Label-Free Detection

Technologies, Key Considerations, and Applications

Simplifying Progress

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OCTET R8

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Why Use Label-Free Detection for Binding Interaction Analysis?

Interactions between biomolecules serve as key triggers for many biological processes and, therefore, provide perfect targets for drug discoveries. Biological binding interactions are a dynamic process driven by changes to the environment. Therefore, techniques used to characterize these interactions needs to mirror the level of biological complexity in order to fully understand these interactions.

Label-Free Biosensor Binding Assay Methods

Commonly used label-free binding analysis platforms include biosensor-based Bio-Layer Interferometry (BLI), sensor chip-based Surface Plasmon Resonance (SPR), solution-based Isothermal Titration Calorimetry (ITC) and Microscale Thermophoresis (MST). In this e-book, the four technologies are compared in terms of their capabilities and workflows, and provide guidance for choosing the most suitable platform based on assay needs and application.

BLI and SPR monitor binding interactions based on molecular accumulation that take place during complex formation. The binding complex is established at the biosensor or sensor chip surface by immobilization or capture of one binding partner (ligand) and directly monitoring the binding of the analyte from the sample solution. The complex formation and dissociation are monitored in real time, providing kinetics and affinity data.

Bio-Layer Interferometry (BLI)

BLI is an optical technology that measures the changes in interference pattern between light waves. Sartorius's Octet® BLI platforms measure light interference originating from the tip of the biosensor surface where light wavelengths are made to reflect from two layers: a biocompatible layer at the end of the biosensor surface and an internal reference layer (Figure 1). Incident white light reflecting from the two layers interfere constructively or destructively depending on the thickness of the molecular biolayer at the biosensor tip. The spectral pattern of the reflected light changes as a function of the optical thickness of the molecular layer, i.e. the number of molecules bound to the biosensor surface. This spectral shift is monitored at the detector, and reported on a sensorgram as a change in wavelength (nm shift). Monitoring the interference pattern (i.e. spectral shift) in real time provides kinetics data on molecular interactions.

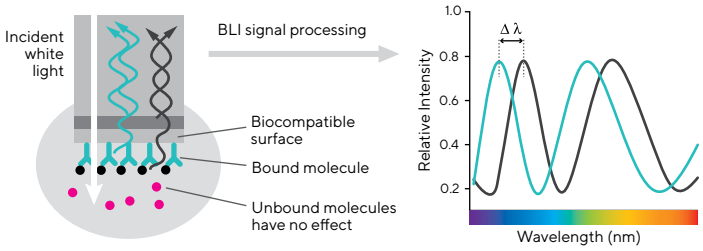


Figure 1: Octet® sytems with BLI technology measure the difference in reflected light's wavelength (Δλ) between the two surfaces on the biosensor.

Label-Free Binding Assays Enable:

- Analysis of biomolecular interactions using conditions close to native biological conformations.
- Faster assay design, development and reagent preparation via use of unmodified reagents and substrates.
- More robust assay signals with less interference from fluorescent or other reporters.
- Direct monitoring of binding complex formation, as secondary detection reagents aren't needed to establish binding responses.

Dip and Read biosensors are core to BLI technology. The biosensor tip is coated with a biocompatible matrix that minimizes non-specific binding while providing a uniform and non-denaturing surface for biomolecules. BLI's ability to characterize interactions directly in complex matrices and non-purified samples is a key advantage. This is possible due to the robustness of the biosensor architecture and that the technology does not use complex fluidic pathways to introduce the sample to the immobilized ligand. The biosensor moves to a 96- or 384-well plate and is 'dipped' into the sample. This provides a robust, flexible, and a simple way to introduce an analyte to the sensor surface to monitor binding.

Octet® BLI Systems Let You:

- Acquire real-time binding kinetics data to measure the rate of association (k_a), the rate of dissociation (k_d) and affinity constant (K_D).
- Generate 1 to 96 data curves simultaneously with fully automated assays.
- Rapidly identify optimal conditions using up to 96 channels to assay multiple conditions and reaction configurations in a single run.
- Characterize molecular interactions in crude or unpurified matrices.
- Easily develop quantitation assays that generate data in real time.
- Detect binding of a wide range of analytes from small molecules to live cells.
- Recover precious or low-availability samples for use in another BLI experiment or other lab analyses as binding reagents are not added directly to the sample and materials are consumed minimally.

Read more about BLI kinetics assay development.

Surface Plasmon Resonance

Surface plasmon resonance is an optoelectronic method that detects changes in the refractive index at the surface of a sensor chip as a result of mass change from biomolecular interactions. The sensor chip is comprised of a glass substrate and a thin gold coating. Light passes through the glass substrate and is reflected off the gold coating. At a certain angle of incidence, a portion of the light energy couples through the gold coating and creates a surface plasmon wave at the sample and gold surface interface. The angle of incident light required to sustain the surface plasmon wave is sensitive to changes in refractive index at the surface. The change in refractive index is directly proportional to mass change that occurs as a result of molecular binding and or dissociation events. In a typical SPR assay, upon immobilization of the ligand, the analyte is supplied to the sensor chip surface using a flow-based fluidics system.

Typical binding assays, whether kinetics or equilibrium-based, require titration of multiple analyte concentrations to accurately establish kinetics and affinity constants. While traditional SPR-based binding platforms utilize fixed concentration injections (FCI) delivered over multiple cycles to derive kinetic rate constants (MCK, multi-cycle kinetics and SCK, single-cycle kinetics), Sartorius' Octet® SF3 SPR platform offers next-generation SPR through OneStep® and NeXtStep™ Injections in addition to MCK capabilities. OneStep® creates an analyte gradient based on the Taylor dispersion principle in an injection line prior to contacting the ligand containing sensor chip surface. The generated analyte concentration gradient consists of a full range of low to high analyte concentrations presented to the sensor surface, circumventing the need for multiple analyte injections. With OneStep® technology, a single analyte concentration gradient injection is sufficient for characterizing kinetics and affinity constants, significantly improving experimental workflows. NeXtStep™ Injections provide a simplified way to perform competition analysis where two back to back gradient injections are performed. The first injection provides the control compound (a known binder) and the second gradient injection assesses binding of a second analyte to the control-ligand bound complex.

SPR Assays on Octet® SF3 SPR Systems Enable:

- Acquisition of real-time binding kinetics data to measure the rate of association (k_a), the rate of dissociation (k_d) and affinity constant (K_D).
- High sensitivity and minimal background noise to detect small molecule and fragment binding.
- Kinetics measurements over a wide range of on- and off-rates.
- Accurate kinetics and affinity values from a single analyte concentration using OneStep® Injections, eliminating the need to prepare a full analyte dilution series.
- High-throughput binding screens with up to 72 hours of unattended operation.

Read more about Next Generation SPR-based interaction analysis.

Biosensor Assays vs. ELISA

Kinetics characterization using fluidic-free BLI or sensor chip-based SPR can readily replace label-dependent methods such as ELISA or fluorometric methods. Real-time biosensor assays allow one to gauge progression of the assay and also dissect binding affinities and mechanism of action with information from association (k_a) and dissociation (k_d) kinetics which ELISA does not provide.

Benefits of Biosensor Assays Over ELISA:

- Biosensor assays provide binding kinetics (k_a) and (k_d) and affinities (k_D) compared to the end point-only affinities with ELISA.
- Kinetic assays can be completed in minutes, and real-time data allows selection of optimized association, dissociation and ligand immobilization step times as appropriate for each experiment. This avoids unnecessarily long or overnight incubation times, and enables analysis of biomolecules that are less stable under assay conditions.
- Low-affinity analytes that can often be washed away in ELISA workflows can be accurately characterized with biosensor assays.

Benefits of ELISA Over Biosensor Assays:

- Low upfront costs to developing a conventional ELISA assay. A simple ELISA can be performed using conventional detection platforms such as a plate reader.
- Commonly needed ELISA reagents such as antibodies needed for capture and detection are readily available.
- Well established legacy method for quantitation and affinity determination.

Calorimetry

Calorimetry is commonly used to characterize molecular binding and structural stability of biomolecules. Isothermal titration calorimetry (ITC), allows the measurement of binding affinity (K_D), stoichiometry (n), change in enthalpy (ΔH) and change in entropy (ΔS) between molecules in solution by directly measuring the heat exchange that occurs during a chemical or biological reaction, such as a binding event. Differential scanning calorimetry (DSC) measures heat capacity changes (ΔCp) associated with temperature-induced unfolding of biomolecules. The resulting temperature-dependent thermogram represents a qualitative and quantitative signature of the protein unfolding. Combining both ITC and DSC data provides a holistic picture of the energetics that can help delineate varying degrees of contributions from thermodynamic properties such as entropy and enthalpy towards the reaction transformation. These parameters can also help uncover information on the binding environment such as conformational changes, solvent interactions and protonation states.

