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Application Note

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From Complexity to Clarity: Simplifying EC_{50} Assay Quantitation

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Abstract

Quantitation and EC_{50} assays are fundamental in drug discovery, serving as essential tools for evaluating the potency and efficacy of therapeutic compounds. Quantitation involves precise measurement of a substance's concentration or activity, crucial for determining a drug candidate's pharmacological profile. Accurate quantitation allows researchers to assess the dose-response relationship, a key aspect of understanding drug-target interactions. These assays are employed in early drug development stages to prioritize compounds for detailed studies, such as *in vivo* testing and clinical trials.

This application note offers insights into the differences between EC_{50} and affinity (K_D) assays and provides initial steps into assay design, including Octet® Analysis Studio software for efficient data analysis and curve fitting. Highlighting advantages over traditional methods like ELISA. With the aim of simplifying EC_{50} assay quantitation, common user questions are answered and highlight the advantages of Octet® BLI over traditional methods like ELISA.

What is the Difference Between EC_{50} and Affinity?

Although at first sight EC_{50} and affinity (K_D) appear to measure the same parameter, they are in fact different. In simple terms the EC_{50} measures drug potency and the affinity measures how tightly a molecule binds to its target.

Affinity – Equilibrium Dissociation Constant (K_D)
The binding of a molecule to its intended target is affected by two parameters, the association rate constant (k_a) and the dissociation rate constant (k_d). At equilibrium, k_d is the overriding parameter and can be used to measure the strength of binding to the target (the lower the k_d value, the tighter the binding and the lower the affinity value). K_D is a concentration independent parameter and as such exceptionally useful in determining the affinity of new drug compounds.

Note: The optimal method to derive accurate kinetics is through analysis of the association and dissociation rate constants using a kinetics-based assay.

(EC_{50}) - Half-maximal Effective Concentration
The Half-maximal Effective Concentration (EC_{50}) is used for molecules that induce a response. The drugs potency is measured by this increase in response and the EC_{50} is the amount of molecule needed to induce a response of 50% (half maximal effective concentration). Like affinity, the lower the EC_{50} value the higher the potency and less drug is needed to achieve the EC_{50}.

Which One to Use?
A common mistake to make when comparing affinity to EC_{50} is to forget the additional parameters that affect their values. Affinity is not affected by experimental conditions (i.e., the strength of binding doesn't matter how many molecules you measure) but EC_{50} does depend on the experimental conditions. As shown in the equation below for solution-based assays, EC_{50} and k_d are linked in a simple 1:1 equilibrium binding model:

$EC_{50} = k_d + 0.5$ [Ligand concentration]

When ligand concentration is much lower than k_d, EC_{50} and kd are comparable but as ligand concentration approaches or is great than kd the EC_{50} can differ significantly and therefore, EC_{50} values are linked to a specific assay setup and do not transfer.

For example, if an interaction between an analyte and ligand has an affinity of 1 nM and the ligand is present at a concentration of 100 nM then:
- EC_{50} = 1 nM + 0.5(100nM)
- EC_{50} = 51 nM

Therefore, two molecules can have the same affinity constant but very different EC_{50} values in different assays therefore, k_d is better for understanding the binding affinity whilst EC_{50} is better for understanding functional activation in a specific assay.

The choice between using EC_{50} or affinity depends on the specific context and what you are trying to measure or understand (See Figure 1):

Use affinity (K_D) when:
- You want to understand the strength of the interaction between an analyte and its ligand (target). Affinity is crucial for studying the binding characteristics and specificity of an analyte for its target.
- You are designing or optimizing drugs to improve their binding to a specific receptor. High affinity can be desirable for ensuring effective binding and activity.
- You are interested in the molecular interactions and mechanisms underlying the binding process, which can inform drug design and development.

Use EC_{50} when:
- You want to assess the potency of a drug or compound in eliciting a biological response. EC_{50} is useful in dose-response studies to determine how much of a substance is needed to achieve a certain level of effect.
- You are comparing the effectiveness of different drugs or compounds in producing a specific effect. EC_{50} provides a quantitative measure to compare the relative potencies.
- You are interested in the functional outcome of a drug interaction, such as its therapeutic effect or toxicity.

In summary, EC_{50} is more relevant for evaluating the functional potency of a compound, while affinity is important for understanding the molecular interactions and binding strength between a ligand and its receptor. Both metrics are valuable in drug development and pharmacological research, but they serve different purposes. In general, Octet® BLI assays use a set concentration (binding response) of ligand and therefore, can be considered as EC_{50} assays.

Which One to Use?

EC_{50}	Affinity (K_D)
Assess the potency of compound in eliciting a biological response	Assess the strength of the interaction between an analyte and a ligand
Comparing the effectiveness of different compounds	Drug optimization screening panels
Assessing the functional outcome of a drug interaction	Investigate the molecular interactions in the branding process

Figure 1: Beginning with the end in mind can let users determine which assay format is most appropriate for their needs.

Introduction to Dose Response Analysis

Measurement of drug potency or activity is possible on Octet® BLI using common dose response measurements such as EC_{50}, IC_{50} and the Hill coefficient.

In an Octet® BLI dose response experiment, what is typically measured is the binding response upon binding of an analyte molecule to a ligand to form an analyte-ligand complex. Measuring these responses over a wide range of analyte concentrations typically produces a sigmoidal plot (response vs. log concentration) (Figure 2). During the lower asymptote, the observed binding response is minimal due to the low concentration (in comparison to the K_D) of analyte and subsequent low amount of analyte-ligand complex formation. As the analyte concentration increases, the amount of analyte-ligand complex formation increases rapidly and a large increase in binding response is observed (transition region). Finally, in the upper asymptote, the analyte concentration is saturating, and the signal once again becomes insensitive to small concentration changes.

