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Glycoproteomics

TECHNICAL GUIDE

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Deglycosylation Enzymes

Several classes of glycans exist, including *N*-linked glycans, *O*-linked glycans, glycolipids, *O*-GlcNAc, and glycosaminoglycans. *N*-linked glycosylation occurs when glycans are attached to asparagine residues on the protein. *O*-linked glycans are most commonly attached to serine or threonine residues through the *N*-Acetylgalactosamine residue. Removal of oligosaccharides from glycoproteins, termed deglycosylation, is often used to simplify analysis of the peptide and/or glycan portion of a glycoprotein. Detailed knowledge of the glycan structures helps to correlate them to their respective function. To do this, tools are required for highly sensitive analysis of glycan chains. Both chemical and enzymatic methods exist for removing oligosaccharides from glycoproteins. However, chemical methods such as β-elimination with mild alkali (1) or mild hydrazinolysis (2) can be harsh and results in the degradation of the protein; whereas enzymatic methods are much gentler and can provide complete sugar removal with no protein degradation.

ENDOGLYCOSIDASE SELECTION CHART

	PROTEIN DEGLYCOSYLATION MIX II (#P6044)	*O*-GLYCOSIDASE (#P0733 & #E0540)	PNGASE A (#P0707)	PNGASE F (#P0704 & #P0705)	REMOVE-IT PNGASE F (#P0706)	RECOMBINANT PNGASE F (#P0708 & #P0709)	RAPID PNGASE F (#P0710)	RAPID PNGASE F, (NON-REDUCING FORMAT) (#P0711)	ENDO H (#P0702) & ENDO H_F (#P0703)	ENDO S (#P0741)	ENDO D (#P0742)	ENDO F2 (#P0772)	ENDO F3 (#P0771)
Deglycosylation of glycoproteins (*N*- and *O*-glycans)	•												
Removal of *O*-glycans	•	•											
Removal of *N*-glycans from glycoproteins	•		•	•	•	•	•	•	•	•	•	•	•
Removal of high mannose and hybrid *N*-glycans (leaving a GlcNAc attached to Asn)									•				
Optional removal of the enzyme from the reaction					•					•	•	•	•
Removal of paucimannose *N*-glycans (GlcNAc attached to Asn)						•			•		•		
Removal of *N*-glycans from IgGs (leaving a GlcNAc attached to Asn)									•	•			
Analysis of therapeutic glycoproteins, compliance with regulatory agencies						•	•	•					
High throughput *N*-glycan analysis of monoclonal antibodies, regulatory compliance							•	•					
Glycomics				•	•	•	•	•	•	•	•	•	•
Proteomics				• (only GF)	•	• (only GF)	•	•	•	•	•	•	•
Determine *N*-glycan sites									•	•	•	•	•
Removal of *N*-glycans from plant and insect glycoproteins			•										

GF = Glycerol Free

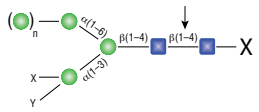
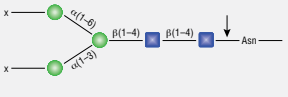
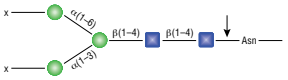
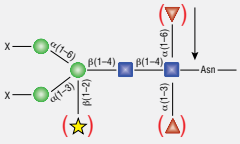
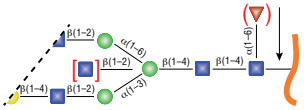
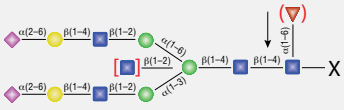
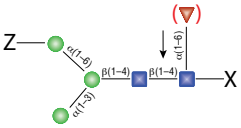
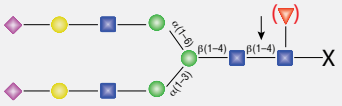
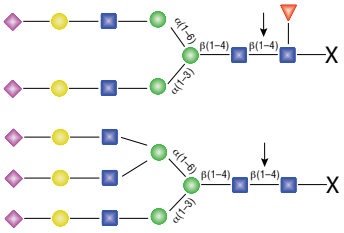
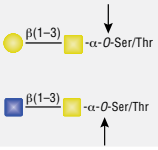
References

1. Kakehi, K. et al. (1994) *J. Chromatogr. A.* 680, 209–215.
2. Royle, L. et al. (2002) *Anal. Biochem.* 304, 70–90.

Endoglycosidases

Endoglycosidases cleave entire glycan groups from glycoproteins. There are a variety of endoglycosidases that are active on *N*-linked glycans, and fewer that are active on *O*-linked glycans. The table below lists the endoglycosidases available from NEB, along with their specificities.

ENDOGLYCOSIDASE SELECTION CHART

ENDOGLYCOSIDASE	NEB #	SOURCE	SPECIFICITY
Endo H Endo H_f	NEB #P0702 NEB #P0703	*Streptomyces picatus*	
PNGase F PNGase F Glycerol Free Remove-iT PNGase F	NEB #P0704 NEB #P0705 NEB #P0706	*Elizabethkingia miricola*	
PNGase F, Recombinant PNGase F Glycerol Free, Recombinant	NEB #P0708 NEB #P0709	*Elizabethkingia miricola*	
PNGase A	NEB #P0707	*Oryza sativa*	
Rapid PNGase F Rapid PNGase F (non-reducing format)	NEB #P0710 NEB #P0711		
Endo S	NEB #P0741	*Streptococcus pyogenes*	
Endo D	NEB #P0742	*Streptococcus pneumoniae*	
Endo F2	NEB #P0772	*Elizabethkingia miricola*	
Endo F3	NEB #P0771	*Elizabethkingia miricola*	
O-Glycosidase	NEB #P0733	*Enterococcus foecalis*	
Protein Deglycosylation Mix II	NEB #P6044		A mixture of endoglycosidases to remove all *N*-linked and many common *O*-linked glycans

Deglycosylation with the Protein Deglycosylation Mix II

REACTION PROTOCOLS

The quantity of enzyme recommended is sufficient for the deglycosylation of 100 µg of a glycoprotein. Reactions may be scaled-up or down linearly to accommodate other amounts of glycoprotein or different reaction volumes. Optimal incubation times may vary for particular substrates. For most glycoproteins, deglycosylation is more extensive under denaturing conditions. Both protocols are compatible with downstream mass spectrometry analysis.

Denaturing Reaction Conditions

1. Dissolve 100 µg of glycoprotein into 40 µl H_2O.
2. Add 5 µl of Deglycosylation Mix Buffer 2.
3. Incubate at 75°C for 10 minutes, cool down.
4. Add 5 µl Protein Deglycosylation Mix II, mix gently.
5. Incubate reaction at 25°C (room temperature) for 30 minutes.
6. Transfer reaction to 37°C, incubate for 1 hour

   Note: most glycoproteins will be deglycosylated after 1 hour at 37°C. However, some complex glycoproteins may require a longer, 16 hour incubation.
7. Analyze by method of choice

Note: *The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS-PAGE gels. To prepare samples for MS, we recommend a buffer exchange by dialysis or microcentrifugation.*

Non-denaturing Reaction Conditions

When deglycosylating a native glycoprotein it is recommended that an aliquot of the glycoprotein is subjected to the denaturing protocol to provide a positive control for the fully deglycosylated protein. The non-denatured reaction can then be compared to the denatured reaction to determine the extent of reaction completion.

1. Dissolve 100 µg of glycoprotein into 40 µl H_2O.
2. To the native glycoprotein add 5 µl 10X Deglycosylation Mix Buffer 1.
3. Add 5 µl Protein Deglycosylation Mix II, mix gently.
4. Incubate reaction at 25°C (room temperature) for 30 minutes.
5. Transfer reaction to 37°C, incubate for 16 hours.
6. Analyze by method of choice.

Note: *The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS-PAGE gels.*

ADVANTAGES

- Fast reaction setup
- Enzyme mixture ensures effective deglycosylation of *N*-linked and many simple *O*-linked glycans
- Can be used under native and denaturing conditions
- Enzymatic deglycosylation leaves *N*-glycan and *O*-glycan core oligosaccharides intact and suitable for further analysis
- All reagents are mass spectrometry compatible

FREQUENTLY ASKED QUESTIONS

Q. I tried the Protein Deglycosylation Mix II on my glycoprotein and didn't see removal of the carbohydrate. What could be the problem?

A. The Protein Deglycosylation Mix II includes the enzymes necessary to remove carbohydrates attached to Asparagine residues, simple core 1 and core 3 *O*-linked carbohydrates attached to Ser/Thr, and decorated core 1 and core 3 *O*-glycans (with lactosamine extensions). There is the possibility that the carbohydrate may be resistant to PNGase F (a rare occurrence). This happens when the core *N*-Acetylglucosamine is modified by an α1-3fucose (often found in plant or insect proteins). Also, *O*-glycans extended with fucose, alpha-galactose, and other residues will be resistant to this cocktail, unless additional exoglycosidase enzymes are added.

If possible, use 10X Deglycosylation Mix Buffer 2 to reduce the protein prior to deglycosylation (this buffer is compatible with HPLC & mass spectrometry). The secondary and tertiary structure of proteins can prevent endoglycosidases from reaching their substrate, thus making reduction a crucial step in efficient cleavage. If you do not want to reduce then consider adding more enzyme and using longer incubation times.

The sample itself could cause enzyme inactivation. Avoid sample buffers containing SDS as this detergent inhibits PNGase F, *O*-Glycosidase, and β1-4 Galactosidase S. The sample could also cause a drop (or rise) in pH, particularly if large volumes are used (in those cases, it would be ideal to exchange the sample in a low molarity, neutral buffer).

Confirm that your target protein should be glycosylated. Some glycosylation predictors might annotate sites that are not occupied in nature. Also, glycosylation patterns change among different tissues, organisms, and/or growth stages. Likewise, different expression systems might result in proteins with unexpected changes in glycosylation.

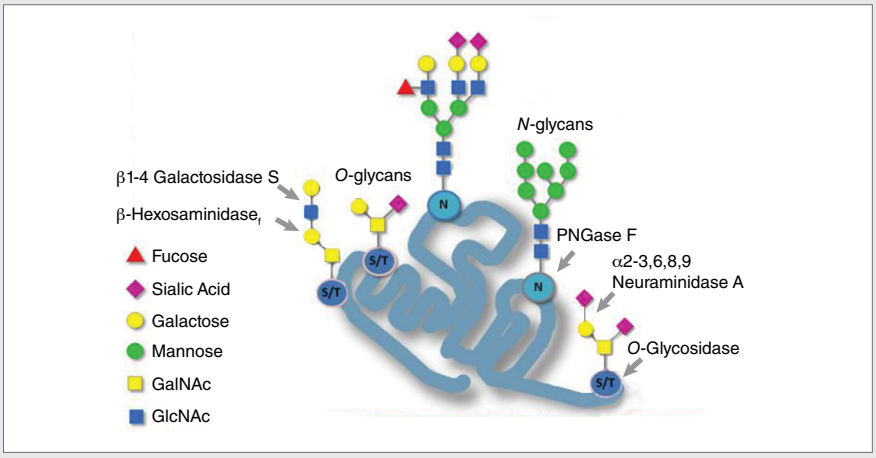
Finally, the shift in mobility of your protein (SDS-PAGE, Western blot) may be difficult to visualize (particularly when a small carbohydrate has been removed from a large protein). If possible, run side by side negative controls. Optimize loading and running conditions to facilitate the detection of subtle mass changes.

Analysis of a Fusion Protein using the Protein Deglycosylation Mix II and Mass Spectrometry

Paula Magnelli Ph.D. and Colleen McClung, New England Biolabs, Inc.

Mass spectrometry (MS) is widely used for the identification of proteins in model systems, elucidation of interactions, and characterization of protein structure, including post-translational modifications. In eukaryotic cells, a common post-translational modification is the attachment of glycans. Glycosylation determines many critical properties of proteins essential to physiological functions (e.g., immunity, endocrine regulation, development, etc). Although *N*-glycosylation sites can be predicted, *O*-glycan modifications have to be empirically demonstrated, since they cannot be accurately determined from the primary sequence. New developments in instrumentation have allowed protein characterization by MS to become commonplace. During the development and manufacturing of biotherapeutics, multi-attribute methods (MAM) by MS are required to comply with the quality-by-design (QbD) approaches recommended by many regulatory agencies (1). However powerful, characterization of glycoproteins by MS presents unique analytical challenges. Analysis is simplified when heterogeneity is reduced after deglycosylation. Enzymatic reagents that completely remove *N*-and *O*-glycans under mild, MS-compatible conditions increase coverage and confidence, and improve quantitation. This application note describes streamlined methods using the Protein Deglycosylation Mix II (NEB #P6044) which, in parallel with PNGase F (NEB #P0709), readily shows whether *N*- or *O*-glycans are present in the protein of interest. Well-characterized biotherapeutics, were used as models for validation.

FIGURE 1: ***N*- and *O*-glycan diversity**



MATERIALS

- Orencia (abatacept) from Bristol-Myers Squibb
- Enbrel (enteracept) from Amgen Inc., manufactured by Immunex Corp
- Protein Deglycosylation Mix II (NEB #P6044) provided with Deglycosylation Mix Buffer II
- PNGase F (Glycerol-free), Recombinant (NEB #P0709)
- α2-3,6,8,9 Neuraminidase A (NEB #P0722)
- Trypsin-ultra, Mass Spectrometry Grade (NEB #P8101)
- Slide-A-Lyzer mini 10K MWCO (Pierce #69570)
- Buffer: 50 mM Tris, 150 mM NaCl, pH 8.0
- PCR tube strips, screw cap microcentrifuge tubes
- DTT
- Acetone
- Formic acid (proteomics grade)
- Acetonitrile (mass spec grade)
- 1M Ammonium acetate buffer
- 200 mM Ammonium bicarbonate
- Iodoacetamide, single use (Thermo, #90034)
- C18 micro spin column (Nest Group, 5-60 µg capacity, #SEM SS18V)
- Trifluoroacetic acid (TFA) (protein sequencing grade)

GENERAL PROTOCOL 1

Deglycosylation and Intact Mass Analysis of Therapeutic Glycoproteins

Samples are treated with different combinations of glycosidases to determine the presence of N- and O-glycosylation.

A. Deglycosylation

1. Prepare four replicas of both abatacept and etanercept, labeling each vial reaction 1, 2, 3 or 4 (Note 1). Adjust each sample to 50 µg (Note 2) by addition of 18 µl MilliQ® water. Add 2 µl of Deglycosylation Mix Buffer 2 (Note 3).
2. Incubate for 5 minutes at 75°C (Note 1), cool down.
3. Add the following:

   Reaction 1: 0.5 µl PNGase F

   Reaction 2: 0.5 µl PNGase F and 0.5 µl α2-3,6,8,9 Neuraminidase A

   Reaction 3: 1 µl Protein Deglycosylation Mix II

   Reaction 4: 1 µl water (negative control)
4. Incubate for 16 hours at 37°C.

B. Buffer Exchange

1. To improve MS signal, exchange buffer by dialysis (Note 4) against 150 mM NaCl, 50 mM Tris-HCl pH 8.0 using mini-dialysis cups.
2. Reduce samples by addition of 10 µl of 200 mM DTT, incubate for 30 minutes at room temperature (25°C). Add formic acid to 0.1% v/v.

C. Liquid Chromatography/Electrospray Ionization Time-Of-Flight Mass Spectrometry (LC/ESI-TOF MS)

Samples are analyzed using a custom reverse-phase chip (Note 5) on an Agilent® 1200 series nano-LC connected directly to an Agilent 6210 series ESI-TOF MS.

1. Equilibrate the chip with 0.1% formic acid in 5% acetonitrile (ACN)
2. Inject samples (1 µl): load the chip trap column at 2 µl /min, develop the separation column at 500 nl/min with a 15 minutes linear gradient from 5% to 95% ACN, followed by 5 minutes at 95% ACN. Protein typically elutes ~10 minutes after injection.
3. Extract and deconvolute spectral data.

GENERAL PROTOCOL 2

N- and *O*-glycan Site Identification

The peptide map of the control sample is compared to the deglycosylated samples, to identify regions where N- and O-glycans are present.

A. Deglycosylation

1. Using screw cap microcentrifuge tubes, prepare two samples of abatacept for digestion with Protein Deglycosylation Mix II, as described in Section A, Steps 1-4.

B. Acetone Precipitation

1. After deglycosylation, precipitate each sample with four volumes (80 µl) of cold acetone, incubate for 30 minutes at -20°C.
2. Spin down samples for 5 minutes in microcentrifuge at maximum speed.
3. Carefully remove supernatant and air dry each pellet for 10 minutes (Note 6).
4. Dissolve each pellet with 50 µl of 50 mM NaOH; quickly neutralize with 50 mM HCl (Note 7).

C. Reduction and Alkylation

1. Reduce samples by addition of 10 µl of 200 mM DTT, incubate for 30 minutes at room temperature (25°C). Add formic acid to 0.1% v/v.
2. Prepare fresh iodoacetamide solution: add 132 µl of 200 mM ammonium bicarbonate to one vial of iodoacetamide (9.3 mg). Add 5 µl of iodoacetamide solution to each sample, incubate for 30 minutes at room temperature in the dark.

D. Trypsinization

1. Add 6 µl of 1 M ammonium acetate buffer to every sample.
2. Resuspend one vial (20 µg) of Trypsin-ultra, Mass Spectrometry Grade in 100 µl highly purified water (Note 8).
3. Add 2.5 µl Trypsin-ultra solution (0.5 µg) to each sample (substrate:Trypsin ratio 100:1).
4. Incubate for 16 hours at 37°C.

NOTES

1. Small PCR tubes incubated in a thermocycler provide good temperature control, minimizing evaporation. Alternatively, 1.5 ml micro-centrifuge tubes as well as an incubator or heat block can be used.
2. Commercial antibodies often contain stabilizers or excipients (e.g., detergents, sorbitol, glycerol). If necessary, dilute or exchange to a suitable buffer.
3. Two buffers are provided with the Protein Deglycosylation Mix II: a native buffer (Deglycosylation Mix Buffer 1) and a denaturing buffer (Deglycosylation Mix Buffer 2, used here).
4. Detailed protocols can be found at: https://www.neb.com/protocols/2014/10/28/glyco-proteomics-buffer-exchange-protocols-p0710.
5. The custom reverse phase chip consists of an integrated trapping column (40 nl), separation column and nano-ESI emitter (75 µm x 150 mm both packed with PLRP-S, 5 µm particles, 1000 A pore size). The mass spectra were acquired from 150 to 3200 m/z, one cycle/sec and 10,000 transients per scan using an ionization energy of 1800 V, fragmentor of 215 V and drying gas of 325°C at 4.0 l/min
6. Do not heat or over-dry.
7. Pellets dissolve rapidly in NaOH, vortex or pipet carefully to break down any particles.
8. Aliquot and store frozen in solution at -20°C for up to 2 weeks.

E. Peptide Cleanup

Using two C18 SPE mini-columns:

1. Condition by washing three times with 200 µl ACN, spin for 30 seconds at 1200 rpm.
2. Equilibrate by washing three times with 200 µl 0.1% TFA, spin for 1 minute at 1500 rpm.
3. Add 1.2 µl 10% TFA to each peptide sample (final concentration of TFA is 0.1%).
4. If needed, spin samples to remove insoluables. Load each sample onto a C18 mini-column, spin for 3 minutes at 1000 rpm.
5. Wash five times with 200 µl 0.1% TFA, spin for 1 minute at 1700 rpm.
6. Using a fresh microcentrifuge vial to collect liquid, elute samples with 50 µl of fresh elution solution (Note 9). Spin for 1 minute at 1500 rpm. Repeat this step once, for a total elution volume of 100 µl.
7. Add 200 µl water to each eluted sample, freeze and lyophilize.
8. Resuspend dried peptides in 100 µl water. Measure concentration (i.e., with a nanodrop spectrophotometer)

F. LC-MS

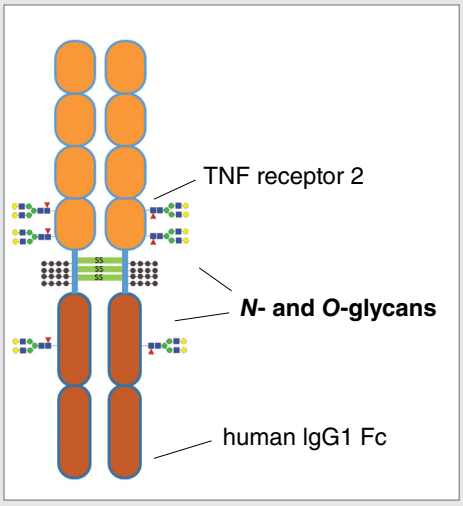
1. Dilute samples to a concentration of 1 µg/µl in 5% acetonitrile, 0.1% formic acid.
2. Inject two microliters (2 µg) of peptide onto a reverse phase analytical column (Note 10) using a Proxeon EASY n-LC 1000 (Thermo Scientific®).
3. Analyze samples using a Q Exactive mass spectrometer with a nano-electrospray ionization source (Thermo Scientific) (Note 11).
4. MS data processing: using PEAKS 7.5 (Bioinformatics Solutions) and filtered by a parent mass error tolerance of 10 ppm and a fragment mass error tolerance of 0.02 Da (Note 12).

RESULTS

Serial deglycosylation of glycoproteins

Etanercept and abatacept are examples of therapeutic fusion proteins containing multiple *N*- and *O*-glycosylation sites (Figure 2). Compared with many antibodies, where only one *N*-glycan is present on each heavy chain, the number and diversity of glycan modifications make these fusion proteins a difficult target for intact mass analysis by MS.

FIGURE 2:
Etanercept structure



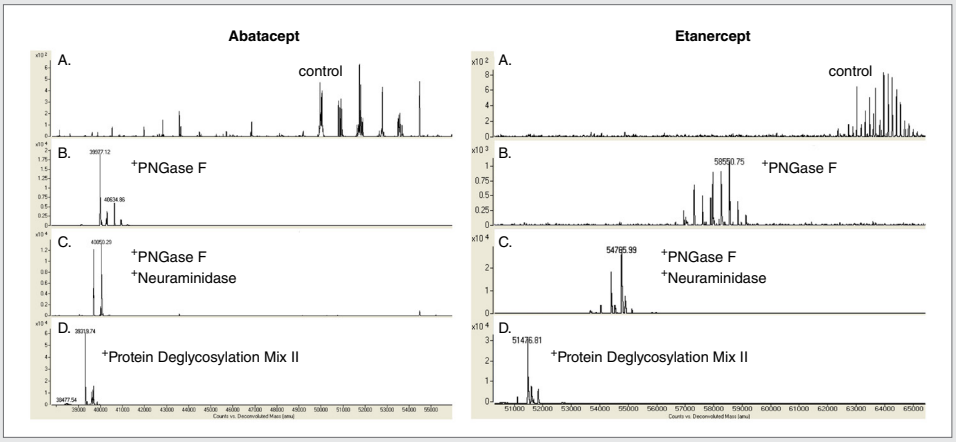
To simplify the analysis, etanercept and abatacept were digested with different enzymes: PNGase F, α2-3,6,8,9 Neuraminidase A or the Protein Deglycosylation Mix II (Figure 3). The untreated glycoproteins are large and polydisperse (panel A). Enzyme digestion resulted in dramatic changes in size. Treatment with PNGase F (panel B) and PNGase F plus α2-3,6,8,9 Neuraminidase A (panel C) renders an intermediate form where only core O-glycans remain. Treatment with the Protein Deglycosylation Mix II results in a fully deglycosylated sample (panel D). Comparing panels B and C (PNGase F and α2-3,6,8,9 Neuraminidase A) spectra with the spectra of a fully deglycosylated sample (panel D) the presence of *O*-glycan modifications can be readily demonstrated.

NOTES

9. 50% acetonitrile, 0.1% formic acid
10. Self-packed 24 cm, 100 ID, Aqua 3 µ C18 packing material.
11. Peptides elute over a 120 minutes 4-28% B gradient followed by 5 minutes 28-50% B gradient (Buffer A: 0.1% formic acid in water, Buffer B: 80% acetonitrile, 10% trifluoroethanol, 0.08% formic acid in water). Ions are chosen for HCD fragmentation during a 140,000 resolution scan with an m/z range of 300-1750. Samples are run in duplicate and the data is combined for analysis.
12. Data processing allows for two missed cleavages, nonspecific cleavage at one end of the peptide, and the following modifications: carbamidomethylation (C), oxidation (M), and deamidation (NQ). Results are filtered to a 1% false discovery rate and ≥ 1 unique peptide for each protein identified.

FIGURE 3: **Deconvoluted spectrum of the intact mass of two fusion proteins (etanercept and abatacept) after serial glycosidase digestions.**

The comparison of Panel C (*N*-glycan and terminal sialic acid removal) to Panel D (complete removal of *N*- and *O*-glycans), indicates the presence and abundance of *O*-glycan sites. Notice the moderate mass decrease for abatacept, which contains 2 *O*-glycans (2), compared to the larger mass decrease in etanercept, which contains up to 7 *O*-glycan sites (3).



Peptide Mapping, identification of regions with *N*- and *O*-glycosylation

Abatacept is a fusion of the extracellular domain of CTLA-4 with the Fc region of human IgG1 used in the treatment of rheumatoid arthritis (4). In order to eliminate native Fc cross link as well as Fc effector properties, four residues of the IgG1 hinge region were engineered (5). The introduction of three serines creates a consensus for *O*-glycosylation, which is otherwise absent in native IgG1. Abatacept also contains the two conserved *N*-glycan sites from CTLA-4 and the Fc conserved *N*-glycan site from IgG1 (2) (Figure 4).

Using this molecule as a model, regions with glycosylation were identified using the Protein Deglycosylation Mix II. As shown in Figure 5, a peptide with confirmed *O*-glycosylation (4) was absent from controls, but it was detected in treated samples. Similarly, the *N*-glycosylation sites of the CTLA-4 domain are within two peptides that were not detected in the controls, but appear in samples treated with Protein Deglycosylation Mix II.

More interestingly, these results show that the conserved *N*-glycan site from the Fc region is partially aglycosylated. The peptide EEQYNSTYR was found in the control sample with high confidence (Note 11). The fact that the abundance of this peptide was much higher in the deglycosylated sample, confirms this is a site of *N*-glycosylation (Note 13).

FIGURE 4:
Abatacept sequence

CTLA-4 sequence (blue) and IgG sequence (black). Hinge region mutation (red) introduce sites for *O*-glycosylation (highlighted in blue). *N*-glycan sites highlighted in yellow.

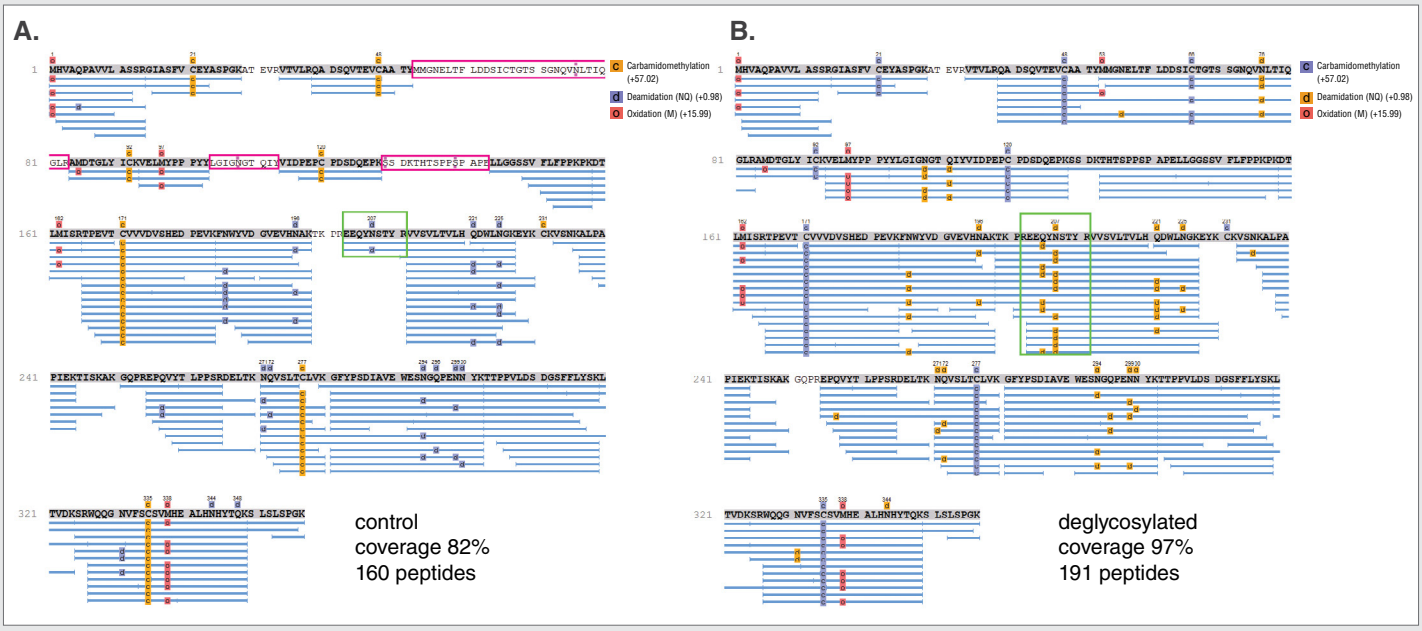
MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAAT
YMMGNELTFLDDSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPY
YLGIGNGTQIYVIDPEPCPDSDQEPKSSDKTHTSPPSPAPELLGGSSVFLF
PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

NOTES

13. There were 7 spectral counts for EEQYNSTYR in the control sample, within 2 ppm mass error. In comparison, there were 177 spectral counts for EEQYN(d)STYR in the deglycosylated sample.

