TECH NOTE

Quantifying monoclonal antibodies in serum with Alto™ Digital SPR

Overview

As the first digital microfluidic (DMF) powered surface plasmon resonance (SPR) instrument in the market, Alto provides users with a quantitation assay to quantify proteins of interest in various mediums, including crude samples. Commonly used quantitative methods, such as Bradford assays, can be time-consuming, require extensive amounts of manual work from the user, and are limited to working with purified samples. With Alto's fully automated system, users can easily generate 8 standard curves and estimate concentrations of up to 40 unknown samples using the quantitation assay.

This note will demonstrate how Alto accurately quantifies monoclonal antibodies (mAb) in serum specific to the H3N2 hemagglutinin (HA) protein at varying concentrations. H3N2 is a variant of the H1N1 Influenza virus and uses HA, a surface glycoprotein, for viral entry into the target cell. The receptor-binding domain of HA is a popular target for antibodies due to their ability to directly inhibit binding of the virus to the host cell receptor.

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) + 10 mM EDTA, 1 mg/mL BSA
- Cleaning, normalization, activation: Alto Carboxyl Surfacing Kit (ALTO-R-CBX-SURF)
- Immobilization buffer: Sodium Acetate pH 5.5 (ALTO-R-IMB-5.5)
- Regeneration buffer: Gly-HCl pH 2.0 (ALTO-R-GLYHCl-2.0)
- Ligand and analyte:
  - Influenza A H3N2 Hemagglutinin / HA (A/Brisbane/10/2007) Protein (His-Tag): Sino Biological, Cat# 11056-V08H
  - Influenza A H1N1 (A/California/07/2009) Nucleoprotein / NP Protein (His Tag); Sino Biological, Cat# 40205-V08B
  - 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

Method

Experiment

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 channels (8 reference channels and 8 active channels). The experimental method was designed on the Nicosystem using the direct quantitation application. The completed method 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.

Alto's quantitation assay includes the following activities: Normalization, Clean, Build Ligand, Quantitation Standards, Quantitation of Unknown Samples.

Firstly, all 16 carboxyl sensors were exposed to high and low RI droplets during the normalization activity.

Sensors were then cleaned with 10 mM HCl, activated with the Carboxyl Surfacing Kit and immobilized with H3N2 HA ligand on the response channels and H1N1 NP on the reference channels. Sensors were blocked to quench any remaining carboxyl groups (figure 1).

A 3X serial dilution on purified samples of H3N2 HA mAb was repeated 10 times to generate a standard curve of 10 different concentrations (figure 2). Following the standard curves, all sensors were exposed to spiked serum samples of H3N2 HA mAbs at various concentrations.

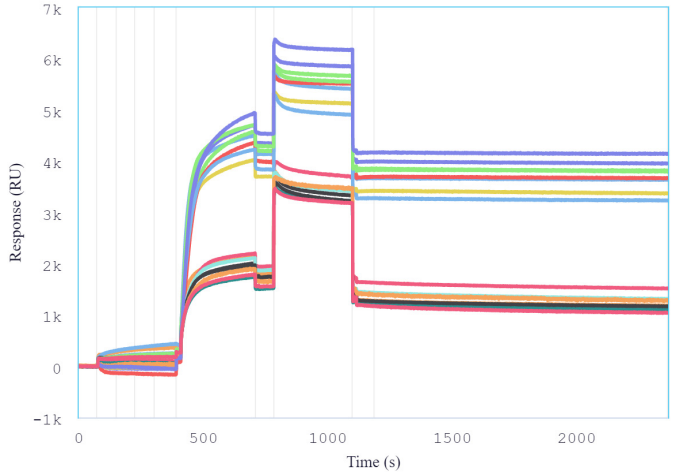

Figure 1: *Activation of response channels with EDC/NHS from Nicoya's Surfacing Kit, followed by immobilization of 50 µg/mL H3N2 HA antigen in Sodium Acetate pH 5.5 in the active channels, and 50 µg/mL H1N1 NP antigen in Sodium Acetate pH 5.5 in the reference channels and blocking of sensors with 1 M Ethanolamine.*

Analysis

Standard Curve

Nicosystem's analysis software automatically processes curves in each lane (e.g., y-align, reference subtract and overlay) to generate an interactive standard curve consisting of 10 different concentrations, each 3X serially diluted (figure 2). Users can crop, y-align, x-align, de-spike, and exclude certain standards from their analysis. Average response values at each known concentration are taken from an interval at the end of the association phase (default) using the Report Point Tools. This interval can be adjusted by the user to create a calibration curve that better suits their data. The average response value of this interval is used to generate the calibration curve.

