

OneStep lead characterization of high affinity biologic interactions with Pioneer SPR systems

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Introduction

Biopharmaceutical drug discovery and development have celebrated the approval of breakthrough treatments in diseases of inflammation, cancer and infectious disease in recent years. As the biopharma industry sees more therapeutic successes, the problems left to solve in medicine will prove increasingly more difficult. Protein therapeutics, or biologics, are known for having good specificity for their therapeutic targets and this often affords fewer side effects than small molecule drugs. Biologics are a viable route for targeting protein–protein interactions, cell surface receptors, and other difficult-to-drug therapeutic targets. The discovery process for biologics has been well established, especially for common molecular classes such as monoclonal antibodies, and newer classes of biologics are being sought for enhanced cell uptake, membrane permeability and serum half-life.

The process for biologics drug discovery from hit to lead involves screening (primary, secondary and tertiary) to identify hits, followed by lead characterization including target binding characterization. Primary screening techniques for biologics normally focus on speed and throughput to identify hits from libraries containing 10^4 – 10^5 crude protein samples. Traditionally, primary screening has been performed with ELISA, biochemical assays, and/or label-free techniques such as the BLI-based Octet® systems. Secondary and tertiary screens can be used to further test the hits for functional binding effects or cross-reactivity. Once a panel of advanced hits has been compiled, they can be expressed and purified in small scale for in depth characterization before *in vivo* testing.

Target binding characterization is an important analytical step for the selection of high affinity ($K_D < 1$ nM) and highly specific biologics regardless of the types of molecules. Kinetic analysis further describes the components of association and dissociation that comprise the overall affinity interaction. A biologics discovery lab may have two lead candidates with similar affi-

ty (K_D) but their differences in kinetic rate constants of association and dissociation can be used to estimate which will be more useful *in vivo*. Accurate analysis of these kinetic rate constants is therefore important information for lead selection and predicting the efficacy of protein therapeutics.

The Pioneer Next Generation SPR platform from ForteBio improves the efficiency of the characterization process over traditional SPR by determining the kinetics and affinity in a single step. The next-generation OneStep® gradient injection featured on the Pioneer platform dramatically increases the speed of affinity characterization while maintaining accuracy and high confidence in results. In this application note, we explore the utility of OneStep Injections in kinetic characterization of biologics in both research and drug discovery.

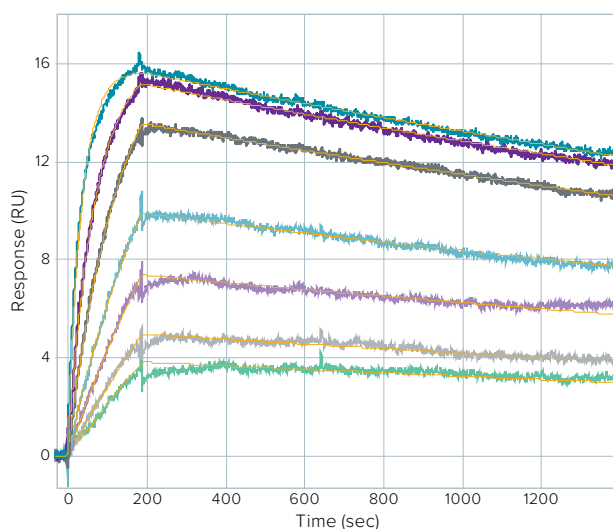


Figure 1: Traditional kinetic analysis of analyte binding to an immobilized ligand with seven concentrations of analyte.

OneStep: The next generation of SPR analysis

Kinetic analysis of biomolecular interactions with traditional SPR techniques involves the testing of different concentrations of analyte in solution binding to the immobilized ligand. Each analyte concentration must be injected over the immobilized molecule briefly (1–10 min each) and then dissociation of bound analyte must be monitored under buffer flow. Complex formation is observed as a function of time and at known analyte concentrations to determine the rate constants of association (k_a) and dissociation (k_d) and the equilibrium dissociation constant (K_D). An example of this analysis is shown in Figure 1 where seven concentrations of analyte are injected over an immobilized molecule to observe the time-resolved and concentration-dependent interaction parameters. Global kinetic analysis is performed by least-squares fitting a kinetic model to the binding data to find the best-fit values for k_a , k_d and R_{max} .

The data in Figure 1 show that seven concentrations spaced two-fold apart are required to effectively test the ‘kinetic space’ of this interaction and determine the association and dissociation rate constants. Traditional SPR requires researchers to prepare a dilution series of analyte that is subsequently injected one after the other — adding preparation and analysis time to the workflow.

