N-Glycan Sequencing Kit

NEB #E0577S

20 reactions

Version 2.0_4/20

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N-Glycan Sequencing Kit Includes

Kit components are stored at multiple temperatures. Please store at temperature indicated on each vial.

α2-3,6,8,9 Neuraminidase A

α2-3 Neuraminidase S

α1-3,4,6 Galactosidase

β1-4 Galactosidase S

β-N-Acetylglucosaminidase S

α1-2,3,6 Mannosidase

α1-2,4,6 Fucosidase O

GlycoBuffer 1 (10X)

Zinc (10X)

Required Materials Not Included

Rapid PNGase F (NEB #P0710)

Fluorophore labeling reaction components:

- 2-aminobenzamide (2AB) (Sigma A89804)
- Procainamide (Sigma P9391)
- Sodium Cyanoborohydride (Sigma 156159)
- DMSO (Sigma 472301)

HILIC columns

Acetonitrile (ACN) HPLC/MS Grade

50 mM NH_4 Formate Buffer (pH 4.4)

Glacial Acetic Acid

HILIC Microspin Column

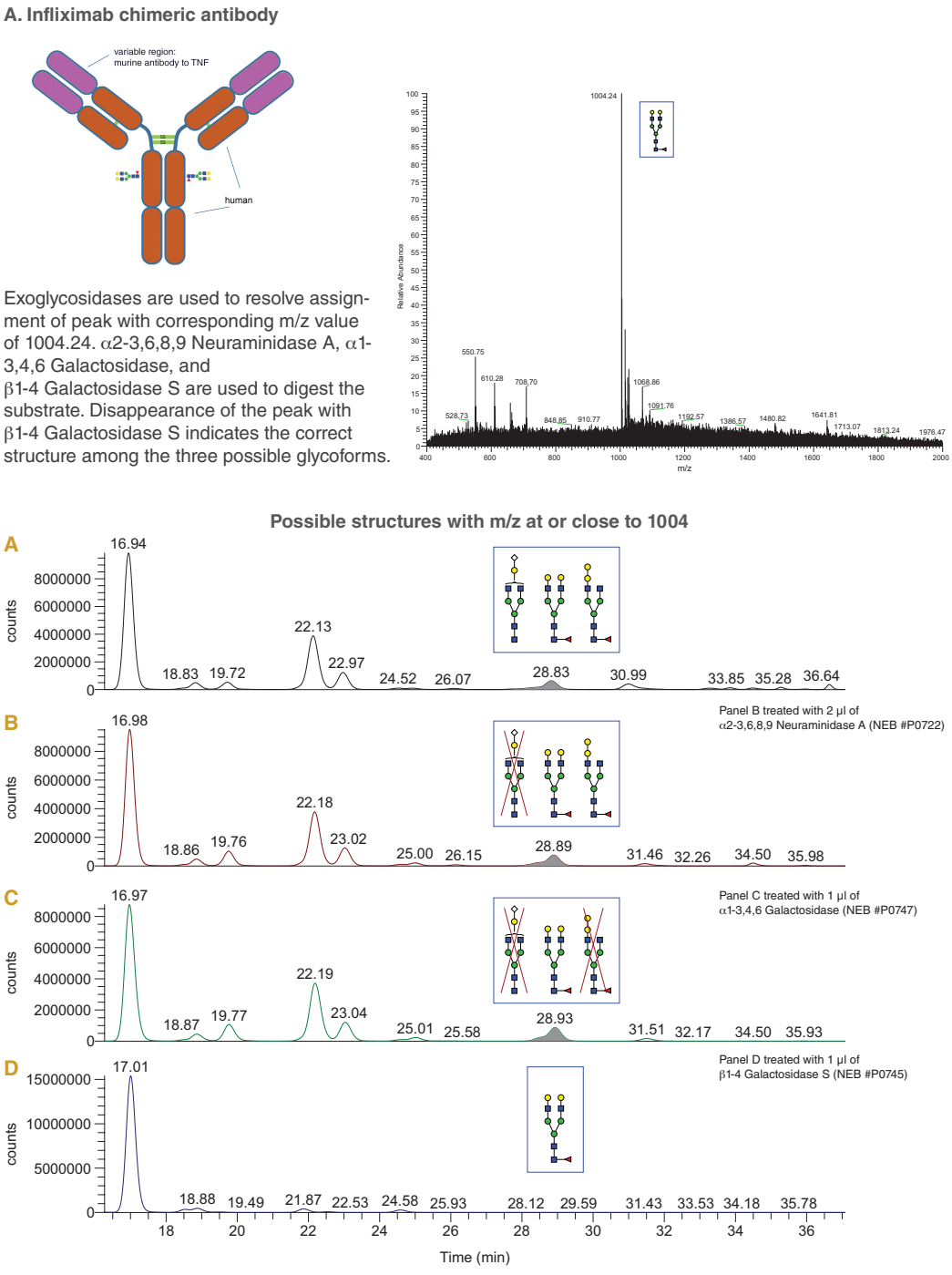
Introduction

The *N*-Glycan Sequencing Kit consists of seven well characterized, highly pure, recombinant exoglycosidase enzymes selected to simplify the process of characterizing typical *N*-linked glycan structures. The extreme diversity of glycan structures on a glycoprotein makes elucidation of the profile challenging and often a number of orthogonal approaches are employed to verify the individual structures. The use of sequential or tandem exoglycosidase digestion of oligosaccharides followed by mass spectrometry (MS) or capillary electrophoresis (CE) analysis provides detailed carbohydrate sequence information and resolves ambiguities (Figure 1, page 3). The exoglycosidases are all active in a universal buffer system, GlycoBuffer 1, and can be used in single digests or in combination.

