APPLICATION NOTE

nicoya

Label-free Characterization of Complementary Oligonucleotide Sequences using Alto™

Summary

Oligonucleotides offer a promising vector for the treatment of diseases related to gene expression and have recently transformed the vaccine industry with the resounding success of mRNA-based COVID-19 vaccines. In this application note, we demonstrate Alto's applicability to the development of oligonucleotide systems in various therapeutic capacities. Alto is used to accurately and precisely measure affinities and provide on/off-rates equivalent to those obtained with conventional SPR.

Introduction

Oligonucleotides, or oligos, are short, single-stranded sequences of synthetic DNA or RNA. In the past couple of decades, advances in the development of low-cost synthesis of oligonucleotides have resulted in their widespread adoption as PCR primers and probes in bioassays such as microarrays and in situ hybridization. Because they are synthesized in a stepwise process, oligos are highly customizable, both in nucleotide sequence and in various types of chemical modifications, which can confer great stability and versatility. Oligonucleotides are also highly resistant to most environmental conditions, making them very easy for researchers to store and handle.

One of the key features of nucleic acids is their ability to bind their reverse complement sequences with a high degree of specificity and affinity. This property can be leveraged for therapeutic uses by targeting disease-related DNA or RNA sequences. Crucially, they can be used to modulate gene expression in a number of different ways, including gene silencing by RNAi, targeted degradation by RNAse H, inhibition by miRNA and transcriptional gene activation. As of the end of 2021, there were 15 FDA-approved oligonucleotide therapeutics, and antisense oligonucleotides in particular continue to be an emerging area of therapeutic research.[1,2] The most recent oligonucleotide drugs include casimersin, an antisense oligonucleotide used to treat Duchenne muscular dystrophy, and inclisiran, an siRNA used to treat atherosclerotic cardiovascular disease and heterozygous familial hypercholesterolemia.

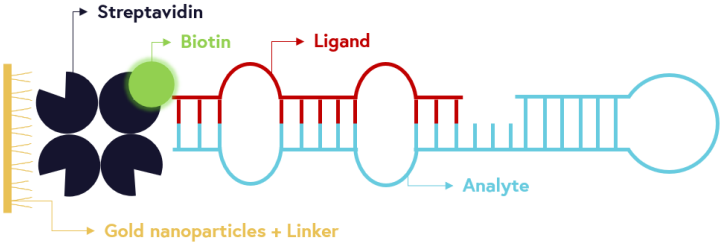

Figure 1: *Overview of Nicoya oligo system.*

In 2020, a significant advance in oligonucleotide research was achieved with the first FDA-approved mRNA vaccines.[3] Human cells take up the mRNA and use it to synthesize the spike protein of SARS-CoV-2, which elicits an antibody response. This achievement is revolutionary for vaccine development, and further corroborates the importance of nucleic acid therapies in medicine.

Representative DNA Oligonucleotide System

In this application note, a representative oligonucleotide pair is tested to show the applicability of Alto in the aforementioned applications. Typical nucleotide binding consists of a 1:1 binding interaction between one sequence and its reverse complement sequence. The ligand in this case is a 15-nucleotide DNA sequence, with a 5' biotin tag. The biotin tag will be used to immobilize the ligand on a streptavidin surface, as shown in Figure 1. The analyte is a 53-nucleotide sequence, which contains two main segments, the reverse complement segment and the bulking segment. The reverse complement segment is at

the 3' end, and contains the reverse complement sequence to the ligand, but has two mismatched base pairs. The bulking segment serves to increase the molecular weight of the analyte, thereby increasing Rmax. This bulking sequence was designed to be self-complementary, reducing the potential of non-specific binding (NSB).

Characterizing Oligonucleotides with SPR

To facilitate the development of the applications outlined above, highly selective and specific bioassays that adequately reflect the reverse complementary binding of oligonucleotides are needed from discovery, through development, and into production. Surface plasmon resonance (SPR) is a powerful tool that meets these needs and is well-suited for analyzing oligonucleotide interactions. Acquisition of real-time binding data enables deeper insight into the binding kinetics of oligonucleotides, compared to affinity data alone.

