Detailed Characterization of Antibody Glycan Structure using the *N*-Glycan Sequencing Kit

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Introduction

Characterization of glycans on therapeutic IgGs is critical as the stability, half-life, and clinical efficacy are affected by the glycoforms present on the molecule. The inherent complexity of protein glycosylation poses a daunting analytical challenge. Multiple orthogonal methods are often used to elucidate structure, but even with techniques such as LC-MS, which has the advantage of an associated mass corresponding to each chromatographic peak, there can be ambiguities when assigning structures. There are often several possible glycan isoforms associated with an identical mass.

The use of sequential exoglycosidase digestion of oligosaccharides followed by LC-MS or CE analysis provides detailed carbohydrate sequence information and resolves ambiguities. Highly specific exoglycosidases cleave monosaccharides from the non-reducing end of an oligosaccharide and can yield information about the linkage, stereochemistry and configuration of the anomeric carbon.

Enzymes can be used in various combinations to simplify glycan profiles and highlight the overall level of a specific epitope. An example is fucosylation, which can potentially influence antibody-dependent, cell-mediated cytotoxicity (ADCC)(1). Exoglycosidases can be used in combination to trim the glycan to the trimannosyl core, with or without fucose. Multiple digestions reduce complex data to a simplified panel that can be more easily quantitated. Mannosidases, such as α1-2,3,6 Mannosidase (NEB #P0768) can be used to monitor the ratio of high mannose in a glycan profile, an epitope that can lead to a higher clearance rate of a given therapeutic(2). Neuraminidases, such as α2-3,6,8,9 Neuraminidase A (NEB #P0722) and galactosidases such as α1-3,4,6 Galactosidase (NEB #P0747), can be used to monitor the presence of *N*-glycolylneuraminic acid (Neu5Gc) and alpha-linked galactose residues, potentially immunogenic, non-human epitopes that are present in murine derived antibodies.

Here we used the *N*-Glycan Sequencing Kit, which contains seven of the most commonly used exoglycosidases for *N*-Glycan sequencing, to precisely characterize glycans on the Fc domain of therapeutic antibodies and dimeric fusion proteins. The workflow described includes glycan release with Rapid™ PNGase F (NEB #P0710), direct labelling of released glycans with procainamide (PCA) or 2-aminobenzamide (2AB), clean-up of labeled glycans and a 3 hour enzymatic digestion with exoglycosidases. This protocol is designed for completion within an 8 hour time frame to allow for subsequent LC or LC-MS analysis overnight.

Materials

Remicade (Infliximab) from Imclone, LLC

Enbrel (entanercept) from Amgen Inc., manufactured by Immunex Corp

Rapid PNGase F (NEB #P0710)

N-Glycan Sequencing Kit (NEB #E0577)

α2-3,6,8,9 Neuraminidase A (NEB #P0722)

α1-3,4,6 Galactosidase (NEB #P0747)

β1-4 Galactosidase S (NEB #P0745)

β-*N*-Acetylglucosaminidase S (NEB #P0744)

α1-2,4,6 Fucosidase O (NEB #P0749)

α1-2,3,6 Mannosidase (NEB #P0768)

HILIC Plate: The Nest Group, Inc. part #SNS-HiL

HILIC Microspin™ Column: The Nest Group Inc. part #SEM HiL

Procainamide (PCA) (Sigma P9391)

2-aminobenzamide (2AB) (Sigma A89804)

Sodium cyanoborohydride (Sigma 156159)

Dimethyl sulfoxide (DMSO)

Glacial Acetic Acid

Acetonitrile (ACN) HLPC/MS grade

50 mM NH_4 Formate Buffer (pH 4.4)

Glycan Sample Preparation Workflow:

