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Application Note

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Mannose Glycans Content Screening for Human-mAb Samples Using the Octet® GlyM Kit

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Introduction

Glycosylation refers to the formation of linkages with glycosyl groups, covalently attaching a carbohydrate to another molecule. Most proteins undergo glycosylation as a post-translational process with N-Glycosylation, O-Glycosylation, Sialylation and Fucosylation being the most common forms. Glycosylation is a critical quality attribute (CQA) and imparts properties such as stability, folding, solubility, cell-cell interaction and adhesion to proteins. Antibody glycosylation for example is critical due to the role carbohydrates play in modulating effector functions which affect safety and efficacy of therapeutic antibodies. The induction of antibody dependent cell mediated cytotoxicity (ADCC) for example, requires the binding of the Fc region of the antibody to Fcy receptors typically present on the surface of effector cells. The effector functions of therapeutic antibodies are strongly affected by the specific glycans added to the Fc domain during post-translational processing. Antibodies bearing high levels of N-linked mannose-5 glycan (Man5) have been reported to exhibit enhanced ADCC compared with antibodies with fucosylated complex or hybrid glycans.[1]

Variability in the glycosylation patterns of a protein, caused by differences in manufacturing processes can adversely affect their biological activity and stability. Mannose-specific lectins such as cyanovirin-N (CVN), microvin (MVN) and microcystis viridis lectin (MVL) amongst others have been found to efficiently neutralize human immunodeficiency virus (HIV) infection and to prevent viral entry into host cells. A similar approach to the neutralization of HIV infection has been used to investigate inhibitory activity of specific lectins on hepatitis C virus (HCV) due to its high mannose glycans content.

Current methods for the detection of glycans include staining procedures such as SDS-PAGE and affinity based procedures such as specific lectin binding, antibody binding or enzymatic cleavage methods. While staining procedures are fairly straight forward, they are however plagued with high propensity for non-specific binding making them less reliable as tools for specific glycan detection. Affinity based approach is therefore more reliable as it utilizes molecules that bind specifically to a given glycan. Once detected, complete structural elucidation of glycoprotein oligosaccharides can be performed using a chemical, enzymatic, and chromatographic (HPLC) techniques combined with mass spectrometry (MS). While both MS and HPLC remain the most popular platforms for the characterization of glycosylation on proteins, they lack the throughput needed for early screening of CQA and are therefore not optimal for early clone selection. Moreover, they require sample purification, a process that can be prohibitively long.

Sartorius's Octet® platform on the other hand is an easy to use and high-throughput platform that when used with the glycosylation detection kit offers cell line development and upstream bioprocessing scientists an alternative with scalable throughput to measure up to 96 samples in parallel. Unlike HPLC or MS techniques, the Octet® platform can also monitor other functional CQAs such as specific receptor binding from the same samples.

The Octet® GlyM Kit is composed of biosensors pre-immobilized with Mannose specific lectin, buffers and reagents necessary for the detection of mannose in purified and non-purified samples. The lectin used is highly specific towards terminal alpha mannose species, including oligomannose-type N-glycan and hybrid-type N-glycans. It also binds strongly to biantennary complex-type N-glycan, but not tri- and tetra-antennary complex-type N-glycans.

The kit is aptly designed to allow for early screening of mannosylation during the subcloning and top clone selection stages of cell line development and upstream bioprocessing. It allows users to rank order screened clones based on total mannose content. It is not meant to be used for distinguishing between different mannose structures. In this application note, we showcase an approach to screen for mannose content in human mAb samples using a well characterized monoclonal antibody from the National Institute of Standards and Technology (NIST) as well as in-house samples developed at Novartis.

