Improving sample workflow: Rapid PNGase F for accurate antibody characterization.

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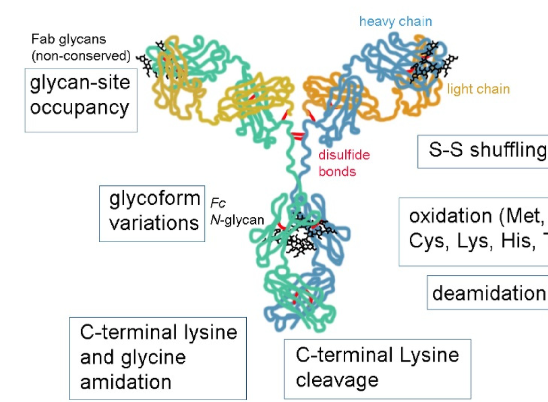
Introduction

The characterization of glycoprotein structure is becoming increasingly sophisticated, as regulatory agencies require multiple attributes to be measured during development, production, and formulation of biological drugs.



Precise determination of *N*- and *O*-glycosylation, site occupancy, disulfide shuffling, misassembly, deamidation, oxidation, etc, require robust methods for sample preparation, to facilitate mass spectrometry analysis.

Enzymes for glycan removal, along with specific proteases, are critical to these studies. Improved methods where glycosidases are combined, and/or coupled with labeling reactions or protease digestion, maximize reproducibility by eliminating handling errors. These methods, in turn, permit a more stringent definition of an original, biosimilar, or biobetter, facilitating formulation and process development innovations.

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**

We present in this poster new glycan removal protocols, including fast deglycosylation (using Rapid PNGase F) and deglycosylation of intact plant-derived glycoproteins (using PNGase Ar). These reactions were coupled with a simplified and versatile glycan labeling reaction by reductive amination, suitable for glycans lacking a glycosylamine end group.

Also, glycosidase combinations were tested for complete *N*- and *O*-glycan removal, to facilitate proteomic analysis for glycoproteins that are heavily glycosylated.

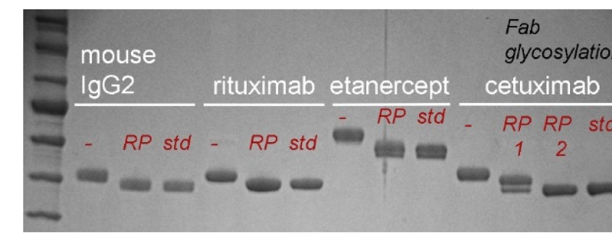
Deglycosylation and trypsin digestion	Trypsin + PNGase F Peptide preparation
Glycoprotein Denaturation (DTT) 30 min Alkylation (IAA) 45 min	Glycoprotein + Trypsin Buffer 95C 5 min
+ PNGase F (2h to ON)	+ PNGase F + Trypsin (1:100) 37C **3h**
+ Trypsin 2h to ON	
Peptide extraction	SPE C18

Finally, an enzyme mix containing PNGase F and Trypsin was used to prepare an IgG sample for peptide mapping. This abbreviated workflow maintained sensitivity and reproducibility.

Results

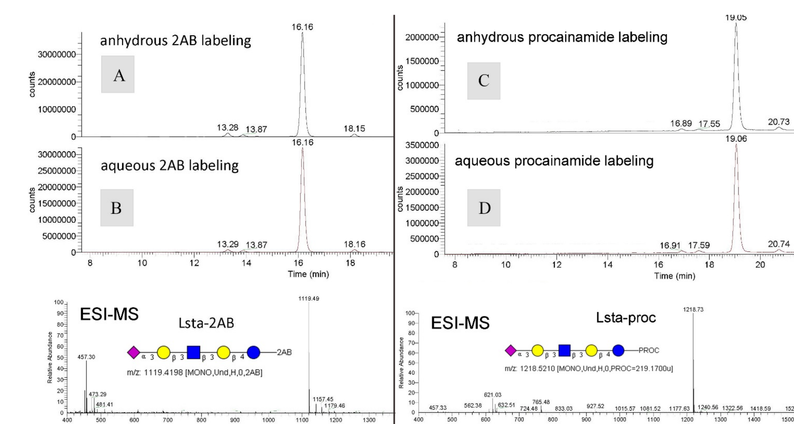
Rapid PNGase F: *complete and fast deglycosylation*

