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The End of End-Point Assays

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Abstract

The determination of accurate kinetics, affinity, titer, and other critical quality attributes plays an increasingly important role in both up-stream and down-stream bioprocessing. It is important that any systems developed for these purposes match not only the high throughput needs of the user but also their sensitivity needs, allowing assays to be performed earlier in the workflow using minimal amounts of precious samples. This faster time-to-results allows assessment of accurate and precise data earlier in the workflow and therefore quicker decisions can be made on lead candidates to promote.

Bio-Layer Interferometry (BLI) is widely used and accepted in research and assay development and has been rapidly adopted as an important analytical tool in laboratories that work with biological molecules, either as drug products, vaccines, or diagnostic reagents.

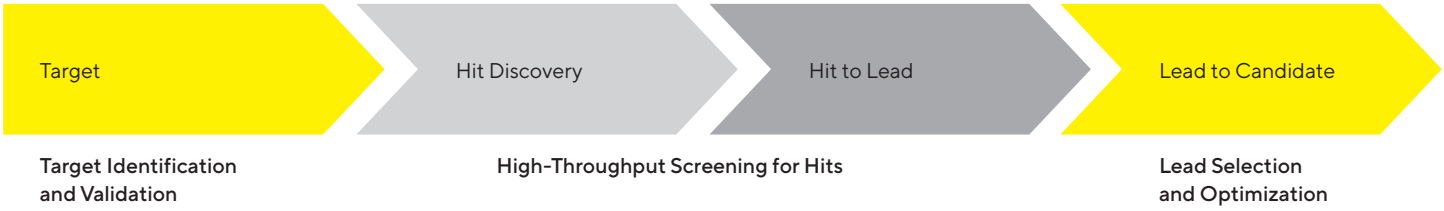
This white paper highlights the recent developments and performance of the three modular Octet® R series configurations with either 2, 4 or 8 channels and shows results demonstrating that all three configurations show similar performance in the quantitation and kinetics characterization of proteins as well as with protein-small molecules interactions. Unique to the Octet® R series is the ability to field upgrade your system configuration to the next modular level when required, allowing you to have full confidence that your system can grow with your future requirements.

Find out more: www.sartorius.com/octet

Introduction

General Introduction

Drug development is predicated on the identification of therapeutic targets, typically proteins or nucleic acids, that play a causal role in a disease and are 'druggable', i.e., amenable to pharmacological action by the drug. The drug development process is generally long, risky, and costly and typically takes longer than 10 years from discovery to approval of a new drug. Moreover, only a very small percentage of the many drug candidates under development each year make it to clinical trials and are approved by regulatory bodies. Identifying and isolating a therapeutic target and characterizing its properties and targeted interactions requires multiple processes and characterization techniques that contribute to long development timelines. Modern label-free and plate-based analytical techniques such as BLI are designed for real-time analysis and high-throughput capabilities, which can significantly reduce the time to the discovery, streamlining the selection of optimal drug candidates with the best chances of success downstream.



BLI is a non-fluidic format and can offer users key distinct advantages over traditional fluorescence-based techniques, including higher throughput, and better sample versatility, including the ability to analyze crude samples and an increased tolerance to diverse sample matrices. 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. Since they do not generally require labelled reagents, label-free analytical platforms speed up assay development and offer distinct advantages in early drug discovery. Combined with ease of use, low maintenance requirements, intuitive data and analysis software with the option of 21 CFR Part 11 compliant software and generating high-quality data, the Octet® BLI R series platform decreases time-to-results throughout the drug development process.

