SARTORIUS

Octet® AHC2 Biosensors

For Quantitation and Kinetic Characterization of Human Fc-Region Containing Proteins

Technical Note

Introduction

Human antibodies and Fc-region containing proteins are the most important drug candidates among biotherapeutics. The quantitation and kinetic characterization of these proteins is of paramount importance for scientists in both downstream and upstream applications. The Octet® AHC2 Biosensors are the next generation of Octet® anti-human IgG Fc capture biosensors that offer significantly improved performance and versatility by enabling both quantitation and kinetic characterization of human IgG antibodies and IgG-derived Fc-fusion proteins.

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The Octet® AHC2 Biosensors exhibit high specificity towards all four human IgG (hIgG) subclasses (IgG1, IgG2, IgG3, and IgG4) and do not interact with human Fab-fragments or IgGs from other species, such as mouse, rat, rhesus, and cynomolgus monkey (Figure 1). Furthermore, these biosensors can be cost-effectively regenerated up to 20 times for both kinetics and quantitation applications. Thus, Octet® AHC2 Biosensors are extremely useful for a wide range of high-throughput applications, including lead identification and optimization, cell line development, process development, and QC in both crude and purified protein samples. This Technical Note describes kinetic and quantitation assay workflows that utilize the Octet® AHC2 Biosensors for characterization of human IgGs and Fc-region containing proteins.

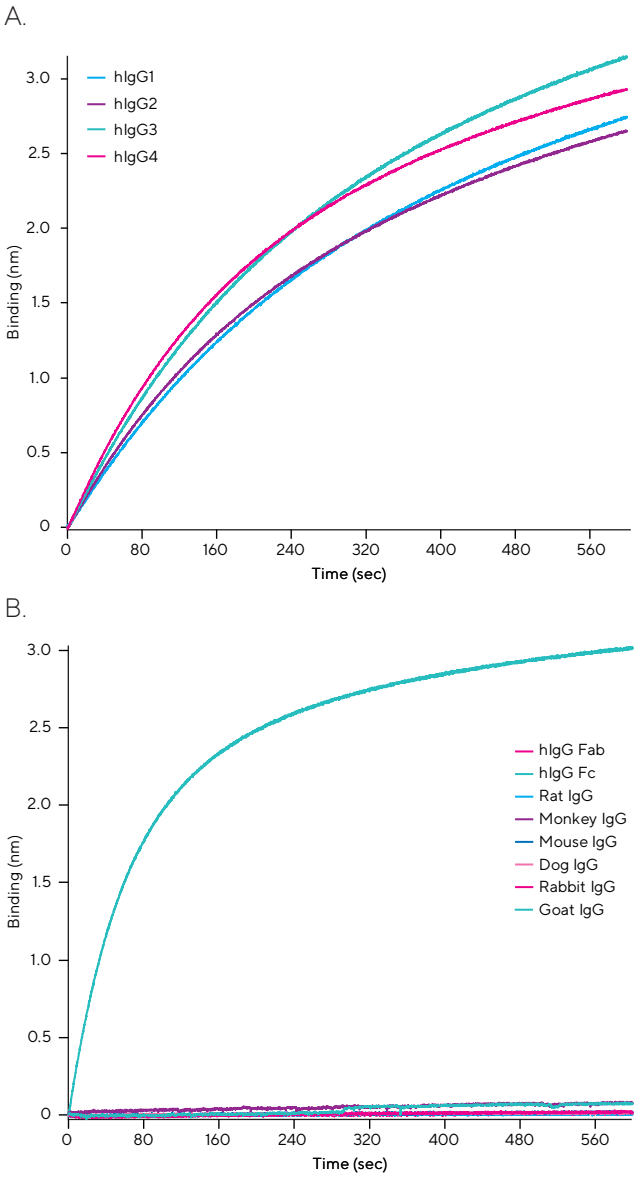


Figure 1: Specificity of the Octet® AHC2 Biosensors. (A) Strong binding to all four hIgG subtypes via Fc-region. (B) No binding to IgGs from mouse, rat, rhesus, and cynomolgus monkey. All samples were tested at concentrations of 5 µg/mL.

Kinetic Assay Workflow

The Octet® AHC2 Biosensors are pre-immobilized with a new anti-human Fc-specific antibody, which enables the immobilization of human IgG and human Fc-region containing proteins directly from a crude or purified samples. The Octet® AHC2 Biosensors provide up to 2-fold increased binding capacity for human IgG and Fc-region containing proteins when compared to the first generation of the AHC biosensors, making the second generation particularly suitable for analysis of proteins at low concentrations and for characterization of small proteins. An example assay workflow utilizing the Octet® AHC2 Biosensors to characterize the interaction between an analyte and a human IgG is outlined in Figure 2.

