SARTORIUS

Application Note

March, 2022

Keywords or phrases:
Octet®, OneStep®, SF3, Kinetics Determination

Kinetics Determination of High Affinity Molecular Interactions Using OneStep® Injections

Introduction

Drug development using protein-based therapeutics has become particularly important in medical research and is predicated on the identification of therapeutic targets. The drug development process is generally a long and costly process and only a very small percentage of drug candidates under development make it through to approval by the regulatory bodies.

Determination of accurate kinetics and affinity along with other critical quality attributes play an ever-increasing important role in identifying and isolating therapeutic molecules to drug-target. It is important therefore, that any systems developed for these purposes can match not only the high throughput needs of the user but also their sensitivity needs; allowing assays to be performed earlier in the workflow with minimal amounts of precious samples. This faster time to results allows assessment of accurate and precise data earlier in the workflow and as such quicker decisions can be made on which lead candidates to promote.

Modern label-free analytical techniques allow kinetics interactions to be monitored with high resolution in real-time, which when combined with high-throughput capabilities, can significantly reduce the time to the discovery, streamlining the selection of optimal drug candidates with the best chances for success downstream.

Typically, during high-throughput screens (HTS) drug candidates are prepared as a single concentration and injected across the target and as such only the binding levels are evaluated. As no reliable kinetic parameters can be determined from such a limited data set the HTS is reduced to 'yes/no' binding depending on whether the observed response falls within a pre-set value range and doesn't indicate additional non-specific interactions with the target or sensor chip surface. Therefore, a major restriction of this method is that no quantitative information about kinetic parameters can be determined and that any hits need subsequent characterization using a more laborious multi-concentration assay, which can often lead to a labor-intensive assay development period.

Due to these limitations, the use of surface plasmon resonance (SPR) in HTS is often limited to hit validation as screening multiple concentrations of the drug candidate is not suitable for primary screening due to an extended time frame for screening and preparing samples. Additionally, there are other considerations, such as how many regeneration cycles the drug target can tolerate and space limitations as to how many samples can be accommodated in the system.

Based on these restrictions there has always been a tradeoff between throughput and precision but this can be remedied using OneStep® injections which is a feature unique to the Octet® SF3 SPR system. OneStep® injections offer a unique single concentration injection profile that provides kinetics and affinity across a range of concentration values in a fraction of the time and with substantial savings in sample and buffer usage compared to standard multi-cycle kinetics (MCK).

Here, we demonstrate that reliable kinetics and affinity for high-affinity interactions can be obtained from significantly less data than is commonly used and the number of measurements necessary for accurate kinetics and affinity determination can be reduced to a single measurement by using OneStep® injections, increasing sample throughput and saving sample material.

This removes the barrier for SPR as a tool for large screens and allows rapid progression for additional assay development of potential therapeutics.

Methods

Instrument and Reagents

All assays were performed using an Octet® SF3 SPR system. Hepes buffered saline with 0.05% Tween 20 (HBS-EP+), pH 7.4 was used as running buffer throughout. Unless indicated, all assays were performed at 37 °C.

Recombinant biotinylated HER2, Vascular endothelial growth factor (VEGF) 165 and 121a were purchased from Sino Biological. Herceptin (Trastuzumab) was purchased from Midwinter Solutions and anti-VEGF (bevacizumab biosimilar) was purchased from Absolute Antibody. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Thermo Fisher Scientific. All other reagents were purchased from Sigma Aldrich and prepared in-house.

Kinetics and Affinity Determination

HER2

Recombinant streptavidin was immobilized on an Octet® SPR CDL Sensor Chip using standard amine coupling chemistry. A 50:50 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.1 M N-Hydroxysulfosuccinimide (NHS) was injected across flow cells 1, 2 and 3 using a flow rate of 10 µl/min for 7 minutes. Recombinant streptavidin (5 µg/mL in sodium acetate pH 4.0) was then injected across flow cells 1, 2 and 3 using a flow rate of 10 µl/min for 7 minutes. The surface was then deactivated by injecting 1 M ethanolamine HCl pH 8.5 across flow cells 1, 2 and 3 using a flow rate of 10 µl/min for 7 minutes. Approximately 400 RU of recombinant streptavidin was immobilized on each flow cell.

