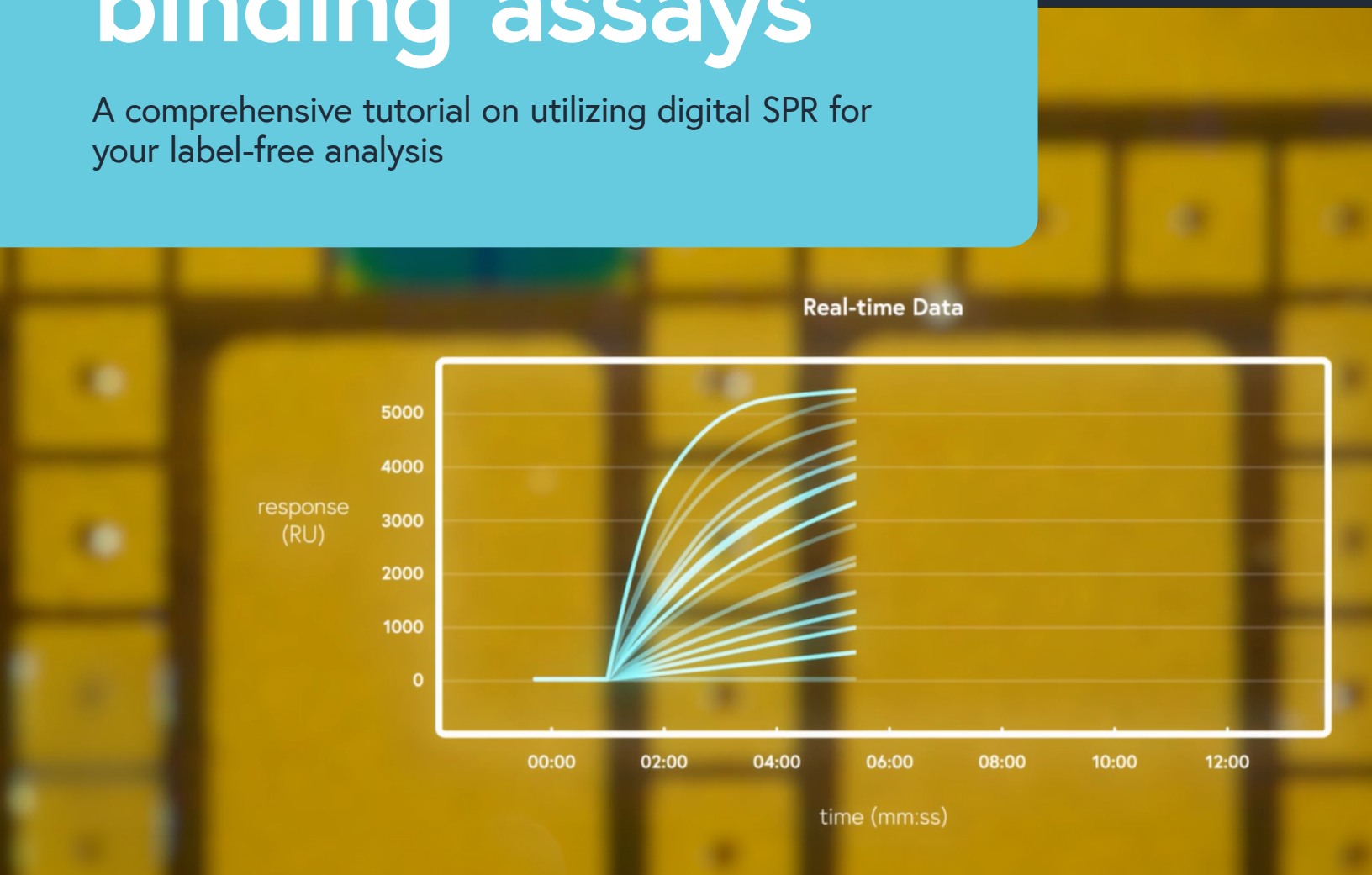
nicoya

EBOOK

Mastering kinetic binding assays

A comprehensive tutorial on utilizing digital SPR for your label-free analysis



Contents

Summary

Characterization of biomolecular interactions is imperative for the discovery, development and manufacturing of biotherapeutics. Label-free assays provide a robust and efficient medium to determine the binding affinity, kinetics and specificity of biomolecules with a high degree of accuracy and reliability. SPR is the gold standard for label-free binding assays, and Alto™ is the world's most user-friendly SPR instrument. Integrated with digital microfluidics (DMF) and nanotechnology-based biosensors, Alto provides the reliability and sensitivity of classical SPR without the hassle and maintenance of conventional fluidic-based SPR systems. Here, we demonstrate how Alto is an ideal platform for binding kinetic characterization and provide guidance and best practices in assay development, optimization, data analysis, and reporting so that you can get the most out of your Alto system.

About Nicoya

A single idea from the human mind can change the world. Nicoya was established in 2012 as a vision from nanotechnology student, Ryan Denomme. While completing his Masters' research at the University of Waterloo, he encountered a recurring problem – researchers don't have access to the cutting-edge technology needed to advance their discoveries. It became his goal to use nanotechnology to help reduce the cost and complexity commonly associated with scientific instruments, making them accessible to every scientist. As CEO, Ryan leads the team at Nicoya and continues to bring his vision to life.

Today, Nicoya's products have been installed in 500+ organizations and cited in 300+ publications. Located in the heart of Canada's Silicon Valley, Nicoya is proud to call Kitchener-Waterloo its headquarter and to be globally helping scientists succeed in over 50 countries.

Our mission

At Nicoya, our mission is to improve human life by helping scientists succeed. Many of us have been impacted by diseases such as cancer and Alzheimer's. Globally, there are millions of researchers (like yourself!) who are working relentlessly to better understand these diseases, and we are here to help

Introduction

Biomolecular interactions are often at the heart of therapeutic discovery as they promote the regulation and execution of most biological processes in the body. Thus, early drug discovery relies heavily on the characterization of these interactions to validate new targets and observe their binding to new drugs. Surface plasmon resonance (SPR) is a label-free technique used to detect biomolecular interactions in real time without the need for labels or markers. It has been widely adopted in biopharmaceutical and life science research as a critical tool for characterizing and quantifying biomolecular interactions. As a unique technique that allows for determination of both binding affinity and kinetics, it lends itself well to applications such as ligand-receptor and enzyme-substrate interaction studies, drug screening, epitope mapping, protein conformation studies, and label-free immunoassays. The detailed insights provided by SPR on the strength and stability of key interactions are critical to advancing our knowledge of human diseases and how to treat them.

Nicoya's Alto system **(Figure 1)** uses digital microfluidics (DMF) to deliver automatically diluted sample droplets to SPR sensors for effortless real-time characterization of biomolecular interaction analysis including quantitation, screening, epitope binning and binding kinetics. Alto has 16 independently addressable sensors for high throughput. Complete kinetics analysis can be done using only 2 µL of crude or pure samples. There are no manual dilutions, tagging, degassing, cleaning, or strenuous assay setup. Alto has powerful experimental design, data visualization and analysis software.

This eBook will walk through the stepwise acquisition and analysis of a kinetics assay as performed on the Alto platform. Additionally, guidance on how to design, optimize, run and analyze a binding kinetics assay will be provided in great detail.

Figure 1: Nicoya's Alto digital SPR system

Surface plasmon resonance

SPR is an optical phenomenon that occurs when an incident light is directed at a metal surface and stimulates the electrons of the metal's conductive band, generating special electromagnetic waves. SPR biosensors exploit these waves to measure changes in the refractive index at the metal surface. Thus, these biosensors can be used to monitor real time binding events that occur at the surface and later derive the binding constants of the interaction. SPR instruments are made up of an optical measurement system to detect changes in refractive index, a fluid handling system for sample delivery, and a sensor with a functionalized surface.

The sensor is a gold film coated on a substrate that has been chemically modified to make it easier to immobilize one of the binding partners onto the surface. The molecule that is immobilized is known as the ligand, and the molecule in solution is known as the Analyte **(Figure 2).**

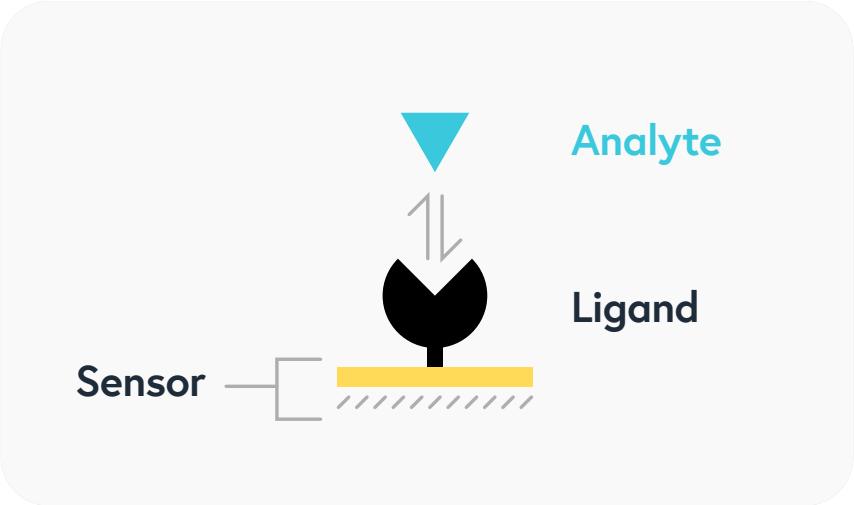


Figure 2: Molecular interaction on SPR. Ligand is immobilized on the gold sensor, and is interrogated with an analyte in solution.

