Understanding ForteBio's Sensors for High-Throughput Kinetic and Epitope Screening for Purified Antibodies and Yeast Culture Supernatant

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Abstract

Real-time and label-free antibody screening systems are becoming more popular because of the increasing output of purified antibodies and antibody supernatant from many antibody discovery platforms. However, the properties of the biosensor can greatly affect the kinetic and epitope binning results generated by these label-free screening systems. ForteBio human-specific ProA, anti-human IgG quantitation (AHQ), anti-human Fc capture (AHC) sensors, and custom biotinylated-anti-human Fc capture (b-AHFc) sensors were evaluated in terms of loading ability, regeneration, kinetic characterization, and epitope binning with both purified IgG and IgG supernatant. AHC sensors proved unreliable for kinetic or binning assays at times, whereas AHQ sensors showed poor loading and regeneration abilities. ProA sensors worked well with both purified IgG and IgG supernatant. However, the interaction between ProA sensors and the Fab region of the IgG with VH3 germline limited the application of ProA sensors, especially in the epitope binning experiment. In an attempt to generate a biosensor type that would be compatible with a variety of germlines and sample types, we found that the custom b-AHFc sensors appeared to be robust working with both purified IgG and IgG supernatant, with little evidence of sensor-related artifacts.

Introduction

During the early stages of antibody discovery, it is important to have an efficient and accurate way to assess the success or failure of a particular discovery effort. First, binding affinity to the target is a critical criterion for the success of the antibody. Second, epitopic coverage or antagonistic activity (ligand blocking) is also important during the very early stages of discovery, for without targeting the relevant epitope, even the highest affinity antibody may not have the desired activity. For in vitro discovery technologies in particular, these assessments at a very early stage can drastically speed up the discovery process.

Adimab's yeast-based system provides the ability to screen libraries ($>10^{10}$) of fully human, full-length IgGs against a given target. The nature of the platform also allows for expression and purification of the IgG directly from the yeast host. However, as the demand for antibodies and number of druggable targets has increased, so too has the demand for purified IgGs for characterization, presenting a bottleneck at the purification stage. In turn, this slows the characterization-selection feedback loop by delaying characterization and further selections. To circumvent this bottleneck, supernatant production of IgGs in a crude culture was investigated as an alternative to purified IgG. At this stage, crude sample screening would be able to identify binders from nonbinders, to qualitatively evaluate the binding kinetics, and to evaluate the epitopic coverage of the output. Yeast supernatant screening is most advantageous within the early discovery process, before

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identifying antibodies for purification, and before more thorough quantitative analyses.

In contrast to a purified sample, working with crude samples from any host has a number of limitations arising from the relatively low concentrations of antibody and high level of impurities within the samples. Enzyme-linked immunosorbent assay (ELISA), AlphaScreen, MSD, and Luminex systems have all been reported to identify binding of a crude antibody sample to its target, but such systems do not give real-time kinetic information.[1] In addition, binning information can be misrepresented by these systems that report only an endpoint signal. Contrary to plate-based or bead-based endpoint measurements, surface plasmon resonance (SPR), the newer surface plasmon resonance imaging (SPRi) technology, and biolayer interferometry (BLI) are reported to be compatible with crude samples from a variety of hosts for real-time kinetic assays.[2–4] However, with crude samples, potential clogging in flow-based system such as SPR or SPRi instruments is of considerable concern.[5]

ForteBio's Octet RED384 and HTX BLI-based instruments are suitable for supernatant screening in that its non–flow-based platform has no fluidics that can become clogged. The sensors can be loaded with samples offline, allowing for longer capture times of the antibody to the sensor, especially for supernatant with low antibody concentration (<10 μg/mL). Only 50 μL of supernatant is required for online loading and 100 μL for offline loading. In either case, the supernatant can be reused for multiple loading cycles and multiple assays. Also, the non–flow-based system enables the reuse and recovery of antigen or analyte, which is especially appealing when screening large numbers of antibody against the same target.[6] Using a 384-well tilted-bottom plate, as little as 50 μL volume is needed per well, using only 1 to 2 nmol antigen to fill 96 wells, which can be reused to screen several hundred antibodies. In addition, ForteBio's HTX 96-channel mode allows for 96 individual kinetic measurements or 96 independent epitope binning assays during one unattended run.[7]

