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Amine Reactive Biosensors

Octet Biosensors for Label-Free Detection of protein binding
Product Codes: 18-5011, 18-5014, 18-5015

Read the entire product insert fully before beginning the assay.
For research use only, not for use in diagnostic procedures.

OVERVIEW

The Amine Reactive Biosensors enable the coupling of proteins to carboxylate groups on the biosensor surface via accessible amine groups. The coupling procedure is a simple three step protocol based on well characterized amide bond formation chemistry. Amine coupling can be fully automated online or can be batch immobilized offline.

INTENDED USE

ForteBio Amine Reactive Biosensors, in conjunction with the Octet System, are designed for kinetic analysis of protein:protein interactions from cell culture screening to validation during the process development and production of protein and antibody therapeutics.

PRINCIPLE

The Amine Reactive Biosensor surface is a biocompatible layer with many available carboxylic acid groups. Treatment of this surface with an EDC/NHS mixture activates the surface toward nucleophillic attack. Subsequent exposure of a protein at a pH below its pI will result in an amide bond formation between the primary amine of the protein and the carboxylate of the biosensor surface. The protein is covalently bound to the biosensor and can be further analyzed for interactions with other proteins for kinetic analysis.

KIT CONTENTS

Amine Reactive Biosensors for the Octet System contain:

Product Ordering Code		18-5011	18-5014	18-5015
1	Tray of 32 biosensors compatible with amine reactive chemistry	1x32	5x32	20x32

Store Amine Reactive Biosensors in a dry place at room temperature away from direct sunlight. Dispose of Amine Reactive Biosensors as sharps. Upon receipt, Amine Reactive Biosensors are stable for 6 months.

ADDITIONAL MATERIALS REQUIRED

The following additional materials are required:

- **Immobilization Buffer:** MES hydrate (Sigma C/N M2933)
- distilled deionized water (ddH_2O)
- EDC, Sigma (C/N E7750)
- NHS, Aldrich (C/N 130672)
- **Quench Buffer:** Ethanolamine, pH 8.5 (Aldrich C/N 23681)
- Octet Instrument and Software version 3.0 or higher
- **Ligand :** purified protein stock that is free of carrier protein in an amine free buffer
- **Assay Buffer:** PBS is strongly recommended
- 2) 96-well, black, flat bottom, polypropylene microplates (Greiner Bio-one # 655209)

TECHNIQUES FOR OPTIMAL PERFORMANCE

1. Amine Reactive Biosensors are not compatible with Acetate Buffer. **DO NOT USE Acetate Buffer** with Amine Reactive Biosensors.
2. Equilibrate reagents and samples to room temperature prior to preparation. For frozen samples, thaw and mix thoroughly prior to use.
3. Hydration of the sensors is required prior to assaying on the Octet System.
4. A minimum volume of 200 µL/well is required for both the assay samples and the biosensor hydration solution.
5. 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.
6. Set the sample plate temperature. In the Octet Software select: **File ➔ Experiment ➔ Set plate temperature.** Enter the desired temperature between room temperature to 40°C. ForteBio recommends assaying at 30°C. Assaying at other temperatures may require different assay times than discussed in this protocol.

PROTOCOL OVERVIEW

1. Prepare buffers, samples, and coupling reagents. Mix EDC:NHS reagent just prior to use.
2. Transfer 200 µL of an appropriate hydration solution to the 96-well plate. Insert the hydration plate, followed by the biosensors onto the biosensor tray to hydrate the sensors.
3. Transfer 200 uL for each sample, buffers or coupling reagents into the appropriate wells of a 96-well plate.
4. Equilibrate both the hydrated biosensor assembly and sample plate for 5 minutes on the Octet instrument.
5. Set up the experiment using Octet software. Select "**shake the plate prior to start**" at the end of programming.
6. Start the assay.
7. Perform data analysis and save the results.

