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Optimizing Assay Performance for IgG1, IgG2, and IgG4 Monoclonal Antibodies

A Guide to Fcγ Receptor Binding Studies

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Abstract

Monoclonal antibodies (mAbs) selectively target a range of diseases and are therefore one of the most commonly used drug development platforms. Therapeutic mAbs play a key role in cancer therapeutics due to their ability to recognize target antigen proteins and subsequently interact through their fragment crystallizable (Fc) domain with membrane bound Fcγ receptors. This interaction can induce multiple cell-killing mechanisms such as antibody dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). Fcγ receptors are essential in regulating both the innate and adaptive immune responses, making them a focal point of testing during multiple phases of therapeutic antibody drug development.

This application note provides insights into the significance of Fcγ receptors binding studies and outlines the assay design and development considerations necessary to effectively analyze the binding kinetics and affinities of IgG1, IgG2, and IgG4 antibodies with Fcγ receptors.

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Introduction

IgG monoclonal antibodies (mAbs) are a rapidly growing class of biopharmaceuticals used to treat a variety of diseases, including cancer, autoimmune disorders, and infections. They function by recognizing and binding to specific pathogens or tumor-associated antigens through the variable region of the antibody, forming immune complexes that initiate various effector responses.

While the variable region is known for these primary functional activities, immune checkpoint antibodies have also demonstrated clinical benefits in cancer treatments, whether used alone or in combination therapies. Despite this, some tumors remain resistant, and merely blocking immune suppression pathways may not be sufficient. The constant region of the antibody, particularly the crystallizable fragment (Fc), is increasingly recognized for its role in therapeutic efficacy by mediating effector functions through Fcγ receptor interactions.[1]

These effector functions include complement-dependent cytotoxicity (CDC) antibody-dependent cellular phagocytosis (ADCP), antibody dependent cellular cytotoxicity (ADCC), and cytokine induction, as summarized in Table 1. Additionally, the binding of IgG to Fcγ receptors is a critical quality attribute (CQA) for the safety and efficacy of biopharmaceutical drugs, and its monitoring is essential.

IgG-Fcγ receptor interactions can be classified into high (FcγRI, CD64), medium (FcγRIIa, CD32a and FcγRIIIa, CD16a), and low (FcγRIIb, CD32b and FcγRIIIb, CD16b) affinity interactions. However, despite numerous publications, there can be a large variability in reported affinities due to the use of different reagents and experimental setups, and there is no consensus on the optimal assay setup. Additionally, glycosylation-induced heterogeneity in Fcγ receptors and antibodies adds complexity to the analysis of interaction kinetics.

In addition to the Fcγ receptors above, IgG1, IgG2 and IgG4 also bind the human neonatal Fc receptor (FcRn) in a strictly pH-dependent manner. A fundamental understanding of the binding and release mechanism of FcRn allows design of a biological-based model that utilizes the pH change that an IgG undergoes during cellular internalization and subsequent degradation. Interaction with FcRn plays a crucial role in determining the half-life of a therapeutic antibody and therefore plays a key role in development of therapeutic antibodies.

This document aims to provide standardized guidelines for assay design, development, performance, and evaluation using Fcγ receptor assays on the Octet® bio-layer interferometry (BLI) platform.

Table 1: *Binding Affinities of IgG Isotypes to Human Fcγ Receptors and Resulting Biological Effector Functions*

	IgG1		IgG2		IgG4	
Fcγ receptor	**Expected Affinity**	**Effector Function**	**Expected Affinity**	**Effector Function**	**Expected Affinity**	**Effector Function**
FcγRI, (CD64)	High	ADCP	None	None	High	ADCP, Cytokine Release
FcγRIIa R167, (CD32a R167)	Medium		Low	Myeloid-cell induced ADCC	Low	Receptor Clustering
FcγRIIa H167, (CD32a H167	Medium		Low		Low	
FcγRIIb, (CD32b)	Low	Immune Complex Clearance	Low	None	Low	Immune Complex Clearance
FcγRIIIa V176, (CD16a V176)	Medium	ADCC	Low		Low	None
FcγRIIIa F176, (CD16a F176)	Medium		Low		Low	
FcγRIIIb NA1, (CD16b NA1)	Low	Immune Complex Clearance	Low | None		Low | None	
FcγRIIIb NA2, (CD16b NA2)	Low		Low | None		Low | None	
FcRn	Medium	N.A	Medium	N.A	Medium	N.A

Note. (ADCP) antibody-dependent cellular phagocytosis, (ADCC)antibody dependent cellular cytotoxicity. Affinity values are based on IgG binding to Fcγ receptors and adapted from Bruhns et al and Chen et al. [2, 3]

IgG Isotypes and Fcγ Receptors

Therapeutic IgG antibodies are categorized into subclasses IgG1, IgG2, IgG3 and IgG4. Each subclass has unique binding affinities to Fcγ receptors and different expression patterns on immune cells, resulting in varied and tightly controlled immune responses as detailed in Table 1. IgG1 is known for its high affinity to all Fcγ receptors and the complement component C1q, which enables it to effectively mediate immune effector functions such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC).

IgG3, which has the capability for competent effector functions, is less commonly used in antibody therapeutics due to its long hinge region, complex disulfide bonds, and high polymorphism, which may increase the risk of immunogenic reactions. IgG2 and IgG4 are associated with weaker or absent ADCC and CDC activities. Despite this, the overall structures of IgG1, IgG2, and IgG4 share over 90% sequence homology, with the major differences in the hinge region and CH2 domain, which are crucial to Fcγ receptor binding.

IgG4 is unique in that it can form "half-antibodies" due to a specific amino acid, S228, in its hinge region, which allows for interchangeable disulfide bond configurations. To prevent this, most approved therapeutic IgG4 antibodies contain an S228P mutation. IgG2 can exist in different disulfide bond isomers, namely IgG2A, IgG2B, and IgG2A/B, influenced by factors like cell culture conditions or thermal stress.

The structural and functional differences among Fcγ receptors and IgG subclasses contribute to the diversity of antibody responses in therapeutic applications. As shown in Table 1, the affinity of each IgG isotype for an Fcγ receptor can range from a low or no affinity interaction to a high affinity interaction. Consequently, a single assay format is not appropriate for all Fcγ receptors.