To save sensor preparation time and for ease of use, companies that provide SPR- and BLI-based platforms offer a range of costly prefunctionalized sensors/chips that can be regenerated. Although information on SPR sensor chips is widely available,[8] there is very little information in the literature on the construction and robustness of the various ForteBio sensors. In developing a high-throughput method for analyzing both purified and crude samples, sensor type drastically affected the accuracy and cost of both kinetic-screening and epitope-binning experiments. Anti-human IgG quantitation (AHQ) sensors and Protein A (ProA) sensors are recommended by ForteBio for quantitation.[9] Anti-human Fc capture (AHC) sensors are recommended for kinetic screening and epitope binning. However, as previously reported by ourselves and others, the construction of the sensors/chips themselves can interfere with the experiment.[10] ProA sensors have significantly higher loading capacity than either AHC or AHQ sensors, making them best suited for sensitive applications such as small antigens and crude materials that have low concentrations of analyte. AHQ sensors showed little capacity for regeneration, and AHC sensors showed significant variability during kinetic and binning assays. ProA is known to interact with the variable region of VH3 IgGs as well as the Fc region, potentially preventing the paratope of the IgG from binding the antigen.[11] In addition, using ProA sensors for binning with a VH3 IgG greatly complicated epitope binning assays (**Suppl. Table S1**). For fast kinetic profiling and binning, a sensor that would provide robust responses for either purified or crude samples after multiple regenerations would be ideal. In an attempt to address these shortcomings, custom biotinylated-anti-human Fc capture (b-AHFc) sensors were generated and are herein demonstrated to be more robust and versatile than currently available commercial sensors.

Materials and Methods

Antibody Production and Purification

Full-length human antibodies (IgG1) were produced using Adimab's proprietary platform. Antibodies were secreted from yeast into the media and were harvested by pelleting the cells at 3000 rpm for 5 min to obtain IgG-containing supernatant. To obtain purified IgG, IgG-containing supernatant was purified using ProA resin.

Generation of b-AHFc Sensors

Goat anti-human Fc antibodies (109-005-088; Jackson ImmunoResearch, West Grove, PA) were biotinylated using EZ-Link NHS-PEG$_{12}$-Biotin (Thermo Scientific, Rockford, IL) at 0.5 mg/mL in DMSO following the manufacturer's recommendations. A molar ratio of 1:15 of goat anti-human Fc antibody to NHS-PEG$_{12}$-Biotin was mixed and incubated for 2 h at 25 °C. After incubation, excess labeling reagent was removed by 40 kDa Zeba spin desalting columns (Thermo Scientific, Rockford, IL). Complete removal of free biotin molecules was checked by size exclusion chromatography (Agilent, Santa Clara, CA). The concentration of the b-AHFc antibody solution was determined by measuring the absorption at 280 nm on a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and prepared in an assay buffer of phosphate-buffered saline (PBS) pH 7.3 with 0.1% bovine serum albumin (PBSF). Then, the b-AHFc antibodies (800 nM) in PBSF were loaded to streptavidin (SA) sensors (18-5021; ForteBio, Menlo Park, CA) for 600 s to achieve a 5 nm response.

Loading and Regeneration Assay

To test the loading and regeneration abilities for ForteBio biosensors, repetitive loading and regeneration assays were

performed on ForteBio Octet HTX (ForteBio) in 96-channel mode. Twenty-four of each sensor type—AHQ (18-5005; ForteBio), ProA (18-5013; ForteBio), AHC (18-5064; ForteBio), and b-AHFc sensors—were run within the same experiment.[12] A stock solution of 24 in-house–produced and –purified IgGs were normalized to 100 nM in PBSF for loading. Glycine (10 mM, pH 1.7) was used for regeneration. Each loading and regeneration cycle consists of three steps: (1) Loading: sensors were dipped into 24 different IgGs at 100 nM for 180 s to capture the IgGs onto the sensors. (2) Regeneration: sensors were alternately dipped into pH 1.7 glycine solution and pH 7 PBSF every 10 s for a total of 60 s to remove the loaded IgGs. (3) Equilibration: sensors were dipped in pH 7 PBSF for 300 s to be equilibrated. A total of 19 loading and 18 regeneration cycles were performed.

Biotinylated Antigen Binding Assay

To examine if the SA sites are exposed on each sensor type, a biotinylated antigen (13 kDa, degree of labeling [biotin:protein] is 1.5) binding assay was performed using a ForteBio Octet HTX. AHQ sensors, ProA sensors, AHC sensors and b-AHFc sensors were tested. Each sensor type was dipped into PBSF for 60 s to establish a baseline and then exposed to biotinylated antigen solution (100 nM) for 180 s to check binding. This was followed by a dissociation step into PBSF for 180 s.

