STANDARD OPERATING PROCEDURE

Title: Kinetics and Affinity Evaluation of Monoclonal Antibodies and Protein Interactions Using Biolayer Interferometry (BLI) on the Sartorius Octet Red96e

SOP#: M-141

Version #: 1 **Authors: T. Caceres**

Date Approved: August 31, 2021 **Date Modified:**

1. PURPOSE

This procedure will describe how to measure the equilibrium constant, K_D, the association rate constant, K_a, and the dissociation rate constant, K_d, of purified proteins or peptides and their purified cognate monoclonal antibodies by Biolayer Interferometry using a Sartorius Octet 96e instrument.

2. SCOPE

This procedure will describe sample preparation and operation of the Sartorius Octet Red96e instrument using either a capture or amine coupling immobilization method to measure affinity and kinetics parameters.

3. RESPONSIBILITY

It is the responsibility of person(s) performing this procedure to be familiar with lab safety procedures. It is the responsibility of person(s) performing this procedure to have read the references listed at the end of this document for proper training. Data analysis must be done by a person trained in the procedure and trained in the interpretation of results using the Data Analysis HT software. It is the responsibility of the analyst to follow the procedure steps as written and to document any deviations, problems, and observations during an experiment in their laboratory notebook.

4. EQUIPMENT

4.1. Octet Red96e™System; Pall ForteBio, Part No: 30-5051

4.2. Rainin Pipet plus LTS Pipettor, adjustable 100-1000 µL

4.3. Rainin Pipet plus LTS Pipettor, adjustable 20-200 µL

4.4. Rainin Pipet plus LTS Pipettor, adjustable 2-20 µL

4.5. Rainin Pipet plus LTS Pipettor, adjustable 0.1-2 µL

4.6. ForteBio Biosensor Dispenser (1.0 uL – 10.0 uL) for biosensor(s) transfer

4.7. Multichannel pipettor, 20 uL – 200 uL

4.8. Evaporation cover (white), Sartorius, Part #: 18-5132

5. MATERIALS

5.1. Octet® Anti-Mouse Fc Capture (AMC) Biosensors, Sartorius, Part #: 18-5088

5.2. Octet® Amine Reactive 2nd Generation (AR2G) Biosensors, Sartorius, Part #: 18-5092

5.3. Octet® Protein A (ProA) Biosensors, Sartorius, Part #: 18-5010

5.4. Octet® Protein G (ProG) Biosensors, Sartorius, Part #: 18-5082

5.4. Amine Coupling Second Generation Reagent Kit Part #: 18-5095

- 10mM Sodium Acetate pH 4.0 Part #: 18-1068
- 10mM Sodium Acetate pH 5.0 Part #: 18-1069
- 10mM Sodium Acetate pH 6.0 Part #: 18-1070
- 1M Ethanolamine pH 8.5 Part #: 18-1071
- EDC 1g Part #: 18-1033
- Sulfo-NHS 573mg Part #: 18-1067

5.5. 10X Kinetics Buffer, Sartorius, Part #:18-1092

5.6. Bio-Rad ProteOn™ Glycine Buffer, pH 2.0 Cat.#: 176-2221

5.7. PBS 10X Solution Ultra Pure VWR Part #: J373-4L

5.8. Deionized, 0.22 µM filtered H_2O, In-house or equivalent

5.9. 96-well, black microplates Greiner Bio-One Part #: 655209

6.0. 1.5 mL Eppendorf polypropylene tubes, or equivalent

6. REAGENTS

6.1. Protein(s) or peptide(s) corresponding to desired target

6.2. Monoclonal antibody or antibodies corresponding to desired cancer related Protein or peptide

7. PROCEDURE

7.1. **Preparation of the Sartorius Octet Red96e instrument**

7.1.1. Turn on the computer.

7.1.2. Turn the Octet instrument on using the power switch located on the external electrical box. The 8-channel biosensor manifold will move within the instrument as the instrument completes a self-check. Wait until this self-check finishes.

7.1.3. Launch the Octet System Data Acquisition software Version 11.1 by double-clicking on the Data Acquisition desktop icon.

7.1.4. Select *New Kinetics Experiment (Basic Kinetics)* from the *Experiment Wizard*

7.1.5. Click the green arrow to open a *Blank Experiment* template.

7.2. Kinetics Analysis Using the Anti-Mouse IgG Fc Capture (AMC) or Protein A/G Biosensor

7.2.1. On the bench, pipette 200 uL/ well of deionized, 0.22 µm H_2O into wells A1 – H1 and A2 – H2 of a new Greiner Bio-One black 96 well plate (hydration tray).

7.2.2. Open a new pack of anti-mouse IgG Fc capture (AMC) biosensors if analyzing mouse antibody kinetics or a new pack of Protein A/G biosensors if analyzing rabbit antibody kinetics (this biosensor can also be used for mouse antibody kinetics if the AMC biosensor is unavailable). Or alternatively, a pack that has already been opened can also be used if it is has been stored properly with the opening sealed shut.

7.2.3. Gently place the green biosensor tray containing sixteen AMC biosensors (or Protein A/G biosensors) that correspond to wells A1 – H1 and A2 – H2 on top of the hydration tray (black) taking care not to touch the tips of the biosensors. If necessary, use the Octet dedicated multichannel pipettor to transfer biosensors to the appropriate wells in the green biosensor tray before placing it on top of the hydration tray.

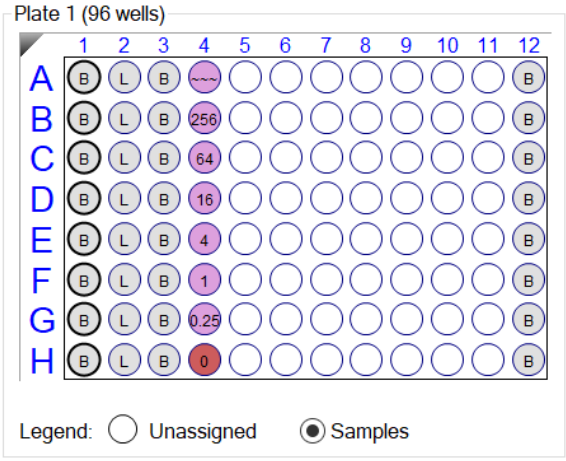
7.2.4. Place the biosensor hydration tray into the Octet Red 96e instrument on the left side. Hydrate the sixteen AMC biosensors (or Protein A/G biosensors) for at least ten minutes.

7.2.5. Remove the 10X kinetics buffer from 4°C and let it equilibrate to room temperature.

7.2.6. In a 50 mL conical tube, dilute 2.0 mL of 10X PBS into 18 mL of 0.22 um filtered H_2O making a 20 mL 1X PBS stock solution.

7.2.7. In a 50 mL conical tube, dilute 2.0 mL of 10X Kinetics Buffer into 18 mL of 1X PBS solution making a 20 mL 1X kinetics buffer stock solution.

7.2.8. Add 200 uL of 1X kinetics buffer to wells A1 – H1 (B), A3 – H3 (B), H4 (Red, *no analyte reference well*), and A12 – H12 (B) of a new Greiner Bio-One black 96 well assay plate. See below.



7.2.9. Remove the test monoclonal antibody (mouse or rabbit) from storage (4°C or -20°C).

7.2.10. Dilute the antibody in 1X Kinetics Buffer to the optimum ligand concentration that was determined in assay development tests or elsewhere. Commonly used concentrations fall in the 5 – 25 ug/mL range to yield a surface density of 5 - 10 nanometers. Each well requires 200 uL of diluted antibody ligand. For eight wells, prepare 1.8 mL total volume of diluted antibody ligand.

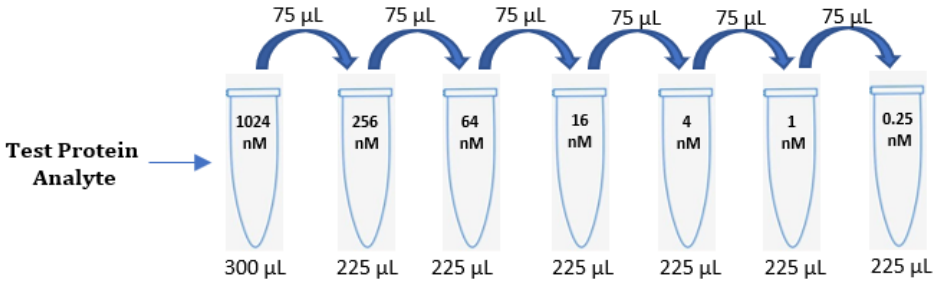
Note: See <u>Appendix A</u> for specific recommendations for identifying an optimum ligand antibody concentration.

