

On the Path to Biobetter Therapeutic Glycoproteins: Simple and Rapid Domain-Specific Screening to Target and Control Optimal Glycan Profiles



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Abstract

A strategy for "Target-directed Product Development" has been described, where multiple glycosylated forms of the protein were generated and evaluated for Critical Quality Attributes¹. In the cited case, a follow-on biological candidate was selected for further development, whose N-glycan profile demonstrated improved efficacy compared to the innovator product. Subsequently this optimal profile was controlled within tight specifications throughout clone selection, cell-culture optimization and formulation in order to speed regulatory approval. Yet significant analytical challenges were reported due to the tedious, manual methods employed and the variability of the results, requiring a significant investment of resources to accomplish.

This poster presents methods for simple and rapid screening of N-glycan profiles suitable for every aspect of drug development and manufacturing, including biocomparability studies and bioreactor monitoring and control (PAT). Use of the GlykoPrep™ platform to dramatically streamline glycoprotein sample preparation, coupled with rapid analysis using a Waters® UPLC®, allows the generation of up to 96, high-quality results overnight.

For some glycoproteins, the information presented in the total N-glycan profile may be insufficiently detailed for Target-directed Product Development. Here we show a domain-specific analysis of the N-glycans of a commercially available Fc-fusion protein. Analysis of the individual glycosylation profiles may be a better indicator to investigate the role glycosylation plays in the efficacy of the protein.

Introduction

Understanding the glycosylation of a therapeutic glycoprotein is important. For example, in antibodies, Fc glycosylation is known to modify antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) where the lack of fucose or the addition of a bisecting GlcNAc increases ADCC potency of IgG^{2,3}. Another example of the relevance of glycosylation is shown by Erythropoietin, where activity and receptor binding ability has been shown to be dependent on the degree of sialylation⁴.

In this poster Enbrel® was chosen as a model therapeutic glycoprotein because it is known to contain N-glycans on the non-Fc domain. Enbrel (etanercept) is a fusion protein composed of the Fc domain of hIgG1 and the p75 tumor necrosis factor receptor (TNFR). It is used to treat a number of long-term inflammatory diseases. Like IgG, which can be separated into the Fc and Fab domains by digestion with proteases such as papain or FabRICATOR®, Enbrel contains the hIgG CH2 and CH3 domains along with the hinge region, and may be separated into its Fc domain and p75 TNFR domain by similar means. Here, we have analyzed the N-linked glycan profile of the separated domains using the GlykoPrep Rapid N-Glycan Preparation System, followed by rapid analysis on the Waters UPLC using a BEH Glycan column, allowing us to compare the N-glycosylation profile for the entire molecule as well as the state of each domain (Figure 1).

