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Application Note

A rapid and reliable method for measuring Total Sialic Acid Content (TSAC) using the Gator® Bio BLI platform

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Scope

An assay for accurate and reproducible total sialic acid content (TSAC) measurement is demonstrated using the Gator Bio biolayer interferometry system. The assay performance is compared with TSAC measurements using HPLC.

Key Takeaways

- **Precise and Reproducible TSAC Measurements:** The direct binding assay ensures high accuracy and reproducibility, making it ideal for IgG characterization and batch-to-batch consistency analysis.
- **Ready-to-use Probes:** Standard probes are adaptable, enabling the execution of a wide range of assays commonly performed in biotherapeutic discovery laboratories.
- **Enhanced Operational Efficiency:** Minimal manual intervention not only streamlines workflows but also significantly reduces the likelihood of human error.

Introduction

Therapeutic antibody constructs not simple antibodies expressed from mammalian hosts - they are complex fusion proteins engineered in ways that improve their efficacy beyond antigen binding affinity. Many Fc fusion proteins are engineered to express additional O- and N-glycosylation sites in order to increase the glycosylation of antibodies. With more glycosylation sites, these fusion proteins can express dramatically higher counts of sialic acid, the terminal component of antibody glycosylation. This increased Sialic Acid content (typically expressed in mols of sialic acid per mol antibody, or mol/mol) has a significant effect on the half-life, immunogenicity, and inflammatory properties of antibodies.

For this reason, Total Sialic Acid Content (TSAC) is a critical quality attribute in antibody development and quality control. However, most traditional methods for assessing sialylation, while accurate, are inefficient and/or expensive. High-Performance Liquid Chromatography (HPLC), Capillary Electrophoresis (CE) and Mass Spectrometry (MS) are each highly sensitive and specific, but are time-consuming, require experienced users, and costly instrumentation that may not always be readily available in a new startup facility. This application note provides an efficient, rapid, and affordable alternative that compares well against these established techniques.

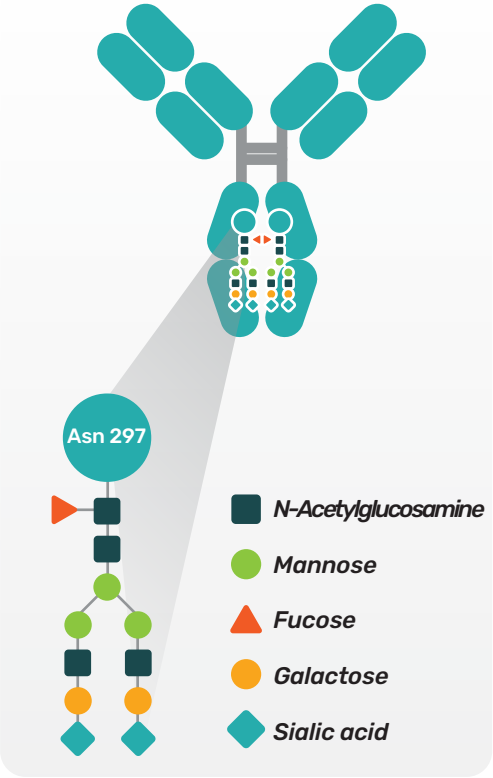

Figure 1: Sialic acids on traditional monoclonal antibodies. Each CH2 region of a monoclonal IgG contains one N-linked glycoylation site at Asparagine 297. Antibodies in this configuration can have at most 4 sialic acids (2 per CH2 region), though only 10-15% of antibodies are actually glycosylated. Modern antibody therapeutics, like those tested in this app note, are often engineered with far more glycosylation sites, increasing the total sialic acid content from 0.10-0.15 mol/mol to 10 mol/mol or more.

Overview of the Gator® Bio BLI Method

The Gator® instruments, widely utilized for antibody quantitation and kinetic studies, present an accessible alternative for measuring sialylation. The Gator® instruments employ Bio-Layer Interferometry (BLI) technology to monitor real-time binding events on biosensors, allowing for label-free analysis of kinetics as molecules in solution interact with the sensor's biochemical base layer. A lamp provides a source of white light, which is reflected from two points near the probe's surface: a reflective layer built into the glass, and the end of the protein layer on the surface. The extra "distance" that the light travels to and from the protein layer's reflection point, a product of the thickness of bound protein and the refractive index of the resultant matrix, creates an offset wave that interferes with the light from the first reflection. This affects the entire visible light spectrum, and as more protein binds to the surface of the probe, the peak of the interference spectrum shifts toward the infrared. This change in the spectrum is measured in real time and plotted as nanometer shift.