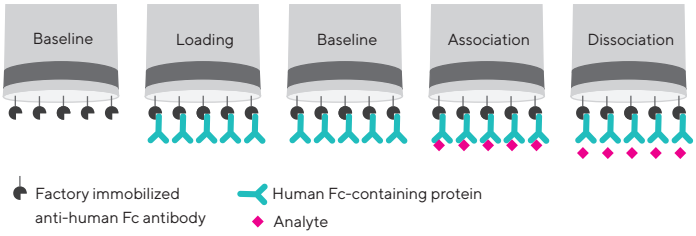


Figure 2: Kinetic assay workflow using the Octet® AHC2 Biosensors typically includes the following steps: 1 – equilibration (baseline), 2 – loading (capture) of hIgG, 3 – baseline, 4 – association kinetics, 5 – dissociation kinetics.

Materials Required

- Octet® instrument with Octet® BLI Discovery and Analysis Studio Software
- Octet® AHC2 Biosensors, Part No. 18-5142
- For all Octet® instruments: 96-well, black, flat bottom microplate, Greiner Bio-One Part No. 655209
- Optional for Octet® RH16 and RH96 instruments:
  - Octet® 384 Well Tilted bottom Plate, Sartorius Part No. 18-5080 (pack); 18-5076 (case)
  - 384-well, black, flat bottom, polypropylene microplate, Greiner Bio-One Part No. 781209
- hIgG or Fc-containing protein for immobilization. It can be present in either a simple buffer or a complex mixture such as cell culture supernatant.
- Analyte protein that interacts with hIgG protein. The analyte proteins can be dissolved in a buffer solution or a complex mixture such as cell culture supernatant. The buffer matrix of the analyte should be identical to that of the baseline buffer immediately prior to the analyte association step, where the concentrations of the bulk components of the baseline buffer and the analyte buffer are the same.
- Octet® Kinetics Buffer 10X, Part No. 18-1105. The AHC2 Biosensor is compatible with a wide range of buffers, although 1X Kinetics Buffer is recommended. It can be prepared by diluting the 10X Kinetics Buffer 10-fold with 1X PBS, pH 7.4.

Assay Optimization Tips

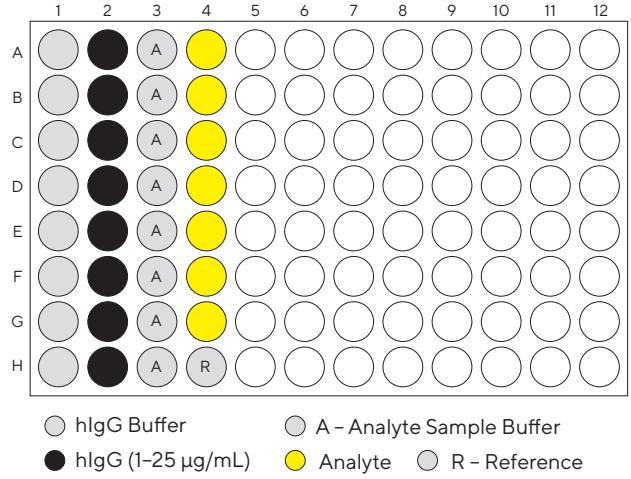
- Use a reference sample in the association step to correct for baseline drift. A reference sample is a biosensor that during the assay experiment has ligand present in loading step, but has buffer only with no analyte present in the association step.
- The baseline and dissociation steps should always be performed in the same microplate well for each biosensor. This enables the inter-step correction feature to align the association and dissociation steps when processing data.

Assay Procedure

For details on setting up a kinetic assay in Octet® software, please refer to the Octet® BLI Discovery Software User Guide. Figure 3 shows an example microplate layout and assay design for a kinetic characterization assay using the Octet® AHC2 Biosensors. For all steps, use a 200 µL sample volume for 96-well plates, and 80–130 µL for standard 384-well plates.

Before the Assay – Warm up all reagents and samples to room temperature before biosensor hydration and assay setup. Pre-hydration of biosensors: Hydrate the AHC2 Biosensors in 200 µL per well of similar or identical buffer matrix as hIgG to be captured. Hydration is performed in a 96-well, black, flat-bottom plate (Greiner 655209) placed in the blue biosensor tray for a minimum of 10 minutes.

A.



B.

Step #	Column #	Description	Step type	Time	Shaking (rpm)
Step 1	1	Equilibration in hIgG buffer	Baseline	180	1000
Step 2	2	Loading of hIgG	Loading	120–600	1000
Step 3	3	Baseline in Analyte sample buffer	Baseline	300–600	1000
Step 4	4	Association of Analyte	Association	300–900	1000
Step 5	3	Dissociation of Analyte	Dissociation	300–3600	1000

Figure 3: (A) Sample Plate map and (B) assay steps with associated parameters for the AHC2 kinetic assay.