Advantages of Alto Digital SPR

Alto, a high-throughput SPR platform enabled with digital microfluidic (DMF) technology, has a number of advantages that make it ideal for SPR characterization of oligos and their targets. With the ability to discreetly control nanolitre-sized droplets of each ligand and analyte, binding assays are simple to implement and provide novel insights into therapeutic performance, while reducing consumption of precious samples by up to 200X. In addition, Alto's 16 independent channels allow for the simultaneous analysis of multiple targets in many different assay formats, while significantly reducing hands-on time with complete assay automation.

In this application note, we demonstrate the applicability of Alto in measuring binding affinities and kinetics of oligos through characterization of a DNA oligonucleotide with a biotin tag and a DNA oligonucleotide analyte containing its reverse complement sequence. We also confirm the accuracy of Alto compared to conventional SPR and highlight Alto's numerous advantages in this important application.

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)
- Regeneration Buffer: 1 mM NaOH (0.1% Tween 20), (ALTO-R-NaOH)
- Nicoya Streptavidin Coupling Kit (ALTO-R-STREPTAVIDIN)
- Ligand: Biotinylated oligo "Bt-oligo" (ALTO-R-BT-OLIGO-L)
- Analyte: Solution oligo "S-oligo" (ALTO-R-OLIGO-A)

Method

Full method is detailed in the Appendix.

Results & Discussion

Kinetic values were calculated based on the sensorgrams obtained on a single cartridge with Alto for each of the SCK and MCK formats. The data were fitted to a Langmuir 1:1 binding model analyzed in the Nicoya Analysis Software. The kinetic parameters for both SCK and MCK experiments, as well as their respective error and % CV, are presented in Table 1. From this analysis, association and dissociation rate constants for the SCK experiment of 4.14×10^4 $M^{-1}s^{-1}$ and 3.16×10^{-4} s^{-1}, respectively, were determined, resulting in a K_D of 7.94 nM (Figure 2). No NSB was observed and full regeneration was achieved by 1 mM NaOH (Figure 3). An example result for SCK showing the data sets obtained across an entire cartridge is included in Figure 4 to demonstrate the excellent reproducibility of the data across all channels and cycles. From the analysis of MCK data (Figure 5), association and dissociation rate constants of 6.89×10^4 $M^{-1}s^{-1}$ and 3.63×10^{-4} s^{-1}, respectively, were determined, resulting in a K_D of 5.38 nM, closely matching the K_D of 7.94 nM obtained from the SCK experiment. As with SCK, no NSB was observed and full regeneration was achieved by 1 mM NaOH (Figure 6).

Table 1: Kinetic parameters measured for full cartridge of oligo using SCK and MCK on Alto.

Type	Rmax (RU)	% CV	k_a ($M^{-1}s^{-1}$)	% CV	k_d (s^{-1})	% CV	K_D (nM)	% CV
SCK	163 ± 12.58	12.58	$4.14 \times 10^4 \pm 6.80 \times 10^3$	16.43	$3.16 \times 10^{-4} \pm 5.57 \times 10^{-5}$	17.61	7.94 ± 2.25	28.39
MCK	163 ± 16.6	10.19	$6.89 \times 10^4 \pm 9.14 \times 10^3$	13.26	$3.63 \times 10^{-4} \pm 6.52 \times 10^{-5}$	17.98	5.38 ± 1.27	23.61

