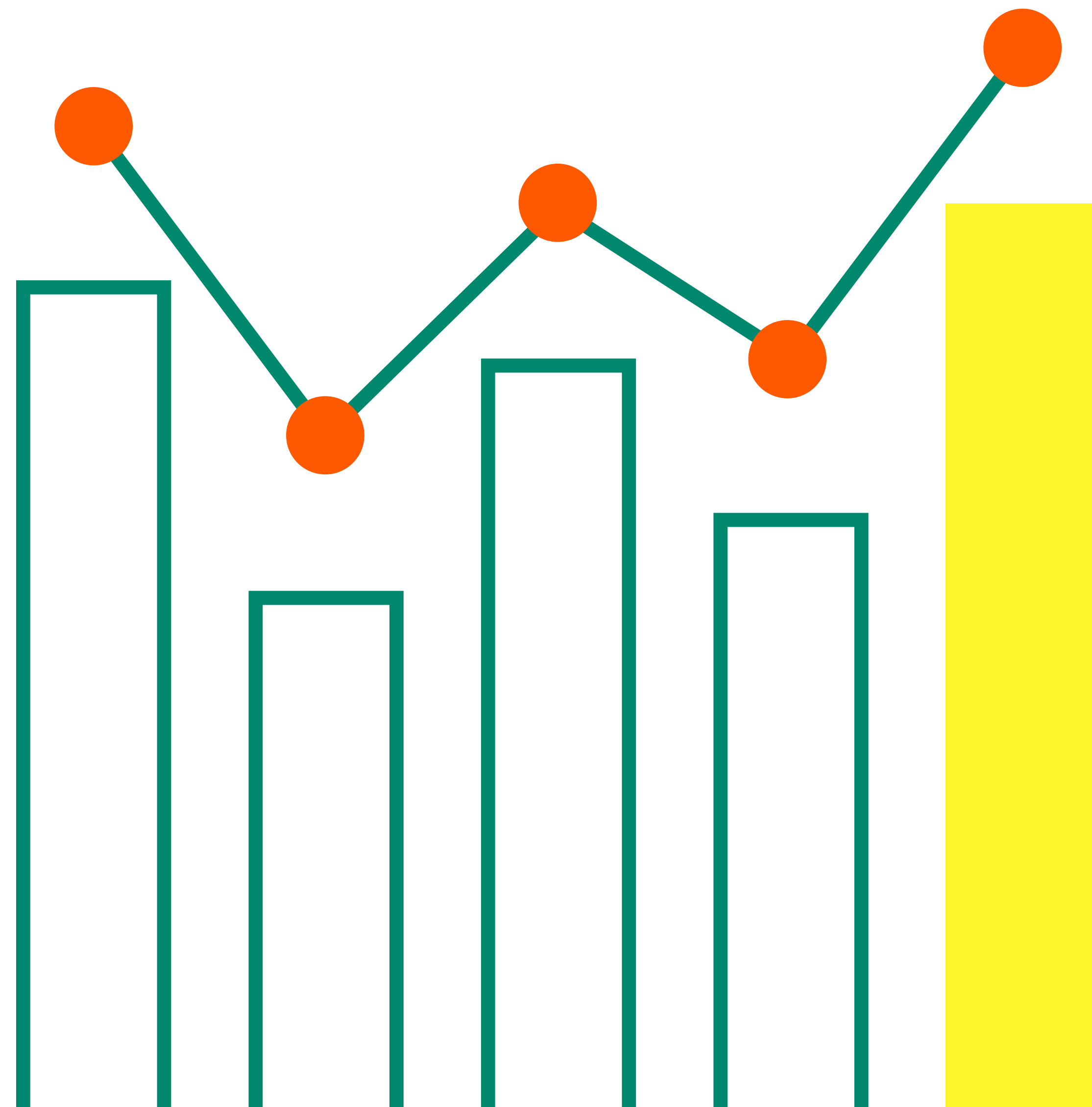


Principles of kinetics and affinity analysis



Introduction

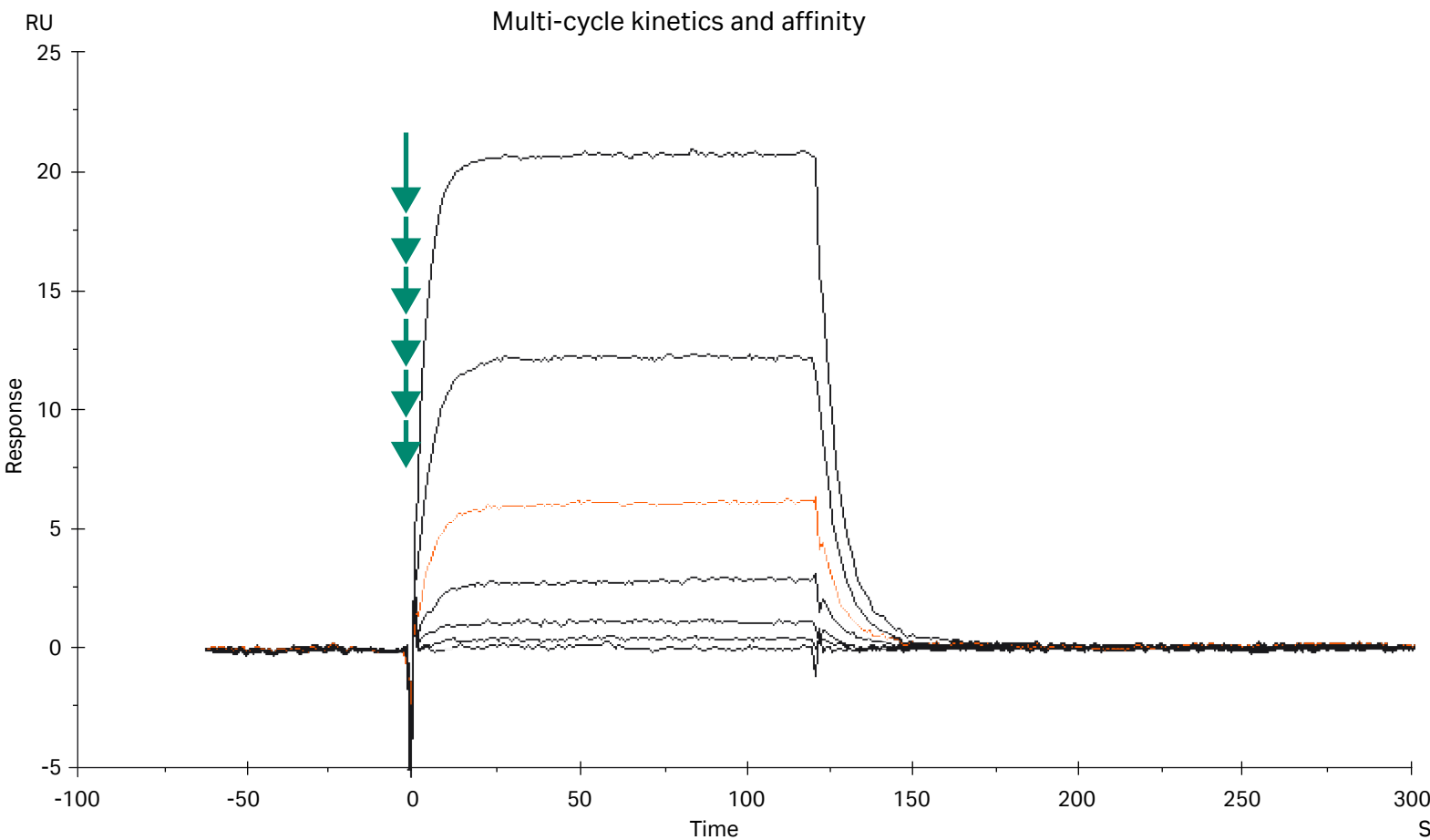
This Application guide gives an overview of the theoretical principles of kinetics and affinity determination with Biacore systems, insofar as the theory is relevant to interpretation of the experimental results.

Practical aspect of kinetics and affinity determination are covered in a separate Application guide.

