SARTORIUS

High Precision Streptavidin Biosensor (SAX)

Quantitation and Kinetic Assays

Technical Note

Scope
This Technical Note provides insight into the utilization of Octet® High Precision Streptavidin (SAX) Biosensors for analyte quantitation and kinetic characterization.

Keywords or phrases:
Octet®, Bio-Layer Interferometry, BLI, protein-protein interaction, affinity analysis, quantitation assay, high precision Streptavidin, SAX, method development, quality control, ligand binding, drug discovery, ligand immobilization

Executive Summary

Ligand binding assays that utilize solid surface chemistries often deploy streptavidin as the molecule of choice for ligand immobilization onto the solid-surface. Streptavidin's biophysical properties enables it to form a high affinity non-covalent bond with biotin allowing for the tight immobilization of biotinylated ligands. Sartorius's High Precision Streptavidin Biosensors (SAX) are developed to be used with Octet® instruments and are qualified for applications in downstream drug discovery and regulated environments. They are QC-tested to meet our precision-controlled coefficient of variation (CV) specification of <4% and can used with any biotinylated molecule for both kinetics and quantitation assays where stringent precision requirements are in place.

Principle

Streptavidin is a 52.8 kDa protein purified from the bacterium *Streptomyces avidinii*. Streptavidin homo-tetramers have an extraordinarily high affinity to biotin. With a dissociation constant (k_d) on the order of ~10^{-6} mol/L, the binding of biotin to streptavidin is one of the strongest non-covalent interactions known in nature. High Precision Streptavidin Biosensors are designed for direct capture of biotin-labeled proteins for both kinetic characterization and quantitation measurements on all Octet® systems.

Materials Required

- Octet® instrument with Octet® BLI Discovery Software and Octet® Analysis Studio Software
- High Precision Streptavidin (SAX) Biosensors: (Sartorius part no. 18-5117 [tray]; 18-5118 [pack]; 18-5119 [case])
- For all Octet® instruments: 96-well, black, flat bottom, polypropylene microplate (Greiner Bio-One part no. 655209)
- Optional for Octet® RH16 instruments:
  - 384-tilted well, black, flat-bottom, polypropylene microplate (Sartorius part no. 18-5080 [pack]; 18-5076 [case])
  - 384-well, black, flat-bottom, polypropylene microplate (Greiner Bio-One part no. 781209)
- **Quantitation assays:** Sample Diluent (Sartorius part no. 18-5028) for dilution of all samples. If undiluted crude samples will be quantitated, a matching blank matrix is required.
- **Kinetic assays:** The SAX Biosensor is compatible with a wide range of buffers, although 1X Kinetics Buffer is recommended. Dilute 10X Kinetics Buffer (Sartorius part no. 18-5032) 1:10 with PBS, pH 7.4). Best results are obtained when all matrices are matched as closely as possible.

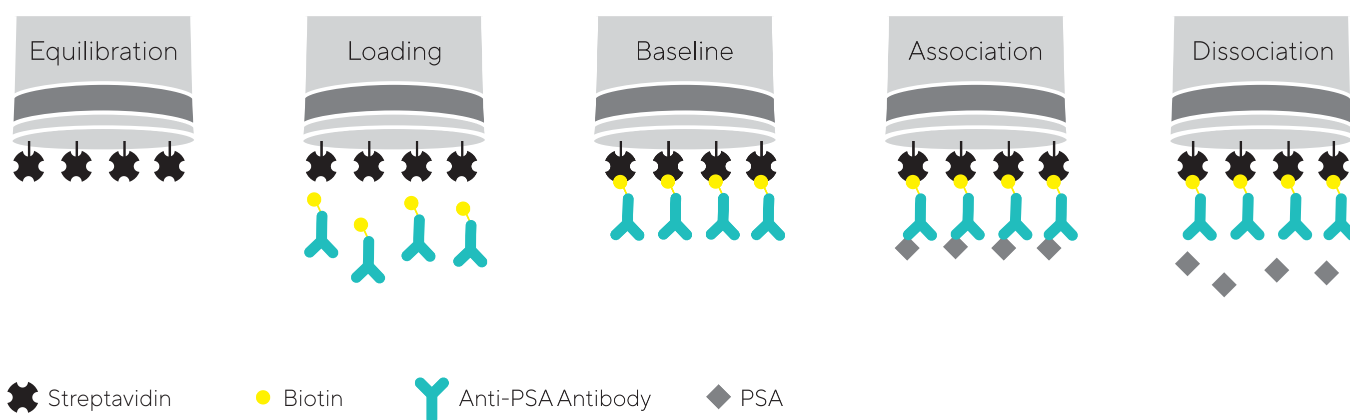

Figure 1: Binding kinetics experiment using SAX Biosensors and PSA model binding pair. After an initial equilibration step in buffer, biosensors are dipped into the solution of biotinylated anti-PSA antibody. A buffer baseline step is performed followed by association and dissociation of PSA protein analyte.

