APPLICATION NOTE

nicoya

The dynamic workflow of Alto™ digital SPR for Influenza vaccine development

Summary

Rapid changes in influenza antiviral target proteins due to antigenic drift result in the cloaking of the influenza virus from the immune system of vaccinated hosts. Hence, annual formulation updates for influenza vaccines and related antibody therapies are required to preserve immune recognition against different influenza subtypes. Rapid development and thorough characterization of antibody vaccine candidates for influenza viral antigens are essential for treating and preventing potential outbreaks. Here, Nicoya's Alto™ digital SPR is used across multiple stages of development including crude library screening, quantitation in serum, epitope binning and kinetics to characterize antibody candidates against Influenza A H5N1 Hemagglutinin (HA).

Introduction

Influenza, or "the flu", is a contagious viral infection caused by the influenza virus. Of the four types (influenza A, B, C, and D), the two main flu viruses impacting humans are influenza A and B, both being highly contagious and routinely causing seasonal epidemics. Furthermore, influenza A viruses are the only known to cause flu pandemics, with all five historical instances since 1900 being associated with influenza A. With the constant evolution of viruses, binding and characterization studies of viral antibodies and antigens are critical to managing and preventing future viral outbreaks. In addition, these studies advance our understanding of viral antigenic drift and the diversity of flu-related antibodies. Surface plasmon resonance (SPR) is a powerful tool for these binding studies, supporting the development and characterization of antibody vaccine candidates.

Alto is the world's first SPR instrument to integrate digital microfluidics (DMF) with nanotechnology-based biosensors. Alto has a 16-channel design that provides high-throughput analysis of up to 48 unique targets while further streamlining user workflows with automated sample dilutions and disposable fluidics. Each test requires only 1 µg of antigen and 100 ng of antibody, which is up to 200X less than what is needed for traditional SPR instruments and bioassays. This application note demonstrates how Alto is an ideal platform for vaccine research through all stages of development, including crude library screening, quantitation in serum, binding kinetics characterization, and epitope binning of Sino Biological's influenza products.

Background

Influenza A Hemagglutinin (HA)

Influenza viral hemagglutinin is an integral membrane glycoprotein critical in viral infection. It binds the virus to the HA protein receptor-binding domain (RBD) on target cells, which will engulf the virus. HA then fuses its membrane with the endosomal membrane of the cell, depositing the viral genome into the cytoplasm. Therefore, the HA protein RBD is critical for vaccine development and a popular target for antibodies due to its ability to directly inhibit the binding of the virus to the host cell receptor. In addition, changes in this protein are essential for the continual development of vaccines. Due to the high frequency of antigenic drift or shift among different influenza strains, broad-spectrum influenza antibodies are particularly desired for flu diagnostics.

Vaccine Development with Alto

The vaccine development process is long, labor-intensive, and expensive. It involves the generation of large-scale libraries of chemical and biochemical compounds, such

as antibodies. Antibody production using hybridoma or phage display technologies occurs in complex media types, including cell lysate and serum. Evaluating the binding activity of these large antibody libraries efficiently can be challenging with traditional SPR instruments or characterization techniques.

Nicoya's Alto system uses digital microfluidics (DMF) to deliver automatically diluted sample droplets to SPR sensors for effortless real-time characterization of biomolecular interaction analysis, including quantitation, screening, epitope binning, and binding kinetics. Alto has 16 independently addressable channels for high throughput. Complete analysis using any experiment method can be done using as little as 2 µL of crude or pure samples. There are no manual dilutions, tagging, degassing, cleaning, or strenuous assay setup. Alto has a diverse suite of pre-configured protocols befitting all stages of vaccine development, as shown in Figure 1.

Materials & Equipment

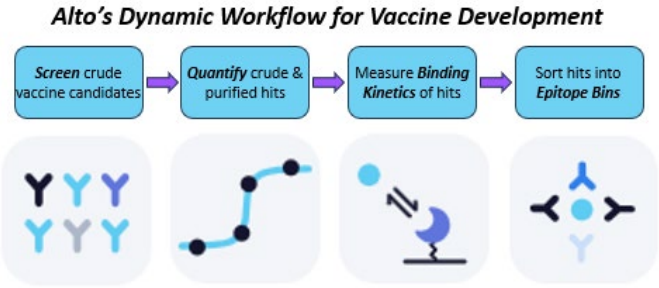

Figure 1: *Alto's dynamic and comprehensive workflow spans across many relevant assay types required for vaccine development, making it a valuable tool for the discovery and characterization of vaccine candidates.*

- 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) and PBS-TE (10 mM EDTA) + 0.1% BSA, pH 7.4
- Alto Carboxyl Surfacing Kit: 10 mM HCl (cleaning), normalization solutions, EDC & NHS (activation), 1 M Ethanolamine (blocking) (ALTO-R-CBX-SURF)
- Immobilization Buffers: 10 mM Sodium Acetate pH 5.5 (ALTO-R-IMB-5.5), 10 mM Sodium Acetate pH 5.0 (ALTO-R-IMB-5.0)
- Regeneration Buffer: 10 mM Gly-HCl pH 1.5 (ALTO-R-GLYHCl-1.5)
- Protein A Capture Kit (ALTO-R-PROA-KIT)
- Reagents
  - Influenza A H5N1 Hemagglutinin/HA Antibodies (Sino Biological Cat# 11048-MM11, 11055-MM08, 11055-RM06, 86001-RM01, 11048-MM04, 11048-MM10, 86001-RM02, 11048-MM03, 11048-RM07, 11048-RM08, 11048-RM09, 11048-MM06, 11048-MM14, 11048-MM01, 11048-MM05, 11715-RP01)
  - Recombinant Influenza A H5N1 Hemagglutinin/HA Protein (Sino Biological Cat# 11048-V08H1)
  - Influenza A H3N2 (A/Brisbane/10/2007) Hemagglutinin/HA Protein (His Tag) (Sino Biological Cat# 11056-V08H)
  - Normal Rabbit Serum (Jackson Immuno Research, Cat # 011-000-120)
  - NanoCaptureLigand human IgG/Rabbit IgG specific VHH, biotinylated (ChromoTek: shurbGB-1-100)
  - NanoCaptureLigand mouse IgG specific VHH, biotinylated (ChromoTek: smsG1B-1-100)

Methods

Screening

The first step in characterizing the vaccine candidates was screening for hits in a yes/no binding assay (Figure 2). Influenza A H5N1 HA antibody candidates were screened using the Alto capture screening protocol. In capture screening, a secondary binder, such as the mouse IgG-specific VHH used here, captures the antibodies out of serum. The antigen is then screened against the captured antibodies for binding activity. All 48 antibodies can be tested against a second control antigen in the same test using the expanded throughput mode.

