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A Division of Pall Life Sciences

# Anti-Human Fab-CH1 (FAB) Biosensor Quantitation Assays

## OVERVIEW

The Anti-Human Fab-CH1 Biosensor consists of a high affinity anti-human CH1 affinity ligand 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 Fab/F(ab')$_2$/IgG quantitation and kinetic analysis. The high specificity of the antibody fragment-based biosensor enables direct analysis of human Fab/F(ab')$_2$/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 kinetic analyses using the Anti-Human Fab-CH1 Biosensor, please see ForteBio Technical Note 34, *Anti-Human Fab-CH1 Biosensor Kinetics Assays*.

## PRINCIPLE

Anti-Human Fab-CH1 biosensors are fiber optic devices coated with a llama antibody fragment recognizing the CH1 domain of human IgG, which enables detection and quantitation of all subclasses (1, 2, 3 and 4) of human IgG and F(ab')$_2$/Fab fragments. This biosensor only binds to the Fab portion of human IgG without recognition of free human light chains and human Fc. The binding of molecules to the biosensor alters the interference pattern of light reflected from the surface, allowing binding events to be monitored in real time using the Octet or BLItz instrument platforms. Higher analyte concentrations result in both faster binding rates and larger signal amplitudes. Unknown concentrations are determined by comparing either kinetic (binding rate) or equilibrium (signal amplitude) data to a standard curve constructed from identical samples of known concentrations.

## MATERIALS REQUIRED

- Octet instrument with Octet 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 tilted-bottom polypropylene microplate (ForteBio part no. 18-5080 [pack]; 18-5076 [case])
  - , polypropylene microplate (ForteBio part no. 18-5080 [pack]; 18-5076 [case])
  - 384-well, black, flat bottom, polypropylene microplate (Greiner Bio-One part no. 781209)
- Standard protein to be used as a calibration standard. For best results, this calibration standard protein should be identical to the protein in the sample.
- Sample Diluent (ForteBio part no. 18-5028) for dilution of all samples. If undiluted crude samples are to be quantified, a matching blank matrix is required.

## TIPS FOR OPTIMAL PERFORMANCE

- The F(ab')$_2$/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 kinetics, the calibration standard protein should be identical to the F(ab')$_2$/Fab present in the unknown sample for best results.
- Typical assay sensitivity ranges from 0.5–1000 µg/mL for assays run at 1000 rpm with a 2-minute read time.

- 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 the Assay Optimization section.
- Use a blank negative control in a matching matrix for reference subtraction. This is especially important when optimizing accuracy and detecting low-concentration analytes.
- Fully equilibrate all reagents, calibration standards and samples to room temperature prior to sample preparation. Thaw frozen samples completely and mix thoroughly prior to use.
- Hydrate the biosensors for a minimum of 10 minutes prior to use.
- Ensure that the Octet instrument is turned on and the lamp is warmed for at least 40 minutes prior to starting the assay.
- Set the sample plate temperature in the Octet software by selecting Experiment > Set Plate Temperature…. Enter the desired temperature. ForteBio recommends 30°C for accurate quantitation. Set the default startup temperature (Octet Software version 6.4 and later) by selecting File > Options. Enter the desired temperature under "Startup".

## ASSAY OPTIMIZATION

The following are recommended each time a new matrix or new Fab protein is analyzed:

1 Determine the minimal dilution factor required to achieve the targeted assay performance.

2 Perform a spike/recovery study to determine assay dynamic range.

3 Determine data analysis parameters.

4 Apply the finalized protocol and data analysis parameters to the routine assay.

### Dilution Factor Determination for Matrix

Differences between matrices can potentially influence assay performance. Diluting the sample matrix using ForteBio's Sample Diluent is a convenient and often effective means of minimizing matrix effects. It is therefore recommended to determine the minimum dilution factor using Sample Diluent that achieves the desired assay performance.

1 Prepare two, 2 mL sample matrix dilutions, at two-fold and ten-fold dilutions in Sample Diluent. General guidelines for dilutions are described in Table 1.

2 Prepare four matrix spiked samples of the Fab protein to be quantitated in Sample Diluent, neat matrix, and the two-fold and ten-fold diluted matrix (prepared in Step 1) by mixing the minimum volume of Fab analyte and 0.5 mL of each matrix. The final concentration of the Fab protein should be in the middle of the desired quantitation range.

3 Transfer each matrix spiked 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 ten-fold diluted matrix should be hydrated in ten-fold diluted matrix). Place the sample plate and the hydrated biosensors into the Octet instrument. The recommended sample plate warm-up in the instrument and biosensor hydration time is 10 minutes. The delay timer can be used to automatically start the assay after 600 seconds.

