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Application Note

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Second Generation Anti-Murine IgG Capture Biosensors (AMC2) Designed for Kinetic Characterization of Murine IgG – Antigen Interactions

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Abstract

Monoclonal antibodies (mAbs) can be derived from a wide variety of scaffolds and are therefore a commonly used tool in diagnostics and as therapeutics in both human and veterinarian treatment regimens. For example, in diagnostics, non-humanized antibodies from mice play a key role while in human therapy discovery, murine mAbs form an important source of leads which can then be further developed through humanization of the antibodies to mitigate unwanted immune response in the human host. The Octet® Bio-Layer Interferometry (BLI) platform offers rapid, label-free and high-throughput characterization of antibodies and has become a workhorse in antibody-based drug discovery and reagent development programs. The second-generation Octet® AMC2 Biosensors enable scientists to extend the usage of the anti-mouse capture biosensors to applications such as measurement of high antibody titer (a critical quality attribute), hybridoma screening, kinetic characterization, epitope binning or diversity assays. AMC2 Biosensors provide higher ligand binding capacity, compatibility with crude and purified samples and increased stability which allows several regeneration cycles, providing a significant reduction in the cost per assay. The increased ligand binding capacity enables the creation of high-density ligand surfaces which allows the user to measure binding kinetics of lower molecular weight biologics such as low molecular weight proteins and peptides while also increasing the dynamic range of quantitation assays. AMC2 Biosensors bind to all subclasses of mouse IgGs (mIgG) and show 2-3 times enhanced binding capacity to mIgG compared to the first-generation AMC (Anti-Mouse Fc Capture) Biosensors. This application note discusses the key advantages of the Octet® AMC2 Biosensors and presents data generated at multiple user sites.

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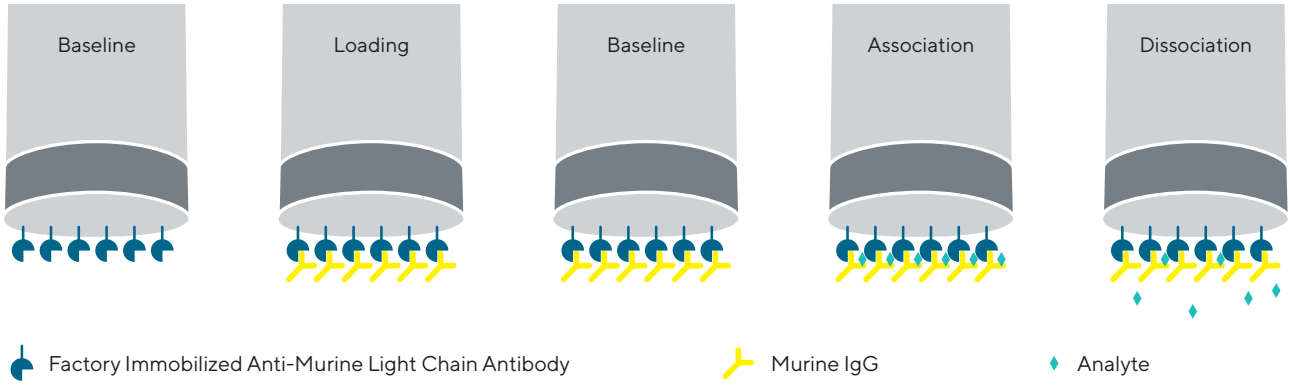
Introduction

Antibodies continue to be an important class of therapeutics being developed within the broader domain of biological therapeutics largely due to their high efficacy, target specificity and lower adverse effects. Mouse derived antibodies have been the backbone of many developments for human use. As of 2022, about 162 antibody therapies have been approved for clinical use globally of which about 50% are of murine origin (humanized, chimeric and whole antibodies)[1]. Further, advancements in the modalities including humanized transgenic mice, single B cell-based antibody discovery and technology improvements around antibody engineering such as bi- and tri-specific antibodies, and antibody drug conjugates (ADCs) continue to make mAbs an attractive therapeutic modality[2].

This application note discusses a second-generation anti-murine antibody-based biosensor, the Octet® AMC2 Biosensor, which can support different stages in antibody development workflows such as early phase discovery, cell-line development, process development and manufacturing. AMC2 Biosensors were developed to serve as a single biosensor for use in both quantitation and kinetics applications. Further, the application note presents and discusses the results of a series of experiments performed at Sartorius and customer sites evaluating the performance of the AMC2 Biosensors and comparing their use with the first generation anti-mouse biosensor (AMC Biosensors) in kinetics applications.

Key features of the AMC2 Biosensors include an increased mouse IgG binding capacity to multiple mouse IgG (mIgG) subclasses, and improved biosensor regeneration and re-use abilities. Figure 1 illustrates the assay workflow for the use of the AMC2 Biosensors in mIgG kinetics applications on the Octet® BLI detection platform.

Figure 1: *AMC2 Kinetics Assay Workflow*



Note: Kinetic assay workflow using the Octet® AMC2 Biosensors typically includes the following steps: 1 – baseline 1 (biosensor equilibration), 2 – loading (capture) of mIgG, 3 – baseline 2, 4 – association phase, 5 – dissociation phase.

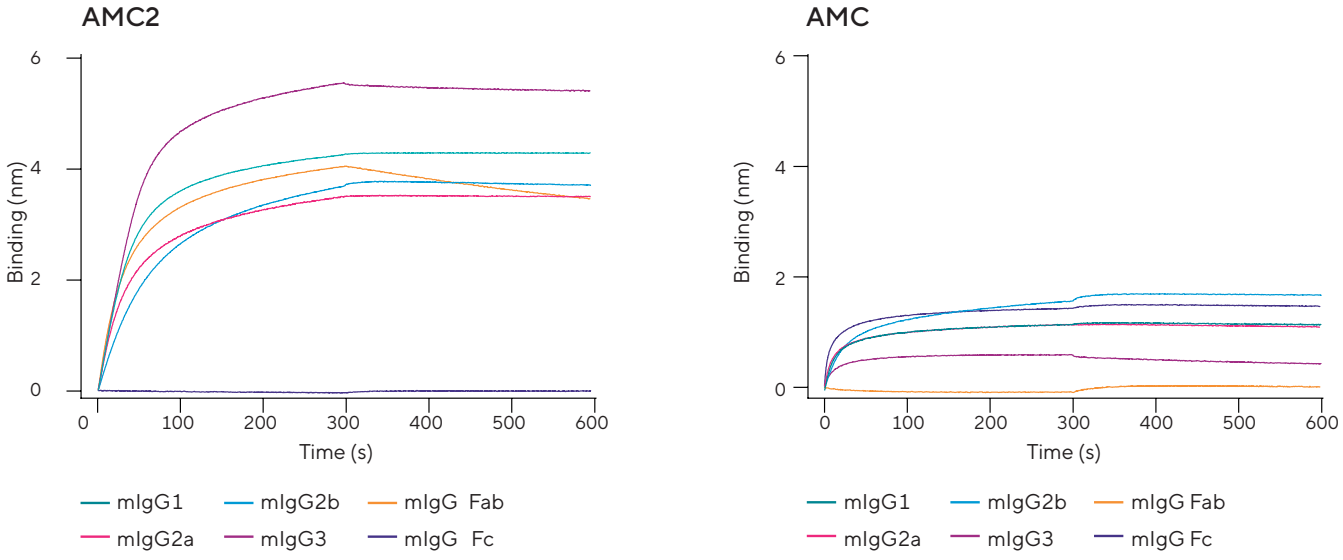
AMC2 Biosensors bind to the constant domain of kappa light chains on all murine IgG subclasses with high specificity. The binding to kappa light chain does not occlude the CH2 (Fc binding domain) of the captured mIgG (**Reference: Octet® AMC2 Biosensors Technical Note**). Table 1 highlights the subclass specificity and compares the key features of the AMC2 and AMC Biosensors. AMC2 Biosensors show a 2-3-fold higher mIgG binding signal for all mIgG subclasses compared AMC and up to 10-fold increased mIgG binding capacity in the case of mouse IgG3 compared to AMC biosensors (Figure 2).