Assay Step 1 – On-line equilibration of the hydrated AHC2 Biosensors in 1X Kinetics Buffer or hIgG custom buffer: Add buffer, media, or diluted lysate to Column A of the Sample Plate according to the map in Figure 3. Note the equilibration buffer must match the buffer matrix of hIgG to be captured.

Assay Step 2 – Capture of hIgG or Fc-containing protein (Loading/Immobilization): Dilute the hIgG or Fc-containing protein to the appropriate concentration in 1X Kinetics Buffer or corresponding sample matrix and add to the Sample Plate. The matrix or buffer used should match the one used for equilibration in Assay Step 1. The typical immobilization concentration is 5–150 nM. The concentration of ligand to use will depend on its affinity for the associating analyte, as well as the size of both ligand and analyte.

For the best kinetic data and most accurate affinity constants, an optimization experiment should be performed to determine the optimal ligand loading concentration and time. Load only enough ligand so that the highest concentration of analyte used has adequate association signal to allow measurement of the dilution series (recommended binding for ligand loading is 2–4 nm). Loading more ligand than what is needed can cause artifacts such as non-specific binding, heterogeneity, or mass transport. For more details on optimization of ligand loading for kinetic assays, refer to Application Note, Biomolecular Binding Kinetic Assays on the Octet® platform.

Assay Step 3 – Baseline step in assay buffer (Baseline): Add 1X Kinetics Buffer or alternative buffer matching the analyte samples being analyzed to the sample plate according to Figure 2. It is important to match the baseline buffer matrix to that of the analyte samples, where the concentrations of the bulk components of the baseline buffer and the analyte buffer are the same. The baseline step should be run for a long enough time until any change in baseline drift has stabilized. We recommend 300–600 seconds of baseline if a new buffer matrix is used in this step. If the buffer is identical to the hIgG ligand buffer, a baseline step of 120-300 seconds should be adequate.

Assay Step 4 – Association to interacting analyte (Association): If detailed kinetic characterization is being performed, the analyte protein must be purified and of known concentration. It is recommended to run a titration series of at least five concentrations of the analyte protein and perform a global fitting of all concentrations to determine k_{on}, k_{off} and K_D values. The highest analyte concentration should not exceed 10–20 times the expected K_D. For screening assays or qualitative interaction analysis, a

single concentration of the interacting protein can be sufficient to characterize the binding. Analyte samples must be diluted in the same buffer used for the baseline and dissociation steps. Include a reference sample, consisting of assay buffer blank with no analyte present, in this step to enable subtraction of background baseline drift.

Assay Step 5 – Dissociation of interacting analyte (Dissociation): The Dissociation step is performed in the same buffer well(s) used for the Baseline step (step 3). Using the same wells for Baseline and Dissociation enables the inter-step correction feature to be used in data analysis for more accurate curve fitting.

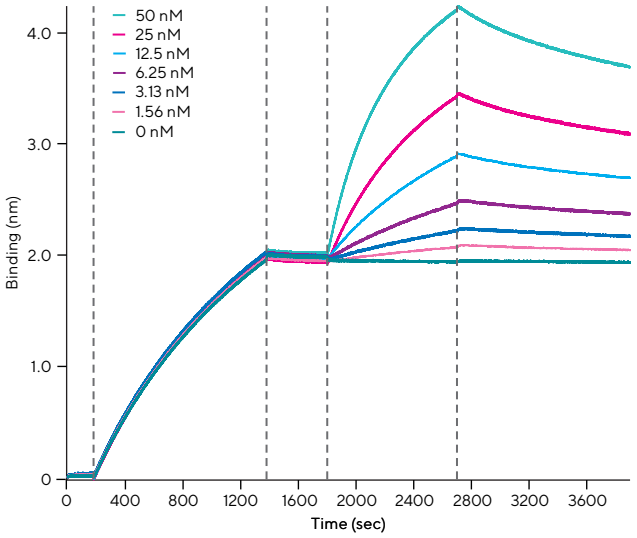
Process and Analyze Data

1. Load data into the Octet® Analysis Studio Software.
2. Process the data by specifying methods for reference subtraction, y-axis alignment by baseline, inter-step correction by dissociation and check the Savitzky-Golay filtering.
3. Analyze the data by specifying steps for analysis, fitting method (local or global) and time window of interest.
4. To export the analyzed data, click Save Report and select desired format, Excel or PDF.

Representative Data

Figure 4 shows kinetic analysis of the interaction between ligand hIgG and an analyte Fab fragment, Goat anti-hIgG (H+L) (50 kDa).