The kit is compatible with *N*-linked glycans released from a variety of CHO and murine derived antibodies, as well as *N*-linked glycans released from other glycoproteins. Optimal incubation times and enzyme concentrations may vary for more complex glycans. The recommended enzyme quantity is sufficient for digestion of up to 130 pmol of released glycans (equivalent to released glycans from approximately 10 µg of antibody) labeled with 2AA, 2AB or procainamide. Other commercially available "Instant labels" may require a larger quantity of enzyme or a longer incubation time for complete digestion.

This protocol describes antibody deglycosylation, glycan labeling, purification and exoglycosidase digestion that can be completed in one day (Figure 2, page 4). The protocol is compatible with subsequent downstream MS and CE analysis.

Figure 1. Elucidation of Structure Associated with Peak 28.83 min (m/z = 1004.4)



Panel A: *Total released glycans from Infliximab*

Panel B: *Total released glycans treated with 2 µl α2-3,6,8,9 Neuraminidase A; peak remains*

Panel C: *Total released glycans treated with 1 µl α1-3,4,6 Galactosidase; peak remains*

Panel D: *Total released glycans treated with 1 µl β1-4 Galactosidase S; results in disappearance of peak, verifying the β1-4 galactose linkage of this glycan*

Gal Glc Man GalNAc GlcNAc Fuc NeuAc NeuGc

Figure 2. Glycan sample preparation workflow

- Deglycosylation with Rapid PNGase F
- Labeling of released glycans by reductive amination or with a fluorophore label
- HILIC clean-up of labeled glycans
- Drying of glycans to concentrate and remove volatile solvents
- Exoglycosidase digestion of labeled glycans
- Sample ready for downstream analysis or enzyme clean up step if necessary

Protocols

Rapid PNGase F Release of Antibody Glycans

For optimal heat transfer, use 0.2 ml thin wall microtubes or alternatively, 1.5 ml centrifuge tubes. A thermal cycler with heated lid, or a microtube heat block, are suitable for incubation.

One-step Protocol:

1. Combine up to 100 µg of antibody and H_2O to a volume of 16 µl.
2. Add 4 µl of Rapid PNGase F Buffer (5X) to make a 20 µl total reaction volume.
3. Add 1 µl of Rapid PNGase F.
4. Incubate 10 minutes at 50°C.

