TECH NOTE

Screening of Unknown Antibodies in Complex Media with Alto™ Digital SPR

Overview

Screening is a common tool used to quickly test activity of a large number of chemical or biological compounds. Commonly seen as one of the first steps within the drug discovery process, screening assays are used on large scale libraries to quickly test and select candidates that bind to or affect a desired target. Surface plasmon resonance (SPR) is often used to conduct high-throughput screening to test for real-time binding activity of a variety of biological and chemical compounds.

Direct screening is one of the high-throughput assays offered on Alto, Nicoya's digital microfluidics (DMF) based SPR instrument (Figure 1). Alto's screening protocol allows users to establish binding activity of up to 48 unique analytes against desired targets in less than 4 hours. This technical note will go through two separate studies, with one demonstrating yes/no binding of various ligands and analytes, and the other presenting how Alto's screening protocol can be used for the discovery of new therapeutic antibodies. The below case studies exemplifies five main qualities of Alto's screening protocol:

- Accurately screen up to 48 analytes
- Test analytes in complex media, such as serum
- Use minimal sample volumes (3 µL)
- Screen against different ligand surfaces
- Minimize assay development time with label-free technology

Specificity and Yes/No Binding Against Various Ligand Surfaces

In Part I of this technical note, we demonstrate the ability of performing a screening assay to test specificity and yes/no binding activity of protein analytes on a variety of ligand surfaces using Alto. The assay demonstrates the correct binding and specificity of 48 protein analytes against four different ligands.

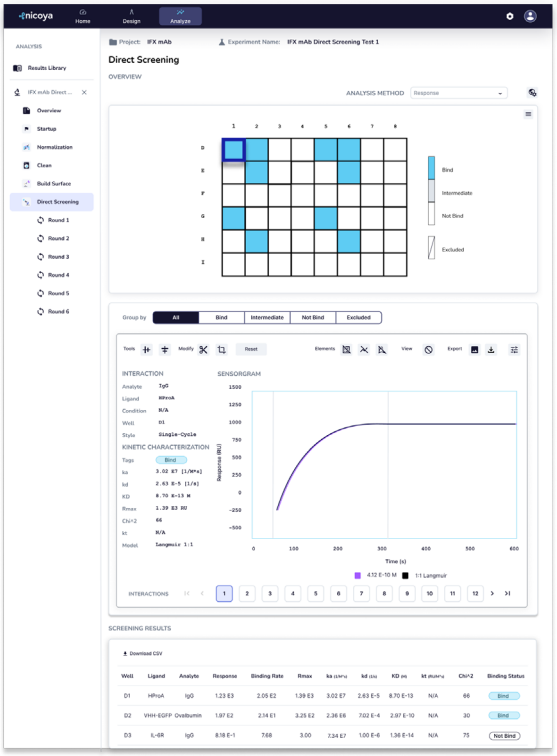

Figure 1: *Direct screening data analysis on Alto's Nicosystem analysis software.*

Antibody Screening Against H3N2 Hemagglutinin

In Part II of this technical note, a blind 48-sample screening test was performed to detect antibodies specific to H3N2 hemagglutinin (HA) at various concentrations. H3N2 is a variant of Influenza virus H1N1 and uses HA, a surface glycoprotein, for viral entry into the target cell. The HA

protein receptor-binding domain (RBD) is 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.

Samples consisted of rabbit serum alone and rabbit serum spiked with anti-H3N2 HA rabbit antibody (Ab) at concentrations ranging from 5.6 - 300 nM. This case study showcases how Alto's screening protocol can be used in antibody drug discovery processes where users can screen unknown antibodies in complex media against their desired target.

Materials

A full list of materials is available in the Appendix

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 direct screening application on Alto's user portal, which was 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 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.

In all experiments, 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 minute activation of the 16 carboxyl sensors with a drop consisting of EDC/NHS prepared from Nicoya's Carboxyl Surfacing Kit.

Part I: Various Ligand Assay

During immobilization, the reference channels were exposed to buffer (PBST), and the response channels were exposed to the following ligands for 5 mins:

- Lane 1 & Lane 5: 40 µg/mL Protein A (ProA) in Sodium Acetate pH 5.0
- Lane 2 & Lane 6: 25 µg/mL VHH-Enhanced Green Fluorescent Protein (VHH-EGFP) in Sodium Acetate pH 5.5
- Lane 3 & Lane 7: 20 µg/mL Interleukin-6 Receptor (IL-6R) in Sodium Acetate pH 5.0
- Lane 4 & Lane 8: 30 µg/mL Immunoglobulin G 2A (IgG2A) in Sodium Acetate pH 5.5

Ligands were all immobilized at a high concentration to saturate the sensor surface and maximize analyte response. All channels were then blocked with 1 M Ethanolamine for 5 mins to quench remaining active carboxyl groups.

Each lane screened six different analytes (Row D to Row I). Analytes include different concentrations of IgG, streptavidin, ovalbumin, EGFP, ProA, biotinylated-Mouse Fc gamma RI (FcγRI), VHH-EGFP, and IL-6R (Supplementary Table 1 shows the cartridge layout and conditions used for Part I of this technical study). Samples from Row D were sent simultaneously to both the response and reference channels of their respective lane for 180 s, followed by 500 s of dissociation in running buffer and 60 s of regeneration. This step was repeated five more times for analytes in Row E, F, G, H, and I. The entirety of the test was completed after 3 hours and 36 mins.

