SARTORIUS

Small Molecule Binding Kinetics

Technical Note

Scope
The technical note describes the method set up process for a successful protein-small molecule binding interactions analysis on Octet® R2, R4, R8, RH16, and RH96 instruments. It provides tips on best practices for the preparation of optimal reagents for small molecules binding studies and on data analysis including data referencing procedures.

Abstract

The Sartorius Octet® R2, R4, R8, RH16, and RH96 instruments can be used in combination with the high capacity Super Streptavidin (SSA) Biosensor to perform Protein-small molecules binding studies and to derive relevant kinetics data and affinity constants. The process requires the immobilization of biotinylated protein onto the Octet® SSA Biosensors that are next dipped into the small molecules of interest in a dose dependent manner or at single concentrations when performing high-throughput screening assays. A set of reference biosensors are needed for reference subtraction. For best results, a biotinylated reference protein identical to protein of interest is suggested and should be immobilized onto the reference biosensors at the same density as the chemistry surface.

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Introduction

Small molecule kinetics can be measured on the Octet® R8, RH16, and RH96 instruments. In a typical experiment, a biotinylated protein target is immobilized onto a high-capacity Super Streptavidin (SSA) Biosensor surface, and this surface is exposed to a solution of the small molecule in a microplate well. The association of the small molecule to the target protein on the biosensor is measured over time. Next, the biosensor is moved to a well containing buffer to monitor the dissociation of the small molecule from the target protein. Rate constants are calculated from the binding data, including on-rate (k_{on} or k_a), off-rate (k_{off} or k_d), and equilibrium dissociation constant (K_D).

Ideally, successful analysis requires a robust, well-characterized system including a stable, active, and purified protein, and stable, non-aggregating small molecule compounds. Compounds that bind to the target protein and those with no binding are valuable as positive and negative controls. Successful analysis also requires a biotinylated target protein with ~1 molar equivalent of biotin per protein. Optimal results are obtained when the sample matrix is identical to the assay buffer used for baselines and dissociations.

Loading biotinylated target protein onto SSA Biosensors can be performed and monitored online (i.e., on the Octet® instrument) and small molecule analysis is performed in the same run. If large numbers of biosensors are required, up to 96 biosensors can be prepared in batch mode on a lab bench or in a refrigerator at 4°C. A protocol for batch immobilization of the target protein is described in Appendix C, and supporting information can be found in Sartorius Technical Note, Batch Immobilization of a Biotinylated Ligand.

The solubility of compounds to be tested is a crucial factor—compounds that form aggregates can produce misleading and anomalous results. Additionally, the sample matrix or buffer used to dilute the samples and the assay buffer used for recording baselines and dissociation data should be identical, to avoid introducing artifacts into the data.

This technical note provides guidelines for developing small molecule kinetic characterization methods using SSA Biosensors. It includes procedures for:

- Immobilizing biotinylated target protein onto SSA Biosensors
- Performing small molecule analysis experiments using the Octet® R8, RH16, and RH96 instruments.
- Analyzing and interpreting the small molecule binding data, including differentiating meaningful data from artifacts

Assay Workflow for New Compounds

Successful small molecule analysis involves four phases (shown in more detail in Figure 1):

1. Prepare and qualify biotinylated protein conjugate according to methods described herein. Load biotinylated protein target on SSA Biosensors to saturation.
2. Prepare SSA Biosensors with biotinylated target protein, and reference biosensors blocked with either biocytin, or, with a biotinylated reference protein.
3. Perform K_D scouting experiment. Determine the optimal compound analysis range by determining preliminary kinetic constants of compounds at 10, 1, and 0.1 µM in assay buffer.

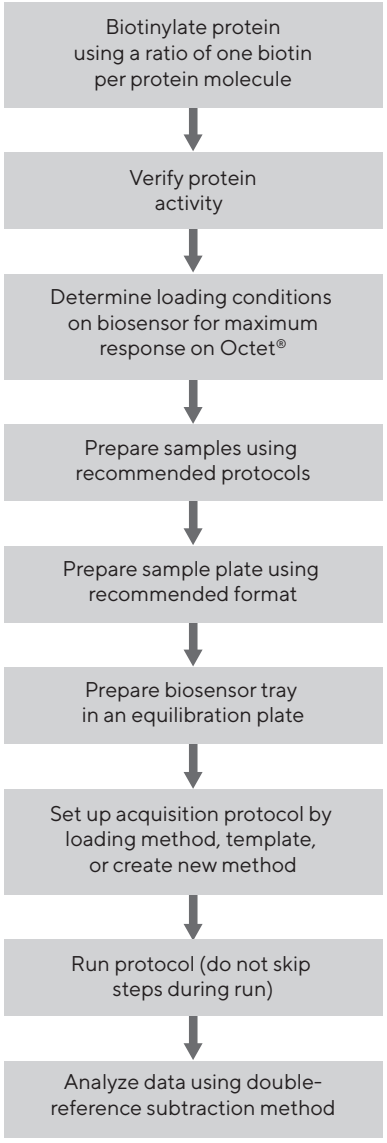

Figure 1: Assay workflow for small molecule analysis using the Octet® R8, RH16, and RH96 systems.

4. Obtain binding data for a 2–4X dilution series in the optimal range to determine the kinetic constants.

Method Templates

As an aid to users, the Octet® software provides method templates. These templates show recommended plate maps and assay protocols for the Octet® R8, RH16, and RH96 instruments. If using the Octet® R8 system, 96-well plates are required. If using the Octet® RH16 or RH916 system, 384-well plate methods are preferred over 96-well plate methods.

Octet® RH16 and RH96 Methods

1. **Kinetic Characterization - 8CH_96W.fmf**
   8-channel method for determining kinetic constants from 2X or 3X dilution series in a 96-well plate. Requires 8 reference biosensors (in column 1), and 8 biosensors labeled with biotinylated target protein (in column 3). Biosensors are prepped offline using the Octet® AS station, or in a separate run. Approximate run time is 2 hours.
2. **Kinetic Characterization_8CH_384W.fmf**
   8-channel method for determining kinetic constants from 2X or 3X dilution series in a 384-well plate. Requires 8 reference biosensors (in column 1), and 8 biosensors labeled with bioti- nylated target protein (in column 3). Biosensors are prepared offline using the Octet® AS station or in a separate run. Approximate time is 4.5 hours.
3. **Kinetic characterization 16CH_384.fmf**
   16-channel method for determining kinetic constants from 2X or 3X dilution series in a 384-well plate. Requires 16 reference biosensors (in columns 1 & 2), and 16 biosensors labeled with biotinylated target protein (in columns 3 & 4). Biosensors are prepared offline using the Octet® AS station, or in a separate run. Approximate run time is 2.5 hours.
4. **TW_Screening_16CH_2X384W.fmf**
   16-channel method for screening up to 288 compounds at a single concentration in a 384-well plate. Requires 16 reference biosensors (in columns 1 and 2) and 16 biosensors labeled with biotinylated target protein (in columns 3 and 4). Biosensors are prepared offline, or in a separate run. This method can be expanded to 336 compounds by adding samples to rows C and D. Approximate time is 4.25 hours.
5. **Sensor prep and screening_8CH_384W.fmf**
   8-channel method for preparing biosensors online and screening 49 compounds at a single concentration (or fewer compounds at multiple concentrations) in a 384-well plate. Requires 16 SSA Biosensors (in columns 1 and 3) of which 8 are reference biosensors (column 1) and the other 8 are loaded with biotinylated target protein (column 3). Approximate time is 4 hours.
6. **Sensor prep and screening_8CH_96W.fmf**
   8-channel method for preparing biosensors online and screening 24 compounds at a single concentration (or fewer compounds at multiple concentrations) in a 96-well plate. Requires 16 SSA Biosensors (in columns 1 and 2) of which 8 are reference biosensors (column 1) and the other 8 are loaded with biotinylated-target protein (column 2). Approximate run time is 2 hours.
7. **Sensor prep_8CH_384W.fmf**
   8-channel method for preparing biosensors online in a 384-well plate using 16 SSA Biosensors in columns 1 and 3. This method prepares 8 reference biosensors (column 1) and 8 biosensors labeled with biotinylated target protein (column 3). Approximate time is 2 hours.
8. **Sensor prep_16CH_384W.fmf**
   16-channel method for preparing biosensors online in a 384-well plate using 32 SSA Biosensors (in columns 1–4). This method prepares 16 reference biosensors (columns 1 and 2) and 16 biosensors labeled with biotinylated target protein (columns 3 and 4). Approximate time is 2 hours.

Octet® R8 Series Methods

1. **Kinetic characterization_8CH_96W.fmf**
   8-channel method for determining kinetic constants from 2X or 3X dilution series in a 96-well plate. Requires 8 reference biosensors (column 1) and 8 biosensors labeled with biotinylated target protein (column 2). Biosensors are prepared offline. Approximate time is 2 hours.
2. **Sensor prep + 10X screen.fmf**
   8-channel method for preparing biosensors online, and screening 24 compounds at a single concentration (or fewer compounds at multiple concentrations) in a 96-well plate. Requires 16 SSA Biosensors (in columns 1 and 2) of which 8 are reference biosensors (column 2) and the other 8 are loaded with biotinylated target protein (column 1). Approximate time is 3 hours.
3. **Assay development small molecule_8CH_96W**
   8-channel method for preparing biosensors online, and optimizing assay conditions such as small molecule concentrations (analyte) for a control molecule or analysis of several small molecule compounds at a single concentration. Requires 16 SSA Biosensors (in columns 1 and 2) of which 8 are reference biosensors (column 2) and the other 8 are loaded with biotinylated target protein (column 1).

