Antibody Accurate Mass Characterization Enabled by Automated Affinity Purification, Deglycosylation, IdeS Digestion, and Reduction

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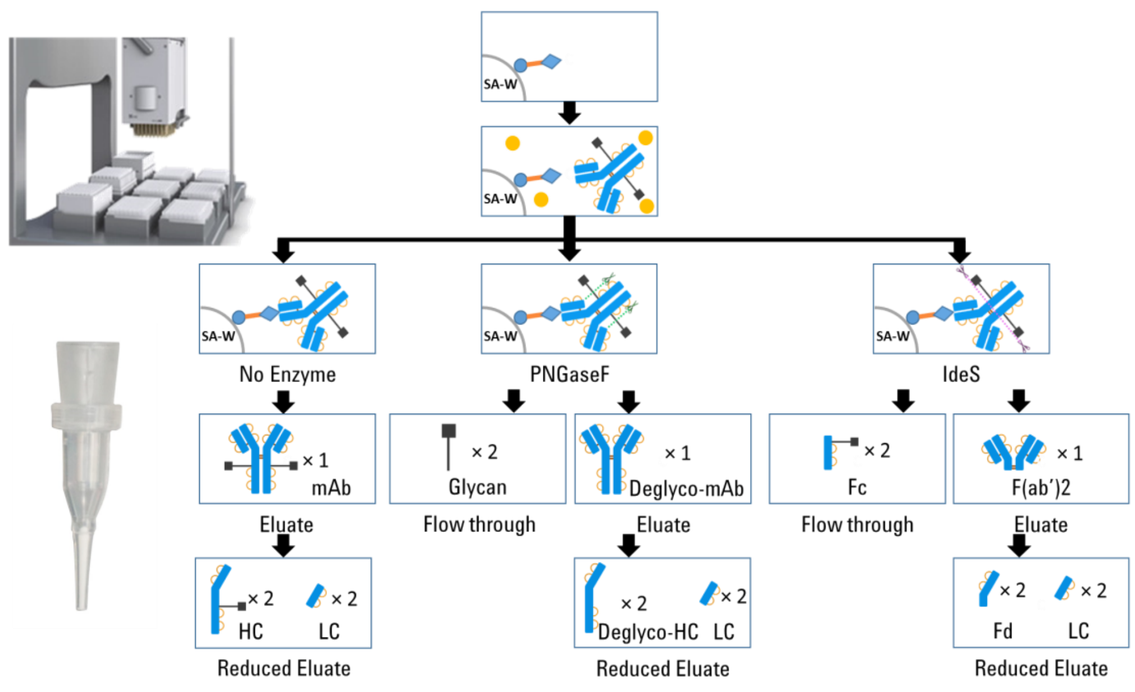
Introduction

Monoclonal antibodies (mAbs) and their related products require extensive characterization. Accurate mass determination 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 difficult. To overcome the challenges associated with antibody mass determination, a number of complementary approaches are typically used. Antibodies can be treated with PGNase 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 measuring the mass. Various combinations of the above approaches can also be used. Here, we demonstrate how these approaches can be streamlined by automation on the AssayMAP Bravo to increase reproducibility, decrease labor, and reduce the probability of human error.

Trastuzumab was affinity purified from cell culture supernatant using biotinylated Her2 extracellular domain (ECD) or biotinylated protein L immobilized on streptavidin cartridges (SA-W); Her2 ECD is the antigen for Trastuzumab and protein L is an affinity reagent for antibody kappa light chains. Immobilized Trastuzumab was either left intact, deglycosylated with PNGase F, or digested with IdeS by flowing the respective enzymes through the cartridges. The glycans and Fc/2 cleaved off the antibody were collected in the flow through. The intact, deglycosylated and F(ab′)$_2$ fragments were eluted from the cartridge into reducing and non-reducing buffers. The Fc/2 fragment was also treated with and without reducing agents. Proteins in both the flow through and the elution were analyzed with a UHPLC coupled to a Q-TOF mass spectrometer to acquire accurate protein mass data.

Antibody Characterization Workflow



Antibody Characterization Sample Preparation Workflow.
MAbs were affinity purified from spent CHO cell medium with affinity cartridges (ECD or Protein L). A subset of the mAbs captured were deglycosylated or proteolyzed with IdeS on-cartridge by flowing heated enzyme solution through the cartridges using the AssayMAP Bravo. The N-glycans or Fc were collected in the flowthrough, and the intact mAb, deglycosylated mAb, or the F(ab′)$_2$ were collected in the eluate. Samples were subsequently analyzed by Agilent 1290 Infinity UHPLC coupled with 6550 Q-TOF mass spectrometer. MS spectra were deconvoluted using MassHunter BioConfirm.

