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Application Note

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Second Generation Anti-human IgG Fc Capture Biosensors for Affinity Characterization of Human IgG-Antigen Interactions and Quantitation of Human IgGs or Human Fc Region Containing Proteins

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Abstract

Sartorius second-generation Octet® AHC2 biosensors have been designed to expand usage of anti-human Fc capture biosensors into a diverse range of applications involving human IgG as the ligand. They have been developed to enhance human IgG ligand loading response and to enable both human IgG titer determination and antigen binding kinetics characterization. The biosensors were tested at different user sites and were found to exhibit 2.0X enhanced ligand capture response over the first generation AHC biosensors with higher antigen binding response observed in all kinetics case studies. The higher antigen response implies better sensitivity and a potential expansion of Octet® applications that utilize anti-human capture biosensors to small antigens such as small proteins and peptides. In addition, the enhanced ligand capture resulted into an expanded dynamic range of up to 5-fold over the first-generation biosensors when performing human IgG quantitation assays. Moreover, these new generation biosensors can be re-used multiple times allowing for significant cost savings.

Introduction

Monoclonal- and antibody-based new modality molecules such as bispecifics (bsAb) and anti-drug antibody conjugates (ADCs) are increasingly popular in biomedical and biopharmaceutical research and development. Biological therapeutics development, as well as development of analytical detection reagents, often involve human antibodies and Fc region-containing proteins. A typical workflow in early phase discovery of antibody therapeutics often starts with the identification of potential lead molecules, follows with target validation, and ends with lead optimization before the development process starts. The selection of a lead molecule is based on the screening of antibody clones for titer and other attributes, while lead molecule optimization may include full characterization of the binding properties of the antibody to target, amongst other properties.

Sartorius Anti-human IgG Fc Capture second generation biosensors, designed for use with the Octet® family of instruments, are developed to enable scientists to characterize lead molecules throughout the discovery and development process workflow when human IgG or human Fc region-containing protein product titer and antigen interaction characterization is desired. They can be used with any of the Octet® family of instruments through a typical Octet® assay format (kinetics workflow shown in Figure 1). These second-generation biosensors differ from the first-generation biosensors in that the same product can be used for both titer and kinetics determination, while two different first-generation biosensor products (AHQ and AHC) are required for quantitation and kinetics characterization respectively. The biosensors have high specificity towards all four human IgG subclasses and do not interact with IgGs from other species such as mouse, rat, rhesus, and cynomolgus monkey IgG (Figure 2). In addition, they are suitable for use with both purified and crude human IgG and human Fc region-containing proteins. In this application note, a series of experiments have been run side-by-side and at different Octet® user sites to compare the performance of AHC2 biosensors against that of AHQ and AHC biosensors in quantitation and kinetics assays, respectively.

Figure 1: *A Typical Workflow for the Use of AHC2 Biosensors in Kinetics Analysis Assay*

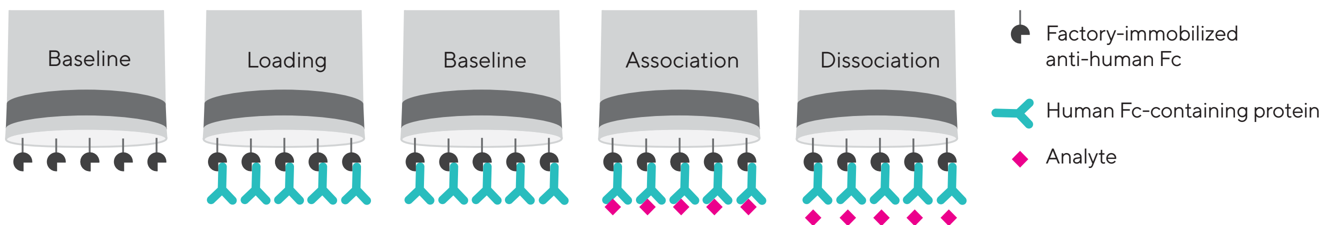
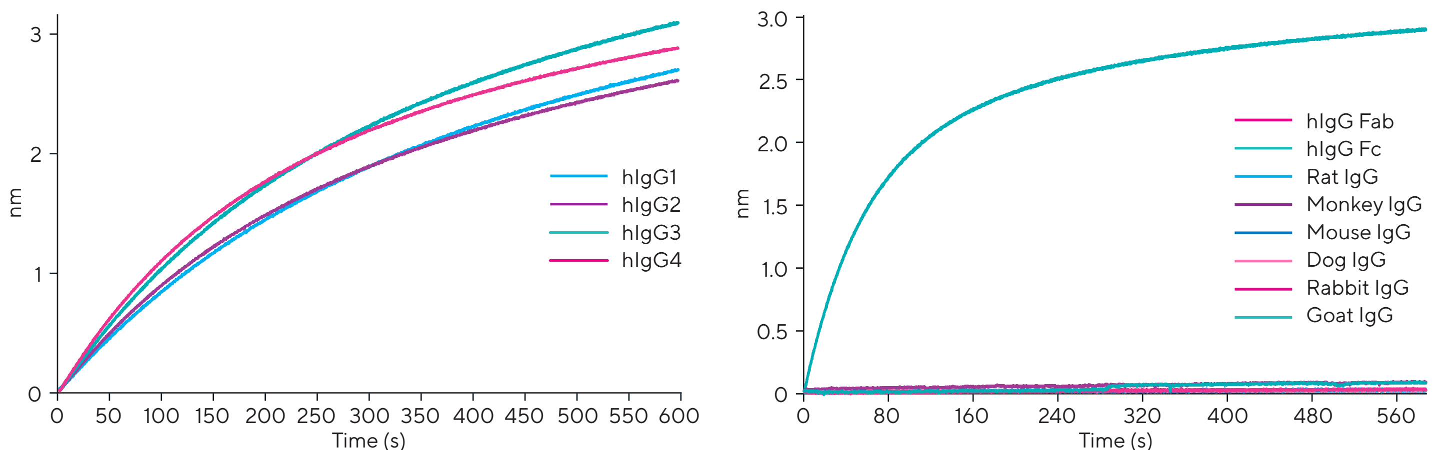


