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

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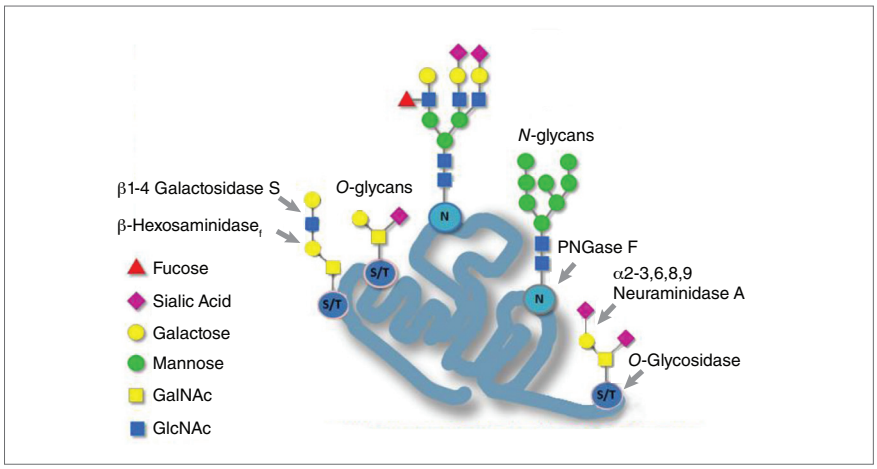
## Introduction

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.

## Notes

**Note 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.

**Note 2:** Commercial antibodies often contain stabilizers or excipients (e.g., detergents, sorbitol, glycerol). If necessary, dilute or exchange to a suitable buffer.

**Note 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).

**Note 4:** Detailed protocols can be found at: https://www.neb.com/protocols/2014/10/28/glycoproteomics-buffer-exchange-protocols-p0710.

**Note 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

## 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 page 2, 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.

#### 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).

## Notes (continued)

**Note 6:** Do not heat or over-dry.

**Note 7:** Pellets dissolve rapidly in NaOH, vortex or pipet carefully to break down any particles.

**Note 8:** Aliquot and store frozen in solution at -20°C for up to 2 weeks.

**Note 9:** 50% acetonitrile, 0.1% formic acid

**Note 10:** Self-packed 24 cm, 100 ID, Aqua 3 µ C18 packing material.

**Note 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.

**Note 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.

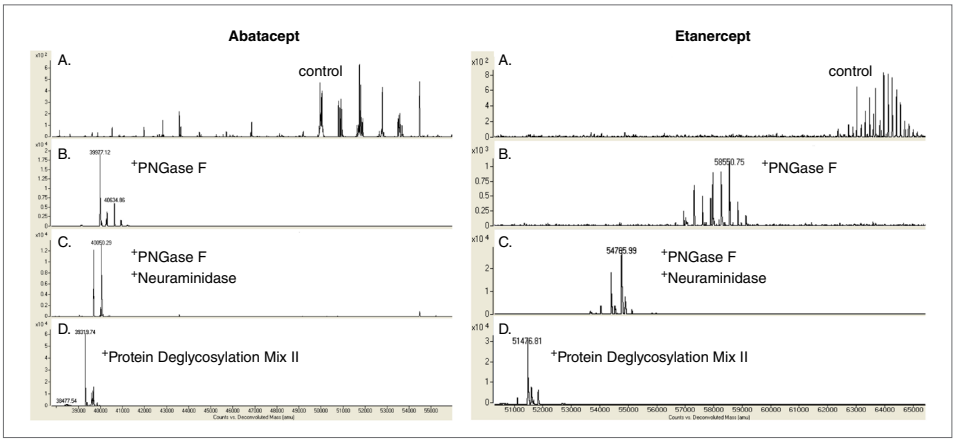
## Results:

### 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.

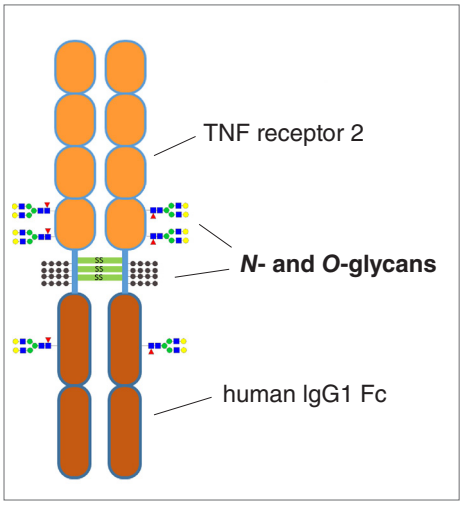
To simplify the analysis, etanercept and abatacept were digested with different enzymes: PNGase F, α2-3,6,8,9 Neuraminidase A or the Protein Deglycosylation Mix II (Figure 3). The untreated glycoproteins are large and polydisperse (panel A). Enzyme digestion resulted in dramatic changes in size. Treatment 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.

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).

FIGURE 2:
**Etanercept structure**




### 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:
**Abatcept sequence**

```
MHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAAT
YMMGNELTFLDDSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPY
YLGIGNGTQIYVIDPEPCPDSDQEPKSSDKTHTSPPSPAPELLGGSSVFLF
PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
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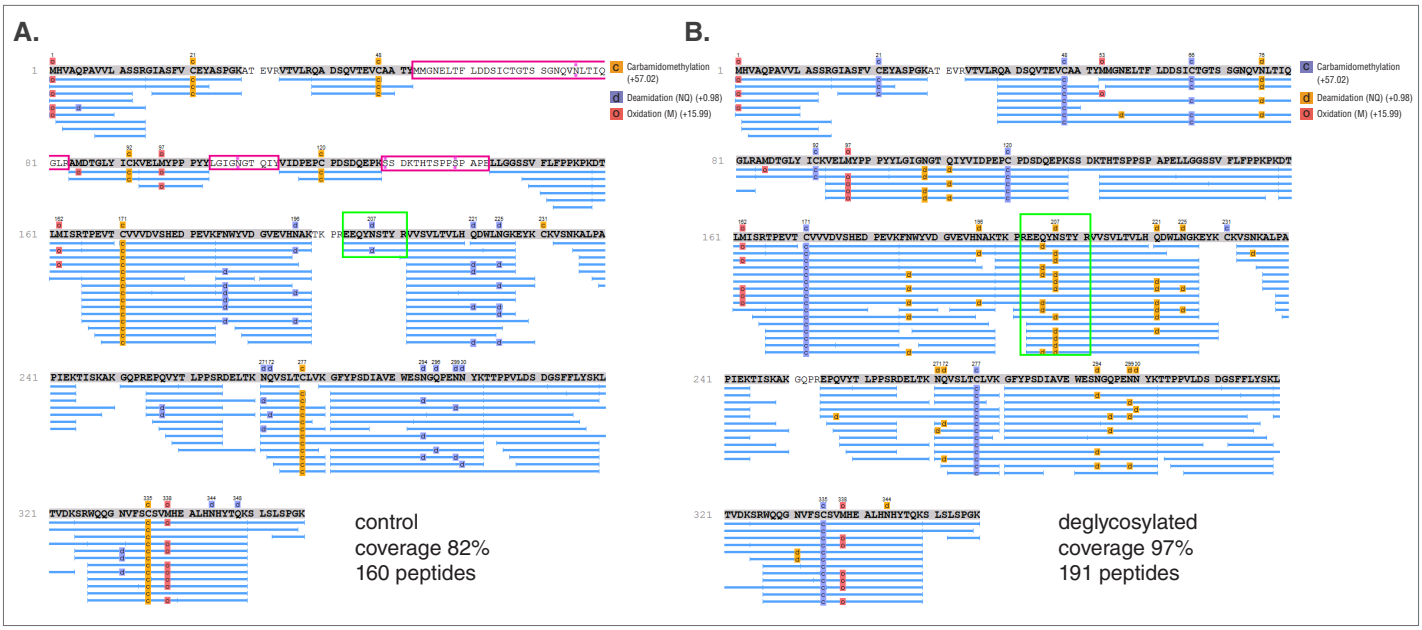
CTLA-4 sequence (blue) and IgG sequence (black). Hinge region mutation (red) introduce sites for *O*-glycosylation (highlighted in blue). *N*-glycan sites highlighted in yellow.

## Notes (continued)

**Note 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:

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