

Amine Reactive Second-Generation (AR2G) Biosensors

Overview

Amine Reactive 2nd Generation (AR2G) biosensors enable kinetic characterization of macromolecular interactions between purified proteins and target analytes (Figure 1). Immobilization of proteins is achieved through standard EDC-catalyzed amide bond formation to create a covalent bond between a reactive amine on the protein and the carboxy-terminated biosensor surface. Covalent immobilization fastens the protein to the biosensor surface for analysis of binding events and kinetic characterization. The AR2G biosensor surface is amenable to a wide range of pH and salt conditions, providing robustness and flexibility during the development of regeneration conditions for higher throughput applications.

Principle

Amine Reactive 2nd Generation (AR2G) biosensors provide a surface with a high density of carboxylic acids and a low propensity for non-specific interactions. The carboxylic acids are activated by reaction with EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and s-NHS (N-hydroxysulfosuccinimide) to generate highly reactive NHS esters. The esters

rapidly react with the primary amines of biomolecules such as proteins and peptides to form highly stable amide bonds. The resulting immobilized peptide or protein biosensor can be used for screening or kinetic experiments. The irreversible immobilization is stable in a wide range of pHs (0.5–11) and salts (up to 5M), providing freedom to explore regeneration conditions. Successful regeneration will remove the analyte binding partner and leave the immobilized protein of interest intact on the biosensor surface.

Materials required

- Octet® instrument and software
- Reactive 2nd Generation (AR2G) biosensors (ForteBio part no. 18-5092 [tray]; 18-5093 [pack]; 18-5094 [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-tilted well, black, flat 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)

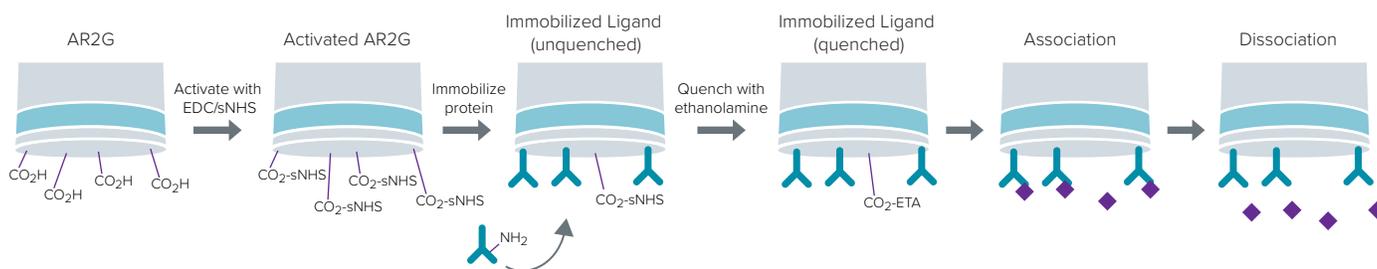


Figure 1: Covalent immobilization of protein on AR2G biosensors with subsequent analyte binding. After activation, immobilization and quenching, the kinetics of association and dissociation between the immobilized ligand and analyte are measured.

- AR2G Reagent Kit (ForteBio part no. 18-5095)
or *equivalent reagents*:
 - EDC stock in water at 400 mM
 - Sulfo-NHS stock in water at 200 mM
 - 1M ethanolamine pH 8.5
 - 10 mM acetate buffer at each pH needed for testing (typically pH 4, 5 and 6)
- Purified protein or peptide to be immobilized. Protein/peptide must be in a buffer without amines (i.e. no Tris or glycine).
- Analyte proteins/peptides that interact with the immobilized protein.
- Running buffer for the assay if different than provided Kinetics buffer. Running buffer should be kept consistent throughout the baseline, association and dissociation steps.
- Optional: Regeneration buffer(s) as needed.

