

Fcγ Receptor assays using Gator HIS XT biosensors

ABSTRACT

Fcγ receptors (FcγRs) mediate antibody effector functions, linking the antibody–target interaction to cellular cytotoxicity, phagocytosis, and inflammatory signaling. Engineering the IgG Fc region to modulate FcγR binding enables antibody engineers to make strategic decisions about whether their biomolecules enhance effector function, only function for certain receptors, or prevent effector activity entirely. Characterizing the interactions between IgG and the activating receptors CD64/FcγRI, CD32/FcγRII, and CD16/FcγRIII is therefore a routine and high-priority measurement across both discovery and development pipelines.

Biolayer interferometry (BLI) is an ideal method for FcγR interaction screening because it offers real-time, label-free kinetic measurements with the throughput and sample efficiency required for iterative Fc engineering campaigns. However, FcγR assays present unique technical challenges that must be carefully addressed. Here we present optimized protocols for measuring IgG–FcγR kinetics on the Gator® platform for FcγRIIA, which can be applied to other Fc receptors as well. We highlight Gator Bio's HIS XT biosensor as a preferred capture surface, as its improved sensitivity enables users to load far less onto the sensor surface, and the Fc-free capture molecule eliminates a key source of assay interference. We also provide practical setup guidance for users new to FcγR kinetics, addressing common pitfalls encountered when working with these receptors for the first time.

INTRODUCTION

What are Fc Receptors? Why are they commonly screened?

Fc gamma receptors (FcγRs) are a family of cell-surface proteins expressed on a variety of lymphoid, myeloid, and non-immune cells that respond to immunoglobulin G (IgG), in order to drive appropriate responses to infections while being transient enough to avoid autoimmunity. The receptor family encompasses three major classes: the high-affinity FcγRI (CD64) and the low-affinity FcγRII (CD32) and FcγRIII (CD16), each with distinct cellular distributions and functions including phagocytosis, release of inflammatory mediators, or antibody-dependent cellular cytotoxicity.

Beyond their roles in basic immunology, FcγRs are of critical importance in the development and optimization of therapeutic antibodies. Fc-mediated effector functions are essential for the therapeutic efficacy of many monoclonal antibodies (mAbs), while avoiding Fc receptor activation is essential for other biologics. Accurately characterizing these interactions across multiple receptor subtypes, IgG isotypes, and clinically relevant polymorphisms is therefore essential for researchers developing biosimilars, discovering therapeutics, studying immune-mediated disease, or engineering next-generation antibody formats.

Bio-Layer Interferometry (BLI) for Fc Receptor binding characterization

Bio-layer interferometry (BLI) is an optical, label-free biosensor method for real-time kinetic analyses and analyte quantitation high throughput. In a BLI experiment, the antibody or receptor of interest is immobilized on the tip of a pre-coated fiber-optic biosensor. When transferred to a well containing the binding partner of interest, shifts in the interference pattern of reflected white light are measured in real time, yielding the association rate (k_{on}), dissociation rate (k_{off}), and equilibrium dissociation constant (K_D) directly from a single experiment. Critically, BLI requires no fluorescent or radioactive labeling, allowing researchers to study interactions in their native state.

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For Fc receptor binding characterization, BLI has emerged as a standard analytical tool [1, 2]. The United States Pharmacopeia has released general chapter <1108> (Assays to Evaluate Fc-Mediated Effector Function), which cites the use of bio-layer interferometry in key ligand binding applications, noting its ability to provide precise kinetic data and measure a relatively wide range of binding affinities. BLI-based Fc γ R binding assays have been shown to overcome interference from sample aggregates (generally, up to 5% [1]), distinguish different Fc Receptor polymorphisms [3, 4], and serve as stability-indicating methods suitable for drug product release and stability testing.

