FORTÉBIO

APPLICATION NOTE 17

# Analysis of Fc-gamma Receptor-IgG interactions on the Octet platform

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## Introduction

Fc gamma receptors (FcγRs) are membrane glycoproteins with affinity for the Fc region of immunoglobulin G (IgG). FcγRs expressed on the surface of immune effector cells play a key role in initiating Fc effector functions such as antibody-mediated cell-dependent cytotoxicity (ADCC)[1], which is a major mechanism of action of therapeutic monoclonal antibodies. There are three classes of FcγRs based on genetic similarity: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). FcγRI is characterized as a high affinity receptor with affinity range of 0.1–10 nM for IgG, while FcγRII and FcγRIII have lower affinity for IgG (0.1–10 μM)[2]. FcγRI binds monomeric IgG as well as immune complexes, while both FcγRII and FcγRIII bind with greater affinity to IgG complexes. Binding can be affected by genetic polymorphisms of the receptors as well as glycosylation patterns in the Fc region of the antibody[3,4].

The ability of therapeutic monoclonal antibodies to bind FcγRs can greatly impact their safety and efficacy. Induction of ADCC by an antibody depends on its binding affinity to both the target and to the FcγR. Therefore efforts to analyze and enhance Fc interactions with FcγRs have become an integral part of biotherapeutic development processes. Throughout drug development, antibodies are selected, engineered, and assayed for either improved or reduced binding to Fc receptors depending on their mechanism of action. Effector function is a critical component of biological characterization and SAR studies of lead candidates. The FDA requires data on Fc effector function for regulatory filings and potency lot release, and Quality Control groups routinely assay in vitro binding and kinetics of therapeutic candidates to Fcγ receptors in parallel with functional cell-based ADCC assays. ELISA, FRET, and SPR are commonly used methods for determining binding affinity of FcγRs to antibodies.

Binding affinities of Fc gamma receptors to monoclonal antibodies can be determined in a high throughput and highly sensitive format using biosensor analysis on the Octet® platform. Here we introduce the Octet® platform as the technology of choice for analyzing Fc gamma receptor-IgG binding interactions. Assay

formatting options and best practices for various biosensor formats 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 Fc gamma receptor/antibody interactions

The Octet family of instruments is based on Bio-Layer Interferometry (BLI), a label-free technology that measures molecular interactions in real time for the purpose of detection, quantitation and kinetic analysis. BLI offers many advantages for detailed characterization of Fc receptor/IgG interactions in terms of throughput, ease of use, specificity and flexibility. Octet instruments can read up 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 performing complete kinetic characterization, steady-state analysis can be used to determine $K_D$, or simple binding assays performed to evaluate relative affinity.

The microplate format combined with Dip and Read™ biosensors enables highly parallel processing on the Octet system in sample volumes as low as 40 µL. Throughput on the Octet system is much higher than SPR instruments, with faster time to results, minimal sample consumption and virtually no instrument maintenance. Reagents in microplate wells can be re-used throughout an assay, enabling the same antibody sample to be measured against multiple FcγRs (or vice-versa) for reagent savings or platform assay development. Multiple Dip and Read biosensor options from ForteBio enable flexible assay formatting. Biosensors are cost-effective and do not require regeneration, saving additional assay development time. In contrast with endpoint assays such as ELISA or FRET which yield only a single readout per sample, real-time biosensor analysis provides significantly more vital information about a molecule's activity than equilibrium data alone. Time savings and simpler processing mean faster time to results when compared with the multiple incubation and wash steps required for ELISA or the complex assay development for FRET assays. Wash steps are not required with BLI due to the high level of specificity provided by biosensor chemistries, eliminating the risk of losing low-affinity binders during repeated wash steps that can occur with ELISA.

The Octet RED96e, RED384 and HTX systems offer the best performance for kinetic analysis and are the recommended platforms for analysis of Fc gamma receptors. These three instruments have suitable sensitivity for measuring low-affinity interactions with fast association rates, which are characteristic of many FcγR-IgG interactions.

## Biosensor selection

A primary consideration when developing Fc gamma receptor-IgG kinetic assays on the Octet system is assay format. In a typical kinetics assay, one of the binding partners is immobilized on the biosensor surface (ligand) and the other remains in solution (analyte) and associates to the immobilized molecule. Selection of the appropriate biosensor for an assay and choice of which molecule to immobilize will depend on several factors. Since FcγR-IgG interactions are often relatively low affinity, concentrations of analyte for association may need to be quite high, often in the micromolar range. In contrast, the ligand molecule immobilization step is typically performed at lower concentrations. Therefore, limitations on availability of reagents will need to be considered when choosing assay orientation.

Several biosensor options are available for studying FcγR-IgG kinetics, depending on the desired format. When immobilizing HIS-tagged FcγRs on the surface of the biosensor, Ni-NTA biosensors can be used. Ni-NTA biosensors have QIAGEN's Tris-NTA charged with nickel (Ni2+) pre-immobilized onto the tip. Ni-NTA will bind to a HIS-tag attached to recombinant proteins. In this format, the Fc gamma receptor protein is loaded onto the biosensor as the ligand, followed by association with IgG. A sample workflow for a Ni-NTA kinetic assay is shown in Figure 1. Since nickel can also bind weakly to non HIS-tagged proteins, non-specific binding to the IgG analyte may occur when using Ni-NTA biosensors to immobilize FcγRs. Blocking and buffer conditions can be optimized to minimize these interactions, and are discussed in the Buffer conditions and non-specific binding section.

Risk of non-specific interactions can be reduced by using an antibody specific for HIS-tagged proteins to capture FcγRs on the biosensor. QIAGEN's Penta-HIS antibody is highly sensitive and specific for HIS-tagged proteins. This antibody can easily be biotinylated for immobilization onto High Precision Streptavidin (SAX) biosensors, or purchased pre-conjugated to biotin. Penta-HIS antibody immobilized on SAX biosensors can then be used to capture HIS-tagged FcγR ligand in the loading step, followed by association with IgG analyte. An example workflow illustrating use of biotinylated Penta-HIS antibody for capture of HIS-tagged FcγRs in a kinetics assay is shown in Figure 2.

For the reverse assay orientation in which IgG is immobilized on the biosensors and FcγR protein remains in solution as analyte, the recommended biosensor selection is Anti-Human Fab-CH1 (FAB). The FAB biosensor incorporates a high-specificity anti-human Fab-CH1 ligand molecule pre-immobilized on the surface. The high specificity of the biosensor towards the CH1 region of human IgG allows the capture and immobilization of all four subclasses of human IgG. This capture method is highly specific and reliable for analyzing FcγR-IgG kinetics, and is more conducive to a platform-approach when testing multiple IgGs and receptors. Capture of IgG is oriented, creating a more homogeneous surface on the biosensor. The Fc hinge region is exposed with minimal steric hindrance for receptor binding (Figure 3).

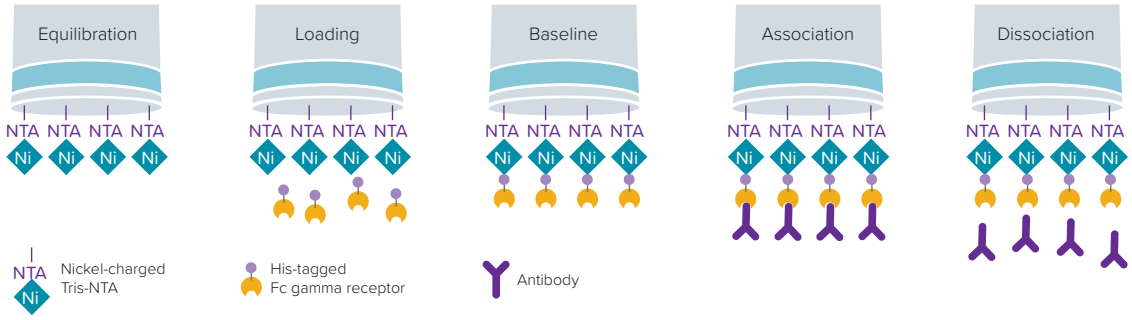



Figure 1: Sample workflow for Fc gamma receptor kinetic assay on Ni-NTA biosensors.