Tips for Optimal Performance

- Fully equilibrate all reagents, samples and calibrators (for quantitation assays) to room temperature prior to sample preparation. Thaw frozen samples completely and mix thoroughly prior to use.
- Hydrate the biosensors for a minimum of 10 minutes prior to use. Hydrating the biosensors in a buffer consistent with the buffer used throughout the assay is recommended.
- Ensure that the Octet® instrument is turned on and the lamp is warmed to room temperature for at least 40 minutes prior to starting the assay.
- Set the sample plate temperature in Octet® BLI Discovery Software by selecting **File > Experiment > Set Plate Temperature**.... Enter the desired temperature. Sartorius recommends running assays at 30°C, using other temperatures may require modifying the assay times discussed in this protocol. Set the default startup temperature (software versions 6.4 and later) by selecting **File > Options**. Enter the desired temperature under "Startup".
- Quantitation assays only:
  - Match the matrix of the samples, standards, references, and hydration solution as closely as possible.
  - Perform a dilution study and a dynamic range study as outlined in the Assay Optimization section.
  - Use a blank negative control in a matching matrix for reference subtraction. This is especially important when optimizing accuracy and detecting low-concentration analytes.

Quantitation Assays

Assay Optimization

High Precision Streptavidin (SAX) Biosensors can be customized with any biotinylated molecule for quantitation use in a simple and straightforward manner. Such customization can be done by dipping SAX Biosensors into a solution containing 20 µg/mL of biotinylated ligand for 300 – 600 seconds with shaking at 1000 rpm (actual optimal biosensor coating parameters might vary and can be adjusted by user depending on material or time requirements). The following assays are recommended each time a new matrix or new protein is analyzed.

Overview

- Determine the minimal required dilution (MRD) factor to achieve the targeted assay dynamic range.
- Perform a spike | recovery study to determine assay recovery.
- Determine data analysis parameters.
- Apply the finalized protocol and data analysis parameters in routine assays.

Dilution factor determination for matrix

Differences between matrices can potentially influence assay performance. Diluting the sample matrix using Sartorius' Sample Diluent is a convenient and often effective means of minimizing matrix effects. It is therefore recommended that the minimum required dilution factor be determined using Sample Diluent to achieve the desired assay performance.

1. Prepare 2 mL of sample matrix diluted both two-fold and 10-fold in Sample Diluent. General guidelines for dilutions are described in Table 1.

Table 1: Recommended minimum dilution for common sample types. In all cases, the matrix for the diluted samples, standards and biosensor hydration solution should be matched as closely as possible.

Sample type	Minimum recommended dilution in sample diluent
Purified proteins	Dilute into assay range
Samples from column eluents	Dilute into assay range
Serum-free cell culture supernatants media	Neat or 10-fold
Serum-containing cell culture supernatants	Neat
Bacterial cell pellet lysed by sonication	10-fold
Bacterial cell pellet lysed by B-PER	20-fold

2. Set the assay temperature to run at 30°C by selecting **File > Experiment > Set plate temperature**.

3. Prepare a spiked sample of the protein to be quantitated in: Sample Diluent, neat matrix, two-fold diluted matrix and 10-fold diluted matrix by mixing the minimum volume of analyte and 0.5 mL of each matrix (four samples total). The final concentration of the protein should be in the middle of the desired quantitation range.

4. Transfer each sample to a 96 or 384-well sample plate in duplicate (eight wells total).

5. Hydrate biosensors in the sample matrix that matches each sample type (*e.g.,* biosensors to be used in wells with 10-fold diluted matrix should be hydrated in 10-fold diluted matrix). Place the sample plate and the hydrated biosensors into the Octet® instrument. Recommended sample plate warm-up in the instrument and biosensor hydration time is 10 minutes. The delay timer can be used in Octet® software to automatically start the assay after 600 seconds.