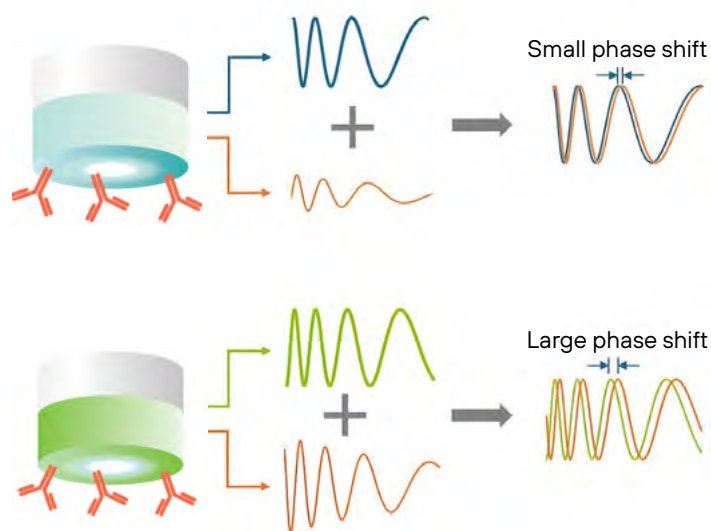


Figure 1: Schematic of standard (top) and XT (bottom) biosensors and their resulting signal intensity. Gator XT Biosensors capture more light from the protein layer, resulting in a greater overall interference. This translates to a larger nm shift in the raw data, providing 3x or greater signal-per-molecule on the surface of the biosensor.

HIS XT as the ideal capture surface for Fc Receptor interaction study

Much of the publicly available BLI and SPR data online show sharply angled, distorted curves for the low-affinity Fc receptors Fc γ RII (CD32) and Fc γ RIII (CD16). These artifacts are often caused because an extremely low ligand loading step is required to visualize 1:1 binding kinetics during Fc receptor-antibody interaction, but classical anti-His biosensors use bivalent capture molecules (anti-His antibodies from rabbit or mouse), and lack sensitivity to visualize a low loading step. Moreover, clear

protocols for performing these experiments are uncommon and often include poorly optimized conditions.

In this application note, we will provide guidelines and give examples of Gator BLI data featuring Fc γ RIIA/CD32a (R167) performed in a manner that avoids these signal artifacts. In particular, this application note will use the new Gator HIS XT biosensor and take advantage of its two major improvements. The first major improvement is the use of an AI-derived capture molecule called a NovoBody™. This single-domain capture molecule, generated in partnership with Monod Bio, has high affinity and minimizes potential avidity and rebinding effects. Its XT surface captures more light reflected from the protein layer, increasing total signal per molecule. These two advantages make the Gator HIS XT biosensor the ideal choice for Fc receptor studies using BLI.

MATERIALS AND METHODS

Reagents and equipment

Item	Origin	Specification / Part Number
Gator® Pivot or Plus+	Gator Bio	PN 110055
BLI 384-Tilt Plate, Polypropylene	Gator Bio	PN 130161
Anti-His (HIS) XT Probes	Gator Bio	PN 160050
K Buffer	Gator Bio	PN 120011
Regen buffer (No salt, pH 2.0)	Gator Bio	PN 120008
Antibody	User supplied	-
Fc γ RIIA/CD32a [R167]	ACROBiosystems	CD0-M36A2 (see appendix 1)

BLI Fc γ Receptor binding assay workflow

The Fc γ R workflow is structured in a five-step process immobilizing a his-tagged Fc gamma receptor (in this case, Fc γ RIIA/CD32a [R167]), followed by antibody association as explained below. Immobilizing the Fc γ R is the ideal choice for this method because researchers typically have an excess of antibody on hand but only limited amounts of Fc receptor, and cannot afford to use micromolar amounts of Fc receptor as analyte.

The HIS XT biosensor was selected for all experiments for reasons enumerated in the previous section.

Unless otherwise noted, all steps were performed on a Gator® Pivot instrument at 30°C, 400 RPM shaker speed at 10 Hz data acquisition rate. The reduction in shaker speed improves the quality of the signal during the beginning of both the association and dissociation phases by slowing the total collision rate, similar to how adjusting flow rate affects mass transport in SPR. Some experiments below were also performed on a Gator Plus+, which has improved temperature control, enabling experiments at 10°C. Remarkably, this change does not introduce mass transport limitation at the tested flow rate regardless of assay temperature (Figure 6). For a visual overview of the full experiment, consult Figure 5.