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Assay Design Considerations

As mentioned above, despite multiple publications on Fcγ receptors and IgG interactions, there is an overall lack of consensus as to how to determine correct kinetics and affinity values and as such, published values may vary up to ten-fold. Here, assay design considerations based upon the Fcγ receptors are presented and applied to IgG1, IgG2 and IgG4 reference medicinal products (RMP). IgG3 is not considered as currently no available therapeutic uses an IgG3 scaffold. A brief overview of how to develop an Fcγ receptor assay is described below, for a complete guide to assay design and development please see A Compendium for Successful BLI and SPR Assays.

As shown in Figure 1, there are two assay formats for analyzing IgG binding to Fcγ receptors:

- Using the Fcγ receptor as the ligand and the antibody as the analyte
- Using the Fcγ receptor as the analyte and the antibody as the ligand

Regardless of the assay orientation, it is recommended to avoid amine coupling of either the Fcγ receptor or IgG as this can create heterogenous surfaces that result in poor intra- and inter-assay precision.

The preferred method is a capture approach that creates a homogenous surface. This approach simplifies assay optimization by eliminating the need to develop specific regeneration conditions for each binding partner.

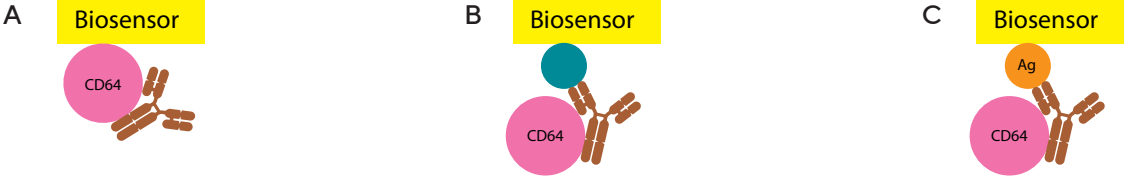

Figure 1: Two Assay Orientations are Possible for Assessing Fcγ Receptors Binding to IgG. (A) The Fcγ receptor as the ligand and the antibody as the analyte and (B) the Fcγ receptor as the analyte and the antibody as the ligand. When using the Fcγ receptor as the analyte (C), care must be taken when selecting the antibody capture molecule due to a change in the observed kinetics and affinity caused by structural restrictions in the antibody , especially when capturing using the antigen (Ag).[4]

Fcγ Receptor as the Ligand and the Antibody as the Analyte
Fcγ receptor as the ligand is the most common assay format due to the high cost associated with purchasing Fcγ receptors for use as analyte. Various Octet® BLI biosensors are amenable for capture assays with Fcγ receptor as the ligand. The final choice of biosensor is dependent upon the affinity tag on the Fcγ receptor.

When using Fcγ receptor as ligand, excipients such as trehalose which are commonly used as cryoprotectants by commercial suppliers do not generally present an issue. They are effectively diluted down in ligand preparation and removed during the baseline step after loading in the capture assay. However, when preparing analyte samples, care must be taken to minimize refractive index differences between the sample and the assay buffer. Using spin columns such as VivaSpin for buffer exchange into the assay buffer is recommended.

Fcγ Receptor as the Analyte and the Antibody as the Ligand
Fcγ receptor as the analyte is less common due to the high costs associated with purchasing Fcγ receptors for use as analyte. Where possible, using Fcγ receptor as the analyte should be considered as this assay orientation can reveal additional information about structural changes in the antibody, and is especially beneficial when assessing stressed samples.

Several Octet® BLI biosensors are suitable for performing capture assays where the antibody is the ligand. However, it is essential to ensure that the antibody capture method does not occlude the Fc receptor binding site. It is advisable to initially assess Fcγ receptor binding to IgG using a common capture molecule such as Octet® FAB2G Biosensors when comparison to published data is desired. This is due to the effect of antibody capture through its antigen causing an increase in affinity to Fcγ receptors due to structural restrictions.[4]

When using the antibody as ligand, excipients commonly found in RMP buffers such as sucrose, dextrose and L-histidine trehalose typically do not present an issue as they are effectively diluted down in ligand preparation and removed during the baseline step after loading in the capture assay. As with the other assay orientation, care must be taken to minimize refractive index differences between the sample and the assay buffer and spin columns such as VivaSpin should be used for buffer exchange into the assay buffer.

Assay Development
Careful assay design can minimize or eliminate many of the artifacts associated with binding data, including challenges fitting data to a simple model. A well-designed assay is fundamental to producing high-quality BLI data that can be easily interpreted to determine meaningful kinetic rate and affinity constants.

A critical parameter in assay design is the inclusion of a suitable reference surface, in addition to the active surface where binding measurements will be performed. Suitable reference surfaces can help correct for multiple artifacts, such as refractive index changes, nonspecific binding, and assay drift.

Prior to assay development, it is important to assess levels of non-specific binding of the assay buffer to the biosensor used in the final assay. This evaluation should initially be performed using a biosensor prepared in the same manner as for the final assay; for example, if AviTag™ ligands are used, Octet® SAX Biosensors should be tested along with a naïve biosensor. Due to the high analyte concentrations used in Fcγ receptor assays, it is also advisable to assess binding of the analyte to the naïve biosensor.

In general, it is important to confirm that the assay setup is suitable using a few well-characterized reference samples prior to performing multiple assays with multiple samples. In the case of Fcγ receptors, it is recommended that assay development is performed with a well-characterized IgG1 antibody, and to apply the same parameters when assessing additional IgG1, IgG2 and IgG4 antibodies.

It is recommended to perform Fcγ receptor assays at 25 °C and with a shake speed of 1,000 RPM. All reagents and samples should be fully equilibrated to room temperature prior to sample preparation. Thaw frozen samples completely and — mix thoroughly prior to use.

Buffers
For all Fcγ receptor assays except FcRn, it is recommended to use 1X Kinetics Buffer (KB) as a suitable starting buffer to assess non-specific binding and for use in further assay development. For FcRn BSA is not recommended as a buffer component, since some cross-reactivity may occur. A phosphate-based assay buffer at pH 6.0 for dilution of analyte samples as well as baseline and dissociation assay steps are recommended. This assay buffer ensures efficient binding of FcRn to IgG under acidic conditions and minimizes non-specific binding.

When using AviTag™ FcRn a phosphate-based assay buffer at pH 6.0 can be used for ligand preparation and loading to help minimize signal drift. If the FcRn ligand is prepared in 1X KB during the loading step, signal drift may occur when transferring to the phosphate-based pH 6.0 assay buffer. To stabilize the signal and minimize drift a baseline step sufficient to equilibrate into the new buffer is necessary. A baseline step lasting one to five minute is usually adequate when switching buffers.