SAMPLES AND AMINE COUPLING REAGENT PREPARATION AND STORAGE

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

The following table lists the recommended reagents and their appropriate handling and storage

Reagent	Function	Handling and Storage
100 mM MES, pH 5.0	Biosensor hydration solution and diluent for coupling reagent preparation	Store at room temperature
0.4 M EDC	With NHS, activates Amine Reactive Biosensor surface	Store aliquots at -20 C for up to 3 months
0.1 M NHS	With EDC, activates Amine Reactive Biosensor surface	Store aliquots at -20 C for up to 3 months
1 M Ethanolamine, pH 8.5	Blocks unused activated carboxylated groups, minimizing non-specific binding on the Amine Reactive Biosensor surface	Store at room temperature
EDC:NHS (1:1) coupling mix	Activates Amine Reactive Biosensor surface	Mix just prior to use. Stable up to 3 hours
Ligand stock	1st protein immobilized onto the biosensor	Dilute 1:20 with MES prior to use
PBS	Recommended Assay Buffer	Per manufacturer

Table 1. Amine Reactive Assay reagents

1. MES Buffer -Prepare a 100 mM MES solution by dissolving 9.8 g of MES free acid (MW 195.2) in 500 mL of ddH_20. Adjust to pH 5.0 with 8 N KOH. Store at room temperature.

2. EDC- Prepare 0.4M EDC solution by dissolving 3.1 g of EDC in ddH_20 to a final working volume of 40 mL. Aliquot 1 mL of the EDC solution into labeled 2 mL eppendorf tubes. Store at -20C.

3. NHS- Prepare 0.1M NHS solution by dissolving 0.46 g of NHS in ddH_20 to final volume 40 mL. Aliquot 1mL of the EDC solution into labeled 2 mL eppendorf tubes. Store at -20C.

4. Ethanolamine (quench) - Prepare quench buffer by dissolving 48.8 g of ethanolamine in water to final volume 500 mL. Adjust pH to 8.5 with 8 N KOH. Solution should be stored at room temperature.

5. Ligand stock - The ligand is the protein that is immobilized onto the biosensor tip surface. It should be a purified protein that is free of carrier protein. It is critical that the protein is in an amine-free buffer of low ionic strength (~ 10 mM) and at a pH below the isoelectric point for the protein

A typical immobilization concentration is 25µg/mL (assay concentration). Ligand stock should be of sufficient concentration to allow for at least a 1:20 dilution into the MES buffer. Be aware that some proteins are not stable for long periods of time at these pHs. It is safest to dilute the proteins into the buffer immediately prior to use.

ONLINE IMMOBILIZATION PROTOCOL

For online immobilization using the Amine Reactive Biosensors, there are a minimum of 7 Assay Steps that are fully automated on the Octet System.

1. Baseline – MES buffer
2. Activation – EDC/NHS mix
3. Loading of ligand – Ligand to be immobilized
4. Quench - Ethanolamine
5. Baseline – assay buffer (ASB)
6. Association of the interacting protein – protein #2
7. Dissociation- assay buffer (ASB)

1. Assay preparation-

EDC:NHS coupling mix: Just prior to preparing the sample plate, prepare the EDC:NHS coupling mix by mixing 1 part EDC stock: 1 part NHS stock.

Note: It is recommended to use the mixture within one hour.

Ligand: Dilute the Ligand stock to 25 µg/mL in 100 mM MES, pH 5. Be aware that some proteins are not stable for long periods of time at these pHs. It is safest to dilute the proteins into the buffer immediately prior to use.

Note: Immobilization can be further optimized by changing protein concentrations and buffer pH. Contact support@fortebio.com for additional information and guidelines.

2. Assay Plate- Transfer 200 µL of each assay reagent into the 96-well black, flat bottom, polypropylene microplate according to the plate map in Table 2. Each reagent is transferred to the 96-well microplate in a column format.

	1	2	3	4	5	6	7
A	MES	EDC/NHS	Ligand	Quench	ASB	Protein	ASB
B	MES	EDC/NHS	Ligand	Quench	ASB	Protein	ASB
C	MES	EDC/NHS	MES only (control)	Quench	ASB	Protein	ASB

Table 2. Example of an online immobilization plate map

3. Biosensor hydration plate preparation - Gently remove the yellow biosensor rack from the biosensor assembly and place on the bench. Place a 96-well plate securely in the blue biosensor tray holder. Transfer 200 µL of the hydration solution into each well in the microplate that matches the number and location of the sensors being used. For online immobilization, use MES Buffer as the hydration solution.

4. Instrument placement of sensors and sample plate Place the sample plate with A1 facing the upper right hand corner of the sample plate stage on the right hand side of the Octet instrument.

