APPLICATION NOTE

nicoya

Rapid, automated capture screening of influenza antiviral targets using Nicoya Alto® digital surface plasmon resonance

Summary

Rapid screening and kinetic characterization of medium-sized libraries is commonly seen as one of the initial steps within the drug discovery process. Screening assays are used to quickly test and select the best candidates that bind to or affect a desired target. Label-free surface plasmon resonance (SPR) is often used to conduct medium to high-throughput screening to test for real-time binding activity of a variety of biological and chemical compounds. Capture screening is a new assay offered on Alto™, Nicoya's digital microfluidics (DMF) based SPR instrument (Figure 1).

The simple, pre-configured capture screening protocol allows users to establish binding activity of up to 96 interactions on one cartridge with only 2 µL of sample. This application note demonstrates that Alto can bring rapid assay development and minimized instrument maintenance to accelerate your antibody identification and characterization flow, with intuitive software features. This feature is tailored to expedite assay development in biopharmaceutical lead discovery, antibody engineering and preclinical assay development.

Introduction

Influenza, or "the flu", is a contagious viral infection caused by the influenza virus. Influenza A viruses are the only known to cause flu pandemics, with all five historical instances of flu pandemics 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. These studies advance our understanding of viral antigenic drift and diversity of flu-related antibodies.

Influenza A Hemagglutinin (HA)

Influenza viral hemagglutinin is an integral membrane glycoprotein that plays a critical role 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 own membrane with the endosomal membrane of the cell, depositing the viral genome into the cytoplasm. The HA protein RBD is therefore critical for vaccine development and a popular target for antibodies due to their ability to directly inhibit binding of the virus to the host cell receptor. Due to the high frequency of antigenic drift or shift among different influenza strains, broad-spectrum influenza antibodies are particularly desired for flu diagnostics and vaccine development.

Traditional Characterization Techniques

Traditional screening techniques require time-consuming assay development and laborious steps. Compared to such traditional techniques, the pre-configured protocol and easy-to-use software provided by Nicoya Alto ® enables increased efficiency in the development of viral diagnostics and therapeutics. The label-free SPR capture screening protocol on Alto provides reliable antibody screening results with only 2 µL of samples.

Other recent techniques in use such as BLI and traditional SPR suffer from various limitations such as the use of complicated fluidic channels that are cumbersome to operate and maintain. These instruments consume a large amount of sample due to the need to load in 96 or 384 well plates, and the need for multiple wells of solution antibody and antigen per screening test.

Capture Screening on Alto

In Alto's capture screening protocol, up to 48 different ligands can be captured from complex matrices including serum and cell lysate on a single cartridge. The antibodies are then screened against an antigen to characterize yes/no binding, kinetics and affinity ranking of candidates all in the same test. This enables users to do a large amount of antibody characterization in an extremely short amount of time. Additionally, a throughput expansion mode has been included, which allows screening of all 48 antibodies against a second antigen, enabling up to 96 interactions in a single screening experiment. This novel feature is highly effective in characterizing the specificity of antibody candidates. This high throughput expansion mode does not require any additional volume of the antibody solutions, minimizing consumption of precious samples. Capture screening studies were conducted on Alto with 11 unique antibodies in serum against an influenza A HA protein, with the results summarized in this application note.

Advantages of Alto

Alto, a high-throughput SPR platform powered with digital microfluidic (DMF) technology, has a number of advantages that make it ideal for SPR characterization of a wide range of proteins. Alto is a label-free method, eliminating the introduction of a design hurdle 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 100X.

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 also minimizes operation time with simple design & analysis software. Alto's sensors provide a versatile surface for capturing targets with protein A, anti-Fc antibodies, anti-histidine tag antibodies and others, allowing for characterization of a wide range of biomolecular systems from discovery to design.

For all of the above reasons, Alto provides an unprecedented ability to study, develop and optimize vaccines & antibody therapeutics among many other applications in the biopharmaceutical lead discovery, antibody engineering and preclinical assay development.