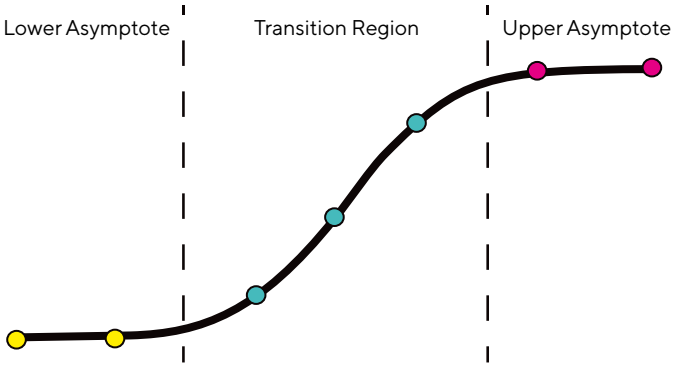

Figure 2: Example of a sigmoidal curve generated from an increasing analyte concentration.

As shown in Figure 3, the mid-point of the assay (~0.75 nm) is found during the transition region and correlates to half of the maximum observed response (~1.5 nm). The extrapolated analyte concentration is the half-maximal effective concentration, or EC_{50} , and is equal to K_D if measured at equilibrium.

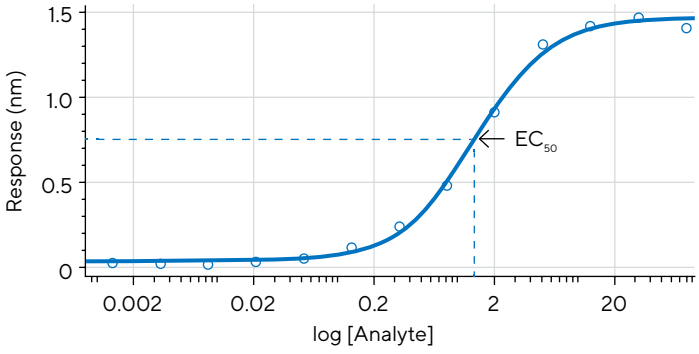

Figure 3: Determining the EC_{50} from a Dose Response Curve.

In addition to EC_{50}, it is possible to design an assay where a second molecule can inhibit or block a binding interaction. Typically, a series dilution of the inhibitor is used where the binding complex is at a fixed concentration. In this case, the midpoint is called the half maximal inhibitory concentration or IC_{50} (Figure 4).

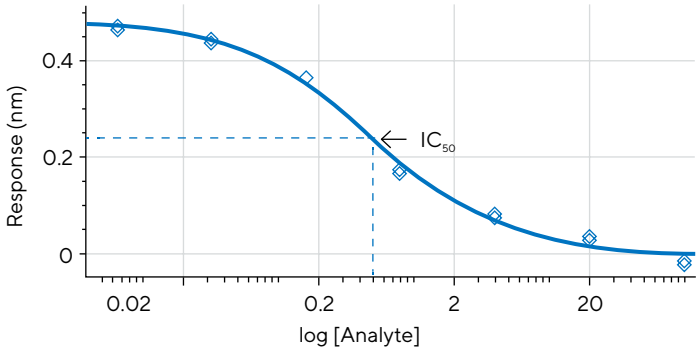

Figure 4: Determining the IC_{50} from a Dose Response Curve.

As discussed above for the EC_{50}, the IC_{50} is the mid-point concentration between the upper and lower asymptotes.

The Hill coefficient can be useful for quality control purposes. Repeated dose response measurements of the same molecules in the same or different lots will yield similar sigmoidal plots but they may vary in the maximal response, or the EC_{50} value. However, they should all have very similar Hill coefficients. A pronounced deviation in the Hill coefficient of a sample can indicate degradation of the sample or contamination.
(see User Examples – Case Number 2
for further information)

Use of Octet® Analysis Studio Software for EC_{50} Assays

Octet® Analysis Studio software provides easy determination of common dose response metrics such as EC_{50}, IC_{50}, and Hill coefficient to support measurement of drug potencies or activity. This section demonstrates how to extract these values and highlight some of the additional features that allow you to analyze less than optimal or unusual data.

Report Point Settings

The dose response curve is plotted in the Fit Graph window. The response value used is directly linked to the user-specified time along the analyte binding curve shown in the Detail Graph on the right-hand side.

The user-specified time is set with Time(s) settings, averaging a number of data points set in # Points to Average. The Time Range shows the effective span of data that is averaged. The number of points to average is symmetrical around the time setting. If the time setting is at or near the beginning or end of the binding curve, the time range is adjusted to ensure the set number points is averaged.

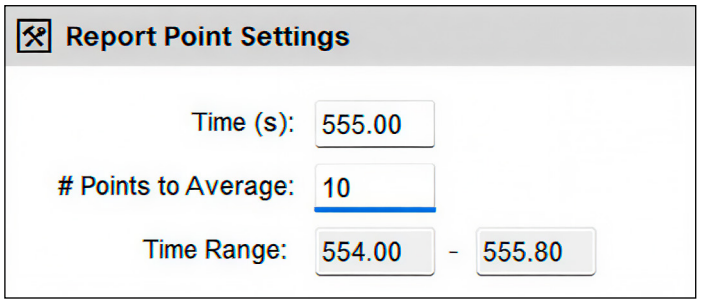

Figure 5: Example of a report point setting set to 555 seconds and an average of 10 data points. At a collection rate of 5 Hz, 5 data points are collected per second, so the time range reflects the time range required to achieve 10 data points to average.

In the binding response curve plot on the far right, a vertical bar shows the center of this averaging window (Figure 6).

Note: that the report point bar in this graph can be moved by Shift + Click anywhere on the graph..