Step 1 – Biosensor equilibration and baseline

1. Pre-wet HIS XT Biosensors in K Buffer for 600 seconds
2. Instrument transfers biosensors to K Buffer “buffer” well
3. Baseline time: 120 seconds

Step 2 – FcγR immobilization

- Instrument transfers biosensors into FcγR well
- Association time: 20 seconds (adjust depending on expected titer)
- FcγR recommended concentration: 0.3 µg/mL

Step 3 – Second baseline

- Instrument transfers IgG-loaded biosensors to K buffer “blank” well
- Second Baseline time: 60 seconds

Step 4 – Antibody association

- Instrument transfers IgG-loaded biosensors to a series of wells containing the antibody of interest beginning at 2.5 µM in a twofold serial dilution in K buffer
- Association time: 60-120 seconds

Step 5 – Antibody dissociation

- Instrument transfers biosensors to well containing only K Buffer to observe antibody dissociation.
- Dissociation time: 60-120 seconds

Biosensor regeneration

- Instrument transfers biosensors between Regen Buffer (no salt, pH 2.0) and K buffer 3-5 times in 5-second pulses
- HIS XT Biosensors can typically be reused for 10 or more cycles with <15% signal loss (see Appendix 2)

Note: Optimization of regeneration conditions should be performed when working with unusual supernatant conditions, Fc receptor variants, or antibody constructs.

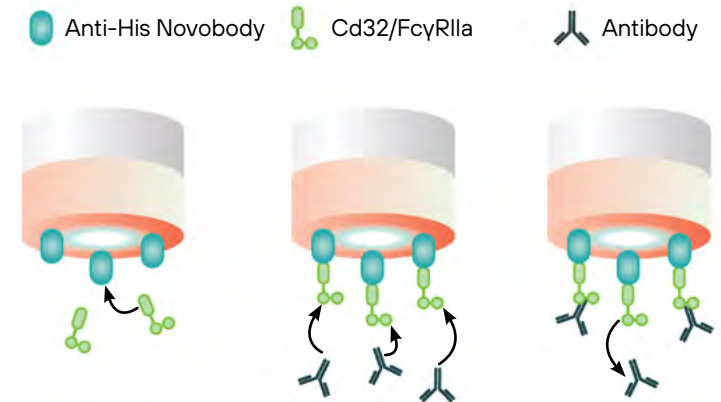


Figure 2: schematic representation of probe surface at each step of Fc receptor kinetics experiment.

Calculating interaction affinity

Unless otherwise noted, assays were analyzed using the following parameters in the GatorOne analysis software:

- Align to association, average 0
- Inter-step correction: On
- Averaging range set to 0 for both association and dissociation
- Single reference subtraction using loaded biosensor interacting with buffer blank
- Note: double-reference subtraction may be required when using a low-quality antibody sample – see appendix 1 for details on how we determined the ideal antibody for use in these example assays, along with advice for reducing NSB using His-Ubiquitin should NSB persist.
- Fitted using a 1:1 Langmuir fitting model.

RESULTS AND DISCUSSION

Fc Receptor do not bind to HIS XT Biosensors as analytes

A key question is whether FcγRs bind non-specifically to the HIS XT biosensor – if they do, any dissociation from the biosensor surface must be allowed to complete in a second baseline step before introduction of antibody analytes. Figure 3 shows that non-his-tagged FcγRIIA/CD32a (R167) does not interact with the sensor surface in any detectable way at concentrations up to 10 µg/mL, the highest concentration tested. This confirms that the single-domain NovoBody™ design on the surface of the biosensor does not contain an Fc or similar off-target binding site.

Identifying appropriate Fc receptor load on HIS XT

Figure 4 shows a loading experiment with titrated 6xHis-tagged Human FcγRIIA/CD32a (R167) ligand loading step, showcasing how at higher ligand loads, both association and dissociation rates are altered due to mass transport limitation, and the dissociation trace does not approach zero as a result of rebinding. High-load samples (red, yellow, green) take 30 seconds or more to reach complete analyte association; low-load samples (purple, magenta) reach the same endpoint in under 10 seconds. During dissociation on high-load samples, dissociation does

not approach zero. This is caused by a rebinding effect, where antibody is unable to adequately dissociate from the sensor surface because the probability to encounter and bind to another CD32 is higher.

In contrast, a load height of 0.5 nm (from purple trace, using 0.3125 µg/mL FcγRIIA/CD32a (R167) or ~10 nM) resulted in binding curves that produced a 1:1 interaction and which dissociate to zero. This represents loading to approximately 8% of maximum load height on HIS XT biosensors, which have a maximum capacity around 6.5 nm shift for this particular CD32 variant, whereas typical anti-His sensors have 2 nm or less. This increase in nm shift capacity is not due to an increase in total number of binding sites, but is instead an optical change from the XT optical improvements, which captures more of the light reflection from the protein layer, resulting in greater interference and measurable nm shift. In practice, this change results in a ~3x increase in total overall signal for the same protein density on the sensor surface – a 0.5 nm shift on a HIS XT biosensor has approximately the same density of captured molecules as a traditional anti-His biosensor loaded to between 0.125 and 0.167 nm shift. With less overall protein loaded onto the HIS XT surface, mass transport and rebinding are less problematic, but sensitivity remains clear.

