APPLICATION NOTE 19

Analysis of FcRn-antibody interactions on the Octet platform

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Introduction

The Fc region of human IgG contributes to a number of beneficial biological and pharmacological characteristics of therapeutic antibodies. One of the most important is prolonging plasma half-life, due to its unique, pH-dependent interaction with the neonatal Fc-receptor (FcRn). Because altered FcRn binding can increase or decrease serum half-life of Fc-containing therapeutics, thereby impacting drug efficacy, FcRn binding interactions are increasingly being assessed at multiple stages of biologic drug development. FcRn-Fc activity and binding assays are performed as part of characterization studies to enhance overall product understanding and demonstrate comparability in the development of biosimilars. Commonly used in vitro methods for analysis of FcRn binding include ELISA, SPR, and bead-based proximity assays.

The Octet® system from ForteBio, in conjunction with disposable Dip and Read™ biosensors, offers users the ability to accurately assess FcRn - IgG binding in high throughput, versatile, and easy to use format. In this application note, assay design and best practices for FcRn-IgG kinetic analysis on the Octet system will be discussed, as well as considerations for assay optimization, data acquisition, curve fitting, and analysis of results.

The Octet platform for analyzing FcRn-antibody interactions

The Octet family of instruments is based on Bio-Layer Interferometry (BLI), a label-free biosensor technology that measures molecular interactions in real time for the purpose of detection, quantitation and kinetic analysis (Figure 1). Octet instruments can read from 2 to 96 samples simultaneously in automated format using a standard microplate for rapid determination of binding affinity constants (K_D), association rates (k_a or on-rate) and dissociation rates (k_d or off-rate). In addition to complete kinetic characterization, equilibrium assays can be used to determine K_D, or simple binding assays performed to rapidly evaluate relative affinity or screen large numbers of samples. BLI biosensors are compatible with a wide range of sample types and buffers, including cell extracts, serum, and media.

The standard microplate format combined with disposable Dip and Read biosensor technology enables automated, highly parallel processing on the Octet system in sample volumes as low as 40 µL. Hands-on time is significantly reduced on the Octet system when compared with the multiple incubation and wash steps required for ELISA or complex assay development required for bead-based assays. A wide array of biosensor specificities enables flexibility in assay formatting. In contrast
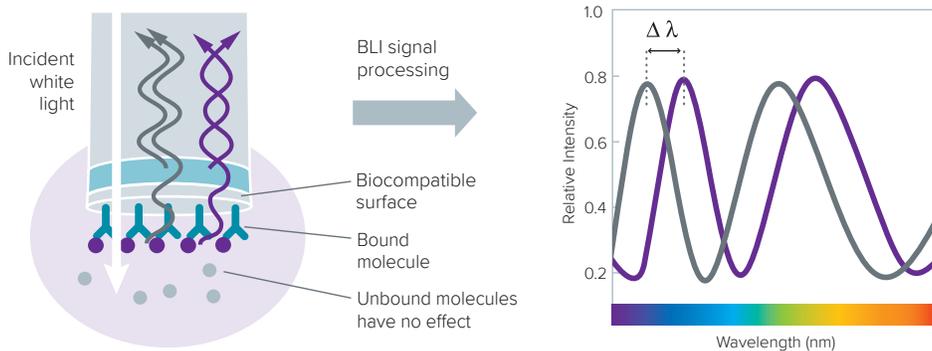
Figure 1: BLI measurement using Dip and Read biosensors. BLI is an optical analytical technique that analyzes the interference pattern of white light reflected from two surfaces. Changes in the number of molecules bound to the biosensor tip causes a shift in the interference pattern that is measured in real time.
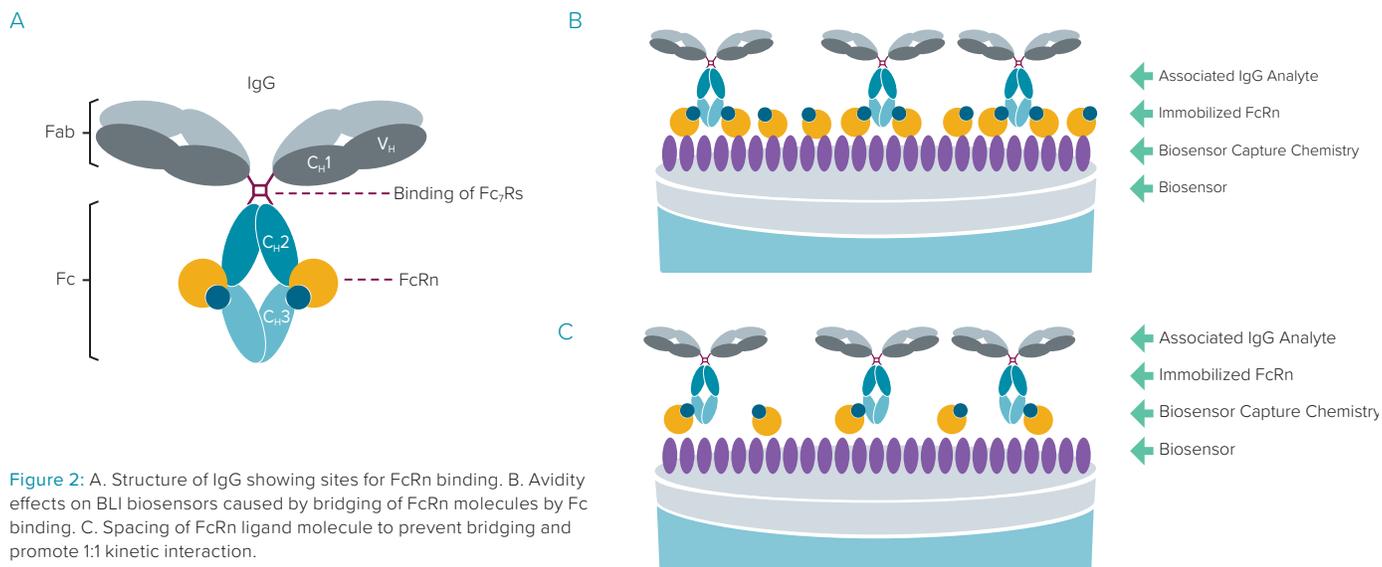
Figure 2: A. Structure of IgG showing sites for FcRn binding. B. Avidity effects on BLI biosensors caused by bridging of FcRn molecules by Fc binding. C. Spacing of FcRn ligand molecule to prevent bridging and promote 1:1 kinetic interaction.

with endpoint assays such as ELISA, which yield only a single readout per sample, kinetic analysis on a biosensor provides binding data in real time, yielding significantly more information about the activity and behavior of a molecular interaction.

For analyzing FcRn binding interactions, which often have fast association rates, use of a higher sensitivity instrument is recommended. The Octet RED96e, Octet RED 384, Octet K2 systems and the Octet HTX system in 8- or 16-channel acquisition mode offer the highest sensitivity of the Octet platform for kinetic analysis and are the suggested instruments for analysis of FcRn-IgG binding interactions.

Biosensor selection and assay orientation

A primary consideration when developing FcRn-antibody kinetic assays on the Octet system is assay format. In a typical binding kinetics assay using BLI, one of the binding partners is immobilized on the biosensor tip surface (ligand) while the other remains in solution (analyte) and associates to the immobilized ligand. Several factors must be considered when choosing which binding partner to immobilize, including stability and size of each molecule as well as the potential for avidity. FcRn receptor itself is a major histocompatibility complex (MHC) class I-like heterodimer that binds to the CH2-CH3 hinge region of both heavy chains of antibody Fc, resulting in a 2:1 binding stoichiometry[1] (Figure 2A). ForteBio offers biosensor chemistries that are suitable for FcRn immobilization. FcRn can be immobilized via biotinylation and capture onto Streptavidin (SAX) biosensors, or captured via polyhistidine (HIS) tag onto Anti-Penta-HIS (HIS1K) biosensors. In each of these approaches, IgG remains in solution as the analyte.