Materials and Methods

Materials

- Octet® instruments: R2, R4, R8, RH16, RH96 (any of these instruments could be used however, for these studies, the Octet® R8 was used).
- Octet® GlyM Kit: Lectin coated Octet® biosensors, detection reagents (Anti-human IgG detection Fab fragment, Glycan detection mix), buffers (Glycan sample buffer, Glycan wash buffer, Glycan buffer A, Detection Fab Fragment Dilution Buffer).
- α-Mannosidase (Sigma, P/N: M7257-1MG, 23U in 0.3mL per vial)
- Samples: NIST hIgG (P/N: RM8671, stock concentration, 10 mg/mL), hIgG test samples developed at Novartis

Method

There are 3 key steps recommended for the mannose screening process; **refer to Octet® GlyM Kit technical note and Octet® GlyM Kit Protocol for assay method details**.
In the first step of the mannose screening process, assay optimization was performed to allow for the determination of the detection range. As part of assay optimization, the NIST hIgG was used to verify dose dependence binding response of the GlyM biosensors. The sample was serially diluted in the range 20 – 3 µg/ml using the Gylcan Sample Prep. Buffer. In tandem with the NIST sample, a Novartis developed hIgG sample with known mannose content was used to determine the optimal IgG titer to be used for test sample screening. To verify and validate the specificity of the mannose assay, samples were either subjected to enzymatic digestion using α-mannosidase enzyme (test) or were incubated without the enzyme (control). Both sample sets were later subjected to the Octet® GlyM binding assay and monitored for binding responses. In the final step of the mannose screening process, screening samples titer was fixed to 2 µg/ml as determined in the optimization step. The selection of this concentration (2 µg/ml) was determined by evaluating the samples with the highest and lowest % of mannose (Novartis IgG), and then selecting titer at which binding signals from both of these samples are within the acceptable detection range.

The general Octet® GlyM Kit assay workflow is shown in Figures 1A and 1B.

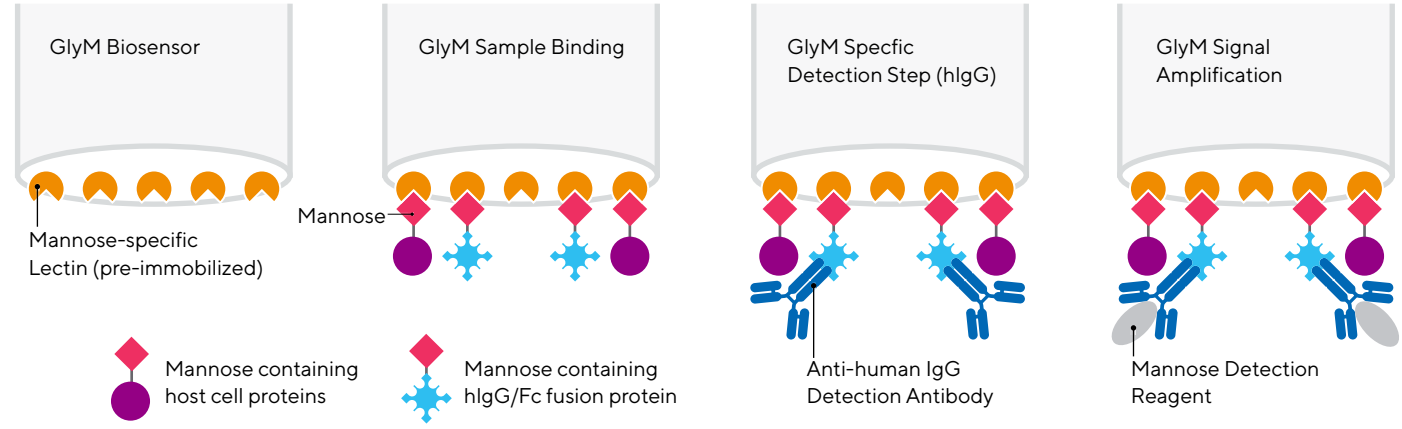

Figure 1A: Octet® mannose assay workflow. Mannose-specific lectin immobilized biosensors and other detection reagents come with the Octet® GlyM Kit. The initial assay step results in the binding of all mannose containing proteins; a secondary step that utilizes an anti-human IgG Fab fragment is used to specifically detect human IgG samples followed by a signal amplification step necessary to achieve sensitivity to low % mannose.