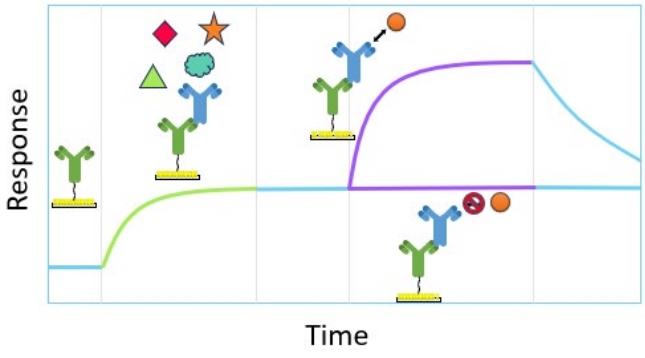

Figure 2: *Sensorgram showing a capture screening test on Alto. The surface is functionalized with an anti-IgG antibody or IgG-specific VHH, which captures antibody candidates out of crude matrices (green curve). The captured candidate is tested against the antigen for binding (purple curve). Blue curves are buffer rinses.*

Quantitation

Alto's quantitation protocol was used to determine the concentration of hits in complex media (Figure 3). In this assay, the antigen is first immobilized on the sensor surface, followed by a purified antibody standard of a known concentration to produce a calibration curve. The calibration curve consists of 10 concentrations created using a 3x serial dilution. Finally, binding curves of up to 40 unknowns are measured. The calibration curve is used to determine the concentration of the unknown based on the binding response measured.

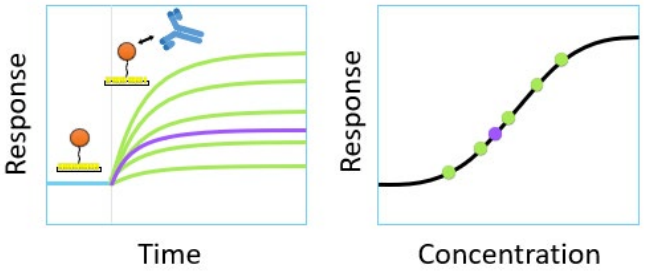

Figure 3: *Sensorgram (left) and calibration curve (right) showing a typical quantitation assay on Alto. Standards are shown in green, and the unknown is shown in purple. Buffer equilibration is shown in blue, and the calculated calibration curve is shown in black.*

Kinetics

After quantifying the hits, the kinetics were characterized on Alto in several different formats (Figure 4). Vaccine candidates were captured using a mouse IgG-specific VHH immobilized on the sensor (capture kinetics). These protocols were run in both single-cycle kinetics (SCK) and multi-cycle kinetics (MCK) formats. An SCK, or titration assay, runs five consecutive analyte concentrations followed by a single, long dissociation phase then regeneration, resulting in a single curve for each interaction. An MCK, or standard format assay, runs five analyte concentrations but with a dissociation phase and a regeneration between each one, between each one, creating five separate curves for each interaction. Purified antibodies were used in all kinetic assays to maximize the accuracy of the kinetic data.

Binning

The epitope diversity of vaccine candidates was evaluated with a classical sandwich assay (Figure 5). Epitope binning is an assay used to characterize the simultaneous binding of antibodies to an antigen, tested pairwise, to determine whether they block one another's binding to the same epitope of the antigen. If the binding of one antibody to the antigen prevents the binding of another antibody, both antibodies can be clustered into groups (or bins) that compete for the same or similar epitope. If both antibodies can bind to the antigen, then they are considered to bind to distinct, non-overlapping epitopes. The Alto epitope binning protocol utilizes a classical sandwich format. In the classical sandwich assay, the first antibody (surface antibody) is immobilized onto the sensor. This is followed by the injection of the antigen and, finally, the second antibody (solution antibody).

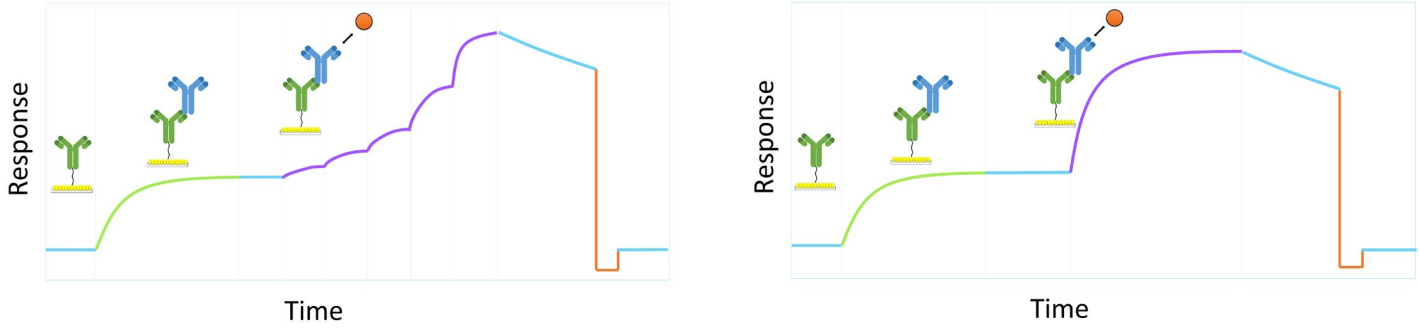

Figure 4: *Here are two sensorgrams showing a typical capture single-cycle kinetics assay (left) and a single round of a capture multi-cycle kinetics assay (right) on Alto. For single cycle kinetics, the surface is regenerated once after all five analyte concentrations, and for multi-cycle kinetics the surface is regenerated between each of the five analyte concentrations. In both cases, the surface is functionalized with an anti-IgG antibody or IgG-specific VHH, followed by the capture of the antibody candidates. The captured candidates are tested against the antigen, and binding curves are fit to determine the interactions' on rate, off rate, and affinity.*

