

PNGase F N-glycan Releasing Kit

Catalog Number EA006

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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PRINCIPLE OF THE ASSAY

N-Glycosylation found on the asparagine residue of the sequon NXT/S is one of the most common post-translational modifications of proteins in eukaryotic cells. N-glycans are initiated as high-mannose glycan, which are converted to hybrid and complex N-glycans during the maturation process in the Golgi apparatus. N-glycosylation involves in myriad of biological functions such as receptor binding, cell signaling, immune recognition, inflammation, and pathogenicity. To study these N-glycans, it is essential to release them from proteins. PNGase F is a peptide N-glycosidase F from *Flavobacterium meningosepticum* and the most used enzyme to extensively release various N-glycans, including high-mannose, hybrid and complex types of N-Glycans, except those N-glycans with the innermost GlcNAc (GlcNAc1 in figure 1) modified with a core-3 linked fucose. PNGase F digestion results in the release of entire N-glycans and conversion of the underlying asparagine residue (N) to an aspartic residue (D), allowing identification of the glycosylation site by mass spectrometry. The PNGase F N-glycan Releasing Kit provides PNGase F and necessary buffers for gel analysis as well as mass spectrometry analysis. The receptor binding domain of SARS-2 spike protein that is fluorescently labeled on N-glycans is provided as a convenient control, allowing monitoring the deglycosylation process in gel through fluorescent imaging.

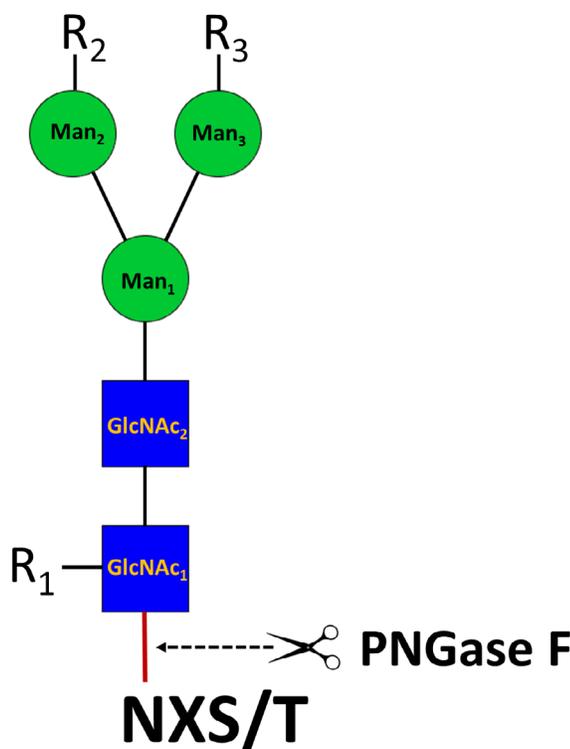


Figure 1: PNGase F is an amidase that cleaves the amide bond between the GlcNAc1 residue of an N-glycan and the underlying asparagine residue. Only the pentasaccharide core of an N-glycan is pictured here for clarity. The pentasaccharide can be extended at R1, R2 and R3 positions. R1 can be an α -6 fucose but not an α -3 fucose. R2 and R3 can be any of common monosaccharides or oligosaccharides. Fluorescent tags can be introduced to R1, R2, and R3 with no obvious change on the sensitivity to PNGase F digestion.

