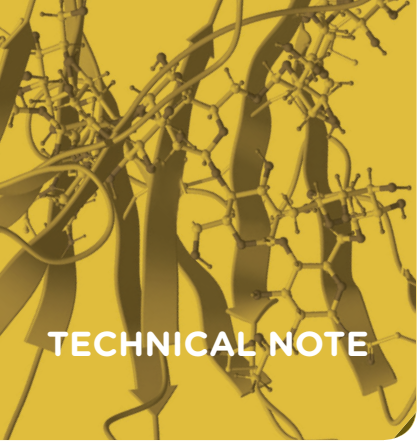

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InstantPC

InstantAB

InstantQ

2-AB

APTS

PhycoLink

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RPE & APC Conjugates

Streptavidins

Rapid N-Glycan Sample Preparation Workflows for Liquid Chromatography and Capillary Electrophoresis Platforms

John Yan, Aled Jones, Emily Dale, Andres Guerrero, Michael Kimzey, Vaishali Sharma, Tom Rice, Justin Hyche, Ted Haxo, Sergey Vlasenko

SUMMARY

- Analysis of N-glycans released from biotherapeutics frequently relies on the addition of a fluorescent label.
- ProZyme Gly-*X* in-solution deglycosylation chemistry allows for the rapid release of N-linked glycans suitable for labeling with both glycosylamine reactive InstantDyes (InstantPC and InstantQ) as well as traditional reductive amination dyes such as 2-AB.
- Fluorescent dye labeled N-glycans are typically analyzed by liquid chromatography (LC) (InstantPC and 2-AB) or capillary electrophoresis (CE) (InstantQ) separation along with fluorescence detection.
- InstantPC, InstantQ and 2-AB labeled N-glycans from MabThera (rituximab) and Enbrel (etanercept) result in comparable relative percent areas for major glycoforms including G0F, G1F[6]/[3], G2F, A2 and A2F.

Keywords

Biotherapeutic
Enbrel
MabThera
Fluorescent Dye
UHPLC
Capillary Electrophoresis
InstantPC
2-AB
InstantQ
N-Glycan labeling

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. N-glycans do not contain a chromophore or fluorophore suitable for online detection with standard liquid chromatography (LC) or capillary electrophoresis (CE) separation techniques. Typically, enzymaticallyreleased glycans must be derivatized with a tag to allow for fluorescence detection prior to analysis, a process that often requires numerous hours or days to complete (2). ProZyme Gly-*X* chemistry releases N-glycans from glycoproteins with PNGase F in 5 minutes, and released N-glycans are labeled with a choice of fluorescent dye: InstantPC, 2-AB, or InstantQ (Figure 1). The sample preparation workflow and labeled glycan separation and analysis for each dye is presented.

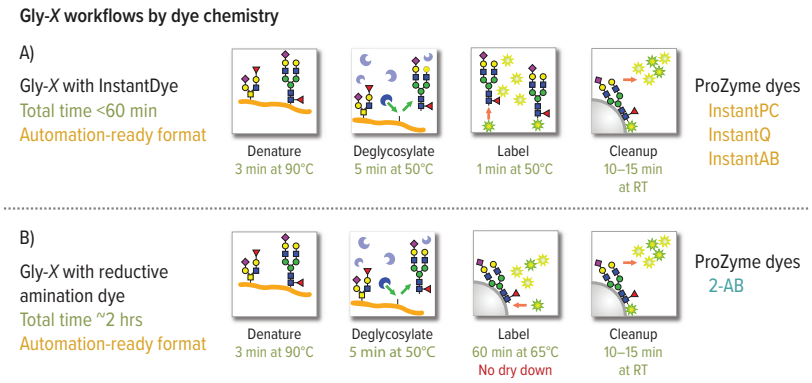

Figure 1: Gly-*X* in-solution deglycosylation technology and dye options. A) InstantDye workflow with in-solution deglycosylation and labeling followed by on-matrix cleanup; B) Reductive amination workflow with in-solution deglycosylation followed by on-matrix labeling and cleanup.

METHODS AND MATERIALS

Materials

MabThera lot # H0120B03, Enbrel lot # 1076038

N-Glycan sample preparation

Labeled N-glycans from samples (40 µg) of MabThera and Enbrel were prepared with the following instructions for the commercially available kits from ProZyme Inc.