Figure 2: *AHC2 Biosensors Binding to 5 µg/mL of Different Species of Human IgG (Left) and Cross Reactivity Assessment Against Other Host IgGs (Right)*



Materials and Methods

Materials

Instruments:

- Octet® instruments R8, RH16, RH96
- Octet® BLI Discovery and Analysis Studio Software

Biosensors:

- Octet® Anti-human IgG Fc capture (AHC) for kinetics Cat. Nos. 18-5060, 18-5063, 18-5064
- Octet® Anti-human IgG Fc (AHQ) for quantitation Cat. Nos. 18-5001, 18-5004, 18-5005
- Octet® Anti-human IgG Fc capture 2 (AHC2) for kinetics and quantitation assays Cat. Nos. 18-5142, 18-5143, 18-5144

For all Octet® instruments:

- 96-well, black, flat bottom microplate, Greiner Bio-One Cat. No. 655209

Optional for Octet® RH16 and RH96 instruments:

- Octet® 384-Well Microplates with Tilted Bottom, Cat. Nos. 18-5076, 18-5080
- 384-well, black, flat bottom, polypropylene microplate, Greiner Bio-One Cat. No. 781209

Buffers:

The biosensors are compatible with a wide range of buffers. 1X Kinetics Buffer (1XKB) from Sartorius is recommended. It can be prepared by diluting the Octet® Kinetics Buffer 10X, 10-fold with 1X PBS, pH 7.4

Methods

For the discussed case studies, the specific methods were established at the user sites. In general, each site followed the typical Octet® analysis workflow in which the biosensor is first hydrated for at least 10 minutes in the assay matrix before use. Biosensor regeneration for re-use was done by dipping the biosensors into 10 mM glycine buffer, pH 1.7 for 5–10 seconds followed by a dip into neutral pH assay buffer for 5–10 seconds. This process is repeated for 3 cycles.

Results and Discussions

The performance of the AHC2 biosensors in quantitation and kinetics assays was evaluated at various Octet® user sites. Performance check studies compared the capability of these new biosensor surfaces to that of the first-generation biosensors. The AHC/AHQ vs AHC2 comparison studies were done side by side using the same methods and identical samples.

AHC2 Biosensors Exhibit an Expanded Quantitative Dynamic Range

Human IgG or Fc region-containing protein quantitation using the second generation AHC2 biosensors followed the well-established Octet® sample plate format that allows for a streamlined workflow and enables rapid quantitation of antibodies. Using this format, the pre-coated biosensors were simply dipped into the analyte samples in a one-step assay that can take as little as 2 minutes and can analyze from 1–96 samples depending on the throughput of the Octet® instrument used. The resultant binding curves were next fitted using an initial slope of binding fit algorithm in the Octet® Analysis Studio software. To quantify samples, a standard curve was first generated using the binding rates of known concentrations of the same analyte as the unknown. The binding rate of test samples were then used to extrapolate their concentration off the standard curve. IgG quantitation studies were performed at Sartorius and Pfizer Inc respectively.

A human IgG standard sample was used for the in-house studies at Sartorius. A series of the human IgG standard samples were prepared in Octet® Sample Diluent (Cat. No. 18-1104) at 0.5–2000 µg/mL. The simple Dip and Read assay was run on an Octet® R8 with the assay shake speed set at 400 RPM using the first generation AHQ biosensors side by side with the new AHC2 biosensors. Note that assay shake speed when using the Octet® instrument should be set at 1000 RPM for low sample concentrations and at 400 RPM for high sample concentrations.[1] The dose response analysis (Figures 3A and 3C) suggests that binding saturates at a much lower concentration on the AHQ than on the AHC2 biosensors. Further fitting of the data using an initial slope of binding analysis (Figures 3B and D) suggests that the optimal dynamic range for the AHQ biosensors is 0.5–400 µg/mL while that of AHC2 biosensors is 0.5–2000 µg/mL.

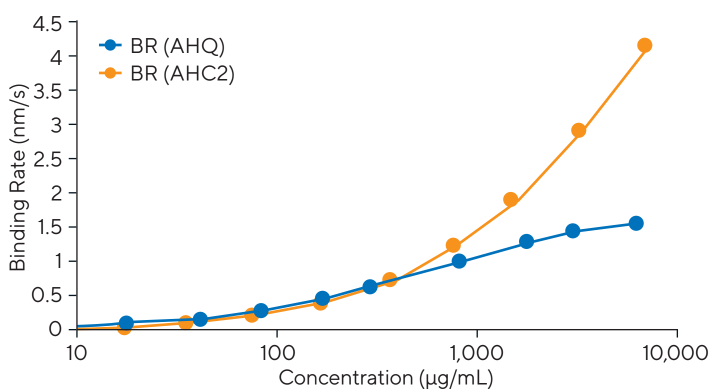
Figure 3

Note. Figures 3A and 3B show the dose response curves for the human IgG sample on the AHQ biosensor and the resultant standard curve (binding rates as a function of sample concentration), respectively, while Figures 3C and 3D show the same assay using the AHC2 biosensors. Binding rates were determined by fitting the initial slope of binding, and the standard curves were fit to a 5PL equations.

In another study to investigate the dynamic range of the new biosensors relative to the AHQ biosensors, scientists at Pfizer used an Octet® HTX (RH96) with a wide range of concentrations of a human IgG Biotherapeutic X drug sample while monitoring the binding rate. Figure 4 shows an overlay of a plot of the binding rates of the drug sample as a function of sample concentration and a direct comparison of the binding rates between the two biosensor surfaces. Similar to the studies at Sartorius, the Pfizer data shows that the IgG sample binding rates plateau on the AHQ biosensors at a much lower concentration than on the AHC2 biosensors, suggesting that the new biosensor surface has a higher capacity hence a wider dynamic range.

In another experiment, two analysts at Pfizer independently tested the two biosensors with QC samples. Here, standard curves were first generated off the two surfaces using known concentrations of the drug sample prepared in a serial dilution in 20% mouse plasma. Assay accuracy was established using QC samples in a spike recovery experiment. The QCs used were spikes of the drug at 32, 16, 4, 0.5, and 0.25 µg/mL in 20% mouse plasma and were recovered off the standard curve. The QC samples yielded comparable and accurate results for both biosensor surfaces (Table 1).

