Simplifying Progress

A Compendium for Successful BLI and SPR Assays

Octet® Label-Free Biosensor Analysis

SARTORIUS

"Designing and performing assays that accurately measure the binding kinetics of biomolecular interactions..."

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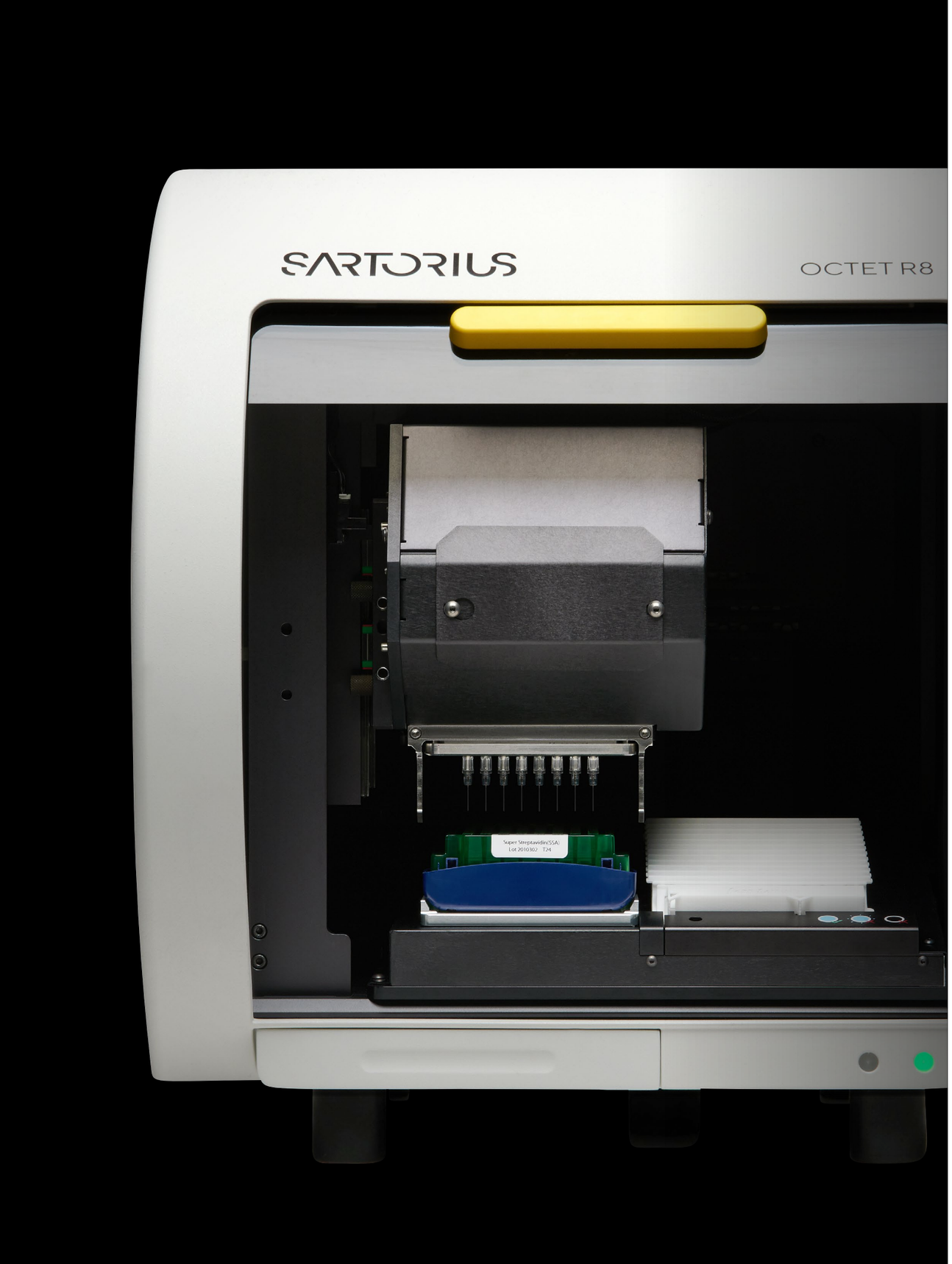
Definitions Table

Term	Description
BLI	Bio-Layer Interferometry
SPR	Surface Plasmon Resonance
Analyte	The solution-phase interaction partner that is injected over the surface-bound ligand.
Assay Buffer	The buffer used during an assay, which may contain surfactants, solvents, or other salts to allow the analyte and ligand to remain solvated or to promote activity of the interaction pair.
Bulk Shift	The observed shift in the refractive index between an analyte solution and the assay buffer.
Immobilization | Capture and Load	The process of tethering a ligand to the biosensor surface covalently or using an affinity tag or capture molecule, respectively.
k_a	The association rate constant that is measured during the association phase of an interaction (e.g., formation of the analyte–ligand complex). The association rate constant has the units $M^{-1}s^{-1}$ and is concentration dependent.
k_d	The dissociation rate constant that is measured during the dissociation phase of an analyte–ligand interaction (e.g., dissociation of the analyte from the ligand into the solution phase). The dissociation rate constant has the units s^{-1} and is concentration independent.
K_D	The equilibrium affinity constant that is calculated as the ratio of k_d to k_a ($K_D = k_d/k_a$). Unit is Molar (M).
Kinetic Analysis	Label-free and real-time monitoring of analyte–ligand complex formation and dissociation. Subsequent analysis of the response curve using a mathematical model allows determination of kinetic rate constants and global affinity.

Term	Description
Ligand	The interaction partner attached to the surface of the sensor. In screening applications, this may also be referred to as the Target.
M	Molar
MW	Molecular weight in Daltons
nm	The spectral shift monitored at the detector during BLI assays and reported on a response curve as a change in wavelength (nm shift).
R	Response in RU
Refractive index	The factor by which the speed and the wavelength of light changes when travelling between two different media.
Regeneration	The process of removing bound analyte or the analyte–ligand complex from the sensor surface.
R_{eq}	Equilibrium response of the analyte–ligand complex in RU
Response Curve | Binding Plot | Sensorgram	Plot of response units versus time that allows the course of the interaction as it occurs in real time to be followed.
Response Units (RU)	The response of the system is measured in RU, where 1 RU = 1 x 10^{-6} change in refractive index ~ 1 pg/mm^2.
R_{ligand}	Immobilization level of the ligand
R_{max}	Maximal response of the analyte–ligand complex in RU



Introduction

Welcome to the Octet® Label-Free Biosensor Analysis Applications Guide for Bio-Layer Interferometry (BLI) and Surface Plasmon Resonance (SPR).

The field of real-time, label-free protein binding analysis has historically been marked with steep learning curves. The Octet® BLI systems were the first systems to challenge this notion by providing scientists with a simple yet powerful user interface in both the system and the software. The recently released Octet® SF3 system aims to achieve the same for the field of SPR with novel injection technologies that dramatically simplify experimental design and decrease time to results.

Designing and performing assays that accurately measure the binding kinetics of biomolecular interactions can seem too specialized and out of reach for many scientists. This applications guide aims to change that by serving as an easy-to-use reference for basic assay design on both BLI and SPR systems. The chapters are designed to answer specific questions about these techniques, complete with relevant application notes and best practices so users are empowered to get the best data in the shortest time possible.

Octet® BLI systems are also well known for having a low entry barrier for new users thanks to a combination of Dip and Read biosensor technology and intuitive software, but still empower even the most experienced users to answer all their scientific questions. At Sartorius, we have a mission to simplify progress and that includes helping scientists access the information they need to design experiments on the Octet® BLI and SPR systems.

Users of our Octet® BLI and SPR systems already have access to a range of relevant application notes, technical notes, and other materials on our website. In this guide, we organize all this information so you can easily find the tips and tricks most applicable to your experiment. Importantly, we designed this guide such that you can skip directly to the topics you need to answer questions about using the Octet® systems and data analysis software.

Note: For any topics that are not covered in this applications guide, please contact your local Field Application Specialist or Octet® Customer Support at **octetsupport@sartorius.com**.

Here's to accelerating your next big discovery and your next big scientific breakthrough!

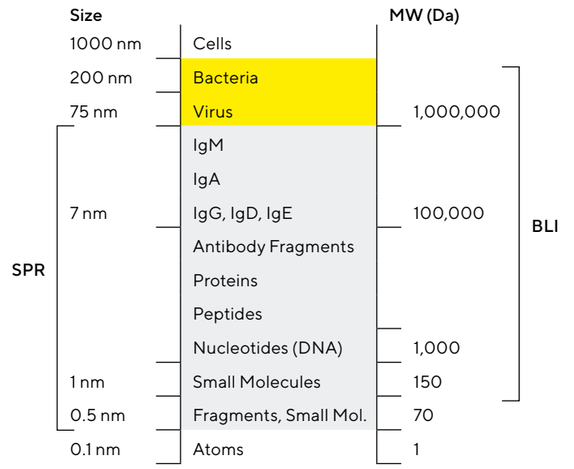


Figure 1

The Octet® brand is synonymous with high quality, accessible systems for answering a wide range of questions about molecular interactions. This figure shows the range of molecules that can be accurately measured using each system, from small molecules and protein fragments, all the way to whole cells.

1 Why Are Real-Time Kinetics and Affinity Important?

Octet® BLI and SPR systems are label-free technologies that allow users to determine real-time kinetics and affinity instead of relying on single points of information that end-point assays offer. But why is that important?

Let's imagine that you are comparing the efficacy of new sleeping aid drug candidates to a reference medicinal product (RMP). An end-point assay would only provide one data point, without additional insight on what happens before and after that time point (Figure 2). This can be misleading as the only bit of data you have is that after 5 hours all the study participants are 100% asleep and it does not provide a complete picture of how the drugs perform over an extended period.

If we were to interrogate the real-time kinetics of the interaction and extrapolate this to a theoretical "percentage asleep" we would see a much different picture. As shown in Figure 3, the RMP allows the subject to fall asleep within an hour and remain fully asleep for several hours, before waking up completely. Drug A (magenta) on the other hand, takes a long time to take effect, but shows a similar wakeup profile to RMP. Drug B (teal) has the least desirable performance as it requires a longer time to fall asleep and makes it hard to wake up following a reasonable amount of sleep. Real-time analysis provides a more complete picture, allowing scientists to make more informed decisions.

End-point assays alone would suggest that the magenta and teal sleeping pills were just as effective as the current market leader, but analyzing real-time kinetics has shown that there is a lot of work left to be done in developing the next great drug.

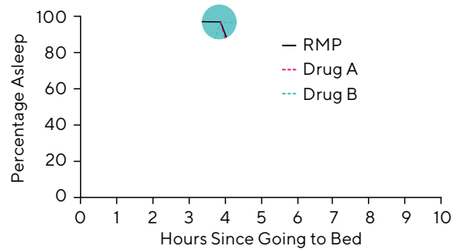


Figure 2
Sleeping Pill Analogy
End-point data gives a very limited amount of data.

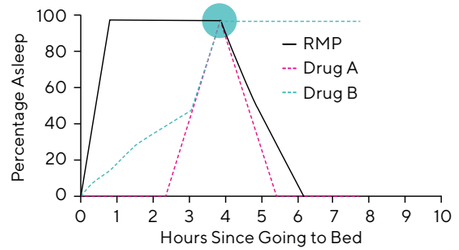
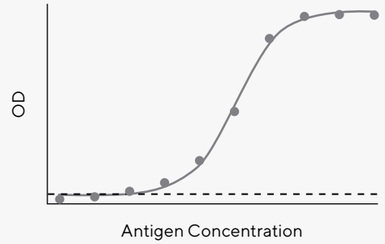
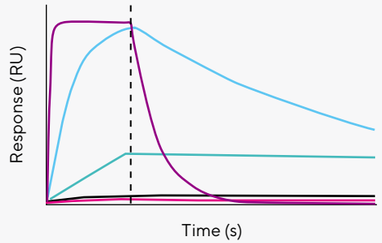


Figure 3
Sleeping Pill Analogy
Real-time assays reveal the route to the final destination and provide a much richer data set for scientists.

A.



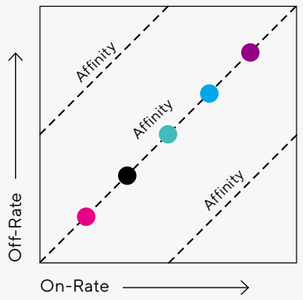
B.



C.

	(k_a) ($M^{-1}s^{-1}$)	(k_d) (s^{-1})	K_D (nM)
●	10^3	10^{-6}	1.0
●	10^4	10^{-5}	1.0
●	10^5	10^{-4}	1.0
●	10^6	10^{-3}	1.0
●	10^7	10^{-2}	1.0

D.



1.0.1 The Advantages of Real-Time and Label-Free Information

Octet® systems utilize label-free technologies, which means that no labeling of any of the interactants is required (Figure 4). This is a major factor in assay development as not all molecules are amenable to labeling and may require structural modification to allow for site-specific labeling. Each step of labeling can require optimization and therefore take a long time. There is also the risk that the label may interfere with binding between the interactants and also contribute to an increase in non-specific binding (NSB).

Figure 4

Standard end-point assays that only contain a single point of information provide only very basic information of global affinity. In the end point assay (A) all molecules would show the same resultant data fit. In a kinetics-based assay (B) you can see that the molecules show very different kinetics. Some compounds have extremely high on-rates, meaning there is very little energy needed to form the interaction, but also very high off-rates, meaning once the interaction is formed, it is relatively weak. Despite these differences in kinetics, because global affinity is a function of the association (k_a) and dissociation kinetics (k_d), the same affinity is observed upon fitting the kinetic data (C) and can be clearly seen when plotted on an affinity isotherm (D). See sections **4** and **5** for more information on kinetics and fitting of data.

1.1 What Is in the Octet® Range?

The Octet® brand has more than 15 years of market experience in offering label-free solutions and services for academic, biopharma, and biotechnology customers. The Octet® systems for label-free binding interaction analysis and concentration measurements include both Bio-Layer Interferometry (BLI) systems and a surface plasmon resonance system (SPR) (Figure 5).

These choices allow the user to select a system based on their requirements for throughput, sensitivity, automation capabilities, and price point. Octet® has an install base of over 2,500 systems globally and is a trusted supplier of consumables, software, user training, and post-sale services and support. The Octet® is included in over 5,000 publications, patents, and citations of the technology and is also cited in the in US Pharmacopeia (USP) <1108> for use in key ligand-binding applications.

1.1.1 Why Choose Octet®?

Octet® systems are designed with both the novice and experienced user in mind. The flexibility of choosing between both BLI and SPR systems puts you in control of selecting the system that is right for your application. While the systems are powerful enough to answer a range of questions about interaction kinetics, the user-friendly systems and software let you optimize your assays, run samples, and analyze your data much faster than traditional methodologies. The ability to analyze crude samples (e.g., cell lysates) is a major benefit of label-free analysis. The systems also require very little sample, so precious samples remain undamaged and can be used in subsequent orthogonal assays. The key features of Octet® BLI systems include:

- **Flexibility** with individual biosensor tips
- Integrated **automation**-ready systems
- **Reduced** assay time and running cost

A.

Octet® RH16 Octet® RH96

Octet® R2 Octet® R4 Octet® R8

Octet® N1

B.

Octet® SF3

Figure 5

The Octet® series includes: (A) Bio-Layer Interferometry (BLI) Systems and (B) a surface plasmon resonance (SPR) System.

With the introduction of the Octet® SF3 SPR system, the entry barrier to SPR has been lowered and even novice users can quickly determine accurate kinetics and affinity data using OneStep® injections. The key features of the Octet® SF3 system include:

- **Robust**, low-maintenance SPR
- **High-sensitivity** measurement for small and large molecule assays
- **Accurate** kinetics and affinity from a single analyte concentration using OneStep® injections

1.1.2 GxP-Ready Systems

GxP compliance is critical within the pharmaceutical industry and ensures that regulated industries manufacture products that are safe and fit for use, meeting strict quality standards throughout the entire process of production. Unsurprisingly, Octet® systems play an ever-expanding role due to their ease of use and reduction in the risk of errors, which can enhance productivity within operations.

Octet® BLI systems offer GxP packages (Figure 6) to support GMP-compliant implementation for installation and operational qualification (IQ|OQ) protocols and kits, and performance qualification (PQ) protocols and kits. GxP software is critical and the Octet® software is 21 CFR Part 11 compliant with full audit trails and software validation packages available.

In addition to system and software GxP support, Octet® also offers a biosensor validation service. This service enables the user to sample multiple lots of a biosensor during assay qualification and validation and reserve a well-characterized lot for purchase. Customers can then order up to five different biosensor lots, up to 20 trays from each lot for evaluation, and reserve up to 40 additional trays for future purchase.

Packages for Octet® BLI include:

- Octet® Instrument with user guide
- IQ|OQ kits and manuals**
- PQ-Q and PQ-K kits bundle and manuals*
- 21 CFR Part 11 Software
- Software validation package
- IQ|OQ service**
- PQ-Kinetics and PQ-Quantitation service*
- PMOQ service**

* Currently available for Octet® R8 and Octet® RH16 packages
** Also available for the Octet® SF3

Figure 6
Octet® Systems Ready for GxP
GxP packages are available for Octet® R8 and Octet® RH16 and Octet® RH96.

2 What Can Label-Free, Real-Time Assays Tell You?

Both Octet® BLI and SPR systems allow you to monitor a wide variety of molecular interactions across a range of molecular sizes (Figure 7). As Octet® uses label-free binding assays, this allows:

- Analysis of biomolecular interactions using conditions that are close to their native biological conformations.
- Faster assay design, development, and reagent preparation through the use of unmodified reagents and substrates. Assays that require labeling can contain multiple labeling-specific steps that each require their own optimization, resulting in increased development times and eventual time to results. In addition, data quality can be negatively impacted due to false positives that arise due to interference from fluorescent labels. Label-free analytical platforms speed up assay development and offer distinct advantages in early drug discovery.
- The lack of fluorescent labels and other reporters reduces the chance of interference from secondary molecules in your assays, resulting in higher quality data.
- In addition to being label-free, Octet® systems measure interactions in real time, which allows direct monitoring of complex formation. No secondary detection reagents are required for establishing binding responses.

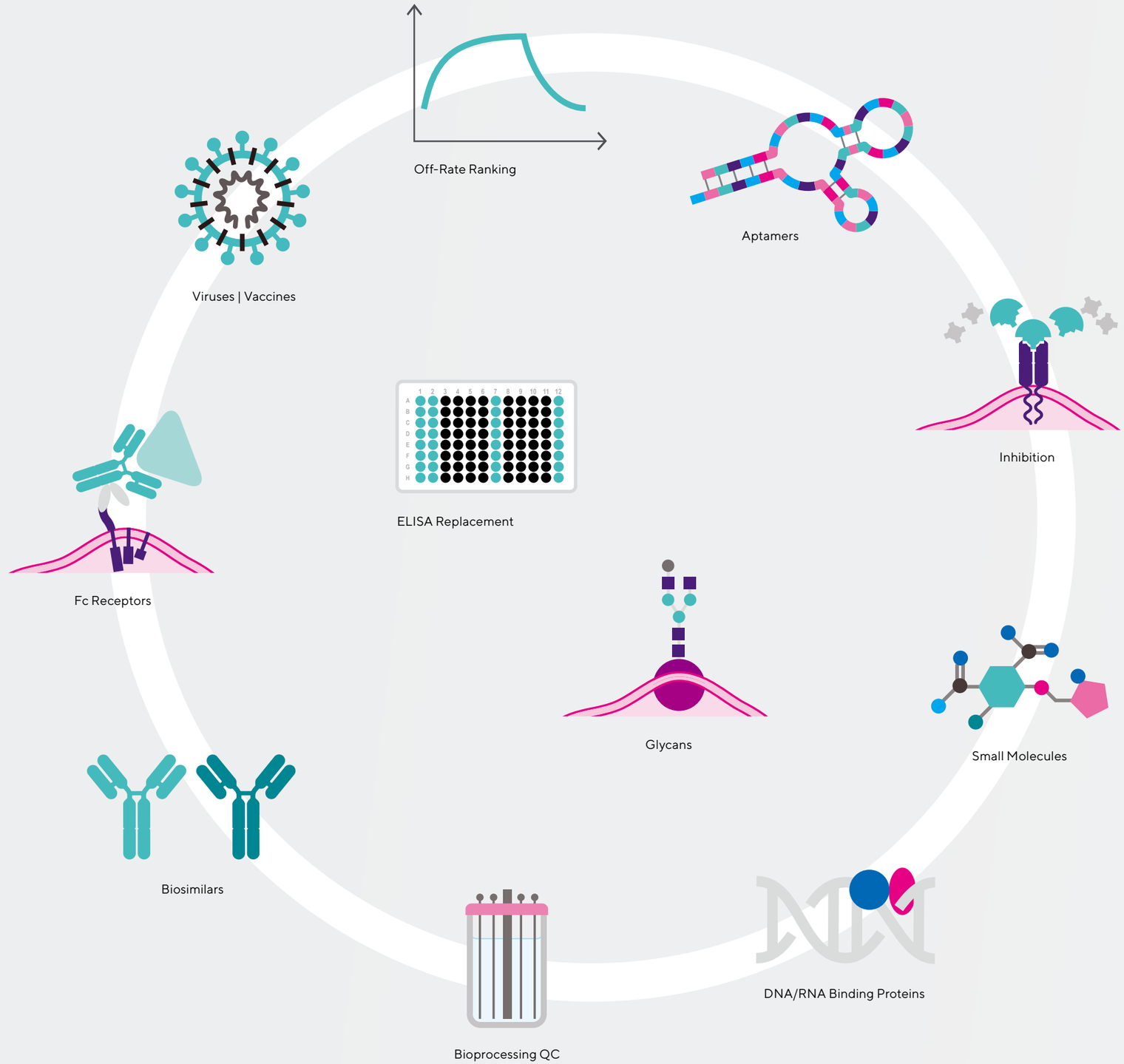


Figure 7
Monitor a Wide Variety of Interactions
Octet® systems allow you to perform a range of assay formats, allowing you to monitor a wide variety of molecular interactions across a range of molecular sizes.

2.0.1 Binding Kinetics

Octet® systems allow for the measurement of the association and dissociation rates of your molecular interaction. From these, the kinetic affinity (K_D) can be calculated. This is ideal for:

- Antibody-antigen characterization
- Protein-protein interactions
- Antibody off-rate ranking
- Drug discovery

2.0.2 Steady State Affinity

Using equilibrium titrations, Octet® can also help determine the steady state affinity (K_D) of your interaction. This is ideal for:

- Antibody-antigen interactions
- Antibody affinity ranking
- Protein-protein interactions
- Drug discovery

2.0.3 Sample Quantification

Octet® systems can quantify active target concentrations in complex biological samples, such as serum, hybridoma supernatant, or cell lysate. This is ideal for:

- Antibody quantification
- Clone selection
- Residual analysis
- Glycan profiling

2.0.4 Epitope Sites

Octet® systems allow for discriminating between antibodies that bind the same antigen but not the same epitope. This is performed using cross-competition assays in which the competitive binding of antibody pairs to a specific antigen is characterized. The Octet® BLI software has a built-in epitope binning module to enable easy assay design, performance, and interpretation. This is ideal for:

- Epitope binning
- In-tandem assays
- Sandwich assays
- Pre-mix assays

2.0.5 Dose-Response Curves

The latest version of the Octet® BLI software contains a software module that allows users to easily assess relative potency using the same assay format currently used for quantitation and kinetic assays, allowing you to measure EC_{50} similarity. This is ideal for:

- Relative binding affinities
- Biosimilar or biobetter similarity assessment
- Drug potency
- Drug efficacy

As shown above, Octet® systems offer a wide range of assay formats to choose from, but what can Octet® assays tell you? Octet® assay formats allow you to assess and analyze a wide range of biological information, including binding specificity, binding affinity, binding kinetics, and concentration analysis (Figure 8).

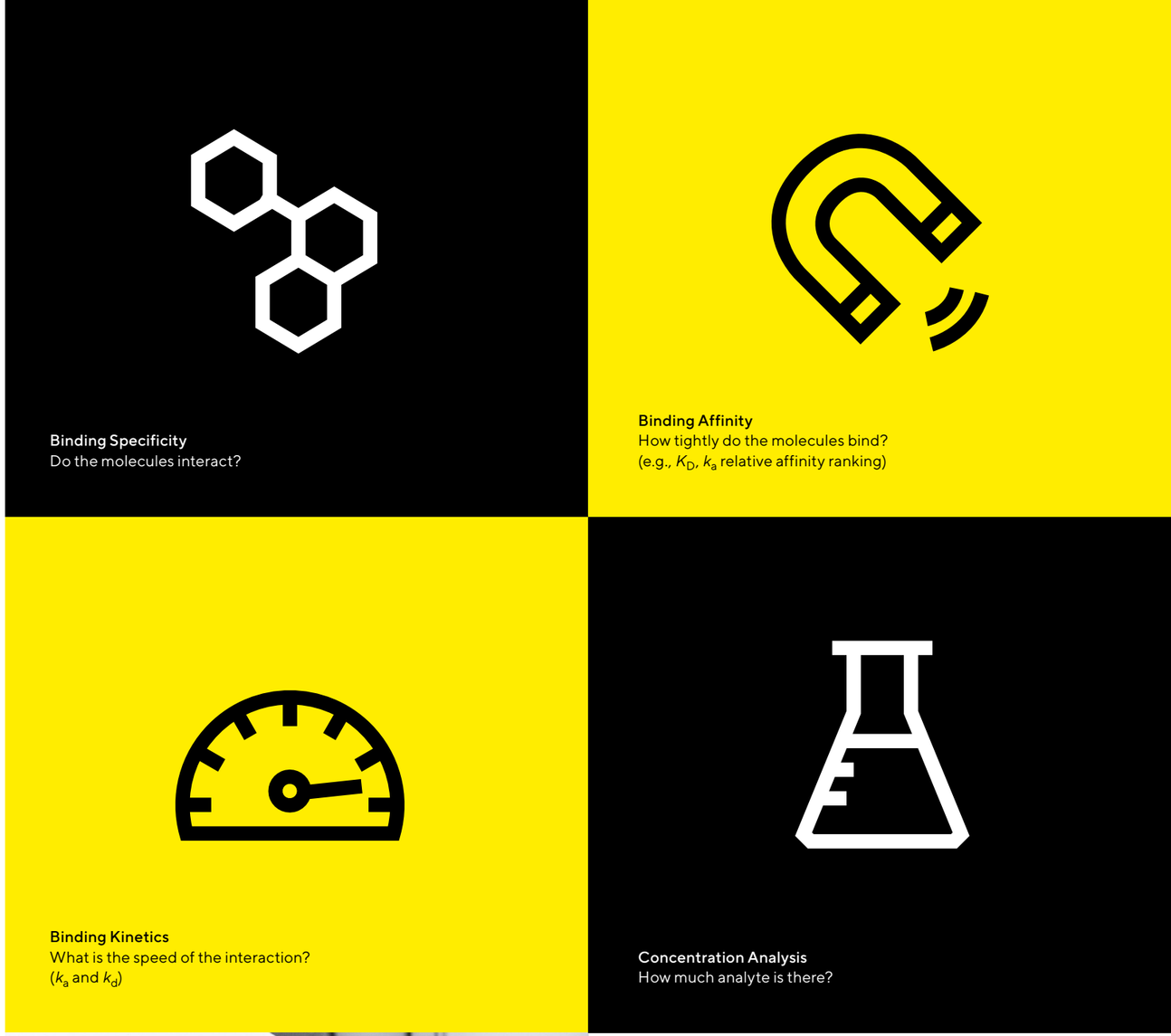


Figure 8
What Can Octet® Assays Tell You?
Octet® assay formats allow you to assess and analyze a wide range of biological information.

2.1 Where Can You Use Label-Free, Real-Time Assays?

Octet® systems can be used at every stage of the drug discovery and development pipeline (Figure 9). Examples are shown below of three stages where Octet® systems can be used to accelerate data generation and save time and money.

