Handbook

Biacore T200 software

cytiva

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01

Introduction

Biacore™ T200 is a high performance system for analysis of biomolecular interactions, based on Cytiva's surface plasmon resonance (SPR) technology. The Control Software supplied with the system offers easy-to-use wizards for assay development and common applications together with flexible facilities for designing custom analysis methods using a graphical interface called Method Builder. Results are evaluated in separate Evaluation Software designed for efficient and flexible evaluation, with dedicated functions for common applications.

This Handbook describes in detail how to use the Control and Evaluation Software.

1.1 System overview

Instrumentation in the Biacore T200 system is described in full in the Biacore T200 Operating Instructions. Important features relevant to software operation include:

- Biacore T200 supports simultaneous analysis in up to four flow cells connected in series. The flow cells are arranged in pairs (Fc1-2 and Fc3-4) with minimum dead volume between the flow cells in a pair to provide accurate reference subtraction.
- The sample compartment accommodates one microplate (96- or 384-well, regular or deep-well capacity) and one reagent rack for reagent vials. A combined sample and reagent rack can be used in place of the separate microplate and reagent rack.
- Material that binds to the sensor surface during sample injection can be recovered in a small volume of liquid for further analysis by e.g. mass spectrometry.
- The temperature in the sample compartment is controlled separately from the analysis temperature, allowing samples to be kept at one temperature while analysis is performed at another. Samples equilibrate to the analysis temperature during injection into the flow cell. The analysis temperature can be varied during a run, and the sample compartment temperature can be set to follow the analysis temperature if desired.
- The system includes a buffer selector valve, allowing analysis to be performed in up to four different buffers in the same unattended run.

1.2 Support for use in regulated environments

Support for use in regulated (GxP[1]) environments is provided in an optional package that adds appropriate functionality to the Biacore T200 software. Functions for GxP support are described in a separate Biacore T200 GxP Handbook. Descriptions of software in the current Handbook apply to installations both with and without the GxP package unless otherwise stated.

1.3 Associated documentation

This Handbook describes Biacore T200 Control Software version 3.2.1 and Evaluation Software version 3.2.1. Any functionality that is added in optional add- on modules is described in separate documentation.

Biacore T200 *Operating Instructions* provides local-language instructions for operating and maintaining the instrument. This publication is provided on CD.

Biacore T200 *GxP Handbook* describes functionality added with the optional GxP package, together with some recommendations for using the system in a regulated environment.

Biacore T200 *Immunogenicity Handbook* describes the use of specialized functions in the software for immunogenicity studies.

Other general handbooks and documentation describing the technology are available from Cytiva. Information may also be found on the Internet at cytiva.com/biacore.

1.4 Biacore terminology

Biacore monitors the interaction between two molecules, of which one is attached to the sensor surface and the other is free in solution. The following terms are used in the context of work with Biacore systems (see Figure 1-1):

- The partner attached to the surface is called the *ligand*. Attachment may be covalent or through high affinity binding to another molecule which is in turn covalently attached to the surface. In the latter case the molecule attached to the surface is referred to as the *capturing molecule*.

Note: *The term "ligand" is applied here in analogy with terminology used in affinity chromatography contexts, and does not imply that the surface-attached molecule is a ligand for a cellular receptor.*

[1] GxP is used as a generic abbreviation for GLP (Good Laboratory Practice), GMP (Good Manufacturing Practice) and GCP (Good Clinical Practice).

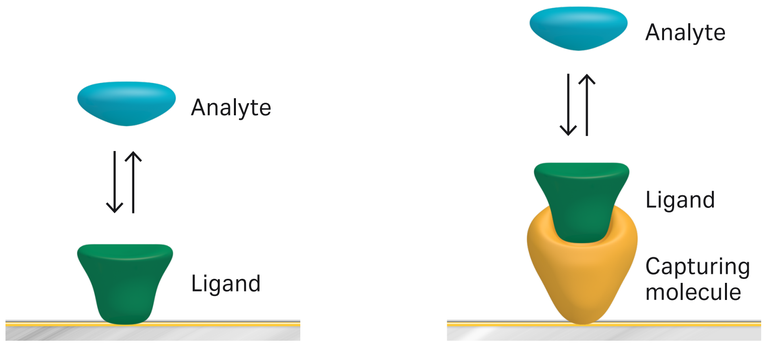


Figure 1-1. Ligand, analyte and capturing molecule in relation to the sensor surface.

- The *analyte* is the interacting partner that is injected in solution over the sensor surface. Commonly, the analyte binds directly to the ligand, although assays may be set up where analyte properties are deduced from the effect on binding of another molecule.
- *Regeneration* is the process of removing bound analyte from the surface after an analysis cycle without damaging the ligand, in preparation for a new cycle.
- Response is measured in *resonance units* (RU). The response is directly proportional to the concentration of biomolecules on the surface.
- A *sensorgram* is a plot of response against time (see Figure 1-2), showing the progress of the interaction. This curve is displayed directly on the computer screen during the course of an analysis. Sensorgrams may be analyzed to provide information on the rates of the interaction.
- In many assay situations, sample passes over two or more flow cells in series, where one flow cell (usually the first) serves as a reference while ligand is attached in the other flow cell(s). Surfaces with ligand are referred to as *active*: blank surfaces used for reference purposes are *reference*.
- A particular sensorgram is referred to as a *curve* in several contexts in the software. This terminology is used to distinguish between different classes of sensorgram that recur within a run: for example, measurements on one active and one reference surface can generate separate curves for each of the two flow cells and a third *reference-subtracted* curve (active minus reference).
- A *report point* records the response on a sensorgram at a specific time averaged over a short time window, as well as the slope of the sensorgram over the window. The response may be absolute (above a fixed zero level determined by the detector) or relative to the response at another specified report point.

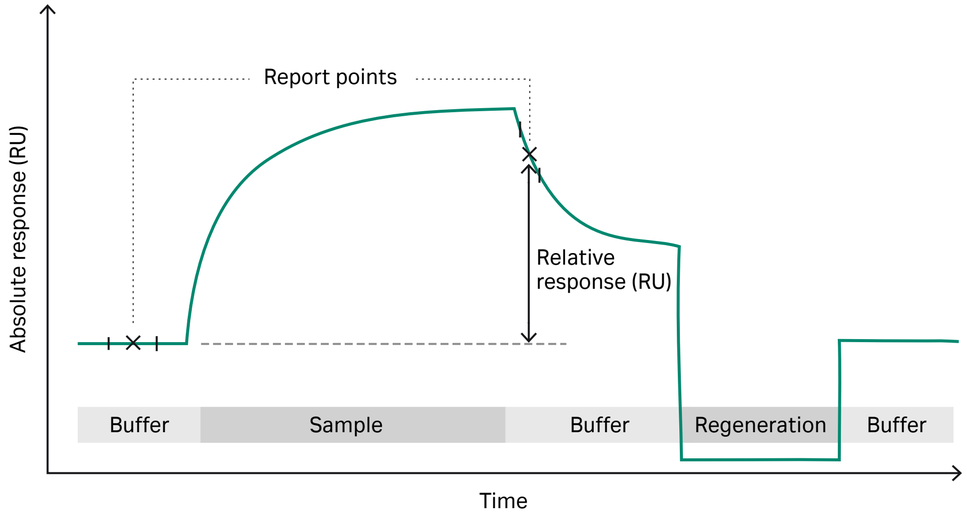


Figure 1-2. Schematic illustration of a sensorgram. The bars below the sensorgram curve indicate the solutions that pass over the sensor surface.

02

Control Software – general features

2.1 Operational modes

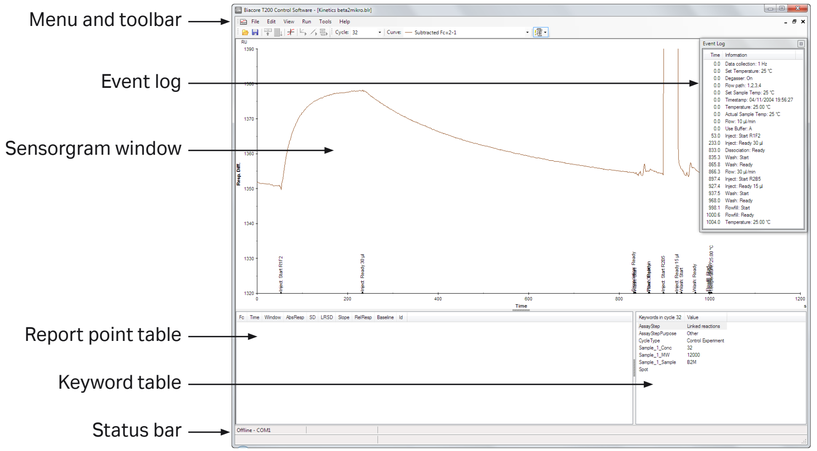
Biacore T200 Control Software offers three modes of operation:

- *Manual run* provides interactive control of the instrument operation, executing commands singly as they are issued. This mode is most useful for *ad hoc* experiments involving one or a few injections, such as testing the response obtained from injection of a single sample.
- *Application wizards* provide guidance in setting up experiments for assay development and execution. Separate wizards are offered for different purposes such as ligand immobilization, concentration determination or measurement of kinetic constants. Each wizard consists of an ordered series of dialog boxes, ensuring that the essential features of the application setup are correctly defined.
- *Methods* provide greater flexibility (and conversely less guidance) in setting up applications, allowing customized applications that are not covered by wizards. Methods are defined in a graphical interface called *Method Builder*, which is designed to provide full flexibility in method definition while retaining a simple interface for running assays based on established methods. Application wizard templates may be opened in Method Builder to provide a starting point for further refinement of application setup. Predefined methods are also provided as help in defining methods for selected purposes (see Appendix B).

Each of these modes of operation is described in more detail in the following chapters.

2.2 User interface

The main screen in the control software is divided into the following areas



- The *menu* and *toolbar* provide access to control commands.
- The *event log* records settings at the start of the run and instrument control events during the run. The event log is displayed in a separate window, opened by clicking on the ***Event Log*** button at the right of the toolbar.
- The *sensorgram window* displays the sensorgrams for the current run or the currently open file.
- The *report point table* lists report points for the currently displayed cycle. Report points record the response at a set time and are defined automatically: custom report points can also be added in methods, or after the run in either the Control Software or the Evaluation Software.
- The *keyword table* lists keywords for the currently displayed cycle. Keywords are defined automatically in wizard runs, or in the method for method runs.
- The *status bar* displays the instrument status, including the temperature of the detector and the sample compartment. The content of the status bar varies between different situations: for wizard- and method-based runs, the elapsed run time and the estimated total run time are included.

2.2.1 Software help

Software help is available at any time from the ***Help*** menu. Context-specific help for dialog boxes is provided through ***Help*** buttons in the boxes.

2.3 Basic operation

2.3.1 Selecting cycles and sensorgrams

During a run, the current cycle is displayed by default. You can choose which cycle to display in the ***Cycle*** selector, but the display will revert to the current cycle when a new cycle is started. For a completed run, choose which cycle to display with the ***Cycle*** selector in the toolbar:

The ***Curve*** selector determines which curve in the cycle is current in the display. Options in the ***View*** menu (Section 2.3.4) control which curves are displayed in the sensorgram window.

2.3.2 File menu

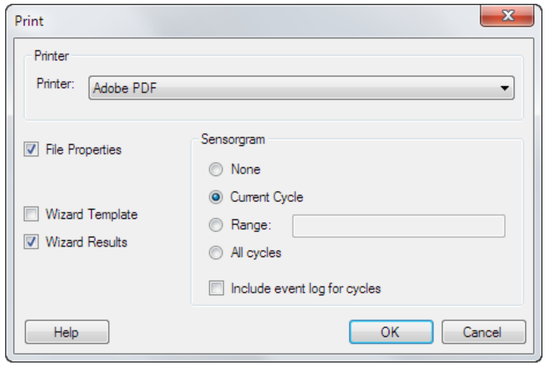
The ***Open/New*** options for wizard templates and methods create new wizard templates and methods, and open existing templates and methods for editing or for starting a run.

Open opens result files. Most result files just display the sensorgrams and tables. Files from immobilization and regeneration scouting wizards also display a summary window showing the results of the run (see Sections 4.5 and 4.6).

Save and ***Save As*** save the results as a Biacore results file (extension .blr).

Export exports the current results to a file in Microsoft® Excel® or XML format, or exports the contents of the report point table to a tab-separated file. See Appendix A for details of the export format.

Print prints a hard-copy of the results. Select the printer to use and check the items you wish to print.



Sensorgrams may be printed as follows:

None No sensorgrams will be printed.

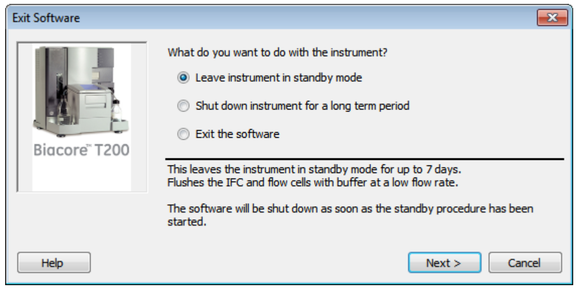
Current cycle The current cycle will be printed with the ***View:Show...*** setting and scale as shown on the screen.

Range and **All cycles** Multiple cycles will be printed. For ***Range***, enter a range or cycle numbers separated by commas (e.g. ***4-16,19,22***). All curves will be included in each cycle regardless of the ***View:Show...*** setting. Sensorgrams will be printed at full scale unless the ***Lock Scale*** box is checked in the sensorgram window, in which case the current scaling will be applied to all cycles (with this setting, some sensorgrams may appear to be empty).

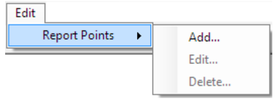
Note: *In order to maintain report layout, the print orientation is fixed regardless of the printer settings in Windows®.*

Properties shows detailed properties of the currently opened run, including the properties of the sensor chip used in the run.

When you close the software with ***Exit*** while the instrument is still switched on, you may choose to shut down the instrument for a shorter or longer period if required. See the Biacore T200 Instrument Handbook or the on-line help for more details.



2.3.3 Edit menu

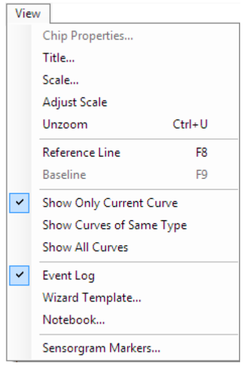


Options in the ***Edit*** menu allow you to add, edit and delete report points. Report points are created automatically and are used in various evaluation contexts. You should in general avoid editing or deleting report points that are created automatically.

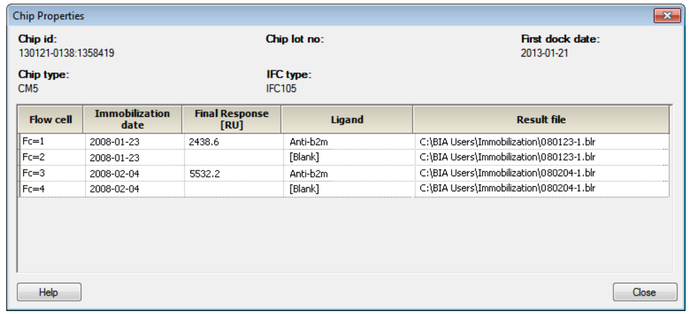
Editing operations for report points in the Control Software may be applied to single report point instances or to the report point on all curves in the current cycle. Note that editing operations are not applied to multiple cycles.

Report points created in the Control Software cannot be edited in the Evaluation Software. The Evaluation Software offers functions for creating and editing custom report points that can be applied to all cycles in the run in a single operation. This is usually preferable to adding report points in the Control Software.

2.3.4 View menu



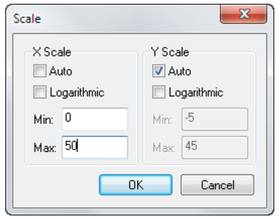
Chip Properties opens a dialog box that displays the properties of the currently docked sensor chip. The ***Ligand*** column is empty for flow cells that have not been used, and shows ***[Blank]*** for flow cells that have been prepared as a blank reference surface by activation and deactivation. The text ***[Incomplete results]*** indicates that the immobilization run was interrupted (by for instance user intervention or power failure) before it could be completed.



Properties for the sensor chip used in a currently open run may be found under ***File:Properties*** (Section 2.3.2).

Title sets a title in the sensorgram window. The default title is the assay step name.

Scale sets the scale of the sensorgram window:



If you set ***Auto scale***, the scale will be adjusted if necessary to accommodate the full data range of the currently displayed cycle. During a run, the scale is adjusted at intervals as more data is collected. Check the ***Lock scale*** box in the top right corner of the sensorgram window to lock the scale to the current settings.

Adjust Scale sets the scale to the full data range. This will not affect the ***Auto scale*** setting in the ***Scale*** dialog. ***Adjust Scale*** overrides but does not turn off the ***Lock scale*** setting.

To scale the sensorgram display interactively, drag with the cursor over the area to be scaled. Double-clicking in the display or choosing ***View:Unzoom*** restores the previous zoom setting.

Reference line toggles display of a movable vertical line in the sensorgram window, together with a separate small window that shows the response and time coordinates at the reference line for the current curve. Use the ***Curve*** selector in the toolbar (see Section 2.3.1) to set the current curve. Drag the reference line to move it. When the reference line is displayed, choosing ***Baseline*** sets a baseline at the current reference line position, and the coordinates window shows the response relative to that baseline.

The options ***Show Only Current Curve, Show Curves of Same Type*** and ***Show All Curves*** control which curves are displayed in the sensorgram window. Curve types distinguish between unsubtracted and reference-subtracted curves.

Choose the ***Event Log*** option or click on the ***Event Log*** button at the right of the toolbar to display the event log window.

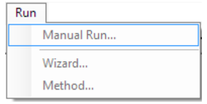
Choose the ***Wizard Template*** or ***Method*** options to display the wizard or method definition for the run. You can edit the definition and save it as a new wizard template or method without affecting the current run. You cannot however change the original definition that is saved together with the result file.

Notebook opens a notebook window where details of the run may be recorded. The notebook is only available during a run or for a completed result file. The run notebook is saved with the result file and can be viewed in the Evaluation Software.

For some wizard runs and for test tools, the ***Wizard Results*** option opens a window showing the results of the run. All other runs are evaluated in the Evaluation Software.

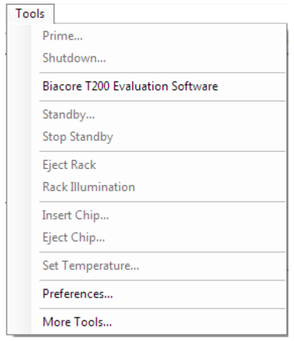
Sensorgram Markers controls display of report point and event markers and labels in the sensorgram window.

2.3.5 Run menu



The options in the ***Run*** menu are used to start the different types of runs (see Chapters 3–5).

2.3.6 Tools menu



Options in the ***Tools*** menu control instrument operations outside the context of runs.

Prime flushes the flow system with fresh buffer. There is an option to include ***Prime*** at the beginning of each wizard- or method-based run. Use the menu option when you want to flush the system at other times (e.g. before a manual run).

Shutdown starts the procedure for shutting down the instrument for long periods of time (more than 7 days). The procedure displays necessary instructions on the screen. Details of the shutdown procedure are given in the Biacore T200 Operating Instructions.

Standby puts the instrument in standby mode, which maintains a low buffer or water flow through the flow system for up to 7 days. Leaving the instrument in standby mode when not in use is generally recommended. The instrument is automatically put in standby mode at the end of a run. Use the menu option if standby has been stopped and you want to restart it.

Stop Standby stops standby mode.

Eject Rack ejects the rack tray from the sample compartment. The rack may be ejected during setup for wizard- and method-based runs, and at any time during a manual run. Use the menu option or the toolbar button when you want to eject the rack at any other time.

> ⚠ **CAUTION**
>
> The rack tray automatically moves into the instrument a preset time after it has been ejected. The time to auto-close is set in ***Tools:Preferences***.
> A timer in the dialog indicates when the rack tray will be automatically moved into the instrument.

Rack Illumination switches the illumination in the sample compartment on or off. The illumination helps you to see in the sample compartment but does not otherwise affect instrument function.

Insert Chip and ***Eject Chip*** are used for docking and undocking the sensor chip respectively. More details are given in the Biacore T200 Operating Instructions.

Set Temperature sets the sample compartment and analysis temperature. More details are given in the Biacore T200 Operating Instructions.

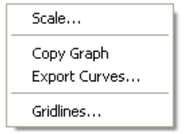
Preferences controls aspects of file storage and data import (see Section 2.5), and the time to automatic retraction of the rack tray.

More Tools provides access to maintenance, test and service tools. Details are given in the Biacore T200 Operating Instructions.

2.3.7 Right-click menus

Right-clicking with the mouse in many windows opens context menus specific for the window.

Sensorgram window



Scale opens the same dialog as the ***View:Scale*** option (Section 2.3.4).

Copy Graph copies the sensorgram window exactly as displayed to the Windows clipboard. Use this option to insert a copy of the sensorgram window into other programs such as presentation software.

Export Curves exports data for the currently displayed curves to a text file. Entire curves are exported regardless of the scale of the display. The exported data includes report points and event marker times if these are displayed in the sensorgram window. See Appendix A for more details of the export format.

Gridlines controls display of gridlines in the sensorgram window.

Report point table

The right-click menu options for the report point table correspond to the ***Edit:Report Points*** menu options.

Notebook

Right-click menu options in the notebook offer standard Windows editing functions.

2.4 Flow cell usage

The Biacore T200 flow cells are designed for use in pairs for optimal performance (Fc1+2 and Fc3+4). If the pairs on one sensor chip are used separately, injected sample passes over only the addressed flow cell pair, but buffer flow during the dissociation phase in Fc3+4 passes over all four flow cells. It is therefore recommended to use Fc3+4 first to avoid possible downstream interference on Fc 3+4 when analysis is run over Fc1+2.

2.5 File storage

2.5.1 Wizard templates and methods

Wizard templates are saved in files with a file name extension ***.bw*****, where ******** represents an abbreviation that identifies the wizard (e.g. a wizard template for concentration analysis has the extension ***.bwConc***).

Methods are saved in files with the file name extension ***.Method***.

Templates and methods may be saved in any location when the optional Biacore T200 GxP Package is not installed. A folder structure under the default location as specified on the ***Folders*** tab in ***Tools:Preferences*** is however recommended, since files in this location are handled preferentially in the ***Open/New*** dialog boxes for wizards and templates (see Section 4.1.1).

2.5.2 Result files

Results are saved in files with the file name extension ***.blr***. Result files from wizard- or method-based runs contain a copy of the wizard template or method as well as the results of the run.

03

Manual run

Manual run allows you to control a run interactively. All settings except temperature and choice of microplate and/or reagent rack can be changed during the run. Commands are placed in a queue if the instrument is busy when a command is issued: queued commands that have not yet been started can be edited or deleted from the queue.

The results of a manual run are saved in a normal result file, and can be evaluated in the Evaluation Software. There are however no predefined keywords associated with the run, and the results cannot be evaluated with the Evaluation Software tools for concentration, kinetics/affinity, thermodynamics or immunogenicity.

3.1 Preparing for a manual run

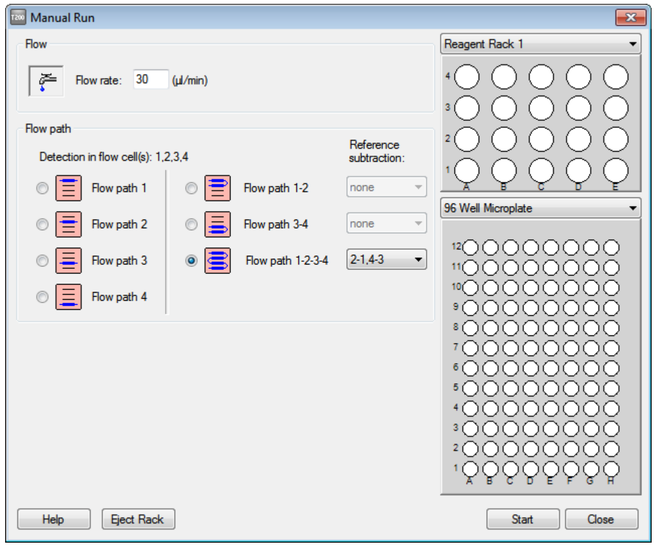
3.1.1 Instrument preparations

The integrated instrument preparation steps that are included with wizard- and method-based runs are not supported for manual run. The instrument should therefore be prepared using options from the ***Tools*** menu.

1. Dock the chip that you want to use, and immobilize ligand on the surface (see Section 4.5) if this has not already been done.
2. Choose ***Tools:Prime*** to flush the flow system with fresh buffer.
3. Choose ***Normalize*** from the ***Maintenance Tools*** section of ***Tools:More Tools*** if the detector has not been normalized since the chip was docked. (In many cases, the detector will have been normalized in connection with ligand immobilization. However, you may need to perform the operation again if the chip has been undocked and re-docked after immobilization.)
4. Choose ***Tools:Set Temperature*** and set the analysis and sample compartment temperatures. Wait until the analysis temperature is stable (as shown in the status bar) before starting the run.
5. Prepare your samples and reagents in the microplate and/or reagent rack. Note the rack positions and volumes of samples that you prepare: there is no software support in manual run for identifying samples or monitoring the volume of liquid in the autosampler positions. You insert the samples as part of the starting procedure for the run. You can also add samples during the run.

3.2 Starting a manual run

Choose ***Run:Manual Run*** to start a manual run.



Choose the initial settings for flow rate, flow path and reference subtraction. You can change the flow rate at any time during the run. You can change the flow path at any time: during a cycle, the available options are restricted by the choice made when the cycle is started.