6. Set up a Basic Quantitation assay according to the Octet® BLI Discovery Software User Guide.

7. Run the assay.

8. Data will be displayed in real time during the assay. Data files, method files and assay pictures (.jpgs) will be saved automatically.

9. Load data into Octet® Analysis Studio Software.

10. Visually inspect the real-time binding traces and determine the dilution required to:

    a. Minimize non-specific binding from the matrix.

    b. Show equivalent analyte binding in the matrix-spiked sample and the Sample Diluent control.

    c. Use this dilution factor for routine assays.

Spike Recovery Assay

To determine the dynamic range and data analysis parameters suitable for a specific analyte, establish a standard curve and spike recovery as described below.

1. Prepare standards in the matrix using the dilution factor determined in the prior dilution factor determination for matrix experiment. The minimum volume needed in each well varies with the plate used:
   - 200 µL/well in a 96-well microplate (all Octet® instruments)
   - 80 µL/well in a 384-well microplate (Octet® RH16 instruments)
   - 40 µL/well in a 384-well, tilted-bottom microplate (Octet® RH16 instruments only)
2. Using the same sample matrix from Step 1, prepare 700 µL of two unknown samples. The concentration of these samples should be within the assay dynamic range.
3. Transfer triplicates of the standards and the unknowns to a sample plate. It is recommended to organize samples in columns, from A–H. Fill at least one well with blank diluted matrix for reference subtraction during data analysis. An example plate map is shown in Figure 2.
4. Hydrate biosensors in the matrix that matches the blank diluted matrix. Place the sample plate and the hydrated biosensors in the Octet® instrument. The recommended sample plate warm-up in the instrument and biosensor hydration time is 10 minutes. The delay timer can be used to automatically start the assay after 600 seconds.
5. Set up a Basic Quantitation assay using the assay parameters used in the prior Dilution Factor Determination for Matrix experiment. Availability of the pre-loaded assay parameters will be software version-dependent. For details on how to set up an assay, see the Octet® BLI Discovery Software User Guide.
6. Run the assay. Data will be displayed in real time during the assay. Data files, method files and assay pictures (.jpgs) will be saved automatically.
7. Load the data into Octet® Analysis Studio Software.
8. If blank matrix was included as a reference, use the reference subtraction option to correct the data as appropriate.
9. Calculate the binding rate.
10. Define a dynamic range by selecting acceptable % CV values for the lower and upper concentration limits. 10% is routinely used as a threshold, but may vary depending on the requirements of each assay.
11. Exclude data points for the standard curve that lie outside the defined dynamic range.
12. Iteratively adjust the following processing parameters and re-calculate the binding rate:
    a. Adjust the read time window if necessary (typically 120 seconds).
    b. Adjust the zero concentration threshold if necessary (recommended 0.0001).
    c. Adjust the low concentration threshold to 0.001 (recommended).
    d. Select the appropriate standard curve equation.
13. Evaluate the calculated concentration value of the unknowns by defining acceptable values of % recovery (accuracy) and % CV (precision). ± 15% recovery with a 10% CV are frequently used threshold values but may vary depending on the requirements of each assay.

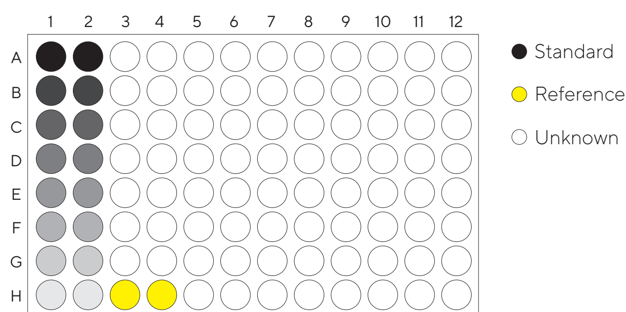

Figure 2: Example plate layout for a routine assay run in a 96-well microplate.

