APPLICATION NOTE

nicoya

Characterization of HuCAL Fab-MH binding kinetics on Alto™ digital surface plasmon resonance using a Twin-Strep-tag® capture system

Summary

In this application note, Alto™ digital SPR was used to characterize HuCAL Fab-MH kinetics using the Twin-Strep-tag® Capture Kit, demonstrating the compatibility of the Strep-TactinXT® as a capture molecule on the Alto™ platform.

Introduction

The streptavidin-biotin interaction is frequently leveraged for purification and detection of biomolecules due to its high affinity and stability. However, the interaction cannot be easily regenerated on biosensor platforms such as SPR or BLI. In order to address this problem, IBA Lifesciences has developed the Twin-Strep-tag® Capture Kit. This kit utilizes Strep-TactinXT®, a modified streptavidin molecule that binds molecules with a Strep-tag®, a short protein fusion tag. This interaction retains the high affinity and stability of the streptavidin-biotin interaction, while also being reversible using a standard SPR regeneration solution.

Streptavidin

Streptavidin is a tetrameric protein found in the bacterium *Streptomyces avidinii*, widely known for its extremely high binding affinity for the molecule biotin (also known as Vitamin H). This affinity is on the order of 10^{-14} mol/L, which is 10^3 to 10^6 times tighter than an antibody-antigen interaction.[1] Streptavidin is used extensively in many biotechnology applications due to its extremely high affinity for biotin and overall high stability.[2-5] One of the few drawbacks of this system, particularly with respect to bioassays such as SPR, is that the interaction is extremely difficult to regenerate. For this reason, IBA Lifesciences created a protein fusion tag, the Strep-tag®, to bind modified streptavidin molecules with affinity in the low picomolar range.[6-7]

IBA Lifesciences Strep-tag®

The Strep-tag® technology offered by IBA Lifesciences uses a short protein fusion tag, similar to a histidine tag or myc-tag. The Twin-Strep-tag® used in this application note includes two Strep-tag® motifs (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) in tandem connected by a ten amino acid linker and therefore consists of 28 amino acids. This Twin-Strep-tag® is captured by a streptavidin molecule with an engineered biotin-binding site, called Strep-Tactin®XT. This Strep-Tactin®XT and Twin-Strep-tag® interaction has high affinity with a dissociation constant, K_D, of around 10 pM , which allows measurements of high affinity binding systems with long dissociation times and slow off-rates. With this binding system, non-specific binding of other solution components is avoided, and therefore ligands are captured efficiently from complex matrices. The biosensor surface is also easy to regenerate, unlike the traditional streptavidin-biotin system.

Green fluorescent protein and HuCAL Fab-MH

To showcase the compatibility of the Twin-Strep-tag® with Alto™, we use the GFP-Twin-Strep-tag® (TST-GFP) provided in IBA's Twin-Strep-tag® Capture Kit as the ligand, and a Human Combinatorial Antibody Library (HuCAL) for GFP as the analyte. Commonly used in antibody based therapeutic selection processes, HuCALs are the first ever fully synthetic human antibody library developed to represent the essential features of the natural human antibody repertoire.[8] Libraries contain consensus frameworks of seven heavy chains and an equal number of light chain germline families that cover more than 95% of the human antibody diversity in a modular format. When combined with the modern antibody selection methods such as phage display selection, HuCAL becomes a highly sophisticated

technology for rapidly selecting antibodies and tuning affinities to a target of interest. Due to its ease of use and speed, HuCAL is commonly used in conventional methods for antibody selection.

In this application note, we have used green fluorescent protein (GFP) with a Twin-Strep-tag® as the target for selection of antibodies from the HuCAL library (Figure 1). We showcase the capability of the Strep-Tactin®XT for use as a capture surface in SPR assays on Alto™ by comparing kinetics determined using this Strep-Tactin®XT surface to those determined with an Alpaca anti-GFP VHH capture surface. The anti-GFP VHH surface acts as a positive control, as data using this capture molecule were published in the previous application note: "Characterization of Biomolecular Interactions using Alto™: Comparison to Conventional SPR."

