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Development of a Rapid 2-AB Sample Preparation Workflow for N-Glycan Release and Labeling

Authors

Vaishali Sharma,
Andres Guerrero, John Yan,
Aled Jones, Michael Kimzey,
Emily Dale, Ted Haxo, and
Sergey Vlasenko
Agilent Technologies, Inc.

Abstract

The characterization of N-glycans is essential to the development of biotherapeutics. Typically, enzymatically released N-glycans are derivatized with a tag to allow for fluorescence (FLR) and mass spectrometry (MS) detection by HILIC UHPLC-FLR and UHPLC/MS. N-glycan sample preparation often requires numerous hours or days to complete. Although newer fluorescent tags such as Agilent InstantPC (formerly ProZyme) provide high FLR and MS sensitivity, 2-AB (2-aminobenzamide) is a tag that has been used to generate N-glycan data for more than 20 years and is well established in many laboratories. Presented herein is the development and application of a rapid N-glycan sample preparation workflow using a five-minute in solution deglycosylation step followed by direct on-matrix 2-AB labeling and cleanup without the need for a dry down step, samples are ready for analysis in approximately 2 hours.

We present a comparison study consisting of two monoclonal antibodies (MabThera, NISTmAb) and one Fc fusion protein (Enbrel), using two different N-glycan sample preparation workflows offered by Agilent: 1) Agilent GlykoPrep Rapid N-Glycan Prep with 2-AB; 2) Agilent AdvanceBio Gly-X N-Glycan Prep with 2-AB Express (formerly ProZyme). Agilent AdvanceBio Gly-X technology and 2-AB Express labeling offers much-improved time to results compared to traditional sample preparation methods.

Introduction

The structure of N-linked glycans can play a critical role in the pharmacology of therapeutic proteins, potentially affecting immunogenicity, pharmacokinetics and pharmacodynamics.[1] This makes the characterization of N-glycans an essential part of the biotherapeutic development process. N-glycans do not contain a chromophore or fluorophore suitable for online detection with standard liquid chromatography (LC) techniques, so they are commonly derivatized with fluorescent tags such as 2-AB by reductive amination chemistry (Figure 1) after enzymatic release with PNGase F. The most commonly used reducing agent used for this purpose is sodium cyanoborohydride.[2] Traditional 2-AB labeling methods require multiple steps including drying released glycans prior to labeling and subsequent cleanup of excess dye label that can take hours if not days to complete.[3] GlykoPrep with traditional reductive amination dyes such as 2-AB streamlined the sample

preparation process allowing the process to be completed in four to five hours. Gly-X N-Glycan Prep with 2-AB Express is a simplified and rapid workflow, where in solution deglycosylation releases N-glycans in five minutes and

2-AB labeling occurs on a solid-state matrix, where excess dye is washed away with acetonitrile before eluting labeled samples with DI water. Samples are typically ready for UHPLC analysis in two hours (Figure 2).
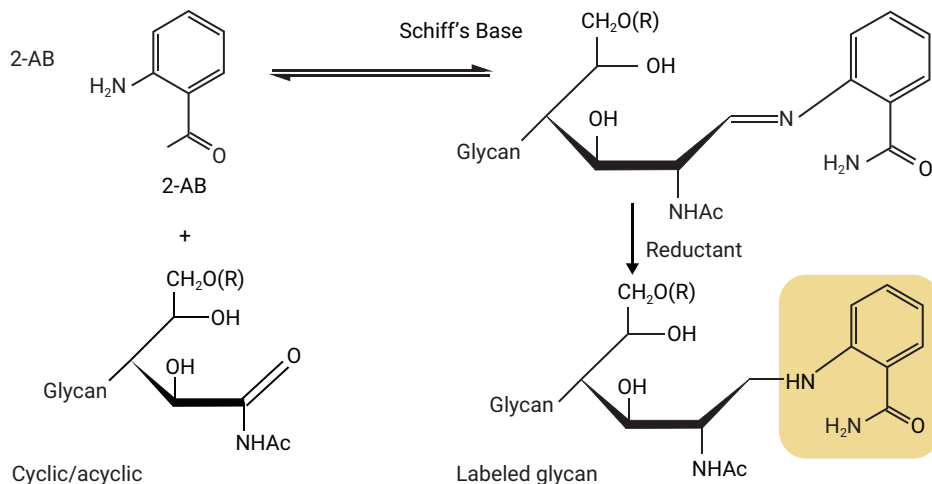
2-AB reductive amination of N-glycans

