

Rapid Analysis of Fab Fragments and IgG with Octet® FAB2G Biosensors



Technical Note

Scope

This technical note walks the reader through the optimal use of Octet® FAB2G Biosensors for the rapid and label-free analysis of Fab fragments or full-length human IgG molecules on the Octet® Bio-Layer Interferometry (BLI) platform.

Abstract

Octet® FAB2G biosensors are coated with a high affinity ligand that binds specifically to the CH1 region of human IgGs and is intended for use in both kinetics characterization and quantitation analysis of Fab fragments or full-length human IgG during lead identification and optimization, cell line screening, process development, and QC applications. The biosensor enables detection and quantitation of all subclasses (1, 2, 3 and 4) of human IgG and Fab fragments of both Kappa and Lambda isotypes from any source material. They have an optimal dynamic range of 0.5 – 1000 µg/mL and can be regenerated for re-use in both kinetic and quantitation assays.

Overview

Octet® FAB2G Biosensors from Sartorius come pre-immobilized with a high-affinity ligand that is specific for the CH1 region of human IgG. In conjunction with the Octet® BLI systems, FAB2G Biosensors provide a rapid and label-free solution for analysis of Fab fragments or full-length human IgG for lead identification and optimization, cell line screening, process development, and QC applications. The high specificity of the anti-human CH1 ligand enables direct analysis of IgG or Fab analytes directly from complex samples such as cell culture supernatants, partially purified samples, and cell lysates, offering a time-saving alternative to traditional analytical methods.

Principle and Applications

Dip and Read Biosensors are fiber optic tips coated with a specific, high-affinity ligand chemistry that enables detection, quantification, and kinetic analysis of a biomolecular target. With Bio-Layer Interferometry (BLI), the binding of the target molecule alters the interference pattern of light reflected from the biosensor tip to a detector, allowing molecular association and dissociation events to be measured in real time with the Octet® BLI system. Higher target concentrations result in both faster binding rates and larger signal amplitudes. Concentration of target molecule in a sample can be determined by comparing either kinetic (binding rate) or equilibrium (signal amplitude) data to a standard calibration curve constructed from identical samples of known concentrations. In addition, detailed kinetic analysis can be performed in real time to determine affinity (k_{on} , k_{off} , K_D) of the target molecule to an analyte binding partner, for example an antibody-antigen interaction or receptor-ligand interaction. The biosensor tip is well suited for capture and analysis directly from complex mixtures as an alternative to chemical protocols such as EDC/NHS and biotinylation.

The capture ligand coated on FAB2G Biosensors recognizes the CH1 domain of human IgG, which enables detection and quantitation of all subclasses (1, 2, 3 and 4) of human IgG and Fab fragments of both Kappa and Lambda isotypes from any source material. The biosensors bind only to the Fab portion with no cross reactivity to free human light chains or human Fc. They are ideal for evaluating the binding affinity of a Fab molecule or full-length monoclonal antibody to its target antigen, for applications such as

off-rate screening, affinity maturation, or lot release testing of therapeutics. FAB2G Biosensors are also recommended for use in analyzing Fc-gamma receptor or FcRn binding to human IgG. In process development, they enable rapid quantification of Fab fragments directly from cell culture supernatants or lysates for cell-line screening and process optimization.

Tips for Optimal Performance

- Equilibrate reagents and samples to room temperature prior to use. For frozen samples, thaw and mix thoroughly prior to use.
- Ensure that the Octet® BLI system is turned on and the lamp is warmed to room temperature for at least 60 minutes prior to starting an assay.
- Hydrate the FAB2G Biosensors for at least 10 minutes in assay buffer before beginning the assay.
- Set the assay temperature to at least 4 degrees above ambient. 30°C is recommended.

Kinetic Assays Only

- Use a reference sample in the association step to correct for baseline downward drift. A reference sample is a buffer-only control 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 use of the inter-step correction feature to align the Association and Dissociation steps when processing data.

Quantitation Assays Only

- The calibration standard Fab should be identical to the Fab molecule present in the unknown sample for best results.
- Match the matrix of the samples, standards, references, and hydration solution as closely as possible.
- Perform a dilution study and a dynamic range study as outlined in "Assay Optimization" on page 6. Typical assay sensitivity for Fabs ranges from 0.5–1000 µg/mL for assays run at 1000 rpm with a 2-minute read time.
- 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.

Kinetic Assays

Figure 1 illustrates two possible formats for kinetic analysis using FAB2G Biosensors: capture of a Fab molecule to measure binding to its target antigen (A) or capture of IgG to measure binding to Fc gamma receptor or FcRn (B). Each of these assays requires no labeling of assay components and can be performed in minutes on the Octet® BLI platform.