Benefits of Biosensor Assays Compared to ITC:

- Affinity ranges from mM to pM can be directly measured compared to a more limited sub-mM to nM range in ITC.
- Real-time association and dissociation kinetics parameters (k_a, k_d) can be measured.
- Biomolecules from unpurified matrices can be captured to perform binding assays, allowing assays to be run in crude samples.
- Biosensor experiments require 10- to 500-fold less sample amounts for analysis compared to ITC.
- While modern ITC instruments enable users to perform up to ~20 titrations per day, biosensor assays can produce hundreds of kinetics profiles depending on the interaction system and platform, allowing them to be widely used across the drug discovery pipeline for assay development, screening, validation and characterization.
- Quantitation assays can be performed with biosensor assays.

Benefits of ITC Assay Over Biosensor Assays:

- Additional thermodynamic parameters for binding such as ΔG, ΔH, and ΔS and ΔCp and T_m (DSC) can be measured.
- Immobilization artifacts aren't an issue as immobilization isn't required - both binding partners interact in solution.
- No mass transfer concerns.

Microscale Thermophoresis

Microscale thermophoresis (MST) is another biophysical method that can be used to measure binding affinities between biomolecules. MST detects variations in fluorescent signals that results from a laser-induced temperature change. The temperature change is often generated by
an infrared laser where the reaction takes place in thin capillaries. Like ITC, binding in MST is monitored in free solution, although a detectable fluorescence signal is required either by intrinsic fluorescence of the molecule (for example tryptophan fluorescence in proteins) or generated via a fluorescent label. Intrinsic fluorescence is the preferred option as detection of the binding interaction using native conformations avoids undesirable artifacts that can arise from chemical labeling.

The MST fluorescent signal is primarily composed of two main components: temperature-related intensity change (TRIC) and thermophoresis. TRIC describes how the fluorescence intensity of a fluorophore responds to a change in local temperature. Thermophoresis describes the movement of molecules along a temperature gradient. The temperature-directed movement of molecules causes a change in the local concentrations, and the migration of a specific molecule depends on the size, charge, hydrodynamic radius and conformation. Formation of a binding complex can change the size of the assembly or cause a change in the solvation sphere, altering the thermophoretic migration pattern that can be quantitated by following the fluorescence signal. Typically, MST experiments are performed by monitoring the fluorescence of a target molecule as a function of concentration of a non-fluorescent ligand.

Key Benefits of Biosensor Assays Compared to MST:

- Kinetics parameters are obtained compared to just end-point measurements with MST.
- Labeling isn't required, which avoids both the experimental artifacts and non-specific binding that can result from the hydrophobic fluorescent labeling molecules MST requires.
- MST is highly sensitive to any change in the binding environment including ionic strength, pH, detergents, and buffer components and require more assay optimization depending on sample conditions.
- Quantitation assays can be performed with biosensor assays.

Benefits of MST Assays Over Biosensor Assays:

- MST has a low sample volume requirement of 3–10 µL compared to a minimum 40 µL for biosensor assays.
- No immobilization is required as binding is performed in solution.

Choosing a Biosensor Platform

Sartorius offers two major biosensor analysis platforms, Octet® BLI systems and Octet® SPR systems for biomolecular interaction analysis. Both offer comprehensive characterization for kinetic and affinity attributes. Biophysical laboratories can take advantage of differences in throughput, detection technologies and sample delivery between the two to establish a wide range of analysis capabilities. For example, the Octet® BLI system's throughput and assay flexibility combined with the Octet® SF3 system's sensitivity to detect molecules as small as 70 Da covers the full spectrum of even the most challenging biomolecular interaction applications.

Application Considerations

Key attributes such as throughput, sample capacity, unattended runtimes, affinity ranges, sensitivity, type of sample matrix and ease of use are important considerations when choosing a biosensor platform to suit your application and workflow needs.

Both the Octet® BLI and Octet® SPR platforms are capable of characterizing a wide range of biomolecular interactions (Table 1). Thanks to its increased sensitivity, the Octet® SF3 system is ideal for small molecule and fragment screening workflows (> 70 Da) due to their exquisite sensitivity and next-generation injection capabilities. Octet® BLI platforms can be used for analytes > 150 Da and applications involving larger molecular weight analytes such as viruses, nanoparticles, liposomes, and cells. Because Octet® BLI systems don't use microfluidics, they are widely recognized as an essential tool for routine analysis of samples in crude lysates or complex matrices. Crude sample compatibility is useful especially in epitope binning, quantitation, off rate ranking or large screening assays where sample purification is neither required or feasible for efficient workflows. Some of the key attributes of Octet® BLI and SPR are shown below for comparison.






	Octet® SF3 System	Octet® R2 System	Octet® R4e System	Octet® R8 System	Octet® RH16 System	Octet® RH96 System
Applications						
Small molecule applications	■□*	■□	■□	■□	■□	■■
Large molecules – e.g. virus, VPL, nanoparticles, cells	□■	■□	■□	■□	■□	■■
DNA, RNA, Peptides, Protein analytes	■□	■□	■□	■□	■□	■■
Measuring weak binding affinities	■□	■□	■□	■□	■□	■■
Measuring tight binding affinities	■□	■□	■□	■□	■□	■■
Screening applications – e.g epitope binning, off-rate ranking	□■	■□	■□	■□	■□	■■
Antibody characterization	■□	■□	■□	■□	■□	■■
Quantitation assays (ELISA replacement)	□■	■□	■□	■□	■□	■■
Antibody titer measurements	■□	■□	■□	■□	■□	■■

Table 1: Featured applications capabilities for Octet® BLI and SPR platforms. Recommendations for each platform is based on assay capabilities, workflow efficiency (■□), and throughput (■■) for each application. A combination of assay capability and required throughput levels should be considered when choosing a label-free instrument. *Octet® SF3 system throughput is significantly increased by using OneStep®.

Assay capability and workflow efficiency ■□ → ■■

Throughput ■□ → ■■

Rating Low → High

Visit the website to view publications on additional applications on the Octet® systems.

Key Octet® BLI System Attributes

Throughput
Octet® BLI systems are available with anywhere from 2 to 96 channels, which directly correlates to throughput. A 2-channel system can generate data on 2 samples at the same time, and 96-channel systems generate data on up to 96 samples simultaneously. Octet® R2, R8/R4, RH16 and RH96 systems come with 2, 8, 16 and 96 channels respectively. Channel configurations for Octet® RH16 (16-channel) and RH96 (96-channel) systems are shown in Figure 2. The Octet® RH96 system can adapt to a user selectable 8, 16, 32, 48 or 96-channel read-head to tailor assay design. It can also complete a 96-well plate quantitation in 2 minutes, a full plate of off-rate ranking assay in minutes, and a 32 × 32 epitope binning screen in less than 8 hours for example. The 8-channel Octet® R8 and R4 systems are widely used for kinetic characterizations, quantitation assays and medium-throughput screening campaigns. The Octet® R2 system is ideal for labs with lower throughput needs that also require high-performance biomolecular characterization capabilities at an economical price point. While all Octet® BLI platforms are fully automated, unattended operation can be further extended with Octet® RH16 and RH96 systems by integrating tailored robotic configurations for advanced assay workflows.

Sample Capacity
Samples and reagents are housed in 96- and 384-well plates. Octet® R2, R8, and R4 systems hold one 96-well plate and Octet® RH16 and RH96 systems have two plate positions where either 96- and | or 384-well plates can be used. A 200 µL minimum sample volume is required for standard 96- well plates and this can be reduced to 40 µL when using 384 tilted-well plates. The increased sample capacity and flexibility with plate types for assays on Octet® RH16 and RH96 systems allow lower sample volumes to be used when required, and enable analysis on more samples. For complex assay workflows that require multiple reagents, this increased sample capacity can also be beneficial in that the number of user interventions during the assay can be minimized.

Broad Range of Affinities and Kinetics
Octet® BLI systems capture a wide range of kinetic association (10^{1} – 10^{7} $M^{-1}s^{-1}$) and dissociation (10^{-6} – 10^{-1} s^{-1}) rates and measure affinities (K_D) from 1 mM – 10 pM, representing biologically relevant interactions.

Small and Large Molecule Analysis
Octet® BLI platforms analyze a wide molecular weight range, from small molecules (> 150 Da) to large nanoparticles, viruses and cells providing the capability to characterize a multitude of interaction systems using a single platform.

