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Immunogenicity Assessment of Therapeutic Protein on the Octet® BLI System

Technical Note

Scope
This Technical Note describes the workflows for immunogenicity assays that use Octet® BLI systems for the detection of anti-drug antibodies from serum and plasma samples.

Abstract

Therapeutic proteins have revolutionized medicine in the last 30 years. However, an unfortunate side-effect of these therapeutic proteins is an unwanted immune response upon drug administration in patients where anti-drug antibodies (ADA) are produced. These ADAs can alter the pharmacodynamics (PD) and/or the pharmacokinetics (PK) of the drug. The detection of these ADAs and other factors that may lead to immunogenicity is therefore critical during the therapeutic development process. A wide range of ADA affinities and concentrations can be produced during the immune response. To accurately detect these polyclonal antibodies, the assessment assay and system must have exquisite sensitivity, be able to withstand a wide range of free drug concentrations and, importantly, have minimal matrix effects so samples in sera can be assessed. This Technical Note highlights the ease of assay design and setup offered by the dip-and-read format on Sartorius' Octet® Biolayer Interferometry (BLI) systems, and provides a step-by-step guide to ADA quantitation directly from samples contain serum or plasma.

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Introduction

A wide range of polyclonal antibody ADAs affinities and concentrations can be produced during an immune response to therapeutic proteins. To accurately detect these polyclonal antibodies, the assessment assay and system must have exquisite sensitivity, be able to withstand a wide range of free drug concentrations and, importantly, have minimal matrix effects so samples in sera can be assessed. Automated immunoassays on Sartorius' Octet® Biolayer Interferometry (BLI) systems provide a high level of sensitivity in multiple media, tolerance to drug, and flexibility to detect both high and low affinity ADA by providing multiple methods without any plate washing steps. These methods 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.

Octet® BLI 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 Octet® BLI systems. Higher 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 was used to 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® system's 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® RH16/RH96 BLI system. Often, this method provides enhanced sensitivity compared to the direct binding method.

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.

Figure 1
Schematic of the Enzyme-Linked Bridging Assay.

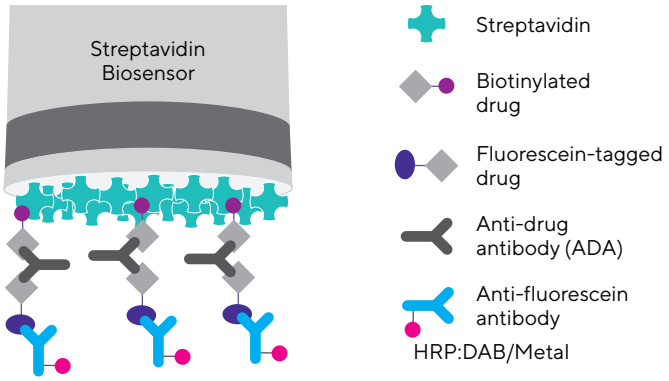
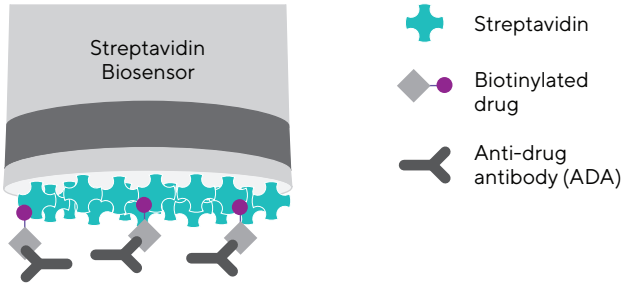


Figure 2
Schematic of the Direct Binding Assay.



