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TECHNICAL NOTE

Gly-Q

Gly-X

GlykoPrep

Glyko Enzymes

Glyko Standards

InstantPC

InstantAB

2AB

APTS

PhycoLink

PhycoPro

RPE & APC conjugates

Streptavidins

Automated N-Glycan Sample Preparation with an Instant Glycan Labeling Dye for Mass Spectrometry

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SUMMARY

The characterization of glycans is an essential part of the biotherapeutic development process. Analysis typically involves the labeling of 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 detection, mass spectrometry is also often utilized.

Presented here is the use of an automated N-glycan sample preparation platform with a glycosylamine-reactive dye (InstantPC) that provides increased fluorescence and MS sensitivity. Automated N-glycan sample preparation provides reproducible data between replicates and reduced operator involvement.

Keywords

Automation

Biotherapeutic

Enbrel

Fluorescent dye

Fc glycosylation

Glycan labeling

HILIC

LC-MS

N-Glycans

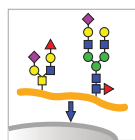
Rituxan

Sample preparation

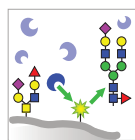
INTRODUCTION

The GlykoPrep (1) platform streamlines N-glycan sample preparation with a simple and reliable cartridge-based format. Glycan release, purification, labeling and cleanup can be completed in as little as 3 hours using the cartridge spin method. Integration of GlykoPrep with the Agilent AssayMAP Bravo provides out-of-the-box automation for increased precision and walkaway time.

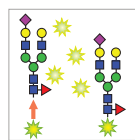
A recent addition to the GlykoPrep platform (Figure 1) is the new MS-compatible InstantPC Dye (2, 3, 4). InstantPC (Figure 2) is a glycosylamine-reactive instant glycan labeling dye that was developed for enhanced fluorescence and MS ionization properties. To demonstrate the full integration of this new dye/labeling protocol onto the AssayMAP Bravo, N-glycans from monoclonal IgG Rituxan (rituximab) and Fc-fusion protein Enbrel (etanercept) were released and labeled with InstantPC using the GlykoPrep workflow automated on the Agilent AssayMAP Bravo. GlykoPrep InstantPC-labeled N-glycans were then separated using Hydrophilic interaction liquid chromatography (HILIC) and analyzed by Q-ToF MS. The results from the automated workflow are compared to the GlykoPrep InstantPC spin format.



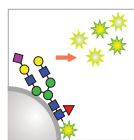
Denature & Immobilize
(30-60 minutes)



Digest with N-Glycanase
(15-60 minutes)



Fluorescent Labels
(1 min-1 hour)



Cleanup & Elute
(15-30 minutes)



Agilent AssayMAP Bravo
Liquid Handling Platform

SPIN		AUTOMATED
Microfuge Format	Plate Format	Automation Format
<ul style="list-style-type: none"> Up to 24 samples Microfuge tubes Microcentrifuge ~3 hours Attended 	<ul style="list-style-type: none"> Up to 4 x 96 samples 96-well microplates Centrifuge ~3 hours Attended 	<ul style="list-style-type: none"> n x 96 samples 96-well microplates AssayMAP Bravo ~3 hours per plate Walk-away



Figure 1: GlykoPrep N-Glycan Sample Prep Platform. N-glycan labels available are 2-AB, APTS, InstantAB, InstantPC.

MATERIALS AND METHODS

Sample preparation

Enbrel lot # 1058467, Rituxan lot # 938802

GlykoPrep-plus Rapid N-Glycan Sample Preparation with InstantPC (96-ct) [GPPNG-PC]

GlykoPrep Rapid N-Glycan Preparation with InstantPC (96-ct) [GP96NG-PC]

GlykoPrep Rapid N-Glycan Preparation with 2-AB (96-ct) [GP96NG-AB]

GlykoPrep Rapid N-Glycan Preparation with InstantAB (96-ct) [GP96NG-LB]

UHPLC 40-Minute Method

- Column: Amide, 2.1 x 150 mm, 1.7 μ m
- Flow rate: 0.4 ml/min
- Gradient: 20–38% 50 mM Ammonium Formate pH 4.4 in 30 minutes
- Temperature: 45 °C
- Excitation and Emission:
 - InstantPC 285 nm, 345 nm
 - InstantAB 278 nm, 344 nm
 - 2-AB 360 nm, 428 nm

MS conditions

Q-TOF + 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.