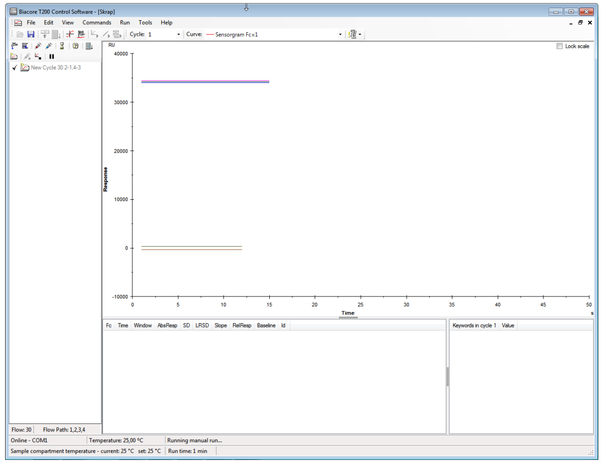
Choose the rack and microplate settings. These settings will apply throughout the run and cannot be changed.

Click ***Eject Rack*** to eject the rack tray so that you can load your samples.

Click ***Start*** to start the run. You will be asked to specify a result file name before the run actually starts.

3.3 Controlling a manual run

Control the manual run from the command buttons in the main window or the options in the ***Command*** menu:



Commands are executed immediately if the instrument is idle. With a few exceptions (noted in the detailed descriptions below), commands issued when the instrument is busy are placed at the end of a queue. The queue is listed in the left-hand panel, with commands that have been executed in gray text and those that are pending in black text. The command currently being executed is marked with a "working" icon.

Right-click on a pending command for a menu with options for:

- editing the command
- inserting a new command before the selected command (you choose the command to insert from a dialog box)
- deleting the command

You can also use the right-click menu to copy selected command or commands and paste them elsewhere in the queue. The ***Copy*** function works with both completed and pending commands.

Flow rate

Sets the flow rate to a new value.

Flow path

Changes the flow path. During a cycle, you can only select a flow path within a range allowed by the setting chosen when the cycle was started (for example, if the current setting is ***Flow path 1-2***, you cannot extend it to ***Flow path 1-2-3-4***).

Sample injection

Injects sample or reagent. Choose the position from which the sample will be taken and specify a contact time. Positions that can be chosen are determined by the rack settings in the manual run start-up dialog. Check ***High performance*** to optimize the injection performance (see Section 5.6.1 for more details). Make sure that the chosen position contains enough sample for the injection. The required volume for the specified contact time is indicated in the dialog box.

Regeneration injection

Injects regeneration solution. Choose the position from which the solution will be taken and specify a contact time. Positions that can be chosen are determined by the rack settings in the manual run start-up dialog. Make sure that the chosen position contains enough solution for the injection. The required volume for the specified contact time is indicated in the dialog box.

Check ***High viscosity solution*** if your regeneration solution has a relative viscosity higher than about 3 (corresponding to about 35% glycerol or 40% ethylene glycol at 20°C). This will adjust the injection procedure to ensure correct handling of viscous solutions, and will limit the maximum contact time that can be specified.

Wait

Inserts a ***Wait*** command in the queue, causing the instrument operation to pause for the specified time period. Buffer continues to flow over the sensor surface during the ***Wait*** period and data collection continues.

Eject Rack Tray

Ejects the rack tray so that you can load more samples. Do not change the type of microplate or reagent rack on the tray.

This command is inserted immediately after the command currently under execution, rather than at the end of the queue, so that the rack tray will be ejected as soon as the current command is completed. If you want to place the command later in the queue, use the right-click menu in the queue panel to insert the command at the appropriate place.

New Cycle

Starts a new cycle. You can choose a new flow path and reference subtraction setting for the new cycle, independently of the setting in the current cycle.

Stop <command>

Stops the command currently being executed. The icon changes to show the command that will be stopped, or is gray if the current command cannot be stopped (e.g. it is not possible to stop an ***Eject Rack Tray*** command).

Stop Run

Finishes the run.

Pause Run

Pauses the run until a ***Resume Run*** command is issued. Buffer continues to flow over the sensor surface while the run is paused. Data collection continues during the pause.

Resume Run

Resumes a run that is paused.

Add report point

Adds a report point to the sensorgram.

Help

Displays help for the manual run.

3.4 Ending a manual run

To end a manual run:

1. Issue a ***Stop Run*** command. The command will normally be placed at the end of the queue. If you want to stop the run before the queue is completed, use the right-click menu in the queue panel to delete commands from the queue or to insert the ***Stop Run*** command in the appropriate position.
2. Choose ***Tools:Eject Rack*** to eject the rack tray and remove your samples and reagents.
3. Choose ***Tools:Eject Chip*** to undock the chip if desired.

04

Application wizards

Application wizards guide you through the procedure of setting up common applications, with recommendations and settings based on Cytiva's expertise in the field of SPR-based interaction studies. Wizards are an ideal starting point for inexperienced or infrequent users, since they offer a structured sequence of settings that covers all essential aspects of the assay in question. Wizard settings can be saved in templates for later use. Advanced users can open wizard templates in Method Builder for more flexible assay design (see Chapter 5).

4.1 Wizard templates

An application wizard consists of a series of dialog boxes that takes you through the steps in setting up the application. Settings in the dialog boxes may be saved in *wizard templates*, so that opening a template will present the saved settings in each dialog box.

Normally, a wizard template is saved when all steps have been defined, so that the template represents a complete assay definition including sample details if desired. If a wizard sequence is closed before reaching the last step, however, you are given an opportunity to save the template, which will then contain settings as far as they have been defined.

4.1.1 Creating and editing wizard templates

To create a new wizard template or edit an existing template, choose ***File:Open/New Wizard Template*** and select the type of wizard in the dialog box. Click ***New*** to create a new template, or navigate to the folder where your template is stored, select the template and click ***Open*** to edit an existing template.

The top-level folder for wizard templates is defined under ***Tools: Preferences*** (see Section 2.5). You can navigate between subfolders under the top level in the dialog box, but you cannot access templates outside the top-level folder directly from within the dialog box. Click ***Browse*** to navigate freely in the computer file structure and open wizard templates stored in other locations.

Note: *The **Open/New Wizard Template** dialog box only lists templates of the selected type, but the **Browse** dialog may list all types. Template types are identified by the file extension, which may or may not be displayed according to your Windows Explorer settings (see Section 2.5.1).*

4.1.2 Running wizards

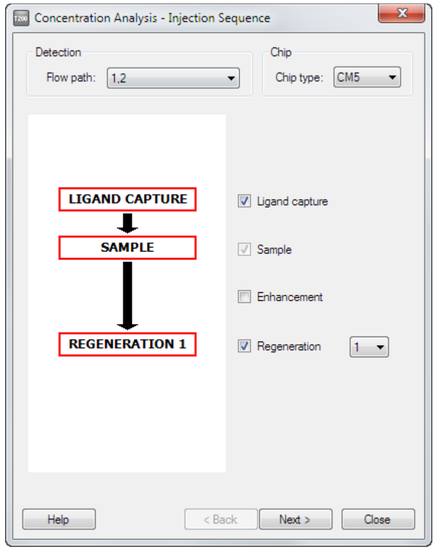
When you start a run based on a wizard template, the template settings are displayed as you step through the wizard. You can change settings for the particular run if desired: the changes are not saved in the wizard template unless you explicitly request this with the ***Save wizard template*** option. Settings used for the run are stored in the result file and can be examined and saved as a new wizard template from the completed run.

4.2 Common wizard components

Several dialogs are common to a number of wizards, with equivalent functions and only minor differences. These dialogs are described in the current section. Wizard-specific variations in these common components are described in the sections on the respective wizards below.

4.2.1 Injection sequence

This dialog determines the sequence of injections in the wizard analysis cycle. Some injections are not supported in certain wizards (e.g. the kinetics wizard does not support enhancement injections).



Detection settings

Select the flow path for the analysis. The setting will apply throughout the whole wizard run. The available flow paths vary between the different wizards according to wizard purpose.

The detection is automatically set to the same settings as the flow path, so that sensorgrams are recorded only from the flow cells used.

When reference subtraction is used together with ligand capture (Section 4.2.1), the captured ligand passes over the active surface but not the reference surface (for example, with ***Flow path*** set to ***2-1***, the ligand is injected in flow cell 2 but not flow cell 1). If the ***Flow path*** setting does not use reference subtraction, ligand is injected in all flow cells included in the flow path.

Chip type

Select the sensor chip type for the analysis. This choice will determine certain assay settings in accordance with the requirements of the sensor chip (for example, selecting Sensor Chip NTA will automatically check the ***Ligand capture*** option and include an injection of nickel before the ligand capture step, and will suggest 0.35 M EDTA as a conditioning solution).

Choose chip type ***Custom*** if you are using a chip type that is not listed.

Injections

Check the injections that you want to include. Injection purposes are listed below. There may be additional injections for some sensor chip types (for example injection of nickel for Sensor Chip NTA).

Ligand capture Intended for ligand solution in applications that use a capturing approach to attach the ligand to the surface. The same solution will be used for the capture injection in all cycles: you cannot vary the captured ligand within the context of one wizard run.

The flow path for capture solution depends on the settings for detection (see above).

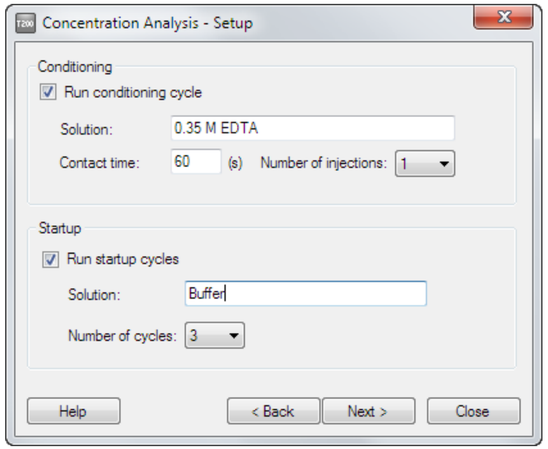
Sample This is the sample to be analyzed. The solution used for the sample injection is normally different in different cycles, and is specified in the sample table at a later stage in the wizard. The sample injection is required in all wizards.

Enhancement Intended for injection of a secondary reagent that binds to analyte on the surface, typically used either to amplify the response obtained from the analyte or to enhance the specificity of analyte detection. The same solution will be used for the enhancement injection in all cycles in the run.

Regeneration One or two regeneration injections may be included, which may use the same or different solutions. The same regeneration procedure is used throughout the run.

4.2.2 Assay setup

Common features of the assay setup dialog are choice of conditioning and start-up cycles at the beginning of the run.



Conditioning cycle

A conditioning cycle prepares the sensor chip for the assay by washing with one or more injections of the specified solution. The surface is not regenerated after the conditioning injections. The conditioning cycle is run once at the beginning of the assay.

Conditioning cycles are recommended for certain chip types, to prepare the surface before starting the assay. Examples are Sensor Chip NTA which should be conditioned with 0.35 M EDTA to remove any bivalent metal ions, Sensor Chip L1 and HPA which may be conditioned with for example octylglucoside to remove any lipids on the surface, and Sensor Chip CAP which should be conditioned with regeneration solution. Conditioning cycles are generally not appropriate for sensor chips where the ligand or capturing molecule is attached to the surface before the assay wizard is started.

If you run several assays after each other without undocking the chip between assays, conditioning is generally only required for the first assay.

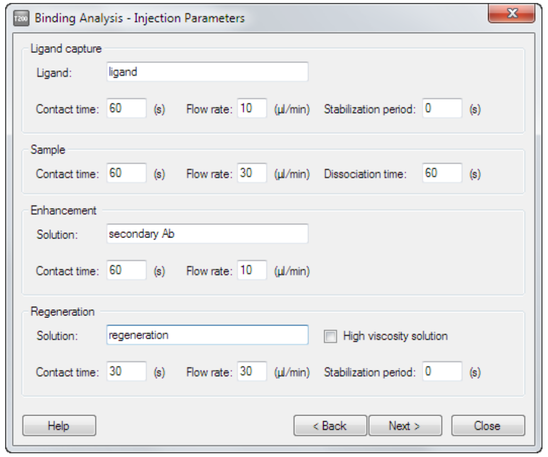
Start-up cycles

Start-up cycles are identical to analysis cycles except that the sample may be replaced by a dummy sample such as buffer. The response from a newly prepared or newly docked sensor chip often shows some instability during the first few cycles, and start-up cycles allow the response to stabilize before the first analysis cycle is performed. Three start-up cycles are generally recommended for most assay purposes, to ensure a stable response in the analysis. Start-up cycles are treated separately from analysis cycles in the evaluation software.

Start-up cycles are run at the beginning of the experiment (after the conditioning cycle), and also directly after buffer change (in the ***Buffer Scouting*** wizard) and temperature change (in the ***Thermodynamics*** wizard).

4.2.3 Injection parameters

The ***Injection parameters*** dialog specifies details of injections selected in the ***Injection sequence***. Injections for which the conditions are fixed in the software are not listed.



Details of this dialog box vary according to the injections selected and the particular wizard. Some features may be generalized:

Parameter limits

Flow rates can be set between 1 and 100 µL/min in increments of 1 µL/min.

Permitted ranges for injection contact times are determined by the flow rate together with the limits for injected volumes, which are 2–350 µL for normal solutions and 5-100 µL for viscous regeneration solutions (see below).

Note: *The injected volume of solution is determined by the combination of flow rate and contact time, rounded to the nearest whole number. At low flow rates, this can result in actual contact times that differ from the requested times: for example, at 1 µL/min a requested contact time of 200 s (requiring 3.3 µL solution) will result in an actual contact time of 180 s (solution volume rounded to 3 µL).*

Stabilization time after injection

This function is available after a capture injection and after the last injection in the sequence. For capture injections, a stabilization time can be useful if a fraction of the ligand dissociates rapidly. Including a stabilization time to allow for such dissociation can help to improve reproducibility.

A stabilization time may be used after the last injection instead of regeneration for systems where analyte dissociates sufficiently rapidly from the surface.

Exposure of the surface to regeneration solution can often lead to transient changes in the baseline. Inclusion of a stabilization time after regeneration helps to ensure a stable baseline for the next cycle.

Sample injection

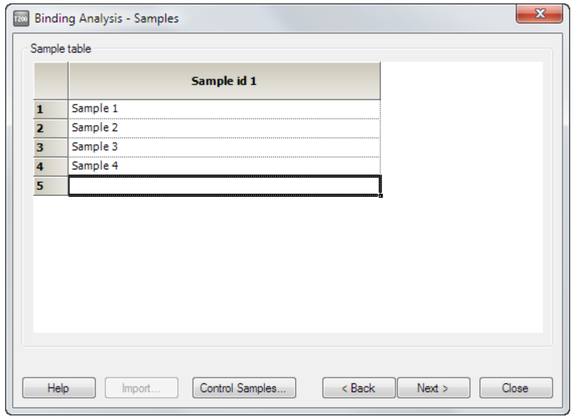
Normally, the injected sample solution is specified in a separate sample table. Some wizards (e.g. ***Surface Performance***) use only a single sample solution that is specified together with the other injection parameters in this dialog box.

Regeneration

The parameters for regeneration include a check-box for ***High viscosity solution***. Check this box if the regeneration solution has a relative viscosity higher than about 3 (corresponding to about 35% glycerol or 40% ethylene glycol at 20°C). This will modify the injection procedure for better handling of viscous solutions. The maximum injected volume is limited to 100 µL when this option is checked.

4.2.4 Sample and control sample tables

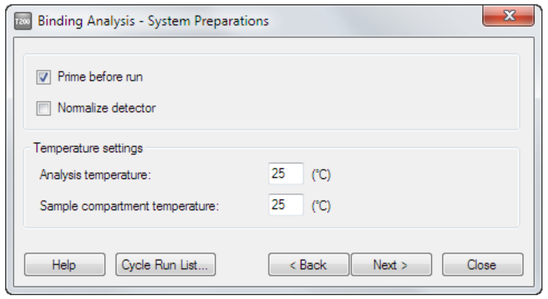
Details of samples and control samples (where applicable) are entered in the ***Sample*** and ***Control Samples*** steps respectively. The details of these steps differ according to the wizard purpose, but the following general features may be noted. Further details are given in the respective wizard descriptions later in this chapter.



- The number of completed rows in the sample and control sample tables determine the number of cycles that will be run in the assay. The rack position requirements and the required volumes of common solutions such as regeneration (Section 4.2.6) are calculated on the basis of the number of samples that will be run.
- For some wizards, control samples are defined in a step in the dialog box sequence. For others, the ***Control samples*** dialog is accessed through a button in the ***Samples*** step.
- Sample details can be imported from an external file if this option is enabled under ***Tools:Preferences***. See Appendix A for details of import formats and procedures.

4.2.5 System preparations

This dialog box specifies how the system will be prepared before the first cycle.



Prime before run

This option flushes the flow system with running buffer to make sure that all tubing contains fresh buffer. You should generally prime the system before each run to ensure fresh buffer throughout the flow system.

Normalize

This option adjusts the detector response to compensate for small variations in reflectance characteristics between individual sensor chips. For best results, you should normalize the detector whenever the chip is changed. You do not need to run normalization if the same chip remains docked between runs.

Normalization injects BIAnormalizing solution (70% glycerol) over the surface: if your ligand does not withstand exposure to this solution, normalize the detector before you immobilize the ligand.

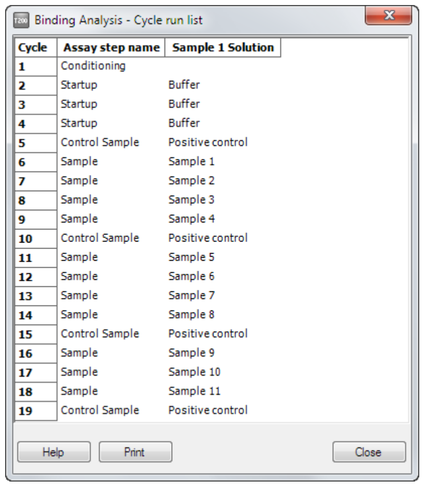
Temperature settings

The ***Analysis temperature*** is the temperature at the flow cell. If the specified value differs from the current temperature, the system will wait at the beginning of the run until the analysis temperature is stable at the new value. You can choose to ignore temperature instability, but the response will drift as the temperature stabilizes. The absolute response decreases by about 150 RU for a 1°C increase in temperature.

The ***Sample compartment temperature*** is the temperature in the sample compartment. Equilibration of the sample compartment to a new temperature will start when the run is started. The system will not wait for a stable sample compartment temperature at the beginning of the run: samples equilibrate to the analysis temperature during passage through the IFC, so that the sample compartment temperature is not critical for the measured SPR response.

Note: *Both analysis temperature and sample compartment temperature can be set in advance with the* ***Set Temperature*** *option from the main* ***Tools*** *menu (Section 2.3.6), to allow the temperature to equilibrate before setting up the assay wizard.*

Cycle run list

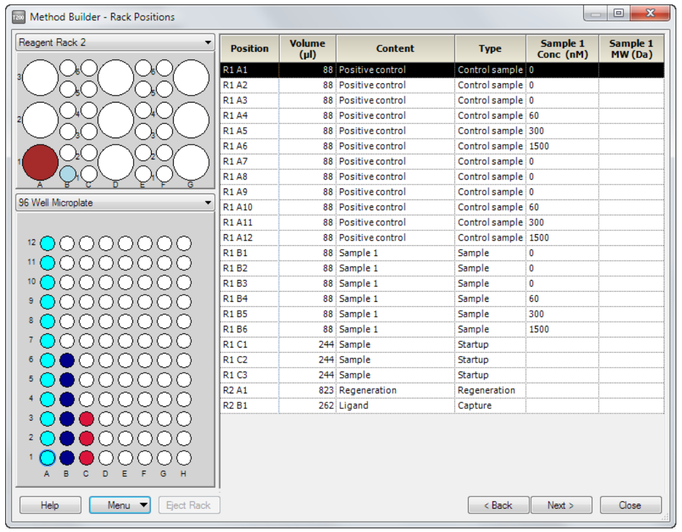


Click ***Cycle Run List*** to display a preview of the cycles that will be run in the assay.

The ***Assay step name*** is generated automatically as a type identifier for each cycle. Other keywords may also be generated according to the wizard purpose. Keyword information is used in the Evaluation Software.

4.2.6 Rack positions

This dialog box shows where samples and reagents are to be placed in the microplate and/or rack. Positions are color-coded by region according to sample and reagent categories: you can change the color-coding in the ***Automatic positioning*** dialog, accessed through the ***Menu*** button.



Use the pull-down lists above the respective illustrations to change the reagent rack and microplate types. If you change a rack or microplate type, all positions in the affected rack or plate will be cleared and must be reassigned either manually or automatically.

Positions are described by tool tips (hold the cursor on the position to display the tool tip). Empty positions show the position capacity and dead volume. Used positions show in addition the content name and the volume that will be used.

Note: *The volumes listed in the table are minimum volumes. Use slightly larger volumes if possible to allow for slight variations in the dead volume in microplates and vials.*

You can change sample and reagent positions manually in two ways:

- Click on a sample or reagent in the sample plate and rack illustration and drag it to a new (empty) position. You cannot drag to a position that does not have sufficient capacity for the required volume of sample or reagent.
- Enter an unused position directly in the ***Position*** column in the table.

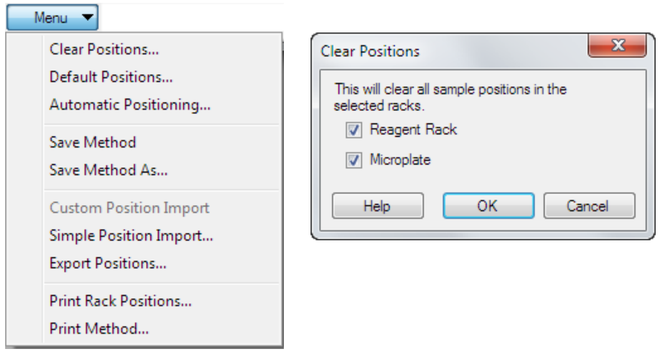
Positions can also be reorganized using the ***Automatic positioning*** dialog (see below).

Menu functions

Use the ***Menu*** button to access additional functions for rack positioning. These functions are not available if the positions have been locked.

Clear Positions

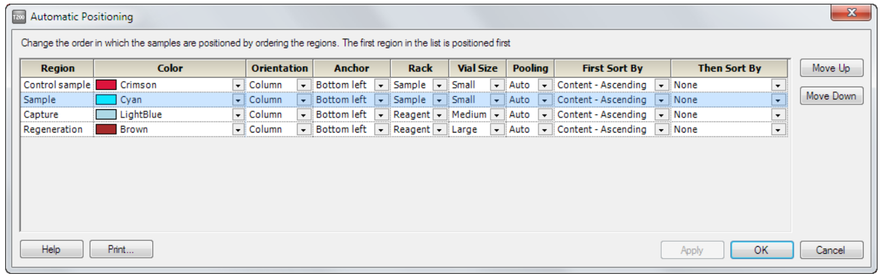
This option clears the entries in the ***Positions*** column for the selected rack or plate.



Positions that are cleared must be reassigned before the run can be started. To reassign positions one by one, select a row in the positioning table and click on the required (empty) position in the illustration. To reassign all positions in one operation, choose ***Default Positions*** or ***Automatic positioning*** from the menu.

Default Positions
This option restores all entries to default positioning. The default positioning is determined from the type and volume of solution in combination with the currently selected rack type. Choosing ***Default Positions*** overrides any changes that have been made in the rack positions, even if the changed positions have been saved in the wizard template.

Automatic Positioning
This option controls the way samples and reagents are positioned automatically. Samples and reagents are placed by region, and samples within regions are kept together as far as possible.



Region This column lists the sample and reagent regions.

Color This option controls the display color for the region.

Orientation This column determines whether samples are arranged by ***column*** (vertically in the rack and plate diagram) or ***row*** (horizontally in the diagram).

Anchor This column determines the position for the first sample in the region.

Rack This option controls whether the samples and reagents will be placed in the reagent rack or the sample microplate. If ***Auto*** is chosen, placement is decided on the basis of number and volume of solutions in the region.

Vial size Use this option to determine the vial size for reagents. If ***Auto*** is chosen, placement is decided on the basis of the volume of solution. The vial size is ignored for rack type ***Sample***.

Pooling This option allows you to combine solutions with the same name into one position or to split combined solutions into separate positions for each cycle. Choose ***Yes*** to pool solutions if suitable vial positions are available, or ***No*** if you always want separate positions for each cycle. Choose ***Auto*** to set the pooling according to the default settings for the type of region.

Sort by Solutions within a region may be sorted by one or two parameters.

Use the ***Move up*** and ***Move down*** buttons to change the order in which regions are listed. Regions are placed in the specified rack or plate in the order listed, so that changing the order of the table can change the automatic positioning of samples and reagents.

Save Wizard Template/Save Wizard Template As
Saves the wizard template, with either the same or a different file name. The corresponding function is also available for methods.

Custom Position Import
This option imports positioning information from an external source. The option is only available if ***Enable custom position import*** is checked in ***Tools:Preferences***. Choosing the option first exports the rack positions table to a temporary tab-separated text file which is processed by the import program specified in the ***Tools:Preferences*** dialog. The output of the import program is then imported to the ***Rack Positions*** table, replacing the existing positioning information. See Appendix A for more details.

Simple Position Import
Imports positioning details from an external file. Details of the import settings and file format are described in Appendix A.