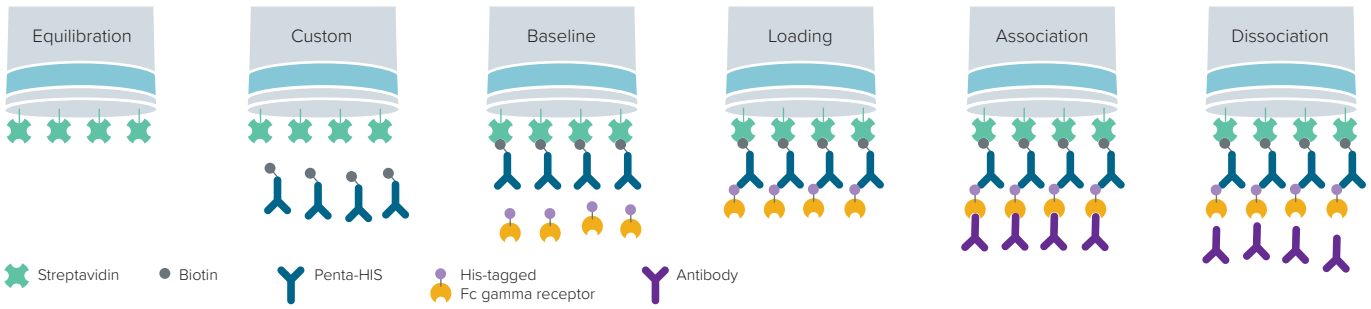



Figure 2: Sample workflow for Fc gamma receptor kinetic assay on High Precision Streptavidin (SAX) biosensors using the Penta-HIS antibody.

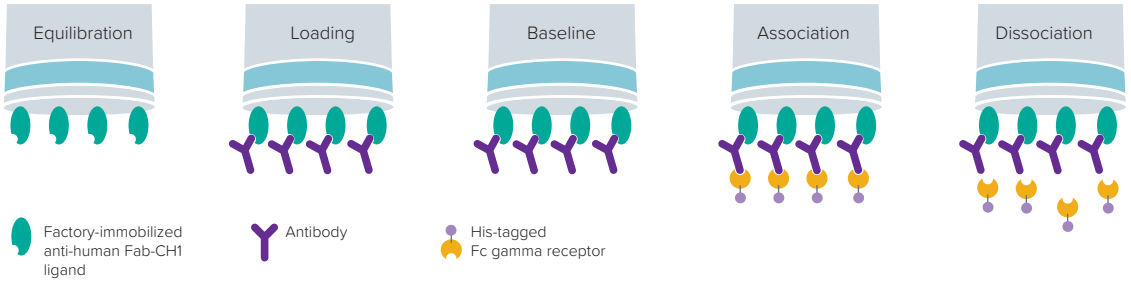



Figure 3: Sample workflow for Fc gamma receptor kinetic assay on FAB biosensors.

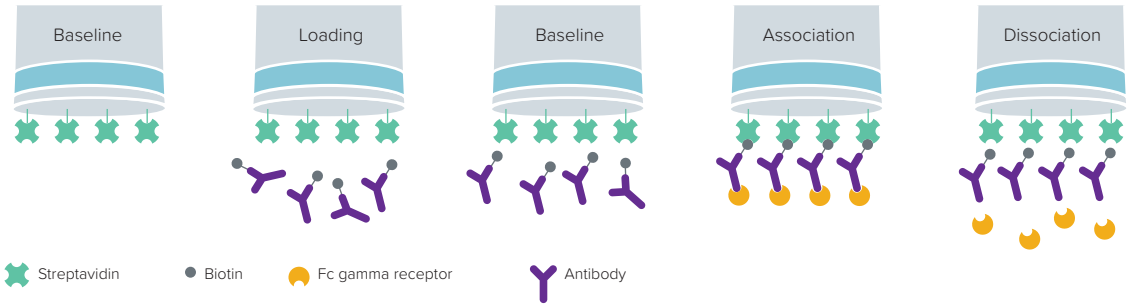



Figure 4: Sample workflow for Fc gamma receptor kinetic assay on High Precision Streptavidin (SAX) biosensors, with biotinylated IgG immobilized on the biosensor tip.

An alternative to capture via HIS tag or Fab region is biotinylation of IgG or receptor for immobilization directly onto SAX biosensors (Figure 4). This approach is particularly useful when the protein being immobilized is not a full-length human IgG, but another Fc-containing protein such as an Fc-fusion protein or an antibody from another species. The biotin-streptavidin coupling method creates a stable surface on the biosensor similar to covalent coupling, yet requires minimal optimization. Biotinylation is simple to perform, gentle on proteins and performed in solution phase at neutral pH. The reaction is easily controlled by regulating the number of biotin molecules added to the reaction per target protein, thus preventing activity loss due to over-modification. A 1:1 molar coupling ratio is recommended. Refer to ForteBio Technical Note 28, *Biotinylation of Proteins for Immobilization onto Streptavidin Biosensors* for information and procedures for protein biotinylation.

Use of SAX biosensors is recommended over the amine covalent coupling approach using AR2G biosensors when working with Fc receptors. Immobilization with amine coupling is difficult to control, involves several steps, and requires a pH optimization step. The resulting random orientation of receptor molecules on the biosensor surface can interfere with activity via steric restriction of access to binding sites or even conformational changes in binding sites. This interference is often reflected in low signal and sub-optimal kinetics[5]. Oriented capture via HIS tag on the Fc receptor protein offers a more robust and homogeneous binding kinetics assay. If the antibody is to be immobilized, FAB biosensors or SAX biosensors with biotinylation are preferred over AR2G.

## Assay optimization

As with any kinetics assay, it is crucial to perform proper assay development when analyzing FcγR-IgG interactions so that resulting affinity and kinetic constants will be accurate, reliable and reproducible. Quality of 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 results are in agreement with what is known about receptor and target and the nature of the binding, and evaluated for secondary, non-specific interactions.

## Quality of reagents

Reagent quality is a critical factor with any kinetics assay, especially for FcγR-IgG interactions. Aggregation of the antibody or receptor can impact kinetics due to increased avidity. Dimeric and multimeric IgG have been shown to dissociate much more slowly from FcγRs and require lower concentration for the same level of binding than monomeric IgG[6,7]. Antibody samples should be fully evaluated for purity, activity and quality using analytical techniques before use 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 storage conditions and handling of receptor proteins and multiple freeze-thaw cycles should be avoided.

## Ligand loading step

The amount of ligand immobilized (loaded) onto the biosensor can have significant impact on the results of a kinetic assay. For either Fc gamma receptor assay orientation — receptor immobilized or antibody immobilized — loading as much protein as possible onto the biosensors in order to maximize signal is not necessarily the best approach. An excess of ligand molecules bound to the biosensor can lead to data artifacts due to crowding, steric hindrance, avidity and mass transport effects. Weaker, non-specific interactions can also be favored, especially at higher analyte concentrations, when the biosensor is over-loaded. These artifacts can impact the observed binding kinetics. If, however, not enough ligand is immobilized, the signal for the analyte association step may be too low, giving poor signal-to-noise ratio. Ideally, loading levels should be optimized for every receptor and biosensor format used.

We have found that when analyzing FcγR-IgG kinetics, ligand loading strategy will differ depending on the assay orientation and the biosensor used. The density of the ligand molecules on the biosensor tip depends on ligand concentration and loading step time. When capturing HIS tagged FcγR protein on the biosensor using Ni-NTA or the biotin-Penta-HIS approach, using a low density of receptor ligand will yield better results. Using the biotin-Penta-HIS approach, biotinylated Penta-HIS antibody is first immobilized onto SAX biosensors using a concentration 5 µg/mL for 10 minutes. For the subsequent Fc gamma receptor loading step, using 50–100 nM receptor and a loading step time of five minutes is recommended. The loading response should reach no more than 0.3–0.4 nM. This relatively low density of receptor on the biosensor tip will improve association-dissociation kinetics and help mitigate avidity effects and/or non-specific binding. Use the 'threshold' setting in Octet Data Acquisition software to assure receptor loading does not progress too far. Figure 5 shows kinetic analysis of FcγRI and IgG1 on SAX biosensors with Penta-HIS antibody using decreasing FcγRI loading concentrations, which yield decreasing signal response in the loading step and progressively lower density of receptor molecules on the biosensor tip. The 1:1 kinetic curve fitting is improved with lower loading density, as indicated by how closely the curve fit lines follow the data traces.

When using FAB biosensors to immobilize IgG, *i.e.* the reverse assay orientation, optimal results are achieved with a higher level of ligand loading. The recommended concentration of antibody to use for loading onto FAB biosensors is 200–500 nM, with loading for 5–10 minutes to achieve a 2.0–3.0 nM response. We have found that for the FAB assay format, the kinetics and curve fitting results obtained using higher loading levels are similar to those using lower loading levels, with improved reproducibility at higher loading.