A.



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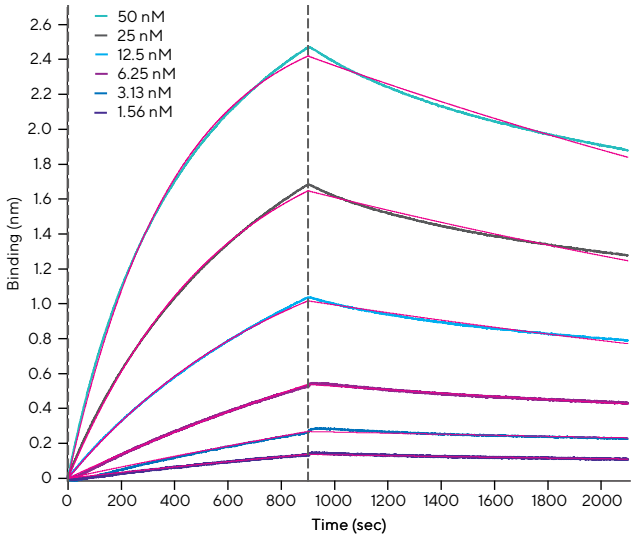


Figure 4: Kinetic analysis of the interaction between a ligand hIgG (150 kDa) and an analyte Fab fragment, goat anti-hIgG (H+L) specific (50 kDa), on the Octet® platform. (A) The raw data for a full assay. 1X Kinetics Buffer was used as a buffer matrix throughout the assay. (B) The association and dissociation traces after data processing (including reference subtraction using the 0 nM trace and fitting using a 1:1 binding model). The kinetic results are reported in Table 1.

Table 1: Kinetic results for the interaction between the ligand hIgG (150 kDa) and an analyte Fab fragment, goat anti-hIgG (H+L) specific (50 kDa), using the Octet® AHC2 Biosensors for the data shown in Figure 4.

k_{on} (1/Ms)	k_{off} (1/s)	K_D (M)
4.82E+04	2.33E-04	4.84E-09 M

Quantitation Assay Workflow

The Octet® AHC2 Biosensors have high specificity towards all four hIgG subclasses (IgG1, IgG2, IgG3 and IgG4) and can be used to quantitate both crude and purified hIgGs with concentrations in the range of 0.1–2,000 µg/mL depending on the assay conditions. For hIgG samples with concentration range of 0.5–2,000 µg/mL, it is recommended to use shaking speed of 400 RPM with 2-min assay read time. However, if hIgG concentration is in the range of 0.1–50 µg/mL, it is typically recommended to use 96-well plate as a sample plate, shaking speed of 1,000 RPM and a longer 5-min assay time for improved sensitivity.

Materials Required

- Octet® instrument with Octet® BLI Discovery and Analysis Studio Software
- Octet® AHC2 Biosensors, Part No. 18-5142
- For all Octet® instruments: 96-well, black, flat bottom microplate, Greiner Bio-One Part No. 655209
- Optional for Octet® RH16 and RH96 instruments:
  - Octet® 384-tilted well, black, flat bottom, polypropylene micro- plate, Part No. 18-5080 (pack); 18-5076 (case)
  - 384-well, black, flat bottom, polypropylene microplate, Greiner Bio-One Part No. 781209
- Purified standard protein (that is of the same molecule as the unknown samples) to be used as a calibration standard.
- Octet® Sample Diluent (Sartorius Part No. 18-1104) for dilution of all samples. If undiluted crude samples are to be quantified, a blank buffer (that is free of the molecules of interest) with the same buffer matrix is required.

Assay Optimization Tips

The following optimization steps are recommended each time when the quantitation assay involves a new matrix or a new human Fc containing protein.

- The calibration standard should be identical to the molecule present in the unknown sample for best results.
- Concentrations of the calibration standards should cover the range of concentrations in the unknown samples.
- Match the matrix of the samples, standards, references, and hydration solution as closely as possible.
- Use a blank negative control in a matching matrix for background signal subtraction. This is especially important when optimizing accuracy and detecting low-concentration analytes.
- Determine the minimal dilution factor required to achieve the targeted assay performance (applicable only when the target protein is in a complex matrix such as cell lysate).
- Perform a spike/recovery study to determine the assay dynamic range.
- Establish data analysis parameters in Octet® Analysis Studio Software.
- Apply the finalized protocol and data analysis parameters in routine assays.

Dilution Factor Determination for Sample Matrix

Components in complex matrices such as cell culture media can potentially interfere with assay performance. Diluting the sample matrix using the Octet® Sample Diluent is an effective means of minimizing matrix effects. Dilution factor guidelines for various sample types are described in Table 2. However, before running a quantitation assay it should be empirically determined whether dilution of samples is needed.

