Guidelines for OneStep® Assay Design

For Octet® SF3 SPR System

Technical Note

Scope

This technical note shows how the Octet® SF3 system and Octet® SPR Discovery software with OneStep® Injections allows users to determine the kinetics and affinity for a wide range of drug sizes and affinities from a single analyte concentration.

Abstract

The design and development of novel biologics requires many developability characteristics to be considered, including the affinity of the drug for its intended target. One of the fastest growing classes of biologics is therapeutic monoclonal antibodies (mAbs) that can be rapidly developed to bind the intended target with high affinity. High affinity interactions play a key factor in the overall potency of the drug and are characterized by a highly stable antibody-antigen complex, which is due to a very low dissociation rate constant (k_d).

Analysis of high affinity biologics is important for lead selection and predicting the efficacy of protein therapeutics and therefore, with the ability to measure dissociation periods for up to 12 hours, the Octet® SF3 allows users to accurately assess high affinity protein therapeutics from the earliest lead selection stages onwards without the need for orthogonal assays.

Find out more: www.sartorius.com

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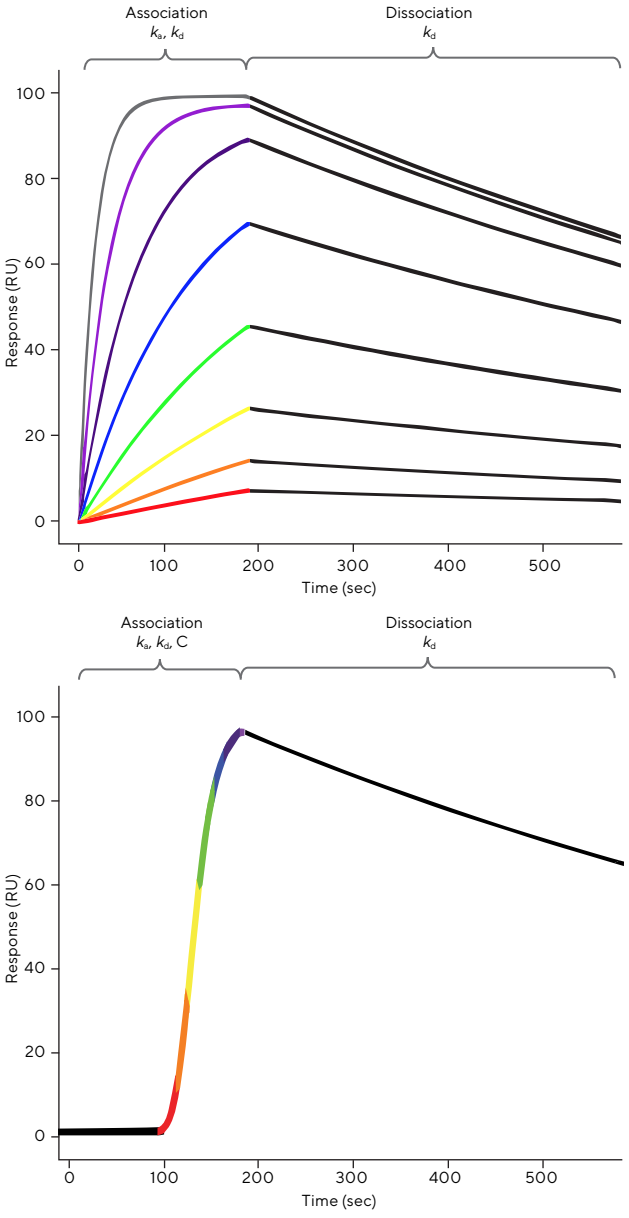
OneStep® Overview

The Octet® SF3 SPR system by Sartorius, employs an innovative gradient injection technology based on Taylor dispersion called OneStep®. A OneStep® injection produces a sigmoidal concentration gradient of analyte from a single sample vial by dispersing the sample through a buffer-filled capillary line that flows into the SPR flow cell. From this single injection, kinetic analysis can be performed to accurately determine the kinetic rate constants (k_a and k_d) and affinity (K_D) of the interaction between an analyte in solution and an immobilized ligand on the sensor chip surface. In contrast, traditional, multi-cycle kinetics (MCK) SPR methods require a dilution series of analyte that typically spans two orders of magnitude from high to low concentration. A comparison of the traditional and OneStep® methods is shown in Figure 1.

Traditional MCK kinetic analysis to determine the association and dissociation rate constants (k_a and k_d, respectively) of binding requires global fitting of a kinetic model to binding responses measured at multiple analyte concentrations. Best practices suggest that six to eight samples diluted in two- or three-fold concentration steps is sufficient for most kinetic assays. The simulated binding curves in Figure 1 (top panel) show eight concentrations in two-fold dilutions. The samples are each injected for a fixed period of time (association) to bind immobilized ligand and each injection is followed with a period of running buffer flow where analyte concentration is zero (dissociation). In practice, the pseudo first-order reversible binding model is fit to the binding curves using non-linear least squares regression to determine best-fit values for k_a, k_d, and R_{max}. The analyte concentration for each injection is input as a constant assigned to its respective binding curve.

Figure 1

Analysis Components of a MCK SPR Assay (Top) and a OneStep® Assay (Bottom).



Note. Color coding illustrates a MCK range of discrete concentrations tested in the assay (top) while a similar concentration range is continuously tested in the OneStep® assay (bottom). Analyte concentration is zero in the dissociation phase for both MCK and OneStep® assays to independently measure the dissociation rate constant, k_d.

With OneStep® next generation kinetic analysis, the analyte concentration is dynamic versus time. The concentration function (Taylor dispersion equation) is therefore incorporated into the numerical integration of the binding model to determine the best-fit values for k_a, k_d, and R_{max} from a single OneStep® injection. Global kinetic analysis of traditional kinetic data combines the observed binding and dissociation at six to eight analyte concentrations to unambiguously determine the kinetic rate constants. OneStep® measures changes in binding response over a wide range of concentrations in one injection, therefore giving comparable certainty in the kinetic rate constants (Figure 1, bottom panel).

OneStep® technology was first described in 2012[1,2], where it was referred to as a "sigmoidal Taylor Dispersion injection". It is recommended that expert OneStep® users be familiar with these references for a better understanding of the mathematical principles of Taylor Dispersion and the analysis of kinetics from a OneStep® gradient injection.

The OneStep® assay method shares some similar considerations with multi-cycle kinetics SPR assay methods in analyte top concentration, assay orientation, and ligand immobilization level. Generally, the top concentration required for a OneStep® assay is no different than assays performed using a conventional, multi-cycle kinetics dilution series. In both cases, the top concentration is recommended to be 10-fold of the equilibrium dissociation constant, K_D. Similar basic assay design parameters, such as assay orientation and ligand immobilization level, may be followed when comparing OneStep® methods to multi-cycle kinetics. For example, there are no unique recommendations on immobilization method when using OneStep® compared to conventional characterization methods. It is recommended that both OneStep® and conventional kinetics assays have an R_{max} no greater than 50 RU for best kinetic results and for best chances of avoiding surface avidity issues.

Table 1
Kinetic Terms Glossary.

Term	Definition
Analyte	The binding partner in solution
Ligand	The binding partner immobilized to the sensor chip
k_a	Association rate constant (units in $M^{-1}s^{-1}$)
k_d	Dissociation rate constant (units in s^{-1})
R_{max}	Maximum binding response at surface saturation (units in RU)

This technical note describes the best practices for designing assays with OneStep® when migrating from traditional, multi-cycle kinetics SPR assays. Differences in method design are most notable when the user considers injection contact time, flow rate and dissociation time. Common OneStep® applications are defined and the method design recommendation is provided and explained in detail in this document.

Key Components of OneStep® Gradients

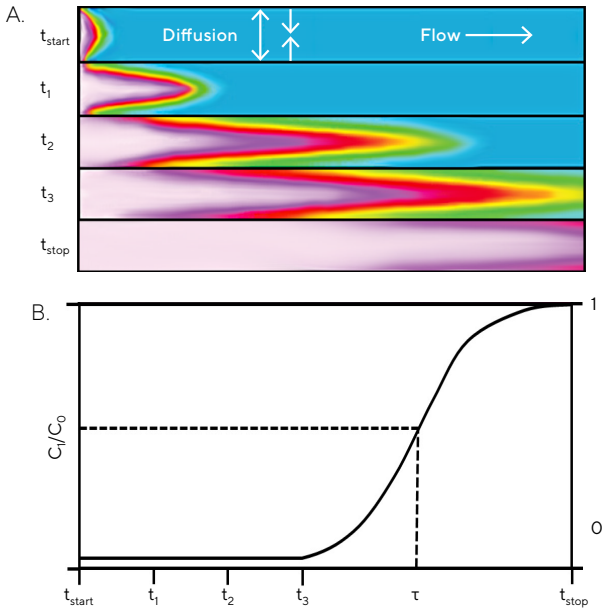
A basic understanding of how OneStep® gradients are produced and flowed over the sensor chip surface is essential for designing effective assays for kinetics and screening. OneStep® is a gradient injection where analyte sample is dispersed through a capillary line filled with buffer *en route* to the SPR flow cell where the ligand is immobilized. Therefore, concentration of analyte at the sensor chip surface varies gradually and continuously from low (three to four orders of magnitude below the full sample concentration) to high (full sample concentration) as the sample gradient is flowed over the sensor chip. The OneStep® injection process, based on Taylor dispersion, produces a sigmoidal concentration gradient of analyte that is mathematically described as a function of time, capillary geometry, flow velocity, and analyte diffusion coefficient. Figure 2 shows an illustration of the OneStep® capillary line at key time points and the analyte concentration observed at the SPR flow cell versus time.

A simple way to understand the OneStep® injection process is to imagine that a fixed volume of buffer is between the sample and and the sensor chip and the sample is injected interfacing with the buffer, displacing it past the biosensor as the sample ultimately reaches the flow cell. The interface between sample and buffer disperses and spreads creating the concentration gradient of analyte, as shown in Figure 2.

The analyte diffusion coefficient affects the shape of the concentration profile such that different diffusing analytes have different sigmoidal slopes but they converge at the time to reach 50% of the assessed analyte concentration, which is referred to as tau (τ). The Octet® SF3 Analysis Software accounts for this by using the analyte's molecular weight (MW) to estimate the analyte diffusion coefficient. As an internal control for the gradient dispersion process, an injection of 3% sucrose prepared in the same buffer as used for the analyte and running buffer is performed at least once in each OneStep® assay to ensure proper gradient formation is occurring and to calibrate for buffer

Figure 2

OneStep® Gradient Injections.



