Application Note

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Preparation of Released N-Glycan Samples from Monoclonal Antibodies Using Agilent AdvanceBio Gly-*X* 2-AB Express for LC-Fluorescence Analysis

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Abstract

This Application Note describes the preparation of N-glycans from biotherapeutic glycoproteins for released glycan analysis. N-Glycan analysis is critical to the development and production of therapeutic proteins, as glycan composition may directly impact the safety and efficacy of the product. This protocol describes the use of the Agilent AdvanceBio Gly-*X* 2-AB Express kit for release of glycans using PNGase F, labeling through reductive amination, and cleanup of free dye within two hours rather than a full day or longer. The label used is 2-aminobenzamide (2-AB), valued for its well established use and consistency with large bodies of historical glycan analysis data.

Introduction

Glycosylation is a common feature of many biotherapeutic proteins that can affect pharmacokinetics, pharmacodynamics, and immunogenicity[1], and is frequently a critical quality attribute[2]. As such, biotherapeutic glycosylation must be carefully characterized and monitored throughout the development and production process.

Glycans are commonly derivatized prior to analysis, as they are not inherently UV-absorbing or fluorescent, and ionize poorly for MS detection. A handful of fluorescent labels have become widely used, including 2-AB and 2-AA, which modify released N-glycans through reductive amination[3]. Labels with both higher fluorescence and MS sensitivity have been introduced recently[4].

Earlier protocols for 2-AB labeling are often long, with multiple prolonged incubation periods. This not only consumes a large amount of the user's time, but also makes it impossible to obtain results and make decisions based on those results quickly. Deglycosylation was often incomplete without a long incubation period, therefore it became common to allow digestion to take place overnight. Reductive amination to affix a 2-AB label included a preceding step to dry the glycans prior to labeling, and labeling reactions were often allowed to incubate for hours. Additionally, older cleanup cartridges for removal of excess 2-AB reagent prior to LC analysis were cumbersome, and not suited for high-throughput or automated workflows.

The protocol for the Agilent AdvanceBio Gly-*X* 2-AB Express kit includes all the high-level steps for N-glycan sample preparation: denaturation, deglycosylation, labeling, and sample cleanup, as illustrated in Figure 1.

Experimental

Materials

HPLC grade acetonitrile was purchased from Sigma-Aldrich. Water was purified using a Milli-Q A10 water purification system (Millipore).

