Immunogenicity Assays on the Octet Platform

OVERVIEW

Immunogenicity testing, the measurement of an immune response to a therapeutic drug, is an integral part of drug development. The immune system may respond to drug administration in patients by producing anti-drug antibodies (ADA). ADA can alter the pharmacodynamics and/or the pharmacokinetics of the drug, so detecting them is essential during the development process. ADA can be produced in a wide range of concentrations and with a wide range of affinities. To accurately detect these polyclonal antibodies, the system must have exquisite sensitivity, withstand a wide range of free drug concentrations, and have minimal matrix effects. Automated immunoassays on Pall ForteBio's Octet instruments provide a high level of sensitivity, tolerance to drug, and flexibility to detect both high and low affinity ADA by providing multiple protocols without any plate washing steps. These protocols work across the many drug types in the market today such as antibodies, proteins, and peptides and can be used with both human and animal samples.

INTENDED USE

Pall ForteBio Immunogenicity assays enable the detection of ADA as a part of an immune response to a protein or antibody therapeutic. This technical note describes protocols, biosensors and basic reagents required to run two different assay configurations: an enzyme-linked bridging assay and a direct binding assay. The assays are intended for use with human, primate and other animal serum and plasma samples.

PRINCIPLE

BLI biosensors are fiber optic tips coated with specific ligand chemistries that enable detection, quantification, and kinetic analysis of a biomolecular target. The binding of the targeted molecule alters the interference pattern of light reflected from the biosensor tip to a detector, allowing molecular association and dissociation events to be measured in real time with the Octet instrument. Higher

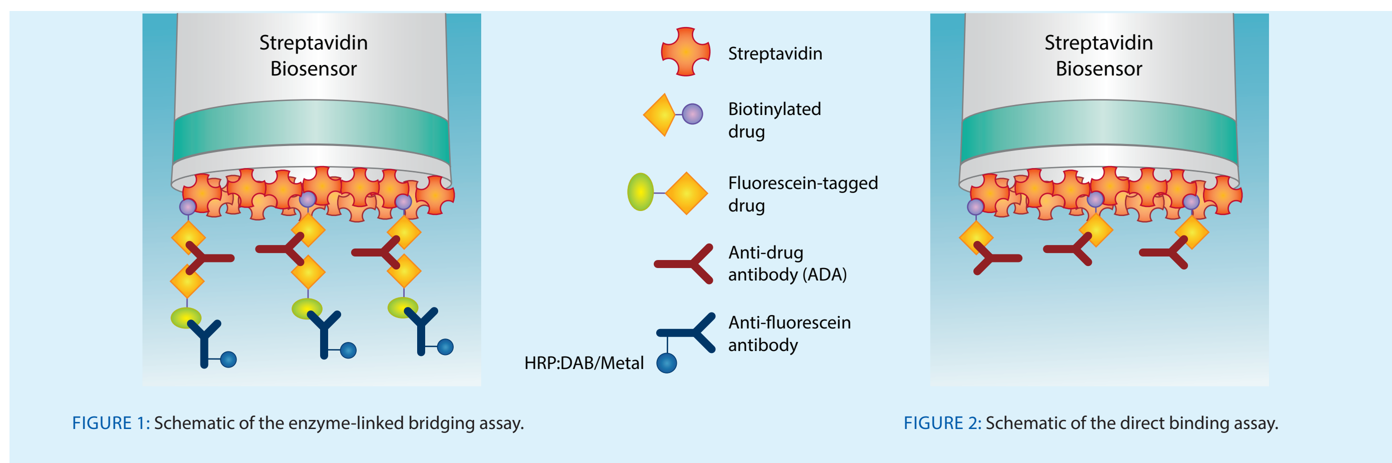

FIGURE 1: Schematic of the enzyme-linked bridging assay.

FIGURE 2: Schematic of the direct binding assay.

target concentrations result in both faster binding rates and larger signal amplitudes. Signal amplification can be achieved on BLI biosensors using multiple molecular layers and addition of enzyme-catalyzed substrate precipitation step (Figure 1).

In the case of the enzyme-linked bridging assay, a Streptavidin (SAX) Biosensor is used to rapidly capture the biotin-drug/ADA/fluorescein-drug complex out of the treated sample mixture (Figure 1). By using a standard HRP-linked antibody and precipitating substrate, the Octet instrument's Biolayer Interferometry (BLI) technology provides a rapid, sensitive and precise readout. Using the enzyme-linked assay a 96-well plate can be read in as little as 30 minutes using the Octet QK384/RED384/HTX system. Often, this protocol provides enhanced sensitivity compared to the direct binding protocol.