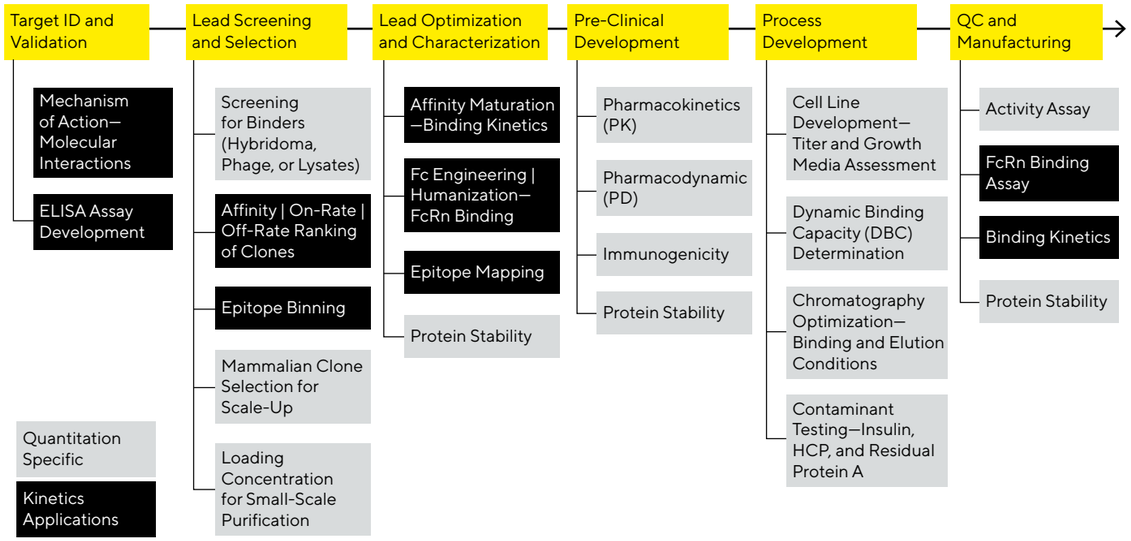


Figure 9
Applications of Octet® Instruments in Drug Research and Process Development

2.1.1 Stage: Target ID and Validation—ELISA Assay Development

Quantitation assays performed on the Octet® platform have many similarities to enzyme-linked immunosorbent assays (ELISA), as both are performed on a solid support on which the capture molecule is immobilized and the analyte is bound from solution.

Assays performed on Octet® systems can be considered as automated forms of ELISA, but are much easier to develop and have numerous advantages over standard ELISA formats, including generating and displaying data in real time (Figure 10).

	BLI (Octet® R8) + Protein A Biosensors	BLI (Octet® R16) + Protein A Biosensors	BLI (Octet® R96) + Protein A Biosensors	ELISA
Pictorial Representation				
Time to Results	< 30 min per 96-well plate	< 15 min per 96-well plate	2 min per 96-well plate	4–6 min per 96-well plate
Operator Time	Approx. 10 min	Approx. 10 min robotics compatible	Approx. 10 min robotics compatible	2–3 hr
Precision (% CV)	< 5%	< 5%	< 5%	15–20%
Dynamic Range	0.01–2,000 µg/mL	0.01–2,000 µg/mL	0.1–700 µg/mL	1–100 ng/mL Requires > 1,000X dilutions
Sample Type	Crude cell culture supernatant (without centrifugation or filtration)	Crude cell culture supernatant (without centrifugation or filtration)	Crude cell culture supernatant (without centrifugation or filtration)	Cell culture supernatant
Sample Preparation	None	None	None	Sample dilution 1:5,000–50,000
Assay Type	Real time	Real time	Real time	End point

Figure 10
IgG Titer Comparison: ELISA vs. Octet®

The conversion of an ELISA assay to an Octet® assay often simply involves re-optimizing and/or validating the conditions and configurations of the existing assay, and offers the following benefits:

1. Choose from a number of assay formats (label-free direct binding, sandwich, sandwich followed by signal amplification, etc.) to suit detection limit requirements (Figure 11).
2. Detect low-affinity analytes often missed by ELISA due to being washed away in wash steps due to their low affinity.
3. There is no need to optimize the labeling of secondary detection molecules.
4. Minimize handling via automated and wash-free steps.
5. Fully recover and re-use samples and reagents.
6. Regenerate the assay surface and re-use for some binding pairs (e.g., Protein A and human IgG).
7. Get faster time to results and better detection of less-stable analytes thanks to real-time analysis.
8. Assay signal is either directly or inversely proportional to the amount of bound analyte.

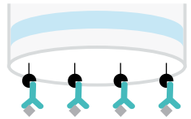
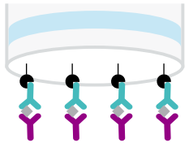
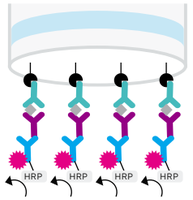
Assay Features	1-Step	2-Step	3-Step
Pictorial Representation			
Assay Steps	▪ Bind analyte	▪ Bind analyte ▪ Bind secondary reagent	▪ Bind analyte—secondary antibody complex ▪ Bind HRP-loaded antibody ▪ Incubate in precipitating substrate for HRP
Typical Assay Time	▪ 30 min (Octet® R4, R8) ▪ 15 min (Octet® 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

Figure 11
Octet® Can Replace Lengthy Conventional ELISAs

Epitope binning assays may also be useful in selecting reagents for sandwich or ELISA-type assays, such as those used for biomarker testing or pharmacodynamic assays to identify good antibody pairs that bind to the antigen simultaneously.

2.1.2 Stage: Lead Screening and Selection—Epitope Binning

All Octet® systems are capable of performing epitope binning. (See Application Note Cross-competition or Epitope Binning Assays on the Octet® RH96 System.)

In early drug development, cross-competition (epitope binning) assays are used to characterize hundreds of antibody clones and can be performed with hybridoma supernatants, phage lysates, or purified samples. The term epitope binning is used to describe segmentation of a panel of monoclonal antibodies (mAbs) into "bins" based upon the antigen region, or epitope, bound by each antibody and is performed using cross-competition assays in which the competitive binding and blocking of antibody pairs to a specific antigen is characterized (Figure 12).

Octet® BLI systems are ideally suited to run cross-competition assays due to their combination of assay speed, versatility of assay design, and parallel, independent "Dip and Read" biosensor format. Of the systems, the RH96 system has the highest throughput and versatility.

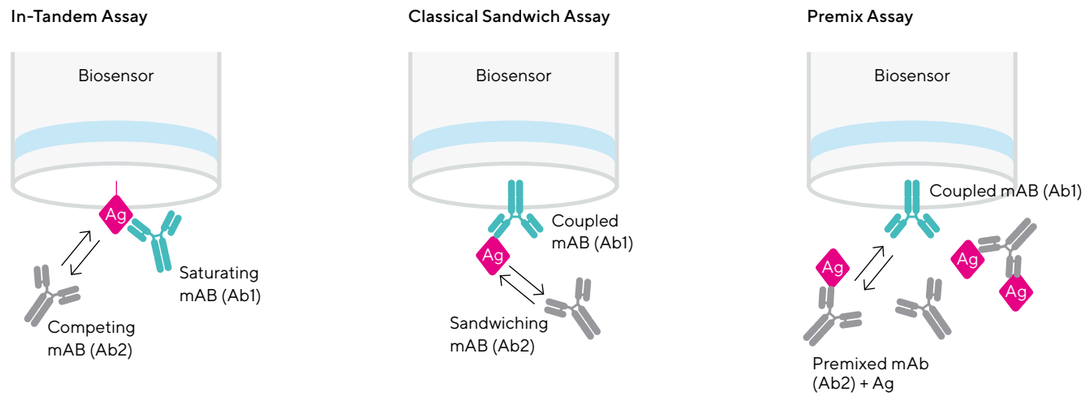


Figure 12
Epitope Binning on the Octet® BLI System
All Octet® systems can perform binning in all three binning formats.

2.1.3 Stage: Process Development—Monitoring Titer and Glycosylation

Cell line development (CLD) typically includes the screening of thousands of clones in an effort to find the few that are stable, grow as expected, and produce high yields of the protein of interest. (See Application Guide **Cell Line Development: Accelerating Antibody Discovery by Monitoring Titer and Glycosylation With the Octet® Platform**.)

The time taken from engineering an optimal cell line to the production of the target biologic can be prohibitive and may differ from molecule to molecule. Determining the expression levels using titer screening is carried out early on, whereas other critical quality attributes (CQA) such as glycan characterization are often assessed later in the development process due to a lack of appropriate and high-throughput analytical techniques that can be used to perform quick screens.

Octet® BLI systems allow rapid titer of antibody clones to enable quick selection of high-producing clones. BLI is sensitive to changes in the amount of analyte bound to an immobilized ligand and therefore are ideal for measurements in complex matrices such as crude supernatant. As only a specific analyte will bind to the immobilized ligand and induce a response, sample preparation beyond an optional dilution step is essentially eliminated.

Drug product glycosylation is a CQA due to its potential impact on pharmacokinetics properties and stability of the product, therefore knowledge of sialic acid and mannose content earlier on in CLD is advantageous. The Octet® GlyS and GlyM kits enable high-throughput relative screening of sialic acid and mannose content respectively in crude and purified samples (Figure 13). It is also possible to combine the Octet® GlyS and GlyM kits and Octet® ProA Biosensors (or any of the Sartorius quantitation biosensors) to perform titer and sialic acid content screening on the same samples using Octet® systems.

When combined, this knowledge allows CLD scientists to better select optimal clones that are both high producers and have desirable sialic acid content. High-throughput Octet® models such as the Octet® RH16 or RH96 systems can process up to 96 samples simultaneously.

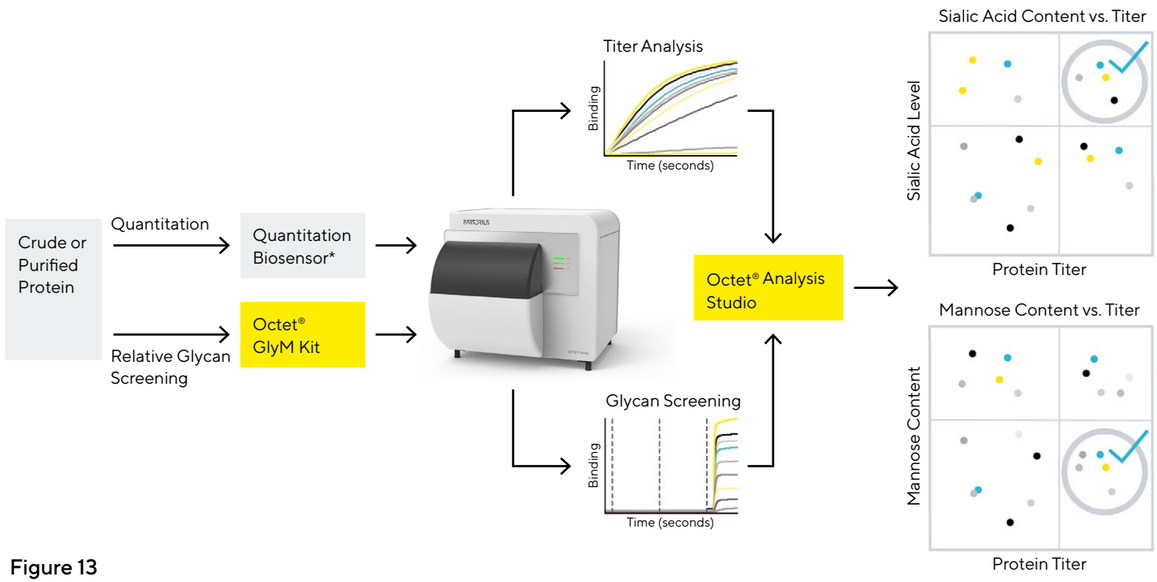


Figure 13
Cell Line Development—Faster Decision-Making
Combine titer and glycosylation screening from the same sample.

"BLI biosensors provide a fluidic-free design, handling crude, unpurified samples with ease."

3 Label-Free Interaction Analysis

3.1 Principles of Label-Free Analysis

The Octet® BLI and SPR instruments present real-time binding data to the user in a binding chart commonly called a "response curve" or "sensorgram." This application guide will use the term "response curve" in reference to both BLI and SPR data representation.

In both Octet® BLI and Octet® SPR analysis, one interactant is immobilized on the surface of the biosensor (ligand) and the other remains in solution (analyte) (Figure 14).

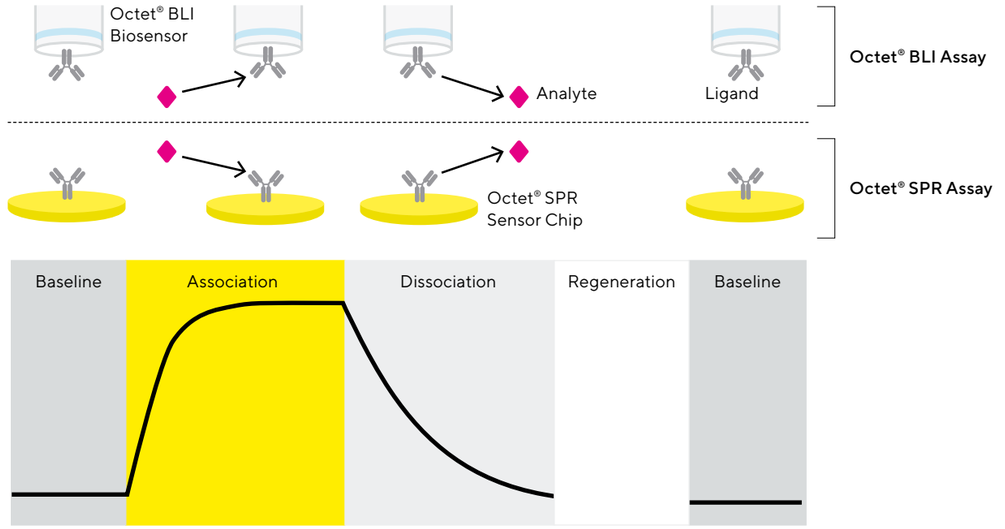


Figure 14
Phases of a Response Curve
BLI and SPR are different label-free technologies, but they share the common features of a chemically-reactive surface. In both cases, ligand can be immobilized or captured on a Biosensor or Sensor Chip prior to determining the binding properties of the solution-based analyte. The baseline phase determines the response of the ligand alone to the assay buffer. During association, analyte is introduced and an analyte-ligand complex is formed. During dissociation, no free analyte is introduced, so no additional complex is formed and the natural dissociation of the analyte from the ligand is observed.

A typical response curve consists of three phases (Figure 14):

- A baseline phase that captures the response to the assay buffer being used.
- An association phase where the analyte binds to the ligand.
- A dissociation phase where the analyte naturally unbinds from the ligand.

Additional phases may also be observed including a loading phase, where ligand is captured onto the sensor surface, and a regeneration phase, where an additional reagent is added to rapidly remove the analyte from the ligand and return the signal back to baseline. Although not always observed, the association step may reach a plateau (steady state affinity) during the injection (Section 3.4.2), where the number of molecules binding to the surface is the same as the number of molecules unbinding, resulting in a zero net change in mass and constant signal.

3.1.1 Baseline

During the baseline phase in a response curve, the biosensor or sensor chip is in contact with the assay buffer. As the assay buffer contains no binding components, a stable baseline is observed. This baseline can be used to normalize all data generated at a later stage (Section 4.1.2).

3.1.2 Loading

Although the term "loading" is not universal to both BLI and SPR, it will be used throughout this applications guide when discussing assays that use a ligand with a regeneratable affinity tag. In such cases, a separate loading step may be included to present fresh ligand for subsequent analyte binding assays. A second baseline phase is included, which corresponds with the loading step and ensures that the surface is stable for accurate measurement of kinetics and affinities. The ligand is then removed by regeneration and fresh ligand is loaded for subsequent analysis. Loading phases are also used in assays using a non-regeneratable ligand with an affinity tag, but require optimization for efficient removal of the analyte from the ligand (Section 4.8).

3.1.3 Association

The baseline and loading phases are essential to ensuring high-quality data is produced during the subsequent measurement of analyte binding to ligand (Section 4.7). This interaction occurs during the association phase, where the analyte solution is introduced to the ligand by either injecting it over the immobilized ligand (SPR) or by moving the biosensor tip into the analyte containing solution (BLI). During association, if the analyte binds to the ligand, there is a corresponding increase in the amount of analyte-ligand complex at the surface, which causes a change in the response. This change is measured in response units (RU) for SPR and nanometers (nm) for BLI. Continued binding of the analyte to the ligand causes further changes in response until the ligand approaches saturation with the analyte and the amount of analyte binding to the ligand matches the amount unbinding from the ligand. This is called steady state affinity and a stable response is observed. Due to the higher amount of analyte-ligand complex present, the observed stable response is at a higher response level than the (non-analyte containing) assay buffer baseline alone.

The association phase is critical in determining the kinetics and affinity of the interaction as the rate of association of the analyte with the ligand is determined by the association rate constant between the two molecules. Since the association rate constant is concentration dependent, knowing the accurate analyte concentration is key for determining accurate kinetics and affinity.

3.1.4 Dissociation

Dissociation occurs when analyte is no longer being presented to the ligand. During this phase, the assay buffer is presented to the ligand, which leads to a decrease in the amount of analyte complexed to the ligand and a decrease in the response signal. Unlike association, dissociation is a concentration-independent event and does not require accurate determination of analyte concentration. This makes dissociation (off-rate) ranking popular for end-point assays, but does not allow for determination of global affinity from the ratio of k_d to k_a (Section 5.2)

According to accepted guidelines for accurate determination of dissociation kinetics, a decrease of 5% or more in response should be observed prior to performing regeneration.

However, the total response must be considered when applying this 5% rule. For example, 5% of 100 RU is 5 RU, while 5% of 30 RU is only 1.5 RU, which may fall within assay noise and drift. To account for this, it is preferable that a visual drop in the response is observed in addition to a measured response decrease of 5%.

For high-affinity interactions, in can take up to 14 hours to see a 5% decrease in response (Table 1). Since temperature plays a key role in determining the dissociation rate, it is recommended that very high-affinity interactions are measured at 37 °C. A chemical regeneration can also help speed up the process, allowing assays to be performed on a sensible timeframe (Section 4.8).

K_d (s^{-1})	% Complex Dissociated per Second	Time to 5% Dissociation	Time to 50% Dissociation	Time to 95% Dissociation
1	100	0.05 s	0.7 s	3 s
0.1	10	0.5 s	7 s	30 s
1×10^{-2}	1	5 s	70 s	5 min
1×10^{-3}	0.1	51 s	12 min	50 min
1×10^{-4}	0.01	8 min 33 s	1 hr 56 min	8 hr 19 min
1×10^{-5}	0.001	1 hr 25 min	19 hr 15 min	3 days 11 hr
1×10^{-6}	0.0001	14 hr 15 min	8 days	35 days 16 hr

Table 1

Analyte–Ligand Complex Dissociation Times

Comparison of how k_d values correlate with half-life (the time it takes for 50% of the complex to dissociate), plus the required times for 5% and 95% dissociation at 25 °C as calculated from the equation: $t_{1/2}=ln2/k_d$

3.1.5 Regeneration

To determine accurate affinity values, the ligand surface must remain consistent across runs in the same study. This means all analyte molecules must unbind from the ligand prior to assessing subsequent samples or analyte concentrations—OneStep® injections only require regeneration between new samples, but not for different analyte concentrations in the same study (Section 3.4.3).

When measuring low-affinity interactions (high nM to low µM), it is possible to allow the dissociation phase to continue until all analyte has unbound from the ligand and the buffer baseline response is achieved. This is not a practical approach when measuring high-affinity interactions (pM to low nM), so a chemical intervention is required for regeneration (Figure 15).

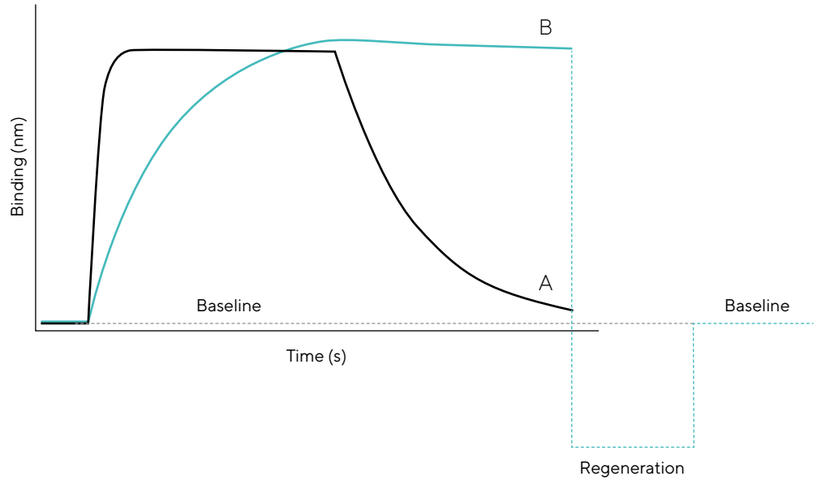


Figure 15
Response Curves Can Exhibit Similar Affinity Constants but Different Binding Profiles
During dissociation, low-affinity interactions (A) can often be left long enough to return to baseline without a chemical intervention for regeneration. High-affinity interactions (B) that do not dissociate to baseline over a reasonable timeframe require the use of a regeneration solution to remove bound analyte and return to baseline signal.

3.1.6 Putting It All Together

When the response phases are combined and repeated over numerous cycles, these label-free, real-time assays allow the user to determine vital information about the interaction (Figure 16).

Association—Yes | No Binding
The Yes | No binding is a fast way to screen libraries. Different analytes are presented to the same ligand, allowing ranking of binding partners prior to further assessment.

Association—Concentration Analysis
This test is used to determine how much analyte is present and, most importantly, how much of it is correctly folded and able to bind the ligand. Standard absorbance-based measurement of protein concentration is a reflection of how much material is present, but does not indicate the amount of biologically-active molecules that can interact specifically with the ligand of interest. Octet® assays can be used to assess the amount of properly-folded protein by comparing the observed response to a known standard. This provides the correct concentration of active analyte being used and aids in accurate measurement of kinetics.

Association—Kinetic Rate Analysis
When all other parameters, such as the active concentration, are kept constant, it is simple to assess the association rate of the molecules by simply injecting the same concentration of each analyte and determining their kinetic association rate. Analyzing the response curve allows the user to determine whether the analyte is a tight binder with slow association kinetics or a lower-affinity binder with faster kinetics.

Dissociation—Dissociation Analysis
The dissociation phase allows users to rapidly perform off-rate ranking and get a first look at the analyte-ligand kinetics. As discussed in Section 3.1.4, dissociation is a concentration-independent event, so it is not necessary to know the protein concentration in advance. This means users can simply allow the analyte to complex with the ligand and then observe the dissociation. Fitting the data to an off-rate–only equation provides the first insight into affinity. As discussed in Section 3.1.4, high-affinity molecules dissociate slowly, while lower affinity molecules dissociate more rapidly, allowing a return to the baseline over the course of the assay.

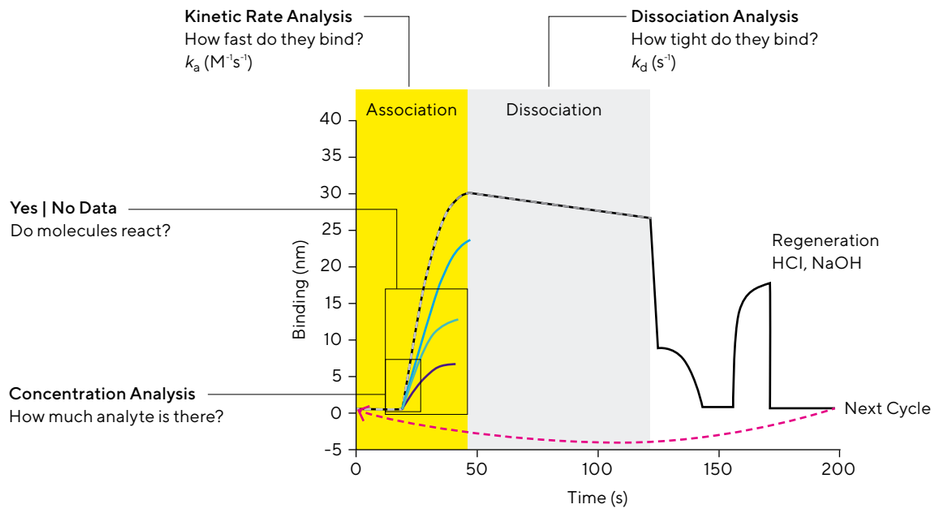


Figure 16
Advantages of Real-Time Assays Compared to End-Point Assays
Octet® SPR and BLI systems allow a wide range of assay formats to be performed, which allows key questions to be answered rapidly.

3.2 How Does BLI Work?

BLI is an optical, label-free, real-time technology that measures the changes in interference pattern between light waves.

The Sartorius 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 17).

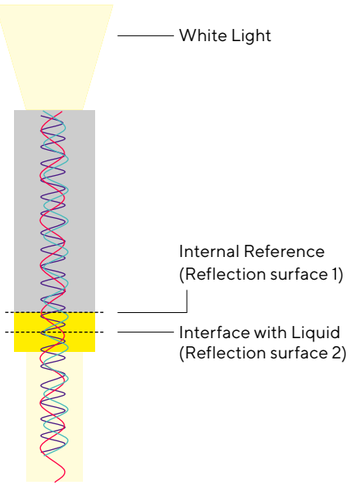


Figure 17
BLI Is an Optical Analytical Technique That Analyzes The Interference Pattern of White Light Reflected from Two Surfaces
Dip and Read biosensors contain two optical interfaces at the biosensor tip: the internal reference layer (optical layer) and the surface biocompatible matrix which interfaces with liquid and upon which ligand molecules are immobilized.

Incident white light that reflects from the two layers contains a mixture of wavelengths that show either constructive, partially constructive, or destructive interference. This relative intensity output depends on the thickness of the molecular biolayer at the biosensor tip and, therefore, BLI is sensitive to changes in the amount of analyte bound to an immobilized ligand (Figure 18). Initially, a baseline wave is observed when the biosensor is dipped into assay buffer.

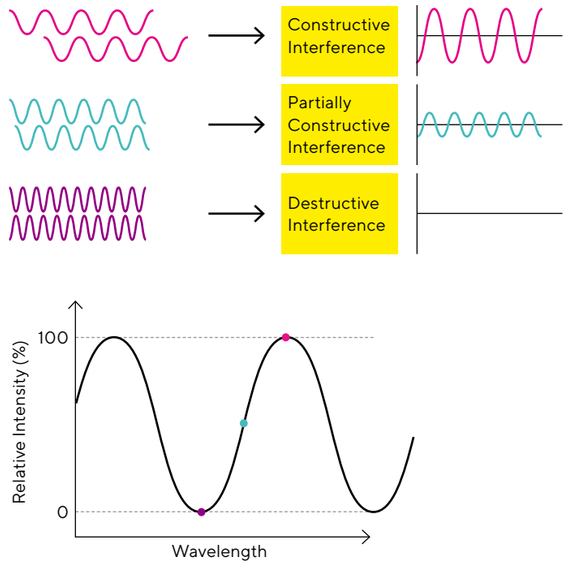


Figure 18
Interferometry: Measurement of Interference of Light Between Two Waves
Incident white light reflects from the two layers and contains a mixture of wavelengths that show either constructive, partially constructive, or destructive interference.

The spectral pattern of the reflected light changes as a function of the optical thickness of the molecular layer (i.e., the number of analyte molecules bound to the ligand at the biosensor surface). As the assay progresses and the ratio of free and bound ligand changes, the spectral shift is monitored at the detector and reported on a response curve as a change in wavelength (nm shift) (Figure 19).