MATERIALS PROVIDED & STORAGE CONDITIONS

PART	PART #	QUANTITY	FORMULATION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
PNGase F Protein	897028	1 x 100 µL	(Yellow cap) 0.5 µg/µL in 25 mM Tris, 150 mM NaCl, pH 7.5.	Aliquot and store for up to 12 months at ≤ -70 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
Cy5-Neu5Ac Labeled RBD Protein (Cy5-RBD)	897027	1 x 100 µL	(Green cap) 0.2 µg/µL in 25 mM Tris, 150 mM NaCl, pH 7.5. <i>Light sensitive (amber tube)</i>	
PNGase F Assay Buffer (10X)	897024	2 x 1 mL	(Clear cap) 1 M Tris, pH 7.5.	
Denaturing Buffer (10X)	897025	1 x 1 mL	(Orange cap) 5% (w/v) Sodium Dodecyl Sulfate, 0.8 M 2-mercaptoethanol.	Store for up to 12 months at ≤ -70 °C in a manual defrost freezer.*
Renaturing Buffer (10X)	897026	1 x 1 mL	(Blue cap) 10% Nonidet™ P-40 (NP-40) Substitute.	
6X SDS Gel Loading Dye	897029	2 x 1 mL	(Brown cap) 0.6% (w/v) SDS, 30% Glycerol, 1 M 2-Mercaptoethanol, 0.04% Bromophenol Blue and 1 M Glycine.	

* Provided this is within the expiration date of the kit.

OTHER MATERIALS REQUIRED

- 37 °C incubator
- Microcentrifuge tubes or equivalent
- Pipettes and pipette tips
- Deionized or distilled water
- Equipment to run SDS-PAGE gel
- Mass Spectrometry Instrumentation
- Fluorescent imager

PRECAUTION

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

ASSAY PROTOCOL

Denaturing Assay Protocol

