APPLICATION NOTE

Characterization of complementary oligonucleotide sequences with Alto Digital SPR

Overview

Oligonucleotides hold great potential for treating diseases linked to gene expression and have recently revolutionized the vaccine industry with the remarkable success of mRNA-based COVID-19 vaccines. In this application note, we demonstrate the applicability of Alto™, Nicoya's Digital surface plasmon resonance™ (SPR) instrument, 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,

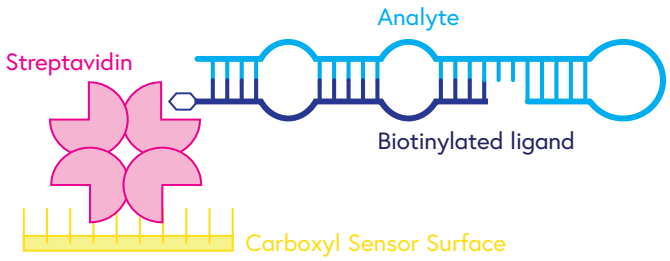

Figure 1: *Schematic representation of the Nicoya oligo system.*

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. 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.

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. 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 100X. Alto's 16 independent channels allow for the simultaneous analysis of up to 48 interactions and the complete assay automation significantly reduces hands-on time.

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 referred to as the bt-Oligo. The biotin tag is used to immobilize the ligand on a streptavidin surface, as shown in Figure 1. The analyte oligo, referred to as Oligo, is a 53-nucleotide sequence that contains the 15-nucleotide reverse complement of bt-Oligo within its sequence. The oligo binding pair is also tested on a conventional SPR instrument to verify the accuracy of the kinetics measured by Alto.

Materials & Equipment

- Alto 16-Channel Instrument with Nicosystem Pro Software (ALTO16)
- Alto 16-Channel Carboxyl Cartridge (KC-CBX-CMD-16)
- Running Buffer: PBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-PBST)
- Alto Carboxyl Surfacing Kit: cleaning, normalization, activation (ALTO-R-CBX-SURF)
- Regeneration Buffer: 1 mM NaOH (0.1% Tween 20), (ALTO-R-NaOH)
- Nicoya Streptavidin Kit (ALTO-R-STREPTAVIDIN)
- Bt-Oligo (ALTO-R-BT-OLIGO-L)
- Oligo (ALTO-R-OLIGO-A)

Method

Alto assay protocol

The following steps were completed automatically by Alto with no operator supervision.

1. Carboxyl sensors were normalized with normalization solutions and primed with 10 mM HCl for 60 s.
2. Carboxyl sensors were activated with 200 mM EDC/NHS for 600 s.
3. The streptavidin from the Streptavidin Kit diluted in 10 mM Sodium Acetate, pH 5.0 was immobilized onto all sensors for 600 s.
4. All sensors were blocked with the 1 M ethanolamine for 300 s to quench any remaining active carboxyl groups.
5. 300 nM samples of Bt-oligo in the running buffer (PBS-T) were introduced to each even-numbered active sensor for 300 s.
6. All sensors were conditioned for 60s with 1 mM NaOH.
7. Alto executed five automated Oligo serial dilutions on the cartridge. Each sample was diluted from 600 nM stock, producing 2.47 nM, 7.41 nM, 22.2 nM, 66.6 nM, and 200 nM solutions in the running buffer.
8. The lowest Oligo concentration was exposed to each sensor for 180 s, followed by dissociation in the running buffer for 600 s, and a 60 s regeneration step with 1 mM NaOH.
9. Step 8 was repeated for the remaining four Oligo analyte concentrations, which constitutes a full multi-cycle kinetics (MCK) round. In single-cycle kinetics (SCK), all five analyte concentrations are exposed to the sensor surface in tandem, followed by a single long dissociation phase and then regeneration. This constitutes a single round of SCK kinetics.

