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Remove-iT[®] PNGase F: Effective Release and Recovery of Neutral and Sialylated *N*-glycans

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Protein glycosylation, the covalent attachment of sugar residues to a polypeptide, is the post-translational modification that generates the greatest functional and structural variation from a single polypeptide (1). Most glycoproteins are typically produced by cells as a collection of glycoforms that differ in mass, charge, conformation, ligand affinity, *in vivo* half life, etc. Therefore, *in vitro* manipulations are necessary to obtain a homogeneously glycosylated (or deglycosylated) protein for functional studies. Because most applications require an active protein, glycosidases are ideal tools for glycoprotein remodeling (2,3,4,5).

Peptide-*N*-Glycosidase F (PNGase F) is able to remove high mannose, hybrid, and complex *N*-linked glycans. Although it is more effective on denatured proteins, this enzyme can be used under native conditions, preserving the integrity of the protein of interest.

Remove-iT PNGase F, a new version of this enzyme, has a chitin binding domain (CBD) tag for easy removal of the enzyme after deglycosylation. The procedure leaves the target protein and the released *N*-glycans ready for analysis (i.e., mass spectrometry, cell based assays, crystallography, etc). Enzyme removal is also an attractive alternative to inactivation by heat or acid, which can have a negative affect on protein stability.

In the following application note we show how Remove-iT PNGase F effectively cleaves *N*-glycans from Bovine Fetuin. Specifically, we demonstrate that the enzyme removal step using chitin magnetic beads does not interfere with the recovery of either neutral or sialylated glycans.

We demonstrate that Remove-iT PNGase F has identical activity to PNGase F, while presenting the additional advantage of being readily eliminated from the protein sample.

General Protocols:

Deglycosylation:

The protein sample is denatured with DTT and heat (avoiding detergents which are not compatible with downstream MS analysis). Alternatively, samples are deglycosylated under native conditions (protein is not denatured).

1. For each sample, mix 10 μ l of Fetuin (100 μ g), 10 μ l 10X DTT and 70 μ l of H₂O in a micro-centrifuge tube.
2. Denature at 55°C for 10 minutes and cool on ice.
3. For deglycosylation under native conditions, mix 10 μ l of Fetuin and 80 μ l of H₂O (do not heat).
4. To all samples, add 10 μ l of 10X GlycoBuffer 2 and 2 μ l of Remove-iT PNGase F or PNGase F (Glycerol-free).
5. Incubate for 1 hour at 37°C. (Longer incubation times may be required for complete deglycosylation of other proteins under native conditions).

Materials

- Fetuin (NEB #P6042)
- Remove-iT PNGase F (NEB #P0706), supplied with 10X DTT and 10X G7 Reaction Buffer
- PNGase F (Glycerol-free) (NEB #P0705), supplied with 10X Glycoprotein Denaturing Buffer, 10% NP-40 and 10X GlycoBuffer 2
- Chitin Magnetic Beads (NEB #E8036)
- 2-Tube Magnetic Separation Rack (NEB #S1510)
- Supelclean[™] ENVI-Carb[™] SPE Tube 100 mg, 1 ml (Sigma, 57109-U)
- Acetonitrile (ACN) HPLC/MS grade
- 50 mM NH₄ Formate buffer pH 4.4 (See Note 1)
- 2-aminobenzamide (2AB, anthranilamide) (Sigma, A89804-5G)
- Sodium cyanoborohydride (Sigma, 156159)
- Dimethyl sulfoxide (DMSO)
- Glacial acetic acid
- Spin SPE HILIC columns (Nest Group Inc., SEM-HIL)

General Protocols (*continued*):

PNGase F Removal using Chitin Magnetic Beads:

In this step, Remove-iT PNGase F is eliminated from the reaction. After the removal of the enzyme, the corresponding samples contain only deglycosylated protein and free glycans. Control samples are processed without performing this step, to determine whether chitin beads affect N-glycan recovery.

