Improving antibody characterization: Rapid PNGase F for accurate *N*-glycan analysis

Paula Magnelli, Beth Mc Leod, Alicia Bielik, Colleen McClung, Stephen Shi, and Ellen Guthrie.

New England Biolabs Inc., Ipswich, MA, USA.

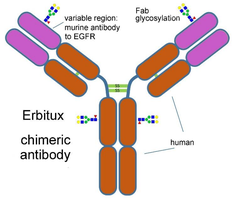
Abstract

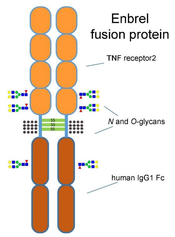
Rapid PNGase F was developed for full deglycosylation of antibodies in only minutes. A variety of antibodies were tested for validation: in each case (including antibodies with more than one glycosylation site), all *N*-glycans were effectively removed.

For convenience, a labeling protocol was designed that allows derivatization directly after delgycosylation. We show that eliminating extraction steps improves reproducibility and yields: sensitivity and accuracy were not compromised by a fast workflow.

Introduction

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 affect recognition, half life, and inflammatory reactions.





It has become increasingly important to monitor antibody glycosylation during development and production. Effective monitoring requires complete and accurate *N*-glycan profiling obtained in the shortest time possible.

Standard deglycosylation	Rapid PNGase F
Denaturation (DDT, heat) 15-45 min	One vial 5 minutes
Alkylation 30 min	
PNGase F digestion 2-16 h (long incubations required for completion)	

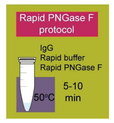
Complete glycan removal requires hours. We report here a protocol using NEB's new Rapid PNGaseF reagent, wich allows complete deglycosylation of antibodies in minutes and is compatible with LC-MS applications.

Finally, reductive amination was tested in the absence of an extracting step by adding the reagents for labeling directly to the deglycosylation reaction

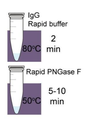
Standard protocol	Streamlined protocol
Denaturation (DDT, heat) 15-45 min	Rapid PNGase F 5 minutes
Alkylation 30 min	
PNGase F digestion 2-16 h (long incubations required for completion)	
SPE (i.e. PGC)	+ labeling reagents 1h 65C
dry	
Reductive amination 2h 65 C	
SPE cleanup	SPE cleanup Hydroxy-aspartamide HILIC cartridge

Results

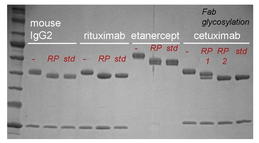
Fig 1: Rapid, extensive deglycosylation



Rapid PNGase F can deglycosylate up to 100µg of antibody in one step for 5-10 min at 50°C



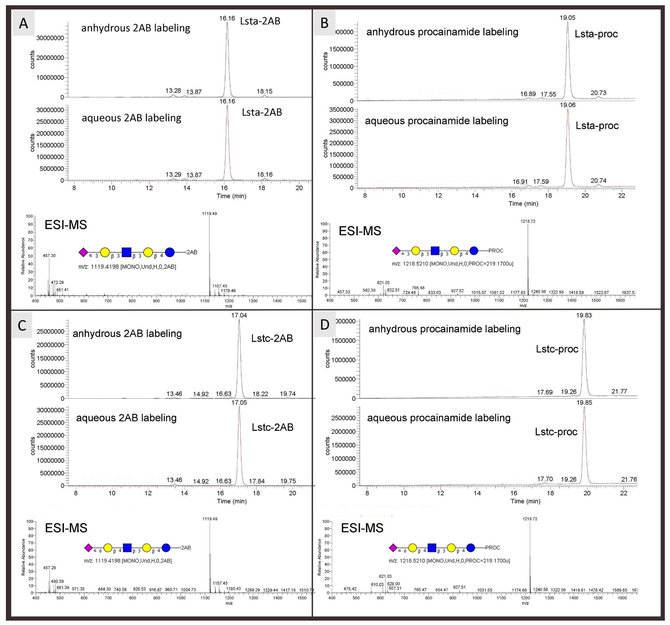
Some antibodies may require 2 min pre-incubation at 80°C



Antibodies treated for 5 min with **Rapid PNGase F** (*RP*). Compare with control (-), and with standard deglycosylation reaction (*std*).

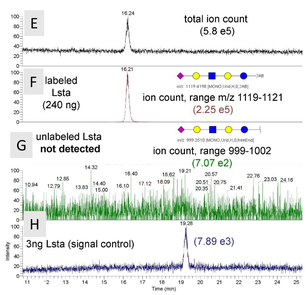
Mouse IgG2, rituximab, and etanercept were efficiently deglycosylated in 5 min at 50˚C. Cetuximab (Fab *N*-glycans) required a 2 step protocol (compare partial shift down in *RP1*, vs *RP2*)

Fig 2: streamlined labeling by reductive amination: fast and complete



To determine whether it is possible to avoid purification (SPE + drying) prior to reductive amination, Lsta glycan (α2-3 sialylated) and Lstc glycan (α2-6 sialylated) were labeled with either 2AB or procainamide under typical (anhydrous) conditions or alternatively by adding labeling reagent directly (to a mock PNGase F reaction), for 1h at 65°C.