Figure 4: *AHQ vs AHC2*



Note. An overlay of binding rates as a function of drug sample concentration for AHQ (blue) and AHC2 (red) biosensors. AHC2 biosensors exhibited an expanded dynamic range for quantitation with no biosensor saturation observed at the highest drug concentration examined.

Table 1
A. Using AHQ Biosensors

Sample Concentration (µg/mL)	0.25	0.50	4.00	16.00	32.00
Analyst 1	0.17	0.38	3.44	16.10	33.40
Analyst 2	0.21	0.43	3.80	17.10	34.70
Analyst 2 rep	0.20	0.44	3.88	16.80	36.50
Mean	0.20	0.42	3.71	16.67	34.87
SD	0.02	0.03	0.23	0.51	1.56
% CV	11.20	7.80	6.30	3.10	4.50
% Bias	-22.00	-16.70	-7.30	4.20	9.00

B. Using AHC2 Biosensors

Sample Concentration (µg/mL)	0.25	0.50	4.00	16.00	32.00
Analyst 1	0.17	0.40	3.49	16.50	34.10
Analyst 2	0.27	0.49	3.68	16.50	34.20
Analyst 2 rep	0.25	0.53	3.88	16.70	34.00
Mean	0.23	0.47	3.68	16.57	34.10
SD	0.05	0.07	0.20	0.12	0.10
% CV	22.20	14.40	5.30	0.70	0.30
% Bias	-7.30	-5.40	-7.90	3.50	6.60

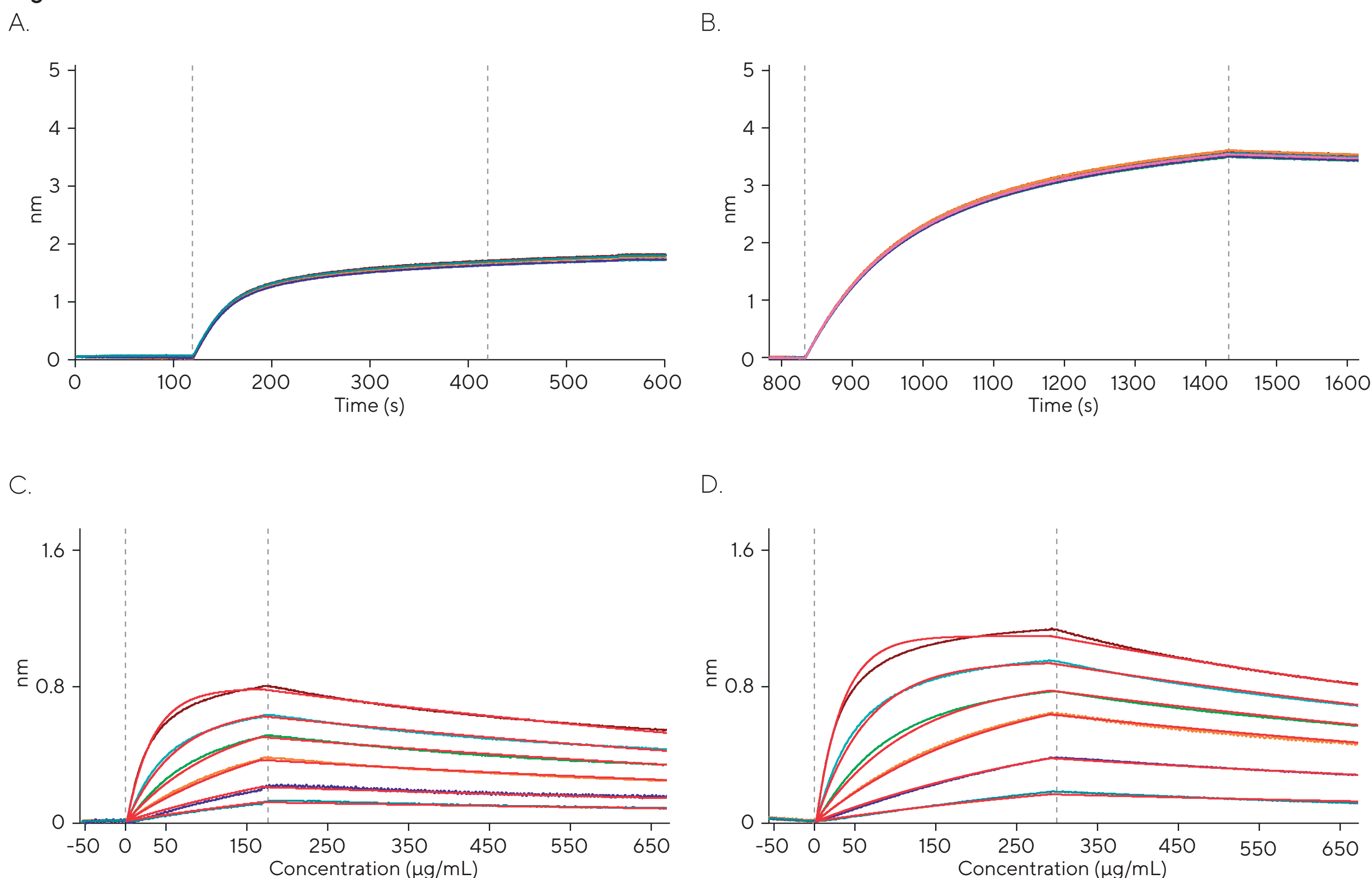
Note. Detection of drug in mouse K2 EDTA plasma (20%) using QC samples: Assay was run by two different analysts using AHQ (A) and AHC2 (B) biosensors. Data show comparable performance using the two biosensor surfaces. Numbers highlighted in yellow fell outside the established acceptance criteria of +/- 30% of nominal concentration

AHC2 Biosensors Exhibit an Increased Ligand Loading Capacity for Kinetics Characterization of Human IgGs

Label-free, biosensors-based technologies require that a ligand be captured onto the biosensor surface followed by antigen dose-response binding analysis to enable the determination of affinity constants and other kinetics parameters. To avoid avidity effects that may lead to artificially slow off-rates often observed from a second-step binding of the second "arm" of an antibody once the first "arm" is bound, it is often recommended that the antibody be used as the ligand in antibody-antigen binding characterization studies. Anti-human capture biosensors can therefore be used to capture human IgG as ligands on the biosensor. A typical kinetics assay requires a ligand (antibody) capture optimization step to determine the optimal concentration of the antibody that leads to accurate antigen binding analysis.[2] Kinetics assay studies comparing the performance of the AHC2 biosensors were done at Sartorius, Adimab and Duke University.

