SARTORIUS

Octet® High Precision Streptavidin 2.0 (SAX2) Biosensors

Technical Note

Scope
This technical note discusses best practices and recommendations in the use of Octet® High Precision Streptavidin 2.0 (SAX2) Biosensors for kinetics characterization and quantitation of target molecules when using biotinylated ligands.

Abstract

Streptavidin is often deployed as the molecule of choice for covalent linking to solid surfaces for ligand binding assays applications. Streptavidin's biophysical properties enables it to form a high affinity non-covalent bond with biotin allowing for the tight immobilization of biotinylated ligands. Octet® High Precision Streptavidin 2.0 (SAX2) Biosensors are developed to be used with the Octet® Bio-Layer Interferometry (BLI) platform and are qualified for applications in downstream drug discovery and regulated environments. They are QC-tested to meet precision-controlled biotinylated-ligand loading coefficient of variation (CV) specification of <4% within a lot and CV range of 20% across different lots and can used with any biotinylated molecule for both kinetics and quantitation assays where stringent precision requirements are in place.

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Overview

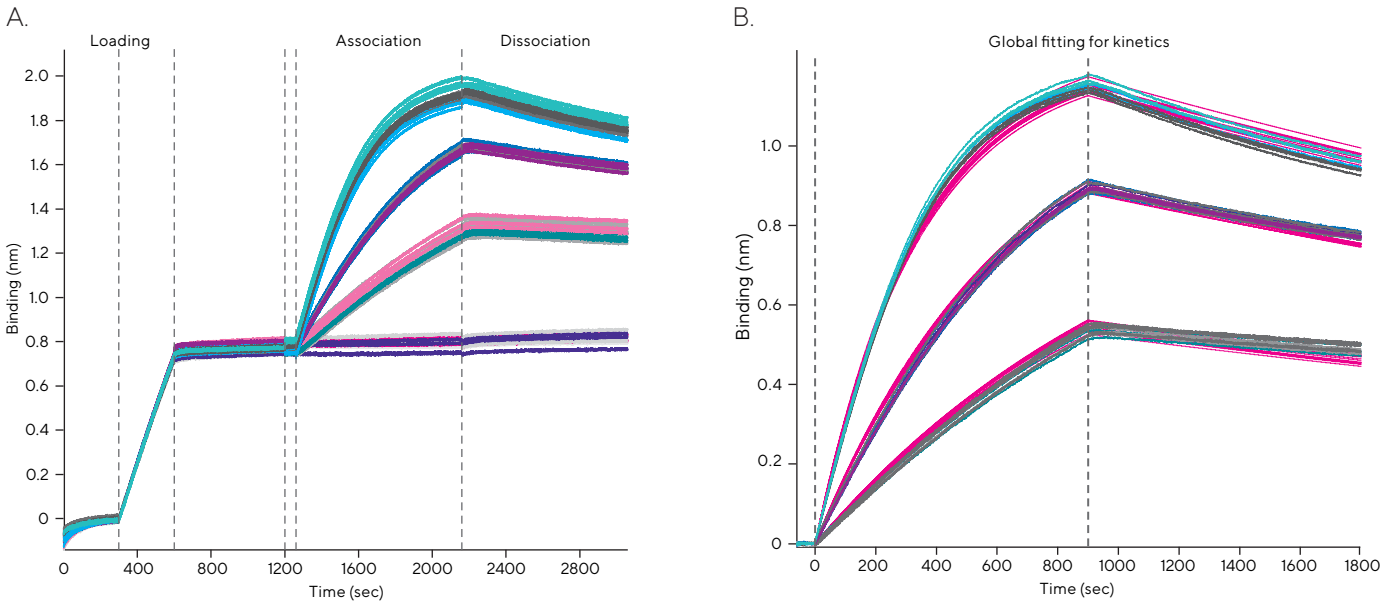
Streptavidin-coated surfaces are widely used as a simple and straight-forward method of molecular immobilization in a variety of applications. Combined with Bio-Layer Interferometry (BLI), the High Precision Streptavidin 2.0 Biosensor (SAX2, PN 18-5136) is one of Sartorius' offering for direct immobilization of biotin-tagged molecules for binding kinetics and custom quantitation on Octet® BLI systems. Octet® SAX2 Biosensors are QC-tested for minimal variance within a lot and across different lots, making it suitable for stringent assay precision requirements in downstream drug discovery and regulated environments.

The SAX2 Biosensor is developed and qualified for applications in manufacturing, quality control, and GxP labs that have stringent assay precision requirements. SAX2 is designed and optimized for assays that require lot-to-lot consistency in consumables to confidently detect variance in samples. SAX2 is QC-tested to meet precision-controlled biotinylated-ligand loading coefficient of variation (CV) specification of <4% within a lot and CV range of 20% across different lots. Overlaid example data from three different lots of SAX2 in a kinetics assay is shown in Figure 1 which shows tight CVs for loading of biotin-Fcγ receptor on the SAX2 Biosensors. CV calculations between lots for the same are shown in Table 1.

Example Data

Figure 1

Kinetics Assay Using 3 Different SAX2 Lots.



Note. (A) Kinetic analysis of the interaction between a ligand biotin-Fcγ Receptor (~55 kDa) and analyte hIgG (150 kDa) with SAX2 Biosensors, overlaying three Biosensor lots and raw data aligned at the ligand loading step. (B) Data was processed and curve fitted using a 1:1 binding model. Pink lines represent fitted curves; other colors represent raw curves.

