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Advancing Glycosciences

Development of a 5-Minute Deglycosylation Method and Instant Labeling Dye for High Throughput N-Glycan Analysis by Mass Spectrometry

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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.

Analysis of N-glycans typically involve the labeling of enzymatically-released glycans with a tag to allow for fluorescence detection; a process that often requires numerous hours or days to complete. In addition to fluorescence (FLR) detection, mass spectrometry (MS) is also often utilized. Unfortunately, many of the commonly-used fluorescent tags are limited with regard to MS sensitivity.

We present a novel N-glycan sample preparation workflow, Gly-*X*™, which uses a 5-minute in-solution digestion, instant labeling, and cleanup of excess label and denaturant prior to analysis.

- The Gly-*X* N-glycan sample preparation workflow can be completed in as little as 45 minutes
- The workflow includes InstantPC™, a new instant glycan labeling reagent that provides markedly increased MS and FLR sensitivity
- The InstantPC glycan label is suitable for hydrophilic interaction liquid chromatography (HILIC) utilizing both FLR and MS detection, allowing flexibility for screening applications as well as in-depth characterization of N-glycans

METHODS

Materials:

Etanercept (Enbrel®), Cetuximab (Erbitux®)

Sample Preparation:

- Gly-*X* N-Glycan Rapid Release and Labeling with InstantPC Kit [2, 3, 4]
- Waters GlycoWorks RapiFluor-MS N-Glycan Kit
- GlykoPrep® Digestion Module
- GlykoPrep 2-AB and developmental Procainamide Labeling Modules

HILIC Chromatography:

- Waters H Class UPLC System
- Waters BEH Glycan columns

The Gly-*X* workflow and kit components (product code GX96-IPC) are shown in Figure 1. The protocol may be completed in as little as 45 minutes (16 samples).

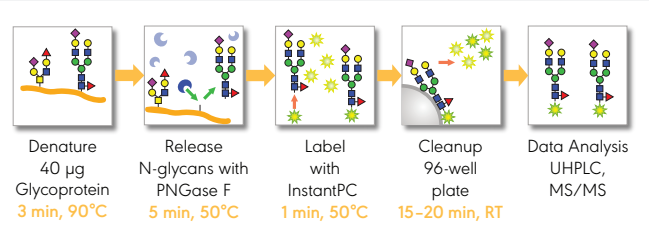

FIGURE 1: Gly-*X* with InstantPC workflow and kit components.

RESULTS

Gly-*X* 5-Minute Deglycosylation

Glycoproteins are incubated with a proprietary denaturant prior to incubation with PNGase F for 5 minutes at 50°C. The denaturant and elevated temperature allowed by optimized buffer conditions speed up deglycosylation. Figure 2 shows the deglycosylation of Cetuximab, a monoclonal antibody with Fc and Fab N-linked glycosylation sites. Performance is comparable to deglycosylation with the Waters RapiFluor-MS Kit, which includes the reagent RapiGest.

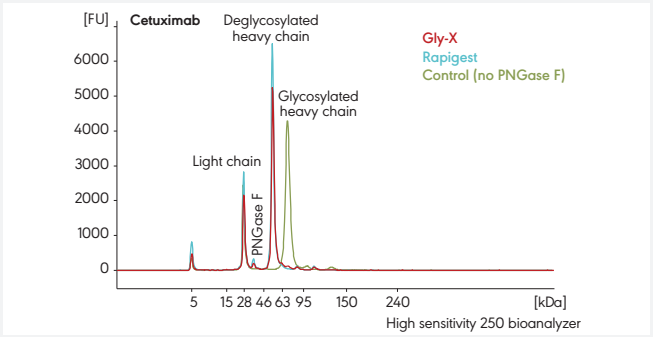

FIGURE 2: Removal of Cetuximab N-glycans with PNGase F. Cetuximab was digested with either the Gly-*X* protocol (——) or the Waters RapiGest (——) protocol. Untreated control (——) is also shown. Proteins were separated using an Agilent High Sensitivity 250 Bioanalyzer.

InstantPC Labeling of N-Glycans

InstantPC is a novel instant glycan label for HILIC-FLR and LC-MS/MS. The InstantPC structure (Figure 3) is an activated form of procaine which labels glycosylamines released by PNGase F digestion. Attachment of InstantPC forms a stable urea linkage with the N-glycan. InstantPC will add a monoisotopic mass of 261.14773 Da to a reducing end. Using the MS conditions provided, the $[M+2H]^{+2}$ adduct is the most abundant ion for InstantPC-labeled bianetannary glycans, such as those from Enbrel.

