

Octet® GlyM Kit

For High-Throughput Mannose Glycans Screening of
Crude and Purified mAb and Non-mAb Protein Samples



Technical Note

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Section 1: Introduction

Introduction and Purpose of the Product

During cell line and drug development, post-translational modifications (PTMs) greatly impact functional characteristics of the protein of interest (POI). One of the major PTMs is glycosylation, which can affect POI's safety and efficacy and is considered a key critical quality attribute (CQA) of biological drugs. Among various glycans, Mannose is especially important, as it affects protein's plasma clearance and thus may reduce its *in vivo* half-life with a significant negative impact on drug efficacy. While process conditions, media, and feed formulations are critical factors in the optimization of protein mannosylation, optimal cell line selection can be a limiting step. Early screening for mannose glycans content can facilitate the selection of clones that produce proteins with favorable glycan profiles during cell line development (CLD).

The Octet® GlyM kit has been designed for a quick high-throughput relative Mannose glycans content screening of both purified and crude cell culture samples during early clone selection stages in CLD. The kit can be used to screen and rank both mAb and non-mAb samples and provides reliable results for samples with mannose content differing by 4% or more. When combined with titer analysis (which can be also performed on the Octet® platform), it facilitates the selection of the top performing cell lines producing proteins with the most favorable glycan (Mannose) profile. Figure 1 shows the effective combination of relative Mannose screening and titer determination on the same sample. It allows verified decisions to be made about which clones are the most suitable to move downstream for further optimization. Reliable data at this early stage supports shorter lead times and prevents later failures.

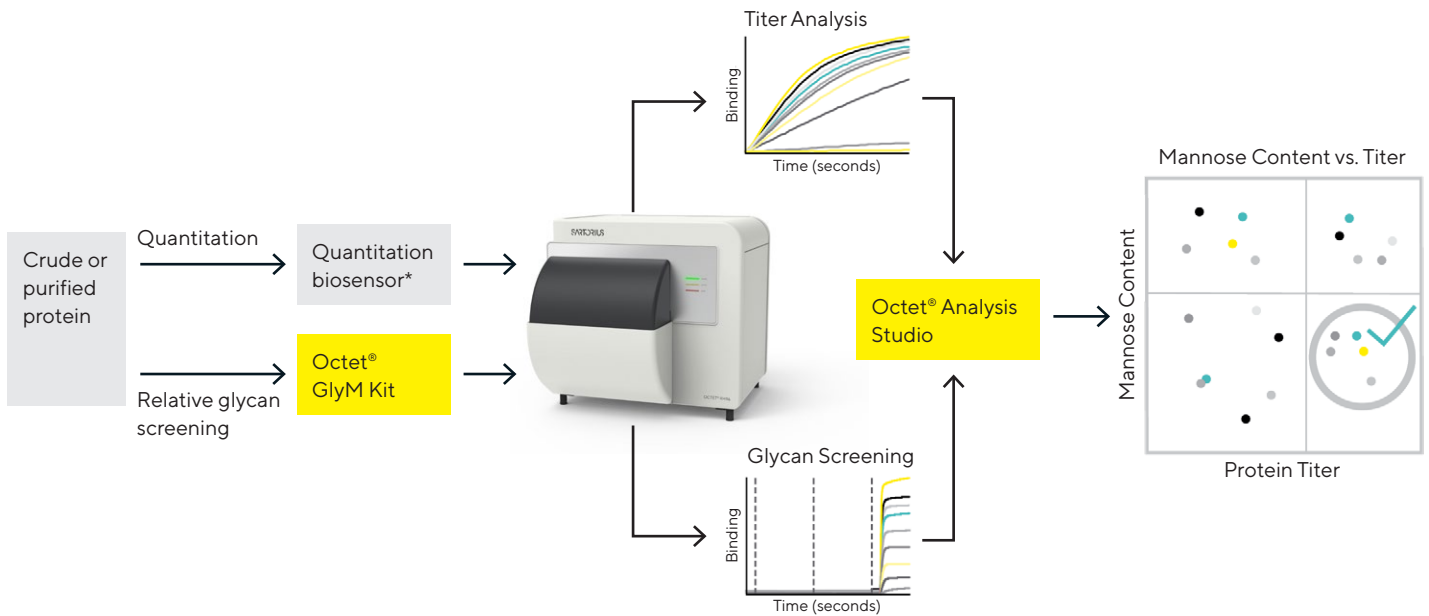


Figure 1: Schematic showing overall Mannose screening workflow using GlyM Kit in combination with titer determination to rank the samples by best producer vs. the desired mannose content.

*Not included with GlyM Kit

The kit is not intended for absolute Mannose quantitation. It is for relative glycan screening at a very early stage and is intended for research use only and not for diagnostic use.

This Technical Note describes the GlyM Kit principle and outlines protocols for high-throughput relative Mannose content screening in purified and crude cell culture mAb and non-mAb samples.

Kit Contents and Storage

Octet® GlyM Kit Component	Part Number	Content	Storage Conditions
Octet® GlyM Biosensors	18-0054	1 tray of 96 biosensors	Room temperature in a dry location
Octet® Glycan Buffer A	18-1141	50 mL	2–8° C
Octet® Glycan Sample Prep Buffer	18-1142	100 mL (2 x 50 mL)	2–8° C
Octet® Anti-hIgG Detection Fab	18-1157	0.3 mL	-20° C
Octet® Anti-hIgG Detection Fab Buffer	18-1158	50 mL	2–8° C
Octet® Glycan Detection Substrate	18-1144	0.5 mL	2–8° C
Octet® Glycan Detection Buffer	18-1145	0.72 mL	2–8° C
Octet® Glycan Wash Buffer	18-1146	46 mL	2–8° C

Other Materials Required

- An Octet® instrument with Octet® BLI Discovery and Analysis Studio software version 12.2 or higher.
- An Octet® AS station (Sartorius, P/N: 30-5011) is recommended to improve throughput if using 8- or 16-channel instruments.
- Black polypropylene 96-well or 384-well microplates (Greiner Bio-One, P/N: 655209 or 781209).
- PBS buffer without sodium azide (Gibco, P/N: 10010023 or equivalent). Not included in the kit.
- User's reference samples with known mannose content. Not included in the kit.
- α-Mannosidase enzyme (Sigma-Aldrich, P/N: M-7257-1MG). Not included in the kit.
- Optional: NIST hIgG sample (NIH, P/N: RM 8671 or Sigma-Aldrich, P/N: NIST8671).
- Optional: RNaseB glycoprotein (Sigma-Aldrich, R7884-500uG).

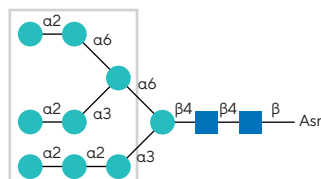
GlyM Kit Principle and Assay Workflow

The GlyM Kit is based on the use of a lectin immobilized on the biosensor that binds to mannose glycans in the protein of interest (POI). The lectin used is highly specific towards terminal alpha mannose glycans, including oligomannose-type N-glycan and Hybrid-type N-glycans (Figure 2).¹ It also binds strongly to biantennary complex-

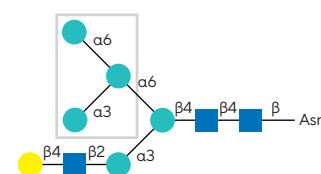
type N-glycan, but not tri- and tetra-antennary complex-type N-glycans (Figure 2). The strength of the GlyM binding signal is proportional to the combined content of these mannose glycans in the POI. The kit does not distinguish or provide information regarding individual Mannose glycoforms (e.g. Man5 or Man9).

Figure 2: Examples of N-glycans recognized by GlyM lectin biosensor with it exhibiting highest preference towards the top structure. The determinants required for binding are indicated in the boxed areas.

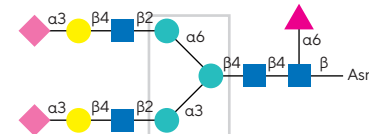
Oligomannose-type N-glycan



Hybrid-type N-glycan



Biantennary complex-type N-glycan but not tri- and tetra-antennary complex-type N-glycan



- Mannose (Man)
- ▲ Fucose (Fuc)
- ◆ N-Acetylneuraminic acid (Neu5Ac)
- Galactose (Gal)
- N-Acetylglucosamine (GlcNAc)

The GlyM kit enables relative ranking of POI samples based on mannose glycans content by comparing GlyM binding signals for these samples. For optimal ranking results, the assay should meet the following requirements:

- All analyzed POIs should have the same amino acid sequence.
- The glycans on the POIs need to be sufficiently exposed to lectin binding. To achieve this, analyzed samples should be diluted minimum 10-fold with Octet® Glycan Sample Prep Buffer (GSB, included in the kit). The buffer has been specifically formulated to relax folded proteins without denaturing them.

- All samples must be analyzed at the same concentration (fixed titer). This is needed to ensure that the measured differences in GlyM binding signals are due to mannose content variation, but not due to titer variation in analyzed samples.

The GlyM Kit assay workflow depends on the type and purity of samples to be analyzed. For purified samples, there is no binding signal amplification required, as the biosensor binds only to the Mannose available on the POI. This 1-step direct binding assay is recommended for screening of both purified mAb and non-mAb samples with high Mannose content (Figure 3). If the protein's level of glycosylation is low, the amplification assay is recommended.

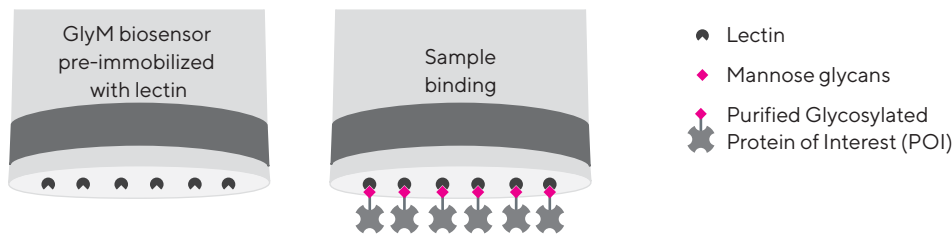


Figure 3: The GlyM Kit assay workflow for purified samples: 1-step assay with direct binding of GlyM biosensor to Mannose on POI. The assay has been tested using purified non-mAb samples with 75% Mannose content.

However, for crude samples, since there is likely competition from Mannose glycans present on Host Cell Proteins (HCP) to bind to GlyM biosensors, the assay provides necessary steps to selectively amplify only signals from a POI's Mannose and not from HCP (Figure 4). This assay with selective amplification of the signal from Mannose glycans on POIs not only increases assay sensitivity, but also eliminates the need for any

sample purification or enzymatic digestion, significantly simplifying the Mannose screening workflow. The Fab Fragment in the kit is optimized for analysis of crude hIgG mAb samples. If users need to analyze crude non-mAb samples, the Fab Fragment might need to be modified to ensure that it binds to the POI.