Note. (A) OneStep® gradient formation in the injection line, with (B) the corresponding analyte concentration measured within the flow cell. Blue indicates the running buffer and pink indicates the analyte. 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 injection line at different times, and shows that a single injection can be used to assess a full analyte concentration series.

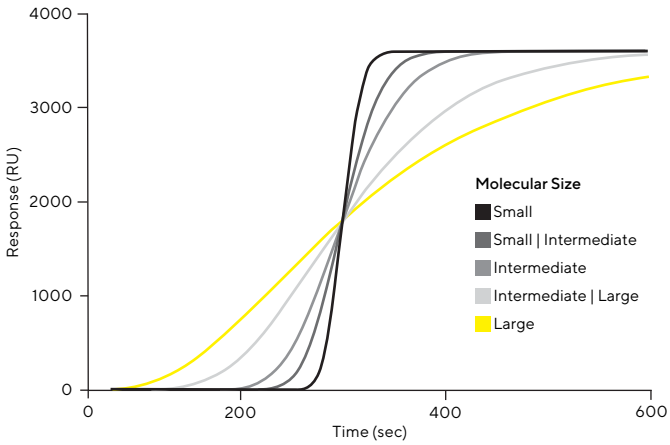
viscosity. No special OneStep® assay or method design considerations apply regarding analyte MW or diffusion coefficients. The OneStep® concentration vs. time slope will become more gradual as molecular weight increases, and for small molecules the concentration slope is steeper. Figure 3 shows the concentration response vs. time relationship for various diffusion coefficients (Figure 3). The majority of analytes (small to large molecules) will have diffusion coefficients between 10^{-9}–10^{-10} m^2/s.

Therefore, the key components specific to OneStep® assay design are injection volume and flow rate, which will be discussed in sections below.

Each OneStep® injection is equal to a fixed volume of buffer (100 µL) plus the analyte sample volume. The analyte sample volume can be 50–200% of the 100 µL capillary loop. The percentage (%) in this case is equivalent to µL since the capillary loop volume for Octet® SF3 SPR systems is 100 µL. The difference in gradient shape when using the 50, 75, 100, 150, or 200% setting is negligible and the only difference is observed by the contact time at full concentration, as shown in Figure 4. Injection volume is therefore a setting that can be selected to suit the

Figure 3

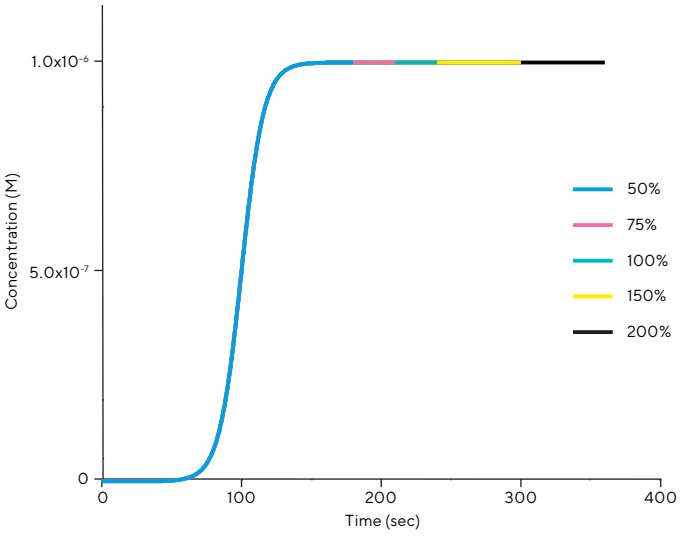
OneStep® Gradient Injection of Different Analytes with Varying Molecular Weights.



Note. The black line corresponds to the smallest molecular weight molecule, while the yellow line highlights molecules with higher molecular weights or greater sample aggregation. The diffusion coefficient of analyte causes the concentration to rotate around a center inflection point, tau (τ).

Figure 4

Illustration of OneStep® Injections at Each Sample Volume Setting.



Note. Sample full concentration was 1 µM at a nominal flow rate of 50 µL/min.

OneStep® assay requirements. The expected dissociation rate (k_d) is commonly used as a constant when deciding which injection volume to select. Assuming a constant association rate (k_a), a molecule with a fast dissociation rate takes less time to reach equilibrium than a molecule with a slow dissociation rate. Therefore, less time at full concentration is required to reach equilibrium and the

Table 2

Effect of Flow Rate on OneStep® Injection Time (Sum of Base Injection Time and Time at Full Concentration).

			50% of Loop		75% of Loop		100% of Loop		150% of Loop		200% of Loop
Flow Rate (µL/min)	Base Injection Time (sec)		Time at Full Conc. (sec)		Time at Full Conc. (sec)		Time at Full Conc. (sec)		Time at full Conc. (sec)		Time at full Conc. (sec)
10	600	+	300	or	450	or	600	or	900	or	1200
25	240	+	120	or	180	or	240	or	360	or	480
30	200	+	100	or	150	or	200	or	300	or	400
50	120	+	60	or	90	or	120	or	180	or	240
75	80	+	40	or	60	or	80	or	120	or	160
100	60	+	30	or	45	or	60	or	90	or	120
125	48	+	24	or	36	or	48	or	72	or	96
150	40	+	20	or	30	or	40	or	60	or	80
175	34	+	17	or	26	or	34	or	51	or	68
200	30	+	15	or	23	or	30	or	45	or	60

50% loop volume setting can be used. In contrast, samples with slower dissociation rates need more time to reach equilibrium and therefore 75% (for samples that require minutes to dissociate) or 100% (for samples that require hours to dissociate) loop volume settings can be used.

Flow rate is an important parameter when considering assay design using OneStep®, as it directly alters the length of injection and the time at full concentration. Slower flow rates (5–10 µL/min) will require longer to inject the OneStep® gradient than faster flow rates (100–200 µL/min). Table 2 shows common flow rates and the respective total injection time (time from injection start to injection stop) and the approximate contact time at full concentration.

Table 2 can be a guide for matching similar analyte contact times between new OneStep® methods and traditional SPR methods to be converted.

- The longest analyte contact times will be possible using the larger volume setting (>100%) and slower flow rates.
- The shortest analyte contact times will be possible using the smaller volume setting (<100%) and faster flow rates.

As with conventional, label-free kinetic experiments, flow rate is a variable affecting the mass transport of analyte from bulk (in the flow cell) to the sensor chip surface where it can interact with the immobilized or captured ligand. Faster flow rates will result in a marginally faster mass transport coefficient (k_m), and this should be balanced with desired contact time (time at full concentration). Interactions with faster association rate constants (k_a of >10^6 $M^{-1}s^{-1}$) are more commonly at risk for mass transport limitation and therefore should be tested at higher injection flow rates (>50 µL/min).

Before Getting Started: Ligand Immobilization

The following sections describe the assay design recommendations for testing various types of analytes binding ligand using OneStep®. Before analytes may be tested, the ligand must be immobilized on the sensor chip surface such that it is active and stable for the duration of the planned experiment. Immobilization methods will vary depending on the ligand's stability and its available form. Since SPR is a quantitative, optical measurement, Equation 1 can be used to guide the immobilization step in assay development to give an appropriate analyte binding R_{max} (see Table 1). Appropriate analyte R_{max} will also vary depending on the application, and these are described in the following sections for each application type.

$$R_{max}\,(RU) = \frac{MW_{analyte}}{MW_{ligand}} \times R_{ligand} \times Stoichiometry_{ligand}$$

Equation 1: R_{max} calculation where R_{ligand} is quantity of immobilized ligand in SPR response (RU), $MW_{analyte}$ is molecular weight of Analyte and MW_{ligand} is molecular weight of ligand and $Stoichiometry_{ligand}$ is the anticipated stoichiometry of the interaction between the analyte and ligand. MW values are in common units (Da, kDa, etc.).

Assay development for SPR involves demonstrating immobilization of ligand yielding a surface that is active, reproducible and stable. If ligand regeneration is required,

conditions to regenerate the analyte-ligand complex will be tested and stability after multiple binding and regeneration rounds will be demonstrated in the development phase. In some cases, an affinity capture method will be utilized such that the ligand is reversibly captured to some component of the biosensor chip (e.g. Protein G, Ni-NTA, etc.) and regeneration removes both analyte and ligand, requiring fresh capture of ligand in every assay cycle. For more information on assay design and optimization, see the following guides:

- Octet® Applications Guide 'A Compendium for Successful BLI and SPR Assays'
- Octet® SPR Chemistry User Guide

Octet® SF3 Discovery Software Method Design Guidelines

The Octet® SF3 Discovery Software Method Setup tab is flexible in sample placement such that samples, controls, buffer blanks, etc. can be placed in any vial or plate well location. Therefore, after the position and type of sample rack being used has been configured, sample placement should be considered arbitrary and open to the user's preference. The identification of sample location is performed at the Initial (rack) Setup screen, where sample locations can be dragged as individuals or in groups to the Sample Table. Sample Identity, Concentration, Volume and Molecular Weight can also be entered as individuals or in groups, and standard Windows® copy/paste commands can be applied. Figure 5 shows the Initial Setup interface where samples and reagents are identified for the automated method.

Sample location can be changed in the Initial Setup screen by dragging individual wells or vials from the original location to the new location. If a method has been designed, the new location will be updated in subsequent method steps. If individual sample locations need to be removed, click the small grey box to the left of the sample row and press the **Delete** key. The Import dialog (button at bottom of Figure 5) can be used to easily copy and paste sample information from plate maps, Microsoft® Excel spreadsheets, etc.