5 Set up a Basic Quantitation assay according to the Octet Data Acquisition User Guide. Use one of the provided method template files for the Anti-Human Fab-CH1 Biosensor or use the Modify button in the Plate Definition tab to choose the appropriate Anti-Human Fab-CH1 parameter file (parameter file availability will depend on software version number).

**Anti-Human Fab-CH1 Biosensor assay:** Loads parameters for a 120-second assay at 1000 rpm, typically suited for a 0.5–1000 µg/mL dynamic range (varies with Fab analyte and sample matrix).

6 Run the experiment. Data will be displayed in real time during the assay. Data and method files will be saved automatically.

7 Load the data into Octet Data Analysis software.

| 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 two-fold (2X) |
| Serum-containing cell culture supernatants | Neat |
| Bacterial cell pellet lysed by sonication | Neat |
| Bacterial cell pellet lysed by B-PER | Fifty-fold (50X) |

TABLE 1: Recommended minimum dilution for common sample types. In all cases, the matrix for the diluted samples, standards and biosensor hydration solution should be matched as closely as possible.

8 Inspect the real-time binding traces and determine the dilution required to:

   a Minimize non-specific binding from the matrix.

   b Show equivalent Fab analyte binding in the matrix spiked samples and the Sample Diluent control.

9 Use this dilution factor for routine assays.

### Spike Recovery Assay

To determine the dynamic range and data analysis parameters suitable for specific Fab analytes, establish a standard curve and spike recovery as described below.

1 Prepare a series of Fab standards in the matrix using the dilution factor ascertained in the Dilution Factor Determination for Matrix experiment. The typical assay range of the series spans 0.5–1000 µg/mL at 1000 rpm. Recommended concentrations for the standard curve are shown in Figure 2. The minimum volume needed in each well varies with the plate used:

   - 200 µL/well in a 96-well microplate (all Octet instruments)
   - 80 µL/well in a 384-well microplate (Octet 384 instruments)
   - 40 µL/well in a 384-well, tilted-bottom microplate (Octet 384 instruments)

2 Using the same sample matrix as in Step 1, prepare 700 µL of two unknown samples. The concentration of these samples should be within the assay dynamic range.

3 Transfer triplicates of the standards and the unknowns 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 1.

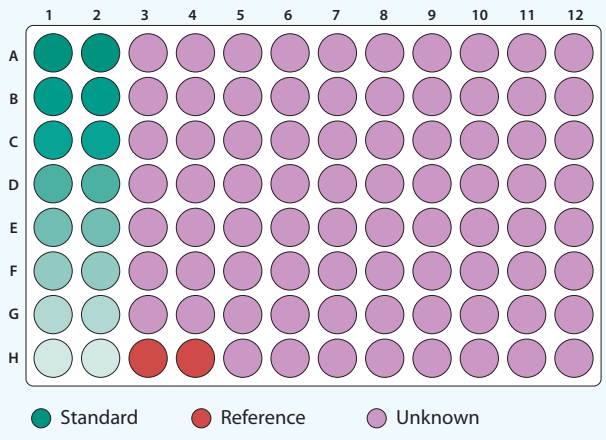


FIGURE 1: Example plate layout for a routine assay run in a 96-well microplate.

4 Hydrate biosensors in the matrix that matches the blank diluted matrix. Place the sample plate and the hydrated biosensors in the Octet instrument. The recommended sample plate warm-up in the instrument and biosensor hydration time is 10 minutes. The delay timer can be used to automatically start the assay after 10 minutes (600 seconds).

5 Set up a Basic Quantitation assay using the assay parameters that were used in the Dilution Factor Determination for Matrix experiment. Availability of pre-loaded Anti-Human Fab-CH1 assay parameters will be software version dependent.

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 Data Analysis software.

8 If blank matrix was included as a reference, use the reference subtraction option to correct the data as appropriate.

9 Calculate the binding rate by initial slope.

10 Define a dynamic range by selecting acceptable % CV values for the lower and upper concentration limits. 10% is routinely used as a threshold, but may vary depending on the assay requirements.

11 Exclude data points for the standard curve that lie outside the defined dynamic range.

12 Iteratively adjust the following processing parameters and recalculate the binding rate:

   a Adjust the read time window if necessary (typically 120 seconds).

   b Adjust the zero concentration threshold if necessary (0.0001 recommended).

   c Adjust the low concentration threshold to 0.001 (recommended).

   d Select the appropriate standard curve equation.

13 Evaluate the calculated concentration value of the unknowns by defining acceptable values of % recovery (accuracy) and % CV (precision). ± 15% recovery and 10% CV are frequently used threshold values but may vary depending on the requirements of each assay. See Figure 2 and Table 2 for representative data.