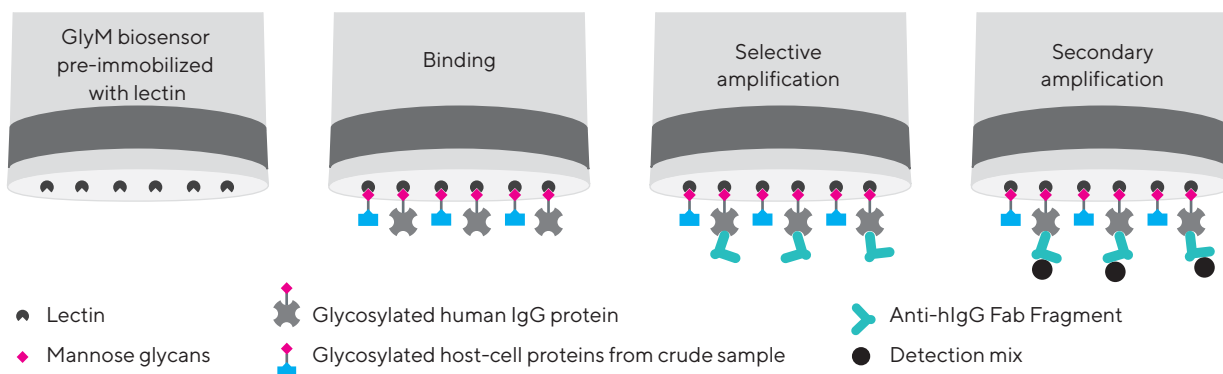


Figure 4: GlyM Kit assay workflow for crude samples: 3-step assay with selective amplification of POI's Mannose binding signal. In the first step GlyM biosensor binds to Mannose glycans in the POI as well as in HCP (Binding step). In the second step, HRP-based Anti-hlgG Detection Fab fragment selectively binds to the POI and not the HCP on the biosensor (Selective amplification step). In the final step, peroxide-based Detection Mix amplifies the signal of the Detection Fab Fragment bound to the POI (Secondary amplification step).

Additionally, the GlyM Kit assay can be performed on 8-, 16- and 96-channel Octet® instruments. Depending on the capacity of your Octet® system and the availability of an Octet® AS system, the assay steps can be performed online and offline (using Octet® AS) depending on the throughput

needs. Table 1 shows 3-step amplification assay times for analysis of 96 samples depending on the instrument capacity. For example, 96 crude samples can be screened using 3-step amplification assay in 31 min on the Octet® RH96 system in 96-channel mode.

Acquisition Mode	96-Channel Mode (No Octet® AS)		16-Channel Mode + Octet® AS		8-Channel Mode + Octet® AS	
Number of analyzed samples per run	96		96		96	
Assay step	Online Octet® AS	Duration (min)	Online Octet® AS	Duration (min)	Online Octet® AS	Duration (min)
▪ Baseline step (Glycan Sample Prep Buffer)	All steps online	3	Octet® AS	3	Octet® AS	3
▪ Binding step (user sample)		10	Octet® AS	10	Octet® AS	10
▪ Selective amplification step (Anti-hIgG Fab Fragment)		10	Octet® AS	10	Octet® AS	10
▪ Wash step (Glycan Wash Buffer)		3	Online	18	Online	36
▪ Secondary amplification step (Glycan Detection mix)		5	Online	30	Online	60
Total time	31		71		119	

Table 1: Total time required for analysis of 96 samples using 3-step amplification assay depending on the Octet® instrument capacity.

GlyM Kit Assay Development and Optimization

The GlyM assay kit requires sample concentration optimization and validation of GlyM binding signal specificity to Mannose glycans in the POI. As a result, the mannose screening process requires 3 key steps as outlined in Figure 5.

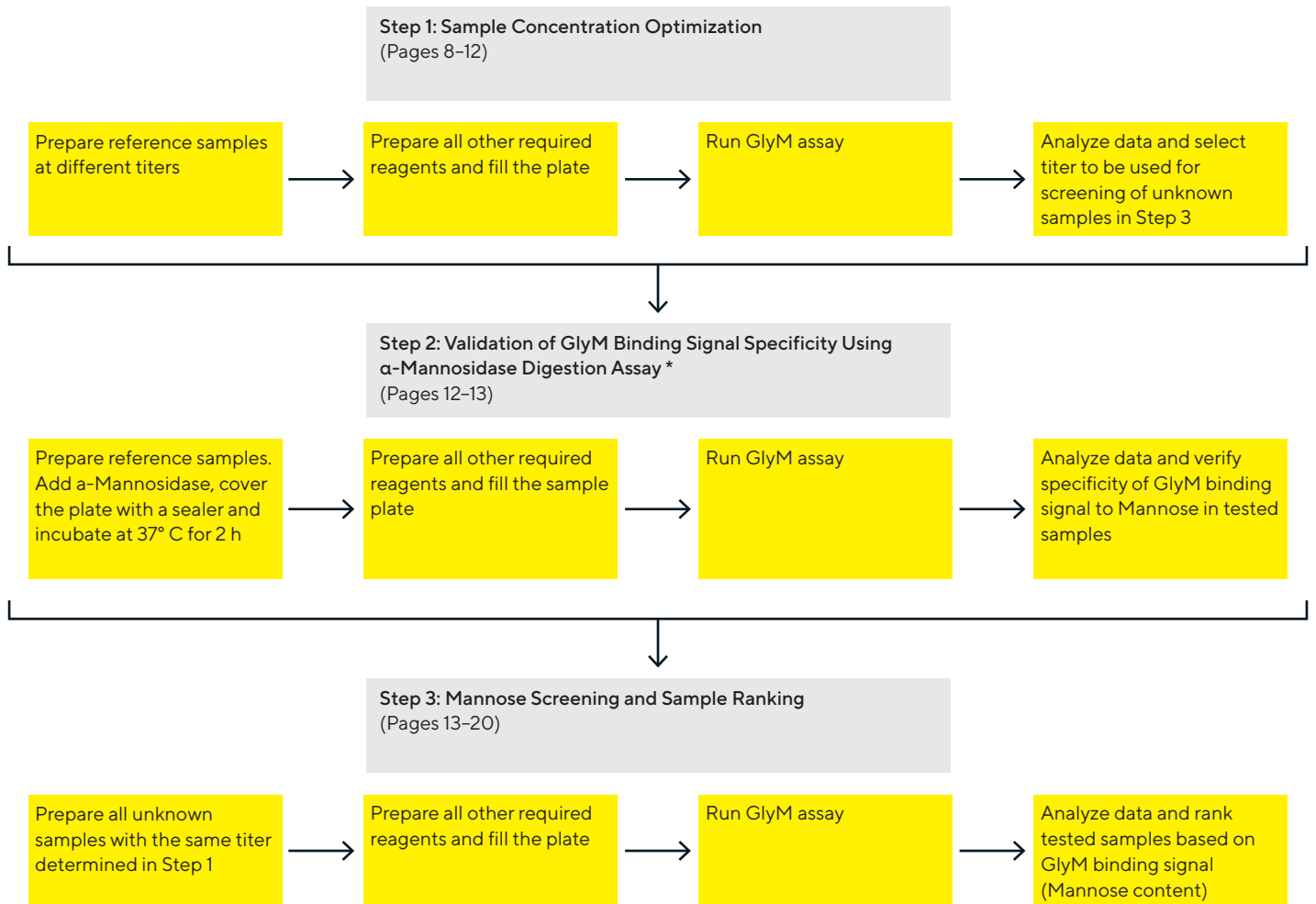


Figure 5: Steps of the Mannose screening and sample ranking process using GlyM kit. See details for each step in the following sections.

*Step 2 is recommended, but not required for Mannose screening process. For this step, the same reference samples solutions can be used as in Step 1.

- The 1st step of the process is the optimization of sample concentration with the goal to identify one concentration (fixed titer) that all samples will be diluted to in Step 3 of the Mannose screening process.
- The 2nd step is validation of GlyM binding signal specificity to Mannose in user's POI using α -Mannosidase digestion assay. This step is recommended, but not required for mannose screening process.
- The 3rd step is Mannose screening and ranking of user's samples.

These steps are described in detail in the following sections. It is highly recommended to perform all these steps for accurate Mannose screening when analyzing crude cell culture samples.

Section 2: Protocol for Mannose Screening and Ranking of Crude mAb samples using GlyM Kit

Step 1: Sample Concentration Optimization

Before You Begin

Reference samples with known Mannose content are required for this step, but are not included in the GlyM Kit and should be supplied by the user.

The optimization of sample concentration is required in order to identify one concentration (fixed titer), which all samples must be diluted to for mannose glycans screening in Step 3. This is needed to ensure that:

- The measured differences in GlyM binding signals are due to mannose content variation in analyzed samples, but not due to titer variation
- GlyM binding signals from all tested samples are within the acceptable detection range, which is 10–65 nm shift in response for the GlyM kit amplification assay. The low end is 10 nm shift to maintain signal to background (i.e. signal from GSB buffer only) ratio of > 2. The high end is 65 nm shift as it is the detection limit of the instrument.

To optimize sample concentration, users are required to have reference samples available. These samples should meet the following requirements:

- Should have the same amino acid sequence as samples, which will be analyzed during Mannose screening in Step 3
- Should be well-characterized and have known Mannose content

It is recommended that users have at least two reference samples:

- Reference sample No. 1 with low mannose content (e.g., < ~1-2%)
- Reference sample No. 2 with high mannose content (e.g., 15-20%)

In addition to the reference samples, users could use commercially available well-characterized NIST Humanized IgG1k Monoclonal Antibody at varying concentrations for side-by-side comparison and additional verification of the assay. NIST mAb is not included in the kit and can be purchased from NIST (P/N: RM8671) or from Sigma-Aldrich (P/N: NIST8671).

To determine the fixed titer, users should perform a titer scouting experiment by testing reference samples at a minimum of 8 various concentrations from the Suggested

Titer Scouting Range (Table 2). It is recommended to use 2-fold serial dilution when preparing test concentrations.

Note: It is required that at all selected test concentrations the reference samples must be diluted in Glycan Sample Prep Buffer at least 10-fold. This dilution ratio is needed to fully relax the POI and to expose all mannose structures for binding to GlyM biosensors.

Sample Type	Relative Mannose Content	GlyM Kit Assay Type	Suggested Titer Scouting Range
mAb (e.g., hlgG)	1-20%	Amplification	0.1-20 µg/mL
Non-mAb (e.g., RNaseB protein)	> 10%	Direct	2-500 µg/mL

Table 2: Suggested Titer Scouting Range for typical mAb and non-mAb samples with varying mannose content.

Finally, to select the fixed titer, users need to identify one concentration at which:

- GlyM binding signals from all reference samples are within the acceptable detection range, which is 10–65 nm shift in response for the Amplification assay.
- The separation between GlyM binding signals from reference samples is the most significant: the signal from a reference sample with the lowest Mannose is as close to the lower limit of the acceptable detection range (10 nm shift) as possible, and the signal from the sample with the highest Mannose content is as close to the upper limit as possible (65 nm shift).
- All samples that need to be analyzed in Step 3 would have to be diluted with Glycan Sample Prep Buffer (GSB) at least 10-fold. This dilution ratio is required to fully relax the POI and to expose all Mannose structures for binding to GlyM biosensors.
- For example, if you have a pool of samples with concentrations ranging 10–100 µg/mL. The highest fixed titer that could be selected for this pool is 1 µg/mL since at this titer all samples would be diluted at least 10-fold. If higher fixed titer is selected (e.g., 2 µg/mL) the samples with the lowest concentrations in this pool (e.g., 10 µg/mL) would be diluted only 5-fold, which is not sufficient to fully relax the POI.

The sample concentration optimization step can be performed on an 8-, 16- or 96-channel instrument. The protocol below describes an example of sample concentration optimization using 8-channel mode.