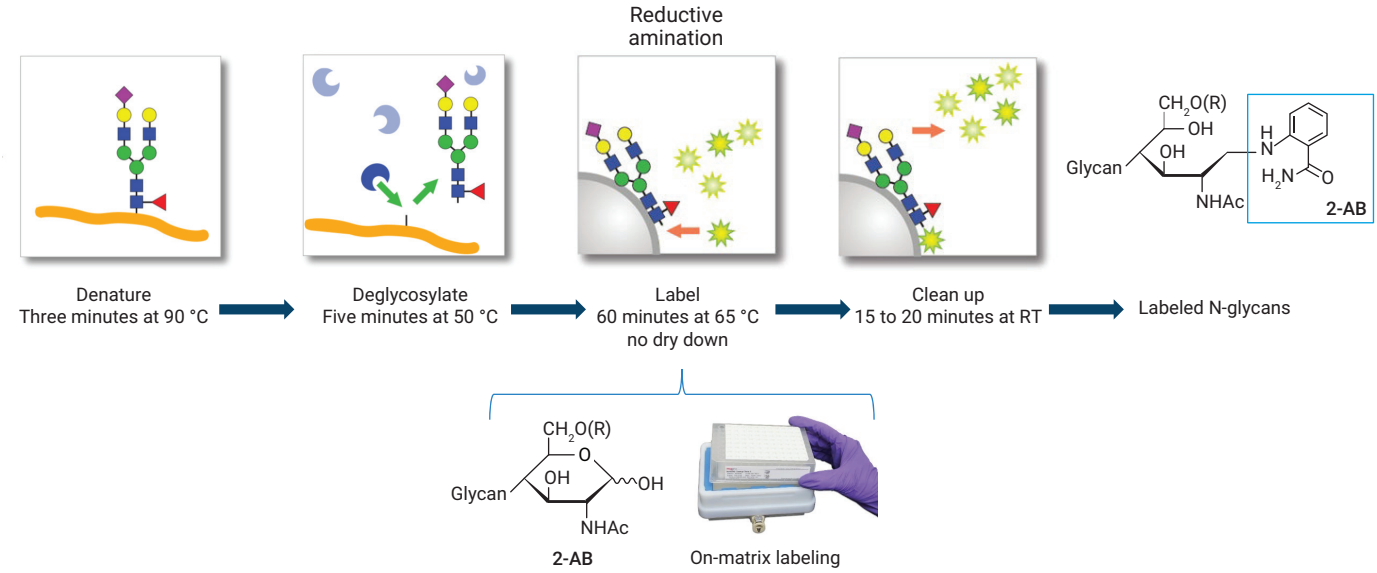

Figure 1. AdvanceBio Gly-*X* 2-AB Express workflow for release and labeling of N-glycans.

N-Glycan sample preparation

Labeled N-glycan samples were prepared using the AdvanceBio Gly-*X* 2-AB Express kit (p/n GX96-2AB). Figure 2 shows the kit components.

AdvanceBio Gly-*X* N-glycan sample preparation involves a series of enzymatic and chemical steps, beginning with denaturation of the target protein (Figure 3). A denaturing reagent is added, and the sample is incubated at 90 °C for three minutes. Effective unfolding of the protein allows for highly efficient, in-solution cleavage of N-glycans using the enzyme PNGase F in only five minutes[4]. PNGase F is specific to N-linked glycans, so only N-glycans are removed from the protein (Figure 4), while any O-linked glycans and nonenzymatic glycosylation remain attached to the protein.

The labeling and cleanup steps take place on a HILIC-based solid phase stationary support. Released N-glycans are converted to -OH form in solution prior to loading onto the stationary phase, followed by the 2-AB labeling reagents, and the phase is incubated for one hour at 65 °C (Figure 5). After labeling is complete, the excess reagents are rinsed away through a series of acetonitrile washes. The labeled N-glycans are then eluted with water (Figure 6). On-matrix labeling eliminates the need to dry the released glycans prior to 2-AB labeling.

Instrumentation

Samples were analyzed using an Agilent AdvanceBio Glycan Mapping column on an Agilent LC composed of:

- Agilent 1290 Infinity II high speed pump (G7120A)
- Agilent Infinity multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1260 Infinity fluorescence detector (G1321B)

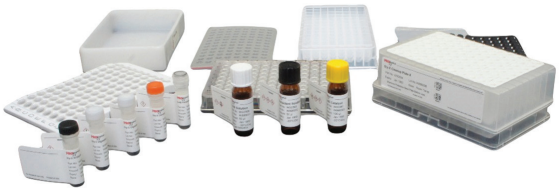

Figure 2. Components of the AdvanceBio Gly-*X* 2-AB Express N-glycan sample preparation kit.

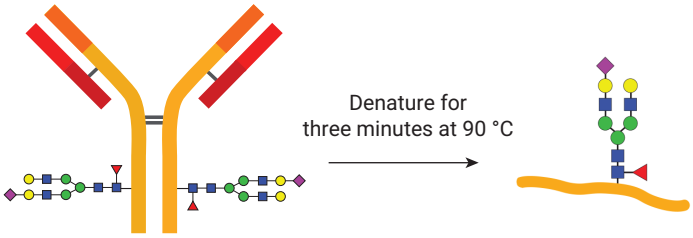

Figure 3. The sample protein is first denatured to effectively deglycosylate in the subsequent step.