Tips for optimal performance

- Equilibrate reagents and samples to room temperature prior to preparation. For frozen samples, thaw and mix thoroughly prior to use.
- Use EDC and s-NHS within 1 hour of mixing them together. Use or refreeze EDC and s-NHS aliquots within 10 hours of thawing.
- Hydrating the biosensors is required prior to using them in an assay. Hydrate the sensors in nanopure water.
- Ensure that the Octet instrument is turned on and the lamp is warmed up to room temperature for at least 40 minutes prior to starting the assay.
- Set the sample plate temperature in the Octet software by selecting File > Experiment > Set plate temperature and entering the desired temperature. ForteBio recommends assaying at 30°C. Assaying at other temperatures may require different assay times than discussed in this protocol.
- If immobilization conditions on AR2G biosensors for protein/peptide are unknown, optimization of immobilization conditions is recommended.
- For optimal performance in determining kinetic rates, a reference biosensor should be included. A reference biosensor should have the protein/peptide immobilized and should be run with an assay buffer blank for the association and dissociation steps.

Assay protocol

OVERVIEW

- 1 Determine immobilization conditions.
- 2 Perform screening or kinetic assay.
- 3 Perform data processing and analysis.

DETERMINE IMMOBILIZATION CONDITIONS

For screening assays that don't require optimal signal to noise ratios, most proteins will immobilize sufficiently using 20 mM EDC and 10 mM sulfo-NHS in 10 mM Acetate pH 5 buffer (included in the AR2G reagent kit). For assays of this type, proceed to the "Perform Screening or Kinetic Assay" section below.

For kinetic assays or assays in which optimal performance is critical, it is recommended to scout various immobilization conditions as described below.

The volumes and plate configuration (refer to Figure 2) given in the following example are for a 96-well plate. If a 384-well tilted bottom (384TW) or standard 384-well plate will be used, sample volumes and placements should be adjusted accordingly (80 µL per well for a standard 384-well plate and 40 µL per well for a 384TW plate).

- 1 Prepare reagents according to the AR2G kit instructions. EDC and Sulfo-NHS aliquots should be stored at -20°C and thawed immediately prior to use. Thawed aliquots should be used or refrozen within 10 hours of thawing. Mixtures of EDC and s-NHS should be used within 1 hour of mixing.
- 2 For a standard immobilization scouting assay, prepare the sample plate as shown in Figure 2.
 - a Pipette 200 µL/well water into column 1 of a 96-well plate.
 - b Pipette 200 µL/well 1M ethanolamine pH 8.5 into column 4.
 - c Pipette 200 µL/well assay running buffer into column 5. Use the provided Kinetics Buffer (dilute the 10X Kinetics Buffer 10-fold in PBS) or other running buffer as desired (HBS, PBS, PBS-T, etc). The assay running buffer should be used to dilute the analyte.
 - d Prepare a minimum of 1.3 mL of analyte in the running buffer to be used for the kinetic assay. A starting analyte concentration 10X over the expected K_d is recommended when scouting immobilization conditions. Pipette 200 µL/well of the analyte into column 6.
 - e Prepare the protein/peptide to be immobilized. Protein should be prepared in each of the 10 mM acetate buffers at pH 4, 5 and 6. A starting ligand concentration of at least 20 µg/mL is recommended. The ligand concentration may be decreased as low as 5 µg/mL if the immobilization time is extended; some performance loss is expected at lower ligand concentrations. Prepare 500 µL at each pH. Pipette 200 µL/well into column 3 as shown in Figure 2.
 - f Thaw EDC and s-NHS aliquots. Prepare 1300 µL of a 20 mM EDC and 10 mM s-NHS working reagent mix by adding 65 µL each of the stock EDC and s-NHS reagents to 1170 µL of water. Mix thoroughly. Pipette 200 µL/well of the EDC/s-NHS mixture into column 2.

