BIOPTIX ANALYTICAL

FRAGMENT SCREENING ON THE BIOPTIX 404pi

APPLICATION NOTE + 404pi™

INTRODUCTION

Fragment-based drug discovery (FBDD) has become prevalent in the pharmaceutical industry as a way to identify molecular scaffolds that can be developed efficiently into lead compounds. By screening small molecular weight fragments (~100-300 Da), the researcher can sample more chemical space with low affinity molecular frameworks that bind protein targets of interest. Subsequently, these "hits" can be combined chemically resulting in a higher affinity lead drug molecule. Label-free biophysical platforms such as biosensors, NMR, and X-ray crystallography are commonly employed to screen fragment libraries for binders. The BiOptix 404pi biosensor based on Surface Plasmon Enhanced Common Path Interferometry (SPE-CPI) is an important and useful tool in the screening of fragment libraries due to its excellent detection capabilities, low sample consumption, and ability to collect kinetic or steady-state data in real time. We demonstrate below the screening of 330 fragments from the commercially available Maybridge Ro3 Diversity Fragment Library against two proteins, bovine carbonic anhydrase II and neutravidin.

EXPERIMENTAL

The running buffer in all experiments was phosphate-buffered saline with 0.05% Tween 20 (PBS-T), pH 7.4 with 2% DMSO. All fragments were injected for 12 seconds (s) and dissociation was followed for 12 s. No regeneration of the surfaces was required. Sensorgram data was processed and fit in Scrubber 2.0 software. The steady-state titrations for the confirmatory studies on the neutravidin fragment hits were fit in Prism 7 to a steady-state model with a baseline offset. Carbonic anhydrase II was from Sigma and neutravidin was from Thermo Fisher. Fragments were purchased from Maybridge and consisted of 330 fragments chosen randomly from the Ro3 Diversity Fragment Library.

Unreacted (bare) or EDC/NHS-activated and ethanolamine-blocked CMD200m sensor chips with no ligand immobilized were used for pre-screening experiments of the fragment library to identify "sticky" fragments that bind to only the sensor surfaces. The BiOptix 404pi was run in 4x1 mode for these experiments since no referencing was required to identify fragment binding to the surface only; a report point 58 s into the dissociation phase was sufficient for identifying sticky fragments. All 330 fragments were screened at 332 µM in the pre-screen and primary binding screen of the library.

For primary binding screen experiments to identify fragments that bound specifically to protein, a CMD200m chip was activated using standard amine coupling chemistry and carbonic anhydrase II (1.3 mg/mL) and neutravidin (530 µg/mL) were immobilized on flow cells (Fc) 4 and 3 in 10 mM sodium acetate buffer pH 4.5 and pH 5 to 13770 and 14380 RU, respectively. After ligand immobilization, the surfaces

were blocked by ethanolamine. Reference Fc 1 and 2 were activated and blocked as above with no ligand immobilized. DMSO calibration curves were constructed in PBS-T from 1.2-3.10% DMSO. A DMSO calibration curve with six standards was collected after every 56th injection of fragment. A buffer blank was injected every 13th injection for double-referencing. A control for CAII, 4-aminomethyl-benzenesulfonamide (ABS), was injected every 57th injection. For neutravidin, the obvious choice for a positive control, biotin, is not viable due to its slow dissociation kinetics. Fortunately, a fragment, AC26811, was identified early in the screen that could be used as a positive control and was also injected every 57th injection to validate activity of neutravidin.

In confirmatory binding experiments, CAII was immobilized to 10,220 RU and neutravidin to 14000-16000 RU on a CMD200m chip using standard amine coupling chemistry. Fragment hits identified in the primary binding screen were flowed over their respective protein target surface to generate high-resolution kinetic and steady-state binding data at 50 µL/min using the concentrations and dilutions outlined in Table 4.