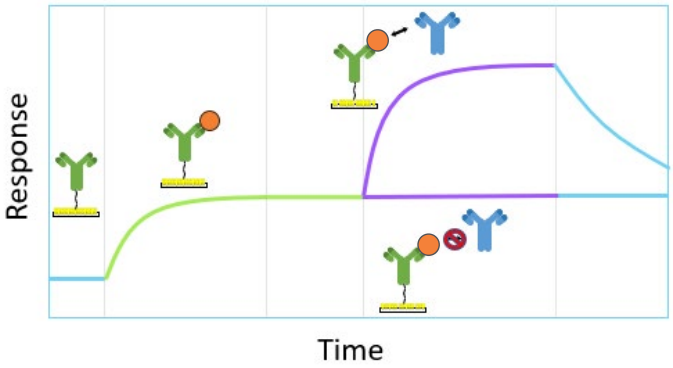

Figure 5: *A sensorgram of a typical binning assay on Alto is shown here. The binning assay follows a classical sandwich format, where sensors are functionalized with an antibody that captures the antigen. A second antibody is then tested against the captured antigen.*

Results & Discussion

Screening

In the capture screening assay, seven anti-H5N1 mouse antibodies spiked into normal rabbit serum were tested in triplicate. Serum-only and anti-H3N2 HA rabbit antibody spiked serum were used as negative controls. The mouse IgG-specific VHH was immobilized at an average of 3117 RU (Figure 6). Figure 7 shows an example of a sensorgram for lane 1 of the capture screening test. Lanes with anti-H5N1 HA mouse antibodies show capture of the antibody and subsequent binding of the HA antigen, and negative controls do not show any antibody capture or antigen binding. A corrected curve of the H5N1 HA antigen binding to the capture anti-H5N1 HA antibody ligand is shown in Figure 8. A threshold the user may adjust determines whether or not a sample binds. In this case, the threshold was set to 40 RU.

As illustrated in Figure 9, Alto's capture screening assay determined with 100% accuracy which samples contained an anti-Influenza A H5N1 HA mouse antibody and which samples were negative controls. The expansion throughput mode was turned on, and Influenza A H3N2 HA was used as a negative control antigen to ensure all antibodies detected were specific for the H5N1 strain. No false positive binding was observed. The summary table (Table 1) provided in the Nicosystem™ analysis software allowed hits to be ranked by their affinity. The highest affinity hit determined was MM11 (Ab#14), with an average K_D of 7.08 x 10-12 M.

Quantitation

After hits were discovered and ranked from the screening assay, they were quantified using the Alto quantitation assay. Here, the highest ranking hit is quantified using a known concentration of 25 nM spiked into rabbit serum across an entire cartridge of 40 replicates, highlighting the data reproducibility of Alto.

Figure 10 shows the sensorgram view of the corrected binding curves acquired on Alto. This sensorgram includes both the standard concentrations of purified MM11 as well as the 'unknown' MM11 samples in serum. The corrected binding responses of the standard concentrations are used to produce the calibration curve shown in Figure 11. This curve is fit to a 5-parameter logistic model and used to calculate the concentration of the 'unknown' samples based on their binding response.

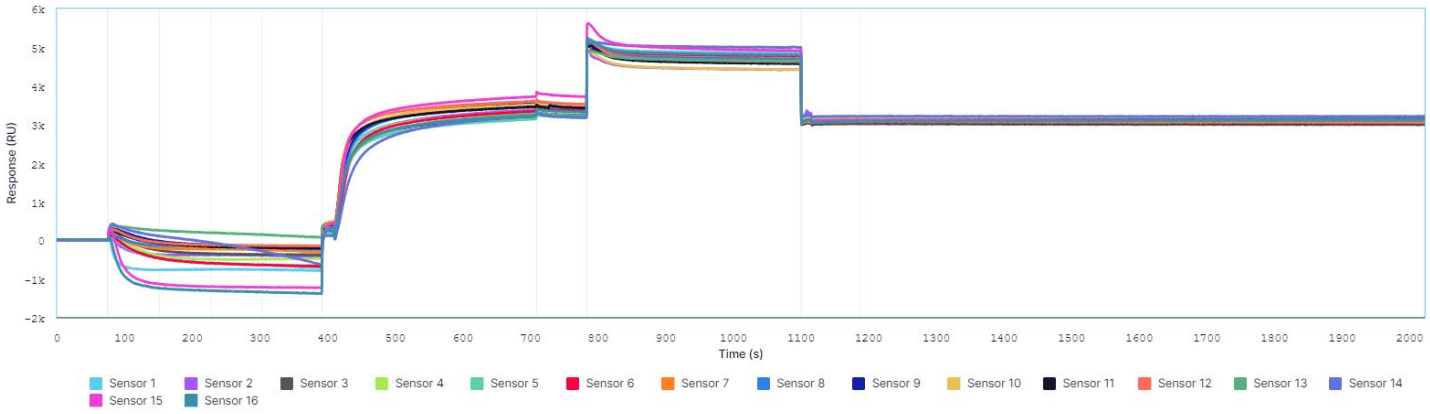

Figure 6: *Activation of response channels with EDC/NHS from Nicoya's Surfacing Kit, followed by immobilization of mouse IgG-specific VHH in 10 mM Sodium Acetate, pH 5.5, and blocking of sensors with 1 M Ethanolamine.*