Table 1

CV Calculations for Loading of Fc-gamma Receptor onto SAX2 Biosensor Shown in Figure 1.

	Lot 1	Lot 2	Lot 3	SAX2 Specification
Loading CV within lot	1.5%	1.3%	1.3%	<4%
Loading CV across lots		0.5%		<20%
K_D CV within lot	3.4%	2.7%	4.1%	Dependent on the sample pair and type
K_D range across lots		4%		

Best Practices for Kinetics Assay Development with SAX2

Proper assay technique and optimization as well as use of high quality, active, and stable reagents are key to obtaining accurate data from a binding kinetics experiment. Below are some best practices for developing a kinetics assay using the SAX2 Biosensor .

Biotinylation of Ligand for Immobilization on SAX2 Biosensor

To immobilize a ligand onto a SAX2 Biosensor , it must first be biotinylated. *In vivo* site-specific biotinylation methods that place one streptavidin binding site at a carefully chosen location on the ligand are recommended. However, when opting to perform biotinylation in the lab, the proteins to be biotinylated must be purified, carrier protein-free and in a buffer that does not contain primary amines, such as Tris or glycine.

A variety of biotinylation reagents targeting different functional groups are available commercially that allow for simple and efficient attachment of biotin to antibodies, proteins or peptides. The most commonly used are NHS-esters of biotin that target primary amines such as the amine group of free lysine residues in a protein or peptide. Spacer arms or linkers of different lengths are sometimes necessary to reduce steric hindrance and for efficient capture of biotinylated molecules. We recommend reagents such as EZ-Link™ NHS-PEG4-Biotin (Thermo Scientific, PN 21362) and EZ-Link™ Sulfo-NHS-LC-LC-Biotin (Thermo Scientific, PN 21338). Peptides can be synthesized with a biotin with a long linker incorporated. An optimal ratio of biotin reagent:ligand is a 1:1 molar coupling ratio (MCR). Over-biotinylation does not improve biosensor loading levels and has the potential to reduce protein activity due to excess biotin on the binding sites. A higher MCR may be necessary if the stock protein is at a concentration of less than 500 µg/mL and in this case a 3:1 or 5:1 ratio may be used. After biotinylation, the reaction must be desalted to remove excess biotin reagent as it may compete for binding sites on the streptavidin surface. Gel filtration spin columns are a rapid and efficient option for desalting. Alternatively, dialysis into PBS buffer with an appropriate molecular weight cutoff membrane or cartridge can be used for gentle buffer exchange of more sensitive proteins (use 100 kDa MW cutoff for antibodies). Once the protein has been biotinylated and desalted into neutral pH buffer, a ligand loading optimization experiment can be performed to determine optimal loading concentration.

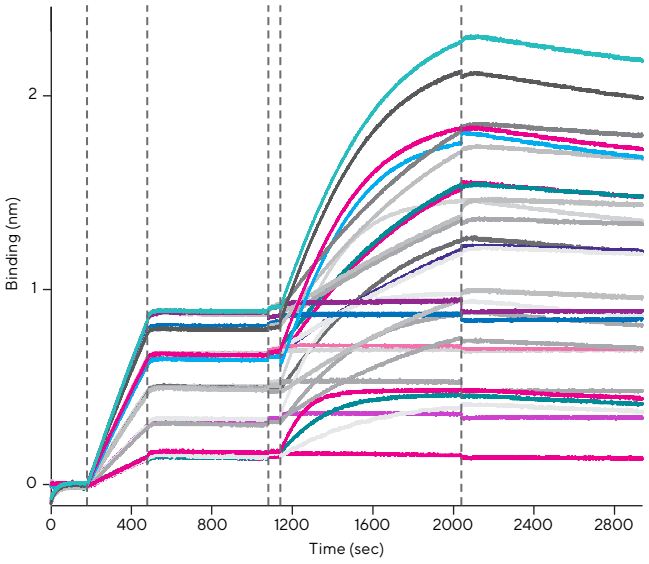
Determine Optimal Conditions for Ligand Capture

Loading an optimal density of immobilized ligand on the SAX2 Biosensor surface is critical to obtaining quality kinetics data. Scout the ligand loading conditions with various concentrations. The optimal concentration can range from 1–100 nM dependent on ligand size. Usually 3–4 fold dilutions are recommended for determining an optimal concentration. The goal is to maximize signal but not overload/saturate the surface. It is recommended to keep ligand loading in the linear range. Also, low ligand concentration loading over a longer period of time is recommended to avoid crowding, steric hindrance and possible aggregation of ligand on the sensor surface. An association step should be performed for each ligand concentration using a high concentration of analyte (10-20X K_D) and a zero ligand biosensor as a control. Select a ligand loading concentration that does not saturate the biosensor, but still provides a strong analyte signal. Ideally, for a 150 kDa antibody, the signal in the loading step should reach about 1.0 nm after 10 minutes of loading.

In Figure 2, an example for scouting loading concentration of biotin-ligand is shown. As seen in the binding trace, loading at too low concentration results in low nm shift and would give unreliable kinetics data. Similarly, loading at a too high concentration results in linear kinetics binding curves and will hence result in unreliable K_D.

Figure 2

Example for Scouting the Ligand Loading Conditions with Various Concentrations.