Assay Protocol

1. Pre-load the biosensors with custom biotinylated ligand which specifically recognizes the molecule to be analyzed.
2. Prepare samples, calibration standards and hydration solutions according to the information in Table 1. The minimum volume needed in each well varies with the plate used:
   - 200 µL/well in a 96-well microplate (all Octet® instruments)
   - 80 µL/well in a 384-well microplate (Octet® RH16 instruments)
   - 40 µL/well in a 384-well, tilted-bottom microplate (Octet® RH16 instruments only)

3. Pipette standards, controls and samples into a black polypropylene microplate (see Figure 2 for sample plate layout).

4. Pipette biosensor hydration solution into the wells of a 96-well, black, flat-bottom microplate corresponding to the number and position of the biosensors to be used.

5. Place the biosensor tray with the hydration plate in the Octet® instrument. Place the sample plate in the Octet® instrument. Warm the sample plate in the instrument and hydrate the biosensors for 10 minutes prior to starting the experiment. The delay timer can be used to automatically start the assay after 10 minutes (600 seconds).

6. Set up a Basic Quantitation assay according to the Octet® BLI Discovery Software User Guide. An example plate map is shown in Figure 2.

7. Run the assay. Data will be displayed in real time during the assay. Data files, method files and assay pictures (.jpgs) will be saved automatically.

8. Load data into Octet® Analysis Studio Software.

9. If blank matrix was included as a reference, use the reference subtraction option to correct the data as appropriate.

10. Calculate the binding rate.

11. Define a dynamic range by selecting acceptable % CV values for the lower and upper concentration limits. 10% is routinely used as a threshold, but may vary depending on the requirements of each assay.
12. Exclude data points for the standard curve that lie outside the defined dynamic range.

13. Iteratively adjust the following processing parameters and re-calculate the binding rate:

    a. Adjust the read time window if necessary (typically 120 seconds).

    b. Adjust the zero concentration threshold if necessary (recommended 0.0001).

    c. Adjust the low concentrations threshold to 0.001 (recommended).

14. To export the analyzed data, use the Save Report button to generate a Microsoft® Excel® report.

Representative Data

Figure 3 shows detection of a hIgG standard using High Precision Streptavidin (SAX) Biosensors on the Octet® RH16 system with assay parameters (1000 rpm for 2 minutes; 15 minutes run time) for a standard dynamic range. Figure 3A shows the hIgG dose response and Figure 3B the resulting calibration curves. Sample diluent was used as a matrix for all samples. See Table 2 for the statistical analysis of data from Figure 3.

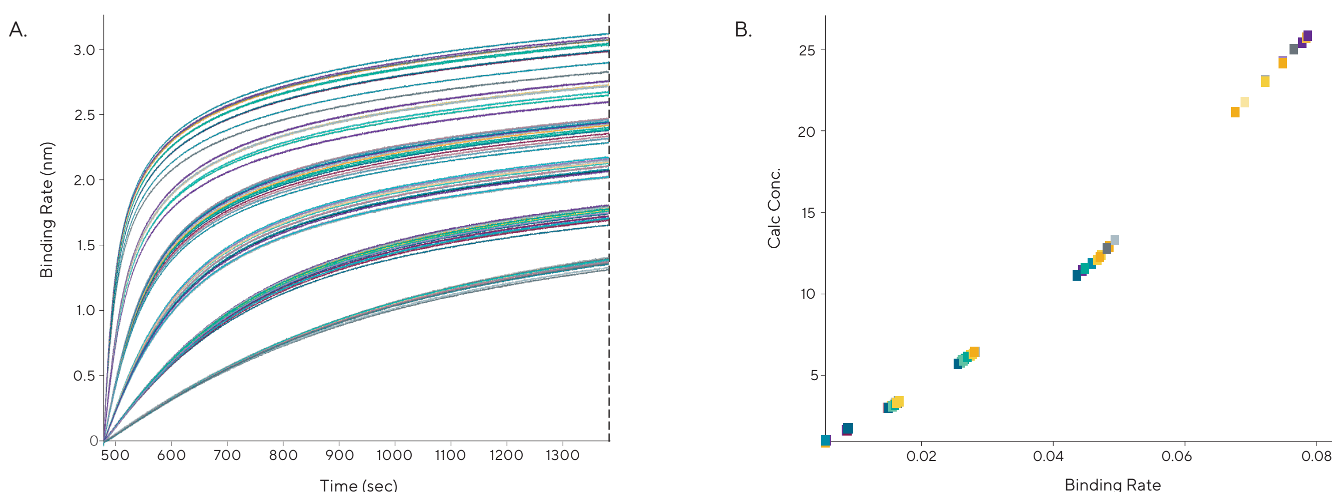

Figure 3: Detection of a hIgG standard using High Precision Streptavidin biosensors (SAX) on the Octet® RH16 system with assay parameters (1000 rpm, 2 min; as shown 15 min) for a standard dynamic range. A) hIgG dose response. B) Representative resulting calibration curves from A). Sample diluent was used as a matrix for all samples.