Further experiments in this application note use this loading concentration (0.3 µg/mL, 20 seconds load, 400 RPM shaker speed) and also use the GatorOne software feature to set a loading threshold of 0.5 nm, to prevent human error.

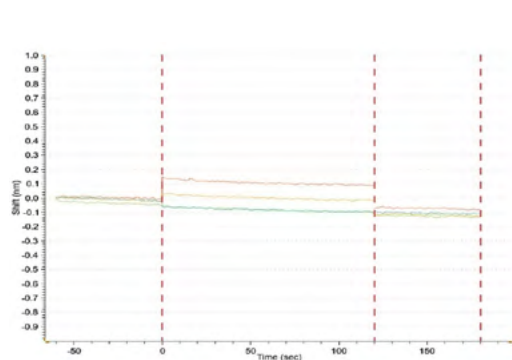


Figure 3: TST-tagged (non-his tagged) Human FcγRIIA/CD32a (R167) at 10 µg/mL (red), 5 µg/mL (yellow), and 2.5 µg/mL (green) against HIS XT biosensors.

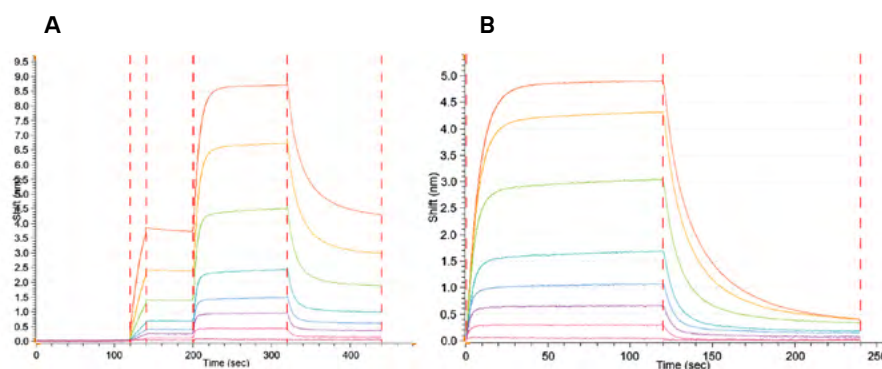


Figure 4: Effect of load height on Antibody binding to FcγRIIA/CD32a (R167). A: Full assay overview, with loading heights ranging from 0.2-3.5 nm shift, each interacting with Rituximab at 625 nM as analyte. B: magnification of association and dissociation steps all setting beginning of association step to 0.

FcγRIIA-Antibody kinetics show a 1:1 binding curve

After loading height normalization to 0.5 nm shift, antibodies were titrated from 2500-125 nM to ascertain the interaction affinity constant, K_D . As expected, some minor non-specific binding of antibody to the biosensor surface was observed representing less than 10% of the total overall response. This is not just specific to the HIS XT biosensor – a change in refractive index due to high Ab concentration as well as antibody NSB to the surface at concentrations greater than 1000 nM are both possible [4]. To mitigate this issue, we elected to use double-reference subtraction with an unloaded biosensor as reference. Example of these traces is contained in Figure 5. Kinetics results agree with established literature, which places the K_D for the CD32-IgG1 between 0.5 and 2.5 μM . Moreover, the curves show well-resolved association kinetics, approaching saturation rather than plateauing immediately, giving greater context to on- and off-rate fitting.