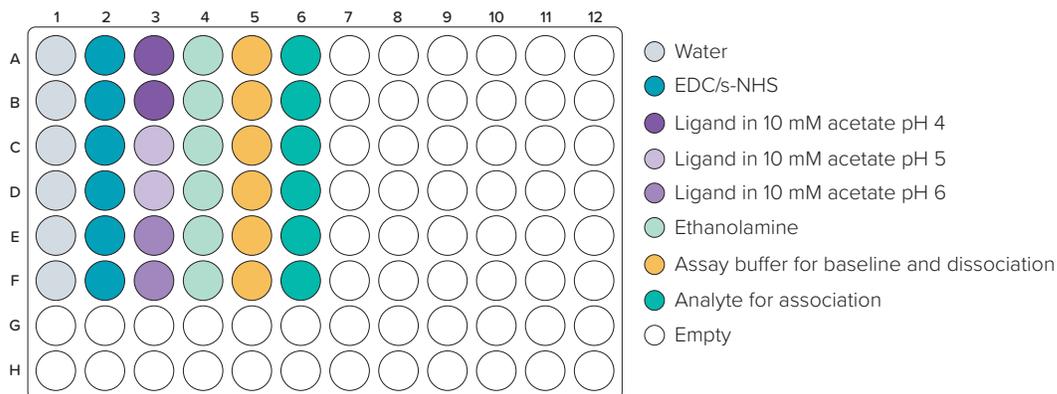


Figure 2: Example sample plate layout for scouting ligand immobilization conditions. Use of the same buffer wells for baseline and dissociation enables use of inter-step correction during data processing.

Step #	Step name	Time (sec)	Shake speed	Step type	Sample plate column (see Figure 2)
1	Equilibration	60	1000	Custom	1
2	Activation	300	1000	Activation	2
3	Immobilization	600	1000	Loading	3
4	Quench	300	1000	Quench	4
5	Baseline	120	1000	Baseline	5
6	Binding	300	1000	Association	6
7	Dissociation	300	1000	Dissociation	5

Table 1: Example assay steps and associated parameters.

3 Hydrate the biosensors:

- Place 6 AR2G biosensors in the top 6 positions of column 1 in an empty biosensor tray.
- Pipette 200 μ L/well water into the wells of a 96-well plate corresponding to the positions of the biosensors to create the hydration plate.
- 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. Alternatively, hydrate the biosensors in water in the Octet instrument according to instrument instructions. Allow biosensors to hydrate for a minimum of 10 minutes (it is critical to hydrate the biosensors for at least 10 minutes)

4 Place both the sample plate and hydration plate/biosensors into the Octet instrument.

5 Step up the assay shown in Table 1 in the Data Acquisition software:

6 Use the Delayed Experiment Start (if needed) to ensure biosensors have been hydrated for at least 600 seconds.

7 Start assay by clicking GO.

8 Optimal immobilization conditions can be determined by analysis of the conditions that generated the desired signal during association.

See Figure 3 for example data. If protein does not immobilize sufficiently or no analyte signal is seen, see the troubleshooting table for tips.

