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How Low Can You Go?
A New Era of Sensitivity in Octet® BLI

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Abstract

Sensitivity in biophysical assays is a critical parameter, particularly in techniques like Biolayer Interferometry (BLI). Octet® BLI systems are widely used to study molecular interactions in real-time without the need for labeling, making them a powerful tool in drug discovery, protein-protein interactions, and biomolecular research. The sensitivity of BLI assays directly impacts the ability to detect and quantify low-abundance analytes, which is essential for accurate and reliable data.

Octet® BLI accommodates various sample types and experimental conditions, which makes it a flexible tool for diverse research applications. With enhanced sensitivity, Octet® BLI can provide the precision needed for intricate studies while maintaining its operational advantages, offering researchers a powerful alternative to SPR that combines efficiency, scalability, and ease of use.

This document highlights best practices for data generation across a range of compounds and molecular weights that highlights the increased sensitivity of the recently released Octet® R8e system, compared to legacy BLI systems.

Introduction

Detection of molecular interactions using biolayer Interferometry (BLI) has traditionally faced challenges with sensitivity compared to techniques such as Surface Plasmon Resonance (SPR) due to fundamental differences in BLI's detection mechanisms.

Sensitivity is a cornerstone of assays, driving advancements in biophysical research and enabling the discovery of novel biomolecular interactions. This inherent limitation in sensitivity means that BLI may struggle to detect low-abundance analytes

or weak interactions, which are often critical in detailed kinetic studies and drug discovery processes.

Octet® BLI offers several advantages that make it a preferable choice for researchers. One of the primary benefits of BLI is its operational simplicity and cost-effectiveness. BLI systems typically require less complex instrumentation and maintenance compared to SPR, making them more accessible and easier to use, especially for laboratories with budget constraints or those new to biophysical assays.

Thanks to recent advancements in Octet® BLI technology(Octet® R8e), sensitivity levels comparable to SPR can be achieved. In addition to detecting low-abundance analytes, sensitivity contributes to the precision and accuracy of measurements. It enables the differentiation between specific and non-specific binding (NSB), reducing background noise and enhancing the signal-to-noise ratio. This precision is crucial for obtaining reliable kinetic data and understanding the mechanistic aspects of interactions.

High sensitivity systems allow researchers to observe interactions at very low concentrations, which is crucial when dealing with scarce or precious samples. This capability is particularly important in the early stages of drug development, where detecting weak interactions can lead to the identification of potential therapeutic candidates. Moreover, sensitivity is vital for characterizing accurate binding kinetics and affinities, providing insights into the strength and duration of molecular interactions. Increased sensitivity also supports the study of complex biological systems, where multiple interactions may occur simultaneously, allowing for the detection of subtle changes in binding events, facilitating the exploration of dynamic processes in real-time.

The Octet® R8e allows users to choose between 96-well or 384-well sample microplates which increases the ability to perform high-throughput screening. This is a significant advantage as it allows researchers to analyze multiple samples simultaneously, accelerating the pace of experiments and enabling large-scale studies that are essential in drug discovery and development. This capability is particularly beneficial when screening compound libraries or studying numerous interactions in parallel.

Here, we demonstrate how with improvements across multiple modalities, in addition to increased sensitivity Octet® BLI is becoming the preferred choice for applications requiring high sensitivity and precision, particularly in scenarios where detecting minute changes in molecular interactions is crucial.

Materials and Methods

Material	Supplier	Product Number
10X Kinetics Buffer (KB)	Sartorius	18-1105
Octet® SAX Biosensors	Sartorius	18-5118
Octet® Evaporation Covers	Sartorius	19-0081
96-well, black, flat bottom microplate	Greiner Bio-One	655209
10X PBS	Gibco	14190-094
20X HBS-EP+	Teknova	H8022
Human IL-15 (Tag Free)	ACRObiosystems	IL5-H4117
Human IL-2 Receptor beta (CD122) (His & Avitag™)	ACRObiosystems	ILB-H82E3
Human Insulin Solution	Merck	I9278
Human Insulin Receptor (28-944) (CD220) (His & Avitag™)	ACRObiosystems	INR-H82E6
Opdivo®	Bristol Myers Squibb	Supplied by WEP Clinical
Human PD-1 (His & Avitag™)	ACRObiosystems	PD1 H82E4
Human GLP-1 Recombinant Protein (7 – 37)	PeproTech	130-08
Recombinant Human GLP1R Protein (His & Avitag™)	SinoBiological	13944-H49H-B
Vivaspin® 500 Centrifugal Concentrators	Sartorius	VS0102

Table 1: Materials required for performing binding assays.