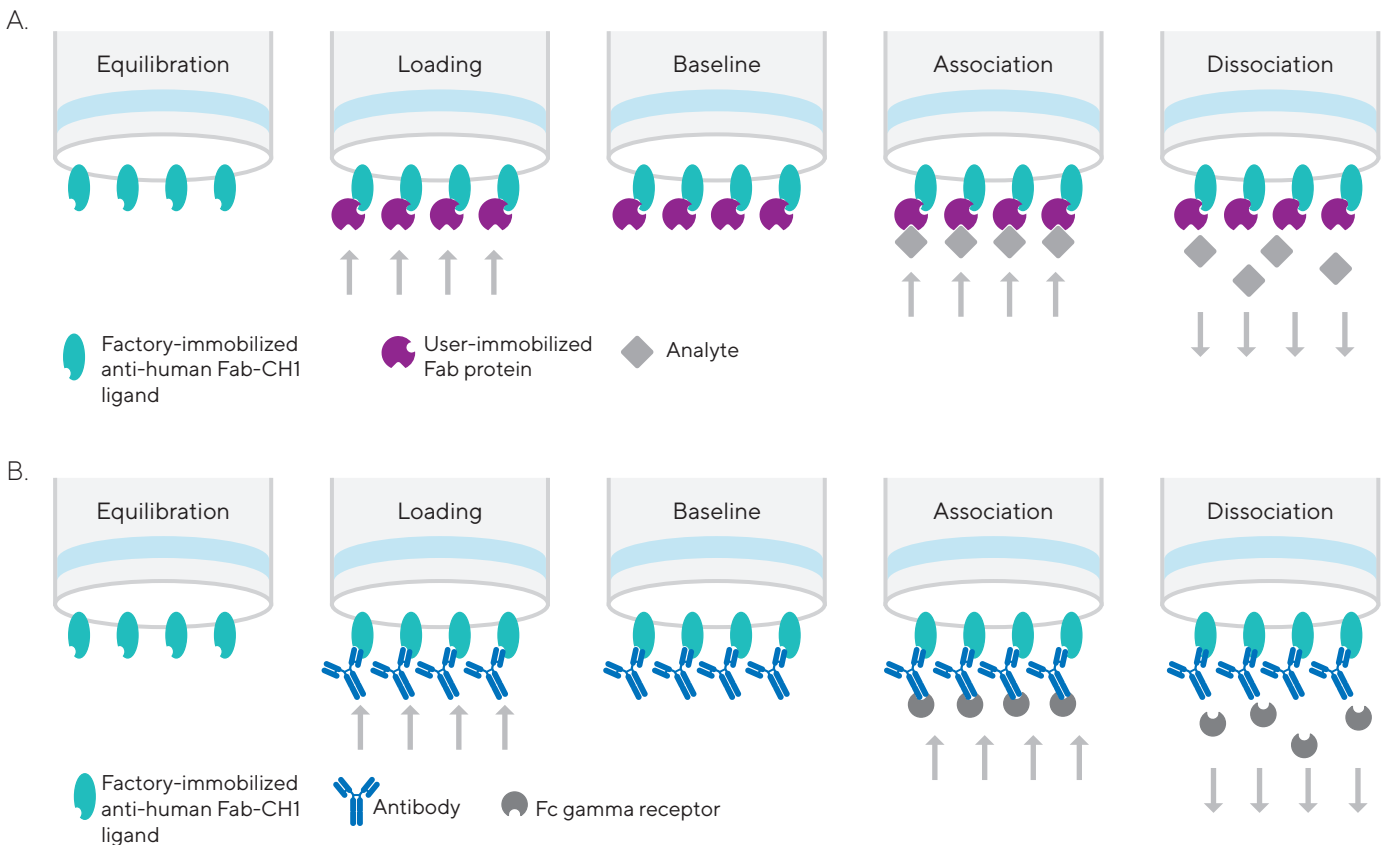
Materials Required

- Octet® BLI system with Octet® BLI Discovery and Analysis Studio Software
- FAB2G Biosensors (Sartorius part no. 18-5125 [tray of 96 biosensors]; 18-5126 [pack of 5 trays]; 18-5127 [case of 20 trays])

- For all Octet® BLI systems: 96-well, black, flat bottom, polypropylene microplate (Greiner Bio-One part no. 655209)
- Optional for Octet® RH16 and RH96 BLI systems:
 - 384-well tilted-bottom, black, polypropylene microplate (Sartorius part no. 18-5080 [pack]; 18-5076 [case])
 - 384-well, black, flat bottom, polypropylene microplate (Greiner Bio-One part no. 781209)
- **Fab or IgG for immobilization.** The Fab protein can be present in either buffer or a complex mixture such as cell culture supernatant.
- **Analyte protein that interacts with Fab protein.** The analyte proteins can be dissolved in buffer matrix or a complex mixture such as culture supernatant.
- **10X Kinetics Buffer (Sartorius part no. 18-5032).** The FAB2G Biosensor is compatible with a wide range of buffers, although 1X Kinetics Buffer is recommended. Dilute 10X Kinetics Buffer 10-fold with PBS, pH 7.4.