Note. In this example, different concentrations of biotin-hCD64 was loaded onto SAX2 Biosensor and hIgG1k at 4, 2 and 1nM was analyzed for each loading condition.

Determine Optimal Buffer Type and Conditions

Though the Octet® BLI system offers a great deal of flexibility in choice of assay and sample matrix, be sure to select an assay buffer that is appropriate for the experimental system, and use the same solution throughout the assay. For example, if the analyte is in culture media, make analyte dilutions in the same media and use this media for the baseline and dissociation steps as well. For kinetic assays using purified samples it is recommended to use Sartorius's Kinetics Buffer as a sample buffer, which is available as a 10X solution (PN 18-1105). This buffer contains a blocking agent, bovine serum albumin (BSA) and surfactant to inhibit non-specific binding to surfaces and other proteins. It is also encouraged to use other buffers as needed to optimize the assay based on specific sample type or pairs.

Target Association and Dissociation

When finalizing the ligand loading concentration, load a column of wells with the final ligand concentration. The binding interaction of analyte to immobilized ligand can then be measured on the same plate. **A dilution series of at least 3–5 analyte concentrations** should be measured in order to obtain an accurate K_D, including a reference at zero analyte concentration. It should ideally range from a concentration of about **10-20X K_D down to 0.1X K_D**, using 2-fold or 3-fold dilutions. For fast-binding target, a short association time is adequate, however, for slower reaction it is critical to run longer for obtaining some curvature in data traces. For dissociation step, run long enough for high affinity binders (to have confidence in the K_{off} value, it is recommended that **at least 5% of complex must dissociate**).

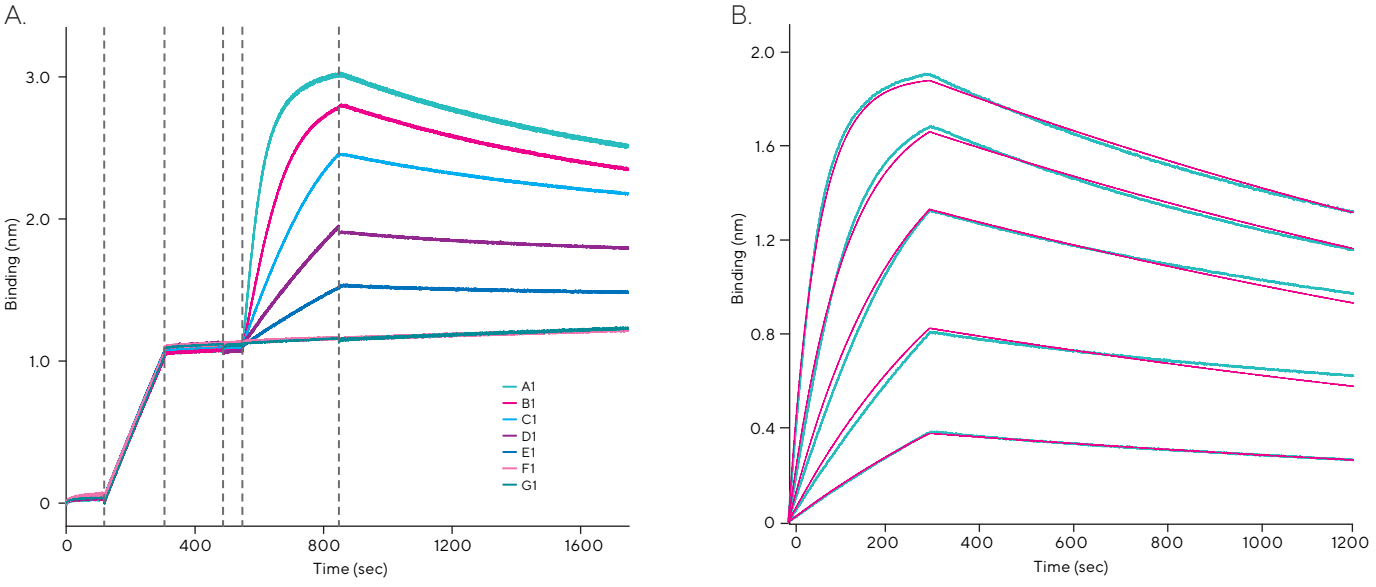
If the expected K_D is in the nM range, an association of 15 minutes and a dissociation of 15–30 minutes may be sufficient to obtain kinetic constants with low error.

If the K_D is <1 nM, an association of 15–30 minutes and a dissociation of one hour or more may be necessary to obtain kinetic constants with low error.

If the K_D is unknown, use the 'Extend Current Step' and 'Skip to Next Step' buttons during the experiment to optimize the analyte binding times.

An example binding interaction is shown in Figure 3 where (A) shows kinetic curves for 5 different concentrations between 20X–0.1X K_D. (B) shows good overlay from 1:1 global fitting of the data. Fitted curves are shown in pink and raw curves are in teal.

Figure 3
Example Binding Interaction of a Dilution Series.



Note. Performed using analyte Herceptin at 0, 3.2, 6.3, 12.5, 25, 50 nM to immobilized ligand biotin-hCD64 on SAX2.

