

## High-Throughput Sialylation Screening on Octet® Label-Free Kit on Instrument for Expediting Clone Selection Process in Cell Line Development

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### Abstract

A high-throughput method for relative screening of terminal sialic acid content was developed on the Octet® platform to expedite cell line development. The method is based on the use of Octet® Sialic Acid (GlyS) Kit to bind sialic acid on glycoproteins. The GlyS Kit can specifically screen the sialylation levels of secreted proteins in crude cell culture samples and does not require purified samples. Using this method, 96 crude cell culture samples can be screened for sialylation in 60 minutes or less. Here we used the GlyS Kit to determine the relative sialylation level of representative glycoproteins including erythropoietin (EPO), standard human mAb samples (NIST mAb), fetuin glycoprotein, as well as crude biosimilar cell culture samples using Octet® RH96 system. In a comparison study between the Octet® GlyS Kit method and HPLC, a linear correlation ( $R^2 > 0.9$ ) was observed between the GlyS Kit binding signal (nm/ $\mu\text{g}$ ) obtained using an Octet® RH96 system and the total sialic acid content measured by HPLC. This GlyS Kit method enables users to screen relative sialylation levels in crude samples in a high throughput manner for the samples ranging between 1–25 mol of total sialic acid per mol of protein, accelerating the process to monitor quality attributes early in cell line development for therapeutic proteins.

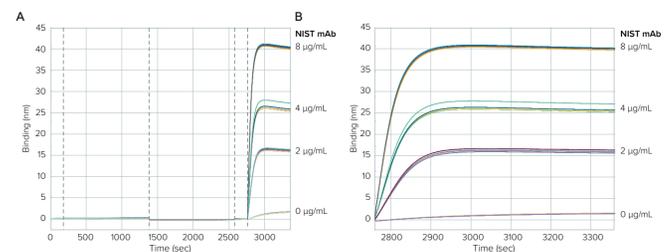
### Introduction

Glycosylation is considered among the most important post-translational modifications when developing new biologics. Having a significant impact on product performance and variability, glycosylation is a critical quality attribute (CQA) influencing product safety and efficacy. Protein glycosylation can affect isolation and purification steps (process consistency), pharmacokinetics (half-life) properties and in vitro stability (product shelf-life). Sialylation is especially important as it significantly affects the safety and efficacy of therapeutic glycoproteins<sup>1</sup>. Many commercial recombinant therapeutic glycoproteins are produced with cell lines from CHO (Chinese Hamster Ovary) host systems because of well-established robust processes. The glyco-forms of therapeutic glycoproteins produced in CHO are similar to human glyco-forms<sup>2</sup>. It is important to note that human glycoproteins contain (α-2,6)-linked sialic acid residues whereas the CHO cell derived antibodies contain (α-2,3)-linked sialic acid residues<sup>3</sup>. The measurement of sialic acid during early stages of cell line screening is a challenging task because of low concentrations and volumes of secreted proteins. The current high-throughput analytical methods for quantifying sialic acid require high concentrations of purified proteins and the methods can only be applied during late stages of cell line screening<sup>4</sup>. Here we use the Sialic Acid (GlyS) Kit which specifically binds terminal sialic acid for high throughput screening to determine relative sialylation levels in therapeutic glycoproteins. We outline the Sialic Acid (GlyS) Kit method, which can be combined with the titer biosensor method such as ProA using Octet® Data Analysis HT software version 11.1 or higher to funnel through proteins of interest that are high producers and have desirable sialic acid content.