Alto data analysis

1. The test was opened under the analysis tab in the Nicosystem™ User Portal.
2. The Build Avidin Surface and Immobilize Biotin Ligand activities were checked to assess streptavidin immobilization and biotin-ligand capture levels across all 8 lanes in the cartridge to ensure sufficient and/or optimal levels.

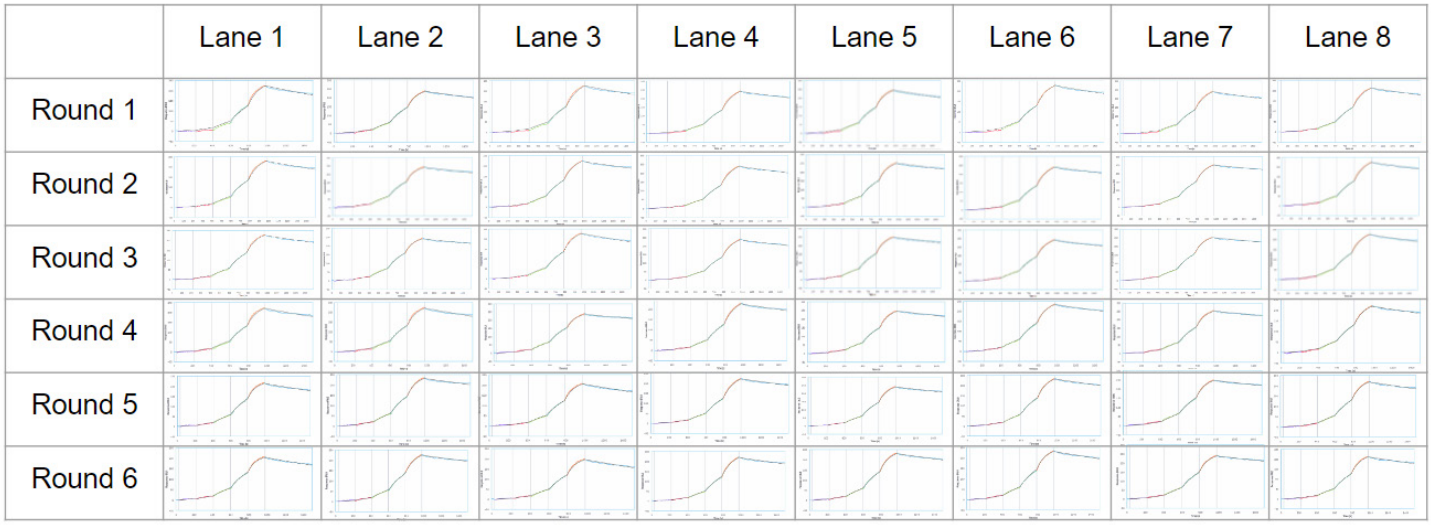

Figure 2: *A full cartridge of 48 SCK interactions of Oligo (analyte) binding to immobilized Bt-oligo (ligand) on Alto. Analyte was titrated from 2.47 nM to 200 nM. Black curve is the Langmuir 1:1 binding fit model analyzed in the Nicosystem software.*

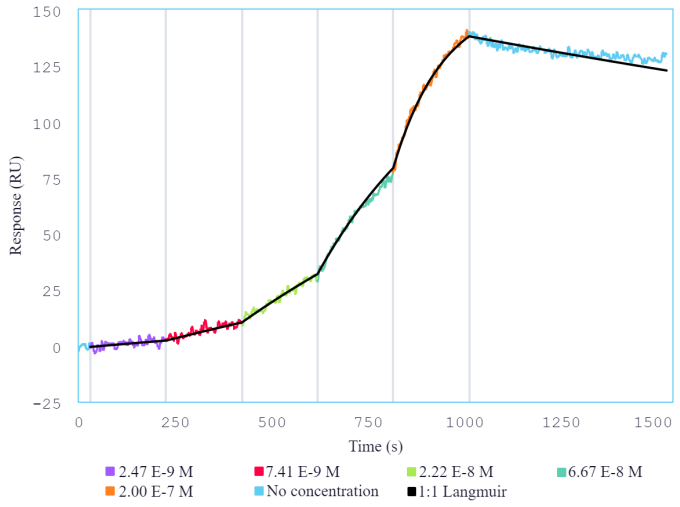

Figure 3: *Single-cycle kinetics of Oligo (analyte) binding to immobilized Bt-oligo (ligand) on Alto. Analyte binding curves were acquired for 5 concentrations ranging from 2.47 nM to 200 nM. Black curve represents the Langmuir 1:1 binding fit model generated by the Nicosystem software.*