FIGURE 5: **Peptide coverage map for Abatacept in control samples (A) and deglycosylated samples (B), showing percentage of coverage and number of peptides for each sample.**

The boxes in pink indicate regions which are not detectable in control samples (predicted glycosylation sites). The boxes in green indicate the conserved glycosylated Fc site. The peptide is present in low abundance in the control samples, and becomes more prominent after deglycosylation.



CONCLUSION

In this report we have combined the simplicity of enzyme digestion with more sensitive detection methods by LC-MS, to facilitate in-process therapeutic protein characterization. Comparing samples (protein and peptides) before and after complete removal of *N*-and *O*-glycans with the Protein Deglycosylation Mix II, rapidly reveals the presence, abundance, and location of glycan modifications.

Although intact glycopeptides can be identified and characterized in a peptide mapping experiment, this requires careful sample preparation, ETD fragmentation, and time-consuming data processing (to allow for enough variable modifications). This approach is unrivaled for in-depth characterization of a biotherapeutic, but it is not suited for routine screenings, clone selection, in-process control, and other time-limited analysis.

In contrast to glycopeptide characterization, a peptide mapping experiment can be performed relatively fast (typically only allowing deamidation and oxidation). As shown in this report, the comparison of control samples and deglycosylated samples reveals the type and number of glycans present. Although the precise position of the *O*-glycans is not identified by this method, the strategy is suited to uncover *O*-glycosylated regions, a first step to guide further structural characterization.

This simple approach is also useful to identify sites with partial *N*-glycosylation. It is known that therapeutic antibodies, depending on the clone and culture conditions, can have substantial amounts of aglycosylation (similar to naturally occurring antibodies) (6). Process control will minimize these undesired variants. Nevertheless, antibodies produced under optimized conditions will have minimal, yet detectable amounts of non-occupied Fc sites. In Orencia in particular, our analysis reveals that the Fc conserved site seems to have a low proportion of aglycosylated species, while the two sites in the CTLA-4 regions appear with a full occupancy rate.

References:

1. Alt, N., et al. (2016) *Biologicals,* 44(5), 291–305.
2. Bongers, J., et al. (2011) *J. Chromatogr. A.,* 1218(45), 8140–8149.
3. Houel, S., et al. (2014) *Anal. Chem.,* 86(1), 576–584.
4. Fiocco, U., et al. (2008) *Autoimmun. Rev.,* 8(1), 76–82.
5. Korhonen, R. and Moilanen, E. (2009) *KR ME Basic Elin Pharnocol Toxicol,* 104(4), 276–284.
6. Rustandi, R.R., et al. (2008) *Electrophoresis.,* 29(17), 3612–3620.

N-Linked Deglycosylation Enzymes

For structural analysis of asparagine-linked carbohydrates (*N*-linked glycans), sugars are released from the protein backbone by enzymes such as PNGase F, PNGase A, Endoglycosidase S, Endoglycosidase D, Endoglycosidase H, Endo F2 and Endo F3.

REACTION PROTOCOLS

PNGase F Denaturing Protocol

1. Combine 1-20 µg of glycoprotein, 1 µl of 10X Glycoprotein Denaturing Buffer and H_2O (if necessary) to make a 10 µl total reaction volume.
2. Denature glycoprotein by heating reaction at 100°C for 10 minutes.
3. Make a total reaction volume of 20 µl by adding 2 µl 10X GlycoBuffer 2, 2 µl 10% NP-40, H_2O and 1 µl PNGase F.

Note: *PNGase F is inhibited by SDS, therefore it is essential to have NP-40 in the reaction mixture under denaturing conditions. Failure to include NP-40 in the denaturing reaction may result in loss of activity.*

4. Incubate reaction at 37°C for 1 hour.
5. Analyze by method of choice.

Note: *The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS-PAGE gels.*

PNGase F Non-denaturing Protocol

1. Combine 1-20 µg of glycoprotein, 2 µl 10X GlycoBuffer 2, H_2O and 2-5 µl PNGase F to make a total reaction volume of 20 µl.
2. Incubate reaction at 37°C for 4 hours to overnight.
3. Analyze by method of choice.

Note: *The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS-PAGE gels. We recommend limiting PNGase F (NEB #P0704/P0708) to 1/10 (or less) of the total reaction volume to keep final glycerol concentration equal to (or less than) 5%. Reaction may be scaled-up linearly to accommodate large amounts of PNGase F and larger reaction volumes.*

Remove-iT PNGase F Protocol

1. Combine 10–20 µg of glycoprotein, 1 µl of 10X DTT and H_2O (if necessary) to make a 10 µl total reaction volume.
2. Denature glycoprotein by heating reaction at 55°C for 10 minutes.
3. Make a total reaction volume of 20 µl by adding 2 µl 10X GlycoBuffer 2, H_2O and 1–5 µl Remove-iT PNGase F.
4. Incubate reaction at 37°C for 1 hour.
5. Eliminate Remove-iT PNGase F from the reaction using Chitin Magnetic Beads (NEB #E8036) or analyze deglycosylation reaction by method of choice. See page 31 for Chitin Bead Protocol.

Note: *To deglycosylate a native glycoprotein, longer incubation time, as well as more enzyme, may be required. If using Remove-iT PNGase F under typical denaturing conditions, it is essential to have NP-40 in the reaction mixture as Remove-iT PNGase F is inhibited by SDS. The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS-PAGE gels.*

Endo F2 Reaction Protocol

1. Combine 20 µg of glycoprotein, 1 µl of GlycoBuffer 4 (10X) and H_2O (if necessary) in a total reaction volume of 10 µl.
3. Add 1 µl of Endo F2.
4. Incubate reaction at 37°C for 1 hour.

Endo F3 Reaction Protocol

1. Combine 20 µg of glycoprotein, 1 µl of GlycoBuffer 4 (10X) and H_2O (if necessary) to make a 10 µl total reaction volume.
2. Add 1 µl of Endo F3.
4. Incubate reaction at 37°C for 1 hour.

References
1. Maley, F. et al. (1989) *Anal. Biochem.,* 180, 195–204.
2. Tretter, V. et al. (1991) *Eur. J. Biochem.* 199, 647–652.

N-Linked Deglycosylation Enzymes (Cont.)

FREQUENTLY ASKED QUESTIONS

Q. Can NEB's glycosidase enzymes be used with live cells?

A. NEB's glycosidase enzymes are highly pure and can be used in conditions that are compatible with live cells. When treating live cells with glycosidases use non-denaturing reaction conditions (do not use SDS or DTT). Most enzymes tolerate moderate changes in the reaction buffer, thus the supplied reaction buffer can be replaced by a buffer of choice that provides osmotic support (maintain pH close to enzyme's optimal if possible). Some of NEB's glycosidases retain activity in culture media; however, it would have to be determined empirically if a given enzyme works in certain culture conditions.

A standard glycosidase reaction requires 1 µl of enzyme per 20-100 µl reaction with 20-200 µg of glycoprotein. However, a larger volume of enzyme may be needed when treating cells because folded proteins are difficult targets to deglycosylate. A typical incubation time is 1 hour. However, longer incubations, up to 24 hours, may be required. If extensive deglycosylation is needed, a mix of exoglycosidases or a mix of Endo H, Endo F2 and Endo F3 may be more optimal than PNGase F. This can be very effective for trimming glycans to a minimum core (which likely will not have physiological function).

The following article used an NEB exoglycosidase (neuraminidase) on live cells; it could serve as a starting point to design your experiments: "Sialylation of beta1 integrins blocks cell adhesion to galectin-3 and protects cells against galectin-3-induced apoptosis." Zhuo, Y., Chammas, R., Bellis, S.L. (2008) *J. Biol. Chem.* 283, 22177-85. This second publication uses two exoglycosidases to treat live cells: Use of novel mutant galactosyltransferase for the bioconjugation of terminal N-acetylglucosamine (GlcNAc) residues on live cell surface. Mercer N, Ramakrishnan B, Boeggeman E, Verdi L, Qasba PK. *Bioconjug Chem.* 2013 Jan 16;24(1):144-52.

NEB Neuraminidases (NEB #P0720 and #P0722) and β1,4 Galactosidase S (NEB #P0745) can effectively remove terminal sialic acid and galactose from live cells; as measured by the reduction in specific lectin binding. These are the conditions used: Jurkat cells (10^5 cells/well), 5 µl of enzyme (alone or in combination), 50 mM sodium citrate pH 6, 100 mM NaCl (for osmotic support), incubated for 1 hour at 37°C. However, another cell type (Caco) was quite resistant to this treatment, presumably because it is a highly confluent cell line. Cells had to be detached (using EDTA), to ensure enzyme access to the cell surface.

Different cell types will require protocol optimization. Bear in mind that for some samples it might not be possible to obtain maximum glycan removal with acceptable preservation of cell viability and integrity.

TECHNICAL TIPS

PNGase F

- Can be used under native or denaturing conditions
- Under native conditions more enzyme and longer incubation times may be necessary
- Activity is inhibited by SDS, under denaturing conditions it is essential to have NP-40 present in the reaction mixture in a 1:1 ratio.
- *N*-linked glycans containing core α1-3 fucose will not be cleaved (PNGase A should be used for this application)
- A positive control substrate is RNase B

Q. What is the difference between PNGase F and Remove-iT® PNGase F?

A. PNGase F and Remove-iT PNGase F are purified from the same source with identical specificity. Remove-iT PNGase F has been expressed with a chitin binding domain (CBD) tag to allow for easy removal of the enzyme from a reaction. The tag does not change or alter the specificity or activity of the enzyme. If used under denaturing conditions, PNGase F and Remove-iT PNGase F have the same unit concentration. However, Remove-iT PNGase F is sold with a modified denaturing unit (DTT only, no SDS) to make it compatible with mass spectrometry.

Q. What is the difference between Endo F2 and Endo F3?

A. Endo F2 removes *N*-linked glycans within the chitobiose core of glycoproteins containing only biantennary and (at a reduced rate) high mannose oligosaccharides. Whereas, Endo F3 has a high specificity for removing *N*-linked glycans within the chitobiose core of glycoproteins containing fucosylated-biantennary and triantennary oligosaccharides.

TECHNICAL TIPS

Endo F2/F3

- Endo F2 & Endo F3 are tagged with a chitin binding domain (CBD) for easy removal from a reaction using chitin magnetic beads (NEB #E8036)
- Endo F2 & Endo F3 should be used under non-denaturing (native) conditions, detergents, such as SDS, inhibit activity.
- The optimal pH for cleavage of biantennary & triantennary glycans is pH 4.5 (10X GlycoBuffer 4)
- Fucosylated-biantennary glycans can be cleaved at both pH 4.5 (10X GlycoBuffer 4) and pH 6.0 (10X GlycoBuffer 3).

Characterization of Glycans from Erbitux, Rituxan and Enbrel using PNGase F (Glycerol-free), Recombinant

Beth McLeod, New England Biolabs, Inc.

Glycosylation is a post-transcriptional modification that is essential for a wide range of biological processes, including cell attachment to the extracellular matrix and protein-ligand interactions in the cell. Detailed characterization of glycans on therapeutic proteins is critical, as the type and degree of glycosylation can have a profound impact on the stability, activity and effector function of the drug. The microheterogeneity of IgG glycans affects biological functions, such as complement-dependent cytotoxicity (CDC), antibody-dependent cytotoxicity (ADCC), binding to various Fc receptors, and binding to C1q protein (1). Since IgG glycans are typically highly heterogeneous and some glycoforms much less abundant than others, it is critical that the enzymatic reaction and downstream MS analysis be efficient and unbiased so that all species are represented.

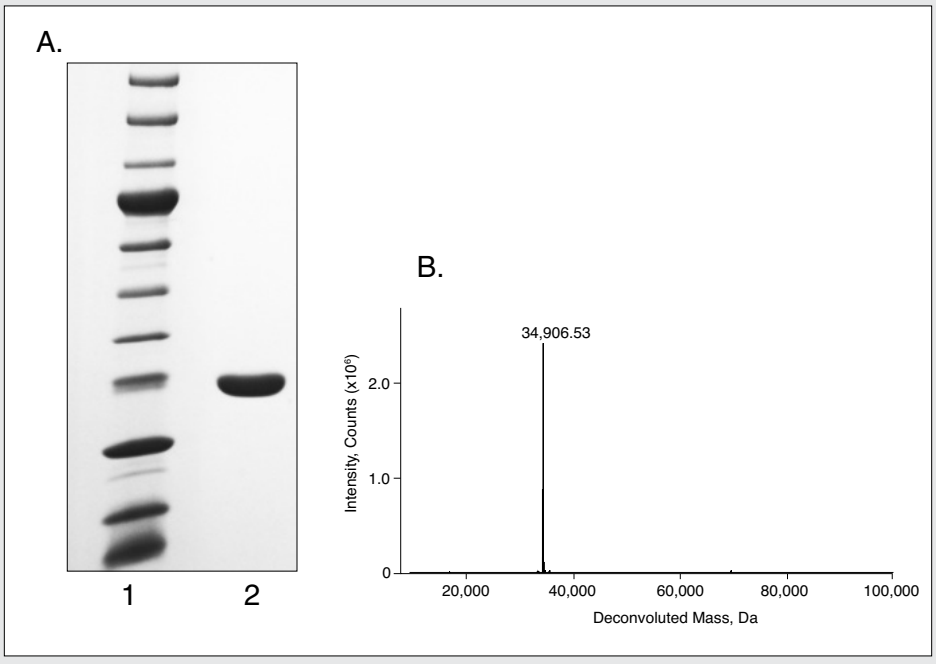
PNGase F or Peptide-N-Glycosidase F, is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins. PNGase F efficiently removes glycans from a variety of glycoproteins including IgGs, and is used extensively in conjunction with Mass Spectrometry to elucidate the structure of the intact released glycan. Recombinant PNGase F (rPNGase F) is expressed in *E. coli*, highly purified to meet stringent quality control standards, and is produced using no animal products. rPNGase F is used here, in conjunction with downstream LC/MS analysis, to characterize the glycans of three therapeutic glycoproteins: Erbitux, Rituxan and Enbrel.

MATERIALS

- Erbitux (cetuximab) from Imclone, LLC
- Rituxan (rituximab) from Genentech, a member of the Roche Group, and Biogen Idec, Inc.
- Enbrel (enteracept) from Amgen Inc., manufactured by Immunex Corp
- PNGase F (Glycerol-free), Recombinant (NEB #P0709), supplied with 10X Glycoprotein Denaturing Buffer, 10% NP-40 and 10X GlycoBuffer 2
- Supelclean™ ENVI-Carb™ SPE Tube 100 mg, 1 ml (Sigma-Aldrich, cat. #57109-U)
- Acetonitrile (ACN) HLPC/MS grade
- 50 mM NH_4 Formate buffer, pH 4.4 (See Note 1)
- 2-aminobenzamide (2AB, anthranilamide) (Sigma #A89804-5G)
- Sodium cyanoborohydride (Sigma #156159)
- Dimethyl sulfoxide (DMSO)
- Glacial acetic acid
- Spin SPE HILIC columns (Nest Group Inc., SEM-HIL)

Figure 1: **SDS-PAGE and mass spectrometry show that recombinant PNGase F is highly purified and subjected to stringent quality control assays.**

SDS-PAGE Analysis (A) ESI-TOF Analysis (B) of PNGase F (Glycerol-free), Recombinant (MW: 34,906.53 Daltons). SDS PAGE analysis; Lane 1: 15 µl Protein Ladder (NEB #P7703); Lane 2: 5 µl PNGase F (Glycerol-free), recombinant (NEB #P0709). Mass determination by an Agilent 6210 TOF LC/MS.



GENERAL PROTOCOLS

Deglycosylation

The protein sample is denatured with DTT and heat (avoiding detergents, which are not compatible with downstream MS analysis). Alternatively, samples are deglycosylated under native conditions (protein is not denatured).

1. To 100 µg of Erbitux, Rituxan or Enbrel, add DTT to a final concentration of 40 mM in a final volume of 100 µl for each sample.
2. Denature at 55°C for 10 minutes, cool on ice.
3. To all samples, add 10 µl of 10X GlycoBuffer 2 and 2 µl of Recombinant PNGase F.
4. Incubate for 24 hours at 37°C.

N-glycan Purification

5. Condition an ENVI-Carb SPE tube with 3 ml of acetonitrile (ACN), followed by 1 ml of 50% ACN (See Note 1). Equilibrate with 3 ml of water.
6. Apply deglycosylated sample from Step 4. Discard flow through, column will retain N-glycans. Wash twice with 1 ml of water. Discard flow through.
7. Elute with 300 µl of 40% ACN/60% 50 mM NH_4 Formate, pH 4.4 (See Note 2). Collect *N*-glycans in a 1.5 ml tube.
8. Lyophilize or dry in speedvac (See Note 3).

Fluorescent labeling with 2-aminobenzamide (2AB)

9. To dried sample, add 10 µl of 2AB Labeling Reagent (See Note 4) and 1 µl 50% acetic acid, and mix.
10. Transfer to 0.2 ml PCR tubes. Incubate at 65°C for 2 hours (See Note 5).

Cleanup

11. Condition a HILIC spin column with 350 µl ACN (spin at 1100 rpm for 1 minute, discard flow through), followed by 350 µl of 50 mM NH_4 Formate, pH 4.4 (spin at 3,000 rpm for 1 minute, discard flow through). Add another 50 µl of 50 mM NH_4 Formate, pH 4.4, spin at 1,000 rpm for 5 minutes, discard flow through.
12. Equilibrate the column with 350 µl of 90% ACN/50 mM NH_4 Formate, pH 4.4 (spin at 1,100 rpm for 1 minute, discard flow through). Repeat a second time.
13. Dilute sample from Step 10 to 85% ACN; add 60 µl of ACN and mix (See Note 6). Apply to prepared HILIC column, spin at 700 rpm for 3 minutes, discard flow through.
14. To remove unbound fluorescent label, wash column with 350 µl of 90% ACN/10% 50 mM NH_4 Formate, pH 4.4, spin at 1,100 rpm for 1 minute, discard flow through. Repeat five times.
15. Spin at 3,000 rpm for 2 minutes to dry the column.
16. Elute 2AB-labeled *N*-glycans with 100 µl of 50 mM NH_4 Formate, pH 4.4, spin at 3,000 rpm for 1 minute. Collect in centrifuge tube.

Liquid Chromatography/Mass Spectrometry (LC/MS)

Hydrophilic Interaction Liquid Chromatography (HILIC) in line with mass spectrometry has been successfully used to separate and identify glycoconjugates (2). The fluorescent label at the glycan reducing end (1:1 molar ratio) is crucial for precise quantitation, while it also facilitates electrospray ionization (ESI) for MS. Data is interpreted based on known *N*-glycan biosynthetic pathways, allowing for the identification of individual glycan species (See Note 7).

17. A sample of labeled *N*-glycans (40 µl) was diluted with 160 µl of ACN in an autosampler vial. The 2AB-labeled *N*-glycans were separated using a XBridge™ BEH Amide column (Waters) on a Dionex UltiMate® LC equipped with fluorescent detection (See Note 8), in line with a LTQ™ Orbitrap Velos™ Spectrometer equipped with a heated electrospray standard source (HESI-II probe) (See Note 9).

NOTES

1. Apply gentle positive pressure, or use a vacuum manifold.
2. Low pH is needed to elute sialylated glycans.
3. To prevent sialic acid loss, do not overheat.
4. Dissolve 5 mg of 2 AB in 20 µl of DMSO, mix. Add 80 µl of water. Add this solution to 6 mg of $NaCNBH_4$. Use immediately. Discard unused solution following safety regulations.
5. A thermocycler provides excellent temperature control, minimizing evaporation. However, any other suitable incubator can be used for this step.
6. It is critical to maintain a dilution in 85% acetonitrile; higher organic content might cause some glycans to precipitate out of solution, and lower organic content will prevent glycans from binding to the HILIC column.
7. Since various isomers can be present, unequivocal assignment of structure is only possible following extensive analysis, such as MS/MS, which is not described here.
8. The glycans were separated using a gradient of 70%:30% to 62%:38% ACN:50 mM NH_4 Formate, pH 4.4, for 48 minutes at 350 µl/min, 2 AB fluorescence was measured at 350 (ex)/420 nm (em). Injection vol:100 µl
9. Optimized settings for positive mode detection of 2 AB-labeled N-glycans: A) ESI: spray voltage, 3.5 kV; capillary temperature, 250°C; sheath gas, 11 psi; Aux gas and sweep gas flow rates, 0; S-lens RF level %, 66. B) Ion Optics settings: Multiple 00 offset, 2.5 V; Lens 0 voltage, 6.5 V; Multiple 0 offset, 7.0 V; Lens 1 voltage, 16 V; Multiple 1 offset, 6.5 V; Multiple RF Amplitude, 600; Front lens, 7.75 V.

RESULTS:

Figure 3: **LC/MS Analysis of Erbitux (cetuximab) Glycans released by PNGase F, Recombinant**

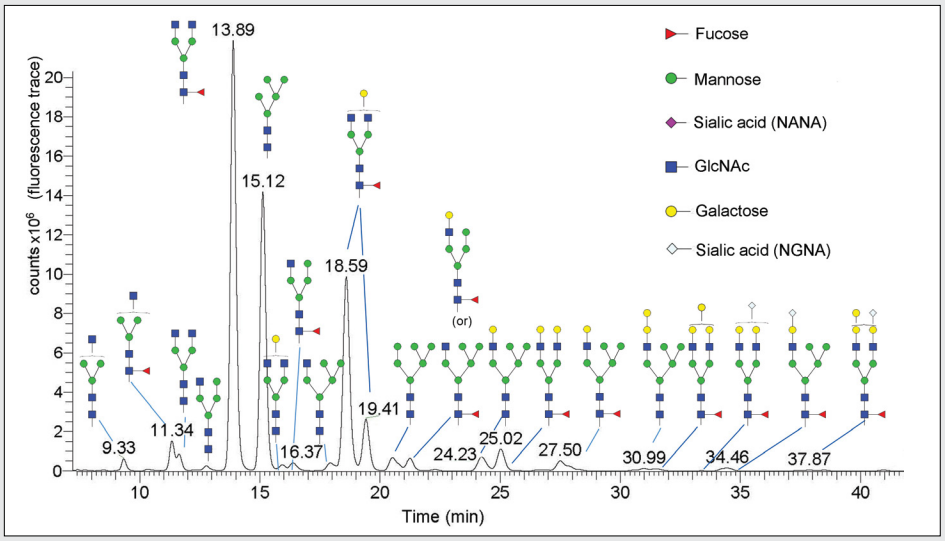
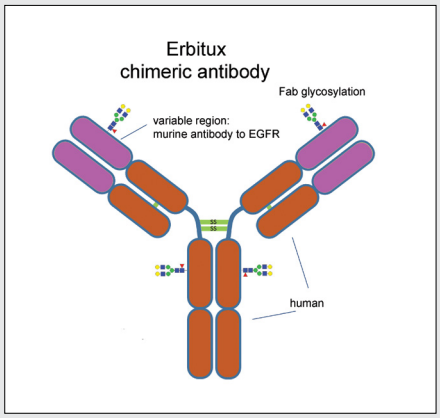


Figure 4: **Erbitux functional domains**



Erbitux, is a recombinant human-mouse chimeric monoclonal antibody that binds specifically to the extracellular domain of the human epidermal growth factor receptor (EGFR) and is produced in mammalian cell (murine myeloma) culture. Erbitux has two glycosylation sites on each heavy chain, one at Asn 299 in the conserved Fc portion and the other present at Asn 88 in the Fd domain. Masses corresponding to G0F, G1F and G2F glycans are detected as well as high mannose and hybrid structures. Low levels of G2FGal1 are found as well as three glycan species with NGNA sialic acids. The NGNA sialic acids oligosaccharides are likely to have been released from the Fd domain of the antibody, although the majority of the glycoforms observed here are species associated with the conserved glycosylation site in the Fc domain (3).

Figure 5: **LC/MS Analysis of Rituxan Glycans released by PNGase F, Recombinant**

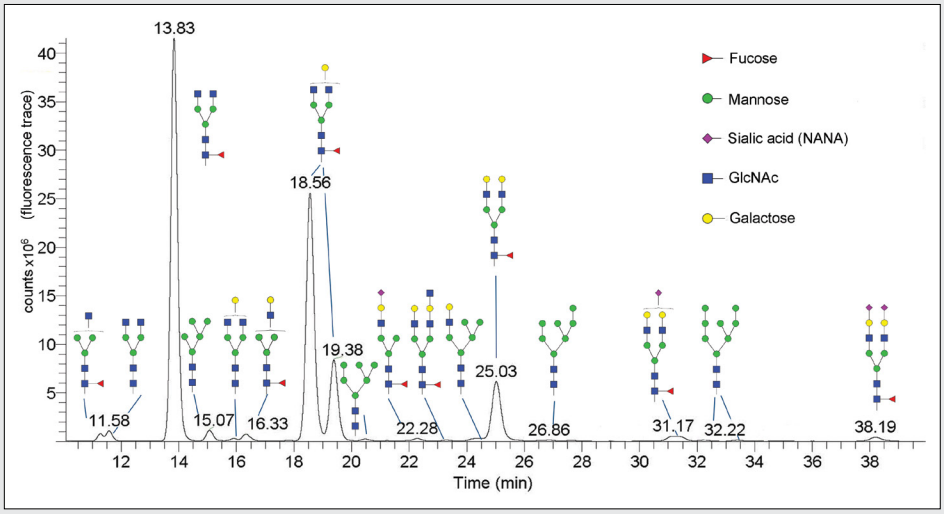
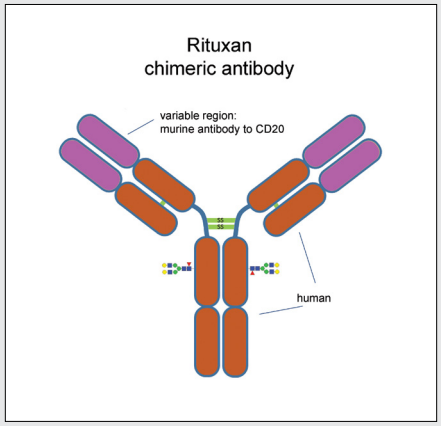


Figure 6: **Rituxan functional domains**



Rituxan is a genetically engineered chimeric human/mouse monoclonal IgG1 kappa antibody directed against the CD20 antigen which is primarily found on the surface of immune system B cells. Rituxan is produced by mammalian cell (Chinese Hamster Ovary) culture. The most abundant for Rituxan species are G0F, G1F, and G2F. Low levels of high mannose and NANA sialic acid species were detected as well.