FIGURE 1:
Infliximab and Enbrel Structures

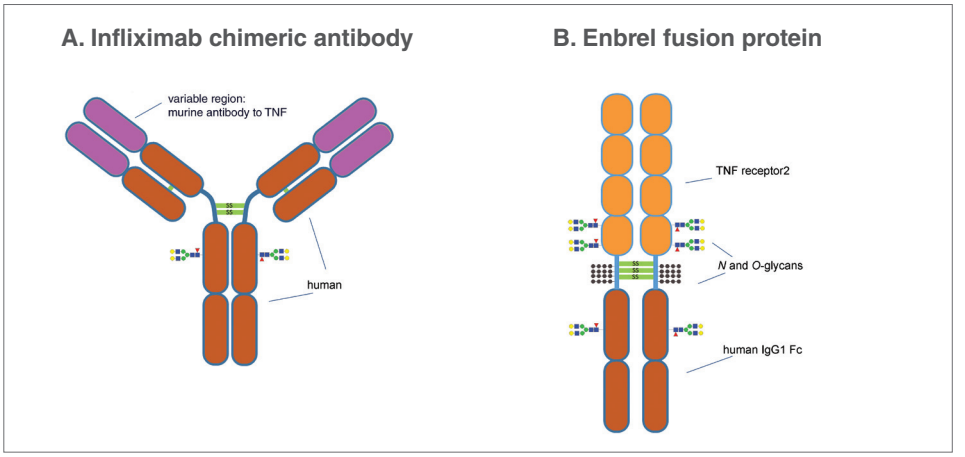
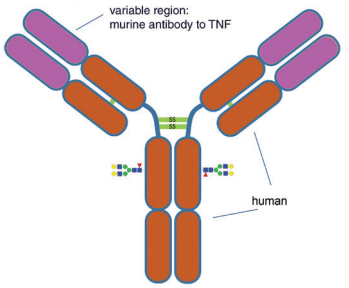
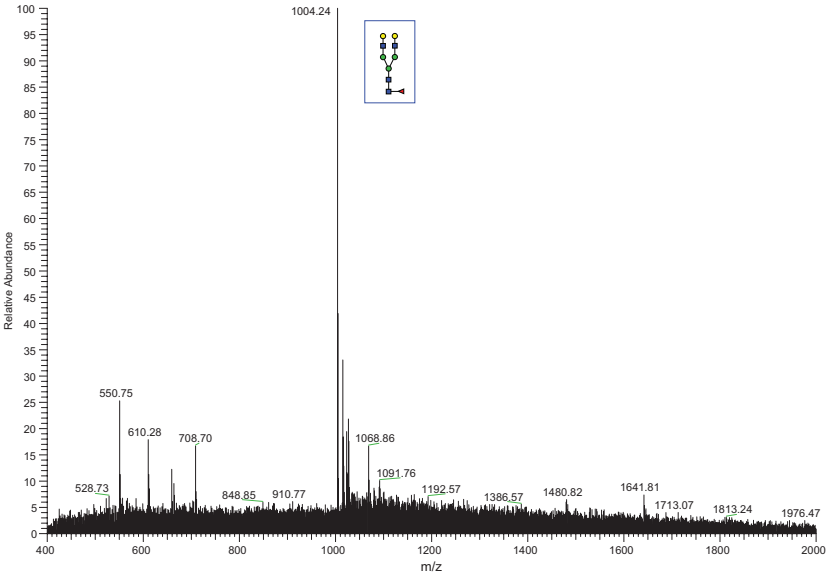