Reference Correction

Minimizing the variability of background signals is a key parameter to the success of demanding applications such as small molecule analysis where signals are typically less than 0.3 nm. Controlling the reproducibility of background/reference signals is critical since in many cases the lower limit of detection (LLOD) of a positive signal is defined as a value that is higher than the background signal by 3 times its standard deviation. By reducing the variability in background signal, smaller positive signals can successfully be resolved to provide improved assay sensitivity.

For small molecule analysis on the Octet® platform, referencing is required in order to obtain precise and accurate data. Two types of references are generally recommended. First, to remove system artifacts and minor buffer inconsistencies, a set of 2–8 biocytin or reference protein-loaded reference biosensors is run using a protocol identical to that used for the biosensors labeled with the protein target. A second type of reference correction is applied to remove signal drift associated with the target protein-loaded biosensor. To perform this correction, one or two target protein-loaded biosensors are exposed to buffer in parallel with the those undergoing sample analysis. Any drift is corrected by baseline subtraction during data analysis. Signal drift on target protein-loaded biosensors can be further minimized by incubating the biosensors in buffer for 30 to 60 minutes prior to the start of the experiment.

Reference biosensors may be prepared from SSA Biosensors by blocking all biotin-binding sites with either biocytin or with a biotinylated reference protein. When loaded with biocytin, the optical properties of the reference biosensor surface are distinct from those of the target biosensor surface, presumably due to the difference in optical thickness in the presence of the protein layer on the target biosensor. For high sensitivity applications this difference in optical properties can introduce minor artifacts into the raw data, resulting in higher variability when assessing compounds or negative controls.

If a biotinylated protein is loaded on the reference biosensor, the optical properties of the reference biosensor can be made similar to those of the target biosensor. Using a protein reference, the standard deviation of the signals resulting from buffer controls for a model system was found to be ~1–3 pm lower than that produced by a biocytin reference biosensor. This result is significant, since 1–3 pm of standard deviation translates to 3–9 pm of variances in lower limit of detection (LLOD), which can be important when discriminating true signals from those close to background levels.

Choosing a Reference Protein

The optimal reference protein is one that is identical to the protein target, but that does not bind the molecules of interest. Some groups are fortunate and have protein mutants that are inactive, and these are ideal reference proteins for creating reference biosensors. For best results, the biotinylated reference protein should be loaded onto the SSA Biosensors at a density similar to the target protein. More often, inactive versions of proteins are not available, and a surrogate must be used. A biotinylated and blocked form of streptavidin (SAB4) is a useful surrogate. By adding a layer of blocked streptavidin to the SSA Biosensor used as a reference, the optical properties of the target and the reference biosensor can be made more similar. A protocol that describes the preparation of SAB4 and example data obtained using SAB4 loaded SSA Biosensors as reference biosensors are presented in Appendix D.

Protein Biotinylation

Small molecule analysis is performed on the Octet® R8, RH16, and RH96 systems using Super Streptavidin Biosensors. The steps involved in performing biotinylation on a target protein apply equally to reference protein as well. Sufficient signal-to-noise ratios for small molecule analysis can be obtained for proteins labeled either by *in vivo* biotinylation methods or with reagents such as Biotin-LCLC-NHS. When using the latter reagents, the molar coupling ratio is important, and is optimal when using ratios of one biotin to one protein at protein concentrations of ~1–30 µM. Other reagents (e.g., Biotin-PEO_4-NHS) can be substituted.

After biotinylation, unreacted biotin should be removed by dialysis or with size exclusion columns such as PD10 or spin columns. After purification, verifying protein activity in an independent assay will ensure that activity of the protein has not been altered significantly by the biotinylation and purification processes.

Protocols for biotinylation are outlined in Technical Note, Biotinylation of Protein for Immobilization onto Streptavidin Biosensors. Biotinylate using either biotin-LCLC-NHS (Pierce, part no. 21343) or biotin-PEO_4-NHS (Pierce, part no. 21329).

After biotinylation, Sartorius recommends loading the biotin-protein conjugate onto a minimum of two biosensors online, so that the loading process can be monitored. In a typical qualification run, the biotinylated protein samples are placed in the wells of a Greiner black microplate at

25–50 µg/mL, and the plate is placed on the Octet® instrument which has also been set up with SSA Biosensors. Incubation for 15–30 minutes at 1000 RPM typically results in biosensor saturation.

Benchtop (Offline) Loading of Biotinylated Protein onto Super Streptavidin Biosensors

Offline protein loading is best performed on a Octet® AS Offline Biosensor Immobilization Station. The Octet® AS station is an accessory to Sartorius' Octet® family of real-time label-free biomolecular interaction analysis instruments (Sartorius' part no Octet-AS). It enables simultaneous and uniform loading of reagents onto all 96 biosensors in a biosensor tray.

Target analyte and other reagents that do not require online signal monitoring can be loaded onto biosensors on the Octet® AS station. The Octet® AS station can be used independently or in collaboration with another Octet® system.

Proteins can be coated at temperatures from ambient + 4°C to 40°C. Protein loading is typically performed on the Octet® AS station using conditions found to be optimal for online protein loading. In the absence of an Octet® AS station, offline protein loading may be performed on a lab bench or in a refrigerator and offers several advantages over online loading: ability to use lower protein concentrations, lower volumes, and longer incubation times.

If the protein is stable, biosensors can be prepared in bulk, then preserved using a 15% sucrose solution, and stored. Since each biosensor picks up only nanograms of protein, the protein solution can be reclaimed, or used for loading additional biosensors.

Note: When placing biosensors in microplates, the tip should not come in contact with the sides of the plate. Transferring tips from tray to tray requires a steady hand and some practice. To assist in rapid tip transfer, Sartorius offers an 8-tip biosensor dispenser tool (Sartorius' part no. 18-5016).

Typically, the protein is loaded on the biosensors in a buffer that is optimized for the protein. PBS is commonly used.

If biosensors are to be prepared offline, the loading conditions should be first monitored online in the absence of flow (0 RPM) to mimic benchtop conditions. After a loading protocol has been developed that produces sufficient signal, biosensors can be prepared offline. Typically, doubling the loading time determined via online monitoring is sufficient to compensate for differences in loading rates due to differences in temperature when loading online versus loading on the benchtop. Adequate biotinylated protein loading is achieved using 25–50 µg/mL incubated for 3–5 hours at room temperature, or >10 µg/mL if incubated overnight at 4°C.

Protein-loaded biosensors sometimes show signal drift when incubated in buffer, which can impact data analysis. The magnitude of the drift is protein-dependent. In general, this drift can be corrected using a reference biosensor (see below). Drift can be minimized by equilibrating the biosensors on a benchtop or at 4°C. For example, biosensors incubated in buffer for 3 hours after protein loading and blocking with biocytin show ~8-fold lower drift than biosensors incubated in PBS for 5 minutes after protein loading and biocytin blocking. Since drift and stability are protein-dependent, it may be necessary to optimize the protocol for each protein.

For some proteins, drift is due to non-specific binding to the biosensors, which may be pH-dependent. In such cases, optimization of the protein loading protocol may be required. For instance, loading biotinylated carbonic anhydrase in 100 mM acetate (pH 4.75), MES (pH 6.00), HEPES (pH 7.40), and bicine (pH 8.00) buffers shows that drift depends on the loading buffer. For carbonic anhydrase, loading in bicine produces 3-fold lower drift than that observed for biosensors prepared in HEPES, and 8-fold lower drift than that observed for biosensors prepared in MES or acetate buffers.

Sample and Buffer Preparation

Sample preparation is important for successful small molecule analysis. Although some variation in composition can be tolerated between the sample buffer and the assay buffer used for baselines and dissociations, best results are obtained when the sample and assay buffers are matched. DMSO should be 99.9% pure, anhydrous. Sample dilution is best performed by diluting in one of two ways:

1. Dilute samples (i.e., 20 mM in DMSO) to the desired DMSO concentration in assay buffer (without DMSO), then perform subsequent dilutions in assay buffer with DMSO at the desired concentration (i.e., 0.5–5%).
2. Perform serial dilutions of compound in DMSO, then dilute all solutions with assay buffer to give the desired DMSO concentration.

Buffers that are compatible with the target protein are generally compatible with Super Streptavidin Biosensors. DTT and some detergents such as 0.005% Tween-20, Zwittergents Z-12 (10 mM) and Z-14 (3 mM), and DMSO from 0.5–5% (vol/vol) are compatible with this biosensor. CHAPS is incompatible. When preparing samples for analysis, best results are obtained when the sample diluent and assay buffer are identical. Triton X-100 and NP-40 may interfere with small molecule analysis because they contain small hydrophobic side chains that can bind to hydrophobic binding pockets on the target protein.

Variation in DMSO concentration (1–1.5% difference) can generally be corrected with referencing techniques (described below). Buffers with differing DMSO compositions can create signal artifacts and therefore should be used with caution. Samples should be prepared fresh, or the sample integrity should be controlled or known prior to analysis.

When adding detergents or other additives to the sample buffer, be sure to match the compositions of the sample and assay buffers to prevent artifacts in data acquisition.

Typical sample volume for the Greiner black polypropylene 96-well plates is 200 µL, and for the Greiner black polyproplyene 384-well plate, the minimum volume is 120 µL (at sensor depth of 6 mm). Further improvements are observed when using 130 µL with a sensor depth of 7 mm.