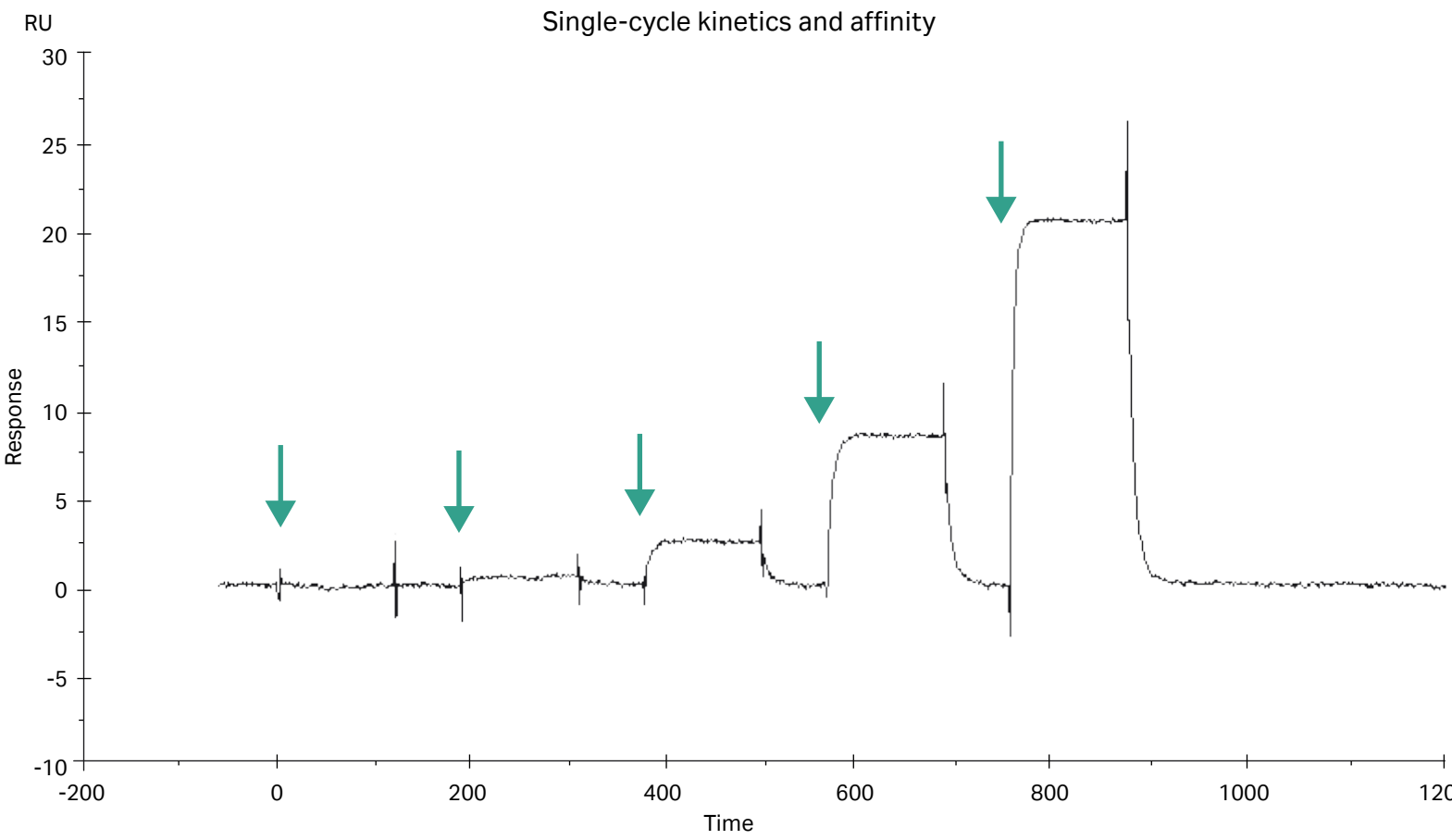
Kinetic determination

Interaction kinetics are determined from the change in response as a function of time, as represented in the sensorgram. Sensorgrams are recorded for a series of analyte concentrations and evaluated together as one data set, and a mathematical model of the interaction is fitted to the experimental data.

The analyte concentrations may be injected in separate cycles with surface regeneration between the cycles (multi-cycle analysis) or sequentially in a single cycle with no regeneration between injections (single-cycle analysis), as illustrated below. In either case, a separate fitted curve is obtained for each analyte concentration.