Figure 1

Example Workflow Steps for FaB Protein (A) and Human IgG (B) Kinetics Studies.



Note. A) Example five-step workflow for kinetic characterization of the interaction between a Fab molecule and target antigen. 1) Equilibration in buffer or media 2) loading (capture) of Fab molecule via pre-immobilized anti-CH1 ligand 3) baseline in assay buffer 4) association of antigen (analyte) 5) dissociation of antigen in assay buffer. B) Example workflow for Fc-receptor binding interaction with human IgG. 1) Equilibration in buffer or media 2) loading (capture) of antibody via pre-immobilized anti-CH1 ligand 3) baseline in assay buffer 4) association of Fc gamma receptor or FcRn (analyte) 5) dissociation of receptor in assay buffer.

Assay Procedure

For details on setting up a kinetic assay in Octet® Software, please refer to the Octet® Software User Guide. Figure 2 shows an example microplate layout and assay design for a kinetic characterization assay on FAB2G Biosensors. For all steps, use a 200 µL sample volume for 96-well plates, and 80 µL for standard 384-well plates.

Before the Assay – Pre-hydration of biosensors: Hydrate FAB2G Biosensors in 200 µL per well of similar matrix as Fab/ IgG to be captured. Hydration is performed in a 96-well plate placed in the blue biosensor tray for a minimum of 10 minutes.

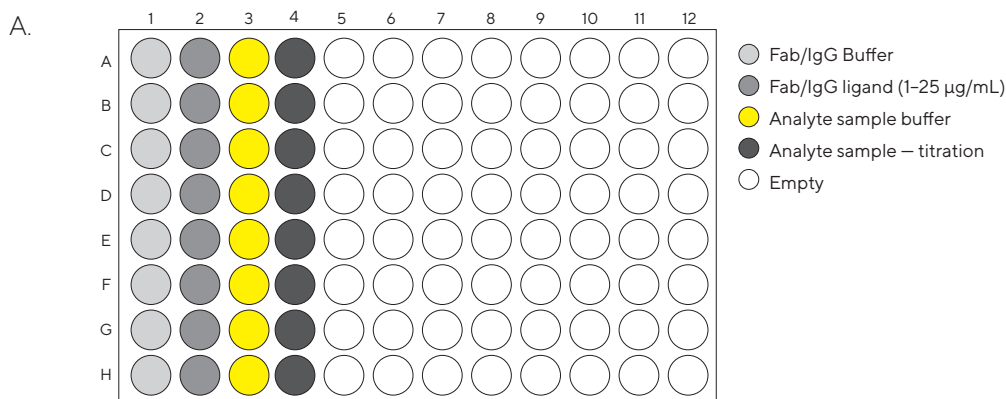
Assay Step 1 – On-line equilibration of hydrated FAB2G Biosensors in Fab/IgG buffer (Custom): Add buffer, media, or lysate (must match the matrix of Fab or IgG to be captured) to Column A of the Sample Plate according to the map in Figure 2.

Assay Step 2 – Capture of Fab or IgG ligand (Loading): Dilute the Fab or IgG 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 1–25 µg/mL.

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, a loading optimization experiment should be performed to determine the optimal Fab/IgG ligand loading concentration and time. As a rule, the least amount of ligand possible should be immobilized. Load only enough so that the highest concentration of analyte used has adequate association signal at equilibrium (4–5 nm recommended). Loading more Fab or IgG than is needed can cause artifacts such as non-specific binding or mass transport. For more details on optimization of ligand loading for kinetic assays, refer to the 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 the analyte samples. The baseline step should be run for a long enough time so that the biosensors are equilibrated in a new buffer matrix and 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 Fab/IgG ligand buffer, a baseline step of 180 seconds should be adequate.

Figure 2
Sample Plate Map and Assay Steps.



B.

Step #	Column #	Description	Step type	Time	Flow
Step 1	1	Equilibration in Fab/IgG buffer	Custom	60	1000
Step 2	2	Loading of Fab or IgG	Loading	120–600	0–1000
Step 3	3	Baseline in Analyte sample buffer	Baseline	180–600	1000
Step 4	4	Association of Analyte	Association	300–600	1000
Step 5	3	Dissociation of Analyte	Dissociation	300–1800	1000

Note. Sample Plate map and (A) and assay steps with associated parameters (B) for a FAB2G kinetic assay.