Kinetic Characterization Assay

AHQ, ProA, AHC, b-AHFc, and Amine Reactive 2nd Generation sensor (AR2G, 18-5095; ForteBio) were tested in a qualitative, single-concentration kinetic assay. Assays were performed on a ForteBio Octet HTX. AR2G sensors were activated and quenched in advance. After 180 s online loading of purified antibody 1 (mAb 1, 100 nM), each sensor was soaked in PBSF for 20 min to achieve equilibrium. The kinetic assay was performed with a baseline (60 s) in PBSF, association with 100 nM antigen A (61 kDa) for 300 s, and dissociation into PBSF (300 s). Data analysis and fitting were performed using ForteBio's Data Analysis Software version 8.1. Solution equilibrium kinetic exclusion K_D measurement (MSD-SET) and the kinetic exclusion assay (KinExA) were performed using mAb 1 according to Estep et al.[13] to measure the binding affinity.

Epitope Binning Assay

Unidirectional binning assays were performed in the traditional sandwich format as described by Abdiche et al.[14] A pairwise competition assay (performing the assay with the analyte antibody on the sensor and then the analyte antibody in solution) was not performed because impurities in the supernatant would produce sensor interference. AHQ, ProA, AHC, and custom b-AHFc sensors were tested on a ForteBio Octet HTX. Two different IgGs that bind to antigen B (160 kDa) were evaluated for binning profile versus a reference antibody (mAb 4, VH3-23): antibody 2 (mAb 2) was purified using ProA resin, and antibody 3 (mAb 3) was produced as IgG supernatant. Reference antibody mAb 4 was also purified using ProA resin to avoid sensor interference from the supernatant. Both the purified mAb 2 (100 nM) and unpurified mAb 3 were loaded online to each sensor type for 180 s and 15 min, respectively. After online loading, the sensors were soaked in an irrelevant IgG1 solution (0.5 mg/mL) for 10 min to block remaining Fc binding sites. The sensors were then soaked in PBSF for at least 20 min to achieve equilibrium. Each binning assay consisted of (1) baseline measurements in PBSF for 30 s; (2) a sensor-binding check control step, in which sensors were dipped into mAb 4 (100 nM) for 90 s to check for nonspecific binding of the mAb 4 to the loaded and blocked sensors; (3) a second baseline measurement in PBSF for 60 s; (4) an association phase with antigen B (100 nM) for 180 s; and, lastly, (5) a binning step, in which sensors were exposed to mAb 4 for 90 s. If mAb 2 or 3 on the sensor blocks the interaction between antigen B and the reference antibody mAb 4, no response would be expected during the binning step. All of the data analysis was perform by using ForteBio Data Analysis software version 8.1.

Results and Discussion

Without accurate, reliable kinetic and bin information, it is difficult to inform selection strategies in real time and confirm that affinity and activity goals are being met during the early stages of discovery. To increase speed and reduce strain on purification groups, crude samples such as IgG-containing supernatant could be an alternative for high-throughput screening. As such, developing a method to analyze a large number of diverse crude samples at potentially low concentrations is demanded. Pall's ForteBio Octet HTX has the throughput to collect affinity and epitope binning data for several hundred antibodies per day for both purified and crude samples. To make this screening more reliable, we evaluated prefunctionalized AHQ, ProA, and AHC sensors using both purified and unpurified samples. Meanwhile, we developed our custom b-AHFc sensor, which is preferred when working with diverse crude samples.