Reaction volumes can be increased or decreased proportionally. It is recommended to start with concentrated samples so that less sample volume is required, therefore less denaturing buffer will be applied to final reactions to minimize the negative effects of SDS on PNGase F. PNGase F can be directly used or diluted in 1X PNGase F Assay Buffer before usage. A titration on PNGase F maybe performed to determine amount of enzyme is needed for a specific sample protein. For negative controls, replace PNGase F with 1X Assay Buffer. Make sure to mix well all components in each step.

REAGENT PREPARATION

Prepare 1X Assay Buffer by diluting the 10X PNGase F Assay Buffer with deionized or distilled water.

A. Sample Preparation

1. In a centrifuge tube add the following items stepwise, 10 μ L Sample Protein or Cy5-RBD and 1 μ L Denaturing Buffer (10X).
2. Heat at 95 °C for 5 minutes and then chill on ice. Briefly centrifuge condensation.
3. Add 1 μ L Renaturing Buffer (10X)

Note: *The sample protein may be diluted with 1X PNGase F Assay Buffer to a desired concentration here.*

B. PNGase F Digestion

1. Add 5 μ L of Denatured Sample Protein or Cy5-RBD, 14 μ L of 1X Assay Buffer, and 1 μ L PNGase F to a new centrifuge tube.
2. Incubate at 37 °C for 60 minutes to overnight.
3. Add 4 μ L of 6X SDS Gel Loading Buffer to the tube.

C. SDS-Gel Separation and Imaging

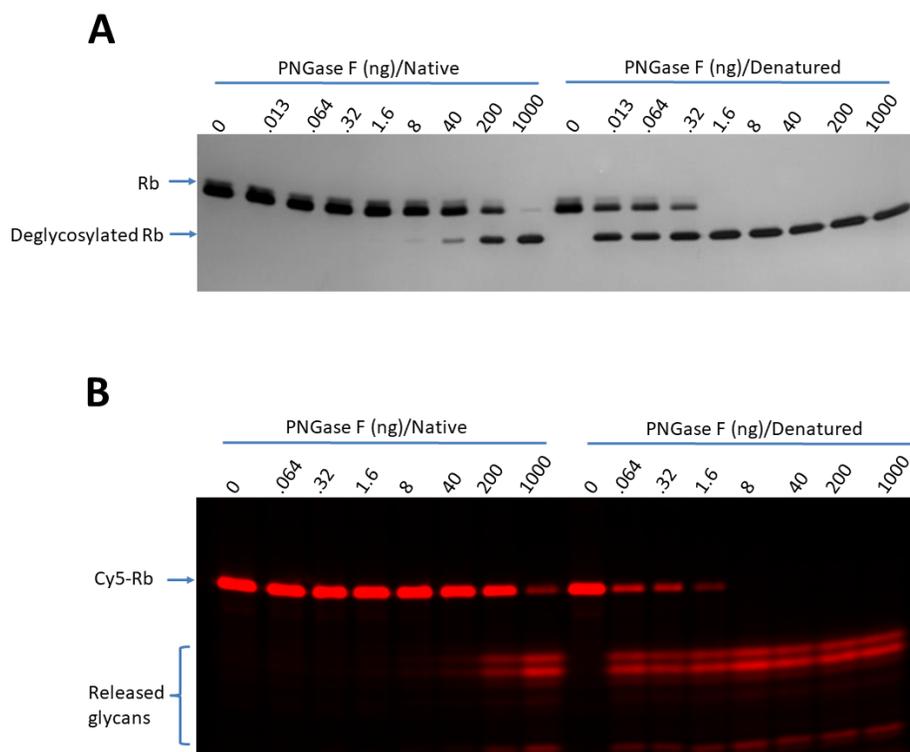
1. Load half of the final digestion volume to an SDS Gel.
2. Run the gel until the dye front reaches the end of the gel.
3. Perform fluorescent imaging first if Cy5-RBD is used as a control.
4. Perform regular protein imaging via Silver, TCE or Coomassie® Blue staining.