Materials & Equipment

- Nicoya Alto 16-Channel Instrument (ALTO16)
- Alto 16-Channel Carboxyl Cartridge
- Running Buffer: PBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-PBST) + 3mM EDTA and 1% bovine serum albumin (BSA)
- Alto CBX Surfacing Kit: cleaning, normalization, activation (ALTO-R-CBX-SURF)
- Regeneration Buffer: Gly-HCl pH 1.5 (ALTO-R-GLYHCl-1.5)
- Immobilization Buffer: Sodium Acetate pH 5.5 (ALTO-R-IMB-5.5)
- Sino Biological Reagents
  - Recombinant Influenza A H5N1 Hemagglutinin (HA) antigen (Cat# 11048-V08H1)
  - Recombinant Influenza A H3N2 Hemagglutinin (HA) antigen (Cat# 11056-V08H)
  - Influenza A H5N1 Hemagglutinin/HA Antibodies (Refer to Table 1)
- Normal Rabbit Serum: Jackson Immuno Research: (Cat # 011-000-120)
- NanoCaptureLigand mouse IgG specific VHH, biotinylated (Chromatek: smsG1B-1-100)

Method

This label-free SPR assay was performed using Alto, the first and only DMF powered SPR instrument. 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 pre-configured capture screening application on Alto's user portal, requiring only the input of the sample name and concentration, and then 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 and samples were pipetted into the cartridge wells following software-directed prompts. The experiment was then initiated on Alto, followed by all subsequent steps being automated to completion. The cartridge layout used for the test is shown in Figure 1.

Normalization of sensors with high and low RI droplets was performed first. The sensors were then cleaned with

	1	2	3	4	5	6	7	8	Vol. (µL)
R (Regen)	Regeneration, Glycine-HCl pH 1.5				PBSTE + 1% BSA	20 µg/mL mouse IgG specific VHH in acetate pH 5.5	HCl	PBST	65
A (Reagent)	Low RI Normalization Solution				EDC		NHS		4
B (Reagent)	High RI Normalization Solution				Quench (1 M Ethanolamine)				4
C (Analyte)	60 nM H5N1 HA in PBST	60 nM H5N1 HA in PBST	60 nM H5N1 HA in PBST	60 nM H5N1 HA in PBST	60 nM H5N1 HA in PBST	60 nM H5N1 HA in PBST	60 nM H5N1 HA in PBST	60 nM H5N1 HA in PBST	5
D (Ligand)	20 nM anti-H5N1 HA Ab #7	20 nM anti-H5N1 HA Ab #5	25X diluted rabit serum	20 nM anti-H5N1 HA Ab #6	25X diluted rabit serum	20 nM anti-H5N1 HA Ab #9	25X diluted rabit serum	20 nM anti-H5N1 HA Ab #8	2
E (Ligand)	20 nM anti-H5N1 HA Ab #14	25X diluted rabit serum	20 nM anti-H5N1 HA Ab #11	20 nM anti-H5N1 HA Ab #15	20 nM anti-H5N1 HA Ab #10	20 nM anti-H5N1 HA Ab #4	20 nM anti-H5N1 HA Ab #2	25X diluted rabit serum	2
F (Ligand)	20 nM anti-H5N1 HA Ab #7	20 nM anti-H5N1 HA Ab #5	25X diluted rabit serum	20 nM anti-H5N1 HA Ab #6	25X diluted rabit serum	20 nM anti-H5N1 HA Ab #9	25X diluted rabit serum	20 nM anti-H5N1 HA Ab #8	2
G (Ligand)	20 nM anti-H5N1 HA Ab #14	25X diluted rabit serum	20 nM anti-H5N1 HA Ab #11	20 nM anti-H5N1 HA Ab #15	20 nM anti-H5N1 HA Ab #10	20 nM anti-H5N1 HA Ab #4	20 nM anti-H5N1 HA Ab #2	25X diluted rabit serum	2
H (Ligand)	20 nM anti-H5N1 HA Ab #7	20 nM anti-H5N1 HA Ab #5	25X diluted rabit serum	20 nM anti-H5N1 HA Ab #6	25X diluted rabit serum	20 nM anti-H5N1 HA Ab #9	25X diluted rabit serum	20 nM anti-H5N1 HA Ab #8	2
I (Ligand)	20 nM anti-H5N1 HA Ab #14	25X diluted rabit serum	20 nM anti-H5N1 HA Ab #11	20 nM anti-H5N1 HA Ab #15	20 nM anti-H5N1 HA Ab #10	20 nM anti-H5N1 HA Ab #4	20 nM anti-H5N1 HA Ab #2	25X diluted rabit serum	2
BF	PBSTE + 1% BSA								180