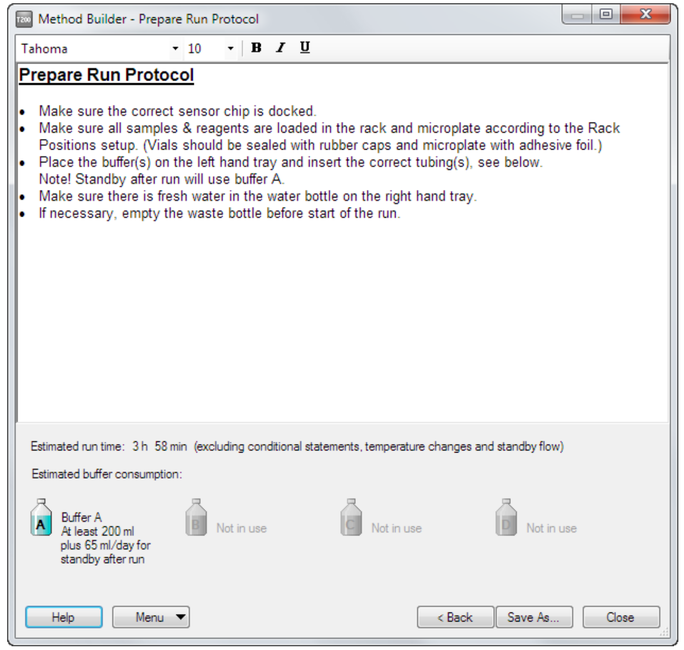
Export Positions
Exports the data in the positioning table to a tab-separated text file. See Appendix A for details of the exported file format.

Print Rack Positions
Prints a copy of the rack positions diagram and table.

Print Wizard Template/Print Method
Prints a copy of the currently open wizard template or method (excluding rack positions).

4.2.7 Prepare Run protocol

This dialog box allows you to enter a run protocol to provide instructions to the user when the run is started. The text in the ***Prepare Run Protocol*** is saved with the wizard template. A suggested general protocol is provided.



Select text and use the controls at the top of the dialog box to control the appearance (typeface, size and style) of the text.

The estimated run time and buffer requirement are shown at the bottom of the dialog box. For all wizards except buffer scouting, only buffer A is used. For the buffer scouting wizard (Section 4.7) and Method Builder-based runs (Section 5.4), buffer names are shown in the ***Prepare Run Protocol*** dialog.

Notes: *The estimated run time and buffer consumption are minimum values, that do not include any time that cannot be predicted when the wizard is set up. This includes time for temperature equilibration at the beginning of the run or between cycles, for ligand injection in immobilization runs using* ***Aim for immobilized level*** *(Section 4.5) and for conditional statements (Section 5.6.1) in Method Builder-based runs.*

The estimated buffer requirement includes a dead volume of at least 50 mL in the buffer bottle and is rounded up to the nearest 100 mL. The actual consumption will often be considerably less than the estimate.
Make sure there is sufficient buffer in the bottle to allow for standby time after the run.

The ***Menu*** button provides options for saving and printing the wizard template (Section 4.2.6).

4.3 Wizard groups

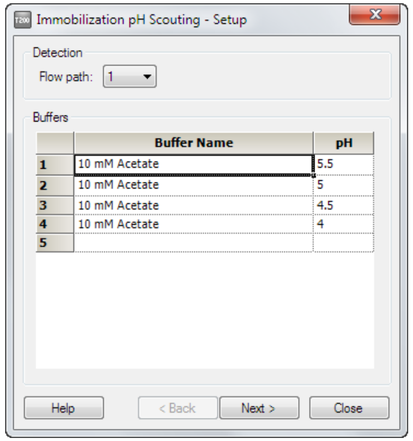
The application wizards are organized into 5 groups:

- ***Surface preparation***, covering immobilization pH scouting and immobilization
- ***Assay development***, covering regeneration scouting, buffer scouting and surface performance tests
- ***Control experiments*** for kinetic measurements, covering tests for linked reaction mechanisms and mass transfer limitation
- ***Assay wizards***, covering kinetics/affinity, binding analysis, concentration analysis and thermodynamics
- ***Immunogenicity***, covering screening, confirmation and isotyping for immunogenicity investigations.

4.4 Immobilization pH scouting

The ***Immobilization pH scouting*** wizard helps you to find the optimal pH for immobilizing your ligand, by testing ligand pre-concentration at a range of pH values. See cytiva.com/biacoregetstarted for more information about ligand immobilization. The injection sequence for immobilization pH scouting is fixed.

Step 1. Setup

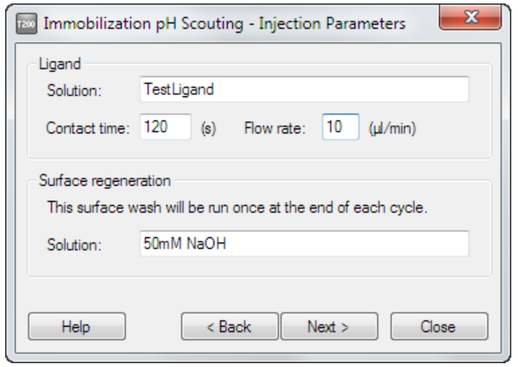


Choose the flow path for the pH scouting. Immobilization pH scouting is restricted to a single flow cell within a run. The sensor surface in the flow cell should be unmodified.

Enter the buffers and pH values to be used for scouting. The default list covers sodium acetate buffers in the pH range 4 to 5.5, available as ready-to-use solutions from Cytiva. Buffers will be tested in the order listed.

Note: *The buffers listed here are buffers in which the ligand should be prepared. They are not used as running buffers: you should use the same running buffer for pH scouting as you intend to use during immobilization.*

Step 2. Injection parameters



Enter the name of the ligand to be tested and the contact time and flow rate. Recommended settings are a contact time of 120 seconds at 5 or 10 µL/min: you may need to use a longer contact time if preconcentration of ligand on the sensor surface proves to be slow.

The surface is washed with a "regeneration" injection at the end of each cycle to remove any ligand that might remain on the surface. The recommended solution for this procedure is 50 mM NaOH.

Step 3. System preparations

See Section 4.2.5. Run immobilization pH scouting at the same temperature as you intend to run the immobilization (electrostatic preconcentration is however usually fairly insensitive to temperature).

Step 4. Rack positions

See Section 4.2.6. Immobilization pH scouting requires one position for ligand solution at each pH tested and one for the surface wash solution. Accept or change the rack positions for the various solutions required (see Section 4.2.6).

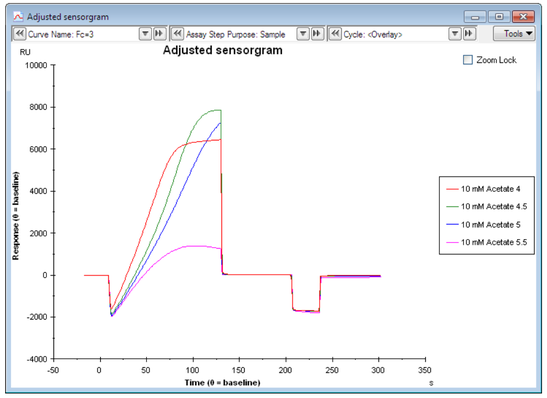
Step 5. Prepare run protocol

Edit the ***Prepare Run Protocol*** text if desired (Section 4.2.7). This text will be displayed at the start of a run when the wizard template is used. Save the wizard template if required and start the run.

pH scouting results

When the wizard run is completed, the Evaluation Software is opened automatically with an ***Adjusted sensorgrams*** item containing an overlay plot of the sensorgrams adjusted to the start of the sample injection. The same overlay plot is created when a saved run from ***Immobilization pH Scouting*** is opened in the Evaluation Software.

Note: *Opening a saved run in the Control Software does not generate the overlay plot automatically.*



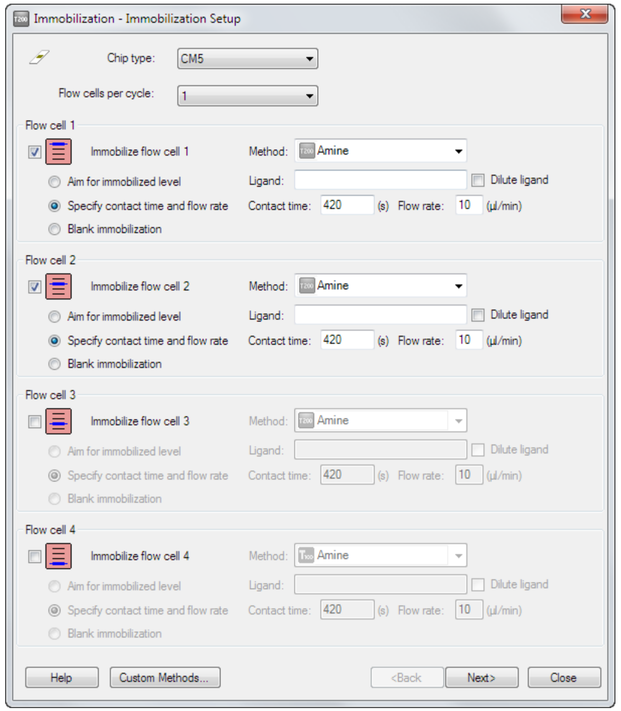
Choose the optimum buffer pH on the basis of the binding behavior: at pH suitable for immobilization, the ligand binds rapidly to the surface during the injection and dissociates completely after the end of the injection. The optimum is generally the highest pH value (i.e. the mildest condition) that gives sufficient ligand binding, not necessarily the value that gives the highest ligand binding. Beware of conditions that give irregular sensorgrams with incomplete dissociation: this behavior often indicates aggregation or denaturation of the ligand.

4.5 Immobilization

The ***Immobilization*** wizard supports immobilization of ligand in any combination of the four flow cells in one run. Immobilization may be performed independently in a separate cycle for each flow cell, in flow cell pairs (Fc 1-2 and 3-4) or in all four flow cells in one cycle. See cytiva.com/biacoregetstarted, for more information about ligand immobilization.

Note: *Performing immobilization in multiple flow cells in a single cycle does not guarantee identical immobilization levels in each flow cell. In general, immobilization yields tend to be lower in flow cells later in the flow path.*

Step 1. Immobilization setup



The choice of ***Chip type*** determines the predefined methods that are available for immobilization. The type chosen when the chip was docked is chosen by default: if you change the chip type you will be able to create and save an immobilization wizard template, but you must dock a corresponding chip type before the immobilization can be performed.

Choose the number of ***Flow cells per cycle*** and check the flow cells where you want to perform immobilization. Set the immobilization parameters as follows:

Choose the immobilization method. Predefined methods are provided for standard immobilization chemistries. Customized methods can be defined by clicking on the ***Custom Methods*** button (see below). Predefined methods are marked with a T200 icon ( ) in the selection lists.

Choose the way in which immobilization will be controlled:

- If you choose ***Aim for immobilized level***, you specify a target level. The immobilization procedure will attempt to reach this level as described below. This option is not available for immobilization in multiple flow cells in a single cycle.
- If you choose ***Specify contact time and flow rate***, enter the settings in the respective fields.
- If you choose ***Blank immobilization***, the surface will be activated and deactivated in accordance with the immobilization method but no ligand will be injected.

Enter the ligand name. To dilute the ligand solution immediately before injection, check ***Dilute ligand*** and enter a percentage value and a solution name. This option can be used for ligands that have limited stability in immobilization buffer, and that are diluted from a stock solution just before immobilization. A setting of 90% will mix one part of ligand solution with 9 parts of the specified diluent.

Aiming for immobilized level

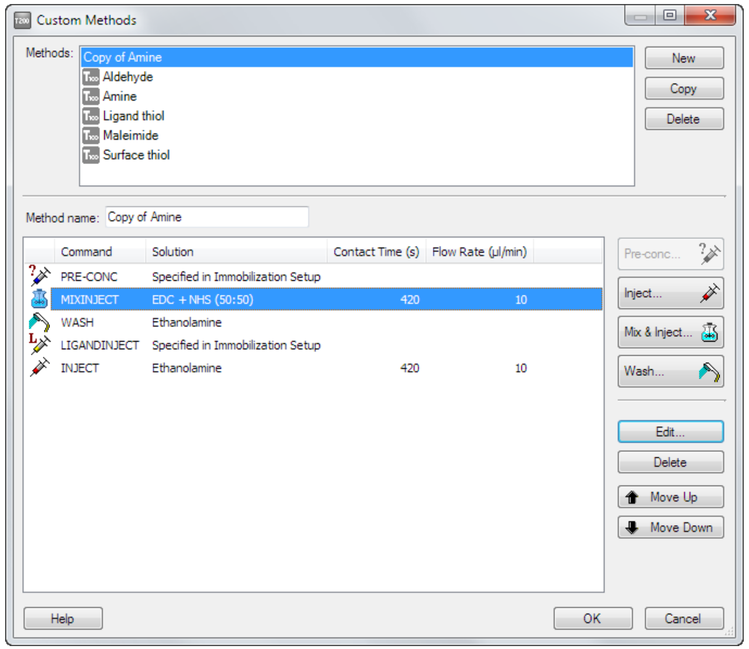
The option ***Aim for immobilized level*** injects a pulse of ligand over the unactivated surface in order to estimate the rate of preconcentration. The surface is washed to remove traces of ligand and then activated. The procedure then uses ligand contact times based on the preconcentration estimate to attempt to reach the specified target level. If preconcentration is either too fast or too slow to permit the target level to be reached, this will be reported and immobilization will not be performed.

The preconcentration step injects 10 µL ligand solution at a flow rate of 5 µL/min, giving a contact time of 2 minutes. This injection is included in predefined methods for CM-series sensor chips but is optional in customized methods (see below).
If ***Aim for immobilized level*** is chosen together with a custom method that does not include a preconcentration injection, the immobilization procedure will activate the surface and then inject short pulses of ligand until either the target level or the maximum total ligand volume of 150 µL is reached. This can be used to conserve valuable ligand without losing the benefits of aiming for a target immobilization level, and can be useful for sensor chips where preconcentration cannot be performed, such as Sensor Chip SA and Sensor Chip Protein A.

Aim for immobilized level is not available for immobilization in multiple flow cells in a single cycle.

Custom methods

Click ***Custom Methods*** to define customized immobilization methods.



Click ***New*** to create a new blank method. Select an existing method and click ***Copy*** to make a copy of the method or ***Delete*** to delete the method. You cannot delete the predefined methods (marked with a T200 icon).

For a new method, enter a name in the ***Method name*** field. Construct the sequence of injections for the immobilization method using the buttons to the right of the main panel. The ligand injection is created automatically and cannot be deleted: solution and contact time for the ligand injection are specified in the main wizard dialog. A method may only contain one ligand injection. Other injections have the following functions:

Pre-conc injects 10 µL of ligand solution at 5 µL/min to estimate the rate of preconcentration. This step is only performed if the option ***Aim for immobilized level*** is chosen when the immobilization method is used. A method may only contain one ***Pre-conc*** injection. The ***Pre-conc*** injection should always be placed before surface activation: it will usually be first in the method, although it may be preceded by a surface conditioning injection if required. If you place the ***Pre-conc*** injection after the surface activation, it will be executed there and the ligand will be immobilized on the activated surface.

After the ***Pre-conc*** injection, the surface is washed with a solution specified in the immobilization setup dialog, to remove any ligand that may remain on the surface.

Do not use a ***Pre-conc*** injection with Sensor Chip SA, since biotinylated ligand will bind to the surface and cannot be removed.

Inject performs an injection of a specified solution with a specified contact time and flow rate. Values are entered in the dialog box that appears when you click ***Inject.***

Mix & Inject mixes two specified solutions and performs an injection of the mixture. Details are entered in the dialog box that appears when you click ***Mix & Inject.***

Wash washes the flow system (but not the sensor surface). The wash solution is specified in the dialog box that appears when you click ***Wash.***

Select an injection and use the ***Edit***, ***Delete***, ***Move up*** and ***Move down*** buttons to edit the injection details, remove the injection from the method and change the order of injections in the method.

Custom methods are stored in the immobilization wizard template: if you need the same or slightly modified method in a different template, save a copy of the template and then edit the method.

Step 2. System preparations

Check the ***System preparations*** options as required (see Section 4.2.5).

Step 3. Rack positions

Accept or change the rack positions for the various solutions required (see Section 4.2.6).

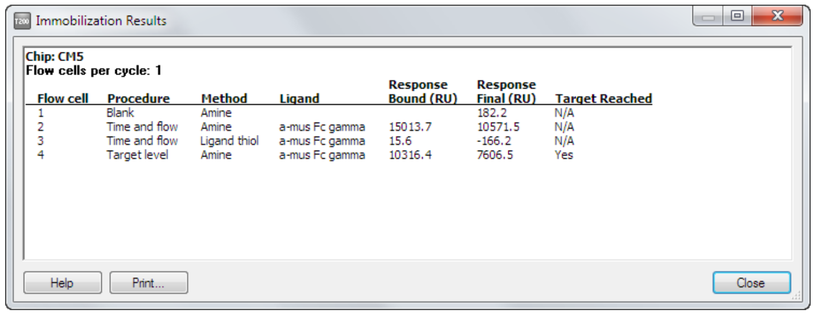
Step 4. Prepare run protocol

Edit the ***Prepare Run Protocol*** text if desired (Section 4.2.7). This text will be displayed at the start of a run when the wizard template is used. Save the wizard template if required and start the run.

Immobilization results

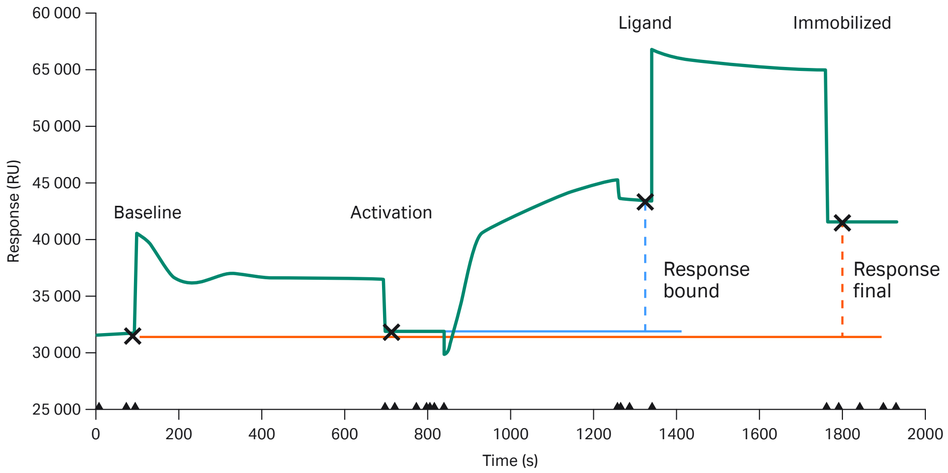
The results of an immobilization run are summarized in the Control Software and logged in the ***Chip Properties*** (see Section 2.3.4) when the run is completed.

Note: *The same information can be accessed under* ***File:Properties*** *in the Evaluation Software.*



The summary lists the procedure and method, the name of the ligand and whether the target was reached with ***Aim for immobilized level***. Two response values are reported, one directly after the ligand immobilization and one after the deactivation injection. The difference between these values is an indication of the amount of non-covalently bound ligand that is washed from the surface by the deactivation injection.

Note that the ***Response bound*** value does not include the contribution from activation by EDC/NHS. For low ligand levels, this value can give a better indication of the amount of ligand immobilized.



Result files from immobilization can also be opened in the Evaluation Software if you want to prepare other sensorgram displays or plots (see Chapter 7).

4.6 Regeneration scouting

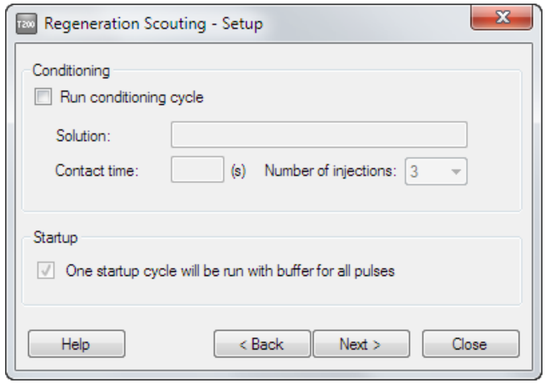
The ***Regeneration Scouting*** wizard guides you through the process of finding suitable regeneration conditions for your sensor surface. See cytiva.com/biacoregetstarted, to learn more about the principles of regeneration scouting. Briefly, regeneration scouting is performed by testing repeated cycles of analyte injection and regeneration over a range of regeneration conditions, and assessing the results on the basis of trends in analyte response and baseline levels. The analyte concentration should be relatively high for best results. The analyte response reflects the binding capacity (ligand activity) of the surface, while the baseline level indicates the extent of regeneration. Each condition should be tested for at least 3 cycles in sequence (recommended number 5) in order to detect trends in the regeneration behavior with the given condition. When testing multiple conditions, start with the mildest conditions to minimize the risk of losing ligand activity at the beginning of the scouting series.

Step 1. Injection sequence

Choose the detection settings, chip type and injection sequence for the regeneration scouting (see Section 4.2.1). One sample injection and one or two regeneration injections are required. Most sensor surfaces can be adequately regenerated with a single injection, but some situations may benefit from using multiple injections.

Step 2. Setup

Specify a conditioning cycle if required.

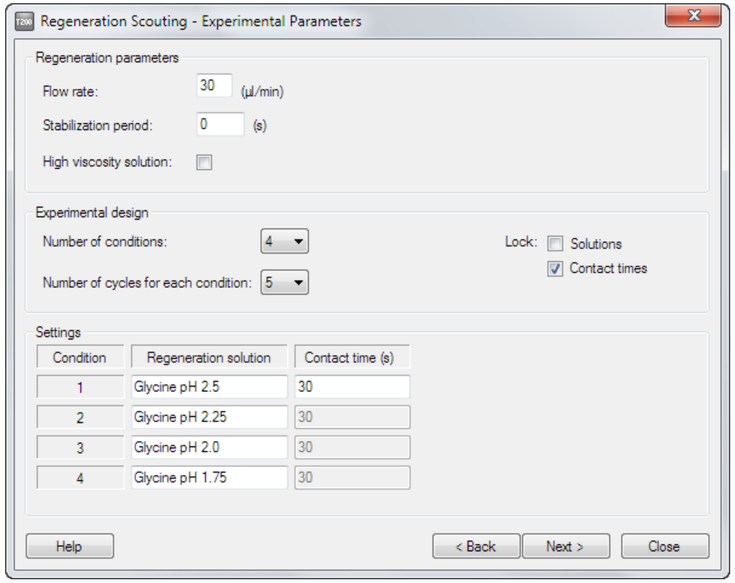


Regeneration scouting always includes one startup cycle with the same injection sequence as the scouting cycles but with injection of buffer for all injections. Note that this differs from the startup cycle construction in the other application wizards.

Step 3. Injection parameters

Specify the injection parameters for each injection in the cycle (see Section 4.2.3). The same sample will be used for all cycles.

Step 4. Experimental parameters



This dialog box determines the design of your regeneration scouting. Set the number of conditions to test and the number of cycles for each condition and specify the conditions in the ***Settings*** frame. The default number of cycles for each condition is five. You may use fewer cycles to shorten the total run time for exploratory work, but five cycles are recommended for fine-tuning conditions in order to reveal trends in the regeneration performance.

Use variants of the same kind of regeneration conditions (e.g. different pH values or different concentrations of ethylene glycol) within the same run. Results are most easily interpreted if you use a separate wizard run with a new flow cell or sensor chip for each kind of regeneration condition that you test, so that the outcome with one kind of condition is not affected by the history of exposing the ligand to another condition.

You may choose to lock the solutions or the contact time used for regeneration tests, so that all conditions will use the same setting for the locked parameter. Do not vary both the solution and the contact time at the same time: the results may be difficult to interpret clearly.

Check ***High viscosity solution*** if any of the regeneration solutions tested has a relative viscosity higher than about 3 (corresponding to about 35% glycerol or 40% ethylene glycol at 20°C). This will modify the injection procedure for better handling of viscous solutions.

Step 5. System preparations

Check the ***System preparations*** options as required (see Section 4.2.5).

Step 6. Rack positions

Accept or change the rack positions for the various solutions required (see Section 4.2.6).

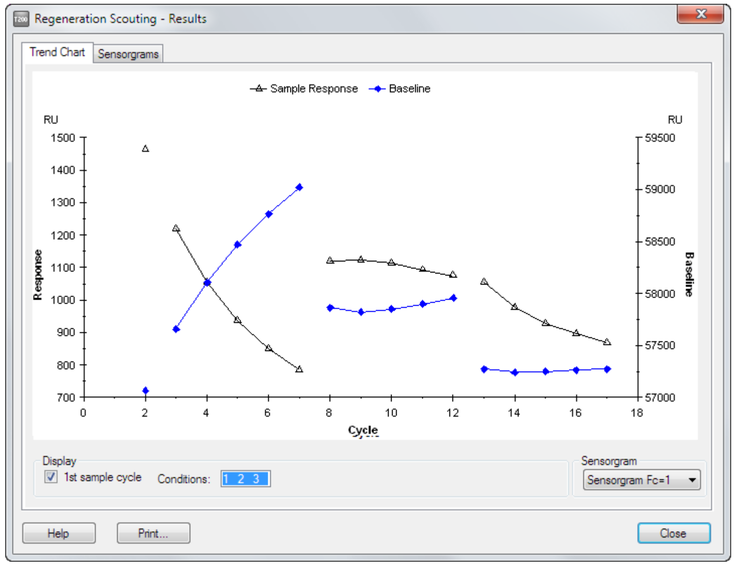
Step 7. Prepare run protocol

Edit the ***Prepare Run Protocol*** text if desired (Section 4.2.7). This text will be displayed at the start of a run when the wizard template is used. Save the wizard template if required and start the run.

Regeneration scouting results

Regeneration scouting results are presented in the Control Software when the run is completed.

