

# Streamlining affinity analysis for accelerated lead screening, characterization, optimization and final selection

## Introduction

Antibody and other protein therapeutics are a major focus in drug discovery pipelines today. The overall process for developing protein therapeutics encompasses target selection and validation, library screening to generate early candidates (hits), follow-up characterization for lead selection, lead optimization, and clinical candidate selection. During lead selection, molecules identified as hits are subjected to screening via multiple analytical methods to select a few candidates for progression to the next stage of the development process. This is typically followed by detailed characterization for confirmation of binding and functional activities via biochemical and biophysical analyses.

## Finding the right characterization technique

One of the common molecular analyses performed during characterization is the determination of a lead molecule's binding kinetics and affinity to the target(s).

By assuming the interaction to be a simple bimolecular reversible interaction, the affinity, expressed as  $K_D$ , of the interaction is derived from the ratio of dissociation rate constant ( $k_d$ ) over the association rate constant ( $k_a$ ) and describes the overall strength of the reversible association molecules between A and B.

$$\frac{[A][B]}{[AB]} = \frac{k_d}{k_a} = K_D$$

The lower the magnitude of  $K_D$ , driven by either a high  $k_a$ , indicating a fast formation of AB, or a low  $k_d$ , indicating a slow dissociation of AB, or driven by a combination of both a high  $k_a$  and a low  $k_d$ , the more stable or tighter the interaction between A and B, is referred to as a high affinity interaction. Often,  $k_a$  and  $k_d$  are also referred to as on-rates and off-rates of molecules, respectively.

Lead molecules selected from a naïve human library using a display technology have relatively low (10–100 nM) target binding affinities. After selection, these molecules are then enhanced using various affinity maturation techniques to reach a desired affinity range - normally 0.01–10 nM.

There are several challenges associated with generating reliable kinetic and affinity information of hits at various stages of the lead selection process. The needs and challenges change as hits progress from the lead screening to characterization, optimization and to final selection stages. Some of the common challenges include:

- 1 Multiple instruments needed to run large screens all the way to high-quality characterization rapidly to eliminate unsuitable candidates.
- 2 The ongoing increase in number and complexity of molecules with distinct assay requirements.
- 3 Reliably measuring affinity for lead candidates with slow off-rates and/or high affinities.
- 4 Maintaining activity of temperature-sensitive targets during analysis.
- 5 Sample consumption, especially during early stages of screening when sample quantities are low.
- 6 Need for expert operators.

## Bio-Layer Interferometry eliminates challenges

Drug molecules are increasing in complexity with bispecific, tri-specific antibodies, fusion proteins and as antibody-drug conjugates gaining prominence within drug discovery programs. Most analytical techniques are unable to measure sequential binding events to evaluate a single molecule's specificity and affinity to multiple targets in one assay set up that is an absolute

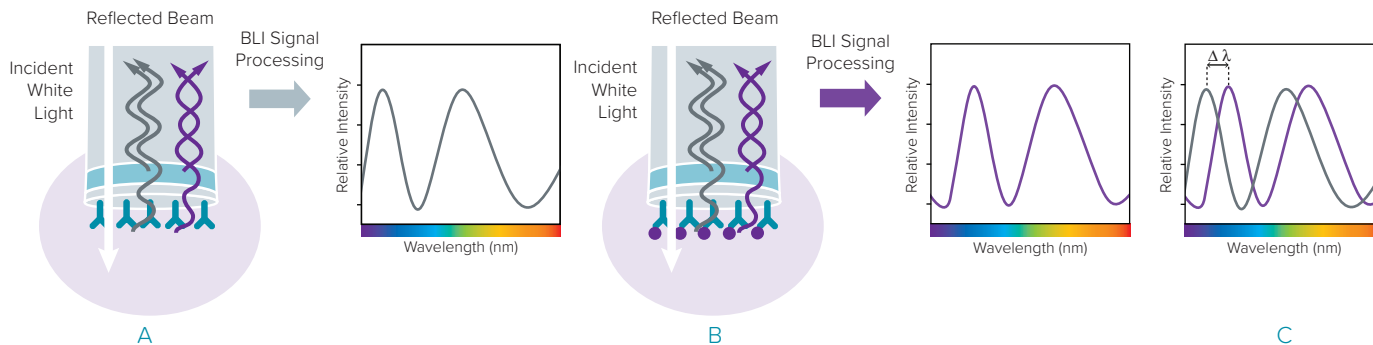


Figure 1: Principles of Bio-Layer Interferometry (BLI).