FIGURE 2:
Infliximab glycan profile

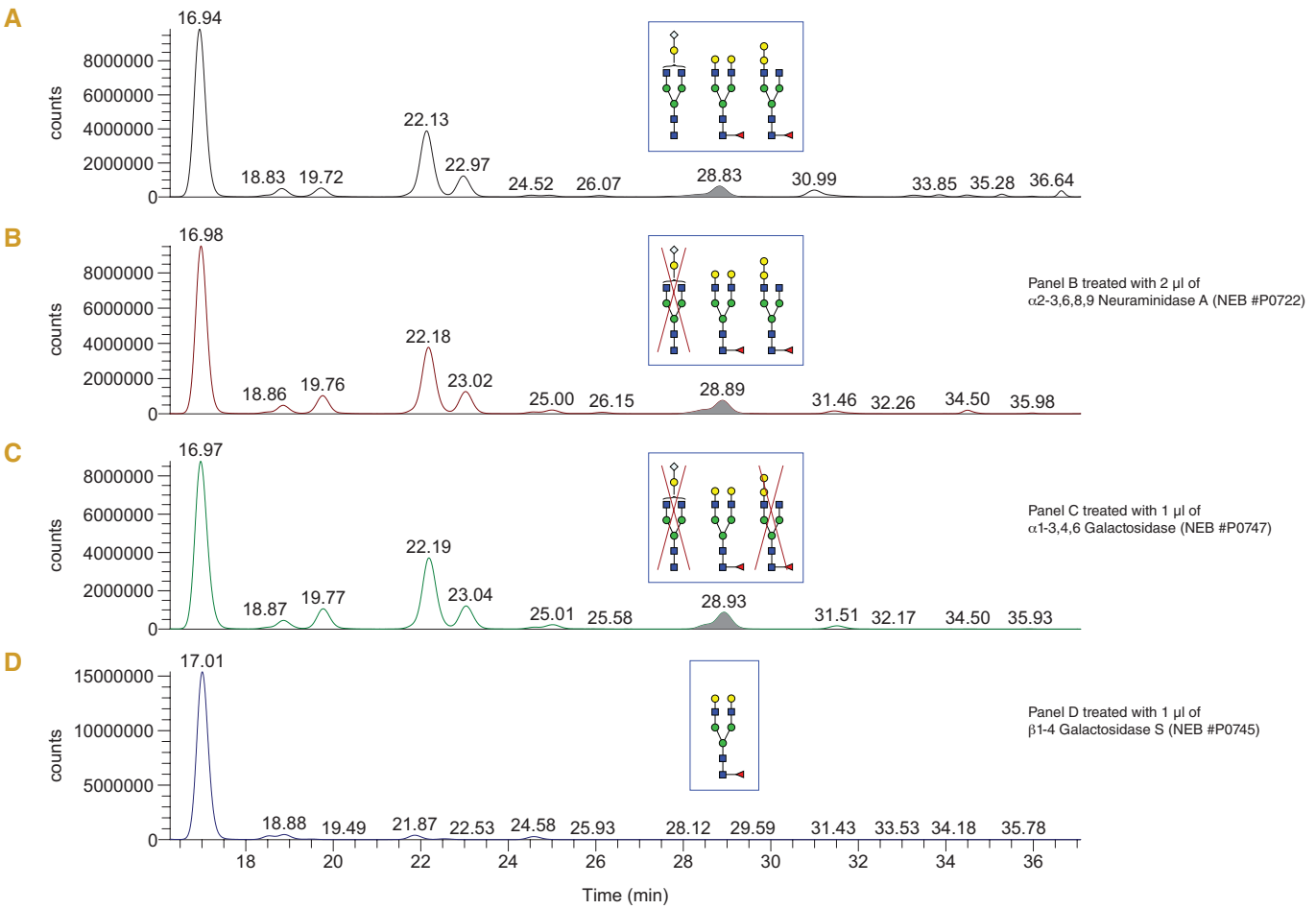
A. Infliximab chimeric antibody



Exoglycosidases are used to resolve assignment of peak with corresponding m/z value of 1004.24. α2-3,6,8,9 Neuraminidase A, α1-3,4,6 Galactosidase, and β1-4 Galactosidase S are used to digest the substrate. Disappearance of the peak with β1-4 Galactosidase S indicates the correct structure among the three possible glycoforms.



Possible structures with m/z at or close to 1004



Panel B treated with 2 µl of α2-3,6,8,9 Neuraminidase A (NEB #P0722)

Panel C treated with 1 µl of α1-3,4,6 Galactosidase (NEB #P0747)

Panel D treated with 1 µl of β1-4 Galactosidase S (NEB #P0745)

Disappearance of peak with β1-4 Galactosidase S allows assignment of glycan isomer.

Gal Glc Man GalNAc GlcNAc Fuc NeuAc NeuGc

General Protocol

Rapid Deglycosylation

The antibody sample is treated with Rapid PNGase F using the two-step deglyosylation protocol

1. Using PCR tubes (200 µl), add 30 µg of monoclonal antibody (see Note 1) to a final volume of 16 µl
2. Add 4 µl Rapid PNGase F Buffer and mix
3. Incubate mixture at 80°C for 2 minutes and cool
4. Add 1 µl of Rapid PNGase F
5. Incubate for 10 minutes at 50°C in a thermocycler or heat block

Fluorescent labeling with procainamide (PCA) or 2-aminobenzamide (2AB)

1. Add 18 µl of acidified PCA or 2AB labeling reagent (see Note 2) and 24 µl cyanoborohydride reagent to the deglycosylation reactions
2. Incubate for 45 minutes at 65°C in a thermocycler
3. Cool reactions to room temperature

Glycan purification with a 96-well HILIC plate

1. Add 350 µl Acetonitrile (ACN) to the labeled reactions to a final concentration of 85% ACN
2. Set up a HILIC elution plate with shims or spacer and waste tray if necessary
3. Condition well with 200 µl of H_2O
4. Equilibrate well with 200 µl of of 85% ACN
5. Load PCA or 2AB labeled samples diluted with ACN (~410 µl) onto the HILIC plate
6. Wash wells with 3 x 200 µl of 1% formic acid, 90% ACN
7. Replace waste tray with collection plate
8. Elute glycans with 3 x 30 µl of SPE buffer (see Note 3) into the collection plate
9. Dry the 90 µl sample in a speed vac or lyophilize overnight (see Note 4)
10. Resuspend the sample in 30 µl of H_2O for subsequent exoglycosidase reactions