Preparation of Reference Samples and Reagents for GlyM Kit Assay

All reference samples and GlyM kit assay reagents should be prepared immediately before you run the assay. All buffers and diluents used should be azide-free. The presence of azide can interfere with the detection chemistry of HRP-based Anti-hlgG Detection Fab Fragment. Freshly prepared Glycan Detection Mix should be stored at 4° C until ready to use and must be used within 3–4 hours.

In the protocol below, proposed sample and buffer volumes are sufficient not only for Sample Concentration Optimization step, but also for Validation of GlyM Binding Signal Specificity step in the Mannose screening process.

1. Remove all reagents from 4° C and -20° C and allow to equilibrate to room temperature on the bench top.
 2. Prepare users' reference samples in GSB buffer at different concentrations using 2-fold serial dilution considering the following recommendations:
 - a. All reference samples should be diluted to the concentrations selected from the Suggested Titer Scouting Range in Table 2.
 - b. At all selected concentrations, the samples must be diluted with GSB at least 10-fold.
 - c. Once diluted with GSB, the samples must be equilibrated for 10 min at room temperature.
- If NIST hlgG is included in the test, recommended concentrations are listed in Table 3.

NIST hlgG Target Conc., µg/mL	Proposed Prep. Volume of NIST Solutions
20	5 mL
8	5 mL
3	5 mL
0	5 mL of GSB

Table 3: Recommended concentrations of NIST hlgG for Sample Concentration Optimization.

3. Prepare Octet® Anti-hlgG Detection Fab Fragment solution as follows: Check the dilution factor on the product label of Anti-hlgG Detection Fab Fragment and calculate the amount of this reagent that should be added to Anti-hlgG Detection Fab Buffer to obtain desired volume of the solution with 1X concentration. For example, if the dilution factor is 270X, 0.1 mL of Anti-hlgG Detection Fab will be needed to make 27 mL of the working solution with concentration of 1X. Working solutions should be stored at 4° C and used within 8 hours.
4. Prepare Glycan Detection Mix should be prepared as follows: Add 576 µL of Glycan Detection Buffer and 360 µL of Glycan Detection Substrate to 23.064 mL of PBS buffer to make a total of 24.0 mL of Detection Mix. The Detection Mix should be prepared freshly before each assay, stored at 4° C in a dark place and used within 3–4 hours.

Please refer to the Safety Data Sheet (SDS) for safety information on the Glycan Detection Substrate and Glycan Detection Buffer. Dispose of used and unused reagents in accordance with all local, state, and federal guidelines. Proper personnel safety measures should also be taken when handling hazardous materials.
5. Prepare buffers for GlyM kit assay as outlined in Table 4.

Buffers Required for GlyM Kit Assay	P/N	Volume Required for 96-Well Plate (200 µL/well)
Octet® Glycan Sample Prep Buffer	18-1142	24 mL
Octet® Glycan Wash Buffer	18-1146	24 mL
Octet® Glycan Buffer A	18-1141	24 mL

Table 4: Buffers required for GlyM assay.

6. Prepare the Sample Plate (example plate layout is shown in Figure 6, which depicts modified Sample Plate configuration template form "Glycan Screening_Mannose_8CH_96W_NoSidekick.fmf" method in Octet® BLI Discovery software version 12.2 or higher):
 - a. Pipette 200 µL of each solution into the wells of a 96-well microplate.
 - b. Pipette NIST hlgG reference samples in Column 2 and user's reference samples S1 and S2 in Column 8 (in this example, sample S1 has the lowest Mannose content available, sample S2 has the highest Mannose content the user needs to screen for).

Note: In this example, the reference samples are pipetted in 2 replicates to minimize variation. Also, included are wells with GSB buffer only for background signal subtraction.

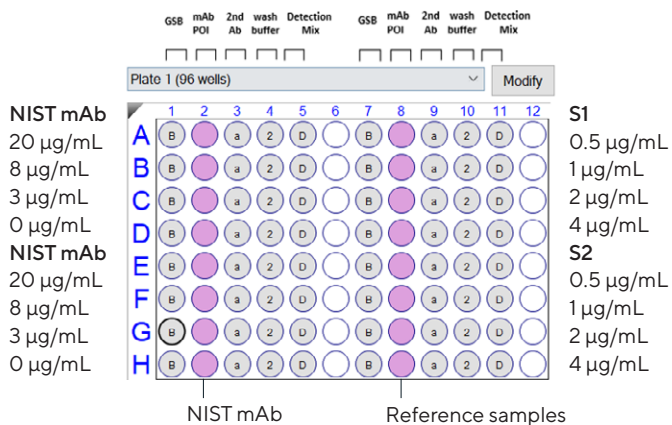


Figure 6: Example of the 96-well plate map for optimization of sample concentration step. In this example, the tested concentrations for NIST mAb are much greater compared to those for S1 and S2 samples. The reason for such difference is that NIST mAb has much lower mannose content (~1%) compared to S1 and S2 samples (2.5% and 16% respectively) and it is mainly used to verify that the assay is working properly. The test concentrations should be selected by the user based on relative Mannose content in user's reference samples (see detailed guidelines in the text above).

7. Prepare the Octet® GlyM Biosensor Tray. Biosensor locations should correspond to filled wells in the Hydration Plate.
8. Prepare Hydration Plate by pipetting 200 µL of Octet® Glycan Buffer A into wells corresponding to biosensor locations in Octet® GlyM Biosensor Tray.
9. Carefully disassemble the green biosensor tray from its blue holder tray.
10. To prevent over-hydration of the biosensors, do not place the sensors to the hydration plate yet. Wait until the experiment is ready to run.

Note: The GlyM sensor should be hydrated for 10 min prior to start of the assay. Use Delay Start Time in Octet® acquisition software before Run start in the instrument (see section "Run GlyM Assay" below).

Run GlyM Kit Assay

1. Place the Sample Plate and the Hydration plate into the Octet® Instrument on the defined plate stations.
2. Launch Octet® BLI Discovery Software and choose the Advanced Quantitation option in the Experiment Wizard. Use the advanced quantitation template "Glycan Screening_Mannose_8CH_96W_NoSidekick.fmf" for this format (Figures 7 and 8).

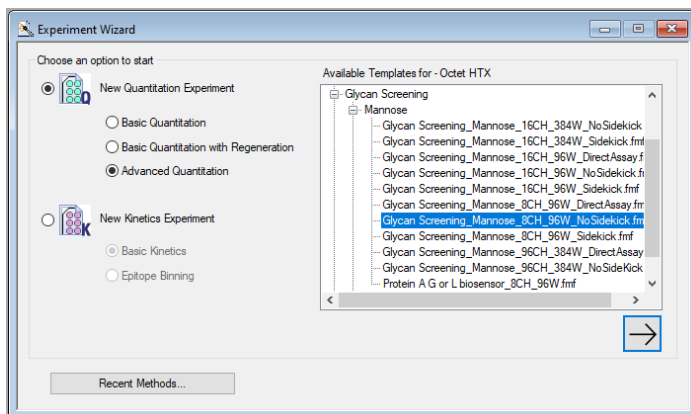


Figure 7: Experimental Wizard window from Octet® BLI Discovery software version 12.2 or higher

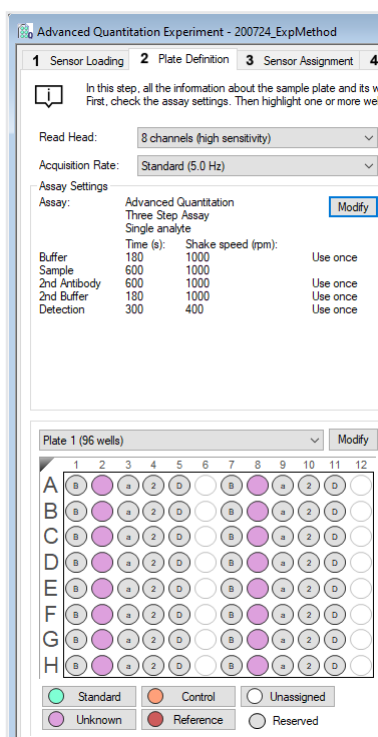


Figure 8: Settings for advanced Quantitation assay using GlyM kit on the Octet® RH96 system in 8-channel mode. The panel shows the modified Sample Plate configuration template from "Glycan Screening_Mannose_8CH_96W_NoSidekick.fmf" method in Octet® BLI Discovery software version 12.2 or higher.

3. Enter Sample ID and Sample information in the Plate Definition tab and the Sensor Assignment tab as desired.
4. In the Run Experiment tab, enter a delay time of 600 seconds in order to give the plates at least 10 min inside the Octet® instrument to equilibrate to assay temperature as well as hydrate the GlyM biosensors.
Note: It is important to keep this hydration time consistent between the assays (~10 min).
5. Enter a location and file name for saving the data.
6. Click Go to run the assay. The run time is about 1.5 h when using 8-channel mode on the Octet instrument.

Data Analysis

The Octet® Analysis Studio software version 12.2 or higher is recommended, which contains GlyM sensor methods and data analysis functionality. The analysis of the data obtained on the Octet® RH96 instrument in 8-channel mode is identical to the analysis of data obtained on other Octet® instruments. To analyze the data:

1. In the Octet® Analysis Studio software, load the data folder to be analyzed.
2. Select wells with GSB buffer only and perform background subtraction if needed.
3. Sample ID, Sensor Type and Sample information can be modified in the Table of the Preprocessed Data Window if needed (Figure 9).



Figure 9: Preprocessed Data Window from Octet® Analysis Studio software version 12.1

4. In the Quantitation Analysis Window, click Mannose Data Tab (Figure 10):
 - a. In the Octet® BLI Discovery software version 12.2 or higher, the default Binding Rate Equation for GlyM sensor is called “End-point” and will read the binding signal at the set time point at the end of the detection time as the output signal. In older versions of the Octet® software, which do not have “End-point” in the dropdown menu, please select “R-equilibrium” as the binding rate equation for GlyM sensor.

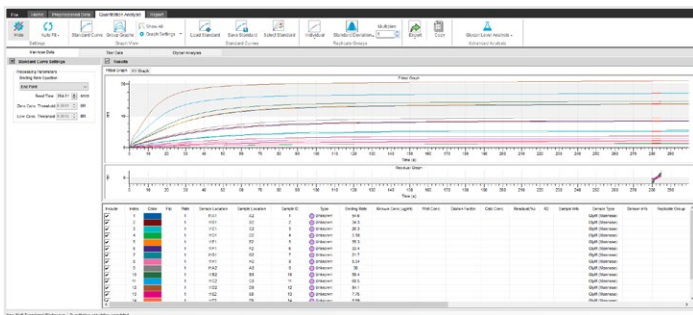


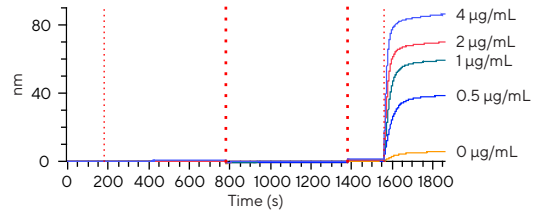
Figure 10: Mannose Data Window from Octet® Analysis Studio software version 12.2

- b. Results will be displayed automatically in the Table in Octet® Analysis Studio software version 12.2. (In older versions of the software, click Calculate Binding Rate.)
- c. Click Export Icon under Quantitation Analysis Window and > Export to generate a Microsoft Excel report file in Octet® Analysis Studio software version 12.2 or higher. (In older versions of the software, click Save Report or select File > Save Report to generate Microsoft Excel report file.)