Part II: Antibody Screening Assay

Ligand H3N2 HA (20 µg/mL in sodium acetate pH 5.5) was immobilized over 8 response channels for 5 mins, while the reference channels had buffer (PBS-T, 10 mM EDTA, 1 mg/mL BSA) passed over them. All channels were then blocked with 1M Ethanolamine for 5 mins to quench remaining active carboxyl groups.

Each lane screened six analytes, consisting of unknown concentrations of anti-H3N2 HA rabbit Ab in 20x diluted rabbit serum. Samples from each row were sent to both the response and reference channels of their respective lane for 180 s, followed by 500 s of dissociation in running buffer and 60 s of regeneration. The entirety of the test was completed after 4 hours and 5 mins.

Results & Discussion

Part I: Various Ligand Screening Assay

ProA, VHH-EGFP, IL-6R and IgG2A ligand molecules were immobilized via amine coupling onto the carboxy sensor surface. Average immobilization of ProA, VHH-EGFP, IL-6R and IgG2A were 1313 RU, 2738 RU, 873 RU and 2183 RU, respectively (Figure 2). High immobilization levels are ideal in screening assays, as they will lead to the detection of lower analyte concentrations within the sample.

Six different analytes were screened against each ligand (Table 1). For each ligand-analyte interaction, the software subtracts the reference channel from the response channel to generate the "corrected curve". The corrected curves are then used to create a screening heat map to indicate which analytes bind or do not bind to their respective ligands (Figure 3). Upon selecting a well from the screening map, the corrected binding curve for that interaction is shown (Figure 4). The binding status tag, estimates of kinetic information (k_a, k_d, and K_D), calculated R_{max}, Chi^2, and fitting model are shown on each interaction card.

The presence of binding events is determined by the response level of the analyte. Users may set a binding threshold to determine which responses are not considered binding events. For this study, a binding threshold of 20 RU was used to detect binding of proteins at lower concentrations. Out of 48 analytes, 10 showed positive binding events (Figure 3).

Supplementary Table 2 illustrates the expected and observed binding behavior of the screening assay. All 48 analytes demonstrated the expected binding activity. Both 1 nM and 100 nM IgG showed positive binding to ProA; 37 nM EGFP showed binding to VHH-EGFP; 300nM IL-6 showed positive binding to IL-6R and 100 nM FcγRI showed binding to IgG2A (Supplementary Figure 1). Other analytes including ovalbumin, streptavidin, ProA, IgG, FcγRI, and VHH-EGFP did not not show binding to any of their non-target ligands as expected (Supplementary Figure 2). Overall, this experiment highlights Alto's capacity of performing screening assays against various ligands using minimal analyte volumes and concentrations as low as 0.15 ppm (1 nM of IgG).

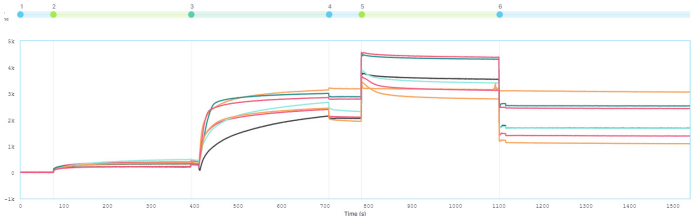

Figure 2: *Activation of response channels with EDC/NHS from Nicoya's Surfacing Kit, followed by immobilization of 40 µg/mL ProA in Sodium Acetate pH 5.0, 25 µg/mL VHH-EGFP in Sodium Acetate pH 5.5, 20 µg/mL of IL-6R in Sodium Acetate pH 5.0 and 30 µg/mL of IgG2A in Sodium Acetate pH 5.5 and blocking of sensors with 1 M Ethanolamine. Image was generated by Nicosystem analysis software.*

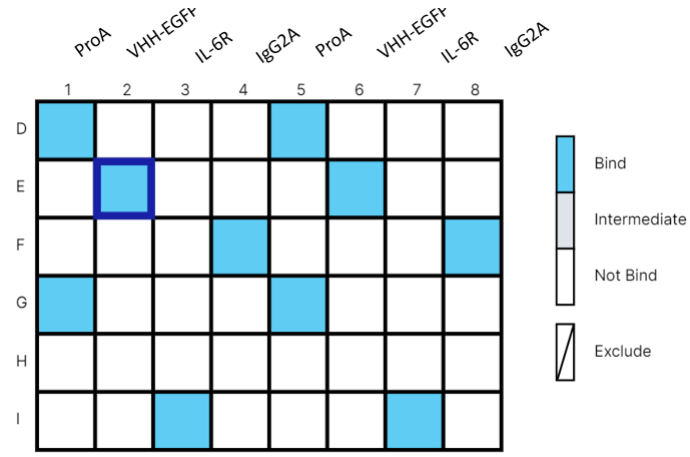

Figure 3: *Display of screening heat map which shows the layout of analyte wells from Row D to I for each lane. Blue indicates that the analyte binds to the ligand, white represents no binding event occurred with the ligand, and gray indicates a weak binding interaction. Binding thresholds (i.e. both bind threshold and not-bind threshold) were set to 20 RU. Yes/no binding events are determined by analyte response units (RU). D1 & D5 = 100 nM IgG; E2 & E6 = 37 nM EGFP; F4 & F8 = 100 nM FcγRI; G1 & G5 = 1 nM IgG; I3 & 17 = 300 nM IL-6. Image was generated by Nicosystem analysis software (excluding sample names).*