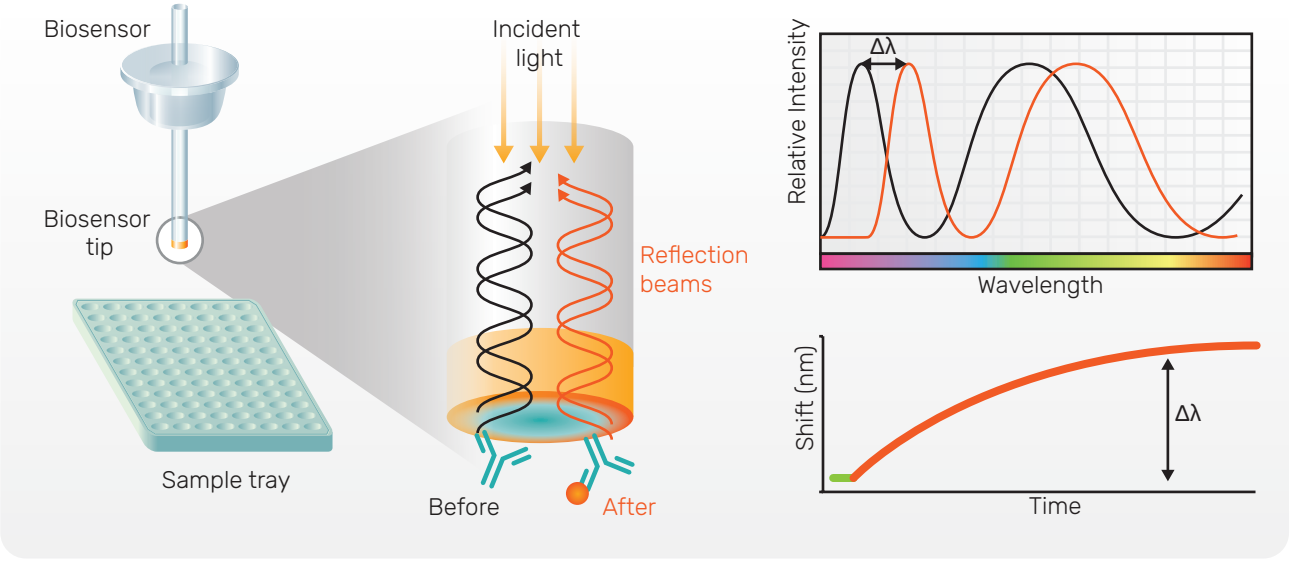

Figure 2: Biolayer interferometry (BLI) Overview. BLI is a label free detection method based on reflection of white light from the surface of a biosensor tip. It analyzes the changes in interference pattern of white light reflected from the tip when biomolecules bind to it. This change is recorded in real time and is expressed as nanometer shift.

Gator BLI analysis offers several advantages, including real-time analysis, ease of use, low cost, and rapid results. Many laboratories use Gator® instruments for antibody quantitation, competition assays, and real-time kinetics for antibody characterization, but may not be aware of the potential of Gator® instruments for assessing glycosylation parameters, specifically Total Sialic Acid Content (TSAC). By utilizing Gator® instruments for sialylation assessment, laboratories can achieve accurate and efficient measurement of TSAC, thereby enhancing their capacity for antibody characterization and ensuring the production of high-quality therapeutic antibodies, without the need for additional instrumentation.

This application note provides a detailed procedure for TSAC measurement using Gator® instruments, leveraging their existing capabilities to extend their utility beyond traditional quantitation and kinetics. The protocol is accompanied by empirical data from a customer laboratory, showcasing the performance and reliability of Gator® instruments in TSAC determination.