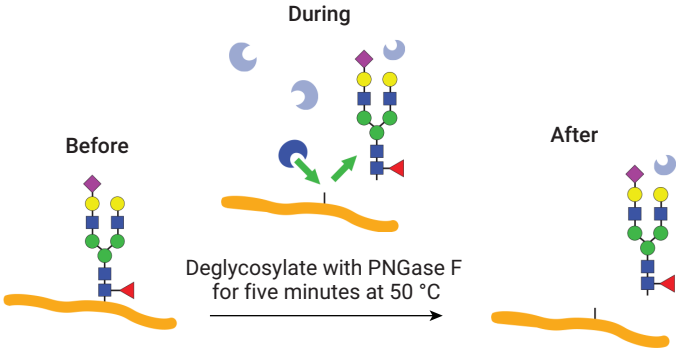

Figure 4. N-glycans are rapidly cleaved from the protein using PNGase F.

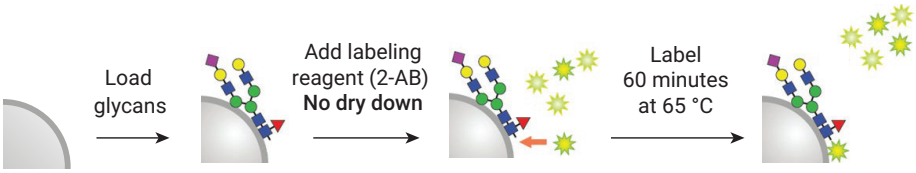

Figure 5. The sample is loaded onto a HILIC-based solid phase support on a vacuum-based cleanup plate. The 2-AB labeling reagents are then added to the solid phase support and incubated for one hour. No dry-down step is necessary.

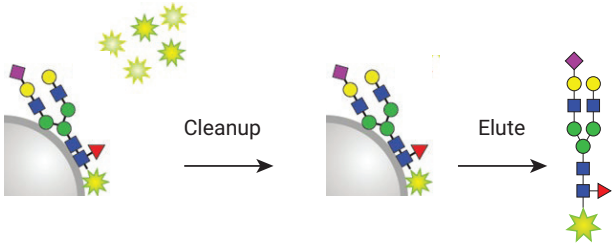

Figure 6. Excess labeling reagents are rinsed away, and labeled glycans are eluted from the solid stationary phase.

Software

- Agilent MassHunter Acquisition
- Agilent MassHunter Qualitative Analysis software

Results and discussion

MabThera and Enbrel 2-AB N-glycan samples were analyzed by LC/FLD. Figure 7 shows representative chromatograms. MabThera has a simpler glycosylation pattern, with Enbrel showing higher relative levels of sialylated glycans. Reproducibility of the sample preparation is of utmost importance, so samples may be compared across production lots. Variability measured needs to truly originate from changes in the sample, rather than as an artifact of sample handling or analysis.

Table 1. LC method.

Parameter	Value
Column	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)
Column Temperature	40 °C
Mobile Phase	A) 50 mM ammonium formate, pH 4.5 B) acetonitrile
Flow Rate	0.5 mL/min
Gradient Program	Time (min) %B Flow rate (mL/min) 0.0 82 0.4 2.0 82 0.4 2.5 77 0.4 48.0 62 0.4 49.0 40 0.4 51.5 40 0.4 52.0 82 0.4 54.0 82 0.6 58.0 82 0.6 58.5 82 0.4
Injection Volume	1 µL (equivalent to glycans from 0.4 µg of protein)
Detection	Agilent 1290 Infinity II FLD Excitation 260 nm Emission 430 nm

