APPLICATION NOTE

nicoya

Characterization of Biomolecular Interactions using Alto: Comparison to Conventional SPR

Summary

Label-free analysis of biomolecular interactions is a critical tool for the development of biologics. Conventional label-free analytical instruments require relatively large amounts of sample thereby making it challenging to use in the early stages of biologics discovery and development. Many of the conventional systems require highly trained personnel to produce high quality data. Further, systems that employ conventional fluidics based on tubing, pumps and valves, are prone to higher risk of reliability issues. This, at the same time, limits sample type utility due to issues such as clogging, thereby rendering them to have limited utility with crude samples. Nicoya's Alto addresses these challenges through the use of digital microfluidics and integrated LSPR sensors to perform label-free analysis in sub-microliter droplets.This application note demonstrates the use of Alto for measuring biomolecular interactions and compares it to a conventional SPR platform. The results show excellent agreement of the kinetics and affinity between Alto and the conventional SPR platform, while using only 1% of the sample and needing under 30 minutes of hands-on time. All of these make Alto an extremely efficient and user-friendly platform for biologics discovery and development.

Introduction

Label-free analytical methods are used in target molecule identification to lead selection and optimization during therapeutic discovery workflow. Kinetic parameters collected by label-free analytical instruments are essential for understanding the mode of action of the candidate therapeutic against its target and its advancement in the discovery pipeline. Conventional label-free instruments utilize the surface plasmon resonance (SPR) phenomenon to detect and quantify binding interactions of biomolecules immobilized on gold surfaces. These instruments require injections of large volumes of samples and buffers, which are handled via complex networks of pumps, valves, tubing and flow cells. As a result of possible sedimentation when using non-homogenous samples, conventional label-free instruments are prone to clogging. Hence, they are not always compatible with crude samples, and require regular maintenance that can be expensive. All of this creates down-time in instrument availability and slows down the discovery process.

The Alto platform uses a digital microfluidics-powered localized SPR (LSPR) system for label-free characterization of biomolecule interactions. In Alto, all experimental reagents such as samples and buffers are loaded and self-contained into a disposable cartridge. Voltage is applied across electrodes to dispense, move, mix, and conduct dilution series on 350 nL droplets inside the cartridge. When the experiment is complete, the cartridge is easily discarded and data can be readily analyzed with the click of a button. All together, Alto requires minimal training to use, is compatible with crude samples, and requires only 2 µL sample volumes for full kinetic characterization.

Commonly used in antibody based therapeutic selection processes, Human Combinatorial Antibody Libraries (HuCAL) is the first ever fully synthetic human antibody library developed to represent the essential features of the natural human antibody repertoire. Libraries contain consensus frameworks of seven heavy chain and an equal number of light chain germline families that cover more than 95% of the human antibody diversity in a modular format. When combined with the modern antibody selection methods such as phage display selection, HuCAL becomes a highly sophisticated technology for rapidly selecting antibodies and tuning affinities to a target of interest. Due to its ease of use and speed,

HuCAL is commonly used in conventional methods for antibody selection. In this application note, we have used enhanced green fluorescent protein (eGFP) as the target for selection of antibodies from the HuCAL library (Figure 1) to demonstrate the Alto platform's superior ease of use and sample requirements while producing equivalent data quality for affinity and kinetics selection when compared to conventional SPR platforms.

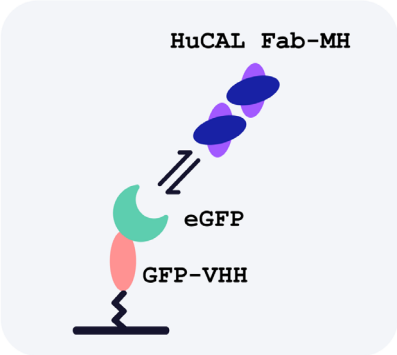


Figure 1: *Schematic demonstrating experimental binding setup*

Material and Equipment

- Alto Instrument (ALTO16)
- Alto CBX Cartridge (KIN-CART-CBX-16)
- Alto Running Buffer: PBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-PBST)
- Alto Regeneration Buffer: 10mM Glycine-HCl pH 2.0 (ALTO-R-GLYHCl-2.0)
- Alto Amine Coupling Kit (ALTO-R-EDCNHS)
- Capture molecule: GFP-VHH (Alpaca anti-GFP VHH, purified recombinant binding protein): Chromotek (CAT# GT-250)
- Ligand: Purified recombinant Enhanced Green Fluorescent Protein (eGFP): Chromotek, (CAT# EFP-250)
- Analyte: HuCAL Fab-MH (monovalent human) Negative Control: Bio-Rad, (CAT# HCA051).