Figure 1. Labeling of enzymatically released N-glycans with 2-AB. The primary amine of the dye attacks the vcarbonyl carbon of the acyclic reducing sugar to form a partial Schiff's base. The imine group of the Schiff's base is chemically reduced to give the labeled glycan.
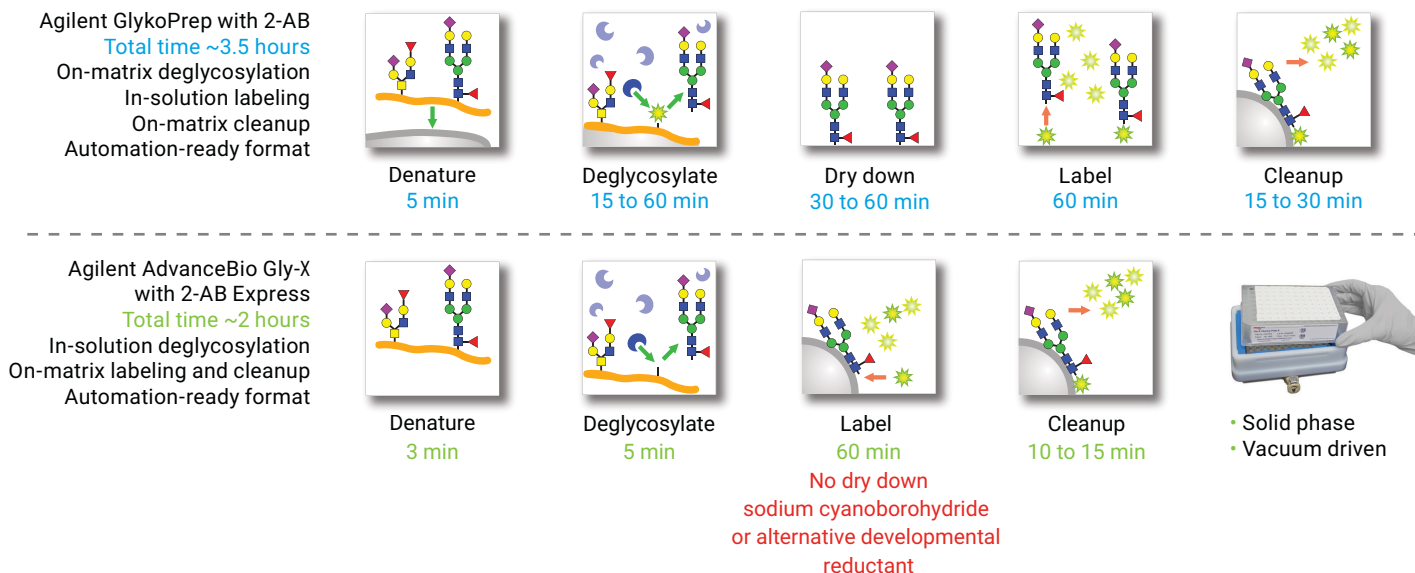
N-glycan sample prep workflows

Figure 2. N-glycan sample preparation workflows: Agilent GlykoPrep and Agilent AdvanceBio Gly-X with 2-AB Express.

Methods

Sample preparation

N-Glycan samples from MabThera (lot no. H0102B03), Enbrel (lot no. R170724), and NIST monoclonal antibody reference material 8671 (NISTmAb) (lot no. 14HB-D-002) were prepared with Agilent's Glyko-Prep Rapid N-Glycan Prep with 2-AB (GP96NG-AB) and Agilent AdvanceBio Gly-X N-Glycan Prep with 2-AB Express (GX96-2AB) (formerly Prozyme) kits, following standard recommended protocols using 50 µg of protein per preparation. All samples were prepared in triplicate.