Since a single IgG can bind to two FcRn molecules, there is potential for avidity to affect kinetic rates when FcRn is used as the immobilized ligand. Figure 2B illustrates how IgG analyte can

potentially bind to multiple immobilized FcRn molecules during the association step, creating a bridging effect. When analyte bridging occurs, dissociation kinetics can be altered resulting in artificially low calculated dissociation constant (k_d).

Two different strategies can be utilized to minimize avidity in a biosensor assay where one binding partner is multivalent: 1) significantly reduce ligand (FcRn) loading density or 2) reverse the assay orientation. If FcRn is immobilized as described above using SA or HIS1K biosensors, the effective surface density of FcRn ligand must be low enough to allow adequate spacing between receptor molecules. This will help to prevent analyte bridging (Figure 2C) and help ensure each IgG molecule binds to a single FcRn molecule. Lowered ligand density can be accomplished by either reducing the concentration of FcRn used in the loading step or by shortening time of the loading step, or both. However, fewer ligand molecules on the surface means fewer available sites for analyte binding, which reduces assay signal. Maintaining adequate signal can therefore limit how much FcRn loading density can be reduced (see Ligand Loading Optimization section).

The second, more reliable, strategy is to reverse the assay orientation so that the bivalent partner (IgG) is immobilized on the biosensor tip surface instead of FcRn. The biosensor we recommend for this assay format is Anti-Human Fab-CH1 (FAB2G). FAB2G biosensors come pre-immobilized with a high affinity ligand that is specific for the CH1 region of human IgG Fab. This capture method is highly specific and reliable for characterizing FcRn-hIgG kinetics with all four subclasses of human IgG, and has the advantage of being more conducive to use as a platform approach when testing multiple IgGs against FcRn and other Fc receptors. Capture of IgG is oriented, creating a more homogeneous surface on the biosensor with the Fc region exposed for receptor binding (Figure 3).
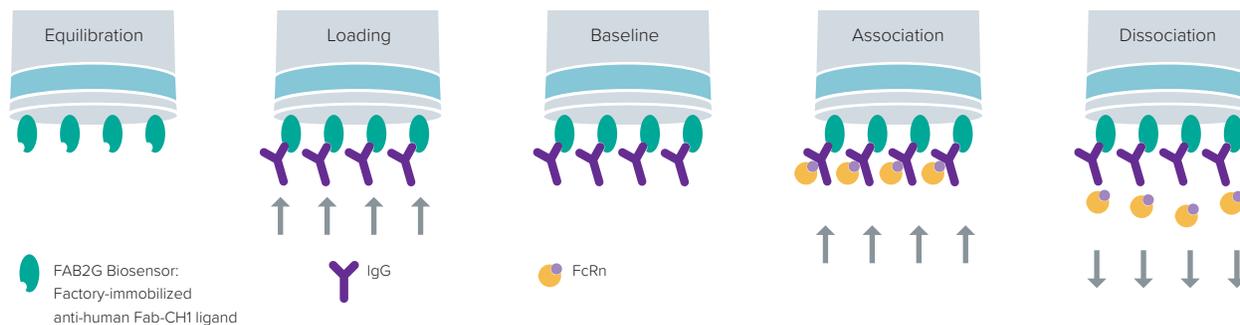
Figure 3: Workflow for FcRn-hIgG kinetic assay on Anti-Human FAB-CH1 (FAB2G) biosensors.

Assay optimization

As with any kinetic assay, it is critical to perform proper assay development when analyzing FcRn binding interactions so that resulting affinity and kinetic constants will be accurate, reliable and reproducible. Quality of the kinetic data depends on using optimal conditions for the biosensor format and the binding pair. Consideration must be given to assay components such as ligand loading density, analyte concentrations, buffer conditions, and assay step times. The data must be examined to ensure the results are in agreement with what is known about the sample and receptor, and evaluated for non-specific interactions.

Quality of reagents

Reagent quality is a critical factor with any kinetics assay. Aggregation of the antibody or receptor can impact kinetics due to increased avidity and non-specific binding. Antibody samples should be fully evaluated for purity, activity and quality using analytical techniques before using in a kinetics experiment. Reagents that have been stored at 4°C for long periods, especially at very high or very low concentrations, should not be used. Careful consideration should be given to purification, storage conditions and handling of receptor proteins and multiple freeze-thaw cycles avoided. Commercially available FcRn can vary in quality, so determining a reliable source of high quality FcRn is critical to good assay performance. FcRn used for experiments described in this application note was provided by Immunitrack. We have found this reagent to be consistent in quality and highly active. More information on FcRn from Immunitrack can be found at http://immunitrack.com.

Assay buffer

The strict pH dependence of the binding interaction between IgG and FcRn is fundamental for efficient recycling and rescue of IgG from intracellular degradation. For FcRn receptor kinetics assays with BLI we recommend using phosphate-based assay buffer at pH 6.0 for dilution of analyte samples as well as baseline and dissociation assay steps (100 mM Sodium Phosphate, 150 mM NaCl, 0.05% Tween-20, pH6.0). This assay buffer ensures efficient binding of FcRn to IgG under acidic conditions and also minimizes non-specific binding. BSA is not recommended as a buffer component, since some cross-reactivity may occur with

FcRn. The ligand loading step can be run in ForteBio's 1x Kinetics buffer (which contains BSA) for best immobilization results. When changing buffers between assays steps - i.e. changing from 1x kinetics buffer in the loading step to pH 6 assay buffer in baseline and association step — be sure to run the baseline for enough time to allow biosensors to equilibrate in the new buffer and for any signal drift to stabilize. A one to five minute baseline step is usually adequate when switching buffers.

Ligand loading

The amount of ligand immobilized (loaded) onto the biosensor — whether FcRn or IgG — can have significant impact on the results of an assay in terms of signal strength, apparent kinetic behavior, and non-specific binding. Although loading as much protein as possible in the loading step will certainly maximize the assay signal, this approach also has the potential to create undesirable artifacts. Effects such as molecular crowding, avidity, non-specific binding, and/or mass transport can all impact the observed binding kinetics. Conversely, if too little ligand is immobilized, assay signal may be very low, resulting in poor separation of data traces and inadequate signal-to-noise ratio. Therefore ligand loading levels should be optimized for every assay and biosensor format.