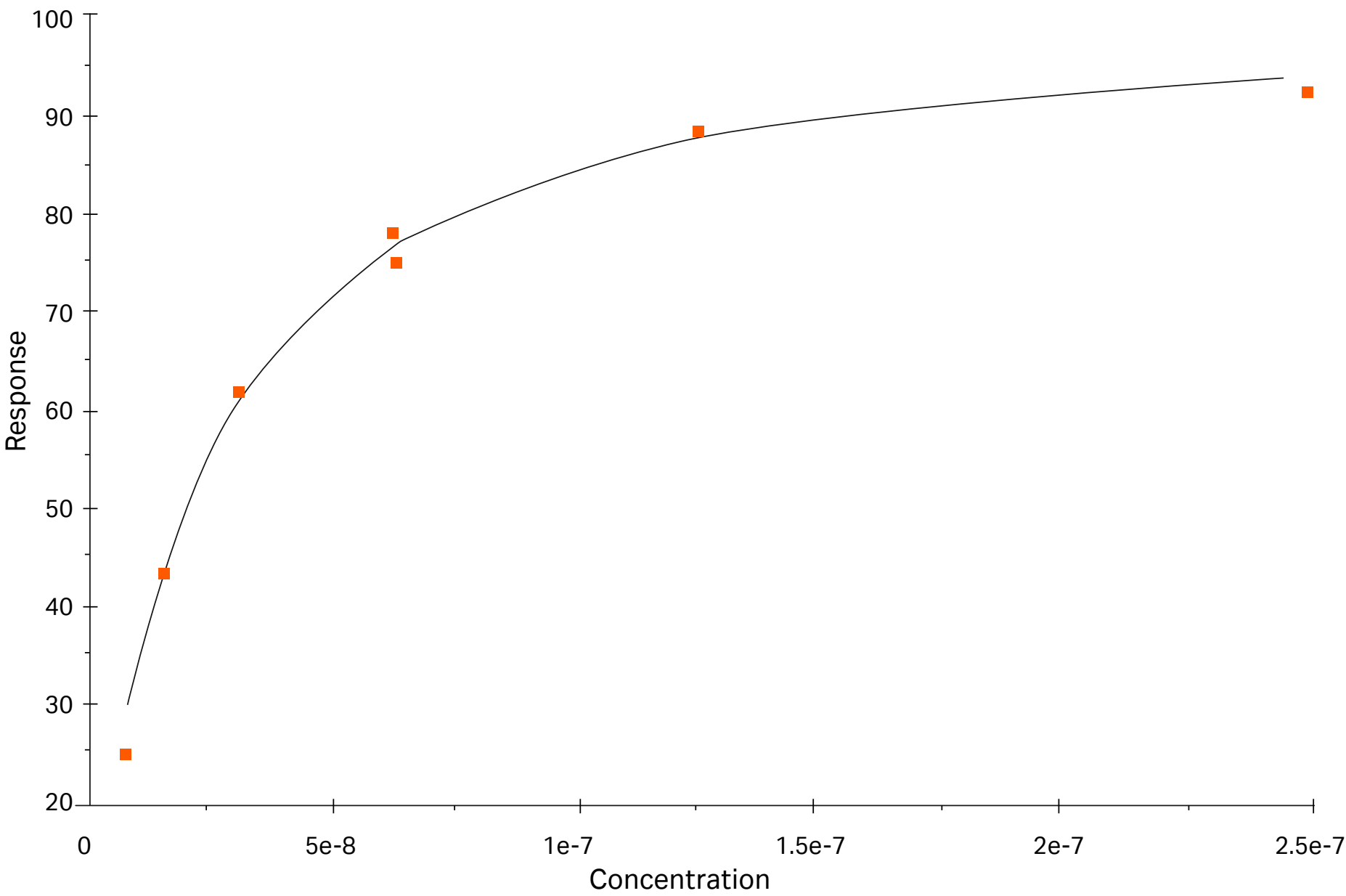
In multi-cycle kinetics and affinity determinations, each sample is injected in a separate cycle. The concentration series is presented as an overlay plot aligned at the start of the injection in the evaluation software. Arrows in the illustration mark the start of sample injections.



In single-cycle determinations, the samples are injected sequentially in the same cycle. Arrows in the illustration mark the start of sample injections.

Affinity determination

For affinity determination, sensorgrams are recorded for a series of analyte concentrations, and the steady state affinity is determined from a plot of the steady state response against concentration (usually referred to as ***R_{eq}* versus C**). One single curve is fitted to the whole data set. Like kinetics, affinity may be measured using single-cycle or multicycle format.



Curve fitting principles

Introduction

Both kinetics and affinity are evaluated by fitting a mathematical model of the interaction to the experimental data. While a close fit between the model and the data provides some confidence in the numerical results, obtaining a good fit is not in itself evidence that the model describes the physical reality of the interaction. The fitting procedure does not have any "knowledge" of the biological significance of parameters in the model equations, and it is wise always to examine the results obtained for reasonableness of the values obtained. In addition, any mechanistic conclusions drawn for the interaction from fitting results (e.g., concerning multiple interaction sites or conformational changes) should ideally be tested using independent techniques.