requirement for these types of leads. With increasing complexity in the molecular library, along with an increase in the number of drug development programs that are run in parallel, higher throughput affinity measurement methods are continuously sought to keep pace with specialized assay requirements for these drug candidate's characterization.

Octet® instruments eliminate many of the common issues associated with detailed characterization of binding and functional activities. These systems utilize the label-free technique Bio-Layer Interferometry (BLI), which enables scientists to obtain accurate information over a large range of binding rates and affinities during drug-target interaction studies. BLI is an optical analytical technique that 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), which is a direct measure of the change in thickness of the biological layer.

To meet the unique needs of different laboratories, the Octet platform includes multiple instruments, biosensor types, reagents and assay kits for analysis of biomolecular interactions. Assays can be run in both 96- and 384-well microplates. The Octet RED96e system, offers increased utility in the lead selection and optimization process as it (i) expands the range of targets that can be studied to include temperature-sensitive proteins (ii) broadens biophysical characterization capability to include measurement of thermodynamic parameters of a binding interaction, and, (iii) allows binding kinetics analysis of a greater number of higher affinity samples in one unattended run. This is achieved with precise temperature control of samples in one 96-well microplate, a wider temperature range that allows samples to be cooled to 15°C, and the use of a microplate cover that extends the unattended run time of a single 96-well plate to 12 hours.

## Pushing past limitations in the lead selection and optimization process

### Rapid large screens can be run directly in complex matrices.

Unfavorable hits with weak or sub-optimal target binding affinities, low target selectivity, poor biochemical or biophysical attributes need to be eliminated early in the development process. This part of the process may become a bottleneck if these large numbers of hybridoma or phage display samples need to be purified prior to analysis. Most techniques are unable to measure accurately in crude matrices due to high non-specific binding or low resolution. Label-free techniques are better suited for these types of samples but for systems that use fluidics, crude samples may often cause fluidic blockages.

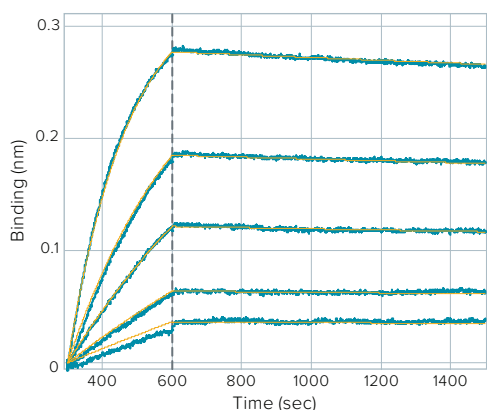
When using BLI assays, only molecules binding to or dissociating from the biosensor can shift the interference pattern. Unbound molecules in the solution matrix or changes in the refractive index of the surrounding medium do not affect the interference pattern. This is a unique characteristic of BLI, and extends its capability to perform kinetic measurements in crude samples useful in applications for protein-protein binding screens, quantitation, affinity, and kinetic ranking.

Scientists can cater Octet RED96e instrument to the rigor of generating the binding information necessary at various screening to characterization stages. Often the progression for determining which antibody candidates have the tightest affinity to their purified antigen target begins with Octet instrumentation performing high-throughput screening of up to 64 samples in only 1.5 hours. Here, the screening data can allow for rapid affinity ranking of dozens of unpurified antibodies in supernatant and yield qualitative epitope binning information on multiple antibodies. Following the screening experiment, a higher resolution approach may be used to evaluate more rigorous kinetic information from the 5 to 10 highest affinity antibody (mAb) supernatants to determine the 1 to 3 best candidates. These selected mAb candidates are then purified and subjected to the high quality and detailed resolution with the most rigorous antibody-antigen kinetics measurements.