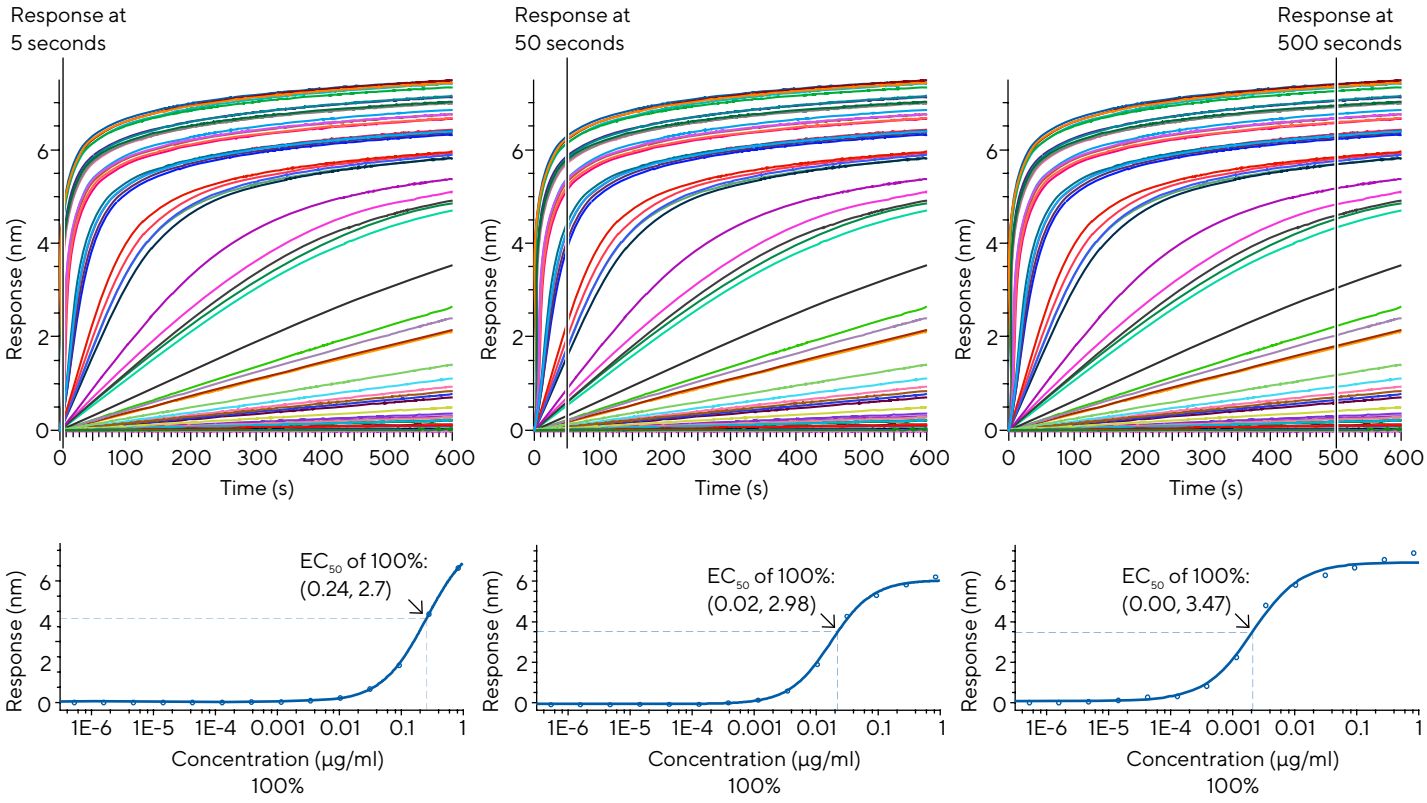

Figure 6: Center of Averaging Window in Binding Response Curve Plot at times 5, 50 and 500 seconds. Note the subsequent change in the shape of the plotted response data and modeled fit.

Grouping, Selecting Standard Groups, and Normalization

Grouping allows you to organize the data for fitting, designating which data groupings to fit in one model versus data which are distinct, and fit to independent models.

As an example of how to use this option, let us assume we have an experiment where the binding of the human IgG1 antibody trastuzumab to Protein A biosensors was assessed. Five different replicate groups representing 80, 90, 100, 110 and 120% of a standard trastuzumab concentration series were prepared and assessed.

There are five replicates organized in columns in the sample plate. In the experiment, all the data have the same Sample ID but different Replicate Group information. In the Dose Response Analysis, if we click the "Group By..." button and select Sample ID only in the pop-up window (Figure 7).

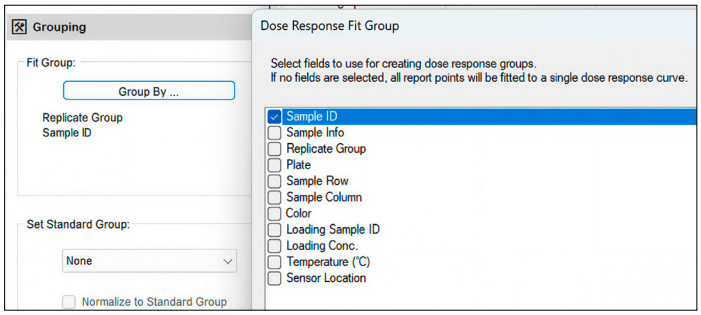

Figure 7: Grouping by Sample ID.

Then in a dose response plot, there will be a single fitted dose response curve with five replicates around each concentration representing the 80, 90, 100, 110 and 120% concentration (Figure 8).

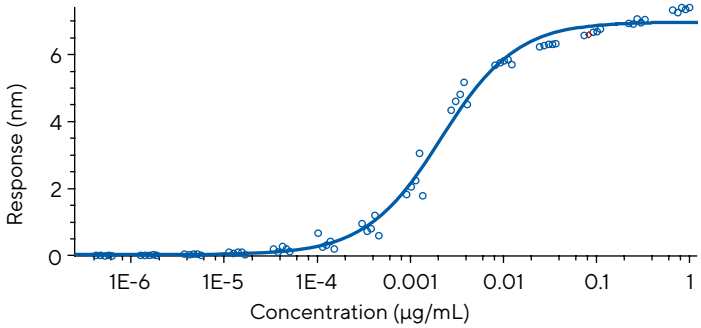

Figure 8: Dose response plot of replicates grouped by Sample ID.

