N-glycan Labeling and Detection Kit

Catalog Number EA007

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

Glycosylation is commonly found on membrane and secreted proteins of eukaryotic cells. N- and O-glycans are among the most common glycans. N-glycans refer to glycans attached to the asparagine residues in the sequon of NXS/T (Asn-X-Ser/Thr, where X can be any amino acid except Pro) of a glycoprotein. N-glycans are initiated as high-mannose glycan in endoplasmic reticulum and are converted to hybrid and complex N-glycans during the maturation process in the Golgi apparatus. In hybrid N-glycan, terminal mannose residues in one branch of the glycan are replaced with a GlcNAc residue. In complex N-glycans, all terminal mannose residues are replaced with GlcNAc residues. The GlcNAc residues are usually elongated with Gal residues that are further terminated with sialic acids. O-glycans usually refer to O-GalNAc and all its derivatives. O-glycans are initiated by attaching a single GalNAc residue to a Ser/Thr residues on proteins via polypeptide GalNAc transferases (ppGalNAcTs). O-GalNAc can be further extended by other sugar residues and become different subtypes, such as Core-1 and Core-2 O-glycans. In some glycoproteins, glycan components can account to more than 50% of their mass. Despite the abundance, labeling and detection of these glycans are extremely difficult.

This kit offers reagents for N-glycan labeling. N-glycan specific labeling is achieved through direct incorporation of Cy3-conjugated Neu5Ac (Cy3-Neu5Ac) or replacement of existing sialic acids with Cy3-Neu5Ac via N-glycan specific sialyltransferase ST6Gal1 (Figure 1 on the next page). Following the provided Assay Protocol, the kit provides sufficient reagents to perform 100 N-glycan labeling reactions. In addition, recombinant human ACE-2 is provided as assay control and Cy3-Neu5Ac labeled recombinant human ACE-2 (Cy3-ACE-2) is provided as a gel control.

In addition to N-glycan/glycoprotein labeling and detection, the kit can also be used to evaluate sialylation levels on glycoproteins.

N-glycans can be labeled with other types of conjugated sialic acids, such as CMP-Cy5-Sialic Acid (R&D Systems®, Catalog # ES302) and CMP-C9-Biotin-Sialic Acid (R&D Systems®, Catalog # ES201). Check our website for the availability of any new activated conjugated sialic acids.