## Methods and Results

### NIST Human mAb Terminal Sialic Acid Detection

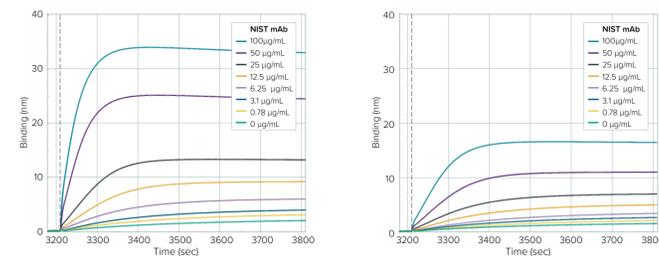
In this study, NIST human mAb reference material 8761 (RM8671, NIH) was chosen as a representative N-glycan human IgG sample to determine the terminal sialic acid content using the GlyS Kit on the Octet® platform. Several different concentration of NIST human mAb (8  $\mu\text{g}/\text{mL}$ , 4  $\mu\text{g}/\text{mL}$ , 2  $\mu\text{g}/\text{mL}$  and 0  $\mu\text{g}/\text{mL}$ , n=4 per each concentration) in Glycan Sample Prep Buffer (GSB) buffer were used in the Octet® binding study. We observed a significant binding signal from single-digit  $\mu\text{g}/\text{mL}$  NIST mAb samples using the GlyS Kit method as shown in Figure 1. Note that the concentrations depend on the specific protein type and level of glycosylation, but a similar concentration series can be performed for IgG samples to determine optimal binding signal.



**Figure 1: Detection of terminal sialic acid from NIST human mAb samples.** A) Binding signals (nm) of varying concentration of NIST mAbs to the GlyS lectin biosensor. A standard amplification assay workflow for human-IgG samples without the Sidekick Station was used via 16-channel mode on the Octet® RH96 instrument. B) Zoomed-in view of the detection step signal.

### Detection of Terminal Sialic Acid Content from NIST Human mAb Sample in the Presence of the Crude Host CHO Cell Supernatant

To see the effect of the crude host cell glycoproteins on the NIST mAb binding to the biosensor, the same NIST mAb samples were diluted in GSB (Figure 2A) or CHO cell supernatant (Figure 2B), which doesn't express human IgG, in a side by side manner. The experiment was designed to mimic the crude sample IgG binding condition to the GlyS lectin biosensor. The dynamic range of the binding signals of NIST mAb, which was diluted in CHO cell supernatant (crude NIST mAb, Figure 2B), was compressed compared to that of GBS (purified NIST mAb, Figure 2A) indicating the CHO host cell glycoproteins were directly competing with NIST mAb to bind to the GlyS biosensor. This was expected as host cell glycoproteins also contain terminal sialic acids. The GlyS Kit was designed to specifically monitor the binding signal of the protein of interest by using a secondary antibody specific to the protein of interest (anti-human-IgG Ab), negating the host cell glycoprotein binding signals in the sample. As shown in Figure 2B, the relative binding ranking of varying concentration of NIST mAb in crude CHO cells was consistent to that of the purified NIST mAb data (Figure 2A). This data indicates the GlyS Kit can be used for crude sample screening without a sample purification step.



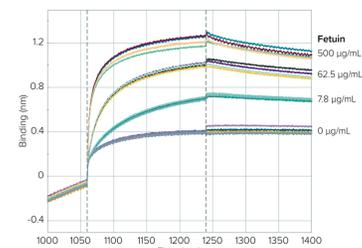
**Figure 2: Detection of terminal sialic acid from crude human mAb samples.** A) Binding signals (nm) of varying concentrations of NIST mAb from 0.78 to 100  $\mu\text{g}/\text{mL}$  (purified human IgG, NIST.gov, mAb-RM8671) to the GlyS biosensor were obtained using the Octet® RH96 system. Zoom-in view of the detection step signals are shown. B) Binding signals (nm) of the same NIST mAb diluted in the crude CHO cell supernatant (-Ab). Experiment 2A and 2B were done side by side. Even in the presence of host cell glycoproteins, the relative binding ranking of varying concentrations of NIST mAb was consistent compared to that of the purified NIST mAb data (2B vs. 2A).