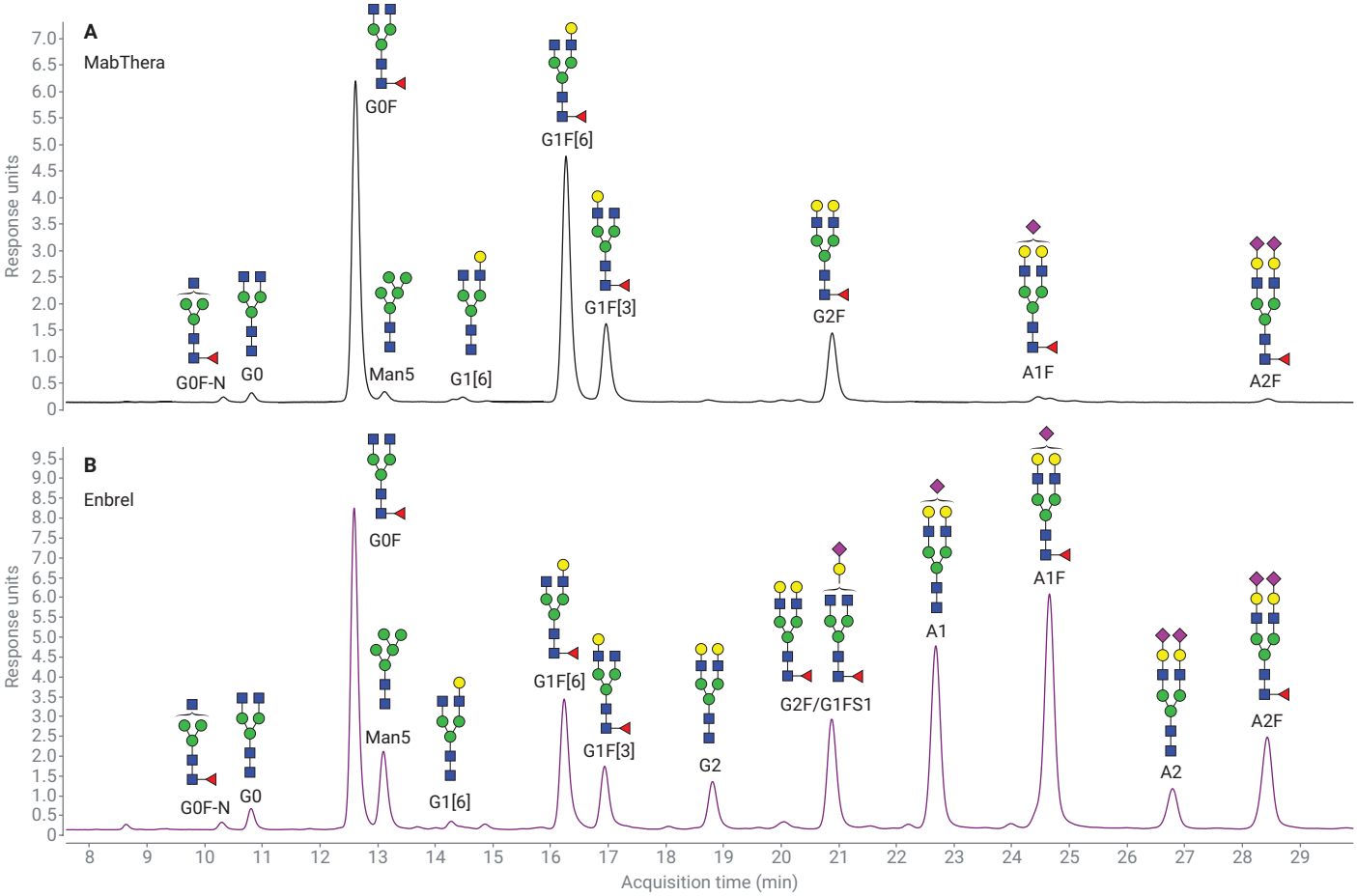

Figure 7. Representative chromatograms showing separation of 2-AB labeled N-glycans from A) MabThera and B) Enbrel.

Table 2 shows the relative percent area of the major N-glycan species detected in three preparations of a MabThera sample. The average percent area, along with standard deviation and relative standard deviation (%CV), are reported. The variations between sample preparations are all low, except for the lowest abundance glycans. Precision is more challenging near the limit of detection, so higher variation is to be expected for these peaks.

For any researcher to change sample preparation methods, the data obtained must be either equivalent, or in some way superior to the results of the previous method. With other labeling chemistries available, a major driver behind continuing to use 2-AB is the ability to compare results to older data obtained with other 2-AB labeling protocols. Figure 8 shows the relative percent area of the N-glycans detected in triplicate preparations of Enbrel. The relative abundances are very similar between the samples prepared using AdvanceBio Gly-*X* 2-AB Express and ProZyme GlykoPrep 2-AB, an earlier generation of 2-AB sample preparation available from ProZyme.