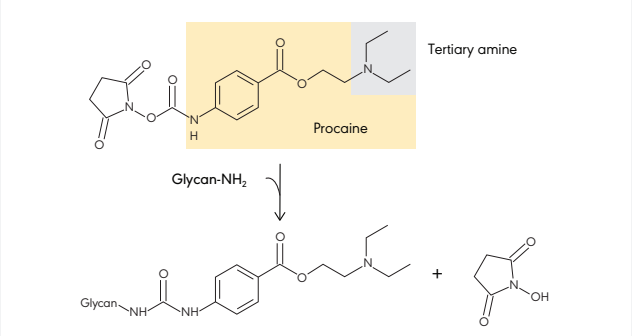

FIGURE 3: Structure of InstantPC. A novel glycan label.

Cleanup of InstantPC-Labeled N-Glycans

The Gly-*X* workflow includes a cleanup step to remove free dye and denaturant from labeled glycans, in a 96-well, vacuum-driven format. Gly-*X* cleanup preserves more than 95% of labeled glycans (data not shown). Sialylated glycan species are preserved as shown in Figure 4A. In addition to removing free label, the cleanup also removes denaturant from the labeled glycans. The denaturant is undetectable by LC-MS (Q-TOF) (Figure 4B). This is a useful feature for LC-MS analysis.

HILIC-FLR Profiles of InstantPC-Labeled N-Glycans

HILIC elution profiles for Enbrel InstantPC N-glycans are shown in Figure 5. The elution order is similar to other glycan labels such as 2-AB (data not shown). HILIC methods of varying length can be used depending on the complexity of the glycan profile – (A) shows Enbrel N-glycans separated with a 15-minute method, (B) shows Enbrel N-glycans separated with a 60-minute method. The free dye peak is minimal, as Gly-*X* cleanup removes more than 99.97% of free dye (data not shown). The relative % peak area data obtained from samples prepared with Gly-*X* is highly reproducible (Table 1).

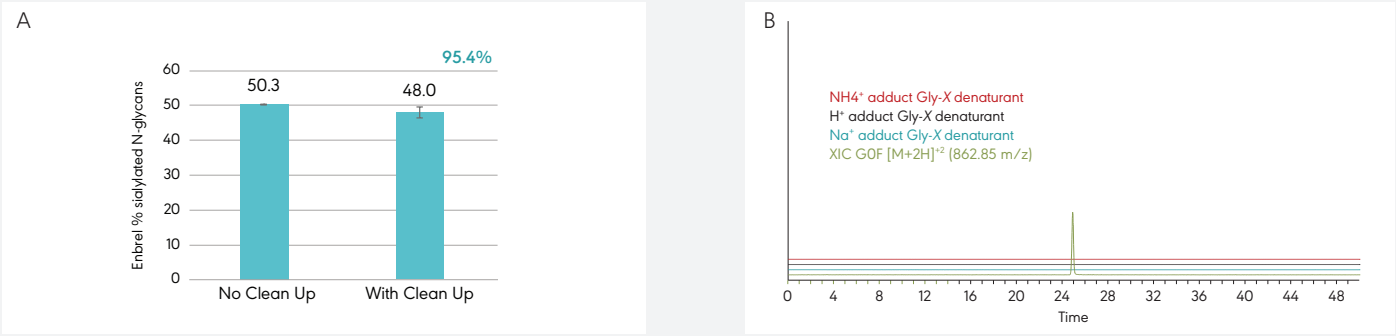

FIGURE 4: Gly-*X* Cleanup. (A) The relative % area of sialylated Enbrel N-glycans was calculated for glycans prepared using Gly-*X* with InstantPC kit with and without the final cleanup step. (B) LC-MS (Q-TOF) analysis of Enbrel InstantPC-labeled glycans from Gly-*X*. The top three traces are extracted ion chromatograms (XIC) for the expected region of Gly-*X* denaturant, (no MS signal is observed in this region as Gly-*X* denaturant is removed by Gly-*X* cleanup). For comparison, the green trace is an XIC for InstantPC-labeled G0F glycan.