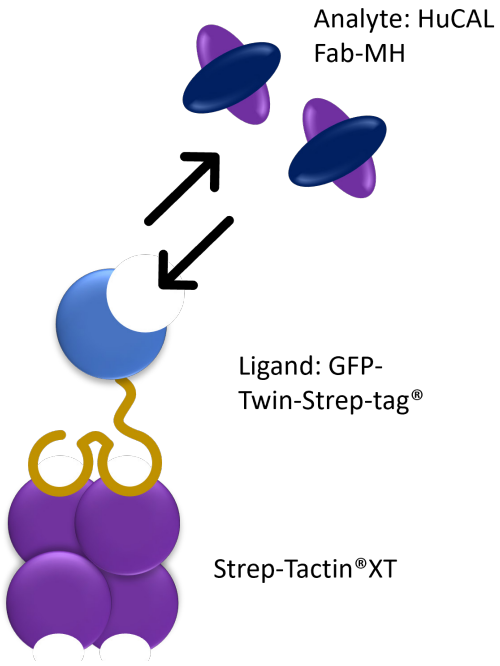

Figure 1: *Schematic representation of the molecules used in the IBA-Lifesciences Twin-Strep-tag® Capture Kit*

Advantages of Alto™

Alto™, a high-throughput SPR platform enabled with digital microfluidic (DMF) technology, has a number of advantages that make it ideal for SPR characterization of difficult-to-express proteins. Alto™ offers a label-free method, eliminating design hurdles and reducing cost. With the ability to discreetly control nano-liter sized droplets of each ligand and analyte, binding assays are simple to implement and provide novel insights into therapeutic performance, while reducing consumption of precious samples by up to 200 times.

In addition, Alto's 16 independent channels provide the ability to simultaneously analyze multiple targets in many different assay formats, while significantly reducing hands-on time with complete assay automation. Alto™ minimizes operation time with simple design and analysis software. Acquisition of real-time binding data enables deeper insight into the binding kinetics of desired targets rather than just the affinity. Alto's sensors provide a versatile surface for capturing targets with protein A, streptavidin, Strep-Tactin®XT and others, allowing for characterization of a wide range of biomolecular systems from discovery to design. In this application note, we demonstrate the compatibility of the IBA Lifesciences Twin-Strep-tag® Capture Kit with Alto™ by measuring binding affinities and kinetics of a HuCAL Fab-MH using the Strep-Tactin®XT technology as a capture surface.

Materials & Equipment

- Nicoya Alto® 16-Channel Instrument (ALTO16)
- Alto™ 16-Channel Carboxyl Cartridge
- Alto™ running Buffer: PBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-PBST)
- Alto™ CBX Surfacing Kit: cleaning, normalization, activation (ALTO-R-CBX-SURF)
- Alto™ regeneration Buffer: Gly-HCl pH 1.5: (ALTO-R-GLYHCl-1.5)
- Alto™ immobilization Buffer: Sodium Acetate pH 5.5 (ALTO-R-IMB-5.5)
- Capture molecule: anti-GFP VHH (Alpaca anti-GFP VHH, purified recombinant binding protein): Chromotek (CAT# GT-250)
- Analyte: HuCAL Fab-MH (monovalent human) Negative Control: Bio-Rad, (CAT# HCA124)
- IBA-Lifesciences Twin-Strep-tag® Capture Kit
  - Capture molecule: Strep-Tactin®XT
  - Ligand: GFP-Twin-Strep-tag® (TST-GFP)
  - SPR Immobilization Buffer: Sodium Acetate pH 4.5 (with 0.1% Tween-20 added for Alto™, also sold as ALTO-R-IMB-4.5)
  - Regeneration Buffer: 3 M Guanidine-HCl (with 0.1% Tween-20 added for Alto™

Methods

This label-free SPR assay was performed using Alto™, the first and only DMF-powered SPR. Alto™ uses a cartridge-based, gold nanostructure sensor with 16 channels (8 reference channels and 8 active channels). The experimental method was designed using the direct screening application on Alto's user portal, which was automatically uploaded to the instrument.

First, a 16-channel Carboxyl Cartridge was loaded into Alto™ followed by dispensing of the cartridge fluid into the cartridge. Reagents were pipetted into the cartridge wells following software-directed prompts. The experiment was then initiated by selecting the "Run Method" command on Alto™. All subsequent steps were automated by Alto™. The contents of each well are shown in Table 1.