### Detection of Terminal Sialic Acid from FC-Fusion Tagged Erythropoietin Sample (EPO): Non-mAb Therapeutic Protein Sample

In this study, an Fc-fusion tagged erythropoietin sample was chosen as a representative Fc-fusion tagged glycoprotein sample (non-mAb) to determine the terminal sialic acid using the GlyS Kit on the Octet® platform. Varying concentrations of EPO protein from 0.15  $\mu\text{g}/\text{mL}$  to 2.5  $\mu\text{g}/\text{mL}$  were used in the binding assay together with the rabbit Anti-EPO Polyclonal antibody as a secondary antibody specific to EPO protein. We observed the significant dose-dependent binding signals of EPO to the GlyS biosensor with ng/mL of protein using the amplification assay.

### Fetuin Glycoprotein Direct Assay (Purified Highly Sialylated Proteins: Non-mAb Sample)

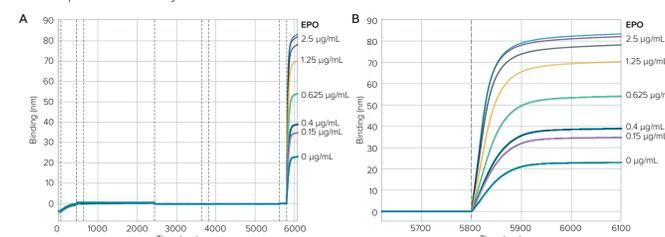
In this study, bovine serum fetuin was chosen as a representative non-mAb glycoprotein sample, which contains both N-glycans and O-glycans, to determine the terminal sialic acid content using the GlyS Kit on the Octet® platform. As fetuin is well known to contain high sialic acid, a direct binding assay was conducted using several different concentrations of fetuin glycoprotein (500  $\mu\text{g}/\text{mL}$ , 50  $\mu\text{g}/\text{mL}$ , 5  $\mu\text{g}/\text{mL}$  and 0  $\mu\text{g}/\text{mL}$ ) in GSB with four replicates per each concentration (n=4). We observed the significant dose-dependent fetuin binding signal to GlyS sensor in less than 5 minutes of total assay time using this direct binding assay method.



**Figure 3: Detection of terminal sialic acid from fetuin glycoprotein (non-mAb sample)** Binding signals (nm) of varying concentration of fetuins to the GlyS lectin sensor. A direct binding assay workflow for fetuin without amplification was used via 16-channel mode on the Octet® RH96 instrument.

### Detection of Terminal Sialic Acid from FC-Fusion Tagged Erythropoietin Sample (EPO); non-mAb Therapeutic Protein Sample

In this study, an Fc-fusion tagged erythropoietin sample was chosen as a representative Fc-fusion tagged glycoprotein sample (non-mAb) to determine the terminal sialic acid using the GlyS Kit on the Octet® platform. Varying concentrations of EPO protein from 0.15  $\mu\text{g}/\text{mL}$  to 2.5  $\mu\text{g}/\text{mL}$  were used in the binding assay together with the rabbit Anti-EPO Polyclonal antibody as a secondary antibody specific to EPO protein. We observed the significant dose-dependent binding signals of EPO to the GlyS biosensor with ng/mL of protein using the amplification assay.

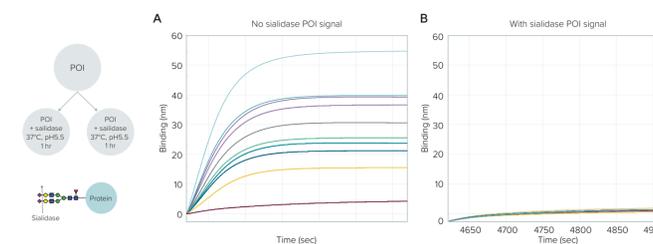


**Figure 4: Detection of terminal sialic acid from Fc-fusion tagged erythropoietin sample (EPO).** Binding signals (nm) of varying concentrations of Fc-fusion EPO proteins (Creative Biomart, PN#EPO-01H) to the GlyS biosensor. A standard amplification assay workflow for human-IgG samples was used via 16-channel mode on the Octet® RH96 system. Rabbit Anti-EPO Polyclonal antibody (Invitrogen, PN#PA5-77965) was used as a secondary antibody specific to EPO protein for detecting specific EPO binding signals. A) Binding signals (nm) of varying concentrations of EPO proteins to the GlyS biosensor on Octet® RH96 instrument. B) Zoomed-in view of the detection step signal of EPO.