RESULTS AND DISCUSSION

Pre-screen to identify fragments binding to the sensor surface. All 330 fragments were injected over bare and activated and blocked CMD200m surfaces to eliminate fragments that bound to the carboxymethyldextran hydrogel before the primary binding screen to target protein was performed. The BiOptix 404pi was run in 4x1 mode for this large initial screen since no reference surface is required to identify sticky compounds. Fragments that bound to the sensor surfaces were found by taking a report point 58 seconds after the end of the 12 s injection. Any fragment that had >10 RU of binding signal remaining at 58 s was deemed a sticky binder and was excluded from the subsequent binding screen to target protein. Figure 1A and B show the fragments that had response units >10 RU (shown in blue). Table 1 shows that 23 fragments bound to the bare surfaces while only 19 fragments bound to the activated and blocked surfaces. In addition, 95% of the sticky fragments (shown in bold) observed binding to the activated and blocked surfaces were the same as those seen binding to the bare surfaces. Five additional fragments were also excluded from subsequent binding screens because they were insoluble in PBS-T, 2% DMSO and are given in Table 2. For all subsequent screens activated and blocked reference surfaces were used since fewer fragments bound to those surfaces and were a more proper reference surface since the protein-immobilized surfaces had been activated and blocked.

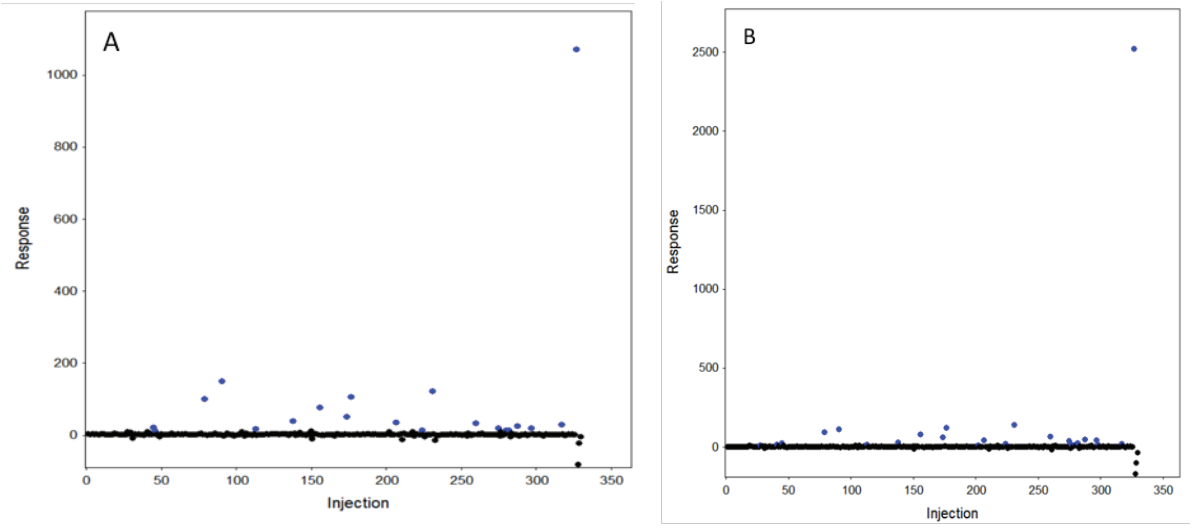


Figure 1. Trend plots that exhibit fragments (blue) that have ≥10 RU 58 s after the start of dissociation. (A) a trend plot on a bare CMD200m surface. (B) a trend plot on a CMD200m surface that has been activated and blocked. Negative RU values are caused by a very sticky fragment preceding the injection of fragments that correspond to the negative values.

Table 1. Fragments that bind to bare and activated and blocked sensor surfaces

Bare Surface	Activated and Blocked Surfaces
ML00416	ML00416
RJC02489	RJC02489
XBX00215	XBX00215
CC39486	CC39486
RH00961	RH00961
TL00838	TL00838
CC01418	CC01418
SEW05507	SEW05507
GK03251	GK03251
CC24118	CC24118
CC50413	CC50413
S05057	S05057
CD00708	CD00708
SPB06169	SPB06169
S00168	S00168
CC53446	CC53446
SPB05974	SPB05974
RH00665	RH00665
CD03760	RJF00203
CC44914	
CC45909	
RJF01201	
BTB05094	