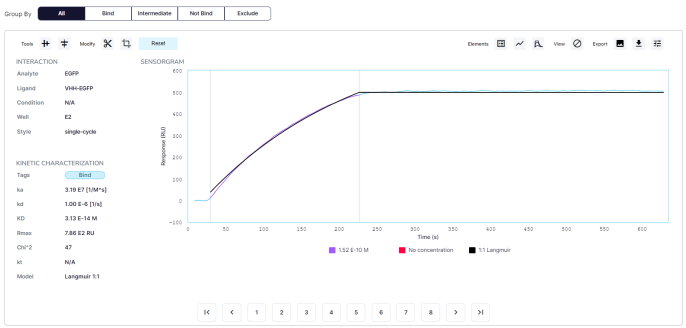

Figure 4: *Corrected binding curve of EGFP in well E2 binding to VHH-EGFP as the ligand. The software indicates a positive binding event between EGFP (analyte) and VHH-EGFP (ligand). Using a 1:1 Langmuir model, the software generates estimates of k_a, k_d, K_D and R_{max} values. Image was generated by Nicosystem analysis software.*

Table 1: Layout of immobilized ligands (in yellow) and analytes (in white).

40 µg/mL ProA (Lane 1 & 5)	25 µg/mL VHH-EGFP (Lane 2 & 6)	20 µg/mL IL-6R (Lane 3 & 7)	30 µg/mL IgG2A (Lane 4 & 8)
100 nM IgG	300 nM IL-6	100 nM IgG	72 nM VHH-EGFP
166 nM Streptavidin	37 nM EGFP	556 nM Ovalbumin	166 nM Streptavidin
556 nM Ovalbumin	166 nM Streptavidin	100 nM FcγRI	100 nM FcγRI
1 nM IgG	100 nM IgG	80 nM ProA	556 nM Ovalbumin
37 nM EGFP	80 nM ProA	72 nM VHH-EGFP	100 nM IgG
72 nM VHH-EGFP	100 nM FcγRI	300 nM IL-6	37 nM EGFP

Part II: Antibody Screening Assay

Immobilization levels of H3N2 HA on response channels averaged at 988 RU (Supplementary Figure 3). Supplementary Table 3 illustrates the analyte map with known concentrations for each lane. Comparing Figure 5 with Supplementary Table 3, the screening activity of the assay was able to determine with a 100% accuracy which samples contained anti-H3N2 HA Ab and which samples were negative controls. Binding thresholds were set to 20 RU to detect lower analyte concentrations. Supplementary Figure 4 illustrates the corrected curves of different analyte concentrations binding to the ligand. Binding responses range from 100 RU in the presence of 5.6 nM of analyte, to 566 RU in the presence of 200 nM of analyte. Wells that only contained serum (negative controls) showed little to no response, highlighting Alto's low susceptibility to non-specific binding (NSB) (Supplementary Figure 5).

The therapeutic discovery process is a long, labor intensive, and expensive process that involves generation of large scale libraries of chemical and biochemical 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 of 0.84 ppm (5.6 nM anti-H3N2 HA Ab) in complex media types with high accuracy and minimal NSB.

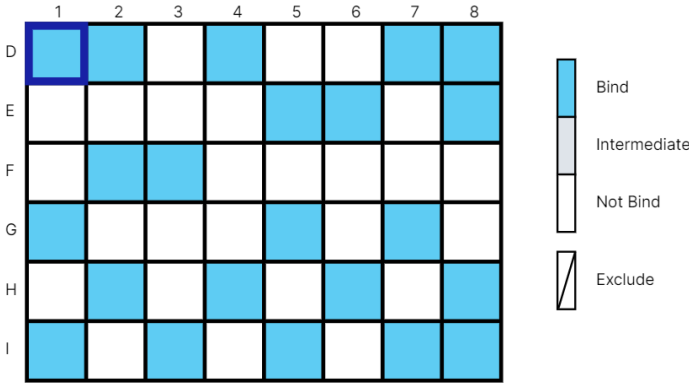

Figure 5: *Screening heat map illustrates which wells had serum containing anti-H3N2 HA Ab (analyte). Blue indicates analyte binding to H3N2 HA (ligand) and white indicates no binding was detected. Image was generated by Nicosystem analysis software.*

Conclusion

Researchers can easily integrate Alto into their therapeutic drug discovery process to identify potential candidates faster and further characterize their properties. Alto's new screening application allows users to screen up to 48 unique analytes against multiple ligand targets in under 4 hours. With 100-times lower sample volume requirements, short experimental times, minimum maintenance needs and crude sample compatibility, Alto can be used to screen for binding activity of various proteins, antibodies and other biotherapeutic agents.

Supplementary Figures

Table 1: Screening assay cartridge layout with protein and reagent conditions. "-" indicates an empty well.