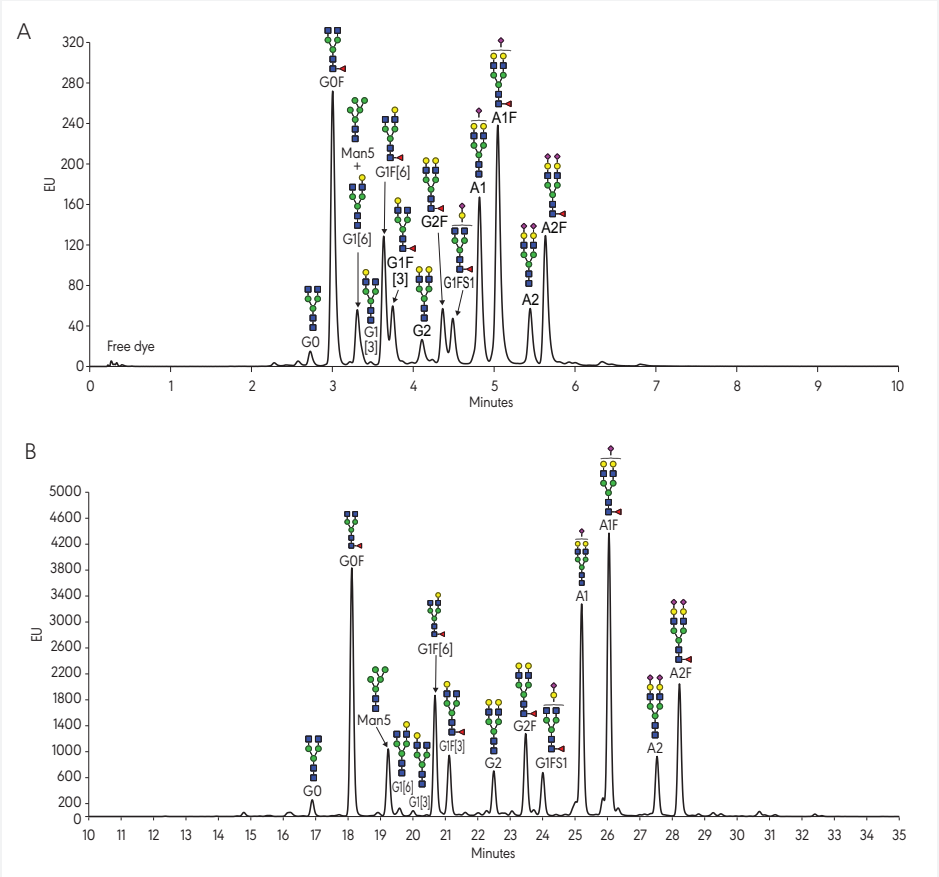

FIGURE 5: HILIC-FLR profiles of Enbrel InstantPC-labeled N-glycans prepared utilizing the Gly-*X* with InstantPC Kit. (A) a 15-minute gradient (B) a 60-minute gradient.

TABLE 1: Relative % peak area for N-glycans with >2% total peak area prepared from Enbrel, n = 8.

Glycan	% Area	% Area CV
G0F	19.8	2.1
Man5	4.7	1.5
G1F[6]	9.4	1.5
G1F[3]	4.8	2.4
G2	2.5	2.4
G2F	4.5	1.0
G1FS1	4.1	0.9
A1	13.7	2.5
A1F	20.3	0.9
A2	4.8	0.3
A2F	10.7	2.2

Comparison of InstantPC to Other Glycan Labels

Most commonly used labels for glycan analysis ionize poorly, so fluorescence is typically the only choice for detection of low abundance glycans. InstantPC has the highest LC-FLR signal of all glycan labels tested (Figure 6A). The next best label for fluorescence was Procainamide, which was prepared by reductive amination, a more time-intensive workflow. In addition to high FLR signal in LC, InstantPC contains a tertiary amine which generates high MS signal in positive mode (Figure 6B).

MS conditions: Waters Xevo G2-S QTof, + mode, capillary voltage 2.8 kV, cone voltage 30 V, source temperature 120°C, desolvation temperature 350°C, scan time 0.8 second, m/z range 300–2000 Da.

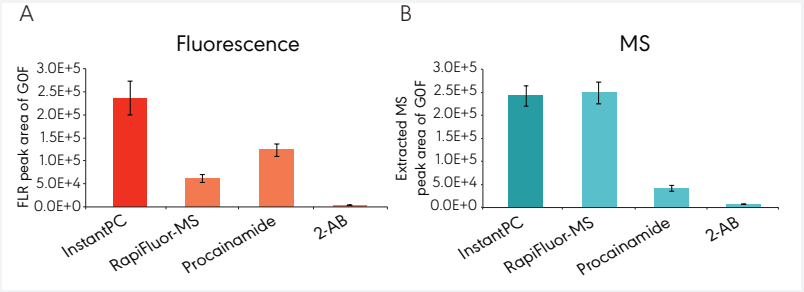

FIGURE 6: Comparison of FLR (A) and MS (B) Response. N-glycans from equal amounts glycoprotein sample (Enbrel) were prepared with GlykoPrep digestion module, labeled with InstantPC, RapiFluor-MS, procainamide and 2-AB according to manufacturer instructions, and measured by UHPLC. Bars represent peak area of G0F N-glycan species.