NATIVE ASSAY PROTOCOL

Native assay protocol is specifically for samples that are intended for mass spec analysis. Reaction volumes can be increased proportionally. Proteins under native conditions are much more resistant to PNGase F digestion. To completely deglycosylate proteins under native conditions, high concentration PNGase F and longer incubation time are recommended.

DATA AND APPLICATIONS

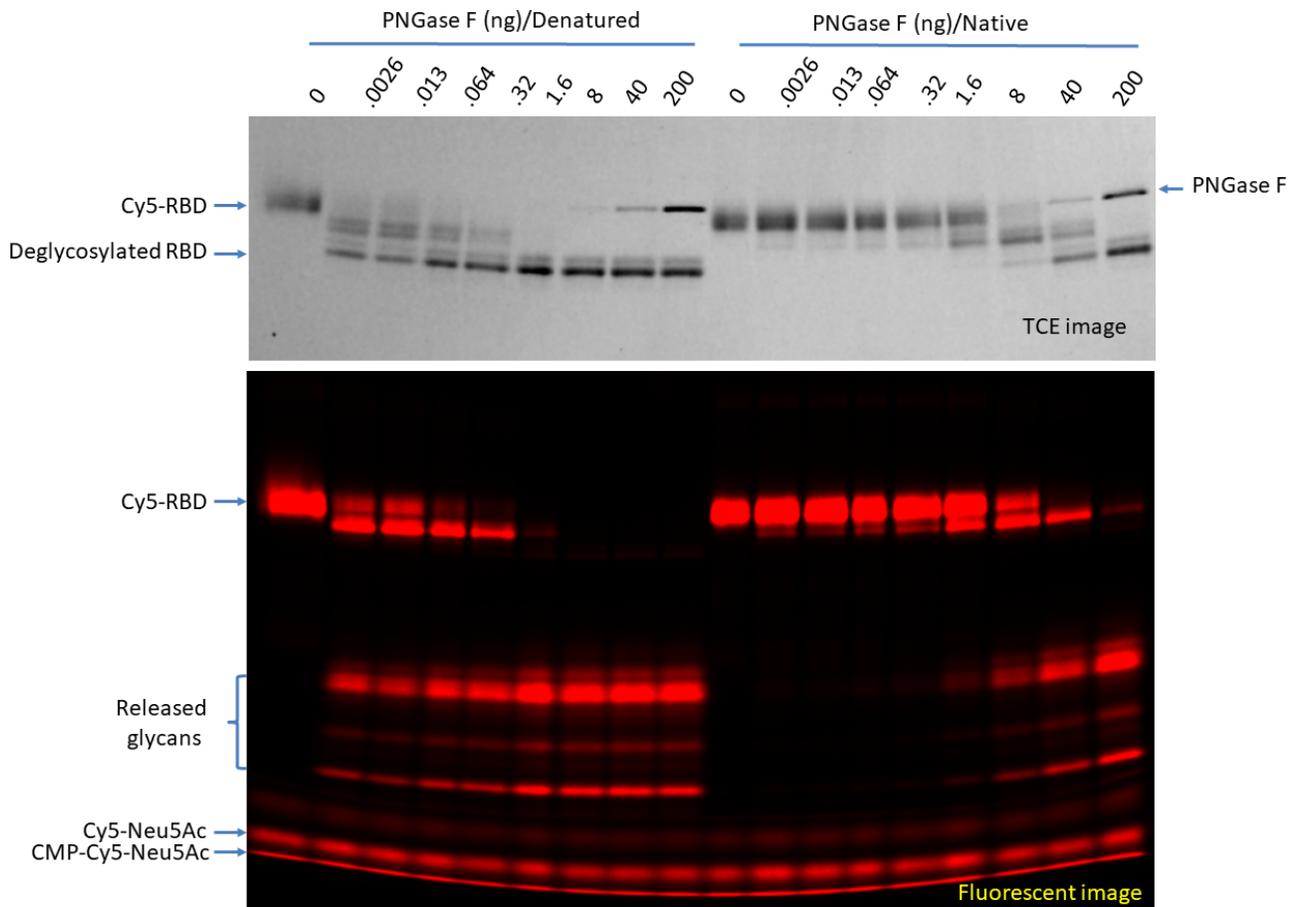
Sample Data 1: Titration of PNGase F on RNase B (Rb) and Cy5-Labeled RNase B (Cy5-Rb) using both native and denaturing assay protocols.