Assay Step 4 – Association to interacting analyte

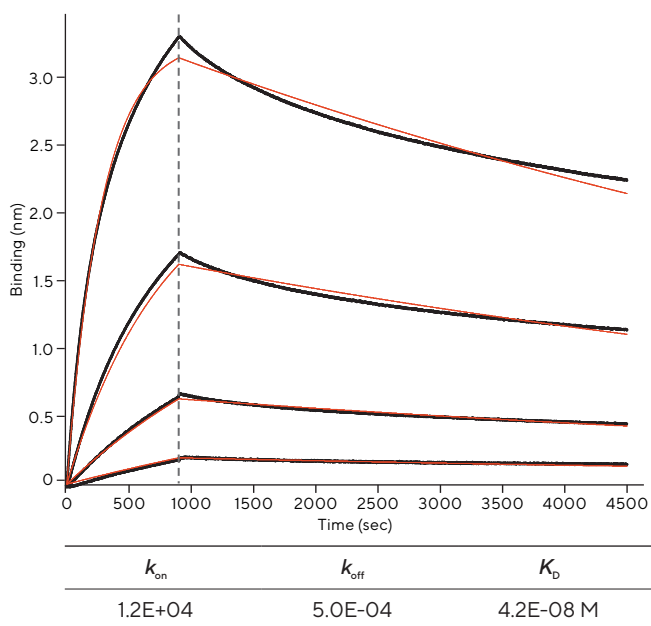
(Association): If detailed kinetic characterization is being performed, the analyte protein must be purified and of known concentration. It is recommended to run a titration series of at least four concentrations of the analyte protein, and perform a global fitting of all concentrations to determine K_D values. The highest analyte concentration should be approximately 10 times the expected K_D . For example, concentrations of 90 nM, 30 nM, 10 nM and 3 nM would be recommended for an analyte with low-nanomolar affinity towards an immobilized Fab or IgG. 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 assay downward 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. 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. Dissociation should be run long enough to see at least 5–10% decrease in signal for accurate curve fitting.

Figure 3

Kinetics Analysis of Human IgG1 Interaction with FcRn.



Note. Kinetic analysis of the interaction between ligand HlgG Fab (50 kDa) and an analyte Fab fragment, Goat anti-HlgG (H+L) specific (50 kDa) on the Octet® RH16 BLI system. 1X Kinetics Buffer was used as the matrix throughout and the assay temperature was 30°C. Data were processed and the curve fit using a 1:1 binding model.

Process and Analyze Data

1. Load data into the Octet® Analysis Studio or BLI Analysis Software.
2. Process the data by specifying methods for reference subtraction, y-axis alignment, inter-step correction and Savitzky-Golay filtering.
3. Analyze the data by specifying steps for analysis, fitting method (local or global) and window of interest.
4. To export the analyzed data, click **Save Report** to generate a Microsoft® Excel® report.

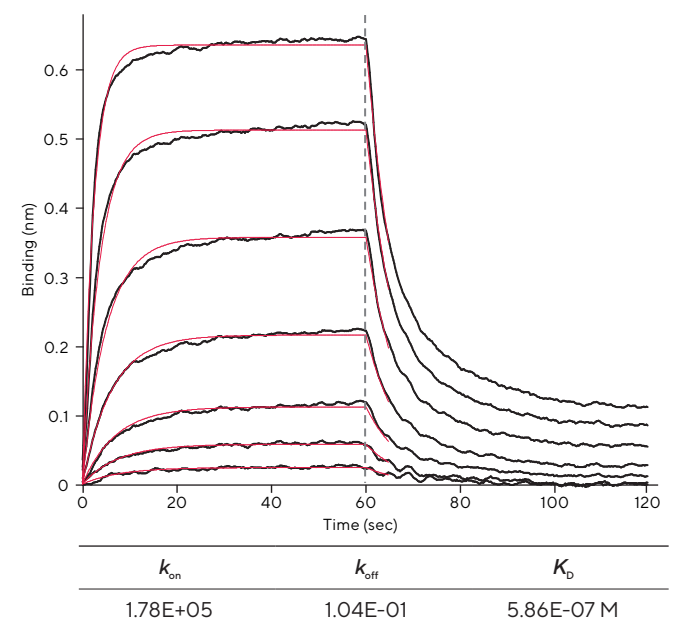
For more details on analyzing kinetic data sets, refer to the Octet® Software User Guide or the Sartorius Application Note, Biomolecular Binding Kinetic Assays on the Octet® System.