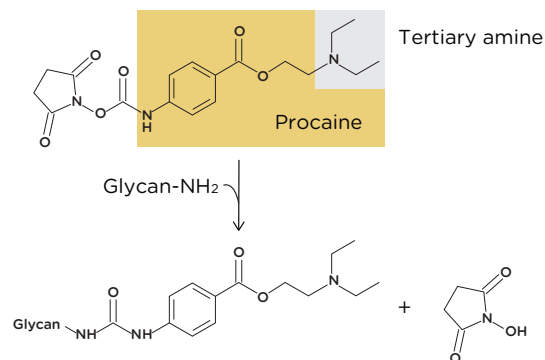


Figure 2: Structure of InstantPC. A novel glycan label.

RESULTS

InstantPC Dye Structural features

InstantPC is a novel (2, 3, 4) instant glycan label for UHPLC, LC/MS and MS/MS. The InstantPC structure (Figure 2) is an activated form of procaine which labels glycosylamines released by PNGase F digestion. InstantPC attachment 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 for InstantPC-labeled biantennary glycans, such as those from Enbrel.

UHPLC and MS Performance

Most commonly used labels for glycan analysis ionize poorly, so fluorescence is typically the only choice for analysis of low abundance glycans. InstantPC has the highest LC-fluorescence (FLR) of all glycan labels tested (Figure 3A). 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 3B).

When comparing glycans labeled with different dyes, dye structure affects the relative retention times. As shown in Figure 4, using this UHPLC method, 2-AB-labeled glycans elute first followed by InstantPC and then InstantAB. Selectivity differences for various glycans can also be observed, for example, with the Man5/G1 pairs and with A1F (5). Labeled glycan standards (including InstantPC, InstantAB and 2-AB) are available to verify peak assignments. The relative peak intensities for the same amount of glycan also varies by the dye selected for labeling (Figure 4, inset).

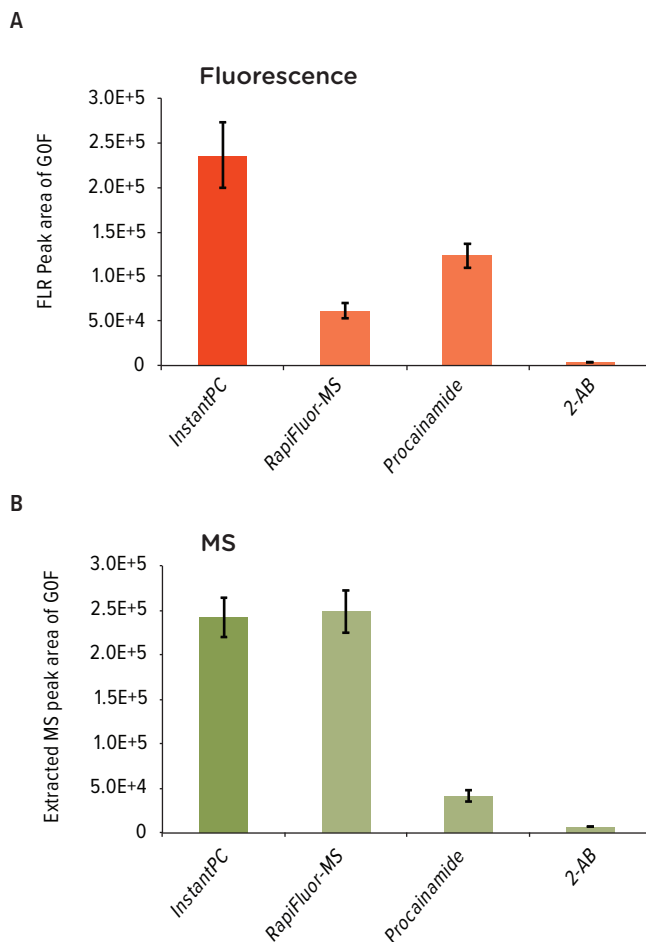


Figure 3: Comparison of FLR (A) and MS (B) Response. Glycans from equal amounts glycoprotein samples were prepared with GlykoPrep sample prep, labeled with InstantPC, RapiFluor-MS, procainamide and 2-AB according to manufacturer instructions, and measured by UHPLC. Bars represent peak area of GOF N-glycan species.