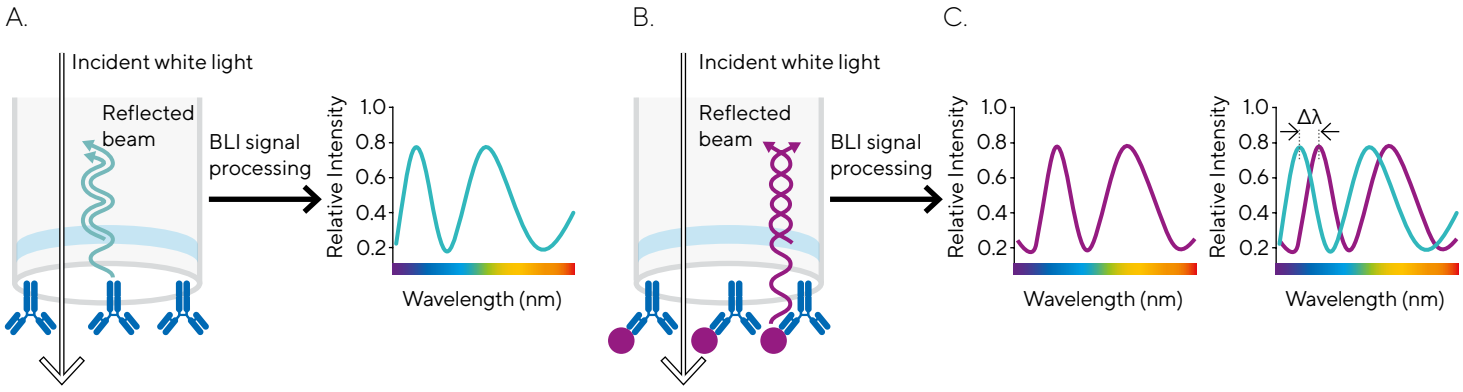
Sartorius' Octet® BLI systems utilize BLI technology to monitor biomolecular interactions in real time. They utilize the robust and easy to use Dip and Read biosensor format and provide faster time-to-results relative to technologies like ELISA and HPLC. Octet® BLI systems are ideal for the quantitation of a diverse array of biological molecules including antibodies and recombinant proteins and are especially suitable for product potency lot release assays. The Octet® R8 platform is particularly well-suited for GxP and QC laboratories.

Bio-Layer Interferometry (BLI) Technology

Bio-Layer Interferometry (BLI) translates biomolecular interactions into response signals in real-time, providing researchers with additional capabilities to characterize binding mechanisms. These systems can be used for kinetics characterization, concentration determination and biomolecular interactions screening among other applications. Of major importance is the ability of label-free technologies to provide on- and off-rates in kinetic characterization experiments which are key determinants in affinity constant derivation and information not available with end-point analysis techniques such as ELISA. Bio-Layer Interferometry can be applied across a range of applications at various stages of drug development, including antibody and protein quantitation and allows the user to circumvent limitations of ELISA and HPLC platforms, enabling informed decisions to be made earlier in bioprocess development.

Bio-Layer Interferometry (BLI) analyzes the interference pattern of white light reflected from two surfaces: a layer of immobilized protein on the biosensor tip and an internal reference layer (Figure 1A). Any change in the number of molecules bound to the biosensor tip causes a shift in the interference pattern that can be measured in real time (Figure 1A and 1B). The binding between a ligand immobilized on the biosensor surface and an analyte in solution produces an increase in optical thickness measured as a wavelength shift, $\Delta\lambda$ (Figure 1C).

Figure 1



Note. Relative intensity of the light reflection pattern from the two surfaces on the bionsensor. Octet® systems with BLI technology measure the difference in the wavelength of reflected light (Δλ) between the two surfaces.