To perform a loading optimization experiment, several dilutions of ligand molecule are loaded in parallel onto biosensors. An association step is performed for each ligand concentration using the same, high concentration of analyte (10–20x the estimated K_D). A zero-ligand biosensor should also be run as a control for determining whether the analyte binds non-specifically to the biosensor surface. The concentration of ligand to select for the optimized assay should be the lowest concentration of immobilized ligand that yields an acceptable signal response in the analyte association step (typically between 0.4 nm and 0.6 nm). Figure 4 illustrates a loading optimization experiment for an FcRn-IgG binding assay using FAB2G biosensors. The optimal IgG loading concentration to use in this example is the one that yields between 0.4–0.6 nm shift response in the association step, in this case around 3 µg/mL IgG. Also note that the shape of the association step curves is improved at the lower loading concentrations. The binding curves flatten out at equilibrium and show less heterogeneity - indicative of reduced secondary, non-specific interactions and better 1:1 kinetics.
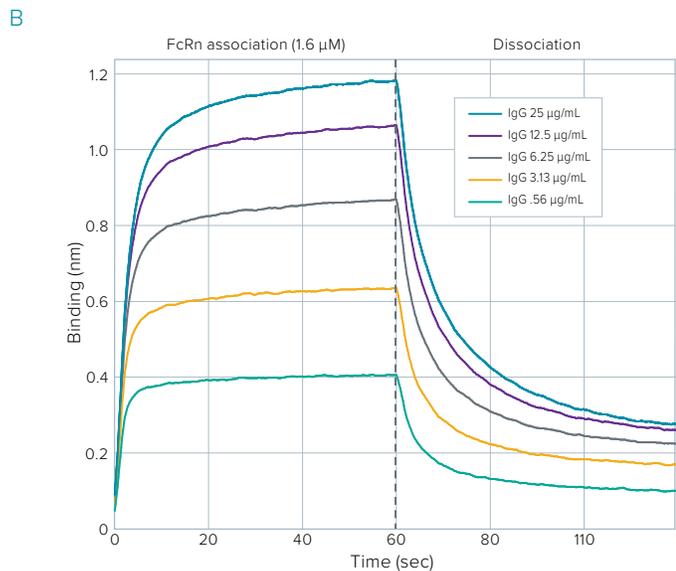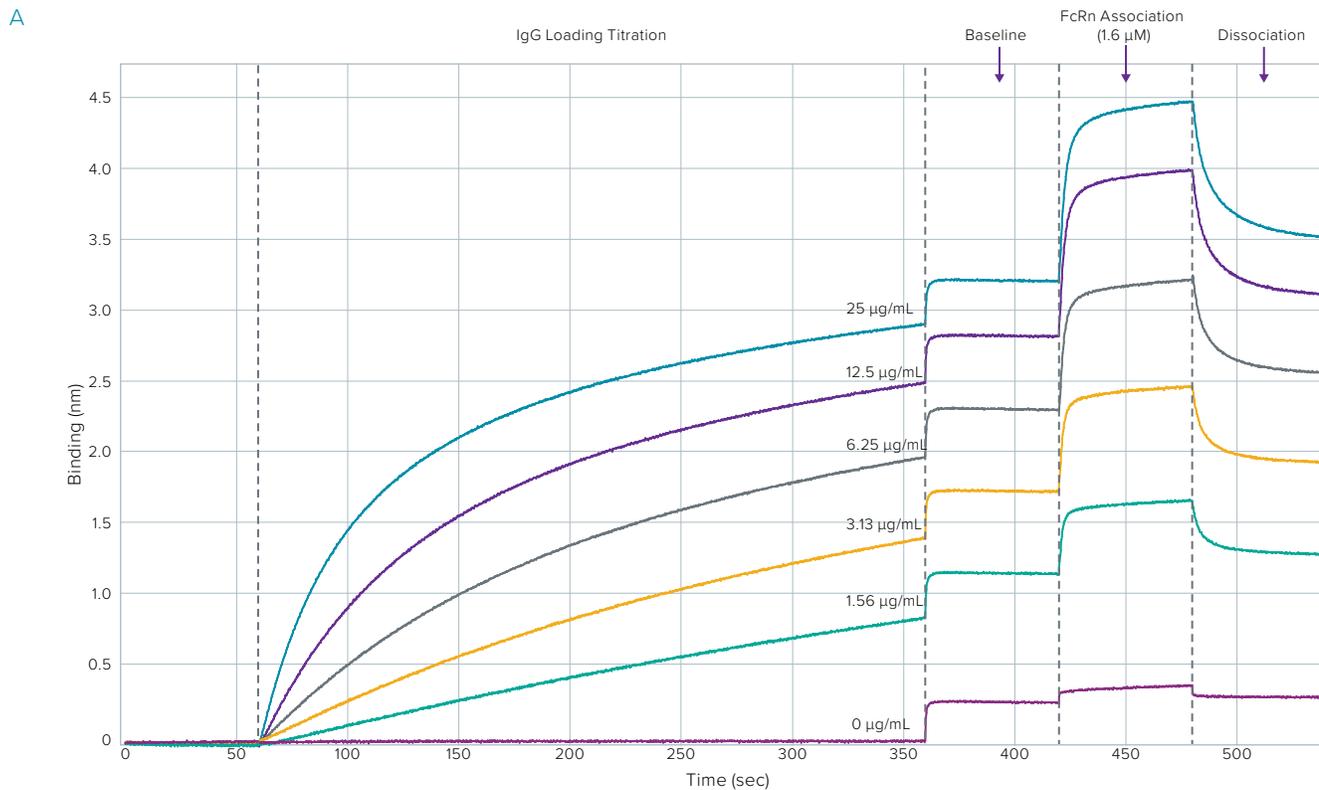
Figure 4: IgG loading optimization for FcRn assay on FAB2G biosensor.
A) Raw data traces. In the loading step, FAB2G sensor is loaded with a 2-fold dilution series of IgG (ligand) starting at 25 µg/mL. A zero-IgG control is also run to assess non-specific binding of FcRn to the biosensor. In the analyte association step, FcRn is run with each IgG concentration at a single concentration of 1.6 µM. B) Overlay of FcRn association step for each IgG loading concentration.

Analyte concentration

In the association step, the rate of binding of the analyte to immobilized ligand is measured. Measuring a single analyte concentration can be sufficient for simple binding assays or qualitative analysis. However, when accurate kinetic and affinity constants are required, a dilution series of at least four to six analyte concentrations must be measured in the association step. Multiple analyte concentrations enable global curve fitting, where all the curves in a data set are fit simultaneously to yield one set of results. The analyte concentration range to use will depend on the sensitivity of the assay and affinity of the interaction, however it should typically range from concentration of about 10x the estimated K_D down to about 0.5x K_D, using 2-fold or 3-fold dilutions.

At acidic pH (pH 6−6.5), FcRn has a low micromolar to nano-molar affinity for the Fc region of IgG.[2] When affinity is high, it may not be possible to see signal at concentrations at or below

