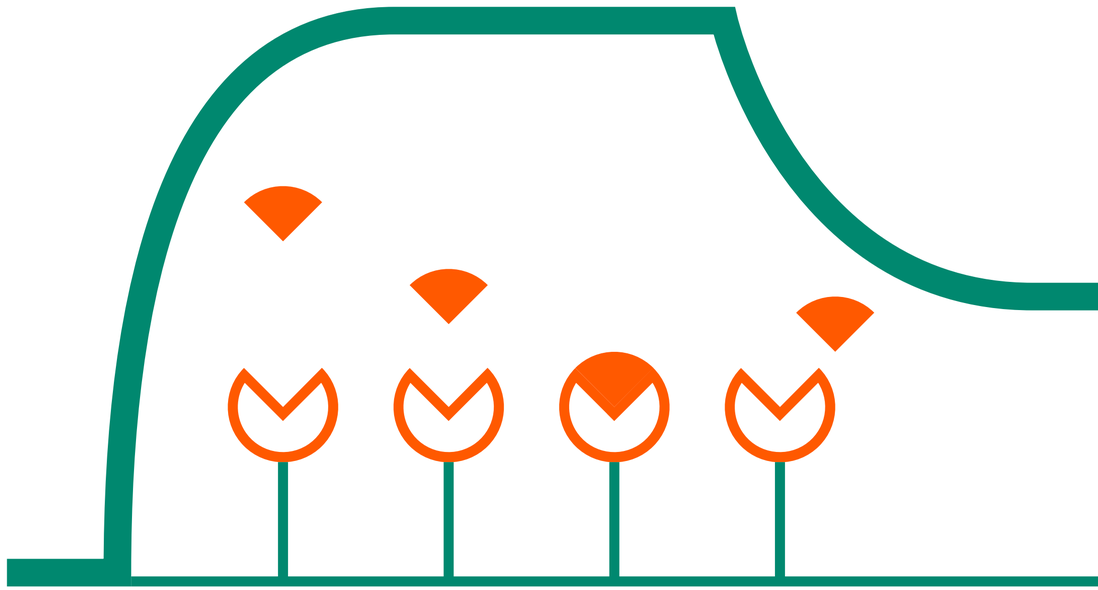
Application guides

Kinetics and affinity measurements with Biacore systems



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Introduction

Characterization of the kinetics and affinity of an interaction is of fundamental interest in all areas of biological research and development including drug development. Binding kinetics describes the speed at which a molecule binds to and dissociates from a binding partner such as an antibody or a receptor. Affinity describes the strength of a binding but not the rate at which it occurs.

The label-free real-time detection in Biacore™ systems allows interactions to be monitored with high resolution as they happen, and the results can be interpreted in relation to a mathematical model of the interaction mechanism to evaluate kinetic parameters (association and dissociation rate constants). The association phase (during sample injection) contains information on both association and dissociation processes, while only dissociation occurs during the dissociation phase (after sample injection, when buffer flow removes dissociated analyte molecules). Affinity constants can be derived either from the ratio of the rate constants or from analysis of the level of binding at steady state.

Theoretical aspects of kinetics and affinity determination are covered in the separate Application guide *Principles of kinetics and affinity analysis.*

Note: The term kinetics in this Application guide refers to interaction kinetics. Kinetics of other processes such as enzyme-catalyzed reactions are not normally amenable to study in Biacore systems.

Scope of this Application guide

This Application guide considers setup and evaluation of experiments designed to measure the kinetics and/or affinity of one or a few interactions. Aspects of kinetic and affinity measurements specific to screening applications are considered in the separate guides for antibody and small molecule/fragment screening.

Careful kinetic measurements may give opportunities for elucidating mechanisms of action. For many purposes, however, it may be sufficient to get a general idea of interaction rates, for example to compare the behavior of mutant and wild-type molecules. Many of the more stringent requirements and recommendations may be relaxed according to the purpose of the investigation.

Range of kinetic and affinity measurements

The range of kinetic and affinity constants that can be determined with Biacore systems covers much of the range found in biomolecular interactions. The precise range varies with both system performance and experimental conditions. In general, systems with higher sensitivity and higher signal-to-noise ratios can measure faster processes. For all systems, however, careful assay development, sensor surface preparation and sample preparation are necessary for achieving the highest sensitivity. Constants that lie outside the range of the instrument cannot be determined reliably.

Interaction constant	Typical range
Association rate (k_a)	10^3 to 10^7 $M^{-1}s^{-1}$
Dissociation rate (k_d)	10^{-5} to 0.5 s^{-1}
Affinity (K_D)	pM to mM

Terminology

Term	Meaning
k_a	Association rate constant, units $M^{-1}s^{-1}$ Governs the rate at which a complex is formed.
k_d	Dissociation rate constant, units s^{-1} Governs the rate at which a complex dissociates.
k_D [1]	Equilibrium dissociation constant, units M. Describes the strength of the interaction.
k_t	Mass transport coefficient, normalized for molecular weight and adjusted approximately for the conversion of surface concentration to RU.[2]
RI	Contribution of bulk refractive index to the analyte response (RU).
R_{max}	Maximum analyte binding capacity of the surface (RU).

Term	Meaning
offset	Parameter in evaluation of steady state affinity representing the response at zero analyte concentration.
avidity	Accumulated strength of multiple affinities of individual interactions involving multivalent molecules. Avidity occurs when the analyte presents multiple sites for binding to the ligand so that release of the analyte molecule from the surface requires simultaneous dissociation of several interactions.
multi-cycle kinetics (MCK)	Assay format where each analyte concentration is injected in a separate cycle.
single-cycle kinetics (SCK)	Assay format where analyte concentrations are injected in sequence in a single cycle.
parallel kinetics	Assay format where each analyte concentration is injected in a different channel in the same cycle. Only applicable to multi-channel Biacore systems.

[1] The equilibrium dissociation constant K_D is the inverse of the equilibrium association constant K_A. Both terms are used in the literature.
[2] See the Biacore Application guide *Principles of kinetic and affinity analysis* for details.

Note: The convention of using lowercase letters to denote rate constants and uppercase for affinity constants is used as far as possible in documentation of Biacore systems. Other formats may be used in the literature.

Tips for kinetic and affinity measurements

- For kinetics and affinity experiments, match running buffer with sample buffer to minimize bulk refractive index contributions
- For kinetics experiments, use low immobilization or capture levels to reduce mass transport limitations and to facilitate data interpretation. As a rule of thumb, aim to use ligand levels that result in maximum analyte responses of 10-30 RU or lower.
- Using low ligand levels can help to suppress heterogeneity in an interaction
- Calculate suitable ligand levels based on theoretical R_{max} values
- Use results obtained during assay development as a guide to suitable association and dissociation times in the final experiments
- If ligand is captured on the surface, make sure that the capture is stable enough so that dissociation of ligand does not affect the evaluation
- The simplest model (i.e., fewest parameters) should always be the default choice before moving on to models describing more complex interactions
- Do not “model surf” just because the fit is poor. Poor fitting to a simple model does not in itself justify moving on to a more complex one without proper verification. However, testing other models or initial parameter settings for exploratory purposes can be useful, as long as the results are treated with care.
- Remember that fitting models are mathematical constructions, that do not necessarily describe reality. Interaction constants obtained by model fitting are only valid in the context of the model.
- For fast kinetics, set the RI parameter to zero to prevent fast binding events from being assessed as bulk contribution. Note that this approach assumes careful refractive index matching of samples and running buffer.
- If capture levels vary between cycles, use local fitting for the R_{max} parameter
- When evaluating steady state affinity using the 1:1 binding model with no additional constraints such as constant R_{max} (see *Choice of fitting model*, on page 32), treat reported values for K_D that are higher than half the highest analyte concentration with caution

General considerations

Work carefully for best results

Careful determinations that claim to measure true kinetic or affinity properties may require considerable effort in assay development and preparation. On the other hand, such effort may be wasted if the aim of the experiment is approximate comparative determination. Consider the following points in assay development for careful measurements. Adjust the assay development requirements according to your needs. Remember that assay development may be an iterative procedure: for example, the best choice of ligand attachment method may depend on the regeneration performance.

- Use carefully purified ligands and analytes. Purification should not only eliminate contaminants but also reduce aggregation as far as possible. Use non-denaturing techniques to determine purity so that aggregates are detected.
- Choose the sensor chip type and ligand attachment method according to the known properties of the interactants
- Scout for optimal ligand attachment conditions such as pH, buffer composition, ligand concentration
- Prepare the sensor surface using techniques that ensure consistent results on repeated occasions
- Determine the analyte binding capacity of the surface and compare with the theoretical value. If the surface activity is low, review the ligand preparation and attachment procedures.
- Scout for optimal regeneration conditions, or plan to use a single-cycle format that does not require regeneration
- Use the cycle settings recommended for your Biacore system. Several systems have sample injection types that are optimized for kinetic measurements.
- Perform approximate determinations to get an idea of the kinetics and affinity, then adjust conditions such as ligand level, analyte concentrations, and contact times for optimal performance
- Always perform several replicate analyses before reporting results. In extreme cases replicates may be run on different instruments and/or by different operators to ensure reliability.

Kinetics or affinity?

Determination of interaction kinetics and steady state affinity place different demands on the experimental data:

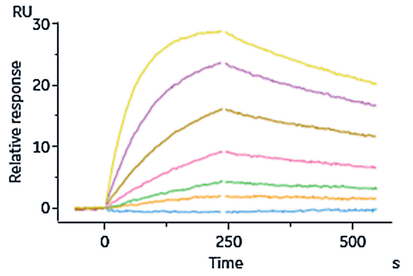
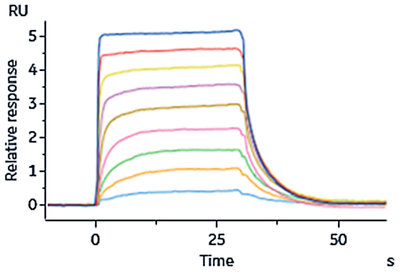
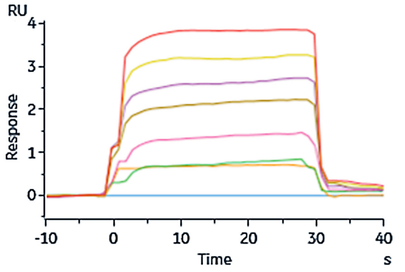
- Kinetic determination requires that the sensorgrams show sufficient curvature during both association and dissociation phases
- Steady state affinity requires that the sensorgrams reach steady state during sample injection

It is usually not possible to meet both these requirements in the same experiment. Interactions that are fast enough to reach steady state within reasonable injection times often show too little sensorgram curvature for kinetic measurement. Sensorgrams that are appropriate for kinetics, steady state affinity and possibly both determinations are illustrated below. Whether both kinetics and affinity can be obtained from the intermediate example must be judged from the evaluation results.