1. Prepare 1 mL of each sample matrix (without target protein) diluted both 2-fold and 10-fold in the Octet® Sample Diluent.
2. Add target protein to the neat matrix, each of the matrix dilutions, and to Sample Diluent as a control. The final concentration of target protein in each of the four samples should be in the middle of the desired quantitation range.
3. Transfer each sample to a 96- or 384-well sample plate in duplicate (eight wells total).
4. Hydrate biosensors in the sample matrix that matches each sample type (e.g., biosensors to be used in wells with 10-fold diluted matrix should be hydrated in 10-fold diluted matrix).
5. Set up a Basic Quantitation assay according to the Octet® BLI Discovery Software User Guide.
6. Run the assay.
7. Data will be displayed in real-time during the assay. Data and method files will be saved automatically to a location specified by user on the C: drive.
8. Load data into Octet® Analysis Studio Software.
9. Visually inspect the real-time binding traces and determine the dilution required to:
   a. Minimize non-specific binding of matrix components.
   b. Show equivalent binding in the matrix spiked sample and the Sample Diluent control.
10. Use this dilution factor for routine assays.

Table 2: Recommended minimum dilution for common sample types. In all cases the matrix for the diluted samples, the standards and the biosensor hydration solution should be matched as closely as possible.

Sample type	Minimum recommended dilution in sample diluent
hIgG in CHO media	Neat
hIgG in DMEM	2-fold
hIgG in DMEM with 10% FBS	Neat
hIgG in Octet® Sample Diluent	Neat
hIgG in PBS	2-fold

Recovery and Precision Assay To Determine Quantitation Range

1. Prepare a series of protein standards in the appropriate matrix diluent using the dilution factor determined in the Dilution Factor Determination for Sample Matrix experiment. The dilution series should span the entire range of the assay based upon user experimental goal, such as 0.5–2000 µg/mL for assays run at 400 RPM.
2. Using the same matrix diluent as in Step 1, prepare two protein samples of known concentration for recovery measurement. The concentration of these samples should be within the range of the standard curve being generated, preferably one at the low end and one at the high end. These will be defined as unknown samples in the assay for calculating recovery.
3. Transfer triplicates of the prepared standards and the samples and standards to a sample plate. It is recommended to organize samples in columns, from A–H. Fill at least one well with blank diluted matrix for reference subtraction during data analysis. An example plate map is shown in Figure 5.
4. Hydrate biosensors for 10 minutes in matching matrix diluent.
5. Set up a Basic Quantitation assay using the same assay parameters that were used in the Dilution Factor Determination for Matrix experiment. Define sample Replicate Groups in order to calculate replicate averages and %CVs.
6. Run the experiment. Data will be displayed in real time during the assay. Data files, method files and assay pictures will be saved automatically.
7. Load the data into Octet® BLI Analysis Software.
8. If a blank matrix was included as a reference, use the reference subtraction option to correct the data as appropriate.
9. Calculate the binding rate. The results table will populate with calculated concentrations and data statistics.
10. Define assay dynamic range by selecting acceptable %CV values for the lower and upper concentration limits in the standard curve.
11. Exclude data points for the standard curve that lie outside the defined dynamic range if necessary.
12. Select the appropriate equation to fit the standard curve. Linear point to point or 5PI with weighted Y2 are recommended for the AHC2.
13. Evaluate the accuracy and precision of the assay using calculated concentration value of the unknowns to determine % recovery and %CV.

Running the Assay to Quantify Proteins of Interest

1. Prepare samples, calibration standards and hydration solution according to the conditions determined in optimization steps in the prior sections.
2. Set up a Basic Quantitation assay using the parameters described previously in the optimization experiments. See Figure 6 for example assay set up.
3. Run the assay.
4. Load data into Octet® Analysis Studio Software. Analyze as in previous optimization steps to determine concentration of samples and data statistics.
5. To export the analyzed data, click Save Report and select desired format, Excel or PDF.

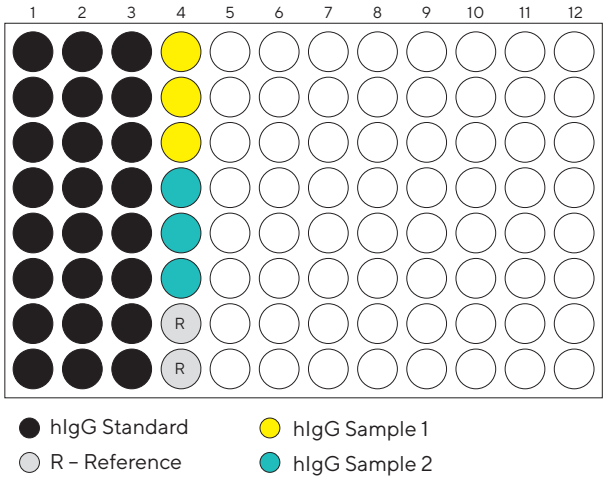


Figure 5: Example plate layout for a spike recovery assay.