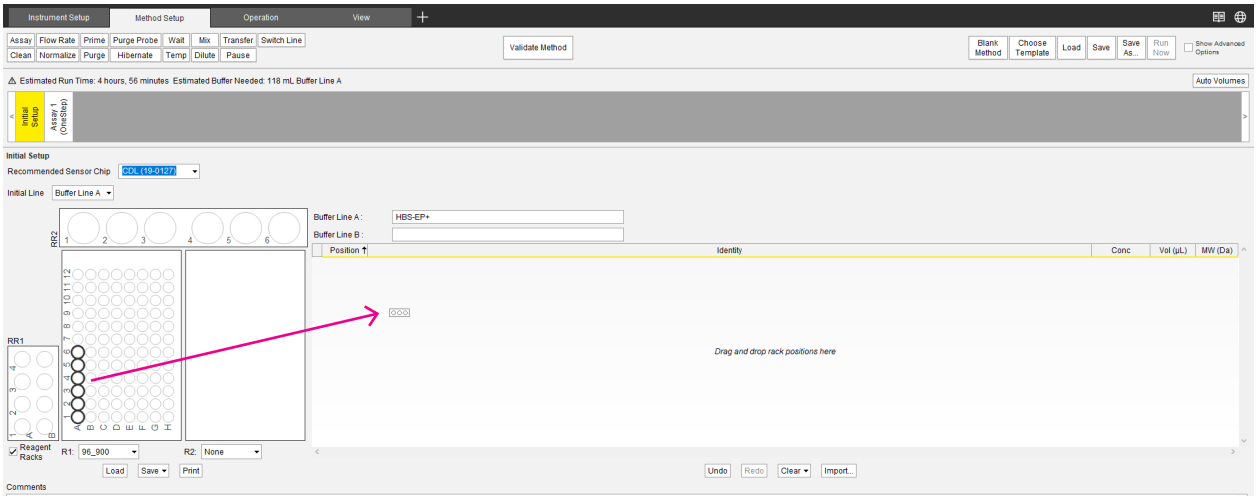
Once samples are defined in the initial rack setup, it is recommended to begin any kinetic or screening assay with a prime function to equilibrate the system fluidics in fresh buffer. Drag the Prime function button into the method timeline to the right of the Initial Rack Setup. Choose a 3X Prime for standard methods.

The Octet® SPR Discovery Software has the feature "Auto Volumes", which is located at the top right side of the Method Setup page. The Auto Volumes feature allows the user to construct the method as detailed above without having to input sample volumes at the start of method writing. As the required volumes are dependent upon several factors such as flow rate, number of replicates and association/dissociation time, the final volumes may not be known at the start.

To use the Auto Volumes feature, the user can simply click the button and the software will calculate the minimum volumes required, including the dead volumes of the vials or plates used.

Figure 5

Initial Rack Setup Showing the Drag-Drop of Six Sample Vials into the Sample Table.



It is a good practice to use the Validate Method tool after writing a new method file (Figure 6). This tool will check that settings in the method are within the proper ranges for the system. Invalid entries are flagged for correction. Validation will also simulate volume dispensing and consumption from the sample racks, and present warnings for any rack positions that may become empty during the method. The volume tracking is a convenience function. Volume tracking errors will not prevent a method from being executed on the system. Also, validation is performed automatically when a method is loaded for execution on the Operation page. Only errors due to invalid parameters are flagged at that point.

The Method Validation report (example in Figure 7) will indicate general errors, volume tracking errors, and additional warnings. The Num column indicates where the error was in the method timeline. The first number represents the function order and the second number indicates the sub-step of that function order. For example, the initial rack setup is the first function in any method and is therefore listed as "1.0", the next function in the method would be listed as "2.0", and, and, if it is an assay, its sub-steps (purge, flow rate, and inject) would be 2.1, 2.2, and 2.3, respectively. The Name column of the erroneous step further identifies the location of the error in the method. Finally, the error message reports the source and nature of the issue.

Common sources of issues when preparing methods include missing information or incompatible settings and these must be corrected for a method to be launched. Volume tracking errors shows cases where sample consumption is expected to exceed the available sample volume, potentially causing injection air spikes or otherwise poor injection performance. A method may be launched with a volume tracking error but it is not advisable for best assay performance. Additional warnings indicate best practices recommendations that can be heeded or ignored per user preference. Examples include buffer blank dissociation time that does not match the longest analyte dissociation time or setting an Analyte injection without a reference channel. Additional warnings are intended to prevent minor errors in method design and can be ignored if not applicable.

Figure 6
Validate Method Button.

Figure 7
Method Validation Error Report

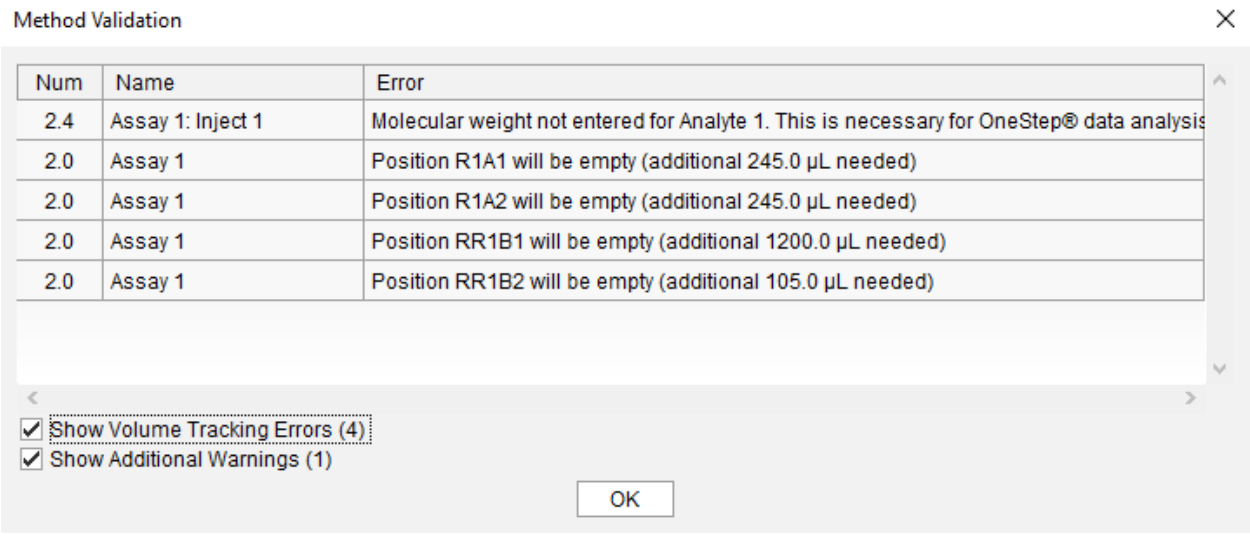


Figure 8
Method Validation Error Report – No Errors Detected.

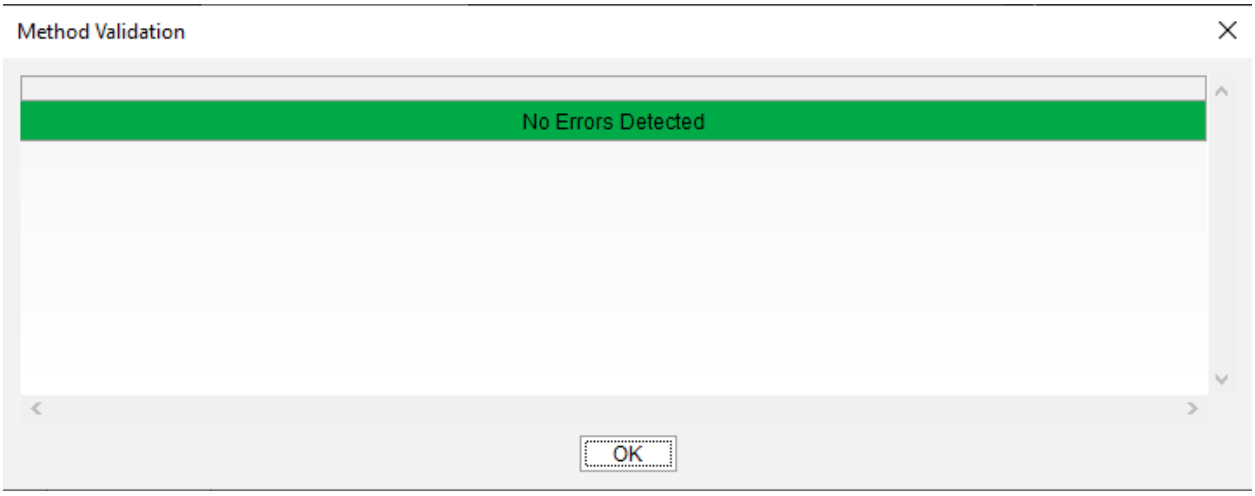


Figure 8 shows a Method Validation Error Report example where no errors were detected. It also shows the estimated run time of the entire method and the estimated buffer required to complete the method.

Small Molecule & Fragment Screening

The recommendations in this section can be applied to small molecule screening assays such as fragment screening or other weak affinity library screening. Due to their weak affinity, fragments that bind immobilized ligand normally do not require surface regeneration and dissociate completely within 10 seconds. Therefore, fragments can be quickly injected, one after another, and the OneStep® responses analyzed for hit selection and ranked by kinetics and affinity.

The ligand of interest and any control ligands must be immobilized prior to beginning the screen. See the Before Getting Started section for more information on assay development. For small molecule screening, high immobilization densities (~8,000 RU) of most target molecules are common for obtaining measurable signals of the small MW analytes. For best results, the expected R_{max} of the smallest MW analyte should be 10 RU or greater to allow for some inactive ligand or activity decay.

From the Method Setup tab, double click on the **OneStep Small Molecule Screen** pre-written method and drag the Prime function to after the Initial Setup (see Octet® SF3 Discovery Software Method Design Guidelines). Use the following method template and settings to design the screening method.

Assay Template: OneStep® Screening

Fragment screening assay recommendations

- Assay Settings:
  - Cycle Order: Sequential
  - Sampling Rate: 20 Hz
  - Replicates: 1
  - Blank Cycles: Every 12–24 cycles
  - Startup: 5–10 cycles
  - Positive Control Cycles: Periodic: Every 12–24 cycles; Leading Samples
  - Negative Control Cycles: Periodic: Every 12–24 cycles; Leading Samples
  - Bulk Std. Cycles: Fixed Quantity: Exactly 2 cycles
  - Micro-calibration: High+Low
  - Micro-calibration Number of Cycles: 3–4; Run After Startup Blanks
  - Wash Cycles: Every 12–24 cycles; clean parameter 'The analyte injection, but the cleaning solution does not enter the flow cell'
- Assay Cycle Settings:
  - Analyte Inject Flow Rate: 150 µL/min
  - Dissociate: 30 sec
  - Analyte Injection Type: OneStep®
  - OneStep® Sample Volume: 50% of Loop
  - High-throughput mode

Click the **Assay function** in the method timeline to set the OneStep® screening assay settings (Figure 9). The General tab is used to give the assay a name, set the cycle run order, reference channel, number of replicates and the data sampling rate. If there is no bias in the order of sample plating, set the **Cycle Order** to **Sequential**. If plating bias is a concern, select **Random**. The weak affinity of fragments to their targets normally consists of a very fast dissociation rate constant (k_d) resulting in rapid equilibrium binding for most hit compounds. A fast sampling rate (20 Hz) is therefore beneficial in accurately characterizing the fast dissociation of hit compound from target biomolecule.