Experimental

Generation of Antibody Affinity Cartridges

- Human Epidermal Growth Factor Receptor (Her2) extracellular domain and Protein L were biotinylated using EZ-Link™ Sulfo-NHS-LC biotin kit.
- 4 µg of biotinylated Her2 ECD and 16 µg of Protein L was immobilized on each streptavidin (SA-W) cartridge using the AssayMAP Bravo.

Antibody Affinity Purification and On-Cartridge Deglycosylation and Digestion

- Commercially obtained Trastuzumab was reconstituted in deionized water to 5 mg/mL, aliquoted and stored at -80 ^{0}C until use.
- Trastuzumab was spiked into spent CHO cell medium at 40 µg/mL; 50 µl was loaded on each affinity cartridge at 3 µL/min, followed by 50 µl HEPES buffer wash, and 50 µl deglycosylation (20mM Tris, pH=8.0) or IdeS proteolysis buffer (50mM Tris, 150mM NaCl, pH=6.6) wash at 10 µl/min.
- 4 µl of heated (37 ^{0}C) rapid PNGase F (1:12), IdeS solution (4U/µL) or a buffer control was aspirated onto each Trastuzumab captured cartridge at 10 µL/min; an additional 2 µl of heated enzyme solution or buffer control was aspirated through each cartridge over the course of 30 minutes.
- 10 µl of respective reaction buffer was aspirated through each cartridge and combined with the enzyme solution that had passed over the cartridge to collect the released glycans or the Fc.
- Each cartridge was washed with three 50 µl washes (1 M NaCl in HEPES buffer, HEPES buffer and water) at 10 µl/min.
- The purified mAb, deglycosylated mAb or F(ab′)$_2$ were eluted with 15 µl of 1% formic acid per cartridge into an existing volume of 15 µl 0.5% ammonium hydroxide with or without 20 mM TCEP to neutralize and reduce the eluates.