Methods

All assays were performed in either 1X KB or 1X HBS-EBT, which was prepared from 20X HBS-EP+ supplemented with 1 mg/mL BSA.

All assays used Octet® SAX Biosensors and were hydrated for at least 10 minutes at room temperature prior to use in either 1X KB or 1X HBS-EBT in a 96-well, black, flat bottom microplate (Greiner Bio-One, 6552091). All assays were performed at 25 °C unless stated differently.

The Octet® BLI assay followed a general assay flow of:

Assay Step	Step Name	Time (s)	Shake Speed (RPM)
1	Baseline	60	1,000
2	Loading	Analyte Specific	1,000
3	Baseline	60	1,000
4	Association	Analyte Specific	1,000
5	Dissociation	Analyte Specific	1,000

Table 2: General Octet® BLI assay flow for Octet® R8e binding assays.

Ligand and Analyte Preparation

Low response assay, recombinant AviTag™ Human PD-1 was prepared to a final concentration of 0.063 µg/mL in 1X HBS-EBT. Opdivo® was buffer exchanged into 1X HBS-EBT using a Vivaspin® 500 centrifugal concentrator and a final analyte concentration series of 0.2469 – 20 nM used. Extended association assay recombinant AviTag™ Human PD-1 was prepared to a final concentration of 0.1 µg/mL in 1X HBS-EBT. Opdivo® was buffer exchanged into 1X HBS-EBT using a Vivaspin® 500 centrifugal concentrator and a final analyte concentration series of 0.0823 – 20 nM used. Avidity assay, a concentration series of recombinant AviTag™ Human PD-1 of 0.006 – 5 µg/mL in 1X HBS-EBT was prepared. Opdivo® was buffer exchanged into 1X HBS-EBT using a Vivaspin® 500 centrifugal concentrator and a final analyte concentration of 20 nM used.

Recombinant AviTag™ Human IL-2 Receptor beta was prepared to a final concentration of 2.5 µg/mL in 1X HBS-EBT and a concentration series of 2.06 – 500 nM Human IL-15 used.

Recombinant AviTag™ Human Insulin Receptor (28-944) was prepared to a final concentration of 2.5 µg/mL in 1X KB and a concentration series of 15.6 – 1,000 nM Human Insulin used.

A reference sample of assay buffer was used in all assessments in order to correct for baseline drift. Each assay step was also replicated using ligand-unloaded biosensors which allowed for double reference subtraction.

Assays were performed using an Octet® BLI R8e system using Octet® BLI Discovery Software version 13.1.0.25. Data was fitted using Octet® Analysis Studio software 13.1.0.38 to a global 1:1 model.

Results and Discussion

Establishing Optimal Conditions to Measure Binding

It is important to ensure that optimal assay conditions are used for different types of assays. For example, quantitation assays benefit from a high ligand load in order to optimize the sensitivity of the assay for low analyte concentrations. Kinetics and affinity assays on the other hand benefit from a much lower ligand concentration, which reduces the potential of mass transfer limitation and off-target binding and NSB occurring, which may lead to incorrect values being determined during data analysis.

As response levels are lowered in assays it is important that the aim of the assay are determined prior to assay development. If, for example, determination of accurate kinetics and affinity is desired then the assay development time and effort will in general need to be higher than if a quantitative assessment of a sample is required.

See **A Compendium for Successful BLI and SPR Assays** for a general approach to assay development.

For kinetics and affinity measurements at low response levels, best practices should be adhered to in order to generate the highest standard of data possible. These include:

- Assay buffer
- Analyte and ligand quality
- Ligand immobilization/capture level
- Double reference subtraction

Assay Buffer

In BLI, 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.