Glycan Purification with a HILIC spin column

1. Add 350 µl Acetonitrile (ACN) to the labeled reactions for a final concentration of 85% ACN
2. Use either a vacuum manifold or centrifuge adaptor (following manufacturer's instructions), condition a HILIC spin column with 350 µl of water
3. Equilibrate column with 350 µl of 85% ACN
4. Load PCA labeled samples diluted with ACN onto the HILIC column
5. Wash column with 5 x 300 µl of 1% formic acid, 90% ACN
6. Elute glycans with 3 x 30 µl of SPE into a collection tube for a final volume of 90 µl
7. Dry the 90 µl sample in a speed vac at 35°C or lyophilize overnight (see Note 4)
8. Resuspend the sample in 30 µl of water for subsequent exoglycosidase reactions

Digestion of PCA labeled glycans with exoglycosidases

Exoglycosidases can be used in single digests or in combinations to elucidate information about the total glycan profile

1. In PCR tubes (200 µl), mix 5 µl of PCA-labeled *N*-glycans (equivalent to 5 µg of starting material) from previous step with 2 µl 10X Glycobuffer 1, the recommended volume of exoglycosidase (see Table 1) and water to a final reaction volume of 20 µl
2. Incubate reactions for 3 hrs at 37°C
3. Add 10 µl of 50 mM NH_4 Formate Buffer pH 4.4 and 90 µl acetonitrile to each 20 µl reaction for a final acetronitrile concentration of 70%
4. Samples are now ready for LC or LC-MS analysis. In this experiement, *N*-glycan samples were separated using a XBridge™ BEH Amide column (Waters) on a Dionex UltiMate® LC equipped with fluorescent detection in line with a LTQ™ Orbitrap Velos™ Spectrometer equipped with a heated electrospray standard source (HESI-II probe)

TABLE 1:
Exoglycosidase digestion panel

COMPONENT	RXN 1	RXN 2	RXN 3	RXN 4	RXN 5	RXN 6
PCA-Labelled *N*-Glycans	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
10X Glycobuffer 1	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
H_2O	13 µl	11 µl	10 µl	9 µl	8 µl	6 µl
α2-3,6,8,9 Neuraminidase A (NEB #P0722)		2 µl	2 µl	2 µl	2 µl	2 µl
α1-3,4,6 Galactosidase (NEB #P0747)			1 µl	1 µl	1 µl	1 µl
β1-4 Galactosidase S (NEB #P0745)				1 µl	1 µl	1 µl
β-*N*-Acetylglucosaminidase S (NEB #P0744)					1 µl	1 µl
α1-2,4,6 Fucosidase O (NEB #P0749)						2 µl
Total	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**

Notes

Note 1: 2-AB labeled glycan signal is typically not as intense as PCA and may require more labeled substrate to get an adequate MS signal

Note 2: Stock solutions of PCA (550 mg dissolved in 1 ml DMSO), 2AB (250 mg dissolved in 1 ml DMSO), and sodium cyanoborohydride (200 mg/ml H_2O) can be kept at -20°C and thawed prior to use (reagents remain stable for several weeks and numerous freeze/thaw cycles). Prepare acidifed PCA or 2-AB solution by adding one volume of glacial acetic acid to eight volumes of PCA or 2AB stock solution.

Note 3: SPE buffer: 200 mM Ammonium Acetate

Note 4: Samples eluted with SPE buffer can be aliquoted before using the speed vac to decrease drying time

Results:

FIGURE 3A:

_N_-Glycans released from Infliximab, labeled with PCA, digested for 3 hours with exoglycosidases.

Digestion of Infliximab with a sequential panel of exoglycosidases serves as a tool to elucidate and verify glycan profile. Refer to Table 1 for reaction conditions.