the K_D when loading conditions are optimized. In this case, choose a series of analyte concentrations that cover the range of the assay, for example from 20−50x K_D down to the limit of measurement. When affinity is low, e.g. in the micromolar range, using analyte concentration 10x above the K_D may be so high as to be impractical and cause non-specific binding. In this case it is best to choose an analyte concentration range that works for the particular interaction, for example starting at 2 to 5x K_D and titrating down to limit of measurement. As a general guideline, nm shift signal for the highest analyte concentration used should equilibrate at 0.4−0.6 nm. The lowest concentration signal should be higher than 0.01 nm so as to be adequately above the noise level of the instrument. Adjusting the ligand loading level can bring analyte signals into the optimal range (see Ligand Loading Optimization section). Figure 8 shows an example of processed data from optimized FcRn-IgG kinetic assays on FAB2G biosensors.
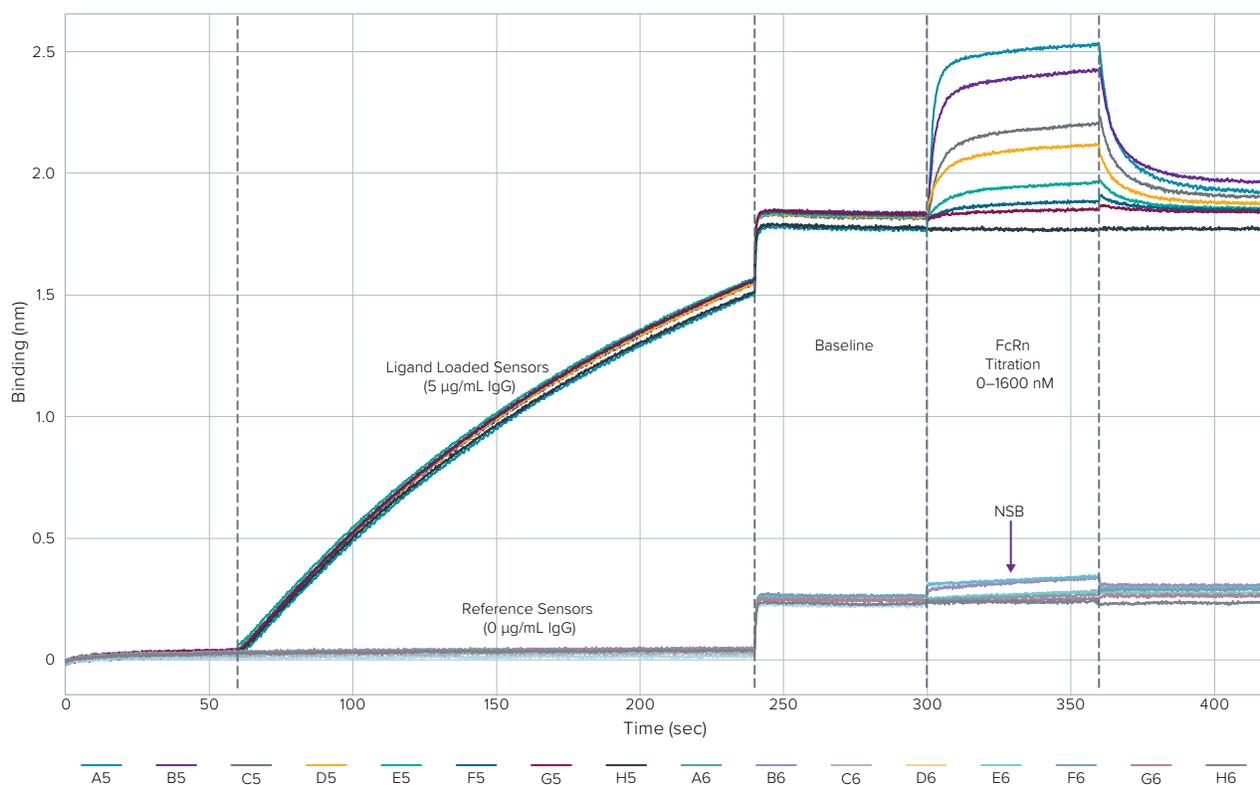
Figure 5: Non-specific binding (NSB) of FcRn to FAB2G biosensors. Loading step for bottom (non-ligand-loaded) trace was run in 1x Kinetics buffer instead of IgG1 ligand. A high concentration of FcRn was associated to the non-ligand-loaded biosensors. NSB is indicated by positive signal in the FcRn association step for the non-ligand-loaded biosensors, indicated by the arrow.

Assay step times

FcRn-IgG interactions often have fast on-rates (>1E5 M-1s-1), where the primary binding interaction will reach equilibrium quickly. When the interaction is fast, the association step should be run only for enough time for the interaction to equilibrate (indicated when binding traces flatten out). This time can be as short as 60 seconds. As long as there is curvature in the association step data traces and equilibrium is reached for higher analyte concentrations, a shorter association step can improve kinetic fitting with a 1:1 binding model.

Non-specific binding

For any biosensor kinetic assay, non-specific binding (NSB) of analyte to the biosensor in the association step must be minimized since it can alter analyte binding profile and interfere with accurate calculation of kinetic rates. In order to test for non-specific binding of analyte, run a preliminary experiment where the concentration of ligand in the loading step is zero, and analyte is run at a single concentration that is well above the K_D (i.e. 10–20x estimated K_D). This test can be easily included in the ligand loading optimization experiment described previously. A positive signal in the association step indicates the analyte is binding non-specifically to the biosensor (Figure 5).

If the NSB signal is minimal, it can be subtracted during data analysis by referencing (see Referencing section). However, NSB nm shift signal that is more than 20% of maximum assay signal should be minimized by optimizing assay conditions. Steps that can be taken to mitigate NSB include modifying the assay buffer and/or adding a blocking step after ligand loading. Increasing the amount of Tween-20 (up to 0.05% v/v) in the buffer or increasing salt concentration can improve the strin-
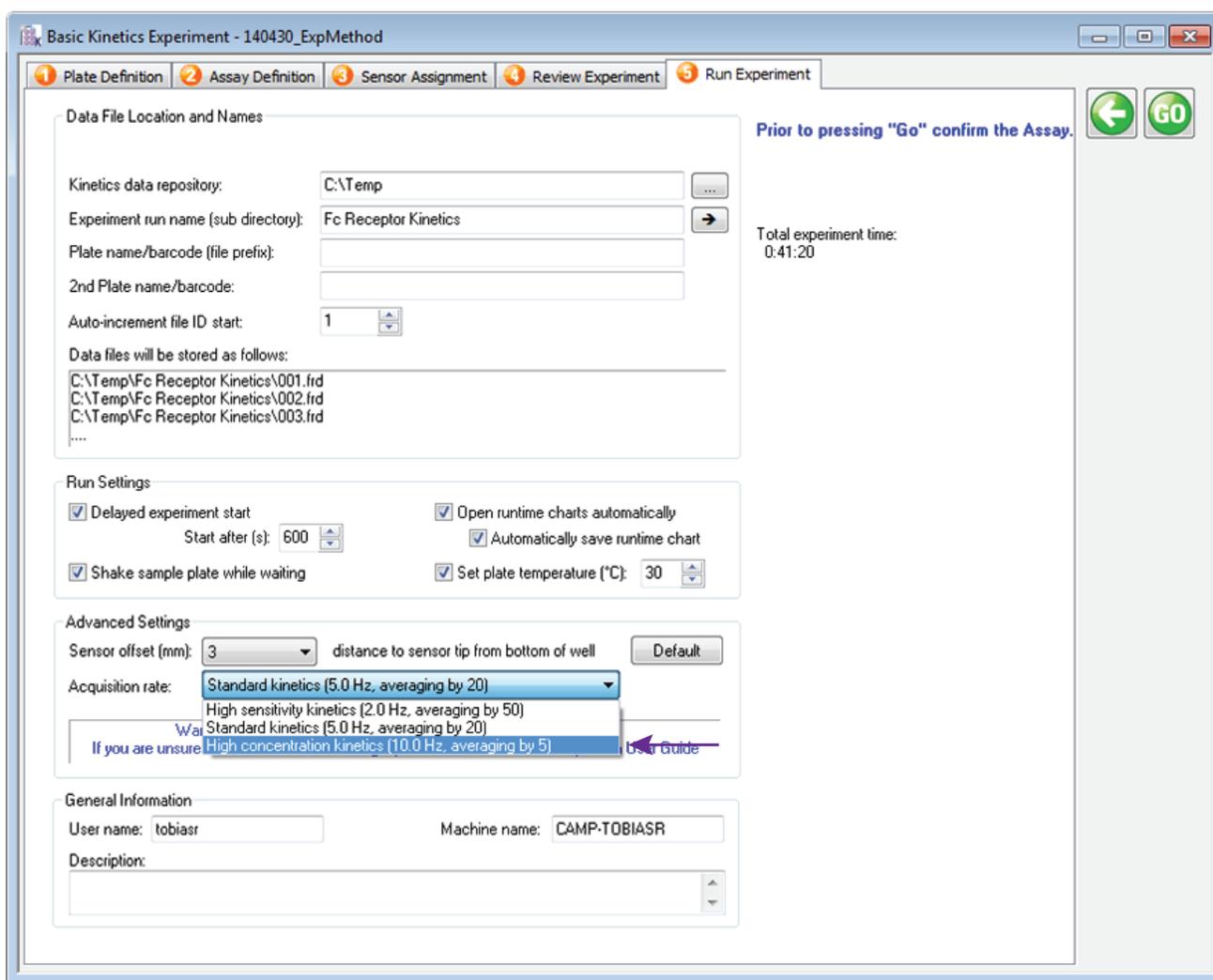
Figure 6: Changing the data acquisition rate. In Octet System Data Acquisition software under the Run Experiment tab, click the pull-down menu for Acquisition rate and select High Concentration Kinetics (10 Hz, averaging by 5). Acquisition rate should be determined based on binding rate, amount of signal generated and experimentation.

gency of binding and eliminate non-specific signal. Adding a five-minute blocking step after loading and before the kinetic baseline step using up to 0.2% casein in PBS can in some cases be effective as well.