In some cases, a direct binding assay may be more appropriate, particularly when detecting low-affinity ADA. In this assay the biotin-drug is immobilized onto the streptavidin biosensor surface and the ADA is captured and detected directly out of the diluted sample (Figure 2). A 96-well plate can be read as little as 60 minutes if the biotin-drug immobilization and blocking steps are performed off-line.

PROTOCOL 1: ENZYME-LINKED BRIDGING ASSAY

Materials Required

- Octet system with software version 6.1 or later
- Samples to be analyzed (including positive and negative controls), 100 µL per test
- Purified drug for immobilization and detection: 1 mg needed for biotinylation and fluorescein labeling; 4 µg used per test
- High Precision Streptavidin (SAX) Biosensors (Pall ForteBio Part No. 18-5117)
- Immunogenicity Reagent (See Appendix for recipe)
- Sample Diluent (Pall ForteBio Part No. 18-1048)
- Phosphate Buffered Saline (PBS)
- 96-well microplates (Greiner Bio-One, Part No. 655209)
- *Optional* 384-well microplates for Octet QK384/RED384/HTX systems (Greiner Bio-One, Part No. 781209)
- EZ-Link NHS-LC-LC-Biotin (Thermo, Part No. 21343)
- DMF (Thermo, Part No. 20672)
- PD-10 desalting column (GE Healthcare, Part No. 17-0851-01)
- NHS-Fluorescein (Thermo, Part No. 46410)
- Stable Peroxide Substrate Buffer (Thermo, Part No. 1855910)
- DAB/Metal Concentrate (10X) (Thermo, Part No. 1856090)
- Rabbit anti-FITC:HRP (AbDSerotec, Part No. 4510-7864)

Tips for Optimal Performance

- A cut point analysis is strongly recommended to establish a ~5% false positive (FP) rate for each sample matrix type.[1,2]
- Fully equilibrate all reagents and samples to room temperature prior to preparation. For frozen samples, thaw and mix thoroughly prior to use.
- Biosensors should be hydrated in Immunogenicity Reagent for at least 15 minutes prior to use.
- A minimum volume of 200 µL/well in a 96-well microplate (80 µL/well in a 384-well microplate) is required for the diluted samples, reagents and the biosensor hydration solution.
- Turn on the Octet instrument at least 40 minutes prior to starting the assay, to allow the lamp to warm up.
- Set the sample plate temperature in the Octet software by selecting File > Experiment > Set plate temperature, then entering the desired temperature. Pall ForteBio recommends 30 °C for immunogenicity assays.

Protocol Overview

1. Prepare the reagents, including conjugation of biotin and fluorescein tag to the drug. Reagents can be prepared ahead of time and stored for future experiments.
2. Prepare the assay plate by combining samples, biotin-drug and fluorescein-drug in Immunogenicity Reagent. Incubate 1–3 hours in the microplate on a shaker at room temperature. Add detection reagents to the assay plate (or use a separate reagent plate if using an Octet QK384/RED384/HTX system).
3. Run the experiment: transfer the microplate(s) into the Octet instrument. Hydrate the biosensors and equilibrate the sample plate for 15 minutes on the Octet instrument before starting the assay run. Total time for a full 96-well plate = 1 hour for Octet QK^{e}/RED96 systems; 30 minutes for Octet QK384/RED384/HTX systems.
4. Perform data analysis and save the results.

Prepare Reagents

1. Equilibrate reagents to room temperature prior to preparation and mix thoroughly.
2. **Biotinylated drug**. The drug must be minimally biotinylated to enable capturing the bridging complex from solution by the Streptavidin biosensors.
   A. Prepare NHS-LC-LC-Biotin in DMF. Determine the concentration of biotin reagent needed in order to insure the volume of DMF does not exceed 3% of protein drug solution to be biotinlyated.

      NOTE: NHS compounds are easily hydrolyzed in aqueous solutions higher than pH 7. Prepare this solution immediately prior to use. Vortex the solution until all the solids are completely dissolved.

B Prepare solution of drug to be biotinylated in PBS (the recommended volume and concentration are 0.5 mL or more at 1 mg/mL). If a lower concentration or smaller volume is used then purification by dialysis instead of by a PD-10 column may be necessary.

C A molar coupling ratio (MCR) of 1 is recommended (1 EZ-Link NHS-LC-LC-Biotin molecule for every 1 drug molecule), but can be optimized for each drug molecule. Calculate the volume of biotin solution to add to the aliquot of drug to give a solution with an MCR = 1.

$$\text{mL of biotin reagent} = \frac{\text{mg drug}}{\text{drug MW (mg/mmol)}} \times \text{MCR} \times \frac{\text{Biotin-NHS MW}}{\text{mg/mL Biotin-NHS in DMF}}$$

D Add the calculated volume of biotin reagent to sample. Incubate 1 hour at room temperature (20–25 °C). During incubation prepare PD-10 columns for buffer exchange with PBS according to the manufacturer's instructions.