Deglycosylation of Rb (A) and Cy5-Rb (B) with PNGase F N-glycan Releasing Kit (R&D Systems[®], Catalog # EA006) using both the native and denaturing assay protocols. >95% deglycosylation was achieved with 1000 ng and 1.6 ng of PNGase F under native and denaturing conditions, respectively. Digested samples were separated on 17% SDS gel. **A.** Rb (1 μ g per digestion) was digested with indicated amounts of PNGase F and imaged with silver staining. **B.** Cy5-Rb (0.2 μ g for digestion) was digested with indicated amounts of PNGase F. Labeling on RNase B was introduced by serial treatment with Recombinant Human N-Acetylglucosaminyltransferase1/MGAT1, CF (R&D Systems[®], Catalog # 8334-GT)/UDP-GlcNAc, Recombinant Human B4GalT1 Protein, CF (Catalog # 3609-GT)/UDP-Gal, and Recombinant Human ST6GAL1 (aa44-406) Protein, CF (Catalog # 7620-GT)/CMP-Cy5-Sialic Acid (Catalog # ES302) (Wu, Z. *et al.* (2020) *Glycobiology* **30**:970). Denaturing increased the PNGase F sensitivity by 500-fold.

DATA AND APPLICATIONS *CONTINUED*

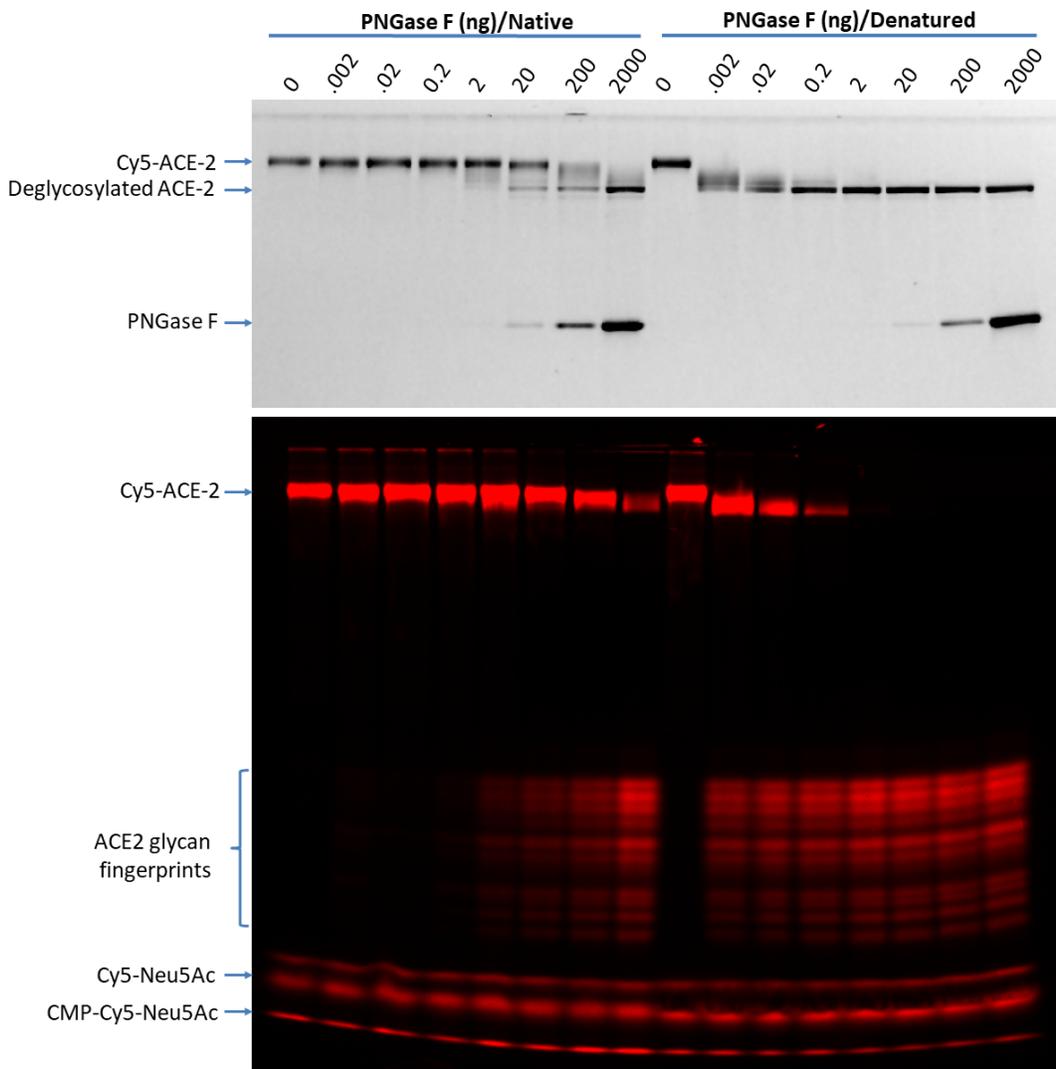
Sample Data 2: Titration of PNGase F on Cy5-Neu5Ac Labeled RBD Protein (Cy5-RBD) using both native and denaturing assay protocols.



Deglycosylation of Cy5-RBD with PNGase F N-glycan Releasing Kit (R&D Systems[®], Catalog # EA006) using both the native and denaturing assay protocols. Cy5-RBD (1 μ g per digestion) was treated with indicated amounts of PNGase F in 20 μ L under both denaturing and native conditions at 37 $^{\circ}$ C for 60 minutes then separated on 17% SDS gel and imaged by trichloroethanol (TCE) staining (top panel, only proteins were visible, and the lower portion was cropped out) and fluorescent imaging (lower panel). >95% deglycosylation was achieved with 200 ng and 1.6 ng of PNGase F under native and denaturing conditions, respectively.