Small Molecule Data Acquisition Protocol Parameters

Number of Samples
In a typical experiment, two rows on the sample plate are used for referencing, and half of the plate contains assay buffer for obtaining baseline and dissociation data. This setup allows analysis of 36 individual samples per plate.

Alternatively, it is possible to increase the number of samples analyzed to 42 by using only one reference row (Kinetic Characterization_8CH_96W.fmf). Further increases in the number of samples can be achieved by using some of the buffer wells more than once. This is possible because carry-over of sample from one well to the next by the biosensor is estimated at only ~80 nL. In a total well volume of 200 µL, this is a 2500-fold dilution, which generally ensures the concentrations are well below those required for observable binding.

Acquisition Parameters
Fifteen minutes are required for instrument equilibration, which occurs after placing biosensors and samples on the instrument and closing the door. When biosensors are moved to the sample plate, biosensor equilibration of 10 minutes is recommended to equalize the temperature of the sample plate and that of the biosensor plate. For compounds with affinities on the order of ~1 µM, baseline, association, and dissociation times of ~30, 90, and 120 seconds are sufficient. For compounds with affinities in the range of 10–500 nM, dissociation times of ~6–8 minutes are generally long enough to allow the compound to dissociate fully prior to analysis of the next sample.

When writing protocols, the total time should be kept under 3–4 hours to prevent artifacts due to sample evaporation. Evaporation control in Octet® R8 systems can provide longer unattended run times up to 12 hours. Shaker speeds of 1000 RPM are sufficient for accurate kinetic measurements, and increasing the RPM beyond 1000 has minimal impact on kinetic constants. For small molecule screening applications, better results may be obtained at 500 RPM. A run temperature of 30°C is preferred, and the temperature can be as low as 4°C above ambient temperature. Total assay time per plate should be less than 4 hours for methods developed all Octet® systems described above except when using the Octet® R8 system with evaporation control where assay times can be up to 12 hours.

Biosensors

Each biosensor is used for multiple analyses, since most small molecules have affinities greater than 1 nM and thus dissociate fully after several minutes. To analyze six rows containing compounds, a total of 16 biosensors, including two reference biosensors, is preferred. Analyzing a single set of compounds in a single row requires a minimum of four biosensors. In both cases, column 1 contains SSA Biosensors blocked with biocytin or biotinylated reference protein, and column 2 contains SSA Biosensors labeled with biotinylated protein target. If additional protein targets are screened in the same run, additional columns of biosensors with the additional targets are added to the biosensor tray.

Referencing

Referencing is required in order to obtain precise and accurate data. Two types of references are generally recommended:

- To remove system artifacts and minor buffer inconsistencies, a set of 2–8 biocytin or reference protein-loaded reference biosensors should be run using a protocol identical to that used for the biosensors labeled with the protein target.
- To correct for signal drift associated with the target protein, one or two reference biosensors exposed to buffer are analyzed in parallel with the biosensors undergoing sample analysis. Any drift is corrected by baseline subtraction during data analysis. Drift can be further minimized by incubating biosensors in buffer for 30–60 minutes prior to analyzing the sample plate.

K_D Scouting: Protocol for Single-Run Protein Loading and Small Molecule Analysis

This method is used to obtain preliminary K_D values, and to identify the range required for obtaining an accurate K_D value from a subsequent run using a 3X dilution series. The materials and method are described in Appendix A.

Typical Results

Typical results for a loading protocol is shown in Figures 2 and 3. In this example, the response for loading the biotinylated protein is 7–8 nm, and subsequent associations and dissociations of a concentration series of furosemide are shown in Figure 3.

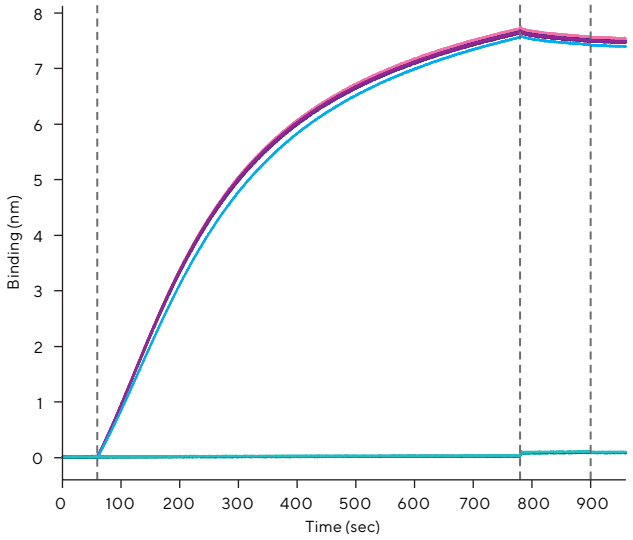

Figure 2: Raw data loading of carbonic anhydrase.

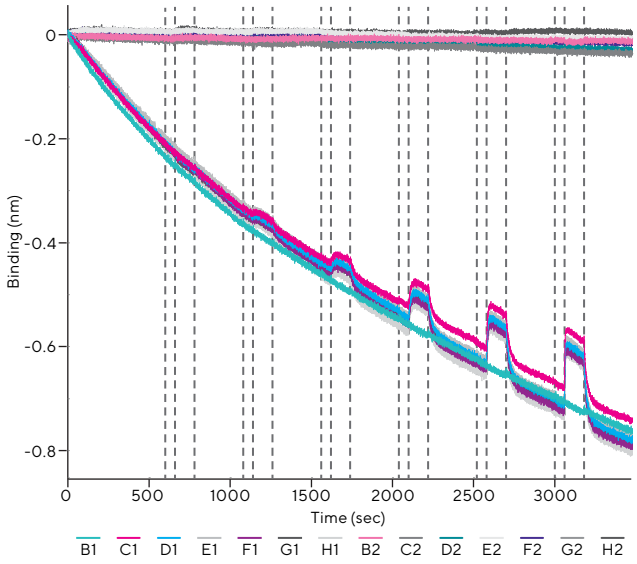

Figure 3: Raw data for biosensors prepared offline by loading biotinylated carbonic anhydrase onto Super Streptavidin biosensors.

Correlating response of protein loading to small molecule signals

When small molecule binding responses are obtained after monitoring the protein loading, the responses of the small molecule are proportional to the protein loading. For the carbonic anhydrase model, the signals associated with sulfonamide inhibitors are generally predictable when normalizing responses for molecular weight. These comparisons require the determination of R_{max}, the maximum signal obtainable for a given surface, at the point at which response is no longer concentration-dependent. This value is typically obtained from the plateau in plots of response vs. concentration. For the carbonic anhydrase model, this relationship with the sulfonamide derivatives is linear, with an R^2 value of 0.9.

Table 1: Precision of furosemide analysis with carbonic anhydrase.

Reproducibility of furosemide analysis on the Octet® R8 system								
Analysis	R_{max} (Δnm)	R_{max} Error	k_{off} (1/s)	k_{on} (1/Ms)	k_{on} Error	K_D (M)	Chi^2	R^2
1	0.0975	0.0001	7.83E-02	6.49E+04	4.57E+02	1.21E-06	0.053	0.99
2	0.1017	0.0001	7.88E-02	5.75E+04	3.84E+02	1.37E-06	0.050	0.99
3	0.0951	0.0002	8.52E-02	6.76E+04	5.66E+02	1.26E-06	0.067	0.99
4	0.0976	0.0002	7.97E-02	6.20E+04	4.69E+02	1.28E-06	0.059	0.99
5	0.0931	0.0001	8.32E-02	8.72E+04	6.97E+02	9.54E-07	0.063	0.99
Avg	0.097	0.0002	0.081	67836	515	1.22E-06	0.058	0.989
SD	0.003	0.0002	0.003	11453	121	1.57E-07	0.007	0.002

For example, carbonic anhydrase was loaded to a level of 7000 picometers (pm) of signal. The ratio of signal to molecular weight is 7000 pm/30,000 Daltons (0.23 pm/Da). The subsequent R_{max} value for furosemide binding to this surface is 75 picometers, which would be predicted based on the binding of carbonic anhydrase (0.23 pm/Dalton x 330 Daltons for furosemide = 76 pm calculated). Deviations from linearity may occur as a result of an inaccurate R_{max}, due to non-specific binding (NSB), and changes in reflectivity when changing compound structure classes.

Protocol for Small Molecule Analysis with Biosensors Prepared Offline

This method generally provides precise and accurate results that correlate to SPR methods, and is compatible with compounds with dissociation rate constants (k_d) >0.01/s. The materials and method are described in Appendix B.

This method gives better data quality than the method in which biosensors are prepared in the same run, because drift can be minimized by equilibrating the biosensors prior to the run. Also, protein consumption can be significantly reduced because longer loading times can be used with lower protein loading concentrations (~1–10 µg/mL), and biosensors can be loaded at 4°C. If the dissociation rate constant is lower than 0.01/s, another method is required (see slow dissociation kinetics templates).

Typical results

Figure 3 shows raw data for the binding of furosemide (330 Daltons) to carbonic anhydrase. The carbonic anhydrase biosensor data drift downward, but not all proteins show this behavior. In certain instances sensor drift can be reduced by longer biosensor equilibration times post immobilization. The binding of furosemide at 0.12, 0.37, 1.1, 3, 10, and 30 µM is visible. The biocytin-blocked reference biosensors are at the top of the plot, and show minor variability. The dashed lines separate assay steps including biosensor equilibration, followed by cycles of baseline, association, and dissociation. The precision for a typical run is shown in Table 1.