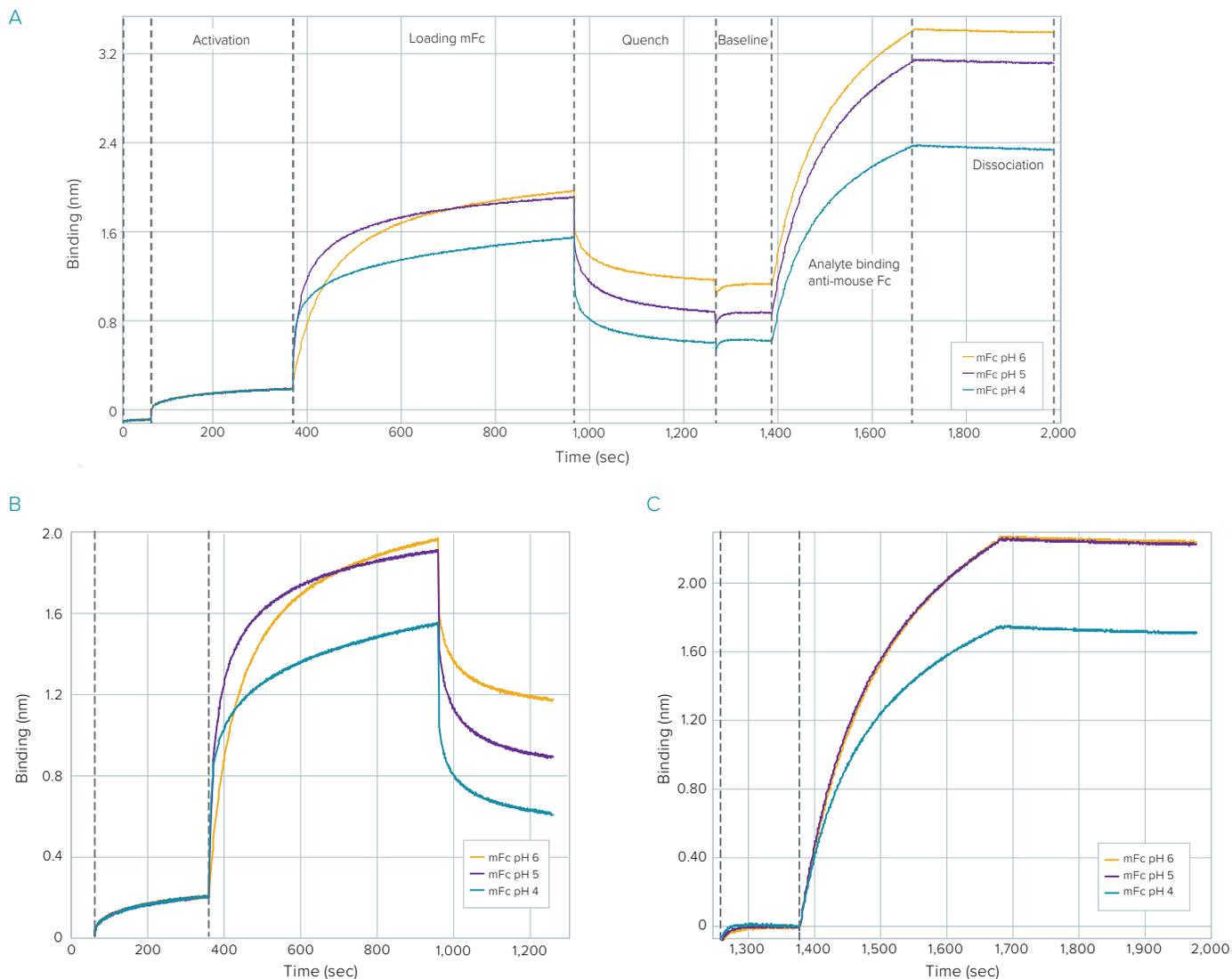


Figure 3: (A) Determination of optimal immobilization conditions for a mouse Fc to the AR2G biosensor (performed on an Octet RED96 System). The Mouse Fc ligand concentration was 20 $\mu\text{g}/\text{mL}$ in 10 mM acetate at pH 4, 5 and 6. Baseline, association and dissociation steps were performed in 1X Kinetics Buffer. Anti-mouse Fc antibody at 8 $\mu\text{g}/\text{mL}$ was used as the analyte. (B) Analysis of ligand immobilization scouting at pH 4, 5 and 6. The loading step (immobilization of ligand) was overlaid and aligned to the baseline. pH 6 generated the maximum signal intensity of approximately 2.0 nm and 1.2 nm after quenching. Signal intensity at pH 5 was slightly less during the loading and quenching steps. (C) Analysis of analyte detection scouting at pH 4, 5 and 6. The association step (detection of analyte) was overlaid and aligned to the baseline. pH 5 and 6 generated maximum analyte signals of approximately 2.3 nm. Performance at pH 4 was slightly less with a maximum nm shift of approximately 1.75 nm. pH 6 was selected as the final immobilization pH because it produced a signal equivalent to pH 5 under milder conditions.

PERFORM SCREENING OR KINETIC ASSAY

Volumes given below and the sample plate layout in Figure 4 are for a 96-well plate. If a 384TW or standard 384-well plate will be used, sample volumes and placements should be adjusted accordingly (80 μL per well for a standard 384-well plate and 40 μL per well for a 384TW plate).

