TECH NOTE

Temperature dependence of antibody-antigen binding kinetics on Alto Digital SPR

Abstract

Biomolecular interaction assays are often conducted at subphysiological temperatures due to equipment limitations, however, temperature significantly influences biomolecular behavior, structure, function, and interactions. For certain applications, such as vaccine development, determining binding kinetics at physiological temperature is critical. In this application note, we demonstrate the ability of the Alto™ Digital SPR™ platform to evaluate the temperature dependence of binding kinetics for an Influenza A hemagglutinin–antibody pair by measuring binding at both 25°C and 37°C. These results demonstrate Alto's ability to capture physiologically relevant kinetics and highlights the importance of measuring at physiological temperatures during development.

Introduction

Molecular binding interactions are fundamental in biological systems, and characterizing their binding kinetics and affinity is essential for biosensing, drug development, and therapeutic applications. Due to molecular dynamics, these interactions have an intrinsic dependency on temperature. Proteins may misfold, aggregate, or alter their conformation at different temperatures, impacting the conclusions from vital assays. For this reason, biomolecular characterization at physiological temperature is critical for obtaining accurate, biologically relevant insights into the behavior, structure, and function of biomolecules. This is particularly important for therapeutic interactions such as antibodies targeting viral epitopes, which facilitate the development of effective, life-saving vaccines.

Altering assay temperature can also allow for improved kinetic fitting of high affinity interactions that have prohibitively slow off-rates. In order to conduct high-quality kinetic analysis of a binding curve, it is recommended to use a dissociation time long enough to observe a 5% decrease in response. This is straightforward for binding interactions with faster dissociation rates, but can be challenging for interactions where the dissociation rate is slower. For example, if the dissociation rate constant (k_d) of a binding interaction is 10^{-6} s^{-1}, it would take 14.2 hours of dissociation time to measure 5% dissociation. Experiment times of this length are not practical, so this rule is not followed in these instances. This is a well-known limitation of SPR and kinetic binding assays in general that can be overcome by conducting assays at higher temperatures. Running tests at physiological temperature rather than room temperature influences the interactions kinetics, often by speeding up dissociation, allowing for more accurate measurement of high affinity binding interactions. This is particularly useful when studying antibody-antigen interactions, which can have affinities in the low pM range.

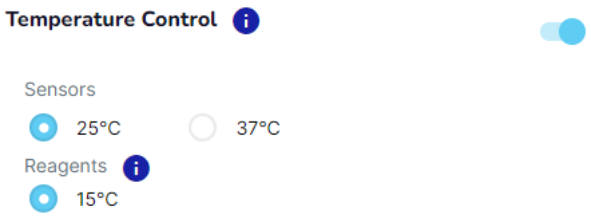

Figure 1: *Temperature options available on the Nicosystem software.*

Nicoya's Alto Digital SPR platform has the option to run assays at 25°C or 37°C (Figure 1), providing researchers the flexibility to characterize binding interactions for both *in vitro* and *in vivo* applications. This application note demonstrates the utility of measuring high-affinity

biomolecular interactions at physiological temperature, specifically, kinetic analysis is performed for an antibody against Influenza A H5N1 hemagglutinin (HA), a popular target for influenza vaccine development (Figure 2). Experiments performed at both 25°C and 37°C demonstrate a decrease in binding affinity under clinically relevant conditions.

Materials & Equipment

Materials

- Alto 16-Channel Instrument with Nicosystem Pro Software (ALTO16)
- Alto 16-Channel Carboxyl Cartridge (KC-CBX-CMD-16)
- Running Buffer: PBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-PBST) + 5 mM EDTA
- Carboxyl Surfacing Kit: cleaning, normalization, activation (ALTO-R-CBX-SURF)
- Human/rabbit IgG VHH Capture Kit (ALTO-R-VHH-HR-KIT)
- Regeneration Solution: 10 mM Glycine-HCl, pH 1.5 (ALTO-R-GLYHCl-1.5)
- Influenza A H5N1 Hemagglutinin/HA rabbit antibody (Sino Biological Cat# 86001-RM02)
- Recombinant Influenza A H5N1 Hemagglutinin/HA Protein (Sino Biological Cat# 11048-V08H1)

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, a Capture Kinetics experiment with multi-ligand format was designed and saved in the Nicosystem. Sensor zone temperature was set to 25°C or 37°C where applicable, while the reagents zone was kept at 15°C to maintain sample integrity.
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".