Protocol for Analysis of Compounds with Slow Off-Rates

Compounds that have off-rates <0.01/s generally do not dissociate to baseline within 5 minutes, and determining the kinetic constants from a dilution series requires using separate biosensors for each concentration. Biosensors should be prepared offline to minimize biosensor drift. The biosensor tray map is shown in Figure 7, but the sample plate map is different. In a typical experiment, buffer is placed in column 1, and a dilution series is placed in column 2 (i.e., 0, 1, 3, 10, 30, 100, 300, 1000 nM in wells A2, B2, C2, etc., respectively). A typical protocol would include delay (15 min), equilibration (column 1, 10 min), baseline (column 1, 1 minute), association (column 2, 3 minutes), and dissociation (column 1, 5 minutes). Data analysis methods are described below. For this protocol, a unique sensor is used for each concentration, and since each sensor has minor variances in binding capacity, R_{max} is unlinked during global fitting to obtain accurate kinetic constants.

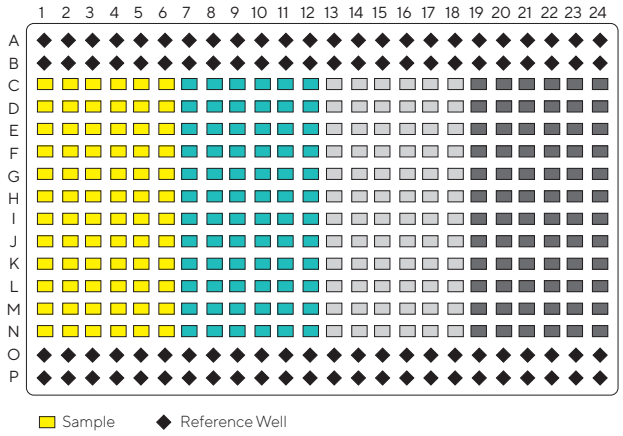

Figure 4: Sample plate map for a protocol that uses two 384-well plates. The reagent plate, which generally contains assay buffer only, is not shown. In a typical protocol, the biosensors move to well A1 in the reagent plate for baseline, to A1 in the sample plate to acquire association data, and back to A1 in the reagent plate for dissociation.

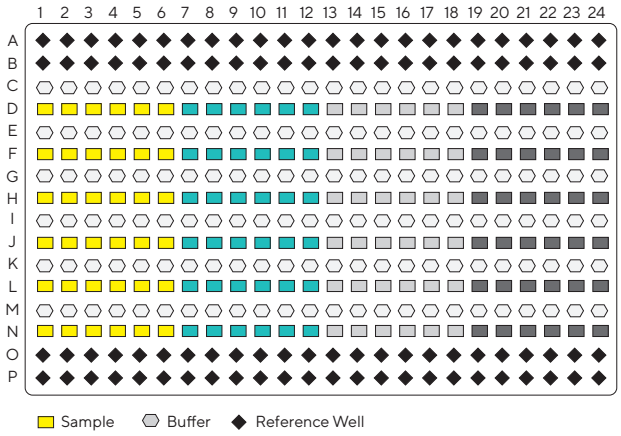

Figure 5: Sample plate map for a protocol that uses a single 384-well plate. In a typical protocol, the biosensors move to well A1 for baseline, to B1 to acquire association data, and back to A1 for dissociation.

Typical 384-Well Plate Maps

Octet® RH16 and RH96 systems are compatible with 384-well plates. Octet® 384 well tilted bottom plates (18-5080) are recommended for small molecule assays. For small molecule screening, a two-plate format is preferred, and for determining kinetic constants from a dilution series, a single plate format is preferred.

When using two 384-well plates, the sample plate map in Figure 4 is recommended. A second plate containing assay buffer (not shown) is used for baselines and dissociations. Figure 4 shows assay buffer in rows A, B, O and P, and samples in the rest of the wells. Buffer is placed in rows A and B because Octet® instruments read every other well due to biosensor spacing requirements. Thus, buffer in row A is used as a reference for samples in rows C, E, G, etc., and buffer in row B is used for samples in rows D, F, H, etc. Buffer for referencing must be placed in an entire row. In addition to rows A and B, a backup set of reference rows, O and P, is recommended. Reference rows can be moved to other locations such as C/D, E/F, etc., but not B/C, D/E, etc. With four rows of buffer, the plate capacity is 288 samples. With two rows, capacity is 336 samples. In a typical two-station A1/A1 protocol, the biosensor moves to well A1 in the reagent plate for baseline, to A1 in the sample plate to acquire association data, and back to A1 in the reagent plate for dissociation. Because of the biosensor placement requirements, every other sample well is analyzed (i.e., A, C, E, G, I, K, M, O or B, D, F, H, J, L, N, P) at any given time.

When determining kinetic constants, a single 384-well plate formatted as shown in Figure 5 is preferred. The rules for using reference rows are the same as those for the two-station protocol. Because baseline, association, and dissociation are obtained on the same plate, alternating rows of buffer and sample are recommended. A typical A1/B1 protocol for this plate map involves obtaining baseline data in A1, C1, E1, etc., followed by association data in B1, D1, F1, etc., and finally, dissociation data in A1, C1, E1, etc.

Data Analysis

Reference corrections

To obtain accurate and precise kinetic constants using the Octet® Analysis Studio Software, reference corrections and global fitting of a dilution series are required. Local fitting of individual biosensorgrams can be used to obtain K_D estimates. Partial fitting is not compatible with small molecule analysis.

Reference corrections are required to remove potential optical artifacts and drift from the raw data prior to data analysis. In a typical experiment, two data sets are generated, including one for the biosensors with the target protein, and one with reference biosensors blocked with the biotin derivative. Reference corrections are performed by subtracting the data set for the reference biosensors from the biosensors with protein target, to correct for systematic artifacts including optical variances due to plate reflections, and artifacts due to the presence of the analyte, including non-specific binding to the biosensor. Next, the row containing only buffer is subtracted to correct for drift, possibly due to gradual loss of protein over time. Thus, a reference-corrected data set is obtained by subtracting biosensor data sets as follows: (A1–A2) – (H1–H2), where A1 is a biosensor containing protein that binds compounds in row A, A2 is the biocytin or biotinylated reference

protein-blocked reference biosensor that is subsequently run in the same wells as biosensor A1 to measure system artifacts in row A, H1 is the biosensor containing protein target that is run in parallel to biosensor A1 in a control row of buffer, and H2 is the biotin-blocked reference biosensor that is run in parallel to biosensor A2, which measures system artifacts in row H of the sample plate. This process is known as double reference subtraction. After these corrections are performed, the data set is ready for analysis.

Data analysis method

Kinetic constants are obtained with the Octet® Analysis Studio Software using reference corrections, and global fitting. After reference corrections, data can be imported into Scrubber, if desired.

1. Load a data set into the Analysis software and open the Processing tab.
2. For the Subtraction Method, choose Parallel Double Reference and select the Align Y Axis checkbox.
3. In the Biosensor Plate Map, define biosensors as ligand or reference by selecting the appropriate biosensors, and using a right click to label. Ligand biosensors contain the target protein, and Reference biosensors generally contain biocytin or biotinylated reference protein.
4. In the Sample Plate Map, define sample plate wells as sample or reference wells.
5. In the lower pane of the Processing tab, select Ligand Biosensors for analysis. Doing so will cause a list of reference subtractions to appear in the Sensors to be Analyzed box at the left side of the tab. If this does not happen, check to see that the Reference Well box is checked.
6. Click the Process Data button to perform reference subtractions.
7. Go to the Analysis tab and determine kinetic constants using the appropriate method (see below). Wells can be grouped by color coding sample IDs, concentrations, etc.

Inter-step correction in the processing tab

Inter-step correction is an option in the data processing tab that can be used, with caution, to correct for artifacts in the first data point that are not removed by reference correction. This feature should not be used with fragments, since the kinetics can be too fast for adequate correction. For small molecule analysis, check the Align to Dissociation box (never use Align to Baseline for the inter-step correction). This feature aligns the last data point in the association phase with the first data point in the dissociation phase. This correction works well for compounds with dissociation constants less than ~1/s. If the dissociation constant is greater than ~1/s, there are