Next, biotinylated HER2 (bHER2) was prepared to a final concentration of 1.25 µg/mL in HBS-EP+. Using the manual mode function of the Octet® SF3 bHER2 was injected at 10 µl/min across flow cell 1 until a capture level of ~150 RU was reached.

Kinetics and affinity of the interaction between Trastuzumab and bHER2 was performed using standard multi-cycle kinetics and OneStep® injections, which are unique to the Octet® SF3.

Trastuzumab was prepared to a final top concentration of 25 nM in HBS-EP+ running buffer and a 6-fold concentration series prepared using a 1:3 dilution into HBS-EP+. Samples were placed into Octet® SPR 0.9 mL vials and placed into a mixed format sample rack. 3% sucrose was prepared using HBS-EP+ as the bulk reference standard for OneStep® injections and 100 mM HCl was used for regeneration injections. The sample rack was sealed using resealable septa and placed in the sample tray set to 15 °C.

The Octet® SF3 system was primed 3 times into HBS-EP+ running buffer and the sensor chip hydrated and conditioned using injections of HBS-EP+ and 100 mM HCl. A short and long assay format was used (Katsamba 2006) with a common association time of 180 sec at 50 µL/min used for multi-cycle kinetics and a 'long' dissociation of 3600 sec used for the highest concentration and a 'short' dissociation of 420 sec used for all other concentrations. Association parameters for OneStep® are fixed based upon the volume of the injection loop used, which was set to 100% here and the same dissociation parameters used as for multi-cycle kinetics. A buffer blank injection was performed for each analyte concentration in order to generate accurate double referenced data.

The trastuzumab-bHER2 complex was regenerated with a single injection of 100 mM HCl at 50 µL/min for 60 sec, followed by a 180 sec stabilization period.

Data were analyzed using the standard short and long method of determining the dissociation rate constant (kd) for the highest concentration (3600 sec dissociation) and constraining the lower concentrations (420 sec dissociation) to the same value. All data was locally fitted to a simple 1:1 interaction model.

VEGF 165 and VEGF 121a

Recombinant VEGF 165 and VEGF 121a were immobilized on flow cells 1 and 3, respectively of an Octet® SPR CDL Sensor Chip using standard amine coupling chemistry. A 50:50 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.1 M N-Hydroxysulfosuccinimide (NHS) was injected across flow cells 1, 2 and 3 using a flow rate of 10 µl/min for 7 minutes. Recombinant VEGF 165 (0.25 µg/mL in sodium acetate pH 5.0) was then injected across flow cell 1 and Recombinant VEGF 121a (0.25 µg/mL in sodium acetate pH 5.0) across flow cell 3 until a response of 35 RU was achieved for both ligands. The surface was then deactivated by injecting 1 M ethanolamine HCl pH 8.5 across flow cells 1, 2 and 3 using a flow rate of 10 µl/min for 7 minutes.

Kinetics and affinity of the interaction between a bevacizumab biosimilar and VEGF 165 and VEGF 121a was performed using standard multi-cycle kinetics and OneStep® injections, which are unique to the Octet® SF3. The bevacizumab biosimilar was prepared to a final top concentration of 100 nM in HBS-EP+ running buffer and a 6-fold concentration series prepared using a 1:3 dilution into HBS-EP+. Samples were placed into Octet® SPR 0.9 mL vials and placed into a mixed format sample rack. 3% sucrose was prepared using HBS-EP+ as the bulk reference standard for OneStep® injections and 100 mM HCl was used for regeneration injections. The sample rack was sealed using resealable septa and placed in the sample tray set to 15 °C.