Miniaturization of SPR

The optical system consists of a light source and a detector. The light source illuminates the gold surface, and the detector measures the unique optical spectrum produced by the SPR phenomenon. Most SPR instruments utilize a laser that shines through a prism beneath the sensor chip, generating a total internal reflection condition. When this occurs, a plasmonic wave is generated on the surface of the gold, with the electrical field of this wave extending hundreds of nanometers into the dielectric space above the surface of the sensor. The reflected light will have a characteristic dip in intensity at a certain angle due to the plasmonic wave, which is measured with a detector.

A main difference between Alto and many other SPR instruments is that it uses localized surface plasmon resonance (LSPR). Compared to traditional SPR which uses a continuous gold film, LSPR uses a surface coated with gold nanoparticles. LSPR produces a strong resonance absorbance peak in the visible range of light, with its position being highly sensitive to the local refractive index surrounding the particle. Therefore, it measures small changes in the wavelength of the absorbance position, rather than the angle of incidence as in traditional SPR. This is advantageous because LSPR can use a spectrometer for detection, eliminating the need for a prism beneath the sensor chip which

couples the light for detection in conventional SPR. This significantly decreases the size of the instrument and reduces sensitivity to vibrational and mechanical noise, allowing Alto to be a compact, robust and maintenance-free benchtop SPR instrument. A schematic outlining the Alto system is shown in **Figure 3**.

Although SPR sensors have a much higher refractive index sensitivity than LSPR sensors, their sensitivity towards biomolecular binding events is similar. This is due to the much shorter electromagnetic decay length of nanoparticles compared to gold films, which confines the response to a smaller sensing volume.

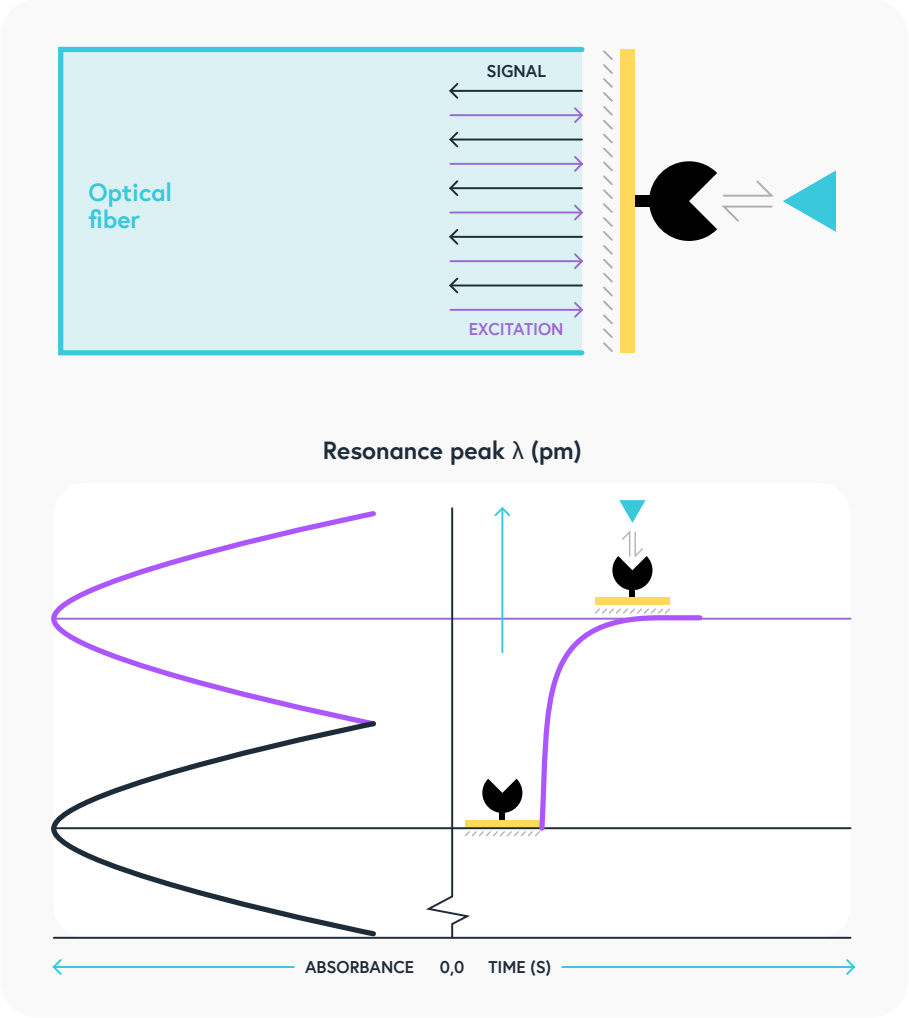


Figure 3: The Alto optical system (top view) consists of an optical fiber that transmits light from an LED source to illuminate the sensor surface and the reflected signal is transmitted back to the spectrometer to measure the absorbance. (Bottom) Example of binding signal detected when a molecule binds to the surface of the sensor caused by a change in the wavelength of the absorbance position.

Digital SPR

NEXT GENERATION SPR WITH AUTOMATED SAMPLE HANDLING

Digital microfluidics (DMF) is a method of moving and manipulating a liquid droplet by using a set of electrodes in a grid. Electrowetting, or the modulation of wettability through voltage, provides the basis of DMF technology. Aqueous droplets tend to form beads on hydrophobic surfaces, but they can be spread through the application of

voltage by modulating the contact angle. DMF devices leverage this simple concept through the intelligent stacking of multiple layers to form a simple circuit. The anatomy of a closed DMF system consists of two parallel plates. The bottom plate contains a substrate, a patterned array of individually controllable electrodes, a dielectric insulator to increase capacitance, and a hydrophobic layer to decrease the wettability of the surface. The top plate consists of a hydrophobic layer and a ground electrode.

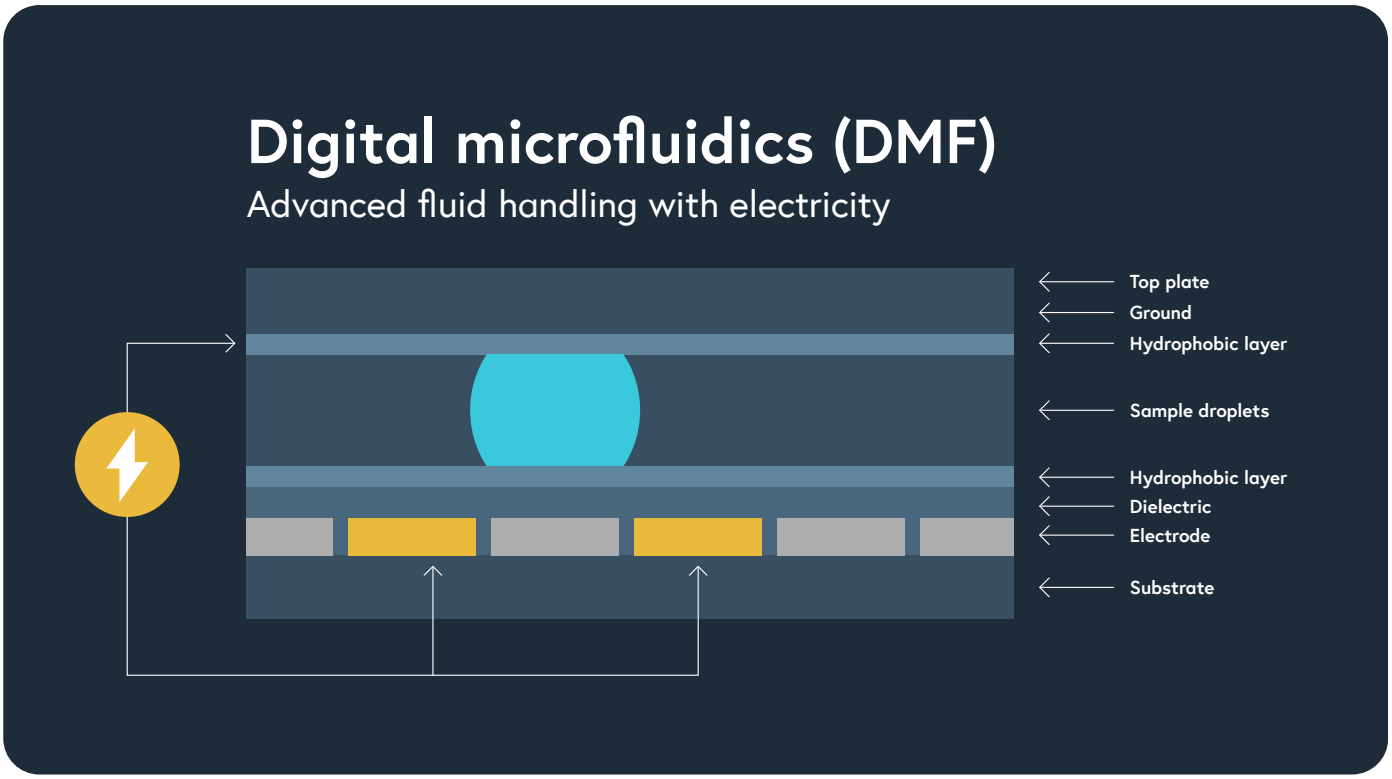


Figure 4: Digital microfluidics uses electricity instead of a pump for fluidic handling. Droplets are moved by activating the voltage supply to electrodes adjacent to the droplet and simultaneously deactivating the electrode under the droplet.

