D) Mass spectra of intact and reduced NISTmAb. 1C, 2C, 2E) Deconvoluted spectra of intact and reduced NISTmAb. LC = light chain; HC = heavy chain.

For both deglycosylated mAbs, the deconvoluted masses were approximately 3 Da more than the theoretical value, with an approximately 20 ppm mass shift. This could be nonspecific adduct not completely desolvated in the ion source and partially resolved on the intact mass level. The deconvoluted masses of the light and heavy chains provided a clearer picture of the mAb. The reduced samples were analyzed with an 8.5-minute gradient (Table 2). The deconvoluted spectra in Figure 7-2C and Figure 10-2C show neutral masses of 23,439.35 (4.1 ppm) for Herceptin light chain and 23,124.12 (7.7 ppm) for NISTmAb light chain. The results were consistent with the results from Figure 6-2C and Figure 9-2C. The deconvoluted spectra in Figure 7-2E and Figure 10-2E gave neutral masses of 49,152.77 (2 ppm) for Herceptin heavy chain and 49,457.87 (12.16 ppm) for NISTmAb heavy chain. Notice that, after deglycosylation, it appeared that TCEP was able to reduce the two intra-chain disulfide bonds within the two heavy chains. This could be because the deglycosylation changed the overall folding of the heavy chain and opened more space for TCEP reduction. This is a different result than that obtained from the deconvoluted mass for the glycosylated heavy chains, where only one disulfide bond was reduced (Figure 6-2E and Figure 9-2E).

The on-cartridge IdeS reaction generated Fc and F(ab')2 fragments for both mAbs within 30 minutes. The AssayMAP Bravo application allowed the user to collect Fc in the flowthrough or combine it with the F(ab')2 in the eluate plate using the combine with eluate function (Figure 5). In this experiment, we chose to collect Fc in the flowthrough, and the F(ab')2 fragments in the elution plate. Figure 8-1A and Figure 11-1A show the overlay of total ion chromatogram (TIC) of Fc and F(ab')2 from both mAbs. The deconvoluted spectra gave neutral masses of 25,232.39 (2.52 ppm) for Herceptin Fc (G0F) and 25,232.41 (2 ppm) for the NISTmAb Fc (G0F). The two mAbs actually have the same amino acid sequence in the Fc region, which was further confirmed by the experimental results. The deconvoluted spectra gave a neutral mass of 97,630.17 for Herceptin F(ab')2 (Figure 8-1E) with 2.27 ppm, compared to the theoretical value of 97,629.95. The deconvoluted NIST F(ab')2 (Figure 11-1E) gave a neutral mass of 97,610.84 with 2.97 ppm, compared to the theoretical value of 97,610.55.

Reduction of the F(ab')2 sample generated light chains and Fd' fragments from both mAbs with good mass accuracy. The deconvoluted spectra (Figure 8-2E) gave a neutral mass of 23,439.36 (4.62 ppm) for the Herceptin light chain, which is consistent with Figure 6-2C and Figure 7-2C. The deconvoluted spectra (Figure 11-2E) also gave a neutral mass of 23,124.02 (3.24 ppm) for the NISTmAb light chain, which is consistent with Figure 9-2C and Figure 10-2C. The TIC for Herceptin Fd' showed a split peak at retention times 2.55 and 2.62 minutes corresponding to the two disulfide forms of the Fd' (Figure 8-2A).

The peak at retention time 2.55 minutes gave a neutral mass of 25,379.87 with 4.6 ppm to the theoretical value of 25,379.75. This peak was the Fd' with two intra-chain disulfide bonds (Figure 8-2G). The peak at retention time 2.62 minutes is the Fd' with one intra-chain disulfide bond, having a neutral mass of 25,381.61 with 6.3 ppm (spectrum not shown). The NISTmAb Fd' (Figure 11-2A), which gave a deconvoluted mass of 25,685.34 with 0.9 ppm, contains two intra-chain disulfide bonds (Figure 11-2G).

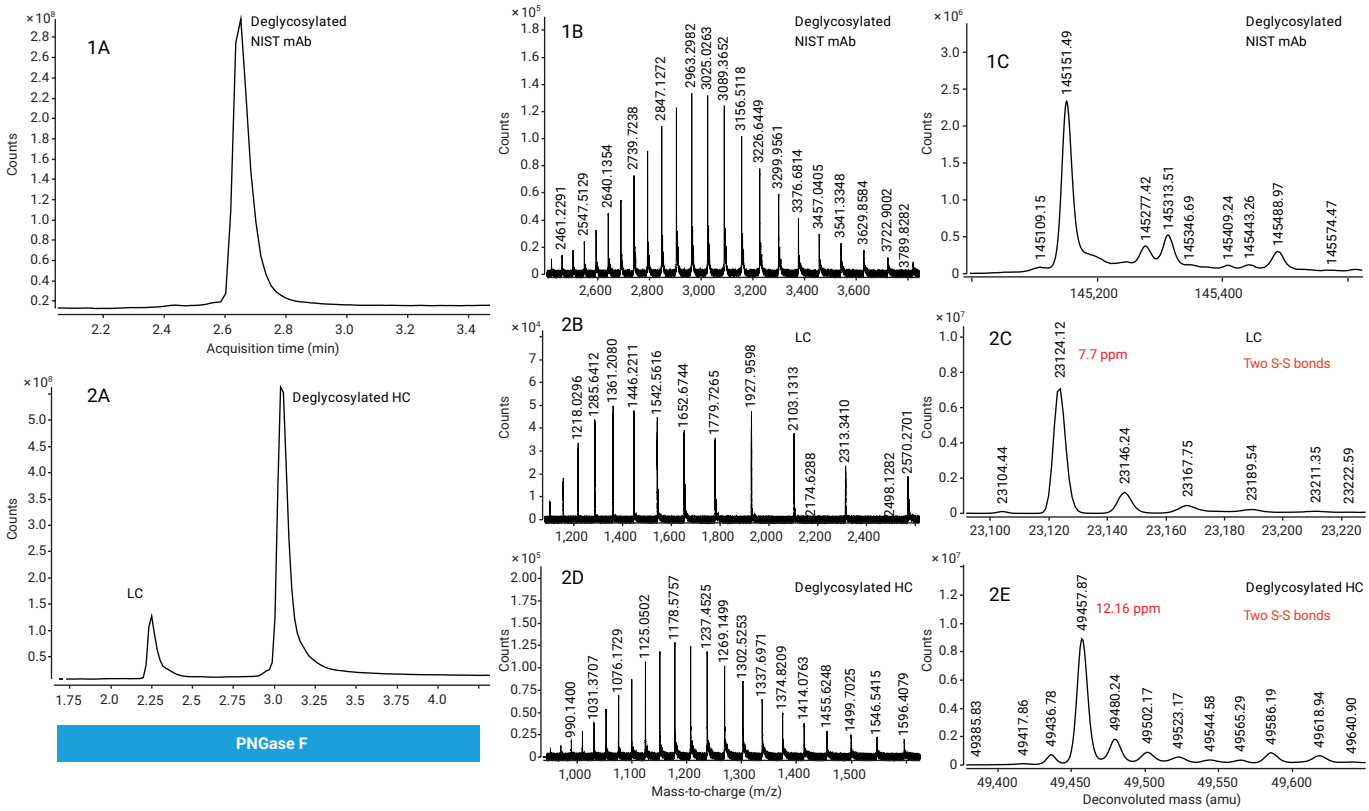

Figure 10. 1A, 2A) TIC of Protein L purified intact and reduced NISTmAb after on-cartridge deglycosylation with PNGase F. 1B, 2B, 2D) Mass spectra of deglycosylated NISTmAb, LC and HC. 1C, 2C, 2E) Deconvoluted spectra of deglycosylated NISTmAb, LC and HC. LC = light chain; HC = heavy chain.

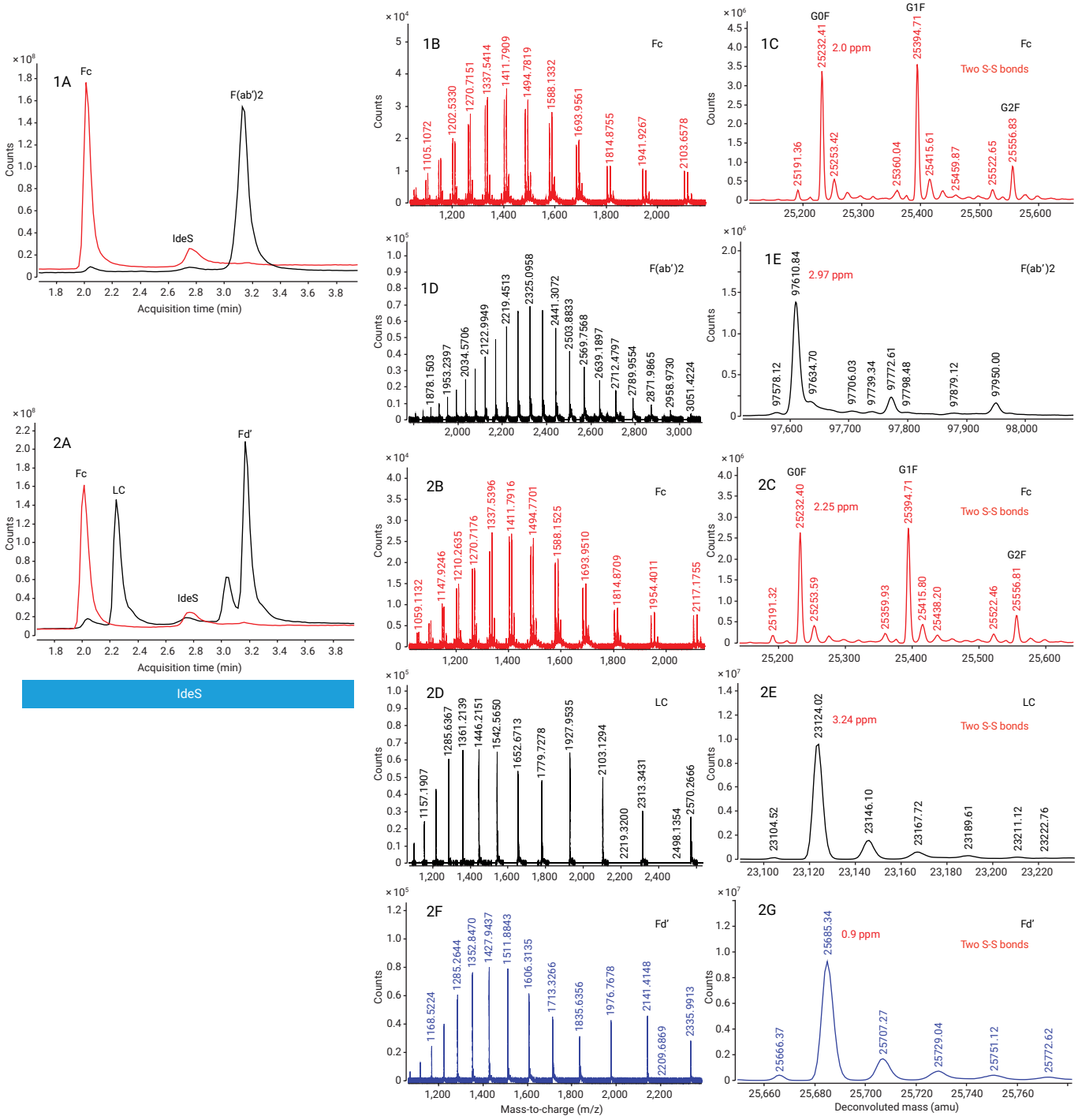

Figure 11. 1A and 2A) TIC of subunits from Protein L purified NISTmAb. 1B, 1D, 2B, 2D, and 2F) Mass spectra of NISTmAb subunits. 1C, 1E, 2C, 2E, and 2G) Deconvoluted spectra of NISTmAb subunits.

Conclusion

The Agilent AssayMAP Bravo platform is a key component of an integrated workflow for monoclonal antibody characterization, including comprehensive intact mAb mass measurement. It automates sample preparation to reduce human error, assure reproducibility, and allow the analyst to walk away and perform other tasks (Figures 1 and 2). Using the AssayMAP Bravo, the current study required an overall time of 5.5 hours to complete the four columns of sample preparation. The same number of samples prepared manually would take at least 1 day. If all 12 columns of sample preparation (a whole plate) is needed, the processing time required on the AssayMAP Bravo would still remain approximately 5–6 hours. However, manual sample preparation of the whole plate will require >1 day of constant bench work time. The AssayMAP Bravo provides an easy-to-use platform that would automate the entire workflow and accelerates the time to results. AssayMAP Bravo is specifically designed for protein and peptide sample preparation using microchromatography cartridges, simple and reliable automated processes, and an application-based user interface. Agilent offers a complete solution for antibody characterization by integrating automated affinity purification and enzymatic digestion on the AssayMAP Bravo with ultrahigh performance liquid chromatography, the Agilent AdvanceBio Q-TOF, and easy-to-use Agilent MassHunter BioConfirm software.

This workflow is also versatile, providing both intact antibody and subunit protein mass analysis. To meet the needs of a comprehensive characterization study, it also provides the flexibility to perform on-cartridge deglycosylation, proteolysis with the IdeS protease, or reduction to release the subunits, as well as all three steps together. Both ECD and Protein L can purify mAb from spent CHO cell medium with high purity.

This integrated approach also enables high-throughput analysis for batch-to-batch comparison of antibody preparations. Superior chromatographic resolution enables fast and efficient separation of intact antibodies and their light and heavy chain subunits, including different disulfide forms. The Agilent AdvanceBio Q-TOF generates high-resolution spectra to achieve high mass accuracy for protein mass analysis. The MassHunter BioConfirm data analysis software enables a complete protein analysis workflow, including automated data extraction, deconvolution, and sequence matching.

Acknowledgement

We would like to thank our former co-worker, Jing Chen, for her work on this project.

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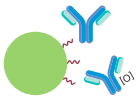
Intact Protein Analysis using Hydrophobic Interaction Chromatography (HIC)

Introduction

Recombinant proteins are subject to many post translational modifications (PTMs) during processing, delivery, and storage[1]. Some of the most PTMs found in therapeutic monoclonal antibodies (mAbs) include glycosylation, disulfide bond formation, and proteolytic cleavage of the protein backbone (e.g., oxidation, deamidation, glycation, pyroglutamate [pyro-Glu] formation). Hydrophobicity-based HPLC methods, such as reversed-phase liquid chromatography (RPLC) and hydrophobic interaction chromatography (HIC), are often used to characterize these mAb variants. HIC technique is often used for large scale protein purification for removing protein aggregates and contaminants such as host-cell proteins [2, 3]. In more recent years, gained popularity for small scale analytical separations. Due to its unique selectivity, HIC is complementary to techniques like size exclusion, ion exchange, and RP. Of these, HIC is most like RP, in that separation is strongly correlated with protein hydrophobicity. But there are two key differences between HIC and RP: 1) HIC separations are driven by a decreasing gradient of salt, rather than an increasing gradient of organic solvent as in RP. 2) HIC separations maintain the native state of a protein, and thus the protein remains biologically active following the separation. This allows collection of purified peaks for downstream potency assays, for example. Furthermore, the native state of the protein means that features on the surface of a protein tend to drive selectivity in HIC mode. Therefore, HIC is particularly well suited to separating PTMs or degradation products of a protein, which is sometimes very difficult to achieve by other modes of chromatography.



Intact Native Protein Analysis

Hydrophobic interaction chromatography

Resolves various protein variants (PTMs) including oxidation in mAbs and drug-antibody species observed in ADcs

AdvanceBio HIC

Designed to Address the Separation of Challenging Biomolecules applications

Attribute	Advantage
Single Chemistry	Multiple applications with better peak shape
Faster method development and higher throughput for greater productivity	Meet vital deadlines
Enhanced robustness, batch-tobatch consistency	Reduce rework

HIC separates analytes in order of increasing hydrophobicity; in other words, less hydrophobic species typically elute first. Both enthalpic (i.e., hydrophobic/hydrophilic interactions) and entropic (i.e., hydration shell) interactions are responsible for retention[4,5]. A weakly hydrophobic stationary phase is used to bind the analyte in the presence of high concentrations of polar salts. A mobile phase gradient of decreasing salt concentration is applied. As the concentration of salts decreases, analytes desorb into the mobile phase. This phenomenon relates to the "salting out" often observed with proteins. Salts can screen the electrostatic and dipole–dipole interactions as well as disrupt the solvation shell of the molecules. Figure 1 presents the effects of various salts on the stability and solubility of proteins in solution. Chaotropic salts appear on the right (e.g., $NaClO_4$) and will improve the protein's solubility, but tend to disrupt protein folding where they unravel from their tertiary structure. Salts on the left, however, often stabilize the protein's structure, but increase the likelihood of adsorption and precipitation. Ammonium sulfate is the most commonly used salt for HIC because of its ability to induce adsorption onto the column. There is also an entropy-driven component to the separation. Water tends to form an ordered hydration layer around the protein as well as the stationary phase. These layers become disrupted and disordered when the protein approaches the stationary phase. The resultant increase in entropy makes retention favorable. Furthermore, selection of a hydrophobic stationary phase, salt type, temperature, pH and use of organic modifier mobile phase, can be used to adjust the selectivity of the separation for the particular analyte or analytes of interest.

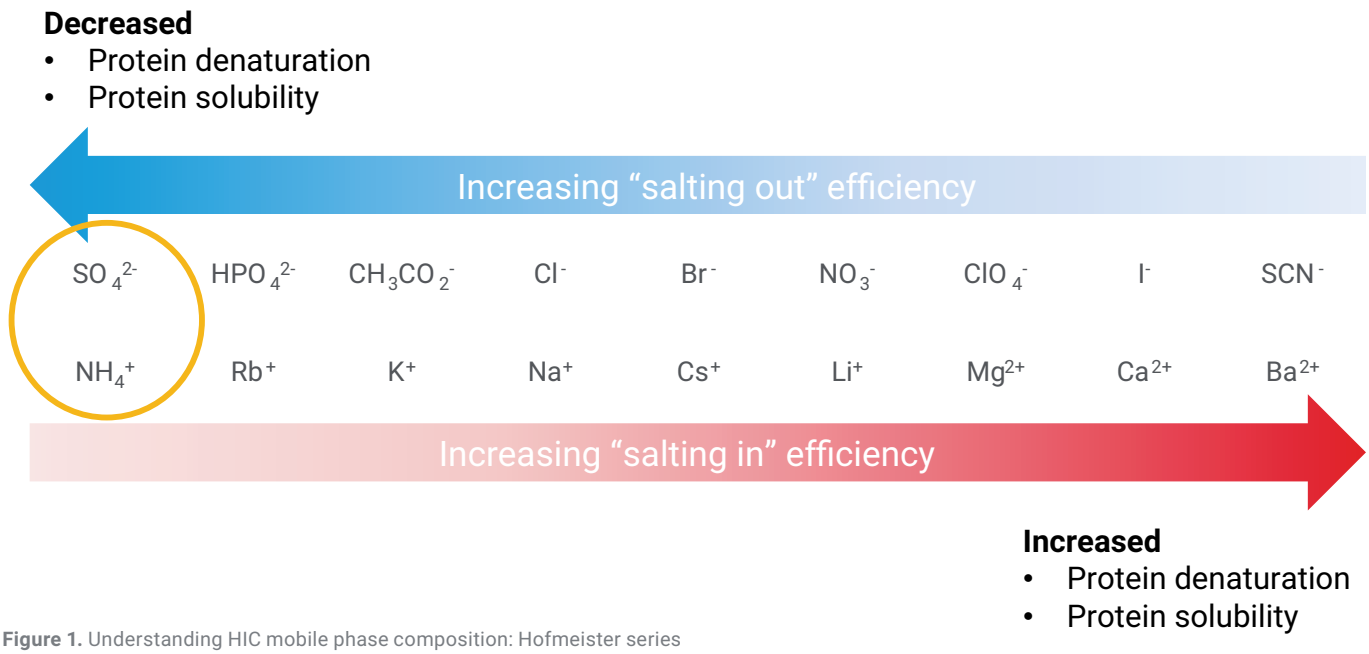


Figure 1. Understanding HIC mobile phase composition: Hofmeister series

With careful column and experimental design, HIC can be characterized by very good repeatability, both injection to injection, and column to column. Like reversed phase (or any chromatography), good mass transfer is essential to having high efficiency and narrow peaks. To accommodate the large molecular size of the simple species, HIC columns tend to have much larger pore sizes, up to 450 angstroms or more. The column capacity is kept high by using fully porous particles. The viscosity of the mobile phase presents some unique challenges. The slow mass transfer associated with large molecules in a viscous environment would make smaller particles the preferred choice, but the high viscosity puts pressure limits on how small the particles can become. Most columns use between 3 µm-5 µm particle size as the optimal compromise. Because of the drastic changes in viscosity that result from the variation in salt concentrations, it is not recommended to rapidly switch back to the initial mobile phase at the end of the gradient, as is typically done in reversed phase chromatography. Such a sharp change could damage the column. Rather, a relatively slow reverse gradient over several minutes should be used, followed by a 2–3 column volumes to complete the re-equilibration.

The ability of HIC to separate even closely related proteins makes it useful application in the analysis of the purity of protein samples. This method could be used to analyze stressed samples (e.g., heat, light) that might cause an increase in hydrophobic variants. Because this method is performed under conditions that do not irreversibly impact the structure of the antibody, collection of variants and testing by potency- or binding-related biological assays could be conducted to further understand the impact and significance of these species. One of the main success stories relates to ADC characterization [4]. In this case, HIC can be used to separate ADCs based on the number of conjugated small molecule drugs, and is thus useful for calculating the drug to antibody ratio or DAR [5]. HIC has also been shown to separate Asp/IsoAsp isomers as well as succinimide variants [6] and oxidation variants of methionine and tryptophan [7,8]. Bispecific mAbs, a modality of increasing importance, can be analyzed using HIC, in particular for assessing the population of parental mAbs vs the intended bispecific product [9]. Relative hydrophobicity of proteins can also be assessed, as a potential indicator of aggregation [4].

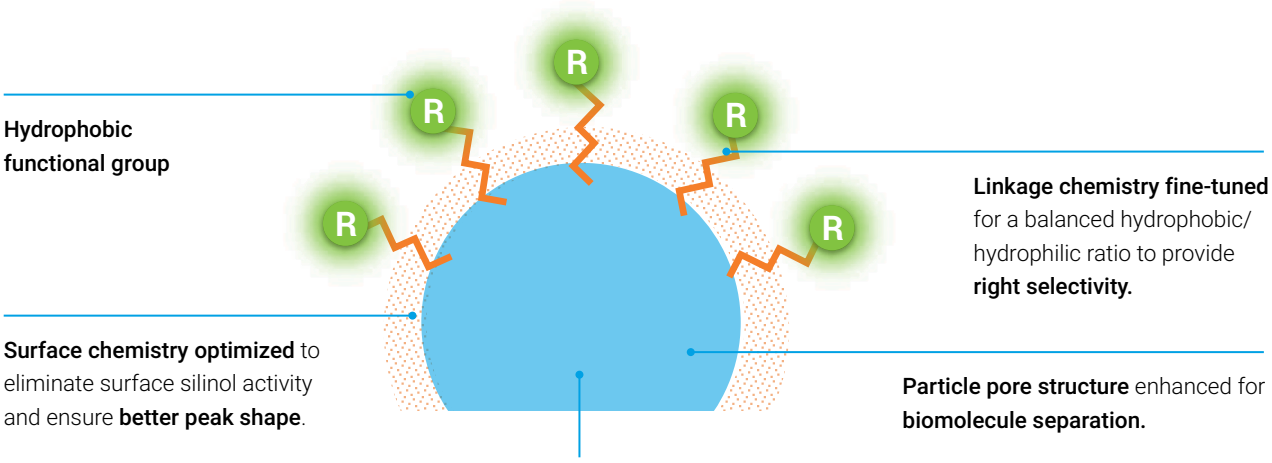
The Application Note (5994-0199EN) describes the separation of oxidized NISTmAb variants from their native form using the Agilent AdvanceBio HIC column.

Column Specifications

Pore Size	Particle Size	Temperature Limit	pH Range	Pressure Limit	Flow rate*
450Å	3.5 µm	60 C (at pH 7)	2.0 - 8.0 (at 35 C)	400 bar (Typical operating pressure <200 bar)	0.5 - 1.0 mL/min (4.6 mm id)

* In some cases, lowering the flow rate to 0.3 ml/min and extending gradient time may further improve resolution.

Novel hydrophobic interaction chemistry optimized to ensure best selectivity and robustness



AdvanceBio HIC columns deliver high resolution, robust, and reproducible separations of native proteins at the intact level. Built using the capabilities of the ZORBAX fully porous particles and proprietary bonding technology, these columns provide new levels of hydrophobicity and versatile single chemistry to address particularly challenging molecules such as monoclonal antibodies (mAbs), antibody drug conjugates (ADCs), and other recombinant proteins.

Together with the 1260 Infinity II Bio-inert LC system, the AdvanceBio HIC provides uncompromised performance and data consistency during characterization and validation.

- Optimized selectivity: ideal for mAb oxidation and ADC DAR ratios.
- Single chemistry: reduces the need for multiple-column screening for different CQAs.
- Enhanced robustness: improved column lifetime for ultimate confidence in your data.
- Proven performance: every batch of media is tested with NISTmAb.
- High quality: each column is individually tested to ensure packing efficiency.
- Greater productivity: shorter columns reduce analysis time while maintaining separation performance

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AdvanceBio HIC Columns

Agilent
Trusted Answers

Quick Reference Guide

In this document, Agilent applications chemists share their recommendations for an optimum LC system and its configuration for performing hydrophobic interaction chromatography (HIC).

They offer guidance on generic methods for protein and monoclonal antibody separations, and for separation of antibody drug conjugates (ADCs). These methods can then be modified to meet your exact requirements.

Additional application information is available at **www.agilent.com/chem/advancebio-hic**

Introduction

Hydrophobic interaction chromatography (HIC) relies on the effect of "salting out" to cause proteins to absorb onto the weakly hydrophobic HIC column. The most common salt used in this technique is ammonium sulfate. This is readily soluble at a concentration of 2 M, which is required in the analysis of many proteins. It is necessary to use a regular buffer to maintain protein solubility and to stabilize pH. For this purpose, sodium phosphate at neutral pH in concentrations from 20 to 100 mM is recommended. Proteins are eluted from the column in order of increasing hydrophobicity using a gradient from high to low ammonium sulfate concentration.

Operating Guidelines

An Agilent 1260 Infinity II Bio-inert LC is recommended due to the high salt concentrations used. Gradients should typically last from 10 to 20 column volumes for good resolution. Flushing and re-equilibration should last at least five column volumes. High salt concentrations can cause issues with some LC systems. Use of a fully bio-inert LC is recommended, ideally with a quaternary pump so that additional channels can be used for flushing. It is not advisable to leave 2 M ammonium sulfate solution in either the HIC column or the LC; flush the column with sodium phosphate buffer after use. Additional precautions, such as pump seal washing and needle washing, should be taken where possible.

Mobile phase solutions should be UV transparent and have little background absorption, allowing detection at low wavelengths for maximum sensitivity. However, it is advisable to use the highest solvent grade available. The Agilent AdvanceBio HIC column is available in two formats: 4.6 × 30 mm for fast separations, and 4.6 ×100 mm for higher resolution separations. For proteins, the optimum flow rate is typically around 0.4 to 0.5 mL/min. It is important to also take the viscosity of the mobile phase into consideration.

Maintaining temperature control is also vital: Samples should be kept in a refrigerated autosampler to avoid deterioration. Temperature control is also recommended during HIC separation: Many proteins are temperature sensitive, and changes in retention time and peak shape may be observed at different temperatures.

Storage

AdvanceBio HIC columns are shipped containing 100 % acetonitrile. **It is important that the columns are returned to 100% acetonitrile for storage after use.** Care must be taken not to mix 100 % acetonitrile with high salt mobile phase in case of precipitation.

Please refer to the User Guide at **www.agilent.com/chem/advancebio-hic-userguide** for column conditioning, use, and storage.

Reagents and chemicals

All reagents should be HPLC grade or higher.

Sample preparation

Dissolve samples in high concentration ammonium sulfate solution. A final concentration of 1 mg/mL should be sufficient for most needs.

Mobile phase preparation

Care should be taken to ensure that all salts are fully dissolved and the pH has been adjusted to its target value. It is necessary to filter all mobile phase solutions through a 0.22 µm membrane filter before use. Do not leave mobile phase on the instrument longer than necessary, and replace regularly.

Instrumentation

A 1260 Infinity II Bio-inert LC is recommended.

Gradients should typically last from 10 to 20 column volumes for good resolution. Flushing and re-equilibration should last at least five column volumes.

Agilent AdvanceBio HIC Columns

Description	Part No.
AdvanceBio HIC, 4.6 x 100 mm, 450 Å, 3.5 µm	685975-908
AdvanceBio HIC, 4.6 x 30 mm, 450 Å, 3.5 µm	681975-908

Suggested starting conditions

HPLC Conditions		
Column	Agilent AdvanceBio HIC, 4.6 × 100 mm, 450 Å, 3.5 µm (p/n 685975-908)	
Mobile phase	Eluent A:	50 mM sodium phosphate, pH 7.0
	Eluent B:	2 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0
	Eluent C:	propan-2-ol
	Eluent D:	water (for flushing)
Flow rate	0.5 mL/min	
Temperature	25 °C	
Injection volume	1 to 10 µL	

Gradient profile: Proteins

Time	%A	%B	%C
0	0	100	0
20	100	0	0
25	100	0	0
30	0	100	0
40	0	100	0

After all samples have been completed, flush the column with eluent A

Gradient profile: Monoclonal antibodies (mAbs)

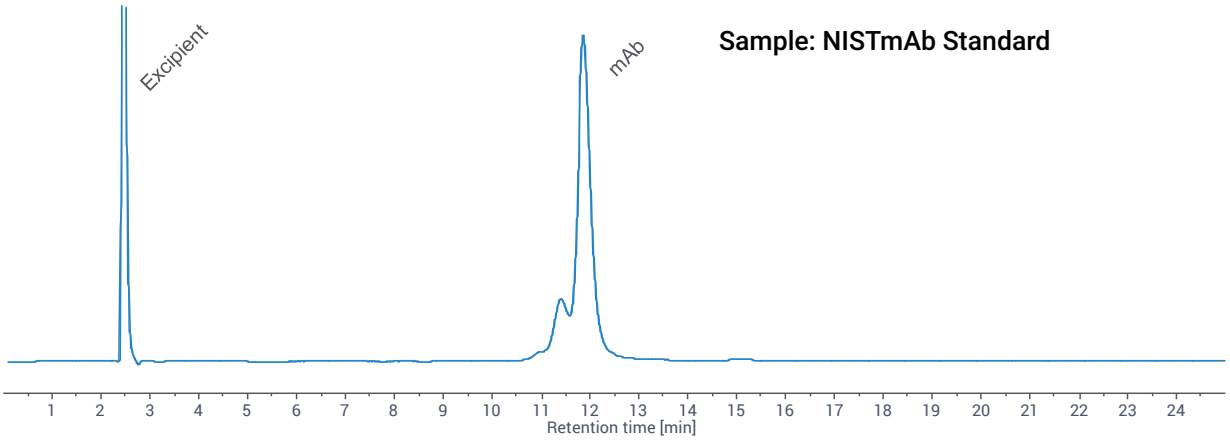
Time	%A	%B	%C
0	50	50	0
20	100	0	0
25	100	0	0
30	50	50	0
40	50	50	0

After all samples have been completed, flush the column with eluent A

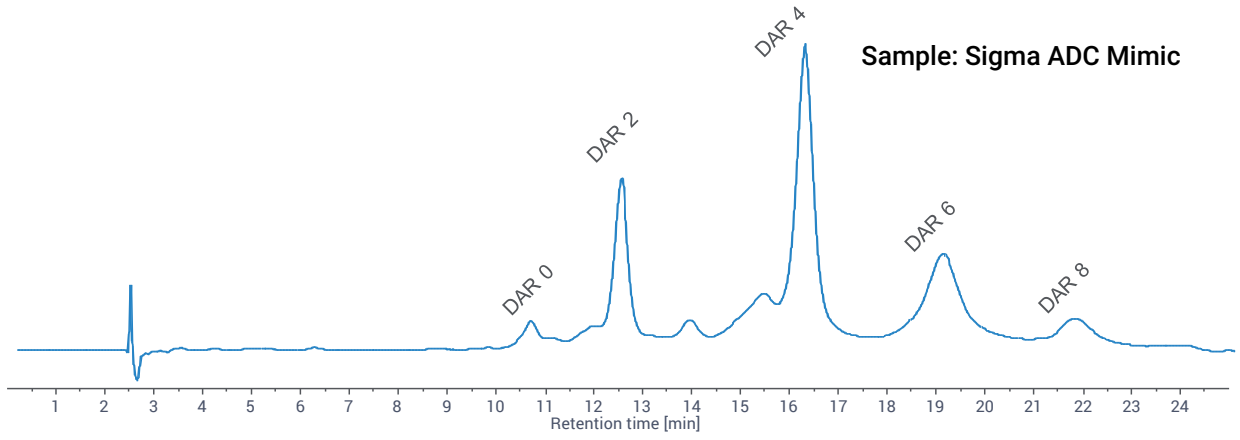
Gradient profile: Antibody drug conjugates (ADCs)

Time	%A	%B	%C
0	45	50	5
20	75	0	25
25	75	0	25
30	45	50	5
40	45	50	5

After all samples have been completed, flush the column with eluent A



Sample: NISTmAb Standard



Sample: Sigma ADC Mimic

Application Note

Biologics Research

Agilent

Trusted Answers

AdvanceBio HIC: a Hydrophobic HPLC Column for Monoclonal Antibody (mAb) Variant Analysis

Using the Agilent 1260 Infinity II Bio-Inert LC

Authors

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Sandeep Kondaveeti
Agilent Technologies, Inc.

Introduction

This Application Note describes the separation of oxidized monoclonal antibody (mAb) variants from their native form using the Agilent AdvanceBio HIC column. Oxidation of exposed amino acid side chain residues such as methionine, cysteine, and tryptophan is a common degradation pathway for monoclonal antibodies, and presents a major analytical challenge in biotechnology. Often, oxidized mAbs have decreased potency compared to their native form1. Therefore, to ensure the therapeutic efficacy of the mAb products, analysis of such degradation is critical. Oxidation of amino acid residues on an mAb can alter the hydrophobic nature of the mAb by the increase in polarity of the oxidized form, or also due to resulting conformational changes2. HPLC methods for separating biomolecules based on differences in hydrophobicity include reversed-phase and hydrophobic interaction chromatography (HIC). HIC can be applied to characterize mAb variants resulting from post-translational modifications (PTMs). The AdvanceBio HIC column provides excellent resolution of oxidized mAb variants from unmodified forms, and can resolve oxidized species without mAb digestion into subunits or other sample preparation methods.

Introduction

mAbs and related products such as antibody drug conjugates (ADCs) and bispecific antibodies (bsAbs) are the fastest growing classes of biotherapeutics. Recombinant mAbs are subject to many PTMs during processing, delivery, and storage. Among these modifications, oxidation of exposed amino acid side chains such as methionine (Met) and tryptophan (Trp) is a common occurrence. Various researchers have reported that oxidation of mAbs has an adverse effect on product shelf life and bio-activity[1,2]. Therefore, developing analytical methods to detect oxidized mAb variants has gained interest. The sulfoxide and sulfone side chains of methionine-oxidized mAb products are larger and more polar compared to the native form, which may alter protein structure, stability, and biological function. Hydrophobicity-based HPLC methods, such as reversed-phase liquid chromatography (RPLC) and HIC, are often used to characterize mAb variants. Recently, several studies have indicated that HIC can be applied to monitor oxidation of recombinant mAbs with reasonable selectivity and ease, as an excellent alternative to RPLC[3].

HIC is similar to RPLC in that separation of analytes is based on hydrophobic interactions with the stationary phase. The elution order in HIC enables proteins to be ranked based on their relative hydrophobicity. Unlike RPLC, HIC employs nondenaturing conditions, does not require the use of organic solvents or high temperatures, and separations are carried out at physiological pH, allowing for the preservation of protein structure. Thus, conformational changes in the native form of the protein may be analyzed using HIC[4].

AdvanceBio HIC is a silica-based HPLC column designed for the separation of mAbs and related products. Its unique proprietary bonded phase chemistry provides high resolution and desired selectivity for the analysis of mAbs and mAb variants. This Application Note describes the separation of oxidized NISTmAb variants using an AdvanceBio HIC column.

Experimental

Equipment and Materials

All chemicals and reagents were HPLC grade or higher, and were obtained from Sigma-Aldrich (now Merck) or VWR Scientific. Humanized IgG1k mAb sample (product item no. 8671) was obtained from NIST SRM Standards. Water was purified using a Milli-Q A10 water purification system (Millipore).

Instrumentation

Agilent 1260 Infinity II bio-inert LC comprising:

- Agilent 1260 Infinity II bio-inert pump (G5654A)
- Agilent 1260 Infinity II bio-inert multisampler (G5668A) with sample cooler (option no. 100)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A) with bio-inert heat exchanger (option no. 019)
- Agilent 1260 Infinity II diode array detector WR (G7115A) with bio-inert flow cell (option no. 028)

Software

Agilent OpenLab 2.2 CDS

mAb Oxidation with t-BHP treatment

A solution of 1 mL of NISTmAb (1 mg/mL) in formulation buffer was incubated with 0.2 % (v/v) of 70 % tert-butyl hydroperoxide (t-BHP) solution at room temperature for 24 hours. Residual oxidant was removed using Amicon Ultra-10 centrifugal filters with a molecular weight cut-off of 10 kDa. Samples were then buffer exchanged with 50 % mobile phase B.

Reaction conditions used to obtain Figure 6 data: 2 % (v/v) of 70 % t-BHP solution was added to a 1-mL sample of NISTmAb (1 mg/mL), and the reaction mixture was injected onto the column. The sample vial was held at 7 °C, and multiple injections from the same vial were carried out.

mAb Oxidation with H_2O_2 treatment

A solution of 1 mL of NISTmAb (1 mg/mL) in formulation buffer was incubated with 0.2 % (v/v) of 50 % hydrogen peroxide (H_2O_2) solution at room temperature for 24 hours. Residual oxidant was removed using Amicon Ultra-10 centrifugal filters with a molecular weight cut-off of 10 kDa. Samples were then buffer exchanged with 50 % mobile phase B

Results and discussion

Protein oxidation is frequently monitored in stability studies or stressed samples during formulation development (for example, samples exposed to a chemical oxidant such as hydrogen peroxide (H_2O_2), UV light, or metal ions). In this study, t-BHP and H_2O_2 were used as chemical oxidants to promote oxidation of NISTmAb samples. It was previously reported that both of these reagents tend to specifically oxidize Met side chain residues of the mAb. H_2O_2 more readily oxidizes less accessible, buried residues, whereas t-BHP is known to target more surface-exposed Met residues7. Figure 1 illustrates the reaction scheme for Met oxidation induced by chemical oxidants.

The NISTmAb (humanized IgG1k) amino acid sequence in Figure 2 shows that there are six possible surface-accessible Met residues located on both heavy chains of the mAb. Based on prior studies for most human IgG1-subclass antibodies, Met residues localized to the CH2 and CH3 domains of the antigen binding, or Fc, region are known to be highly susceptible to oxidation[5]. In the case of NISTmAb, Met 255 and Met 431 correspond to the amino acid residues prone to oxidation. This is depicted by the illustration in Figure 3.

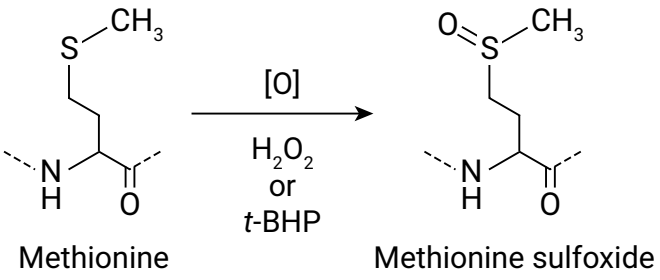


Figure 1. Methionine oxidation induced by chemical oxidant.