Figure 1: *Cartridge layout for the capture screening test. All antibody ligands are suspended in 25x rabbit serum diluted in PBSTE + 1% BSA.*

10 mM HCl for 60 s, followed by a 5 min activation of the 16 carboxyl sensors with drops consisting of 25 mM EDC and 25 mM NHS prepared from Nicoya's Surfacing Kit. For creation of the capture surface, each sensor was exposed to 20 µg/ml of NanoCaptureLigand mouse IgG specific VHH in sodium acetate pH 5.5 for 5 min. All sensors were then blocked with 1 M Ethanolamine for 5 min to quench remaining active carboxyl groups.

Each lane screened six different ligands (Row D to Row I), for a total of 48 unique ligands, against 2 different antigens. 2 µL of each ligand, which include 20 nM of various H5N1 Influenza A hemagglutinin antibodies in 25x rabbit serum, as well as controls with 25x rabbit serum only, were loaded in the D through I wells. 5 µL of 60 nM of H5N1 Influenza A hemagglutinin was loaded into wells C1-C4 and 5 µL of 60 nM H3N2 Influenza A hemagglutinin was loaded into wells C5-C8. The analyte is automatically diluted on cartridge by 3x to create the necessary volume for all the interactions. Ligands in wells D1-D4 through I1-I4 (to be referred to as lanes 1-4) are tested against the H5N1 antigen in C1-C4 and the ligands in wells D5-D8 through I5-I8 (to be referred to as lanes 5-8) are tested against the H3N2 antigen in C5-C8. With throughput expansion mode enabled this is followed by ligands in lanes 1-4 tested against the H3N2 antigen in C5-C8 and the ligands in lane 5-8 tested against the H5N1 antigen in C1-C4. A full explanation of the layout is shown in Figure 2.

Each ligand from Row D was introduced in the response channel of their respective lane for 5 min, followed by a wash step and a buffer baseline step. The analytes, 20 nM (after the 3X dilution) of H5N1 and H3N2 Influenza A hemagglutinins, were then passed over the captured ligands on their respective sensors for 5 min, followed by a dissociation time of 15 min. After completion of the dissociation step the sensor surface was regenerated with a 1 min exposure of glycine-HCl pH 1.5. This resulted in complete removal of the antibodies and antigens.

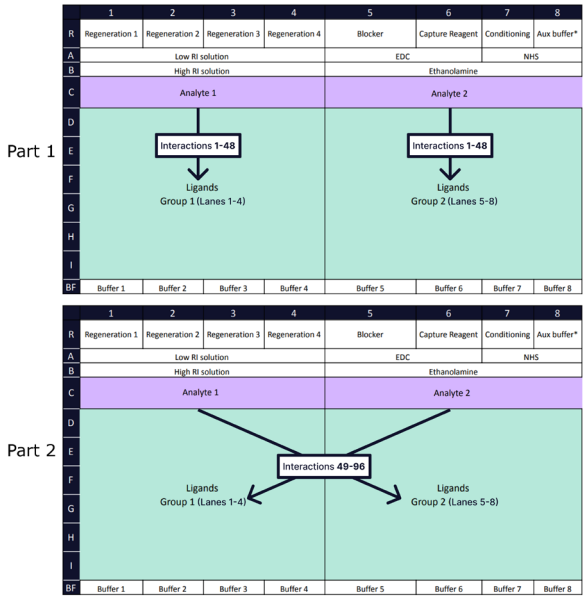

Figure 2: *Schematic of the expanded throughput mode. In Part 1 of the protocol Analyte 1 is interrogated against Group 1 ligands in lanes 1-4 and Analyte 2 is interrogated against Group 2 ligands in lanes 5-8, accounting for interactions 1 through 48. In Part 2 of the protocol Analyte 1 is interrogated against Group 2 ligands in lanes 5-8 and Analyte 2 is interrogated against Group 1 ligands in lanes 1-4 accounting for interactions 49 through 96.*

