

## Development of an Instant Glycan Labeling Dye for High Throughput Analysis by Mass Spectrometry

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## INTRODUCTION

Glycosylation of biotherapeutic proteins is frequently a critical quality attribute; therefore the characterization of glycans on biotherapeutics is an important activity in the development process. A common approach is to release and label N-glycans with a tag to allow for fluorescence detection; a process that often requires numerous hours to complete. Unfortunately, many of the most commonly used fluorescent tags have poor MS sensitivity.

- We present a novel instant glycan labeling reagent, InstantPC™ (IPC) that provides markedly increased MS and FLR sensitivity
- The workflow utilizes a rapid in-solution digestion, instant labeling, and cleanup of excess IPC label
- IPC is shown to lend itself to both rapid (5-minute) and high-resolution (60-minute) HILIC methods for N-glycan separation, allowing flexibility for screening applications and in-depth characterization

## METHODS

### Materials

Enbrel® lot # 1036862, Herceptin® lot # B1629B11

### N-Glycanase digestion, IPC labeling and cleanup

A developmental protocol was used to release N-glycans with a 5-minute in-solution digest. In ProZyme's proprietary method [1], a solution of 20 µl of glycoprotein at 2 mg/ml (40 µg total) was treated with a shelf-stable master mix consisting of buffer, reductant, and denaturant. The sample was briefly heated, prior to incubation with N-Glycanase for 5 minutes at 50 °C. Released glycosylamines were labeled with 5 µl of IPC, and the labeled N-glycans were cleaned up and eluted in aqueous buffer using a modified protocol for GlykoPrep® CU cartridges.

### 5-Minute screening UHPLC method

Agilent AdvanceBio Glycan Mapping column, 2.1 x 100 mm 2.7 µm, flow rate 1.4 ml/min, 23–40% 100 mM Ammonium Formate pH 4.4 in 4 minutes, column temperature 35 °C, excitation 285 nm, emission 345 nm.

### 60-Minute high-resolution UHPLC method

Agilent AdvanceBio Glycan Mapping column, 2.1 x 150 mm 2.7 µm, flow rate 0.4 ml/min, 20–46% 50 mM Ammonium Formate pH 4.4 in 43.5 minutes, column temperature 45 °C, excitation 285 nm, emission 345 nm.

### 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.

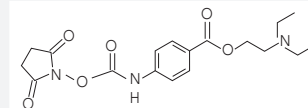
### MS/MS conditions

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.

## RESULTS

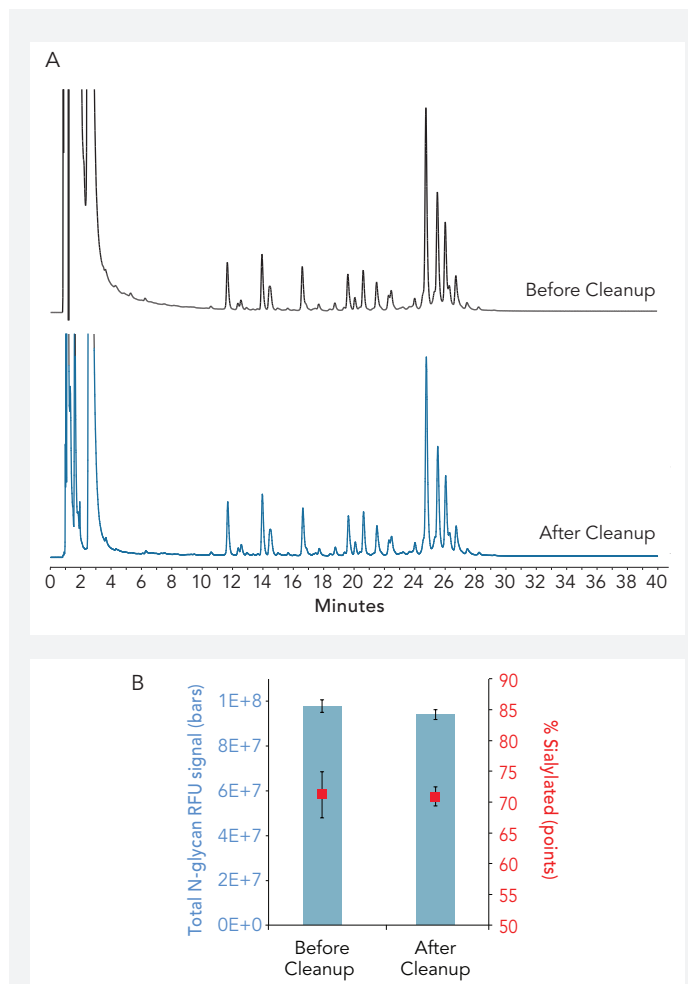
### Structural features of IPC.

