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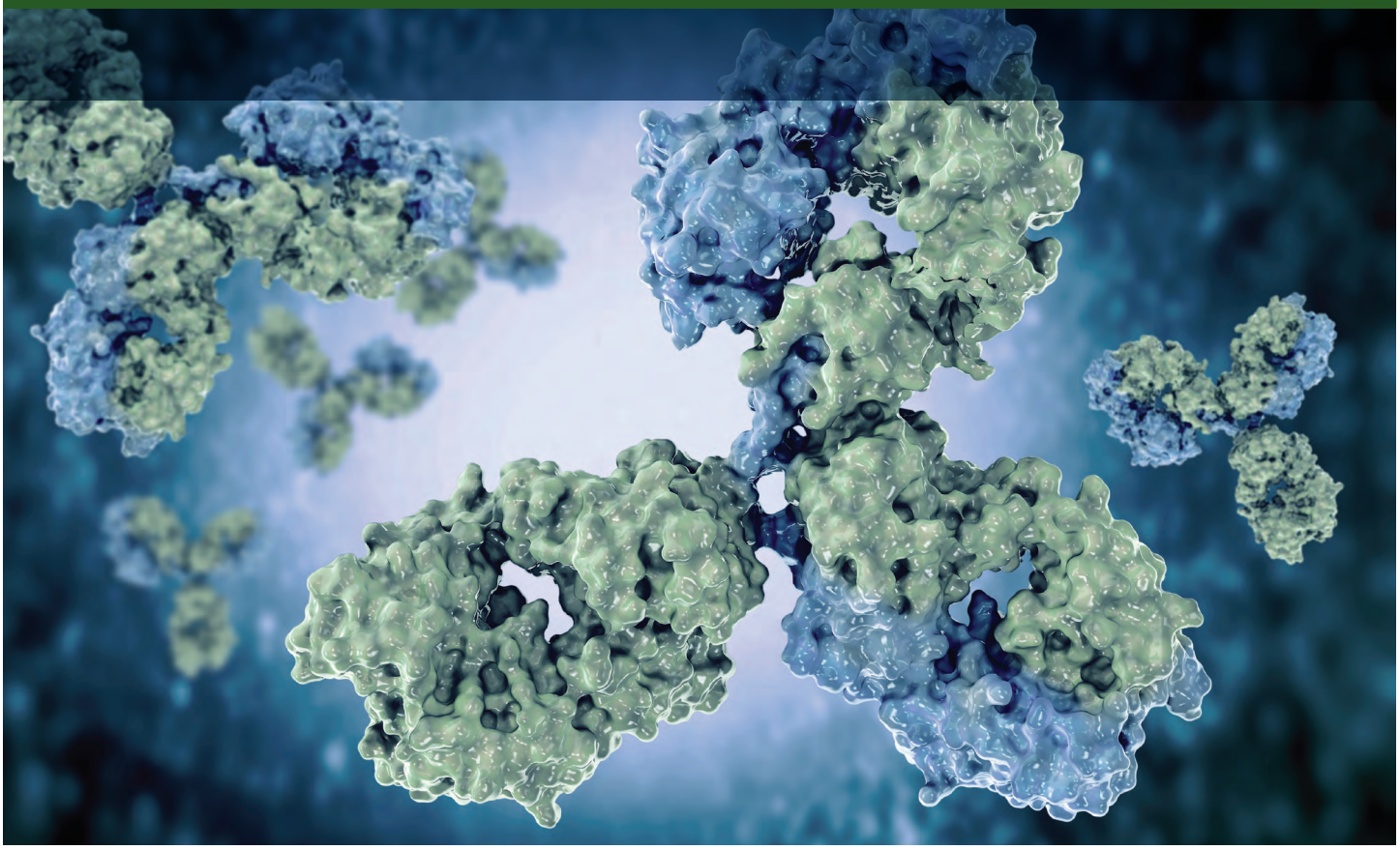
Agilent-NISTmAb

Intact & Subunit Analysis Using Reversed Phase

Agilent BioHPLC Columns
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.

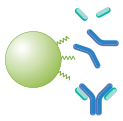
Intact and Subunit Analysis- Reversed Phase Chromatography

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.

Back To Contents Page



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.

Back To Contents Page

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.

Back To Contents Page

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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.

Back To Contents Page

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

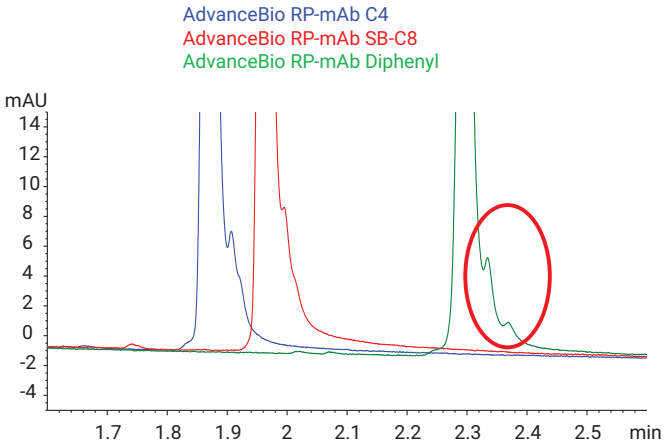
Suggested gradient for resolution

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

Back To Contents Page

Intact mAb Analysis

Fast and high-resolution separation

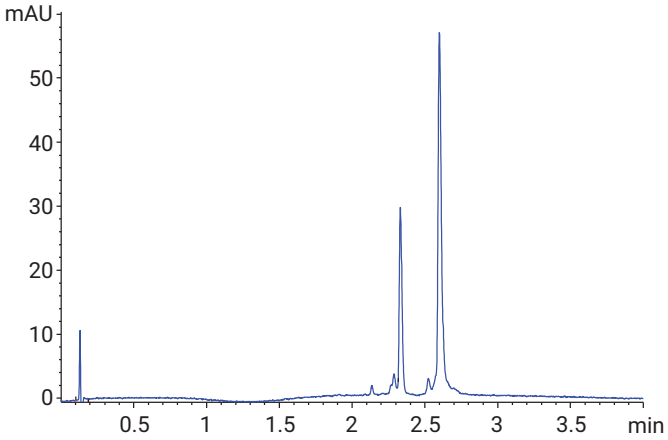


Conditions

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

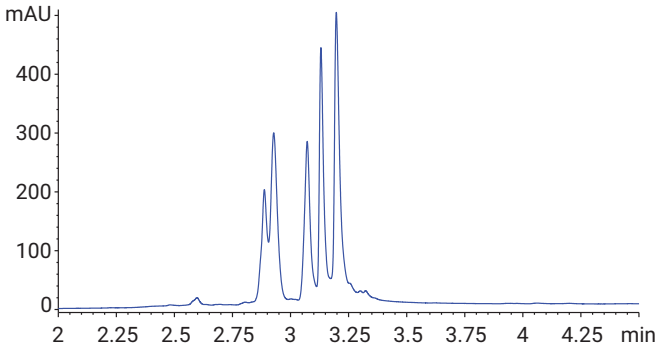
Chemical digestion – heavy chain/light chain