The sample droplet forms the building block of a DMF-enabled assay. The sample droplet and associated buffers are sandwiched between the plates and subjected to an electric field, while the electrode, capacitive dielectric layer, and droplet form the total impedance of the circuit. Droplets are then moved by activating the voltage supply to electrodes adjacent to the droplet and simultaneously deactivating the electrode under the droplet **(Figure 4)**. This manipulation of fluid enables automation of advanced fluidic protocols through the movement, mixing, and splitting of droplets, allowing users to carry out an endless array of bioassays.

Alto harnesses this technology into a disposable cartridge where the entire experiment takes place. This eliminates the need for traditional fluidics systems of pumps, valves, and tubing found in conventional SPR systems, and requires no cleanup **(Figure 5)**. The integration of DMF allows for the same protocols to be performed on Alto with a minuscule volume of 2 µL compared to the large volumes of samples and buffers used in classical SPR systems, while still outputting data that closely resemble those of conventional SPR. Alto's versatile fluid handling makes it a powerful tool for a variety of SPR assay designs and applications to generate high precision binding kinetics, including novel and complex workflows not currently (or physically) available in traditional fluidic systems.

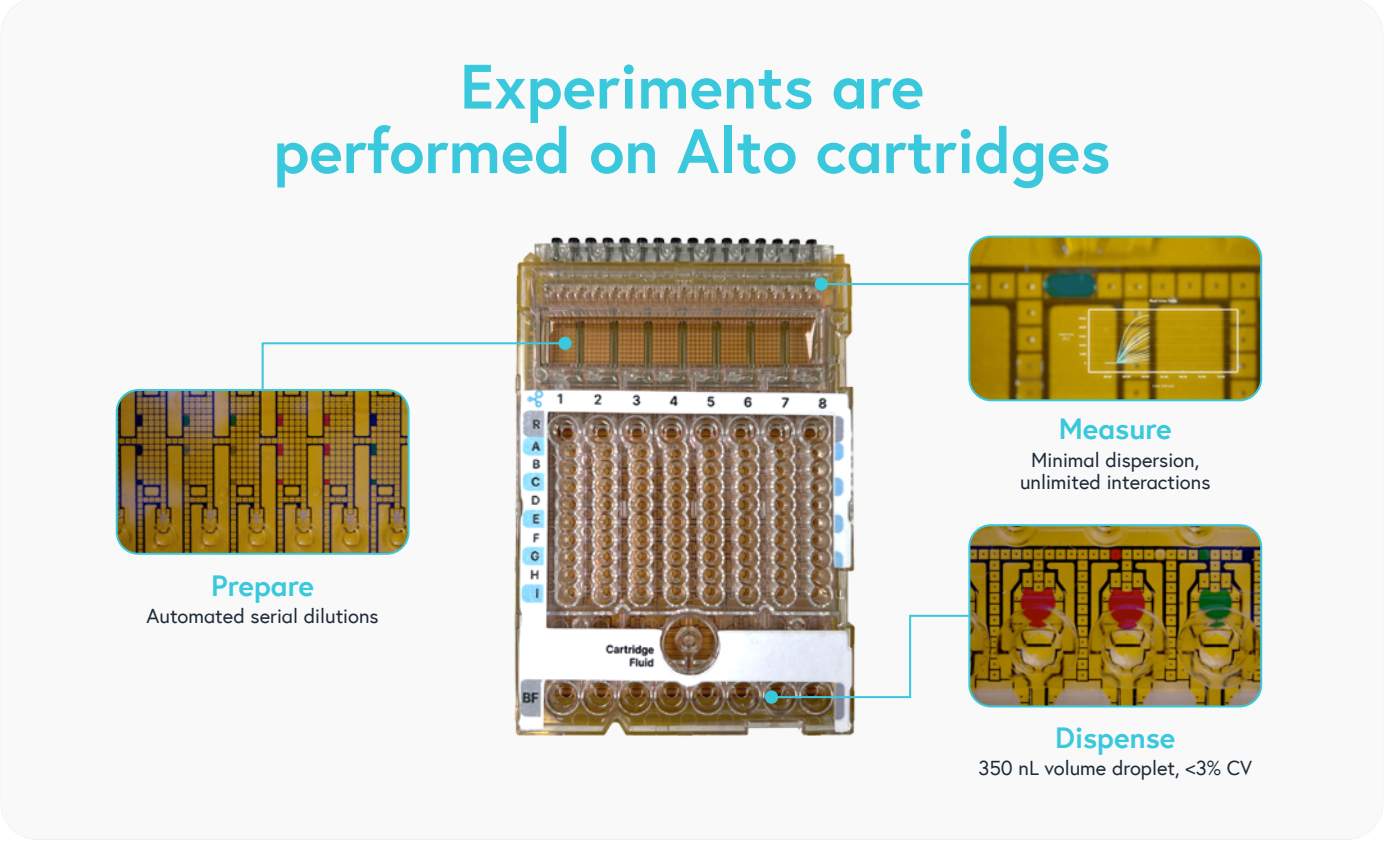


Figure 5: Alto experiments are performed on a disposal cartridge equipped with DMF technology, enabling the movement, mixing, and splitting of droplets.

Binding kinetics

Basic principles

The study of biomolecular interactions is critical to our understanding of biological systems and the forces involved in driving biological action. Kinetic analysis enables us to examine how specific biomolecules interact by providing important information about their mechanism and behavior in living systems. Observing how quickly a biomolecular complex forms (association rate of the interaction) and comes apart (the dissociation rate) provides insights about the roles of different biomolecules, residence times for antibody and receptor-target complexes, competitor drugs' mechanisms, and much more.

SPR is a powerful tool to study biomolecular interactions as it is one of few techniques that allow for accurate and real-time determination of binding kinetics, providing researchers with more detailed information about the molecules and systems being studied. **Figure 6** depicts a typical SPR binding curve showing a plot of SPR response against time, also known as a sensorgram.

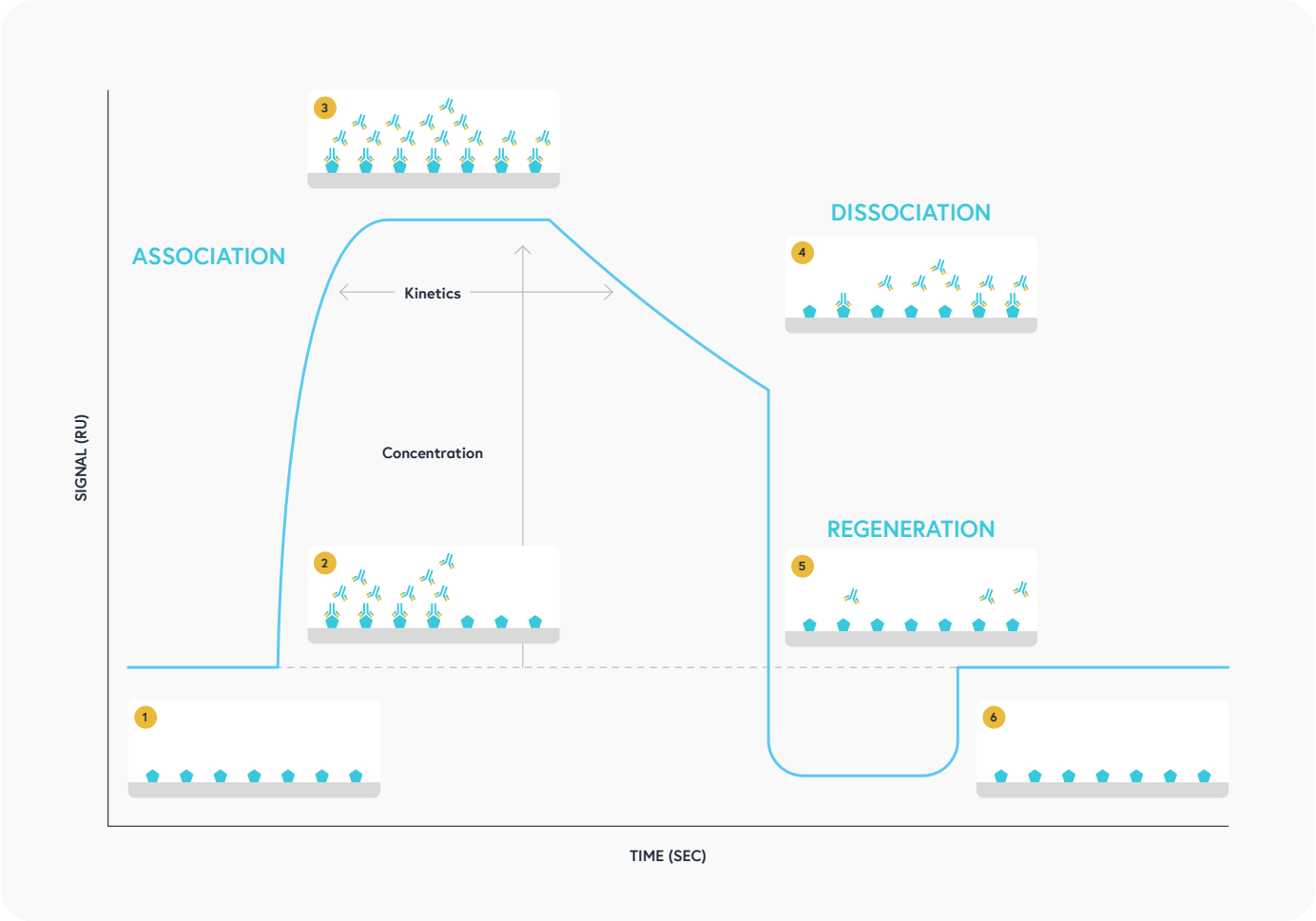
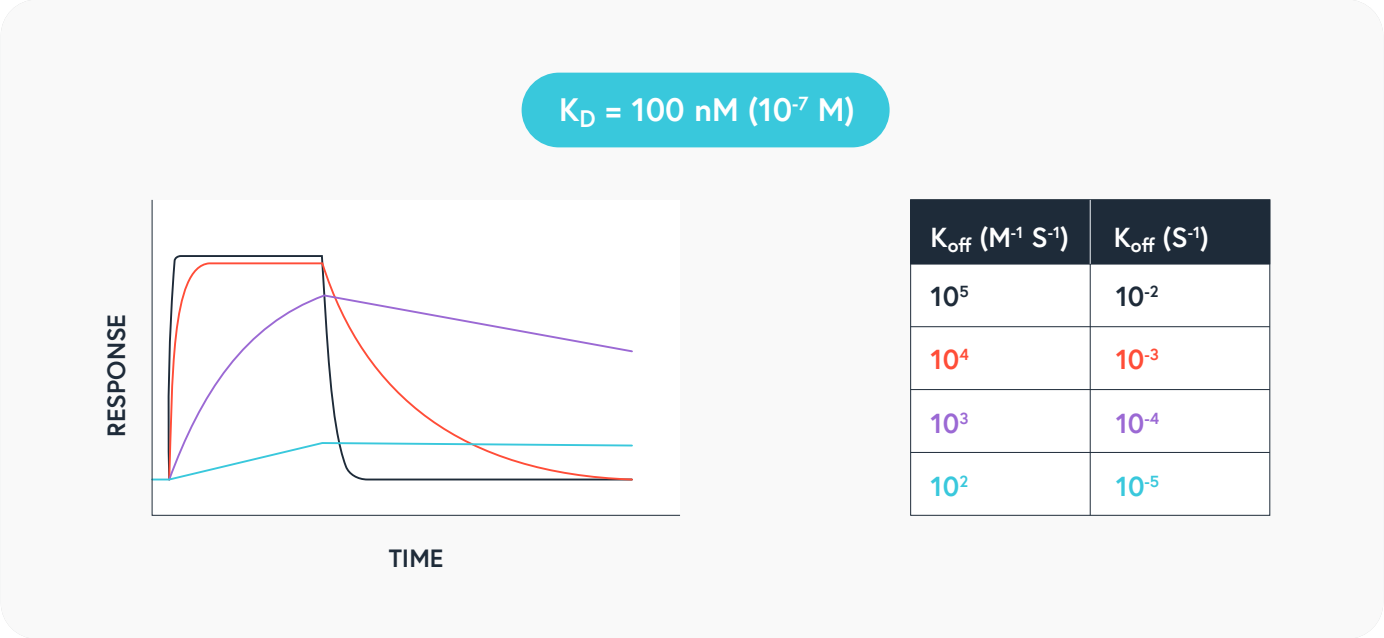