The IPC structure (Figure 1) is an activated form of Procaine [2,3,4], which labels glycosylamines released by PNGase F digestion. IPC attachment forms a stable urea linkage with the N-glycan.



**FIGURE 1: Structure of InstantPC.** A novel instant glycan label for MS [2,3,4].

**Sample preparation.** Glycoprotein preparation with IPC uses an N-Glycanase digestion time of 5 minutes. The short digestion time was enabled by ProZyme's new proprietary methods, which use denaturants that are both enzyme- and MS-friendly [1]. The denaturant is convenient and rugged, as the solution is stable at room temperature and does not require special handling procedures. To illustrate the cleanup, a mixture of human IgG and bovine fetuin was labeled with IPC. Half of the samples went through the cleanup and the other half did not. Figures 2A and 2B show unbiased and near-complete recovery of this complex mixture of IPC-labeled glycans.

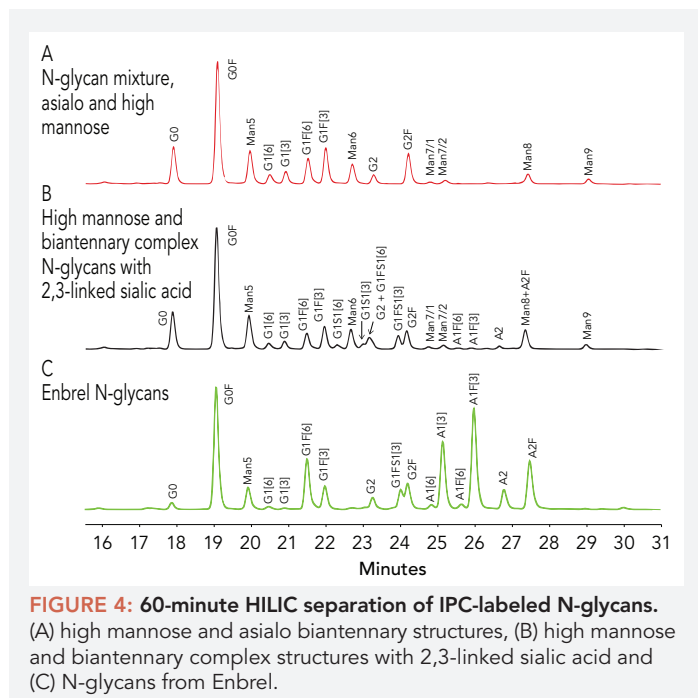
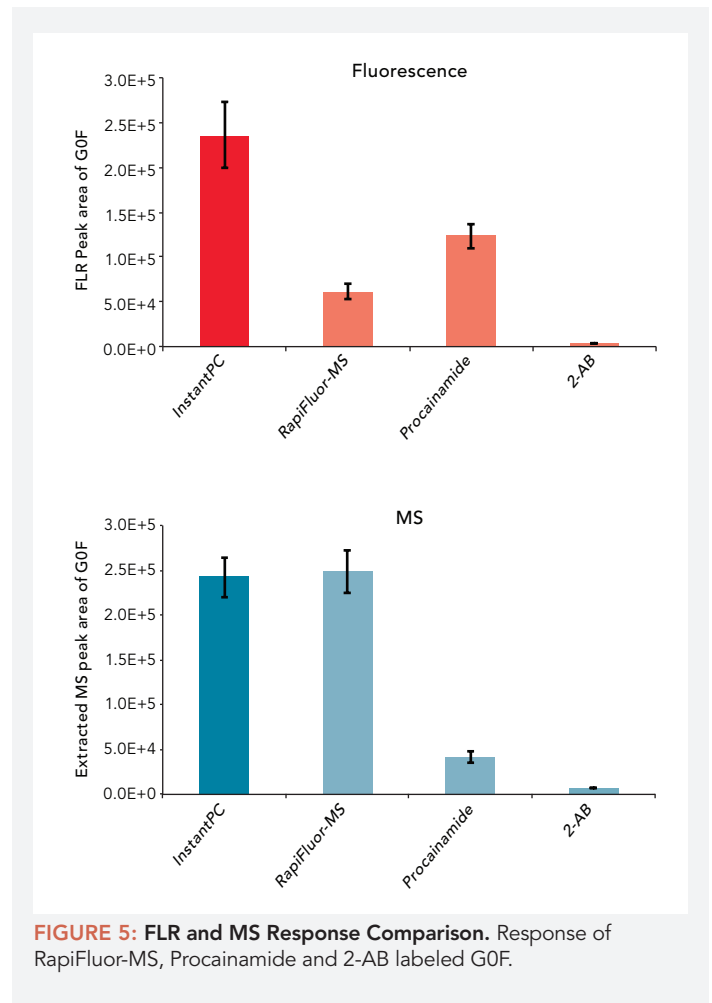
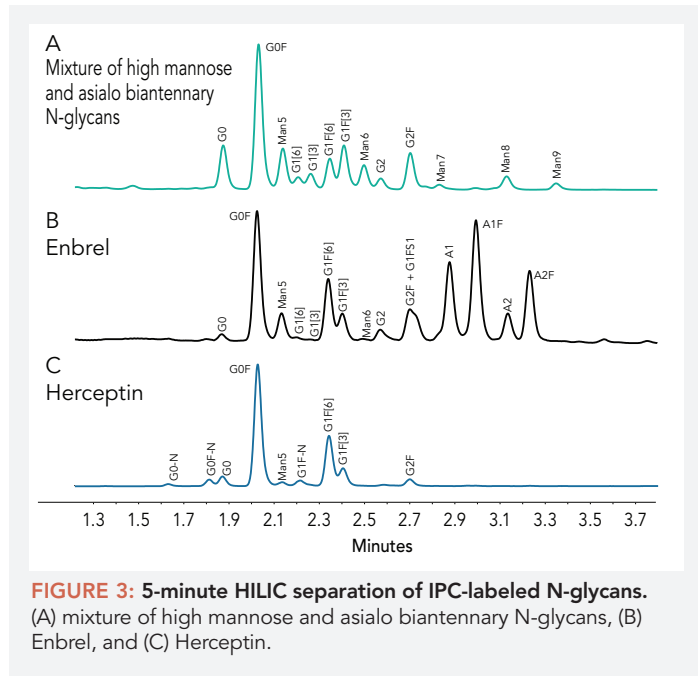