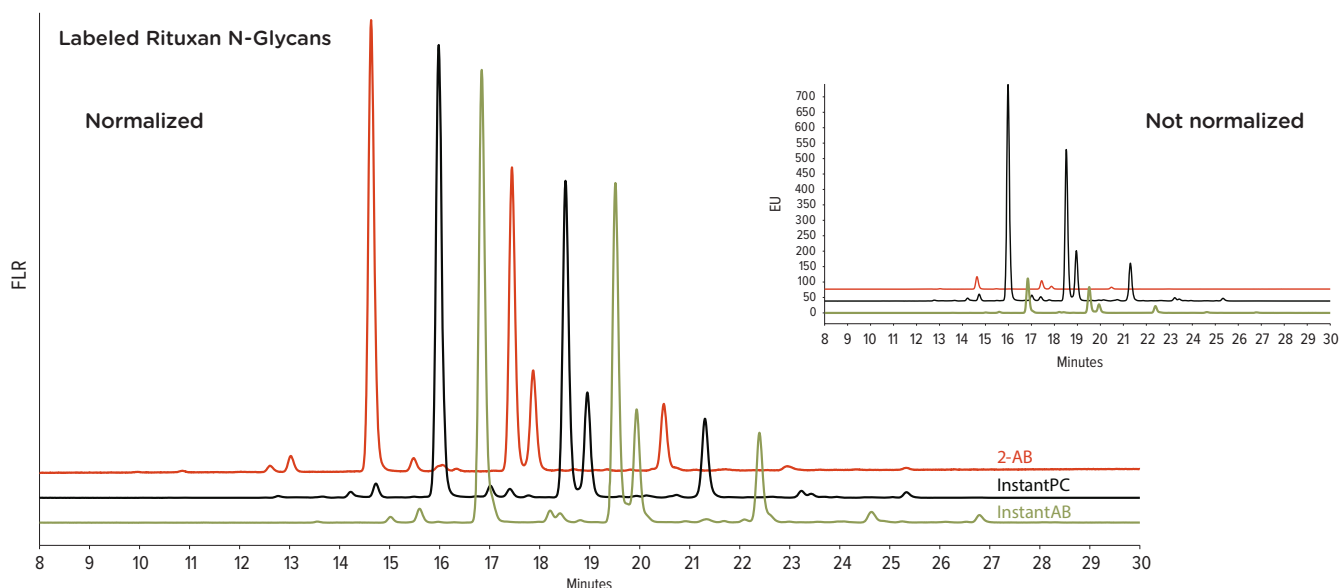


Figure 4: Comparison of InstantPC HILIC retention time to other N-glycan dyes. Separation of glycans released from Rituxan and labeled with 2-AB (green); InstantPC (blue); InstantAB (black) by HILIC chromatography. Data normalized to the peak height of the most abundant glycan species, G0F. Inset: unnormalized chromatograms.

GlykoPrep InstantPC Automation

GlykoPrep workflows allow sample processing flexibility and have been optimized to yield quantitative deglycosylation, high labeling efficiency and the effective removal of excess labeling reagents without loss or degradation of N-glycans. Two concerns that often arise regarding automation are the maintenance of sample fidelity during preparation and the reproducibility of results. The results below demonstrate that the automation of sample preparation with GlykoPrep InstantPC and the AssayMAP Bravo have addressed these concerns.

Peak tables were assembled based upon the retention and migration times of the N-glycans from Rituxan (Figure 5A) and Enbrel (Figure 5B) for the two GlykoPrep InstantPC methods: 1) Plate-based spin method and 2) Automated AssayMap Bravo method. All peaks with relative peak areas below 0.5% were excluded from the final profile.

Percent peak areas across platforms showed close correlation with the average of the difference varying less than 0.6% for peaks greater than 1% (Figures 6 and 7). Results from Enbrel show that the relative abundance of sialylated glycans, typically most sensitive to degradation and bias, were also in close alignment across methods (Figure 7). Both GlykoPrep methods were found to be reproducible with average CV's of 1.5% (Spin) and 1.6% (Bravo).