Materials Required

The Gator Prime instrument was used for this study, however any Gator BLI instrument can be used for TSAC analysis. Additional materials for the Gator TSAC assay include:

- **Protein A (ProA) Probes (Catalog No. 160001), Gator Bio**
- **Maackia amurensis lectin II (MAL II) which binds to (a-2,3)-linked sialic acids (Catalog No. L-1260), Vector Laboratories, Inc., Burlingame, CA**
- **DPBS, no calcium, no magnesium (Catalog No. 14190144), Thermo Fisher Scientific, Waltham, MA**
- **96- well plates, Grenier Bio-One, Monroe, NC (Catalog No. 655209) & Gator Bio (Catalog No. 130150)**
- **Max Plate (Catalog No. 130062), Gator Bio**
- **Neuraminidase (Sialidase), MilliporeSigma, Burlington, MA (Catalog No. 11080725001)**

Methods

Samples and standards

Thirty-five samples consisting of IgG's at various stages of the purification process and across several different lots were used for analysis. Samples were diluted for loading to 100 ug/mL using DPBS. MAL II lectin was diluted to 250 ug/mL with DPBS prior to use. Samples used for specificity tests were desialylated using neuraminidase.

TSAC assay using the Gator platform

The BLI TSAC Assay is performed using Gator ProA sensors, which bind to the Fc region of most immunoglobulins. Antibodies thus bound to the probe are carried to the next well, containing MAL II lectin, which binds to all O-Glycans on the surface of the protein. MAL II binding causes a second change in interference. The final endpoint nm shift as a result of MAL-II binding is proportional to the total sialic acid content of the protein (note: MAL-II binds only to O-glycans. This customer independently verified that N- and O-glycans on their constructs remained at a consistent ratio using HPLC methods. If N-glycans need to be specifically monitored, consider using a MAL I lectin instead). Figure 3 contains the assay steps and times shown to work with this lectin.

The steps to perform the TSAC assay are shown in Figure 3. After prewetting the ProA probe in DPBS (Step 1), a baseline measurement is performed in the DPBS buffer (Step 2). Once the baseline has been established, the ProA probe is loaded with the sample (Step 3) and then washed to remove any residual non-binding components from the probe (Step 4). The last step involves binding of the MAL II lectin to the sample now adhered to the ProA probe (Step 5). The resultant nm shift is indicative of the total sialic acid content of the bound sample.

- **Step-1**, Prewet: ProA probes are prewet in 250 µL of medium for 10 min in Max Plate.
- **Step-2,** Baseline: The ProA probes are dipped into DPBS buffer for 180 sec to establish a baseline reading.
- **Step-3,** Loading: Samples are loaded onto the ProA probes at 400 rpm for 480 sec.
- **Step-4,** Wash: The ProA probe is washed in 200 µL of DPBS for 180 sec at 400 rpm.
- **Step-5,** Association: The MAL II lectin (200 µL of MAL II lectin diluted to 250 µg/mL with DPBS) is loaded onto the ProA probes for 480 sec at 400 rpm.