The Octet® SF3 system was primed 3 times into HBS-EP+ running buffer and the sensor chip hydrated and conditioned using injections of HBS-EP+ and 100 mM HCl. A short and long assay format was used (Katsamba 2006) with a common association time of 180 sec at 50 µL/min used for multi-cycle kinetics and a 'long' dissociation of 3600 sec used for the highest concentration and a 'short' dissociation of 420 sec used for all other concentrations. Association parameters for OneStep® are fixed based upon the volume of the injection loop used, which was set to 100% here and the same dissociation parameters used as for multi-cycle kinetics. A buffer blank injection was performed for each analyte concentration in order to generate accurate double referenced data.

The bevacizumab VEGF complex was regenerated with two injections of 100 mM HCl at 50 µL/min for 30 sec, followed by a 180 sec stabilization period.

Data were analyzed using the standard short and long method of determining the dissociation rate constant (kd) for the highest concentration (3600 sec dissociation) and constraining the lower concentrations (420 sec dissociation) to the same value. All data was globally fitted to a simple 1:1 interaction model.

Results and Discussion

The objective was to demonstrate that the kinetic and affinity constants determined using OneStep® injections compared favorably to those derived from standard multi-cycle kinetic assays, which require multiple analyte concentration injections. In addition, as the affinity of the drug – therapeutic is often unknown, the flexibility in determining the initial analyte concentration choice is considered.

Kinetic analysis trastuzumab binding HER2

High affinity kinetic interactions are often poorly defined due to the lack of curvature in the association phase and may require in excess of an hour to approach equilibrium. Therefore, the resolving power of a single analyte injection must not be at the expense of resolution or throughput.

Initial analysis of the trastuzumab HER2 kinetics and affinity determined by multi-cycle kinetics on the Octet® SF3 are shown in Table 1 and the associated sensorgram shown in figure 1.

Table 1

	ka ($M^{-1}s^{-1}$)	kd (s^{-1})	KD (M)
Trastuzumab	$1.30*10^{6}$	$1.00*10^{-5}$	$7.38*10^{-12}$

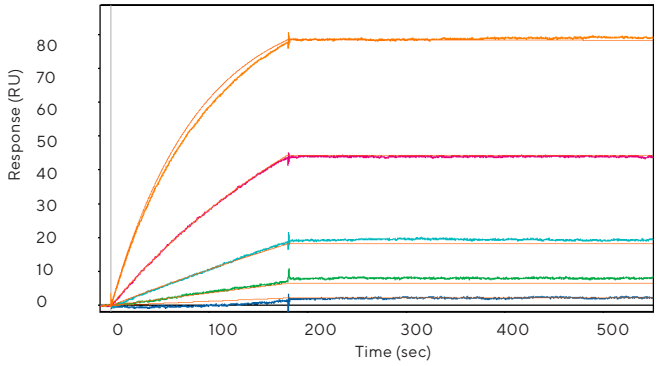


Figure 1

A single injection of each analyte concentration was then injected across the same surface using OneStep® injections. 0.3 nM was rejected as the dissociation phase was not well described by the observed kd at higher concentrations in both multi-cycle kinetics and OneStep® injection. It is important to note that multiple OneStep® injections are not required in normal screening and the rationale of performing multiple injections here was to determine the tolerance levels between standard multi-cycle kinetics and OneStep® injections. Kinetics parameters for local fits of each concentration are shown in Table 2 with corresponding sensorgrams in figure 2a – d (note: the full 3600 sec dissociation phase is shown for 25 nM).

Table 2

	Concentration (nM)	ka ($M^{-1}s^{-1}$)	kd (s^{-1})	KD (M)
Trastuzumab	25	$1.06*10^{6}$	$1.00*10^{-5}$	$9.85*10^{-12}$
	8.33	$1.18*10^{6}$	$1.00*10^{-5}$	$8.49*10^{-12}$
	2.78	$1.45*10^{6}$	$1.00*10^{-5}$	$11.73*10^{-12}$
	0.926	$1.16*10^{6}$	$1.00*10^{-5}$	$8.60*10^{-12}$