3. The Direct Kinetics activity was opened and a 1:1 Langmuir binding model was automatically applied to the data.
4. Processing tools were used as required.
5. Final images and .CSV files were downloaded.

Conventional SPR protocol

1. 500 mL of PBS-T buffer was prepared, followed by pipetting all the common reagents and samples into a 96-well plate.
2. 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.
3. The sensor was docked and the surface was primed with the running buffer. Needles were washed and flow cells were degassed.
4. 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 executed automatically by the conventional SPR instrument for the MCK experiment, with the operator being completely hands-off until data analysis.
5. Carboxyl sensors were primed with 10 mM HCl for 60 s.
6. Carboxyl sensors were activated with 0.4 M EDC/0.1 M NHS for 5 min.
7. 10 µg/mL streptavidin in 10 mM sodium acetate, pH 5.0 was immobilized for 6 min on all sensors.
8. All sensors were blocked using a 1 M ethanolamine blocking solution for 5 min to quench the remaining active carboxyl groups.

9. Carboxyl sensors were preconditioned with 10 mM NaOH for 60 s.
10. Bt-oligo ligand at 300 nM in PBS-T was passed over the active channel and captured by the streptavidin for 300 s.
11. A buffer blank was passed over the active and reference channels with a 200 s association time and a 600 s dissociation time.
12. The lowest Oligo concentration was exposed to each sensor for 200 s, followed by dissociation in the running buffer for 600 s, and a 60 s regeneration step with 1 mM NaOH.
13. Step 12 was repeated for the remaining four Oligo analyte concentrations, which constitutes a full MCK round.

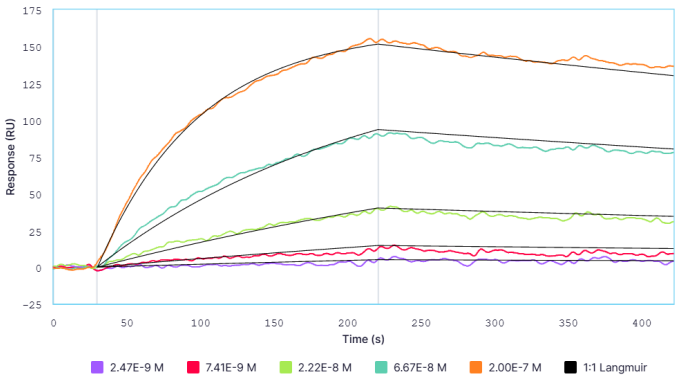

Figure 4: *Multi-cycle kinetics of Oligo (analyte) binding to immobilized Bt-oligo (ligand) on Alto. Analyte binding curves were acquired for 5 concentrations ranging from 2.47 nM to 200 nM. Black curves represent the Langmuir 1:1 binding fit model generated by the Nicosystem software.*