Materials

Material	Supplier	Product Number
10X KB	Sartorius	18-1105
Octet® SAX Biosensors	Sartorius	18-5118
Octet® Evaporation Covers	Sartorius	19-0081
96-well, black, flat bottom microplate	Greiner Bio-One	655209
1X PBS	Gibco	14190-094
Herceptin® (trastuzumab)	Roche	Supplied by WEP Clinical
Prolia® (denosumab)	Amgen	Supplied by WEP Clinical
Opdivo® (nivolumab)	Bristol-Myers Squibb	Supplied by WEP Clinical
Biotinylated Human Fc gamma RI/CD64 Protein, His, Avitag™	ACRObiosystems	FCA-H82E8
Biotinylated Human Fc gamma RIIA/CD32a (R167) Protein, His, Avitag™	ACRObiosystems	CDA-H82E5
Biotinylated Human Fc gamma RIIA/CD32a (H167) Protein, Avitag™, His	ACRObiosystems	CDA-H82E6
Biotinylated Human Fc gamma RIIB/C (CD32b/c) Protein, Avitag™, His	ACRObiosystems	CDB-H82E0
Biotinylated Human Fc gamma RIIIA/CD16a (V176) Protein, Avitag™, His	ACRObiosystems	CDA-H82E9
Biotinylated Human Fc gamma RIIIA/CD16a (F176) Protein Avitag™, His	ACRObiosystems	CDA-H82E8
Biotinylated Human Fc gamma RIIIB/CD16b (NA1) Protein, His, Avitag™(	ACRObiosystems	CDB-H82E4
Biotinylated Human Fc gamma RIIIB/CD16b (NA2) Protein, His, Avitag™	ACRObiosystems	CDB-H82Ea
Biotinylated Human FcRn/FCGRT&B2M Heterodimer Protein, His, Avitag™	ACRObiosystems	FCM-H82W7

General Assay Development Workflow

For the purpose of this document and the general stages of assay development, an assay format of Fcγ receptor as the ligand was chosen using AviTag™ Fcγ receptors captured onto Octet® SAX Biosensors (Figure 1).

The general assay development workflow consisting of five steps is shown in Figure 2. Here Octet® SAX Biosensors are used in an assay format using AviTag™ FcγRI (CD64, ligand) binding to Herceptin® (analyte), with the aim to generate an assay with a binding response of < 0.3 nm that fits well to a 1:1 kinetic model.

A lower surface density was chosen as an excess of ligand bound to the sensor can lead to data artifacts due to crowding, steric hindrance, and possible aggregation on the biosensor surface. Over-saturation of the sensor may also promote weaker, non-specific interactions at higher analyte concentrations, or analyte rebinding. Together, these artifacts may significantly impact observed binding kinetics.

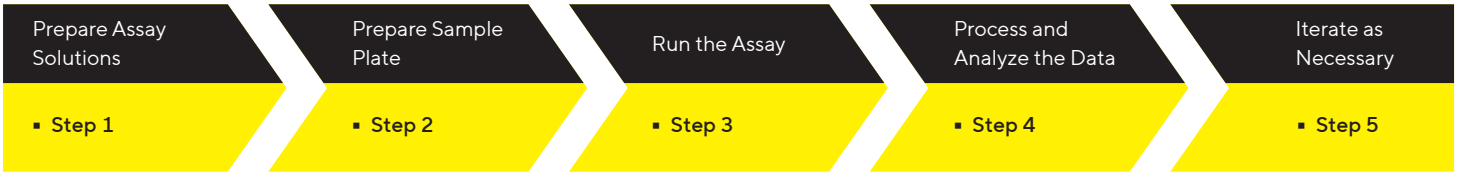

Figure 2: General Assay Workflow for Development of Fcγ Receptors Assay.

Step 1 – Prepare Assay Solutions

FcγRI (CD64) and Herceptin® were prepared according to the manufacturer's recommendations. Prior to preparation, all reagents were equilibrated to room temperature and mixed thoroughly.

Step 2 – Prepare Sample Plate

Initially the loading level of FcγRI (CD64) must be determined empirically. In order to minimize assay development time, it is recommended to assess as many samples as possible in a single assay. Here, an Octet® BLI R8 was used for assay development and therefore eight unique ligand concentrations were assessed (0.0625 – 8 µg/mL) in a single assay. Figure 3 shows a typical assay sample plate layout containing multiple ligand concentrations and assay buffer (1X Kinetics Buffer).

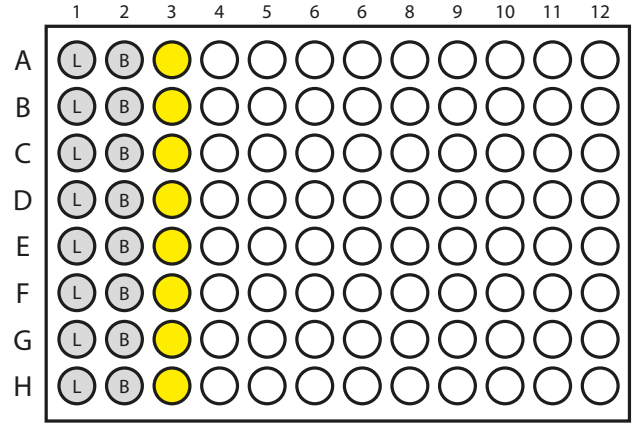

Figure 3: Typical Plate Layout Used During Assay Development. Careful planning of ligand and analyte concentrations can help yield maximum usable data from a single assay plate.

Step 3 – Run the Assay

Ensure that the Octet® BLI instrument is turned on and the lamp has been warmed for at least three hours prior to starting the assay.

Pipette 200 µL of biosensor hydration solution into each well of a 96-well black, flat-bottom microplate corresponding to the number and the positions of biosensors to be used.

Step 4 – Process and Analyze the Data

As shown in Figure 4 a ligand concentration between 0.0625 – 8 µg/mL for FcγRI (CD64) returned a binding response between ~ 0.05 – 1.7 nm over a period of 60 s.

Step 5 – Iterate as Necessary

With initial ligand load parameters determined, the assay can be further developed by the introduction of an analyte (Herceptin®). The subsequent iterations for a successful assay design are described below.

Iteration 1 - Ligand Load Optimization: As shown in Figure 5a, an initial assessment of a fixed concentration of Herceptin® (150 nM) binding to FcγRI (CD64) ligand concentrations assessed above (0.0625 – 8 µg/mL) was performed and the binding responses observed.

