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A Division of Pall Life Sciences

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Anti-Human Fab-CH1 Biosensor Kinetics Assays

OVERVIEW

The Anti-Human Fab-CH1 Biosensor consists of a high-specificity anti-Human Fab-CH1 antibody fragment pre-immobilized on a ForteBio biosensor. In conjunction with the Octet® or BLItz™ systems, the Anti-Human Fab-CH1 Biosensor provides a rapid and label-free method for Human F(ab')$_2$/Fab/IgG quantitation and kinetic analysis. The high specificity of the antibody fragment-based biosensor enables direct analysis of Human F(ab')$_2$/Fab/IgG analytes in crude lysates, column eluents, cell lysates and cell culture supernatants, serving as a time-saving alternative to traditional analytical methods. For more information on quantitation analyses using the Anti-Human Fab-CH1 Biosensor, please see ForteBio Technical Note 33, *Anti-Human Fab-CH1 Biosensor Quantitation Assays*.

PRINCIPLE

Anti-Human Fab-CH1 biosensors are fiber optic sensors coated with a llama antibody fragment recognizing the CH1 domain of human IgG, which enables detection of interactions between Human F(ab')$_2$/Fab/IgG and biomolecular analytes. The high specificity of the biosensor towards the CH1 region of human IgG allows the capture and immobilization of all four subclasses of human IgG, Fab, and F(ab')$_2$, independent of the type of light chain present. The surface is well suited for capture and analysis directly from complex mixtures as an alternative to chemical protocols such as EDC/NHS and biotinylation.

Figure 1 depicts the equilibration, Fab capture, analyte association and dissociation steps that take place on the biosensor during a kinetic assay. Anti-Human Fab-CH1 biosensors can be regenerated up to 10 times via a standard low-pH protocol in as little as two minutes for select applications such as acquisition of replicate data (same ligand/analyte pair) and "bucket"-based screening applications. Regeneration dissociates the Fab molecule from the factory-immobilized anti-Fab antibody fragment, allowing additional analyses. However, for the highest quality affinity and kinetic results, using a new Anti-Human Fab-CH1 Biosensor for each unique capture ligand is recommended.

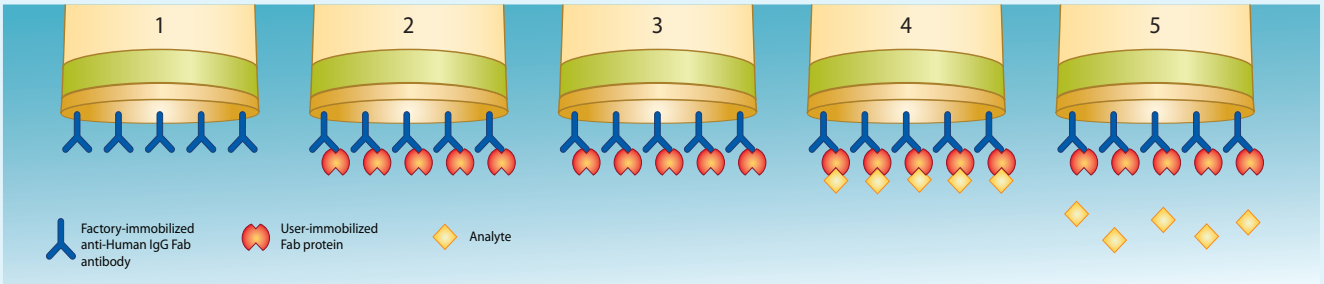

FIGURE 1: Example workflow for kinetic characterization of the interaction between a Fab fusion protein and a target analyte. The assay consists of five assay steps. Step 1: equilibration, Step 2: loading (capture) of Fab protein, Step 3: baseline, Step 4: association kinetics, Step 5: dissociation kinetics.