- 1 Prepare reagents according to AR2G kit instructions. EDC and Sulfo-NHS aliquots should be stored at -20°C and thawed immediately prior to use. Thawed aliquots should be used or refrozen within 3 hours of thawing. Mixtures of EDC and s-NHS should be used within 1 hour of mixing.

- 2 For a standard kinetics assay, prepare the sample plate as shown in Figure 4.
 - a Pipette 200 μL /well water into column 1.
 - b Pipette 200 μL /well 1M ethanolamine pH 8.5 into column 4.
 - c Pipette 200 μL /well assay running buffer into column 5. Use the provided Kinetics Buffer (dilute the 10X Kinetics Buffer 10-fold in PBS) or other running buffer as desired (HBS, PBS, PBS-T, etc). The assay running buffer should be used to dilute the analyte.

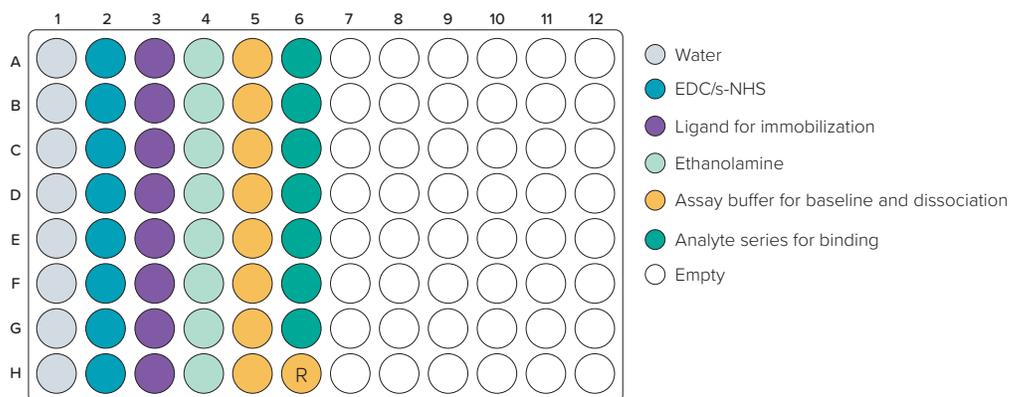


Figure 4: Example sample plate layout for a kinetic assay using the AR2G biosensor. Well H6 is used as a reference well “R” during the assay. Use of the same buffer wells for baseline and dissociation enables use of inter-step correction during data processing.

Step #	Step name	Time (sec)	Shake speed	Step type	Sample plate column (see figure 4)
1	Equilibration	60	1000	Custom	1
2	Activation	300	1000	Activation	2
3	Immobilization	600–1200	1000	Loading	3
4	Quench	300	1000	Quench	4
5	Baseline	120	1000	Baseline	5
6	Binding	300–900	1000	Association	6
7	Dissociation	600–1800	1000	Dissociation	5

Table 2: Example assay steps and associated parameters.