Two-step Protocol:

Some antibodies (i.e., Fab *N*-glycans) require a preheating step for efficient deglycosylation.

1. Combine up to 100 µg of antibody and H_2O to a volume of 16 µl.
2. Add 4 µl of Rapid PNGase F Buffer (5X) to make a 20 µl total reaction volume.
3. Incubate at 80°C for 2 minutes, cool down.
4. Add 1 µl of Rapid PNGase F.
5. Incubate 10 minutes at 50°C.

Fluorescent Labeling with Procainamide (PCA), or 2-aminobenzamide (2AB)

1. Add 18 µl of acidified PCA or 2AB labeling reagent and 24 µl cyanoborohydride reagent to the deglycosylation reaction and incubate for 45 minutes at 65°C.

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

2. Cool reactions to room temperature.

Glycan Purification with a HILIC Spin Column

Released, labeled glycans can be purified from the free label using various methods. Purified glycans must be free of organic solvents prior to enzyme digestion.

1. Add 350 µl Acetonitrile (ACN) to the labelled reactions to a final concentration of 85% ACN.
2. Using either a vacuum manifold or centrifuge adapter (following manufacturer's instructions), condition a HILIC spin column with 350 µl H_2O, followed by 350 µl of 50 mM ammonium formate, pH 4.5.
3. Equilibrate column with 350 µl of 85% ACN/15% ammonium formate.
4. Load sample onto the HILIC column, spin.
5. Wash column with 300 µl of 1% formic acid, 90% ACN, repeat 5 times.
6. Elute glycans into a fresh collection tube with 30 µl of 50 mM ammonium formate, pH 4.4. Repeat 3 times for a final volume of 90 µl.

7. Dry the 90 µl glycan sample in a speed vac with temperature no greater than 4°C or lyophilize overnight

   Note: To minimize drying time, samples can be aliquoted into several tubes prior to placing in speed vac.

8. Resuspend the sample in 100 µl H_2O for subsequent exoglycosidase digestion reactions. The resuspension volume can be modified to accommodate subsequent exoglycosidase reactions.

Exoglycosidase Digestion of Labelled Glycans

Exoglycosidase reactions can be assembled with single or multiple enzymes. The amount of labeled glycan should be determined based on the downstream method of analysis (i.e., HPLC, UPLC, MS, CE). Considerations include the instrument range of detection and the signal to noise ratio of the fluorophore used. This protocol allows for the digestion of up to 130 pmol (equivalent to 10 µg of antibody) using the recommended amounts of exoglycosidase(s).

1. Combine up to 130 pmol of labeled, purified glycans (10 µg antibody) and H_2O to a total reaction volume of 20 µl.

   Note: Labeled glycans can be increased to a maximum of 330 pmol (25 µg of antibody) using the recommended amount of exoglycosidases with an incubation time of 18 hr at 37°C.

2. Add 2 µl of 10X GlycoBuffer 1.

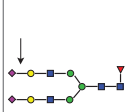
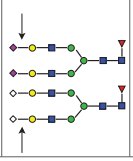
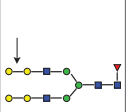
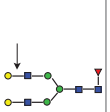
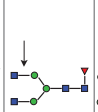
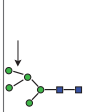
3. Add the recommended volume of each exoglycosidase (single or in combination) to yield the specific structures, as shown in Table 1.

4. Incubate reactions for 3 hours at 37°C.

   Note: If digestion is not complete, increase incubation time to 18 hours at 37°C.

   Samples can be analyzed directly with MS or CE or can be further purified to remove enzymes from the reactions if necessary.