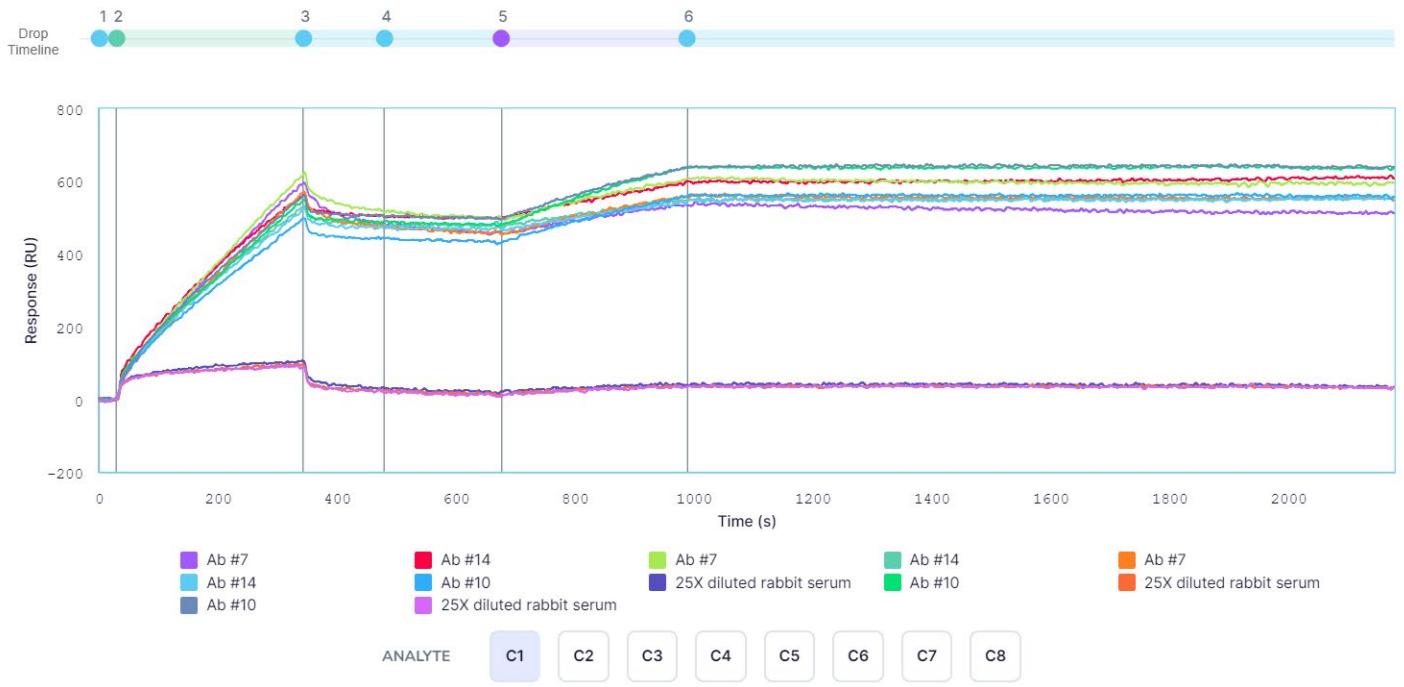

Figure 7: *Series of sensorgrams collected for a single lane of the capture screening assay with high throughput expansion turned on. The sensorgrams show the ligand's capture (or absence of capture) followed by washing with buffer and then antigen binding (or absence of binding). Antibodies loaded in wells D1-I1 are tested against both antigens, resulting in an overlay of twelve curves for each lane of the test and 96 overall.*

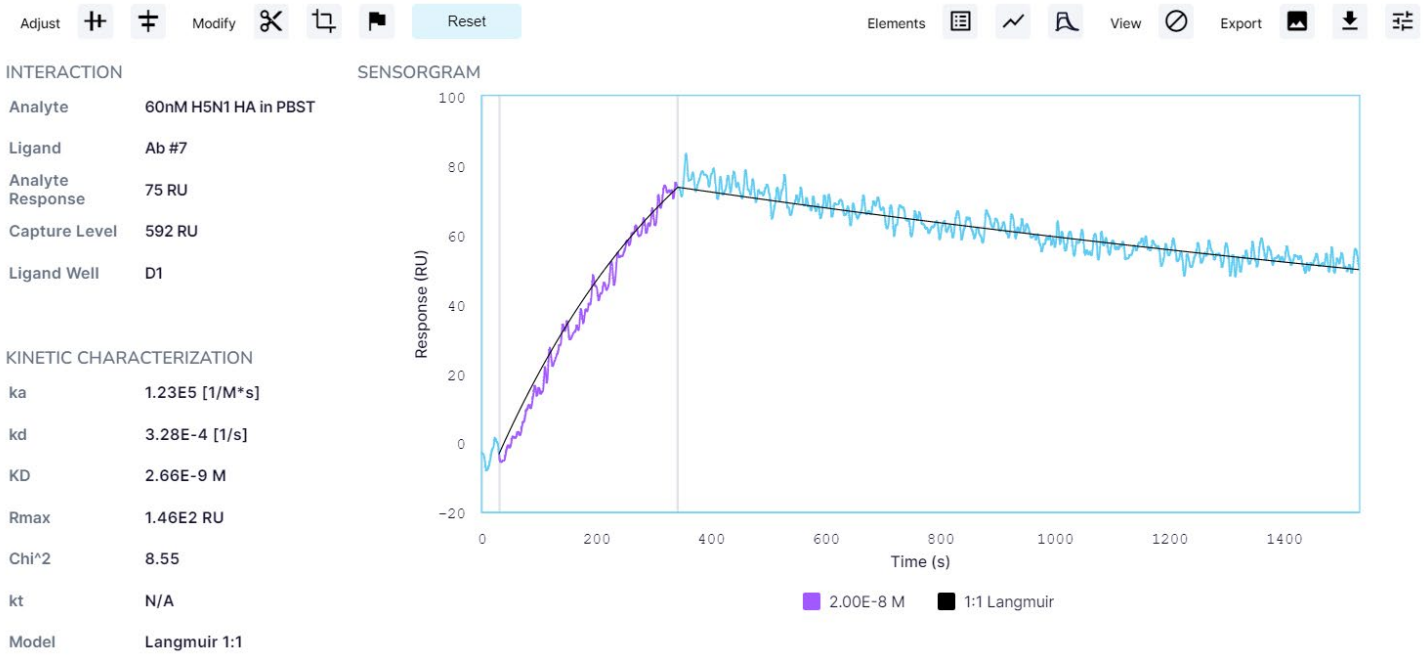

Figure 8: *Sensorgram showing a single antigen interaction from the capture screening test in the Nicosystem analysis software. The association is shown in purple, and the dissociation is shown in light blue. The data were fit to a Langmuir 1:1 binding model analyzed in the Nicosystem analysis software.*