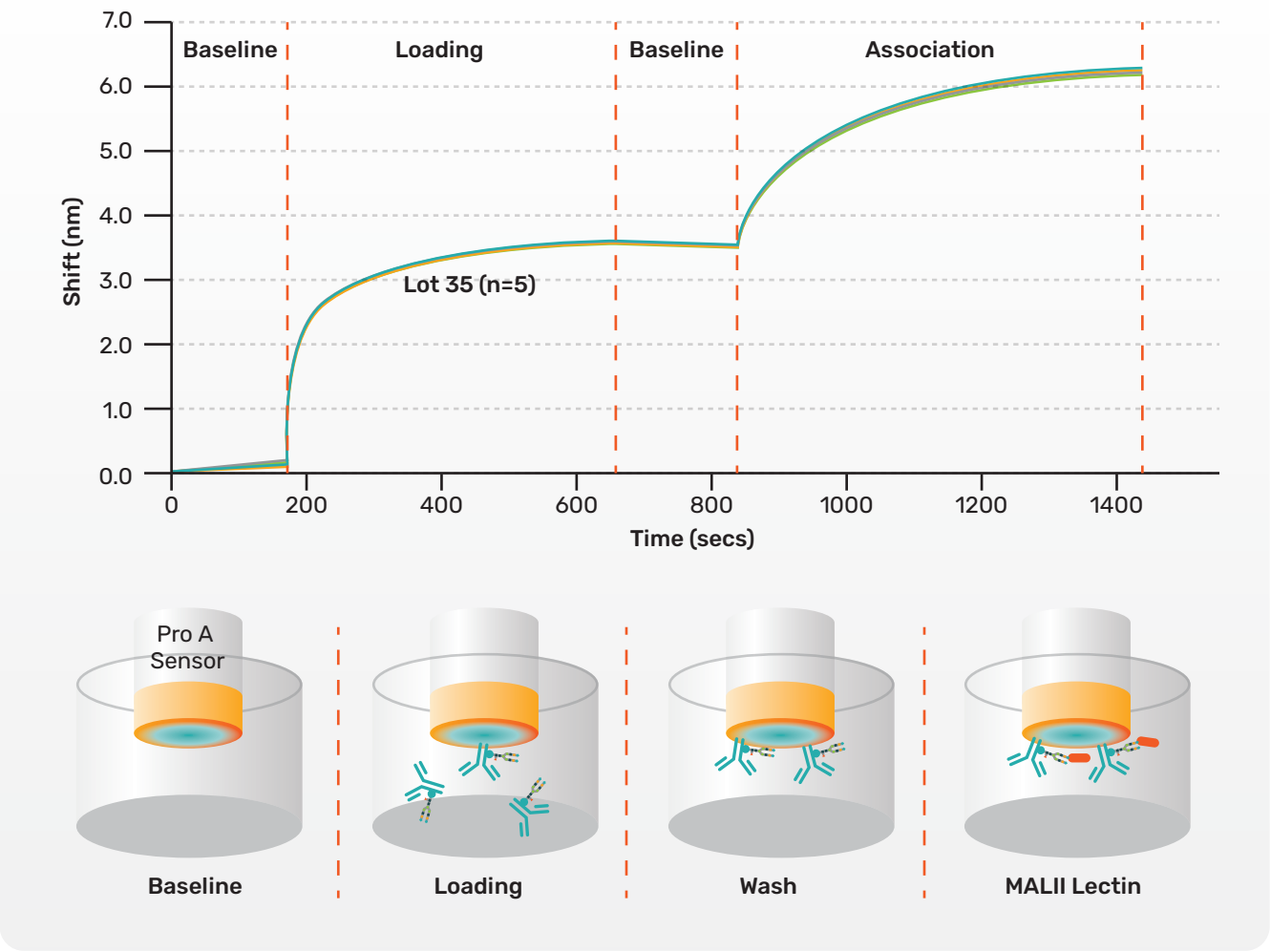

Figure 3: The sialylation analysis is based on the binding rate of MAL II lectin to (a-2,3)-linked sialic acids on proteins that are bound to ProA probes. ProA probes are first dipped into a solution containing the sample to allow samples to bind to the probe. The probes are then moved to a wash solution to remove any residual non-binding components. Finally, the probes are dipped into the solution containing the MAL II lectin to enable binding to sialic acid on samples.

Results

Gator TSAC assay response

Using several different lots of Fc fusion proteins with known TSAC amounts, a standard curve was generated on the Gator® BLI system. Table 1 shows the samples that were used for the standard curve. Figure 4 shows the standard curve that was generated from these samples. The amount of total sialic acid (TSA) is expressed as moles of sialic acid per mole of antibody (mol /mol).

Sample	TSAC mol/mol	Response with 250 µg/ml MAL II
1	6.8	1.58
2	9.0	2.08
3	9.5	2.18
4	10.2	2.35
5	11.0	2.50
6	12.4	2.78
7	13.9	2.98