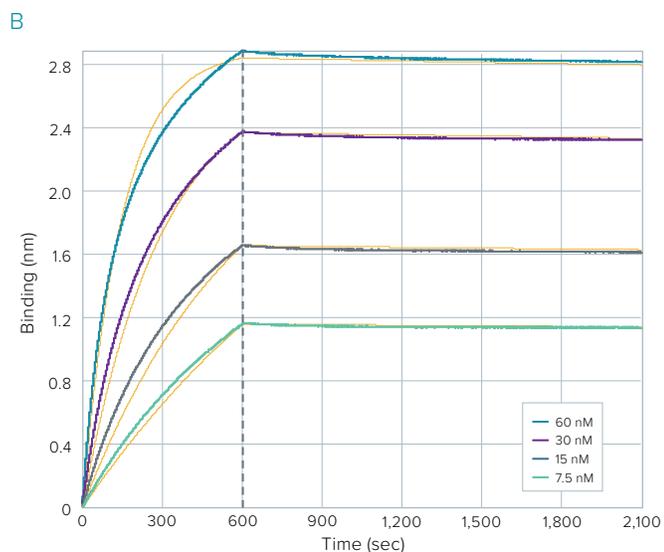
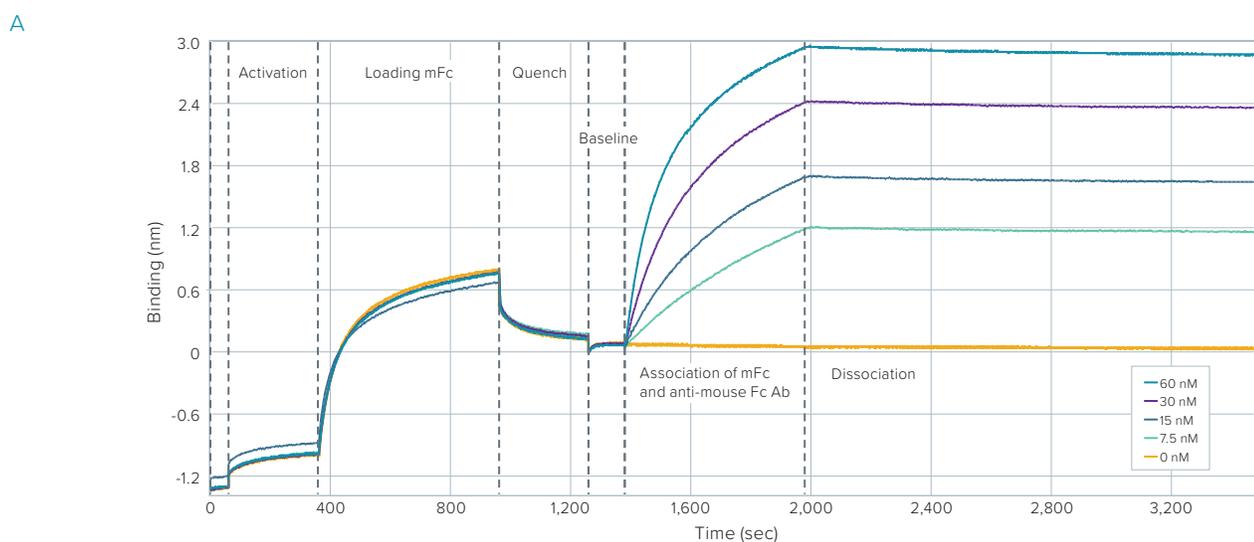
- d Prepare analyte in the assay running buffer used for the kinetic binding. A 2-fold dilution series starting at 10X over the expected K_d is recommended for most proteins. Prepare at least 220 μL at each concentration. If replicates are desired, prepare enough added volume to fill each well with 200 μL .
 - e Pipette 200 μL /well of the analyte into wells A–G of column 6. For optimal performance, pipette 200 μL /well running buffer into well H of column 6 to use as a baseline reference.
 - f Prepare the protein/peptide to be immobilized. Protein should be prepared in the 10 mM acetate buffers at the optimal pH identified above. Best performance will be seen with a concentration of at least 20 $\mu\text{g}/\text{mL}$, however as little as 5 $\mu\text{g}/\text{mL}$ can be used with some loss in binding signal. If the concentration of protein is lower than 20 $\mu\text{g}/\text{mL}$, the immobilization time should be extended. Prepare at least 1.7 mL to fill 8 wells. Pipette 200 μL /well in column 3.
 - g Thaw EDC and s-NHS aliquots. Create a 20 mM EDC and 10 mM s-NHS working reagent mix by adding 100 μL each of EDC and s-NHS to 1800 μL of water and mixing thoroughly. Pipette 200 μL /well of the EDC/s-NHS mixture into column 2.
- 3 Hydrate the biosensors:
 - a Place 8 AR2G biosensors in column 1 of an empty biosensor tray.
 - b Pipette 200 μL /well of water into the wells of a 96-well plate corresponding to the positions of the biosensors to create the hydration plate.
 - c 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. Alternatively, hydrate the biosensors in water in the Octet instrument according to instrument instructions. Allow biosensors to hydrate for a minimum of 10 minutes (it is critical to hydrate the biosensors for at least 10 minutes).