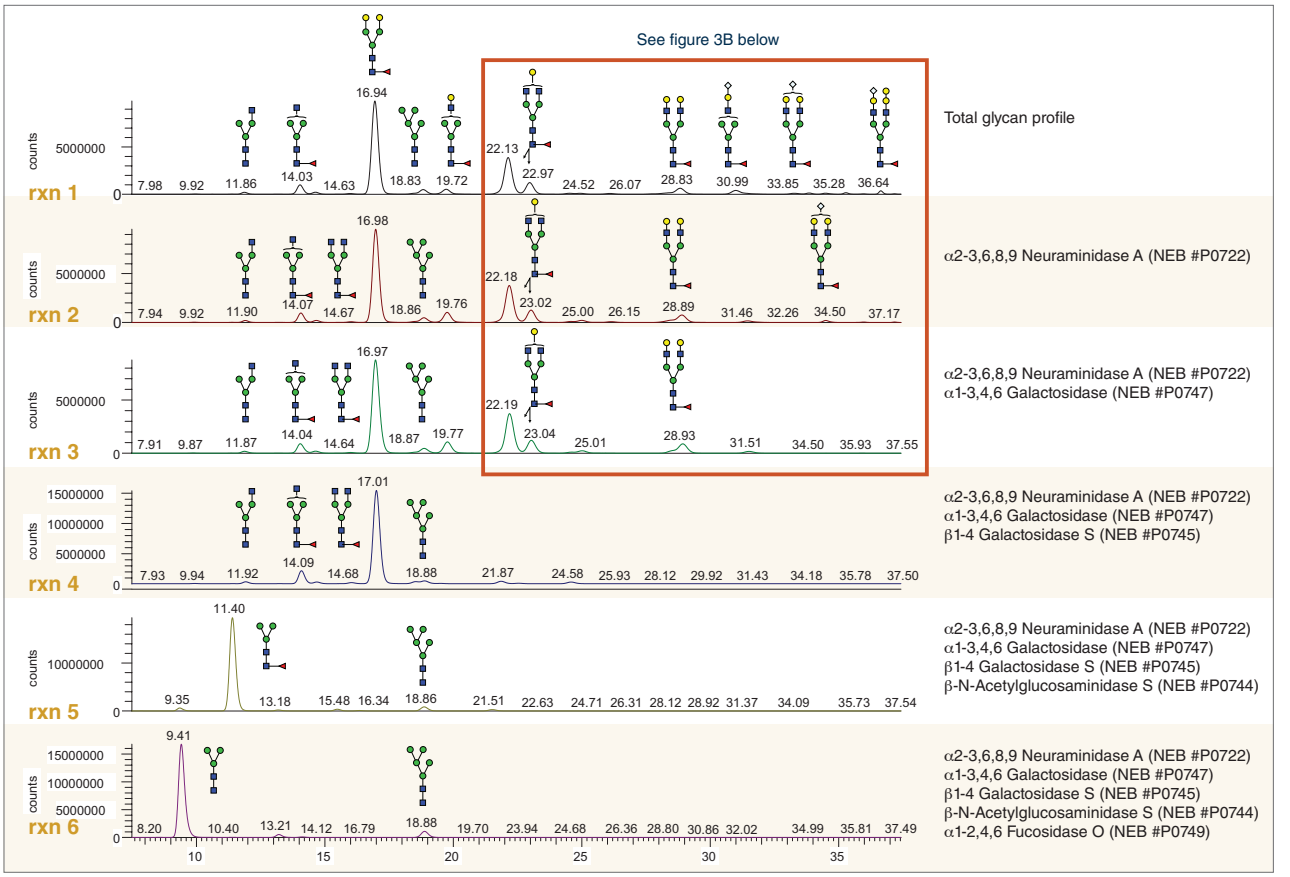
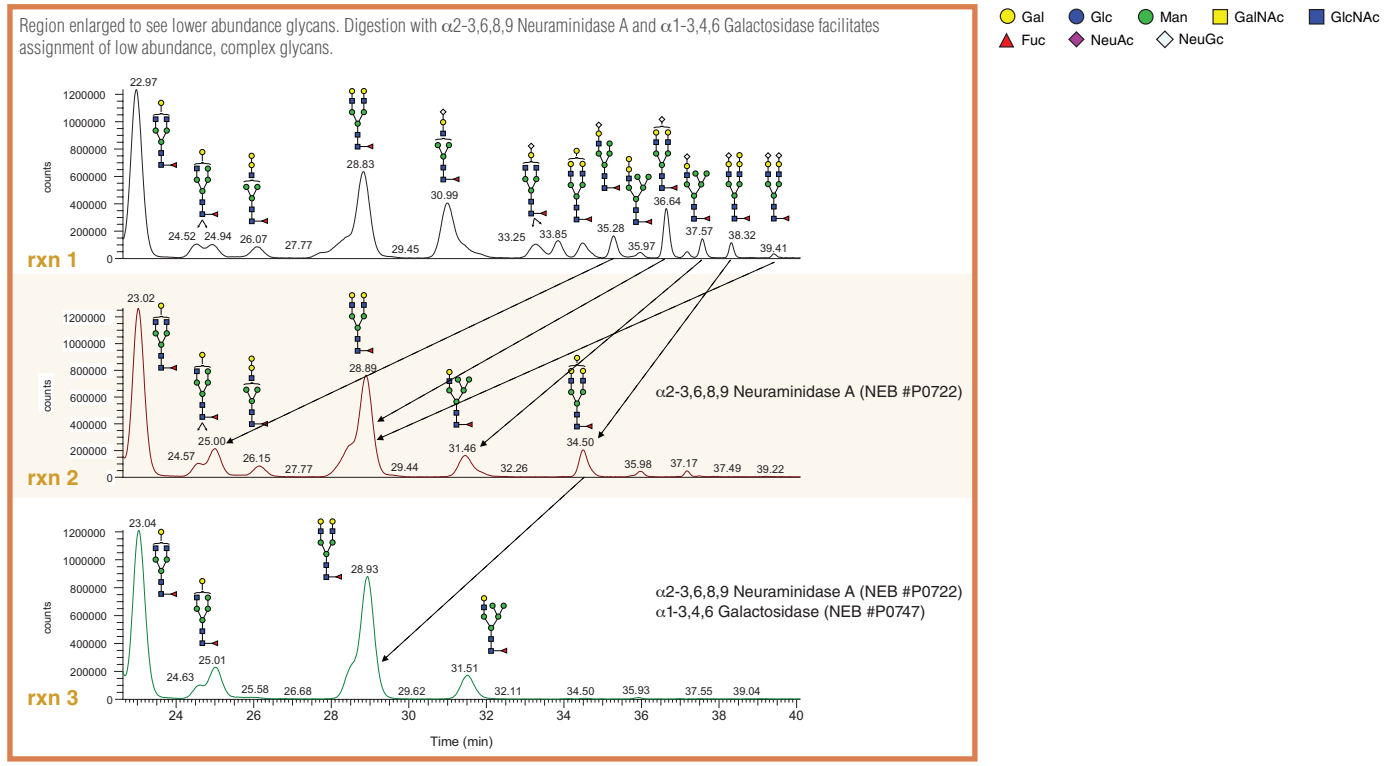