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



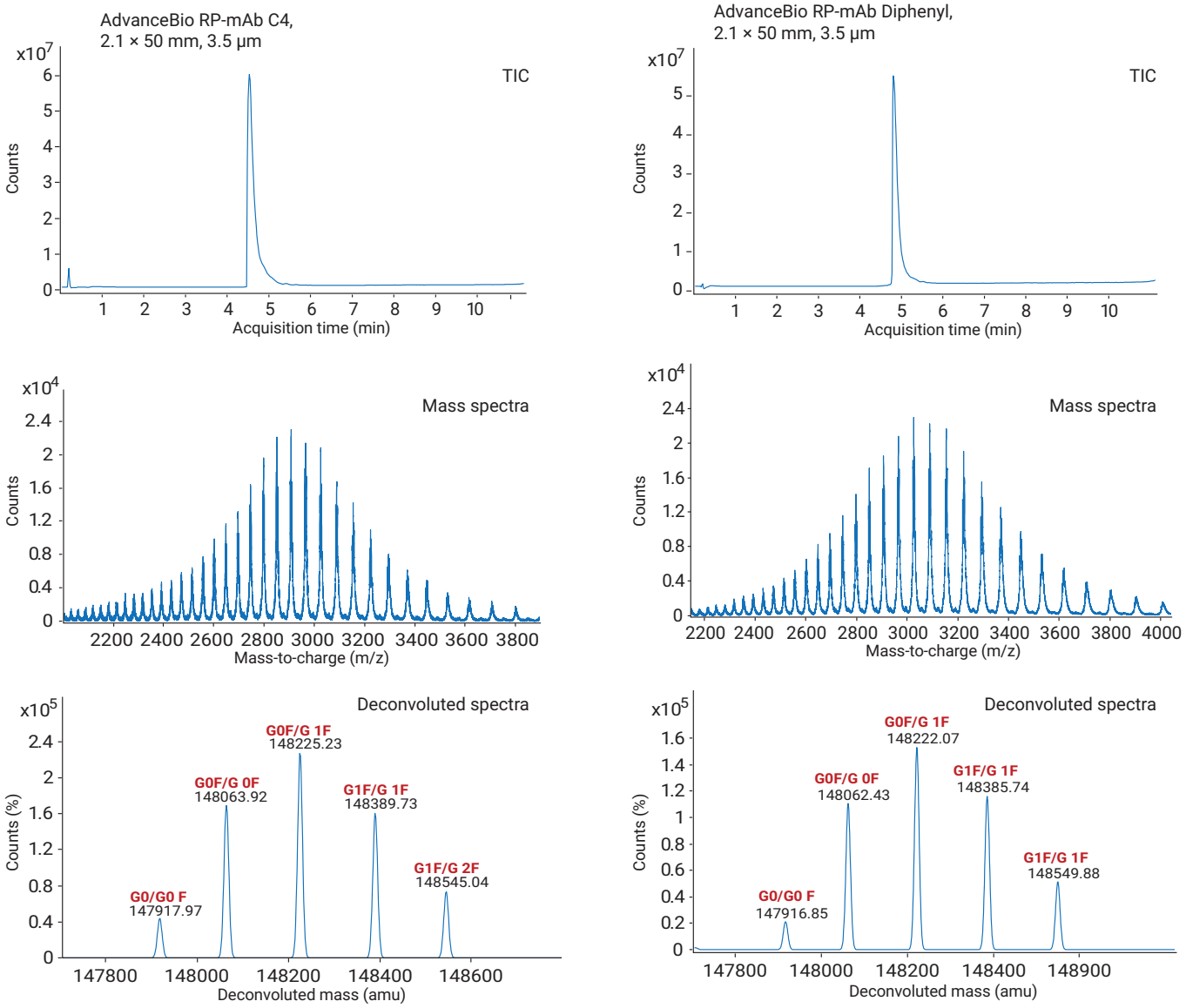
Conditions

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

Back To Contents Page

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

Back To Contents Page

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.

Back To Contents Page

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

Back To Contents Page

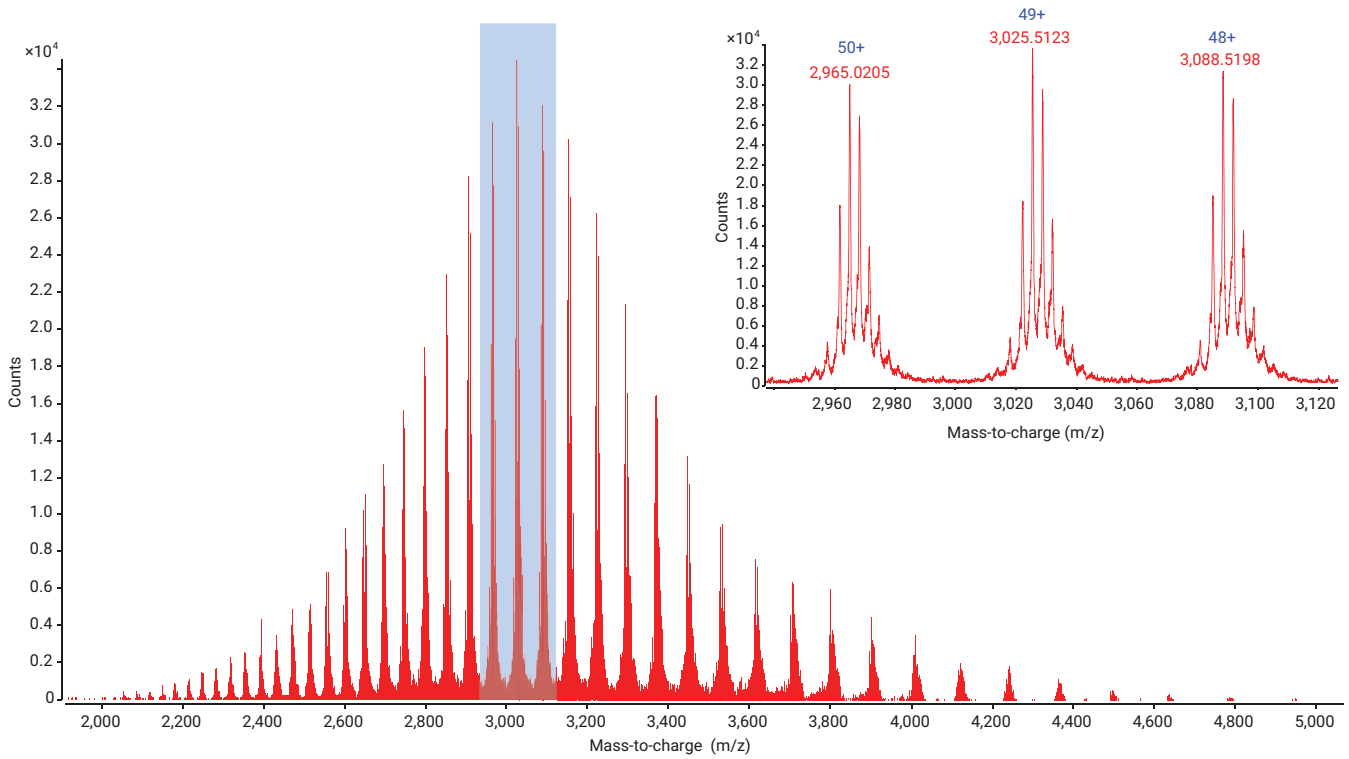

Figure 2. Intact NISTmAb analysis (0.5 µg injection).