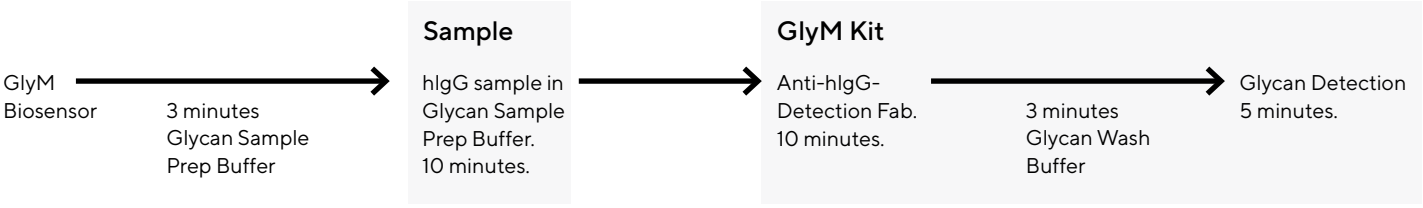

Figure 1B: General schematic for the assay including sample assay step times. The glycan sample prep buffer step is used for the relaxation of antibody samples to ensure the exposure of mannose to the lectin on the biosensor for binding. The step requires a minimum of 10 minutes for sample equilibration.

Results and Discussions

Assay Optimization

Assays development and optimization is a critical and necessary step in method development for biologics analytical platforms. The Octet® GlyM Kit is designed for screening and rank ordering of molecules that belong to the same class of biologics such as antibody clones. The accuracy of the rank ordering process therefore assumes a normalized and identical concentration or titer of the test samples. In this assay, method optimization required the assessment of optimal antibody titer while monitoring the range for mannose detection. Two reference samples were used for this assessment; first the well characterized NIST mAb was dose titrated to include low (3ug/ml) and high (20 µg/ml) titer (Figure 4). These samples were used to probe the GlyM

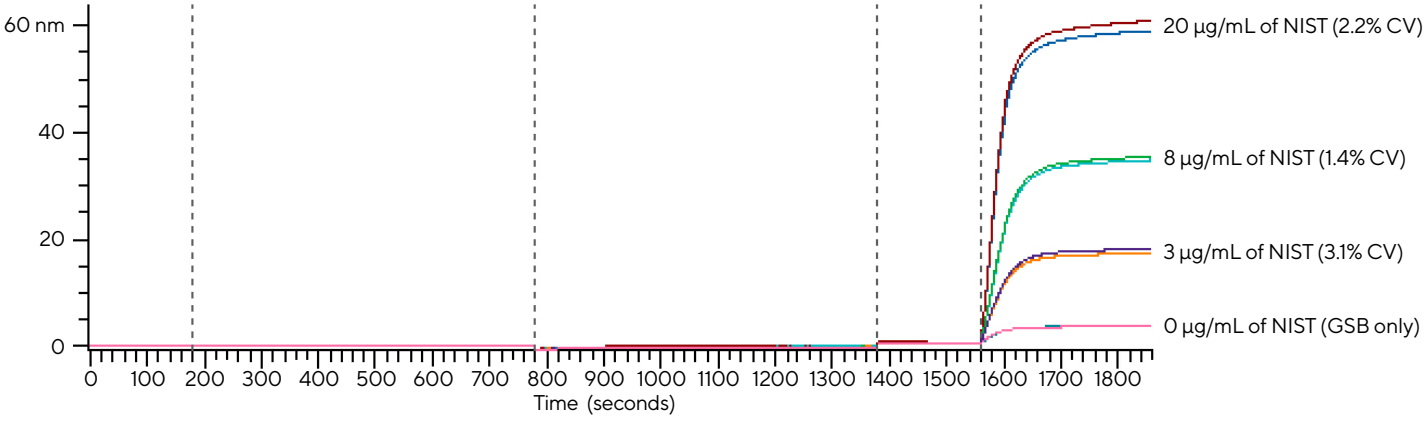

Figure 4: GlyM biosensors binding response signals for NIST hIgG samples at different concentrations. The assay was run in replicates and exhibits dose response with good precision.

biosensors ability to detect mannose binding in a dose response manner. In addition to NIST, in-house reference samples with two extreme % mannose content were used (the % of mannose content is a % of high mannose structures among all glycan structures in the IgG sample). These samples were S1 (90% mannose) and S2 (2 % mannose) and were dose titrated in the range 4 – 0.5 µg/ml (Figure 5A, B). While the NIST sample is standard and is a generic representation of well-characterized biologics that may or may not contain optimal mannose content, the in-house reference sample should be as representative of the subsequent test (screening samples) as possible. As a result, the response signals from the in-house samples were plotted against sample titer (Figure 6) and were used to determine the optimal fixed or normalized titer for the screening assay.