In contrast, OneStep Injections are a powerful method to determine the kinetic and affinity parameters of a binding interaction using only a single sample of analyte and one injection. OneStep Injections take a sample of known concentration and disperse it through a volume of buffer (held within the OneStep capillary line) as it is flowed to the sensor surface. This method, based on Taylor dispersion¹, produces a sigmoidal concentration gradient of analyte in the capillary fluidic line (Figure 2). As the sample gradient flows from the capillary to the sensor, binding data are collected in real time incorporating the full range of analyte concentrations presented to the surface from low to high.

For OneStep Injections, the analyte concentration at the flow cell over time is described by the Taylor dispersion theory as a function of the analyte diffusion coefficient, flow rate, and dispersion line geometry. Pioneer analysis software (Qdat) automatically recognizes OneStep Injections and calculates the analyte concentration over time using the system-given constants (flow rate, geometry data, and analyte diffusion coefficient). As an internal control for the gradient dispersion process, an injection of 3% sucrose dissolved in the assay buffer is performed in each OneStep assay to ensure proper gradient formation is occurring and to calibrate for buffer viscosity. The result is a binding response with kinetic curvature which defines the association and dissociation rate constants when analyzed as a function of time and concentrations, as shown in Figure 3.

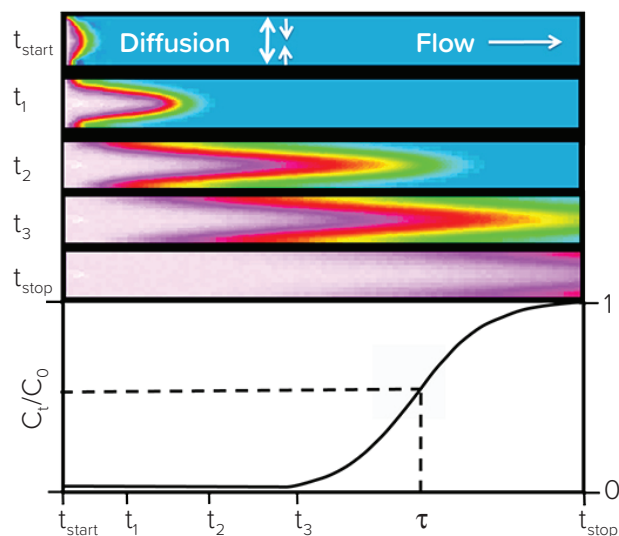


Figure 2: OneStep gradient formation in the Pioneer capillary line (top) and analyte concentration measured at the flow cell (bottom). Blue indicates buffer and pink indicates sample. The gradient formation and its relationship to analyte concentration at the flow cell is illustrated using five simulated snapshots ($t_{start} - t_{stop}$) of the capillary line at different times.

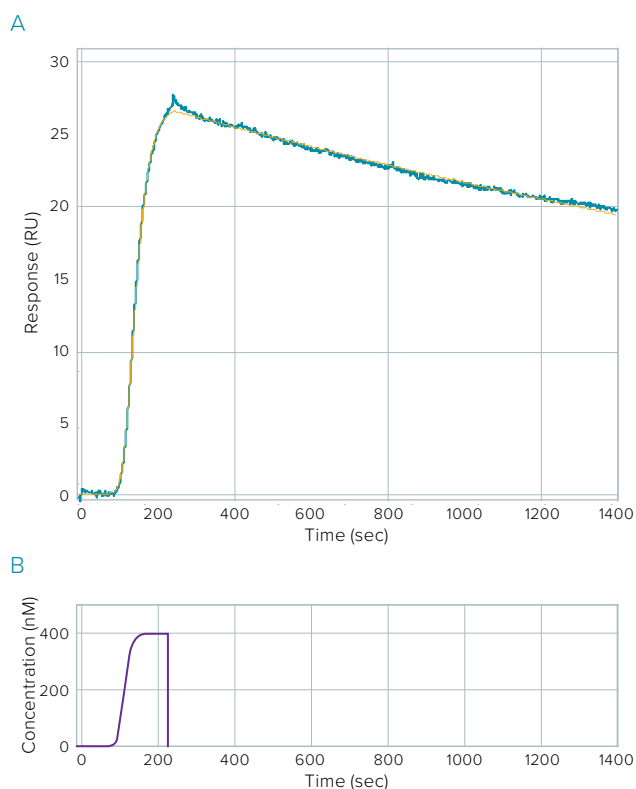


Figure 3: OneStep binding curve of analyte (gradient from 0–400 nM) binding immobilized ligand. (A) Binding data are shown in blue and the 1:1 kinetic model curve is shown in red. (B) The analyte concentration gradient is illustrated.