It is critical to hydrate the Amine Reactive Biosensors for **5 minutes** on the Octet System. Prior to running, replace the biosensor rack by aligning it over the hydration plate, careful not to scrape or touch the bottom of the sensors. Place the biosensor tray on the biosensor plate stage on the left hand side of the Octet instrument.

Ensure that both the biosensor tray and sample plate are securely in place. Go to **"Instrument preparation and programming".**

INSTRUMENT PREPARATION AND PROGRAMMING

Ensure that the Octet instrument and its computer are turned on. It is essential that the lamp is warmed up for at least 40 minutes.

Double click on the Octet User Software icon and allow the Octet to complete initialization. From the menu, select **Experiment ➔ New Kinetics Experiment** (Ctrl+K). Fill in the required information for each tab in the following order:

1 Sensor <-> Sample Assignment

1. Use the mouse to highlight the sensors (squares) being used in the biosensor plate.
2. Use the mouse to highlight the wells which contain samples or reagents. Sample information can be entered directly into the table.
3. Enter in the concentration values in [M] for the protein whose kinetics is under investigation. *E.g. 1 E-8*

2 Assay Definition

In the Table "**Step data setup**" is an example list of the programmable steps with the associated parameters. The table below lists the order of steps for online immobilization.

Step#	Step name	Time (sec)	Flow (rpm)	Step type
1	Activation	300	1000	Activation
2	Loading	300-600	1000	Loading
3	Quench	180-300	1000	Quench
4	Baseline	180-600	1000	Baseline
5	Protein binding	600-1800	1000	Association
6	Dissociation	600-3600	1000	Dissociation

Table 5. Available kinetic assay steps and associated parameters

1. Click on the "**+**" or "ADD" the number of assay steps needed to perform the assay.
2. Define each assay step.
   - 2.1. Under the "data name", double click to change the name of the step
   - 2.2. Under "Assay Time", enter the time (seconds) for the specific assay step
   - 2.3. Under "Flow Rate", enter the rpm for the orbital flow shaker. E.g. 1000
   - 2.4. Under "Type", select the step type from the pull down menu.
3. Assign the biosensors, samples and their associated assay steps in the order they should be sampled.
   - 3.1. Select the biosensors. Hold the mouse down for multiple biosensors in a column.
   - 3.2. Select the defined assay step from the **Step data setup**
   - 3.3. Double-click on the samples for the selected biosensors and assay step. The step will appear in the "Assay Setup List"
   - 3.4. Complete this for the remaining assay steps prior before selecting the next biosensors.

3 Run Experiment

1. Select the browse button to specify a location to save the raw data. It is strongly recommended to save the data to the local drive(C:/). Once the run is complete, the data can then be transferred to a network drive.
2. Enter a unique Experiment Run Name (sub-directory), which creates a new folder for all raw data files and real-time binding charts related to the experiment. Enter a plate name if default name is not acceptable.
3. Check the "Delay the experiment start". Enter 300 seconds and check **"Shake sample plate while waiting to start". This is a critical step to insure optimal performance.**
4. Default settings will ensure the runtime charts will be saved automatically.
5. Start the assay by clicking GO.

DATA ANALYSIS

The Octet System has the capability to proceed to data analysis upon completion of the assay or the data can be analyzed offline.

After the assay is complete, skip to **Step 2 – Data Visualization.**

Offline data analysis –
From the menu, select **Data Analysis ➔ New Kinetics Data Analysis** (Ctrl_Shift+K). Fill in the required information for each tab in the following order:

1 Data File Selection

1. Locate and load your raw data files (filename.frd) by double clicking on the folder or individual biosensor files.
   - 1.1. To easily locate kinetic files, select the "Show Explorer" button at the bottom of the window. Kinetic folders are colored peach .
   - 1.2. To remove files, simply click the check box under file #.

2 Data Visualization

In the Data Visualization screen, the raw data results for all sensors assayed are presented in graphical form. This section allows for customization of how the data is graphically presented and selection of the time frame for kinetic data analysis.

