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Application Note

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Fragment-Based Drug Discovery (FBDD) Using Octet® SPR OneStep® and NeXtStep™ Injections

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Abstract

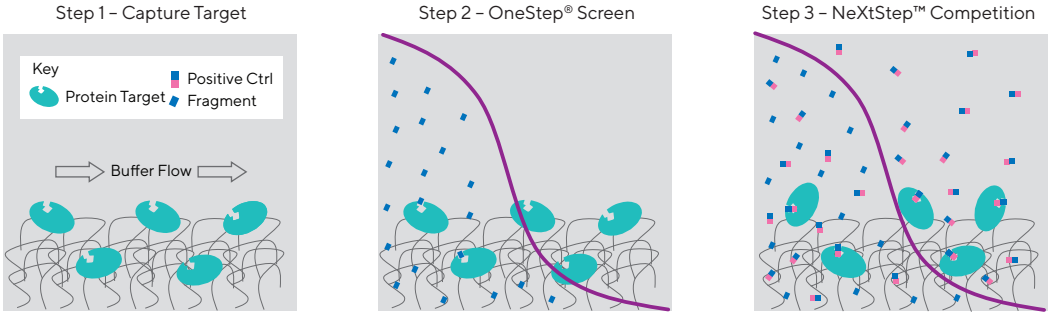
Fragment-based drug design (FBDD) has become an increasingly popular platform for the identification of lead candidates in drug discovery programs. The detection and characterization of fragment binding events is facilitated by sensitive biophysical technologies capable of detecting low affinity interactions of low molecular weight compounds. This application note demonstrates how the Octet® SF3 system with OneStep® Injections has the extraordinary advantage of directly influencing the rate at which fragment screening is accomplished compared to other SPR-based biosensor technologies.

The implementation of high-throughput small molecule and fragment-based drug design screens is a critical step in the identification of compounds with suitable kinetics and affinities from large initial libraries. Determination of which compounds to progress from initial FBDD screens can be hampered by low molecular weights, weak affinities and solubility limitations, which make it impractical to test for binding using sample concentrations above the K_D of the interaction. Over the last decade, approaches such as nuclear magnetic resonance (NMR), X-ray crystallography, differential scanning fluorimetry (DSF), and surface plasmon resonance (SPR) have become core technologies in many pharma and biotech settings for the identification of these low-affinity fragment compounds. In particular, SPR-based biosensors have sufficient sensitivity and throughput to provide complete fragment screens on libraries of several thousand compounds in just a few weeks per target.

Sartorius has recently launched the Octet® SF3 SPR system which exclusively offers OneStep® and NeXtStep™ gradient injection technologies. OneStep® Injections use a continuous analyte titration method that provides reliable affinity measurements in a single injection, and is also suitable for rapid screening in both direct and competitive binding formats. It offers improvements in screening time and provides higher content information that allows for confident, rapid characterization of hits. NeXtStep™ Injections for competition analysis enable rapid determination of whether a fragment binding is competitive, non-competitive, or un-competitive. OneStep® and NeXtStep™ Injection methodologies advance binding characterization in multiple application areas throughout the drug discovery process. When used together in tandem, OneStep® and NeXtStep™ provide a highly streamlined, accurate, and cost-effective method to screen and characterize fragment libraries (Figure 1).

Figure 1

A Workflow for Fragment Screening on the Octet® SF3 System.