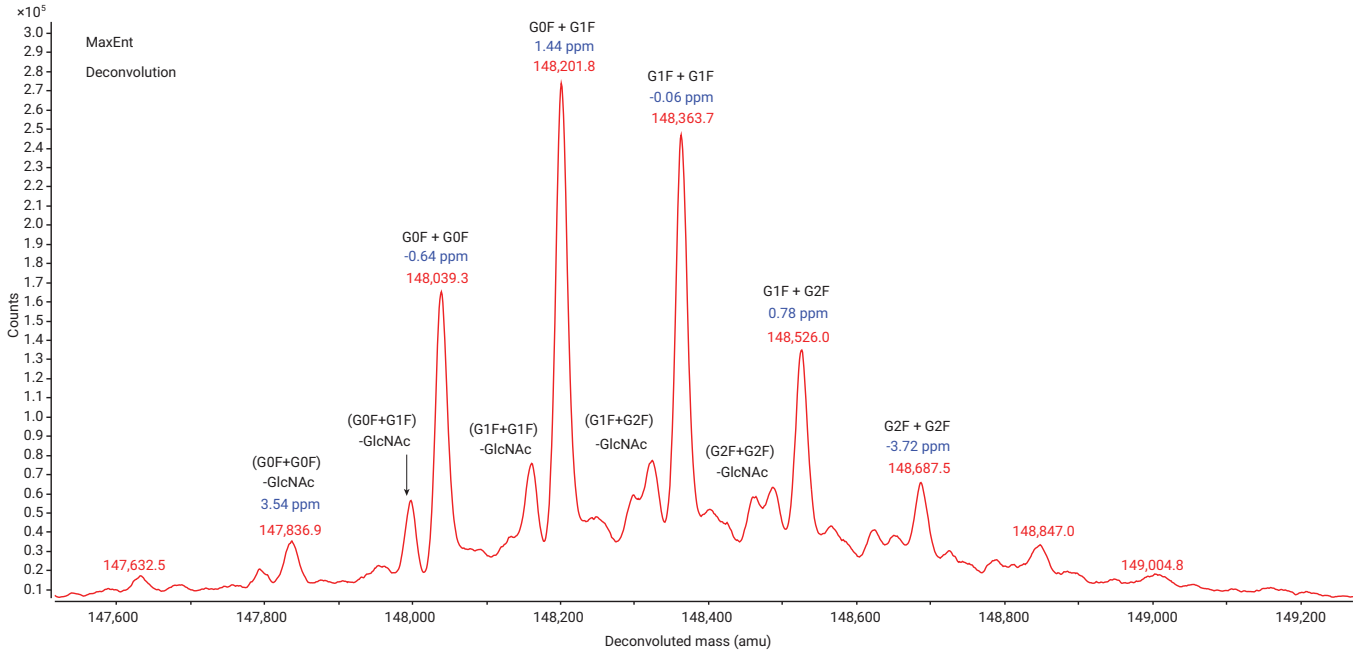

Figure 3. MS Deconvolution of intact NISTmAb (0.5 µg injection).

Back To Contents Page

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

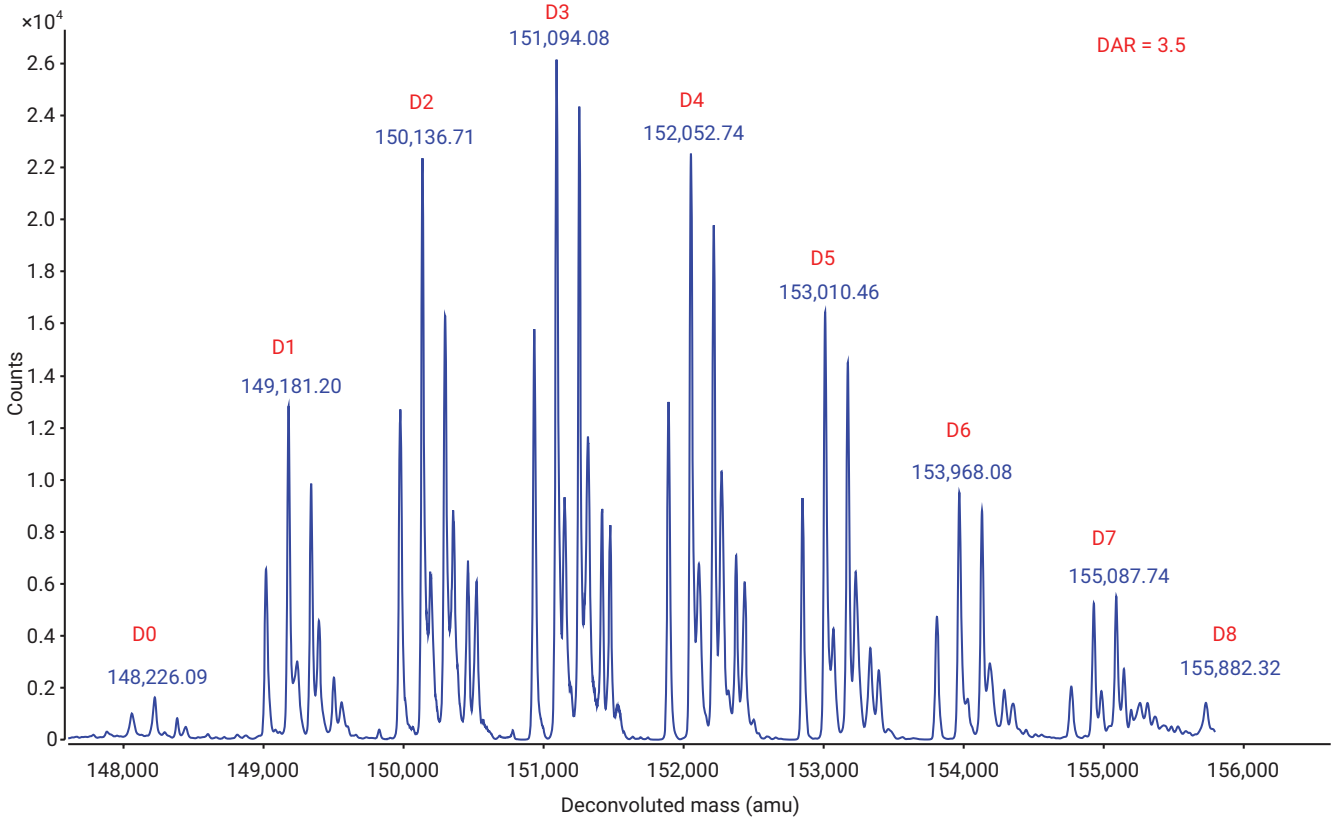

Figure 4. Intact T-DM1 analysis (0.5 µg injection).

Back To Contents Page

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.