Table 1: Comparison of the Performance Features of the Octet® AMC2 and Octet® AMC Biosensors

Performance Feature	Octet® AMC2 Biosensor	Octet® AMC Biosensor
Specificity	mouse IgG (IgG1, IgG2a, IgG2b and IgG3) and rat IgG (IgG1, IgG2a, IgG2b and IgG2c) subclasses	mouse IgG1, IgG2a, IgG2b and IgG3*
Biosensors' Binding Site on Antibody	Kappa light chains**	Fc domain
IgG Loading Capacity	High	Low
Biosensor Regeneration	Yes	Yes

* Octet® AMC Biosensors binds to mIgG3 with low affinity. It is recommended to evaluate the binding of mIgG3 to AMC Biosensors on a case-by-case basis.
** Octet® AMC2 Biosensors do not bind to antibodies with lambda light chains.

Figure 2: *mIgG Binding Capacity and IgG Subclass Specificity of AMC2 vs AMC*



Note: A binding assay was performed to test the ligand binding capacity and binding specificity of mouse IgG subclasses (mIgG1, mIgG2a, mIgG2b and mIgG3), mIgG Fab and mIgG-Fc domain to AMC2 and AMC biosensors. All samples were tested at 20 µg/mL.

Material and Methods

Materials

Instruments:

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

Biosensors:

- Octet® AMC (Anti-mouse IgG Fc capture) Biosensors for kinetics assays, Cat. Nos. 18-5088 (tray); 18-5089 (pack); 18-5090 (case)
  Octet® AMC2 Biosensors for kinetics and quantitation assays Cat. Nos. 18-5163 (tray), 18-5164 (pack), 18-5165 (case)

For all Octet® BLI systems:

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

Optional for Octet® RH16 and RH96 BLI systems:

- 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

Assay Buffers:
The biosensors are compatible with a wide range of buffers. For kinetics assays, Octet® 10X kinetics Buffer (Sartorius part no. 18-1105) or 1X Kinetics Buffer (dilute 10X Kinetics buffer 1:10 with PBS, pH 7.4) are recommended. Best results are obtained when all matrices are matched as closely as possible.

Methods

The kinetics assays used in the different studies followed the recommended Octet® BLI workflow, which included an initial biosensor hydration step of the AMC2 Biosensor performed prior to the start of an assay where, the biosensors are dipped in assay buffer for a minimum of 10 minutes. The hydrated biosensors were used in the assay as designed in Octet® BLI Discovery software.

All biosensors were regenerated at the end of each kinetic measurement, where the biosensors were dipped for 3-5 cycles alternating between the regeneration buffer and assay buffer. Appropriate reference sensors and buffer blank controls were included in the assays.

Note: It is recommended that regeneration optimization is performed prior to sample measurement and the default regeneration parameters modified within the Octet® BLI Discovery software as required.

Binding Kinetics Assay

The kinetics assay workflow with AMC2 Biosensors followed the typical assay steps used with an affinity capture strategy (Figure 1). As noted previously, prior to the start of the assay, AMC2 Biosensors were hydrated in the assay buffer for a minimum of 10 minutes.

The kinetics assay setup and analysis workflow included the below steps

1. Initial biosensors equilibration step (baseline 1): 180s
2. Capture of the ligand (loading): 120-600s
3. Wash / baseline 2 step in assay buffer (to monitor the stability of the ligand capture): 300-600s
4. Association phase to measure the binding of the antigen (using a 2- or 3-fold dilution series) to the captured mIgG: 300-900s
5. Dissociation phase of the IgG-Antigen complex in assay buffer: 300-3600s.
6. Biosensor regeneration for re-use: Biosensors were dipped into 10mM - 100mM phosphoric acid buffer for 60 seconds followed by a dip into assay buffer for 5s. This process was repeated for 3-5 cycles.
7. All kinetics assays were performed at a shake speed of 1000 rpm at 25°C-30°C..
8. Data was analyzed with the Octet® Analysis Studio software using standard data pre-processing steps followed by applying a suitable model fitting algorithm (1:1 model with local or global fitting).

Note: The specific assay steps times used at the different test sites were modified depending on the assay conditions and model system used.

Results and Discussion

The capabilities of the Octet® AMC2 Biosensors were evaluated to assess the performance in kinetic assays and the results were compared to the first-generation anti-mouse Fc capture biosensor (AMC).

Kinetic Characterization of Mouse IgG - Antigen Interactions

A key attribute considered during the development of the AMC2 biosensors was an increase in binding capacity compared to first-generation AMC Biosensors. The ability to bind a higher amount of mIgG enabled the design of mIgG quantitation assays with a broad dynamic range as well as to allow the users to design kinetic assays to characterize lower molecular weight biologics interaction to the mIgGs, which typically requires a higher density of the mIgG captured onto the biosensors to yield a

measurable binding response. When designing binding assays to determine the kinetic rate constants (association, k_a and dissociation, k_d) and affinity (K_D), assay design aspects such as avidity, assay orientation, ligand density (load) on the biosensors, binding valency, baseline stability etc., should be carefully considered (**See Application Note: Optimizing Kinetics Assays to Avoid Avidity Effects**).

The recommended assay orientation when studying antibody-antigen interactions is to have the antibody captured onto the biosensors such that the two arms of the antibody are available to bind to the antigen in a 1:1 binding stoichiometry. With a capture-based ligand loading strategy (as with AMC2 Biosensors), where an affinity capture molecule on the biosensor is employed to capture (load) the ligand molecule (mIgG). A stable capture of the ligand molecule with minimal baseline drift is imperative to generate robust kinetics assays. AMC2 Biosensors provide a stable capture of mIgG and were observed to have a better baseline stability (post capture of the ligand molecule) as compared to the first-generation AMC Biosensors. Further, a loading optimization assay would have to be performed to determine the ideal load density of the ligand on the biosensor and the increased ligand binding capacity enables one to test a broader range of ligand densities.

Note:

1. AMC2 Biosensors show weak binding to human and monkey IgG and therefore, this should be considered if human or monkey antibodies are to be used as the analyte. No cross-reactivity was observed with IgGs from other species, such as bovine, rabbit, goat and sheep.

2. Readers are encouraged to refer to the **Octet® AMC2 Biosensors Technical Note** for a detailed discussion on quantitation assays on the AMC2 Biosensors.

Kinetic assay studies comparing the performance of the AMC2 Biosensors were performed at Sartorius (USA and China), Adimab (USA) and Eli Lilly and Co., (USA). The kinetic assay setup followed the workflow employed with affinity capture biosensors (as described in the Methods section).

Binding Kinetics as a Function of Load Density (Load Response)

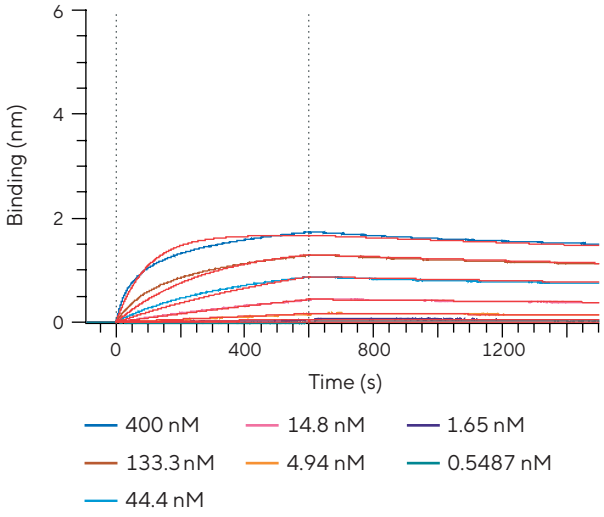
Ligand density on the biosensor plays an important role in the outcome of the binding kinetics of a biomolecular interaction. It is therefore good practice to perform a load optimization assay to determine the optimal ligand density prior to the setup of a full kinetic characterization assay. In addition, the ligand loading optimization assay provides information on the assay conditions, optimal analyte concentration, analyte binding response, non-specific binding (NSB) of the analyte to the biosensors surface and non-ideal binding behavior. Typically, ligand load optimization is performed by testing the binding of the analyte at a single concentration (~10x K_D) to different ligand densities (**See Application Note: Biomolecular Binding Kinetics Assays on the Octet® BLI Platform**). However, to assess the effect of ligand density on the binding kinetics, a detailed analysis of binding kinetics using a 3-fold diluted concentration series (400 nM – 0.54 nM) of anti-mIgG Fab (50kDa) was tested to generate the dose response sensorgrams (Figure 3A).