The plot of the signal response for S1 (high mannose %) and S2 (low mannose %) samples as a function of sample titer suggests that the binding response separation between the two increases with increasing titer. For this kit, an acceptable binding response dynamic range of 10 – 65 nm is suggested hence a fixed sample titer in the 1-2 µg/ml was recommended.

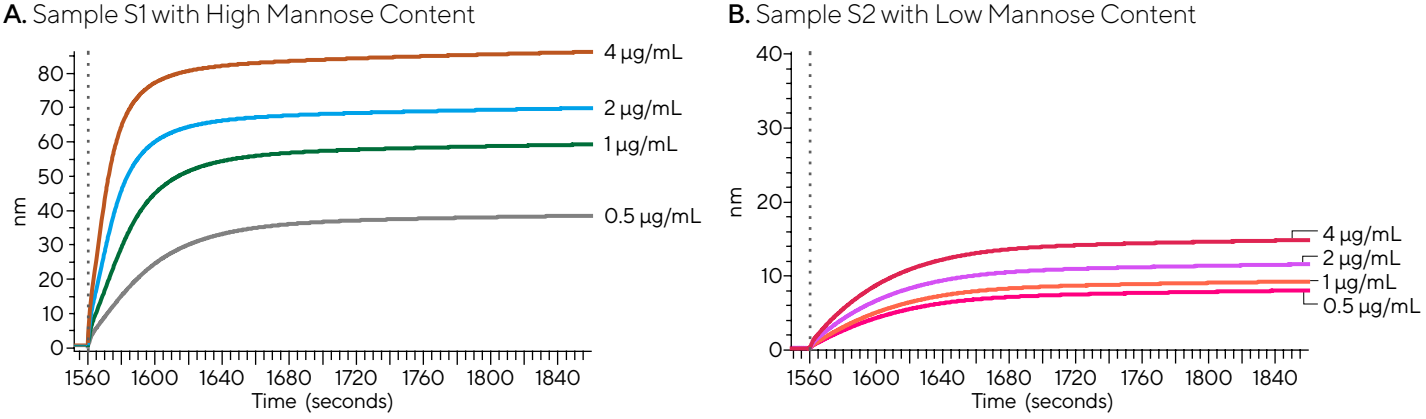

Figure 5: GlyM biosensors detection of human IgG samples: (A) hIgG sample (S1) at varying concentration while containing high mannose content (90%). (B) hIgG sample (S2) at varying concentrations while containing low mannose content (2 %). The assay exhibits a clear difference in response between samples with high % mannose and those with low % mannose.

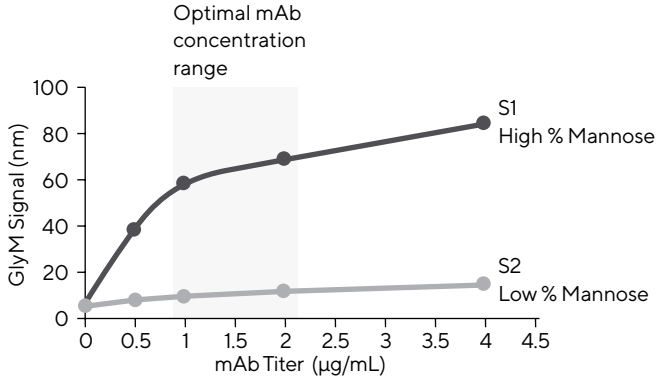

Figure 6: Binding response of S1 (high % mannose) and S2 (low % mannose) as a function of mAb titer. Experiment used to determine optimal mAb titer for mannose screening. Optimal mAb concentration range is determined to 1 – 2 µg/mL.