If we click "Group By..." and instead select Sample ID and Replicate Group in the dose response plot, there will be five sets of points with five independent dose response curve fits (Figure 9).

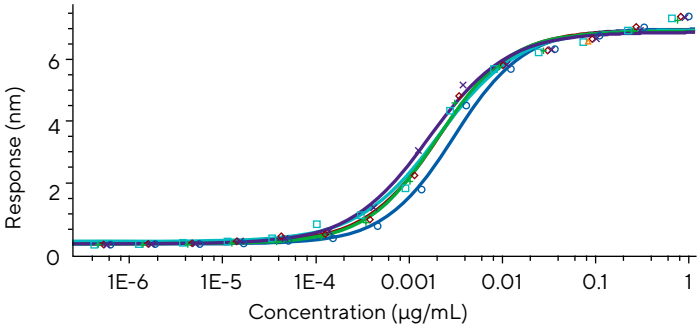

Figure 9: Dose response plot of replicates grouped by Sample ID and Replicate Group.

Standard Group

If the choice of grouping options have created multiple dose response curves, it is possible to set one as a standard by selecting one group in the Set Standard Group option (Figure 10).

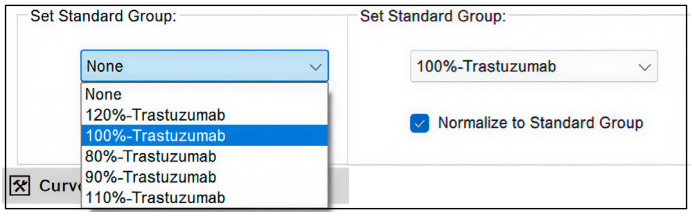

Figure 10: Setting a Standard Group. (A) Select the desired Standard Group and (B) if required, data can be normalized to that Standard Group.

Choosing one standard enables the Pair View where you can see individual dose response curves overlaid on the standard curve. Choosing a standard also enables the Normalize to Standard Group checkbox. This normalization option rescales the response. Rescaling sets the minimum response of the standard group to 0 and the maximum response is set to 100. All other response values are scaled according to this transformation (Figure 11).

Note: Global Fit in the curve fitting section must be selected for rescaling to take effect.

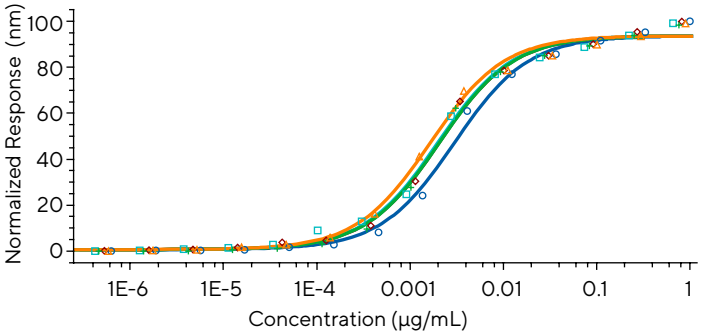

Figure 11: Dose response plot of replicates grouped by Sample ID and Replicate Group with the Standard Group set to 100% and the Normalize to Standard Group checkbox ticked. Note the minimum response is set to 0 and the maximum response set to 100.

Curve Fitting

The Curve Fitting panel collects the options for tailoring the dose response model fit to achieve the best statistics.

The Octet® BLI Analysis Studio Software allows for two types of fits to the data, Independent and Global.

In an **Independent Fit**, each is fitted with the same model function, but the variables of all curves are independent of one another as shown in Figure 9 (assuming multiple datasets are present).

In a **Global Fit**, if there are multiple datasets, each is fit with the same model function with the constraints that all curve fits converge to the same upper and lower asymptotes, the same Hill coefficient, and same asymmetry parameter as shown in Figure 11.

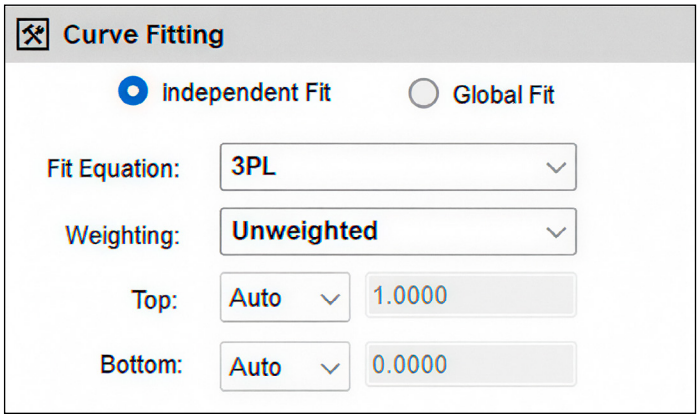

Figure 12: Curve fitting panel.

Note: Only the EC_{50}/IC_{50} values differ between datasets. For the linear and semilog fits, the slope is forced to be the same for each group, while the intercept term is allowed to differ between groups.

The fit equation dropdown offers five model functions:

1. 3PL
2. 4PL
3. 5PL
4. Linear
5. Semilog line

The differences between the models are discussed below.

3PL (Three-Parameter Logistic Model):
This model is used to fit a curve to data where the relationship is expected to be logistic. It includes three parameters: the lower asymptote, the upper asymptote, and the growth rate. The 3PL model uses the same equation as the 4PL model but the Hill Coefficient is fixed to the value of 1.0 (i.e., the h parameter is fixed to the value of 1 in Equation 1).

4PL (Four-Parameter Logistic Model):
The 4PL model allows for more flexibility in fitting data as the Hill Coefficient is a variable parameter . The 4PL equation therefore, includes parameters for the lower asymptote, upper asymptote, inflection point, and Hill coefficient (slope) (Figure 13).