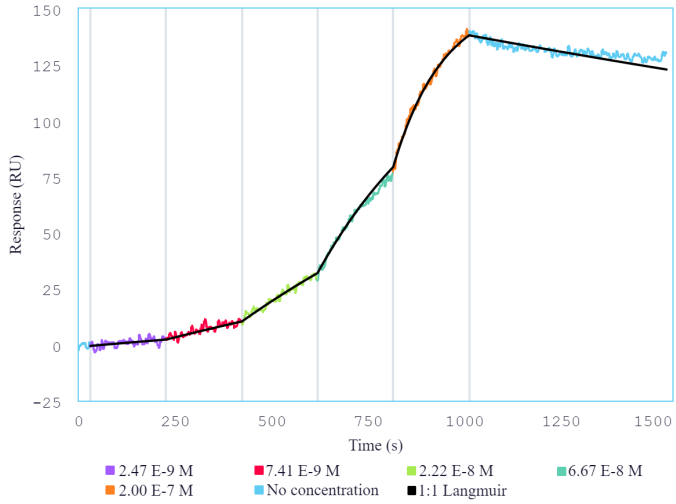

Figure 2: *Single-cycle kinetics of S-oligo (analyte) binding to immobilized Bt-oligo (ligand) on Alto. Analyte was titrated from 2.47 nM to 200 nM. Black curve is the Langmuir 1:1 binding fit model analyzed in the Nicoya Analysis Software.*

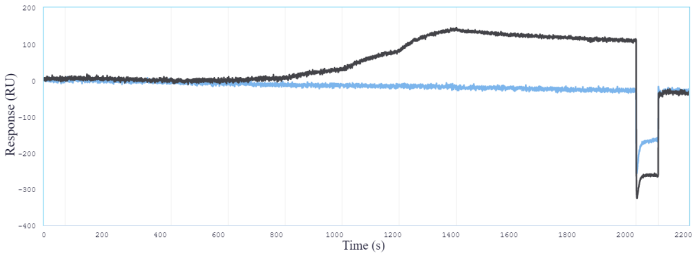

Figure 3: *Reference (blue trace) and active channel (black trace) binding data showing binding of S-oligo to the Bt-oligo. Full regeneration was achieved by 1 mM NaOH.*

