

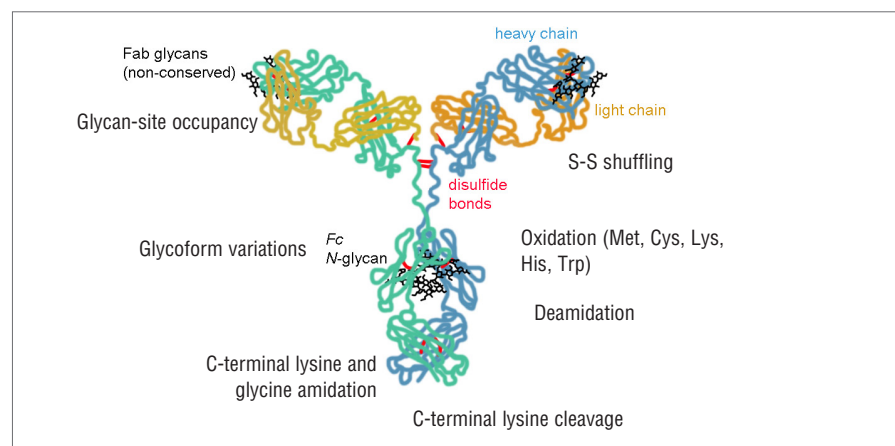
Proteomics: Fast and Efficient Antibody Deglycosylation using Rapid PNGase F

Paula Magnelli and Colleen McClung

Introduction

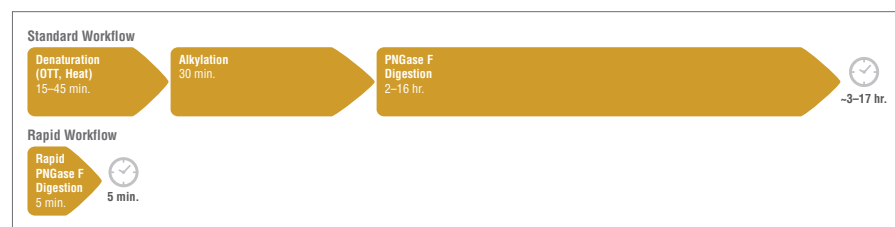
Innovations in process development and manufacturing of therapeutic monoclonal antibodies have been critical for their clinical and economic success. Along with these advances, methods for quality control are constantly evolving to guarantee the safety and effectiveness of these drugs. During development 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

Erbix (cetuximab) from Imclone, LLC.

Rituxan (rituximab) from Genentech, a member of the Roche Group, and Biogen Idec, Inc.

Enbrel (entercept) 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 Protocols

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

Note 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>.

Note 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.

Note 3: Equivalent to 2-4 µg protein.

Note 4: 4 µl of 1.25 M DTT, 130 µl 3X Blue Loading Buffer

Note 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.

Note 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-protocols-p0710>.

Note 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.

Note 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).

Note 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

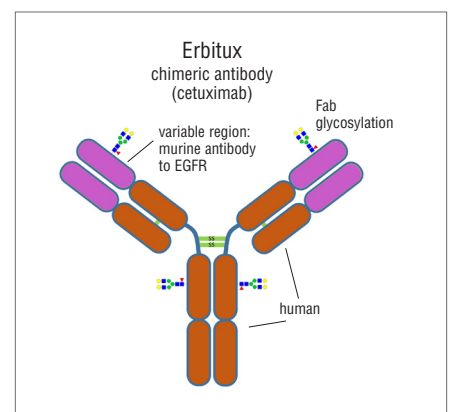
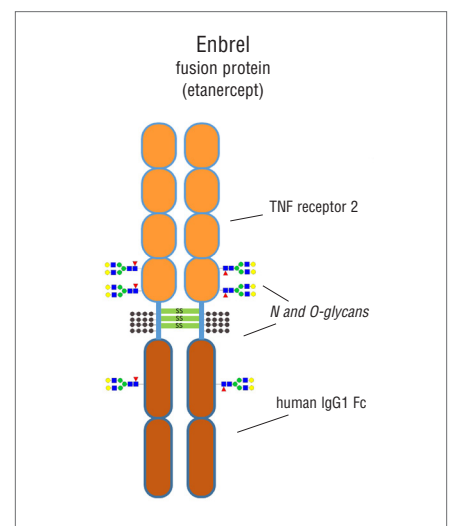
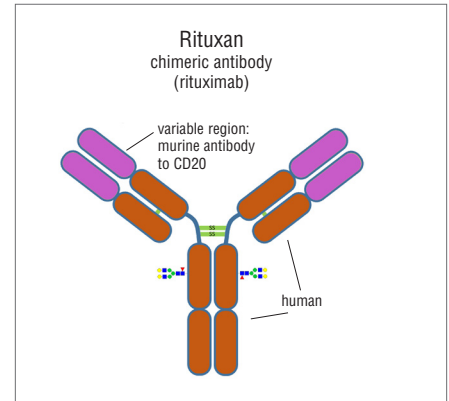
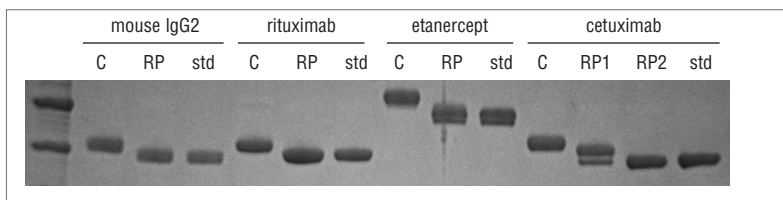
Results