Although BLI is less refractively sensitive to changes in buffer conditions than techniques such as SPR, at lower response

levels differences between the assay buffer and sample containing buffer can cause refractive index (RI) issues (also called bulk shift). Therefore, it is important that the composition of assay and sample-containing buffer are as closely matched as possible. This is best ensured through use of purifying columns that allow the sample to be buffer exchanged into the same buffer as being used for the assay buffer.

The matching of assay buffer to sample buffer is important in kinetics and affinity assays as the determination of accurate kinetic parameters requires removal of any buffer effects from the observed response.

For further information on single and double reference subtraction see **Section 4.12 of A Compendium for Successful BLI and SPR Assays** and **Prioritizing Data Quality by Implementing Double Reference SubtractionDouble Reference Subtraction**.

Analytes and Ligands

Of primary importance at low response levels is the use of purified ligands and analytes as the observed response is directly related to binding at the biosensor surface, any non-specific or off-target binding will impact the overall observed response.

It is important that analyte concentration series are based upon assay development and that due to lower ligand levels, previously used analyte concentration series may be inappropriate for low-level assays. This may also apply to association and dissociation times as well.

Where possible, use orthogonal techniques to assay the concentration and aggregation of samples prior to use. All ligands and analytes should be as close to their native form as possible through the removal of aggregates.

Ligand Immobilization/capture Level

Use low ligand level immobilization and/or capture. This helps to reduce mass transport limitations and ensures that, due to a reduction in molecular crowding, affinity rather than avidity is determined during data analysis.

If using a capture approach, it is important to ensure that the capture is stable and the dissociation of the ligand from the biosensor does not affect the performance of the assay. This is assessed by capturing the ligand at the concentration used during the assay and assessing its dissociation in assay buffer.

Mass Transport Considerations

For binding to occur in Octet® BLI assays the sample must diffuse through the sample containing assay buffer to the biosensor surface through the process of mass transfer. As with SPR assay design, avoiding mass transfer limitation is vital in kinetics and affinity assays in order to determine accurate rate constants and therefore, affinity (for further information see **Section 4.11 A Compendium for Successful BLI and SPR Assays**).

A simple way to test whether the shake speed is appropriate for an assay is to perform the assay at multiple shake speeds and determine the effect on the association rate constant. As shown in figure 1, shake speeds below 1,000 RPM effect the binding rate of human IgG (hIgG) to Protein A biosensors but shake speeds greater than 1,000 RPM exhibit no such change. Therefore, shake speeds of ≥1,000 RPM are suitable for kinetics and affinity assays as there is no change in binding rates above that speed but below that value, the binding rate is affected by shake speed. Quantitation assays are less affected by shake speed as analysis is performed on equilibrium assessments.

Double Reference Subtraction

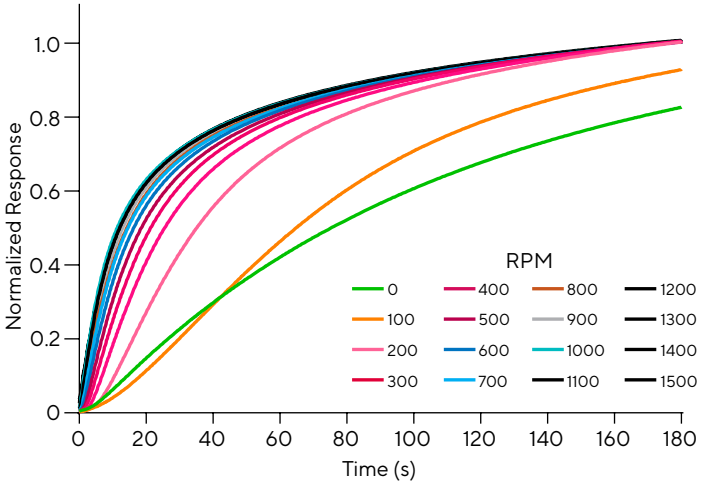

Figure 1: Effect of shake speed upon the binding rate and final response. Normalized response calculated for binding of 100 µg/mL Human IgG to for Octet® ProA biosensors in a 96 well microplate with a well volume of 200 µL.