RESULTS (continued)

Figure 7: **LC/MS Analysis of Enbrel Glycans released by PNGase F, Recombinant**

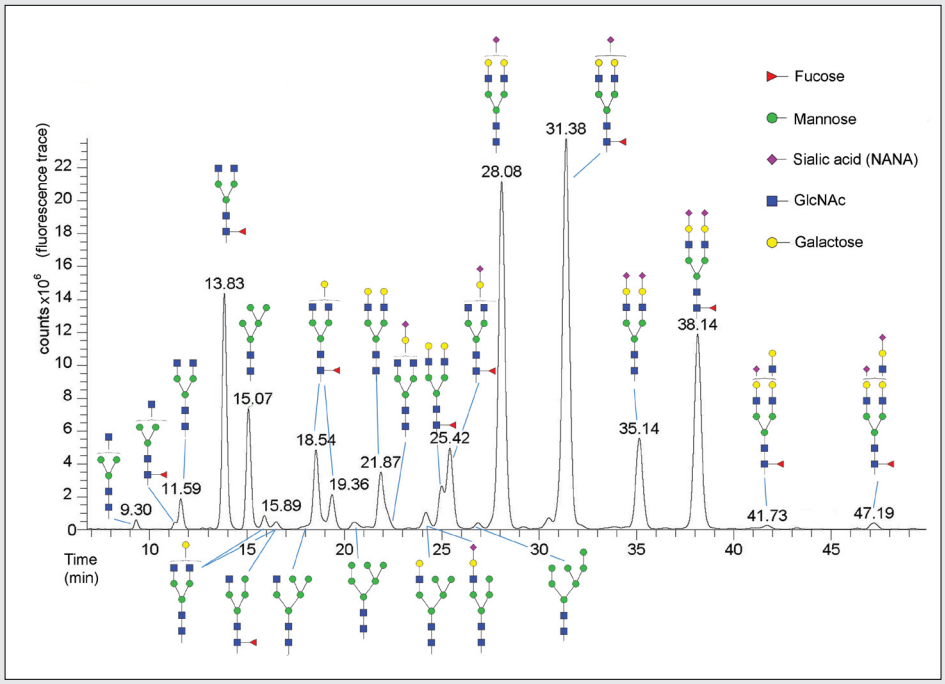
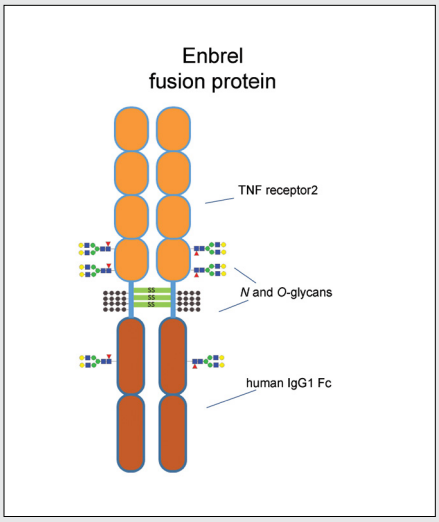


Figure 8: **Enbrel functional domains**



Enbrel is a dimeric fusion protein consisting of the extracellular ligand binding portion of the human tumor necrosis factor receptor (TNFR) linked to the Fc portion of human IgG1. The Fc component of Enbrel contains the CH2 domain, the CH3 domain and hinge region, but not the CH1 domain of IgG1. Enbrel is produced by recombinant DNA technology in a Chinese hamster ovary (CHO) mammalian cell expression system.

Glycoforms identified by LC/MS analysis of intact Enbrel (etanercept). Enbrel contains many NANA-sialylated species, the most abundant being G2 and G2F monosialylated. Low levels of high mannose and hybrid species are detected as well.

In each chromatogram the identity of each *N*-glycan peak was annotated manually according to the peak retention time in comparison to known standards, and the corresponding m/z value as determined by ESI-MS, in agreement with the known metabolic pathways of antibody producing expression systems. The elucidated *N*-glycan structures are shown above each peak using the conventional glycan nomenclature of the Consortium for Functional Glycomics.

CONCLUSION

Endoglycosidases are critical tools to investigate the nature of protein glycosylation. PNGase F, Recombinant is used here with LC/MS analysis to assign glycans released from three well-characterized therapeutic glycoproteins, Erbitux, Rituxan and Enbrel. A wide range of glycan structures is easily detected using this enzyme including high mannose and sialylated species. PNGase F, Recombinant efficiently deglycosylates these biotherapeutic proteins with results that are reproducible and unbiased with regard to glycan species released. These glycosylation profiles are in agreement with published glycan studies of Erbitux, Rituxan and Enbrel. Finally, it should be noted that protein samples treated with enzymes remain intact, and therefore are ready for downstream proteomic analysis.

References:

1. Wright, A. and Morrison, S. L. (1997) *Trends Biotechnol.* 15(1): 26–32.
2. Wuhrer, M., de Boer, A. R. and Deelder, A. M. (2009) *Mass Spectrom Rev.* 28, 192–206.
3. Ayoub, et al. (2013). Correct primary structure assessment and extensive glyco-profiling of cetuximabby a combination of intact, middle-up, middle down and bottom-up ESI and MALDI mass spectrometry techniques. mAbs 5:5, 699–710.

Remove-iT PNGase F: Effective Release and Recovery of Neutral and Sialylated *N*-glycans

Alicia Bielik and Paula Magnelli, New England Biolabs, Inc.

Protein glycosylation, the covalent attachment of sugar residues to a polypeptide, is the post-translational modification that generates the greatest functional and structural variation from a single polypeptide (1). Most glycoproteins are typically produced by cells as a collection of glycoforms that differ in mass, charge, conformation, ligand affinity, *in vivo* half life, etc. Therefore, *in vitro* manipulations are necessary to obtain a homogeneously glycosylated (or deglycosylated) protein for functional studies. Because most applications require an active protein, glycosidases are ideal tools for glycoprotein remodeling (2,3,4,5).

Peptide-*N*-Glycosidase F (PNGase F) is able to remove high mannose, hybrid, and complex *N*-linked glycans. Although it is more effective on denatured proteins, this enzyme can be used under native conditions, preserving the integrity of the protein of interest.

Remove-iT PNGase F, a new version of this enzyme, has a chitin binding domain (CBD) tag for easy removal of the enzyme after deglycosylation. The procedure leaves the target protein and the released *N*-glycans ready for analysis (i.e., mass spectrometry, cell based assays, crystallography, etc). Enzyme removal is also an attractive alternative to inactivation by heat or acid, which can have a negative affect on protein stability.

In the following application note we show how Remove-iT PNGase F effectively cleaves *N*-glycans from Bovine Fetuin. Specifically, we demonstrate that the enzyme removal step using chitin magnetic beads does not interfere with the recovery of either neutral or sialylated glycans.

We demonstrate that Remove-iT PNGase F has identical activity to PNGase F, while presenting the additional advantage of being readily eliminated from the protein sample.

GENERAL PROTOCOLS

Deglycosylation

The protein sample is denatured with DTT and heat (avoiding detergents which are not compatible with downstream MS analysis). Alternatively, samples are deglycosylated under native conditions (protein is not denatured).

1. For each sample, mix 10 µl of Fetuin (100 µg), 10 µl 10X DTT and 70 µl of H_2O in a microcentrifuge tube.
2. Denature at 55°C for 10 minutes and cool on ice.
3. For deglycosylation under native conditions, mix 10 µl of Fetuin and 80 µl of H_2O (do not heat).
4. To all samples, add 10 µl of 10X GlycoBuffer 2 and 2 µl of Remove-iT PNGase F or PNGase F (Glycerol-free).
5. Incubate for 1 hour at 37°C. (Longer incubation times may be required for complete deglycosylation of other proteins under native conditions).

PNGase F Removal using Chitin Magnetic Beads:

In this step, Remove-iT PNGase F is eliminated from the reaction. After the removal of the enzyme, the corresponding samples contain only deglycosylated protein and free glycans. Control samples are processed without performing this step, to determine whether chitin beads affect N-glycan recovery.

6. Using a magnetic separation rack, rinse (twice) a 50 µl aliquot of Chitin Magnetic Beads with 500 µl with 50 mM NH_4 Formate pH 4.4 (See Note 1).
7. Add 100 µl of the deglycosylation reaction containing Remove-iT PNGase F and rock for 10 minutes at 4°C. Back on the magnetic separation rack, allow the chitin beads to separate. Remove and save the supernatant transferring it to a fresh tube.
8. Wash the magnetic chitin beads three times with 100 µl of 50 mM NH_4 Formate pH 4.4 (or buffer of choice), removing and saving the supernatant after each wash.
9. Combine all supernatants (See Note 2).

MATERIALS

- Fetuin (NEB #P6042)
- Remove-iT PNGase F (NEB #P0706), supplied with 10X DTT and 10X GlycoBuffer 2
- PNGase F (Glycerol-free) (NEB #P0705), supplied with 10X Glycoprotein Denaturing Buffer, 10% NP-40 and 10X GlycoBuffer 2
- Chitin Magnetic Beads (NEB #E8036)
- 2-Tube Magnetic Separation Rack (NEB #S1510)
- Supelclean ENVI-Carb SPE Tube 100 mg, 1 ml (Sigma, 57109-U)
- Acetonitrile (ACN) HLPC/MS grade
- 50 mM NH_4 Formate buffer pH 4.4 (See Note 1)
- 2-aminobenzamide (2AB, anthranilamide) (Sigma, A89804-5G)
- Sodium cyanoborohydride (Sigma, 156159)
- Dimethyl sulfoxide (DMSO)
- Glacial acetic acid
- Spin SPE HILIC columns (Nest Group Inc., SEM-HIL)

GENERAL PROTOCOLS

Glycan Preparation:

Salts and proteins are removed, purified glycans are labeled with a fluorescent tag.

N-glycan Purification:

10. Condition an ENVI-Carb SPE Tube with 3 ml of acetonitrile (ACN) following by 1 ml of 50% ACN (See Note 3). Equilibrate with 3 ml water.
11. Apply deglycosylated sample from Step 9. Discard flow through, graphite will retain *N*-glycans. Wash twice with 1 ml water. Discard flow through.
12. Elute with 300 µl of 40% ACN/60% 50 mM NH_4 Formate pH 4.4 (See Note 4). Collect N-glycans in a 1.5 ml tube.
13. Lyophilize or dry in speedvac (See Note 5).

Fluorescent labeling with 2-aminobenzamide (2AB):

14. To dried sample, add 10 µl of 2AB Labeling Reagent (See Note 6) and 1 µl 50% acetic acid, mix.
15. Transfer to 0.2 ml PCR tubes. Incubate at 65°C for 2 hours (See Note 7).

Cleanup:

16. Condition a HILIC spin column with 350 µl ACN (spin at 1,100 rpm for 1 minute, discard flow through), followed by 350 µl of 50 mM NH_4 Formate pH 4.4 (spin at 3,000 rpm 1 minute, discard flow through). Add another 50 µl of 50 mM NH_4 Formate pH 4.4, spin at 1,100 rpm 5minutes, discard flow through.
17. Equilibrate with 350 µl of 90% ACN/50 mM NH_4 Formate pH 4.4 (spin at 1,100 rpm for 1 minute, discard flow through). Repeat a second time.
18. Dilute sample to 85% ACN: add 60 µl of ACN, mix (See Note 8). Apply to HILIC column, spin at 700 rpm for 3 minutes, discard flow through.
19. To remove unbound fluorescent label, wash column with 350 µl 90% ACN/10% 50 mM NH_4 Formate pH 4.4, spin at 1,100 rpm for 1 minute, discard flow through. Repeat five times.
20. Spin at 3,000 rpm for 2 minutes to dry the column.
21. Elute 2AB-labeled *N*-glycans with 100 µl 50 mM NH_4 Formate pH 4.4., spin at 3,000 rpm for 1 minute. Collect in centrifuge tube.

Liquid Chromatography/Mass Spectrometry (LC/MS)

Hydrophilic Interaction Liquid Chromatography (HILIC) in line with mass spectrometry has been successfully used to separate and identify glycoconjugates (6). The fluorescent label at the glycan reducing end (1:1 molar ratio) is crucial for precise quantitation, while it also facilitates electrospray ionization (ESI) for MS. Data is interpreted based on known N-glycan biosynthetic pathways, allowing the identification of individual glycan species (See Note 9).

22. A sample of labeled *N*-glycans (40 µl) was diluted with 160 µl of ACN, in an autosampler vial. The 2AB-labeled *N*-glycans were separated using a TSK gel Amide-80 column (Tosoh Bioscience LLC) on a Dionex Ultimate LC equipped with fluorescent detection (See Note 10), in line with a Velos LTQ Pro Mass Spectrometer equipped with a heated, electrospray-standard source (HESI-II probe) (See Note 11).

NOTES

1. To prepare 50 mM Ammonium Formate Buffer add 1.91 ml formic acid to 1000 ml H_2O, adjust pH to 4.4 dropwise with ammonium hydroxide. Filter using a 0.2 micron filter.
2. A detailed protocol can be found at https://www.neb.com/protocols/2013/01/23/remove-it-pngase-f-magnetic-chitin-bead-protocol-p0706.
3. If flow is too slow, apply gentle positive pressure.
4. Low pH is needed to elute sialylated glycans.
5. To prevent sialic acid loss, do not overheat.
6. Dissolve 5 mg 2 AB in 20 µl of DMSO, mix. Add 80 µl water. Add this solution (100 µl) to 6 mg $NaCNBH_4$. Use immediately. Discard unused solution following safety regulations.
7. A thermocycler provides excellent temperature control and minimizes evaporation. However, any other suitable incubator can be used for this step.
8. It is critical to maintain a dilution in 85% acetonitrile: higher organic content might cause some glycans to precipitate out of solution, lower organic content will prevent glycans from binding to the HILIC column.
9. Since various isomers can be present, unequivocal assignment of structure is only possible following extensive analysis, such as MS/MS, which is not described here.
10. The glycans were separated using a gradient of 80%:20% to 40:60% ACN:50 mM NH_4 formate pH 4.4 for 34 min at 350 µl/min, 2 AB fluorescence was measured at 350 (ex)/420 nm (em). Injection vol: 100 µl
11. Optimized settings for positive mode detection of 2 AB-labeled *N*-glycans.

    A) ESI: spray voltage 3.5 kV; capillary temperature 250°C; sheath gas 11 psi, Aux gas and Sweep gas Flow rates 0, S-lens RF level % 66.

    B) Ion Optics settings: Multiple 00 offset 2.5 V, Lens 0 voltage 6.5 V, Multiple 0 offset 7.0 V, Lens 1 voltage 16 V, Multiple 1 offset 6.5 V, Multiple RF Amplitude 600, Front lens 7.75 V.

RESULTS:

Remove-iT PNGase F: Chitin Beads and *N*-glycan Recovery

It was previously determined that the PNGase F removal step is extremely efficient. Under the conditions described, there is no residual Remove-iT PNGase F in the supernatant (detectable by activity assay or mass spectrometry). Additionally, we observed complete recovery of the target protein (data not shown). In order to demonstrate that no *N*-glycan species, neutral or sialylated, are lost to nonspecific binding or entrapment to the chitin magnetic beads, the *N*-glycan-containing supernatant (after Remove-iT PNGase F removal) was compared with a control where chitin beads were not used.

Figure 1 shows the fluorescent trace of these samples, which have identical profiles. These experiments were run in triplicate, to calculate the average abundance of each glycoform. As shown in the lower profile, the overall recovery was around 85%, showing that very little material is lost during the handling of the chitin beads. In summary, there is neither bias or significant loss of *N*-glycan forms after the sample reaction is incubated in the presence of chitin.

Each glycoform species was identified according to its m/z: all the known fetuin *N*-glycans were observed (7).

Detailed Analysis of *N*-glycan Profiles from Remove-iT PNGase F and Standard PNGase F: Glycan Release Under Denaturing and Native Conditions

As shown in Figure 1, Remove-iT PNGase F removes all expected *N*-glycans from Bovine Fetuin. However, the presence of the CBD tag could result in subtle activity changes. These would become apparent as a bias in the relative abundance of the *N*-glycans released from a given sample. To investigate whether the CBD tag itself imposes constrains in the activity of the enzyme, a series of side-by-side comparisons of Remove-iT PNGase F (used without the removal step) versus PNGase F were performed. Experimental triplicates were used in order to detect minor changes in the relative abundance of the glycans released.

Figure 2 and Figure 3 show the *N*-glycans released under native and denaturing conditions, respectively. In both cases, the performance of Remove-iT PNGase F is identical to the standard enzyme, quantitatively and qualitatively.

Figure 1: **Glycan profile is identical between samples with and without enzyme removal.**

N-glycan was incubated with (A) no chitin beads, and (B) with Remove-iT PNGase F according to recommended conditions. Results indicate 85% recovery after chitin removal, with no loss of sample due to non-specific binding.

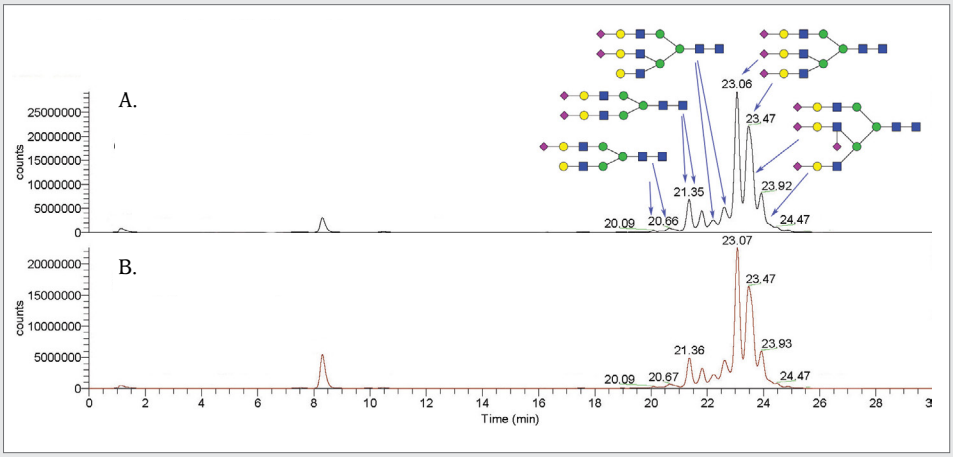


Figure 2: **Remove-iT PNGase F has Identical Activity as the Native Enzyme on Native Proteins.**

Deglycosylation of native Fetuin was performed using (A) Remove-iT PNGase F and (B) PNGase F, (Glycerol-free). Similar elution patterns were seen in both cases, demonstrating that Remove-iT PNGase F displays similar activity as the native enzyme.

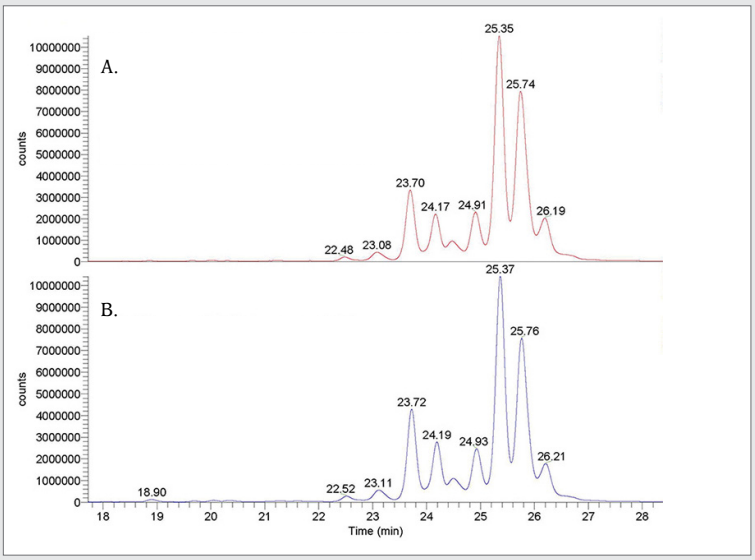
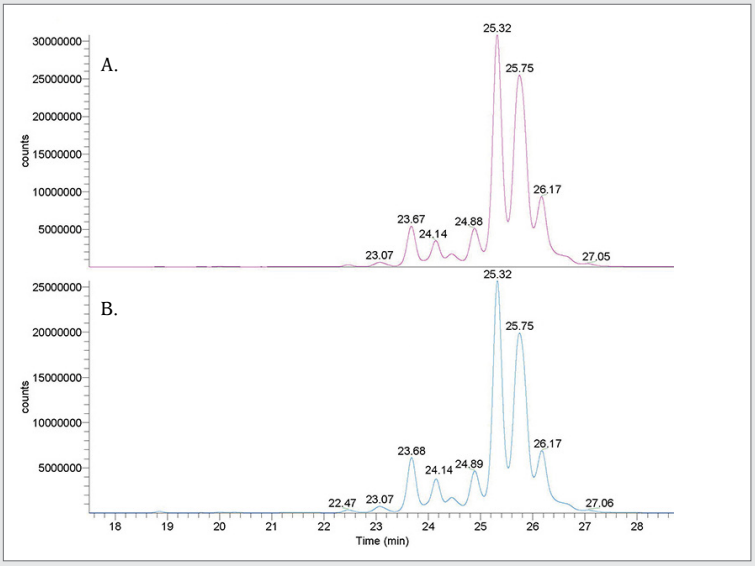


Figure 3: **Remove-iT PNGase F has Identical Activity as the Native Enzyme on Denatured Proteins.**

Deglycosylation of denatured Fetuin was performed using (A) Remove-iT PNGase F and (B) PNGase F, (Glycerol-free). Similar elution patterns were seen in both cases, demonstrating that Remove-iT PNGase F displays similar activity as the native enzyme.



CONCLUSION:

We investigated in detail the properties of Remove-iT PNGase F in comparison to the standard reagent PNGase F; the presence of a CBD tag does not interfere with *N*-glycan release. Additionally, we demonstrated that the convenient enzyme removal step has no effect on the recovery of the target protein, nor does it alter the composition or yield of the *N*-glycan products.

References:

1. Varki, A. and Sharon, N. (2009). Historical Background and Overview. In A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart and M.E. Etzler (eds.), *Essentials of Glycobiology*, (2nd ed.), (Chapter 1). New York: Cold Spring Harbor Laboratory Press. PMID: 20301255.
2. Sackstein, R. et al. (2008) *Nat. Med.* 14, 181–187. PMID: 18193058
3. Rich, J.R. and Withers, S.G. (2009) *Nat. Chem. Biol.* 5, 206–215. PMID: 19295526.
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Rapid PNGase F

A growing number of antibodies and antibody fusions are currently used as therapeutic agents. A conserved *N*-glycan at Asn297 of the Fc region of IgG is critical for functional activity. Moreover, some antibodies have additional *N*-glycans that, together with the conserved site, affect recognition, half-life, and immune reactions. Antibody glycosylation is heterogeneous, and variables in cell culture can increase glycan diversity. Monitoring glycosylation during production is essential to obtain the correct glycoprotein forms.

PNGase F is the most effective enzymatic method for removing almost all *N*-linked oligosaccharides from glycoproteins. PNGase F digestion deaminates the asparagine residue to aspartic acid, and leaves the oligosaccharide intact for further analysis.

The growing importance of protein glycosylation in both pharmaceutical and clinical science, as well as basic research, is placing new demands on the quality of reagents for glycan analysis. NEB offers a selection of PNGase F reagents, all highly pure, to support a variety of applications.

Rapid PNGase F is an improved reagent that allows the complete and rapid deglycosylation of antibodies and immunoglobulin-fusion proteins in minutes. All *N*-glycans are released rapidly and without bias, and are ready to be prepared for downstream chromatography or mass spectrometry analysis. Rapid PNGase F creates an optimized workflow which reduces processing time without compromising sensitivity or reproducibility.

TECHNICAL TIPS

- Before incubation, make sure the Rapid Buffer, IgG, and Rapid PNGase F are properly mixed
- For high throughput applications reactions can be prepared at room temperature. The deglycosylation reaction will begin once the temperature is raised to 50ºC.
- Typically, the reaction is completed after 10 minutes at 50ºC. Incubation longer than 15 minutes will not result in further deglycosylation. If the reaction is still incomplete, try the 2 step protocol.
- Avoid buffers containing SDS, as it inhibits Rapid PNGase F. Common stabilizing reagents such as Tween, Triton X-100, NP-40, octyl glucoside and non-detergent sulfobetaine, as well as traces of organic solvents, can prevent optimal rapid deglycosylation

REACTION PROTOCOLS

The optimal amount of starting material will be determined by the nature of a sample (glycan diversity) and the particular downstream analysis that will be performed. Reactions may be scaled-up linearly to accommodate larger amounts of antibody or glycoprotein and/or larger reaction volumes.

Rapid PNGase F 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.
5. Prepare *N*-glycans for derivatization (i.e., reductive amination) for downstream analysis. To prepare a deglycosylated protein for mass spectrometry analysis, exchange the buffer by micro dialysis or micro filtration. Refer to **www.neb.com** for more detail.

Rapid PNGase F Two-step Protocol

Some antibodies (i.e. Fab N-glycans) require a pre-heating 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.
6. Prepare *N*-glycans for derivatization (i.e., reductive amination) for downstream analysis. To prepare a deglycosylated protein for mass spectrometry analysis, exchange the buffer by micro dialysis or micro filtration. Refer to **www.neb.com** for more detail.

Rapid PNGase F (Non-reducing Format) Protocol

1. Combine up to 10 µg of antibody and H_2O to a volume of 8 µl.
2. Add 2 µl of Rapid PNGase F (non-reducing format) Buffer (5X) to make a 10 µl total reaction volume.
3. Incubate at 75°C for 5 minutes, cool down.
4. Add 1 µl of Rapid PNGase F (non-reducing format).
5. Incubate 10 minutes at 50°C.
6. Prepare antibody sample for SDS-PAGE or mass spectrometry analysis.

Note: *The amount of Rapid PNGase F (non-reducing format) Buffer can be increased up to 4 µl to facilitate rapid deglycosylation of complex substrates*

FREQUENTLY ASKED QUESTIONS

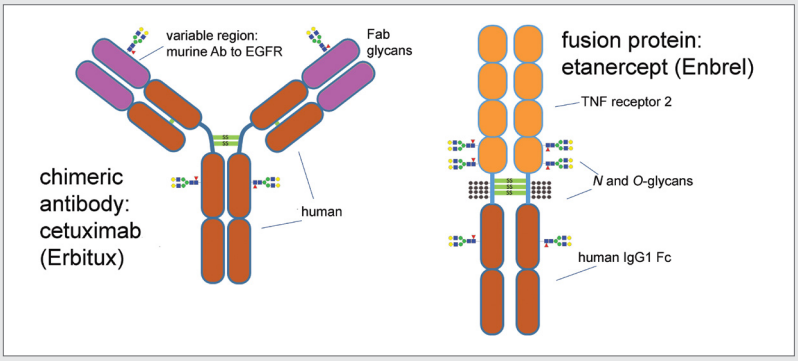
Q. How do I know whether to follow the "One-step" or the "Two-step" protocol?

A. 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.

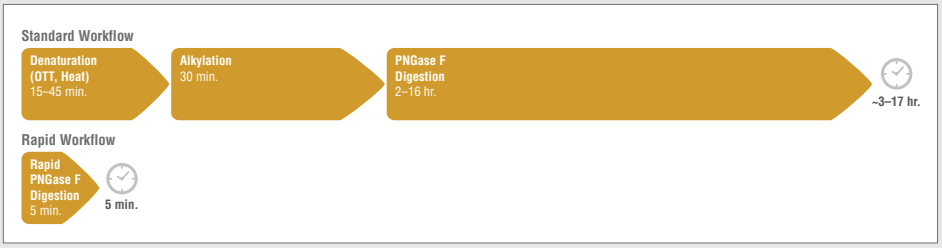
Unbiased and Fast IgG Deglycosylation for Accurate *N*-glycan Analysis using Rapid PNGase F

Paula Magnelli, New England Biolabs, Inc.

A growing number of monoclonal antibodies and antibody chimeras are in development as therapeutic agents. The Fc region of IgG carries a conserved *N*-glycan, which is critical for biological activity. Also, some IgGs and IgG fusions have additional *N*-glycans that, together with the conserved Asn297 *N*-glycan, affect recognition, half life and inflammatory reactions.



It has become increasingly important to monitor antibody glycosylation during development and production to obtain the right glycoforms, while keeping undesired glycans (e.g., Galα1-3Gal epitope) at trace levels. Effective monitoring requires that a complete and accurate *N*-glycan profile be obtained in the shortest time possible. Enzymatic release of *N*-glycans with PNGase F typically takes at least a few hours, which is only the first step in a process involving glycan derivatization and analysis by liquid chromatography (LC) and/or mass spectrometry (MS).



Rapid PNGase F allows complete deglycosylation of therapeutic monoclonal antibodies in minutes, and is compatible with LC-MS applications. Results obtained using this enzyme were in accordance with published data, demonstrating that sensitivity and accuracy are not compromised by a faster and more convenient glycoprotein characterization workflow.

MATERIALS

- Erbitux (cetuximab) from Imclone, LLC.
- Rituxan (rituximab) from Genentech, a member of the Roche Group, and Biogen Idec, Inc.
- Enbrel (enteracept) from Amgen, Inc., manufactured by Immunex Corp
- Rapid PNGase F supplied with 5X Rapid PNGase F Buffer (NEB #P0710)
- PCR tube strips or centrifuge tubes
- Acetonitrile (ACN) HLPC/MS grade
- 50 mM NH_4 Formate buffer, pH 4.4
- 2-aminobenzamide (2AB, anthranilamide) Sigma cat. #A89804-5G)
- Sodium cyanoborohydride (Sigma, cat. #156159)
- Dimethyl sulfoxide (DMSO)
- Glacial acetic acid
- Spin SPE HILIC columns (Nest Group, Inc., cat #SEM-HIL)

GENERAL PROTOCOLS:

Rapid deglycosylation

The antibody sample is treated with Rapid PNGase F at its optimal temperature of 50°C.

