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

## Application Note

Biotherapeutics and Biosimilars


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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).

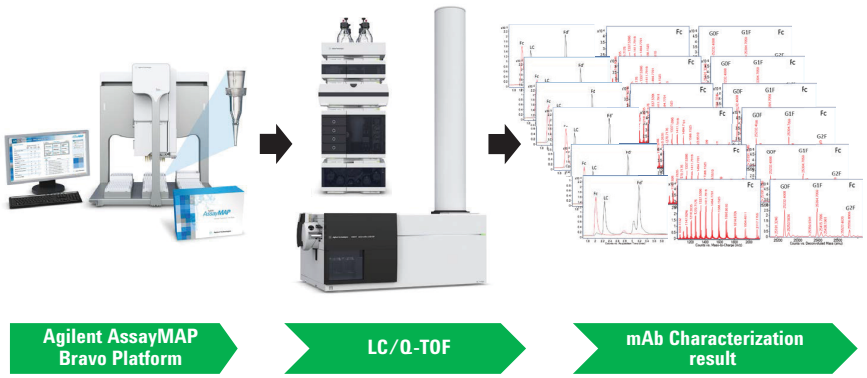


Figure 1. Integrated workflow for automated antibody characterization using Agilent AssayMAP Bravo.

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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 (NIST mAb) bound to AssayMAP streptavidin (SA-W) cartridges. Protein L is an affinity reagent for antibody kappa light chains. Both affinity purified Herceptin and NIST mAb 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

### Material

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.

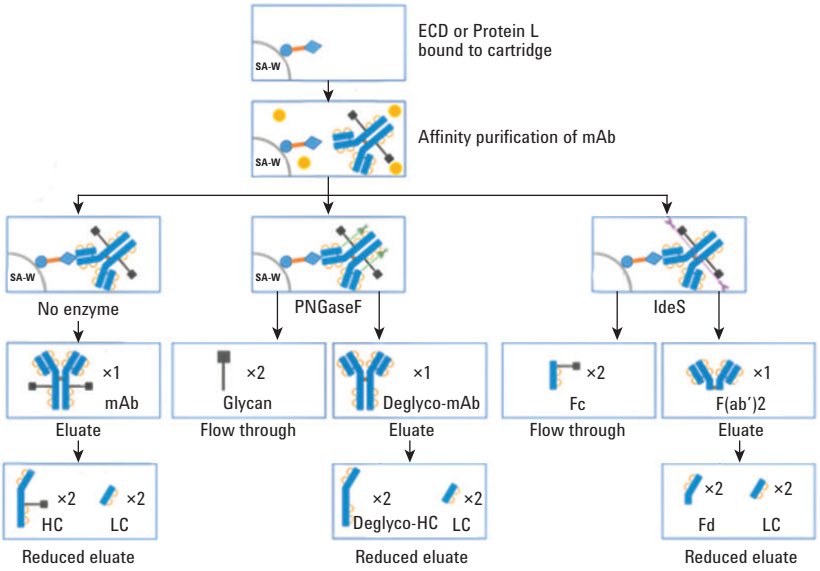


Figure 2. Antibody sample preparation performed by the Agilent AssayMAP Bravo.

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) + Herceptin (2 µg/100 µL) | Step 1: Protein L -biotin(2 µg/100 µL) + NIST mAb (2 µg/100 µL) | Step 3 |
|---|---|---|---|---|
| | | **Columns 1 and 2** | **Columns 3 and 4** | |
| Step 2: 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 |
| Step 2: PNGase F | C | Herceptin without glycan | NIST mAb without glycan | |
| | D | Herceptin LC/HC | NIST mAb LC/HC | TCEP |
| Step 2: IdeS | E | Herceptin F(ab')2 | NIST F(ab')2 | |
| | F | Herceptin Fd', LC | NIST Fd', LC | TCEP |
| | G | | | |
| | H | | | |