Lane / Row	1	2	3	4	5	6	7	8
R (Regen)	Gly-HCl pH 1.5	Gly-HCl pH 2.0	Gly-HCl pH 3.0	Gly-HCl pH 1.5	PBST	-	Cleaning	PBST
A (Reagent)	Low RI Normalization				Activation			
B (Reagent)	High RI Normalization				Blocking			
C (Ligand)	40 µg/mL ProA in Na Acetate pH 5.0	25 µg/mL VHH-EGFP in Na Acetate pH 5.5	20 µg/mL IL-6R in Na Acetate pH 5.0	30 µg/mL IgG2A in Na Acetate pH 5.5	40 µg/mL ProA in Na Acetate pH 5.0	25 µg/mL VHH-EGFP in Na Acetate pH 5.5	20 µg/mL IL-6R in Na Acetate pH 5.0	30 µg/mL IgG2A in Na Acetate pH 5.5
D (Analyte)	100 nM IgG	300 nM IL-6	100 nM IgG	72 nM V HH-EGFP	100 nM IgG	300 nM IL-6	100 nM IgG	72 nM VHH-EGFP
E (Analyte)	166 nM Strep	37 nM EGFP	556 nM Ovalbumin	166 nM Strep	166 nM Strep	37 nM EGFP	556 nM Ovalbumin	166 nM Strep
F (Analyte)	556 nM Ovalbumin	166 nM Strep	100 nM FcγRI	100 nM FcγRI	556 nM Ovalbumin	166 nM Strep	100 nM FcγRI	100 nM FcγRI
G (Analyte)	1 nM IgG	100 nM IgG	80 nM ProA	556 nM Ovalbumin	1 nM IgG	100 nM IgG	80 nM ProA	556 nM Ovalbumin
H (Analyte)	37 nM EGFP	80 nM ProA	72 nM VHH-EGFP	100 nM IgG	37 nM EGFP	80 nM ProA	72 nM VHH-EGFP	100 nM IgG
I (Analyte)	72 nM VHH-EGFP	100 nM FcγRI	300 nM IL-6	37 nM EGFP	72 nM VHH-EGFP	100 nM FcγRI	300 nM IL-6	37 nM EGFP
Buffer	PBST							

Table 2: Layout of ligand (in yellow) on each lane and the analytes that were screened against the ligand. Under "Expected Outcome", analytes highlighted in blue are those that were expected to show binding to their ligand, and highlighted in white were expected to not bind to the ligand. Under "Observed Outcome", analytes highlighted in blue were samples that showed binding, and highlighted in white were the analytes that did not bind to their ligand.

Expected Outcome			
40 µg/mL ProA (Lane 1 & 5)	**25 µg/mL VHH-EGFP (Lane 2 & 6)**	**20 µg/mL IL-6R (Lane 3 & 7)**	**30 µg/mL IgG2A (Lane 4 & 8)**
100 nM IgG	300 nM IL-6	100 nM IgG	72 nM VHH-EGFP
166 nM Streptavidin	37 nM EGFP	556 nM Ovalbumin	166 nM Streptavidin
556 nM Ovalbumin	166 nM Streptavidin	100 nM FcγRI	100 nM FcγRI
1 nM IgG	100 nM IgG	80 nM ProA	556 nM Ovalbumin
37 nM EGFP	80 nM ProA	72 nM VHH-EGFP	100 nM IgG
72 nM VHH-EGFP	100 nM FcγRI	300 nM IL-6	37 nM EGFP
Observed Outcome			
40 µg/mL ProA (Lane 1 & 5)	**25 µg/mL VHH-EGFP (Lane 2 & 6)**	**20 µg/mL IL-6R (Lane 3 & 7)**	**30 µg/mL IgG2A (Lane 4 & 8)**
100 nM IgG	300 nM IL-6	100 nM IgG	72 nM VHH-EGFP
166 nM Streptavidin	37 nM EGFP	556 nM Ovalbumin	166 nM Streptavidin
556 nM Ovalbumin	166 nM Streptavidin	100 nM FcγRI	100 nM FcγRI
1 nM IgG	100 nM IgG	80 nM ProA	556 nM Ovalbumin
37 nM EGFP	80 nM ProA	72 nM VHH-EGFP	100 nM IgG
72 nM VHH-EGFP	100 nM FcγRI	300 nM IL-6	37 nM EGFP

Table 3: Layout of analyte concentrations (anti-H3N2 HA rabbit Ab in 20x diluted rabbit serum) in each lane. "-" indicates samples with 20x diluted serum and no antibody.

Lane Row	1	2	3	4	5	6	7	8
D	150 nM	8 nM	-	75 nM	-	-	25 nM	50 nM
E	-	-	-	-	16.7 nM	5.6 nM	-	200 nM
F	-	300 nM	150 nM	-	-	-	–	-
G	100 nM	-	-	-	5.6 nM	-	100 nM	-
H	-	16.7 nM	-	200 nM	-	150 nM	-	300 nM
I	5.6 nM	-	25 nM	-	50 nM	-	75 nM	8 nM