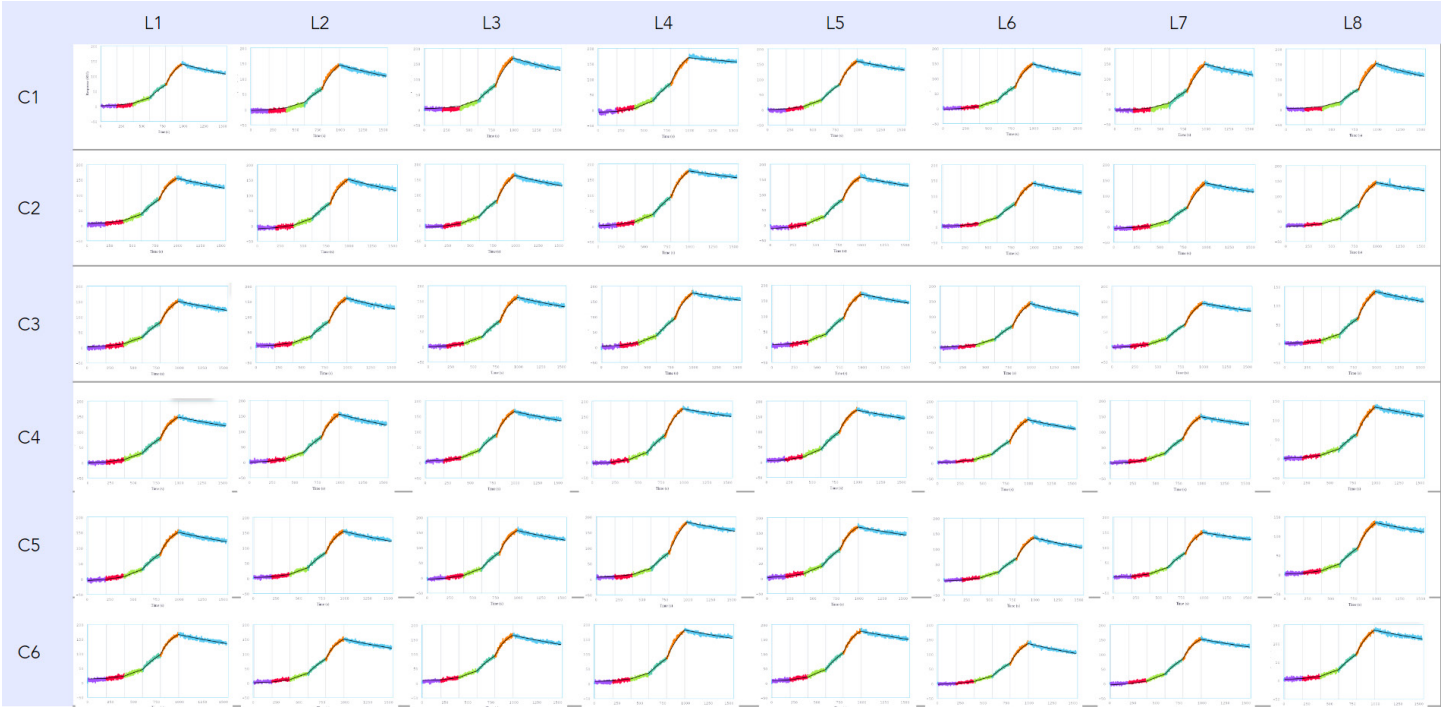

Figure 4: *A full cartridge of 48 SCK data points of S-oligo (analyte) binding to immobilized Bt-oligo (ligand) on Alto. Analyte was titrated from 2.47 nM to 200 nM. Black curve is the Langmuir 1:1 binding fit model analyzed in the Nicoya Analysis Software.*

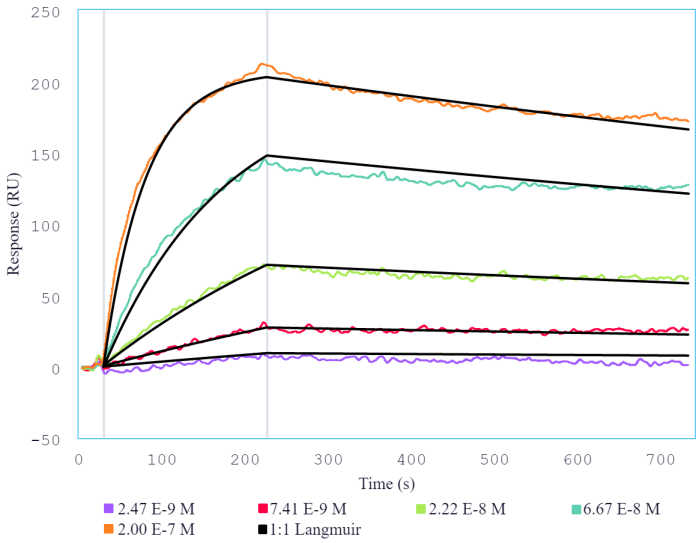

Figure 5: Multi-cycle kinetics of S-oligo (analyte) binding to immobilized Bt-oligo (ligand) on Alto. Analyte was titrated from 2.47 nM to 200 nM. Black curves are the Langmuir 1:1 binding fit model analyzed in the Nicoya Analysis Software.

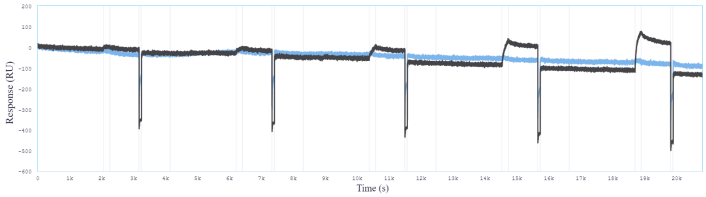

Figure 6: Reference (blue trace) and active channel (black trace) binding data showing binding of S-oligo to the Bt-oligo analyte for each analyte concentration. Following the dissociation of each analyte concentration, full regeneration was achieved by 1 mM NaOH.