Protocol 1: Enzyme-Linked Bridging Assay

Materials Required

- Octet® BLI system with software version 6.1 or later (version 12 for Octet® R Series BLI systems)
- Samples to be analyzed (including positive and negative controls), 100 µL per test or 200 µL per test when using Octet® R Series BLI systems or when working with 96-well microtiter plates
- Purified drug for immobilization and detection: 1 mg needed for biotinylation and fluorescein labeling; 4 µg used per test
- Vivaspin® 6 Centrifugal Concentrator with an appropriate molecular weight cut-off (MWCO) for the drug being assessed (shop.sartorius.com)
- Octet® High Precision Streptavidin (SAX) Biosensors (Part No. 18-5117)
- Immunogenicity Reagent (See Appendix for components)
- Octet® Sample Diluent Buffer (Part No. 18-1104)
- Phosphate Buffered Saline (PBS)
- 96-well microplates (Greiner Bio-One, Part No. 655209)
- Optional 384-well microplates for Octet® RH16/RH96 BLI systems (Greiner Bio-One, Part No. 781209)
- EZ-Link NHS-LC-LC-Biotin (Thermo Fisher Scientific, Part No. 21343)
- DMF (Thermo Fisher Scientific, Part No. TS-20672)
- NHS-Fluorescein (Thermo Fisher Scientific, Part No. 46410)
- Stable Peroxide Substrate Buffer (10X) (Thermo Fisher Scientific, Part No. 34062)
- DAB/Metal Concentrate (10X) (Thermo Fisher Scientific, Part No. 34065)
- Rabbit anti-FITC:HRP (Bio-Rad, 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® BLI system at least 40 minutes prior to starting the assay, to allow the lamp to warm up.
- Set the sample plate temperature in the Octet® BLI software by selecting Experiment > Set plate temperature, then entering the desired temperature. Sartorius 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 assays.
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® RH16/RH96 BLI system).
3. Run the assay: transfer the microplate(s) into the Octet® BLI system. Hydrate the biosensors and equilibrate the sample plate for 15 minutes on the Octet® BLI system before starting the assay run. Total time for a full 96-well plate is 60 minutes for Octet® R8 BLI systems; 30 minutes for Octet® RH16/RH96 BLI 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 7.0. 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 Vivaspin® centrifugal concentrator 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 for 1 hour at room temperature (20–25°C). During incubation prepare Vivaspin® centrifugal concentrators for buffer exchange with PBS according to the manufacturer's instructions.

e. Remove the excess Biotin-LC-LC-NHS using Vivaspin® centrifugal concentrators according to the manufacturer's instructions, except elute and collect only 90% of the volume recommended by the manufacturer, to ensure minimal contamination by free biotin (x mL in the equation in step f).
f. Calculate the concentration of biotinylated drug assuming a 90% yield:

[biotin-drug (mg/mL)] = (mg of drug/x 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 7.0. 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 1 mg/mL). If lower concentration or less volume is used then purification by dialysis instead of by a Vivaspin® centrifugal concentrator 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 Vivaspin® centrifugal concentrators for buffer exchange with PBS according to the manufacturer's instructions.
   e. Remove the excess Fluor-LC-LC-NHS using Vivaspin® centrifugal concentrators according to the manufacturer's instructions, except elute and collect only 90% of the volume recommended by the manufacturer, to ensure minimal contamination by free fluorescein (x mL in the equation in step f).
   f. Calculate the concentration of labeled drug assuming a 90% yield:

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

Prepare Assay Plate

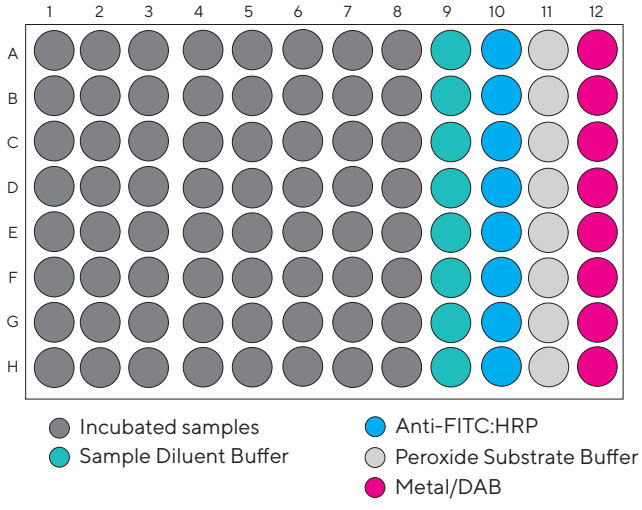
Equilibrate samples, reagents and buffers to room temperature and mix thoroughly prior to use. When filling the microplates, a minimum of 200 µL should be put in each well of a 96-well microplate (all systems), and a minimum of 80 µL in each well of a 384-well microplate (Octet® RH16/RH96 BLI systems 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 the 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.

   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® R8 BLI system (see Figure 3) and 96 on the Octet® RH16/RH96 BLI systems.

Figure 3

Sample 96-Well Plate Map for Use in Octet® R8 BLI systems.



3. Cover and incubate the microplate on a plate shaker (500–1000 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.
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® R8 BLI systems – 96-well plate only
   - 2 mL anti-FITC:HRP antibody conjugate diluted 1:1000 in the Octet® Sample Diluent Buffer.
   - 2 mL Metal/DAB substrate diluted 1:10 in Peroxide Substrate Buffer.