7.2.11. Vortex.

7.2.12. Add 200 uL of the diluted antibody ligand to wells A2 – H2 (L) of the assay plate.

7.2.13. Remove the test protein or peptide analyte(s) from storage. Let the test analyte(s) equilibrate to room temperature. If necessary, reconstitute the test protein or peptide analyte(s) according to the commercial vendor instructions.

7.2.14. Using 1.5 mL Eppendorf tubes, prepare the test analyte at a concentration range that is approximately 0.1X – 100X the K_D of the binding interaction. If no knowledge of the kinetics is available, dilute the analyte to 1024 nM in 1X kinetics buffer. Serially dilute the 1024 nM to prepare 4-fold dilutions as seen below.



7.2.15. Add 200 uL of the diluted test analyte protein to sample wells A4 – G4 (Pink) of the assay plate (7.2.8).

7.2.16. Carefully place the assay plate inside the Sartorius Octet Red96e instrument on the right side. Use the arrow indicator to guide the correction positioning of the plate.

7.2.17. After the plate has been inserted properly in the instrument, proceed to set up the assay method (use of the evaporation cover is optional for overnight assays or higher than room temperature experiments).

7.2.18. **Kinetics Assay Setup on the Octet**

7.2.18.1. Click on File -> Open Method File…

7.2.18.2. Navigate to Share -> Instrumentation -> Octet -> SOP Protocols, and open the ***AMC_Kinetics_Template***

7.2.18.3. In the *Plate Definition* tab, highlight the *Test Ligand* in Wells A2-H2 under the Sample ID Column.

7.2.18.4. Right click after selecting all wells and select *Set Well Data.*

7.2.18.5. Input the appropriate name of the test antibody ligand.

7.2.18.6. The test antibody ligand concentration default setting is 5 ug/mL. Modify the concentration if necessary.

7.2.18.7. Highlight the *Test Analyte* in Wells A4 – H4 under the Sample ID column.

7.2.18.8. Right click after selecting all wells and select *Set Well Data.*

7.2.18.9. Input the appropriate name of the test analyte.

7.2.18.10. Input the molecular weight of the test analyte (kDa).

7.2.18.11. Input the molar concentration of the test analyte (nM) that was prepared. Input '0' for the molar concentration of the buffer *reference well*.

NOTE: Be sure to include a no analyte reference well (red well).

Well	Sample ID	Replicate Group	Type	Conc (µg/ml)	MW (kD)	Molar Conc (nM)	Information
B A1	1X Kinetics Buffer		Buffer				
B B1	1X Kinetics Buffer		Buffer				
B C1	1X Kinetics Buffer		Buffer				
B D1	1X Kinetics Buffer		Buffer				
B E1	1X Kinetics Buffer		Buffer				
B F1	1X Kinetics Buffer		Buffer				
B G1	1X Kinetics Buffer		Buffer				
B H1	1X Kinetics Buffer		Buffer				
L A2	Test Ligand		Load	5			
L B2	Test Ligand		Load	5			
L C2	Test Ligand		Load	5			
L D2	Test Ligand		Load	5			
L E2	Test Ligand		Load	5			
L F2	Test Ligand		Load	5			
L G2	Test Ligand		Load	5			
L H2	Test Ligand		Load	5			
B A3	1X Kinetics Buffer		Buffer				
B B3	1X Kinetics Buffer		Buffer				
B C3	1X Kinetics Buffer		Buffer				
B D3	1X Kinetics Buffer		Buffer				
B E3	1X Kinetics Buffer		Buffer				
B F3	1X Kinetics Buffer		Buffer				
B G3	1X Kinetics Buffer		Buffer				
B H3	1X Kinetics Buffer		Buffer				
A4	Test Analyte		Sample	102.4	100	1024	
B4	Test Analyte		Sample	25.6	100	256	
C4	Test Analyte		Sample	6.4	100	64	
D4	Test Analyte		Sample	1.6	100	16	
E4	Test Analyte		Sample	0.4	100	4	
F4	Test Analyte		Sample	0.1	100	1	
G4	Test Analyte		Sample	0.025	100	0.25	
H4	Test Analyte		Referen	0	100	0	

7.2.18.12. Click on the Assay Definition tab. Verify the assay settings are as seen below:

Step Data List

Add... | Copy | Remove | Regeneration Params | Threshold Params

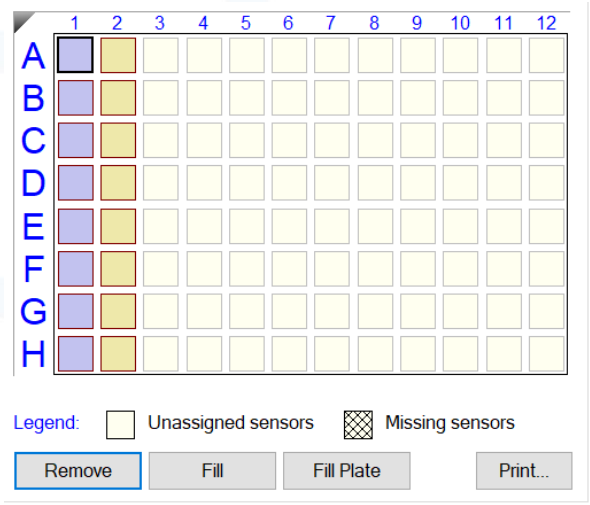
	Name	Time	Shake speed	Type	Threshold
→	Baseline	180	1000	Baseline	☐
	Loading	300	1000	Loading	☐
	Equilibration	600	1000	Baseline	☐
	Association	600	1000	Association	☐
	Dissociation	600	1000	Dissociation	☐

Assay Steps List

New Assay | Move Up | Move Down | Remove | Replicate | Edit Step | Info Table

Assay	No.	Sample	Step Name	Step Type	Sensor Type	Assay Time	Comment
1	1	1	Equilibration	Baseline	AMC (Anti-mIgG Fc Capture)		
1	2	2	Loading	Loading	AMC (Anti-mIgG Fc Capture)		
1	3	3	Baseline	Baseline	AMC (Anti-mIgG Fc Capture)		
1	4	4	Association	Association	AMC (Anti-mIgG Fc Capture)		
1	5	3	Dissociation	Dissociation	AMC (Anti-mIgG Fc Capture)	0:38:40	
2	1	1	Equilibration	Baseline	AMC (Anti-mIgG Fc Capture)		
2	2	12	Loading	Loading	AMC (Anti-mIgG Fc Capture)		
2	3	3	Baseline	Baseline	AMC (Anti-mIgG Fc Capture)		
2	4	4	Association	Association	AMC (Anti-mIgG Fc Capture)		
2	5	3	Dissociation	Dissociation	AMC (Anti-mIgG Fc Capture)	0:38:40	

7.2.18.13. Click on the Sensor Assignment tab. Verify the biosensors appear as seen below. Optionally, check the box above the tray image to return the sensors back to its original location rather than to the waste compartment.



Note: The yellow biosensors (Column 2) are the reference biosensors. They are required to test for non-specific binding. If the absence of non-specific binding of the analyte for the biosensor has already been determined, these biosensors may be omitted from the assay.

7.2.18.14. Click on the Review Experiment tab. Review the assay steps by moving the blue slider.

7.2.18.15. Click on the Run Experiment tab. In the “Data File Location and Names” section, assign a primary directory folder destination in the “Kinetics data repository” field but clicking on the ellipses and creating folder in that destination. Assign a secondary “Experiment run name” folder within the primary destination clicking on the arrow and creating folder in that destination. In the “Run Settings” section check the “Delayed experiment start” box and get the timer for 600 seconds. Check the box with the option to “Shake sample plate while waiting”. Check the box with the option for “Open runtime charts automatically” as well as the sub

option “Automatically save runtime chart”. Check the option “Set plate temperature (*C) to 25. Within the “Advanced Settings” location, set the acquisition rate to “Standard kinetics (5.0Hz, averaging by 20) “. Then proceed to start the experiment by clicking on the green “Go” button.