Data Analysis

In the Octet® Analysis Software window, processed data can be analyzed by fitting to one of the available curve fit models. Fitting the experimental data to a model involves some consideration. Many interactions studied do not fit a simple 1:1 binding model. When deviation from this model is a function of the type of interaction, rather than an experimental artifact, additional pre-programmed curve fitting models are available in the Octet® Analysis Studio Software:

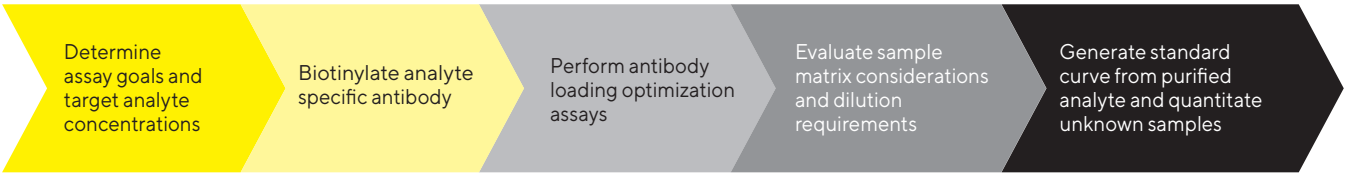
- 2:1 HL Model (heterogeneous ligand)
- 1:2 Bivalent Analyte Model
- Mass Transport

The most accurate kinetic and affinity constants are obtained when performing a global fit using several analyte concentrations. Global fit analysis includes all binding curve data in the group using a full fit option. R_{max} should remain unlinked by biosensor when separate biosensors are used for each individual analyte concentration. When R_{max} is linked, the theoretical maximum response is calculated assuming equal binding capacity between biosensors. Different biosensors will have slight variability in surface capacity. R_{max} can be linked if the same biosensor is used for every analyte concentration in the series. This strategy is typically used in small molecule analyses, where dissociation is rapid and complete and enables re-use of the same biosensor for a new sample concentration. In standard large molecule kinetics assays, where each sample is run on a new or regenerated biosensor, R_{max} should be unlinked to enable calculation of separate R_{max} for each sample.

Best Practices for Quantitation Assay Development with SAX2

Similar to Sartorius's currently available SA, SAX, and SSA Biosensors, the High Precision Streptavidin 2.0 (SAX2) Biosensor can be customized with biotinylated molecules for quantitation use. A summary of the work flow for customized quantitation is illustrated in Figure 4. One example of two-step quantitation of PSA standards is shown in Figure 6. Such customization can be done by dipping SAX2 Biosensors into a solution containing 15 µg/mL of biotinylated ligand for 300–600 seconds with shaking at 1000 rpm (actual optimal biosensor coating parameters might vary and can be adjusted depending on material or time requirements).

Figure 4
A Summary of the Workflow for Custom Quantitation Assays.



The following assays are recommended each time a new matrix or new protein is analyzed.

Biotinylation of Ligand for Immobilization onto Octet® SAX2 Biosensor

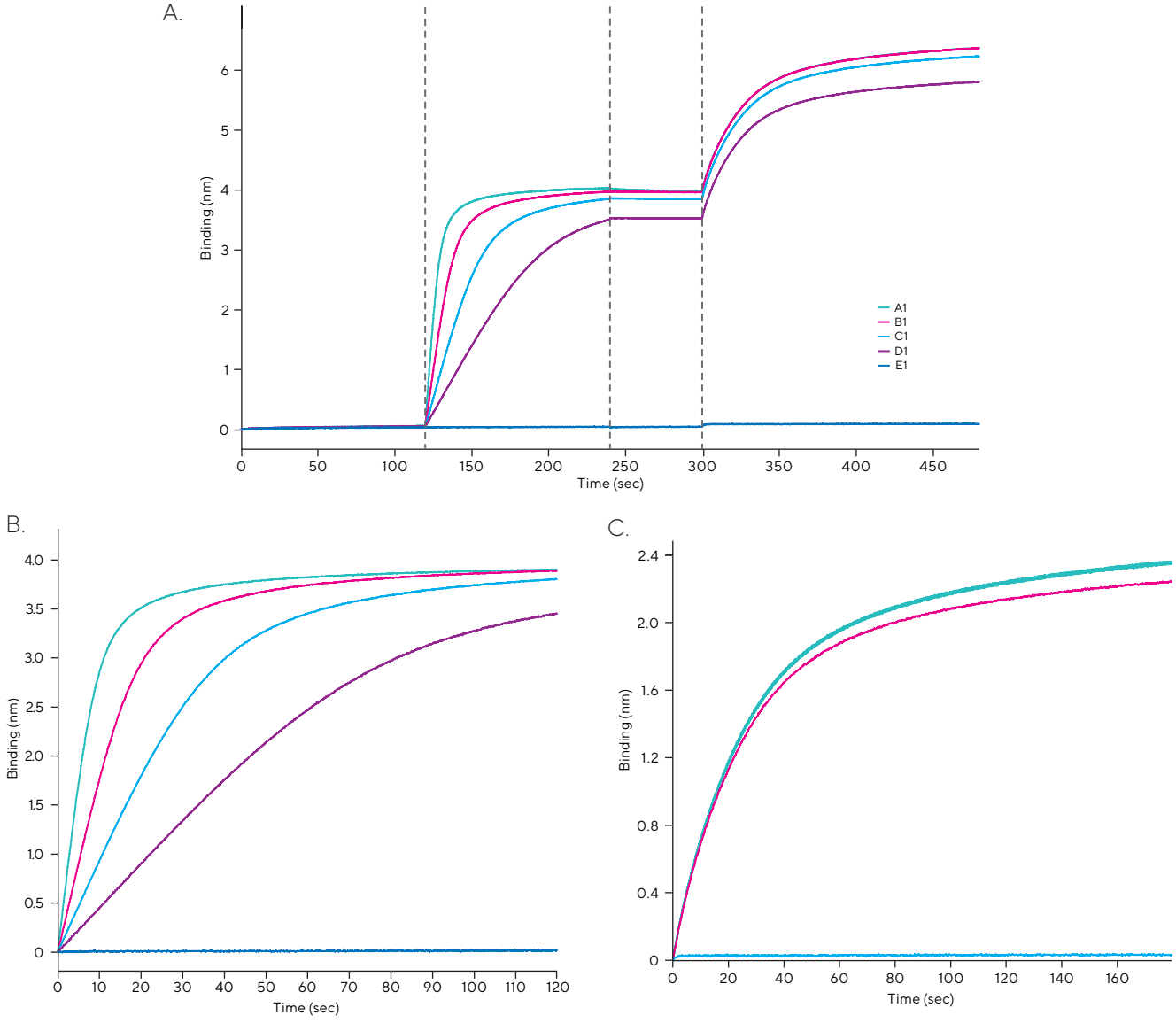
See recommendations under the kinetics assay section