Antibodies treated for 5 min with **Rapid PNGase F** (*RP*). Compare with control (-), and with a standard deglycosylation reaction (*std, 1h at 37C in the presence of SDS*). Mouse IgG2, rituximab, and etanercept were efficiently deglycosylated in 5 min at 50°C. Cetuximab (containing resistant Fab *N*-glycans) required a 2 step protocol (compare partial shift down in *RP1*, vs *RP2*) which still required less than 10 minutes.



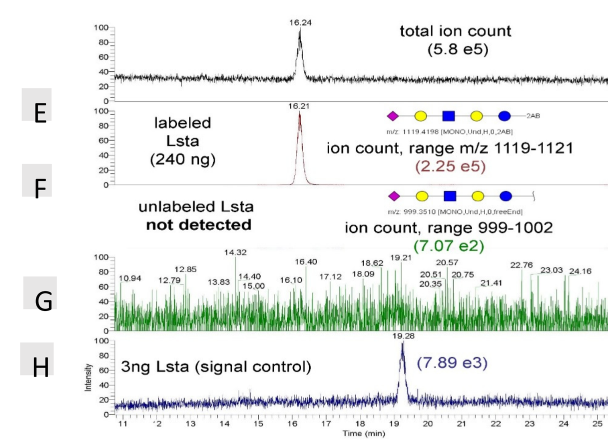
Direct Labeling: *versatile, complete labeling*

Glycan purification (prior to reductive amination) can be avoided, minimizing losses. The simplified labeling protocol yields complete derivatization in 1h, and the glycan integrity is preserved. The protocol was tested with two different labels, and in principle is applicable to a variety of derivatization reagents.



Dried Lsta glycan standard was labeled under anhydrous conditions with 2AB or procainamide (A, C). Alternatively, the glycan was labeled under high aqueous conditions (B, D) by adding labeling reagent directly to a (mock) PNGase F reaction.
Compare A-B and C-D. The glycan remained intact, no hydrolysis of labile groups detected.
Identical results were obtained with a similar α2-6 sialylated glycan (Lstc)

To confirm that direct labeling is complete after only 1h, the mass spectrum (TIC, panel E) was scanned for the presence of labeled product (panel F), and unlabeled reagent (panel G). The ion count is indicated. The unlabeled precursor was not present: as low as 3ng of glycan (1% of the total sample) could be easily detected (panel H), demonstrating that the direct, high aqueous, reductive amination reaction is as effective.

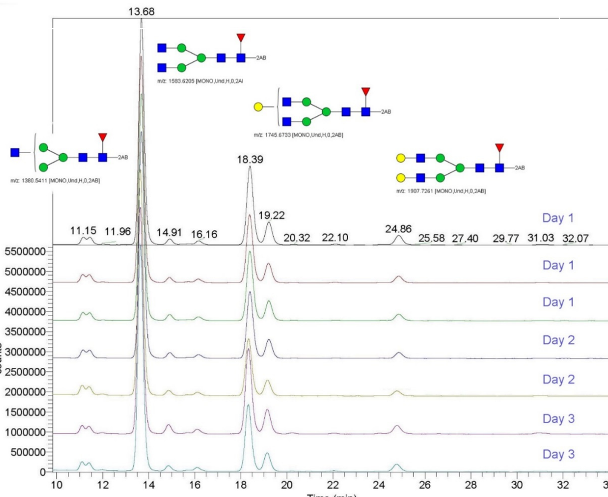


Rapid PNGase F + Direct Labeling: *reproducibility*

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 by different operators.

The composition of major *N*-glycans was highly reproducible from day to day and among different operators. 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.

