Epitope characterization of influenza antiviral targets using Alto™ digital surface plasmon resonance

Summary

Rapid changes in influenza antiviral target proteins due to antigenic drift result in 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. As such, characterizing the binding kinetics and epitope diversity of various antibodies to influenza viral antigens is essential for treating and preventing potential outbreaks. In collaboration with Sino Biological, a global leader in recombinant technology, we use Nicoya's Alto digital surface plasmon resonance (SPR) system to perform kinetic analysis and epitope characterization of 16 antibodies against Influenza A hemagglutinin (HA), using only 1 µg of antigen and 100 ng of antibody. Alto now is introduced with a simplified 16x16 epitope binning application for biotherapeutics research, galvanizing your productivity by reducing your cost and effort.

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 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. In addition, 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 receptor-binding domain (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. In addition, changes in this protein are essential for 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.

Traditional Characterization Techniques

Traditional techniques such as ELISA and western blot (WB) require time-consuming washing and incubation steps, and depend on the use of tags for analysis. Compared to such traditional techniques, the information content provided by Alto enables increased efficiency in the development of viral diagnostics and therapeutics. Alto provides complete kinetic characterization of antibody-antigen interactions to understand, rank, and optimize antibody therapeutics, without the use of tags or labels. In addition, it offers the unique advantage of epitope binning capabilities to better understand epitope diversity and overlap.

Other recent techniques in use such as BLI and traditional SPR suffer from various limitations. Traditional SPR instruments typically have a large footprint with complex mechanics that in some cases, are more suited to large binning experiments; and in other cases, they are slower than other platforms. In all cases, they involve the use of complicated fluidic channels that are cumbersome to operate and maintain. BLI 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 binning run.

Kinetic Analysis and Epitope Characterization with Alto

Alto provides digital microfluidic (DMF) powered SPR with an unprecedented ability to study, develop and optimize vaccines & antibody therapeutics among many other applications. Full kinetic characterization with SPR enables a deeper understanding of both antibody efficacy and antigen drift. Alto accelerates analysis with sample volumes as low as 2 µL and the execution of automated on-cartridge serial dilutions with this volume. Kinetic characterization with Alto enhances the study, development, and optimization of vaccines & antibody therapeutics, among many other applications, as it enables a deeper understanding of both antibody efficacy and antigen drift.

Epitope binning is a competitive assay used to characterize the simultaneous binding of monoclonal antibodies (mAbs) to an antigen, tested in a pairwise manner, for determining whether they block one another's binding to the same epitope of the antigen. If the binding of one mAb to the antigen prevents the binding of another mAb, both mAbs can be clustered into groups (or bins) that all compete for the same or similar epitope. If both mAbs are able to 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 (Figure 1). In the classical sandwich assay, the first mAb (surface antibody) is immobilized onto the sensor. This is followed by injection of the antigen and then finally the second mAb (solution antibody).

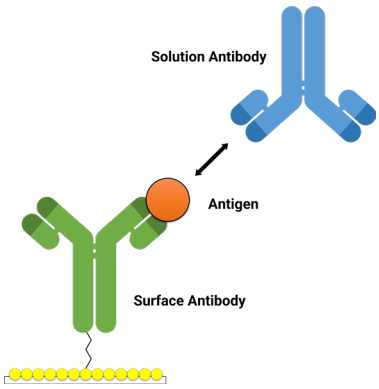

Figure 1: *In a classical sandwich assay, the surface antibody is immobilized onto the sensor, followed by injection of the antigen and then the solution antibody. If one antibody blocks the binding of another, they can be grouped together into a bin, which indicates they compete for the same or a similar epitope.*

Sino Biological provides recombinant antigen products, including hemagglutinin (HA), neuraminidase (NA) and nucleoprotein (NP) proteins, for all WHO-recommended vaccine strains, for use in SPR-based assays. In collaboration with Sino Biological, epitope characterization studies were conducted on Alto in a classical sandwich binning format with 16 unique antibodies against an influenza A HA protein, with the results summarized in this application note. Nicoya's digital SPR platform, Alto, collapses 16x16 epitope binning runs into pre-designed, automated assays using just 1 µg of antigen and 100 ng of antibody and one-click data analysis to reduce complexity, costs and effort and thereby galvanize productivity across small and large biotherapeutics research projects.

