APPLICATION NOTE

Accelerating influenza antibody therapeutics with Alto™ digital microfluidic-powered epitope binning and kinetics analysis

Summary

Annual formulation updates for influenza vaccines and related antibody therapies are required to preserve immune recognition against different influenza subtypes. 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 our Alto system to perform kinetic analysis and epitope characterization of several antibodies against influenza A hemagglutinin (HA), using DMF-powered surface plasmon resonance (SPR) technology. Alto provides a streamlined automated assay to achieve accurate kinetics data with 200x less sample consumption.

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 is the only known type 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 RBD is therefore critical for vaccine development and a popular target for antibodies due to its 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 these techniques, the information content provided by SPR enables increased efficiency in the development of viral diagnostics and therapeutics. SPR 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, 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 and 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 and antibody therapeutics, among many other applications.

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. 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).

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. Devised in collaboration with Sino, this application note features kinetic and epitope characterization studies conducted simultaneously on Alto with eight unique antibodies against an influenza A HA protein. Alto uses just 1 µg of antigen and 400 ng of antibody, offers one-click data analysis to reduce complexity, costs and effort, and galvanizes productivity across small and large biotherapeutics research projects.

Materials & Equipment

- Nicoya Alto 16-Channel Instrument (ALTO16)
- Alto 16-Channel Carboxyl Cartridge (KC-CBX-PEG-16)
- Alto CBX Surfacing Kit: cleaning, normalization, activation (ALTO-R-CBX-SURF)
- Running Buffer: PBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-PBST)
- Regeneration Buffer: 10mM Glycine-HCl pH 2.0 (ALTO-R-GLYHCl-2.0)
- Nicoya Amine Coupling Kit (ALTO-R-EDCNHS)
- Ligands and analytes: All viral products were supplied by Sino Biological. For a full list of products and their Catalog #, refer to Table 3.

Method

Antibody Binning and Kinetics using Alto's Capture Kinetic Application

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 the instrument. All subsequent steps were automated by Alto. A full cartridge layout showing the 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 1.

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 pair of sensors was exposed to 2 µL of one of the antibodies at

20 µg/mL in sodium acetate pH 5.5 for 3 min, creating duplicates of eight 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, the sensors were incubated in PBS-T for 15 min to collect blank injection curves.

The antigen was then captured on all eight surface coupled antibody sensors for 3 min. Alto executed five automated 3-fold serial dilutions on cartridge per influenza A HA antibody used as the "secondary antibody" sample from the 300 nM stock concentration, producing 1.2 nM, 3.7 nM, 11 nM, 33 nM and 100 nM analyte samples. Influenza A HA antibody secondary antibody samples were introduced in increasing concentrations with an association time of 180 s, without dissociation or regeneration between each sample, starting from the lowest concentration. The last (highest) analyte concentration was followed by a 1200 s dissociation. The sensor surface was regenerated with a 60 s exposure of glycine-HCl pH 2.0. This step ended with regeneration using 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 for each additional Pan influenza A HA antibody. Upon completion of the test, all binding curves were fitted to a 1:1 binding model to determine kinetic and affinity constants (Table 2).

Results & Discussion

Epitope Characterization

The epitope diversity of Sino Biological's reagents were evaluated by performing a sandwich assay utilizing capture method. Surface antibodies were immobilized via amine coupling onto the carboxyl sensor. Immobilization levels of these antibodies varied between 1800 and 3500 Response Units (RU) (Figure 2). High immobilization levels on the sensor surface are ideal in epitope binning assays to maximize the detection signal for target molecule binding.

Eight unique HA antibodies were screened against the HA antigen in a pairwise fashion as listed in Table 3. In addition to epitope characterization, in the same experiment, kinetic values corresponding to each antibody were also obtained with on-cartridge serial dilutions. An example of the epitope binning and kinetic characterization is shown in Figure 3, as illustrated by the capturing of the influenza A HA antigen to the immobilized surface HA antibody followed by binding of the five automated 3-fold serial dilutions of the solution antibody.

Determination of secondary antibody binding was done by analyzing the maximum response of the solution antibody binding to the capture HA antigen. 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 between 30-50 RU was classified as "intermediate" binding. These thresholds can be determined by the user to suit the response level of their samples. Figure 4 illustrates the heat map of the eight antibody epitope bins sorted by groupings of binders and non-binders. "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 were "binned" together. Antibodies that bound to the antigen to form a sandwich complex were inferred to have non-overlapping epitopes.