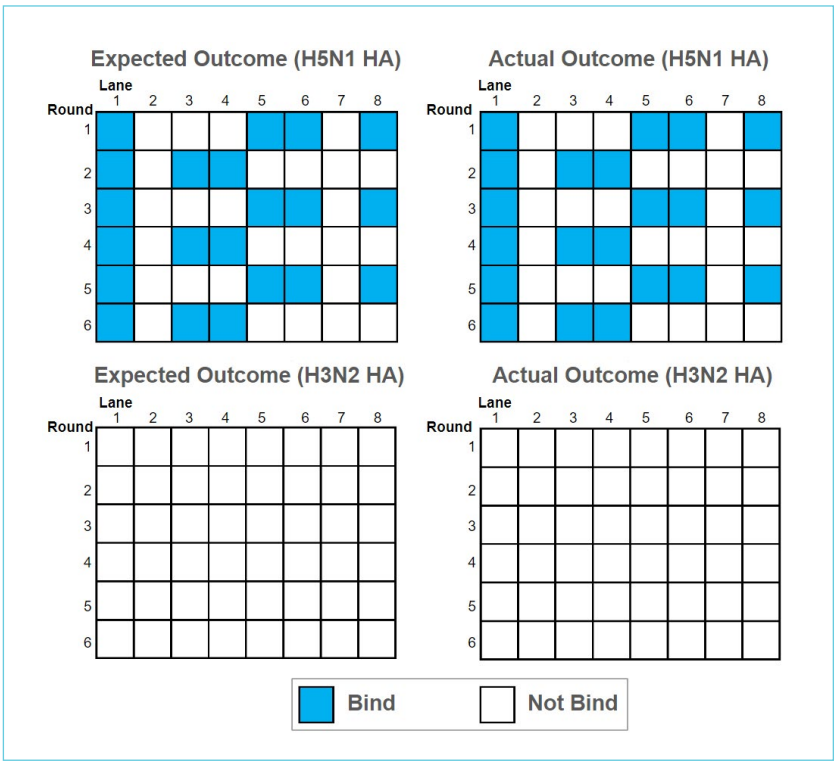

Figure 9: *Capture screening heatmaps showing the expected and observed outcomes for the antibodies tested against the Influenza A H5N1 HA antigen. Expected and observed outcomes for the Influenza A H3N2 HA antigen are also shown below. Interactions were labeled as 'bind' if the analyte response was ≥ 40 RU.*

Well	Sample	Vol (µL)	Conc (C)	C units	Calc Conc	Response	Time (RP 1)	Time (RP 2)	Residual %
E2	Ab 14 in 25x R.S.	3	N/A	nM	26.88	1007.62	216.62	226.62	N/A
F2	Ab 14 in 25x R.S.	3	N/A	nM	26.02	995.22	216.62	226.62	N/A
G2	Ab 14 in 25x R.S.	3	N/A	nM	25.37	985.4	216.62	226.62	N/A
H2	Ab 14 in 25x R.S.	3	N/A	nM	25.15	981.95	216.62	226.62	N/A
I2	Ab 14 in 25x R.S.	3	N/A	nM	25.15	981.95	216.62	226.62	N/A

Table 1: *An example of a screening results table as provided by the Nicosystem analysis software. Only the first nine interactions are depicted in this figure, but the user can scroll through all 96 interactions in the table or export it as a .csv file. The interactions may be sorted by K_D to rank the affinity of the hits.*

Nicosystem's analysis software generates a summarized data table (Table 2), showing calculated concentrations for unknown samples E2-I2. The calculated concentrations ("Calc Conc") of the unknown sample shown had a residual percentage of 2.24% from the actual concentration (Table 3). The average % coefficient of variation (CV) for a single sensor (5 rounds) was 1.90%, and the %CV across an entire cartridge was determined to be 6.59%.

Kinetics

The kinetics of all hits were evaluated in both SCK and MCK formats using a mouse IgG-specific VHH and a rabbit IgG-specific VHH capture surface, depending on the host species of each particular antibody. These tests were run using purified samples to maximize the accuracy of the kinetics determined, but this may also be done in crude samples if desired. Examples of kinetics plots for MM11 in both SCK and MCK format are shown in Figure 12. The Influenza A H5N1 HA antibody was reproducibly captured after each regeneration of the test, and full regeneration was achieved after each cycle using 10 mM Glycine-HCl, pH 1.5, with 0.1% Tween 20.

Kinetic values were calculated based on the sensorgrams obtained in four Alto kinetics assays, with each Influenza A H5N1 HA antibody tested in both SCK and MCK formats. Each test consisted of eight antibodies, one in each of the 8 lanes. Each antibody was tested in a single lane for up to 6 cycles, depending on experiment format. The data were fit to a Langmuir 1:1 binding model analyzed in the Nicosystem analysis software. Kinetic parameters, including k_a, k_d, and K_D, were averaged for all replicates in each lane, and the calculated K_D values are reported and ranked in Table 4. As expected, MM11 is one of the highest affinity binders, although the more robust kinetics determined by these assays show that RM08 is the highest affinity binder.