A range of ligand densities from 0.26 nm to 3.58 nm were tested. No significant difference was observed in the association rate (k_a), dissociation rate (k_d) and affinity (K_D) of the interaction (Table 2) suggestive of no/minimal interference from assay artifacts such as avidity, NSB etc. (as described previously). For example, avidity driven interactions typically show a change in the dissociation rate (k_d) at different ligand density, progressing from a pseudo slow off-rate, an artifact of higher ligand density to a faster and measurable value at low ligand density (**See Application Note: Optimizing Kinetics Assays to Avoid Avidity Effects**). Figure 3B shows the trend in the kinetics parameters of affinity (K_D), association rate (k_a) and dissociation rate (k_d) as a function of different mIgG load densities. The coefficient of variance (%CV) of the kinetics parameters measured were observed to be 8.80% (k_a), 2.98% (k_d) and 8.50% for the affinity (K_D).

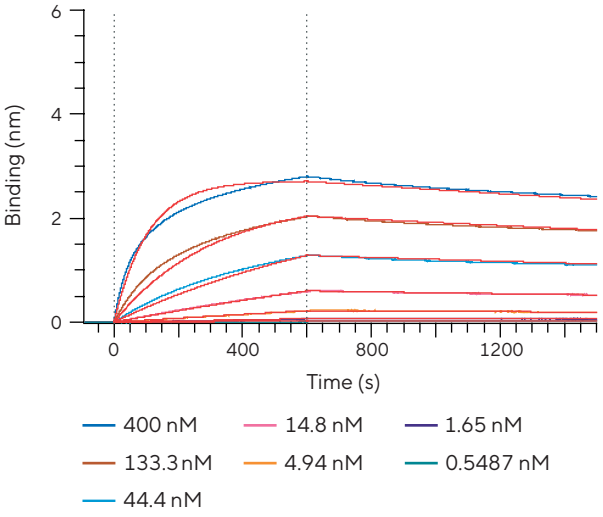
Figure 3: *Effect of Loading Density on the Binding Kinetics Parameters*

A. *Average Load Response (Loading Density):*

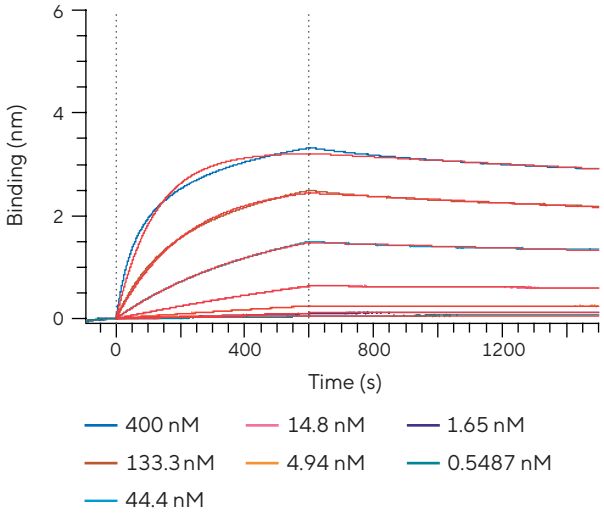
I. Load response: 0.26nm:



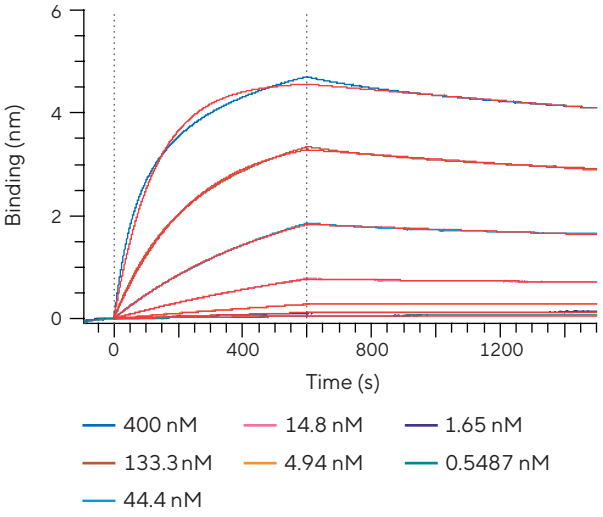
II. Load response: 0.47nm:



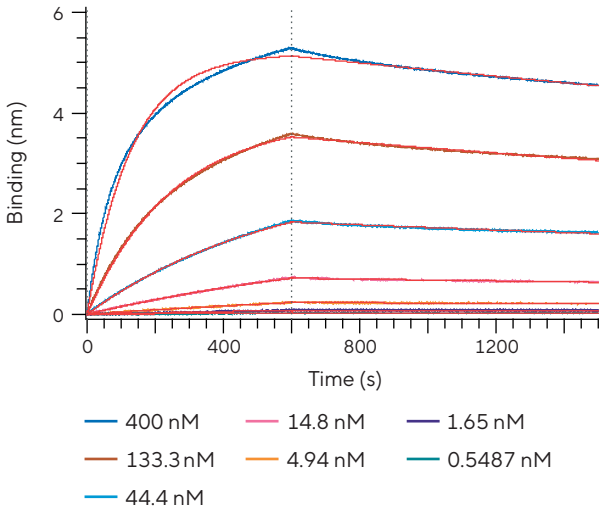
III. Load response: 1.02nm:



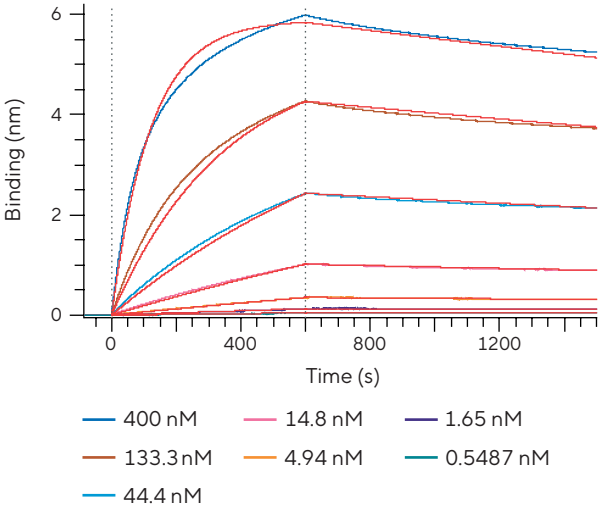
IV. Load response: 1.63nm:



V. Load response: 2.31nm:



VI. Load response: 3.58nm:



B. *Effect of Loading Density on the Measured Binding Affinity (K_D), Association rate (k_a) and Dissociation rate (k_d)*