1. Using PCR tubes (200 µl), adjust each sample of monoclonal antibody (Erbitux 32 µg, Rituxan 60 µg, or Enbrel 50 µg) to a final volume of 16 µl with Milli-Q water.
2. Add 4 µl Rapid Buffer and mix.
3. Add 1 µl of Rapid PNGase F (see Note 1).
4. Incubate for 5 minutes at 50°C in a thermocycler or heat block (see Note 2).

Fluorescent labeling with 2-aminobenzamide (2AB)

5. To Rapid PNGase F reaction, add 20 µl of 2AB Labeling Reagent (see Note 3) and 1 µl glocial acetic acid, and mix.
6. Incubate at 65°C for 1 hour (see Note 4).

Cleanup

7. Condition a HILIC spin column with 350 µl ACN (spin at 1,100 rpm for 1 minute, discard flow through). Then add 350 µl of 50 mM NH_4 Formate, pH 4.4 and spin at 3,000 rpm for 1 minute, discarding flow through. Add another 50 µl of 50 mM NH_4 Formate, pH 4.4, and spin at 1,000 rpm for 5 minutes, discarding flow through.
8. Equilibrate the column with 350 µl of 90% ACN/50 mM NH4 Formate, pH 4.4. Spin at 1,100 rpm for 1 minute, and discard flow through. Repeat a second time.
9. Dilute sample from Step 12 to 85% ACN by adding 60 µl of ACN and mix (see Note 5). Apply to conditioned HILIC column, spin at 700 rpm for 3 minutes, and discard flow through.
10. To remove unbound fluorescent label, wash column with 350 µl of 90% ACN/10% 50 mM NH_4 Formate, pH 4.4, spin at 1,100 rpm for 1 minute, and discard flow through. Repeat five times.
11. Spin at 3,000 rpm for 2 minutes to dry the column.
12. Elute 2AB-labeled *N*-glycans with 100 µl of 50 mM NH_4 Formate, pH 4.4, spin at 3,000 rpm for 1 minute. Collect in centrifuge tube.

Liquid Chromatography/Mass Spectrometry (LC/MS)

Hydrophilic Interaction Liquid Chromatography (HILIC), in line with mass spectrometry, has been successfully used to separate and identify glycoconjugates (2). The fluorescent label at the glycan-reducing end (1:1 molar ratio) is crucial for precise quantitation, while it also facilitates electrospray ionization (ESI) for MS. Data is interpreted based on known N-glycan biosynthetic pathways, allowing for the identification of individual glycan species (see Note 6).

13. A sample of labeled *N*-glycans (40 µl) was diluted with 160 µl of ACN in an autosampler vial. The 2AB-labeled *N*-glycans were separated using a XBridge BEH Amide column (Waters) on a Dionex UltiMate LC equipped with fluorescent detection (see Note 7), in line with a LTQ Velos Pro Mass Spectrometer equipped with a heated electrospray standard source (HESI-II probe) (see Note 8).

NOTES

1. Some antibodies (e.g., Fab glycans) require a pre-incubation of 2 min. at 80°C before addition of Rapid PNGase F.
2. Small PCR tubes incubated on a thermocycler provide excellent temperature control, minimizing evaporation. However, any other suitable incubator can be used for this step.
3. Dissolve 5 mg of 2AB Labeling Reagent in 20 µl of DMSO, add 30 µl H_2O. Add this solution to 6 mg of $NaCNBH_4$. Use immediately. Discard unused solution following safety regulations.
4. The heated lid of a thermocycler prevents condensation and volume losses. Yet, any other incubator or microcentrifuge tubes can be used in this step.
5. It is critical to maintain a dilution in 85% acetonitrile; higher organic content might cause some glycans to precipitate out of solution, and lower organic content will prevent glycans from binding to the HILIC column.
6. Since various isomers can be present, unequivocal assignment of structure is only possible following extensive analysis, such as MS/MS, which is not described here.
7. The glycans were separated using a gradient of 70%:30% to 62%:38% ACN:50 mM NH_4 Formate, pH 4.4, for 48 minutes at 350 µl/min, 2 AB fluorescence was measured at 350 (ex)/420 nm (em). Injection vol:100 µl.
8. Optimized settings for positive mode detection of 2 AB-labeled *N*-glycans: A) ESI: spray voltage, 3.5 kV; capillary temperature, 250°C; sheath gas, 11 psi; Aux gas and sweep gas flow rates, 0; S-lensRF level %, 66. B) Ion Optics settings: Multiple 00 offset, 2.5 V; Lens 0 voltage, 6.5 V; Multiple 0 offset, 7.0 V; Lens 1 voltage, 16 V; Multiple 1 offset, 6.5 V; Multiple RF Amplitude, 600; Front lens, 7.75 V.

RESULTS 1: Reproducibility and Sensitivity

Rituximab samples were treated for 5 min. with Rapid PNGase F. Released *N*-glycans were labeled with 2AB and analyzed by LC-MS. Results show seven replicates analyzed on 3 different days. The composition of *N*-glycans was highly reproducible from day to day (Fig. 1). There was negligible variation in the levels of low abundance *N*-glycans, as well (Fig. 2). All major and minor species previously reported in the literature were found. Relative abundance was within previously reported ranges (1) .

Figure 1: **Rituximab *N*-glycans released with Rapid PNGase F and labeled with 2AB.**

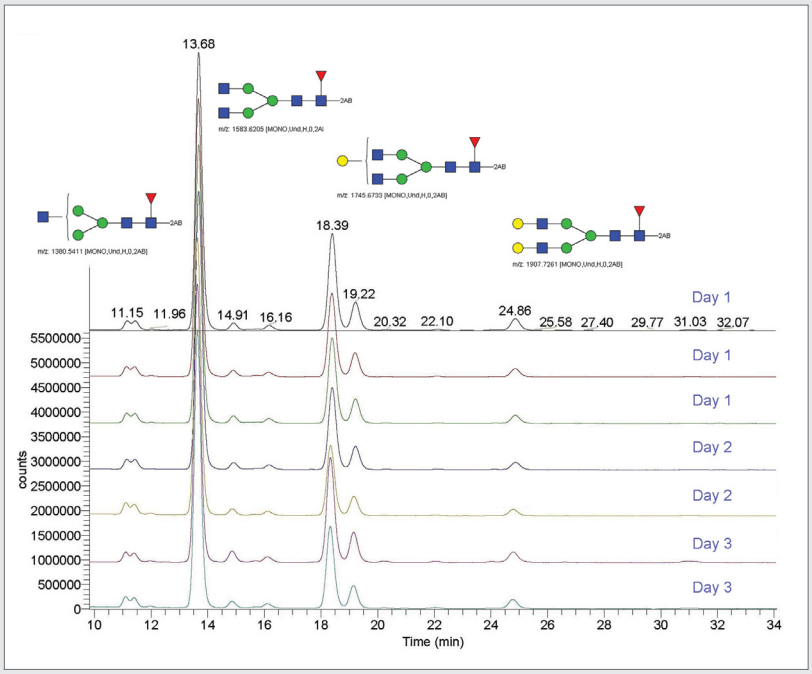
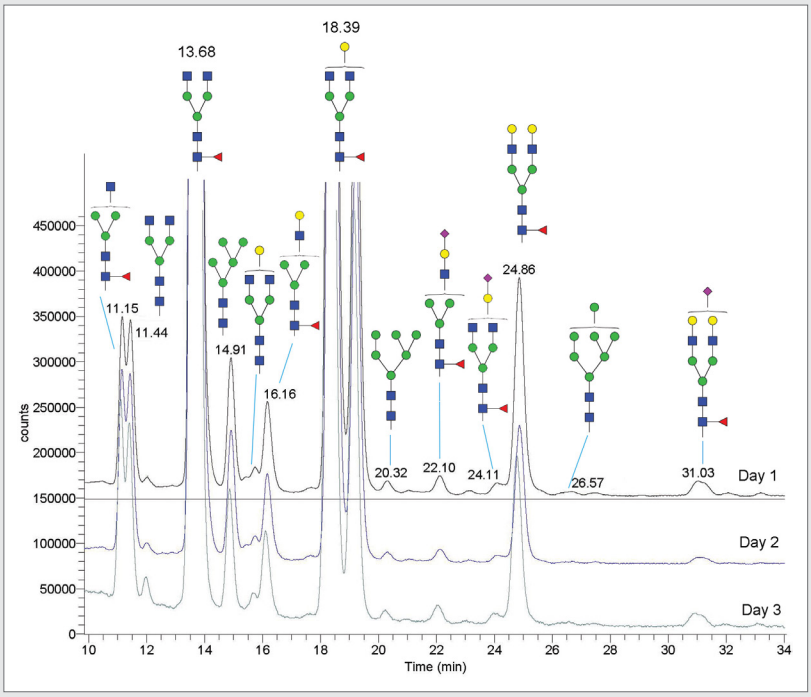


Figure 2: **Rituximab *N*-glycans zoomed view to visualize low abundance peaks.**

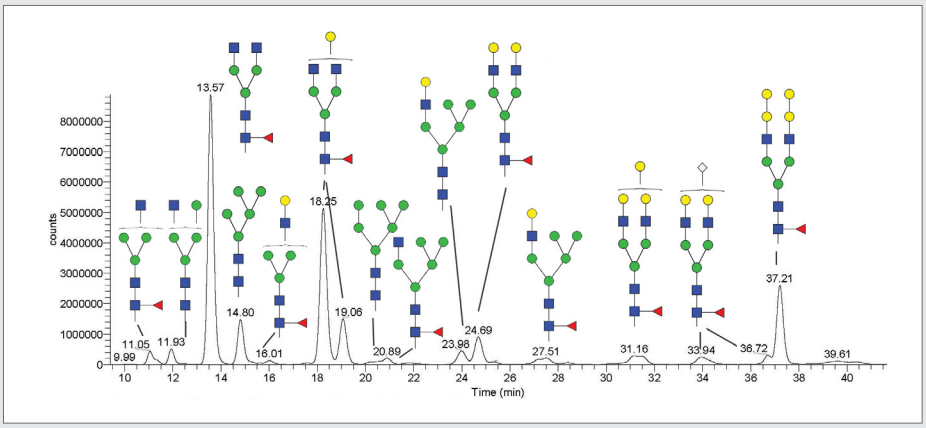


Gal Glc Man GalNAc GlcNAc Fuc NeuAc NeuGc

RESULTS 2: Therapeutic Antibodies with Additional *N*-glycan Sites

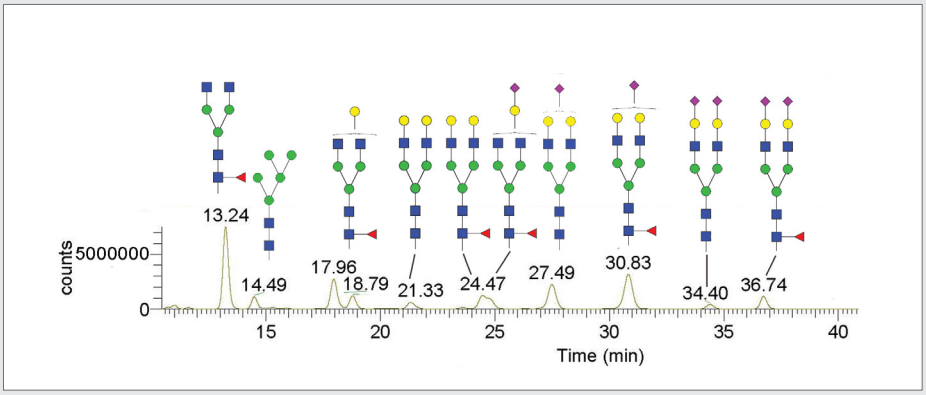
A sample of cetuximab (32 µg) was diluted in Rapid Buffer, pre-incubated 2 min. at 80°C, and treated for 5 min. with Rapid PNGase F (Fig. 3). Released glycans were analyzed as before. Abundance of major and minor peaks, known to be present in either Fc or Fab sites, was similar to previous studies (2).

Figure 3: **Cetuximab: *N*-glycans released with Rapid PNGase F (5 min. reaction).**



A sample of etanercept (50 µg) was diluted in Rapid Buffer, and treated for 5 min. with Rapid PNGase F. Released glycans were analyzed as before (Fig. 4). All expected *N*-glycans (from either conserved Fc site or from TNF domain) were found, in relative abundance as previously reported (3).

Figure 4: **Etanercept: *N*-glycans released with Rapid PNGase F (5 min. reaction).**



CONCLUSION

NEB's Rapid PNGase F reagent can achieve complete and unbiased removal of *N*-glycans from antibodies in minutes. This reaction, which occurs in solution and requires minimal setup, is amenable to high throughput and automation, and is compatible with downstream glycomics analysis by LC/MS.

References:

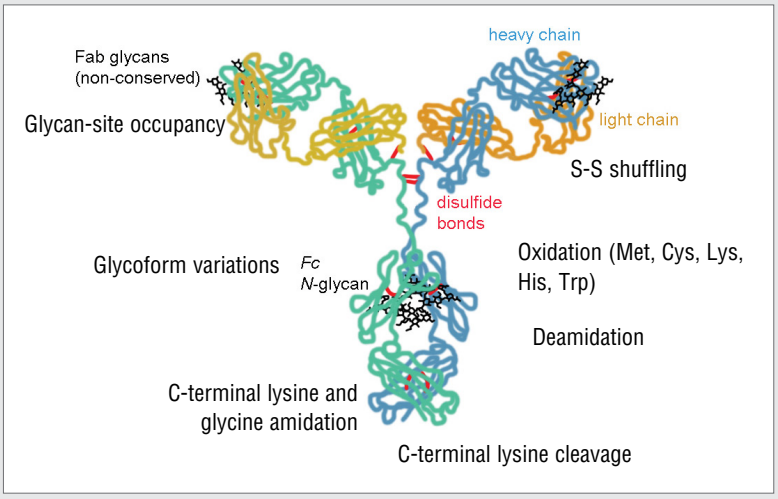
1. Visser J, et al. (2013). *BioDrugs.* 27, 495–507.
2. Ayoub, et al. (2013). *mAbs* 5, 699–710.
3. Houel S, et al. (2014). *Anal Chem.* 7, 576–584.

Proteomics: Fast and Efficient Antibody Deglycosylation using Rapid PNGase F

Paula Magnelli and Colleen McClung, New England Biolabs, Inc.

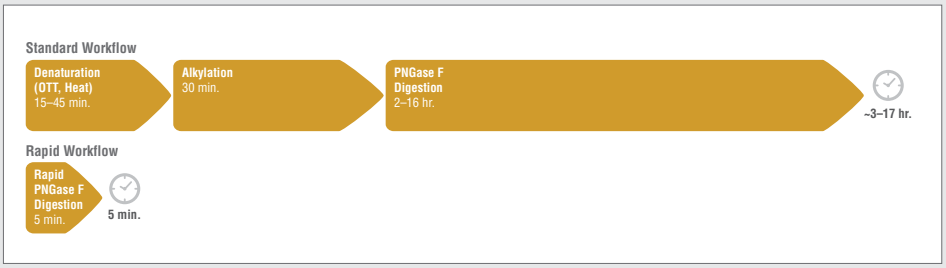
Innovations in process development and manufacturing of therapeutic monoclonal antibodies have been critical for their clinical and economic success. Along with these advances, methods for quality control are constantly evolving to guarantee the safety and effectiveness of these drugs. During development and production, mass spectrometry (MS) "top-down" methods (e.g. analysis of intact or reduced proteins) complemented by "bottom-up" approaches (e.g. peptide and glycopeptide mapping) are used to verify structural attributes of monoclonal antibodies.

Figure 1: **Critical attributes for monoclonal antibodies therapeutic products**



Removal of *N*-glycans with PNGase F simplifies MS analysis. However, complete deglycosylation of IgG typically requires lengthy protein denaturation and PNGase F incubation steps, because for a given site (i.e., Fc conserved glycosylation) some *N*-glycans are easier to remove than others. Moreover, non-conserved *N*-glycan sites (i.e., Fab) can be particularly resistant to PNGase F, and are only fully removed after several hours of incubation. To address these limitations, NEB has developed Rapid PNGase F. This novel product completely removes *N*-glycans from antibodies in just minutes and is compatible with high throughput proteomics applications.

Figure 2: **A standard deglycosylation workflow compared with Rapid PNGase F**



We demonstrate that all targets were deglycosylated extensively and without bias in less than 10 minutes. Moreover, Rapid PNGase F efficiently removes *N*-glycans from antibodies with additional glycosylation sites.

MATERIALS

- Erbitux (cetuximab) from Imclone, LLC.
- Rituxan (rituximab) from Genentech, a member of the Roche Group, and Biogen Idec, Inc.
- Enbrel (enteracept) from Amgen, Inc., manufactured by Immunex Corp
- Anti-MBP Monoclonal Antibody (NEB #E8032)
- Rituximab Isotype collection (Invivogen, San Diego, CA)
- PCR tube strips or centrifuge tubes
- Rapid PNGase F (NEB #P0710, supplied with 5X Rapid PNGase F Buffer)
- PNGase F (Glycerol-free) Recombinant (NEB #P0709, supplied with 10X Protein Denaturing Buffer, 10X NP40, and 10X GlycoBuffer 2).
- Blue Loading Buffer Pack (NEB #B7703, supplied with 1.25 M DTT)
- Protein Ladder (NEB #P7703)
- Precast Tris-Glycine mini-gels, 10-20%
- Coomassie Blue protein gel stain
- 400 M DTT
- Formic acid (proteomics grade)
- Acetonitrile (mass spec grade)

GENERAL PROTOCOL:

Rapid deglycosylation one-step protocol:

The antibody sample is treated with Rapid PNGase F at 50°C.

1. Using PCR tubes (200 µl), adjust each sample of antibody (20-100 µg, See Note 1) to 16 µl with Milli-Q Water. Add 4 µl of Rapid Buffer, mix. Add 1 µl of Rapid PNGase F. Incubate at 50°C for 5 minutes (See Note 2).

Rapid deglycosylation two-step protocol:

Some antibodies (i.e., Fab glycans) require a preincubation of 2 minutes at 80°C before addition of Rapid PNGase F.

1. Using PCR tubes (200 µl), adjust antibody sample (20-100 µg, See Note 1) to 16 µl with Milli-Q Water. Add 4 µl of Rapid PNGase F Buffer and mix. Incubate at 80°C for 2 minutes (See Note 2), and then cool.
2. Add 1 µl of Rapid PNGase F. Incubate at 50°C for 5 minutes (See Note 2).

Standard denaturing deglycosylation:

This protocol (harsh denaturation, long incubation) completely removes N-glycans. SDS is not compatible with MS; it is used here to compare migration on SDS-PAGE.

1. Using PCR tubes (200 µl), adjust antibody sample (20-100 µg) to 16 µl with Milli-Q Water. Add 2 µl Protein Denaturing Buffer and mix. Incubate at 95°C for 2 minutes (See Note 2), and then cool.
2. Add 2 µl of 10% NP-40 and 2 µl of 10X GlycoBuffer 2. Add 1 µl of PNGase F (Glycerol-free), Recombinant. Incubate at 37°C for 60 minutes.

Standard (only DTT) deglycosylation:

This protocol (mild denaturation with DTT, long incubation) is compatible with MS analysis. Extensive N-glycan removal requires overnight incubation. Some resistant sites (i.e., Fab) may not reach completion, even in the presence of high amounts of enzyme.

1. Using PCR tubes (200 µl), adjust antibody sample (20-100 µg) to 16 µl with Milli-Q Water. Add 2 µl 400 mM DTT. Incubate at 95°C for 2 minutes (See Note 2), and then cool.
2. Add 2 µl of 10X GlycoBuffer 2. Add 1 µl of PNGase F (Glycerol-free), Recombinant. Incubate at 37°C for 60 minutes.

SDS-PAGE analysis:

The migration of the target protein is compared before and after deglycosylation with Rapid PNGase F, and with a standard denaturing reaction with PNGase F.

1. After deglycosylation, take a 3 µl aliquot for SDS-PAGE (See Note 3). Prepare fresh 3X Blue Loading Buffer (See Note 4). Add 17 µl of Milli-Q Water and 10 µl of 3X Loading Buffer to each sample, mix and incubate at 94°C for 2 minutes.
2. Load 7-10 µl on a 10–20% Tris–Glycine gel, load a MW ladder lane, run at 120-200 V (See Note 5). Stain with Coomassie Blue following manufacturer's instructions.

Liquid Chromatography/Electrospray Ionization Time-Of-Flight Mass Spectrometry (LC/ESI-TOFMS):

Samples are prepared for Top-Down analysis by ESI-TOF in order to detect the intact mass of the heavy chain.

1. To improve MS signal, samples from Steps 1 or 2 from the Rapid Deglycosylation Protocol were subjected to buffer exchange by drop dialysis (See Note 6) against 150 mM NaCl, 50 mM Tris-HCl, pH 8.0 (See Note 7). Protein was reduced using 10 mM DTT for 30 minutes at room temperature. Finally, formic acid was added to 0.1% v/v.
2. Samples were analyzed using a custom reverse-phase chip (See Note 8) on an Agilent 1200 series nano LC connected directly to an Agilent 6210 series ESI-TOF MS. The chip was equilibrated with 0.1% formic acid in 5% acetonitrile (ACN). Samples (1 µl) were injected, the chip trap column was loaded at 2 µl/min and the separation column developed at 500 nl/min with a 15 minute linear gradient from 5% to 95% ACN, followed by 5 minutes at 95% ACN. Protein was found to elute at approximately 10 minutes after injection. The spectra were extracted and deconvoluted (See Note 9).

NOTES

1. Commercial antibodies often contain stabilizers or excipients (e.g., detergents, sorbitol, glycerol) which interfere with deglycosylation and/or analysis. If necessary, dilute or exchange in a suitable buffer. Suggested protocols can be found at: https://www.neb.com/protocols/2014/10/28/glycoproteomicsbuffer-exchange-protocols-p0710.
2. Small PCR tubes incubated in a thermocycler provide good temperature control, and minimize evaporation. Alternatively, any other incubator or heat block can be used.
3. Equivalent to 2-4 µg protein.
4. 4 µl of 1.25 M DTT, 130 µl 3X Blue Loading Buffer
5. Add 3X Blue Loading Buffer to empty wells. Run until blue front reaches the bottom of the gel. For 10-20% gels running at 200 V, it takes approx 1 hour.
6. Drop dialysis is an inexpensive method for buffer exchange (although it requires careful manipulation). However, dialysis in a small device is also appropriate, as well as microfiltration. Detailed protocols can be found at: https://www.neb.com/protocols/2014/10/28/glycoproteomics-buffer-exchange-protocolsp0710.
7. Buffer of choice should be compatible with the instrument and method used for analysis. For instance, direct infusion require solutions to be free of salts, Tris, detergents. A low molarity volatile buffer can be used (ammonium bicarbonate, acetate, formate) instead.
8. The reverse phase chip consisted of an integrated trapping column (40 nl), separation column and nano-ESI emitter (75 µm x 150 mm both packed with PLRP-S, 5 µm particles, 1000 Å pore size).
9. The mass spectra were acquired from 150 to 3200 m/z, one cycle/sec and 10,000 transients per scan using an ionization energy of 1800 V, fragmentor of 215 V and drying gas of 325°C at 4.0 l/min

RESULTS

Extensive deglycosylation with Rapid PNGase F

Incomplete *N*-glycan removal is a concern because some species might be removed faster than others, resulting in a biased composition. Following a traditional protocol, overnight treatment is often required to achieve complete conversion of IgGs to their deglycosylated form (not shown). In contrast, glycan removal is complete with a 5 minute incubation using Rapid PNGase F (Figure 3).

Figure 3: **Deglycosylation with Rapid PNGase F**

Antibodies treated for 5 minutes with Rapid PNGase F (RP), in comparison with an untreated control (C), and with a standard denaturing reaction with SDS (std). Mouse IgG2, rituximab, and etanercept were efficiently deglycosylated in 5 minutes. Cetuximab (which carries Fab *N*-glycans) required a 2 step protocol (RP2): compare with the partial shift down observed with a one step (RP1).

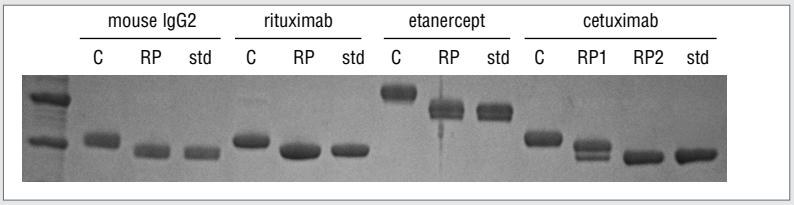
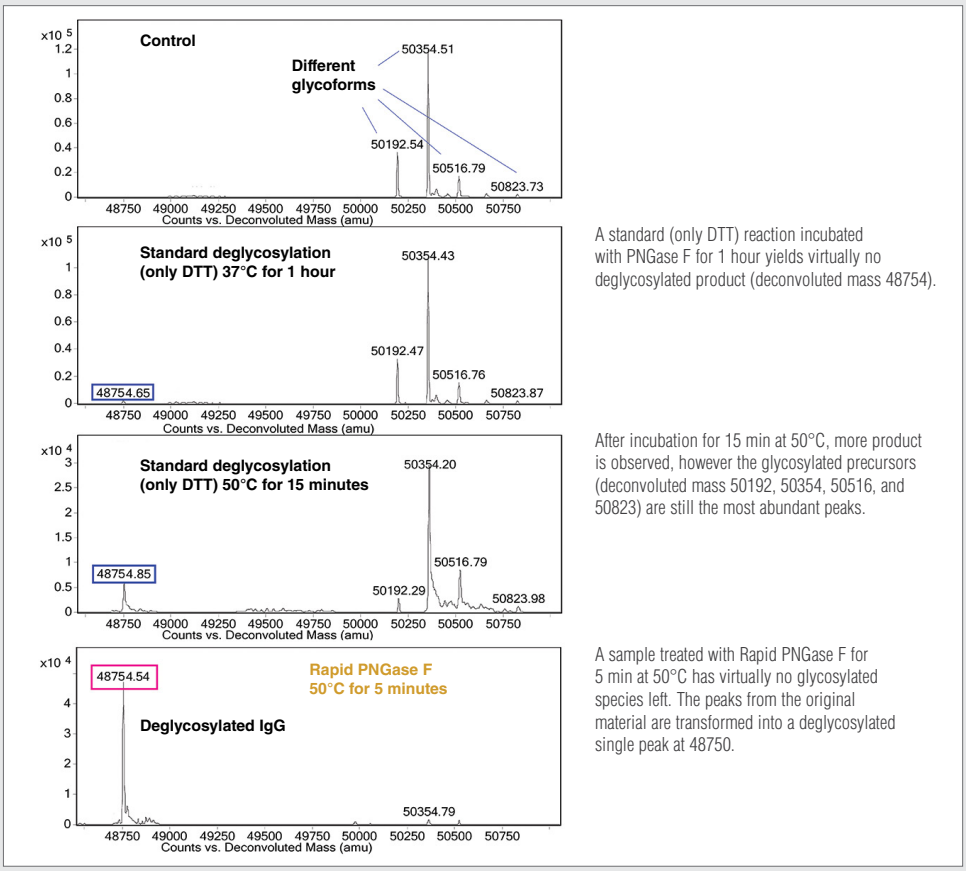


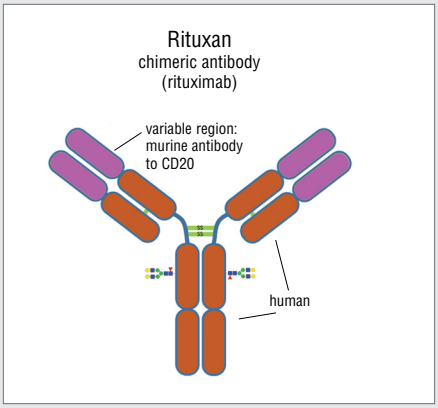
Figure 4: **The ESI-TOF deconvoluted spectra of a mouse IgG2 sample before (control) and after several deglycosylation treatments.**

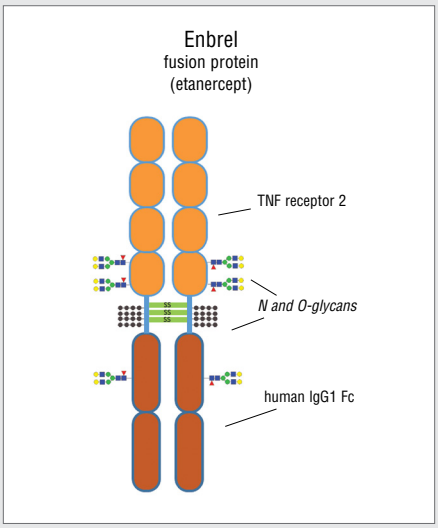


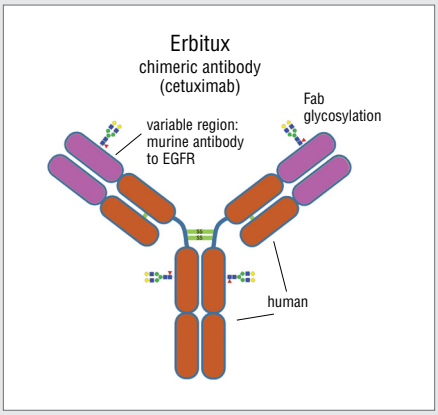
A standard (only DTT) reaction incubated with PNGase F for 1 hour yields virtually no deglycosylated product (deconvoluted mass 48754).