The kinetics assays were performed using the typical Octet® kinetics assay workflow where the biosensor was first hydrated in assay buffer. The ligand was next captured onto the biosensor surface, followed by a short baseline step prior to the binding of the antigen in a dose response manner. The Sartorius studies involved a human anti-HER2 antibody binding to a HER2 antigen. The antibody was captured onto both AHC and AHC2 biosensors at 10 µg/mL, while the antigen was serially diluted from 20–0.313 µg/mL. A buffer-only reference biosensor was used as negative control (Figure 5). Data fit to a 1:1 fit model generated affinity constants that were nearly identical for both biosensor surfaces (2.45 nM and 2.79 nM for AHC and AHC2 biosensors, respectively). However, on further examination of the data, the ligand response on the AHC2 was 2.0X that of AHC. In turn, the analyte response when using the AHC2 biosensors was observed to be much higher. Since the affinity constant is a function of the on- and off- rates of binding, these differences in response did not affect the affinity constant. One key advantage of an enhanced ligand response however is that the AHC2 biosensors should enable users to extend applications to the analysis of smaller molecules where enhanced ligand loading response may be necessary.

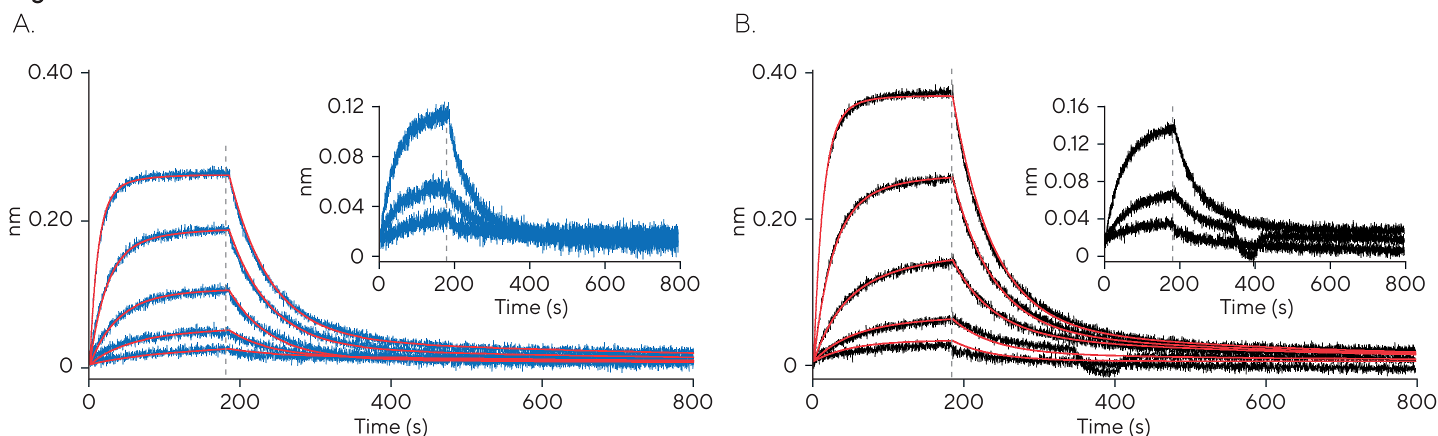
Figure 5



Note. A comparison of the capture response levels of anti-HER2 antibody between AHC (A) and AHC2 (B) biosensors. 10 µg/mL of the antibody was captured in both cases. The capture step for the AHC biosensor saturated within 300 seconds, while the enhanced capacity for the AHC2 biosensors necessitated a longer capture step to allow the response to approach saturation. Panels C and D show the subsequent dose response binding analysis (2-fold serial dilutions of HER2 antigen; 20 µg/mL–0.313 µg/mL) for AHC and AHC2 biosensors respectively.

It should be noted that the Bio-Layer Interferometry (BLI) technique exhibits binding response that may be influenced by concentration, conformation, and size of the analyte. A similar kinetics study to that with HER2 was performed at Adimab using an antigen of a much lower molecular weight (Hen Lysozyme (15kDa); HER2 is 185kDa). Hen Lysozyme was expected to exhibit much lower response upon binding to human IgG than HER2. The study at Adimab corroborated the expected increased response for both ligand capture and antigen binding on AHC2 when compared to AHC. Similar to the HER2 study, the observed affinity constants were comparable (50.4 nM and 63.2 nM for AHC and AHC2 biosensors, respectively), while the binding response data were higher for AHC2. Since lysozyme is a smaller antigen with an expected low response, AHC2 biosensors data exhibited a relatively higher signal-noise ratio (Figure 6) than that seen with AHC biosensors, suggesting that the new AHC2 biosensors could be used in situations where the AHC biosensors may not be suitable. This would possibly include characterization of small proteins and peptides. In addition, with AHC2 biosensors, less ligand samples may be required to achieve a targeted loading response than is the case with the first-generation biosensors.

Figure 6



Note. A comparison of response signals of Hen Lysozyme (15kDa) binding to human IgG using AHC biosensors (A) and AHC2 biosensors (B). Insets are the binding curves of the lowest 3 concentrations.

The two biosensor types were also used to screen for the binding of a panel of 4 mAbs against a gp120 antigen at Duke. The mAbs consisted of anti-Flu mAb 1 and anti-HIV mAbs 2-4. In each experiment, the gp120 antigen was used in a dose-dependent, 2-fold serially-diluted samples from 100 µg/mL to 1.56 µg/mL. Anti-Flu mAb1 was used as negative control, while anti-HIV mAbs 2-4 were characterized for binding to gp120. The data were analyzed using either a 1:1 model or a 2:1 heterogeneous ligand model fit. The results suggest different kinetics properties (Table 2) for the different mAbs. Data further suggest similarity between the two biosensor surfaces. The AHC2 biosensors, however, exhibited better signal-to-noise ratio than the AHC biosensors.