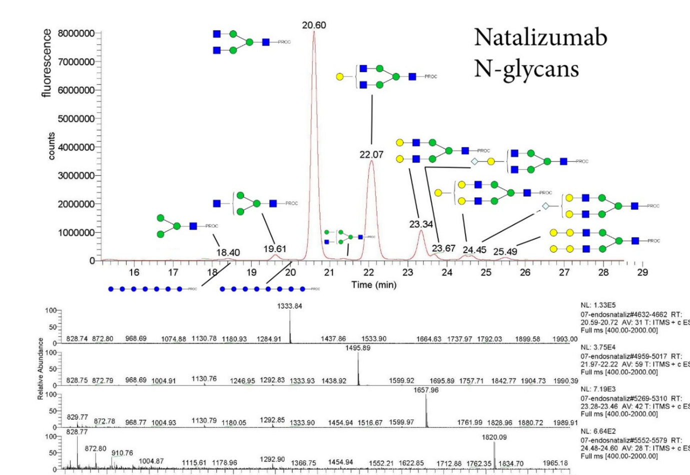


Direct Labeling: *endoglycosidases, low pH amidases.*

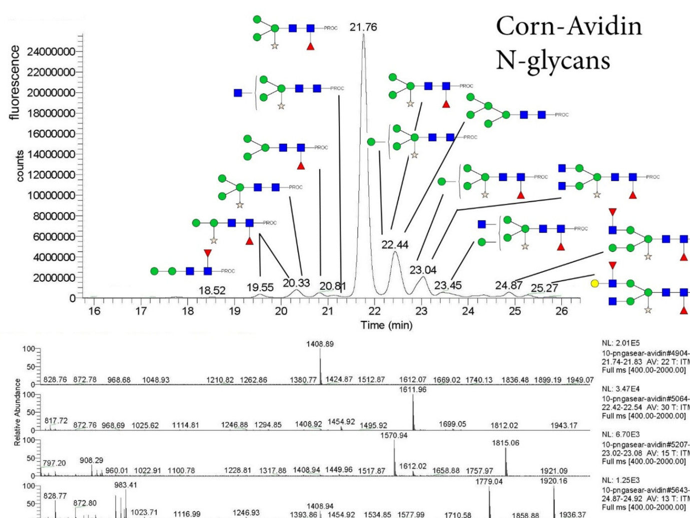
A simplified reductive amination protocol is useful when instant labeling methods (requiring an intact glycosylamine) cannot be used. For instance, *N*-glycans released with EndoS (a true endoglycosidase), or with PNGase Ar an amidase able to remove glycans from proteins expressed in plants or insect cells (glycosylamines are unstable at its low optimum pH).

Deglycosylation reactions with EndoS (on natalizumab) or PNGase Ar (on corn-expressed avidin) were labeled directly with procainamide. Reproducibility was very good (not shown) with variations of less than 1% in relative composition.

N-glycans released with EndoS



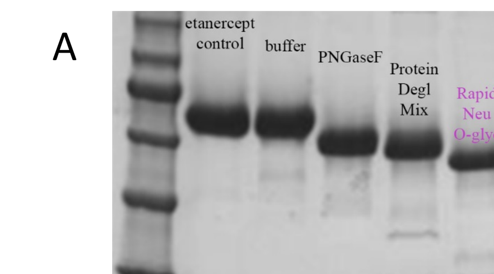
N-glycans released with PNGase Ar



Rapid PNGase F, O-glycosidase, neuraminidase: *N- and O-glycan removal*

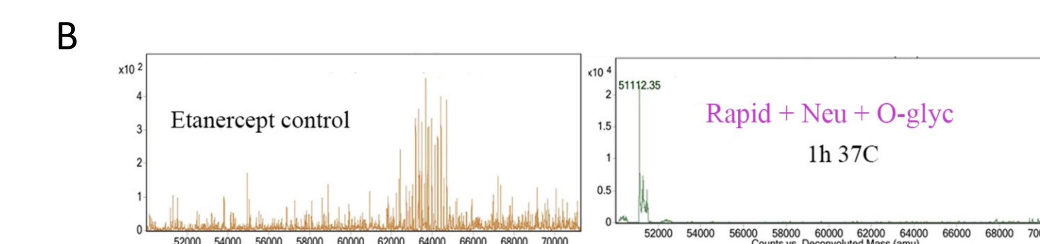
Rapid PNGase F, in combination with O-glycosidase and neuraminidase, allows complete *N*- and *O*-glycan removal under conditions that are compatible with mass spectrometry analysis.

