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Fragment based drug discovery on Pioneer Systems using Next Generation SPR analysis

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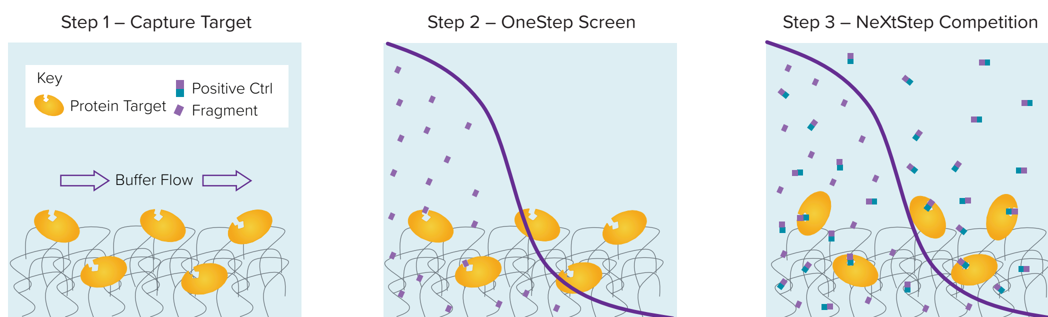
Fragment-based drug design (FBDD) has become an increasingly popular platform for the identification of lead candidates in drug discovery programs. The detection and characterization of fragment binding events is facilitated by sensitive biophysical technologies capable of detecting low affinity interactions of low molecular weight compounds. 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.

ForteBio has recently introduced the next generation in SPR biosensor analysis for fragments. Exclusively available on Pioneer systems, Next Generation SPR consists of OneStep® and NeXtStep™ gradient injection technologies. OneStep Injections use a continuous analyte titration method that provides



	Step 1 – Capture Target	Step 2 – OneStep Screen	Step 3 – NeXtStep Competition
Assay steps	1 Capture protein target to sufficient surface density 2 Verify target activity with a positive control	1 Screen fragment library using OneStep Injections 2 Periodically verify target activity during the run	1 Prepare fragment hits mixed solution with control 2 Compete fragment hits with control using NeXtStep Injections 3 Confirm competitive fragments
Typical step time	• 30 min – 1 hr	• 768 fragments in 24 hrs (Pioneer FE system)	• 96 fragment hits in 9 hrs
Advantages	• **Low protein sample requirement:** Most immobilization methods use <20 µg material • **Label-free:** No tags required for detection • **Flexible:** Pioneer systems can accommodate screening in diverse 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, 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 shows a fragment's "competition K_D" which is beyond a yes/no answer

Figure 1: A workflow for fragment screening with Next Generation SPR on the Pioneer FE System

A

B

Figure 2: A) Optimized workflow using Next Generation SPR. 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.

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 competiitive, 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).

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 how SPR biosensor assays are performed 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). Fragment screening with OneStep:

- Allows users to make decisions early on by obtaining reliable affinity (K_D) and kinetics (k_a, k_d) data directly from the primary screen
- Provides fast time to first result, is fully automated and requires minimal assay development to arrive at the correct fragment candidate(s)
- 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 are often fast enough to allow for a steady-state approximation. The Pioneer 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 and Next Generation SPR using OneStep Injections was performed using a Biacore S51 (GE Healthcare) and ForteBio's Pioneer FE instrument. Biotin-tagged multidomain non-kinase phosphoribosyl transferase (biotinylated during expression using the Avitag system) was captured in high capacity on a biosensor 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 Pioneer FE system all samples were injected using OneStep Injections at a flow rate of 200 µL/min and a dissociation time of 10 sec. Experimental analyses were performed at Genentech in South San Francisco (a member of Roche Group). The Biacore S51 was used to screen the same compounds against the target using the conventional fixed concentration injection (FCI) method. The Pioneer FE system data was processed and fit with a real-time equilibrium binding model using Qdat 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 Biacore S51 is used only for the identification of the fragment hits, the data from the Pioneer FE system 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 Pioneer FE system data (Figure 3 inset) than in the Biacore S51 presumably due to less exposure of the biosensor surface to high compound concentrations. The number of compounds whose binding signals exceed the control response is higher in the Biacore S51 (Figure 4 inset), again presumably due to lower exposure time of the high concentration samples to the biosensor surface in the Pioneer FE system.