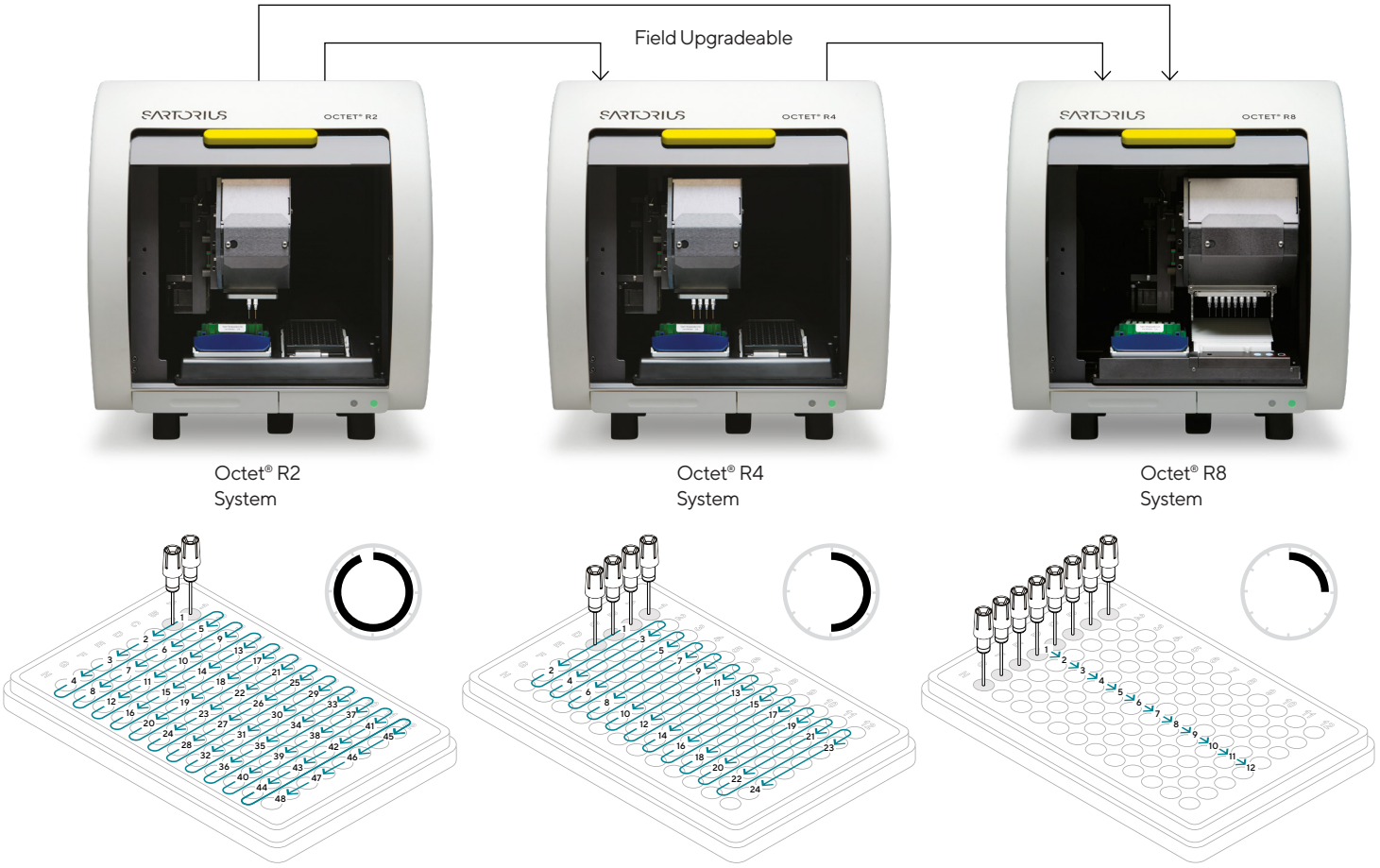
Octet® BLI systems utilize a standard microplate format, enabling high-throughput, automated binding analysis of samples directly from 96-well plates and greater flexibility in assay design. In addition, sample consumption during analysis is minimal and due to its non-destructive technique, precious samples can be recovered for use in other analyses, maximizing process economy. Octet® BLI systems offer the best quantitation and kinetics performance for a diverse range of molecules over a broad dynamic range and with sensitivity to detect molecules as small as 150 Daltons and as large as 1000 KDa. The systems can measure both high- and low-affinity interactions and detect fast binding interactions including protein-small molecule binding.

The newest addition to the Sartorius portfolio of Octet® BLI systems is the modular Octet® R series, available in three different configurations of 2, 4 or 8 channels (Figure 2). Compared to previous versions, all the systems feature improved sensitivity, which allows users to analyze a broader range of molecule sizes and higher data acquisition rates (2, 5 and 10 Hz), meaning the user can analyze faster interactions. The introduction of a cooling sample plate stage to all R series systems allows the user to control sample temperature between 15–40 °C in 1 °C increments; allowing kinetics and affinity to be determined at different temperatures and ensuring that thermodynamics can also be easily studied. In addition, the R8 includes an evaporation cover that allows experiments to be extended to up to 12 hours. Thanks to hardware improvements and the fluidics-free format of the Octet ® R series there are minimal requirements for maintenance and as the 2- and 4-channel Octet® R2 and Octet® R4 systems are field upgradable to the 8-channel Octet® R8 system, no future system trade-in is required, meaning you maintain the value of your initial investment.