Method conditions

	HPLC Conditions
Column	AdvanceBio HIC, 4.6 × 100 mm (p/n 685975-908)
Mobile phase	Eluent A) 50 mM sodium phosphate, pH 7.0 Eluent B) 2 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0
Flow rate	0.3 to 0.5 mL/min
Column temperature	25 °C
Injection volume	5 µL
Final sample concentration	1 mg/mL
Detection	UV, 220 nm
Gradient profile	Flow rate: 0.5 mL/min Time %A %B 0 50 50 20 100 0 25 100 0 30 50 50 40 50 50

Heavy chain

QVTLRESGPA LVKPTQTLTL TCTFSGFSLS TAGMSVGWIR QPPGKALEWL ADIWWDDKKH YNPSLKDRLT
ISKDTSKNQV VLKVTNMDPA DTATYYCARD MIFNFYFDVW GQGTTVTVSS ASTKGPSVFP LAPSSKSTSG
GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS
NTKVDKRVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW
YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ
VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
QQGNVFSCSV MHEALHNHYT QKSLSLSPGK

Light chain

DIQMTQSPST LSASVGDRVT ITCSASSRVG YMHWYQQKPG KAPKLLIYDT SKLASGVPSR FSGSGSGTEF
TLTISSLQPD DFATYYCFQG SGYPFTFGGG TKVEIKRTVA APSVFIFPPS DEQLKSGTAS VVCLLNNFYP
REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSSTLTL SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC

Figure 2. NISTmAb amino acid sequence.

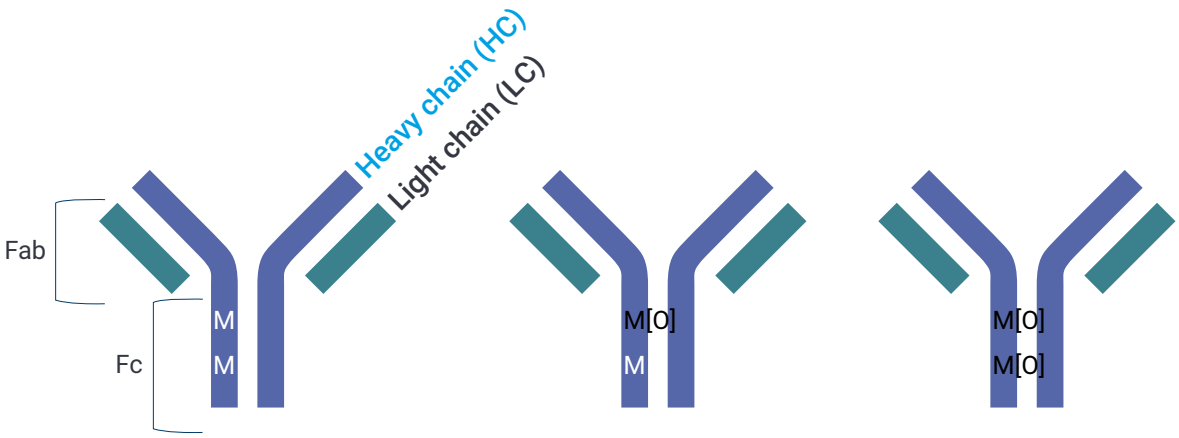
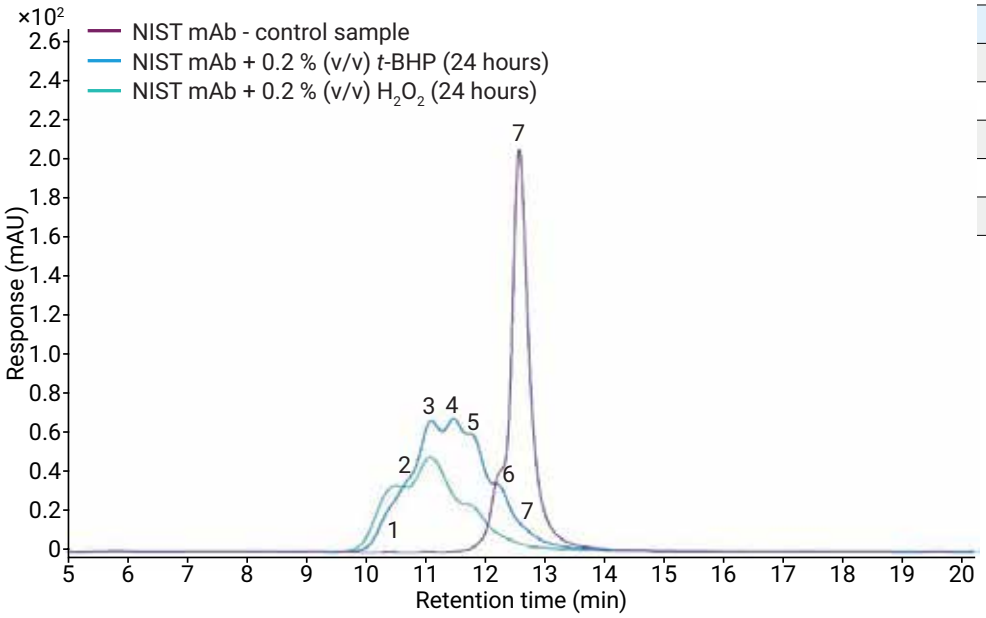


Figure 3. Methionine residues located in Fc region are most susceptible to oxidation in human IgG1 mAbs.

An AdvanceBio HIC column was able to differentiate oxidized mAb variants from the untreated mAb sample under low salt starting conditions. Oxidation of the NISTmAb with t-BHP under reported experimental conditions resulted in multiple peaks with shorter retention times, presumably due to conformational change. The HIC chromatogram (Figure 4) showing earlier retained peaks labeled 1 to 6 likely indicates the result of oxidized Met residues on the mAb, and peak 7 with a retention time of approximately 12.6 minutes, corresponds to nonoxidized mAb. For the H_2O_2-treated mAb sample, complete oxidation occurred, with three peaks eluting in a shorter retention time, indicating more aggressive oxidation of Met residues. These differences in the chromatograms of the IgG1k mAb sample incubated with two different oxidation reagents suggest that reactivity is governed by solvent accessibility of the Met residues and steric limitations of the oxidizing agent, as previously reported[6].

To further improve the resolution, a slower and shallower gradient was used. Using a flow rate of 0.3 mL/min and a starting ammonium sulfate concentration of 1.2 M with a lower gradient rate of 25 mM/min, better resolution was achieved with a relatively short analysis time (Figure 5). In this chromatogram, multiple mAb-oxidized species are clearly observed from the untreated mAb sample.



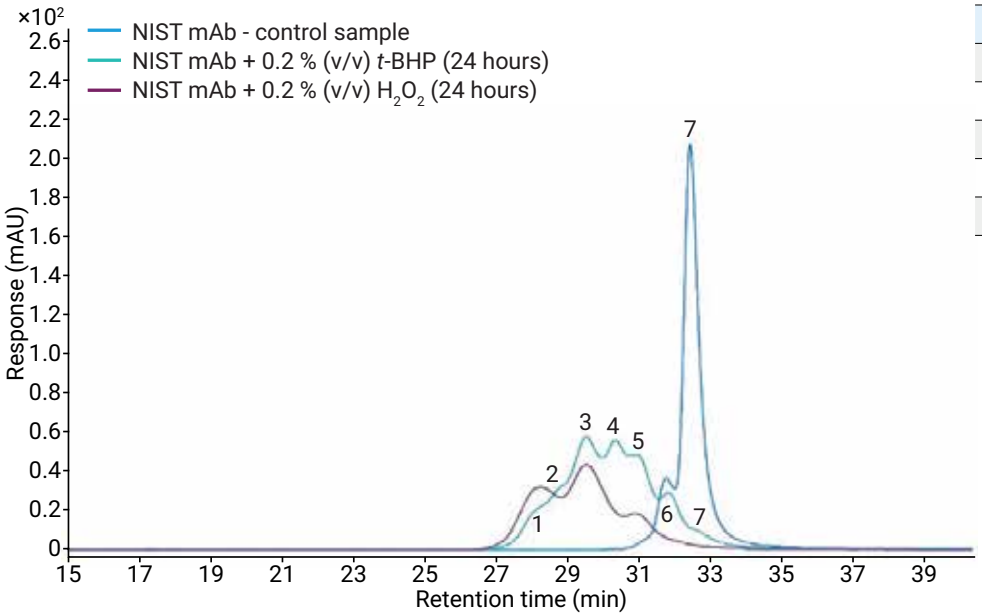
Time	%A	%B
0	50	50
20	100	0
25	100	0
30	50	50
40	50	50

50 mM/min

Figure 4. Separation of oxidized NISTmAb variants using lower starting salt concentration.

In Figure 6, the NISTmAb sample was incubated with 2 % (v/v) t-BHP, and the oxidation reaction was monitored at various time points using shallower gradient conditions. As represented by an overlay of chromatograms, the mAb oxidation progressed with t-BHP incubation time. Multiple mAb oxidation species were observed within a few hours of the oxidation reaction. This suggested that surface-accessible Met residues in both heavy chains of the mAb sample might be oxidized randomly, which was previously reported[5].

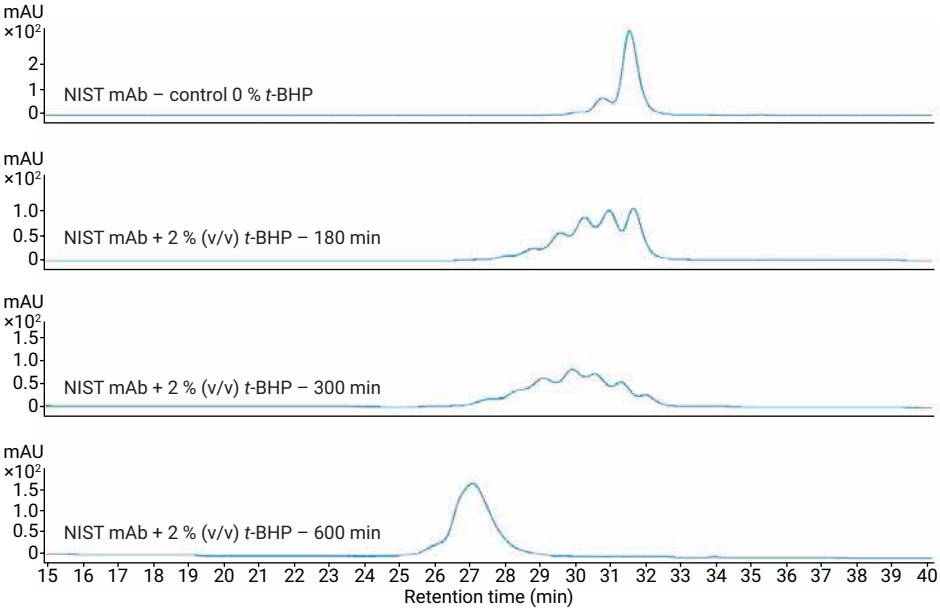
Further oxidation of the mAb sample after 10 hours of reaction led to a broad peak, indicating forced oxidation. It has previously been speculated that oxidation of deeply buried Met residues can lead to a more dramatic structural change, which may cause the mAb to partially unfold[7]. Partially unfolded mAb is likely to have more conformational variation, resulting in a broader peak with a large retention time shift.



Time	%A	%B
0	40	60
40	90	10
45	90	10
50	40	60
60	40	60

25 mM/min

Figure 5. Separation of oxidized NISTmAb variants using a shallow gradient.



Time	%A	%B
0	40	60
40	90	10
45	90	10
50	40	60
60	40	60

25 mM/min

Figure 6. Monitoring the t-BHP oxidized mAb reaction.

Conclusion

The AdvanceBio HIC column demonstrated the separation of oxidized mAb variants from its native form. Using the AdvanceBio HIC column, optimal separation of oxidized mAb variants can be achieved using slower flow rates and shallower gradient conditions, while maintaining relatively short analysis times.

References

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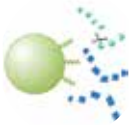
7. Liu, D.; et al. Structure and stability changes of human IgG1 Fc as a consequence of methionine oxidation. *Biochemistry* **2008**, *47(18)*, 5088–5100.

Peptide Mapping

Introduction

Peptide mapping – an invaluable tool for biopharmaceuticals – is a very powerful method and the most widely used identity test for proteins, particularly those produced by recombinant means. Peptide mapping can be used to comprehensively identify a protein primary structure. It is also possible to distinguish the exact position of a variant within the protein. It most commonly involves enzymatic digestion (usually using trypsin) of a protein to produce peptide fragments, followed by separation and identification of the fragments in a reproducible manner, allowing the detection and monitoring of single amino acid changes, oxidation, deamidation, and other degradation products. It also enables the direct detection of common monoclonal antibody variants such as N-terminal cyclization, C-terminal lysine processing, and N-glycosylation, as well as other post-translational modifications.

Using this approach, mAb sample will be broken into fragments and a high resolution reversed-phase separation should be able to separate these out into a classic “fingerprint” chromatogram. Combining the separation with mass spectrometry detection should make it possible to correlate the actual peaks observed in the peptide mapping chromatogram with the expected fragments predicted by the analysis software. Different proteins will give different peptide “fingerprints”, and these will include a wide range of sizes (from individual amino acids and dipeptides up to much larger polypeptides), with varying degrees of hydrophobicity. The recommended column for this type of separation is therefore a C18 reversed phase in either superficially porous or totally porous particles.



Peptide Mapping

Reversed-phase chromatography (<150 Å)

Reliably characterizes primary sequence and detects PTMs

AdvanceBio Peptide Mapping

Protein identification and PTM analysis

Attribute	Advantage
Endcapped C18 bonded phase	Good retention of hydrophilic peptides
Superficially porous particles	UHPLC-like efficiency at modest back pressure

A peptide map is a fingerprint of a protein and the end product of several processes that provide a comprehensive understanding of the protein being analyzed. It involves four major steps: isolation and purification of the protein; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and validated analysis of the peptides.

Peptide mapping is considered a comparative procedure that confirms the primary structure of the protein and detects alterations in structure. Additionally, it demonstrates process consistency and genetic stability. A peptide map should include positive identification of the protein, maximize coverage of the complete peptide sequence, and provide additional information and sequence identification beyond that obtained at the non-digested protein level.

The selection of a chromatographic technique to separate peptides and generate peptide maps depends on the protein, experimental objectives, and anticipated outcome. However, the excellent resolving power of reversed-phase chromatography (RPC) makes this technique the predominant HPLC technique for peptide mapping separations. It is also ideal for both analytical and preparative separations because of the availability of volatile mobile phase eluents. It is important to note that the preferred columns for peptide mapping separations are similar to those used for small molecules, but because most peptide mapping separations are performed at low pH and elevated temperature, columns with excellent pH stability and minimal silanol effects are routinely used.

Careful inspection of the complete characterization strategy is required to generate successful peptide maps. A profile may consist of over 100 peaks representing individual peptides and their derivatives, so it requires knowledge of sample preparation methods, powerful separation techniques and validated protocols. Having the skill and information to develop a successful peptide map will help you achieve the best possible separation of your proteolytic digests and deliver a successful and reliable peptide characterization outcome. Optimum peak shape is obtained using trifluoroacetic acid as ion pair reagent and for this separation the AdvanceBio Peptide Mapping column is the preferred choice.

This column contains a 120 Å pore size Poroshell particle and provides excellent resolution and peak capacity without the need for UHPLC instrumentation. For extremely hydrophilic, small peptides AdvanceBio Peptide Mapping is recommended for best retention. For applications where MS detection will be used, it is often preferable to use formic acid as ion pairing reagent.

The featured application note in this chapter highlight high-throughput workflow that uses the Agilent AssayMAP Bravo liquid-handling robot, the Agilent 1290 Infinity II UHPLC system coupled to Agilent 6545XT AdvanceBio LC/Q-TOF, and automatic data analysis using Agilent BioConfirm software for complete NISTmAb sequence mapping analysis.

Quick Start Guide:

Reduce Peptide Mapping time without losing resolution

Biopharmaceutical discovery and development require you to characterize a target molecule's primary sequence to confirm its identity, and/or determine amino acid substitutions or modifications that may occur during manufacturing. It is currently necessary to use high-resolution techniques to resolve individual peptides for identification and quantitation. Accordingly, conventional peptide mapping with fully porous HPLC columns can take 60 minutes or longer to complete.

Agilent AdvanceBio Peptide Mapping columns let you quickly resolve and identify amino acid modifications in primary structure.

These advanced biocolumns feature a 120Å pore size with superficially porous 2.7 µm particles. They are specially tested with a challenging peptides mix to ensure reliable peptide mapping performance. In addition, AdvanceBio Peptide Mapping columns deliver exceptional resolution and speed for UHPLC, and excellent results for conventional HPLC too.

- Greater analytical confidence: Each batch of AdvanceBio Peptide Mapping media is tested with a rigorous peptide mix to ensure suitability and reproducibility, and to enable the identification of key peptides in complex peptide maps.
- Save time: 2 to 3 times faster than fully porous HPLC columns.
- Every instrument works harder: 4.6, 3.0, and 2.1 mm id columns are stable to 600 bar, enabling you to get the most from your UHPLC instruments. They can also deliver excellent performance for your legacy 400 bar instruments, too.
- Increased flexibility: Achieve increased MS sensitivity with formic acid mobile phases on any HPLC.

AdvanceBio columns are rigorously tested to ensure reproducibility and confidence in your results. They are also backed by Agilent's 60-day full satisfaction warranty.

Agilent AdvanceBio Peptide Mapping BioHPLC Columns.

With their 2.7 µm particles and C18 functionality, Agilent AdvanceBio Peptide Mapping columns provide excellent retention, resolution, and peak shape for basic hydrophobic peptides.

To learn more, visit **agilent.com/chem/AdvanceBio**

Quickly confirm the identity of target proteins and peptides

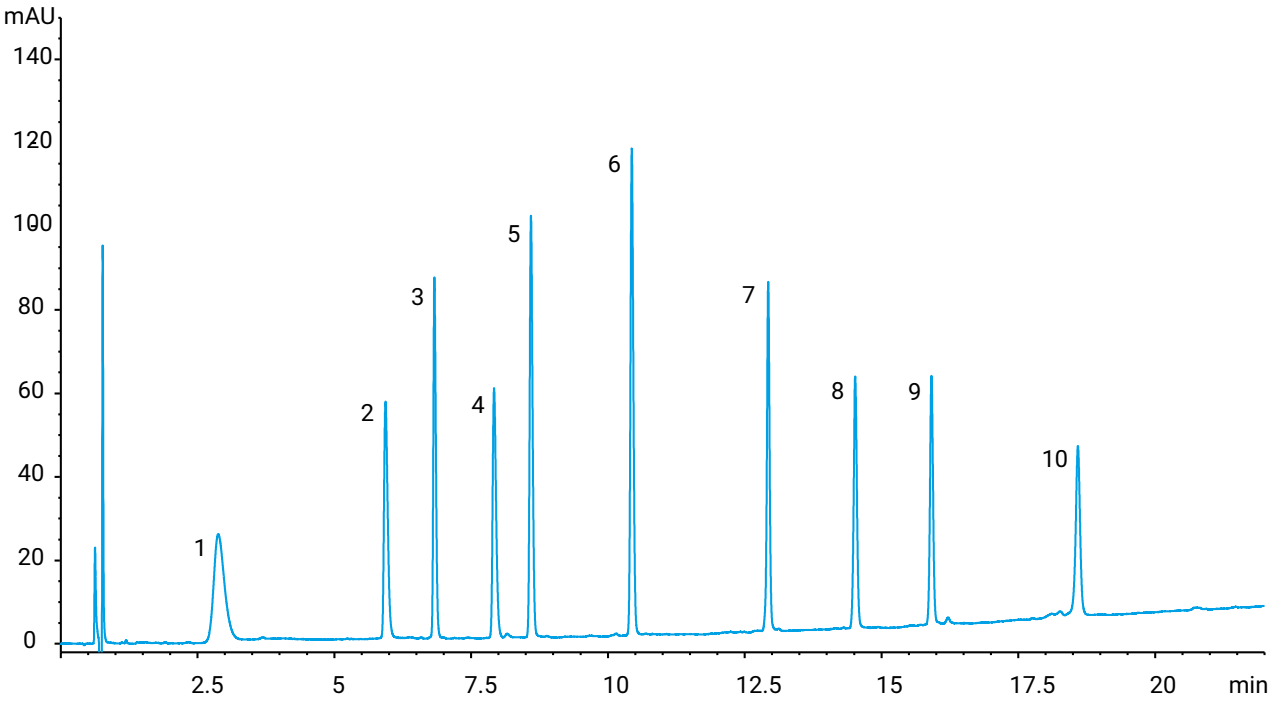
Quality Assurance Testing with Agilent Peptide Mix

Test mix used for every batch of AdvanceBio Peptide Mapping media. The mixture contains 10 hydrophilic, hydrophobic, and basic peptides, ranging in molecular weight from 757 to 2845 Da. Every column is also tested with a small-molecule probe to ensure efficiency.

Conditions

Parameter	Value
Column:	AdvanceBio Peptide Mapping, 2.1 x 150 mm, 2.7 µm, p/n 653750-902
Flow rate:	0.5 mL/min
Injection:	3 µL
Temp:	55 °C
Detection:	220 nm
Gradient:	A, water (0.1% TFA), B, ACN (0.1% TFA), 0-25 min,15-65% B; 25-26 min, 65-95% B
Sample:	Agilent Peptide Mapping Standards Mix (0.5-1.0 µg/µL per peptide) p/n 5190-0583

Pk no.	Peptide
1	Bradykin frag (1-7)
2	Bradykin Acetate
3	Angiotensin II
4	Neurotensin
5	Angiotensin I
6	Renin
7	[Ace-F-3,-2 H-1] Angiotensinogen (1-14)
8	Ser/Thr Protein Phosphotase (15-31)
9	[F14] Ser/Thr Protein Phosphotase (15-31)
10	Mellitin (Honey bee venom)



Separation of Agilent Peptide Mix standard on AdvanceBio Peptide Mapping column

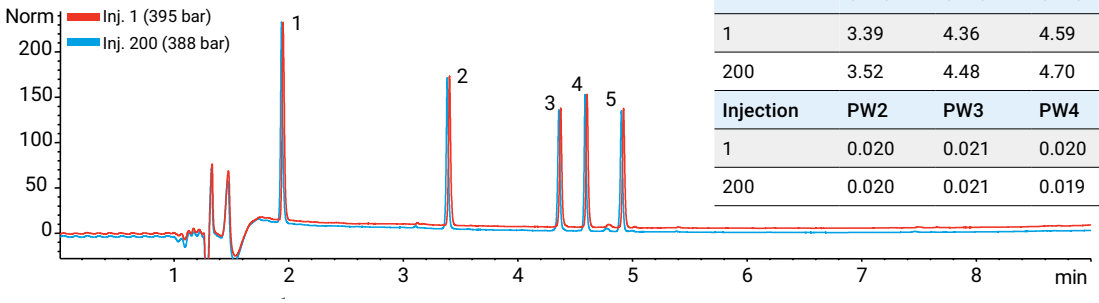
Lot-to-Lot Reproducibility after 200 Injections

Superior reproducibility, lot-to-lot and run-to-run. A 2.1 x 250 mm AdvanceBio Peptide Mapping column was used for maximum resolution.

Conditions

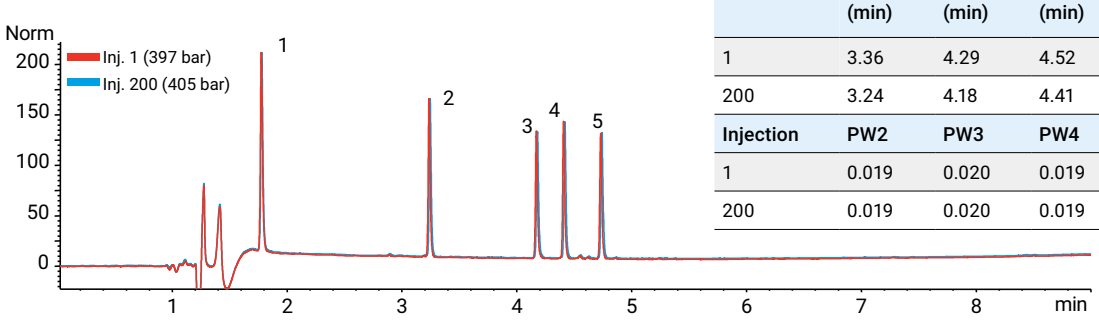
Parameter	Value
Column:	AdvanceBio Peptide Mapping, 2.1 x 250 mm, 2.7 µm, p/n 651750-902
Flow rate:	0.5 mL/min
Injection:	1 µL
Temp:	55 ºC
Detection:	220 nm
Gradient:	A, water (0.1% TFA), B, ACN (0.08% TFA), 0-8 min, 10-60% B; 8.1-9 min, hold 95% B
Sample:	Sigma HPLC peptide standards: 1-Gly-Tyr, 2-Val-Tyr-Val, 3-Met Enk, 4-Angio II, 5-Leu Enk

Silica lot PEP1227229



Injection	RT2 (min)	RT3 (min)	RT4 (min)	RT5 (min)
1	3.39	4.36	4.59	4.90
200	3.52	4.48	4.70	5.02
Injection	**PW2**	**PW3**	**PW4**	**PW5**
1	0.020	0.021	0.020	0.022
200	0.020	0.021	0.019	0.021

Silica lot B12169



Injection	RT2 (min)	RT3 (min)	RT4 (min)	RT5 (min)
1	3.36	4.29	4.52	4.85
200	3.24	4.18	4.41	4.74
Injection	**PW2**	**PW3**	**PW4**	**PW5**
1	0.019	0.020	0.019	0.020
200	0.019	0.020	0.019	0.020

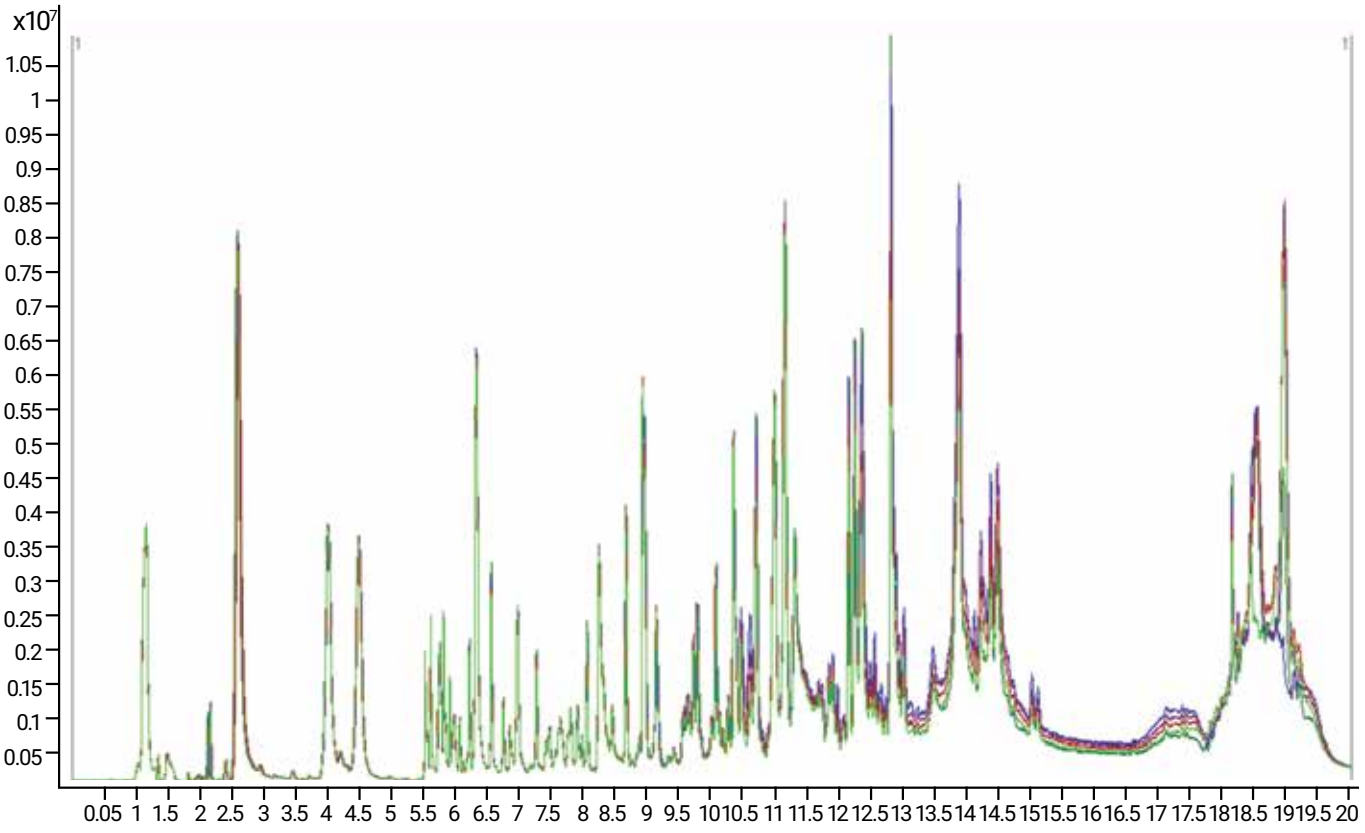
Chromatograms represent superior reproducibility on multiple batch lots of AdvanceBio Peptide Mapping column

LC /MS Reproducibility

Excellent reproducibility of peak heights and retention times for more accurate identification of target peptides. The entire IgG1 tryptic peptide map was completed in just 20 minutes (n=5).

Conditions

Parameter	Value
Column:	AdvanceBio Peptide Mapping, 3.0 x 150 mm, 2.7 µm, p/n 653950-302
	LC /MS (Agilent 6520 Q-TO F) P arameters: Dry gas: 10 L/min, Vcap: 4000 V, fragmentor: 150 V
Flow rate:	0.3 mL/min
Injection:	1 µL
Temp:	40 °C
Detection:	220 nm
Gradient:	A, water (0.1% FA), B, ACN (0.10% FA), 0-3 min, 2% B; 3-13 min, 2-45% B; 13-15 min, 45-65% B; 15.1-17 min., hold 90% B
Sample:	Stratagene mAb, in-house tryptic digestion



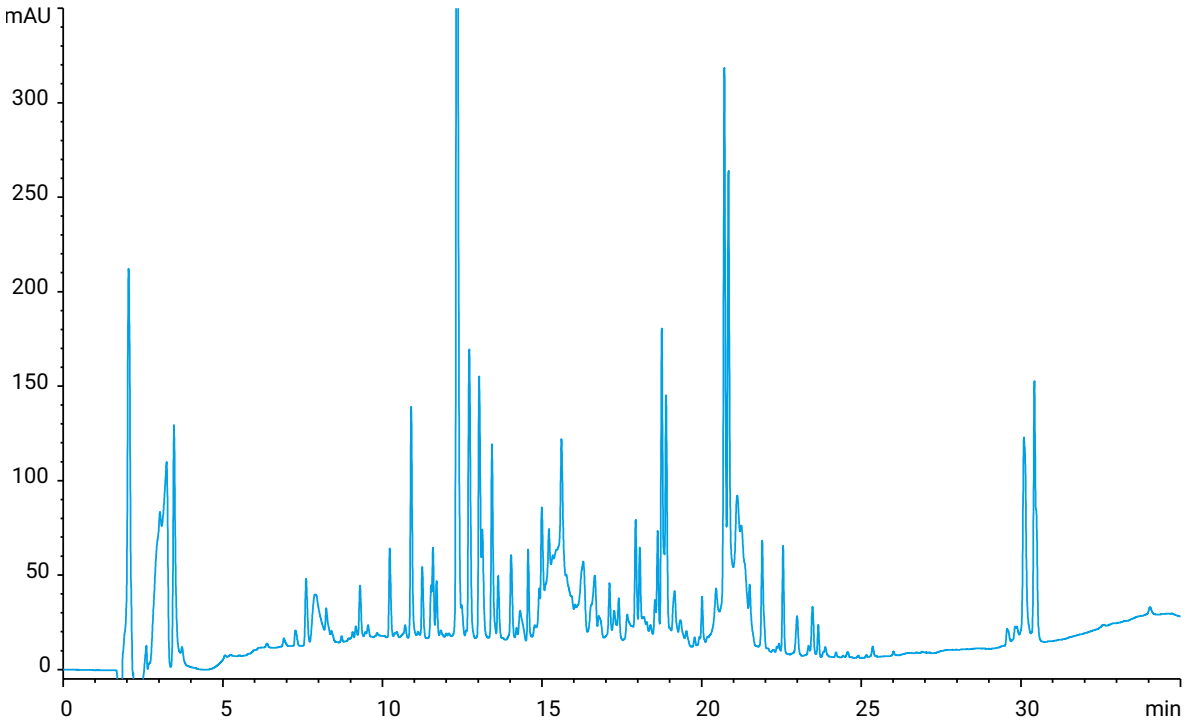
Peptide Map of a Biosimilar EPO

The top chromatogram shows a peptide map of a highly glycosylated EPO from a biosimilar. Note the excellent resolution achieved for small peptide fragments using UV detection. The bottom chromatogram shows the same separation using mass spectroscopy to determine the sequence coverage (100%). UV detection is used for comparing peptide maps, while MS is ideal for identifying amino acid substitutions and modifications.

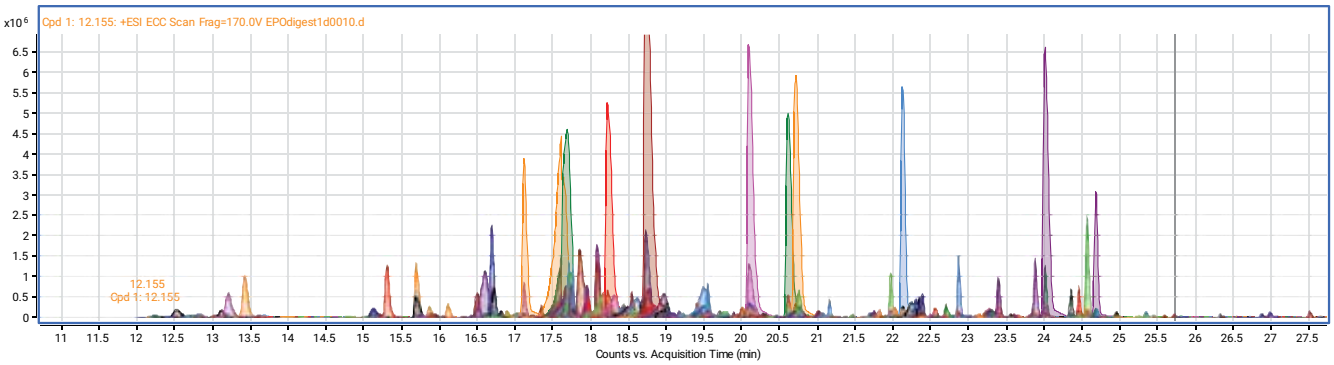
So, you can easily confirm protein identity, and identify any post-translational modifications, using the AdvanceBio Peptide Mapping column.

Conditions

Parameter	Value
Column:	AdvanceBio Peptide Mapping, 2.1 x 250 mm, 2.7 µm, p/n 651750-902
Flow rate:	0.4 mL/min
Injection:	5 µL (2.0 mg/mL)
Temp:	55 °C
Detection:	220 nm
Gradient:	A, water (0.1% FA); B, ACN (0.1% FA), 0-28 min, 3-45% B; 28-33 min, 45-60% B; 33-34 min, 60-95% B



EPO digest, Peptide sequence coverage achieved using UV Detection



EPO digest, LC/MS TOF 100% sequence coverage achieved using MassHunter Workstation software

Agilent AdvanceBio columns:

For faster, more consistent biopharmaceutical analysis

AdvanceBio Peptide Mapping columns are part of Agilent's growing state-of-the-art family of biocolumns. They are designed to deliver consistent, exceptional performance for the separation and characterization of peptides and proteins, antibodies, conjugates, new biological entities, and biopharmaceuticals. The science behind AdvanceBio columns helps to advance accuracy and productivity that support faster analysis and efficiency in your lab.

Agilent AdvanceBio Peptide Mapping Columns

Description	Part Number
4.6 x 150 mm, 2.7 µm	653950-902
3.0 x 150 mm, 2.7 µm	653950-302
2.1 x 250 mm, 2.7 µm	651750-902
2.1 x 150 mm, 2.7 µm	653750-902
2.1 x 100 mm, 2.7 µm	655750-902
4.6 mm Fast Guard*	850750-911
3.0 mm Fast Guard*	853750-911
2.1 mm Fast Guard*	851725-911

* Fast Guards extend column lifetime without slowing down the separation or affecting resolution.

Peptide sample preparation for mass spec analysis, intelligently automated

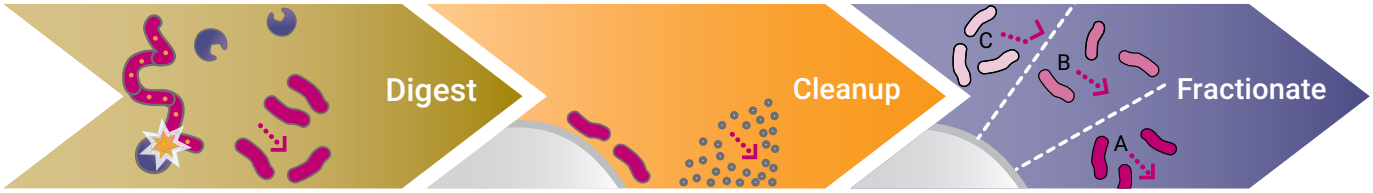
Manual sample preparation of peptides is a time-consuming process. If you are doing peptide mapping applications on MS, you are likely looking for increased throughput. And you are going to be reliant on a highly reproducible end-to-end workflow to ensure your results are consistent. AssayMAP transforms digestion, cleanup, and fractionation workflows to enable previously unachievable precision and throughput:

- Improved reproducibility, due to decreased human error – <5 % CVs
- Increased throughput – up to 384 samples each day
- Significantly reduces hands-on time – freeing up scientists to do analytical work
- Faster method development – the automated platform enables you to quickly optimize methods

AssayMAP Peptide Sample Prep Solution is based on the powerful combination of miniaturized, packed bed chromatography, the state-of-the-art Bravo Liquid Handling Platform and a simple, applications-based user interface that creates an open access environment for both novices and experienced users and simplifies the most challenging sample preparation workflows.

AssayMAP peptide sample prep solution

For Mass Spec Analysis



Digestion:

- In-solution digestion with user-supplied reagents
- Parallel process up to 4x96-well plates
- 1 manual pipetting step

Benefits:

- Reduce user variability
- Improve throughput and reproducibility

Cleanup:

- Quantitative separation method using reversed-phase cartridges
- Parallel process 1x96-well plate

Benefits:

- 10 µL elution equals short dry down times or "dilute and shoot" method
- Process control – every sample is treated identically

Fractionation:

- Strong cation exchange (SCX) cartridges generate up to 6 fractions to simplify the sample using step-wise elution with pH or salt
- Parallel process 1x96-well plate

Benefits:

- Increases LC/MS throughput by taking fractionation offline, reducing long LC gradient times
- Powerful enrichment tool for simplifying samples and isolating target peptides prior to analysistreated identically

Total workflow benefit:

- User interfaces for workflows are standardized for ease-of-use and linked for workflow integration.
- AssayMAP reduces the need for sample replicates and requires fewer repeated samples.

Achieve total workflow reproducibility with Agilent AssayMAP solution for sample prep before mass spec analysis

The AssayMAP Peptide Sample Prep Solution was used to digest 64 replicates each of two sample types: BSA in urea and guanidine HCL. The samples were cleaned using AssayMAP reversed-phase cartridges and analyzed using an Agilent AdvanceBio Peptide Mapping column, Agilent 1290 Infinity LC, and an Agilent 6550 iFunnel Q-TOF mass spectrometer. The experiment was repeated on day two to examine reproducibility. %CV was determined for 25 peptides within each sample as shown in Table 1. The different %CV bins are shown. Illustrating the contributions of the total average %CV. To further showcase the reproducibility, peak area for representative peptides are shown in Figure 12.