Note: *This result presentation is not shown if the run is opened in the Evaluation Software.*



The ***Trend chart*** tab shows the results as a plot of baseline and sample response for each cycle in the run, grouped by regeneration conditions. Conditions are identified in tool tips for the data points (place the cursor on a point for a couple of seconds to display the tool tip).

Note: *Report points are set before the sample injection for baseline and shortly after the sample injection for sample response. Thus the points for the first cycle indicate the starting values, while those for subsequent cycles each indicate the effect of the previous cycle.*

Check ***1st cycle*** to include the starting values derived from the first sample cycle in the plot. (This cycle is shown as cycle number 2 or 3: conditioning and startup cycles are numbered but not shown.)

Select which conditions to display in the ***Conditions*** box. Use Shift-click to make multiple adjacent selections, Ctrl-click to make multiple non-adjacent selections. The scale of the display will be adjusted according to the number of cycles displayed.

Select which curves to display in the ***Sensorgram*** box.

The ***Sensorgrams*** tab shows the sensorgrams for regeneration scouting. Select the conditions and cycles to display in the respective boxes. Check ***Zoom lock*** to keep the scale fixed when the choice of sensorgrams is changed.

Result files from regeneration scouting can also be opened in the Evaluation Software if you want to prepare other sensorgram displays or plots (see Chapter 7). See the Biacore Sensor Surface Handbook for a discussion of how to interpret the results of regeneration scouting.

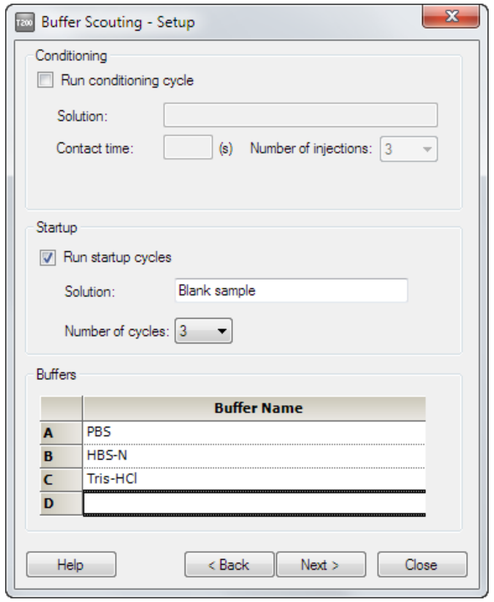
4.7 Buffer scouting

The ***Buffer Scouting*** wizard helps you to test the effect of up to four different buffers on your assay, using the buffer selector valve to switch running buffers.

Step 1. Injection sequence

Choose the detection settings, chip type and injection sequence for the buffer scouting (see Section 4.2.1). The flow path can either be chosen explicitly (in which case the same flow path will be used for each buffer), or set to vary with the buffer (in which case a single flow cell will be used for each buffer, with flow cells 1, 2, 3 and 4 for buffers A, B, C and D respectively).

Step 2. Setup



Specify the conditioning and start-up cycles as required. If start-up cycles are chosen, separate rack positions will be created for the start-up sample solution in each buffer tested, and the start-up cycles will be run at the start of each buffer test. Conditioning if used will only be run once at the start of the experiment.

Specify the buffers you want to test. You can enter up to 4 different buffers. The buffers will be tested in the order given.

Note: *If you use less than 4 buffers, positions A, B and C will be used in order.*

If you enter buffer names on non-consecutive rows, the table will be adjusted when you leave the dialog box.

Step 3. Injection parameters

Specify the injection parameters for each injection in the cycle (see Section 4.2.3).

Step 4. Samples

Enter the samples to be tested in the buffer scouting. The scouting procedure will work through the sample table for the first buffer before switching to the next buffer.

Ligands for capture, samples and enhancement reagents should be prepared in each of the buffers tested. Separate rack positions will be created for samples in each buffer (for example, running buffer scouting with 4 buffers and 5 samples will require 20 sample positions).

Step 5. System preparations

Check the ***System preparations*** options as required (see Section 4.2.5).

Step 6. Rack positions

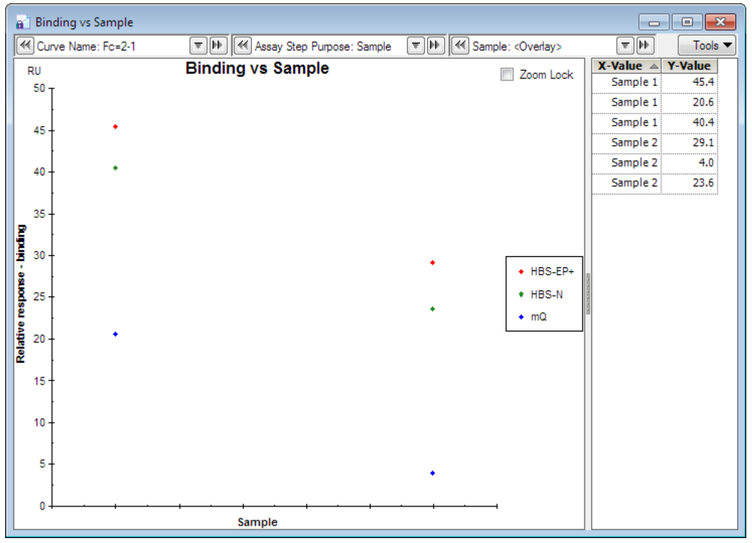
Accept or change the rack positions for the various solutions required (see Section 4.2.6).

Step 7. Prepare run protocol

Edit the ***Prepare Run Protocol*** text if desired (Section 4.2.7). This text will be displayed at the start of a run when the wizard template is used. Save the wizard template if required and start the run.

Buffer scouting results

When the wizard run is completed, the results are opened automatically in the Evaluation Software. In addition to the general predefined plots (Section 6.4), plots of binding and stability against sample are created to visualize the behavior in the different buffers.



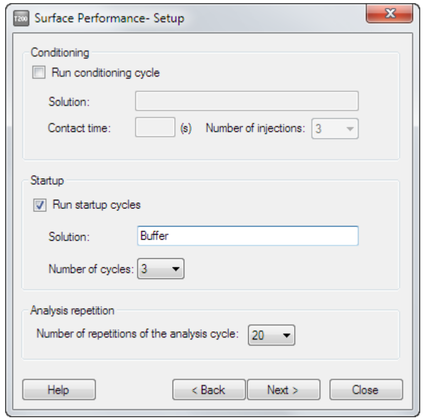
4.8 Surface performance

The ***Surface Performance*** wizard allows you to test the robustness of your surface by performing repetitions of the same analysis cycle. The software allows up to 100 cycles, but the number of repetitions may in practice be limited by the capacity of the sample plate. The cycle requires one sample injection, and can also include capture and enhancement steps and one or two regeneration steps. Use this wizard for example when you want to confirm that the regeneration conditions that you identified in regeneration scouting hold good for an extended number of cycles.

Step 1. Injection sequence

Choose the detection settings, chip type and injection sequence for the surface performance test cycle (see Section 4.2.1).

Step 2. Setup



Specify conditioning and start-up cycles (see Section 4.2.2).

Set the number of repetitions of the analysis cycle according to the purpose of the surface performance test. As a general guide, the test should run for at least as many cycles as will be used normally in the assay.

Step 3. Injection parameters

Specify the injection parameters for each injection in the cycle (see Section 4.2.3).

Step 4. System preparations

Check the ***System preparations*** options as required (see Section 4.2.5).

Step 5. Rack positions

Accept or change the rack positions for the various solutions required (see Section 4.2.6).

Step 6. Prepare run protocol

Edit the ***Prepare Run Protocol*** text if desired (Section 4.2.7). This text will be displayed at the start of a run when the wizard template is used. Save the wizard template if required and start the run.

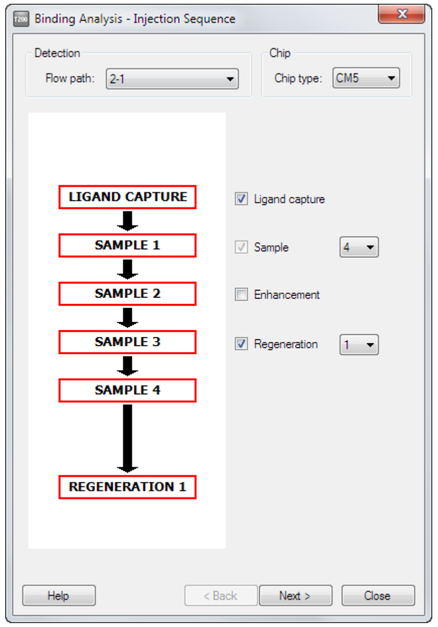
Surface performance results

When the wizard run is completed, the results are opened automatically in the Evaluation Software. Examine the plots of baseline and sample response against cycle number. The response values should ideally be unchanged throughout the run.

4.9 Binding analysis

The ***Binding Analysis*** wizard supports injection of up to four samples in series, in addition to ligand capture, enhancement and regeneration steps. This wizard is suitable for analysis of applications like multi-component complex formation and pair-wise epitope mapping, as well as simple applications like screening for binding partners to an immobilized ligand.

Step 1. Injection sequence

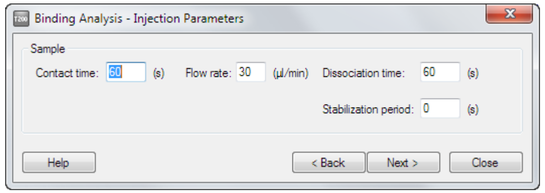


Choose the detection settings, chip type and injection sequence for the binding analysis (see Section 4.2.1). Up to four sequential sample injections may be included in each cycle.

Step 2. Setup

Specify the conditioning and start-up cycles (see Section 4.2.2).

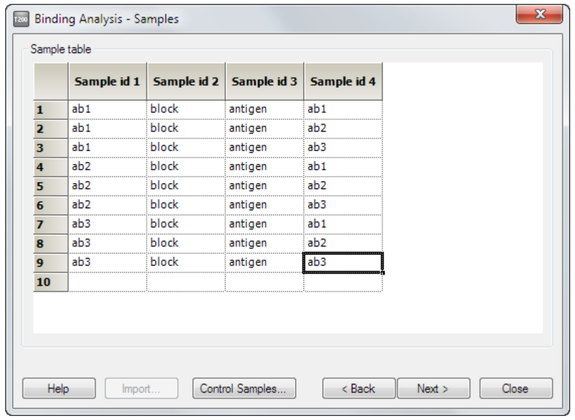
Step 3. Injection parameters



Specify the injection parameters for each injection in the cycle (see Section 4.2.3). One sample injection panel will be created for each sample

injection in the injection sequence. The sample injection for binding analysis has an additional setting for dissociation time. This is the time for which dissociation will be monitored after the end of the injection without disturbances from flow system washing procedures.

Step 4. Samples

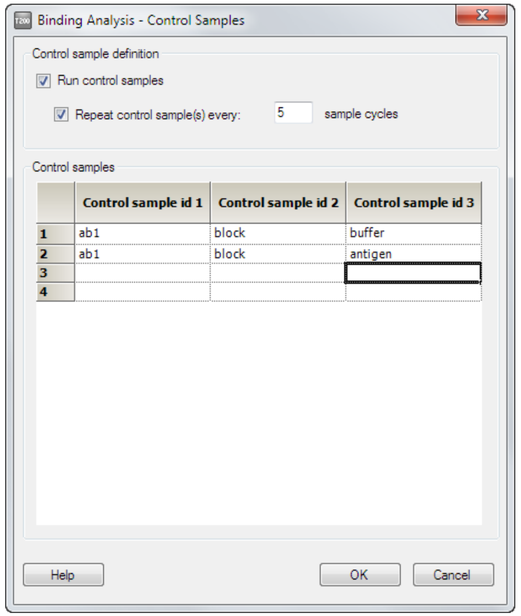


	Sample id 1	Sample id 2	Sample id 3	Sample id 4
1	ab1	block	antigen	ab1
2	ab1	block	antigen	ab2
3	ab1	block	antigen	ab3
4	ab2	block	antigen	ab1
5	ab2	block	antigen	ab2
6	ab2	block	antigen	ab3
7	ab3	block	antigen	ab1
8	ab3	block	antigen	ab2
9	ab3	block	antigen	ab3
10				

The sample table contains one column for each sample injection in the injection sequence. New rows are created as you enter data in the table. (The illustration above shows how the wizard could be used to set up a pair-wise epitope mapping experiment.)

Click ***Import*** to import the sample data from an external file. Import of sample information must be enabled in ***Tools:Preferences*** to use this function. See Appendix A for details of import functions and file formats.

Click ***Control Samples*** to enter control samples for the run.



	Control sample id 1	Control sample id 2	Control sample id 3
1	ab1	block	buffer
2	ab1	block	antigen
3			
4			

Specify the details of control samples and the frequency with which they should be run. If you check only ***Run control samples***, the controls will be run once only at the start of the assay. If you check ***Repeat control samples every...***, the controls will be run at the start of the assay and then at the specified interval, and again at the end of the assay. Use the ***Cycle Run List*** option in the ***System Preparations*** step to verify where control samples will be run during the assay.

Step 5. System preparations

Check the ***System preparations*** options as required (see Section 4.2.5).

Step 6. Rack positions

Accept or change the rack positions for the various solutions required (see Section 4.2.6).

Step 7. Prepare run protocol

Edit the ***Prepare Run Protocol*** text if desired (Section 4.2.7). This text will be displayed at the start of a run when the wizard template is used. Save the wizard template if required and start the run.

Binding analysis results

When the wizard run is completed, the results are opened automatically in the Evaluation Software. Predefined plots (Section 6.4) are created for each sample.

4.10 Concentration analysis

The ***Concentration Analysis*** wizard helps you to set up an assay for determining analyte concentration in samples with the help of a calibration curve using known concentrations. Control samples may be included at intervals to monitor the stability of the assay.

Note: *Analyte concentrations can be determined without reference to a calibration curve using the calibration-free concentration assay approach, supported in Method Builder (see Section B.2).*

Step 1. Injection sequence

Choose the detection settings, chip type and injection sequence for the concentration analysis (see Section 4.2.1).

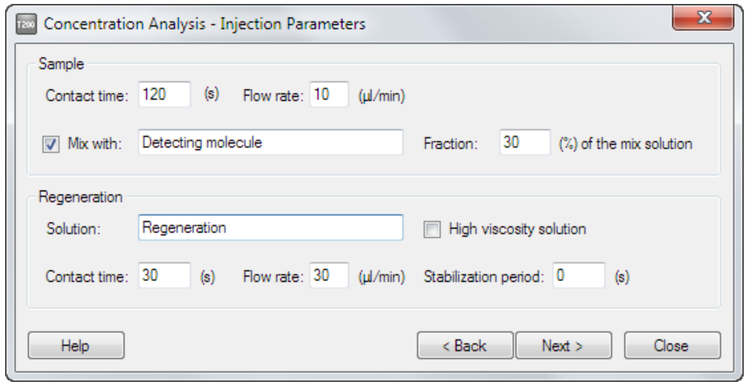
Step 2. Setup

Specify the conditioning and start-up cycles (see Section 4.2.2). Start-up cycles are recommended for concentration assays to ensure that the initial response drift that may occur with a new chip does not interfere with the first measurements.

Step 3. Injection parameters

Specify the injection parameters for each injection in the cycle (see Section 4.2.3).

The sample injection for concentration analysis can be extended to include a mixing function, whereby sample is mixed with a specified proportion of a second fixed solution. This feature enables inhibition assay formats where samples are mixed with a constant proportion of a detecting molecule solution. The value specified for ***Fraction*** refers to the proportion of the fixed component in the final mixture: for example, a value of 30% will mix 7 parts of sample with 3 parts of the specified solution.

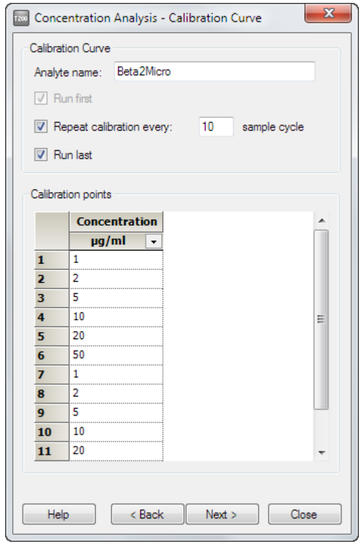


The volumes of sample and mixing solution used are determined automatically so that the final volume of mixed solution is sufficient for the injection.

Notes: *Mixing in the autosampler is very reproducible, but high accuracy cannot be guaranteed. If your application requires accurate mixing proportions, mix the samples outside the autosampler.*

Mixing is not supported in 384-well microplates. The wells on these plates are too small for reliable mixing in the autosampler.

Step 4. Calibration curve



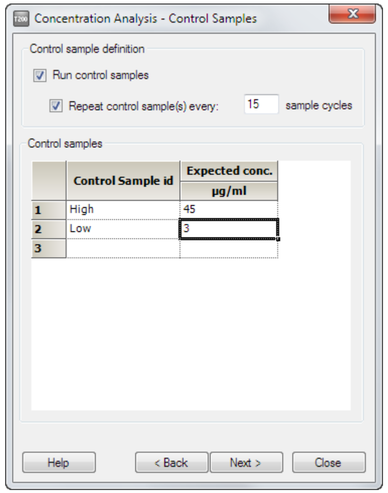
Specify the details of the calibration curve for the concentration measurement.

Check ***Repeat calibration*** and enter a repeat interval to repeat the calibration curve at regular intervals during the assay. The calibration curve will be run at the beginning of the assay (as indicated by the checked option ***Run first***, which cannot be changed) and at the specified interval. Thus specifying a repeat every 15 sample cycles and running 35 samples will result in calibration curves at the beginning and after samples 15 and 30. Use at least 4 calibration curves with one at the beginning and one at the end of the sample series if you intend to use calibration trends for evaluation (see Section 9.2.2).

Check ***Run last*** to include a calibration curve as the last cycle in the assay. Use this option if you intend to use calibration trends in the evaluation to compensate for drift in the calibration curve (see Section 9.2.2).

Enter the concentrations for the calibration points on the curve. You must enter at least two concentrations for the calibration curve. (Two concentrations are sufficient for a linear calibration curve, but if you intend to use the recommended four-parameter fitting function for the calibration curve, you need at least four points.) To run replicate concentrations, enter the same concentration on multiple rows. Calibration points will be run in the order entered. You can choose a different concentration unit if required from the pull- down list in the table header.

Step 5. Control samples

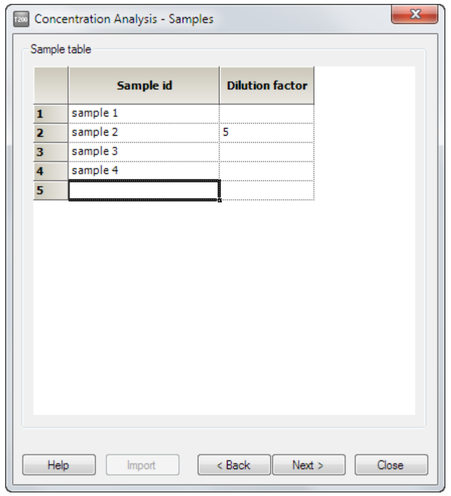


Specify the details of control samples and the frequency with which they should be run. You may choose not to run control samples at all: however, including control samples is generally recommended as an aid in assessing the performance of the assay.

If you check only ***Run control samples***, the controls will be run once only at the start of the assay. If you check ***Repeat control samples every...***, the controls will be run after the first calibration curve and then at the specified interval for sample cycles (not counting repeated calibration curves), and once at the end of the assay. Use the ***Cycle Run List*** option in the ***System Preparations*** step to verify where control samples will be run during the assay.

Control samples are specified in terms of sample ID and expected concentration. The expected concentrations should lie within the range covered by the calibration curve.

Step 6. Samples



Enter the details of the samples to be analyzed. Each sample is defined by a sample ID and a dilution factor: the dilution factor is used during evaluation to calculate the measured concentration in the original undiluted sample. For undiluted samples (dilution factor 1), this column may be left blank.

Samples will be analyzed in the order entered. To analyze replicate samples, enter same sample on multiple rows.

Click ***Import*** to import the sample data from an external file. Import of sample information must be enabled in ***Tools:Preferences*** to use this function. See Appendix A for details of import functions and file formats.

Step 7. System preparations

Check the ***System preparations*** options as required (see Section 4.2.5).

Step 8. Rack positions

Accept or change the rack positions for the various solutions required (see Section 4.2.6).

Step 9. Prepare run protocol

Edit the ***Prepare Run Protocol*** text if desired (Section 4.2.7). This text will be displayed at the start of a run when the wizard template is used. Save the wizard template if required and start the run.

Evaluation of concentration assays

Chapter 9 describes how to evaluate concentration assays.

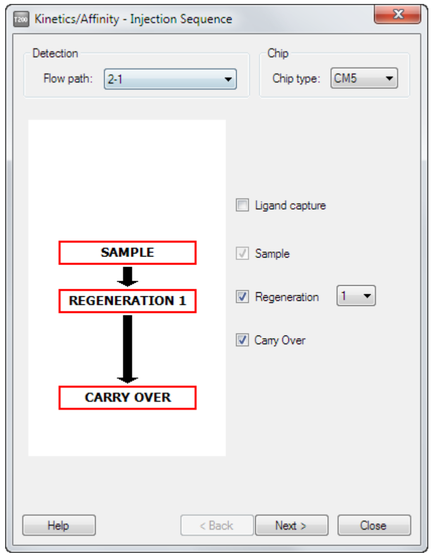
4.11 Kinetics/Affinity

The ***Kinetics/Affinity*** wizard guides you through the setup of experiments to determine kinetic constants or affinity constants for an interaction. Wizards for control experiments relevant to kinetic analysis are described in Section 4.13.

Notes: *The Kinetics/Affinity wizard supports kinetic determinations in multi-cycle format, where each analyte concentration is injected in a separate cycle and analyte is allowed to dissociate fully or is removed by regeneration between cycles. An alternative format that does not require regeneration or full dissociation is single-cycle kinetics, supported in Method Builder (see Section B.14).*

The Kinetics/Affinity wizard is not recommended for kinetics and affinity screening applications. Use a method defined in Method Builder instead (see Appendix B for examples).

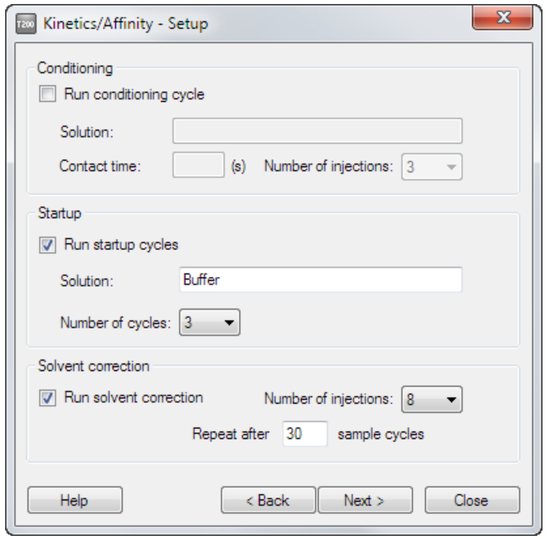
Step 1. Injection sequence



Choose the detection settings, chip type and injection sequence for the assay (see Section 4.2.1). Only reference subtracted detection using either Fc2-1 or Fc4-3 is available for kinetic analysis. The Kinetics wizard supports capture but not enhancement injections.

A ***Carry Over*** injection is also available in the ***Kinetics/Affinity*** wizard. This performs an injection of buffer as the last injection in the sample analysis cycle, to check that the flow system is clean from residual analyte that would otherwise carry over to the sample injection in the next cycle. The ***Carry Over*** injection is fixed as a 30-second injection at 40 µL/min, regardless of other settings in the wizard. Report points are set automatically for the carry-over injection to allow evaluation of potential carry-over problems.

Step 2. Setup

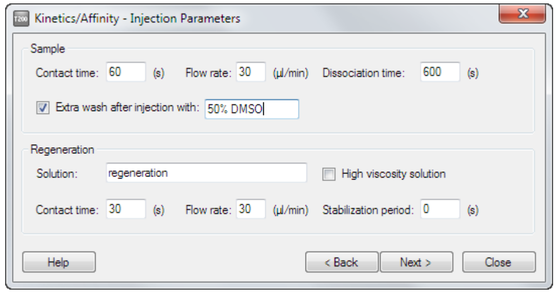


Specify the conditioning and start-up cycles (see Section 4.2.2). Start-up cycles are recommended for kinetics experiments to ensure that the initial response drift that may occur with a new chip does not interfere with the first measurements.

Check ***Run solvent correction*** to include solvent correction cycles in the run. Solvent correction adjusts response values for the effects of varying bulk refractive index contribution between samples, and may improve the quality of the results for analyses using small organic analytes that require dimethyl sulfoxide (DMSO) in the sample buffer to maintain solubility. The principles and application of solvent correction are described in Section 6.7.

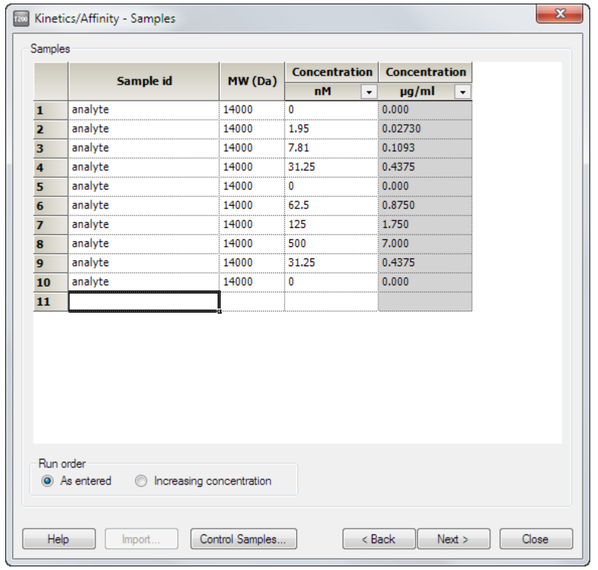
Set the required number of injections per cycle and the frequency of solvent correction cycles. The default settings use 8 injections per cycle and repeat the solvent correction cycle every 30 sample cycles.