Table 1. Standard curve data for the Gator TSAC assay

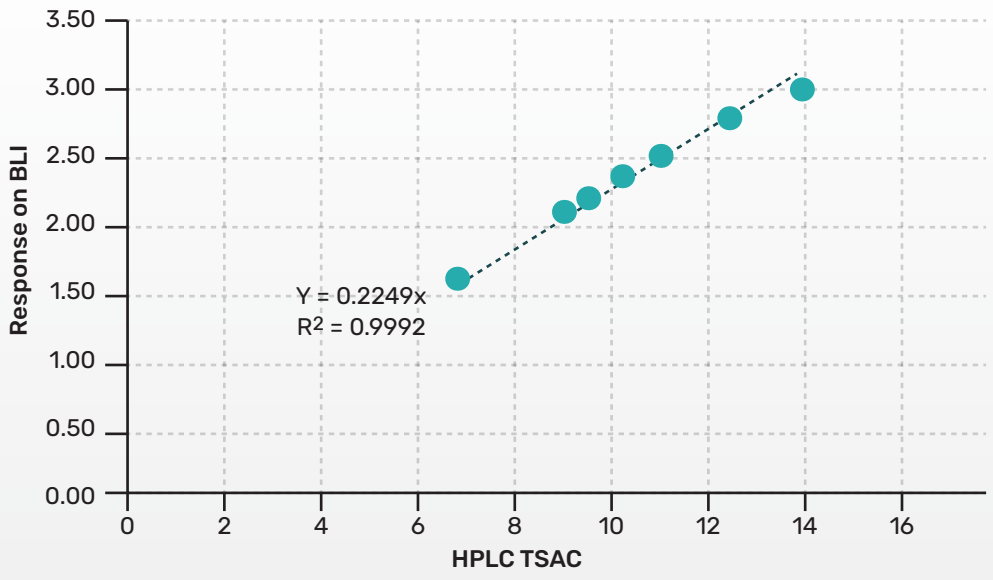

Figure 4: Standard curve. Standard curve generated using 7 different batches of a fusion protein with differing sialic acid content.

The response was shown to be linear, and a fit curve was calculated using Microsoft Excel.

$$\frac{MAL\ II\ response\ \left(\frac{nm}{s}\right)}{0.2249} = TSAC\ \left(\frac{mol}{mol}\right)$$

The y-intercept is set to zero, because treatment with sialidase completely removes all MAL II binding (as discussed further down).

Tips for obtaining maximum consistency with a TSAC assay

Calibration curves for TSAC may need to be tailored to each specific protein due to differences in Protein A binding affinities – a recombinant or engineered antibody may not bind with the same affinity as a classical antibody. Thus, the calibration curves may need to be protein specific. In this study different variations of the same antibody were used for generation of the standard curve and the results were broadly applied across a range of antibody constructs with identical Fc portions and heavy chains.

Additionally, the concentrations of the proteins used during the loading and association steps should be adjusted to ensure accurate binding and measurement. Proteins with lower Protein A affinity may dissociate in the assay's lectin well, leading to inaccurate TSAC measurements. This necessitates careful consideration of binding affinities and appropriate adjustments of assay conditions. As shown in Figure 5, Protein 3 has a lower binding affinity for Protein A, so dissociation is observed in the binding step of MAL II as Protein 3 dissociates from the ProA probe. In this case, the assay can be adjusted by increasing the concentration of Protein 3.