Experimental Details

Experimental steps are listed below for both Alto and the conventional SPR system.

Alto

Experimental design was remotely completed on Alto's User Portal, followed by run initiation on the instrument:

1. Using a laptop, the experiment (Run Method) was designed in the Nicosystem Software and saved on the User Portal.
2. On the instrument, the designed experiment was selected to launch Alto's on-screen setup wizard.
3. When prompted, a Carboxyl Cartridge was loaded and all reagents were pipetted according to the on-screen wizard.
4. The experiment was initiated by selecting the 'Run Method' command.

The remainder of the experiment was executed automatically by Alto for the multi-cycle kinetics (MCK) experiment, with the operator being completely hands-off:

5. The sensors in the cartridge were primed with 10 mM HCl for 40 seconds.
6. The carboxyl surface was activated with EDC/NHS for 5 minutes.
7. The GFP VHH capture molecule was prepared in acetate pH 5.0 and immobilized at 25 µg/ml for 10 minutes on all Sensors.
8. All sensors were blocked using an ethanolamine blocking solution for 5 minutes to quench the remaining active Carboxyl groups.
9. Five automated 3-fold serial dilutions were executed per monovalent human HuCAL analyte sample from the 300 nM stock concentration, producing 1.25 nM, 3.7 nM, 11.1 nM, 33.3 nM and 100 nM analyte samples.
10. The eGFP protein ligand at 0.5 ug/mL in PBS-T was passed over the active channel and captured by the GFP VHH.
11. The first concentration of the HuCAL analyte sample was introduced with an association time of 300 seconds, followed by a 300 seconds dissociation
12. A 60 seconds regeneration of Glycine-HCl pH 2.0 was performed to remove the captured eGFP ligand and HuCAL analyte complex.
13. Steps 9 through 11 were repeated for the next four concentrations of HuCAL.

14. Upon completion of the test, binding curves were fitted to a 1:1 diffusion corrected binding model to determine kinetic and affinity constants.

Conventional SPR

1. Prepared minimum 500 mL of required buffer.
2. All common reagents and samples were pipetted into a 96-well plate. HuCAL analyte samples were prepared via 3-fold serial dilution on a 96-well plate producing 1.25 nM, 3.7 nM, 11.1 nM, 33.3 nM and 100 nM analyte samples. At least, 200 µL of each sample dilution and reagent was prepared.
3. The sensor was installed.
4. The surface was primed with the running buffer.
5. The tubing in the instrument was degassed.
6. A plate map was created for experiment based on the prepared 96 well plate containing the samples. The plate map was saved and loaded into a new method.
7. A method was manually built in the software which allows the user to select well, reagent type (ligand, common or analyte), volume to be used per sample, flow rate, and dissociation time. The method was then saved prior to execution.
8. The 96-well plates containing the samples were loaded into the sample compartment of the instrument.
9. The method was sent to the instrument for execution.

The remainder of the experiment was executed automatically by the conventional SPR instrument for the multi-cycle kinetics (MCK) experiment, with the operator being completely hands-off until data analysis:

10. The sensor was primed with 10 mM HCl for 60 seconds.
11. The sensor surface was activated with 0.4 M EDC/0.1 M NHS for 5 minutes.
12. The GFP VHH capture molecule was prepared in acetate pH 5.5 buffer and immobilized at 50 µg/ml for 6 minutes on all sensors.
13. All sensors were blocked using an ethanolamine blocking solution for 5 minutes to quench the remaining active Carboxyl groups.
14. The sensor surface was preconditioned with 10 mM HCl for 60 seconds.
15. First the eGFP protein ligand at 1 ug/mL in PBS-T was passed over the active channel and captured by the GFP VHH.
16. A buffer blank was passed over the active and reference channels with an association time of 180 seconds and a 1200 seconds dissociation time.
17. The first concentration of the HuCAL analyte sample was introduced with an association time of 180 seconds, followed by a 1200 seconds dissociation.
18. Step 6 was repeated for the next four concentrations of HuCAL.
19. Upon completion of the test, the data was opened in the corresponding analyser software. The data corresponding to the tested analyte concentrations was manually selected.The data was pre-processed by selecting the active and reference channels to subtract background binding and the buffer blank was subtracted to create a double referenced dataset.
20. Binding curves were fitted to a 1:1 diffusion corrected binding model to determine kinetic and affinity constants.