Step 3. Injection parameters



Specify the injection parameters for each injection in the cycle (see Section 4.2.3). The sample injection for kinetics measurement has an additional setting for dissociation time. This is the time for which dissociation will be monitored after the end of the injection without disturbances from flow system washing procedures. An extra wash after the sample injection with 50% DMSO is recommended for work with low molecular weight analytes. This wash solution does not pass over the sensor surface.

Step 4. Samples



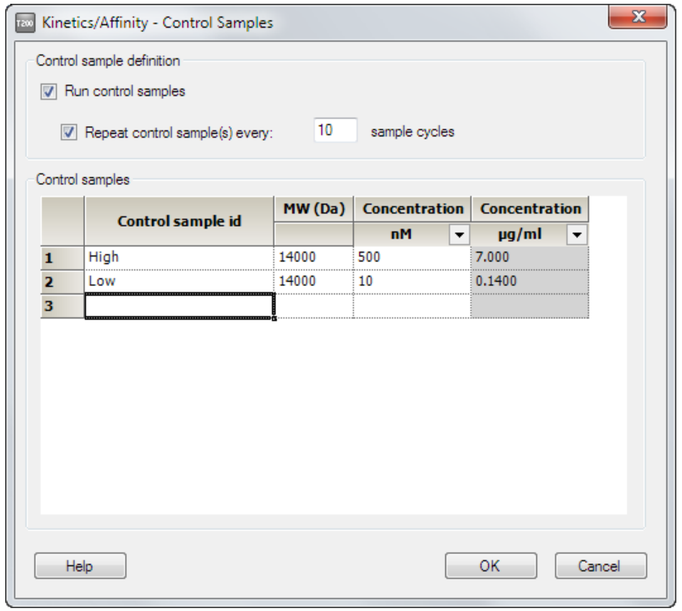
Enter the details of the samples for kinetic or affinity determination. For each analyte, a zero concentration sample and at least four non-zero concentrations, one of which is run in duplicate, are strongly recommended. Concentrations are entered in the left-hand ***Concentration*** column, either in molar or weight-based units. Choose the concentration units from the pull-down list in the column header. If a weight-based concentration unit is chosen (e.g. µg/mL) a molecular weight must also be specified. When a molecular weight is entered, the right- hand ***Concentration*** column displays the conversion from molar to weight- based or *vice versa*. Samples with the same sample name may not be given different molecular weights.

The samples may be analyzed either in the order entered in the table or sorted in increasing concentration. The order displayed in the sample table is not affected by the choice of run order.

If you enter samples with different names, they will be handled as separate concentration series regardless of the order in which they are entered. The samples will be run as separate concentration series even if the order is mixed in this dialog: thus samples entered in the order A, B, A, A, B, A, B, B... will be run in the order A, A, A, A, B, B, B, B... The ***Run order*** setting applies within each concentration series.

Click ***Import*** to import the sample data from an external file. Import of sample information must be enabled in ***Tools:Preferences*** to use this function. See Appendix A for details of import functions and file formats.

Click ***Control Samples*** to enter control samples for the run.



Specify the details of control samples and the frequency with which they should be run. If you check only ***Run control samples***, the controls will be run once only at the start of the assay. If you check ***Repeat control samples every...***, the controls will be run at the start of the assay and then at the specified interval, and again at the end of the assay. Use the ***Cycle Run List*** option in the ***System Preparations*** step to verify where control samples will be run during the assay.

Note: *Control samples do not require a concentration series and will not be evaluated for kinetics and affinity. These samples are intended as check on the consistency of binding levels based on single report point measurements.*

Step 5. System preparations

Check the ***System preparations*** options as required (see Section 4.2.5).

Step 6. Rack positions

Accept or change the rack positions for the various solutions required (see Section 4.2.6).

Step 7. Prepare run protocol

Edit the ***Prepare Run Protocol*** text if desired (Section 4.2.7). This text will be displayed at the start of a run when the wizard template is used. Save the wizard template if required and start the run.

Evaluation of kinetics/affinity assays

Chapter 14 describes how to evaluate kinetics/affinity assays.

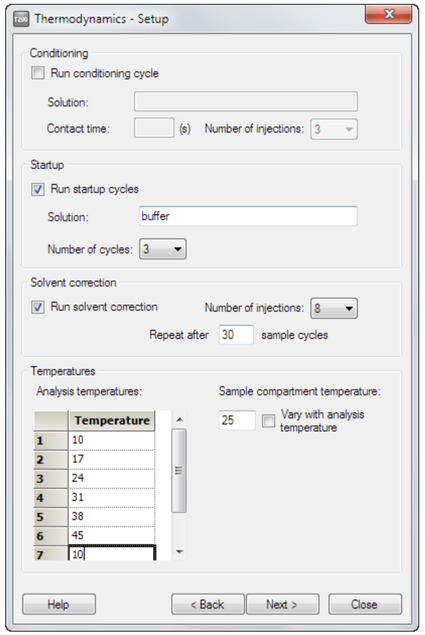
4.12 Thermodynamics

The ***Thermodynamics*** wizard supports kinetic and affinity determinations over a range of temperatures. The corresponding evaluation software extracts thermodynamic data from the dependence of rate and affinity constants on temperature (see Section 15.1).

Step 1. Injection sequence

Choose the detection settings, chip type and injection sequence for the assay (see Section 4.2.1). Options for detection settings are the same as for kinetics/affinity determinations (Section 4.11). The thermodynamics wizard supports capture but not enhancement injections. The same injection sequence will be used at all temperatures.

Step 2. Setup



Specify the conditioning and start-up cycles (see Section 4.2.2). Start-up cycles are recommended for thermodynamics experiments to ensure that the response drift that may occur with a new chip or when the temperature is changed does not interfere with the first measurements. Start-up cycles will be run at each temperature.

Check ***Run solvent correction*** to include solvent correction cycles in the run. Solvent correction adjusts response values for the effects of varying bulk refractive index contribution between samples, and may improve the quality of the results for analyses using small organic analytes that require dimethyl sulfoxide (DMSO) in the sample buffer to maintain solubility. The principles and application of solvent correction are described in Section 6.7.

Set the required number of injections per cycle and the frequency of solvent correction cycles. The default settings use 8 injections per cycle and repeat the solvent correction cycle every 30 sample cycles. Separate solvent correction cycles will be run at each temperature.

Enter the temperatures at which the measurements are to be performed. For most purposes, 5-7 temperatures will be adequate: fewer points make the determination of thermodynamic parameters uncertain, while more points increase the run time. Distribute the points evenly over the widest temperature interval that the ligand and analyte tolerate. Start from the lowest temperature to minimize the time needed for temperature equilibration between measurements (increasing the analysis temperature takes less time than decreasing it). The system will wait for a stable temperature between each determination. As an additional control, you may want to include a replicate of the first temperature at the end of the run.

Note: *For robust determination of standard thermodynamic parameters (ΔG°, ΔH° and ΔS°), arrange the analysis temperatures so that 25°C is in the middle of the range (e.g. 10–40°). See Section 15.1 for further details.*

Specify the sample compartment temperature, or check ***Vary with analysis temperature*** to change the sample compartment temperature automatically when the analysis temperature is changed.

Step 3. Injection parameters

Specify the injection parameters for each injection in the cycle (see Section 4.2.3). The sample injection for thermodynamics measurement has an additional setting for dissociation time. This is the time for which dissociation will be monitored after the end of the injection without disturbances from flow system washing procedures.

Step 4. Samples

Enter the details of the samples. This dialog box is equivalent to the concentration series dialog for kinetics determination (see Section 4.11, step 4). Separate rack positions will be created for each sample at each temperature.

Step 5. System preparations

Check the ***System preparations*** options as required (see Section 4.2.5). The settings for analysis temperature and sample compartment temperature cannot be changed here: the values for the first temperature as specified in step 2 are shown.

Step 6. Rack positions

Accept or change the rack positions for the various solutions required (see Section 4.2.6).

Step 7. Prepare run protocol

Edit the ***Prepare Run Protocol*** text if desired (Section 4.2.7). This text will be displayed at the start of a run when the wizard template is used. Save the wizard template if required and start the run.

Evaluation of thermodynamics assays

Section 15.2 describes how to evaluate thermodynamics assays.

4.13 Control experiments

Two control experiments are currently supported by wizards, both for kinetic analyses:

- The ***Mass transfer*** control experiment analyses the interaction of one or more analyte concentrations at three different flow rates, to establish whether the observed binding rate varies with flow rate. A dependence of binding rate on flow rate indicates that the binding is limited to some extent by mass transfer of analyte to the sensor surface. If mass transfer limitations are too dominant, reliable kinetic data cannot be obtained (see Section 12.4.2).
- The ***Linked reactions*** control experiment analyses the interaction of one or more analyte concentrations for different contact times, to identify a particular kind of deviation from a 1:1 binding mechanism. Variation of the dissociation behavior after the end of the injection with the contact time indicates that the observed binding consists of at least two processes, one where the analyte binds to the surface and a second where the surface- attached complex undergoes alteration such as a conformational change.

The control experiment wizards have the same basic structure as the kinetic analysis wizard, but some of the analysis settings are fixed.

4.13.1 Mass transfer control

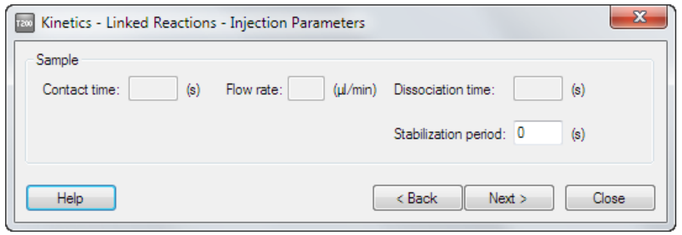
Each sample entered in the ***Samples*** step will be analyzed three times, at fixed flow rates of 5, 15 and 75 µL/min, with a contact time of 1 minute and dissociation time of 2 minutes. Use an analyte concentration that gives readily measurable initial binding rates. Mass transfer limitation is not affected by analyte concentration, but dependence of binding rate on flow rate may be difficult to detect if the binding rate is too low or too high.

Note: *All predefined kinetic evaluation models include a term for mass transfer, and the original purpose of the mass transfer control experiment has largely been superseded by functions in the evaluation software. The control experiment may however still be useful to confirm suspected mass transfer limitations if desired.*

4.13.2 Linked reactions control

Each sample entered in the ***Samples*** step will be analyzed at a flow rate of 10 µL/min with fixed contact times of 0.5, 3 and 10 minutes and a dissociation time of 10 minutes. Use one or more fairly high analyte concentrations, preferably so that steady state is approached or reached within the shortest contact time. The experiment is easiest to interpret if the interaction is maintained at steady state for varying lengths of time, so that the starting response for dissociation is constant.

Note: *The **Injection Parameters** step allows you to enter a stabilization time after each sample injection, but all other settings in this dialog are fixed in the wizard.*

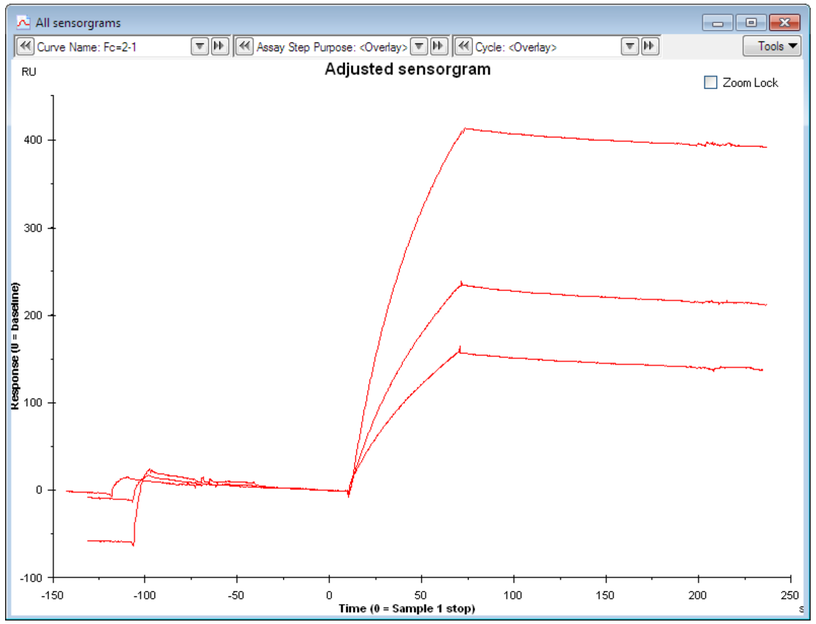


4.13.3 Evaluation of control experiments

When the wizard run is completed, the results are opened automatically in the Evaluation Software. An overlay plot of adjusted sensorgrams appropriate to the control experiment is created.

Mass transfer

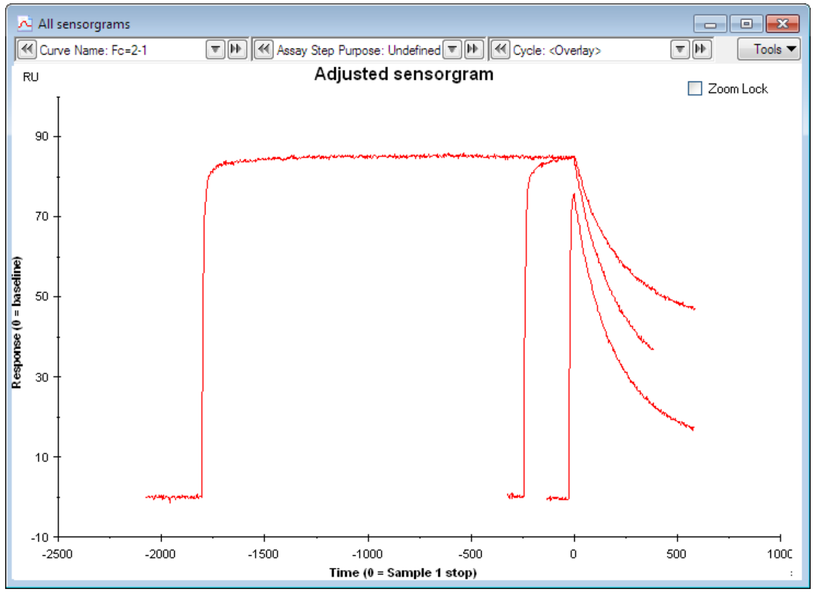
The sensorgrams are adjusted to zero response and time at the baseline report point. Compare the observed binding rates at the different flow rates. If the observed binding rate during sample injection varies with flow rates, there is some degree of mass transfer limitation in the data. Note that it may still be possible to obtain kinetic information from the sensorgrams (see Chapter 14).



This example shows a clear dependence of binding rate on flow rate, indicating mass transfer limitations in the observed binding. It may however still be possible to extract kinetic information from the sensorgrams.

Linked reactions

The sensorgrams are adjusted to zero response at the baseline report point and to zero time at the end of the sample injection. Compare the observed dissociation rates at the different contact times. Variation of the observed dissociation rate after sample injection with contact time indicates linked reactions in the interaction model.



This example shows a clear dependence of dissociation rate on contact time, indicating linked reactions in the interaction model.

4.14 Immunogenicity

Biacore T200 Control Software includes application wizard support for three areas of immunogenicity studies:

- ***Immunogenicity Screening***, designed for detection of immune response to administered drugs. Enhancement reagents can be used to reduce false positive results by confirming that observed responses derive from antibodies. This wizard can also be used to provide an estimate of the binding stability of detected antibodies to the drug.
- ***Immunogenicity Confirmation***, designed for confirming the specificity of detected antibodies through inhibition of the observed response by excess drug added to the sample
- ***Immunogenicity Isotyping***, designed to assist in identifying the isotype of detected antibodies through the use of isotyping reagents as enhancement reagents.

Both ***Confirmation*** and ***Isotyping*** wizards can be used for direct analysis of serum samples or to detect antibodies in the presence of drug interference, where antibodies are complexed with excess drug in the sample and would not be detected by a standard direct analysis.

Details of support for immunogenicity studies are provided in the separate Biacore T200 Immunogenicity Handbook.

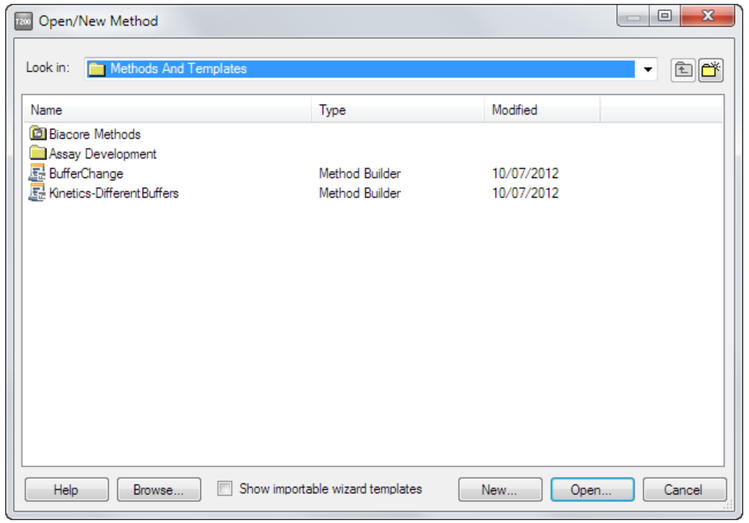
05

Methods

Methods in Biacore T200 offer flexibility in instrument control, providing support for applications that cannot conveniently be handled with wizards. Methods are constructed with the Method Builder tool as described in this chapter. Templates from application wizards can be opened in Method Builder (Section 5.1) to provide a starting point for development of customized applications. Method examples are provided with the software installation.

5.1 Opening methods

To open an existing method or create a new method, choose ***File:Open/New Method.***



Select a method and click ***Open*** to open the method, or click ***New*** to create a new method. Predefined methods for common applications are provided in the folder ***Biacore Methods***. If you make changes to a predefined method, you must save your changed method under a new name.

Check ***Show importable wizard templates*** to display wizard templates that can be opened in Method Builder. Opening a wizard template imports all wizard settings into a method and allows you to add functionality that is not supported in the wizard. Templates from all wizards except immobilization can be imported into Method Builder.

The top-level folder for methods is defined under ***Tools:Preferences*** (see Section 2.5). You can navigate between subfolders under the top level in the dialog box, but files outside the top-level folder are not listed in the dialog box. Click ***Browse*** to navigate freely in the computer file structure and open methods stored in other locations.

5.2 Method structure

Methods are handled in Method Builder in a series of sections representing different aspects of the method definition.

Overview

The ***Overview*** screen summarizes the method definition. Use this information as an aid in checking that the method is correctly built.

General settings

Here you define general parameters such as the concentration unit for samples, sample compartment temperature, data collection rate, detection mode and buffer names.

Assay steps

An assay step represents a specific function in the assay, defined in terms of what the step is intended to achieve. Assay steps may for example be start-up operations, solvent correction, sample analysis or control sample analysis.

Steps can be run singly or repeated within the context of other steps: for example, start-up operations are typically performed once at the start of a run, while control samples may be repeated at intervals during the sample analysis. Analysis temperature and buffer selection can be set individually for each assay step.

Cycle types

Cycle types define the details of how assay steps will be performed, in terms of sample and reagent injections. Each assay step is linked to one cycle type, but the same cycle type can be used in multiple assay steps. For example, sample and control sample analysis are two assay steps that will typically use the same cycle type, ensuring that controls are analyzed in exactly the same way as samples.

Parameters for injections in a cycle type definition may be variable, so that they can be assigned a series of different values when the method is used. Sample names will typically be variable. The number of values for variable parameters together with assay step repetition determines the number of cycles that will be run.

Report points can also be defined for each cycle type.

Variable Settings

This section determines how values for variable parameters are specified. You can choose whether values are specified in the method or at run-time: this can be used to restrict the number of parameters that have to be entered when the method is run while at the same time maintaining flexibility for method development purposes.

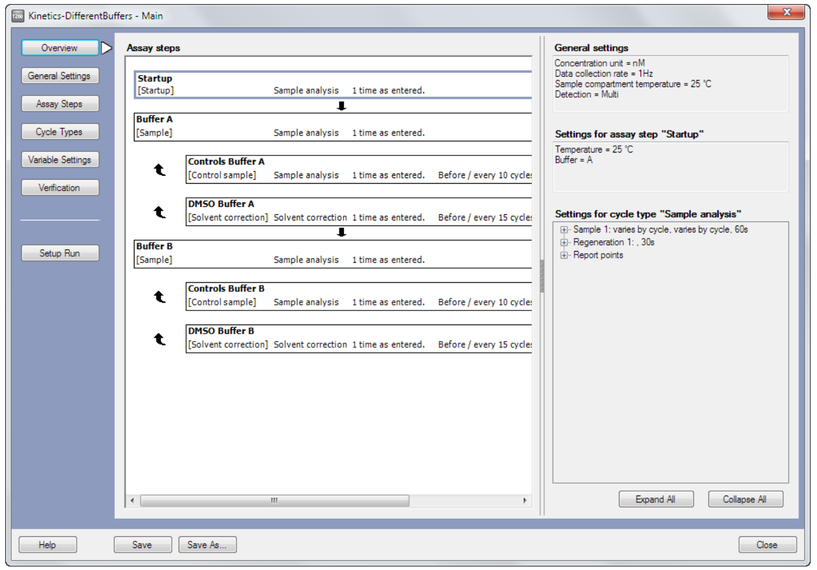
Verification

Once the method has been defined in full, this section verifies that all aspects are consistent and completely specified. The verification results are reported in the work area. A method that does not pass verification can be saved but cannot be run.

Note: *Verification only checks the consistency and completeness of the method. It does not in any way verify that the method is suitable for the intended purpose.*

Each aspect of Method Builder is described in detail in the following sections.

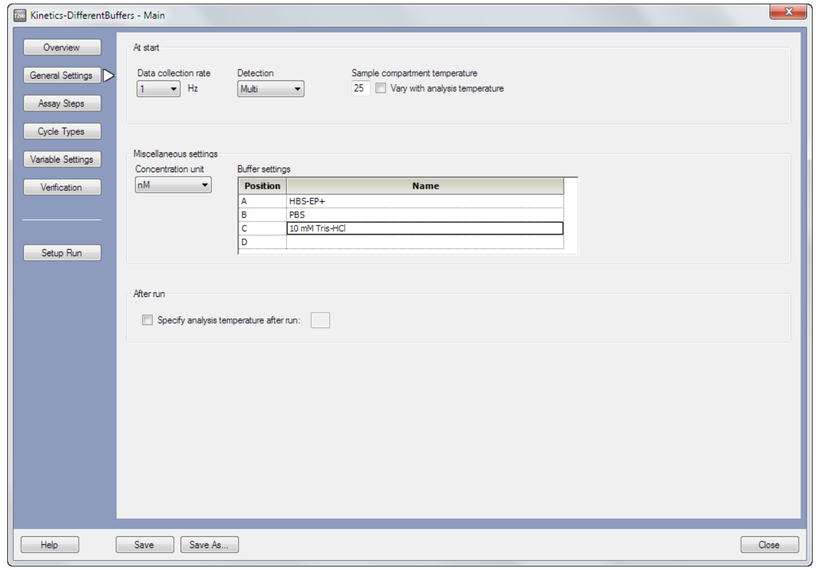
5.3 Method overview



This screen provides a summary of the method. The main panel shows the assay steps in the method (see Section 5.5). Click on an assay step to show the settings for the step and the details of the cycle definition (see Section 5.6) in the panels to the right. The cycle definition is listed as a series of injection commands: to see command details, expand individual commands by clicking on the **+-** marking or use the ***Expand All*** button to expand all commands in the panel.

This screen is for information only: settings cannot be changed here.

5.4 General settings



The settings that are specified here are:

Data collection rate
Choose between 1 and 10 Hz for data collection. The higher setting will provide better resolution for kinetic analysis of fast interaction processes, but will result in larger result files.

Detection
Choose the detection setting for the run:

Single	Records data from one flow cell according to the chosen flow path. Data is not recorded from the other flow cells.
Dual	Records data from one flow cell pair (1,2 or 3,4) according to the chosen flow path. Data is not recorded from the other flow cell pair.
Multi	Records data from all four flow cells.

This setting affects the choice of flow path that can be made for each injection command in the cycle types definitions (see Section 5.6.1). Use the setting ***Multi*** if you are not sure what you need: this will provide maximum flexibility for the injection settings.