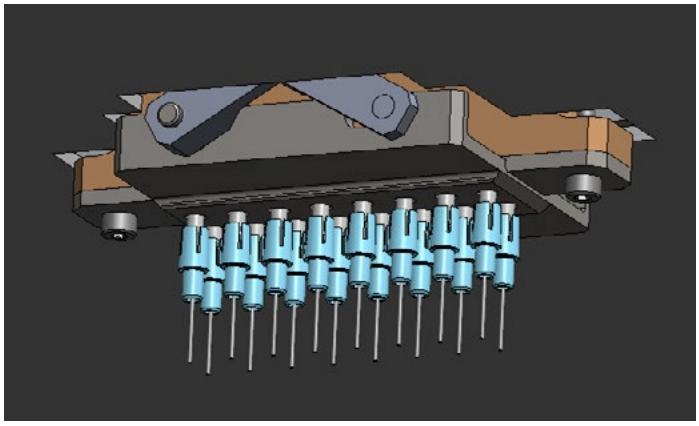
Sample Delivery
Sample introduction with Octet® BLI systems is accomplished by dipping biosensors directly into the samples, no fluidics are used. This provides greater flexibility in sample preparation and use of different sample types with none of the concerns of clogged fluidics associated with other methodologies.

Walk-In Ready
No prior instrument preparation such as cleaning, priming or equilibration is required to run assays on Octet® systems. Simply design the experiment, load samples and run.

Learn more about Octet® systems.

Learn more about robotic automation of Octet® assays for high-throughput ligand screening.

Octet® RH16 platform



Octet® RH96 platform

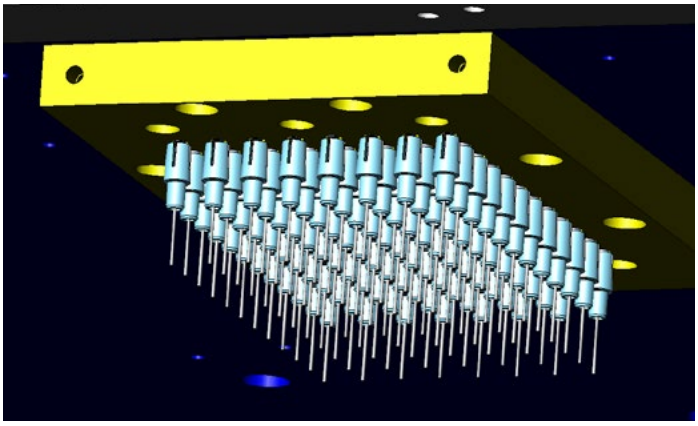


Figure 2: Biosensor channel configurations in Octet® BLI systems.

Key Octet® SF3 SPR System Attributes

Throughput
The Octet® SF3 system has three flow cells, two of which are typically used for detecting interactions, with the third being used as a reference. This allows kinetic data from two ligand targets to be generated simultaneously.

Simplified Workflows
Unlike traditional kinetics and affinity analysis workflows, Octet® SF3 systems don't require a full dilution series with multiple analyte concentrations to characterize a binding interaction. Unique to the Octet® SF3 are OneStep® Gradient Injections that provide a continuous analyte concentration gradient of 3–4 orders of magnitude in a single injection. This reduces the number of analyte concentrations that have to be analyzed (Figure 3A and B).

Sample Capacity
Samples and reagents are introduced to the sensor chip surface using a tightly-controlled automated fluidic system. The Octet® SF3 system can hold up to two 96- and | or 384-well plates with two reagent racks providing the capacity to analyze up to 768 fragments in a single unattended assay.

Broad Range of Affinities and Kinetics
The Octet® SF3 system can capture a wide range of kinetic association (10^2 – 10^9 $M^{-1}s^{-1}$) and dissociation (10^{-6} – 2.5 s^{-1}) rates and measure affinities (K_D) from 1 mM – 1 fM.

Small and Large Molecule Analysis
The Octet® SF3 system provides the sensitivity and baseline stability needed to detect binding interactions for a wide range of analytes, from biologics to small molecule fragments as low as 70 Da.

Typical Assay Workflow

Despite differences in detection and sample delivery, assay workflows for measuring kinetics and affinity constants between BLI- and SPR-based systems is similar (Figure 4). The main assay steps include:

Immobilization and Ligand Loading to the Biosensor or Sensor Chip
One of the binding partners (ligand) can be irreversibly immobilized using suitable chemistry such as amine coupling or captured via a suitable affinity tag. For example, a His-tagged protein can be captured using a Ni-NTA Biosensor or HisCap sensor chip, or biotin-labeled proteins or nucleic acids can be loaded to a streptavidin-coated biosensor or SADH sensor chip. It's critical to choose a suitable immobilization chemistry to present the ligand favorably towards the analyte molecule.

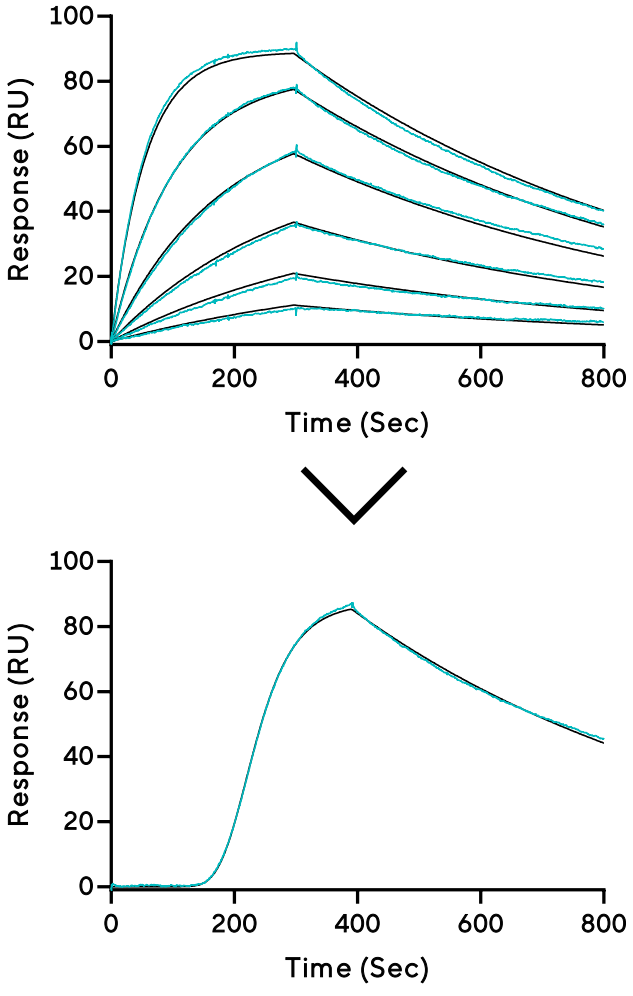


Figure 3: (A) Analysis of data obtained from conventional multi-cycle kinetics (MCK). Multiple analyte titrations are necessary for accurate determination of kinetic and affinity constants. (B) Analysis of data obtained from a OneStep® Injection. As well as offering a significant time saving, a single analyte concentration is sufficient to calculate binding kinetics and affinities.