### Confirmation of the GlyS Binding Signal to Terminal Sialic Acid of the Glycoprotein using Sialidase Enzyme Digestion Assay

To determine whether the observed binding signals were coming from the terminal sialic acid of the protein of interest, a sialidase enzymatic digestion assay was conducted. Neuraminidase (sialidase) was used to cleave terminal N-acetyl neuraminic acid (sialic acid) from a variety of glycoproteins. Figure 5 shows the representative example data from crude IgG samples. In this study, sialidase enzyme from Clostridium perfringens (Sigma, N2876-25U), which cleaves terminal sialic acid residues which are α-2,3-, α-2,6 or α-2,8-linked sialic acid residues, was used.

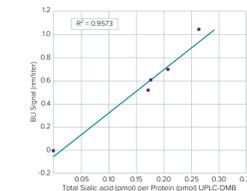
As shown in Figure 5A, the non-treated sample exhibits a strong binding signal using the GlyS Kit. However, the sialidase-treated crude sample exhibits a diminished binding signal, confirming the binding specificity of the GlyS biosensor towards terminal sialic acid.



**Figure 5: Confirmation of the GlyS binding signal to the terminal sialic acid of the glycoprotein using a sialidase enzyme digestion assay.** Sialidase digestion workflow example (left) and sialidase digestion data are shown (A, B). Sialidase was from Sigma (N2876-25U). Two sets of crude IgG samples were prepared in the sialidase enzyme buffer (Sigma, N2876). One set of samples were prepared in the absence of sialidase (A) and the other set were prepared in the presence of sialidase (B). 10  $\mu\text{L}$  of 25 U/mL sialidase (B) or the digestion buffer alone (A) was added to the protein of interest (POI) in the digestion buffer and both sets of samples were incubated in 37 °C for 1 hr. The experiment was done under side by side comparable conditions. After incubation, the GlyS Kit binding assay was conducted. GlyS biosensor binding signals were abolished with the sialidase digestion (B) view of the detection step signal of EPO.

### Correlation of Octet® GlyS Kit Sialylation to Total Sialic Acid by UPLC/DMB/FLR Analysis

To verify the binding signal obtained from the GlyS Kit, an independent analytical HPLC method was conducted. Purified biosimilar human IgG protein fractions (P1–P4) were diluted in a CHO cell supernatant (which doesn't express human IgG) and were analyzed using the GlyS Kit method on the Octet® RH96 system. The same samples were hydrolyzed by mild acid hydrolysis to release the unbiased total sialic acid (TSA) and the hydrolyzed samples were then analyzed by HPLC for total sialic acid as described in reference (4). The amount of total sialic acid is expressed as moles of sialic acid per mole of protein (mol mol<sup>-1</sup>). A linear correlation ( $R^2 > 0.9$ ) was observed between the GlyS biosensor binding signal and TSA by HPLC.

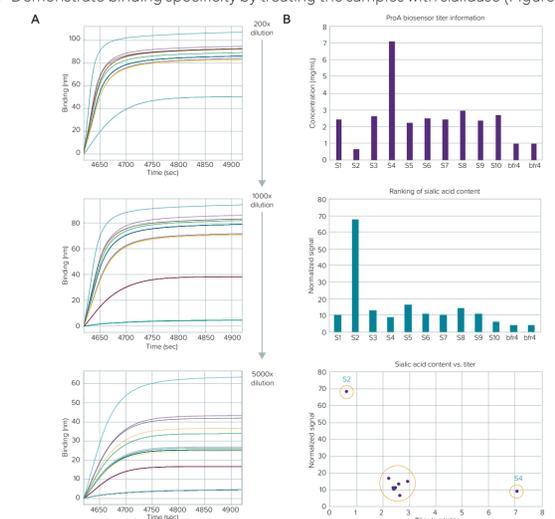


Biosimilar IgGs	HPLC TSA mol/mol	BLI binding nm/titer
Buffer	0	0
P1	0.24	1.06
P2	0.19	0.61
P3	0.22	0.7
P4	0.15	0.5