7.2.18.16. Skip to section 7.4 for Data Analysis.

7.3. Kinetics analysis using the Amine Reactive 2nd Generation (AR2G) Biosensor

Note: The use of AR2G biosensors is recommended for interactions with very strong (pM affinity) binding.

7.3.1. On the bench, pipette 200 uL/ well of deionized, 0.22 µm H_2O into wells A1 – H1 of a new Greiner Bio-One black 96 well plate (hydration tray).

7.3.2. Open a new pack of Amine Reactive 2nd Generation (AR2G) biosensors. Or alternatively, a pack that has already been opened can also be used if it is has been stored properly with the opening sealed shut.

7.3.3. Gently place the green biosensor tray containing eight AR2G biosensors that correspond to wells A1 – H1 on top of the hydration tray (black) taking care not to touch the tips of the biosensors. If necessary, use the Octet dedicated multichannel pipettor to transfer biosensors to the appropriate wells in the green biosensor tray before placing it on top of the hydration tray.

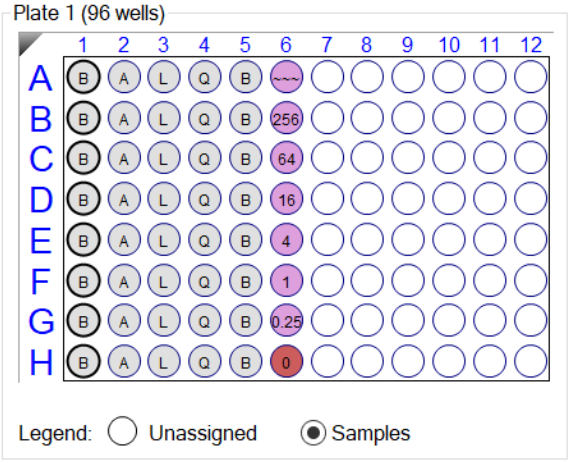
7.3.4. Place the biosensor hydration tray into the Octet Red 96e instrument on the left side. Hydrate the eight AR2G biosensors for at least ten minutes.

7.3.5. Remove the 10X kinetics buffer from 4°C and let it equilibrate to room temperature.

7.3.6. In a 50 mL conical tube, dilute 2.0 mL of 10X PBS into 18 mL of 0.22 um filtered H_2O making a 20 mL 1X PBS stock solution.

7.3.7. In a 50 mL conical tube, dilute 2.0 mL of 10X Kinetics Buffer into 18 mL of 1X PBS solution making a 20 mL 1X kinetics buffer stock solution.

7.3.8. Add 200 uL of 0.22 um filtered H_2O to wells A1 – H1 (B) in a new Greiner Bio-One black 96 well assay plate. See below.



7.3.9. Remove one aliquot each of EDC (400 mM) and s-NHS (200 mM) from the -20C. Thaw at room temperature.

7.3.10. Vortex briefly after thawing.

7.3.11. Prepare 1800 µL of a 20 mM EDC and 10 mM s-NHS working reagent mix by adding 90 µL each of the stock EDC and s-NHS reagents to 1620 µL of water. Mix thoroughly. Pipette 200 µL/well of the EDC/s-NHS mixture into wells A2 – H2 (A).

Note: Use or refreeze EDC and s-NHS aliquots within 10 hours of thawing. Use EDC and s-NHS within 1 hour of mixing them together.

7.3.12. Add 200 uL of 1X kinetics buffer to wells A5 – H5 (B) and H6 (Red, *no analyte reference well*).

7.3.13. Remove 1M ethanolamine pH 8.5 from 4C.

7.3.14. Add 200 uL of 1M ethanolamine pH 8.5 to wells A4 – H4 (Q).

7.3.15. Remove the test monoclonal antibody (mouse or rabbit) from storage (4°C or -20°C).

7.3.16. Dilute the antibody in 10 mM sodium acetate pH 5.0 to the optimum ligand concentration that was determined in assay development tests or elsewhere. Commonly used concentrations fall in the 5 – 25 ug/mL range, or a surface density of 5 – 10 nanometers. Each well requires 200 uL of diluted antibody ligand. For eight wells, prepare 1.8 mL total volume of diluted antibody ligand.

Note: See Appendix A for specific recommendations for identifying an optimum ligand antibody concentration.

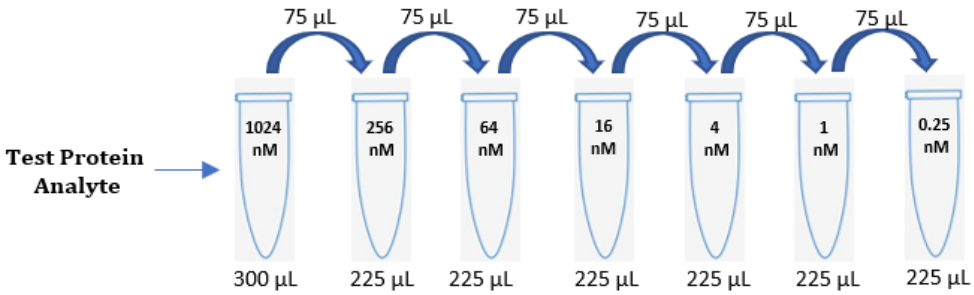
Note: It may be necessary to optimize the pH of the antibody immobilization prior to running a kinetics assay. See Appendix A for specific recommendations.

7.3.17. Vortex.

7.3.18. Add 200 uL of the diluted antibody ligand to wells A3 – H3 (L) of the assay plate.

7.3.19. Remove the test protein or peptide analyte(s) from storage. Let the test analyte(s) equilibrate to room temperature. If necessary, reconstitute the test protein or peptide analyte(s) according to the commercial vendor instructions.

7.3.20. Using 1.5 mL Eppendorf tubes, prepare the test analyte at a concentration range that is approximately 0.1X – 100X the K_D of the binding interaction. If no knowledge of the kinetics is available, dilute the analyte to 1024 nM in 1X kinetics buffer. Serially dilute the 1024 nM to prepare 4-fold dilutions as seen below.



7.3.21. Add 200 uL of the diluted test analyte protein to sample wells A6 – G6 (Pink) of the assay plate.

7.3.22. Carefully place the assay plate inside the Sartorius Octet Red96e instrument on the right side. Use the arrow indicator to guide the correction positioning of the plate. Optional use of the evaporation cover will be dependent on temperature or delay run time of the assay.

7.3.23. After the plate has been inserted properly in the instrument, proceed to set up the assay method.

7.3.24. **Kinetics Assay Setup on the Octet**

7.3.24.1. Click on File -> Open Method File…

7.3.24.2. Navigate to Share -> Instrumentation -> Octet -> SOP Protocols, and open the ***AR2G_Kinetics_Template***

7.3.24.3. In the *Plate Definition* tab, highlight the *Test Ligand* in Wells A3-H3 under the Sample ID Column.

7.3.24.4. Right click after selecting all wells and select *Set Well Data.*

7.3.24.5. Input the appropriate name of the test antibody ligand.

7.3.24.6. The test antibody ligand concentration default setting is 5 ug/mL. Modify the concentration if necessary.

7.3.24.7. Highlight the *Test Analyte* in Wells A6 – H6 under the Sample ID column.

7.3.24.8. Right click after selecting all wells and select *Set Well Data.*

7.3.24.9. Input the appropriate name of the test analyte.

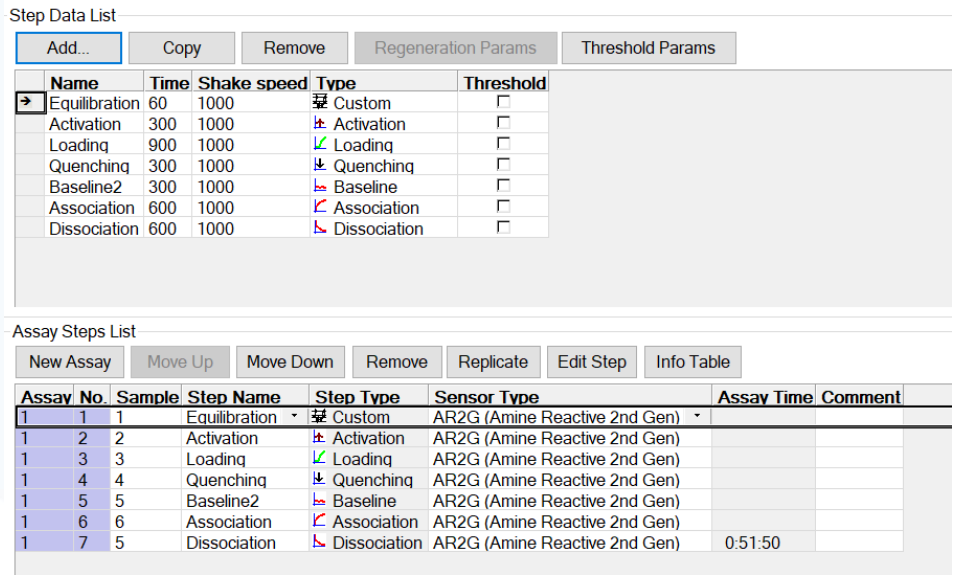
7.3.24.10. Input the molecular weight of the test analyte (kDa).