Table 2

A. 1:1 Fit Model

Sensor Type	Ligand	Sample ID	Fit Model	ka (1/Ms)	kd (1/s)	KD (M)
AHC	mAb2	gp120	1:1	2.09E+04	1.14E-02	5.44E-07
AHC2	mAb2	gp120	1:1	2.58E+04	1.11E-02	4.30E-07
AHC	mAb3	gp120	1:1	7.16E+03	4.42E-04	6.17E-08
AHC2	mAb3	gp120	1:1	7.53E+03	4.71E-04	6.26E-08
AHC	mAb4	gp120	1:1	3.08E+04	3.26E-05	1.06E-09
AHC2	mAb4	gp120	1:1	2.04E+04	9.58E-05	4.69E-09

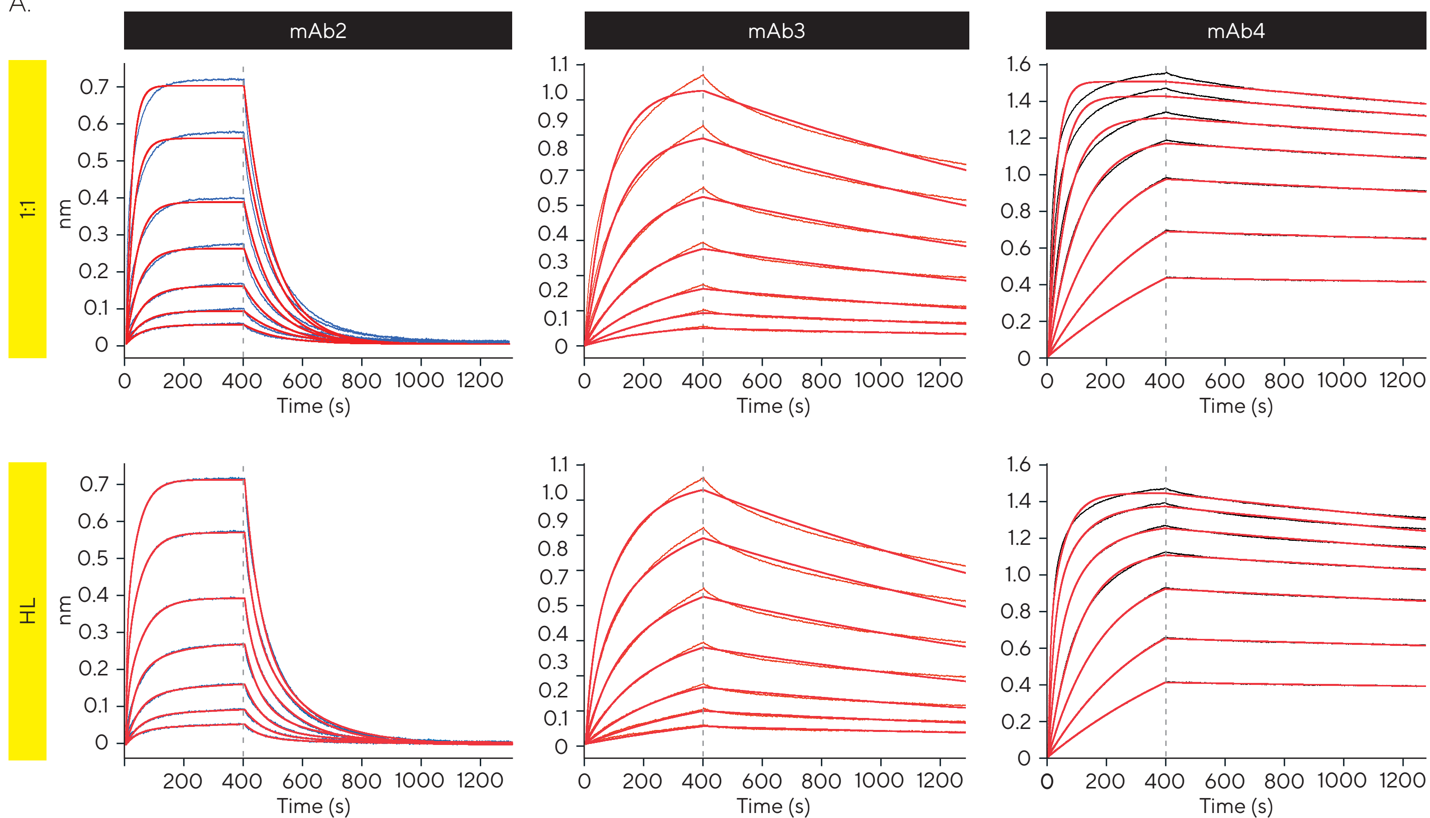
B. 2:1 Fit Model

Sensor Type	Ligand	Sample ID	Fit Model	ka (1/Ms)	kd (1/s)	KD (M)
AHC	mAb2	gp120	2:1	2.43E+05	4.12E-02	1.70E-07
AHC2	mAb2	gp120	2:1	2.72E+05	3.70E-02	1.36E-07
AHC	mAb3	gp120	2:1	2.03E+04	5.28E-04	2.61E-07
AHC2	mAb3	gp120	2:1	3.39E+04	2.83E-03	8.33E-08
AHC	mAb4	gp120	2:1	4.98E+04	9.80E-05	1.97E-09
AHC2	mAb4	gp120	2:1	4.74E+04	2.02E-04	4.26E-09