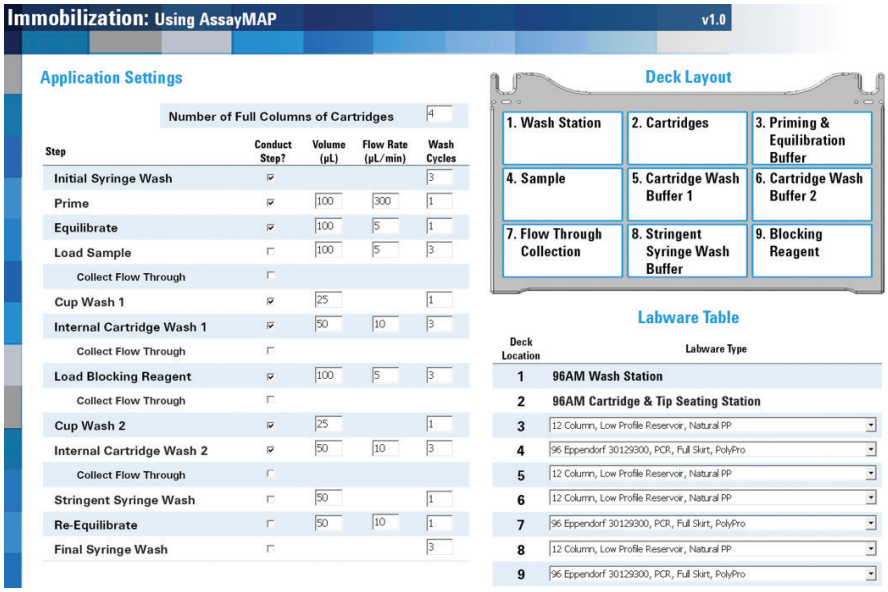


Figure 3. User interface for Agilent AssayMAP Bravo, showing the setup screen for the Immobilization application on AssayMAP Bravo used to generate antibody affinity cartridges.

## Antibody purification

Commercially obtained Herceptin (Trastuzumab) and NIST mAb were reconstituted in deionized water to 20 mg/mL, aliquoted, and stored at –80 °C. Just before use, Herceptin and NIST mAb 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 NIST mAb 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.

## On-cartridge deglycosylation and IdeS digestion

Following capture of the Herceptin and the NIST mAbs, 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).

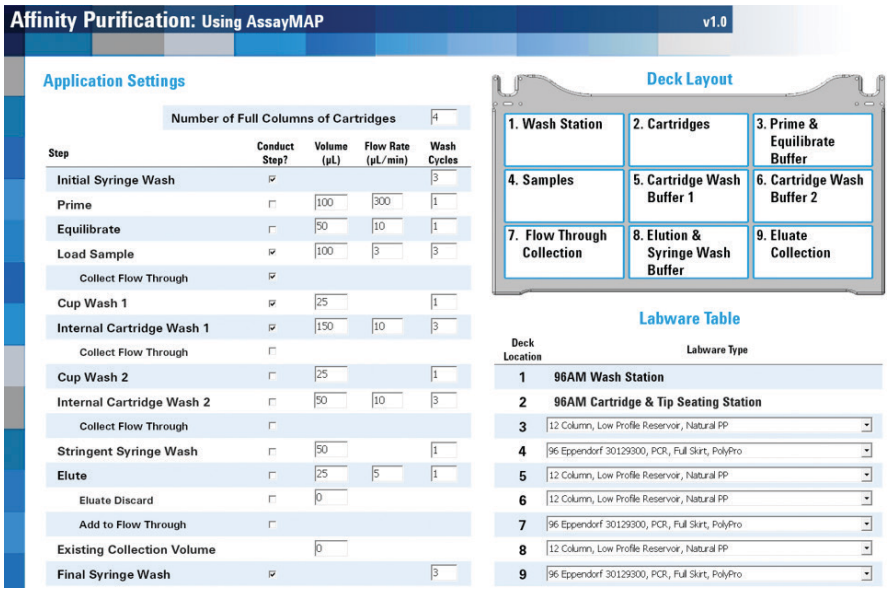


Figure 4. The setup screen for the Affinity Purification application on Agilent AssayMAP Bravo used to purify antibody from cell culture supernatant.