Table 2. Insoluble fragments

Fragment
KM03516
BTB13068
SEW03743
BTB07813
BTB07014

Primary binding screen to two target proteins. The two target proteins chosen in this study were carbonic anhydrase II (CAII) and neutravidin (NA). Each protein serves as a control protein to the other since it is well known that fragments usually bind to all proteins to a certain extent and the key in fragment screening is to identify *specific* fragments to the target protein of interest. This primary binding screen on the BiOptix 404pi was conducted in 2x2 mode since proper referencing is required. The 306 remaining fragments that were not eliminated because of binding to the sensor surface or insolubility were screened at 332 µM with a 12 second injection. A report point was taken at 7 s into the association phase for each fragment injection. The report points for CAII and NA were plotted in a Versus Plot™ to determine fragments that bind specifically to CAII or NA (figure 2). In a Versus Plot most of the fragments fall on the diagonal since the majority bind both proteins to varying extents. Fragments that are specific, or relatively specific for a target protein, fall off the diagonal. A Versus Plot is a powerful visualization tool to quickly identify lead fragments. Two fragments were identified as hits to CAII and three fragments were examined and confirmed as specific binders to NA. Many more fragments bound specifically to varying extents to NA than CAII as evidenced by the greater number of fragments falling off the diagonal. Table 3 gives the Maybridge identifier for the fragment hits and also the chemical name for the fragment. Figure 3 illustrates the chemical structures for the fragment hits for CAII and NA. NA and CAII lost 8% and 50% of their respective activity during the primary binding screen over two days as concluded from the positive control injections throughout the screen.

Confirmatory binding screen to CAII and NA. To confirm that the fragments in Table 3 do, indeed, bind specifically to their target proteins, a full kinetic experiment with 7-11 concentrations injected in triplicate with each fragment was performed. Figure 4 shows the results for the three titrations with NA. These experiments confirm that all three fragments bind specifically to NA and have equilibrium dissociation constants, K_D, from 83-309 µM as shown in Table 4. No kinetic information was available for these three interactions from the sensorgrams due to the lack of curvature in the association and dissociation phases. The sensorgram data for the two CAII fragments is given in Figure 5. For both CAII fragments, kinetic binding information could be extracted from the sensorgrams (Table 4) and a K_D from a steady-state titration could be determined for AC14849. No steady-state K_D could be calculated for SEW01355 because the sensorgrams did not reach steady-state. The K_D for the CAII fragments ranged from 13-850 nM, which is ~100-6000-fold tighter than those observed for the NA fragments. This is not totally unexpected since upon identifying the CAII fragment hits both were determined to be sulfonamides, which are well-known binders of CAII. The strength of binding of SEW01355 was surprising because the K_D for most inhibitors of CAII are in the triple digit nM to single- to double-digit µM range. SEW01355 also has a fast k_a, appreciably faster than most other CAII inhibitors.

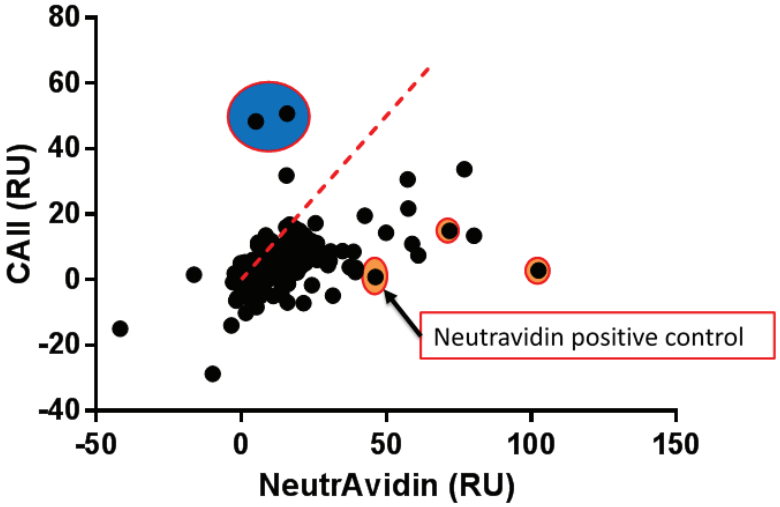


Figure 2. Versus Plot™ of report points at 7 s from the primary binding screen of 306 fragments against CAII and NA. The two fragments in the blue are hits for CAII and the three fragments shown in orange are hits for NA. Versus Plot is a trademark of Biosensor Tools LLC.