- Using a magnetic separation rack, rinse (twice) a 50 μ l aliquot of Chitin Magnetic Beads with 500 μ l with 50 mM NH₄ Formate pH 4.4 (See Note 1).
- Add 100 μ l of the deglycosylation reaction containing Remove-iT PNGase F and rock for 10 minutes at 4°C. Back on the magnetic separation rack, allow the chitin beads to separate. Remove and save the supernatant transferring it to a fresh tube.
- Wash the magnetic chitin beads three times with 100 μ l of 50 mM NH₄ Formate pH 4.4 (or buffer of choice), removing and saving the supernatant after each wash.
- Combine all supernatants (See Note 2).

Glycan Preparation:

Salts and proteins are removed, purified glycans are labeled with a fluorescent tag.

N-glycan Purification:

- Condition an ENVI-Carb™ SPE Tube with 3 ml of acetonitrile (ACN) following by 1 ml of 50% ACN (See Note 3). Equilibrate with 3 ml water.
- Apply deglycosylated sample from Step 9. Discard flow through, graphite will retain N-glycans. Wash twice with 1 ml water. Discard flow through.
- Elute with 300 μ l of 40% ACN/60% 50 mM NH₄ Formate pH 4.4 (See Note 4). Collect N-glycans in a 1.5 ml tube.
- Lyophilize or dry in speedvac (See Note 5).

Fluorescent labeling with 2-aminobenzamide (2AB):

- To dried sample, add 10 μ l of 2AB Labeling Reagent (See Note 6) and 1 μ l 50% acetic acid, mix.
- Transfer to 0.2 ml PCR tubes. Incubate at 65°C for 2 hours (See Note 7).

Cleanup:

- Condition a HILIC spin column with 350 μ l ACN (spin at 1,100 rpm for 1 minute, discard flow through), followed by 350 μ l of 50 mM NH₄ Formate pH 4.4 (spin at 3,000 rpm 1 minute, discard flow through). Add another 50 μ l of 50 mM NH₄ Formate pH 4.4, spin at 1,100 rpm 5 minutes, discard flow through.
- Equilibrate with 350 μ l of 90% ACN/50 mM NH₄ Formate pH 4.4 (spin at 1,100 rpm for 1 minute, discard flow through). Repeat a second time.
- Dilute sample to 85% ACN: add 60 μ l of ACN, mix (See Note 8). Apply to HILIC column, spin at 700 rpm for 3 minutes, discard flow through.
- To remove unbound fluorescent label, wash column with 350 μ l 90% ACN/10% 50 mM NH₄ Formate pH 4.4, spin at 1,100 rpm for 1 minute, discard flow through. Repeat five times.
- Spin at 3,000 rpm for 2 minutes to dry the column.
- Elute 2AB-labeled N-glycans with 100 μ l 50 mM NH₄ Formate pH 4.4., spin at 3,000 rpm for 1 minute. Collect in centrifuge tube.

Liquid Chromatography/Mass Spectrometry (LC/MS)

Hydrophilic Interaction Liquid Chromatography (HILIC) in line with mass spectrometry has been successfully used to separate and identify glycoconjugates (6). 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 the identification of individual glycan species (See Note 9).

- A sample of labeled N-glycans (40 μ l) was diluted with 160 μ l of ACN, in an autosampler vial. The 2AB-labeled N-glycans were separated using a TSK gel Amide-80 column (Tosoh Bioscience LLC) on a Dionex Ultimate LC equipped with fluorescent detection (See Note 10), in line with a Velos LTQ Pro Mass Spectrometer equipped with a heated, electrospray-standard source (HESI-II probe) (See Note 11).

Notes:

- To prepare 50 mM Ammonium Formate Buffer add 1.91 ml formic acid to 1000 ml H₂O, adjust pH to 4.4 dropwise with ammonium hydroxide. Filter using a 0.2 micron filter.
- A detailed protocol can be found at <https://www.neb.com/protocols/2013/01/23/remove-it-pngase-f-magnetic-chitin-bead-protocol-p0706>.
- If flow is too slow, apply gentle positive pressure.
- Low pH is needed to elute sialylated glycans.
- To prevent sialic acid loss, do not overheat.
- Dissolve 5 mg 2 AB in 20 μ l of DMSO, mix. Add 80 μ l water. Add this solution (100 μ l) to 6 mg NaCNBH₄. Use immediately. Discard unused solution following safety regulations.
- A thermocycler provides excellent temperature control and minimizes evaporation. However, any other suitable incubator can be used for this step.
- It is critical to maintain a dilution in 85% acetonitrile: higher organic content might cause some glycans to precipitate out of solution, lower organic content will prevent glycans from binding to the HILIC column.
- 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.
- The glycans were separated using a gradient of 80%:20% to 40:60% ACN:50 mM NH₄ formate pH 4.4 for 34 min at 350 μ l/min, 2 AB fluorescence was measured at 350 (ex)/420 nm (em). Injection vol: 100 μ l
- Optimized settings for positive mode detection of 2 AB-labeled N-glycans.
 - ESI: spray voltage 3.5 kV; capillary temperature 250°C; sheath gas 11 psi, Aux gas and Sweep gas Flow rates 0, S-lens RF level % 66.
 - 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.

Results:

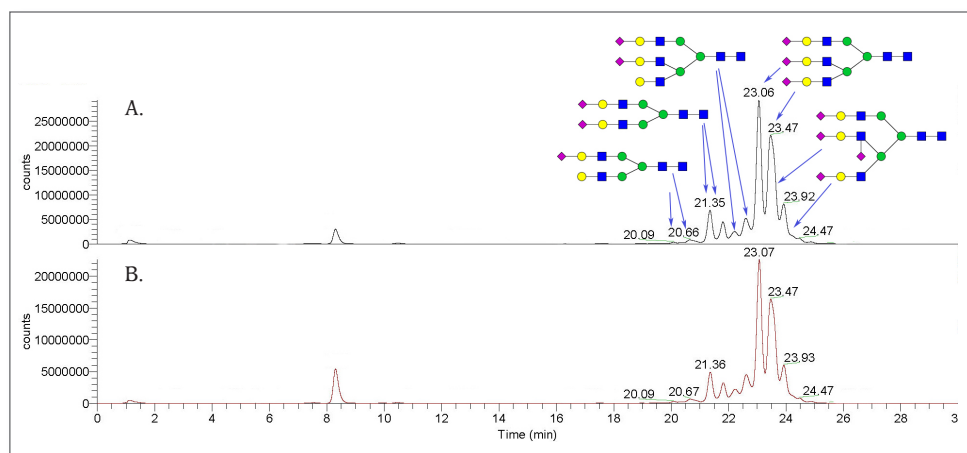
Remove-iT PNGase F: Chitin Beads and *N*-glycan Recovery

It was previously determined that the PNGase F removal step is extremely efficient. Under the conditions described, there is no residual Remove-iT PNGase F in the supernatant (detectable by activity assay or mass spectrometry). Additionally, we observed complete recovery of the target protein (data not shown). In order to demonstrate that no *N*-glycan species, neutral or sialylated, are lost to non-specific binding or entrapment to the chitin magnetic beads, the *N*-glycan-containing supernatant (after Remove-iT PNGase F removal) was compared with a control where chitin beads were not used.

Figure 1 shows the fluorescent trace of these samples, which have identical profiles. These experiments were run in triplicate, to calculate the average abundance of each glycoform. As shown in the lower profile, the overall recovery was around 85%, showing that very little material is lost during the handling of the chitin beads. In summary, there is neither bias or significant loss of *N*-glycan forms after the sample reaction is incubated in the presence of chitin.

Each glycoform species was identified according to its *m/z*: all the known fetuin *N*-glycans were observed (7).

Figure 1. Glycan profile is identical between samples with and without enzyme removal.