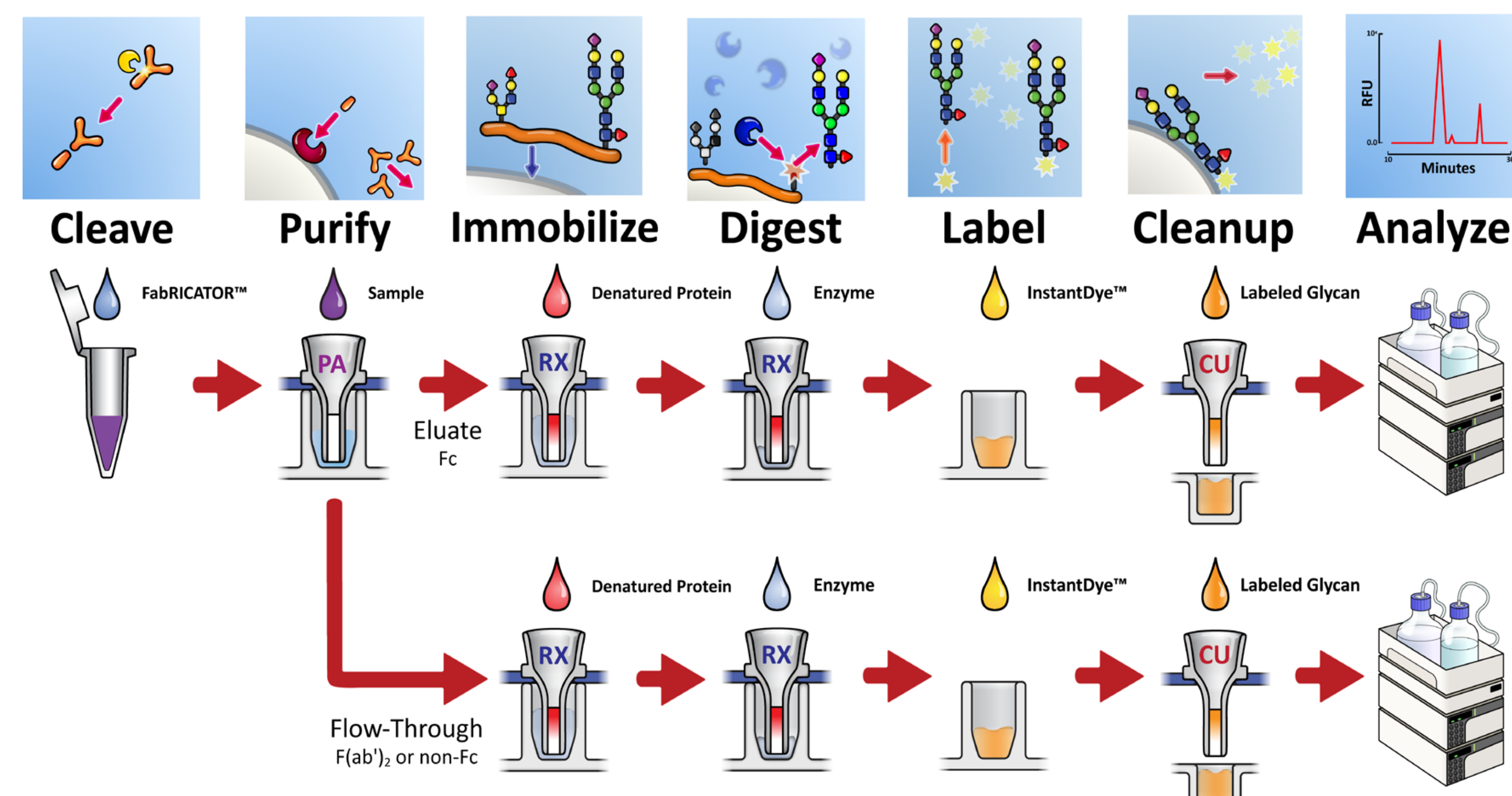


Figure 1 - Proposed Domain-specific Workflow

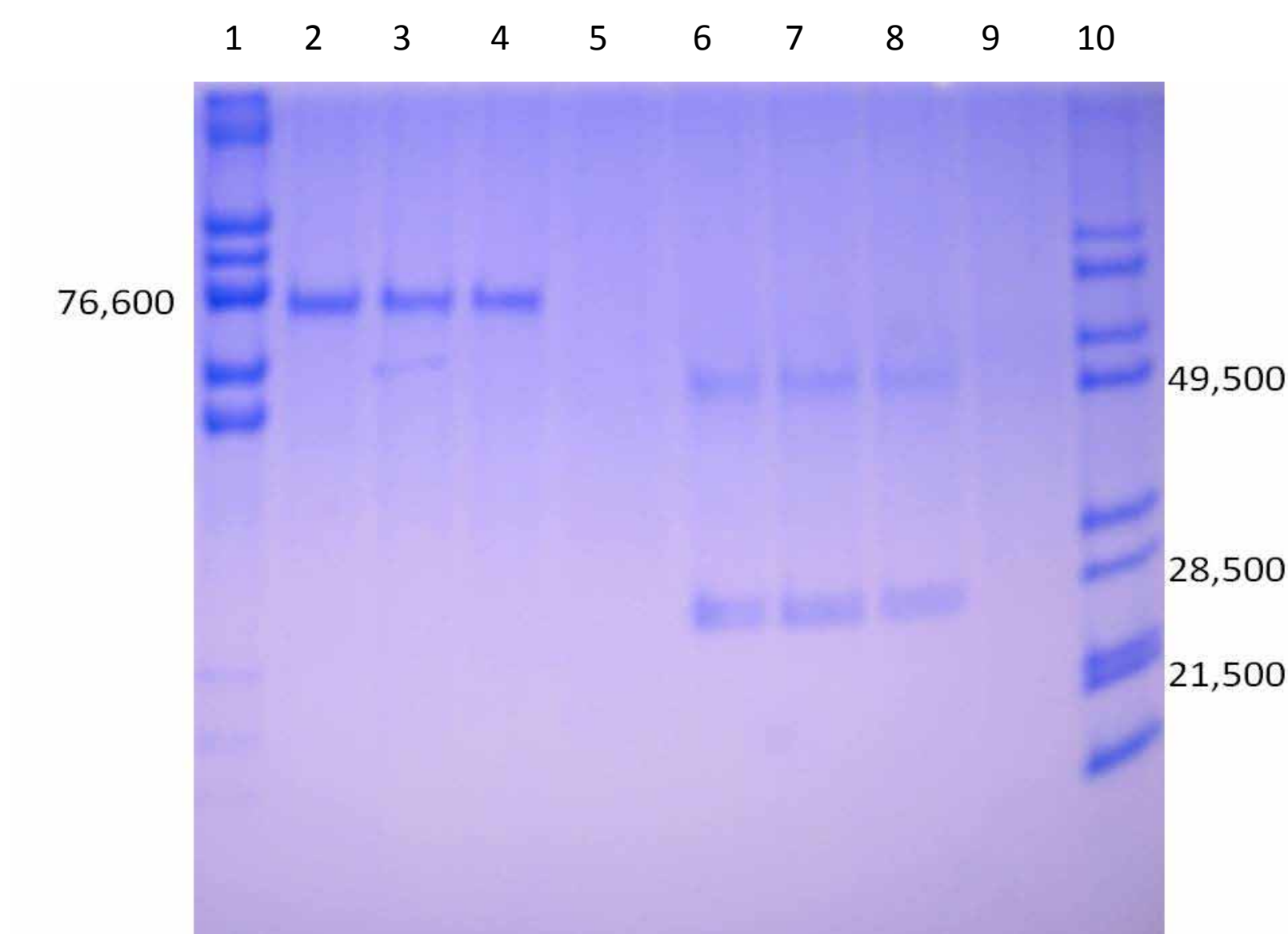


Figure 2 - Digestion of Enbrel® into Fc and TNFR Fragments
Description of Lanes: 1) High MW marker; 2) Enbrel lot 1023954, undigested; 3) Enbrel lot 1014063, undigested; 4) Enbrel lot A60610, undigested; 5) blank; 6) Enbrel lot 1023954, FabRICATOR digest; 7) Enbrel lot 1014063, FabRICATOR digest; 8) Enbrel lot A60610, FabRICATOR digest; 9) FabRICATOR only; 10) Low MW marker