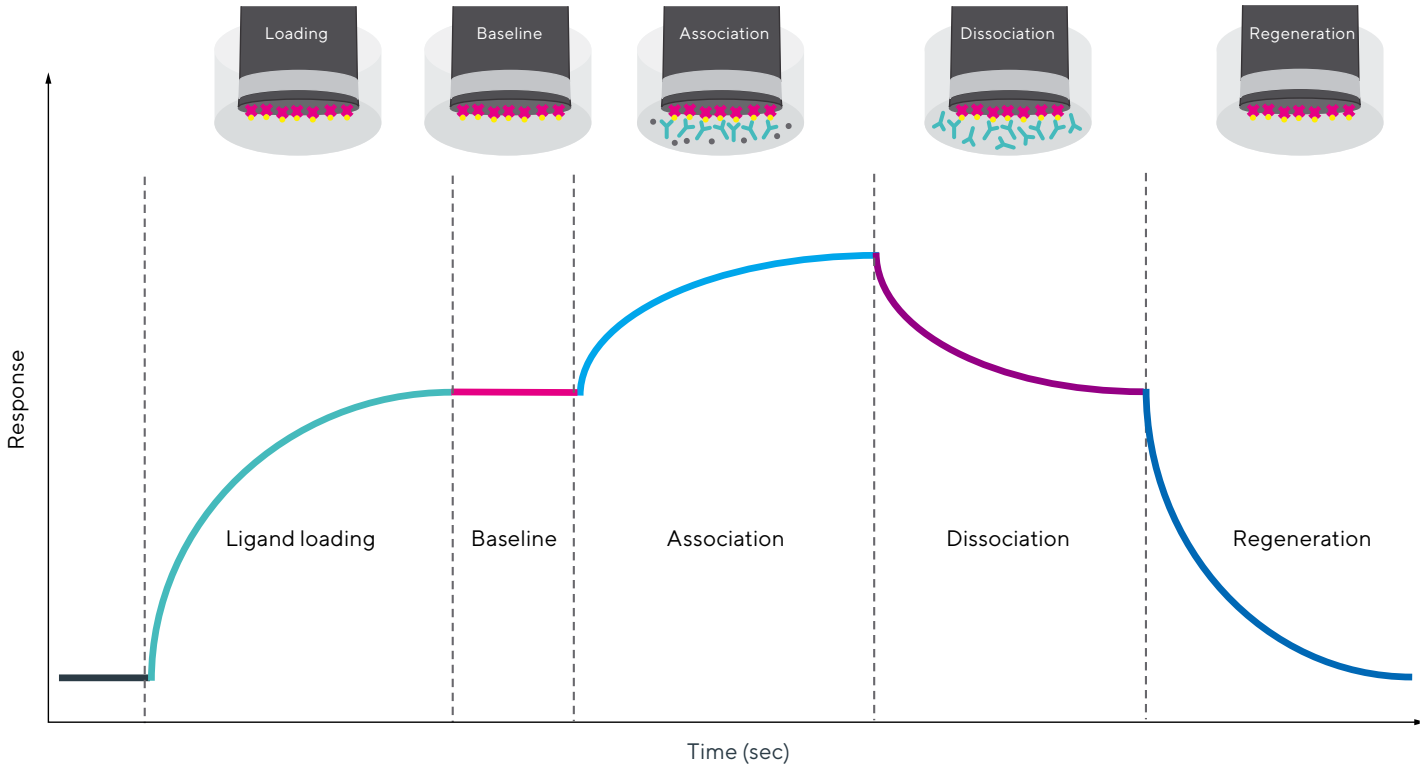


Figure 4: Typical assay steps in a multiple analyte concentration kinetic binding experiment to measure binding kinetics (association and dissociation)

Post-Immobilization Baseline
After immobilization, the ligand is introduced to binding | assay buffer to equilibrate and wash away loosely bound ligand molecules.

Analyte Association
Analyte is introduced for binding analysis. Association kinetics are measured in this step. While the analyte concentration is required for calculating association rates, in the absence of this information, the presence of a binding (Yes | No) interaction can be tested. Octet® systems can also utilize the association phase for quantitation purposes in lieu of ELISA.

Analyte Dissociation From the Ligand
The bound complex is dissociated in assay buffer. Unlike the association process, the dissociation is independent of reagent concentration. This enables accurate calculation of dissociation kinetics in this phase, even when the analyte concentrations are unknown.

Regeneration
After data collection, the regeneration process strips the bound analyte from the immobilized ligand which prepares the surface for another round of analyte binding. For ligands immobilized by affinity capture methods, for example with Ni-NTA or Protein A Biosensors, regeneration usually removes the ligand molecule as well. In these scenarios, recapture of the ligand is required prior to analyte binding.

The total assay time for generating a kinetic curve using multiple analyte concentrations depends on the time of each individual step, which in turn depends largely on the kinetic properties of the interaction itself. An interaction system with a rapid association can reach equilibrium within a few minutes, a slow dissociating complex can require a much longer time at the dissociation phase. For a typical interaction, an estimated time for a kinetic trace would be about 5–30 min of the total experimental time. It's also well established that when using standard methods to characterize interactions, multiple analyte concentrations

are required in order to correctly sample the kinetic space, preferably below and above K_D concentrations (Figure 5). Kinetic curves are then fitted as a group (global fitting) to calculate for kinetics parameters. While traditional assays require generating multiple kinetic curves representing several analyte concentrations, due to OneStep® injections the Octet® SF3 system only requires one analyte concentration, which generates one kinetic curve to characterize kinetics and affinity values.

See our BLI biosensor chemistry selection guides for a full list of available biosensors.

See our SPR biosensor chemistry selection guides for a full list of available biosensors.

Learn how to develop a kinetic binding assay on the Octet® platform.

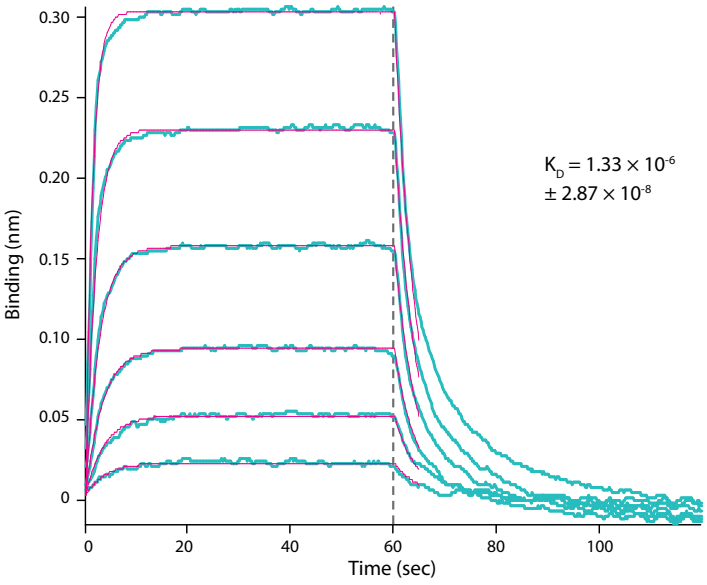


Figure 5: Analyte titration series to calculate kinetics and affinity constants. A six point analyte titration (teal) and 1:1 binding model fits (magenta) are shown. Data were fitted using Octet® Analysis Studio Software by global fitting unifying all kinetic traces to fit for association (k_a), dissociation (k_d) kinetics and affinity (K_D).

Featured Applications

Analysis on FC-Gamma Receptor Interactions in Octet® Platforms

Fc gamma receptors (FcγRs) are membrane glycoproteins found on certain types of cell surfaces that contribute to immune system functions. With high binding specificities to Fc regions of immunoglobin G (IgGs), Fc-receptors bind to IgGs displayed on pathogenic cells to trigger antibody mediated cell-dependent cytotoxicity (ADCC) or phagocytosis. Fc-receptor interactions bind IgGs with a broad range of affinities based on the type of receptor involved. FcγRI (CD64) affinities to IgGs are characterized at 0.1 nM–10 nM, while FcγRII (CD32) and FcγRIII (CD16) interactions are measured at 0.1–10 µM affinities. In addition, binding properties can be affected by genetic polymorphisms of the receptors as well as glycosylation patterns in the Fc region on the antibody.

Octet® BLI platforms are utilized for characterizing Fc-gamma receptors due to their:

- Ability to rapidly measure binding interactions across a wide range of affinities (1 mM–10 pM).
- Detect variabilities in affinities due to variations such as glycosylation patterns.
- Availability of a wide range of biosensor chemistries for ligand immobilization.
- High-throughput, and lower consumable costs.

An example workflow for performing an FcRn-human IgG (hIgG) kinetic assay is shown in Figure 6.

Learn more about BLI on the Octet® platform.