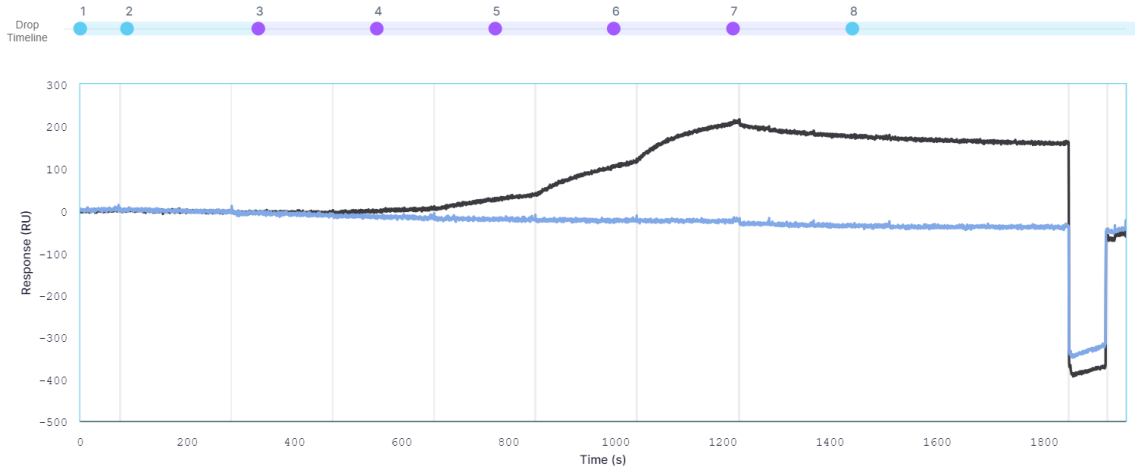

Figure 5: *Reference (blue trace) and active channel (black trace) binding data showing SCK binding of Oligo to the Bt-oligo. After dissociation data was collected the surface was fully regenerated with 1 mM NaOH.*

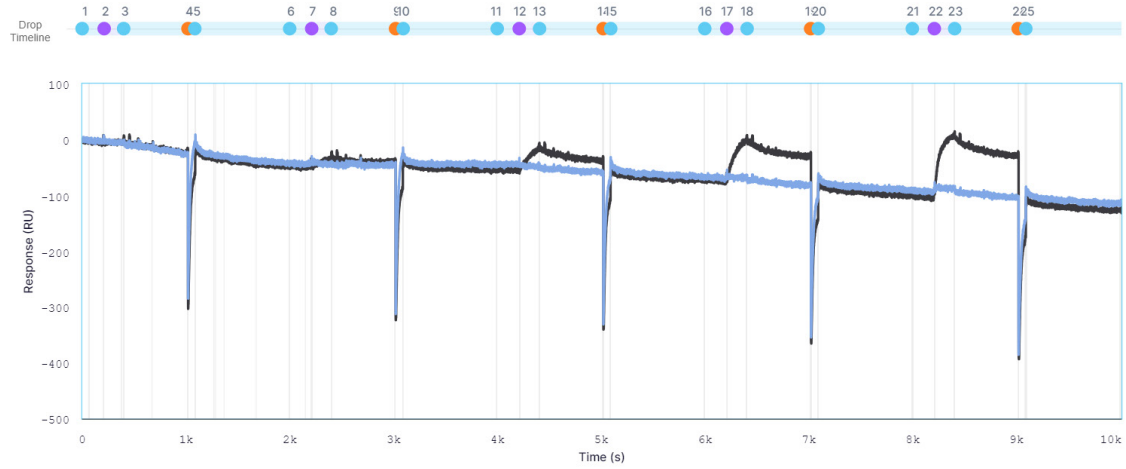

Figure 6: *Reference (blue trace) and active channel (black trace) binding data showing MCK binding of Oligo to the Bt-oligo for each analyte concentration. Following the dissociation of each analyte concentration, full regeneration was achieved by 1 mM NaOH.*

Conventional SPR data analysis

1. The data were opened in the analysis software and results of tested analyte concentrations were manually selected.
2. 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.
3. Streptavidin and biotin ligand immobilization was assessed to ensure sufficient and/or optimal levels.
4. Binding curves were fitted to a 1:1 diffusion corrected binding model to determine kinetic and affinity constants.
5. Processing tools were used as required.
6. Final images and .CSV files were downloaded.