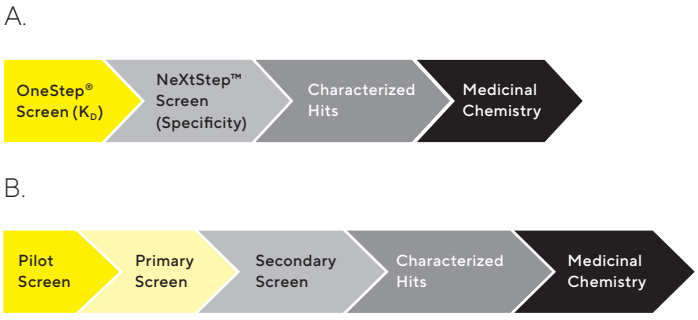


Assay Steps	a. Capture protein target to sufficient surface density b. Verify target activity with a positive control	a. Screen fragment library using OneStep® Injections b. Periodically verify target activity during the run	a. Prepare fragment hits mixed solution with control b. Compete fragment hits with control using NeXtStep™ Injections c. Confirm competitive fragments
Typical Step Time	▪ 30 min–1 hr	▪ 768 fragments in 24 hrs	▪ 96 fragment hits in 9 hrs
Advantages	▪ **Low protein sample requirement:** Most immobilization methods use <20 µg of material. ▪ **Label-free:** No tags required for detection. ▪ **Flexible:** The Octet® SF3 system can accommodate screening in a diverse range of buffers and temperatures to enhance protein stability.	▪ **Fast:** Assay setup, run-time, and analysis are rapid steps. ▪ **Information-rich :** OneStep® screens simultaneously identify and characterize (k_a, k_d, and K_D) fragment hits. ▪ **Intuitive:** User-friendly software enables confident analysis and identification of ideal/non-ideal fragments.	▪ **Fast:** Assay setup, run-time and analysis are rapid steps. ▪ **Information-rich:** NeXtStep™ competition easily shows if fragments are competitive, non-competitive, or un-competitive. ▪ **New data:** NeXtStep™ Injections reveal a fragment's actual competition K_D, giving more than a yes/no answer.

Fragment Screening with OneStep® and NeXtStep™ Injection Techniques

Fragment screening is typically hampered by a host of technical challenges including large numbers of samples, low molecular weight analytes (< 300 Da), and weak affinity interactions (K_D: 10 µM to 10 mM). Low molecular weight, weak affinity, and solubility limitations make it impractical to test for binding using sample concentrations above the K_D and frequently results in making decisions based on inappropriate (small, square-shaped) sensorgrams. Increasing sample throughput and data content through the generation of gradients, while decreasing sample preparation time, OneStep® Injections can dramatically improve SPR-based fragment screening. The technique is designed to streamline binding analysis by testing a full concentration series in a single injection. This not only saves sample preparation time and materials, but also reduces human error by eliminating the preparation of multiple sample dilutions. OneStep® Injections also represent a significant improvement in assay performance by streamlining the efficiency of the workflow process. Secondary screening can be completely avoided as fragment candidate selection is optimized during primary screening (Figure 2).

Figure 2
Workflow using OneStep® and NeXtStep™ Injections vs. Conventional Fragment Screening.



A) Optimized workflow using OneStep® and NeXtStep™ injections. Initial compound screening is followed by a specificity test analysis that leads to full characterization of the identified hits. The selected compounds can then be used in various applications in medicinal chemistry. B) Conventional fragment screening workflow. An initial screening process is followed by a secondary screening process where samples have to be prepared at different concentrations and analyzed separately to allow for affinity characterization and fragment hit confirmation.

Fragment screening with OneStep®:

- Allows users to make decisions early on by obtaining reliable kinetics (k_a, k_d) and affinity (K_D) data directly from the primary screen.
- Fully automated and requires minimal assay development to arrive at the correct fragment candidate(s), providing faster time to results.
- Provides reproducible identification of fragment actives.
- Provides high throughput fragment actives analysis: up to 768 samples in 24 hours.

OneStep® Injections eliminate the need for multiple sample preparations by generating a continuous concentration gradient using the sample and the running buffer. The data generated in a weak-affinity binding typically associated with fragments resembles a dose response plot and can be fitted with a real-time equilibrium binding model. The fragment off-rates (k_d) are often fast enough to allow for a steady-state approximation. The Octet® SPR Analysis Software provides models incorporating kinetics, mass-transport corrections, and multi-site binding parameters that can adequately describe different interactions. Therefore, primary screening data are ready for K_D analysis without the need for laborious secondary screening and extra sample preparation steps.