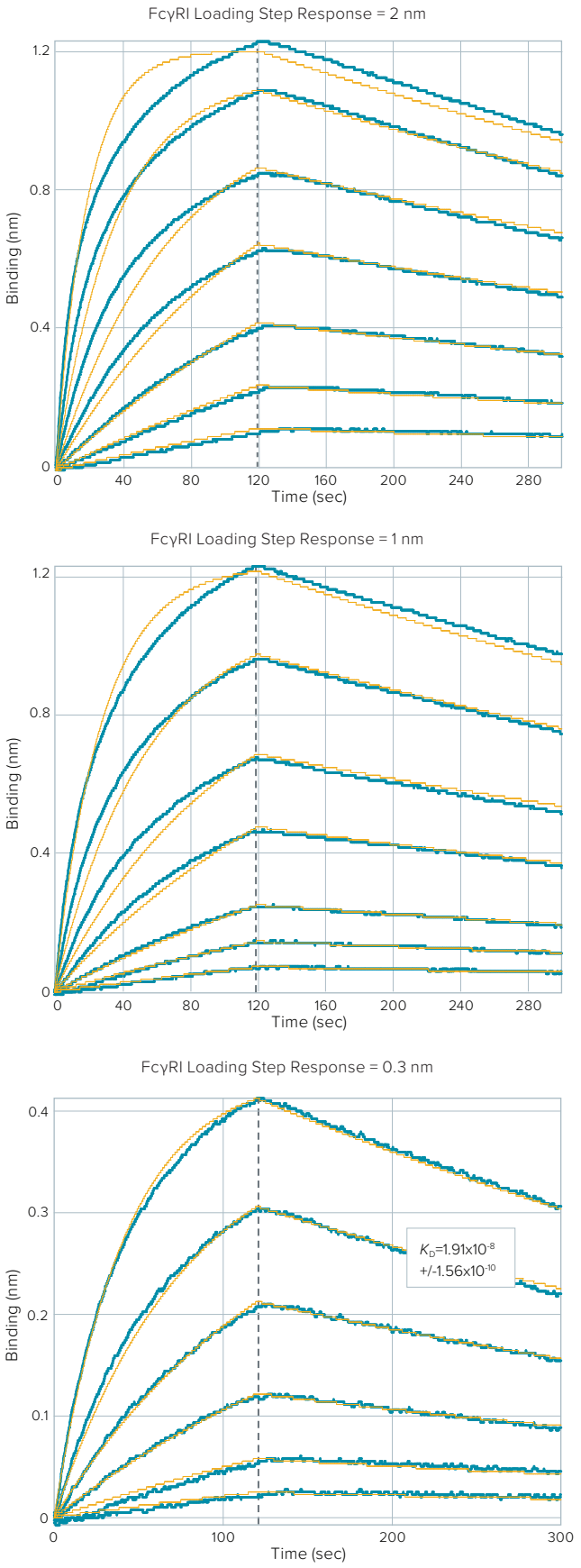



Figure 5: Association/dissociation kinetics of IgG to FcγRI with decreasing loading densities of FcγRI ligand. SAX biosensors with biotin-Penta-HIS antibody format was used. Comparison of data fitting using 1:1 model shows visually improved fitting with lower loading levels.

When loading biotinylated IgG or FcγR directly onto SAX biosensors, loading conditions should be optimized for each experimental system. An assay development step or scouting step in which the biotinylated ligand is titrated on the biosensors is recommended. To perform a loading optimization experiment, load several concentrations of biotinylated ligand on the biosensor for 5–10 minutes. Following a 2–5 minute baseline step in buffer, perform an association step for each ligand concentration using a single high concentration of analyte (5- to 10-fold above the $K_D$), followed by dissociation in assay buffer. The optimal loading concentration to select for a detailed kinetic analysis is the lowest ligand concentration that yields an acceptable response in the analyte association step (see the Analyte Concentration/Association Step section). The selected loading concentration should not saturate the biosensor or cause changes in the association or dissociation kinetics compared to lower concentrations. Examples of such changes are the appearance of secondary binding in the association curve (caused by non-specific interactions) or decreased dissociation rate (caused by avidity). Generally, the less ligand loaded on the biosensor the better, as long as analyte signal is sufficient. Ligand loading strategies are listed according to biosensor format in Table 1.

## Analyte concentration/association step

In the association step, the rate of binding of the analyte to the immobilized ligand is measured. For simple binding assays or qualitative analyses, measuring binding curves for a single analyte concentration can be sufficient. However, when accurate kinetic and affinity constants are required, a dilution series of four to six analyte concentrations must be measured in the association step. Multiple analyte concentrations enable global curve fitting, where all the traces in a data set are fit simultaneously to yield one set of results. This assures more accurate and reliable results. The analyte dilution series in a full kinetic characterization should ideally range from concentrations of about 10*$K_D$ down to about 0.5*$K_D$, using 2-fold or 3-fold dilutions. Running several concentrations will also show how well the fitted binding model applies over a concentration range around the $K_D$. The analyte concentration range to use will also depend on the sensitivity of the assay and affinity of the interaction. For high-affinity FcγR-IgG interactions, it may not be possible to see signal at or below the $K_D$. In this case, choose a series of analyte concentrations that cover the range of the assay, from 10–20 fold above the $K_D$ down to the limit of measurement.

Low-affinity FcγR-IgG interactions tend to be in the 0.1–10 µM $K_D$ range. Using analyte concentrations well above the $K_D$ may be high enough to create some artifacts in the data. It is best to choose an analyte concentration range that works for the binding pair, for a low affinity binding pair this may start as low as 5-fold above the $K_D$ with 2-fold dilutions down to the limit of measurement. FcγR-IgG interactions tend to also have fast on-rates (>1E5 $M^{-1}s^{-1}$), where the primary binding interaction

| Biosensor | Anti–Human Fab–CH1 (FAB) | SAX + Biotin–Penta–HIS antibody | Ni–NTA (NTA) | High Precision Streptavidin (SAX) | Anti–Penta–HIS (HIS) | Amine Reactive Second-Generation (AR2G) |
|---|---|---|---|---|---|---|
| **Recommended for** | High and low affinity receptors. Check for cross–reactivity with FcγRIIa and FcγRIIIb. | High and low affinity receptors, except FcγRIIa. | High and low affinity receptors. Lower affinity receptors may give low signal in association step. | High and low affinity receptors, including FcγRIIa. | Not recommended, though sometimes used off–label for kinetics assays. Use for high and low affinity receptors, except FcγRIIa. | Not recommended. |
| **Molecule immobilized on biosensor (ligand)** | IgG | *HIS*–tagged FcγR | *HIS*–tagged FcγR | Biotinylated FcγR or biotinylated IgG | *HIS*–tagged FcγR | |
| **Ligand loading strategy** | Higher Loading<br><br>*eg.* 200–500 nM for 5–10 min | Lower Loading<br><br>50–100 nM for 5 min | Lower Loading<br><br>50–100 nM for 5 min | Depends on ligand. For biotinylated IgG immobilization start with 200–300 nM for 10 min. Use lower concentration for biotin–receptor immobilization. | Lower Loading<br><br>50–100 nM for 5 min | |
| **Recommended loading response** | 2.0–3.0 nM | 0.3–0.4 nM | 0.3–0.4 nM | Must be optimized. | 0.3–0.4 nM | |
| **Recommended buffer** | 1X Kinetics Buffer | 1X Kinetics Buffer | 1X Kinetics Buffer, may need to be optimized by increasing salt and/or Tween-20 to reduce NSB. | 1X Kinetics Buffer, may need added BSA (up to 1%) and/or Tween-20 (up to 0.05%). | 1X Kinetics Buffer | |
| **Blocking** | Typically not required | Typically not required | May be required, recommend dipping into 0.2% casein in assay buffer for 5 minutes after loading step. | Typically not required | Typically not required | |
| **Regeneration conditions** | Regenerate down to original biosensor chemistry with 10 mM glycine, pH 1.5. | Regenerate down to Penta–*HIS* antibody with 10 mM glycine, pH 1.5. | Regenerate with with 10 mM glycine, pH 1.5 and NiCl2 re–charging. Small loss in loading signal can be expected with each cycle. | Can be regenerated down to immobilized ligand. Regeneration conditions may require optimization. | Regenerate down to original biosensor chemistry with 10 mM glycine, pH 1.5. | |
| **Regeneration cycles** | 6–10 cycles | 6–10 cycles | Depends on receptor stability, must be tested. | Depends on ligand stability, must be tested. | 6–10 cycles | |
| **Special considerations** | May need to use High Concentration Kinetics mode (10 Hz data acquisition rate) to capture initial data for fast on–rates. | • Prior to HIS–tagged receptor loading, immobilize biotin– penta–HIS antibody by loading at 5 µg/mL for 10 min.<br>• For low affinity receptors, use short association step time, 30–60 seconds. | • Check for non–specific binding. Optimize buffer and blocking conditions if needed.<br>• If signal is low in association step, increase loading concentration or step time.<br>• For low affinity receptors, use short association step, 30–60 seconds. | • Biotinylate ligand protein using 1:1 molar coupling ratio.<br>• Loading conditions should be optimized. | Can be used like SAX + Penta–HIS antibody, however these biosensors are qualified for quantitation applications only and may have higher drift and variability in kinetics assays. | |

Table 1: Summary of Fc gamma receptor assay strategies for various biosensor formats.

will quickly reach equilibrium. When the interaction is fast, the association step should not be run for too long — particularly when formatted in the receptor-immobilized orientation (SAX with Penta-HIS antibody or Ni-NTA biosensors). During a long association step, weaker non-specific interactions may begin to be favored once the primary interaction has equilibrated. This secondary binding can lead to heterogeneity in the binding curves, which will impact the data fitting. To avoid heterogeneous association curves caused by secondary binding, it is best to limit the association step to 30–60 seconds, as long as curvature is observed in the traces at higher analyte concentrations. Limiting the time of the association step can improve fitting with a 1:1 binding model.