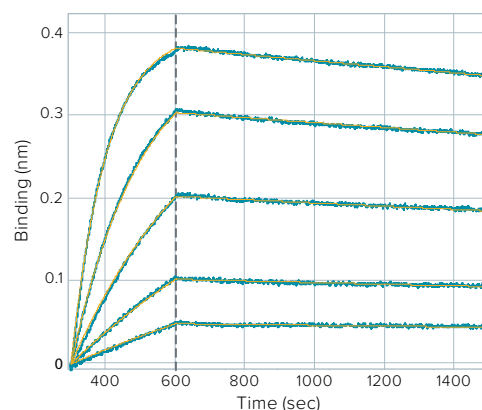
**Expanded assays with temperature-sensitive proteins.** An important consideration in selecting an appropriate assay is ensuring the target protein is active following its attachment to the binding surface. This is especially difficult to achieve when working with temperature-sensitive proteins. Typically, these experiments are run at lower than room temperature to get accurate kinetic and affinity information. Measuring an interaction over a series of temperatures, including lower than room temperature, also enables thermodynamic characterization, indicating whether the interaction is enthalpically or entropically driven.

With the Octet RED96e system, samples that degrade quickly at room temperature can be assayed at lower temperatures, ensuring the protein remains active for a longer period (Figure 2). This allows more active protein to be loaded on the biosensor, enabling these difficult protein targets to be assayed against a large number of samples reliably and quickly.

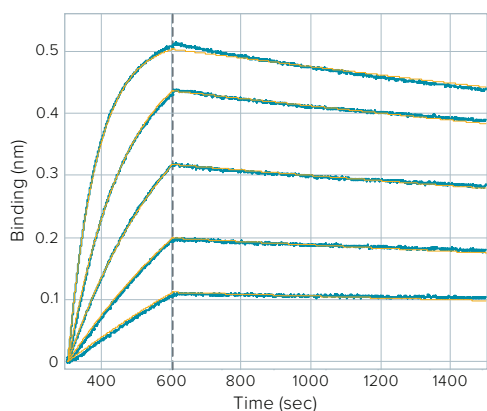
**High resolution and low noise to measure high affinity interactions.** A critical parameter to be considered in choosing the right technology for the measurement of high affinity binding interactions is low baseline noise and signal drift. Some label-free binding techniques are not well suited for measuring these interactions as they suffer from low resolution due to high noise and/or baseline drift when performing long high-quality characterization studies of the lead candidates. RMS noise in the data can result from various factors, such as temperature changes and matrix effects to name a few. Low off-rate measurements show very small changes in signal in the dissociation step, requiring the analysis of a very small change in signal in comparison to the baseline noise and drift signals. In Figure 3, high-resolution kinetics of an interaction between Human Epidermal Growth Factor 2 (HER2) protein and anti-HER2 monoclonal antibody (Herceptin) was analyzed using Octet RED96e system to demonstrate the reliable measurement of the on- and off-rates of a very high affinity binding interaction.



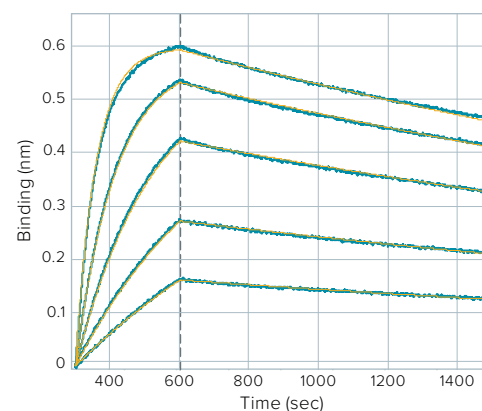
Temperature	$k_a$ ( $M^{-1}s^{-1}$ )	$k_d$ ( $s^{-1}$ )	$K_D$ (M)
15°C	2.87E+04	4.51E-05	1.57E-09



Temperature	$k_a$ ( $M^{-1}s^{-1}$ )	$k_d$ ( $s^{-1}$ )	$K_D$ (M)
20°C	4.86E+04	1.02E-04	2.09E-09