A



Panel A shows the SDS-PAGE gel of etanercept (a fusion protein with 3 *N*-glycans and up to 13 *O*-glycan sites) digested with only PGNase F or Protein Deglycosylation Mix (in the presence of SDS and DTT), compared with Rapid PNGase F in combination with O-glycosidase and Neuraminidase. The sample digested with a Rapid PNGase F cocktail is not only compatible with downstream mass spectrometry, but glycan removal is more extensive as indicted by a further downshift in MW

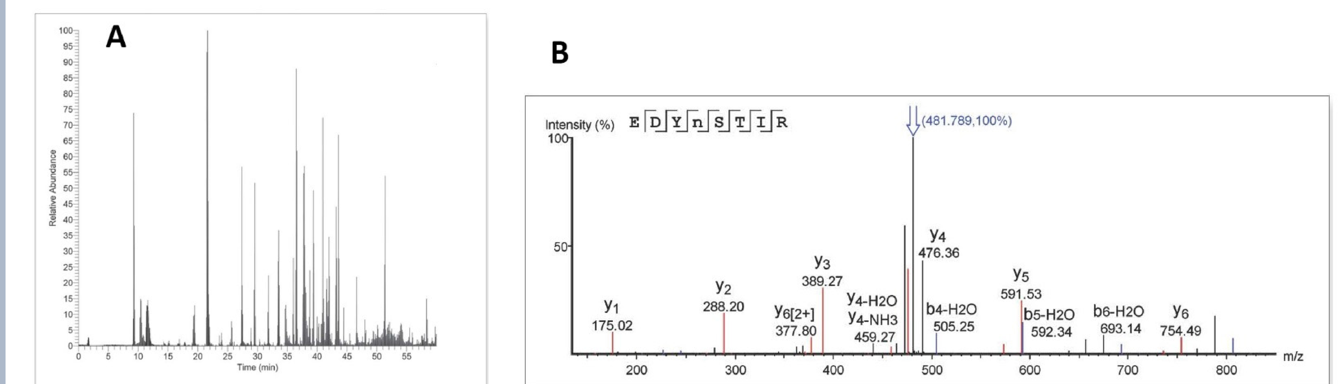
B



Panel B shows the ESI-TOF profile of etanercept before (control, av MW 64KD) and after (MW 51KD) demonstrating that indeed all *N*- and *O*-glycan groups have been removed

PNGase F and Trypsin-ultra™: *peptide mapping*

A murine monoclonal antibody (IgG2) was simultaneously treated with PGNase F and Trypsin-ultra™, Mass Spectrometry Grade. This fast protocol did not compromise sensitivity, resulting in optimal peptide yields.