Figure 2a. *25 nM*

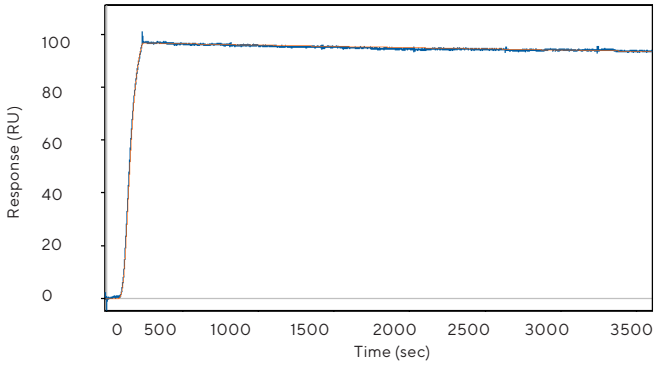


Figure 2b. *8.33 nM*

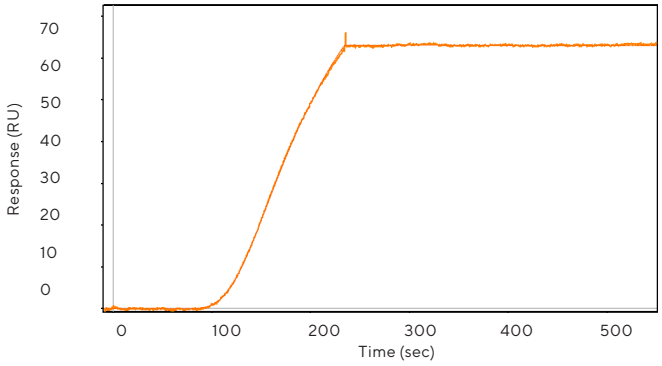


Figure 2c. *2.78 nM*

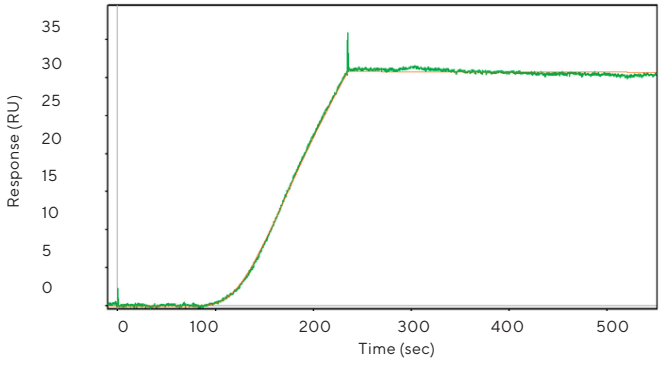
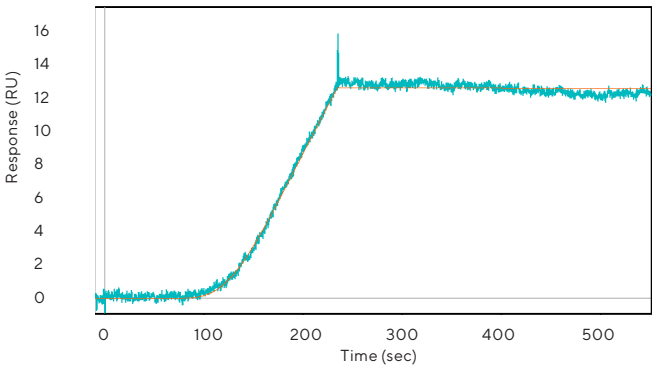


Figure 2d. *0.926 nM*



It is important to define what an acceptable level of precision is for defining kinetic parameters prior to comparing data.

Four landmark studies have reported benchmarks for variability in the measurements of acceptable association and dissociation rate constants based on interactions characterized by multiple independent laboratories (Cannon 2004, Katsamba 2006, Papalia 2006, Navratilova 2007). The variability between 59 independent measurements was approximately 20% for both the association and dissociation rate constants. Therefore, kinetic constants that deviate by less than 20% are considered acceptable in this assessment of OneStep® vs multi-cycle kinetics.