As the initial aim of assay development was to generate an assay with a binding response of < 0.3 nm, it is clear that ligand concentrations greater than 1 µg/mL are inappropriate (Figure 5b) and 0.25 – 0.5 µg/mL would be appropriate for further assay development.

Iteration 2 - Analyte Top Concentration Optimization:
A concentration of 0.25 µg/mL was selected for further assay development as 0.5 µg/mL is close to the desired assay binding response and the optimum maximum analyte concentration has not been determined.

It is recommended to test a wide range of analyte concentrations to determine the optimum maximum concentration for use in subsequent analyte concentration series. A concentration series of Herceptin® (3.13 – 400 nM) was assessed as shown in Figure 6. At high analyte concentrations (200 and 400 nM) the association response is too rapid and an extended time period is spent at steady state equilibrium, which increases the risk of binding events occurring due to low-affinity interactions. Although dissociation is a concentration independent event, it is important to note that surface saturation can lead to an increase in surface crowding and non-specific binding events.

Conversely, at lower analyte concentrations (3.13 and 6.25 nM) where the binding response is essentially linear and therefore, lacks sufficient information about kinetic events. In both cases, especially with a low analyte concentration series inaccurate kinetics would be determined.

The optimal top analyte concentration for a subsequent series shows sufficient curvature to determine accurate kinetic rate constants but does not reach steady equilibrium for extended periods of time, which reduces binding events that may occur due to low-affinity interactions. In this scenario, 25 and 50 nM would be good choices for top concentration, with 50 nM being chosen for further assay development.

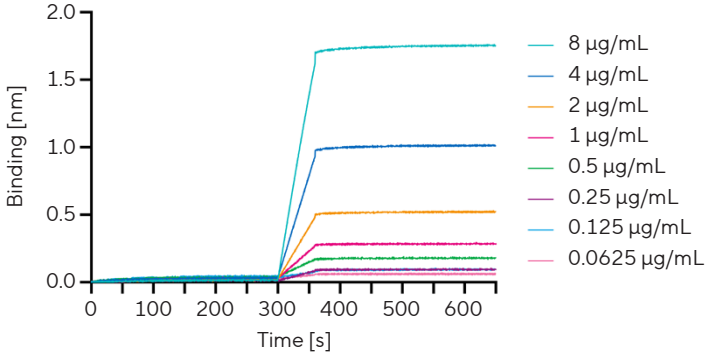

Figure 4: Ligand Capture Levels Assessment Using 0.0625 – 8 µg/mL for FcγRI (CD64).

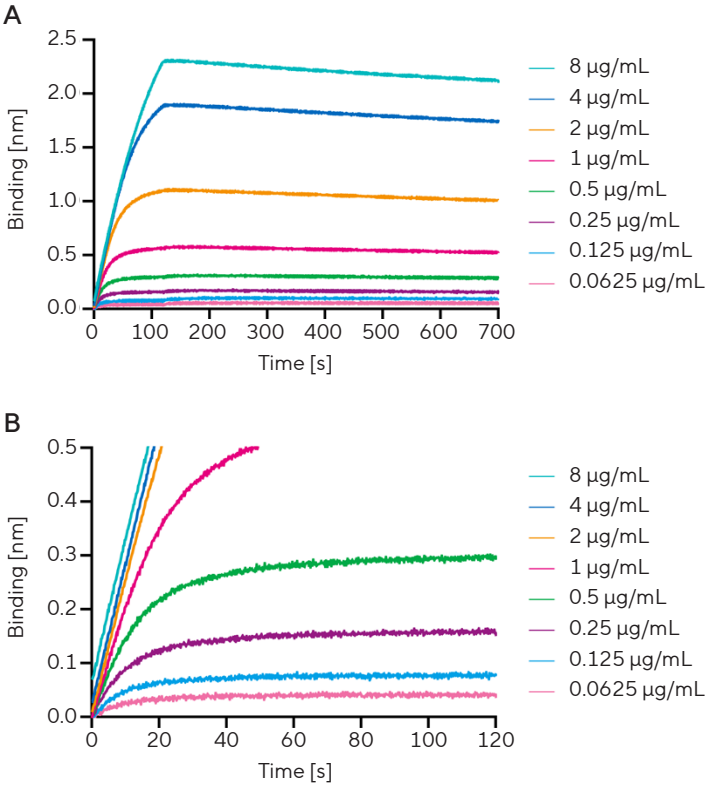

Figure 5: (A) Ligand load optimization using a fixed concentration of analyte 150 nM Herceptin®. (B) Assessment of the initial 120 s of the association phase allows suitable ligand concentrations to be rapidly determined.

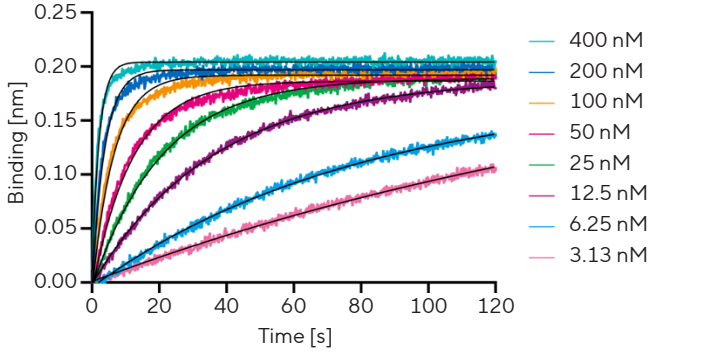

Figure 6: It is recommended to test a wide range of analyte concentrations as possible to determine the optimum maximum concentration for use in subsequent analyte concentration series.

Choosing the Correct Analyte Concentration Range

One of the most critical parameters in determining accurate kinetics and affinity is the selection of an appropriate analyte concentration series for fitting to an appropriate kinetic or steady state model. Unfortunately, this crucial step is often overlooked, leading to the use of unsuitable analyte concentration series.

As shown in Figure 7, applying different analyte concentration series to the same ligand load can yield substantially different responses. The first concentration series (Figures 7a and 7b, 0.206 – 50 nM) meets several key parameters for the optimal determination of kinetics and affinity (See A Compendium for Successful BLI and SPR Assays, Biomolecular Binding Kinetics Assays on the Octet® Platform and Octet® BLI Kinetics Assay: Method Development Guideline for further information):

- The association phases are not limited by mass transport.
- There are minimal to no refractive index jumps; ideally association and dissociation should connect after referencing.
- The top analyte concentration should reach saturation (where possible) and curves below should contain sufficient curvature.
- The association curves are spaced out appropriately and cover a wide concentration range.
- The dissociation phase is long enough to allow sufficient response change for accurate fitting.
- The observed response is low, such that R_{max} is low and avidity effects are minimized.

