APPLICATION NOTE

Kinetic analysis of a recombinant antibody in undiluted serum on Alto Digital SPR

Abstract

Characterizing the binding and kinetics of antibody-antigen interactions in serum is critical for advancing the development of vaccines, diagnostics, and other biotherapeutics. Screening these interactions without extensive purification is highly desirable as it reduces cost and hands-on time. In traditional label-free binding assays, serum samples need to be diluted and analyzed using standard running buffers such as PBS. In this application note, we demonstrate a novel approach using Alto™ Digital SPR™ for measuring binding kinetics and affinity of antibody-antigen interactions that allows experiments to be conducted in serum without the need for dilution or purification.

Introduction

The ability to perform label-free kinetic analysis on unpurified antibody samples is highly valuable, as purification can be labor-intensive, costly, and often impractical, especially for high-throughput screening. Moreover, it is crucial to characterize binding kinetics in the original sample matrix—such as serum or plasma—to ensure results accurately reflect antibodies' behaviour in their native environment. For instance, diluting an antibody in a standard running buffer like PBS, as is done in typical label-free assays, may alter its activity due to the absence of essential matrix components, leading to kinetics that differ from those in the native matrix. Complex matrices better simulate the *in vivo* environment, where competing molecules, inhibitors, and stabilizers affect molecular diffusion, stability, and interaction kinetics. Studying interactions in these relevant matrices helps account for macromolecular crowding effects, and previous studies have shown that kinetics, particularly the off-rate, can change as serum content increases[1-2].

Analyzing biomolecular interactions by surface plasmon resonance (SPR) in complex matrices is not always feasible due to the high chance of clogging traditional microfluidic systems. Even when tests can be completed, instruments need to undergo significant maintenance that often leads to lengthy program delays. Unlike traditional SPR instruments, Alto Digital SPR employs innovative, disposable digital microfluidics (DMF). This allows all reagents to be contained on a disposable cartridge and enables the movement of ultra-low sample and reagent volumes without the possibility of clogging. For this reason, Alto is uniquely compatible with unpurified samples, such as those in serum, making complete kinetic analysis in physiologically relevant matrices possible and providing superior insight compared to traditional SPR technologies.

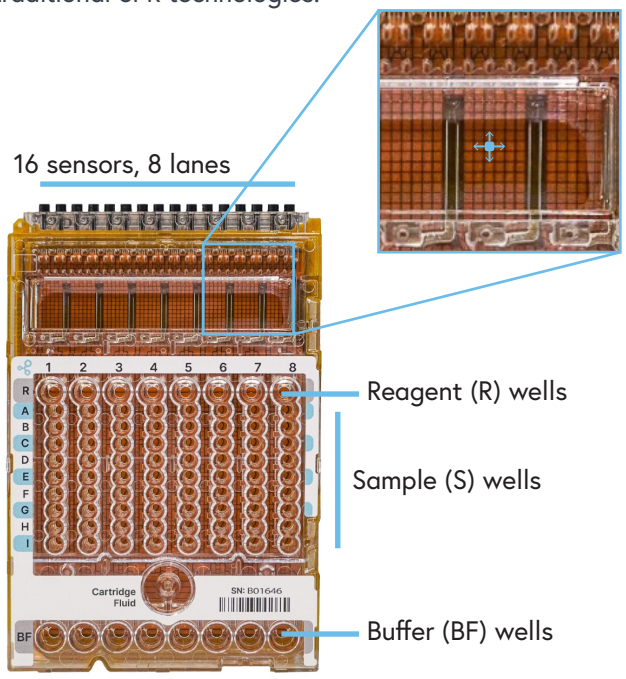

Figure 1: *Alto experiments are performed on disposal cartridges equipped with DMF technology. Using a grid of electrodes, small volumes of samples loaded into the catridge wells can be moved, mixed, spilt, and incubated with integrated SPR sensors.*