As shown in Table 3, all OneStep® injections of trastuzumab show acceptable association kinetic values compared to the association kinetic rate constant determined by multi-cycle kinetics (Table 1). Therefore, even with high affinity interactions a single OneStep® injection is sufficient to determine accurate association kinetics compared to multi-cycle kinetics.

Table 3

	Concentration (nM)	ka ($M^{-1}s^{-1}$)	Acceptable association (k_a) range ($M^{-1}s^{-1}$)
Trastuzumab	25	$1.06*10^6$	$1.04*10^6$ - $1.56*10^6$
	8.33	$1.18*10^6$	
	2.78	$1.45*10^6$	
	0.926	$1.16*10^6$	

Kinetic analysis bevacizumab binding VEGF 165 and VEGF 121a

Unlocking the potential of high throughput screening involves generating accurate data quickly that allows you to progress potential candidates quickly through downstream stages. As shown for trastuzumab binding to HER2, OneStep® injections are able to produce accurate association kinetics and a single concentration injection that accurately resolves association and dissociation kinetics is highly desirable in terms of saving precious sample, time and importantly, how many samples can be assessed in a single run.

VEGF 165 and VEGF 121a were immobilized as described in the methods section and its binding to a bevacizumab biosimilar assessed using standard multi-cycle kinetics and a single top concentration OneStep® injection.

Initial analysis of the bevacizumab biosimilar VEGF 165 and VEGF 121a kinetics and affinity determined by multi-cycle kinetics are shown in Table 4 and the associated sensorgram shown in figures 3a (VEGF 165) and b (VEGF 121a).

Table 4

	ka ($M^{-1}s^{-1}$)	kd (s^{-1})	KD (M)
VEGF 165	$8.22*10^4$	$6.12*10^{-5}$	$744.4*10^{-12}$
VEGF 121a	$9.40*10^4$	$3.93*10^{-5}$	$417.9*10^{-12}$

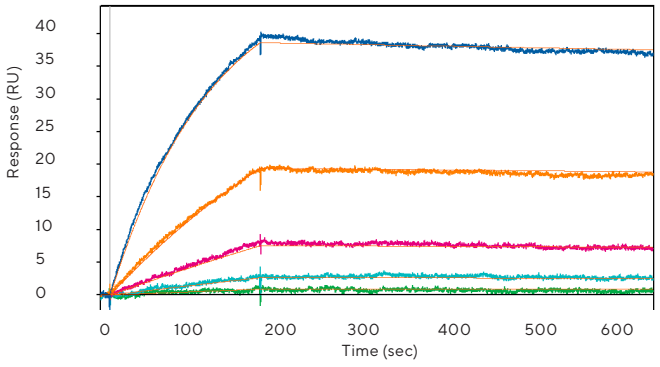
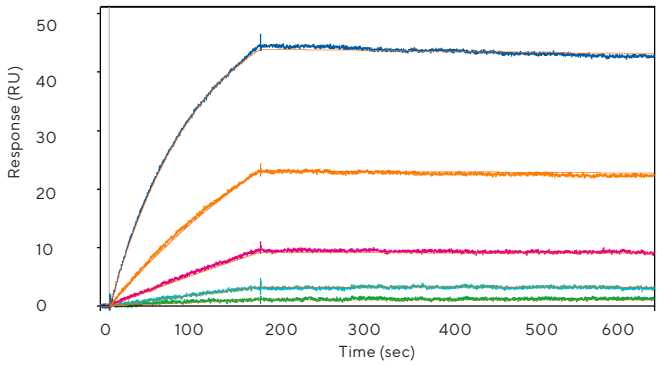


Figure 3a

Figure 3b



A single injection of the top concentration assessed in multi-cycle kinetics (100 nM) was then injected across the same surfaces using OneStep® injections. Kinetics parameters for local fits of each concentration are shown in Table 5 and the corresponding sensorgrams in figure 4a (VEGF 165) and 4b (VEGF 121a).