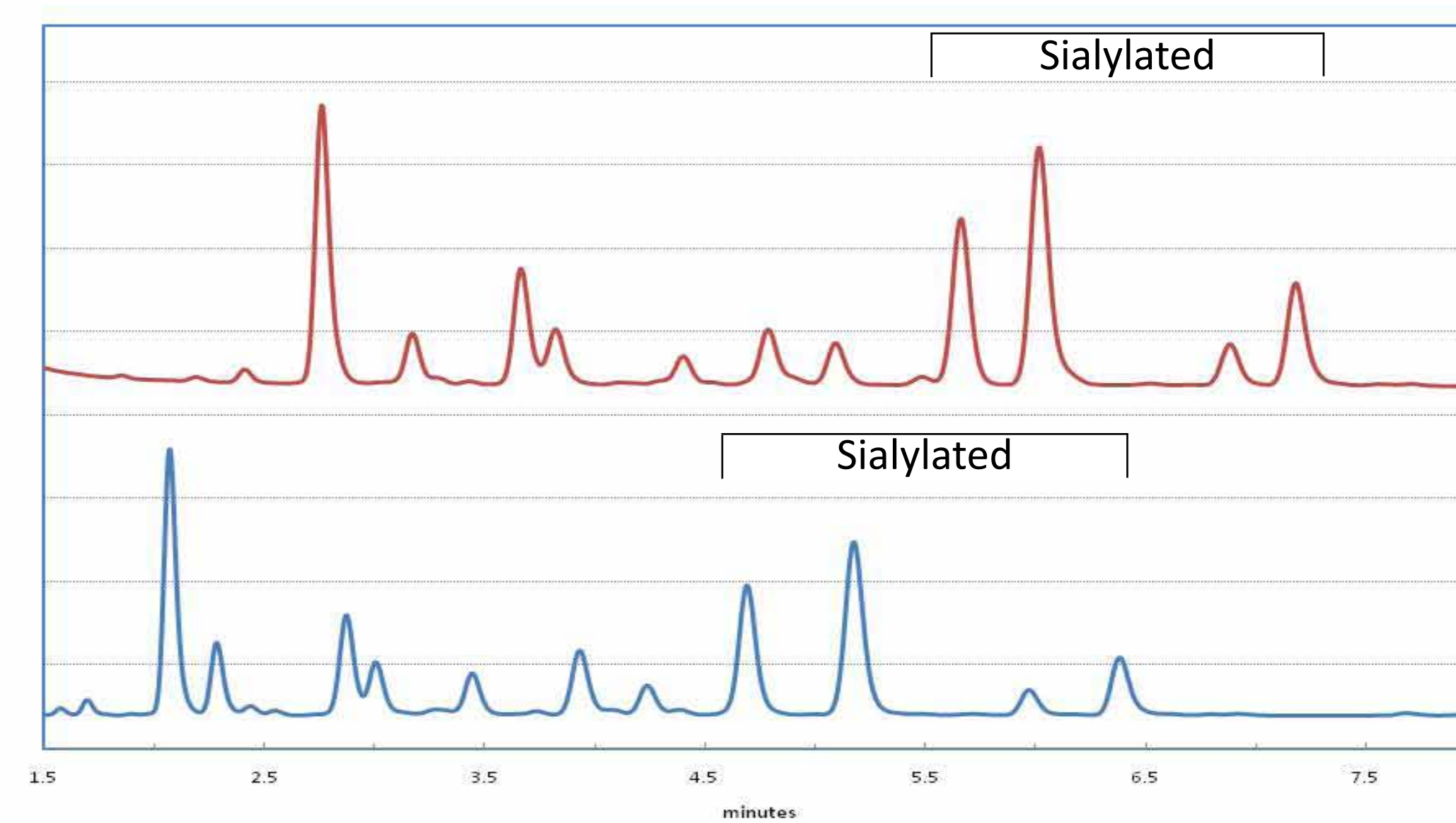


Figure 3 - Comparison of InstantAB™ Enbrel® N-Glycan Labeling (red trace) and Standard 2-AB Labeling (blue trace) by Reductive Amination