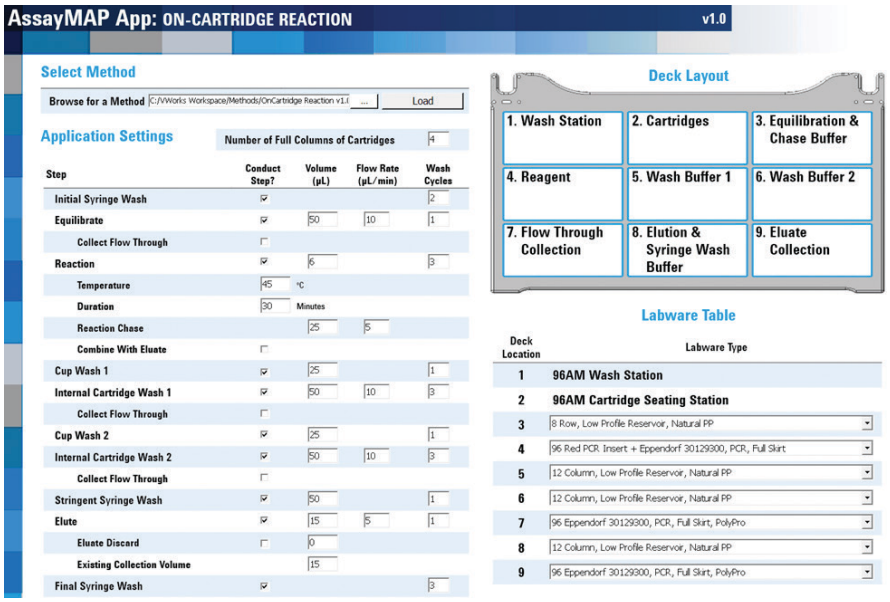


Figure 5. The setup screen for the On-Cartridge Reaction application, used in this case for deglycosylation (PNGase F) and proteolysis (IdeS) of the mAbs.

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).

## 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 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 | | | |

Table 3. Mass spectrometer parameters.

| | Agilent 6545XT AdvanceBio Q-TOF | | |
|---|---|---|---|
| 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) |

## 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 mAb | 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 NIST mAb 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 NIST mAb 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.