Buffers

For best performance in kinetics and affinity experiments, the composition of sample and running buffer should be closely matched so that association (during sample injection) and dissociation (after the end of the injection) occur in the same environment. In addition, evaluation is more robust if sample and running buffer are matched in refractive index, so that bulk shifts at the beginning and end of the sample injection are kept to a minimum.

Variations in bulk refractive index between different sample concentrations can if necessary be compensated by evaluating kinetics with the bulk contribution RI as a local parameter. For affinity, however, it is important that any bulk contribution is the same at all sample concentrations.

Sensorgrams	Kinetics	Affinity
	√	X
	√	√
	X	√

Sensor surface preparation

Ligand purity

It is important for careful kinetic and affinity measurements that the ligand is pure and homogeneous. Impurities may be immobilized together with the ligand, reducing the ratio between observed and expected analyte binding capacity. Heterogeneity (including the presence of contaminants that can bind analyte) lead to more complex binding behavior and may preclude meaningful evaluation.

Ligand attachment approach

Ligand attachment may be permanent (immobilization) or reversible (capture). Covalent immobilization is generally preferable.

The choice of immobilization method may affect homogeneity of the immobilized ligand. Methods that exploit common groups in the ligand molecules (including amine coupling, the most commonly used approach for immobilization) may introduce a mixture of ligand orientations on the surface even if the ligand solution has been rigorously purified. Approaches directed towards less common groups, such as thiol coupling, may help to reduce surface heterogeneity.

A capturing approach can provide on-chip purification of the ligand as well as resulting in consistent ligand orientation. However, it is important that the capturing interaction is sufficiently stable so that dissociation of ligand during the course of an analysis cycle is negligible. Ligand dissociation will affect both the observed response and the analyte binding capacity of the surface, and is not included in any of the standard evaluation models.

Theoretical ligand levels

Suitable immobilization levels can be calculated in advance from the relative molecular weights of ligand and analyte and the desired maximum analyte response R_{max}, according to the expression below.

$$R_{ligand} = \frac{R_{max}}{n} \times \frac{MW_{ligand}}{MW_{analyte}}$$

Term	Meaning
R_{ligand}	Ligand immobilization level (RU)
R_{max}	Theoretical maximum analyte binding capacity (RU)
MW	Molecular weight
n	Interaction stoichiometry (analyte/ligand)

This estimation assumes that the ligand on the surface is 100% active. In practice, the activity is usually lower. The amount of immobilized ligand should be adjusted accordingly.

Example

To characterize the interaction between an antibody (molecular weight 150 000) and antigen (molecular weight 15 000), the bivalent antibody is immobilized on the sensor surface. Each antibody molecule can bind two antigen molecules independently of each other. To achieve a theoretical R_{max} of 20 RU, the target level of immobilized antibody should be (20/2) × (150 000/15 000) = 100 RU. If the antibody is expected to be 80% active after immobilization, aim for a target level of 125 RU.

Suitable ligand levels in practice

For evaluation of kinetics and affinity measurements, both the analyte response and the surface concentration of ligand are expressed in resonance units (RU). In consequence, the amount of immobilized ligand does not have to be known in absolute terms. The immobilization level is however one factor that determines the analyte response levels and is important for the quality of the results.

For kinetic analysis, the amount of immobilized ligand should be kept low, so that the maximum response (R_{max}) from analyte binding is typically in the region of 10 to 30 RU or lower. There are two main reasons for this:

- Low ligand levels reduce limiting effects of mass transfer. Mass transfer refers to the diffusion-controlled supply of analyte molecules to the surface from bulk solution. If transfer is slow in relation to the association rate, the observed binding will be dominated by the diffusion process rather than the interaction rate. Mass transfer is discussed in more detail in the Application guide *Principles of kinetics and affinity analysis*.
- Low ligand levels help to simplify data interpretation by minimizing artifacts that can arise from crowding of molecules on the surface. Crowding effects are difficult to quantitate and can give rise to unnecessarily complex binding behavior.

Steady state binding levels are not affected by mass transport limitations. Ligand levels for affinity measurements do not need to be kept low. Use levels that give confidently measurable responses at the lowest analyte concentration.

A recommended workflow for ligand attachment is summarized in *Ligand attachment workflow,* see below.

Ligand attachment workflow

A suggested workflow for preparing a suitable sensor surface for an uncharacterized interaction is summarized to the right. The procedure may consume one or more sensor chips, flow cells or channels. Adapt the workflow to your needs and your prior knowledge of how the interacting molecules behave under the experimental conditions.

Reference surface

Kinetic and affinity measurements should normally be performed with reference-subtracted data. The reference surface may be untreated, activated and deactivated, or prepared with an inactive protein to mimic the physical properties of the active surface. When working with capture, the reference surface should be immobilized with the capture molecule in question.

Step	Action
1	Calculate the required theoretical ligand level. If you know that the immobilized ligand will not be fully active, adjust the target level accordingly.
2	Perform a trial immobilization. Some Biacore systems support immobilization using an Aim for immobilized level procedure. General recommendations may be found in the *Biacore Sensor Surface Handbook* and in the system documentation.
3	Inject high concentrations of analyte over the surface to test the binding capacity.
4	Repeat the trial immobilization if necessary until conditions are found that give a suitable immobilization level. The main parameters that can be adjusted to control the level of immobilized ligand are the ligand concentration and the surface activation time (for amine coupling, activating the surface with 20% EDC:80% NHS can also help to achieve lower immobilization levels). Working with conditions where the amount of ligand immobilized varies with ligand contact time is not recommended, since it may be difficult to obtain reproducible immobilization results.
5	Perform multiple cycles of analyte binding and regeneration to confirm that the immobilized ligand withstands regeneration without loss of binding capacity. Note that regeneration performance may differ at different immobilization levels.

Sample preparation

Introduction

Preparation of samples can be a critical factor in the quality of kinetic and affinity measurements. Ideally but not crucially, both ligand and analyte should be pure and homogeneous, to simplify the interpretation of the results as far as possible.

It is important that non-specific binding of sample components to the sensor surface is kept to a minimum, and that the analyte is homogeneous and non-aggregated. Aggregation results in differently sized binding species that will give different responses, and potentially in multivalent binding behavior. Use non-denaturing analysis methods to check analyte homogeneity.

In many cases, analyte samples may be prepared by dilution from stock solutions. Samples may be diluted directly into running buffer from stock solution if the dilution factor is sufficiently high so that residual stock solution components can be ignored. Alternatively, samples may be prepared using buffer exchange techniques.

Prepare concentration series of analyte using careful serial dilution techniques. Ideally, use fixed-volume pipettes to avoid introducing errors from adjustment of pipette volumes.

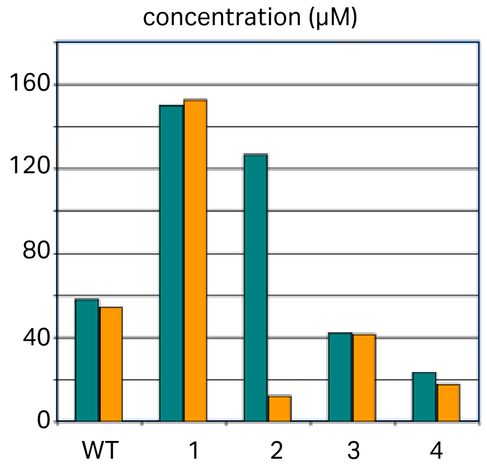
Sample concentrations

Evaluation of association rate constants and affinity constants from the experimental data requires that values are provided for the analyte concentrations (either in molar units or in weight units together with the molecular weight). Different kinds of errors may be distinguished:

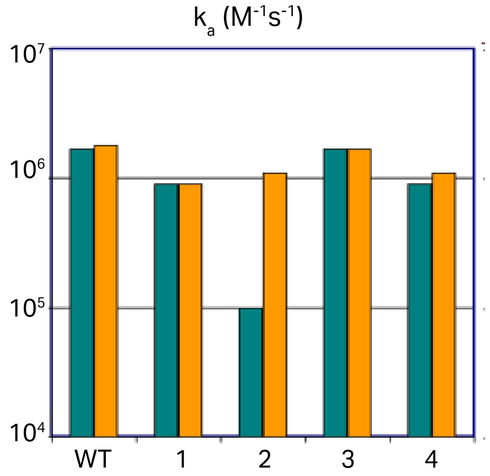
- Random errors in diluting samples from stock solutions, such as pipetting errors, will make the evaluation less robust but not necessarily incorrect
- Systematic errors that are propagated through the dilution series will make the evaluation less robust. In the worst case, the fitting model may be judged inappropriate.
- Errors in the concentration of the stock solution will not affect the fitting quality but will give incorrect values for the association rate constant and the affinity constant (but not for the dissociation rate constant)

Ideally, the analyte concentration should reflect the amount of analyte capable of interacting with ligand, which may not be the same as the total concentration (for example, if the analyte is only partially active). Calibration-free concentration analysis (CFCA) provides an approach to concentration measurement that is directly related to the amount of analyte that can bind to the ligand and is recommended where possible in critical measurements. Note that CFCA is not supported in all Biacore systems.

The illustration to the right shows the importance of using correct analyte concentrations. The experiment compares the kinetics of wild type and four mutants of a papain inhibitor binding to papain. Using concentrations obtained from A_{280} measurements, mutant number 2 had an association rate constant about one order of magnitude lower than the wild type and the other mutants. However, CFCA measurements showed that only a fraction of mutant 2 was active in terms of binding to the surface. Using concentration values obtained with CFCA, association rate constants obtained were similar for all variants of the inhibitor.



Analyte concentrations determined by A_{280} (green bars) and CFCA (yellow bars).