Table 1. Exoglycosidase Digestion Panel

EXOGLYCOSIDASES	RXN 1	RXN 2	RXN 3	RXN 4	RXN 5	RXN 6	RXN 7
α2-3 Neuraminidase S (NEB #P0743)	1 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*-Acetylglucos-aminidase S (NEB #P0744)					1 µl	1 µl	
α1-2,3,6 Mannosidase* (NEB #P0768)							2 µl
α1-2,4,6 Fucosidase O (NEB #P0749)						2 µl	
Glycan Product							

*Supplement reaction with final reaction concentration of 1X Zinc for optimal activity.

Example 1: Characterization of Infliximab Glycans using Exoglycosidase Panel

Infliximab (30 µg) was deglycosylated with Rapid PNGase F, released glycans were labeled with procainamide (PCA) and purified as described in the general protocol. PCA-labeled glycans were lyophilized and resuspended in 30 µl of water.

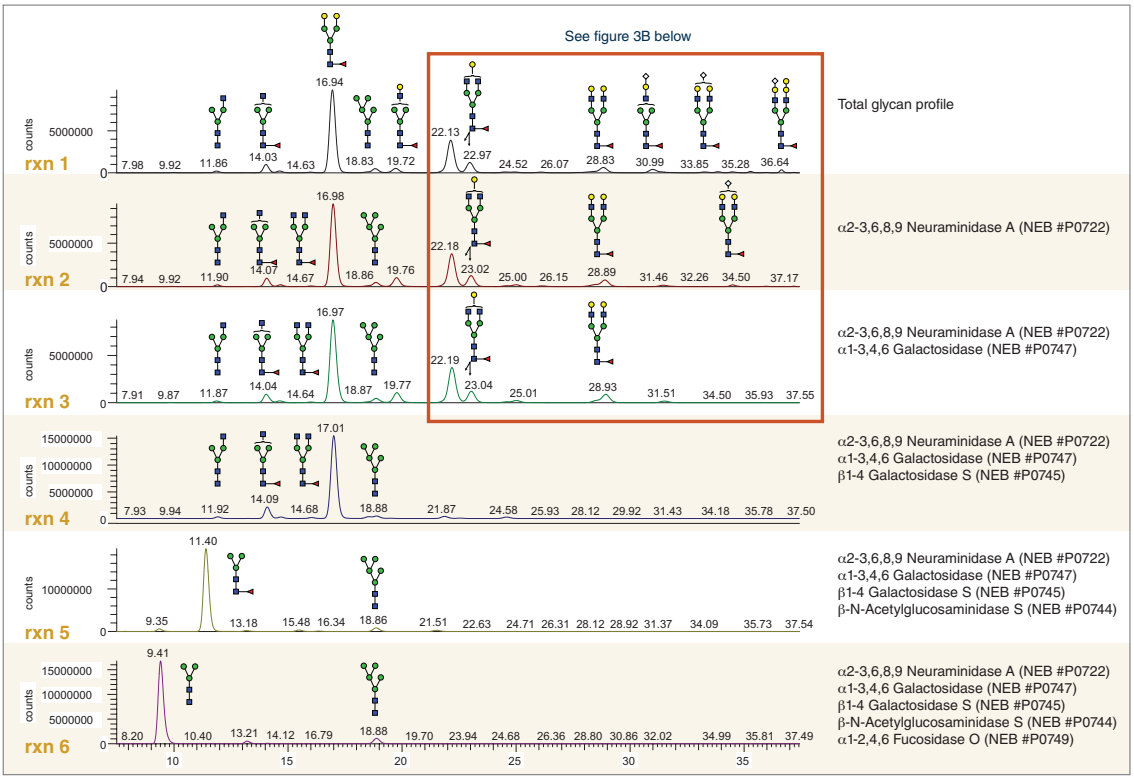
1. Prepare exoglycosidase reactions as described in Table 2:

Table 2. Exoglycosidase Digestion Panel

COMPONENT	RXN 1	RXN 2	RXN 3	RXN 4	RXN 5	RXN 6
N-glycans-PCA Labelled	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
Glycobuffer 1 (10X)	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