Table 5

	ka ($M^{-1}s^{-1}$)	kd (s^{-1})	KD (M)
VEGF 165	$8.20*10^4$	$6.12*10^{-5}$	$746.2*10^{-12}$
VEGF 121a	$1.05*10^4$	$3.93*10^{-5}$	$371.14*10^{-12}$

Figure 4a

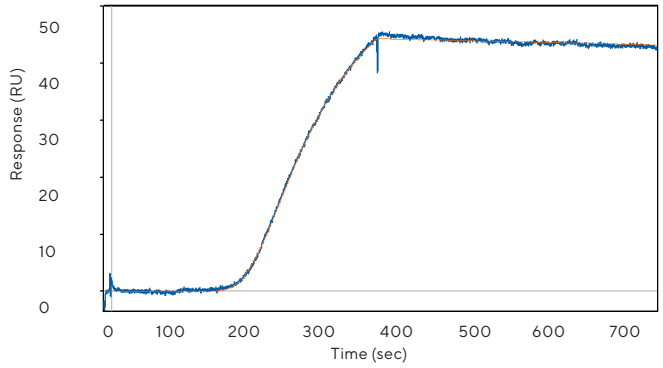
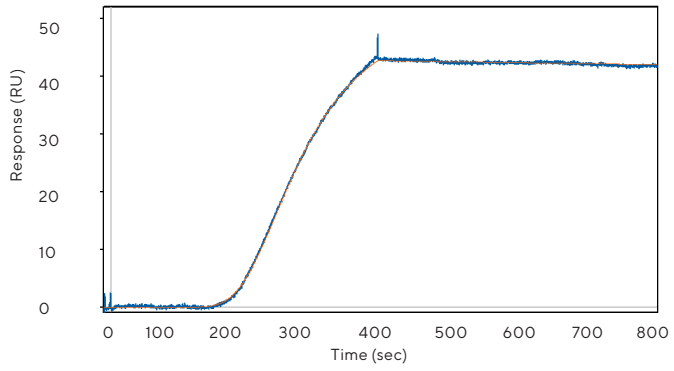


Figure 4b



Applying the same accuracy measure for the association kinetic parameters as discussed for trastuzumab binding to HER2, the association kinetics determined from a single OneStep® injection are within the acceptable range of accuracy for the interaction compared to multi-cycle kinetics.

Table 6

	ka ($M^{-1}s^{-1}$)	Acceptable association (ka) range ($M^{-1}s^{-1}$)
VEGF 165	$8.20*10^4$	$6.58*10^4$ - $9.87*10^4$
VEGF 121a	$1.05*10^4$	$7.52*10^4$ - $1.13*10^5$

Previous attempts at determining the minimum data set required for multi-cycle kinetics have shown that in a confidently measurable region, significant curvature is required (Onell and Andersson 2005) to determine accurate kinetics, and even then, a screening process is required before hand to determine the necessary concentrations. As shown here for trastuzumab binding to HER2 and a bevacizumab biosimilar binding to VEGF 165 and VEGF 121a and also exemplified in (Quinn 2012), OneStep® injections provide significant curvature during the single injection thanks to the analyte gradient formation, which allows accurate association kinetics to be determined for even high-affinity interactions. As shown here, a single top/high concentration matching that used for multi-cycle kinetics is sufficient to derive accurate kinetics and affinity. Importantly, as shown for trastuzumab binding to HER2, even if the affinity of the interaction is unknown, a wide range of analyte concentrations can be used to determine association and dissociation kinetics, these concentrations are typically linear in multi-cycle kinetics and therefore, yield no usable kinetic information.

In addition to a significant sample saving of ~50% due to the lack of necessity for serial dilutions, OneStep® injections offer significant time savings. As a fair comparison, multi-cycle kinetics assays here were performed using short and long dissociation times which collect dissociation information for the highest analyte concentration which is then combined with short dissociation times for lower concentrations. This method works because the reduction in the observed signal at the highest analyte concentration is sufficient (>5%) to establish the dissociation rate constant (kd) for the entire assay as the dissociation phase is concentration independent. This shortens the time required for a multi-cycle assay significantly. The total time taken for the multi-cycle kinetics assay was approximately 4 hours and 30 min and 2 hours and 50 min for the OneStep®. This represents a significant saving it time of over 90 mins for a single analyte, which would only be compounded at a higher number of samples.