The large numbers of compounds tested in a fragment screen warrant a brief assay cycle which can be achieved by selecting the high-throughput mode option to enable the next sample to load during the previous sample injection (Figure 9). A single replicate per analyte is common for most fragment screens to keep the assay times practical. Subsequent tests will confirm activity of fragment hits from the primary screen.

The next tab, Periodic Cycles, sets buffer blanks, positive (and negative) controls, and bulk standard (Sucrose) injections as shown in Figure 10. Buffer blank cycles should be performed at least every 24 cycles for longer screens (two 384-well plates) and every 12 cycles for shorter screens. Blank cycles can be set to a fixed quantity or periodically. The fixed quantity setting is more often used for shorter assays where the total number of blanks is practical to consider. The periodic setting is used for longer assays like fragment screens where the number of cycles between each blank is more practical to consider. Startup cycles are additional blank cycles which are performed at the beginning of the assay to stabilize the blank response into a reproducible rhythm. Five to ten startup cycles are appropriate for most fragment screening assays, but more can be configured if biosensor stability is a concern.

Positive and negative controls should be performed every 12–24 cycles and a minimum of 5 positive control cycles should be performed for each screening assay to enable the hit selection feature. Positive and negative controls, like controls can be set to fixed quantity or periodic.

Figure 9
General Assay settings – Fragment Screening. Highlighted Areas Show the High-Throughput Mode Checkbox and General Tab.

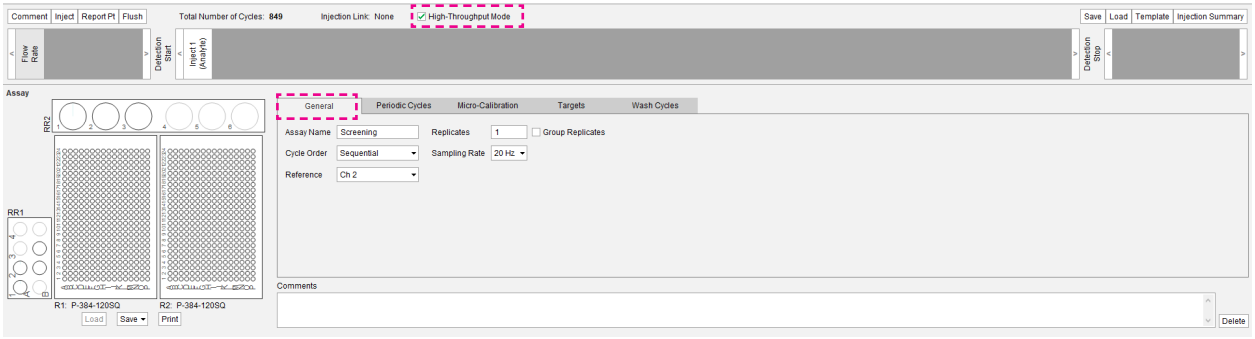
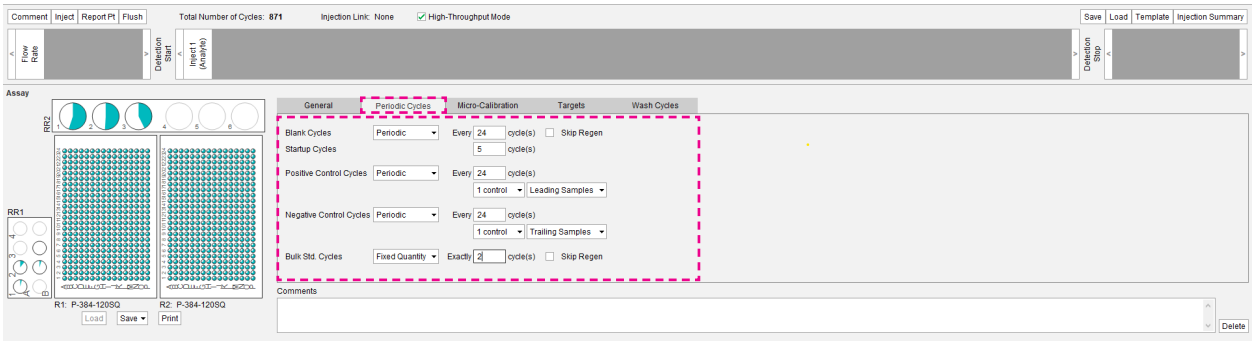


Figure 10
Periodic Cycles Settings – Fragment Screening.



Positive and negative controls can also be set to "Lead" or "Trail" Samples, meaning the control will be tested either before (Leading) or after (Trailing) a respective block of samples. For example, if a positive control was set to periodic: Every 24 cycles with leading samples, then the Positive control cycle would be run first, followed by 24 analyte cycles, the next positive control cycle, etc. positive control analytes are ideally similar in MW to the fragment analytes and dissociate readily (within 2–4 min). Negative controls may be either a non-binding molecule or a DMSO blank and ideally will be prepared in the same format as the analytes (i.e. in the same plate and prepared with the same pipetting procedure). Negative controls should have a non-zero concentration in the Octet® SF3 Discovery Software so they are assumed to be samples (rather than blanks) in the Octet® SF3 Analysis Software. Up to three positive or negative controls can be set if more than one ligand is being screened (control A for ligand A, control B for ligand B, etc.) or if more than one type of control is desired for a single ligand.

The bulk standard cycle refers to the OneStep® injection of sucrose, (prepared at 3% *w/w* in the DMSO assay buffer) which is used to internally calibrate the dispersion process for each assay. A minimum of one Bulk Standard injection is required for any assay where OneStep® is the analyte injection type. For fragment and small molecule screens, set the Bulk Std. Cycles setting to Fixed Quantity with one cycle as more than one bulk standard response will be averaged for accurately calibrating the dispersion process.

Micro-calibration is the automated refractive index offset correction method exclusive to Octet® SF3 systems. It produces a refractive index calibration curve from two standards where one is above the refractive index of the buffer and the second has a lower refractive index than buffer. Fragment screening buffer conditions commonly contain a low percentage of DMSO to ensure solubility of fragments which are normally stored as stocks in 100% DMSO. Therefore, a refractive index (RI) correction is important to ensure that ligand channels and reference channels are calibrated similarly, to generate data with less RI artifacts after reference channel subtraction.

Micro-calibration standards are recommended to contain a ±1% DMSO concentration difference from the concentration in the assay buffer. For example, if the assay buffer contains 4% DMSO the high/low standards should be 3% or 5% (±1%), DMSO in assay buffer.

The setup of the micro-calibration procedure is simple, and only requires entering the number of calibration cycles to be performed in the run and the vial positions of the high and low standards. In the Micro-Calibration tab, drag the low and high micro-calibration standards vial positions into the table for the Low RI Standard and High RI Standard, respectively (Figure 11). Fragment screens of one 384-well plate should be calibrated 3 times, and screens with two 384-well plates should be calibrated 4 times. The Octet® SF3 Discovery Software will divide the calibration cycles to evenly distribute them in the assay, and will automatically apply the calibration to respective regions of the assay. Repeat calibration allows for potential sensitivity changes at the biosensor (i.e. protein decay, non-specific binding of analytes, etc.) to be recalibrated and their effects on the data minimized. Choosing to run the first micro-calibration after the startup cycles will allow the biosensor to stabilize before calibrating which should be more accurate.

The Targets tab configures the identities and additional information for the immobilized or captured ligands. If the ligand is immobilized prior to beginning the assay, select **Fixed – Use table for all cycles** and enter the **Identity**, **Molecular Weight** (in Da or kDa), and **Immobilization Density** (in RU).

Figure 11
Assay Micro-calibration Settings – Fragment Screening.

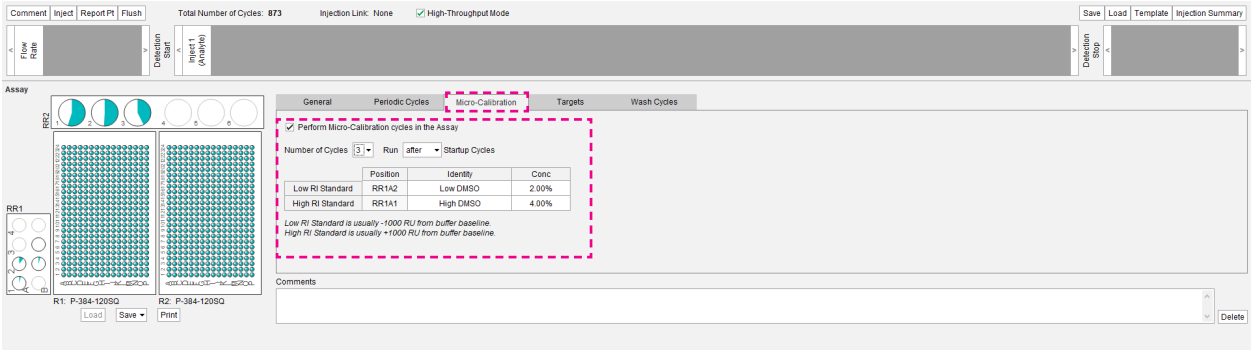
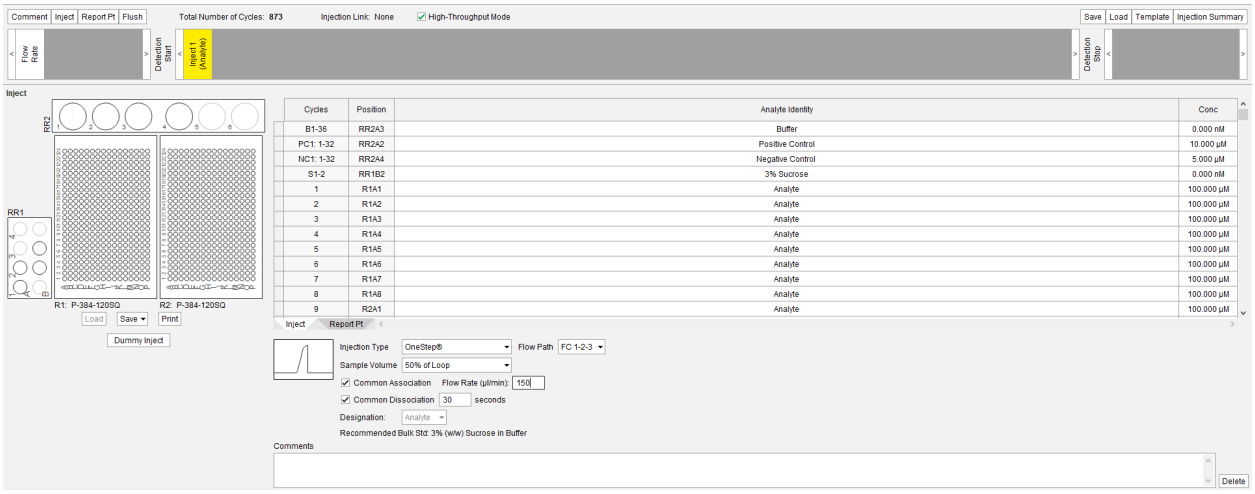


Figure 12
Analyte Injection Settings – Fragment Screening.