E Remove the excess Biotin-LC-LC-NHS using PD-10 columns according to the manufacturer's instructions, except elute and collect only 3 mL instead of the 3.3 mL recommended by the manufacturer, to ensure minimal contamination by free biotin.

F Calculate the concentration of biotinylated drug assuming a 90% yield:

[biotin-drug (mg/mL)] = (mg of drug/3 mL) X 90%

3 **Fluorescein-tagged drug**. An aliquot of the drug must be conjugated to fluorescein to serve as a tag to bridge to the enzyme-linked detection. This can be performed concurrently with the biotinylation protocol.

A Prepare a solution of NHS-Fluorescein in DMF. Determine the concentration of fluorescein reagent needed in order to insure the volume of DMF does not exceed 3% of protein drug solution.

NOTE: NHS compounds are easily hydrolyzed if exposed to air in aqueous solutions higher than pH 7. Prepare this solution immediately prior to use. Vortex the solution until all solids are completely dissolved.

B Prepare solution of drug to be labeled in PBS (recommended volume is 0.5 mL or greater at 1mg/mL). If lower concentration or less volume is used then purification by dialysis instead of by PD10 may be necessary.

C A molar coupling ratio (MCR) of 15 is recommended (15 fluorescein-NHS molecules for every 1 drug molecule), but can be optimized for each drug molecule. Calculate the volume of fluorescein reagent solution to add to the aliquot of drug to give a solution with an MCR = 15.

$$\text{mL of fluorescein reagent} = \frac{\text{mg drug}}{\text{drug MW (mg/mmol)}} \times \text{MCR} \times \frac{\text{Fluorescein-NHS MW}}{\text{mg/mL Fluorescein-NHS in DMF}}$$

D Add the calculated volume of fluorescein reagent to sample. Incubate 1 hour at room temperature (20–25 °C), protected from light. During incubation prepare PD-10 columns for buffer exchange with PBS according to the manufacturer's instructions.

E Remove the excess Fluor-LC-LC-NHS using PD-10 columns according to the manufacturer's instructions, except elute and collect only 3 mL instead of the 3.3 mL recommended by the manufacturer, to ensure minimal contamination by free fluorescein.

F Calculate the concentration of labeled drug assuming a 90% yield:

[fluorescein-drug (mg/mL)] = (mg of drug/3 mL) X 90%

Prepare Assay Plate

Equilibrate samples, reagents and buffers to room temperature and mix thoroughly prior to use. When filling the microplates, a minimum of 200 µL should be put in each well of a 96-well microplate (all instruments), and a minimum of 80 µL in each well of a 384-well microplate (Octet RED384, QK384 and HTX systems only).

1 Prepare a solution containing 20 µg/mL biotin-drug + 20 µg/mL Fluorescein-drug in Immunogenicity Reagent.

2 In a black 96-well microplate:

A Combine 100 µL of sample to be tested + 100 µL of the biotin-drug/fluorescein-drug mixture to generate 200 µL of diluted sample. Samples should be diluted a minimum of 1:1 in the final mixture. Keep dilution consistent for all samples to be test.

B Repeat for all samples to be tested, filling the plate in columns starting at A1–H1 then progressing to A2–H2, etc.

C For reference samples, treat negative control serum and positive spike controls identically to samples as described above.

NOTE: the maximum number of samples that can be run in a 96-well plate is 64 on the Octet QK^e or RED96 systems (see Figure 3) and 96 on the Octet QK384, RED384 or HTX systems.

3 Cover and incubate the microplate on a plate shaker (> 500 rpm < 1000 rpm) for 1–3 hours at room temperature (time may need to be further optimized depending on the shaker) taking care not to splash the samples out of the wells. Incubation time can be reduced if plate shaker is at 30 °C. If no shaker is available, incubate for 4 hours at room temperature.

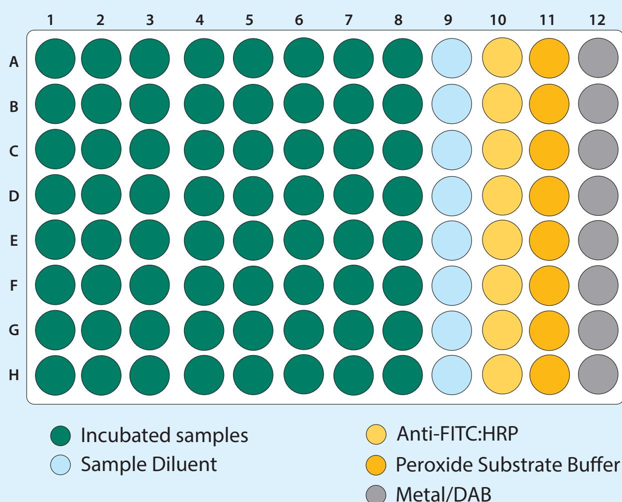

FIGURE 3: Sample 96-well plate map for use in Octet QK/RED systems.