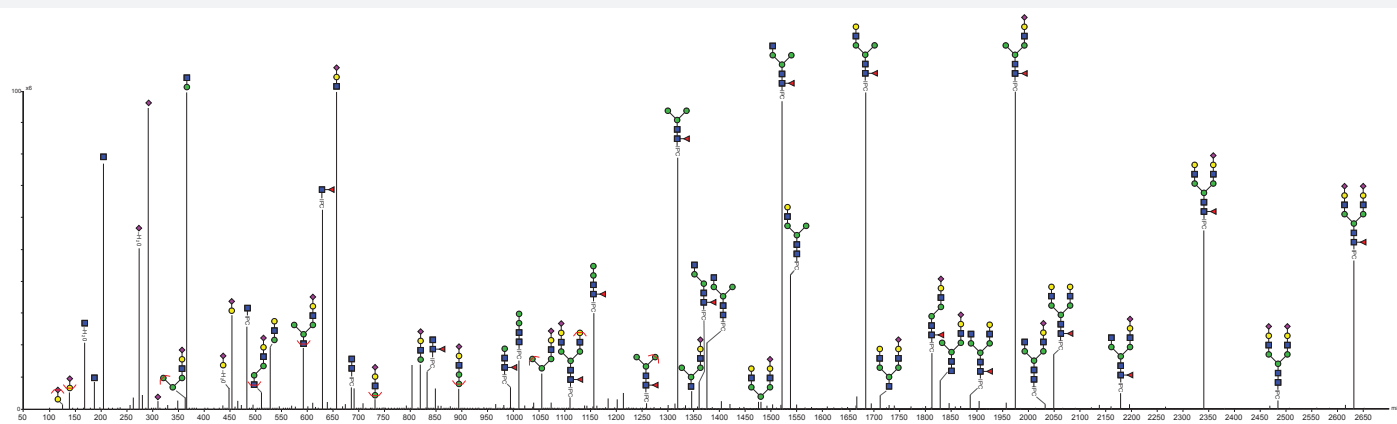
**FIGURE 2: Cleanup of IPC-labeled N-glycans.** (A) HILIC profiles of N-glycans from human IgG (75%) and bovine fetuin (25%), ± cleanup. Neutral peaks 10–18 minutes, sialylated peaks 18–30 minutes. (B) Triplicate samples before and after cleanup of free IPC. Total glycan fluorescence in blue bars, % sialylated peaks on secondary axis.

**UHPLC performance.** One of the features of IPC compared to Procainamide or 2-AB is that IPC is less polar, so IPC-labeled glycans elute earlier on HILIC. This results in shorter retention times, so the gradient must be adjusted to start with a higher percentage of acetonitrile. AdvanceBio Glycan Mapping columns (2.7  $\mu\text{m}$ ) are particularly useful for IPC-labeled N-glycan analysis, as they provide a key separation of Man5 from G1[6] which may be important for screening mAbs during cell-culture optimization (Figure 3). If greater resolution is required, a 60-minute gradient may be used (Figure 4).

**MS performance.** IPC contains a tertiary amine which generates high MS signal in positive mode. IPC will add a monoisotopic mass of 261.14773 Da to a reducing end. Most commonly used labels for glycan analysis ionize poorly, so FLR is typically the only choice for analysis of low abundance glycans. IPC-labeled glycans, however, have such an improved ionization efficiency that the MS signal is comparable to or better than FLR sensitivity. For the N-glycans on Enbrel, most IPC-labeled biantennary glycan ions are  $[M+2H]^{2+}$  using the conditions provided.

**Comparison to other labels.** IPC has the highest LC-fluorescence of all glycan labels tested. The next best label for fluorescence was Procainamide, which was prepared by reductive amination (Figure 5). The MS response of IPC was comparable to the glycosylamine reactive label RapiFluor-MS, yet RapiFluor-MS is not suitable for screening using this method, as Man5 does not separate from G1 (Figure 7 - see next page). Software programs such as UNIFI are not recommended for quantification of coeluting species such as Man5/G1[6] because it will assign the relative peak area to the most intense m/z only.