Association rate constants determined using concentration values from A_{280} (green bars) and CFCA (yellow bars).

Sample concentration range

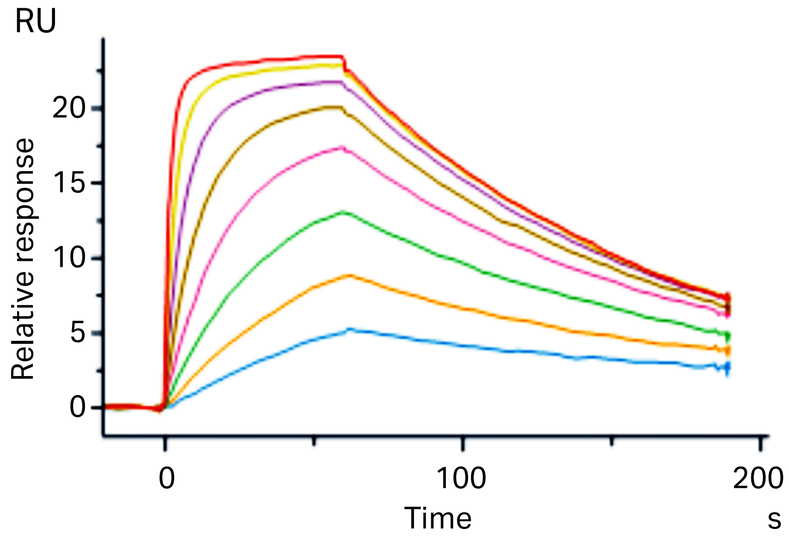
Kinetic determinations

In principle, kinetic constants can be estimated from analysis of a single analyte concentration. However, analysis over several (typically 5 to 9) concentrations gives a better foundation for discovering deviations from the simplest interaction models and for revealing concentration-dependent artefacts such as aggregation of analyte with increasing concentrations.

Ideally, concentrations for kinetic measurements should cover a wide range centered on the value for the dissociation equilibrium constant K_D (for example, for an interaction with K_D = 10 nM, concentrations from 1 to 100 nM are appropriate). A good starting point is usually to let the concentration series range from well below to at least 10 times higher than the expected affinity, giving up to 90% binding site occupancy at steady state.

In practice, the K_D value is often not known. In such cases, run pilot experiments with widely spaced concentrations (for example, 5 concentrations in the range 2 to 1500 nM) may be run to give a first indication (or, in the best case, a determination) of the kinetic and affinity properties. Adjust these values as necessary if the range of concentrations that can be used may be limited by availability or solubility of the analyte. A pilot experiment can be set up to cover a wider range of analyte concentrations using 2D kinetics (see *2D kinetics*, on page 16) on systems that support this mode.

An example of sensorgrams from kinetic analysis with a good range of analyte concentrations is shown to the right.



Affinity determinations

Determination of affinity constants requires at least three analyte concentrations (of which one may be zero) in order to evaluate the data. As a general recommendation, use five non-zero concentrations in addition to zero concentration.

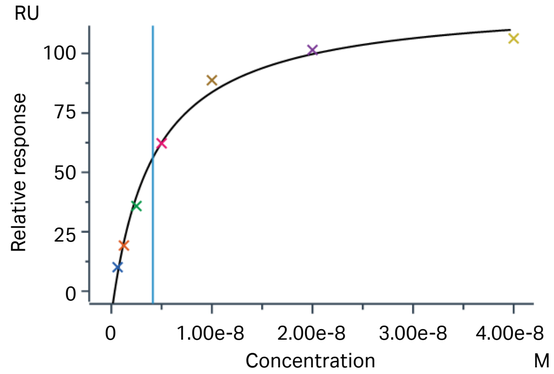
Ideally, the concentration range should extend beyond at least twice the value of the equilibrium dissociation constant K_D: otherwise, the plot of R_{eq} against concentration will have insufficient curvature for robust evaluation. Reported values for K_D value higher than half the highest analyte concentration should be treated with caution. Some Biacore systems support evaluation with a fixed value for the maximum binding capacity R_{max}, which can help in evaluating low affinity (high K_D) interactions where the recommendation cannot be met. See the Application guide *Fragment and small molecule screening* for more details.

Examples of affinity determination with good and poor ranges of analyte concentrations are shown to the right. The K_D value is marked with a vertical blue line.

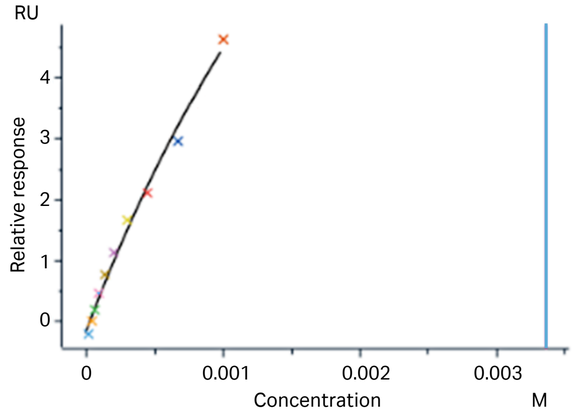
Zero concentration samples (blanks)

Both kinetic and affinity determinations should include at least one (preferably several) blank cycle with zero analyte concentration. For single-cycle experiments, the blank cycle(s) should include the same number of injections as the sample cycles. Blank cycles are subtracted from non-blanks during evaluation, to eliminate any irregularities that appear in all cycles. If multiple blank cycles are included in the experiment, an average of selected cycles may be used for blank subtraction.

Blank subtraction is important for kinetic determination since evaluation relies on all points in the sensorgram, and deviations that are not corrected by blank subtraction can distort the results. Blank subtraction is less critical for affinity measurements since evaluation is based on average responses over a defined time at steady state. Blank subtraction should nevertheless always be applied to sensorgrams for affinity measurements to improve the robustness of the evaluation.



Good concentration range. The K_D value lies within the range.



Poor concentration range. The K_D value lies well outside the range.

Experimental formats

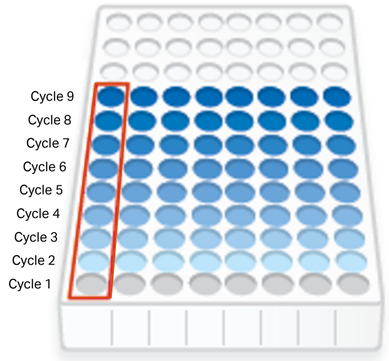
There are several ways of setting up a kinetics or affinity experiment on a Biacore system:

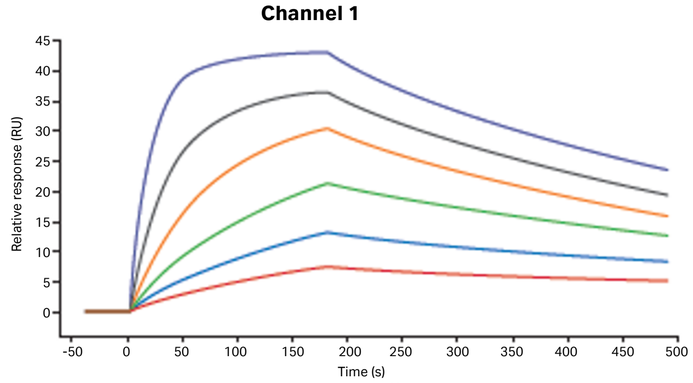
- In *multi-cycle* experiments, each analyte concentration is run in a separate cycle, regenerating the surface after each sample injection
- In *single-cycle* experiments a series of analyte concentrations is injected sequentially in one cycle, with no regeneration between sample injections
- In *parallel* kinetics each analyte concentration is run in the same cycle but in parallel channels. Parallel kinetics is only available in Biacore systems with multiple parallel channels, and is not recommended for affinity determination.
- In *2D* kinetics, different series of the same analyte-ligand interaction are run in single-cycle mode in separate channels, and the results are evaluated as a single data set. Either or both of the ligand level and the analyte concentrations can be varied between series. In this way, a much wider range of concentrations can be covered in one experiment, concentrations that are outside the suitable range can be excluded from the evaluation. *2D* kinetics is only available in Biacore systems with multiple parallel channels.

Multi-cycle kinetics and affinity

In the multi-cycle approach, each analyte concentration (including one or more zero concentration blanks) is run in a separate cycle with regeneration after each injection. To keep the surface properties consistent from cycle to cycle, it is important that the regeneration is complete. At least one blank cycle with running buffer should be included in the run. Multi-cycle kinetics is suitable for running many samples against one ligand.

An example of how a multi-cycle kinetics experiment is set up for a multi-channel Biacore system is shown to the right. Cycle 1 (colored gray in the illustration) is a zero concentration (blank) cycle, providing a separate blank for each channel.

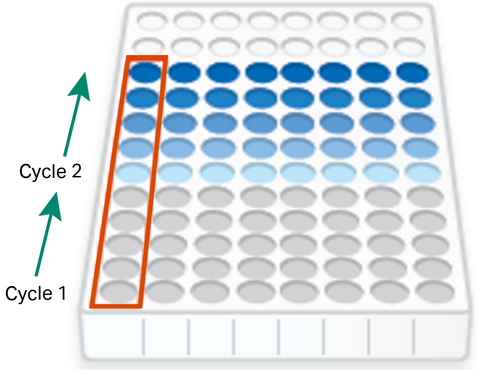


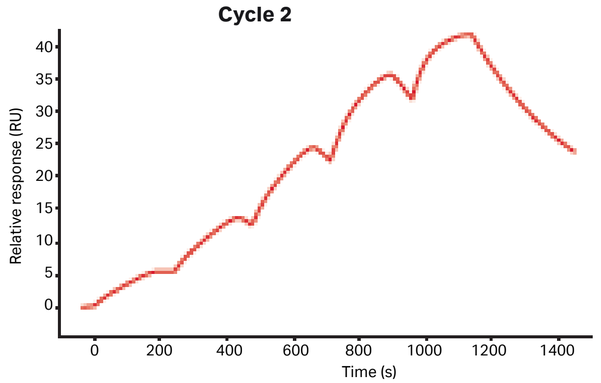


Single-cycle kinetics and affinity

In single-cycle experiments a series of analyte concentrations is injected sequentially in one cycle, with no regeneration between sample injections. Separate cycles are run for zero concentration blanks, replicate measurements, or different interactions. This approach is valuable in situations where acceptable regeneration cannot be achieved, but is more sensitive to response drift since the cycle time is longer. A single dissociation period is used in each cycle, allowing longer dissociation times with less impact on the total run time.