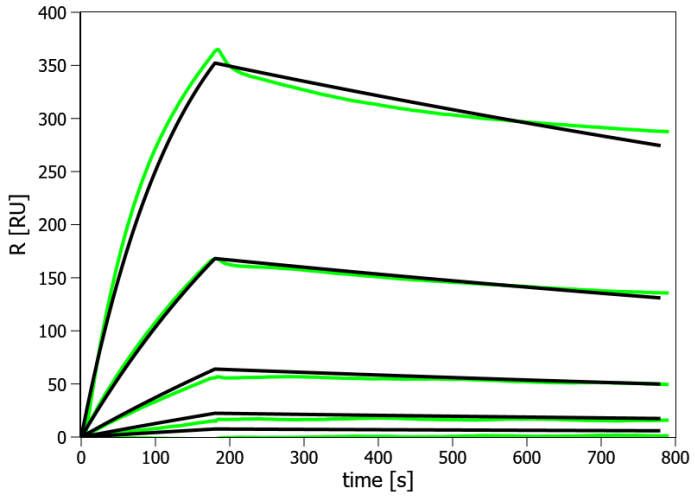

Figure 7: Multi-cycle kinetics of S-oligo (analyte) binding to immobilized Bt-oligo (ligand) on conventional SPR. Analyte was titrated from 2.47 nM to 200 nM. Green curves are the raw data, black curves are the Langmuir 1:1 binding fit model analyzed.

Table 2: Kinetics parameters generated by Alto compared to traditional SPR.

Instrument	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
Alto SCK	4.14×10^4	3.16×10^{-4}	7.94
Alto MCK	6.89×10^4	3.63×10^{-4}	5.38
Conventional SPR	3.53×10^4	4.16×10^{-4}	11.7

Accuracy of Alto Compared to Traditional SPR

To demonstrate the accuracy of kinetics obtained by Alto, analogous experiments were run on a conventional fluidics-based SPR instrument. The Alto kinetics were calculated with a 1:1 Langmuir fit which resulted in an excellent fit of the data. Similarly, for conventional SPR, a 1:1 global fit was used. Comparison of the kinetic values obtained by the Alto and conventional SPR MCK assays revealed similar results, as shown in Table 2. Visual comparison of the MCK data sets obtained with Alto and conventional SPR showed binding curves and fit overlays to be comparable for each analyte concentration (Figure 7). In summary, Alto generated kinetic data and binding curves comparable to the conventional SPR instrument.

Conclusion

Alto generated accurate kinetics and affinity values of a DNA oligonucleotide ligand with a biotin tag and a DNA oligonucleotide analyte containing the reverse complement sequence of the ligand. This system showed the high repeatability of Alto with % CVs below 18% for R_{max}, k_a and k_d with both SCK and MCK experimental setups. Furthermore, all parameters obtained were closely comparable to those determined by traditional SPR.

Compared to traditional SPR, Alto's unique and fully automated digital microfluidic platform enabled analysis of up to 48 data points in a single cartridge, significantly reducing the time, sample volume and overall cost of kinetic analysis. The SCK experiments were completed in under 5 hours, requiring only 30 minutes of hands-on time for setup and analysis.

The analysis performed in this application note also highlights the applicability of Alto in oligonucleotide research, particularly in sense-antisense interactions which are used in applications such as siRNA and RNAi.

References

1. Roberts TC, Langer R, Wood MJA. Advances in oligonucleotide drug delivery. *Nat Rev Drug Discov*. 2020 Aug 11; 19: 673-94. doi:10.1038/s41573-020-0075-7. PMID: 32782413; PMCID: PMC7419031.
2. Igarashi J, Niwa Y, Sugiyama, D. Research and development of oligonucleotide therapeutics in Japan for rare diseases. *Future Rare Diseases*. 2022 Feb 28; 2(1): 1-12. doi: 10.2217/frd-2021-0008.
3. Polack FP, Thomas SJ, Kitchin N, Absalon J, Gurtman A, Lockhart S, Perez JL, Marc GP, Moreira ED, Zerbini C, Bailey R, Swanson KA, et al. Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N Engl J Med*. 2020 Dec 31; 383: 2603-2615. doi: 10.1056/NEJMoa2034577

Appendix

Method

This label-free SPR assay was performed using Alto, the first and only digital microfluidic (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 remotely using Alto's cloud-based user portal, the Nicosystem, which was uploaded to the instrument with the click of a button.