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).

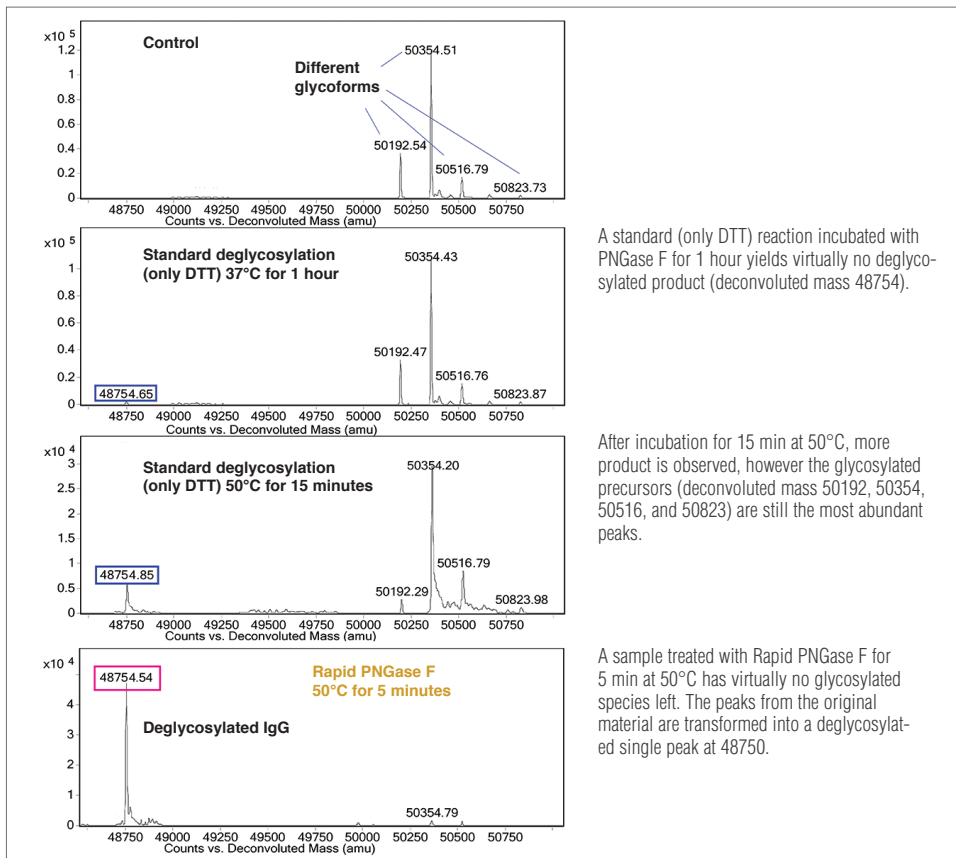
FIGURE 3:
Deglycosylation with Rapid PNGase F

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).



"Top-down" Proteomic Analysis with Rapid PNGase F

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



Results (cont.)

Analysis of Different Isotypes, Subclasses and Sources

FIGURE 5:
Deglycosylation IgG subclasses and sources

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).