Figure 6: An SPR sensorgram consists of three major steps: association of the analyte to the ligand, dissociation of the analyte from the ligand and regeneration of the ligand surface, stripping off any remaining analyte.

To perform an SPR experiment on the Alto, one of the interactants, referred to as the ligand, is immobilized on a sensor surface, and the second interactant, referred to as the analyte, is passed over the surface via DMF. The response change over time is plotted, and the resulting sensorgrams are analyzed using analysis software equipped with various fitting algorithms. Experiments are performed in replicates, with 3-5 concentrations of each analyte being studied. A typical sensorgram includes a baseline phase where a buffer drop is oscillated over the immobilized ligand on the sensor and a baseline response is recorded. Next, a drop of analyte is oscillated over the immobilized ligand, and the binding interaction of the analyte to ligand (association phase) is measured. This is followed by the dissociation phase, during which a buffer drop is oscillated over the sensor, and the dissociation of analyte from the immobilized ligand is measured. In cases where dissociation is too slow to achieve completion on a reasonable experimental time scale, a regeneration step is performed to remove the analyte from the sensor without damaging the ligand. From the resulting sensorgram, the association rate (k_{on}), dissociation rate (k_{off}), and affinity constant (K_D) of the interaction can be determined.

There are many techniques available, such as ELISAs and fluorescence assays, that can provide scientists with the affinity of an interaction between two biomolecules. While K_D is an important value, it does not tell the whole story of an interaction between two biomolecules.

Consider the examples of real-time binding curves measured using SPR in **Figure 7**. All 4 curves have the exact same affinity, so a comparison of K_D would characterize all as identical. However, when examining the k_{on} and k_{off} rates, or the kinetics of the interaction, one can see that each interaction gets to the same affinity (K_D) in very different ways. The binding constants of each interaction differ by 4 orders of magnitude, yet all have the same affinity. This type of information is critical for understanding the biological system that is being studied, especially for applications like drug discovery and understanding molecular disease mechanisms.



K_{off} (M^{-1} S^{-1})	K_{off} (S^{-1})
10^5	10^{-2}
10^4	10^{-3}
10^3	10^{-4}
10^2	10^{-5}

Figure 7: Different binding pairs can have the same affinity even though the binding constants vary by 4 orders of magnitude

Developing a binding kinetics assay

Choosing a kinetic experiment method

The Alto platform walks a user through the experimental planning process. Depending on the objective of the testing, which could be looking for optimization or kinetic parameters, users have the choice of a few different experimental parameters as outlined below:

Cycle formats

1. Single-cycle kinetics (SCK)
2. Multi-cycle kinetics (MCK)

Immobilization strategy

3. Direct kinetics
   - Carboxyl (CBX)
   - Carboxyl (CBX) with streptavidin (STV) strategy

4. Capture kinetics
   - Multi-analyte: many analytes against one ligand for binding (many on one)
   - Multi-ligand: one analyte against many ligands for binding (one on many)

Single-cycle and multi-cycle kinetic formats

In an SPR experiment, there are two common experimental methods for kinetic analysis: multi-cycle kinetics and single-cycle kinetics **(Figure 8)**. Before selecting a format for an experiment, it is important to understand how they differ and which is best for a specific interaction.

Kinetic analysis performed with alternating cycles of analyte injections and surface regeneration (as described above) is known as multi-cycle kinetics (MCK). MCK is the most common strategy for determining interaction kinetics. Each injection of an analyte is done in a separate sequence, and a single SPR curve is generated per analyte concentration. However, surface regeneration requires conditions that can sometimes damage the functionality of the ligand or irreversibly inactivate it. For systems where total regeneration is difficult to achieve, single-cycle kinetics may be a more optimal approach.

Kinetic titration, also known as single-cycle kinetics (SCK), consists of sequential injections of increasing concentrations of the analyte over the ligand, without dissociation or regeneration between each sample concentration. The last (highest) analyte concentration is then followed by a single, long dissociation phase. This experimental format has been independently validated and is an accepted method for performing SPR analysis. Alto is uniquely suited for SCK analysis as it enables the exchange of droplets on the sensor surface without the need for buffer flow between samples.

Figure 8: Comparing sensorgrams for SCK and MCK experiment formats. Both formats have five analyte concentrations but SCK only has one regeneration step after all five analyte concentrations and MCK has a regeneration step after each analyte concentration.

Numerous biological systems have been characterized with both single-cycle and multi-cycle kinetics on Alto and the kinetic binding constants obtained with the SCK method are consistent with those measured with the MCK assay for the same interaction and analyte concentrations. Selecting the best assay format for an interaction allows for expansion and optimization of kinetic studies and enables one to dig deeper into the roles and behaviors of a wide range of biomolecular targets.

The advantages of each method are outlined in the table below:

Format	Advantages
SCK	• Shorter experiments (one dissociation phase per five concentrations instead of five) • More information on sensitive and non-regenerable ligands • Higher throughput per cartridge
MCK	• More informational content and more robust data • Possible to omit unrepresentative concentrations from fit

Table 1: Advantages of SCK and MCK experiment formats

Immobilization strategies

Alto's 16-sensor Carboxyl Cartridge is a single-use cartridge containing 16 carboxyl-functionalized sensors. It is used for immobilization of biomolecules containing free primary amine groups (proteins, antibodies and more) onto the sensor surface for analysis in Alto instruments. The 16 sensors are arranged into 8 lanes with two sensors each, an active sensor and a reference sensor. The active sensor is where the binding interaction takes place, and the reference sensor simply flows analyte over the surface without ligand to acquire a background signal which will be subtracted from analyte response in the active sensor.

There are four main kinetic formats that are offered on Alto. These are direct kinetics, direct kinetics with streptavidin, multi-analyte capture kinetics and multi-ligand capture kinetics. The difference between a direct and capture format is illustrated in **Figure 9**.

Figure 9: Direct kinetics is when the ligand is directly immobilized onto the sensor (left). Capture kinetics uses another molecule to capture the ligand onto the surface (right). An example of this would be a Protein A surface used to capture antibody ligands.