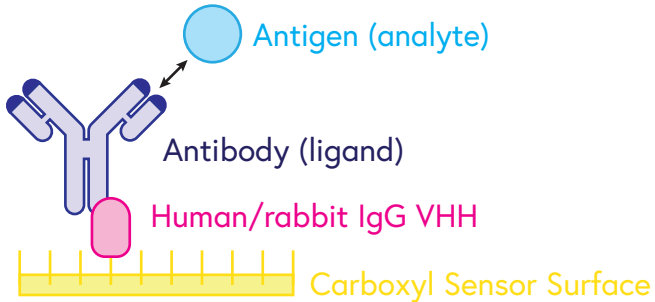

Figure 2: *Schematic representation of the interaction characterized in the tech note.*

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 200 mM EDC/NHS for 600 s.
4. 2.5 µg/mL human/rabbit IgG VHH diluted in 10 mM MES, pH 6.0 was immobilized onto all sensors for 600 s.
5. All sensors were blocked with 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. 0.1 µg/mL samples of Anti-H5N1 HA antibody RM02 in the running buffer were introduced to each even-numbered active sensor for 300 s.
8. Alto executed five automated H5N1 HA serial dilutions on the cartridge. Each sample was diluted from 150 nM stock, producing 0.617 nM, 1.85 nM, 5.55 nM, 16.6 nM, and 50 nM solutions in the running buffer.
9. The lowest H5N1 concentration was exposed to each sensor for 300 s, followed by dissociation in the running buffer for 600 s at 37°C and 900 s at 25°C, The dissociation step is followed by a 60 s regeneration step with 10 mM glycine-HCl, pH 1.5.
10. Step 9 was repeated for the remaining four H5N1 HA analyte concentrations, which constitutes a full multi-cycle kinetics (MCK) round.

Results & Discussion

In this study, Nicoya's Human/rabbit IgG VHH Capture Kit was immobilized via amine coupling onto both the reference and response sensors of the carboxyl cartridge. Figure 3 shows an immobilization overlay for the cartridge run at 25°C, with an average immobilization level of 3185 RU for the human/rabbit IgG VHH. Table 1 summarizes the average immobilization level and standard deviation for this step as well as the ligand capture step. Data acquired for VHH immobilization at 37°C are listed in Table 1, but not displayed as the results are analogous to the 25°C data. The low standard deviation (<4% in both tests) of the immobilization averages highlights the reproducibility of this step.

After the capture surface was created, MCK assays using H5N1 HA antigen binding to a captured rabbit IgG (RM02) were performed. The recapture of the rabbit IgG ligand was consistent across all five analyte concentrations and regenerations, with a sample cycle shown for each temperature in Figure 4A and 4B. This figure also demonstrates the low susceptibility of the sensor surface for non-specific binding, as evidenced by the minimal response in the reference channel for the H5N1 HA analyte. Complete regeneration of the ligand and bound analyte was achieved with 10 mM glycine-HCl, pH 1.5 demonstrating the reusability of the capture surface.

Temperature	2.5 µg/mL human/ rabbit IgG VHH	0.2 µg/mL RM02
25°C	3185 ± 64 RU	141.3 ± 14.0 RU
37°C	3123 ± 124 RU	98.2 ± 13.4 RU

Table 1: *Immobilization level average and standard deviation of human/rabbit IgG VHH and capture of RM02 across entire cartridge.*

Kinetic values were calculated based on a single multi-ligand MCK test with 16 total interactions for each temperature. A representative example of sensorgrams acquired at 25°C and 37°C are shown in Figure 5A and 5B, respectively. The data were fit to a Langmuir 1:1 binding model applied by the Nicosystem software. Kinetic parameters for data obtained are reported in Table 2 and demonstrated excellent reproducibility across all replicates. From the kinetic analysis, association (k_a) and dissociation (k_d) rate constants for MCK at 25°C were determined to be $3.15 \times 10^4 \pm 6.63 \times 10^3$ $M^{-1}s^{-1}$ and $3.10 \times 10^{-5} \pm 8.02 \times 10^{-6}$ s^{-1}, respectively, resulting in a K_D of 0.984 ± 0.155 nM. For the MCK experiment at 37°C, k_a and k_d were determined to be $7.18 \times 10^4 \pm 1.13 \times 10^4$ $M^{-1}s^{-1}$ and $1.75 \times 10^{-4} \pm 2.13 \times 10^{-5}$ s^{-1}, respectively, resulting in a K_D of 2.45 ± 0.244 nM.

