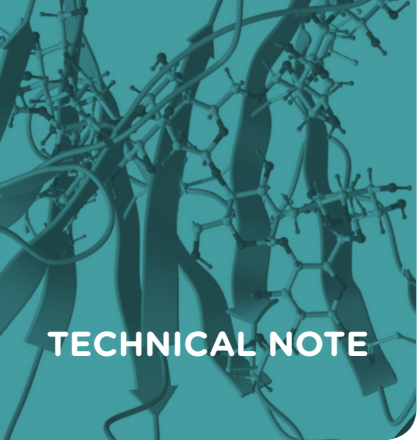

TECHNICAL NOTE

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RPE & APC Conjugates
Streptavidins

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

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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 InstantPC 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 utilizing a 5-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 ProZyme: 1) GlykoPrep® with 2-AB; 2) Gly-X™ with 2-AB Express. Gly-X with 2-AB Express offers much-improved time to results compared to traditional sample preparation methods.

Keywords

2-AB
Biotherapeutic
Enbrel
Glycan Labeling
Glycoprotein
HILIC
N-Glycan Labeling
Rituximab

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 4 to 5 hours. ProZyme Gly-*X* with 2-AB Express™ is a simplified and rapid workflow, where in solution deglycosylation releases N-glycans in 5 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 2 hours (Figure 2).

2-AB reductive amination of N-glycans

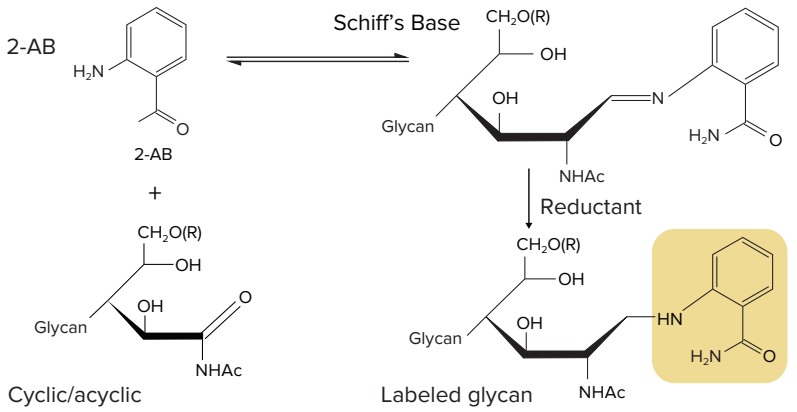


Figure 1: Labeling of enzymatically released N-glycans with 2-AB. The primary amine of the dye attacks the vcarbonyl carbon of the acyclic reducing sugar to form a partial Schiff's base. The imine group of the Schiff's base is chemically reduced to give the labeled glycan.

METHODS

Sample Preparation

N-Glycan samples from MabThera (lot # H0102B03), Enbrel (lot # R170724) and NIST monoclonal antibody reference material 8671 (NISTmAb) (lot # 14HB-D-002) were prepared with ProZyme's GlykoPrep Rapid N-Glycan Preparation with 2-AB (GP96NG-AB) and Gly-*X* N-Glycan Rapid Release and Labeling with 2-AB Express Kit (GX96-2AB) kits, following standard recommended protocols utilizing 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 a Waters Glycan BEH Amide, 2.1 x 150 mm, 1.7 µm column using 25–38% 50 mM ammonium formate pH 4.4, between 2.5–50 minutes at a flow rate of 0.4 mL/minute. Labeled glycans were monitored by fluorescence detection Ex/Em (nm) 360/428. Glycan assignments were made by comparison to existing ProZyme data, mass determination by LC-MS (4,5), and for the NISTmAb, published data (6,7).

