Application Note

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A Workflow for Rapid Sample Preparation and Exoglycosidase Characterization of N-Glycans in Antibodies

Authors

Yongjing Xie,
Letícia Martins Mota, and
Michael Butler
National Institute for
Bioprocessing Research and
Training (NIBRT),
Foster Avenue, Mount Merrion,
Blackrock, Co.
Dublin, Ireland

Bethan Morgan and
Aled Jones
Agilent Technologies, Inc.

Abstract

This application note describes use of the Agilent AdvanceBio Gly-X N-glycan prep with InstantPC kit, 96 ct (GX96-IPC) for high-throughput N-glycan profiling of Immunoglobulin G (IgG), a polyclonal antibody purified from human serum. This kit uses an innovative 96-well-plate-based workflow for rapid glycoprotein denaturation, deglycosylation, and instant glycan fluorescent derivatization. This is followed by on-matrix glycan purification prior to hydrophilic interaction liquid chromatography with fluorescence detection (HILIC/FLD) analysis. The N-glycan assignment was further cross-validated by exoglycosidase sequential digestion. This workflow enables reproducible high-throughput, high-sensitivity N-glycan profiling, and holds great potential for routinely monitoring glycosylation-based critical quality attributes (CQA) of biotherapeutics in biopharmaceutical development.

Introduction

Protein glycosylation has a significant impact on the structure and function of biotherapeutic glycoproteins, as it influences stability, solubility, protein folding, and the mediation of protein-protein interactions. Additionally, protein glycosylation of biopharmaceutical drugs plays a vital role in assuring product quality, safety, and potency. Glycosylation contributes to modulating a variety of biological properties, such as immunogenicity, serum clearance, *in vivo* circulating half-life, and anti-inflammatory activity.[1,2] Therefore, a comprehensive knowledge of protein glycosylation is necessary to ensure successful biomanufacturing of efficacious therapeutics and is of great importance for determining the functional role in a biological context. To this end, continuous development of fast and sensitive strategies for glycomic analysis of aberrant glycoproteins is decidedly critical. However, many of the commonly used glycan preparation procedures using 2-aminobenzamide (2-AB) or other fluorescent dyes[3,4] are time-consuming and usually require several hours (or even days) for glycan sample preparation, and ultimately yield poor reproducibility and low sensitivity.

This study presents a streamlined workflow for N-glycan sample preparation, which takes advantage of 3-minute glycoprotein denaturation, 5-minute in-solution deglycosylation, 1-minute instant fluorescent derivatization, and on-matrix glycan purification prior to further HILIC/FLD analysis. All preparations are in a 96-well plate format and are ready for automation.[5] This application is demonstrated in N-glycan profiling of Immunoglobulin G (IgG), a polyclonal antibody purified from human serum. This is followed by a detailed validation of glycan structural assignments by exoglycosidase sequential digestion.

This workflow enables reproducible, high-throughput sample preparation for high-sensitivity N-glycan profiling and is believed to hold great potential for routinely monitoring glycosylation-based CQAs of biotherapeutics or biosimilars in biopharmaceutical development.

Experimental

Materials

The Agilent AdvanceBio Gly-X N-glycan prep with InstantPC kit, 96 ct (GX96-IPC) consists of three modules, including the Gly-X deglycosylation module (GX96-100), Gly-X InstantPC labeling module (GX96-101), and Gly-X InstantPC cleanup module (GX96-102). Agilent AdvanceBio exoglycosidases Sialidase A (GK80040), β (1-4)-Galactosidase (Streptococcus pneumoniae, GKX5014), and β-N-acetylhexosaminidase (GK80050) were used. Agilent AdvanceBio InstantPC Maltodextrin ladder (GKPC-503) was used as a calibration standard. IgG from human serum (14506-10MG) was purchased from Sigma-Aldrich. HPLC-grade acetonitrile was purchased from Sigma-Aldrich, and Milli-Q water was used in all preparations. All common chemicals were purchased from Sigma-Aldrich.

Rapid in-solution antibody denaturation and enzymatic deglycosylation

The in-solution enzymatic deglycosylation of human serum IgG was carried out according to the instructions provided with the AdvanceBio Gly-X N-Glycan Prep InstantPC kit. Human serum IgG (40 µg) was diluted with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.0) to make a final volume of 20 µL. Gly-X denaturant (2 µL) was added to the 20 µL of human IgG solution, mixed thoroughly and incubated at 90 °C for 3 minutes. After leaving at room temperature for 2 minutes, 2 µL of N-Glycanase working solution was added, mixed thoroughly, and incubated at 50 °C for 5 minutes.