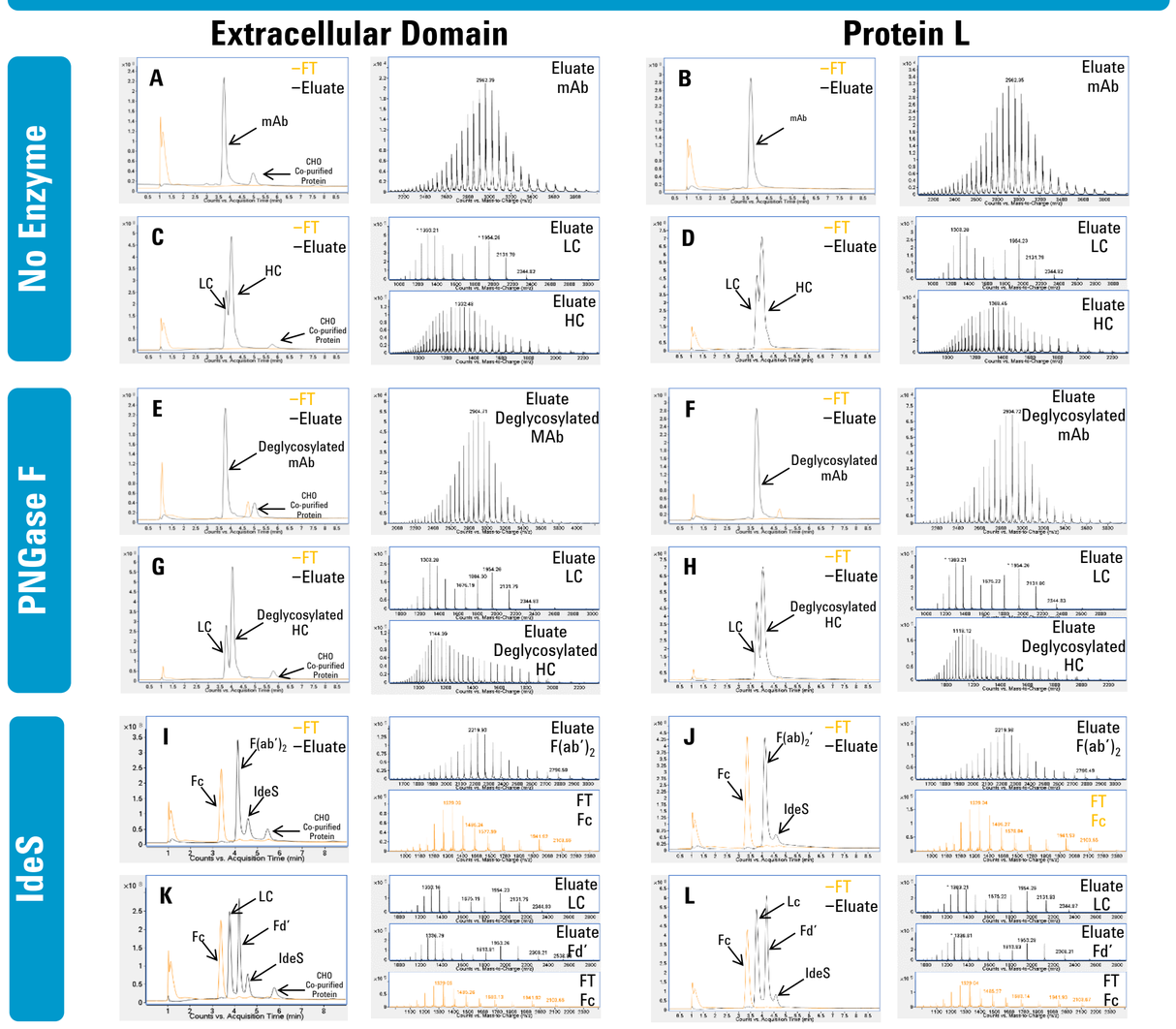
LC/MS Analysis

- LC/MS analyses were conducted using an Agilent 1290 Infinity UHPLC system (Santa Clara, CA) with a PLRP-S column (PL1912-3802) coupled to an Agilent iFunnel Accurate Mass 6550 Q-TOF equipped with a Dual Agilent Jet Stream ESI source.
- LC gradient was held at 25%B (0.1% Formic Acid in ACN) for 1 min, increased from 25%-50% B in 6.5 min, held at 50% B for 1 min, and returned to 25% B in 0.5 min. Column temperature was set at 80^{0}C for intact and deglycosylated mAbs and at 40^{0}C for the other samples.

Data Analysis

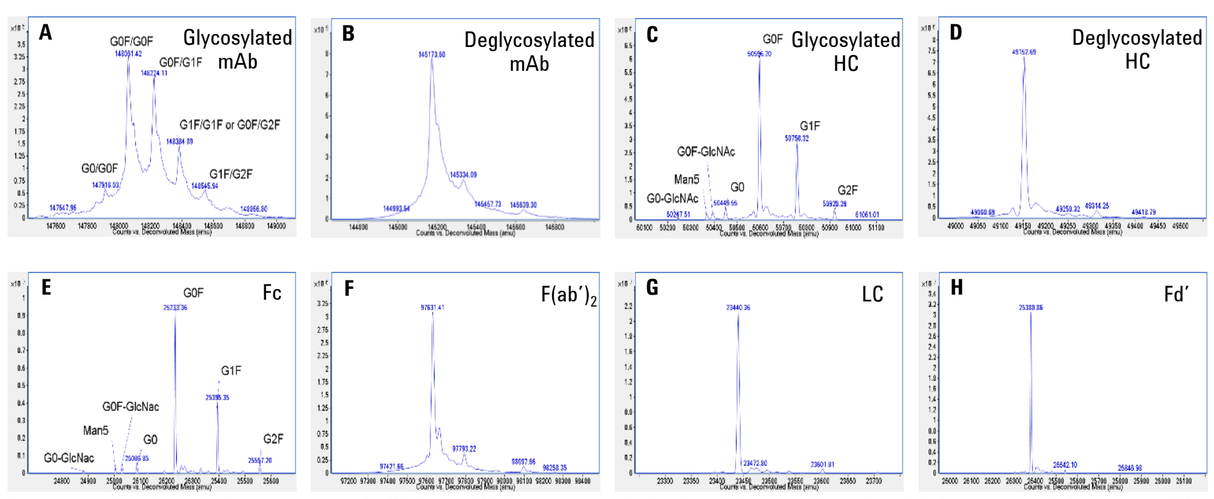
- Spectra were extracted for each TIC peak and deconvoluted using MassHunter BioConfirm Maximum Entropy Algorithm.