Alto avoids the heavy cleaning and instrument maintenance that is required when analyzing unpurified samples on traditional SPR by integrating all fluidic handling into a disposable cartridge (Figure 1). Each cartridge contains 16 fibre-optic SPR sensors and a grid of sample wells following standard well-plate form. Once samples, reagents, and buffers are pipetted into the cartridge, their movement and contact with the SPR sensors are controlled by DMF technology. This removes the need for any physical pumps, valves, and tubes prone to clogging and avoids any sample contact with the system's permanent hardware. A further benefit of using DMF is the unique ability to control extremely small sample volumes, as low as 350 nL, allowing a five-concentration kinetic analysis to be performed with just 2 µL samples. With the capacity to measure up to 48 kinetic interactions in one test, the cartridge requires only 1.44 mL of running buffer for the entire experiment, enabling users to use a crude matrix, such as serum, as the running buffer. Beyond binding kinetics applications, the Alto platform can also be used for faster screening experiments to determine yes/no binding results.

In this application note, the binding kinetics between eGFP and a HuCAL Fab-MH, a recombinant antibody for eGFP, are measured in buffer and 100% human serum. This study demonstrates that Alto, with its maintenance-free digital microfluidics technology, delivers robust kinetic analysis directly from serum.

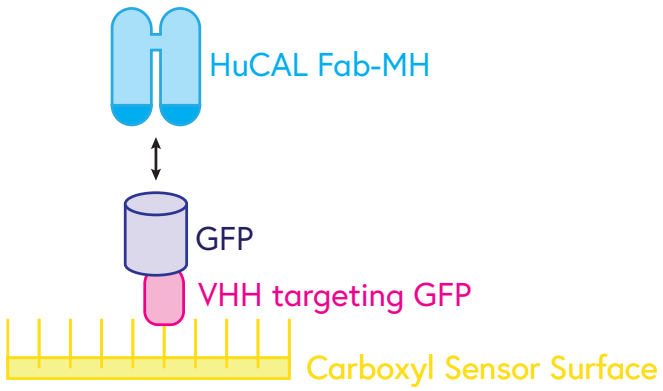

Figure 2: *Schematic representation of the assay performed. Anti-GFP VHH is immobilized on Nicoya's Carboxyl Sensor as a capture molecule. eGFP is used as the ligand and a HuCAL Fab-MH recominant antibody for GFP is used as the analyte.*

Materials & Equipment

Materials

- Alto 16-Channel Instrument with Nicosystem Pro Software (ALTO16)
- Alto 16-Channel Carboxyl Cartridge (KC-CBX-PEG-16)
- Running Buffer: PBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-PBST)
- Carboxyl Surfacing Kit: cleaning, normalization, activation (ALTO-R-CBX-SURF)
- Immobilization Buffer: 10 mM MES, pH 6.0 (ALTO-R-IMB-6.0)
- Regeneration Solution: 10 mM Glycine-HCl, pH 1.5 (ALTO-R-GLYHCl-1.5)
- Serum: Human serum (Sigma, CAT# H3667)
- Capture molecule: Alpaca anti-GFP VHH, purified recombinant binding protein (Chromotek, CAT# GT-250)
- Ligand: Purified recombinant Enhanced Green Fluorescent Protein (Chromotek, CAT# EFP250)
- Analyte: HuCAL Fab-MH monovalent human Negative Control (Bio-Rad, CAT# HCA051)

Methods

Experiment setup

The experimental setup was completed remotely on Alto's Nicosystem™ user portal, followed by run initiation on the instrument.

1. From a laptop, the experiment was designed and saved in the Nicosystem.
2. On the instrument, the designed method was selected to launch Alto's on-screen setup guide.
3. An Alto 16-Channel Carboxyl Cartridge was placed in the instrument, and samples were loaded into the cartridge following the experiment setup guide.
4. The experiment was initiated on the Alto device by selecting "Run Method".

Assay protocol

The following steps were completed automatically by Alto with no operator supervision.