N-glycan labeling with InstantPC and purification

InstantPC dye solution was prepared by dissolving one vial of InstantPC dye with 150 µL of the accompanying solvent and mixing well. The dye solution (5 µL) was added to the above-prepared sample and incubated at 50 °C for 1 minute. The Load/Wash solution (150 µL of 2.5% formic acid/97.5% acetonitrile) was added to each sample, then the entire sample (179 µL) was transferred to each well of the Gly-X Cleanup plate containing 400 µL of Load/Wash solution. After applying a vacuum to pass the solution through the cleanup plate, samples were washed with 600 µL of Load/Wash solution three times. InstantPC-labeled N-glycans were eluted with 100 µL of Gly-X InstantPC eluent (160 mM ammonium formate/10% (v:v) acetonitrile, pH 4.4). The collected N-glycan solutions were either analyzed immediately without the need for further concentration, or alternatively, stored at –20 °C for future analysis.

The procedure for Exoglycosidase sequential digestions for InstantPC-labeled N-glycan assignment was as follows:

1. InstantPC-labeled N-glycans were prepared from human serum IgG according to the above glycan preparation protocol, and the glycans were dried using a vacuum concentrator.
2. N-glycans were dissolved in distilled water and combined to make a final volume of 60 µL (54 µL H_2O + 6 µL 10 x ammonium acetate, pH 5.5). For the entire exoglycosidase sequential digestion panel, the InstantPC-labeled N-glycan content isolated from 40 µg of antibody was adequate.

3. The reaction solutions were prepared according to the exoglycosidase sequential digestion panel (Table 2).
4. The reaction solutions were mixed well and incubated at 37 °C for 24 hours.
5. Each reaction solution (1 µL) was injected directly into the HPLC for HILIC/FLD analysis without further treatment.

HILIC/FLD analysis of InstantPC-labeled N-glycans

The profiles of InstantPC-labeled N-glycans from human serum IgG were determined by hydrophilic interaction liquid chromatography with fluorescence detection (HILIC/FLD) using an Agilent 1260 Infinity II system equipped with an Agilent AdvanceBio Glycan Mapping column (120 Å, 2.1 × 150 mm, 2.7 µm, p/n 683775-913) under the control of Agilent OpenLab ChemStation software. The system consisted of a quaternary solvent pump, autosampler, and fluorescence detector. The detector was set with excitation and emission wavelengths of 285 and 345 nm, respectively (for InstantPC). The InstantPC-labeled glycan samples were injected at a volume of 1 µL, without further treatment prior to injection. The N-glycans were separated with 50 mM ammonium formate (pH 4.4) as solvent A, and acetonitrile as solvent B. After equilibrating the HPLC system with 50 mM ammonium formate (pH 4.4) and acetonitrile (27:73 v:v) for 1.5 minutes at a flow rate of 0.5 mL/minute, the separation was carried out by a linear gradient of 73 to 62% acetonitrile (v:v) in a 30-minute analytical run at a flow rate of 0.5 mL/minute. Samples were maintained at 5 °C before injection and the column temperature was set at 60 °C. The system was calibrated using the Agilent AdvanceBio InstantPC Maltodextrin ladder (GKPC-503). The glucose unit (GU) value and retention time T (min) data were fitted to a 5th order polynomial curve to obtain the standard curve.

Results and discussion

AdvanceBio Gly-X technology for express Glycan preparation

As demonstrated in Figure 1, the AdvanceBio Gly-X N-glycan prep with InstantPC kit used in this study enables streamlined N-glycan sample preparation in a 96-well plate format workflow. The kit features a 3-minute glycoprotein denaturation at 90 °C, followed by a 5-minute deglycosylation at 50 °C, which enables the release of glycans from the targeting glycoproteins in an efficient way. Additionally, with the introduction of the InstantPC fluorescent label (an active form of procaine), the released glycosylamine intermediates are attached to InstantPC via activated carbamate chemistry to form a stable urea linkage. It takes less than an hour from glycoproteins to glycan profiling, which uses this glycan preparation workflow to ensure complete qualitative and quantitative glycan analysis. Of particular significance is the thermally sensitive sialic acid linkage, which suffers less degradation under these mild conditions.