Note: *If you set detection to **Multi** and then inject sample over fewer than all four flow cells, buffer will remain stationary in the unused flow cells and data recorded from them will be meaningless.*

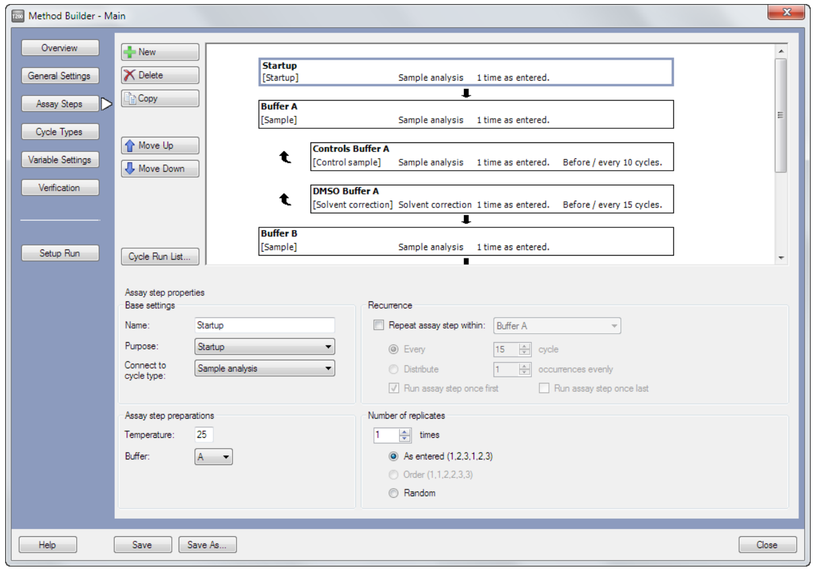
Sample compartment temperature
This is the temperature in the sample compartment (not the analysis temperature at the flow cell, which is set for each assay step). Check the ***Vary with analysis temperature*** box to set the sample compartment temperature automatically to the same value as the analysis temperature.

Concentration unit
This setting defines the unit for entering sample concentrations. The unit must be specified here, and cannot be changed at any other step in the assay definition. The unit can however be changed in the Evaluation Software when the results of the run are evaluated.

Buffer settings
Enter names if desired for the buffers in bottles A through D. Names entered here will be displayed in the ***Prepare Run Protocol*** (Section 4.2.7). Different buffers may be chosen for different assay steps (Section 5.6).

Specify analysis temperature after run
Check this option and enter a temperature to set the analysis temperature when the run is completed. The rack temperature will also be reset if the ***Vary with analysis temperature*** box is checked. This setting provides automated control of the chip and detector environment after the completion of a run, for example in preparation for another run at a different temperature.

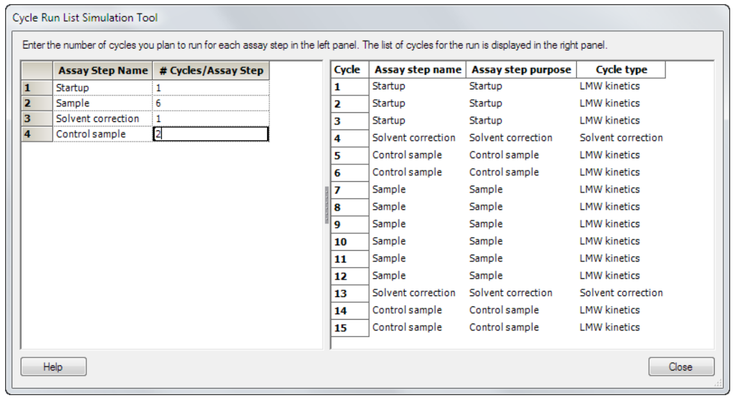
5.5 Assay steps



This screen determines the main structure of the method in terms of assay steps. Steps at the top level (i.e. not indented or marked with the symbol ↳) are executed in the order given. Nested steps (marked with the symbol ↳) are executed within the context of the level in which they are placed, as specified by the settings for ***Recurrence***.

To create a new assay step, click ***New Step***. The step will be created with default settings at the end of the current method. Move the step in the method with the ***Move Up/Down*** buttons until it is in the required position in the method. You can make a copy of the currently selected step with the ***Copy Step*** button.

The ***Cycle Run List*** button beside the method summary panel allows you to check the number and order of cycles for the method as defined.



Enter the number of cycles to be run in each assay step in the left-hand panel. The cycle list for the run as currently defined is shown in the right-hand panel.

5.5.1 Base settings

Name

This is the name of the assay step. Each step in a method must have a unique name. New steps are by default named ***Assay Step n***, where ***n*** is a serial number: change the name to something that describes the context or intent of the step, to make the method easier to follow.

Purpose

Assay steps are assigned a purpose, used to identify cycles in the evaluation software. The choice of purpose can help to document the method structure, and also determines the way the data is treated in evaluation. Choose the purpose from the list.

An assay step may have one of the following purposes:

Purpose	Usage and restrictions
Calibration	Used for calibration curves in concentration assays and affinity in solution. This assay step should be connected to the same cycle type as the ***Sample*** step so that the calibration and sample analyses are performed in the same way. Set ***Calibration*** to recurring within ***Sample*** to repeatthe calibration at intervals through the assay.
Conditioning	Used to condition the sensor surface at the start ofan assay.
Control sample	Used for control samples. This assay step should be connected to the same cycle type as the ***Sample*** step so that the control sample and sample analyses are performed in the same way. Set ***Control sample*** to recurring within ***Sample*** torepeat the control sample analysis at intervals through the assay.
Sample	Used for sample analysis in all applications. At least one sample step is required for application-specific evaluation.
Solvent correction	Used for solvent correction cycles. This step shouldbe connected to a cycle type designed for solvent correction. Set ***Solvent correction*** to recurring within ***Sample*** torepeat the solvent correction at intervals through theassay.
Startup	Used to condition the flow system at the start of an assay. This assay step will commonly be connected tothe same cycle type as the ***Sample*** step.
Undefined	Used for assay steps that do not fit the predefinedpurposes. Assay steps with ***Undefined*** purpose will not beincluded in application-specific evaluation.

Note: *For simple methods, the assay step name and purpose may often be the same (e.g. Solvent Correction, Sample, Control Sample etc). It is however important to remember that the name is for documentation from the user's perspective and may be chosen freely, while the purpose has significance for the step properties and for evaluation of the run and must be chosen from the predefined list.*

Connect to cycle type
Each assay step is connected to one cycle type, which determines the detailed operation of the step (see Section 5.6). Choose the cycle type from the list of types available in the method.

5.5.2 Assay step preparations

Temperature
This value determines the analysis temperature for the assay step. The setting will also control the sample compartment temperature if the appropriate option is checked under ***General Settings*** (Section 5.4).

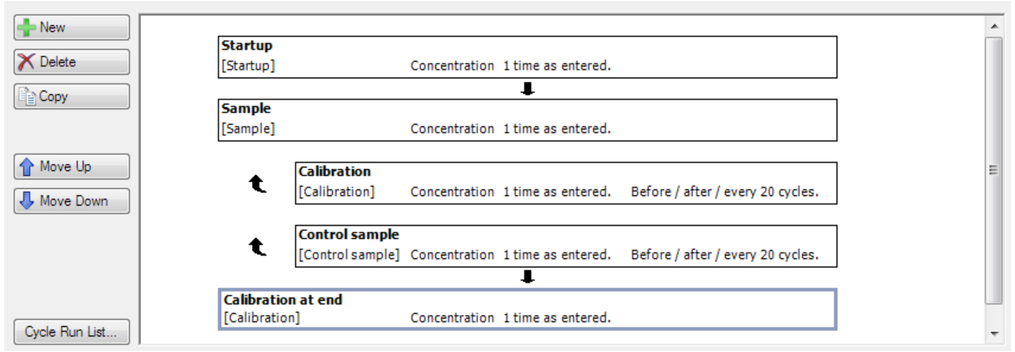
If the actual temperature at the start of an assay step does not match the setting for the step, the system will wait until the set temperature is reached and is stable.

Buffer
Select the running buffer to be used for the assay step. The default buffer is A (corresponding to buffer bottle and tubing A on the instrument).

5.5.3 Recurrence

An assay step can be set to recur at a specified interval within the context of another step. The recurrence can be specified as ***Every ... cycles*** (so that the number of occurrences will depend on the number of cycles in the assay step) or ***Distribute ... occurrences evenly*** (in which case the number of occurrences is fixed and they are distributed as evenly as possible among the cycles in the assay step). In addition, the recurring step can be specified to be executed at the beginning and/or end of the step in which it is set to recur.

Note: *The **Run assay step first/last** options refer to the beginning and end of the context in which the assay step recurs. To ensure that a recurring assay step is run at the beginning and end of a whole assay with several different assay steps, set up a separate copy of the assay step to run once at the beginning and/or end of the entire assay. The illustration below shows an assay step setup that ensures calibration before the first sample, repeated during the samples and at the end of the assay.*



If the step within which another step recurs is run in replicate, the recurring step is distributed among the total number of cycles including replicates. This is illustrated in the table below for a recurring step set to ***Every 5 cycles***:

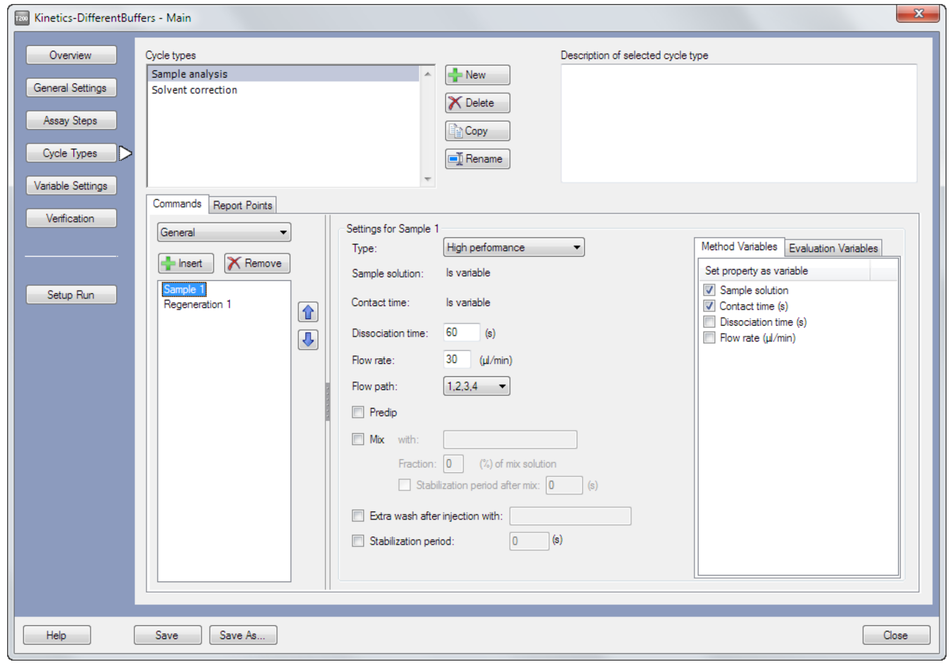
Top level step			
Number of cycles	10	20	10
Number of replicates	1	1	2
Total number of cycles	10	20	20
Number of recurrences for the nested step	2	4	4

5.5.4 Number of replicates

Assay steps can be set to run in replicate, which means that all cycles in the assay step will be repeated the specified number of times. The order in which cycles in the assay step are repeated can be specified:

As entered	performs all cycles in the step once, then repeats the step until the number of replicates is reached (this is represented as ***1,2,3,1,2,3*** to illustrate the order of 3 cycles in a step repeated twice)
Order	performs the first cycle in the step for the specified number of replicates, then the second cycle and so on (represented as ***1,1,2,2,3,3***)
Random	randomizes the order of the cycles within the step until all cycles have been executed the specified number of times. The order is randomized each time the method is run.

5.6 Cycle types



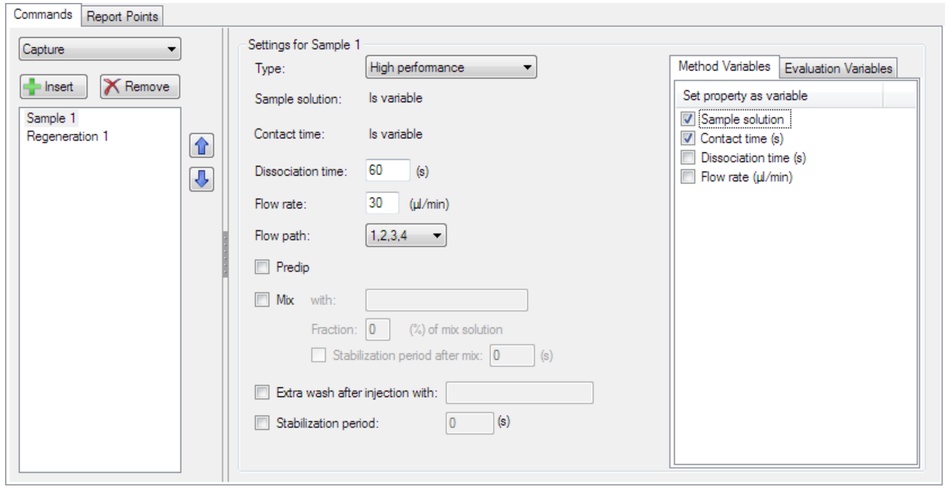
Cycle types define the detailed sequence of operations to be performed in each assay step.

The top panel in the work area lists the cycle types currently available in the method. Use the ***New*** button to create a new cycle type. Mark a cycle type and click ***Delete*** to remove the cycle type from the method. Use the ***Copy*** button to make copies of cycle types in the method: this can be useful if a method requires a number of similar cycle types with small variations. Click ***Rename*** to rename a cycle type.

Enter a description of the cycle type if desired.

Settings for the currently selected cycle type are configured in the lower part of the work area. The settings are divided into ***Commands*** and ***Report Points***, accessed on the respective tabs.

5.6.1 Commands



The commands in a cycle definition correspond to different kinds of injection of liquid over the sensor surface. To add a new command to the cycle definition, choose the command type from the pull-down list and click ***Insert***. The command will be inserted with default parameter settings immediately after the currently marked command (or at the end of the cycle definition if no command is marked). Use the ***Up*** ( ) and ***Down*** ( ) buttons to change the position of the selected command in the cycle, and the ***Remove*** button to remove the selected command from the cycle definition. Commands are executed from top to bottom in the cycle definition.

General command properties

Common features of several commands are check-boxes for ***Predip***, ***Extra Wash After Injection*** and ***Stabilization period.***

Predip — Check this box to dip the needle in a separate position before aspirating the solution to be injected. The predip position will normally contain the same solution as is injected, so that the needle is rinsed briefly to minimize carry-over effects. The same predip position is used for all cycles.

Extra Wash After Injection — Check this box and specify a wash solution to perform an extra wash of the flow system after the injection. The flow system is washed automatically with buffer after each injection, but an extra wash with a different solution can be included if required. This wash solution does not pass over the sensor surface.

Stabilization period — Check this box and specify a time in seconds to introduce a delay before the next command is started. This can sometimes be necessary (for example after regeneration steps) to allow the response to stabilize before performing the next injection.

Capture command

This command is intended for injection of ligand over a capturing molecule at the beginning of a cycle. The injected solution, contact time and flow rate can be set as variables.

Sample command

This command is intended for injection of sample containing analyte. Only ***Sample*** commands are recognized as analyte injections in the Evaluation Software for kinetics, affinity and concentration evaluation. The injected solution, contact time, dissociation time and flow rate can be set as variables. Evaluation variables can also be defined for the ***Sample*** command (see Section 5.6.2).

The ***Sample*** command offers 5 alternative settings under ***Type***:

High performance — Optimizes the injection for high performance by using extra segments of air and sample during aspiration to separate the injected solution from running buffer, thereby minimizing dispersion of sample at the beginning and end of the injection at the expense of additional sample consumption.

Low sample consumption — Optimizes the injection for low sample consumption by using fewer air segments than the High performance setting. The Low sample consumption setting however still achieves a performance that is adequate for most applications except analysis of rapid kinetics.

Single cycle kinetics — Injects a series of sample concentrations in the same cycle, intended for single-cycle kinetics analysis (see Section B.14). The samples are injected in direct sequence, separated only by the time required to prepare the next injection. A dissociation time is included after the last sample injection. With this option checked, an evaluation variable (see Section 5.6.2) will be set up for each of the specified number of concentrations.

Merged injection — Performs simultaneous injection of acidified sample and neutralization solution so that the sample is neutralized during passage through the flow cells. This injection type is specifically designed for use in immunogenicity studies, for dealing with drug interference by neutralization of acidified samples immediately before analysis. See the Biacore T200 Immunogenicity Handbook, Chapter 4, for details of how to use ***Merged injection***.

Double mix Sample is first acidified then neutralized by sequential addition of acid and neutralization solution to the sample in the microplate well. The neutralized sample is then injected over the sensor surface. This injection type is specifically designed for use in immunogenicity studies, for dealing with drug interference by analysis of acidified-and neutralized samples. See the Biacore T200 Immunogenicity Handbook, Chapter 4, for details of how to use ***Double mix***.

Note: *The injection types **Merged injection** and **Double mix** are specifically designed for dealing with drug interference in immunogenicity studies and should only be used in such studies.*

The ***High performance*** and ***Low sample consumption*** options support a ***Mix*** function for mixing sample with a defined solution in the autosampler before injection. Check the ***Mix*** option and enter a mix solution and mixing fraction to use this function. Entering a fraction of e.g. 20% will mix one part of mixing solution with four parts of sample. The sample and mixing solution are taken from respective positions in the autosampler and mixed in a third position. The option ***Stabilization period after mix*** allows you to specify a wait period between the mixing operation and injection of the mixed solution. ***Mix*** is not supported for the ***Single cycle kinetics*** option.

Note: *Mixing is not supported in 384-well microplates.*

Enhancement command

This command is intended for injection of a secondary enhancement reagent following the sample injection. Enhancement reagents are most commonly used to amplify the analyte response and to confirm the identity of the bound analyte. The injected solution, contact time and flow rate can be set as variables.

Regeneration command

This command is intended for injection of a regeneration solution following the sample injection. Check ***High Viscosity Solution*** if the regeneration solution has a relative viscosity higher than about 3 (corresponding to about 35% glycerol or 40% ethylene glycol at 20°C). This will adapt the solution aspiration and injection procedure for higher viscosity. The injected solution, contact time and flow rate can be set as variables.

Carry-over control command

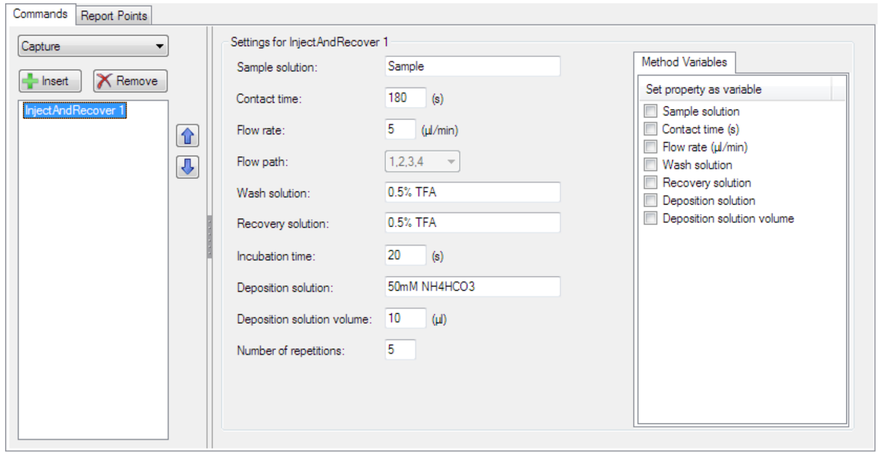
This command injects a 30-second pulse of buffer over the surface at a flow rate of 40 µL/min, in order to check that there is no carry-over of analyte or other material from an injection earlier in the cycle. The injection is suitably placed at the end of the cycle, and can be used in a conditional context (see the ***If...then*** command) to perform additional buffer injections or regeneration steps if carry- over is detected. A plot of the binding response from a carry-over injection against cycle number is created automatically for quality control purposes in the evaluation software (see Section 6.4).

Solvent correction command

This command injects a 30-second pulse of solvent correction solution over the surface at a flow rate of 30 µL/min. A solvent correction cycle should contain 4-8 ***Solvent correction*** commands for the different solvent concentrations used to construct the correction curve (see Section 6.7). ***Solvent correction*** commands should only be used in cycle types that are connected to assay steps with purpose ***Solvent correction***.

Inject and recover command

This command recovers analyte that is bound to the sensor surface, and is intended for use in applications where the bound material is to be analyzed further. Several features of the command are designed specifically for integration of Biacore analysis with mass spectrometry (MS). Most of the parameters for the command can be set as variables. This command can only be used for injection over all four flow cells: normally, the same ligand should be immobilized in each flow cell.



The command initiates a sequence of operations in the instrument:

1. The specified volume of ***Deposition solution*** is transferred in the autosampler to a target position in the sample and reagent rack. Target positions are assigned as required in the ***Rack Positions*** dialog (Section 4.2.6). The deposition solution should be MS-compatible, and may be used for example to neutralize the recovery conditions (which are often acidic) or to add trypsin or another protease to the sample for peptide digestion. The presence of deposition solution also helps to collect the small recovered volume reliably from the autosampler needle.
2. The ***Sample solution*** is injected over the sensor surface with the ***Contact time*** and ***Flow rate*** as specified. The ***Flow path*** is fixed so that sample passes through all four flow cells to maximize the amount of analyte that binds to the surface.
3. The flow system is washed with the specified ***Wash solution***. Distilled water or an MS-compatible buffer should be used as washing solution.
4. A small volume (approximately 2 µL) of ***Recovery solution***, separated from the surrounding buffer by air segments, is injected into the flow cells. The flow is stopped for the specified ***Incubation time*** while the recovery solution is in contact with the sensor surface, to allow the bound analyte to dissociate into the recovery solution.
5. The flow direction over the sensor surface is reversed and the recovery solution containing recovered analyte is deposited in the target position where it mixes passively with the deposition solution.
6. Steps 2-5 are repeated for the specified ***Number of repetitions***. This increases the yield of recovered analyte without requiring additional commands. The same target position is used for recovered analyte from all repetitions. Note that only one aliquot of deposition solution is used, regardless of the number of repetitions.

Notes: *Methods that include the **InjectAndRecover** command require a sample and reagent rack and cannot be used with microplates (see the Biacore T200 Operating Instruction for rack details).*

The contact time for sample, flow rate and number of repetitions determine the total injected volume for both sample and recovery solution. You may need to adjust one or more of these parameters if the method does not pass verification.

General command

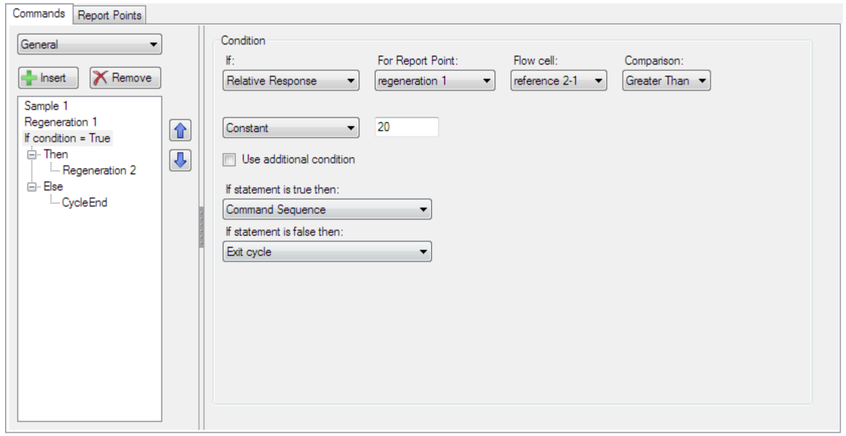
This command is a general-purpose injection that supports the following options under Type:

Dual	Injects two solutions in direct succession, with no intervening automatic wash routines. A dissociation time may be set for the second injection but not for the first.
High performance	Prioritizes high injection performance over sample consumption (see description under ***Sample*** command).
Low sample consumption	Prioritizes low sample consumption over injection performance (see description under ***Sample*** command).

General commands are not recognized as sample injections for evaluation of concentration, kinetics or affinity and may therefore also be used to "hide" injections from the predefined evaluation functions. The injected solution, contact time and flow rate can be set as variables. Evaluation variables can also be defined for ***General*** commands (see Section 5.6.2).

If...then command

This command allows construction of conditional methods, where commands are executed or skipped depending on the outcome of certain conditions. The illustration below shows a cycle which will perform an additional regeneration if the relative response after the first regeneration exceeds a specified value:



To set up a conditional command:

1. Insert an ***If...then*** command at the appropriate place in the cycle definition.
2. Specify the condition. This is defined as the outcome of a comparison between a report point value (absolute response, relative response or slope) and a constant or another report point value with an added or subtracted constant value. Only report points that have already been set in the cycle definition may be used in the condition.

   Check ***Use additional condition*** to combine two conditions, using either AND (both conditions must be fulfilled) or OR (fulfillment of one condition is sufficient) as a logical operator.

   The available comparison conditions are ***Greater than*** and ***Less than***. The conditions do not include ***Equal to*** since exact equality is an unpredictable condition in view of noise in the SPR response. To construct an equality condition, combine one ***Greater than*** and one ***Less than*** condition so that a window of tolerance is created. For example, the combined condition *A greater than B-1 AND A less than B+1* is equivalent to *A equals B* with a tolerance of ±1.

3. Choose the actions to be taken when the condition is met and when it is not met. You may choose to execute a command sequence, terminate the cycle or the method, or introduce a stabilization period.

   If you choose a command sequence for either the ***True*** or ***False*** outcome, click on the appropriate branch of the command (***Then*** or ***Else*** respectively) and insert the commands you wish to be executed. If you leave the branch empty, the cycle will simply continue with the next command following the ***If...then*** construction.

   You can use the ***Move up*** and ***Move down*** buttons to rearrange the order of commands within a branch, but you cannot move commands outside the branch in which they are placed.

   If you have chosen a command sequence for an action and have entered commands, you must delete the commands before you can change to a different action.