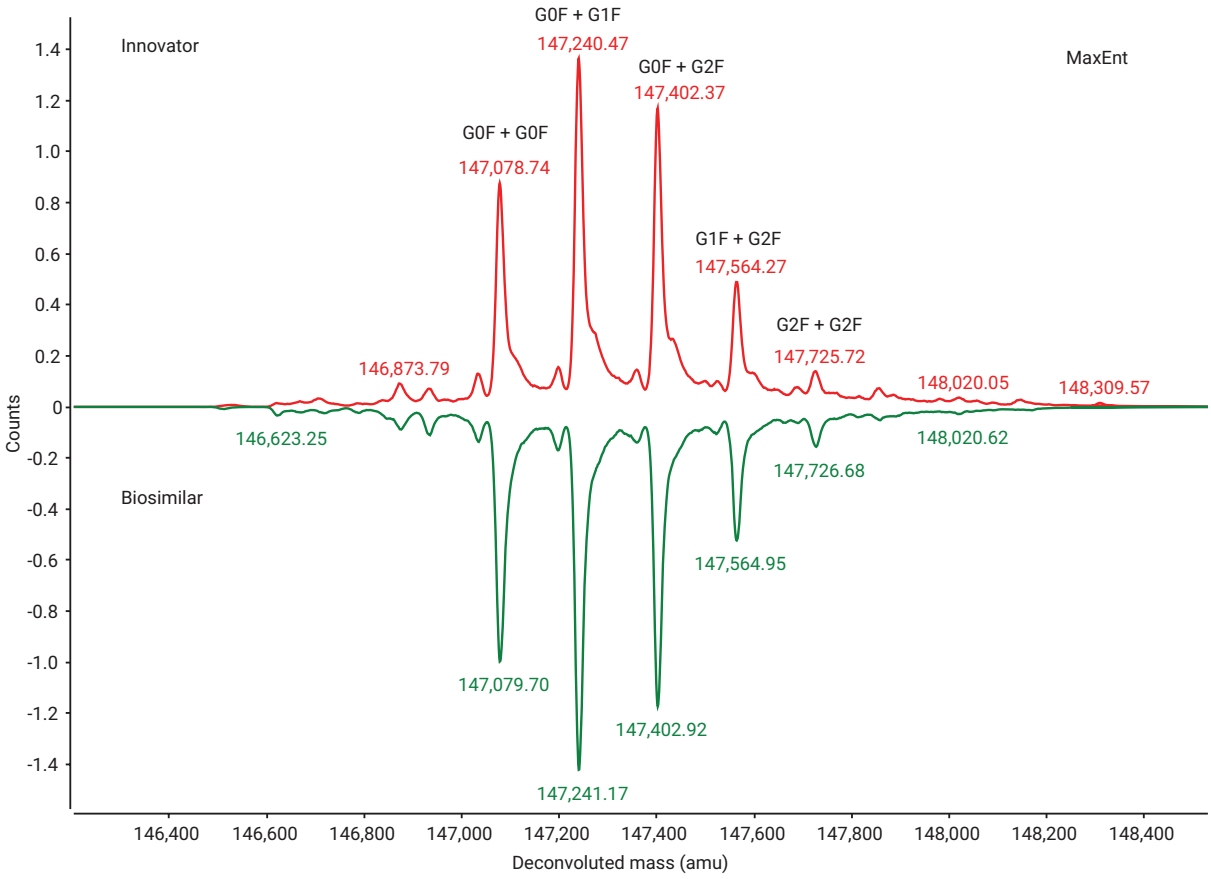

Figure 5. Intact Rituximab analysis (Innovator versus Biosimilar) (0.5 µg).

Back To Contents Page

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.

Back To Contents Page

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).

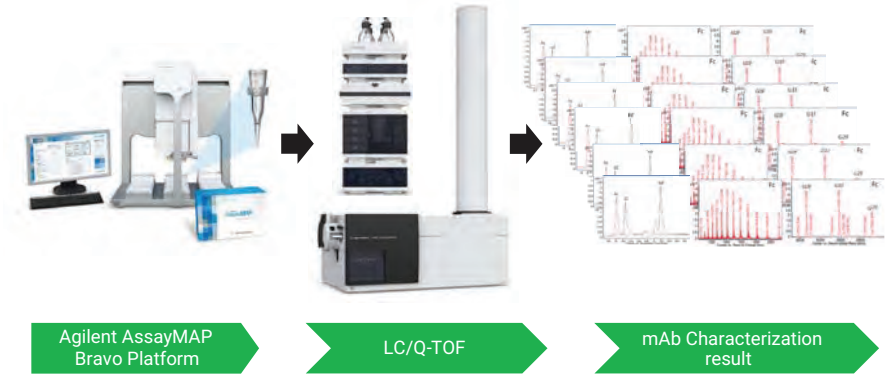

Figure 1. Integrated workflow for automated antibody characterization using Agilent AssayMAP Bravo.

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.

Back To Contents Page

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.

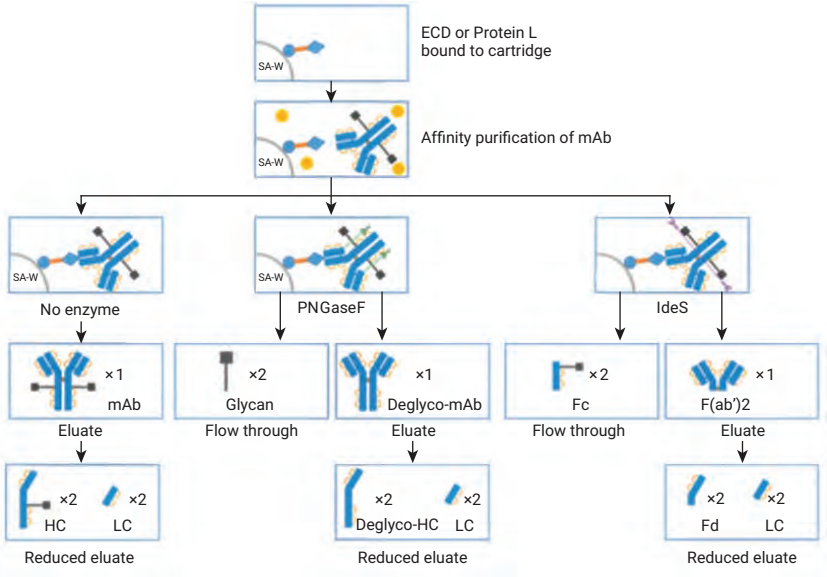

Figure 2. Antibody sample preparation performed by the Agilent AssayMAP Bravo.

Back To Contents Page

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)	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			

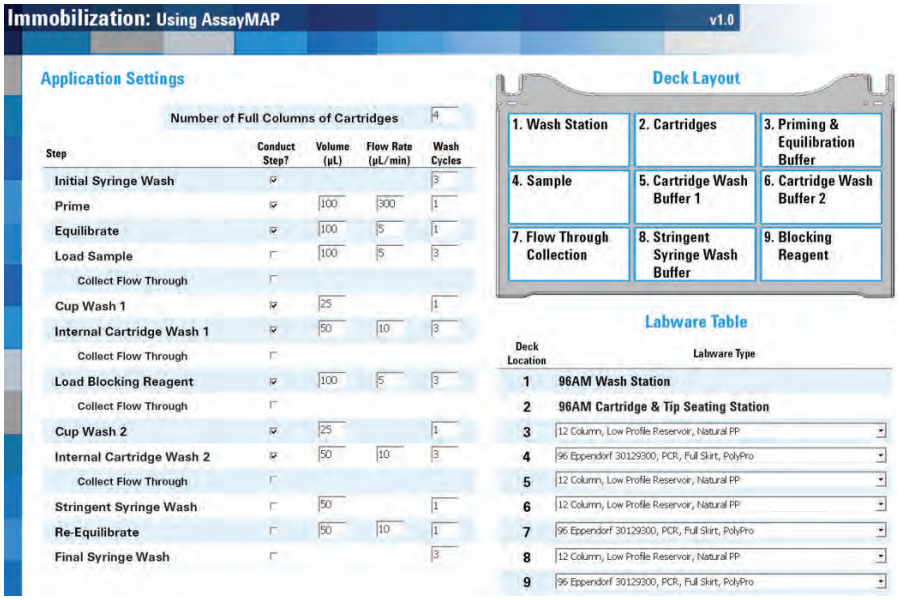

Figure 3. User interface for Agilent AssayMAP Bravo, showing the setup screen for the Immobilization application on AssayMAP Bravo used to generate antibody affinity cartridges.

Back To Contents Page

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.