- Place both the sample plate and hydration plate/biosensors in the Octet instrument.
- Step up the assay shown in Table 2 in the Data Acquisition software.
- Immobilization, Binding and Dissociation times can be extended if low concentrations are used or slow binding or dissociation is expected.
- Use the Delayed Experiment Start (if needed) to ensure biosensors have been hydrated for at least 600 seconds.
- Start assay by clicking GO.

See Figure 5 for example kinetics data. If the protein does not immobilize or no analyte signal is seen, refer to the troubleshooting table for tips.

PROCESS AND ANALYZE THE DATA

- Load data into the Octet Data Analysis software.
- Process the data by specifying methods for reference subtraction, y-axis alignment, inter-step correction and Savitzky-Golay filtering. For details on each processing parameter, refer to the *Octet Data Analysis User Guide*.
- Analyze the data by specifying steps for analysis, fitting method (local or global) and window of interest. For details on each analysis parameter, refer to the *Octet Data Analysis User Guide*.
- To export the analyzed data, use the Save Report button to generate a Microsoft Excel report. For details on data exporting, refer to the *Octet Data Analysis User Guide*.



C

K_D (M)	k_{on} (1/Ms)	k_{on} Error	k_{dis} (1/s)	k_{dis} Error
9.19E-11	1.13E+05	2.74E+02	1.04E-05	4.58E-07

Figure 5: (A) Kinetic characterization between a mouse Fc domain and an anti-mouse antibody on the AR2G biosensor. Mouse Fc was immobilized at 20 µg/mL in 10 mM acetate at pH 6 (optimal conditions determined based on data in Figure 3). Baseline, dissociation and analyte were run in 1X Kinetics Buffer. The association and dissociation kinetics of the analyte, anti-mouse Fc antibody, were performed at concentrations of 7.5, 15, 30 and 60 nM. (B) The kinetic dataset was globally fit using a 1:1 binding model.

Troubleshooting tips

Symptom	Solutions
Sensor failure error during assay.	<ul style="list-style-type: none">• Make sure biosensor is immersed in the hydration solution in the instrument.• Make sure to only use black, polypropylene plates for the hydration plate and sample plate. The Octet system adjusts lamp levels in the hydration plate to insure optimal light intensity is used during the assay.• Hydrate the biosensor for the recommended time.• Make sure all wells have the recommended volume in them (at least 200 μL for 96-well plates, 80 μL for 384-well plates and 40 μL for 384TW well plates).
Low immobilization density of protein/peptide.	<ul style="list-style-type: none">• Ensure protein to be immobilized is in buffer without amine containing contaminants (<i>i.e.</i> no Tris or ammonium sulfate). If amine containing salts are present, dialyze protein into PBS. If protein contaminants are present, purify protein using standard methods.• Run optimization assay to determine best pH for immobilization. If pH needs to be further optimized, create 10 mM acetate buffers at finer gradations of pH (4.5, 4.75 etc).• In rare cases the EDC/s-NHS concentration may need to be optimized. In these cases, run the immobilization optimization experiment at several concentrations of EDC/s-NHS. A useful series is to run a 1:1 mixture of the EDC and sNHS stocks and a 1:50 dilution to give 200:100 mM and 4:2 mM EDC:sNHS respectively.• Increase the concentration of the protein to be immobilized and/or increase the immobilization (loading) step time.
Analyte signal is low.	<ul style="list-style-type: none">• Ensure immobilization of ligand is optimized.• Determine if analyte binds to the ligand. A useful test, if pure analyte is available in amine free buffer, is to reverse the orientation and try to immobilize the analyte. Alternatively, use an orthogonal assay method to test activity.• Increase the concentration of the analyte to test for weaker than expected binding. Time for the binding (association step) can also be increased.
Binding activity is lower or lost after regeneration.	<ul style="list-style-type: none">• Incomplete regeneration can result in lower binding. See ForteBio Technical Note 8 for general guidelines on developing regeneration conditions.



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