An example of how a single-cycle kinetics experiment may be set up for a multi-channel Biacore system is shown to the right. Cycle 1 (colored gray in the illustration) is a zero concentration (blank) cycle, providing a separate blank for each channel. Note that the blank cycle has the same number of injections as the sample cycle, and therefore occupies significant space in the microplate.

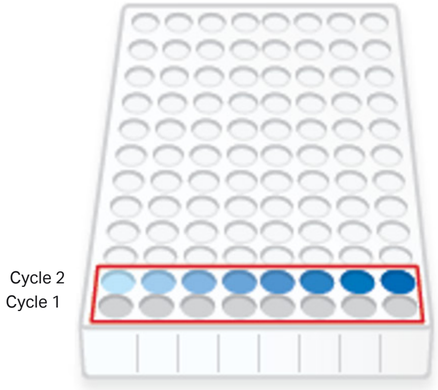


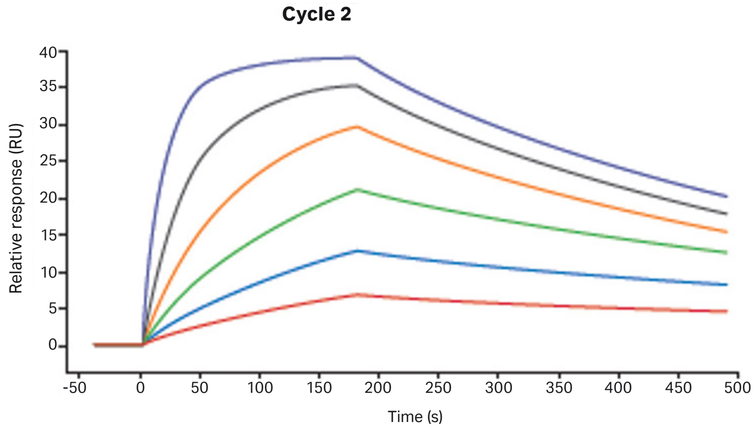


Parallel kinetics

In parallel kinetics, supported in multi-channel Biacore systems, the ligand is immobilized in multiple channels and different concentrations of analyte are injected in each channel in a single cycle. Regeneration is performed between cycles so that the surface can be reused for different analytes. Blanks are run in a separate cycle, providing a separate blank sensorgram for each channel. The number of analyte concentrations is limited by the number of channels in the instrument (for example, the 8-channel instrument Biacore 8K supports up to 8 concentrations over one ligand, or up to 4 concentrations over two different ligands). This approach is valuable for small samples sets or when there is no regeneration available. As with single-cycle kinetics, a single dissociation period is used in each cycle, allowing longer dissociation times with less impact on the total run time. The parallel format is not recommended for affinity measurements.

An example of how a parallel kinetics experiment may be set up for an 8-channel Biacore system is shown to the right. Cycle 1 (colored gray in the illustration) is a zero concentration (blank) cycle, providing a separate blank for each channel.





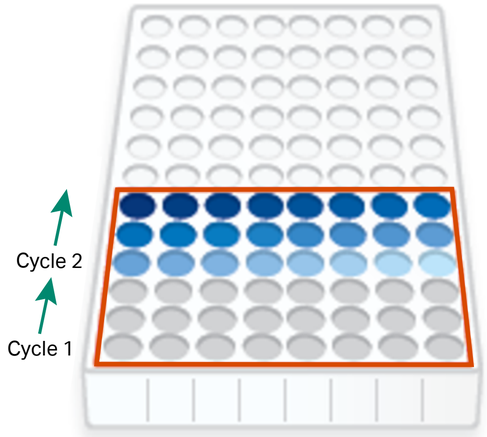
Note: Parallel kinetics is run in a single cycle for each analyte concentration series, but should not be confused with single-cycle kinetics. The term single-cycle kinetics is reserved for the experimental format where analyte concentrations are injected sequentially in the same flow channel.

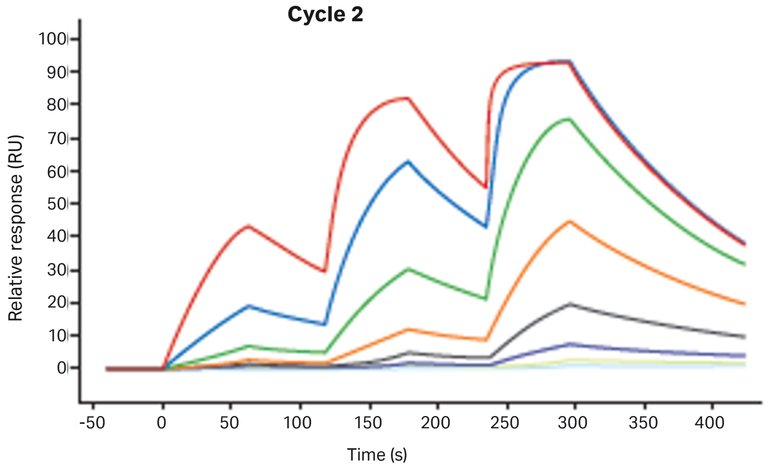
2D kinetics

2D kinetics is a combination of single-cycle and parallel kinetics, and is only supported in multi-channel Biacore systems. Sample dilutions are placed in a 2-dimensional matrix of wells in the microplate, and all samples are evaluated together. Ligand levels may be constant or varied between channels. Typically, single-cycle series covering different concentration ranges are run in each channel, using fewer analyte injections in each cycle than in single-cycle kinetics run in a single channel. The approach can cover a wide analyte concentration range in a single run, and is valuable for previously uncharacterized interactions. The 2D format is not recommended for affinity measurements.

An example of how a 2D kinetics experiment may be set up for a multi-channel Biacore system is shown to the right. Cycle 1 (colored gray in the illustration) is a zero concentration (blank) cycle, providing a separate blank for each channel.

Note: According to the suitability of the analyte concentrations used, the results from a 2D kinetics experiment may be evaluated as single-cycle (by channel), parallel (by cycle) or 2D (channel and cycle combined).





Kinetic analysis

Workflow and settings

The general approach to kinetic analysis is summarized below. The procedure assumes that appropriate assay development has been completed to determine suitable surface preparation and regeneration conditions.

Step	Action
1	Dock a sensor chip and immobilize the ligand or capture molecule. The reference surface can be left untreated, activated/deactivated or immobilized with an irrelevant/inactive ligand. When the ligand is captured, capturing molecule should be immobilized on both the reference and active surfaces, and ligand injected over the active surface only.
2	Run up to 3 startup cycles using buffer as analyte and, if applicable, regeneration to equilibrate the system. Startup cycles should use the same cycle settings as the analysis cycles[1]. For capture assays, run the startup cycles using the selected ligand.
3	Include solvent correction cycles for runs with low molecular weight analytes (see the *Solvent Correction* Application guide).
4	Include zero-concentration (blank) cycle(s) with identical setup as the analysis cycles.
5	Run the sample concentration series from low to high concentration. For multi-cycle experiments, replicates of at least one concentration may be run early and late in the concentration series, to provide a check on the stability of the surface during the course of the experiment.

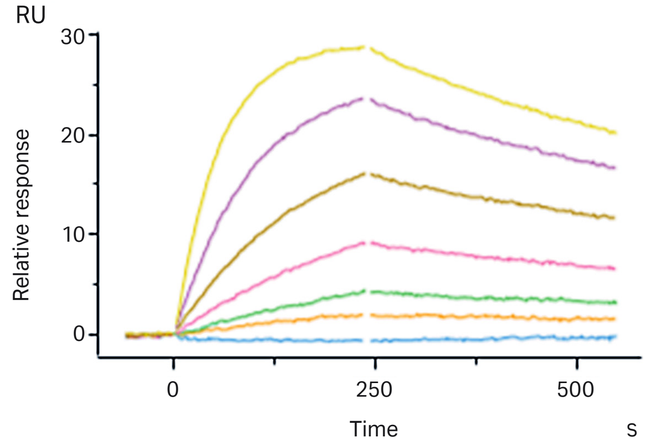
[1] For single-cycle experiments, a single buffer injection may be used instead of a single-cycle injection in the startup cycle, to conserve space in the microplate. Include regeneration in the startup cycles if the surface will be regenerated for use with multiple analytes.

Recommended conditions for typical kinetic experiments are listed to the right. Conditions may need to be adjusted according to the interaction properties (for example, longer dissociation times may be necessary for interaction with very slow dissociation). Many of the settings are discussed in more detail to the right.

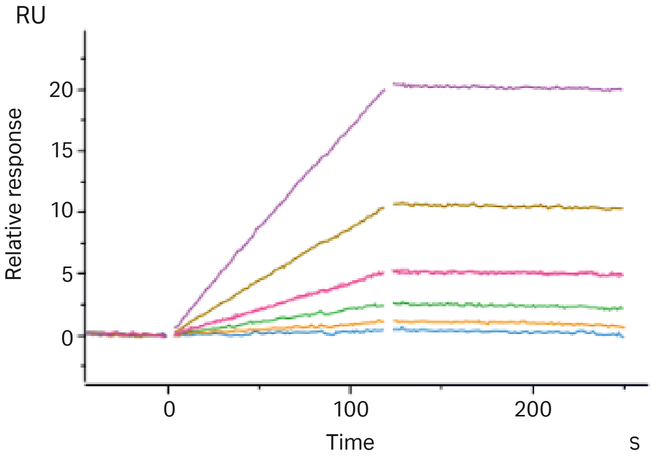
Parameter	Recommendation	Comments
Flow rate for capture	10 µL/min	Minimize reagent consumption
Contact time for capture	60 to 180 s	According to assay requirements
Flow rate for analyte	30 µL/min (default)	Compromise between sample consumption and mass transport limitations
Contact time for analyte (association time)	60 to 180 s	Long enough to give sufficient curvature of the sensorgrams
Dissociation time	Minutes for low affinity interactions. Up to hours for higher affinities.	Interaction dependent
Sample pooling in microplate	Yes for ligand capture. No for samples.	Ligand solution for capture can be pooled to minimize reagent consumption. Do not pool samples, even for replicate measurements. The analyte concentration is critical, and pooling can lead to contamination and evaporation of used microplate positions.
Molecular weight	Required if analyte concentrations are in weight units.	Molar analyte concentrations required for evaluation of association rate constants.
Startup cycles	1 to 3	Sufficient to stabilize the surface
Analyte concentrations	Use molar units.	
Number of analyte concentrations	Typically 5 to 9	More for 2D kinetics
Blank cycles	1 to 3	Use multiple blank cycles for more careful analysis.
Regeneration time	30 to 180 s	As determined by regeneration scouting. If dissociation is rapid, regeneration may be replaced by longer dissociation time.