As shown in Figure 2, the Octet® R series provides users the flexibility to select a system that satisfies their current requirements and upgrade the system as their needs change and still experience comparative data across each system. This means that users can seamlessly carry methods and workflows across the Octet® R series without having to spend time and money redesigning and qualifying assays and reagents.

To compare the performance of the three Octet R® Series systems, a series of quantitation and kinetics experiments were performed. In all cases the samples and assay conditions used were identical, however some minor differences in the workflow were necessary due to the differences in each system's throughput.

Figure 2



	Octet® R2 System	Octet® R4 System	Octet® R8 System
Throughput	2 channels	4 channels	8 channels
Temperature Control	15–40 °C in 1 °C increments*	15–40 °C in 1 °C increments*	15–40 °C in 1 °C increments*
Upgradeability	To 4 and 8 channels	To 8 channels	No
GMP/GXP Compatibility	No	No	Yes

Differences Are More Than Just In Throughput

	Microplate Compatibility	Sample Volume	Molecular Weight Detection	On-rate (k_a) Range ($M^{-1}s^{-1}$)	Off-rate (k_d) Range (s^{-1})	Affinity (K_D) Range	Data Collection Rate	Orbital Flow Capacity
Octet® R Systems	96-well plate	200 µL/well, non-destructive testing	> 150 Da	10^{1}–10^{7}	10^{-6}–10^{-1}	1 mM–10 pM	2, 5 or 10 Hz	Static or 100–1500 rpm

Note. The new Octet® R series consists of three different configurations that allows you to go from a 2-channel to 4-channel or 8-channel system from a single visit field upgrade. Among the R series, the Octet® R8 system offers the highest throughput, allowing you to analyze a 96-well plate in the least amount of time. The Octet® R4 system strikes the right balance between throughput and price, and the Octet® R2 system provides throughput at the most affordable price. As represented by the clock face, the relative time for measuring the same number of samples and replicates is lowest on the R8 (R8 < R4 < R2).

* The Octet® R8 includes an evaporation cover that allows experiments to be extended to up to 12 hours.

Data Section and Results

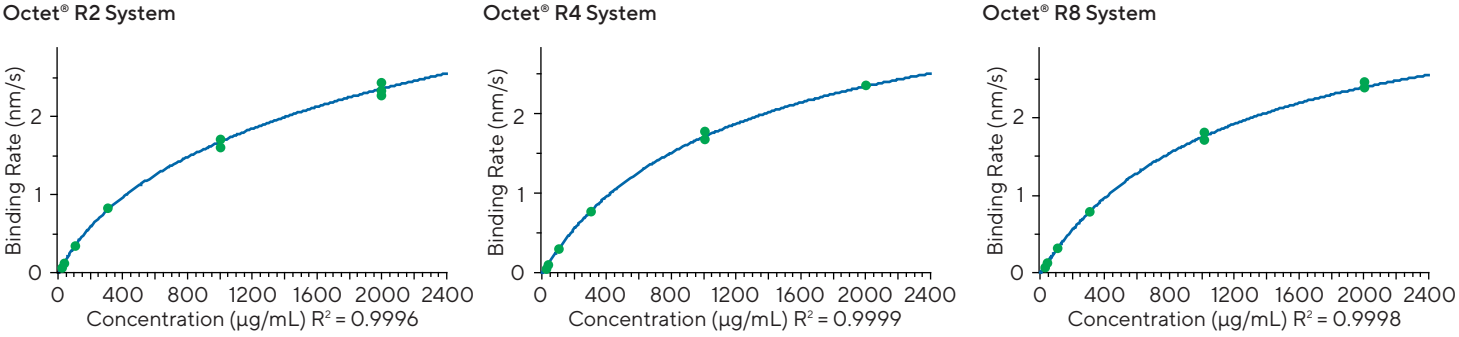
Quantitation Assay Setup for Performance Comparison
Generating accurate kinetics and affinity data requires knowledge about the concentration of your proteins and therefore, quantitation of protein concentration prior to performing kinetic-based assays is a critical step. Thanks to its plate-based format it is possible to rapidly quantitate antibodies and other biologics using a simple assay workflow on the Octet® R series modules.