Binning

Sixteen unique Influenza A H5N1 HA antibodies were screened against the Influenza A H5N1 HA in a classical sandwich binning format. A buffer blank was subtracted

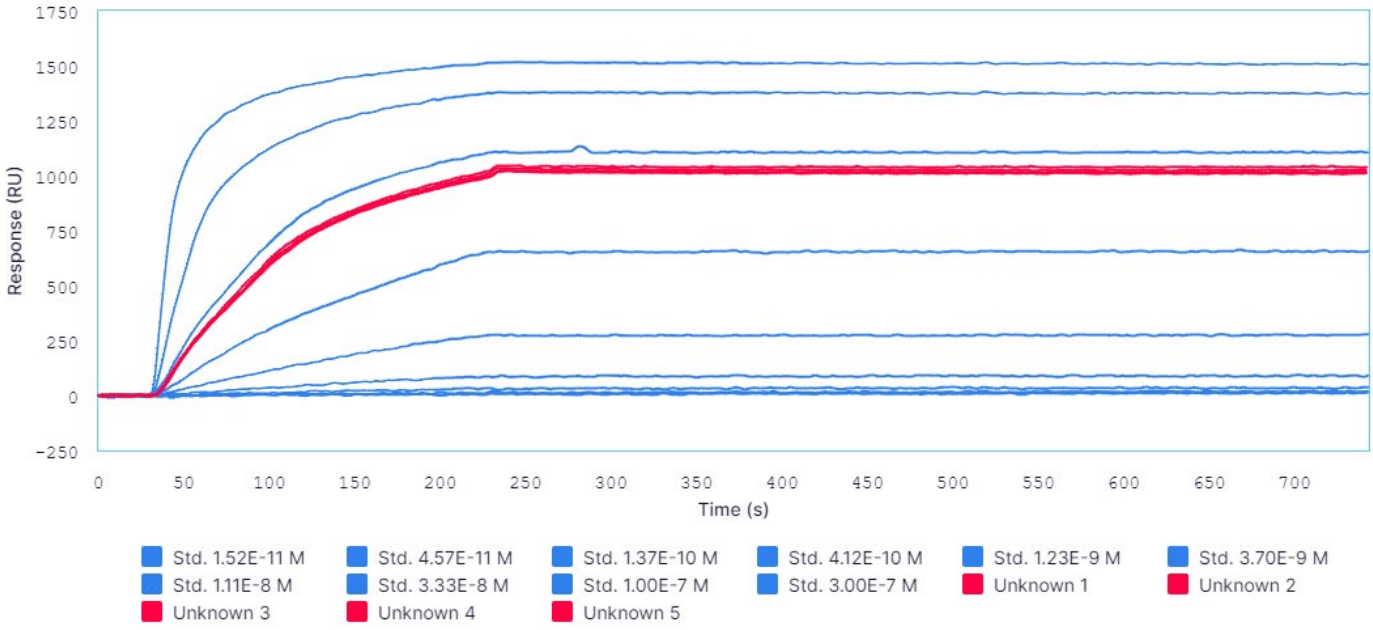

Figure 10: *Sensorgram view of corrected binding curves of Influenza A H5N1 HA antibody MM11 binding to the immobilized Influenza A H5N1 antigen, containing ten three-fold dilutions of the known standard analyte sample (blue curves) and five unknown analyte samples (red curves).*

Well	Sample	Vol (µL)	Conc (C)	C units	Calc Conc	Response	Time (RP 1)	Time (RP 2)	Residual %
E2	Ab 14 in 25x R.S.	3	N/A	nM	26.88	1007.62	216.62	226.62	N/A
F2	Ab 14 in 25x R.S.	3	N/A	nM	26.02	995.22	216.62	226.62	N/A
G2	Ab 14 in 25x R.S.	3	N/A	nM	25.37	985.4	216.62	226.62	N/A
H2	Ab 14 in 25x R.S.	3	N/A	nM	25.15	981.95	216.62	226.62	N/A
I2	Ab 14 in 25x R.S.	3	N/A	nM	25.15	981.95	216.62	226.62	N/A

Table 2: *Summary table from an Alto quantitation assay, showing a single lane of five MM11 replicates. The actual concentration of the 'unknown' antibody used was 25 nM. The time report points indicate the period of time from which the average response was calculated.*

Antibody Cat#	Detected conc., full cartridge (nM)	Residual % (for known conc. of 25 nM)	Average % CV for a single sensor	% CV (all sensors, full cartridge)
MM11	25.56	2.24	1.90	6.59

Table 3: *Quantitation accuracy of Alto for the Influenza A H5N1 HA antibody MM11.*

Sino Cat #	K_D (M)	Sino Cat #	K_D (M)	Sino Cat #	K_D (M)
RM08	5.53E-12	RM09	1.33E-11	RM02	1.37E-10
MM14	7.18E-12	MM04	1.35E-11	MM08	1.73E-09
MM01	1.10E-11	MM06	2.16E-11	RM06	3.11E-09
MM11	1.13E-11	RM07	6.02E-11	MM05	3.15E-09
MM03	1.22E-11	MM10	1.10E-10	RM01	6.54E-09

Table 4: *Kinetics determined for each Influenza A H5N1 HA antibody, sorted by K_D*

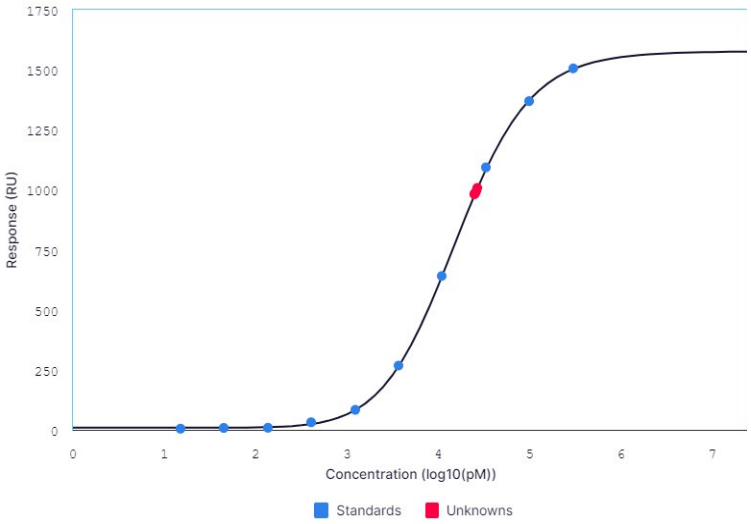

Figure 11: *Calibration curve (black curve) created from plotting the log10 of the concentration for the ten standard analyte concentrations against their binding response (blue points). The unknown concentrations are plotted on the calibration curve (red points), and their concentration is solved using the 5-parameter logistic model.*