DATA AND APPLICATIONS *CONTINUED*

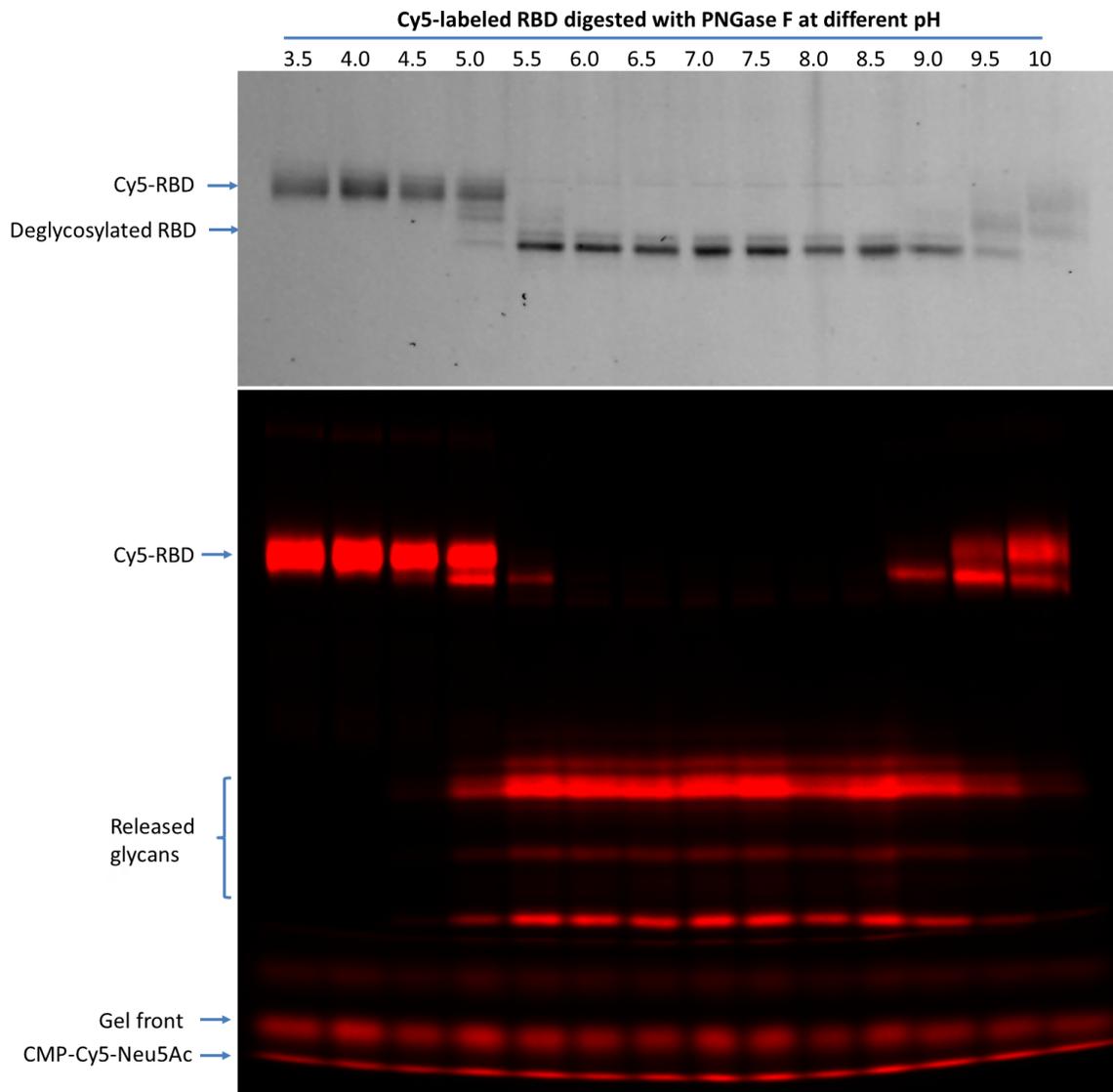
Sample Data 3: Titration of PNGase F on Cy5-Neu5Ac Labeled ACE-2 (Cy5-ACE-2) using both native and denaturing assay protocols.