1. A thumbnail graphic of each individual biosensor is presented on the left had side of the screen. Select or deselect the check box the biosensors for analysis
2. Under **Data Tracking**, select how the biosensors should be identified in data tables and graphs.
   a. biosensor Loc – biosensor location, e.g. B1
   b. Sample loc – sample location
   c. Sample ID
3. Under **Assay Step Times,** define the desired time frame for analysis. Octet System software gives the option of selecting either the time that is stored in the method file or overriding the programmed time for either association or dissociation.
   - 3.1. Octet System software for kinetic analysis is based on a 1:1 model. Select full or partial dissociation based on the experiment and understanding of the interaction
4. For better visualization, the data can be aligned by assay step.
   - 4.1. Select the step to align using the pull down menu
   - 4.2. Select which steps you would like presented
   - 4.3. Select which axis to align
   - 4.4. The image and the raw data can be exported as bmp and text file, respectively.
5. To calculate kinetic constants click .

ForteBio Kinetic Analysis

The Octet System software has standard kinetic analysis and reporting based on the selected data established in the Data Visualization section.

1. In order to calculate affinity constants, a molar concentration is required. Enter the molar concentration [M] for protein whose kinetic constants are being calculated. *e.g. 4 E-8 is equivalent to 40 nm*
2. Select the Data Analysis settings for inclusion in the Microsoft® Word report. The available options are listed in the Table 6.

#	Data Analysis Setting	Description
1	Create stacked graph	For each biosensor selected, displays individual on/off binding data and associated residuals
2	Create one overlay graph	Displays binding data and residuals in a single graph
3	Draw fitted curve on the graph	Displays the non-linear fitted curve for each biosensor
4	Show curve legend	Displays a legend
5	Show residual curve	Displays the difference between the actual binding rate and the curve fit
6	Create results chart	A table of all calculated data for each biosensor.

Table 6. Available kinetic data analysis settings for reporting

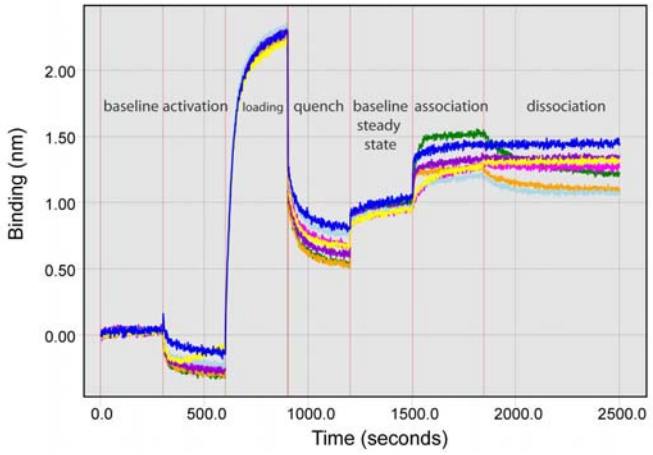
   2.1. Select the settings for inclusion in the report.
   2.2. Click on the "**Do Analysis"** button.
   2.3. After the analysis is complete, you can save the report and are given the following options:
      a. Tables and graphs
      b. Graphs only
      c. Tables only

Note: For more advanced kinetics analysis the data can be analyzed in Origin®. Refer to the Origin manual for more information

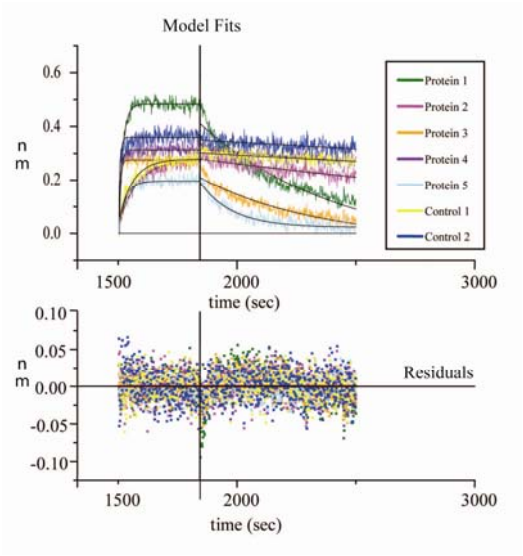
Technical Support: **Toll Free (888) OCTET-QK**
Phone (650) 322-1360 option 3

Representative Data

Example of an Octet System real-time binding chart using Amine Reactive Biosensors.



Data visualization of the model fits with their associated residuals.



Kinetic constants graphed by affinity (bar graph) and kdissoc/kassoc rate mapping.