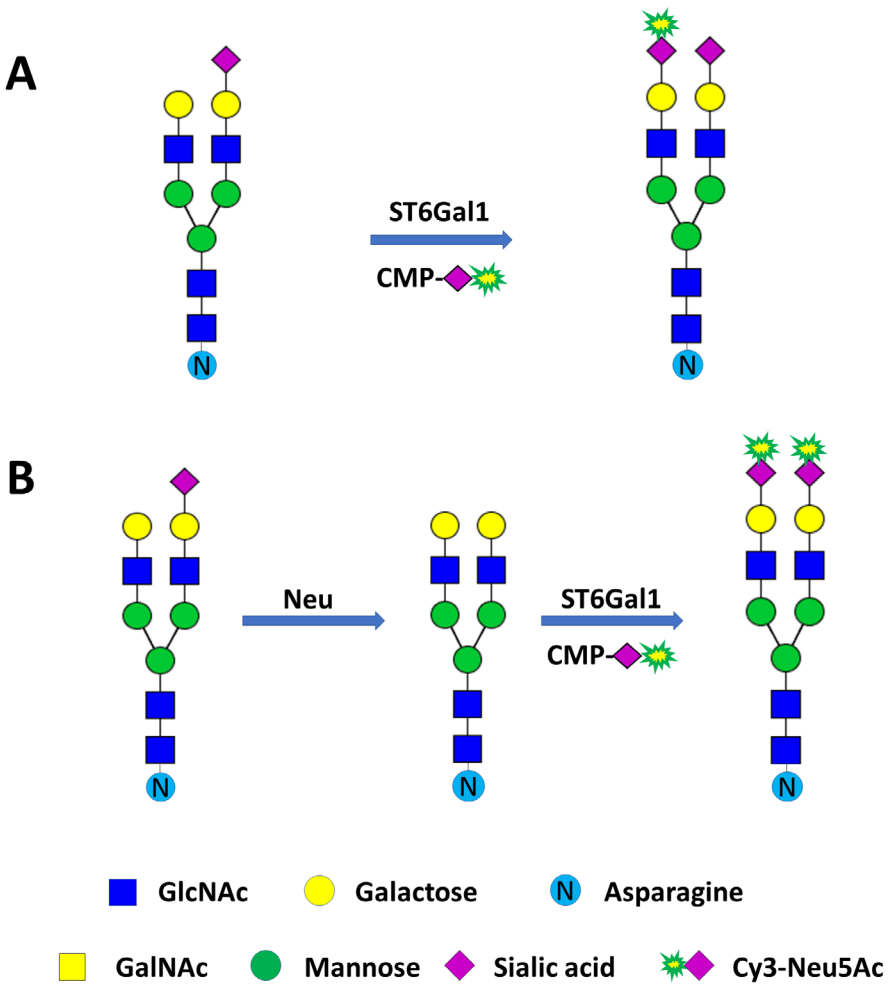


Figure 1: Strategy for N- glycan labeling. Glycan labeling is achieved through direct incorporation of Cy3-conjugated Neu5Ac (Cy3-Neu5Ac) **(A)** or replacement of natural sialic acids with Cy3-Neu5Ac **(B)**. In both strategies, Cy3-Neu5Ac is introduced via recombinant ST6Gal1. In strategy B, samples are first desialylated with recombinant *C. perfringens* Neuraminidase (Neu) that specifically removes α2-3 and α2-6 linked sialic acids. Alternative fluorophore conjugated Neu5Ac donor substrates and sialyltransferases may be used for the labeling.

MATERIALS PROVIDED & STORAGE CONDITIONS

PART	PART #	QUANTITY	FORMULATION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
ST6Gal1 Protein	897066	1 x 100 µL	**(White cap)** 0.2 µg/µL	Aliquot and store for up to 12 months at ≤ -70 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
Neuraminidase Protein	897068	1 x 100 µL	**(Yellow cap)** 0.05 µg/µL	
CMP-Cy3-Neu5Ac	897070	1 x 100 µL	**(Green Cap)** 0.2 mM Light sensitive (amber tube)	
Cy3-Neu5Ac Labeled ACE-2 Protein	897071	1 x 100 µL	**(Green Cap)** 0.2 µg/µL Light sensitive (amber tube)	Store for up to 12 months at ≤ -70 °C in a manual defrost freezer. *