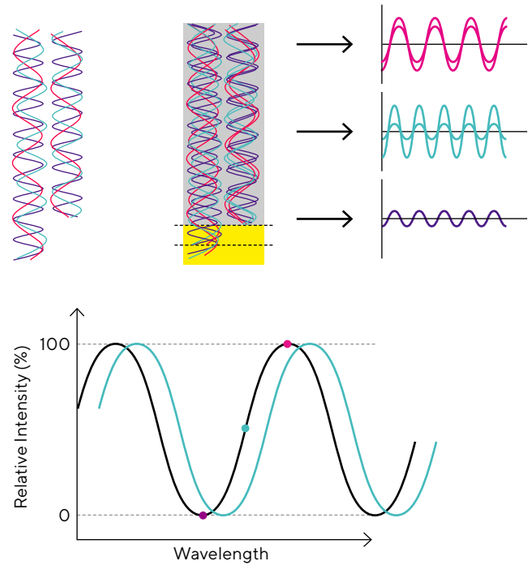


Figure 19
Interference Pattern Changes with Increased Molecular Thickness
The relative intensity output depends on the thickness of the molecular biolayer at the biosensor tip. Changes in the ratio of free and bound ligand produce a spectral shift that is reported on a response curve as a change in wavelength (nm shift).

Monitoring the interference pattern (i.e., spectral shift) in real time provides kinetics data on molecular interactions.

Dip and Read biosensors are fundamental 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 the lack of complex fluidic pathways for introducing 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 simple way to introduce an analyte to the sensor surface to monitor binding.

3.3 How Does SPR Work?

Like BLI systems, SPR is an optical, label-free, real-time technology used to measure molecular interactions. In simple terms, SPR occurs under conditions of total internal reflection (TIR), where incident light is reflected off a thin metal film. The presence of free electrons at the interface between the metal film and a second layer is required to generate surface plasmons. Gold is typically used in the metal layer due to the presence of abundant free electrons, while glass is the material most often used as the second interface. When TIR occurs at a specific angle, some of the energy from the incident light is lost. This occurs when the light interacts with delocalized electrons (plasmons) in the thin metal film, which causes a reduction in the reflected light intensity (Figure 20).

These surface plasmons can be thought of as electro-magnetic waves that move parallel to an interface, which is normally metal and glass. This localization of the wave to the boundary and external medium (e.g., assay buffer) makes SPR highly sensitive to any changes that occur within the field of the wave, such as a small molecule binding to a ligand.

In prism-coupled SPR instruments, the thin gold film is coated onto a glass chip that is in optical contact with the TIR prism. The gold side of the SPR chip is coated with an appropriate surface chemistry and is in fluid contact with a nanoliter-scale flow cell through which assay buffer flows.

Gold is the most commonly used metal in sensor chips due to a favorable combination of SPR characteristics combined with excellent chemical stability. The gold sensor chip surface has two important functions:

- It provides the physical conditions needed to produce the SPR signal.
- It provides a scaffold for the necessary sensor chip chemistry at the chip surface.

With the gold layer in place, light photons have the required energy and momentum to produce surface plasmons and an enhanced evanescent field.

The evanescent field that enables the energy transfer into the metal film exhibits exponential decay with distance from the gold interface and is therefore sensitive to a penetration depth of ~210 nm from the sensor surface. Therefore, the penetration depth of the evanescent field determines how thick the solution layer

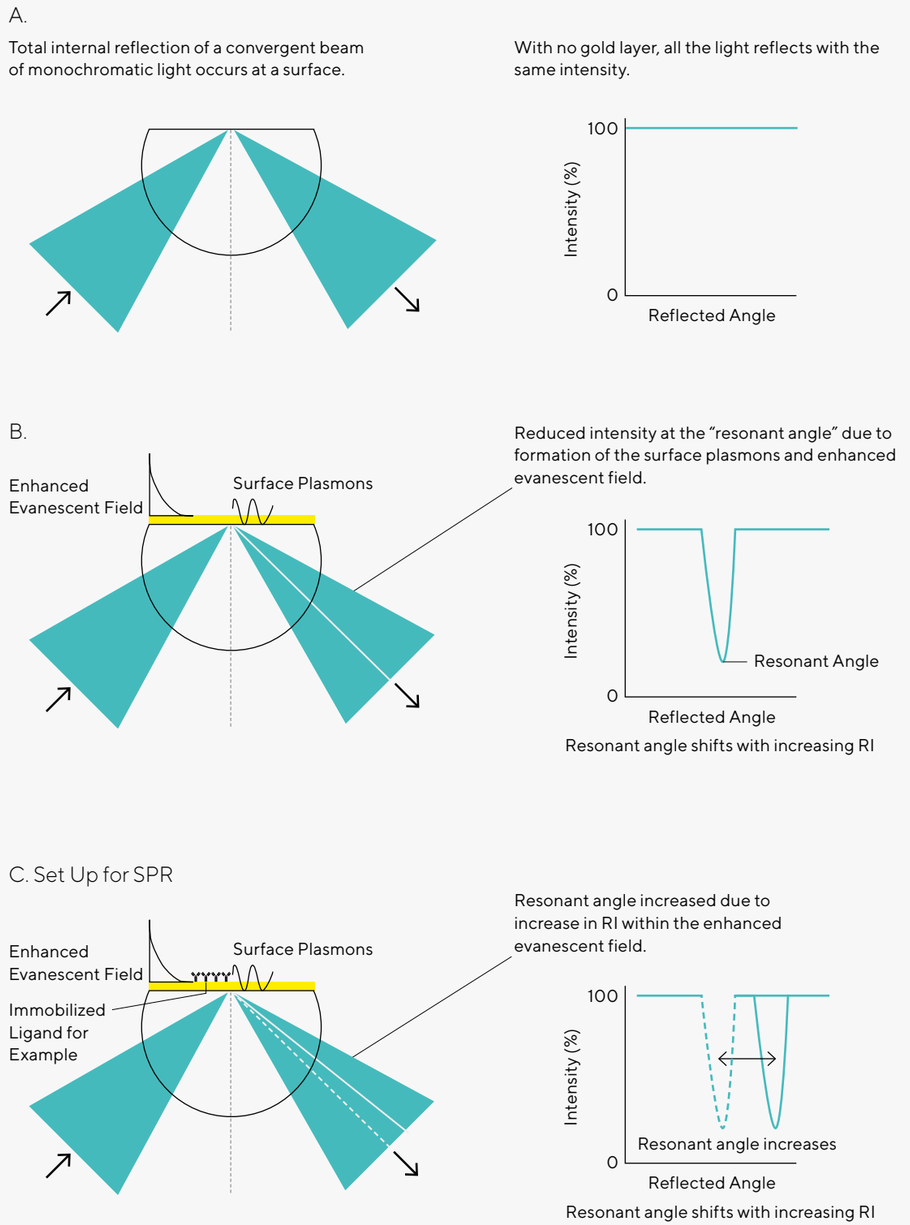


Figure 20
Fundamentals of the SPR Detection System
(A) With no gold layer present, all the incident light from a convergent beam of monochromatic source reflects with the same intensity. (B) Once a gold layer is in place, light photons at a specific incident angle have the correct energy and momentum to produce surface plasmons and the enhanced evanescent field. The energy used to create the surface plasmons is missing in the reflected light and, therefore, a dip in intensity is observed. (C) Upon change in the refractive index of the solution (e.g., ligand immobilization) within the enhanced evanescent field, a new resonance situation occurs. The "resonant angle" increases and a reduction in intensity is observed due to the increase in refractive index within the enhanced evanescent field.

can be since only the refractive index changes within that range are monitored (Figure 21).

Therefore, in an SPR assay, one of the participants in the interaction must be immobilized or captured close to the gold sensor surface (the ligand) within the penetration depth and the sample (analyte) is then injected across this surface.

The change in refractive index is directly proportional to the mass change that occurs as a result of binding and/or dissociation events and is measured in response units (RU). This allows SPR to be used to assess events based on molecular mass. As a general guideline, a 1 RU change equates to the accumulation of 1 pg mm^{-2} of protein at the sensor chip surface. In a typical SPR assay, upon immobilization and capture of the ligand, the analyte is supplied to the sensor chip surface using a flow-based fluidics system and the interactions can be monitored by tracking the SPR response.

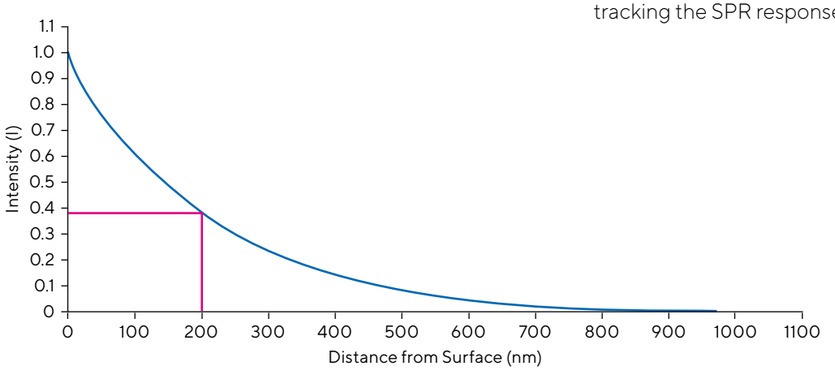


Figure 21
SPR Penetration Depth Decays Exponentially With Distance From the Sensor Surface
Penetration depth is commonly determined as 1/e or 37% for the exponential decay. The penetration depth for most commercial SPR systems is < 210 nm.

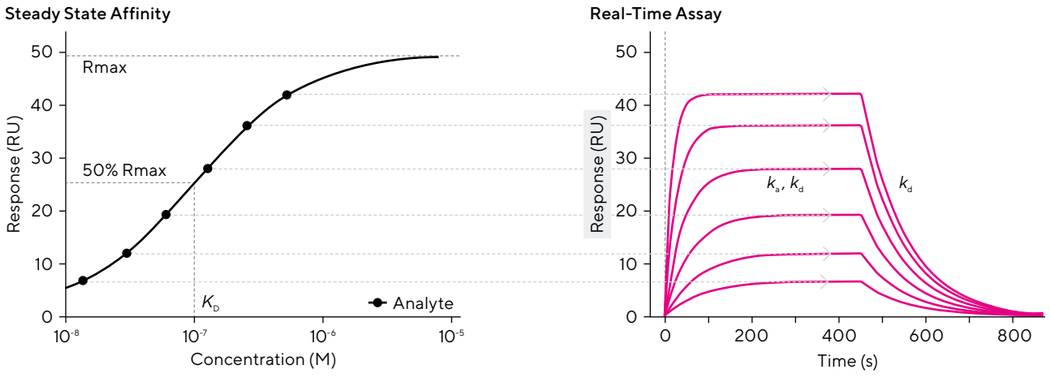


Figure 22
Real-Time Assays Provide Kinetic Information
End-point assays use equilibrium data points to produce affinity data while real-time assays (SPR, BLI) show binding progressing to reach equilibrium and also provide kinetic rate constants. An important caveat for equilibrium analysis is that all concentrations must reach equilibrium as shown in the binding response on the real-time assay response plots

3.4 Overview of Kinetics and Affinity

3.4.1 Multi-Cycle Kinetics

Multi-cycle kinetics is the most common type of kinetics experiment performed on SPR or BLI systems and is characterized by multiple injections of an analyte. Each injection shares a common association and dissociation time but has a discrete and fixed concentration of analyte. The concentrations of analyte used often require assay development such that the highest concentrations of analyte contain sufficient curvature to determine accurate binding kinetics when fitted globally. Additionally, the dissociation phase must exhibit a response decrease of > 5% so that accurate dissociation kinetics can be determined. At the end of the assay, these discrete association and dissociation injections are combined into a single response curve and fitted to a suitable kinetics model, ideally 1:1 (Section 5.2).

An alternative format to multi-cycle kinetics is single-cycle kinetics (SCK), where analyte concentrations are injected sequentially without regeneration between injections. A single dissociation phase is included after the final, highest, analyte injection. MCK and SCK share the common feature that a discrete analyte concentration is required for each injection, occupying significant space in the microplate, and reducing the number of samples that can be assessed in a single run.

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3.4.2 Steady State Affinity

While the association and dissociation rate can be extrapolated in a kinetic assay, in an equilibrium assay (often called steady state affinity) the interaction affinity may be weak and defined by rapid association and dissociation phases outside the detection range of current systems.

In general, these rapidly-forming and dissociating complexes reach equilibrium during the association phase, so it is still possible to determine the global affinity by fitting the response from each analyte concentration. The global affinity is represented by the analyte concentration required to saturate half the binding sites on the immobilized ligands.

An important caveat for equilibrium analysis is that all concentrations must reach equilibrium (Section 5.1.3), where the number of association events is the same as the dissociation events (Figure 22). At equilibrium, the response of the complex is directly proportional to that of the analyte concentration and only then can an accurate equilibrium dissociation constant be derived. Although it is not necessary to fully saturate the ligand binding sites, it is important that the resultant log plot of the analyte concentration series displays enough curvature for correct fitting. This is usually achieved by ensuring a saturation range between 10%–90%, such that when plotted with a linear analyte concentration, a plateau in the top responses is observed.

3.4.3 OneStep® Injections

OneStep® gradient injections are unique to the Octet® SF3 SPR system (Figure 23). Based on the Taylor dispersion theory, OneStep® injections diffuse a single concentration of analyte into a moving stream of buffer to create an analyte concentration gradient of at least three orders of magnitude, allowing an accurate measurement of molecular kinetics and affinity from a single analyte concentration. Unlike multi-cycle kinetics that requires several analyte concentrations to be prepared, OneStep® injections require only a single analyte concentration and can generate accurate kinetics and affinity using much less sample than is commonly required. This has the extraordinary advantage of increasing sample throughput and data content while decreasing sample preparation time and reducing human error by eliminating the preparation of multiple sample dilutions. Using a single sensor chip and fewer reagents also lowers assay costs significantly.

The Octet® SF3 and OneStep® injections remove the guess work from high-throughput screens and allow a full kinetic panel to be determined in a fraction of the time compared to standard techniques that require initial screening and then potentially extensive assay development. Full kinetic information early in drug discovery allows rapid progress of potential therapeutics through the development pipeline and forms a solid foundation for any additional assay development.

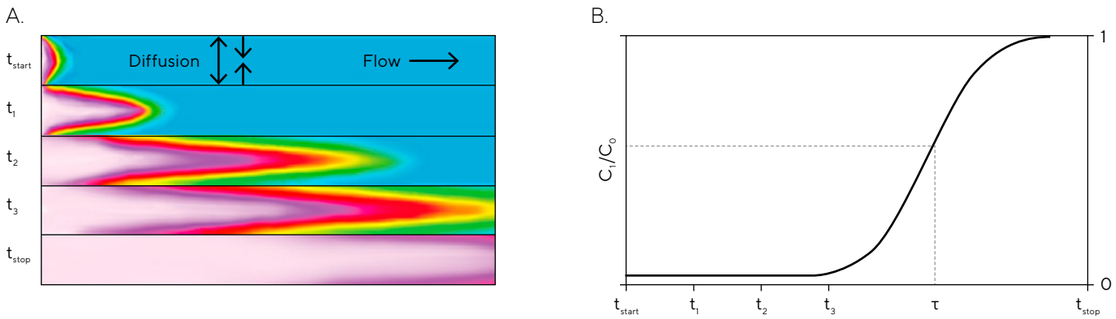


Figure 23

OneStep® Injections Eliminate the Need to Prepare a Dilution Series of Each Analyte

(A) OneStep® gradient formation in the injection line with (B) the corresponding analyte concentration measured within the flow cell. The blue color indicates the running buffer and the pink color indicates the analyte. The gradient formation and its relationship to analyte concentration at the flow cell is illustrated using five simulated snapshots (t start—t stop) of the injection line at different times and shows that a single injection can be used to assess a full analyte concentration series.

"...producing consistent, high quality kinetic binding profiles from biological samples."

4 Experimental Design

While setting up a kinetic assay using the Octet® platform is simple and straightforward, there are many practical considerations involved in producing consistent, high-quality kinetic binding profiles from biological samples. Proper assay technique and optimization, use of high quality, active reagents, and appropriate choice of biosensor are all key to obtaining accurate kinetic constants and affinities. Use of unstable or inactive proteins, improperly characterized reagents, or inappropriate buffer conditions will negatively impact results. Performing interaction analysis on an active and stable ligand surface facilitates generation of robust data.

Here, we describe important considerations for developing kinetic assays in various formats, including how to design an effective assay, optimize each step, and minimize artifacts due to non-specific binding.

4.1 What to Consider Before Starting Experimental Design

Before starting an assay, it is important to know as much information as possible about the molecules that are being investigated in the study. Below are some useful parameters to know about your molecules:

- Primary amino acid sequence
- Molecular weights of the ligand and analyte
- pI of the ligand and analyte
- Purity
- Activity
- Expected K_D
- Solubility

The basic information about the molecules is then considered during assay development following this general sequence (Figure 24):

- Assigning the ligand and analyte
- Biosensor and sensor chip selection and hydration
- Ligand optimal density scouting
- Assay buffer optimization
- Analyte binding optimization
- Assay robustness and specifications testing

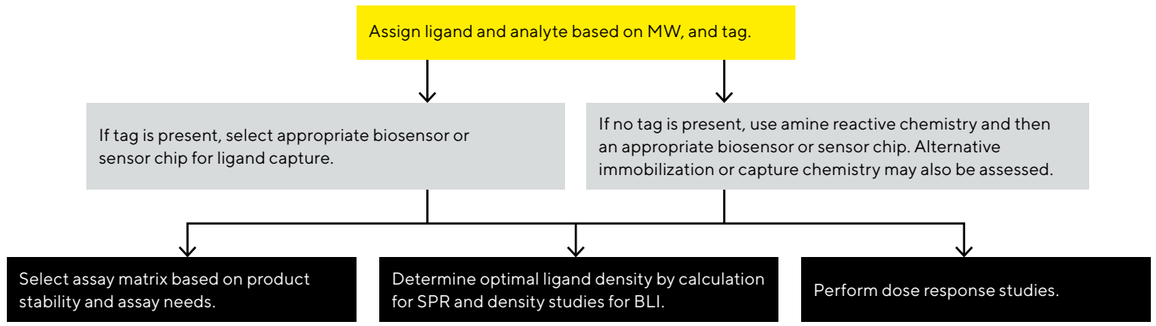


Figure 24
General Assay Development Sequence
Overview of suggested ligand binding assay sequence using the Octet® systems.

4.2 Assigning the Ligand and Analyte

The first step of assay design is determining which member of a binding pair to immobilize or capture (ligand) and which member to leave in solution (analyte). Protein stability, size, and valency are big factors in this decision. More sensitive proteins may not tolerate the relatively harsh conditions imposed by immobilization on amine reactive surfaces. In this case, the less sensitive molecule must be immobilized or a different biosensor surface chemistry can be considered (Section 4.3). Size is another factor as smaller molecules produce smaller signals upon binding. Therefore, the molecular size and the instrument sensitivity must also be considered when deciding which molecule to immobilize. When working with a small molecule, such as a peptide, one way to increase assay signal is to immobilize the peptide onto the sensor surface, leaving the larger binder in solution. With this strategy, adding a linker to the captured molecule before immobilization can prevent steric hindrance and make the binding site more available to its analyte partner.

Avidity is an important consideration in kinetics experiments as it can affect the overall calculation of K_D. While the affinity of a molecule to its binding partner is defined as the strength of the non-covalent association between one ligand binding site with one analyte binding site, the avidity of a molecule is determined by the total strength of all the binding associations possible between the two molecules. (See Application Note Optimizing Kinetics Assays to Avoid Avidity Effects.) Avidity effects can create non-ideal binding profiles and result in an artificially high affinity measurement. Regardless of orientation or assay format, proper assay development is a necessity for obtaining reliable kinetic data with any system. For example, an antibody is bivalent, and can potentially interact with two antigen molecules on the surface (Figure 25). Therefore, bivalent molecules such as antibodies should be immobilized on the surface of the biosensor whenever possible to avoid avidity effects.

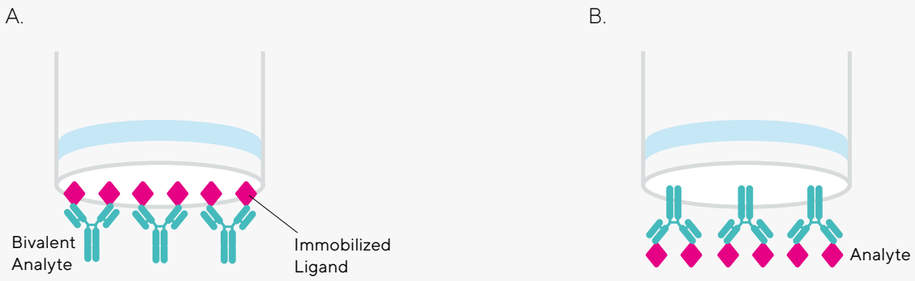


Figure 25

Avidity is an Important Consideration in Kinetics Experiments

(A) A bivalent analyte in solution that binds to immobilized ligand has the potential to bind to two ligand molecules at once. This can result in artificially low apparent off-rates. (B) These effects can be prevented by reversing the assay format or by lowering the ligand density on the biosensor.

4.3 Biosensor and Sensor Chip Selection

4.3.1 How to Choose a Suitable Attachment Approach for Your Ligand

Several attachment methods for the ligand are available and choosing the right one is a critical step in assay development. Binding partners that carry an intrinsic affinity tag, such as an HIS or GST tag, should be assigned as the ligand and used with an appropriate anti-tag biosensor. However, in cases of high affinity binding, some anti-tag biosensors may not be appropriate. In such cases, we recommend biotinylation of one of the partner molecules and use of a high-precision Streptavidin surface.

Changes in the refractive index of solutions are fundamental to SPR measurements, but less important in BLI measurements. In both cases, a matrix is required to provide a suitable environment to shield the protein reagents from the sensor surface (gold sensor surfaces used in SPR can bind and denature proteins). The matrix also provides a suitable chemical environment for immobilization of ligands and subsequent molecular interactions to be observed (Figure 26).

In general, the unmodified sensor surface for BLI and SPR is stable in extreme pH and temperature ranges, and also in relatively high concentrations of organic solvents. However, after immobilization or capture of a ligand, the stability of the active surface depends on the properties of the ligand.

Choosing the correct sensor surface chemistry is a critical step in assay design and a number of key factors should be considered. Although each assay will likely require some assay development, choosing the most appropriate surface can rapidly accelerate this development.

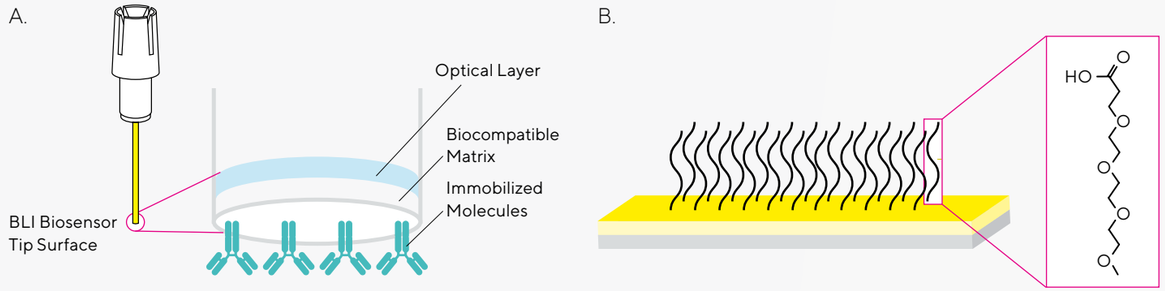


Figure 26

Octet® BLI and SPR Utilize Different Biosensor Technologies

A Dip and Read biosensor (A) and SPR Sensor Chip (B), illustrating the two optical interfaces and the surface biocompatible matrix on which ligand molecules are immobilized for BLI and SPR, respectively.

4.3.2 Covalent Immobilization

Covalent immobilization to sensors is one of the most frequently used approaches in SPR and is the method of choice for attaching affinity capture molecules, where pre-made sensors are not available. In general, the level of ligand immobilized to the sensor surface during covalent immobilization using amine coupling is affected by five main parameters:

- Chip capacity—planar sensor chips have a lower capacity than their hydrogel counterparts and therefore, it is important to choose the correct sensor chip capacity for SPR assays. In general, low-capacity sensor chips are preferable for protein–protein kinetic measurements, but higher capacity carboxymethyl dextran sensor chips may be needed for small molecule assays to ensure a large enough signal change.
- Activation time (EDC/NHS mixture)—EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride)/NHS (N-hydroxysuccinimide) activation of carboxylic acid groups creates a reactive species that reacts with primary amines. For a high immobilization level, a higher activation time with EDC/NHS can be used, whereas for a low immobilization level, a shorter activation time may be preferable. It is also possible to vary the ratios of EDC/NHS to create different reactive surfaces for amine coupling.
- pH of injected ligand mixture—preconcentration of the ligand to the sensor surface is a critical step in ensuring efficient immobilizations and must be optimized prior to immobilization (Section 4.4.1).
- Ligand concentration—higher concentrations of starting ligand correlate linearly to the observed immobilization level. This is caused by more ligand molecules preconcentrated to the sensor surface being available to react with the reactive succinimide esters.
- Ligand injection/contact time—as with EDC/NHS, the longer the injection time, the larger the potential for the ligand to react with the activated surface and create a stable covalent bond. Different ligand density surfaces can be created from the same solution by varying the contact time.

The Octet® SPR sensor chip and BLI biosensor Amine Reactive 2G (AR2G) are the basis for direct immobilization of ligands using amine coupling, where the carboxylic groups on the sensor surface are activated through standard EDC/NHS-catalyzed amide bond formation, which generates reactive succinimide esters that can then create a covalent bond between a reactive amine on the ligand and the carboxy-terminated sensor surface (Table 2). The immobilization is done in a series of steps (Figure 27):

1. Carboxylic acid groups on the biosensor surface are first activated by reaction with EDC and either NHS or sulfo-NHS (N-hydroxysulfosuccinimide) to generate highly reactive NHS esters.
2. The esters rapidly react with the primary amines of the ligand biomolecule in the coupling step to form highly stable amide bonds.
3. Excess NHS or sulfo-NHS esters are quenched using ethanolamine.