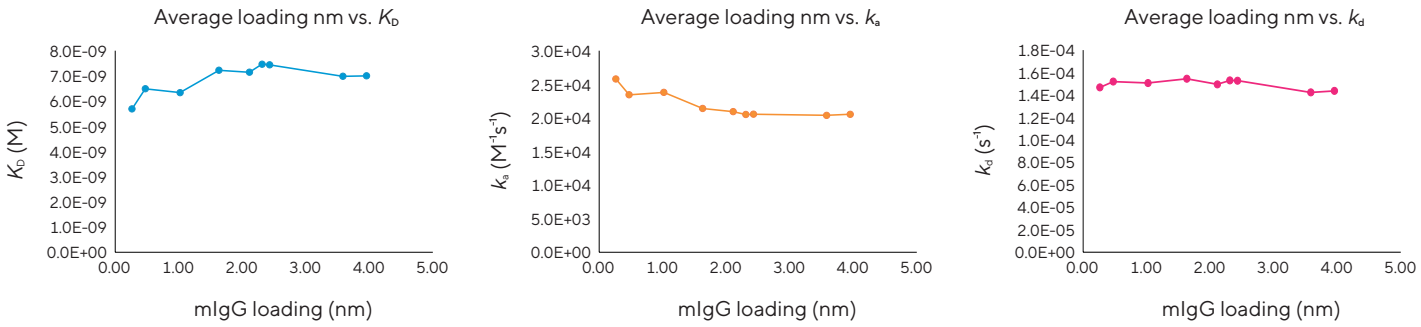


Table 2: Effect of Loading Density on the Binding Kinetics Parameters

mIgG loading (nm)	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
0.26	2.6E+04	1.5E-04	5.7
0.47	2.3E+04	1.5E-04	6.5
1.02	2.4E+04	1.5E-04	6.3
1.63	2.1E+04	1.5E-04	7.2
2.11	2.1E+04	1.5E-04	7.1
2.31	2.1E+04	1.5E-04	7.5
2.43	2.1E+04	1.5E-04	7.4
3.58	2.0E+04	1.4E-04	7.0
3.95	2.1E+04	1.4E-04	7.0
Average	**2.20E+04**	**1.48E-04**	**6.9**
Standard Deviation	**1.94E+03**	**4.41E-06**	**0.6**
%CV	**8.80**	**2.98**	**8.50**

Note: A. Ligand load optimization assay. mIgG was captured onto AMC2 Biosensors at a concentration of 10-20 µg/ml with a loading time of 20s, 60s, 150s, 250s and 470s to achieve different mIgG ligand densities. The antigen (anti-mIgG Fab, 50 kDa) was prepared by a 3-fold serial dilution in 10x KB from 400 nM - 0.54 nM to generate the dose-response curves. B. Plot showing the effect of loading density on the kinetics parameters of affinity (K_D), association rate (k_a) and dissociation rate (k_d). The assays were performed on an Octet® RH96 at 30°C at a shake speed of 1000rpm. Kinetics parameters were determined by applying a 1:1 Global fit model. Data generated at Sartorius, USA.

Demonstration of the Binding Specificity of AMC2 to mIgG Subclasses

The broad mIgG subclass specificity of the AMC2 biosensors allows the user to assess a wider range of mIgGs. To illustrate the high binding specificity of the AMC2 Biosensors to mIgG3 subclass compared to the AMC biosensors, which show weak to variable affinity to mIgG3s, an assay was set up to test the binding kinetics of anti-CD314 mIgG3 to CD314 (NKG2D) a C-type lectin-like receptor involved in CD8+ T-cell-mediated adaptive immune responses[3]. While the kinetics parameters of k_a, k_d, K_D measured on AMC and AMC2 Biosensors were comparable, the statistical parameters (R^2, Chi^2, residual and model fit) were observed to be improved in the assay format using AMC2 Biosensor (Figure 4, Table 3). An approximately 10-fold higher mIgG3 ligand load was achieved on the AMC2 Biosensors (load ~5.3 nm) as compared to the AMC Biosensors (load ~0.55 nm), which resulted in a higher analyte binding response with better signal-to-noise, which enabled an improvement in overall data fitting, especially at lower analyte concentrations on AMC2 Biosensors compared to AMC Biosensors.

Figure 4: *Binding Kinetics of 23kDa Antigen (CD314) to Immobilized Anti-CD314 mIgG3 on AMC and AMC2 Biosensors*

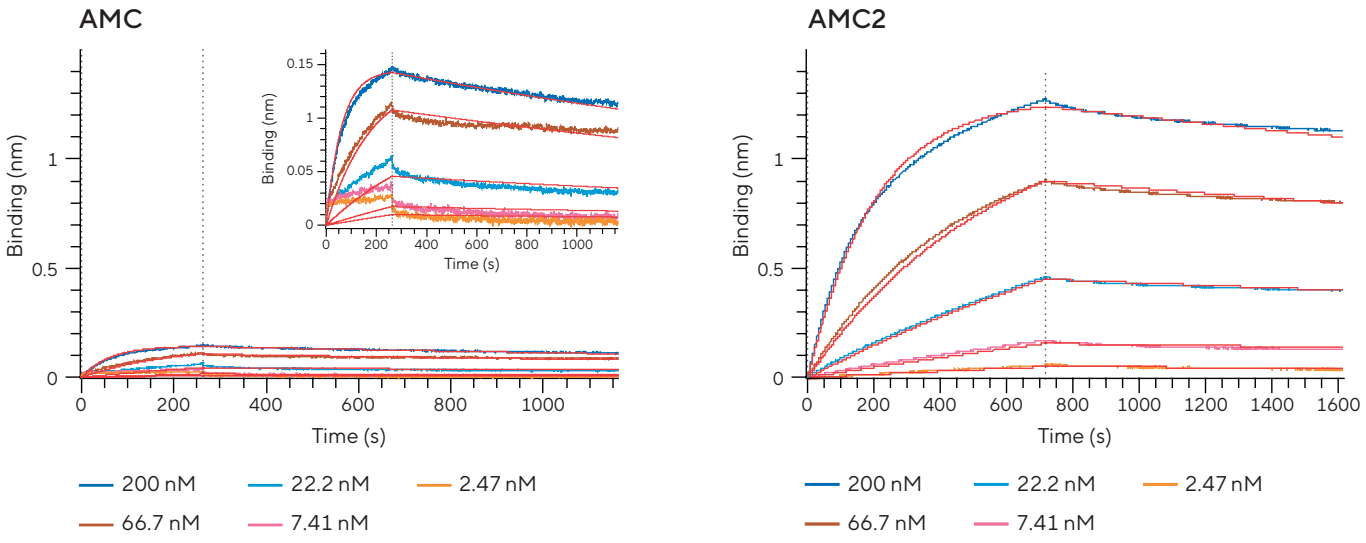


Table 3. Kinetic Characterization of the Binding of Anti-CD314 mIgG3 to CD314 Antigen

Biosensor type	Sample ID	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	Full R^2	Avg. Load response (nm) at 10 µg/ml of mIgG ligand	Analyte binding response (nm) at 200 nM
AMC	CD314	8.12E+04	3.05E-04	3.75E-09	0.9689	0.51	0.14
AMC2	CD314	2.45E+04	1.33E-04	5.43E-09	0.9989	5.14	1.35

Note: Binding kinetics of CD314 antigen (23kDa) to captured anti-CD314 mIgG3 ligand on AMC and AMC2 Biosensors. The mIgG3 was captured at 10 µg/ml while the CD314 antigen was 3-fold serially diluted in 10X KB from 200 nM – 2.46 nM to generate the dose-response curves. The assay was performed on the Octet® RH96 (8-channel mode) at 30°C and a shake speed of 1000 rpm. The kinetics parameters were determined by applying a 1:1 Global fit model. Data generated at Sartorius, USA.

Binding kinetics of the interaction between mIgG2a anti-CD147 and its antigen CD147 is shown in Figure 5. CD147, also known as basigin or EMMPRIN is a transmembrane glycoprotein of the immunoglobulin superfamily. CD147 and is known to participate in tumor development, plasmodium invasion, and bacterial and virus infection. Recent studies have shown that CD147 could be an alternative route for SARS-CoV2 infection in the lungs[4]. Blocking CD147 with an antibody (Meplazumab, a humanized anti-CD147 immunoglobulin 2 (IgG2) monoclonal antibody) was shown to reduce SAR-CoV2 nfectivity[5].

In the Octet® BLI kinetics assay, a higher analyte binding response (~3X) was observed with AMC2 Biosensors, which is commensurate with the higher ligand binding capacity of the Biosensor (Table 4). The binding kinetics of the interaction is independent of biosensor type used as AMC and AMC2 Biosensors generated comparable values (Table 4). Thus, providing the user the choice of two biosensor types to pick from depending on the IgG-antigen samples tested.