Kinetic Characterization

The binding and kinetic fits of the influenza A HA antibodies to the captured influenza A antigen allows for evaluation of the strength of the antibody pairs in a sandwich format. The kinetic constants and K_D values were determined for binding of the five automated 3-fold serial dilutions of the solution antibody to the captured antigen (Figure 4). Resulting SCK fits are presented in Figure 5. The data were fit to a 1:1 binding model and the calculated kinetic constants for a subset of the tighter binding sandwich pairs are presented in Table 3. The kinetic data allow for the determination of high affinity sandwich pairs of antibodies.

Conclusion

Alto successfully characterized Sino Biological's highly specific influenza antibodies by conducting kinetic and epitope analysis studies with DMF-powered SPR. Compared to traditional methods of characterization, SPR accelerates analysis of multiple interactions by eliminating the need for labels and reducing time-consuming preparation steps. Alto's capture kinetics application facilitates simultaneous kinetics analysis and epitope binning by streamlining experiments with pre-designed, automated assays that require just 1 µg of antigen and 400 ng of antibody, further enabled by one-click data analysis. This supports Alto's use in both small and large biotherapeutics research projects to reduce the complexity, cost and effort of influenza diagnostics and vaccine development.

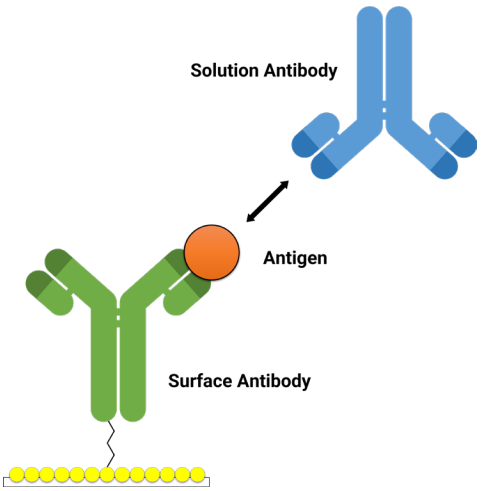

Figure 1: *In a classical sandwich assay, the surface antibody is immobilized onto the sensor, followed by injection of the antigen and then the solution antibody. If one antibody blocks the binding of another, they can be grouped together into a bin, which indicates they compete for the same or a similar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

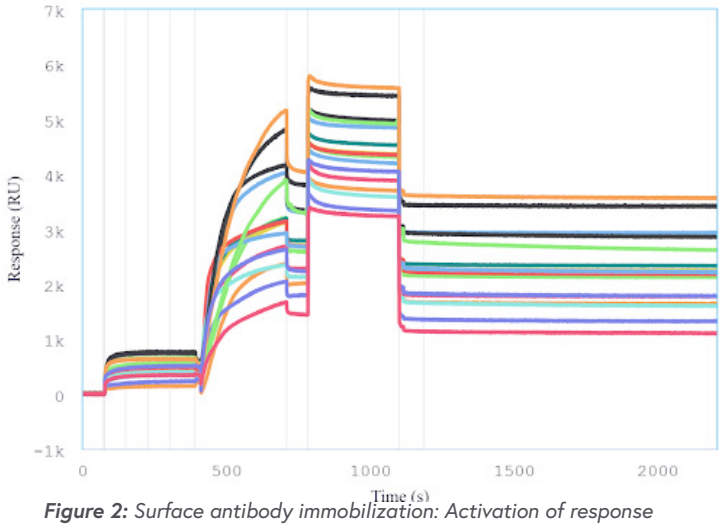

Figure 2: *Surface antibody immobilization: Activation of response channels with EDC/NHS from Nicoya's Surfacing Kit, followed by immobilization of 20 µg/mL of each surface antibody in Sodium Acetate pH 5.0 and blocking of sensors with 1 M ethanolamine. Image was generated by Nicosystem analysis software.*

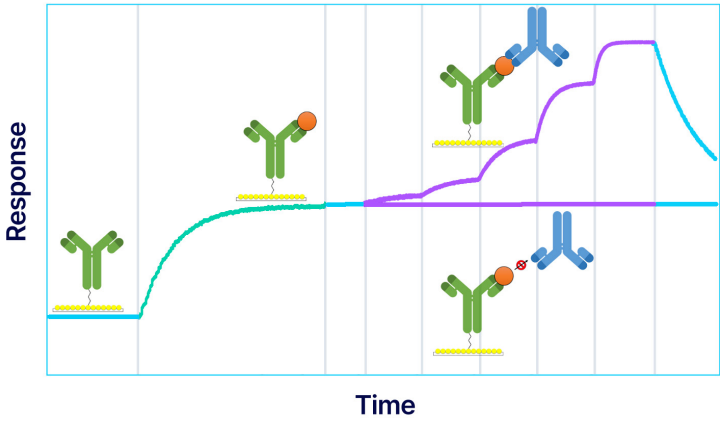

Figure 3: *Epitope characterization showing capturing of the influenza A HA antigen to the immobilized surface HA antibody (green curve) followed by binding of the five automated 3-fold serial dilutions of the solution antibody to a different epitope of the antigen (top purple curves). A lack of binding of the five automated 3-fold serial dilutions of the solution antibody indicates epitope overlap of the surface antibody (bottom purple line).*