As discussed above, as the response level is lowered the effect of off-target and non-specific binding (NSB) must be minimized and corrected for. Best practice is to perform double reference subtraction in order to control for the effects of the biosensor surface. For the purpose of double reference subtraction, the biosensors used to correct for NSB purposes should either contain a non-specific ligand that has a similar composition to that on the active biosensor or have no ligand present. For capture assays, the reference surface should be immobilized with the same capture molecule as the active (ligand capture) surface. For further information on single and double reference subtraction see **Section 4.12 of A Compendium for Successful BLI and SPR Assays** and **Prioritizing Data Quality by Implementing Double Reference SubtractionDouble Reference Subtraction**.

Optimizing Kinetics Assays to Avoid Avidity Effects

Affinity and avidity are key concepts in biomolecular interactions, particularly in antigen-antibody studies. Affinity measures the strength of a single binding interaction between a ligand and its analyte, typically described in a 1:1 interaction model. This provides a clear understanding of the kinetics parameters that define their behavior. Avidity, on the other hand, refers to the combined strength of multiple affinities between biomolecules, arising from multiple interaction sites. It is especially relevant when the analyte has multiple binding sites that can interact with the ligand simultaneously, forming a bridging complex. This is exemplified in antigen-antibody interactions, particularly when the antigen is immobilized on the biosensor as the ligand and the antibody is the analyte. See **Optimizing Kinetics Assays to Avoid Avidity Effects** for further information.

In non-1:1 interaction, such as those with immobilized antigens, interactions are linked, meaning the formation and dissociation of complexes are interdependent. Multiple binding interactions prevent the molecule from diffusing away upon transient unbinding, often resulting in unreliable association and dissociation rate constants.

A robust assay design for affinity aims to provide accurate measurements of kinetics parameters for a 1:1 binding interaction, promoting specific interactions unless avidity is the intended measurement.

To minimize avidity effects, as shown in figure 2 and table 3 using low immobilization levels of human PD-1 is beneficial, reducing the chance of one analyte (bivalent antibody Opdivo®) binding to multiple ligand molecules. Testing for avidity involves assessing kinetics over sensor surfaces with varying ligand levels, where reduced avidity is seen as increased dissociation rates. This approach helps determine accurate dissociation constants (k_d) for off-rate ranking.

Moreover, having the antigen as the antibody for screening purposes offers advantages. Low ligand levels reduce avidity issues, ensuring accurate kd determination for off-rate ranking, enhancing the reliability of assay results and providing valuable insights into binding dynamics. As shown in figure 2, it should also be noted that low ligand levels show significant curvature during the association phase, which allows accurate determination of association kinetics.

See section **It's All About Kinetics – Extended Association Time** for further information.

Loading Response (nm)	k_d (s^{-1})
1.48	$< 1.0 * 10^{-7}$
0.76	$1.2 * 10^{-6}$
0.30	$3.1 * 10^{-5}$
0.10	$4.9 * 10^{-5}$
0.04	$1.7 * 10^{-5}$
0.02	$3.3 * 10^{-4}$
0.01	$8.7 * 10^{-4}$

Table 3: Reduced ligand immobilization levels causes a reduction in the observed loading response, which causes an increase in the dissociation rate due to the assay shifting from avidity-based to affinity-based.