Sensor Loading and Regeneration

Regeneration ability is a very important criterion when evaluating biosensors to keep consumable costs low, and loading capacity can be markers of sensor stability and sensitivity. To test the loading and regeneration ability of
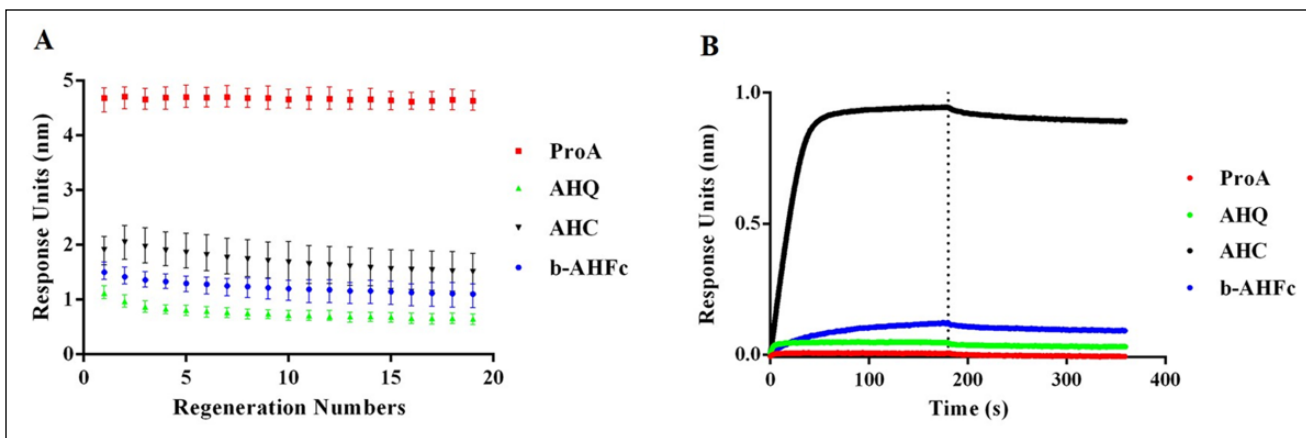
Figure 1. Biosensor regeneration and biotinylated antigen binding assays. (**A**) Average loading response for 19 loading (18 regenerations) cycles. Each data point is the average loading response of 24 IgGs (100 nM) on 24 sensors. Upper error bars represent the highest loading response and lower error bars represent the lowest loading response among 24 sensors in each loading. (**B**) Biotinylated antigen binding assay. Sensors were exposed to 100 nM biotinylated antigen (13 kDa, degree of labeling: 1.5) solution for association (0–180 s) and to PBSF for dissociation (180-360s). Sensorgrams show biotinylated antigen binding to AHC sensor strongly and the dissociation was slow. ProA, AHQ and b-AHFc showing minimal binding.

several prefunctionalized biosensors, we performed 19 loading and 18 regeneration cycles consecutively with AHQ, ProA, AHC, and custom b-AHFc sensors. ProA sensors gave the highest average loading response (~4.68 nm) and did not show an obvious decrease (~1%) after 18 cycles of regeneration. However, the average loading response decreased 42% for AHQ (from 1.12 to 0.65 nm), 21% for AHC (from 1.91 to 1.51 nm), and 26% for b-AHFc (from 1.50 to 1.10 nm) after 18 cycles of regeneration (**Fig. 1A**). Thus ProA sensors have the highest consistent loading response, whereas AHC and b-AHFc sensors are both moderately affected by each regeneration cycle. AHQ's low loading response after regeneration limits the use of the sensors to only one or two assays per sensor to ensure reproducible results.

Biotinylated Antigen Binding Assay

Biotinylation of proteins is a useful tool to label proteins, particularly for flow cytometry experiments and purification techniques. Because biotinylated antigens are commonly used, it is necessary to confirm there is no undesirable interaction between the biotinylated materials and the sensor surface. As shown in **Figure 1B**, AHC sensors show a strong response (~0.95 nm) due to the binding of a biotinylated antigen (13 kDa, degree of labeling: 1.5), which suggests that the sensor's functionalization includes an SA moiety that has not been blocked. Compared with AHC sensors, AHQ and b-AHFc sensors show only marginal binding (0.05 nm and 0.11 nm, respectively) to the biotinylated antigen, whereas ProA sensors show no interaction with the biotinylated antigen. Although minor interactions of antigen with sensors can be subtracted out of kinetic data, it is

significantly more challenging to do so with very strong responses. As a result, AHC sensors would be incompatible with biotin-containing analytes without additional blocking of the sensor.