Here, a standard curve of human IgG (hIgG) reference samples was prepared over a wide range of concentrations (0.025–2,000 µg/mL) at 30 degrees Celsius and a shake speed of 400 RPM using biosensors coated with the capture molecule Protein A (Protein A Biosensors (18-0004)). The initial binding rate of the standards were fitted to a 5PL unweighted curve, and all curves showed an R^2 value > 0.999 across all R series systems (Figure 3A). In a typical quantitation assay, biosensors coated with capture molecules are simply dipped into analyte samples of unknown concentration and the resultant binding, which is a function of sample concentration, is then analyzed using the initial rate of binding (or the equilibrium of binding), which depends on the concentration of the unknown sample.

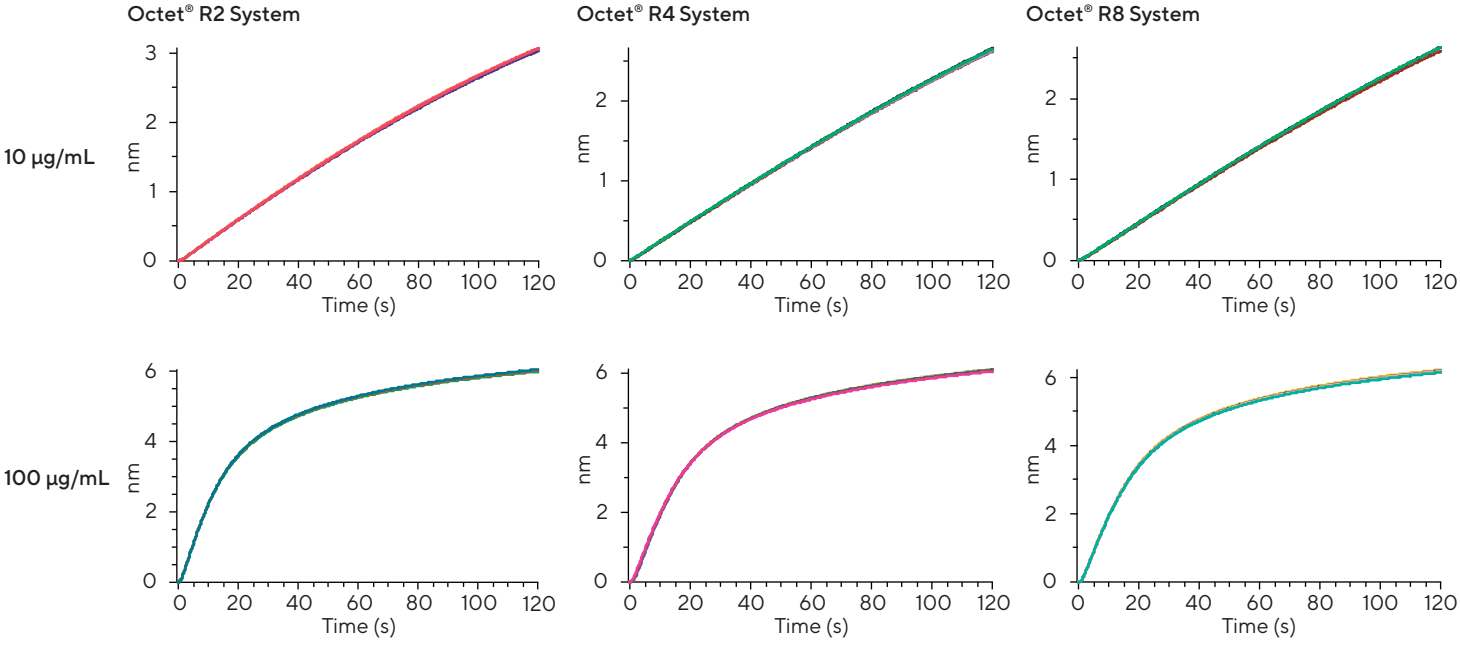
Here, samples of human IgG of known concentrations, 10 µg/mL (n=4) and 100 µg/mL (n=4), were spiked into the assay at 400 RPM and their concentration determined by comparing their initial rate of binding to the standard curve (Figure 3B).

Figure 3

A.



B.



The three R series modules showed excellent precision at 10 µg/mL and 100 µg/mL with % CV < 0.91% and < 1.89%, respectively (Figure 3B, Table 1) with a concentration accuracy range of –7.75 to 11.18% for 10 µg/mL and 1.56 to 3.93% for 100 ug/mL (Table 1). Therefore, all three Octet® R series modules show comparable quantitation performance.