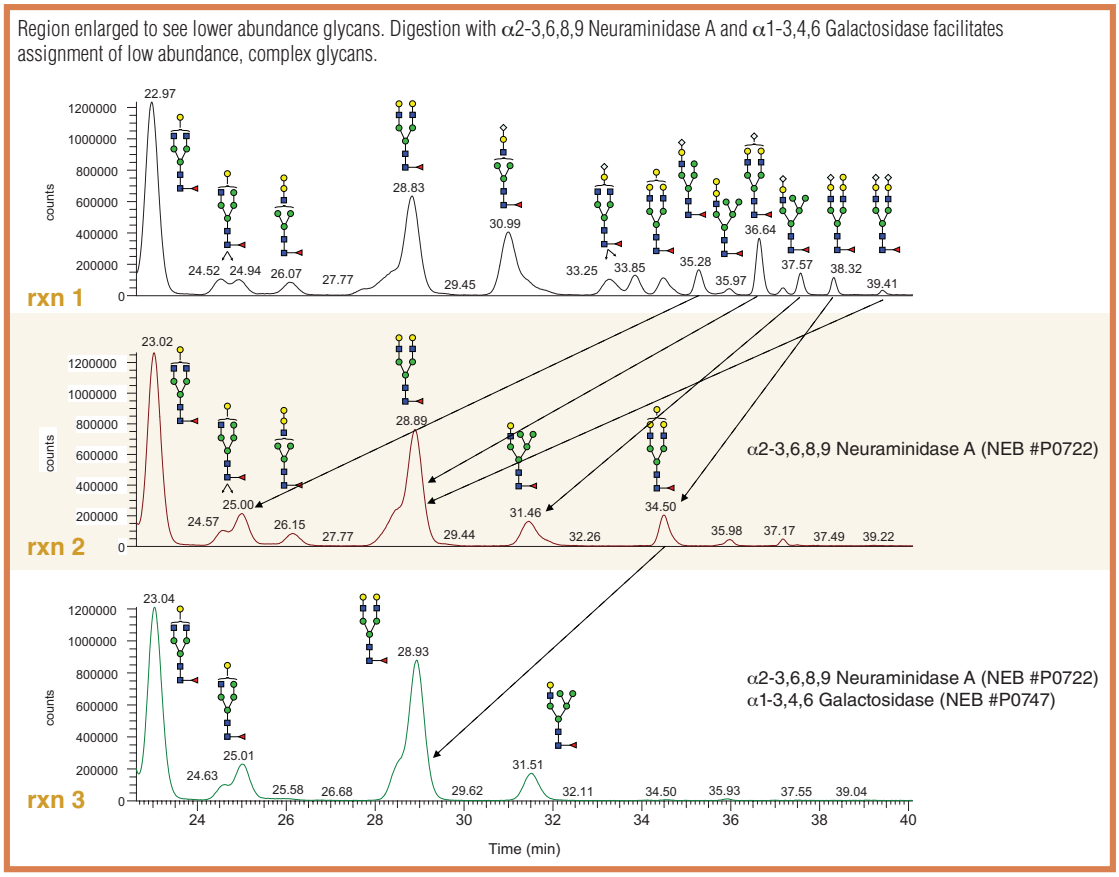
2. Incubate samples for 3 hours at 37°C
3. Add 10 µl of 50 mM NH_4 formate buffer pH 4.4 and 90 µl acetonitrile to each reaction for a final acetonitrile concentration of 70%.
4. Analyze by LCMS, results shown in Figure 3A and 3B: *N*-glycan samples are separated using a XBridge® BEH Amide column (Waters) 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).

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 2 for reaction conditions.

Figure 3B: Expanded lower abundance profile of Infliximab glycan analysis.



Example 2: Exoglycosidase Combinations to Isolate and Quantitate Potentially Immunogenic Low Abundance Isotopes such as Neu5Gc and α1-3 Galactose in Infliximab, a Murine-derived Therapeutic

Using combinations of enzymes can be useful to simplify the glycan profile data and isolate a species of interest. Glycans were released from 15 µg of Infliximab using Rapid PNGase F and labelled with procainamide (PCA). Purified, labelled glycans were resuspended in 15 µl of H_2O.