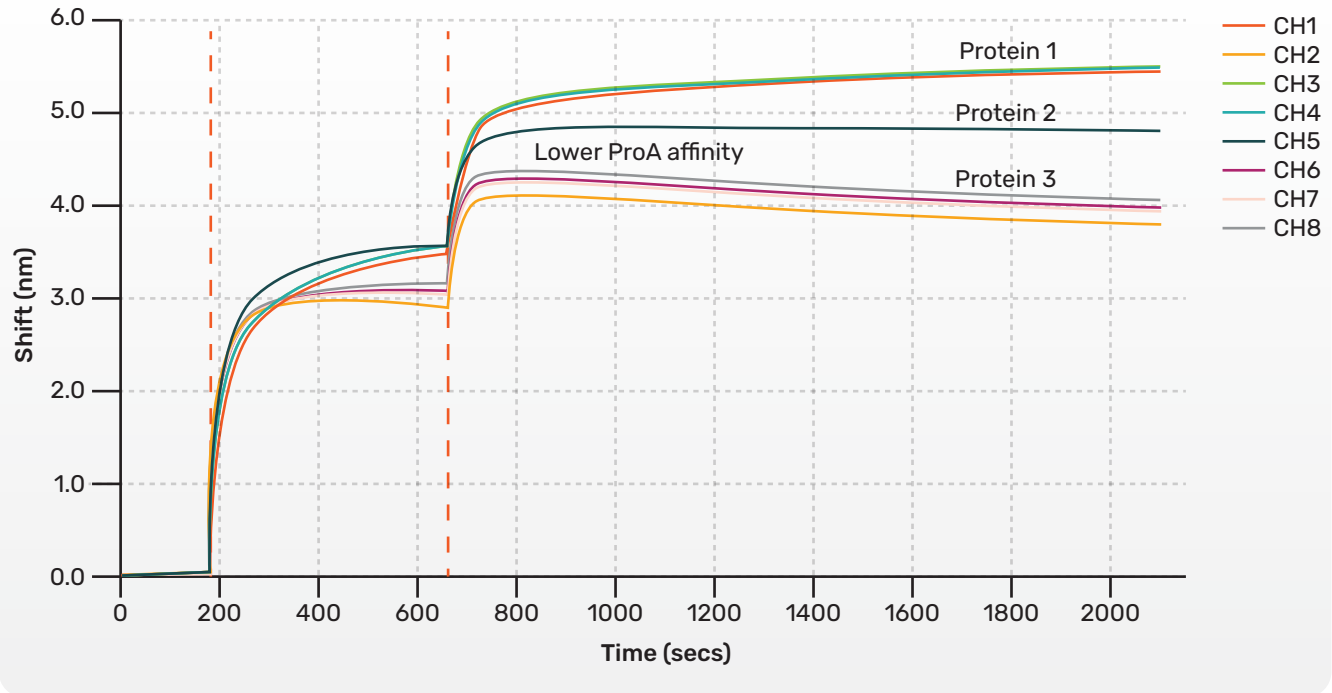

Figure 5: Importance of protein concentration. Proteins with lower Protein A (ProA) affinity, such as Protein 3, tend to dissociate from the probe when placed in the lectin well. This dissociation hinders the accurate measurement and calculation of TSAC values.

Another important aspect of the TSAC assay is that captured protein/IgG may dissociate from the ProA probe during the association step to MAL II, so the lectin wells should not be reused. When these wells are reused for a new sample, the residual proteins from within the well from previous samples may interfere with the MAL II binding to the proteins on the Pro A probe causing inaccurate results.

Specificity of the Gator TSAC assay

Desialylation is the process of removing sialic acid residues from glycoproteins or glycolipids. To evaluate the specificity of the assay, samples that were previously run were desialylated and analyzed again. Figure 6 indicates that after desialylation with neuraminidase, the lectin (MAL II) does not bind to the sample on the ProA sensor. This lack of binding confirms that the lectin was specifically recognizing sialic acid residues on the bound proteins, demonstrating the specificity of the lectin-binding assay. Additionally, this also confirms that the lectin binding is specifically due to the presence of sialic acids, rather than other non-specific interactions.