Method	k_a ($\times 10^4$ M ⁻¹ s ⁻¹)	k_d ($\times 10^{-4}$ s ⁻¹)	K_d (nM)	Sample required (μ l)	Characterization time per analyte (hrs)
OneStep Injections	9.46 \pm 0.01	2.717 \pm 0.002	2.874 \pm 0.004	150	0.68
Traditional dose response	7.14 \pm 0.01	2.054 \pm 0.002	2.878 \pm 0.006	400	4.73

Table 1: Comparison of kinetics and affinity characterization results with OneStep and dose response methods.

A basic requirement for conventional kinetic characterization is the observation of time-dependent binding of multiple, constant analyte concentrations to accurately determine k_a and k_d . OneStep Injections accomplish this requirement in a single injection by measuring the time-dependent binding of analyte where concentration is also time-dependent. OneStep binding data therefore gives the time- and concentration-dependent information required to determine k_a and k_d for a binding interaction in a single injection. Table 1 shows the kinetic rate constants and equilibrium dissociation constants calculated from the data in Figure 3 (OneStep Injections) and Figure 1 (dose response) using the 1:1 kinetic model.

The comparison between the traditional injection method and the OneStep method show good agreement in the kinetic rate constants (<30% difference) and the equilibrium dissociation constants (<1% difference) determined for a nanomolar affinity interaction. Other comparisons of OneStep Injections with traditional dose response have shown good agreement for interactions up to high micromolar^{2,3} affinity. Characterization with the OneStep method required one sample versus seven samples with the traditional method. In addition, the OneStep injection completed in roughly one-seventh the time required for the traditional method (Table 1).

Pioneer systems can perform either the traditional method or OneStep Injections to characterize kinetics and affinity. However the OneStep method offers distinct advantages in time, reagent and labor savings without compromising data accuracy and reproducibility.

Pioneer platform basics

Pioneer systems are fully automated SPR instruments using a Kretschmann configuration optics to address three distinct flow channels for immobilizing molecules or for reference subtraction. The flow channels can be addressed individually for immobilization or in series for analyte characterization which enables in-line reference subtraction. Samples are stored in temperature-controlled sample racks (4–40 °C) using either glass vials or standard 96- or 384-well microplates, and up to 768 samples can be run unattended. Sample delivery to the sensor is achieved using an XYZ autosampler and buffer for the flow sys-

tem is degassed in-line. The simple fluidic system is comprised of PEEK tubing for durability and chemical resistance. Tubing is connected to switching router valves that have excellent lifetimes, offering years of usage without required parts replacement. Simple method design enables setup for standard inject types or next-generation injections (i.e. OneStep, NeXtStep™, etc.) in a common workflow with Pioneer software.

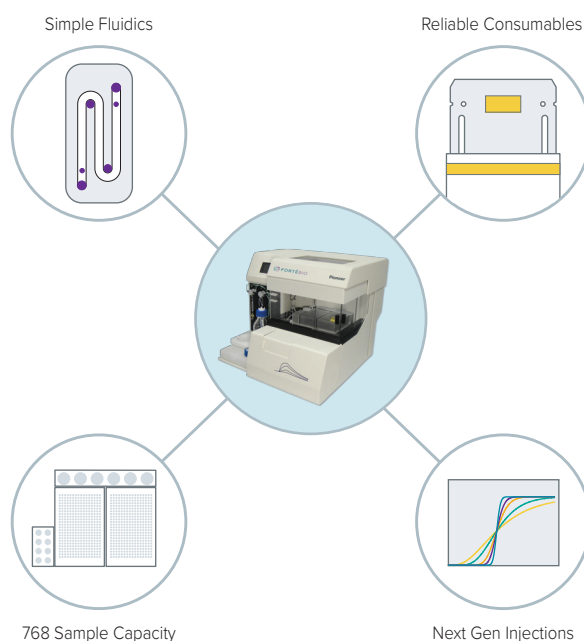


Figure 4: The Pioneer platform allows streamlined analysis through a combination of several key hardware and assay attributes.

OneStep assay design to characterize advanced hits for lead selection

Kinetic characterization of biologic advanced hits can be performed in several assay formats depending on the valency of the molecules and their ability to be specifically captured on a sensor surface. The following examples provide assay design guidelines and tips to make optimal use of OneStep Injections for kinetic analysis on the Pioneer platform.