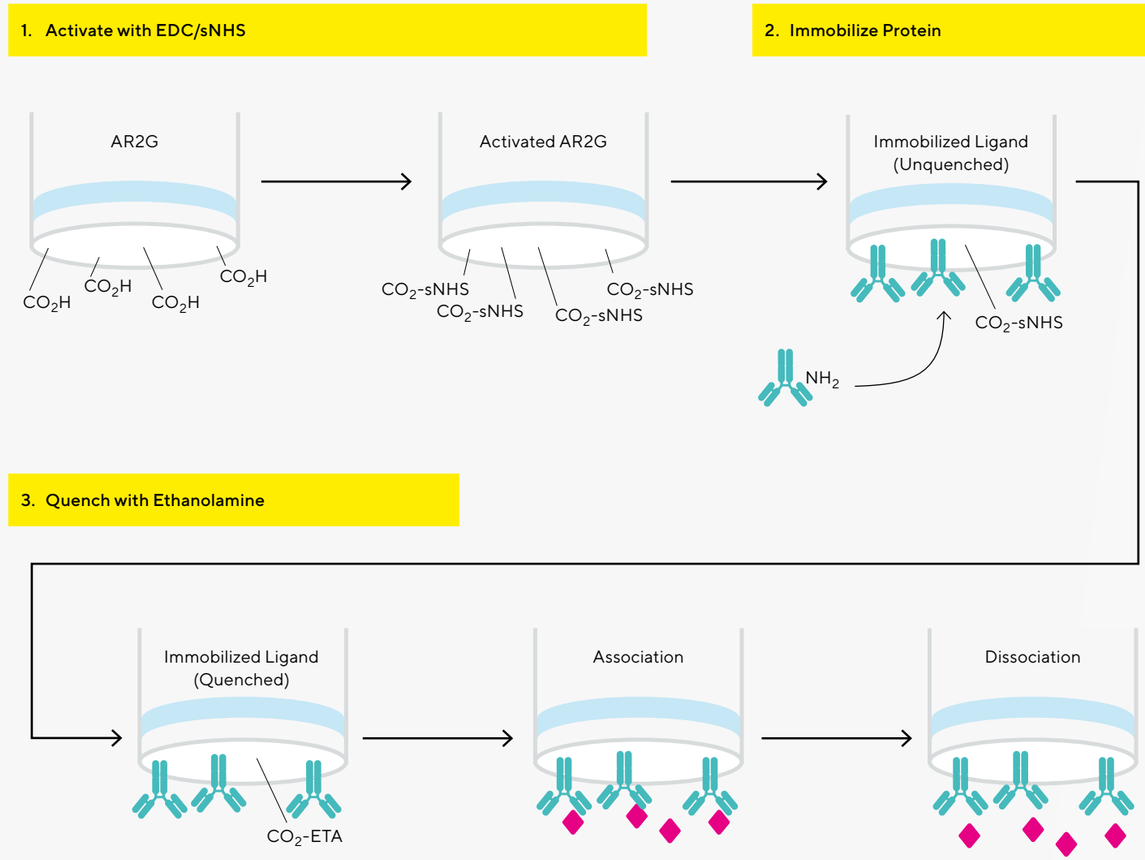


Figure 27
Workflow for a Kinetic Assay Using an Amine Reactive Surface (AR2G Shown Here)
Ligand molecules are covalently attached to the activated amine-reactive surface. After activation, immobilization and quenching, the kinetics of association and dissociation between the immobilized ligand and analyte are measured.

For covalent immobilization of the ligand to be successful:

- The ligand must retain its activity after immobilization on the surface.
- If necessary, the protein must be robust enough to undergo surface regeneration to remove bound analyte at the end of the analysis cycle (Section 4.8.4).

The benefits of amine coupling include its stability and the fact that the protein does not have to be modified. In addition, the reaction requires a small amount of ligand and generates reproducible immobilization levels in subsequent assays.

Many proteins have accessible primary amines on their surface, which means that numerous orientations of the protein can be covalently attached to the sensor chip surface. Therefore, the main drawback associated with amine coupling is the creation of a heterogenous sensor chip surface. As shown in Figure 28, this can cause obstruction or blocking of binding sites. Additionally, the required low pH solutions that drive the protein electrostatically towards the chip surface during amine coupling can cause protein degradation. Another drawback associated with amine coupling is the need for high purity ligands. Amine-bearing impurities can co-immobilize to the sensor chip surface and reduce the binding capacity.

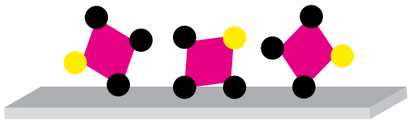


Figure 28
Amine Coupling Can Create Heterogenous Surfaces
Primary amines (black dots) on the ligand can be covalently attached to carboxymethyl groups present on the sensor chip surface in multiple orientations, which can hinder or block the binding site (yellow dot).

If chemical modification strategies affect the analyte-binding activity of the ligand or cannot be used, an affinity capture is an alternative solution (Section 4.3.3).

Octet® System	Product Name	Surface Chemistry	Applications
BLI	AR2G	Second generation amine reactive	Amine coupling Protein binding assays
SPR	COOH1	PEG	Amine coupling Protein binding assays
SPR	CDL	Carboxymethylated dextran	Amine coupling Protein binding assays
SPR	CDH	Carboxymethylated dextran	Amine coupling Protein binding assays
SPR	PCH	Linear polcarboxylate	Amine coupling Protein binding assays

Table 2
Covalent Immobilization
A wide range of biosensors and sensor chips are suitable for amine-coupling reactions on Octet® systems.

4.3.3 Affinity Capture Approaches

The most common solution to amine coupling problems is to introduce an affinity tag into the ligand. This strategy orients all molecules in the same way on the surface, which helps prevent differences in binding site presentation. In addition, unlike amine coupling, which requires high-purity reagents, affinity tags allow lower-purity preparations to be used. During affinity capture, only those molecules bearing the affinity tag will be captured, while impurities remain unbound.

In affinity capture methods, a molecule that is covalently immobilized on the surface uses an affinity interaction to capture the ligand (Figure 29).

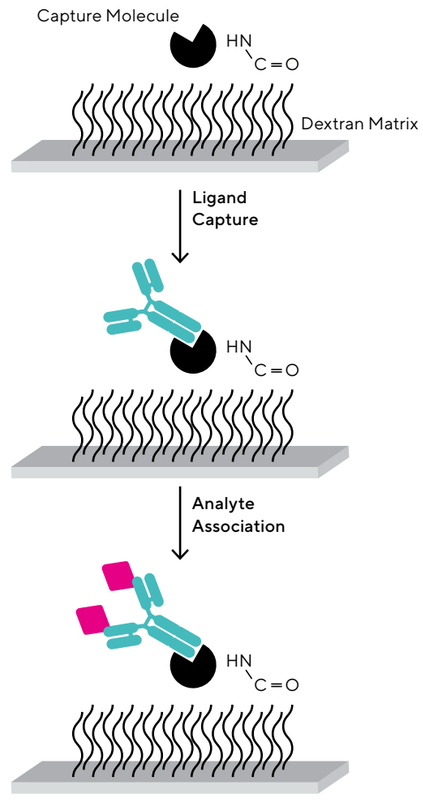


Figure 29
Affinity Capture Illustration
During affinity capture, only those molecules bearing the affinity tag will be captured.

In order for capture-based assays to be accurate, the dissociation rate between the capture molecule and the ligand must be slow enough so that only insignificant quantities of ligand dissociate from the surface during an analysis cycle; otherwise, baseline drift can occur and prevent accurate measurement.

The affinity capture approach has several advantages over covalent immobilization, including:

- The ligand is usually not modified except for any conformational changes introduced by the interaction between the ligand and capturing molecule.
- Generally, the ligand is attached to the surface in a specific, homogeneous orientation, which is determined by where it is bound by the capturing molecule (an exception to this is if a polyclonal antibody is used as capture molecule).
- The affinity capture approach allows the capture of specific ligands from crude samples, such as cell culture media, as the capture molecule/ligand interaction acts to purify the ligand from the sample matrix.
- A major advantage of capture-based assays is that regeneration of the surface is feasible and ensures that fresh ligand can be used for each new assessment during an assay. Regeneration of the capture surface involves removal of the ligand (with bound analyte) from the capturing molecule and capture of fresh ligand in preparation for the next analysis cycle.

Disadvantages of the affinity capture approach are the increased consumption of ligand as fresh ligand is required for each cycle and the potential complications introduced in kinetic analysis by dissociation of ligand during an interaction cycle.

The most frequently used variations of the affinity capture approach are antibody-based capture, capture of tagged proteins, and capture based on protein A, protein G, or protein A/G (Table 3). The affinity capture paradigm may be used with any ligand that is bound by a secondary molecule at a site independent of the analyte binding site.

Binding partners with an intrinsic affinity tag should be assigned as the ligand. In this case, an anti-tag biosensor should be selected for use. However, in cases of high affinity binding, some anti-tag biosensors may not be appropriate. In such cases, it is recommended that one of the partner molecules be biotinylated and a Streptavidin sensor be used for the studies (Section 4.3.4).

Octet® System	Product Name	Surface Chemistry	Applications
BLI	AHQ	Anti-Human IgG Fc	hIgG quantitation
BLI	AMQ	Anti-Murine IgG Fv	mIgG quantitation
BLI	ProA	Protein A	IgG quantitation (various species)
BLI	ProG	Protein G	IgG quantitation (various species)
BLI	ProL	Protein L	IgG quantitation (various species)
BLI	AHC	Anti-hIgG Fc Capture Surface	hIgG-Ag binding assays/screening
BLI	AHC2	Anti-hIgG Fc Capture Surface	For quantitation and kinetic analysis of human IgGs and IgG-derived Fc-fusion proteins
BLI	AMC	Anti-Mouse Fc Capture	mIgG-Ag binding assays/screening
BLI	FAB2G	Anti-Human Fab-CH1	Fab-Ag binding assay, Fc-R binding assay
BLI	HIS2	Second Generation Anti-HIS	His-tagged protein quantitation
BLI	HIS1K	Anti-Penta-HIS	His-tagged protein quantitation and kinetics
BLI	NTA	NI-NTA	His-tagged protein quantitation and kinetics
BLI	GST	Anti-GST	GST-tagged protein quantitation and kinetics
BLI	APS	Aminopropylsilane	Protein-protein binding assay
SPR	HisCap	NI-NTA	His-tagged protein quantitation and kinetics

Table 3
Affinity Capture
A wide range of biosensors and sensor chips are suitable for affinity capture approaches on Octet® systems.

4.3.4 Streptavidin-Based Capture

Octet® offers numerous surface chemistries that utilize the extremely high affinity of streptavidin for biotin (K_D ~10^{-14} M) to create a stable surface for molecular analysis (Table 4). This affinity is high for a noncovalent binding interaction, so a negligible dissociation of biotinylated ligand occurs during an interaction cycle. The main disadvantage of using streptavidin-based capture is the difficulty in regenerating the surface, because of the very low equilibrium dissociation constant.

The introduction of biotin into a range of molecules (nucleic acids, lipids, proteins, and carbohydrates) is easy; with many convenient and easy-to-use kits available.

To immobilize a ligand onto a streptavidin 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 following recommendations should be followed. 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 available, and are necessary to reduce steric hindrances and for efficient capture of biotinylated molecules. (See Application Note Biomolecular Binding Kinetics Assays on the Octet® Platform and Technical Note Biotinylation of Protein for Immobilization onto Streptavidin Biosensors for further information on biotinylation of protein ligands.) Affinity capture of biotinylated ligands onto the immobilized streptavidin surface is then easy and reliable.

It is important to consider that like direct immobilization, using amine coupling (Section 4.3.2) chemically biotinylated proteins can be captured in a similar "random" orientation if they contain multiple primary amines. However, even if the protein contains only one primary amine suitable for labeling, there is always the possibility that capture via the biotin may occlude the binding site.

Assay development is therefore required to generate an appropriate regeneration strategy that removes the analyte, but leaves the (active) biotinylated ligand on the surface.

Octet® System	Product Name	Surface Chemistry	Applications
BLI	SA	Streptavidin	Protein | DNA | peptide-protein binding assays
BLI	SAX	High Precision Streptavidin	Protein-protein binding assays, QC activity assays
BLI	SAX 2	High Precision Streptavidin 2.0	Protein-protein binding assays, QC activity assays
BLI	SSA	Super Streptavidin	Small molecule binding assays | screening
SPR	SADH	Streptavidin	Protein-protein binding assays, QC activity assays

Table 4
Streptavidin-Based Capture
A wide range of biosensors and sensor chips are suitable for streptavidin-based capture approaches on Octet® systems.

"Octet® stands for biomolecular interactions analysis in a versatile, label-free, and easy-to-use format."

4.4 Ligand Optimal Density Scouting

4.4.1 pH Scouting

For assays that use amine coupling, preconcentration of the ligand to the biosensor surface is critical for efficient immobilization and optimal signal-to-noise ratio. Prior to knowing how much ligand needs to be immobilized, the protein must be preconcentrated at the sensor surface for the reaction to occur. In preconcentration, the positively-charged ligand is concentrated at the negatively-charged surface by electrostatic attraction prior to covalent coupling. For these electrostatic interactions to occur, the pH of the coupling buffer must be higher than the pKa of the biosensor surface (~4.0 for carboxymethyl dextran surfaces), but slightly lower than the isoelectric point of the ligand. The ligand buffer should have low ionic strength as the high concentration of ions in high ionic strength buffer will tend to shield the ligand and surface from one another. For many proteins, diluting 10 mM acetate buffer at pH 4.0–6.0 works well, however an optimization step, or pH scouting, is recommended to identify the optimal buffer in which to perform the immobilization.

Determine the optimum pH and ionic strength for immobilization by observing the level of charge-mediated concentration of ligand onto the sensor surface in varying buffer conditions. Initially, choose three to four buffers differing by 0.5 or 1.0 pH unit; for example, 10 mM sodium acetate at pH 4.0, 4.5, and 5.0. Ideally, the proteins attracted to the sensor will dissociate freely after the injection ends and return to the assay buffer. Some proteins will precipitate onto the sensor and require regeneration injections. After the injection, use a high ionic strength neutral pH buffer to wash the non-covalently bound ligand from the surface. This is in preparation for testing with the next sample. As many proteins precipitate at low pH, causing irregularities in the response curve, test higher pH buffers first. The ideal charge preconcentration allows proteins to rapidly attract to the sensor surface and immediately dissociate upon return to assay buffer condition. When selecting buffer conditions, select a slower or lower preconcentration signal over a higher signal, which leaves more protein adhered to the sensor post-injection.

Some ligands cannot be efficiently drawn to the sensor chip surface by charge-mediated concentration. They may be too electronegative, even at an acidic pH. Ligand modification may reduce the acidity of the ligand if, for example, the acidic carboxyl groups are replaced by less acidic or non-acidic reactive groups. Affinity capture approaches that do not depend on charge-mediated concentration may provide a solution for negatively-charged ligands. Physiological salt buffers reduce the effects of electrostatic repulsion of acidic ligands and may be helpful in affinity capture approaches.

Note: If a satisfactory charge-mediated concentration does not occur at any combination of pH and ionic strength, and increasing the ligand concentration does not help, a different coupling procedure may be used.

4.4.2 How Much to Immobilize or Capture?

For both SPR and BLI assays, the observable response is proportional to the amount of binding at the sensor surface. The best rule of thumb is that sufficient ligand must be immobilized or captured to see a measurable response that is above the noise of the system at the lowest analyte concentration. Simply loading as much ligand as possible to maximize signal is not recommended.

An excess of ligand bound to the sensor can lead to data artifacts due to crowding, steric hindrance, and possible aggregation on the surface. Over-saturation of the sensor may also promote weaker, non-specific interactions at higher analyte concentrations, or analyte "walking" or "rebinding" effects at lower analyte concentrations. These artifacts may significantly impact observed binding kinetics. Without enough immobilized ligand, however, the signal in the analyte association step may be too low to detect.

When performing the loading step in a kinetic assay, slow loading for longer time is preferable to rapid ligand immobilization. Ideally, the binding curve in the loading step will show a gradual increase in signal and should not be allowed to reach saturation. A steep, initial increase in signal may lead to uneven loading and assay artifacts.

For SPR assays, the analyte binding capacity of a given surface is related to the amount of ligand immobilized and the relative molecular weights of the analyte and ligand. Assuming a 1:1 binding stoichiometry, the analyte binding capacity of a surface can be expressed as:

$$R_{max}\ (RU) = \frac{MW_{analyte}}{MW_{ligand}} \times R_{ligand} \times Stoichiometry_{ligand}$$

Equation 1: *Equation to Determine the Theoretical R_{max} in an SPR Assay*

The observable response in SPR is directly proportional to the masses of the analyte and ligand in addition to the amount of ligand present and how many analyte molecules the ligand can bind.

For example, if the ligand and analyte molecular weights are 150,000 Daltons and 30,000 Daltons, respectively, immobilizing 500 RU of ligand will result in a theoretical analyte binding capacity of 100 RU, if the ligand is 100% active.

R_{max} *= (30,000 Da * 500 RU)/150,000 Da = 100 RU*

The theoretical binding capacity can be achieved only when the analyte concentration and contact time reach infinity.

In actual experiments, the effective binding capacity, as represented by the maximum SPR response observed, is affected by:

- The activity of the ligand (< 100%)
- Available analyte concentration
- Analyte binding kinetics
- Real-world limitations on the maximum contact time

The theoretical binding capacity equation is a useful guide to the percentage activity of the immobilized ligand. It can be rearranged to provide a guide of the concentration of ligand (RU) that can be immobilized or captured to achieve a certain R_{max} value.

$$R_{ligand}\ (RU) = \frac{MW_{ligand}}{MW_{analyte}} \times \frac{R_{max}}{Stoichiometry_{ligand}}$$

Equation 2: *Equation to Determine the Theoretical R_{ligand} Required in an SPR Assay*

Rearrangement of Equation 1 allows SPR users to calculate the theoretical amount of ligand required for immobilization or capture to achieve a desired observed response (R_{max}).

Using the values from the examples above we can see that to achieve a predicted R_{max} of 100 RU:

R_{ligand} *= (150,000/30,000) * 100 = 500 RU*

The aim should be an immobilization or capture level of 500 RU. The surface is then amenable for empirical testing using the analyte concentration to be used in the final assay. If the K_D and thus the analyte concentration are not known, it is recommended that OneStep® injections are used with a high analyte concentration and a further 1:10 dilution of analyte into assay buffer to determine the apparent K_D, before optimizing the analyte concentration.

Unlike SPR, determining the ideal concentration of ligand to immobilize or capture for a BLI experiment requires experimentation with both the ligand and the analyte. For BLI assays, ligand load density must be optimized as Equations 1 and 2 are not applicable to BLI assays and the observed analyte response is coupled to the ligand density on the biosensor.

The microplate format used on the Octet® BLI platform allows for rapid testing of several experimental parameters at once, minimizing time spent on optimizing ligand density during assay development.

To perform a loading optimization experiment, several concentrations of ligand are loaded onto the biosensor. A typical immobilization concentration for a ligand molecule, such as an IgG1 molecule (150 kDa), is ~50–300 nM, based on a five-minute loading time. If the ligand concentration is low (e.g., < 50 nM), a longer loading time may be required for sufficient immobilization signal. Overnight incubation in ligand solution may also be performed at 4 °C. Overnight incubation can greatly improve results in cases where capture biosensors are being used to capture a ligand molecule from a dilute supernatant or cell culture sample.

An association step is subsequently performed for each ligand concentration using a high concentration of analyte (10–20X K_D). If the K_D is unknown, a high analyte concentration may be used. A zero-ligand biosensor should also be run as a control for determining whether the analyte binds non-specifically to the biosensor. The recommended sequence for the optimization assay is:

1. Baseline
2. Loading
3. Baseline 2
4. Association
5. Dissociation

The Baseline 2 step time should be optimized to establish a step time with minimal drift (i.e., extend assay step time) to achieve minimal drift prior to the biosensor dipping into analyte for association time (Figure 30A). Assay step times should ultimately be developed and will depend on the expected K_D of the binding partners.

The loading concentration to select for an assay should be the lowest concentration of immobilized ligand that yields an acceptable signal in the analyte association step. Figure 30A shows the raw data trace for a ligand-loading optimization experiment. In the loading step, higher concentrations of ligand quickly saturate the binding sites, as evidenced by the binding curve leveling off in the 100 µg/mL sample, while the lowest concentrations do not reach saturation. When the data are processed so that the analyte association step is aligned to the baseline (Figure 30B), the relative signal of the analyte binding at each corresponding ligand concentration can be clearly observed. At 10 µg/mL (67 nM), the desired loading curve characteristics (significant loading signal with slow initial binding that does not reach saturation) and a high signal in the association step are observed. This concentration would then be selected as the optimal loading concentration for this example.

The amount of ligand that is required is dependent on the assay format and appropriate levels are suggested below.

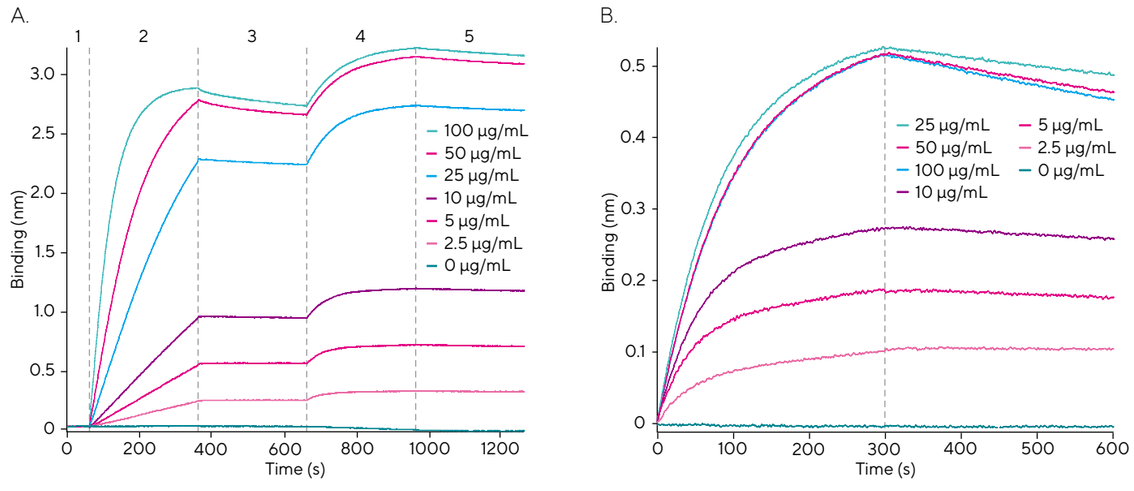


Figure 30

Raw Response Curve Data for Ligand Loading Optimization Experiment on the Octet® BLI Instrument.

(A) After an initial baseline step (1) biotinylated anti-PSA antibody was loaded onto streptavidin biosensors at several concentrations in 1X Kinetics Buffer (2). After a baseline 2 step (3), PSA antigen was associated to the immobilized ligand at a concentration of 200 nM (4), followed by the dissociation step in 1X Kinetics Buffer (5). (B) Aligned sensorgram traces showing association and dissociation steps. The difference in analyte signal at each ligand concentration can be easily observed when data is aligned.

4.4.3 Kinetic Assays

In kinetic assays, the goal is to minimize the effects of mass transport limitation (MTL) of analyte to the surface.

Immobilization of small quantities of ligand minimizes the effects of MTL and improves measurement of kinetics-related observations. Due to the mass-dependence of SPR, higher concentrations of ligand should be immobilized when the analyte has low molecular weight. The primary consideration in experimental planning should be the anticipated analyte binding levels, not the exact quantity of ligand immobilized. Use Equation 2 to calculate the theoretical amount of ligand required.

As a general rule, an R_{max} of < 50 RU avoids most MTL issues and generates ideal kinetic data for assays performed using SPR. For BLI, we recommend a ligand load density between 0.5–1.5 nm shift (y-axis). This is approximately equivalent to an immobilization density that would produce an R_{max} of about 50–100 RU in an SPR assay.

For BLI assays, agitating the sample plate in a fluidic-free format creates a turbulent flow over the biosensor, which is not subject to laminar forces and is highly efficient at replacing the volume close to the surface of the biosensor. If mass transport effects are an issue, the supply of analyte to the surface must effectively be raised. This can be accomplished by reducing the level of immobilized ligand or increasing the shaking speed during the assay to increase flow rate.

4.4.4 Concentration Assays

For concentration assays, the analyte binding response at a given sample concentration is directly related to the level of immobilized ligand. A high immobilization level allows measurements at lower analyte concentrations.

Also, high levels of immobilized ligand result in rapid analyte binding, which promotes mass transport-limited binding and reduces the impact of the analyte–ligand affinity on the concentration measurement.

When the ligand is large and the analyte is very small, the observed responses is low even at high analyte concentration. The sensitivity of a direct concentration assay may be limited even at very high ligand immobilization levels. In this case, it is best to redesign the concentration assay as an inhibition assay.

If the ligand is small and the analyte is large, it is necessary to moderate the amount of ligand immobilized in order to avoid steric crowding effects at high analyte concentrations.

4.5 Sensor Conditioning

4.5.1 The Importance of Sensor Hydration and Achieving a Stable Baseline

Sensor hydration and conditioning are critical to creating a stable baseline and minimizing baseline drift caused by expansion of the sensor matrix during the assay. A baseline step must be performed prior to analyte association to remove unbound ligand from the biosensor and to assess drift caused by non-specific binding, aggregation, or buffer effects. The baseline must be run in the buffer (or media) that will be used for the association and dissociation steps. In many cases this solution differs from what is used in the ligand immobilization step. Switching to a different buffer solution can create matrix effects due to non-specific binding of new buffer components, changes in refractive index, or drift from dissociation of ligand molecules from the surface (especially for capture biosensors) that must be allowed to equilibrate. It is important to establish a stable baseline with minimal signal drift before proceeding to the association step. An unstable baseline will impact measurement of the dissociation phase, especially if the baseline drift is high relative to the dissociation rate or is drifting in a non-linear fashion. Baseline drift also impacts maximum binding capacity, or R_{max}, which is a factor in calculating rate constants and K_D in both kinetic and equilibrium analyses, so it is important that R_{max} remain constant throughout the assay.

Octet® SPR sensor chips and BLI sensors are supplied in a sealed packet containing an inert gas and should be stored according to the package instructions upon delivery. Prior to installation, allow the sensors to equilibrate to room temperature without opening the packet (this requires ~10 min). Once temperature-equilibrated, the sensor chip or sensor is ready for hydration. In general, all biosensors apart from planar SPR sensor chips contain a matrix that expands upon exposure to fluids. This causes a change in the apparent density of ligand in the vicinity of the sensor surface and can lead to data accuracy issues due to assay drift. As both SPR sensor chips and BLI sensors are supplied dry, they must be hydrated prior to use.

4.5.2 BLI Biosensor Hydration

Biosensor hydration is typically recommended for a minimum of 10 minutes, but the actual biosensor hydration time will depend on the instrument equilibration setting used prior to the start of the experiment.

Optimal hydration results in minimal drift in the baseline step of the assay. To optimize hydration, select three different hydration times. Use at least two biosensors per condition to allow for replicates and stagger the hydration to enable simultaneous evaluation on the instrument.

Hydration is typically set at 10 minutes, but can be evaluated by pipetting 200 µL of 1X Kinetics Buffer into the biosensor hydration plate and an appropriate volume into corresponding wells of the sample plate. Perform a baseline step assay (recommended times are 5, 10, and 20 minutes, but can be longer). Based on the data, select the hydration time and baseline step time conditions that are associated with minimal baseline drift. Use these parameters as the optimal hydration time for your remaining studies.

Note: Sartorius recommends the use of 1X Kinetics Buffer in BLI hydration plates, though binding molecule stability and activity should dictate the selection of assay buffer.

Note: Use fresh hydration buffer for every biosensor.

Note: Once biosensors are wet, they should not be allowed to dry to preserve their activity. They may be stored at 4 °C, with the tips of the biosensors submerged in hydration buffer for up to 24 hours. The total storage time depends on the stability of the proteins on biosensors, which should be validated beforehand.

4.5.3 SPR Sensor Chip Hydration

Unlike BLI biosensor hydration, SPR sensor chip hydration is performed *in situ* after sensor chip docking. Prior to sensor chip hydration, it is important to normalize the sensor chip as individual sensor chips contain slight differences in their surface properties. Therefore, it is important to adjust the system's detector to compensate for these differences. The Octet® SF3 system allows rapid normalization of sensor chips using > 99.5% DMSO and the Normalize function on the system.

Sensor chip hydration is typically recommended for a minimum of three priming cycles, which takes approximately 10 minutes. As with BLI biosensors, hydration should be optimized to produce minimal drift in the baseline step of the assay. To optimize hydration, conditioning of the sensor chip should also be performed using the fast injection command in manual mode. In general, for carboxymethyl dextran-based sensor chips, three to five injections of 1 M NaCl 50 mM NaOH for 15 seconds at 100 µL/min should be assessed and repeated until no baseline drift occurs.

Following conditioning, it is best practice to perform a single prime with the assay buffer to help ensure a stable baseline prior to performing further surface preparation.

Note: Sartorius recommends the use of 1X HBS-EP+ or PBS-T for SPR sensor chip hydration, though binding molecule stability and activity should dictate the selection of assay buffer.

4.6 Assay Buffer Optimization

Assay buffer/matrix can have a large effect on assay performance and therefore, it is advisable to perform a mini design of experiment (DOE) to scout for optimal assay buffer.