ACE-2 Protein	897069	1 x 100 µL	**(Blue Cap)** 0.2 µg/µL	
6X SDS Gel Loading Dye	897029	1 x 1 mL	**(Brown cap)**	
Glycan Labeling Assay Buffer (10X)	897067	1 x 1 mL	**(Clear cap)**	

* 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
- Fluorescent imager
- 95 °C Heat block (Optional)
- PNGase F N-glycan Releasing Kit (R&D Systems®, Catalog # EA006) (Optional)

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

ST6Gal1 is used for N-glycan labeling. If the sample protein is partially sialylated, Neuraminidase may be omitted from the labeling reaction.

REAGENT PREPARATION

Prepare 1X Glycan Labeling Assay Buffer by diluting the 10X Glycan Labeling Assay Buffer with deionized or distilled water.

N-GLYCAN LABELING REACTION

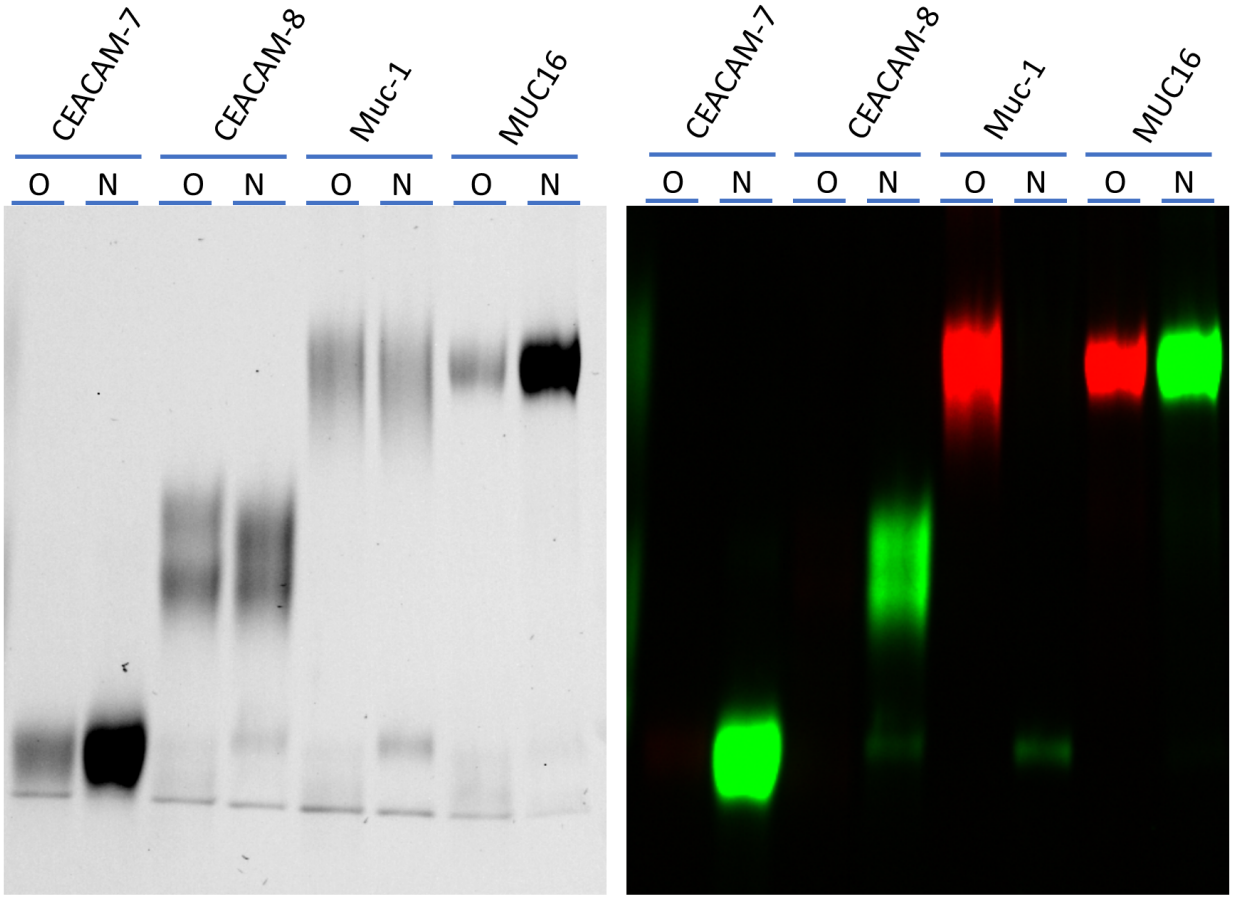
1. Add 1-5 µg of a sample protein, 1 µL CMP-Cy3-Neu5Ac, 1 µL ST6Gal1, and 1 µL Neuraminidase to a test tube.
2. For assay control, assemble a new reaction by replacing the sample protein with 1 µL ACE-2 Protein.
3. Add 1X Assay Buffer to a total reaction volume of 20 µL.
4. Incubate at 37 °C for 60 minutes.
5. N-glycan labeling is complete, proceed to downstream applications such as SDS-PAGE, Cell-based Assay, or PNGase F N-glycan Releasing Kit (R&D Systems®, Catalog # EA006).