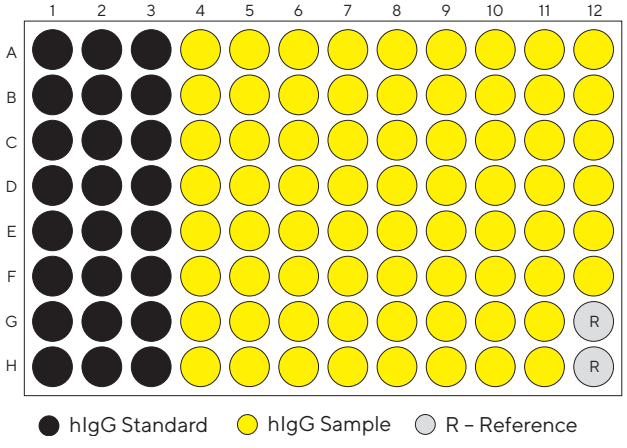
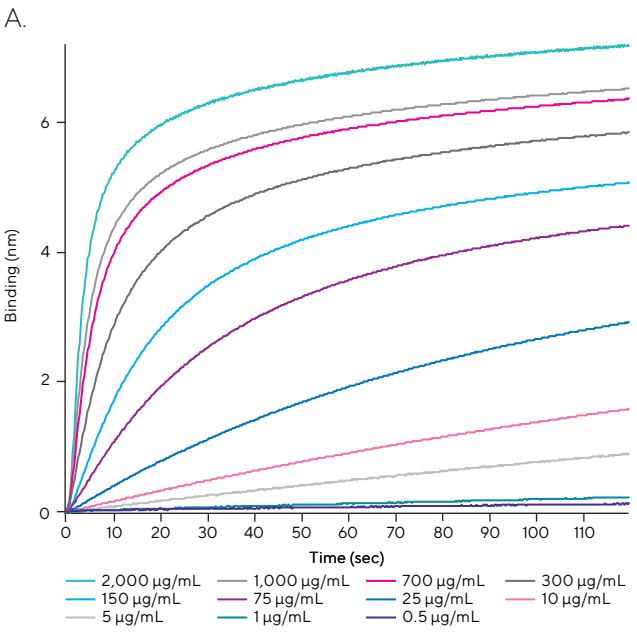


Figure 6: Example plate layout for a routine quantitation assay run in a 96-well microplate.

Representative Data

Figure 7 shows the detection of hIgG using the AHC2 biosensors on the Octet® RH16 system. A standard curve was run to demonstrate quantitation dynamic range (0.5–2,000 µg/mL).

When the sample concentration is in the range of 0.1–50 µg/mL, it is highly recommended to use 96-well plate as sample plate, 1,000 rpm shaking speed and a longer 5-min assay time for increased sensitivity. Figure 8 shows the detection of hIgG at low concentration using the AHC2 Biosensors on the Octet® RH16 system.



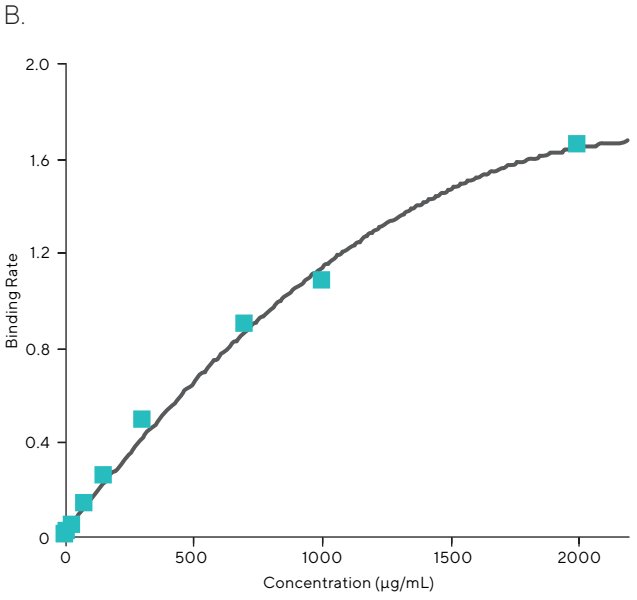
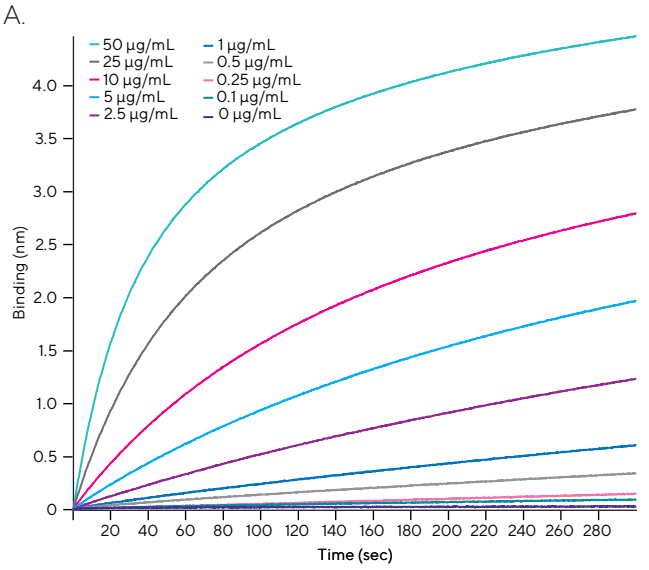