Association time

The association time should be long enough to allow for sufficient curvature of the sensorgrams during at least some of the analyte injections. Ideally the highest concentration should approach steady state. In practice, the injection time is limited by flow rate and the maximum injection volume supported by the Biacore instrument. The actual time required will depend on the kinetic characteristics of the interaction. In most cases an association time of 1 to 3 minutes is adequate to obtain enough data. The illustration to the right shows an example with good curvature on the top and too little curvature on the bottom.



Ideally, the sensorgram for at least one analyte concentration should approach steady state during sample injection.



If no sensorgrams approach steady state, use a longer sample injection time.

Dissociation time

Dissociation of a 1:1 complex is an exponential process, independent of the analyte concentration used. For reliable evaluation of the dissociation rate constant, the dissociation time that allows a drop in response of about 10% or more of the starting value is recommended. Comparative estimates and dissociation ranking can be performed with dissociation amounts as low as 1%, provided that the relative response at the start of dissociation is sufficiently high. Accurate measurement of slow dissociation rates (k_d 10^{-6} s^{-1} or less) still requires long dissociation times, and it is important that the instrument is well-maintained and solutions carefully prepared to ensure high performance.

Times required to achieve 1% and 10% dissociation for different dissociation rate constants are listed in the table below. Use the table in combination with expected response levels as an approximate guide to suitable dissociation times. For example, an interaction with a k_d of 10^{-6} s^{-1} starting with a response level of 100 RU will take approximately 3 h for dissociation of 1 RU.

A rough estimate of the dissociation rate can often be obtained from assay development work. If no estimate is available, a dissociation time of 10 minutes is generally recommended as a starting point.

Dissociation rate constant k_d (s^{-1})	Time for 1% dissociation	Time for 10% dissociation
10^{-2}	1 s	11 s
10^{-3}	10 s	1 m 48 s
10^{-4}	1 m 40 s	1 min 35 s
10^{-5}	16 m 45 s	2 h 46 m
10^{-6}	2 h 47 m	29 h 20 m

Mass transport considerations

For interaction with the surface-bound ligand to occur, analyte must be transported from the bulk solution to the surface, a process referred to as mass transport. Mass transport is a diffusion-limited process, which can be described in strict mathematical terms under the conditions of laminar flow in the Biacore flow cell. Details may be found in the Application guide *Principles of kinetics and affinity analysis*.

The observed rate of analyte binding is the net result of mass transport and interaction rate. Three situations may be broadly distinguished:

- If mass transport is fast compared with interaction, the observed binding will be limited by the interaction rate. Evaluated rate constants will be relevant to the interaction.
- If mass transport and interaction rates are of similar orders of magnitude, the observed binding will represent both processes. Evaluation is possible provided that the interaction model takes mass transport processes into account.
- If mass transport is slow compared with the interaction, the observed binding will be limited by mass transport. Evaluated rate constants will reflect mass transport processes and will not be relevant to the interaction.

Ways of assessing the relevance of reported rate constants are given in the Application guide *Principles of kinetics and affinity analysis*.

From these considerations, it is clear that complete mass transport limitation should be avoided in kinetic determination. To minimize the effect of mass transport limitation, analyses should be run at a high flow rate and with a low ligand level (so that absolute rate of analyte binding is low and less likely to be limited by mass transport).

Note: It may seem that lowering the analyte concentration should reduce the mass transport effect. However, both the rate of mass transport and the absolute interaction rate are directly proportional to the analyte concentration, so that changing the concentration has no effect on mass transport limitations.

A test to see whether an interaction is mass transport limited is to run the assay at multiple flow rates and see how this affects the obtained rate constants. If the constants remain unaffected by flow rate changes, the interaction is probably not mass transport limited. If faster kinetics is obtained with a higher flow rate, the interaction is limited with respect to mass transport. Note that the mass transport rate is proportional to the flow rate raised to the power of 1/3, so that an eightfold change in flow rate results in only a twofold change in mass transport rate.

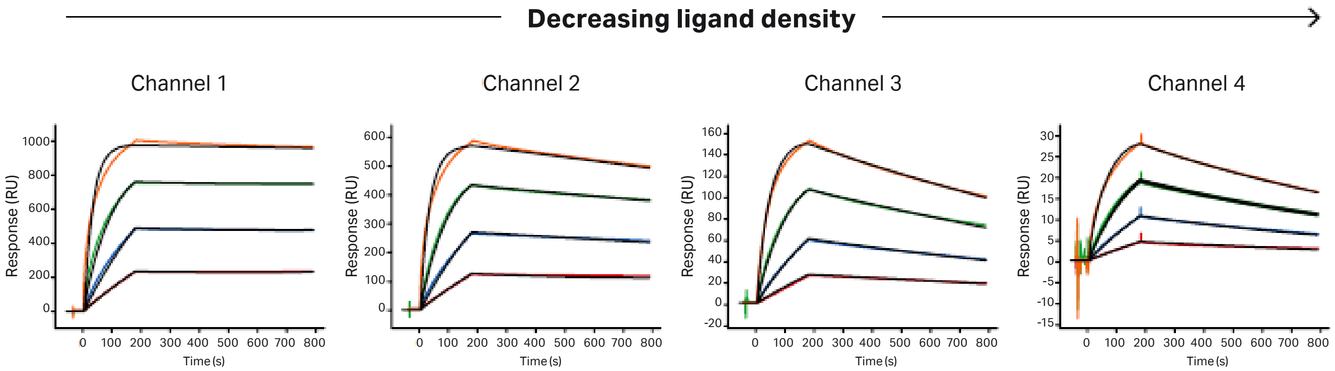
Multivalent analytes (avidity)

Interactions between monovalent surface-bound ligands and multivalent analytes such as antibodies generate avidity effects that are difficult to analyze. This is because release of an analyte molecule from the surface requires simultaneous dissociation at multiple sites, so that the observed dissociation rate is slower than a corresponding 1:1 interaction.

Interactions in which one of the interactants is multivalent are best handled by immobilizing the multivalent molecule on the surface and injecting its binding partner as the analyte. Multiple sites on the ligand behave as independent binding sites.

Avidity effects can be minimized by using extremely low immobilization levels to reduce the possibility of one analyte binding to multiple ligand molecules. Flat sensor surfaces such as Sensor Chip C1 or Sensor Chip PEG can be useful in achieving low ligand immobilization levels.

To test for avidity, determine the kinetics over a series of sensor surfaces with varying ligand levels. As the ligand level decreases, reduction in avidity will be seen as an increase in dissociation rate. The example below illustrates the interaction of a dimeric analyte with a ligand. The figure clearly shows how the dissociation rate increases as avidity is reduced with decreasing immobilization level.



Channel	Immobilization level (RU)	Apparent dissociation constant (s^{-1})
1	373	3.1×10^{-5}
2	169	2.3×10^{-4}
3	34	6.7×10^{-4}
4	3.4	8.9×10^{-4}

Note: The y-axis is scaled automatically in each plot according to the response range.

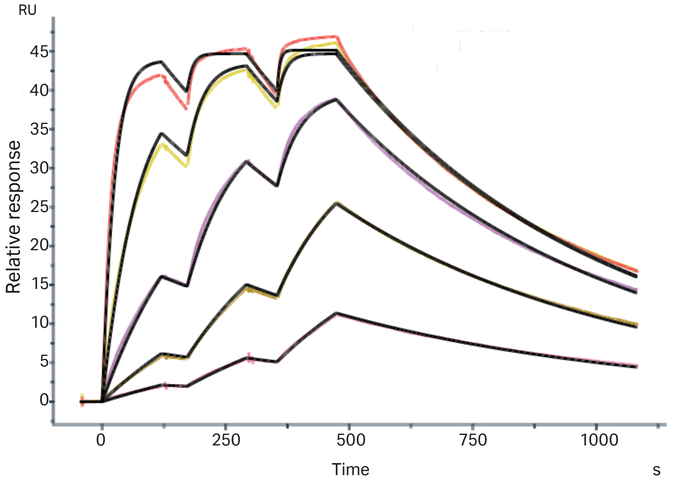
Heterogeneous ligand

When ligand on the sensor surface is heterogeneous (containing multiple analyte binding sites with different kinetic characteristics), the observed sensorgram represents the sum of the individual binding events. The evaluation software can resolve kinetic constants for two distinct ligand sites but not more.

Take the following precautions to reduce or eliminate heterogeneous binding behavior:

- Purify the ligand thoroughly
- Use directed immobilization chemistry (such as thiol coupling) to reduce heterogeneity introduced by the immobilization procedure. A capturing approach may in some cases result in a more homogeneous ligand population on the sensor surface.
- Use low immobilization levels and low analyte concentrations to reduce the contribution from low affinity binding sites

In some cases, heterogeneous behavior may be eliminated during evaluation of the results by excluding the highest analyte concentrations. The illustration to the right shows a 2D kinetics experiment where heterogeneity is apparent at the highest analyte concentrations. A good fit to the 1:1 interaction model was obtained when the highest analyte concentrations were excluded from the evaluation.