Low affinity interactions can also exhibit low binding response in the association step. Assay signal is dependent on several factors, including affinity of the interaction, size of the analyte, size of the ligand, reagent quality, ligand loading density, buffer components and biosensor chemistry. For protein kinetics, responses that equilibrate at 0.01 nM or below are approaching the noise level of the instrument and generally cannot be fit reliably. When running a full kinetic analysis where four to six analyte concentrations must be measured for global fitting, enough signal must be generated that even the lowest analyte concentration is still above the detection limit of the assay. When responses are low, minor changes in the data can significantly skew results and fitting becomes less reliable. As a general guideline, the association signal for the highest analyte concentration should be 0.4 nM or higher. Optimization steps can be taken to improve low binding response such as increasing ligand loading concentration, increasing analyte concentration, or optimizing buffer conditions. Orienting the assay so that the Fc receptor is immobilized on the biosensor and IgG is analyte in solution can also be an effective way to improve signal in the association step.

## Buffer conditions and non-specific binding

For Fc gamma receptor kinetics assays, we recommend using ForteBio Kinetics Buffer as a sample buffer, which is available as a 10X solution. This buffer contains the blocking agent bovine serum albumin (BSA), and a surfactant (Tween-20) to inhibit non-specific binding to the biosensor tip and to other proteins. All samples (ligand and analyte) should be diluted in 1X Kinetics Buffer, and baseline and dissociation steps should be run in this buffer as well.

Non-ligand-loaded FAB biosensors and SAX biosensors with biotin-Penta-HIS antibody tend to exhibit minimal non-specific binding to IgG in Kinetics Buffer, primarily because the method of ligand capture is highly specific. Adding BSA (up to 1%) or Tween-20 (up to 0.05%) can sometimes improve data quality when using SAX biosensors with biotin-IgG directly immobilized. Since any histidine-containing protein can bind weakly to Ni-NTA, Ni-NTA biosensors are prone to binding non-specifically to IgG and other proteins. If Ni-NTA biosensors are used to capture HIS-tagged FcγR, it is important to check for non-specific binding (NSB) of IgG analyte to the biosensor surface. Run a preliminary experiment where IgG is associated to a biosensor that is not loaded with FcγR ligand. A positive signal in the association step indicates the analyte is binding directly to the biosensor (Figure 6).

If background NSB signal is minimal, it can be subtracted during data analysis by double referencing (see Referencing section). However, significant NSB will impact the apparent kinetics of the interaction and must be mitigated by adjusting assay conditions, such as modifying the assay buffer and/or adding a blocking step after ligand loading. Adding a greater amount of Tween-20 (up to 0.05%), or increasing the salt concentration can increase the stringency of the binding and help decrease non-specific signal. However, these approaches may also decrease binding

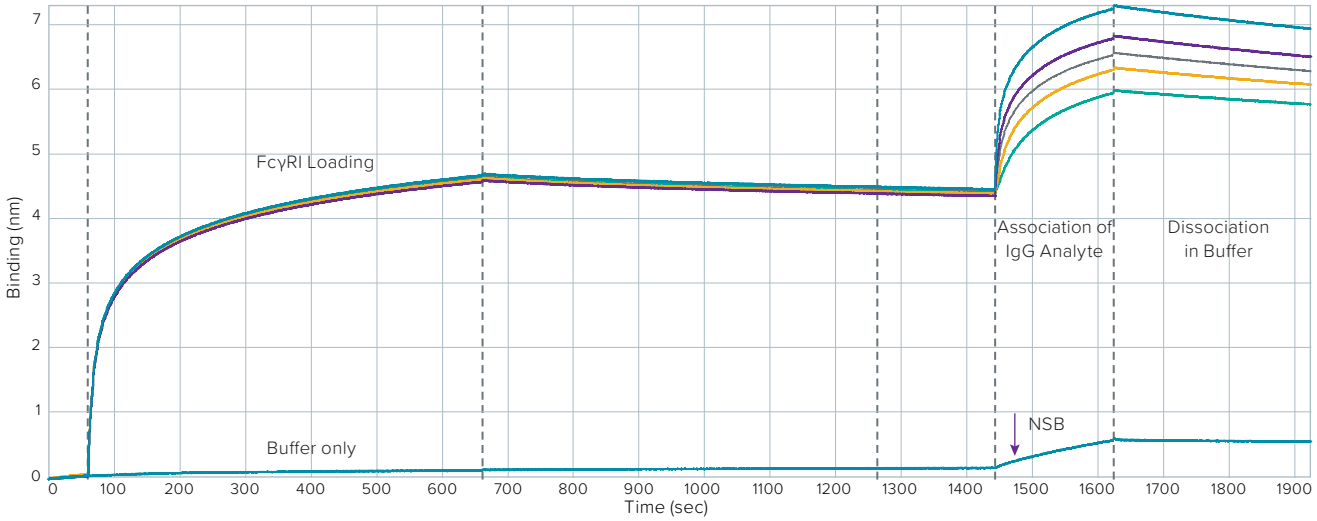



Figure 6: Non-specific binding (NSB) of IgG to Ni-NTA biosensors. Loading step for bottom (non-ligand-loaded) trace was run in Kinetics Buffer instead of FcγRI ligand. The high concentration of IgG was associated to the non-ligand-loaded biosensors. NSB is indicated by positive signal in the IgG association step for the non-ligand-loaded biosensor, shown by arrow.

| Buffer/blocking agent | Loaded biosensor (response in nm) | Unloaded biosensor (repsonse in nm) | Loaded/unloaded response ratio (signal/nsb) |
|---|---|---|---|
| Kinetics buffer | 0.13 | 1.13 | 0.1 |
| Kinetics buffer with 0.2% Casein blocking | 0.41 | < 0.01 | > 41 |
| Kinetics buffer with his-ubiquitin blocking | 0.22 | < 0.01 | > 22 |
| 10 Mm imidazole in kinetics buffer | 0.10 | 0.05 | 2.0 |
| 1 M nacl in kinetics buffer | 0.14 | 0.19 | 0.7 |
| 0.5 M nacl in kinetics buffer | 0.10 | 0.31 | 0.3 |
| 0.05% Tween in kinetics buffer | 0.20 | 1.32 | 0.2 |
| 0.1% Tween in kinetics buffer | 0.20 | 1.45 | 0.1 |

Table 2: Performance of different buffer and blocking conditions to reduce non-specific binding of IgG to Ni-NTA biosensors in a FcγRI kinetic assay. Maximum signal response from the IgG association step (nm) is listed for both FcγRI-ligand-loaded and non-FcγRI-loaded Ni-NTA biosensors. IgG association response for biosensors without FcγRI represents the level of non-specific binding (NSB). While several methods decreased NSB response, most also decreased binding to FcγRI, yielding a poor signal/NSB ratio. The most effective method in this example was blocking with 0.2% casein in assay buffer, which eliminated NSB while preserving the actual binding to the receptor.

of antibody to the receptor in the association step, so must be tested prior to running the assay. Increasing BSA concentration or blocking with BSA is not recommended when FcγR is immobilized, since the large size of the albumin protein compared to FcγR could result in steric interference. We have not had good results with added BSA. Dipping the loaded biosensor into a solution of blocking agents such as casein (0.2%) or HIS-tagged ubiquitin (0.5 µg/mL, EMD Millipore) after loading the receptor ligand can be very effective in reducing NSB.

Table 2 summarizes results from an experiment testing multiple buffer and blocking conditions for reducing non-specific binding of IgG to Ni-NTA biosensors. The extent of the non-specific binding in this particular assay is apparent in the Kinetics Buffer sample. In this sample, IgG binding to the unloaded biosensor (no FcγRI) is nearly 10-fold higher than binding response on the FcγRI-loaded biosensor. Increasing sodium chloride to 0.5 M or 1 M significantly reduced NSB, however the binding response of IgG to the FcγRI loaded biosensor is unacceptably low in these buffer conditions. Adding a low concentration of imidazole also lowered NSB but affected loading of receptor onto the biosensor (not shown). Increasing Tween-20 did not affect NSB. When a 5-minute blocking step with 0.2% casein in assay buffer was added to the assay after the loading step, non-specific binding was eliminated with adequate signal maintained for IgG association (>0.4 nM). Different buffer conditions can be more or less effective depending on the receptor and antibody binding pair, so testing a small panel of conditions, as demonstrated in this experiment, is recommended. A combination of buffer modifications, such as increasing both salt and Tween-20 by a smaller amount may also be helpful. Since the Octet instrument enables processing of 8 to 16 samples at a time, this entire optimization can be done in a single experimental run (approximately a 20-minute assay).