1. Carboxyl sensors were normalized with normalization solutions.
2. Carboxyl sensors were cleaned with 10 mM HCl for 60 s.
3. Carboxyl sensors were activated with 25 mM EDC/NHS for 300 s.
4. 20 µg/mL anti-GFP VHH diluted in 10 mM MES, pH 6.0 was immobilized onto all sensors for 600 s.
5. All sensors were blocked with the 1 M ethanolamine for 300 s to quench any remaining active carboxyl groups.
6. All sensors were conditioned for 60 s with 10 mM glycine-HCl, pH 1.5.
7. 10 nM samples of eGFP in PBS-T were introduced to each even-numbered active sensor for 300 s.
8. Alto executed five automated HuCAL serial dilutions on the cartridge. Each sample was diluted from 150 nM stock using the solution from the buffer wells, producing 0.617 nM, 1.85 nM, 5.56 nM, 16.7 nM, and 50 nM. HuCAL was spiked into either PBS-T or 100% serum.
9. From lowest to highest, the five HuCAL concentrations were exposed to each sensor in tandem for 180 s, followed by dissociation in the running buffer for 600 s, and a 60 s regeneration step with 10 mM glycine-HCl, pH 1.5. This constitutes one round of single-cycle kinetics.

Data analysis

1. The test was opened under the analysis tab in the Nicosystem user portal.
2. The "Build Capture Surface" tab was opened to assess VHH immobilization levels across 4 lanes of the cartridge to ensure sufficient and/or optimal levels.
3. The "Raw data" tab was opened to ensure that the ligand capture levels and regeneration are sufficient and/or optimal.
4. The "Capture Kinetics" tab was opened and a 1:1 Langmuir binding model was automatically applied to the data.
5. Processing tools were used as required.
6. Final images and .CSV files were downloaded.