4 During sample incubation, make certain that all HRP reagents, buffers and substrates are equilibrated to room temperature. During the last 10 minutes of the incubation prepare the following solutions:

For Octet QKe/RED96 systems – 96-well plate only

- 2 mL anti-FITC:HRP antibody conjugate diluted 1:1000 in Sample Diluent (ProClin®300).
- 2 mL Metal/DAB substrate diluted 1:10 in Peroxide Substrate Buffer.

For Octet QK384/RED384/HTX systems – 96-well plate only

- 4 mL anti-FITC:HRP antibody conjugate diluted 1:1000 in Sample Diluent (ProClin®300).
- 4 mL Metal/DAB substrate diluted 1:10 in Peroxide Substrate Buffer.

For Octet QK384/RED384/HTX systems – 384-well plate only

- 2 mL anti-FITC:HRP antibody conjugate diluted 1:1000 in Sample Diluent (ProClin®300).
- 2 mL Metal/DAB substrate diluted 1:10 in Peroxide Substrate Buffer.

5 After the sample incubation is complete, remove the plate from the shaker and add reagents in each well as necessary. Array the reagents in columns (single columns for the 8 channel Octet QKe and Octet RED96 systems, duplicate columns for the Octet QK384/RED384/HTX systems). Reagents can be placed in any column. Figure 3 depicts a plate set up to be used with the Octet RED96 system, having reagents in columns 9–12.

NOTE: when using Octet QK384/RED384/HTX systems, reagents must be arrayed to accommodate the 16 channel layout (See the User Guide for a complete description of working in 16-channel mode).

6 Hydrate the biosensors in Immunogencity Reagent for a minimum of 15 minutes prior to the first measurement. In a 96-well microplate, pipet 200 µL Immunogenicity Reagent into each well corresponding to the locations of the biosensors to be used. One biosensor is needed for each sample to be assayed.

7 Place the hydrated biosensors and sample/reagent plates in the Octet instrument. Make certain the A1 position on the plate is at the back right corner of the plate holder. Ensure that both the biosensor tray and sample plate are securely in place and sit flat on the holder. Equilibrate the samples to the assay temperature (30 °C) for at least 15 minutes prior to the first measurement.

Run Experiment

1 Ensure the Octet instrument and associated computer are turned on. It is critical that the lamp is warmed up for at least 40 minutes prior to the first measurement.

2 Start the Data Acquisition software (version 6.1 or later).

3 From the Experiment Wizard (ctrl+N) choose the Advanced Quantitation option.

4 In Tab 1:

A Click the Modify button and choose the Immunogenicity - Enzyme-Linked assay file from the list on the left. The settings in this list are default settings. To modify the settings use the up/down arrows. Modifications only apply to the current assay and are saved as part of the assay's method file. To create a new assay file, see Help > User Guide > Edit Assay Parameters.

B Right-click on the plate map and define locations for reagents, samples, standards and references.

C Enter Sample ID, concentrations and dilution factors as appropriate into the table.

5 In Tab 2:

A Define the locations of the biosensors. Default locations are shown automatically and can be changed by highlighting the biosensors and using the Remove, Fill or Fill Plate buttons.

B Choose Streptavidin from the Sensor Type drop-down menu. Add biosensor Information in the table on the right, if desired.

6 In Tab 3:

A Enter the location and folder name in which the data should be saved.

B Enter a delay if needed to complete the 15 minute minimum sample equilibration and biosensor hydration time.

7 Click the GO button to start the assay. The default settings ensure the data, method and runtime charts are saved automatically.

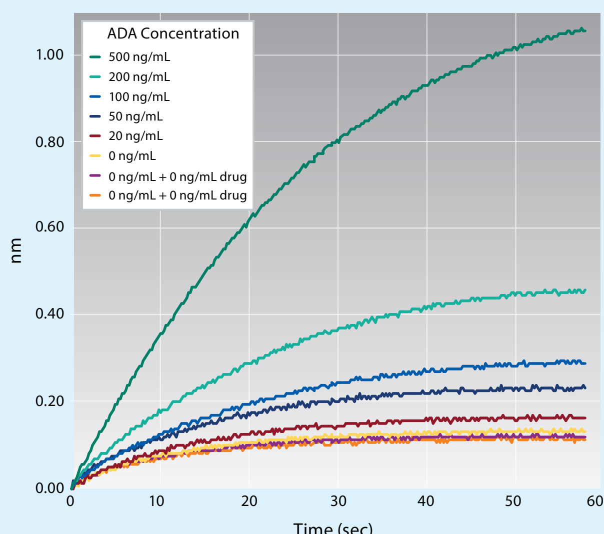

FIGURE 4: Typical concentration response curve for detecting ADA (sheep anti-goat IgG) in the presence of 10 µg/mL of drug (HIgG) in the sample (data taken on the Octet RED system at 1000 rpm).