Table 2: Average calculated concentration and % CVs for hIgG (n=15).

	hIgG	
Expected conc. (µg/mL)	Avg. conc. µg/mL (N=15)	% CV (N=15)
24	24.3	6.0%
12	12.1	5.8%
6	6.0	4.1%
3	3.0	4.5%
1.5	1.5	2.8%
0.75	0.75	1.6%

Assay Protocol-Precision

1. Prepare biotin-Protein A conjugate (1 µg/mL), buffer, hIgG (6 µg/mL), and hydration solutions according to the information in Table 3.

2. Pipette standards, controls and samples into a black polypropylene microplate (see Figure 4 for a sample plate layout).

3. Pipette biosensor hydration solution into the wells of a 96-well black flat-bottom microplate corresponding to the number and position of the biosensors to be used.

4. Place the biosensor tray with the hydration plate in the Octet® instrument. Place the sample plate in the Octet® instrument. Warm the sample plate in the instrument and hydrate the biosensors for 10 minutes prior to starting the experiment. The delay timer can be used to automatically start the assay after 10 minutes (600 seconds).

5. Set up a Basic Quantitation assay according to the Octet® BLI Discovery Software User Guide. See Table 3 for assay setup parameters, and Figure 4 for example plate maps.

6. Run the assay. Data will be displayed in real time during the assay. Data files, method files and assay pictures (.jpgs) will be saved automatically.

7. Load data into Octet® Analysis Studio Software.

8. Calculate the nm shift of biotin-Protein A and hIgG.

Representative Data

Experimental raw data showing 96 replicates of the biotin-Protein A conjugate quantitative precision assay are shown in Figure 5.

Table 3: Precision assay setup parameters.

Step	Step name	Time (s)	Sample	Shake speed	Step type
1	Equilibration	120	3	1000	Custom
2	Loading	300	1	1000	Loading
3	Baseline	60	3	1000	Baseline
4	Association	900	2	1000	Association

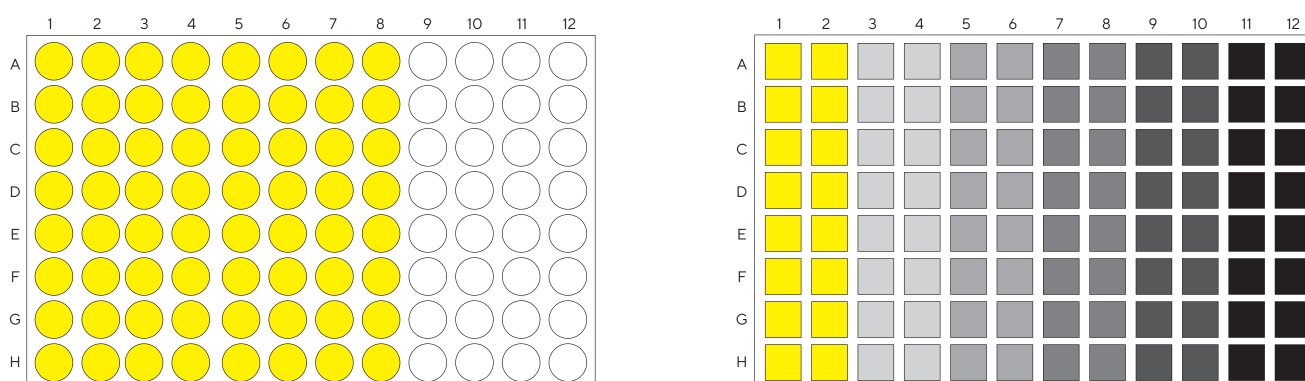

Figure 4: Sample and biosensor plate layout (left and right respectively) for the precision assay.