The equation generally looks like:

$$y = R_{Bot} + \frac{(R_{Top} - R_{Bottom})}{1 + \left(\frac{x}{A}\right)^{h}}$$

Equation 1

Where (Figure 13):
y and ***x*** are plotting coordinates of the analyte binding concentrations and responses, respectively.

R_{Top} is the upper asymptote
R_{Bottom} is the lower asymptote
A is the x-value at the mid-range concentration inflection point
h is the Hill coefficient

Note: EC_{50} is the midpoint concentration between the upper and lower asymptotes.

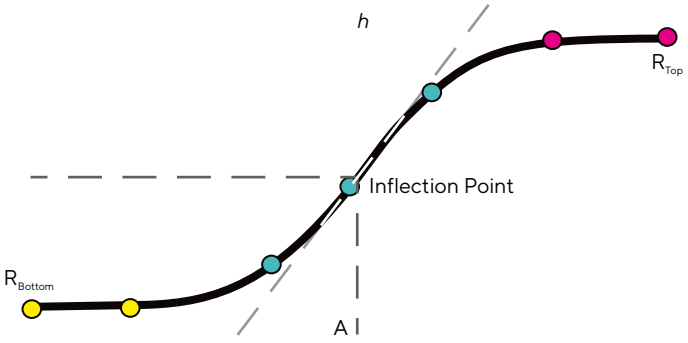

Figure 13: Hill equation parameters model on a sigmoidal curve. R_{Bottom} and R_{Top} are the lower and upper asymptotes, A is the x-value at the mid-range concentration inflection point, and *h* is the Hill coefficient.

5PL (Five-Parameter Logistic Model):
The 5PL model further extends the 4PL model by adding a parameter that allows for asymmetry in the curve. It is used when the data shows asymmetrical behavior. The equation generally looks like:

$$y = R_{Bot} + \frac{(R_{Top} - R_{Bottom})}{\left(1 + \left(\frac{x}{A}\right)^{h}\right)^{b}}$$

Equation 2

Where:
y and ***x*** are plotting coordinates of the analyte binding concentrations and responses, respectively

R_{Top} is the upper asymptote
R_{Bottom} is the lower asymptote
A is the x-value at the mid-range concentration inflection point
h is the Hill coefficient
b is the asymmetric factor

Note: EC_{50} is the midpoint concentration between the upper and lower asymptotes.

The **Linear Model** fits to the standard linear model of:

$$y = a^{*}x + b$$

Equation 3

Finally, the **Semilog Line** model fits to the standard semi-logarithmic line model of:

$$y = a^{*}log(x) + b$$

Equation 4

Which one to use?

With so many models it can be confusing as to which model should be chosen to fit your data. The simple rule of thumb is to pick the model that fits the data the best with the fewest number of floating parameters i.e., if the data looks like a symmetric sigmoidal curve with clear upper and lower asymptotes, start with the 4PL model.

When dealing with truncated data, the most appropriate model to use is 3PL (Figure 14). The 4PL model is symmetrical and therefore, most appropriate for biological data sets that display symmetry with a clearly defined sigmoidal shape (Figure 14). Finally, the 5PL model is most appropriate for complete data sets that do not exhibit symmetry. As discussed above, the 5PL contains an additional parameter (b) that corrects for asymmetry as shown in Figure 14.

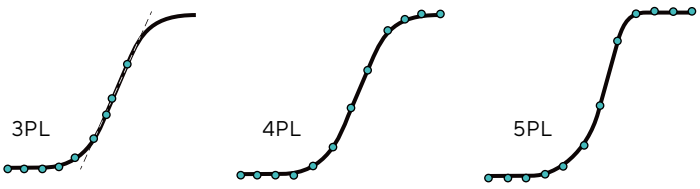

Figure 14: Examples of 3PL, 4PL and 5PL data sets. 3PL is a reduced 4PL model with the Hill coefficient set to 1.0 in order to accommodate truncated data sets with no upper asymptote. 4PL is the most commonly used model for fitting data sets that exhibit a symmetrical sigmoidal dose response. 5PL contains an additional parameter to accommodate asymmetrical data sets (ceiling or floor effects).

Which One to Use?	
3PL	Truncated data with no upper asymptote
4PL	Symmetrical data with a lower and upper asymptote
5PL	Asymmetrical data with a lower and upper asymptote

Weighting

Why Use Weighted Models?

Weighted models are particularly useful when the precision of measurements varies across different doses. For example, low doses might have higher variability than high doses, and weighting can help to account for this. By using weights, the model can provide a better fit to the data, leading to more reliable estimates of parameters.

In the context of dose-response analysis, the difference between unweighted and weighted dose-response models lies in how the data points are treated during the fitting process.

In general, in an **unweighted dose-response**, all data points are treated equally during the curve fitting process. This means that each observation contributes equally to the determination of the best-fit parameters. This approach assumes that the variability or error associated with each data point is the same across all doses. Unweighted models are simpler but may not be appropriate if the variability in the data changes with the dose level.

In a **weighted dose-response**, different weights are assigned to data points based on certain criteria, such as the variance or standard deviation of the measurements at each dose level. Weighting is used to give more importance to data points with lower variability or higher reliability, and less importance to those with higher variability. This approach can improve the accuracy of the model by accounting for heteroscedasticity (non-constant variance) in the data, which is common in biological assays where variability often increases with dose.

In summary, the choice between unweighted and weighted models depends on the characteristics of the data and the assumptions about variability across different dose levels. Weighted models are often preferred when there is significant heteroscedasticity in the data.

The Octet® BLI Analysis Studio Software allows for the following weighting:

Unweighted: A symmetrical dose response curve. No points are weighted during the curve fitting.

Weighted Y: A symmetrical dose response curve with weighting applied as 1/Y (as Y increases, weighting decreases).