Kinetic characterization with biologic as analyte

One standard assay format is to immobilize the target and test the biologic (IgG, Fab, Fv, etc.) binding as the analyte as illustrated in Figure 5. The method of target immobilization can be either irreversible (amine, biotin, or thiol coupling) or reversible (His-tag or other anti-tag capture). Flexibility in immobilization method is helpful as some targets may require specific conditions to maintain activity during and after the immobilization process. In the example shown in Figure 7, the target was immobilized via amine coupling and a simple regeneration of the surface with 10 mM HCl was used after each binding cycle.

Table 2 gives general method design guidelines for OneStep assay protocol steps. If the ligand is captured using a reversible method, then each assay cycle can capture ligand (Experiment Step #3 in Table 2), OneStep Injection of analyte, and use a regeneration injection which removes ligand at the end of each cycle.

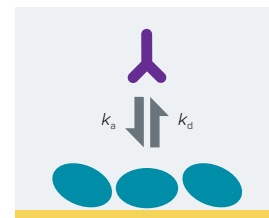


Figure 5: Illustration of immobilized target (semicircle) binding biologic (IgG) as analyte.

The assay design guidelines can be modified to suit the nature of different samples, target molecules, ligand capture method, or other experimental variables.

Experiment step	Protocol method settings	Notes
1 General Assay Settings	<ul style="list-style-type: none"> Replicates: 2 Sample rate: 5 Hz Periodic cycles: <ul style="list-style-type: none"> Blank cycles: periodic, every 4 cycles Leadoff blanks: 3 Bulk std. cycles: 1 <div style="border: 1px solid gray; padding: 2px; margin-top: 5px;"> Bulk Std. Cycles <input type="text" value="Fixed Quantity"/> Exactly <input type="text" value="1"/> cycle(s) <input checked="" type="checkbox"/> Skip Regen </div>	Duplicate injections are recommended for most characterizations. 5 Hz data sampling provides good resolution for the association phase while maintaining minimal file size for long assays.
2 Assay Pre-cycle	<ul style="list-style-type: none"> Purge Flow rate <div style="border: 1px solid gray; padding: 2px; margin-top: 5px;"> Buffer Flow Rate <input checked="" type="checkbox"/> Match Analyte Injection Flow Rate </div> Wait: 60 sec 	Pre-cycle procedure of purging fluidics washes out sample from the previous cycle. Setting flow rate ensures the analyte inject flow rate is configured and the wait allows a 1 min period for baseline stability prior to starting the cycle.
3 Capture Injection (optional)	<ul style="list-style-type: none"> Inject: <ul style="list-style-type: none"> Inject volume/flow rate (per assay development) Flow path: typically FC 1 or FC 3 	Optional ligand capture injection for fresh immobilization of ligand in each cycle. Ligand solution must be stable for the duration of the assay. Concentration, inject volume, and flow rate are per assay development. Use a dummy or buffer inject for the Sucrose Cycle (S1).
4 Analyte Injection	<ul style="list-style-type: none"> OneStep: <ul style="list-style-type: none"> Flow rate: 50 μL/min Flow path: FC 1-2-3 Sample volume: 75% of loop for moderate affinity and 100% of loop for high affinity Dissociation: 300 sec minimum, (30 sec for Sucrose Cycle) 	Requires at least 250 μ L of analyte solution for 2 replicates. Sucrose Cycles (S1) need minimal dissociation as only data from the association phase are used.
5 Regeneration Injection	<ul style="list-style-type: none"> Inject: <ul style="list-style-type: none"> Inject volume/Flow rate: typically 25 μL at 25 μL/min Flow path: typically FC 1-2-3 Dissociation: 60 sec 	Regeneration scouting and validation is a common part of assay development. The general regeneration suggestion is to use the shortest injection times that efficiently regenerate the surface to retain ligand/surface activity.

Table 2: Assay design guidelines for kinetic characterization with OneStep Injections.

The assay method designed in Figure 6 was performed with 10 Fab hits injected using the OneStep method and a simple 1:1 kinetic model was fit to the data via non-linear least squares fitting.

Ten Fabs (of advanced hits) binding to the immobilized target were characterized using OneStep Injections with binding traces show in in Figure 7, with the goal of selecting the best candidates for lead optimization. The best-fit values for association and dissociation rate constants from the results in