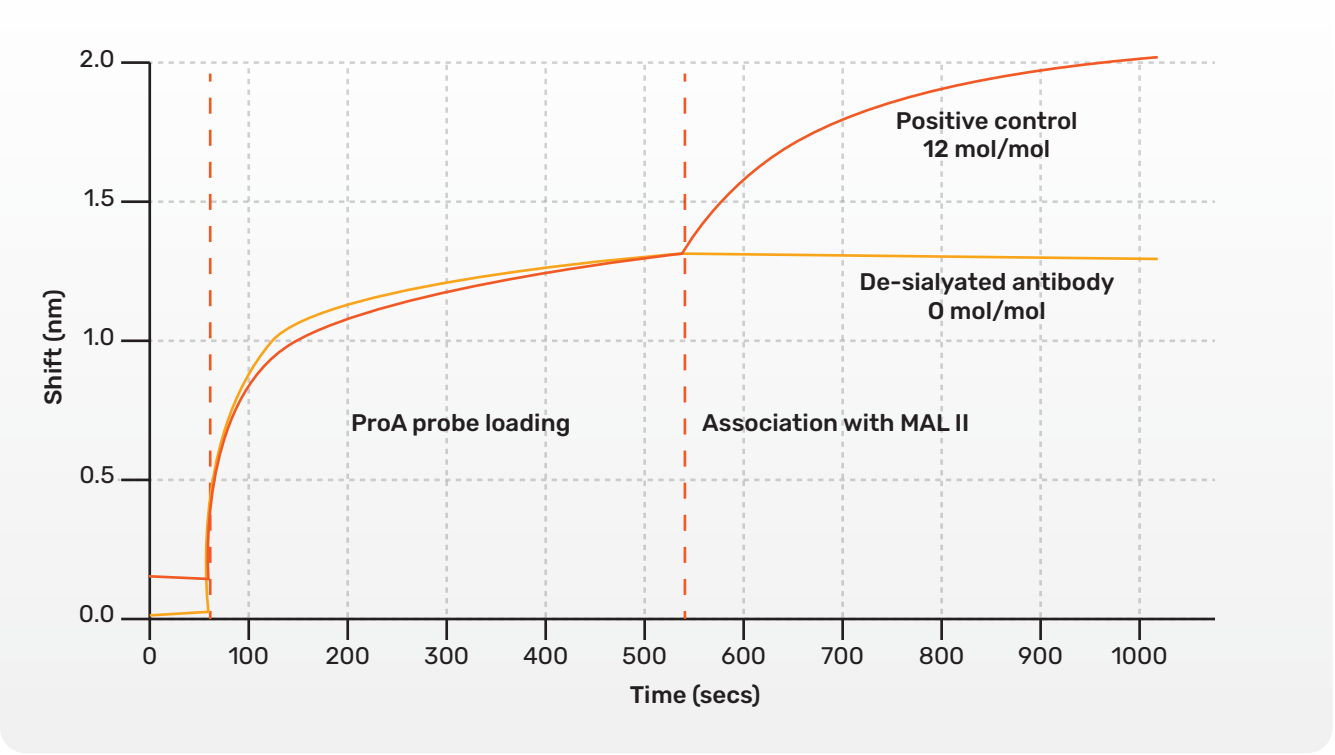

Figure 6: No binding of MAL II is observed to the desialylated version of the same sample, indicating the specificity of the method.

Comparison with HPLC

Although the initial standards were generated using HPLC TSAC data as a calibrator , Gator results were compared with HPLC results to further establish the validity and accuracy of the Gator Bio BLI TSAC assay. This involves comparing the mean values, standard deviations, and %CV of the Gator results with those obtained from HPLC.

Using the standard curve generated from the Gator BLI assay, the HPLC and Gator TSAC values were directly compared across 35 samples. As shown in Figure 7, the two assays show strong correlation with each other indicating the two assays produce highly similar results ($R^2 > 0.92$).