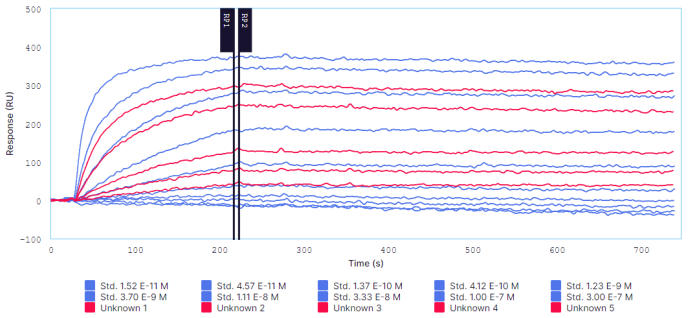

Figure 2: *Sensorgram View of corrected binding curves of H3N2 HA mAb binding to the immobilized H3N2 antigen, containing ten three-fold dilutions of the known standard analyte sample (blue curves) and five unknown analyte samples (red curves). The report point flags are shown for the time interval used to collect the response reading for each concentration/unknown measurement.*

Calibration Curve

Each standard curve generates a calibration curve, where the following 5PL logistic equation is used to fit the data:

5PL

$$y = d + \frac{(a-d)}{\left[1+\left(x/c\right)^{b}\right]^{g}}$$

To generate the best fit, least-square regression technique is used to minimize the sum of the square of the distance between the data points (responses of each concentration) and the model. This is repeated over a 100 iterations to get the closest fit to the model. Various parameters of the 5PL model, including inflection point, maximum and minimum asymptote, slope, and asymmetry factors can be obtained from the calibration curve.

Using the model, unknown samples are plotted against the fit to determine their concentration (figure 3).

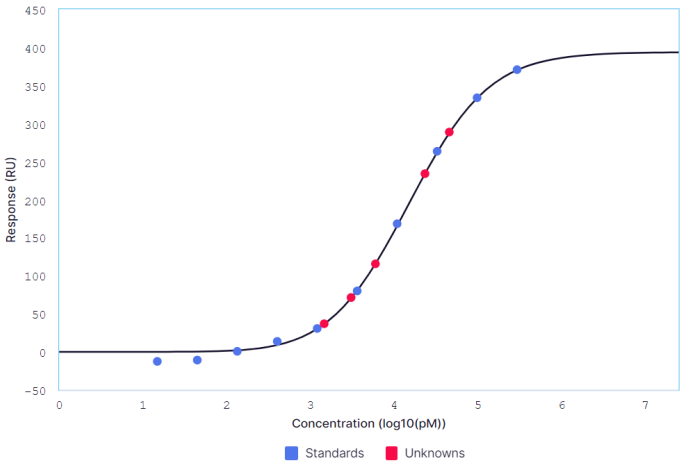

Figure 3: *5-parameter logistic (5PL) calibration curve (black curve) created from plotting the log10 of the concentration for the ten standard analyte concentrations against their binding response (blue points). The unknown concentrations are plotted on the calibration curve (red points) and their concentration is solved from the 5PL model.*

Quantitation of Unknown Samples

Nicosystem's analysis software generates a summarized table of data, as shown in Table 1. The table shows a snippet of Lane 3's standard curve (Well D3) and calculated concentrations for unknown samples E3-I3. Unknown samples are spiked serum samples with varying concentrations of H3N2 mAb (2 nM, 4 nM, 8nM, 32 nM and 64 nM). The calculated concentrations of the unknown samples ("Calc Conc") had a maximum difference of 10% from the actual concentration.

Conclusion

Alto's fully automated quantitation assay allows users to accurately determine concentrations of proteins in complex media with minimal time in the lab. Alto can successfully generate a processed 10-concentration standard curve, a calibration curve using a 5PL logistics curve to fit the data, and a results table that accurately quantifies up to 40 samples.

Table 1: Summarized table of Quantitation results* generated in Nicosystem's analysis software.

Well	Sample	Vol (µL)	Conc (C)	C units	Calc Conc	Response	Time (RP 1)	Time (RP 2)	Residual %
D3	R104	4	0.14	nM	0.58	4.01	63.57	68.97	324.02
D3	R104	4	0.41	nM	N/A	2.23	63.57	68.97	N/A
D3	R104	4	1.23	nM	1.07	6.26	63.57	68.97	13.52
D3	R104	4	3.7	nM	3.83	20.77	63.57	68.97	3.44
D3	R104	4	11.11	nM	10.97	54.64	63.57	68.97	1.26
D3	R104	4	33.33	nM	33.59	125.92	63.57	68.97	0.77
D3	R104	4	100	nM	99.54	220.16	63.57	68.97	0.46
D3	R104	4	300	nM	300.57	307.45	63.57	68.97	0.19
E3	32nM	3	N/A	nM	30.98	119.56	63.57	68.97	N/A
F3	64nM	3	N/A	nM	67.97	186.21	63.57	68.97	N/A
G3	2nM	3	N/A	nM	1.74	9.7	63.57	68.97	N/A
H3	4nM	3	N/A	nM	3.86	20.9	63.57	68.97	N/A
I3	8nM	3	N/A	nM	7.88	40.9	63.57	68.97	N/A

*Well: D wells are known standards, E to I wells are unknowns; Conc: Known Concentration (standards only); C units: Units for concentration; Calc Conc: Calculated Concentration; Response: Average Response Value in time segment selected by Report Point Tools; Report Time Points (RP1 and RP2); and Residual %: Sum of the squared distance between the data point and model.