	RM06	RM02	RM07	RM08	RM09	MM14	MM01	MM11
RM06								
RM02			600 pM	348 pM	171 pM	222 pM	324 pM	180 pM
RM07	595 pM	6.5 pM			4.2 pM	6.3 pM	4.6 pM	6.1 pM
RM08	116 pM	245 pM	25 pM			15.8 pM	217 pM	2.3 pM
RM09	251 pM	207 pM	2.7 pM	35.5 pM				3.4 pM
MM14	232 pM		4.8 pM	7.7 pM				3.9 pM
MM01	192 pM	8.31 pM	4.2 pM	8.0 pM	15.7 pM	256 pM		
MM11							1.21 nM	

Not Tested
Bind
Intermediate
Not Bind

Figure 4: *Epitope characterization analysis for influenza A HA antibodies binding to influenza A H5N1 HA from the Nicosystem analysis software. 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). K_D values are shown in each of the "bind" wells in the heat map. The K_D values were determined for binding of the five automated 3-fold serial dilutions of the solution antibody to the captured antigen. Self blocking is confirmed by the diagonal, which shows no binders.*

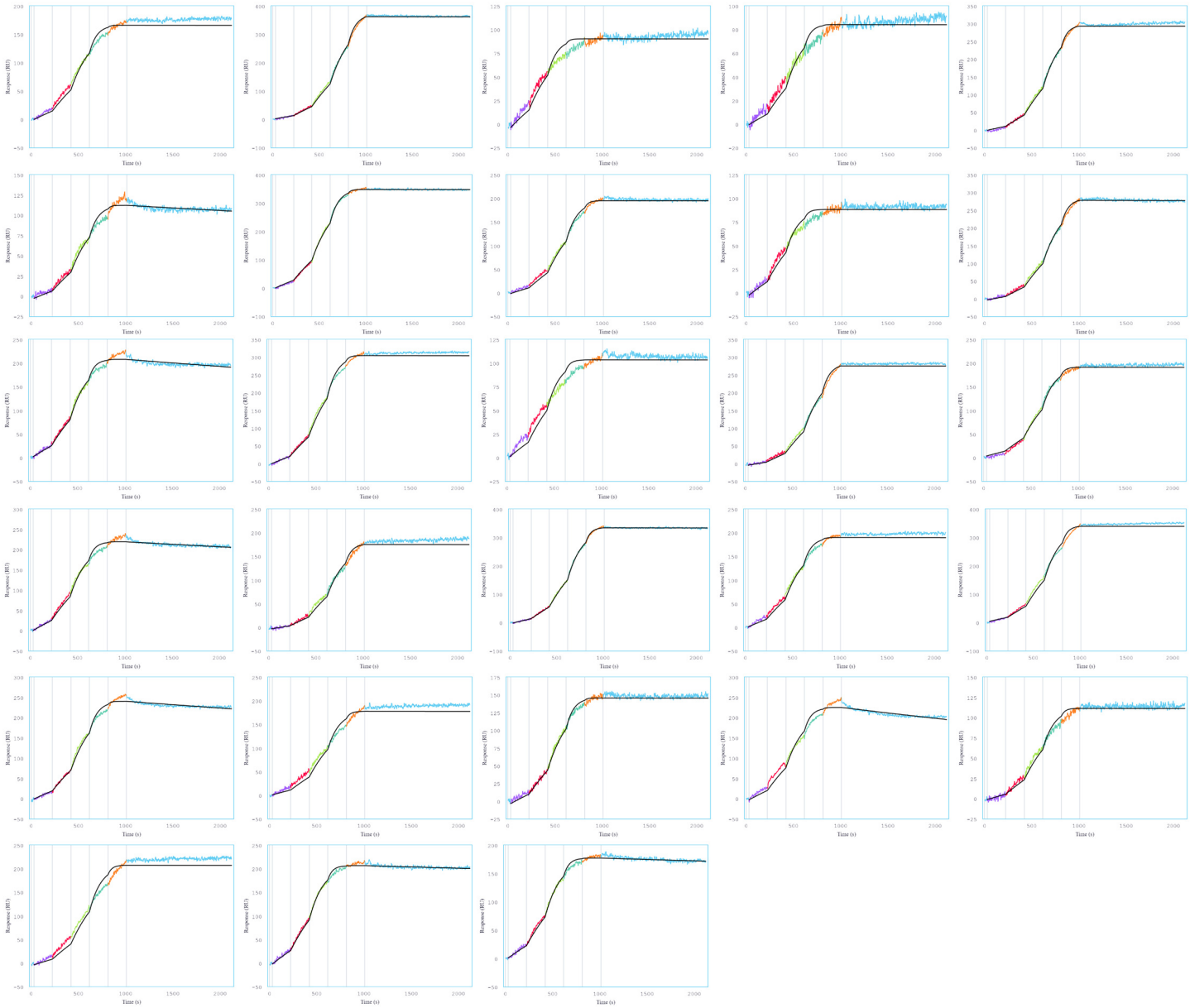

Figure 5: *Single-cycle kinetics of the five automated 3-fold serial dilutions of the solution antibody binding to the captured HA antigen on Alto. The solution HA antibody was titrated from 1.2 nM to 100 nM. Black curve is the Langmuir 1:1 binding fit model analyzed. Image was generated by Nicosystem analysis software.*

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				Empty	300 nM HA	10 mM HCl	PBST	65	
A	4% glycerol				EDC		NHS		4	
B	32% glycerol				Quench (1 M Ethanolamine)				4	
C	Surface RM02	Surface RM06	Surface RM07	Surface RM08	Surface RM09	Surface MM01	Surface MM11	Surface MM14	2	20 µg/mL
D	Solution RM06	Solution RM07	Solution RM08	Solution RM09	Solution MM01	Solution MM11	Solution MM14	Solution RM02	2	300 nM
E	Solution RM07	Solution RM08	Solution RM09	Solution MM01	Solution MM11	Solution MM14	Solution RM02	Solution RM06	2	300 nM