1. Prepare exoglycosidase reactions as described in Table 3:

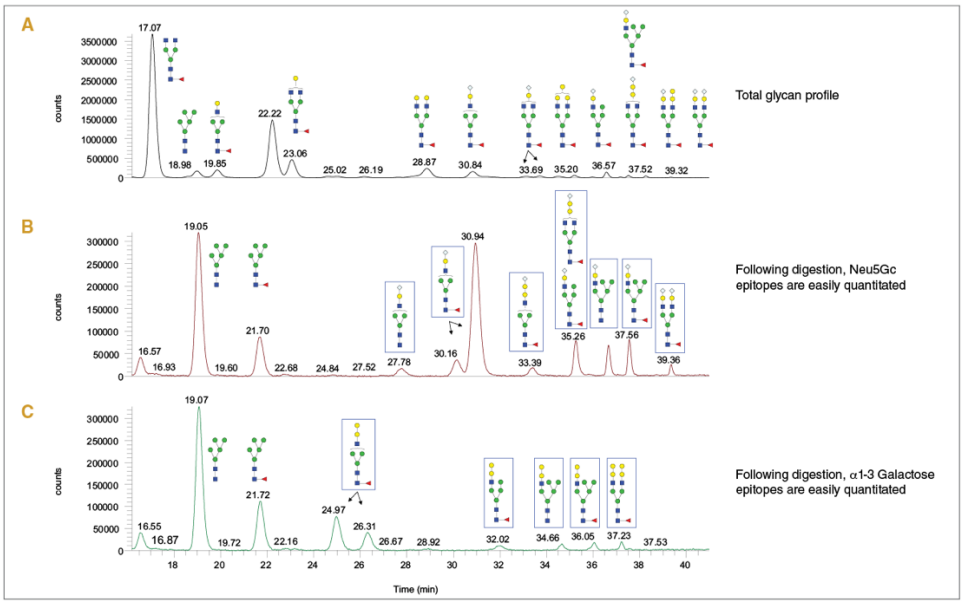
Table 3. Exoglycosidase Digestion Panel

COMPONENT	RXN A	RXN B	RXN C
N-glycans-PCA Labelled	5 µl	5 µl	5 µl
Glycobuffer 1 (10X)	2 µl	2 µl	2 µl
H_2O	13 µl	8 µl	7 µl
α2-3,6,8,9 Neuraminidase A (NEB #P0722)			2 µl
α1-3,4,6 Galactosidase (NEB #P0747)		1 µl	
β1-4 Galactosidase S (NEB #P0745)		2 µl	2 µl
β-*N*-Acetylglucosaminidase S (NEB #P0744)		2 µl	2 µl
Total	20 µl	20 µl	20 µl

2. Incubate samples for 3 hours at 37°C
3. Add 10 µl of 50 mM NH_4 format buffer pH 4.4 and 90 µl acetonitrile to each reaction for a final acetonitrile concentration of 70%.

4. Analyze by LCMS, results shown in Figure 4: *N*-glycan samples are 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).

Figure 4: Enzyme combinations help isolate and quantitate potentially immunogenic low abundance isotopes such as Neu5Gc and α1-3 Galactose in Infliximab, a murine-derived therapeutic.



RXN A: *Total glycan profile.*

RXN 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.*

RXN 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.*

Gal Glc Man GalNAc GlcNAc Fuc NeuAc NeuGc

Example 3: Exoglycosidase Digestion of Enbrel Glycans to Quantitate Overall Level of Fucosylation and High Mannose Structures

Glycans were released from 15 µg of Enbrel using Rapid PNGase F and labelled with procainamide (PCA) as described in the general protocol. Purified, labelled glycans were resuspended in 15 µl of H_20.