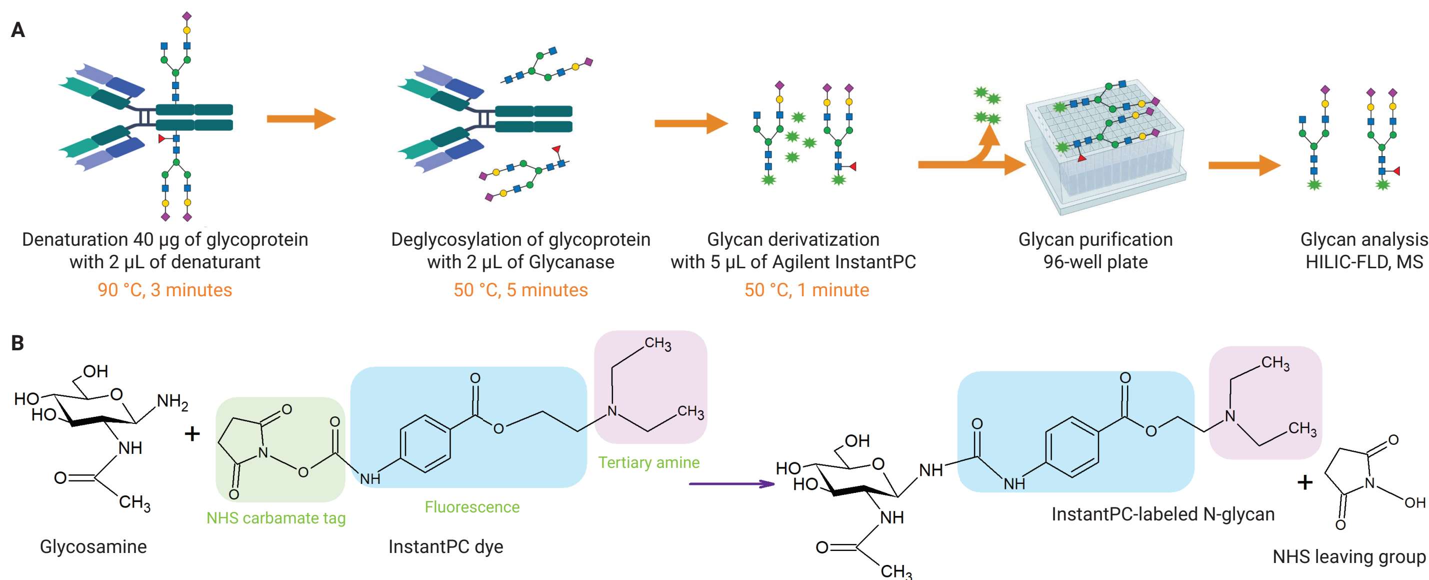

Figure 1. (A) Workflow for glycosylamine release and Agilent InstantPC derivatization. (B) Activated carbamate chemistry-based InstantPC-labeling of glycosylamine.

Human serum IgG InstantPC-labeled glycan profiling by HILIC/FLD

Human serum IgG was selected as the model glycoprotein and the released glycosylamine intermediates were labeled with InstantPC for qualitative and quantitative analysis by HILIC/FLD. Under the developed chromatographic conditions (Table 1), the InstantPC-labeled N-glycans from human serum IgG resulted in well resolved peaks for all major N-glycan species (Figure 2). The developed HILIC/FLD method for glycan profiling shows good reproducibility (data not shown). For other glycoproteins with simplified glycan profiles, the HILIC/FLD method can be adjusted to under 10 minutes.

The GU values of the InstantPC-labeled Maltodextrin ladder were fit to a 5th order polynomial curve to generate a standard curve between glycan GU values and HILIC/FLD retention time, which was used to generate GU values of peaks from the IgG separation and ascribe structural assignments. As shown in Figure 2 and Table 2, human serum IgG possesses a variety of N-glycan species, consisting predominantly of high fucose glycans, sialic acid glycans, GlcNAc-bisected glycans, and some neutral galactose and GlcNAc containing glycans. The most abundant glycan is FA2G1 (24.74%), followed by FA2 (18.11%), FA2G2 (12.90%), and FA2G2S1 (11.28%), respectively.

Table 1. HILIC/FLD conditions for Agilent InstantPC-labeled N-glycan profiling.