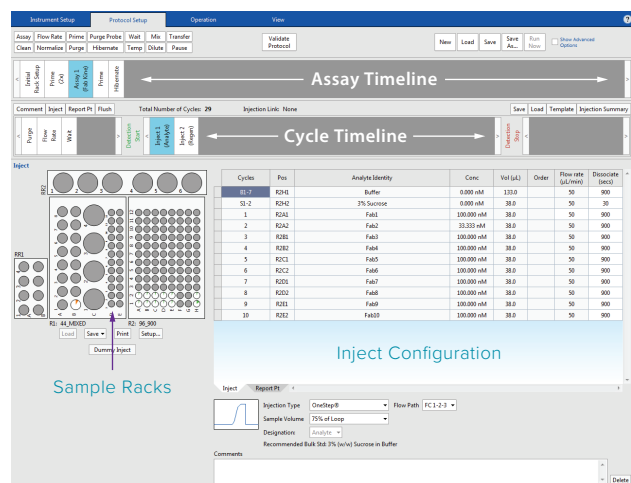
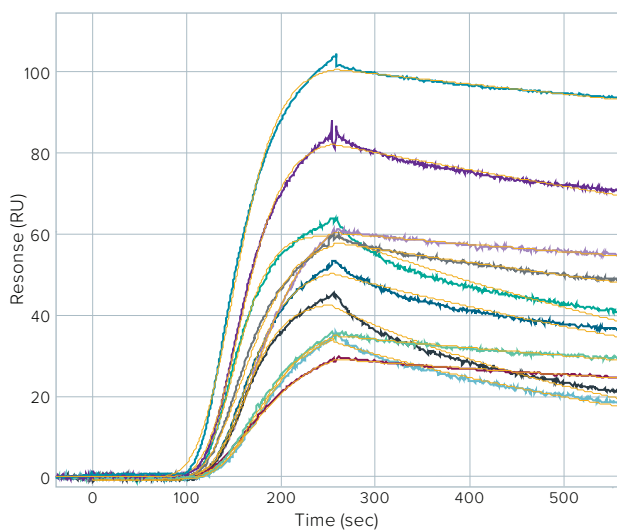


Figure 6: Pioneer Control Software showing the OneStep Injection of biologic hits in OneStep kinetics protocol.



3.7 hrs	Time required for the OneStep protocol including buffer blank and a sucrose cycle
24.5 hrs	Time required for the traditional dose response protocol including 7 analyte concentrations and buffer blank cycles

Figure 7: OneStep kinetic characterization of biologic advanced hits for lead selection. Assay format is biologic as analyte. Binding data are least-squares fit with the simple 1:1 kinetic model (fitted curves shown in red).

Figure 7 were presented in an affinity plot (k_d vs k_a) to visualize the kinetic differences of each Fab binding target (Figure 8).

The affinity plot (Figure 8) helps visualize how kinetics comprise affinity and to compare both kinetics and affinity of hits. OneStep Injections capture this level of information from a single injection per analyte. The OneStep-derived kinetics of biologics can be reviewed with other important biophysical and functional properties to select a lead or leads to optimize.

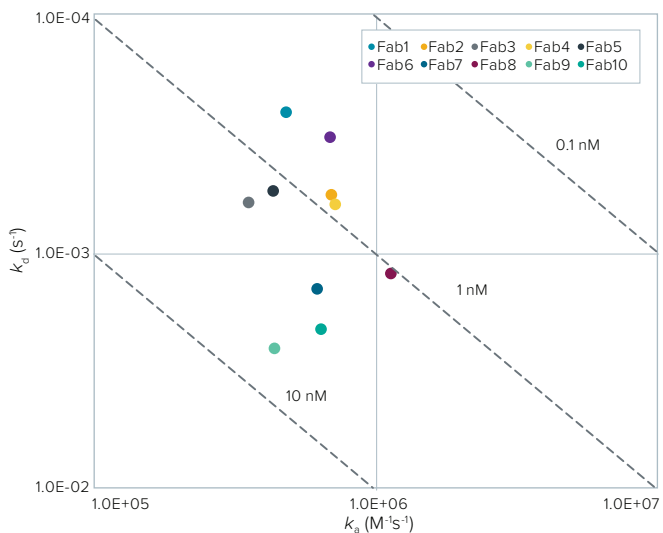


Figure 8: Affinity plot (k_d vs k_a) for biologics binding to immobilized target characterized by OneStep assays. Dashed lines indicate common affinity relationships with different kinetic rate constants.

Kinetic characterization with target as analyte

A second characterization assay format is to immobilize the biologic(s) to the sensor surface and bind the target in solution (Figure 9). This approach can be easily applied to biologics which have common domains that serve as a capture epitope, such as the Fc domain on IgG. Immobilizing the biologic is also beneficial if the molecule is multivalent or if the target is unstable when immobilized. Capture of IgG can be accomplished by pre-immobilizing an anti-Fc antibody or a recombinant Protein A or G on the sensor surface via amine coupling. These capture molecules can be used to capture IgG and orient the captured IgG to bind target in solution. Other specific capture antibodies can be used when biologics are not Immunoglobulins. Regeneration of the biologic from capture antibody or Protein A may require injection of 10 mM glycine HCl pH 1.5 or 3 M MgCl₂, while regeneration of IgG from Protein G may require injection of 10 mM NaOH, with appropriate assay development as needed.