Kinetic Characterization Assay

Real-time kinetic characterization in a high-throughput fashion is essential for ranking large numbers of antibodies by affinity during the early-stage discovery process. Purified mAb 1 was loaded onto AHQ, ProA, AHC, AR2G, and b-AHFc sensors (loading responses were 1.8 nm, 5.0 nm, 2.7 nm, 1.6 nm, and 1.7 nm, respectively). A monomeric binding assay was then performed on the Octet HTX system with antigen A (61 kDa, monomer). The K_D for mAb 1 for antigen A was 2.2 nM on ProA sensors (**Fig. 2A**) and 0.96 nM on the AHQ sensor (**Fig. 2C**). Covalently bound mAb 1 on AR2G sensors confirmed tight binding of mAb 1 to antigen A ($K_D < 0.6$ nM; **Fig. 2D**). The k_{off} on the AR2G sensor was very slow and could not be determined beyond a theoretical limit of 1E-04 s^{-1}. The K_D measured on b-AHFc sensors correlated well with AHQ and AR2G sensors at 0.67 nM. Contradictory to the nanomolar affinities given by AHQ, ProA, b-AHFc, and AR2G sensors, the K_D given by AHC sensor was 165 nM (**Fig. 2B**). Two solution equilibrium kinetic exclusion K_D measurements (MSD-SET and KinExA) provided <18 pM affinity measurements, in apparent agreement with all sensors other than AHC. When comparing the profiles generated by AR2G and ProA sensors, it is clear that the affinity of mAb 1 to antigen A exceeds the affinity between ProA on the sensors to the Fc and that the dissociation observed is primarily due to the Fc dissociating from the sensor. The discrepancy between the
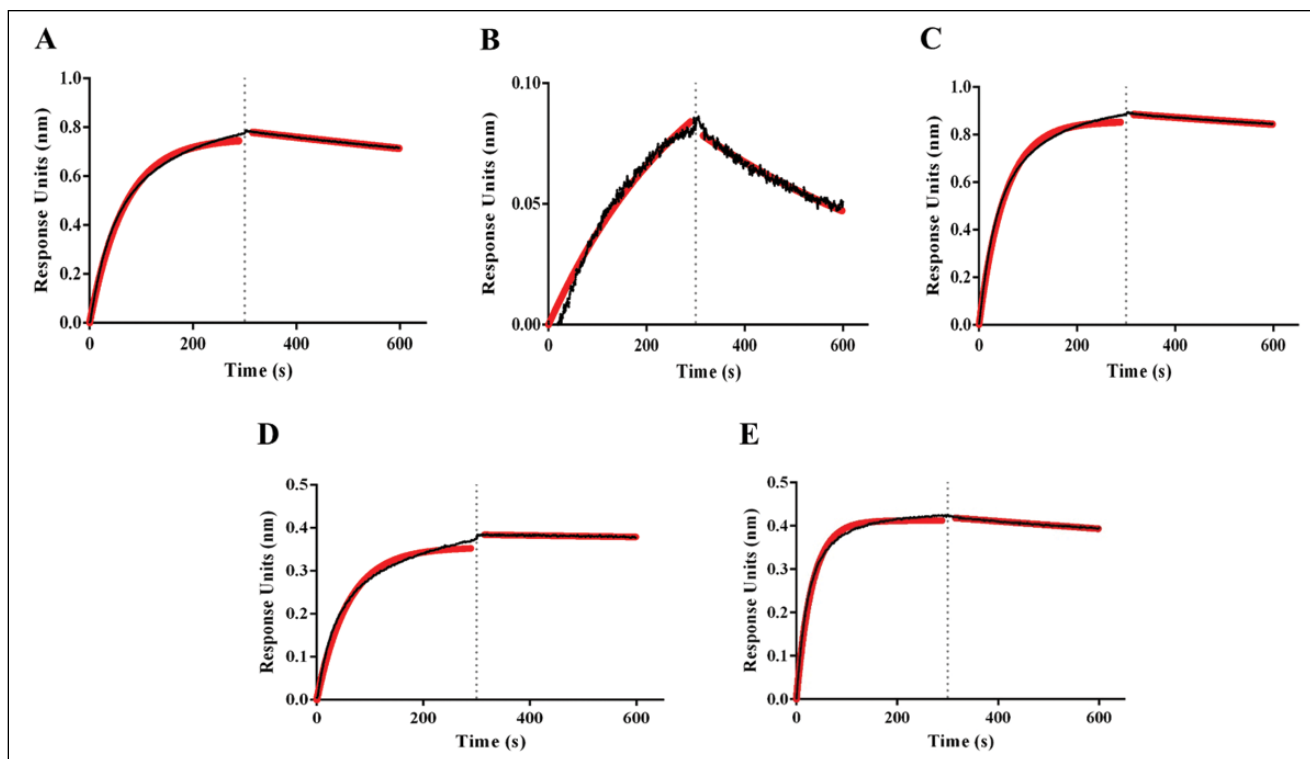
Figure 2. ForteBio kinetic characterization assay. Purified antibody mAb 1 was loaded to the sensors previously (sensorgram not shown). Sensors than were exposed to 100 nM antigen A (61 kDa) solution for association (0–300 s) and to PBSF for dissociation (300–600 s). Association and dissociation steps are divided by the dotted line. Sensorgrams are in black. Fitting curves used for affinity calculation are in red. (**A**) ProA sensor (K_D = 2.2E-9 M). (**B**) AHC sensor (K_D = 1.65E-7 M). (**C**) AHQ sensor (K_D = 9.6E-10 M). (**D**) AR2G sensor (K_D = 5.9E-10 M). (**E**) b-AHFc sensor (K_D = 6.7E-10 M). Response units are not normalized.