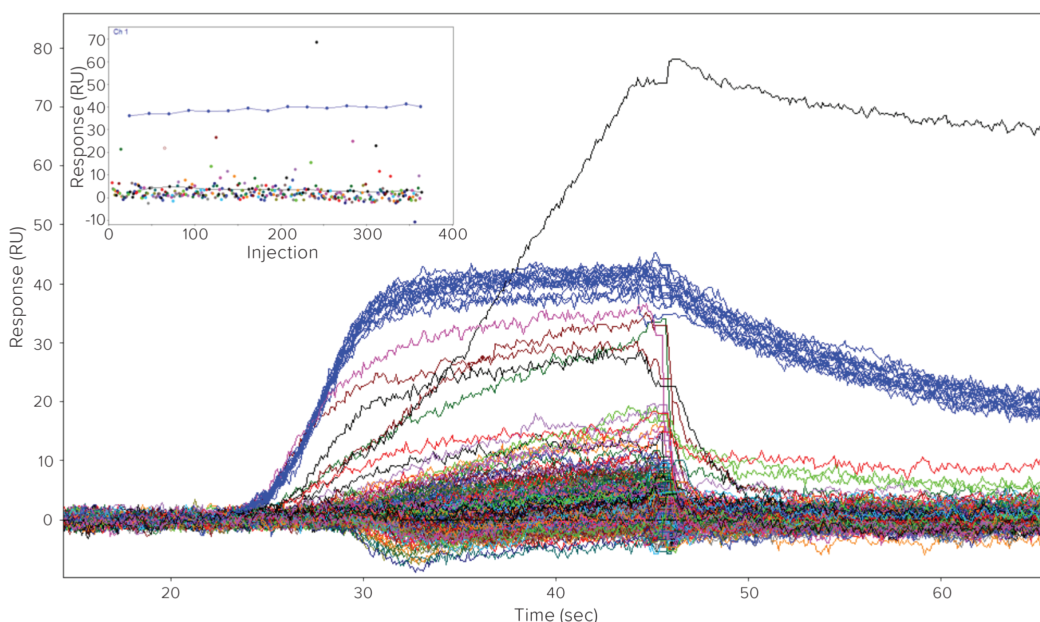


Figure 3: OneStep assay primary screening results using ForteBio's Pioneer FE system. The positive control compound was run in 16 replicates and is shown in blue. The inset shows the equilibrium response of each fragment versus assay cycle number with the control compound's 16 replicates connected in blue.

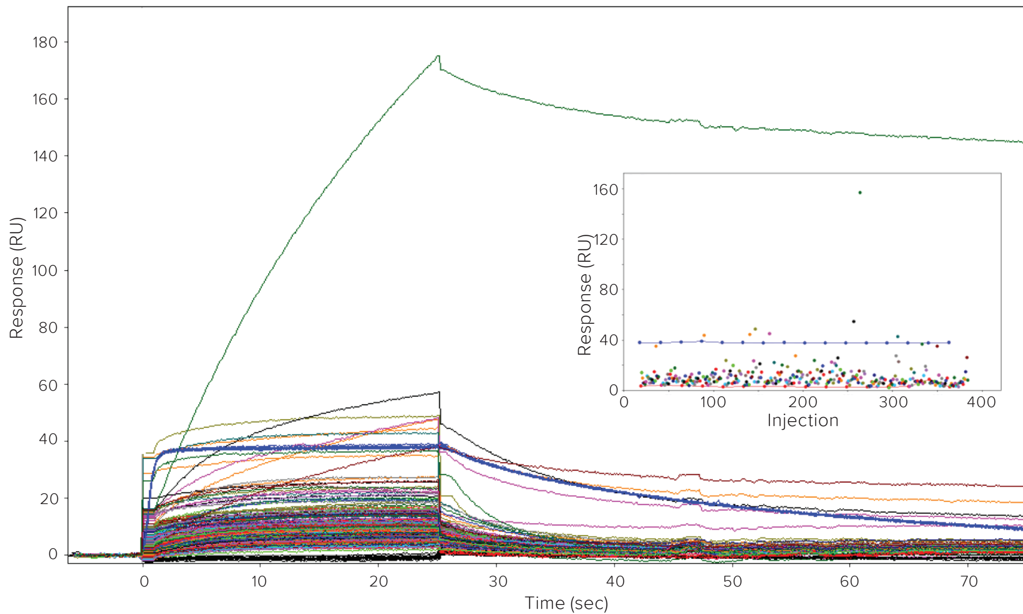


Figure 4: Fixed concentration injection (FCI) primary screening results using the Biacore S51. Positive control compound is shown in blue with 16 replicates. The inset shows the equilibrium response of each fragment versus assay cycle number with the control compound's 16 replicates shown in blue.