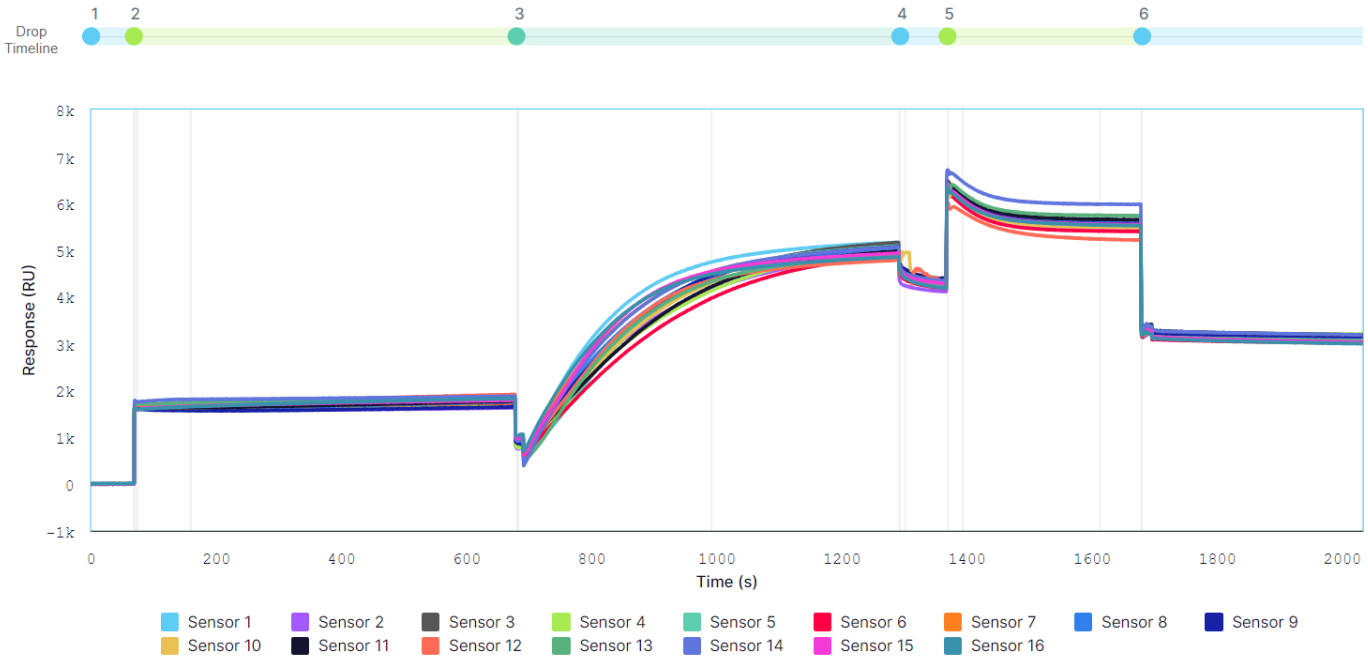

Figure 3: *Activation of the 16 channels with 200 mM EDC/NHS from Nicoya's Carboxyl Surfacing Kit, followed by immobilization of 2.5 µg/mL of human/rabbit IgG VHH in 10 mM MES, pH 6.0 and blocking of sensors with 1 M ethanolamine. This sensorgram was generated at 25°C, equivalent data was generated at 37°C as listed in Table 2. The image was generated in the Nicosystem software.*