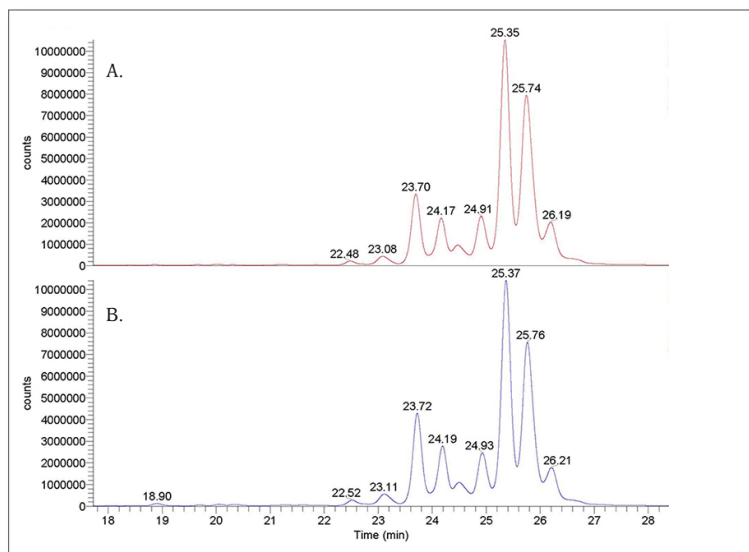
N-glycan was incubated with (A) no chitin beads, and (B) with Remove-iT PNGase F according to recommended conditions. Results indicate 85% recovery after chitin removal, with no loss of sample due to non-specific binding.

Detailed Analysis of *N*-glycan Profiles from Remove-iT PNGase F and Standard PNGase F: Glycan Release Under Denaturing and Native Conditions

As shown in Figure 1, Remove-iT PNGase F removes all expected *N*-glycans from Bovine Fetuin. However, the presence of the CBD tag could result in subtle activity changes. These would become apparent as a bias in the relative abundance of the *N*-glycans released from a given sample. To investigate whether the CBD tag itself imposes constraints in the activity of the enzyme, a series of side-by-side comparisons of Remove-iT PNGase F (used without the removal step) versus PNGase F were performed. Experimental triplicates were used in order to detect minor changes in the relative abundance of the glycans released.

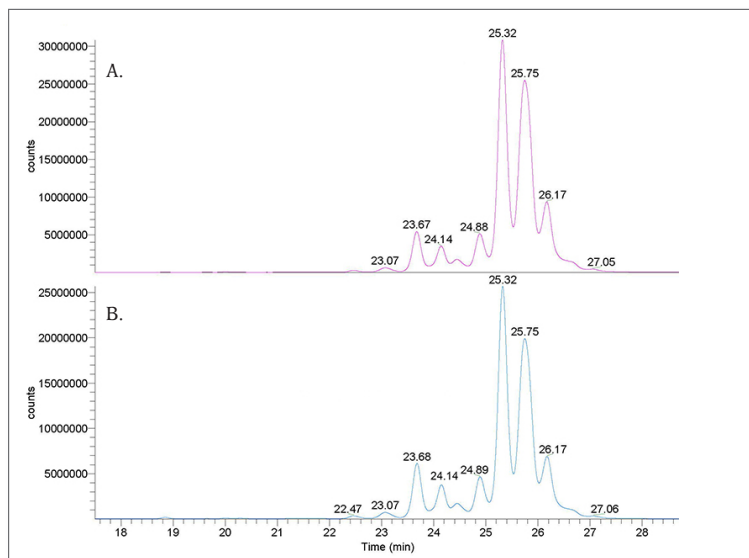
Figure 2 and Figure 3 show the *N*-glycans released under native and denaturing conditions, respectively. In both cases, the performance of Remove-iT PNGase F is identical to the standard enzyme, quantitatively and qualitatively.

Figure 2. Remove-iT PNGase F has Identical Activity as the Native Enzyme on Native Proteins.



Deglycosylation of native Fetuin was performed using (A) Remove-iT PNGase F and (B) PNGase F, (Glycerol-Free). Similar elution patterns were seen in both cases, demonstrating that Remove-iT PNGase F displays similar activity as the native enzyme.

Figure 3. Remove-iT PNGase F has Identical Activity as the Native Enzyme on Denatured Proteins.



Deglycosylation of denatured Fetuin was performed using (A) Remove-iT PNGase F and (B) PNGase F, (Glycerol-Free). Similar elution patterns were seen in both cases, demonstrating that Remove-iT PNGase F displays similar activity as the native enzyme.

Conclusion:

We investigated in detail the properties of Remove-iT PNGase F in comparison to the standard reagent PNGase F; the presence of a CBD tag does not interfere with *N*-glycan release. Additionally, we demonstrated that the convenient enzyme removal step has no effect on the recovery of the target protein, nor does it alter the composition or yield of the *N*-glycan products.

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