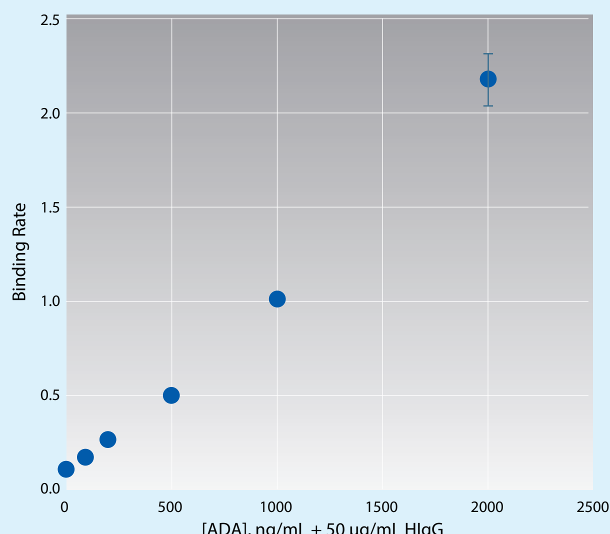

FIGURE 5: In this example, the bridging assay format can tolerate > 500-fold molar excess of drug in these samples.

[ADA], (ng/mL)	Run 1	Run 2	Run 3	Mean	SD	CV%
250	5.936	4,572	5,361	5,290	0,685	12.9
100	1.667	1.630	1.524	1.607	0.074	4.6
50	0.775	0.743	0.712	0.744	0.031	4.2
25	0.508	0.437	0.439	0.461	0.040	8.8
10	0.271	0.259	0.257	0.262	0.008	2.9
5	0.251	0.231	0.225	0.235	0.014	5.8
0	0.162	0.158	0.156	0.165	0013	7.8
0	0.173	0.188	0.154	0.172	0.017	9.8

TABLE 1: Typical binding rates for a titration of an ADA (no drug present).

[Drug] (µg/mL)	[free ADA] (ng/mL)	Run 1	Run 2	Run 3	Mean	SD	CV%
50	2000	2.020	2.275	2.245	2.180	0.139	6.4
	1000	1.023	1.020	0.999	1.014	0.014	1.3
	500	0.518	0.493	0.509	0.507	0.013	2.5
	200	0.275	0.265	0.262	0.267	0.006	2.4
	100	0.174	0.188	0.167	0.176	0.011	6.0
	0	0.117	0.114	0.106	0.112	0.006	5.0
0	0	0.118	0.120	0.115	0.111	0.009	7.9
0	0	0.101	0.099	0.110	0.103	0.006	5.6

TABLE 2: Example binding rates for an ADA in the presence of 50 µg/mL of drug.

Analyze Data

1 Start the Data Analysis software (version 6.1 or later).

2 In Tab 1:

   A Load data by locating the data folder in the explorer, right-clicking it, then choosing Load Folder from the pop-up menu.

   B Select data to analyze by clicking on the data folder in the Quantitation pane at the top of the window. Multiple data sets can be selected for analysis. All selected data sets will be analyzed as a group.

   C Once the data is loaded, sample ID, group, concentration and dilution factors can be modified in the table if needed.

3 In Tab 2:

   A Select the desired standard curve (optional). If no standards were included in the experiment, then the output will be binding rates only.

   B Select R equilibrium as the binding rate equation. This equation will fit the binding curve generated during the experiment and calculate a response at equilibrium as the output signal.

   C Click Calculate Binding Rate. Results will be displayed automatically in the table.

   D Click the Save Report button or select File> Save Report to generate a Microsoft® Excel formatted report.

Representative Data

Figure 4 and Table 1 show data from assays set up on the Octet RED96 system according to the enzyme-linked bridging assay protocol. The binding curves shown in Figure 4 are typical response curves for detecting ADA in the presence of drug. The data shown in Table 1 are for three independent assays run with an identical setup and assay protocol. Data show clear detection of 5 ng/mL ADA (sheep anti-human polyclonal antibody) over 3 standard deviations above background.

Table 2 and Figure 5 contain example binding rates for an ADA in the presence of 50 µg/mL of drug (polyclonal human IgG). Binding rates shown were calculated from data run on the Octet RED system using the enzyme-linked bridging assay protocol described above. The signal of the 100 ng mL ADA in the presence of 50 µg/mL drug is 3 standard deviations over background. This data shows a tolerance of at least 500-fold higher concentration of drug over ADA.