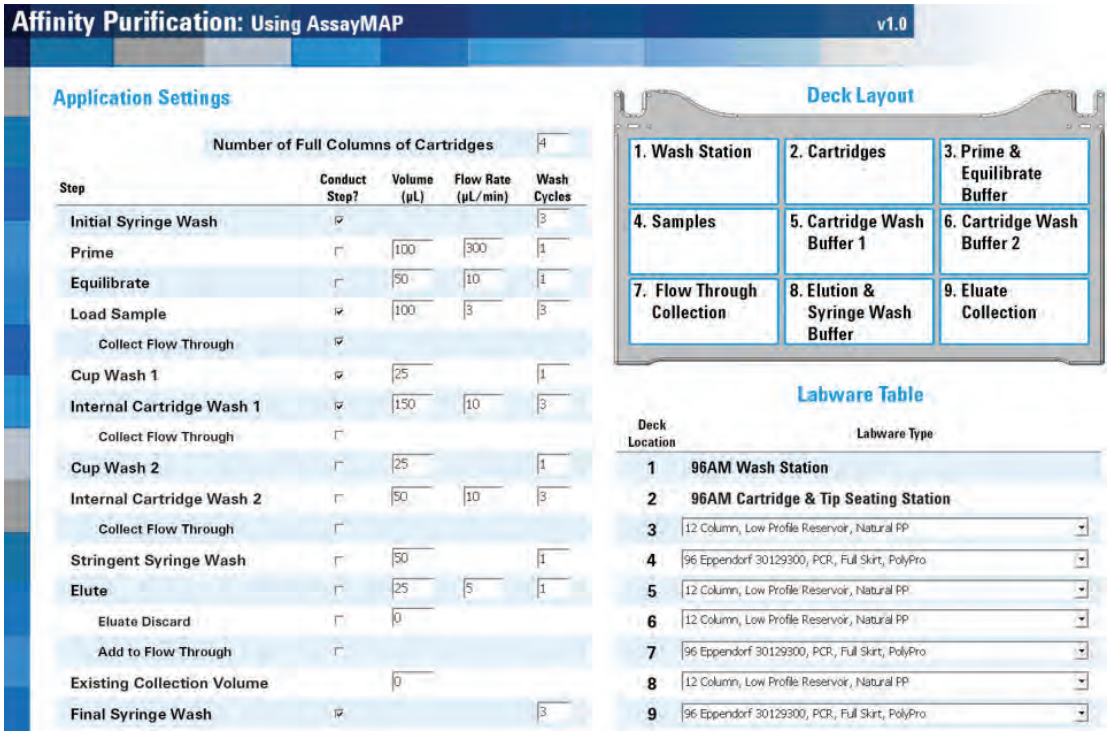

Figure 4. The setup screen for the Affinity Purification application on Agilent AssayMAP Bravo used to purify antibody from cell culture supernatant.

Back To Contents Page

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).

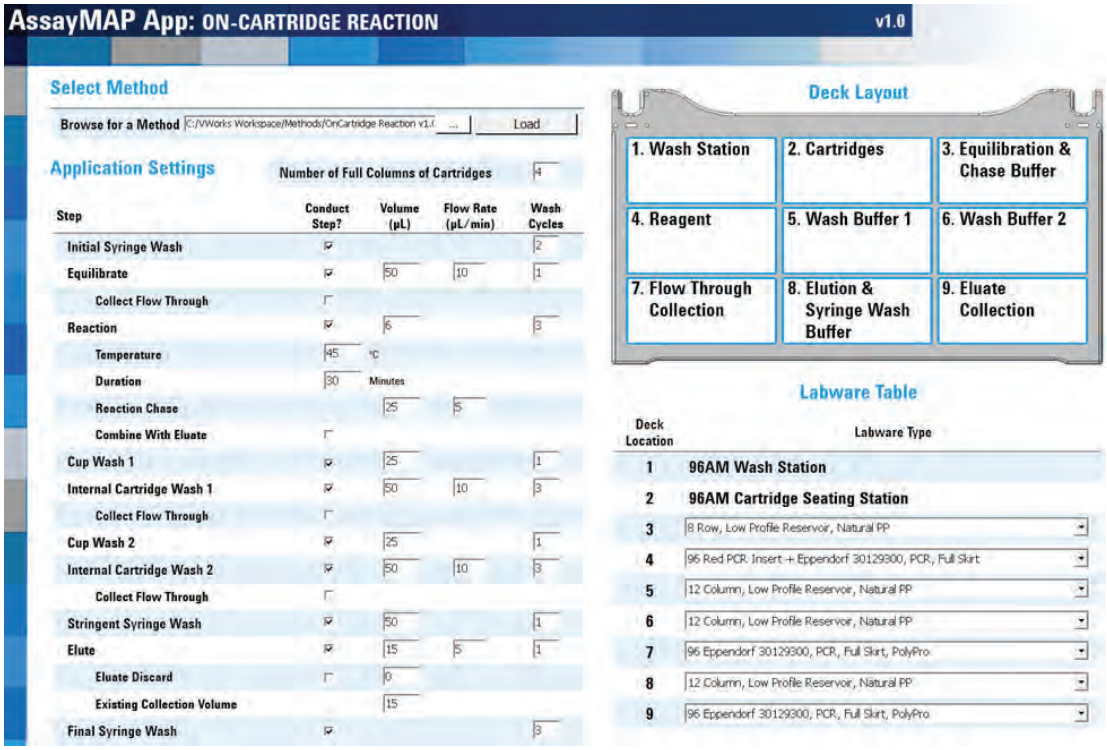

Figure 5. The setup screen for the On-Cartridge Reaction application, used in this case for deglycosylation (PNGase F) and proteolysis (IdeS) of the mAbs.

Back To Contents Page

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		

Back To Contents Page

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.