Table 3. Fragment hits that bind to CAII and NA

Maybridge Identifier for NA Hits	**Chemical Name**
AC26811 (positive control)	3-Azatricyclo[4.2.1.0^{2,5}]non-7-en-4-one
KM05322	3-[(2-hydroxyethyl)thio]-6,6-dimethyl-4-oxo-4,5,6,7-tetrahydrobenzo(c)thiophene-1-carbonitrile
S14674	3-[(4-methylphenyl)methyl]-2-(pyridin-3-yl)-1,3-thiazolidin-4-one
Maybridge Identifier for CAII Hits	**Chemical Name**
AC14849	Benzenesulfonamide
SEW01355	2-chloro-4-(trifluoromethyl)benzenesulfonamide

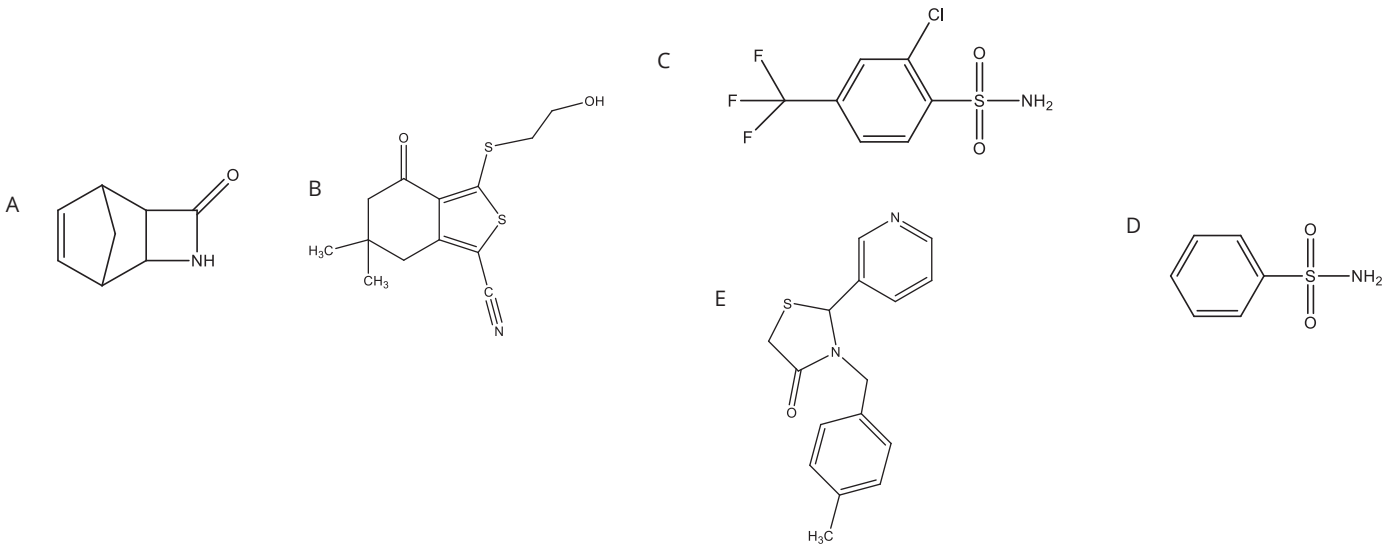


Figure 3. Chemical structures of fragment leads for NA (A-C) and CAII (D, E). (A) 3-Azatricyclo[4.2.1.0^{2,5}]non-7-en-4-one. (B) 3-[(2-hydroxyethyl) thio]-6,6-dimethyl-4-oxo-4,5,6,7-tetrahydrobenzo(c)thio-phene-1-carbonitrile. (C) 3-[(4-methylphenyl)methyl]-2-(pyridin-3-yl)-1,3-thiazolidin-4-one. (D) Benzenesulfonamide. (E) 2-chloro-4-(trifluoromethyl)benzene-sulfonamide.

Table 4. Experimental conditions and results for the fragment confirmatory studies.

Ligand	Fragmant Analyte	MW (Da)	Analyte concentration range (µM)	k_a (x10^5) $M^{-1}s^{-1}$	k_d (s^{-1})	K_D (µM)	Steady-state K_D(µM)
NA	AC26811	135.2	1000-0.98, 2-fold	ND	ND	ND	83
	KM05322	281.4	1100-1.1, 2-fold	ND	ND	ND	309
	S14674	284.4	1000-0.98, 2-fold	ND	ND	ND	104
CAII	AC14849	157.2	12-0.016, 3-fold	1.1	0.092	0.85	890
	SEW01355	259.6	1.1-0.0015, 3-fold	41	0.053	0.013	ND