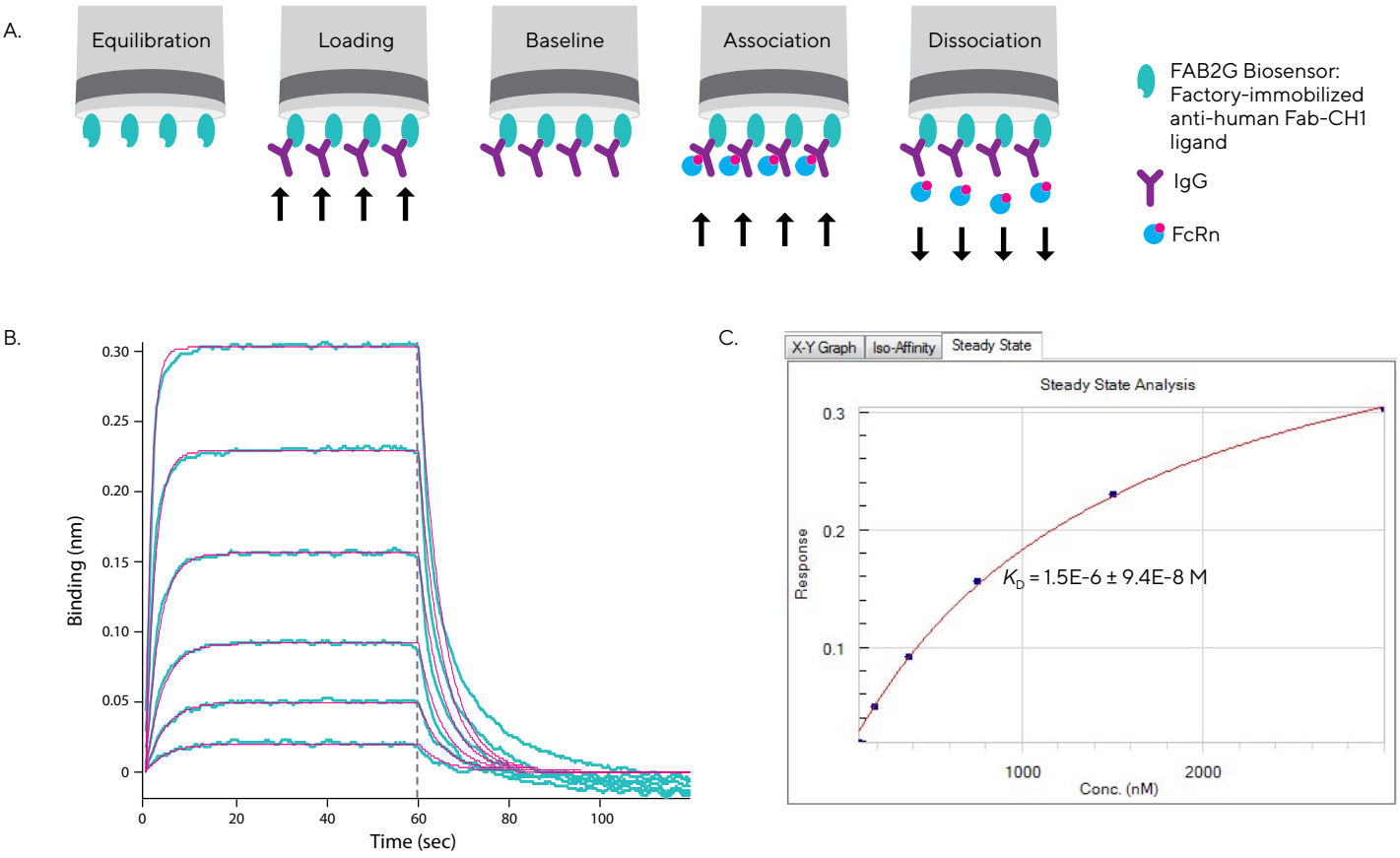


Figure 6: Workflow of a FcRn-hIgG kinetic assay using anti-human FAB2G Biosensors. (A) hIgG is captured and FcRn is introduced as the analyte. Other commonly used biosensor chemistries are Ni-NTA and Streptavidin. (B) Kinetic analysis using a 1:1 interaction model to determine kinetics and affinity. (C) Steady state analysis using data represented in (B). Steady state (end-point) analysis can also be useful especially in low affinity interactions or for systems that produces very fast association rates.

% Deglycosylation	% Glycosylation	K_D (M)
0	100	8.68E-07
10	90	9.89E-07
25	75	1.13E-06
50	50	1.52E-06

Table 2: Binding of FcγRIIIa is affected by glycosylation and can be measured by an Octet® BLI binding assay. Monoclonal hIgG with various levels of glycosylation profiles binding to FcγRIIIa. Interaction becomes weaker with reduced glycosylation levels.

Learn more about analyzing Fc-gamma receptor-IgG interactions.

Learn more about analyzing FcRn-antibody interactions.

Quantitation on Octet® BLI Platforms

Octet® BLI platforms offer an excellent solution to replace assays that are currently performed as ELISA. Similar to ELISA, Octet® assays are carried out on an immobilized surface (solid support in ELISA), and the analyte is bound from solution. However, Octet® assays are fully automated, require much less user intervention, and provide a simplified workflow. In addition, they provide researchers with the flexibility to choose the most efficient assay format based on assay needs such as sensitivity, dynamic range, and workflow (Table 3).

Advantages of Octet® quantitation assays over ELISA:

- Multiple assay formats – depending on the required sensitivity of the assay, several assay formats can be adapted: a single step, sandwich or the 3-step assay which provides the highest sensitivity.
- Quantitation assays are easily developed for a variety of analytes such as IgG, recombinant proteins, vaccines and viruses, and more.
- Detection of low affinity analytes that can often be washed away during ELISA procedures due to their faster dissociation rates.
- Real-time data generation to assess experimental progression.
- Faster time to results which is advantageous in detecting less stable analytes.
- Samples and reagents can be fully recovered and reused.
- Lower assay costs as biosensors can be regenerated.

Learn how to convert your ELISA assays to Octet® assays.

Selecting an assay format

The choice of an assay format is dependent on the concentration range of analyte to be quantified (Table 3). Direct binding quantitation assays (1-step) typically can measure concentrations from low mg/mL to low ng/mL depending on the analyte. Larger analytes provide higher sensitivity compared to lower molecular weight analytes. 1-step assays are simpler, faster and eliminate the need for secondary reagents and steps needed for detection.

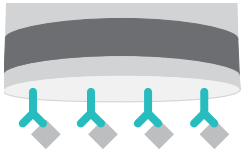
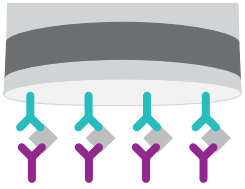
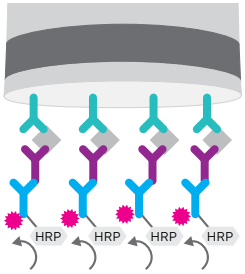
Assay features	1-step	2-step	3-step
Pictorial representation			
Assay steps	▪ Bind analyte	▪ Bind analyte ▪ Bind secondary reagen	▪ Bind analyte – secondary antibody complex ▪ Bind HRP-loaded antibody ▪ Incubate in precipitating substrate for HRP
Typical assay time	▪ 30 min (Octet® R4, R8) ▪ 15 min (Octet® RH16) ▪ 2 min (Octet® RH96)	▪ 1 hr 30 min (Octet® R4, R8) ▪ 1 hr 15 min (Octet® RH16) ▪ 1 hr (Octet® RH96)	▪ 2 hr (Octet® R4, R8) ▪ 1 hr 30 min (Octet® RH16) ▪ 1 hr (Octet® RH96)
Typical concentration range	▪ Low mg/mL to low ng/mL	▪ Low ng/mL to low pg/mL	▪ Low ng/mL to low pg/mL
Advantages	▪ Single incubation step – fast, easy, reduces reagent expenses ▪ Low affinity analytes detected – even those missed by ELISA ▪ No labeled reagents ▪ Kinetic parameters can be measured ▪ Allows regeneration and re-use of biosensor in most cases	▪ Two incubation steps – still fast, easy, reduces reagent expenses in comparison to ELISA ▪ Higher sensitivity of detection, down to low pg/mL, depending on assay ▪ No labeled reagents ▪ Automated and no-wash assay minimizes handling	▪ Similar to most ELISA assays in format – but faster and easier ▪ Excellent sensitivity – down to low pg/mL, depending on assay ▪ Automated and no-wash assay minimizes handling

Table 3: Octet® quantitation assay formats and features.

In addition, they enable researchers to reuse biosensors after regeneration in most cases. Multi-step assays (2- and 3-step) are used when enhanced sensitivities are required up to low pg/mL concentrations (Figures 7 and 8). Since Octet® detection relies on the optical thickness generated at the biolayer, increasing the binding layer by 2 or 3 step analytes increases the binding signal and sensitivity. The 2-step format typically includes the capture of the analyte by the biosensor followed by sandwiching the analyte by a second antibody. For higher signal amplification, an enzyme-linked sandwich assay (3-step assays) captures analyte bound by two separately-labeled capture molecules to the biosensor in the first step, binds an HRP-conjugated antibody to the complex in the second step, and precipitates a substrate directly onto the biosensor surface further increasing the mass added in the third step.

Read about how you can build you own custom quantitation assays.