Advantage of OneStep Injections

In conventional SPR analysis, a standard injection (FCI) provides a flow of uniform analyte concentration. The Pioneer FE system is also capable of performing these standard injections. However, the Pioneer FE system differentiates itself from conventional SPR by diffusing the compound solution into a moving stream of buffer to create a sample concentration gradient during OneStep Injections. These features make it possible to vary the analyte concentration during the 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 instrumentation 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.

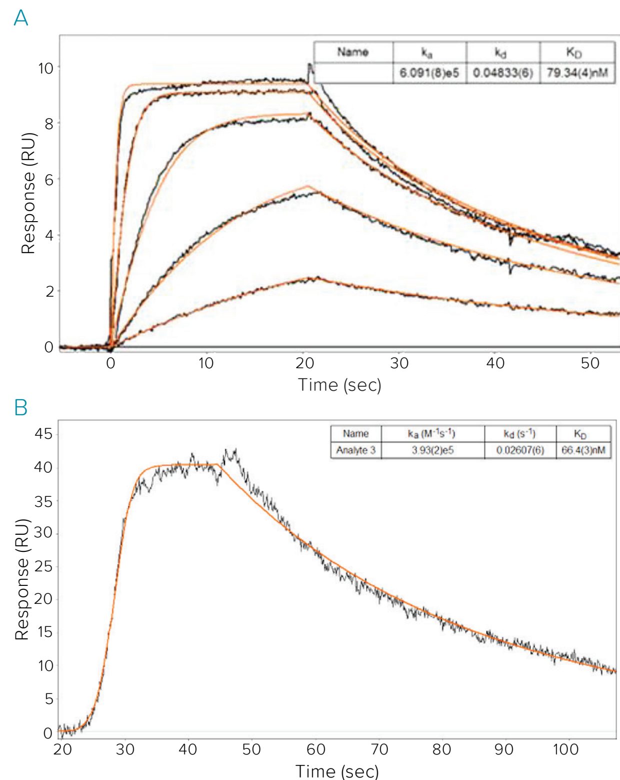
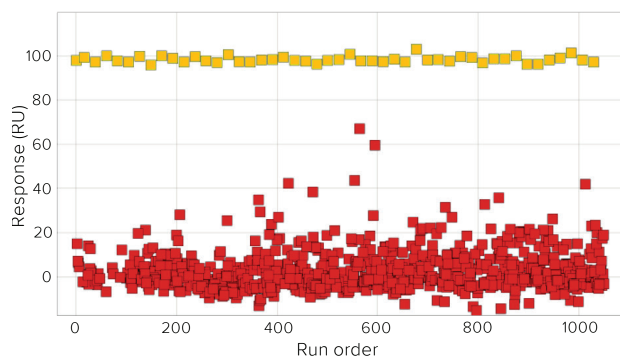


Figure 5: Control compound binding the target. (A) A six-point FCI assay with kinetic model fit to the data in orange. (B) A OneStep assay with kinetic model fit to the data in orange. The time to collect these data was 20 minutes on the Biacore S51 or 4 minutes on Pioneer FE system. OneStep Injections are capable of rapidly delivering kinetic rate and affinity constants similar to the classical approach.



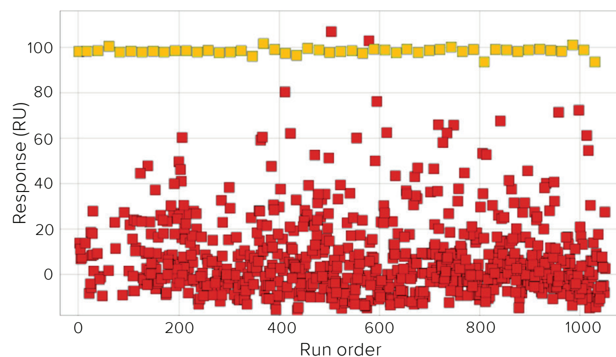


Figure 6: 1000 fragments tested on the Pioneer FE system using OneStep Injection (left) and the Biacore S51 (right). Data scaled according to fractional binding occupancy. There is less apparent scatter in the Pioneer system experiment suggesting a more stable target environment.