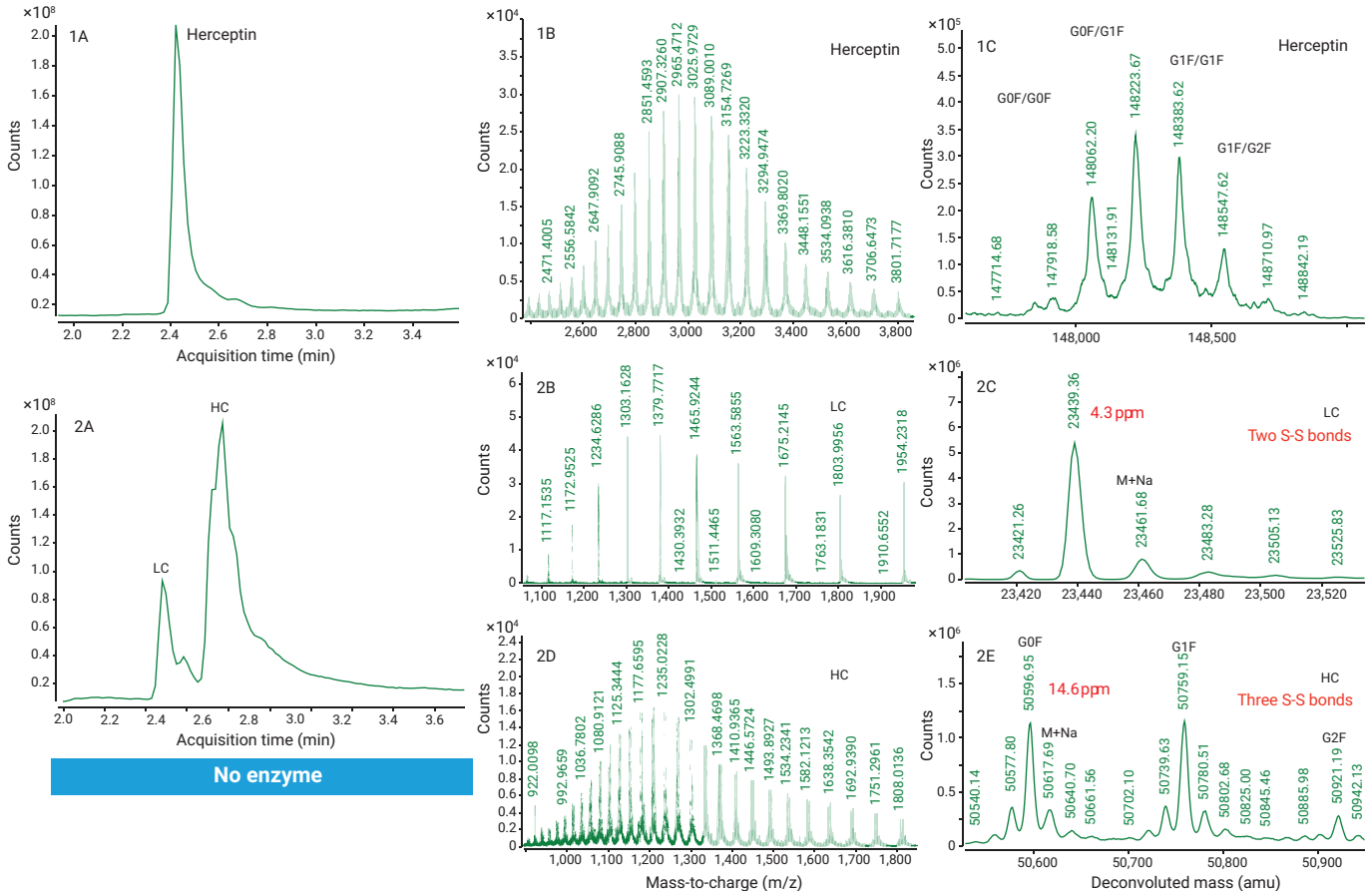

Figure 6. 1A and 2A) TIC of ECD purified intact and reduced Herceptin. 1B, 2B, and 2D) Mass spectra of intact and reduced Herceptin. 1C, 2C, and 2E) Deconvoluted spectra of intact and reduced Herceptin. LC = light chain, HC = heavy chain.

Back To Contents Page

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).

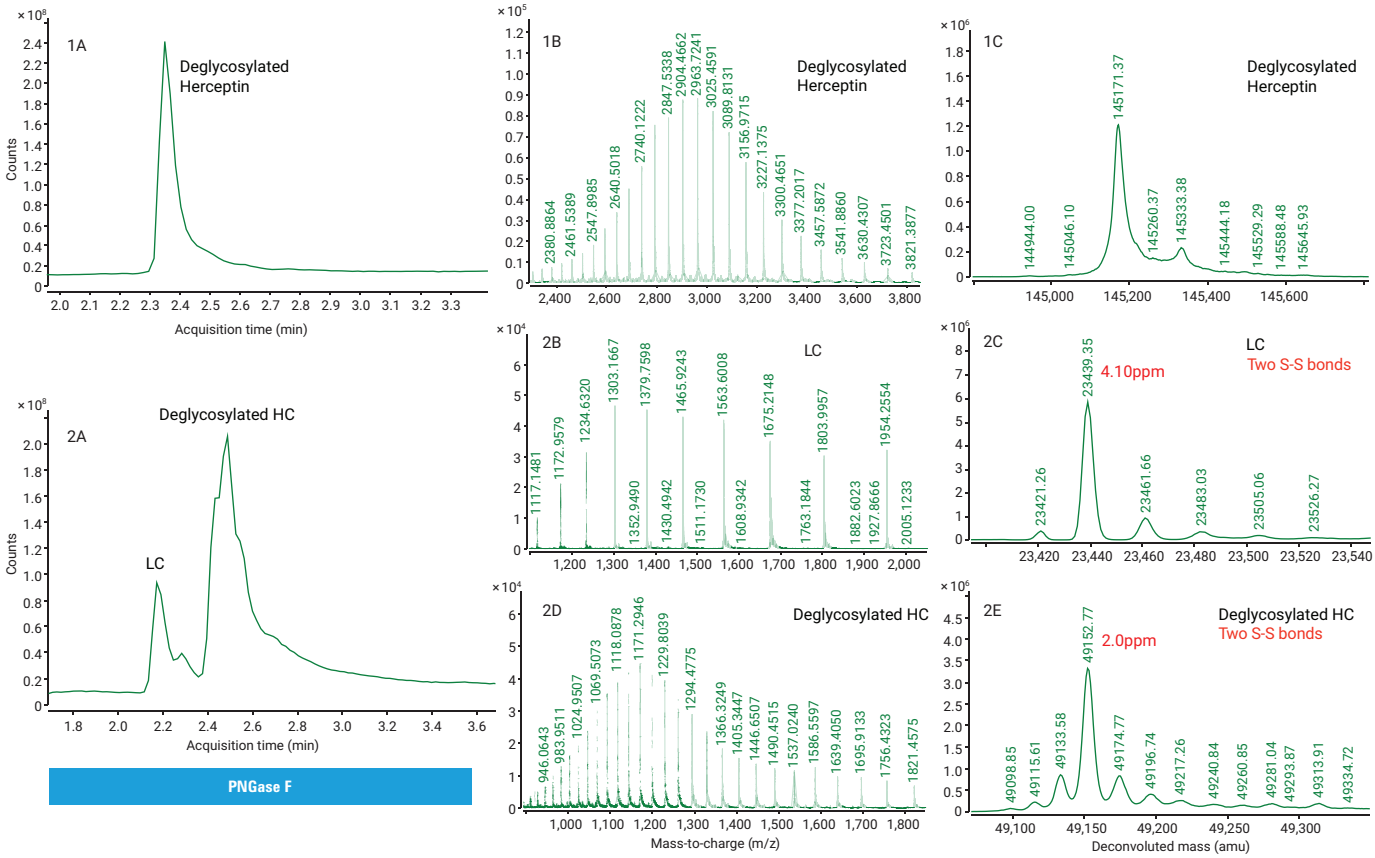

Figure 7. 1A and 2A) TIC of ECD purified intact and reduced Herceptin after on-cartridge deglycosylation with PNGase F. 1B, 2B, and 2D) Mass spectra of deglycosylated Herceptin, LC and HC. 1C, 2C, and 2E) Deconvoluted spectra of deglycosylated Herceptin, LC and HC. LC = light chain, HC = heavy chain.