7.3.24.11. Input the molar concentration of the test analyte (nM) that was prepared. Input ‘0’ for the molar concentration of the buffer *reference well*.

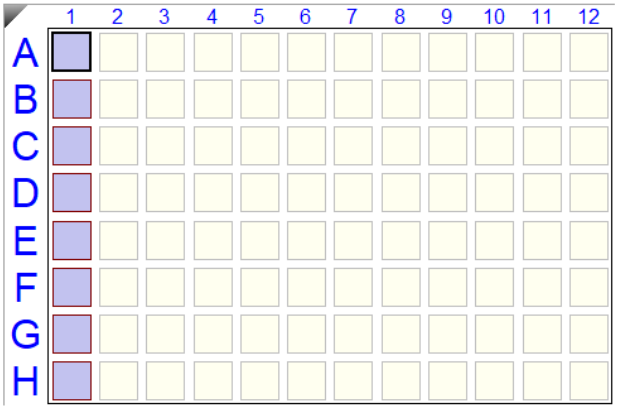
NOTE: Be sure to include a no analyte reference well (red well).

Well	Sample ID	Replicate Group	Type	Conc (µg/ml)	MW (kD)	Molar Conc (nM)	Information
B A1	WATER		Buffer				
B B1	WATER		Buffer				
B C1	WATER		Buffer				
B D1	WATER		Buffer				
B E1	WATER		Buffer				
B F1	WATER		Buffer				
B G1	WATER		Buffer				
B H1	WATER		Buffer				
A A2	EDC+s-NHS		Activation				
A B2	EDC+s-NHS		Activation				
A C2	EDC+s-NHS		Activation				
A D2	EDC+s-NHS		Activation				
A E2	EDC+s-NHS		Activation				
A F2	EDC+s-NHS		Activation				
A G2	EDC+s-NHS		Activation				
A H2	EDC+s-NHS		Activation				
L A3	Test Antibody		Load	5			in 10 mM sodium acetate pH 5.0
L B3	Test Antibody		Load	5			in 10 mM sodium acetate pH 5.0
L C3	Test Antibody		Load	5			in 10 mM sodium acetate pH 5.0
L D3	Test Antibody		Load	5			in 10 mM sodium acetate pH 5.0
L E3	Test Antibody		Load	5			in 10 mM sodium acetate pH 5.0
L F3	Test Antibody		Load	5			in 10 mM sodium acetate pH 5.0
L G3	Test Antibody		Load	5			in 10 mM sodium acetate pH 5.0
L H3	Test Antibody		Load	5			in 10 mM sodium acetate pH 5.0
Q A4	1M Ethanolamine		Quench				
Q B4	1M Ethanolamine		Quench				
Q C4	1M Ethanolamine		Quench				
Q D4	1M Ethanolamine		Quench				
Q E4	1M Ethanolamine		Quench				
Q F4	1M Ethanolamine		Quench				
Q G4	1M Ethanolamine		Quench				
Q H4	1M Ethanolamine		Quench				
B A5	1X Kinetics Buffer		Buffer				
B B5	1X Kinetics Buffer		Buffer				
B C5	1X Kinetics Buffer		Buffer				
B D5	1X Kinetics Buffer		Buffer				
B E5	1X Kinetics Buffer		Buffer				
B F5	1X Kinetics Buffer		Buffer				
B G5	1X Kinetics Buffer		Buffer				
B H5	1X Kinetics Buffer		Buffer				
A6	Test Analyte		Sample	102.4	100	1024	
B6	Test Analyte		Sample	25.6	100	256	
C6	Test Analyte		Sample	6.4	100	64	
D6	Test Analyte		Sample	1.6	100	16	
E6	Test Analyte		Sample	0.4	100	4	
F6	Test Analyte		Sample	0.1	100	1	
G6	Test Analyte		Sample	0.025	100	0.25	
H6	Test Analyte		Reference	0	0	0	

7.3.24.12. Click on the Assay Definition tab. Verify the assay settings are as seen below:



7.3.24.13. Click on the Sensor Assignment tab. Verify the biosensors appear as seen below. Check the box above the sensor tray image to return the sensors for reuse once the assay is complete, rather than the waste container.



Note: Include a set of reference biosensors if there is non-specific binding of the analyte for the biosensor.

7.3.24.14. Click on the Review Experiment tab. Review the assay steps by moving the blue slider.

7.3.24.15. Click on the Run Experiment tab. In the “Data File Location and Names” section, assign a primary directory folder destination in the “Kinetics data repository” field but clicking on the ellipses and creating folder in that destination. Assign a secondary “Experiment run name” folder within the primary destination clicking on the arrow and creating folder in that destination. In the “Run Settings” section check the “Delayed experiment start” box and get the timer for 600 seconds. Check the box with the option to “Shake sample plate while waiting”. Check the box with the option for “Open runtime charts automatically” as well as the sub option “Automatically save runtime chart”. Check the option “Set plate temperature (*C) to 25. Within the “Advanced Settings” location, set the acquisition rate to “Standard kinetics (5.0Hz, averaging by 20) “. Then proceed to start the experiment by clicking on the green “Go” button.

7.4. Data Analysis

7.4.1. Open the ForteBio Data Analysis HT 11.1.3.50 software. Click on the new workspace icon at the left of the screen.

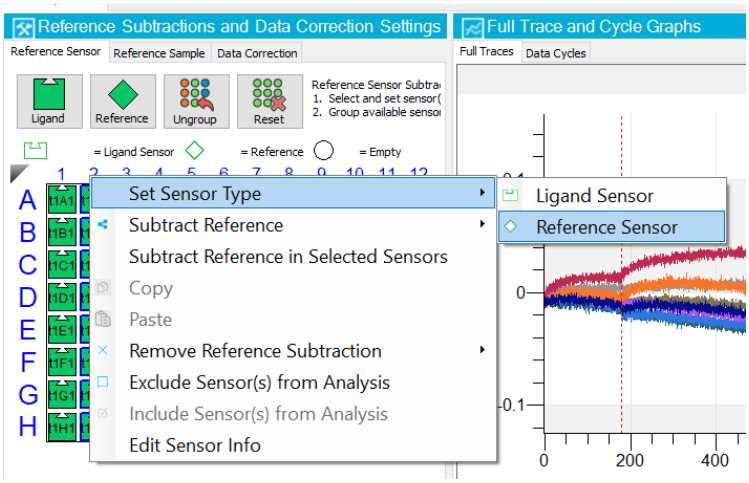
7.4.2. Browse to the project folder where the run was saved. Drag the pink results folder to the middle of the new workspace.

7.4.3. After the sensorgrams from the assay are loaded, click on the “Pre-Processed Data” tab at the top of the page.

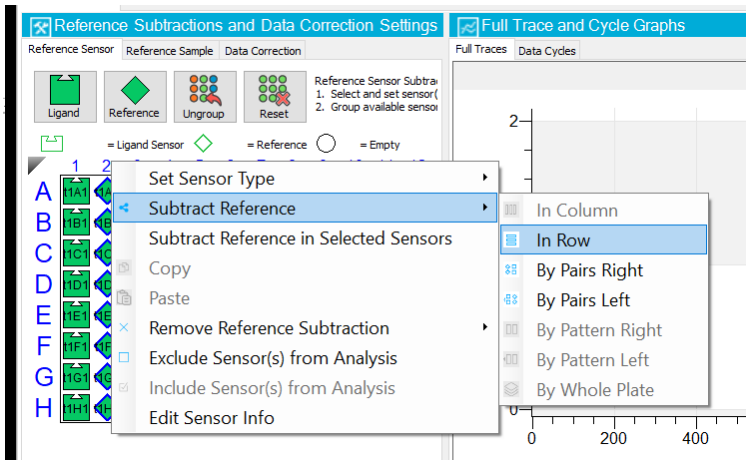
7.4.4. Select “Full Traces” in the middle of the screen. Highlight the sample biosensor A1 and the reference biosensor A2 simultaneously to examine the sensorgrams. Determine the presence or absence of non-specific binding of the analyte for the no-ligand biosensor. The reference biosensor should show no signal compared to the sample biosensor. If signal is observed in the reference biosensor (>10% of the sample signal), the reference biosensors must be subtracted from

the sample biosensors. If the reference biosensor shows signal < 10% of the sample signal, reference biosensors do not need to be used in the assay.