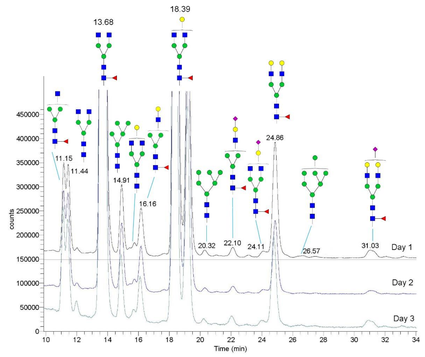
Panels A to D show that the integrity of the glycan is preserved: no significative sialic acid loss was observed. Moreover yields were slightly higher, illustrating the advantage of minimizing handling by avoiding purification steps.



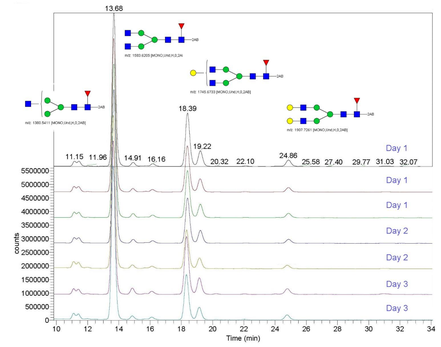
To confirm that alternative labeling conditions do not result in underlabeling, MS spectrum was interrogated for the presence of labeled product (panel F), and unlabeled reagent (panel G). The ion count for each mass range is indicated, no signal for unlabeled glycan is observed.
Panel H shows that as low as 3ng of glycan (1% of the total sample used for labeling) can be easily detected, which indicates that a streamlined labeling protocol is not only fast but allows complete derivatization.

Fig 3: Reproducibility

Rituximab samples (80µg) 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.



(1) Visser J, et al., BioDrugs. 2013 Oct;27(5):495-507.



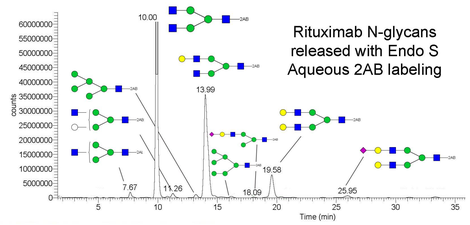
The composition of *N*-glycans was highly reproducible from day to day (upper panel). There was negligible variation in the levels of low abundance *N*-glycans as well.

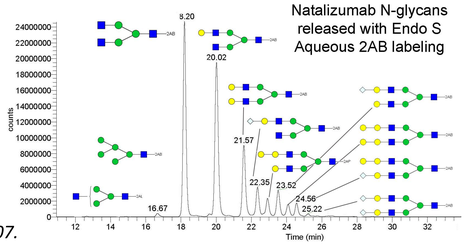
All major, and minor species previously reported in the literature were found. Relative abundance was within previously reported ranges (1) .

Fig 4: labeling Endo S-released glycans

Rituximab (80µg) or Natalizumab (50µg) were treated under native conditions for 1h with **EndoS**. Released *N*-glycans were labeled with 2AB and analyzed by LC-MS.

All major, and minor species previously reported in the literature were found. Relative abundance was within previously reported ranges (1) .





(1) Visser J, et al., BioDrugs. 2013 Oct;27(5):495-507.

Materials and Methods

Rapid deglycosylation: Protein sample (20-100µg), 4µl Rapid buffer, 1µl of Rapid PNGase F (*NEB #P0710*), and water (to 20µl) were incubated at 50°C for for 5 to 10 min. Alternatively, samples (protein, Rapid buffer, water to 20µl) were pre-incubated 2 min at 80°C, and then 1µl of Rapid PNGase F was added before incubating at 50°C for 5 to 10 min. **Endo S deglycosylation**: Protein sample (20-100µg) was mixed with reaction buffer and incubated with 1ul Endo S (NEB *#P0741*) for 1h at 37°C. **SDS-PAGE:** Protein samples (2-3µg) were heated with loading buffer (*NEB # B7703S*) at 94°C for 2 min. Samples and a MW marker (*NEB # P7703S*) were run on a 10–20% Tris–Glycine gel at 200 V for 1h, and developed with Coomasie stain.

N-glycan labeling: Dried glycan standards were labeled with 2-AB (5mg 2AB or 11mg procainamide, 6mg NaCNBH4) in 70% DMSO 30% acetic acid, for 1h at 65°C. **Direct N-glycan labeling**: glycan standards were diluted in 20ul of Rapid PNGase F buffer, to which 20ul of concentrated labeling reagent (10mg 2AB or 22 mg procainamide, 12mg NaCNBH4 in 40% DMSO, 1% acetic acid) were added, reaction was incubated for 1h at 65°C. **HILIC cleanup**: Excess label was removed with an HILIC SPE hydroxyaspartamide cartridge (Nest Group, SEM-HIL). **LC-MS:** A sample of labeled glycans (24µl) was diluted with 96µl of ACN. The labeled glycans were separated using a Amide 80 (Tosoh) column, on a Dionex UltiMate® LC with fluorescent detection, in line with a LTQ™ Orbitrap Velos™ Spectrometer equipped with a heated electrospray standard source (HESI-II probe). Structures (CFG notation) assigned based on retention time, m/z, and in accordance with known biosynthetic pathways.

Conclusions

NEB's new Rapid PNGaseF reagent can achieve complete and unbiased removal of *N*-glycans from antibodies in minutes. This reaction, which occurs in solution and requires minimal setup, can be coupled with an optimized labeling procedure. The reaction conditions allowed use of different labels, and are compatible with other glycosidases such as Endo S. The complete protocol is compatible with downstream analysis by LC/MS.

www.neb.com

This work was supported by New England Biolabs

Special thanks to Dr. Don Comb for his lasting support and commitment to basic research at NEB