Selection of the Fixed Titer

1. To select the fixed titer it is recommended to plot GlyM binding nm shifts for the reference samples as a function of the sample titer as shown in Figure 11.

Sample S1 With High Mannose Content



Sample S2 With Low Mannose Content

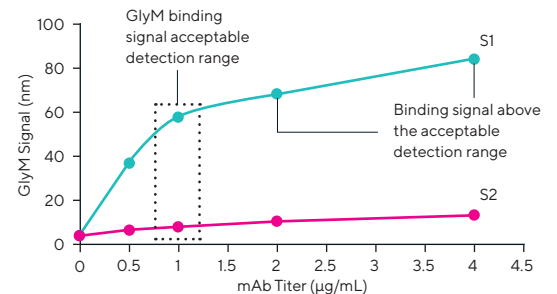
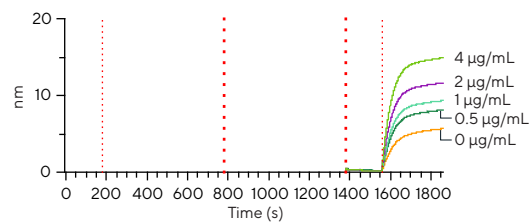


Figure 11: Selection of the fixed titer: (top) Sensorgrams showing GlyM binding signals from user's S1 and S2 reference samples (Note: y-axis scale for S1 and S2 is different due to difference in mannose content.); (bottom) Graph showing GlyM binding signal vs. Titer for S1 and S2 samples. Dotted box depicts acceptable GlyM biosensor detection range (10–65 nm) and an acceptable titer range for the fixed titer selection.

2. Select one concentration (fixed titer) that meets concentration selection criteria stated on page 8 in Before You Begin section. In the example in Figure 11, the selected fixed titer is 1 µg/mL as at this concentration the separation between reference samples is the highest, S1 and S2 are closest to the respective upper and lower limits of the acceptable detection range, and all samples that need to be analyzed during mannose screening in Step 3 have sufficient quantity to be diluted 10-fold with GSB buffer.
3. All samples that will need to be analyzed in Step 3 of the Mannose screening process will need to be diluted to the selected fixed titer. (In the example in Figure 11, the fixed titer is 1.0 µg/mL.)

Step 2: Additional Validation of GlyM Binding Signal Specificity to Mannose Glycans Using α-Mannosidase Digestion Assay

Before You Begin

α-Mannosidase enzyme is required for this step, but is not included in the GlyM Kit and should be purchased from one of the major suppliers (for example, from Sigma-Aldrich, P/N M-7257-1MG).

The α-Mannosidase digestion assay is designed to verify specificity of GlyM binding signal to Mannose glycans in user's samples. It provides additional confirmation that the observed GlyM binding signal is mainly due to binding to Mannose in POI and not due to non-specific binding (NSB). However, this assay will not provide information regarding the specific Mannose glycans GlyM biosensor binds to in user's samples.

Although this step is recommended to be performed during initial stages of GlyM assay development, it is optional and is not required for Mannose screening process.

To perform this assay, one set of user's samples should be prepared with the addition of α-Mannosidase enzyme and the other set without the enzyme. Both sets of samples should be incubated at 37° C for 2 h and then analyzed side-by-side using GlyM Kit. The samples with the enzyme should have no or significantly reduced GlyM binding signal compared to samples not treated with the enzyme. This result confirms that GlyM biosensor specifically binds to mannose glycans in user's samples.

It is recommended to perform this assay using the same user's reference samples as in Step 1 of the Mannose screening workflow.

Preparation of Samples and Reagents for GlyM Kit Assay

All reference samples and GlyM assay reagents should be prepared immediately before you run the assay. All buffers and diluents used should be azide-free. Freshly prepared Glycan Detection Mix should be stored at 4° C until ready to use and must be used within 3-4 hours.

1. Remove all reagents from 4° C and -20° C and allow to equilibrate to room temperature on the bench top. No dilution of α-Mannosidase (Sigma, P/N: M7257-1MG, 23U, in 0.3mL per vial) is needed.
2. Prepare fresh reference samples and GlyM assay reagents as described on p. 9-10 for the Sample Concentration Optimization step.
3. Prepare the Sample Plate (example plate layout is shown in Figure 12):
 - a. First, pipette only NIST hlgG samples in Column 2 and the reference samples with the highest Mannose content (Sample S2) in Column 8. Do not pipette buffers and detection mix yet.
 - b. Pipette 20 µL of the α-Mannosidase solution to the NIST hlgG samples and user's reference samples S2 in rows E-H only as shown on Figure 12

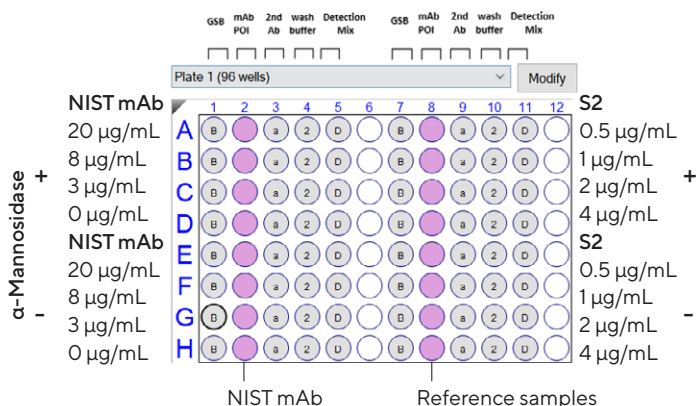


Figure 12: Example of the 96-well plate map for α-Mannosidase digestion assay. It is recommended to use NIST and reference samples at the same concentrations as in Step 1 (see p. 9-11 for detailed guidelines).

4. Cover the plate with a sealer (standard film ELISA plate sealer or equivalent) to minimize the evaporation during incubation.
5. Incubate the samples in the plate at 37° C for 2 h.
6. Cool the sample plate to Room Temperature for 20 min and then remove the sealer.

7. Prepare the GlyM Biosensor Tray. Biosensor locations should correspond to filled wells in the Hydration Plate.
8. Prepare Hydration Plate by pipetting 200 μL of Octet[®] Glycan Buffer A and the Sample plate by adding Octet[®] anti-HlgG Detection Fab and Glycan Detection mix.
9. Carefully disassemble the green biosensor tray from its blue holder tray.
10. To prevent over-hydration of the biosensors, do not place the sensors to the hydration plate yet. Wait until the experiment is ready to run.

Note: The GlyM sensor should be hydrated for 10 min prior to start of the assay. Use Delay Start Time in Octet[®] acquisition software before Run start in the instrument. (See section "Run GlyM Kit Assay" below.)

Run GlyM Kit Assay

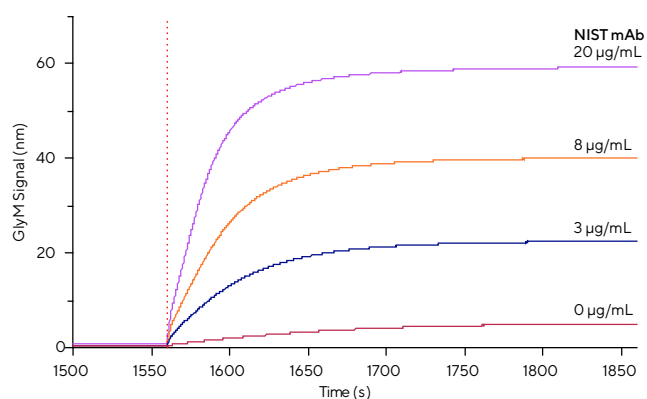
1. Place the Sample Plate and the Hydration plate into the Octet[®] Instrument on the defined plate stations.
2. Launch Octet[®] BLI Discovery software and choose the Advanced Quantitation option in the Experiment Wizard. Use the advanced quantitation template "Glycan Screening_Mannose_8CH_96W_NoSidekick.fmf" for this format.
3. Set up the method and run the assay as described on p. 11-12 in the previous section, Sample Concentration Optimization.

Data Analysis

The data analysis procedure is the same as described on p. 12-14 for the Sample Concentration Optimization step.

Example results from the α -Mannosidase digestion assay are shown in Figure 13. The samples that were treated with the enzyme showed significantly reduced GlyM binding signal compared to the undigested samples. This result confirms that GlyM biosensor primarily binds to Mannose glycans in NIST samples.

No α -Mannosidase POI Signal



Step 3: Mannose Screening and Sample Ranking

Before You Begin

In this step of the Mannose screening process users can analyze and rank hundreds of crude cell culture samples with unknown Mannose content in a matter of hours. To perform the assay, all samples should be diluted with Glycan Sample Prep Buffer (GSB) to the fixed titer determined in Step 1 of the Mannose screening process.

It is recommended to include reference samples with known Mannose content in this assay in order to increase the accuracy when ranking and grouping samples depending on Mannose content (e.g., high, medium, low).

Depending on the capacity of your Octet[®] system and the availability of an Octet[®] AS station, the GlyM Kit assay steps can be performed online or offline (using Octet[®] AS) depending on the throughput needs. However, irrespective of how the assay steps are performed (online or offline), the protocols for sample preparation and data analysis are nearly the same. Please refer to Table 1 for the assay times required to analyze 96, 64, 32 and 16 samples depending on the instrument capacity.

The protocol below describes how to run GlyM kit assay with:

- All steps performed online using the Octet[®] RH96 instrument in 96-channel mode
- The initial steps performed offline on the Octet[®] AS station and the secondary signal amplification steps performed online using the 8- or 16-channel Octet[®] instrument

With α -Mannosidase POI Signal

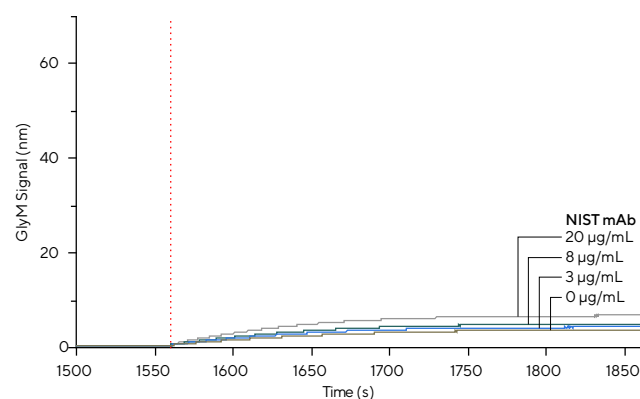


Figure 13: Example results from α -Mannosidase digestion assay using NIST hlgG samples

Preparation of Samples and Reagents for GlyM Kit Assay

All samples and GlyM kit assay reagents should be prepared immediately before you run the assay. All buffers and diluents used should be azide-free. The presence of azide can interfere with the detection chemistry of Anti-hlgG Detection Fab Fragment. Freshly prepared Glycan Detection Mix should be stored at 4° C until ready to use and must be used within 3–4 hours.

1. Remove all reagents from 4° C and -20° C and allow to equilibrate to room temperature on the bench top.
2. Prepare user's samples in Glycan Sample Prep Buffer at the same fixed titer determined in Step 1 of the Mannose screening workflow.

Note: All samples must be diluted with GSB at least 1:10 v/v.