Results and Discussion

Precision

In this capture kinetic experiment, GFP VHH capture molecules were immobilized via amine coupling onto the carboxy sensor surface. Following this step, eGFP ligand was captured on each immobilized sensor surface and the HuCal analyte was brought to the sensor surface to collect binding kinetics. For each cycle of the MCK, regeneration of the VHH sensor surface was performed to remove the captured eGFP ligand and HuCAL analyte complex, then the eGFP ligand was recaptured onto the sensor surface prior to the next concentration of the analyte.

Overall analysis of surface recapture levels showed that Alto maintains a high level of ligand capture level with a less than 10% CV for recapture on average, as shown in Table 1. Further, a consistently similar binding curve profile was observed after each recapture step as displayed in Figure 2.

eGFP capture

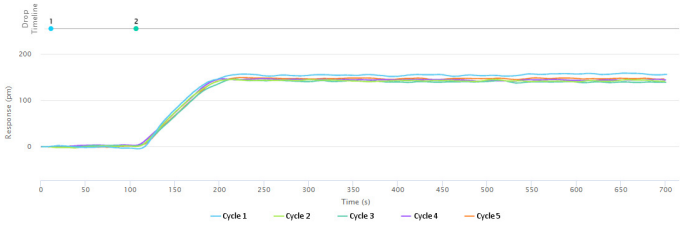


Figure 2: *Overlay of the recaptured eGFP ligand onto the VHH capture surface for each cycle of the multi-cycle kinetic experiment. Drop 1 on the drop timeline is a buffer drop, and Drop 2 is the eGFP ligand.*

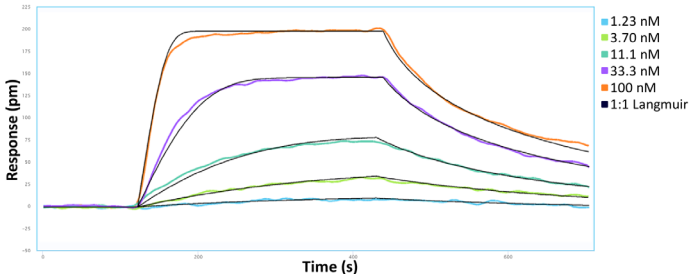
Table 1: eGFP capture for each cycle of the multi-cycle kinetic experiment ran in 5 replicates of the same sample

		Sensor				
		1	2	3	4	5
MCK Cycle (pm)	1	120.3	86	110.2	144.5	174.9
	2	116.1	103.2	109.3	125.1	153.7
	3	108.7	100.8	101.5	126.9	152.6
	4	99.2	104	91.9	131.9	154.4
	5	81.8	107.4	83.8	129.7	149.2
Average		105.2	100.3	99.3	131.6	156.9
STD		15.3	8.3	11.4	7.7	10.2
%CV		14.60%	8.30%	11.50%	5.80%	6.50%

Data Quality

Kinetic values were calculated based on the sensorgrams generated by the Alto and conventional SPR instrument. Visual comparison of the two sensorgrams (Figure 3) showed comparable binding curves for each analyte concentration. Comparison of the kinetic values obtained by Alto and the conventional SPR instrument revealed similar results as shown in Table 2. In summary, Alto was able to generate kinetic data results and binding curves that compare very well with the conventional SPR instrument.

A: Alto



B: Conventional SPR

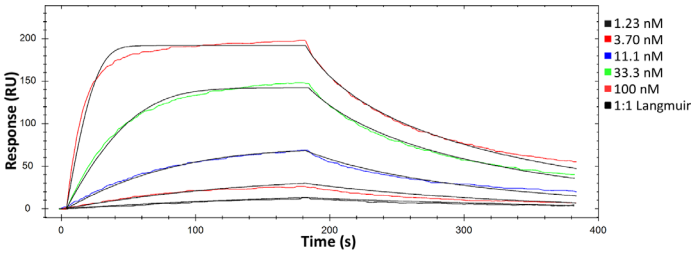


Figure 3: *Multi-cycle kinetics of HuCAL (analyte) binding to captured GFP (ligand) on (A) Alto and (B) conventional SPR. Analyte was titrated from 1.23 nM to 100 nM. Black curve represents the Langmuir 1:1 diffusion corrected binding fit.*

Table 2: Comparison of the kinetic data parameters calculated from the sensorgrams collected by Alto and conventional SPR experiments.