It is important to keep in mind that both the analyte and ligand must be properly folded, active, and stable in the chosen assay buffer and, ideally, the assay should be run using analyte sample matrix. However, is some cases sample dilution may be necessary if the sample matrix causes interference with the assay. This is especially prevalent in SPR assays, where the analyte storage buffer elicits a large refractive index change and can result in a large bulk response that is not consistent between samples in a concentration series. This effect is especially prevalent in small molecules that are solubilized in DMSO and require further dilution to achieve concentrations suitable for assessment in SPR assays. These molecules include sucrose, histidine, and trehalose and should be avoided where possible by using carrier-free proteins. Note that analyte instability due to suboptimal buffer conditions can result in non-specific binding (NSB), hence another important reason for buffer optimization is to minimize NSB.

The fluidic-free format of the Octet® BLI system allows for greater flexibility in selecting an assay buffer compared to SPR. Because biosensors are based on BLI technology, only molecules binding to or dissociating from the surface of the biosensor cause a shift in the interference pattern of the reflected light and generate a response. In BLI, unbound molecules or changes in refractive index of the surrounding solution do not affect the interference pattern, enabling measurements in crude or complex samples, such as cell lysates or culture supernatants, and in solutions containing high refractive index components, such as glycerol or DMSO.

Though Octet® systems offer a great deal of flexibility in the choice of the 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, analyte dilutions should be prepared in the same media and this media used for the baseline and dissociation steps as well. For kinetic assays using purified samples, Sartorius Kinetics Buffer is recommend for BLI assays as it contains bovine serum albumin (BSA), and surfactant (Tween-20) to inhibit non-specific binding to surfaces and other proteins. For Octet® SPR assays, it is recommended that buffer optimization is started in either 1X HBS-EP+ or PBS-T and the optimization process is performed as detailed below.

As discussed in Section 4.1, it is essential before starting any assay to know as much information as possible about the molecules that you are going to assess as this plays a key role in determining which buffers or sample matrix conditions to evaluate for assay performance. For example, when the molecules are highly charged, changes in pH and the ionic strength of the buffer may be evaluated to determine optimal conditions. Whereas when either molecule is highly hydrophobic, different detergents should be evaluated and spiked into the base buffer.

To assess these buffers, an initial DOE, which may initially be wide in scope but narrowed after initial optimization. The ligand molecule density should be as determined in Section 4.4.2, and the analyte concentration should be high enough (typically 10–20X of the expected K_D) to produce optimal binding.

The analyte sample is then prepared in each buffer or matrix condition and a full binding experiment performed, ensuring that a stable baseline is observed with no drift and association (5–10 minutes) and dissociation (2–10 minutes) is observed. Actual binding assay step time will depend on the analyte kinetics and affinity, but should be kept constant for assay buffer optimization. For each condition it is important to include a negative control where all conditions are identical except the analyte concentration should be 0 M (buffer or matrix without product/analyte). It is also critical to include a ligand-free reference surface (either an unmodified biosensor for BLI or reference channel for SPR to ensure that the chosen buffer does not increase NSB).

4.7 Analyte Association and Dissociation Optimization

Following optimization of the assay buffer with a generic concentration of analyte (typically 10–20X of the expected K_D) the concentration of the analyte must be optimized to determine accurate kinetics and affinity. For ease, multi-cycle kinetics (MCK) are discussed here as the main form of kinetics used, but OneStep® injections on the Octet® SF3 can simply be performed with the top concentration of analyte determined optimal for MCK.

4.7.1 Kinetic Screening and Dealing with Unknown K_Ds

The association step measures the binding interaction of the analyte to immobilized ligand. For screening purposes or qualitative analyses, measuring binding curves for a single analyte concentration is often sufficient, although an appropriate signal response curvature is essential for determining kinetic estimates using a fixed single concentration (Figure 31). If the K_D of the interaction is not known, either through experimentation or literature, an initial analyte scouting step is recommended. Perform the assay using analyte concentrations that span a wide range and use a 10-fold dilution between concentrations, following the recommendations made in Figure 31 with regards to assessing kinetics and affinity by an appropriate level of curvature. This approximation of K_D can then be used to run a full characterization using an expanded concentration series.

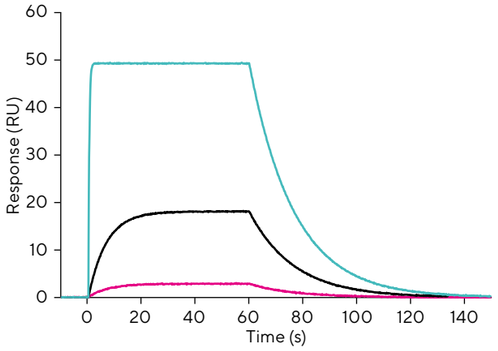


Figure 31
Kinetics is Curvature
When using a single concentration, it is important that sufficient curvature is observed in order to determine reliable kinetics. Here, the teal curve shows rapid association and equilibrium, which would result in inaccurate kinetics determination. The magenta curve shows a low level of response and curvature, which would also result in poor kinetics determination. The black curve shows a good level of curvature during the association phase and also reaches equilibrium, and should generate acceptable estimates of kinetics and affinity from a single concentration assessment.

4.7.2 Choosing the Optimal Concentration Series

When reliable, accurate kinetic constants are required, a dilution series of at least five analyte concentrations should be measured during the association step. An ideal binding profile should have at least two data points below the expected K_D and two data points above the expected K_D. The analyte dilution series measured should ideally range from a concentration of about 0.1–10 fold of the expected K_D, using 2-fold or 3-fold dilutions. For example, an interaction that has an observed K_D of 100 nM would use a concentration series ranging from ~ 10–1000 nM (12.3, 37.0, 111.1, 333.3, and 1000.0 nM).

The affinity constant (K_D) is defined as the concentration of analyte at equilibrium that occupies 50% of the available binding sites on the sensor surface. This range will assure that the assay will span from about 90% R_{max} down to the limit of detection (Figure 32). Running several concentrations will also show how well the fitted binding model applies over a concentration range around the K_D.

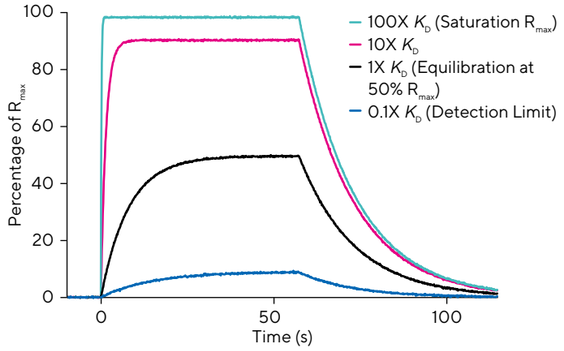


Figure 32
The Relationship Between R_{eq}, R_{max}, and K_D
All curves, if left for long enough, will reach equilibrium. Equilibrium level relative to R_{max} will depend on concentration relative to K_D. Only at concentrations around 100X K_D will the equilibrium be saturating.

It is important when assessing kinetics to use an optimal range of analyte concentrations (Figure 33). In Figure 33A, the binding signals span the dynamic range of the assay from near the limit of detection to just below saturation. The even spacing between the association curves indicates the ligand on the biosensor has not been saturated with analyte. If the analyte concentrations tested in a kinetic assay are too high, ligand binding sites can be saturated, increasing the potential for artifacts and non-ideal performance related to non-specific binding or aggregation (Figure 33B). The lower range of analyte concentration is not represented, though this is the range where the most accurate data are produced. If analyte concentrations are too low, however, signal may be weak and/or the resulting binding rates may be diffusion limited (Figure 33C).

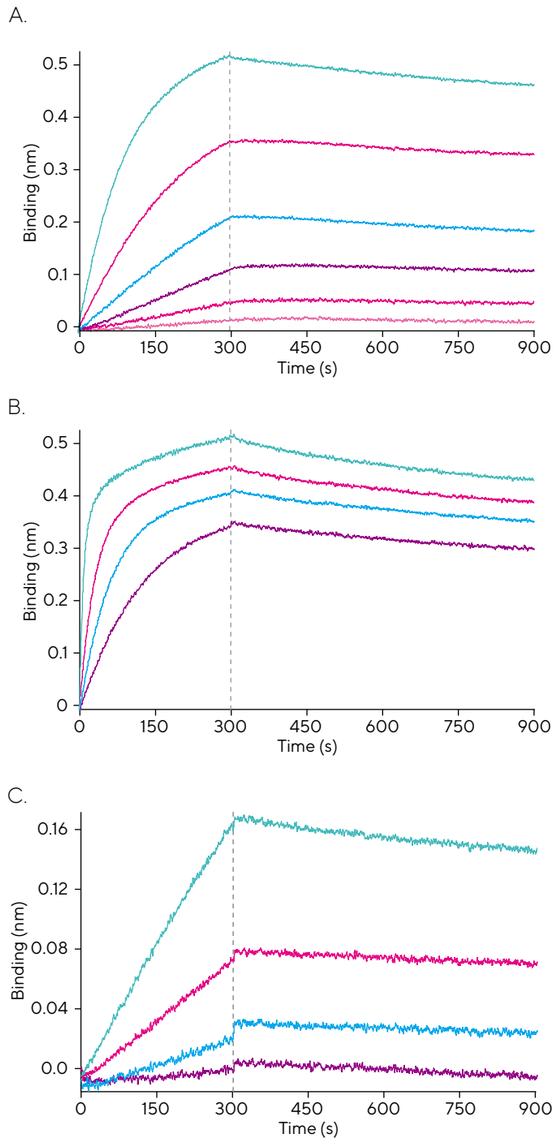


Figure 33
Binding Response Data Illustrating Varying Ranges of Analyte Concentrations
(A) Ideal range of analyte concentrations for a kinetics experiment, with signals spanning the dynamic range of the assay. Curve fits generated using such data will likely give reliable kinetic constants. (B) The range of analyte concentrations utilized is too high. Note that the curves are close together and exhibit heterogeneity most likely due to non-specific binding. The lower range of concentrations is not represented, which tends to give the most accurate data. (C) The range of analyte concentrations here is too low and data is limited by mass transport.

4.7.3 Dealing With High-Affinity Binders

In practice, the observed binding rate will be very low for a high-affinity binder run at low concentrations at analyte concentrations near or below the K_D value. As ligand and analyte binding is a second-order binding event, the two molecules must come together prior to being able to bind. If the concentration of the analyte is low, then the binding reaction will also be slow. This effect is compounded in high-affinity interactions where interactions take a long time to reach equilibrium and the low analyte concentration near the K_D amplifies the observed linear binding.

If the binding signal below the K_D is not measurable, it is best to run a dilution series beginning at 100X K_D and titrate down using 2-fold or 3-fold dilutions until there is no measurable signal. This strategy will enable testing over a range of concentrations, still producing valid kinetic and binding constants.

The association step should be run long enough to see some curvature in the data traces, but not so long that the curves flatten out for an extended period of time. In general, a 5–10-minute association step is recommended. When using impure solutions, avoid running the association step for too long and allowing binding curves to reach equilibrium as this leaves opportunity for weaker, non-specific interactions to occur.

When running a full kinetic profile with several analyte concentrations, data are analyzed globally by fitting both association and dissociation phases for several analyte concentrations simultaneously using the same set of rate constants (Section 5). Global analysis of a wide range of analyte concentrations provides robust analysis and accurate estimation of binding constants. Knowing the correct analyte concentration is critical, as this value also has a direct impact on calculated constants.

4.7.4 Optimizing the Dissociation Step

Although analyte association is critical in determining accurate kinetics, it only provides half of the information required to determine the global affinity of the interaction (Equation 4 in Section 5). The dissociation step provides the remaining required information and should be optimized during assay development.

In the dissociation step, the analyte is not present due to a return of the sensor surface to assay buffer and, therefore, dissociation of the bound analyte–ligand complex on the surface of the sensor is observed. For Octet® BLI, it is recommended that the dissociation step is performed in the same microplate well as the baseline for each sample. Running these two steps in the same buffer in the same well can prevent step artifacts that may result from subtle differences in buffer, volume, or shape of the well. While these artifacts can be corrected for, it is best to minimize them for accurate data analysis. The dissociation step can be run in the same well for every sample in an assay, as long as the buffer matrix is identical for each sample. It is acceptable to dip into the same well of buffer for every dissociation step because the number of molecules coming off the biosensor surface is negligible, even when the biosensor is saturated and dissociation rate is high (Table 5).

For Octet® SPR assays, dissociation is a much simpler process as the microfluidics constantly pass fresh assay buffer across the flow cells being measured. This unbound analyte is either then removed to waste or can be captured in a separate well of a microplate during a recovery injection. It is important that the flow rate used during dissociation matches the flow rate used during association. For both BLI and SPR, the dissociation step should be run long enough to observe decay in the binding response; meaning the length of the dissociation step will depend on the affinity of the interaction.

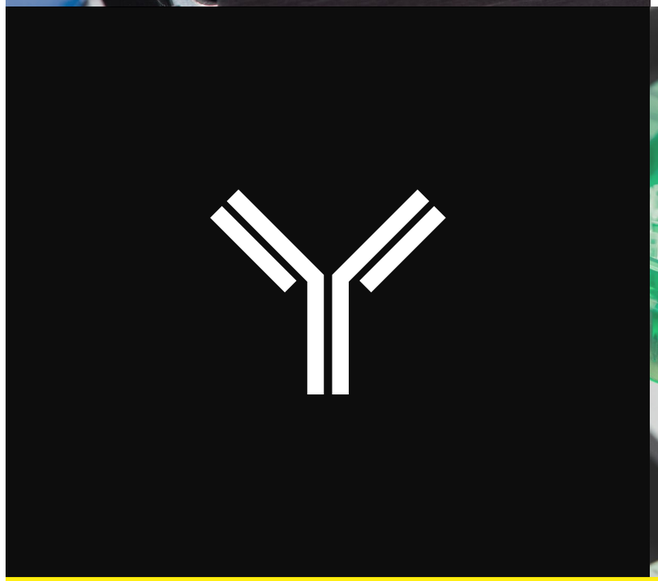
As discussed previously, it is generally accepted in the kinetics community that determining accurate dissociation kinetics requires a > 5% decrease in response during dissociation in order to determine accurate K_D values. It is preferable that a visual drop in the response is also observed in addition to a mathematical decrease of 5% prior to performing regeneration (Section 3.1.4).

Extent of Dissociation	Number of Molecules Going into Solution	Moles Going into Solution	Volume/Well (µL)	Molarity in Solution (M)	pM at End of Dissociation
100% dissociation (unlikely unless very weak interaction)	1.00E+09	1.66E-15	200	8.30E-12	8.3
10% dissociation	1.00E+08	1.66E-16	200	8.30E-13	0.83
1% dissociation	1.00E+07	1.66E-17	200	8.30E-14	0.083

Table 5

Effect of Dissociation of Molecules from the Biosensor on Concentration in Buffer Solution.

The number of molecules coming off the biosensor is negligible even when the same well is used for multiple samples in the dissociation step. It may be beneficial to use separate wells for dissociation of each sample in cases where dissociation rates are extremely fast and molecules dissociate fully from the biosensor.



"Regeneration provides an efficient and cost-effective solution for high-throughput applications."

4.8 Surface Regeneration, Ligand Regeneration | Scouting

Analysis of replicates is essential when analyzing samples using any technology, including SPR and BLI. Ideally, for the most accurate and precise data, the user should keep all conditions identical between replicates. In the case of SPR and BLI assays, to successfully re-use the same sensor surface, the surface must be regenerated so that bound analyte is removed without any deleterious effects on the immobilized capture molecule, ligand, or other aspects of the surface.

Depending on the nature of the ligand, an SPR sensor chip surface can be regenerated up to 100 times and a BLI biosensor up to 10 times. Regeneration of the surface is sometimes unnecessary if the analyte–ligand interaction has a fast dissociation rate. In this case, all analyte will dissociate off the surface in a short period of time (ideally < 10 minutes) just by washing with assay buffer.

Determining and optimizing regeneration conditions is dependent on the ligand immobilization strategy. For example, if the ligand is immobilized using a covalent immobilization strategy, regeneration involves removing the analyte without destroying ligand activity.

If the ligand is immobilized using an affinity capture approach, regeneration usually removes the ligand, with bound analyte, from the capture molecule. As the ligand will be replaced in the next cycle, it is irrelevant whether the ligand tolerates the regeneration conditions used.

It is important to invest sufficient time and resources in developing suitable regeneration conditions as this will ensure the reproducibility and success of the assay. Developing a regeneration procedure essentially involves searching for appropriate conditions and confirming the effectiveness of these conditions.

Note: Although Dip and Read biosensors for Octet® BLI systems are disposable and cost effective for single-use applications, many are also regenerable. In some cases, especially in kinetic screening, it may be advantageous to assay several samples using the same ligand-coated biosensor. This practice can save some cost on biosensors; however, these savings should be carefully weighed against the costs involved in optimizing regeneration conditions (Figure 34).

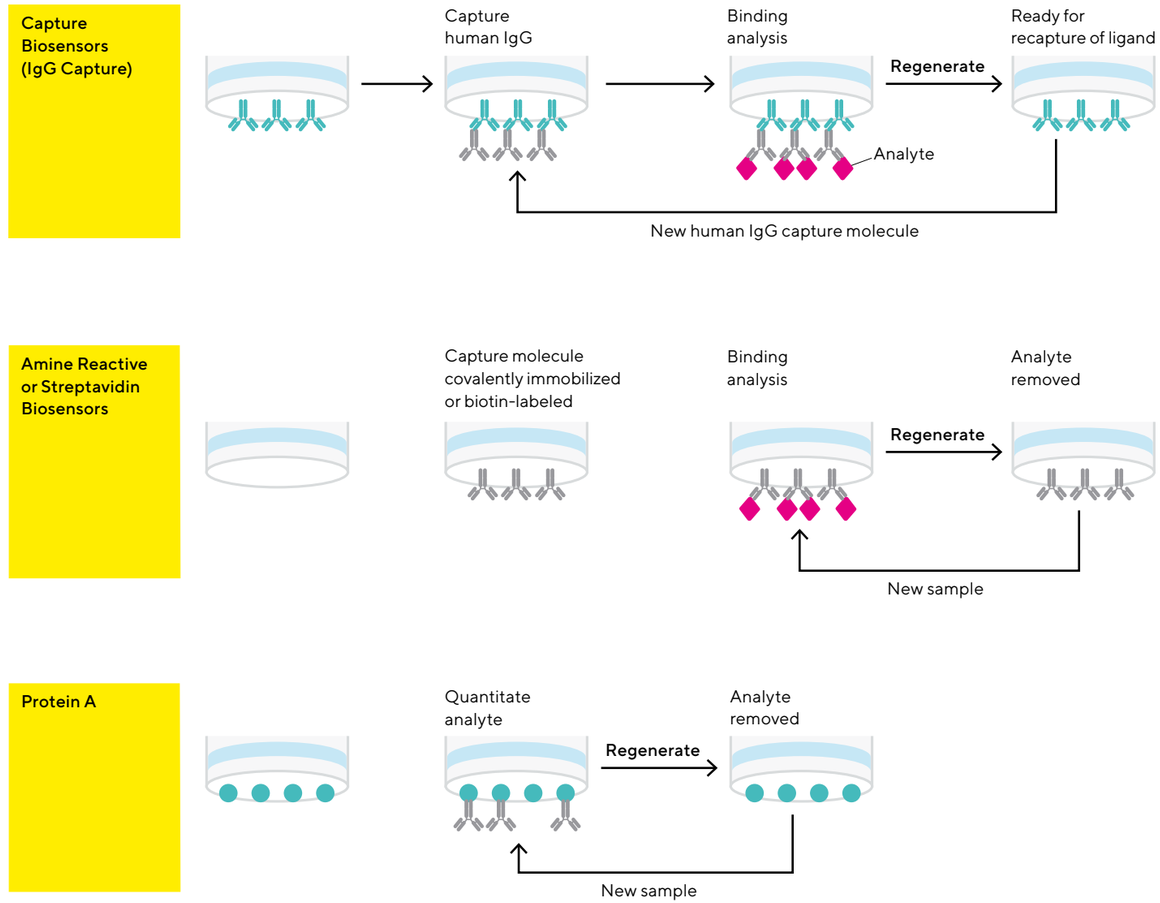


Figure 34
Efficient Regeneration Requires Removing the Bound Analyte Without Affecting Ligand Activity
For successful regeneration, biosensor surface chemistry and the immobilized ligand must be stable under the regeneration conditions. The analyte–ligand protein interaction must dissociate fully during regeneration and the immobilized ligand must retain activity over multiple regeneration cycles.

4.8.1 Determining Suitable Regeneration Conditions

Empirical research has shown that regeneration of the analyte–ligand protein interaction often involves denaturation of the analyte while the ligand remains stable. Ligand stability is due in part to its immobilization on the surface. Therefore, determination of the best regeneration conditions for an interaction can only be achieved by testing candidate conditions during an empirical process, in which the user tests each set of potential conditions for a small number of cycles and assesses analyte binding and ligand regeneration. A suitable set of regeneration conditions can then be determined, followed by fine-tuning to give the optimal conditions.

In searching for regeneration conditions, it is important to:

- Use the same type of sensor surface chemistry that will be used in the actual assays.
- Use all available information about the ligand and analyte to select the first set of regeneration conditions to be tested.
- Note that regeneration of an interaction is a non-ideal solution and should be avoided where possible; using affinity capture avoids regenerating immobilized ligand and subsequent denaturing of the binding site.
- Immobilize the same quantity of ligand on an unused sensor chip using the same immobilization chemistry that will be used in the assay (e.g., in capture assays, capture the same quantity of ligand on a sensor chip immobilized with the affinity capture molecule that will be used in the assay).
- Use a high concentration of analyte. When ≥ 50% of the ligand binding sites are filled, the user may have greater confidence in the results observed. In addition, regeneration conditions established for low concentrations of analyte may not work as well for higher concentration, but the reverse is not necessarily true.
- Test conditions using actual experimental samples. This is especially important with complex samples (e.g., culture media or body fluids), where non-specific binding of non-analyte components can occur.
- Test the least aggressive regeneration conditions before trying harsher treatments in order to reduce the possibility of damaging the surface before all tests are completed.
- Assess the results of multiple replicates of a set of regeneration conditions.
- Remember that antibodies, particularly monoclonal antibodies, are generally sensitive to regeneration and particularly sensitive to acidic regeneration.

Note: After determining the optimal regeneration conditions for Octet® SPR assays, it is recommended to post-condition the sensor chip surface with the regeneration solution prior to assessing analyte binding. This can be achieved performing three to five cycles of analyte injection using the lowest concentration of analyte and regeneration solution. Clean the sensor chip surface before ligand immobilization and analyte binding to condition the system to the regeneration solutions. (See Octet® SPR Best Practice Guide Octet® SPR Sensor Chip Preparation.)

Note: For Octet® SPR assays, it is best to employ two injections instead of one (e.g., two 30-second injections instead of one 60-second injection) as the sensor chip surface experiences a pH shock during regeneration.

4.8.2 Issues Affecting Regeneration

The analyte–ligand interaction is the key factor in finding good regeneration conditions, but optimum conditions are affected by other factors:

- Sensor type—optimal regeneration conditions can vary for the same analyte–ligand interaction on different sensor chip types.
- Coupling chemistry—optimal regeneration conditions can vary slightly with different coupling chemistries.
- Ligand density—the quantity of ligand immobilized can significantly affect the optimal regeneration conditions; for example, monoclonal antibodies regenerated with glycine-HCl require lower pH values at lower ligand densities.
- Analyte binding level—higher levels of bound analyte necessitate harsher conditions.
- Temperature—temperature can have a marked effect on regeneration performance, and regeneration should be optimized for the temperature at which the assay will be run.

4.8.3 Determining Regeneration Conditions

Although regeneration should be empirically determined, common starting points can be used for optimization. (See Application Note Biomolecular Binding Kinetics Assays on the Octet® Platform and Technical Note Regeneration Strategies for Streptavidin Biosensors on the Octet® Platform for more information.) For example, most antibody–protein interactions can be disrupted by a series of short incubations in low pH buffer (pH 1.5–4.0), such as 10 mM glycine (pH 1.5–2.0), followed by neutralization in assay buffer. However, if the sensor surface is not efficiently regenerated with a low-pH buffer, other conditions may be tested such as high salt concentrations, detergent, or high-pH buffer. There are different modes of interaction at work between different analyte–ligand pairs, such as hydrophobic forces, van der Waals forces, and ionic binding. An understanding of the nature of the proteins involved and the type of non-covalent forces that dominate the interaction is important in developing an effective regeneration protocol. Therefore, for a successful regeneration, the following conditions must be met:

- Biosensor surface chemistry must be stable under the regeneration conditions and retain activity over multiple regeneration cycles.
- The immobilized ligand must be stable under the regeneration conditions and retain activity over multiple regeneration cycles.
- Analyte–ligand protein interaction must dissociate fully during regeneration.

The number of regeneration cycles that a ligand can withstand, and the efficiency of the regeneration also, greatly depend on the proteins being disrupted. Some ligands can be regenerated ten or more cycles, while others tolerate far fewer cycles or cannot be regenerated at all. A worked example using the Octet® BLI system's high-throughput format is shown here (Figure 35), but the same generic principles apply to optimizing regeneration conditions using Octet® SPR.

As shown in Figure 35, biotinylated Protein A was bound to eight streptavidin biosensors and a human IgG assessed as the analyte. Therefore, the aim of regeneration is to determine the optimum conditions that remove the human IgG from the Protein A without damaging the Protein A for further capture of the analyte.

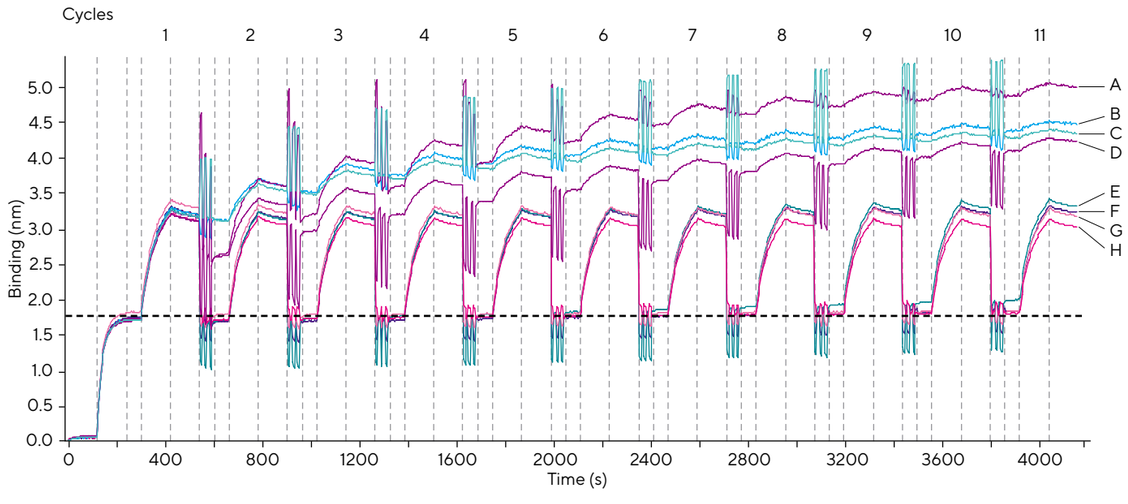


Figure 35

Real-Time Results from Regeneration Scouting and Validation Experiment

Data shown are from an Octet® R8 instrument where eight streptavidin biosensors (A1–H1) each correspond to a different regeneration solution. Biotinylated Protein A was captured, followed by 11 cycles of binding to human IgG with regeneration.