- Gly-*X* N-Glycan Rapid Release and Labeling with InstantPC Kit (96-ct) [GX96-IPC]
- Gly-*X* N-Glycan Rapid Release and Labeling with 2-AB Express Kit (96-ct) [GX96-2AB]
- Gly-*X* N-Glycan Rapid Release and Labeling with InstantQ Kit (96-ct) [GX96-IQ]

N-Glycan separation

InstantPC and 2-AB labeled N-glycans from MabThera and Enbrel were analyzed with HILIC UHPLC separation in conjunction with fluorescence detection. InstantPC labeled N-glycans were also analyzed by HILIC UHPLC with detection by mass spectrometry (MS). InstantQ labeled N-glycans were analyzed with Gly-Q, a capillary electrophoresis instrument with LED-induced fluorescence (LEDIF) detection.

HILIC separation conditions

- 60-minute UHPLC high resolution•• method
- Column: Amide, 2.1 x 150 mm, 1.7 µm
- Flow rate: 0.4 mL/minute
- Gradient: 25–38% 50 mM ammonium formate pH 4.4, between 2.5–50•• minutes
- Column temperature: 60°C

MS conditions

Q-TOF analysis was performed in positive mode using a capillary voltage of 2.8 kV, cone voltage 30 V, source temperature 120°C, desol-vation temperature 350°C, scan time 0.8 second, m/z range 400–2000 Da.

Gly-Q analysis conditions

- Instrument: ProZyme Gly-Q Glycan Analysis System [GQ2100]
- Instrument method: N-glycans Pro-Zyme (10 kV, 120 second separation)
- Processing methods: Rituxan_Meth-od / Enbrel_Method

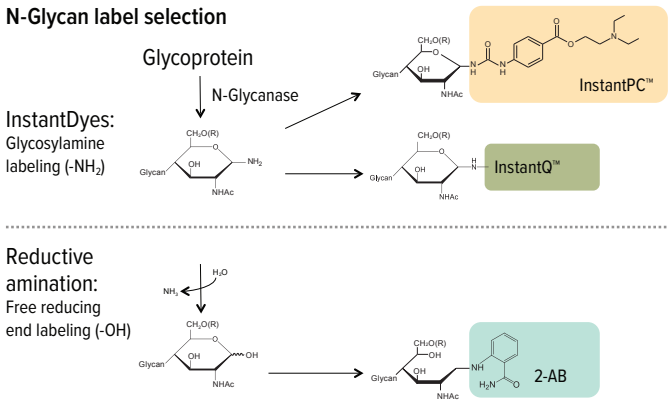

Figure 2: Comparison of InstantDye labeling and traditional reductive amination. InstantDyes react with glycosylamines released by PNGase F, while reductive amination proceeds via the free reducing end hydroxyl (-OH).

Three N-glycan dye options were used to label N-glycans released from MabThera and Enbrel and (Figure 2). The entire sample preparation protocol can be completed in less than 1 hour for instant dyes (InstantPC, InstantQ) or ~2 hours for reductive amination dye (2-AB), and the protocol can be automated on common laboratory liquid handlers (3).

- InstantPC is an N-glycan dye that allows for separation with hydrophilic interaction liquid chromatography (HILIC) with fluorescence (FLR) detection for relative quantitation of glycan species. The increased fluorescence signal of InstantPC in conjunction with its favorable properties for mass spectrometry (MS) allows for the detection of low abundance glycans (4).
- The fluorophore 2-AB (2-aminobenzamide) has been used to generate labeled N-glycan data for over 20 years and is well established in many laboratories. Traditionally, labeling with 2-AB requires the sample to be dried down prior to the labeling reaction, a step that has been eliminated with Gly-*X* 2-AB Express on-matrix labeling.
- Finally, InstantQ is a charged N-glycan dye that facilitates separation of labeled N-glycans on the Gly-Q CE system, using a run time of 2 minutes per sample with LED-induced fluorescence detection (LEDIF) (5). The Gly-Q system enables relative N-glycan quantification for up to 96 purified cell culture samples within a single workday. ProZyme supports Gly-X sample preparation workflows with a range of labeled N-glycan standards.