Data acquisition rate

Because FcRn-IgG interactions often have fast binding rates, the standard rate of data acquisition (5.0 Hz, averaging by 20) in the Octet System Data Acquisition software may not be the ideal setting. When data is acquired by the Octet system, there is a delay from when the biosensor dips into sample and the first data points are reported to allow the software to average the collected data. This delay can cause the reported signal for the association step to initiate well above the baseline in a fast binding interaction, leading to inaccuracies. If this effect is observed and impacts data fitting, the data acquisition rate can be increased to enable more rapid reporting of binding data. The data acquisition rate refers to the number of binding signal data points reported by the Octet system per second and is reported in Hertz. A higher acquisition rate generates more data points per second with less averaging, and monitors fast binding events better than a slower acquisition rate. The rate setting can be changed in the Advanced Settings box in the Run Experiment tab in Data Acquisition software (Figure 6). Select the acquisition rate for High Concentration Kinetics (10.0 Hz, averaging by 5). Data collected at a higher acquisition rate may have lower signal-to-noise ratio and appear noisier than data collected at standard rate. Acquisition rate setting should always be decided based on the binding rates, the amount of signal generated, as well as experimentation with the settings.

Data analysis

The most accurate kinetic and affinity constants are determined when using global data fitting method with data from several (four to six) analyte concentrations run in parallel. The Octet System Data Analysis software offers several pre-programmed curve fitting models for global analysis of binding data. The kinetic and affinity constants that are calculated depend upon the model selected. Although the solution stoichiometry of the interaction of FcRn with IgG Fc is 2:1, in a properly optimized biosensor assay, the kinetic profile would be expected to follow a single-stoichiometric binding curve representative of a single analyte molecule per binding site – as illustrated in Figure 2B. Therefore, the 1:1 binding model is the most relevant for fitting FcRn-IgG interaction with BLI.

FcRn-IgG dissociation profiles typically display more complex kinetics, however — even in an optimized assay (Figure 7). The dissociation step typically appears biphasic. A possible explanation can be found in crystal structures of FcRn-Fc complex, which suggest two potential conformations for FcRn bound to the Fc region[3–5]. In one, FcRn binds singly, one to each binding site on opposite sides of Fc, as illustrated in Figure 2A. Binding in this conformation would be expected to dissociate via 1:1
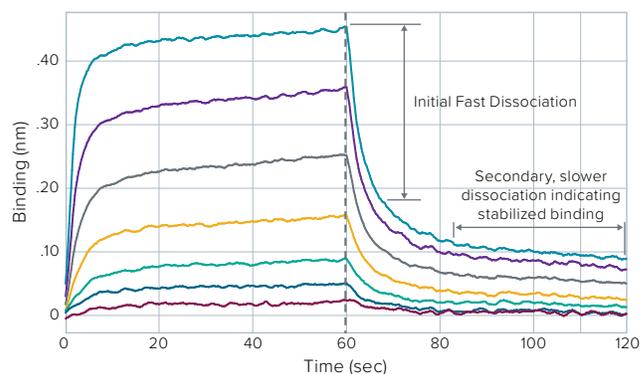
Figure 7: Characteristics of an optimized FcRn kinetic profile on FAB2G biosensors. Processed data shown after reference sample subtraction and alignment to baseline. Association step represents a 2-fold dilution series of FcRn, where equilibrated signal ranges from 0.45 nm at highest concentration, down to 0.02 nm for the lowest concentration. The biphasic dissociation step in FcRn-Fc binding is typical and may be due to dimerized FcRn molecules stabilizing the interaction.

kinetic profile. The other conformation has two FcRn molecules binding a single Fc site asymmetrically as a dimer, with one FcRn molecule involved primarily in the binding interaction and the other contributing to stabilization of the complex. Dimerization is not required for binding in this model, but the presence of an FcRn homodimer bound to Fc increases the affinity of the interaction over monomer binding. This model may explain the biphasic nature of the dissociation step observed in biosensor analysis of FcRn binding to Fc. After an initial rapid dissociation of singly-bound FcRn, the secondary, higher-affinity interaction of dimerized FcRn bound to Fc begins to dominate the kinetic profile in a two-step binding interaction.

The 1:1 kinetic binding model can be used to fit this more complex interaction when only the first portion of the dissociation step is included in the analysis. Truncating the dissociation step fitting to 5–10 seconds enables the initial dissociation rate in the biphasic curve to be captured so that fitting is improved and reproducible off-rate and K_D values can be calculated (Figure 8A, 8B, 8D, 8E). Using this method for curve fitting is most useful for ranking purposes, when FcRn binding is being compared between IgG samples or to a reference material in order to determine loss or gain of activity.

For the purpose of reporting or publishing affinity of an interaction displaying complexity in dissociation, the equilibrium binding constant can be calculated using Steady State analysis tools in the Octet System Data Analysis software. The steady state responses (where the binding trace plateaus, or equilibrates, in the association step) for the various analyte concentrations are calculated using the R-equilibrium (R_{eq}) function and plotted against analyte concentration. The resulting binding isotherm is fitted using the Langmuir model to calculate equilibrium constant K_D (Figure 8C, 8F). Steady state K_D is also useful for ranking experiments or calculating percent activity against a reference sample. Note that the kinetic K_D's using the truncated dissociation step fitting and the steady state K_D's calculated in the experiment in Figure 8 closely match.
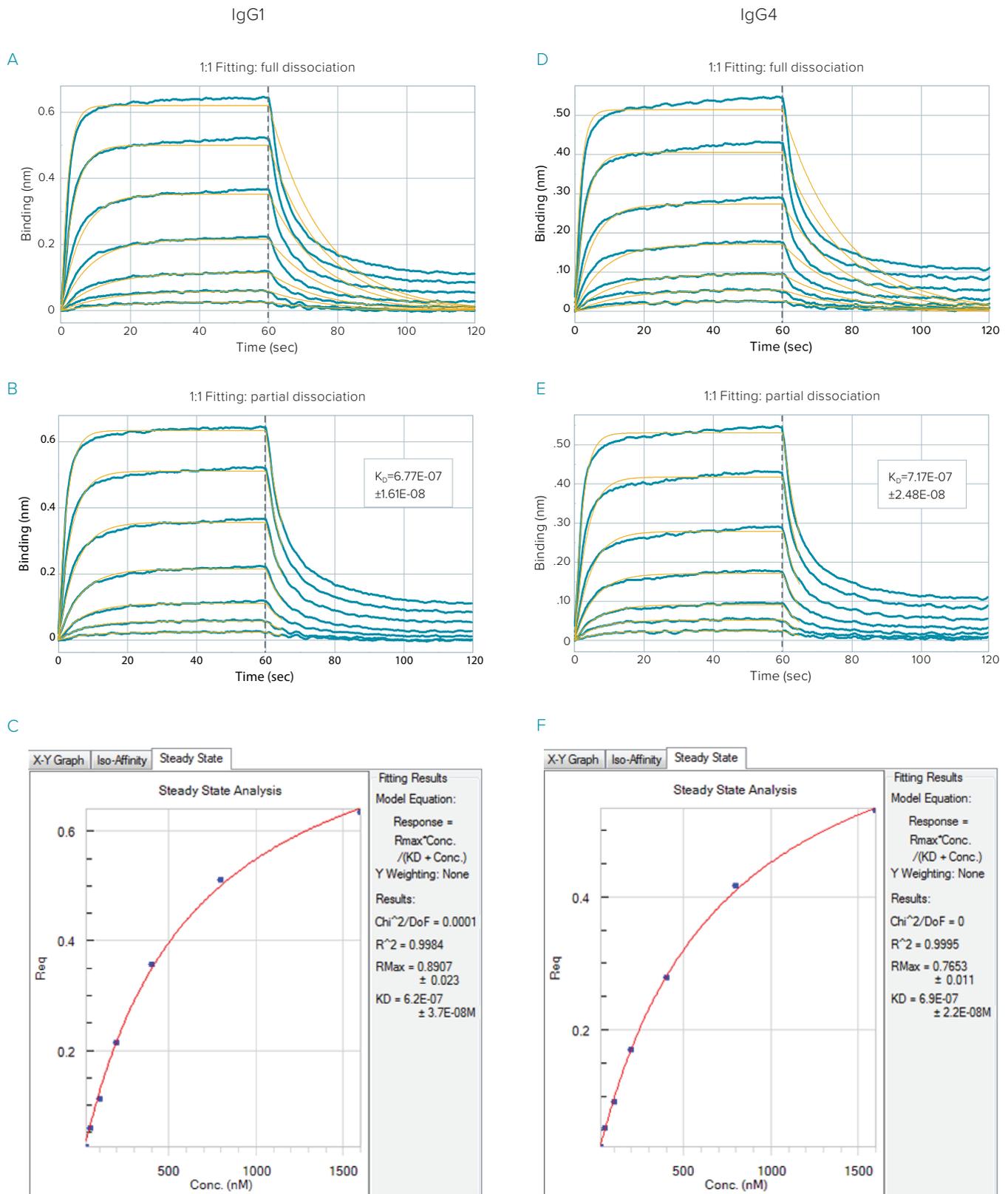
Figure 8: Comparison of kinetic data fitting strategies. FAB2G biosensors were used to capture hIgG1 and hIgG4 for a binding kinetics assay with FcRn using several concentrations (A, B, C for IgG1; D, E, F for IgG4). The 1:1 model with global fitting and 5 seconds of the dissociation step (fit lines are in red) were used to determine affinity constant. K_D, using the full dissociation step (A, D) and 5 seconds of the dissociation step (B, E). Steady-state analysis of data was also used to determine equilibrium K_D (C, F).