The AssayMAP sample prep took about four hours per day, with only two hand-on hours per day. Manual sample prep for the same workflow would take about eight hours per day, with four hands-on hours each day.

Total workflow CVs were <4%. The full workflow included AssayMAP Peptide Sample Prep system, an Agilent AdvanceBio Peptide Mapping Column, the 1290 Infinity LC System, and an Agilent 6550 iFunnel Q-TOF mass spectrometer.

For more details about this application, see Agilent publication 5991-2474EN.

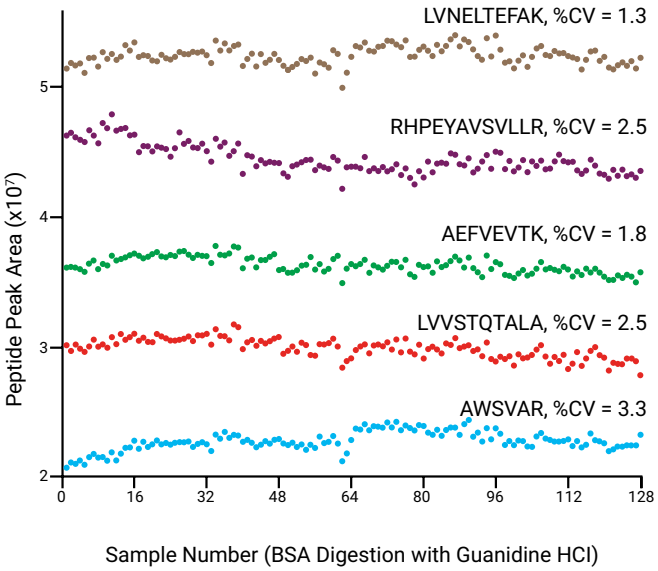


Figure 12. Scatter plots showing peak area of 4 peptides over 2 days.

Table 1. – %CV by day with different %CV bins.

	Urea (n=64, 62)		Guanidine HCl (n=64, 62)	
25 Peptides	**Day 1**	**Day 2**	**Day 1**	**Day 2**
Average Peak Area %CV	3.3	3.7	2.3	2.6
Peptides with %CV<5	23	21	25	23
Peptides with 5>%CV<10	2	3		1
Peptides with %CV>10		1		1

Application Note

Biotherapeutics and Biosimilars

Agilent

Trusted Answers

Making Peptide Mapping Routine with the Agilent 6545XT AdvanceBio LC/Q-TOF

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Madison, WI, USA

Introduction

Monoclonal antibodies (mAbs) comprise a rapidly growing group of protein-based biomolecules being researched. Due to the heterogeneous nature of protein drugs, extensive analytical characterization is required.

Peptide mapping by the combination of liquid chromatography and electrospray mass spectrometry (LC/MS) is a well-established technique used by the biopharmaceutical industry for the confirmation of the primary sequence of an mAb. The comprehensive characterization provides not only the complete amino acid sequences of mAbs and their variants, but also the information on post-translational modifications (PTMs) and locations1-3. However, the lack of automatic workflow in the data processing and result interpretation has been the rate-limiting step for most biopharmaceutical analytical or clinical research laboratories.

In this study, we have developed a high-throughput workflow that uses the Agilent AssayMAP Bravo liquid-handling robot, the Agilent 1290 Infinity II UHPLC system, the Agilent 6545XT AdvanceBio LC/Q-TOF, and automatic data analysis using Agilent BioConfirm software for complete sequence mapping analysis.

Figure 1. Agilent 6545XT AdvanceBio LC/Q-TOF system.

Experimental

Materials and methods

Monoclonal antibody (mAb) standard RM 8671 was purchased from National Institute of Standards and Technology (NIST). DL-Dithiothreitol (DTT), iodoacetamide (IAA) and guanidine-hydrochloride were purchased from Sigma-Aldrich. High quality mass spec grade Trypsin/Lys-C enzyme mix was obtained from Promega. AssayMAP C18 cartridges were from Agilent Technologies.

The Agilent AssayMAP Bravo liquid handling system was used to dilute, digest, and desalt the NISTmAb sample. Samples were then dried down and resuspended with 0.1 % TFA in DI water. Approximately 0.5 µg of mAb digested sample was injected for each LC/MS/MS analysis.

LC/MS analysis

LC/MS analyses were conducted on an Agilent 1290 Infinity II UHPLC system coupled with an Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with an Agilent Dual Jet Stream ESI source. LC separation was obtained with an Agilent AdvanceBio Peptide Mapping column (2.1 × 150 mm, 2.7 µm). Tables 1 and 2 list the LC/MS parameters used.

Data processing

Raw data acquired from LC/MS/MS were processed using Agilent MassHunter BioConfirm B.08.00 software. This powerful algorithm simplifies downstream data analysis, enabling the automatic identification of peptides and PTMs when compared to a theoretically digested NISTmAb sequence.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II UHPLC System	
Column	AdvanceBio Peptide Mapping column, 2.1 x 150 mm, 2.7 µm (p/n 653750-902)
Thermostat	4 °C
Solvent A	0.1 % Formic acid in water
Solvent B	0.1 % Formic acid in 90 % acetonitrile
Gradient	0–15 minutes, 0–40 % B 15–18 minutes, 40–90 % B 18–20 minutes, 90 % B
Column temperature	60 °C
Flow rate	0.4 mL/min
Injection volume	3.0 µL

Table 2. MS Acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System	
Gas temperature	325 °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	500 V
Fragmentor	175 V
Skimmer	65
Quad AMU	95
Reference mass	121.0509, 922.0098
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
Min MS/MS acquisition rate	3 spectra/sec
Isolation width	Narrow (~ 1.3 *m/z*)
Precursors/cycle	Top 10
Collision energy	3.6*(*m/z*)/100–4.8
Threshold for MS/MS	3,000 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

A comprehensive peptide mapping of an antibody can be a complex and time-consuming process due to the necessary sample preparation and data analysis for hundreds of peptides with various modifications. We used the high-throughput AssayMAP Bravo liquid handling system, Agilent Infinity II UHPLC, and Agilent accurate-mass AdvanceBio Q-TOF system to overcome these challenges. In addition, the automatic data processing workflow by Agilent MassHunter BioConfirm B.08 software improved the overall data mining and resulting accuracy significantly.

The Figure 2 illustrates the extracted compound chromatogram (ECC) of peptides from Trypsin/Lys-C digested NISTmAb. Excellent chromatographic resolution was achieved with a short 15-minute gradient. Each identified peptide from the NISTmAb light chain and heavy chain are labeled with their corresponding sequence numbers.

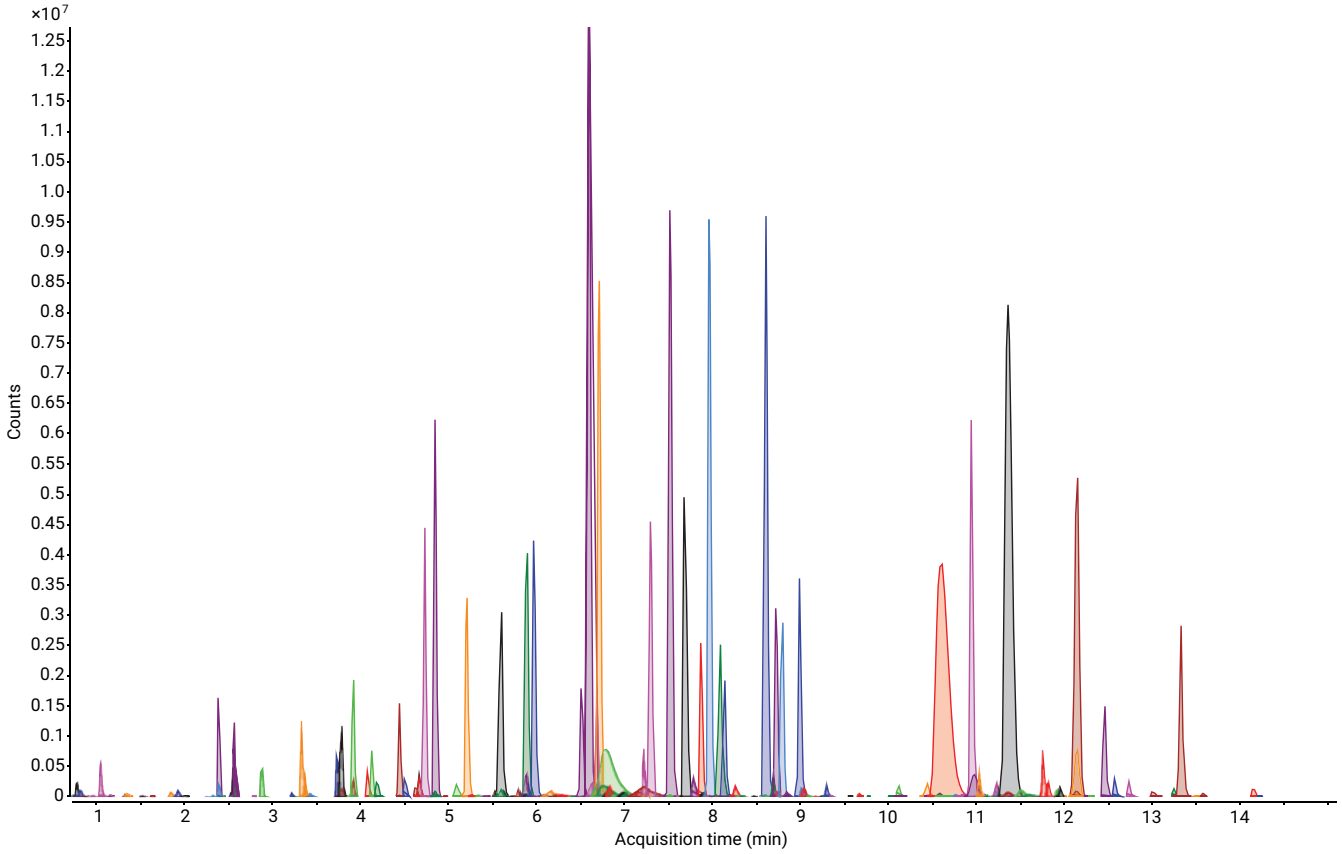

Figure 2. ECC of peptides from Trypsin/Lys-C digested NISTmAb standard RM 8671, separated using an Agilent AdvanceBio Peptide Mapping column.

In our peptide mapping workflow, all matched peptides were required to have < 5 ppm MS mass error and have at least one confirmatory MS/MS spectrum. The BioConfirm scoring algorithm considers factors such as: the presence of b and y fragment ions, immonium ions, mass accuracy, MS/MS peak intensity, and other parameters. Figure 3A highlights the detailed example of the identified peptides by the BioConfirm software. The majority of identified peptides displayed excellent mass accuracy, with errors less than 1 ppm. After the peptides were identified, an mAb sequence coverage map was reported automatically. Figure 3B shows a sequence coverage of 99.4 % on the NISTmAb, achieved using the 15-minute UHPLC gradient.

The peptide mapping result summary (Figure 4) in BioConfirm allows quick review of detailed peptide information including mass, retention time, matched peptide sequence, modifications, and matching score. It allows users to review the TIC of the sample as well as the individual peptide MS and MS/MS spectra. In addition, the abundances of the precursor molecule along with its fragment ions are also provided for relative quantitation analysis.

Score	Mass	RT	Score (MFE)	Seq Loc	Tgt Seq Mass	Diff (Bio, ppm)
88.15	1796.8876	12.1442	100	A(126-141)/ C(126-141)	1796.888	-0.18
84.09	1723.9	6.7992	87.7	B(344-358)/ D(344-358)	1723.9006	-0.33
78.65	1285.6665	7.2351	80	B(348-358)/ D(348-358)	1285.6667	-0.12
77.35	1080.5225	4.7314	100	A(19-28)/ C(19-28)	1080.5234	-0.78
77.03	2101.119	10.6051	100	A(107-125)/ C(107-125)	2101.1208	-0.85
75.94	1320.6706	7.9666	100	B(137-150)/ D(137-150)	1320.6708	-0.14
75.12	1923.0328	6.1898	80	B(342-358)/ D(342-358)	1923.0326	0.07
75.07	3043.3936	7.6842	100	B(418-442)/ D(418-442)	3043.393	0.19
74.63	1501.7518	8.9926	100	A(169-182)/ C(169-182)	1501.7512	0.44
72.94	1806.9981	11.7542	100	B(305-320)/ D(305-320)	1806.9992	-0.61
72.84	785.4405	4.5012	99	A(53-60)/ C(53-60)	785.4396	1.18
71.52	1945.0193	11.6124	80.3	A(108-125)/ C(108-125)	1945.0197	-0.21
71.46	1891.8935	7.8711	100	A(1-18)/ C(1-18)	1891.8946	-0.54
71.22	6712.3081	13.3296	100	B(151-213)/ D(151-213)	6712.3072	0.14
69.7	1872.9134	10.9458	100	B(396-412)/ D(396-412)	1872.9146	-0.6
69.36	1185.6395	9.04	100	B(125-136)/ D(125-136)	1185.6394	0.13
69.19	1160.6214	8.6107	100	B(364-373)/ D(364-373)	1160.6223	-0.81
68.93	1797.872	10.7793	100	A(126-141)/ C(126-141)	1797.872	-0.01
68.64	1676.794	8.7257	100	B(278-291)/ D(278-291)	1676.7947	-0.4
68.24	1847.7825	7.2197	100	B(84-99)/ D(84-99)	1847.7818	0.36
68.23	951.5279	7.5208	100	A(45-52)/ C(45-52)	951.5277	0.16
67.57	1787.889	12.5731	100	B(46-59)/ D(46-59)	1787.8883	0.38
67.42	659.3488	7.2981	100	B(443-449)/ D(443-449)	659.349	-0.28
67.13	1923.0327	6.3056	63.1	B(342-358)/ D(342-358)	1923.0326	0.04
66.7	2228.1847	11.2323	100	B(305-323)/ D(305-323)	2228.1841	0.26
65.35	834.4266	6.6893	100	B(252-258)/ D(252-258)	834.4269	-0.42
65.15	2138.0206	8.795	100	B(259-277)/ D(259-277)	2138.0202	0.22
64.64	487.3003	3.7822	100	A(103-106)/ C(103-106)	487.3006	-0.59
64.38	559.3119	4.4421	100	A(145-148)/ C(145-148)	559.3118	0.06
64.1	574.3323	3.9175	100	B(413-417)/ D(413-417)	574.3326	-0.6

Figure 3A. An example of an Agilent MassHunter BioConfirm B.08 peptide mapping results table, summarizing the details of all matched peptides from the NISTmAb digest. The majority of identified peptides posted less than 1 ppm of mass accuracy (red box).

Sequence Coverage Map: Intact NIST mAB (Protein Digest) (99.40%)

NIST mAb Digest_250 ng-uL_01.d - Intact NIST mAB

A: NIST mAb_LC Monoisotopic mass: 23113.3043 Average mass: 23127.9774 Molecular formula: C1020H1578N270O330S7

```
  1   N-term  DIQMTQSPSTLSASVGDRVTITCSASSRVGYMHWYQQKPGKAPKLLIYDTSKLASGVPSR   60
 61  FSGSGSGTEFTLTISSLQPDDFATYYCFQGSGYPFTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS  130
131  VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL  200
201  SSPVTKSFNRGEC   C-term                                                  213
```

B: NIST mAb_HC Monoisotopic mass: 49430.7257 Average mass: 49462.5065 Molecular formula: C2212H3430N580O673S17

```
  1   N-term  QVTLRESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIRQPPGKALEWLADIWWDDKKH   60
 61  YNPSLKDRLTISKDTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQGTTVTVSSASTKGPSVFP  130
131  LAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQT  200
201  YICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS  270
271  HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS  340
341  KAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY  410
411  SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG   C-term                        449
```

Figure 3B. Summary of sequence coverage of NISTmAb.

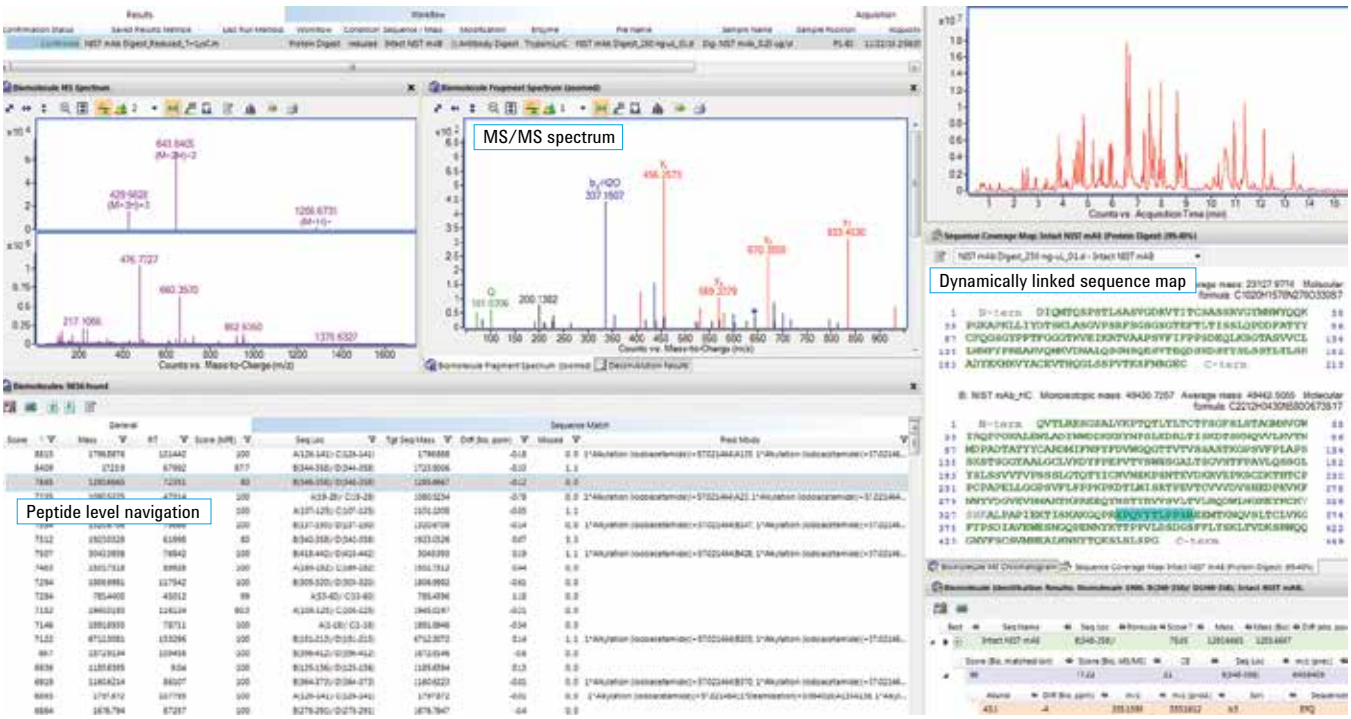

Figure 4. Screenshot of Agilent MassHunter BioConfirm B.08 software with representative peptide mapping results and protein sequence coverage.

Figure 5 illustrates the MS/MS spectra comparison of the native (precursor at m/z = 631.6385, +3) and Met-oxidized peptides (precursor at m/z = 636.9698, +3). The major differences (+15.99 Da) in the b4–b7 fragment ions (green box) clearly distinguished the native species from the modified forms, and indicate that the Met-4 in light chain is the location of oxidation.

Similarly, Figure 6 shows the MS/MS spectrum of the native and the deamidated peptides, where the b2–b3 fragment ions (purple boxes) all show the signature mass shift of 0.98 Da, clearly indicating the presence of deamidation. Moreover, as most of the y ions (y4–y8, highlighted in red) remain the same (except the y10 ion) as in the native form (top panel), it is clear that the deamidation occurred at the heavy chain Asn-364 position.

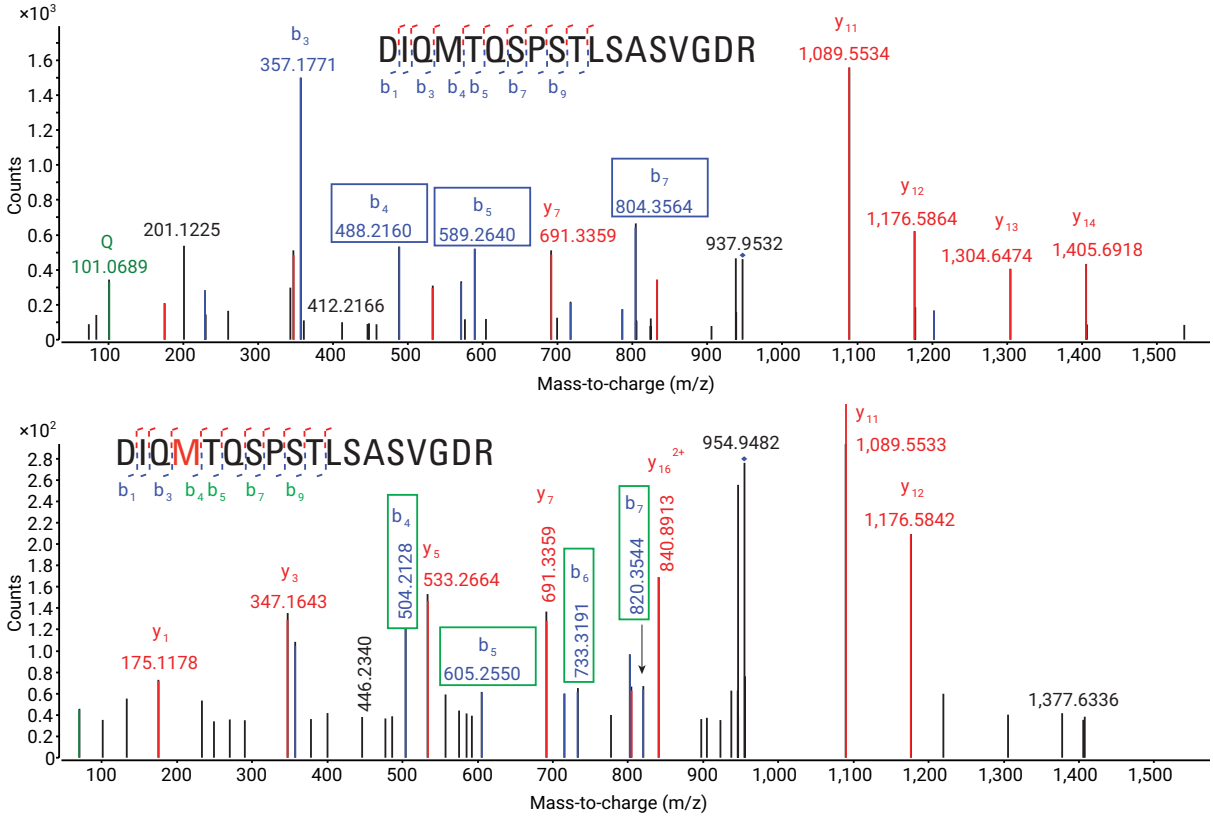

Figure 5. Post-translational modification (methionine oxidation) analysis. MS/MS spectrum of native and Met-oxidized peptides (light chain peptide 1-18). Top: native peptide, Bottom: oxidation at Met 4 (confirmed fragment ions in green boxes).

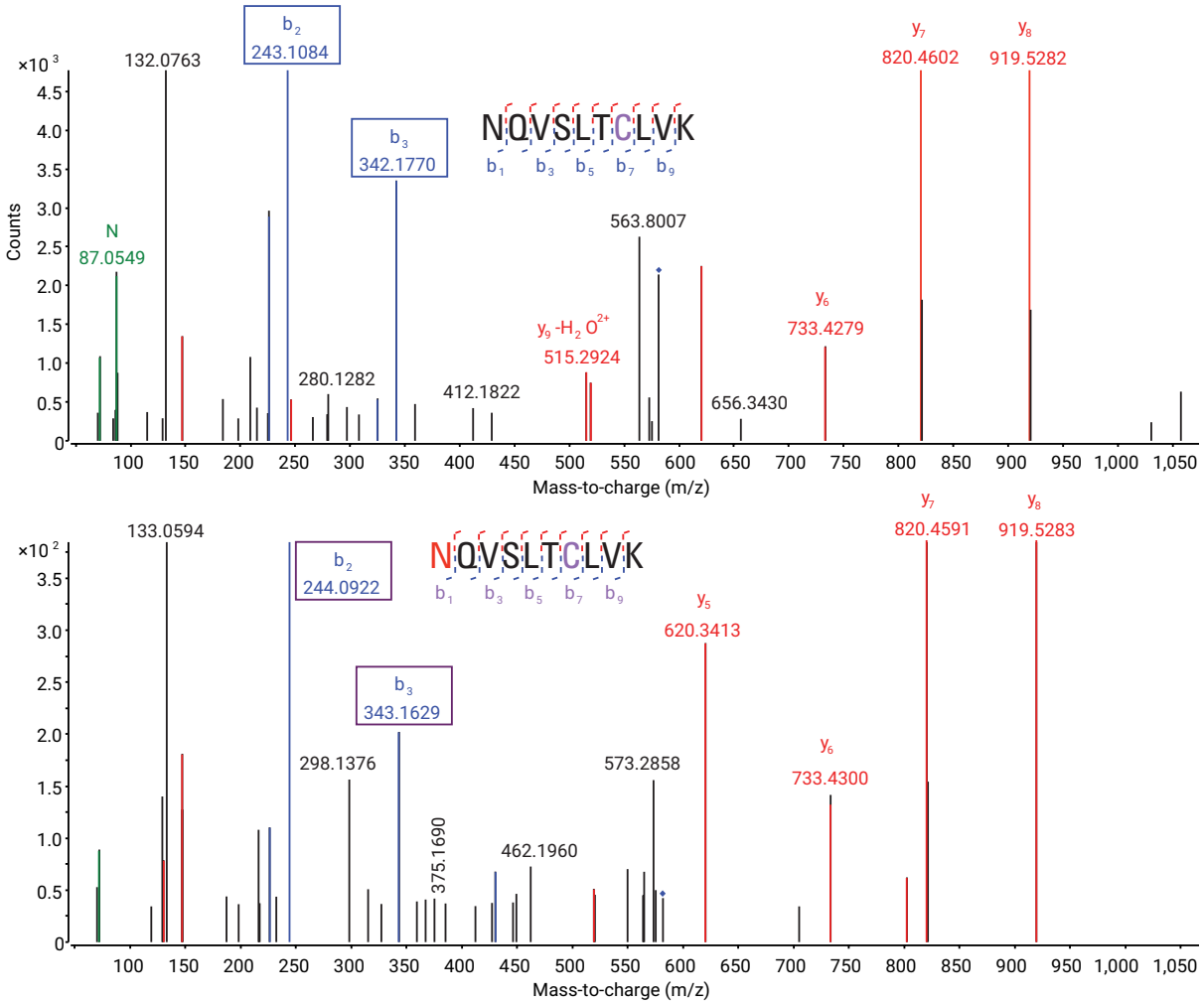

Figure 6. Post-translational modification (deamidation) analysis. MS/MS spectrum of native and deamidated peptides (heavy chain: 364-373). Top: native peptide, Bottom: deamidation at Asn 364.

Conclusion

The combination of automated sample preparation, rapid separation, confident detection, and streamlined processing changes the process of peptide mapping from a time-consuming and tedious effort into a routine workflow. This is possible due to the reliable nature and high performance of each component in this process, starting with Agilent AssayMAP Bravo through the data processing in Agilent MassHunter BioConfirm B.08. Total analysis time is significantly condensed by the separation capabilities of the Agilent 1290 Infinity II UHPLC and the Agilent AdvanceBio Peptide Mapping column as well as the automated processing capability of MassHunter BioConfirm. The combination of accuracy and resolution provided by the Agilent 6545XT AdvanceBio LC/Q-TOF is demonstrated by the uniformly precise results seen when analyzing a complete protein digest.

References

1. Automation for LC/MS Sample Preparation: High Throughput In-Solution Digestion and Peptide Cleanup Enabled by the Agilent AssayMAP Bravo Platform, Agilent Technologies, publication number 5991-2957EN.
2. Fast and Efficient Peptide Mapping of a Monoclonal Antibody (mAb): UHPLC Performance with Superficially Porous Particles, Agilent Technologies, publication number 5991-3585EN.
3. High Resolution and Rapid Peptide Mapping of Monoclonal Antibody Using an Agilent 1290 Infinity UHPLC and an Agilent 6550 iFunnel Q-TOF LC/MS System, Agilent Technologies, publication number 5991-3600EN.

Application Note

Pharma & Biopharma

Agilent

Trusted Answers

Monitoring Product Quality Attributes of Biotherapeutics at the Peptide Level Using the Agilent InfinityLab LC/MSD XT System

Authors

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Agilent Technologies, Inc.

Introduction

Single quadrupole (SQ) LC/MS has been adopted in the biopharmaceutical QC labs for its low-cost, robustness, and simple operation. This Application Note describes a simple, generic method for routine biotherapeutic peptide map analysis using the Agilent InfinityLab liquid chromatography/mass selective detector XT (LC/MSD XT), an SQ system with an extended mass range up to m/z 3,000, in combination with an Agilent 1290 Infinity II LC System and Agilent OpenLab ChemStation software. Streamlined data processing and reporting were demonstrated for pre-identified peptides of a recombinant monoclonal antibody (mAb), including complementary-determining regions (CDR) peptides, deamidated peptides, oxidized peptides, and glycopeptides using OpenLab ChemStation. This study serves as a proof of concept for monitoring multiple product quality attributes (PQAs) using an SQ LC/MS system with software that is recommended for laboratories requiring regulatory compliance.

Introduction

In the biotherapeutic industry, optically based chromatographic methods have widely been used for quality control (QC). However, protein-based biotherapeutics are generally very complex, making an orthogonal detection method (for example, mass spectrometry) very attractive or necessary to assess product quality attributes at a molecular level. Therefore, SQ-based LC/MS has been adopted in the QC environment. Due to the product complexity, comprehensive analysis of protein-based therapeutics often requires running a panel of analytical methods. The concept of using a single LC/MS analytical method to monitor multiple PQAs has gained momentum in the biopharmaceutical industry. Therefore, it is valuable to develop an SQ-based LC/MS assay for monitoring multiple PQAs.

In the QC environment, an important need is to support regulatory compliance. OpenLab ChemStation in combination with central data storage (OpenLab ECM or OpenLAB Server) provides functionality that labs need to achieve compliance: controls for managing system access, audit trail, versioning of data, electronic signature, secured records and data archival.1,2

This Application Note develops a simple, untargeted, generic LC/MS method for routine biotherapeutic peptide map analysis using the InfinityLab LC/MSD XT system, coupled with a 1290 Infinity II LC and OpenLab ChemStation software. In a stress study using NIST monoclonal antibody (NISTmAb), we demonstrate that this compliance-ready system allows streamlined data processing and reporting for multiple PQAs in a single analysis, such as product identification confirmation, post translation modification (PTM) analysis, and glycopeptide analysis.

Table 1. LC conditions.

Agilent LC Parameters		
Column	Agilent ZORBAX RRHD 300Å StableBond C18, 2.1 × 150 mm, 1.8 µm (p/n 863750-902)	
Mobile Phase A	H_2O with 0.1% (v/v) formic acid	
Mobile Phase B	Acetonitrile with 0.1% (v/v) formic acid	
Flow Rate	0.25 mL/min	
Injection volume	5.0 µL	
Gradient	Time (min) 0 5 6 70 72 77 79 81	%B 1 1 10 35 90 90 1 1
Column temperature	50 °C	

Experimental

Materials

All reagents and solvents were LC/MS grade. The NISTmAb reference material was purchased from National Institute of Standards and Technology.

Sample preparation

To induce asparagine deamidation, NISTmAb was exposed to elevated temperature (37 °C) in a Tris-HCl buffer system at pH 8.7 for six days. To induce methionine oxidation, NISTmAb was incubated in Tris-HCl buffers containing 0.002% (v/v) oxidizing agent H2O2 overnight at room temperature. Both reference and stress-induced NISTmAb were denatured, reduced, alkylated, and trypsin-digested followed by desalting using the Agilent AssayMAP Bravo platform.3 Digested samples were injected at a concentration of approximately 0.5 µg/µL onto the LC/MS system.

LC/MS analysis

LC separation was carried out using an Agilent 1290 Infinity II LC, consisting of an Agilent 1290 Infinity II High-Speed Pump (G7120A), an Agilent 1290 Infinity II Multisampler (G7167B) with sample cooler (option 100), and an Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with an Agilent ZORBAX 300StableBond C18 column (2.1 × 150 mm, 300 Å, 1.8 µm, p/n 863750-902) (Table 1). The MS system used was the Agilent InfinityLab LC/MSD XT system (G6135BA) with the Agilent Jet Stream source (G1958-65138). Agilent OpenLab ChemStation (version C 01.09) was used for data acquisition, processing, and reporting. The data were acquired in positive scan mode ranging from m/z 360 to 1,400 (Table 2).

Table 2. MS conditions.

Agilent MSD XT Parameters		
Drying Gas Flow	11 L/min	
Drying Gas Temperature	325 °C	
Sheath Gas Flow	10 L/min	
Sheath Gas Temperature	325 °C	
Nebulizer Pressure	35 psi	
Capillary Voltage	4,000 V	
Nozzle Voltage	0 V	
Peak Width	0.07 minutes	
Scan	360 to 1,400 *m/z* in positive mode from 5 to 80 minutes, step size 0.1	
Gradient	Mass 300 2,000	Value 125 V 200 V
Cycle Time	0.62 sec/cycle	

Results and discussion

Monitoring multiple PQAs in a single analysis

To evaluate the InfinityLab LC/MSD XT system for monitoring multiple attributes of biomolecules, NISTmAb was stressed under two conditions to induce deamidation and oxidation, respectively. The LC/MS method using MS positive scan mode described above was applied to collect the full peptide map for each sample. Figure 1 shows the total ion chromatogram of the peptide map data with 2.5 µg of NISTmAb digest loaded on-column, showing the sample complexity, as well as the high sensitivity and ultrafast scan speeds of the MSD within the InfinityLab LC/MSD XT system. The full scan of the NISTmAb peptide map allows monitoring of multiple attributes of interest using customized data processing methods. The scan also avoids re-acquiring data if additional attributes are of interest in the future.

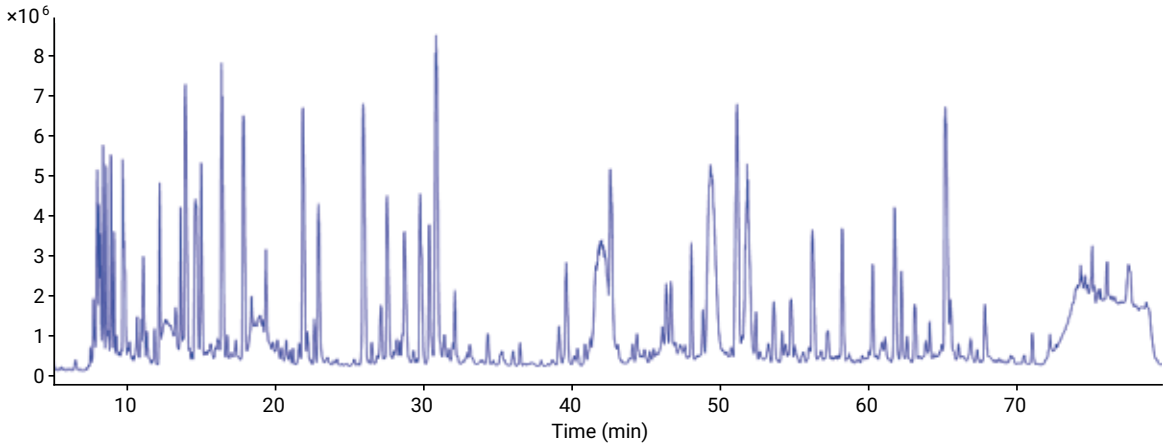


Figure 1. Total ion chromatogram of peptide map detection by Agilent LC/MSD XT with positive scan.

OpenLab ChemStation software supports automated data processing and reporting. To avoid manual extraction and integration of each peptide, a processing method can be created for extracted ion chromatograms (EICs) of multiple peptides of interest. Figure 2 shows screen captures of the EIC method setup for multiple peptides by the following steps:

1. MS chromatograms for the peptides of interest are defined with targeted m/z, then the targeted MS chromatograms are extracted accordingly (Figure 2A).
2. These targeted EICs are added to the processing method with adjustable retention time windows for automatic signal extraction and loading (Figure 2B).
3. The compound names, associated retention times, and EIC signals are linked through the Calibration Table setup (Figure 2C).

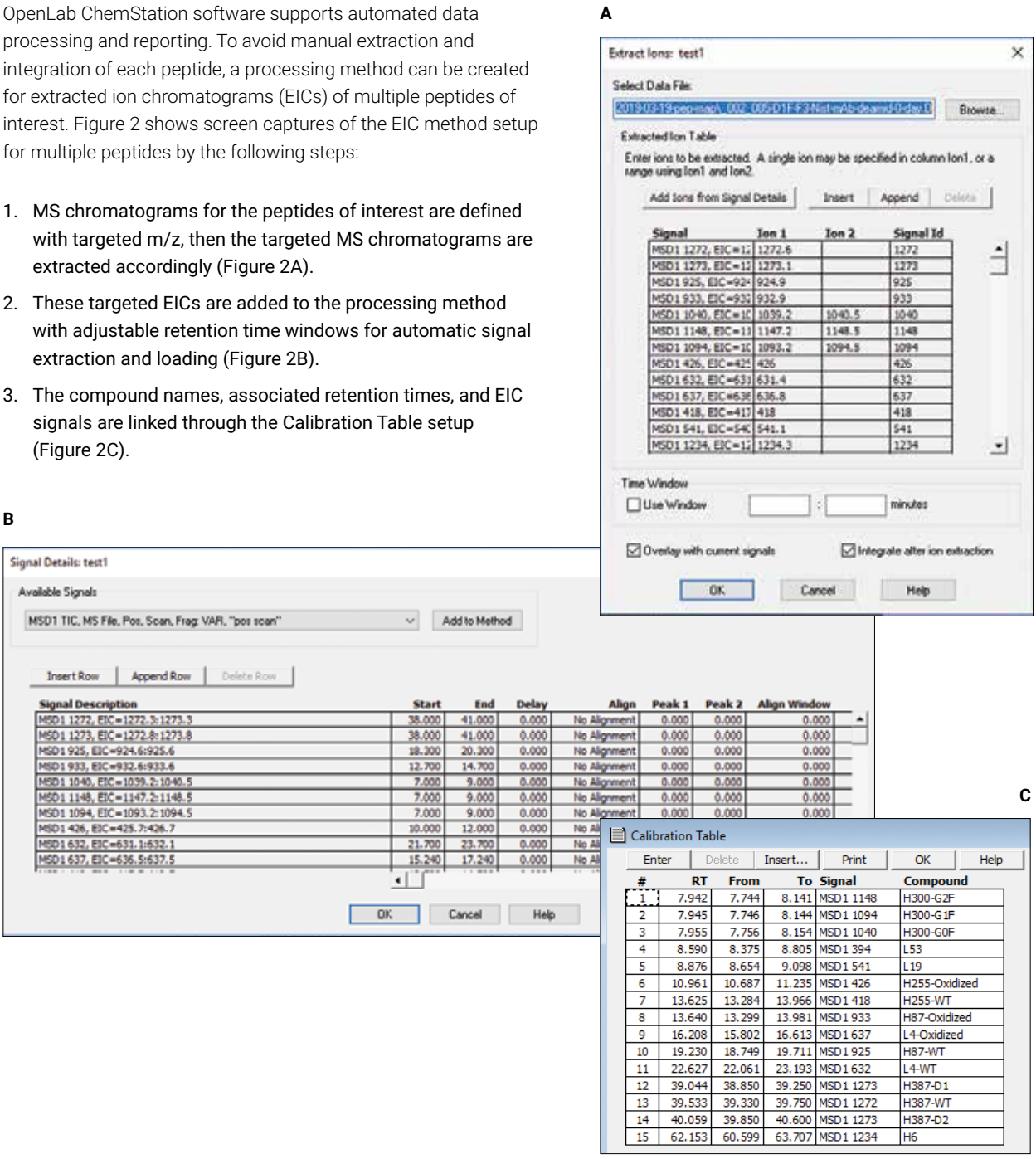


Figure 2. ChemStation screen captures of EIC method setup for multiple peptide attributes.