Representative Data

Figure 3 shows kinetic analysis of the interaction between ligand HlgG Fab (50 kDa) and an analyte Fab fragment, Goat anti-HlgG (H+L) (50 kDa). In Figure 4, an Fc receptor binding assay was performed on FAB2G Biosensors to analyze human IgG1 binding to FcRn.

Figure 4

Kinetics Analysis of Human IgG FAB Interactions with an Analyte.



Note. Kinetic analysis of interaction of human IgG1 with FcRn. FAB2G Biosensors were used to capture hlgG1 and associated with FcRn using several concentrations. The 1:1 model with global fitting and 5 seconds of the dissociation step (fit lines are in pink) were used to determine the affinity constant. The assay was run in phosphate buffer, pH 6.0.

FAB2G Quantitation Assays

Fab molecules can be directly quantified using FAB2G Biosensors by comparison of either initial binding rate or equilibrium signal data to a standard calibration curve constructed from identical samples of known concentrations. The Fab portion of human IgG will possess different binding kinetics due to amino acid sequence variations and differing steric environments. Since the quantitation performance is typically based on binding rate kinetics, the calibration standard should be identical to the Fab in the sample. To run a quantitation assay, a set of standards must be generated in matrix matching that of the samples.

Materials Required

- Octet® BLI system with Octet® BLI Discovery and Analysis Studio Software
- FAB2G Biosensors (Sartorius part no. 18-5125 [tray of 96 biosensors]; 18-5126 [pack of 5 trays]; 18-5127 [case of 20 trays])
- For all Octet® BLI systems: 96-well, black, flat bottom, polypropylene microplate (Greiner Bio-One part no. Optional for Octet® RH16 and RH96 BLI systems:
 - 384-tilted well, black, flat bottom, polypropylene microplate (Sartorius part no. 18-5080 [pack]; 18-5076 [case])
 - 384-well, black, flat bottom, polypropylene microplate (Greiner Bio-One part no. 781209)
- **Standard Fab.** To be used as a calibration standard.
- **Sample Diluent (Sartorius part no. 18-5028) for dilution of all samples.** If undiluted crude samples are to be quantified, a matching blank matrix is required.

Table 1

Recommended Minimum Dilution for Common Sample Types.

Sample Type	Minimum Recommended Dilution in Sample Diluent
Purified proteins	Dilute into assay range
Samples from column eluents	Dilute into assay range
Serum-free cell culture supernatants media	Neat or 2-fold
Serum-containing cell culture supernatants	Neat
Bacterial cell pellet lysed by sonication	Neat
Bacterial cell pellet lysed by B-PER	50-fold

Note. In all cases the matrix for the diluted samples, the standards and the biosensor hydration solution should be matched as closely as possible.

Assay Optimization

The following optimization steps are recommended each time a new matrix or new Fab is quantified.

1. 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).
2. Perform a spike/recovery study to determine the assay dynamic range.
3. Establish data analysis parameters in Octet® Software.
4. 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 Sartorius' Sample Diluent is an effective means of minimizing matrix effects. Dilution factor guidelines for various sample types are described in Table 1. However, before running a quantitation assay it should be empirically determined whether dilution of samples is needed.

1. Prepare 1 mL each of sample matrix (without target protein) diluted both 2-fold and 10-fold in Sample Diluent.
2. Add target His-tagged protein to each of the matrix dilutions, and also to neat matrix 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).

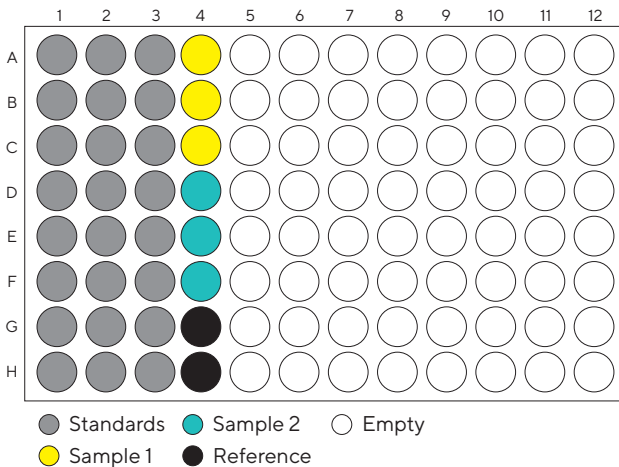
- Set up a Basic Quantitation assay according to the Octet® Software User Guide.
- Run the assay.
- Data will be displayed in real time during the assay. Data and method files will be saved automatically.
- Load data into Octet® Analysis Studio or BLI Analysis Software.
- Visually inspect the real-time binding traces and determine the dilution required to:
 - Minimize non-specific binding of matrix components.
 - Show equivalent binding in the matrix spiked sample and the Sample Diluent control.
- Use this dilution factor for routine assays.