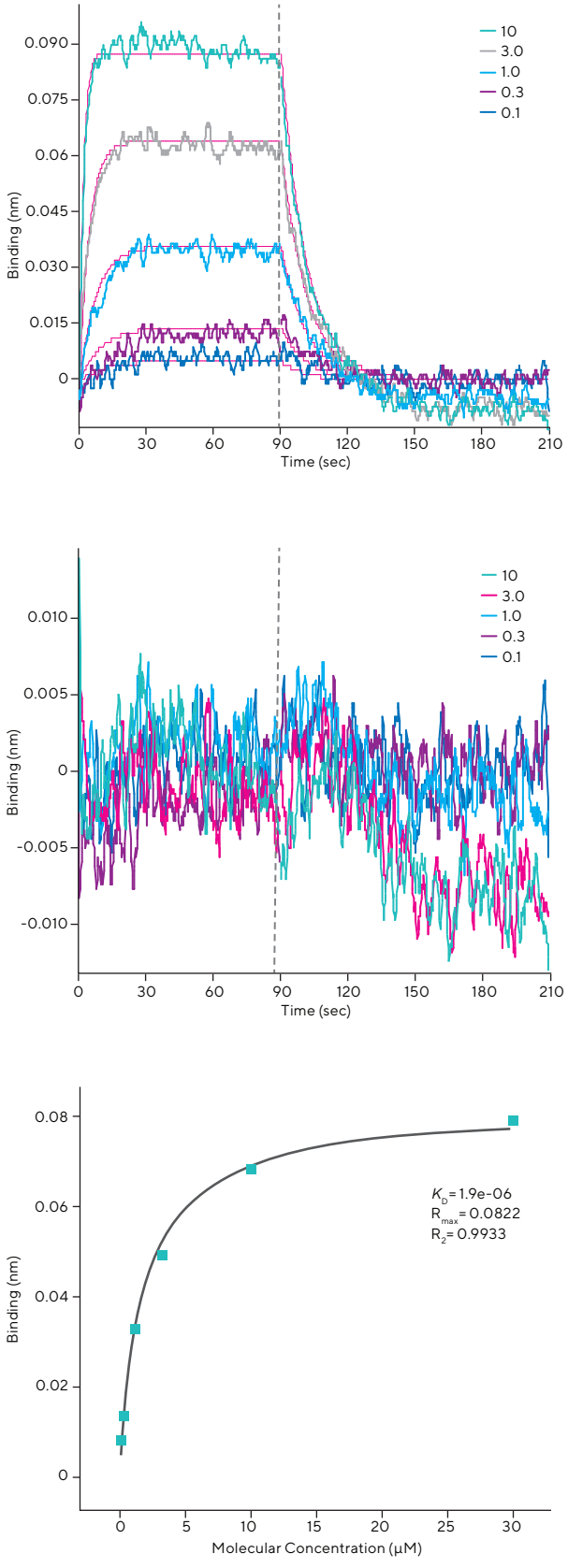

Figure 6: Global analysis of small molecule binding data at 0.12–30 µM (top plot), residuals for curve fitting (center), and the steady-state plot (bottom plot).

not enough data points to allow for an adequate correction. Use of the feature requires using only two wells for an experiment, where baseline and dissociation occur in the same well.

User-defined response points (report points) for compound screening

The Report Points feature of the Octet® Analysis Studio Software can be used for screening compounds. It allows the user to set defined time points to generate signal responses at a given time point (or points). This data can be exported and plotted in other programs.

Kinetic analysis of individual curves

If analysis of individual curves consisting of association and dissociation is desired, then choose Local Fitting to analyze the data.

Steady-state analysis

The K_D is derived from equilibrium responses. This method does not generate k_{on} and k_{off} values. The experiment requires a concentration-dependent response, and dilutions of 2–4X are recommended for a six-point concentration series (minimum). An example is shown in Figure 6 (right plot).

Global analysis

Global analysis derives a single set of parameters including R_{max}, k_{on}, k_{off}, and K_D from a set of association and dissociation curves from a concentration series (see reference for more information). This method generates more precise and accurate data than results obtained from association and dissociation data from a single concentration. An example of global fitting of a titration series is shown in Figure 6.

Carry-over

When moving from one well to the next, about 80 nL is attached to the tip of the biosensor. If 200 µL of liquid is used, this represents a 2500-fold dilution. For most analyses, this dilution results in final concentrations that are not detectable in the well. With respect to bound analyte liberated from the biosensor, this represents about 1 ng, which also represents a negligible amount of material.

Mass transport

Mass transport occurs at the biosensor surface, but generally does not have an impact on small molecule binding. Most compounds have association rates that are unaffected by mass transport, but in rare cases, the association rates are faster than the mass transport rate to the biosensor surface. When this occurs, K_D is unaffected, but k_{on} and k_{off} are generally significantly different from values obtained from reference methods that use a mass transport term. Kinetic analysis with mass transport can be performed in Scrubber.

Qualification of curve-fitting results

Visual inspection of the curve-fitting results in the processed data is the best way to evaluate the validity of curve fitting results. R^2 values can be misleading for data sets that contain non-ideal behavior or kinetics. Good results for 1:1 modeling of data contain <5–10 pm of deviation from processed data.

Troubleshooting

Non-specific binding

Non-specific binding (NSB) to a biosensor can be a significant issue in small molecule analysis. Primary screening assays such as HTRF or enzymatic assays may be affected by NSB, and most often, compounds are carried through to secondary screening as hits. Some of these compounds may show non-specific binding in label-free assays.

The magnitude of NSB signals can be similar to that expected for small molecule signals, or they may sometimes be greater. The raw data for every run should be examined to check for NSB in either the reference biosensor or the ligand-coated biosensor. If NSB is present, some strategies to eliminate it include lowering the concentration of analyte, adding detergents to the buffer, or increasing DMSO concentration. However, it is possible that the compound is a promiscuous binder (see references). Such compounds are generally problematic.

Detergents

If non-specific binding is observed, detergents such as Tween-20 (0.005%), Zwittergent Z-12 (10 mM) or Z-14 (3 mM) may reduce non-specific signals due to aggregation. However, some compounds are strong aggregators, and detergents may reduce signals due to non-specific binding, but will not eliminate them completely. Additionally, detergents will usually have an impact on the kinetic constants for the compound binding to the target because the detergent will reduce the effective concentration of the compound.

When choosing a detergent, the stability of the target protein must be confirmed using a positive control compound. The concentration of detergent is generally set below the critical micelle concentration (CMC), but higher concentrations may be required to reduce non-specific binding, or to match the molar concentration of the drug. For some compounds, detergents increase non-specific binding on both Octet® and SPR platforms.

Poor correlation to reference methods

IC_{50} values are related to K_i values for inhibition assays by the Cheng-Prusoff equation (see Biochem. Pharmacol. 22:3099–3108, 1973), where K_i is the K_D of the inhibitor of interest, IC_{50} is the IC_{50} value for the inhibitor of interest, D is the concentration of the competing ligand, and K_d is the K_d for the competing ligand. Deviations can occur if the concentration of the competing ligand is much greater than the K_d for this ligand. The relationship is as follows:

$$K_i = \frac{IC_{50}}{1 + \frac{D}{K_d}}$$

Correlation of data obtained on the Octet® system to SPR results is generally good, and K_D values agree within three-fold. If a ligand-small molecule system is affected by mass transport, then the association and dissociation rate constants may differ significantly from reference methods if a mass transport term is not included in the analysis algorithms used to determine the rate constants. Additional deviations may occur when the assay configuration is significantly different from the reference method, including the method of immobilization. Reagent stability (including the target protein and the compound stability), can affect results significantly, and for accurate comparisons, these issues must be addressed prior to analysis.

Compound solubility can also have a major impact on performance and hence the storage matrix is important. Aqueous solutions may precipitate or aggregate over time. To detect aggregation, which can be invisible to the naked eye, light scattering is commonly used to detect aggregation in aqueous solutions of organic compounds. If aggregation occurs, the effective concentration will be significantly lower than expected. Additionally, the aggregates may bind to the biosensor surface and generate an anomalous, imprecise response.

Negative signals

Negative signals are observed in some experiments. The most common source of negative signals is variability in raw data that is not corrected adequately by reference data. These signals are typically between 0 and –10 pm. Larger negative signals can occur if non-specific binding to the reference biosensor is greater than the non-specific binding to the target biosensor.

More rarely, negative signals arise in the raw data as a result of small molecule binding to the protein. This can sometimes result from changes in protein conformation that occurs upon ligand binding. Conformational changes upon binding of proteins are known for MDM2, and conformational changes are also known for firefly luciferase, and myokinase. Such effects depend directly on the biological system under study and are not a reflection on the Octet® system.

Biphasic responses

Biphasic responses may be observed for non-stoichiometric binding of the compound to the target protein, or for complex binding mechanisms. They are also observed when non-specific binding occurs. A 2:1 heterogeneous ligand (2:1 (hL)) binding model is available in Octet® Analysis Studio Software to fit curves that show one type of biphasic response. The 2:1 (hL) model fits the binding of one analyte in solution to two different binding sites on the surface. kinetic parameters are calculated for two interactions (k_{on1}, k_{on2}, k_{dis1}, k_{dis2}, K_{D1}, K_{D2}).

Drift

Drift due to immobilized protein leaching off the biosensor may occur, and is typically protein-dependent. As mentioned above, optimization of the protein loading conditions can have a significant impact on drift. However, drift is usually not an issue for small molecule data analysis since it can be readily removed from the final data using reference subtraction methods.

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Appendix A
K_D Scouting: Protocol for Single-Run Protein Loading and Small Molecule Analysis

Materials

- Super Streptavidin Biosensors (Sartorius, part no. 18-5065)
- Octet® R8 instrument
- Biotinylated target protein (with ~1 biotin per protein), 50–100 µg/mL
- DMSO, 99.9% pure, anhydrous
- Protein buffer
- Assay buffer (with DMSO)
- Compounds (~20 mM in DMSO)
- 96-well black flat bottom, polypropylene microplate (Greiner Bio-one, part no. 655209)

Assay description

Protein loading and subsequent small molecule binding can be performed in a single run as shown in the following example. Protein concentrations of 50–100 µg/mL are typically required to ensure protein loading to saturation. Typical biosensor and sample plate maps are shown in Figure 7. In this method, two sets of Super Streptavidin Biosensors are used, and the type, number, and duration of steps must be identical for the two acquisition protocols associated with these biosensors. For the reference biosensors, incubation in buffer is substituted for incubation in the biotinylated protein solution, with all other conditions being the same. In this example, up to 6 compounds are screened in a 10X dilution series after loading biotinylated target protein onto the biosensors. If the Octet® RH16 instrument is used, the biosensor depth required is 6 mm.

When writing methods in the data acquisition software, be sure to label the step type for baselines, associations, and dissociations accordingly, and do not eliminate any of these steps.