EICs for monitoring product attributes

To To evaluate the performance using the InfinityLab LC/MSD XT, 15 precharacterized peptides were selected for identification and quantification analysis for the NISTmAb stress study (Table 3).4,5 The identity and retention time of these peptides was predetermined using a high-resolution LC/Q-TOF system with the same LC gradient as in Table 1. A processing CH3method, including all 15 peptides, was created using the steps described earlier, and a single dominant charge state was used to identify each of the peptides. If desired, the user could sum up additional charge states for each peptide.

The peptides listed in Table 3 can be separated into three categories according to the different monitoring purposes. The first category is the CDR peptides including peptides L4, L19, L53, H6, and H87. During product monitoring, an important need is to confirm the identity of a given biomolecule product. The sequences of CDR peptides are variable among different mAbs and can be used to confirm the product identity. Figure 3 shows the EIC of the CDR peptides that can be used to confirm protein identity.

Table 3. Peptide information for monitored attributes.

Peptide	Peptide sequence	Modification	Calculated *m/z*	Charge state (z)	Expected retention time (min)	mAb region
L4	DIQMTQSPSTLSASVGDR	Oxidation	637.0	3	16.24	CDR
L4	DIQMTQSPSTLSASVGDR	WT	631.6	3	22.68	CDR
L19	VTITCSASSR	WT	541.3	2	8.966	CDR
L53	LASGVPSR	WT	393.7	2	8.353	CDR
H6	ESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIR	WT	1234.3	3	62.105	CDR
H87	VTNMDPADTATYYCAR	WT	924.9	2	13.64	CDR
H87	VTNMDPADTATYYCAR	Oxidation	932.9	2	19.23	CDR
H255	DTLMISR	Oxidation	426.2	2	11.01	CH2
H255	DTLMISR	WT	418.2	2	13.71	CH2
H300	TKPREEQYNSTYR	G0F	1039.5	3	7.97	CH2
H300	TKPREEQYNSTYR	G1F	1093.5	3	7.97	CH2
H300	TKPREEQYNSTYR	G2F	1147.5	3	7.98	CH2
H387	GFYPSDIAVEWESNGQPENNYK	Deamidation	1273.1	2	39.07	CH3
H387	GFYPSDIAVEWESNGQPENNYK	WT	1272.6	2	39.56	CH3
H387	GFYPSDIAVEWESNGQPENNYK	Deamidation	1273.1	2	40.06	CH3

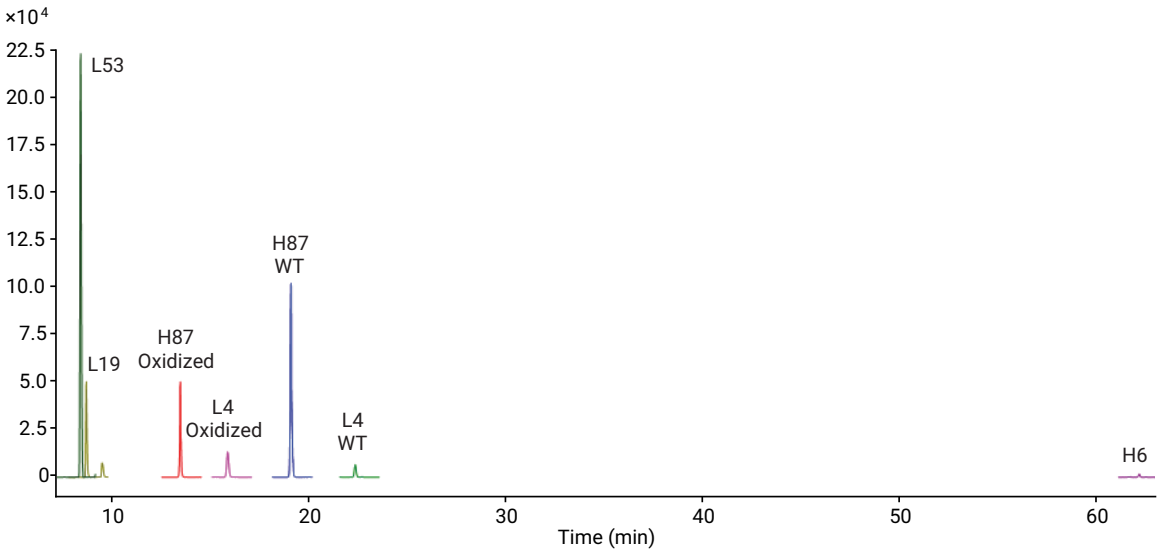


Figure 3. EICs of the CDR peptides.

The second category is peptides with variable modification sites, which are responsive for chemically induced deamidation and oxidation (L4, H87, H255, and H387).6,7 PTMs such as asparagine deamidation, aspartate isomerization, and methionine oxidation lead to degradation products typical for recombinant antibodies. Process changes during manufacturing or storage conditions can affect the rate and extent of these modifications, which could potentially impact the stability and function of the protein drug. Therefore, these PTMs are closely monitored during process development and drug production. Figure 4A shows EICs of the wild type H387 peptide and its deamidation forms, which is also called the PENNY peptide, in the reference and deamidated samples. The deamidated forms of H387 are elevated after deamidation induction. Figure 4B shows the overlaid EICs of the wild type peptide and its oxidized form from peptide H87 in both NISTmAb reference and oxidized samples. As expected, the extent of oxidation of H87 peptide was increased after oxidation induction.

The third category is glycopeptide (H300). Relative abundance of each glycopeptide can provide valuable information about the abundance of protein glycoforms. According to a previous publication on glycoanalysis in the NISTmAb tryptic digest using high-resolution LC/MS/MS, the glycopeptide located at heavy chain 292–304 (TKPREEQYNSTYR) was chosen as the dominant tryptic form5. Figure 5 shows the overlaid EICs of three glycopeptides (G0F, G1F, and G2F) used for determining their relative abundance. This result is consistent with a previous report on the relative abundance of these NISTmAb glycopeptides obtained using high-resolution LC/MS/MS.5

Intelligent reporting

OpenLab ChemStation software enables automated intelligent reporting. Intelligent reporting provides superior flexibility and allows the user to customize their report templates as desired. Figures 6A and 6B show examples of intelligent reports generated for monitoring multiple attributes.

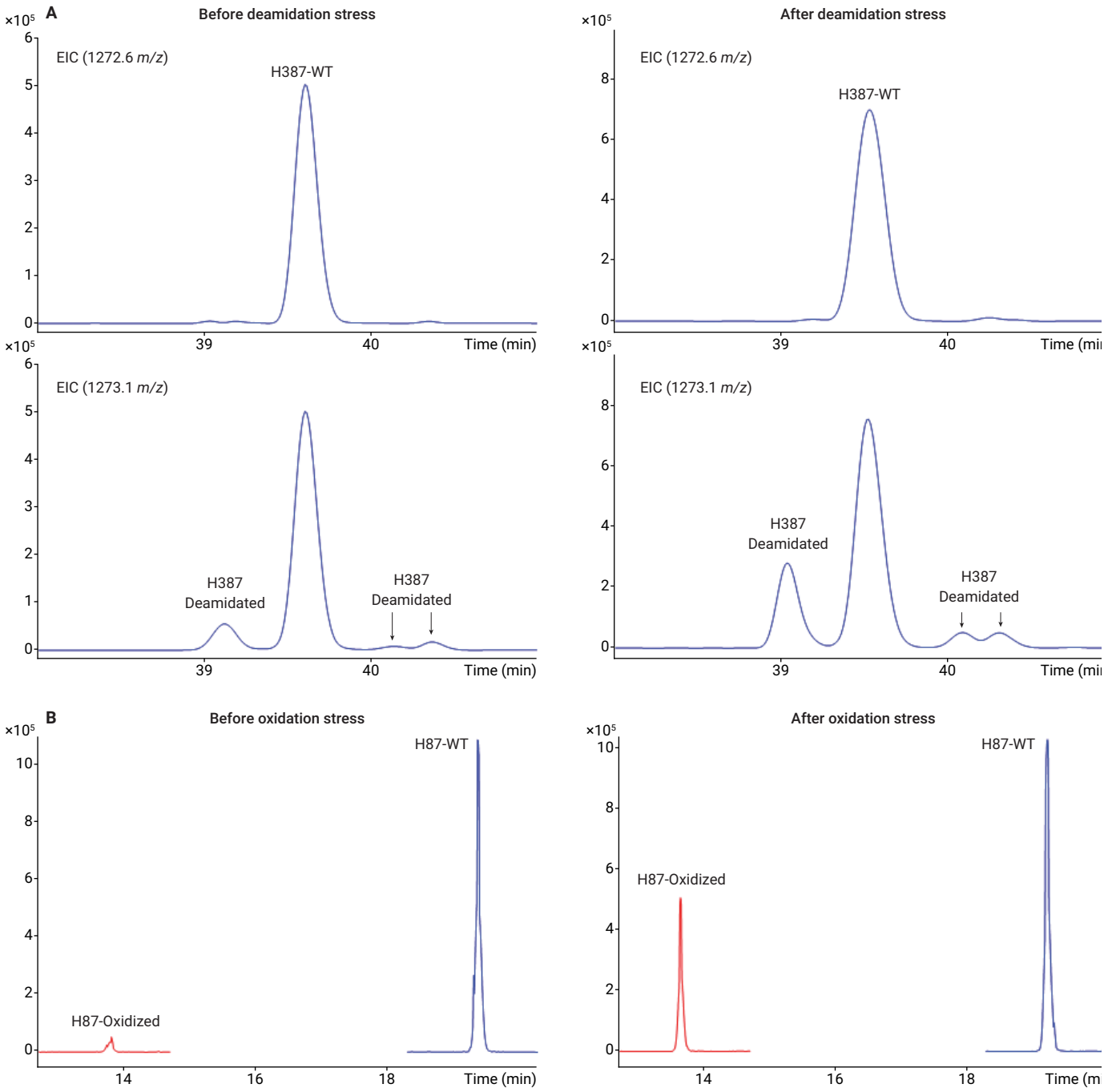


Figure 4. EICs of the peptides with variable PTMs. A) EIC comparison of the WT and deamidated H387 peptides before and after deamidation stress induction. B) EIC comparison of the WT and oxidized H87 peptides before and after oxidation stress induction.

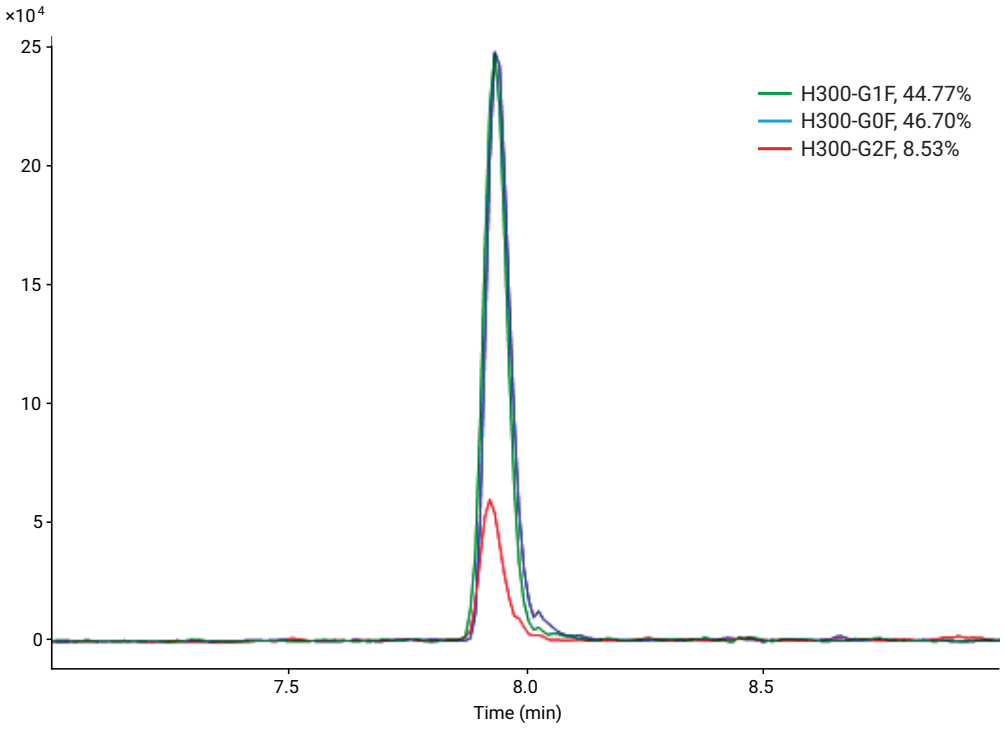


Figure 5. EICs of the three glycopeptides for determining relative abundance.

Single Injection Report

Agilent | Trusted Answers

Data file:
Sample name:
Description:
Sample amount:

Instrument:
Injection date:
Acq. method:
Analysis method:

Location:
Injection:
Injection volume:
Acq. operator:

Analyst: _____

Date: _____

Pass/Fail: _____

Peak Summary Table: Glycopeptides

Name	RT [min]	Area	Area Percent
H300-G0F	7.955	1207180	46.70%
H300-G1F	7.945	1157171	44.77%
H300-G2F	7.942	220399	8.53%
	AreaSum	2584749	

Peak Summary Table: Deamidation

Name	RT [min]	Area	Area Percent	P/F
H387-D1	39.112	146688	2.86%	Pass
H387-WT	39.592	4914364	95.93%	Pass
H387-D2	40.123	61809	1.21%	Pass
	AreaSum	5122861		

Peak Summary Table: Oxidation

Name	RT [min]	Area	Area Percent	P/F
H87-Oxidized	13.811	113448	3.47%	Pass
H87-WT	19.367	3160505	96.53%	Pass
	AreaSum	3273952		

Name	RT [min]	Area	Area Percent	P/F
L4-Oxidized	16.208	105370	9.00%	Pass
L4-WT	22.627	1065867	91.00%	Pass
	AreaSum	1171237		

Name	RT [min]	Area	Area Percent	P/F
H255-Oxidized	10.961	1967116	16.80%	Fail
H255-WT	13.625	9738910	83.20%	Fail
	AreaSum	11706026		

Figure 6A. Intelligent reporting by Agilent OpenLab ChemStation. Example of a single injection report. Peak tables summarize the relative abundance of WT and PTM forms for each peptide sequence by custom calculation.

Sequence Summary Report

Agilent | Trusted Answers

Sequence name:

Acquisition date:

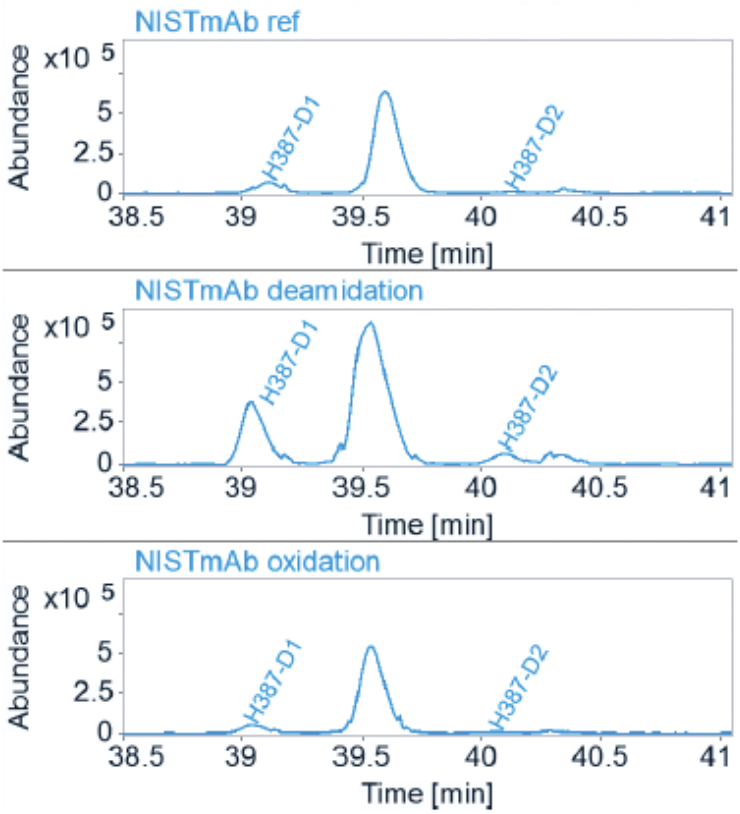
Acquired by:

Approved by: _____

Signature: _____

Date: _____

Extracted Ion Chromatogram **EIC=1272.8 :1273.8**



			H387-D1	H387-WT	H387-D2
			RT (min)	RT (min)	RT (min)
1	NISTmAb ref	_001_005-D1F-F3-Nist-mAb-deamid-0-day.D	39.11	39.59	40.12
2	NISTmAb deamidation	_002_006-D1F-F4-Nist-mAb-deamid-6-day.D	39.04	39.53	40.10
3	NISTmAb oxidation	_003_003-D1F-F2-Nist-mAb-H2O2-002pct.D	39.04	39.53	40.06

Figure 6B. Intelligent reporting by Agilent OpenLab ChemStation. Example of a sequence summary report comparing a NISTmAb reference sample, deamidated sample, and oxidized sample for the EIC and retention time of H387 peptide.

Conclusion

The Agilent InfinityLab LC/MSD XT system provides a simple and cost-effective solution for monitoring multiple PQAs in a development and quality control environment, assuming those attributes that have been precharacterized using a high-resolution MS instrument. This Application Note demonstrates that the InfinityLab LC/MSD XT system can deliver quantitative analysis for monitoring multiple attributes of biotherapeutics at the peptide level, including CDR peptides, oxidized and deamidated peptides, and glycopeptides in a single analysis. Automated data processing and reporting through Agilent OpenLab ChemStation software avoid manual interrogation and allow high-throughput analysis. OpenLab ChemStation in combination with central data storage provides a compliance solution for chromatography and mass spectrometry data collected in compliant environments.

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5. Dong, Q.; *et al.* In-Depth Characterization and Spectral Library Building of Glycopeptides in the Tryptic Digest of a Monoclonal Antibody Using 1D and 2D LC–MS/MS. *J. Proteome Res*. **2016**, *15(5)*, 1472–1486.
6. Quantitation of Chemical-Induced Deamidation and Oxidation on Monoclonal Antibodies Using Agilent 6545XT AdvanceBio LC/Q-TOF and Agilent MassHunter BioConfirm Software. *Agilent Technologies*, publication number 5994-0406EN.
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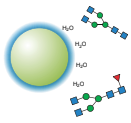
Glycan Analysis

Introduction

The structure of N-linked glycans on biotherapeutic glycoproteins can play a critical role in protein recognition and cellular signaling, therefore it is not surprising that glycosylation on therapeutic proteins can significantly impact the safety, efficacy, and half-life of these drug species[1]. For example, high mannose glycans can increase clearance, impacting the pharmacokinetics of thebiotherapeutic[2]. Regulatory authorities consider glycosylation to be one of the critical quality attributes (CQA) of biomolecules. This makes N-linked glycan characterization and relative quantification within acceptable range, an essential part of the drug development process.

Protein glycosylation is a complex post-translational modification (PTM) involving attachment of glycans at specific sites on a protein, most commonly at Asn (N-linked) or Ser/Thr (O-linked) residues. The N-linked glycosylation occurs at the consensus sequence of Asn-X-Ser/Thr (where X is any amino acid except proline)[3]. Protein glycosylation is influenced by the type of host cells and fluctuations in fermentation conditions (e.g., media, pH, temperature, agitation)[4]. Depending on the sample type and detailed information necessary, glycosylation profile can be analyzed at the intact protein level, glyco-peptide, or as released glycans.

Analysis of released N-glycans is very common in characterizing therapeutic glycoproteins, and in many cases is considered a product quality attribute. In order to detect released N-glycans effectively, they are most commonly derivatized with a fluorescent dye, as they are not optically active and ionize poorly for mass spectrometric (MS) detection. Traditionally reductive amination labelling chemistry with labels such as 2-aminobenzamide (2-AB) or 2-aminobenzoic acid (2-AA) has been the gold standard for many years. In more recent years, glycosylamine reactive labels such as InstantPC with higher fluorescence signal and strong ionization for MS detection have entered the market. With the integration of ProZyme, Agilent can now offer convenient and easy-to-use all in one kit for N-glycan sample preparation with any of these labels, such as the AdvanceBio Gly-X N-glycan prep with InstantPC and AdvanceBio Gly-X N-glycan prep with 2-AB Express.



Glycan Analysis

Hydrophilic interaction chromatography

Fast, high-resolution, reproducible glycan separation

AdvanceBio Glycan Mapping

An amide HILIC column

Attribute	Advantage
2.7 µm superficially porous particle	High resolution at low back pressure
1.8 µm totally porous particles	Maximum resolution
Fluorescence and MS compatible	Easy method transfer

Column Selection

AdvanceBio Glycan Mapping	Amide bonded phase for rapid equilibration and enhanced selectivity for glycans.
1.8 µm	Based on a fully porous particle for high speed separations and high throughput applications. Stability to 1200 bar for use with the Agilent 1290 Infinity II LC.
2.7 µm	Based on Poroshell technology to give a superficially porous particle with reduced diffusion distances to give high resolution separations at lower pressures and enable the use of longer column lengths for increased separation efficiency.

Labeled N-glycan mixtures are most commonly separated via HILIC separations. HILIC, or hydrophilic interaction chromatography, uses reversed-phase type eluents with gradients starting at high organic solvent content. The mechanism of interaction of analytes with the stationary phase is a partitioning from the high organic eluent into the aqueous layer present on the surface of the stationary phase. Water is a strong eluting solvent and it is therefore important to minimize the amount of water present in the sample matrix and to allow enough time for the column to re-equilibrate and stabilize at the end of each gradient. Agilent's biocolumn portfolio offers an amide bonded phase-HILIC column for analysis of released glycans, the AdvanceBio Glycan Mapping column. This HILIC based column are available in two formats, a superficially porous columns with 2.7 µm particle size suitable for use on all HPLC instruments, and in fully porous 1.8 µm columns for maximum resolution designed to use on highly optimized UHPLC instruments. AdvanceBio Glycan Mapping column is batch tested with a glycan mixture to ensure its performance for glycan analysis. Furthermore, Agilent AdvanceBio Glycan Mapping solution provides an optimized workflow designed to deliver reproducibility in the analysis of labeled glycans, for accurate identification and quantification.

In relatively rare, or niche cases, other chromatography types may be used, such as reversed-phase for 2-AA labeled glycans, or mixed mode separations. Although it is becoming less common, capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) is still used in several labs for glycan analysis. For chromatographic separations of labelled N-glycans, fluorescence detection is the dominant detection method. MS is also commonly used in development or discovery settings; however, QA/QC settings rely heavily on fluorescence detection.

Most monoclonal antibodies (mAbs) are produced in mammalian expression systems, such as Chinese hamster ovary (CHO) cells or Murine Myeloma (NS0) Cell lines[2].The NISTmAb material is a recombinant humanized IgG1κ expressed in murine suspension culture. Cell type of origin can dramatically impact the glycosylation pattern on a protein.

As both cell lines are mammalian, the glycosylation pattern of mAbs generated from these expression systems will have similarities to human glycosylation, but as they are ultimately different species and cell types there will be appreciable differences. The NISTmAb has a more complex glycan profile than many CHO-derived biotherapeutics, both in number and diversity of glycan species[4]. For example, the NISTmAb has glycans containing Neu5Gc and α-Gal structures, which are not synthesized in humans are considered immunogenic and required to be monitored. The featured application note (5994-0372EN) highlights various glycoform/glycan quantitative analysis LC/MS workflows.

Featured Application Notes for IPC-labeled Glycan analysis:

The three application notes featured in this section each offer a different perspective on released glycan analysis as they present data on the glycans found in the NISTmAb standard as well as a mAb produced in-house at Agilent. All three use a HILIC-based separation of InstantPC-labeled glycans using the AdvanceBio Glycan Mapping column and the Agilent 1290 Infinity II LC system, selected primarily for its minimal system dead volume. The first application note (5991-8550EN) illustrates a total workflow solution for glycan analysis, including automated sample preparation on the AssayMAP Bravo, detection with the Agilent 6545XT AdvanceBio Q-TOF, and data analysis using Agilent MassHunter BioConfirm software.

The second application note (5991-6958EN) compares results obtained by fluorescence to those obtained using a QTOF mass spectrometer, and demonstrates the power of a high resolution QTOF workflow to identify unknown glycans using accurate mass and tandem MS. This application note contains data from Agilent's contribution to the glycan analysis inter-lab study hosted by NIST in 2015-20165. Comparing the results Agilent obtained to the aggregate results of the round robin study[6] illustrates the strength of Agilent's glycan analysis tools.

The third application note (5991-8071EN) shows the suitability of a more cost-effective, rugged single quadrupole MS instrument for glycan analysis for those settings where a high-power, large-footprint instrument is not ideal.

Featured Application Notes for 2-AB labeled Glycan analysis:

The application note (5994-0682EN) featured here demonstrates analysis of released N-glycans from an in-house Agilent CHO mAb and a fusion protein (Enbrel) that have been labeled with 2-AB and prepared using the Agilent AdvanceBio Gly-X 2-AB Express N-Glycan Sample Preparation kit. Samples were analyzed using a very classic approach – a HILIC separation on the AdvanceBio Glycan Mapping column with fluorescence detection using the Agilent 1290 Infinity II LC.

References

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2. D. Reusch and M.L. Tejada, Glycobiology 25 (12), 1325-1334 (2015).
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6. M. L. A. De Leoz, D. L. Duewer, S. E. Stein, NIST Interlaboratory Study on the Glycosylation of NISTmAb, a ***Monoclonal Antibody Reference Material*** June **2015** to February **2016** https://nvlpubs.nist.gov/nistpubs/ir/2017/NIST.IR.8186.pdf

Agilent
Trusted Answers

Quick Reference Guide

Glycan Mapping Workflow

In this document Agilent applications chemists share their recommendations for an optimum LC system and its configuration for characterizing biomolecules. They also offer guidance on a generic method to get you started, and how this method can be further optimized to meet your specific separation goals. Additional application information is available at **www.agilent.com/chem/advancebio**

Agilent AdvanceBio Glycan Mapping 1.8 µm Columns

- AdvanceBio Glycan Mapping products include sample preparation, labelled and unlabelled standards and 1.8 µm and 2.7 µm columns.
- Both gradients provide 1.25%/mL slope.
- It may be necessary to adjust the start and end point to obtain highest resolution for samples containing different types of glycan.
- Larger glycan structures may require 75 to 55% acetonitrile gradient for optimum results for example.

AdvanceBio Glycan Mapping column, 1.8 µm, stable to 1200 bar

Description	Part No.
2.1 x 100 mm	858700-913
2.1 x 150 mm *	859700-913
Fast Guard, 2.1 mms	651750-913

* Recommended initial column size

Agilent 1290 Infinity UHPLC System

Mobile phases

Eluent A: 100 mM ammonium formate, pH 4.5
Eluent B: acetonitrile
(mass spec compatible).

Detection (G1321B)

Agilent 1260 Infinity Fluorescence Detector,
ex 260 nm, em 430 nm, 8 µL cell.

Column compartment (G1316C)

40 °C gives longer column life; 60 °C gives sharper peaks but significantly reduces lifetime. Selectivity and resolution may change with temperature.

Sample injection (G4226A)

1 to 2 µL injection for maximum resolution. Samples should first be dissolved in H2O then made up to 70:30 ACN:Water. Chiller should be used.

Pump (G4220A)

0.5 mL/min for high resolution separations; up to 1.0 mL/min for high speed. High aqueous clean up should ALWAYS be run at reduced flow rate.

Suggested gradient for resolution

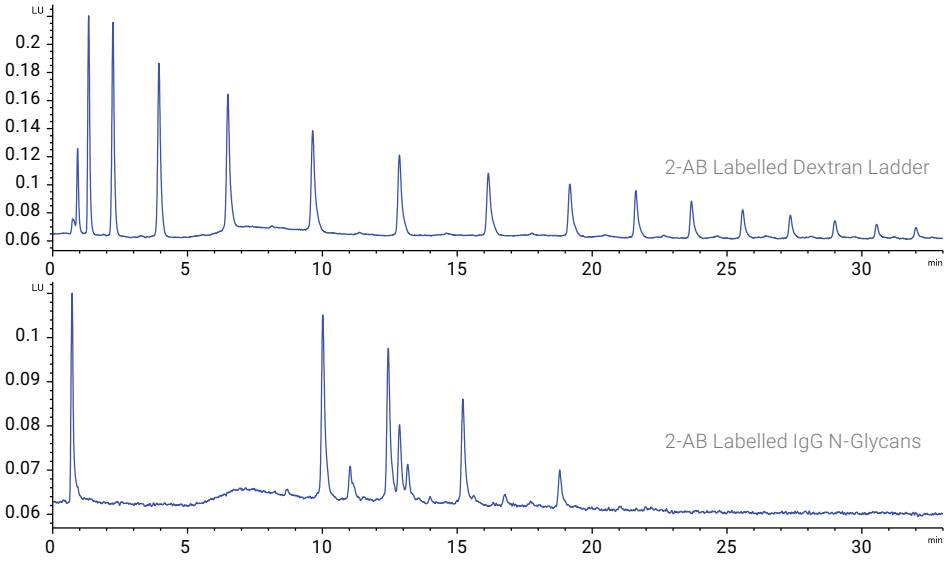
Time	Eluent A	Eluent B	Flow
0	20%	80%	0.5 mL/min
32	40%	60%	0.5 mL/min
33	80%	20%	0.5 mL/min
35	80%	20%	0.5 mL/min
36	20%	80%	0.5 mL/min
45	20%	80%	0.5 mL/min

Suggested gradient for speed

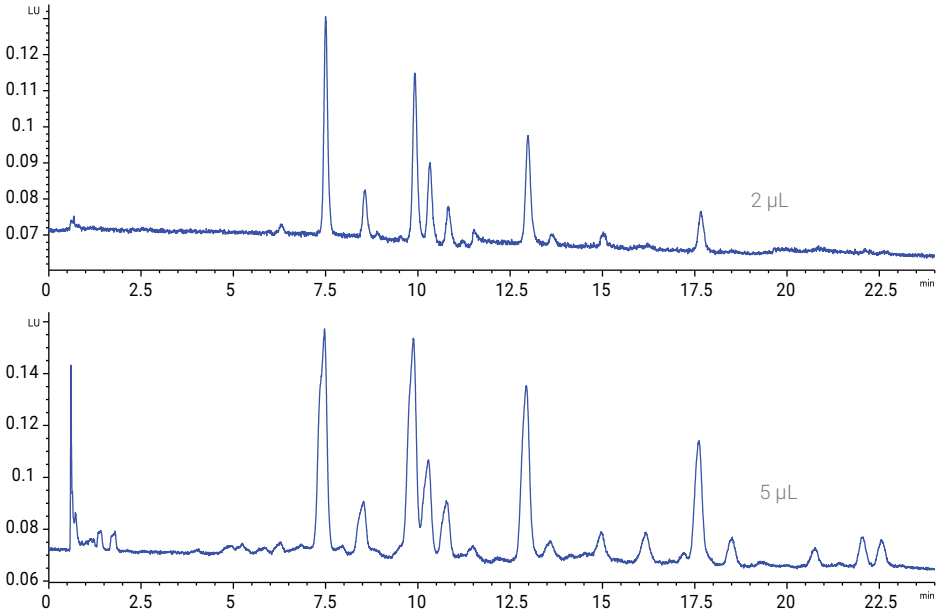
Time	Eluent A	Eluent B	Flow
0	25%	75%	1.0 mL/min
12	40%	60%	1.0 mL/min
12.5	80%	20%	0.5 mL/min
13.5	80%	20%	0.5 mL/min
14	25%	75%	0.5 mL/min
15	25%	75%	1.0 mL/min
20	25%	75%	1.0 mL/min

Time	Eluent A	Eluent B	Flow
0	20%	80%	0.5 mL/min
32	40%	60%	0.5 mL/min
33	80%	20%	0.5 mL/min
35	80%	20%	0.5 mL/min
36	20%	80%	0.5 mL/min
45	20%	80%	0.5 mL/min

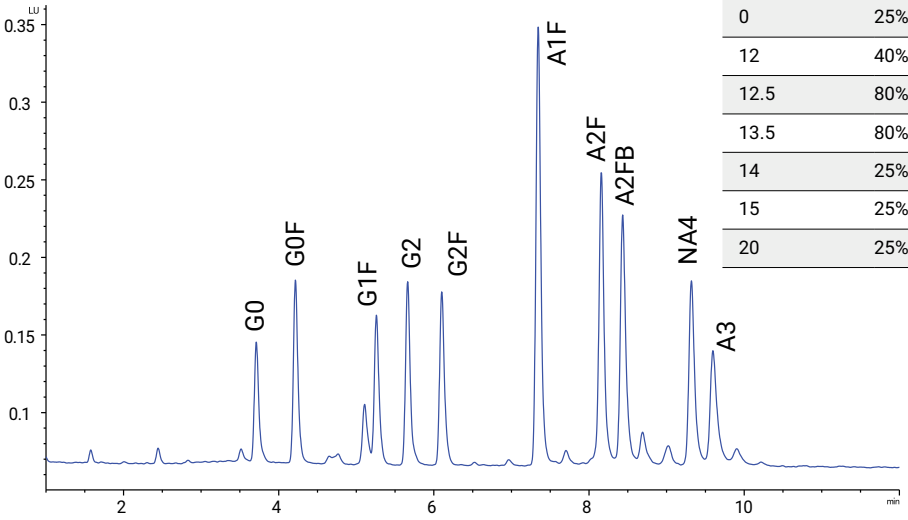
Samples should be prepared by dissolving in water and then adding acetonitrile to give a final composition 30:70 water:acetonitrile. The small column dimension, 2.1 x 150 mm, still requires small injection volumes. The figure below on the left demonstrates the outcome from injecting 5 µL – peaks become broader and resolution is lost – compared to 2 µL injection.



High-resolution separation of 2-AB Labeled Dextran Ladder (p/n 5190-6998) and 2-AB Labeled Human IgG N-Glycan Library (p/n 5190-6996).

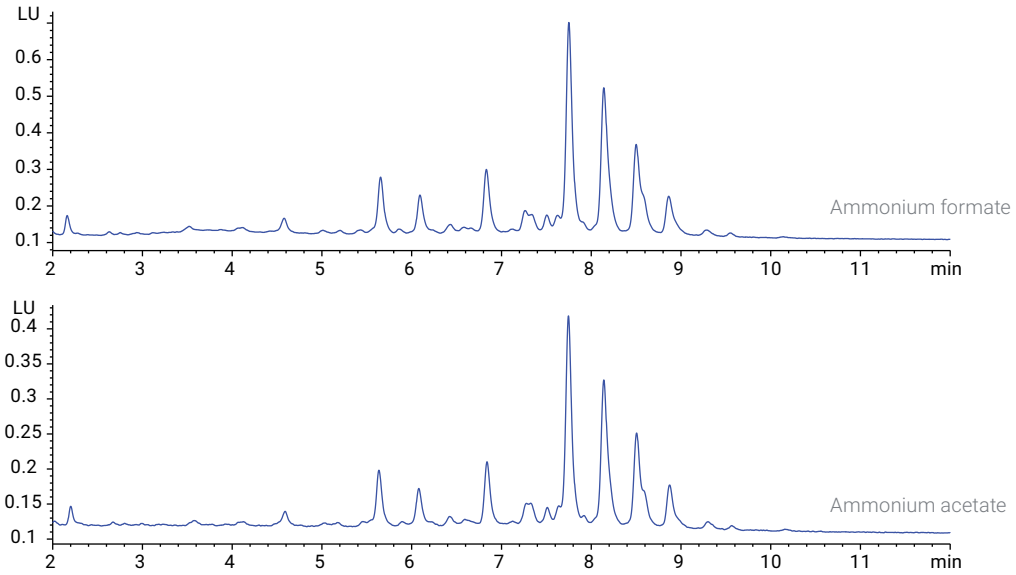


Over-injection of 2-AB Labeled Human IgG N-Glycan Library (2 µL vs. 5 µL).



Time	Eluent A	Eluent B	Flow
0	25%	75%	1.0 mL/min
12	40%	60%	1.0 mL/min
12.5	80%	20%	0.5 mL/min
13.5	80%	20%	0.5 mL/min
14	25%	75%	0.5 mL/min
15	25%	75%	1.0 mL/min
20	25%	75%	1.0 mL/min

High speed separation of 2-AB Labeled N-Glycans (tentative peak assignment).



Glycans, such as those found in bovine fetuin, can be eluted with ammonium formate or ammonium acetate mobile phases.

Agilent AdvanceBio Glycan Mapping 2.7 µm Columns

AdvanceBio Glycan Mapping, 2.7 µm, stable to 600 bar

Description	Part No.
2.1 x 100 mm	685775-913
2.1 x 150 mm *	683775-913
2.1 x 250 mm	651750-913
Fast Guard, 2.1 mm, 2.7 µm	821725-906

* Recommended initial column size

Description	Part No.
4.6 x 100 mm	685975-913
4.6 x 150 mm	683975-913
4.6 x 250 mm	680975-913

AdvanceBio Glycan Mapping products include sample preparation, labeled and unlabelled standards, and 1.8 µm and 2.7 µm columns.

Agilent 1260 Infinity Bio-Inert LC System

Mobile phases

Eluent A: 100 mM ammonium formate, pH 4.5
Eluent B: acetonitrile
(mass spec compatible)

Pump (G5611A)

0.5 mL/min for high resolution separations; up to 1.0 mL/min for high speed. High aqueous clean up should ALWAYS be run at reduced flow rate.

Sample injection (G5667A)

1 to 2 µL injection for maximum resolution. Samples should first be dissolved in H_2O then made up to 70:30 ACN:Water. Chiller should be used.

Column compartment (G1316C)

40 °C gives longer column life; 60 °C gives sharper peaks but significantly reduces lifetime. Selectivity and resolution may change with temperature.