Parameter	Value
Instrument	Agilent 1260 Infinity system
Column	Agilent AdvanceBio Glycan Mapping column, 120 Å, 2.1 × 150 mm, 2.7 µm (p/n 683775-913)
Column Temperature	60 °C
Mobile Phase	A) 50 mM Ammonium formate (pH 4.4) B) Acetonitrile
Gradient Program	Time (min) / A (%) / B (%) / Flow Rate (mL/min) 0 / 27 / 73 / 0.5 1.5 / 27 / 73 / 0.5 31.5 / 38 / 62 / 0.5 34 / 60 / 40 / 0.5 35 / 27 / 73 / 0.25 36 / 27 / 73 / 0.5 50 / 27 / 73 / 0.5
Injection Volume	1 µL
Detection	FLD λ_{Ex} 285 and λ_{Em} 345 nm

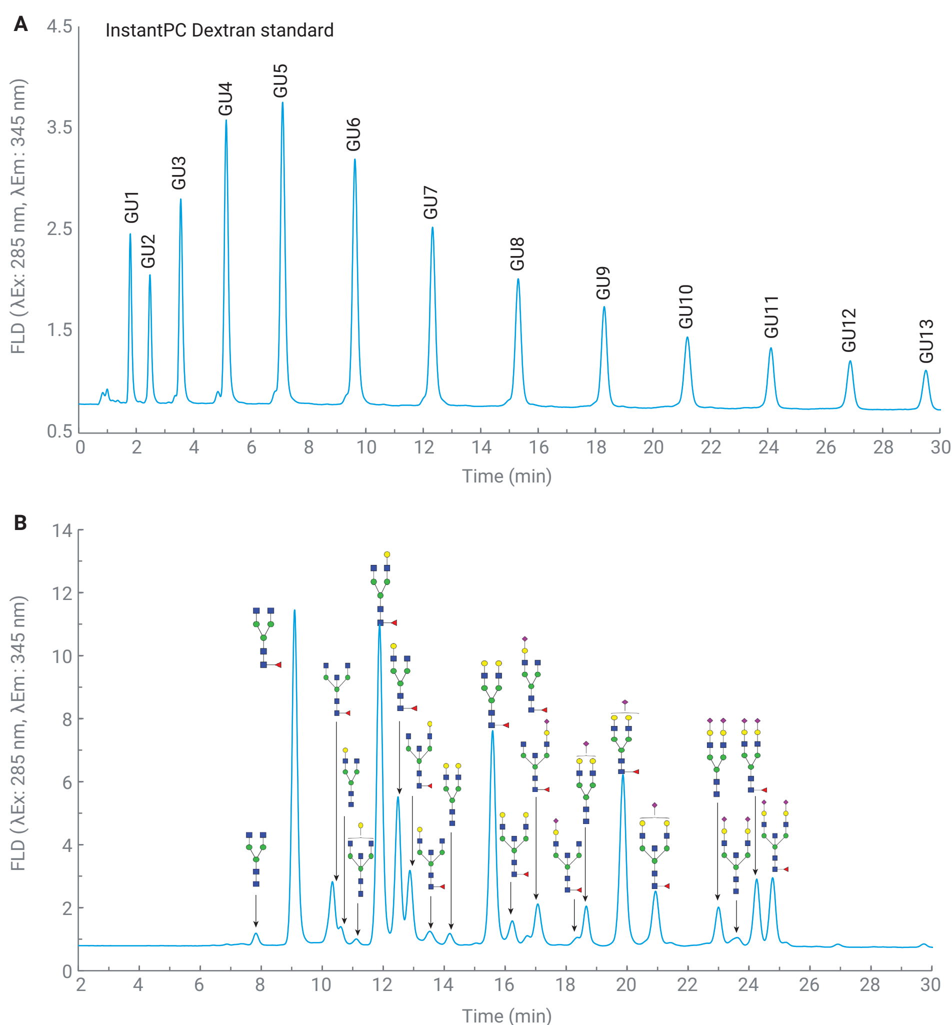

Figure 2. HILIC/FLD chromatograms of Agilent InstantPC-labeled Maltodextrin ladder (A) and human IgG N-glycans (B). GU values from this Maltodextrin ladder can fit to a 5th order polynomial curve to obtain a standard curve: $GU = a + bT + cT^2 + dT^3 + eT^4 + fT^5$, where GU is glucose unit, and T is retention time (in minutes). Only major glycan peaks are displayed.

Table 2. Human serum IgG N-glycan profiling labeled with InstantPC.