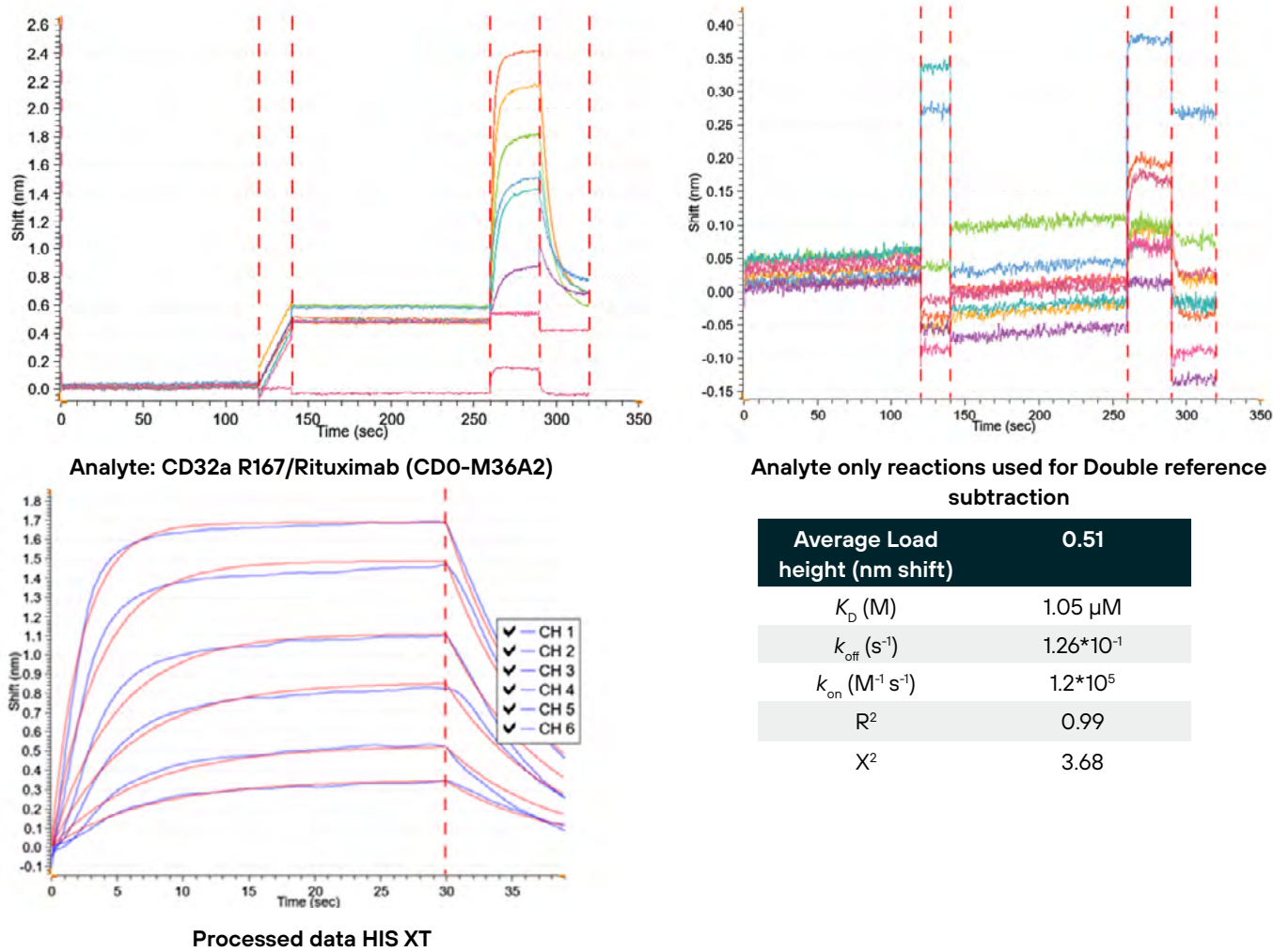
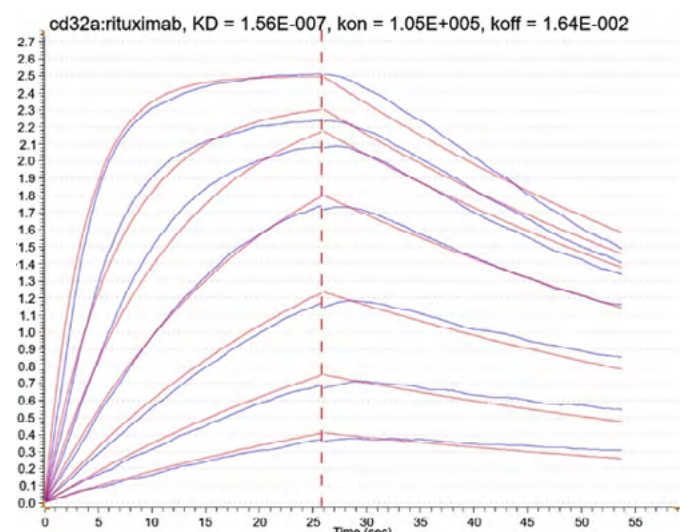