Background binding can also occur as a result of cross-reactivity between some FcγRs and the molecules pre-immobilized on the biosensors for ligand capture. Human FcγRIIa is known to bind to mouse IgG1[8], and therefore will bind to the Penta-HIS antibody, which is mouse IgG1. FcγRIIa and FcγRIIIb also appear to cross-react with the antibody fragment pre-immobilized on FAB biosensors. Some of this cross-reactivity may be mitigated by buffer optimization or blocking, but should also be taken into consideration when choosing an assay format. For FcγRIIa assays, using Ni-NTA or SAX biosensors with biotinylated IgG or a biotinylated receptor as ligand is recommended. Recommended buffer and blocking conditions are summarized by biosensor in Table 1.

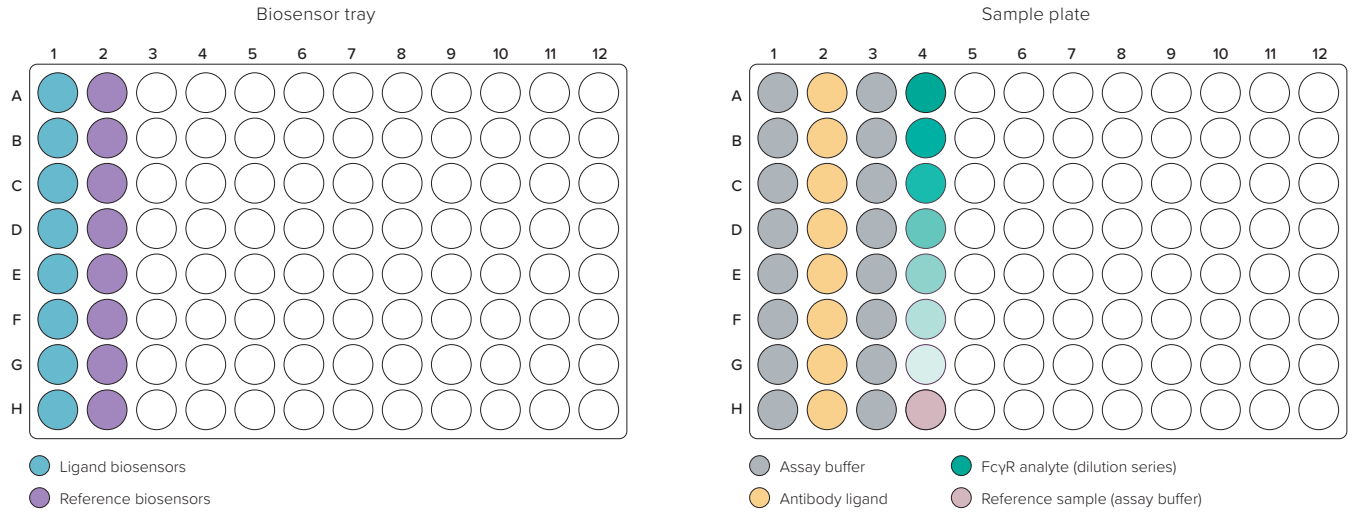
## Referencing

A reference sample must always be included in a FcγR-IgG kinetic assay to allow subtraction of assay drift. A reference sample is a sample with no analyte added in the association step, *i.e.* a buffer-only negative control. The reference sample is run on a biosensor that is ligand-loaded. When using a capture-based biosensor such as FAB, or when immobilizing biotin-Penta-HIS antibody onto SAX biosensors, there will be some background level of dissociation of the captured IgG ligand from the pre-immobilized capture molecule. This background dissociation must be subtracted out using a buffer-only reference sample. Double referencing with both a reference sample and a reference biosensor can be performed when there is a small amount of non-specific binding. A reference biosensor is a biosensor dipped into buffer or irrelevant protein instead of ligand during the loading step. It is then run through a replicate assay using the same analyte samples as the ligand-loaded biosensors (Figure 7). Reference biosensors enable subtraction

of non-specific binding of analyte to the biosensor. A separate reference biosensor should be included for every sample/analyte concentration when performing double referencing.

## Data acquisition rate

Because Fc gamma receptor-IgG interactions often have fast binding rates, the standard rate of data acquisition (5.0 Hz, averaging by 20) in Octet Data Acquisition software may not be the ideal setting for measuring kinetics. When data is acquired by the Octet system, there is a small delay from when the biosensor dips into the sample to when the first data points are reported to allow the software to average the collected data. When binding is very fast, this delay can cause the reported signal for the association step to initiate well above the baseline, leading to inaccuracies with curve fitting in data analysis. If this effect is observed and affects curve 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 faster 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 Octet Data Acquisition software (Figure 8). 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 the standard rate. Acquisition rate should always be determined based on consideration of the binding rate, the amount of signal generated in the assay as well as experimentation with the settings.




| 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 7: Assay protocol for an experiment on FAB 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 Data Analysis software Processing window, both Reference Sample and Reference Biosensor data will be subtracted from sample data.

## 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 the 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 FcγR-IgG interactions is exposure to a 10 mM glycine (pH 1.5) regeneration buffer for five seconds followed by assay buffer for five seconds, and repeating for a total of four exposures to regeneration buffer. Utilizing this protocol on FAB biosensors will remove IgG ligand-FcγR complex, restoring the original biosensor chemistry. New IgG samples can then be loaded for measuring another interaction.

When using SAX biosensors with Penta-HIS antibody for capturing HIS-tagged FcγR, the receptor ligand and IgG analyte will be removed, regenerating down to the level of the Penta-HIS antibody. New Fc receptor ligand can be loaded immediately after regeneration without loading more biotin-Penta-HIS antibody. FcγR-IgG complex can be removed from Ni-NTA biosensors with the same procedure. After regeneration, Ni-NTA biosensors must be recharged with 10 mM NiCl2 for one minute, then loaded with new HIS-tagged receptor. Regeneration information is summarized by biosensor in Table 1.

If regeneration is successful, the analyte binding curves following each regeneration cycle will overlay with minimal change in response when compared to earlier binding cycles. Figure 9 shows the overlay of data for association of IgG1 to FcγRIIIa immobilized on SAX biosensors with Penta-HIS antibody. Six kinetic assay cycles were run using the regeneration procedure described earlier performed between each cycle. When association and dissociation steps for each cycle are overlaid, binding response does not decrease but remains consistent across all assay 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 one regeneration cycle is performed before beginning the assay. The number of regeneration cycles that can be performed successfully in an assay will be biosensor- and reagent-dependent, and should be tested for each experimental system.