SDS-PAGE GEL SEPARATION AND IMAGING (OPTIONAL)

1. For a gel control, add 1 µL of Cy3-Neu5Ac Labeled ACE-2 Protein and 19 µL 1X Assay Buffer to a new test tube.
2. Add 6X SDS Gel Loading Buffer to all the above tubes.
3. Heat the tubes at 95 °C for 2 minutes.
4. Load reaction to SDS-PAGE Gel.
5. Run the gel until the dye front reaches the end of the gel.
6. Perform fluorescent imaging first.
7. Perform regular protein imaging via Silver, TCE or Coomassie® Blue staining.

DATA AND APPLICATIONS

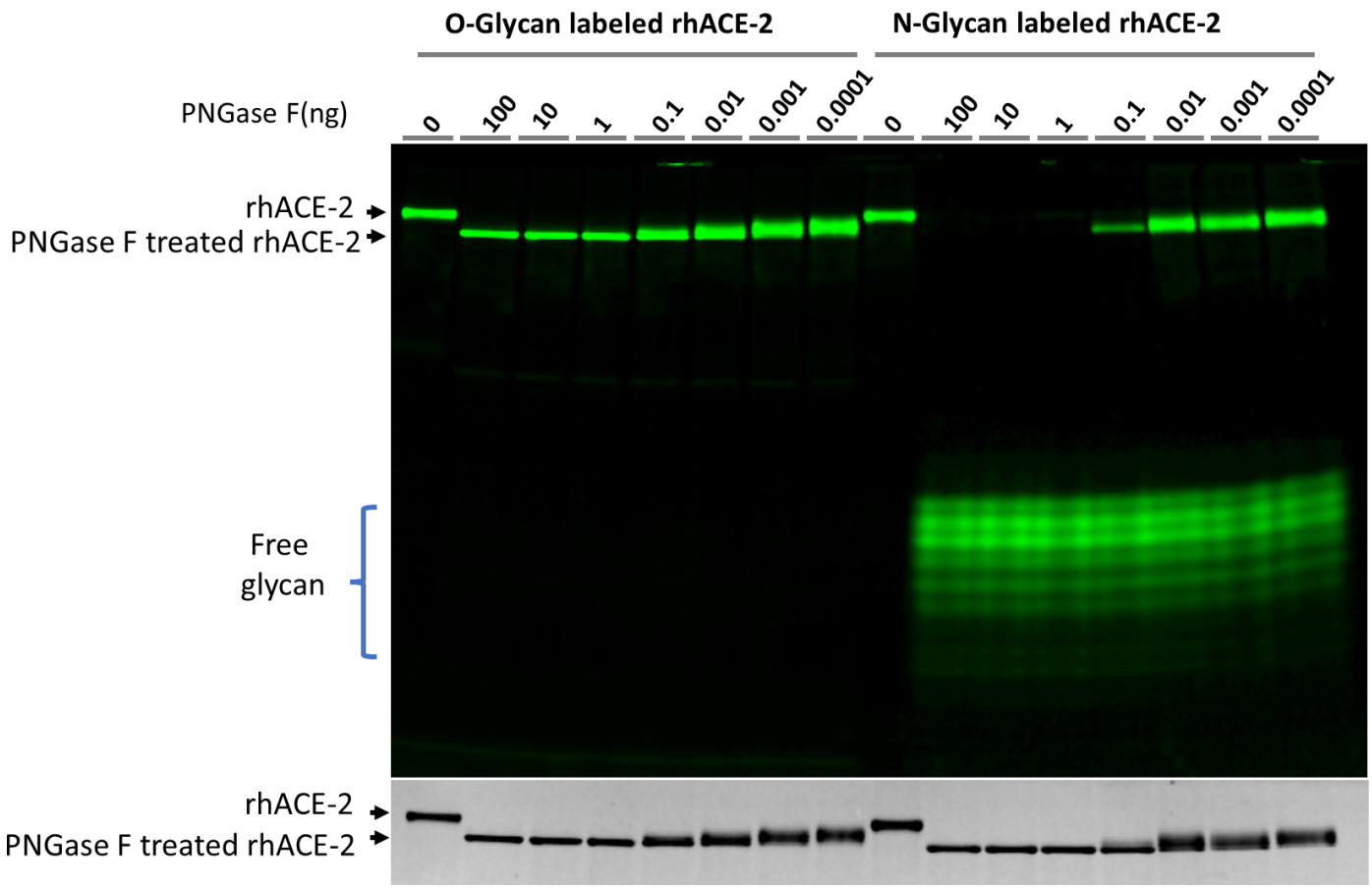
Specific detection of N- and O-glycans on different glycoproteins.