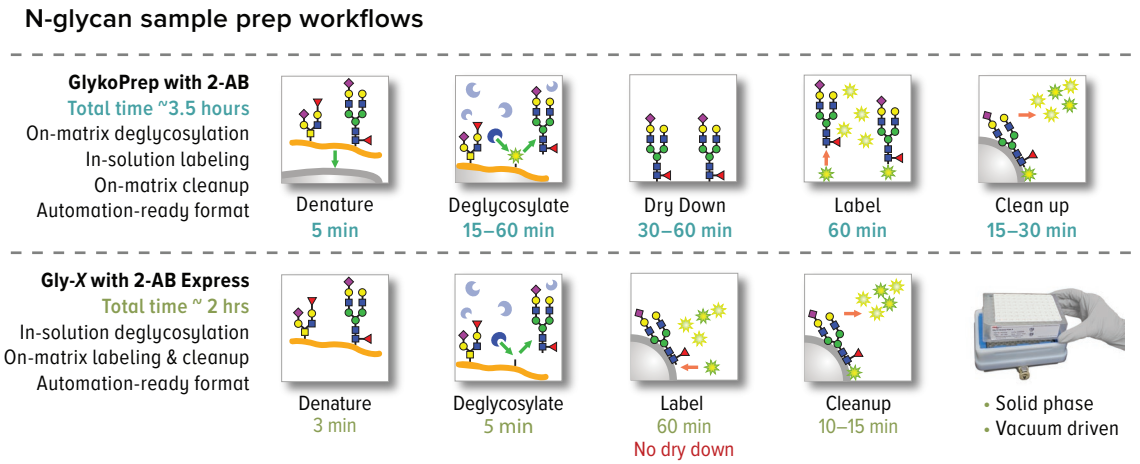


Figure 2: N-glycan sample preparation workflows: GlykoPrep and Gly-*X* with 2-AB Express.

RESULTS

- 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)
  - 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 and 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)
- Higher total fluorescence signal was observed for Gly-*X* sample preparation vs 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)

MabThera 2-AB

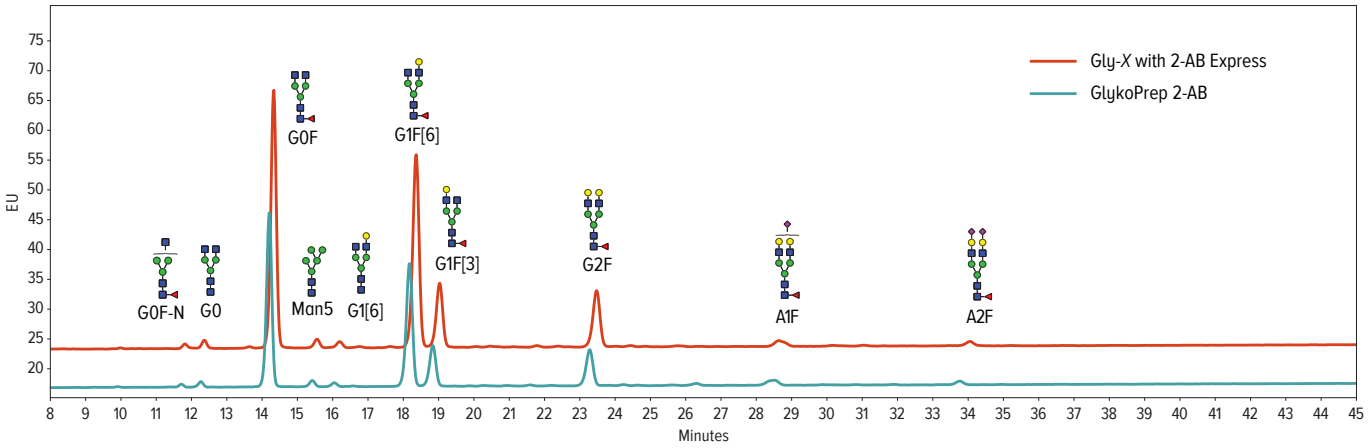


Figure 3: Overlay of 2-AB UHPLC fluorescence profiles of N-glycans from MabThera prepared with GlykoPrep with 2-AB and Gly-*X* Express.