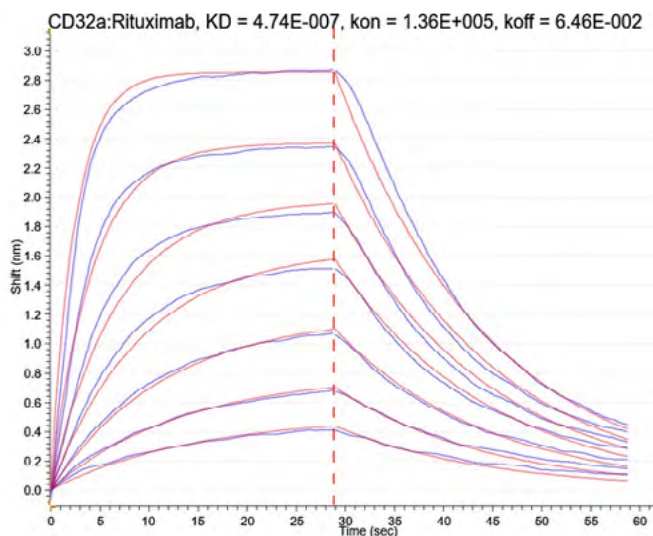
Figure 5: Rituximab (analyte) interacting with his-Human FcγRIIA/CD32a (R167) (ligand) on HIS XT biosensors. Biosensors were loaded to ~0.5 nm shift, after which these results were collected. Results are aligned to beginning of association step and double-reference subtracted. Kinetics results are shown in the table and align with experimental data on supplier’s website for the interaction (1.4 μM on competitor BLI; 2.3 μM on SPR).

Temperature-dependent kinetics of FcγRIIA/CD32a are consistent with established literature

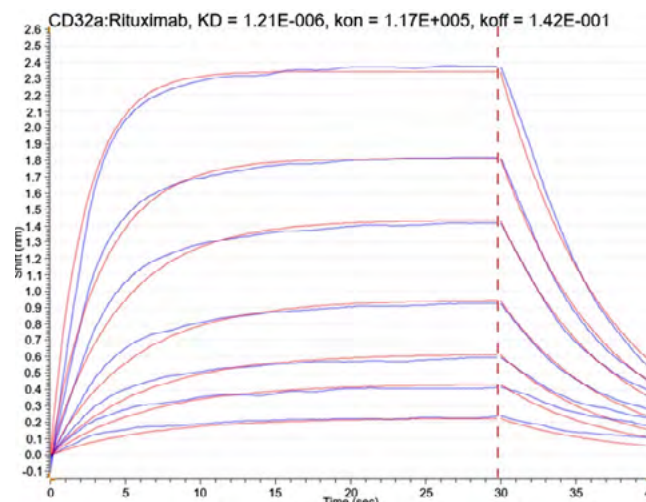
Assays performed across different temperatures showed small deviations in overall K_D as a result of assay temperature which align with previously-established kinetics values measured by SPR [5]. Importantly, the on-rate remains largely steady among the assay temperatures tested, while k_{off} is dramatically affected by temperature. From a practical assay design perspective, this means that Gator BLI using HIS XT provides kinetics results that align with other techniques regardless of temperature, allowing users flexibility in choosing assay temperatures depending on experimental needs. Often, lower temperatures are used to slow the binding interaction for accurate curve fitting, and these data support that changing temperature does not introduce any unusual variation to the kinetics, with R2 values consistently above 0.99 (see Figure 6).



Assay run at 10°C



Assay run at 25°C



Assay run at 40°C

	10°C	25°C	40°C
K_D (M)	1.56×10^{-7}	4.74×10^{-7}	1.21×10^{-6}
k_{on} ($M^{-1}s^{-1}$)	1.05×10^5	1.36×10^5	1.17×10^5
k_{off} (s^{-1})	1.64×10^{-2}	6.46×10^{-2}	1.42×10^{-1}
R^2	0.9925	0.9956	0.9966

Kinetics report table

Figure 6: Fc receptor assays run at different temperatures on the Gator HIS XT biosensor at 0.5 nm shift and 400 RPM shaker speed. Even at 40°C, the assay provides a clean 1:1 fitting, highlighting how HIS XT improvements to sensitivity and monovalent capture provide dramatically improved data quality when following the protocol outlined above.