Method

1. Prepare reagents (protein solution, protein buffer, assay buffer, samples).
2. Prepare biosensor and sample plates. Be sure to match the composition of the assay buffer and sample buffer.
3. Equilibrate biosensors with protein buffer in a biosensor tray.
4. Place the biosensor tray and sample plate in the Octet® system.
5. Set up the assay protocol in the Octet® BLI Discovery Software, or load template sensor prep + 10X screen_8CH_96W.fmf
   a. Enter compound and biosensor information.
   b. If a template is not used, create the small molecule analysis protocol using Tables 2 and 3 (below). Use the Replicate feature to ensure that the protocols for the first and second set of biosensors are identical, with the following exception: for the loading step, direct the biosensors to protein buffer (column 3) instead of protein solution. Parameters are shown in Table 2, and the protocol steps are shown in Table 3.
   c. Verify the protocols for the target protein biosensors and control biosensors. Check to see that the steps are labeled appropriately. Protein loading, biocytin quenching, washing and equilibration should be labeled with Step Type Custom and baseline, association, and dissociation steps should be labeled baseline, association, and dissociation.
   d. Define data folders.
   e. Set delay to 300 seconds and start data acquisition.

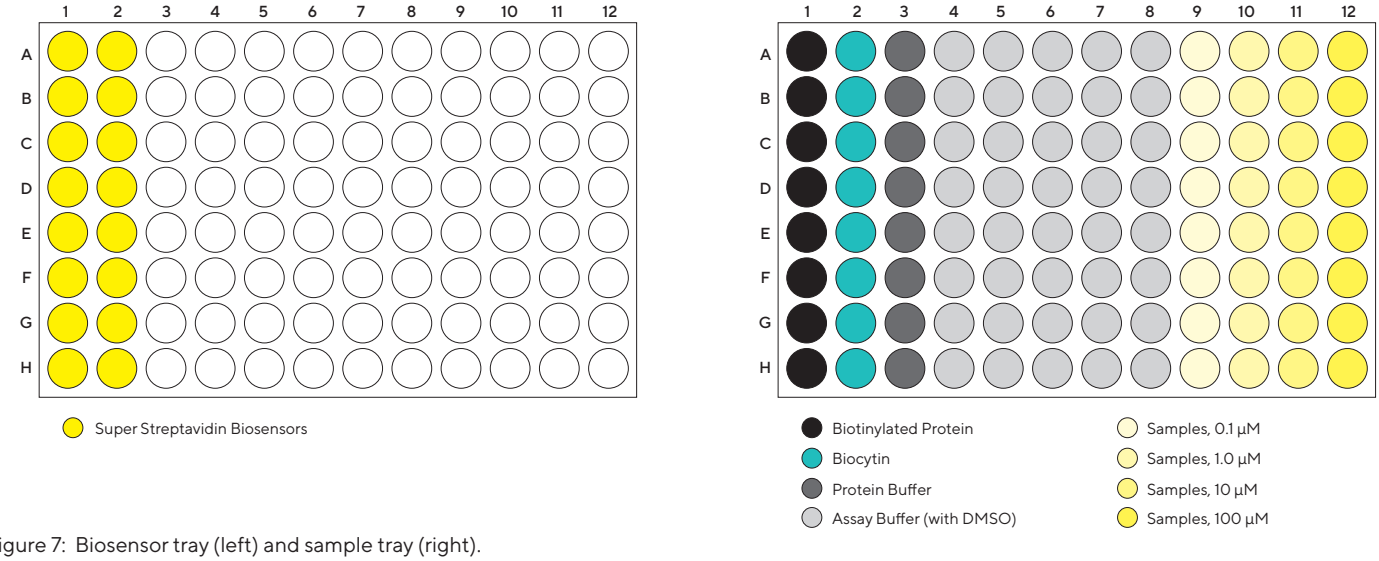

Figure 7: Biosensor tray (left) and sample tray (right).

Table 2: Assay definition.

Name	Time (s)	Flow rate (RPM)	Type
Loading	1200	1000	Custom
Biocytin Quenching	60	1000	Custom
Wash	1800	1000	Custom
Equilibration	300	1000	Custom
Baseline1	60	1000	Baseline
Association1	120	1000	Association
Dissociation1	300	1000	Dissociation

Data analysis for K_D scouting

1. Process data in Octet® Analysis Studio Software using the parallel double reference method.
2. Examine raw data in the sensor summary mode
   a. Is compound binding observed on the reference biosensor? If so, exclude result.
   b. Is compound binding to the protein biosensor?
3. Examine the overlay plots.
   a. Did compound dissociate back to baseline in the dissociation phase?
   - If not, longer times are needed for dissociation.
   - If a compound has a very slow off-rate, then run this compound using a different concentration in the same column (i.e., A2, B2, C2, D2, E2), instead of the same row.

   b. Is there a dose-response relationship?
4. Analyze data to determine kinetic constants.
   a. For preliminary K_Ds, local fitting of data from the preliminary run (0.1, 1, 10, 100 µM) can give an approximate K_D. If data set is good, global fitting may be used.
   b. For accuracy and precision, global fitting of a 3X dilution series is required. Do not use partial fitting.
   c. Are the fits OK? They should match the raw data within ~5–10 pm. Refer to Figure 6.

Table 3: Protocol definition for K_D scouting. Each assay represents a different set of biosensors, and the Sample refers to the sample column in which the biosensors are placed.

Assay	Sample	Step name	Step type	Biosensor type
1	1	Loading	Custom	SSA
1	2	Biocytin quenching	Custom	SSA
1	3	Wash	Custom	SSA
1	4	Equilibration	Custom	SSA
1	5	Baseline1	Baseline	SSA
1	9	Association1	Association	SSA
1	5	Dissociation1	Dissociation	SSA
1	6	Baseline1	Baseline	SSA
1	10	Association1	Association	SSA
1	6	Dissociation1	Dissociation	SSA
1	7	Baseline1	Baseline	SSA
1	11	Association1	Association	SSA
1	7	Dissociation1	Dissociation	SSA
1	8	Baseline1	Baseline	SSA
1	12	Association1	Association	SSA
1	8	Dissociation1	Dissociation	SSA
2	3	Loading	Custom	SSA
2	2	Biocytin Quenching	Custom	SSA
2	3	Wash	Custom	SSA
2	4	Equilibration	Custom	SSA
2	5	Baseline1	Baseline	SSA
2	9	Association1	Association	SSA
2	5	Dissociation1	Dissociation	SSA
2	6	Baseline1	Baseline	SSA
2	10	Association1	Association	SSA
2	6	Dissociation1	Dissociation	SSA
2	7	Baseline1	Baseline	SSA
2	11	Association1	Association	SSA
2	7	Dissociation1	Dissociation	SSA
2	8	Baseline1	Baseline	SSA
2	12	Association1	Association	SSA
2	8	Dissociation1	Dissociation	SSA

Appendix B
K_D Determination from a Six-Point Dilution Series: Protocol for Small Molecule Analysis with Biosensors Prepared Offline

K_D after scouting experiments. This protocol generally gives better data quality because drift is minimized by preparing the biosensors offline, on a benchtop. If the Octet® RH16 instrument is used, the biosensor depth required is 6 mm. When writing methods in Octet® BLI Discovery Software, be sure to label the step type for baselines, associations, and dissociations correctly, and do not eliminate any of these steps.

Materials

- Super Streptavidin Biosensors (Sartorius, part no. 18-5065)
- Biotinylated target protein (with ~1 biotin per protein)
- DMSO
- Carbonic anhydrase
- Compounds (~20 mM in DMSO)
- Assay buffer
- 96-well, black, flat bottom, polypropylene microplate (Greiner Bio-one, part no. 655209)

Method summary

1. Load biotinylated protein onto Super Streptavidin Biosensors offline, prior to run (see Appendix C).
2. Prepare reagents (assay buffer, samples). Be sure to match the composition of the sample buffer with the assay buffer.
3. Prepare biosensor and sample plates. A 3X dilution series bracketing the K_D is recommended. See Figure 8 for recommended plate maps.
4. Equilibrate biosensors with assay buffer in a biosensor tray for 30 minutes.
5. Place biosensor tray and sample plate in the Octet® system.
6. Set up the assay protocol in the Octet® BLI Discovery Software.
   a. Load template or method if desired. Enter compound and biosensor information.
   b. If a new method is desired, create the small molecule analysis protocol. See Tables 4 and 5 for parameters.
   c. Verify the protocols for the target protein biosensors and control biosensors. They should be identical.
   d. Define data folders.
   e. Start data acquisition.