Oxford Notation Name	N-glycan Structure	Retention Time (min)	GU	AUC	AUC (%)
A2		7.841	5.314	4.872	0.641
FA2		9.107	5.836	137.615	18.110
FA2B		10.341	6.305	28.361	3.732
A2[3]G1		10.613	6.405	5.802	0.763
A2BG1		11.130	6.590	1.924	0.253
FA2[6]G1		11.892	6.857	132.885	17.487
FA2[3]G1		12.500	7.066	55.132	7.255
FA2[6]BG1		12.896	7.200	27.680	3.642
FA2[3]BG1		13.549	7.420	5.303	0.698
A2G2		14.205	7.638	4.722	0.621
FA2G2		15.612	8.104	98.053	12.903
FA2BG2		16.260	8.319	10.428	1.372
FA2[3]G1S1		17.098	8.597	20.580	2.708
FA2[3]BG1S1		18.396	9.032	2.863	0.377
A2G2S1		18.686	9.130	18.876	2.484
FA2G2S1		19.893	9.540	85.744	11.283
FA2BG2S1		20.964	9.909	30.837	4.058
A2G2S2		23.029	10.633	20.049	2.638
A2BG2S2		23.642	10.851	4.969	0.654
FA2G2S2		24.282	11.080	30.866	4.061
FA2BG2S2		24.805	11.268	32.372	4.260

– InstantPC-labeled Maltodextrin ladder standard curve: $GU = -0.01117 + 1.063T - 0.07027T^2 + 0.003248T^3 - 0.00007251T^4 + 0.0000006418T^5$.

– The relative abundance of each N-glycan was calculated with the following equation: $\text{FLR AUC (\%)} = \text{FLR AUC}_{\text{Glycan i}}/\text{sum}(\text{FLR AUC}_{\text{Glycans}}) \times 100$.

– The retention time, GU, area under the curve (AUC), relative abundance (AUC%) were calculated by averaging three different measurements.

Exoglycosidase sequential digestion of InstantPC-labeled glycans of human IgG

Exoglycosidase sequential digestion was subsequently carried out to further validate the assignment of the InstantPC-labeled human IgG N-glycan profiles. A variety of enzymes of Sialidase A, β(1-4) Galactosidase, and β-N-acetylhexosaminidase were used to remove N-acetylneuraminic acid (NANA), galactose, and N-acetylglucosamine (GlcNAc), respectively. A complete description of the exoglycosidase enzymes, including full name, product code, short name, specificity, and cleavage sites is displayed in Table 3. An exoglycosidase sequential digestion panel for InstantPC-labeled glycans is displayed in Table 4.

Table 3. Exoglycosidase enzymes, along with example glycans and substrate specificity.

Exoglycosidase [Product Code]	Product Code	Short Name	Specificity
Sialidase A (Recombinant from *Arthrobacter ureafaciens*, expressed in *E. coli*)	GK80040	ABS	α (2-3,6,8,9) N-acetylneuraminic acid linkages
β(1-4)-Galactosidase (*Streptococcus pneumoniae*)	GKX-5014	SPG	β (1-4) galactose linkages
β N-Acetylhexosaminidase (*Streptococcus pneumoniae*)	GK80050	SPH	β (1-2,3,4,6) N-acetylglucosamine (GlcNAc) linkages

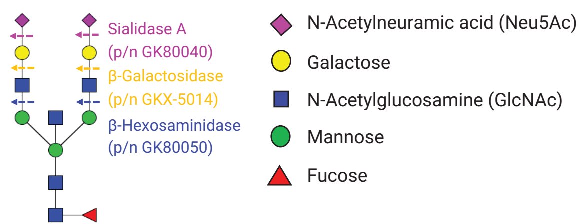

Table 4. Exoglycosidase sequential digestion panel for InstantPC-labeled glycans.

Reagent (μL)	Rxn 1	Rxn 2	Rxn 3	Rxn 4
InstantPC-labeled glycan in buffer	10	10	10	10
H_2O	10	8	6	4
α(2-3,6,8,9) Sialidase A	0	2	2	2
β(1-4)-Galactosidase	0	0	2	2
β(1-2,3,4,6)-N-acetylhexosaminidase	0	0	0	2
Total Volume	20	20	20	20

As shown in Figure 3, the enzymatic arrays shifted the peaks in each chromatogram predictably and were in accordance with the assigned glycan structures. Therefore, it is reasonable to deduce that the N-glycan peak assignments for the human IgG are accurate.

Conclusion

The AdvanceBio Gly-X N-Glycan Prep with InstantPC kit can achieve high-throughput glycan preparation and fluorescent derivatization for biotherapeutic N-glycan analysis. The fluorescent dye, InstantPC, labels the glycosylamine intermediates in an "instant" manner with high fluorescence signal. The streamlined 96-well plate-based format for glycan sample preparation is robust and can be completed in approximately one hour. The developed HILIC/FLD method separates InstantPC-labeled N-glycans from human IgG into well resolved peaks. Sequential exoglycosidase digestion with a variety of AdvanceBio enzymes is used to assign structures to InstantPC-labeled glycans.