Direct kinetics

Direct kinetics is the simplest kinetic format, which involves direct amine coupling of one binding partner on the surface. The ligand is only immobilized on active sensors in a direct format on an activated carboxyl surface. It is possible to run most systems in both orientations, meaning that if desired in an antibody-antigen system, the antigen can be directly immobilized on the surface and analyzed against the antibody in solution or vice-versa. Analyte response is dependent on molecular weight, therefore it is recommended that where possible, the lower molecular weight molecule be used as the ligand. Alto's direct kinetics protocols can measure up to 8 irreversibly immobilized ligands against up to 48 unique analytes per experiment.

It is recommended to use direct kinetics if:

- At least one binding partner has primary amine groups available (such as N-terminus or lysine residues)
- There are no tags (such as biotin, his-tag, AVI-tag) available for capture
- Both binding partners have the same tag
- There is no capture molecule (such as a a protein-specific mAb) readily available

Direct kinetics with streptavidin

Direct kinetics with streptavidin is an assay format specifically for studying biomolecules with biotin tags. In this format, streptavidin is directly amine coupled to the carboxyl surface. The biotinylated ligand then irreversibly binds to the streptavidin, effectively immobilizing the ligand on the surface. The analyte will then be analyzed against the ligand surface. In this format, streptavidin is also immobilized on reference sensors as it is more representative of the active sensor surface. Because the biotin-streptavidin interaction is non-regenerable, the ligand is not reapplied to the surface after each regeneration step, as it would be in a capture kinetics format. For example, a biotinylated antigen could be used as the ligand, and a non-biotinylated antibody as the analyte. Streptavidin surfaces are extremely robust and have reliable, consistent capture of biotin tags.

It is recommended to use direct kinetics with streptavidin if:

- One binding partner has a biotin tag
- At least one binding partner is a nucleic acid (does not have primary amines, but can be easily, inexpensively and site-specifically biotin-tagged)

Capture kinetics

In a capture kinetics format, a capture biomolecule with a primary amine is immobilized on the sensor surface. A capture molecule is one that has a high affinity for a tag on a corresponding ligand of interest. This format removes the need for ligand modification (in most cases), as well as enabling selective orientation and homogeneity of the ligand. Some of the most common capture molecules used for SPR are Protein A or anti-IgG/Fc surfaces to capture antibodies via their Fc region, or Strep Tactin-XT to capture twin-strep tagged proteins. After each cycle, the ligand is regenerated from the capture surface and another ligand may be captured. It is re-captured each cycle throughout the test.

It is recommended to use capture kinetics if:

- An antibody or a protein with a tag such as twin-strep-tag or his-tag are used as one of the binding partners
- The ligand is in a crude matrix such as serum or lysate

- The ligand does not have primary amines
- Binding partner is known to be inactive upon immobilization
- The binding partner is known to become inactive upon immobilization

Multi-analyte vs multi-ligand capture kinetics

Alto's capture kinetics experiments can be set up in two different ways depending on the user's needs. Alto's multi-analyte capture kinetics protocols can measure 48 unique analytes in solution against a single captured ligand ("many on one"). Alto's Capture Kinetics Multi-ligand protocol can measure a single analyte against up to 48 unique ligands captured via an immobilized capture molecule ("one on many").

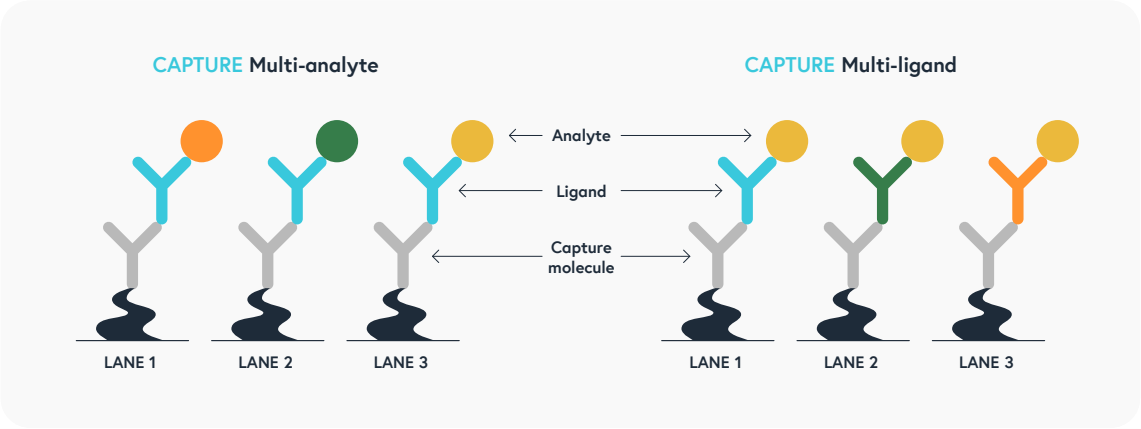


Figure 10: The difference between multi-analyte vs. multi-ligand capture kinetics experiement is illustrated here.

Optimizing experimental conditions

Immobilization

Nicoya provides a surface activation kit suitable for most protein applications. This kit includes activation and blocking reagents as well as a variety of immobilization buffers at different pH for easy test setup

The isoelectric point (pI) of a ligand or capture reagent can be used to select the immobilization buffer pH and optimize the amount of immobilization on the surface.

Typically, an immobilization buffer pH should be:

$$pH = ligand\ pI - 0.75 \pm 0.25$$

For example, if the pI is 6, try pH 5 and pH 5.5.

Given the 8 lanes per Alto cartridge, it is recommended to test a maximum of four unique ligand conditions per cartridge to allow for duplicates and orthogonal testing of other variables. This is suggested for any system not previously characterized on SPR.

Ligand density

In general, a good starting point for ligand concentration is between 10-50 µg/mL. Using too low of a concentration risks failure to observe binding of the analyte to the ligand, whereas immobilizing too high of an amount of ligand risks introduction of artifacts that can make the interpretation of results challenging. The size of the analyte is also a factor for how much immobilization is required. Typically, increasing the immobilization level can be advantageous for smaller analytes. Depending on the size of the analyte and the signal observed after the initial ligand immobilization, the ligand concentration can be increased or decreased as required in subsequent tests.

Limiting mass transfer effects is key to getting good data. In SPR, the analyte in a solution must first diffuse from the bulk to the sensor surface to interact with the immobilized ligand. If the diffusion rate is slower than the association rate, mass transfer effects can be observed in the data **(Figure 11)**.

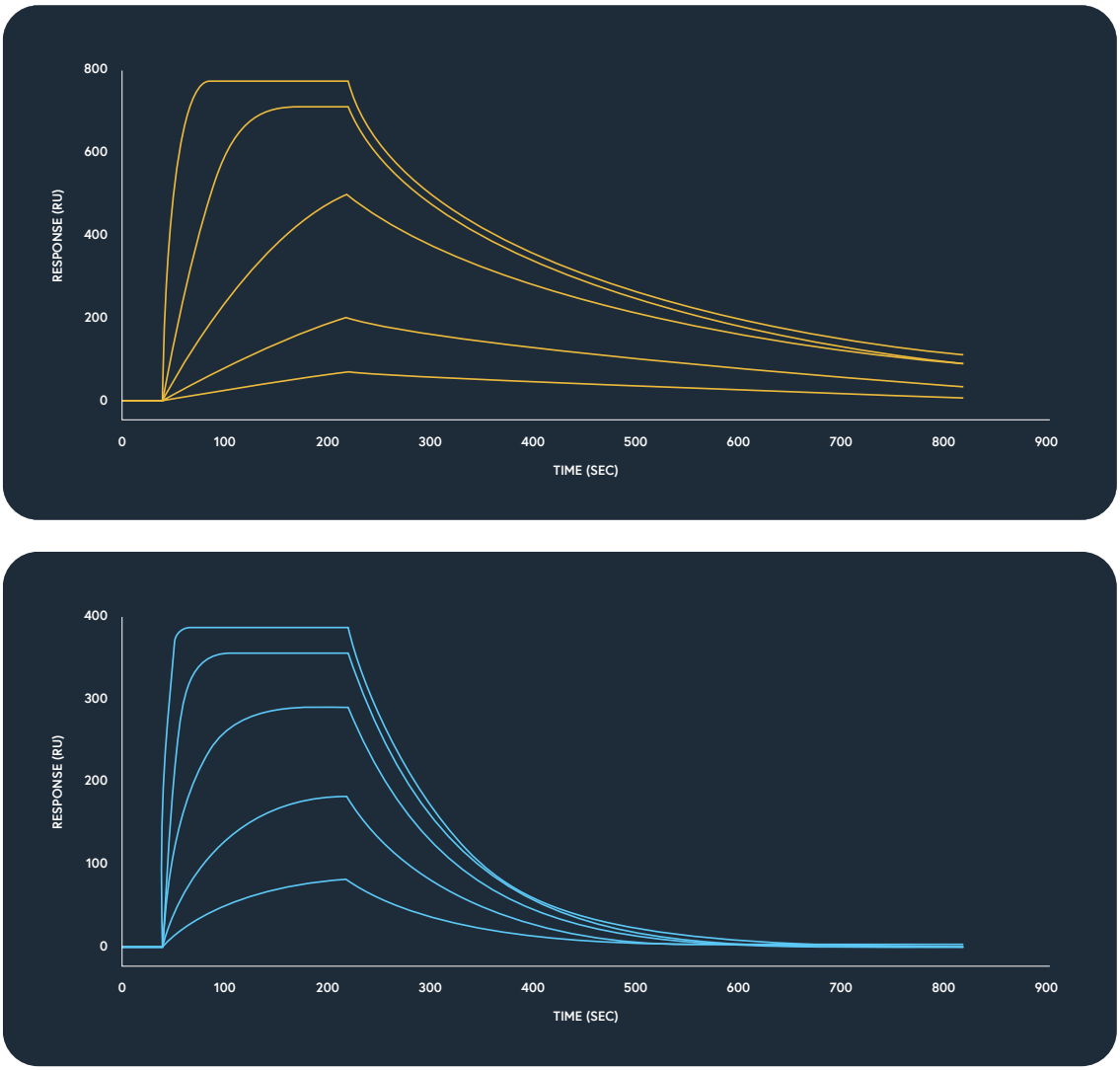


Figure 11: The sensorgram on the top shows a binding pair suffering from mass transport limitation. This is characterized by a dissociation that plateaus before the previous baseline. The sensorgram on the bottom shows the same system with a decreased ligand concentration, that returns to the appropriate baseline.