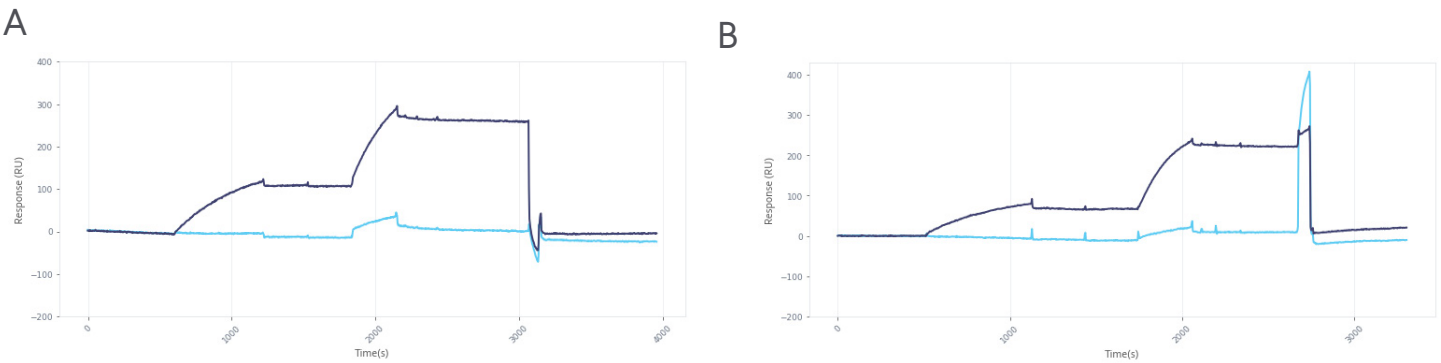

Figure 4: *Reference (light blue trace) and active channel (dark blue trace) binding data showing binding of 50 nM H5N1 HA to captured RM02 antibody at A) 25°C and B) 37°C. Longer dissociation time was used at 25°C due to slower off-rate. Following the dissociation of the analyte concentration, regeneration was achieved with glycine-HCl, pH 1.5.*