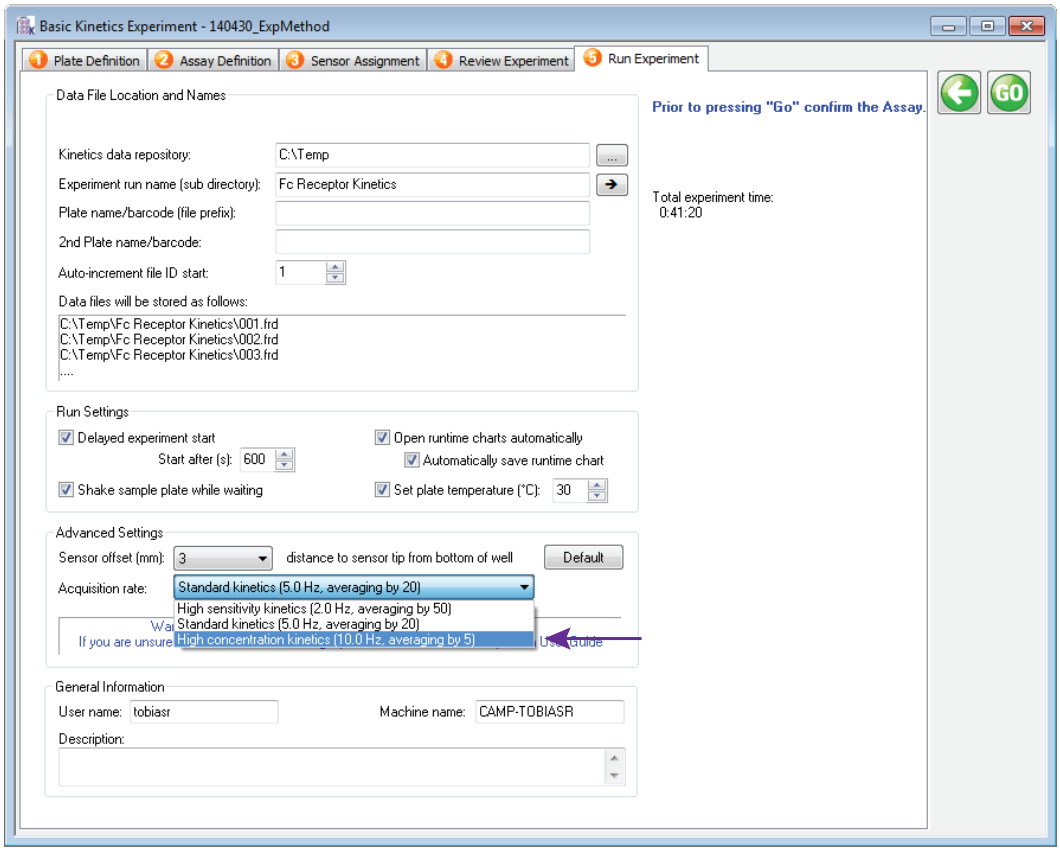
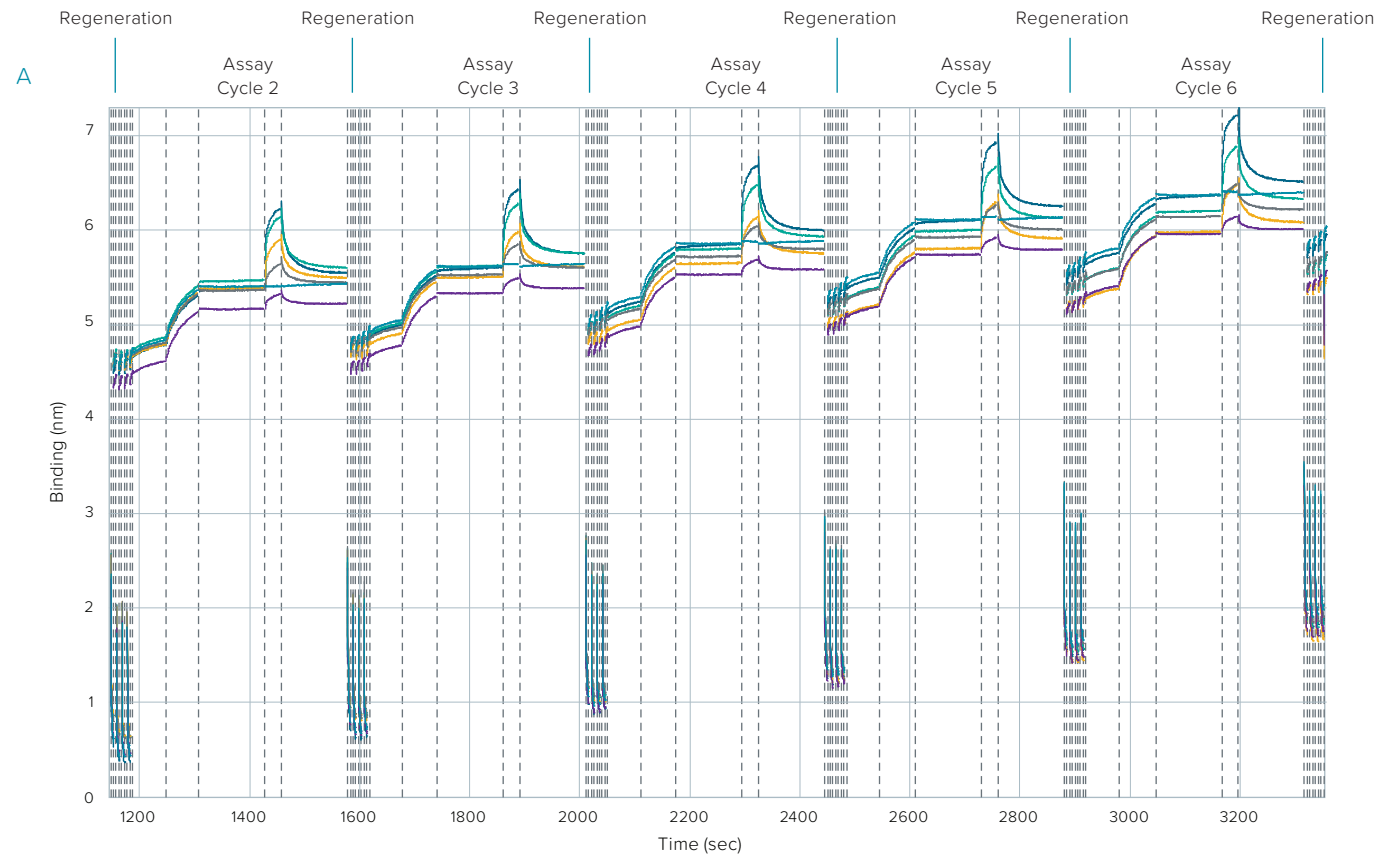
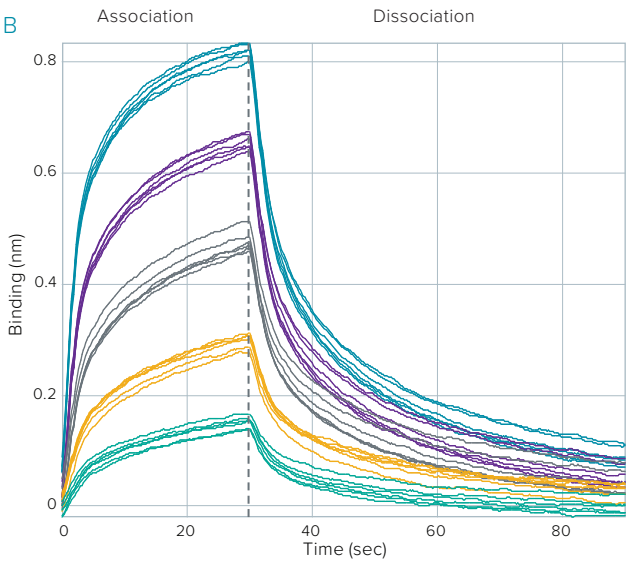
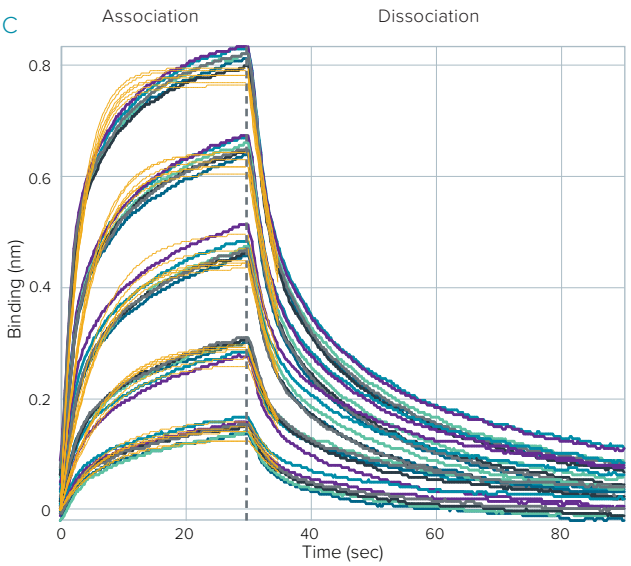



Figure 8: Changing the data acquisition rate. In Octet Data Acquisition software under the Run Experiment tab, click the pull-down menu for Acquisition rate and select Fast Kinetics (10 Hz, averaging by 5). Acquisition rate should be determined based on binding rate, amount of signal generated and experimentation.




D

| | Average | %CV |
|---|---|---|
| $K_D$ | 1.95E-06 | 7.9 |
| $K_{on}$ | 6.36E+04 | 5.7 |
| $K_{off}$ | 0.124 | 3.1 |

Figure 9: Biosensor regeneration in Fc gamma receptor kinetic assays. A) Raw data: biotin Penta-HIS antibody was immobilized on High Precision Streptavidin (SAX) biosensors, followed by a pre-conditioning step, then six kinetic assay cycles with regeneration. For each kinetic assay, HIS-tagged FcγRIIIa was first captured onto Penta-HIS antibody, then association and dissociation steps performed to a 2-fold dilution series of IgG1. B) Overlay of IgG1 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 orange). D) Table of average kinetic and affinity constants with CV's for the 6 regeneration cycles.

## Data analysis

The most accurate affinity constants are determined in a kinetic assay when global data fitting is used with several (four to six) analyte concentrations. Octet Data Analysis software offers several pre-programmed curve fitting models for analysis of binding data.