3. Prepare Octet® Anti-hlgG Detection Fab Fragment solution as follows: Check the dilution factor on the product label of Anti-hlgG Detection Fab Fragment and calculate the amount of this reagent that should be added to Anti-hlgG Detection Fab Buffer to obtain desired volume of the solution with 1X concentration. For example, if the dilution factor is 270X, 0.1 mL of Anti-hlgG Detection Fab will be needed to make 27 mL of the working solution with concentration of 1X. Working solutions should be stored at 4° C and used within 8 hours.
4. Prepare Glycan Detection Mix should be prepared as follows: Add 576 µL of Glycan Detection Buffer and 360 µL of Glycan Detection Substrate to PBS buffer to make a total of 24 mL Detection Mix. The Detection Mix should be prepared freshly before each assay or stored at 4° C in a dark place for same-day assays.
5. Prepare buffers required for GlyM kit assay as outlined in Table 5.

Reagents Required for GlyM Kit Assay	P/N	Volume Required for 96-Well Plate (200 µL/well)
Octet® Glycan Sample Prep Buffer	18-1142	24 mL
Octet® Glycan Wash Buffer	18-1146	24 mL
Octet® Glycan Buffer A	18-1141	24 mL

Table 5: Buffers required for GlyM kit assay. Scale up or down as needed based on the number of user's sample.

Run GlyM Kit Assay

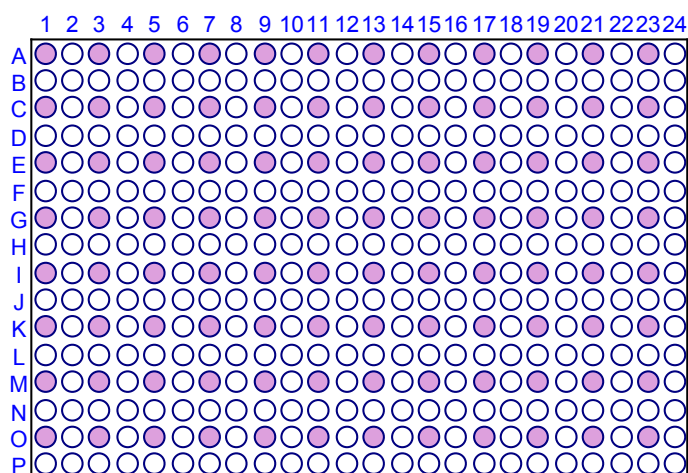
Depending on the capacity of your Octet® system and availability of an Octet® AS system, GlyM kit assay steps can be performed in online and offline formats depending on your throughput needs. Both formats are described in detail below.

GlyM Kit Assay With All Steps Online: A Complete Walk-Away Assay Format

In this format, all assay steps are performed online inside the instrument without the need for any manual processing or user intervention. This assay format eliminates any potential user-related data variation and allows for the most streamlined and efficient complete walk-away workflow.

Depending on the capacity of your Octet® system and throughput needs, GlyM kit assay in this format allows you to analyze up to 96 samples with excellent assay precision in 33 min using Octet® RH96 system in the 96-channel mode.

1. Prepare the Sample Plate (example plate layout is shown in Figure 14):
 - a. Pipette 80 µL of each sample prepared in Glycan Sample Prep Buffer into the wells of a 384-well microplate (Sample Plate).



● Samples Prepared in Glycan Sample Prep Buffer

Figure 14: Sample Plate layout for 96 biosensor mode in 384-well plate for Octet® RH96 system

2. Prepare the Reagent Plate (example plate layout shown in Figure 15):
 - a. Pipette 80 µL of Octet® Glycan Sample Prep buffer into the wells of a 384-well microplate (Reagent Plate).
 - b. Pipette 80 µL of the diluted to 1X Octet® Anti-hlgG Fab Fragment solutions into the wells of a 384-well microplate (Reagent Plate).
 - c. Pipette 80 µL of Octet® Glycan Wash Buffer into the wells of a 384-well microplate (Reagent Plate).
 - d. Pipette 80 µL of Octet® Glycan Detection Mix into the wells of a 384-well microplate (Reagent Plate).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D
B	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2
C	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D
D	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2
E	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D
F	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2
G	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D
H	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2
I	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D
J	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2
K	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D
L	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2
M	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D
N	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2
O	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D	B	D
P	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2	a	2

- (B)** Octet® Glycan Sample Prep Buffer
- (a)** Octet® Anti-hlgG Fab Fragment diluted to 1X concentration using Octet® Anti-hlgG Detection Fab Buffer
- (2)** Octet® Glycan Wash Buffer
- (D)** Octet® Glycan Detection Mix

Figure 15: Reagent plate layout for 96 biosensor mode in 384-well plate for Octet® RH96 system

3. Prepare the GlyM Biosensor Tray. Biosensor locations should correspond to filled wells in the Hydration Plate.
4. Prepare Hydration Plate by pipetting 200 µL of Octet® Glycan Buffer A into wells corresponding to biosensor locations in GlyM Biosensor Tray (one biosensor for each filled well in the Sample Plate).
5. Carefully disassemble the green biosensor tray from its blue holder tray.
6. Place the hydration plate into the blue holder tray. Do not place the sensor to the hydrate plate yet. Wait until the experiment is ready to run.

Note: The GlyM sensor hydration time needs to be kept consistent between assays (e.g., 10 min). Use Delay Start Time in Octet® acquisition software before Run start in the instrument (see section “Run GlyM Kit Assay” below).

7. Place the Sample Plate and Detection Plate in the Octet® RH96 instrument on the defined plate stations.
8. Launch Octet® BLI Discovery Software and choose the Advanced Quantitation option in the Experiment Wizard (Figures 16 and 17). Use the advanced quantitation template “Glycan Screening_Mannose_96CH_384W_NoSidekick.fmf” for this format.

Octet® BLI Discovery software version 12.2 or higher would be recommended which contain GlyM sensor default assay setting. Please refer to the Octet® BLI Discovery Software manual for guidance where to download the most updated software.

If you are also running the titer assay with ProA sensor using the same samples and plan to compare that data with the GlyM data in the Octet® Analysis Studio software, please use the same sample IDs for both ProA and GlyM experiments in Octet® BLI Discovery software. Please refer to Data Analysis section below for details.

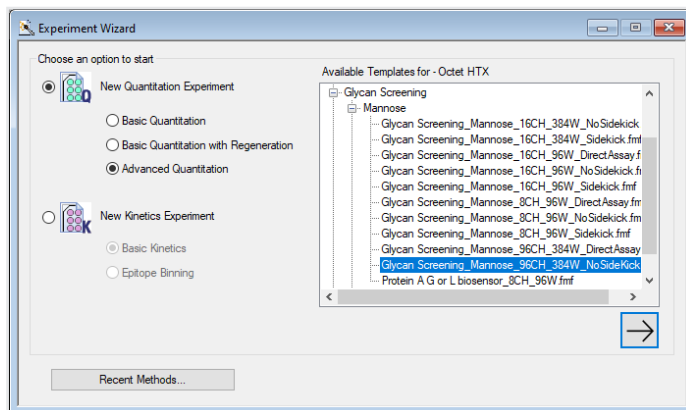


Figure 16: Experimental Wizard window from the Octet® BLI Discovery Software v. 12.2

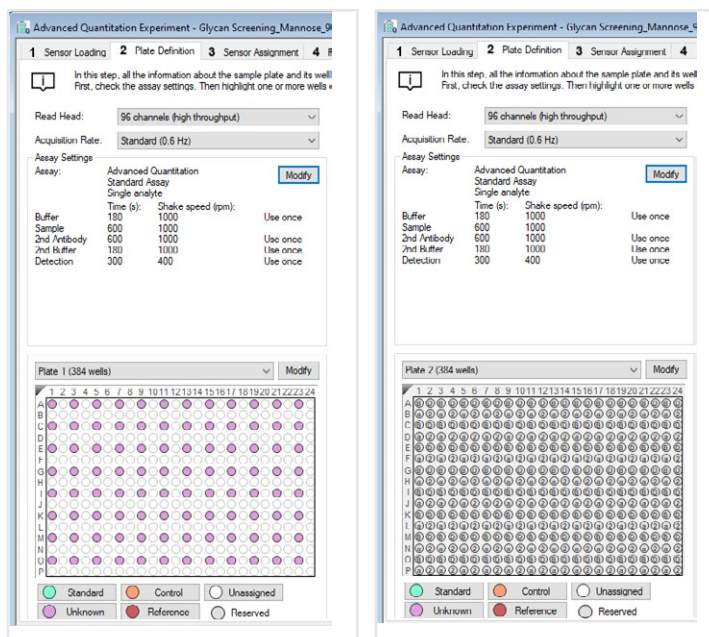


Figure 17: Settings for GlyM kit assay on the Octet® RH96 system. The panel on the left shows the Sample Plate configuration and the assay settings in 96-channel mode. The panel on the right shows the Reagent Plate and the assay settings in 96-channel mode. This is the default assay setting from template “Glycan Screening_Mannose_96CH_384W_NoSidekick.fmf” in Octet® BLI Discovery Software v. 12.2.

9. Enter Sample ID and Sample information in the Plate Definition Tab and the Sensor Assignment tab. It is required to use the same sample IDs for both ProA (or other biosensor type used for titer measurement) and GlyM experiment in order to be able to analyze and rank samples based on titer and Mannose content in the Octet® Analysis Studio software.
10. In the Run Experiment tab, enter a delay time of 600 seconds in order to give the plates at least 10 min inside the Octet® instrument to equilibrate to assay temperature as well as hydrate the GlyM sensor. It is important to keep this hydration time consistent between the assays (e.g., 10 min).
11. Enter a location and file name for saving the data.
12. Click Go to run the assay. Run time for all 96 sensors is 33 min.

GlyM Kit Assay With Offline Steps Using Octet® AS Station

An Octet® AS station is recommended to be used with 8- and 16-channel Octet® instruments as it allows to process the initial incubation assay steps outside of the instrument (offline) and to perform the remaining steps on the Octet® instrument. This helps to increase sample throughput for these systems.

The GlyM kit assay for 8- and 16-channel Octet® instruments using Octet® AS station is performed according to the steps shown in Figure 18. The initial 4 steps of the assay, including incubation with Pre-wet plate, Preconditioning Plate, Sample Plate, and Anti-hlgG Detection Fab Plate, are performed on using the Octet® AS station. The remaining detection steps are performed on the Octet® instrument.