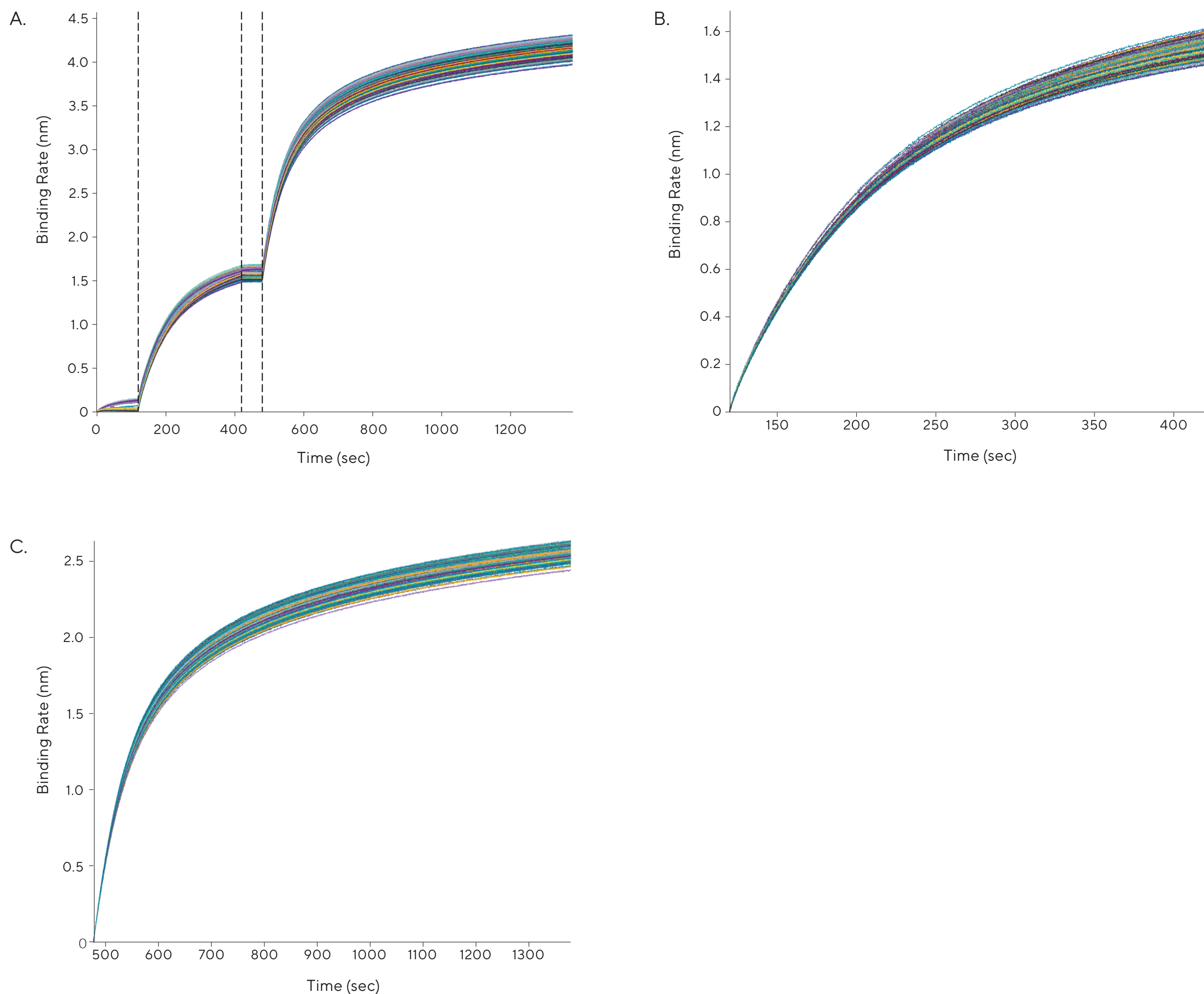

Figure 5: (A) Full experimental raw data showing the same steps measured on 96 replicates. (B) 96 replicates of the second step in the measurement, biotinylated Protein A loading onto 96 SAX Biosensors. (C) 96 replicates of the 4th step in the measurement, hIgG binding to Protein A.