Figure 5: *Binding Kinetics of 21 kDa Antigen (CD 147) to Captured Anti-CD147 mIgG2a Tested on AMC and AMC2 Biosensors*

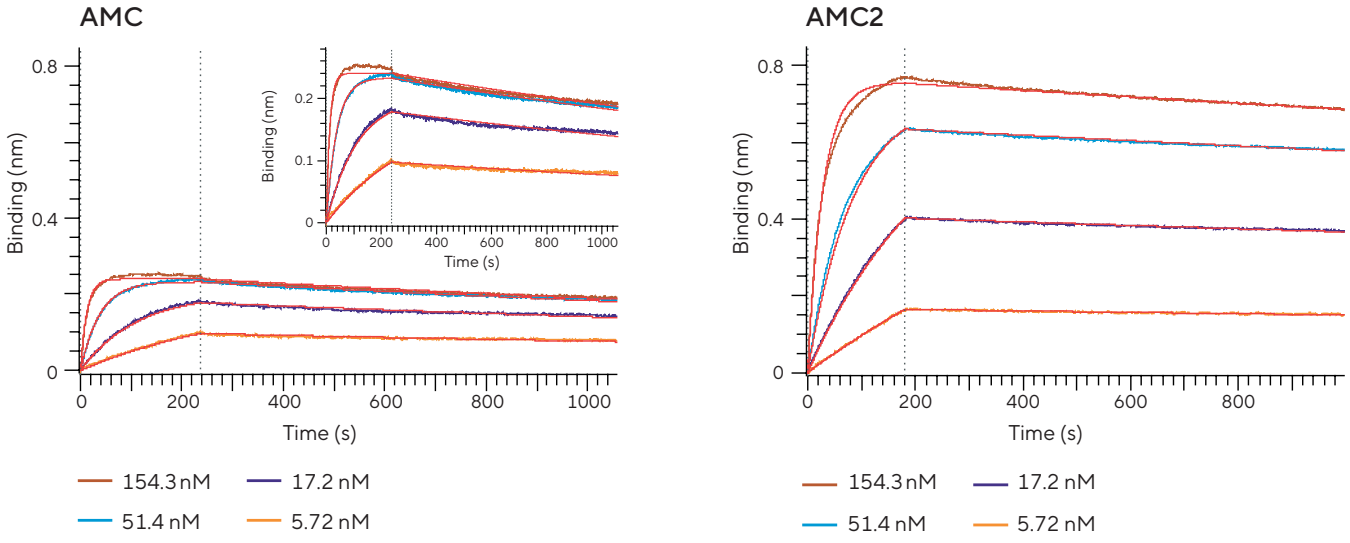


Table 4. Kinetic Characterization of Anti-CD147 mIg2a to CD147 Antigen

Biosensor type	Sample ID	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	Full R^2	Avg load response (nm) at 10 µg/ml of mIgG	Analyte binding response (nm) at 154 nM
AMC	CD147	5.090E05	3.066E-04	6.023E-10	0.9957	1.47	0.24
AMC2	CD147	2.277E05	1.158E-04	5.086E-10	0.9991	3.37	0.76

Note: Binding kinetics of CD147 antigen (21.6 kDa) to captured mIgG2a anti-CD147 ligand on AMC and AMC2 Biosensors. Anti-CD147 mIgG2a was captured at 10 µg/ml and CD147 antigen was prepared using a 3-fold serial dilution in 10X KB from 463 nM – 5.71 nM to generate the dose-response curves. The assay was performed on an Octet® RH96 (8-channel mode) at 30°C and a shake speed of 1000 rpm. The kinetics parameters were determined by applying a 1:1 Global fit model. Data generated at Sartorius, USA.

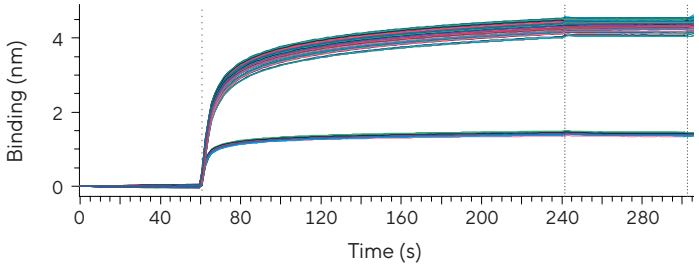
Kinetic Characterization of High Affinity mIgG-Antigen Interactions

Affinity capture provides a strategy to create a homogenous 'oriented ligand surface', where the ligand is captured onto the biosensors via a specific binding site, for example, via the Fc, Fab or Fv domains of an antibody or site directed tags such as N- or C-terminal His-tags and biotin tags. This is different to direct immobilization techniques such as amine reactive chemistry where the ligand is covalently attached onto the biosensors in a random orientation, which creates a heterogenous surface. A critical factor to consider when working with high affinity interactions is to ensure a stable capture of the ligand to the affinity capture surface. A mIgG screening experiment was performed at Adimab, USA to evaluate the performance of AMC and AMC2 Biosensors to screen for high affinity antibodies produced in CHO cells. To exemplify the screen, three mIgG1 produced in CHO cells were purified and captured onto AMC and AMC2 Biosensors and the binding kinetics to a 38 kDa protein antigen assessed. Figure 6A shows the binding of the three mIgG1s to AMC2 and AMC Biosensors. Both biosensor types showed a stable capture of the mIgG1s, however, the load density was observed to be ~3x higher on the AMC2 biosensor.

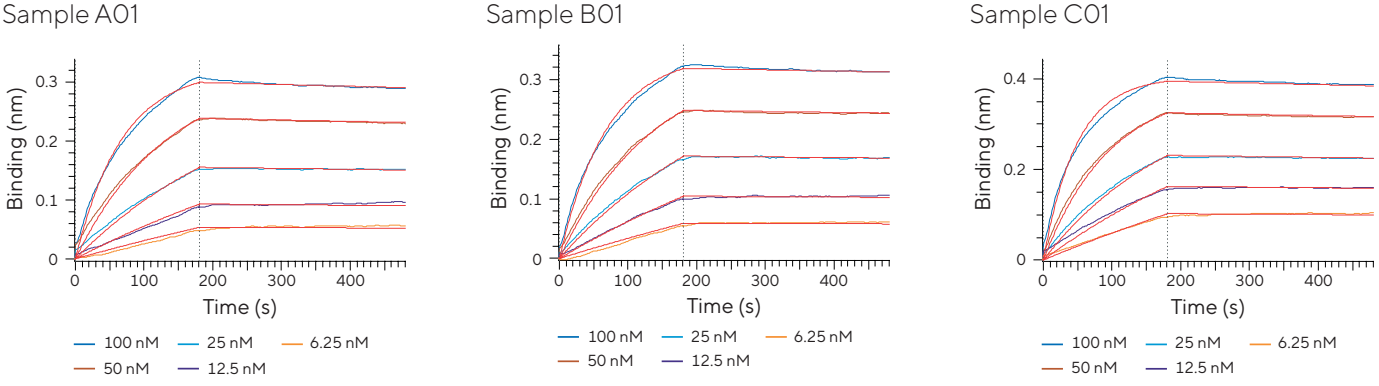
The three mIgG samples tested showed a high affinity to the antigen with nanomolar to picomolar affinity (Figure 6B). Table 5 presents the kinetics parameters determined using AMC2 and AMC Biosensors. The affinity (K_D) measured with AMC and AMC2 biosensors were comparable. However, the binding kinetics (k_a and k_d) were observed to be different with the AMC and AMC2 biosensors. Closer evaluation of the data showed that the differences in the binding kinetics could be attributed to poor data fitting due to low analyte binding response with the AMC biosensor (Data not shown). AMC2 biosensors provided good estimates of the relative K_D values for the high affinity antibodies tested in the study.