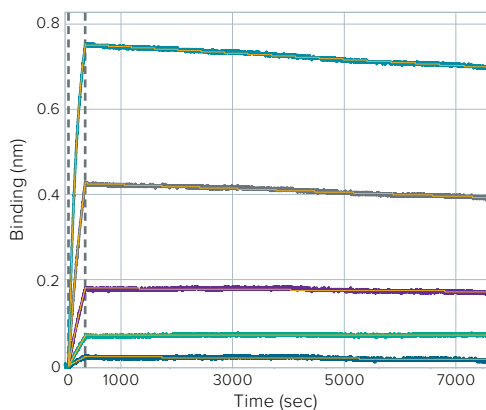


Temperature	$k_a$ ( $M^{-1}s^{-1}$ )	$k_d$ ( $s^{-1}$ )	$K_D$ (M)
25°C	5.90E+04	1.44E-04	2.44E-09



Temperature	$k_a$ ( $M^{-1}s^{-1}$ )	$k_d$ ( $s^{-1}$ )	$K_D$ (M)
30°C	8.45E+04	2.89E-04	3.42E-09

**Figure 2:** Demonstration of the Octet RED96e system's capability to run biomolecular interactions at a wider range of temperatures. Data was generated for human prostate specific antigen (PSA, MW 30kDa) binding to a biotinylated anti-human PSA mouse monoclonal antibody loaded onto Streptavidin biosensors. Interactions were monitored across a range of temperatures from 15°C, 20°C, 25°C to 30°C and can be thermodynamically characterized. For this interaction, both association and dissociation rates are increasing at higher temperatures.

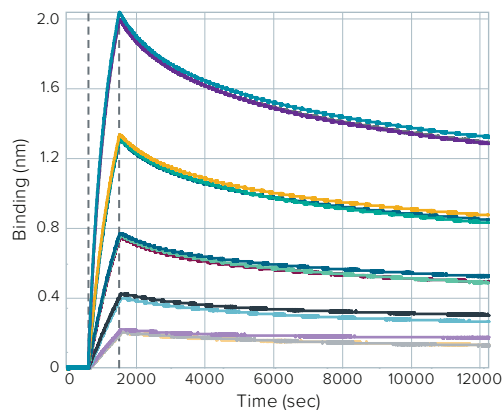


Sample ID	$k_a$	$k_d$	$K_D$
HER2-Anti HER2 antibody	$1.820 \cdot 10^5 \text{ M}^{-1}\text{s}^{-1}$	$1.024 \cdot 10^{-5} \text{ s}^{-1}$	56.25 pM

**Figure 3:** High-resolution kinetic characterization of Human Epithelia Growth Factor 2 (HER2) antigen binding to biotinylated anti-HER2 monoclonal antibody onto High Precision Streptavidin (SAX) biosensors measured at 25°C.

**Accurate measurement of multiple high-affinity interactions over long run times.** After promising hits are selected, they are often engineered or affinity matured to reach the desired potency and pharmacokinetic (PK) properties. A major consideration with technologies used for binding kinetics characterization of these lead candidates is the requirement for assay design and development because measuring  $K_D$ ,  $k_a$ , and  $k_d$  for high affinity binding interactions requires specialized assay conditions, depending on the underlying mechanism. If the time to reach equilibrium for the drug candidate-target protein complex is on the order of days, the  $k_d$  can be extremely low. In such a case, techniques that enable long (>1-3 hour) periods of data acquisition per sample are necessary to accurately measure off-rates ( $k_d$ ) to calculate the affinity of the interaction.

Measuring such long off-rates for up to 10 samples equates to a 10 to 12-hour experiment, which typically isn't feasible as evaporation over the long run time changes sample composition. The Octet RED96e system enables simultaneous measurement of up to 8 samples at once in a single unattended run via its 8-channel parallel processing format. This combined with a microplate cover that keeps samples intact by minimizing evaporation enables scientists to collect more accurate measurements with evaporation control for up to 12 hours.