The kinetic and affinity constants that are calculated will depend upon the binding model selected. The stoichiometry of the interaction of FcγRIIIa and FcγRII with IgG Fc has been demonstrated to be 1:1 in structural and sedimentation studies9,10,11. Asymmetrical binding of a single receptor to Fc to both heavy chains causes minor conformational changes that prevent a second receptor molecule from binding12. Since other FcγRs are thought to bind Fc by a similar mechanism with one receptor to one IgG stoichiometry, a 1:1 binding model is the most biologically relevant model for fitting Fcγ receptor-IgG interactions. However, the data sensorgrams for these interactions, especially FcγRIIIa-IgG, often show complex binding attributes which confound 1:1 model fitting. Some of the complex or biphasic binding exhibited can be eliminated experimentally by lowering the amount of receptor or antibody ligand on the biosensor and by shortening the association step to 30–60 seconds. Even with these adjustments, however, the 1:1 binding model may not provide a good quality fit. Some researchers opt for using the 2:1 heterogeneous ligand model because the fitting tends to be improved over the 1:1 model. However, selecting the 2:1 model assumes that the immobilized binding partner (ligand) has two independent binding sites with different affinities for the analyte. This assumption is not consistent with what has been demonstrated with FcγR interactions with IgG. Data for FcγRIIIa-IgG kinetics will also fit a 'conformational change' or Two-State model (currently not provided in Octet Data Analysis software) yet it is unclear that this model is the correct way to interpret the kinetics. While minor conformational changes have been shown to occur at the binding site between FcγRIIIa, b and IgG Fc upon complex formation, there is limited evidence to show that conformational change is part of the mechanism of signal transduction for Fc receptors. Rather, cross-linking of receptor molecules upon binding to multivalent immune complexes appears to be the primary mechanism for intracellular activation[3,10,13]. The complexity in the binding traces may be caused by multiple factors such as non-specific interactions or aggregation of the antibody creating sub-populations of higher-affinity multimers.

With appropriate assay optimization and data analysis tools, some of which have been discussed above, kinetic off-rates and on-rates can be calculated even for difficult to fit interactions. The 1:1 binding model can often be fit successfully to complex and low-affinity FcγR-IgG data when the data acquisition rate is increased and only a portion of the dissociation step is included in the analysis. Truncating the dissociation step to 5–10 seconds enables the initial dissociation rate in a biphasic curve to be captured so that fitting is improved and reproducible off-rate and $K_D$ values can be calculated (Figure 10a, b). Using a truncat-

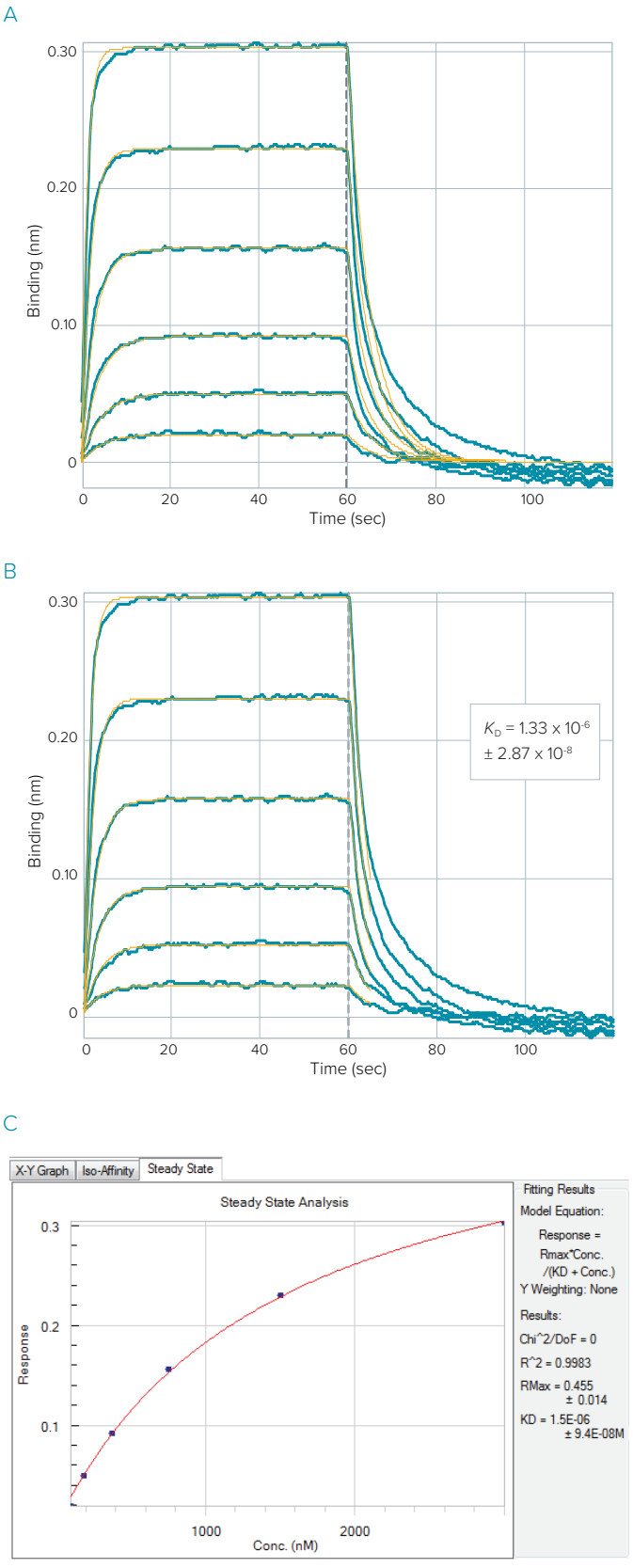



Figure 10: Comparison of data fitting strategies. FAB biosensors were used to capture human IgG for a binding kinetics assay with FcγRIIIa using several concentrations. The 1:1 model was used to determine affinity constant, $K_D$, using the full dissociation step (A) and 5 seconds of the dissociation step (B). Steady-state analysis of data was also used to determine $K_D$ (C).

ed dissociation step for curve fitting is most useful for ranking purposes, when reagents are being compared to each other or to a reference sample to examine loss or gain of activity.

Steady state values are often used when reporting $K_D$s for low-affinity receptors such as FcγRII or FcγRIIIa. Steady-state analysis can provide an accurate $K_D$ value, and is especially useful for analyzing interactions that are of low affinity or have very fast on-rates. Steep initial slopes or complexity in the binding curves are often more reliably analyzed using steady state rather than kinetic analysis. However, it is important to consider that the steady state method is an endpoint analysis that delivers an equilibrium binding constant. Desirable kinetic information is left out, and on-rates and off-rates are not calculated. Tools are available in Octet Data Analysis software for performing steady-state data analysis. The steady-state responses (where the sensorgram plateaus in the association step) for various analyte concentrations are calculated using the R-equilibrium (Req) function and plotted vs. analyte concentration. The resulting binding isotherm is fitted using the Langmuir model to calculate the equilibrium constant $K_D$ (Figure 10c).

## Evaluating quality of the data and fitting

When developing an assay it is important to look closely at data traces and assess the quality of the data being analyzed. Consider noise level, shape of the traces, signal level at equilibrium, number of data points being assessed, and whether there is even spacing between curves in a titration. Look for heterogeneity in the binding which can be reduced by optimizing experimental conditions. Primary considerations in assay development are loading concentration/loading density, buffer composition, and assay step times. Check for non-specific binding to the biosensor. Be sure reagents are of good quality and have not been stored under conditions that would cause degradation or aggregation.