HILIC-UHPLC analysis

Eluted samples were adjusted to a final volume of 100 µL prior to analysis (1 µL injection). Glycans were separated by hydrophilic interaction liquid chromatography (HILIC). HILIC-UHPLC separation was performed on an Amide 2.1 × 150 mm, 1.7 µm column using 25 to 38% 50 mM ammonium formate pH 4.4, between 2.5 to 50 minutes at a flow rate of 0.4 mL/min. Labeled glycans were monitored by fluorescence detection Ex/Em (nm) 360/428. Glycan assignments were made by comparison to existing Agilent data, mass determination by LC/MS,[4,5] and for the NISTmAb, published data.[6,7]

Results and discussion

HILIC-UHPLC analysis of 2-AB labeled N-glycans showed comparable results between the two different sample preparation methods GlykoPrep with 2-AB and, Gly-X with 2-AB Express

- **MabThera:** Like most monoclonal antibodies produced in CHO cells, the glycosylation pattern of MabThera consists of mostly neutral biantennary glycans where G0F, G1F[6]/G1F[3] and G2F predominate with low levels of siaylated and high mannose species (Figure 3).
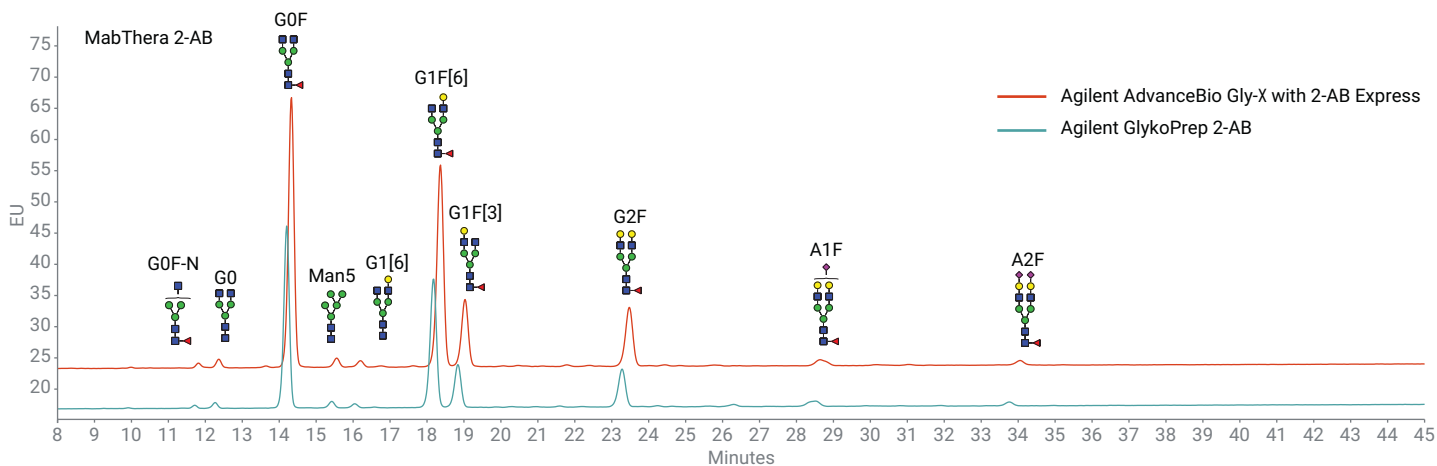

Figure 3. Overlay of 2-AB UHPLC fluorescence profiles of N-glycans from MabThera prepared with Agilent GlykoPrep with 2-AB and Agilent AdvanceBio Gly-X with 2-AB Express labeling.

- **Enbrel:** In contrast to MabThera, the glycosylation profile of Enbrel contains much higher levels of sialylation as well as afucosyl glycans as seen with A1F, A2F, A1, and A2 (Figure 4).