Referencing

Two methods of referencing are used in biosensor kinetic assays: 1) A **reference sample** is run on a biosensor that has ligand present in the loading step but with zero analyte in the association step, *i.e.* a buffer-only negative control. When using a capture-based biosensor such as FAB2G, some background level of dissociation of the captured IgG ligand from the sensor will occur. This background dissociation, or assay drift, can be subtracted out using the reference sample. 2) **Reference biosensors** refer to zero-ligand biosensors that are dipped into buffer or irrelevant protein during the loading step. Reference biosensors are run through the same analyte samples as the ligand-loaded biosensors in a replicate assay (Figure 9). A separate reference biosensor should be included for each analyte concentration used. Reference biosensors

enable subtraction of non-specific binding of analyte to the bare surface, and are considered optional in a BLI protein kinetic assay.

Double referencing can be performed during data analysis, where signal from reference biosensors and reference sample are subtracted from sample data. We have found that in FcRn assays, subtracting out the small amount of NSB via double referencing generates improved kinetic profiles and data fitting over single referencing with a reference sample only. To illustrate, Figure 10 shows the FcRn-IgG kinetic fits generated from single vs. double referencing and respective calculated kinetic and affinity constants. NSB signal that is more than 20% of maximum association signal should not be subtracted out, but should instead be reduced by optimizing assay conditions (see Non-Specific Binding section).
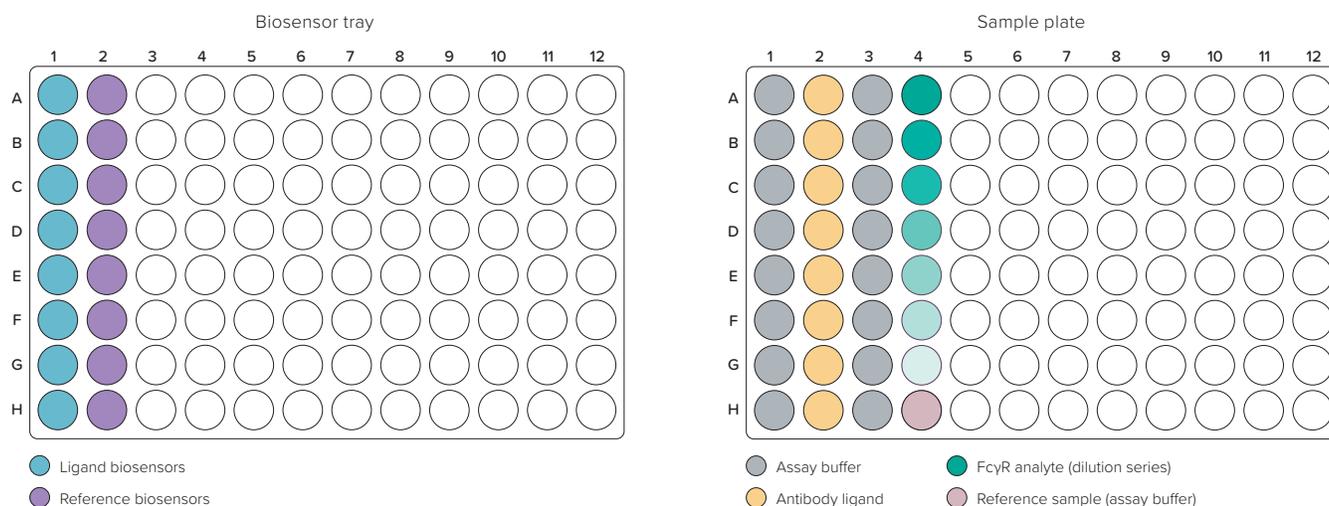
Assay	Step	Sensor column	Step name	Sample column	Step type
1	1	1	Equilibration	1	Custom
1	2	1	Loading	2	Loading
1	3	1	Baseline	3	Baseline
1	4	1	Association	4	Association
1	5	1	Dissociation	3	Dissociation
2	1	2	Equilibration	1	Custom
2	2	2	Loading	1	Loading
2	3	2	Baseline	3	Baseline
2	4	2	Association	4	Association
2	5	2	Dissociation	3	Dissociation

Figure 9: Assay protocol for an experiment on FAB2G biosensors with the Octet RED96 instrument that utilizes double referencing. A reference sample is included in the association step that contains assay buffer with no analyte to correct for baseline drift. Reference biosensors enable subtraction of non-specific binding, and are an additional set of biosensors that are run through a replicate assay. All steps are repeated on reference biosensors except the ligand loading step, which is performed in buffer. When Double Reference is selected in the Octet System Data Analysis software Processing window, both Reference Sample and Reference Biosensor data will be subtracted from sample data.