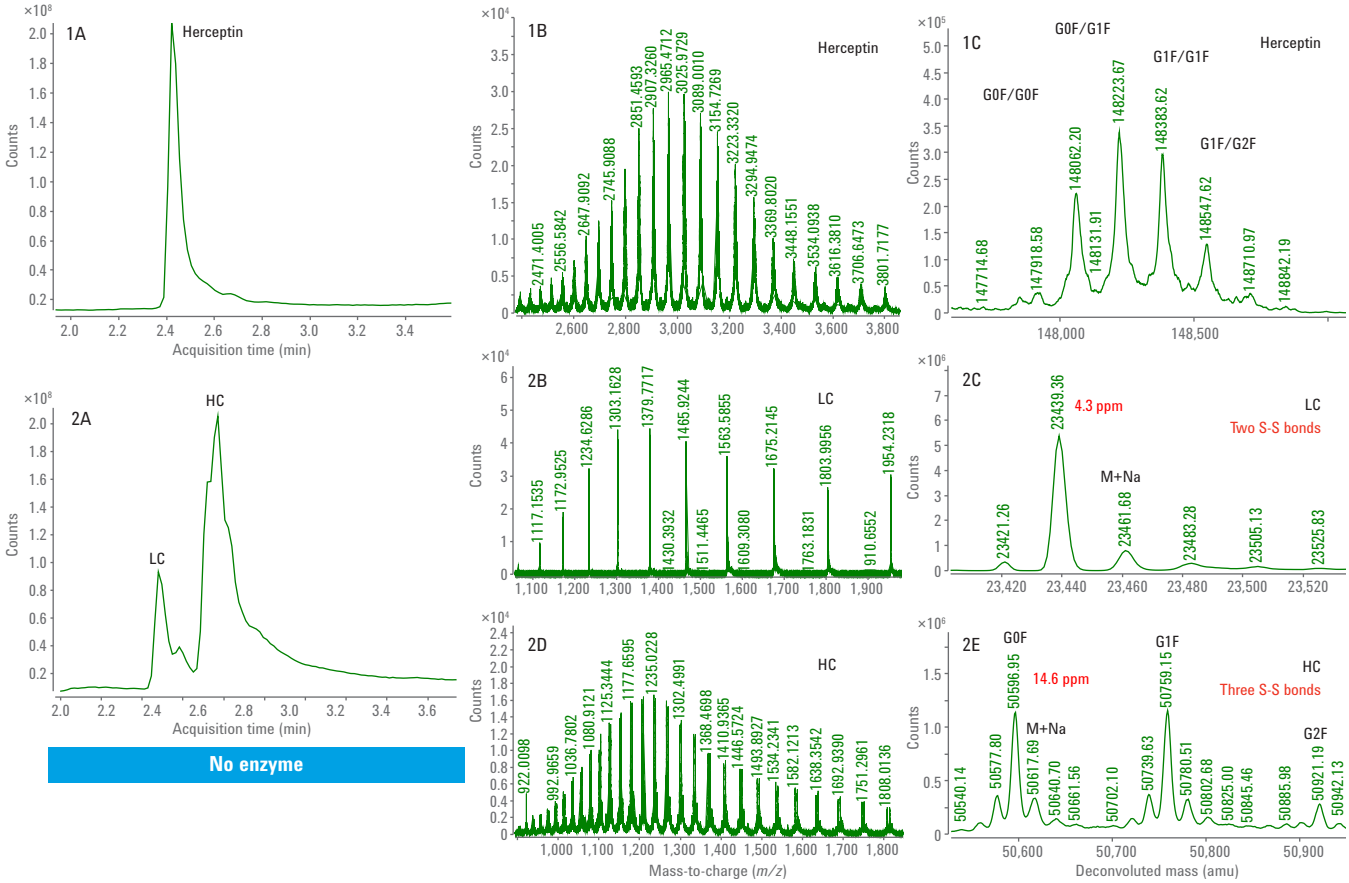


Figure 6. 1A and 2A) TIC of ECD purified intact and reduced Herceptin. 1B, 2B, and 2D) Mass spectra of intact and reduced Herceptin. 1C, 2C, and 2E) Deconvoluted spectra of intact and reduced Herceptin. LC = light chain, HC = heavy chain.

The purified intact Herceptin (Figure 6-1) and NIST mAb (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 NIST mAb. 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 NIST mAb 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 NIST mAb 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 NIST mAb (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 NIST mAb showed one major peak without the glycans (Figures 7-1C and 10-1C).