Recovery and Precision Assay to Determine Quantitation Range

- Prepare a series of Fab 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, generally 0.5–1000 µg/mL for assays run at 1000 rpm.
- Using the same matrix diluent as in Step 1, prepare two Fab 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.

Figure 5

Example of a Sample Plate Layout for a Spike Recovery Assay.

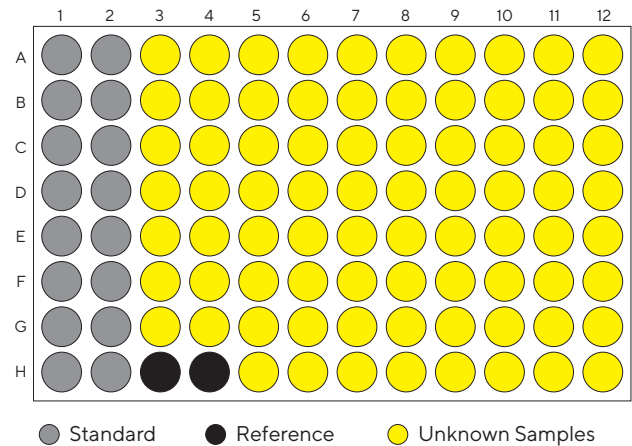


Note. Example plate layout for a spike recovery assay.

- 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.
- Hydrate biosensors for 10 minutes in matching matrix diluent.
- Set up a Basic Quantitation assay using the same assay parameters that were used in the Dilution Factor Determination for Sample Matrix experiment. Define sample Replicate Groups in order to calculate replicate averages and CVs.
- Run the experiment. Data will be displayed in real time during the assay. Data files, method files and assay pictures will be saved automatically.
- Load the data into Octet® Analysis Studio or BLI Analysis Software.
- If a blank matrix was included as a reference, use the reference subtraction option to correct the data as appropriate.
- Calculate the binding rate. The results table will populate with calculated concentrations and data statistics.
- Define assay dynamic range by selecting acceptable %CV values for the lower and upper concentration limits in the standard curve.
- Exclude data points for the standard curve that lie outside the defined dynamic range if necessary.
- Select the appropriate equation to fit the standard curve.
- Evaluate the accuracy and precision of the assay using calculated concentration value of the unknowns to determine percent recovery and percent CV.

Figure 6

Example of a Full Plate Sample Layout for a Quantitation Assay.



Note. Example plate layout for a routine quantitation assay run in a 96-well microplate.