The Wash Cycles tab allows users to automate the insertion of wash cycles within the assay. Wash cycles are required during assays that may contain 'sticky' analytes that may affect subsequent cycles, such as assays with small molecules. Options to select the frequency and flow path allow full flexibility as to what the wash cycle solution contacts during the method.

Figure 12 shows the analyte injection settings for a large screen of two 384-well plates with the OneStep® Screening assay settings. Vial positions for buffer blank cycles, positive controls, negative controls, bulk standard, and all analyte samples need to be dragged into their respective positions in the Inject table. The OneStep® injection type is selected with a 50% of Loop sample volume, which is standard for weak affinity interactions as described in the Key Components of OneStep® Gradients section. The flow path is fixed for all OneStep® injections to FC1-2-3. The maximum injection flow rate that still enables the high-throughput mode is 150 µL/min. Dissociation times of 20–30 sec are sufficient for most fragment hits to completely dissociate from the immobilized biomolecule.

When the method is ready for error-checking, click the **Validate Method** button to ensure there are no critical errors. Make note of the assay run time to ensure the ligand(s) and samples are active for that length of time, and that the buffer is available in sufficient quantity and stability for the full run time.

Fragment Screening Assay Notes

Fragment screening without a positive control is a fairly common scenario as natural ligands may not be available or may have unsuitable binding properties. To setup a screening assay without a positive control, design the method as described previously and change the **Positive Control** setting to **None**.

Kinetic Characterization (Moderate Affinity)

The recommendations in this section can be applied for analytes (e.g. small molecule, peptide, nucleic acid, protein, lipid, etc.) that bind an immobilized ligand with a moderate affinity (K_D) between 10 µM–10 nM. This range of affinity can be grouped together because, from the SPR assay design perspective, these interactions can be accurately measured (especially the dissociation phase) in a 10–30 minute assay cycle without requiring special considerations for very long cycle times. Interactions in this moderate affinity range may require the regeneration of bound analyte from immobilized ligand for repeat or multiple analyte tests. If the ligand is to be recaptured in each assay cycle, the cycle would require three injections: (1) ligand capture, (2) analyte (OneStep®), and (3) regeneration.

The ligand of interest and any control ligands must be immobilized prior to beginning the screen. See the Before Getting Started section for more information on assay development. For kinetic characterization, the analyte R_{max} is recommended to be less than 50 RU for best results. In some cases, the R_{max} should be much lower than 50 RU, closer to 5–10 RU if, for example, avidity binding is expected. Refer to Equation 1 for estimating the expected analyte R_{max} as a function of ligand density.

In the **Method Setup**,double click on the **OneStep Triplicate** pre-written method. Where required, methods with regeneration and capture pre-written are also available to the user.

Assay Template: OneStep® Kinetics (Optional: with Regeneration)

Kinetic Characterization Assay Recommendations

- Assay Settings:
  - Cycle Order: Sequential
  - Sampling Rate: 5 Hz
  - Replicates: 2–3
  - Blank Cycles: Every 2–4 cycles
  - Startup cycles: 5–10 cycles
  - Pos Control Cycles: None
  - Neg Control Cycles: None
  - Bulk Std. Cycles: Fixed Quantity: Exactly 1 cycles

If DMSO or glycerol is present in buffer:

- Micro-Calibration: High+Low
- Micro-calibration Number of Cycles: 1; Run After startup cycles
- Assay Cycle Settings:
  - Optional: Capture Inject(s) for affinity capture ligands
  - Analyte Inject Flow Rate: 50 µL/min
  - Dissociate: 300 sec (30 sec for sucrose S1 cycle)
  - Analyte Injection Type: OneStep®
  - OneStep® Sample Volume: 75% of loop
  - Optional: Regeneration Inject(s)

Kinetic characterization of biomolecular interactions is commonly performed using SPR technology with 6 or more analyte concentration injections for full characterization. The OneStep® gradient enables full kinetic characterization in one injection per analyte and these conditions will suit most kinetic characterization assays for moderate affinity interactions.

Click the Assay function in the **Method** timeline to set the OneStep® kinetics assay settings (Figure 13). The General tab is used to give the assay a name, set the cycle run order, reference channel, number of replicates and the data sampling rate. If there is no bias in the order of samples, a sequential cycle order can be selected. If plating bias is a concern, use a random order. A 5 Hz sampling rate is sufficient for capturing the kinetic information of a moderate affinity interaction. Performing a duplicate or triplicate injection for each analyte is an important practice for demonstrating interaction reliability and the certainty of the kinetic rate constants determined from the binding curves.

Figure 13
Assay General Settings – Kinetic Characterization Moderate Affinity.

The Periodic Cycles tab (Figure 14) is used to set buffer blank cycles, positive and negative control cycles, and bulk standard cycles. The number of blank cycles can be adjusted depending on the number of analytes to be tested in the assay using either a fixed quantity of periodic blank setting. For short assays (one to two analytes), a blank every cycle is recommended. For longer assays (10–30 analytes), a blank every 4 cycles is recommended. It is common practice to have at least 5–10 startup cycles for kinetic characterization assays to stabilize the SPR response.

If a regeneration injection is included in the assay cycle, it is recommended to allow the blank cycles to perform regeneration so they are identical in procedure to analyte cycles. Positive and negative controls are normally not required for kinetic characterization assays, however, they can be included in the method, if applicable.

A single bulk standard cycle (Fixed Quantity: Exactly 1 Cycle) will be sufficient for most characterization assays. If a regeneration injection is included in the assay cycle, it is recommended to select the **Skip Regen** box next to the **Bulk Std Cycle** setting.

If the assay method requires a ligand capture inject in every cycle, this injection can be added by dragging an Inject to the left of the analyte inject. The **Injection Type** can be set to either **Inject or Fast** (for faster load time and slightly less sample consumption). The **Flow Path** should be over the channel to capture ligand only. The **Designation** should be set to **Capture** indicating the ligand is being captured and the applicable channel (**Ch**) box should be selected where ligand is captured. **Flow Rate**, and Common Dissociation time should be set per results of assay development experiments. An example is shown in Figure 15 for a 90 sec ligand inject over FC1 in every cycle before analyte and regeneration. *Note.* Ligand sample is used for the capture inject of blank cycles (B1–5) and analyte cycles (1–6) but a "DUMMY" sample is used for the capture inject of the bulk standard cycle (S1). The DUMMY position is dragged from the **Dummy Inject** button below the sample racks, and represents buffer taken directly from the buffer bottle. This is configured to skip ligand capture for the bulk standard cycle as it is not necessary to capture ligand in the bulk standard (sucrose) cycle for OneStep®.

Figure 14
Assay Periodic Cycle Settings – Kinetic Characterization Moderate Affinity.

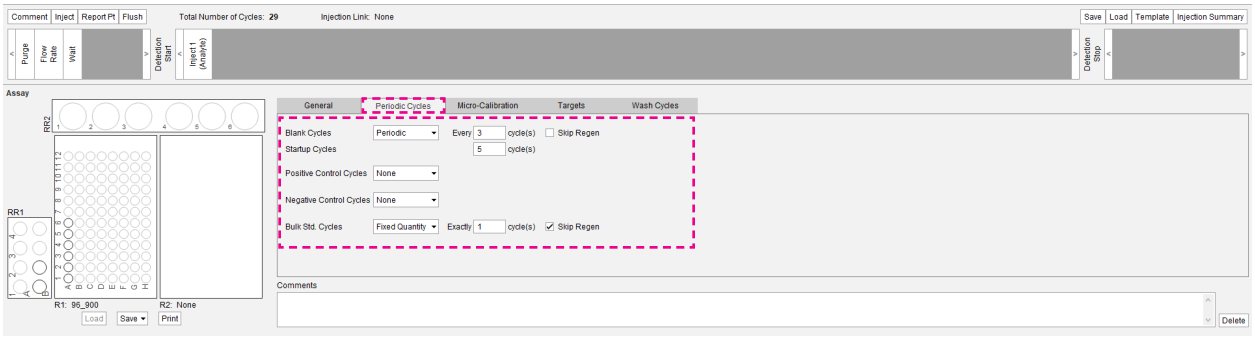
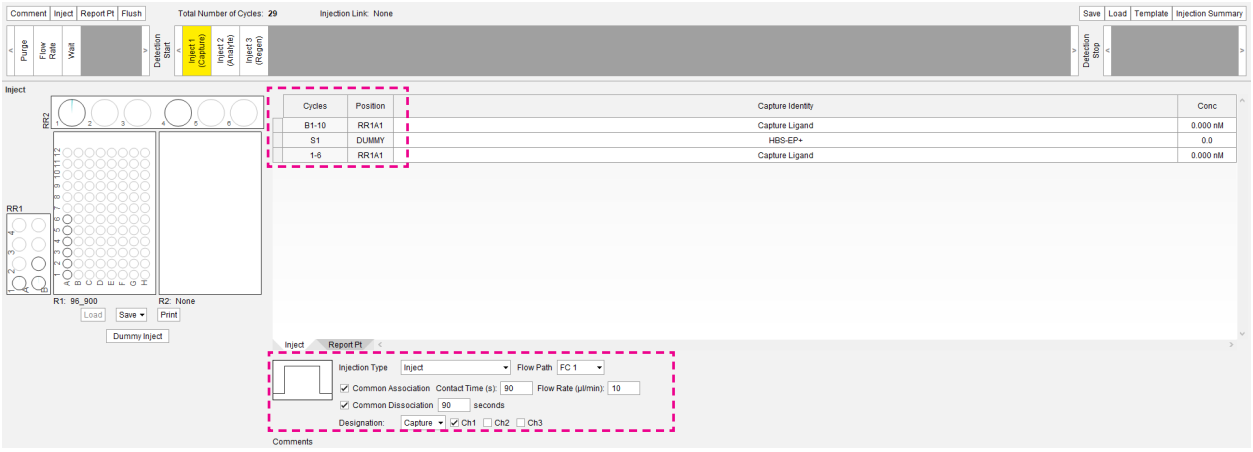


Figure 15
Capture Inject Settings – Kinetic Characterization Moderate Affinity.