It is important to note that quantitation is molecule specific and assay parameters such as shake speed can influence the assay resolution. This is especially important when low concentrations of analyte are being assessed.

Table 1

	Modular Octet® Configuration		
	Octet® R2 System	Octet® R4 System	Octet® R8 System
Human IgG Concentration	Coefficient of Variation (CV)		
10 µg/mL	0.91%	0.49%	0.75%
100 µg/mL	1.61%	1.89%	0.70%
	Concentration Accuracy*		
10 µg/mL	11.18%	-2.70%	-7.75%
100 µg/mL	3.93%	1.13%	1.56%

* Negative concentration accuracy value denotes a value lower than the accepted value and a positive value denotes a value higher than the accepted value.

Accelerating Cell Line Development

In addition to monoclonal antibodies and recombinant proteins (Read more in the Sartorius Application Note: MAb Quantitation: Protein A HPLC vs. Protein A Bio-Layer Interferometry), the Octet® BLI platform can also be used to accelerate cell line development by quantifying critical quality attributes such as mannose and sialic acid glycan content of Crude and Purified mAb and Non-mAb Protein Samples (GlyM (18-5139) and GlyS (18-5135) kits) and measuring residual contaminants such as host cell protein (HCP (18-5141)) and Residual Protein A (RPA). The detection and removal of process-related Residual Protein A from antibody drug molecules is an essential requirement to ensure the safety of antibody-based therapeutics.

Residual Protein A detection kits (18-5128) are available for all Octet® BLI systems and enable sensitive detection of leached Protein A with a sensitivity as low as 100 pg/mL. Here we compared the detection and quantitation of RPA using of four different MabSelect Sure standards using the three modular Octet® R series systems.

As can be seen in table 2, there is a significant time and reagent saving when comparing the R8 to the R4 and R2 due to the increased number of channels present in the R8 and subsequent throughput. Therefore, it is important to consider which system is most appropriate for current and future assay needs.

Table 2

	Modular Octet® Configuration		
	Octet® R2	Octet® R4	Octet® R8
Number of Residual Protein A Biosensors	40	40	40
Number of detection reagents usage times	10	10	5
Total run time (hours)	2	1	0.5

Accurate Kinetic and Affinity Analysis of Biological Interactions

The specific recognition and binding of biological molecules by antibodies and other proteins is fundamental to many processes in biology and the determination of accurate kinetic rate constants (association (ka) and dissociation (kd)) provides further information about interaction mechanisms, as well as the global affinity constant (KD). This information is critical when characterizing functional properties and is a necessary analytical step in target molecule identification, lead selection, optimization, and subsequent production.

These parameters allow researchers to better understand the potential mechanisms of action (MOA) of the drug candidate against its target and provides information needed to select optimal therapeutic candidates to advance through the development cycle. In general, the full characterization of lead molecules is typically performed using purified molecules; however, lead selection, as well as off-rate ranking in screening experiments, can be performed with non-purified samples.

Kinetics analysis is used to measure association and dissociation rate constants and to determine the affinity of such interactions. Octet® BLI kinetic analysis begins with the selection of a biosensor from a list of multiple chemistries provided by Sartorius. Read more in the Sartorius Application Note: Biomolecular Binding Kinetics Assays on the Octet® Platform. The process involves the immobilization of one interactant, commonly known as the ligand, on the surface of the biosensor while the other (analyte) remains in solution.