Weighted 1/Y²: A symmetrical dose response curve with weighting applied as $1/Y^2$.

In addition to the 3PL, 4PL, and 5PL models and weighting, users have some control over the independent variables such as the upper and lower asymptotes, the Hill coefficient, and the asymmetry. For each independent variable the user can select:

Auto: The curve fit code will make an initial estimate of this variable and run the iterative fitter, optimizing this variable to minimize the difference between the model and data.

Fixed: Here you can assign a variable to a set value and the iterative fitter will treat it as a constant. This option is useful for the asymptote values if your assay doesn't include controls at very high and very low concentrations.

Seed: If the data is sparse and noisy, the initial estimate of the variable may not be good and the iterative fitter will have trouble optimizing the model. You can override the automatic initial estimation by selecting Seed and entering your best guess.

Alerts

The use of alerts in the Octet® BLI Analysis Studio Software enables the user to flag cycles that may need to be excluded from the analysis due to variability in loading. This is achieved by enabling the Loading Z-Score alert. The z-score is computed with this formula:

$$y = \left(y\left(\mathrm{i}\right) - \bar{y}\right)/\sigma$$

Equation 5

Where y(i) is the response, or nm shift, of an individual loading step, $\bar{y}$ is the average response of the group of loading steps, and σ is the standard deviation of this group. A group is defined as loading steps that have the same Sample ID and loading concentration so that the data are comparable.

The z-score is a measure of the distance of a data point from the mean in units of standard deviation of the sampled population. If the distribution of values about the mean is normal, also known as Gaussian, 99.7% of random samples should fall withing 3 σ of the mean.

Typically, if a loading step z-score is more than 3 σ, the assay is considered suspect and is flagged for inspection. If a flag is raised, the data is not automatically excluded from the analysis.

Next Page: **User Examples** >

User Examples – Case Number 1

Question:
I'm a scientist in a formulation department. My protein is an antibody fragment (no Fc region). I'd like to test the impact of long-term storage into different formulations.
1. How should I proceed ?
2. What are the different options in terms of assay design?

Answer:
Prior to testing the impact of long-term storage it is important to design, develop, and where necessary, qualify a suitable assay (see Harmonizing Regulatory Guidelines for Assay Validation: A Focus on Kinetics-Based Assays Using Octet® BLI Systems and Octet® Potency Assay: Development and Validation Strategies for further information on assay qualification and validation). As the protein has no Fc domain, typical biosensors such as anti-human capture (AHC2) and Protein A (ProA) or Protein G (ProG) are not suitable for quantification assays. Instead, Protein L (ProL, Item No.: 18-5085) and Anti-Human Fab-CH1 (FAB2G, Item No.: 18-5125) that bind to the CH1 region should be assessed (see Figure 15).

Once a suitable biosensor has been determined a one-step (direct binding to the biosensor) or two-step (assessment of the molecule bound to the biosensor) can be performed to assess the current binding levels of the protein to the biosensor and/or antigen, respectively (see Designing and Measuring Drug Potency Using the Octet® BLI Ligand Binding Methods for further information).

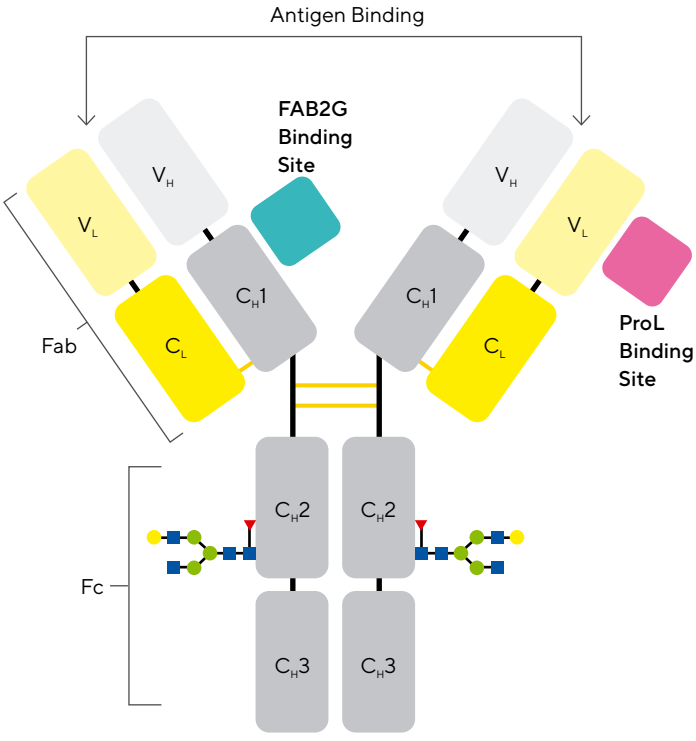

Figure 15: Suggested Octet® BLI Biosensor for binding to antibody fragments that lack an Fc domain.

In addition, direct binding to the antigen captured or immobilized to the biosensor surface may also be performed to assess the impact of long-term storage. In both cases where the antigen is assessed the user must be sure that the antigen is also stable under long-term storage or that a fresh supply of active protein can be produced.

User Examples – Case Number 2

Question:
My antibody is a full-length IgG. I have already performed my EC_{50} experiment using AHC2 with my IgG at different concentrations and the antigen at a saturating concentration.
1. Looking at the detection responses (test vs standard) over the IgG sample concentration range, what does it tell me if the Hill coefficient is different?
2. Looking at the detection responses (test vs standard) over the IgG sample concentration range, what does it tell me if the response level is different?

Answer:
A difference in the EC_{50} Hill coefficient can indicate several things when assessing binding and these include a change in cooperativity. Where binding of one ligand increases the affinity of the receptor for additional ligands a Hill coefficient of greater than 1 will be observed and the opposite for negative cooperativity, where a Hill coefficient less than 1 will be observed.