In the next tab, micro-calibration is not required for assays performed in buffer without a high refractive index component. If the assay buffer contains some percentage of DMSO, glycerol, or other high refractive index component then micro-calibration should be performed to correct for potential refractive index offsets in the referenced data. Buffer standards containing ±1% of the high RI component should be used as high and low limit standards to calibrate RI around the buffer baseline. For example, if the assay buffer contains 2% DMSO, a low standard should be buffered with 1% DMSO and the high standard should be buffered with 3% DMSO. Drag the low and high micro-calibration standards vial positions into the table for the Low RI Standard and High RI Standard, respectively. Set **Number of Cycles** to 1 for for most characterization assays (or 3 cycles for long assays) and run **After** the startup cycles.

Targets configures the identities and additional information for the immobilized or captured ligands. If the ligand is immobilized prior to beginning the assay, select **Fixed – Use** table for all cycles and enter the identity, molecular weight (Da or kDa), and immobilization density (in RU). If the assay method is designed with a ligand capture inject, the target identity can be linked to the identity of ligand captured in each cycle. Click the **Assay** and click the **Targets** tab. Select **Dynamic – Use the Capture Injection(s**) from the **Source** drop-down menu so that the identity of ligand captured in each cycle will be the target name transferred to the Octet® SPR Analysis Software.

The analyte injection settings can be configured by clicking the **Analyte Inject** in the assay cycle (Figure 16). The OneStep® injection flow rate can be 30–70 µL/min where a standard kinetic characterization flow rate is 50 µL/min. As shown in Table 2, the slower flow rates give a slower injection time and a longer contact time of analyte at full concentration. The user might choose a slower flow rate if a slow k_a (<10^4 $M^{-1}s^{-1}$) and/or a slow k_d (<10^{-4} s^{-1}) is expected. A faster flow rate may be chosen if a fast k_a (>10^6 $M^{-1}s^{-1}$) is expected in order to minimize potential mass transport limitation. The faster flow rate will serve to enhance the transport of analyte from bulk to the surface and thereby encourage the measurement of unhindered kinetics.

The optional regeneration injection can be configured as either an inject or fast (to save time and sample) using inject volume, flow rate, and dissociate values determined from assay development. Regeneration injects are usually injected over the ligand channel(s) and reference channel, so a FC1-2-3 is common. If the regeneration solution is highly concentrated or tends to adhere in fluidic channels (i.e. SDS), following the regeneration inject with an additional purge (1X) will ensure the solution is flushed from the system fluidics.

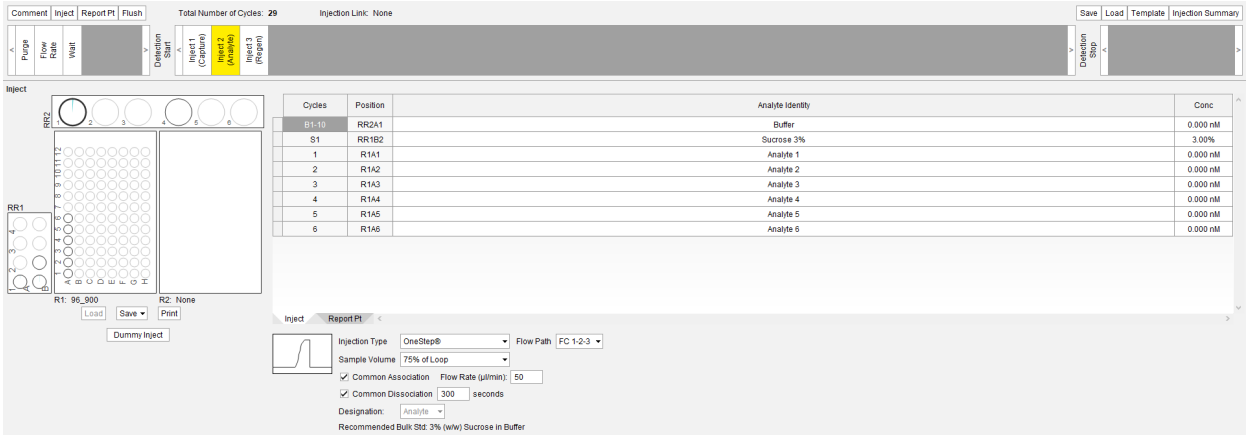
When the method is ready for checking, click the **Validate Method** button to ensure there are no critical errors. Make note of the assay run time to ensure the ligand(s) and samples are active for that length of time and that the buffer is available in sufficient quantity and stability for the full run time.

Kinetic Characterization (Moderate Affinity) Assay Notes

Analyte retention

Small molecule compounds with MW >450 Da sometimes have more hydrophobic character than smaller compounds and this can result in hydrophobic adsorption of the analyte to fluidic tubing. The effect of compound adsorption on a OneStep® injection is a systematic shift in the concentration vs. time profile of the injection. In the data analysis software this is referred to as analyte retention and

Figure 16
Analyte Injection Settings – Kinetic Characterization Moderate Affinity.



model fits can accommodate this artifact in binding data. The adsorption of analyte may cause sample carryover between assay cycles, causing specific binding or non-specific binding (NSB) in subsequent cycles as the analyte slowly dissociates from the fluidic tubing rather than being washed out after the injection.

Prevention of sample carryover

Preventing sample carryover is simple, and is performed by including a cleaning solution (usually 50% DMSO) in the initial rack setup and including wash cycles to remove adsorbed material that is not completely washed out with buffer.

Wash cycles can be found in the wash cycles tab and perform a clean and purge using a user specified wash solution and flow path (as shown in Figure 17). In this example, wash cycles are programmed to be performed after every cycle and the analyte OneStep® injection will be performed, followed by a wash cycle with 50% DMSO, then the next cycle will be started. Where desired, the clean function can also be dragged into the post cycle after an injection (by placing the clean command to the right of the detection stop) and the desired flow path being chosen. The cleaning solution is selected by dragging its sample location into the position cell (Pos). The clean volume should be slightly larger (+5 µL) than the OneStep® inject volume (e.g. 75 µL if the inject volume is 75% of loop) to ensure the cleaning is effective and the flow rate is fast enough to quickly discard the cleaning solution. Choosing the OneStep® path as the Flow Path to Clean setting indicates the cleaning solution will be injected via the OneStep® capillary line. ***Note.*** the injection of cleaning solution using the OneStep® path will exit the flow cell via the waste port upstream of flow channel 1 thereby avoiding the active flow channel and preventing any damage to the immobilized ligand.

The inclusion of wash cycles or a clean function in the assay cycle will add time to the method but will improve data quality when analytes are expected or predicted to show non-specific adsorption to the system fluidics.

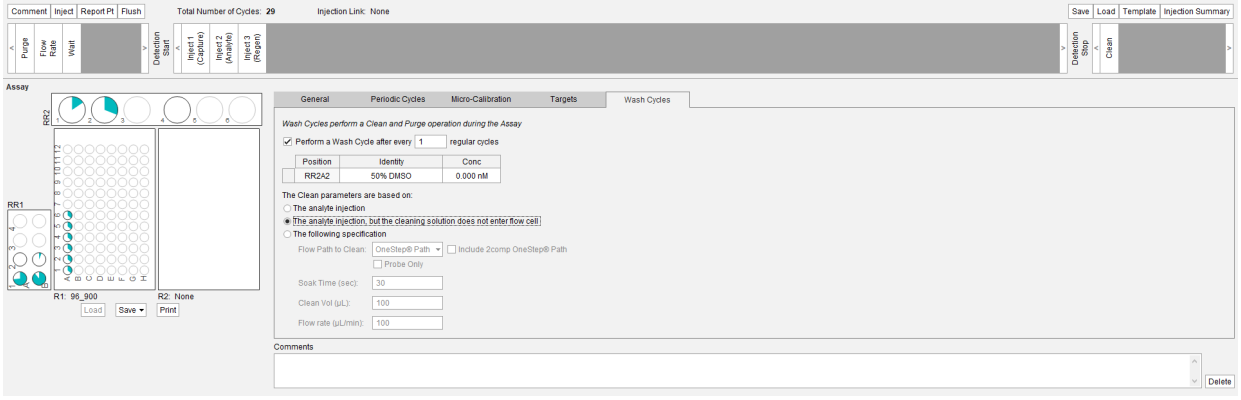
Kinetic Characterization (High Affinity)

The recommendations in this section can be applied for analytes (e.g. small molecule, peptide, nucleic acid, protein, lipid, etc.) that bind the immobilized ligand with an equilibrium dissociation constant (K_D) of < 1 nM. Interactions with such high affinity will require some form of surface regeneration after each analyte injection to perform replicate injections or test multiple analytes. Regeneration can be optimized by finding a condition that removes the analyte from the ligand or that removes the analyte-ligand complex from the biosensor and recaptures fresh ligand every cycle.

The challenge in characterizing very high affinity interactions is most often in accurately measuring the dissociation, which is very gradual. It is generally accepted in the kinetics community that to determine accurate dissociation kinetics a decrease of >5% response should be observed during dissociation in order to determine accurate k_d values. Though it is preferable that a visual drop in the response is also observed in addition to a mathematical decrease of 5% prior to performing regeneration. The assay must therefore be

Figure 17

Clean Function Settings – Kinetic Characterization (Moderate Affinity).



stable and sensitive to changes in response equal to or greater than 5% of equilibrium response (R_{EQ}) over the course of 5+ hours in certain cases. As shown in Table 3, the minimum time to measure dissociation increases dramatically between 10^{-5} and 10^{-6} s^{-1}.