Figure 6: *Binding Kinetics of 38 kDa Protein Antigen to mIgG1 Captured on AMC2 and AMC Biosensors*

A. *Loading of mIgG on AMC and AMC2 Biosensors*



B. *Binding Kinetics of mIgG1 to a 38 kDa Protein Antigen*



Note: A. Loading of purified mIgG1 samples expressed in CHO cells onto AMC2 and AMC Biosensors. B. Binding kinetics of 38 kDa protein antigen to captured mIgG1 ligand on AMC and AMC2 Biosensors. The antigen was 2-fold serially diluted in 1X KB from 400 nM – 6.25 nM to generate the dose-response curves. The assay was performed on an Octet® RH96 (32-channel HT mode) at 25°C and a shake speed of 1000 rpm. The kinetics parameters were determined by applying a 1:1 Global fit model. Data generated at Adimab, USA.

Table 5. Kinetic Characterization of mIgG Produced in CHO cells

Biosensor type	Load sample	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})*	K_D (M)	RSS	Full R^2	Avg Load Response (nm)	Analyte binding response (nm) at 100 nM
AMC	A01	8.36E+04	1.71E-04	2.05E-09	0.0259	0.9784	1.34	0.10
AMC	B01	4.24E+04	1.71E-04	4.03E-09	0.0159	0.9883	1.36	0.10
AMC	C01	2.92E+04	1.71E-04	5.86E-09	0.0319	0.9846	1.18	0.11
AMC2	A01	1.47E+05	1.71E-04	1.16E-09	0.0303	0.9976	3.75	0.31
AMC2	B01	1.45E+05	1.71E-04	1.18E-09	0.0217	0.9985	4.04	0.32
AMC2	C01	2.06E+05	1.71E-04	8.30E-10	0.0568	0.9971	3.89	0.40

* The dissociation rate (k_d) values shown in the table correspond to a minimal decay in response during the dissociation phase and the assigned k_d values correspond to a response decay of 5.0%. The data was processed based on the customers internal analysis protocol.

Note: When testing high affinity interactions, it is recommended to design a kinetics assay with a longer dissociation time (usually 600-3600 seconds) to allow at least a 5% decrease in the overall dissociation response which is required by the software's fitting algorithm to generate a measurable and reliable dissociation rate.

High ligand Binding Capacity of AMC2 Biosensors: Evaluation of the Binding Kinetics of mIgG - Low Molecular Weight Protein Interaction

The binding response observed on Octet® BLI systems is a function of the size of the analyte, amount of ligand on the biosensor surface, conformation and amount of analyte bound to the ligand. The increased ligand binding capacity of AMC2 Biosensors allows the user to test the binding kinetics of low molecular weight protein analytes to mIgG, a challenge faced when working with AMC Biosensors. To evaluate the suitability of AMC2 Biosensors to measure low molecular weight protein binding, experiments were performed at Eli Lilly, USA to determine the kinetics of the interaction between a 6 kDa protein antigen with mIgG1. Figure 7 presents the results of the binding assay. The mIgG1 ligand was loaded on to AMC and AMC2 Biosensors at a concentration of 10 µg/ml. An average mIgG1 load of 4.60 nm was achieved on AMC2 Biosensors compared to 1.0 nm on AMC Biosensors, resulting in a higher analyte binding response, a better signal-to-noise ratio and cleaner dose-response curve on AMC2 Biosensors. The antigen binding response achieved with AMC2 Biosensors was observed to be approximately 2x higher than that achieved with AMC Biosensors and not reflective of the higher ligand density achieved on AMC2 Biosensors (Table 6). This difference could be attributed to the factors contributing to the response signal on the Octet® BLI system. As discussed earlier, the Octet® BLI binding signal is a function of the thickness and density of the biolayer bound on the tip of the biosensor. Therefore, we could hypothesize that the binding of the 6kDa antigen on the mIgG1 is oriented such that it does not produce a large increase in the optical thickness / density resulting in a small binding signal.

The affinity (K_D) of the interaction showed a 10-fold difference between the measurement on AMC Biosensors (K_D: 1.84E-10) vs AMC2 Biosensor (K_D: 2.13E-09) (Table 6). A closer evaluation of the kinetics parameters reveals a significant difference in both the association rate (k_a) and dissociation rate (k_d) measured with the AMC and AMC2 Biosensors. Further, the increased k_a and slower k_d with AMC Biosensors were observed to be an artifact of lower binding response and the model fitting process on the data generated using AMC Biosensors.

Figure 7: *Binding Kinetics a 6 kDa Protein Antigen to Captured mIgG1 on AMC and AMC2 Biosensors*

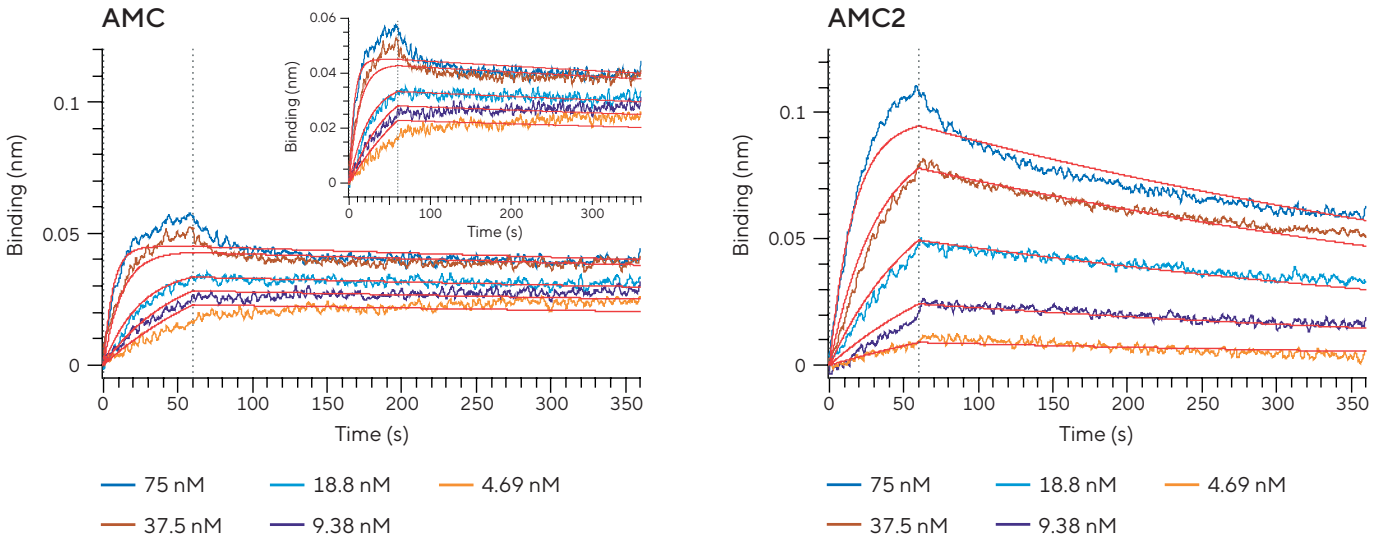


Table 6. Kinetic Characterization of the Binding of mIgG1 – Antigen (6 kDa)

Biosensor	k_a ($M^{-1}s^{-1}$)*	k_d (s^{-1})	K_D (M)	RSS	Full R^2	Avg. Loading response (nm) at 10 µg/ml of mIgG	Analyte binding response (nm) at 75 nM
AMC	2.15E+06	3.96E-04	1.84E-10	0.0652	0.9283	1	0.06
AMC2	7.86E+05	1.67E-03	2.13E-09	0.0832	0.9874	4.89	0.11