Advantages of Epitope Binning with Alto

- 16 x 16 epitope binning with 256 pairwise binding interactions on a single cartridge.
- Classical sandwich bin with 10 µL, <100 ng per antibody, and ~1 µg of antigen.
- Real-time sensorgrams show sandwiching mAbs and blocking mAbs.
- Self-referencing of solution mAb to surface.
- Go from sample prep to analysis in less than 16 hours, with only 20 mins of hands-on time.
- Robust platform with minimal downtime and full automation to reduce hands-on time.
- Label-free technology reduces cost of sample prep and purification.
- Sensors, fluidics and reagent integrated into a single, disposable cartridge.
- Flexible software, access your data from anywhere with the cloud.

Materials & Equipment

- Nicoya Alto 16-Channel Instrument (ALTO16)
- Alto 16-Channel Carboxyl Cartridge (KIN-CART-CBX-16)
- 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)
- Immobilization Buffer: Sodium Acetate pH 5.5 (ALTO-R-IMB-5.5)

- Regen Buffer: Gly-HCl pH 1.5: (ALTO-R-GLYHCl-1.5)
- Sino Biological Reagents
  - Recombinant Influenza A H5N1 Hemagglutinin/HA Protein (Cat# 11048-V08H1)
  - Influenza A H5N1 Hemagglutinin/HA Antibodies (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, 11056-RP01)

Method

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 individually addressable channels. The experimental method was designed using the epitope binning application on Alto's user portal, which was automatically uploaded to the instrument.

First, a 16-channel carboxyl cartridge was loaded onto 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 cartridge layout pre-designed on Alto is shown in Figure 2. A full cartridge layout showing reagent map that guides addition of reagents to wells in the cartridge is illustrated in Table 1. Information about the surface and solution antibodies that are loaded into the cartridge can be found in Table 2.

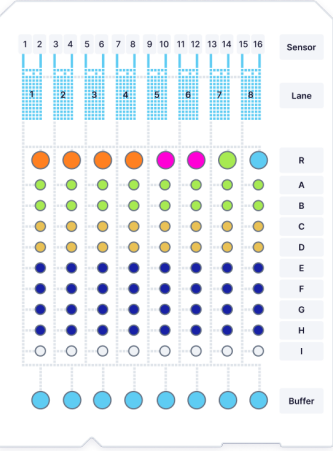

Figure 2: *Alto cartridge layout for epitope binning reagents. Orange wells (R1-4) are regeneration solution, pink (R5-6) are antigen, green (R7, A-B) are reagents, light blue (R8 & Buffer) are buffers, yellow (C-D) are surface antibodies and dark blue (E-H) are solution antibodies.*

Normalization of sensors with high and low RI droplets was performed first. The sensors were then cleaned with 10 mM HCl for 60 s, followed by a 5 min activation of the 16 carboxyl sensors with a drop consisting of EDC and NHS prepared from Nicoya's Surfacing Kit.

During surface antibody immobilization, each sensor was exposed to 2 µl of one of the antibodies at 20 µg/ml in sodium acetate pH 5.5 for 3 min, creating 16 unique surface coupled antibodies. All sensors were then blocked with 1 M Ethanolamine for 5 min to quench remaining active carboxyl groups.

After immobilization of the surface antibody, 16 rounds of the classical sandwich assay were performed. First, one solution antibody was passed over each of the 16 surface coupled antibody sensors for 3 min, to be used as a referencing step (to detect any non-specific binding), then the surface was regenerated for 1 min with 10 mM glycine HCl pH 1.5. The antigen was then captured on all 16 surface coupled antibody sensors for 3 min, followed by the same solution antibody used in the referencing step for 3 min. This step ended with regeneration with 10 mM glycine HCl pH 1.5 for 1 min to remove both the solution antibody and the antigen bound to the surface antibody. This was repeated in a pairwise manner for the remaining 15 solution antibodies. A buffer blank was also performed following the same procedure as above, except using a buffer drop in place of the solution antibody, to give an idea of the baseline response of the buffer when the antigen is bound. The entirety of the test was completed in about 14 hours without any need for manual intervention.

Results & Discussion

The epitope diversity of the influenza A HA antibodies were evaluated with a classical sandwich assay. Surface antibodies were immobilized via amine coupling onto the carboxy sensor surface. Immobilization levels of these antibodies varied between 2000 and 3200 RU (Figure 3). High immobilization levels on the sensor surface are ideal in epitope binning assays, to maximize the detection signal for target molecule binding.