RESULTS

UHPLC Performance

UHPLC-FLR/MS chromatograms for InstantPC and 2-AB labeled N-glycans (Figures 3–5). When comparing the HILIC separations for these dyes, InstantPC produces a much stronger fluorescent response than 2-AB for an equivalent amount of labeled glycan injected on column. For example, peak heights of labeled N-linked glycans from MabThera are substantially higher for InstantPC compared to 2-AB (Figure 6). The strong fluorescence signal of InstantPC also allows for the detection of low abundance peaks such as G1[6] from Enbrel. InstantPC also allows the resolution of smaller peaks, for example A1F[6] from A1F[3] in both MabThera and Enbrel, while 2-AB labeling results in co-elution when analyzed under same UHPLC conditions. Overall, both dyes provide similar glycan profiles and major % peak area assignments (Table 1), by LC. InstantPC also provides a high MS signal compared to 2-AB, supporting both LC and LC/MS workflows (3).

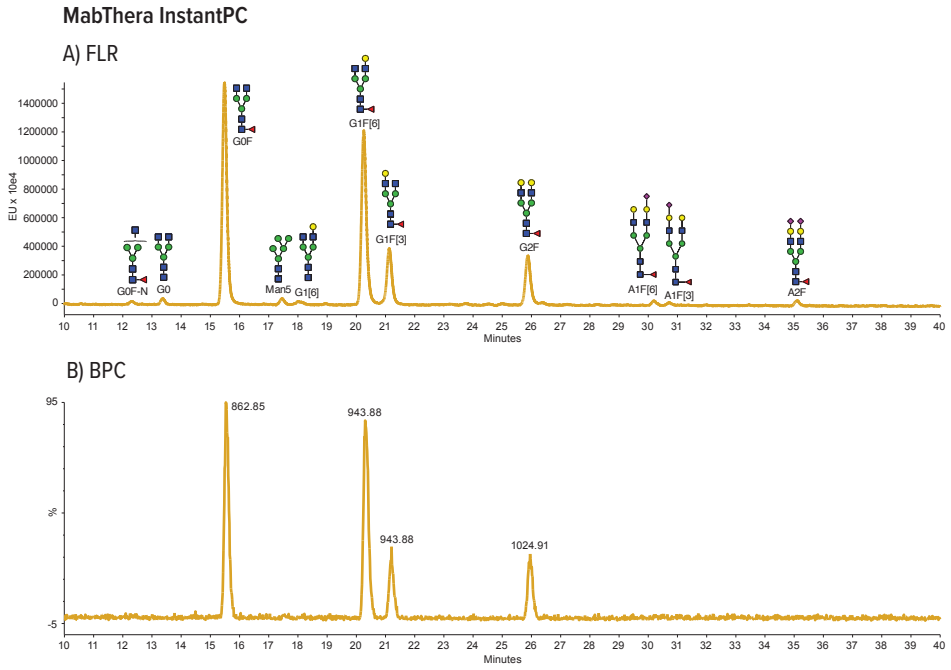

Figure 3: UHPLC fluorescence – MS profile of MabThera N-glycans labeled with InstantPC. A) Fluorescence profile (FLR), B) base peak chromatogram (BPC).