Running the Assay to Quantify Fabs of Interest

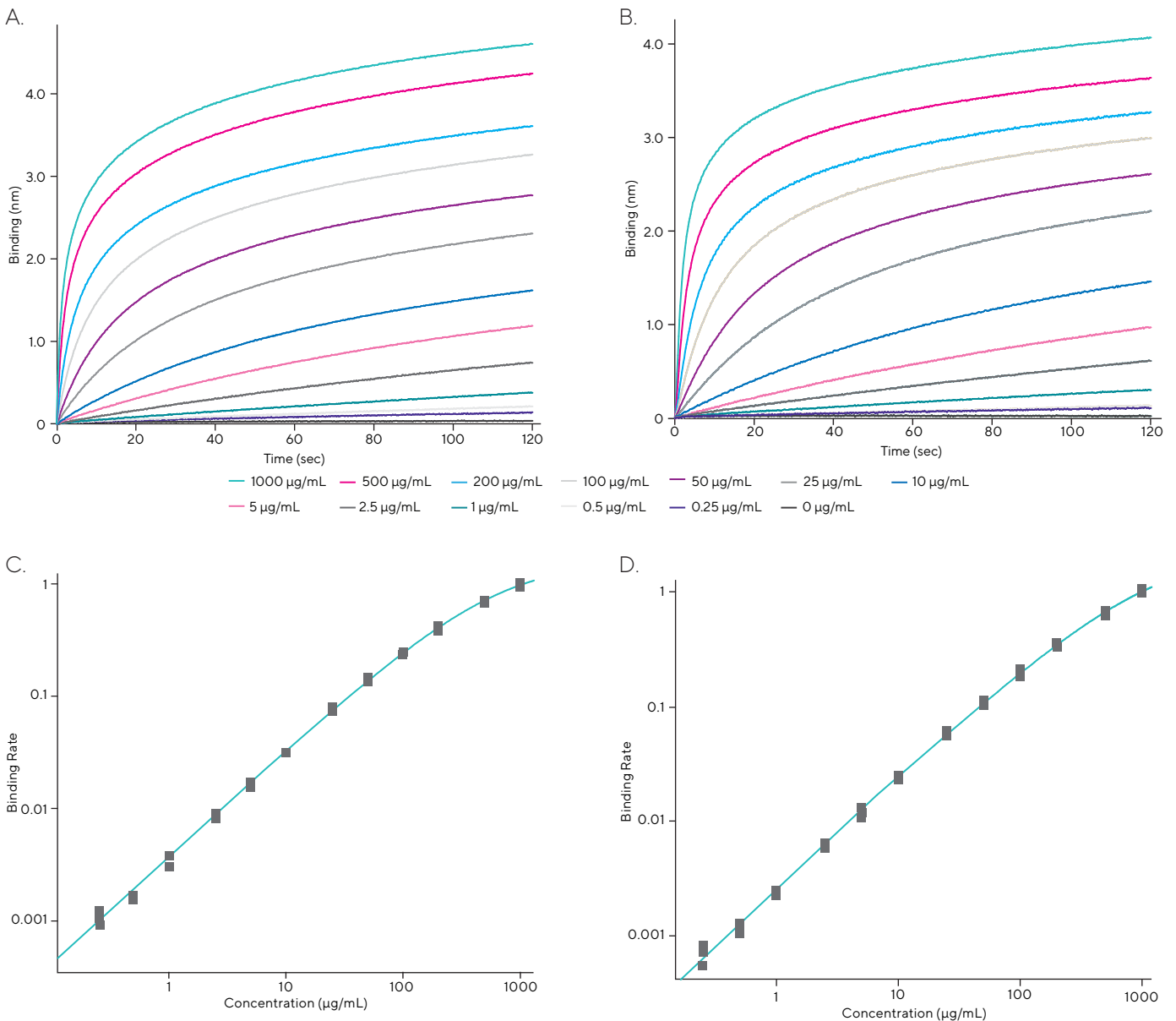
1. Prepare samples, calibration standards and hydration solution according to the conditions determined in optimization steps in the prior section.
2. Set up a Basic Quantitation assay using the parameters described previously in the optimization experiments. See Figure 6 for example assay set up.
3. Run the assay.

4. Load data into Octet® Analysis Studio or BLI Analysis Software. Analyze as in previous optimization steps to determine concentration of samples and data statistics.
5. To export the analyzed data, click **Save Report** to generate a Microsoft® Excel® report.

For more information on quantitation using Octet® system, refer to the Octet® Software User Guide, as well as the Application Note, Developing a Robust Quantitation Assay for Monoclonal Antibodies and Other Proteins on the Octet® Platform.

Figure 7

Dose Response of Fab and IgG.



Note. Dose response of Fab and IgG using FAB2G Biosensors on the Octet® RH16 BLI system with assay parameters (1000 rpm, 2 min) for a standard dynamic range. A) Fab raw data traces. B) IgG raw data traces. C) and D) represent the resulting titration curves from A) and B) respectively using triplicate samples. Sample Diluent was used as a matrix for all samples.

Representative Data

Figure 7 shows detection of a Fab and IgG using FAB2G Biosensors on the Octet® RH16 BLI system. A standard curve for each molecule was run to demonstrate quantitation dynamic ranges.

Regeneration of Biosensors

FAB2G Biosensors can be regenerated in both kinetic and quantitation assays. Regeneration provides a cost-saving solution for generating replicate data for ligand-analyte pairs, or for analyzing large numbers of samples in sequence. The captured Fab (quantitation) or bound complex (kinetics) can be removed from the biosensors after an assay cycle by dipping into a solution of 10 mM glycine, pH 1.7, for 5 seconds, followed by a dip in assay buffer for 5 seconds to neutralize. These regeneration steps should be repeated 3–4 times in sequence to fully remove bound Fab or interaction complex. After regeneration, the biosensor can be reloaded with Fab or IgG for a new analysis. For best results, run the regeneration protocol before loading the first ligand as a biosensor pre-conditioning step. A small loss in binding capacity may occur after each regeneration cycle. This loss should not affect results within a limited number of assay-regeneration cycles.

Regeneration results will depend somewhat on the molecule being captured. For each new Fab or IgG molecule, regeneration conditions should be tested. The number of possible regeneration cycles should also be determined experimentally. When data are aligned for multiple assay cycles run on the same set of biosensors, data traces for each assay should overlap with no decrease in signal detected as the number of regeneration cycles increases. Figure 8 shows aligned association-dissociation data for IgG1-FcRn kinetic assay (A) and a Fab quantitation assay (B) with ten and six assay-regeneration cycles respectively. In both experiments the data traces overlap closely, with low variability between calculated binding and affinity constants from cycle to cycle in the kinetic assay.