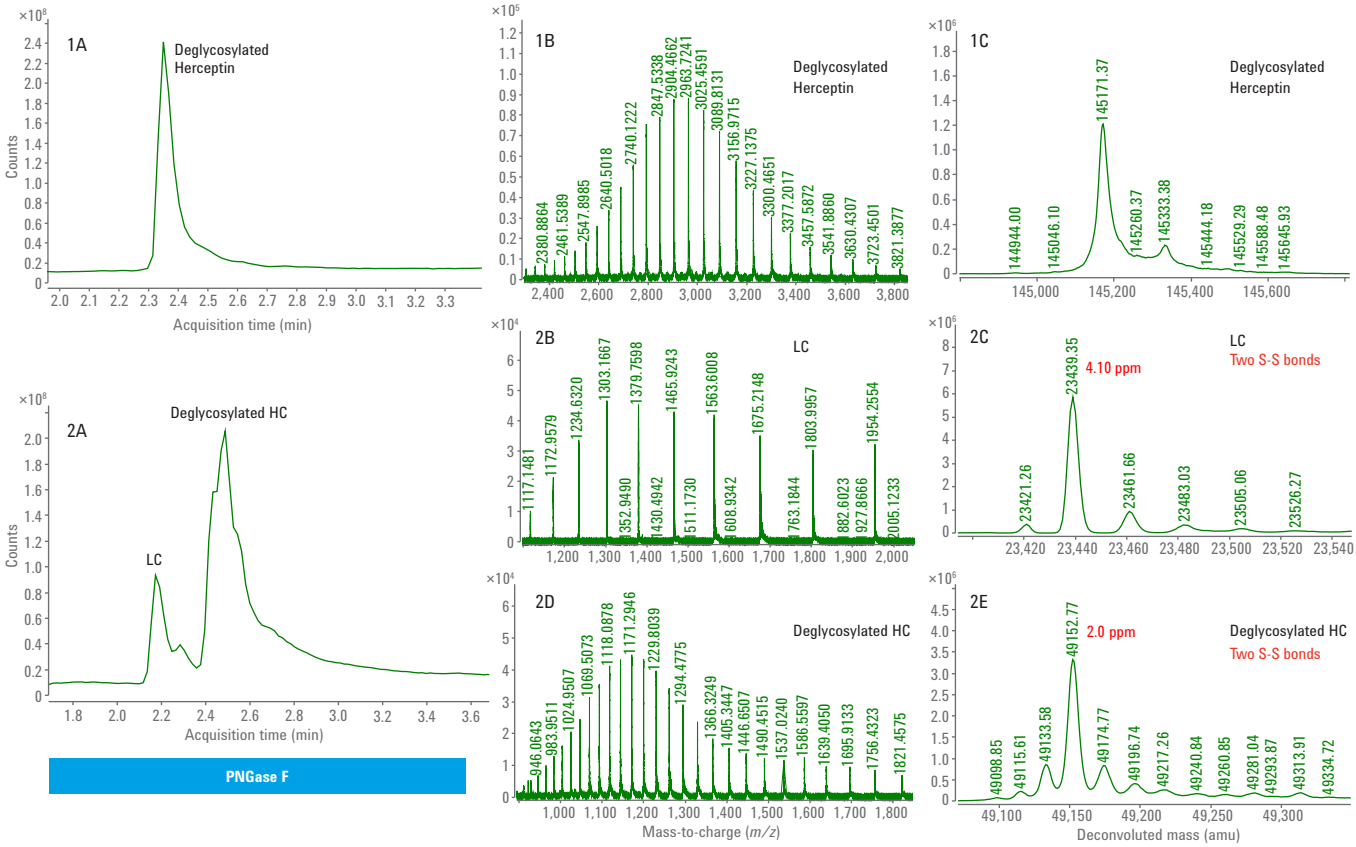


Figure 7. 1A and 2A) TIC of ECD purified intact and reduced Herceptin after on-cartridge deglycosylation with PNGase F. 1B, 2B, and 2D) Mass spectra of deglycosylated Herceptin, LC and HC. 1C, 2C, and 2E) Deconvoluted spectra of deglycosylated Herceptin, LC and HC. LC = light chain, HC = heavy chain.