Table 4: Biotin-Protein A and hIgG precision % CV (n=96).

n=96	Biotin-Protein A (1 µg/mL)	hIgG (6 µg/mL)
Mean	1.53	2.58
SD	0.04	0.05
% CV	2.6%	1.8%

Kinetic Assays

Assay Protocol

Overview

- Prepare assay solutions
- Prepare the sample plate
- Equilibrate both the hydrated biosensor assembly and the assay plate for 10 minutes on the Octet® instrument
- Run the assay
- Process and analyze the data
- Save the results

Prepare the Samples and the Calibration Standards

- Equilibrate reagents and samples to room temperature prior to preparation and mix thoroughly.
- The PSA model binding pair provides a reliable, consistent antibody-antigen model binding pair for use in binding kinetics assays on the Octet® platform. This pair consists of an anti-human PSA mouse monoclonal antibody and human PSA (Prostate Specific Antigen, molecular weight 30 kDa). The affinity of the interaction between the two proteins is in the low nanomolar range and follows a 1:1 binding kinetics model. This binding pair can be used with any Octet® instrument and the new High Precision Streptavidin (SAX) Biosensor.
- Biotinylate ligand that specifically binds to the analyte that will be analyzed. Biotinylate according to the protocol outlined in Technical Note 6, Biotinylation of Protein for Immobilization onto Streptavidin Biosensors using either biotin-LC-LC-NHS (Pierce, part no. 21343) or biotin-PEO4-NHS (Pierce, part no. A39259). The long linkers in these two reagents typically produce good quality assays.
- Dilute the biotin-anti-PSA antibody ligand to 50 µg/mL by adding 70 µL of the stock antibody solution to 1330 µL of 10x Kinetics Buffer (Sartorius part no 18-5032).
- Prepare 400 µL of the PSA antigen solution. Add 4 µL of the PSA antigen to 396 µL 10X Kinetics Buffer to make a 200 nM solution. Mix gently. This solution will be the top concentration in a dilution series of PSA antigen.
- Perform four serial two-fold dilutions of the PSA antigen solution: add 200 µL of 200 nM PSA antigen solution from Step 2 to 200 µL 10X Kinetics Buffer to make a 100 nM solution, mixing gently. Repeat this dilution to make 50 nM, 25 nM, and 12.5 nM PSA solutions.

Running the Assay

1. Ensure that the Octet® instrument is turned on and the lamp has been warmed to room temperature for at least 40 minutes prior to starting the assay.

2. Pipette 200 µL/well of biosensor hydration solution into the wells of a 96-well black, flat-bottom microplate corresponding to the number and the positions of biosensors to be used.

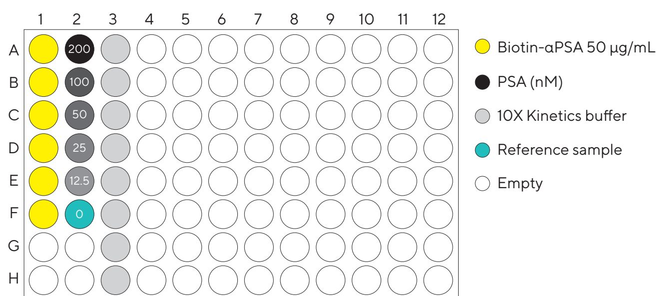

Figure 6: Sample kinetic assay plate setup.

Table 5: Kinetic assay setup parameters.

Step	Step name	Time (s)	Sample	Shake speed	Step type
1	Equilibration	60	3	1000	Custom
2	Loading	600	1	1000	Loading
3	Baseline	120	3	1000	Baseline
4	Association	300	2	1000	Association
5	Dissociation	900	3	1000	Dissociation

3. Insert the hydration plate into the biosensor tray. Align the biosensor rack over the hydration plate and lower the biosensors into the wells.

4. Transfer 200 µL of each assay reagent into the appropriate wells of a black polypropylene microplate. Place the biosensor hydration assembly in the Octet® instrument on the left stage. Ensure that both the biosensor tray and sample plate are securely in place.

5. Equilibrate the plates in the instrument for 10 minutes prior to starting the experiment. The delay timer can be used to automatically start the assay after 10 minutes (600 seconds).

6. Set up a kinetic assay. For details, see the Octet® BLI Discovery Software User Guide. Table 5 shows an example kinetic assay consisting of equilibration, ligand loading, baseline, association and dissociation steps. Figure 6 shows an example plate map for a typical kinetic assay.

7. Run the assay.

Process and Analyze the Data

1. Load data into the Octet® Analysis Studio Software.

2. Process the data by specifying methods for reference subtraction, y-axis alignment, inter-step correction and Savitzky-Golay filtering.