Fitting procedure

Kinetic and affinity parameters are extracted from experimental data by an iterative process that finds the best fit for a set of equations describing the interaction. The fitting process begins with initial values for the parameters in the equations, and optimizes the parameter values according to an algorithm that minimizes the sum of the squared residuals.

The interaction equations may be created from a description of the interaction model or entered as mathematical expressions. Equations for kinetic analysis are essentially differential equations (describing the rate of change of response with time) that cannot be mathematically integrated.

Local and global parameters

Parameters in the fitting equations are treated as *local variables*, *global variables*, or *constants* as described in the table to the right.

When the data set contains multiple curves, fitting can be performed with *local* or *global* parameter settings. This applies primarily to kinetic analysis. Steady state affinity determination results in a single curve for the data set, so that the local/global distinction is not relevant.

- Local parameters are assigned independent values for each curve in the data set
- Global parameters have the same value for all curves in the data set

Evaluating kinetics with global rate constants gives a more robust value for the rate constants, although the curves may fit the experimental data more closely if all parameters are fitted locally. This is because local fitting allows variation between the constants obtained from different curves: when the constants are fitted globally, this variation appears in the closeness of fit rather than the reported values. Rate constants are always global in predefined kinetic models.

In general, rate constants should be fitted as global parameters and bulk refractive index contribution as a local parameter. The analyte binding capacity of the surface R_{max} is normally set to global, but may be evaluated as a local parameter if there is reason to believe that the surface capacity may vary between cycles in serial mode or channels in parallel mode.

Parameter type	Description
Local variables	Assigned an independent value for each curve in the data series (or sample injection in single-cycle kinetics).
Global variables	Have one single value that applies to the whole data series.
Constants	Have a fixed value that is not changed in the fitting procedure.

Statistical parameters

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

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). $\text{chi-square} = \frac{\sum_1^n (r_f - r_x)^2}{n - p}$ where r_f is the fitted value at a given point r_x is the experimental value at the same point n is the number of data points p is the number of fitted parameters
Standard error (SE)	A measure of the parameter significance, reported separately for each parameter. The parameter significance indicates the extent to which a change in the parameter value affects the closeness of fit. A parameter with low significance can have a wide range of values without affecting the fit.
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. The U-value is determined by testing the dependence of the fit on correlated variations in pairs of parameters, and is reported as a single value for the whole fitting. 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.

Fitting models for kinetics

Mass transfer in kinetic models

Analyte must reach the sensor surface in order to interact with the surface-bound ligand. Free analyte is depleted at the surface by interaction with ligand, and is replenished by diffusion-driven transfer from the bulk solution. If transfer is slow compared with binding of analyte to the ligand, the transport process will limit the observed binding rate, at least partially. All kinetic models except **1:1 dissociation** include a term for mass transfer of analyte, allowing rate constants to be extracted from partially mass transfer limited data.

Mass transfer parameters

Mass transfer is described in terms of transfer of analyte (A) between bulk solution and the surface, with the same rate constant in both directions. Only analyte at the surface can interact with ligand (B). As an example, the simple 1:1 interaction scheme may be represented as



The rate of mass transfer under the conditions of non-turbulent laminar flow that prevail in the flow cell is characterized by the *mass transfer coefficient* k_m (units $\text{m} \cdot \text{s}^{-1}$):

$$K_m = 0.98 \left(\frac{D^2 \cdot f}{0.3 \cdot h^2 \cdot w \cdot l} \right)^{1/3}$$

Parameter	Description
D	Diffusion coefficient of the analyte ($\text{m}^2 \cdot \text{s}^{-1}$)
f	Volume flow rate of solution through the flow cell ($\text{m}^3 \cdot \text{s}^{-1}$)
h, w, l	Flow cell dimensions (height, width, length in m)

The mass transfer coefficient can be normalized for molecular weight and adjusted approximately for the conversion of surface concentration to RU, to give a parameter referred to as the mass transfer constant k_t (units $\text{RU} \cdot \text{M}^{-1} \cdot \text{s}^{-1}$):

$$k_t = k_m \times \text{MW} \times G$$

where G is the conversion factor from surface concentration to RU. The value of G is approximately 10^9 for proteins on Sensor Chip CM5.