As shown in Figure 35, solutions A, B, C, and D perform very poorly in removing the bound human IgG from the Protein A, which is shown by a failure to return to the assay baseline (black dashed line). Due to the human IgG remaining on the Protein A, the binding response increases each cycle as more human IgG binds to the Protein A, ultimately reaching saturation, where minimal further binding of the human IgG is possible to the Protein A. In comparison, solutions E–H show excellent ability to remove the bound human IgG from Protein A and return to the assay baseline. The importance of assessing multiple cycles of regeneration is shown by solution H after Cycle 7, where a failure to return to the assay baseline can be observed unlike solutions E–G, which would be taken forward for optimization.

In addition to observing baseline responses, the suitability of the regeneration solution can also be shown by aligning the binding responses and assessing the association response. As shown in Figure 36A, when a non-optimal regeneration solution is used, a decrease in the observed analyte binding response is observed due to the Protein A being damaged during regeneration and unable to bind the human IgG at the same level. Figure 36B shows an optimized regeneration where the analyte response level is precise between replicates, indicating that the previous human IgG was removed from the Protein A without damaging the ligand.

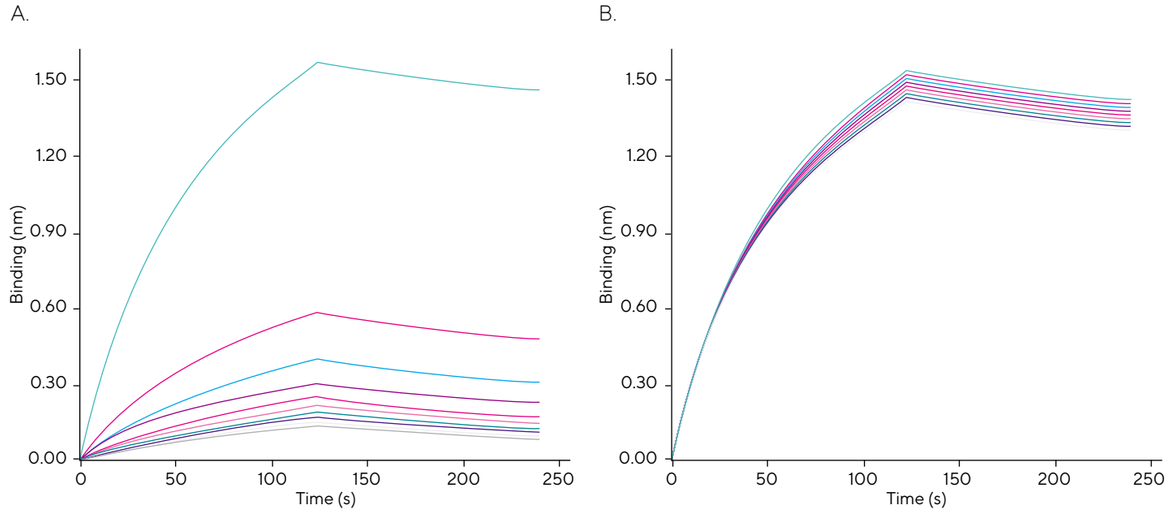


Figure 36

Aligned Sensorgram Traces Showing Association and Dissociation Steps for Two Regeneration Conditions

(A) shows data from non-optimal regeneration reagent (NaOH, pH 11.0) where the association signal of the same analyte declines with each subsequent regeneration cycle. (B) shows optimal regeneration condition (10 mM glycine, pH 2.0), where binding activity of the ligand is maintained through all regeneration cycles.

For capture-based biosensors, such as Anti-Human IgG Fc Capture or Anti-Mouse Fc Capture, the surface can be regenerated to the level of the original surface chemistry. Both the captured ligand and the analyte are removed during regeneration, which is typically performed using a 10 mM glycine (pH 1.7) regeneration buffer. Three to five cycles are performed consisting of a 5-second incubation in regeneration buffer followed by 5-second incubation in assay buffer. The biosensor can then be re-loaded with ligand for a new kinetic assay. Depending on the protein being captured, however, regeneration buffer formulation may require optimization. For recommended regeneration conditions for a specific capture-based biosensor, refer to the technical note for the specific biosensor on the Sartorius website and to the Application Note Biomolecular Binding Kinetics Assays on the Octet® Platform.

When potential regeneration conditions have been identified, it is important to verify the regeneration process by repeating analyte injection and regeneration multiple times. Generally, with good regeneration conditions the analyte response should not vary by > 5%–10% over 20 cycles for SPR. As there is often some reduction in activity in the first few cycles as discussed earlier, the final assessment of a set of regeneration conditions should not include the first five cycles in a set of 25 cycles.

Note: In some cases, binding capacity may decrease during the first regeneration cycle but stabilize through subsequent cycles. This decrease may be due to many factors, including loss of a small amount of immobilized protein during the first exposure to regeneration buffer. To minimize the impact of this initial change on the final data, it is common practice to run a conditioning step before the assay. After determining the optimal regeneration conditions for Octet® assays, it is recommended to post-condition the sensor chip surface with the regeneration solution prior to assessing analyte binding. For SPR, this can be achieved performing three to five cycles of analyte injection using the lowest concentration of analyte and regeneration solution. Clean the sensor chip surface before ligand immobilization and analyte binding to condition the system to the regeneration solutions.(See Best Practice Guide Octet® SPR Sensor Chip Preparation.)

For Octet® BLI assays, a single cycle of regeneration is recommended.

4.8.4 Interpreting Experiments to Optimize Regeneration

Ideal regeneration conditions should remove all bound analyte but leave the ligand intact and not denatured or inactivated. Experiments to optimize regeneration involve binding the analyte then attempting to remove it. Consequently, the observed response in one cycle reflects the efficiency of regeneration in the previous cycle. Preparing an overlay plot (Figure 37) of the analyte response and baseline phase from regeneration test cycles can be helpful in revealing changes in the interaction behavior. The response curve shape should not be affected by the regeneration treatment.

As shown in Figure 37A–C, during the first cycle, the surface is exposed to analyte before ever having been exposed to any regeneration solutions and a baseline response is observed that provides a reference for the maximum level of binding possible. In subsequent cycles, the baseline response obtained indicates how much analyte was removed from the surface in the previous regeneration cycle. The analyte response shows how much analyte-binding activity was retained by the ligand.

Regeneration conditions that do not remove all analyte result in an increasing baseline response and decreasing analyte binding response due to a decrease in potential analyte binding sites (Figure 37A). Conditions that are too harsh may remove all bound analyte but cause a decrease in analyte binding capacity as the ligand becomes denatured or inactivated (Figure 37B). Suitable conditions should remove all bound analyte and not cause a decrease in analyte binding capacity or baseline response (Figure 37C).

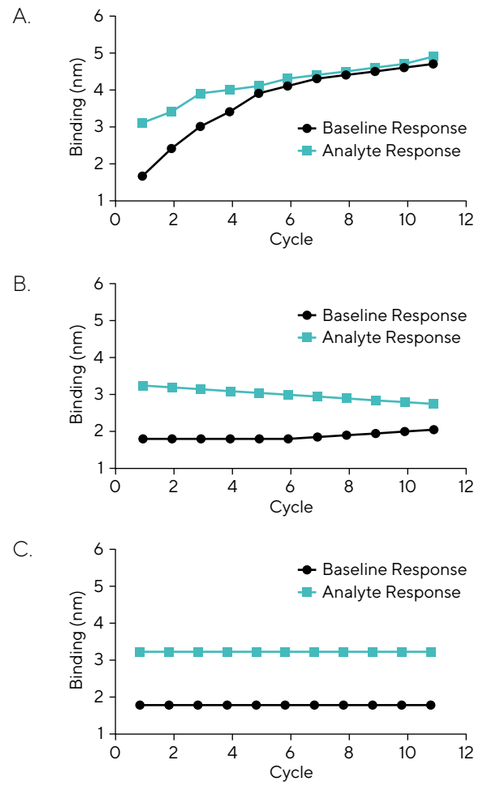


Figure 37
Immobilized Ligands Vary Significantly in Their Ability to Retain Activity After Regeneration
Regeneration response plots of baseline and analyte response can help visualize whether regeneration conditions are optimal.

Therefore, regeneration conditions should be assessed on the basis of trends in the analyte response, both within and between tested conditions, and on the basis of trends in the baseline response. The following general guidelines apply:

- The analyte binding response should not decrease. A decreasing response indicates that the ligand is becoming denatured or inactivated due to overly harsh regeneration conditions; or that some ligand moieties are not being regenerated, indicating overly mild regeneration conditions.
- The post-regeneration baseline should not increase. Increases in the baseline indicate that material is accreting on the surface and regeneration is too mild.
- The post-regeneration baseline may fall. Decreases in the post-regeneration baseline are acceptable during the first few cycles if the analyte response is not affected.

The ideal behavior of an analyte–ligand interaction with respect to regeneration is rare:

- The analyte response after regeneration often differs from the value obtained before any regeneration solutions have been introduced. The analyte binding capacity often varies by 5%–10% during the first few cycles on a newly-prepared sensor chip. A consistent analyte response during repeated cycles of binding and regeneration is of higher importance. When only a relatively small fraction of the activity is lost in the first few cycles and the analyte response remains relatively constant in later cycles, the regeneration conditions used can be accepted for use.
- When analyte accumulates on the surface due to insufficient regeneration conditions and harsher conditions result in recovery of this binding capacity, this indicates that those regeneration conditions are preferable and potentially close to ideal.
- Trends in the absolute baseline level can usually be ignored as long as the analyte response relative to baseline is constant. Even following optimization of regeneration, a slight downward drift in baseline is common.

4.9 Non-Specific Binding

Biological molecules tend to interact with surfaces; therefore, non-specific binding (NSB) is a concern with any assay under any conditions. Non-specific binding can occur as a result of many factors, such as cell culture media components, BSA or serum proteins, or charged species in the sample or buffer. With careful consideration, NSB can be minimized in most common assays.

4.9.1 Electrostatic and Non-Electrostatic Non-Specific Binding

Two common types of non-specific binding are observed in SPR and BLI assays:

- Electrostatic NSB
- Non-electrostatic NSB

Electrostatic NSB is characterized by attraction of the positively-charged analyte to the negatively-charged sensor surface (Figure 38). This is often exacerbated when using carboxymethyl dextran-based sensor chips in SPR due to their net-negative charge. Attraction of the positively-charged analyte to the sensor chip surface often occurs when the analyte has a higher isoelectric point than that the assay buffer.

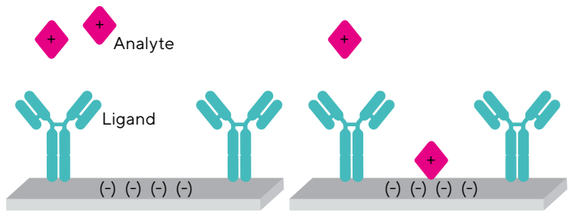


Figure 38
Electrostatic Binding
Electrostatic NSB is characterized by attraction of the positively-charged analyte to the negatively-charged sensor chip surface.

Non-electrostatic NSB is characterized by interaction of the analyte or components of the sample to the sensor chip through interactions such as hydrophobic, hydrogen bonding, or binding to exposed gold patches on the sensor chip (Figure 39). Non-electrostatic NSB is particularly noticeable with thiol-containing small molecules and "sticky" or crude samples that contain patches of hydrophobicity or charge on the protein's surface.

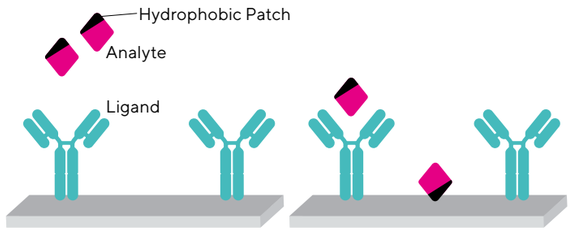


Figure 39
Non-Electrostatic Binding
Analyte containing large hydrophobic patches (black regions) are susceptible to non-electrostatic NSB.

4.9.2 Assay Design to Minimize Non-Specific Binding

In general, the simplest way to determine a molecule's proclivity for non-specific binding is to inject the highest concentration of the analyte across an unmodified sensor chip surface. For accurate test results, it is critical to use the same sensor chip that will be used during the assay.

In general, strategies for addressing with non-specific binding can be separated into two basic strategies:

- Sensor chip optimization
- Buffer optimization

Sensor Optimization for Octet® SPR and BLI Assays

As discussed previously, the net negative charge of carboxymethyl dextran-based sensor chips can cause electrostatic non-specific binding to be observed and therefore, it is recommended that different sensor chip types with fewer functional groups are tested for a reduction in NSB. For example, if a high-capacity Octet® SPR sensor chip produces an observable level of non-specific binding, then a lower capacity Octet® SPR sensor chip, which contains fewer carboxylic groups, should be tested. A planar sensor chip, which contains no carboxymethyl dextran, should also be considered (though the user must consider the required immobilization levels when assessing alternative sensor chips).

Correct reference surfaces have a large impact on data quality in addition to having a noticeable effect on non-specific binding. Where possible, the reference surface should contain the same immobilization conditions as the active surface and contain a non-interacting protein that should be immobilized to the same response level as the active surface. The non-interacting protein does not directly reduce non-specific binding, but masks the charge on the sensor chip surface and allows the non-specific binding to be "referenced" out. Where a positively-charged analyte is being assessed, it is possible to lower the sensor chip's net negative charge during amine coupling-based immobilizations by using the same assay parameters and inactivating remaining succinimide esters with ethylenediamine instead of ethanolamine. This reduces the net negative charge on the sensor chip surface and decreases the potential for nonspecific binding.

Octet® BLI biosensors contain a biocompatible layer that greatly mitigates non-specific binding and a stronger emphasis can be placed on optimizing the buffer to minimize non-specific binding. As with Octet® SPR sensor chips, it is important to include a reference biosensor that ideally includes the same ligand being assessed in the rest of the assay. BSA should be avoided as the non-active protein is prone to non-specific binding interactions.

Assay Buffer Optimization for Octet® SPR and BLI Assays

The choice of assay buffer is very important in SPR and BLI assays as it is the carrier of the analyte and therefore, any composition, pH, or other excipient issues can affect the interaction between the analyte and ligand. For SPR assays, assessment of non-specific binding should be performed in a suitable starting buffer, such as HBS-EP+. For BLI assays, 1X Kinetics Buffer is a suitable starting buffer to assess non-specific binding.

When non-specific binding occurs, proper assay optimization and buffer selection can minimize the effects. Modifying the assay buffer can reduce such binding. In most assays that measure protein-protein interactions, blocking agents such as BSA (up to 1%–2%) and/or non-ionic detergents, such as Tween-20 (up to 0.09%) can be added. Other blocking agents, such as casein, PEG, or gelatine can also be substituted. Salt concentration can also be modified. Raising the salt concentration to 150–500 mM (physiological concentrations and higher) can increase assay stringency and therefore reduce non-specific binding.

Where possible, the analyte should be as pure as possible to ensure that contaminants that may cause non-specific binding are removed. Where it is not possible to eliminate non-specific binding using the techniques shown here, orientating the assay with the "sticky" protein as the ligand should be considered. Attention must be paid to the immobilization process as the activity of the immobilized ligand may be affected by its non-specific binding.

Always include a reference sample with every kinetics experiment to allow subtraction of background signal and assay drift. A reference sample is run in the association step using a ligand-loaded sensor and should consist of assay buffer minus the target analyte. Double referencing with both a reference sample and a reference biosensor can be performed when background signal is an issue, or in small-molecule binding assays, where the signal is very small in relation to noise. Reference sensors are typically loaded with a non-active protein that is similar to the ligand and run through the assay with the same analyte samples as the ligand-loaded sensors. The reference sensor is used to subtract non-specific binding of analyte to the biosensor.

4.10 General Assay Optimization

Careful assay design can minimize or eliminate many of the artifacts associated with binding data, including challenges fitting data to a simple model. A well-designed assay is fundamental to producing high-quality SPR and BLI data that can be readily interpreted to deduce meaningful kinetic rate and affinity constants.

Adhering to some basic "good practices" and acquiring hands-on experience are fundamental to producing high-quality SPR and BLI data that can be readily interpreted to deduce meaningful kinetic rate and affinity constants.

Whether using low-throughput Octet® systems as a secondary characterization tool to study binding interactions, or high-throughput systems as a preliminary screening tool to survey hundreds of binding interactions in a single assay, there are some established best practices that aid in improving the quality of biosensor data and minimizing artifacts. Here, we provide a synopsis of these recommendations.

4.10.1 Reference Surface

As discussed in Section 4.9.2, one of the most critical parameters in assay design can be the inclusion of a suitable reference surface, in addition to the active surface where binding measurements will be performed. It has been shown that a suitable reference surface can help correct for multiple artifacts, such as refractive index changes, non-specific binding, assay drift, and injection noise.

Where possible, the reference surface should contain the same immobilization conditions as the active surface. Both the active and reference surfaces should have the capture molecule immobilized to provide a more relevant reference surface for data analysis (Figure 40A) compared to an unmodified reference surface that is either unmodified or activated/deactivated (Figure 40B).

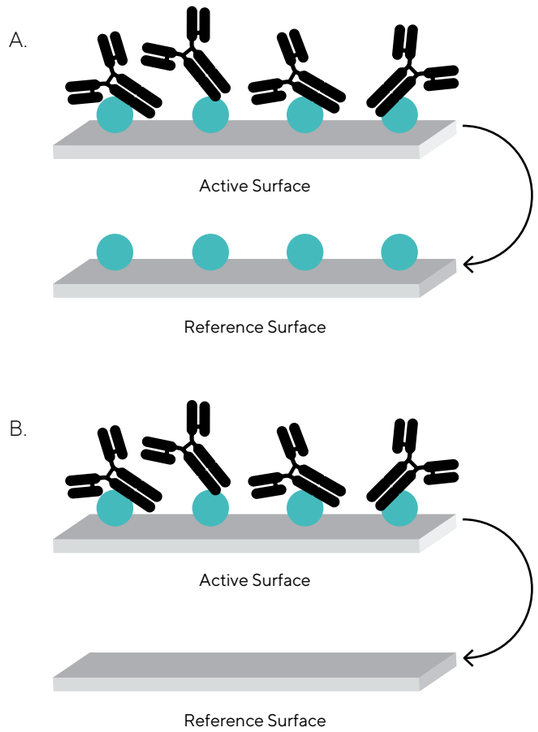


Figure 40
Correct Reference Surfaces Have a Large Impact on Data Quality
The capture molecule (teal) should ideally be present on both the active and reference surfaces to ensure the best data quality possible.

Where a capture assay is not possible, a non-interacting protein, such as a non-binding variant, should be immobilized on the reference surface to the same response level as the active surface.

4.10.2 Shake Speed

The recommended shake speed for Octet® BLI binding assays is 1,000 RPM. However, the shake speed may require optimization depending on sample type. Shake speed evaluation should aim at achieving differentiation of analyte binding response as a function of concentration. To evaluate shake speed, keep all assay conditions constant while changing the shake speed.

4.11 Mass Transport Limitation

For binding to occur in SPR assays, the analyte must be transported laterally (diffusion) in the flow cell from the bulk solution to the surface before any interaction with a ligand can take place (Figure 41). This is referred to as mass transport and is a critical component of SPR as a technique. As discussed above, low surface capacities minimize multiple artifacts associated with SPR, but also play a key role in minimizing the risk of mass transport limitation.

Mass transport limitation (MTL) occurs when the analyte cannot diffuse to the sensor chip surface rapidly enough and the measurement becomes diffusion-limited rather than kinetics-limited. This is where the analyte can diffuse to the sensor chip surface rapidly enough and there is an abundance of analyte for accurate kinetics determination.

To minimize MTL, it is important to not only consider the surface capacity during assay development, but also the analyte flow rate.

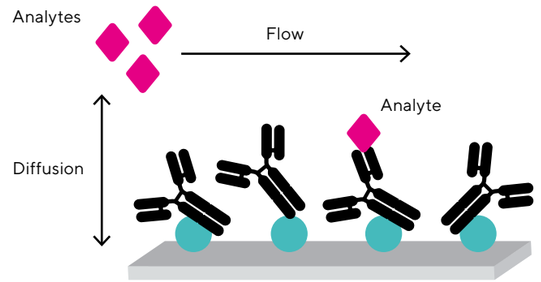


Figure 41
Mass Transport is a Critical Component of SPR
Measurement of binding interactions requires diffusion of the analyte from the bulk flow to the sensor chip surface before binding can be observed (Mass Transport). Mass Transport Limitation (MTL) occurs when the analyte cannot diffuse to the sensor chip surface rapidly enough.

When designing an SPR assay, it is important to consider that at low flow rates and high surface capacity there is an increased risk of experiencing MTL (Figure 42). Although this assay setup can have a role in determining the active concentrations of the analyte, it is not suitable for determining accurate kinetics as with high immobilization levels, transport can be limiting and the analyte concentration at the surface varies with the flow rate. This means the analyte concentration is different at the sensor chip surface than the analyte concentration in the bulk of the sample being used in the assay. Since observed binding rates are proportional to analyte surface concentration, this reduction in surface concentration will result in slower-than-expected observed binding rates.

As discussed above, use of a planar sensor chip reduces the surface density and can minimize or remove the avidity effects observed with a 3D-hydrogel sensor chip. This is due to a reduced immobilization level (and subsequent decrease in R_{max}). However, it is clear that flow rate also plays an important role in preventing MTL and allowing accurate kinetic data (Figure 43) to be derived. In general, for biologics assays, a flow rate of 50 µL/min should be used (with 30 µL/min being the lowest flow rate), but higher flow rates can be tested if needed. Higher flow rates should generally be used for small-molecule assays.

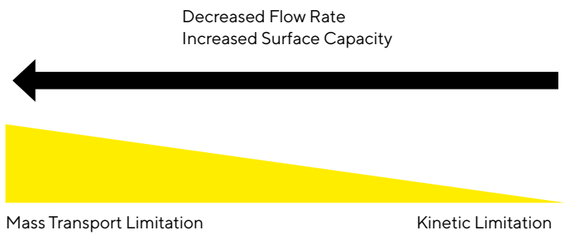


Figure 42
Low Flow Rates and High Immobilization Levels Can Cause Mass Transport Limitation
During mass transport limitation (MTL), measurements become diffusion limited rather than kinetics limited and can lead to the determination of incorrect kinetics and affinity.

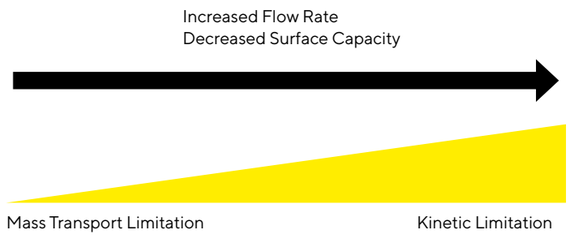


Figure 43
High Flow Rates and Low Immobilization Levels Minimize the Risk of Mass Transport Limitation
Decreasing the surface capacity and increasing the flow rate moves the assay away from mass transport limitation (MTL) and towards measuring accurate kinetics and affinity.

4.12 Double Reference Subtraction

Data processing of SPR and BLI response curves is necessary to eliminate systematic artifacts such as injection noise, non-specific binding, baseline drift, and bulk refractive index (RI) changes between the assay buffer and analyte. This is especially important for SPR assays, which are more sensitive to the features listed. After correcting for these artifacts, high-quality data can be generated for further data analysis and fitting to appropriate kinetic models using the Octet® Analysis software.

4.12.1 The Purpose of Referencing

A commonly misunderstood concept of SPR and BLI data is the importance of double reference subtraction during data analysis. Assay orientation and minimization of assay artifacts using a suitable reference surface contribute to the ability to collect high-quality data on the Octet® systems (Sections 4.9 and 4.10).

Correcting for these surface-to-surface artifacts between the active (ligand immobilized) and reference surfaces for the analyte-containing sample solution involves a processing step called "referencing." A second referencing step is required to subtract the highly reproducible and systematic deviations in the data collected for buffer blanks, occurring within the active and reference flow cells. Performing the above two referencing steps is referred to as "double referencing." While artifacts related to buffer referencing may be subtle and only noticeable in data collected on low-capacity surfaces, it is advisable to apply double referencing routinely regardless of the capacity of the immobilized ligand surface. This helps to ensure the highest quality data input for use in fitting routines.

4.12.2 Double Reference Subtraction

Subtraction of the analyte response to the active and reference surface, and the assay buffer blank (no analyte) response to the same two surfaces is known as double reference subtraction and is recommended to be performed to determine the "true" binding response (Figure 44).

In the first step, the reference surface is subtracted from the active surface for the analyte-containing sample solution. This single reference subtraction allows the binding response of the analyte to the ligand on the active flow cell to be determined, as the ligand is not present on the reference flow cell and the analyte interaction with the sensor chip surface with/without a reference protein is removed by subtracting the reference flow cell. This single subtraction can remove most issues with injection noise. In double reference subtraction, the binding response of the assay buffer blank is corrected for across the active and reference flow cells and this is subtracted from the analyte-containing sample buffer to correct for any refractive index changes caused by the sample buffer. This double subtraction can remove most issues with refractive index changes.

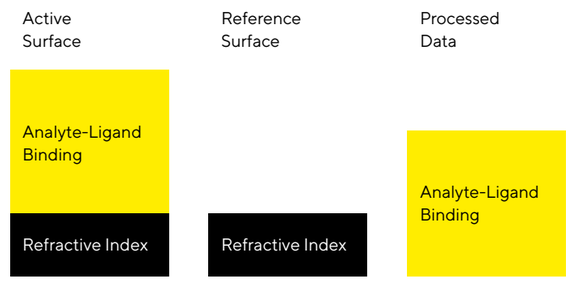


Figure 44
Double Reference Subtraction Allows Accurate Values to be Determined
In experiments where the analyte is in a sample buffer that exhibits a low refractive index, reference subtraction allows the "true" analyte-ligand binding to be assessed.

4.12.3 Solution Types

To understand the necessity of double reference subtraction, the role of the solutions must be considered. In the simplest case, two different solutions are prepared during an SPR or BLI assay: the analyte-containing sample solution and the assay buffer (Figure 45). These solutions are then sequentially exposed to an active sensor surface or reference sensor surface (Figure 46).

As shown in Figure 45, the analyte-containing sample solution may contain additional excipients or impurities that are not present in the assay buffer (that will subsequently be used for buffer blanks). Therefore, buffer exchange of the sample solution into the assay buffer (it is best practice to discard the assay buffer that the sample has been exchanged into) ensures that excipients are removed and do not affect the observed data through refractive index bulk effects (Section 4.12.4). Buffer exchange is especially important when performing multi-cycle kinetics, as the excipient level is diluted along with the sample and can potentially cause a different refractive index response at each concentration. This cannot be normalized out through reference subtraction and it is best practice to have no jump present at the start or end of the association phase.

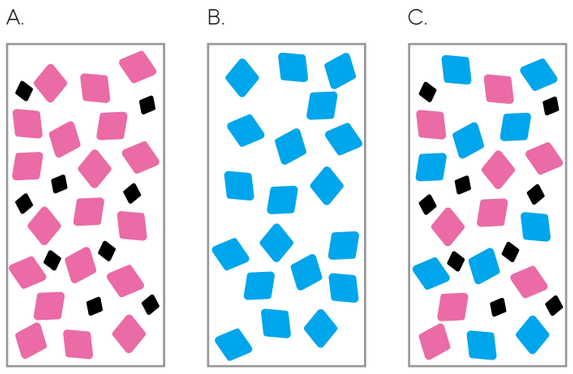


Figure 45
Excipients and Impurities Cause Changes in Refractive Index Bulk Effects
The stock sample solution (A) contains the desired analyte (pink diamonds) and may also contain excipients or impurities that will be carried over to the sample preparation (black diamonds). The stock sample solution is mixed into running buffer (B) (blue diamonds) to create the analyte-containing sample solution at the desired concentration (C).