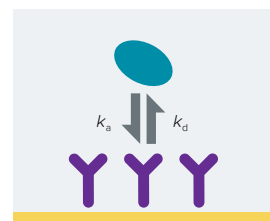
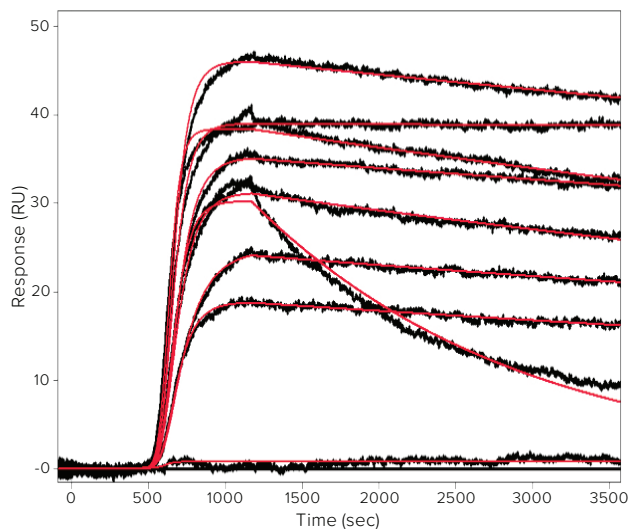


Figure 9: Illustration of immobilized biologic (IgG) binding target (semicircle) as analyte.

The example for target as analyte kinetic characterization in Figure 10 uses an anti-Fc antibody to capture IgG biologics (not shown) followed by a OneStep Injection of target over each captured biologic.



19.7 hrs	Time required for the OneStep protocol including buffer blank and a sucrose cycle
62.8 hrs	Time required for the traditional dose response protocol including 7 analyte concentrations and buffer blank cycles

Figure 10: OneStep kinetic characterization of biologic advanced hits for lead selection. Assay format is target as analyte. Binding data (black curves) are least-squares fit with the simple 1:1 kinetic model (fitted curves shown in red).

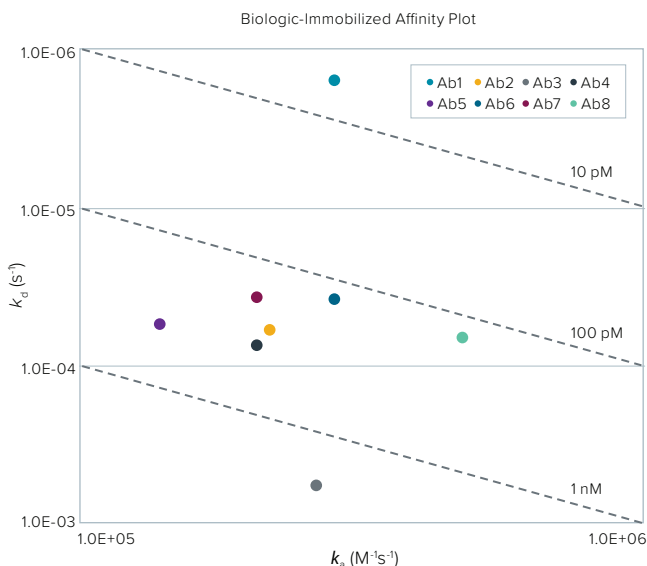


Figure 11: Affinity plot (k_d vs k_a) for target binding immobilized biologics characterized by OneStep assay. Dashed lines indicate common affinity relationships with different kinetic rate constants.

The best-fit kinetic rate constants from the characterization example in Figure 10 were plotted in the affinity plot, Figure 11. Kinetic rate constants derived from this OneStep characterization can be combined with other biophysical and functional data to select the best biologic hits to progress.

Pioneer systems can characterize binding interactions with OneStep Injections to determine the important kinetic and affinity constants for making informed lead selections using either the biologic or target as analyte assay formats. Once a lead is selected, the next phase of drug discovery includes lead optimization and in-depth characterization. The target as analyte assay format is often chosen where humanization (if required) and affinity maturation are performed to optimize the target binding properties of a lead.