Normalization of sensors with high and low RI droplets was performed first. The sensors were then cleaned with 50 mM NaOH for 60 s, followed by a 5 min activation of the 16 carboxyl sensors with a drop consisting of 1 mM EDC and 1 mM NHS prepared from Nicoya's Surfacing Kit. For creation of the capture surface, 3 µL of Strep-Tactin®XT at 50 µg/µL in sodium acetate pH 4.5 was loaded into each capture molecule well. Each sensor was exposed to one of these drops for 15 min. The immobilization level may be adjusted, if necessary, by changing the contact time or concentration of Strep-Tactin®XT. For a more stable baseline after capture of Twin-Strep-tag® protein it is recommended to create a high density of Strep-Tactin®XT on the sensor surface. All sensors were then blocked with 1 M Ethanolamine for 5 min to quench remaining active carboxyl groups.

This test used a multi-ligand format, where 2 µL of the TST-GFP ligand was passed over each of the 8 active channels for 15 min at 10 nM in PBS-T, pH 7.4. For kinetic experiments it is recommended to optimize the capture level of the ligand to the lowest level of immobilized ligand that produces a detectable response signal in the analyte association step. Lower concentrations of Twin-Strep-tag® protein and shorter contact times can be used to lower the capture level of the ligand.

For the HuCAL Fab-MH analyte, 65 µL of 150 nM solution was loaded into the reagent well, and the mixing of five 3-fold serial dilutions per analyte was automated by Alto™. The concentrations of HuCAL Fab-MH used for binding were 617 pM, 1.85 nM, 5.56 nM, 16.7 nM and 50 nM. For kinetic experiments, the analyte concentration range should typically range from 0.1X to 10X the expected K_D.

For the single cycle kinetics (SCK) format, the analyte was introduced from lowest to highest concentration with five subsequent association times of 180 s, followed by a single dissociation time of 600 s after the analyte titration series. The sensor surface was regenerated with a 60 s exposure of 3 M guanidine HCl after the dissociation, which resulted in complete removal of the TST-GFP ligand and analyte. The entirety of the test was completed in less than 6 h.

For the multi-cycle kinetics (MCK) format, the analyte was introduced from lowest to highest concentration for 180 s each, with a 600 s dissociation step following each concentration. The sensor surface was regenerated with a 60 s exposure of 3 M guanidine HCl after the dissociation of each concentration of analyte. This resulted in the complete removal of the TST-GFP ligand and analyte. The entirety of the test was completed in less than 8 h.

Upon completion of each test, binding curves were automatically fitted to a 1:1 binding model in the Nicosystem™ analysis software to determine kinetic and affinity constants.

Table 1: Well layout with reagents and concentrations for single-cycle capture kinetics

	1	2	3	4	5	6	7	8	Vol. (µL)	Conc.
R	Regeneration, 3 M Guanidine HCl				PBST	150 nM HuCAL	HCl	PBST	65	
A	Low RI solution				1 mM EDC		1 mM NHS		4	
B	High RI solution				Quench (1 M Ethanolamine)				4	
C	Strep-Tactin®XT	Strep-Tactin®XT	Strep-Tactin®XT	Strep-Tactin®XT	Strep-Tactin®XT	Strep-Tactin®XT	Strep-Tactin®XT	Strep-Tactin®XT	3	50 µg/mL
D	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	2	10 nM
E	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	2	10 nM
F	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	2	10 nM
G	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	GFP-Twin Strep-tag	2	10 nM
H	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	-	-
I	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	-	-
BF	PBST	PBST	PBST	PBST	PBST	PBST	PBST	PBST	180	

Results & Discussion

The capability of the Strep-Tactin®XT was evaluated for use as a capture surface in SPR assays. Strep-Tactin®XT was reproducibly immobilized via amine coupling onto the CBX sensor surface with immobilization levels of 1500 RU obtained (Figure 2). High immobilization levels on the sensor surface are ideal to maximize the detection signal for Twin-Strep-tag® ligand capture. The immobilization level may be adjusted, if necessary, by changing the contact time or concentration of Strep-Tactin®XT.