Comparison of OneStep® and Conventional SPR in FBDD

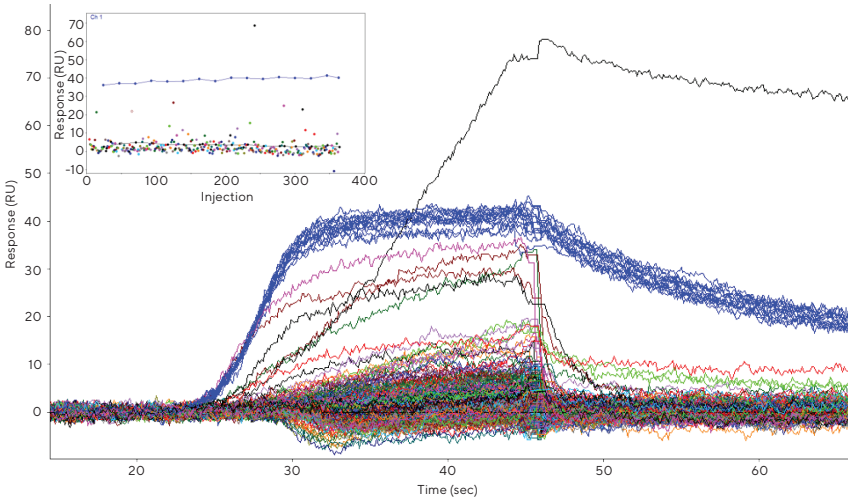
A comparison study in fragment screening between conventional SPR using OneStep® Injections was performed. Biotin-tagged multidomain non-kinase phosphoribosyl transferase (biotinylated during expression using the Avitag system) was captured in high capacity on a sensor chip coupled with NeutrAvidin (Pierce). 384 fragments were prepared in assay buffer containing 5% DMSO to a fragment concentration of 100 µM. Buffer and positive control injections were performed every 16 assay cycles. On the Octet® SF3 system all samples were injected using OneStep® Injections at a flow rate of 200 µL/min and a dissociation time of 10 seconds. A competitor SPR system was used to screen the same compounds against the target using a single, conventional, fixed single concentration injection (FCI) as commonly used in multi-cycle kinetics. Data was processed and fit with a real-time equilibrium binding model using Octet® SPR Analysis Software.

Results

The data generated between the two platforms revealed identical compound hits. Hits were identified using a box-whisker analysis of the LOESS normalized data as previously described[1]. In addition, the assay time for the primary screens is comparable. However, while the data from the competitor SPR system can be used only for the identification of the fragment hits, the data from the OneStep® Injections are ready for K_D analysis. Moreover, the scatter data around the baseline (high scatter renders it difficult to accurately determine hits) shown in the Figure 3 and 4 inset is lower in the OneStep® system data (Figure 3 inset) than in the single, fixed concentration injection data presumably due to less exposure of the sensor chip surface to high compound concentrations. The number of compounds whose binding signals exceed the control response is higher in the single, fixed concentration injection data (Figure 4 inset), again presumably due to lower exposure time of the high concentration samples to the sensor chip surface in the Octet® SF3 system.

Figure 3

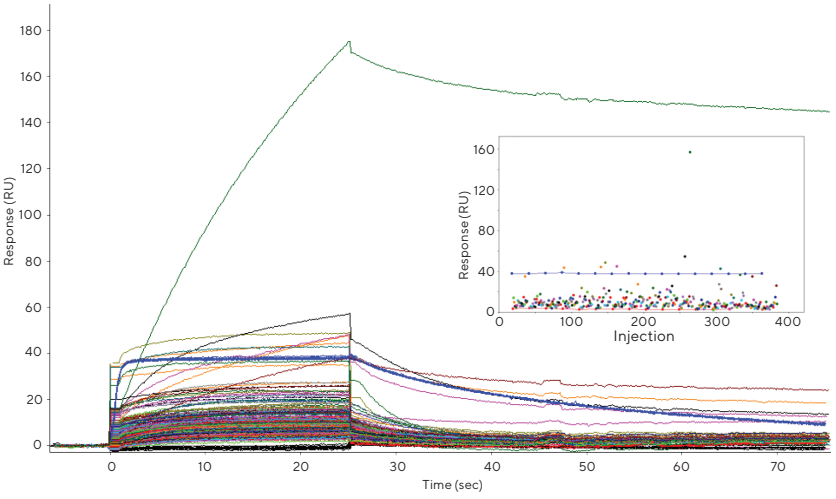
OneStep® Assay Primary Screening Results Using Octet® SF3 System.



Note. The positive control compound was run in 16 replicates and is shown in blue. The inset shows the equilibrium response of each fragment versus assay cycle number with the control compound's 16 replicates shown in blue.

Figure 4

Fixed Concentration Injection (FCI) Primary Screening Results Using a Competitor SPR System.