ND=not determined

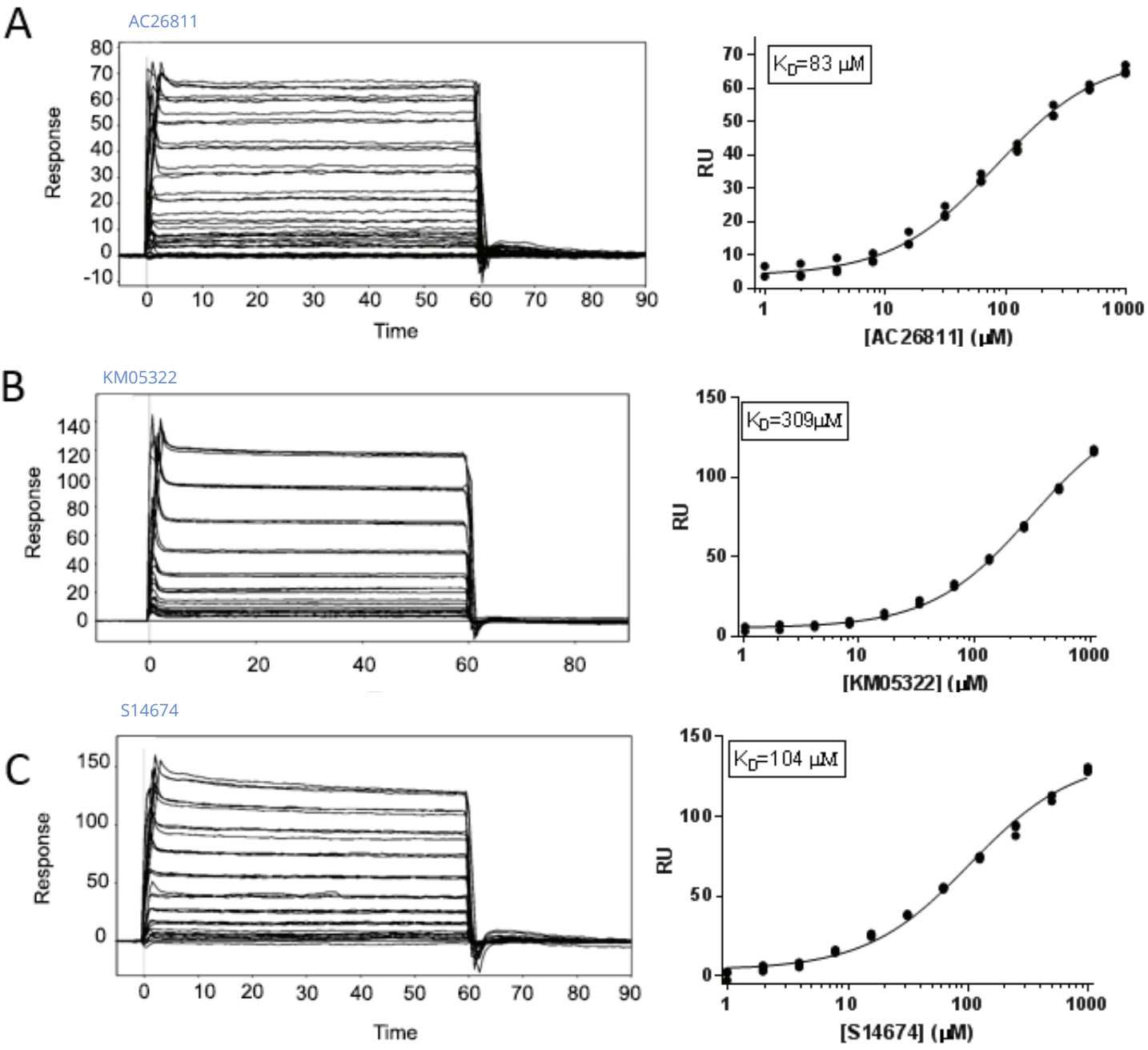
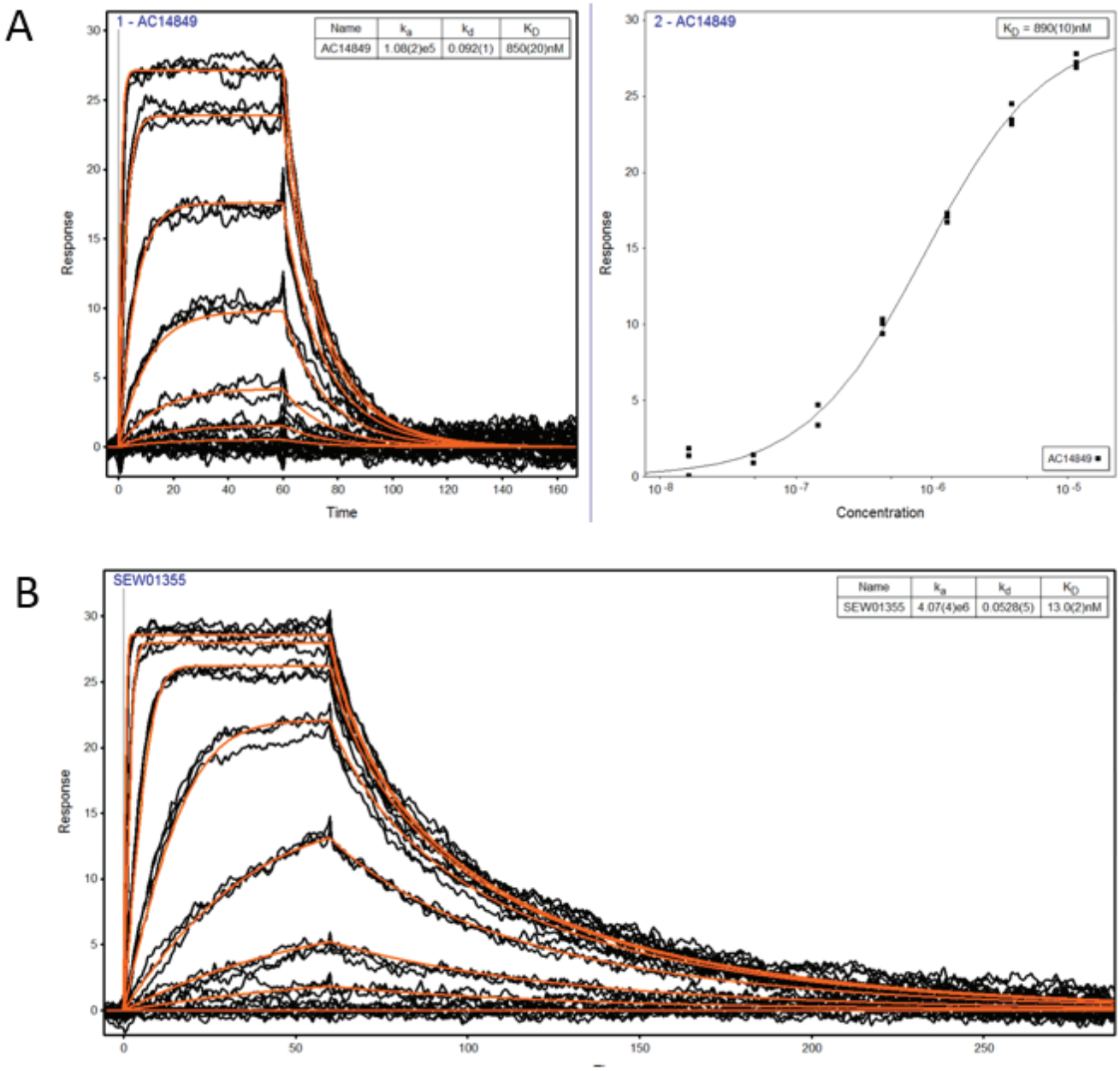


Figure 5. Sensorgram data and steady-state titration curves for CAII fragments (A) AC14849 and (B) SEW01355



CONCLUSIONS

In this application note, a 330 fragment library was screened and several fragments were identified as hits to NA and CAII. Subsequently, these hits were confirmed as specific binders by kinetic and steady-state titration. The throughput of the 404pi for primary fragment screening in 2x2 mode is 168 fragments/day when two target proteins are utilized with each one serving as a control protein to the other, or 336 fragments/day if no control protein is used. The 4x1 mode also allows for four-fold higher throughput than serial flow cell biosensors with only one injector during the pre-screening of fragments, further enhancing the throughput of pre-screens to 864 fragments/day. The BiOptix 404pi™ is a highly capable biosensor for fragment screening with excellent sensitivity and detection capabilities for fragments with molecular weight as low as 95 Da.