MS/MS

InstantPC is suitable for Collision Induced Dissociation (CID) MS/MS. As with other positively charged tags such as Procainamide, the CID profile contains mostly glycosidic cleavages with some cross-ring fragmentation. A2F (aka G2FS2 or F(6)A2G(4)2S(3)2) from Enbrel is a disialylated fucosylated biantennary glycan with α(2,3)-linked NANA (Figure 7).

MS/MS conditions Collision energy ramp of 40–60 V for +1; 15–30 V for +2; 15–25 V for +3; 1.0 second scan time, m/z range 50–2000 Da.

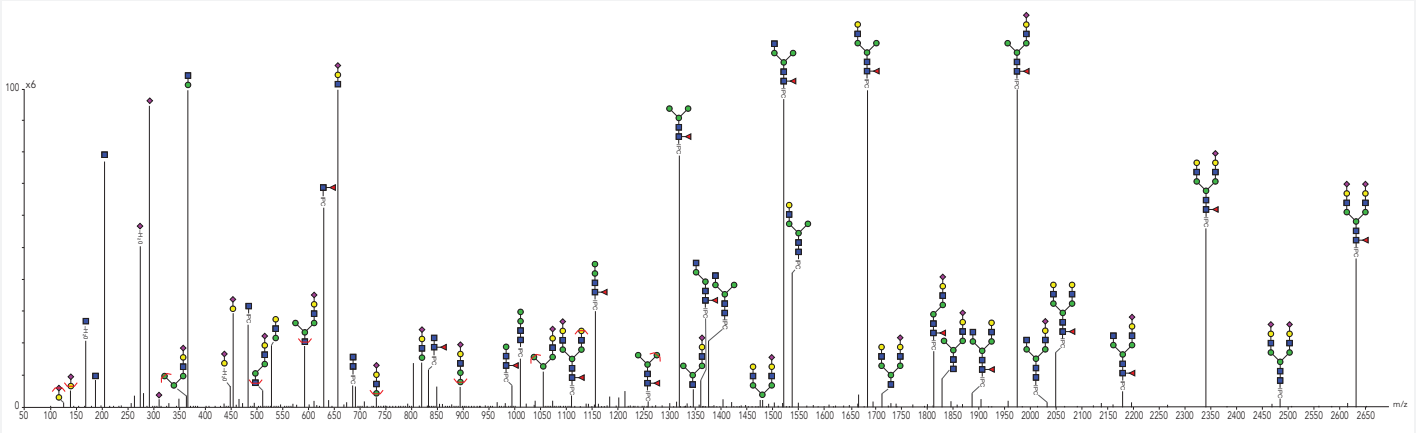

FIGURE 7: CID MS/MS of IPC-labeled A2F.

Labeled Glycan Standards

ProZyme supports users of InstantPC by making labeled N-glycan standards available. Table 2 shows a provisional list.

TABLE 2: InstantPC-labeled N-glycan standards.

Glycan + Structure	Product Code	Glycan	Product Code
Human IgG N-glycan library	GKPC-005	A1F	GKPC-315
GU Ladder	GKPC-503	A2	GKPC-312
G0	GKPC-301	A2F	GKPC-313
G0F	GKPC-302	Man5	GKPC-103
G1	GKPC-317	Man6	GKPC-104
G1F	GKPC-316	Man7	GKPC-105
G2	GKPC-304	Man8	GKPC-106
G2F	GKPC-305	Man9	GKPC-107

CONCLUSIONS

- The Gly-*X* platform uses a 5-minute deglycosylation, InstantPC labeling, and rapid vacuum cleanup allowing labeled N-glycans to be prepared in as little as 45 minutes
- In addition to removing free dye (99.97%), Gly-*X* cleanup removes Gly-*X* denaturant below detectable limits by LC-MS (Q-TOF)
- InstantPC has the highest fluorescence response of any N-glycan label tested
- InstantPC generates high MS response, superior to 2-AB and Procainamide, and comparable to RapiFluor-MS
- N-glycans labeled with Instant PC are suitable for CID MS/MS in positive mode

REFERENCES

1. Liu, *J Pharm Sci.* 2015; 104: 1866-1884
2. US Patent 8124792
3. US Patent 8445292
4. Patents Pending

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