Unbiased and fast IgG deglycosylation for accurate *N*-glycan analysis using Rapid PNGase F

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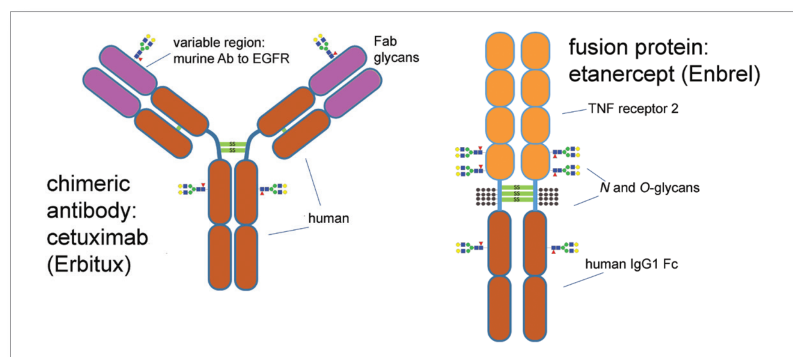
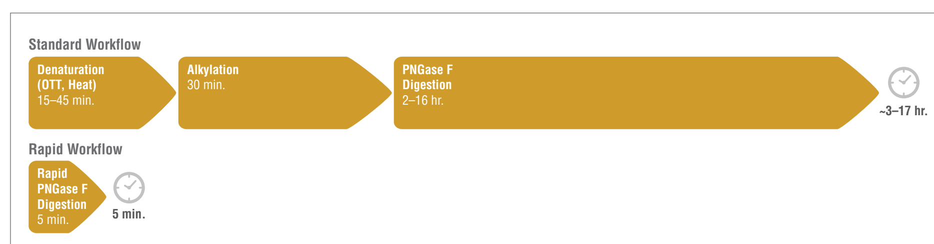
A growing number of monoclonal antibodies and antibody chimeras are in development as therapeutic agents. The Fc region of IgG carries a conserved *N*-glycan, which is critical for biological activity. Also, some IgGs and IgG fusions have additional *N*-glycans that, together with the conserved Asn297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

General Protocol

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).

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)

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.

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General Protocol *(continued)*

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)

Hydrophylic 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 *(continued)*

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% acetronitrile; 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 NH4 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.

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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) .

Symbols

- Fucose
- Mannose
- Sialic acid (NANA)
- GlcNAc
- Galactose
- Sialic acid (NGNA)

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

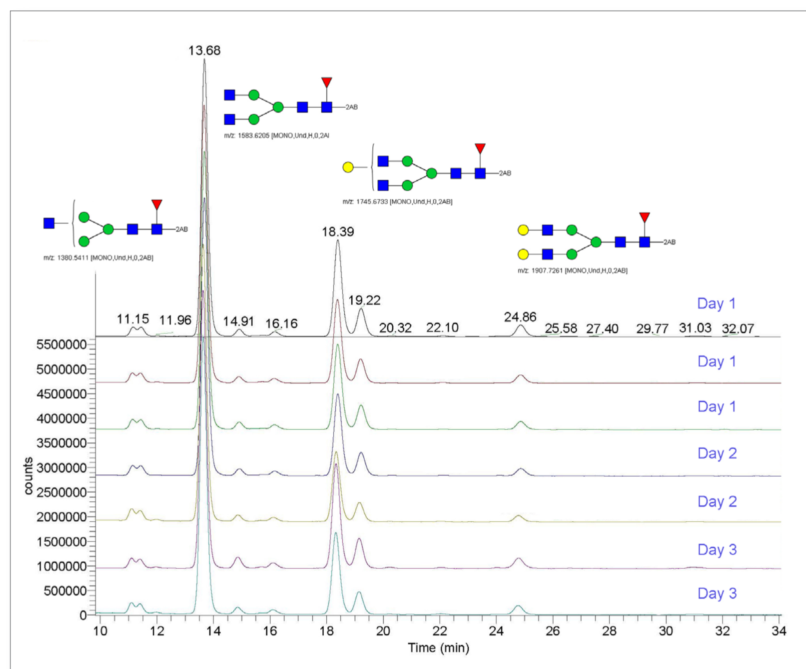
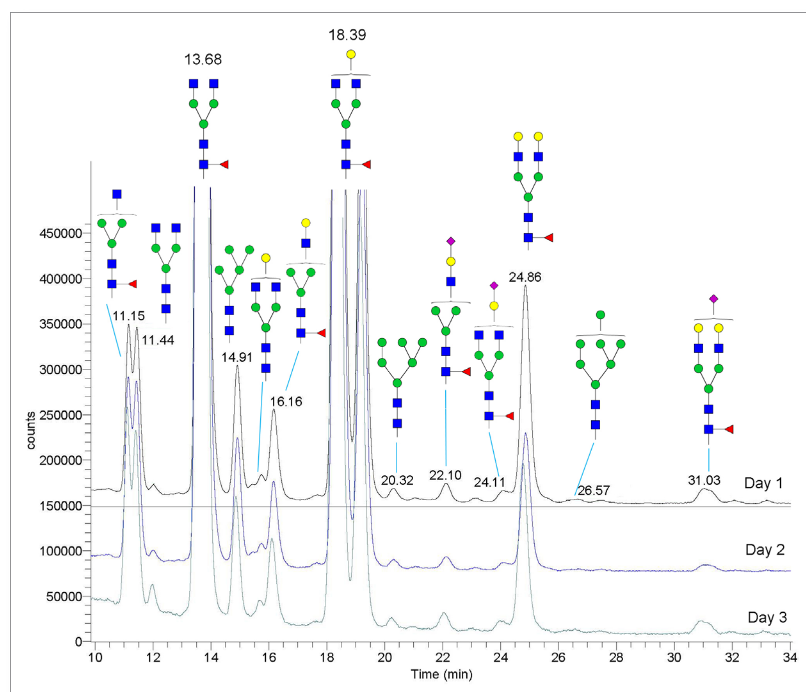