A   Single referencing

B   Double referencing

	Single referencing	Double referencing
K_D	5.86E-07	6.77E-07
K_{on}	1.78E+05	1.83E+05
K_{off}	1.04E-01	1.24E-01
K_D error	2.24E-08	1.61E-08
R^2	0.9972	0.9990
χ^2	0.0700	0.0226
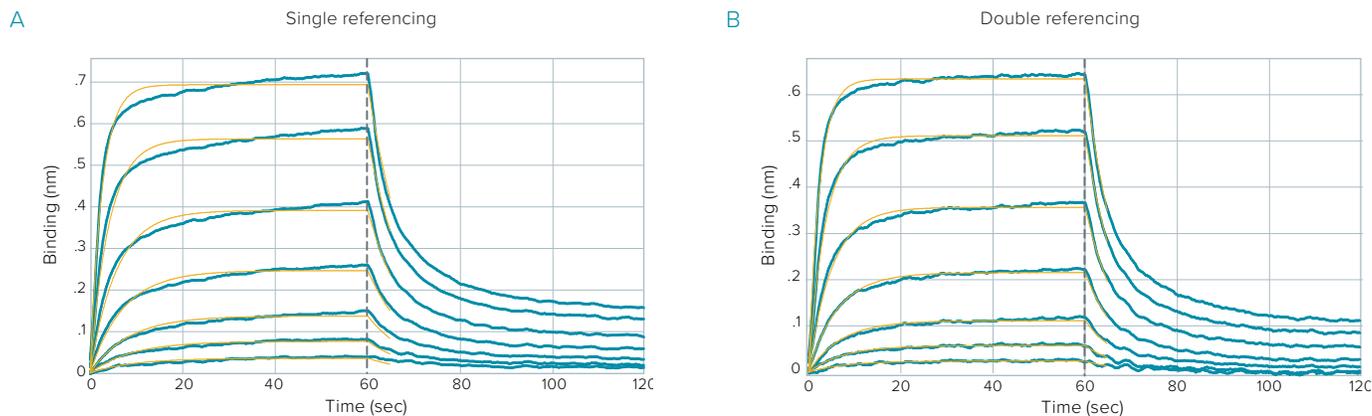
Figure 10: Comparison of kinetic fitting using single referencing with reference sample vs. double referencing with both reference sample and reference biosensors. FAB2G biosensors were used to capture Herceptin (HIgG1) for a binding kinetics assay with FcRn using several concentrations. The 1:1 model with global fitting and 5 seconds of the dissociation step (fit lines are in red) were used to determine affinity constant. A) Kinetic fitting using single reference. B) Kinetic fitting using double reference. C) Kinetics constant and fitting statistics calculated from single reference and double reference data analysis.

Biosensor regeneration

Regeneration of biosensors in kinetic analysis can offer savings on biosensor costs and provide a cost-effective method for generating replicate data for ligand-analyte pairs. Efficient regeneration requires removing the bound analyte or ligand/analyte complex without affecting activity of the biosensor. The number of regeneration cycles that can be withstood is biosensor and protein dependent; some can be regenerated ten or more cycles, while others tolerate far fewer cycles or cannot be regenerated at all. A standard regeneration procedure for biosensors used for studying FcRn-IgG interactions is exposure to 10 mM glycine pH 1.7 for five seconds followed by assay buffer for five seconds, repeating four times for a total of four exposures to regeneration buffer. Utilizing this protocol on FAB2G biosensors will remove IgG ligand-FcRn complex and restore the original biosensor chemistry. New IgG sample can then be loaded for measuring another interaction.

If regeneration is successful, the analyte binding curves following each regeneration cycle will overlay with minimal change in responses when compared to earlier binding cycles. Figure 11

shows the overlay of data for association of FcRn to IgG1 immobilized on FAB2G biosensors. Ten assay cycles were run, with regeneration procedure described above performed between each cycle. When association/dissociation steps for each cycle are overlaid, binding response does not decrease but remains consistent with increasing number of cycles. The CVs for the kinetic and affinity constants calculated for each assay cycle are well below 10%.

In some cases binding capacity may decrease during the first regeneration cycle but stabilize for the remaining cycles. To avoid this initial change, a pre-conditioning step is recommended, where the regeneration protocol is performed on the unused biosensors before beginning the assay. The number of regeneration cycles that can be performed successfully in an assay will be biosensor and format-dependent, and should be tested for each experimental system. Regeneration can also be used to improve reproducibility within an assay, since it enables multiple samples to be run on an identical biosensor surface.
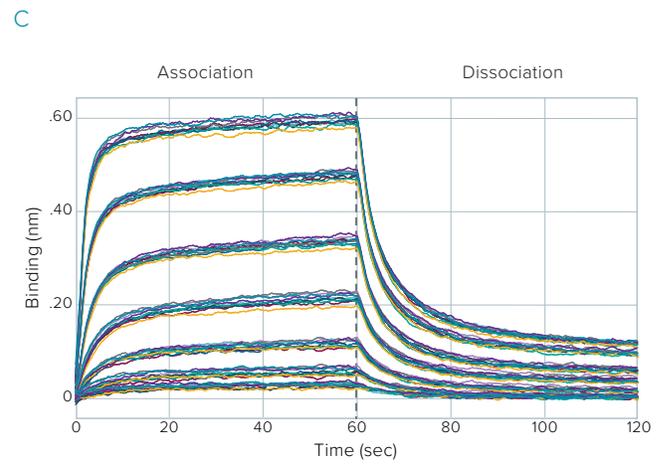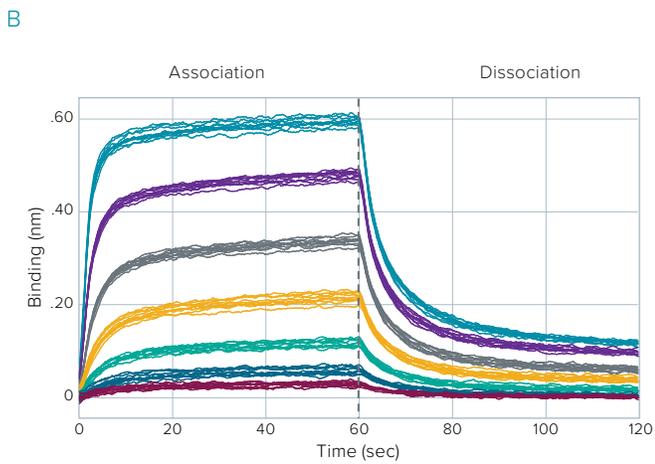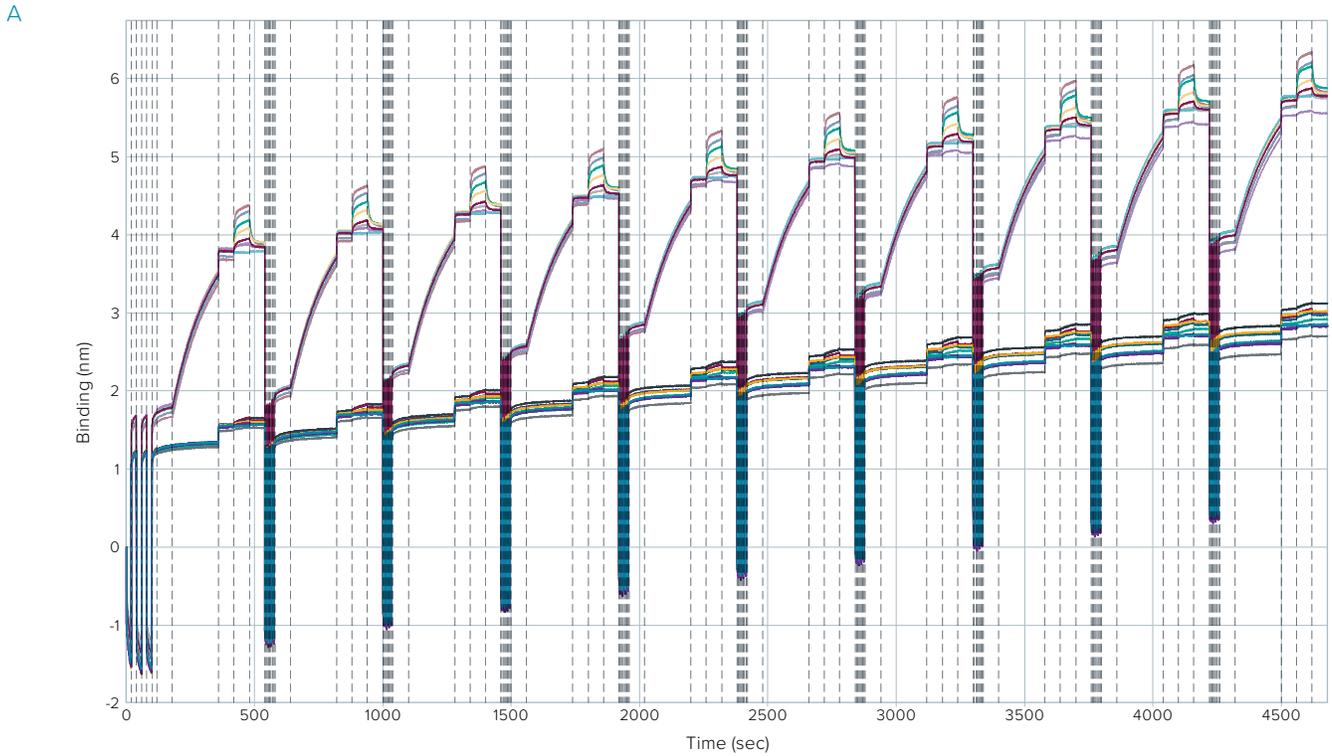
Figure 11: Biosensor regeneration in FcRn kinetic assays. A) Raw data: FAB2G sensors went through pre-condition step, then 10 kinetic assay cycles with regeneration. Reference sensors without ligand loading were also included to determine the non-specific binding of FcRn to FAB2G sensors. For each kinetic assay, association and dissociation steps performed to a 2-fold dilution series of FcRn. B) Overlay of FcRn association/dissociation steps. C) Curve fitting of data traces, using a 1:1 model with global fitting and a 5-second dissociation step (fit lines are in red). D) Table of average kinetic and affinity constants with CV's for the 10 regeneration cycles.