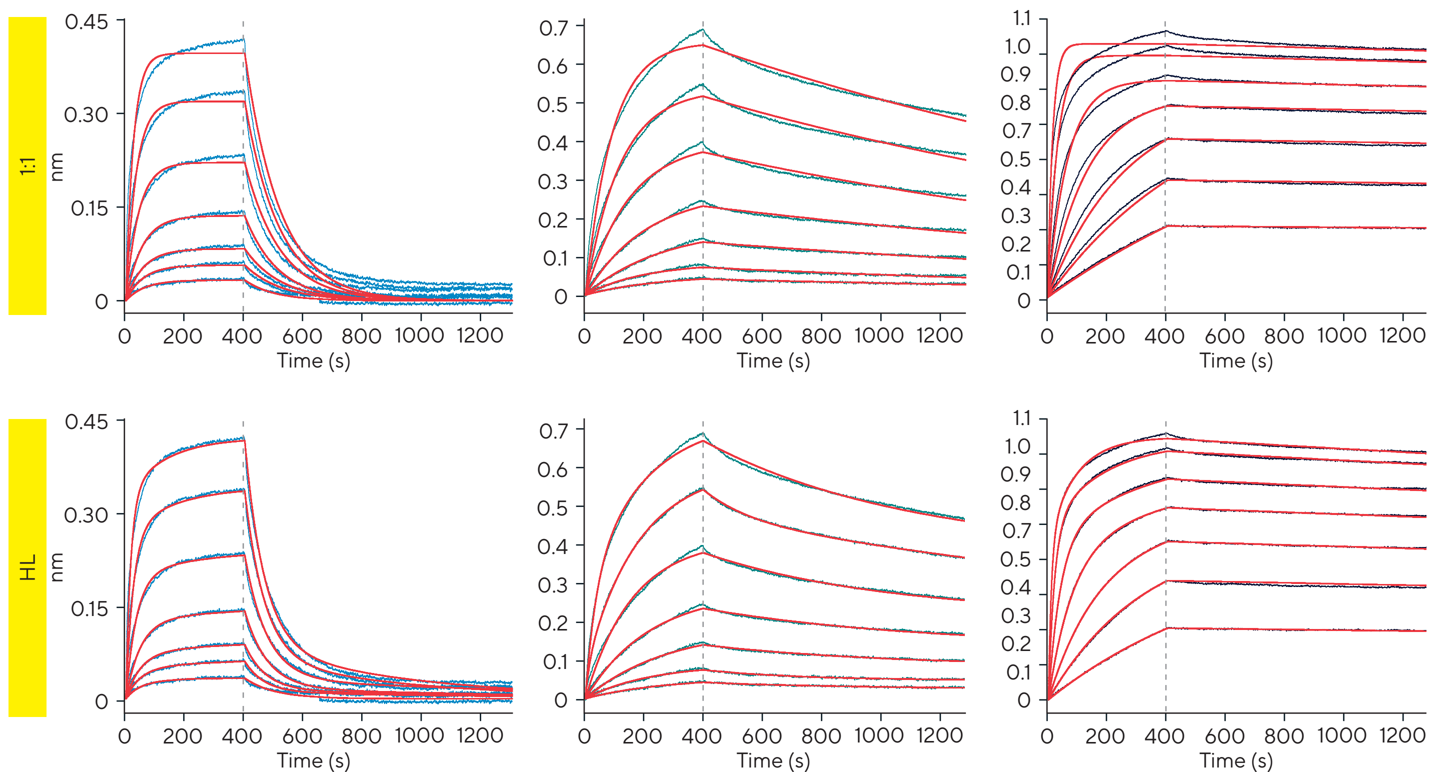
Note. Kinetic parameters for the binding of 3 anti-HIV mAbs to the HIV envelope protein gp120 analyzed using (A) 1:1 fit model or (B) 2:1 heterogeneous ligand fit model in the Octet® Analysis Studio software.

Figure 7

A.



B.

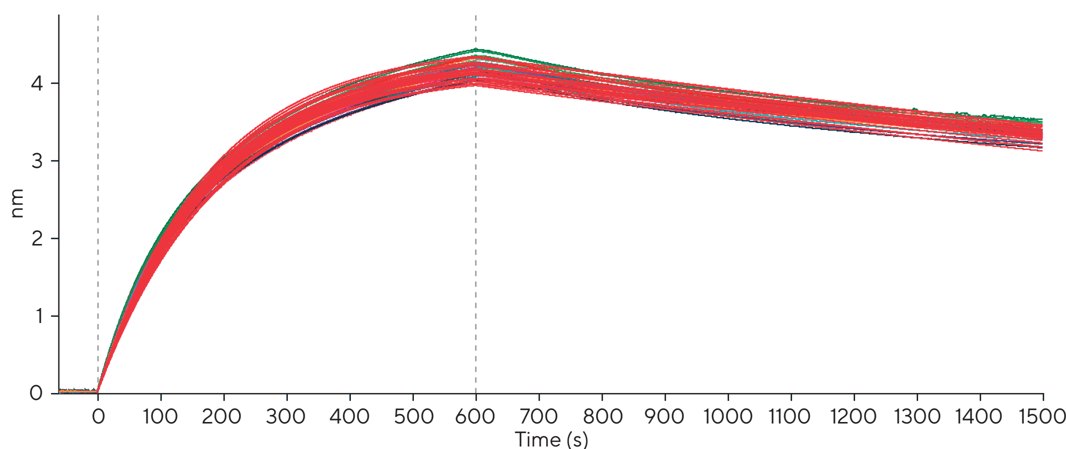


Note. Anti-HIV mAbs binding to gp120 protein as analyzed using the AHC2 (A) and AHC (B) biosensors. The 3 mAbs exhibit distinctly different binding properties.

AHC2 Biosensors Stability in Matrix

A key advantage of the Dip and Read format on the Octet® platform is that biosensors can be dipped directly into capture ligand without the need for purification. One challenge with this approach for label free platforms is that matrix effect may sometimes necessitate assay optimization. To verify the stability and suitability of the AHC2 biosensors in different matrices, a Fab anti-human IgG sample was spiked into a series of commonly used matrices at 10 µg/mL followed by the binding of antigen (also spiked in the various matrices). The same sample in 1XKB was used as a reference sample for kinetics parameters comparison. The resultant data (Figure 8 and Table 3) show insignificant differences in kinetics, thereby validating the stability of the AHC2 biosensors in different but commonly used buffer systems.

Figure 8



Note. Binding analysis of a Fab molecule binding to a Fab anti-human IgG in different matrix. The IgG was spiked in each matrix at 10 µg/mL. Each sample was captured onto the AHC2 biosensor followed by the binding of the antigen at an identical concentration for each matrix. The resultant binding curves were then analyzed in the Octet® Analysis Studio software (See Table 2).