Figure 2. Rituximab *N*-glycans zoomed view to visualize low abundance peaks.



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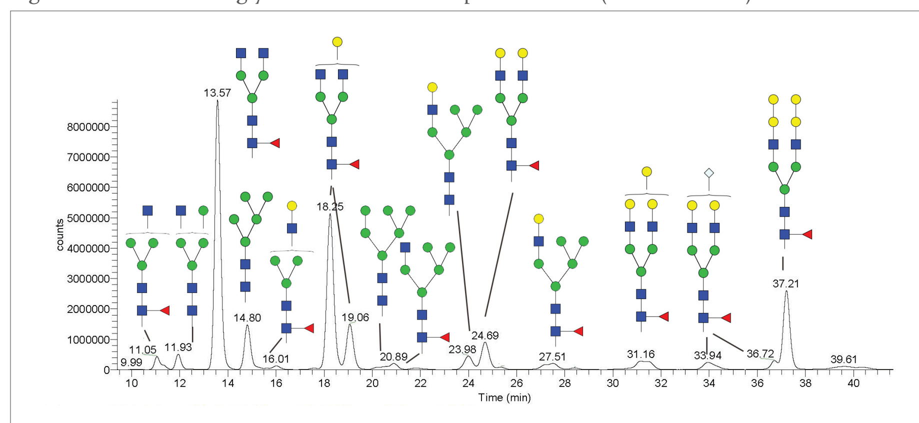
Results 2: Therapeutic Antibodies with Additional *N*-glycan Sites

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).

Symbols

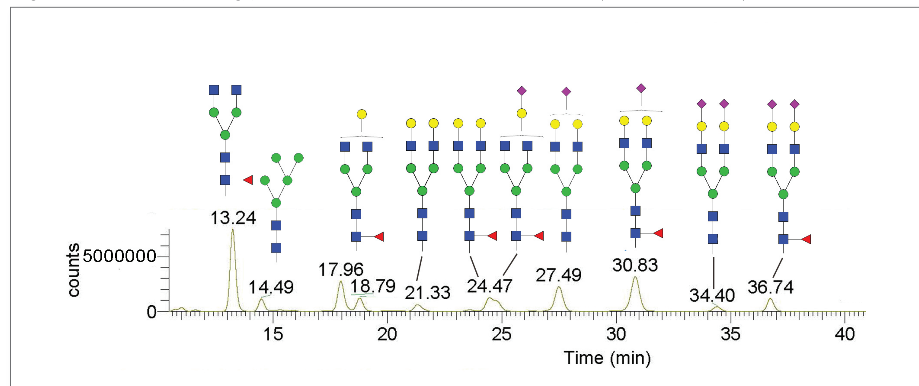
- Fucose
- Mannose
- Sialic acid (NANA)
- GlcNAc
- Galactose
- Sialic acid (NGNA)

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.

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