Determine Optimal Conditions for Ligand Capture

A loading scouting experiment is recommended to ensure the optimal ligand concentration is selected. An initial load scouting assay (Figure 8), where the ligand concentration is varied, typically around 100 nM or 15 µg/mL with concentrations above and below 15 µg/mL while the analyte concentration is left constant should be performed. The goal is to saturate the biosensor surface and select the lowest ligand concentration (devoid of assay artifacts) that yields maximum and reproducible analyte response signals.

Figure 5

Ligand Loading Scouting.

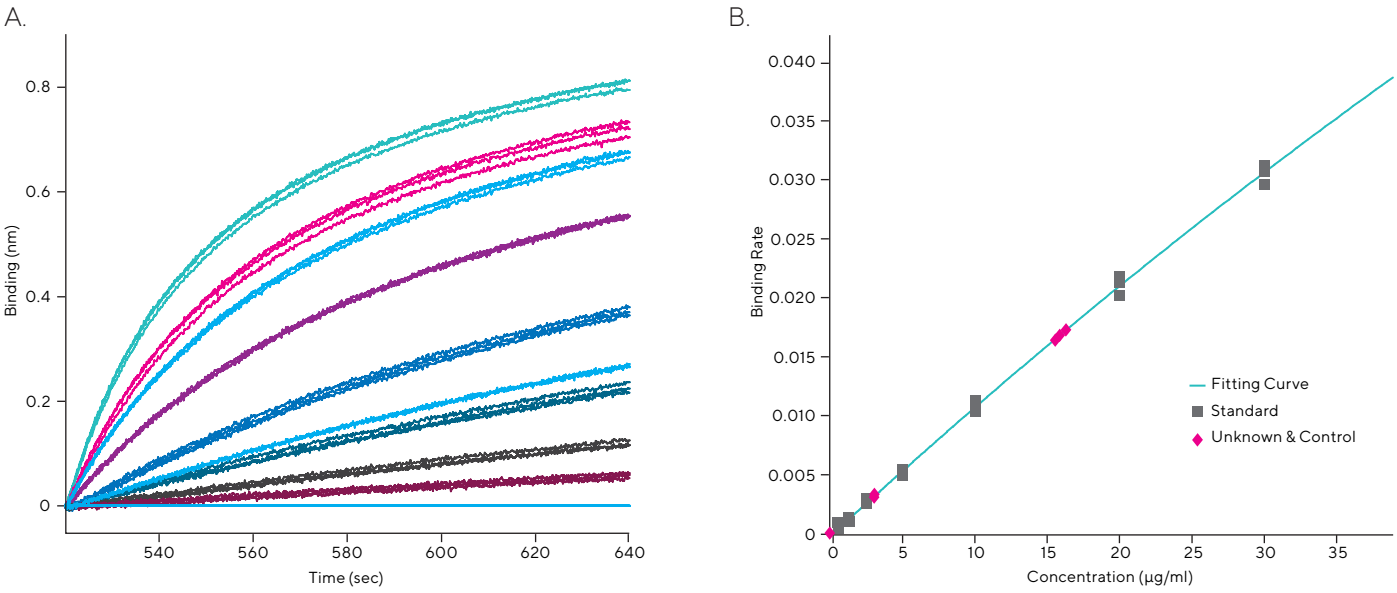


Note. Biotinylated anti-insulin loading was screened at 20, 10, 5 and 2.5 µg/mL. (A) shows raw data obtained when 5 µg/mL of recombinant insulin was used to probe for binding at the different antibody concentrations. Note: NSB was not observed when insulin was bound to biosensor surface with no antibody loaded (curve E1) (B) - antibody and (C) - antigen show the respective aligned response signals at 20 µg/mL (dark blue), 10 µg/mL (red), 5 µg/mL (blue), and 2.5 µg/mL (green). No discernible difference in signals was observed at the top three concentrations implying that 5 µg/mL is optimal for anti-insulin antibody loading. Similar studies were performed for the other recombinant proteins with 5 µg/mL deemed optimal in all cases.

Custom Quantitation Assay

Figure 6

Detection and Quantitation of Prostate Specific Antigen (PSA), Binding Curves (A) and Standard Curve (B).