Sample Data 1: Recombinant Human CEACAM-7 (R&D Systems®, Catalog # 9010-CM), Recombinant Human CEACAM-8/CD66b Protein, CF (Catalog # 9639-CM), Recombinant Human MUC-1 Fc Chimera Protein, CF (Catalog # 10332-MU), Recombinant Human MUC16 Protein, CF (Catalog # 5609-MU) were labeled on N-glycan (N) by ST6Gal1 with Cy3-Neu5Ac according to the protocol and on O-Glycan (O) by Recombinant Human ST3Gal2 Protein, CF (Catalog # 7275-GT) with Cy5-Neu5Ac and then separated on 4-20% gradient SDS-PAGE gel. Only N-glycans were detected on CEACAM-7 and CEACAM-8, and only O-glycans were detected on MUC-1, both N- and O-glycans were detected on MUC16. The left part is the TCE image of the gel and the right part is the fluorescent image.

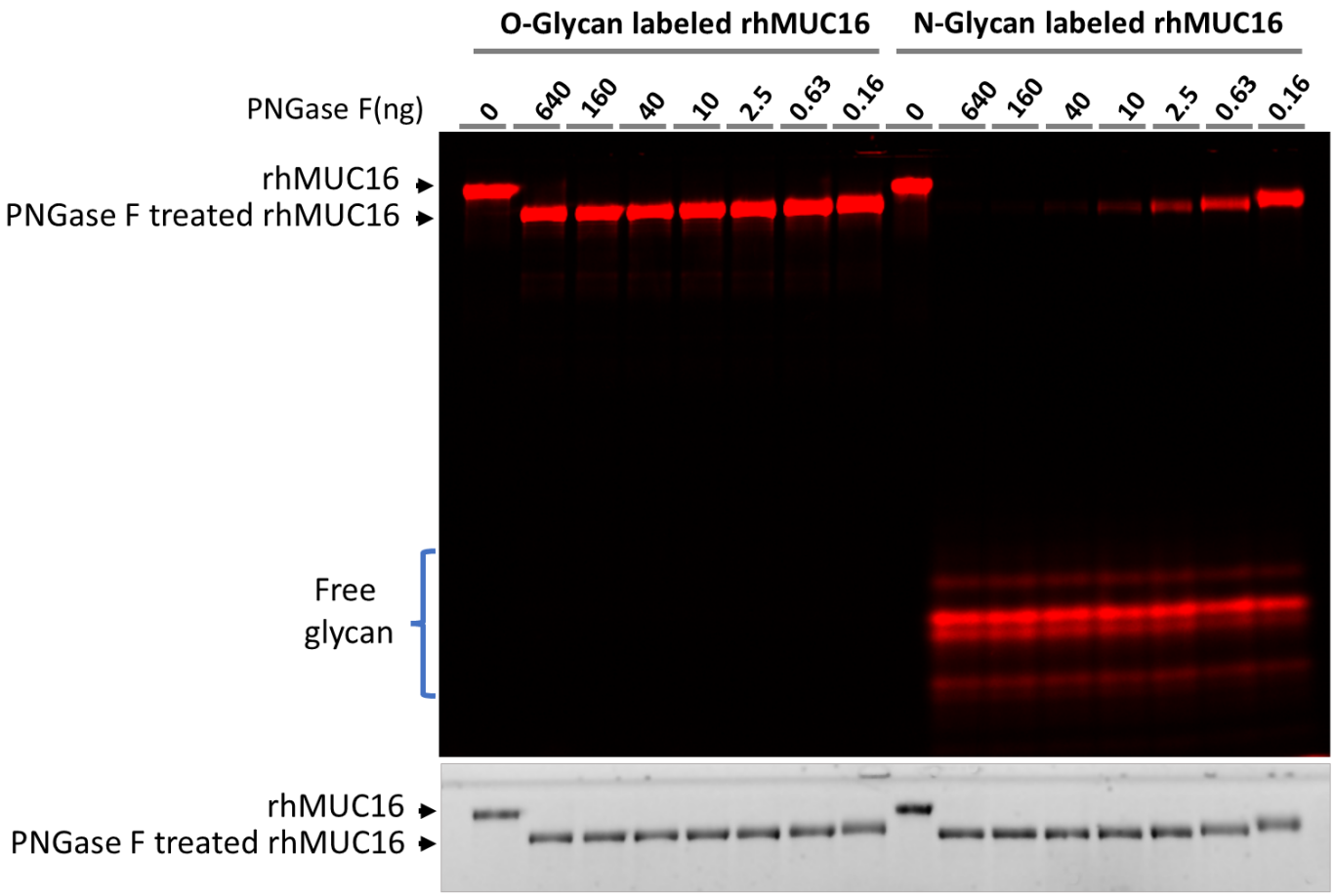
DATA AND APPLICATIONS *CONTINUED*

N-glycan and O-glycan specific labeling of recombinant human ACE-2.



Sample Data 2: N-glycan labeled recombinant human (rh) ACE-2 was prepared according to the above protocol and O-glycan labeled rhACE-2 was prepared in the same way except that ST6Gal1 was replaced with O-glycan specific ST3Gal2. The labeled samples were then treated with 10-fold serial dilution of PNGase F starting with 100 ng using the PNGase F N-glycan Releasing Kit (R&D Systems®, Catalog # EA006). The digestions were separated on 17% SDS-PAGE gel. The upper part is the fluorescent image and the lower part is the TCE image of the gel. While all labeled N-glycans could be released by PNGase F N-glycan Releasing Kit, no labeled O-glycans were released by PNGase F, demonstrating the specific labeling on N- and O-glycans by ST6Gal1 and ST3Gal2, respectively.

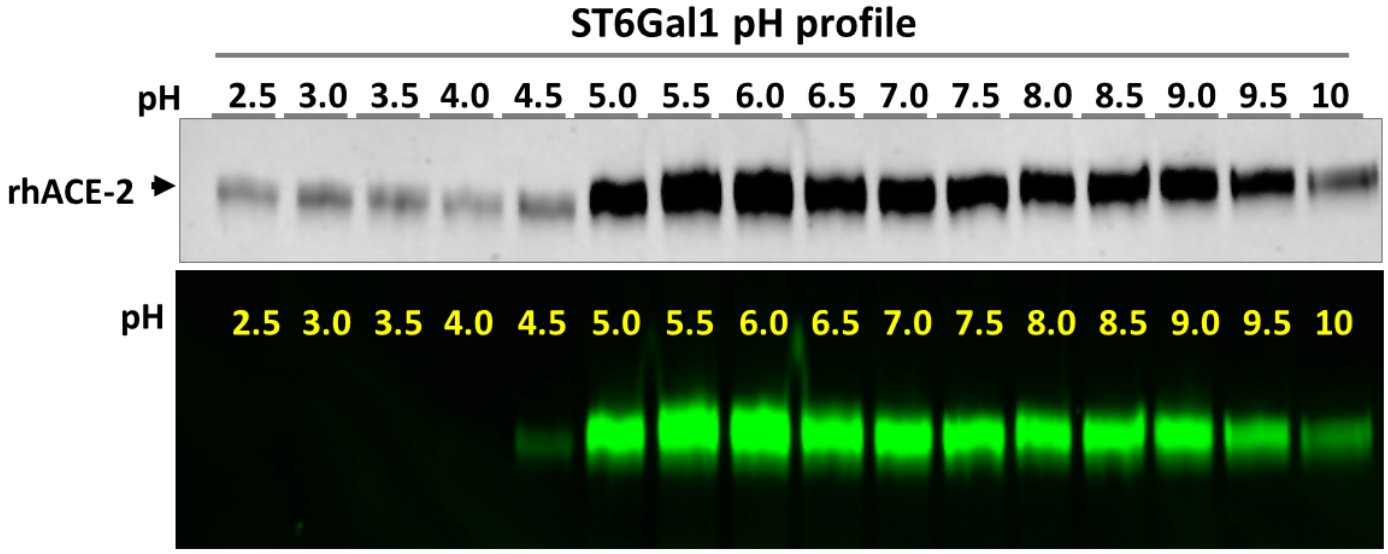
N-glycan and O-glycan specific labeling of recombinant human MUC16.