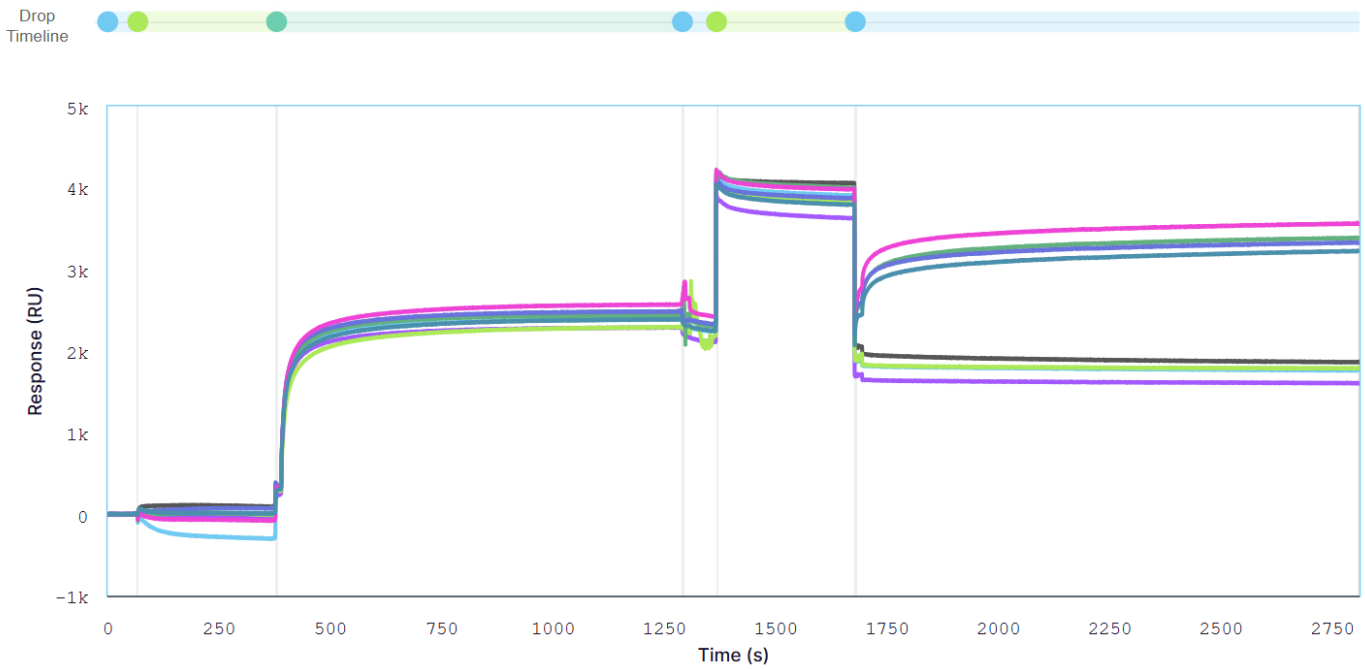

Figure 3: *Activation of the 8 sensors of the Alto cartridge used for this experiment with 25 mM EDC/NHS from Nicoya's Carboxyl Surfacing Kit, followed by immobilization of 20 µg/mL of anti-GFH VHH in 10 mM MES, pH 6.0 and blocking of sensors with 1 M ethanolamine. Four sensors use PBS-T as the running buffer both before and after the blocking step. The other 4 sensors use PBS-T until the blocking step, then switch to serum, resulting in the observed baseline shift. The image was generated in the Nicosystem software.*

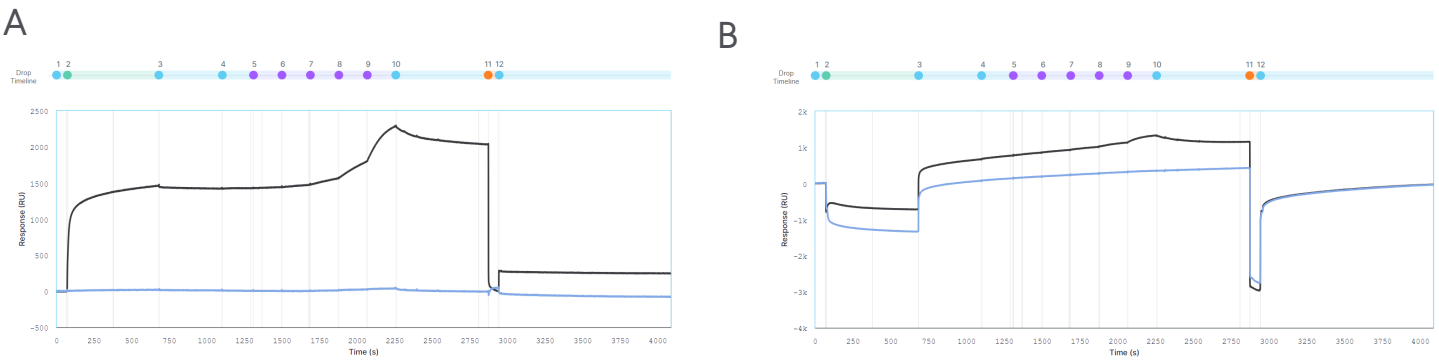

Figure 4: *Ligand loading, analyte binding, and regeneration for the eGFP-HuCAL interaction using (A) PBS-T or (B) 100% serum + 0.1% Tween 20 as the running buffer. Anti-GFP VHH was immobilized on Nicoya Carboxyl Sensors followed by two rounds of HuCAL binding kinetics. For each cycle, 10 nM of eGFP was captured on the VHH surface followed by association of HuCAL at concentrations of 0.617 nM, 1.85 nM, 5.56 nM, 16.7 nM and 50 nM. The analyte was allowed to dissociate in the running buffer and the VHH surface was regenerated with 10 mM glycine-HCl, pH 1.5.*