Note. Detection of PSA standards and unknowns after Biotin-anti-PSA loading (30 µg/mL, 400s) using SAX2 on the Octet® RH16 BLI system with assay parameters (1000 rpm, 120s). (A) PSA dose response and unknown response. 4PL was used for the standard curve. (B) Representative resulting calibration curve and unknowns prediction from A.

Table 2

Average Calculated Concentration, %CVs and Recovery for PSA.

PSA	Expected Concentration (µg/mL)	Avg. Concentration (µg/mL) (n=3)	%CV (n=3)	% Recovery (n=3)
Standards	30	30.2	4.5%	100.6%
	20	19.9	6.5%	99.6%
	10	10.0	0.8%	100.4%
	5	5.0	2.6%	99.7%
	2.5	2.5	4.5%	100.6%
	1.25	1.2	4.3%	99.6%
	0.625	0.6	6.3%	100.0%
Unknowns	15	16.1	3.1%	107.5%
	3	3.1	0.9%	104.3%

Dilution Factor Determination for Matrix

Differences between matrices can potentially influence assay performance. Diluting the sample matrix using Octet® Sample Diluent (PN 18-1104) is a convenient and often effective means of minimizing matrix effects. It is therefore recommended that the minimum required dilution factor be determined using Sample Diluent to achieve the desired assay performance. Other assay buffers can be explored as desired.

Prepare 2 mL of sample matrix diluted both two-fold and 10-fold in Sample Diluent. General guidelines for dilutions are described in Table 3. Prepare a spiked sample of the protein to be quantitated in: Sample Diluent, neat matrix, two-fold diluted matrix and 10-fold diluted matrix by mixing the minimum volume of analyte and 0.5 mL of each matrix (four samples total). The final concentration of the protein should be in the middle of the desired quantitation range. Hydrate biosensors in the sample matrix that matches each sample type (e.g. biosensors to be used in wells with 10-fold diluted matrix should be hydrated in 10-fold diluted matrix). Place the sample plate and the hydrated biosensors into the Octet® BLI system. Always hydrate the biosensors for at least 10 minutes in the appropriate assay matrix. Note that in some cases, it may be necessary to hydrate the biosensors for longer than 10 minutes. A good rule of thumb is to hydrate the biosensors for approximately 20 minutes in cell culture spent media if the analyte is to be analyzed in its crude matrix during cell line expression monitoring. The delay timer can be used in the Octet® Software to automatically start the assay after 600 seconds. Once the quantitation assay is completed, visually inspect the real-time binding traces and determine the dilution required to:

a. Minimize non-specific binding from the matrix.
b. Show equivalent analyte binding in the matrix-spiked sample and the Sample Diluent control.
c. Use this dilution factor for routine assays.

Table 3

Recommended Minimum Dilution for Common Sample Types.

Sample type	Minimum Recommended Dilution in Sample Diluent
Purified proteins	Dilute into assay range
Samples from column eluents	Dilute into assay range
Serum-free cell culture supernatants media	Neat or two-fold
Serum-containing cell culture supernatants	Neat
Bacterial cell pellet lysed by sonication	10-fold
Bacterial cell pellet lysed by B-PER	20-fold

Note. In all cases, the matrix for the diluted samples, standards and biosensor hydration solution should be matched as closely as possible.

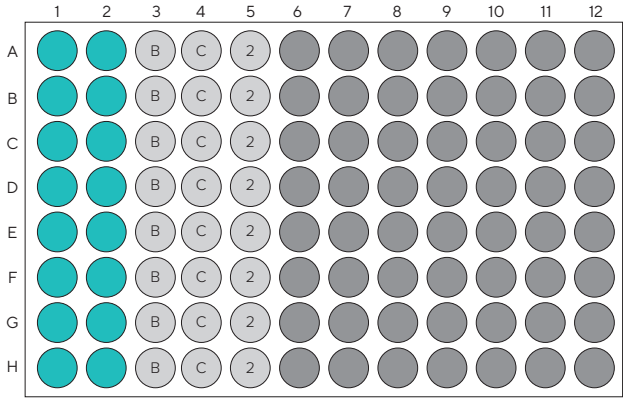
Customized Quantitation Assay Set Up

During assay development, make sure to test for potential non-specific binding interactions (NSB) by running reference biosensors and samples. A reference analysis is two-fold: first test for NSB of the analyte to a biosensor blocked with an irrelevant antibody; second test for NSB between the assay media and the coated antibody. In the first case, the presence of NSB should suggest further assay development while in the second case the NSB can be subtracted using reference biosensors.

Protein concentration determination on Octet® BLI systems requires the development of a standard reference curve. A standard reference curve is obtained using a purified sample of the respective protein. Known concentrations of the purified standard sample are spiked into the matrix of interest and are serially diluted within the desired concentration range using an analyte depleted assay matrix. Example sample plate is shown in Figure 7 and the method setup is illustrated in Table 4. Note that for recombinant protein quantitation, the recommended assay shaking speed is 1000 RPM. However, there may be analytes where a different shaking speed may be required. 1000 RPM is typically recommended in tandem with a longer assay time to improve assay sensitivity. In general, the higher the RPM, the better the sensitivity. In cases where the analyte is high in concentration, the assay dynamic range may be expanded by lowering the shaking speed to the default 400 RPM.