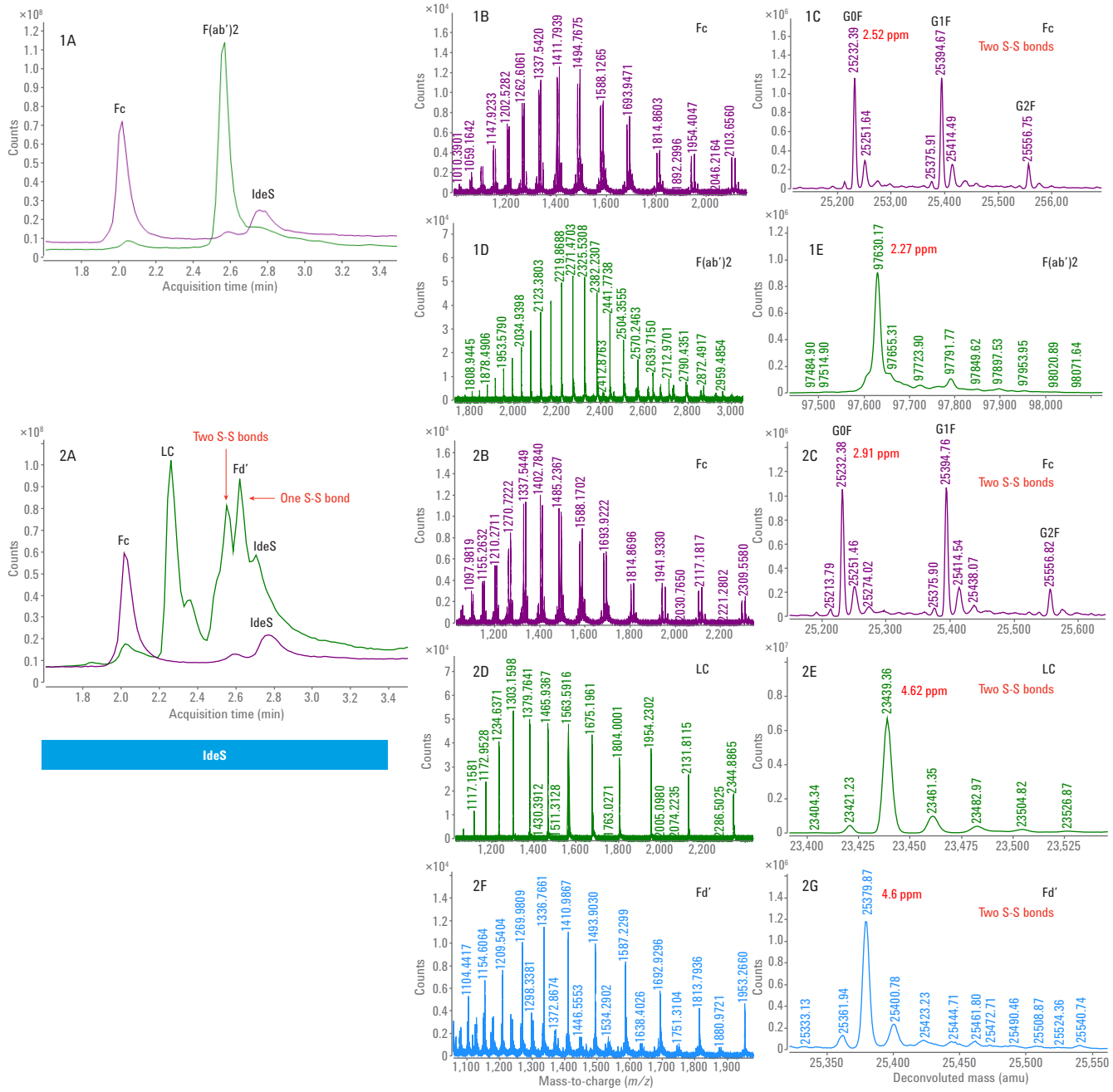


Figure 8. 1A and 2A) TIC of subunits from ECD-purified Herceptin after on-cartridge IdeS reaction and reduction. 1B, 1D, 2B, 2D, and 2F) Mass spectra of Herceptin subunits. 1C, 1E, 2C, 2E, and 2G) Deconvoluted spectra of Herceptin subunits.

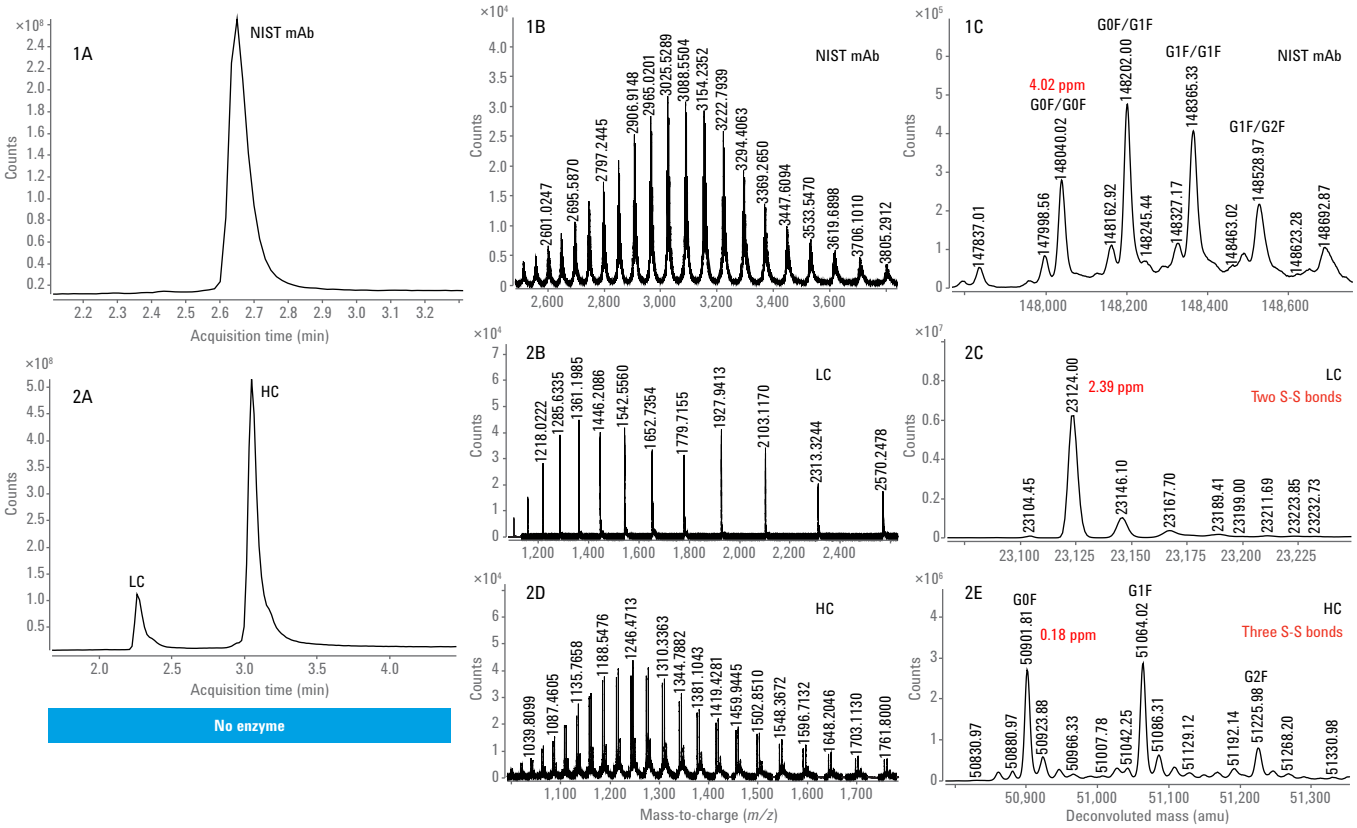


Figure 9. 1A, 2A) TIC of Protein L purified intact and reduced NIST mAb. 1B, 2B, 2D) Mass spectra of intact and reduced NIST mAb. 1C, 2C, 2E) Deconvoluted spectra of intact and reduced NIST mAb. LC = light chain; HC = heavy chain.