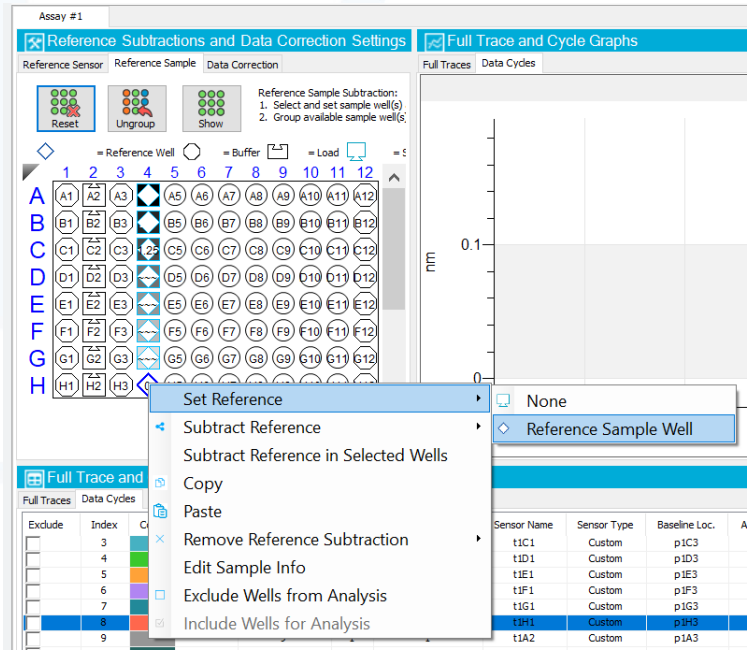
7.4.5. Look at the “Reference Sensor” tab within the **Reference Subtractions and Data Correction Settings** panel on the left. If non-specific binding is observed, right click on the Column 2 reference biosensors. Select “Set Sensor Type” and then select “Reference Sensor”. The square ligand symbol will change to a diamond symbol. If non-specific binding is not observed, right click on the Column 2 reference biosensors. Select “Exclude Sensor(s) from Analysis



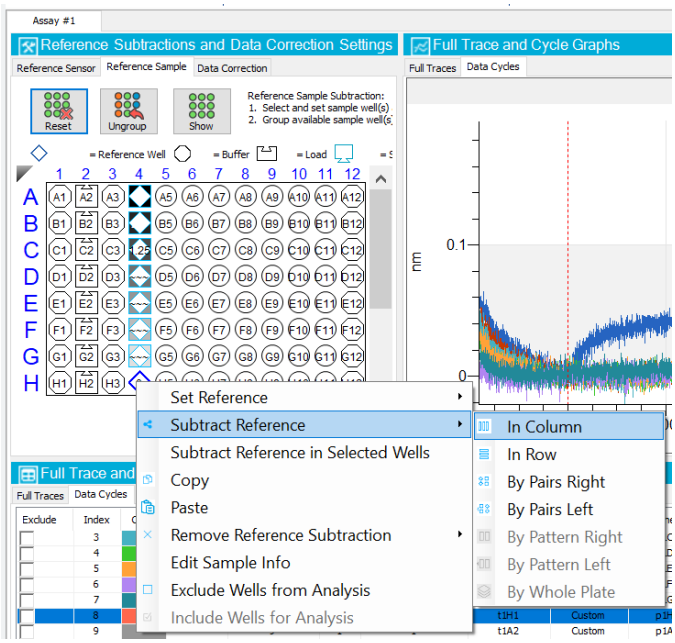
7.4.6. Subtract the reference biosensor as follows:



7.4.7. Look at the “Reference Sample” tab within the **Reference Subtractions and Data Correction Settings** panel on the left. Right click on the reference well, zero concentration analyte, and select “Set Reference” -> “Reference Sample Well”.



7.4.8. Right click on the reference well, zero concentration analyte, and select “Subtract Reference” -> “In Column”.



7.4.9. Finally, click on the “Data Correction” Tab. Perform the data correction as follows:

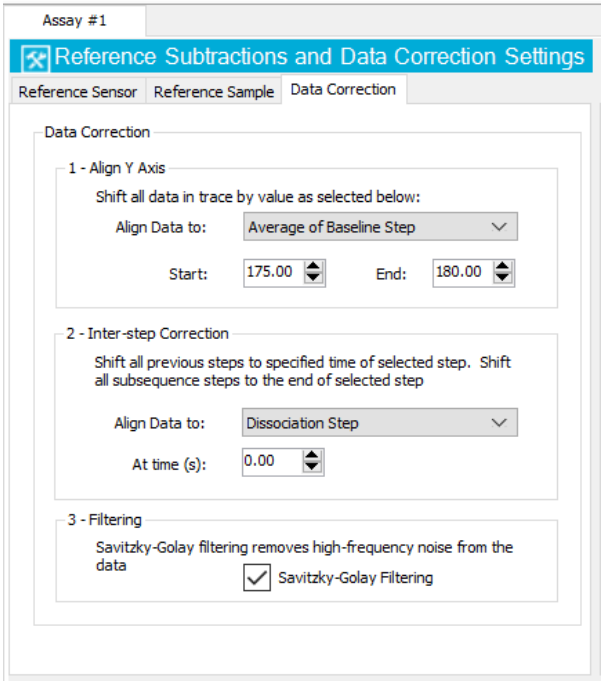
7.4.9.1. Align Y-Axis: Average of Baseline Step

7.4.9.2. Inter-step Correction: Dissociation Step

Note: Only select this option if the baseline and dissociation wells are the same in the assay plate.

Note: This option is only recommended when there is discontinuity between the association and dissociation sensorgram curves. It is not required.

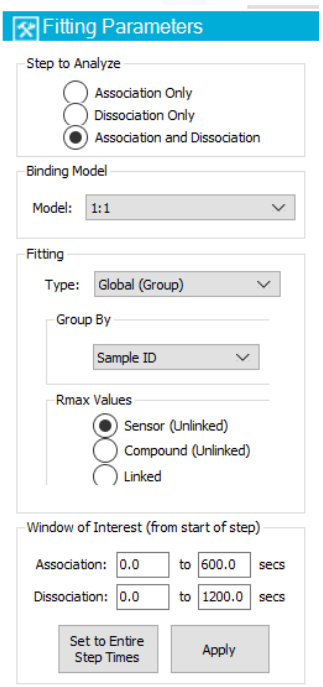
7.4.9.3. Filtering: Check mark the Savitzky-Golay Filtering



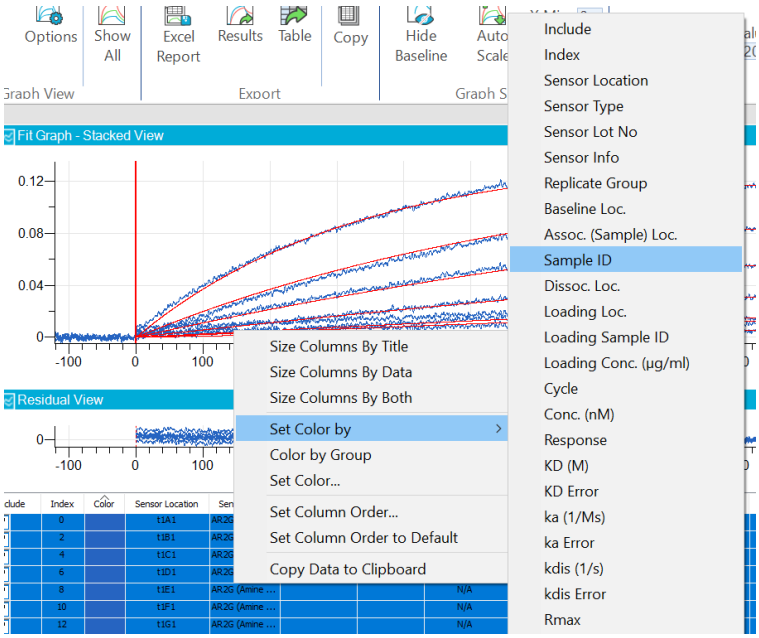
7.4.10. The Full Trace and Cycle Graphs panel in the middle of the screen should display the reference subtracted and data corrected sensorgrams. Examine the Full Traces and Data Cycle curves.