Sixteen unique HA antibodies were screened against each HA antigen ligand as listed in Figure 2 and Table 2. A buffer blank is subtracted from each interaction, where the buffer is used in place of the antibody. An example of the

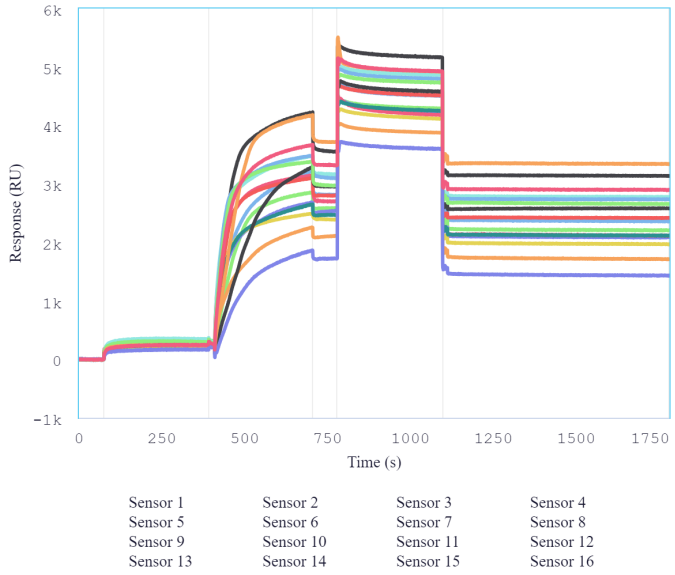

Figure 3: *Activation of response channels with EDC/NHS from Nicoya's Surfacing Kit, followed by immobilization of 20 µg/mL of each surface antibody in Sodium Acetate pH 5.0 and blocking of sensors with 1 M Ethanolamine. Image was generated by Nicosystem analysis software.*

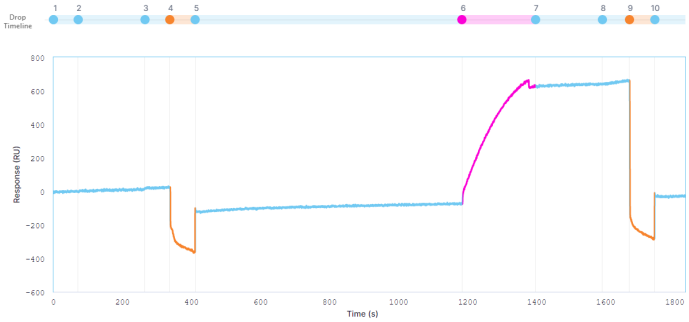

Figure 4: *A representative sensorgram overlay showing antigen binding and then buffer in place of solution Ab. This acts as the buffer blank due to all 16 channels being used as active channels in the epitope binning protocol. Image was generated by Nicosystem analysis software.*

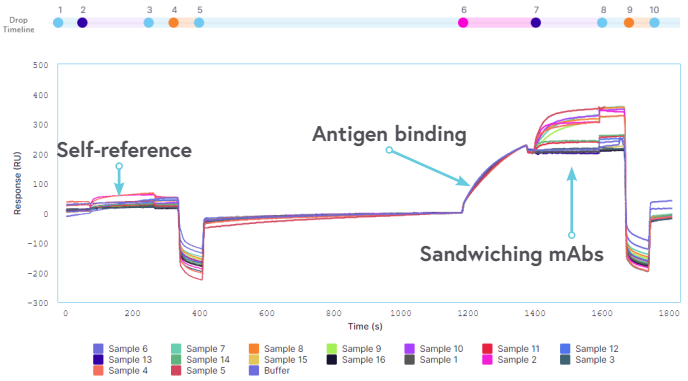

Figure 5: *A representative sensorgram overlay showing all sixteen solution antibodies and buffer blank interacting with a single surface antibody. Solution antibody (or buffer) is passed over the sensor and used for referencing. The surface is then regenerated. This is followed by antigen, solution antibody (or buffer), and finally a second regeneration. Image was generated by Nicosystem analysis software.*

buffer blank sensorgram is shown in Figure 4. Additionally, for each sandwich interaction, the software subtracts the solution antibody's signal without antigen binding from the solution antibody's signal following antigen binding. Using this self-referencing therefore generates data which is double referenced. The self-referencing can be toggled on or off in the Nicosystem software. The response level of the secondary antibody was normalized using the antigen binding level across all curves, since binding activity of the antigen to the surface can change over time. The referencing and normalization together created the 'corrected curves' which are used for binding analysis (Figure 5).