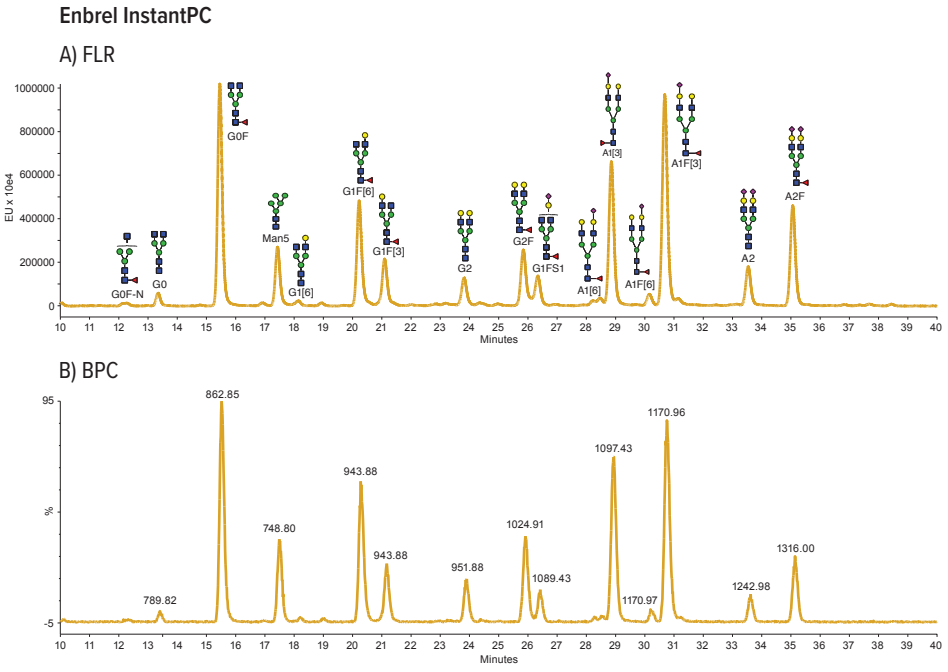

Figure 4: UHPLC fluorescence – MS Profile of Enbrel N-glycans labeled with InstantPC. A) Fluorescence profile B) base peak chromatogram.

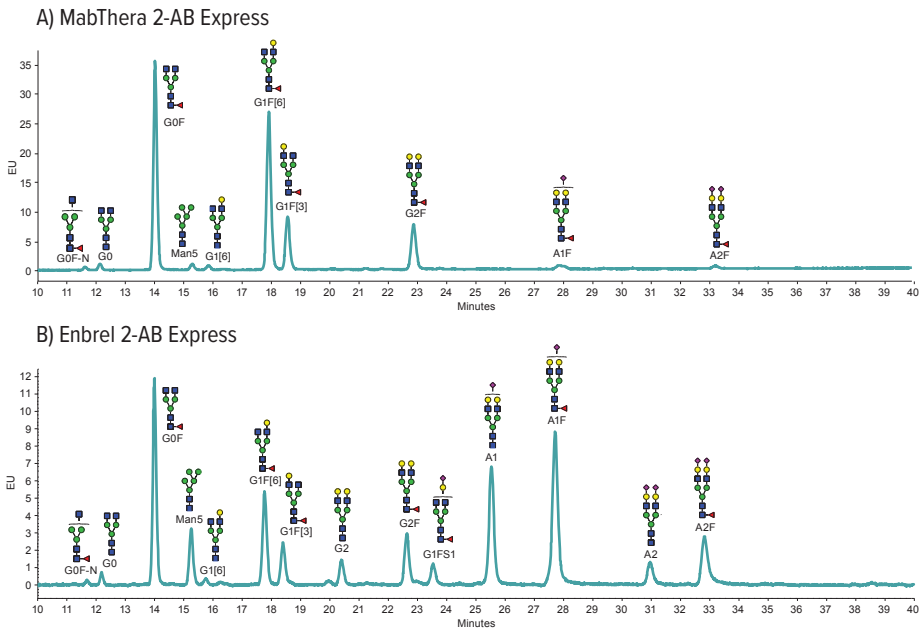

Figure 5: UHPLC fluorescence profile of 2-AB labeled glycans from A) MabThera and B) Enbrel.

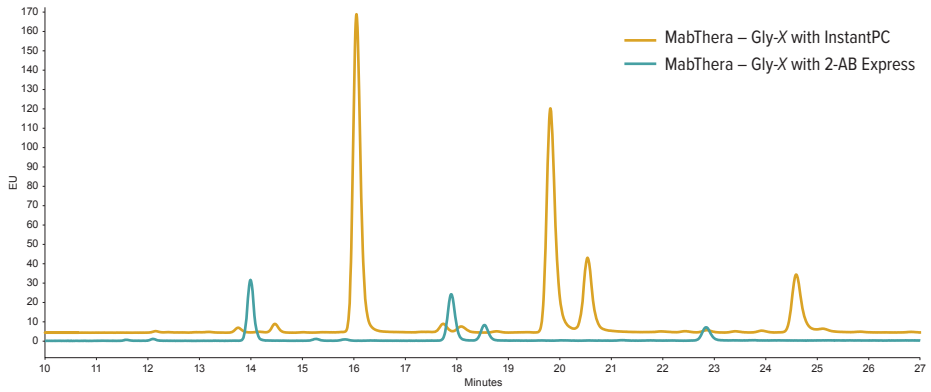

Figure 6: UHPLC-FLR chromatograms (not normalized), overlay of MabThera N-linked glycans labeled with InstantPC (——) and with 2-AB Express (——).