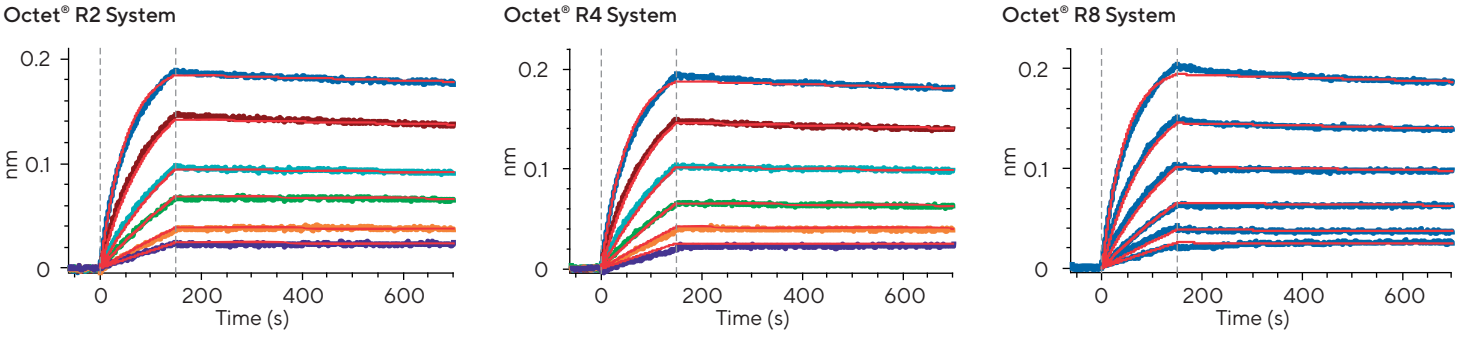
Here, the Octet® R2, R4, and R8 systems were used to assess the binding of a biotinylated anti-HER2 antibody (ligand) to the HER2 receptor protein (analyte) at 25 °C using SAX2.0 biosensors (18-5136). A dissociation time of 1800 seconds was used to ensure an observed decrease in binding > 5% but only the first 700 seconds of the dissociation are shown here in order to highlight the association kinetics.

As shown in Figures 4A, 4B, and 4C the Octet® R series exhibit good visual agreement between systems and analysis of the binding interaction using a 1:1 kinetic model (Table 3) shows a variability of less than 5% CV for the association phase and less than 10% CV for the global affinity. All variability is lower than the levels recommended by regulatory organizations (ICH and FDA).[1,2] Therefore, even if you start with an Octet® R2 system and field-upgrade to an R4 or R8 system, you can be sure that your data will be directly comparable without the need for any assay development.

Table 3

	Modular Octet® Configuration			
	Octet® R2 System	Octet® R4 System	Octet® R8 System	% CV
Ka ($M^{-1}s^{-1}$)	6.83E+05	6.76E+05	6.24E+05	4.9
Kd (s^{-1})	6.57E-05	6.87E-05	5.47E-05	11.7
KD (M)	9.62E-11	1.02E-10	8.76E-11	7.4
χ^2	0.1525	0.2125	0.3397	NA
R^2	0.9991	0.9988	0.9984	NA

Figure 4



Kinetics Performance Comparison of Small Molecules on the Modular Octet® R Series

Of the drugs approved by the FDA in 2021 over 70% were non-protein based and therefore, the ability to measure the kinetics and affinity of a range of molecular sizes is critical. BLI can measure molecular sizes as low as 150 Da and here, carbonic anhydrase II was loaded onto SSA (Super Streptavidin (18-5057)) Biosensors and the association and dissociation kinetics of the analyte Furosemide (330 Da) determined.

As with the large molecule kinetics performance, good reproducibility between the replicates was seen across the Octet® R series modules (Figure 5) and broad agreement with literature values of 0.5 to 1.0 µM for the affinity of Furosemide for carbonic anhydrase II. In addition, comparable loading nm shift, KD, ka and kd were seen across the Octet® R series modules, with all intra- and inter-assay % CV below the recommended acceptable level for ligand binding assays (Table 4).[2]

As shown in Table 5, there is a clear saving of time and reagents when performing multi-cycle kinetic assays using the R4 and R8 systems compared to the R2 system. Fewer biosensors were needed for the R4 and R8 compared to the R2 (16, 16 and 24, respectively) and due to the increased number of channels on the R8 compared to the R2, a 6-fold decrease in time taken to perform the assay was observed. When taken together, this allows the user to generate more data per day with the need for fewer consumable reagents.