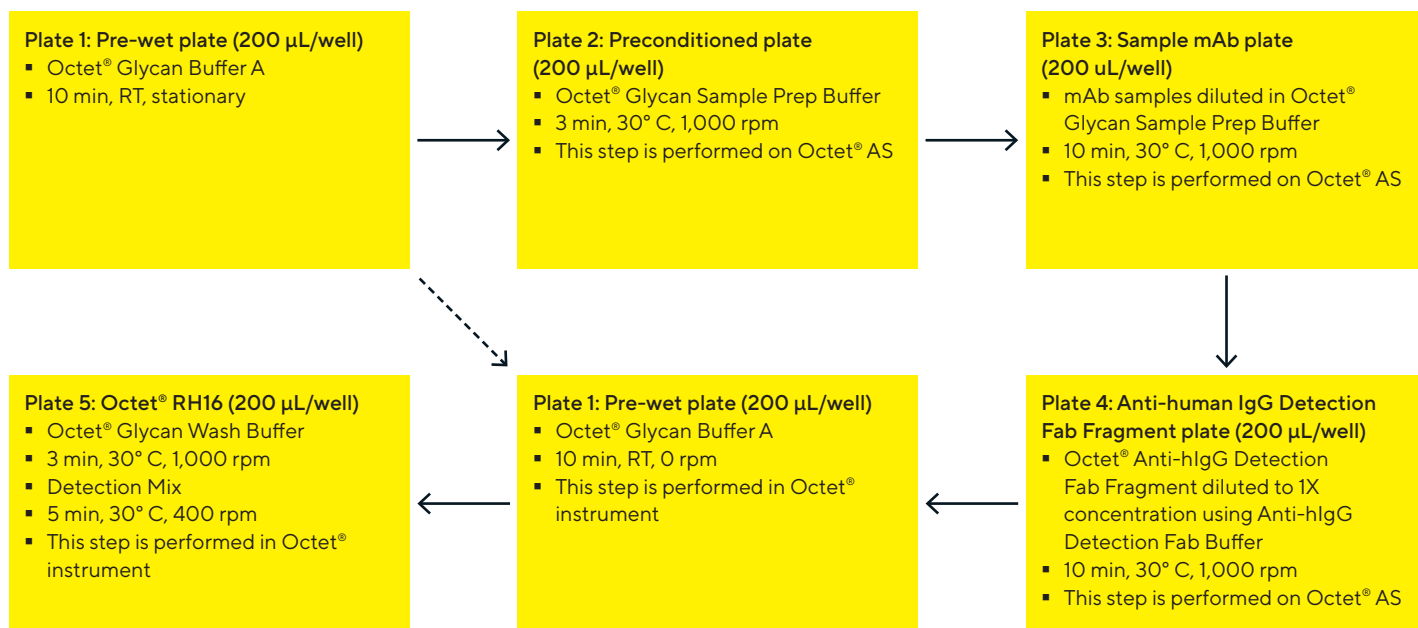


Figure 18: Flow chart of GlyM kit assay steps on 8- and 16-channel Octet® systems with the Octet® AS Offline Station.

1. Prepare the Reagents, Sample and Detection Plates (example plate layout shown in Figure 19): Pipette 200 µL of each reagent into each well of the 96-well reagent plate for Octet® AS incubation.
 - 1-Plate: Octet® Glycan Buffer A Buffer Plate (Hydration Plate, used twice)
 - 2-Plate: Octet® Glycan Sample Prep Buffer Buffer Plate
 - 3-Plate: hlgG Sample Plate (Protein of Interest (POI))
 - 4-Plate: Octet® Anti-hlgG Detection Fab Fragment Plate
 - 5-Plate: Octet® Glycan Detection Plate containing Glycan Wash Buffer and Glycan Detection Mix. It is recommended to make this detection plate fresh right before use.
2. Prepare the Biosensor Tray. Biosensor locations should correspond to filled wells in the Sample Plate (one biosensor for each filled well in the Sample Plate).
3. For the Hydration Plate containing Octet® Glycan Buffer A (1st Plate), the filled well locations should correspond to biosensor locations in Biosensor Tray.

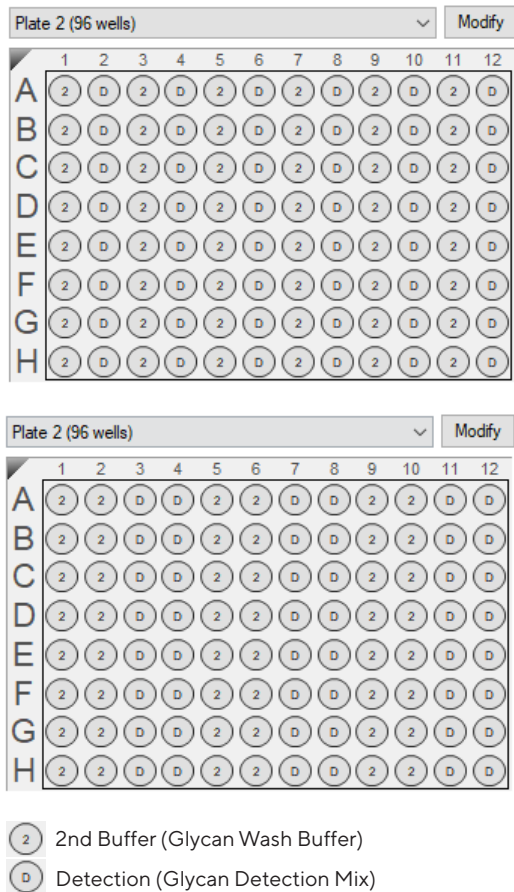


Figure 19: Detection plate loading guide for (top) Detection Plate of 8-channel Octet® instrument, and (Bottom) Detection Plate of 16-channel Octet® instrument. For Octet® AS method, use template “Glycan Screening_Mannose_8CH_96W_Sidekick.fmf” or “Glycan Screening_Mannose_16CH_96W_Sidekick.fmf”. Glycan Wash buffer and Glycan Detection Mix can be dipped twice, which is sufficient to cover all 96 samples.

4. Place the biosensor tray onto the Hydration plate (1st Plate). Keep stationary for 10 min on the lab bench. Please keep this hydration time consistent between different GlyM kit assay runs.
5. On the Octet® AS Station, place the GlyM biosensors in the Preconditioned plate with GSB buffer (2nd plate). Shake the plate at 1000 rpm and 30° C for 3 min.
6. Incubate pre-wet biosensors in the mAb Sample Plate (3rd Plate) on the Octet® AS Station at 1000 rpm and 30° C for 10 min.
7. After 10 min of sample incubation, replace the Sample Plate with the Anti-hIgG Detection Fab Fragment Plate (4th Plate). Incubate on the Octet® AS Station at 1000 rpm and 30° C for 10 min.
8. During this incubation, prepare the Detection Plate as shown in Figure 19. Pipette 200 µL of each reagent specified into all wells of one column (when using 8-channel format) or into two columns (when using 16-channel format) in the Detection Plate.
9. After 10 min incubation with the Anti-hIgG Detection Fab Fragment plate, promptly transfer the green Biosensor Tray to the Octet® instrument and place it over the Hydration Plate containing Octet® Glycan Buffer (1st plate, the same plate used at the beginning of the Octet® AS procedure).
10. Place the Detection Plate into the Octet® Instrument.
11. Launch Octet® BLI Discovery Software and choose the Advanced Quantitation option in the Experiment Wizard. Use the advanced quantitation template “Glycan Screening_Mannose_8CH_96W_Sidekick.fmf” or “Glycan Screening_Mannose_16CH_96W_Sidekick.fmf” for this format. The assay parameters can be modified if needed in the Assay Setting window by clicking Modify (Figure 20). Click OK once the assay parameters have been defined.

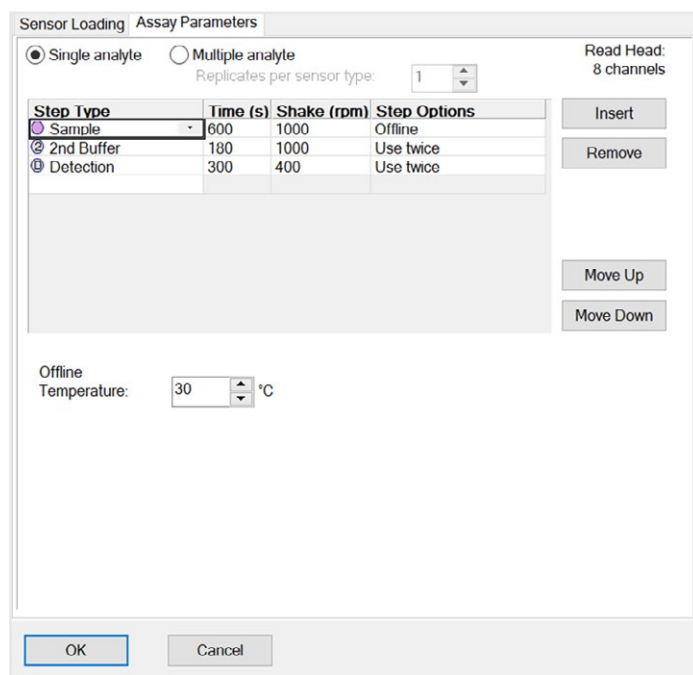


Figure 20: Assay Parameters window from Octet® BLI Discovery Software version 12.2.

- Define the Sample and Detection Plate layouts. Enter Sample ID and Sample information in the Plate Definition Tab and the Sensor Assignment tab as desired. Please use the same sample ID for both Titer and GlyM experiments if you want to compare the same samples titer level and the glycan level.

Note: Sample Plate Layout needs to be defined in the Octet® BLI Discovery Software even though the Sample step has been performed offline as this enables the software to assign the correct Sample IDs to the biosensors during analysis.

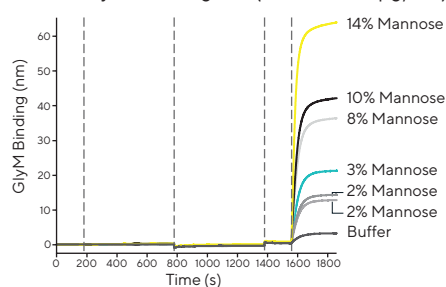
- In the Run Experiment tab, enter a delay time of 600 seconds in order to give the plates at least 10 min inside the Octet® instrument to equilibrate to assay temperature.
- Enter a location and file name for saving the data.
- Click GO to run the assay.

Data Analysis

The data analysis procedure for the Online and Offline GlyM assay formats is the same, and is as described on p. 10–12 in the previous section, Sample Concentration Optimization.

Example results from screening of crude hIgG samples using GlyM Kit on the Octet® RH96 instrument are shown in Figure 21. (All samples were diluted using GSB buffer to the same titer of 2 µg/mL.)

Octet® GlyM Sensorgram (Fixed titer: 2 µg/mL)



Relative Mannose Glycans Content (% by HPLC) vs. GlyM Signal (nm) (Fixed titer: 2 µg/mL)

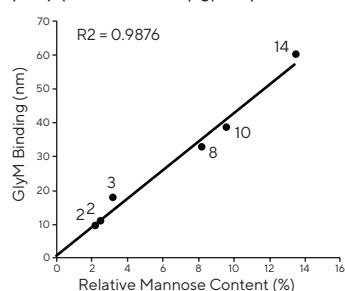


Figure 21: Screening of crude human mAb samples using GlyM Kit on the Octet® RH96 system: (top) raw sensorgram of GlyM binding signals of mAb samples with varying amount of mannose and (bottom) correlation of GlyM binding signal (nm) with relative % of Mannose determined by HPLC/MS analysis.

Sample Ranking Based on Mannose Content and Titer Data

For sample ranking based on Mannose content and Titer, GlyM data can be combined with the titer data in the Octet® Analysis Studio software version 12.2 or higher.

Note: For this functionality to work, titer should be measured using Octet in a separate experiment. Sample ID information in the titer measurement experiment must be identical to that in the GlyM experiment. If sample IDs do not match, please go back to “Preprocessed Data” Tab in the BLI Discovery Software and add | edit your sample ID information as follows:

- Open Preprocessed Data tab in BLI Discovery Software and then select wells on the Sample Plate (top left Sample Plate map) by right-clicking and selecting Edit Sample Info. Alternatively, sample ID can be edited in the Table (bottom right) directly under the sensorgram (Figure 23).
- In addition to the sample ID, sensor type can also be modified in the Preprocessed Data tab in BLI Discovery Software by clicking the Show icon on the top, followed by selecting the sensor well (top left Sensor Tray map), right-clicking and selecting Edit Sensor Info. Use the dropdown menu to choose the right sensor type (Figure 22).