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

Materials and Methods

Deglycosylation: 1) Rapid PNGase F: Protein (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. **2) Endo S**: Protein (20-100µg) was mixed with reaction buffer and incubated with 1ul Remove-iT Endo S (NEB #*P0741*) for 1h at 37°C. **3) PNGase Ar**: Protein (20-100µg), 4µl PNGase Ar Buffer (pH 6.0), and water (to 20µl) were incubated at 80°C for for 5 min, cooled, and 1µl of PNGase Ar (recombinant, cloned from rice) was added before incubating at 37°C for 1h. **4) Standard reaction PNGase F**: samples were treated with SDS-DTT at 95C, and deglycosylated with PNGase F (*NEB# P0709*) for 1h at 37C. **SDS-PAGE**: Protein (2-3µg) was mixed with loading buffer (*NEB # B7703S*). For non-reducing SDS-PAGE, loading buffer did not contain DTT. Samples were run on a 10–20% Tris–Glycine gel at 200 V for 1h, stained with Coomasie. **Glycan labeling:** Dried glycans were labeled with 2-AB (5mg 2AB or 11mg procainamide, 6mg NaCNBH4) in 70% DMSO 30% acetic acid, for 1h at 65°C. **Direct glycan labeling**: glycans (in 20ul deglycosylation reactions) were mixed with 20ul of concentrated labeling reagent (10mg 2AB or 22 mg procainamide, 12mg NaCNBH4 in 40% DMSO, 1% acetic acid), reaction was incubated for 1h at 65°C. **HILIC cleanup**: Excess label was removed with an HILIC SPE cartridge (Nest Group, SEM-HIL), sample load in 90% ACN/NH4formate, glycans eluted in 50ul NH4 formate. **LC-MS:** A sample of labeled glycans (24µl) was diluted with 96µl of ACN. Labeled glycans were separated using Amide 80 (Tosoh) or BEH-XBridge (Waters) columns, on a Dionex UltiMate® LC with fluorescent detection, in line with a LTQ™ Orbitrap Velos™ Spectrometer (HESI-II probe). Structures (CFG notation) were assigned based on retention time, m/z, and in accordance with known biosynthetic pathways.

***N*- and *O*-glycan removal**: Protein (25µg), 4µl Rapid Buffer, and water (to 20µl) were pre-incubated 5 min at 75°C, after cooling 1µl of Rapid PNGase F, 1ul of O-glycosidase (*NEB# P0733*), and 1ul of Neuraminidase A (*NEB# P0722*) were added before incubating at 37°C for 1h. Alternatively, samples were treated with standard PNGase F or with Protein Deglycosylation Mix (*NEB# P6039*) in the presence of SDS and DTT. **Intact mass:** After deglycosylation, the buffer exchanged protein was reduced (10 mM DTT, 30 min RT) and adjusted to 0.1% formic acid. Samples were run on a custom reverse-phase chip with a 1200 series nano LC in line with a 6210 series ESI-TOF MS (Agilent). Protein eluted 10 minutes after injection. The spectra were extracted and deconvoluted. **Simultaneous PNGase F/Trypsin Digestion**: 25 µg of murine IgG were mixed in 25 µl of 2X Trypsin Buffer, and incubate at 95°C for 5 minutes. After cooling, 6 µl of PNGase F and 250 ng of Trypsin-ultra™ (*NEB #P8101*) were added, reaction was incubated at 37°C for 3 hours. **Peptide MS and MS/MS:** 400 ng (1 µl) of sample was injected onto a 20 cm 100 ID analytical column (Aqua 3µ C18) using a Proxeon EASY-nLC (Thermo) and separated using a 60 min 5-35% FB linear gradient (FA = 0.1% formic acid, FB = CH3CN, 0.1% formic acid, flow rate 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 nano-ESI (Thermo). Data was analyzed with Proteome Discoverer™ 1.4 (Thermo) and PEAKS7, and searched using a SwissProt FASTA database. Theoretical tryptic peptides (2 missed cleavages max) were considered. Precursor and product mass tolerances were set to ± 10 ppm and ± 0.01 Da. Modifications (N to D after PNGase F removes an N-glycan) were allowed. Data was validated using a reverse database decoy search to a false discovery rate of 1%.

Conclusions

- NEB's Rapid PNGaseF reagent completely and quickly removes all *N*-glycans from antibodies. This in-solution reaction could be coupled with an optimized labeling procedure, compatible with different labels and other glycosidase reactions. The complete protocol gave highly reproducible results.
- An enzyme cocktail containing Rapid PNGase F, O-glycosidase and neuraminidase completely deglycosylated a therapeutic *N*- and *O*-glycoprotein. The reaction was compatible with downstream MS proteomic analysis.
- An abbreviated, simultaneous, Trypsin and PGNase F reaction was validated for mass spectrometry applications (peptide mapping).

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