**Figure 4:** Utilization of a novel evaporation cover unique to the Octet RED96e system enables the measurement of long assay times per sample, for up to 12 hours of experimental run time with minimal sample evaporation. Here, the high reproducibility of running a full kinetic characterization of antibody-antigen interaction is demonstrated. The experiment was run three times with a total run time of 11 hours, each assay was 3.67 hours.

The measurement of one high-affinity interaction measured in triplicates in a long dissociation experiment on the Octet RED96e system is demonstrated in Figure 4. Full kinetic characterization of an antibody-antigen interaction is run three times with a total run time of 11 hours, each assay was 3.67 hours. During measurement, one column of sample wells in a 96-well microplate was opened while the other 11 sample columns remained covered — reducing their exposure to open air and evaporation. This data demonstrates that multiple high-affinity interactions can be measured with high accuracy and precision in a single unattended run.

**Precious samples can be recovered post-analysis.** Common binding characterization techniques consume large amounts of sample during analysis and they are often not recoverable. With precious samples, the number and type of analyses must be chosen carefully in order to obtain more detailed characterization of potential lead candidates.

A majority of samples can be recovered post-analysis for short assays with the Octet platform. With longer assays like those for high-affinity interactions on the Octet RED96e system, recovery of precious samples after analysis can be maximized with the evaporation cover, allowing re-assaying for additional parameters.

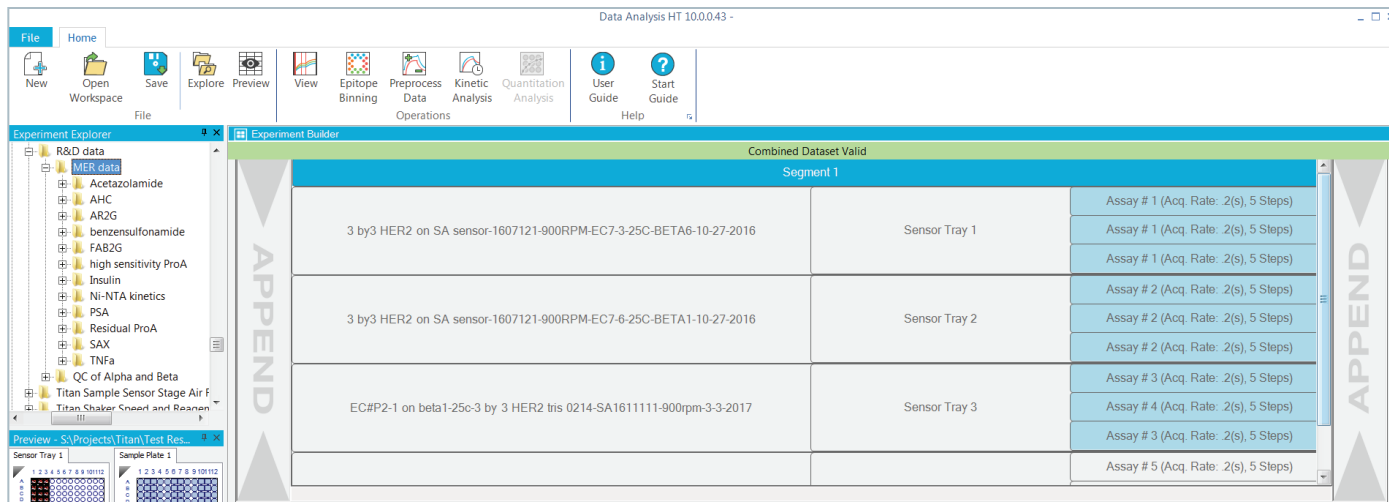


Figure 5: Octet Data Analysis HT software v10 reduces analysis time when working with large data sets. Features for appending and merging assay data enable comparisons between experiments and assay conditions.

**New users up and running in less than a day.** Because the instrumentation and software used to generate binding data and calculate affinity in lead characterization is often quite complex, highly specialized personnel are needed for operation and analysis. This can add delays to the selection progress as scientists involved in decision making are unable to generate and analyze the data they need, when they need it.