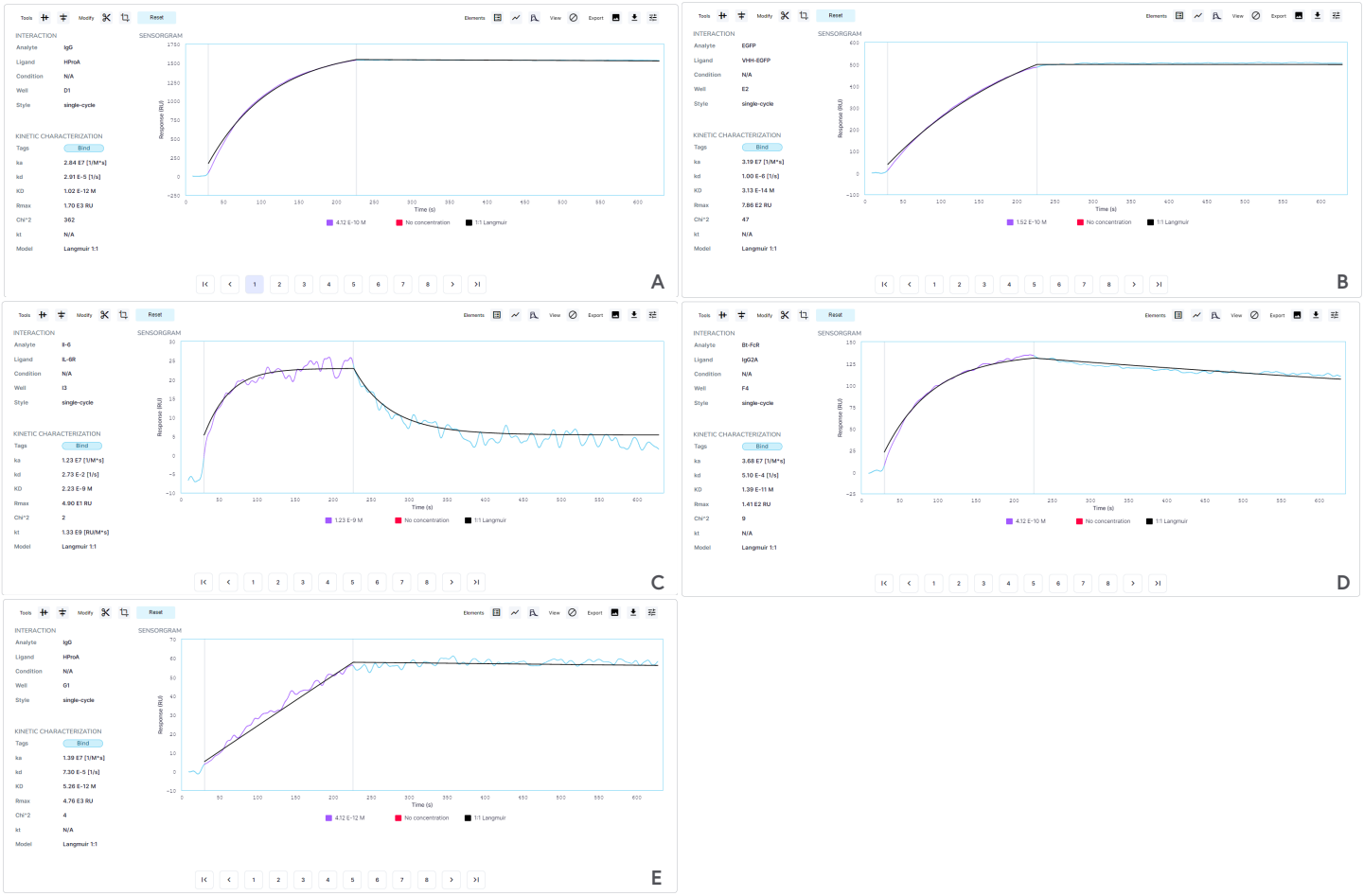

Figure 1: *Sample data of corrected curves of ligand-analyte pairs that showed binding. A) 100 nM IgG binding to ProA; B) 37 nM EGFP binding to VHH-EGFP; C) 300 nM IL-6 binding to IL-6R; D) 100 nM FcγRI binding to IgG2A and E) 1 nM IgG binding to ProA. Images were generated by Nicosystem analysis software.*