Detection (G1316C)

Agilent 1260 Infinity Fluorescence Detector, ex 260 nm, em 430 nm, 8 µL cell

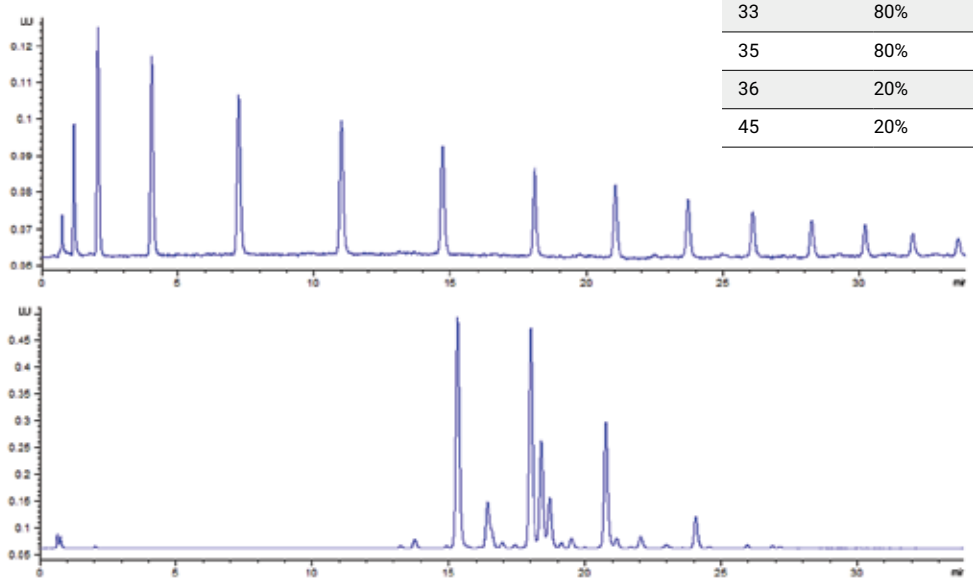
Suggested gradient for resolution

Time	Eluent A	Eluent B	Flow
0	20%	80%	0.5 mL/min
32	40%	60%	0.5 mL/min
33	80%	20%	0.5 mL/min
35	80%	20%	0.5 mL/min
36	20%	80%	0.5 mL/min
45	20%	80%	0.5 mL/min

Suggested gradient for speed

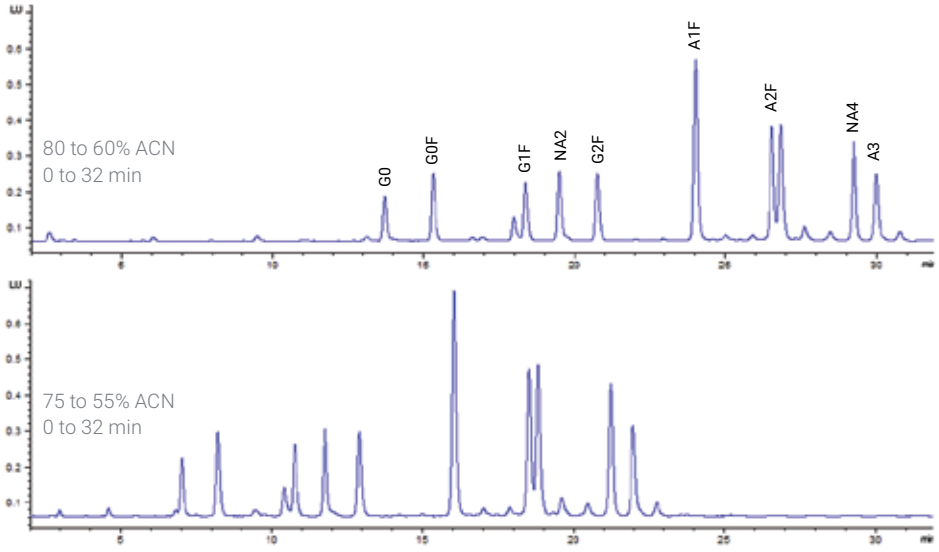
Time	Eluent A	Eluent B	Flow
0	20%	80%	0.7 mL/min
12	40%	60%	0.7 mL/min
12.5	80%	20%	0.5 mL/min
13.5	80%	20%	0.5 mL/min
16	20%	80%	0.5 mL/min
17	20%	80%	0.7 mL/min
20	20%	80%	0.7 mL/min

Time	Eluent A	Eluent B	Flow
0	20%	80%	0.5 mL/min
32	40%	60%	0.5 mL/min
33	80%	20%	0.5 mL/min
35	80%	20%	0.5 mL/min
36	20%	80%	0.5 mL/min
45	20%	80%	0.5 mL/min



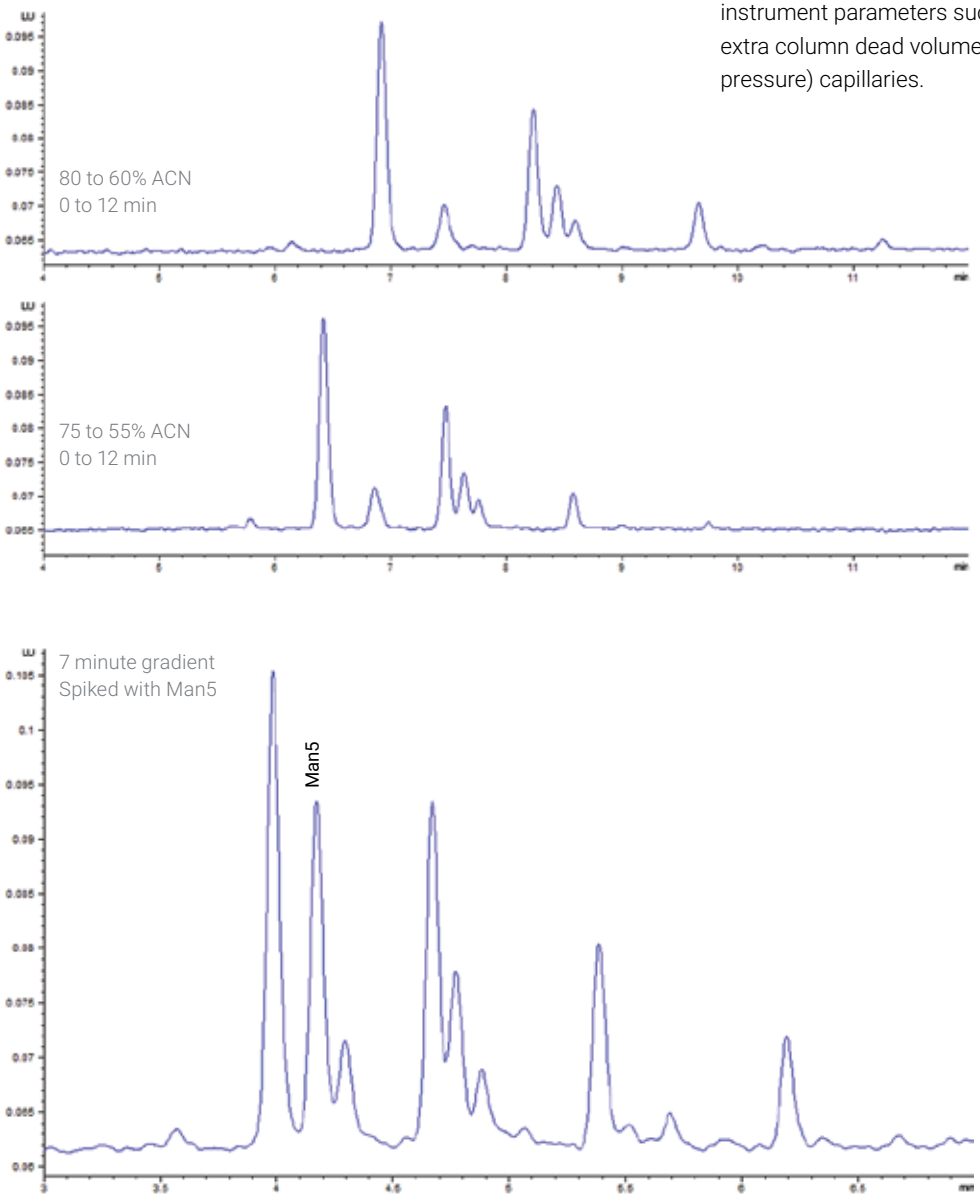
High-resolution separation of 2-AB Labeled Dextran Ladder (p/n 5190-6998) and 2-AB Labeled Human IgG N-Glycan Library (p/n 5190-6996).

Time	Eluent A	Eluent B	Flow
0	25%	75%	0.5 mL/min
32	45%	55%	0.5 mL/min
33	80%	20%	0.5 mL/min
35	80%	20%	0.5 mL/min
36	25%	75%	0.5 mL/min
45	25%	75%	0.5 mL/min



Effect of gradient adjustment on larger (later eluting) 2-AB Labeled N-Glycans

High speed separations can be more difficult to achieve due to instrument parameters such as pump gradient dwell time, and extra column dead volume through use of wider bore (lower pressure) capillaries.



High speed and very high speed separations of 2-AB Labeled Human IgG N-Glycan Library (p/n 5190-6996).

Application Note

Biotherapeutics and Biosimilars

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Glycopeptide Characterization for Various Monoclonal Antibodies Using the Agilent 6545XT AdvanceBio LC/Q-TOF

Authors

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Introduction

Monoclonal antibodies (mAbs) and their derivatives represent a very complex but important class of biopharmaceutical molecules with a wide range of applications. As mAbs are heterogeneous molecules by nature, comprehensive analytical characterization is required. The full range of biotherapeutics characterization usually includes confirmation of the protein sequences, protein post-translational modification (PTM) locations, and their relative quantitative information. Protein glycosylation is one of the major PTMs of an mAb, and is involved in many biological regulatory processes as well as therapeutic efficacy and immunogenicity. Therefore, it is important to understand the various glycans' distribution and composition for pharmaceutical bioprocessing.

Quadrupole time-of-flight (Q-TOF) liquid chromatography/mass spectrometer (LC/MS) systems are often used to analyze intact mAbs and mAb subunits, perform mAb peptide sequence mapping, and characterize PTMs due to their excellent mass accuracy and resolution in the high-mass range[2,3].

Typically, four levels of LC/MS workflows for glycoform/glycan characterization are used:

- Levels 1 and 2 focus on the analysis of glycoforms on intact and reduced mAb molecules. The intact mAb workflow provides rapid assessment of the major glycoforms of the intact mAb, while the mAb subunit workflow offers detailed quantitative information about individual glycans such as G0F, G1F, and G2F.
- Level 3 is the analysis of glycopeptides generated from the proteolytical digestion of mAbs, commonly part of the peptide sequence mapping workflow[4]. This workflow shows results not only in glycan-relative quantitation, but also N-glycosylation site(s) information.
- Level 4 is the characterization of glycans that have been released by enzymatic cleavage or other mechanisms. It provides high analytical sensitivity and the best quantitation for glycan analysis (Figure 1)[5].

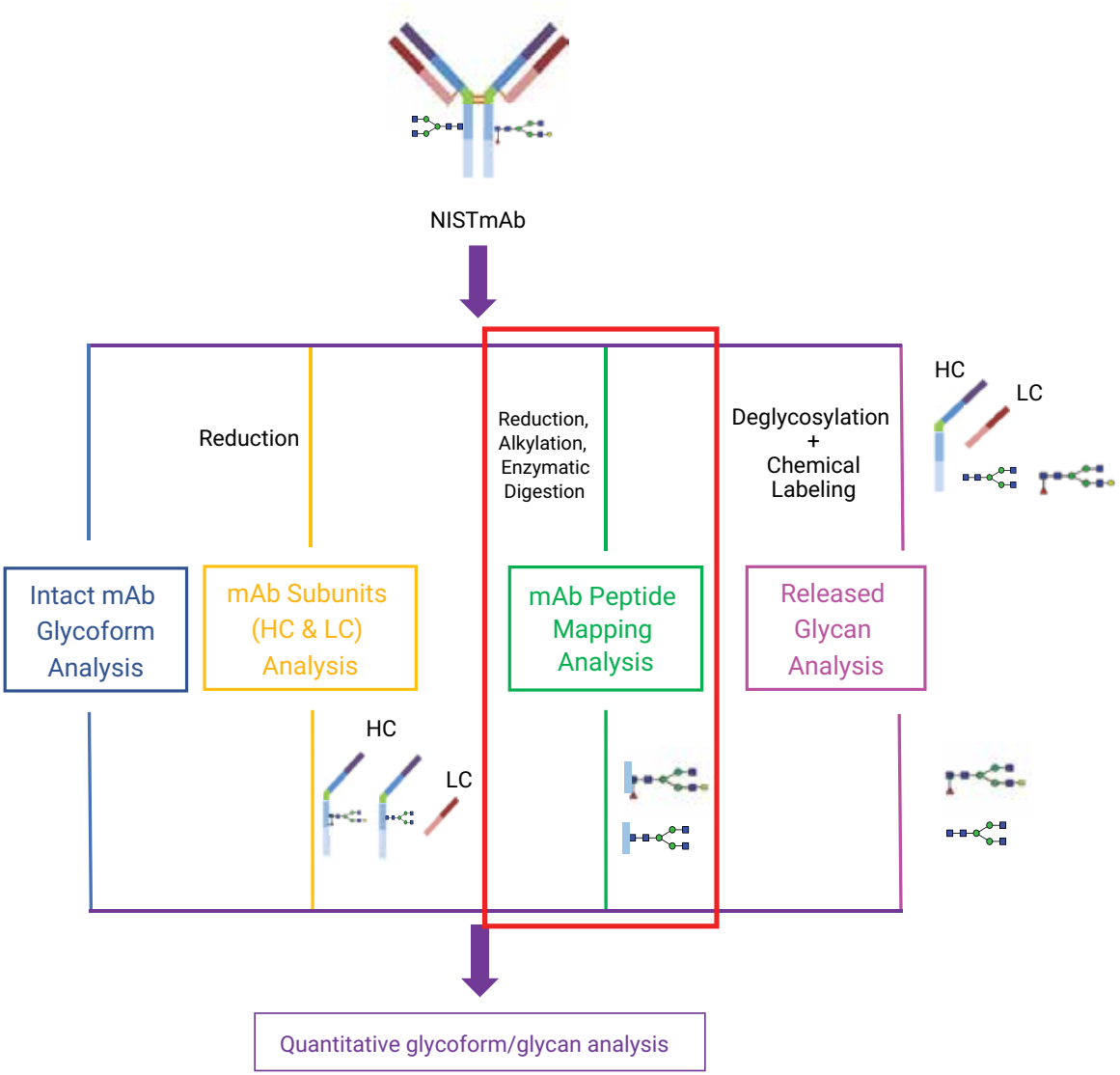


Figure 1. Various glycoform/glycan quantitative analysis workflows. The glycopeptide workflow is highlighted in the red box.

Peptide mapping of mAbs has widely been used as an analytical technique for the comprehensive characterization of protein biotherapeutics. This technique provides not only the complete amino acid sequences of mAbs and their variants, but also information on PTMs and locations. In routine analysis, peptides resulting from proteolytic digestion are typically separated by reversed-phase (RP) chromatography. RP-C18 or C8 columns are the most commonly used due to their excellent chromatographic separation power for regular peptides as well as peptides with PTMs such as oxidation and deamidation.

However, some protein modifications are not so easy to resolve through RP-type separation. Glycopeptides, which post relatively higher hydrophilicity, demonstrate very low retention and poor resolution on RP columns. In this case, hydrophilic interaction liquid chromatography (HILIC) with an amide-bonded stationary phase is often used as it can provide significantly more retention for glycosylated peptides.

This work demonstrates an optimized LC/MS workflow for mAb glycopeptide characterization (level 3) using the Agilent AssayMAP Bravo liquid-handling robot, the Agilent 1290 Infinity II LC system, the Agilent 6545XT AdvanceBio LC/Q-TOF, and automatic data analysis using Agilent MassHunter BioConfirm software for various glycopeptide identification and their relative quantitation (Figure 2). HPLC separation of glycopeptides from three different mAbs (NISTmAb, Trastuzumab, and A CHO cell cultured human IgG1 mAb) were compared on both the Agilent AdvanceBio Peptide Mapping (RP-C18) column and the Agilent AdvanceBio Glycan Mapping (HILIC) column.

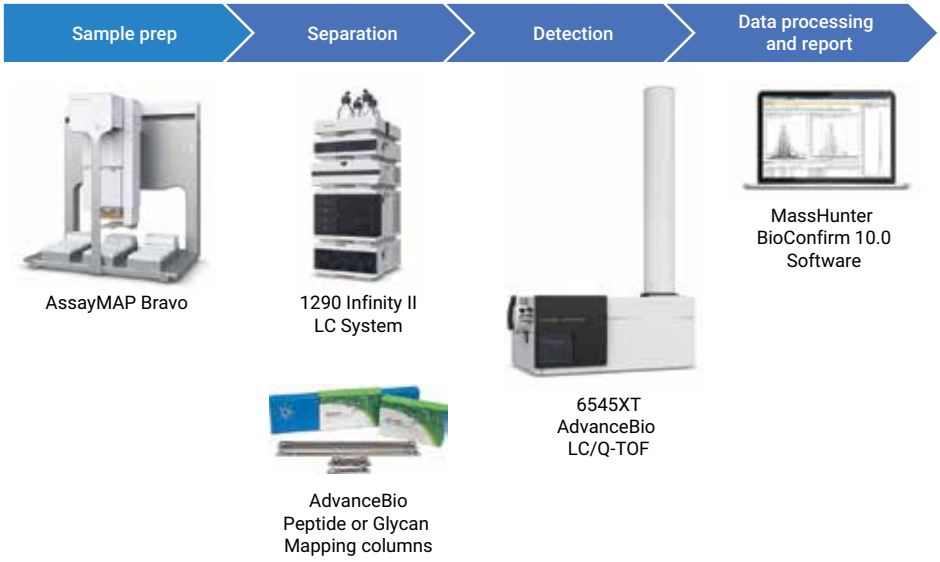


Figure 2. Analytical components of the mAb glycopeptide characterization workflow.

Experimental

Materials and methods

Three mAb samples were used in this study:

- The mAb standard, RM 8671, was from National Institute of Standards & Technology (NIST), aka NISTmAb.
- Formulated Herceptin (Trastuzumab) was from Genentech (So. San Francisco, California, USA). CHO mAb1 (A-mAb) was obtained from a collaborator.

2,2,2-Trifluoroethanol (TFE), DL-dithiothreitol (DTT), iodoacetamide (IAA), and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. High-quality mass spec grade Trypsin/Lys-C enzyme mix was obtained from Promega. AssayMAP C18 cartridges were from Agilent Technologies.

All mAb samples were diluted with DI water to 1.0 µg/µL prior to sample preparation using the AssayMAP Bravo liquid handling system.

Instrumentation

Three mAb samples were used in this study:

- Agilent AssayMAP Bravo system (G5542A)
- 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)
- Agilent 6545XT AdvanceBio LC/Q-TOF

Sample preparation

The AssayMAP Bravo liquid handling system was used to dilute, digest, and desalt the mAb samples. Samples were then dried down and resuspended with 0.1 % formic acid (FA) in DI water for analysis on the Peptide Mapping column. The digested samples that needed to be analyzed by the AdvanceBio Glycan Mapping (HILIC) column were resuspended with 80 % acetonitrile solution, which allowed effective sample loading and better chromatographic separation.

LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II LC system coupled with a 6545XT AdvanceBio LC/Q-TOF system with a Dual Agilent Jet Stream source. LC separation was obtained with either an AdvanceBio Peptide Mapping column (2.1 × 150 mm, 2.7 µm) or an AdvanceBio Glycan Mapping column (2.1 × 150 mm, 2.7 µm). Tables 1–3 list the LC/MS parameters used. Approximately 2 µg of protein digest was injected onto the AdvanceBio Peptide Mapping column, and 5 µg of protein digest was used on the AdvanceBio Glycan Mapping column for the glycopeptide analyses.

Two separate sample data acquisitions were run for glycopeptide quantitative analysis: one with MS/MS data acquisition mode (using the shaded parameters in Table 3) for peptide identification; the other, with MS-only acquisition mode, was for glycopeptide quantitation.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC System		
Column	Agilent AdvanceBio Peptide Mapping, 2.1 × 150 mm, 2.7 µm, (p/n 653750902)	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 2.7 µm, (p/n 683775913)
Thermostat	4 °C	4 °C
Solvent A	0.1 % Formic acid in water	0.1 % Formic acid in acetonitrile
Solvent B	0.1 % Formic acid in acetonitrile	0.1 % Formic acid in water
Gradient	0–15 minutes, 0–10 %B 15–45 minutes, 10–40 %B 45–55 minutes, 40–90 %B	0–30 minutes, 5–40 %B 30–40 minutes, 40–60 %B 40–55 minutes, 60–90 %B
Column temperature	60 °C	50 °C
Flow rate	0.4 mL/min	0.4 mL/min
Injection volume	8.0 µL	2.0 µL
Amount on column	2 µg	5 µg

Table 2. MS Acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF system	
Gas temperature	250 °C
Drying gas	10 L/min
Nebulizer (psig)	25
Sheath gas temperature	250 °C
Sheath gas flow	12 L/min
VCap	3,500 V
Nozzle voltage	0 V
Fragmentor	170 V
Skimmer	65 V
Quad AMU	95
Reference mass	121.0509 922.0098

Data processing

Raw data acquired from LC/MS/MS were processed using MassHunter BioConfirm 10.0 software. This software simplifies data analysis, enabling automatic identification and relative quantitation of targeted biomolecules for all major biopharma workflows.

Table 3. MS/MS Acquisition parameters.

Parameter	Value
Acquisition mode	Extended Dynamic Range (2 GHz)
Mass range	m/z 150–1,700
Acquisition rate	8 spectra/sec
Auto MS/MS range	m/z 50–1,700
Min MS/MS acquisition rate	3 spectra/sec
Isolation width	Narrow (~ 1.3 m/z)
Precursors/cycle	Top 10
Collision energy	3.6*(m/z)/100–4.8
Threshold for MS/MS	2,000 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

Data acquired for glycopeptide quantitation analysis used the MS-only acquisition mode. Highlighted parameters were used for peptide identification.

Results and discussion

mAb glycoform profiling through the routine peptide mapping approach has been a widely used method. To demonstrate the effectiveness of glycopeptide separation by the HILIC column compared to the conventional RP-C18 column, three humanized IgG-1 type of mAbs were selected in this study. All mAbs were reduced, alkylated, and digested with a Trypsin + Lys-C enzymes mix using the same protocol in the AssayMAP Bravo liquid handling system. The digested mAb samples were then injected and separated by both the RP-C18 and the HILIC columns with the same HPLC run time (60 minutes). Figures 3 and 4 illustrate the chromatographic retention differences between the regular peptides and glycopeptides in the RP-C18 and the HILIC conditions.

Under routine RP HPLC conditions, peptides are separated by their hydrophobicity. The less hydrophobic peptides elute earlier than the more hydrophobic peptides. Since our HPLC gradient was optimized for the mAb tryptic digested samples, most of the peptides were separated nicely in the HPLC run. The glycopeptides are more hydrophilic and, thus, had shorter retention on the RP column. Figure 3 shows that all glycopeptides were eluted in the early gradient, with an approximately one-minute retention time window.

The HILIC separation is an orthogonal method to the RP, where the HPLC gradient is reversed. The lyophilized mAb digests should be dissolved in a high organic content solution to have better sample loading retention. High resolution in separation was achieved, and all major glycopeptide peaks were eluted between 28–34 minutes, as shown in Figure 4.

For LC/MS data analysis, the Peptide Digest Workflow in MassHunter BioConfirm 10.0 software was used. This software program enables the quick setup for batch sample analysis. A modification file of most major PTMs, including oxidation, deamidation, and many glycans imported from a personal compound database (PCD), can be generated easily. The Agilent proprietary Peptide Feature Extraction (PFE) algorithm was used for the identification of biomolecules, which were then confirmed by matching the measured masses with theoretical masses based on the known mAb sequences in the protein database. The relative quantitation on all identified peptides (including the glycopeptides) was also automatically calculated using either peak heights or peak areas of the mass spectra. Figure 5 is a screen capture of the BioConfirm 10.0 software layout showing the compound list of matched glycopeptides of NISTmAb. This program allows quick review of detailed peptide information including mass, retention times, sequences, modifications, scores, and quantitative results by either peak heights or peak areas. One feature of the BioConfirm 10.0 software is that users have the ability to select or deselect certain peptides for grouping in relative quantitation analysis, with the results shown in histogram format.

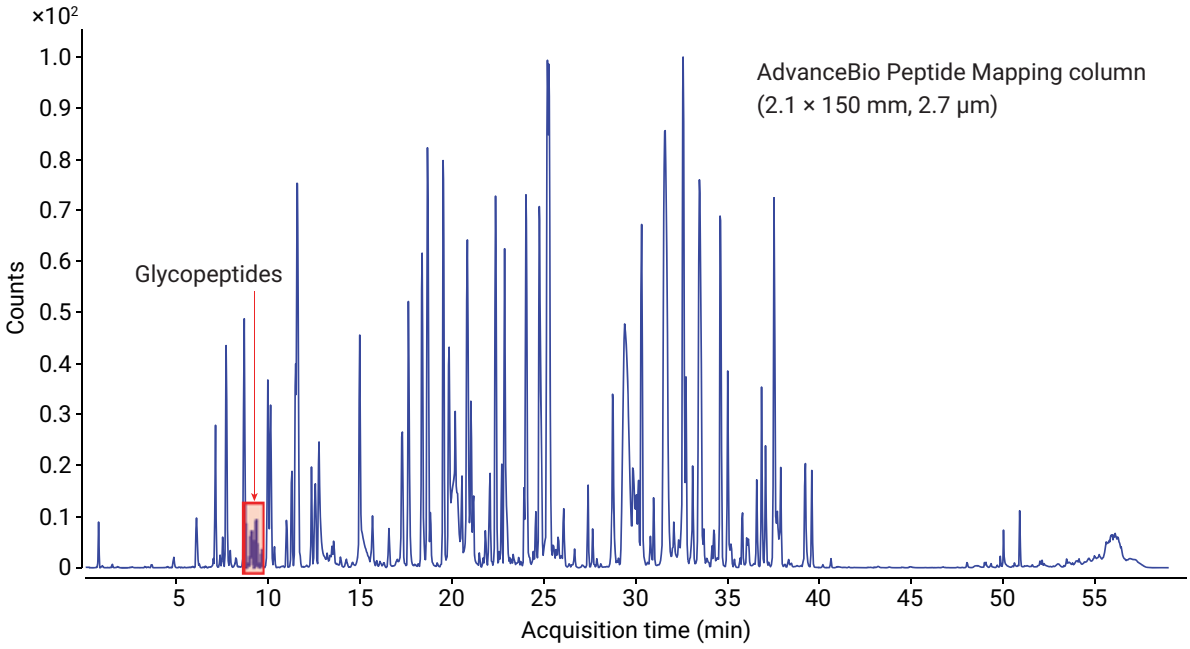


Figure 3. MS TIC of peptides from Trypsin/Lys-C digested NISTmAb on the AdvanceBio Peptide Mapping (RP-C18, 2.1 × 150 mm, 2.7 µm) column.

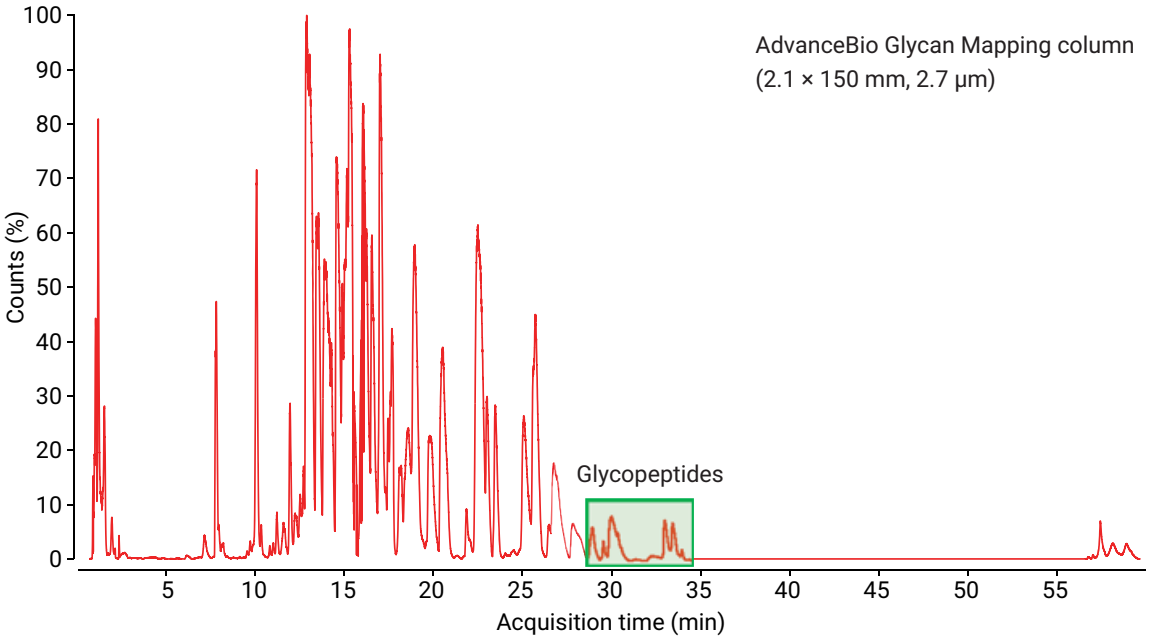


Figure 4. MS TIC of peptides from Trypsin/Lys-C digested NISTmAb on the AdvanceBio Glycan Mapping (HILIC, 2.1 × 150 mm, 2.7 µm) column.

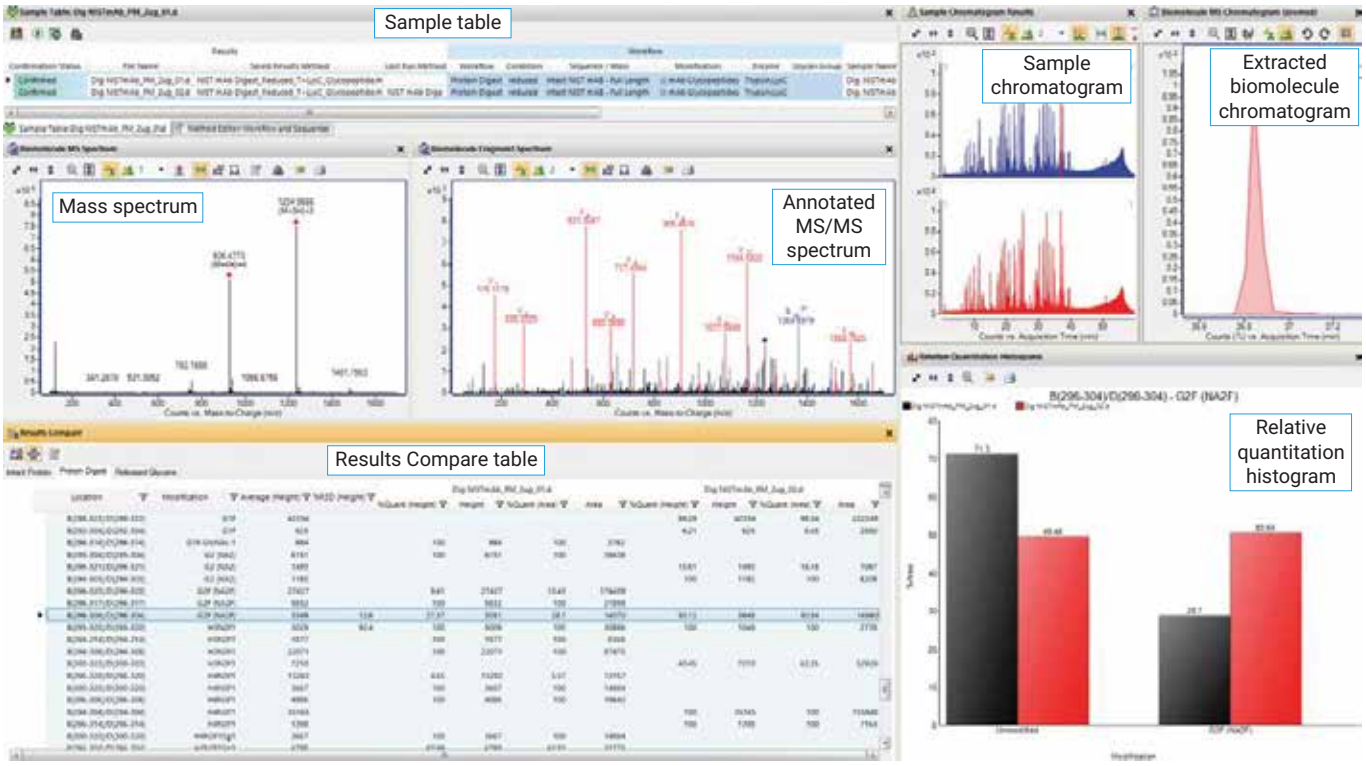
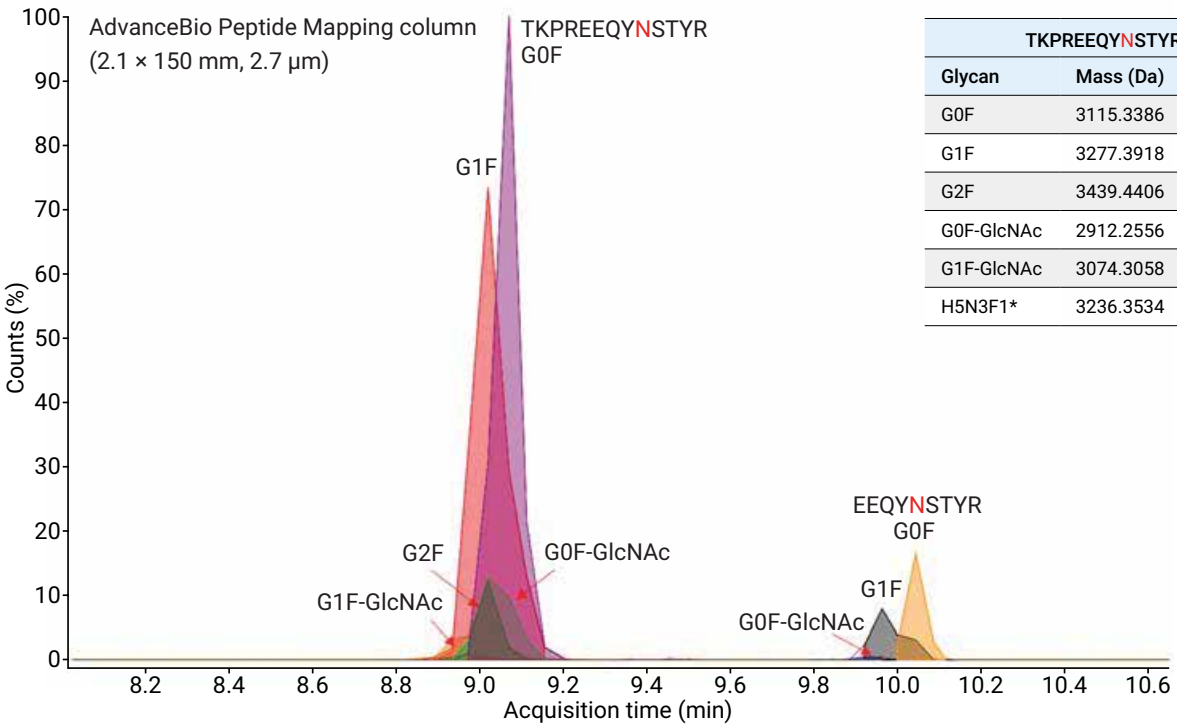


Figure 5. Screen capture of MassHunter BioConfirm 10.0 software with representative glycopeptide profiling results and histogram of relative quantitation on glycopeptides.

For LC/MS data analysis, the Peptide Digest Workflow in MassHunter BioConfirm 10.0 software was used. This software program enables the quick setup for batch sample analysis. A modification file of most major PTMs, including oxidation, deamidation, and many glycans imported from a personal compound database (PCD), can be generated easily. The Agilent proprietary Peptide Feature Extraction (PFE) algorithm was used for the identification of biomolecules, which were then confirmed by matching the measured masses with theoretical masses based on the known mAb sequences in the protein database. The relative quantitation on all identified peptides (including the glycopeptides) was also automatically calculated using either peak heights or peak areas of the mass spectra. Figure 5 is a screen capture of the BioConfirm 10.0 software layout showing the compound list of matched glycopeptides of NISTmAb. This program allows quick review of detailed peptide information including mass, retention times, sequences, modifications, scores, and quantitative results by either peak heights or peak areas. One feature of the BioConfirm 10.0 software is that users have the ability to select or deselect certain peptides for grouping in relative quantitation analysis, with the results shown in histogram format.

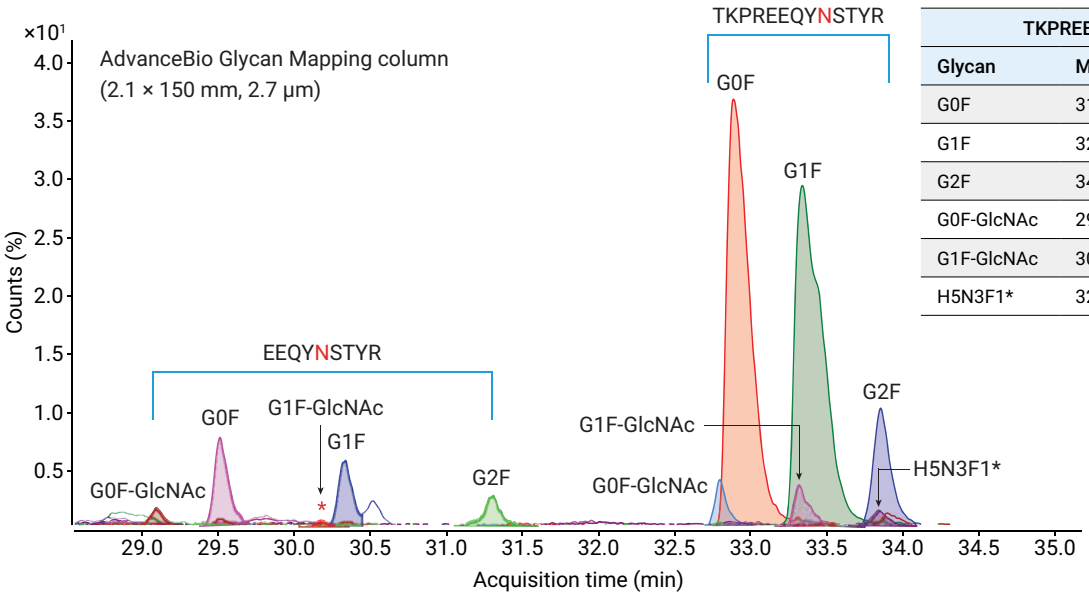
Detailed inspection of raw MS data from Figures 3 and 4 reveals that there were two major group of glycopeptides (EEQYNSTYR and TKPREEQYNSTYR) with various glycans attached at the asparagine (N300 of heavy chain) position. In the RP separation, three glycopeptides with sequence of EEQYNSTYR, and six glycopeptides in TKPREEQYNSTYR were identified (Figure 6). However, the same group of glycopeptides were coeluted, and poor chromatographic resolution was observed. Conversely, the HILIC column demonstrated great resolution for the separation of the same sets of glycopeptides (Figure 7).

Although more than nine glycopeptides (Figures 6 and 7) were detected and identified in different LC conditions, a set of six major abundant glycopeptides with the sequence of TKPREEQYNSTYR were selected for relative quantitation analysis (tables in Figures 6 and 7) to have fair comparison results.



TKPREEQYNSTYR (292-304)			
Glycan	**Mass (Da)**	**RT (min)**	**Quant (%)**
G0F	3115.3386	9.066	41.82
G1F	3277.3918	9.016	41.70
G2F	3439.4406	9.016	9.08
G0F-GlcNAc	2912.2556	9.016	2.87
G1F-GlcNAc	3074.3058	9.016	3.35
H5N3F1*	3236.3534	8.930	1.18

Figure 6. MS extracted compound chromatograms (ECCs) and relative % quantitation of the identified glycopeptides from RP LC separation. H5N3F1* may be denoted as FM4A1G1 or FA1G1Ga1 in other publications.



TKPREEQYNSTYR (292-304)			
Glycan	**Mass (Da)**	**RT (min)**	**Quant (%)**
G0F	3115.3386	32.923	43.07
G1F	3277.3918	33.334	39.89
G2F	3439.4406	33.877	9.31
G0F-GlcNAc	2912.2556	32.797	3.10
G1F-GlcNAc	3074.3058	33.321	3.37
H5N3F1*	3236.3534	33.864	1.25

Figure 7. MS ECCs and relative % quantitation of the identified glycopeptides from HILIC separation.

Figure 8 summarizes the relative quantitation and reproducibility results of the six major glycopeptides of the NISTmAb from three replicate sample injections of 2 µg (RP-C18) and 5 µg (HILIC) on-column, respectively. The quantitative results from the peak area of the RP method were similar to those from the HILIC method. However, due to the better glycopeptide separation, the HILIC results represented higher quantitation accuracy and smaller average standard deviations (SDs) for all glycopeptides (<0.2 %); the average SDs of the RP method results were approximately 0.56 %.