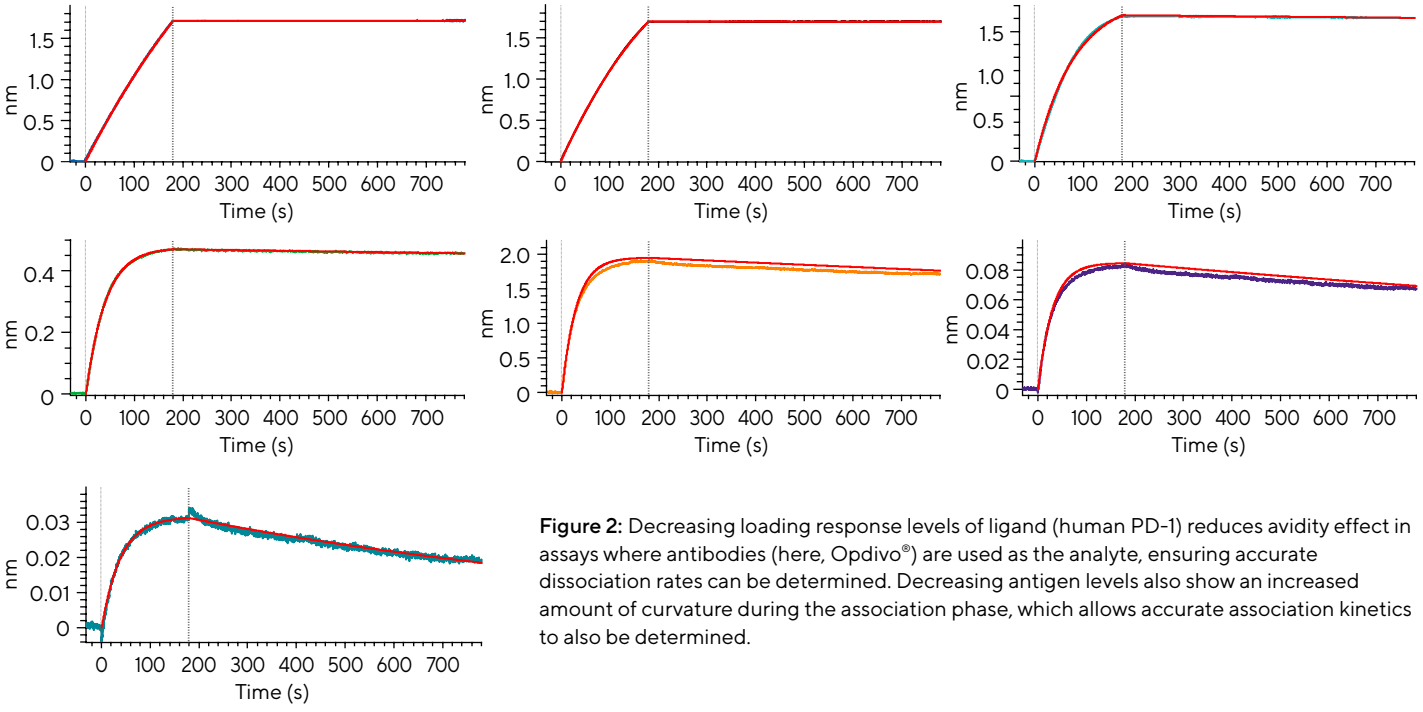

Figure 2: Decreasing loading response levels of ligand (human PD-1) reduces avidity effect in assays where antibodies (here, Opdivo®) are used as the analyte, ensuring accurate dissociation rates can be determined. Decreasing antigen levels also show an increased amount of curvature during the association phase, which allows accurate association kinetics to also be determined.

It's all about Kinetics – Extended Association Time

Binding of an analyte to a ligand is a second order binding process and therefore, to determine association rate constants curvature is required in the generated data. For low nanomolar (nM) interactions, this in general occurs over a timescale that is amenable to typical assay conditions. As the affinity of the interaction increases, the transition state energy barrier to forming the correctly associated analyte-ligand complex becomes higher and typical association times lead to linear responses that lack the required curvature to determine accurate kinetics.

Therefore, for high affinity interactions, the advice has been to ensure that sufficient curvature is present in the top one or two analyte concentrations, but this can lead to determination of inaccurate association rates.

In order to generate data with the desired curvature in standard SPR assays a large volume of analyte must be injected over the sensor chip surface from a large capacity vial or the analyte must be added to the running buffer[1]. This is a major drawback for injection-based systems as the injection parameters (vial size, flow rate) limits the association time that high affinity or equilibrium experiments can be collected for.

The Octet® R8e has reduced baseline drift compared to previous BLI systems and in combination with the newly designed evaporation cover allows easy assessment of extended association times. As shown in figure 3A, a standard 180 second association time of the analyte IgG4 antibody Opdivo® binding to the ligand human PD-1 shows minimal curvature for anything but the top analyte concentration. Due to Octet® BLI dip and read technology, no additional analyte is required when measurement of extended association time is required and as shown in figure 3B, analyte concentrations that were previously linear now show curvature that would allow accurate determination of association rate constants.

Octet® R8e assays can be performed in either a 384 or 96 well microplate and use a sample volume between 40 – 200 µL. Typical SPR assays for assessment of kinetics and affinity use a flow rate between 30 – 50 µL/min. To collect the data shown in figure 3B with an association phase of 3600 seconds would require between 40 – 200 µL for Octet® BLI and between 1,800 – 3,000 µL for stand SPR assays therefore, Octet® BLI offers a 900 – 7,500% reduction in the volume of analyte required.