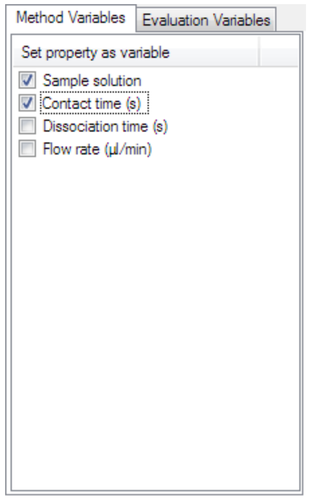
5.6.2 Variables

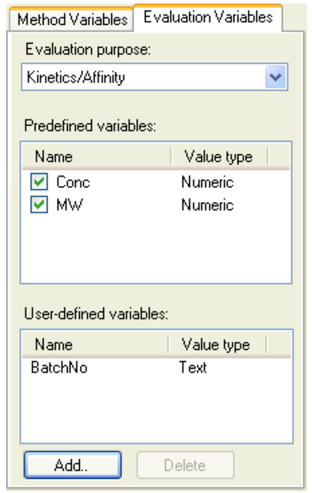
Parameters for many of the commands in a cycle definition may be set as variables. Values for variables are entered in either the ***Variable Settings*** in the method or the ***Setup Run*** step when the method is run (Sections 5.7 and 5.9.2), and determine the number of cycles that will be performed in the run. Variables fall into two broad classes:

- *Method variables* such as sample name or contact time control the way the cycle will be performed. Parameter values that are not set as variables are defined in the main command panel.
- *Evaluation variables* such as concentration or analyte molecular weight are used in evaluation of the data. Some evaluation variables are required for correct functioning of application-specific evaluation procedures (for example, kinetic evaluation requires a variable called ***Conc*** which holds the analyte concentration). These are selected from a predefined list specific to the purpose of the application. Other evaluation variables may be freely defined by the user, to hold information that is relevant to the assay but not required by an application-specific evaluation procedure (for example the sample batch number). Evaluation variables may only be defined for ***Sample*** and ***General*** commands.

Variables are set in the list at the right in the command panel. For method variables, check a parameter to set it as variable. ***Sample solution*** is checked by default for ***Sample*** commands. For evaluation variables, choose the purpose of the assay to display an appropriate list of predefined variables and check the variables you want to use. Click ***Add*** to set up user-defined variables.

Note: *For specific assay purposes, you should generally check all suggested variables. If you leave some variables unchecked, the assay-specific evaluation may not work.*





Predefined evaluation variables for different assay purposes are described in the table below (see also Section 5.10).

Evaluation purpose: General, Kinetics/Affinity, Thermodynamics

Conc	Analyte concentration. Multi-cycle kinetics requires a single concentration variable. Single-cycle kinetics requires one concentration variable for each sample injection in the cycle.
MW	Analyte molecular weight: required for molecular weight adjustment of report points, and for kinetic evaluation when the concentration is entered in weight-based units.

Evaluation purpose: Kinetics (heterogeneous analyte)

Conc1, Conc2	Analyte concentrations for the two analytes.
MW1, MW2	Molecular weight for the two analytes: these variables are required even if concentrations are entered in molar units, to determine the relative contributions of the two analytes to the observed response.

Evaluation purpose: Concentration (using calibration curve)

Conc	Analyte concentration: required for calibration and control samples, left blank for unknowns.
Dilution	Dilution factor: used for unknown samples to calculate original concentrations.

Evaluation purpose: Calibration-free concentration analysis

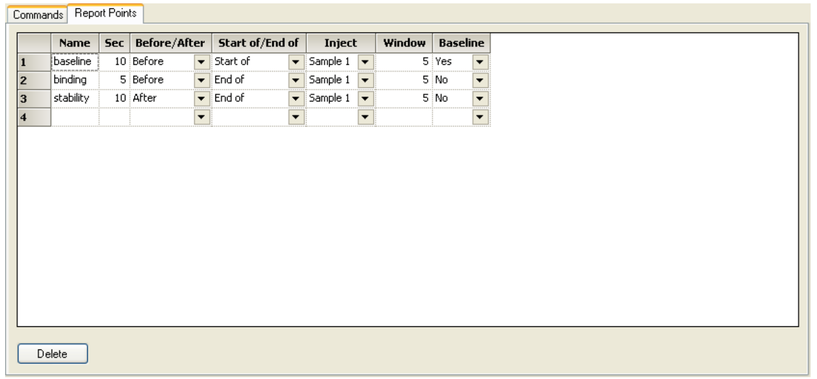
MW	Analyte molecular weight: used in calculation of concentration from binding rates (empty for blank injections).
D(20°C)	Diffusion coefficient of the analyte at 20°C (empty for blank injections). A tool for calculating diffusion coefficients from molecular size and shape is available on cytiva.com/biacoretools
Blank	Identifies blank cycles for blank subtraction purposes. Blank cycles have the value y or yes (upper or lower case): any other value identifies the cycle as non-blank.

Evaluation purpose: Affinity in solution*

ConcB- calibration	Concentration of interactant B used to construct a calibration curve.
ConcB-fix	Concentration of interactant B in the sample mixture (the concentration of B is kept constant).
ConcA-variable	Concentration of interactant A in the sample mixture (the concentration of A is varied).

*See Section 16.1.1 for details of how this assay is set up.

5.6.3 Report points



The ***Report points*** tab lists the report points in the cycle type definition, ordered as far as possible in the order they will appear in the cycle. Several injection commands have a predefined set of report points that are added to the list when the command is included in the cycle type. You can add your own report points by filling in the details in the empty row at the bottom of the table. A new empty row is added whenever you create a report point.

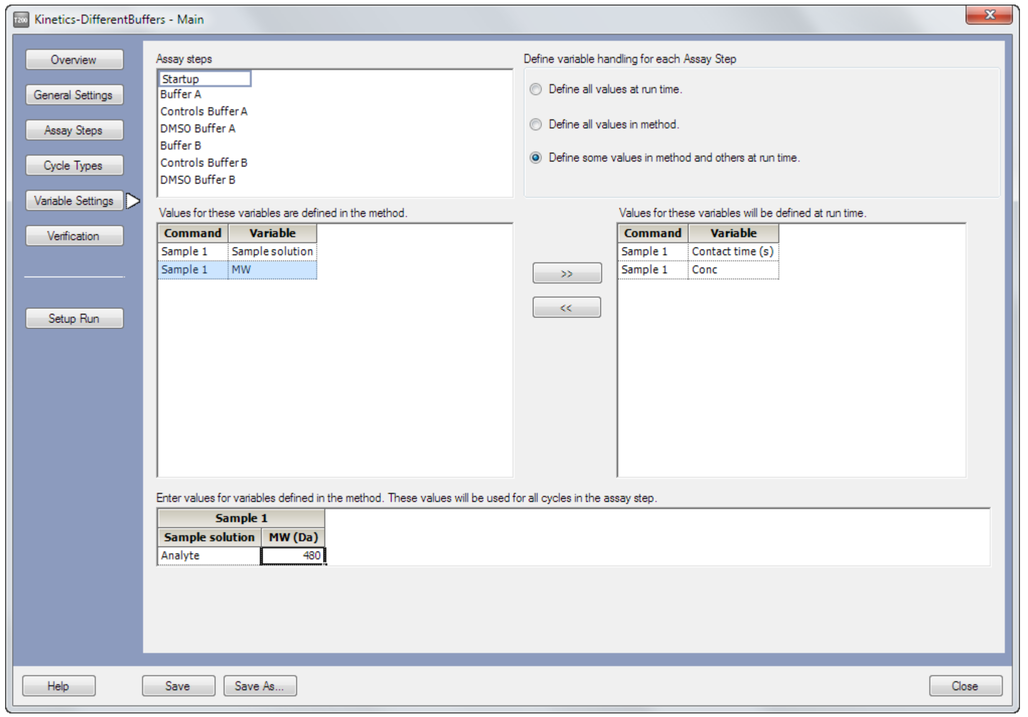
Report points are set at defined times in relation to the start or end of injections in the cycle. Report points that are set outside the time range for the cycle (i.e. a significant time before the start of the first injection or after the end of the last injection) will not be created.

Note: *Do not position report points far away from events, so that they lose their relevance to the event, or so close to an event so that the report point window overlaps the event itself.*

Enter the report point details as follows:

Name	The report point name must be unique within the cycle type. Choose a name that reflects the function of the report point.
Sec	Enter the time in seconds between the report point and the event.
Before/ After	Choose whether the report point is to be placed before or after the event.
Start of/ End of	Choose whether the report point is to be placed relative to the start or end of the injection.
Inject	Choose the injection to which the report point will be related.
Window	Set the window for the report point calculation. The report point will be placed at the center of the window, and the reported response will be an average of the response values within the window. A window of 5 seconds is adequate for most purposes.
Baseline	Choose whether the report point will be defined as a baseline or not. Response values for report points that are not defined as a baseline will be calculated relative to the closest preceding baseline value.

5.7 Variable settings



This screen determines whether variable parameter values are specified in the method or at run time. You may choose to specify all values in the method, all at run time, or a mixture of the two. Values that are specified in the method are saved with the method and cannot be changed at run time. You can change the level at which values are specified without changing the cycle type definition, so that the same cycle type can be used for different assay steps with different sets of run-time variables. An example of this may be found in the predefined method for affinity in solution (Section B.1).

Variables are configured independently for the different assay steps (even if the assay steps use the same cycle type).

If you choose to specify values for all variables in the method, the values are entered in this screen. For each assay step, one row of variable values represents one analysis cycle (the cycle may be repeated if the ***Repeat*** property is set in the ***Assay Step*** screen). A new empty row (marked with an asterisk) is created automatically at the bottom of the table as soon as data is entered. Columns in the table correspond to variables for the cycle type used in the assay step, and are grouped according to commands in the cycle type definition. Use the right mouse button in the variables table to access functions for copying and pasting cell contents and for inserting and removing rows. When all variables are specified at run time, variables are handled in the same way in the ***Setup Run*** step (Section 5.9).

To specify that some variables are specified in the method and others at run time, check the appropriate option and then distribute the variables as required between the method and run-time lists. This mode can be used to hide variables at run time that are not relevant for the assay step. Fill in values for variables that are specified in the method: those specified at run time are filled in the ***Setup Run*** step. Note that in this mode only one value can be given for each variable that is specified in the method. These values will be used for all cycles: the number of cycles is determined by the number of rows of variable values in ***Setup Run***.

Depending on how the method is defined, there may be variable tables for several assay steps. Variable handling must be defined for all steps before the method will pass verification.

5.8 Verification

This step checks that the method is correctly and completely defined. A method that does not pass the verification step can be saved but cannot be run.

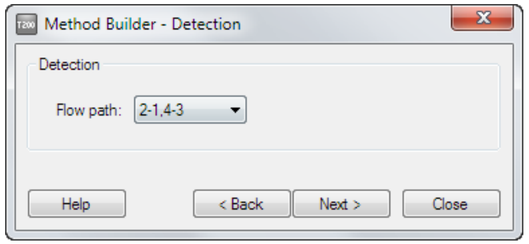
Verification may fail because parameters are missing (e.g. variables that are specified in the method have not been assigned values) or because the method construction is invalid (e.g. an assay step is not connected to a cycle type).

Note: *The verification step does not check whether the run fulfils the requirements for any assay-specific evaluation (see Section 5.10).*

5.9 Setup Run

5.9.1 Detection

Set the flow path for the method in the ***Detection*** dialog.



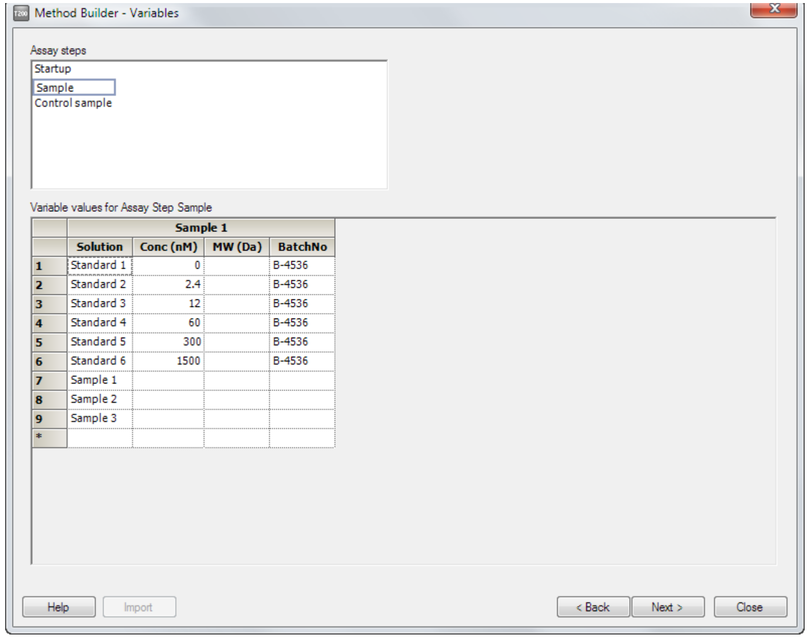
You can only choose a flow path that is consistent with the ***Detection*** setting for the method (see Section 5.4).

5.9.2 Variables

In this step you assign values to variables that are to be defined at run time (typically a sample table, see Section 5.7). Each row in the variables table corresponds to a cycle in the run. A new empty row (marked with an asterisk) is created automatically at the bottom of the table as soon as data is entered.

Columns in the table correspond to variables for the cycle type used in the assay step, and are grouped according to commands in the cycle type definition.

Depending on how the method is defined, there may be variable tables for several assay steps. Method variable values must be entered in all tables before you can continue to the next step. Evaluation variables may be left blank if desired at this step and values entered in the Evaluation Software.



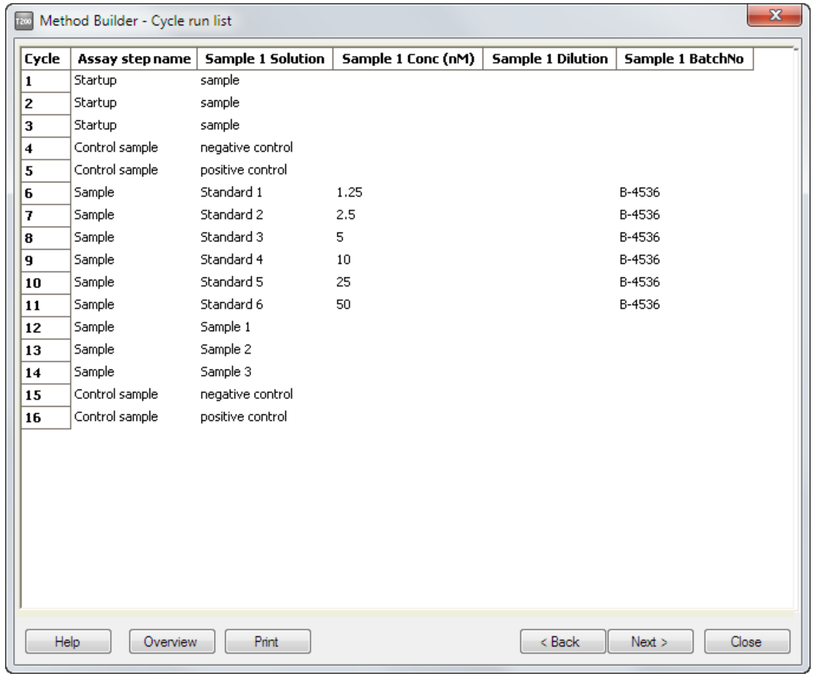
	Sample 1			
	Solution	Conc (nM)	MW (Da)	BatchNo
1	Standard 1	0		B-4536
2	Standard 2	2.4		B-4536
3	Standard 3	12		B-4536
4	Standard 4	60		B-4536
5	Standard 5	300		B-4536
6	Standard 6	1500		B-4536
7	Sample 1			
8	Sample 2			
9	Sample 3			
*				

Use the right mouse button in the variables table to access functions for copying and pasting cell contents and for inserting and removing rows. The columns in the table are listed in the order they are defined in the method (see Section 5.6.2).

Click ***Import*** to import the variables table from an external file. See Appendix A for details of the import format.

5.9.3 Cycle run list

When you have completed the variables table, click ***Next*** to view a complete summary of the cycles that will be performed in the run. This view is for information only and cannot be edited. Check through the cycle list to confirm that the variable tables are correctly filled in.

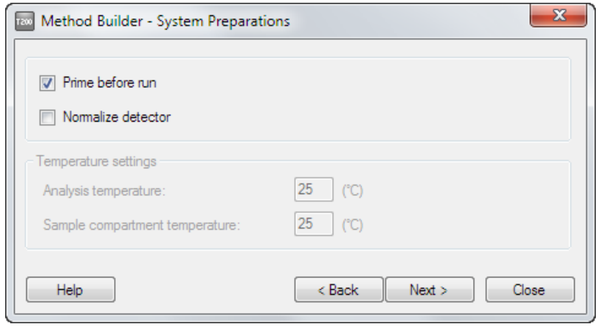


Cycle	Assay step name	Sample 1 Solution	Sample 1 Conc (nM)	Sample 1 Dilution	Sample 1 BatchNo
1	Startup	sample			
2	Startup	sample			
3	Startup	sample			
4	Control sample	negative control			
5	Control sample	positive control			
6	Sample	Standard 1	1.25		B-4536
7	Sample	Standard 2	2.5		B-4536
8	Sample	Standard 3	5		B-4536
9	Sample	Standard 4	10		B-4536
10	Sample	Standard 5	25		B-4536
11	Sample	Standard 6	50		B-4536
12	Sample	Sample 1			
13	Sample	Sample 2			
14	Sample	Sample 3			
15	Control sample	negative control			
16	Control sample	positive control			

Click ***Overview*** to display the method overview (Section 5.3).

5.9.4 System preparations

Choose which preparation steps should be executed before the method starts.



System preparations are equivalent to those for wizard-based runs (see Section 4.2.5). The temperature settings are taken from the first assay step in the method and cannot be changed here: they are shown for information only.

5.9.5 Rack positions

See Section 4.2.6 for a description of the ***Rack positions*** dialog box.

5.9.6 Prepare Run Protocol

See Section 4.2.7 for a description of the ***Prepare Run Protocol*** dialog box. Save the method at this step to include all settings including the Prepare Run protocol in the saved method.

5.9.7 Starting the run

When the positions are finalized, the sample plate is prepared and loaded into the instrument and a chip is docked, choose ***Start Run*** to start the run. You will be asked to specify a file name for the results.

5.10 Requirements for assay-specific evaluation

This section describes the requirements and recommendations if assay-specific evaluation is to be applied to method-based runs.

5.10.1 Concentration analysis

See Chapters 9 and 10 for a description of concentration evaluation.

Using calibration

- At least one assay step is required with purpose ***Calibration*** and one with purpose ***Sample***. An assay step with purpose ***Control Sample*** is also required in order to create trend plots for control samples.
- Assay steps ***Calibration***, ***Sample*** and ***Control Sample*** must be connected to a cycle type that includes one ***Sample*** command. The three assay steps will normally be connected to the same cycle type.
- Samples in the ***Calibration*** step must have concentrations specified in the variable ***Conc***. At least two different concentrations are required for linear calibration curves and at least four for 4-parameter fitting.
- Full use of the ***Calibration trends*** function (Section 9.2.2) requires calibration curves run before the first sample cycle and after the last.
- Samples in the ***Sample*** step will normally not have specified concentrations. If concentrations are specified, they will be ignored.

Calibration-free

- At least one assay step is required with purpose ***Sample*** or ***Control Sample***, connected to a cycle type that includes one ***Sample*** injection of type ***High performance*** with flow rate set to ***Variable***.
- Each sample must be injected at least twice using different flow rates.
- Evaluation variables ***D(20°C)***, ***MW*** and ***Blank*** should be included for the ***Sample*** injection. If these variables are not included, they must be added to the keyowrd table in the evaluation software before the results can be evaluated.

5.10.2 Kinetics and affinity screening

- For kinetic screening, optimal conditions represent a compromise between the number of concentration for each sample and the overall throughput of the assay. Three to five sample concentrations are recommended for reliable kinetic determination, although successful screening can be performed with fewer.
- For affinity screening, at least three unique non-zero sample concentrations are required for evaluation of the steady state affinity data.

5.10.3 Kinetics/Affinity

See Chapter 14 for a description of kinetics and affinity evaluation.

- At least one assay step is required with purpose ***Sample***, connected to a cycle type that includes one ***Sample*** command.
- Sample concentration must be specified in the variable ***Conc***. If weight- based units are used, a molecular weight for the analyte must be specified in the variable ***MW***.
- As a recommendation for multi-cycle kinetics and affinity, there should be a concentration series with at least four non-zero analyte concentrations and one zero concentration. At least one of the non-zero concentrations should be measured in duplicate. Kinetic evaluation can be applied to runs with fewer sensorgrams but the results will generally be less reliable if these recommendations are not followed. Steady state affinity evaluation requires at least three unique non-zero concentrations
- Five sample concentrations are recommended for single-cycle kinetics and affinity, injected in order of increasing concentration. Duplicate cycles are recommended to ensure robust evaluation. For each determination, there should be a blank cycle corresponding to the sample cycle, with buffer replacing the sample for each injection.

5.10.4 Sensorgram comparison

Evaluation of sensorgram comparison is described in Chapter 11.

Single-cycle kinetic analysis is recommended. It is important that all sensorgrams to be compared are obtained using identical method settings.

5.10.5 Thermodynamics

See Chapter 15 for a description of thermodynamics evaluation.

Thermodynamics evaluation requires that kinetics or affinity (see Section 5.10.3) is determined at two or more (recommended 5) temperatures.

5.10.6 Affinity in solution

See Chapter 16 for a description of affinity in solution evaluation.

- At least one assay step is required with purpose ***Calibration*** and one with purpose ***Sample***. Both assay steps must be connected to a cycle type that includes one ***Sample*** command. The two assay steps will normally be connected to the same cycle type.
- Samples in the ***Calibration*** step must have concentrations specified in the variable ***ConcB-calibration***. At least two different concentrations are required for linear calibration curves and at least four for 4-parameter fitting. These samples should contain only component B.
- Samples in the ***Sample*** step must have concentrations specified in the variables ***ConcB-fix*** and ***ConcA-variable***. At least 3 samples with the same concentration of component B mixed with different concentrations of component A are required.

5.10.7 Immunogenicity

Evaluation of immunogenicity experiments is described in the separate Biacore T200 Immunogenicity Handbook.

- For evaluation of specificity confirmation by addition of excess drug, sample analysis cycles must use the option to mix samples with a specified solution in the autosampler. For correct evaluation, each sample should be analyzed twice, once mixed with drug and once with buffer.
- For evaluation of isotyping, isotyping reagents must be injected with the ***Enhancement*** command.

5.10.8 Other requirements

Application of solvent correction (see Section 6.7) requires an assay step with purpose ***Solvent Correction***, connected to a cycle type that includes at least four ***Solvent Correction*** commands for different solvent concentrations.

06

Evaluation software – general features

Biacore T200 Evaluation Software offers general functions for presentation of results as sensorgrams, report point plots and bar charts, and specific evaluation functions for applications such as concentration, sensorgram comparison, kinetics and affinity screening and analysis, thermodynamics and immunogenicity. There is also a function that corrects for solvent effects that can sometimes distort the results from analyses with low molecular weight analytes that give low response levels and require organic solvents (e.g. dimethyl sulfoxide (DMSO)) to maintain solubility.

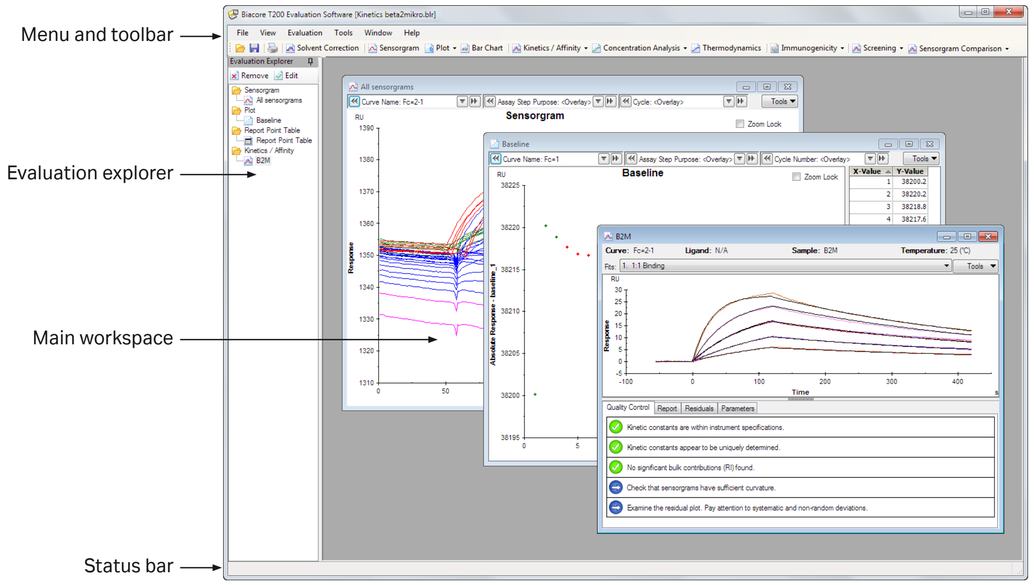
This chapter describes the organization of the evaluation software. The various evaluation functions are described in detail in the following chapters.

Result files can also be opened and evaluated in Biacore Insight Evaluation Software, available as a separate product.

6.1 User interface

6.1.1 Organization

The Biacore T200 Evaluation Software screen is divided into four main regions:



- The *menu and toolbar* provide access to the evaluation functions.
- The *Evaluation Explorer* at the left of the screen lists the evaluation items (sensorgrams, plots, bar charts and other result displays) that have been created in the current session. See below for a description of how to use the Evaluation Explorer.
- The *main workspace* displays the currently open items. Each item is shown in a separate window that is independent of the other items.
- The *status bar* indicates the progress of current operations such as application of evaluation methods.