Figure 7

96-well Plate Design for Advanced Quantitation on the Octet® BLI System.



Note. Showing standard samples (yellow), buffer (B), capture antibody (C), 2nd buffer (2) and unknown concentration samples (dark gray). At least two replicates are required for the standard sample.

Table 4

Octet® Advanced Quantitation Assay Method Step Setup for the Customized Quantitation of Recombinant Proteins.

Step	Step Type	Time (s)	Shaker (rpm)
1	Buffer	180	1000
2	Capture Antibody	300	1000
3	2nd Buffer	180	1000
4	Sample	300	1000

Data Analysis

Octet® Analysis Studio Software allows users to select between one of two binding rate analysis models: an initial slope of binding rate analysis and an end-point R-equilibrium analysis. The initial slope option should always be used when the analyte binding step is a simple single step post antibody loading. If the assay is modified to allow for a signal amplification step such as what would be used in a higher sensitivity assay, the detection step should be allowed to reach equilibrium, and the R-equilibrium binding rate model should be used to analyze the data. The analyzed data can be fit using one of several models; however, care should be taken to ensure that accurate analyte concentration is determined off the linear portion of the standard curve. The unknown analytes concentration is extrapolated off the generated standard curve.

Instrument Usage and Workflow Recommendations

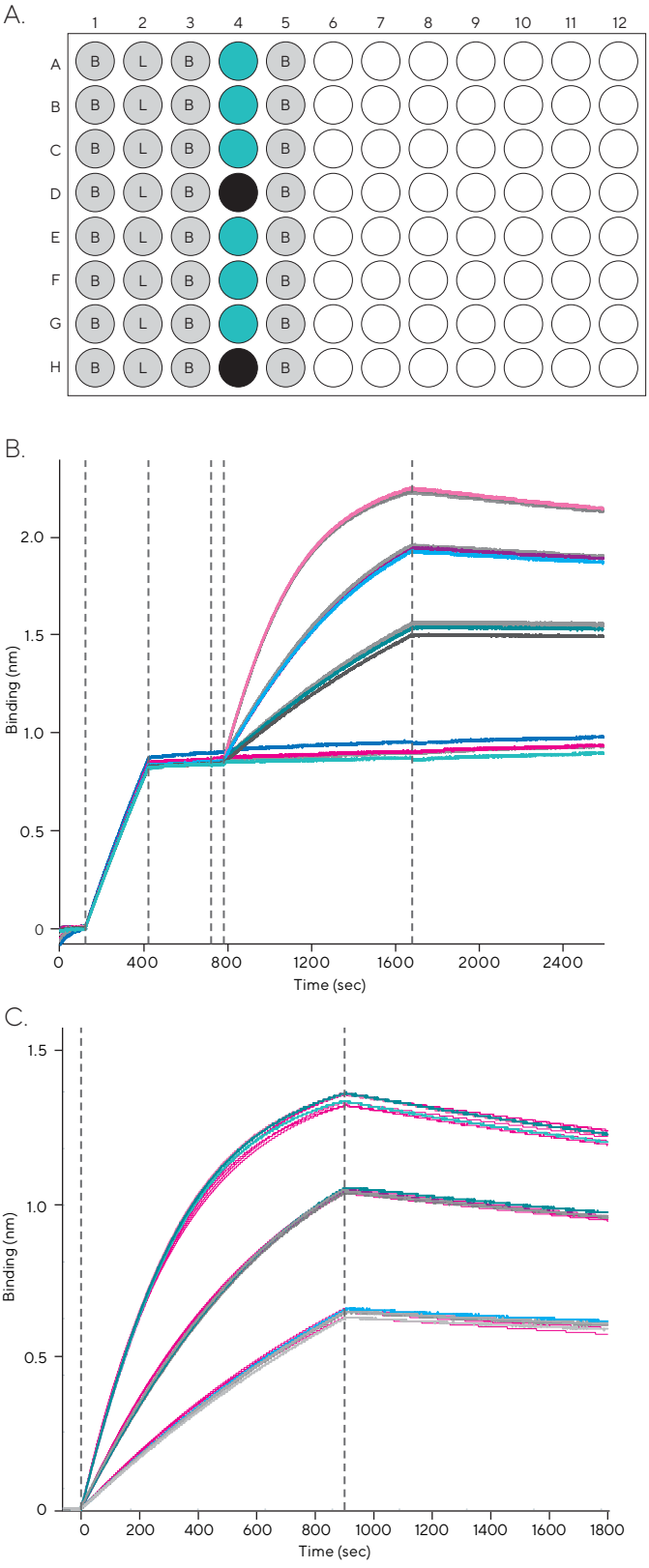
Performance Maintenance (PM) from Sartorius's Service team should be carried out at least once per year for the instrument to maintain a quality performance. Sartorius offers Installation/Operational Qualification for all Octet® BLI systems, and a Performance Qualification kit (PN 18-5134) validated especially for the Octet® R8 BLI system. If changing instrument type, a bridging study to re-validate the assay is recommended.