To test the ability of Strep-Tactin®XT immobilized sensors to capture and regenerate Twin-Strep-tag® ligands we tested multiple rounds of TST-GFP capture followed by regeneration with 3 M GuHCl in both the SCK and MCK assay formats. In the SCK format, the recapture of the TST-GFP ligand was consistent throughout each of the four rounds tested (Table 2), as shown in Figure 3. 100% regeneration of the ligand and bound analyte was achieved with 3 M GuHCl. The GFP Twin-Strep-tag® shows minimal dissociation following the binding of Strep-Tactin®XT, highlighting the rigidity of this capture system.

In the MCK format, the TST-GFP was reproducibly captured for each of the five concentration cycles in each round of the test; one set of the five concentration cycles is shown overlaid in Figure 4. Full regeneration was also achieved after each cycle using 3 M GuHCl with 0.1% Tween 20. The data for the capture and regeneration rounds for each of the SCK and MCK assays closely align when overlaid, demonstrating that Strep-Tactin®XT coated sensors can be regenerated several times without loss of capture activity.

Kinetic values for SCK kinetics were calculated based on the sensorgrams obtained on one cartridge with Alto™, tested across 8 lanes and 4 rounds. Kinetic values for MCK kinetics were calculated based on the sensorgrams obtained on one cartridge with Alto™, tested across 8 lanes and 2 rounds. The data were fit to a Langmuir 1:1 binding model analyzed in the Nicoya® analysis software. Kinetic parameters for data obtained using both capture molecules are reported in Table 3 and demonstrated excellent reproducibility across all channels and rounds.

From the kinetic analysis, association and dissociation rate constants when Strep-Tactin®XT was used as the capture molecule in an SCK format were determined to be 2.62×10^{5} $M^{-1}s^{-1}$ and 1.32×10^{-3} s^{-1}, respectively, resulting in a K_D of 5.08 nM (Figure 5). Association and dissociation rate constants when anti-GFP VHH was used as the capture molecule in an SCK format were determined to be 1.80×10^{5} $M^{-1}s^{-1}$ and 7.97×10^{-4} s^{-1}, respectively, resulting in a K_D of 5.20 nM (Figure 6). Additionally, association and