These steps were repeated five more times each for ligands in Row E, F, G, H, and I. Figure 3 provides an example of a sensorgram for a single interaction which includes all of the steps listed above. The sequence of drops is explained in further detail in Table 1.

Next, in the throughput expansion mode, the same antibodies starting from Row D are once again introduced to the sensors. They are then tested against the opposite antigen from the one they were tested against in part 1 of the method. I.e. Ligands in lanes 1-4 are tested against the analyte in C5-C8 and ligands in lanes 5-8 are tested against the analyte from C1-C4. The exact same step sequence is used as above and is then repeated five more times each for ligands in Row E, F, G, H, and I. Note that the use of the second antigen is not required, and the absence of this throughput expansion will decrease the time required to complete the test by approximately half. Without throughput expansion the analytes loaded in C1-C8 are used to test the ligands loaded in their respective lanes 1-8 only once. Upon completion of each test, binding curves were automatically fit to a 1:1 binding model in the Nicosystem analysis software to determine kinetic and affinity constants.

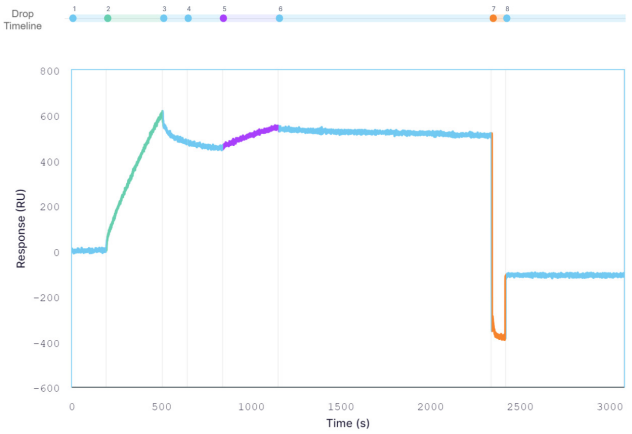

Figure 3: *Raw data for a single interaction of the capture screening assay. The drop sequence for this assay is explained in detail in Table 2.*

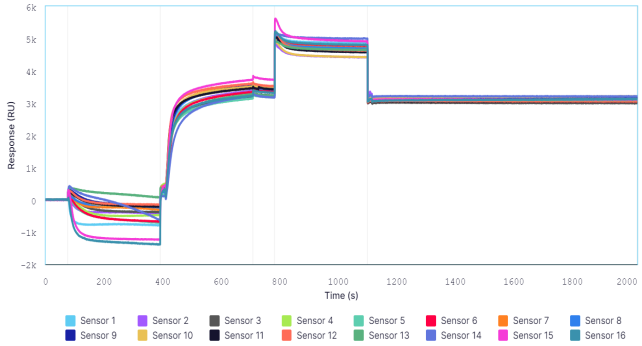

Figure 4: *Activation of response channels with EDC/NHS from Nicoya's Surfacing Kit, followed by immobilization of 20 µg/mL mouse IgG specific VHH in Sodium Acetate pH 5.5 and blocking of sensors with 1 M Ethanolamine. Image was generated by Nicosystem analysis software.*

Results & Discussion

Mouse IgG specific VHH capture molecules were immobilized via amine coupling onto the carboxy sensor surface. Average immobilization was 3117 ± 57 RU (Figure 4). High capture molecule immobilization levels are ideal in screening assays, as they will lead to the detection of lower ligand concentrations within the sample. The low standard deviation (<2%) of the immobilization averages highlights the reproducibility of this step.