From the **Method Setup** tab, double click on the **OneStep Triplicate with regeneration** pre-written method.

Table 3
Minimum Required Dissociation Times by Dissociation Rate Constant.

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

Assay Template: OneStep® Kinetics with Regeneration

High affinity kinetic characterization assay recommendations

- Assay Settings:
  - Cycle Order: Sequential
  - Sampling Rate: 2–5 Hz
  - Replicates: 2–3
  - Group Replicates: ☑ Checked
  - Blank Cycles: None (Configured manually)
  - Startup cycles: None
  - Pos Control Cycles: None
  - Neg Control Cycles: None
  - Bulk Std. Cycles: Fixed Quantity: Exactly 1 cycles

If DMSO or glycerol is present in buffer:

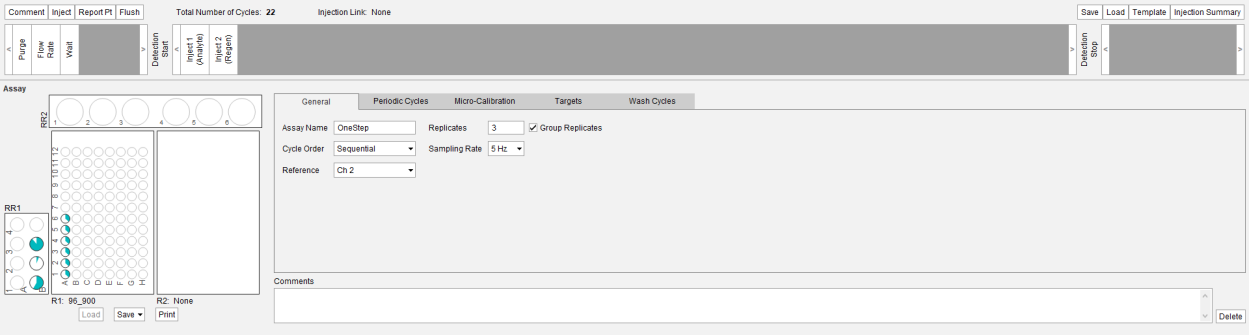
- Micro-calibration: High+Low (only if DMSO or glycerol is present in buffer)
- Micro-Calibration Number of Cycles: 1; Run After startup cycles

- Assay Cycle Settings:
  - Pre-Cycle Wait Time: 5 min
  - Analyte Inject Flow Rate: 30–50 µL/min
  - Dissociate: 3600–43,200 sec (30 sec for sucrose S1 cycle)
  - Analyte Injection Type: OneStep®
  - OneStep® Sample Volume: 100–200% of Loop

Kinetic characterization of high affinity biomolecular interactions is commonly performed using SPR technology with 6 or more analyte concentration injections for full characterization. The OneStep® gradient enables full kinetic characterization in one injection and these conditions will suit most kinetic characterization assays for high affinity interactions.

Click the **Assay function** in the **Method** timeline to set the OneStep® kinetics with regeneration assay settings (example in Figure 18). The General tab is used to set the assay name, the cycle run order, reference channel, number of replicates and the data sampling rate. A sequential cycle order should be selected as the sample injection order will be specifically organized in this assay type. A 2–5 Hz sampling rate is recommended as data files for high affinity interactions are often larger and the kinetics occur over longer periods thus requiring fewer data points per second for accurate analysis. Similar to moderate affinity characterizations, a duplicate or even triplicate injection is important for establishing high certainty in the kinetic rate constants determined. Grouping replicates ensures that duplicate injections within a group are performed one after

Figure 18
Periodic Cycles Settings – High Affinity Kinetic Characterization.



another, rather than in sequence throughout the assay with all other samples. This is important for the high affinity assay type because the dissociation times for blank cycles are configured by run order and the Group Replicates option ensures the run order is consistent while still performing a duplicate for every injection.

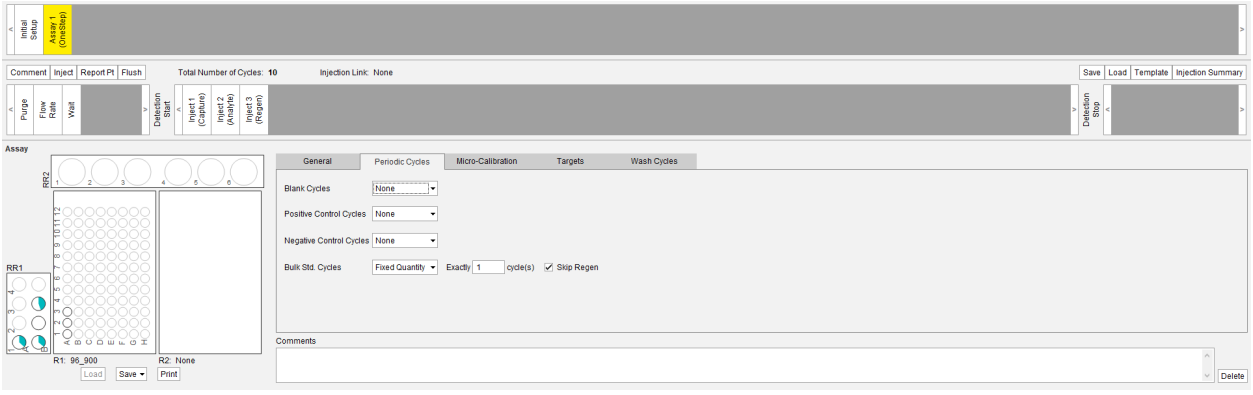
The Periodic Cycles tab (Figure 19) is used for configuring the bulk standard cycle only for the high affinity assay type. Buffer blank cycles can be configured manually as discussed in the next section (High Affinity Assay Blank Configuration) and can be set to None. Positive and negative controls are normally not required for kinetic characterization assays, however they can be included in the method, if desired. A single bulk standard cycle (Fixed Quantity: Exactly 1 Cycle) will be sufficient for the high affinity characterization assay. Select the **Skip Regen** box to skip the regeneration injection for the bulk standard cycle.

In the next tab, Micro-calibration is not required for assays performed in buffer without a high refractive index component. If the assay buffer contains some percentage of DMSO, glycerol, or other high refractive index component then a High+Low micro-calibration should be performed to correct for potential refractive index offsets in the referenced data. Buffer standards containing ±1% of the high RI component should be used as high and low limit standards to calibrate RI around the buffer baseline. For example, if the assay buffer contains 2% DMSO, a low standard should be buffered with 1% DMSO and the high standard should be buffered with 3% DMSO. Drag the vial positions for high and low micro-calibration standards into the position (Pos) cells for the respective Limit. For most characterization assays, set the **Number of Cycles** to 1 and to run before the startup cycles.

The Targets tab configures the identities and additional information for the immobilized or captured ligands. If the ligand is immobilized prior to beginning the assay, select **Fixed – Use table for all cycles** and enter the identity, molecular weight (Da or kDa), and immobilization density (in RU). If the assay method is designed with a ligand capture inject, the target identity can be linked to the identity of ligand captured in each cycle. Click the **Assay** and click the **Targets** tab. Select **Dynamic – Use the Capture Injection(s)** from the **Source** drop-down menu so that the identity of ligand captured in each cycle will be the target name transferred to the analysis software.

The assay cycle for a high affinity characterization will typically include two or more injections: (1) ligand capture (optional), (2) analyte OneStep®, and (3) regeneration ligand. Ligand Capture in every cycle is an option, but as a high affinity characterization requires a very stable baseline to measure the slow dissociation response, the ligand capture would need to be extremely stable for a reliable kinetic analysis. An increased pre-cycle wait time of 5 min is recommended to allow the biosensor to stabilize prior to recording SPR signal and thereby giving best possible baseline stability. Click **Wait** in the pre-Cycle and ensure the **Wait Time** is set to **5 min** (Figure 19).

Figure 19
Periodic Cycles Settings – High Affinity Kinetic Characterization.



The optional capture injection should have the Ligand sample for the analyte cycles (1 – 7) but a "DUMMY" sample is used for the capture inject of the bulk standard cycle (S1). The DUMMY position is dragged from the Dummy Inject button below the sample racks and represents buffer taken directly from the buffer bottle (Figure 20). This is configured to skip ligand capture for the bulk standard cycle as it is not necessary to capture the ligand in the bulk standard (sucrose) cycle for OneStep®. Therefore, when subtracting the blank signal from the analyte signal, any drift due to the ligand will be subtracted from the response.

The analyte injection settings can be configured by clicking the **Analyte Inject** in the assay cycle (Figure 21). A slower OneStep® flow rate (30–50 µL/min) is chosen in high affinity characterizations as it will give more contact time for the interaction to near or reach equilibrium. A minimum flow rate of 30 µL/min should be chosen to avoid inducing mass transport limitations on the observed kinetic rates. Dissociation time for the analyte injections should be chosen by the expected dissociation rate constant (dissociation time for blank injections is discussed in the next section). Table 3 shows the minimum time required for accurate dissociation measurements per k_d rate constant.

Note. The dissociation time for the bulk standard cycle (Figure 21, Cycle S1) is set to 30 seconds rather than 43,200 seconds. This will avoid a long dissociation where it is not required and save assay time. The Injection Type selected is OneStep® with a Sample Volume of 100%. The largest Sample Volume is selected to give the high affinity interaction more time at full analyte concentration to reach equilibrium prior to beginning dissociation. Where necessary, loop volume can be increased to 150% or 200% if equilibrium is not achieved.

Figure 20
Optional Capture Injection Settings – High Affinity Kinetic Characterization.