7.4.11. Select the “Kinetics Analysis” tab at the top of the screen. The software will complete a preliminary data analysis and will try to fit the data.

7.4.12. Analyze the kinetics data by selecting the appropriate Fitting Parameters as follows:



7.4.13. Examine the data table at the bottom of the middle panel. Select each of the set of curves in the kinetics analysis. Right click on the selection and select the following:



Note: It is recommended to use a global 1:1 binding model. However, for covalent immobilization using the AR2G biosensors, a 2:1 heterogeneous binding model may provide better fitting. The signal should not have an on rate so fast that it saturates the sensor immediately and not be so slow that it cannot generate enough of an appropriate signal to fit properly. If there is contamination or impurities a 2:1 Heterogeneous ligand binding model may be more effective.

7.4.14. After global fitting, the calculated KD will be identical for each analyte concentration. Examine the Full X^2 value. Ideally, this value should be < 3. Examine the R^2 value. Ideally, this value should be > 0.95. Examine the residuals of the fit. Residuals should not be > +/- 10% nm of the maximum fitted curve. Re-analyze the data set if necessary, to fit the data. When analysis is complete, document the kinetics parameters, K_a and K_d, and the affinity KD value in the appropriate laboratory notebook.

7.4.15. Export the data analysis results to excel using the Export options at the top of the panel. Print out a copy of the sensorgrams for documentation.

7.5. Post Experimental Clean-up.

7.5.1. Discard tips after kinetics have been determined and remove plates from the instrumentation. Use a Kimwipe with 70% IPA to gently clean the light emitting sensor head.

8. MAINTENANCE

8.1. Make sure that spills are cleaned up as soon as they are discovered.

8.2. Remove any “stray” sensors that may have dropped into the instrument.

8.3. Make sure that the sensor “waste container” is emptied on a regular basis.

8.4. The waste container will hold 5 trays, or 480 sensors and the software will remind you to empty the tray after five runs –it is best to empty it after each run.

8.5. If possible, keep the Octet system on at All Times. Turn the system on / off will shorten the lifespan of the Lamp. At the end of the day, just close the Data Acquisition Program and keep the computer & Octet on.

8.6. Close the Data Acquisition Program at the end of the day (after the last experiment) and initiate the system on the following day to ensure "connectivity" of Octet & Software

8.7. Refresh/Reboot computer monthly to refresh internal memory

8.8. It is advised to have a yearly system check by a ForteBio Engineer performing a Preventive Maintenance.

8.9. Sensor manifold may accrue buffer salt residue, impeding the light source and effecting the sensorgram acquisition. Wiping the manifold with 70% IPA with a soft paper towel at least once a month will prevent the build-up of unwanted contaminants.

9. REFERENCES

9.1. Octet Red96e™ Octet® System Data Acquisition User Guide, Release 11.1

9.2. Operating Guildelines for the Octet Red96e™ Octet® System

9.3. Biomolecular Binding Kinetics Assays on the Octet Platform, Application Note #14

9.4. Octet® and BLItz® Systems: Optimize ligand loading density, Published 03/04/2019 06:28 PM | Updated 03/04/2019 06:35 PM

9.5. ForteBio Amine Reactive Second Generation (AR2G) Biosensors Technical Note #26

10. APPENDIX A

10.1. Optimize ligand loading density

10.1.1. On the bench, pipette 200 uL/ well of deionized, 0.22 µm H_2O into wells A1 – H1 of a new Greiner Bio-One black 96 well plate (hydration tray).

10.1.2. Open a new pack of anti-mouse IgG Fc capture (AMC) (or Protein A/G) biosensors. Or alternatively, a pack that has already been opened can also be used if it is has been stored properly with the opening sealed shut.

10.1.3. Gently place the green biosensor tray containing eight AMC biosensors that correspond to wells A1 – H1 on top of the hydration tray (black) taking care not to touch the tips of the biosensors. If necessary, use the Octet dedicated multichannel pipettor to transfer biosensors to the appropriate wells in the green biosensor tray before placing it on top of the hydration tray.

10.1.4. Place the biosensor hydration tray into the Octet Red 96e instrument on the left side. Hydrate the eight AMC biosensors for at least ten minutes.

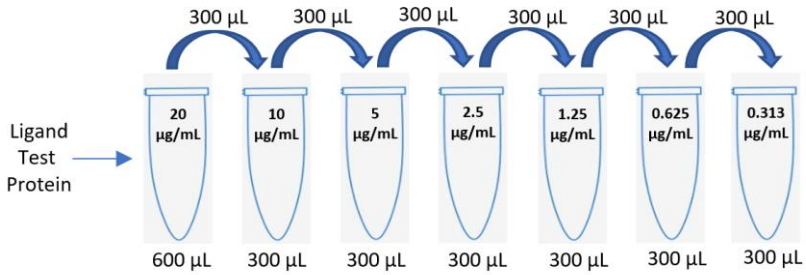
10.1.5. Remove the 10X kinetics buffer from 4°C and let it equilibrate to room temperature.

10.1.6. In a 50 mL conical tube, dilute 2.0 mL of 10X PBS into 18 mL of 0.22 um filtered H_2O making a 20 mL 1X PBS stock solution.

10.1.7. In a 50 mL conical tube, dilute 2.0 mL of 10X Kinetics Buffer into 18 mL of 1X PBS solution making a 20 mL 1X kinetics buffer stock solution.

10.1.8. Remove the test monoclonal antibody (mouse or rabbit) from storage (4°C or -20°C) and let it equilibrate to room temperature.

10.1.9. Using 1.5 mL Eppendorf tubes, serially dilute the mouse or rabbit monoclonal antibody two-fold from 20 ug/mL to 0.313 ug/mL in 1X kinetics buffer as seen below.

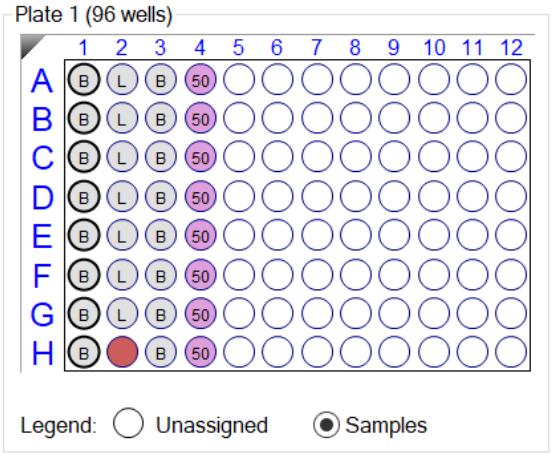


10.1.10. Remove the test protein or peptide analyte(s) from storage. Let the test protein or peptide analyte(s) equilibrate to room temperature.

10.1.11. Dilute the test protein or peptide analyte(s) to a single high concentration ~ 20 x KD in 1X kinetics buffer for a total volume of 1.6 mL. Or, dilute the test protein or peptide analyte(s) to 50 nM if the KD is not known.

10.1.12. Pipette 200 uL each of 1X kinetics buffer, mouse or rabbit monoclonal antibody, and protein test analyte into a new black 96-well, Greiner Bio-One microplate as seen below:

NOTE: Be sure to include a no ligand reference biosensor (red well) to test for non-specific binding.