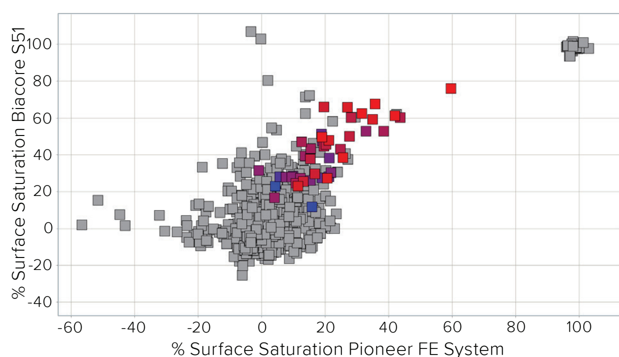


Figure 7: Similar sets of confirmed hits (colored squares) would be selected by both the Biacore S51 and the Pioneer FE system, but OneStep data gave K_D information directly from the screen.

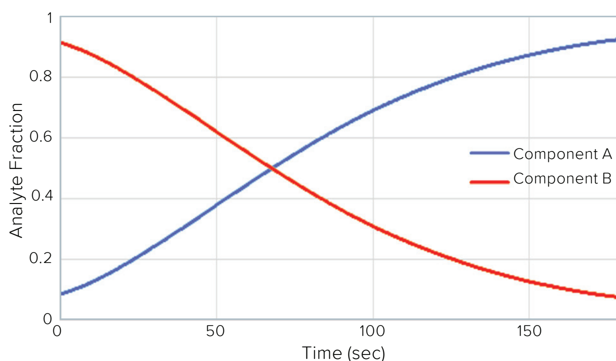


Figure 8: An illustration of NeXtStep Injections. Rapid dispersion of two analyte components forming a sigmoidal concentration gradient.

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 Pioneer FE 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 biosensor 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. Like OneStep Injections, NeXtStep Injections enable full kinetic analysis in single competition injection and competition is clearly seen as a lack of or reduction in binding in the presence of the competitor. A single NeXtStep Injection covers a wide analyte concentration range with one dissociation and is used to determine full kinetics and affinity data in the presence of a competitor.