Conclusion

N-Glycan samples can be fully prepared for analysis within two hours with Agilent AdvanceBio Gly-*X* 2-AB Express, versus older methods that take a full day, including an overnight incubation and dry down prior to labeling. Data produced for a variety of glycoproteins are highly reproducible, and consistent with data obtained from older 2-AB sample preparation methods.

Table 2. Relative % area of major N-glycan species from three preparations of a MabThera sample.

Glycan	RT	Relative % Area					
		1	2	3	Average	Standard Deviation	%CV
G0F-N	10.32	0.56	0.57	0.57	0.57	0.01	1.02
G0	10.81	1.09	1.01	0.98	1.03	0.06	5.54
G0F	12.62	39.85	39.31	39.33	39.50	0.31	0.78
Man5	13.12	0.74	0.62	0.69	0.68	0.06	8.82
G1[6]	14.47	0.66	0.6	0.62	0.63	0.03	4.88
G1F[6]	16.27	34.65	34.81	34.67	34.71	0.09	0.25
G1F[3]	16.96	10.65	10.47	10.6	10.57	0.09	0.88
G2F	20.89	10.15	10.78	10.83	10.59	0.38	3.58
A1F	24.45	1.13	1.26	1.17	1.19	0.07	5.61
A2F	28.44	0.52	0.57	0.54	0.54	0.03	4.63

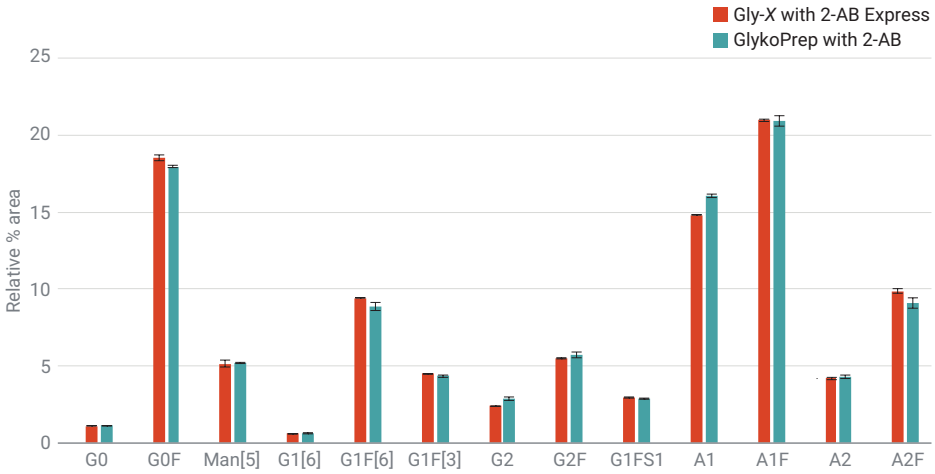

Figure 8. Samples produced using the AdvanceBio Gly-*X* 2-AB Express kit produce data equivalent to samples prepared using older methods, such as ProZyme GlykoPrep 2-AB, shown here for N-glycans from Enbrel. n = 3 for all data.

References

1. Liu, L. Antibody Glycosylation and its Impact on the Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies and Fc-Fusion Proteins. *J. Pharm. Sci.* **2015**, *104(6)*, 1866–1884.
2. Reusch, D.; Tejada, M. L. Fc Glycans of Therapeutic Antibodies as Critical Quality Attributes. *Glycobiology* **2015**, *25(12)*, 1325–1334.
3. Ruhaak, L. R.; *et al.* Glycan Labeling Strategies and Their Use in Identification and Quantification. *Anal. Bioanal. Chem*. **2010**, *397(8)*, 3457–3481.
4. Kimzey, M.; *et al.* Development of a 5-Minute Deglycosylation Method for High Throughput N-Glycan Analysis by Mass Spectrometry. *ProZyme Technical Note*, Bulletin 4001, Rev E.

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