Modern SPR systems offer space for up to seven 4.0 mL vials and therefore, extended associations as shown above are severely limited as only single concentrations can be assessed at one time and generating the data shown in figure 3B would take over seven hours. When compared with the plate-based Octet® BLI assays; the same assay would take one hour, or 12 samples can be assessed in a single assay using the Octet® AE Evaporation Cover.

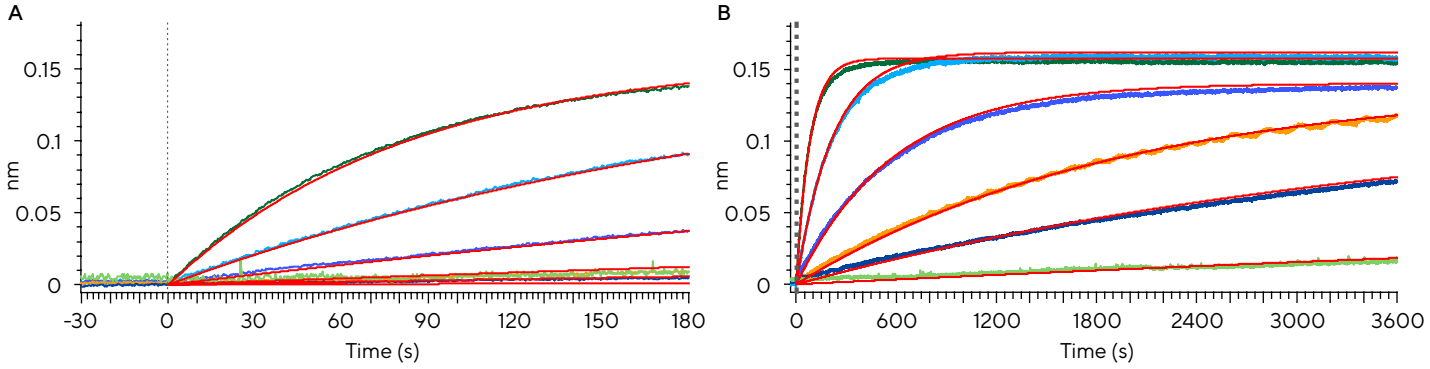

Figure 3: Extended association times utilizing the newly designed evaporation cover for the Octet® R8e. (A) Standard association time of 180 seconds of the analyte IgG4 antibody Opdivo® binding to the ligand human PD-1. (B) Extended association time of 3,600 seconds reveals curvature from previously linear response, which allows for more accurate association kinetics for high affinity interactions to be determined. Vertical dotted line represents 180 seconds association time.

Determine Accurate Kinetics and Affinity at the Lowest Response Levels

Opdivo® (143,597 Da)
The Octet® R8e allows for accurate assessment of antibody-antigen kinetics and affinity at low ligand density, which allows the bivalent antibody to be used as the analyte without the risk of avidity effects. As shown in figure 4A, Opdivo® can bind to human PD-1 with an affinity of 4.2 nM, which is exceptionally close to the value reported in the European public assessment report (EPAR) for Opdivo® binding to PD-1 of 3.06 nM[2].

Brown *et al* performed a multi-SPR system study of PD-1 binders, including nivolumab, and the results are shown in Table 4 with the Octet® R8e results added for comparison[3]. Importantly, all SPR affinity results reported in Table 4 were generated using an Fc capture approach where Opdivo® was captured via an anti-Fc capture molecule and human PD-1 was assessed as the analyte. This assay orientation was chosen to eliminate avidity effects but can significantly increase assay costs. Due to the high sensitivity of the Octet® R8e system, avidity effects can be minimized and a precise affinity value determined with the antibody as the analyte, which leads to a significant reduction in assay cost, especially when considering screening multiple antibodies.

Interleukin-15 (17,000 Da)
Interleukin-15 (IL-15) is a cytokine that binds to the IL-2 receptor beta (IL-2βR) chain and is involved in immune system regulation. It stimulates the proliferation and activation of natural killer (NK) cells and T-cells, enhancing immune responses. IL-15 plays a crucial role in immune surveillance and has potential therapeutic applications in cancer and infectious disease treatments due to its immunostimulatory properties.