PROTOCOL 2: DIRECT BINDING ASSAY

Materials Required

- Octet system with software version 6.1 or later
- Samples to be analyzed (including negative and positive controls), 40 µL per test
- Negative pool serum, for blocking the immobilized biosensors.
- Purified drug for immobilization: 0.5 mg needed for biotinylation; 10 µg used per test
- High Precision Streptavidin (SAX) Biosensors (Pall ForteBio Part No. 18-5117)
- Immunogenicity Reagent (See Appendix for recipe)
- Sample Diluent (Pall ForteBio Part No. 18-1048)
- Phosphate Buffered Saline (PBS)
- 96-well microplates (Greiner Bio-One, Part No. 655209)
- *Optional* 384-well microplates for Octet QK384/RED384/HTX systems (Greiner Bio-One, Part No. 781209)
- EZ-Link NHS-LC-LC-Biotin (Thermo, Part No. 21343)
- DMF to dissolve biotin and Fluorescein-NHS (Thermo, Part No. 20672)
- PD-10 desalting column (GE Healthcare, Part No. 17-0851-01)
- Sucrose (Sigma, Part No. S0389); prepare as a 15% (w/v) solution in distilled deionized water
- Pall ForteBio Technical Note 10: *Batch Immobilization of a Biotinylated Ligand onto Streptavidin Biosensors*

Tips for Optimal Performance

- A cut point analysis is strongly recommended to establish approximately a 5% false positive (FP) rate with each sample matrix type.[1,2]
- Equilibrate reagents and samples to room temperature prior to preparation. For frozen samples, thaw and mix thoroughly prior to use.
- Biosensors must be hydrated for at least 5 minutes before use.
- Blocking the immobilized biosensors with a matrix similar to that used for the samples will improve assay sensitivity. Block the biosensors for at least one hour.
- A minimum volume of 200 µL/well in a 96-well microplate (80 µL/well in a 384-well microplate) is required for the diluted samples, reagents and the biosensor hydration solution.
- Turn on the Octet instrument at least 40 minutes prior to starting the assay, to allow the lamp to warm up.
- Set the sample plate temperature in the Octet software by selecting File > Experiment > Set plate temperature, then entering the desired temperature. Pall ForteBio recommends 30 °C for the immunogenicity assay.

Protocol Overview

1. Prepare the reagents, including biotinylating the drug. Reagents can be prepared ahead of time and stored for future experiments. Total time = 1.5 hours
2. Load Streptavidin biosensors with biotin-drug. This process can be performed in batch mode and the biosensors preserved for future use. See Technical Note 10: *Batch Immobilization of a Biotinylated Ligand onto Streptavidin Biosensors* for complete details on optimizing the immobilization.
3. Block the immobilized biosensors in a matrix similar to that used for the samples.
4. Prepare the assay plate by pipetting samples, controls and references into a microplate. Total time = 15 minutes
5. Run the experiment: transfer the microplate(s) into the Octet instrument. Hydrate the biosensors and equilibrate the sample plate for 5 minutes on the Octet instrument before starting the assay run.
6. Analyze the data and save the results.

Prepare Reagents

1. Equilibrate reagents to room temperature prior to preparation and mix thoroughly.
2. **Biotinylated drug.** The drug must be minimally biotinylated to enable immobilization to the Streptavidin Biosensor.

   A. Prepare NHS-LC-LC-Biotin in DMF. Determine the concentration of biotin reagent needed in order to insure the volume of DMF does not exceed 3% of protein drug solution to be biotinlyated.

      NOTE: NHS compounds are easily hydrolyzed in aqueous solutions higher than pH 7. Prepare this solution immediately prior to use. Vortex the solution until all the solids are completely dissolved.

   B. Prepare solution of drug to be biotinylated in PBS (the recommended volume and concentration are 0.5 mL or more at 1 mg/mL). If a lower concentration or smaller volume is used then purification by dialysis instead of by a PD-10 column may be necessary.

C A molar coupling ratio (MCR) of 1 is recommended (1 EZ-Link NHS-LC-LC-Biotin molecule for every 1 drug molecule), but can be optimized for each drug molecule. Calculate the volume of biotin solution to add to the aliquot of drug to give a solution with an MCR = 1.

$$\text{mL of biotin reagent} = \frac{\text{mg drug}}{\text{drug MW (mg/mmol)}} \times \text{MCR} \times \frac{\text{Biotin-NHS MW}}{\text{mg/mL Biotin-NHS in DMF}}$$

D Add the calculated volume of biotin reagent to sample. Incubate 1 hour at room temperature (20–25 °C). During incubation prepare PD-10 columns for buffer exchange with PBS according to the manufacturer's instructions.

E Remove the excess Biotin-LC-LC-NHS using PD-10 columns according to the manufacturer's instructions, except elute and collect only 3 mL instead of the 3.3 mL recommended by the manufacturer, to ensure minimal contamination by free biotin.