Mass transfer limitations are most common for fast binding reactions, as diffusion limits the association rate. Diffusion-limited binding looks linear rather than exponential. The easiest way to limit mass transfer effects is to reduce the amount of ligand immobilized to the sensor. This will help reduce diffusion-limited effects as less analyte needs to diffuse for the interaction to occur.

Additionally, one can use a fitting model that includes mass transport in the overall reaction equations to mathematically account for mass transport effects. Most kinetic processing software, including Alto's Nicosystem™, include this model as an option when fitting data.

Analyte concentration range

A general guideline is that the highest analyte concentration should be ~10-20 times the K_D. If the analyte and ligand interaction has been studied before, either through SPR or an orthogonal technique like BLI or ELISA, there may exist an affinity constant that can be used to guide analyte concentrations. Alternatively, if the K_D is unknown, an educated guess can be drawn from similar systems. Otherwise, one may pick a concentration and adjust accordingly based on the results. An advantage of the Alto cartridge is the ability to test multiple analyte ranges on one cartridge to help quickly optimize tests.

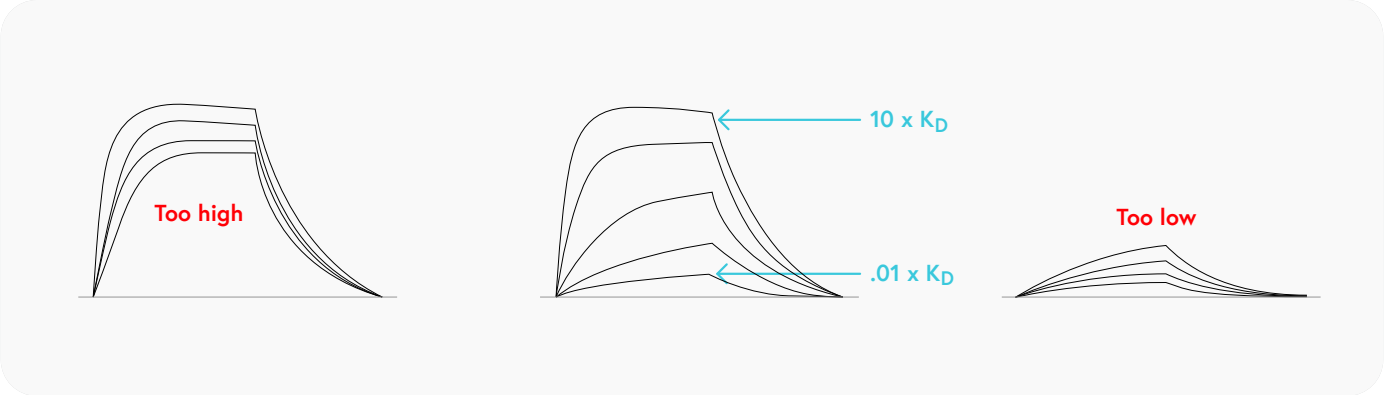


Figure 12: Analyte concentrations should cover an appropriate range on either side of the K_D. The sensorgram on the left shows a concentration range that is too high, the sensorgram on the right shows a concentration range that is too low, and the sensorgram in the middle is just right.

Analyte concentrations should cover a wide range around the K_D **(middle, Figure 12)**. If the concentrations are too high or too low, the curves may be tightly grouped and the K_D value will be skewed too high or too low, respectively.

Another benefit of Alto is the automated on-cartridge dilution of analytes. A user can simply load a single concentration of each sample into the cartridge, and Alto will automatically and accurately perform a series of serial dilutions of each sample. For example, if the K_D of the binding pair being tested is 10 nM, utilizing 100 nM as the highest analyte concentration would result in the Alto performing five 3x dilutions and utilizing 100, 33, 11, 3.7 and 1.2 nM for the full kinetic analysis. This would put the highest analyte concentration at 10x the K_D and the lowest analyte concentration at 0.1x times the K_D, covering an excellent dynamic range for this particular analyte. We recommend that two of the five analyte concentrations tested are below the K_D to ensure accurate kinetics.

Choosing a regeneration solution

Regeneration refers to the application of a solution to the surface to disrupt the binding of the analyte to the ligand, removing the analyte from the sensor surface. Regeneration is important for optimizing binding kinetics because all of the analyte must be removed from the sensor surface to ensure the same number of ligand binding sites are available for the next concentration of analyte injected. Removing the bound analyte allows the sensors to be re-used for multiple ligand-analyte interactions. Different ligands, buffers, and analytes, along with their concentrations, can require different regeneration conditions. If the natural off-rate of the ligand-analyte complex is fast enough, a regeneration solution may not be needed.

There are three possible outcomes when regeneration is being optimized, which are summarized in **Table 2**.

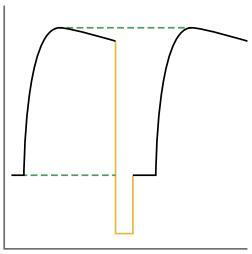
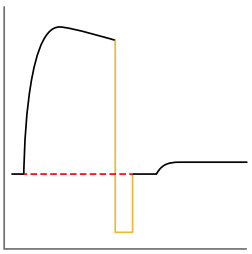
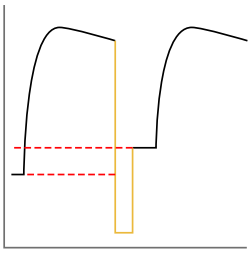
Format	What does the curve look like?	
	Images	Features
Optimal		Signal returns to a level similar to the baseline prior to the analyte injection. The analyte was removed but ligand remains on the surface. Analyte response is consistent between rounds. This is optimal regeneration.
Too harsh		Regardless of where the baseline stabilizes after the regeneration injection, the analyte injections after regeneration yield a binding response much lower than before regeneration. This indicates the regeneration condition is too harsh and is inactivating the ligand. Use a milder regeneration solution.
Too mild		An increase in the baseline and height of subsequent analyte injections at the same concentration indicates some of the analyte was not removed from the ligand and there are fewer binding sites available than before. Use a stronger regeneration solution.

Table 2: Examples of binding curves for regeneration optimization

It is critical to choose a regeneration solution that is strong enough to remove all of the analyte, but mild enough to avoid damaging ligand activity after multiple regeneration cycles. If ligand activity is compromised, the analyte response will decrease significantly with future injections. **Table 3** highlights common regeneration solutions and the systems they are used on.

Type	Chemicals	Harshness index (low to high)	Type of binding
Acidic	Glycine HCl (pH 1-3, 10 mM)	Medium-high	• Proteins • Antibodies • Lipids
Basic	NaOH (10 mM)	High	• Nucleic acids
Electrostatic	NaCl or KCl (0.5-5 M)	Low-high (depends on concentration)	• Peptides • Small molecules
Chaotropic	$MgCl_2$ (1-4 M)	Low-medium	• Peptides • Proteins • Nucleic acids

Table 3: Examples of common regeneration solutions

Non-specific binding (NSB)

In SPR, non-specific binding (NSB) refers to the binding of analyte molecules to non-target sites on the sensor surface or the immobilized ligand **(Figure 13)**. NSB gives rise to a false-positive signal; therefore, it is necessary to control NSB in SPR experiments. NSB can be reduced by disrupting the molecular interactions which stabilize the analyte on the non-target sites through the addition of detergents, salt, and blocking additives to the running buffer, immobilizing a blocking molecule, or adjusting the pH. **Table 4** summarizes how to overcome NSB based on the molecular interactions involved.

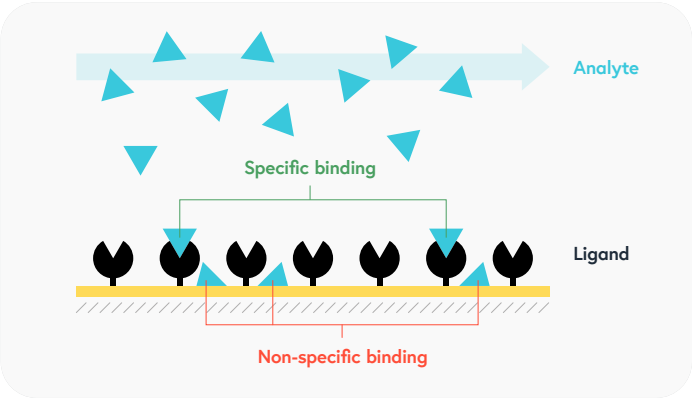


Figure 13: Example of non-specific binding of the analyte to the sensor surface.