## REGENERATION

When tested with commercially-sourced human IgG, human Fab, and human $F(ab')_2$ purified samples, the biosensor is able to withstand 5 regeneration cycles while maintaining reproducibility of less than 10% CV when in the concentration range of 5–500 µg/mL and using binding rate for analysis.

- The optimal regeneration buffer is 10 mM glycine at pH 1.7
- The neutralization buffer should be the same as the biosensor pre-wet buffer

- The concentration range of 5–500 µg/mL is analyte- and matrix-dependent
- Users should evaluate their own analyte samples in crude matrices to determine an acceptable concentration range and respective % CV values

### Regeneration Protocol

1 From the Experiment Wizard, select "New Quantitation Experiment" and then "Basic Quantitation with Regeneration."

2 In the first tab of the setup window, click "Modify" and choose "Anti-Human Fab-CH1 (FAB) regeneration" from the built-in assay protocols. If this option is not available in your version of the software, click "Modify" to change the shake speed from the default 400 rpm to 1000 rpm for the Quantitation, Regeneration, and Neutralization steps, then click "OK".

- The regeneration buffer is 10 mM glycine pH 1.7.
- The neutralization buffer should be the same as the biosensor pre-wet buffer.
- Recommended biosensor reuse time is 5.

## ASSAY PROTOCOL

1 Prepare samples, calibration standards and hydration solutions according to the information contained in Table 1. The minimum volume needed in each well varies with the plate used:

- 200 µL/well in a 96-well microplate (all Octet instruments)
- 80 µL/well in a 384-well microplate (Octet 384 instruments)