In contrast, the second concentration series (Figure 7 C, 0.156 – 5 nM) meets some key parameters but the top concentrations do not show sufficient curvature and appear almost linear (Figure 7D). This can lead to inaccurate kinetics and affinity. It is important to consider as well that if analyte concentrations are too low, signals may be weak and | or the resulting binding rates may be diffusion limited.

The third concentration series (Figure 7E and F, 12.5 – 400 nM) contains multiple issues. Several of the highest analyte concentrations reach saturation steady state, which increases the risk of binding events occurring due to low-affinity interactions. In addition, although curvature is observed in all analyte concentrations, the association curves are not spaced out appropriately and the lower range of analyte concentration is not adequately represented. However, this is the range where the most accurate data are produced, therefore inaccurate kinetics and affinity would be determined.

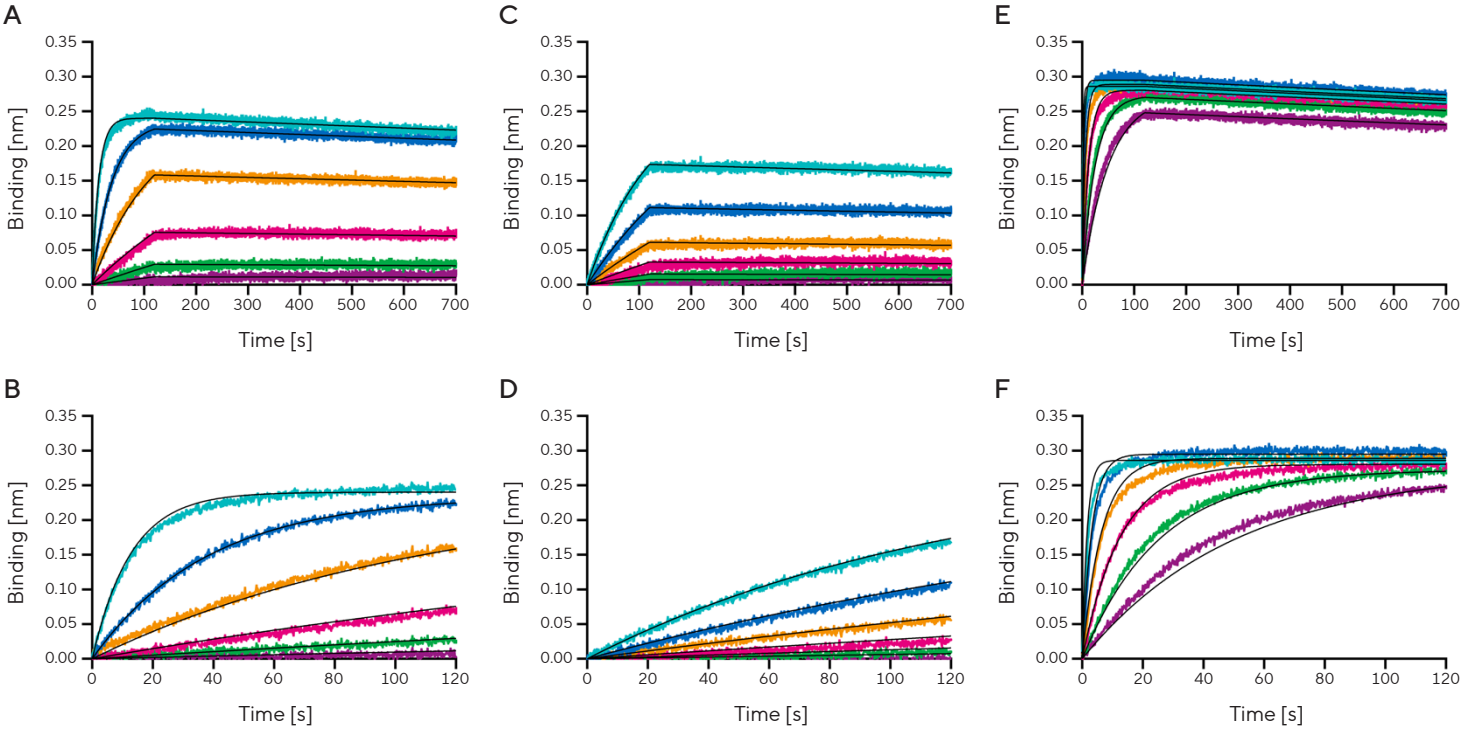

Figure 7: It is important when assessing kinetics to use an optimal range of analyte concentrations (A, B). Low analyte concentrations do not contain sufficient curvature and inaccurate kinetics and affinity would be determined (C,D). If the analyte concentrations tested in a kinetic assay are too high, ligand binding sites can be saturated, increasing the potential for artifacts and non-ideal performance related to non-specific binding or aggregation (E,F).

Suggested Assay Parameters for Fcγ Receptors

Based on assay development parameters discussed above. The following method parameters are suggested for studying the interaction between IgG1, IgG2 and IgG4 antibodies and Fcγ receptors. Representative data and assessments are shown in Figures 8, 9 and Table 2. They also serve as suitable starting parameters for assessing other antibody-based variants, such as bispecifics and antibody drug conjugates (ADCs).