Sample Data 3: Recombinant Human MUC16 Protein, CF (R&D Systems®, Catalog # 5609-MU) was prepared according to the above protocol except CA125/MUC16 that Cy3 was replaced with Cy5 and ST6Gal1 was replaced with ST3Gal2 for O-glycan labeling. The labeled samples were treated with 4-fold serial dilution of PNGase F starting with 640 ng using the PNGase F N-glycan Releasing Kit (R&D Systems, Catalog # EA006). The digestions were separated on 17% SDS-PAGE gel. The upper part is the fluorescent image and the lower part is the TCE image of the gel. While all labeled N-glycans could be released by PNGase F N-glycan Releasing Kit, no labeled O-glycans were released by PNGase F, demonstrating the specific labeling on N- and O-glycans by and ST6Gal1 and ST3Gal2, respectively.

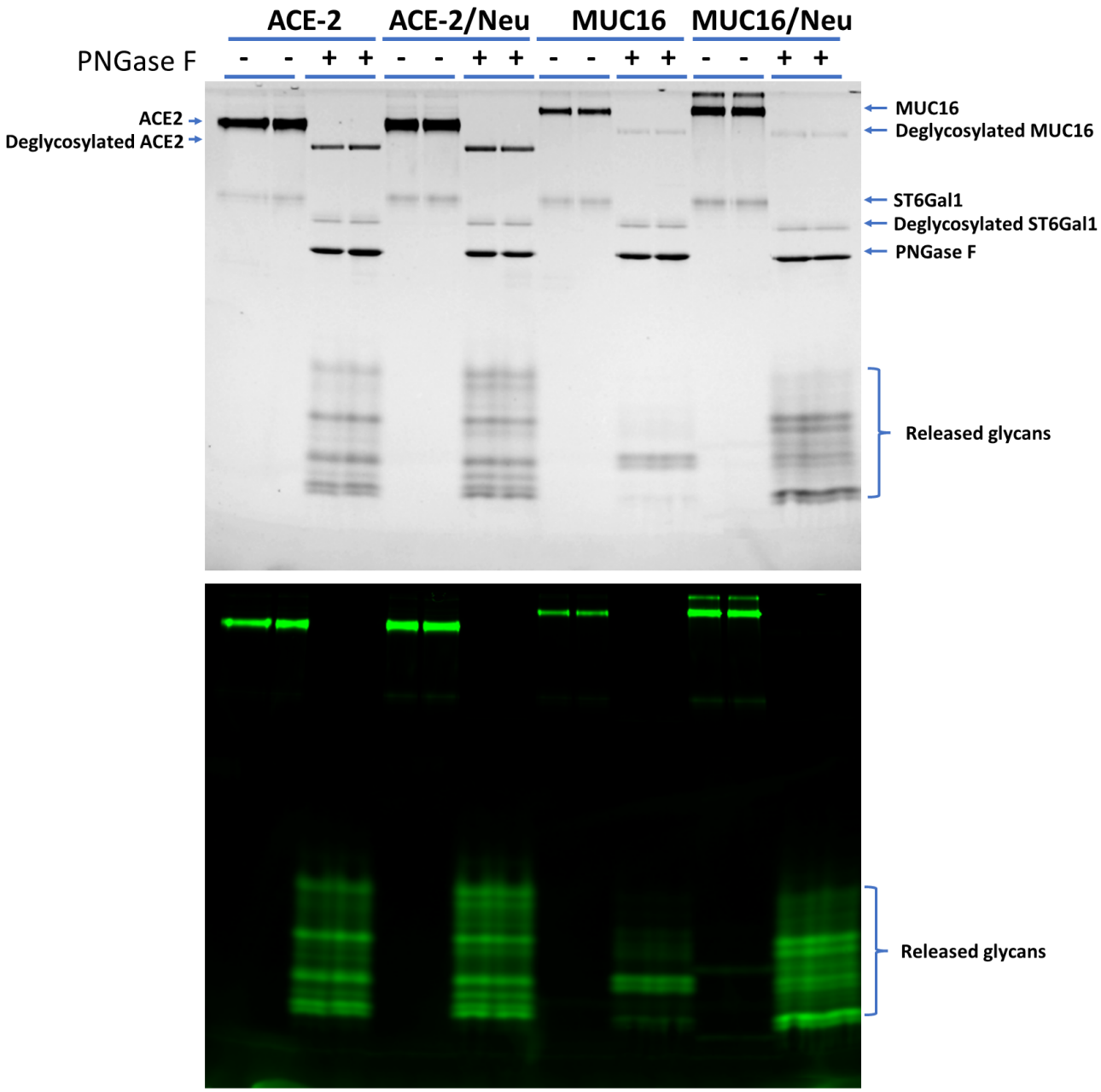
DATA AND APPLICATIONS *CONTINUED*

Determining the optimal pH for ST6Gal1 labeling.



Sample Data 4: Optimal pH determination of a sialyltransferase was an extremely difficult task in the past. Using the N-glycan Labeling and Detection Kit (R&D Systems®, Catalog # EA007), this task becomes rather easy. In this figure, recombinant human ACE-2 was labeled by ST6Gal1 at different pH following the provided protocol except with different buffers. The labeled reactions were then separated on a 4-20% gradient SDS-PAGE gel. Both TCE protein image and fluorescent image are shown. Incorporation of Cy3 to rhACE-2 resulted much intensified band absorption in TCE images. While ST6Gal1 is active from pH 4.5-10, its optimal pH ranges from 5-9.