Note. Positive control compound is shown in blue with 16 replicates. The inset shows the equilibrium response of each fragment versus assay cycle number with the control compound's 16 replicates shown in blue.

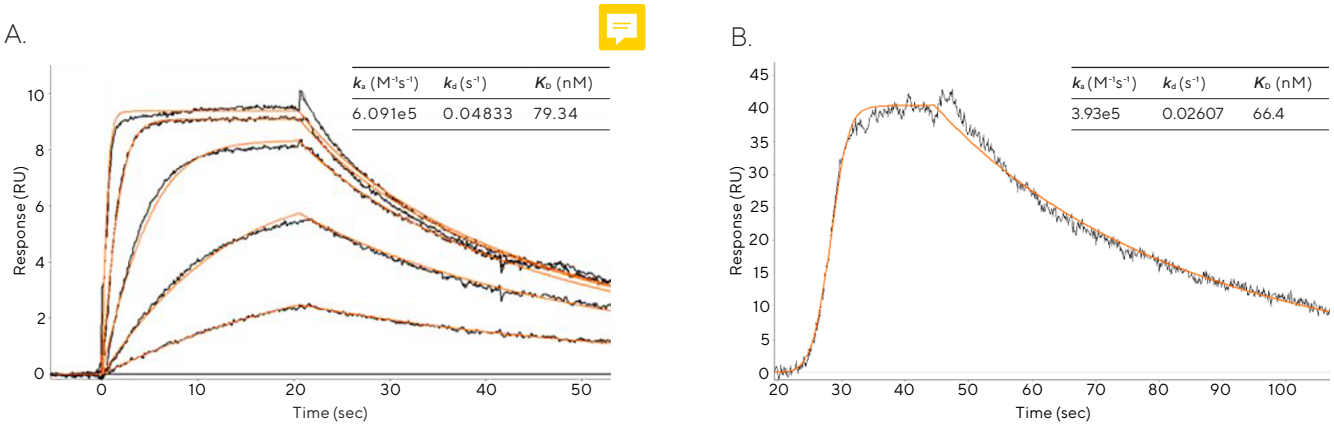
Advantages of OneStep® Injections

In conventional, multi-cycle kinetics SPR analysis, a single, conventional, fixed concentration injection (FCI) provides a flow of uniform analyte concentration. The Octet® SF3 system is also capable of performing these standard injections. However, the Octet® SF3 system differentiates itself from conventional SPR system by diffusing the sample solution into a moving stream of buffer to create a sample concentration gradient during OneStep® Injections. These features make it possible to create a variable analyte concentration during a sample injection. Using this dynamic approach, an analyte titration of three orders of magnitude can be recorded in one continuous injection. There is no need to prepare a full dilution series as is the case with other SPR systems because OneStep® Injections actively test analyte concentrations sufficient to fully characterize kinetic and affinity interactions (Figure 5).

In a typical fragment screen, both conventional FCI and OneStep® Injections confirm similar hits (Figures 6 and 7). However, as stated previously, active fragment candidates are identified directly from the primary screen in data generated using the OneStep® method. Also, less scatter is readily visible in data generated using OneStep® Injections, thus making the decision of which fragments to work with further downstream that much easier.

Figure 5

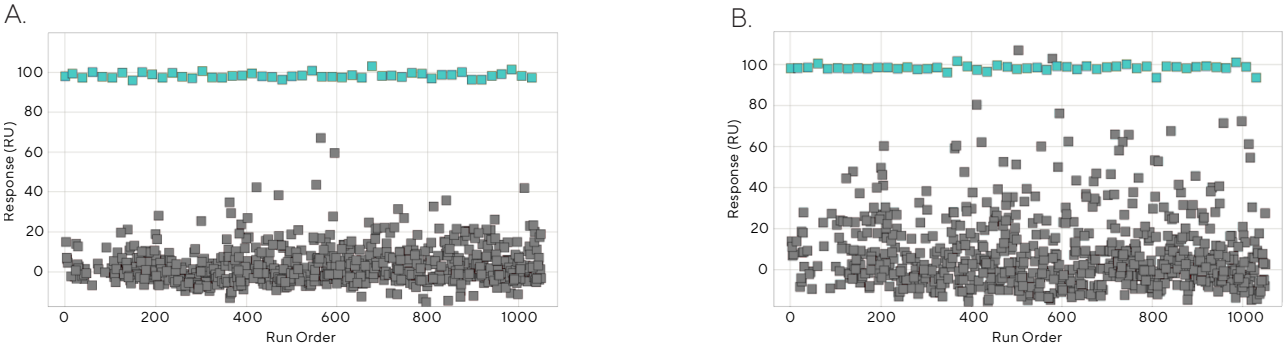
Control Compound Binding the Target.