F Calculate the concentration of biotinylated drug assuming a 90% yield:

[biotin-drug (mg/mL)] = (mg of drug/3 mL) X 90%

Load the Biosensors

The biotinylated drug can be immobilized onto the Streptavidin biosensors in batch mode. Using a batch mode approach allows the biosensors to be prepared in advance and stored for later use. A general outline for a batch-loading procedure is given below. This procedure may need optimization depending on the biotinylated molecule used.

NOTE: For complete details on how to develop and validate batch loading procedures, see Pall ForteBio Technical Note 10: *Batch Immobilization of a Biotinylated Ligand onto Streptavidin Biosensors.*

1 Prepare a 50 µg/mL solution of biotin-drug in Sample Diluent (~20 mL are needed to load 96 biosensors simultaneously).

2 In a 96-well microplate, pipet 200 µL of the biotin-drug solution into each of the wells corresponding to the locations of the biosensors to be used.

3 In a second 96-well microplate, pipet 200 µL of Sample Diluent into each of the wells corresponding to the locations of the biosensors to be used.

4 Place the plate containing Sample Diluent in the lower portion of a biosensor tray. Carefully lower the biosensors into the liquid by lowering the top portion of the tray into the bottom portion. Allow to hydrate 5 minutes.

5 Remove the plate with the Sample Diluent and replace with the plate containing biotin-drug. Lower the biosensors into solution. Incubate overnight (8–16 hours) at 4 °C.

6 The next day prepare 2 plates containing Sample Diluent and one plate containing 15% sucrose (w/v in distilled, deionized water). Each plate should contain 200 µL of the appropriate solution in the wells corresponding to the locations of the biosensors.

7 Remove the biotin-drug plate from the biosensor tray bottom and replace with a plate containing Sample Diluent. Incubate the biosensors in the Sample Diluent 2–5 minutes. Replace the first Sample Diluent plate with the second one and incubate again for 2–5 minutes for a total of 2 washes.

8 Preserve the biosensors by replacing the plate containing Sample Diluent with the plate containing 15% sucrose. Incubate the biosensors in the sucrose 2–5 minutes. Remove the sucrose plate and allow the biosensors to dry. Store coated biosensors in the original re-sealable bag. Biosensors must be rehydrated prior to use.

Block Biosensors and Prepare Assay Plate

Equilibrate all samples and buffers to room temperature and mix thoroughly prior to use. When filling microplates, use a minimum of 200 µL/well in a 96-well microplate (all instruments), and a minimum of 80 µL/well in a 384-well microplate (Octet QK384/RED384/HTX systems only).

1 Prepare the blocking solution for the biosensors. Blocking solution should be a matrix as similar to the samples as possible (typically 20% serum in Immunogenicity Reagent). 200 µL of blocking solution is needed per biosensor.

   A Pipet 200 µL of the blocking solution into wells in a 96W microplate that correspond to the number and position of biosensors to be treated.

   B Place the plate with the blocking solution into the lower portion of the biosensor tray. Carefully lower the biosensors into the liquid by lowering the top portion of the tray into the bottom portion. Allow to block for at least 1 hour.

2 While the biosensors are in the blocking solution:

   A Dilute samples a minimum of 1:5 in Immunogenicity Reagent to give a final serum concentration of 20% (use the same dilution for samples and blocking solution).

   B If a reference sample is desired, treat negative control serum identically to the sample.

   C Pipet the diluted samples and references into the appropriate wells filling the plate in columns starting at A1–H1 then progressing to A2–H2, etc.

   NOTE: when using QK384/RED384/HTX systems, reagents must be arrayed to accommodate the 16 channel layout (See the User Guide for a complete description of working in 16-channel mode).

3 Place the blocked biosensors and sample plates in the Octet instrument with the A1 position on each plate toward the back right corner of the plate holder. Ensure that both the biosensor tray and sample plate are securely in place and sit flat on the holder. Equilibrate the samples to the assay temperature (30°C) for a minimum of 5 minutes prior to the first measurement.

Run Experiment

1 Ensure the Octet instrument and associated computer are turned on. It is critical that the lamp is warmed up for at least 40 minutes prior to the first measurement.

2 Start the Data Acquisition software (version 6.1 or later).

3 From the Experiment Wizard (ctrl+N) choose the Basic Quantitation option.

4 In Tab 1:

A Click the Modify button and choose the Immunogenicity - Direct assay file from the list on the left. The settings in this list are default settings. To modify the settings use the up/down arrows. Modifications only apply to the current assay and are saved as a part of the assay's method file. To create a new assay file, see Help > User Guide > Edit Assay Parameters.

B Right-click on the plate map and define locations for samples, standards and references.

C Enter Sample ID, concentrations and dilution factors as appropriate into the table.