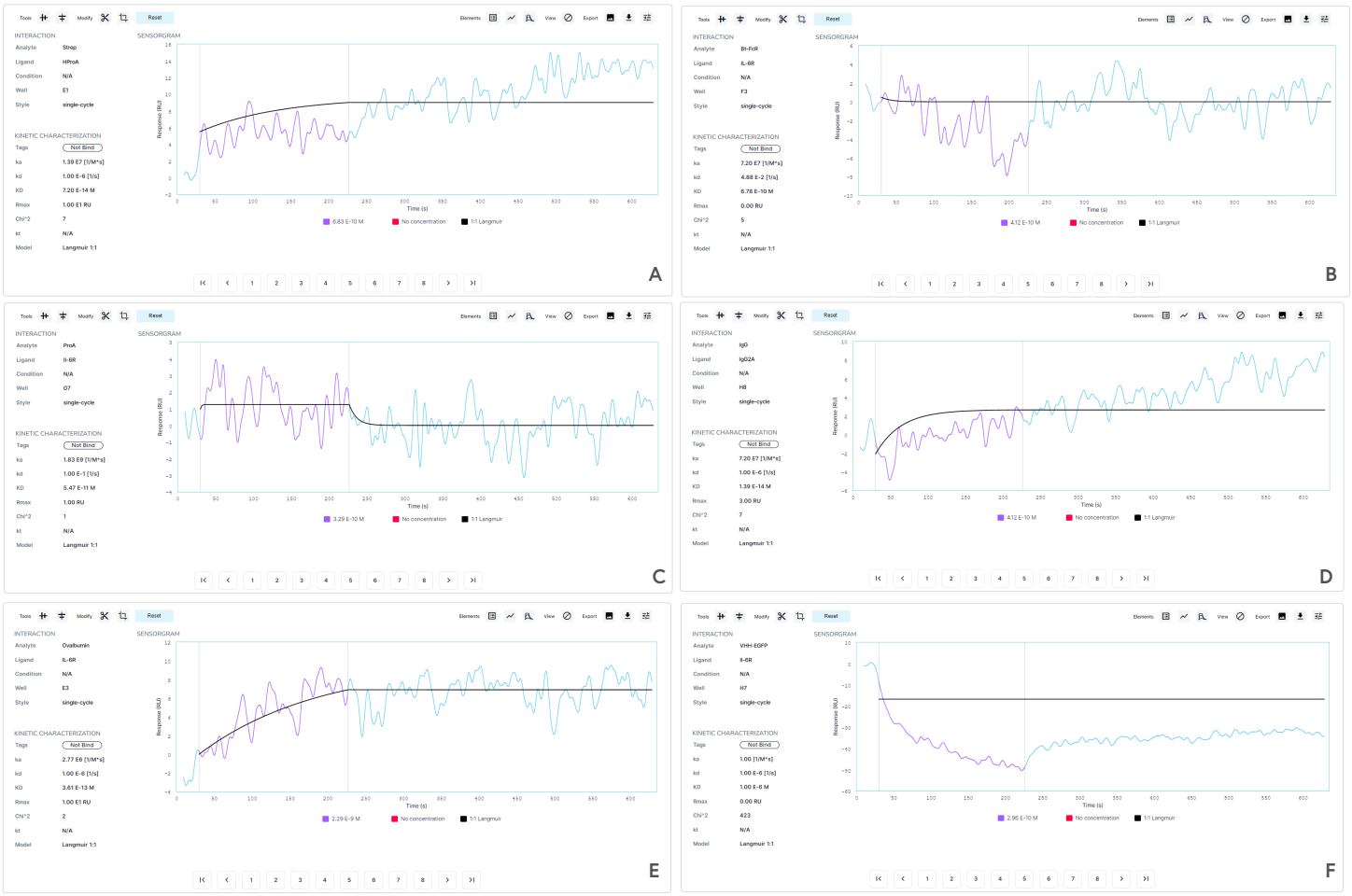

Figure 2: *Sample data of corrected curves of ligand-analyte pairs that did not show binding. A) Streptavidin showed no binding to ProA; B) FcγRI showed no binding to IL-6R; C) ProA showed no binding to IL-6R; D) IgG showed no binding to IgG2A; E) Ovalbumin showed no binding to IL-6R and F) VHH-EGFP showed no binding to IL-6R. Images were generated by Nicosystem analysis software.*

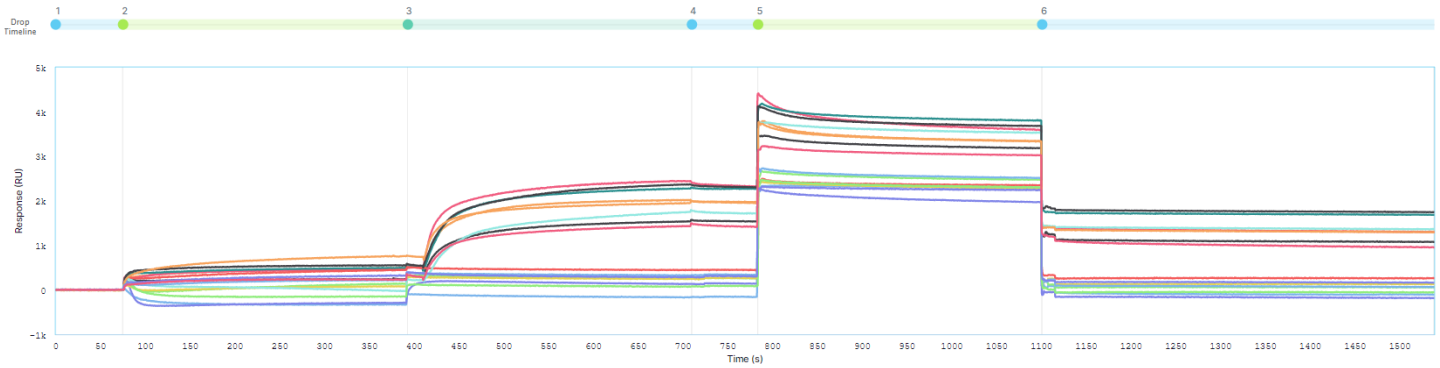

Figure 3: *Activation of response channels with EDC/NHS from Nicoya's Surfacing Kit followed by immobilization of H3N2 HA and blocking with 1 M Ethanolamine. Image was generated by Nicosystem analysis software.*