Assessment of IL-15 binding to IL-2βR at a response level below 0.05 nm using a 1:1 kinetics model displays an affinity of 52 nM and excellent agreement between the observed data and the 1:1 kinetics model (Figure 4B).

Insulin (5,808 Da)
The human insulin receptor is a transmembrane protein crucial for regulating glucose metabolism. It binds insulin, triggering a cascade of intracellular signaling pathways that promote glucose uptake and utilization. This receptor plays a vital role in maintaining blood sugar levels and is a key target in diabetes treatment strategies. As shown in figure 4C, assessment of insulin binding to the insulin receptor on the Octet® R8e at a response level below 0.05 nm results in an affinity value of 223 nM and 214 nM when determined using a 1:1 kinetics model or steady state affinity, respectively. Both values are close to those observed in literature.

GLP-1 (7-37) (3,355 Da)
GLP-1 (7-37) is an active form of the glucagon-like peptide-1 hormone, which binds to the GLP-1 receptor. This interaction enhances insulin secretion, inhibits glucagon release, and slows gastric emptying, contributing to glucose homeostasis. GLP-1 (7-37) is significant in diabetes treatment due to its role in regulating blood sugar levels. An affinity value of 1800 nM was determined when assessing GLP-1 (7-37) binding to the GLP-1 receptor at a response level below 0.05 nm (Figure 4D).

System	Sensor Type	Sensor Chemistry	Assessment Type	K_D (nM)
Octet® R8e	SAX	Streptavidin	Multi-cycle Kinetics	4.2
Octet® SF3	CDL	CMD – 50 nm	OneStep®	4.1
Carterra® LSA®	CMD-P	Planar	Multi-cycle Kinetics	7.2
Biacore® 8K	CM5	CMD – 100 nm	Multi-cycle Kinetics	7.4
Biacore® 8K	C1	Planar	Multi-cycle Kinetics	3.5

Table 4: Comparison of Opdivo® - PD-1 Affinity. Affinity values taken from Brown et al[3].

Ligand	Analyte
PD-1	Opdivo® (143,597 Da)
IL-2β Receptor	IL-15 (17,000 Da)
Insulin Receptor	Insulin (5,808 Da)
GLP Receptor	GLP-1 (7-37) (3,355 Da)