K_D values given by the ForteBio Octet and solution-based assays is partially due to the mass transport effects and lack of sensitivity inherent in BLI technology.[13]

Epitope Binning Assay

Early identification of a library's breadth of epitopic diversity and individual antibodies' epitope bin is crucial to antibody selection efforts. Adimab's yeast-based discovery platform is capable of discovering four to five bins per antigen on average, requiring many rounds of binning per panel of antibodies. We investigated the best application of sensors for binning in the sandwich format using both purified antibody (mAb 2) and antibody from supernatant (mAb 3). As can be seen in **Figure 3A** (step 2), ProA sensors registered a very large response during the sensor binding check step, resulting from the interaction of mAb 4 (VH3-23 antibody) and the blocked ProA sensor. As a result, the response observed in the binning step (**Fig. 3A**, step 5) is inconclusive. A control experiment with a VH4 germline antibody as the reference antibody did not show any binding during the sensor binding check step for all the sensor types (data not shown). When performing the same experiment on AHC

sensors, the signal response was low (~0.2 nm) for the antigen association step (**Fig. 3B**, step 4), and there was no apparent binding of mAb 4 to antigen B (**Fig. 3B**, step 5), leading to the conclusion that mAb 4 is a competitor with respect to mAb 2. Finally, to reconcile these different results, AHQ and b-AHFc sensors were used to perform the experiment. There is minimal response during the sensor binding check step and a large response during the binning step. The sensors showed identical responses during each step, and **Figure 3C and 3D** clearly show that that mAb 4 is a noncompetitor to mAb 2.

Because low concentrations of sample in supernatant limits the amount of antibody that can be loaded to sensors, an evaluation of prefunctionalized sensors in terms of binning with IgG supernatant was performed. To avoid interference from media components, IgG supernatant mAb 3 was loaded to sensors and then the binning assay was performed as described above. For supernatant mAb 3, the loading responses after 15 min were 2.72 nm (ProA), 1.65 nm (AHC), 0.97 nm (AHQ), and 1.28 nm (b-AHFc; data not shown). ProA sensors have a strong signal response (~0.8 nm) in the sensor binding check step (**Fig. 4A**, step 2) due to the mAb 4 variable region interaction with the ProA
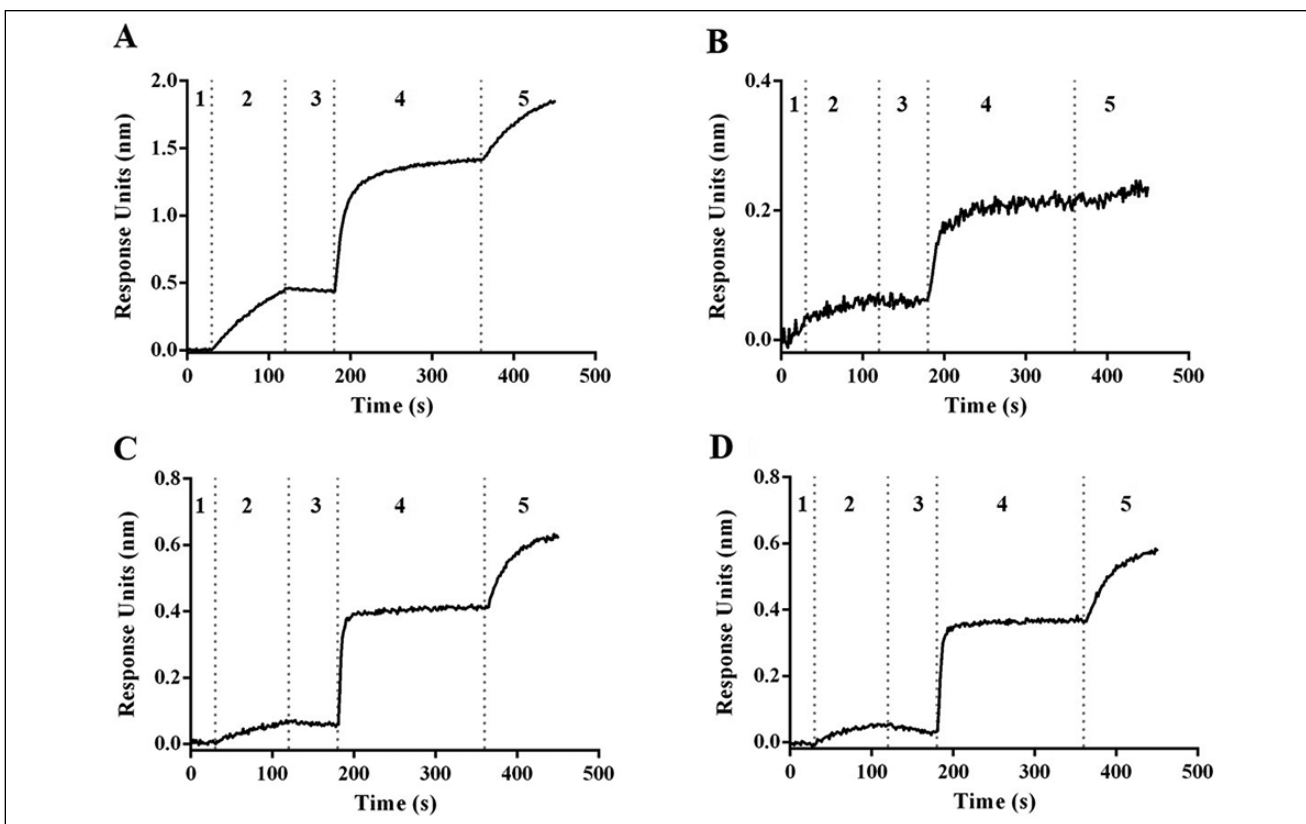
Figure 3. ForteBio epitope binning assays using purified antibody 2 (mAb 2). Dotted lines and numbers represent different steps in the assay: baseline (step 1, 0–30 s), mAb 4 (reference antibody, VH3) sensor binding check (step 2, 30–120 s), second baseline (step 3, 120–180 s), 100 nM antigen B (160 kDa) association (step 4, 180–360 s), and mAb 4 binning (step 5, 360–450 s). (**A**) ProA sensor. Unable to determine competitor or noncompetitor because of the strong sensor binding of mAb 4 in step 2. (**B**) AHC sensor. False mAb 4 competitor profile due to weak antigen binding in step 4. (**C**) AHQ sensor. mAb 4 noncompetitor profile. (**D**) b-AHFc sensor. mAb 4 noncompetitor profile.