MATERIALS REQUIRED

- Octet instrument with Octet Data Analysis software
- Anti-Human Fab-CH1 biosensors (ForteBio part no. 18-5104 [tray]; 18-5105 [pack]; 18-5106 [case])
- For all Octet instruments: 96-well, black, flat bottom, polypropylene microplate (Greiner Bio-One part no. 655209)
- Optional for Octet RED384 and Octet QK384 instruments:
  - 384-well, black, tilted-bottom polypropylene microplate (ForteBio part no. 18-5080 [pack]; 18-5076 [case])
  - 384-well, black, flat bottom, polypropylene microplate (Greiner Bio-One part no. 781209)
- **Fab for immobilization:** the Fab can be present in either buffer or a complex mixture such as culture supernatant.
- **Analyte molecules that interact with Fab:** the analyte can be dissolved in buffer matrix or a complex mixture such as culture supernatant.
- **Kinetics Buffer:** the FAB biosensor is compatible with a wide range of buffers; however, 1X Kinetics Buffer is recommended (dilute 10X Kinetics buffer 1:10 with PBS pH 7.4). Best results are obtained when all matrices are matched as closely as possible.
- **Regeneration Buffer:** Regeneration Buffer (10 mM glycine pH 1.7) is required for surface conditioning and regeneration. Further evaluation of the most appropriate pH and optimization may be required.

TIPS FOR OPTIMAL PERFORMANCE

- To improve baseline and surface stability, condition the biosensors before the first loading step. Typically, conditioning consists of three 20-second exposures to Regeneration Buffer alternating with 1X Kinetics Buffer.
- "Priming" the biosensor surface with a binding and regeneration cycle is recommended for the most rigorous kinetic applications. The priming binding cycle consists of capturing Fab protein from solution and then regenerating the biosensors back to the original surface with the regeneration protocol. Biosensor priming should follow biosensor conditioning.
- The inter-step correction software processing feature corrects for misalignment between the association and dissociation steps. For the most effective inter-step correction, the baseline and the dissociation steps of an assay cycle should be performed in the same microplate well.
- Typically, the biosensor surface can be regenerated by cycling it between 10 mM glycine pH 1.7 (5 seconds) and 1X Kinetics Buffer (5 seconds) five times. Depending on the Fab protein being used, the regeneration buffer formulation (buffering element and pH) may require optimization.
- Use of a reference biosensor to correct for drift is recommended. A reference biosensor should be loaded with the Fab protein and run with a buffer blank for the association and dissociation steps.
- Equilibrate reagents and samples to room temperature prior to preparation. For frozen samples, thaw and mix thoroughly prior to use.
- Ten minutes of biosensor hydration is required prior to an assay. Hydrating the biosensors in a buffer consistent with the buffer used throughout the assay is recommended.
- Ensure that the Octet instrument is turned on and the lamp is warmed to room temperature for at least 40 minutes prior to starting the assay.
- Set the sample plate temperature in the Octet Data Analysis software by selecting File > Experiment > Set Plate Temperature and entering the desired temperature. ForteBio recommends assaying at 30°C. Using other temperatures may require modifying the assay times discussed in this protocol.
- Some systems can intermittently display a small "bump" artifact during the transition from the association step to the dissociation step. Increasing the concentration of the BSA carrier protein by utilizing 10X Kinetics Buffer rather than 1X Kinetics Buffer typically removes this artifact.

ASSAY PROTOCOL

Overview

1 Prepare assay solutions.

2 Prepare the sample plate.

3 Equilibrate both the hydrated biosensor assembly and the assay plate for 10 minutes on the Octet instrument.

4 Run the assay.

5 Process and analyze the data.

6 Save the results.

Prepare Samples and Calibration Standards

Equilibrate reagents and samples to room temperature prior to preparation and mix thoroughly.

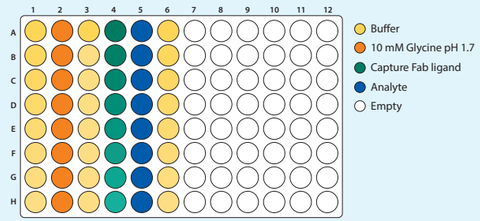

FIGURE 2: Example plate map for a kinetic assay that includes a pre-conditioning cycle, ligand loading, association, dissociation and a regeneration cycle. An assay step list using the sample plate is described in Table 1. The same sample wells (column 3) are used for the baseline and dissociation steps for optimal use of the inter-step correction processing feature.