FcγRI, CD64

Step Name	Parameter	Suggested Value
	Assay Buffer	1X KB
Equilibration		
	Time	300 s
	Shake Speed	1,000 RPM
Loading		
	Ligand	CD64 – 0.25 µg/mL in 1X KB
	Time	Threshold Signal Change: 0.1 nm
	Shake Speed	1,000 RPM
Baseline		
	Time	300 s
	Shake Speed	1,000 RPM
Association		
	Analyte	0, 0.2058 – 50 nM
	Time	120 s
	Shake Speed	1,000 RPM
Dissociation		
	Time	1,800 s
	Shake Speed	1,000 RPM
Analysis		
	Binding Model	1:1

FcγRIIa R167, CD32a R167

Step Name	Parameter	Suggested Value
	Assay Buffer	1X KB
Equilibration		
	Time	300 s
	Shake Speed	1,000 RPM
Loading		
	Ligand	CD32a R167 – 0.5 µg/mL in 1X KB
	Time	Threshold Signal Change: 0.07 nm
	Shake Speed	1,000 RPM
Baseline		
	Time	300 s
	Shake Speed	1,000 RPM
Association		
	Analyte	0, 82.3 – 20,000 nM
	Time	60 s
	Shake Speed	1,000 RPM
Dissociation		
	Time	60 s
	Shake Speed	1,000 RPM
Analysis		
	Binding Model	SSA — Response Average 50 – 55 s

FcγRIIa H167, CD32a H167

Step Name	Parameter	Suggested Value
	Assay Buffer	1X KB
Equilibration		
	Time	300 s
	Shake Speed	1,000 RPM
Loading		
	Ligand	CD32a H167 – 0.5 µg/mL in 1X KB
	Time	Threshold Signal Change: 0.07 nm
	Shake Speed	1,000 RPM
Baseline		
	Time	300 s
	Shake Speed	1,000 RPM
Association		
	Analyte	0, 82.3 – 20,000 nM
	Time	60 s
	Shake Speed	1,000 RPM
Dissociation		
	Time	60 s
	Shake Speed	1,000 RPM
Analysis		
	Binding Model	SSA – Response Average 50 – 55 s

FcγRIIb, CD32b

Step Name	Parameter	Suggested Value
	Assay Buffer	1X KB
Equilibration		
	Time	300 s
	Shake Speed	1,000 RPM
Loading		
	Ligand	CD32b – 0.2 µg/mL in 1X KB
	Time	Threshold Signal Change: 0.07 nm
	Shake Speed	1,000 RPM
Baseline		
	Time	300 s
	Shake Speed	1,000 RPM
Association		
	Analyte	0, 82.3 – 20,000 nM
	Time	60 s
	Shake Speed	1,000 RPM
Dissociation		
	Time	60 s
	Shake Speed	1,000 RPM
Analysis		
	Binding Model	SSA – Response Average 50 – 55 s

FcγRIIIa V176, CD16a V176

Step Name	Parameter	Suggested Value
	Assay Buffer	1X KB
Equilibration		
	Time	300 s
	Shake Speed	1,000 RPM
Loading		
	Ligand	CD16a V176 – 0.2 µg/mL in 1X KB
	Time	Threshold Signal Change: 0.05 nm
	Shake Speed	1,000 RPM
Baseline		
	Time	300 s
	Shake Speed	1,000 RPM
Association		
	Analyte	0, 6.859 – 5,000 nM
	Time	120 s
	Shake Speed	1,000 RPM
Dissociation		
	Time	300 s
	Shake Speed	1,000 RPM
Analysis		
	Binding Model	SSA – Response Average 110 – 115 s

FcγRIIIa F176, CD16a F176

Step Name	Parameter	Suggested Value
	Assay Buffer	1X KB
Equilibration		
	Time	300 s
	Shake Speed	1,000 RPM
Loading		
	Ligand	CD16a F176 – 0.2 µg/mL in 1X KB
	Time	Threshold Signal Change: 0.05 nm
	Shake Speed	1,000 RPM
Baseline		
	Time	300 s
	Shake Speed	1,000 RPM
Association		
	Analyte	0, 6.859 – 5,000 nM
	Time	120 s
	Shake Speed	1,000 RPM
Dissociation		
	Time	300 s
	Shake Speed	1,000 RPM
Analysis		
	Binding Model	SSA – Response Average 110 – 115 s

FcγRIIIb NA1, CD16b NA1

Step Name	Parameter	Suggested Value
	Assay Buffer	1X KB
Equilibration		
	Time	300 s
	Shake Speed	1,000 RPM
Loading		
	Ligand	CD16b NA1 – 0.5 µg/mL in 1X KB
	Time	Threshold Signal Change: 0.07 nm
	Shake Speed	1,000 RPM
Baseline		
	Time	300 s
	Shake Speed	1,000 RPM
Association		
	Analyte	0, 82.3 – 20,000 nM
	Time	60 s
	Shake Speed	1,000 RPM
Dissociation		
	Time	60 s
	Shake Speed	1,000 RPM
Analysis		
	Binding Model	SSA— Response Average 50 – 55 s

FcγRIIIb NA2, CD16b NA2

Step Name	Parameter	Suggested Value
	Assay Buffer	1X KB
Equilibration		
	Time	300 s
	Shake Speed	1,000 RPM
Loading		
	Ligand	CD16b NA2 – 0.5 µg/mL in 1X KB
	Time	Threshold Signal Change: 0.07 nm
	Shake Speed	1,000 RPM
Baseline		
	Time	300 s
	Shake Speed	1,000 RPM
Association		
	Analyte	0, 82.3 – 20,000 nM
	Time	60 s
	Shake Speed	1,000 RPM
Dissociation		
	Time	60 s
	Shake Speed	1,000 RPM
Analysis		
	Binding Model	SSA— Response Average 50 – 55 s

FcRn

Step Name	Parameter	Suggested Value
	Assay Buffer	1X PBS-T pH 6.0 1X PBS-T pH 7.4
Equilibration		
	Time	300 s
	Shake Speed	1,000 RPM
	Assay Buffer	1X PBS-T pH 6.0
Loading		
	Ligand	FcRn – 0.1 µg/mL in 1X PBS-T pH 6.0
	Time	Threshold Signal Change: 0.2 nm
	Shake Speed	1,000 RPM
Baseline		
	Time	300 s
	Shake Speed	1,000 RPM
	Assay Buffer	1X PBS-T pH 6.0
Association		
	Analyte	0, 4.115 – 3,000 nM
	Time	60 s
	Shake Speed	1,000 RPM
	Assay Buffer	1X PBS-T pH 6.0
Dissociation		
	Time	60 s
	Shake Speed	1,000 RPM
	Assay Buffer	1X PBS-T pH 6.0 (Biological Model) 1X PBS-T pH 7.4 (Kinetic Model)
Analysis		
	Binding Model	SSA— Response Average 50 – 55 s