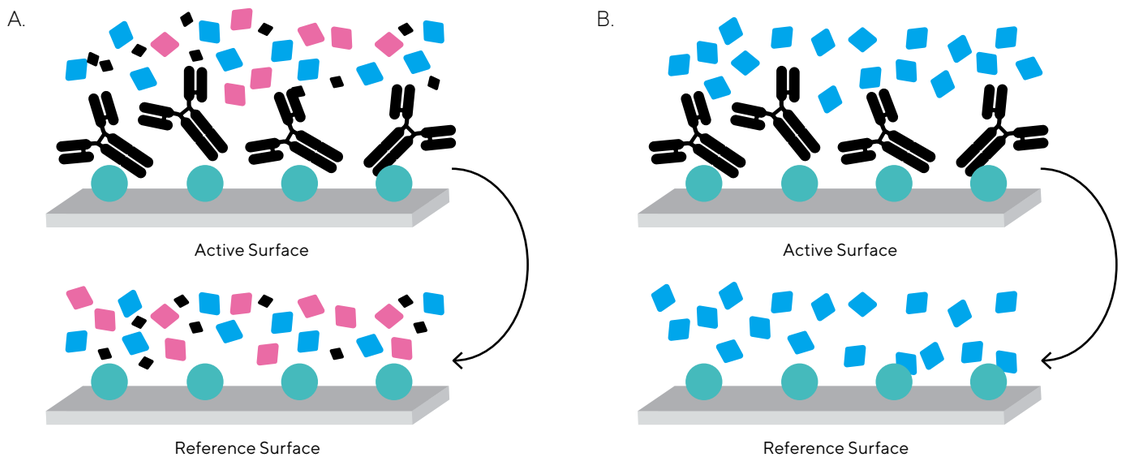


Figure 46
Inclusion of a Suitable Reference Surface Improves the Quality of Binding Data
Analyte-containing sample solution is injected across the ligand-containing active surface and a suitable reference surface (A), which allows reference subtraction of the reference surface. Buffer blank is also injected across the same active and reference surfaces (B) and the observed response is used for double reference subtraction, which helps compensate for bulk, non-specific binding and baseline drift.

4.12.4 Refractive Index

In typical macromolecular SPR assays, the analyte is highly soluble and is resuspended in the same buffer as is used for the running buffer, which should not contain molecules with a high refractive index (e.g., sucrose, histidine, and trehalose).

Refractive index "spiking" or "jumps" are commonly observed at the start and end of sample solution injections, especially during SPR assays, which are more sensitive to refractive index changes compared to BLI assays. These are caused by a mismatch in the refractive index between the sample solution and the buffer solution (Figure 47).

Since the response signal from macromolecules is large compared to that of the running buffer, these refractive index differences can be partially referenced out during double-reference subtraction prior to kinetic data analysis. However, the best practice is to ensure samples are buffer-exchanged into the assay buffer prior to assessment. This is especially relevant for commercially-sourced lyophilized recombinant proteins where excipients that contribute to the observed response, such as trehalose, are included as cryoprotectants.

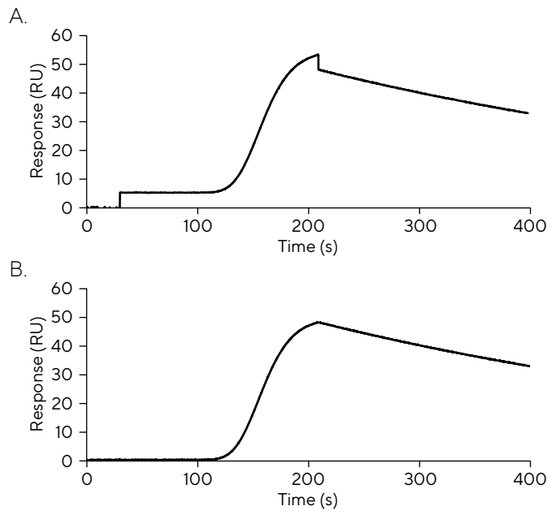


Figure 47
Differences in the Refractive Index Between Sample and Buffer Causes "Spiking"
(A) Sample OneStep® sensorgram with a 5 RU refractive index jump between the sample solution and the running buffer. (B) Buffer exchange of the sample solution into the running buffer can minimize or remove refractive index jump without the need for double reference subtraction.

In assays where the analyte is not soluble, such as in small molecule or fragment screens, organic solvents are required to ensure the analytes remain solubilized throughout the assay. Commonly used organic solvents, such as DMSO, contribute large bulk refractive changes compared to the small response from the analyte. Therefore, any differences in the bulk refractive index of the samples can cause errors in data analysis.

As shown in Figure 48, when the analyte is resuspended in a solution with a high refractive index, such as DMSO, the refractive index response between the active and reference flow cell is different, with the reference flow cell exhibiting a higher response. This effect is known as the "excluded volume effect" and is caused by a different concentration of DMSO at the sensor chip surface on the reference flow cell compared to the chip surface on the active flow cell. This happens because the immobilized ligand on the active flow cell allows for fewer DMSO molecules to get closer to the sensor chip surface.

The refractive index on reference flow cell is higher so the processed double reference-subtracted data shows an incorrect value for the analyte–ligand binding leading to errors in the reference subtracted-data, unless these variations are controlled.

Theoretically, if all samples within an assay contained the same concentration of DMSO, they would exhibit the same refractive index and the reference-subtracted response would be consistent across the assay, requiring no correction. Small differences in DMSO concentration can have a large effect on the observed response with a 0.1% difference in DMSO concentration resulting in a ~100 RU difference. Although variability in sample preparation can be minimized through standard good pipetting practices, solvent correction must be applied within the assay to account for residual variability.

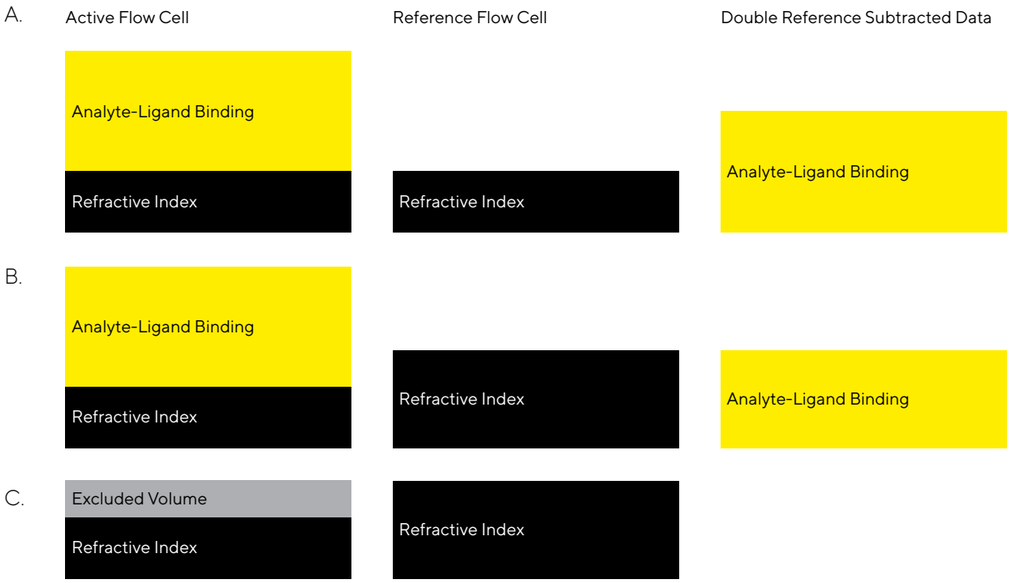


Figure 48
The Excluded Volume Effect Causes Incorrect Binding Data Generation
(A) In standard SPR assays, the refractive index response is the same between the active and reference flow cell as the running buffer contains no molecules with a high refractive index. The correct kinetics and affinity can then be determined from the analyte-ligand binding data using double reference subtraction. (B) In situations where a solvent with a high refractive index such as DMSO is used, more molecules are present closer to the reference flow cell surface than the active flow cell surface and, without correction, inaccurate kinetics and affinity would be determined. (C) The large bulk refractive index difference between the reference and active flow cell surface causes the excluded volume effect when refractive index correction is not performed.

4.12.5 Solvent Correction Curves

Standard solvent correction curve preparation involves injecting a series of DMSO reference solvent samples that span the expected range of the solvent concentration in the running buffer and samples. The response data is then plotted as the reference-subtracted active surface (y-axis) against the reference surface (x-axis) and a linear correlation is applied. The linear correlation can then be used to correct differences in bulk responses for samples. For example, an observed response of 200 RU on the reference flow cell would lead to a correction of ~ 5 RU on the reference-subtracted flow cell (Figure 49).

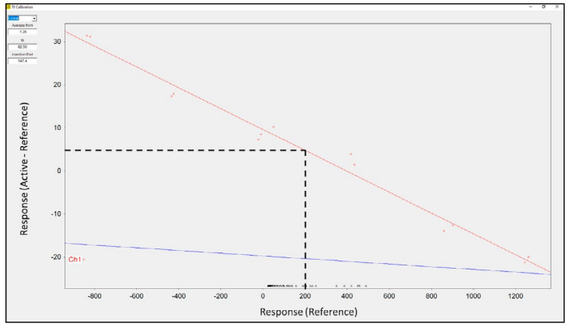


Figure 49
Linear Calibration Curve
Linear calibration curves are classically used to correct for the difference in response between the active flow cell and the reference flow cell. As shown here, a difference of 200 RU would lead to a correction of ~ 5 RU on the reference-subtracted active flow cell.

Unlike standard SPR systems that rely on user-prepared linear calibration curves, the Octet® SF3 allows a much simpler and rapid solvent correction called "micro-calibration" that makes use of its dispersion loop to create a continuous DMSO concentration between two standards (Figure 50). Micro-calibration on the Octet® SF3 system is a dynamic procedure that produces the standard curve in a single cycle before an assay is run and then normalizes refractive index sensitivity for the three channels in real time. When an assay is recorded after micro-calibration, the resulting data is already calibrated for refractive index effects.

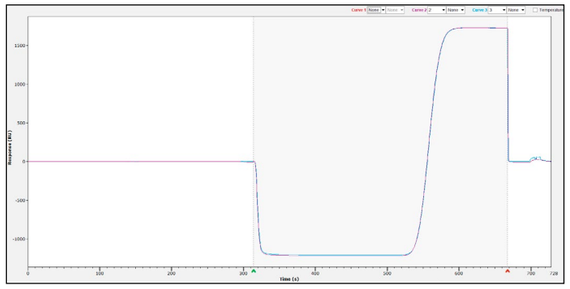


Figure 50
An Example Micro-Calibration Curve on the Octet® SF3 System
Unlike standard linear calibration curves that require multiple DMSO concentration standards, micro-calibration only requires two standards to be prepared and a continuous calibration curve is then generated using the dispersion loop.

Double reference subtraction forms a critical part of processing sensorgrams for subsequent data analysis through the removal of system artifacts and assay artifacts. However, it must be noted that double reference subtraction requires high-quality response curves before performing data analysis.

Proper assay design is critical to ensure that reference surface (single referencing) and buffer referencing (double referencing) can be performed and should show minimal baseline drift or bulk effects, which is observable by no refractive index jump between the end of the association phase and the start of the dissociation phase.

It is also best practice to ensure that the parameters for buffer referencing match that of the analyte-containing sample solution; these include the flow rate and association and dissociation times. By following best practices during assay design and development, double referencing allows the user to prepare high-quality sensorgrams that are suitable for data analysis.

4.12.6 Preparing Running Buffer and Calibration Solutions

Best practice guidelines for working with DMSO include:

- DMSO can vary in quality from supplier to supplier and therefore, it is important to choose a high quality DMSO with minimal H_2O impurity. For example, ACS grade 99.9% DMSO (≤ 0.1% water impurity) and anhydrous 99.9% DMSO (≤ 0.005% water impurity) are excellent choices.
- DMSO is hygroscopic and therefore quickly absorbs water from the atmosphere. This accelerates the degradation of DMSO and can lead to a difference in DMSO concentrations if the same solution is used on different days. Therefore, it is best to purchase smaller quantities of DMSO and use fresh bottles for optimal results.
- It is important to prepare calibration solutions and negative controls using the same buffer as the one used for the running buffer. It is important that independently- prepared negative controls are prepared in the same manner as calibration solutions and that running buffer is not used. This helps to account for pipetting and sample preparation errors.
- Where a septum is used, calibration solutions and negative controls may be pooled as the septum will reseal after the needle has removed the solution. Where a plate seal is used, calibration solutions and negative controls should be pipetted into individual wells as the plate seal will be pierced and not reseal, allowing evaporation and variability in the DMSO concentration.

"The Octet® platform allows unparalleled speed of analysis for protein–protein and protein–small molecule interactions."

5 Kinetic Data Analysis

Binding response curves obtained during assays contain a lot of information on the interaction between molecules and allow the user to answer simple questions on the presence or absence of a binding interaction, or perform complex analyses to fully characterize interactions. Kinetic data are then interpreted from the assay data and are based on a mathematical model of the interaction, from which kinetic (k_a and k_d) and equilibrium binding constants (K_D) can be calculated based on rates of association and dissociation.

The Octet® Analysis Studio Software is a powerful, yet simple-to-use software for analyzing assays and determining kinetics and affinity data. In this section, the features of the data analysis software are described, including steps in analyzing data, and considerations for interpreting results. Refer to the appropriate user guide for in-depth instructions on how to use each systems data analysis (Octet® SPR Analysis and Octet® BLI Analysis Studio) .

Kinetic data are interpreted based on a mathematical model of the interaction, from which kinetic and equilibrium binding constants can be calculated based on rates of association and dissociation. This type of analysis can be performed either locally, where kinetic parameters are determined based on a single analyte concentration, or globally, where constants are derived simultaneously from all analyte concentrations available. Alternatively, the equilibrium dissociation constant (K_D) can be determined using data at equilibrium from each available analyte concentration using steady state analysis.

5.1 Initial Visual Data Evaluation

It is vital to visually inspect the assay data prior to processing. While all Octet® systems are exceptionally sensitive systems, they are blind to the biology of an interaction and merely report on the observed change in binding. Without visual assessment it is impossible to determine whether the assays were run correctly, although the implementation of artificial intelligence will simplify this task in the future. This highlights the importance of assay design (Section 4) in order to generate high quality data since a good mathematical fit to the data should not be taken as an implication of the biological interaction.

5.1.1 Buffer Blanks

As discussed in Section 4.12.2, buffer blanks play a key role in double reference subtraction and allow the user to minimize the effect of systematic drift and refractive index changes observed during the assay. As the buffer blanks allow you to correct for systematic changes during the assay, we recommend initially using the closest buffer blank to the analyte-containing sample. It is important to remove buffer blanks that show non-ideal behavior as errors in data fitting and analysis will be observed.

Ideally the response of the buffer blank cycle should be close to zero compared to the assay buffer, although small refractive changes (< 10 RU) are usually observed in SPR assays. Buffer blanks should also not drift. If using a capture assay approach, any drift observed is usually caused by fluctuations in temperature and/or lose of surface integrity (e.g., the captured ligand is dissociating during the assay).

5.1.2 Binding Data

In addition to buffer blanks, the binding data should also be visually assessed prior to fitting to either a kinetic or affinity model. As shown in Figure 51, a visual check can reveal a large amount of information from a binding response curve. In general, the features described below are universal to BLI and SPR, although it is to be expected that the effects of refractive index changes are more likely to be experienced in SPR assays and should be optimized in advance (Section 4.6).

Before fitting the data, it is important to visually assess the following parameters:

- Stable baseline with no drift prior to the association phase (Section 4.5)
- Association phases are free from mass transport limitation (Section 4.11)
- Minimal to no refractive index jumps; ideally association and dissociation should connect after referencing (Section 4.12)
- The top analyte concentration should reach saturation (where possible) and curves below should contain sufficient curvature (Section 4.7.1)
- The association curves are spaced out appropriately and cover a wide concentration range (Section 4.7.2)
- The dissociation phase is long enough to allow sufficient response change for accurate fitting (Section 4.7.4)
- The observed response is low, such that R_{max} is low and avidity effects are minimized (Sections 4.4.3 and 4.11)
- Replicates response curves overlay with each other

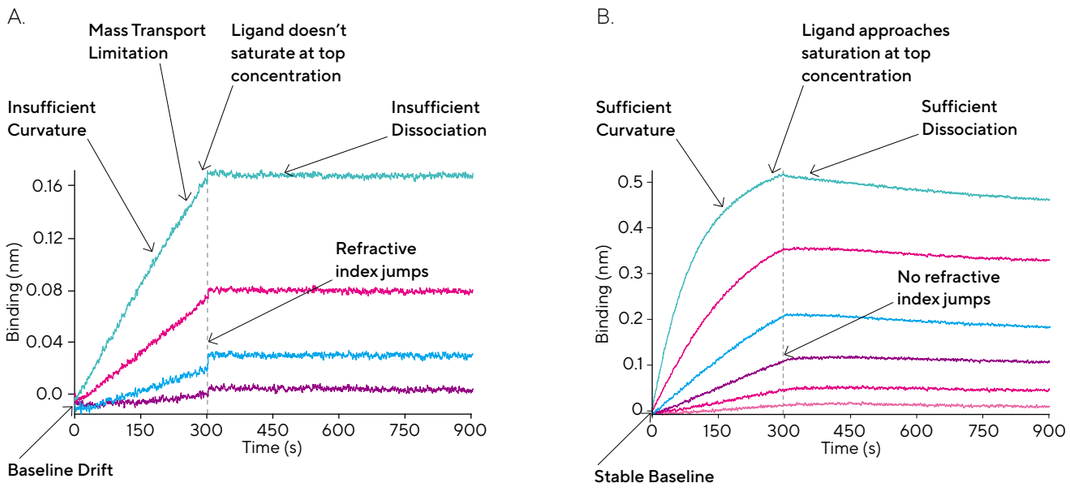


Figure 51

Visual Checks Form a Critical Part of Data Analysis and Should Always Be Performed Prior to Fitting

Features of poor data are shown in (A), while features of good data are shown in (B).

5.1.3 Kinetics or Affinity?

As discussed in Section 3.4, the requirements for fitting to a kinetic or affinity model can be different and a visual check of the data should be performed prior to fitting. As shown in Figure 52, a kinetics model should only be fitted to data that contains a concentration series with signals that have sufficient curvature during the association phase and decrease during the dissociation phase (Figure 52A–B). Kinetic models should not be applied to response curves with rapid association and dissociation phases because they lack the requisite curvature for determining kinetics (Figure 52C).

A common error by BLI and SPR users is the application of a steady state affinity model to data that is not appropriate to the model. As shown in Figure 52, an important caveat for equilibrium analysis is that all concentrations must reach equilibrium (number of associations events is the same as dissociation events). At equilibrium the response of the complex is directly proportional to that of the analyte concentration, which is when an accurate equilibrium dissociation constant can be derived.

In general, a single assay cannot yield kinetic and steady state affinity data as interactions that are fast enough to reach steady state often display little curvature, which is required for accurate kinetic assessment.

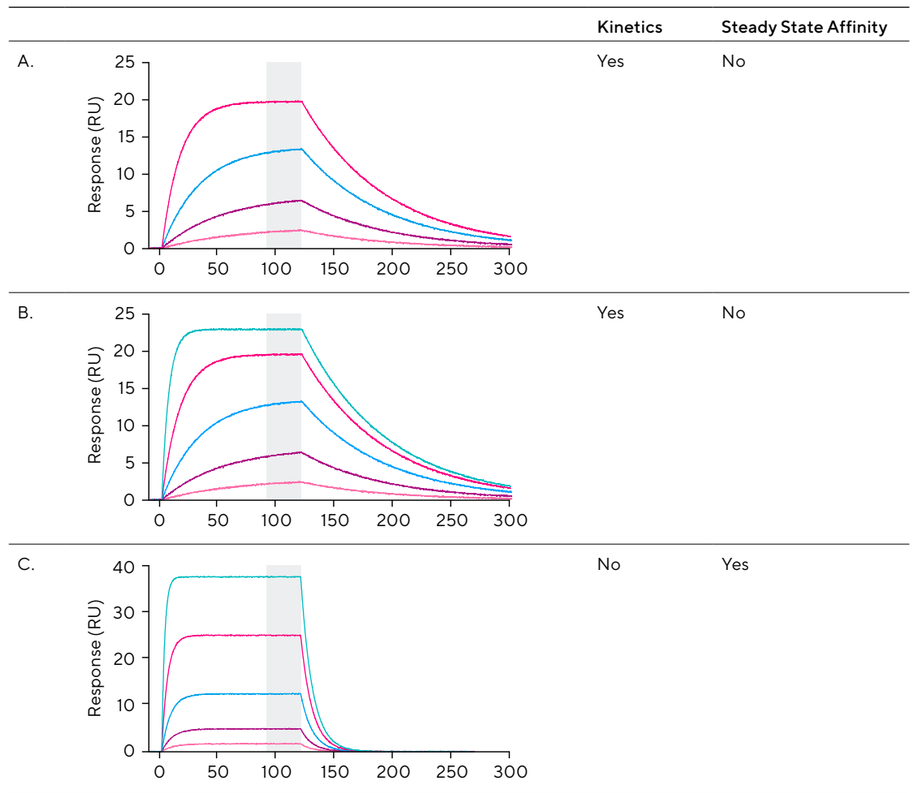


Figure 52

It Is Important That Only the Correct Model Is Fitted to Data and Model Hunting to Fit the Data Is Not Performed

Figures (A) and (B) do not reach equilibrium at all of the analyte concentrations and therefore, steady state affinity analysis is not suitable and a kinetic model should be assessed. Figure (C) reaches equilibrium at all analyte concentrations, but exhbits a rapid association phase that makes kinetic assessment unreliable and a steady state model should be assessed.

5.1.4 Choosing an Appropriate Model

Both Octet® SPR and BLI Analysis software offer a variety of models based on the heterogeneity of the analyte or ligand, but as discussed in Section 4.10, assay optimization should always be driven to the simplest model possible. Therefore, a 1:1 binding model is appropriate, but depending on the binding molecules in question, more complex models may be observed. More complex models should not be used to generate a better data fit (unless the interaction is expected to be none 1:1) as this leads to overfitting of the data and false conclusions about the kinetics and affinity of the interaction may be drawn.

A general workflow for fitting and assessing data is described below:

- Select a model for data analysis ranging from a 1:1 binding model to a heterogenous binding model. For most binding pairs, the 1:1 model is appropriate, however, depending on the binding molecules in question, more complex models may be observed.
- First fit the data using the local fit model.
- Evaluate the kinetics output for each sample concentration to determine if outliers exist that may not satisfy acceptable statistical parameters. Ideally, the kinetics parameters should pass the coefficient correlation requirement of > 95%.
- If outlier analyte concentrations have been determined and eliminated, the assay should be repeated in replicates using the acceptable analyte concentrations. The new data should be fit to the global fit algorithm to determine the reportable association and dissociation constants in addition to the global affinity.

5.1.5 Visual Assessment of Data Fitting

Visual assessment of the fitted data is critical to ensure that the assay design was suitable to generate the desired results. Check whether the fitted model line (Figure 53A) overlays well on the assay data or if there are systematic differences in the fitted data to the assay data. If the fitted model line overlays the assay data, then it is a suitable model (as above the 1:1 model should, in general, be assessed first). If the fitted model line does not overlay the assay data, then alternative models may need to be assessed. However, in general, assay optimization should be performed where a 1:1 interaction is expected.

If the fitted model line does overlay the assay data, then the accuracy of the data fit is shown visually in the residual plots (Figure 53). Residuals are a representation of a fit of the mathematical model to the experimental data (in simple terms, a measure of the distance between the data fit and the assay data). As shown in Figure 53B for MCK, the residuals are not randomly distributed, indicating the 1:1 model does not accurately explain the assay data. In comparison, Figure 53A residuals show random distribution around zero and the 1:1 model is an appropriate fit.

After visual assessment of the fit, various statistical parameters can be used to further analyze the fit including X^2. This is a measure of error between the experimental data and the fitted line, where a smaller X^2 indicates a better fit and R^2 indicates how well the fit and the experimental data correlate. In general, R^2 values above 0.95 are considered as a good fit for large molecule kinetics.

In the event that the model fit is not of high quality, repeat the visual data checks described in section 5.1.1–5.1.3 and check the reference surface for any unexpected non-specific binding as binding to the reference surface causes complications in correctly reference subtracting the data for assessment. Where non-specific binding is not observed, the analyte sample should be checked for the presence of contaminants. As outlined in Section 4, performing in-depth assay development should minimize any issues at the data analysis stages.

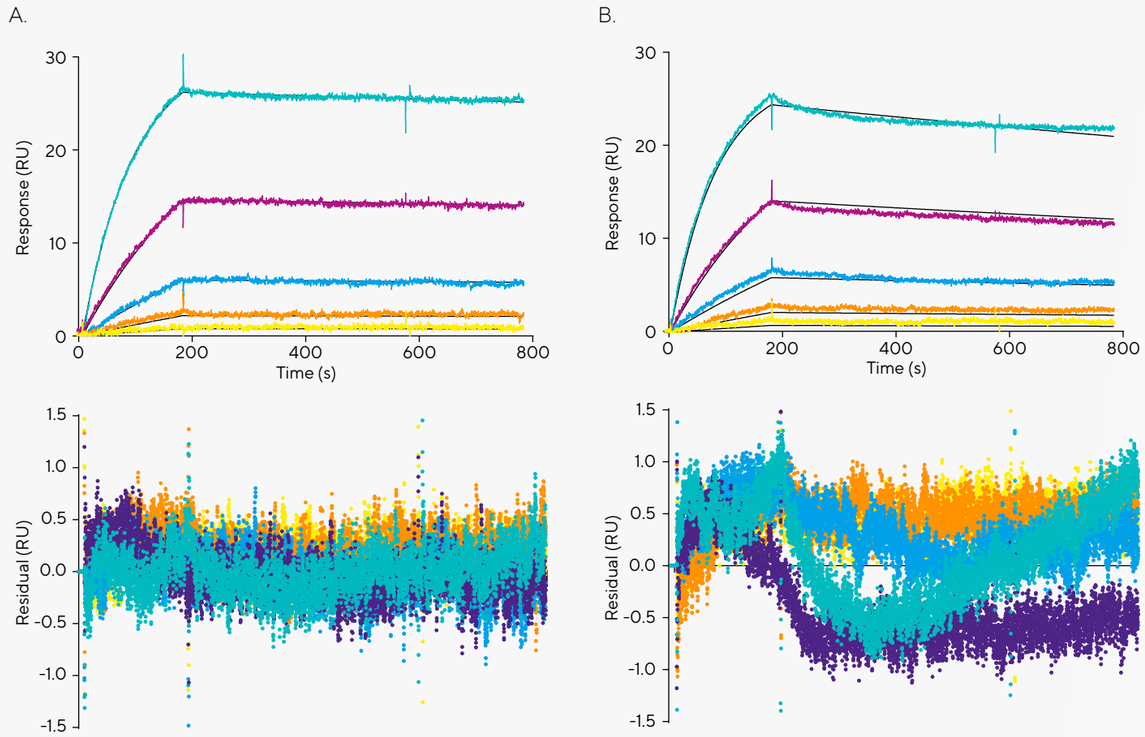


Figure 53

Comparison of the Assay Data to the Fitted Model

(A) Shows a good model fit line overlay of the assay data and random distribution of the residual around zero, indicating a good assay design. (B) A poor model fit line overlay of the assay data and inspection of the residuals shows non-random distribution, indicating more assay development may be required.

5.2 Binding Models

5.2.1 1:1 Kinetics

In a 1:1 bimolecular interaction, both the association and dissociation phases display a time-resolved signal that is described by a single exponential function. Analyte molecules bind at the same rate to every ligand binding site. The association curve follows a characteristic hyperbolic binding profile, with exponential increase in signal (association) followed by a leveling off to plateau as the binding reaches equilibrium (steady state) and the dissociation curve follows single exponential decay with the signal eventually returning to baseline. (Figure 54). Each of these phases can be described using appropriate equations.