After incubation for 15 min at 50°C, more product is observed, however the glycosylated precursors (deconvoluted mass 50192, 50354, 50516, and 50823) are still the most abundant peaks.

A sample treated with Rapid PNGase F for 5 min at 50°C has virtually no glycosylated species left. The peaks from the original material are transformed into a deglycosylated single peak at 48750.





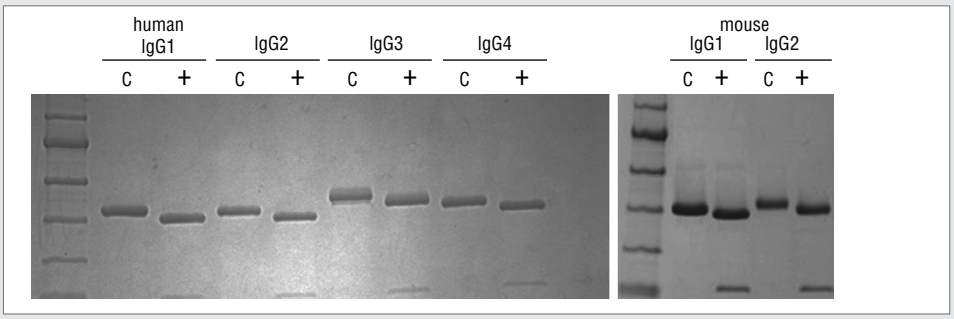


RESULTS (continued)

Analysis of different isotypes, subclasses and sources

Figure 5: **Deglycosylation IgG subclasses of rituximab**

The rituximab IgG subclass collection (Figure 5) was treated for 10 min with Rapid PNGase F (+), compare with control (C). Rapid PNGase F effectively deglycosylates all IgG forms, despite their structural differences (see diagrams to the right side).



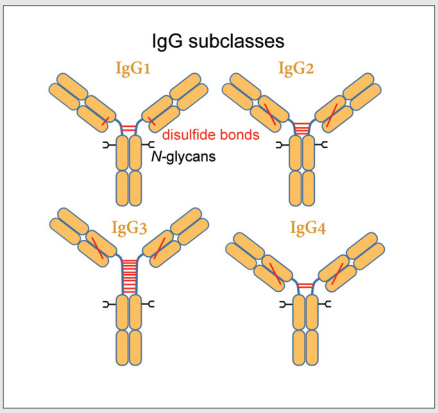
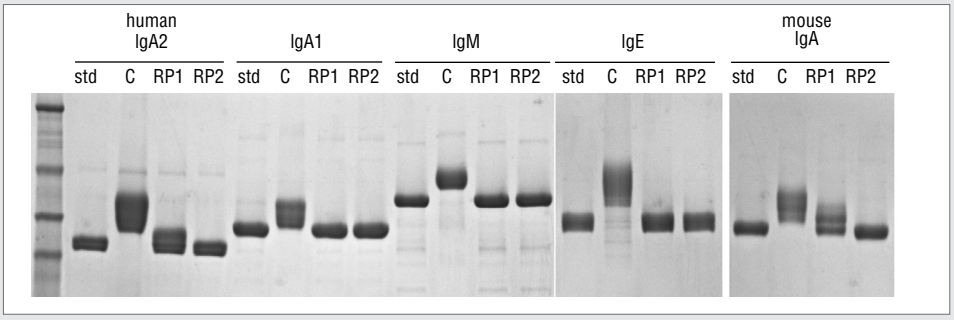
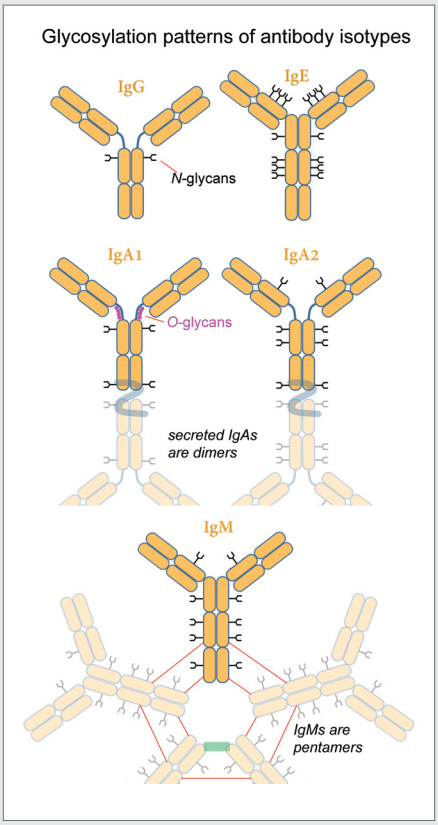


Figure 6: **Deglycosylation of various isotypes of rituximab**

The corresponding rituximab isotype collection (Figure 6) was also treated for 10 min with Rapid PNGase F in a 1-step (RP1) or 2-step (RP2) protocol. Compare with standard SDS denaturing reaction (std) and control (C). Human IgA2 and mouse IgA required a 2-step protocol for complete conversion illustrating how some *N*-glycans sites are less accessible and require additional relaxation of the protein structure (see diagrams to the right side).





CONCLUSION:

NEB's Rapid PNGase F reagent can achieve complete and unbiased removal of *N*-glycans from antibodies, a requisite for accurate measurement of critical quality attributes by Mass Spectrometry. This reaction, which occurs in solution and requires minimal setup, is remarkably fast, amenable to high throughput and automation, and is compatible with downstream proteomics analysis by LC/ESI-MS.

References:
1. Khawli LA, et al. (2010). *mAbs* 2, 613–624.
2. Tang L, et al. (2013). *mAbs* 5, 114–125.
3. Zauner G, et al. (2013). *Cell Proteomics* 12, 856–865s.

PNGase A

REACTION PROTOCOLS

PNGase A Denaturing Reaction Conditions

1. Combine 1-5 µg of glycoprotein, 1 µl of Glycoprotein Denaturing Buffer (10X) and H_2O (if necessary) in a total reaction volume of 10 µl.
2. Denature glycoprotein by heating reaction at 100°C for 10 minutes.
3. Chill denatured glycoprotein on ice and centrifuge 10 seconds.
4. Make a total reaction volume of 20 µl by adding 2 µl GlycoBuffer 3 (10X), 2 µl 10% NP-40 and 6 µl H_2O.

Note: *PNGase A is inhibited by SDS; therefore, it is essential to have NP-40 in the reaction mixture under denaturing conditions. Failure to include NP-40 into the denaturing protocol will result in loss of enzymatic activity.*

5. Add 1 µl PNGase A, mix gently.
6. Incubate reaction at 37°C for 1 hour.
7. Analyze by method of choice

PNGase A Reduced Reaction Conditions

When deglycosylating a DTT reduced glycoprotein it is recommended that an aliquot of the glycoprotein is subjected to the denaturing protocol to provide a positive control for the fully deglycosylated protein. The DTT reduced reaction can then be compared to the denatured reaction to determine the extent of reaction completion.

1. Combine 1-5 µg of glycoprotein, 2 µl of GlycoBuffer 3 (10X), 4 µl of 0.2 M DTT and H_2O (if necessary) to make a 20 µl total reaction volume.
2. Reduce glycoprotein by heating reaction at 55°C for 10 minutes.
3. Add 2-5 µl PNGase A, mix gently.
4. Incubate reaction at 37°C for 4-24 hours.

Note: *To deglycosylate a DTT reduced glycoprotein, longer incubation time as well as more enzyme may be required.*

5. Analyze by method of choice.

Note:*The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS-PAGE gels.*

TECHNICAL TIPS

- Can cleave glycoproteins, there is no need for tryptic digest prior to deglycosylation.
- Can cleave *N*-linked glycans containing core α1-3 fucose.
- Activity is inhibited by SDS, under denaturing conditions it is essential to have NP-40 present in the reaction mixture in a 1:1 ratio.
- A positive control substrate is recombinant avidin from maize or HRP.

FREQUENTLY ASKED QUESTIONS

Q. What is the difference between PNGase F and PNGase A?

A. PNGase F and PNGase A both cleave between the innermost GlcNAc and asparagine residues of *N*-linked glycans on both glycoproteins and glycopeptides. PNGase F can cleave almost all *N*-linked glycans from high mannose, hybrid, and complex oligosaccharides. However, PNGase F cannot cleave *N*-glycans with core α1-3 fucosylation. In contrast, PNGase A cleaves *N*-linked glycans from high mannose, hybrid, and short complex oligosaccharides such as those found in plant and insect cells. PNGase A differs from PNGase F in that it cleaves *N*-linked glycans with or without α(1,3)-linked core fucose residues.

Endoglycosidase H/H_f

REACTION PROTOCOLS

Endo H/H_f Denaturing Reaction Conditions

1. Combine 1–20 µg of glycoprotein, 1 µl of 10X Glycoprotein Denaturing Buffer and H_2O (if necessary) to make a 10 µl total reaction volume.
2. Denature glycoprotein by heating reaction at 100°C for 10 minutes.
3. Make a total reaction volume of 20 µl by adding 2 µl 10X GlycoBuffer 3, H_2O and 1–2 µl Endo H/H_f.
4. Incubate reaction at 37°C for 1 hour.
5. Analyze by method of choice.

Note: *The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS-PAGE gels.*

Endo H/H_f Non-Denaturing Conditions

1. Combine 1–20 µg of glycoprotein, 2 µl 10X GlycoBuffer 3, H_2O and 2–5 µl Endo H/H_f to make a total reaction volume of 20 µl.
2. Incubate reaction at 37°C for 4 hours to overnight.
3. Analyze by method of choice.

Note: *The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS-PAGE gels Reaction may be scaled-up linearly to accommodate large amounts of glycoprotein and larger reaction volumes. The pH range of Endo H/H_f is very specific and can effect activity of the enzyme, therefore we recommend using the supplied* GlycoBuffer 3 *which has a pH of 6.0.*

Endo S/Endo D

REACTION PROTOCOLS

Endo S Reaction Protocol

1. Combine 100 µg of native IgG, 1 µl of 10X GlycoBuffer 2 and H_2O (if necessary) to make a 10 µl total reaction volume.
2. Add 1 µl Endo S.
3. Incubate reaction at 37°C for 1 hour.
4. Eliminate Endo S from the reaction using Chitin Magnetic Beads (NEB #E8036) or analyze deglycosylation reaction by method of choice.

Endo D Reaction Protocol

1. Combine 10–20 µg of glycoprotein, 1 µl of 10X DTT and H_2O (if necessary to make a 10 µl total reaction volume.
2. Denature glycoprotein by heating reaction at 55°C for 10 minutes.
3. Make a total reaction volume of 20 µl by adding 2 µl 10X GlycoBuffer 2, H_2O and 1–5 µl Endo D.
4. Incubate reaction at 37°C for 1 hour.
5. Eliminate Endo D from the reaction using Chitin Magnetic Beads (NEB #E8036) or analyze deglycosylation reaction by method of choice.

Note: *The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS-PAGE gels. To deglycosylate a native glycoprotein, longer incubation time as well as more enzyme may be required. Endo D is not recommended for use with Glycoprotein Denaturing Buffer containing both SDS and DTT, as Endo D is inhibited by SDS and unlike other endoglycosidases, NP-40 does not counteract the SDS inhibition.*

FREQUENTLY ASKED QUESTIONS

Q. What is the difference between PNGase F, Endo S and Endo D?

A. PNGase F cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins. Endo S has a high specificity for removing N-linked glycans within the chitobiose core of native IgG. Whereas, Endo D cleaves within the chitobiose core of paucimannose N-linked glycans from glycoproteins and glycopeptides, with or without extensions in the antennae.

Q. Will SDS inhibit Endo D?

A. Yes, Endo D is inhibited by SDS and unlike other endoglycosidases, a non-ionic detergent, such as NP-40, does not counteract the SDS inhibition. Endo D is therefore not recommended for use with NEB's Glycoprotein Denaturing Buffer which contains both SDS and DTT. The enzyme is supplied with a 10X DTT solution for denaturation purposes without SDS.

Remove-iT PNGase F/Endo S/Endo D Magnetic Chitin Beads

PROTOCOL

Remove-iT PNGase F/Endo S/Endo D Magnetic Chitin Bead Protocol

1. Pipette 50 µl Chitin Magnetic Beads (NEB #E8036) into an eppendorf tube and place the eppendorf in a Magnetic Separation Rack. Let the magnet attract the chitin beads, then pipette off the liquid supernatant and discard.
2. Wash the magnetic chitin beads with 500 µl of 50 mM NH_4 Formate pH 4.4 (or buffer of choice) and pull the beads to the side of the tube using the Magnetic Separation Rack. Pipette off the supernatant and discard. Repeat.
3. Add the deglycosylated glycoprotein sample into the eppendorf with magnetic chitin beads.
4. Rock the deglycosylated glycoprotein sample with the magnetic chitin beads for 10 minutes at 4°C.
5. Place the eppendorf back on the magnetic separation rack, and allow the magnet to attract the chitin beads. Pipette off the supernatant and keep.
6. Wash the magnetic chitin beads 3 x 100 µl with 50 mM NH_4 Formate pH 4.4 (or buffer of choice). Pipette of the supernatant from each wash and keep.
7. Combine all supernatants from Steps 5 & 6, as these are the deglycosylated glycoprotein.
8. Analyze sample by method of choice

Note: *Elimination of Remove-iT enzymes from the deglycosylation reaction can be scaled up linearly with larger magnetic chitin bead volumes. The ideal reaction volume for 50 µl of chitin beads is in the range of equal volume to no more than 5X bead bed volume. The Magnetic Chitin Beads binding capacity is approximately 0.4 mg/ml of CBD-tagged protein. This binding capacity is calculated in mg of protein per bed volume of resin. The chitin magnetic beads are a 50:50 slurry. Therefore, 50 µl of slurry will yield 25 µl bed volume of resin.*

Glycan Analysis of Murine IgG by Enzymatic Digestion with Endo S and PNGase F, Followed by Mass Spectrometric Analysis

Beth McLeod and Colleen McClung, New England Biolabs, Inc.

Immunoglobulin Gs (IgGs) are antibody molecules that are composed of four peptide chains — two heavy chains and two light chains. There are four IgG subclasses (IgG1, 2a, 2b, and 3) in mice. The heavy chains are known to be glycosylated, while the light chains are not. The *N*-glycan moiety attached to the asparagine 297 residue in the Fc domain of the antibody is critical for the structure and biological activity of the molecule (1). A growing number of monoclonal IgG antibodies are currently being developed and used as therapeutic agents and it is apparent that there are many variables in cell culture systems that can greatly influence the heterogeneity of the glycans on IgGs. Therefore, it has become increasingly important to monitor the glycosylation profiles of these molecules in the production process.

Endoglycosidase S (Endo S) isolated from *Streptococcus pyogenes* has been shown to specifically and completely cleave the biantennary complex *N*-glycan at asparagine 297 of IgG under native conditions (2). This enzyme removes the *N*-glycan moiety after the first *N*-acetylglucosamine (GlcNAc) residue on the chitobiose core, leaving only a GlcNAc with or without a core fucose residue on the protein. In contrast, PNGase F cleaves between the innermost GlcNAc and asparagine residue of high mannose, hybrid and complex oligosaccharides from *N*-linked glycoproteins.

Endo S is cloned and expressed in *E. coli* as a fusion to the chitin binding domain (CBD). The specificity of Endo S is identical to Endo S with the added benefit of the CBD tag to remove the enzyme following a deglycosylation reaction if needed.

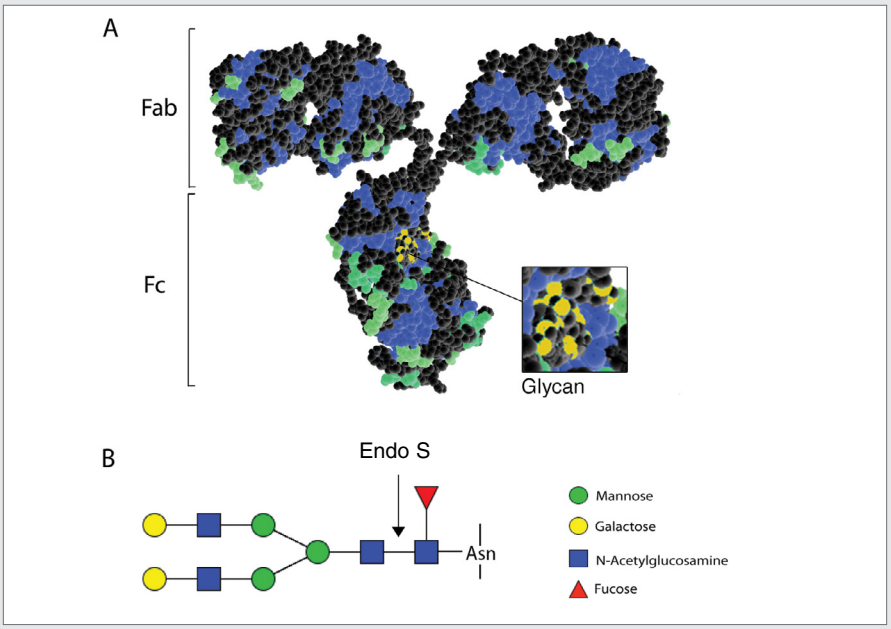
This application note compares the enzymatic removal of glycans on murine IgG using Endo S and PNGase F Glycerol free under native conditions. Endo S is a more robust enzyme for this purpose, completely removing the sugar residues from monoclonal mouse IgG. Conversely, the PNGase F digest does not result in a complete digestion under native conditions.

MATERIALS

- Endo S (NEB #P0741)
- PNGase F (Glycerol-free) (NEB #P0705)
- Anti-MBP Monoclonal Antibody (Murine IgG2a) (NEB #E8032)
- GlycoBuffer 2 [10X, supplied with PNGase F (Glycerol-free) and Endo S]
- 3K Millipore Amicon Ultra Filter Unit (cat. #UFC500324)
- Dilution Buffer: 20 mM Tris HCl pH 7.5, 50 mM NaCl, 1 mM EDTA
- Protein Ladder (NEB #P7703)
- Agilent 6210 TOF MS with both 1200 Series Capillary and Nano pumps with a ChipCube. Custom PLRP-S Chip (75 µm x 150 mm with 40 nl trap) or equivalent TOF or Q-TOF and nanoLC system

Figure 1: **Structure and glycosylation of a murine IgG**

(A) Structural model of murine IgG. In the IgG, beta-sheets are colored blue, loops are colored black and the helices are colored green. The brackets indicate the antigen-binding Fab portion and the Fc effector portion of IgG. The inset highlights the two conserved glycans (yellow) attached to Asn-297 of the heavy chains. The model was generated using JMOL 12.2.23 from a model deposited in the Protein Data Bank by L. Harris (University of California, Riverside). (B) Schematic representation of the fully substituted IgG heavy-chain glycan and the location of the Endo S cleavage.



GENERAL PROTOCOLS

Deglycosylation of IgG using Endo S occurs optimally under native conditions. Deglycosylation using PNGase F occurs optimally under denaturing conditions (using SDS, DTT and heat); however, this is not optimal for downstream mass spectrometry analysis and thus the reaction needs to be performed under native conditions without the addition of detergents.

Removal of Glycerol from Murine IgG Substrate

1. Dilute 50 µl of 1 mg/ml Anti-MBP Monoclonal Antibody (Murine IgG2a) with 450 µl of the Dilution Buffer: 20 mM Tris HCl pH 7.5, 50 mM NaCl, 1 mM EDTA.
2. Apply to a 0.5 ml 3K Millipore Amicon Ultra Filter Unit and spin in a microcentrifuge for 30 minutes at 12,000 rpm.
3. Discard flow-through and add an additional 450 µl of Dilution Buffer to the sample. Spin in a microcentrifuge for 30 minutes at 12,000 rpm.
4. Place the Amicon filter device upside-down in a clean microcentrifuge tube and spin for 2 minutes at 1,000 rpm to transfer glycerol-free murine IgG to the tube.

Deglycosylation of IgG with Endo S

1. Add 10 µl prepared glycerol free Anti-MBP Monoclonal Antibody (Murine IgG2a) at 1 mg/ml (10 µg total) to a 200 µl tube. Add 5 µl of 10X GlycoBuffer 2 (500 mM NaPhosphate pH 7.5) (see Note 1). Add 34 µl of water, and add 1 µl (200 units) of Endo S. Mix with pipette and incubate at 37°C for 1 hour.
2. Reserve 10 µl for SDS-PAGE gel analysis, if desired. Analyze remainder by nanoLC-TOF MS.

Deglycosylation of IgG with PNGase F (Glycerol-free)

1. Add 10 µl prepared glycerol free Anti-MBP Monoclonal Antibody (Murine IgG2a) at 1 mg/ml (10 µg total) to a 200 µl tube. Add 5 µl of 10X GlycoBuffer 2 (500 mM Sodium Phosphate pH 7.5) (see Note 1), 34 µl of water and 1 µl (500 units) of PNGase F (Glycerol-free).
2. Mix with pipette and incubate at 37°C for 1 hour. Reserve 10 µl for SDS-PAGE gel analysis if desired.
3. Analyze remainder by nanoLC-TOF MS.

Liquid Chromatography/Electrospray Ionization Time-of-Flight Mass Spectrometry (LC/ESI-TOF MS):

1. Protein was denatured using 10 mM DTT for 30 minutes at room temperature. Finally, formic acid was added to 0.1% v/v.
2. Samples were analyzed using a custom reverse-phase chip (see Note 2) on an Agilent 1200 series nano LC connected directly to an Agilent 6210 series ESI-TOF MS.
   a) The chip was equilibrated with 0.1% formic acid in 5% acetonitrile (ACN).
   b) Samples (1 µl) were injected, the chip trap column was loaded at 2 µl/min and the separation column developed at 500 nl/min with a 15 minute linear gradient from 5% to 95% ACN, followed by 5 minutes at 95% ACN. Protein was found to elute at approximately 10 minutes after injection.
   c) The spectra were extracted and deconvoluted (see Note 3).

NOTES

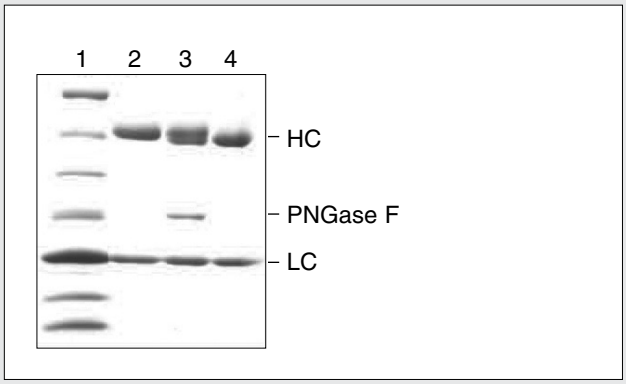
1. Previous versions of this protocol called for other reaction buffers. As of 2015, a universal buffer system was introduced (10X GlycoBuffer 1 for exoglycosidases and 10X GlycoBuffer 2 for most endoglycosidases). You can find more information at: https://www.neb.com/faqs/2015/04/01/why-have-the-neb-glycosidase-enzymes-changed-reaction-buffers-what-are-the-new-reaction-buffers-a
2. The reverse phase chip consisted of an integrated trapping column (40 nl), separation column and nano-ESI emitter (75 µm x 150 mm both packed with PLRP-S, 5 µm particles, 1000 A pore size).
3. The mass spectra were acquired from 150 to 3200 m/z, one cycle/sec and 10,000 transients per scan using an ionization energy of 1800 V, fragmentor of 215 V and drying gas of 325°C at 4.0 l/min.

RESULTS:

N-glycan removal under native conditions

Endo S has a high specificity for removing the *N*-glycan moiety of IgG under native conditions (Figure 3). Digestion of IgG with PNGase F (Glycerol-free) under native conditions (Lane 3) is not complete, as seen by the presence of a doublet band of the heavy chain (HC). Endo S (Lane 4) yields a complete deglycosylation of IgG under native conditions as shown by a complete shift of the band compared with the control (no enzyme, Lane 2).

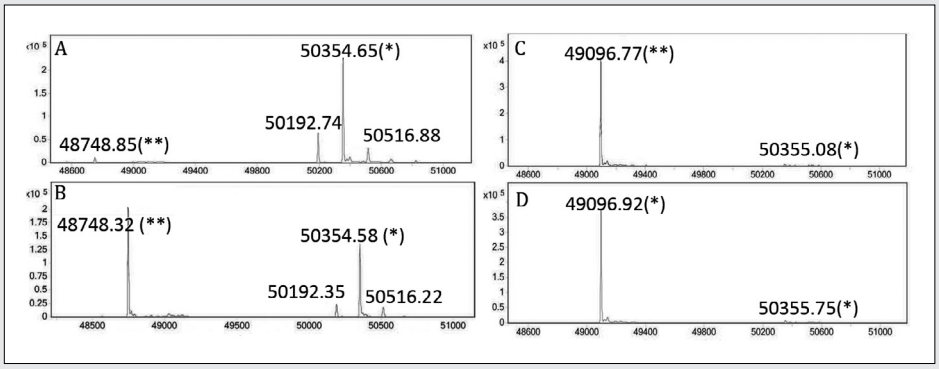
Figure 2: **IgG deglycosylation (native) with Endo S or PNGase F (Glycerol-free)**



To confirm these results, samples were analyzed by ESI-TOF MS. Figure 3 shows the analysis of IgG digested under native conditions with PNGase F (Glycerol-free) (Figure 3A-B), or Endo S (Figure 3C-D).

Figure 3: **ESI-TOF analysis of deglycosylation**

(A,B) ESI-TOF MS of a PNGase F digested monoclonal mouse IgG incubated for 1 hour at 37°C (A) or 24 hrs at 37°C (B), where (*) indicates IgG with intact glycan at MW 50,354, and (**) indicates deglycosylated heavy chain of IgG at MW 48,748; (C,D). Endo S digested monoclonal mouse IgG incubated 1 hour (C) or 24 hrs (D), where (*) indicates IgG with intact glycan at MW 50,355, and (**) indicates deglycosylated heavy chain of IgG MW 49,096.



CONCLUSION:

Endo S is a superior choice for glycobiology applications that demand rapid and reliable deglycosylation of IgG under native conditions.

References:

1. Arnold, J.N. et al. (2007) *Annual Rev. Immunol.* 25, 21-50.
2. Colin, M. and A. Olsén (2001) *EMBO Journal* 20, 3046-3056.
3. Vollmer, M and van de Goor (2009) *Methods in Molecular Biology* 544, 3-15.

Glycan Analysis of Murine IgG2a by Enzymatic Digestion with PNGase F and Trypsin, Followed by Mass Spectrometric Analysis

Alicia Bielik, Colleen McClung and Cristen Ruse, New England Biolabs, Inc.

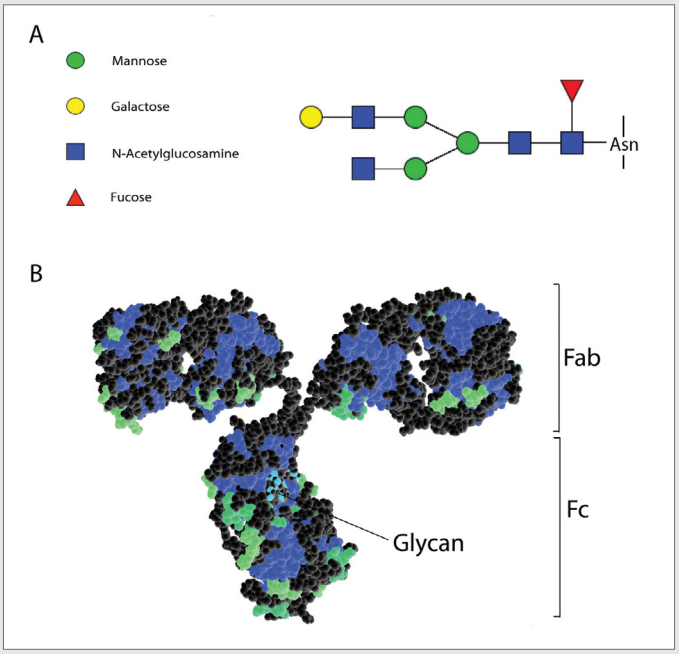
Proteomics, the systematic study of proteins in biological systems, has expanded the knowledge of protein expression, modification, interaction and function. However, in eukaryotic cells, the majority of proteins are post-translationally modified (1). A common post-translational modification, essential for cell viability, is the attachment of glycans. Glycosylation defines the adhesive properties of glycoconjugates and it is largely through glycan-protein interactions that cell-cell and cell-pathogen contact occurs.