Antibody Purification and On-cartridge Deglycosylation/IdeS Proteolysis



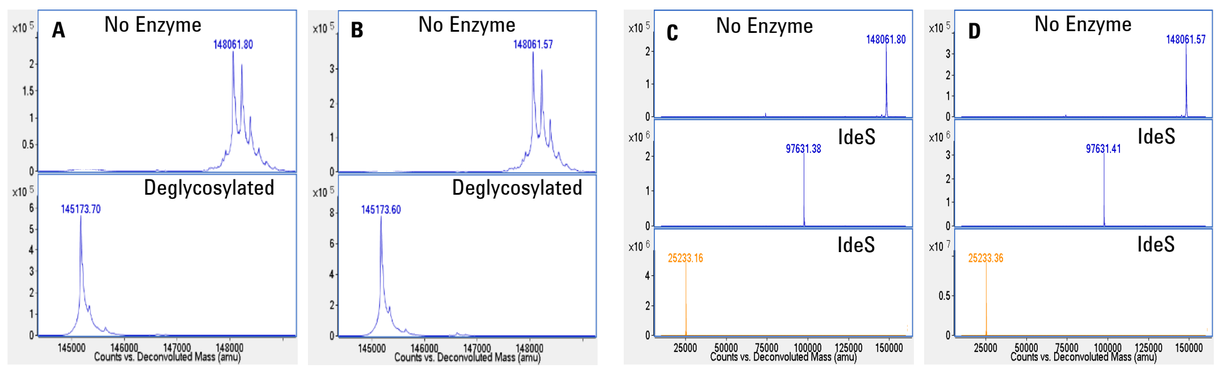
Total Ion Chromatography (TIC) and Extracted Spectra of Affinity Purified mAb Samples. Fifty microliters of spent CHO cell medium containing 40 µl/mL mAb were affinity purified with affinity cartridges (ECD or Protein L). A subset of samples were deglycosylated with rapid PNGase F or proteolyzed with IdeS on-cartridge. 10% of the enzyme flowthrough and eluate was injected for mass spectrometry analysis for each affinity purified sample. Average spectra of each TIC peak was extracted. Representative TIC and extracted spectra of (A) ECD purified intact mAb, (B) protein L purified intact mAb, (C) ECD purified reduced mAb, (D) protein L purified reduced mAb, (E) ECD purified intact deglycosylated mAb, (F) protein L purified intact deglycosylated mAb, (G) ECD purified reduced deglycosylated mAb, (H) protein L purified reduced deglycosylated mAb, (I) ECD purified IdeS proteolyzed mAb, (J) protein L purified IdeS proteolyzed mAb, (K) ECD purified IdeS proteolyzed reduced mAb, (L) protein L purified IdeS proteolyzed reduced mAb. FT: enzymatic reaction flow through; mAb: monoclonal antibody; LC: light chain; HC: heavy chain.

Deconvoluted Spectra of mAb and Fragments



Representative deconvoluted spectra of affinity purified glycosylated, deglycosylated mAb and its IdeS proteolyzed fragments. Fifty microliters of spent CHO cell medium containing 40 µl/mL mAb were affinity purified with affinity cartridges (ECD or Protein L). A subset of samples were deglycosylated with rapid PNGase F or proteolyzed with IdeS on-cartridge. Half of these samples were reduced. 10% of the enzyme flowthrough and eluate was injected for mass spectrometry analysis for each affinity purified sample. Average spectra of each TIC peak was extracted and deconvoluted using BioConfirm. (A) Glycosylated mAb. (B) Deglycosylated mAb. (C) Glycosylated heavy chain. (D) Deglycosylated heavy chain. (E) Fc. (F) F(ab′)$_2$. (G) light chain. (H) Fd′. mAb: monoclonal antibody; LC: light chain. HC: heavy chain;

Evaluation of Deglycosylation/IdeS Proteolysis



Evaluation of deglycosylation/IdeS proteolysis completeness. Comparison of deconvoluted spectra of affinity purified Trastuzumab without and with PNGase F on-cartridge deglycosylation show complete deglycosylation of (A) ECD captured mAb and (B) protein L captured mAb. Comparison of deconvoluted spectra of affinity purified Trastuzumab without and with IdeS on-cartridge proteolysis show complete fragmentation of (C) ECD captured mAb and (D) protein L captured mAb. Blue traces indicate elute and yellow traces indicate flowthrough.

Conclusions

Agilent provides a rapid and versatile antibody characterization solution that includes automated affinity purification and on-cartridge enzymatic reaction with the AssayMAP Bravo. Subsequently, spectra were acquired with the 1290 Infinity UHPLC couple to a Q-TOF 6550, and were deconvoluted with the MassHunter BioConfirm software. This protein characterization solution:

- Decreases variability, probability of human error while simultaneously increasing scalability with minimal additional labor
- Purifies mAb from spent CHO cell medium with high yield and purity.
- Generates high resolution spectra.
- Provides easy antibody intact mass characterization.

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