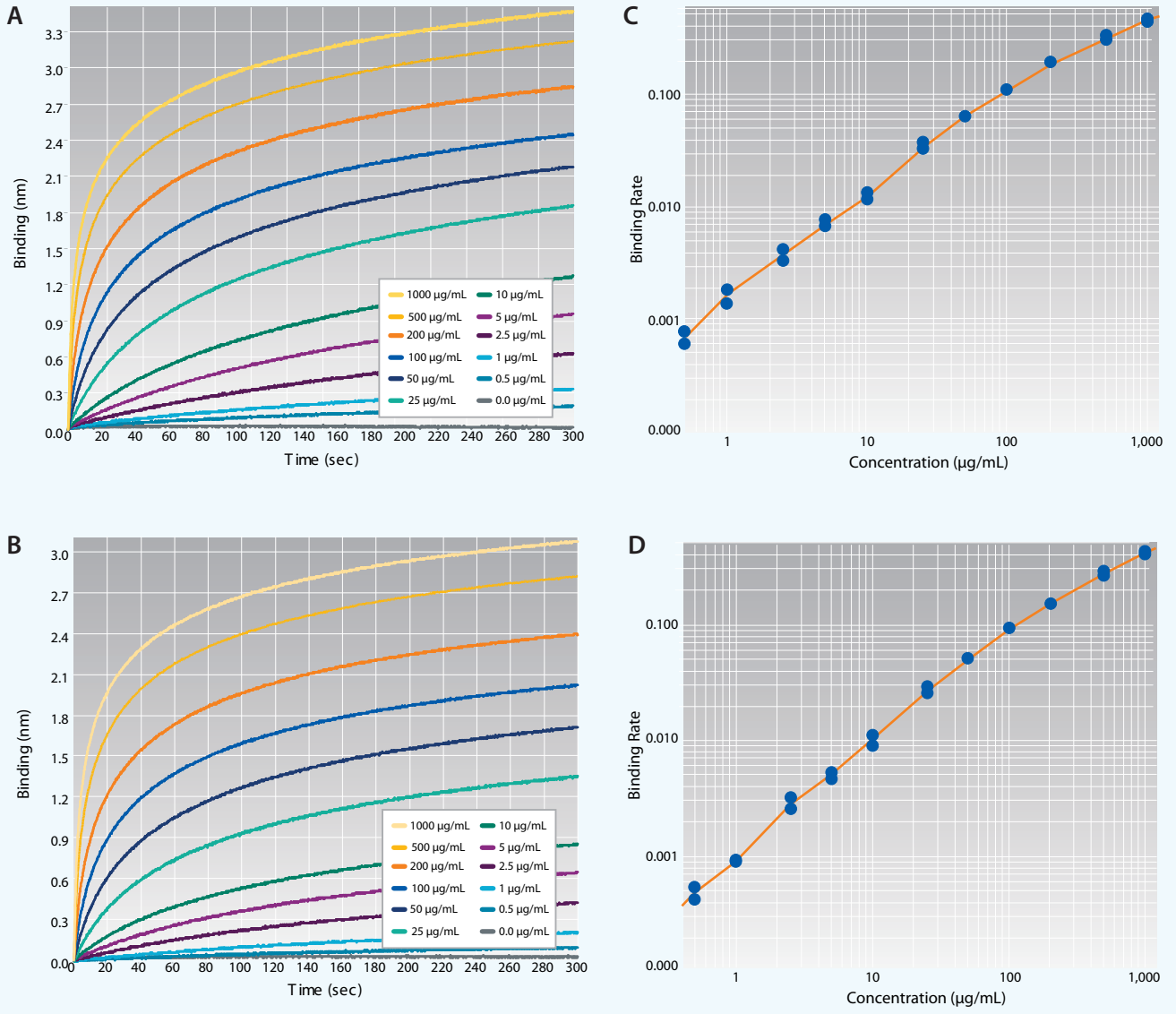


FIGURE 2: Detection of a F(ab')$_2$/Fab standard using Anti-Human Fab-CH1 biosensors on an Octet RED384 system (1000 rpm, 2 minutes) for a standard dynamic range. A) F(ab')$_2$ dose. B) Fab dose. C and D represent the resulting calibration curves from A and B respectively. Sample diluent was used as a matrix for all samples.

- 40 µL/well in a 384-well, tilted-bottom microplate (Octet 384 instruments)

2 Pipette standards, controls and samples into a black polypropylene microplate (see Figure 1 for an example plate layout).

3 Pipette biosensor hydration solution into the wells of a 96-well black, flat-bottom microplate corresponding to the number and position of the biosensors to be used.

4 Place the biosensor tray with the hydration plate in the Octet instrument, then insert the sample plate. Warm the sample plate in the instrument and hydrate the biosensors for 10 minutes prior to starting the experiment. The delay timer can be used to automatically start the assay after 10 minutes (600 seconds).

5 Set up a Basic Quantitation assay (an example plate map is shown in Figure 1). For details on how to set up an assay see the Octet Data Acquisition User Guide.

6 Run the assay.

7 Load data into Octet Data Analysis software.

8 If blank matrix was included as a reference, use the reference subtraction option to correct the data as appropriate.

9 Calculate the binding rate by initial slope.

10 Define a dynamic range by selecting acceptable % CV values for the lower and upper concentration limits. 10% is routinely used as a threshold, but may vary depending on the requirements of each assay.

11 Exclude data points for the standard curve that lie outside the defined dynamic range.

12 Iteratively adjust the following processing parameters and re-calculate the binding rate:

- **a** Adjust the read time window if necessary (typically 120 seconds).
- **b** Adjust the zero concentration threshold if necessary (0.0001 recommended).
- **c** Adjust the low concentration threshold to 0.001 (recommended).
- **d** Select the appropriate Standard Curve Equation.

13 To export the analyzed data, click "Save Report" to generate a Microsoft® Excel® report.

## REPRESENTATIVE DATA

Figure 2 shows detection of a F(ab')$_2$/Fab standard using Anti-Human Fab-CH1 biosensors on an Octet RED384 system with assay parameters of 1000 rpm, 2 minutes for a standard dynamic range. A) F(ab')$_2$ dose, B) Fab dose. C and D represent the resulting calibration curves from A and B respectively. Sample diluent was used as a matrix for all samples. See Table 2 for the statistical analysis of data from Figure 2.

| Expected Concentration (µg/mL) | F(ab')$_2$ Avg. Conc. µg/mL (N = 3) | F(ab')$_2$ % CV (N = 3) | Fab Avg. Conc. µg/mL (N = 3) | Fab % CV (N = 3) |
|---|---|---|---|---|
| 1000 | 1000.0 | 3.0% | 1000.0 | 5.2% |
| 500 | 501.8 | 2.2% | 506.9 | 7.3% |
| 200 | 201.4 | 3.6% | 201.5 | 4.9% |
| 100 | 100.2 | 2.7% | 100.1 | 1.1% |
| 50 | 50.2 | 3.5% | 50.0 | 0.9% |
| 25 | 25.1 | 3.1% | 25.1 | 6.0% |
| 10 | 9.94 | 5.0% | 10.6 | 3.4% |
| 5 | 5.01 | 2.6% | 4.98 | 7.4% |
| 2.5 | 2.50 | 7.3% | 2.59 | 6.8% |
| 1 | 1.05 | 7.4% | 1.00 | 2.6% |
| 0.5 | 0.50 | 6.9% | 0.57 | 5.1% |

TABLE 2: Average calculated concentration and % CV of triplicates of F(ab')$_2$ and Fab calibration standards for the data from Figure 2. Results may vary with individual Fab analytes and assay matrices.

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