NSB caused by	Suggested solution(s)
Electrostatic interactions	• Increase the salt concentration (up to 0.5 M) • Adjust buffer pH • Block the sensor surface with ethylenediamine instead of ethanolamine
Hydrophobic interactions	• Adjust concentration of surfactants (e.g. Tween 20) • Reduce the salt concentration • Change buffer types (e.g. HEPES vs PBS vs Tris)
Other protein-protein NSB	• Include up to 1% BSA in the running buffer
Nucleic acids	• Add $MgCl_2$ (up to 0.1 M) or EDTA (up to 1 mM) depending on surface charge and concentrations

Table 4: Common sources of NSB with suggested solutions

Immobilizing blocking molecules

For reference sensors, the ligand immobilization step is skipped but having a similar density with immobilization of a blocking molecule/protein allows for the reference surface to be representation of what the analyte sees in the active channel. The blocking molecule sterically blocks the sensor surface from interacting with the analyte, reducing NSB. Some examples of common blocker molecules and how to use them are shown in **Table 5**.

Blocking molecule	Concentration	Immobilization buffer pH
Bovine serum albumin	1 mg/mL	Sodium acetate, pH 4.5
Streptavidin	100 µg/mL	Sodium acetate, pH 5.0
Nonspecific isotype control antibody	10 µg/mL	Sodium acetate, pH 5.0

Table 5: Common examples of blocker molecules for the reference sensor

Choosing an appropriate buffer

The Alto platform is compatible with Good's buffers and a wide variety of buffer additives including salts, proteases, preservatives (e.g. trehalose) and solvents (e.g. DMSO). Nicoya offers several commonly used running buffer solutions to use on the Alto platform. These include phosphate buffered saline with 0.1% Tween 20 (PBS-T), pH 7.4, HEPES buffered saline with 0.1% Tween 20 (HBS-T), pH 7.4, and Tris buffered saline with 0.1% Tween 20 (TBS-T), pH 7.4.

Up to 8 unique running buffers can be tested per cartridge to compare the effects of running buffers on the experiment.

Note that it is important for the analyte buffer and running buffer to be the same, including pH, ionic strength and concentration of buffer additives. Discrepancies between buffers will result in bulk shift effects being observed.

Bulk shifts

Bulk shift, or solvent effect, occurs as a result of a difference between the refractive index (RI) of the analyte solution and running buffer. This will create a tell-tale 'square' shape in the sensorgram due to a large, rapid response change at the start and end of the injection **(Figure 14)**. The shifts may be positive or negative, depending on the RI difference detected.

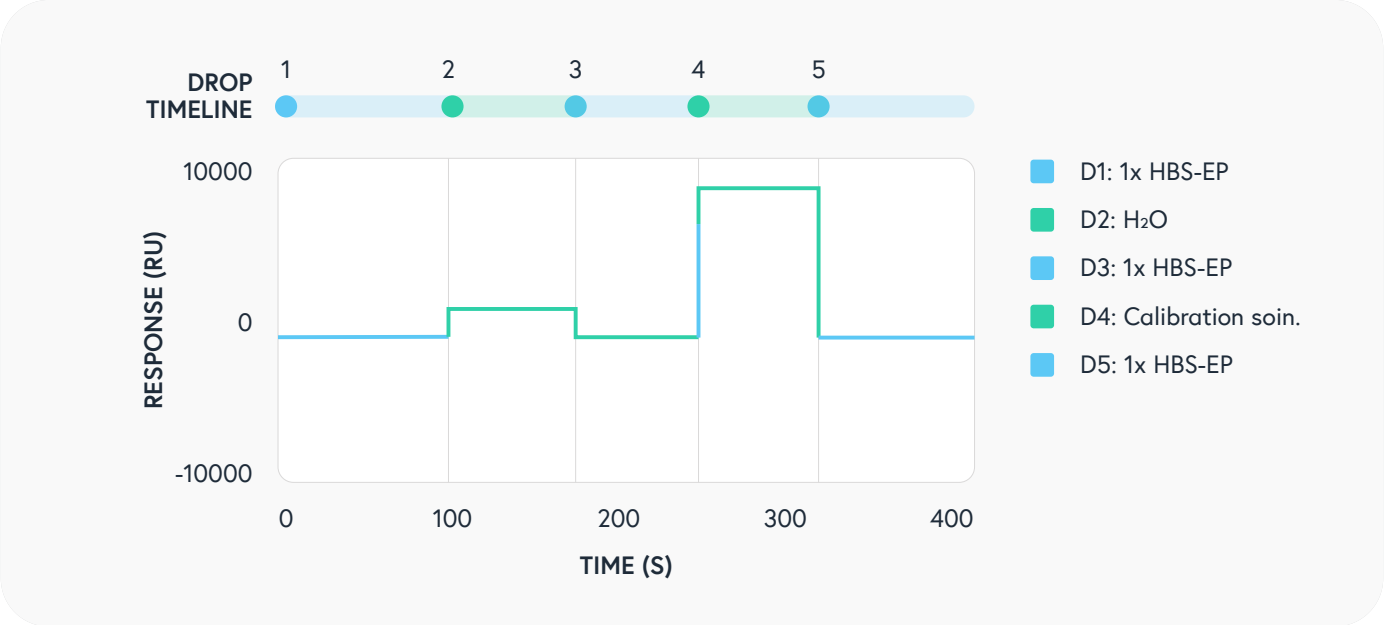


Figure 14: Bulk shifts are caused by an RI difference between the sample buffer and running buffer. They have a characteristic square shape.

When bulk shifts occur, they are present on both the reference and active sensors. Subtracting the reference sensor response from the active sensor response will reduce the impact for bulk shifts in the data, but this correction may not be enough to suppress the bulk effect entirely. While the presence of bulk shift does not change the inherent kinetics of the binding partners, it is challenging to differentiate small binding-induced signal changes (response) from a high refractive index background. It is crucial to minimize bulk shifts by matching the components of the analyte buffer to the running buffer and, wherever possible, avoid buffer components that cause this effect. Some recommendations for common buffer components that cause bulk shifts are outlined in **Table 6.**

Helpful tips and tricks for buffer matching:

- Buffer exchange the sample to match the running buffer
- Dilute the sample at least 10x in the running buffer
- Use the sample buffer as the running buffer

Component	Recommendations
DMSO	Analytes or ligands that require DMSO should be run in a buffer containing equal concentrations of DMSO. It is recommended not to use >10% DMSO.
Glycerol	Analytes or ligands obtained from stocks containing glycerol should be dialyzed into the running buffer to remove glycerol. If it is required for stability, it must not exceed 10%, and the running buffer should contain an equal concentration of glycerol.
Other molecules	The best practice is solvent/buffer matching, as the addition of buffer additives could change the intrinsic refractive index of the sample.

Table 6: Recommendations to reduce bulk shift for common buffer components

Example of an initial optimization test

There are many approaches to optimize a new system from scratch on Alto. We suggest scouting 2 different immobilization buffer pHs with 4 different regeneration solutions to have the best chance of identifying multiple high impact conditions in a single cartridge. As seen in **Figure 15,** multiple test configurations can be scouted simultaneously in a single round on the Alto system, allowing users to pinpoint optimal test conditions after just one test.

Figure 15: Simultaneous analysis of 4 immobilization buffers and 4 regeneration conditions in a single test. A pH scout for ligand immobilization with sodium acetate buffer shows pH 5.5 to be optimal (left). Full regeneration for this ligand-analyte pair is achieved with 10 mM glycine-HCl pH 2.5 (right)

The example in **Figure 15** tests 4 different immobilization buffers, ranging from pH 4.5 to pH 6.0 in 0.5 pH unit increments. Viewing the results, the optimal levels are achieved with pH 5.5 buffers, as evidenced by the greater increase in ligand signal in the data. A subtle difference between pH 5.5 and pH 6.0 is noted. Next, regeneration success for these tests can be examined in the kinetics portion of the test. In this example, 4 regeneration conditions are also tested. The data shows that pH 1.5 glycine-HCl is too harsh of a regeneration for these samples, as seen by the extreme drop in the baseline after the regeneration step, whereas pH 3.0 is too mild, as seen by the baseline being greater than zero after the regeneration step. In this example, pH 2.5 is the optimal regeneration. Thus, Alto has run 8 different conditions on one test to quickly optimize the immobilization and regeneration conditions for this ligand-analyte pair.

Subsequent rows on the cartridge could be used to assess ideal analyte concentration in the same test to further streamline experimental optimization.

Helpful tips and tricks for optimizing a kinetics assay on Alto

- Analyte response is dependent on molecular weight, so use the larger molecule as the analyte where possible
- Select a ligand concentration that does not saturate the biosensor, but still provides a strong analyte signal. This will limit mass transport effects
- The buffer used for baseline and dissociation steps must match the matrix of the analyte sample as closely as possible
- The highest analyte concentration should be at least 10x higher than the expected K_D value

Running a binding kinetics assay

Alto's Nicosystem is a comprehensive software that provides a centralized hub for acquisition and analysis of real-time binding data. Nicosystem offers flexibility in kinetic assay design, including the use of a capture molecule or streptavidin surface, single-cycle and multi-cycle, and full customization of contact times for each step of the experiment. Alto's plug-and-play design makes it easy for anyone in a lab to confidently operate SPR pre-designed experiments that are readily accessible via a touch-screen interface.