3. Analyze the data by specifying steps for analysis, fitting method (local or global) and window of interest.

4. To export the analyzed data, use the **Save Report** button to generate a Microsoft® Excel® report.

   Note: For details on processing and analysis parameters and data exporting, refer to the Octet® Analysis Studio Software User Guide.

Table 6: Affinity characterization between biotin anti-PSA and PSA shown in Figure 7.

n=12	K_D (M)	k_{on}(1/Ms)	k_{dis}(1/s)
Mean	6.08E-09	5.84E+04	3.55E-04
Error	6.10E-12	4.42E+01	2.34E-07

Representative Data

Figure 7 shows the kinetic analysis of the interaction between ligand biotin-anti-PSA (150 kDa) and an analyte PSA (30 kDa). High Precision Streptavidin Biosensors (SAX) were hydrated for 10 minutes in 1X Kinetics Buffer prior to analysis. Assays steps included: 1 minute of equilibration, 10 minutes of biotin-anti-PSA loading (50 µg/mL), 2 minutes of baseline stabilization, 5 minutes of ligand:analyte association and 15 minutes of ligand:analyte dissociation. Analyte concentrations were 0, 12.5, 25, 50, 100 and 200 nM. 1X Kinetics Buffer was used as the matrix throughout and the assay temperature was 30°C. Data was analyzed using the data using Global (Full) fitting, specifying a 1:1 binding model. Examples of raw and fitted data are shown in Figure 7A and 7B.

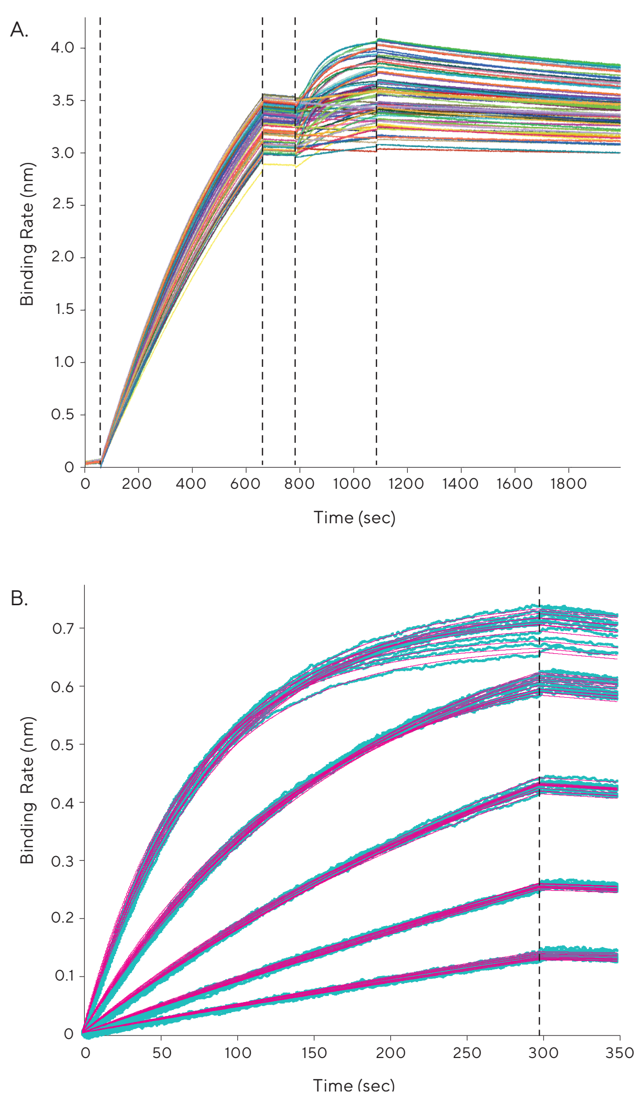

Figure 7: Kinetic analysis of the interaction between ligand biotin-anti-PSA (150 kDa) and an analyte PSA (30 kDa). 1X Kinetics Buffer was used as the matrix throughout and the assay temperature was 30°C. Data were processed and curve fit using a 1:1 binding model. The kinetic results are reported in Table 6 (n=12).

Germany
Sartorius Lab Instruments GmbH & Co. KG
Otto-Brenner-Strasse 20
37079 Goettingen
Phone +49 551 308 0

USA
Sartorius Corporation
565 Johnson Avenue
Bohemia, NY 11716
Phone +1 888 OCTET 75
Or +1 650 322 1360

For further information, visit
www.sartorius.com/octet-support

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