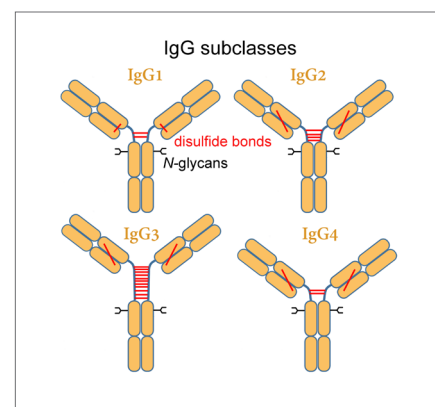
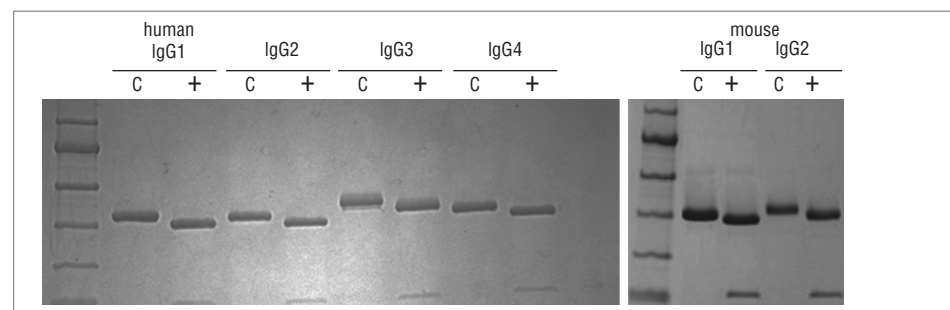
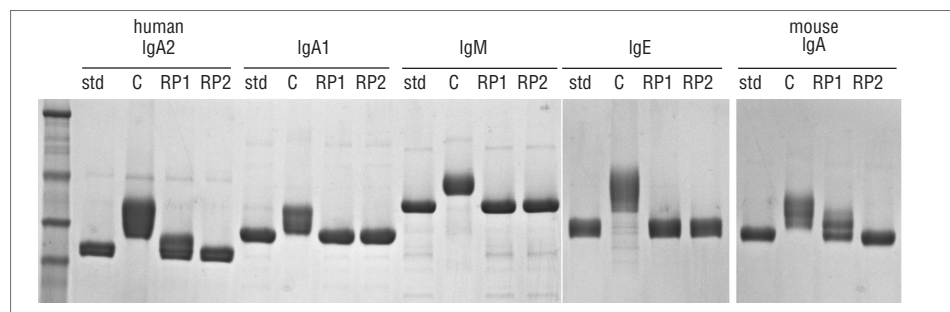
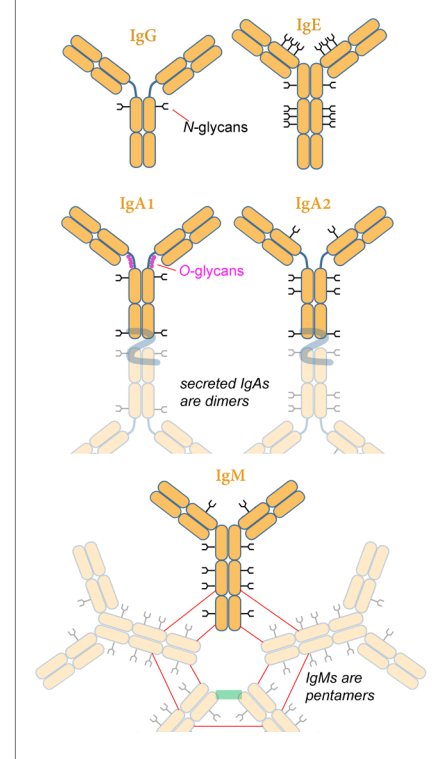


FIGURE 6:
Deglycosylation of various isotypes of rituximab

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).



Glycosylation patterns of antibody isotypes



Concluding Remarks

NEB's Rapid PNGaseF 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.

One or more of these products are covered by patents, trademarks and/or copyrights owned or controlled by New England Biolabs, Inc. For more information, please email us at gbd@neb.com. The use of these products may require you to obtain additional third party intellectual property rights for certain applications.

Your purchase, acceptance, and/or payment of and for NEB's products is pursuant to NEB's Terms of Sale at www.neb.com/support/terms-of-sale. NEB does not agree to and is not bound by any other terms or conditions, unless those terms and conditions have been expressly agreed to in writing by a duly authorized officer of NEB.

ERBITUX® is a registered trademark of ImClone, LLC.
 RITUXAN® is a registered trademark of Genentech, Inc. and Biogen Idec, Inc.
 ENBREL® is a registered trademark of Amgen, Inc.
 XBRIDGE® is a registered trademark of Waters.
 ULTIMATE® is a registered trademark of Thermo Scientific.
 SUPELCLEAN™ and ENVI-CARB™ are trademarks of Sigma-Aldrich Co.
 LTQ™ and VELOS™ are trademarks of Thermo Scientific.
 MILLI-Q® is a registered trademark of Millipore Corporation.

© Copyright 2018, New England Biolabs, Inc.; all rights reserved.



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.