**Figure 6: Linear correlation of sialylation determined by the GlyS biosensor binding signal and HPLC total sialic acid.** The free sialic acids from the samples were removed using a 3 kDa centrifuge filter and washed with water six times. The concentrated samples were diluted with water to make a volume of 100  $\mu\text{L}$ , and another 100  $\mu\text{L}$  of 4 M acetic acid was added to the protein samples and then the mixture was incubated at 80 °C for 2 hr. The pretreated samples were labeled using DMB for UPLC/FLR detection with Waters BEH C18 (4.6 mm x 250 mm, 3.5  $\mu\text{m}$ ) and sialic acids standard molecules as described<sup>4</sup>. The amount of total sialic acid is expressed as moles of sialic acid per mole of protein (mol mol<sup>-1</sup>). (A) GlyS biosensor binding signal (nm/titer) on the y-axis plotted against the HPLC TSA on the x-axis. UPLC/FLR/DMB analysis were conducted by the Service Lab (ChemilyUS, LLC).

### Example Workflow of Unknown Crude Human mAb Samples

1. Quantitate the crude samples with the appropriate titer biosensor (such as a ProA biosensor) to get the titer information.
2. Dilute the crude samples with >10x with Glycan Sample Prep Buffer (i.e. 1/10x, 1/100x, 1/1000x, 1/10,000x etc.) Take an initial screening test with the GlyS biosensor (Figure 7A) to find the optimum dilution range for the protein of interest in the linear binding range.
3. Further optimize the dilution factor for high enough sensitivity and linear response (60 nm shift as shown below in Figure 7A).
4. Normalize the sialic acid binding data against titer (Figure 7B).
5. Demonstrate binding specificity by treating the samples with sialidase (Figure 5).



**Figure 7: Example workflow of unknown crude human mAb samples.**

## Conclusion

A high-throughput screening method for sialylated glycoproteins was developed to support cell line development proteins during the primary and secondary stages. This method can specifically quantify the sialylation levels of secreted proteins in crude cell culture samples and does not require purified samples. Interference from host cell proteins was negated by using a secondary antibody which is specific for the protein of interest. Hence, only the specific protein of interest signal was detected as the binding signal. Data comparison confirmed a linear correlation between the GlyS lectin biosensor binding signals obtained using the Octet® RH96 system and total sialic acid measured by the HPLC method. The method was successfully applied to several different types of workflows including those for Fc-fusion tagged protein human mAb (IgG), as well as non-mAb samples for relative sialylation screening. This high-throughput screening method can quantify sialylation levels in a relative manner in crude samples and can readily be used to accelerate cell line development for producing therapeutic proteins with desired product qualities.

## References

1. Carbohydrate analysis of glycoproteins and glycopeptide. N. Jenkins and E. M. Curling, *Enzyme Microb. Technol.*, 1994, 16, 454-464.
2. The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line. X. Xu, H. Nagarajan, N. E. Lewis, S. Pan, Z. Cai, X. Liu, W. Chen, M. Xie, W. Wang, S. Hammond, M. R. Andersen, N. Neff, B. Passarelli, W. Koh, H. C. Fan, J. Wang, Y. Gui, K. H. Lee, M. J. Betenbaugh, S. R. Quake, I. Famili, B. O. Palsson and J. Wang, *Nat. Biotechnol.*, 2011, 29, 745-741.
3. History of lectins: from hemagglutinins to biological recognition molecules. N. Sharon and H. Lis, *Glycobiology*, 2004, 14, 54R-62R.
4. High-throughput sialylation measurement using lectins on an Octet® platform for clone screening. K. N. Jonnalagadda, L. R. A. Markely, B. Guan, C. Alves and S. Prajapati, *Anal. Methods*, 2016, 8, 7193-7198.