Typical biosensor and sample plate maps are shown in Figure 8. In this example, two columns of biosensors are used. The first column contains reference Super Streptavidin Biosensors blocked with biocytin. The reference biosensors could also be loaded with a biotinylated reference protein instead. The second

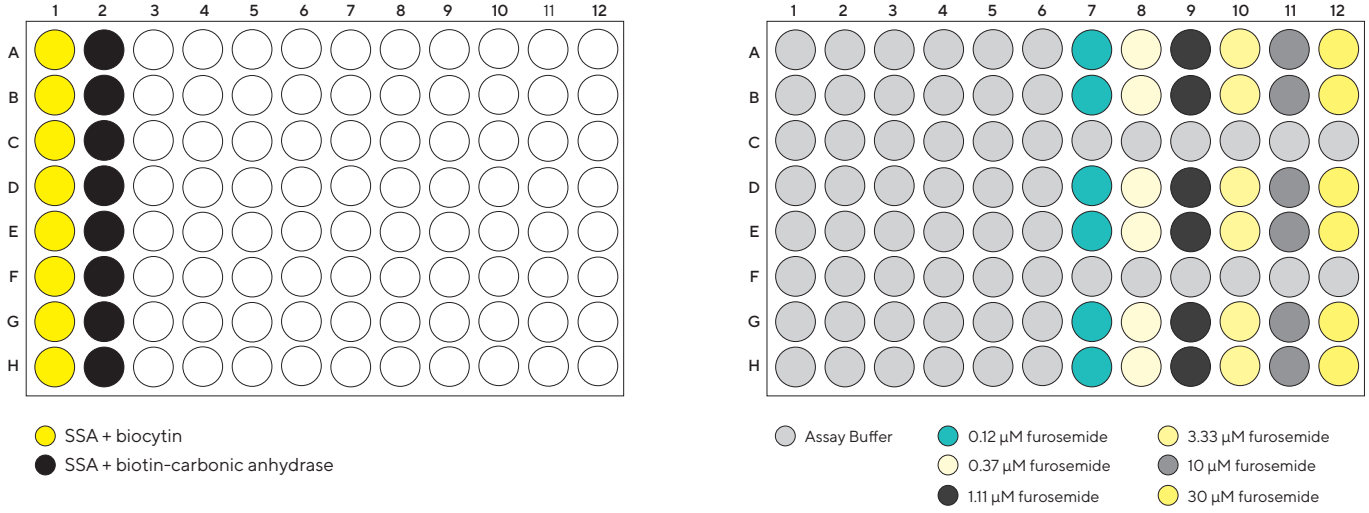

Figure 8: Biosensor tray (left) and sample tray (right).

column contains Super Streptavidin Biosensors pre-loaded with biotin-carbonic anhydrase. This example contains 0.12–30 µM furosemide from a 3-fold dilution series. It's possible to use only one row for reference corrections, but there can occasionally be artifacts in the reference channel that preclude its use during data analysis, so a second reference channel is recommended as a backup.

Reagents and samples

Samples are typically dissolved in DMSO (99.9%) to a concentration of 1–100 µM and subsequently diluted with assay buffer to achieve the DMSO concentration desired for analysis (up to 5%). For subsequent dilutions, use assay buffer containing the desired DMSO concentration. For example, diluting 10 mM samples in DMSO 1:20 with assay buffer results in 0.5 mM solutions containing 5% DMSO. Subsequent 3X serial dilution in assay buffer containing 5% DMSO results in a concentration series of 0.5, 0.167, .056, 0.019, 0.006, and 0.002 mM in 5% DMSO.

Larger dilutions are required for compounds with affinities in the nM range, and dilutions of 2–3 fold are typically recommended for determining kinetic parameters. If the compound affinity is unknown, screening 10-fold dilutions is recommended (i.e., 100 µM, 10 µM, 1 µM, 0.1 µM, etc., Appendix A), followed by a second analysis with 2 to 3-fold dilutions to obtain more accurate kinetic parameters.

When the samples are ready, place them in the appropriate locations in a 96-well ,black, flat-bottom Greiner polypropylene microplate.

Biosensor equilibration with assay buffer

Before use, biosensors must be equilibrated with assay buffer. Place assay buffer in a 96-well microplate, place the microplate in the biosensor tray holder, then carefully place the biosensor tray in the holder without touching the tips of the biosensors. If transferring biosensors from one tray to another, be careful not to touch the surface of the biosensor with fingers, trays, microplates or other objects. Sartorius recommends transferring biosensors with a Sartorius biosensor dispenser.

Table 4: Assay parameters for K_D determination from a dilution series.

Name	Time	Flow rate	Type
Equilibration	600	1000	Custom
Baseline	30	1000	Baseline
Association	90	1000	Association
Dissociation	120	1000	Dissociation

Table 5: Assay protocol for acquisition software. Each assay represents a different set of biosensors, and the Sample refers to the sample column in which the biosensors are placed.

Assay	Sample	Step name	Step type
1	1	Equilibration	Custom
1	1	Baseline	Baseline
1	7	Association	Association
1	1	Dissociation	Dissociation
1	2	Baseline	Baseline
1	8	Association	Association
1	2	Dissociation	Dissociation
1	3	Baseline	Baseline
1	9	Association	Association
1	3	Dissociation	Dissociation
1	4	Baseline	Baseline
1	10	Association	Association
1	4	Dissociation	Dissociation
1	5	Baseline	Baseline
1	11	Association	Association
1	5	Dissociation	Dissociation
1	6	Baseline	Baseline
1	12	Association	Association
1	6	Dissociation	Dissociation
2	1	Equilibration	Custom
2	1	Baseline	Baseline
2	7	Association	Association
2	1	Dissociation	Dissociation
2	2	Baseline	Baseline
2	8	Association	Association
2	2	Dissociation	Dissociation
2	3	Baseline	Baseline
2	9	Association	Association
2	3	Dissociation	Dissociation
2	4	Baseline	Baseline
2	10	Association	Association
2	4	Dissociation	Dissociation
2	5	Baseline	Baseline
2	11	Association	Association
2	5	Dissociation	Dissociation
2	6	Baseline	Baseline
2	12	Association	Association
2	6	Dissociation	Dissociation

Place biosensors and sample plate on the instrument
Place the biosensor and sample plates on the Octet® R8 instrument, close the door, and allow the plates to equilibrate for at least 10 minutes prior to a run.

Set up the assay protocol
While the microplates are equilibrating, set up the assay protocol. In the Octet® BLI Discovery Software, follow the tabs for Kinetics and enter the compound information (including ID) and biosensor information. Define the locations of wells containing biosensors, buffer, and samples (refer to Figure 6). If desired, load a previous method, or a template.

1. Define four new assay steps in the Step Data Setup table by clicking the Add button.
2. Change the step names to biosensor EQ, baseline, association, and dissociation.
3. Set the time for biosensor EQ to 600–900 seconds, and the times for the baseline, association, and dissociation steps to 30, 90, and 120 seconds respectively (Note: these values are for compounds with >0.5 µM K_D values).
4. Use the pull down menu to change the Step Type to match the descriptions above. For equilibration (EQ) steps, label as custom.
   Important: the Step Type labels must be entered exactly as given for the data analysis to work correctly.
5. Define an assay for the target biosensors (with protein) and the reference biosensors (with biocytin) as follows:
   a. Highlight the first column of biosensors in the biosensor plate.
   b. Click on a step in the Step Data Setup table.
   c. Click the Sample Plate column in which the event will occur.
   d. Repeat steps a–c for the remaining assay steps.
   e. When finished, click on Biosensor column 2, and define the same protocol as in steps a–d.
   f. Verify that the information in the Assay Steps List table is identical for the two sets of biosensors, except for the Biosensor column number. Proof-reading the methods is essential, because the data analysis will not work correctly if the step times are different, or if the step labels are not identical.
   g. Define data folders.
6. When ready, press the Go button to start acquisition.

Appendix C
Offline Protein Loading Protocol

Introduction
This protocol can be used to load up to 96 SSA Biosensors (Sartorius, part no. 18-5065) with biotinylated target or reference, on the benchtop in a single batch. It can be modified as required for each reference or target protein. All steps can be performed at room temperature. Protein can also be loaded overnight at 4°C, and at lower concentrations (~10 µg/mL) if longer incubation times are used. This protocol can be modified to prepare full or partial trays of coated biosensors in batch by simply putting each reagent needed in a new 96-well plate and switching out plates for each step using the biosensor tray as a coating station. If the protein is stable, the biosensors can be soaked in 15% sucrose in buffer (PBS) for 3–5 minutes, then dried to preserve them for future use. Allow the biosensors to dry at room temperature for 10 minutes, or in an oven at 37°C for 1 minute.

Offline protein loading protocol
1. Prepare a plate with the following:
   a. Column 1: PBS (or protein buffer)
   b. Column 2: biotinylated target or reference protein (50 µg/mL)
   c. Column 3: biocytin (10 µg/mL)
   d. Column 4: PBS (or protein buffer)
   e. Column 5: assay buffer
2. Place the plate in a blue tray, place 8 biosensors in column 1 of the biosensor tray, and place the biosensor tray in the blue tray. Load protein onto the biosensors by moving the biosensors from column to column as follows:
   a. Column 1 (protein buffer or PBS): 1 minute
   b. Column 2 (biotin-protein): 30 minutes
   c. Column 3 (biocytin): 1 minute
   d. Column 4 (protein buffer or PBS): 30 minutes
   e. Column 5 (assay buffer): 5 minutes
3. Prepare 8 reference biocytin coated biosensors in the same way, but skip the wells containing biotinylated protein.
   a. Column 1 (protein buffer or PBS): 1 minute
   b. Skip column 2, which contains the biotinylated protein
   c. Column 3 (biocytin): 1 minute
   d. Column 4 (protein buffer or PBS): 5 minutes
   e. Column 5 (assay buffer): 5 minutes

Appendix D
Preparing Biotinylated, Blocked Streptavidin (SAB4)

Materials

Preparing the conjugate
- Streptavidin (Scripps Laboratories, part no. S1214)
- Biotin (Pierce Protein Research Products, part no. 29129)
- Biotin-LCLC-sulfo-NHS (Pierce Protein Research Products, part no. 21338)
- PBS (Invitrogen, Gibco, or equivalent)
- 3–12 mL Dialysis cassette, 10k MWCO (Pierce Protein Research Products, part no. 66811)