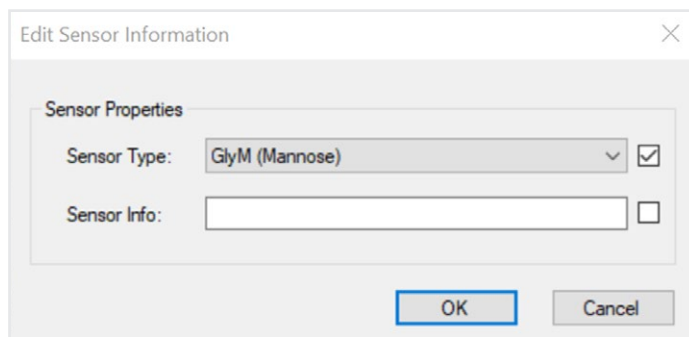


Figure 22: Edit Sensor Information window in the Octet® BLI Discovery Software v.12.2.

To analyze GlyM Titer data follow the following steps:

1. In the Octet® Analysis Studio Software v. 12.2 or higher, load the data folder to be analyzed. You can preview the data by clicking on the experiment data file in the Experiment Explorer window (Figure 23).
2. Select the folder with your glycan data in the Experiment Explorer window and load both GlyM sensor data and ProA Titer sensor data files. To load these files, drag ProA titer data file into the Segment window. Then select the GlyM data file in the Experiment Explorer window and drag it into the Experiment Builder window to overlay the data onto the titer data file.



Figure 23: Octet® Analysis Studio Home tab window view. GlyM sensor data and ProA Titer sensor data files can be overlaid together in the software by dragging them into the Experiment Builder window on the right.

3. Click on the Preprocessed Data tab to view the data. Select Assay No. 1 to view Titer data (Figure 24) or Assay No. 2 to view Mannose data (Figure 25).
4. You can do all reference subtractions by first assigning the reference wells, then right-clicking and selecting the type of subtraction.

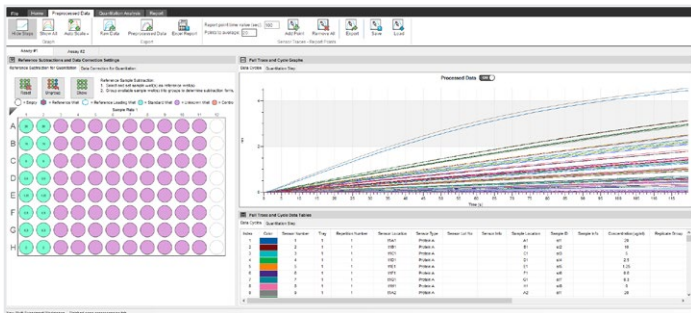


Figure 24: Viewing Assay No. 1 ProA Titer sensor Data in “Preprocessed Data” window.

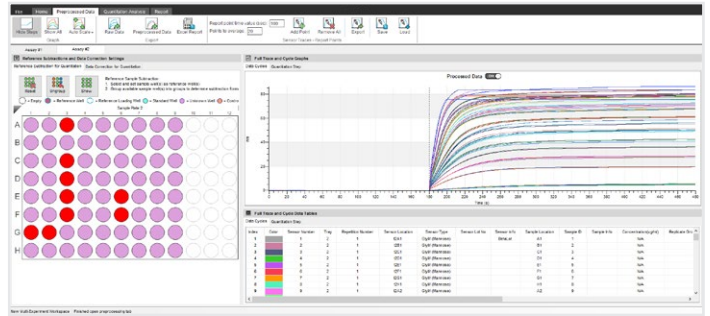


Figure 25: Viewing Assay No. 2 GlyM sensor Data in “Preprocessed Data” window

5. Click on the Quantitation Analysis Window.
6. Click on the Titer Data tab.
7. In the Standard Curve Equation window, select appropriate Standard Curve Equation and then select the source for the standard curve.

Note: All procedure for ProA titer sensor should be followed according to ProA titer measurement standard procedure. A separate standard curve can also be loaded by clicking Standard Curve box on the top in Quantitation Analysis window under Titer Sensor tab.
8. Select Initial Slope as the Binding Rate Equation for ProA Titer sensor (Default setting of ProA for Octet® Analysis Studio Software v.12.2 or higher).
9. Select the titer concentration data with desired dilution factors by unchecking the Include box next to the undesirable rows in the Table (Figure 26).

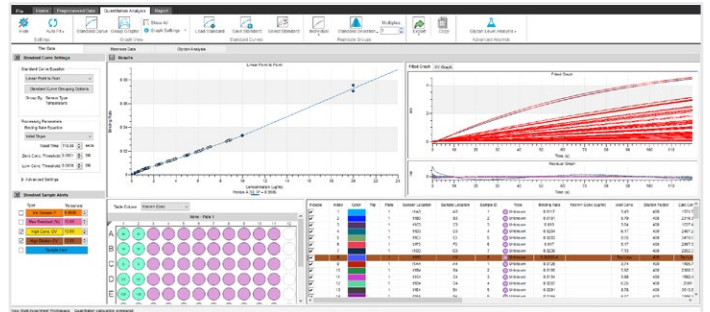


Figure 26: Selecting the Binding Rate Equation, Source for Standard Curve and Titer Concentration Data from ProA sensor.

10. Click (Excel Report) or (Results) on the top bar menu to export the ProA Titer Data in Excel format or .csv format for any custom analysis as desired.
11. In the Quantitation Analysis Window, click Mannose Data Tab.
12. Default Binding Rate Equation for GlyM sensor is “End-point” in Octet® Analysis Studio software v.12.2 or higher version (Figure 27). This equation will read the binding signal at the set time point, which is typically at the end of the detection time as the output signal. For older version software, please select “R-equilibrium” in the Results Tap as the binding rate equation for GlyM sensor.
 Note: It is important to select “initial slope” as Processing Parameter for Titer Sensor Data. It is important to select “End Point or R-equilibrium analysis” for Glycan Lectin sensor data.
13. Click (Excel Report) or (Results) on the top bar menu to export the Mannose Data Tap in Excel format or .csv format for any custom analysis as desired.

14. Click on the Glycan Analysis Data tab to visualize both ProA and GlyM sensor data (Figure 28). To see zoomed-in view, double click on each chart. Also, scroll the mouse over to the bar graph to see more sample information. The sample information can be added during Data Acquisition software step when you run the samples on Octet® instrument, or during data analysis in Preprocessed Data window in Octet® Analysis Studio software v.12.2.

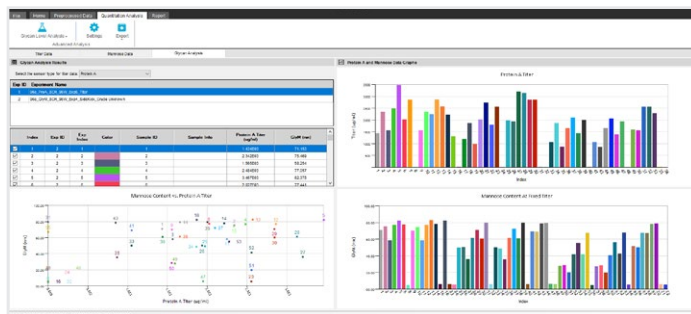
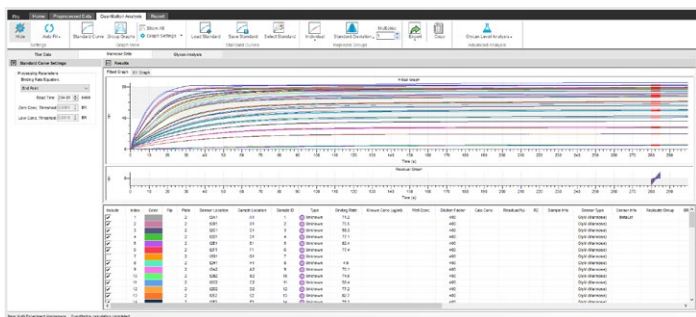


Figure 28: The titer information for the selected samples from ProA sensor data is shown on the top graph on the right. Mannose content for the selected samples from GlyM sensor is shown on the bottom right graph. The comparison chart showing GlyM Signal (nm) at fixed titer vs. Protein A titer (µg/mL) is shown on the bottom left graph.

Figure 27: End Point Data analysis in Mannose Data tab. For older version software, please select “R-equilibrium” in the Results Tap as the binding rate equation for GlyM sensor.

Section 3: Mannose Screening of Purified Non-mAb Glycoproteins Using GlyM Kit Assay

As described in Section 1, the rationale and steps of the Mannose screening and sample ranking process are the same for both GlyM assay workflows (Direct Binding and Signal Amplification), and those were covered in detail in Section 2. Thus, this section will mainly focus on details of GlyM Kit Direct Binding assay that are different from the amplification assay.

This assay is mainly suitable for analysis of purified non-mAb samples with high Mannose content. It is not suitable for analysis of crude cell culture samples since Host Cell Proteins (HCP) in the crude samples are also glycoproteins which will bind to GlyM sensor together with the protein of interest.

Before You Begin

For the Sample Concentration Optimization step, as described on p. 5–6, it is recommended to test at least two user's reference samples with known mannose content and the same amino acid sequence as the samples to be analyzed in Step 3 of the Mannose screening process.

If user's reference samples with known Mannose content are not available, commercially available RNaseB glycoprotein can be used as a reference (not included in the kit). It can be purchased from Sigma-Aldrich (P/N: R7884-500uG).

The sample and reagent preparation protocol for Direct Binding GlyM kit assay is the same for all Octet® Instruments. Below it is outlined for an 8-channel instrument as a representative example.

Preparation of Samples and Reagents for GlyM Kit Assay

All samples and GlyM assay reagents should be prepared immediately before you run the assay.

1. Remove all reagents from 4° C and allow to equilibrate to room temperature on the bench top.
2. Prepare user's samples in Glycan Sample Prep Buffer (see p. 5–13 for reference how to optimize the concentration).

Note: All samples must be diluted with GSB at least 1:10 v/v.

3. Prepare buffers required for GlyM kit assay as outlined in Table 6.

Buffers Required for GlyM Assay	P/N	Volume Required for 96-Well Plate (200 µL/well)
Octet® Glycan Sample Prep Buffer	18-1142	24 mL
Octet® Glycan Buffer A	18-1141	24 mL

Table 6: Buffers required for the Direct binding GlyM assay

4. Prepare Sample Plate (example plate layout is shown in Figure 29):
 - a. Pipette 200 µL of Octet® Glycan Sample Prep Buffer into one column of wells.
 - b. Pipette 200 µL of purified glycoprotein of interest prepared in Glycan Sample Prep Buffer into the next column of wells.

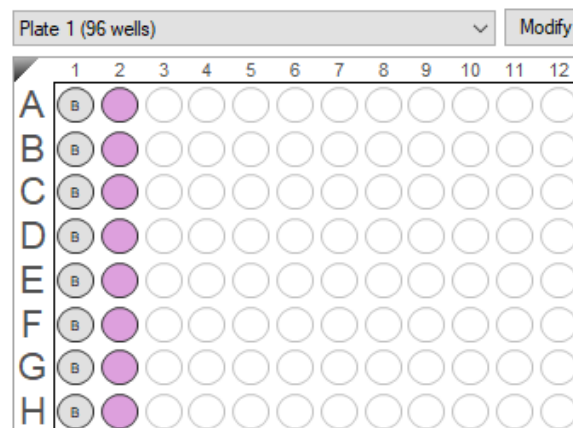


Figure 29: Example Sample Plate layout for GlyM Kit Direct Binding assay.