Table 3

	Matrix	K_D (M)	Ka ($M^{-1}S^{-1}$)	Kd (S^{-1})	Change in K_D
CHO	Neat CHO	9.60E-09	2.64E+04	2.53E-04	-6%
	50% CHO	9.69E-09	2.60E+04	2.52E-04	-5%
	25% CHO	9.78E-09	2.60E+04	2.55E-04	-4%
5% FBS	Neat 5% FBS	9.28E-09	2.61E+04	2.42E-04	-9%
	50% (5%) FBS	9.37E-09	2.61E+04	2.44E-04	-8%
10% FBS	Neat 10% FBS	9.60E-09	2.70E+04	2.59E-04	-6%
	50% (10%) FBS	9.41E-09	2.68E+04	2.52E-04	-7%
	25% (10%) FBS	9.51E-09	2.65E+04	2.52E-04	-6%
DMEM	Neat DMEM	8.40E-09	2.55E+04	2.14E-04	-17%
	50% DMEM	9.40E-09	2.60E+04	2.45E-04	-8%
PBS	Neat PBS	8.62E-09	2.64E+04	2.27E-04	-15%
	50% PBS	9.92E-09	2.61E+04	2.59E-04	-2%
	25% PBS	9.82E-09	2.62E+04	2.57E-04	-3%
Control	1xKB control	1.02E-08	2.62E+04	2.66E-04	

Note. Kinetics analysis of antigen binding to Fab anti-human IgG in different matrices. An analysis of % KD change from the control (1x KB) shows < 10% difference for all but two media types (Neat DMEM and Neat PBS). A 2-fold dilution of the two media types using 1X KB resulted into a significant reduction in KD % differences.

Biosensor Regeneration

Biosensor regeneration is a key step to lowering assay consumables costs. Regeneration is the removal of the captured ligand-analyte complex (kinetics assays) or the removal of the human IgG or Fc containing protein analyte (quantitative assays) and allows for the re-use of the biosensor. As the biosensors are designed to capture human IgGs, the regeneration process is a simple dip into low pH buffers followed by neutralization in neutral pH buffer (see methods section). Regeneration for quantitative assays should be more stringent and must be more complete than in kinetics assays as a slight change in surface capacity has a higher effect on quantitation than in rates of binding.

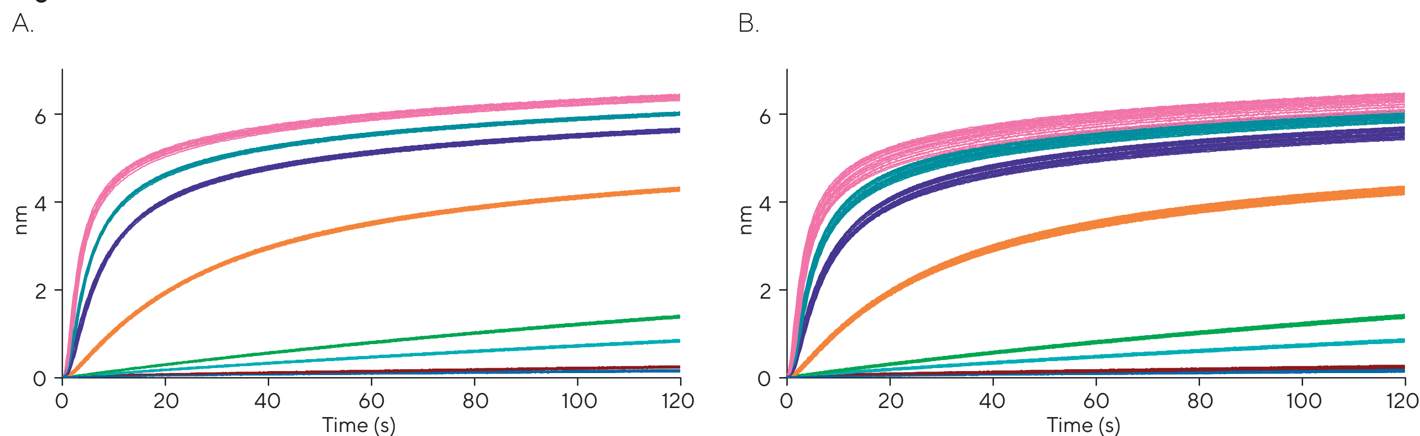
Regeneration Assessment for Quantitation Assays

To assess the regeneration capacity of the AHC2 biosensors, while keeping in mind the more stringent regeneration requirements when the biosensors are to be used for quantitation, two different assays were set up on the Octet® RH96 system; one was a 10-cycle regeneration with the goal of re-using the biosensor 10 times, and the other was a 20-cycle regeneration aimed at > 10 time reuse of the biosensor. The regeneration was performed by dipping the biosensors into a solution of 10 mM glycine at pH 1.7 for 5 seconds followed by a dip in the assay buffer for 5 seconds. This cycle was repeated 3 times in sequence to fully remove bound human IgG from the biosensor surface. Figure 9 shows an overlay of the binding response of the 10- and 20-cycle regeneration process for the full range of concentrations within the established dynamic range for the AHC2 biosensors. An analysis of the resultant data (Table 4) suggests that the biosensors meet acceptance criteria of ≤ 10% CVs for both assays.

Regeneration Assessment for Kinetics Assays

The AHC and AHC2 biosensors used with gp120 protein binding to anti-HIV antibody at Duke were regenerated and assessed for re-use in kinetics assays. Here, the sensors used to assay mAb4 were regenerated after use, and the kinetics assay was repeated 6 more times with regeneration in-between each assay.