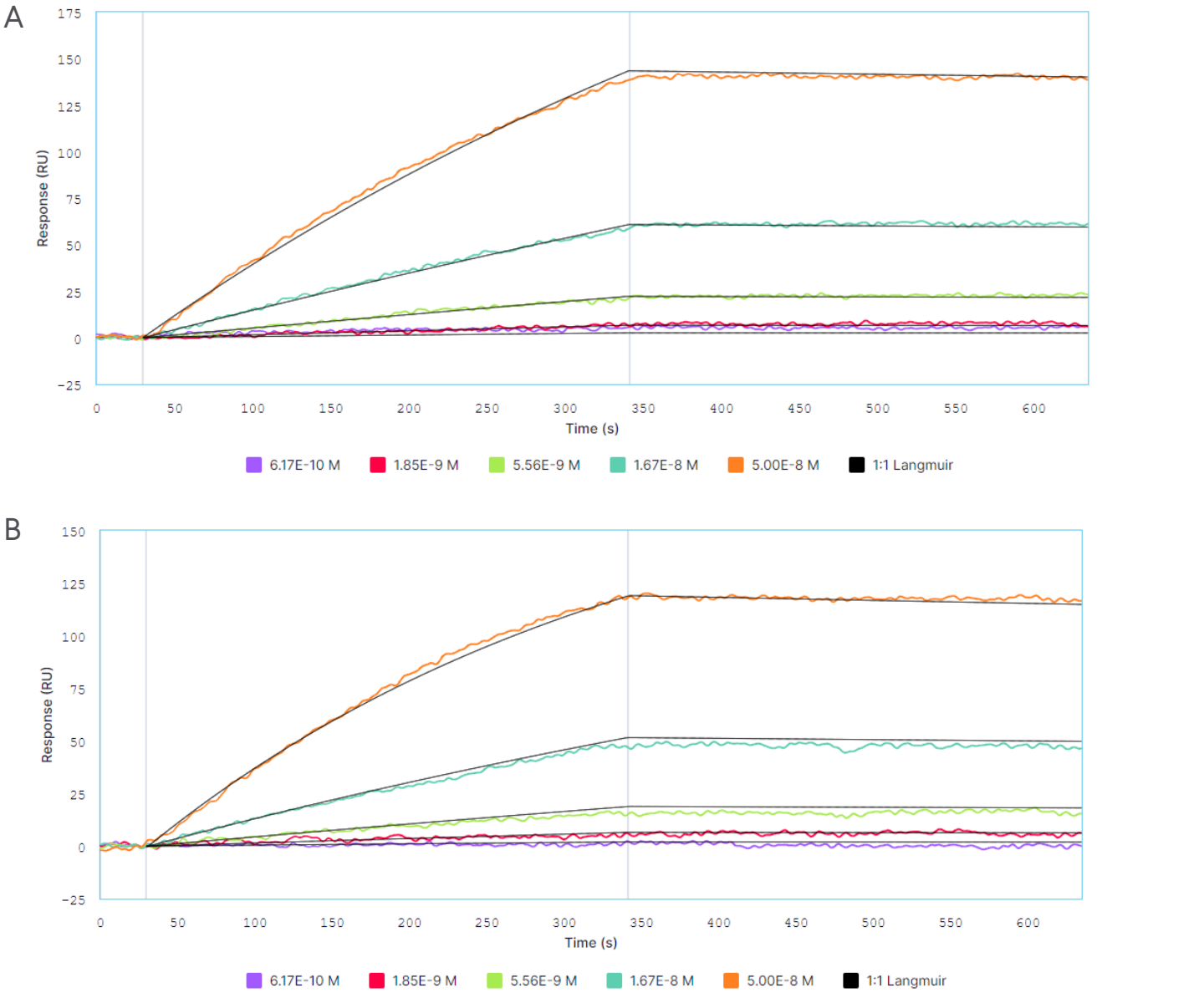

Figure 5: *Multi-cycle kinetics of H5N1 HA (analyte) binding to captured RM02 (ligand) on Alto at A) 25°C and B) 37°C. The analyte was titrated from 0.617 nM to 50 nM. Black curves are the Langmuir 1:1 binding fit model generated by the Nicosystem software.*

Temperature	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
25°C	$3.15 \times 10^4 \pm 6.68 \times 10^3$	$3.10 \times 10^{-4} \pm 8.02 \times 10^{-6}$	0.984 ± 0.155
37°C	$7.18 \times 10^4 \pm 1.13 \times 10^4$	$1.75 \times 10^{-4} \pm 2.13 \times 10^{-5}$	2.45 ± 0.24

Table 2: *Binding kinetic rate constants for H5N1 HA and RM02 antibody on Alto (n=16).*

This binding interaction showed an approximately 2.3-fold increase in k_a and an approximately 5.6-fold increase in k_d with increasing temperature. These two competing differences resulted in a modest decrease in affinity (K_D) of approximately 2.5-fold from 0.984 nM to 2.45 nM when increasing temperature from 25°C to 37°C. This indicates that the antibody-antigen complex is more stable at the lower temperature of 25°C. Using the dissociation rate equation for the k_d measured at each temperature, 5% dissociation is reached after less than 5 minutes at 37°C, while taking almost 30 minutes at 25°C.

The general trend observed of faster association and dissociation rates with increasing temperature is aligned with expected thermodynamic principles; an increase in motion of the molecules results in a faster interaction. The results presented here are consistent with several past studies done using SPR and other binding assays, which have reported an increase in k_a and k_d with increasing temperature. Furthermore, these studies have generally observed a larger increase in k_d than k_a, resulting in a lower overall binding affinity[1-3].

Conclusions

In summary, the temperature dependence of binding kinetics for an Influenza A hemagglutinin and antibody binding pair was demonstrated on Alto Digital SPR by measuring kinetics at both 25°C and 37°C. Our analysis concludes that increasing temperature increases both k_a and k_d, ultimately lowering the binding affinity and overall stability of the interaction due to the greater increase in k_d than k_a. These results highlight the importance of performing biomolecular characterization assays at physiological temperatures to draw accurate conclusions on the behavior, structure, and function of biomolecules. These results also provide evidence that kinetic analysis of high affinity binding interactions may be improved at higher temperature due to the increase in k_d. This study demonstrates that Alto is a robust platform for characterization of high-affinity biomolecular interactions at physiological temperature.

References

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