We used the same HILIC method for glycopeptide relative quantitative comparison among three mAbs (NISTmAb, Herceptin, and A-mAb). Figure 9 shows the relative % quant of the top six most abundant glycopeptides. Unlike the NISTmAb that posted similar abundances of G0F and G1F (43 % and 40 %), the Herceptin sample contained a very high level of G0F (>65 %) and low level of G2F (~2 %). In addition, no H5N3F1 could be detected in either Herceptin or A-mAb samples. Two degraded glycan molecules (G0F-GlcNAc and G1F-GlcNAc) were found at trace levels (<0.5 %) as well in the A-mAb sample.

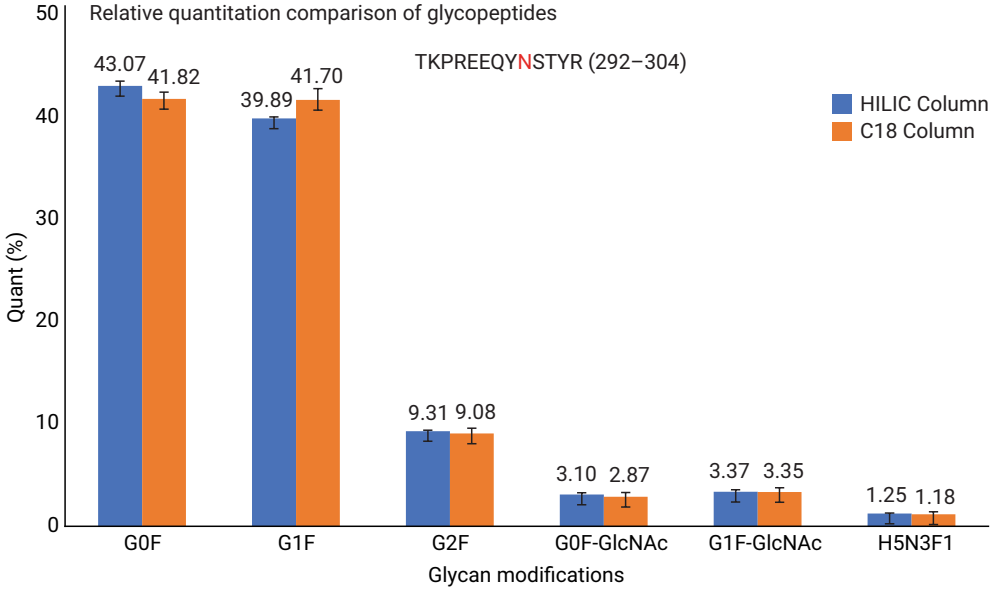


Figure 8. Relative quantitative comparison of NISTmAb glycopeptides analysis (RP-C18 versus HILIC, three replicates).

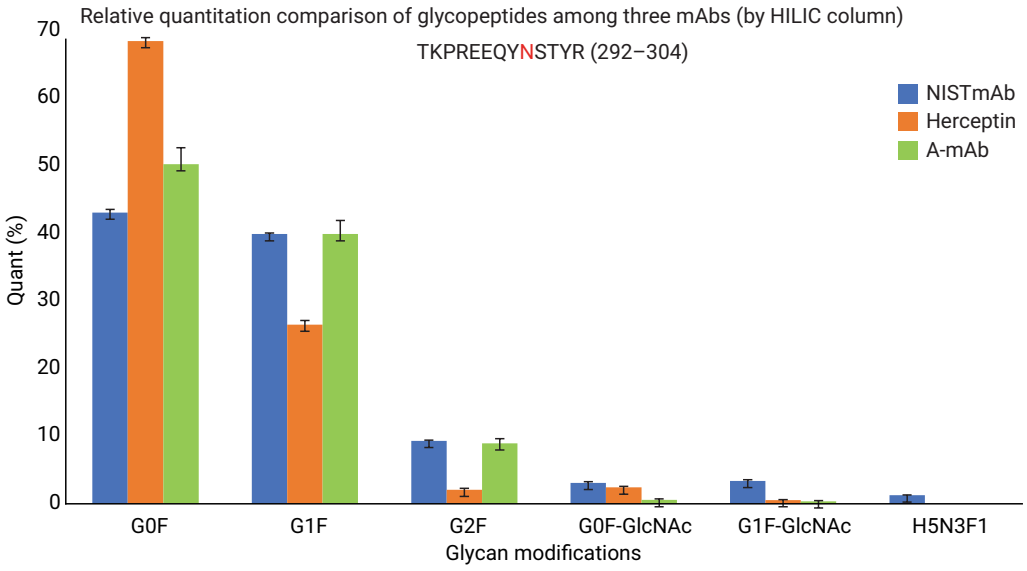


Figure 9. Relative % quantitation of the top six glycopeptides in each of the three mAb samples. All digested mAb samples were separated by the HILIC column (three replicates).

Conclusion

A complete workflow solution for mAb glycopeptide characterization by integrating the AssayMAP Bravo liquid handling platform, 1290 Infinity II LC, 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software has been developed. The major benefits of this new workflow include:

- The AdvanceBio Glycan Mapping (HILIC) column demonstrated strong retention and increased resolution for hydrophilic glycopeptides. Various glycoforms of the same peptide were well resolved.
- The glycopeptide analysis through peptide mapping workflow resulted in not only glycan relative quantitation, but also N-glycosylation site(s) information.
- The automated data processing capability of BioConfirm 10.0 resulted in accurate glycopeptide profiling—identification and relative quantitation. A batch of samples or different mAb digests can easily be analyzed and compared.

References

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7. Highly Confident Peptide Mapping of Protein Digests Using Agilent LC/QTOFs, *Agilent Technologies*, publication number 5991-8552EN.

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Application Note

Biotherapeutics and Biosimilars

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A Comprehensive Approach for Monoclonal Antibody N-linked Glycan Analysis from Sample Preparation to Data Analysis

Authors

David L. Wong, Oscar Potter, Jordy Hsiao, and Te-Wei Chu
Agilent Technologies, Inc.
Santa Clara, CA, USA

Introduction

Monoclonal antibodies (mAbs) and their derivatives comprise a very important class of biopharmaceutical molecules with a wide range of applications. Due to the heterogeneous nature of these mAbs, comprehensive analytical characterization is required. These analyses include determining the complete amino acid sequences of the mAbs and their variants, as well as characterization of post-translational modifications (PTMs) including glycosylation, oxidation, and deamidation. Glycosylation plays an important role in many biological processes. It also affects the therapeutics' efficacy, stability, pharmacokinetics, and immunogenicity[1]. Glycan characterization usually involves techniques such as NMR, HPLC, or mass spectrometry (MS). Since glycans are very diverse in composition/structures and are poorly ionized by electrospray, the MS-based approach for glycan characterization has been challenging. InstantPC is a novel fluorescence tag from ProZyme Inc. (Figure 1) that has been developed to improve MS ionization efficiency, and sensitivity for N-glycan molecules.

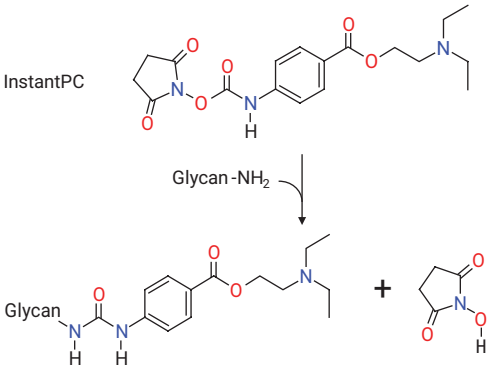

Figure 1. Diagram of InstantPC-labeled N-glycans released from an mAb.

The traditional method of glycan analysis is laborious, and involves many steps, starting with enzymatic glycan release by PNGaseF (overnight), followed by sample cleanup, labeling with a fluorescence tag by reductive amination (2-AB or InstantPC), and finally cleanup of the released labeled N-glycans prior to LC-FLD or LC/MS analysis[2,3]. Despite the significant improvement of MS sensitivity using fluorescent tags, the labor intensiveness of manual sample preparation, low reproducibility, and limitation to scale-up on sample processing have been major issues for the biopharmaceutical industry.

This study demonstrates how to increase sample throughput for glycan characterization workflows using the Agilent AssayMAP Bravo liquid handling platform. The solution incorporates the Agilent 1290 Infinity II LC system, Agilent AdvanceBio Glycan Mapping column, Agilent highly sensitive fluorescence detection (FLD), and the Agilent 6545XT AdvanceBio LC/Q-TOF. The Q-TOF data are analyzed automatically with Agilent MassHunter BioConfirm B.09.00 software (Figure 2). This solution dramatically improves productivity by allowing convenient sample preparation, streamlined data acquisition, and data analysis. This solution provides the flexibility to perform quantitation based on FLD or MS signals with accurate mass peak assignment from an N-glycan mass database.

Experimental

Sample preparation

Four monoclonal antibody (mAb) samples were used in this study:

- The monoclonal antibody standard, RM 8671, was from National Institute of Standards & Technology (NIST) A.K.A. NISTmAb.
- Formulated Herceptin (Trastuzumab) was from Genentech (So. San Francisco, California, USA).
- Sigma SiLu mAb was purchased from Sigma-Aldrich (SiLu Lite, P/N: MSQC4).
- CHO mAb1 was expressed and purified from the Agilent R&D lab.

All mAb samples were diluted with DI water to 1.0 µg/µL prior to sample preparation using the AssayMAP Bravo liquid handling system (G5542A) with the GlykoPrep-plus Rapid N-Glycan Sample Preparation with InstantPC (96-ct) from ProZyme Inc. A detailed procedure for the sample preparation is described in ProZyme's application note (product code: GPPNG-PC). After the final cleanup step, the eluted, released, labeled N-glycans had a final volume of 50 µL, so that each 1 µL of the prepared sample contained N-glycans from 1 µg of mAb.

Figure 2. mAb Glycan characterization workflow.

LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II LC system equipped with an Agilent 1260 Infinity Fluorescence Detector (G1321B), coupled with a 6545XT AdvanceBio LC/Q-TOF system with a Dual Agilent Jet Stream source. The detector was set to λ_{Ex} = 285 nm, λ_{Em} = 345 nm, with PMT gain = 10. Glycans were chromatographically separated with an AdvanceBio Glycan Mapping column (2.1 × 100 mm, 1.8 µm). Tables 1 and 2 list the LC/MS parameters used. Approximately 1–2 µL of each N-glycan sample were injected for LC/MS analysis.

Data processing

The InstantPC-labeled released N-glycans were analyzed using the Released Glycans Workflow of MassHunter BioConfirm B.09.00 software. This analytical workflow uses the Agilent Personal Compound Database (PCD) glycan database. The PCD glycan database provides accurate glycan identification and confirmation. Finally, a summarized report of the analyses was generated in PDF format using the Report Builder program in BioConfirm B.09.00.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC System	
Column	Agilent AdvanceBio Glycan Mapping, 2.1 × 100 mm, 1.8 µm
Thermostat	4 °C
Solvent A	50 mM formic acid adjusted to pH 4.5 with ammonium hydroxide
Solvent B	Acetonitrile
Gradient	0–0.5 minutes, 75–71 %B 0.5–16 minutes, 71–67.5 %B 1–22 minutes, 67.5–60 %B 22–22.5 minutes, 60–40 %B 22.5–23.5 minutes, 40 %B (0.7 mL/min) 23.5–24 minutes, 40–75 %B (0.7 mL/min) 24–30 minutes, 75 %B (0.9 mL/min)
Column temperature	40 °C
Flow rate	0.4 mL/min
Injection volume	2.0 µL

Agilent 1260 Infinity Fluorescence Detector (G1321B) was used. The detector was set to λ_{Ex} = 285 nm, λ_{Em} = 345 nm, with PMT gain = 10.

Table 2. MS Acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF system	
Gas temperature	150 °C
Drying gas	9 L/min
Nebulizer (psig)	35
Sheath gas temperature	300 °C
Sheath gas flow	10 L/min
VCap	3,000 V
Nozzle voltage	500 V
Fragmentor	120 V
Skimmer	65 V
Quad AMU	95
Acquisition mode	Low mass range, HiRes (4 GHz)
Mass range	m/z 300–1,700
Acquisition rate	2 spectra/sec

Results and Discussion

LC-FLD analysis of released labeled glycans is one of the most widely used approaches to determining therapeutic protein glycosylation. We have previously published application notes showing optimized separation of several mAb glycan profiles using various column dimensions and run conditions[4,5]. The separation method in this report represents the best overall performance with maximum peak resolution and excellent robustness for the different mAb N-glycan samples in this study.

Figure 3 shows the representative chromatograms of N-glycans (FLD and MS EIC) from the NISTmAb. The FLD chromatogram (Figure 3 top, zoom in) reveals that more than 15 glycan peaks were detected. The glycosylation pattern of the major abundant glycans, such as the G0F, G1F isoforms, and G2F was comparable between the fluorescent and MS data (Figure 7).

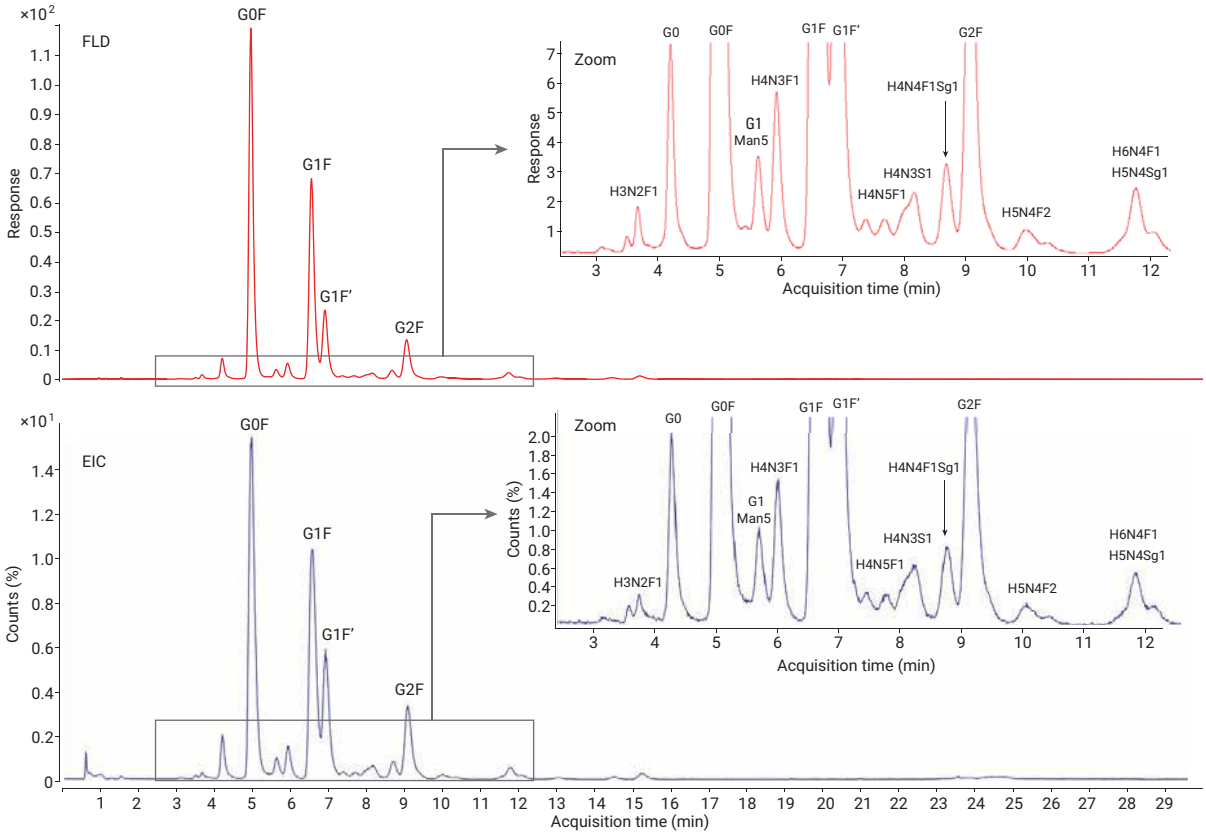

Figure 3. FLD chromatogram and mass spectra (EIC) of InstantPC-labeled N-glycans from NISTmAb.

While fluorescence detection does not allow for direct structure elucidation, MS analysis of mAb glycans can be used to determine glycan monosaccharide composition. In the case of many mAb N-glycans, this composition is sufficient to achieve a high-confidence structural assignment. The combination of the positively charged InstantPC tag and sensitive Agilent Jet Stream (AJS) electrospray ionization (ESI) source technology dramatically increases MS detection sensitivity for N-glycans. In addition, we have optimized the MS parameters to maximize the sensitivity of the InstantPC-labeled N-glycans while minimizing in-source fragmentation of these fragile molecules.

The optimized conditions have significantly improved the MS spectrum quality, leading to accurate N-glycans identification and relative quantification results. Figure 4 shows the MS spectrum of an InstantPC-labeled N-glycan (G2F) where only the doubly charged ions of its protonated form, $[M+2H]^{2+}$, as well as its adducts $[M+H+Na]^{2+}$ and $[M+H+K]^{2+}$ were observed (Note: InstantPC tag causes a mass increment of 261.1477 Da compared to the free reducing end form of the glycan).

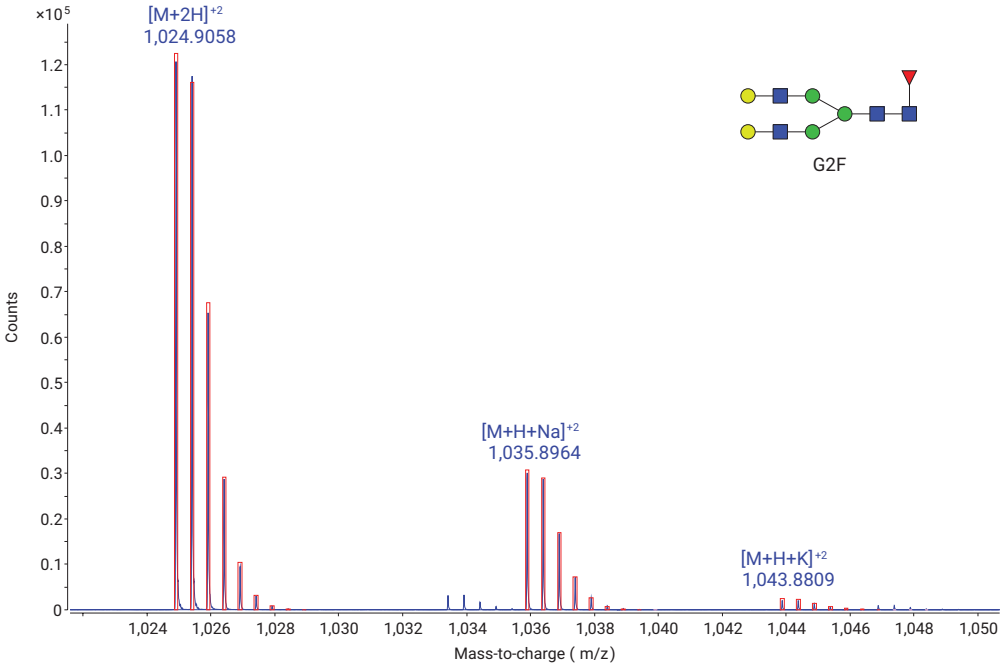

Figure 4. Representative spectrum of an InstantPC-labeled N-glycan (G2F). Excellent isotopic fidelity of the charge states of the InstantPC-labeled G2F glycan and its adducts. The red boxes represent the theoretical isotopic pattern, and the blue lines represent the actual raw MS spectrum.

We have introduced a workflow in MassHunter BioConfirm software for released glycan profiling. This workflow enables the easy setup of sample batch analysis. The software can accommodate many commercial or customized fluorescent tags. A Personal Compound Database (PCD) containing accurate mass and structural information of glycans is used for identification using the Agilent proprietary Find by Formula algorithm. Subsequently, a summary analytical report can be created in a customer-defined report format. Figure 5 shows the extracted ion chromatograms (EICs) of the identified glycans.

The Biomolecules results table (Figure 6) in BioConfirm allows quick review of detailed glycans information including names, mass, retention time, peak area, composition, and database matching score. Multiple IDs are displayed for glycans with possible isoform structures. It also allows users to review the TIC of the sample as well as the individual glycan MS spectra.

In addition, multiple data files can be processed and analyzed in batch mode. The user may use peak areas of the selected glycans in the results table for relative quantitative analysis.

InstantPC-labeled glycans were previously shown to give similar relative quantitation results for MS and FLD analysis[4]. The FLD chromatogram for the CHO mAb1 sample was integrated using the Agilent MassHunter Qualitative Analysis software. The relative sum abundance of the top seven most abundant N-glycans was calculated and compared against the same data from the MS analysis (Figure 7). To get equivalent results, do not saturate the MS detector. An ideal quantity for this workflow would be to inject N-glycans released from approximately 0.5 µg of mAb.

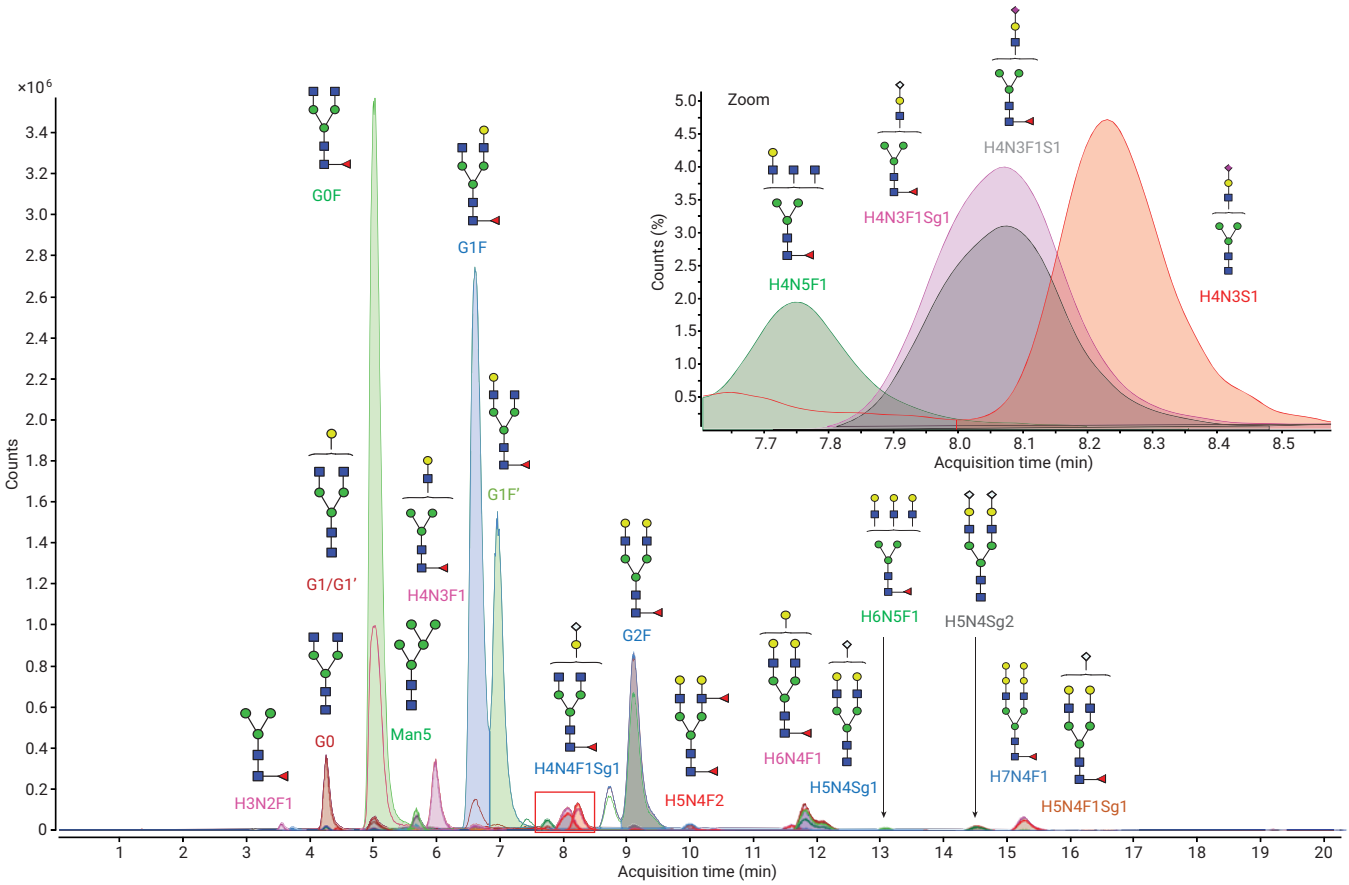

Figure 5. Extracted ion chromatograms of the identified glycans from NISTmAb. Inset: zoom of EICs of identified glycans eluted in the retention time range of 7.6–8.6 minutes.

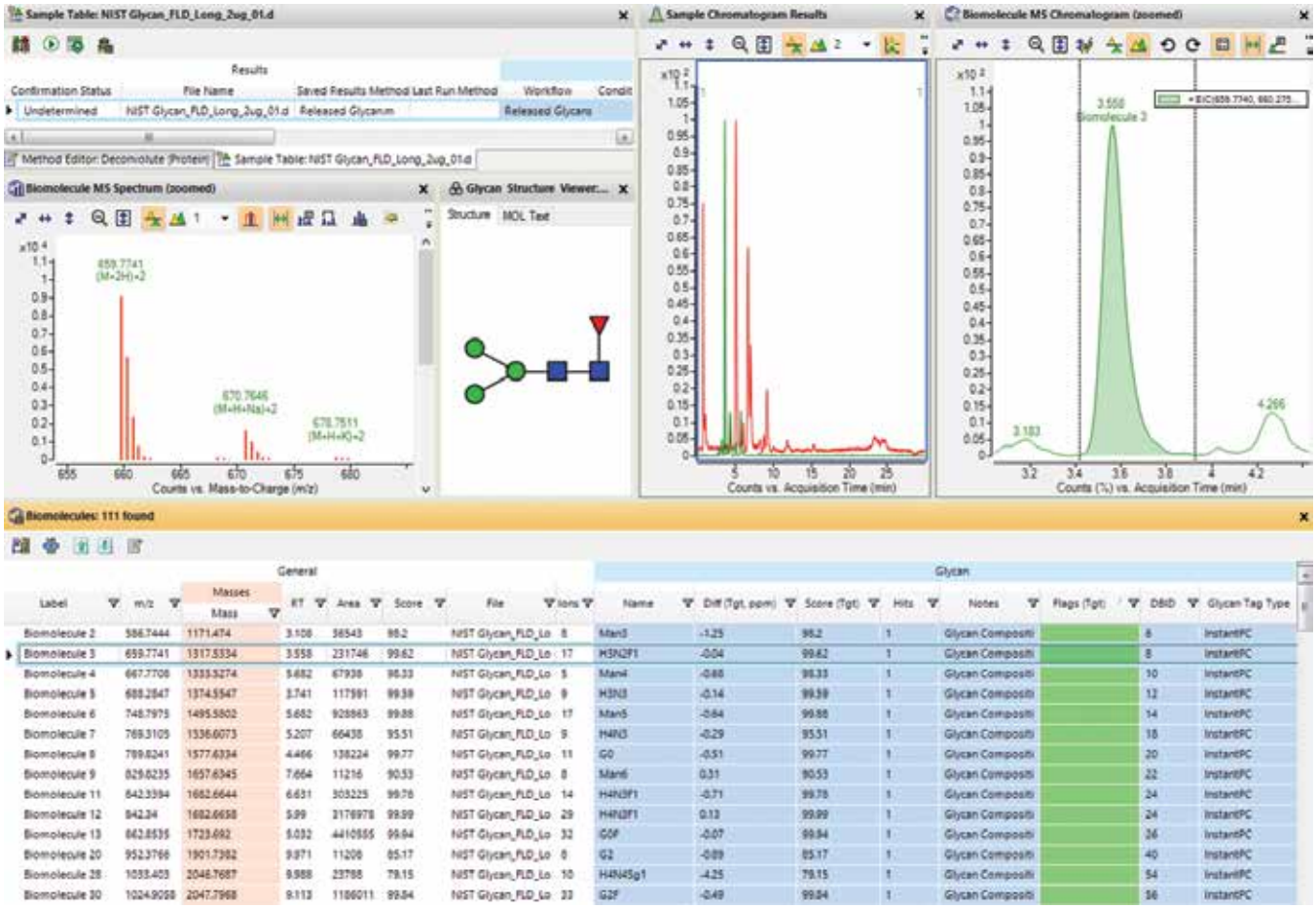

Figure 6. Screenshot of Agilent MassHunter BioConfirm B.09.00 software with representative glycan profiling results.

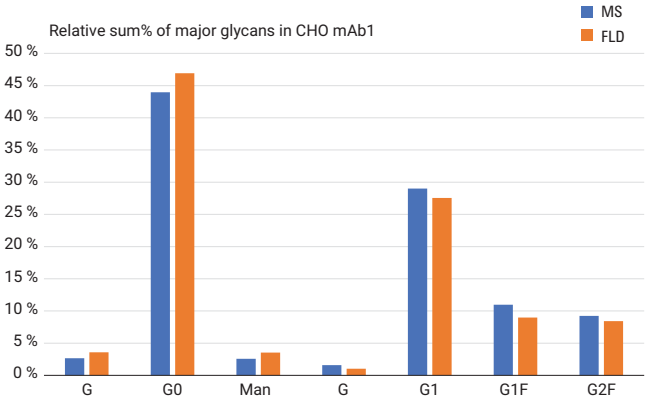

Figure 7. Relative sum % of the major N-glycans in the CHO mAb1 (0.5 µg), comparing results from MS-based quantitation (blue) with FLD-based quantitation (orange).

To summarize and compare the MS results, the top five most abundant N-glycans for each mAb sample were used to calculate relative sum %. Figure 8 presents the data.

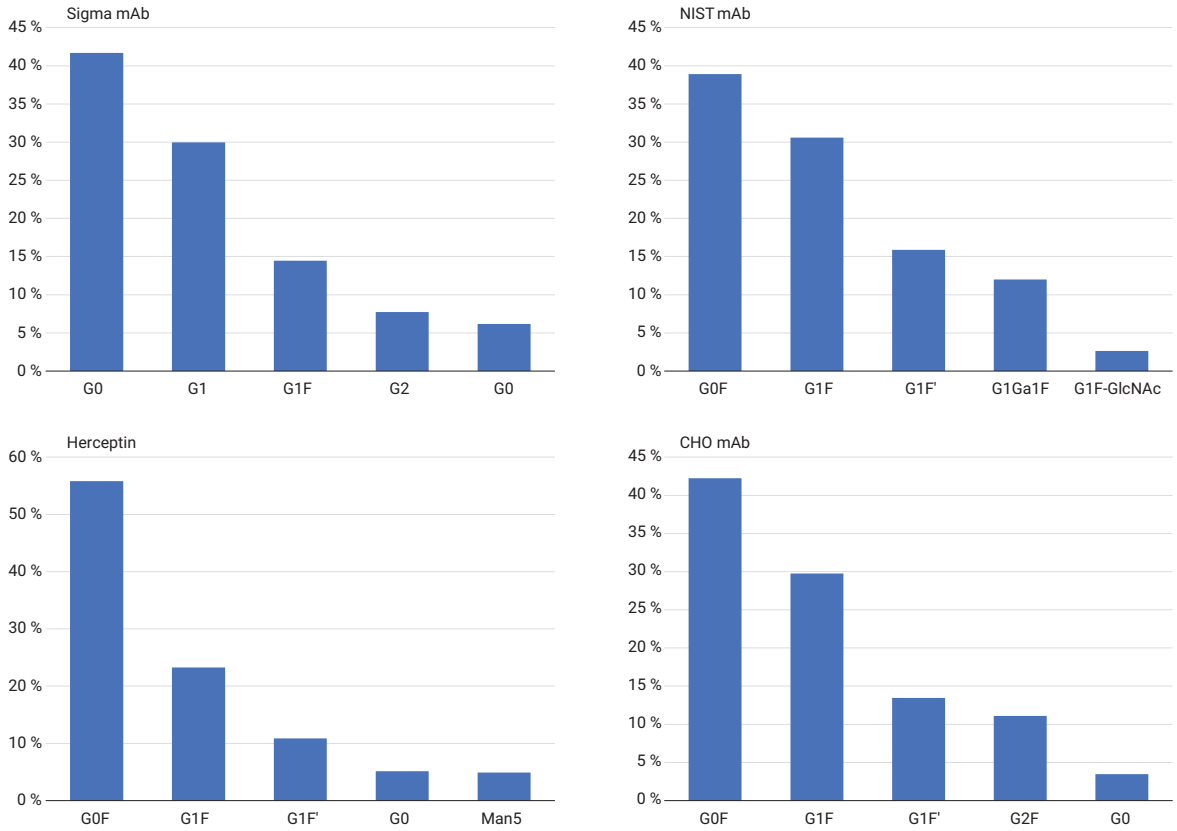

Figure 8. Relative sum % of the top five N-glycans in each of the four mAb samples. Note: The NISTmAb contained a structure suspected to be G1F with an additional alpha-1,3-galactose, and this was labeled as G1Ga1F.

The BioConfirm B.09.00 software allows users to generate their own glycan profile reports using the Report Builder program. Figure 9 shows an example of a released glycan report. In the Report Builder, users can customize the report sections with information such as Sample Information, Sample Chromatogram, Biomolecule Summary, and Biomolecule Details.
The corresponding glycan structures are displayed along with the identified glycans.

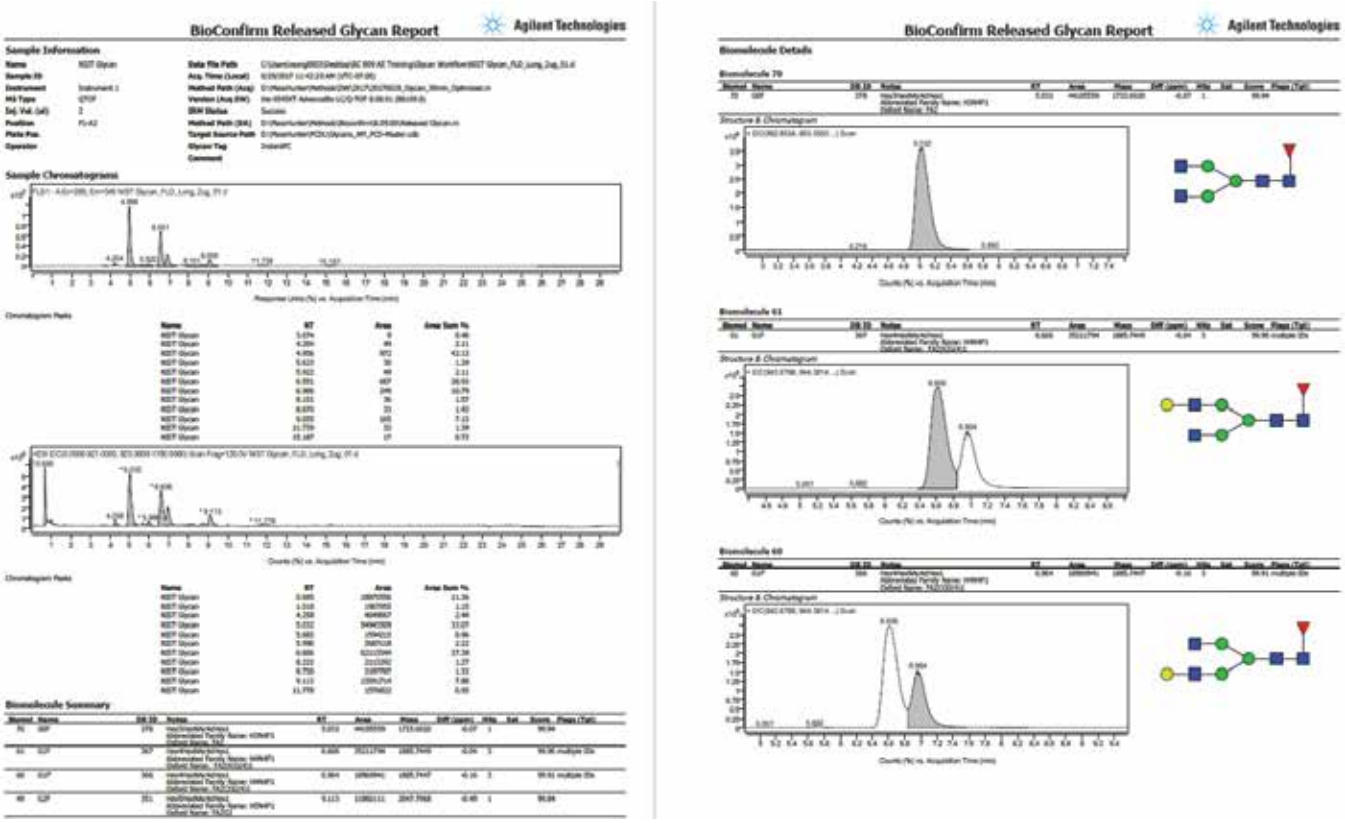

Figure 9. Agilent MassHunter BioConfirm B.09.00 Software – Released Glycan Report.

Conclusion

This study demonstrated the performance of the Agilent AssayMap Bravo, 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software, when used as an integrated solution for released glycan analysis.

- This workflow combines high-throughput sample preparation with excellent chromatographic separation using the Agilent AdvanceBio Glycan Mapping column.
- The easy setup and use of the glycan database included with BioConfirm B.09.00 provided the ability to accurately profile, identify, and perform relative quantification.
- The 6545XT-based glycan analysis generated similar quantitative results to that of fluorescence analysis, making it possible to compare different N-glycans across different mAb samples.
- The Report Builder function in BioConfirm B.09.00 provides the ability to create custom reports.

In conclusion, the Agilent solution automated the entire process of N-linked glycan analysis from sample preparation to data analysis with high precision. This approach provided high sensitivity and best quantitation for glycan analysis using fluorescence and additional identification by mass spectrometric detection.

References

1. Rademacher, T. W; Williams, P; DwekMark, R. A. Agalactosyl glycoforms of IgG autoantibodies are pathogenic. *P. Natl. Acad. Sci.* **1994**, 91, pp 6123-6127.
2. Anumula, K. R. "Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates" *Anal. Biochem.* **2006**, *350*, 1-23.
3. N-Glycan Analysis of mAbs and Other Glycoproteins with UHPLC and Fluorescence Detection, *Agilent Technologies*, publication number 5991-5253EN.
4. Comparison of Relative Quantification of Monoclonal Antibody N-glycans Using Fluorescence and MS Detection, *Agilent Technologies*, publication number 5991-6958EN.
5. Analysis of Monoclonal Antibody N-glycans by Fluorescence Detection and Robust Mass Selective Detection Using the Agilent LC/MSD XT, *Agilent Technologies*, publication number 5991-8071EN.

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Application Note

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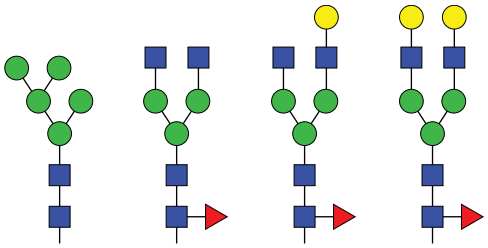
Analysis of Monoclonal Antibody N-glycans by Fluorescence Detection and Robust Mass Selective Detection Using the Agilent LC/MSD XTs

Authors

Oscar Potter, Gregory Staples, Jordy Hsaio, and Te-Wei Chu

Introduction

N-glycosylation of therapeutic proteins is monitored during product development, process development, and QC. Many analysts perform enzymatic N-glycan release followed by labeling with a fluorescent tag to run HILIC-FLD analysis. The InstantPC tag from Agilent (formerly ProZyme) allows manual sample prep within 1 hour, or alternatively, allows high-throughput parallel processing with the Agilent AssayMap Bravo liquid handling platform. High-resolution separation can be completed in short cycle times using the Agilent AdvanceBio Glycan Mapping column (see 5991-4886EN). While fluorescence detection is a popular detection technique, analysts running HILIC-FLD workflows may face difficult challenges in the form of ambiguous peak assignment, particularly in the case of new sample types or unexpected peaks. In such cases, analysts might resort to sending the samples through complementary analyses such as LC-Q-TOF or MALDI-TOF, but this loses time, and incurs additional expenses.