Note. (A) A six-point FCI assay with kinetic model fit to the data in orange. (B) A OneStep® assay with kinetic model fit to the data in orange. The time to collect these data was 20 minutes on the competitor SPR system and 4 minutes for the OneStep® injection. OneStep® Injections are capable of rapidly delivering kinetic rate and affinity constants similar to the standard multi-cycle kinetics approach.

Figure 6

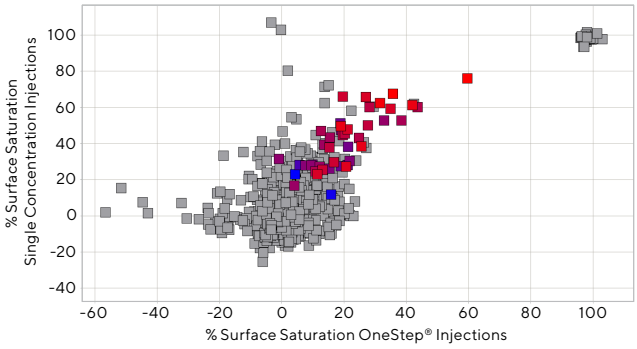
1000 Fragments Tested Using OneStep® Injection (A) and Standard, Single Fixed, Concentration Injections (B).



Note. Data is scaled according to fractional binding occupancy. There is less apparent scatter in the OneStep® Injection data, suggesting a more stable target environment.

Figure 7

OneStep® and Single Fixed Concentration Injections Confirm Similar Sets of Hits.



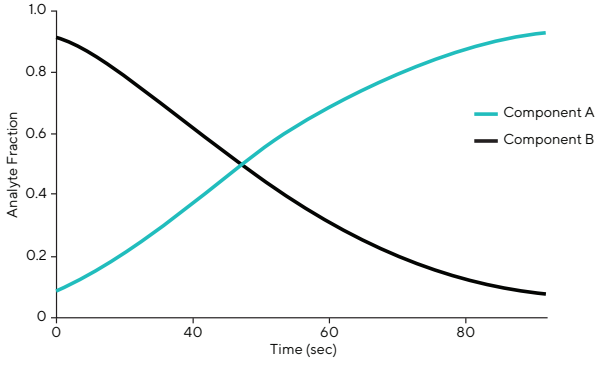
Note. In addition to confirming similar hits to single fixed concentration injections, OneStep® data supplies K_D information directly from the screen, which removes the need for additional secondary screening.

NeXtStep™ Injections in FBDD Competition Assays

Competition assays are very useful in drug discovery. They allow for the ability to find site-selective binders directly by confirming the location(s) of the binding site of a specific compound. The Octet® SF3 system's NeXtStep™ Injection technology has the unique ability to determine full kinetics and affinity in the presence of a competitor. Using a rapid dispersion process (non-Taylor dispersion), two sample components are dispersed and injected over a sensor chip surface. Figures 8 and 9A–C show the experimental setup of a NeXtStep™ competition assay. In summary, a two-part experimental design is demonstrated where fragments are injected using the NeXtStep™ technique in the presence and absence of constant control concentration. Blank NeXtStep™ Injections then consist of either buffer-only or control analyte-only to subtract the respective background signal. NeXtStep™ Injections cover a wide analyte concentration range with a single dissociation and competition is clearly seen as a lack of or reduction in binding in the presence of the competitor.

Figure 8

An Illustration of NeXtStep™ Injections.