10.1.13. Place the sample plate into the right side of the instrument.

10.1.14. Set up the assay on the Sartorius Octet Red96e instrument.

10.1.15. **Ligand Optimization Assay Setup on the Octet**

10.1.13.1. Click on File -> Open Method File…

10.1.13.2. Navigate to Share -> Instrumentation -> Octet -> Training, and open the ***Ligand_Density_Optimization_Template***

10.1.13.3. In the *Plate Definition* tab, highlight the *Test Ligand* in Wells A1-H1 under the Sample ID Column.

10.1.13.4. Right click after selecting all wells and select *Set Well Data.*

10.1.13.5. Input the appropriate name of the test ligand.

10.1.13.6. Verify the titration under the Concentration (ug/mL) column ranges from 20 ug/mL – 0.313 ug/mL.

10.1.13.7. Highlight the *Test Analyte* in Wells A4 – H4 under the Sample ID column.

10.1.13.8. Right click after selecting all wells and select *Set Well Data.*

10.1.13.9. Input the appropriate name of the test analyte.

10.1.13.10. Input the molecular weight of the test analyte (kDa).

10.1.13.11. Input the molar concentration of the test analyte (nM).

Well	Sample ID	Replicate Group	Type	Conc (µg/ml)	MW (kD)	Molar Conc (nM)
B A1	1X Kinetics Buffer		Buffer			
B B1	1X Kinetics Buffer		Buffer			
B C1	1X Kinetics Buffer		Buffer			
B D1	1X Kinetics Buffer		Buffer			
B E1	1X Kinetics Buffer		Buffer			
B F1	1X Kinetics Buffer		Buffer			
B G1	1X Kinetics Buffer		Buffer			
B H1	1X Kinetics Buffer		Buffer			
L A2	Test Ligand		Load	20		
L B2	Test Ligand		Load	10		
L C2	Test Ligand		Load	5		
L D2	Test Ligand		Load	2.5		
L E2	Test Ligand		Load	1.25		
L F2	Test Ligand		Load	0.625		
L G2	Test Ligand		Load	0.313		
H2	1X Kinetics Buffer		Reference	0		
B A3	1X Kinetics Buffer		Buffer			
B B3	1X Kinetics Buffer		Buffer			
B C3	1X Kinetics Buffer		Buffer			
B D3	1X Kinetics Buffer		Buffer			
B E3	1X Kinetics Buffer		Buffer			
B F3	1X Kinetics Buffer		Buffer			
B G3	1X Kinetics Buffer		Buffer			
B H3	1X Kinetics Buffer		Buffer			
A4	Test Analyte		Sample	5	100	50
B4	Test Analyte		Sample	5	100	50
C4	Test Analyte		Sample	5	100	50
D4	Test Analyte		Sample	5	100	50
E4	Test Analyte		Sample	5	100	50
F4	Test Analyte		Sample	5	100	50
G4	Test Analyte		Sample	5	100	50
H4	Test Analyte		Sample	5	100	50

10.1.13.12. Click on the Assay Definition tab. Verify the assay settings as seen below:

	Name	Time	Shake speed	Type	Threshold
	Baseline	180	1000	Baseline	☐
	Loading	300	1000	Loading	☐
➔	Equilibration	600	1000	Baseline	☐
	Association	600	1000	Association	☐
	Dissociation	600	1000	Dissociation	☐

Assay Steps List

New Assay | Move Up | Move Down | Remove | Replicate | Edit Step | Info Table

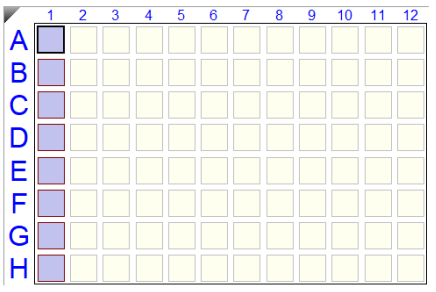
Assay	No.	Sample	Step Name	Step Type	Sensor Type	Assay Time
1	1	1	Equilibration	Baseline	AMC (Anti-mIgG Fc Capture)	
1	2	2	Loading	Loading	AMC (Anti-mIgG Fc Capture)	
1	3	3	Baseline	Baseline	AMC (Anti-mIgG Fc Capture)	
1	4	4	Association	Association	AMC (Anti-mIgG Fc Capture)	
1	5	3	Dissociation	Dissociation	AMC (Anti-mIgG Fc Capture)	0:38:40

10.1.13.13. Click on the Sensor Assignment tab. Verify the biosensors appear as seen below.

1 Plate Definition | 2 Assay Definition | 3 Sensor Assignment | 4 Review Experiment | 5 Run Experiment

In this step, sensors are assigned to samples.
If you have a partial sensor tray it can be accomodated by selecting the missing sensors and clicking 'Remove'.
Only the first sensor tray can be a partial tray. Right click to assign a sensor type to selected sensors.

Sensor Tray



Well	Sensor Type	Lot Number	Information
A1	AMC (Anti-mIgG Fc Capture)		
B1	AMC (Anti-mIgG Fc Capture)		
C1	AMC (Anti-mIgG Fc Capture)		
D1	AMC (Anti-mIgG Fc Capture)		
E1	AMC (Anti-mIgG Fc Capture)		
F1	AMC (Anti-mIgG Fc Capture)		
G1	AMC (Anti-mIgG Fc Capture)		
H1	AMC (Anti-mIgG Fc Capture)		

Legend: Unassigned sensors | Missing sensors

Remove | Fill | Fill Plate | Print...

10.1.13.14. Click on the Review Experiment tab. Review the assay steps by moving the blue slider.

10.1.13.15. Click on the Run Experiment tab. In the “Data File Location and Names” section, assign a primary directory folder destination in the “Kinetics data repository” field but clicking on the ellipses and creating folder in that destination. Assign a secondary “Experiment run name” folder within the primary destination clicking on the arrow and creating folder in that destination. In the “Run Settings” section check the “Delayed experiment start” box and get the timer for 600 seconds. Check the box with the option to “Shake sample plate while waiting”. Check the box with the option for “Open runtime charts automatically” as well as the sub option “Automatically save runtime chart”. Check the option “Set plate temperature (*C) to 25. Within the “Advanced Settings” location, set the acquisition rate to “Standard kinetics (5.0Hz, averaging by 20) “. Then proceed to start the experiment by clicking on the green “Go” button.

10.1.16. **Data Analysis**

10.1.16.1. Open the ForteBio Data Analysis HT software. Click on the new workspace icon at the left of the screen.

10.1.16.2. Browse to the folder where the run was saved. Drag the pink results folder to the middle of the new workspace.

10.1.16.3. After the sensorgrams from the assay are loaded, click on the “Pre-Processed Data” tab at the top of the page.

10.1.16.4. Examine the sensorgrams. Evaluate the stability of the loading of the ligand molecule and the quantity of surface that was loaded onto the biosensors.

Note: For large molecules, it is important to avoid saturation of the biosensor to prevent overcrowding and any stearic hindrance that may occur with the analyte.

10.1.16.5. Select the optimum ligand loading density. It should be low enough to yield a *homogeneous* analyte binding signal of *sufficiently high* magnitude to allow scope for an analyte dilution series during the subsequent kinetics assay.

Note: The following are recommended signals for the biosensors after 5-10 minutes of loading.

AMC: 0.5 – 2 nm

ProA: 1 – 3 nm

ProG: 0.5 – 1.5 nm

AR2G: 0.5 – 2 nm

SAX: 0.5 – 3 nm for large molecule

10.2. Optimize the pH for Ligand Loading

10.2.1. On the bench, pipette 200 uL/ well of deionized, 0.22 µm H_2O into wells A1 – F1 of a new Greiner Bio-One black 96 well plate (hydration tray).

10.2.2. Open a new pack of Amine Reactive Second Generation (AR2G) biosensors. Or alternatively, a pack that has already been opened can also be used if it is has been stored properly with the opening sealed shut.

10.1.16. Gently place the green biosensor tray containing six AR2G biosensors that correspond to wells A1 – F1 on top of the hydration tray (black) taking care not to touch the tips of the biosensors. If necessary, use the Octet dedicated multichannel pipettor to transfer biosensors to the appropriate wells in the green biosensor tray before placing it on top of the hydration tray.

10.1.17. Place the biosensor hydration tray into the Octet Red 96e instrument on the left side. Hydrate the six AR2G biosensors for at least ten minutes.

10.1.18. Remove the 10X kinetics buffer from 4°C and let it equilibrate to room temperature.

10.1.19. In a 50 mL conical tube, dilute 2.0 mL of 10X PBS into 18 mL of 0.22 um filtered H_2O making a 20 mL 1X PBS stock solution.

10.1.20. In a 50 mL conical tube, dilute 2.0 mL of 10X Kinetics Buffer into 18 mL of 1X PBS solution making a 20 mL 1X kinetics buffer stock solution.