We previously demonstrated an enhanced workflow where a HILIC-FLD system was hyphenated online to a high-resolution Q-TOF mass spectrometer (see 5991-6958EN). This approach couples the benefits of robust fluorescence based quantitation with the powerful structural elucidation capabilities of mass spectrometry. However, analysts who seek a routine cost-effective solution may prefer to work with a rugged, small-footprint mass selective detector (MSD) based on single quadrupole technology. Therefore, we present a method where a HILIC-FLD system is coupled to a rugged, sensitive, and stackable Agilent LC/MSD XT single quadrupole. The MSD data provide mass information to solve ambiguous peak assignments and detect coeluting structures. The method operates at less than 600 bar to maintain compatibility with a range of existing LC systems.

Agilent LC/MSD XT

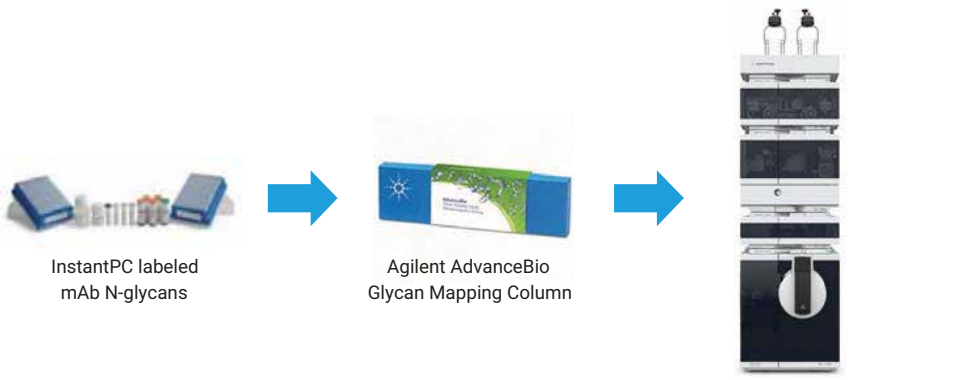


Figure 1. Flow chart of the sample preparation, LC column, and instruments used for identification and quantification of InstantPC labeled N-glycans.

Experimental

Sample preparation

Samples of monoclonal antibody (mAb) were expressed in our own lab (CHO mAb 1) or purchased from Sigma-Aldrich (SiLu Lite P/N MSQC4) and from NIST (NISTmAb, Reference Material 8671). All samples were adjusted to 1 µg/µL prior to processing by GlykoPrep-plus Rapid N-Glycan Sample Preparation with InstantPC (96-ct) from Agilent (formerly ProZyme) (GPPNG-PC). The sample handling was automated using the AssayMap Bravo Liquid Handling Platform (G5542A). The final step of this protocol elutes the labeled glycans in 50 µL, so that each µL of this final sample solution represents N-glycans released from 1 µg of mAb. Conveniently, this workflow has the advantage of not requiring any centrifugation or dry down steps.

Chromatography conditions

Pump

Agilent 1260 Infinity II Binary Pump G7112B

Mobile phase A

50 mM Formic acid adjusted to pH 4.5 with ammonium hydroxide

Mobile phase B

Acetonitrile

Sampler

Agilent 1260 Infinity II Multisampler (G7167A) with thermostat set at 11 °C

Columns

- AdvanceBio Glycan Mapping Column
  1.8 µm, 2.1 × 100 mm used with method A
- AdvanceBio Glycan Mapping Column
  2.7 µm, 2.1 × 150 mm used with method B
- AdvanceBio Glycan Mapping Column
  1.8 µm, 2.1 × 150 mm used with method C

Column heater

Agilent 1260 Infinity II G7116 thermostatic column compartment with G7116-60015 solvent preheater set to 40 °C

Columns were plumbed using 100 µm id SSTL tubing to an Agilent 1260 Infinity Fluorescence Detector (G1321B) with a 8 µL flow cell (G1321-60005). The detector was set to λEx at 285 nm, λEm at 345 nm with PMT gain = 10.

LC/MSD XT Parameters

Parameter	Value
Source	Agilent Jet Stream in positive mode
Sheath gas	300 °C at 10.0 L/min
Dry gas temperature	150 °C at 9.0 L/min
Nebulizer pressure	35 psig
VCap	2,500 V Nozzle: 500 V
Mass range	500–1,400 m/z
Fragmentor	100 V
Gain EMV	1.0
Step size	0.10
Peak width	0.2

Software

OpenLab CDS Chemstation Edition Rev C.01.07 SR3

Reagents

All reagents and solvents used were of the highest purity available.

Gradient tables

Method A (for 2.1 × 100 mm 1.8 µm column)

Time	Eluent A	Eluent B	Flow
0.00	25.0%	25.0%	0.70 mL/min
0.50	27.0%	27.0%	0.70 mL/min
4.00	28.0%	28.0%	0.70 mL/min
9.00	33.0%	33.0%	0.70 mL/min
9.20	50.0%	50.0%	0.70 mL/min
9.70	50.0%	50.0%	0.70 mL/min
10.00	25.0 %	75.0%	0.70 mL/min
11.50	25.0%	75.0%	0.70 mL/min
11.80	25.0%	75.0%	0.80 mL/min
14.50	25.0%	75.0%	0.90 mL/min
15.30	25.0%	75.0%	0.70 mL/min

Stop time = 18 minutes

Method B (for 2.1 × 150 mm 2.7 µm column)

Time	Eluent A	Eluent B	Flow
0.00	27.0%	73.0%	0.50 mL/min
1.00	28.5%	71.5%	0.50 mL/min
9.00	29.5%	70.5%	0.50 mL/min
22.00	41.0%	59.0%	0.50 mL/min
22.50	50.0%	50.0%	0.80 mL/min
23.50	50.0%	50.0%	0.70 mL/min
23.70	27.0%	73.0%	0.70 mL/min
25.00	27.0%	73.0%	0.70 mL/min
25.50	27.0%	73.0%	0.80 mL/min
27.50	27.0%	73.0%	0.90 mL/min
28.00	27.0%	73.0%	0.50 mL/min

Stop time = 30 minutes

Method C (for 2.1 × 150 mm 1.8 µm column)

Time	Eluent A	Eluent B	Flow
0.00	22.0%	78.0%	0.60 mL/min
0.50	26.0%	74.0%	0.60 mL/min
13.00	27.5%	72.5%	0.60 mL/min
28.00	39.0%	61.0%	0.60 mL/min
28.50	50.0%	50.0%	0.50 mL/min
28.60	50.0%	50.0%	0.40 mL/min
28.80	22.0%	78.0%	0.40 mL/min
31.00	22.0%	78.0%	0.50 mL/min
31.50	22.0%	78.0%	0.55 mL/min
33.50	22.0%	78.0%	0.60 mL/min

Stop time = 37 minutes

Results and Discussion

Separation

We optimized the separation of typical therapeutic mAb glycan profiles on three different columns, targeting various cycle times. The methods begin with a shallow gradient that maximizes resolution of the common neutral glycans that appear in most therapeutic mAb samples. The gradient slope was increased midway through the run to elute any larger, acidic glycan structures more efficiently. These methods were all designed to stay well under 600 bar to maximize robustness, as well as allowing for compatibility with a wide range of LC instruments. While all three separation methods are shown in Figure 2, we chose to use Method C for the remainder of the experiments because it was found to give the best overall performance for our three mAb N-glycan samples.

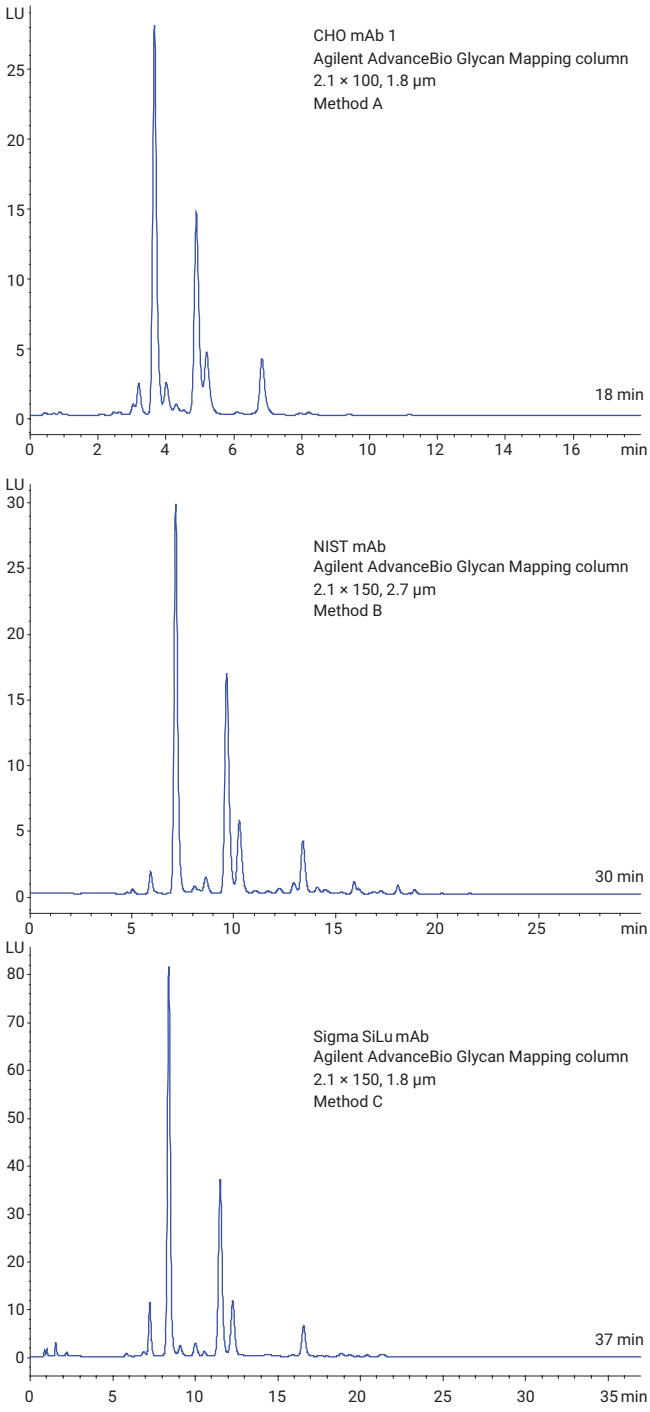


Figure 2. Three separation methods using different column formats with three mAb samples.

Peak assignment

The data from all three samples (using method C) were integrated, and the peaks were assigned to glycan compositions using an approach similar to that shown in application note 5991-5253EN.

Note: InstantPC labeling causes a mass increment of 261.1477 m/z versus the free reducing-end form of the glycans.

Table 1 was constructed showing the composition of the glycans that were detected across the three samples. Glycan compositions are shown in the form: HxNxFxSgx + Core (where H = Galactose or Mannose, N = N-acetylglucosamine, F = Fucose, Sg = N-glycolylneuraminic acid, and Core = trimannosyl, a core common to all N-glycans). In many cases, these compositions allowed us to propose glycan structures by supplementing basic knowledge of therapeutic mAb glycosylation patterns and HILIC retention order. Since most therapeutic mAbs contain a similar, limited set of common N-glycan structures, Table 1 can serve as a useful reference for assigning N-glycans in this workflow.

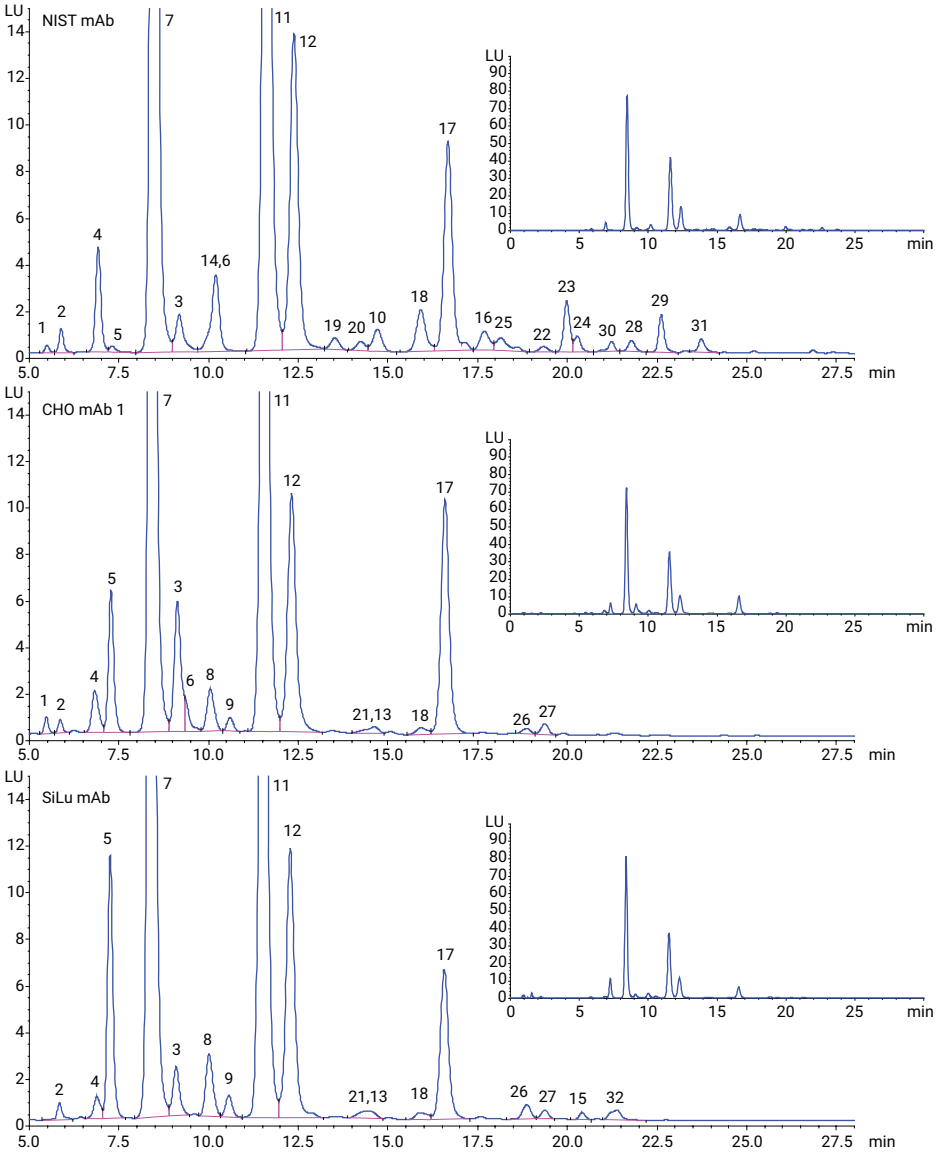


Figure 3. Zoomed FLD chromatograms of the three mAb N-glycan samples showing detection of major and minor glycans for method C. Insets show the zoomed-out data. Annotations refer to Table 1.

LC/MSD XT Sensitivity

Traditionally, analysts have avoided using single quadrupole mass spectrometers for LC/MS analysis of N-glycans due to concerns about sensitivity. However, two technical innovations have led to dramatic improvements in the limit of detection for N-glycans. The first is the availability of a highly sensitive ion source, Agilent Jet Stream, which uses a super-heated sheath gas flow around the electrospray plume to dramatically improve ionization. The second innovation is the availability of a InstantPC tag from Prozyme Inc. that radically increases ionization efficiency versus traditional fluorescent labels.

We optimized the MSD parameters to maximize sensitivity. InstantPC-labeled N-glycans were observed almost exclusively as doubly charged ions of the forms $[M+2H]^{2+}$, with some $[M+NH_4+H]^{2+}$, and $[M+H+Na]^{2+}$. Increasing the dry gas temperature or fragmentor voltage can cause collision-induced dissociation of the N-glycans, offering powerful structural elucidation strategies similar to those achievable with a Q-TOF (see 5991-5253EN).

Diagnostic signals were obtained for glycans making up as little as 0.1 % of the profile. When comparing this sensitivity with alternative workflows, remember that these results were achieved without adding a preconcentrating step to the end of the sample preparation workflow, therefore saving considerable time. Sensitivity could be boosted even further by drying the samples and reconstituting them in a lower volume of sample matrix.

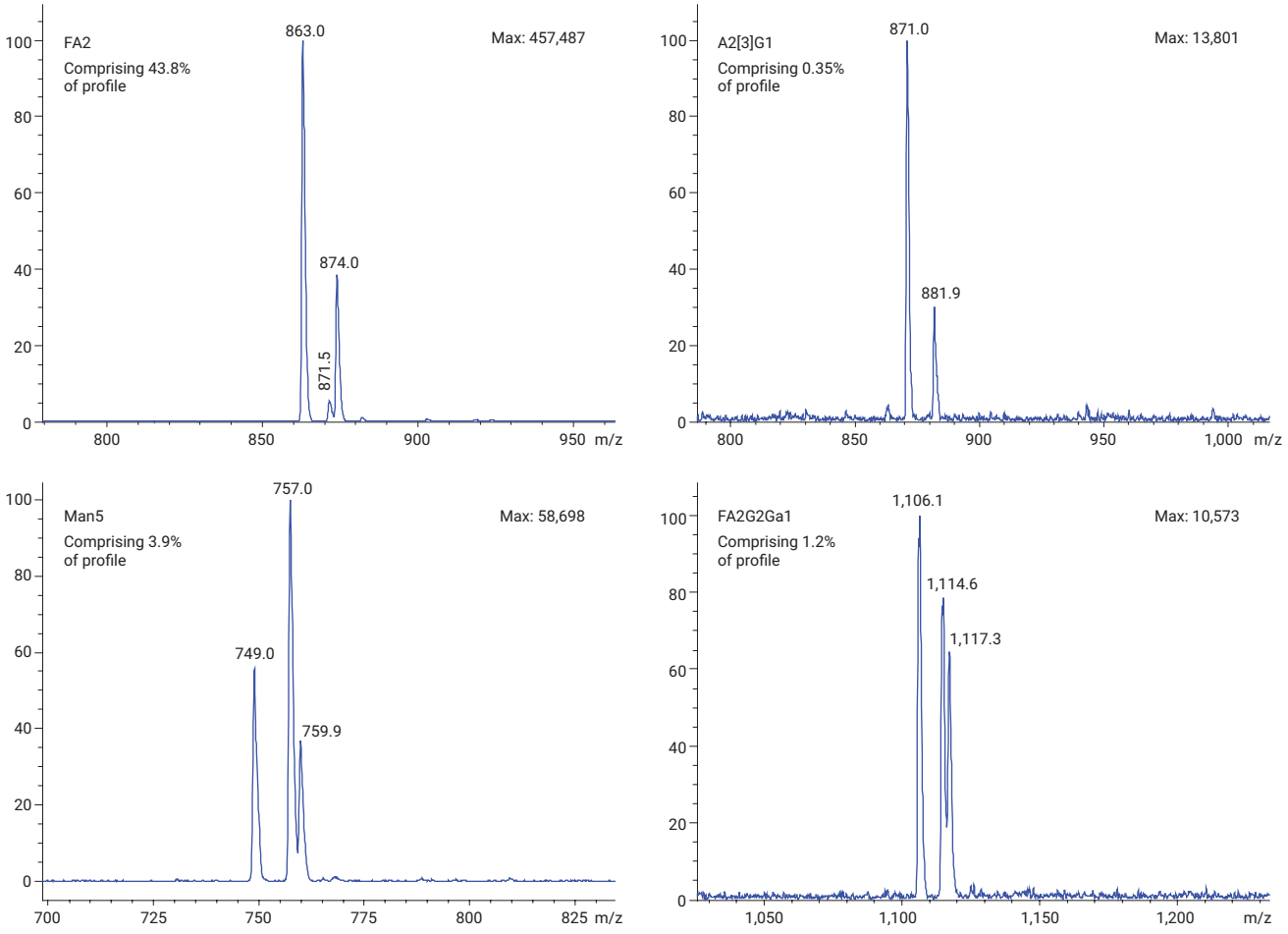


Figure 4. Mass spectra of four representative glycans from 3 µg of mAb at various levels of relative abundance. The signal for A2[3]G1, which makes up just 0.35 % of the glycan profile, is clearly observable with a high signal-to-noise ratio.

Table 1. Glycan Compositions

ID	Observed $[M+2H]^{2+}$	Proposed composition	Theoretical $[M+2H]^{2+}$	Proposed structure	
				Oxford	Alternative
1	659.9	F1+Core	659.774	F1M3	G0F-2GlcNAc
2	688.4	N1+Core	688.284	A1	G1-GlcNAc
3	748.9	H2+Core	748.798	M5	Man5
4	761.5	N1F1+Core	761.313	FA1	G0F-GlcNAc
5	790.0	N2+Core	789.824	A2	G0
6	842.4	H1N1F1+Core	842.340	FA1G1	G1F-GlcNAc
7	863.0	N2F1+Core	862.853	FA2	G0F
8	871.0	H1N2+Core	870.851	A2[6]G1	G1
9	871.0	H1N2+Core	870.851	A2[3]G1	G1'
10	923.6	H2N1F1+Core	923.366	-	-
11	944.0	H1N2F1+Core	943.879	FA2[6]G1	G1F
12	944.0	H1N2F1+Core	943.879	FA2[3]G1	G1F'
13	952.0	H2N2+Core	951.877	A2G2	G2
14	964.7	N3F1+Core	964.393	FA2B	G0FB
15	991.9	H5+Core	991.877	M8	Man8
16	996.1	H1N1Sg1F1+Core	995.885	FA1G1Sg1	G1Sg1F-GlcNAc
17	1025.1	H2N2F1+Core	1024.906	FA2G2	G2F
18	1025.1	H2N2F1+Core	1024.906	FA2G1Ga1	G1F+αGal
19	1045.6	H1N3F1+Core	1045.419	FA2[6]B1G1	G1FB
20	1045.7	H1N3F1+Core	1045.419	FA2[3]B1G1	G1FB'
21	1089.5	H1N1F1S1+Core	1089.427	FA1G1S1	G1S1F-GlcNAc
22	1097.7	H1N2F1Sg1+Core	1097.425	FA2G1Sg1	G1Sg1F
23	1106.1	H3N2F1+Core	1105.932	FA2G2Ga1	G2F+αGal
24	1106.1	H3N2F1+Core	1105.932	FA2G2Ga1 iso	G2F+αGal'
25	1126.5	H2N3F1+Core	1126.446	FA2BG2	G2FB
26	1170.4	H2N2F1S1+Core	1170.454	FA2G2S1	A1F
27	1170.4	H2N2F1S1+Core	1170.454	FA2G2S1 iso	A1F iso
28	1178.7	H2N2F1Sg1 + Core	1178.451	FA2G2Sg1	Ag1F
29	1187.1	H4N2F1+Core	1186.959	FA2G2Ga2	G2F+(αGal)2
30	1207.7	H3N3F1+Core	1207.472	FA2BG2Ga1	G2FB+αGal
31	1259.4	H3N2F1Sg1+Core	1259.477	FA2G2Sg1Gal1	Ag1F+αGal
32	1316.2	H2N2F1S2+Core	1316.001	FA2G2S2	A2F

Result tables

Peaks in the FLD chromatograms were reported as relative sum % of the total glycan profiles. Figure 5 shows the results.

Some of the profiles contained peaks representing coeluting glycan structures. These coelutions involved minor glycans, and quantitation based on FLD alone would report the peak area as the combined contribution of two structures.

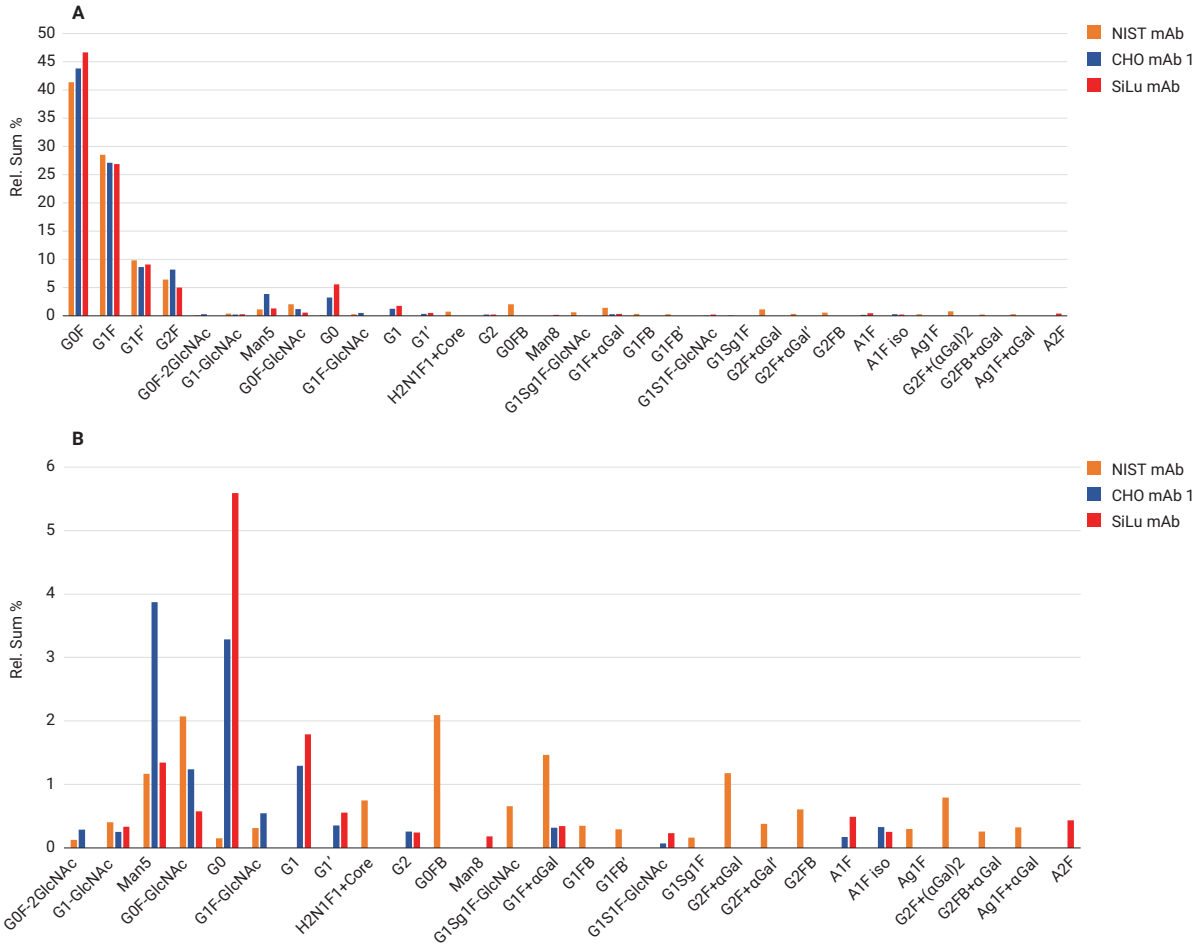


Figure 5. A) Relative abundance of N-glycans in the three mAb samples.
B) Zoomed-in chart showing only the minor components.
Minor glycans were detected easily at less than 1 %.

For analysts who need accurate quantitation of these low abundance coeluting structures, we propose using the LC/MSD XT data to supplement the FLD. The mass spectrum can be integrated across the time period corresponding to the coeluting FLD peaks. The FLD area can then be apportioned to the different structures based on the combined relative intensity of the doubly charged N-glycan ions in this spectrum. We have previously shown that the MS signals for InstantPC labels closely correspond to their true abundance as defined by fluorescence intensity (see 5991-6958EN).

Conclusion

The Agilent LC/MSD XT based on single quad technology can be hyphenated online to a typical UHPLC HILIC-FLD system to provide the option of mass spec-based identification for every peak in every sample. Using an Agilent Jet Stream ion source in combination with the InstantPC glycan tag provides ample sensitivity to detect MS signals for both major and minor peaks in typical antibody samples without needing to concentrate the sample. For typical monoclonal antibody samples, this allows confident assignment of glycan structures. While this application note used the traditional approach of basing relative quantitation on robust fluorescence detection, the MS data can be used to assist quantitation in the case of coeluting peaks.

Application Note

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Comparison of Relative Quantification of Monoclonal Antibody N-glycans Using Fluorescence and MS Detection

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Introduction

This application note describes the analysis of monoclonal antibody N-glycosylation using a novel instant mass tag (InstantPC) that permits detection using both fluorescence and mass spectrometry (MS). We have optimized a HILIC separation method for the purpose of comparing the two detection methods. Our results indicate that fluorescence and MS relative quantification of InstantPC-labeled glycans is highly similar. The sample prep procedure was conducted in quadruplicate for two different samples, and the results showed outstanding reproducibility with low RSDs even for minor components. The high MS sensitivity afforded by the InstantPC label facilitates identification of unknown glycans using accurate mass and tandem MS.

Introduction

Monoclonal antibodies (mAbs) are modified by N-glycans during biosynthesis in cell culture. Typical mAbs contain two N-glycosylation sites, one in each of the Fc regions of the molecule. Some mAbs contain additional glycosylation sites, including N- or O-glycosylation in the Fab region. Glycans can affect the function of the mAb, so it is important to monitor the glycosylation profile using appropriate analytical methods.

Popular methods for glycan analysis involve NMR, CE-LIF, HPLC with fluorescence detection (FLD), and more recently, LC/MS. Both CE-LIF and HPLC-FLD require that the glycans are labeled with a dye to permit optical detection. Conventionally, the dyes that have been used also increase the ionization efficiency of glycans in comparison to the unlabeled species, but only to the point where the most abundant compositions can be detected using MS. More recently, a novel dye (InstantPC from Prozyme Inc., depicted in Figure 1) has been developed, which moderately improves fluorescence activity and greatly improves ionization efficiency for MS analysis. Using such a tag, researchers can now use MS (in the form of accurate mass or tandem MS) for identification of glycans from LC separations. Furthermore, they have the option of relative quantification using MS rather than fluorescence detection.

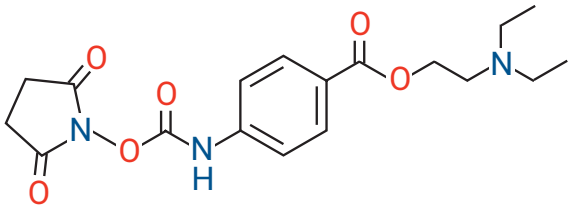

Figure 1. Diagram of InstantPC (ProZyme, Inc), an amine reactive instant label for fluorescence and MS detection of glycans.

This application note investigates the performance of InstantPC in the context of relative quantification of N-glycans released from two mAb preparations. Quadruplicate samples of the mAb samples were processed using the InstantPC kit from ProZyme, Inc. The samples were then separated by HILIC on a UHPLC system using FLD and MS detection. The LC separation conditions were optimized for maximum chromatographic separation. In doing so, the goal was to decrease the number of overlapping peaks that would otherwise not be discernable using FLD detection alone. As a result, we were able to compare the relative quantification results from the two detection methods for nearly all significant glycan structures. Accurate mass and tandem MS spectra were acquired for all glycan compositions, and were used for identification of the glycans present in the mAb preparations. Figure 2 shows the entire workflow.

Figure 2. Workflow used for identification and quantification of InstantPC-labeled N-glycans from mAbs.

Experimental

The Agilent LC/MS System used in this work comprised the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A)
- Agilent 1290 Infinity High Performance Autosampler (G4226A) with an Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Fluorescence Detector (G1321B)

MS system

Agilent 6550 iFunnel Q-TOF LC/MS system with dual-nebulizer AJS source

Columns

Agilent AdvanceBio Glycan Mapping column, 2.1 × 150 mm, 1.8 µm connected to a second AdvanceBio Glycan Mapping column, 2.1 × 100 mm

Software

- Agilent PCDL Manager (Version B.07.00 Build 7024.0) and Agilent Mass Profiler (Version B.07.01 Build 99.0)
- Agilent MassHunter Workstation Software, Version B.05.01, Build 5.01.5125.1

Solvents and samples

All reagents and solvents used were of the highest purity available.

Chromatographic conditions

Parameter	Value
Mobile phase A	50 mM ammonium formate pH 4.4
Mobile phase B	Acetonitrile
Gradient	Time (min) %B 0 75 32 69 48 60 48.5 25 50.5 25 52 75
Autosampler temperature	4 °C
FLD	Ex. 285 Em. 345
Injection	2 µL in 20 % DMSO (1 µg of IgG equivalent)
Column temperature	40 °C
Flow rate	0.4 mL/min

Results and Discussion

FLD chromatograms from both mAb 1 and mAb 2 revealed that each molecule was modified by a very similar set of glycoforms, as shown in Figure 3. Some structures have been annotated in the figures, and represented by symbols according to the guidelines of the Consortium for Functional Glycomics (CFG) [1].

Using accurate mass and tandem mass spectrometry information, FLD peaks were assigned to glycan compositions in the form:

HxNxFxSgx + Core

H = galactose or mannose, N = N-acetylglucosamine, F = fucose, Sg = N-glycolylneuraminic acid, and
Core = trimannosyl core common to all N-glycans.

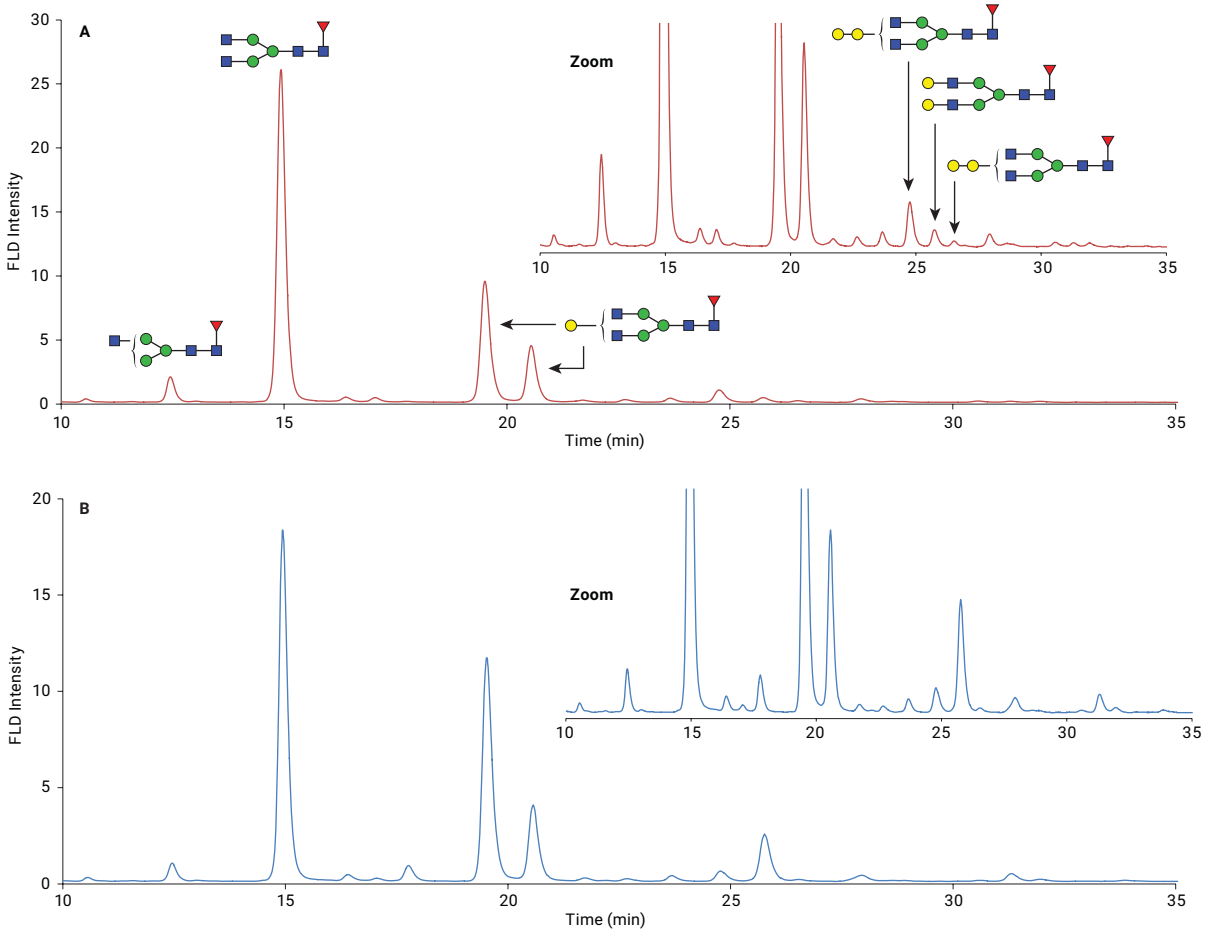

Figure 3. FLD chromatograms of InstantPC-labeled N-glycans released from mAb 1 and mAb 2.
A) FLD chromatogram for mAb 1.
B) FLD chromatogram for mAb 2.

Based on peak area from the FLD chromatograms, each composition was quantified as a relative sum percentage based on the total FLD area for all compositions. The results are shown as a histogram in Figure 4.

Overall, 21 glycan compositions were quantified based on the FLD data. The criteria used for inclusion in the FLD quantification were a relative abundance of 0.1 % or greater, and reasonable resolution from neighboring peaks.

One exception was the pair H2N1F1Sg1 + Core/H2N3F1 + Core. These coeluting compositions were abundant enough to merit inclusion in the FLD quantification. Therefore, the FLD signal area from this peak was divided into two portions according to the relative abundance of each as determined by MS.

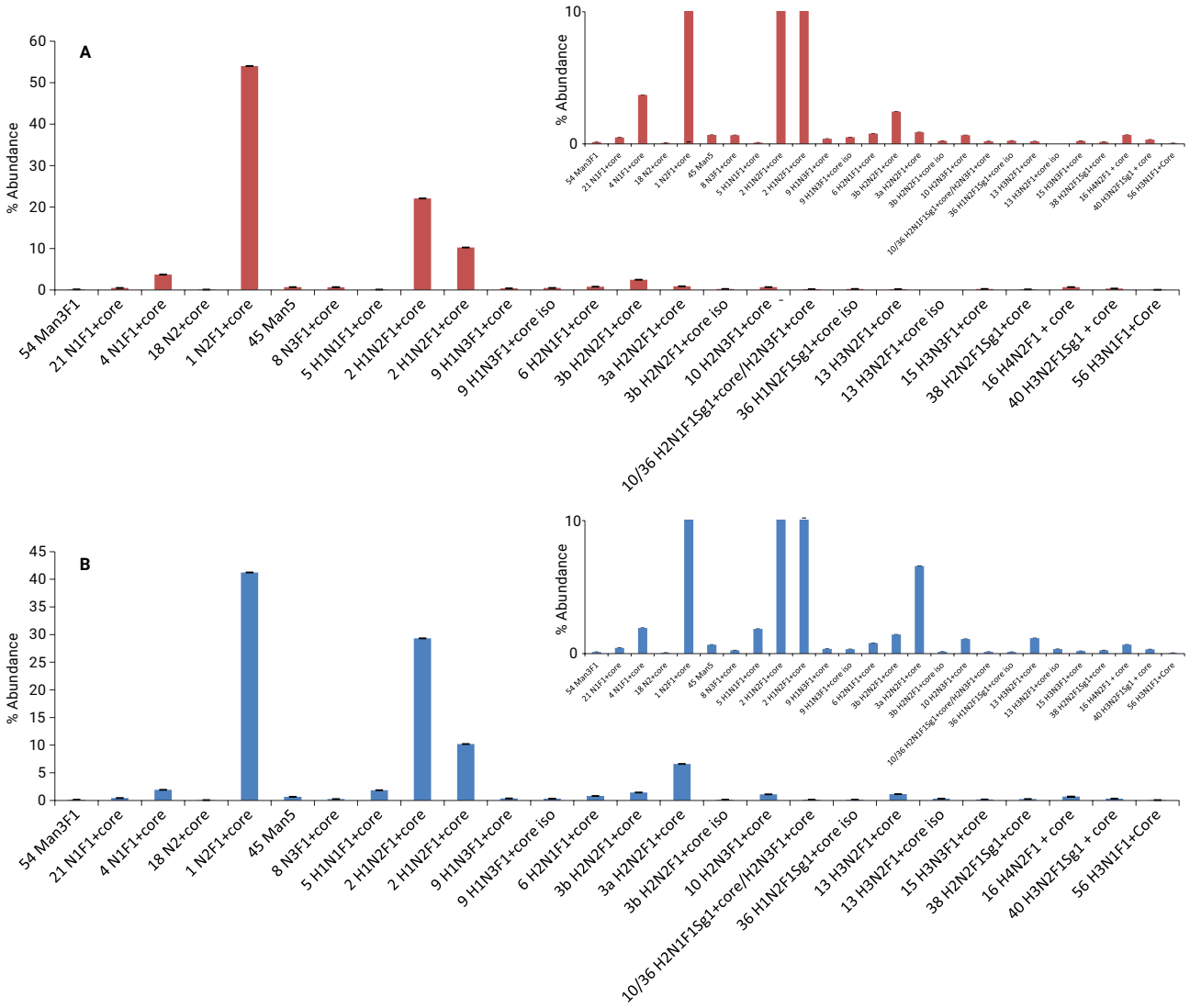

Figure 4. A) Relative FLD quantification of mAb 1 glycans. B) Relative FLD quantification of mAb 2 glycans. Error bars represent ± standard deviation of quadruplicates having gone through the entire workflow. Integration of FLD signals was performed using Agilent MassHunter Qualitative Analysis Software. Insets show the same data zoomed to better display components with <10 % relative abundance.