Analysis of secondary antibody binding was done by setting thresholds for the normalized solution antibody responses (Figure 6). Bind, intermediate and not bind categories were defined based on a 'bind' threshold of 50 RU and a 'not bind' threshold of 30 RU. Anything in between was classified as intermediate binding. These thresholds can be adjusted by the user 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 7).

Figure 8 illustrates the results of the 16x16 epitope bin sorted by groupings of binders and non-binders, as well as displaying the antibody category numbers listed in Table 2. "Bind" results signify a unique epitope targeted by the antibodies. Antibodies that blocked another antibody from binding to the antigen were deemed to have the same target epitope and 'binned' together. Antibodies that bound to the antigen to form a sandwich complex were inferred to have non-overlapping epitopes.

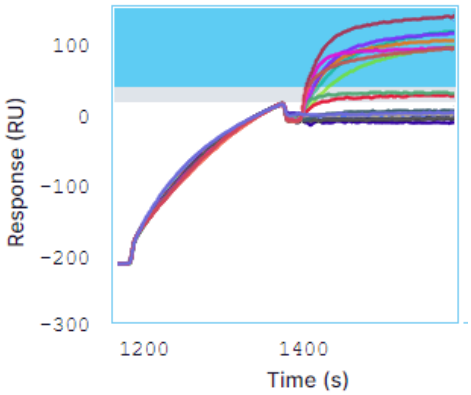

Figure 6: *Overlay of normalized antigen binding followed by solution antibody sandwiching or blocked binding. Binding (blue region) and non-binding (white region) thresholds can be changed by the user.*

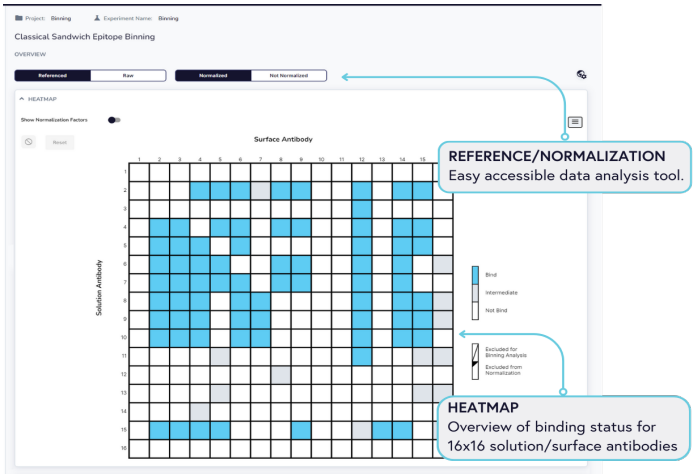

Figure 7: *Epitope characterization analysis for influenza A HA antibodies binding to influenza A H5N1 HA (Cat# 11048-V08H1) from the Nicosystem analysis software. The automatically referenced and normalized algorithm is applied and displayed. The degree of binding displayed in the heat map was determined 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 50 RU and a 'not bind' threshold of 30 RU (Intermediate is a response between 30 and 50).*

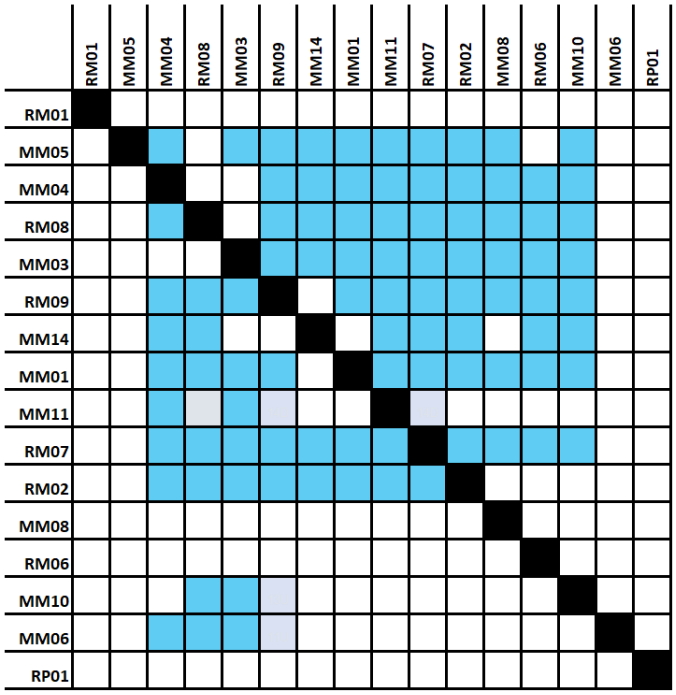

Figure 8: *Epitope characterization analysis for Influenza A HA antibodies binding to Influenza A H5N1 HA (Cat# 11048-V08H1), sorted by groupings of binders and non-binders, as well as displaying the antibody catalog number. Self blocking is confirmed by the diagonal which shows no binders.*