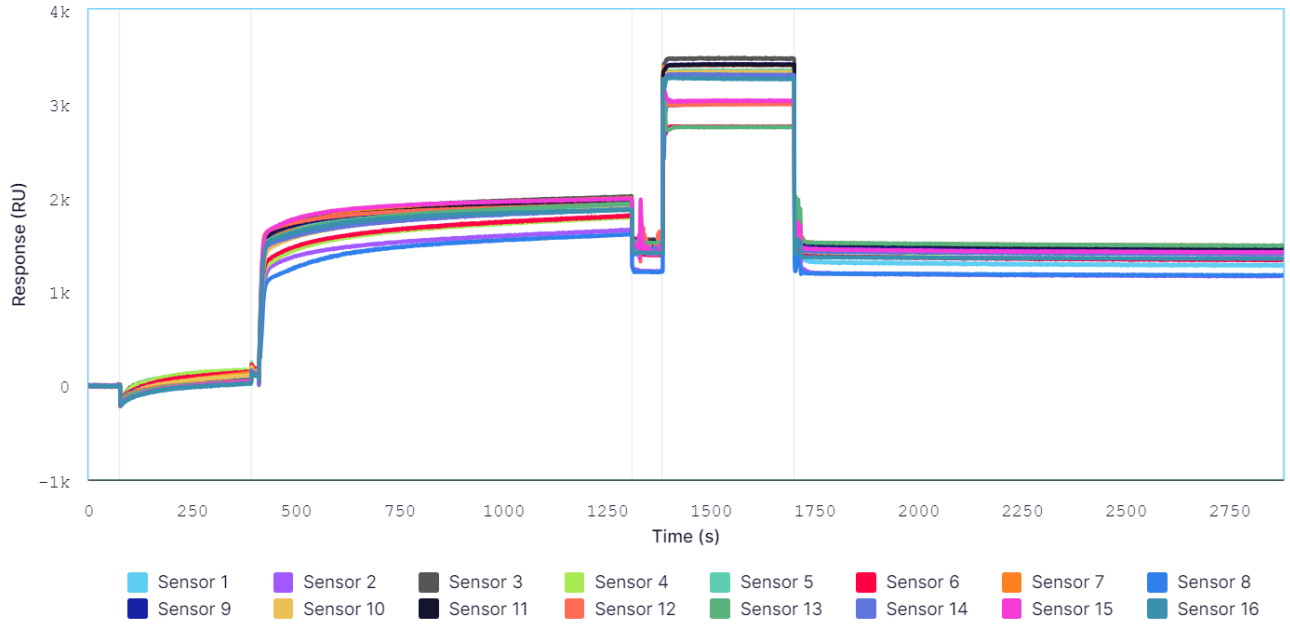

Figure 2: *Immobilization of Strep-Tactin®XT on 16 sensors simultaneously on the carboxyl cartridge. The capture surface building activity includes activation of all 16 channels with 1 mM EDC and 1 mM NHS from Nicoya's Surfacing Kit for 300 s, followed by immobilization of 50 µg/mL Strep-Tactin®XT in sodium acetate pH 4.5 for 900 s, and blocking of sensors with 1 M Ethanolamine for 300 s. Typical immobilization levels of the Strep-Tactin®XT should be ~1500 RU.*

dissociation rate constants when Strep-Tactin®XT was used as the capture molecule in an MCK format were determined to be 2.14 x 10^5 $M^{-1}s^{-1}$ and 1.37 x 10^{-3} s^{-1}, respectively, resulting in a K_D of 6.80 nM (Figure 7). Association and dissociation rate constants when anti-GFP VHH was used as the capture molecule in an MCK format were determined to be 1.12 x 10^5 $M^{-1}s^{-1}$ and 6.10 x 10^{-4} s^{-1}, respectively, resulting in a K_D of 5.44 nM (Figure 8). These data confirm the kinetics are effectively the same using either capture system in either kinetic format (Table 3).

Table 2: Average capture of GFP-Twin-Strep-tag®

10 nM Ligand	GFP Capture (RU)
Average	214.63
STDev	14.19
%CV	6.61%

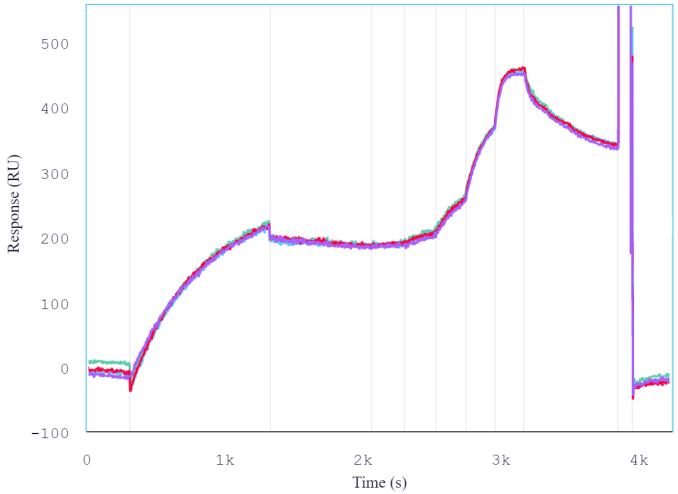

Figure 3: *The Strep-Tactin®XT immobilized sensors provide reproducible results and can be efficiently regenerated several times. 10 nM TST-GFP was captured on a Strep-Tactin®XT sensor followed by sequential association of the HuCAL analytes at concentrations of 617 pM, 1.85 nM, 5.56 nM, 16.7 nM and 50 nM. The sensor surface was regenerated with a 60 second exposure of 3M GuHCl after the dissociation phase (full RI shift of the 3 M GuHCl solution extends off the y-axis and was removed for visual purposes). This capture, analyte binding and regeneration steps were repeated 3 more times and overlaid in the figure. Capture levels of TST-GFP were 214 ± 14 RU, 6.6 %CV with 100% regeneration.*