Assay Verification

To verify that the response signals were specific to mannosylated sites on the antibody samples, an α-mannosidase enzyme was used to cleave off the mannose linked residues on the samples. The cleaved moieties are presumed to be small in size and should therefore not exhibit any significant response to the GlyM biosensors. A side by side comparison of the mannosidase treated samples with the untreated samples (Figure 7) reveals that while the untreated sample (same titer) exhibits high response signals upon binding to the GlyM biosensors, the treated samples show only background response (similar to response from buffer). The results indicate that the GlyM assay as performed in step 1 is highly specific to mannose content on the antibody samples.

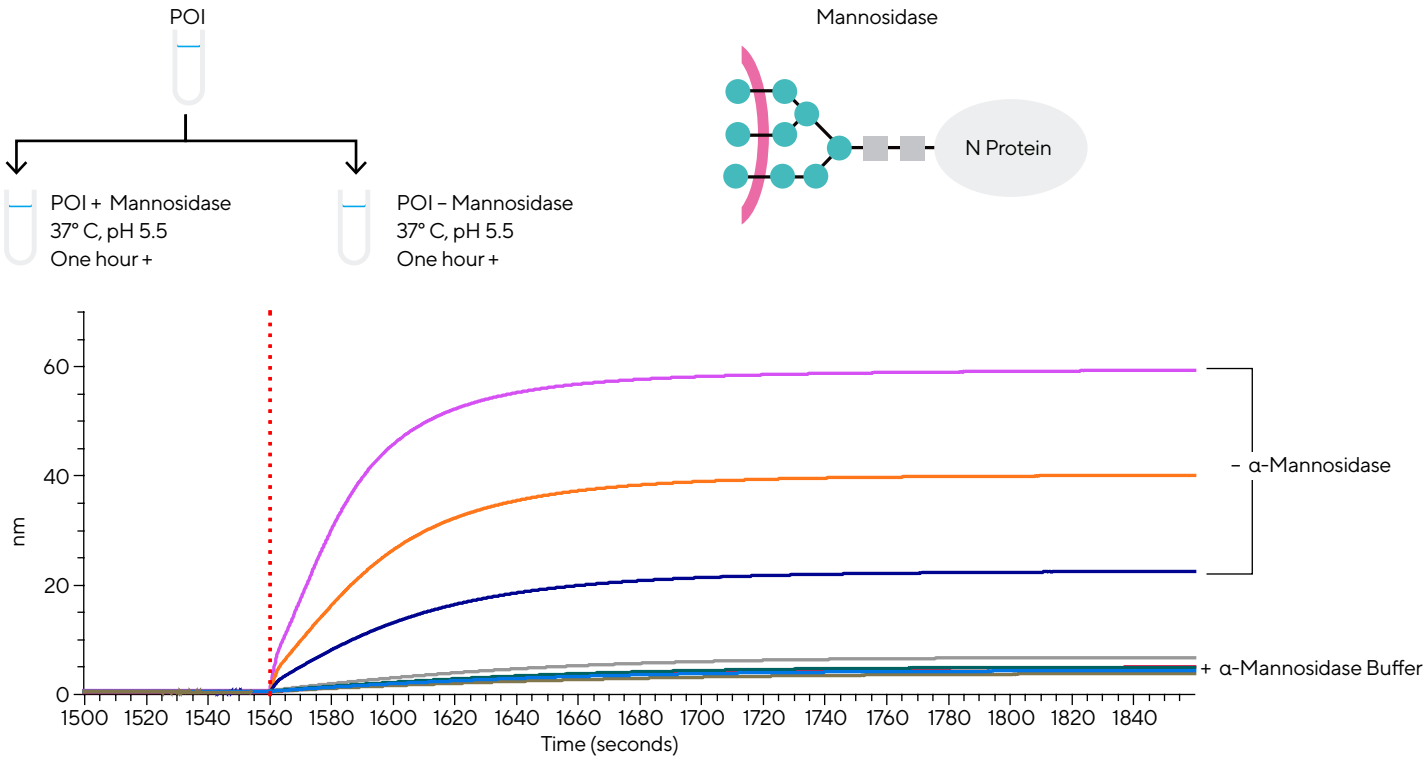

Figure 7: Mannosidase digestion workflow and Octet® GlyM binding validation assay. Samples incubated with α-Mannosidase show diminished binding while α-Mannosidase negative samples exhibit significant binding to the GlyM biosensor.