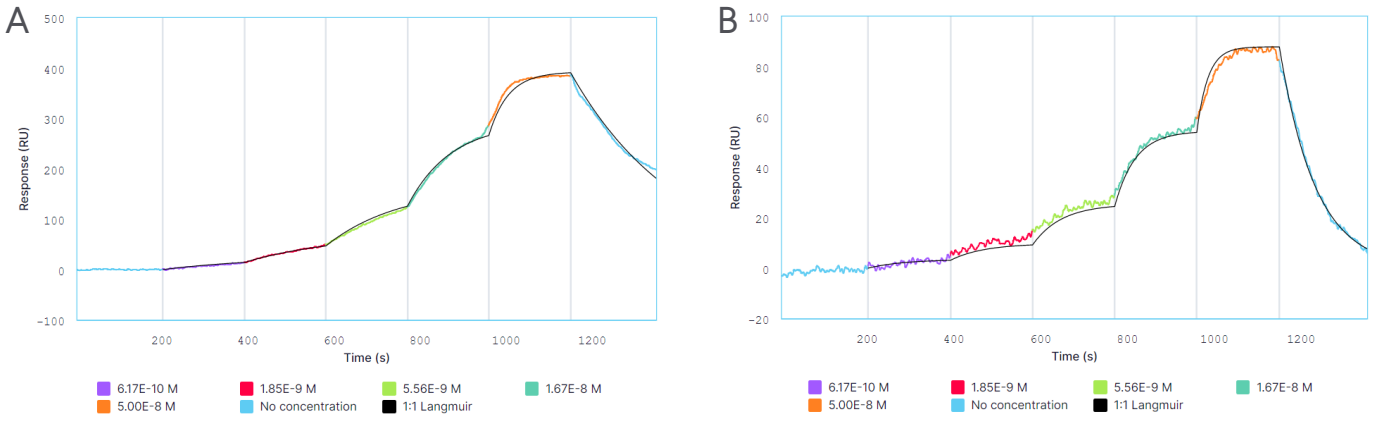

Figure 5: *Kinetic fits for HuCAL binding to eGFP in (A) PBS-T and (B) 100% serum + 0.1% Tween 20. The HuCAL analyte was titrated from 0.617 nM to 50 nM, followed by a dissociation in the respective running buffers. Black curves represent the Langmuir 1:1 binding fit model generated by the Nicosystem software.*

Results & Discussion

The anti-GFP VHH was immobilized on both the reference and active sensors of the cartridge to form the capture surface. Figure 3 shows a representative sensorgram for anti-GFP VHH immobilization. An average immobilization level of 2287 RU was calculated for sensors where PBS-T was used as the running buffer. Average immobilization cannot be determined for sensors where serum is used as the buffer due to a baseline shift that occurs after the quenching step (drop 6). Baseline shifts, or bulk shifts, are known to occur when switching between solutions that have a different composition and refractive index (RI). This baseline shift after quenching occurs because drop 6 is the first exposure of the sensors to serum loaded into the buffer (BF) wells. Prior to this drop, an auxiliary buffer loaded into cartridge position R8 (PBS-T) is used for all buffer drops.

Use of the auxiliary buffer is required when the buffer loaded into the buffer (BF) wells would interfere with immobilization of a molecule on the sensors, as is the case for serum.

To determine kinetics in both buffer and serum, a single-cycle kinetic (SCK) assay was performed using a HuCAL recombinant antibody binding to eGFP. The capture of the eGFP was consistent between all lanes and rounds of the same buffer condition (PBS-T or serum). Similar to the blocking step, a bulk shift is observed for eGFP when using serum as the running buffer due to the RI difference between the serum and the ligand prepared in PBS-T. Figure 4 shows a representative example of ligand capture and analyte binding for active and reference sensors when PBS-T or serum are used as the running buffer.