5 In Tab 2:

A Define the locations of the biosensors. Default locations are shown automatically. Locations can be changed by highlighting the biosensors and using the Remove, Fill or Fill Plate buttons.

B Choose Custom from the Sensor Type drop-down menu. Add biosensor Information in the table on the right, if desired.

6 In Tab 3:

A Enter the location and folder name in which the data should be saved.

B Enter a delay if needed to complete the 5 minute minimum sample equilibration and biosensor hydration time.

7 Click the GO button to start the assay. The default settings ensure the data, method and runtime charts are saved automatically.

Analyze Data

1 Start the Data Analysis software (version 6.1 or later).

2 In Tab 1:

A Load data by locating the data folder in the explorer, right-clicking it, then choosing Load Folder from the pop-up menu.

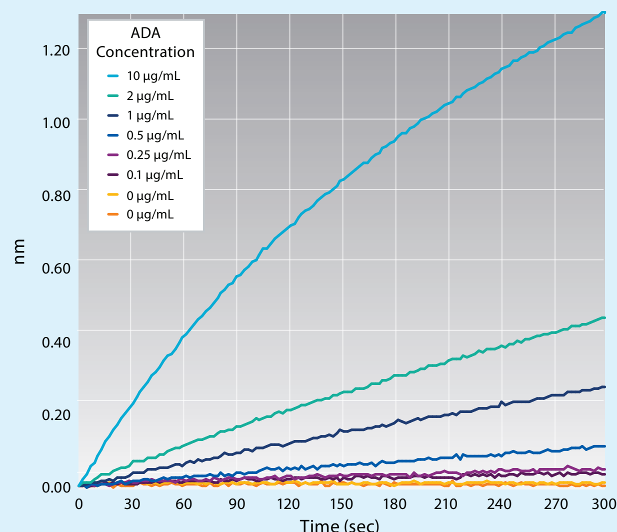

FIGURE 6: Typical ADA (polyclonal sheep anti-human antibody) binding curves obtained using the direct binding assay in the absence of drug.

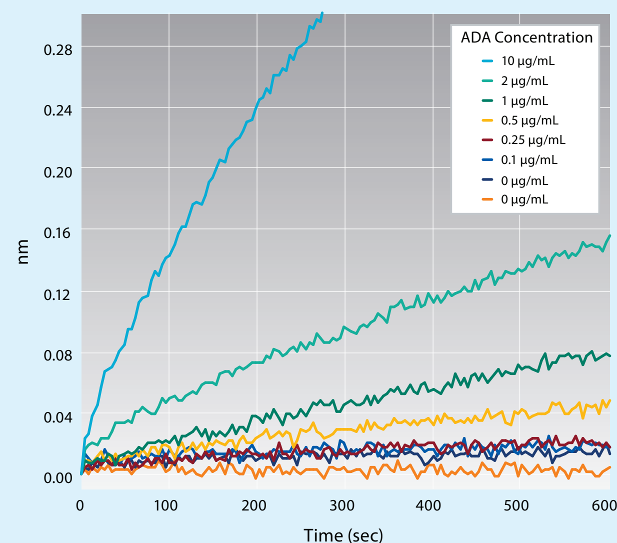

FIGURE 7: Typical ADA binding curves using the direct binding assay format in the presence of 20 µg/mL of drug (polyclonal human IgG).

B Select data to analyze by clicking on the data folder in the Quantitation pane at the top of the window. Multiple data sets can be selected for analysis. All selected data sets will be analyzed as a group.

C Once the data is loaded, sample ID, group, concentration and dilution factors can be modified in the table if needed.

3 In Tab 2:

A Select the desired standard curve (optional). If no standards were included in the experiment, then the output will be binding rates only.

B Select Initial Slope as the binding rate equation. This equation fits the binding curve generated during the experiment and calculates a response at equilibrium as the output signal.

C Click Calculate Binding Rate. Results will be displayed automatically in the table.

D Click the Save Report button or select File> Save Report to generate a Microsoft® Excel formatted report.

Representative Data

Figure 6 shows typical ADA binding curves obtained using the direct binding assay in the absence of drug.

Figure 7 shows typical ADA binding curves in the presence of 20 µg/mL of antibody drug in the sample.

REFERENCES

1 Mire-Sluis, A.R. et al. *Journal of Immunological Methods,* 2004, 289, 1–16.

2 Shankar, G. et al. *Journal of Pharmaceutical and Biomedical Analysis,* 2008, 48, 1267–1281.

APPENDIX

Immunogenicity Reagent recipe:

- 10mM PBS, pH 7.4
- Tween®-20, 0.02%
- Bovine Serum Albumin (≥ 98% purity), 0.10% W/V
- Sodium Hydroxide (2N), 1.80% V/V
- Acetic Acid, 7.50%

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