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 NIST mAb 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 NIST mAb 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 NIST mAb 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 NIST mAb 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 NIST mAb 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).

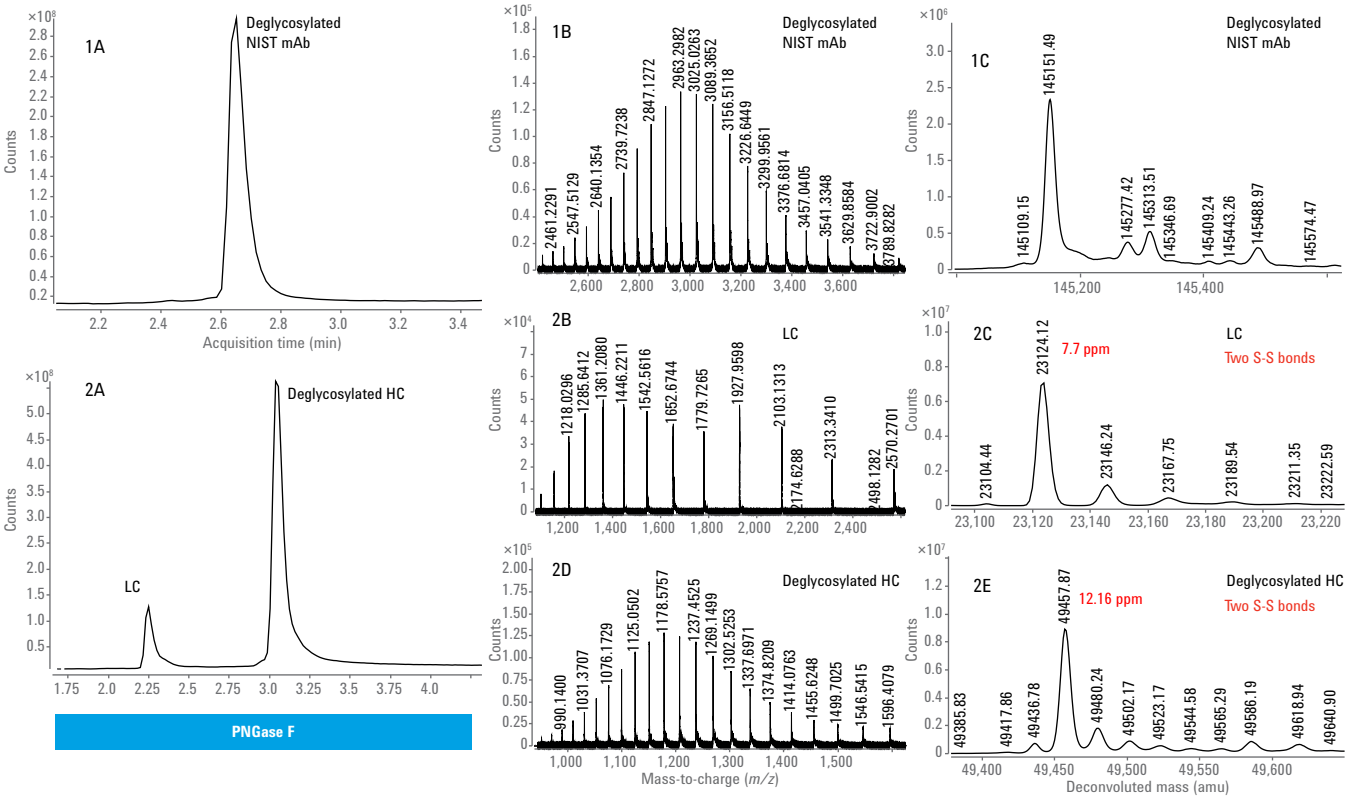


Figure 10. 1A, 2A) TIC of Protein L purified intact and reduced NIST mAb after on-cartridge deglycosylation with PNGase F. 1B, 2B, 2D) Mass spectra of deglycosylated NIST mAb, LC and HC. 1C, 2C, 2E) Deconvoluted spectra of deglycosylated NIST mAb, LC and HC. LC = light chain; HC = heavy chain.