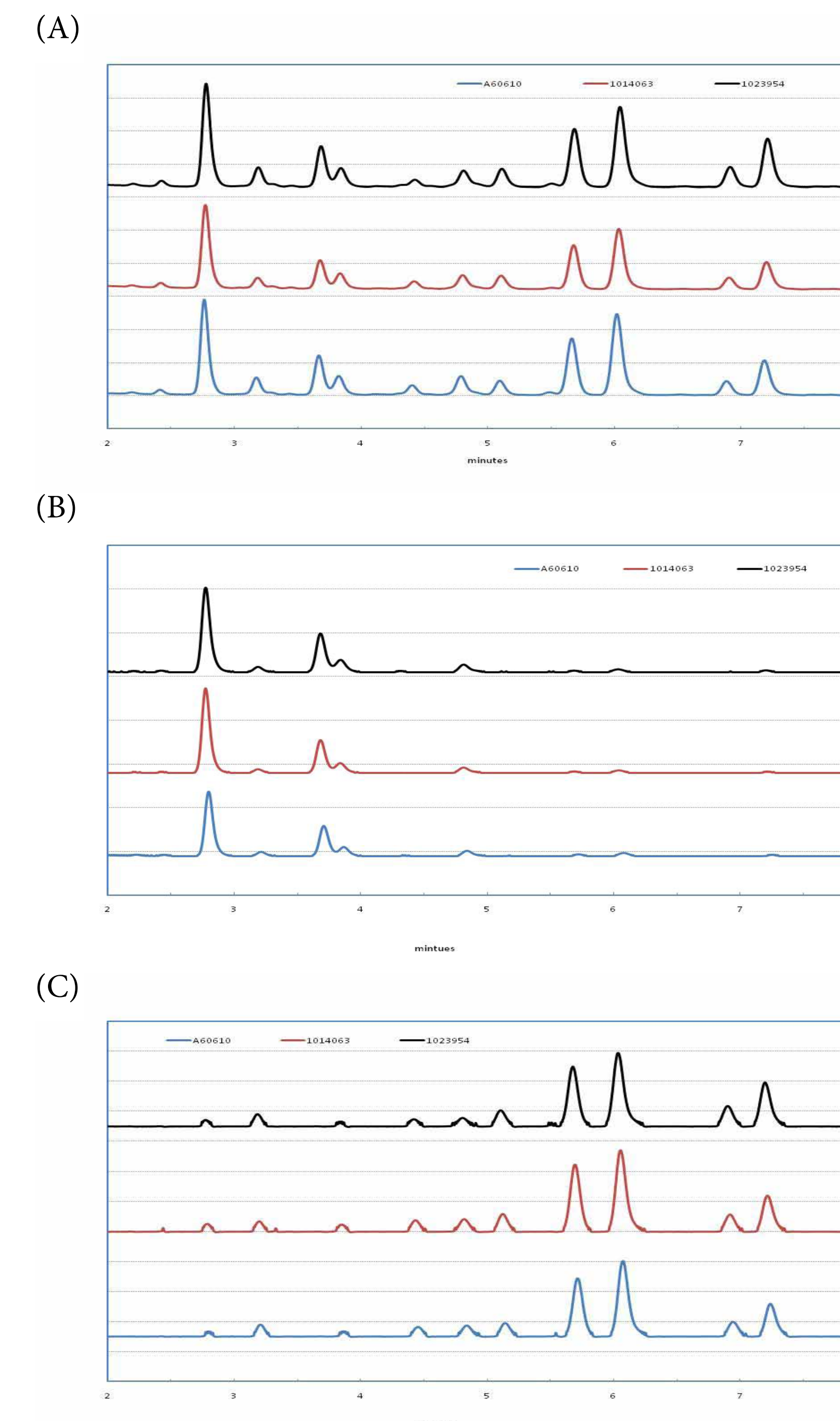


Figure 4 - Total and Domain-specific N-Glycan Profiles of a Representative Lot of Enbrel®
(A) Profile of Enbrel eluted from the PA Cartridge. No N-glycans were found in the Flow-through Samples (data not shown) suggesting no breakthrough of Enbrel on the PA Cartridge. (B) Profile of Fc-Fragment N-glycans eluted from the PA Cartridge. (C) Profile of TNFR-domain N-glycans from PA Cartridge Flow-through.