- **NISTmAb:** Similar to MabThera, the NISTmAb contains mostly G0F, G1F[6]/[3], and G2F. However, the NISTmAb also contains glycans with single and double galactose-α(1,3)-galactose epitopes on neutral species (Figure 5) as in published data.[6]
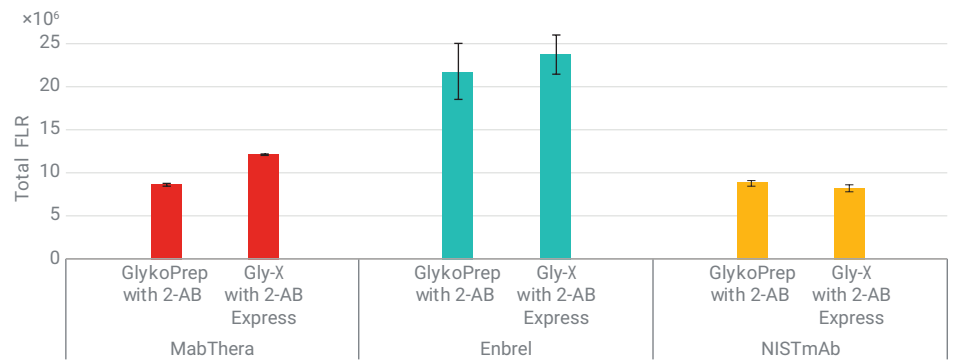

Figure 6. Comparison of 2-AB total fluorescence response. Glycans from equivalent amounts of glycoprotein (MabThera, Enbrel, and the NISTmAb) prepared were prepared with Agilent AdvanceBio Gly-X N-Glycan Prep with 2-AB Express and Agilent GlykoPrep Rapid N-Glycan Prep with 2-AB. All eluted samples were adjusted to 100 μL final volume prior to UHPLC – FLR analysis (1 μL injection).
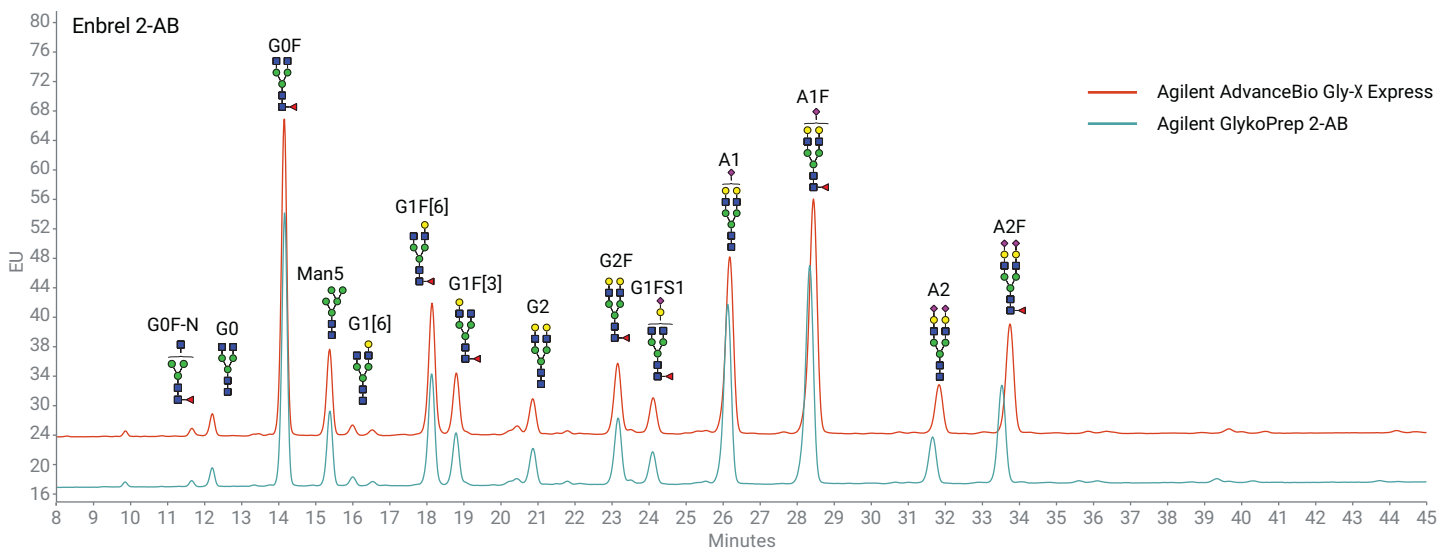