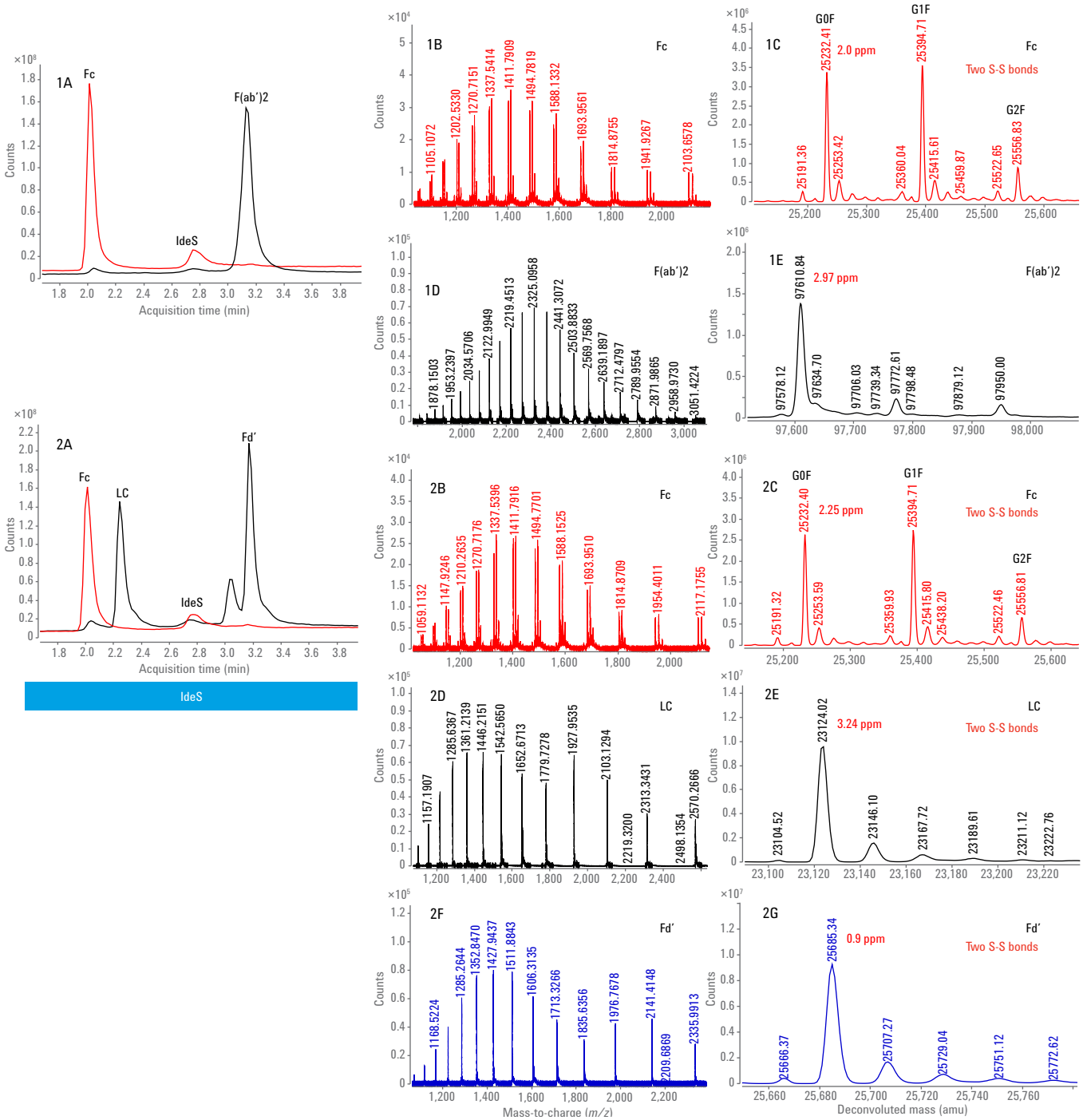


Figure 11. 1A and 2A) TIC of subunits from Protein L purified NIST mAb. 1B, 1D, 2B, 2D, and 2F) Mass spectra of NIST mAb subunits. 1C, 1E, 2C, 2E, and 2G) Deconvoluted spectra of NIST mAb subunits.

## Conclusions

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

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