Glycosylation is also important in the production of therapeutic proteins as it can significantly affect the potency of a biological drug. Producing a homogenously glycosylated protein is very difficult and often impractical. For this reason, development and manufacturing processes are highly monitored to minimize glycosylation variability. Therefore, the ability to determine the presence or absence of a glycan at a particular site is critical to the production of therapeutic proteins. A combination of enzymes (PNGase F and Trypsin) in tandem with mass spectrometry can be used to release the *N*-glycans present on glycoproteins and determine the sites of *N*-glycosylation on the protein.

Immunoglobulin Gs (IgGs) are antibody molecules that are composed of four peptide chains – two heavy chains and two light chains (Figure 1). There are four IgG subclasses (IgG1, 2a, 2b, and 3) in mice. The heavy chains are known to be glycosylated. The glycans present on the heavy chains of IgG are attached to asparagine residues (*N*-linked). *N*-linked glycans are produced by the secretory pathway (ER and Golgi). Synthesis of *N*-glycans begins with the transfer of a common oligosaccharide to a nascent polypeptide in the ER. Some *N*-glycans remain unmodified ("high mannose"), while others are initially trimmed and then extended as the glycoprotein matures in the Golgi ("complex").

Figure 1: **Structure and glycosylation of a murine IgG**

(A) Schematic representation of the fully substituted IgG heavy-chain glycan.
(B) Structural model of murine IgG. In the IgG, beta-sheets are colored blue, loops are colored black and the helices are colored green. The brackets indicate the antigen-binding Fab portion and the Fc effector portion of IgG. The arrow indicates the two conserved glycans (aqua) attached to Asn-180 of the heavy chains. The model was generated using JMOL 12.2.23 from a model deposited in the Protein Data Bank by L. Harris (University of California, Riverside).



MATERIALS

- PNGase F (Glycerol-free) (NEB #P0705)
- GlycoBuffer 2 (10X, supplied with enzyme)
- Glycoprotein Denaturing Buffer (10X, supplied with enzyme)
- Trypsin-ultra, Mass Spectrometry Grade (NEB #P8101)
- Trypsin Buffer (2X, supplied with enzyme)
- Trypsin-digested BSA MS Standard (CAM Modified) (NEB #P8108)
- Anti-MBP Monoclonal Antibody (Murine IgG2a) (NEB #E8032)
- Agilent 6210 TOF MS with both 1200 Series Capillary and Nano pumps coupled with a HPLC-Chip Cube, with a customPLRP-S Chip (75 µm x 150 mm with 40 nl trap) or equivalent TOF or Q-TOF and nanoLC system
- Thermo LTQ Orbitrap™ XL ETD MS with Thermo (Proxeon®) EASY-nLC or equivalent nanoLC and high resolution MS/MS system and a 20 cm C18 reverse phase analytical column
- FASP Protein Digestion Kit (abcam #ab270519)

Here, we describe the enzymatic removal of *N*-linked glycans using PNGase F from a model glycoprotein, murine monoclonal IgG type 2a, expressed in a mouse hybridoma cell line. We also demonstrate the use of trypsin to identify the site of glycosylation. Two mass spectrometers (MS) are used in this protocol: an Agilent 6210 Time-of-Flight (TOF) MS for analysis of the intact protein and a Thermo LTQ Orbitrap XL MS for analysis of the trypsin-digested murine IgG.

GENERAL PROTOCOLS

Deglycosylation occurs optimally under denaturing conditions (using SDS and heat). However it can be performed under native conditions without the addition of detergents.

Keep all enzyme solutions on ice.

Denaturation and PNGase F Digestion

1. Add 25 µl glycoprotein at 1 µg/ml (25 µg total) to a 200 µl tube.
2. Add 2.5 µl of 10X GlycoBuffer 2.
3. Add 5 µl of 10X Glycoprotein Denaturing Buffer.
4. Mix and incubate at 95°C for 5 minutes.
5. Cool on ice for 2 minutes.
6. Add 1 µl PNGase F.
7. Mix and incubate at 37°C for 2 hours.
8. Either remove detergent from reaction using the detergent removal protocol below and analyze by LC-MS or continue with Trypsin Digestion using NEB Trypsin-ultra and the FASP Protein Digestion Kit.

Simultaneous PNGase F & Trypsin Digestion

1. Add 25 µl of glycoprotein at 1 µg/µl (25 µg total) to a 1.5 ml tube.
2. Add 25 µl of 2X Trypsin Buffer.
3. Mix and incubate at 95°C for 5 minutes.
4. Cool on ice for 2 minutes.
5. Add 6 µl of PNGase F.
6. Add 250 ng of Trypsin (1:100 enzyme:substrate).
7. Mix and incubate at 37°C for 3 hours.

Detergent Removal by Acetone Precipitation

Generally, a minimum of 1 µg of protein will produce a visible pellet upon precipitation.

1. Add 50 µl of protein solution to a 1.5 ml microcentrifuge tube.
2. Add 450 µl of acetone to the tube and mix.
3. Place on a dry ice/ethanol slurry (-78°C) for 10 minutes.
4. Centrifuge at 14,000 x g for 20 minutes.
5. Carefully remove and discard the supernatant without disturbing the pellet.
6. Wash the pellet with 100 µl of ice cold 9:1 acetone:water.
7. Place on dry ice/ethanol slurry (-78°C) for 10 minutes.
8. Centrifuge at 14,000 x g for 20 minutes.
9. Carefully remove and discard the supernatant without disturbing the pellet.
10. Remove any remaining supernatant and dry by vacuum centrifugation.
11. Resuspend protein pellet in 1X Trypsin Buffer.

Trypsin Digestion using NEB Trypsin-ultra and FASP Protein Digestion Kit

1. Add 200 µl 8 M Urea/10 mM DTT to 10 µg deglycosylated protein solution. Vortex briefly (See Note 1).
2. Rock at room temperature for 30 minutes.
3. Transfer protein-urea mixture to spin filter (provided in FASP Kit). Centrifuge at 14,000 x g for 10 minutes.
4. Add 200 µl fresh urea solution (no DTT) (See Note 2).
5. Centrifuge at 14,000 x g for 10 minutes.
6. Discard flow-through.
7. Add 10 µl prepared iodoacetamide solution and 90 µl urea solution (no DTT). Incubate without mixing for 20 minutes in the dark (See Note 3).
8. Centrifuge at 14,000 x g for 10 minutes.
9. Add 200 µl urea solution (no DTT). Centrifuge at 14,000 x g for 10 minutes.
10. Discard flow-through.
11. Add 200 µl 50 mM ammonium bicarbonate solution (provided with FASP Kit). Centrifuge at 14,000 x g for 10 minutes.
12. Transfer filter to new collection tube.
13. Add digestion solution. Pipette up and down to mix on top of filter. Incubate at 37°C for 2 hours (no rocking) (See Note 4)
14. Add 80 µl 50 mM ammonium bicarbonate solution. Centrifuge at 14,000 x g for 10 minutes.
15. Add 30 µl 50% acetonitrile/0.1% formic acid. Centrifuge at 14,000 x g for 10 minutes.
16. Add 40 µl 0.1% formic acid/water. Centrifuge at 14,000 x g for 10 minutes.
17. Filtrate contains digested peptides. Total filtrate volume = 150 µl.
18. Analyze the peptides by LC-MS/MS.

NOTES

1. To prepare urea solution: Add 1 ml 100 mM Tris-HCl pH 8.5 (provided with FASP Kit) to one tube of urea (provided with FASP Kit). Vortex until all powder dissolves and add DTT to a 10 mM concentration.
2. To prepare urea solution: Add 1 ml 100 mM Tris-HCl pH 8.5 (provided with FASP Kit) to one tube of urea (provided with FASP Kit). Vortex until all powder dissolves.
3. To prepare iodoacetamide solution: Add 100 µl prepared urea solution (no DTT) to 1 tube iodoacetamide (provided with FASP Kit). Pipette up and down 10-15 times to mix well.
4. To prepare digestion solution: Add 1 ml 50 mM ammonium bicarbonate solution (provided with FASP Kit) to 20 µg Trypsin-ultra (NEB #P8101) to make a 20 ng/µl trypsin solution.
5. Add trypsin solution to top of filter at a 1:50 protein:trypsin ratio, and add 50 mM ammonium bicarbonate solution to bring total digestion solution volume to 100 µl.

RESULTS

Intact Protein MS Data Acquisition

Samples of protein prepared as described above were analyzed by reverse phase liquid chromatography (RPLC) (Figure 2) and electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) (Figure 3). A custom reverse-phase chip, containing an integrated trapping column (40 nl capacity), separation column and nano-ESI emitter (75 µm x 150 mm both packed with PLRP-S, 5 µm particles, 1000 Å pore size) was used for the separation of proteins (1). The chip trap column was loaded at 4 µl/min and the separation column was run at a flow rate of 500 nl/min using an Agilent 1200 series nano LC connected directly to an Agilent 6210 series ESI-TOF MS. The column was equilibrated with 0.1% formic acid in water containing 5% acetonitrile. One to eight microliters of protein sample was injected onto the column and proteins were separated with a gradient of acetonitrile. The acquired spectra were extracted and the protein spectra were deconvoluted.

Figure 2: **PNGase F treated Murine IgG**

Murine IgG-treated with PNGase F, subjected to chromatography, nanoESI and TOF MS. Spectra were deconvoluted and the major peaks were identified.

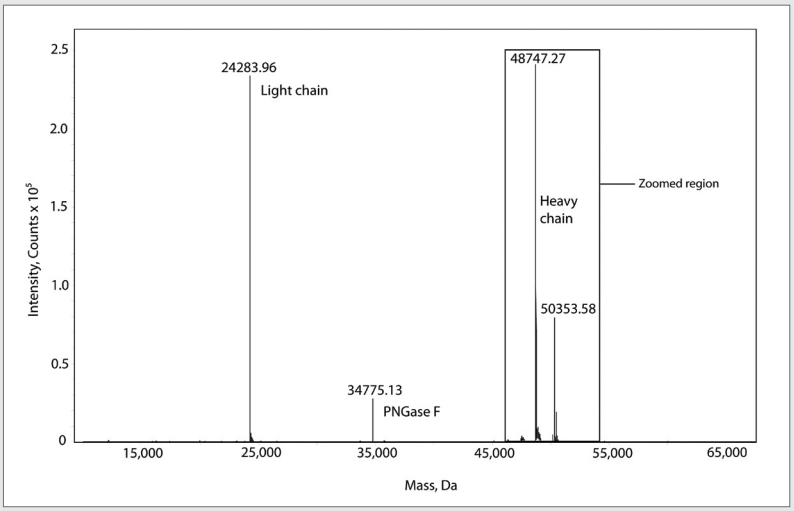
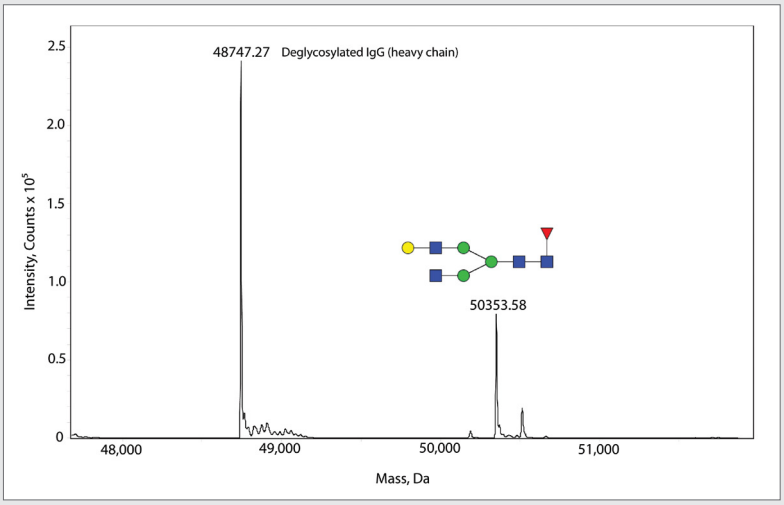


Figure 3: **PNGase F treated Murine IgG Heavy Chain**

The heavy chain region of the above spectra showing the identity and glycan.



The spectra shown are from a PNGase F digestion done under native conditions. The two heavy chain species observed are the glycan-free heavy chain, 48,747.27 Da, and a small amount of remaining glycosylated species, 50,353.58 Da. The most likely structure of the remaining glycan is also shown.

Trypsin Peptide MS and MS/MS Data Acquisition

One microliter (400 ng) of digested sample (simultaneous PNGase F/trypsin digestion) was injected onto a self-packed 20 cm 100 ID analytical column (Aqua 3µ C18 packing material) using a Thermo Scientific (Proxeon) EASY-nLC and separated using a 60 min 5-35% FB linear gradient (FA = 0.1% formic acid, FB = CH_3CN, 0.1% formic acid) at a flow rate of 300 nl/min. Multiply charged peptide ions were automatically chosen during a 30,000 amu resolution scan and fragmented by both CID and ETD in a LTQ Orbitrap XL ETD Mass Spectrometer with a nano-electrospray ionization source (Thermo Scientific). A BSA peptide standard online analysis of trypsin digest of BSA (NEB #P8108) was injected (100 fmol) to test the LC and MS system (see Figure 4,5,6).

Figure 4: **87% sequence coverage of Trypsin-digested BSA MS Standard**

BSA digest solution diluted to 100 fmol/µl with 0.1% formic acid. 1 µl digest solution was injected via a Proxeon Easy n1000 LC System (Thermo Fisher) onto a self-packed C18 column (100 ID x 20 cm, Aqua 3µ C18 packing material). Peptides were separated using a 30 min 5-40% B linear gradient (A = 0.1% formic acid, B = Acetonitrile, 0.1% formic acid) at a flow rate of 400 nl/min and analyzed online by a Q Exactive mass spectrometer (Thermo Fisher) with a nano-electrospray ionization source. Acquisition range was from 400 to 1600 m/z and a source voltage of 2.5 kV was used. 87% sequence coverage was obtained.

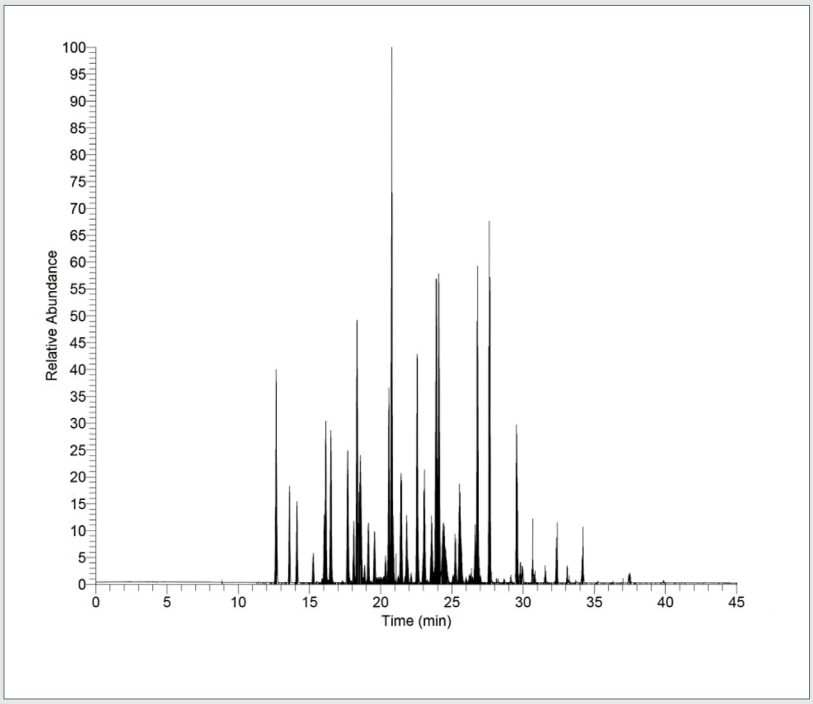
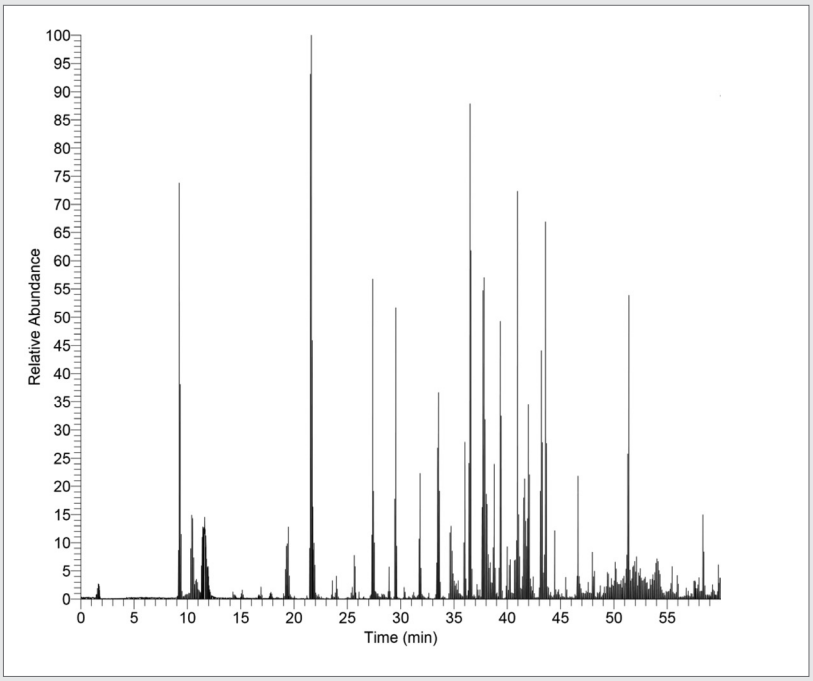
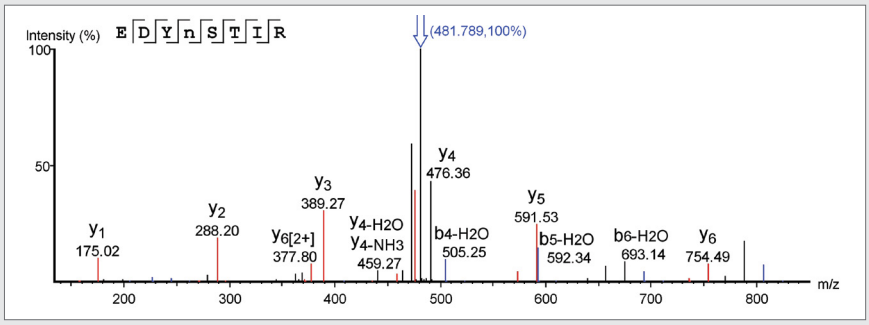


Figure 5: **Online analysis of PNGase F/Trypsin-digested Murine IgG**
LTQ Orbitrap XL base peak chromatogram of 400 ng Murine IgG simultaneously digested with PNGase F & Trypsin.



The MS and MS/MS fragmentation data were analyzed with both Proteome Discoverer™ 1.4 (Thermo Scientific) and PEAKS®7 software. Data was searched using a SwissProt FASTA database. For these analyses, theoretical peptides generated by a tryptic digest with a maximum of two missed cleavages were considered, and the precursor and product mass tolerances were set to ± 10 ppm and ± 0.01 Da, respectively. Variable modifications of asparagine were allowed for (the conversion of asparagine to aspartic acid that occurs when PNGase F removes the glycan). Data was validated using a reverse database decoy search to a false discovery rate of 1%.

Figure 6: **MS/MS spectrum of PNGase F-treated peptide**



CONCLUSION

A search of the data collected from the simultaneous PNGase F/Trypsin digested sample identified a peptide with the characteristic N-X-S/T, with an N to D modification (a mass change of +0.98 amu). The peptide identified was EDYNSTLR from the heavy chain of the murine IgG, and is consistent with a previously observed glycosylation site of murine IgG.

References:

1. Spiro, R.G. (2002) *Glycobiology* 12, 43R-56R.
2. Perkins, D.N., et al. (1999) *Electrophoresis* 20, 3551-3567.

O-Glycosidase

NEB's *Enterococcus faecalis* *O*-Glycosidase, also known as Endo-α-*N*-Acetylgalactosaminidase, catalyzes the removal of core 1 and core 3 disaccharide structures with no modification of the serine or threonine residues. Any modification of the core structures, including sialyation, will block the action of the *O*-Glycosidase. Sialic acid residues are easily removed by a general Neuraminidase. In addition, exoglycosidases such as β(1-4) Galactosidase S, β-*N*-Acetylglucosaminidase S and/or β-*N*-Acetylhexosaminidase$_f$ can be included in deglycosylation reactions to remove other complex modifications often known to be present on the core structures.

REACTION PROTOCOLS

O-Glycosidase Denaturing Reaction Conditions

1. Combine 1–20 µg of glycoprotein, 1 µl of 10X Glycoprotein Denaturing Buffer and H_2O (if necessary) to make a 10 µl total reaction volume.
2. Denature glycoprotein by heating reaction at 100°C for 10 minutes.
3. Make a total reaction volume of 20 µl by adding 2 µl 10X GlycoBuffer 2, 2 µl 10% NP-40, 2 µl α2-3,6,8,9 Neuraminidase A (NEB #P0722), H_2O and 1–5 µl *O*-Glycosidase.
4. Incubate reaction at 37°C for 1 hour.
5. Analyze by method of choice.

O-Glycosidase Non-denaturing Reaction Conditions

1. Combine 1–20 µg of glycoprotein, 2 µl 10X GlycoBuffer 2, 2 µl α2-3,6,8,9 Neuraminidase A (NEB #P0722), H_2O and 1–5 µl *O*-Glycosidase to make a total reaction volume of 20 µl.
2. Incubate reaction at 37°C for 4 hours to overnight.
3. Analyze by method of choice.

Note: *The simplest method of assessing the extent of deglycosylation is by mobility shifts on SDS-PAGE gels. The enzyme can be used under either denaturing or non-denaturing conditions. However, under denaturing conditions the enzyme activity is increased two-fold. This observation is substrate dependent. The reaction may be scaled-up linearly to accommodate large amounts of glycoprotein and larger reaction volumes.*

RNase B/Fetuin Deglycosylation Protocol

1. In a 10 µl reaction, add 2 µl of RNase B or Fetuin, 1 µl of 10X Glycoprotein Denaturing Buffer and 5 µl of H_2O.
2. Incubate at 100°C for 10 minutes.
3. Add 1 µl of 10X GlycoBuffer 2 and 1 µl of 10% NP-40.
4. Add 1 µl of endoglycosidase.
5. Incubate at 37°C for 1 hour.
6. Visualize reaction products on 10–20% SDS-PAGE gel.

FREQUENTLY ASKED QUESTIONS

Q. Can *O*-Glycosidase, Neuraminidase and PNGase F be used together in a simultaneous digest?

A. Yes. The *O*-Glycosidase reaction uses the same reagents as PNGase F (10X GlycoBuffer 2, and if denaturing, 10X Glycoprotein Denaturing Buffer and 10% NP-40). In addition, α2-3,6,8 Neuraminidase and α2-3,6,8,9 Neuraminidase A are compatible with denaturing conditions, have broad pH ranges, and retain full activity in 10X GlycoBuffer 2.

COMPANION PRODUCTS

Fetuin

Fetuin (NEB #P6042) is a glycoprotein containing sialylated *N*-linked and *O*-linked glycans that can be used as a positive control for endoglycosidase enzymes.

Endoglycosidase Reaction Buffer Pack

The Endoglycosidase Reaction Buffer Pack (NEB #B0701) contains 1 ml of every buffer necessary for optimal activity of a deglycosylation reaction including 10X GlycoBuffer 2, 10X GlycoBuffer 3, 10X Glycoprotein Denaturing Buffer and 10% NP-40.

Rapid PNGase F Antibody Standard

Rapid PNGase F Antibody Standard (NEB #P6043) is a murine anti-MBP monoclonal antibody, isotype IgG2a. It is comprised of two heavy chains which are each approximately 49 kDa, as well as two light chains which are each approximately 24.4 kDa. This antibody standard can be used as a positive control for Rapid PNGase F.

RNase B

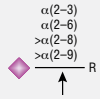
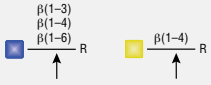
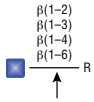
RNase B (NEB #P7817) is a high mannose glycoprotein that can be used as a positive control for endoglycosidases that cleave *N*-linked carbohydrates. RNase B has a single *N*-linked glycosylation site which makes it ideal for SDS-PAGE gel shift assays. It has an intact molecular weight of 17,000 daltons, and a molecular weight of 13,683 daltons after deglycosylation.

Reference
1. Koutsioulis, D., Landry, D. and Guthrie, E.P. (2008) *Glycobiology* 18, 799–805.

Exoglycosidase Enzymes

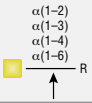
NEB offers a wide selection of exoglycosidases for glycobiology research. Exoglycosidases cleave a monosaccharide from the non-reducing end of an internal glycosidic linkage in an oligosaccharide or polysaccharide. Many of these reagents are recombinant, and all undergo several quality control assays, enabling us to provide products with lower unit cost, high purity, and reduced lot-to-lot variation. All of our glycosidases are tested for contaminants. Since *p*-nitrophenyl-glycosides are not hydrolyzed by some exoglycosidases, we use only fluorescently-labeled oligosaccharides to assay activity and screen for contaminating glycosidases.

EXOGLYCOSIDASE SELECTION CHART

EXOGLYCOSIDASE	NEB #	SOURCE	SPECIFICITY
α2-3,6,8 Neuraminidase	NEB #P0720	*Clostridium perfringens*	
α2-3,6,8,9 Neuraminidase A	NEB #P0722	*Arthrobacter ureafaciens*	
α2-3 Neuraminidase S	NEB #P0743	*Streptococcus pneumoniae*	
β-*N*-Acetylhexosaminidase$_f$	NEB #P0721	*Streptomyces plicatus*	
β-*N*-Acetylglucosaminidase S	NEB #P0744	*Streptococcus pneumoniae*	
α1-2 Fucosidase	NEB #P0724	*Xanthamonas manihotis*	
α1-2,4,6 Fucosidase O	NEB #P0749	*Omnitrophica bacterium*	
α1-3,4 Fucosidase	NEB #P0769	Almond meal	
β1-3 Galactosidase	NEB #P0726	*Xanthamonas manihotis*	
β1-4 Galactosidase S	NEB #P0745	*Streptococcus pneumoniae*	
β1-3,4 Galactosidase	NEB #P0746	Bovine testis	
α1-6 Mannosidase	NEB #P0727	*Xanthamonas manihotis*	
α1-2,3 Mannosidase	NEB #P0729	*Xanthamonas manihotis*	
α1-2,3,6 Mannosidase	NEB #P0768	Jack Bean	

Gal Glc Man GalNAc GlcNAc Fuc NeuAc **R** = any sugar

EXOGLYCOSIDASE SELECTION CHART (CONT.)

EXOGLYCOSIDASE	NEB #	SOURCE	SPECIFICITY
α1-3,6 Galactosidase	NEB #P0731	*Xanthamonas manihotis*	
α1-3,4,6 Galactosidase	NEB #P0747	Green coffee bean	
α-*N*-Acetylgalactosaminidase	NEB #P0734	*Chryseobacterium meningosepticum*	

FREQUENTLY ASKED QUESTIONS

Q. What is the difference between the three Neuraminidase enzymes sold by NEB: α2-3,6,8 Neuraminidase, α2-3,6,8,9 Neuraminidase A and α2-3 Neuraminidase S?

A. 2-3,6,8 Neuraminidase (NEB #P0720) is cloned from *Clostridium perfringens* and cleaves α2-3, α2-6 and α2-8 linked sialic acid residues. α2-3,6,8,9 Neuraminidase A (NEB #P0722) is cloned from *Arthrobacter ureafaciens* and cleaves α2-3, α2-6, α2-8 and α2-9 linked sialic acid residues. It can also cleave branched sialic acid residues that are linked to an internal residue. α2-3 Neuraminidase S (NEB #P0743) is cloned from *Streptococcus pneumoniae* and cleaves only α2-3 linked sialic acid residues.

Q. Do the NEB Neuraminidase enzymes cleave both *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) residues?

A. Depending on the enzyme source and specificity, some neuraminidases cleave both Neu5Ac and Neu5Gc, while some cleave only Neu5Ac. Those that cleave both Neu5Ac and Neu5Gc, tend to have lower efficiency towards Neu5Gc.