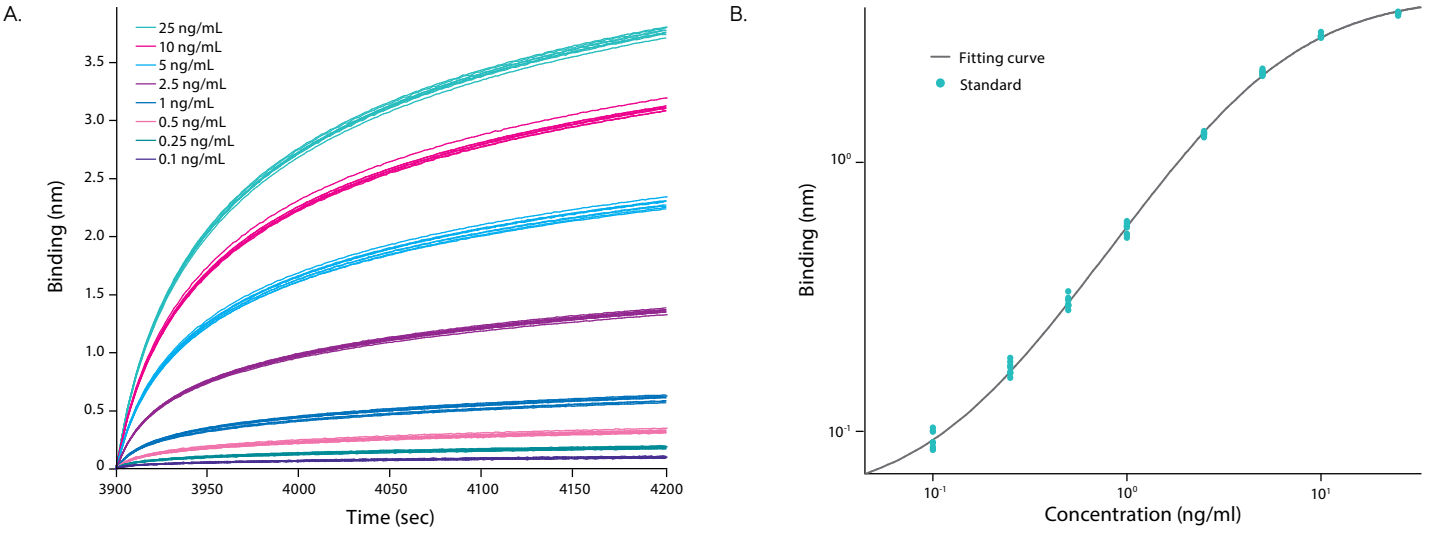


Figure 7: Sandwich assay to quantitate Protein A: Protein A is widely used in purification columns to capture and purify IgGs. However, during the purification process, Protein A can leach off the chromatography matrix and co-elute with IgGs. A sandwich-based (2-step) assay can be used to quantitate protein A co-eluted with IgG. (A) BLI sensorgrams for Protein A standards are generated using protein A concentrations from 25 ng/mL to 0.1 ng/mL (multiple replicates). (B) Binding rates from (A) are plotted for each protein A standard concentration. The standard curve points are fitted to a 4-PL model in Octet® Analysis Studio Software.

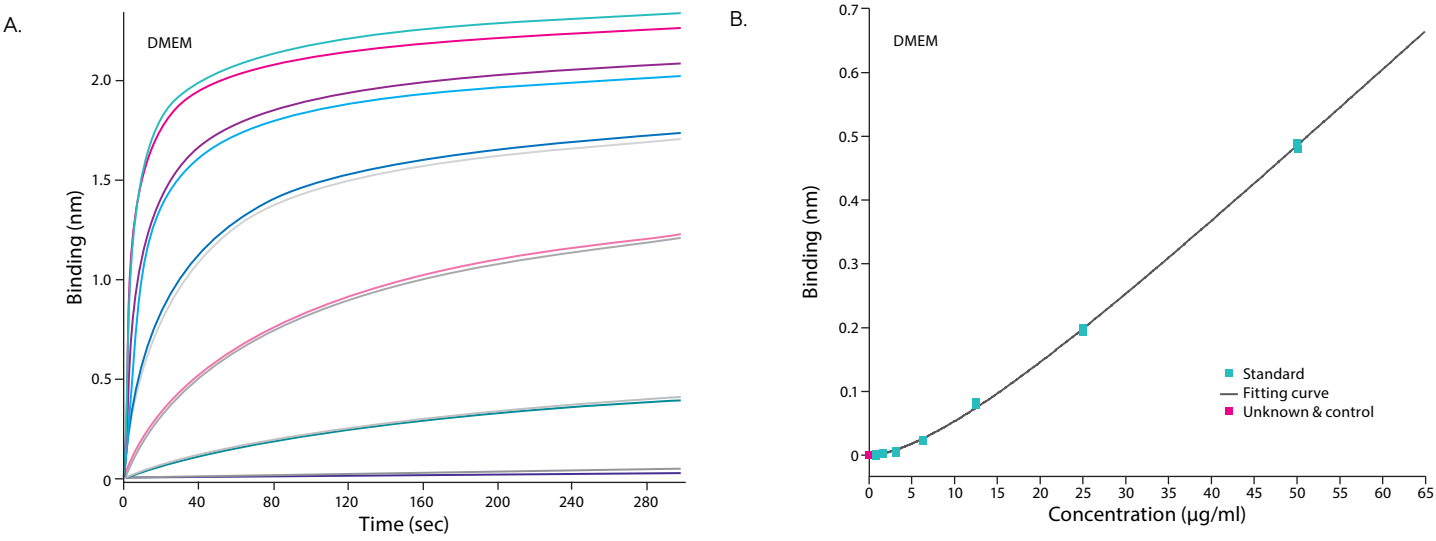


Figure 8: Quantitation of recombinant insulin in DMEM media using a 1-step Octet® quantitation assay. (A) BLI sensorgrams for insulin binding to immobilized biotin labeled anti-insulin antibody at concentrations of 0.78–50 µg/mL. (B) Standard curves generated by plotting initial binding rates from (A) against standard concentrations. Data were fitted using a 5-PL dose-response curve fitting equation in Octet® Analysis Studio Software (replicates). (B) Binding rates from (A) are plotted for each protein A standard concentration. The standard curve points are fitted to a 4-PL model in Octet® Analysis Studio Software.

Epitope Binning or Cross-Competition Assays on Octet® BLI Systems

Current capabilities in antibody engineering allow for rapid generation of large antibody libraries against a therapeutic target. Characterization of the target protein's epitope regions in early stage drug development is essential to understanding the function and mechanism and provides information for a more targeted development of therapeutic antibodies. Epitope binning is a process that enables segmentation of antibodies into bins based on the antigen region or epitope, bound by each antibody. Since monoclonal antibodies (mAbs) in different bins bind to distinct epitopes that can display diverse functional characteristics, epitope binding information can increase the likelihood of choosing lead antibody pools with desired properties. Epitope binning assays also are performed to identify mAbs that bind similar epitopes to a previously characterized mAb for generations of biosimilars or biobetters. These assays can also be useful in reagent development programs to identify antibody pairs that are best suitable for development of ELISA assays.

Octet® BLI systems provide several advantages for these assays:

- High-throughput and assay speed.
- Versatility – choose from three assay formats (Figure 9).
- Availability of a variety of biosensor chemistries suitable for analysis.
- Ability to characterize antibodies from hybridoma supernatants, phage lysates or purified samples.

Learn more about epitope binning assays on Octet® platforms.

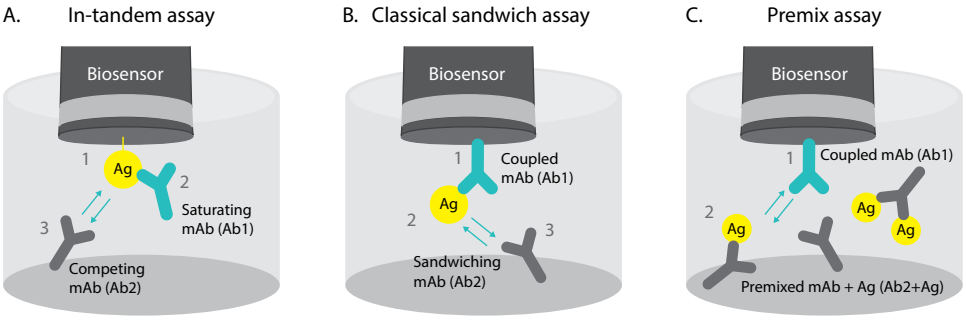


Figure 9: Octet® epitope binning assay formats: (A) In-tandem assay. Antigen is immobilized onto the biosensor followed by binding with saturating conditions on mAb1 and competing with mAb2. (B) Classical sandwich format. mAb1 is immobilized onto the biosensor followed by antigen capture by mAb1. mAb2 is then used to test for antigen binding. (C) Premix assay. One mAb is immobilized on the biosensor and bound with pre-bound antigen-mAb1 complex. Premix assay format is typically used to clarify ambiguous results obtained from other binning formats.