Alto

First, a 16-Channel Carboxyl Cartridge was loaded into Alto followed by dispensing of the cartridge fluid into the cartridge. Since Alto is an automated SPR instrument, all the reagents were pipetted into the cartridge wells.

After all the reagents were loaded into the cartridge, the experiment was initiated by selecting the "Run Method" command on Alto. All subsequent steps were automated by Alto.

Normalization of sensors with high and low RI droplets was first performed. The sensors were then cleaned with 10 mM HCl for 60 s, followed by activation of 16 carboxyl sensors in the cartridge with 25 mM EDC/NHS for 5 min. The capture molecule, streptavidin (60 µg/mL) in 10 mM sodium acetate pH 5.0, was immobilized onto all 16 sensors for 5 min, and all sensors were subsequently blocked with 1 M ethanolamine for 5 min to quench the remaining active carboxyl groups. The biotinylated oligo ligand at 300 nM in PBS-T was passed over the 8 active channels for 5 min and captured by the streptavidin molecule.

For each analyte, 2 µL of 600 nM solution oligo were loaded per well, and the mixing of five 3-fold serial dilutions per analyte was automated by Alto. This resulted in analyte concentrations of 200 nM, 66.7 nM, 22.2 nM, 7.41 nM and 2.47 nM. The analytes were introduced in either a single-cycle kinetics (SCK) or a multi-cycle kinetics (MCK) format. The SCK format used an association time of 200 s, without dissociation or regeneration between each sample, starting from the lowest to highest concentration. A dissociation time of 600 s was set to run after the highest concentration. The sensor surface was regenerated with a 60 s exposure of 1 mM NaOH, which resulted in 100% regeneration. Six cycles of SCK were run and analyzed. The MCK format used an association time of 200 s, with a dissociation time of 600 s after each association. After each dissociation, the sensor surface was regenerated with a 60 s exposure of 1 mM NaOH, which resulted in 100% regeneration. Three cycles of MCK were run and analyzed.

Conventional SPR

First, 500 mL of PBST buffer was prepared, followed by pipetting all the common reagents and samples into a 96-well plate. Next S-oligo analyte samples were prepared via 3-fold serial dilution on a 96-well plate, producing 200 nM, 66.7 nM, 22.2 nM, 7.41 nM and 2.47 nM analyte samples. At least 200 µL of each sample dilution and reagent was prepared.

The sensor was installed and the surface was primed with the running buffer. Next, the tubing in the instrument was degassed.

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. 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. The 96-well plates containing the samples were loaded into the sample compartment of the instrument and the method was sent to the instrument for execution.

The remainder of the experiment was performed automatically by the conventional SPR instrument for the MCK experiment, with the operator being completely hands-off until data analysis.

The sensor was primed with 10 mM HCl for 60 s, followed by sensor surface activation with 0.4 M EDC/0.1 M NHS for 5 min. Then the streptavidin capture molecule, prepared in sodium acetate pH 5.0 buffer, was immobilized at 50 µg/mL for 6 min on all sensors. All sensors were blocked using an ethanolamine blocking solution for 5 min to quench the remaining active carboxyl groups. Next, the sensor surface was preconditioned with 10 mM HCl for 60 s.

First, the Bt-oligo ligand at 300 nM in PBS-T was passed over the active channel and captured by the streptavidin. A buffer blank was passed over the active and reference channels with an association time of 200 s and a 600 s dissociation time. The first concentration of the S-oligo analyte sample was introduced with an association time of 200 s, followed by a 600 s dissociation. After each dissociation, the sensor surface was regenerated with a 60 s exposure of 1 mM NaOH, which resulted in 100% regeneration. This was repeated for the next four concentrations of the S-oligo analytes.

Upon completion of the test, the data were opened in the corresponding analyzer software and results of tested analyte concentrations were manually selected. The data were pre-processed by subtracting background binding and the buffer blank from the active and reference channels to create a double referenced dataset. Binding curves were fitted to a 1:1 diffusion corrected binding model to determine kinetic and affinity constants.