Step	Step Name	Time (s)	Shake Speed	Step Type
1	Equilibration	180–600	1000	Custom
2	Conditioning (10 mM Glycine)	20	1000	Custom
3	Neutralization	20	1000	Custom
4	Conditioning (10 mM Glycine)	20	1000	Custom
5	Neutralization	20	1000	Custom
6	Conditioning (10 mM Glycine)	20	1000	Custom
7	Neutralization	20	1000	Custom
8	Equilibration	300–600	1000	Custom
9	Loading	300–600	0-1000	Custom
10	Baseline	180–600	1000	Baseline
11	Association	600–1800	1000	Association
12	Dissociation	600-3600	1000	Dissociation
13	Regeneration (10 mM Glycine)	5	1000	Custom
14	Neutralization	5	1000	Custom
15	Regeneration (10 mM Glycine)	5	1000	Custom
16	Neutralization	5	1000	Custom
17	Regeneration (10 mM Glycine)	5	1000	Custom
18	Neutralization	5	1000	Custom
19	Regeneration (10 mM Glycine)	5	1000	Custom
20	Neutralization	5	1000	Custom
21	Regeneration (10 mM Glycine)	5	1000	Custom
22	Neutralization	5	1000	Custom

TABLE 1: Example assay steps and associated parameters. After completing step 22, steps 8–22 would be repeated. Step types correspond to Octet software version 5.0 and greater. The same sample wells are used for the baseline and dissociation steps for optimal use of the inter-step correction processing feature.

1 **Fab ligand:** The ligand will be immobilized on the biosensor tip surface. Fab ligands are typically immobilized at a concentration between 5–25 µg/mL. If the Fab ligand is below 5 µg/mL, a longer loading time may be required (60 minutes loading at 1 µg/mL is equivalent to 10 minutes loading at 10 µg/mL). The ligand solution can be recovered from the well after the assay and reused, if desired. If the ligand is captured from a cell culture supernatant, dilution of the supernatant two-fold or greater with 1X Kinetics Buffer can potentially increase data quality. If dilution results in a low total concentration of the ligand, the biosensors can be incubated in the supernatant overnight at 4°C to maximize loading of the Fab ligand. For tips on optimizing overnight loading, see ForteBio Technical Note 10, *Batch Immobilization of a Biotinylated Ligand onto Streptavidin Biosensors*.

2 **Interacting molecule (analyte):** During rigorous kinetic analysis, it is recommended to run a dilution series of at least four concentrations of the analyte protein. The highest 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 ligand. 200 µL/well, 80 µL/well and 40 µL/well of analyte solution are required for 96-well, 384-well and 384-well tilted-bottom plates, respectively. The solution can be recovered from the wells after the assay and reused if desired. For screening assays or qualitative interaction analysis, a single concentration of the interacting protein can be sufficient to characterize the binding.

3 **Conditioning/regeneration solution:** The capture Fab ligand and the analyte can be removed from the biosensors by exposing them to 10 mM glycine pH 1.7 followed by a neutralization buffer (typically 1X Kinetics Buffer). 200 µL/ well, 80 µL/well and 40 µL/well of regeneration and neutralization solutions are required for 96-well, 384-well and 384-well tilted-bottom plates, respectively. After regeneration, the biosensor can be reloaded with Fab analyte samples for a new interaction analysis. A small loss in