As biologic hits are progressed to leads the potencies and especially the binding residence time increases, requiring long dissociation times for typical SPR analysis. Interaction residence time, defined as the inverse of the dissociation rate constant ($1/k_d$), may be reported in seconds, minutes or hours for very potent molecules. When residence time is measured in hours, it is

important for the analytical technique to provide stable measurements for these lengths of time. It has been previously shown⁴ that a dissociation event of 5% or more of bound complex is key to accurate measurement of dissociation rates for label-free biosensor assays. The biosensor

assay must therefore be stable and sensitive to changes in response equal to 5% of equilibrium response (R_{EQ}) over the course of 5+ hrs in certain cases. As shown in Table 3, the minimum time to measure dissociation increases dramatically between 10^{-5} and 10^{-6} s^{-1} ultimately reaching 14 hrs for the slowest off rates.

In characterizations of interactions with slow dissociation rate constants the assay design must be adjusted to collect accurate data while maintaining practical run times. Buffer blank injections must be performed to reference any drift of the immobilized molecule for the same cycle length as the analyte injection (up to 14 hrs in extreme cases). Instrument baseline stability must also be consistent and reproducible for multi-hour recordings stressing the stability of temperature and buffer flow control of the SPR system. In the next section we will address these aspects of high affinity measurements using examples of OneStep analysis of slow dissociation interactions.

k_d (sec^{-1})	Time to 5% dissociation
10^{-3}	51.3 s
10^{-4}	8 m 33 s
10^{-5}	1 h 25 m
10^{-6}	14 h 15 m

Table 3: Minimum required dissociation times by dissociation rate constant

Simplifying high affinity lead characterization with OneStep

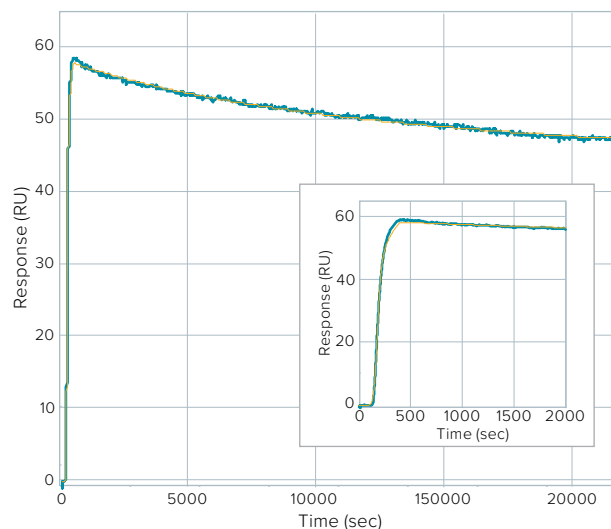
High affinity characterization with OneStep Injections requires system and sensor stabilization time, appropriate gradient design, and sufficient dissociation time (see Table 3) for best results. The Pioneer system should be recently cleaned and have a new biosensor chip installed. The method of immobilization chosen should be irreversible or very high affinity such as biotin-streptavidin to avoid long-term dissociation of the immobilized molecule. The analyte solution for OneStep Injections should be at least 100X the expected K_D and the dissociation time should be long enough for at least 5% dissociation of bound analyte.

The first high affinity example (Figure 12) is a complex kinetic case where both the biologic and target are bivalent. The biologic lead with biotin tag was immobilized via biotin capture using a BioCap sensor (ForteBio Part No. PS03AFB) to a low surface density (50 RU), and the sensor was allowed to stabilize for 1 hr. Alternatively, SADH biosensor can also be utilized in a similar manner (ForteBio Part No. 19-0130). The assay protocol design for this characterization was constructed where the lead was immobilized prior to the kinetic assay and without regeneration.

Leadoff blank injections are common in SPR-based kinetic assays to obtain the best injection reproducibility and referencing, however in high-affinity assays with multiple-hour dissociations they must be truncated for practicality. In this example, the first two blanks are dissociated for 6000 sec to stabilize the sensor and the third blank and analyte injection have the full dissociation time of 21600 sec (6 hrs). This design allows the injection system and sensor chip to give the most reproducible and stable signal for referencing the 6 hr dissociation event. Therefore, the third blank is used as the reference for the full binding and dissociation curve recorded during the analyte inject.

The protocol just described was performed in this first example where 100 nM target solution was injected with the OneStep method using 100% sample loop volume, a flow rate of 30 $\mu\text{L}/\text{min}$, and dissociation was monitored for 6 hrs. The data were processed in Qdat software per standard procedure and analyzed with the bivalent analyte kinetic model as the target and biologic were both bivalent. The data and best-fit model curve are shown in Figure 12.