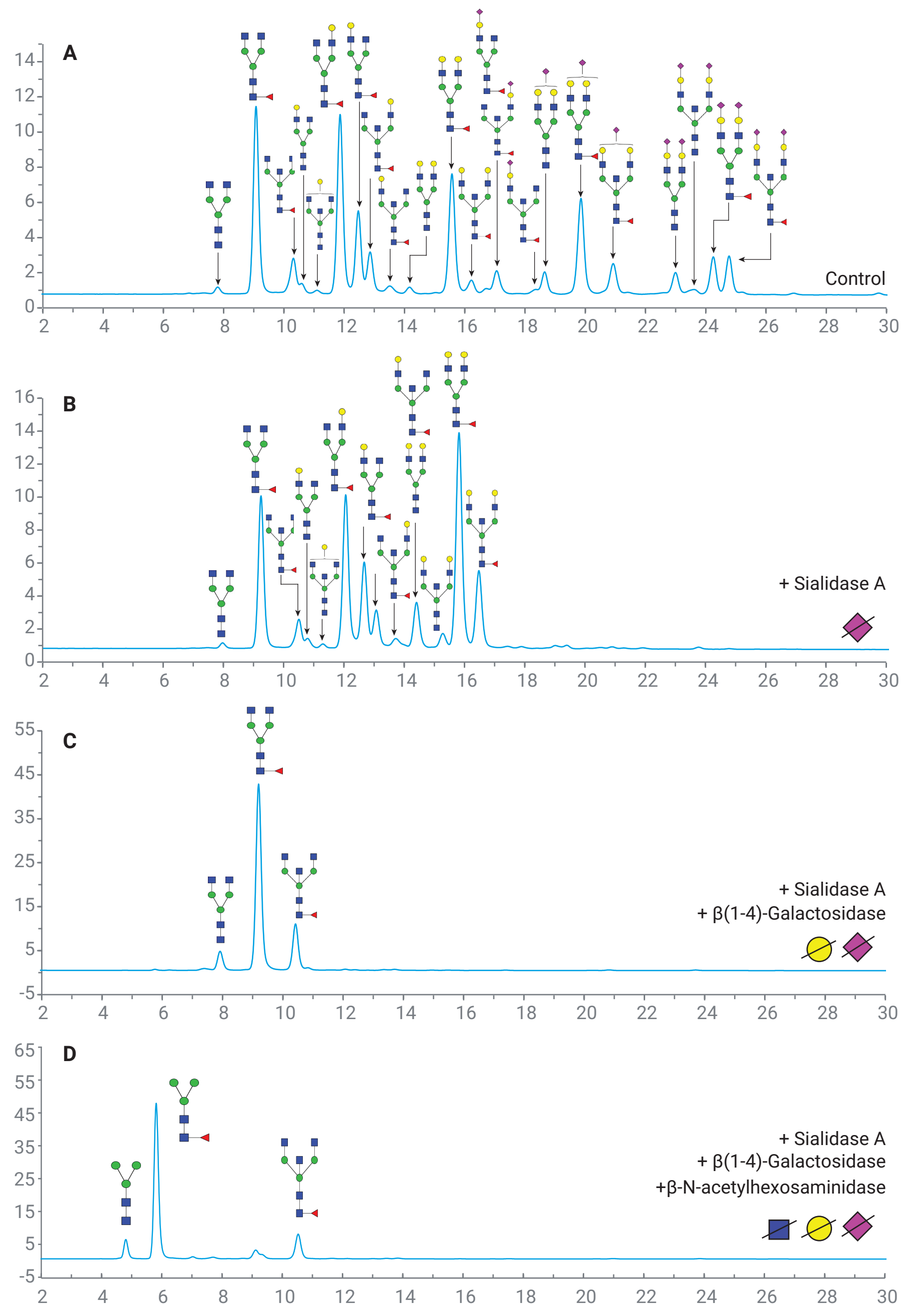

Figure 3. Exoglycosidase sequential digestions of human serum IgG N-glycans labeled with InstantPC: (A) Control (undigested); (B) Sialidase A; (C) Sialidase A + β(1-4)-Galactosidase; (D) Sialidase A + β(1-4)-Galactosidase + β-N-acetylhexosaminidase.

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