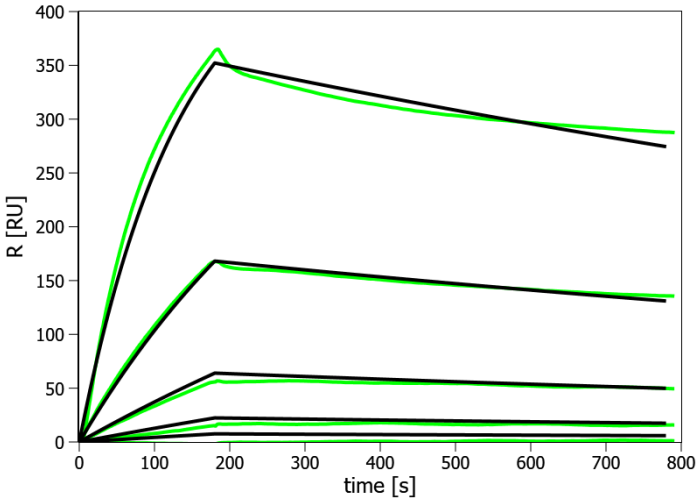

Figure 7: *Multi-cycle kinetics of Oligo (analyte) binding to immobilized Bt-oligo (ligand) on conventional SPR. The analyte was titrated from 2.47 nM to 200 nM. Green curves represent the corrected sensorgram data and black curves represent the Langmuir 1:1 binding fit model generated by the analysis software.*

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 Nicosystem software. SCK data from an entire cartridge (48 interactions) are included in Figure 2 to demonstrate the excellent reproducibility of the kinetic data.

The kinetic parameters for both SCK and MCK experiments are presented in Table 1. From the SCK analysis, association and dissociation rate constants of 4.43 x 10^4 $M^{-1}s^{-1}$ and 3.16 x 10^{-4} s^{-1}, respectively, were determined, resulting in a K_D of 7.27 nM (Figure 3). From the analysis of MCK data (Figure 4), association and dissociation rate constants of 5.79 x 10^4 $M^{-1}s^{-1}$ and 5.49 x 10^{-4} s^{-1}, respectively, were determined, resulting in a K_D of 9.60 nM, closely matching the K_D of 7.27 nM obtained from the SCK experiment.

For both SCK and MCK data, no NSB was observed and full regeneration was achieved by 1 mM NaOH (SCK data shown in Figure 5, MCK data in Figure 6).

Accuracy of Alto compared to conventional SPR

To demonstrate the accuracy of kinetics obtained by Alto, an analogous experiment was 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. Conventional SPR binding curves were fitted to a 1:1 diffusion corrected binding model to determine kinetic and affinity constants.

Comparison of the kinetic values obtained by the Alto and conventional SPR MCK assays revealed similar results, as shown in Table 1. Visual comparison of the MCK dataset 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.

Instrument	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
Alto SCK (n=48)	4.43 x 10^4 ± 5.02 x 10^3	3.16 x 10^{-4} ± 6.22 x 10^{-5}	7.27 ± 1.89
Alto MCK (n=16)	5.79 x 10^4 ± 1.01 x 10^4	5.49 x 10^{-4} ± 1.29 x 10^{-5}	9.60 ± 2.22
Conventional SPR (n=1)	3.53 x 10^4	4.16 x 10^{-4}	11.7

Table 1: *Kinetic parameters measured for Oligo binding to bt-Oligo on Alto and conventional SPR. Average ± Standard deviation are shown for SCK and MCK data where n>1.*

Conclusion

Alto successfully measured kinetics of a biotinylated DNA oligonucleotide ligand and a DNA oligonucleotide analyte containing the ligand's reverse complement sequence. These assays showcase the high repeatability of Alto data using both SCK and MCK experimental setups and demonstrate the accuracy and reliability of the kinetic parameters measured on Alto by comparing results to a conventional SPR instrument.

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. Each interaction required only 2 µL of sample, 100x less than conventional SPR.

The analysis performed in this application note highlights the applicability of Alto Digital SPR in oligonucleotide research, particularly in sense-antisense interactions which are used in several applications including siRNA and RNAi characterization.

References

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