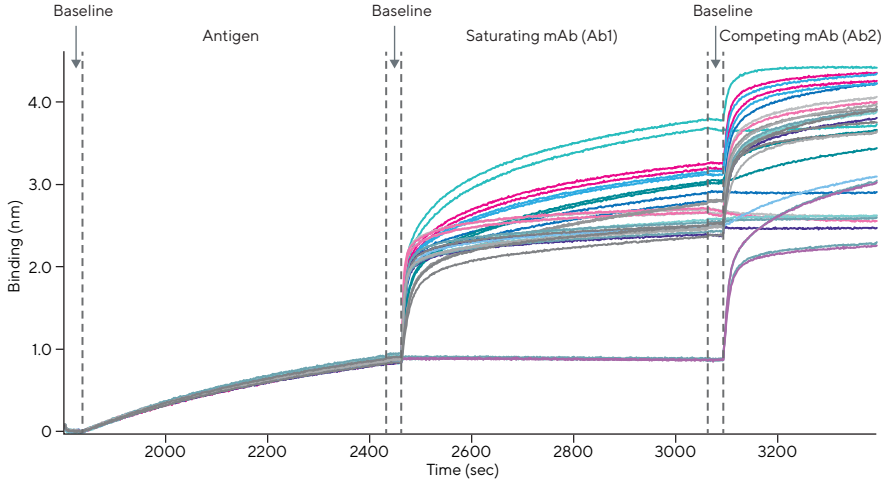


Figure 10: Representative data from an in-tandem assay. Main assay steps include antigen loading, mAb1 binding at saturating conditions followed by competing mAb2 binding. In the competing mAb2 step, responses are either increased indicative of binding mAb2 recognized by a separate epitope binding site or no binding is observed as a result of competitive binding sites to the antigen.

Ab#	mab-2	mAb-50	mab-3	mAb-51	mAb-44	mab-15	mAb-62	mAb-24	mAb-52	mAb-58
mab-2	0.0034	0.0195	0.0093	0.0199	0.0064	0.002	0.0161	0.0123	0.0031	0.0312
mAb-50	0.005	0.0247	0.0367	0.0296	0.0305	0.0005	0.0218	0.0342	0.0049	0.0418
mab-3	0.0108	0.0152	0.0211	0.0125	0.0234	0.0022	0.0051	0.0192	-0.0067	0.0115
mAb-51	0.0239	0.0232	0.0323	0.0315	0.0005	0.0058	0.0127	0.0391	-0.0008	0.0388
mAb-44	0.0105	0.0382	0.0169	0.0314	0.0122	0.0099	0.0225	0.0291	0.018	0.0371
mab-15	0.0084	0.0116	0.0202	0.0146	0.0075	0.0148	0.0261	0.0232	0.0207	0.027
mAb-62	-0.0004	0.0065	0.0232	0.0122	0.0067	0.0175	0.0012	0.0185	0.0243	0.0396
mAb-24	-0.0001	0.0341	0.0125	0.029	0.026	0.0181	0.0165	0.0227	0.0374	0.0587
mAb-52	0.0015	0.0347	0.011	0.0322	0.0078	0.008	0.0162	0.0269	-0.0233	0.0464
mAb-58	0.0386	0.0368	0.0322	-0.0002	0.002	0.0007	0.0216	0.0307	-0.0428	0.0328
mAb-25	0.0267	0.0396	0.0329	0.0399	0.0232	0.0284	0.027	0.0511	0.0135	0.0572
mAb-23	0.0336	0.0398	0.0236	0.0639	0.0245	0.0352	0.0506	0.0566	0.075	0.0698
mAb-38	0.0035	0.0061	0.0188	0.0295	0.021	0.0197	0.0328	0.0352	0.0127	0.0221
mAb-63	0.2239	0.2575	0.2407	0.4791	0.2863	0.16	0.3683	0.2381	0.3513	0.3675
mAb-60	0.252	0.2697	0.257	0.2791	0.3783	0.3022	0.3139	0.3386	0.3618	0.269
mAb-64	0.4397	0.4474	0.4073	0.4464	0.523	0.2777	0.3779	0.4468	0.5822	0.614
mAb-31	0.4097	0.464	0.3968	0.4413	0.4479	0.3386	0.4535	0.4763	0.5579	0.6324
mAb-32	0.4226	0.4729	0.3922	0.4461	0.4641	0.3648	0.4574	0.4765	0.5678	0.6118
mab-12	0.3907	0.434	0.3847	0.4172	0.4008	0.4173	0.3992	0.4208	0.4119	0.4514
mAb-54	0.6306	0.6569	0.6414	0.6693	0.7571	0.6758	0.7236	0.7197	0.8777	0.9317
mAb-37	0.5113	0.5277	0.5461	0.555	0.5725	0.5452	0.552	0.5211	0.5448	0.5779
mAb-17	0.7644	0.7953	0.7066	0.7535	0.796	0.7086	0.8256	0.6222	0.7612	0.7669
mAb-33	0.588	0.5896	0.628	0.6229	0.4952	0.5491	0.5191	0.4923	0.5164	0.5011
mab-1	0.3307	0.3079	0.402	0.3624	0.3639	0.4086	0.3709	0.365	0.3391	0.3852
mAb-49	0.7726	0.7833	0.691	0.7298	0.8354	0.6344	0.7367	0.5978	0.7226	0.7457

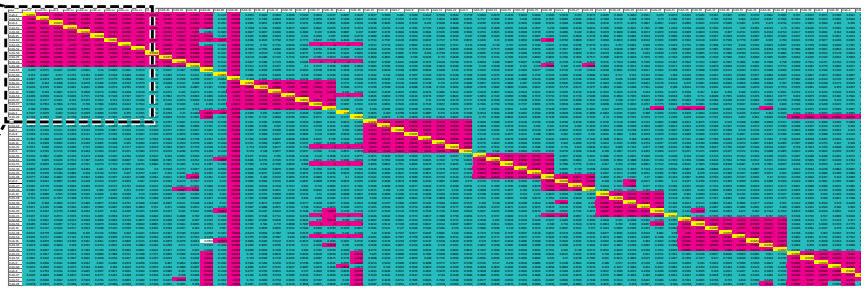


Figure 11: Data analysis. Two-dimensional matrix for a panel of 64 × 64 mAbs profiled in an in-tandem binding assay (4096 total pairing reactions). The saturating antibodies (Ab1) and competing antibodies are listed in rows and columns respectively. Red indicates a blocking interaction (competing) pair and green indicates a binding pair. The matrix diagonal represents self-self interactions for each mAb that is highlighted in orange. Related antibodies were clustered into bins using a Pearson clustering analysis. Data was analyzed using Octet® Analysis Studio Software version 11 that includes a dedicated module for unifying multiple runs and analysis of large epitope binding data sets.

Fragment Screening with OneStep® SPR

Fragment based drug discovery (FBDD) approaches are aimed at identifying chemical scaffolds that interact with a target molecule and alter or inhibit activity. Typical fragment libraries consist of compounds with 6–15 heavy atoms presenting low complexity scaffolds resulting in low affinity binding interactions that are in the range of 10 µM to 10 mM. Sensitive detection technologies are required to successfully capture fragment binding hits. The Octet® SF3 platform is an ideal solution to establish fragment binding screens due to high sensitivity, throughput and the availability of OneStep® and NeXtStep™ Injections that significantly simplifies and reduce screening workflow timelines.

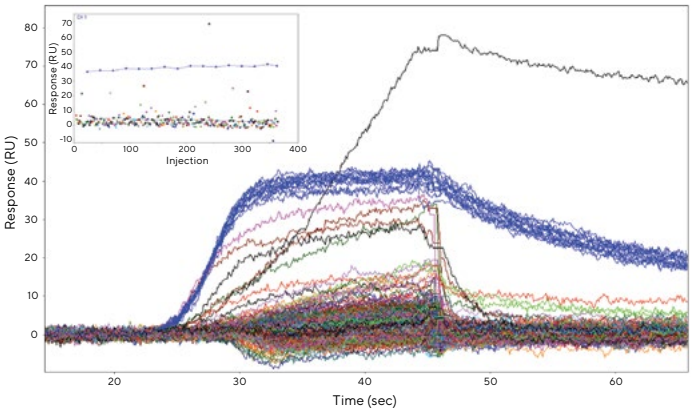


Figure 12: OneStep® fragment screening using the Octet® SF3 system. SPR sensorgrams from a fragment screen are shown. Blue curves represent the positive control compound binding that run intermittently to assess protein activity throughout the screen. The inset depicts equilibrium response values for each fragment vs. cycle number. The hit selection feature in Octet® SPR Analysis software enables identification of fragment hits and calculates kinetics and affinity values.

In fragment screening projects utilizing the Octet® SF3 system, using a single concentration copy of the fragment library researchers are able to:

- Determine kinetics (k_a, k_d) and affinity (K_D) constants from the primary fragment screen with OneStep® Injections (Figure 12).
- Test up to 768 fragments in 24 hrs with OneStep® and eliminate secondary screening that involves dose-response titrations.
- Test up to two therapeutic targets versus a fragment library simultaneously.
- Test fragments for competition mechanism and determine kinetics (k_a, k_d) and affinity (K_D) in the presence of a competitor molecule using NeXtStep™ Injections.
- Accurately select hits from primary screen data using a novel normalization and selection algorithm developed in collaboration with industry leaders.
- Gain significant time and reagent savings compared to conventional SPR-based fragment screening.