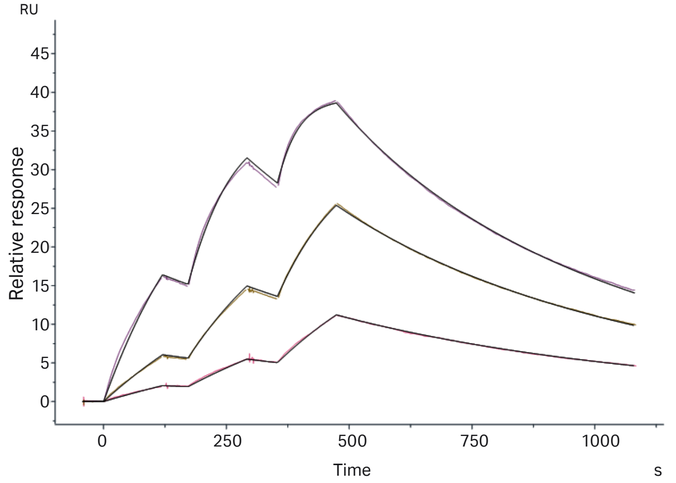
The model does not fit the data for the highest analyte concentrations.

Heterogeneous analyte

If the analyte is heterogeneous, the kinetics can only be evaluated if the relative contributions of the different species to the overall binding behavior are known. A fitting model for heterogeneous analytes is not provided with all Biacore systems.

Comparative kinetic screening

Comparison of relative interaction kinetics can be sufficient for many applications. At the simplest level, this can be achieved by plotting the ratio of response values early and late in the sample injection (for association rates) and in the dissociation phase (for dissociation rates), using a single analyte concentration. This simplified approach is sometimes used in information-rich screening work, where full determination of kinetics and/or affinity is not practicable for throughput considerations. For more information on kinetic screening, see the Application guide *Antibody screening with Biacore systems*.



Excluding two cycles with the highest concentrations results in a good fit.

Evaluation of kinetics

Introduction

Kinetics experiments are evaluated by finding the best fit for a mathematical model of the interaction to the experimental data. Theoretical aspects are described in the Application guide *Principles of kinetics and affinity analysis*.

Note: Fitting is a purely mathematical process. Good fitting of the data set to an interaction model does not imply that the model is a correct physical or biological description of the interaction mechanism.

Workflow for kinetics evaluation

A recommended workflow for evaluation of kinetics is summarized to the right.

Step	Action
1	Prepare the data as required: • Perform solvent correction if included in the experiment • Exclude seriously disturbed sensorgrams. If too many sensorgrams are disturbed, you may need to rerun the experiment. • Exclude transient disturbances such as air spikes and baseline shifts In most Biacore systems, it is not necessary to manually adjust baselines to zero or subtract blank sensorgrams. These steps are performed automatically as part of the evaluation procedure.
2	Evaluate the data using the simplest model (1:1 binding), even if you suspect that the interaction may be more complex. If the 1:1 model fits the data, evaluation with a more complex model will not give additional significant information.
3	Assess the fitting quality (see *Assessing the fit*, on page 25 for details)
4	If the fitting is not acceptable: • Examine the sensorgrams from the reference surface. Binding to the reference surface can complicate evaluation of the reference-subtracted data and should be eliminated as far as possible during assay development. • Review the choice of blank cycles • Review the choice of fitting model. Ideally, use of a complex model should be supported by independent evidence (for example, antibodies that are known to be bivalent may be expected to show bivalent kinetics when used as analytes). Better fitting to a more complex model should not in itself be taken as evidence of a complex interaction mechanism, but may suggest additional experiments to test the complex interaction hypothesis.

Choice of blank cycles

The purpose of blank cycles (cycles with zero analyte concentration) in kinetic analysis is to remove systematic drift and disturbances by subtracting blank sensorgrams from the analyte cycles. For careful determinations, multiple blank cycles run at intervals throughout the experiment are recommended. The evaluation software gives the option of subtracting the nearest preceding blank or an average of all blanks, to accommodate changes in the blank behavior during the course of the experiment.

The response in blank cycles should be close to zero. Do not use blank cycles that are not representative for the analyte cycles (for example, blanks disturbed by baseline shifts or drift). Subtracting non-representative blanks will introduce unnecessary errors in the evaluation.

Available fitting models

Available fitting models for kinetic analysis are listed to the right. The models are described in more detail in the Application guide *Principles of kinetics and affinity analysis*.

Note: The availability of models may differ for different Biacore systems.

Model	Description
1:1 binding	Describes binding of two monovalent interactants to form a binary complex. This is the simplest and most commonly used model.
1:1 dissociation	Describes dissociation of a binary complex. This process is independent of analyte concentration.
Bivalent analyte	Describes binding of a bivalent analyte in solution to monovalent ligand on the surface. One analyte molecule can bind to two separate ligand sites resulting in avidity effects.
Heterogeneous ligand[1]	Describes binding of a homogeneous analyte to a mixture of two independent ligands with different interaction properties (either different molecules or different sites on the same molecule). The observed kinetics is the sum of the kinetics for interaction with the two ligands.
Two-state reaction	Describes a 1:1 interaction where the complex is stabilized by a transformation that occurs after the interaction, on a slower time scale than the interaction itself. This model is necessarily a simplification of the actual mechanisms involved in a two-state reaction.

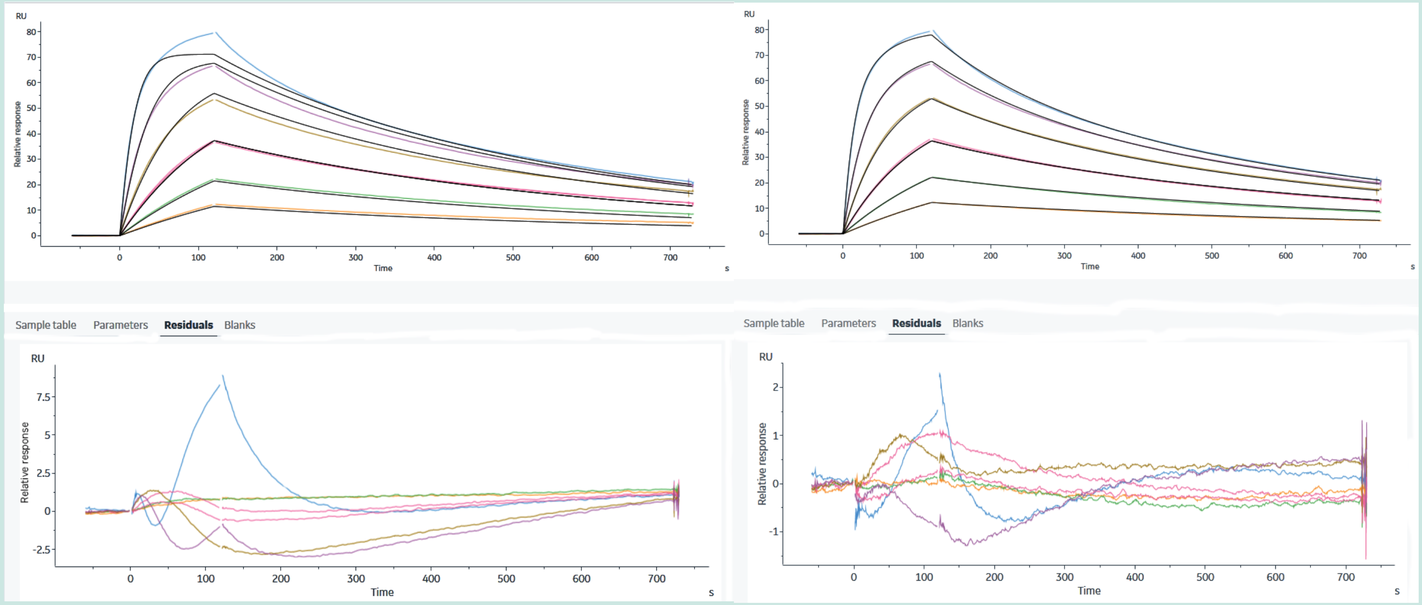
[1] Older Biacore systems also include a model for heterogeneous analyte kinetics. This model requires knowledge of the relative concentrations of the analytes and may not be supported in newer systems.

The 1:1 model should always be used as a starting point for evaluation of previously uncharacterized interactions. More complex models may be tested if the simple model does not fit.

Assessing the fit

Assess the fitting results as follows:

Step	Action
1	Inspect the fit visually. Do the fitted curve(s) follow the experimental data closely, or are there systematic shape differences between experimental and fitted curves? Systematic differences indicate that the chosen model is not precisely appropriate for the experimental data. Decide whether to test other models based on the extent of the differences and the purpose of the experiment. The closeness of fit is visualized in residual plots, which show the difference between fitted and experimental curves for each point. These plots emphasize the shape of deviations between the curves. The illustration below shows sensorgrams and corresponding residual plots for the same data evaluated with unsuitable and suitable models. Note the difference in scale of the residual plots.



Poor fit. Approximate residual range -3 to 8 RU.

Good fit. Approximate residual range -1 to 2 RU

2	Examine statistical parameters and (where provided) the QC report. Statistical parameters are summarized below and described in more detail in the Application guide *Principles of kinetics and affinity analysis*.
3	Assess the contribution of mass transport limitations to the observed kinetics as described below.

Statistical parameters

The closeness of fit between the experimental data and the fitted curve is formally described by a set of statistical parameters, listed in the table to the right.

Mass transport contribution

If the observed interaction is limited by mass transport processes (described in detail in the Application guide *Principles of kinetics and affinity analysis*), values for the rate constants will be reported but will have no significance. Frequently (but not always) the reported values are well outside the range that can be measured with Biacore systems, for example, k_a values of 10^{10} $M^{-1}s^{-1}$ or higher.

Fitting to kinetic models reports a value for t_c, the flow-rate independent component of the mass transport coefficient (see the Application guide *Principles of kinetics and affinity analysis*). As a rule of thumb, mass transport limitations can be ignored if the t_c value is at least 100-fold higher than the reported k_a and the SE value indicates that t_c is not significant. Many interactions show partial mass transport limitation, and the SE values indicate that both t_c and k_a are significant. Rate constants obtained from such experiments can usually be trusted.

To test experimentally whether mass transport limitations are significant, run the samples at several widely different flow rates (for example, 5, 15 and 75 µL/min). Some Biacore systems provide an application wizard to set up such a control experiment. The mass transport rate varies with the cube root of the flow rate, while the interaction rate is independent of flow rate. If the sensorgrams show binding rates that vary with flow rate, the binding is at least partially limited by mass transport. Depending on the extent of the limitation, it may still be possible to obtain valid kinetic constants from the evaluation.

Some Biacore systems provide visual tools to help in assessing the significance of reported kinetic parameters. These tools are described in the documentation for the respective systems.