Enbrel 2-AB

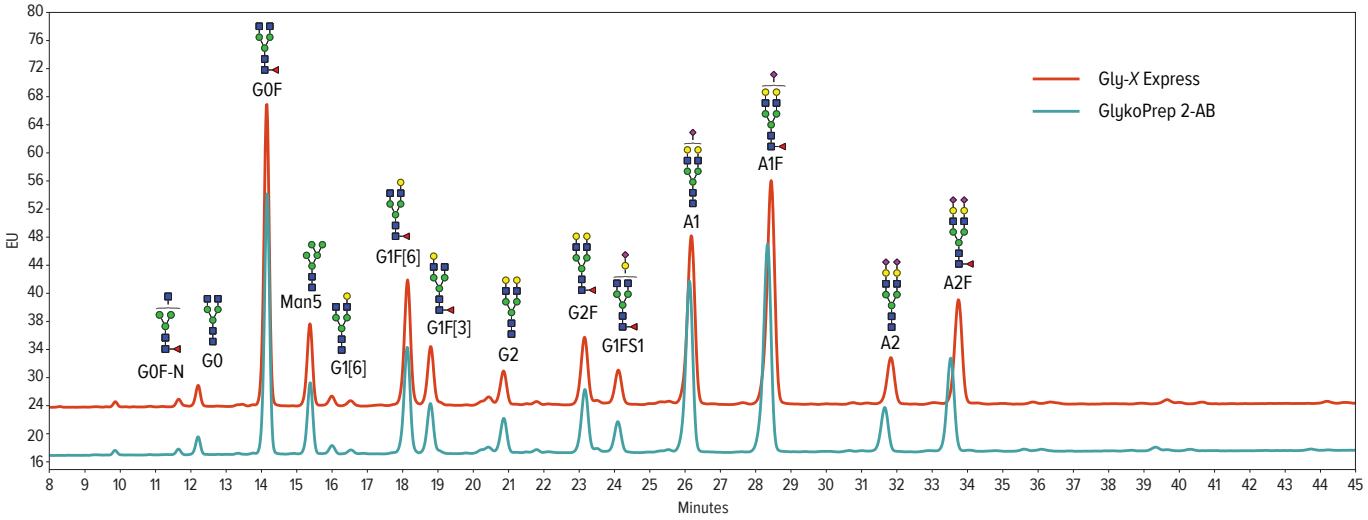


Figure 4: Overlay of 2-AB UHPLC fluorescence profiles of N-glycans from Enbrel prepared with Gly-X 2-AB Express and GlykoPrep 2-AB.