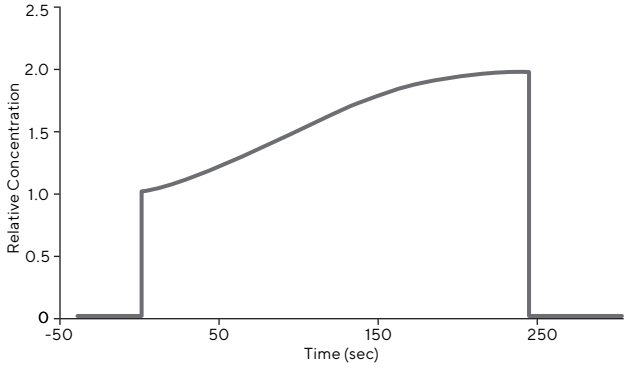


Note. Rapid dispersion of two analyte components forming a sigmoidal concentration gradient.

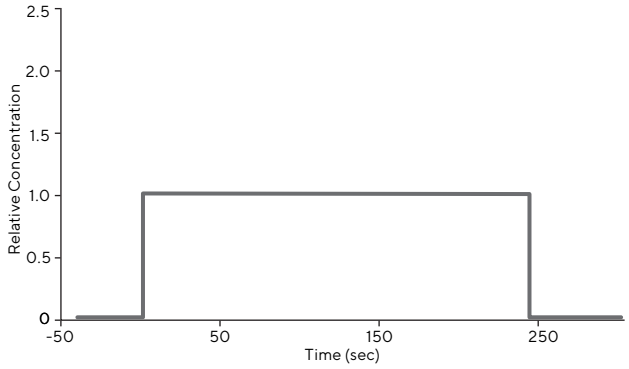
Figure 9

Competition NeXtStep™ Assay Illustrated with Real-time Concentration Graphs.

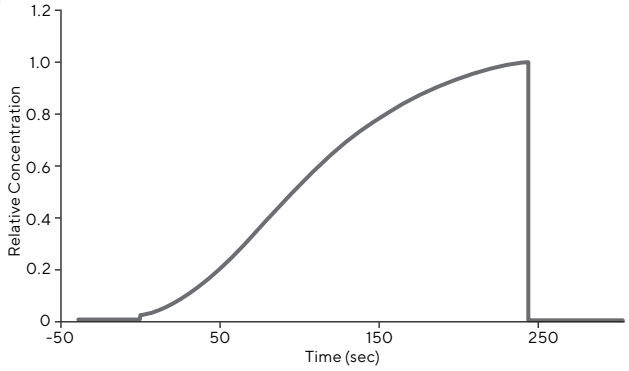
A.



B.



C.



Note. (A) NeXtStep™ Injection control and analyte + control which shows the binding of control plus a gradient dispersing of analyte binding. (B) Control only NeXtStep™ Injection which is the reference (blank) for Figure 9A. (C) By subtracting Figure 9B from Figure 9A, only constant concentration of analyte remains, and is shown in Figure 9C.

NeXtStep™ Direct Competition Fragment Assay

To demonstrate the NeXtStep™ Injection technique, carbonic anhydrase II (CAII) was immobilized on a sensor chip via amine coupling and the system primed in HEPES buffered saline, pH 7.4, with 0.005% Tween-20 (HBS-T). 24 fragment actives were previously identified from the Maybridge Ro3 1,000 compound library using OneStep® Injections and its integrated actives selection in the Octet® SPR Analysis Software. These fragments were prepared in two sample solutions, one in buffer and another in buffer with 100 µM furosemide. NeXtStep™ Injections (50 µL/min) were performed on each sample by dispersing the sample solutions with buffer (Figure 10A) and the furosemide solutions with 100 µM furosemide (Figure 10B). Control injections of buffer and furosemide were also performed for double referencing. Data analysis was performed using in the Octet® SPR Analysis Software and results are shown in Figures 10–13.

Figure 11 shows one binding mode represented by comparing the binding of an analyte in the presence and absence of a control. When a fragment binds the target protein away from the active site, the binding will be unchanged in the presence of the control. Fragment actives are therefore promptly binned without further screening cycles typical of conventional SPR systems.

In a second binding mode, when a fragment binds to the active site, the NeXtStep™ response will decrease in the presence of the control. The decrease in signal is readily identifiable in the Figure 12 examples. Further screening becomes a rate-limiting and unnecessary step. What happens when fragment binding cooperates with the control? In a third binding mode shown in Figure 13, fragments which display cooperative or non-competitive binding with the control will show an increased NeXtStep™ binding response in the presence of the control. The NeXtStep™ competition assay qualitatively identifies the three most common competition binding modes and quantitatively gives a competition K_D for increased certainty beyond a yes/no answer for analyte binding.