Back To Contents Page

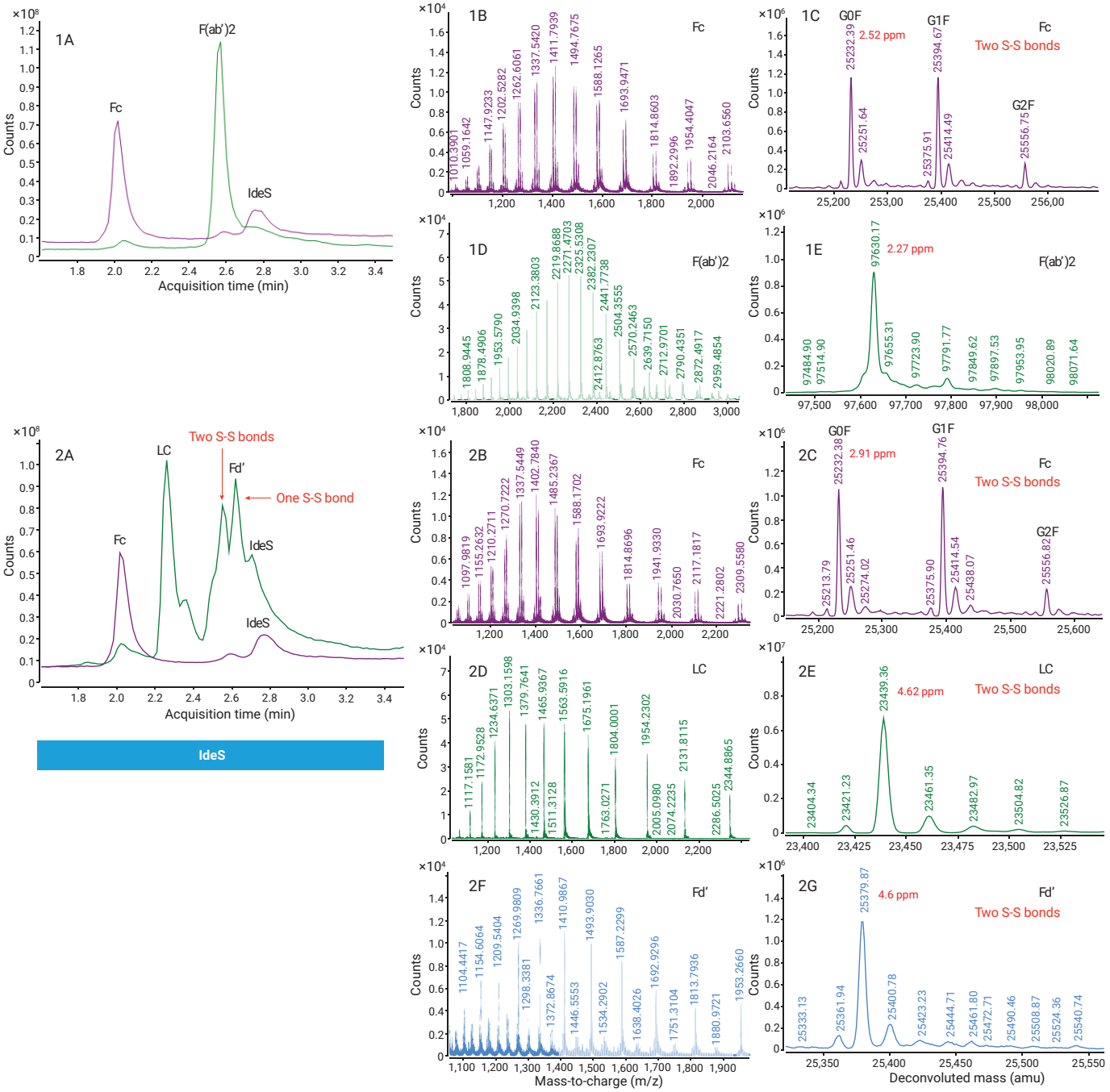

Figure 8. 1A and 2A) TIC of subunits from ECD-purified Herceptin after on-cartridge IdeS reaction and reduction. 1B, 1D, 2B, 2D, and 2F) Mass spectra of Herceptin subunits. 1C, 1E, 2C, 2E, and 2G) Deconvoluted spectra of Herceptin subunits.

Back To Contents Page

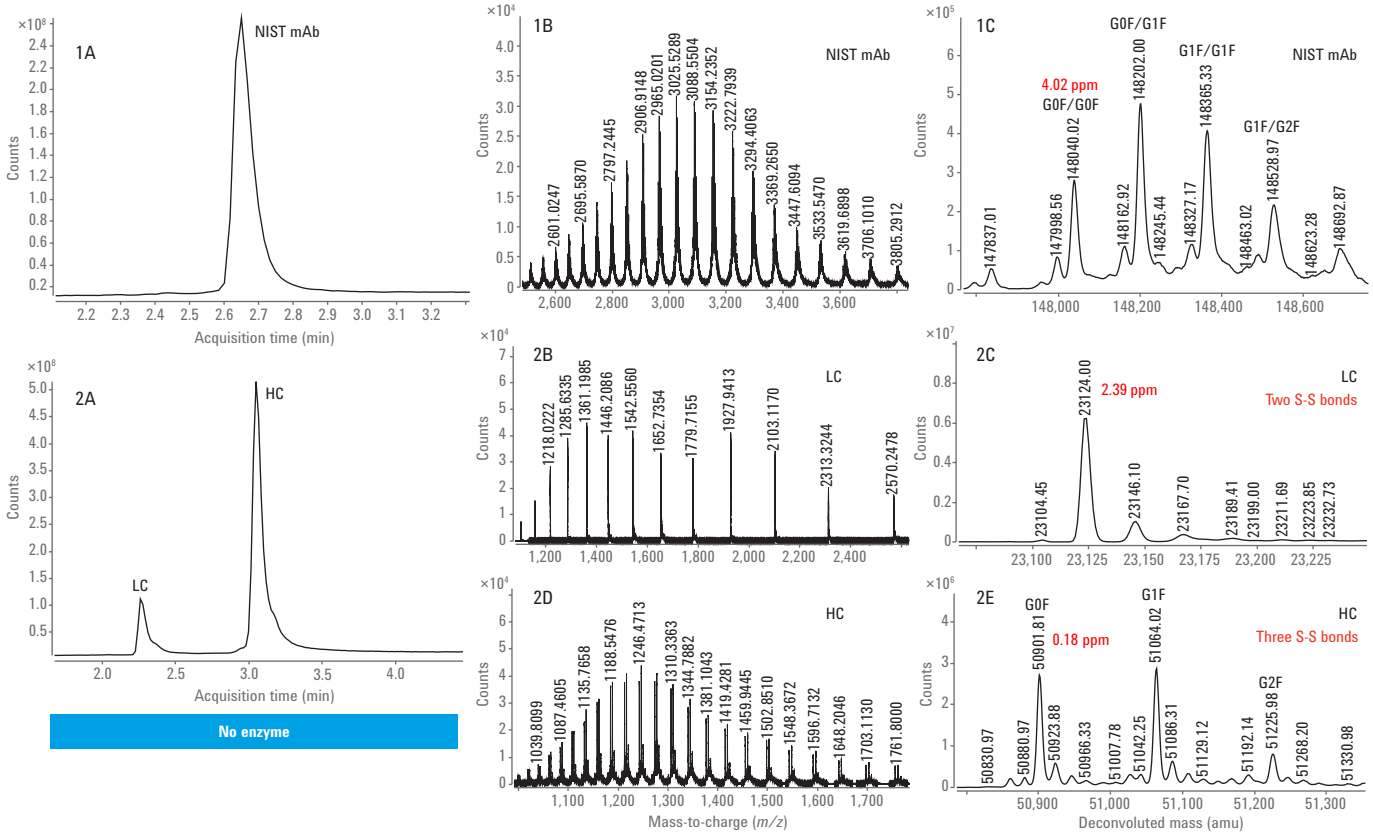

Figure 9. 1A, 2A) TIC of Protein L purified intact and reduced NISTmAb. 1B, 2B, 2D) Mass spectra of intact and reduced NISTmAb. 1C, 2C, 2E) Deconvoluted spectra of intact and reduced NISTmAb. LC = light chain; HC = heavy chain.

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.

Back To Contents Page

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).