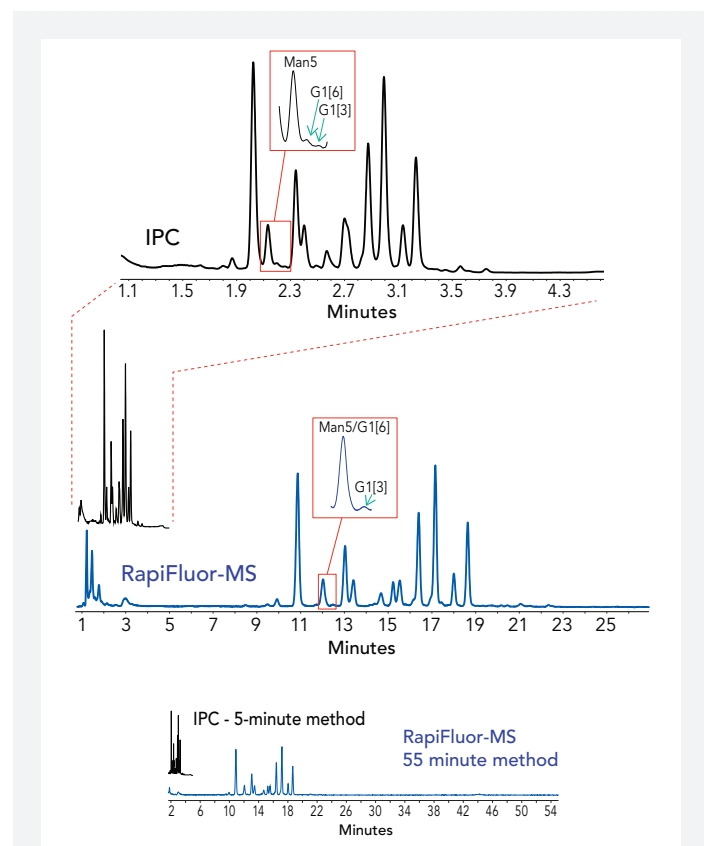




**FIGURE 6:** CID MS/MS of IPC-labeled A2F

**MS/MS.** IPC is suitable for Collision Induced Dissociation 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 FA2G2S2) from Enbrel is a disialylated fucosylated biantennary glycan with  $\alpha$ 2,3-linked NANA (Figure 6).

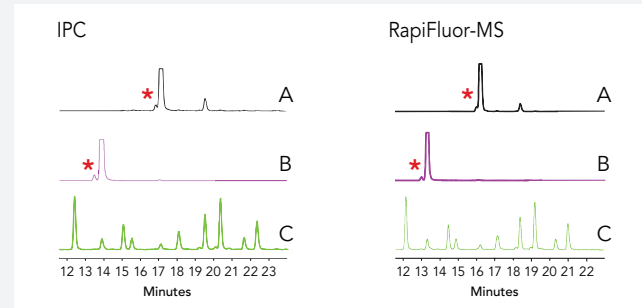
both IPC and RapiFluor-MS, however, share the labeling artifact that is apparent for afucosylated reducing end GlcNAc (Figures 8 and 9). This is a feature of GlcNAc glycosylamine labeling and efforts to understand this artifact are underway.



**FIGURE 7: Run Time and Resolution Comparison.** Resolution of Man5, G1[6], and G1[3] IPC-labeled glycans vs. RapiFluor-MS labeled glycans. IPC-labeled glycans using AdvancedBio Glycan Mapping column vs. RapiFluor-MS labeled glycans using Glycan BEH column.

## REFERENCES

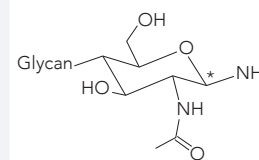
1. Patent Pending
2. US Patent 8124792B2
3. US Patent 8445292B2
4. Patent Pending



**FIGURE 8: Glycosylamine labeling artifact.** Extracted ion chromatograms from (A) G2 and (B) Man5 N-glycans show the labeling artifact (\*) peak for Enbrel. FLR trace is shown in (C). Left is IPC, right is RapiFluor-MS. The artifact is ~1.5% of main peak and is consistent across both glycosylamine reactive labels. This artifact is typically observed only in high resolution methods and coelutes with parent peak with screening and medium resolution gradients.

## FIGURE 9: GlcNAc Glycosylamine.

The early artifact peak results from an isomer, likely at C1 (\*) or an alternative chair conformation of the reducing end glycosylamine ring. C2 epimers (GlcNAc to ManNAc) may be ruled out as they result in later LC retention times.



## CONCLUSIONS

- 1 IPC has the highest fluorescence response of any glycan label tested
- 2 IPC shows high MS response, superior to Procainamide, and comparable to RapiFluor-MS
- 3 IPC rapid LC methods separate Man5 from G1 for mAb screening
- 4 New 5-minute deglycosylation method using MS friendly denaturant enables both screening and in-depth characterization on N-glycans using a single workflow

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