	Average	%CV
K_D	5.67E-07	4.1%
K_{on}	2.23E+05	3.5%
K_{off}	1.26E-01	2.8%

A

H$_2$O$_2$ treatment (hr)	K$_D$ (M)
0	5.05E-07
1	6.55E-07
3	9.20E-07
5	1.32E-06

Figure 12: Impact of Methionine oxidation on the binding of Herceptin-FcRn kinetics. H$_2$O$_2$ treated hIgG1 samples were analyzed for affinity to FcRn in a full kinetic assay. Analysis was performed using 1:1 global fitting with a portion of the dissociation step (5 seconds). Double referencing method was used. A) Table of calculated K$_D$'s for each oxidation time point. B) Calculated K$_D$ plotted against time of H$_2$O$_2$ treatment (hr) showing trend of decreasing binding affinity with increasing oxidation time.

B

K$_D$ (M) increase with H$_2$O$_2$ treatment

y = 2E-07x + 5E-07
R^2 = 0.99007

Measuring afinity of FcRn in antibody engineering, comparability studies, lot release assays and QC

The Octet system provides a user-friendly platform that enables rapid assay optimization and integrates readily into workflows at many stages of antibody drug development - from early phase candidate selection to release of marketed product. Detailed analysis of both kinetics and binding affinity are essential to understanding the activity of biotherapeutic candidates and guiding further design strategies. Equivalence of materials needs to be established in terms of quality, safety and efficacy, including full comparison of immunological properties between biosimilar and a licensed originator product.

The FcRn-IgG kinetic assays described here are well suited as an analytical tool for detailed biotherapeutic characterization as well as assessment of stability, comparability, and lot-to-lot consistency. In order to demonstrate sensitivity of kinetic analysis on the Octet system for determining changes in product quality, the described methods of analysis were used to measure affinity of FcRn to stressed IgG samples compared to control. Methionine
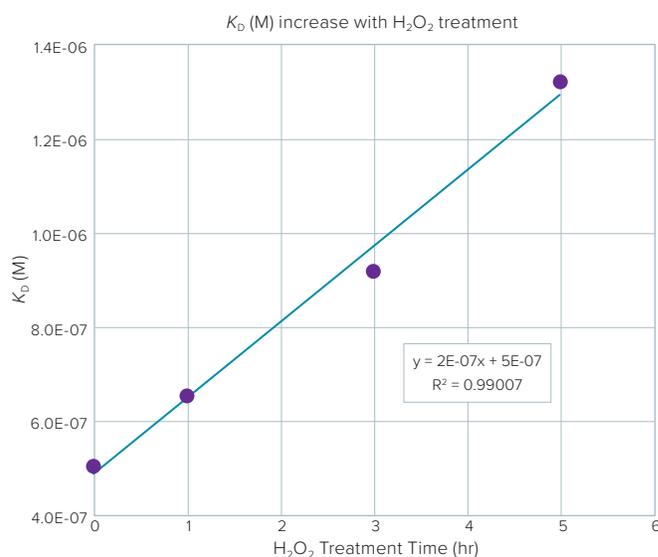
oxidation as a form of product degradation has been demonstrated to reduce the affinity of Fc fragment to FcRn[4]. For this experiment, oxidized antibody samples were generated via time course incubation in 0.3% hydrogen peroxide followed by buffer exchange into low pH assay buffer. The affinity of FcRn binding was measured for each of the treated hIgG1 samples as well as untreated hIgG1 control using FAB2G biosensors on the Octet RED384 instrument. A dilution series of FcRn was associated to immobilized hIgG1 samples and double referencing with 1:1 global data fitting used to calculate K$_D$ and kinetic constants.

Figure 12 shows the fitted data traces and analysis from the kinetics experiment. The results demonstrate that an increase in calculated K$_D$ correlates with increasing degree of oxidation of the sample. The experiment was performed on RED384 instrument using 80 μL of sample per well in a 384-well plate. All samples for every time point can be run in a single walk-away assay with total running time of less than 2 hours. This experiment illustrates the ease with which a sensitive, high throughput characterization assay can be established on the Octet platform for assessing FcRn binding activity to IgG.

| | hIgG1-FcRn | | | | hIgG4-FcRn | | | |
| | Intra-assay | | Inter-assay | | Intra-assay | | Inter-assay | |
	Average	CV%	Average	CV%	Average	CV%	Average	CV%
K_D	5.92E-07	2.0%	5.25E-07	4.2%	6.20E-07	2.0%	6.66E-07	6.0%
k_{on}	2.10E+05	0.6%	2.25E+05	5.1%	2.08E+05	1.7%	2.13E+05	1.4%
k_{dis}	1.24E-01	2.5%	1.18E-01	2.5%	1.29E-01	0.4%	1.42E-01	5.0%

Figure 13: Intra-assay and Inter-assay precision of the HIgG-FcRn binding pairs.

Intra-assay and inter-assay precision

To demonstrate reproducibility of FcRn kinetic assays on the Octet platform, intra-assay and Inter-assay precision of the K_Ds were determined for hIgG1 and hIgG4 binding to FcRn using FAB2G biosensors. Results are shown in Figure 13. Replicate data (n=3) were collected for each binding pair and kinetic and affinity constants calculated using 1:1 binding model with truncated dissociation step (Figure 13). The low percent CV values demonstrate the high level of precision and reliability that can be achieved with kinetic analysis on the Octet platform when proper assay optimization is performed.

Conclusion

Measuring accurate and reliable kinetics of interactions between neonatal Fc receptor and monoclonal antibodies can be challenging, but is a critical application in many stages of biopharmaceutical development. The Octet platform offers a rapid, flexible, and sensitive solution for measuring these interactions, whether performing full kinetic analysis, steady state analysis or measuring relative binding. Here we have described methods for producing high quality FcRn kinetic data on the Octet system using our FAB2G biosensors, with recommendations for assay optimization and data analysis. Careful assay development and experimental design will consistently yield data that is reproducible and of high quality, making the Octet system ideal for cost-effective, label free kinetic analysis to complement functional assays and replace more cumbersome label-free methods such as SPR and immunoassays.

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