α2-3,6,8 Neuraminidase (NEB #P0720) – cleaves both Neu5Ac and Neu5Gc

α2-3,6,8,9 Neuraminidase A (NEB #P0722) – cleaves both Neu5Ac and Neu5Gc

α2-3 Neuraminidase S (NEB #P0743) – cleaves only Neu5Ac

Q. Which exoglycosidase, β-*N*-Acetylhexosaminidase$_f$ or β-*N*-Acetylglucosaminidase S is recommended for the digestion of monoclonal antibodies?

A. *N*-glycans released from common monoclonal antibodies often do not contain terminal GalNAc residues, but instead contain terminal GlcNAc residues in both G0F and G1F, as well as other minor forms. β-*N*-Acetylhexosaminidase$_f$ (NEB #P0721) does **not** cleave GlcNAc residues from G0F or G1F as the enzyme is not able to cleave a non-linear substrate (i.e. *N*-glycans with multiple antennae). However, β-N-Acetylglucosaminidase S (NEB #P0744) can cleave these terminal GlcNAc residues to completion in a 1-hour reaction.

Q. What is the difference between α1-2 Fucosidase and α1-2,4,6 Fucosidase O?

A. α1-2 Fucosidase (NEB #P0724) is cloned from *Xanthomonas manihotis* and is specific for the cleavage of only linear α1-2 fucose residues. α1-2,4,6 Fucosidase O (NEB #P0749) is cloned from *Omnitrophica* bacterium and expressed in *E. coli* and catalyzes the hydrolysis of α1-6, α1-2, and at a lower rate α1-4 linked fucose residues. α1-2,4,6 Fucosidase O is recommended for use in *N*-glycan sequencing arrays as it is compatible with traditional reductive amination labels (2-AB, procainamide) and instant labels.

TECHNICAL TIPS

Fucosidase

- α1-2 Fucosidase is active on only linear α1-2 linked fucose residues
- α1-2,4,6 Fucosidase O can cleave substrates with instant labels

Q. What is the difference between α1-2,3 Mannosidase, α1-6 Mannosidase and α1-2,3,6 Mannosidase?

A. α1-2,3 Mannosidase (NEB #P0729) and α1-6 Mannosidase (NEB #P0727) are cloned from *Xanthomonas manihotis*, and α1-2,3,6 Mannosidase (NEB #P0768) is cloned from *Canavalia ensiformis* (jack bean). α1-2,3 Mannosidase cleaves only α1-2 and α1-3 linked mannose residues, and α1-6 Mannosidase cleaves only α1-6 linked mannose residues. Whereas, α1-2,3,6 Mannosidase (JBM) cleaves all three α1-2, α1-3 and α1-6 linked mannose residues.

Q. When digesting complex substrates that include α1-6 mannose residues, should multiple mannose enzymes be used in combination?

A. Because α1-2,3,6 Mannosidase has a slightly reduced activity towards α1-6 mannose residues (in comparison to α1-2 and α1-3 mannose residues) it may be advantageous to use the enzyme in combination with α1-6 Mannosidase (NEB #P0727). A sequential digest is recommended, in which digestion with α1-2,3,6 Mannosidase is followed by α1-6 Mannosidase (NEB #P0727) treatment

Q. What is the difference between α1-3,6 Galactosidase and α1-3,4,6 Galactosidase?

A. α1-3,6 Galactosidase (NEB #P0731) is cloned from *Xanthomonas manihotis* and is specific for the cleavage of only α1-3 and α1-6 galactose residues. It can also be used for the removal of the antigenic Galα1,3Gal epitope from glycoproteins. Whereas, α1-3,4,6 Galactosidase (NEB #P0747) is cloned from green coffee bean and is a broader specificity enzyme that catalyzes the hydrolysis of α1-3, α1-4, and α1-6 linked galactose residues.

TECHNICAL TIPS

α1-2,3,6 Mannosidase

- The optimal pH for α1-2,3,6 Mannosidase is 4.5. This differs from the majority of exoglycosidases, which have an optimal pH at 5.5.
- α1-2,3,6 Mannosidase activity is enhanced by the presence of 2 mM Zn^{2+} in the reaction.

REACTION PROTOCOLS

Exoglycosidase Reaction Protocol

1. Combine 10 µg of glycoprotein or 100 nM of oligosaccharide and H_2O (if necessary) in a total reaction volume of 9 µl.
2. Add 1 µl of 10X GlycoBuffer 1 to make a 10 µl total reaction volume.
3. Add 1 µl exoglycosidase enzyme.
4. Incubate at 37°C for 1 hour.
5. Analyze by method of choice.

Exoglycosidase Reaction with BSA Protocol

1. Combine 10 µg of glycoprotein or 100 nM of oligosaccharide and H_2O (if necessary) in a total reaction volume of 8 µl.
2. Add 1 µl of 10X GlycoBuffer 1 and 1 µl of 10X BSA (diluted 1:10 from 100X concentration) to make a 10 µl total reaction volume.
3. Add 1 µl exoglycosidase enzyme.
4. Incubate at 37°C for 1 hour.
5. Analyze by method of choice.

Note: *Some exoglycosidase enzymes require a buffer other than GlycoBuffer 1. Use the optimal buffer(s) supplied with each individual enzyme.*

The amount of exoglycosidase enzyme required varies when different substrates are used. Start with 1–2 µl of exoglycosidase for 10–50 µg of glycoprotein or 1 nmol of oligosaccharide. If after a 1 hour incubation there is still undigested material, let the reaction go overnight.

The protocol can be scaled up linearly to accommodate more substrate and larger reaction volumes. It is imperative to increase the amount of enzyme as you increase the amount of substrate.

Exoglycosidases can be used concomitantly with other endo- and exoglycosidases for double digest reactions. Most exoglycosidase double digests can be done using 1X GlycoBuffer 1 (50 mM NaAcetate, pH 5.5, 5 mM $CaCl_2$). Whereas, endoglycosidase reactions coupled with exoglycosidases should be done using 1X GlycoBuffer 2 (50 mM sodium phosphate, pH 7.5). First digest your glycoprotein with PNGase F, Endo H or O-Glycosidase, heat-kill the enzyme at 65°C for 10 minutes, and then treat with the exoglycosidase in the same 1X GlycoBuffer 2.

FREQUENTLY ASKED QUESTIONS

Q. Do detergents inhibit exoglycosidases/endoglycosidases?

A. At moderate levels (0.5-1.0% ionic and non-ionic detergents) most of the glycosidases show satisfactory activity or are unaffected. Exceptions are: PNGase F (all formulations), *O*-Glycosidase, and β-N-Acetylglucosaminidase S, which are inhibited by SDS. It is imperative to add NP-40 to a final concentration of 1% to your endoglycosidase reaction mixture in order to counteract detergent inhibition.

Q. Do exoglycosidases require denaturing conditions to act on glycoproteins?

A. No, in general exoglycosidases can remove sugar residues from native (folded) glycoproteins (hydrophilic glycans face away from the protein backbone). However, the protein folding around a glycan site might affect enzyme accessibility. Exoglycosidase digestion of some proteins will require longer incubation times, or in some cases a mild extent of denaturation.

Q. What is a good method to re-purify a glycan or glycopeptide after exoglycosidase treatment?

A. Filtration with micro-spin filters, or solid-phase extraction (i.e. graphitized carbon or HILIC cartridges).

GLYCOBUFFER COMPOSITIONS

1X GlycoBuffer 1:
50 mM sodium acetate (pH 5.5 @ 25°C), 5 mM $CaCl_2$

1X GlycoBuffer 2:
50 mM sodium phosphate (pH 7.5 @ 25°C)

1X GlycoBuffer 3:
50 mM sodium acetate (pH 6.0 @ 25°C)

1X GlycoBuffer 4:
50 mM sodium acetate (pH 4.5 @ 25°C)

Detailed Characterization of Antibody Glycan Structure using Exoglycosidases for *N*-Glycan Sequencing

Beth McLeod, New England Biolabs, Inc.

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**

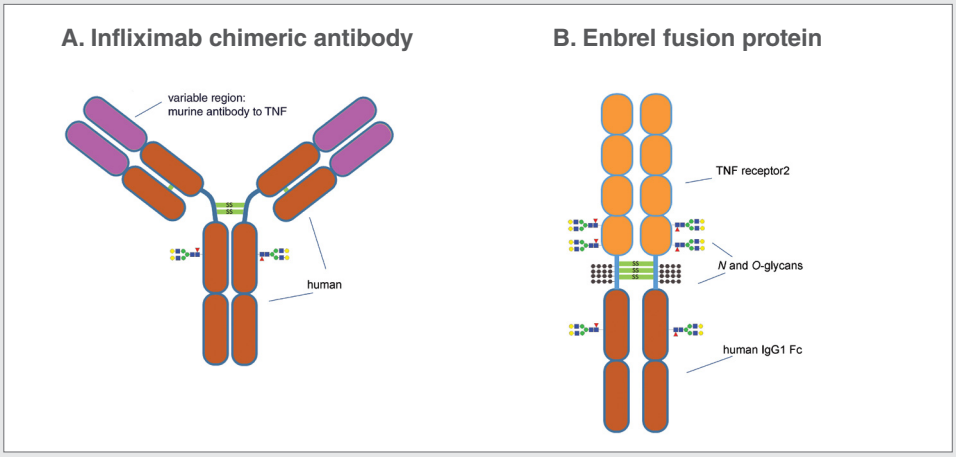
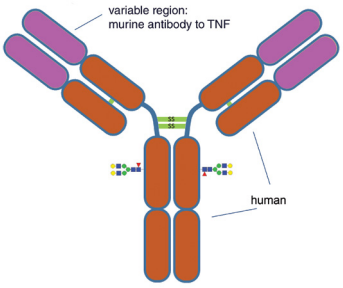
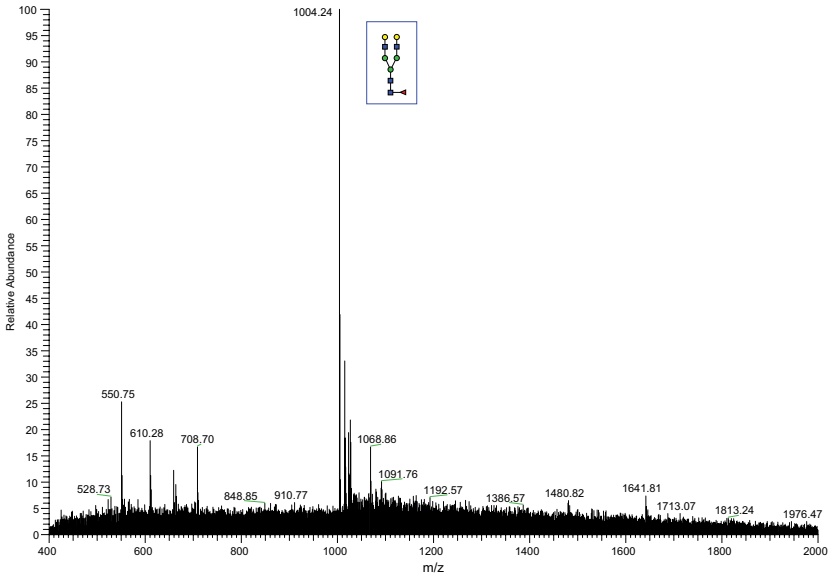


FIGURE 2: **Infliximab glycan profile**

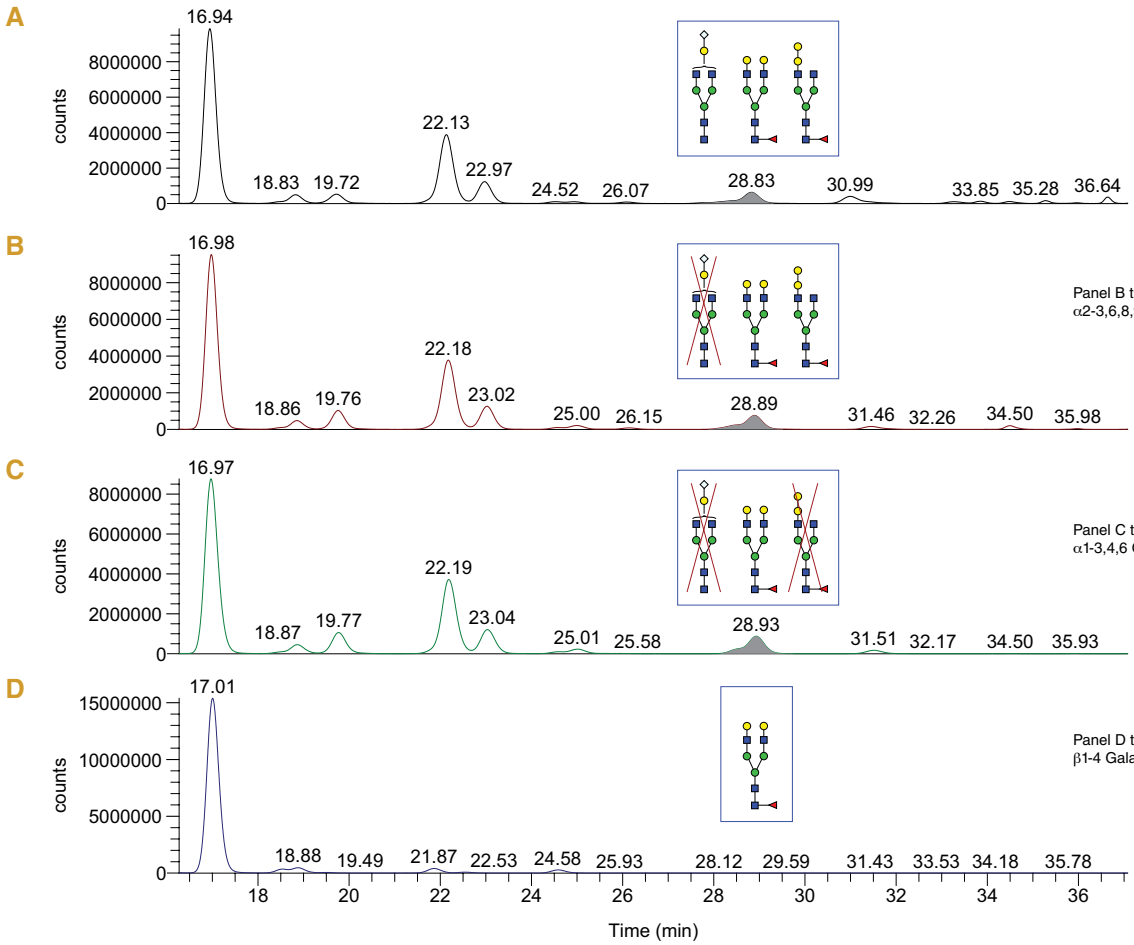
A. Infliximab chimeric antibody



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 PROTOCOLS

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 acetonitrile concentration of 70%
4. Samples are now ready for LC or LC-MS analysis. In this experiment, *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

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
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 acidified PCA or 2-AB solution by adding one volume of glacial acetic acid to eight volumes of PCA or 2AB stock solution.
3. SPE buffer: 200 mM Ammonium Acetate.
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.

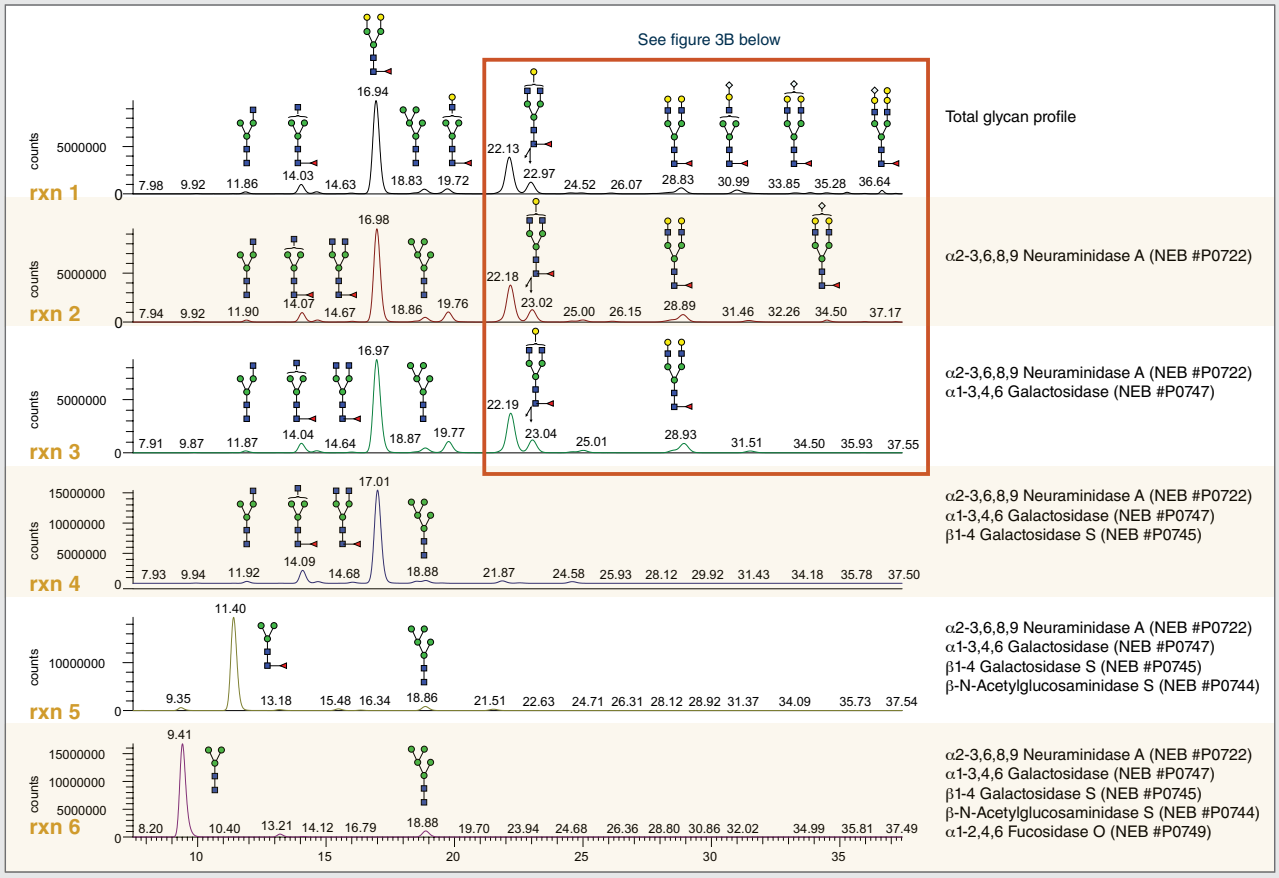
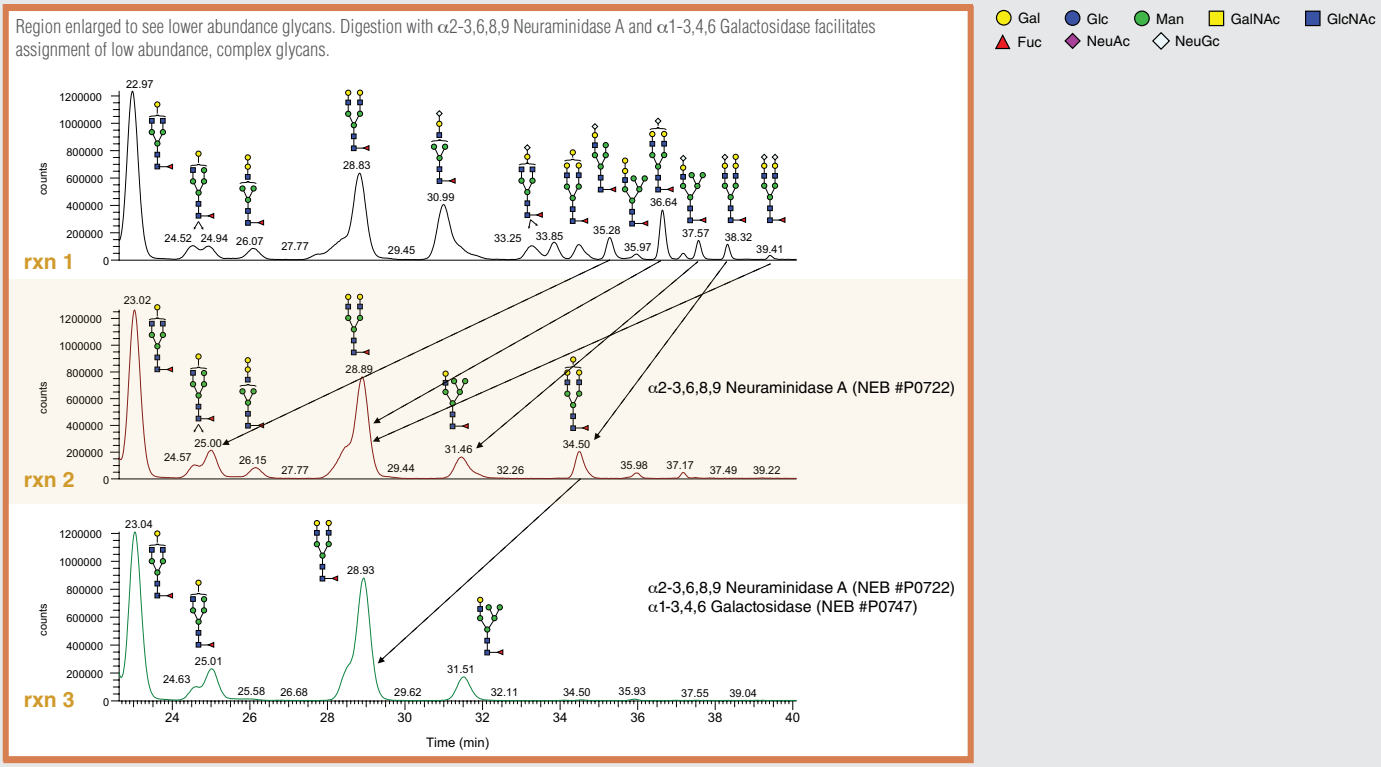


FIGURE 3B: **Expanded lower abundance profile of Infliximab glycan analysis**



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.

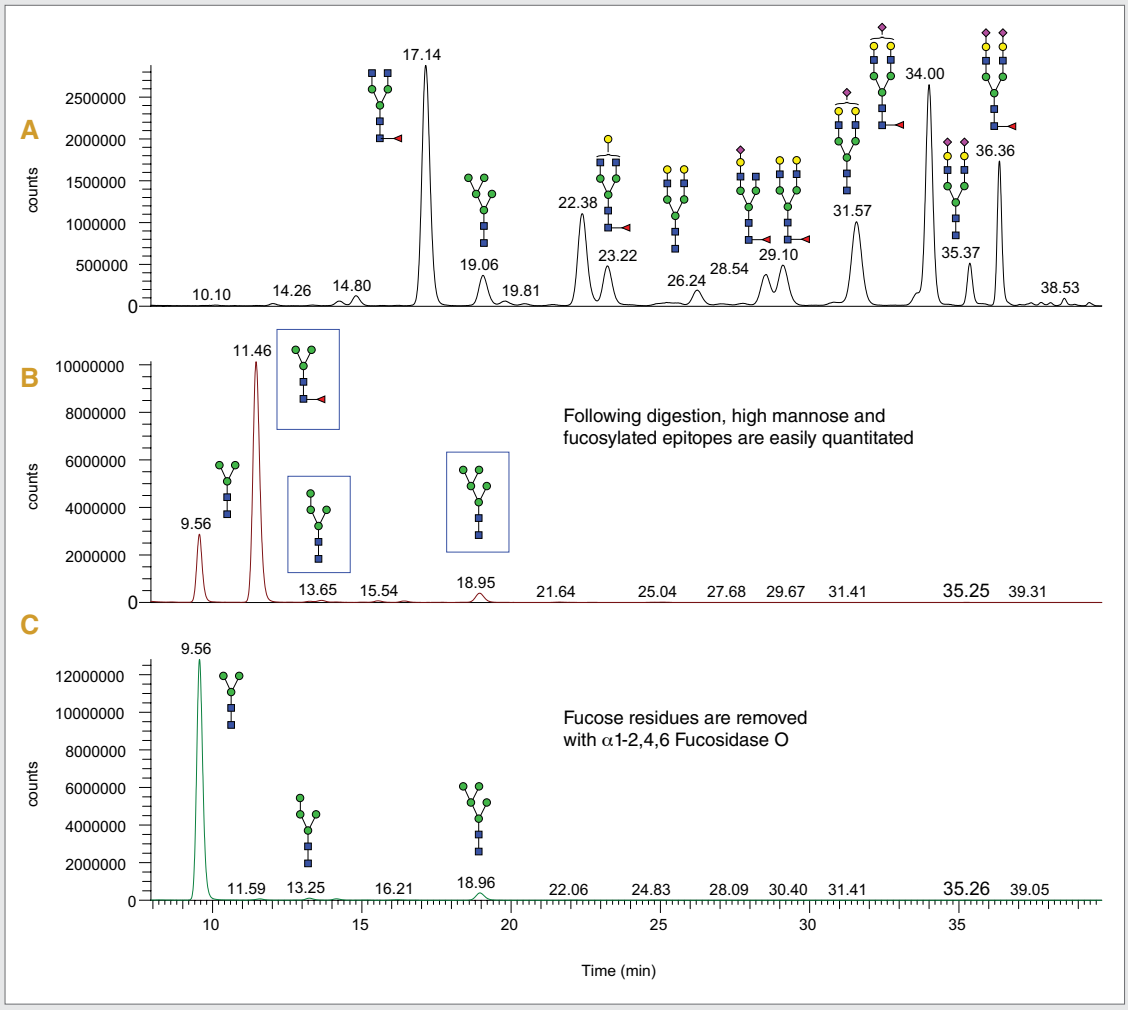


FIGURE 5: **Quantitation of fucosylation and high mannose structures.**

% 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.

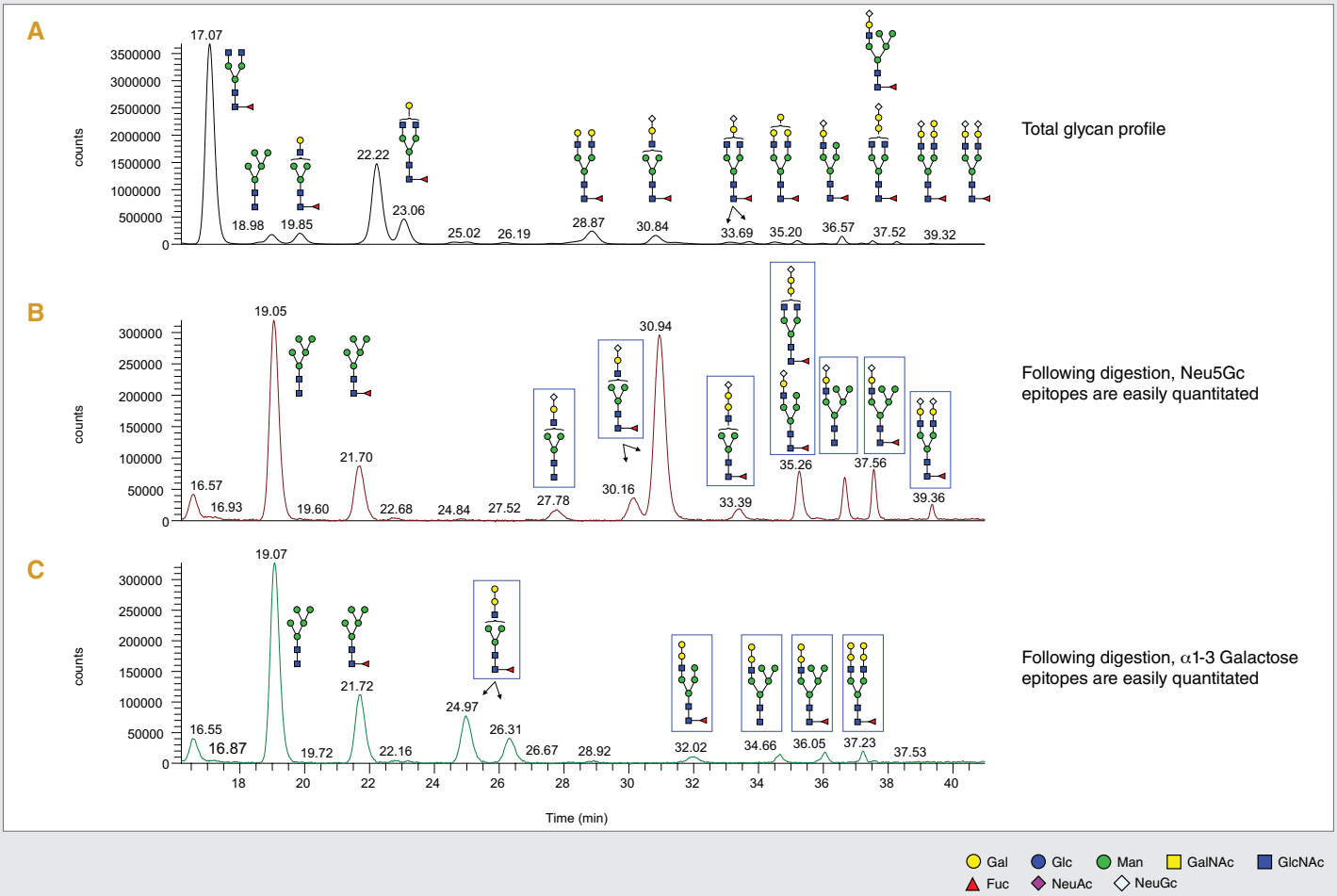


FIGURE 7: **Quantitation of Neu5Gc and α1-3 Galactose epitopes.**

% 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.