Buffer	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
PBS-T	$4.44 \times 10^5 \pm 9.69 \times 10^4$	$4.01 \times 10^{-3} \pm 1.66 \times 10^{-4}$	9.31 ± 1.82
100% Serum	$5.70 \times 10^5 \pm 6.02 \times 10^4$	$8.70 \times 10^{-3} \pm 2.52 \times 10^{-3}$	15.4 ± 4.7

Table 1: *Binding kinetic rate constants for eGFP and HuCAL recombinant antibody on Alto.*

The sensorgram also demonstrates the low susceptibility of non-specific binding (NSB) to the anti-GFP VHH surface as evidenced by the minimal response in the reference channel for the HuCAL analyte. Complete regeneration of the ligand and bound analyte was achieved with 10 mM glycine-HCl, pH 1.5 demonstrating the reusability of the sensor surface for multiple rounds of binding. The ligand, eGFP, shows minimal dissociation following binding to the anti-GFP VHH surface, highlighting the rigidity of the capture surface.

Average kinetic constants and binding affinity were calculated from data obtained on one cartridge with Alto; each condition was run in duplicate lanes across two rounds for n = 4. A representative example of a sensorgram for standard running buffer and serum as running buffer are shown in Figures 5A and 5B, respectively. The data were fit to a Langmuir 1:1 binding model analyzed in the Nicosystem software. Kinetic parameters for both conditions are reported in Table 1 and demonstrated excellent reproducibility across replicates. From the kinetic analysis when PBS-T was used as the running buffer, association and dissociation rate constants (k_a and k_d) measured were $4.44 \times 10^5 \pm 9.69 \times 10^4$ $M^{-1}s^{-1}$ and $4.01 \times 10^{-3} \pm 1.66 \times 10^{-4}$ s^{-1}, respectively, resulting in a K_D of 9.31 ± 1.82 nM. From the kinetic analysis when serum was used as the running buffer, association and dissociation rate constants (k_a and k_d) measured were $5.70 \times 10^5 \pm 6.02 \times 10^4$ $M^{-1}s^{-1}$ and $8.70 \times 10^{-3} \pm 2.52 \times 10^{-3}$ s^{-1}, respectively, resulting in a K_D of 15.4 ± 4.70 nM. The difference in composition of the running buffers showed a 1.65x difference in affinity, predominantly due to the faster off rate observed in serum, which aligns with previous kinetics studies done in serum running buffers[1-2].

Conclusions

The Alto Digital SPR system is uniquely suited to measure binding kinetics for biomolecular interactions in complex matrices, including undiluted human serum. The use of a disposable cartridge prevents common maintenance issues, including clogging and cross-contamination, that occur when performing similar experiments on traditional SPR instruments. Alto's unique and fully automated digital microfluidic platform enables analysis of up to 48 data points in a single cartridge, significantly reducing the time, sample volume, and overall cost of kinetic analysis. Experiments were completed in under 5 hours, requiring only 30 minutes of hands-on time for setup and analysis. Each interaction required only 2 µL of sample, 100x less than conventional SPR. Only 1.44 mL of running buffer total is required for analysis, allowing undiluted serum to be used.

The interaction between eGFP and a HuCAL recombinant antibody for eGFP was analyzed using PBS-T and undiluted serum as the running buffer. Kinetics were determined in running buffer and serum, with affinities of 9.31 nM and 15.4 nM, respectively. We have demonstrated a novel approach to measuring high-quality kinetics of an antibody-antigen interaction using undiluted serum as the running buffer, enabled by Alto's minimal sample volume and maintenance-free microfluidic technology. Analyzing biomolecular interactions in relevant matrices is critical for understanding the mechanism of action of these therapeutics inside the body and Alto's novel technology provides researchers an effective, easy to use and high throughput tool for this type of analysis

References

1. Zhang P, Ma G, Wan Z, Wang S. Quantification of Single-Molecule Protein Binding Kinetics in Complex Media with Prism-Coupled Plasmonic Scattering Imaging. ACS Sens. 2021 Mar 26; 6(3): 1357-1366.
2. Yao D, Yu H, Kantor AB, et al. Differential Binding Kinetics for Evaluating Immune Checkpoint Inhibitors in Serum. bioRxiv; 2021.