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

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SUMMARY

The structure of N-linked glycans can play a critical role in the pharmacology of therapeutic proteins, potentially affecting immunogenicity, pharmacokinetics and pharmacodynamics(1).

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 features:

- 5-minute in-solution enzymatic deglycosylation
- 1-minute labeling of released N-Glycans
- Cleanup of excess label and denaturant prior to analysis by LC or LC-MS
**INTRODUCTION**

Gly-X N-Glycan Rapid Release and Labeling kit with InstantPC (2, 3, 4) is a next generation N-glycan preparation method that features an enhanced workflow combined with an MS compatible dye.

- 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.

**MATERIALS AND METHODS**

**Materials:**
Etanercept (Enbrel), Cetuximab (Erbitux)

**Sample Preparation**
Gly-X N-Glycan Rapid Release and Labeling with InstantPC Kit (GX96-IPC)
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

**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.

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).

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

**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 (data not normalized).

**Figure 3:** Structure of InstantPC, a novel glycan label.
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. 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 biantennary glycans, such as those from Enbrel.

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. Unlike RapiFluor MS, the Gly-X denaturant is undetectable by LC-MS (Q-TOF, positive mode) (Figure 4B, 4C). Commonly observed adducts such as NH4^+, H^+ and Na^+ are not observed in the Gly-X system, making it more ideal 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 GOF glycan. (C) LC-MS (Q-TOF) analysis of Enbrel N-Glycans using a Rapifluor MS kit shows residual denaturant.

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

Labeled Glycan Standards

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

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Table 2: InstantPC-labeled N-glycan standards.

References

2. US Patent 8124792 Patent Pending
3. US Patent 8445292
4. Patents Pending