Deglycosylation of Cy5-ACE-2 with PNGase F N-glycan Releasing Kit (R&D Systems[®], Catalog # EA006) using both the native and denaturing assay protocols Cy5-ACE-2. (1 μ g for each digestion) was treated with indicated amounts of PNGase F in 20 μ L under both denaturing and native conditions at 37 $^{\circ}$ C for 30 minutes then separated on 17% SDS gel and imaged by TCE staining (top panel, only proteins were visible, and the lower portion was cropped out) and fluorescent imaging (lower panel). Multiple bands are visible for the released glycans suggesting the complicated nature of the glycosylation on ACE-2. >90% deglycosylation was achieved with 2000 ng and 0.2 ng of PNGase F under native and denaturing conditions, respectively.

DATA AND APPLICATIONS *CONTINUED*

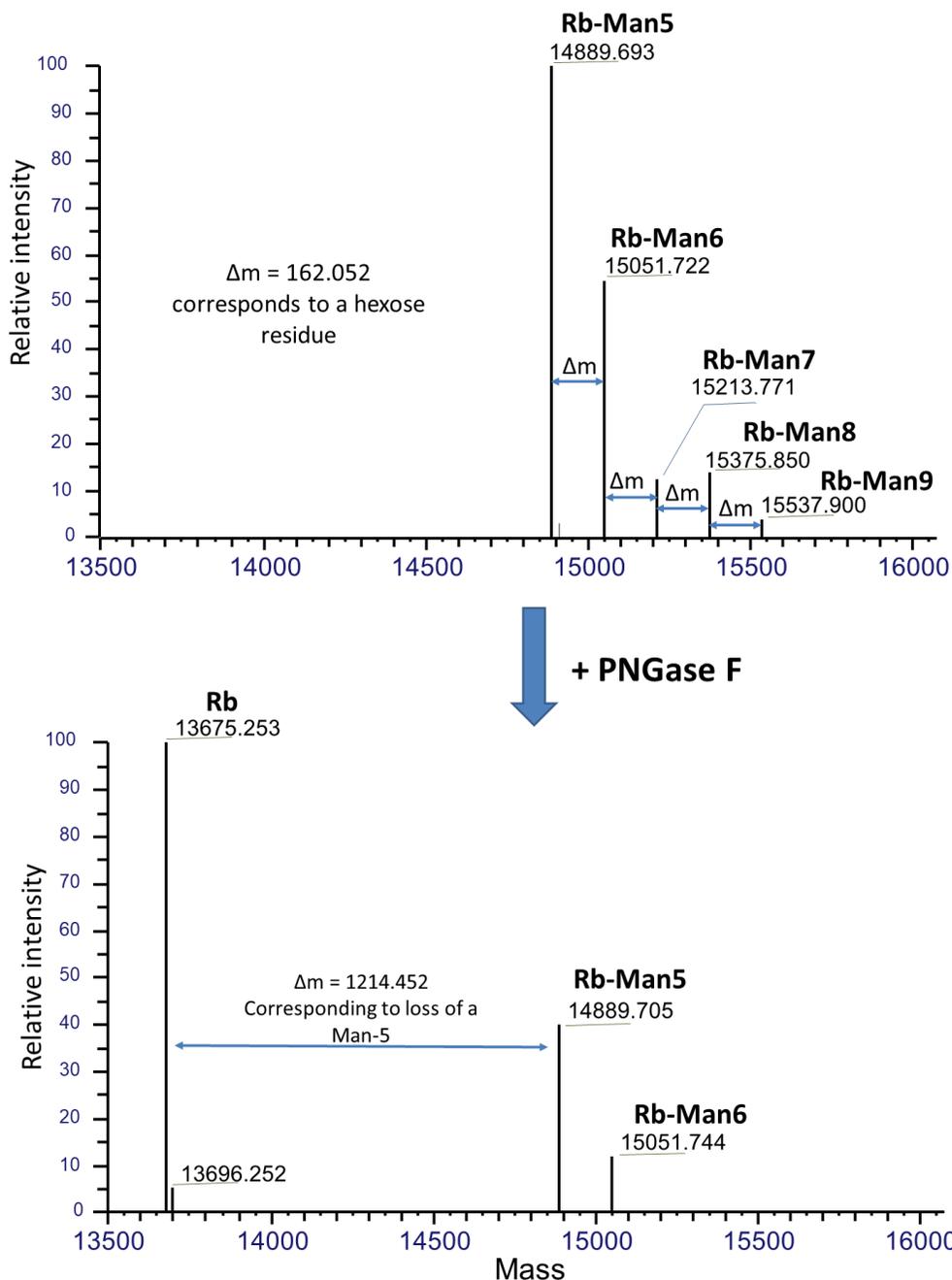
Sample Data 4: PNGase F pH profile test on Cy5-Neu5Ac Labeled RBD Protein (Cy5-RBD) using denaturing assay protocols.