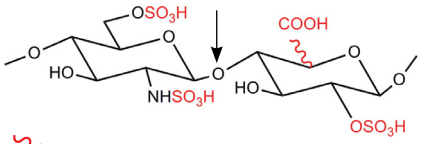
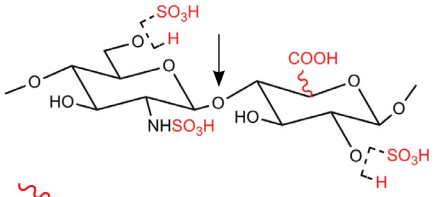
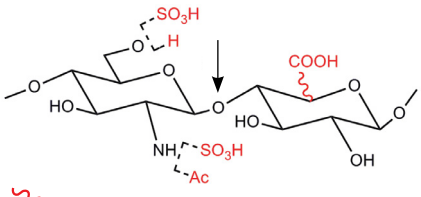
Heparin Lyase Enzymes

Heparin Lyase enzymes, also called Heparinases, are enzymes that cleave the glycosidic linkage between hexosamines and uronic acids and are known to cleave heparin and heparin sulfate (HS) chains selectively, via an elimination mechanism. Heparinase enzymes create a double bond on the non-reducing end of the uronic acid that absorbs at 232 nm and can be used for the detection of oligosaccharide and disaccharide products. Three Heparinase enzymes are available: *Bacteroides* Heparinase I, Heparinase II and Heparinase III. Heparinase I cleaves highly sulfated heparin/HS chains, Heparinase III cleaves less sulfated HS chains, while Heparinase II cleaves domains of both high and low sulfation on both heparin and HS. Heparinase I, II and III used in combination can produce a near-complete depolymerization of heparin/HS polysaccharide chains to disaccharides.

TECHNICAL TIPS

- Avoid repeated freeze-thaw cycles. It is recommended to aliquot the enzyme and store at -80°C.
- *Bacteroides* Heparinase I, II and III are most active between pH 6.5-7.5
- *Bacteroides* Heparinase I, II and III are calcium dependent enzymes and have optimal activity in the presence of 1.5-5 mM $CaCl_2$.

HEPARIN LYASE ENZYMES SELECTION CHART

HEPARINASES	NEB #	DESCRIPTION	SPECIFICITY
***Bacteroides* Heparinase I**	NEB #P0735	Cloned from *Bacteroides eggerthii*, also called Heparin Lyase I, is active on heparin and the highly sulfated domains of heparan sulfate. The reaction yields oligosaccharide products containing unsaturated uronic acids which can be detected by UV spectroscopy at 232 nm.	 Denotes either glucuronic acid or iduronic acid. All structural determinants for enzyme specificity are displayed in red.
***Bacteroides* Heparinase II**	NEB #P0736	Cloned from *Bacteroides eggerthii*, also called Heparin Lyase II, is a low specificity enzyme that is active on both heparin and heparan sulfate. The reaction yields oligosaccharide products containing unsaturated uronic acids which can be detected by UV spectroscopy at 232 nm.	 Denotes either glucuronic acid or iduronic acid. All structural determinants for enzyme specificity are displayed in red.
***Bacteroides* Heparinase III**	NEB #P0737	Cloned from *Bacteroides eggerthii*, also called Heparin Lyase III, is active on both heparin and heparan sulfate. The reaction yields oligosaccharide products containing unsaturated uronic acids which can be detected by UV spectroscopy at 232 nm.	 Denotes either glucuronic acid or iduronic acid. All structural determinants for enzyme specificity are displayed in red.

FREQUENTLY ASKED QUESTIONS

Q. Can *Bacteroides* Heparinase I, II, and III be used together in one digest?

A. Yes, all three Heparinase enzymes are supplied with the same Heparinase Reaction Buffer (1X formulation: 20 mM Tris-HCl, pH 7.0, 100 mM NaCl, 1.5 mM $CaCl_2$), and have optimal incubation temperatures of 30°C.

REACTION PROTOCOL

Heparinase Reaction Protocol

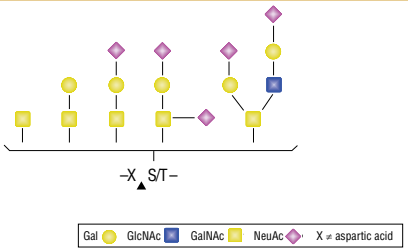
1. Combine 10 µl of 1 mg/ml heparin or heparan sulfate substrate, 10 µl Heparinase Reaction Buffer and H_2O in a total reaction volume of 100 µl.
2. Add 1 µl *Bacteroides* Heparinase enzyme(s).
3. Incubate reaction at 30°C for 1–24 hours (monitor absorbance at 232 nm for determination of partial or complete digestion).

   Note: Optimal incubation times and enzyme concentrations must be determined empirically for a particular substrate.

Glycoproteomics

NEB offers a selection of high purity proteases and standards for use in concert with our glycosidases for proteomic and glycoproteomic applications. Our proteases are of the highest quality, show no lot-to-lot variation and are offered at exceptional value.

GLYCOPROTEOMICS SELECTION CHART

PRODUCT	NEB #	DESCRIPTION	SPECIFICITY
O-Glycoprotease (IMPa)	NEB #P0761	O-Glycoprotease (IMPa), also known as Immunomodulating protease (IMPa), is a broad specificity O-glycoprotease from Pseudomonas aeruginosa. It recognizes mucin-type O-glycans in glycoproteins or glycopeptides, including branched and sialylated structures, and cleaves the peptide bond immediately *N*-terminal to a glycosylated serine or threonine residue. It can be used in glycoproteomics workflows to map O-glycosylated residues and structurally profile O-glycans present at each glycosite. O-Glycoprotease (IMPa) has a 6xHis-tag for easy removal from a reaction using nickel affinity resins.	
IdeZ Protease (IgG-specific)	NEB #P0770	A recombinant antibody specific protease cloned from *Streptococcus equi* subspecies *zooepidemicus* that recognizes all human, sheep, monkey, and rabbit IgG subclasses, specifically cleaving at a single recognition site below the hinge region, yielding a homogenous pool of $F(ab')_2$ and Fc fragments. IdeZ Protease has significantly improved activity against murine IgG2a and IgG3 subclasses compared to IdeS Protease.	human IgG1, IgG3, IgG4: CPAPELLG▼GPSVF human IgG2: CPAPPVA▼GPSVF murine IgG2a: CPAPNLLG▼GPSVF murine IgG3: CPPGNILG▼GPSVF
Trypsin-ultra,™ Mass Spectrometry Grade	NEB #P8101	A serine endopeptidase isolated from Bovine pancreas. Trypsin is the most widely used enzyme in proteomics. It selectively cleaves peptide bonds C-terminal to lysine and arginine residues. Trypsin-ultra is treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) to inactivate any remaining chymotryptic activity. It is modified by acetylation of the ε-amino groups of lysine residues to prevent autolysis. Trypsin-ultra cleaves at Lys-Pro and Arg-Pro bonds at a much slower rate than when Lys and Arg are N-terminal to other residues.	Xxx-Arg▼Xxx Xxx-Lys▼Xxx
α-Lytic Protease	NEB #P8113	α-Lytic Protease (aLP) cleaves after Threonine (T), Alanine (A), Serine (S) and Valine (V) residues. Its specificity makes it an orthogonal and alternative protease to others commonly used in proteomics applications, including trypsin and chymotrypsin. Peptides generated by aLP are of similar average length to those of Trypsin.	XX-T/A/S/V▼XX
Endoproteinase LysC	NEB #P8109	A serine endoproteinase, isolated from *Lysobacter enzymogenes*, that cleaves peptide bonds at the carboxyl side of lysine. LysC is a sequencing grade enzyme and is suitable for proteomics and glycobiology applications.	XX-Lys▼XXX
Endoproteinase AspN	NEB #P8104	A zinc metalloendopeptidase which selectively cleaves peptide bonds N-terminal to aspartic acid residues.	
Endoproteinase GluC	NEB #P8100	A serine proteinase which preferentially cleaves peptide bonds C-terminal to glutamic acid residues. Endoproteinase GluC also cleaves at aspartic acid residues at a rate 100-300 times slower than at glutamic acid residues. *Staphylococcus aureus* Protease V8 gene was cloned and expressed in *Bacillus subtilis*. Endoproteinase GluC is observed as a single band on SDS-PAGE and TOF-MS and is intended for use in mass spectrometry and protein sequencing.	XX▼Asp-XXX
Trypsin-digested BSA MS Standard (CAM-modified)	NEB #P8108	A complex mixture of peptides produced by the tryptic digest of Bovine Serum Albumin (BSA) that has been reduced and alkylated with iodacetamide (CAM modified). This peptide mixture is free of salts, glycerol and detergents and can therefore be used to standardize Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) or Electrospray Ionization (ESI) mass spectrometers (TOF, Q-TOF, Ion Trap, or Orbitrap) using a standardization range of 500-2400 Da.	Xxx-Glu▼Xxx Xxx-Asp▼Xxx

REACTION PROTOCOLS

O-Glycoprotease (IMPa) Non-denaturing Reaction Protocol

Optimal incubation times and enzyme concentrations must be determined empirically for a particular substrate. Typical reaction conditions are as follows:

1. Combine 10 µg of glycoprotein or glycopeptide in 20 mM Tris-HCl pH 8.0 to a total reaction volume of 50 µl.
2. Add 1 µl of O-Glycoprotease (IMPa), mix gently.
3. Incubate at 37°C for 2 hours.

Note: *Some substrates may require extended incubation periods (up to 24 hours at 37°C).*

O-Glycoprotease (IMPa) Denaturing Reaction Protocol

1. Combine 10 µg of glycoprotein in 20 mM Tris-HCl pH 8.0, 40 mM DTT, 0.1% SDS to a total reaction volume of 50 µl.
2. Denature glycoprotein by heating reaction at 95°C for 10 minutes.
3. Cool the reaction to room temperature.
4. Purify the denatured glycoprotein using HiPPR Detergent Removal Resin (Thermo catalog #88306), according to manufacturer's recommended protocol. Recommended equilibration buffer: 20 mM Tris-HCl pH 8.0 or 20 mM HEPES pH 8.0.

Note: *Any detergent removal spin column of choice may be used.*

5. To the detergent-free denatured glycoprotein, add 1 µl of O-Glycoprotease (IMPa), mix gently.
6. Incubate at 37˚C for 2 hours.

Note: *Some substrates may require extended incubation periods (up to 18 hours at 37°C).*

FREQUENTLY ASKED QUESTIONS

Q. Is it necessary to first remove sialic acids from the substrate prior to digest with O-Glycoprotease (IMPa)?

A. No, O-Glycoprotease (IMPa) is fully active on sialylated glycoproteins and glycopeptides.

Q. Is O-Glycoprotease (IMPa) active on TF-antigen and Tn-antigen?

A. O-Glycoprotease (IMPa) efficiently cleaves peptides containing smaller asialylated glycans such as TF-antigen (Galβ1,3GalNAc-α-) and Tn-antigen (GalNAc-α-).

Reaction Conditions for IdeZ Protease (IgG-specific)

Optimal incubation times and enzyme concentrations may need to be determined for a particular IgG. Typical reaction conditions are as follows:

1. Combine 50 µg of native IgG, 2.5 µl of 10X GlycoBuffer 2 and H_2O (if necessary) to make a 25 µl total reaction volume.
2. Add 1 µl of IdeZ Protease (IgG-specific).
3. Incubate at 37°C for 30 minutes.

Note: *Extend incubation time to 2 hours for mouse IgG2a*

Reaction Conditions for Simultaneous Digestion of IgG with IdeZ protease (IgG-specific) and PNGase F (Fragmentation and Deglycosylation)

Optimal PNGase F units may vary for a particular antibody. Typical reaction conditions are as follows:

1. Combine up to 50 µg of human IgG and H_2O to a volume of 25 µl.
2. Add 2.5 µl of 10X GlycoBuffer 2.
3. Add 1 µl (500 units) of PNGase F Glycerol Free (NEB #P0709) and 1 µl (80 units) of IdeZ Protease (IgG-specific).
4. Incubate 2 hours at 37°C.

FREQUENTLY ASKED QUESTIONS

Q. Can Protein A Magnetic Beads be used to create Fc and Fab fragment pools after cleavage with IdeZ Protease?

A. Yes, Protein A Magnetic Beads (NEB #S1425) can be used to create Fc and Fab fragment pools after cleavage with IdeZ Protease. The Fc fragment will bind to the beads and the Fab fragment will remain in the supernatant.

Reaction Conditions for Endoproteinase LysC

For difficult to digest proteins or general proteomics applications, substrate may be reduced, denatured and alkylated prior to LysC digest.

1. Combine 10-100 ng/µl substrate protein, 10 mM DTT, 30 mM iodoacetic acid and 5-8 M urea.
2. Incubate in the dark 30 minutes.
3. Add LysC; 1:20–1:100 LysC : Substrate ratio by mass.
4. Incubate reaction at 37°C for 2-18 hours.

In-gel Digestion Protocol for Endoproteinase LysC

1. Slice out the protein of interest from a band of an SDS-PAGE gel.
2. Dehydrate the gel slice by adding 300 µl acetonitrile and mixing for 30 minutes at room temperature.
3. Remove acetonitrile and vacuum dry 15 minutes.
4. Add 100 µl of 10 mM DTT in 100 mM ammonium bicarbonate.
5. Incubate 1 hour at 50°C.
6. Remove DTT solution and add 100 µl of 50 mM Iodoacetamide.
7. Incubate the gel piece for 45 minutes in the dark with intermittent gentle mixing.
8. Wash the gel piece for 10 minutes with 100 µl of 100 mM ammonium bicarbonate.
9. Dehydrate the gel slice with acetonitrile for ~15 minutes.
10. Add 100 ng Endoproteinase LysC in 100-300 µl 50 mM Tris-HCl, pH 8.5.
11. Desalt and purify peptides with C18 ZipTips prior to MS analysis.

FREQUENTLY ASKED QUESTIONS

Q: How much Endoproteinase LysC is required to digest a protein?

A: A LysC : substrate ratio within 1:50-100 is recommended. However, significant digestion products have been observed using a ratio as low as 1:250. A database search revealed 65% sequence coverage for the latter and > 80% coverage for the former.

Q: Can Endoproteinase LysC be used in a double digest with Trypsin-ultra?

A: Trypsin-LysC co-digests have been found to improve protein coverage in proteomics studies especially among low abundance proteins. Many different conditions have been used successfully. A typical sequential protocol for Trypsin-LysC digest starts with a LysC digest in 8 M urea buffer for 4 hours at 37°C, followed by dilution to 1.5 M urea for a trypsin digest for 4 hours at 37°C. Both digests should use a 1:20-50 protease to substrate protein ratio.

REACTION PROTOCOLS

α-Lytic Protease Typical Reaction Protocol

Reactions may be scaled-up linearly to accommodate larger amounts of substrate and larger reaction volumes. Typical reaction conditions are as follows:

1. Combine 2 µg substrate and 50 mM ammonium bicarbonate pH 8.5 to make a 10 µl total reaction volume.
2. Add α-Lytic Protease in a 1:20 –1:100 (α-Lytic Protease : substrate) ratio by mass.

*Note: For example, add 20–100 ng α-Lytic Protease to 2 µg substrate protein

3. Incubate at 37°C for 1 hour.

*Note: longer incubations (18 hours) or more enzyme may be required, especially at lower temperatures, for complete digestion.

α-Lytic Protease In-gel Digestion Protocol

1. Excise the band from an SDS-PAGE gel corresponding to the protein of interest. Cut the gel slice into three 1 mm pieces and transfer them to a 1.5 ml microcentrifuge tube.
2. Dehydrate the gel slices for 5 minutes in 200 µl of acetonitrile : 50 mM ammonium bicarbonate (1:1 v/v) with intermittent vortex mixing. Discard supernatant.
3. Add 200 µl of acetonitrile, vortex mix for 30 seconds. Discard the supernatant.
4. Vacuum dry for 5 minutes.
5. Add 100 µl of fresh 25 mM DTT in 50 mM ammonium bicarbonate.
6. Incubate for 20 minutes at 55°C.
7. Remove DTT solution and add 100 µl of fresh 50 mM iodoacetamide in 50 mM ammonium bicarbonate.
8. Incubate the gel pieces for 20 minutes at room temperature with intermittent gentle mixing. Minimize exposure of the reaction mixture to light.
9. Remove iodoacetamide solution and wash the gel pieces with 400 µl of water by vortex mixing briefly. Discard supernatant and repeat once.
10. Dehydrate the gel slices for 5 minutes in 200 µl of acetonitrile : 50 mM ammonium bicarbonate (1:1 v/v) with intermittent vortex mixing. Discard supernatant.
11. Add 200 µl of acetonitrile, vortex mix for 30 seconds. Discard the supernatant.
12. Vacuum dry for 5 minutes.
13. Add sufficient volume of 50 mM ammonium bicarbonate, pH 8.5 to just cover the gel slices.
14. Add 100 ng of α-Lytic Protease (dilute 0.4 mg/ml α-Lytic Protease to 0.1 mg/ml in 10 mM sodium acetate, pH 5.0, then add 1 µl to the gel slices).
15. Incubate at 50°C for 1 hour or 37°C for 16 hours.
16. Collect the condensate from the tube walls by centrifuging at 12,000–16,000 × g for 10 seconds.
17. Desalt and purify peptides with C18 ZipTips prior to MS analysis.

FREQUENTLY ASKED QUESTIONS

Q: What reaction buffers and incubation temperatures are recommended for use with α-Lytic Protease?

A: α-Lytic Protease is active in a variety of buffers including ammonium bicarbonate, Tris-HCl, and HEPES. The optimal pH range for α-Lytic Protease digestion is pH 7.5-8.5. The enzyme is active between 40-50°C. Extended incubation times may be necessary for digests at lower temperatures.

Q: Can α-Lytic Protease be used instead of or in combination with Trypsin?

A: Yes. α-Lytic Protease may better digest proteins containing large hydrophobic regions or membrane-associated proteins compared to Trypsin. In addition,site-specific cleavage after lysine and arginine residues by trypsin creates peptides that are positively charged but may be too large for mass spectrometric detection. Performing a double digest of the substrate with Trypsin and α-Lytic Protease creates smaller peptides, thereby increasing both sequence coverage and the likelihood of identifying novel post-translational modifications.

REACTION PROTOCOLS

Trypsin-ultra Typical Reaction Conditions

The following reaction conditions represent a 1:40 enzyme:substrate ratio.

1. Combine 2 µg protein, 7 µl H_2O and 10 µl 2X Trypsin-ultra Reaction Buffer.
2. Add 0.5 µl of 100 ng/µl Trypsin-ultra
3. Incubate at 37°C for 2-18 hours, with gentle shaking.

Note: *Trypsin reactions should be set up with a particular ratio of protein substrate to enzyme. This ratio should be in the range of 1:30 to 1:100. Various proteins are digested at different rates and efficiencies. In many cases, 2-4 hrs at 37°C is sufficient incubation time.*

Trypsin Digestion Protocol using NEB Trypsin-ultra and the FASP Kit

The starting material can be cells, proteome extracts, protein complexes or pure proteins. The total amount depends on goals and complexity: for whole proteomes use between 5–50 µg, for pure proteins use between 0.5–10 µg. Please note the use of too much material can have a negative effect. Never use more than 50 µg of total protein, as filter clogging will occur.

1. Add 200 µl of 1% SDS to each sample, vortex briefly. The use of SDS can be omitted if sample is soluble proteins or complexes.
2. Heat at 60°C for 5 minutes.
3. Allow samples to cool to room temperature.
4. Add 200 µl 100 mM Tris/8 M Urea/10 mM DTT to each sample. Vortex briefly.
    a. To prepare urea solution: Add 1 ml 100 mM Tris-HCl pH 8.5 (provided with FASP Kit) to one tube of urea (provided with FASP Kit). Vortex until all powder dissolves and add DTT to a 10 mM concentration.
5. Rock at room temperature for 30 minutes.
6. Transfer protein-urea mixture to spin filter (provided in FASP Kit). Centrifuge at 14,000 x g for 10 minutes.
7. Add 200 µl fresh 100 mM Tris/8 M Urea solution.

a. To prepare urea solution: Prepare the same as above, omitting DTT.

8. Centrifuge at 14,000 x g for 10 minutes.
9. Discard flow-through.
10. Add 10 µl prepared iodoacetamide solution and 90 µl 100 mM Tris/8 M urea solution. Incubate without mixing for 20 minutes in the dark
    a. To prepare iodoacetamide solution: Add 100 µl of 100 mM Tris/8 M urea solution to 1 tube iodoacetamide (provided with FASP Kit). Pipette up and down 10-15 times to mix well.
11. Centrifuge at 14,000 x g for 10 minutes.
12. Add 200 µl 100 mM Tris/8 M urea solution. Centrifuge at 14,000 x g for 10 minutes.
13. Discard flow-through.
14. Add 200 µl 50 mM ammonium bicarbonate solution (provided with FASP Kit). Centrifuge at 14,000 x g for 10 minutes.
15. Transfer filter to new collection tube.
16. Add digestion solution. Pipette up and down to mix on top of filter. Incubate at 37°C for 2 hours (no rocking).
    a. To prepare digestion solution: Add 1 ml 50 mM ammonium bicarbonate solution (provided with FASP Kit) to 20 µg Trypsin-ultra (NEB #P8101) to make a 20 ng/µl trypsin solution. Add trypsin solution to top of filter at a 1:50 protein:trypsin ratio, and add 50 mM ammonium bicarbonate solution to bring total digestion solution volume to 100 µl.
17. Add 80 µl 50 mM ammonium bicarbonate solution. Centrifuge at 14,000 x g for 10 minutes.
18. Add 30 µl 50% acetonitrile/0.1% formic acid. Centrifuge at 14,000 x g for 10 minutes.
19. Add 40 µl 0.1% formic acid/water. Centrifuge at 14,000 x g for 10 minutes.
20. Filtrate contains digested peptides.
21. Analyze the peptides by 1D or 2D-LC-MS/MS.

Notes: *The FASP Protein Digestion Kit is a product of Expedeon, Inc. (now abcam) and is available here: https://www.abcam.com/fasp-protein-digestion-kit-ab270519.html.*

Trypsin-ultra has been found to have very low levels of autocleavage as compared to other MS grade trypsin. Therefore, using excess Trypsin-ultra will not lower the quality of the data obtained since excess trypsin will remain intact and be retained by the filter.

Volumes may be adjusted based on needs and the concentration of the sample. For 1D analysis with an autosampler, where minimal volume is needed, it is possible to cut elution volumes in half without negative effects.

TECHNICAL TIPS

- Substrate must be in phosphate-free buffer to prevent calcium precipitation with both reconstituted enzyme and buffer.
- Trypsin will self-proteolyze unless calcium is present
- Trypsin-ultra, Mass Spectrometry Grade should be reconstituted by the addition of 20–200 µl of high purity water. Rapid autolysis is a function of enzyme concentration.
- Can be stored frozen in solution at -20°C for up to 2 weeks. For optimal activity, use freshly reconstituted protease.

FREQUENTLY ASKED QUESTIONS

Q: How much Trypsin-ultra is required to digest a protein?

A: A 1:20 Trypsin:substrate ratio is recommended. For proteomics and large scale applications, a 1:50 ratio is recommended. A 1:10 ratio of Trypsin:substrate is the largest amount of trypsin that should be used.

Ordering Information

Deglycosylation Enzymes

PRODUCT	NEB #	SIZE
Protein Deglycosylation Mix II	P6044S	20 reactions
PNGase A	P0707S/L	150/750 units (5,000 units/ml)
Rapid PNGase F	P0710S	50 reactions
Rapid PNGase F (non-reducing format)	P0711S	50 reactions
PNGase F	P0704S/L	15,000/75,000 units (500,000 units/ml)
PNGase F, Recombinant	P0708S/L	15,000/75,000 units (500,000 units/ml)
PNGase F (Glycerol-free)	P0705S/L	15,000/75,000 units (500,000 units/ml)
PNGase F (Glycerol-free), Recombinant	P0709S/L	15,000/75,000 units (500,000 units/ml)
Remove-iT PNGase F	P0706S/L	6,750/33,750 units (225,000 units/ml)
Endo F2	P0772S	480 units (8,000 units/ml)
Endo F3	P0771S	240 units (8,000 units/ml)
Endo S	P0741S/L	6,000/30,000 units (200,000 units/ml)
Endo D	P0742S/L	1,500/7,500 units (50,000 units/ml)
Endo H	P0702S/L	10,000/50,000 units (500,000 units/ml)
Endo H_f	P0703S/L	100,000/500,000 units (1,000,000 units/ml)
O-Glycosidase	P0733S/L	2,000,000/10,000,000 units (40,000,000 units/ml)
O-Glycosidase & Neuraminidase Bundle	E0540S	2,000,000 units *O*-Glycosidase & 2,000 units Neuraminidase

Heparin Lyases

PRODUCT	NEB #	SIZE
Bacteroides Heparinase I	P0735S/L	240 units/600 units (12,000 units/ml)
Bacteroides Heparinase II	P0736S/L	80 units/200 units (4,000 units/ml)
Bacteroides Heparinase III	P0737S/L	14 units/35 units (700 units/ml)

Glycoproteomics

PRODUCT	NEB #	SIZE
O-Glycoprotease (IMPa)	P0761S	200 reactions
IdeZ Protease (IgG-specific)	P0770S	4,000 units
Trypsin-digested BSA MS Standards (CAM Modified)	P8108S	500 pmol
Trypsin-ultra, Mass Spectrometry Grade	P8101S	100 µg
α-Lytic Protease	P8113S/L	20/100 µg
Endoproteinase LysC	P8109S	20 µg
Endoproteinase AspN	P8104S	50 µg
Endoproteinase GluC	P8100S	50 µg

Exoglycosidase Enzymes

PRODUCT	NEB #	SIZE
N-Glycan Sequencing Kit	E0577S	20 reactions
α-N-Acetylgalactosaminidase	P0734S/L	3,000/15,000 units (20,000 units/ml)
β-N-Acetylglucosaminidase S	P0744S/L	100/500 units (4,000 units/ml)
β-N-Acetylhexosaminidase$_f$	P0721S/L	500/2,500 units (5,000 units/ml)
α1-2 Fucosidase	P0724S/L	1,000/5,000 units (20,000 units/ml)
α1-2,4,6 Fucosidase O	P0749S/L	80/400 units (2,000 units/ml)
α1-3,4 Fucosidase	P0769S/L	200/1,000 units (4,000 units/ml)
α1-3,6 Galactosidase	P0731S/L	100/500 units (4,000 units/ml)
α1-3,4,6 Galactosidase	P0747S/L	200/1,000 units (8,000 units/ml)
β1-3 Galactosidase	P0726S/L	500/2,500 units (10,000 units/ml)
β1-3,4 Galactosidase	P0746S/L	400/2,000 units (8,000 units/ml)
β1-4 Galactosidase S	P0745S/L	400/2,000 units (8,000 units/ml)
α1-2,3 Mannosidase	P0729S/L	640/3,200 units (32,000 units/ml)
α1-2,3,6 Mannosidase	P0768S/L	80/400 units (2,000 units/ml)
α1-6 Mannosidase	P0727S/L	800/4,000 units (40,000 units/ml)
α2-3,6,8 Neuraminidase	P0720S/L	2,000/10,000 units (50,000 units/ml)
α2-3,6,8,9 Neuraminidase A	P0722S/L	800/4,000 units (20,000 units/ml)
α2-3 Neuraminidase S	P0743S/L	400/2,000 units (8,000 units/ml)

Companion Products

PRODUCT	NEB #	SIZE
Endoglycosidase Reaction Buffer Pack	B0701S	4 x 1 ml
Fetuin	P6042S	500 µg (10 mg/ml)
RNase B	P7817S	250 µg (5 mg/ml)
Rapid PNGase F Antibody Standard	P6043S	250 µg (5 mg/ml)
Chitin Magnetic Beads	E8036S/L	5/25 ml
6-Tube Magnetic Separation Rack	S1506S	6 tubes
50 ml Magnetic Separation Rack	S1507S	4 tubes
12-Tube Magnetic Separation Rack	S1509S	12 tubes
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