As shown above, kinetics and affinity determined by OneStep® injections compared favorably to those derived from standard multi-cycle kinetic assays. OneStep® injections allow flexibility in assay design as accurate association and dissociation kinetics can be generated from a single top concentration injection without the need to determine an optimum concentration series of analyte, as would be required for multi-cycle kinetics.

OneStep® injections offer additional benefits when assessing binding interactions thanks to the necessity of only a single injection. Surface regeneration is an integral part in assessing high affinity interactions and unlike standard multi-cycle kinetic assays, where several concentrations require an equal number of regenerations, OneStep® injections only require a single regeneration step, which over the course of a high throughput screen is critical to achieving stable baselines regardless of whether the ligand is directly immobilized to the sensor chip surface or a capture molecule is being used.

OneStep® injections remove the guess work from high throughput screens and allows a full kinetics panel to be determined in a fraction of the time compared to standard techniques that require initial screening and then potentially extensive assay development. Full kinetic information early on in the drug development process allows rapid progress of potential therapeutics through the development pipeline and forms a solid ground for any additional assay development.

Conclusions

In conclusion, this application note shows that current common practices for high throughput screening using SPR, which rely on a limited data set of 'yes/no' binding and subsequent calculation of kinetic constants for those molecules deemed hits involves an unnecessarily large amount of sample and lab time. OneStep® injections are shown to generate accurate association kinetics for even high affinity interactions and offer a rational and cost-effective method to determine kinetics much earlier in the drug discovery process.

A neglected source of error in SPR assays is the nature of the sample preparation (Karlsson and Larsson, 2004). Pipetting errors and evaporation can lead to erroneous estimates of the concentration of the binding partner in solution, which affects the accuracy of ka, and subsequently the accuracy of KD. OneStep® injections reduce the risk of this error due to the necessity of preparing only a single analyte concentration with no requirement to prepare a concentration series, meaning fewer errors in sample preparation but also in that the decreased time to generate accurate kinetics means that samples are less prone to experience evaporation or any side-effects from sitting in a sample plate. As shown here, lower analyte concentrations for OneStep® are still capable of generating accurate association kinetics and as such the advantage of these lower concentrations is that adverse effects such as aggregation or solubility issues are minimized.

Therefore, the barrier to the tradeoff between throughput and precision has been removed by OneStep® injections and reliable kinetic estimates can be obtained much earlier in drug discovery with much less data than is normally required; increasing sample throughput and saving sample material, particularly for protein-based therapeutics. Thanks to the flexible sample format of the Octet® SF3 up to 768 unique analytes can be assessed in a single run. Combined with the ability to measure dissociation rates for up to 12 hours, full kinetics for even the highest affinity interactions and high throughput screens are now within easy reach for all SPR users.

References

1. Cannon MJ et al. Anal. Biochem. 2004 1;330(1):98-113
2. Karlsson R and Larsson A. Methods Mol Biol. 2004;248:389-415
3. Katsamba P et al. Anal. Biochem. 2006 15;352(2):208-221
4. Navratilova I et al. Anal. Biochem. 2007 1;364(1):67-77
5. Papalia GA et al. Anal. Biochem. 2006 1;359(1):94-105
6. Onell A and Andersson K. J. Mol Recognit. 2005;18(4):307-17
7. Quinn J. Anal Biochem. 2012 15

Germany
Sartorius Lab Instruments GmbH & Co. KG
Otto-Brenner-Strasse 20
37079 Goettingen
Phone +49 551 308 0

USA
Sartorius Corporation
565 Johnson Avenue
Bohemia, NY 11716
Phone +1 631 254 4249
Toll-free +1 800 635 2906

For further contacts, visit
www.sartorius.com

Publication No.: Octet-SF3-High-Affinity-Interactions-Application-Note-en-L-RevA-4077
Status: 03 | 2022