The first high-affinity characterization example of target binding immobilized biologic shown in Figure 12 gave best-fit values of $4.552 (\pm 0.007) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ for k_a and $5.75 (\pm 0.02) \times 10^{-5} \text{ s}^{-1}$ for k_d for the primary (1:1) interaction. The observed



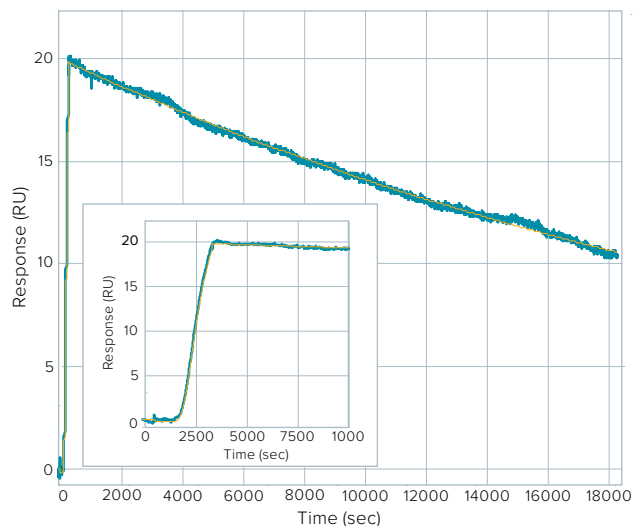
17.1 hrs	Time required for the OneStep protocol including buffer blank and a sucrose cycle
62.6 hrs	Time required for the traditional dose response protocol including 7 analyte concentrations and buffer blank cycles

Figure 12: OneStep kinetic characterization of a high affinity bivalent biologic-target interaction. Figure inset shows the first 2000 sec of data including the full OneStep association (0–400 sec) and the first 1600 sec of dissociation. Binding data are shown in blue and the fitted model curve in red.

affinity (K_D) for the 1:1 interaction (the initial binding event) was $126.4 \pm 0.5 \text{ pM}$. Approximately 19% of the bound target was observed to dissociate during the 6 hr measurement which exceeds the minimum analysis requirement of 5%. Complex kinetic cases such as this bivalent example normally pose a challenge for standard SPR analyses but with the high-concentration resolution of OneStep assays, the bivalent analyte model is fit to the data to easily determine the kinetics of the interaction. The concentration resolution of the OneStep method refers to the continuous concentration gradient wherein every time point during the injection tests a different concentration within a range of 3–4 orders of magnitude. The enhanced kinetic resolution of OneStep assays will be useful as more complex therapeutic mechanisms are explored in the future.

The second high-affinity example (Figure 13) is a simple 1:1 interaction using the target as analyte assay format, where 120 RU of biotin-tagged biologic was captured on a BioCap sensor. 100 nM target was injected with the OneStep method using 100% of sample loop volume at 40 $\mu\text{L}/\text{min}$ with a 5-hr dissociation.

The high-affinity characterization example shown in Figure 13 gave best-fit values of $9.21 (\pm 0.09) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ for k_a and $3.500 (\pm 0.001) \times 10^{-5} \text{ s}^{-1}$ for k_d . The observed affinity (K_D) for the interaction was $380 \pm 4 \text{ pM}$. Nearly 50% of bound target was observed to dissociate in the 5 hr dissociation period. The data demonstrate that the Pioneer platform can resolve the slow dissociation expected for these efficient and potent interactions.



15.1 hrs	Time required for the OneStep protocol including buffer blank and a sucrose cycle
53.6 hrs	Time required for the traditional dose response protocol including 7 analyte concentrations and buffer blank cycles

Figure 13: OneStep kinetic characterization of a simple high affinity biologic-target interaction. Assay format is target as analyte and dissociation time is 5 hrs. Figure inset shows the first 1000 sec of data including the full OneStep association.

Conclusion

Scientists in the biologic drug discovery space are constantly identifying new therapeutic targets and seeking new molecules to combat disease. As new hits are generated and optimized into lead candidates, Pioneer Next Generation SPR systems with OneStep gradient injections can accurately characterize target binding for panels of advanced hits all the way to high affinity leads.

- OneStep characterization is 3- to 6-fold faster than traditional characterization on a 4-channel SPR system
- OneStep Injections use less sample (50% less analyte)
- No analyte dilutions required
- No major adjustments to workflow — current affinity characterization workflows easily migrate to OneStep assays from traditional methods

New classes of biologics with complex binding mechanisms are also more easily analyzed with the higher analytical resolution of OneStep. The OneStep method relies on the robust principles of Taylor dispersion and can be used to confidently determine kinetics and affinity from a single gradient injection. The Pioneer platform provides excellent baseline stability for accurate kinetics characterization of high affinity binding interactions. Baseline stability coupled with the time and injection reducing advantages of OneStep make the Pioneer systems an optimal assay platform for high affinity biologic characterization.

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