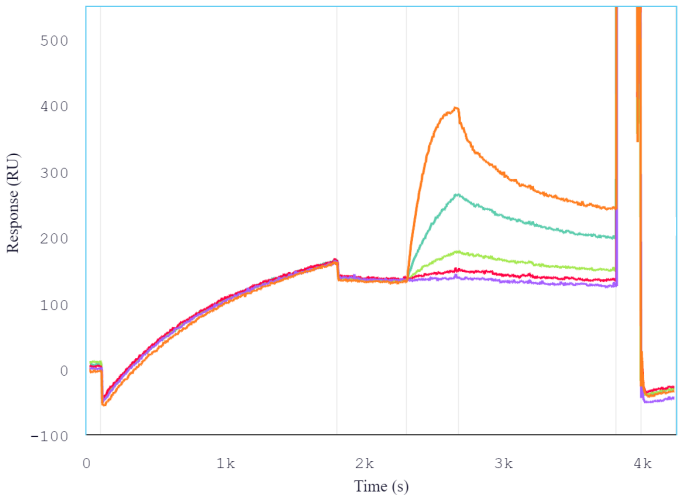

Figure 4: *Binding Kinetics on Strep-Tactin®XT Surface. 10 nM TST-GFP was captured on a Strep-Tactin®XT sensor followed by association of the HuCAL analyte at concentrations of 617 pM, 1.85 nM, 5.56 nM, 16.7 nM and 50 nM. TST-GFP ligand is captured on the surface with a contact time of 900 s, followed by buffer baseline of 150 s, HuCAL analyte association phase of 180 s, dissociation of 600 s and regeneration in 3 M GuHCl of 60 s to remove the captured ligand and analyte from the surface (full RI shift of the 3 M GuHCl solution extends off the y-axis and was removed for visual purposes). Nicosystem™ analysis software was used to fit the interaction to a 1:1 binding model.*

Table 3: Kinetic parameters measured for HuCAL Fab-MH using each capture molecule and assay format

Capture molecule	Assay Format	ka (1/M*s)	kd (1/s)	K_D (nM)
Strep-Tactin®XT	SCK	$2.62x10^5 \pm 1.31x10^4$	$1.32x10^{-3} \pm 9.38x10^{-5}$	5.08 ± 0.579
anti-GFP VHH	SCK	$1.80x10^5 \pm 1.03x10^5$	$7.97x10^{-4} \pm 1.95x10^{-4}$	5.20 ± 1.77
Strep-Tactin®XT	MCK	$2.14x10^5 \pm 6.50x10^4$	$1.37x10^{-3} \pm 1.54x10^{-4}$	6.80 ± 1.58
anti-GFP VHH	MCK	$1.12x10^5 \pm 1.48x10^4$	$6.10x10^{-4} \pm 2.43x10^{-4}$	5.44 ± 1.95

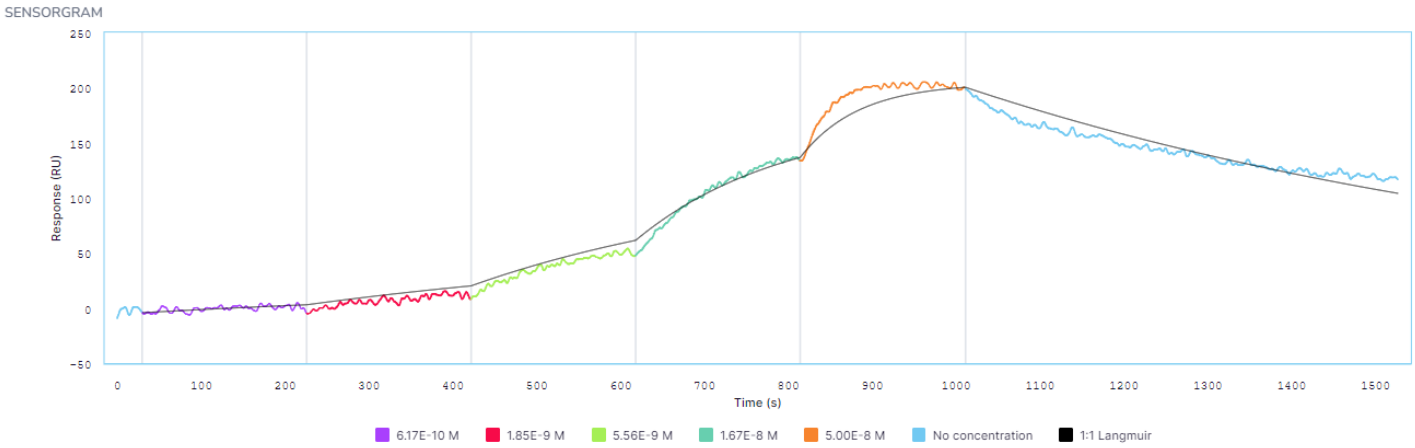

Figure 5: *Sample sensorgrams showing the binding of HuCAL to GFP-Twin-Strep-tag that is captured by Strep-Tactin®XT in an SCK format. Image was generated by Nicosystem™ analysis software.*