Figure 10

NeXtStep™ Injections of Fragment Actives Binding CAII in the (A) Absence and (B) Presence of Furosemide.

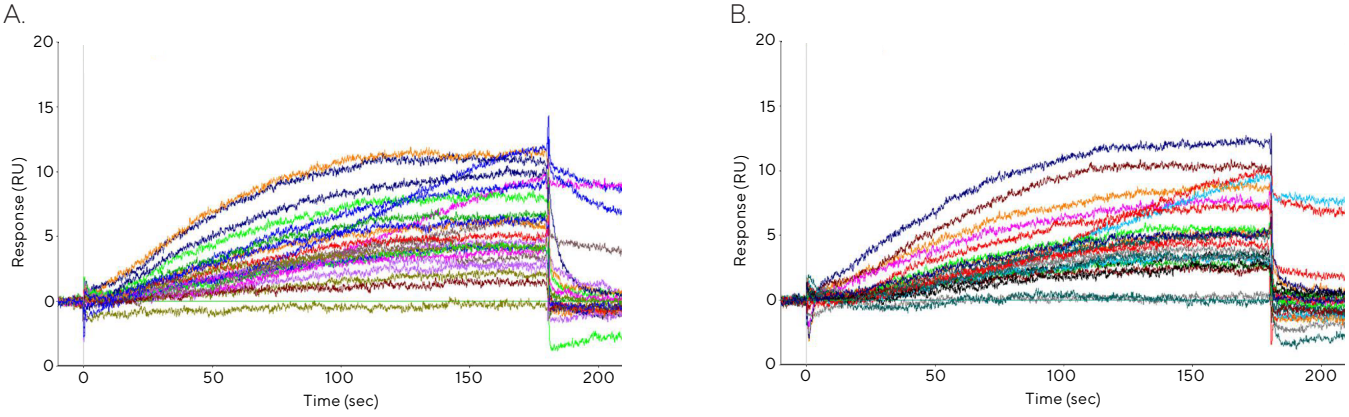
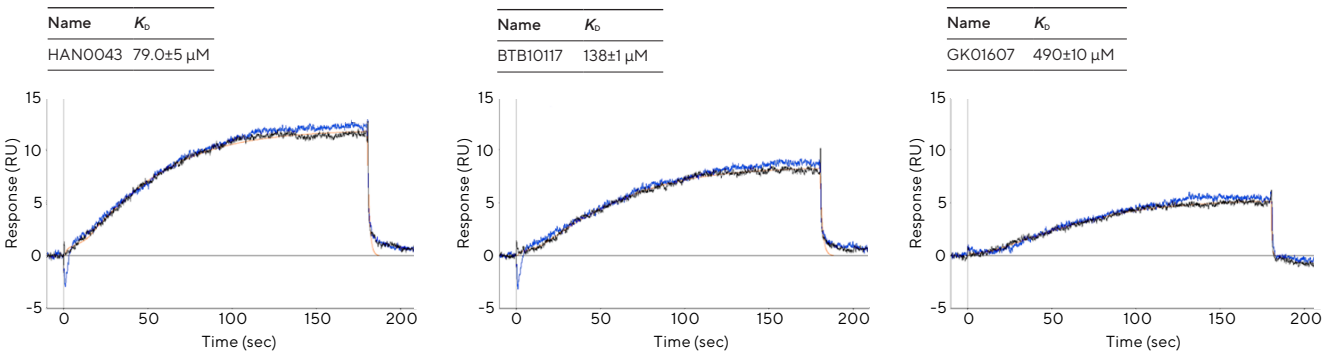