InstantPC imparts high ionization efficiency to N-glycans. Thus, it is possible to perform relative quantification using the peak area from extracted ion chromatograms from MS detection. To assess this possibility, we compared FLD chromatograms with ion chromatograms. Figure 5 shows that the FLD and MS chromatograms were highly similar. There is a corresponding MS peak for every FLD peak that was detected.

Encouraged by the high similarity seen in Figure 5, we performed relative quantification of glycans from mAb 1 and mAb 2 based on the MS data. Ion chromatograms for each feature (defined as a mass-retention time pair, which includes signals from all charge states and adducts) were created using Agilent Mass Profiler software. In this case, no lower threshold for detection was imposed. The features determined using Mass Profiler were identified using a Personal Compound Database (PCD) constructed for these experiments.

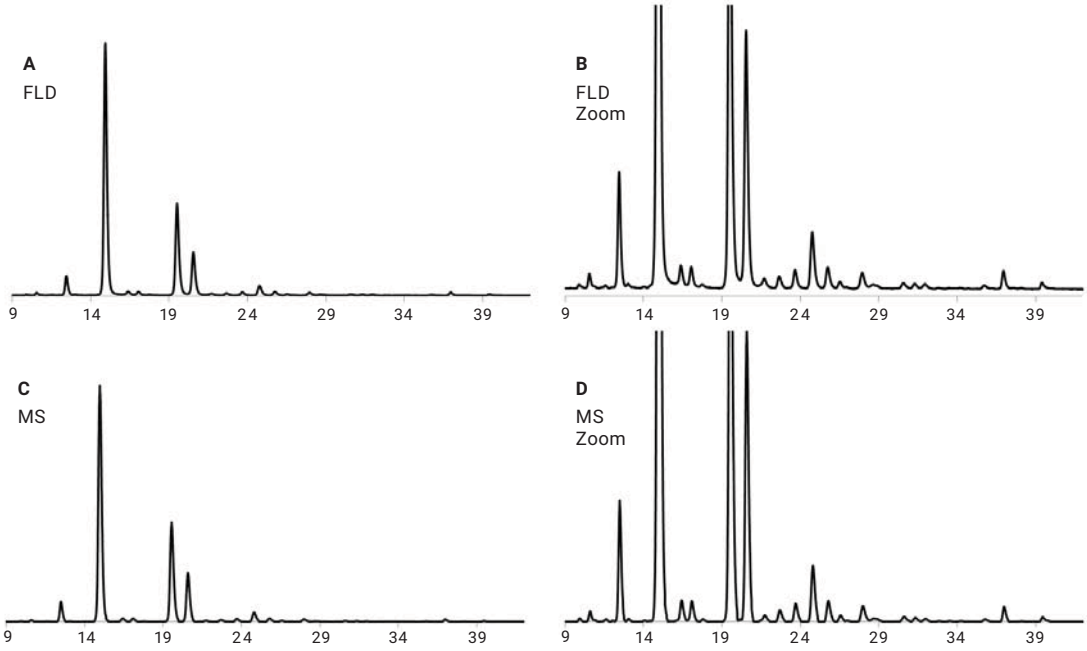

Figure 5. Comparison of FLD and MS chromatograms for mAb 1.
A) FLD chromatogram of mAb 1 glycans.
B) Zoom of FLD chromatogram of mAb 1 glycans.
C) MS chromatogram of mAb 1 glycans.
D) Zoom of MS chromatogram of mAb 1 glycans.

The PCD contains accurate mass and retention time information for mAb glycans. The database was constructed based on a combination of tandem MS information from the current work in addition to knowledge of glycan biosynthetic rules. Figure 6 shows an example of the utility of tandem MS for assigning glycan compositions. In particular, the example shown in Figure 6B illustrates a common case where mass alone may be insufficient for assignment of composition, due to the fact that the mass of NeuGc + fucose is isobaric with that of NeuAc + galactose. Tandem MS resolves the ambiguity, because the presence of the fragment ion at ***m/z*** 673 provides strong evidence that the structure contains an antenna with NeuGc.

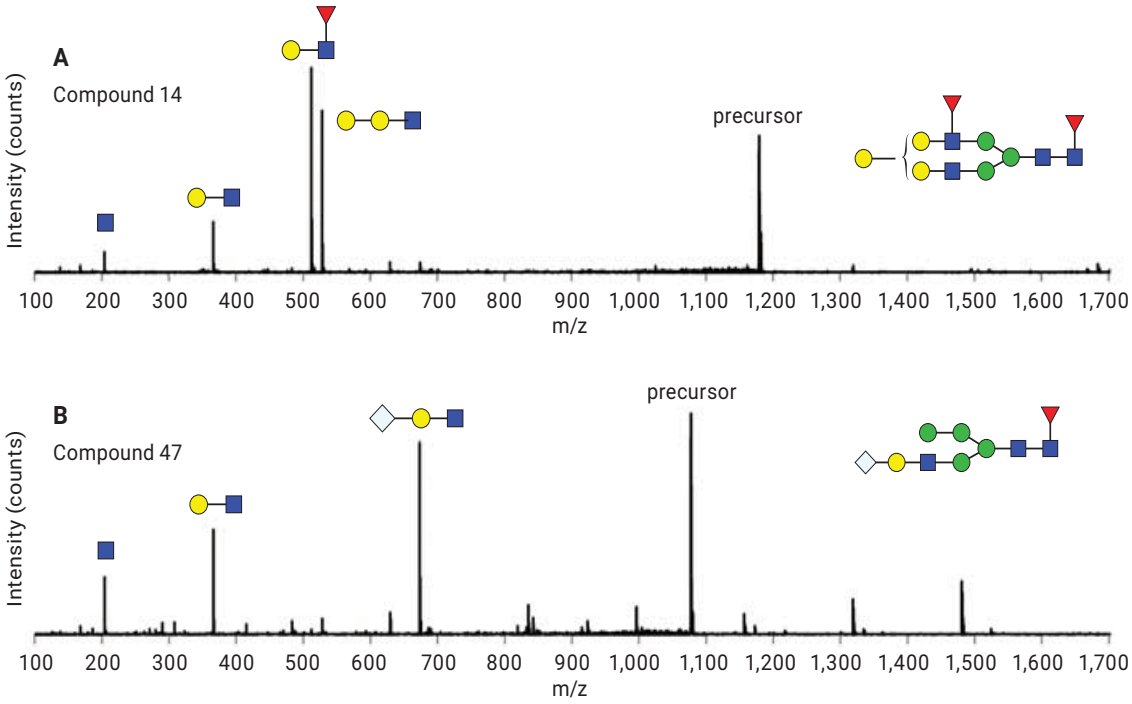

Figure 6. Tandem MS data were acquired for all glycans. MS/MS aided in compound identification when accurate mass was insufficient. The two examples above are consistent with gal-gal and outer arm fucose (A) and NeuGC (B) modifications.

Figure 7 shows the results of the MS-based quantification of mAb 1 and mAb 2 glycans.

As a result of the mass selectivity provided by Q-TOF detection, it was possible to quantify more compositions than from the FLD detection. In this case, a total of 35 compositions were quantified. The average RSD was 3.2 % for mAb 1 and 3.9 % for mAb 2 for all features independent of abundance. For those features equal to or greater than 0.1 % relative abundance, RSDs were 2.7 % and 3.4 % respectively.

Finally, we directly compared the relative quantification of glycans from mAb 1 and mAb 2 using FLD and MS. Figure 8 shows the results from each method plotted on a single histogram.

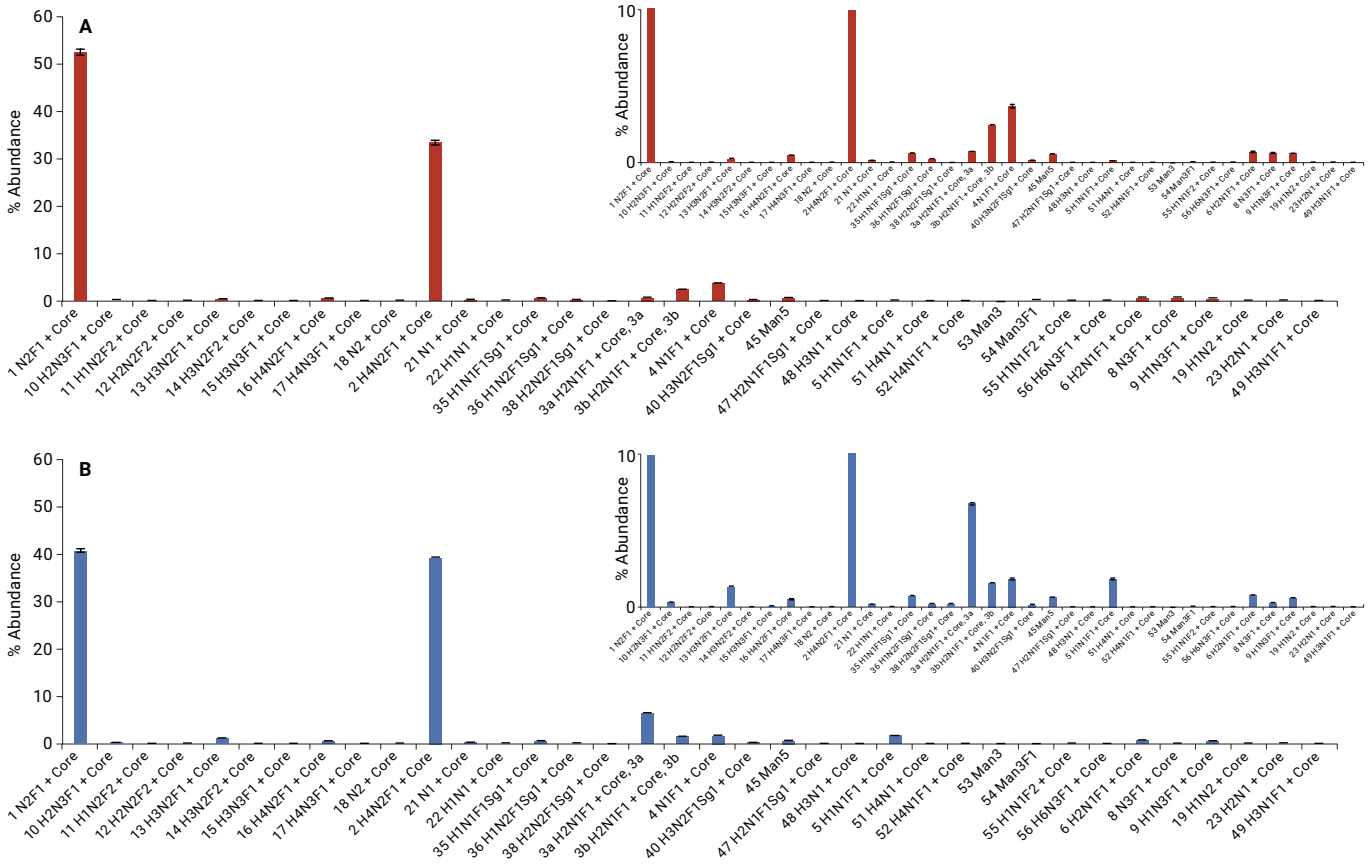

Figure 7. Relative MS quantification of InstantPC labeled N-glycans released from mAb 1 and mAb 2.
A) Relative MS quantification of mAb 1 glycans.
B) Relative MS quantification of mAb 2 glycans.
Error bars represent ± standard deviation of quadruplicates having gone through the entire workflow.
Insets show the same data zoomed to better display components with <10 % relative abundance.

Conclusion

As shown in Figure 8, the relative quantification results from FLD and MS were highly similar. Some small differences in the results from the two methods can be explained by the different numbers of compositions quantified in the two methods (21 from FLD, 35 from MS). Based on the results of this study, the combination of Prozyme's InstantPC label and an Agilent LC/MS system provides the researcher with the capability to perform MS-based quantification of glycans from mAbs. Still, FLD will likely remain a gold standard detection method for this compound class. In that case, high quality Q-TOF MS data greatly facilitate peak assignment by offering accurate mass and tandem mass information for each of the InstantPC-labeled glycans detected using FLD.

Acknowledgements

We would like to thank NIST for providing the two mAb samples used in this work.

References

1. http://glycomics.scripps.edu/CFGnomenclature.pdf

For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.

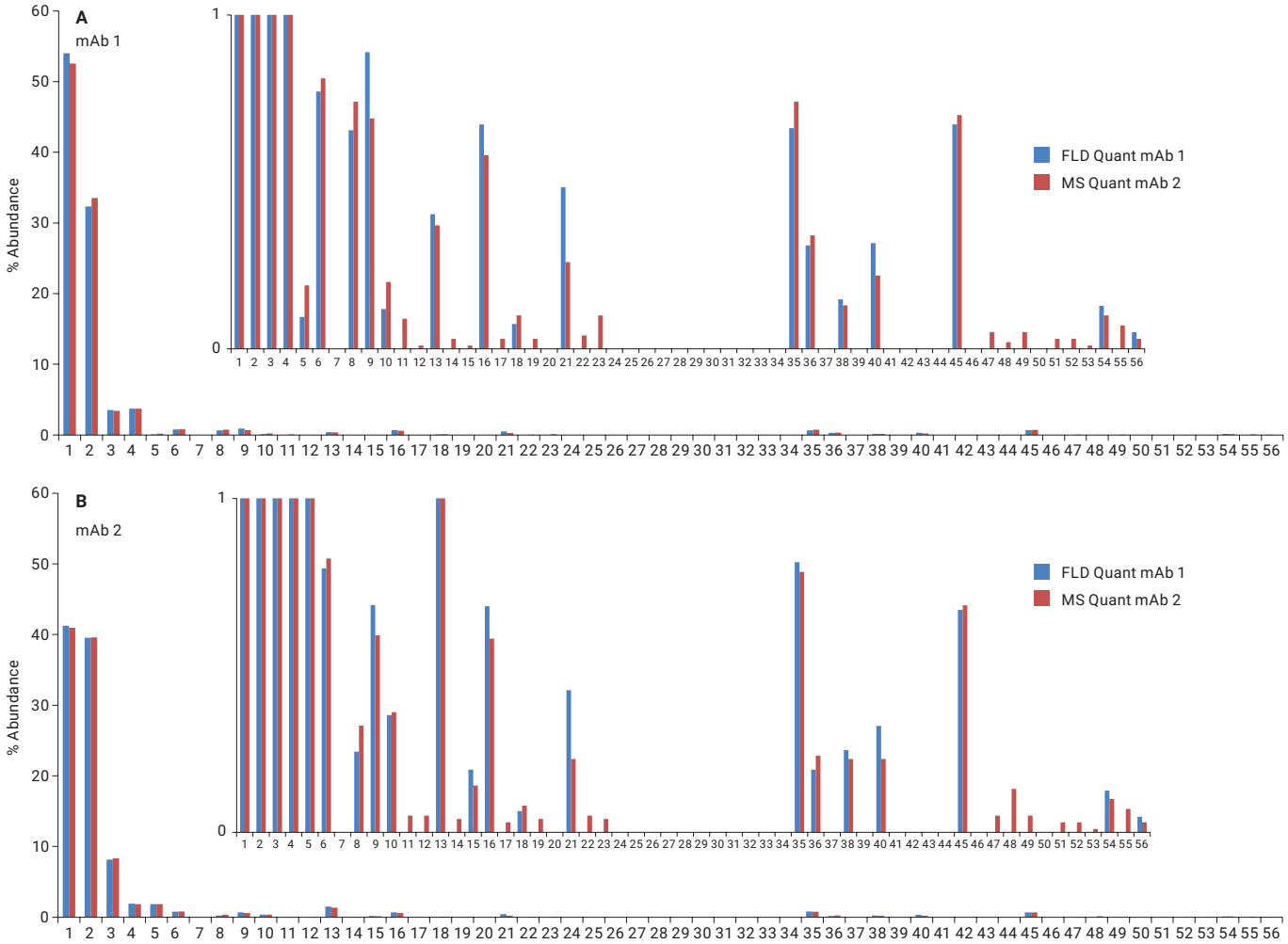

Figure 8. Comparison of fluorescence and relative MS abundance (area sum percentage) of InstantPC-labeled N-glycans from mAb 1 and mAb 2. The X-axis represents individual glycan compositions quantified in the study.

Application Note

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Preparation of Released N-Glycan Samples from Monoclonal Antibodies Using Agilent AdvanceBio Gly-*X* 2-AB Express for LC-Fluorescence Analysis

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Abstract

This Application Note describes the preparation of N-glycans from biotherapeutic glycoproteins for released glycan analysis. N-Glycan analysis is critical to the development and production of therapeutic proteins, as glycan composition may directly impact the safety and efficacy of the product. This protocol describes the use of the Agilent AdvanceBio Gly-*X* 2-AB Express kit for release of glycans using PNGase F, labeling through reductive amination, and cleanup of free dye within two hours rather than a full day or longer. The label used is 2-aminobenzamide (2-AB), valued for its well established use and consistency with large bodies of historical glycan analysis data.

Introduction

Glycosylation is a common feature of many biotherapeutic proteins that can affect pharmacokinetics, pharmacodynamics, and immunogenicity[1], and is frequently a critical quality attribute[2]. As such, biotherapeutic glycosylation must be carefully characterized and monitored throughout the development and production process.

Glycans are commonly derivatized prior to analysis, as they are not inherently UV-absorbing or fluorescent, and ionize poorly for MS detection. A handful of fluorescent labels have become widely used, including 2-AB and 2-AA, which modify released N-glycans through reductive amination[3]. Labels with both higher fluorescence and MS sensitivity have been introduced recently[4].

Earlier protocols for 2-AB labeling are often long, with multiple prolonged incubation periods. This not only consumes a large amount of the user's time, but also makes it impossible to obtain results and make decisions based on those results quickly. Deglycosylation was often incomplete without a long incubation period, therefore it became common to allow digestion to take place overnight. Reductive amination to affix a 2-AB label included a preceding step to dry the glycans prior to labeling, and labeling reactions were often allowed to incubate for hours. Additionally, older cleanup cartridges for removal of excess 2-AB reagent prior to LC analysis were cumbersome, and not suited for high-throughput or automated workflows.

The protocol for the Agilent AdvanceBio Gly-*X* 2-AB Express kit includes all the high-level steps for N-glycan sample preparation: denaturation, deglycosylation, labeling, and sample cleanup, as illustrated in Figure 1.

Experimental

Materials

HPLC grade acetonitrile was purchased from Sigma-Aldrich. Water was purified using a Milli-Q A10 water purification system (Millipore).

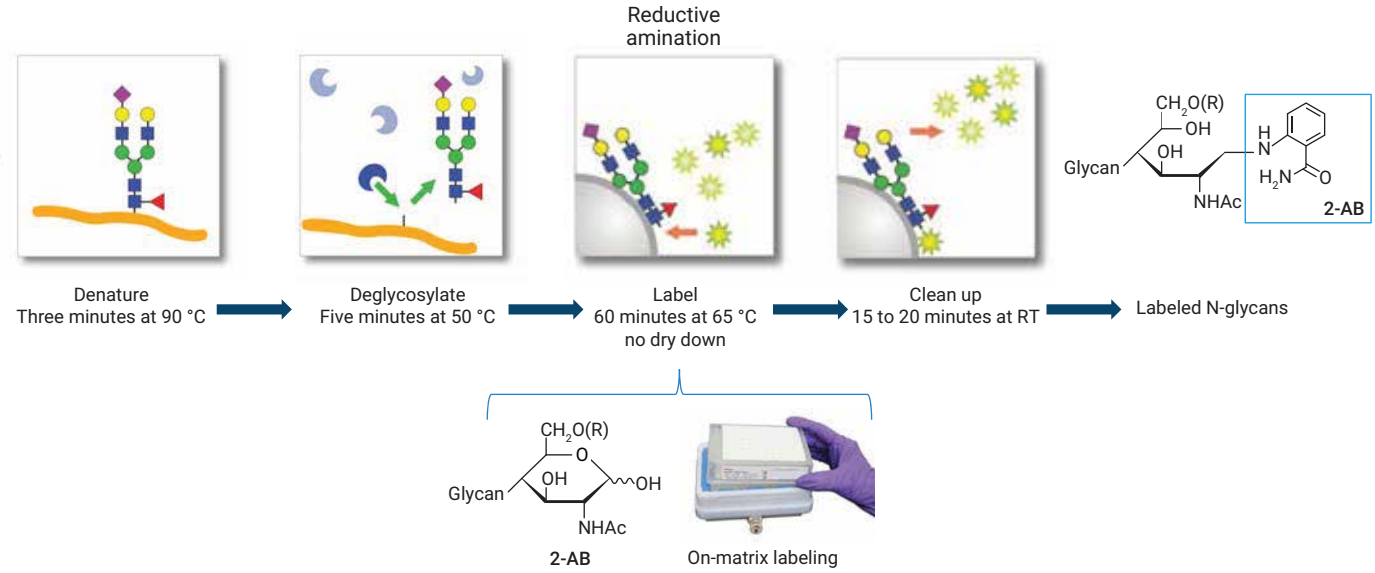

Figure 1. AdvanceBio Gly-*X* 2-AB Express workflow for release and labeling of N-glycans.

N-Glycan sample preparation

Labeled N-glycan samples were prepared using the AdvanceBio Gly-*X* 2-AB Express kit (p/n GX96-2AB). Figure 2 shows the kit components.

AdvanceBio Gly-*X* N-glycan sample preparation involves a series of enzymatic and chemical steps, beginning with denaturation of the target protein (Figure 3). A denaturing reagent is added, and the sample is incubated at 90 °C for three minutes. Effective unfolding of the protein allows for highly efficient, in-solution cleavage of N-glycans using the enzyme PNGase F in only five minutes[4]. PNGase F is specific to N-linked glycans, so only N-glycans are removed from the protein (Figure 4), while any O-linked glycans and nonenzymatic glycosylation remain attached to the protein.

The labeling and cleanup steps take place on a HILIC-based solid phase stationary support. Released N-glycans are converted to -OH form in solution prior to loading onto the stationary phase, followed by the 2-AB labeling reagents, and the phase is incubated for one hour at 65 °C (Figure 5). After labeling is complete, the excess reagents are rinsed away through a series of acetonitrile washes. The labeled N-glycans are then eluted with water (Figure 6). On-matrix labeling eliminates the need to dry the released glycans prior to 2-AB labeling.

Instrumentation

Samples were analyzed using an Agilent AdvanceBio Glycan Mapping column on an Agilent LC composed of:

- Agilent 1290 Infinity II high speed pump (G7120A)
- Agilent Infinity multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1260 Infinity fluorescence detector (G1321B)

Figure 2. Components of the AdvanceBio Gly-*X* 2-AB Express N-glycan sample preparation kit.

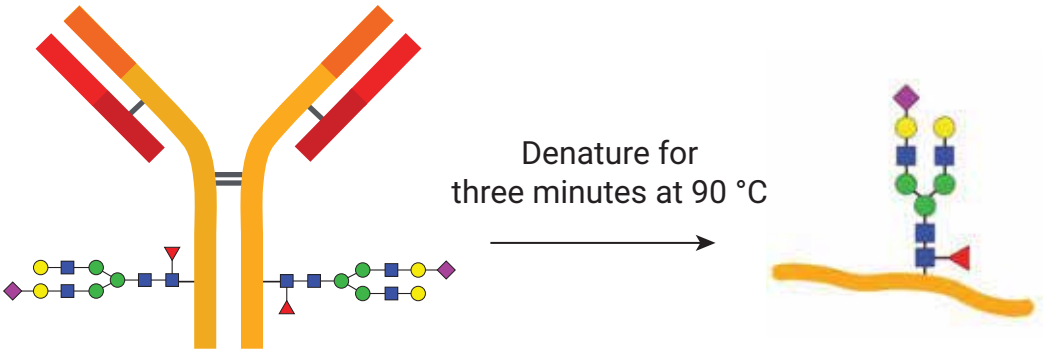

Figure 3. The sample protein is first denatured to effectively deglycosylate in the subsequent step.

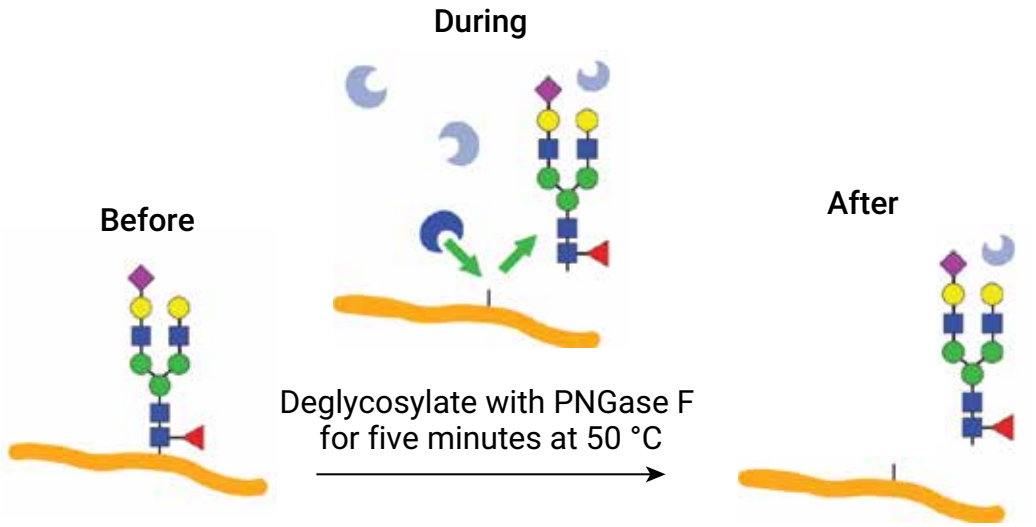

Figure 4. N-glycans are rapidly cleaved from the protein using PNGase F.

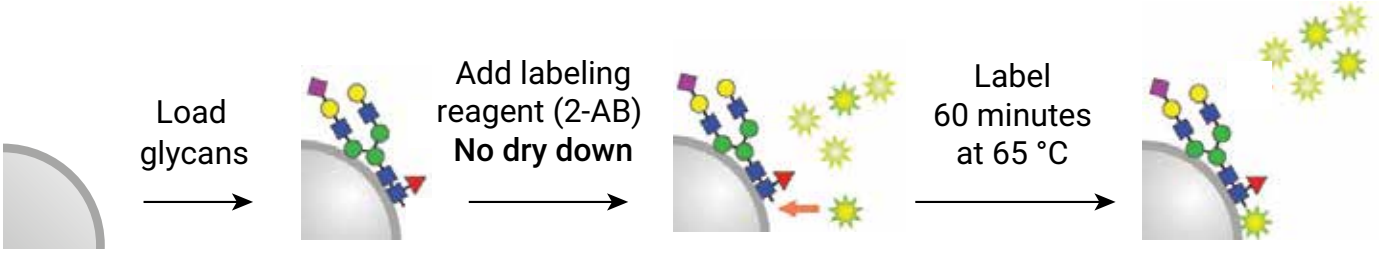

Figure 5. The sample is loaded onto a HILIC-based solid phase support on a vacuum-based cleanup plate.
The 2-AB labeling reagents are then added to the solid phase support and incubated for one hour. No dry-down step is necessary.

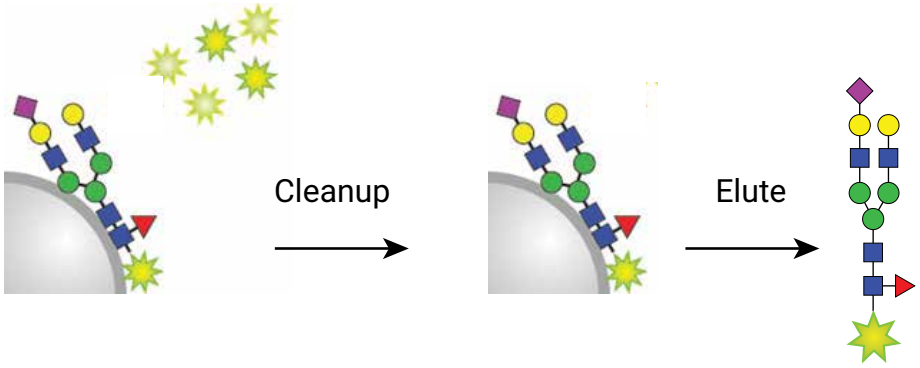

Figure 6. Excess labeling reagents are rinsed away, and labeled glycans are eluted from the solid stationary phase.

Software

- Agilent MassHunter Acquisition
- Agilent MassHunter Qualitative Analysis software

Results and discussion

MabThera and Enbrel 2-AB N-glycan samples were analyzed by LC/FLD. Figure 7 shows representative chromatograms. MabThera has a simpler glycosylation pattern, with Enbrel showing higher relative levels of sialylated glycans. Reproducibility of the sample preparation is of utmost importance, so samples may be compared across production lots. Variability measured needs to truly originate from changes in the sample, rather than as an artifact of sample handling or analysis.

Table 1. LC method.

Parameter	Value
Column	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)
Column Temperature	40 °C
Mobile Phase	A) 50 mM ammonium formate, pH 4.5 B) acetonitrile
Flow Rate	0.5 mL/min
Gradient Program	Time (min) %B Flow rate (mL/min) 0.0 82 0.4 2.0 82 0.4 2.5 77 0.4 48.0 62 0.4 49.0 40 0.4 51.5 40 0.4 52.0 82 0.4 54.0 82 0.6 58.0 82 0.6 58.5 82 0.4
Injection Volume	1 µL (equivalent to glycans from 0.4 µg of protein)
Detection	Agilent 1290 Infinity II FLD Excitation 260 nm Emission 430 nm

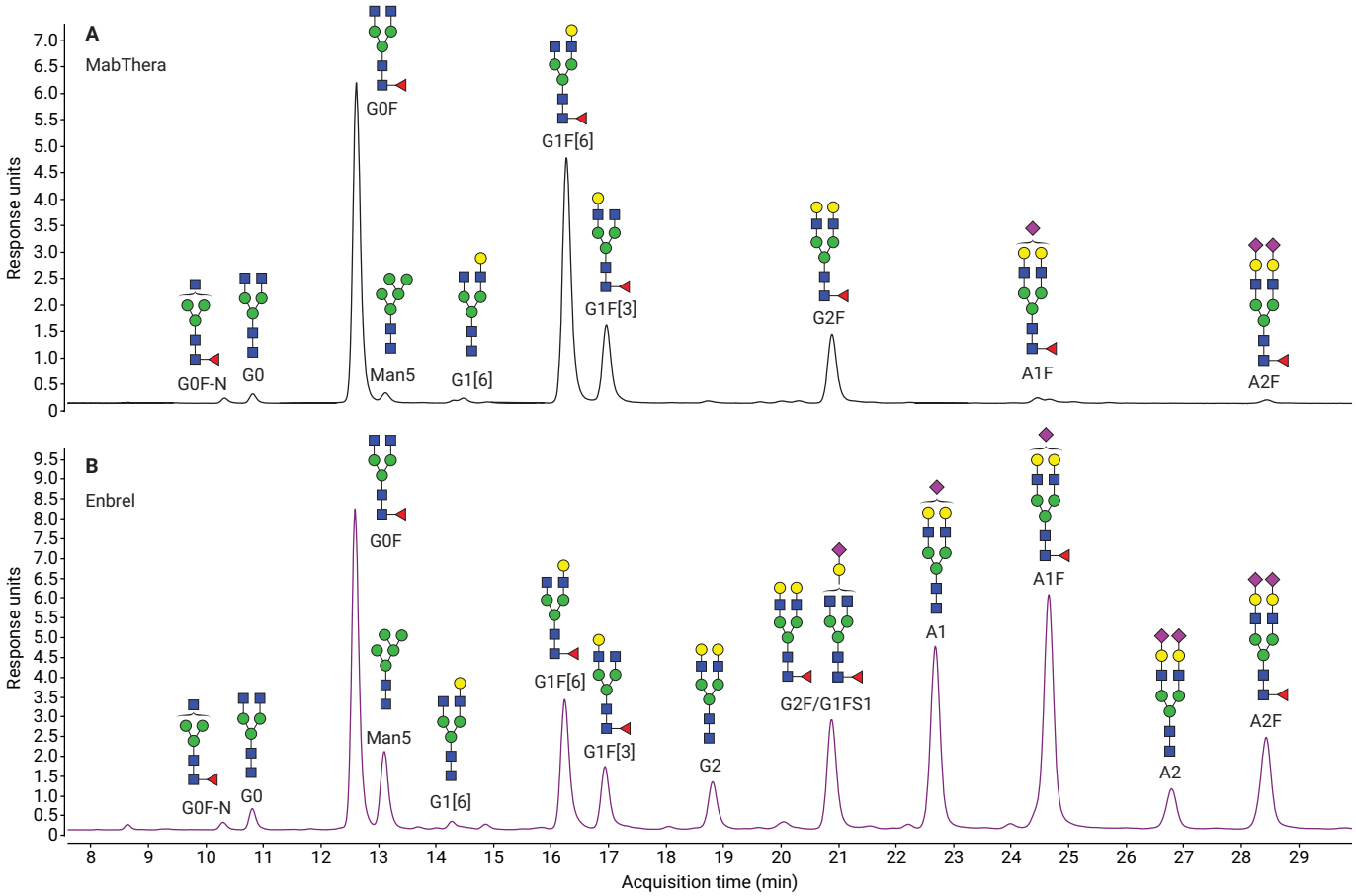

Figure 7. Representative chromatograms showing separation of 2-AB labeled N-glycans from A) MabThera and B) Enbrel.

Table 2 shows the relative percent area of the major N-glycan species detected in three preparations of a MabThera sample. The average percent area, along with standard deviation and relative standard deviation (%CV), are reported. The variations between sample preparations are all low, except for the lowest abundance glycans. Precision is more challenging near the limit of detection, so higher variation is to be expected for these peaks.

For any researcher to change sample preparation methods, the data obtained must be either equivalent, or in some way superior to the results of the previous method. With other labeling chemistries available, a major driver behind continuing to use 2-AB is the ability to compare results to older data obtained with other 2-AB labeling protocols. Figure 8 shows the relative percent area of the N-glycans detected in triplicate preparations of Enbrel. The relative abundances are very similar between the samples prepared using AdvanceBio Gly-*X* 2-AB Express and ProZyme GlykoPrep 2-AB, an earlier generation of 2-AB sample preparation available from ProZyme.

Table 2. Relative % area of major N-glycan species from three preparations of a MabThera sample.

		Relative % Area					
Glycan	RT	1	2	3	Average	Standard Deviation	%CV
G0F-N	10.32	0.56	0.57	0.57	0.57	0.01	1.02
G0	10.81	1.09	1.01	0.98	1.03	0.06	5.54
G0F	12.62	39.85	39.31	39.33	39.50	0.31	0.78
Man5	13.12	0.74	0.62	0.69	0.68	0.06	8.82
G1[6]	14.47	0.66	0.6	0.62	0.63	0.03	4.88
G1F[6]	16.27	34.65	34.81	34.67	34.71	0.09	0.25
G1F[3]	16.96	10.65	10.47	10.6	10.57	0.09	0.88
G2F	20.89	10.15	10.78	10.83	10.59	0.38	3.58
A1F	24.45	1.13	1.26	1.17	1.19	0.07	5.61
A2F	28.44	0.52	0.57	0.54	0.54	0.03	4.63

Conclusion

N-Glycan samples can be fully prepared for analysis within two hours with Agilent AdvanceBio Gly-*X* 2-AB Express, versus older methods that take a full day, including an overnight incubation and dry down prior to labeling. Data produced for a variety of glycoproteins are highly reproducible, and consistent with data obtained from older 2-AB sample preparation methods.

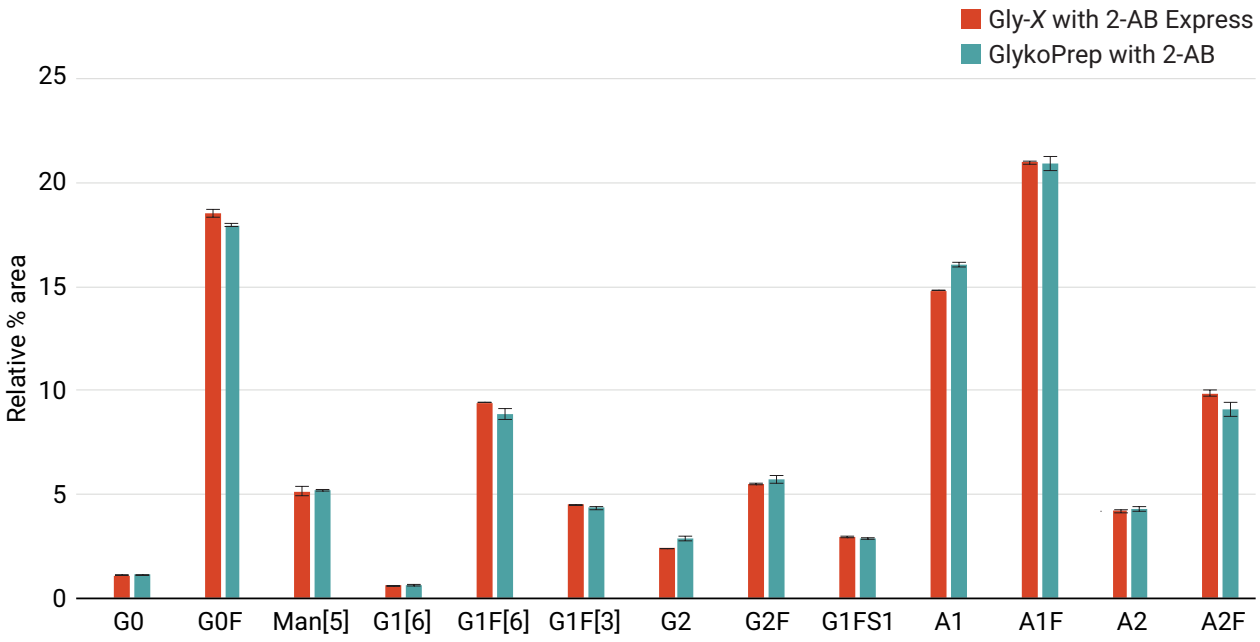

Figure 8. Samples produced using the AdvanceBio Gly-*X* 2-AB Express kit produce data equivalent to samples prepared using older methods, such as ProZyme GlykoPrep 2-AB, shown here for N-glycans from Enbrel. n = 3 for all data.

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