In this study eleven antibody candidates (seven mouse monoclonal antibodies and four rabbit monoclonal antibodies) were screened in this test, and were run in triplicate to confirm reproducibility. The remaining wells had 25x rabbit serum as a negative control. The antibodies were purchased as a purified product and used to spike 25x diluted rabbit serum (diluted with PBST) to show compatibility with complex matrices. The capture molecule is specific for mouse Fc, and therefore only the mouse antibodies should be captured in this test. As depicted in Figure 5, the screening activity of the assay was able to determine with a 100% accuracy which samples contained anti-H5N1 HA mouse mAbs. All antibodies captured were also positively identified as binding the antigen H5N1 HA, as expected. None of these antibodies were able to bind the negative control antigen, H3N2 HA as expected (Figure 5). Figure 6 illustrates the corrected curves of the mouse Abs captured on the anti-Fc surface, followed by antigen binding to the captured mouse mAbs. Binding responses of the bound antigen range from 48 RU to 159 RU. Responses lower than 40 RU are considered not binding. Wells that only contained serum (negative controls) showed little to no ligand capture response, highlighting Alto's low susceptibility to nonspecific binding (NSB). Full regeneration was achieved using glycine-HCl pH 1.5 with 0.1% Tween 20 (Figure 3). The analysis also shows a sensorgram for each antigen interaction from the capture screening test (Figure 7).

The Nicosystem analysis software automatically determines and reports the ligand capture level response, as well as

the antigen binding response as Illustrated in figure 8. The antigen binding curves were fitted to a Langmuir 1:1 binding model analyzed in the Nicosystem analysis software. Kinetic parameters for all data are reported in a table in the Nicosystem as shown in Figure 9. This table can be sorted by affinity ranking of various kinetics parameters, providing hassle-free information on ideal antibody candidates. After evaluating the off-rate ranking of all the binding interactions, the best candidates in this assay were Antibody (Ab) #9 and Ab #14. From the kinetic analysis, association rate constants determined for were 3.78 x 105 M-1s-1 and 3.51 x 105 M-1s-1, respectively, and both Abs had a dissociation rate constant of 1 x 10-6 s-1, resulting in KD values of 2.65 pM and 2.85pM.

The dissociation rate constants could not be measured accurately as the off rates were too slow for the given dissociation time used in this experiment. An increase of the dissociation time beyond 15 minutes would result in more accurate off-rate determination for these antibodies. However, we can conclude from this assay that we are able to rank the interactions based on on-rate, off-rate, affinity, as well as sort by sample names, to understand how to find the best candidates to continue the downstream study.

The therapeutic discovery process is a long, labor intensive, and expensive process that involves generation of large scale libraries of biotherapeutic compounds, such as antibodies. Production of therapeutic antibodies using hybridoma or phage display technologies occur in complex media types, including cell lysate and serum. Evaluating binding activity of these large mAb libraries efficiently can be difficult without the use of a low maintenance, high-throughput and sample-friendly SPR instrument. This study demonstrates Alto's capability in screening up to 48 biological compounds at a low limit of detection in complex media types with high accuracy and minimal NSB.