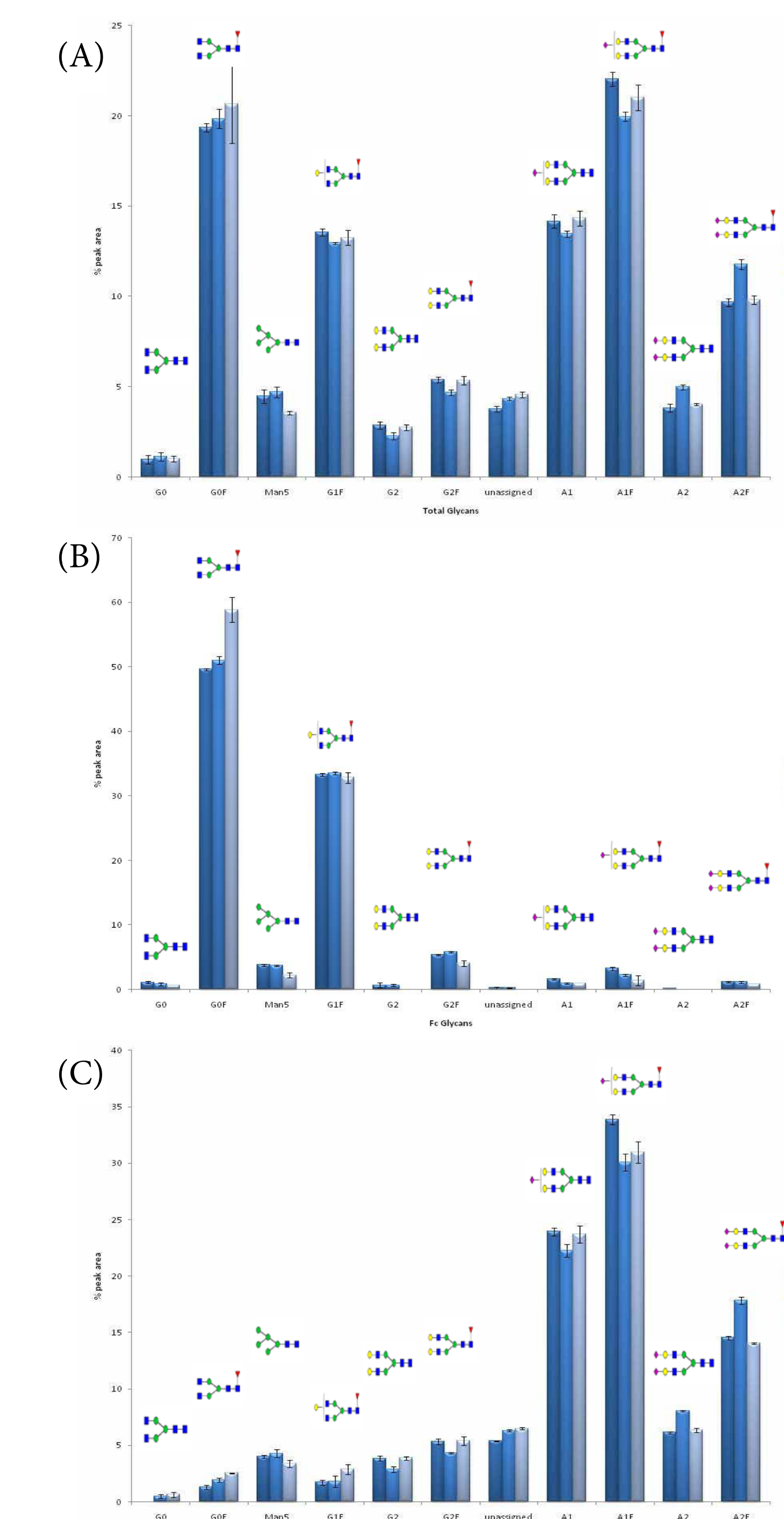


Figure 5 - Total and Domain-specific Enbrel® N-Glycan Peak Areas (%)
(A) Total Enbrel N-glycan Peak Areas; (B) Fc-domain N-glycan Peak Areas; (C) TNFR-domain N-glycan Peak Areas