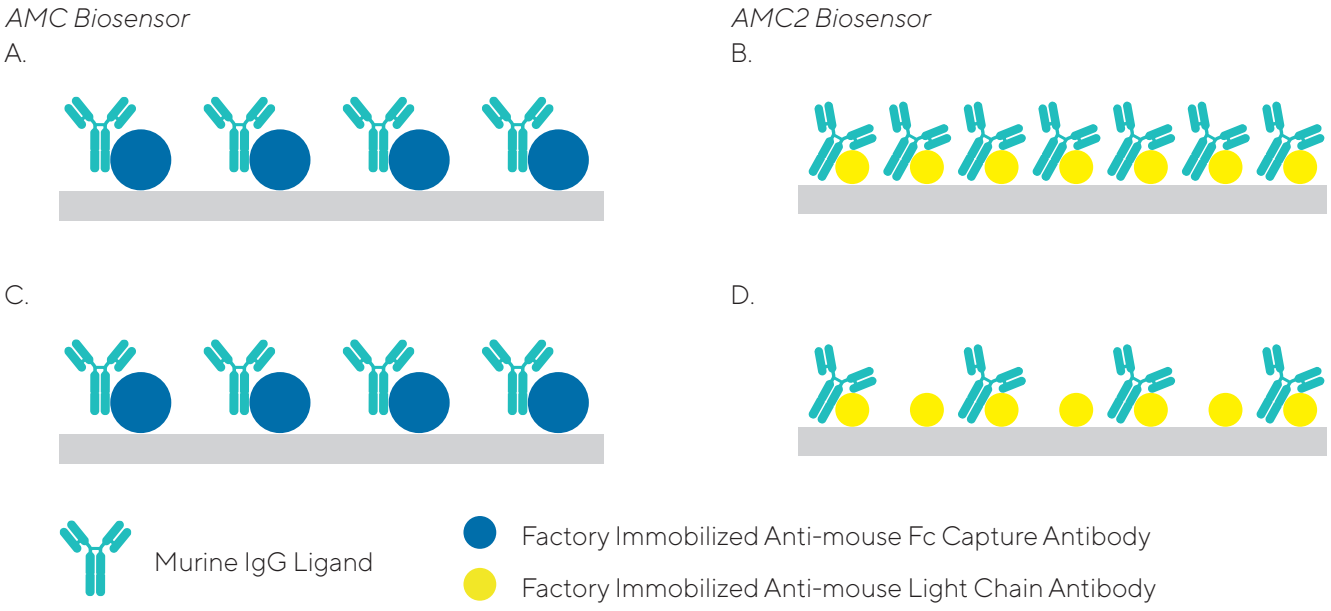
Note: Binding kinetics of a 6 kDa antigen to captured mIgG1 on AMC and AMC2 Biosensors. mIgG1 was captured at 10 µg/ml while the antigen was 2-fold serially diluted in 1X KB from 300 nM – 4.68 nM to generate the dose-response curves. The assay was performed on an Octet® RED96e at 25°C and a shake speed of 1000 rpm. The kinetics parameters were determined by applying a 1:1 Global fit model. Data generated at Eli Lilly, USA.

As discussed above, determining accurate kinetics and affinity for low molecular weight analyte interactions can require the creation of high ligand density surfaces in order to increase the observed binding response. Therefore, determining the optimal ligand density is critical when characterizing binding interactions (**See section on: Binding Kinetics as a Function of Load Density (Load Response)**).

With Octet® BLI, low response signal is typically indicative of a low ligand density on the biosensor surface and increasing the ligand density would cause an increase in the observed response to a particular concentration of analyte. However, given the relatively low ligand binding capacity of the original AMC Biosensors, directly increasing the amount of ligand captured to increase the observed response can cause surface saturation, which potentially leads to artifacts arising due to an increase in surface crowding, steric hinderance, aggregation, heterogenous binding and an increase in avidity effects causing a slower off-rate to be measured than the actual dissociation rate constant.

It is important to note that accepted good practice in the design of a kinetic assay is to create a low-density ligand surface with steric hinderance minimized by optimal spacing of the captured ligand on the biosensor. An increase in spatial separation of the ligand molecules on the biosensor surface helps mitigate many of the artifacts associated with surface saturation. Therefore, the higher capacity of AMC2 Biosensors allows the user to capture larger amounts of ligand required to generate an optimal surface for kinetics and affinity determination while ensuring that the surface has a lower ligand density than is possible with AMC Biosensors (Figure 8). As shown in Figure 8C - D for AMC2 Biosensors, bound ligand is separated by a greater spatial distance than the same load on the original AMC biosensor which leads to a distribution that helps to minimize assay artifacts described above.

Figure 8: *Biosensor Binding Capacity*



Note: (A) Original AMC Biosensors are low capacity compared to AMC2 Biosensors. (B) AMC2 Biosensors allow a much larger amount of ligand to be captured without obstruction of the antigen binding site. (C) Measurement of low molecular weight analytes is potentially problematic as capturing sufficient ligand to ensure a sufficient response for accurate kinetics and affinity to be measured can lead to steric hinderance and other issues. (D) The AMC2 Biosensors' increased capacity, however, allows the user to capture larger amounts of ligand without potential assay artifacts. As shown, the same amount of ligand captured on an AMC2 Biosensor has a lower spatial density compared to AMC Biosensors, which allows users to achieve higher analyte binding response with good signal to noise resolution at low ligand densities.

AMC2 Biosensor Regeneration

The ability to regenerate and re-use biosensors offer a means to reduce the cost per assay. First-generation anti-mouse biosensors namely, AMC and AMQ were not amenable to regeneration as a significant loss in biosensor surface activity (ligand binding capacity) was observed. AMC2 Biosensors however are amenable to regeneration. Prior to the setup of a quantitation or kinetics assay consisting of a regeneration cycle, it is strongly recommended that the user perform a regeneration optimization assay using the test ligand-analyte pair to determine the optimal regeneration buffer and the number of times the AMC2 Biosensors can be regenerated and re-used (**See technical note on Regeneration Strategies for Streptavidin Biosensors**).

Please refer to the **AMC2 Biosensors Technical Note** for more information on the effect of regeneration on quantitation assays. This application note presents data and discusses the effects of AMC2 biosensors regeneration on binding kinetics assays.

Regeneration Assessment of AMC2 Biosensors for Kinetics Assays

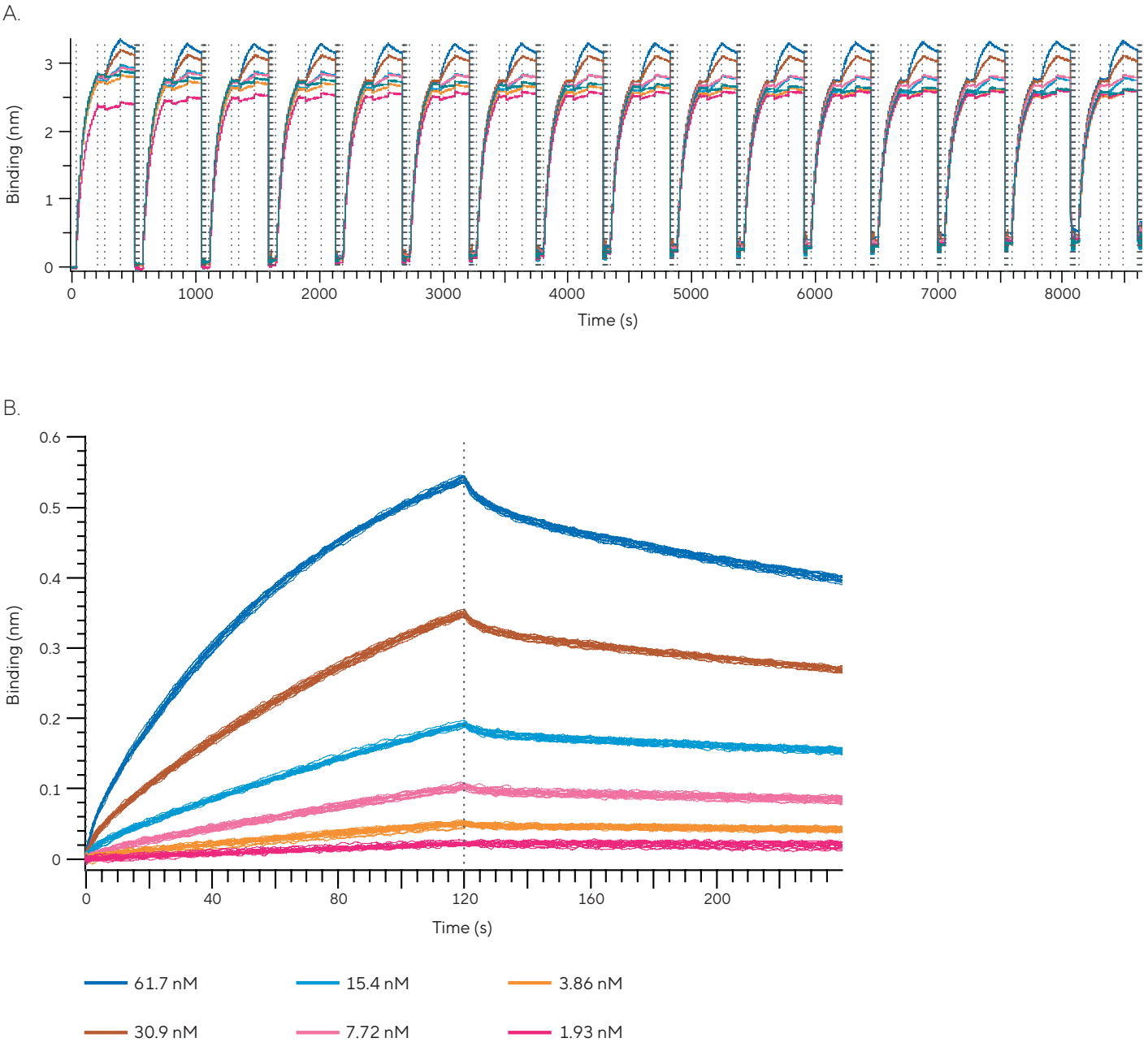
Critical parameters in obtaining high-quality kinetic data when using a ligand regeneration process include regeneration solutions that are too harsh and cause a loss in ligand binding capacity, drift in baseline, and a corresponding decrease in the analyte binding response (an increase in analyte binding response can also be observed when regeneration conditions are too mild and the analyte-ligand complex is not fully regenerated). AMC2 Biosensors were observed to be regenerable approximately 8-10 times with most samples tested. Below is an example of a regeneration optimization assay for a kinetics assay performed at Sartorius, China with samples kindly provided by Beijing Strong (China). The binding kinetics of mIgG1 captured on AMC2 Biosensors to an 81 kDa protein antigen was performed and the assay repeated over 16 cycles of regeneration with 450 mM phosphoric acid (Figure 9).