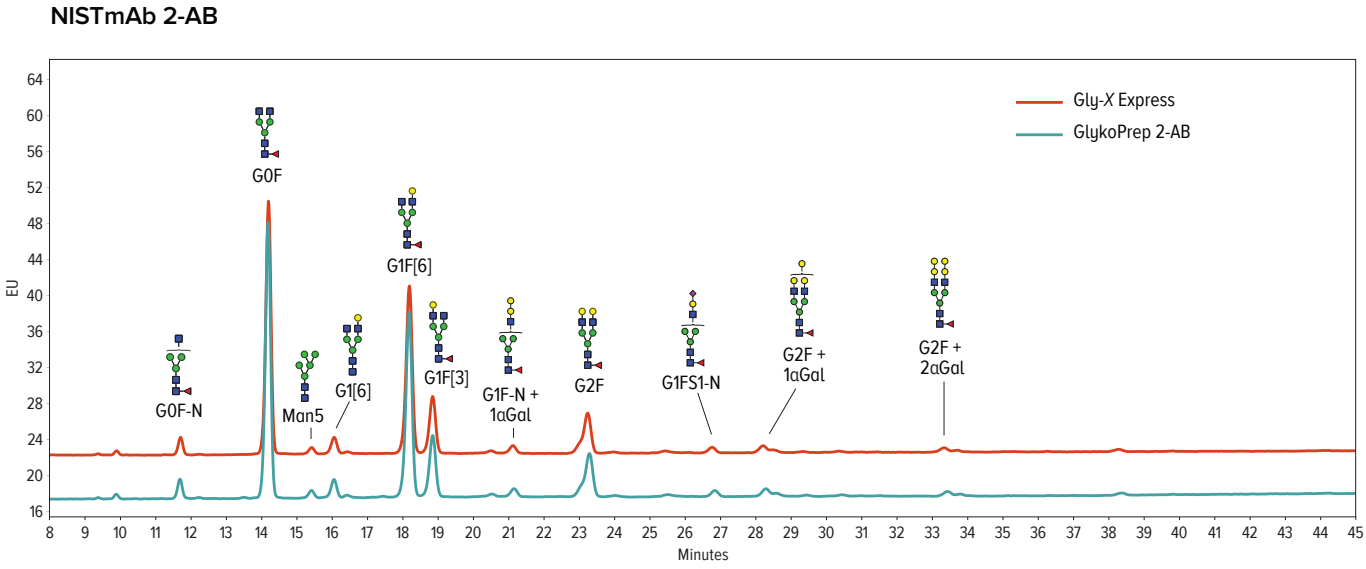


Figure 5: Overlay of 2-AB UHPLC fluorescence profiles of N-glycans from NISTmAb prepared with Gly-X 2-AB Express and GlykoPrep 2-AB.

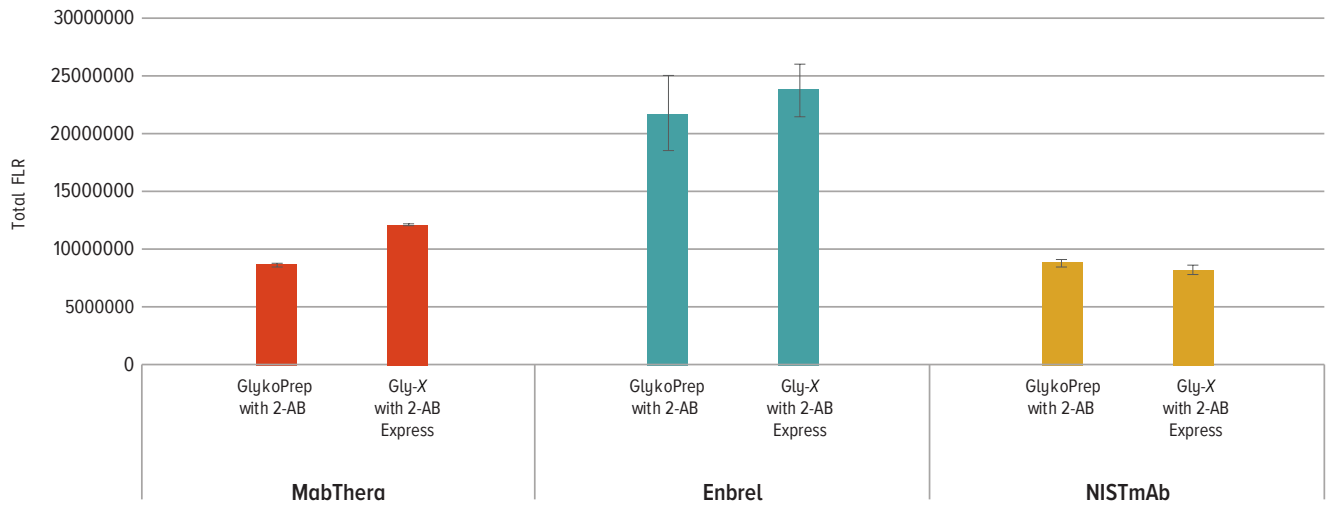
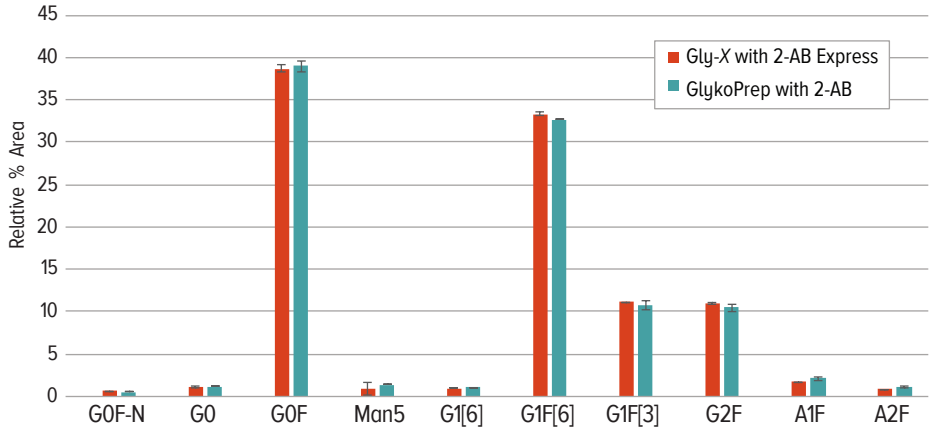
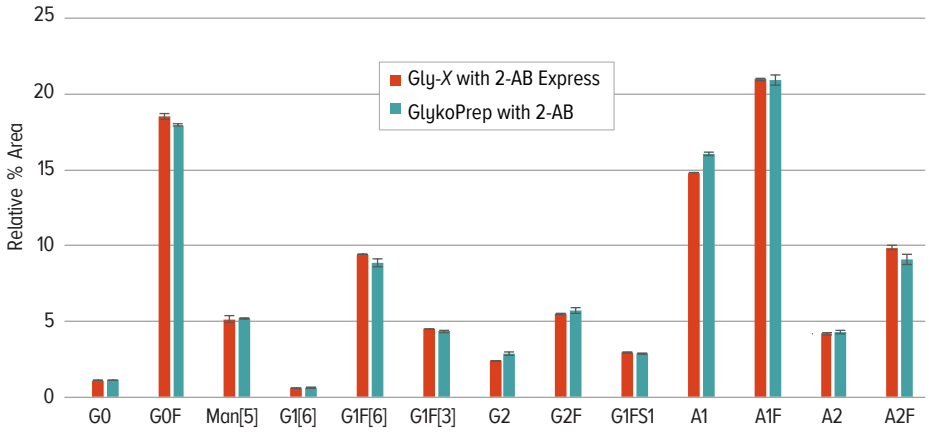