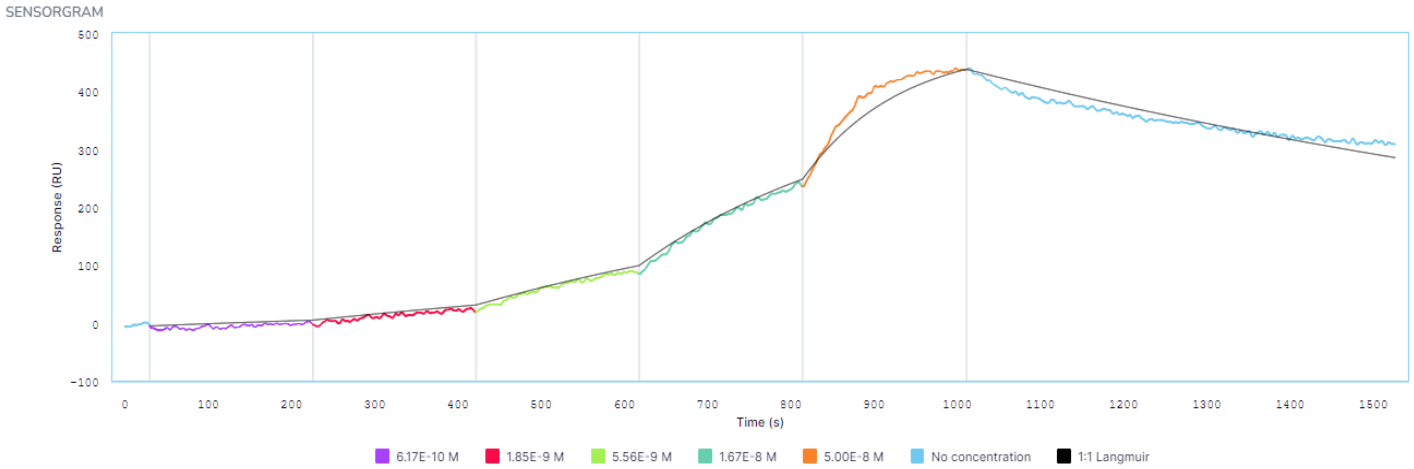

Figure 6: *Sample sensorgrams showing the binding of HuCAL to GFP-Twin-Strep-tag that is captured by anti-GFP VHH in an SCK format. Image was generated by Nicosystem™ analysis software.*