The following is a general assay protocol for Alto **(Figure 16)**:

1. Design experiment on the Nicosystem
2. Prepare buffers, reagents and samples
3. Load cartridge and run the assay

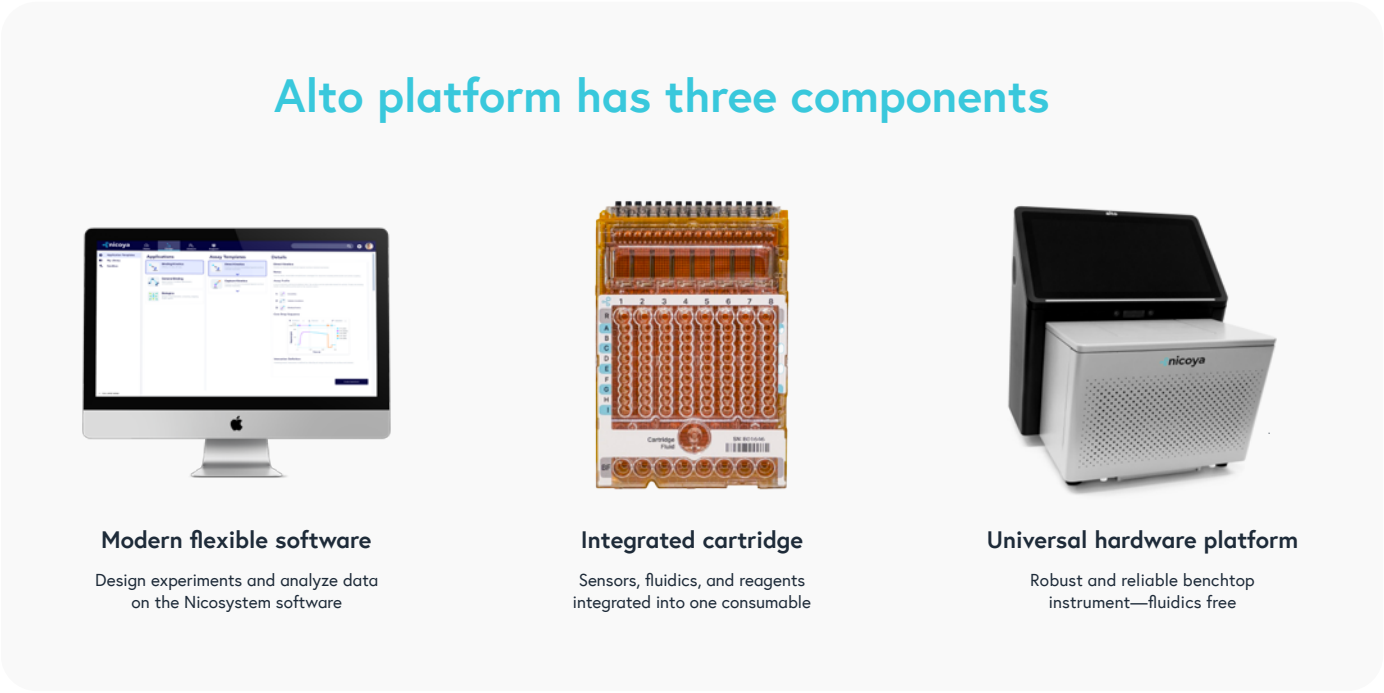


Figure 16: Running an Alto test is as easy as 1-2-3. Design experiment on the Nicosystem, load samples into the cartridge and start the test!

Analyzing a binding kinetics assay

Alto's Nicosystem provides powerful, easy-to-use analysis of real-time binding data. Test results are accessible on the Nicosystem immediately following the completion of an assay. Nicosystem's one-click analysis reduces tedious data uploads and lengthy post-processing. Its seamless end-to-end handling of experiments will empower a user to quickly scale their workflow and output new discoveries.

The following are general analysis steps for kinetic data on Alto:

1. Assess ligand immobilization levels across all 16 sensors in the cartridge simultaneously to ensure optimal levels
2. Open the kinetics tab, a 1:1 Langmuir binding model will be automatically applied to the data
3. Change the fit model and use processing tools as required
4. Download final images, .CSV files and/or report tables

Alto makes data analysis a breeze:

- A diverse range of models and investigative tools are available to accurately interpret data and provide high-quality insights
- Accommodations can be made both to individual interactions and cartridge-wide to refine data and produce high-quality figures
- Export data in a variety of file types. Raw and analyzed interaction data for each activity can be exported as .CSV files and images in one-click for continued data processing, reporting of results, or company databases
- Bulk data download is available for quick and convenient download of all .CSV files and images from the entire experiment

An example of a real test in the Nicosystem is shown in **Figure 16**. The data are automatically fit to a 1:1 binding model as soon as the experiment are accessed and is organized conveniently into tabs. The sensorgram has a legend which includes all relevant sample information as well as comprehensive kinetic characterization, including k_{on}, k_{off}, K_D, Rmax, Chi^2, kt (if using mass transport model), binding model and linear baseline drift indicator. These results are also summarized for all interactions in a table beneath the sensorgram, downloadable in .CSV format.

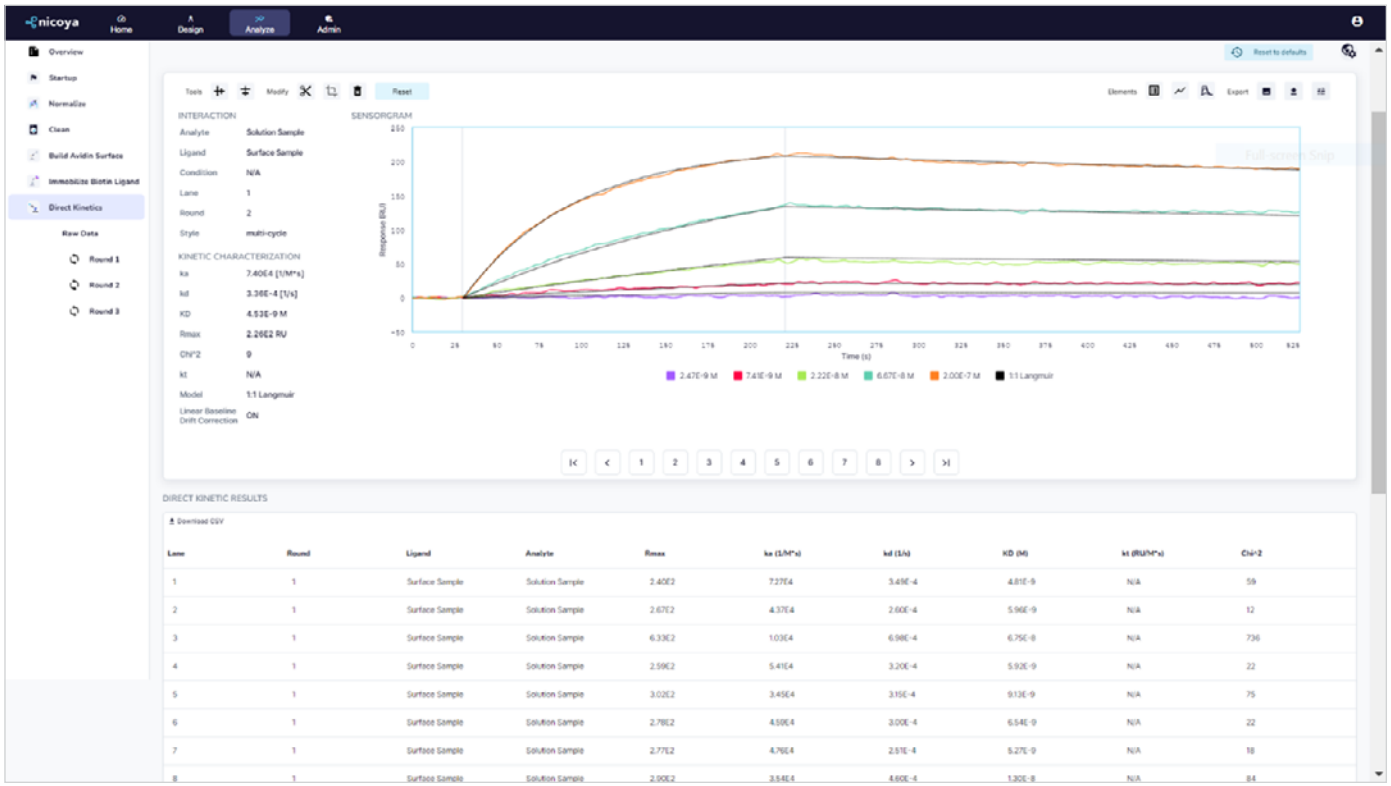


Figure 16: Multi-cycle kinetics assay in the Nicosystem™ analysis software. This assay tests the binding kinetics of an Influenza A H5N1 hemagglutinin analyte against several antibody ligands.

Conclusions

SPR is a critical tool in the discovery and development of novel biological drugs. In Nicoya's mission to improve human life by helping scientists succeed, we developed Alto to overcome the common challenges of using SPR to provide a more cost-effective and efficient solution for biologics discovery and development. Here, we have presented an overview of Alto, the world's first DMF-powered SPR instrument. The DMF platform offers great flexibility in assay design and requires only 2 µL of sample for full kinetics. There are many ways to configure tests depending on the type of molecules being tested and available tags on those molecules. This offers a great deal of versatility in the types of binding pairs that can be characterized on Alto. Effective assay design is critical to running successful assays that give the most reliable data possible. Many factors including ligand density, immobilization pH, analyte concentration and regeneration solutions need to be optimized for each binding pair being analyzed. By properly designing experiments and optimizing critical parameters, kinetic analysis on the Alto digital SPR system will consistently produce robust data and accurate, reliable kinetic parameters.

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