FIGURE 3B:

Expanded lower abundance profile of Infliximab glycan analysis

Region enlarged to see lower abundance glycans. Digestion with α2-3,6,8,9 Neuraminidase A and α1-3,4,6 Galactosidase facilitates assignment of low abundance, complex glycans.



Quantification of specific isoforms can be difficult with a complex glycan panel, especially when looking for less abundant species or epitopes that coelute. This process can be simplified by digesting with exoglycosidase combinations that are selected to trim the panel down to simplified forms while highlighting the species of interest. In the experiment shown below in Figure 4, the glycan profile of Enbrel is reduced to three main peaks corresponding to high mannose, fucosylated and afucosylated species. These peaks are then easily integrated and quantitated.

FIGURE 4:
Glycans released from Enbrel, trimmed to trimannosyl core with exoglycosidases to quantitate overall level of fucosylation and high mannose structures.

Panel A: Total glycan profile.
Panel B: Enbrel glycan digestion with 2 µl of α2-3,6,8,9 Neuraminidase A, 1 µl of β1-4 Galactosidase S, and 1 µl of β-*N*-Acetylglucosaminidase S.
Panel C: Enbrel glycan digestion with 2 µl of α2-3,6,8,9 Neuraminidase A, 1 µl of β1-4 Galactosidase S, 1 µl of β-*N*-Acetylglucosaminidase S, and 2 µl of α1-2,4,6 Fucosidase O.