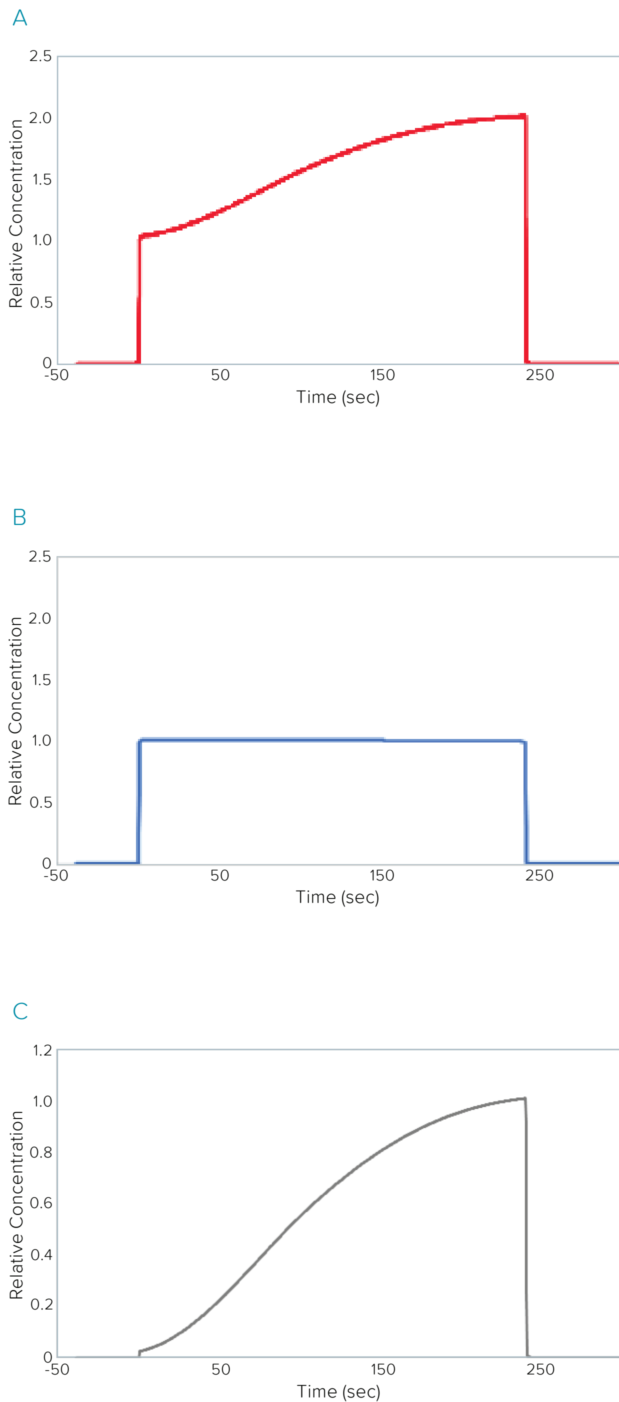


Figure 9: Competition NeXtStep assay illustrated with real-time concentration graphs. (A) NeXtStep Injection control and analyte + control which shows the binding of control plus a gradient dispersing of analyte binding. (B) Control only NeXtStep Injection which is the reference (blank) for Figure 9A. (C) By subtracting Figure 9B from Figure 9A, constant concentration of analyte only is left and is shown in Figure 9C.

NeXtStep direct competition fragment assay

To demonstrate the NeXtStep Injection technique on the Pioneer FE system, carbonic anhydrase II (CAII) was immobilized on a COOHV sensor chip via amine coupling and the Pioneer FE system was primed in HEPES buffered saline, pH 7.4, with 0.005% Tween-20 (HBST). 24 fragment actives were previously identified from the Maybridge Ro3 1,000 compound library using the Pioneer FE system and its integrated actives selection. 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 Qdat software and results are shown in Figures 10–13.

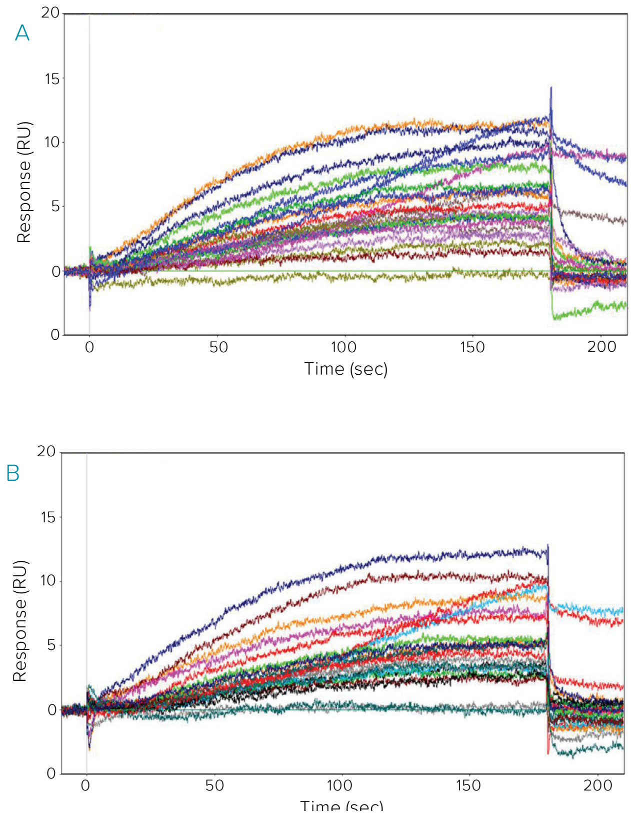


Figure 10: NeXtStep Injections of fragment actives binding CAII in the (A) absence and (B) presence of furosemide using Pioneer FE.