(A) Total N-Glycans

Probable Glycan	Enbrel Lot Number					
	A60610		1023954		1014063	
	Avg Area %	Std. Dev.	Avg Area %	Std. Dev.	Avg Area %	Std. Dev.
GO	0.97	0.23	1.11	0.23	1.01	0.15
G0F	19.35	0.25	19.84	0.52	20.65	2.16
Man5	4.47	0.37	4.69	0.29	3.55	0.08
G1F	13.54	0.19	12.94	0.07	13.25	0.40
G2	2.85	0.20	2.27	0.21	2.73	0.16
G2F	5.39	0.16	4.66	0.16	5.35	0.25
(Unassigned)	3.76	0.16	4.32	0.10	4.56	0.17
A1	14.16	0.38	13.45	0.19	14.32	0.41
A1F	22.03	0.38	19.96	0.25	21.01	0.72
A2	3.82	0.22	4.97	0.14	4.00	0.06
A2F	9.66	0.21	11.77	0.26	9.81	0.23

(B) Fc-domain N-Glycans

Probable Glycan	Enbrel Lot Number					
	A60610		1023954		1014063	
	Avg Area %	Std. Dev.	Avg Area %	Std. Dev.	Avg Area %	Std. Dev.
GO	1.05	0.16	1.03	0.22	0.97	0.19
G0F	49.60	0.18	51.63	0.58	58.87	1.99
Man5	3.77	0.13	3.68	0.15	2.18	0.35
G1F	33.34	0.22	33.54	0.20	32.81	0.82
G2	0.65	0.35	0.65	0.12		
G2F	5.34	0.09	5.81	0.13	4.05	0.41
(Unassigned)	0.23	0.15	0.25	0.04		
A1	1.57	0.08	0.94	0.09	0.90	
A1F	3.21	0.32	2.22	0.19	1.34	0.76
A2	0.12					
A2F	1.13	0.10	1.12	0.13	0.77	

(C) TNFR-domain N-Glycans

Probable Glycan	Enbrel Lot Number					
	A60610		1023954		1014063	
	Avg Area %	Std. Dev.	Avg Area %	Std. Dev.	Avg Area %	Std. Dev.
GO	0.48	0.15	0.63	0.19		
G0F	1.30	0.17	1.91	0.18	2.53	0.03
Man5	4.00	0.12	4.27	0.34	3.38	0.31
G1F	1.72	0.22	1.79	0.50	2.87	0.47
G2	3.84	0.23	2.88	0.26	3.85	0.16
G2F	5.31	0.25	4.31	0.07	5.39	0.39
(Unassigned)	5.37	0.01	6.28	0.09	6.47	0.09
A1	23.93	0.33	22.22	0.56	23.70	0.77
A1F	33.86	0.44	30.09	0.75	30.94	0.95
A2	6.14	0.09	8.06	0.06	6.35	0.18
A2F	14.53	0.15	17.84	0.30	14.05	0.08

Table 1 - Total and Domain-specific N-Glycan Profile Results
Note: Standard deviation not obtained for some of the minor peaks because these peaks were not found in all replicates.

Methods/Discussion

The GlykoPrep platform, powered by AssayMAP® technology, was chosen for sample preparation because of its ability to rapidly handle multiple samples in a single 8-hour day. In this experiment we prepared 54 samples in 6 hours, including the additional digestion time to prepare the domain-specific samples; up to 96 samples could be easily prepared in a single work day.

A 10-minute UPLC method was developed in order to match the throughput of the GlykoPrep sample preparation. The observed N-glycan sample separation suggests shorter methods could be tested for even higher throughput.

Three lots of Enbrel (Lots A60610, 1014063, 1023954) were purchased commercially under prescription. Enbrel is a dimeric fusion protein with an apparent MW of ~150 kDa; it contains the Fc Domain of hIgG and the p75 TNFR Domain. Digestion with FabRICATOR enzyme separates these domains by cleaving in the hinge region of the fusion protein. Samples were analyzed by SDS-PAGE (10% Bis-Tris) under reducing conditions (Figure 2). For samples that have not been treated with FabRICATOR (undigested), Enbrel is present as a dimer, represented by the ~75 kDa band, Lanes 2-4. After digestion with FabRICATOR, Enbrel is cleaved into the TNFR domain (~50 kDa) and Fc fragments (~25 kDa), Lanes 6-8.