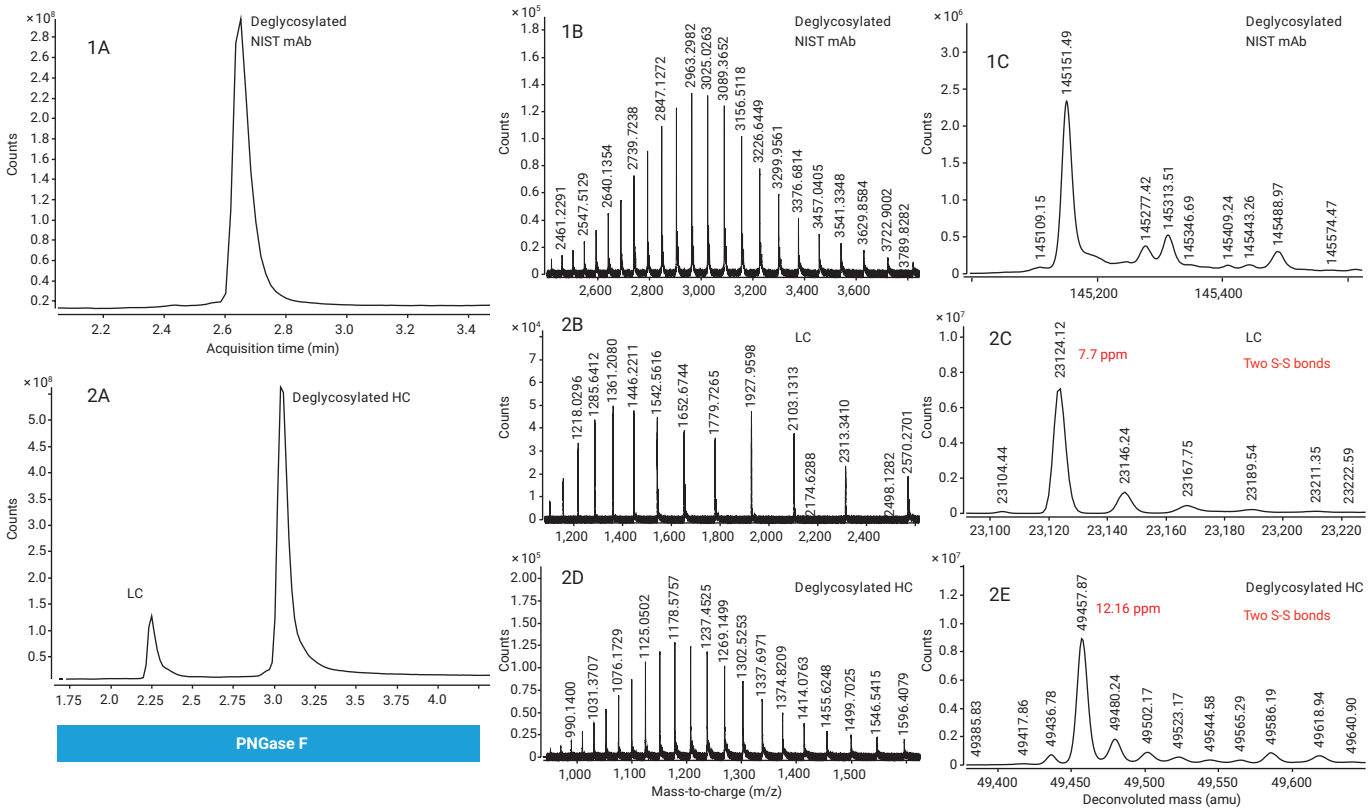

Figure 10. 1A, 2A) TIC of Protein L purified intact and reduced NISTmAb after on-cartridge deglycosylation with PNGase F. 1B, 2B, 2D) Mass spectra of deglycosylated NISTmAb, LC and HC. 1C, 2C, 2E) Deconvoluted spectra of deglycosylated NISTmAb, LC and HC. LC = light chain; HC = heavy chain.

Back To Contents Page

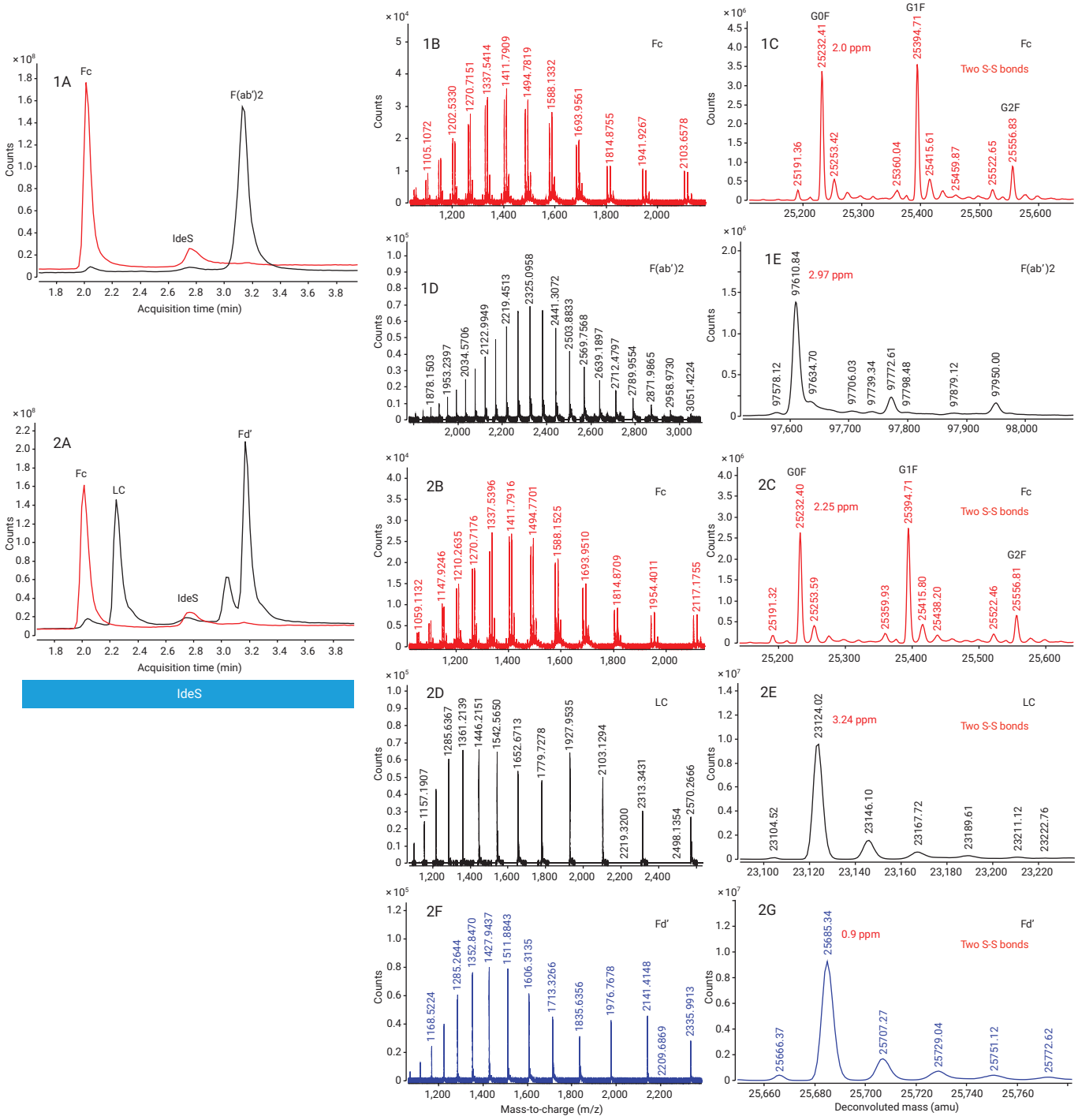

Figure 11. 1A and 2A) TIC of subunits from Protein L purified NISTmAb. 1B, 1D, 2B, 2D, and 2F) Mass spectra of NISTmAb subunits. 1C, 1E, 2C, 2E, and 2G) Deconvoluted spectra of NISTmAb subunits.

Back To Contents Page

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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Back To Contents Page

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