Learn more about performing fragment-based drug discovery screens on Octet® SF3 systems.

Characterizing Mechanism of Binding Using NeXtStep™ Competition Analysis

Competition binding assays can be used to identify site-selective binding or gain more information on the binding mechanism of the identified hits. The Octet® SF3 system utilizes NeXtStep™ Injections to generate full kinetics and affinities in the presence of a competitor. NeXtStep™ consists of two back-to-back gradient injections, in which the first injection results in the formation of the binding complex with a known compound (positive control) at saturating conditions. Once all binding sites are occupied, the second gradient is established with the fragment. If the fragment requires the identical binding site as the positive control, no binding or reduced response can be expected (competitive inhibitors, Figure 13B), while an unperturbed binding response would indicate a non-competitive binding mechanism (Figure 13A).

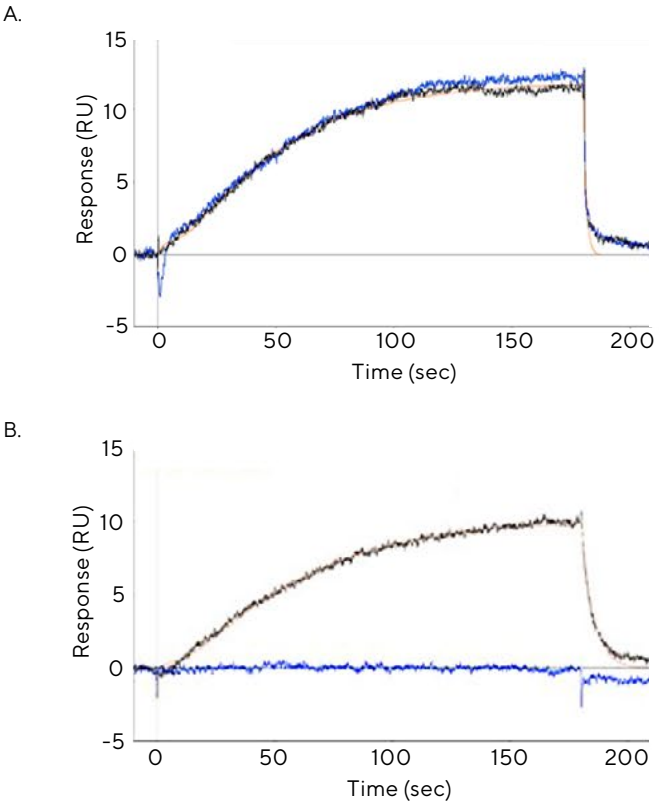


Figure 13: NeXtStep™ Injections with the Octet® SF3 system. Binding profile in the presence of a control (blue) and binding profiles in the absence of control (black). (A) Non-competitive fragment binding shows an unchanged binding response in the presence of a control. (B) Fragments with competitive binding show decreased binding responses in NeXtStep™ Injections in the presence of the control.

Characterization of High Affinity Biologics Interactions with OneStep® on Octet® SF3 Systems

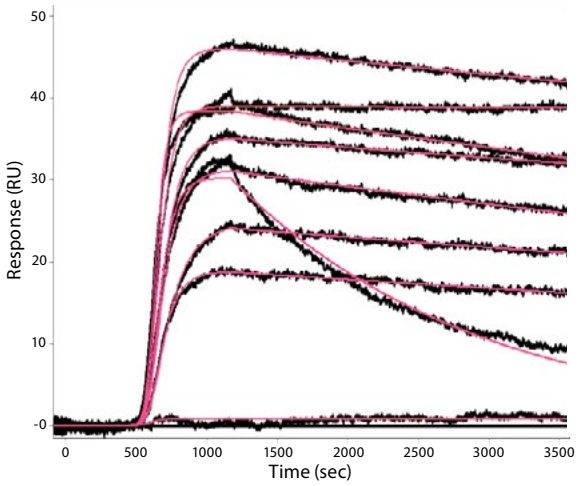
Recent advances in protein engineering technologies have contributed to the development of several breakthrough biologics to treat diseases such as cancer, inflammation, and diabetics. Biologics or protein therapeutics often can provide higher target specificity and reduced side effects compared to small molecule drugs. Today's biologics drug discovery process involves a hit-to-lead campaign where lead candidates are identified by screening thousands of protein samples using biochemical, ELISA or binding screens. Lead identification is followed by target binding characterization, an essential step in lead validation, to select for highly selective biologic candidates with desired kinetics and affinities towards the target.

The Octet® SF3 system is ideal for high-affinity lead characterization and validation due to:

- OneStep® characterization is 3- to 6-fold faster than a traditional characterization on a 4-channel SPR system.
- OneStep® Injections use less sample (50% less analyte).
- No analyte dilutions are required.

With OneStep® gradient injections, binding kinetics and affinity values can be measured using a single analyte injection, which significantly improves the efficiency of characterization workflows over traditional SPR (Figure 14). Since most high-affinity interactions produce slow dissociation rates from the target, longer dissociation analysis times are required for accurate kinetic analysis. When traditional SPR workflows are utilized that require multiple analyte concentrations per target the experimental times can be significantly longer compared to OneStep®, where a single titration is sufficient for analysis (Figure 15).

A.



B.

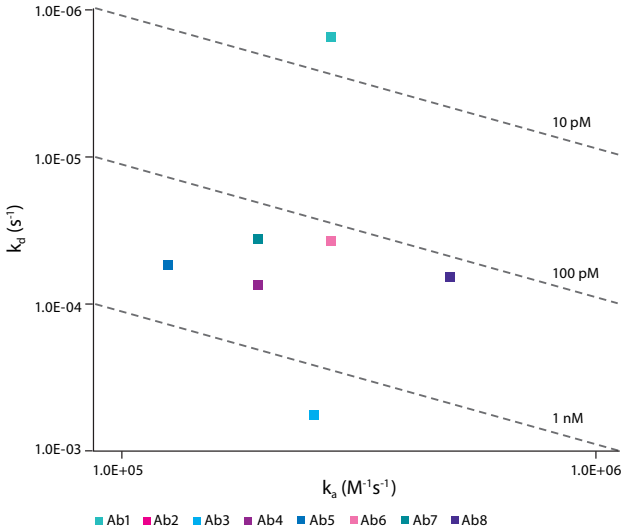


Figure 14: OneStep® Kinetic Characterization of Biologic Advanced Hits for Lead Selection. (A) Eight mAb hit candidates binding against a target protein were assessed. Binding data (black) are least-squares fit to a 1:1 kinetic binding model (pink). Total experimental time for OneStep® binding assays is ~20 hrs hours including buffers and sucrose controls. The identical experiment with seven analyte titrations per target would require ~63 hrs on a traditional SPR system with 7 analyte concentrations and buffer blank cycles. (B) Affinity plot (k_d vs. k_a) for target binding to mAbs characterized by OneStep® SPR. Dashed lines indicate common affinity relationships with different kinetic rate constants.

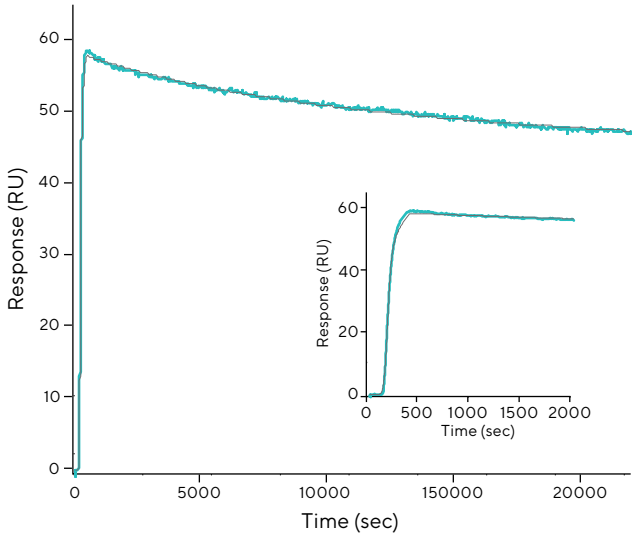


Figure 15: Octet® SF3 systems provide excellent baseline stability for accurate kinetic characterizations of high affinity binding interactions. This is indicated in the OneStep® characterization of a high-affinity binding interaction with an extended dissociation time of 6 hours the Octet® SF3 can measure dissociation periods for up to 12 hours). Figure inset show the first 2000 sec of data including the full OneStep® association (0–400 sec) followed by the first 1600 sec of the dissociation phase.

Octet® Portfolio of Instruments

BLI Systems

Octet® R2 System

Octet® R4 System

Octet® R8 System

Octet® RH16 System

Octet® RH96 System

Octet® N1 System

SPR System

Octet® SF3 System

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