   For Octet® RH16/RH96 BLI systems – 96-well plate only
   - 4 mL anti-FITC:HRP antibody conjugate diluted 1:1000 in the Octet® Sample Diluent Buffer.
   - 4 mL Metal/DAB substrate diluted 1:10 in Peroxide Substrate Buffer.

   For Octet® RH16/RH96 BLI systems – 384-well plate only
   - 2 mL anti-FITC:HRP antibody conjugate diluted 1:1000 in the Octet® Sample Diluent Buffer.
   - 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® R8 BLI system, duplicate columns for Octet® RH16/RH96 BLI systems). Reagents can be placed in any column. Figure 3 depicts a plate set up to be used with the Octet® R8 BLI system, having reagents in columns 9–12.
   Note: when using Octet® RH16/RH96 BLI 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® BLI system. 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® BLI system 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 Octet® BLI Discovery Software.
3. From the Experiment Wizard (ctrl+N) choose the Advanced Quantitation option. Choose the Enzyme Linked Immunogenicity assay file from the expanded view.
4. Plate Definition Tab:
   a. The settings in this method are default settings. To modify the settings click on the modify button contained with the assay setting box. 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 (unknowns), standards and references.
   c. Enter Sample ID, concentrations and dilution factors as appropriate into the table.
5. Sensor Assignment Tab:
   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. Review Experiment Tab:
   a. Review the steps that make up the method by moving the slider to change the active step.
7. Run Experiment:
   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.
8. Click the GO button to start the assay. The default settings ensure the data, method and runtime charts are saved automatically.

Analyze Data

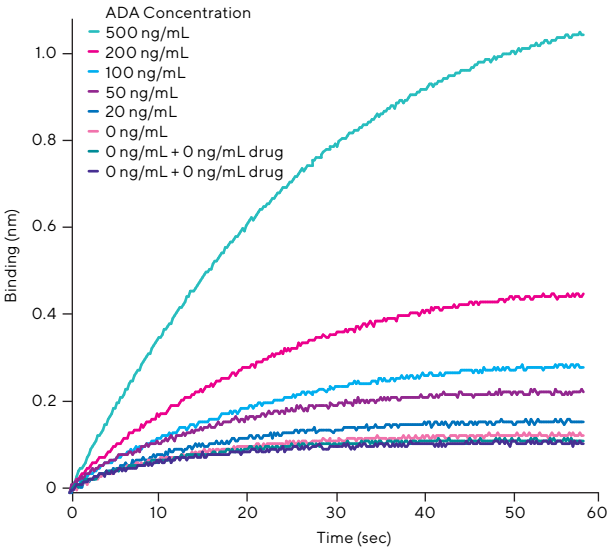
1. In Octet® BLI Analysis Software, load the data folder to be analyzed.
2. In the Preprocessed Data tab: Select the reference wells to perform reference subtraction if needed.
3. Sample ID, Group, Concentration and Dilution factors can be modified in the table if needed.
4. In the Quantitation Analysis tab:
   a. Select the desired Standard Curve Equation (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. Results will be displayed automatically in the table.
   d. Click Export to generate a report file and save results.

Representative Data

Figure 4 and Table 1 show data from assays set up on the Octet® R8 BLI 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.

Figure 4

Typical Concentration Response Curve.



Note. 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 an Octet® BLI system at 1000 rpm).

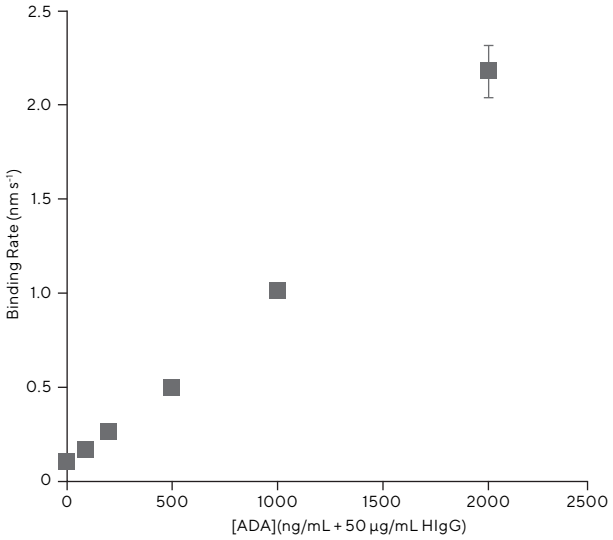
Table 1

Typical Binding Rates for a Titration of an ADA (no drug present).

[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	0.013	7.8
0	0.173	0.188	0.154	0.172	0.017	9.8

Figure 5 and Table 2 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 an Octet® BLI 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.