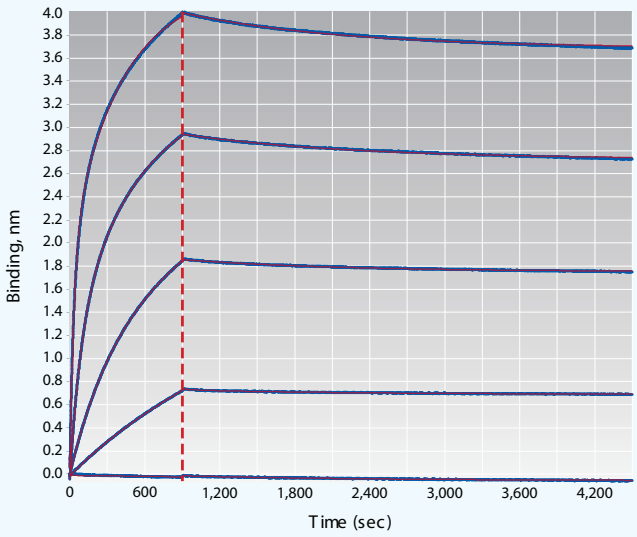

FIGURE 3: Kinetic analysis of the interaction between ligand HIgG F(ab')$_2$ (110 kDa) and an analyte F(ab')$_2$ fragment, Goat anti-HIgG F(ab')$_2$ specific (110 kDa). 1X kinetics buffer was used as the matrix throughout and the assay temperature was 30°C. Data were processed and the curve fit using a 1:1 binding model. The kinetic results are reported in Table 2.

k_{on}	k_{off}	K_D
4.6x10^4 1/Ms	1.0x10^{-5} 1/s	2.2x10^{-10} M

TABLE 2: Kinetic results for the interaction between ligand HIgG F(ab')$_2$ (110 kDa) and an analyte using Anti-Human Fab-CH1 biosensors.

binding capacity after a regeneration cycle is expected. Regeneration provides a cost-effective solution for generating replicate data for ligand-analyte pairs. For the highest quality kinetic results, using a new biosensor to capture each unique ligand is recommended.

Running the Assay

1. Pipet 200 µL/well of biosensor hydration solution into the wells of a 96-well black, flat-bottom microplate corresponding to the number and the positions of the biosensors to be used.
2. Insert the hydration plate into the biosensor tray. Align the biosensor rack over the hydration plate and lower the biosensors into the wells, taking care not to scrape or touch the bottom of the biosensors.
3. Transfer 200 µL of each assay reagent into the appropriate wells of a black polypropylene microplate. Place the assay plate on the sample plate stage with well A1 toward the back right corner.
4. Place the biosensor hydration assembly in the Octet instrument on the left stage. Ensure that both the biosensor tray and sample plate are securely in place.
5. Ensure that the Octet instrument and computer are turned on. It is essential that the instrument lamp warms up for at least 40 minutes before running an experiment.
6. Equilibrate the plates in the instrument for 10 minutes prior to starting the experiment. The delay timer can be used to automatically start the assay after 10 minutes (600 seconds).
7. Set up a kinetic assay. For details, see the Octet Data Acquisition User Guide. Table 1 shows an example kinetic assay consisting of a pre-conditioning cycle, ligand loading, association, dissociation and regeneration steps. Figure 2 shows an example plate map for a typical kinetic assay.
8. Run the assay.

Process and Analyze the Data

1. Load data into the Octet Data 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 processing and analysis parameters or data exporting, please refer to the Octet Data Analysis User Guide.

REPRESENTATIVE DATA

Figure 3 shows the kinetic analysis of the interaction between ligand HIgG F(ab')$_2$ (110 kDa) and an analyte F(ab')$_2$ fragment, Goat anti-HIgG F(ab')$_2$ specific (110 kDa). Anti-Human Fab-CH1 biosensors were hydrated for 10 minutes in 1X Kinetics Buffer prior to analysis. Assays steps included: preconditioning (three 20-second exposures to Regeneration Buffer alternating with 1X Kinetics Buffer), 5 minutes of equilibration, 10 minutes of F(ab')$_2$ loading (10 µg/mL), 5 minutes of baseline stabilization, 15 minutes of ligand:analyte association and 60 minutes of ligand:analyte dissociation. Analyte concentrations were 3.13, 12.5, 50 and 200 nM. 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. The kinetic results are reported in Table 2.

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