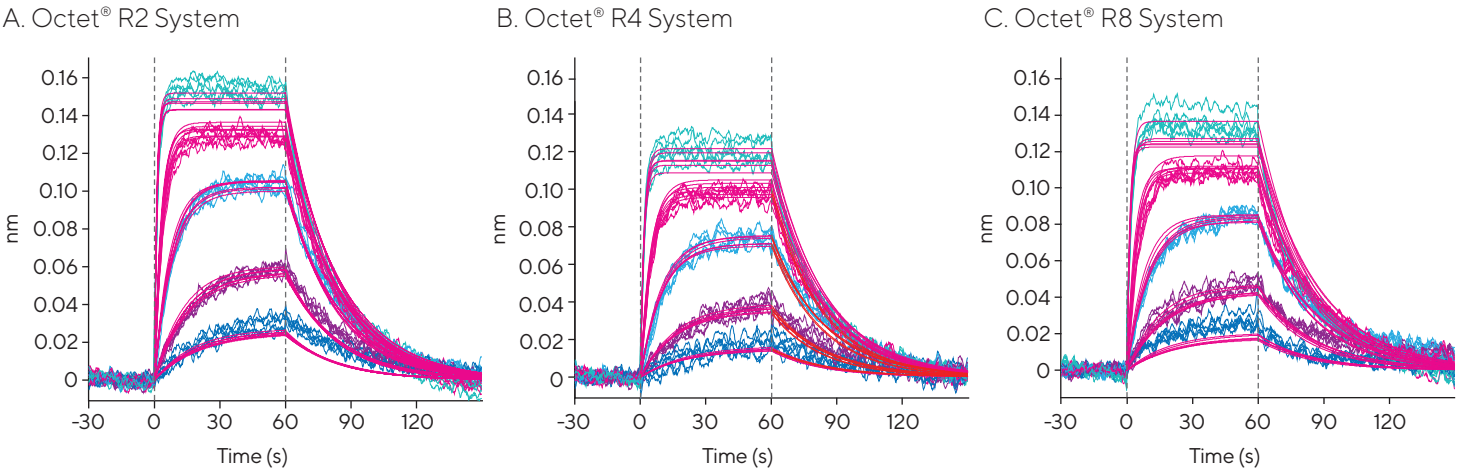
Table 4

Furosemide Kinetics	Octet® R2 System	Octet® R4 System	Octet® R8 System
Ka ($M^{-1}s^{-1}$)	8.10E+04	5.53E+04	6.44E+04
Kd (s^{-1})	4.56E-02	4.48E-02	4.41E-02
KD (M)	5.64E-07	8.13E-07	6.91E-07
pm shift at 30 µM	166.60	135.44	141.19
Ka % CV	7.0%	5.6%	12.3%
Kd % CV	5.7%	3.9%	6.4%
KD % CV	3.3%	7.9%	10.3%

Table 5

	Octet® R2 System	Octet® R4 System	Octet® R8 System
Number of replicates for kinetic rates	6	6	6
Number of sample concentrations for global kinetic analysis	6	6	6
Number of runs needed	2	1	1
Number of biosensors	24	16	16
Total run time (hours)	6	2	1

Figure 5



Note. Carbonic anhydrase II – Furosemide binding characterization comparison data as obtained using the three modular Octet® R series systems. The data represents replicates of a dose response analysis of Furosemide dissolved in PBS/0.5% DMSO buffer. Data was later globally fit to a 1:1 kinetics binding model using the Octet® Analysis Studio Software.

Conclusion

The three new modular configurations, Octet® R2, R4 and R8 systems, show very comparable results for quantitation and kinetic analysis of biomolecules irrespective of the size. All three modules can be used for analyzing a whole 96-well plate of samples and come with sample temperature control for stable processing of temperature-sensitive samples.

However, there is significant difference in the total time required to assay the same set of samples and replicates between the three modules. The Octet® R8 system consists of 8 channels which can simultaneously assay eight independent samples with eight biosensors leading to significant time saving. In contrast, the Octet® R4 and R2 systems have four and two channels each, and can only analyze four and two samples simultaneously, leading to increased time requirement respectively. The Octet® R8 system also comes with an evaporation cover, facilitating longer experimental runs (up to 12 hrs) without any significant loss in the sample volume.

Although all the three systems show comparable performance, the Octet® R2 system is most suited to the labs and workflow steps which have low throughput requirements and the Octet® R4 system is suited to labs with higher throughput. Since these systems are modular, they can be upgraded to the highest-throughput Octet® R8 system when the throughput needs increase, thereby futureproofing the lab and maintaining the initial investment within the low-throughput system. Octet® R8 systems are best suited for labs working on high-throughput biomolecule analysis or within workflow steps requiring parallel processing of a large number of samples.

Also highlighted is the significant time saving associated with using the Octet® R8 system over the Octet® R4 and R2 due to its higher throughput ability.

References

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2. Bioanalytical Method Validation Guidance for Industry

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