Gly-Q CE Analysis

The InstantQ workflow results in labeled glycans that are ready for separation by CE using the Gly-Q system (Figure 7). Unlike UHPLC separations of 60 minutes, the Gly-Q CE separations are completed in 2 minutes with Gly-Q Manager software performing automated peak analysis and glycan assignments from the glycan library. The rapid separation of InstantQ-labeled N-glycans on the Gly-Q system supports cell line screening and in-process monitoring during cell culture scale-up.

Recovery of Sialylated N-glycans

Gly-*X* sample preparation protocols utilizing Enbrel and fluorescent dyes InstantPC, 2-AB and InstantQ result in comparable total relative % areas for sialylated species (Figure 8).

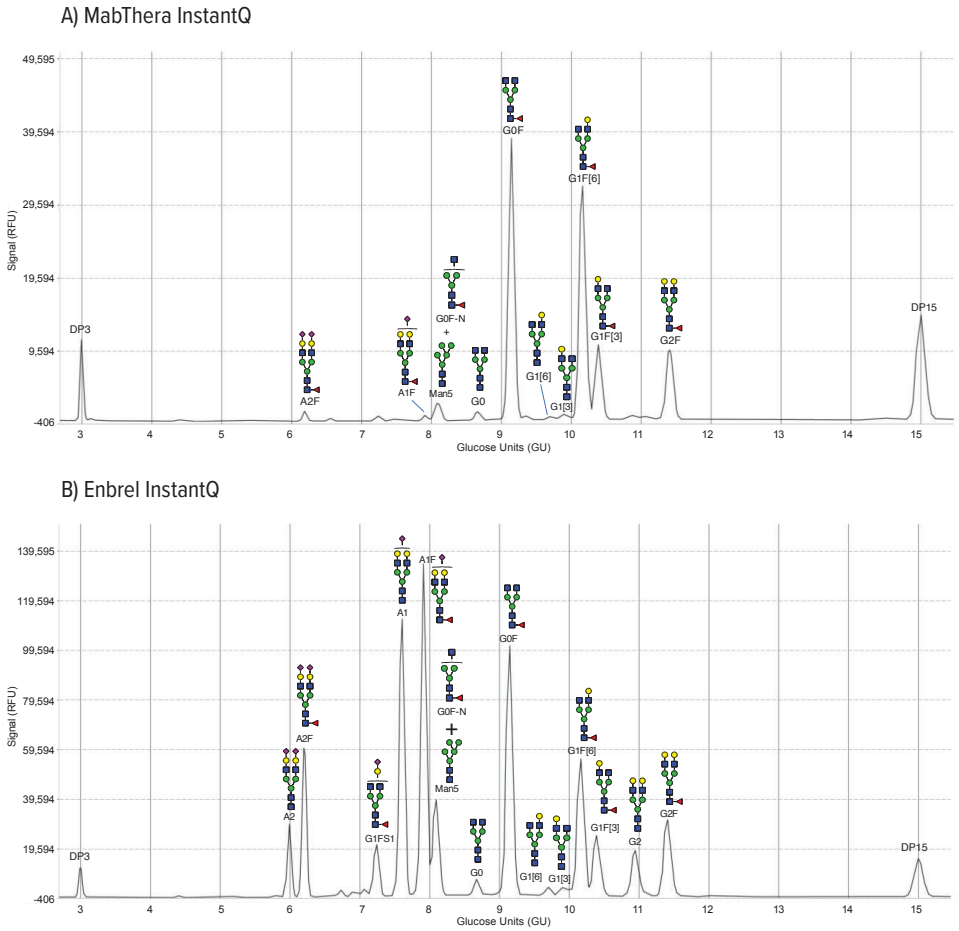

Figure 7: Gly-Q CE analysis of InstantQ labeled N-glycans from A) MabThera and B) Enbrel.

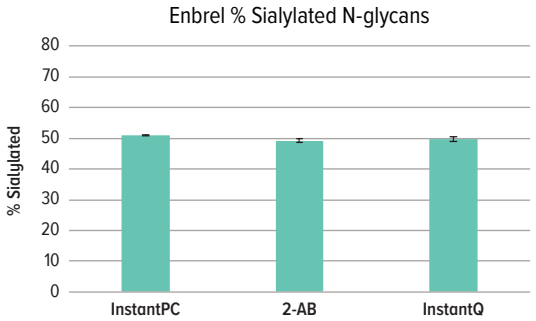

Figure 8: Sum total of sialylated N-glycans (G1FS1, A1[6]/[3], A1F[6]/[3], A2, and A2F) from Enbrel for InstantPC, 2-AB and InstantQ labeled N-glycans analyzed by UHPLC and CE (Gly-Q) respectively.