As the assay is already established and only contains a single antigen at saturating concentration, cooperativity is not relevant and other parameters such as drug potency, efficacy and biological variability must be considered.

As the assay potentially contains two variables (the IgG and the antigen) it is possible that a change in the Hill coefficient is caused by a more biologically active batch of the antigen, which would cause an increase in the Hill coefficient and show a more pronounced effect over a narrower concentration range (Hill coefficient would increase and be greater than 1 compared to the standard). If the batch of antigen was less biologically active than the opposite would be observed, where a shallower slope would occur and the Hill coefficient would decrease and be less than 1 compared to the standard).

The change in Hill coefficient may also be caused by a change in the biological activity of the captured IgG with the same observed effect. A more biologically active batch

of IgG would increase the Hill coefficient due to binding more antigen over a narrower concentration range and a less biologically active batch would decrease the Hill coefficient.

An example of this effect is shown below in a 2-step assay where Octet® AHC2 Biosensors were used to capture a human IgG and a Fab anti-hIgG was used during the detection step. Human IgG Samples were prepared to 130, 100 and 70% of the nominal concentration and captured onto AHC² biosensors before addition of the detection reagent Fab anti-hIgG at a fixed concentration.

As can be seen in Figure 16, fitting the data using an independent fit to a 5PL model highlights the change in observed Hill coefficient. As expected, higher concentrations (130 % of nominal concentration, representing more biologically active material) shifted the dose response curves to the left, while the lower (70 % of nominal concentration, representing less biologically active material) shifted the dose response curves to the right. As can shown in Table 1, in addition to the change in Hill coefficient, a corresponding change in the EC_{50} is observed with 130% exhibiting a lower EC_{50} value than the standard (19.75 vs. 24.31, respectively) due to an increase in biologically active material. The opposite is observed for 70%, where a higher EC_{50} value is observed compared to the standard (37.31 vs. 24.31, respectively).

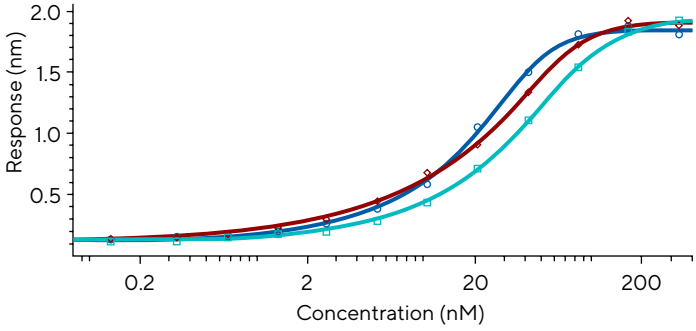

Figure 16: Two- step assessment of 130% (Blue), 100% (Red) and 70% (Cyan) of nominal hIgG sample concentration demonstrates the effect on the Hill coefficient and EC_{50} in the presence of more or less biologically active sample in an assay.

	Hill Coefficient	EC_{50} (nM)
Standard	2.8732	24.31
130%	3.1386	19.75
70%	2.0407	37.31

Table 1: A Summary of Octet® Analysis Studio Software v13.1 fitted parameters for dose-response of Fab anti-hIgG to hIgG with +/-30% change in nominal concentration.

As discussed in depth above, the effect of more, or less, biologically active sample or detection agent (antigen) can have a profound effect upon the observed results. Therefore, it is of key importance that the binding responses of the assay are assessed prior to fitting. For example, using the data set shown above for human IgG Samples prepared to 130, 100 and 70% of the nominal concentration. It is known that all samples were assessed using a single preparation of a detection reagent (Fab anti-hIgG), therefore, any changes in observed response must be due to changes in hIgG capture levels. As shown in Figure 17, assessment of the 3.13 µg/mL (20.9 nM) hIgG capture response shows a clear difference between the standard response (orange) of 0.75 nm and the 130% (yellow) and 70% (burgundy) responses of 0.95 nm and 0.55 nm, respectively. Therefore, a change in response with a standard solution of detection reagent is primarily due to changes in the concentration or biological activity of the capture molecule (here hIgG). Where the capture levels are the same but the detection levels are different, the structure/folding of the capture molecule is most likely to cause the change.

Intra-assay changes in response, are in general simpler to interpret as the detection molecule is generally prepared in bulk and divided between wells. Inter-assay result changes in response can be more difficult to interpret due to the presence of multiple variables. Therefore, it is highly recommended that if batch-batch samples are assessed a qualified or validated assay for this purpose as it will allow the user to determine the root cause of the change in response.
(see User Examples – Case Number 1
for further information)

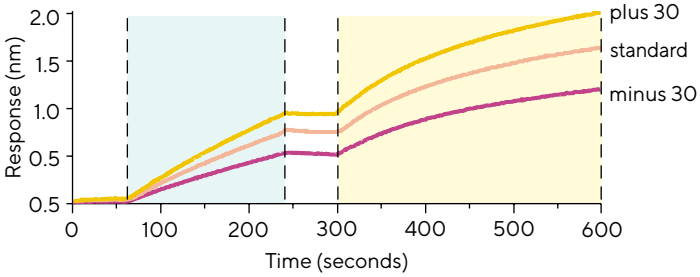

Figure 17: Assessment of hIgG sample concentration 1(30% (yellow), 100% (orange) and 70% (burgundy)) highlights the difference in the observed capture response (cyan box) prior to the detection step (yellow box).

User Examples – Case Number 3

Question:
Currently, I use ELISA to calculate EC_{50}. A typical assay design is the target (antigen) is adsorbed to the wells. Then 8 to 12 concentrations of my Reference and Test samples (mAbs) are tested. For the detection, a secondary anti-Fc or anti-Fab HRP labelled is used followed by TMB (HRP substrate).
What would be the benefit of using Octet® BLI for this application?