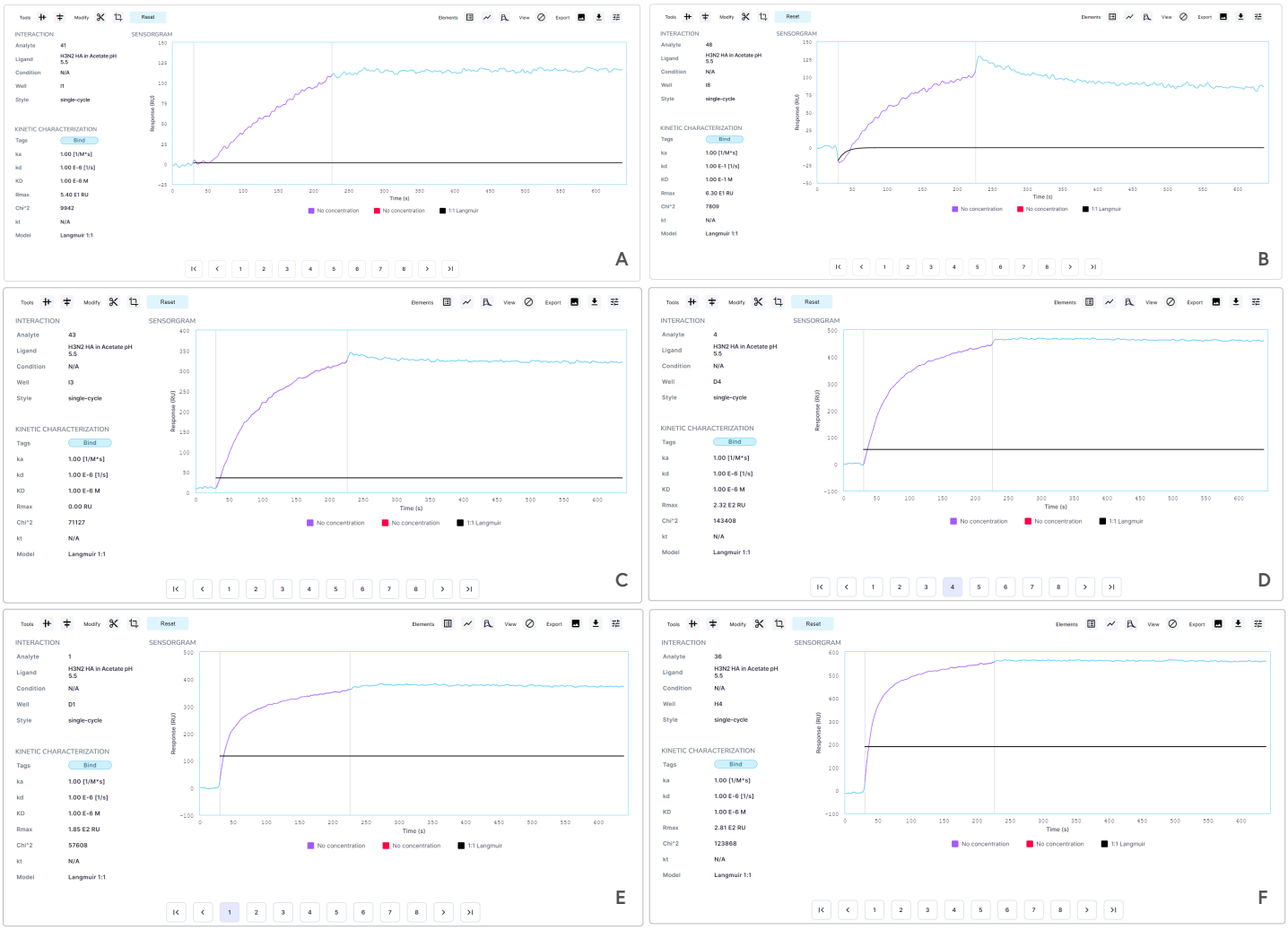

Figure 4: *Corrected curves of diluted serum samples containing analyte. A) 5.6 nM of anti-H3N2 HA Ab showed a response of 108 RU. B) 8 nM of anti-H3N2 HA Ab showed a response of 101 RU. C) 25 nM of anti-H3N2 HA Ab showed a response of 308 RU. D) 75 nM of anti-H3N2 HA Ab showed a response of 438 RU. E) 150 nM of anti-H3N2 HA Ab showed a response of 361 RU. F) 200 nM of anti-H3N2 HA Ab showed a response of 566 RU. No kinetic fits are provided since this test was performed as a blind test, with the user unaware of the analyte concentrations. Images were generated by Nicosystem analysis software.*