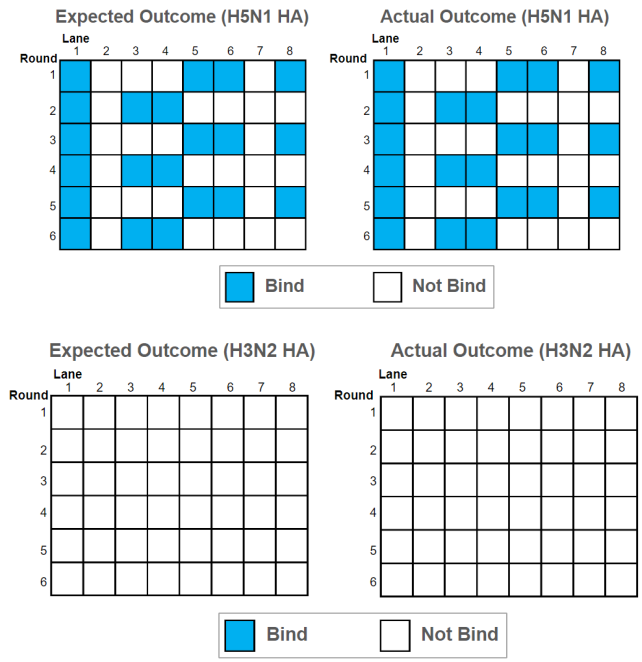

Figure 5: *Heatmap showing the expected and observed outcomes for the antibodies tested against the H5N1 HA antigen (top) and the H3N2 HA antigen (bottom). Interactions were labeled as 'bind' if the analyte response was ≥ 40 RU.*

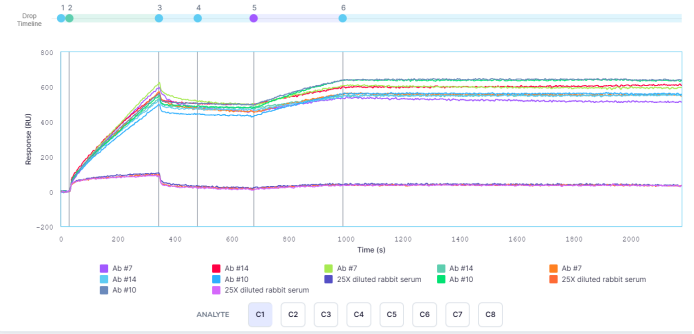

Figure 6: *Series of sensorgrams collected for a single lane of the capture screening assay with high throughput expansion turned on. The sensorgrams show the capture (or absence of capture) of the ligand followed by washing with buffer and then binding (or absence of binding) of the antigen. Antibodies loaded in wells D1-I1 are tested against both antigens, resulting in an overlay of twelve curves for each lane of test, and 96 overall.*

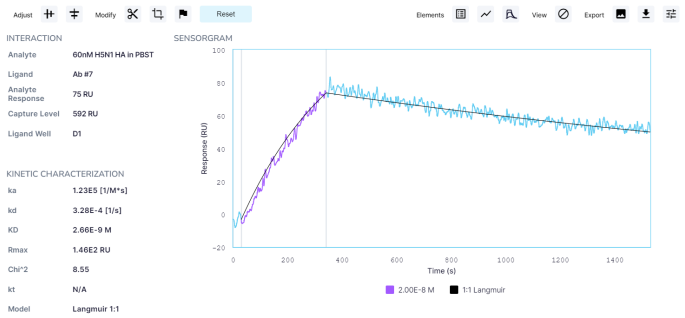

Figure 7: *Sensorgram for a single antigen interaction from the capture screening test in the Nicosystem analysis software.*

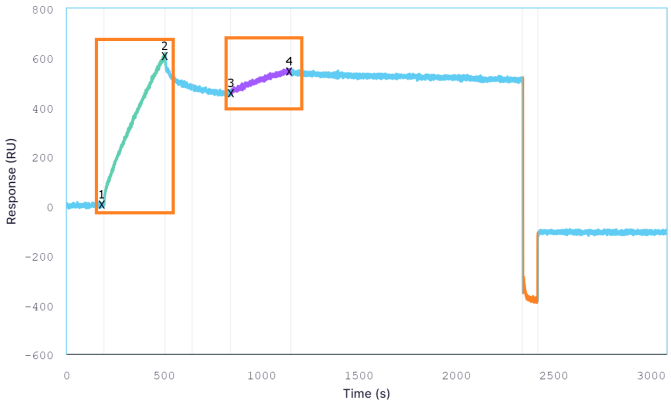

Figure 8: *Diagramatic explanation of how capture level and analyte response are calculated by the Nicosystem analysis software. The ligand capture level is calculated by the subtraction of the report point at position 2 minus the report point at position 1. The analyte binding response level is calculated by the subtraction of the report point at position 4 minus the report point at position 3. The values for these parameters are given in a table as shown in Figure 9.*