Figure 9



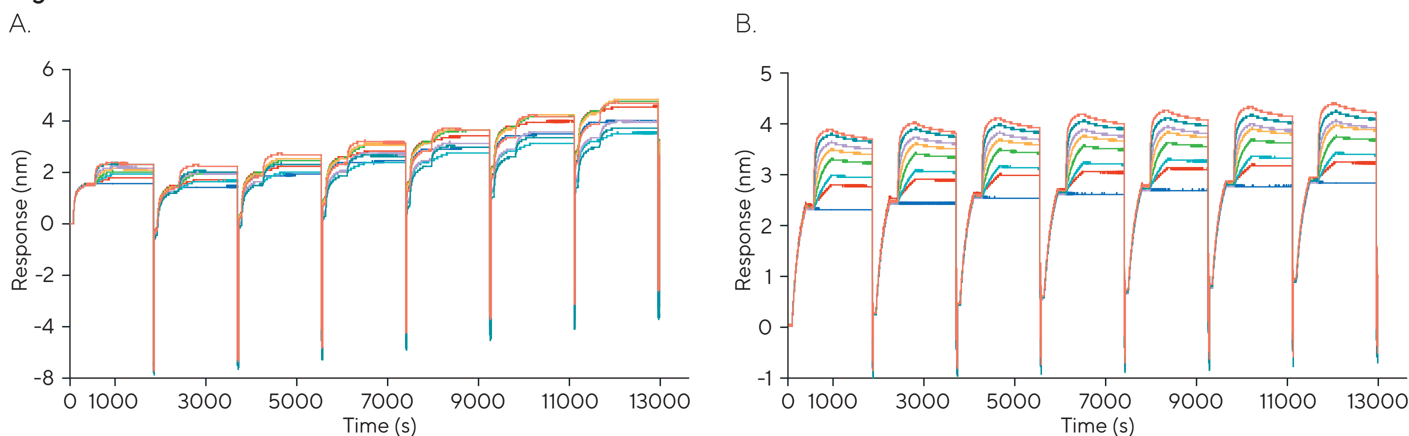
Note. AHC2 biosensors binding curves showing 10 cycles (A) and 20 cycles (B) of low pH biosensor regeneration for hIgG samples in the range 2000 µg/mL to 0.5 µg/mL.

Table 4

	10-Cycle Regeneration			20-Cycle Regeneration		
Known Concentration (µg/mL)	Calculated Concentration (µg/mL) mean = 10	SD	CV	Calculated Concentration (µg/mL) mean = 20	SD	CV
2000.00	2000.00	113.84	5.70%	2000.00	205.88	10.30%
1000.00	1002.62	21.42	2.10%	1009.66	59.87	5.90%
500.00	502.03	8.99	1.80%	505.65	23.60	4.70%
100.00	100.17	1.17	1.20%	100.23	1.66	1.70%
10.00	9.99	0.26	2.60%	9.99	0.29	2.90%
5.00	5.02	0.19	3.80%	5.02	0.17	3.30%
1.00	1.00	0.03	3.30%	1.00	0.04	4.00%
0.50	0.51	0.04	7.20%	0.51	0.04	8.00%

Note. Calculated concentration, standard deviation and % CV analysis of human IgG samples quantitated after multiple cycles of regeneration. Data show acceptable % CV up to 20 regeneration cycles.

Figure 10

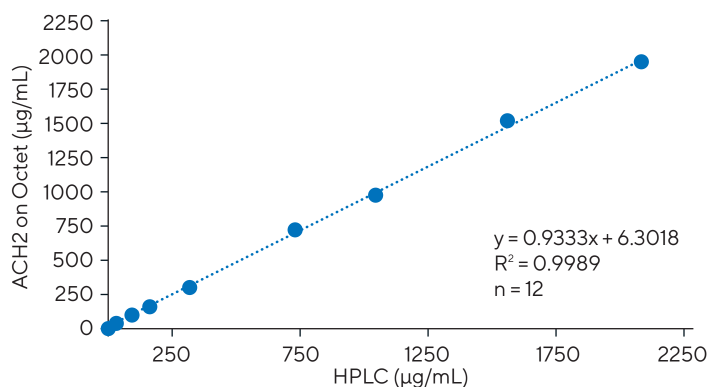


Note. Results for kinetics analysis of anti-HIV mAb 4 binding to gp120 protein using re-cycled AHC (A) and AHC2 (B) biosensors. Although data analysis (data not shown) indicated comparable kinetics parameters, the AHC2 biosensors exhibited better response signal-drift stability over successive re-use cycles.

Comparison to HPLC

Protein A-based HPLC is often used for IgG quantitation. However, it has disadvantages that render it comparatively less cost-efficient when compared to the Octet® platform.[3] To validate the performance of the new AHC2 biosensors in a quantitative assay, we used the same human IgG samples to "determine protein concentrations" on both the Octet® R8 system and the HPLC. Data from both techniques show good correlation, indicating that the Octet® platform could be used interchangeably with the HPLC for IgG quantitation.

Figure 11



Note. Correlation between human IgG quantitation using AHC2 biosensors on the Octet® R8 (y-axis) and HPLC (x-axis).

Summary

The Octet® AHC2 biosensors have been developed to facilitate the kinetics characterization and quantitation of human IgGs and Fc-region containing proteins. They differ from the first generation Anti-human Fc biosensors in that, although they can both be used for both IgG quantitation and kinetics characterization, the first-generation biosensors are divided into two distinct products: AHC for kinetics characterization and AHQ for titer determination. A series of experiments were performed at different user sites to assess the performance of the AHC2 biosensors relative to their first-generation counterparts. Data generated reveal significant improvement in performance with 2.0X enhancement of ligand capture response signals over the AHC biosensors, which resulted in higher responses upon analyte binding. While these increases in signal did not impact the derived kinetics parameters, they improved the generated signal to noise ratio. This would likely lead to an expanded range of analytes that could be used with these new biosensors to include smaller proteins or peptides, where the use of AHC biosensors may not be optimal. Moreover, the higher ligand loading capacity exhibited by these biosensors means that less sample would be needed to achieve similar loading response as with the AHC biosensors. In quantitative determinations, the new biosensors exhibited up to 5-fold wider dynamic range, which should minimize the need for sample dilutions. Moreover, we have shown that the AHC2 biosensors can be regenerated and re-used up to 20X, allowing for significant cost savings. These attributes, combined with the high through-put capability of the Octet® instruments, should enable users in both discovery and bioprocessing applications working with human IgGs to rapidly characterize and | or quantitate their samples.

Acknowledgements

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References

1. Sartorius Technical Note: Octet® AHC2 Biosensors For Quantitation and Kinetic Characterization of Human Fc-Region Containing Proteins
2. Sartorius Application Note 4014 Biomolecular Binding Kinetics Assays on the Octet® Platform
3. Sartorius Application Note 4015: MAb Quantitation: Protein A HPLC vs. Protein A Bio-Layer Interferometry

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