Test Samples Screening and Comparison to HPLC/MS

Samples testing was setup with two examples. In the first example, the assay was setup as described in step 3 in the protocol; samples with known % mannose content (HPLC) were screened (Figure 8) for binding to the GlyM biosensors. The results demonstrate a clear differentiation in response signals for samples containing up to 90 % mannose content. There's however no differences observed in signal response for samples with different types of mannose M5, M7, M8 and M9; all at 90% mannose. We postulate that at 90 % mannose content; the selected titer of 2 µg/ml was too high; hence for high mannose content samples, fixed mAb titer would need to be lowered. It is also noteworthy that structure plays a critical role in the exposure of antibody glycosylated sites; hence sample "relaxation" is a critical step in assay optimization. These results correlate well with HPLC and mass spec analysis results from the same samples (Figure 9) thereby confirming the utility of the Octet® GlyM Kit as a tool for the rapid screening of samples relative mannose content.

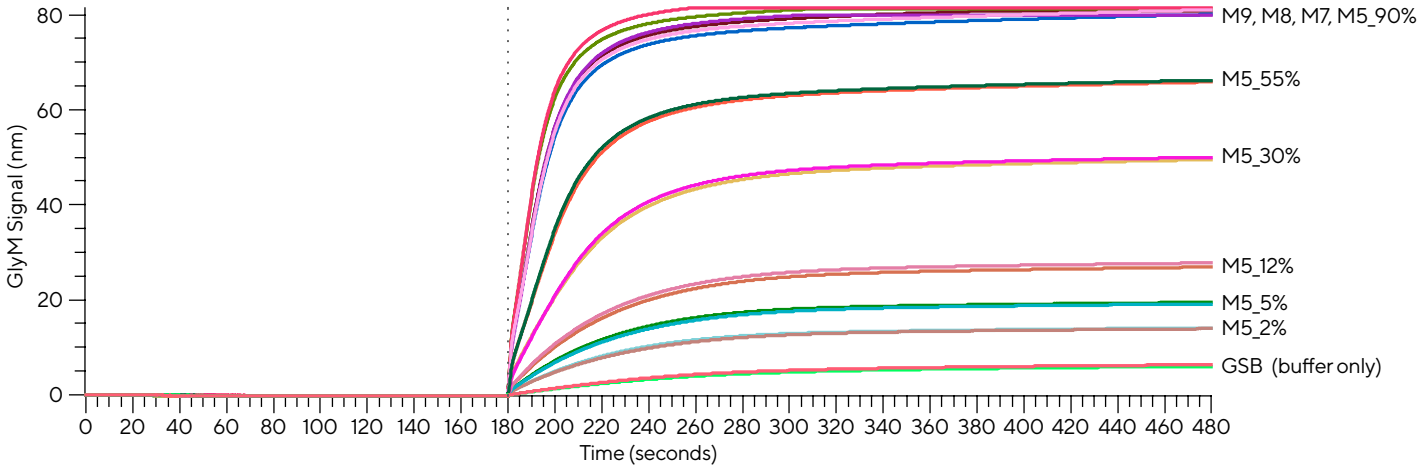

Figure 8: GlyM binding response signals for the screening of samples with known mannose contents. The samples varied from man5 (M5) to man9 (M9) and contained mannose at different % concentrations (ranging from 2 – 90%). Samples were first fixed at 2 µg/ml before screening for binding response. A buffer only reference sample was used to check for background binding response.