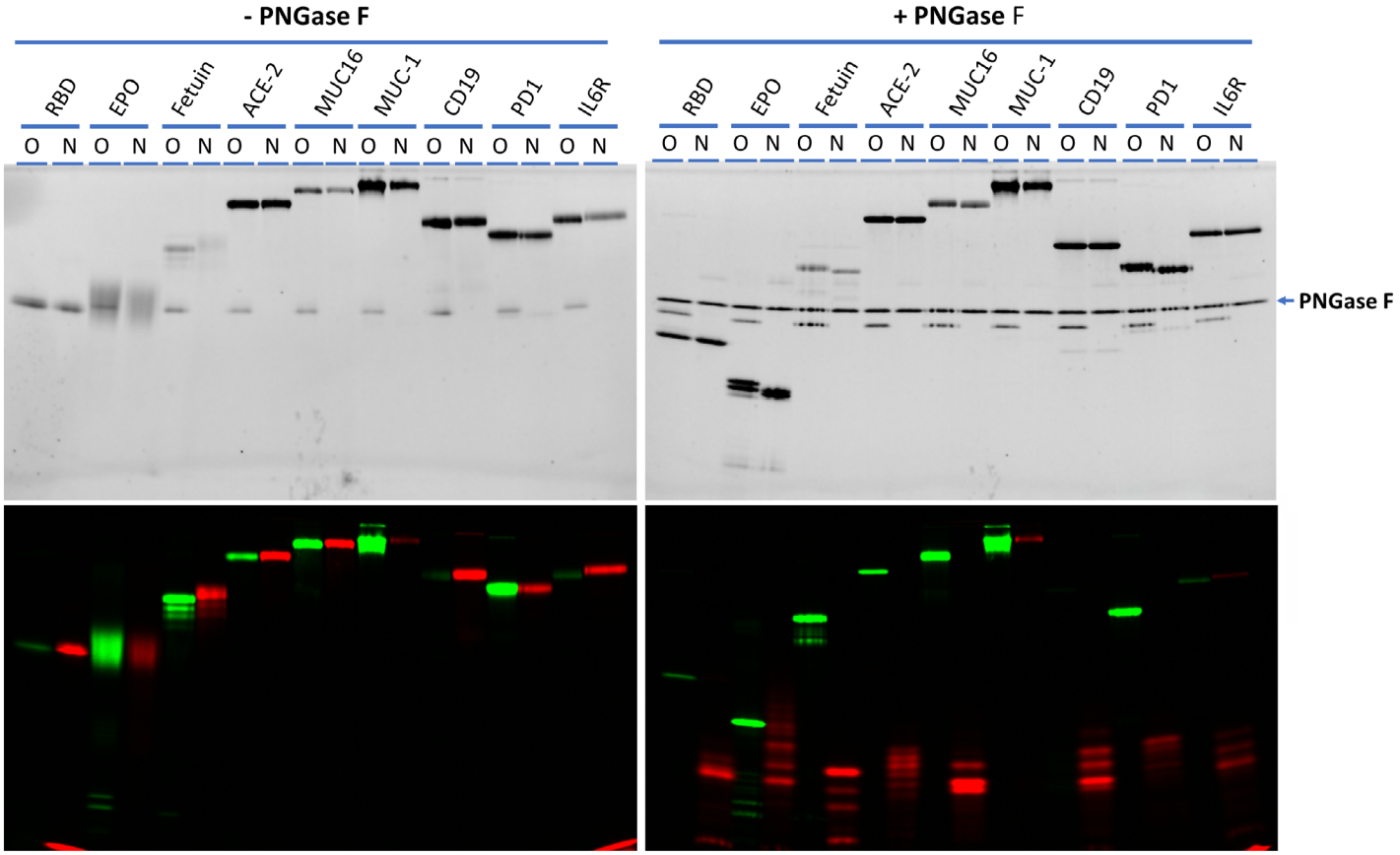
Effect of neuraminidase treatment on N-glycan labeling.



Sample Data 5: Recombinant Human ACE-2 Protein, CF (R&D Systems®, Catalog # 933-ZN), Recombinant Human CA125/MUC16 Protein, CF (Catalog # 5609-MU), without or with Neuraminidase (Neu) treatment were labeled by ST6Gal1, then digested with PNGase F N-glycan Releasing Kit (Catalog # EA006) and finally separated on 17% SDS-PAGE gel. Both the protein image stained with TCE (upper panel) and the fluorescent image (lower panel) are shown. The freed glycans are visible in both images. While Neuraminidase treatment slightly increased labeling on ACE-2, it greatly enhanced the labeling on MUC16, indicating that the majority N-glycans on MUC16 were initially sialylated. This data demonstrates that the kit is also a good tool to evaluate the sialylation levels on target proteins.

DATA AND APPLICATIONS *CONTINUED*

Effect of neuraminidase treatment on N-glycan labeling.