from each interaction, where the buffer was used instead of the solution antibody. Additionally, the software subtracts the solution antibody's signal without antigen binding for each sandwich interaction from the solution antibody's signal following antigen binding. Using both the buffer blank and this self-referencing step generates data that is double-referenced. The self-referencing can be toggled on or off in the Nicosystem analysis software. The response level of the secondary antibody was normalized using the antigen binding level across all curves since the binding activity of the antigen to the surface can change over time. The referencing and normalization together created the 'corrected curves' used for binding analysis (Figure 13).

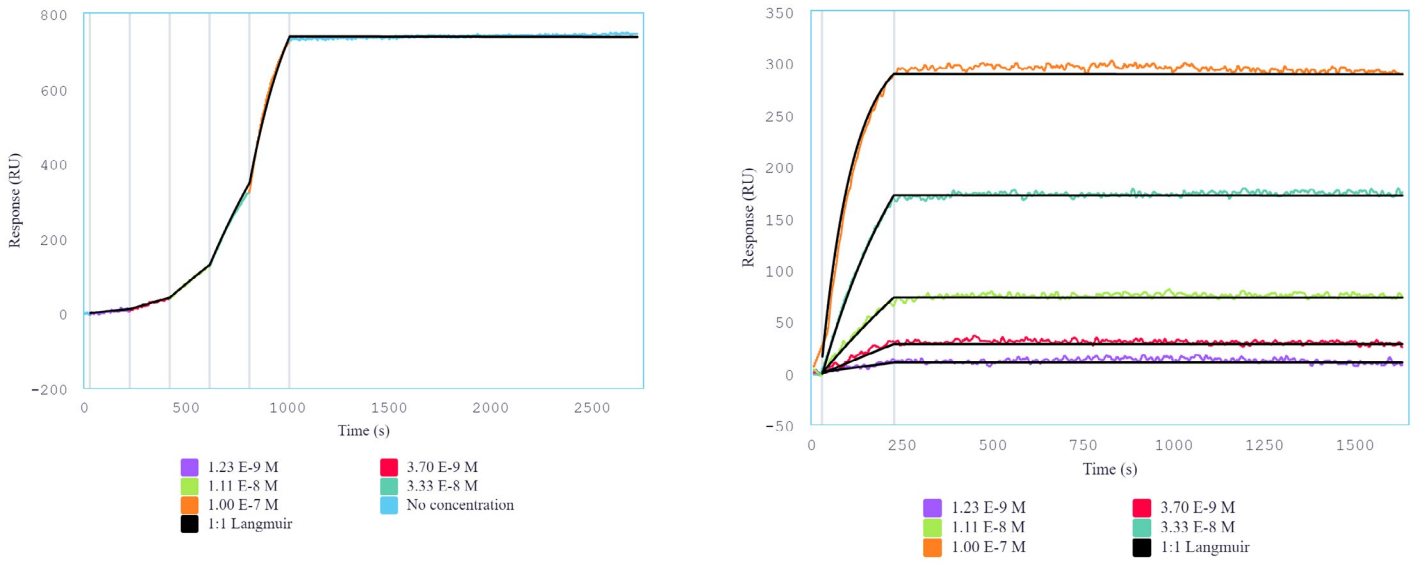

Figure 12: *Single-cycle (left) and multi-cycle (right) kinetics sensorgrams for MM11. Each analyte concentration is represented by a different color, with the light blue in the single-cycle test representing the dissociation. The beginning of dissociation in the multi-cycle plot is denoted by the vertical line at 240 s.*

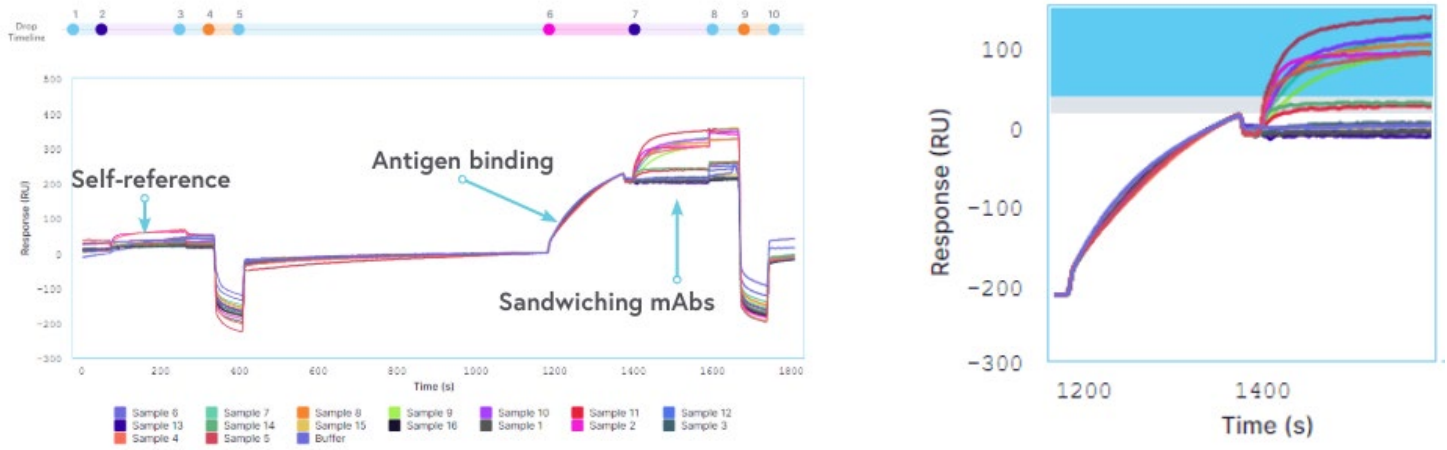

Figure 13: *Left: A representative sensorgram overlay showing all sixteen solution antibodies and buffer blank interacting with a single surface antibody. Solution antibody (or buffer) is passed over the sensor and used for referencing. The surface is then regenerated. This is followed by antigen, solution antibody (or buffer), and a second regeneration. Right: Overlay of normalized antigen binding followed by solution antibody sandwiching or blocked binding. The user can change binding (blue region) and non-binding (white region) thresholds.*