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ID#	Ligand Well	Ligand	Analyte	Ligand Capture (RU)	Analyte Response (RU)	Time (RP 1, RP 2)	ka (1/M*s)	kd (1/s)	KD (M)	Rmax (RU)	Chi^2	kt (RU/M*s)
1	D1	Ab #7	60nM H5...	592	75	(337,342)	1.23E5	3.28E-4	2.66E-9	1.46E2	8.55	N/A
2	E1	Ab #14	60nM H5...	557	100	(337,342)	1.06E5	1.00E-6	9.42E-12	1.98E2	6.59E1	N/A
3	F1	Ab #7	60nM H5...	617	103	(337,342)	1.11E5	1.22E-4	1.10E-9	2.12E2	1.31E1	N/A
4	G1	Ab #14	60nM H5...	526	66	(337,342)	3.51E5	1.00E-6	2.85E-12	6.81E1	6.17E1	N/A
5	H1	Ab #7	60nM H5...	566	100	(337,342)	1.14E5	5.70E-5	4.99E-10	2.01E2	5.87	N/A
6	I1	Ab #14	60nM H5...	519	68	(336,341)	1.12E5	1.00E-6	8.95E-12	1.51E2	2.41E1	N/A
7	D5	Ab #10	60nM H5...	490	128	(337,342)	1.06E5	1.00E-6	9.45E-12	2.68E2	7.87	N/A
8	E5	25X dilut...	60nM H5...	90	19	(337,342)	1.08E5	1.04E-4	9.61E-10	4.59E1	4.98	N/A
9	F5	Ab #10	60nM H5...	538	157	(337,342)	1.08E5	1.00E-6	9.25E-12	3.20E2	6.30	N/A

Figure 9: *Screening results table as provided by the Nicosystem analysis software. Only the first nine interactions are depicted in this figure, but the user is able to scroll through all 96 interactions in the table, or export as a .csv file. The user is also able to sort any parameters shown in the table from highest to lowest value, or lowest to highest value by clicking on the header of the desired parameter.*

Conclusion

High-quality antibody candidates were successfully screened for H5N1 Influenza A hemagglutinin using Alto's new capture screening feature. This application note demonstrates how Nicoya's digital SPR platform, Alto, enables rapid, automated SPR-quality screening and characterization of biologic lead libraries with simple, pre-configured capture screening and binding kinetics characterization assays. It eliminates long, laborious assay development and expensive maintenance of traditional label-free methods in the lead generation process. Users successfully screen up to 48 unique antibodies against a single antigen target in under 5 hours, or two antigen targets in under 12 hours. With 100X lower sample volume requirements, simplified one-click analysis, minimum maintenance needs and crude sample compatibility, Alto can be used to screen for binding activity of various proteins, antibodies and other biotherapeutic agents using a variety of capture molecules. This allows researchers to easily integrate Alto into their therapeutic drug discovery process to identify potential candidates faster and further characterize their properties.

Table 1: List of Influenza A H5N1 Hemagglutinin/HA antibodies used for the capture screening assay. RM denotes rabbit monoclonal antibody and MM denotes mouse monoclonal antibody.

Antibody name	Solution Ab
Ab #2	86001-RM02
Ab #4	11048-RM07
Ab #5	11048-RM08
Ab #6	11048-RM09
Ab #7	11048-MM01
Ab #8	11048-MM03
Ab #9	11048-MM04
Ab #10	11048-MM05
Ab #11	11048-MM06
Ab #14	11048-MM011
Ab #15	11048-MM014

Table 2: Drop sequence for the capture screening assay. The drop numbers in this table correspond to the drop timeline shown above the graph in Figure 3.

Drop #	Description	Composition
1	Baseline 1	Running buffer
2	Capture	Ligand
3	Rinse	Running buffer
4	Baseline 2	Running buffer
5	Association	Analyte
6	Dissociation	Running buffer
7	Regeneration	Regeneration solution
8	Baseline 3	Running buffer