Parameter	Description
Chi-square	A measure of the closeness of fit, calculated as the average squared residual (the difference between the experimental data and the fitted curve).
Standard error (SE)	A measure of the parameter significance, reported separately for each parameter. A parameter with low significance can have a wide range of values without affecting the closeness of fit significantly. **Note:** The value reported for a parameter with low significance will not necessarily vary between different evaluation instances. The fitting algorithm applied to the same data will return the same set of values, even for parameters that are statistically not significant.
T-value	The parameter value divided by the standard error. This can make it easier to compare the significance of parameters with widely differing absolute values.
Uniqueness (U-value)	An estimate of the uniqueness of the calculated values for rate constants and R_{max}. For correlated parameters, the fitting procedure can determine their relative magnitudes but not absolute values. For example, knowing the affinity gives the ratio but not the values for rate constants. U-values above about 25 indicate that two or more of the parameters (rate constants and R_{max}) are correlated and the absolute values cannot be determined. If the U-value is below about 15 the parameter values are not significantly correlated. **Note:** Some Biacore systems do not report a U-value.

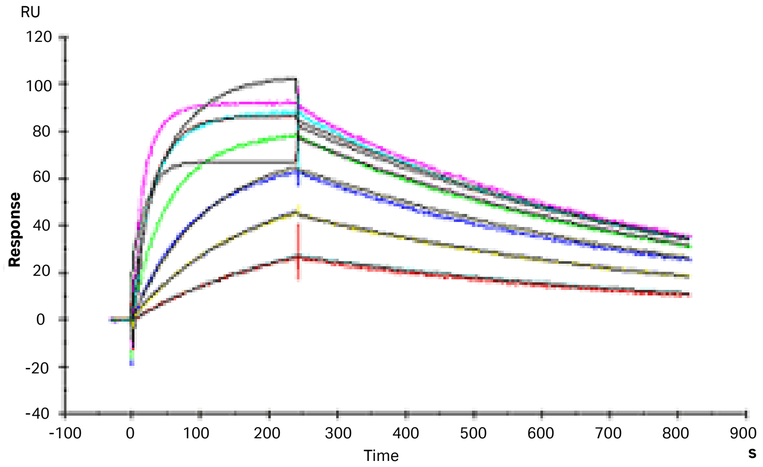
If the fitting is not acceptable

Most commonly, the fitting results are not acceptable because the model is not appropriate for the interaction. It is important to realize that the mathematical models used for curve fitting can only represent simplifications of real-life interaction mechanisms. It is not uncommon to find that the experimental data does not fit any available model satisfactorily, in which case valid kinetic constants cannot be obtained. It may however be possible to perform comparative analyses using apparent constants or sensorgram shape.

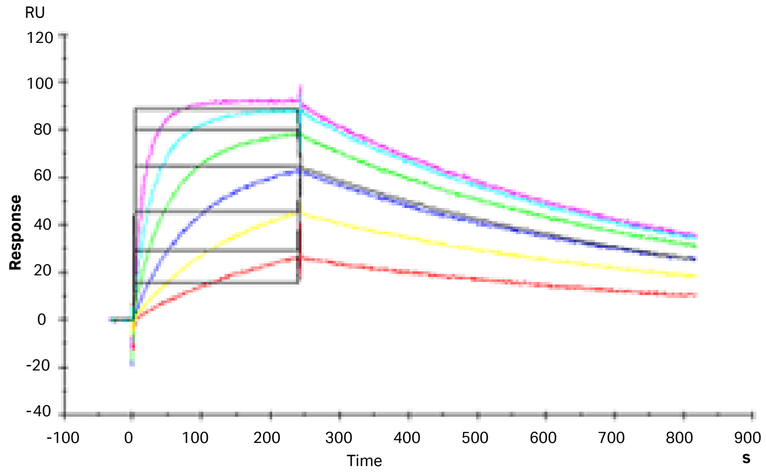
Some unacceptable situations, such as analyte concentrations or initial parameter values that are incorrect by several orders of magnitude, give characteristic fitting results. Two examples are illustrated to the right. These errors can usually be corrected in the evaluation software, either in the variables or keyword table or in the initial values for the fitting model.

The status of fitting parameters (local, global, or constant) can also have important consequences for the quality of fit. For example:

- The analyte binding capacity R_{max} is normally a global parameter, but should be set to local for multi-cycle experiments based on ligand capture, to allow for variations in ligand level between cycles
- Setting the kinetic constants k_a and/or k_d to local parameters can help to reveal progressive changes in the interaction characteristics during the course of an experiment. This kind of information can be useful for troubleshooting kinetic determinations.
- The bulk refractive index parameter RI is normally evaluated as a local parameter, but rapid interaction events can sometimes be wrongly interpreted by the curve fitting algorithm as bulk effects. Setting RI to a constant value of 0 can correct this, provided that refractive index is closely matched.



Pipetting error results in two sample concentrations interchanged in the microplate.



Sample concentrations or initial values incorrect by several orders of magnitude (for example, molar instead of micromolar).

Affinity analysis

Methods of affinity measurement

Analyzing the level of steady state or equilibrium binding as a function of interactant concentrations is the foundation for many standard techniques for determining interaction affinity. In Biacore systems, steady state affinity experiments are evaluated by plotting the response at equilibrium (R_{eq}) against analyte concentration and fitting a model for 1:1 equilibrium binding to the curve. Analysis is generally limited to 1:1 binding. A model for binding at two independent ligand sites can sometimes be useful, but the fitting of more complex models is typically not sufficiently robust for reliable analysis of the data. Classical Scatchard analysis may be applied to data obtained from Biacore systems, to give some indication of the causes of deviation from 1:1 binding behavior.

Steady state affinity analysis requires that binding reaches steady state during sample injection, but does not require resolution of the interaction kinetics. The approach can thus be used for fast interactions where kinetics are not available.

The affinity constant K_D (or K_A) can also be obtained from kinetics analysis as the ratio of the rate constants k_a and k_d. Ideally, values determined from kinetics and from steady state measurements should be the same. In practice, these approaches are generally applicable in different situations that are often mutually exclusive: kinetics analysis can be used for sensorgrams that contain kinetic information but do not necessarily reach steady state, while determination from steady state measurements is most useful for interactions where the kinetics are too fast to measure with confidence, but steady state is reached with relatively short injection times.

Some Biacore systems provide support for determination of affinity in solution, by measuring the free concentrations of interactants in steady state mixtures. This approach is outside the scope of the current Application guide.

Workflow and settings

The general approach to affinity analysis is summarized below. The procedure assumes that appropriate assay development has been completed to determine suitable surface preparation and regeneration conditions.

Note: This section considers affinity analysis based on steady state measurements. Values for affinity constants can also be derived from kinetic measurements, as the ratio of the rate constants for a 1:1 interaction.

Step	Action
1	Dock a sensor chip and immobilize the ligand or capture molecule. The reference surface can be left untreated, activated/deactivated or immobilized with an irrelevant/inactive ligand. When the ligand is captured, capturing molecule should be immobilized on both the reference and active surfaces, and ligand injected over the active surface only.
2	Run up to 3 startup cycles using buffer as analyte and, if applicable, regeneration to equilibrate the system. Startup cycles should use the same cycle settings as the analysis cycles[1]. For capture assays, run the startup cycles using the selected ligand.
3	Include solvent correction cycles for runs with low molecular weight analytes see the *Solvent Correction* Application guide).
4	Include zero-concentration (blank) cycle(s) with identical setup as the analysis cycles.
5	Run the sample concentration series from low to high concentration. For multi-cycle experiments, replicates of at least one concentration may be run early and late in the concentration series, to provide a check on the stability of the surface during the course of the experiment.

[1] For single-cycle experiments, a single buffer injection may be used instead of a single-cycle injection in the startup cycle, to conserve space in the microplate. Include regeneration in the startup cycles if the surface will be regenerated for use with multiple analytes.

Recommended conditions for typical affinity experiments are listed below. Conditions may need to be adjusted according to the interaction properties (for example, longer association times may be necessary for slow interactions to reach steady state). Many of the settings are discussed in more detail below.

Parameter	Recommendation	Comments
Flow rate for capture	10 µL/min	Minimize reagent consumption
Contact time for capture	60 to 180 s	According to assay requirements
Flow rate for analyte	30 µL/min (default)	Lower flow rates may be used to conserve sample.
Contact time for analyte (association time)	60 to 300 s	Long enough to reach steady state
Dissociation time	0	Dissociation time is not relevant unless used instead of regeneration.
Sample pooling in microplate	Yes for ligand capture No for samples	Ligand solution for capture can be pooled to minimize reagent consumption. Do not pool samples, even for replicate measurements. The analyte concentration is critical, and pooling can lead to contamination and evaporation of used microplate positions.
Molecular weight	Required if analyte concentrations are in weight units.	Molar analyte concentrations required for evaluation of affinity constants.
Startup cycles	1 to 3	Sufficient to stabilize the surface
Analyte concentrations	Recommended 1.5 × dilution series	Wider spacing may not properly reveal the shape of the plot of R_{eq} against concentration.
Number of analyte concentrations	Typically 5 to 8	Evaluation requires at least 3 points on the plot of R_{eq} against concentration.
Blank cycles	1 to 3	Use multiple blank cycles for more careful analysis
Regeneration time	30 to 180 s	As determined by regeneration scouting. If dissociation is rapid, regeneration may be replaced by longer dissociation time.

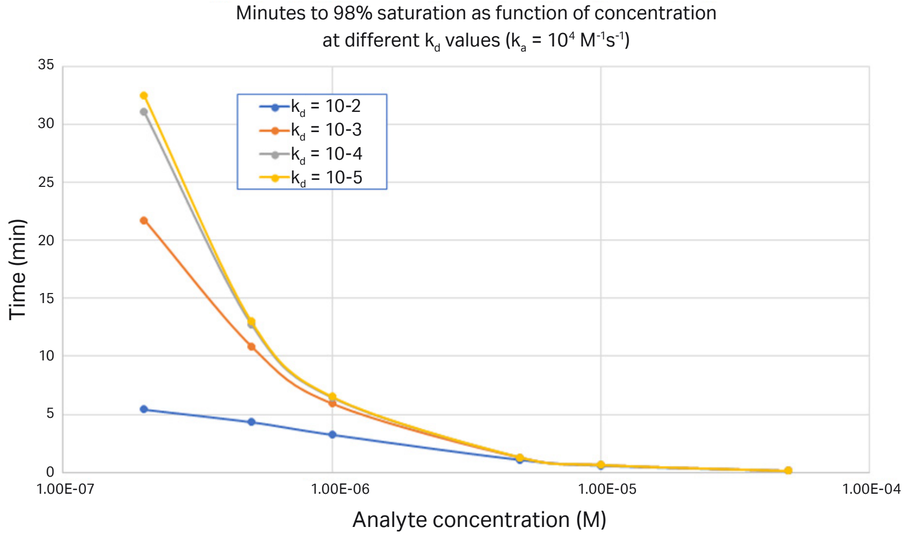
Association time

The association time in an affinity assay should be long enough to allow the response to approach steady state during sample injection. In most cases an association time of 1 to 5 minutes is adequate.