Sample Data 6: A collection of glycoproteins was labeled on O-glycans with Cy3-Neu5Ac (green) by ST3Gal2 and N-glycans with Cy5-Neu5Ac (red) by ST6Gal1. The left side are gel images of labeled samples without PNGase F treatment. The right side are gel images of the samples after treatment with PNGase F N-glycan Releasing Kit (R&D Systems®, Catalog # EA006). While MUC-1 mainly contains O-glycans and CD19 mainly contains N-glycans, the other proteins contain both N- and O-glycans at different levels. All labeled N-glycans in (red) except that on MUC-1 could be released by PNGase F N-glycan Releasing Kit, further confirming the specific labeling. The nature of the labeling on MUC-1 by ST6Gal1 is not clear. All samples were separated on 17% SDS-PAGE gel. Recombinant SARS-CoV-2 Spike RBD His-tag Protein, CF (RBD) (Catalog # 10500-CV), and Recombinant Human Erythropoietin/EPO Protien, Recombinant Human ACE-2 Protein, CF (Catalog # 933-ZN), Recombinant Human CA125/MUC16 Protein, CF (Catalog # 5609-MU), Recombinant Human MUC-1 Fc Chimera Protein, CF (Catalog # 10332-MU), Recombinant Human CD19 Fc Chimera Protein, CF (Catalog # 9269-CD), Recombinant Human PD-1 Fc Chimera Protein, CF (Catalog # 1086-PD) and Recombinant Human IL-6R alpha Protein (Catalog # 227-SR) were from Bio-Techne®. Fetal bovine fetuin was from Sigma Aldrich®.

TECHNICAL GUIDELINES AND LIMITATIONS

Successful labeling is dependent on the unmodified terminal Gal residues on N-glycans. Natural glycoproteins usually contain a certain percentage of unmodified terminal Gal residues, which allows labeling without desialylation. Maximal labeling can be achieved after desialylation with the provided neuraminidase[1].

Glycans such as high mannose N-glycans will not be labeled for lacking terminal Gal residues. To label these glycans, it is necessary to introduce terminal Gal residues, which can be achieved via different strategies. For example, terminal Gal residue can be introduced to oligomannose by successive treatment with Recombinant Human N-Acetylglucosaminyltransferas1/MGAT1 Protein, CF (R&D Systems®, Catalog # 8334-GT) and Recombinant Human B4GalT1 Protein, CF (Catalog # 3609-GT) or Recombinant Human B4GalT2 Protein, CF (Catalog # 7530-GT)[2].

Glycans with terminal α2-8 linked sialic acid is resistant to Recombinant *C. perfringens* Neuraminidase Protein, CF (Catalog # 5080-NM) treatment. To remove α2-8 linked sialic acid, it is recommended to use Recombinant *M. viridifaciens* Neuraminidase Protein, CF (Catalog # 5084-NM).

The labeling protocol provided is not designed for maximal labeling. To achieve maximal labeling, the input of CMP-Cy3-Neu5Ac maybe increased and/or the labeling reaction time may be prolonged.

ST6Gal1 (MW ranges 40-50 kDa) is a glycoprotein itself. It can exhibit some level of self-labeling. If a target protein has similar molecular mass with the labeling enzyme, enzyme only labeling reactions are recommended so to distinguish the labeled target protein from the self-labeled enzyme.

The kit may be used for labeling N-glycans on cell surface, but we do not guarantee the success of the labeling as cells are much more sensitive to buffers and medium and enzyme treatment [3, 4].

In cases when the neuraminidase and the labeling enzyme need to be removed before the next application, they may be precipitated by nickel beads as those enzymes contain 6X His-tag.

Since Cy3 has strong absorption in TCE imaging, incorporation of Cy3 to target proteins will result in intensified protein bands in a TCE image.

For labeling and detection of O-glycans, it is recommended to use O-glycan specific sialyltransferases, such as Recombinant Human ST3Gal1 Protein, CF (Catalog # 6905-GT) or Recombinant Human ST3Gal2 Protein, CF (Catalog # 7275-GT).

REFERENCES

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2. Wu, Z.L., Whittaker, M., Ertelt, J.M., Person, A.D. & Kalabokis, V. Detecting substrate glycans of fucosyltransferases with fluorophore-conjugated fucose and methods for glycan electrophoresis. Glycobiology **30**, 970-980 (2020).
3. Wu, Z.L. Person, A.D., Anderson, M., Burroughs, B., Tatge, T.J., Khatri, K., Zou, Y., Wang, L., Geders, T., Zaia, J., Sackstein, R. Imaging specific cellular glycan structures using glycosyltransferases via click chemistry. Glycobiology **28**, 69-79 (2018).
4. Wu, Z.L. Person, A.D., Zou, Y., Burton, A.J., Singh, R., Burroughs, B., Fryxell, D., Tatge, T.J., Manning, T., Wu, vG., Swift, K.A.D., Kalabokis, V. Differential distribution of N- and O-Glycans and variable expression of sialyl-T antigen on HeLa cells-Revealed by direct fluorescent glycan imaging. Glycobiology **30**, 454-462 (2020).