Response Curves for IgG1, IgG2 and IgG4 Binding to Fcγ Receptors

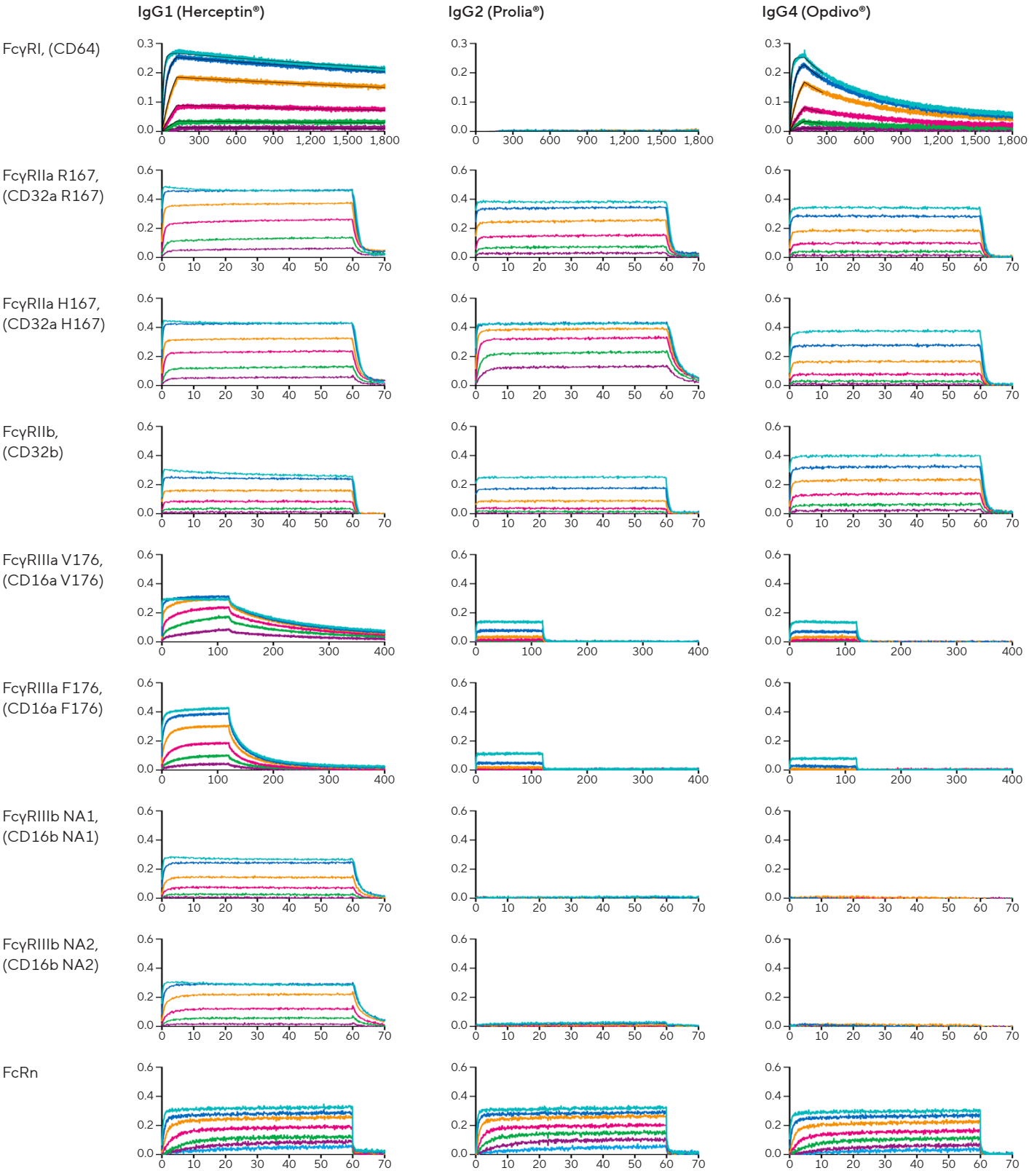

Figure 8: Response Curves Generated Using Assay Parameters and Analyte Concentrations as Shown in Suggested Assay Parameters for Fcγ Receptors. Binding [nm] is represented by the Y-axis and Time [s] is shown by the X-axis.