The time to reach steady state is related to the rate constants (both association and dissociation) and to the analyte concentration. In general, faster association and dissociation rates and higher analyte concentrations require shorter times to reach steady state. The illustration to the right shows the time required to reach steady state (calculated as 98% of the equilibrium level) plotted as a function of analyte concentration for different dissociation rate constants. The same association rate constant applies for all curves.

Note: The time to reach steady state is an inverse function of the expression (k_aC+k_d). The dissociation rate will have less effect if it is small relative to the product of association rate constant and concentration.

In some cases, it is not practically possible to reach steady state during sample injection because of limitations on injection time and sample volume. If some but not all of the concentrations reach steady state, the value obtained for K_D will be close to the true value. If some or all of the concentrations do not reach steady state, the value for K_D will be underestimated.



Dissociation time

Steady state affinity is assessed at equilibrium, i.e., when the rates of complex formation and dissociation are equal. For evaluation purposes, there is therefore no need to include a dissociation phase in the assay. A dissociation phase may however be used to allow complete dissociation in place of regeneration in cases of rapid dissociation.

Evaluation of affinity

Introduction

Affinity experiments are evaluated by plotting the steady state response R_{eq} against the analyte concentration C and fitting the equation describing steady state binding to the experimental data. Details of the equation are described in the Application guide *Principles of kinetics and affinity analysis*.

The simplest equation used for evaluation describes 1:1 binding. The analyte concentration range should cover at least twice the reported K_D value. Two variants used primarily in fragment binding experiments are provided in some Biacore systems:

- 1:1 binding with constant R_{max}, which is used for weak interactions where sufficiently high analyte concentrations cannot be used due to solubility limitations
- 2-site binding with constant R_{max} at one site, which is used for interactions where the analyte binds to one primary site and one or more weak secondary sites on the ligand

Note: It is relatively simple to write an equation that describes 2-site binding with K_D and R_{max} as fitted parameters for both sites. However, fitting such an equation to experimental data is not robust, and evaluation of 2-site binding with variable R_{max} at both sites is not supported in the evaluation software.

Workflow for affinity evaluation

A recommended workflow for evaluation of steady state affinity is summarized below.

Step	Action
1	Prepare the data as required: • Perform solvent correction if included in the experiment • Exclude cycles where the analyte response does not reach steady state • Exclude cycles with disturbances that affect the steady state response measurement. Alternatively, move the measurement region away from the disturbance. In most Biacore systems, it is not necessary to manually subtract blank sensorgrams. This is performed automatically as part of the evaluation procedure.
2	Evaluate the data using the appropriate steady state model (see *Choice of fitting model*, on page 32).
3	Assess the fitting quality (see *Assessing the fit*, on page 32 for details).
4	If the fitting is not acceptable: • Review the choice of blank cycles • Review the choice of fitting model

Choice of blank cycles

The purpose of blank cycles in a steady state affinity experiment is to remove systematic baseline drift. Blank subtraction is not critical for affinity analysis, provided that the drift is constant throughout the experiment. Constant drift will affect the reported offset value (the response at zero concentration) but will have no effect on the K_D or R_{max} parameters. However, if the drift changes during the course of the experiment, blank cycles can be repeated at intervals and blank subtraction performed using the nearest preceding blank.

Choice of fitting model

Choose the fitting model according to the appearance of the plot of R_{eq} against C.

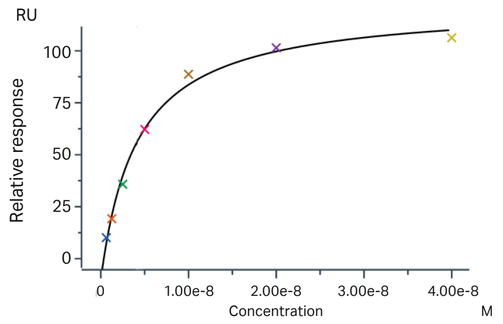
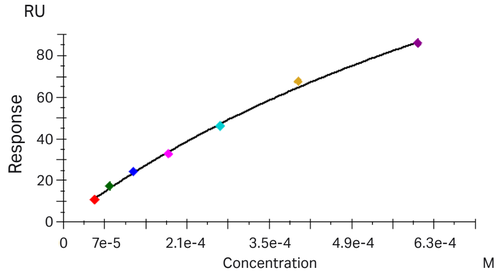
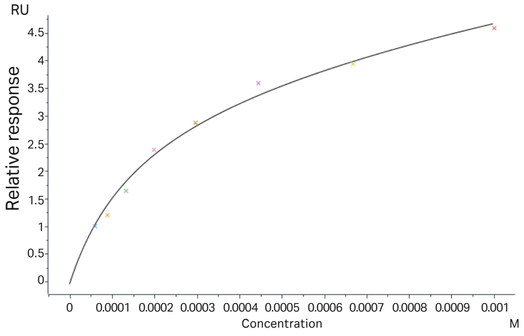
Note: Some models may not be supported in all Biacore systems.

Assessing the fit

Assess the affinity results as follows:

- Confirm that the sensorgrams reach a steady state response
- Examine the plot of R_{eq} against C for curvature and analyte concentration range:
  - The plot should flatten out at the highest analyte concentrations
  - The reported K_D should be less than half the highest analyte concentrations. The K_D value is indicated on the evaluated plot.
- Examine the reported R_{max} value. A high value in relation to theoretical R_{max} may indicate non-specific binding or analyte aggregation. A low R_{max} may indicate loss of activity.
- The offset value should be close to zero. If the offset is high or negative, check reference flow cell and blank cycles. Significant reference and blank responses indicate background binding and/or flow system contamination.

Note: If steady state affinity evaluation is applied to sensorgrams that have not reached steady state, the evaluation model may fit the plot of R_{eq} against C but the K_D will be underestimated.

Plot appearance	Description	Model
	Plot flattens out at high concentrations	1:1 binding
	Plot does not flatten out because sufficiently high analyte concentrations cannot be achieved.	1:1 binding with constant R_{max}
	Plot shows initial curvature followed by a gradual increase in response	Multi-site binding with constant R_{max}

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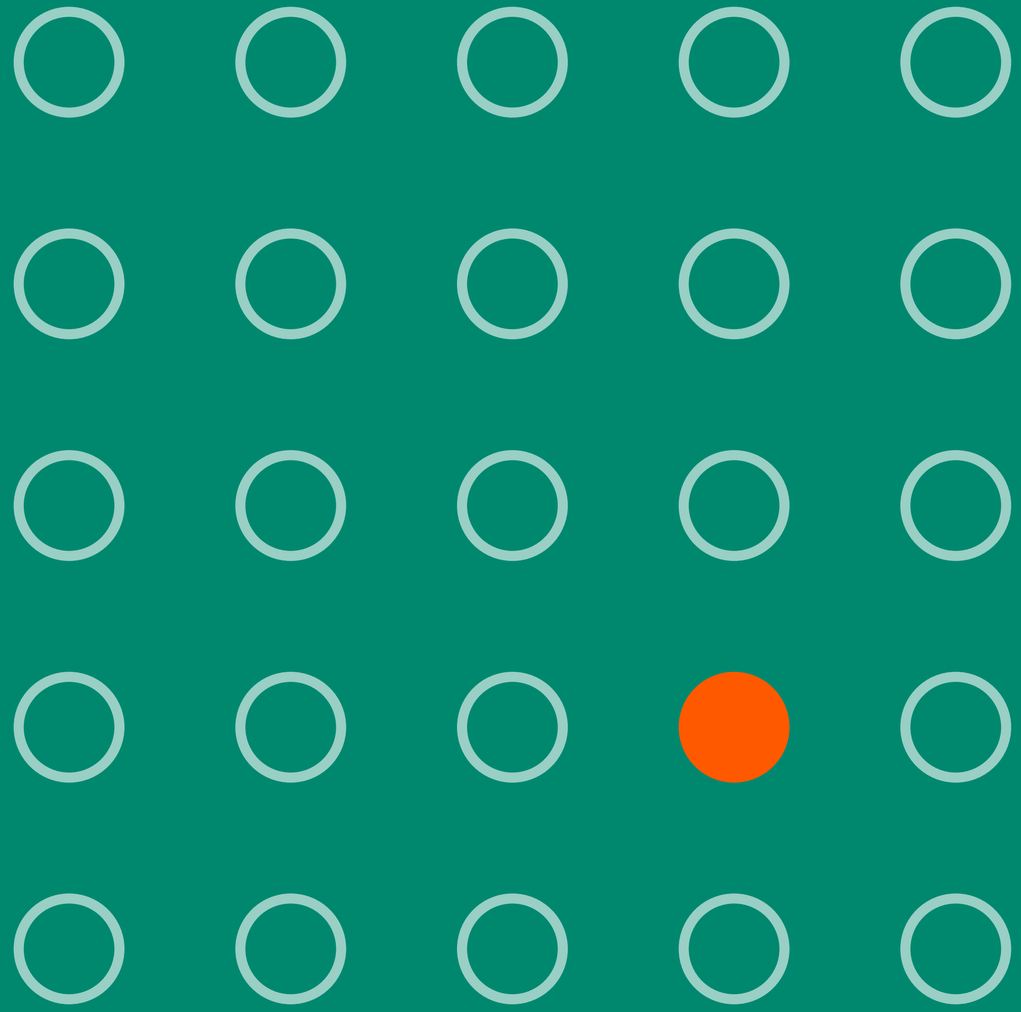

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