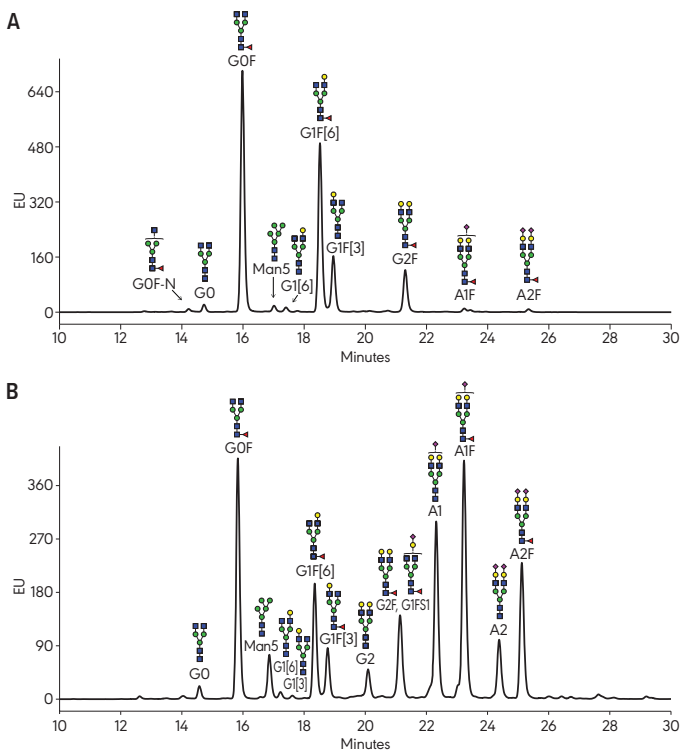


Figure 5: 40-minute HILIC separation of InstantPC-labeled N-glycans. (A) Representative chromatogram, Rituxan (B) Representative chromatogram, Enbrel.

CONCLUSIONS

1. InstantPC has the highest fluorescence response of any glycan label tested
2. InstantPC shows high MS response, superior to Procainamide, and comparable to RapiFluor-MS
3. InstantPC labeling has been adapted to GlykoPrep platform, and results are comparable between manual and automated methods
4. Automation of GlykoPrep InstantPC N-glycan sample preparation on the Agilent AssayMAP Bravo provides reproducible results with increased walk away time

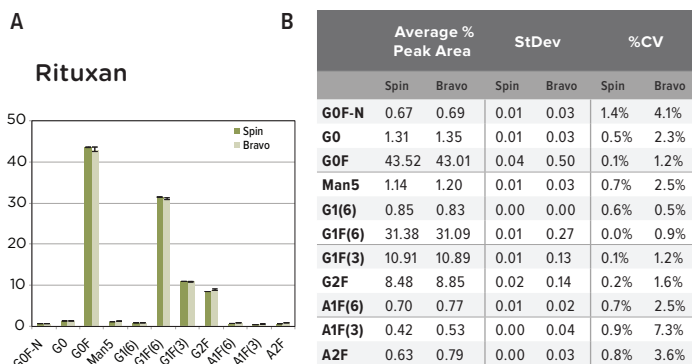


Figure 6: Comparison of manual spin and Bravo automated methods for Rituxan. InstantPC-labeled glycans were detected by fluorescence and average % peak areas calculated for 16 replicates (Panel A). For each set of replicates the Stdev and %CV were calculated (Panel B).

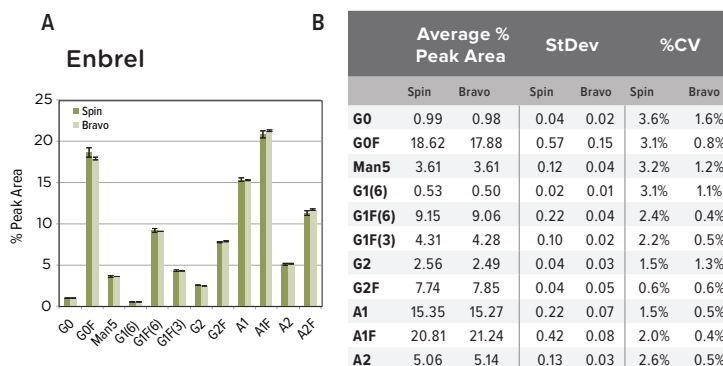


Figure 7: Comparison of manual Spin and Bravo automated methods for Enbrel. InstantPC-labeled glycans were detected by fluorescence and average % peak areas calculated for 16 replicates (Panel A). For each set of replicates the Stdev and %CV were calculated (Panel B).

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1. US Patent 8198063
2. US Patent 8124792
3. US Patent 8445292
4. Patent Pending
5. Development of an Instant Glycan Labeling Dye for High Throughput Analysis by Mass Spectrometry. M. Kimzey et al., Prozyme poster reprint ASMS 2015