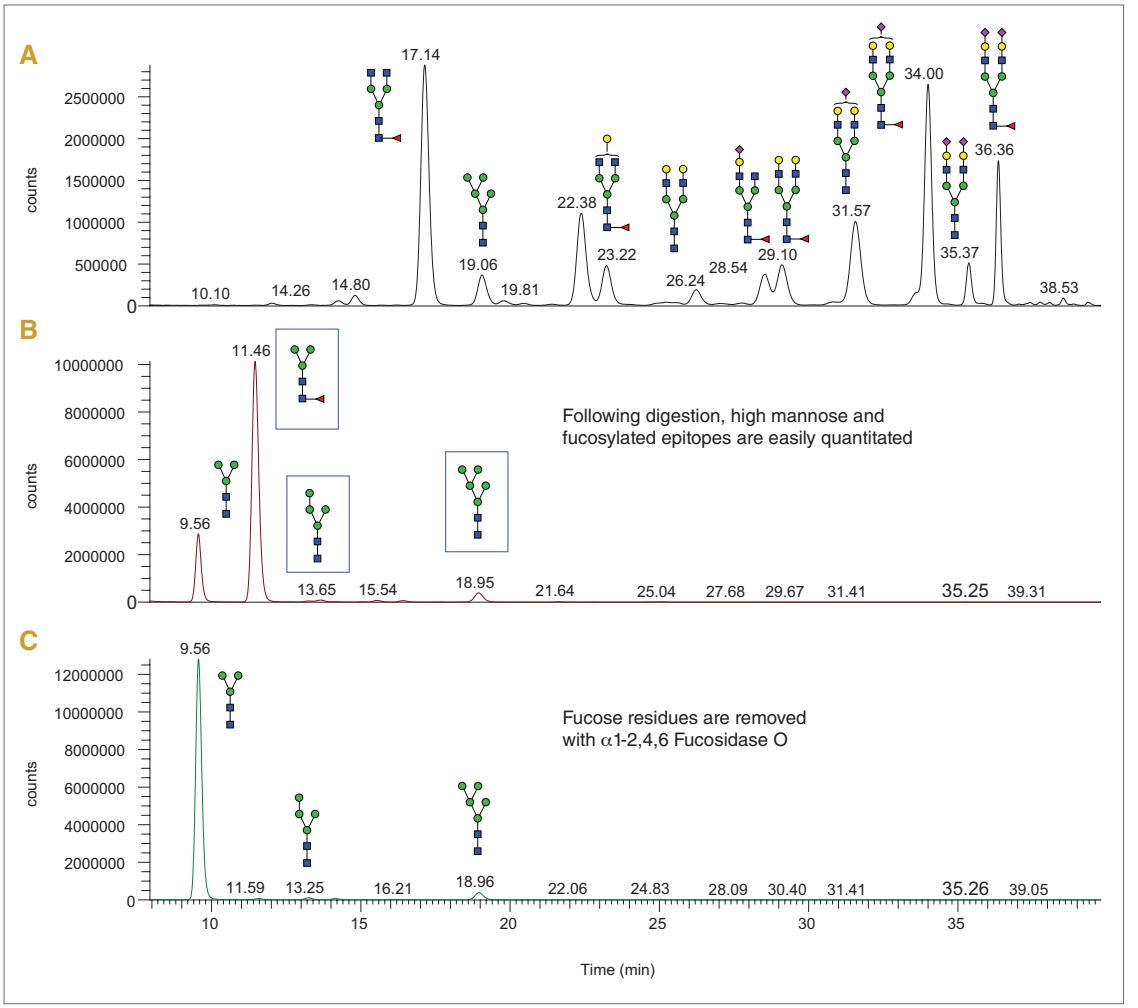


FIGURE 5:
Quantitation of fucosylation and high mannose structures.

% HIGH MANNOSE		% FUCOSYLATION	
Man 4	Man 5	Fucosylated	afucosylated
1.6	2.9	75.6	24.4

Gal Glc Man GalNAc GlcNAc Fuc NeuAc NeuGc

FIGURE 6:

Enzyme combinations to help isolate and quantitate potentially immunogenic low abundance isotopes such as Neu5Gc and α1-3 Galactose in Infliximab, a murine-derived therapeutic.

PANEL A: Total glycan profile.

PANEL B: Infliximab glycan digestion with 1 µl of α1-3,4,6 Galactosidase, 1 µl of β1-4 Galactosidase S, and 1 µl of β-*N*-Acetylglucosaminidase S.

PANEL C: Infliximab glycan digestion with 2 µl of α2-3,6,8,9 Neuraminidase A, 1 µl of β1-4 Galactosidase S, and 1 µl of β-*N*-Acetylglucosaminidase S.