Figure 7: Quantitation of hIgG using the Octet® AHC2 Biosensors. (A) hIgG dose response for concentrations within the dynamic range of 0.5–2,000 µg/mL on the Octet® RH16 instrument with assay parameters: 400 rpm, 2 min. (B) hIgG standard calibration curve generated from triplicate samples and calculated using 5PL (weighted Y2) fitting model. The Octet® Sample Diluent Buffer was used as a matrix for all samples.



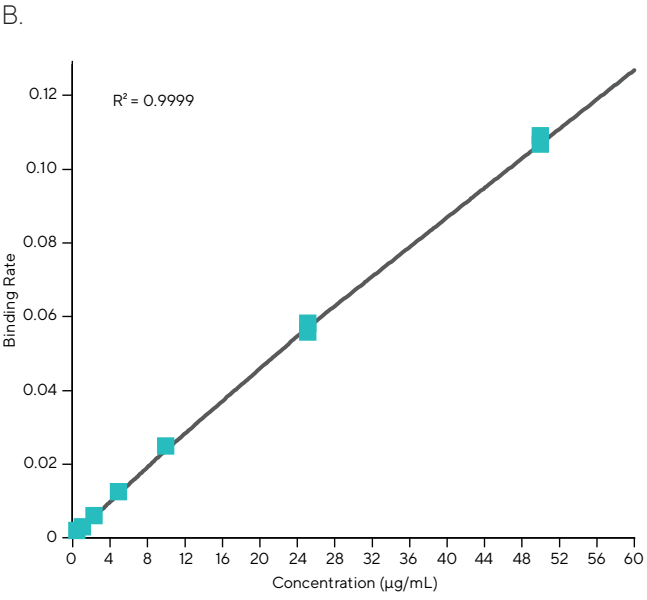


Figure 8: Quantitation of hIgG using the Octet® AHC2 Biosensors. (A) hIgG dose response for concentrations within the dynamic range of 0.1–50 µg/mL on the Octet® R8 system with assay parameters: 1,000 rpm, 5 min. (B) hIgG standard calibration curve generated from triplicate samples and calculated using linear point-to-point fitting model. The Octet® Sample Diluent Buffer was used as a matrix for all samples.

Regeneration of Octet® AHC2 Biosensors

The Octet® AHC2 biosensors can be cost-effectively regenerated and re-used up to 20 times in both kinetic and quantitation assays for generating replicate data for ligand-analyte pairs, or for analyzing large numbers of samples in sequence. The regeneration is performed by dipping the biosensors into a solution of 10 mM glycine with pH 1.7, for 5 seconds, followed by a dip in the assay buffer for 5 seconds. These regeneration steps should be repeated 3–5 times in sequence to fully remove bound hIgG or the interaction complex. After regeneration, the biosensor can be immobilized with hIgG for a new analysis. For best results it is recommended to pre-condition biosensors by running the regeneration protocol prior to loading the ligand the first time.

Regeneration results will depend on the captured molecule and a small loss in binding capacity may occur after each regeneration cycle. The exact number of possible regenerations should be determined experimentally and will depend on assay precision requirements. See example of kinetics and quantitation assays with 10 to 20 regeneration cycles in Figures 9 and 10, and Tables 3 and 4.

Table 3: The K_D and %CV for 20 cycles of regeneration for hIgG1-Fab anti hIgG kinetic assay.