F	Solution RM08	Solution RM09	Solution MM01	Solution MM11	Solution MM14	Solution RM02	Solution RM06	Solution RM07	2	300 nM
G	Solution RM09	Solution MM01	Solution MM11	Solution MM14	Solution RM02	Solution RM06	Solution RM07	Solution RM08	2	300 nM
H	Solution MM01	Solution MM11	Solution MM14	Solution RM02	Solution RM06	Solution RM07	Solution RM08	Solution RM09	2	300 nM
I	Solution MM11	Solution MM14	Solution RM02	Solution RM06	Solution RM07	Solution RM08	Solution RM09	Solution MM01	2	300 nM
BF	PBST	PBST	PBST	PBST	PBST	PBST	PBST	PBST	180	

Table 2: Kinetic values of top 10 antibody binders, ranked by affinity, measured using Alto data with the Nicosystem analysis software.

Surface Ab	Solution Ab	k_a (1/(M*s))	k_d (1/s)	K_D (M)
MM11	RM08	3.65E+05	1.00E-06	2.30E-12
RM07	RM09	3.68E+05	1.00E-06	2.72E-12
MM11	RM09	2.39E+05	1.00E-06	3.35E-12
MM11	MM14	2.38E+05	1.00E-06	3.93E-12
RM07	MM01	2.49E+05	1.00E-06	4.17E-12
RM09	RM07	2.09E+05	1.00E-06	4.22E-12
MM01	RM07	2.24E+05	1.00E-06	4.55E-12
RM07	MM14	3.43E+05	1.00E-06	4.79E-12
MM11	RM07	1.32E+05	1.00E-06	6.08E-12
MM14	RM07	1.91E+05	1.20E-06	6.28E-12

Table 3: List of antibodies from Sino Biological used in epitope characterization of influenza antiviral targets and their assigned sample numbers.

Molecule	Cat #
Influenza A Virus Hemagglutinin / HA Antibody, Rabbit MAb	86001-RM02
Influenza A H1N1 (Swine Flu 2009) Hemagglutinin / HA Antibody, Rabbit MAb	11055-RM06
Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Rabbit MAb	11048-RM07
Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Rabbit MAb	11048-RM08
Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Rabbit MAb	11048-RM09
Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Mouse MAb	11048-MM01
Influenza A H5N1 (Avian Flu) Hemagglutinin / HA Antibody, Mouse MAb	11048-MM11
Influenza A H5N1 (A/Anhui/1/2005) Hemagglutinin / HA Antibody, Mouse MAb	11048-MM14