CONCLUSION

The data presented here demonstrate that the Gator HIS XT biosensor provides a robust and reliable platform for characterizing IgG–FcγR interactions using BLI. By combining an XT optical surface with a single-domain capture molecule, the HIS XT biosensor enables low-density receptor loading that produces clean 1:1 binding kinetics for low-affinity receptors such as FcγRIIA/CD32a – an outcome that has historically been difficult to achieve with conventional anti-His biosensors. The optimized protocol presented here, including loading normalization to 0.5 nm and double-reference subtraction to address non-specific antibody binding at high concentrations, consistently yields well-resolved association and dissociation curves with R^2 values above 0.99.

Temperature-dependent kinetic measurements further confirmed that the HIS XT platform produces results consistent with established SPR literature values, with k_{off} responding predictably to temperature while k_{on} remains stable, giving users flexibility to adjust assay conditions without compromising data quality. These characteristics position the Gator HIS XT biosensor as a practical path to compliance with USP general chapter <1108> (Assays to Evaluate Fc-Mediated Effector Function), which cites BLI as a reference method for Fc receptor binding characterization. The protocols and best practices outlined here are directly applicable to FcγRIIIa/CD16a assay development, where the same low-affinity binding challenges and sensitivity requirements apply. The HIS XT biosensors are also re-usable (See Appendix 2), making them the ideal choice for high throughput screening for iterative Fc engineering workflows.

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APPENDIX 1: TROUBLESHOOTING FC RECEPTOR ASSAYS

Ensure the quality of your antibody reagent

The amount of non-specific binding (NSB) observed on the surface of the sensor is highly dependent on the quality of antibody samples that are used. While preparing this application note, we ordered four different Rituximab samples for use in the experiments. On blank, unloaded HIS XT biosensors, we observed that NSB intensity varied greatly among them. For the purposes of these experiments, ACRO Biosystems CD0-M36A2 (Premium Grade) was found to give the most consistent results, as well as produce the least NSB. Figure 7 shows the NSB from each of the antibodies in the table below at 1 μM, and from the ACRO Biosystems CD0-M36A2 at 2.5 μM in order to showcase the difference in non-specific binding from each sample.

Antibody origin	Catalog number	Concentration tested (μM)	NSB Response (nm shift) at 2 min
ACROBiosystems	MAB9575	1	0.80
R&D Systems	CD0-M36	1	0.23
Antibodies Online	Not numbered	1	0.33
ACROBiosystems	CD0-M36A2 (Premium Grade)	2.5	0.16



Reducing NSB using His-Ubiquitin

When antibodies of high quality are unavailable, a blocking strategy that prevents their non-specific binding (NSB) to the biosensor may be required. Even when antibodies without his-tag are exposed to the biosensor, typical strategies like BSA or detergents only partially reduce antibody NSB. There is likely a residual weak interaction between the capture molecule and the antibodies themselves at concentrations above 1 μM .

To reduce total NSB to the HIS XT sensors, His-Ubiquitin was tested and shown to have a dramatic impact (Figure 7). To incorporate this blocking into the protocol, simply add a step after "Step 2 – Loading" to cover the remaining HIS XT capture molecule positions with his-ubiquitin. Figure 7 further shows that blocking performed at 0.5 $\mu\text{g}/\text{mL}$ of ubiquitin dramatically reduces NSB. Note that with traditional Ni-NTA or anti-his biosensors, this strategy does not work because the his-tagged ubiquitin will displace captured Fc receptor, while HIS XT with its slow dissociation rate does not experience this difficulty.