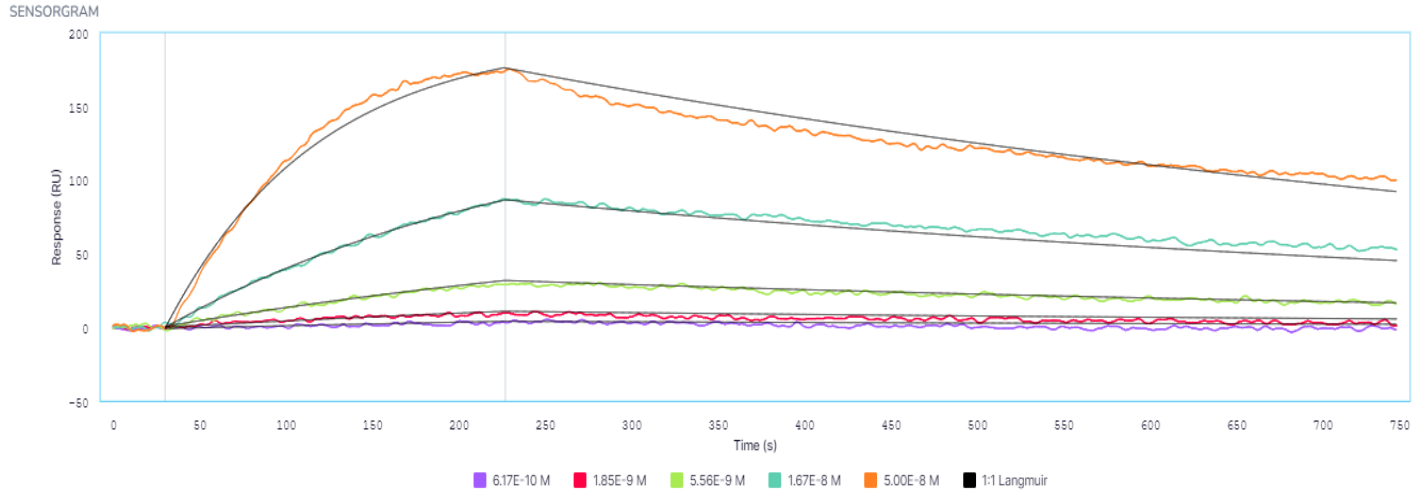

Figure 7: *Sample sensorgrams showing the binding of HuCAL to GFP-Twin-Strep-tag that is captured by Strep-Tactin®XT in an MCK format. Image was generated by Nicosystem™ analysis software.*

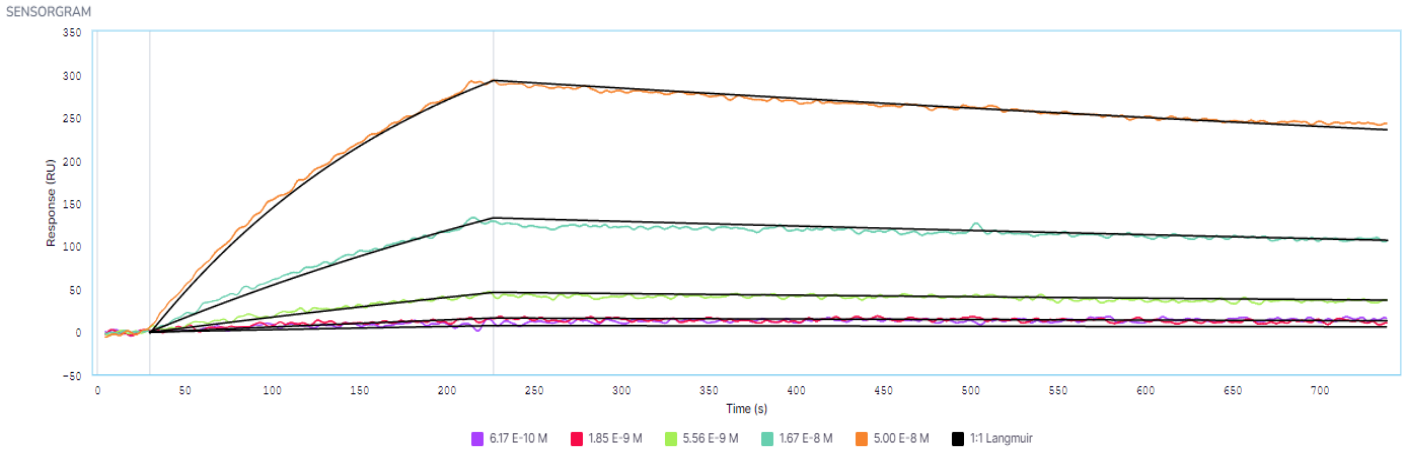

Figure 8: *Sample sensorgrams showing the binding of HuCAL to GFP-Twin-Strep-tag that is captured by anti-GFP VHH in an MCK format. Image was generated by Nicosystem™ analysis software.*

Conclusion

Alto™ successfully characterized a HuCAL Fab-MH using the Twin-Strep-tag® Capture Kit. Kinetic parameters determined using the kit were closely comparable to those determined using traditional capture with a GFP-specific VHH. This demonstrates the compatibility of strep-tag capture systems on Alto™. Compared to traditional SPR, Alto's unique and fully automated digital microfluidic platform enabled analysis of up to 48 data points in a single cartridge, significantly reducing the time, sample volume and overall cost of kinetic analysis. By leveraging DMF technology, Alto™ streamlines SPR analysis by automating sample dilutions, eliminating fluidic maintenance, and reducing sample requirements by up to 200 times.

References

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