Testing the conjugate
- Super Streptavidin Biosensors (Sartorius, part no. 18-5065)
- Octet® R8 or RH16 with Software 6.1 or later
- Black, flat bottom microplates (96-well: Greiner Bio-one, part no. 655209; 384-well: Greiner Bio-one, part no. 781209; 384-well tilted bottom plate Sartorius, part no. 18-5080)

Technical resources
- Technical Note, Biotinylation of Protein for Immobilization onto Streptavidin Biosensors

Conjugate preparation procedure
1. Prepare a solution of biotin (244 Da) by adding 2.5 mg to 25 mL PBS to give a 0.1 mg/mL (400 mM) solution.
2. Dissolve 6 mg of streptavidin (55 k_D) in 6 mL of PBS buffer containing 100 µg/mL biotin to give a 1 mg/mL solution of streptavidin (18 mM).
3. Prepare a solution of biotin-LCLC-sulfo-NHS by adding 5 mg to 5 mL of PBS buffer to give a 1 mg/mL solution (1.5 mM).
4. Add 73.2 µL of 1.5 mM biotin-LCLC-sulfo-NHS (670 Da) to 6 mL of the streptavidin solution containing 100 µg/mL biotin. The final biotin-LCLC-sulfo-NHS concentration is 18 mM. Incubate for 1 hour at room temperature.
5. Add 1 L of PBS to a 1 L beaker, and add a stir bar.
6. Transfer the streptavidin solution to the dialysis cassette according to the manufacturer's instructions. Dialyze against PBS buffer (3 X 1L, 18 hours) in a refrigerator.
7. Transfer to a storage vessel, store at 4°C.

Note: the stability of this conjugate at 4°C or at -20°C has not been assessed, and may require freezing if use extends beyond one week.

Conjugate testing procedure
Using the methods described earlier in this technical note, test the loading of the biotin-reference protein on SSA Biosensors on an Octet® R8 or RH16 instrument. For best results the signal (nm shift) from loading the biotinylated-reference protein should be equivalent to the signal from loading the biotinylated target protein. If loading of the reference protein is higher or lower than that of target protein, then the conditions (concentration, time of loading, etc.) for immobilizing the reference protein should be further optimized.

Sample data
The buffer control data shown in Figures 1 and 2 was generated during a study screening a small molecule library for binders to immobilized carbonic anhydrase. In this screening study, it was important to minimize the variability of the background signals in order to positively identify any small molecules that bound to the target protein, so biotinylated, blocked streptavidin (SAB4) was used as a reference protein.

To determine the baseline variability, and thus the LLOD, in this small molecule screening assay, 16 target biosensors and 16 reference biosensors were used to assay a 384-well plate filled with buffer only using the Octet® RH16 system. The resulting data was processed using a standard double reference subtraction technique that is available in the Octet® Software. This data processing involves using the reference sensors and a set of reference wells to correct for systematic baseline offsets. Ideally, after this data processing the baseline should be stable with no discontinuities. In reality, small artifacts show up as a result of the optical changes from well to well.

For this example, either the biocytin blocked SSA Biosensors or the SAB4 Biosensors (preparation described above) were used as reference for the carbonic anhydrase coated target sensor. The typical loading signal for the biotinylated carbonic anhydrase was 6–10 nm and for the SAB4 conjugate was 4–7 nm. Figure 9 shows the double reference subtracted data from the buffer-only plate when using either of these reference biosensors. When comparing the data resulting using the biocytin blocked sensors and the SAB4 sensors, both the average baseline signal and the standard deviation of the baselines are significantly lower when using the SAB4 sensors as a reference. This improvement is even more apparent when looking at the calculated LLOD of signal for each assay based on these buffer controls (Table 6).

The experiment was repeated using the Sartorius 384-well tilted bottom plate (384TW) and is shown in Figure 10. Using the 384TW plate in conjunction with the SAB4 reference sensors, the LLOD is decreased from 24 to 11 pm (Table 6). This allows for positive signals in the 14-20 pm range to be identified as hits. These signals would have been lost in the baseline noise using the original biocytin blocked reference sensors and normal 384-well plate.

Table 6: Summary of the data resulting from double reference subtraction processing of 32 target biosensor and 16 reference biosensors assaying a 384-well plate of buffer only using the Octet® RH16 system (N=648 for each condition). LLOD was calculated as the [average + 3* standard deviation] of the signals shown in Figures 9 and 10.

Plate	Reference biosensor	Average (pm)	Deviation (pm)	LLOD (pm)
384-well	SSA:Biocytin blocked	7	6	24
	SSA:SAB4	4	5	19
384-well tilted bottom	SSA:Biocytin blocked	3	4	14
	SSA:SAB4	1	3	11

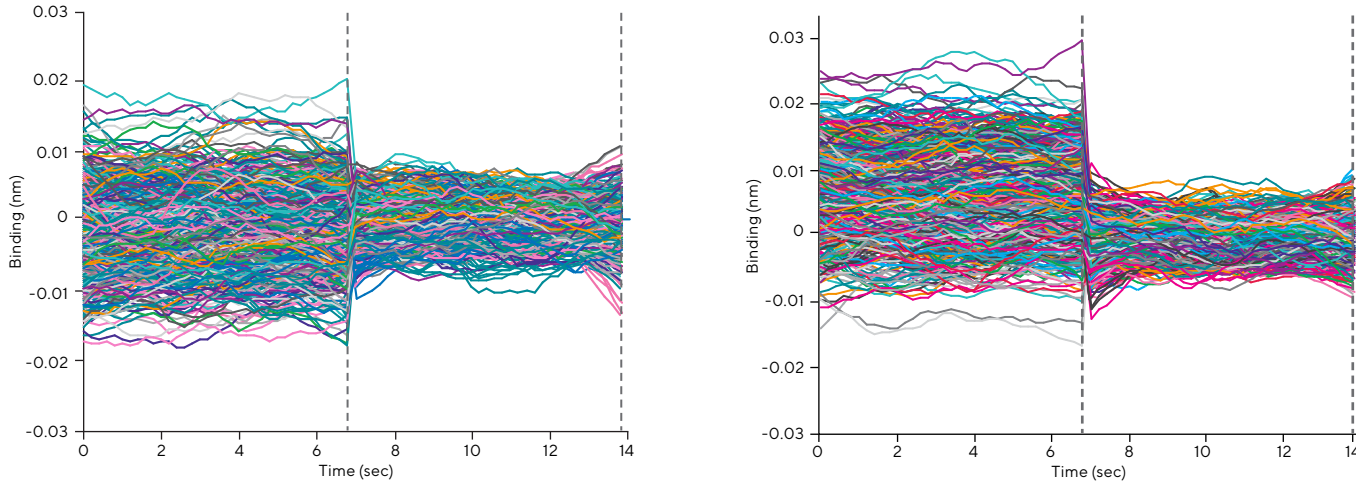

Figure 9: Comparison of the signal responses for the carbonic anhydrase biosensors in PBS buffer (negative controls) with blocked, biotinylated, streptavidin-coated reference biosensors (SAB4, left panel) and biocytin-blocked reference biosensors (BCT, right panel). Assay run using a standard 384-well plate on the Octet® RH16 system. Data processed using double reference subtraction in Data Analysis software 6.3.

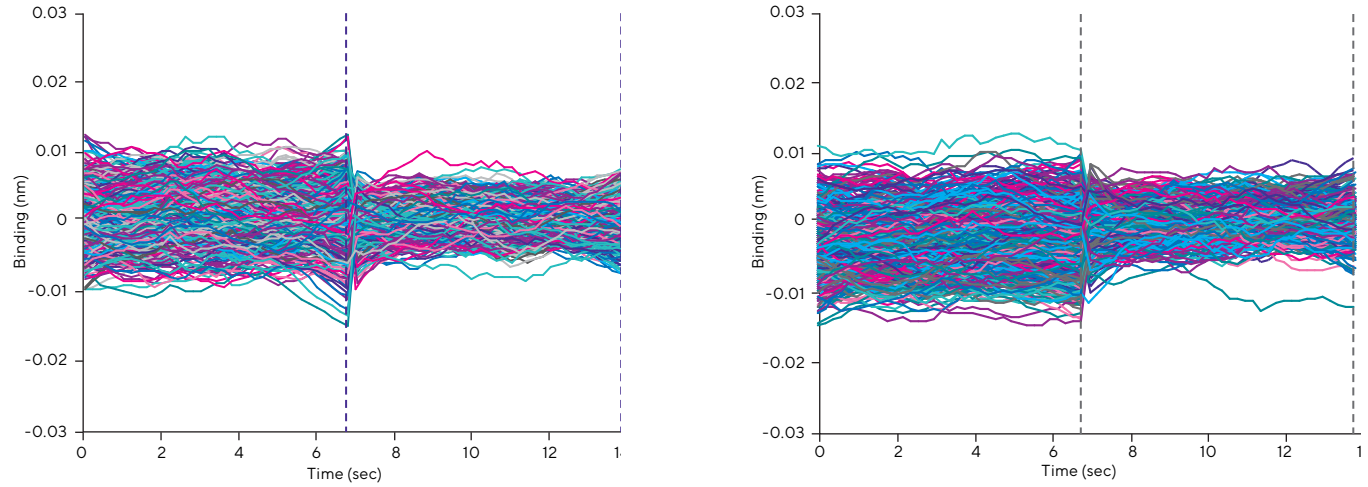

Figure 10: Data from a repeat of the experiment from Figure 1 using the Sartorius 384-well tilted-bottom plate. Use of the 384TW plate further reduces the artifacts in the PBS-only negative controls.

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