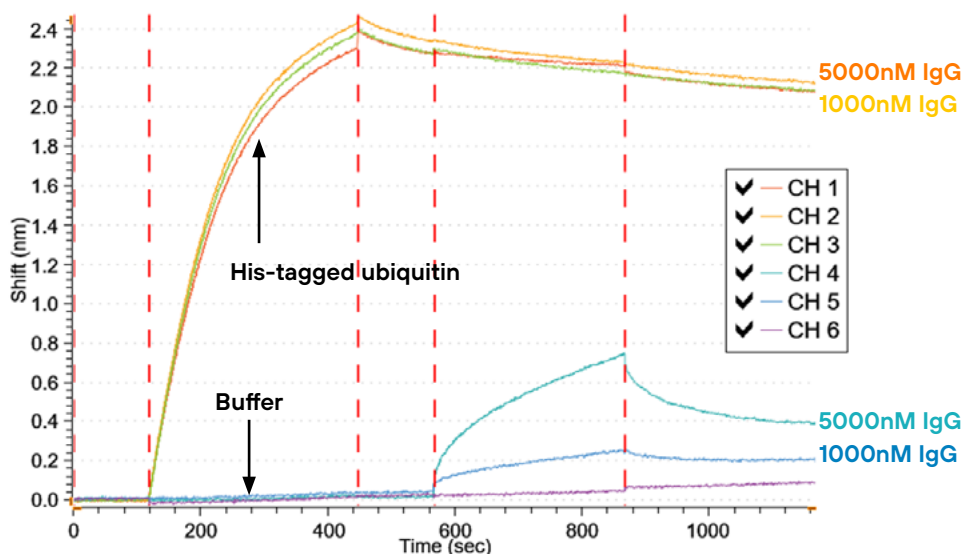


Figure 7: NSB blocking using His-Ubiquitin for less-ideal antibody samples at 5 μM concentrations. Blocking with his-ubiquitin not only blocks the his-tag binding site on the anti-His NovoBody but also blocks sites on the glass or other probe surfaces that may interact with antibody non-specifically.

Appendix 2: Reproducibility after regeneration on HIS XT

Robustness of the regenerable surface and the ability to completely release captured Fc receptor ligand are equally important. To confirm that Fc receptor is completely removed after loading, we performed an Fc receptor binding experiment ten times with the same biosensor, then skipped the Fc receptor load step to directly expose the sensor to the antibody. The premise is that if the antibody bound, it would indicate that the Fc receptor had not been completely removed from the surface, most likely due to incomplete regeneration.

Our result (Figure 8) indicates that the HIS XT biosensor completely releases bound Fc receptor from the sensor surface using the standard Regen Buffer (No Salt, pH 2.0) as recommended in this protocol. After these repeated assays, the sensor surface showed near-zero NSB from antibody at 2.5 μM .

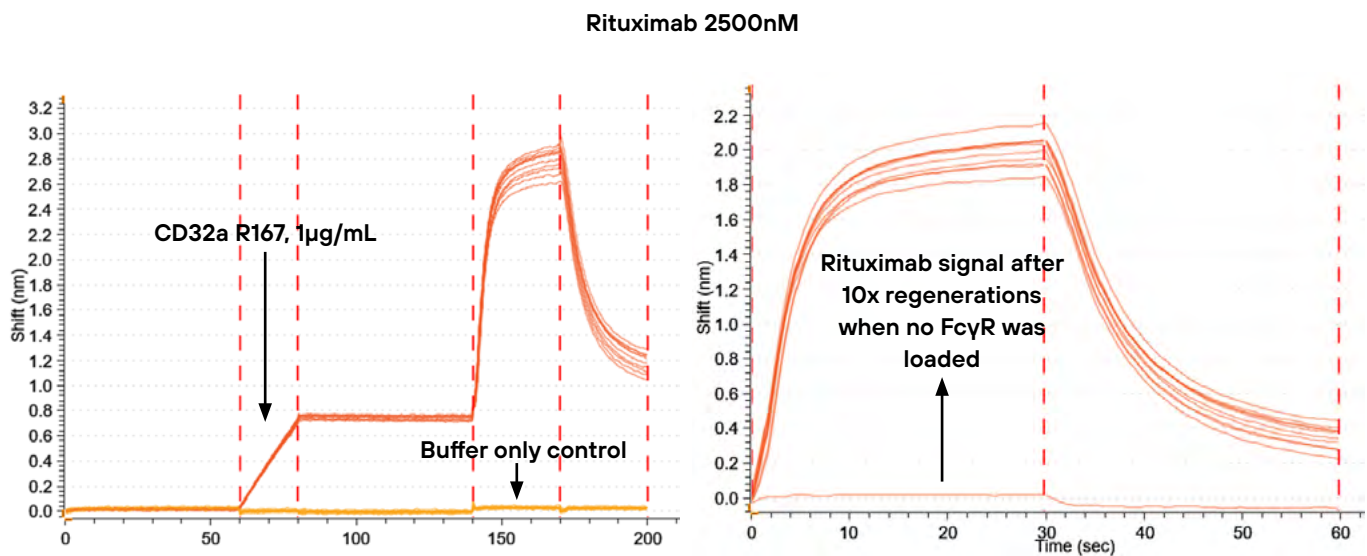


Figure 8: Regeneration efficacy of Fc receptor/antibody interaction study using HIS XT. Top: Raw data from CD32 capture and Rituximab binding across ten studies. Bottom: Rituximab signal after 10x regenerations with no ligand loaded – demonstrating that PN: 120008 Regen Buffer pH 2.0 causes complete removal of bound CD32a even after 10 cycles.

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