Here are some considerations for kinetics assay workflow development based on different instrument types:

- The **Octet® RH16 and RH96 BLI systems** features high throughput with up to 16 and 96 channels per run respectively. It is recommended to design a kinetics assay with at least 3–4 replicates per run to minimize run-to-run variations. **Reuse of reagents is generally not recommended but can be sample type and pair dependent and should be validated during method development.**
- **The Octet® R8 BLI system** features 8 channels with temperature control of 25–40°C. It is recommended to design the kinetics assay with 1–2 replicates per run. Post assay run, overlay runs if necessary in Octet® Analysis Studio Software, process the data using global fit to obtain a robust analysis and accurate estimation of binding constants.
- **The Octet® R2 BLI system** is a two channel system with sample plate temperature at 30°C. It is recommended to use one channel for titration and one for reference. Overlay runs in Octet® Analysis Studio Software and subtract in each run its own reference and **fit the data globally** to obtain a robust analysis and accurate estimation of binding constants.

Figure 8

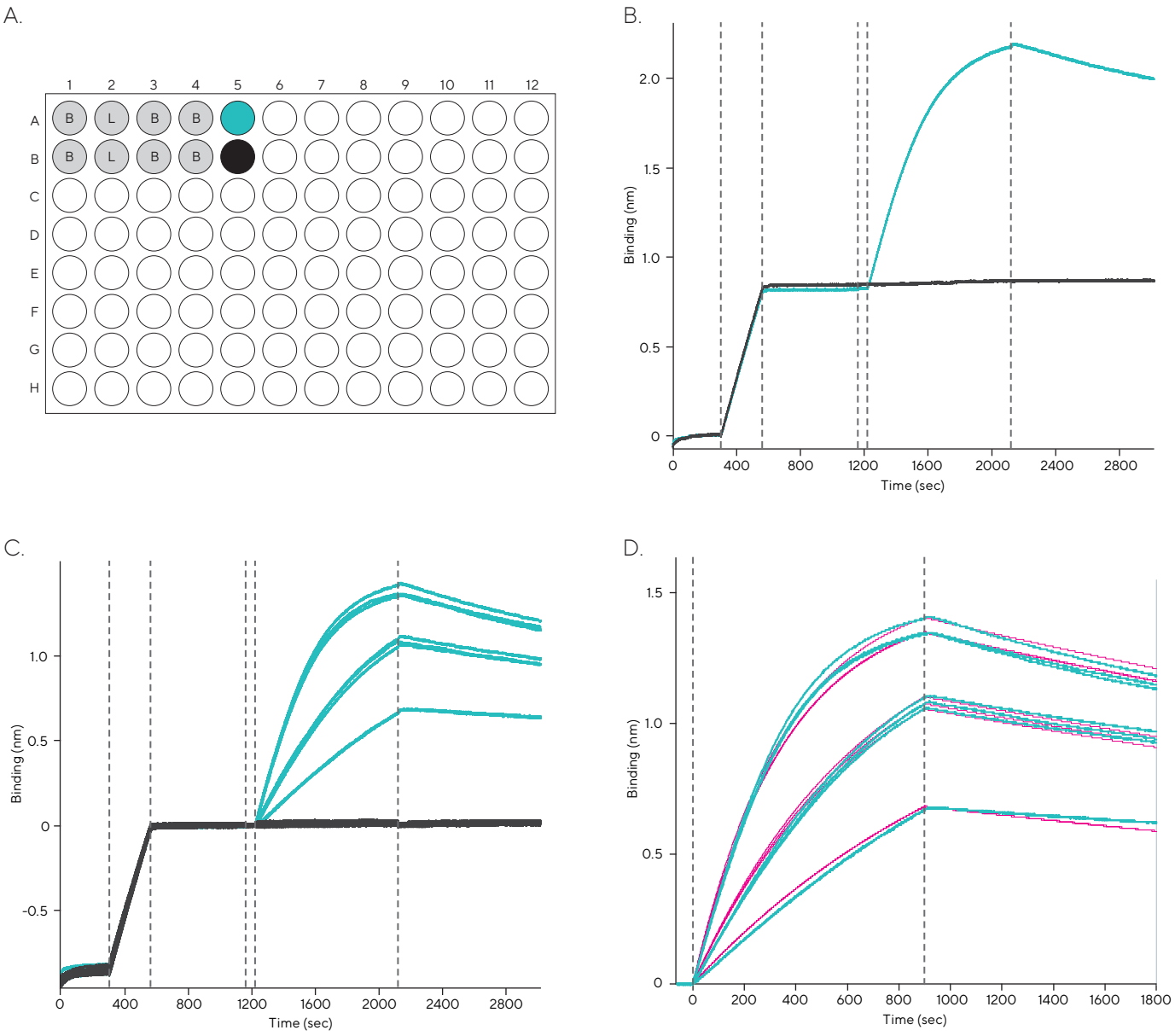
Binding Between Biotin-hCD64 and hIgG1k at 4 nM, 2 nM and 1 nM Concentrations.



Note. (A) A representative method file for one run of kinetics assay on Octet® R8 BLI system (three titrations, one reference and two replication groups). (B) Overlaying two runs of kinetics assay (four replicate groups total) in Octet® Analysis Studio Software. (C) Data processed and curve fit using a 1:1 binding model.

Figure 9

Binding Between Biotin-hCD64 and hIgG1k at 4 nM, 2 nM and 1 nM Concentrations.



Note. (A) A representative method file and sensor trace for one run of kinetics assay in K2 (B) sensor trace for one run of kinetics assay in K2 (one titration plus one reference). (C) Overlaying nine runs of kinetics assay (three titration and three replicates per titration) in Octet® Analysis Studio Software. (D) Data processed and curve fit using a 1:1 binding model.

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