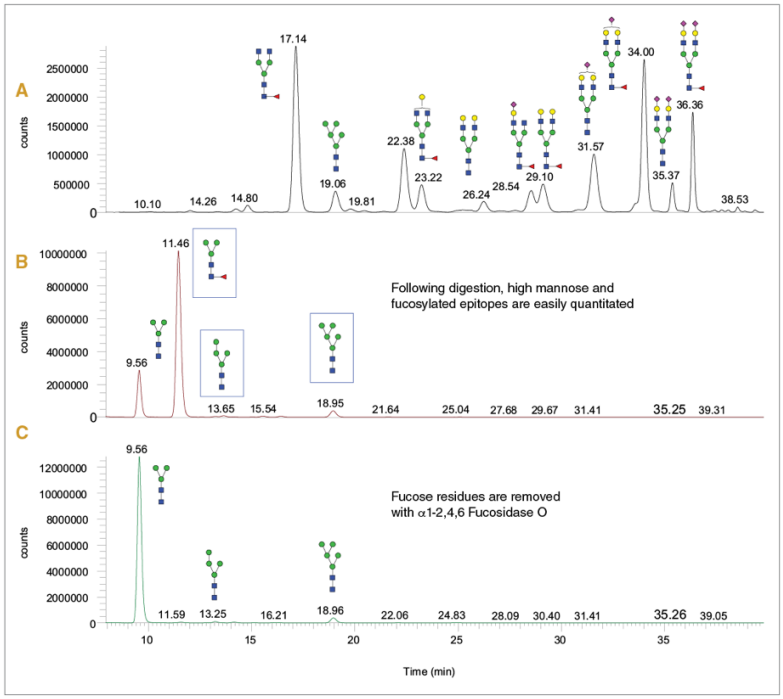
1. Prepare exoglycosidase reactions as described in Table 4:

Table 4. Exoglycosidase Digestion Panel

COMPONENT	RXN A	RXN B	RXN C
N-glycans-PCA Labelled	5 µl	5 µl	5 µl
Glycobuffer 1 (10X)	2 µl	2 µl	2 µl
H_2O	13 µl	8 µl	7 µl
α2-3,6,8,9 Neuraminidase A (NEB #P0722)		2 µl	2 µl
β1-4 Galactosidase S (NEB #P0745)		2 µl	2 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

2. Incubate samples for 3 hours at 37°C
3. Add 10 µl of 50 mM NH_4 formate buffer pH 4.4 and 90 µl acetonitrile to each reaction for a final acetonitrile concentration of 70%.
4. Analyze by LCMS, results shown in Figure 5: *N*-glycan samples are 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).

Figure 5: Glycans released from Enbrel, trimmed to the trimannosyl core with exoglycosidases to quantitate overall level of fucosylation and high mannose structures..



RXN A: Total glycan profile.
RXN 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.
RXN 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.

Frequently Asked Questions (FAQs)

Q1. Is this protocol suitable for digestion of glycoproteins?

A1: Although this protocol has been optimized for digestion of antibodies, it can be utilized with various other glycoproteins. Some glycoproteins are not efficiently deglycosylated with Rapid PNGase F. In this case, the glycoprotein can be treated with PNGase F Glycerol-free, Recombinant (NEB #P0709) using denaturing reaction conditions.

Q1. How do I calculate the molarity of released glycans from my antibody substrate?

A1: Typically an IgG antibody with Fc glycosylation has approximately 13.3 pmol of glycans per 1 µg of antibody.

Q1. Why are two different neuraminidase enzymes provided?

A1: α2-3,6,8,9 Neuraminidase A (NEB #P0722) has a broader specificity, as it cleaves both α2-3,6,8,9 linked Neu5Ac (*N*-Acetylneuraminic acid) and Neu5Gc (*N*-Glycolylneuraminic acid) residues. Neu5Gc epitopes are found in murine derived antibodies. α2-3 Neuraminidase S (NEB #P0743) cleaves only α2-3 linked Neu5Ac residues.