The change in K_D, k_a and k_d was monitored over 10 cycles and 16 cycles of regeneration. The average K_D did not change significantly after 10 cycles (K_D: 8.15E-09 M) and after 16 cycles (K_D: 7.89E-09 M) with a %CV of 4.34% and 5.82% respectively. Similarly, the k_a and k_d values did not

vary significantly after 10 and 16 cycles of regeneration (Table 7) with a %CV within acceptable limit of <10%. The data presented lends credence to the principles of performing regeneration optimization assays and illustrating the possibility of increased number of AMC2 Biosensor regeneration depending on the ligand present in the assay.

It must be noted that a strong regeneration buffer (450 mM Phosphoric acid) was used in the above example. However, the recommended regeneration buffer for AMC2 Biosensors is 10-100 mM phosphoric acid. Additionally, the choice of regeneration buffer and the regeneration condition should be optimized on a case-by-case basis.

Figure 9: *Regeneration Assessment of AMC2 Biosensors for Kinetics Assays*



Note: A. AMC2 Biosensors regeneration optimization. Binding of AMC2 captured mIgG1 to an 81kDa protein antigen 2-fold serially diluted from 61.73 nM – 0.96 nM in 1X KB to generate a dose-response curve was tested and repeated over 16 cycles of biosensor regeneration with 450 mM phosphoric acid. B. Overlay of dose-response binding sensorgrams generated over 16 cycles. Data generated by FAS team (China) with samples kindly provided by Beijing Strong, China.

Table 7: Assessment of AMC2 Biosensors Regeneration

Cycle	k_a ($M^{-1}s^{-1}$)*	k_d (s^{-1})	K_D (M)	Full R^2	Avg Load Response (nm)	Regeneration cycle
1	2.69E+05	2.31E-03	8.59E-09	0.9984	2.68	
2	2.68E+05	2.32E-03	8.66E-09	0.9982	2.60	
3	2.64E+05	2.23E-03	8.46E-09	0.9985	2.57	
4	2.82E+05	2.33E-03	8.24E-09	0.998	2.53	
5	2.77E+05	2.30E-03	8.33E-09	0.9983	2.49	
6	2.81E+05	2.24E-03	7.96E-09	0.9985	2.46	
7	2.83E+05	2.24E-03	7.90E-09	0.9985	2.42	
8	2.88E+05	2.25E-03	7.82E-09	0.9983	2.39	
9	2.91E+05	2.28E-03	7.84E-09	0.9983	2.36	
10	2.91E+05	2.24E-03	7.67E-09	0.9986	2.33	
11	2.86E+05	2.26E-03	7.91E-09	0.9981	2.30	
12	2.97E+05	2.22E-03	7.46E-09	0.9984	2.27	
13	2.96E+05	2.21E-03	7.46E-09	0.9987	2.25	
14	3.00E+05	2.24E-03	7.47E-09	0.9985	2.22	
15	3.12E+05	2.32E-03	7.46E-09	0.9983	2.19	
16	3.17E+05	2.24E-03	7.08E-09	0.9984	2.16	
Mean	2.79E+05	2.27E-03	8.15E-09		2.48	
STDEV	9.77E+03	4.02E-05	3.54E-10		0.11	1-10 Regen cycle
%CV	3.50	1.77	4.34		4.53	
Mean	2.88E+05	2.26E-03	7.89E-09		2.39	
STDEV	1.47E+04	4.15E-05	4.60E-10		0.16	1-16 Regen cycle
%CV	5.12	1.83	5.82		6.52	

Note: Binding kinetics parameters of affinity (K_D), association rate (k_a) and dissociation rate (k_d) determined over 16 regeneration cycles with 450 mM phosphoric acid.

Summary

Understanding the binding kinetics of an antibody-antigen interaction is a key measure during the discovery and development of antibodies for multiple purposes. The Octet® BLI platform with its library of ready to use biosensors enables users to characterize the binding kinetics of a broad range of biomolecules. In this application note we discuss the key features and performance of the new Octet® AMC2 Biosensors. AMC2 Biosensors are a second-generation anti-murine biosensor, which has a broad binding specificity to all mouse IgG subclasses and were developed to support users in both quantitation and kinetics applications with mIgGs workflows. AMC2 Biosensors can be used with all models of the Octet® BLI platform. Compared to the first-generation AMC and AMQ Biosensors which were individually required for kinetics and quantitation respectively, AMC2 Biosensors provide users a single biosensor type for both kinetics and quantitation applications. Further, this application note presents the results of the experiments performed at Sartorius and multiple customers sites evaluating the performance of the AMC2 biosensors. The results show that AMC2 Biosensors provide a higher binding capacity of 2- to 3-fold for mIgGs and 10-fold higher capacity to mIgG3 compared to the original AMC Biosensor. AMC2 biosensors provided a stable capture of the mIgG ligand to enable testing of high affinity IgG-antigen interactions, exemplified by the kinetics data discussed using purified antibodies from CHO cell supernatants. AMC2 Biosensors were designed to allow the testing of samples in crude matrices such as cell culture media (data not show). For information on the compatibility of the AMC2 biosensors for kinetics and quantitation of mIgG in crude matrix, please see the **AMC2 Technical Note**.

It is important to note that reducing ligand density on AMC Biosensor to non-saturating levels/ low density to mitigate assay artifacts may result in a low analyte binding response with poor signal to noise resolution. The increased mIgG binding capacity of the AMC2 Biosensor allows users to study a broader range of ligand densities without concerns about steric hinderance. This ensures a higher analyte binding response with good signal to noise resolution for the study of kinetics and affinity for lower molecular weight proteins, and peptides.

AMC2 Biosensors can be regenerated about 8-10X reducing the cost per assay, which is a significant improvement over the AMC and AMQ biosensors which were not regenerable.

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