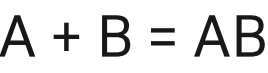
The mass transfer constant can be further modified to give the flow rate-independent component (units $\text{RU} \cdot \text{M}^{-1} \text{s}^{-2/3} \text{m}^{-1}$), referred to as t_c .

$$t_c = \frac{k_t}{\sqrt[3]{f}}$$

Different software versions may report different combinations of k_m , k_t and t_c .

1:1 binding

This is the simplest model for kinetic evaluation, and is recommended as default unless there is good experimental reason to choose a different model. The model describes a 1:1 interaction at the surface:



Default initial values for the **1:1 binding** model are listed below.

Parameter	Description	Fit	Value
ka	Association rate constant (M ⁻¹ s ⁻¹)	Global	1e5
kd	Dissociation rate constant (s ⁻¹)	Global	1e-3
Rmax	Analyte binding capacity of the surface (RU)	Global	Ymax
tc	Flow rate-independent component of the mass transfer constant	Global	1e8
RI	Bulk refractive index contribution	Constant	0

1:1 dissociation

This model fits the dissociation phase of the sensorgrams to an equation for exponential decay, representing dissociation of a homogeneous 1:1 complex. The fitting is independent of analyte concentration. The equation includes an offset term to allow for a nonzero residual response after completion of the dissociation.

$$R = R_0e^{-k_d(t-t_0)} + \text{offset}$$

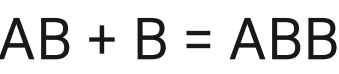
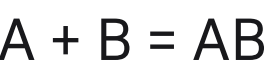
Default initial values for the **1:1 dissociation** model are listed below.

Parameter	Description	Fit	Value
kd	Dissociation rate constant (s ⁻¹)	Global	1e-3
offset	Residual response above baseline after complete dissociation (RU)	Local	0

- Note:** This model cannot handle initial response changes resulting from bulk refractive index contributions. If the sensorgrams show bulk contributions, remove data ranges so that the fitting starts after the bulk change is complete.
- Note:** This model does not take account of the effect of mass transport limitations.
- Note:** Application of this model can be sensitive to initial parameter settings. If a good fit cannot be obtained with apparently reasonable dissociation data, try adjusting the initial values to correspond more closely with expected results.

Bivalent analyte

This model describes the binding of a bivalent analyte to immobilized ligand, where one analyte molecule can bind to one or two ligand molecules. The two analyte sites are assumed to be equivalent. The model may be relevant to studies among others with signaling molecules binding to immobilized cell surface receptors (where dimerization of the receptor is common) and to studies using intact antibodies binding to immobilized antigen. As a result of binding of one analyte molecule to two ligand sites, the overall binding is strengthened compared with 1:1 binding. This effect is often referred to as avidity.



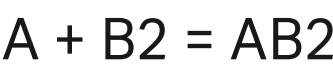
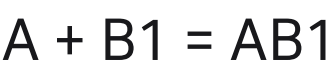
Note: Once analyte is attached to the ligand through binding at the first site, interaction at the second site does not contribute to the SPR response. For this reason, the association rate constant for the second interaction is reported in units of $RU^{-1}s^{-1}$, and can only be obtained in $M^{-1}s^{-1}$ if a reliable conversion factor between RU and M is available. For the same reason, a value for the overall affinity or avidity constant is not reported.

Default initial values for the **Bivalent analyte** model are listed below.

Parameter	Description	Fit	Value
ka1	Association rate constant for the first site ($M^{-1}s^{-1}$)	Global	1e5
kd1	Dissociation rate constant for the first site (s^{-1})	Global	1e-3
ka2	Association rate constant for the second site ($RU^{-1}s^{-1}$)	Global	1e-3
kd2	Dissociation rate constant for the second site (s^{-1})	Global	1e-3
Rmax	Analyte binding capacity of the surface (RU)	Global	Ymax
tc	Flow rate-independent component of the mass transfer constant	Global	1e8
RI	Bulk refractive index contribution	Constant	0

Heterogeneous ligand

This model describes an interaction between one analyte and two independent ligands. The binding curve obtained is simply the sum of the two independent reactions. The relative amounts of the two ligands does not have to be known in advance.



Note: The model is limited to two ligands because the fitting algorithm tends to become unstable with more components, and three or more ligand species cannot be reliably resolved.

Default initial values for the **Heterogeneous ligand** model are listed below.