Equation 3, k_a

$$R_t = \frac{R_{max}[A]}{K_D + [A]}\left(1 - \frac{1}{e^{(k_a[A] + k_d)t}}\right)$$

Equation 9, k_D

$$R_t = \frac{R_{max}[A]}{K_D + [A]}$$

Equation 4, k_d

$$R_t = R_0 e^{-k_d t}$$

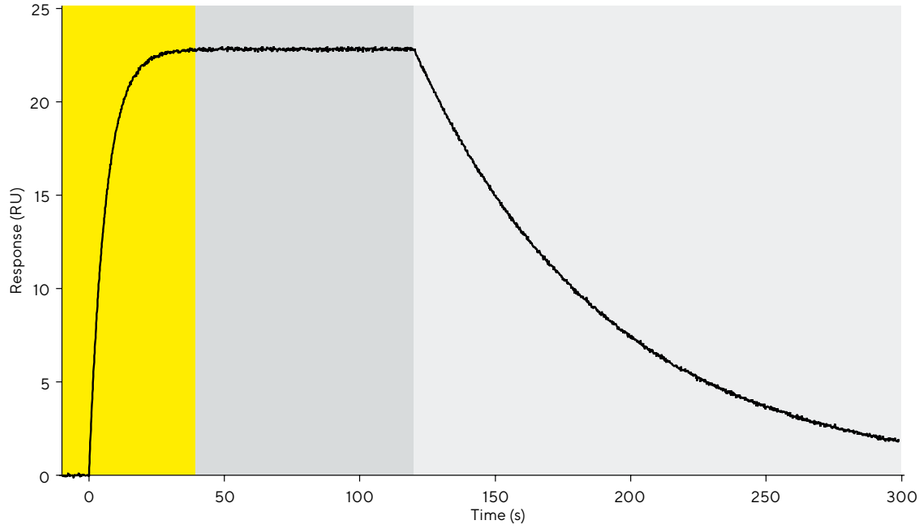


Figure 54

Basic 1:1 Fitting Consists of up to Three Phases: Association, Steady State Equilibrium, and Dissociation

Each of these phases can be defined by a set of basic parameters including the concentration of the analyte and the ligand and the maximal response. From this the kinetic rate constants k_a, k_d and the global affinity K_D can be derived.

The full fitting solution for a 1:1 binding is:

Association phase:

$$R_t = \frac{R_{max}[A]}{K_D + [A]}\left(1 - \frac{1}{e^{(k_a[A] + k_d)t}}\right)$$

Equation 3

Dissociation phase:

$$R_t = R_0\, e^{-k_d t}$$

Equation 4

Unlike multi-cycle kinetics, OneStep® injections do not rely on discrete concentrations to determine kinetics and affinity, but produce a concentration gradient of analyte during the injection. With this in mind, the [Analyte] parameter shown in Equation 3 is not appropriate for OneStep® injections and an alternative model is required:

5.2.2 1:1 Kinetics—OneStep®

The two-compartment model is fundamental to SPR and is a simple model that treats binding as a two step process. In step one, the analyte must be transported to the sensor surface, and in the second step, the analyte interacts with the ligand on the sensor surface (Figure 55). In the two-compartment model the flow cell is divided into two imaginary compartments (Ct and C). Within each compartment the concentrations are uniform in space, but may change in time; for example in multi-cycle kinetic (MCK) injections the concentration of C is initially 0, but over time will increase such that C = Ct.

The concentration in the inner compartment, C, changes because analyte is transported between compartments and binds to, and dissociates from, immobilized ligands on the sensor surface (Figure 55), a process that depends on the mass transport coefficient (k_m).

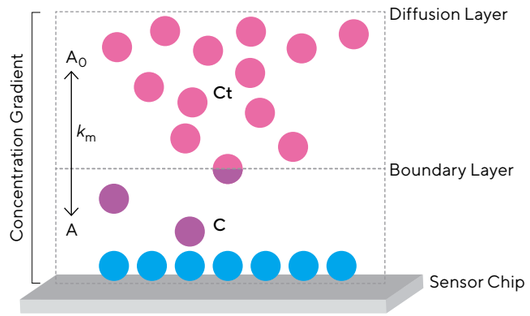


Figure 55
In the Two-Compartment Model the SPR Flow Cell Is Viewed as Containing Two Compartments (Ct and C)
Within each compartment the concentrations are uniform in space, but may change in time. The mass transport coefficient (k_m) describes the diffusive movement of the analyte between the two compartments, and when the association rate of the analyte binding to the ligand is fast compared to the rate of transport, then transport effects influence the observed kinetics and mass transport limitation is observed (Section 4.11).[1]

It is important to bear in mind that the physical process of forming the analyte gradient for OneStep® injections is not linked to the subsequent binding interaction at the sensor surface. Therefore, OneStep® injections observe fundamental parameters as multi-cycle kinetics (*i.e.*, the binding kinetics between the analyte and the ligand are the same and obey the two-compartment model).

However, during OneStep® injections, the progress of the binding interaction is strongly influenced by the availability of analyte (*i.e.*, analyte concentration), which, unlike MCK, changes over time due to dispersion. The analyte concentration profile observed during OneStep® injections is encoded into the resulting analyte binding response curves and, therefore, inclusion of the appropriate Taylor dispersion expressions into the biomolecular binding interaction model of interest accurately accounts for the dispersion process. Users wanting a deeper understanding of OneStep® injections are recommended to read the seminal papers by John Quinn on the use of Taylor Dispersion Injections in SPR.[2,3]

The two-compartment 1:1 pseudo-first-order kinetic interaction model for OneStep® injections is composed of two differential equations (Equation 5 and 6)

- Analyte concentration gradient (dC/dt) as the analyte passes from the bulk liquid through the diffusion boundary layer to the sensing surface (Equation 5)
- Change in the concentration of affinity complexes (dR/dt) at the sensing surface (Equation 6)

$$\frac{dC}{dt} = (-k_a C\,(R_{max} - R) + k_d R + k_m\,(C_{in} - C))$$

Equation 5

$$\frac{dR}{dt} = (k_a C\,(R_{max} - R) - k_d R)$$

Equation 6

In addition to the common parameters (R, R_{max} and km), additional parameters C_{in} (injected analyte concentration (M) (C_{in} = 0 in dissociation)) and C, the concentration (M) of the analyte at the sensor chip surface as shown in Figure 55.

In order for C_{in} to be expressed as a function of time, dispersion terms are included in an affinity interaction model to define the analyte concentration (C) within the flow cell as a function of injection time (Equations 7 and 8), where *erf* is the error function (Gauss error function) for a sigmoid and τ is the mean analyte residence time.

$$C(t) = \frac{C_{in}}{2}\left[1 - erf\,\frac{1 - \frac{t}{\tau}}{2\sqrt{\frac{k}{uL}\frac{t}{\tau}}}\right] \qquad (t < \tau)$$

Equation 7

$$C(t) = \frac{C_{in}}{2}\left[1 + erf\,\frac{1 - \frac{t}{\tau}}{2\sqrt{\frac{k}{uL}\frac{t}{\tau}}}\right] \qquad (t > \tau)$$

Equation 8

The resultant concentration (C) can then be substituted into Equations 5 and 6 to determine the required parameters.

5.2.3 Steady State/Equilibrium Analysis

Under steady state interaction conditions the overall rate of binding is 0 (dR/dt = 0). As analyte is continuously available the system is under steady state and the equilibrium constant can be determined from the ratio of free analyte ([A]) and ligand ([B]) to the bound complex form ([AB]).

As shown in Equation 3

$$R_t = \frac{R_{max}[A]}{K_D + [A]}\left(1 - \frac{1}{e^{(k_a[A] + k_d)t}}\right)$$

The (R_{max}[A]/K_d +[A]) parameter defines at what level equilibrium will occur, while the (1- 1/e(k_a[A] + k_d)t) defines how long the system will take to reach equilibrium.

Therefore, as equilibrium has been achieved, the equation can be simply arranged as Equation 9.

$$R_t = \frac{R_{max}[A]}{K_D + [A]}$$

Equation 9

5.2.4 Dissociation Kinetics

Dissociation kinetics can be independently determined from association kinetics, which allows off rate ranking. This is simply achieved by using Equation 4 above when applied to the dissociation phased of an interaction.

5.2.5 2:1 Heterogeneous Ligand

The 2:1 heterogeneous ligand model assumes analyte binding at two independent ligand sites (Equation 10). Each ligand site binds the analyte independently and with a different rate constant. Two sets of rate constants are given, one for each interaction where A represents the analyte and B represents the immobilized ligand:

$$A + B1 \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} AB1$$

$$A + B2 \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} AB2$$

Equation 10

Mathematically, the equation used to fit a 2:1 binding interaction is a combination of two 1:1 curve fits, with an additional parameter to account for percentage of binding contributed by each interaction.

Note: When the 2:1 heterogenous ligand model is used, the fitting results are presented such that binding site 1 will be the higher-affinity site, and binding site 2 will be the lower-affinity site.

5.2.6 Mass Transport

As previously discuss in sections 4.11 and 5.2.2, in a fluidics-based system, samples pass over the sensor chip surface via laminar flow, where frictional forces from the sides of the tubing and the surface of the biosensor slow the velocity of the liquid close to the surface. In such a system, the decreased flow rate immediately adjacent to the biosensor inhibits efficient exchange of analyte molecules from the surface to the bulk solution. At low concentrations, analyte molecules present near the biosensor surface can bind to the ligand faster than they can be replaced in the surrounding solution (Equation 11). When this occurs, the binding rate becomes dependent on supply of analyte molecules rather than the actual kinetics of the interaction. The shape of the binding curve is determined by the rate at which the analyte diffuses to the surface and changes with flow rate.

As flow increases, so does the apparent binding rate, as the supply of analyte molecules available to bind increases.

$$A_{bulk} \underset{k_m}{\overset{k_m}{\rightleftharpoons}} A_{surface} + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

Equation 11

Mass transport-limited curves are often less steep than expected. Mass transport-limited data can be fit using the Mass Transport binding model, which fits the binding of the analyte taking into account two steps:

- Rate of transport of the analyte from the bulk solution to the surface (k_m)
- Interaction of the analyte with the ligand

Diffusion limitation occurs when k_m is much smaller than k_a[B]:

$$\frac{dR}{dt} = \frac{k_a[A]}{1 + \frac{k_a}{k_m}(R_{max} - R)}(R_{max} - R) - \frac{k_d}{1 + \frac{k_a}{k_m}(R_{max} - R)}R$$

Equation 12

5.2.7 1:2 Bivalent Analyte

A bivalent analyte is one that can bind to one or two ligand molecules. The 1:2 bivalent analyte model fits the binding of one bivalent analyte to a monomeric immobilized ligand. Kinetic parameters are calculated for two interactions (k_{a1}, k_{a2}, k_{d1}, k_{d2}, K_{D1}, K_{D2}).

$$A+B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} AB \longrightarrow AB + B \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} AB_2$$

Equation 13

This model assumes that because of limited distance between two adjacent binding sites on the sensor surface, the bivalent analyte can form a bridged complex. This interaction is linked, meaning that the formation of AB2 complex cannot occur before the formation of AB, and AB cannot dissociate before the dissociation of AB2. This avidity effect results in a slower apparent dissociation rate than would be expected if the interaction followed a 1:1 binding profile.

Two sets of rate constants and K_D values are reported using the 1:2 bivalent analyte model. The first set of values reflects the binding due to the affinity of the interaction. The second set of values represents binding due to avidity.

Three equations are used to fit bivalent analyte curves, the first describing association of A to B (Equation 14), the second is the association of AB to B (Equation 15), and the third describes dissociation of the AB2 complex (Equation 16).

$$\frac{dB}{dt} = -(2k_{a1} * [A] * B - k_{d1} * AB) - (k_{a2} * AB * B - 2k_{d2} * AB_2)$$

Equation 14

$$\frac{dAB}{dt} = -(2k_{a1} * [A] * B - k_{d1} * AB) - (k_{a2} * AB * B - 2k_{d2} * AB_2)$$

Equation 15

$$\frac{dAB_2}{dt} = -(k_{a2} * AB * B - 2k_{d2} * AB_2)$$

Equation 16

As discussed in Section 4.2 and 4.11, changes in the assay format and conditions can reduce avidity effects in a binding interaction. One approach is to lower the density of the immobilized ligand by decreasing ligand concentration or decreasing the loading step time.

A lower ligand density effectively increases the distance between molecules on the surface, minimizing the likelihood of a bound analyte reaching adjacent molecules. This approach may require some optimization because lowering the ligand density decreases the sensitivity of the assay. Alternatively, reversing the assay orientation so that the bivalent molecule is immobilized on the surface eliminates the possibility of analyte bridging. The 1:2 binding model is useful when the bivalent molecule cannot be captured due to issues related to instability under conditions of immobilization, lack of sensitivity in the opposite assay orientation, reagent availability, or if an interaction needs to be tested in multiple formats.

5.2.8 Two-State (SPR Only)

This model describes the 1:1 interaction between an analyte and ligand, where the analyte-ligand complex subsequently undergoes a conformational change. Assumptions of this model are that the conformational change only occurs in the complex AB and not in the ligand alone; and that conformationally-changed complex does not dissociate freely, but must undergo the reverse conformational change first. The binding model is governed by the following set of differential equations:

$$\frac{dB}{dt} = -(k_a * A(t) * B - k_d AB(t))$$
$$B(0) = R_{max}$$

Equation 17

$$\frac{dAB}{dt} = (k_a * A(t) * B - k_d * AB(t)) - (k_{a2} * AB(t) * B - k_{d2} * AB2(t))$$
$$AB(0) = 0$$

Equation 18

$$\frac{dAB2}{dt} = k_{a2} * AB(t) - k_{d2} * AB2(t)$$
$$AB2(0) = 0$$

Equation 19

where:

A(t) = Molar concentration of analyte at the interaction surface at time t

B = Available immobilized ligand

AB(t) = Concentration of complex (analyte-ligand binding) at the interaction surface at time t

AB2(t) = Concentration of complex (analyte-ligand in second state) at the interaction surface at time t

k_a = Association rate constant (single analyte-ligand binding ($M^{-1}s^{-1}$))

k_d = Dissociation rate constant (single analyte-ligand binding (s^{-1}))

k_{a2} = Association rate constant for formation of conformationally changed complex

k_{d2} = Dissociation rate constant for formation of conformationally changed complex

5.2.9 Diffusion (SPR Only)

The diffusion model can be used to fit the apparent diffusion coefficient of an analyte using only the refractive index signal when a diffusion-based injection (*i.e.,* OneStep®) is used. This model assumes a linear relationship between concentration and SPR response exists (*i.e.,* non-binding refractive index response). Therefore, this model should not be used for interactions where kinetic or affinity binding is occurring. For most biomolecules, a concentration of >0.1 mg/mL would be necessary to observe a reliable response. Concentrations of >100 mg/mL analyte can be tested unless significant viscosity or precipitation is an issue.

The fitted parameters for the diffusion model are R_{max} (RU) and D_{app} (m^2/s). R_{max} represents the signal at maximum concentration, which is observed for sigmoidal OneStep®, but is not observed for OneStep® Pulse. Since refractive index is expected to be linear to analyte concentration, R_{max} in this context can be explained as:

$$R_{max} = C * \eta d$$

Equation 20

where:

C is analyte concentration (typically in mg/mL) and ηd is refractive index increment (RU*mL/mg). Alternative units can be used where available. This can be viewed as a quality control metric for the sample response, which should give an R_{max} value within the correct range for a known concentration and ηd.

5.2.10 Aggregation (SPR Only)

The aggregation model can be used to quantify the relative concentrations and diffusion coefficients of components in a bi- or poly-disperse sample using only refractive index signal from a diffusion-based injection (*i.e.,* OneStep®). The basic assumption with the aggregation model is that the sample comprises at least two analyte components that differ by diffusion coefficient (*i.e.,* monomer and heptamer components, etc.). For the aggregation model, the total analyte concentration and the analyte refractive index increment (n_d or dndc) must be known. Fitted parameters of the aggregation model are refractive index increment of monomer ($dndc_m$), diffusion coefficient of monomer (D_m), concentration of aggregate species (C_a), refractive index increment of aggregate species ($dndc_a$), and diffusion coefficient of aggregate species (D_a).

Output parameters from the aggregation model are concentration of monomer species (C_m) and percent aggregate (% agg). Ideally $dndc_m$ and to a lesser extent, $dndc_a$, should be known. Dndc for proteins >10 kDa can typically be assumed to be 188.8 RU*mL/mg and predictions for a wide panel of proteins have shown a range of 180–200 RU*mL/mg.[4] A wider distribution is seen for smaller proteins, but the mean dndc is still similar at 190.2 RU*mL/mg. Note that the Aggregation model enables a separate Retention term for monomer (RF_m) and aggregate (RF_a).

1. Myszka, D. G., He, X., Dembo, M., Morton, T. A., Goldstein, B. (1998). Extending the range of rate constants available from BIACORE: interpreting mass transport-influenced binding data. *Biophysical Journal*, 75(2), 583-594.
2. Quinn, J. G. (2012). Modeling Taylor dispersion injections: determination of kinetic/affinity interaction constants and diffusion coefficients in label-free biosensing. *Analytical Biochemistry*, 421(2), 391–400.
3. Quinn, J. G. (2012). Evaluation of Taylor dispersion injections: determining kinetic/affinity interaction constants and diffusion coefficients in label-free biosensing. *Analytical Biochemistry*, 421(2), 401–410.
4. Zhao, H., Brown, P.H., Schuck, P. (2011). On the distribution of protein refractive index increments. *Biophysical Journal*, 100(9), 2309-2317.

6 References

A number of Octet® publications are referenced within this applications guide but it is not intended as an exhaustive list. For access to the most up to date list of Octet® BLI and SPR publications please visit:

- https://www.sartorius.com/en/pr/octet
- https://www.sartorius.com/en/products/protein-analysis/octet-surface-plasmon-resonance

Octet® information is also available on the Protein Analysis YouTube playlist:

- Protein Analysis—YouTube

6.1 BLI Information

6.1.1 Videos

BLI

- Drop. Read. Done: Label-Free Protein-Protein Interaction Analysis
- Novel High-Throughput Glycoanalytics for Biopharmaceuticals
- Octet® Platform: Bio-Layer Interferometry (BLI) Technology Introduction
- Octet® Platform: BLI Applications
- Octet® Platform: Instrument Showcase
- Octet® RH16 and RH96 | High Throughput Monitoring of Binding of Proteins and Other Biomolecules
- Rapid and Real-Time Results for the Study of Viral Biology and Development of Antiviral Therapies

SPR

- Octet® SF3 SPR—Powered and Prepared with Accurate High-Throughput Surface Plasmon Resonance

6.1.2 Application Guides

- Accurate Measurements on the Octet® Platform Without Added Sample Purification Steps
- Advancing Development of Coronavirus Vaccine and Therapeutics with Octet® Bio-Layer Interferometry Systems
- Cell Line Development: Antibody Discovery by Monitoring Titer & Glycosylation with Octet®
- Characterizing Membrane Protein Interactions by Bio-Layer Interferometry
- Expanding Octet® Applications in Downstream Biologics Characterization: Stability, Formulations and Aggregations Studies
- Label-Free Technologies for Accurate Determination of Affinity and Kinetics Rate Constants
- Octet® Bio-Layer Interferometry as a Tool for Determining Nanoparticle Vaccine Construct Design, Stability and Antigenic Efficiency
- Octet® Kinetics Assay Method Development Guideline
- Octet® Systems in Bioprocessing: Easy-to-Use and Cost-Effective Tools for Multiple Applications
- Octet® Systems in Ligand Binding Assays That Meet Compliance
- Octet® Systems Modernize Biopharmaceutical QC Testing to Increase Efficiency
- Recent Insights into Covid-19 Binding Epitopes
- Simplifying Biotherapeutic Manufacturing and Quality Control with Label-Free Biosensor Technology

6.1.3 Application Notes

- A Fast and High Precision Influenza Vaccine Potency Assay
- A Rapid Method to Quantitatively Screen Bispecific Antibodies Using Protein A and Octet® His1K Biosensors
- Analysis of Fc Gamma Receptor IgG Interactions on Octet® Platform
- Analysis of FcRn-Antibody Interactions on the Octet® Platform
- Best Practices for Performing Quantitation Assays Using the Octet® N1 System
- Biomolecular Binding Kinetics Assays on the Octet® Platform
- CaptureSelect Affinity Ligands for Antibody Detection and Characterization
- Cell Line Development: Accelerating Process Optimization by Combining Ambr® 15 Cell Culture with Octet® Titer Measurements
- Converting an ELISA Assay into an Octet® Quantitation Assay
- Cross-Competition or Epitope Binning Assays on the Octet® RH96 System
- Customized Quantitation of Recombinant Therapeutic Proteins Using High Precision Octet® Streptavidin Biosensors (SAX)
- Enhancing Efficiency and Economics in Process Development and Manufacturing of Biotherapeutics
- Fast Quantitation of Proteins and Antibodies Using the Octet® N1 System
- Generating Reliable Kinetic Data for Protein-Ligand Interactions
- Instant Determination of Protein Presence Using the Octet® N1 System
- MAb Quantitation Protein A HPLC vs. Protein A Bio-Layer Interferometry
- Mannose Glycans Content Screening for Human-mAb Samples Using the Octet® GlyM Kit
- Octet Potency Assay Development Qualification and Validation Strategies
- Optimizing Kinetics Assays to Avoid Avidity Effects
- Optimizing Protein-Protein and Protein-Small Molecule Kinetics Assays
- Rapid, Automated, At-Line AAV2 Virus Quantitation Advances Bioprocessing in Gene Therapy
- Reducing Variability in Small Molecule Screening and Kinetics Applications
- Second Generation Anti-Human IgG Fc Capture Biosensors for Affinity Characterization of Human IgG-Antigen Interactions and Quantitation of Human IgGs or Human Fc Region Containing Proteins
- Strategies for the Development of a High Throughput Octet® Bio-Layer Interferometry Method to Measure Pharmacokinetics of Monoclonal Antibodies in Preclinical Animal Models
- Validated Quantitation and Activity Assay of Antibody Fragment Molecule (Fab) for Process Development and Quality Control

6.1.4 Brochures

- Label-free Assays in a Drop
- Octet® R Series Systems
- Octet® Systems Platform Brochure

6.1.5 Case Studies

- Greatest Hits: A Move to Bispecific Antibodies: From Research to Clinical Application

6.1.6 eBooks

- Accelerating the Development and Manufacture of Therapeutics Using the Octet® Platform
- Accelerating Vaccines and Virus Research
- How Are Label-Free Technologies Used in Early Drug Discovery?
- Label-Free Detection Technologies: Key Considerations and Applications
- Overcoming the Challenges of Infectious Diseases with Label-Free Approaches

6.1.7 Editorials

- Addressing the Challenges of Quality Control Labs in Vaccine Manufacturing
- Therapeutic Antibodies: Advancing the Development of COVID-19 Neutralizing Antibodies

6.1.8 Flyers

- Octet® BLI Biosensor Selection Guide
- Octet® R Series of Systems
- Octet® Service Contracts
- Quality Control of Biotherapeutics Using Octet® Systems
- Top Ten Reasons to Choose Sartorius Octet® BLI Technology to Accelerate Your Research, Development, and Manufacturing

6.1.9 Infographics & Posters

- AAV Capsid Titer Data 80% Faster Compared to ELISA
- High productivity and process economy in GxP applications with Octet
- Influenza Vaccine Titre Determination Bio-Layer Interferometry (BLI)
- Octet® Bio-Layer Interferometry (BLI) Technology
- Octet® BLI Systems—Unmatched Versatility for Discovery, Development and Quality Control
- Understanding Covid-19 Vaccine and Therapeutic Development Research—Infographic

6.1.10 Technical Notes

- Biotinylation of Protein for Immobilization onto Streptavidin Biosensors
- CHO Host Cell Protein Detection
- High Precision Streptavidin Biosensor (SAX) Quantitation and Kinetic Assays
- Host Cell Protein Detection
- Octet® AAVX Biosensors for Quantitation of AAV Capsids
- Octet® ACH2 Biosensors For Quantitation and Kinetic Characterization of Human Fc-Region Containing Proteins
- Octet® Amine Reactive Second-Generation (AR2G) Biosensors
- Octet® Amine Reactive Second-Generation (AR2G) Reagent Kit
- Octet® Anti-Mouse IgG Fc Capture (AMC) Biosensors
- Octet® BLI Quantitation Assays: Method Development Guideline
- Octet® GlyM Kit
- Octet® GST Biosensor Kinetics Assays
- Octet® GST Biosensor Quantitation Assays
- Octet® HIS1K Biosensors for Label-free Analysis of His-tagged Proteins
- Octet® HIS2 Biosensor Quantitation Assays
- Octet® NTA Biosensor Kinetic Assays Technical Note
- Octet® NTA Biosensor Quantitation Assays Technical Note
- Rapid Analysis of Fab Fragments and IgG with Octet® FAB2G Biosensors
- Ready BLI Detection Kit—Residual Protein A
- Regeneration Strategies for Streptavidin Biosensors on the Octet® Platform
- Small Molecule Binding Kinetics

6.1.11 Webinars

- Accelerating Drug Discovery with High-Throughput Biomolecular Interaction Analysis
- Bio-Layer Interferometry as a Strategic Platform to Validate Covalent Proximity Inducing Small Molecules With Synthetic Tumor Immunotherapeutic Applications
- Cell Surface SARS-CoV-2 Nucleocapsid Protein Modulates Host Immunity
- CHO for Accelerated Antibody Development
- Developing Methods for Comparability Studies of Therapeutic Monoclonal Antibodies
- Fragment Based Drug Discovery of Co-Factor Inhibitors for p97
- Integrating Octet® BLI into Early Antibody Discovery Workflows
- Novel High-Throughput Glycoanalytics for Biopharmaceuticals
- Optimers™—Future Ready Antibody Mimetics
- Rapid Characterization and Selection of SARS-CoV-2 Vaccine Candidates by Bio-Layer Interferometry (BLI) Technology
- Utilizing Octet® Platform for Characterizing Critical Reagents in Support of Drug Development for Contemporary Biotherapeutics

6.1.12 White Papers

- Enhanced Productivity and Labor Efficiency in Lot Release and In-Process Testing of Biologics in GxP Laboratories
- Instrument Comparability Assessment: Kinetics Precision Assessment in Ligand Binding Assays on the GxP-Compliant Octet® RED96e and Octet® R8 Instruments
- Investigating Synthetic Immune Recruitment by Proximity Inducing Molecules: Validation of Covalent Immune Recruiter (CIRs) Function Using the Octet® Platform
- Streamlining Affinity Analysis for Accelerated Lead Screening, Characterization, Optimization and Final Selection
- The End of End-Point Assays
- Writing Grant Proposals for Biomolecular Interactions Research

6.2 SPR Information

6.2.1 Application Notes

- Fragment-Based Drug Discovery (FBDD) Using Octet® SPR OneStep® and NeXtStep™ Injections
- Kinetic Curvature: Assessment of Small Molecule Kinetics and Affinity Using OneStep® Injections in SPR Screening
- Kinetics Determination of High Affinity Molecular Interactions Using OneStep® Injections
- OneStep® Lead Characterization of High Affinity Biologic Interactions

6.2.2 Best Practice Guides

- Double Reference Subtraction
- Minimize Artifacts
- Minimizing the Effects of Non-Specific Binding
- Octet® SF3 Assay Orientation
- Octet® SF3 System Maintenance
- Octet® SPR Sensor Chip Preparation

6.2.3 Brochures

- Octet® SF3 Instrument Brochure
- Octet® SPR Sensor Chips

6.2.4 Flyers

- Octet® SF3 Consumables
- Octet® SF3 SPR Instrument
- Octet® SF3 SPR Maintenance
- The Next Generation of SPR-Based Interaction Analysis

6.2.5 Technical Note

- Guidelines for OneStep® Assay Design

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