sensor. Very low responses (<0.05 nm) during the sensor-binding check step with mAb 4 were observed for AHQ (**Fig. 4C**, step 2) and b-AHFc (**Fig. 4D**, step 2) sensors in this step. For all sensors, strong response increases were observed in the binning step, 0.60 nm for ProA (**Fig. 4A**, step 5), 0.40 nm for AHQ (**Fig. 4C**, step 5), 0.39 nm for AHC (**Fig. 4B**, step 5), and 0.45 nm for b-AHFc (**Fig. 4D**, step 5).

Whether with supernatant or purified IgG, throughout this study, we found ProA sensor to be the most robust in terms of regeneration and response. AHC sensors appeared to be fairly robust in terms of regeneration but were unpredictable for kinetics and binning assays. The significant impact AHC sensors have on the response, k_{on}, and k_{off} of mAb 1:antigen A interaction (**Fig. 2B**) is misleading, considering the consensus between other sensors types. AHC's sensor-related artifact may have also affected the binning assay with mAb 2 and antigen B, resulting in a false competitor profile (**Fig. 3B**). Finally, the discovery that AHC sensors use an SA functionalization that has exposed free SA sites (**Fig. 1B**) demonstrates further complications with

the use of AHC sensors. As we have shown, AHC sensors do not seem to be reliable at times. Because of the proprietary nature of AHC sensors construction, we cannot determine the cause of the poor binding and binning profiles, except to say that it seems to be unpredictable and affects very diverse antibodies against different targets. We cannot say that poor binding profiles on AHC sensors is due to germline, sequence, antigen, or any other predictable characteristic. AHQ is very well behaved when working with purified samples, but the low loading response makes it less favorable when working with low titer samples or IgG supernatant, and its poor regeneration makes it less economically efficient. Conversely, ProA sensors' high loading response is desirable as it can increase sensitivity for small antigens, weak interactions, low titer samples, and can be regenerated well over a dozen times before loading response begins to fall. However, the interaction of ProA with VH3 family antibodies prevents its use for antibodies from that family.