5. Prepare the biosensor tray:
 - a. Prepare a hydration plate by pipetting 200 μL of Glycan Buffer A into each well of a 96-well plate. Well locations filled with Glycan Buffer A in the hydration plate should correspond to biosensor locations in biosensor tray.
 - b. Carefully disassemble the green biosensor tray from its blue holder tray.
 - c. Place the hydration plate into the blue holder tray.
 - d. Place the green tray with biosensors back on top of the blue holder tray such that the biosensors are dipping into the wells of the hydration plate.
 - e. Do not place the sensor to the hydrate plate yet. Wait until all the instrument is ready to run.

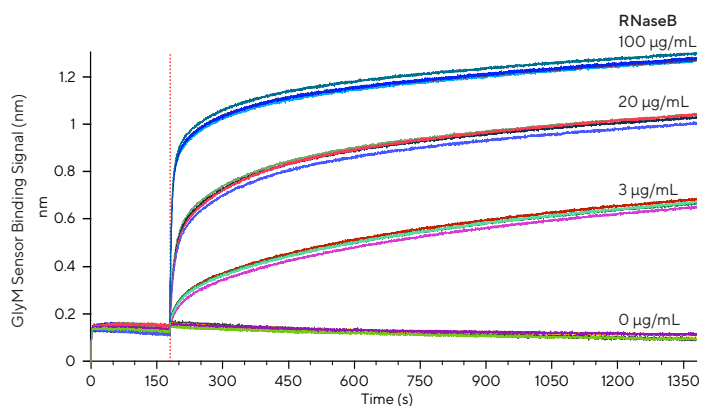
Note: The GlyM sensor hydrate time needs to be kept consistent between assays (e.g., 10 min). Use Delay Start Time in Octet[®] acquisition software before Run start in the instrument. (See section “Run GlyM Kit Assay” below.)

Run GlyM Kit Assay

1. Place the Sample Plate and the Hydration plate into the Octet[®] Instrument on the defined plate stations.
2. Launch Octet[®] BLI Discovery Software and choose the Advanced Quantitation option in the Experiment Wizard. Use the advanced quantitation template “Glycan Screening_Mannose_8CH_96W_DirectAssay.fmf” for this format.
3. Set up the method and run the assay as described on p. 8–9 above.

Data Analysis

The data analysis procedure is the same as described on p. 10–12 in the previous section. Example results from the Direct binding GlyM kit assay are shown in Figure 30.



RNaseB	GlyM (nm)	
	Average	%CV (n= 4)
100 $\mu\text{g/mL}$	1.1	0.9 %
20 $\mu\text{g/mL}$	0.87	2.6 %
3 $\mu\text{g/mL}$	0.5	1.5 %
0 $\mu\text{g/mL}$	0.0	N/A

Figure 30: Example of Mannose screening using Direct binding GlyM kit assay workflow for non-mAb glycoprotein (sample: Bovine RNaseB glycoprotein tested at 3 concentrations 100 $\mu\text{g/mL}$, 20 $\mu\text{g/mL}$ and 3 $\mu\text{g/mL}$ in Glycan Sample Prep buffer).

Section 4: Additional GlyM Kit Assay Guidelines and Troubleshooting Information

Troubleshooting Information

Issue	Possible Reasons	Suggested Solutions
Sample signals show crashed pattern	<ul style="list-style-type: none"> ▪ Signals are too high. 	<ul style="list-style-type: none"> ▪ Dilute the sample using GSB buffer to get the signal below 65 nm.
High variability between runs	<ul style="list-style-type: none"> ▪ Inconsistency in incubation time resulting in antibody relaxation and glycan exposure variation. 	<ul style="list-style-type: none"> ▪ For best results, assay step times should be consistent across all reagents steps including manual intervention, and also sensor hydration time. Use a lab timer to ensure all samples and assay are treated similarly.

Issue	Possible Reasons	Suggested Solutions
Mannose ranking between samples do not make sense	<ul style="list-style-type: none"> ▪ Lectin's binding preference towards different mannose and glycan species. ▪ Assay variables are in play other than mannose content of the samples. ▪ Samples from the different host cells, which may contain different species of mannose, were compared together. ▪ Samples containing different HCP amounts via difference culture stages crude samples (early or late stages) were compared together. ▪ Samples with the different titer amounts were compared together, then followed by the titer normalization. ▪ Samples having the different amino acid sequences were compared together. Different amino acid sequences could result in different MW or different glycosylation. Both can affect the GlyM binding signals. ▪ Inconsistency due to biased analytic method. HPLC N-glycan digestion vs. Acid hydrolysis total glycan (both N- and O-glycan). ▪ Sample may contain Fab glycans as well, but HPLC data is generated via N-glycan analysis via PNGaseF digestion which only digest Fc-glycans. 	<ul style="list-style-type: none"> ▪ Check the α-Mannosidase digestion assay to see the binding signals were from terminal mannose. ▪ GlyM assay works the best for the same amino acid sequence molecules with minimum variables other than mannose contents. For best result, reduce the assay variables by keeping the condition consistent and comparable for a fair comparison. ▪ Do not use Titer normalization method. Use the fixed titer samples to compare the mannose ranking between samples. ▪ When in doubt, use the total glycan release method, such as acid hydrolysis, to minimize the glycan release method related bias for HPLC analysis.
Background signals are too high (> 15 nm)	<ul style="list-style-type: none"> ▪ Glycan Detection Mix was not fresh (old prep). ▪ Detection Fab fragment dilution buffer was not used. 	<ul style="list-style-type: none"> ▪ Make fresh glycan detection mix and use once (or maximum twice only for Octet[®] AS method). ▪ Use appropriate buffer to make the reagent.
No binding signal in amplification assay	<ul style="list-style-type: none"> ▪ Reagent missing or inactive reagents were used during the amplification assay. ▪ Sample amount is too low. ▪ Too much HCP proteins in the crude samples (thick fed batch late stage samples with little IgG expression) compressing the binding signals via direct competition inhibiting the GlyM sensor binding. 	<ul style="list-style-type: none"> ▪ Use standard glycoprotein such as NIST mAb to see the expected binding signals there (see Figures in Amplification Assay section for NIST mAb binding signal example), and to assure if the reagents are all active. If there is a good binding signals from the positive controls, then check your samples to see if the sample is active or titer amount is too low. ▪ Do titer scouting experiment to find the optimum concentration to use.
No binding signal in direct binding assay	<ul style="list-style-type: none"> ▪ Sample amount is too low. ▪ Unpurified crude sample is used. ▪ The protein's level of glycosylation is too low. 	<ul style="list-style-type: none"> ▪ Use standard glycoprotein such as RNaseB to see the expected binding signals there (see Figures in Direction Assay section for RNaseB binding signal example), and to assure that the reagents are all active. ▪ User purified samples for the direct assay. ▪ Add more sample. Do titer scouting assay to find the range. (See Appendix Table for Titer scouting guidance.) ▪ Use amplification assay.
Signal outliers	<ul style="list-style-type: none"> ▪ Glycan Detection Mix is not fresh and/or reused multiple times. 	<ul style="list-style-type: none"> ▪ Make fresh glycan detection mix and use once (or maximum twice only for Octet[®] AS method).
Signal trending	<ul style="list-style-type: none"> ▪ Glycan Detection Mix is not fresh and/or reused multiple times. 	<ul style="list-style-type: none"> ▪ Make fresh glycan detection mix and use once (or maximum twice only for Octet[®] AS method).
Mannosidase digestion does not work (signal not reduced upon enzyme digestion)	<ul style="list-style-type: none"> ▪ Not enough enzyme to digest the samples. Ratio of enzyme and protein may be off. Enzyme amount should be in saturation range over protein amount. ▪ Enzyme is inactive. ▪ Not enough incubation time. ▪ Protein contains not terminal mannose, but more of core mannose. 	<ul style="list-style-type: none"> ▪ Use sufficient enzyme amount and longer incubation time such as overnight incubation. 10–20 μL of enzyme (75 mU/μL) would be sufficient for 96-well 200 μL sample for most of biosimilar mAbs (2 ~ 10 μg/mL). Try overnight incubation time or fresh enzymes. Make sure to use GSB buffer or the recommended buffer by vendor's suggestion for better enzyme efficiency. ▪ Run HPLC/MS analysis.

Additional Assay Guidelines

- For the HPLC/MS data comparison for the mannose content validation, please check the types of glycan digestion method for MS/HPLC data generation. The data from the typical N-glycan digestion method using PNGaseF enzyme is comparable with GlyM.
- To avoid lot-to-lot GlyM Kit binding signal variation, it is recommended to use the same lot of GlyM kit for side-by-side comparison of glycoprotein of interest. Refer to Certificate of Analysis (CoA) for the lot-specific details, including within the lot % CV information.
- Do not mix and match kits and biosensor trays from different lots. Kit reagents are paired with a specific lot of biosensors to perform optimally. Lot numbers for individual components of a kit are printed on the CoA.
- The signal nm shift of more than 70 nm is less accurate, because the algorithm have a relatively less number of data points for the reliable calculation. If the binding signal nm shift is higher than 80 nm, it may exhibit a crashed signal pattern in the sensorgram window. It is recommended to dilute the samples to get a signal lower than 70 nm to increase the confidence in the data quality.
- The tilted well microplates are not supported for GlyM assay.
- GlyM and GlyS sensors simultaneous | overlay run is currently not supported.
- Non-Mab sample amplification assay procedure is currently not supported by standard GlyM kit assay protocol. (Custom assay development would be required. An antibody against your protein of interest (POI) is required. A polyclonal antibody from rabbit can be used. As a detection antibody, Goat-Anti-RabbitIgG Detection Antibody (Jackson ImmunoResearch, PN111-035-144 or equivalent; requires to be HRP-labeled) can be used).
- Host cell glycoprotein interference
Please keep in mind below factors when planning your experiments:
 - a. It is recommended *not* to mix the different host cell types of crude samples in the same experiment for a fair comparison. The host cells from different species (e.g., CHO cells and HEK cells) may contain different mannose amounts and glycan species. For example, the CHO cells' HCPs may compete with your sample differently than that of HEK cells' HCPs.
 - b. It is recommended *not* to mix the early and late-stage samples in the same experiment for a fair comparison. Crude samples from different stages of cell line development (CLD) most likely will contain different amounts of HCPs.
- Media interference
The differences between matrices can influence GlyM kit assay performance. Diluting the sample matrix using GSB buffer is an effective way to minimize matrix effects. If recommended dilution factor of 1:10 (v/v) does not produce suitable results, optimization of the dilution factor might be required. In this case, the reference sample should be diluted with varying amount of GSB and then tested in order to determine the minimum dilution factor required for optimal assay performance.

References

1. Cummings, R.D., & Etzler, M.E. (2009). 'Antibodies and lectins in glycan analysis'. In: A.Varky, R.D. Cummings, & J.D. Esko et al. (Eds.), Essentials of glycobiology (2nd ed., pp. 45.1-45.25). Cold Spring Harbor Laboratory Press.

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