Due to the robust nature of the instrumentation and its ease of use, the Octet RED96e can be operated with minimal training by laboratory personnel. Typically, a half-day training session is sufficient for independent operation of the system by most users.

Octet Data Analysis HT software also simplifies and streamlines data analysis for scientists. It enables multiple experiments and plates to be overlaid and/or appended to be analyzed simultaneously, reducing analysis time significantly (Figure 5). The software also offers more comprehensive and flexible tools for customizable data analysis to cater to users of all levels of experience. These tools include increased reference subtraction options, data correction to remove bulk shifts or artifacts, and report point features to assess loading levels, early and late dissociation levels to name a few. Once data analysis is complete, the software allows scientists to create customized reports of the experiments, combining various data elements such as graphs, text, data tables, company logos, images and experimental details - ready to be uploaded to an electronic notebook or stored in the database (Figure 6).

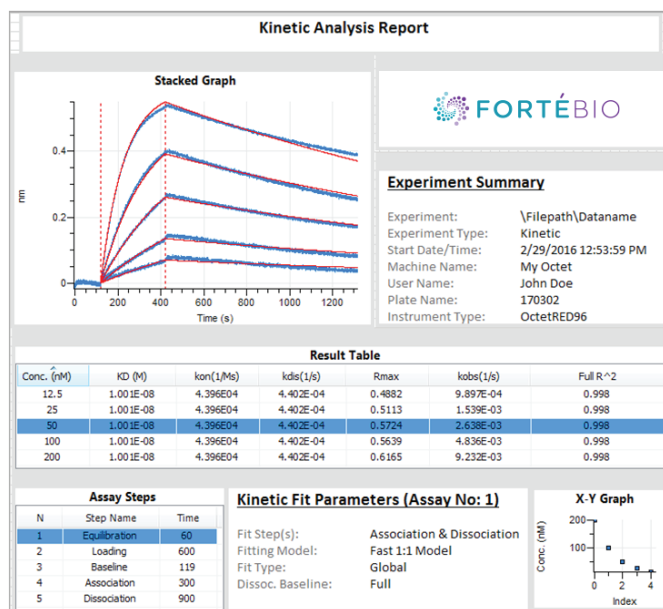


Figure 6: Octet Data Analysis HT software enables making customized reports ready to be added to electronic notebook or to the database.

## Conclusion

The Octet RED96e system can characterize molecules ranging in size from small organic compounds of 150 Da up to mammalian cells which are several microns in diameter. The system can be used across many applications including binding kinetics assays and affinity characterization for a wide dynamic range of rate constants, rapid qualitative screening for yes/no binding, and quantitation assays. Simple one-step assays measure large quantitative ranges of 25 ng/mL to 2 mg/mL of IgG in crude matrices in as little as 2 minutes per sample or 32 minutes for a full 96-well plate. Detection of sub-ng/mL levels possible with 2-step and 3-step assay formats, allowing automated measurement of contaminants such as host cell proteins and residual protein A. The Octet RED96e system also offers the workflow advantages BLI is known for – a microfluidics-free Dip and Read™ format that enables simple assay setup and automated analyses, re-racking of regenerable biosensors for ultra-low operating costs, and non-destructive sampling that allows full sample recovery. Off-the-shelf biosensor chemistries also enable a wide range of applications to be run on a single system at various stages of the lead generation and characterization process.

The Octet RED96e system offers the versatility needed to accelerate and de-risk binding kinetics and affinity analysis in drug discovery and development:

- Eight parallel, independent channels and sample detection in a 96-well plate format offers greater assay speed and flexibility for laboratories characterizing lead candidates
- Extended temperature range and enhanced evaporation control expands the number and types of samples that can be assayed
- An increased assay time of up to 12 hours enables measurement of long dissociation rates on up to 10 high-affinity binders in a single unattended run with no sample degradation
- Maximize recovery of precious samples after analysis with the evaporation cover
- Sample cooling for temperature-sensitive molecules, or to explore the thermodynamic profile of a binding interaction at multiple temperatures

## FOR MORE INFORMATION

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