Parameter	Description	Fit	Value
ka1	Association rate constant for the first ligand (M ⁻¹ s ⁻¹)	Global	1e5
kd1	Dissociation rate constant for the first ligand (s ⁻¹)	Global	1e-3
ka2	Association rate constant for the second ligand (M ⁻¹ s ⁻¹)	Global	1e5
kd2	Dissociation rate constant for the second ligand (s ⁻¹)	Global	1e-3
Rmax1	Analyte binding capacity of the first ligand (RU)	Global	Ymax
Rmax2	Analyte binding capacity of the second ligand (RU)	Global	Ymax
tc	Flow rate-independent component of the mass transfer constant	Global	1e8
RI	Bulk refractive index contribution	Constant	0

Two state reaction

This model describes a 1:1 binding of analyte to immobilized ligand followed by a conformational or other change that stabilizes the complex. To keep the model simple, it is assumed that the changed complex can only dissociate through reversing the conformational change:



Note: Conformational changes in ligand or complex do not normally give a response in Biacore systems. A good fit of experimental data to the two-state model should be taken as an indication that conformational properties should be investigated using other techniques (e.g., spectroscopy or NMR), rather than direct evidence that a conformational change is taking place.

Default initial values for the **Two state reaction** model are listed below.

Parameter	Description	Fit	Value
ka1	Association rate constant for analyte binding (M ⁻¹ s ⁻¹)	Global	1e5
kd1	Dissociation rate constant for the complex (s ⁻¹)	Global	1e-2
ka2	Forward rate constant for the stabilizing change (s ⁻¹)	Global	1e-3
kd2	Reverse rate constant for the stabilizing change (s ⁻¹)	Global	1e-3
Rmax	Analyte binding capacity of the surface (RU)	Global	Ymax
tc	Flow rate-independent component of the mass transfer constant	Global	1e8
RI	Bulk refractive index contribution	Constant	0

Fitting models for affinity

Steady state affinity

This model calculates the equilibrium dissociation constant K_D for a 1:1 interaction from a plot of steady state binding levels (R_{eq}) against analyte concentration (C). The equation includes an offset term which represents the response at zero analyte concentration.

$$R_{eq} = \frac{CR_{max}}{K_D + C} + \text{offset}$$

Note: Reported K_D values that are higher than half the highest analyte concentration used should be treated with caution. If the response against concentration plot does not flatten out sufficiently because the concentrations are not high enough in relation to the K_D value, the reported value may be unreliable.

Default initial values for the **Steady state affinity** model are listed below.

Parameter	Description	Fit	Value
KD	Equilibrium dissociation constant	Global	Xmax
Rmax	Analyte binding capacity of the surface (RU)	Global	Ymax
offset	Response at zero analyte concentration	Global	Ymax/5

Steady state affinity (constant Rmax)

This model uses the same equation as the simple steady state affinity model, but sets the $R_{\max}$ parameter to a constant. The value for $R_{\max}$ is obtained for each analyte from a value entered for a control analyte and the relative molecular weights of control and sample (see the Application guide *Fragment and small molecule screening with Biacore™ systems* for more details. The value may be adjusted for assay drift using repeated control samples.

$$R_{\max_{\text{analyte}}} = R_{\max_{\text{control}}} \times \frac{MW_{\text{analyte}}}{MW_{\text{control}}}$$

Steady state affinity (constant Rmax and multi-site)

This model fits data from interactions that exhibit binding to multiple sites. Two sites are accommodated in the model.

The model uses a constant $R_{\max}$ value for one site, defining the expected stoichiometry, and a fitted value for the other site, which can give an apparent value with undefined stoichiometry.

$$R_{\text{eq}} = \frac{CR_{\max1}}{K_{D1} + C} + \frac{CR_{\max2}}{K_{D2} + C} + \text{offset}$$

Default initial values for the **Steady state affinity (constant Rmax and multi-site)** model are listed below.

Parameter	Description	Fit	Value
KD	Equilibrium dissociation constant for the main (strong) binding (M)	Global	Xmax
KD2	Equilibrium dissociation constant for the secondary (weak) binding (M)	Global	100*Xmax
Rmax	Analyte binding capacity for the main binding (RU)	Constant	(Input) ¹
Rmax2	Analyte binding capacity for the secondary binding (RU)	Global	Ymax
offset	Response at zero analyte concentration	Global	Ymax/5

¹ The default initial value is set to Constant = Ymax if no input value for $R_{\max}$ has been provided.

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