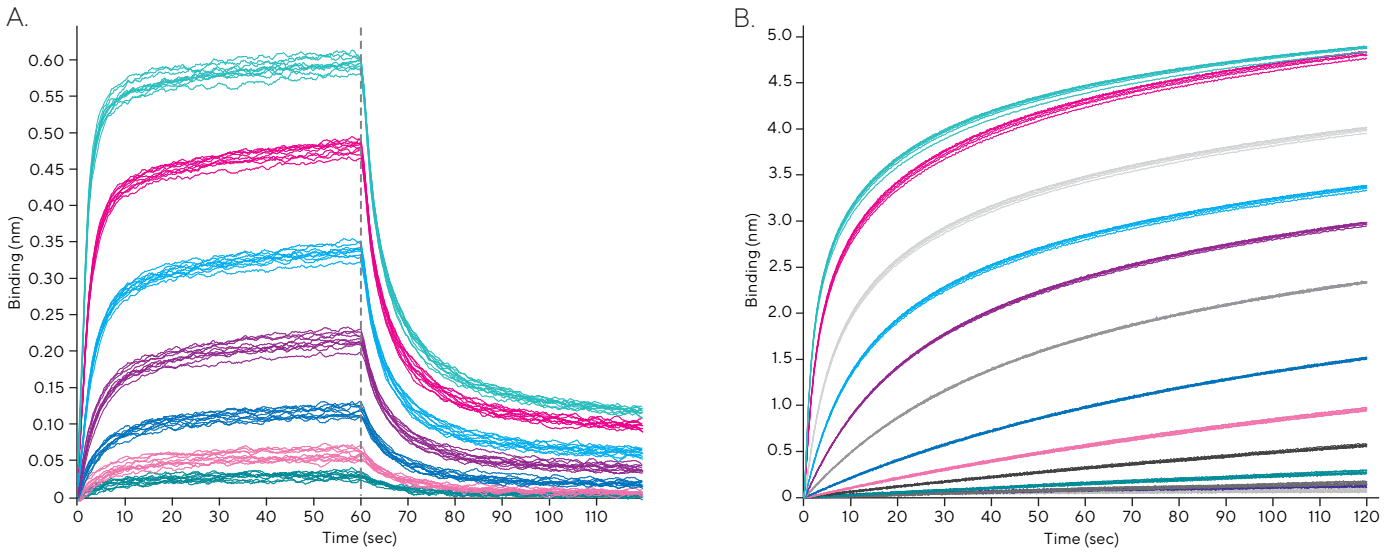
Regeneration Tips

- Regenerate the FAB2G 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 four times.
- Depending on the assay conditions or protein being captured, the regeneration buffer and/or conditions may require optimization.
- 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 once before the first loading step.

Table 2
Concentration Analysis of Fab and F(ab)₂ Calibration Standards.

Expected Conc. (µg/mL)	Fab		F(ab) ₂	
	Avg. Conc. µg/mL (N = 3)	%CV (N = 3)	Avg. Conc. µg/mL (N = 3)	%CV (N = 3)
1000	999.98	6.4%	1000.00	2.2%
500	504.00	3.5%	501.78	2.0%
200	201.43	3.9%	200.70	2.0%
100	100.18	1.6%	100.15	1.6%
50	50.10	2.1%	50.18	4.4%
25	25.05	2.5%	25.00	1.8%
10	10.00	0.8%	10.03	4.1%
5	5.00	3.4%	5.00	2.5%
2.5	2.50	1.9%	2.51	2.2%
1	1.00	13.4%	1.00	3.2%
0.5	0.50	1.6%	0.50	2.7%
0.25	0.25	9.8%	0.26	22.2%

Note. Average calculated concentration and %CV of triplicates of Fab and F(ab)₂ calibration standards for the data from Figure 7. Results may vary with individual Fab analytes and assay matrices.

Figure 8*Biosensor Regeneration on FAB2G Biosensors using the Octet® RH16 BLI System.*

Note. A) Kinetics. Biosensors were pre-conditioned before first assay cycle. HIgG1 was loaded onto biosensors, followed by association of FcRn analyte at several concentrations. Assay was repeated over 10 cycles with regeneration on the same set of FAB2G Biosensors. Alignment of association and dissociation steps shows consistent overlap of traces and low CVs for calculated kinetic and affinity constants. B) Quantitation. Biosensors were regenerated over six cycles of standard curve generation using a Fab molecule diluted in Sample Diluent. Traces at all concentrations overlap with little decline in signal.

	Average	%CV
K_D	5.67E-07	4.1%
k_{on}	2.23E+05	3.5%
k_{off}	1.26E-01	2.8%

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