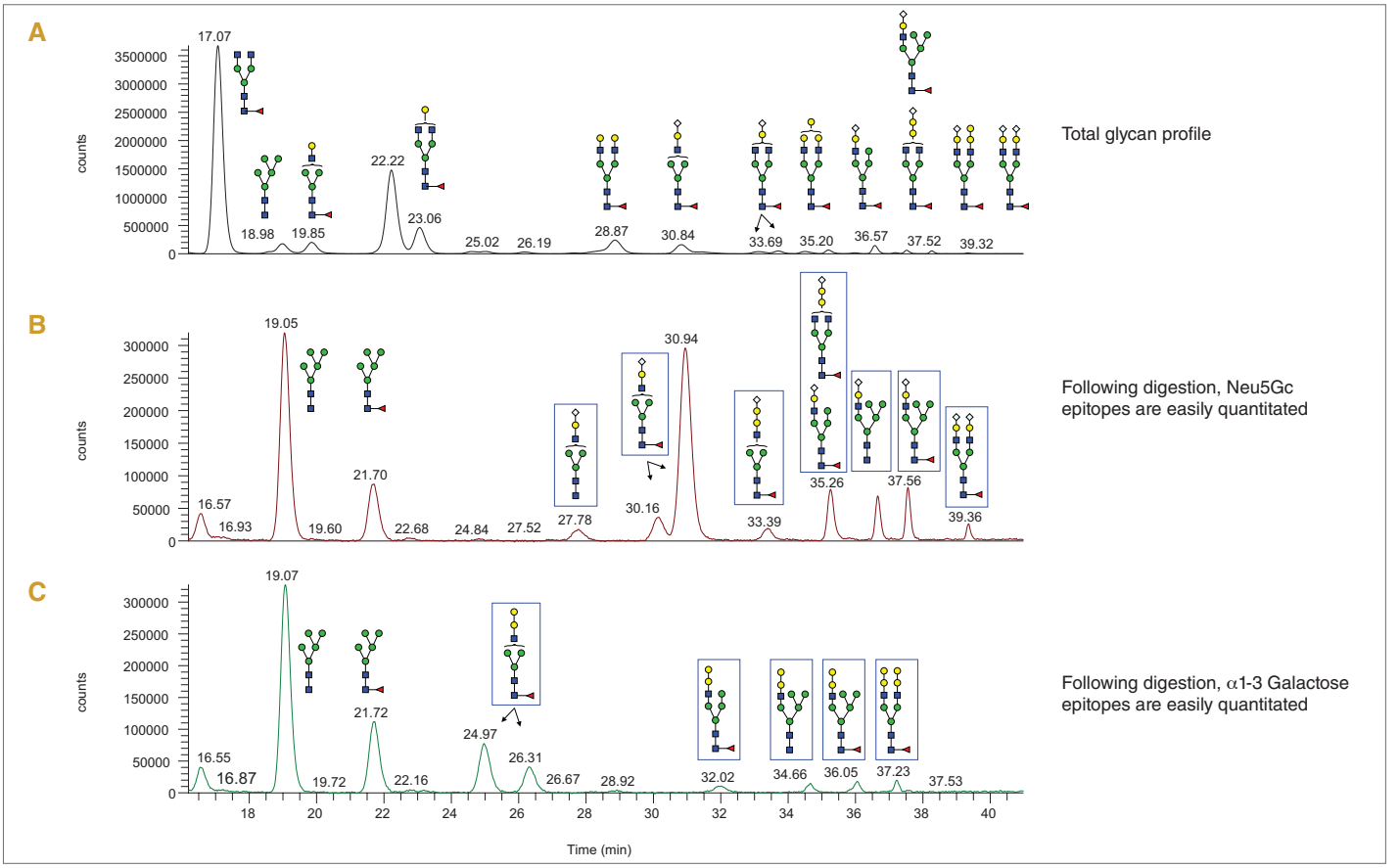


FIGURE 7:

Quantitation of Neu5Gc and α1-3 Galactose epitopes.

% Neu5GC	% α1-3 Galactose
7.0	2.3

Conclusion

Highly purified, specific exoglycosidases are valuable tools for determining the glycan profile of antibodies. Even using LC-MS, where a chromatographic peak has an exact molecular weight assignment, isoforms can make it difficult to accurately assign structures. Combinations of these enzymes can be used to highlight overall fucosylation and high mannose structures. In addition, *N*-glycolylneuraminic acid (Neu5Gc) and alpha-linked galactose residues, potentially immunogenic, non-human epitopes that are present in murine derived antibodies can be quantitated. The method described here has been developed to allow glycan release, labelling and exoglycosidase digestion within a work day to expedite the process of glycan sequencing.

References:
1. Abes, R. et al. *Pharmaceuticals* 3(1) 2010: 146–157.
2. Goetz, AM. et al. (2011) *Glycobiology* (7) 949–959.

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