Figure 5
Binding Rate Plot in the Presence of ADA.



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

Table 2
Example Binding Rates for an ADA in the Presence of 50 µg/mL of Drug.

[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

Protocol 2: Direct Binding Assay

Materials Required

- Octet® BLI system with software version 6.1 or later (version 12 for Octet® R Series BLI systems)
- Samples to be analyzed (including negative and positive controls), 40 µL per test
- Negative pool serum, for blocking the immobilized biosensors.
- Vivaspin® 6 Centrifugal Concentrator with an appropriate molecular weight cut-off (MWCO) for the drug being assessed (shop.sartorius.com)
- Purified drug for immobilization: 0.5 mg needed for biotinylation; 10 µg used per test
- Octet® High Precision Streptavidin (SAX) Biosensors (Part No. 18-5117)
- Immunogenicity Reagent (See Appendix for components)
- Octet® Sample Diluent Buffer (Part No. 18-1104)
- Phosphate Buffered Saline (PBS)
- 96-well microplates (Greiner Bio-One, Part No. 655209)
- Optional 384-well microplates for Octet® RH16/RH96 BLI systems (Greiner Bio-One, Part No. 781209)
- EZ-Link NHS-LC-LC-Biotin (Thermo, Part No. 21343)
- DMF (Thermo Fisher Scientific, Part No. TS-20672)
- Sucrose (Sigma, Part No. S0389); prepare as a 15% (*w/v*) solution in distilled deionized water
- See Technical Note: 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® BLI system at least 40 minutes prior to starting the assay, to allow the lamp to warm up.
- Set the sample plate temperature in the Octet® BLI software by selecting Experiment > Set plate temperature, then entering the desired temperature. Sartorius 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: 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® BLI system. Hydrate the biosensors and equilibrate the sample plate for 5 minutes on the Octet® BLI system 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 ensure 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 7.0. 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 Vivaspin® centrifugal concentrator 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 Vivaspin® centrifugal concentrators for buffer exchange with PBS according to the manufacturer's instructions.
   e. Remove the excess Biotin-LC-LC-NHS using Vivaspin® centrifugal concentrators according to the manufacturer's instructions, except elute and collect only 90% of the volume recommended by the manufacturer, to ensure minimal contamination by free biotin (x mL in the equation in step f).
   f. Calculate the concentration of biotinylated drug assuming a 90% yield:

$$[\text{biotin-drug (mg/mL)}] = (\text{mg of drug/x mL}) \times 90\%$$

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. 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 Technical Note: Batch Immobilization of a Biotinylated Ligand onto Streptavidin Biosensors.

1. Prepare a 50 µg/mL solution of biotin-drug in Sample Diluent Buffer (~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 Buffer into each of the wells corresponding to the locations of the biosensors to be used.
4. Place the plate containing Sample Diluent Buffer 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 Buffer 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 Buffer 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 Buffer. Incubate the biosensors in the Sample Diluent Buffer 2–5 minutes. Replace the first Sample Diluent Buffer 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 Buffer 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 systems), and a minimum of 80 µL/well in a 384-well microplate (Octet® RH16/RH96 BLI 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 96-well 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 Octet® RH16/RH96 BLI 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® BLI system 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 Method

1. Ensure the Octet® BLI system 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 Octet® BLI Discovery Software.
3. From the Experiment Wizard (ctrl+N) choose the Basic Quantitation option. Choose the Direct Detection Immunogenicity assay file from the expanded view.
4. Plate Definition Tab:
   a. The settings in this method are default settings. To modify the settings click on the modify button contained with the assay setting box. 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 (unknowns), standards and references.
   c. Enter Sample ID, concentrations and dilution factors as appropriate into the table.
5. Sensor Assignment Tab:
   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. Review Experiment Tab:
   a. Review the steps that make up the method by moving the slider to change the active step.
7. Run Experiment:
   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.
8. Click the GO button to start the assay. The default settings ensure the data, method and runtime charts are saved automatically.

Analyze Data

1. In Octet® BLI Analysis Software, load the data folder to be analyzed.
2. In the Preprocessed Data tab: Select the reference wells to perform reference subtraction if needed.
3. Sample ID, Group, Concentration and Dilution factors can be modified in the table if needed.
4. In the Quantitation Analysis tab:
   a. Select the desired Standard Curve Equation (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 will fit the binding curve generated during the experiment and calculate a response at equilibrium as the output signal.
   c. Results will be displayed automatically in the table.
   d. Click Export to generate a report file and save results.

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 components:

- 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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TN-3017 Rev C