Figure 4. Overlay of Agilent 2-AB UHPLC (formerly ProZyme) fluorescence profiles of N-glycans from Enbrel prepared with Agilent AdvanceBio Gly-X with 2-AB Epxress labeling and Agilent GlykoPrep with 2-AB.
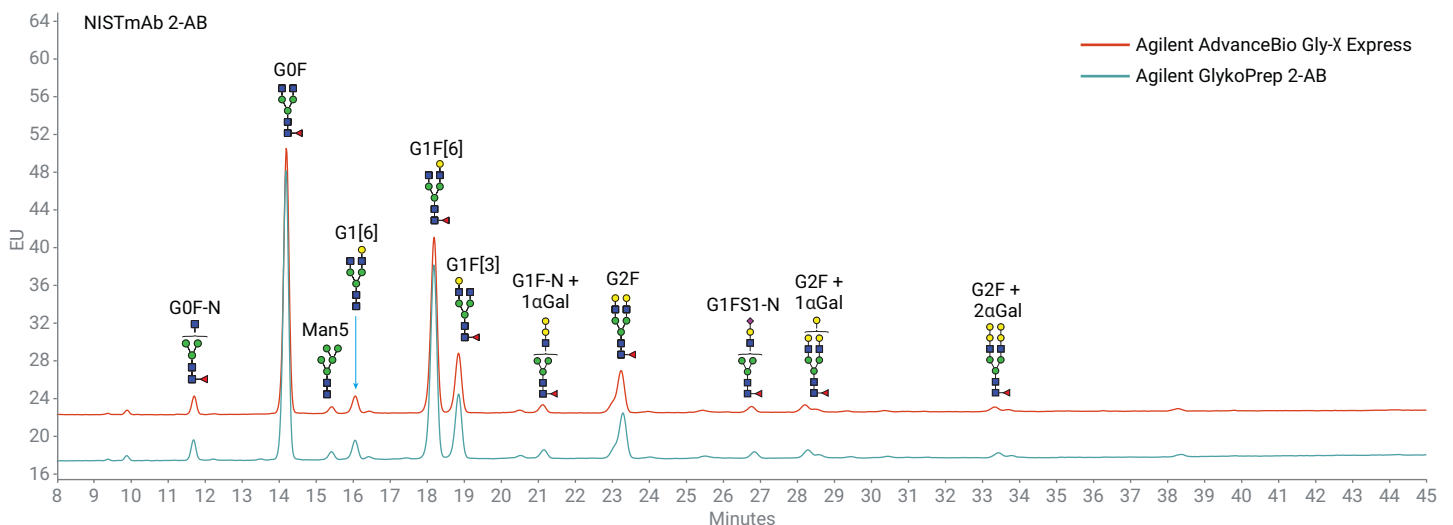

Figure 5. Overlay of 2-AB UHPLC fluorescence profiles of N-glycans from NISTmAb prepared with Agilent AdvanceBio Gly-X N-Glycan Prep with 2-AB Express and Agilent GlykoPrep Rapid N-Glycan Prep with 2-AB.

- Higher total fluorescence signal was observed for Gly-X sample preparation versus GlykoPrep for molecules MabThera and Enbrel. NISTmAb total fluorescence was comparable for both Gly-X and GlykoPrep.