Figure 11

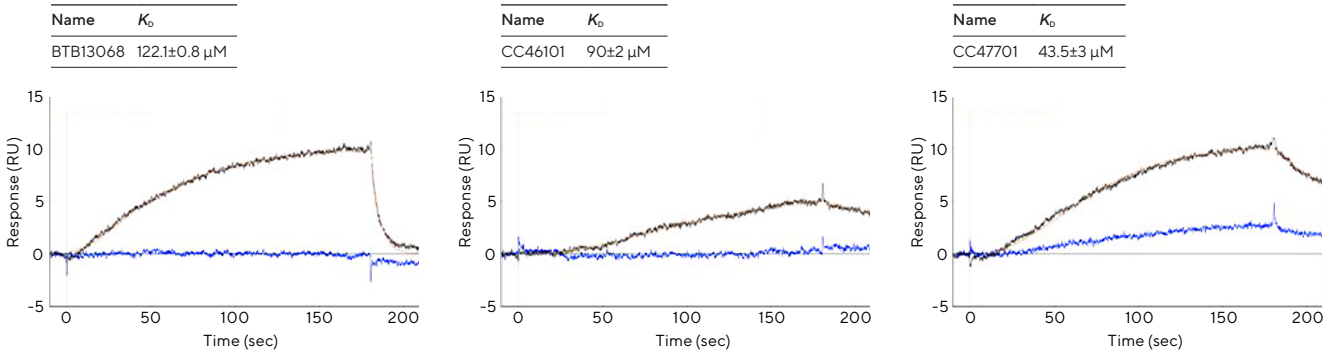
Non-competitive Binding Assessment Using NeXtStep™.



Note. Fragments with a non-competitive binding show an unchanged response in the presence (blue curves) and absence (black curves) of a control molecule. Competition K_D data derived from a single NeXtStep™ Injection.

Figure 12

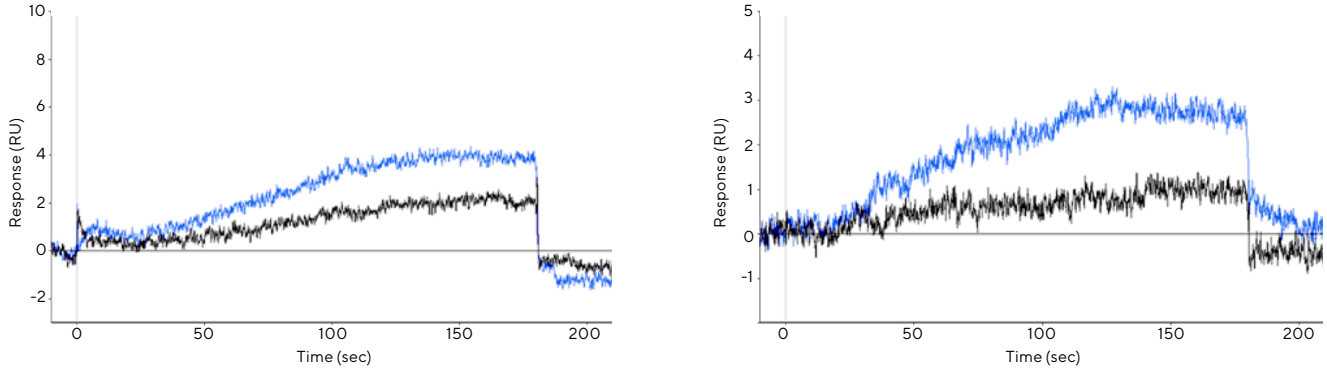
Competitive Binding Assessment Using NeXtStep™



Note. Fragments with competitive binding show a decreased response with NeXtStep™ Injections in the presence of the control allowing for prompt identification.

Figure 13

Cooperative Binding Assessment Using NeXtStep™.



Note. Fragments which bind cooperatively and un-competitive binders will show increased binding in the presence of the control.

Conclusion

The detection and characterization of fragment binding events is dependent upon ultrasensitive technologies that are well suited to detect interactions of weak affinity and low molecular weight compounds. As demonstrated throughout this application note, in comparison to other SPR-based biosensor technologies, the Octet® SF3 system with OneStep® Injections has the extraordinary advantage of directly influencing the rate at which fragment screening is accomplished. Content-rich data can be derived from a primary screen, enabling the rapid selection of active fragments.

The OneStep® gradient injection method provides K_D values from single injections, allowing the Octet® SF3 instrument to eliminate secondary screens required in other SPR techniques — no other SPR systems have this technology. The Octet® SF3 system combines identification and affinity from OneStep® Injections with a specificity before the NeXtStep™ competitive assay to provide rapid and complete evaluation of fragment actives.

References

1. A. M. Giannetti et al. Fragment-Based Drug Discovery, S. Howard, C. Abell, Eds. (Royal Soc. Chem., Cambridge, 2015) chap. 2

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