Figure 6: Comparison of 2-AB total fluorescence response. Glycans from equivalent amounts of glycoprotein (MabThera, Enbrel and the NISTmAb) prepared were prepared with Gly-X 2-AB Express and GlykoPrep 2-AB. All eluted samples were adjusted to 100 µl final volume prior to UHPLC – FLR analysis (1 µl injection).

A) MabThera



B) Enbrel



C) NISTmAb

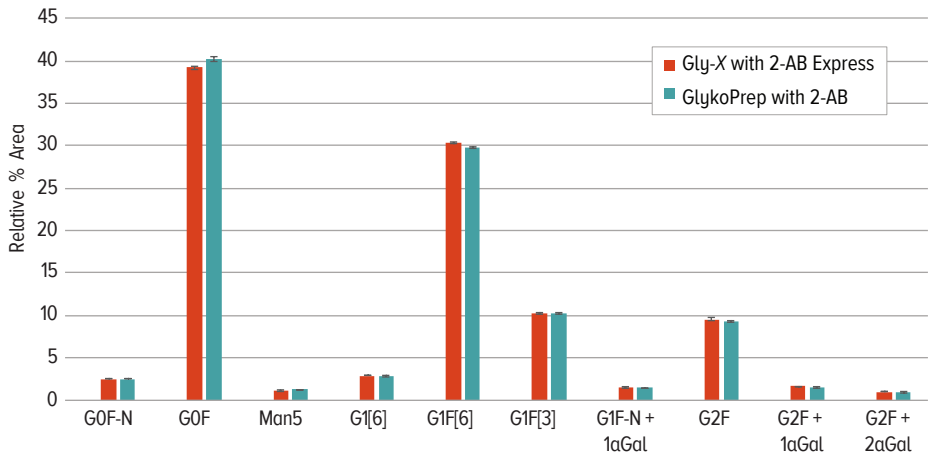


Figure 7: Comparison of relative % areas for N-glycans prepared with GlykoPrep with 2-AB, Gly-*X* with 2-AB Express and Gly-*X* with 2-AB Express (non-sodium cyanoborohydride). (A) MabThera, (B) Enbrel, (C) NISTmAb. Glycans >0.5% relative area reported for MabThera/Enbrel and >1.0% for NISTmAb.

CONCLUSIONS

1. 2-AB labeling of released N-glycans offers reproducible and reliable results that can be directly compared to historic data.
2. Gly-*X* 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.
3. 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.
4. The NISTmAb N-glycan profile is consistent with published data.

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