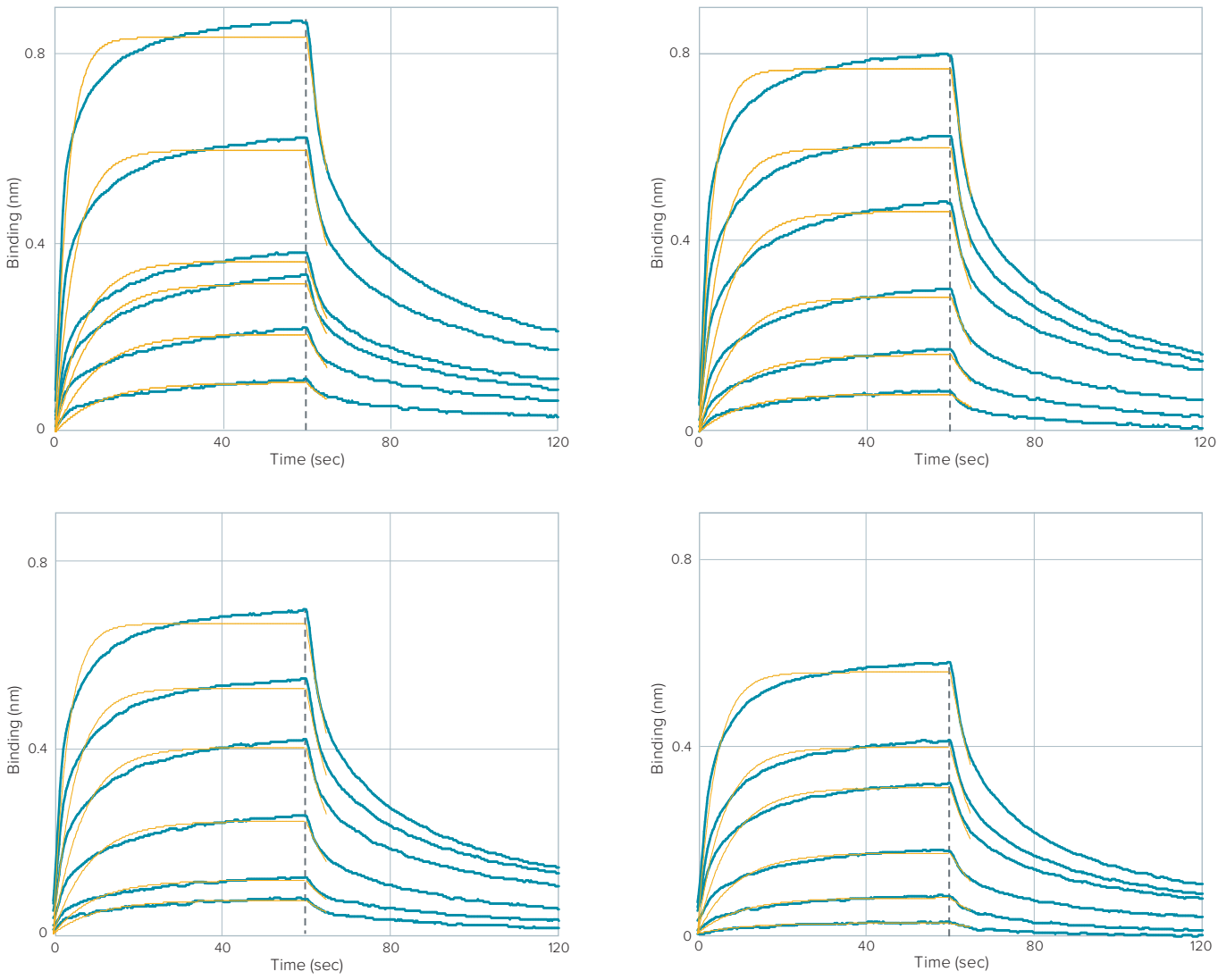
Once good quality data has been acquired and processed and the curve fitting performed, check the analysis for goodness of fit. Visually inspect the data to determine whether the fit lines conform well to the data traces. Individual traces that deviate from the pattern shown by other traces in a titration can be excluded from the analysis, as well as traces that show weak response. Exclusion of analyte concentrations outside the working range of the analyte titration will improve fitting in global analysis. Residual values, which are calculated in the results table and plotted by Octet Data Analysis software, should ideally be less than 10% of the maximum response of the fitted curve. Error values also indicate the reliability of calculated kinetic and affinity constants. Errors are considered acceptable if they are within one order of magnitude of the rate constant value. The $R^2$ value indicates how well the fit and experimental data correlate. $R^2$ values above 0.95 are indicative of a good fit. Chi$^2$ ($X^2$) is the sum of the squared deviations, and is a measure of error between experimental data and the fit line. Chi2 is also provided in the data analysis results table and should generally be a value below three ($X^2 < 3$).

## Measuring affinity of Fc gamma receptors 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. In the development stage, it is important to differentiate the most promising candidates early in the process. Antibody engineering technologies are constantly advancing to improve the clinical effectiveness of monoclonal antibodies. Effector functions may be modified by engineering the Fc region to improve or reduce binding to FcγRs. Detailed analysis of both kinetics and binding affinity to FcγRs is essential to understanding the activity and safety of drug candidates and guiding further design strategies. Studies to demonstrate comparability are of critical importance in development of biosimilar monoclonal antibody products. Equivalence of materials needs to be established in terms of quality, safety and efficacy, including full comparison of immunological properties between a biosimilar and a licensed originator product. Fc-associated functions must be characterized as a component of comparability exercise[14]. Fc gamma receptor binding can be impacted by product degradation. Forced degradation studies are designed to generate product-related variants and develop analytical methods to determine the degradation products formed during long-term storage. In addition, the manufacturing of biotherapeutics requires continual certification of every lot of product produced. GMP-compliant batch release and stability assessments must be performed that address the criteria of purity, concentration, consistency, and safety, including evaluation of effector function. Fc gamma receptor binding kinetics assays, where appropriate, can potentially serve as a surrogate measure of ADCC when correlation can be established.

In order to demonstrate Fc gamma receptor kinetic assays on the Octet system as a feasible platform for performing comparability studies and lot release testing assays, the methods of analysis described here were used to compare affinities of Fc gamma receptors to IgG samples on the Octet RED384 system. First, 'stressed' antibody samples were generated with altered affinity for FcγRIIIa. Humanized monoclonal $IgG_1$ was incubated with EndoS de-glycosylase enzyme (Remove-iT EndoS®, New England Biolabs), which cleaves the chitobiose core of the N-linked glycan at Asn297 of the IgG heavy chain. Removal of this glycan is expected to reduce IgG binding to most Fc gamma receptors due to more closed conformation of the Fc region[14]. After removal of the enzyme and buffer exchange, de-glycosylated hIgG was mixed with un-modified hIgG at varying amounts to generate samples that contained 10%, 25%, and 50% de-glycosylated antibody. The affinity to FcγRIIIa was measured for each of these samples using the Octet RED384 instrument. SAX biosensors with immobilized biotin-Penta-HIS antibody were used to capture HIS-tagged FcγRIIIa (R&D Systems), then associated to

A




B

| % Deglycosylation | $K_D$ (M) |
|---|---|
| 0 | 8.68E-07 |
| 10 | 9.89E-07 |
| 25 | 1.13E-06 |
| 50 | 1.52E-06 |

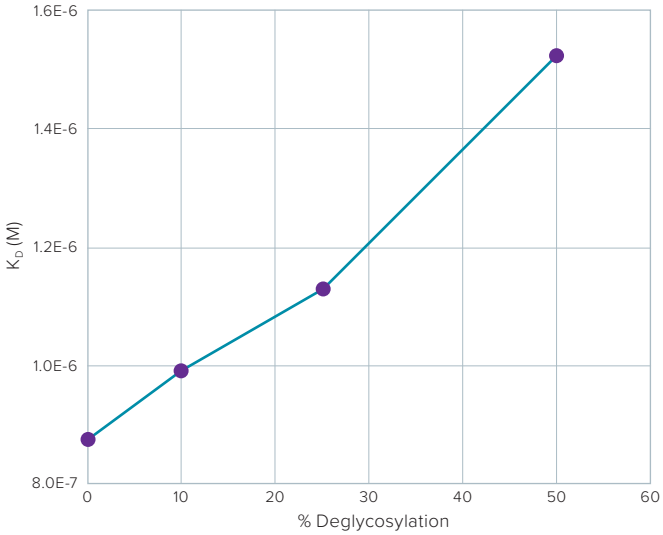



Figure 11: Results of kinetic analysis of deglycosylated hIgG1 binding to FcγRIIIa. Affinity of deglycosylated samples to FcγRIIIa was measured using High Precision Streptavidin biosensors with Penta-HIS antibody to capture the HIS-tagged receptor. Analysis was performed using 1:1 global fitting with a portion of the dissociation step (5 seconds). A) Fitted data for each sample. B) Calculated KD plotted against % deglycosylation, showing a clear relationship between glycosylation level and affinity.

a 2-fold dilution series of each hIgG1 sample. Global fitting was performed to each set of analyte concentrations to calculate $K_D$ and kinetic constants, using a 1:1 fitting model with a portion of the dissociation step (5 seconds).

Figure 11 shows the fitted data and analysis from the kinetics experiment. The results demonstrate that a decrease in calculated $K_D$ correlates with an increased percentage of de-glycosylated IgG in the sample. A change in affinity was measured in this experiment even in the 10% de-glycosylated sample, which showed a 12% decrease in $K_D$ compared to un-modified IgG ($K_D$ = 990 nM vs. $K_D$ = 871 nM). The entire experiment was performed in a single 45-minute automated run on the instrument plus approximately 45 minutes assay set-up time, using 80 µL of sample per well in a 384-well plate. This experiment illustrates the ease with which a sensitive assay can be developed on Octet systems for measuring differences in affinity of FcγR-IgG interactions, even for lower affinity receptors.

## Conclusion

Measuring accurate and reliable kinetics of interactions between Fc gamma receptors 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 FcγR kinetic data on the Octet system using a variety of assay formats, with recommendations for assay optimization and data analysis. Considerations such as biosensor selection, assay orientation, choice of buffers, ligand immobilization and regeneration conditions can all impact results. Fc gamma receptor kinetic assays on the Octet platform can be extremely useful for evaluating Fc receptor binding activity of antibodies produced from different clones, different production processes or prepared in different formulations. We have shown how these methods can potentially be adapted to function in comparability studies and qualified or validated GMP lot release and stability studies. 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.

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