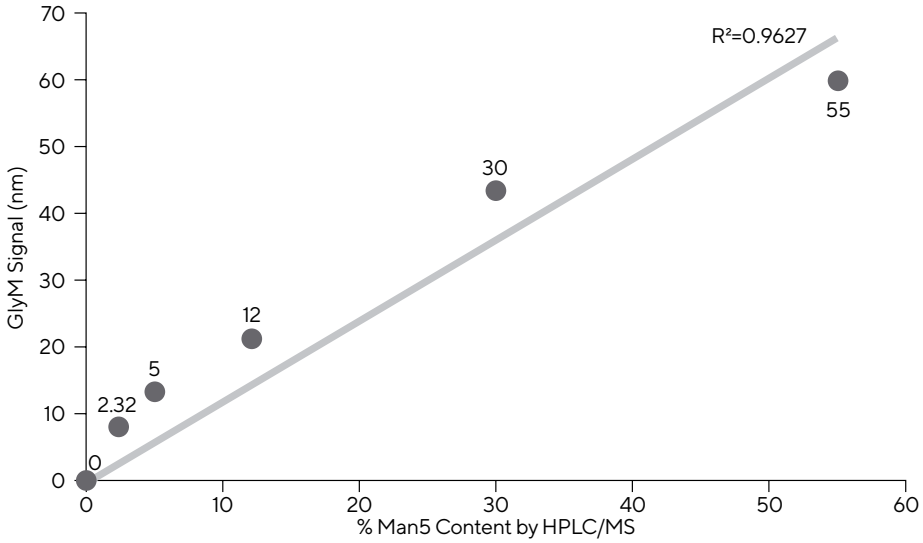

Figure 9: Correlation between % mannose content for hIgG samples as determined by HPLC/MS and Octet® binding response (Figure 8); while the Octet® does not quantify % mannose, it exhibits good rank ordering correlation with HPLC/MS; the higher the % mannose, the higher the binding response.

In the second example, representative of an assay run to screen harvest samples with no known mannose content a series of samples were normalized to an equivalent titer and assayed in a similar method as indicated in example 1 above. In this example, since the titer is normalized to a fixed concentration, the response signals can be used to rank order the samples for mannose content. As can be seen in Figure 10A and B, test sample 6 exhibits the lowest binding response implying that it contains the lowest mannose content and is a good candidate for selection for further development.