Unlike AHC and AHQ sensors, custom b-AHFc sensors behaved well in terms of regeneration ability and maintained
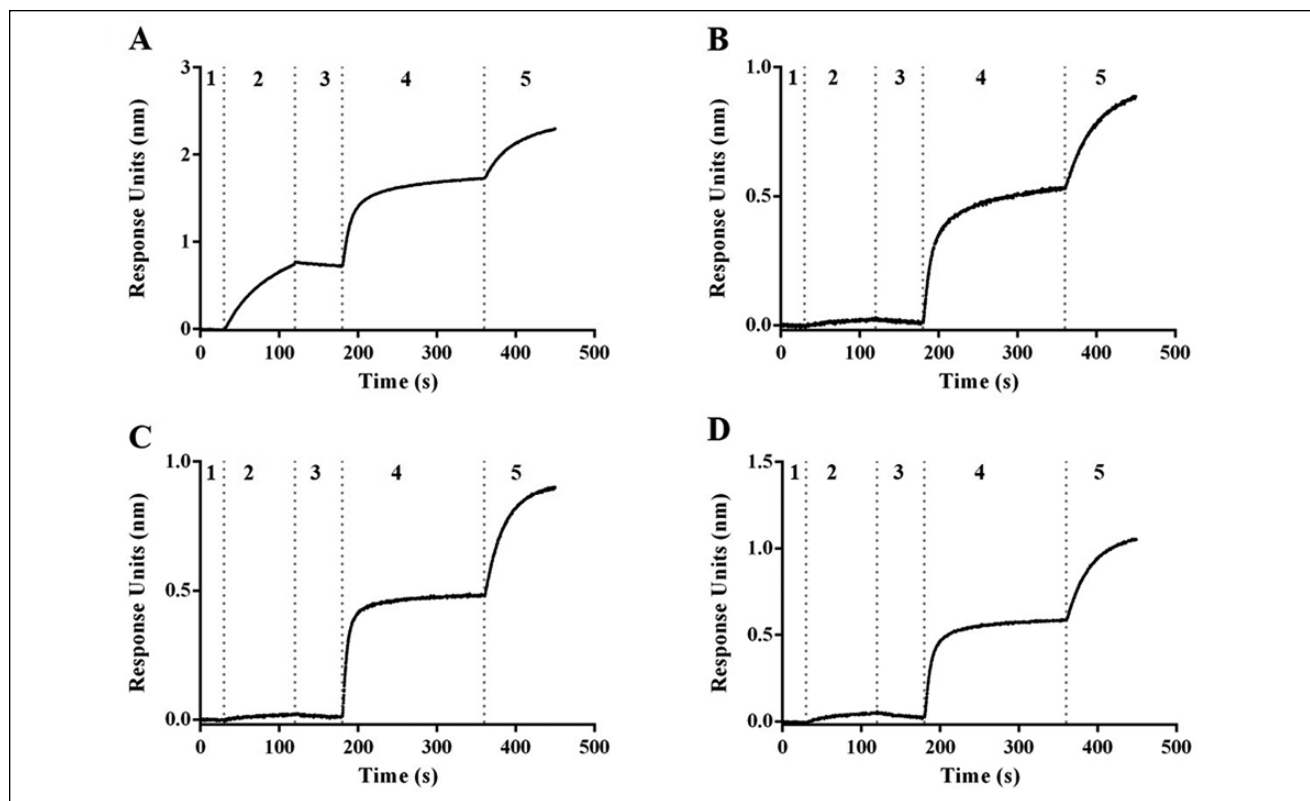
Figure 4. ForteBio epitope binning assays using supernatant antibody 3 (mAb 3). mAb 3 was loaded to the sensors previously (sensorgrams not shown). Sensors went through multiple assay steps (indicated by numbers and divided by dotted lines) including baseline (step 1, 0–30 s), mAb 4 (reference antibody, VH3) sensor binding check (step 2, 30–120 s), second baseline (step 3, 120–180 s), 100 nM antigen B (160 kDa) association (step 4, 180–360 s) and mAb 4 binning (step 5, 360–450 s). (**A**) ProA sensor. Unable to determine competitor or noncompetitor due to strong sensor binding of mAb 4 in step 2. (**B**) AHC sensor. mAb 4 noncompetitor profile. (**C**) AHQ sensor. mAb 4 noncompetitor profile. (**D**) b-AHFc sensor. mAb 4 noncompetitor profile. Response units are not normalized.

74% of its loading capacity after 18 cycles of regeneration (**Fig. 1A**). The strong interaction between the b-AHFc and SA sensors helped to guarantee the good regeneration ability of the b-AHFc sensors, but long-term stability may suffer without chemically cross-linking the anti-human Fc capture antibodies to the SA base. Most importantly, b-AHFc sensors do not interact with the variable region of VH3 IgG, simplifying binning conclusions (**Fig. 3D and 4D**).

Adimab's yeast-based platform and the use of supernatant screening drastically reduces the time spent between antibody selection and characterization. In addition, without the need for laborious purification, the number of samples that can be interrogated and characterized can be increased. The ForteBio Octet HTX system's high-throughput capability fits well in our workflow. However, rigorous sensor selection and characterization for a particular system is paramount to obtain reliable screening data for both purified and crude samples. Through careful analysis of each sensor type, we have demonstrated that our custom b-AHFc sensors is a robust biosensor type for both purified and supernatant antibodies for early stage antibody selection.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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