Answer:
Enzyme-linked immunosorbent assays (ELISA) are routinely used to quantitate molecules and despite their popularity, these assays are labor- and time-intensive. Quantitation assays on the Octet® platform can be considered automated forms of ELISA but allow a myriad of benefits compared to standard ELISA assays.

Quantitation assays on the Octet® platform have many similarities to ELISA. Both are performed on a solid support on which the capture molecule (antigen) is immobilized and the analyte is bound from solution.

The most obvious benefit of using Octet® BLI for EC_{50} determination is the ability to perform a direct binding assay (also called 1-step) (Figure 18), that generally affords a dynamic range of detection from low ng⁄mL to low mg⁄mL, depending on the analyte. The direct binding assay is fast, easy and eliminates the need for secondary reagents and steps. It also allows regeneration of the biosensor, where the analyte is removed from the ligand and allows multiple samples to be assessed using the same ligand-coated biosensors. This is advantageous as it reduces sample requirements while increasing sample throughput in a single assay.

Where an increased dynamic range is required for EC_{50} determination, multi-step assays (Figure 18) provide enhanced sensitivity down to low pg⁄mL. Multi-step assays include sandwich-style assays (also called 2-step assays), which use the biosensor to capture analyte in the first step, followed by use of a second molecule (commonly an antibody) to sandwich the analyte in the second step. For even more signal amplification, an enzyme-linked sandwich assay (also called a 3-step assay) captures analyte bound by two separately-labeled capture molecules to the biosensor in the first step, binds an HRP-conjugated antibody to the complex in the second step, and precipitates a substrate directly onto the biosensor surface in the third step.

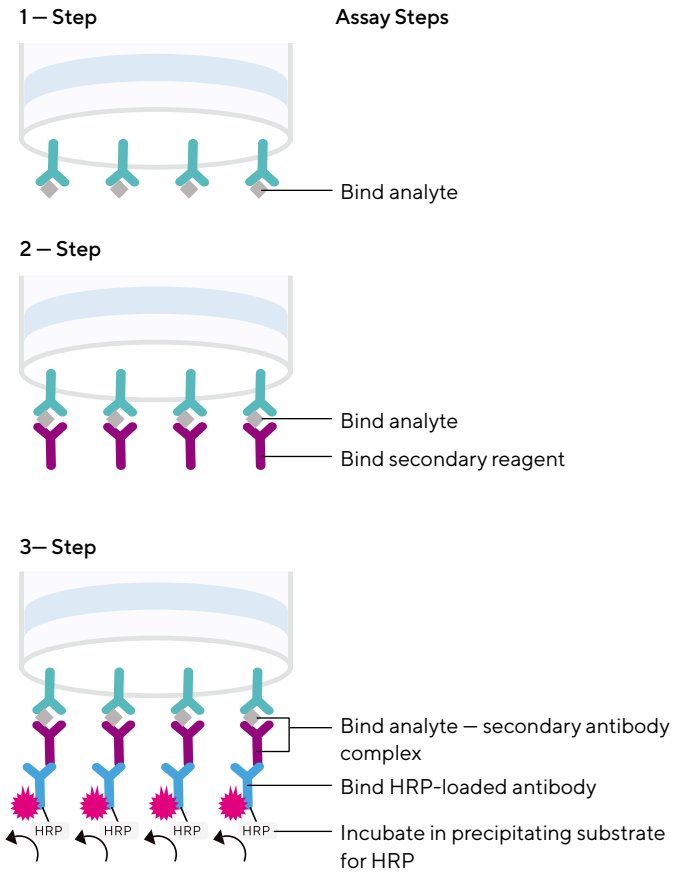

Figure 18: Octet® BLI systems offer the advantage of a direct binding assay while also offering the flexibility of multi-step assays that increase sensitivity.

While ELISA remains a powerful and widely used method, Octet® BLI's label-free, real-time, and flexible nature make it advantageous for precise EC_{50} determination, especially in research and development settings where kinetic analysis and rapid assay development are critical. Therefore, it is often beneficial to convert ELISA assays to the Octet® platform for EC_{50} determination as it allows:

- Label-Free Detection and Reduced Sample Preparation: Unlike ELISA, which typically requires labeling of the analyte or detection molecule, Octet® BLI is label-free, which reduces potential interference from labels. The simplified assay setup for EC_{50} determination does not involve washing steps and therefore, the streamlined 1-step process can save time and reduce potential errors associated with sample handling.

- Real-Time Analysis: Octet BLI provides real-time monitoring of binding interactions, which allows for the direct observation of kinetic parameters such as association and dissociation rates. This real-time

capability allows each step of the assay to be monitored, including antigen binding to the biosensor, which can lead to increased precision across assay. This increased precision is reflected in the determined EC_{50} as BLI allows any changes in binding events and their effects to be assessed.

- Direct Measurement of Binding Interactions: Octet BLI measures binding interactions directly through changes in the interference pattern of light, providing a direct correlation between antibody concentration and binding response. This can lead to more accurate and reproducible EC_{50} values.

- Broad Dynamic Range: The technology often provides a broader dynamic range for detection, which is beneficial for accurately determining EC_{50} across varying analyte concentrations.

- Flexibility and Throughput: Octet BLI systems can analyze multiple samples simultaneously, increasing throughput compared to traditional ELISA. The system can be adapted to different assay formats quickly, allowing for a more flexible approach in experimental design.

In summary, Octet® EC_{50} assays share similarities with ELISA-based assays but the Octet® platform's direct binding assay method (1-step) offers users a simple, fast and accurate method to determine EC_{50}. Where required, multi-step methods offer high sensitivity and expanded dynamic range. Automated assay formats enhance time-to-results and walk-away time and reduce operating expenses. Conversion of a pre-configured ELISA assay to a 1-step assay on the Octet® platform may only require transfer of existing assay conditions (see Converting an ELISA Assay Into an Octet® Quantitation Assay for further information) and where required, assay optimization on the Octet® platform is often similar to those employed in ELISA.

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