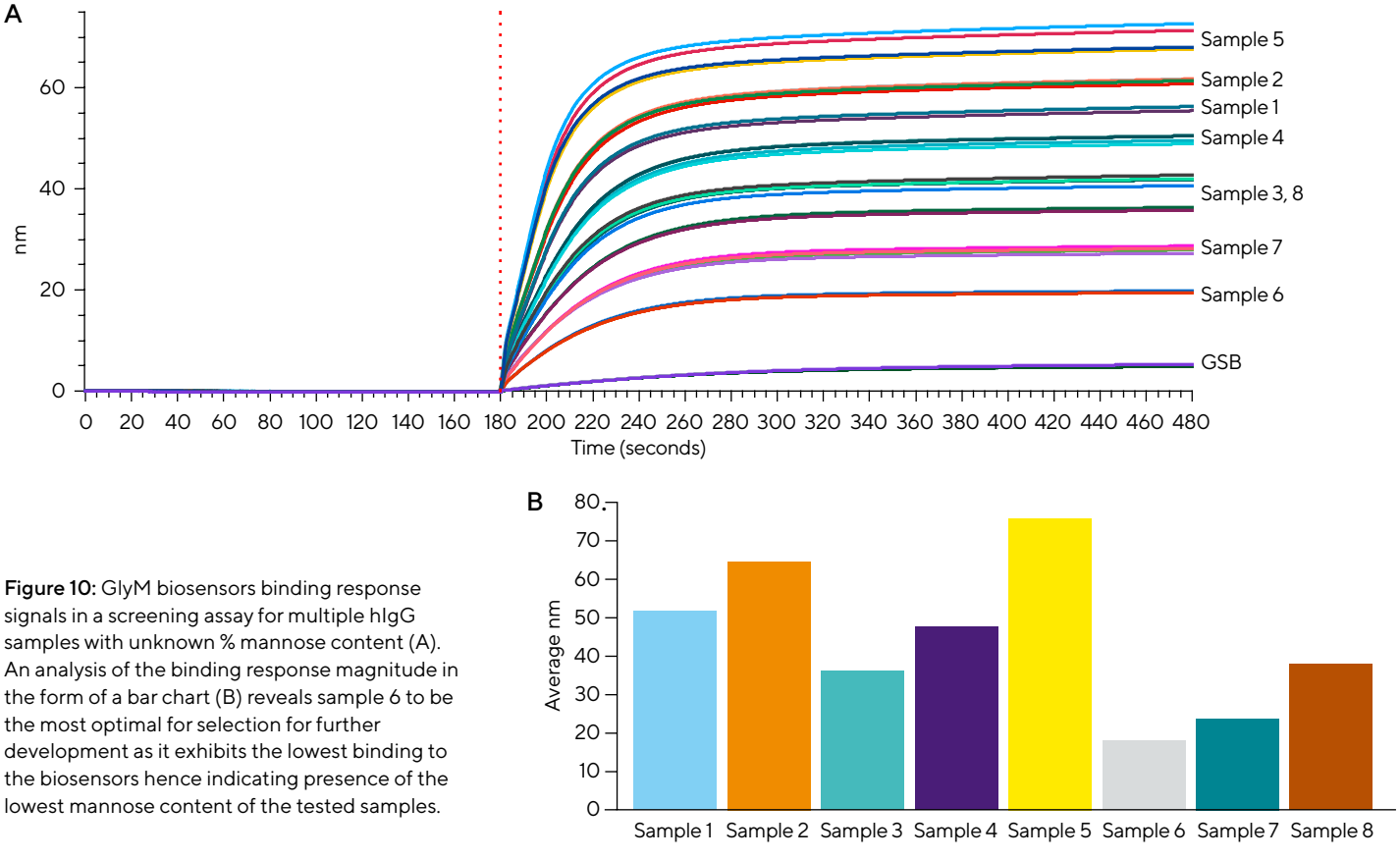

Figure 10: GlyM biosensors binding response signals in a screening assay for multiple hIgG samples with unknown % mannose content (A). An analysis of the binding response magnitude in the form of a bar chart (B) reveals sample 6 to be the most optimal for selection for further development as it exhibits the lowest binding to the biosensors hence indicating presence of the lowest mannose content of the tested samples.

Best Practices for Gly M Assay

1. Let kit reagents equilibrate at ambient temperatures for at least 30 minutes prior to use
2. Use only azide free buffers and reagents
3. Determine a fixed and acceptable mAb concentration to work with through an optimization assay; mAb samples titer can be determined first using Protein AB biosensors prior to performing the mannose screening optimization assay. To perform mAb titer, note that a standard reference curve is needed. Refer to Octet® GlyM Kit Technical Note.
4. Use reference samples with known mannose concentration. Reference samples used during assay optimization should have a high (~15 %) and low (~ 1%) mannose content and should ideally be structurally similar to test samples
5. It is important to "relax" the protein of interest prior to mannose detection; use the provided Glycan Sample Prep Buffer (GSB) to dilute the samples 10-fold and incubate for at least 10 minutes
6. A control specificity experiment to validate the response signals should be done by carrying out a-mannosidase digestion to ensure the binding signals are from terminal mannose (see Sartorius Octet® GlyM Kit Technical Note).[2]
7. Glycan detection mix should be used within 4 hours of preparation; the mix and anti-hIgG detection Fab fragment solutions should be kept at 4°C prior to use.

Conclusion

The Octet® GlyM Kit is designed to provide cell line development and upstream bioprocessing Octet® users with a ready to use kit for the rapid screening of % mannose content of purified and cell harvest antibody samples. The kit correlates well with HPLC and mass spec techniques in rank ordering of samples and has the added advantage that it does not require sample purification or enzymatic digestion prior to sample analysis. When combined with the high-throughput features of Octet® instruments and the flexibility of sample analysis using a variety of off-the shelf biosensors; including for titer determination, specificity to target antigen binding and off-rate analysis (all from the same sample set); it is an added tool for the assessment of critical quality attributes that should facilitate the early selection of optimal clones during cell line development.

References

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