Conclusion

Alto was used to successfully characterize Sino Biological's highly specific influenza antibodies by conducting epitope analysis. Compared to traditional methods of characterization, SPR accelerates analysis of multiple interactions by eliminating the need for labels and reducing time-consuming preparation steps. This supports the use of the Alto for applications in influenza diagnostics and vaccine development and can be used for small and large biotherapeutics research projects. Alto facilitates accurate kinetics analysis and 16x16 epitope binning for accelerated development of antibody therapeutics. By leveraging DMF technology, Alto streamlines 16x16 epitope binning into pre-designed, automated assays using just 1 µg of antigen and 100 ng of antibody and one-click data analysis to reduce complexity, costs and effort.

Table 1: Epitope binning assay cartridge layout with protein and reagent conditions.

	1	2	3	4	5	6	7	8	Vol. (µL)	Conc.
R	Regeneration, Gly-HCl pH 1.5				100 nM Influenza A H5N1 HA		HCl	PBST	65	
A	32% Glycerol				EDC		NHS		4	
B	Quench (1 M Ethanolamine)								4	
C	Surface Ab 1	Surface Ab 3	Surface Ab 5	Surface Ab 7	Surface Ab 9	Surface Ab 11	Surface Ab 13	Surface Ab 15	2	20 µg/mL
D	Surface Ab 2	Surface Ab 4	Surface Ab 6	Surface Ab 8	Surface Ab 10	Surface Ab 12	Surface Ab 14	Surface Ab 16	2	20 µg/mL
E	Solution Ab 1	Solution Ab 3	Solution Ab 5	Solution Ab 7	Solution Ab 9	Solution Ab 11	Solution Ab 13	Solution Ab 15	4	300 nM
F	Solution Ab 1	Solution Ab 3	Solution Ab 5	Solution Ab 7	Solution Ab 9	Solution Ab 11	Solution Ab 13	Solution Ab 15	4	300 nM
G	Solution Ab 2	Solution Ab 4	Solution Ab 6	Solution Ab 8	Solution Ab 10	Solution Ab 12	Solution Ab 14	Solution Ab 16	4	300 nM
H	Solution Ab 2	Solution Ab 4	Solution Ab 6	Solution Ab 8	Solution Ab 10	Solution Ab 12	Solution Ab 14	Solution Ab 16	4	300 nM
I	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty		
BF	PBST	PBST	PBST	PBST	PBST	PBST	PBST	PBST	180	

Table 2: List of antibodies from Sino Biological used in epitope characterization of influenza antiviral targets and their assigned sample numbers.

#	Molecule	Cat#
1	Influenza A Virus Hemagglutinin / HA Antibody, Rabbit MAb	86001-RM01
2	Influenza A Virus Hemagglutinin / HA Antibody, Rabbit MAb	86001-RM02
3	Influenza A H1N1 (Swine Flu 2009) Hemagglutinin / HA Antibody, Rabbit MAb	11055-RM06
4	Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Rabbit MAb	11048-RM07
5	Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Rabbit MAb	11048-RM08
6	Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Rabbit MAb	11048-RM09
7	Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Mouse MAb	11048-MM01
8	Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Mouse MAb	11048-MM03
9	Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Mouse MAb	11048-MM04
10	Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Mouse MAb	11048-MM05
11	Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Mouse MAb	11048-MM06
12	Influenza A H1N1 (Swine Flu 2009) Hemagglutinin / HA Antibody, Mouse MAb	11055-MM08
13	Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Mouse MAb	11048-MM10
14	Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Mouse MAb	11048-MM11
15	Influenza A H5N1 (A/Anhui/1/2005) Hemagglutinin / HA Antibody, Mouse MAb	11048-MM14
16	Influenza A H3N2 Hemagglutinin HA Antibody, Rabbit PAb (IgG)	11056-RP01