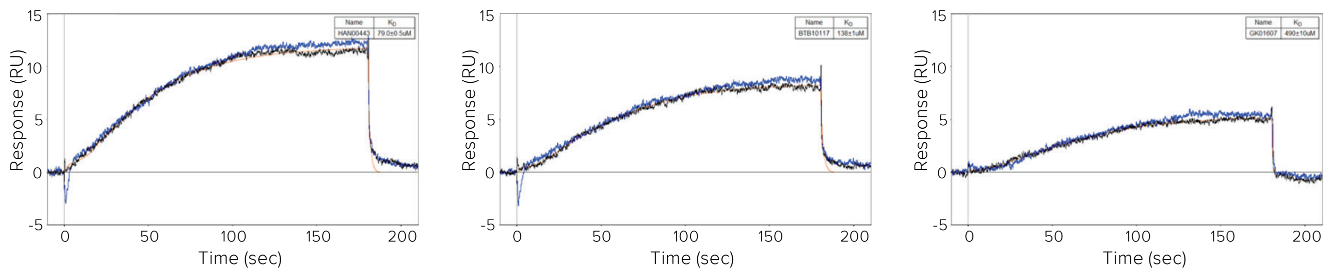


Figure 11: Fragments with a non-competitive binding show an unchanged response in the presence (blue curves) and absence (black curves) of a control molecule. K_D data derived from a single NeXtStep Injection.

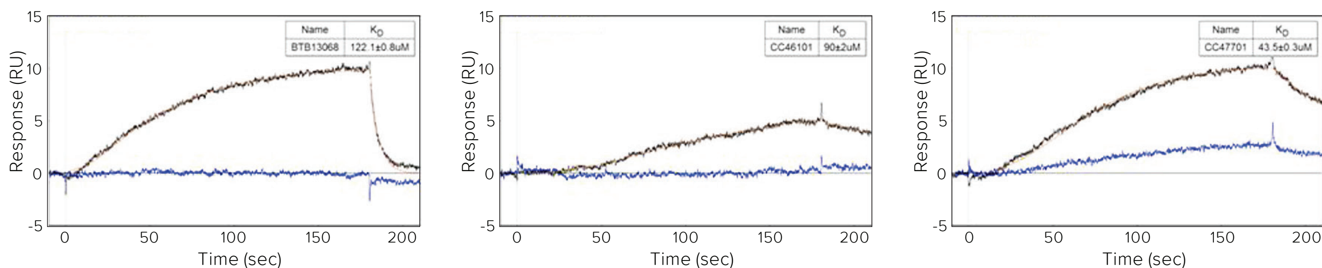


Figure 12: Fragments with competitive binding show a decreased response with NeXtStep Injections in the presence of the control allowing for prompt identification.

As previously stated, the Pioneer platform with NeXtStep Injection technology has the unique ability to determine full kinetics and affinity of a fragment in the presence of a competitor from a single injection. 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 on the Pioneer FE system qualitatively identifies the three most common competition binding modes and quantitatively gives competition K_D for increased certainty of analyte binding modes.

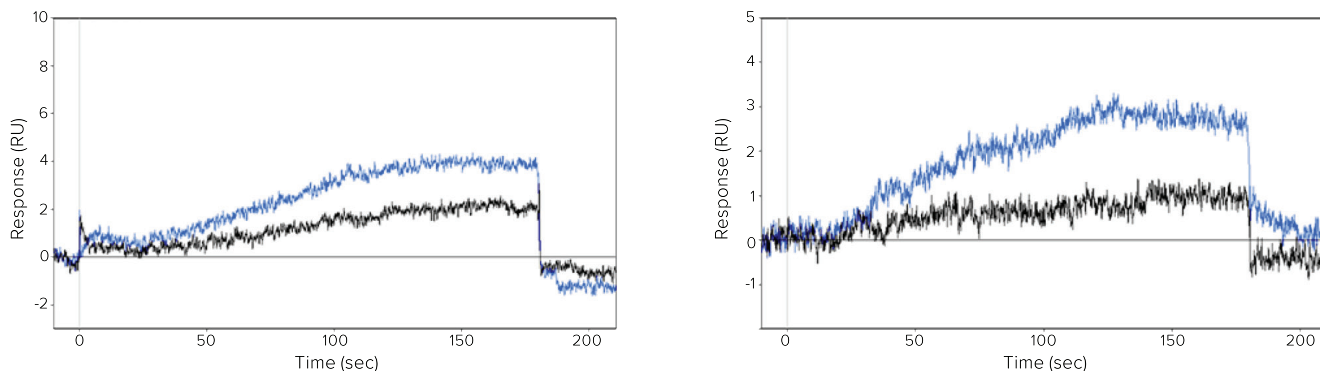


Figure 13: Fragments which bind cooperatively or un-competitive binders will show increased binding in the presence of the control.

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 Pioneer FE 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 Pioneer FE instrument to eliminate secondary screens required in other SPR techniques — no other SPR systems have this technology. Next Generation SPR on the Pioneer FE 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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