- The two sample preparation methods provided similar reported relative % areas for the three molecules tested (Figure 7).
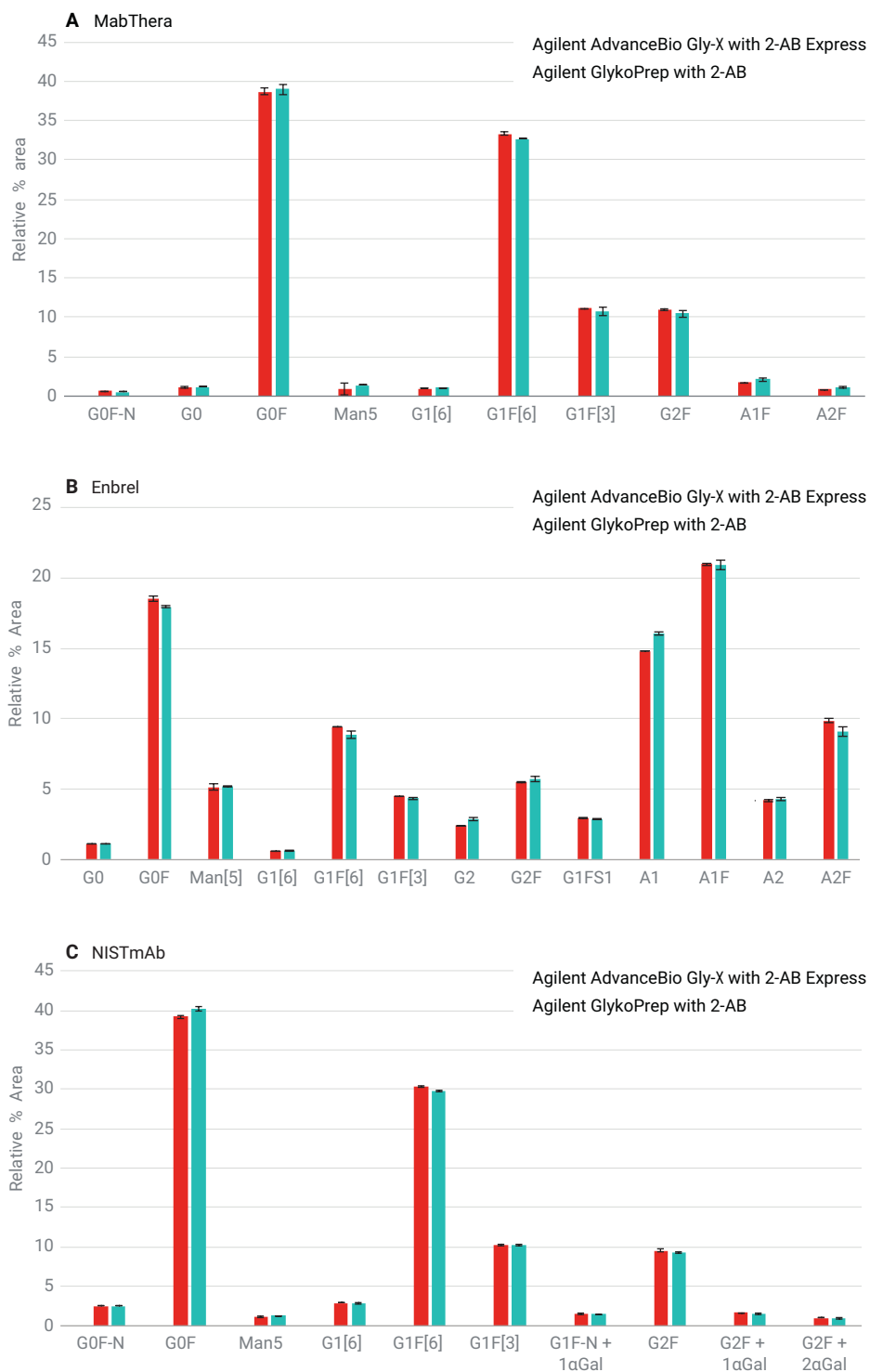

A MabThera

Agilent AdvanceBio Gly-X with 2-AB Express
Agilent GlykoPrep with 2-AB

B Enbrel

Agilent AdvanceBio Gly-X with 2-AB Express
Agilent GlykoPrep with 2-AB

C NISTmAb

Agilent AdvanceBio Gly-X with 2-AB Express
Agilent GlykoPrep with 2-AB

Figure 7. Comparison of relative % areas for N-glycans prepared with Agilent GlykoPrep with 2-AB and Agilent AdvanceBio Gly-X with 2-AB Express. (A) MabThera, (B) Enbrel, (C) NISTmAb. Glycans >0.5% relative area reported for MabThera/Enbrel and >1.0% for NISTmAb.

Conclusion

- Agilent GlykoPrep Rapid N-Glycan Prep with 2-AB labeling of released N-glycans offers reproducible and reliable results that can be directly compared to historic data.

- Agilent AdvanceBio Gly-X N-Glycan Prep with 2-AB Express offers a rapid workflow with traditional 2-AB labeling without the need for lengthy dry down steps, shortening the time to result.

- Preparation of 2-AB labeled N-glycans from MabThera, Enbrel and NISTmAb with GlykoPrep and Gly-X results in comparable data in terms of total fluorescence signal and reported relative percent areas.

- The NISTmAb N-glycan profile is consistent with published data.

References

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