10.1.21. Add 200 uL of 1X kinetics buffer wells A5 – F5 on a new Greiner Bio-One black 96 well assay plate.

10.1.22. Add 200 uL of water to wells A1 – F1 (B).

10.1.23. Remove the test monoclonal antibody from storage (4°C or -20°C) and let it equilibrate to room temperature.

10.1.24. Using 1.5 mL Eppendorf tubes, dilute the monoclonal antibody to 10 ug/mL in 10 mM sodium acetate pH 4.0, pH 5.0, and pH 6.0. Prepare 250 uL of each pH concentration (for 2 wells). Add 200 uL / well of pH 4.0 to wells A3 and B3. Add 200 uL / well of pH 5.0 to wells C3 and D3. Add 200 uL / well of pH 6.0 to wells E3 and F3.

10.1.25. Remove the test protein or peptide analyte(s) from storage. Let the test protein or peptide analyte(s) equilibrate to room temperature.

10.1.26. Dilute the test protein or peptide analyte(s) to a single high concentration ~ 20 x KD in 1X kinetics buffer for a total volume of 1.3 mL. Or, dilute the test protein or peptide analyte(s) to 50 nM if the KD is not known. Add 200 uL / well to wells A6 – F6 (Pink).

10.1.27. Remove one aliquot each of EDC (400 mM) and s-NHS (200 mM) from the -20C. Thaw at room temperature.

10.1.28. Vortex briefly after thawing.

10.1.29. 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

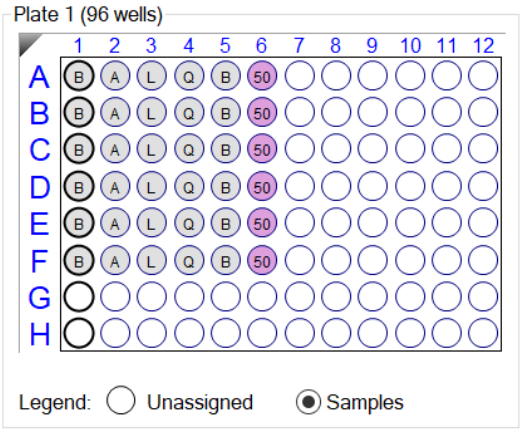
reagents to 1170 µL of water. Mix thoroughly. Pipette 200 µL/well of the EDC/s-NHS mixture into wells A2 – F2 (A).

Note: Use or refreeze EDC and s-NHS aliquots within 10 hours of thawing. Use EDC and s-NHS within 1 hour of mixing them together.

10.1.30. Remove 1M ethanolamine pH 8.5 from 4C.

10.1.31. Add 200 uL of 1M ethanolamine pH 8.5 to wells A4 – F4 (Q).

10.1.32. Verify that the sample plate appears as seen below:



10.1.33. Place the sample plate into the right side of the instrument.

10.1.34. Set up the assay on the Sartorius Octet Red96e instrument.

10.1.35. **pH Loading Optimization Assay Setup on the Octet**

10.1.35.1. Click on File -> Open Method File…

10.1.35.2. Navigate to Share -> Instrumentation -> Octet -> Training, and open the ***AR2G_pH_Scouting_Template***

10.1.35.3. In the *Plate Definition* tab, highlight the *Test Ligand* in Wells A1-F1 under the Sample ID Column.

10.1.35.4. Right click after selecting all wells and select *Set Well Data.*

10.1.35.5. Input the appropriate name of the test ligand.

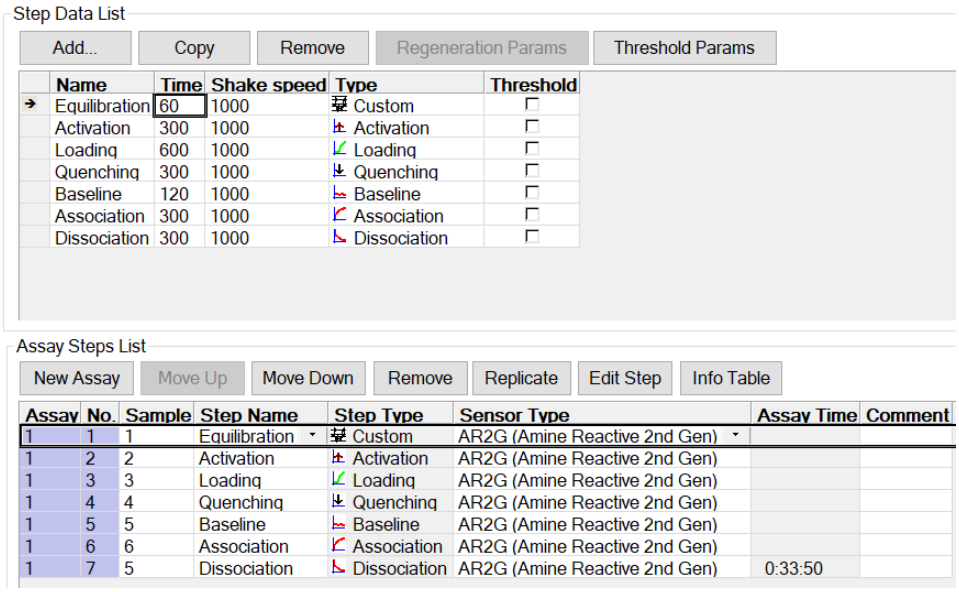
10.1.35.6. Verify the antibody concentration (ug/mL) column. A starting ligand concentration of at least 20 ug/mL is recommended. The ligand concentration may be decreased to as low as 5 ug/mL, however the immobilization time should be increased.

10.1.35.7. Highlight the *Test Analyte* in Wells A4 – H4 under the Sample ID column.

10.1.35.8. Right click after selecting all wells and select *Set Well Data.*

10.1.35.9. Input the appropriate name of the test analyte.
Input the molecular weight of the test analyte (kDa).

10.1.35.10. Verify the Assay Definition as seen below.



10.1.35.11. Review the sensor assignment.

10.1.35.12. Review the Experiment using the blue slider.

10.1.35.13. Click on the Run Experiment tab. In the “Data File Location and Names” section, assign a primary directory folder destination in the “Kinetics data repository” field but clicking on the ellipses and

creating folder in that destination. Assign a secondary "Experiment run name" folder within the primary destination clicking on the arrow and creating folder in that destination. In the "Run Settings" section check the "Delayed experiment start" box and get the timer for 600 seconds. Check the box with the option to "Shake sample plate while waiting". Check the box with the option for "Open runtime charts automatically" as well as the sub option "Automatically save runtime chart". Check the option "Set plate temperature (*C) to 25. Within the "Advanced Settings" location, set the acquisition rate to "Standard kinetics (5.0Hz, averaging by 20) ". Then proceed to start the experiment by clicking on the green "Go" button.

10.1.36. Data Analysis

10.1.36.1. Open the ForteBio Data Analysis HT software. Click on the new workspace icon at the left of the screen.

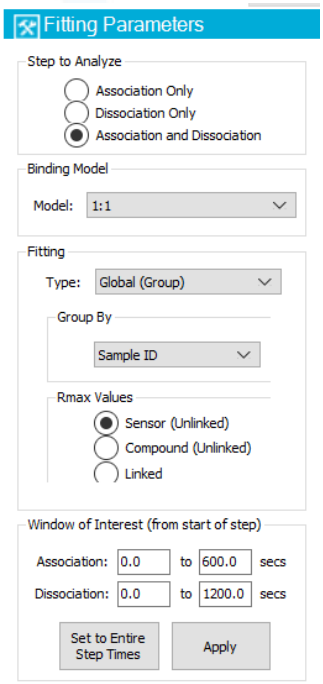
10.1.36.2. Browse to the folder where the run was saved. Drag the pink results folder to the middle of the new workspace.

10.1.36.3. After the sensorgrams from the assay are loaded, click on the "Pre-Processed Data" tab at the top of the page.

10.1.36.4. Examine the sensorgrams. Evaluate the stability of the loading of the ligand molecule and the quantity of surface that was loaded onto the biosensors. The recommended loading surface is 0.5 – 2.0 nm.

10.1.36.5. Click on the "Kinetic Analysis" tab at the top of the page.

10.1.36.6. Perform the fitting according to the parameters seen below:



10.1.36.7. Examine the analyte sensorgrams for stability in the curvature. Identify the pH which results in the maximum signal under the mildest conditions.