Analysis of secondary antibody binding was done by setting thresholds for the normalized solution antibody responses. Bind, intermediate, and not bind categories were defined based on a 'bind' threshold of 40 RU and a 'not bind' threshold of 30 RU. Anything in between was classified as intermediate binding. The user can adjust these thresholds to suit the response level of their samples. The corrected curves use these thresholds to create a heat map to indicate which analytes bind or do not bind to their respective ligands (Figure 14). This heat map illustrates the results of the 16x16 epitope bin on the Nicosystem analysis software. "Bind" results signify a unique epitope targeted by the pair of antibodies. Antibodies that bound to the antigen to form a sandwich complex were inferred to have non-overlapping epitopes. Antibodies that blocked another antibody from binding to the antigen were deemed to have the same target epitope and 'binned' together. Figure 15 shows this data, sorted by groupings or 'bins' of binders and non-binders, and displays the antibody catalog number. Self-blocking is confirmed by the diagonal, which shows no binders.

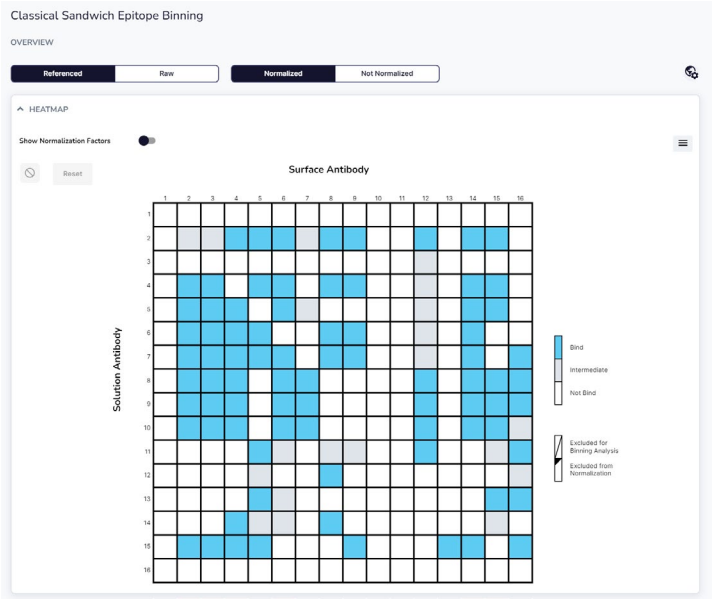

Figure 14: *Epitope characterization analysis for influenza A HA antibodies binding to Influenza A H5N1 HA from the Nicosystem analysis software. The automatically referenced and normalized algorithm is applied and displayed. The degree of binding displayed in the heat map was determined based on the change in response levels after the addition of the solution antibody onto the antigen-bound surface antibody. Bind (blue), intermediate (gray), and not bind (white) categories were defined based on a 'bind' threshold of 40 RU and a 'not bind' threshold of 30 RU (Intermediate is a response between 30 and 40 RU).*

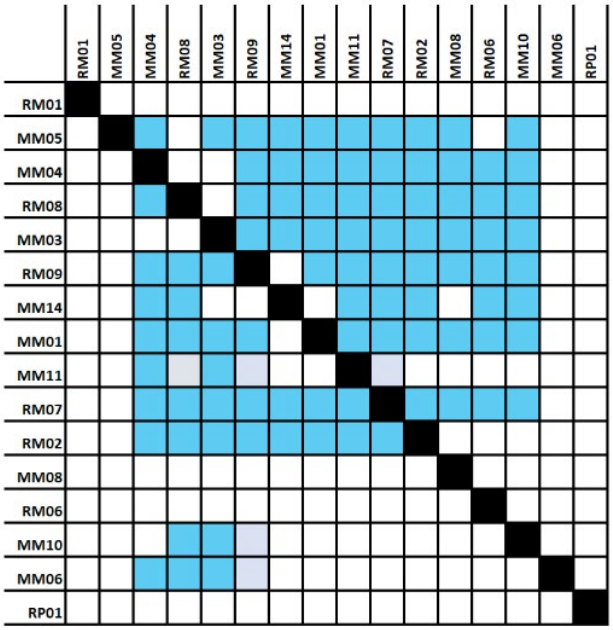

Figure 15: *Epitope characterization analysis for Influenza A HA antibodies binding to Influenza A H5N1 HA, sorted by groupings of binders and non-binders, as well as displaying the antibody catalog number. Self-blocking is confirmed by the diagonal which shows no binders. RP01 is a negative control.*

Conclusions

Using Alto, Nicoya's digital SPR platform, vaccine candidates were successfully screened for hits from serum and ranked by affinity. The highest affinity binder determined from the initial screening test, MM11, was quantified in serum with a residual of 2.24% compared to the expected concentration. Purified hits were further characterized in both SCK and MCK formats to understand the on/off rates of the interactions entirely, and a more robust affinity ranking was determined. From this affinity ranking, it was determined that the MM11 was one of the highest affinity binders, as expected, but RM08 had the highest affinity. Kinetics uses multiple concentrations of analyte, giving more confidence in the measured kinetics than the estimations given by the initial screening assay. Finally, all hits were characterized in an epitope binning experiment. This experiment successfully demonstrated which antibodies had overlapping epitopes.In summary, Alto streamlines SPR analysis by automating sample dilutions, eliminating fluidic maintenance, and reducing sample requirements by up to 200X. Alto's dynamic and comprehensive workflow allows for the discovery and characterization of novel vaccine candidates such as Sino Biological's Influenza A H5N1 HA products.