Q1. Should I use the One- or Two-step Rapid PNGase F deglycosylation method for my antibody?

A1: Rapid PNGase F has been developed for efficient and fast deglycosylation of antibodies in a simple One-step reaction at 50°C. However, some IgGs (i.e., carrying Fab glycosylation) require a pre-denaturing step of 2 minutes at 80°C. We recommend starting with the standard One-step protocol. If there is evidence (i.e., by gel migration or proteomic analysis) that *N*-glycans remain attached to the protein, follow the Two-step protocol.

Q1. The deglycosylation protocol is recommended for up to 100 µg of antibody; How do I determine the amount of starting material I should deglycosylate?

A1: There are several factors to consider. In general, the substrate is prepared in batch and then subdivided for enzyme digestion; therefore the number of final exoglycosidase reactions desired needs to be determined. Furthermore, the sensitivity of the particular fluorescent label used determines the quantity of labelled glycan required for adequate signal detection. Some instant labels, such as Rapifluor, have a sensitive fluorescent detection and good signal intensity for mass detection and so less starting material is required.

Q1. Is it possible to combine the α1-2,3,6 Mannosidase with other exoglycosidases? Are any of the exoglycosidases inhibited by the zinc that is required for optimal mannosidase activity?

A1: Yes, α1-2,3,6 Mannosidase (NEB #P0768) can be combined with any of the exoglycosidases provided; 1X Zinc will not affect the activity of the other enzymes.

Q1. Does ammonium formate inhibit exoglycosidase digestion?

A1: Yes, the exoglycosidases are sensitive to ammonium formate, it is important that no ammonium formate remains after elution. If necessary, dry the sample in a speed vac or lyophilize and resuspend in water prior to exoglycosidase digestion.

Q1. How long do I need to incubate a Rapifluor labelled substrate with the exoglycosidases?

A1: Rapifluor-labelled glycans released from 10 µg or less of antibody are fully digested in 3 hrs with α2-3,6,8,9 Neuraminidase A, α1-3,4,6 Galactosidase, β1-4 Galactosidase S, β-N-Acetylglucosaminidase S and α1-2,3,6 Mannosidase. However, α1-2,4,6 Fucosidase O requires an 18 hour incubation at 37°C for complete digestion.

Q1. Which is the enzyme of choice to release α1-6 fucose residues from antibody glycans: α1-2,4,6 Fucosidase O or α1-2,3,4,6 Fucosidase from bovine kidney?

A1: α1-2,4,6 Fucosidase O cleaves α1-6 fucose residues from antibody glycans more effectively than α1-2,3,4,6 Fucosidase from bovine kidney. α1-2,4,6 Fucosidase O does require an 18 hour incubation at 37°C for complete digestion.

Troubleshooting

Incomplete Digestion of Glycans with Exoglycosidases

- Be sure glycans are fully dried after speed vac or lyophilization step. Residual ACN can inhibit activity of exoglycosidases
- Increase incubation time of exoglycosidase reactions to 18 hours at 37°C if shorter incubation was performed

Low Fluorescent or Mass Spec Signal

- Increase the amount of labelled glycans used in the digestions. Concentrate sample if necessary
- Make fresh 1% formic acid, 90% ACN wash solution, if solution becomes more aqueous, the glycans can elute in the wash step

Ordering Information

NEB #	PRODUCT	SIZE
E0577S	*N*-Glycan Sequencing Kit	20 reactions

COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
P0722S/L	α2-3,6,8,9 Neuraminidase A	800/4,000 units
P0743S/L	α2-3 Neuraminidase S	400/2,000 units
P0747S/L	α 1-3,4,6 Galactosidase	200/1,000 units
P0745S/L	β1-4 Galactosidase S	400/2,000 units
P0744S/L	β-*N*-Acetylglucosaminidase	100/500 units
P0768S/L	α1-2,3,6 Mannosidase	80/400 units
P0749S/L	α1-2,4,6 Fucosidase O	80/400 units

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	1/18
2.0	New format applied	4/20

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