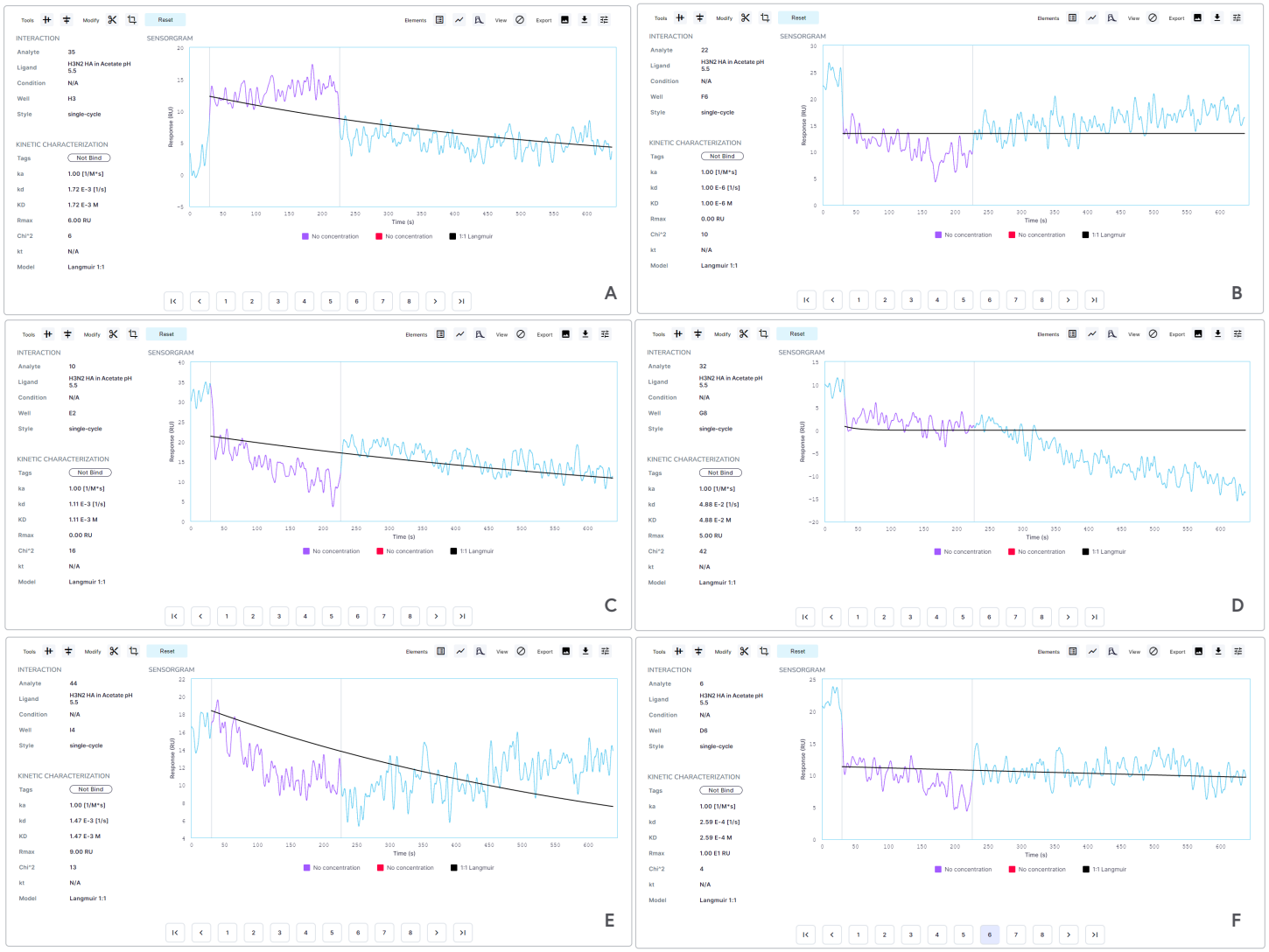

Figure 5: *Sample data of corrected curves of serum samples with no anti-H3N2 HA Ab. Images were generated by Nicosystem analysis software.*

Appendix

Materials

- 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)
  - PBS-T (0.1% Tween 20), 10 mM EDTA, 1 mg/mL BSA
- Alto CBX Surfacing Kit: cleaning, normalization, activation (ALTO-R-CBX-SURF)
- Immobilization Buffers:
  - Sodium Acetate pH 5.0 (ALTO-R-IMB-5.0)
  - Sodium Acetate pH 5.5 (ALTO-R-IMB-5.5)
- Regen Buffers:
  - Gly-HCl pH 1.5: (ALTO-R-GLYHCl-1.5)
  - Gly-HCl pH 2.0: (ALTO-R-GLYHCl-2.0)
  - Gly-HCl pH 3.0: (ALTO-R-GLYHCl-3.0)
- Ligand and analytes:
  - ProteoSure™ Recombinant Protein A, Acid-, Alkali-stable: Marvelgent, Cat# 14-RSPA-10mg
  - IgG from Human Serum: Sigma, Cat# I2511-10MG
  - GFP VHH, recombinant binding protein: Chromatek, Cat# gt-250
  - EGFP, recombinant purified protein: Chromatek; Cat# egfp-250
  - Mouse IgG2A Isotype Control: R&D Systems, Cat# MAB003
  - Biotinylated-Mouse Fc gamma RI (FcγRI)/ CD64 Protein, His,Avitag™: Acrobiosystems, Cat# CD4-M82E7
  - IL-6 Protein, Human, Recombinant: Sino Biologicals, Cat# 10395-HNAE
  - IL-6R Protein, Human, Recombinant (ECD, His & AVI Tag), Biotinylated: Sino Biologicals, Cat# 10398-H49H-B
  - Streptavidin: Cedarlane, Cat# CLPRO283
  - Ovalbumin from chicken egg white: Sigma, Cat# A5503-1G
  - Influenza A H3N2 Hemagglutinin (A/Brisbane/10/2007) Protein (His-Tag): Sino Biological, Cat# 11056-V08H
  - Influenza A H3N2 (A/Brisbane/10/2007) Hemagglutinin / HA Antibody, Rabbit MAb: Sino Biological, Cat# 11056-R104
  - Normal Rabbit Serum: Jackson Immuno Research, Cat # 011-000-120