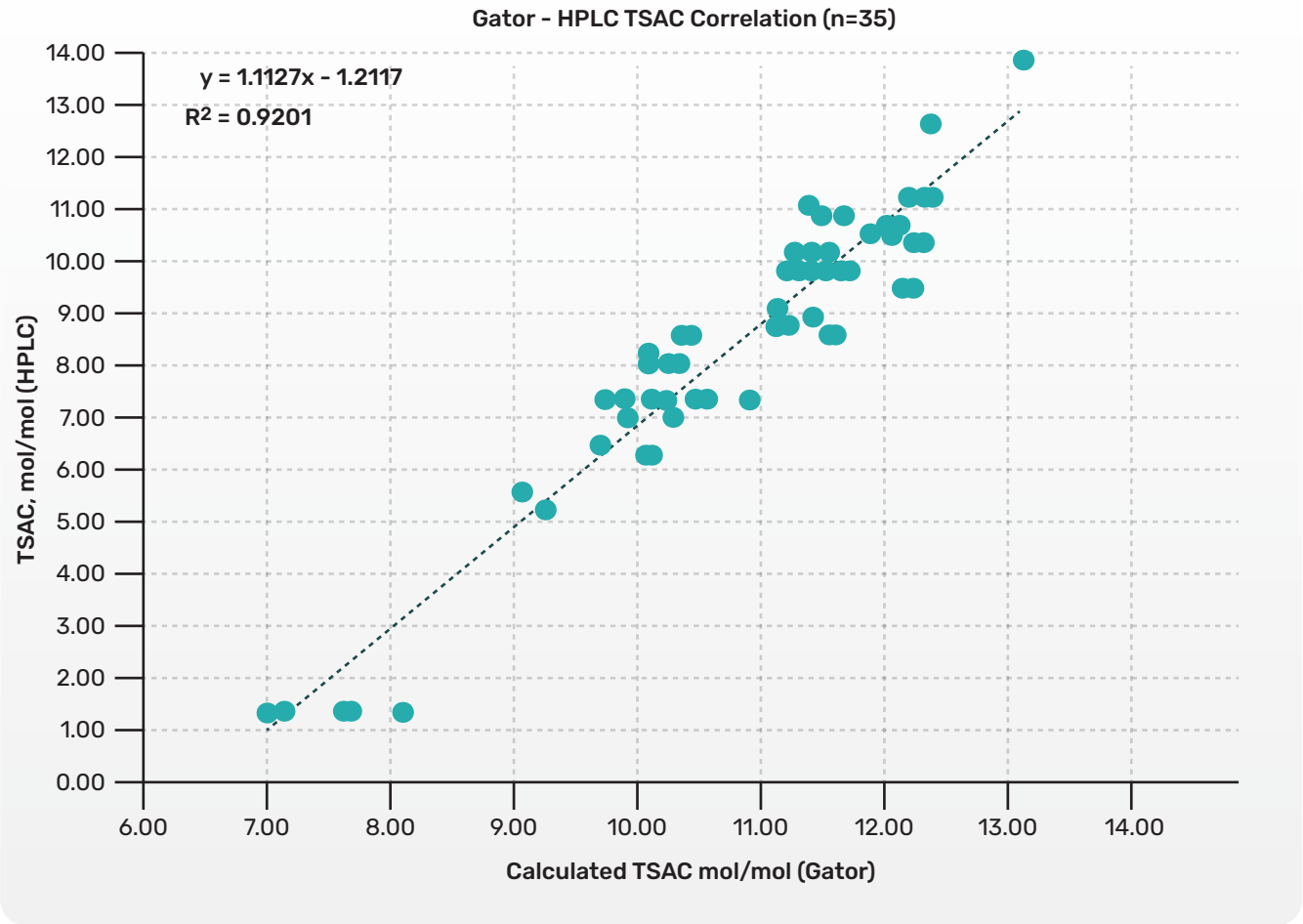

Figure 7: Correlation of Gator BLI with HPLC. Strong correlation was shown for sialic acid levels measured for all 35 samples using Gator BLI and HPLC (R^2>0.92).

In general, multiple replicates of the same sample show excellent reproducibility. As shown in Table 2 for both HPLC and Gator TSAC assays the %CV is < 3% in all cases.

Sample	HPLC			Gator			Ratio
	n	TSAC mol/mol	% CV	n	TSAC mol/mol	% CV	
Protein 6	5	11.94	2.02%	12	11.69	2.30%	97.90
Protein 7	2	11.65	2.48%	4	12.22	0.55%	104.9
Protein 8	1	12.04	N/A	4	12.31	0.70%	99.27
Protein 11	4	10.43	2.52%	4	10.29	2.41%	98.75
Protein 14	1	11.60	N/A	6	11.41	0.61%	98.36

Table 2. Within-sample variation comparison between HPLC and Gator

Additionally, HPLC and Gator instruments agree when calculating the variation between the two assays. As shown for Protein 14 in Table 3, the mol/mol response from both the HPLC and Gator TSAC assays are very similar with less than 2% variation observed. In this case, six different TSAC analysis replicates were performed for Protein 14 using BLI and compared with the single analysis performed using HPLC.

Sample	HPLC	Gator	
	TSAC mol/mol	Response (nm)	TSAC mol/mol
Protein 14, Final	11.60	2.54	11.30
Protein 14, Final	-	2.56	11.39
Protein 14, Final	-	2.56	11.39
Protein 14, Final	-	2.56	11.42
Protein 14, Final	-	2.57	11.46
Protein 14, Final	-	2.58	11.51

Table 3. Comparing mol/mol measurements for HPLC and Gator TSAC assays

Conclusion

This assay employs MAL II lectin binding as a straightforward readout for Total Sialic Acid Content (TSAC). The data demonstrate that the final MAL II binding step height is directly proportional to the TSAC of an Fc fusion protein. The method shows high reproducibility (Table 3) and correlates strongly with a gold-standard HPLC method (Figure 7). Measurements were performed using standard Gator Bio Protein A probes, requiring no specialized kits. Its simplicity allows application to various glycoproteins beyond Fc fusion proteins, and can also be used to measure the content of other glycan species using other lectins.

While yielding comparable results, Gator and HPLC methods differ fundamentally. HPLC detects both O- and N-glycosylated species for TSAC, whereas Gator measures MAL II binding to O-glycans alone. Despite this, results were consistently within 5% (Table 2), indicating a stable O- to N-glycan sialylation ratio in constructs with conserved glycosylation sites. However, since Fc fusion constructs vary greatly, the glycosylation characteristics of your target proteins may vary from those presented herein.

This document introduces an accessible, cost-effective TSAC measurement method that is ideal for quality control during clone selection and expression stages, enhancing batch-to-batch consistency. For assistance in setting up similar experiments, contact support@gatorbio.com.

References

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