	Alto	Conventional SPR
kon (1/(M*s))	1.58E+06	1.81E+06
koff (1/s)	1.29E-02	1.82E-02
KD (M)	8.6 nM	10.0 nM
RMAX (RU)	1.36E+02	2.11E+02
Xi squared	1.35E+01	1.06E+01

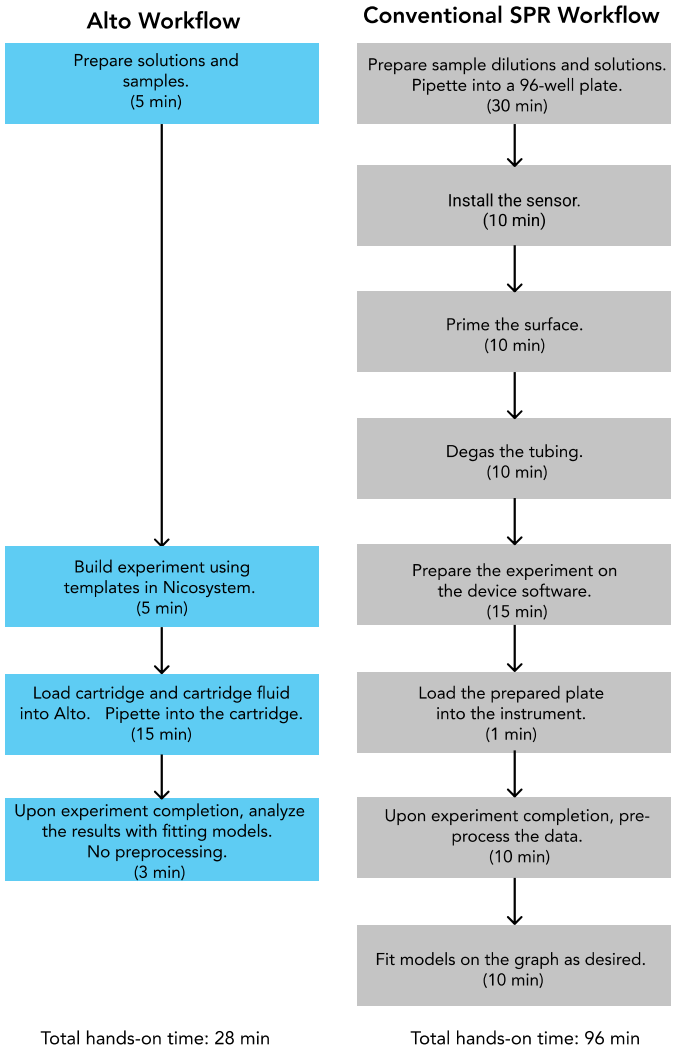


Figure 4: *Flow chart of Alto vs conventional SPR workflow with step time summary. This figure summarizes side by side user workflow steps executed from sample preparation to data analysis. Overall, the same experiment required 28 minutes of hands-on operator time with Alto, instead of 96 minutes with the conventional SPR instrument.*

Ease of Use

The same experiment described above was conducted in Alto and the conventional SPR instrument to compare the performance of the two devices as summarized in Figure 4. This experiment required 28 minutes of operator time with Alto versus 96 minutes with the conventional SPR device. Side by side experimental workflow comparison revealed that 25 minutes of operator time was saved in preparation of sample dilutions with Alto's on-cartridge dilutions. In addition, eliminating sensor installation, priming and degassing of the tubing resulted in 30 minutes of time that is saved from the operator's workflow. Furthermore, Alto's intuitive software, prepared templates and automated analysis tool has saved an additional 27 minutes of operator time. Overall, when compared to the conventional SPR instrument, Alto saved approximately an hour of operator time during this experiment.

Conclusion

When compared to the conventional SPR system, the Alto platform demonstrated equivalent accuracy for obtaining kinetic measurements for kon, koff, KD, RMAX and Xi-squared. While Alto needed only 2 µL of sample volume and under 30 minute hands on time, the conventional SPR system required 200 µL of sample and a corresponding hands on time of over 90 minutes. The Alto workflow significantly decreases the burden of preparing large experimental production batches for biologic screens and accelerates time to discovery. When combined with the ease of use, overall time savings and only 2 µL of sample volume requirement, Alto is well-positioned to be a powerful label-free analysis instrument of choice for therapeutic discovery and development applications.