Steady State Fits for IgG1, IgG2 and IgG4 Binding to Fcγ Receptors

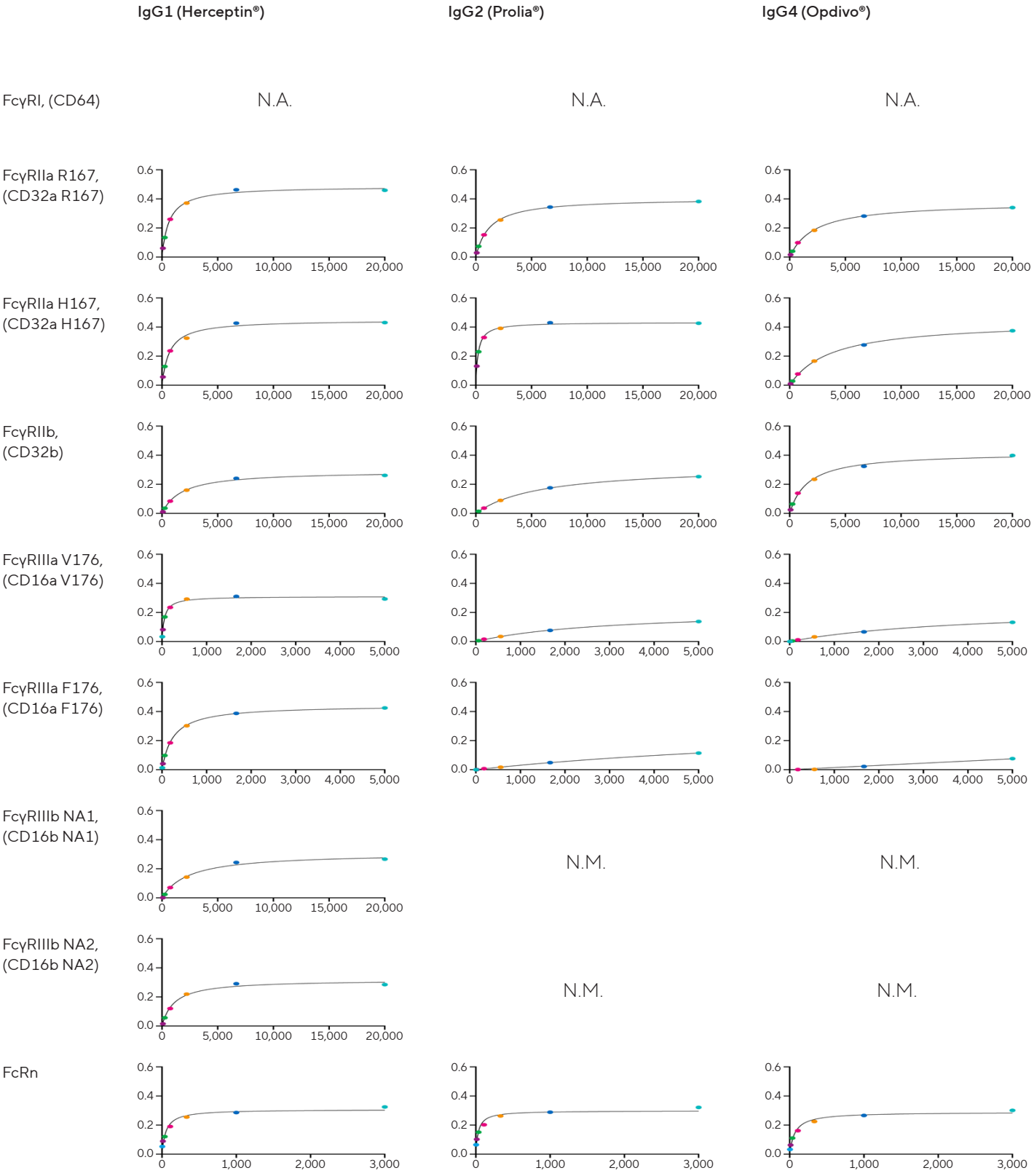

Figure 9: Steady State Affinity Fits Generated Using Assay Parameters and Analyte Concentrations as Shown in Suggested Assay Parameters for Fcγ Receptors. Binding [nm] is represented by the Y-axis and time [s] is shown by the X-axis. N.M. (not measurable).

Kinetics and Affinity Data for for IgG1, IgG2 and IgG4 Binding to Fcγ Receptors

Table 2: *Kinetics and Affinity Data Generated Using Assay Parameters and Analyte Concentrations as Shown in Suggested Assay Parameters for Fcγ Receptors.*

	Analyte	Model Used	k_a [$M^{-1}s^{-1}$]	k_d [s^{-1}]	K_D [M]	R_{max}	R^2
FcγRI, (CD64)	IgG1 (Herceptin®)	1:1	1.60E+06	1.28E-04	7.99E-11	0.2634	0.9983
	IgG2 (Prolia®)	1:1	N.M.	N.M.	N.M.	N.M.	N.M.
	IgG4 (Opdivo®)	1:1	1.77E+06	1.55E-03	8.73E-10	0.2495	0.9974
FcγRIIa R167, (CD32a R167)	IgG1 (Herceptin®)	SSA	N.A.	N.A.	6.41E-07	0.4865	0.996
	IgG2 (Prolia®)	SSA	N.A.	N.A.	1.29E-06	0.4044	0.9989
	IgG4 (Opdivo®)	SSA	N.A.	N.A.	2.18E-07	0.4297	0.9952
FcγRIIa H167, (CD32a H167)	IgG1 (Herceptin®)	SSA	N.A.	N.A.	6.69E-07	0.4489	0.9922
	IgG2 (Prolia®)	SSA	N.A.	N.A.	3.12E-07	0.5036	0.9829
	IgG4 (Opdivo®)	SSA	N.A.	N.A.	3.73E-06	0.4415	0.9994
FcγRIIb, (CD32b)	IgG1 (Herceptin®)	SSA	N.A.	N.A.	1.76E-06	0.2912	0.9969
	IgG2 (Prolia®)	SSA	N.A.	N.A.	5.86E-06	0.3274	0.9992
	IgG4 (Opdivo®)	SSA	N.A.	N.A.	1.63E-06	0.4185	0.9954
FcγRIIIa V176, (CD16a V176)	IgG1 (Herceptin®)	SSA	N.A.	N.A.	5.42E-08	0.3103	0.994
	IgG2 (Prolia®)	SSA	N.A.	N.A.	2.80E-07	0.2203	0.9985
	IgG4 (Opdivo®)	SSA	N.A.	N.A.	4.07E-06	0.239	0.9982
FcγRIIIa F176, (CD16a F176)	IgG1 (Herceptin®)	SSA	N.A.	N.A.	2.48E-07	0.4434	0.9989
	IgG2 (Prolia®)	SSA	N.A.	N.A.	1.46E-06	0.3552	0.9992
	IgG4 (Opdivo®)	SSA	N.A.	N.A.	N.M.	N.M.	N.M.
FcγRIIIb NA1, (CD16b NA1)	IgG1 (Herceptin®)	SSA	N.A.	N.A.	2.42E-06	0.3089	0.992
	IgG2 (Prolia®)	SSA	N.A.	N.A.	N.M.	N.M.	N.M.
	IgG4 (Opdivo®)	SSA	N.A.	N.A.	N.M.	N.M.	N.M.
FcγRIIIb NA2, (CD16b NA2)	IgG1 (Herceptin®)	SSA	N.A.	N.A.	1.09E-06	0.3174	0.9892
	IgG2 (Prolia®)	SSA	N.A.	N.A.	N.M.	N.M.	N.M.
	IgG4 (Opdivo®)	SSA	N.A.	N.A.	N.M.	N.M.	N.M.
FcRn	IgG1 (Herceptin®)	SSA	N.A.	N.A.	5.26E-08	0.3077	0.9564
	IgG2 (Prolia®)	SSA	N.A.	N.A.	3.30E-08	0.2989	0.9464
	IgG4 (Opdivo®)	SSA	N.A.	N.A.	6.97E-08	0.2891	0.975

Note. All affinity values were in good agreement with published data.[2] N.A. (not applicable), N.M. (not measurable), SSA (steady state analysis).

Conclusions

Fcγ receptors provide a gateway for controlling both the innate and adaptive immune systems. As a result, there have been extensive efforts to engineer and tune the Fc domain of IgG monoclonal antibodies to either augment effector function for targeted therapies or attenuate them for checkpoint blockade therapies.

This application note highlights the pivotal role of Fcγ receptor binding in immunology and demonstrates how these interactions can be studied using the Octet® BLI system. Accurate assessment of the interactions between various IgG subclasses and Fcγ receptors is critical for researchers. To support this, the application note provides essential insights into assay design and development. These insights are presented as either complete assays or as key initial parameters for developing assays tailored to specific IgG-based therapeutics.

By following the suggested assay parameters, researchers can gain a deeper understanding of these interactions, and use this application note as a valuable resource enhancing their studies of antibody-receptor binding. Such research is pivotal for further exploration and advancement of this critical area of immunology.

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