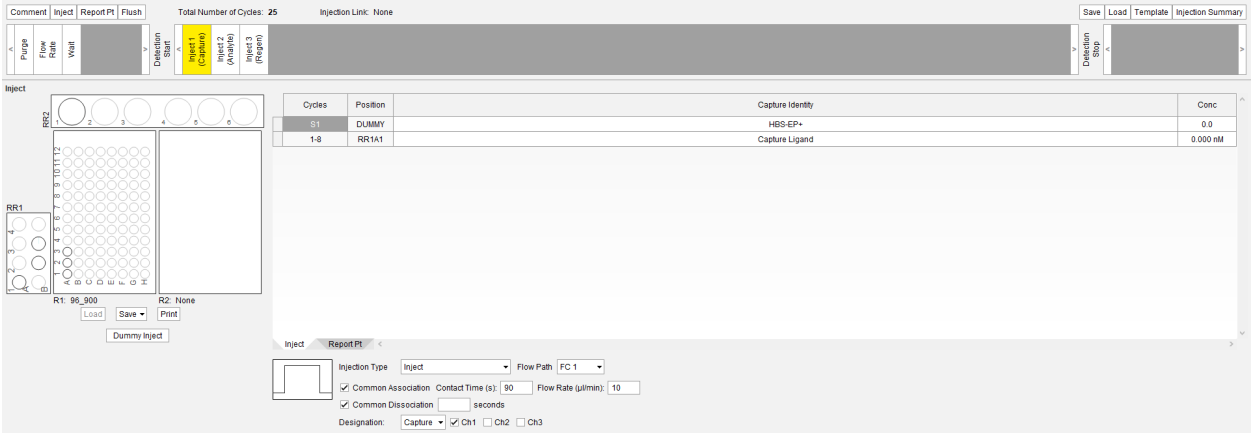
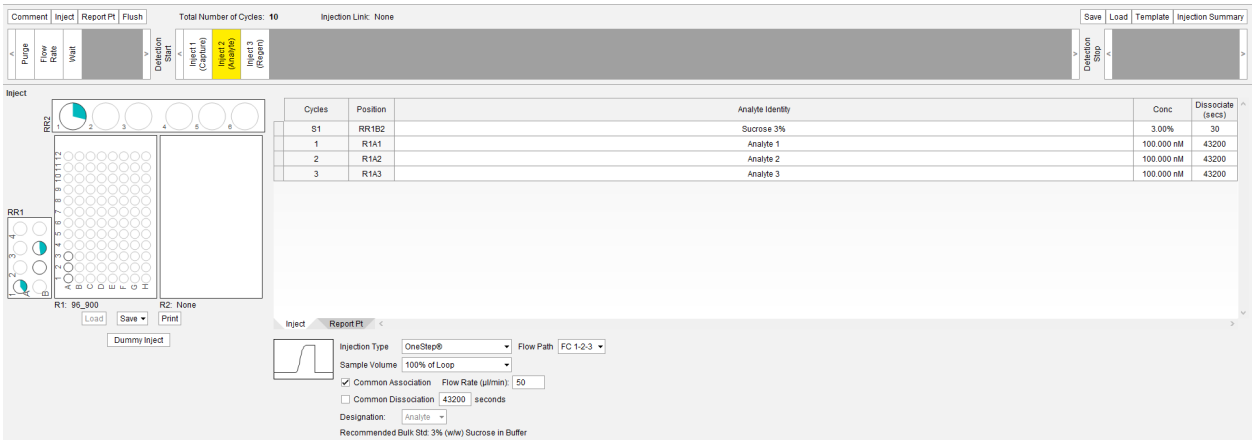


Figure 21
Analyte Inject Settings – High Affinity Kinetic Characterization.



Note. The Common Dissociation box is deselected to allow unique dissociation times to be entered for each analyte.

The regeneration injection can be configured using inject volume, flow rate and dissociate values determined from assay development (example in Figure 22). Regeneration injects are usually injected over the ligand channel(s) and reference channel, so a FC1-2-3 is common. If the regeneration solution is highly concentrated or tends to adhere in fluidic channels (i.e. SDS), following the regeneration inject with an additional Purge (1X) will ensure the solution is flushed from the system fluidics.

High Affinity Assay Blank Configuration

Manual configuration of blank cycles for high affinity assays will allow a variable dissociation time that can be used to run 2–3 blank cycles with a shorter dissociation time and another 1–2 blank cycle(s) with a long dissociation time. With the common dissociation box unchecked, configure blanks accordingly by dragging the buffer blank vial into the Sample Inject table for the analyte inject. A minimum of five blank injects at the beginning of the assay (Cycles 1–5) should be setup as shown in Figure 23. These buffer blank

Figure 22
Regeneration Inject Settings – High Affinity Kinetic Characterization.

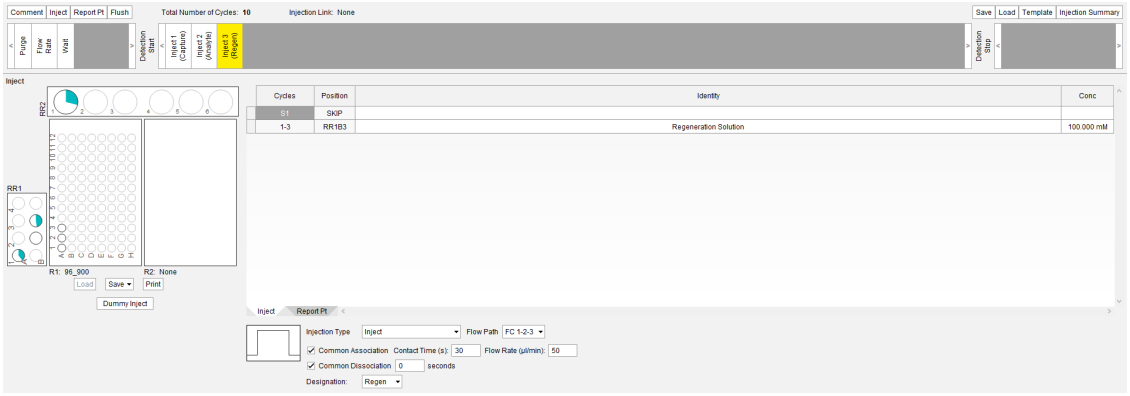
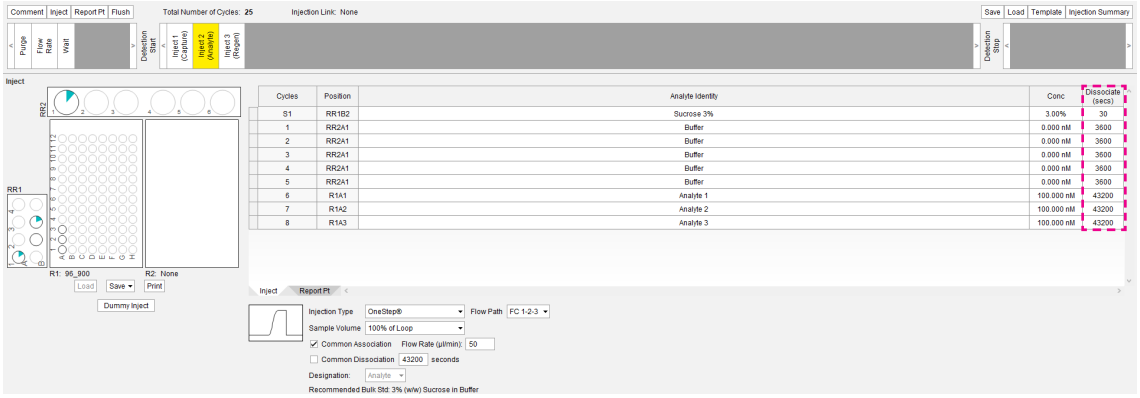


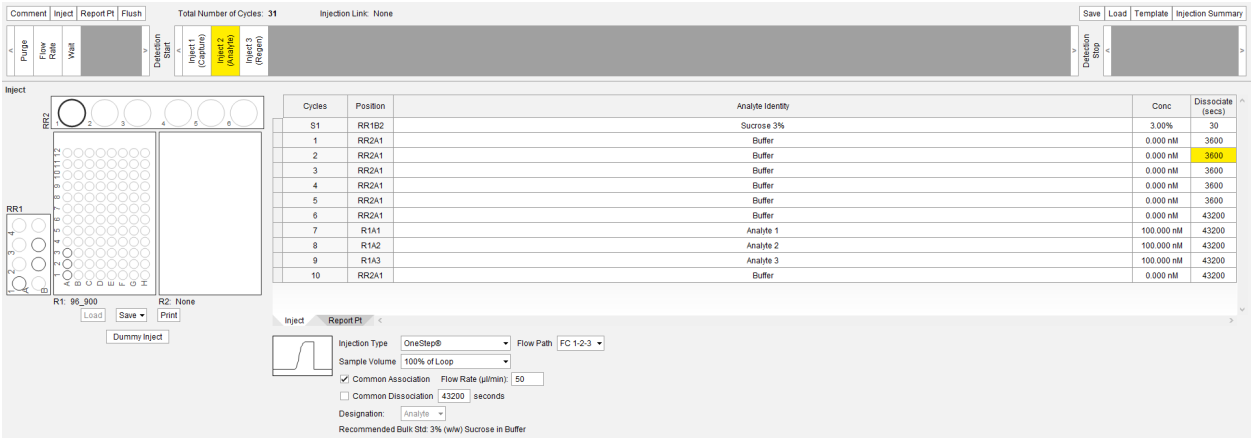
Figure 23
Analyte Inject Dissociation Settings – High Affinity Kinetic Characterization.



injections serve to equilibrate the sensor chip surface prior to assessing analyte binding. Buffer blanks for reference subtraction must also be included and these are added as above (Figure 24). It is recommended that these buffer blanks are performed before and after each analyte, where possible, and, at a minimum, sandwich the analytes as shown in Figure 24. The dissociation time for the first five blanks can be shorter than the blanks that will be used for reference subtraction, which must match the dissociation time for the analyte cycle(s). The short blanks should have a minimum dissociation time of 600 sec and for very long analyte dissociations, should be set to 3,600 sec.

When the method is ready for checking, click the **Validate Method** button to ensure there are no critical errors. Make note of the assay run time to ensure the ligand(s) and samples are active for that length of time and that the buffer is available in sufficient quantity and stability for the full run time.

Figure 24
Analyte Inject Dissociation Settings.



Note. High affinity characterization requires the buffer blanks used for reference subtraction to have the same dissociation settings as the analytes.

Additional Resources

The purpose of this document is to describe the OneStep® method design for the most common binding assays and give guidance on how to create effective OneStep® methods for most applications. It is recommended that the users read the Octet® SPR Discovery, Analysis and Chemistry User Guides for further assay specific information as well as the Octet® Systems Applications Guide. If exceptions are encountered that are not described here or on the website, please contact your local field applications scientist or Octet® support: www.sartorius.com/octet-support

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