Kinetic Assay	K_D (M)
Regen 1	1.38E-08
Regen 2	1.36E-08
Regen 3	1.37E-08
Regen 4	1.38E-08
Regen 5	1.39E-08
Regen 6	1.46E-08
Regen 7	1.39E-08
Regen 8	1.42E-08
Regen 9	1.41E-08
Regen 10	1.41E-08
Regen 11	1.44E-08
Regen 12	1.47E-08
Regen 13	1.45E-08
Regen 14	1.45E-08
Regen 15	1.46E-08
Regen 16	1.47E-08
Regen 17	1.47E-08
Regen 18	1.45E-08
Regen 19	1.44E-08
Regen 20	1.44E-08
Average (20 regenerations)	1.43E-08
%CV (20 regenerations)	2.6%

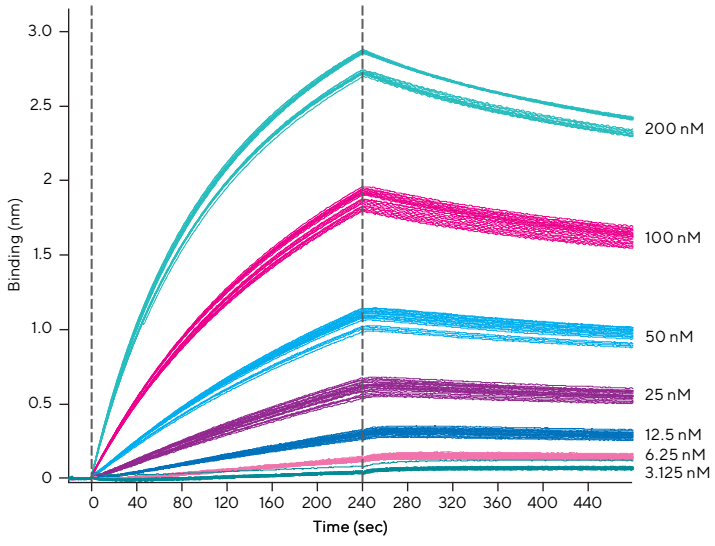


Figure 9: Overlay of association-dissociation curves for hIgG1-Fab anti hIgG kinetic assay after 20 regeneration cycles. The data traces overlap closely, with low variability between calculated binding and affinity constants from cycle to cycle in the kinetic assay.

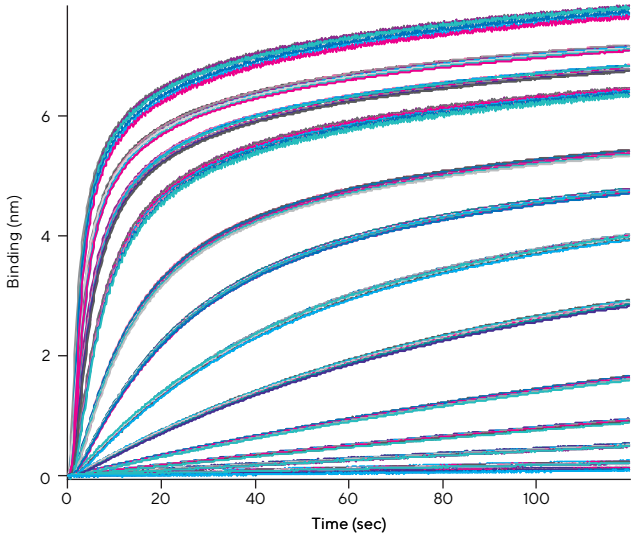


Figure 10: Overlay of binding curves for hIgG quantitation assay after 10 regeneration cycles.

Table 4: Calculated concentrations and %CV for 10 cycles of regeneration for hIgG quantitation assay.

Well Concentration, µg/mL	Average Calculated Concertation after 10 regenerations	%CV after 10 regenerations
2000	2052.29	8%
1000	975.92	5%
500	508.66	3%
200	199.03	2%
100	100.36	1%
50	51.16	1%
25	24.60	1%
10	9.92	1%
5	4.94	2%
2.5	2.61	5%
1	0.98	4%
0.5	0.50	6%

Regeneration Tips

- Regenerate the Octet® AHC2 Biosensor surface after a kinetic or quantitation assay by dipping the biosensors into 10 mM glycine pH 1.7 for 5 seconds followed by neutralization in assay buffer for 5 seconds, then repeating these regeneration steps three to five times.
- Depending on the assay conditions or protein being captured, the regeneration buffer and/or conditions may require additional optimization.
- It is recommended to pre-condition the biosensors before the first assay cycle for most consistent results when incorporating regeneration. Biosensors are pre-conditioned by performing the regeneration procedure one time prior to the first loading step.
- It is important to ensure that the regeneration of biosensors for quantitation applications is complete. This is because the quantitation results are significantly dependent on surface capacity of the sensor. For example, a loss of 20% capacity over multiple regeneration cycles could affect precision of quantitation by 10–20%.

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