In order to compare against standard methods, N-glycans released from Enbrel lot A60610 were labeled with 2-AB according to the instructions in the Signal™ 2-AB Labeling Kit (product code GKK-404, available from ProZyme; 2-hour incubation at 65°C) or labeled with InstantAB according to the instructions in the GlykoPrep N-Glycan Sample Preparation (product code GP96NG-LB, available from ProZyme;

room temperature in water, 5-minute duration). Cleanup of excess dye and labeling reagents was performed for both samples using the CU Cartridge. N-glycans were then separated on the Acquity UPLC™ BEH glycan column (2.1 x 50 mm, 1.7 µm) for 10 minutes using a binary gradient of ammonium formate buffer and acetonitrile with the signal normalized (Figure 3). Although the retention times shift slightly due to the different dye structures, the results are largely comparable.

Figure 4 shows the normalized chromatograms of InstantAB-labeled Enbrel N-glycans. Fifteen µg of each lot of Enbrel was digested with FabRICATOR for one hour, then purified on the PA Cartridge by specific binding to Protein A (one undigested sample, incubated without FabRICATOR); the portion of the digest that did not bind to the PA Cartridge (flow-through) was collected. The eluates and the flow-throughs were processed using the GlykoPrep protocol (deglycosylation and separation of N-glycans (30-minute incubation at 50°C); labeling with InstantAB; and cleanup and desalting). Chromatography was performed as described in the previous paragraph; a representative profile of each Enbrel lot is shown in Figure 4.

Analysis of each of the three Enbrel lots (including digestion, purification, deglycosylation, labeling, cleanup and LC) was performed in quadruplicate; the standard deviation is represented by the error bars in Figure 5, and the numerical results shown in Table 1. When discussing the comparability of these profiles, the question of whether or not the three lots of Enbrel are essentially the same must be answered based on both the statistical significance of lot-to-lot differences in the profiles, and on the roles of the specific N-glycans on product efficacy. We note that there are a number of peaks in both the total profile and the domain-specific profiles for which the glycan abundance for one lot is different from that of the other lots by more than three standard deviations. This assay platform now permits the analysis of a sufficient number of replicates with sufficient reliability and consistency to distinguish significant differences between lots. As a result, the tools are available to effect targeting and control of glycans which are critical for product efficacy.

The total N-glycan profile of Enbrel indicates there is a mixture of fucosylated and non-fucosylated N-glycans and sialylated and non-sialylated N-glycans, that are for the most part bi-antennary structures. The majority of Fc-domain N-glycans are fucosylated and non-sialylated; G0F and G1F are the predominant N-glycans. The absence (or low levels) of non-fucosylated N-glycans may possibly decrease ADCC potency. The majority of the TNFR-domain N-glycans are sialylated, which may play a role in the half life or efficacy

of the drug. An examination focusing on the separate domains indicates an overlap of some N-glycans but clearly reveals major differences. For example, sialylated N-glycans and G2 are found almost exclusively in the TNFR domain. A comparison across the lots shows that the N-glycan profiles are not as consistent lot to lot as the total N-glycan profiles suggest, specifically, a 9% variation was found among lots for the peak area of G0F.

Conclusion

1. The GlykoPrep standard N-Glycan protocol coupled with a rapid LC method successfully allows preparation of samples in a single 8-hour day, and generation of up to 96 high-quality results overnight. The additional steps for the domain-specific sample preparation were easily accommodated in the standard GlykoPrep protocol.
2. Analysis of N-glycan profiles of the two major domains of Enbrel demonstrate substantial domain-specific differences in N-glycans. These differences are consistent across all lots tested.
3. The three tested lots of Enbrel showed low variability and could be considered comparable when the whole molecule N-glycan profile was evaluated. However, some differences were observed, which would warrant further analysis to determine the statistical significance to a higher degree. The large number of samples that can be prepared and analyzed using the GlykoPrep platform now makes such an analysis possible with a reasonable investment in resources.

References

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