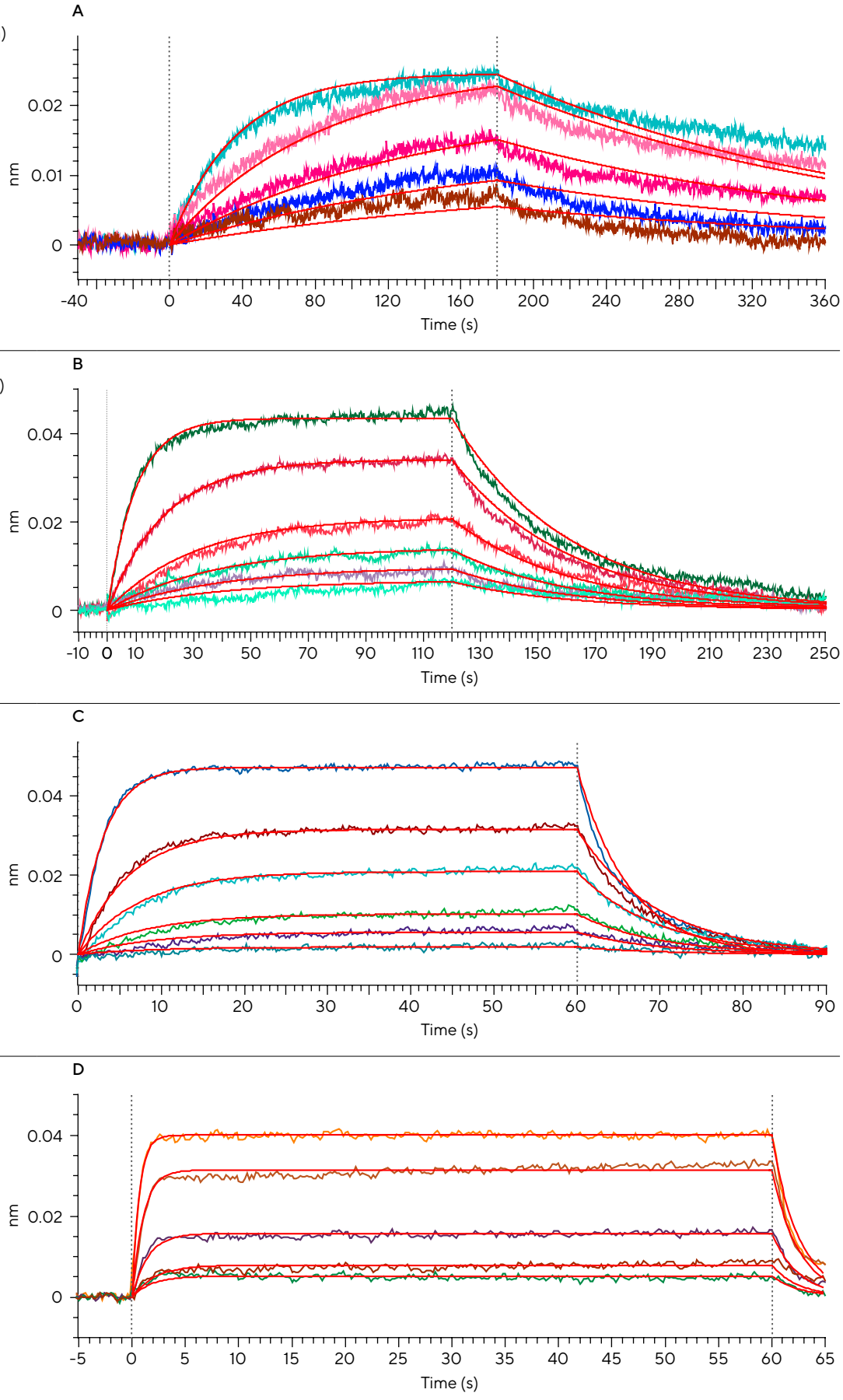

Figure 4: The Octet® R8e allows accurate kinetics to be assessed at low ligand levels across a range of molecular weights.

Conclusions

Assay sensitivity in biomolecular research and drug discovery plays a pivotal role in determining whether an assay or technique can be used, particularly in early pipelines applications. The Octet® R8e BLI system offers enhanced sensitivity that helps to streamline research processes and improve data accuracy.

A notable advantage of increased sensitivity and signal to noise is the ability to use less sample material, which is particularly important in early-stage drug discovery where sample availability can be limited. In combination with the integration of 384-well plates into Octet® BLI assay formats the Octet® R8e system's heightened sensitivity allows researchers to detect and analyze low-abundance analytes with precision in sample volumes as low as 40 µL. This not only conserves valuable resources but also enables the study of rare or precious samples that might otherwise be inaccessible.

In addition to reduced sample volumes, 384-well plates also facilitate performance of higher-throughput screening on the Octet® R8e, allowing researchers to conduct multiple assays simultaneously. This capability accelerates the pace of experiments, making it possible to screen libraries of drugs molecules or study numerous interactions in parallel. The use of 384-well plates, combined with the Octet® R8e's sensitivity, ensures that even subtle binding events are detected, providing comprehensive insights into molecular interactions.

Enhanced sensitivity in BLI, particularly with the Octet® R8e system, effectively addresses challenges related to avidity and affinity. By minimizing avidity effects, it ensures accurate dissociation rate determination and reliable off-rate ranking, crucial for understanding biomolecular interactions. This sensitivity allows precise measurement of affinity at low ligand densities, providing clear data essential for identifying therapeutic candidates in early pipeline applications.

In conclusion, the advancements in Octet® BLI sensitivity, as demonstrated by the Octet® R8e system, are revolutionizing biomolecular research and drug discovery. These improvements help facilitate the exploration of complex biological systems and also contribute to the discovery of novel biomolecular interactions, driving innovation in drug development.

As Octet® BLI technology continues to evolve, its role in advancing scientific understanding and therapeutic discovery will undoubtedly expand, offering new possibilities for researchers worldwide.

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