A) MabThera

	InstantPC		2-AB		InstantQ	
Name	% Area	%CV	% Area	%CV	% Area	%CV
G0	1.00	1.09	1.00	6.98	1.11	1.18
G0F	41.79	0.42	38.41	0.62	37.13	0.64
Man5	0.94	0.46	1.12	13.42	*	*
G0F-N	0.56	1.79	0.49	12.12	*	*
Man5/G0F-N	N/A	N/A	N/A	N/A	2.57	0.84
G1[6]	0.58	23.61	0.81	16.18	0.47	9.29
G1[3]	N/D	N/D	N/D	N/D	0.97	6.68
G1F[6]	33.42	0.38	34.10	0.84	33.32	0.53
G1F[3]	10.78	0.75	11.24	1.73	10.81	0.07
G2F	9.18	1.03	10.74	2.36	10.70	0.42
A1F[6]	0.66	2.75	*	*	*	*
A1F[3]	0.40	18.35	*	*	*	*
A1F[6]/[3]	N/A	N/A	1.51	6.30	0.62	0.89
A2F	0.70	8.97	0.59	18.56	0.93	3.57

Table 1: Comparison of relative % area with different labels. InstantPC and 2-AB Express labeled glycans were separated by HILIC-UHPLC and InstantQ labeled glycans separated by CE on Gly-Q and both detected by fluorescence. Average peak areas calculated for 3 replicates.

* Denotes labeled glycans that co-migrate and subsequently reported as a sum total of two species

B) Enbrel

	InstantPC		2-AB		InstantQ	
Name	% Area	%CV	% Area	%CV	% Area	%CV
G0	1.00	0.43	0.88	7.93	1.13	2.03
G0F	19.79	0.23	20.05	2.4	17.42	0.8
Man5	5.38	0.76	5.59	4.01	*	*
G0F-N	0.35	2.47	0.37	23.87	*	*
Man5/G0F-N	N/A	N/A	N/A	N/A	6.46	0.17
G1-6	0.57	2.15	0.49	22.63	0.68	3.35
G1-3	0.28	3.09	N/D	N/D	0.77	3.44
G1F-6	9.83	0.18	10.32	2.89	9.8	2.19
G1F-3	4.09	0.2	4.38	3.48	4.49	3.34
G2	2.38	0.46	2.75	5.93	3.61	3.55
G2F	5.33	0.2	5.94	2.87	5.84	3.47
G1FS1	3.37	0.21	2.37	4.56	3.1	2.1
A1-6	1.02	1.88	*	*	*	*
A1-3	13.72	0.24	*	*	*	*
A1[6]/[3]	N/A	N/A	15.13	2.19	15.56	0.88
A1F-6	0.79	9.83	*	*	*	*
A1F-3	19.87	0.71	*	*	*	*
A1F[6]/[3]	N/A	N/A	21.39	2.97	19.48	0.61
A2	3.37	0.25	2.83	7.1	3.63	4.1
A2F	8.87	0.42	7.51	2.83	7.32	3.9

CONCLUSIONS

1. Gly-*X* in-solution deglycosylation technology allows for the rapid release of N-linked glycans suitable for labeling with both glycosylamine reactive InstantDyes (InstantPC and InstantQ) as well as traditional reductive amination dyes such as 2-AB.
2. HILIC-UHPLC analysis of InstantPC-labeled N-glycans results in high resolution separation of the glycans for both MabThera and Enbrel. In addition to high fluorescence signal, InstantPC labeling of N-glycans results in high MS response.
3. Gly-*X* with 2-AB Express workflow (total time ~2 hours) results in labeled glycans ready for HILIC separation with fluorescence detection.
4. Analysis of InstantQ labeled N-glycans on the Gly-Q system provides the most rapid separation and detection of glycans of all three dyes presented,
5. Analysis of InstantPC, InstantQ and 2-AB labeled N-glycans from MabThera result in comparable relative percent areas for major glycoforms, G0F, G1F[6]/[3], G2F, A2 and A2F.
6. InstantPC offers both an increased fluorescence signal and increased MS sensitivity, allowing for the detection of low abundance glycans.

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