pH profile of PNGase F on Cy5-RBD with PNGase F N-glycan Releasing Kit (R&D Systems[®], Catalog # EA006). Denatured Cy5-RBD (1 μ g per digestion) was treated with 5 ng of PNGase F at indicated pH in 20 μ L at 37 $^{\circ}$ C for 60 minutes then separated on 17% SDS gel and imaged by TCE staining (top panel, only proteins were visible, and the lower portion was cropped out) and fluorescent imaging (lower panel). PNGase F showed activity from pH 5.0 to 10 with complete glycan removal between pH 6.0 to pH 8.5.

DATA AND APPLICATIONS *CONTINUED*

Sample Data 5: Mass spectrometry analysis of PNGase F treated bovine fetal RNase B



Mass spectrum of PNGase F treated fetal bovine RNase B. Native RNase B of different glycoforms with high-mannose glycans, Man-5 to Man-9, are indicated as Rb-Man5 to Rb-Man9 respectively. Upon PNGase F treatment under native condition, majority of these glycans were removed and resulted in deglycosylated RNase B (Rb). Mass spectrometry was performed using a Thermo Scientific Q Exactive HF system connected to a Thermo Vanquish LC. Samples were separated on a C4 column then analyzed using full scan at 240,000 resolution in intact mode. Data was analyzed using Thermo BioPharma Finder software.

TECHNICAL GUIDELINES AND LIMITATIONS

PNGase F is more efficient on denatured protein samples. To achieve comparable level of deglycosylation on native proteins, hundreds to thousands fold more PNGase F maybe needed. In both situations, levels of deglycosylation can be increased with prolonged incubation time.

For mass spectrometry analysis, it is recommended that the sample proteins to be deglycosylated under native conditions. For samples that can only be deglycosylated under denaturing condition, try to reduce the final input of Denaturing Buffer as much as possible.

Ensure that any precipitation present in buffer are redissolved completely before use.

SDS in the Denaturing Buffer inhibits PNGase F activity and is not compatible to mass spectrometry analysis. To reduce the final input of SDS, it is recommended that the initial sample concentration to be as high as possible, so that less sample volume and therefore less SDS will be applied to final PNGase F digestion.

Denaturation processes could be unique to individual proteins. While some proteins are easily denatured, some proteins are more resistant to denaturation. Our denaturing assay protocol guarantees the success of denaturation for most proteins. Failure to denaturation could result in incomplete deglycosylation.

The freed fluorescent glycans are better separated on high percentage SDS polyacrylamide gel. SDS gel with 15-17% polyacrylamide is recommended for glycan separation.

Glycans cannot be fixed in gel and diffuse much faster than proteins. To capture the images of freed glycans, run at constant 20 volts per centimeter of SDS-PAGE gel and take image as soon as possible after gel separation.

Increasing sample loading volumes will result in increasing signals but decreasing gel band resolutions. Optimal sample loading volumes maybe decided based on desired band resolutions and signal intensities.

In the case that the provided assay buffer is not applicable, make sure that the pH of the sample falls between pH 6.0 to pH 8.5 for best results.

End user may have to adjust volumes of assay protocol based on protein or enzyme input.

Glycan labeling reagents ([Sialic Acid Detection](#)/[Fucose Detection](#)) and labeling enzymes ([Sialyltransferases](#)/[Fucosyltransferase](#)) are available from Bio-Techne®/R&D Systems®.

NOTES

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