6.1.2 The Evaluation Explorer

The Evaluation Explorer lists the sensorgram windows, plots and other evaluation items in the current evaluation session. Items are organized in folders according to type. Double-click on a folder to expand or collapse the folder. Click on an item to display it in the work area. Right click in the explorer area for options for adding new items: right click on an item for additional options relating to that item. You can also remove or edit selected items using the ***Remove*** and ***Edit*** buttons at the top of the explorer panel.

You can hide the Evaluation Explorer panel to increase the useful size of the work area by clicking on the pin icon ( ) in the explorer title bar. If the panel is hidden, it will automatically reappear when you move the mouse over the left- hand edge of the evaluation window.

6.2 Basic operations

6.2.1 Opening files

To open a file in the evaluation software, use the ***File:Open*** menu option. You can open result files from the control software (file extension .blr) and saved evaluation sessions (file extension .bme). If a file is already open in the software, opening a new file with ***File:Open*** will automatically close the first file. You will be asked if you want to save the file if any changes have been made.

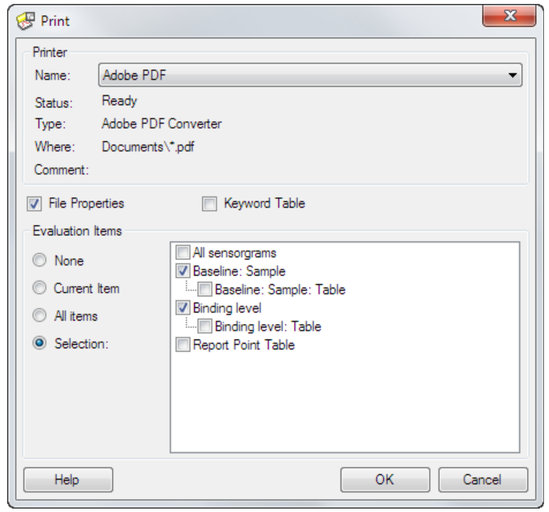
Opening a file automatically creates a number of default plots according to the content of the file (see Section 6.4).

To open multiple result files in the same session, select multiple result files in the ***Open*** dialog or use the ***File:Append Result File*** option. You can only append result files, not evaluation sessions. Appending a file to a session will delete all the user-defined evaluation items in the session.

Note: *The software does not check that appended result files contain compatible data. Beware of appending files from different types of run or from the same type of assay run under different conditions.*

6.2.2 Printing evaluation results

Choose ***File:Print*** to prints a hard-copy of the results. Select the printer to use and choose the items you wish to print.



6.3 Common display functions

6.3.1 Zooming the display

To zoom a display window, drag with the mouse over the area you want to enlarge. To restore the previous zoom level, double-click anywhere in the display window except on the axes or legend, or select ***Unzoom*** from the right-click menu.

Displays are normally rescaled automatically whenever you change the displayed data. To keep the current zoom setting when data is changed, check ***Zoom lock*** in the display window.

You can also set the display scale with the ***Scale*** option from the right-click menu. The display is not rescaled automatically if the scale has been set to specified values.

6.3.2 Right-click menus

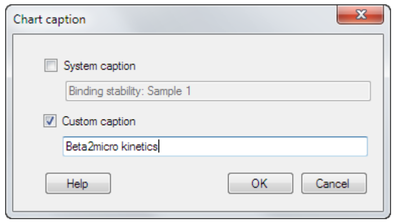
Right-click in display windows for options relating to the display. The available options vary according to the type of window, and also depending on whether you right-click on a point, a curve or elsewhere in the window.

Labels

Displays a label on each point in a plot window, showing cycle number, flow cell and sample name. (Labels may overlap and be difficult to read if the points in a plot are closely spaced.)

Caption

Displays a caption in the item window. The displayed caption can have system defined and user-defined components



Show sensorgram

Displays the sensorgram relating to a point in the plot. This option is only available when you right-click on a point: the sensorgram is displayed in a separate window that must be closed before you can continue with the evaluation.

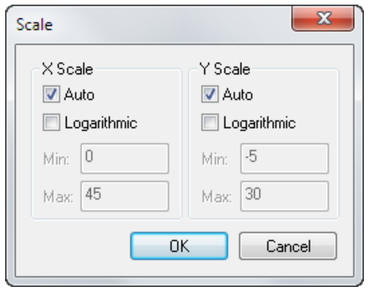
Exclude cycle/curve/point

Excludes data from the evaluation session or item. The data that can be excluded differs according to the type of window. Excluding data in sensorgram, QC plot or bar chart windows affects all other sensorgram, QC plot and chart windows correspondingly: however, result plots and application-specific evaluation items that have already been created are not affected until they are edited and updated. Excluding data in a result plot or application-specific items affects only the item from which the data is excluded.

Right-click on the data to be excluded and choose ***Exclude cycle***. Multiple cycles can be excluded in result plot items and bar charts by selecting in the table instead of the plot. Excluded cycles are shown with a broken line in sensorgrams. Excluded points are not displayed in plots but are listed in red strike-through text in the plot table (see Section 7.2.4). Excluded cycles may be re-included using the right-click menu.

Scale

Sets the scale for the display. You can also access this function by double- clicking on either the x- or y-axis in the display.



Uncheck ***Auto*** and enter minimum and maximum values to specify a scale.

Copy graph

Copies the current display as it appears on the screen to the Windows clipboard as a graphic object, from where it can be pasted into third-party software such as word-processing or presentation programs.

Copy table

Copies the table data in the current display to the Windows clipboard as tab- separated text. All rows in the table are copied (including any header row) regardless of how many rows are visible in the current display. However, in tables where the columns to be displayed can be selected by the user, only columns that are currently shown are copied.

Export curves

Exports the curves in the current display to a tab-separated text file, for import to third-party software. Complete data is exported regardless of the scale setting of the screen display.

Unzoom

Restores the previous zoom setting.

Gridlines

Shows or hides major and minor gridlines in the display window.

Legend

Shows or hides a legend for the display window. Choose the legend placing from the dialog box. In sensorgram and plot windows, the legend corresponds to the ***Color by*** setting for the display.

6.4 Predefined evaluation items

When a result file is opened, a number of evaluation items are created automatically if the results contain the appropriate cycles and report points. This section describes the items created for all result files: special items are also created for certain wizard runs (see Chapter 4).

6.4.1 Sensorgram

An overlay plot of all sensorgrams is created automatically. Except for results from immobilization pH scouting and kinetics control experiments, the sensorgrams are colored by assay step and no adjustments are applied.

6.4.2 QC Plots

QC plots are created for most wizards if the appropriate report point is present in the results. The settings for predefined plots are locked and cannot be edited. Common predefined plots are listed below. Separate plots will be created if there are multiple injections with similar report points (for example baseline for capture and sample injections). The ***Plot Settings*** cannot be changed, but the plot can be modified using the selectors and the ***Tools*** menu (see Section 7.2). Note that changing the selector settings can sometimes defeat the purpose of the plot.

Baseline: Sample	Absolute response for report point ***baseline*** against cycle number.
Baseline: Capture	Absolute response for report point ***capture_baseline*** against cycle number.
Baseline: General	Absolute response for report point ***general_baseline*** against cycle number.
Binding to reference	Relative response for report point ***stability*** against cycle number for the reference flow cell.
Capture	Relative response for report point ***capture_level*** against cycle number for the capture injection.
Carry-over	Relative response for the report point ***co_binding*** against cycle number for the carry-over injection (only for reference- subtracted curves).
Controls, binding	Relative response for the report point ***binding*** against cycle number for control samples (only for reference-subtracted curves).
Controls, stability	Relative response for the report point ***stability*** against cycle number for control samples.
Binding levels	Relative response for the report point ***binding*** against cycle number for samples (only for reference-subtracted curves).
Binding stability	Relative response for the report point ***stability*** against cycle number for samples.
Binding to reference, enhancement	Relative response for report point ***enhance_level*** against cycle number for enhancement injections on the reference surface.
Enhancement	Relative response for the report point ***enhance_level*** against cycle number for enhancement injections on the active surface.

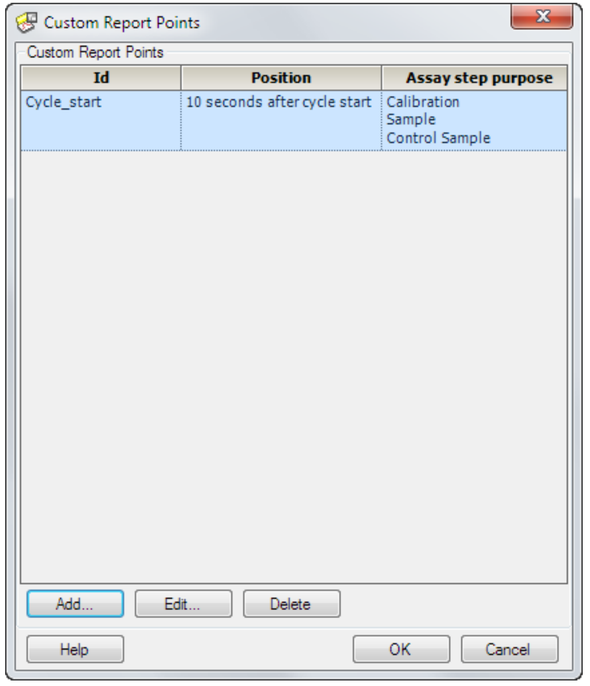
6.4.3 Report point table

A ***Report Point Table*** item is automatically created when the result file is opened. An evaluation session may only contain one report point table.

6.5 Custom report points

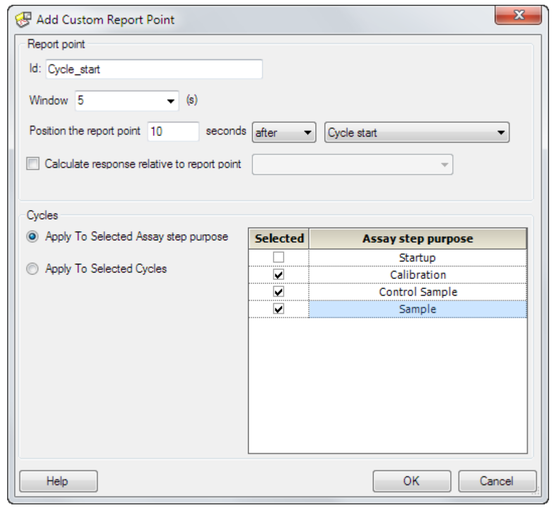
Choose ***Tools:Custom Report Points*** to add and edit custom report points.

Report points are automatically created for all wizard- and method-based runs, placed at strategic positions in relation to injections. These report points, and any other report points that have been created in the Control Software (Section 2.3.3), are not listed in the ***Custom Report Points*** dialog and cannot be edited or deleted in the Evaluation Software.



6.5.1 Adding report points

Click ***Add*** to add a new report point.



Enter a name for the report point (maximum 30 characters) in the ***Id*** field. The report point name must be unique within an evaluation session.

Enter a value between 1 and 35 for the ***Window***. Preset values of 5, 10 and 15 seconds are provided for convenience: a window of 5 seconds is recommended for most purposes. The response value for the report point is the average response over the window, with the report point time at the center of the window.

Use the settings under ***Position the report point*** to define where the report point will be placed. Report points are placed a specified number of seconds before or after the beginning or end of injections or the beginning or end of the cycle.

Notes: *Do not position report points far away from events so that they lose their relevance to the event, or so close to an event so that the report point window overlaps the event itself. You cannot define a report point with settings that would place part of the window outside the time limits of the sensorgram.*

Check ***Calculate response relative to report point*** and select the required report point if you want to calculate the responses at the custom report point relative to another report point. If the box is not checked, the closest preceding baseline report point will be used for calculating relative response values.

You can apply custom report points either to cycles with selected assay step purposes or to cycles selected by cycle number. Choose the appropriate option and check the assay step purpose(s) or cycles to which the report point should apply.

Note: *If you append a result file to the evaluation session after creating custom report points, the custom report points are retained but they are not applied to sensorgrams in the appended file. To apply a custom report point to all files, either append all files before creating the report point or edit the report point definition and re-apply without changing the settings.*

6.5.2 Editing and deleting report points

Select a report point in the list in the ***Custom Report Points*** dialog and click ***Edit*** to edit the report point definition or ***Delete*** to delete the report point.

Notes: *All user-defined evaluation items are deleted from the session when custom report points are edited or deleted, since changes to the report point definitions may affect existing evaluation items. You will be warned when this occurs. To avoid losing work, save your evaluation session before editing or deleting custom report points.*

If you delete a custom report point that is used as baseline for other report points, the relative response can no longer be calculated for the latter points. You will be warned if this situation arises.

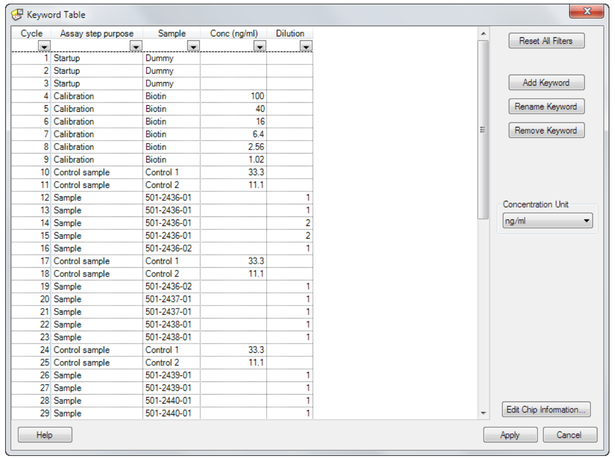
6.6 Keywords

Keywords are assigned to cycles when the run is performed, and are then used for identification and evaluation purposes. Keywords are created automatically for wizard-based runs and may be defined in the method for method-based runs, and include:

- automatically generated identifiers such as cycle number or assay step purpose,
- method variables and predefined evaluation variables such as sample name, concentration and molecular weight,
- user-defined variables (see Section 5.6.2).

You can add and remove user-defined keywords in the evaluation software, and edit the contents of certain keywords. You can also edit the names of the molecules immobilized on the sensor surface. These names are stored in the ***Chip Properties*** when the sensor chip is prepared (see Section 4.5).

Choose ***Tools:Keyword Table*** to open the keyword table. When you save changes to the keyword table, all user-defined items in the evaluation session will be deleted. Save the session before editing the keyword table if you do not want to lose your work. Click ***Cancel*** in the ***Keyword Table*** dialog to close the dialog without applying changes and deleting user-defined evaluation items.



To simplify management of the keyword table, you can sort and filter the table display:

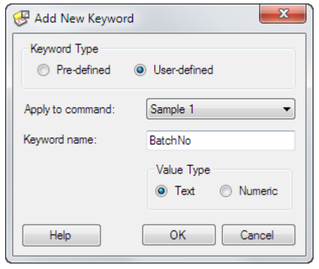
- Click on a column header to sort the table by the contents of that column.
- Click in the filter row (directly below the column header) and select a value to filter the table contents. Click ***Reset All Filters*** to restore all filters to the ***[All]*** setting.

To change a keyword value, simply enter the new value in the appropriate cell. Values for some system-generated keywords (such as ***Assay Step Purpose***) are chosen from a predefined list of values: the list is displayed when you click in such a cell.

To change the units for concentration keywords, choose a new unit from the ***Concentration Unit*** list. This changes the unit but not the numerical value of the keyword. For example, if you change the concentration unit from µM to mM, a concentration entered as 10 (i.e., 10 µM) will be treated as 10 mM. If the evaluation session includes data from multiple files, a table of concentration units for the different files is displayed. Make sure that the unit is correct for all files if data are to be evaluated together.

Note: *The concentration unit affects only predefined concentration keywords. Numerical user-defined keywords are simply numbers, and will not be re- interpreted when you change the concentration unit even if they are intended to hold concentration information.*

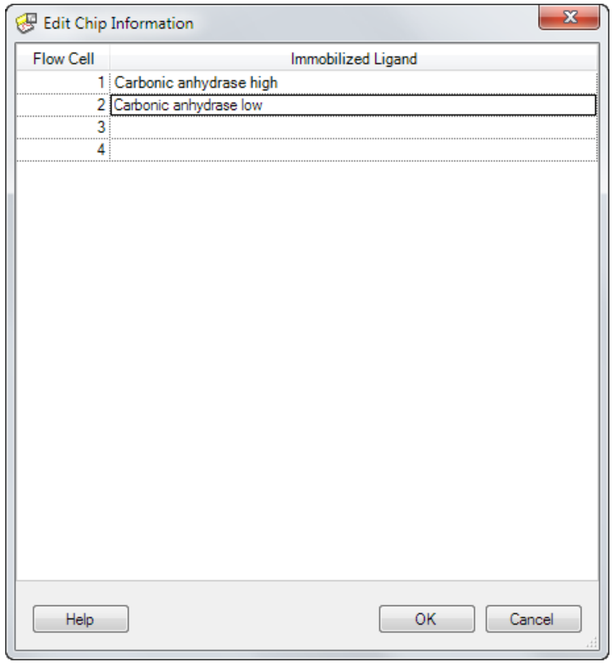
Click ***Add Keyword*** to create a new keyword in the table. You can choose between predefined keywords and user-defined keywords (see Section 5.6.2). If there are multiple ***Sample*** or ***General*** commands in the method from which the data is obtained (see Section 5.6.1), specify the command to which the new keyword should apply.



Enter the required keyword values in the empty column that is created for the new keyword.

To rename or delete a keyword, click the appropriate button, then select the keyword in the dialog box. You cannot remove system-generated keywords such as file number or cycle number, or keywords derived from method variables such as sample name.

Click ***Edit Chip Information*** to edit the ligand names as stored in the chip properties.



Note: *Editing the chip information is relevant only when ligand is immobilized directly on the sensor surface. In capturing experiments, the name of the captured ligand appears as a keyword in the keyword table.*

6.7 Solvent correction

6.7.1 Background

Solvent correction adjusts reference-subtracted responses for small artefacts that can be introduced by variations in the bulk refractive index between samples. The correction is only relevant when variations in the bulk refractive index are of the same order of magnitude as the response: this situation arises commonly in work with small organic analytes that give intrinsically low response values and that often require organic solvents such as dimethyl sulfoxide (DMSO) to maintain solubility.

The need for solvent correction arises because subtraction of the reference response does not exactly eliminate the contribution of the bulk solution to the measured response. Bulk solution is excluded from the volume occupied by ligand on the active surface, so that the bulk contribution to the response on the active surface is slightly smaller than that on the reference surface (Figure 6-1).

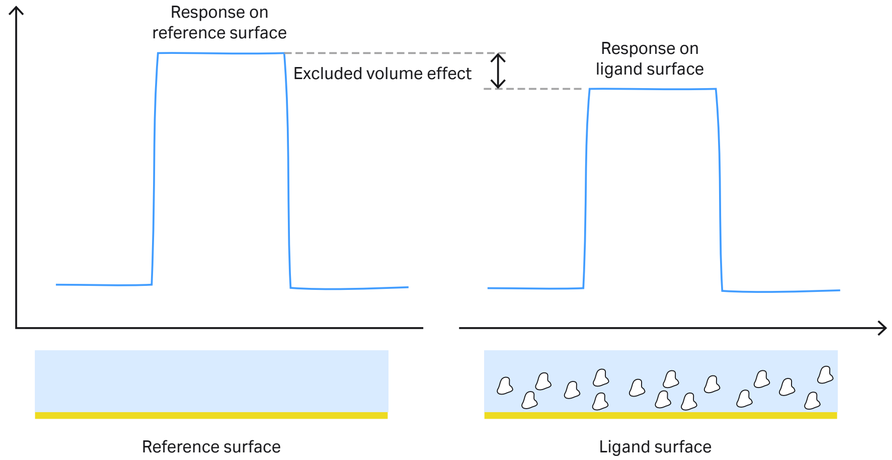


Figure 6-1. *Bulk solution is excluded from the volume occupied by ligand molecules on the ligand surface, so the bulk contribution to the relative response is smaller than on the reference surface.*

As long as the refractive index of the samples is constant, this excluded volume effect introduces a constant error in reference subtraction which may be ignored for practical purposes. However, if the refractive index of the samples varies, the magnitude of the excluded volume effect will also vary.

Organic solvents like DMSO often give a high bulk response (addition of 1% DMSO gives a bulk response of about 1200 RU), so that small variations in the DMSO content lead to significant variations in the bulk response between samples. Such variations are unavoidable in the preparation of diverse samples such as drug candidates for screening applications. The solvent correction procedure corrects for the variations arising from the excluded volume effect in these cases.

A more detailed description of solvent correction background and procedures may be obtained at cytiva.com/biacoregetstarted.

6.7.2 When solvent correction should be used

It is important to bear in mind that solvent correction is only relevant when

- the expected analyte responses are low,
- the ligand is a macromolecule immobilized at a high density (typically 5,000 RU or more relative to the reference surface). Lower ligand densities lead to excluded volume effects that are too small to merit correction,
- the bulk response is subject to variations between samples of at least the same order of magnitude as the measured binding response.

Solvent correction should not be applied in situations that do not meet all three of these criteria. Attempts to use solvent correction in other circumstances may introduce errors that are larger than the solvent effects that the procedure is intended to correct.

6.7.3 How solvent correction works

Solvent correction factors are determined by calibrating variations in the reference-subtracted bulk response on the active surface against the relative response on the reference surface, using a series of blank samples with a range of solvent concentrations. Sample measurements are then corrected from this calibration curve according to their relative response on the blank surface (Figure 6-2).

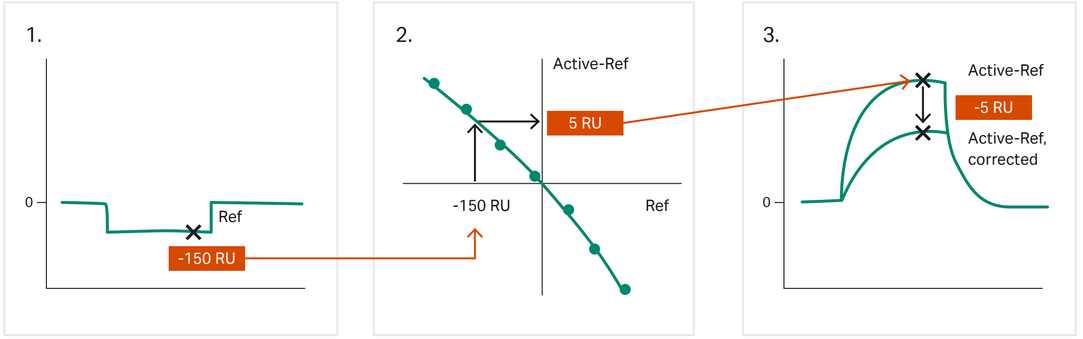
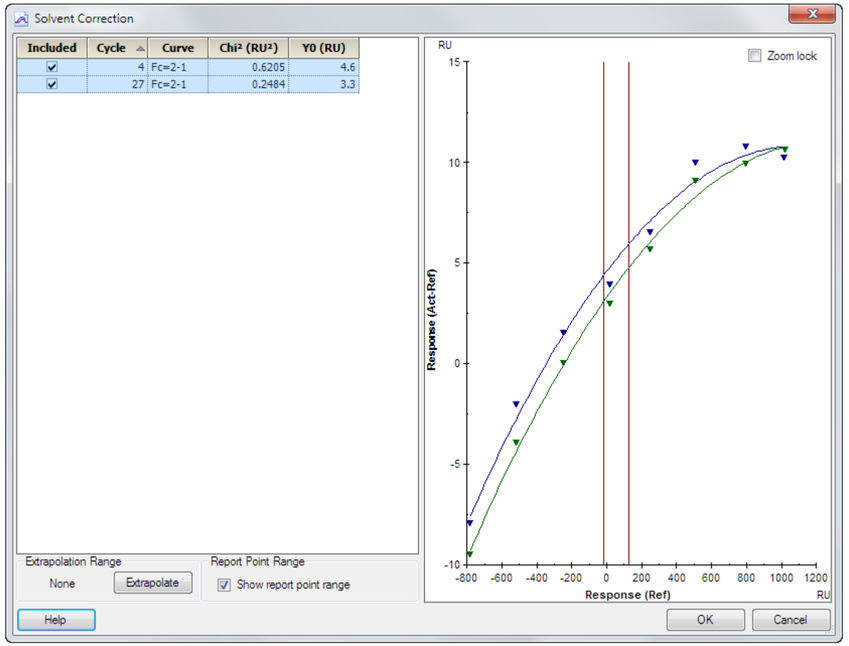


Figure 6-2. *The principle of solvent correction.* ***1****. The sensorgram from the reference flow cell shows a bulk displacement (-150 RU in the illustration) during sample injection because the sample and running buffer are not exactly matched.* ***2.*** *From the solvent correction curve, a displacement of –150 RU in the reference sensorgram corresponds to a solvent error of +5 RU in the reference-subtracted sensorgram.* ***3.*** *The reference-subtracted sensorgram is corrected by subtracting the solvent error. This procedure is applied to every point during sample injection.*

Solvent correction is applied only to response levels during sample injection, since the correction adjusts for differences in the bulk refractive index of the samples compared with running buffer. Solvent correction is meaningless when buffer is flowing over the surface before and after the sample injection.

6.7.4 Applying solvent correction

To apply solvent correction, click the ***Solvent correction*** button on the toolbar or choose ***Add solvent correction*** from the ***Evaluation*** menu. In order to apply solvent correction, the run must include solvent correction cycles (see Section 5.6.1). Once solvent correction has been applied, the item cannot be edited or deleted, and excluding or including solvent correction cycles will have no effect on the correction.



The left-hand panel of this dialog lists the solvent correction curves in the run, and the corresponding curves are shown in the right-hand panel. For each solvent correction cycle, there is one correction curve for each set of reference- subtracted sensorgrams. All cycles are shown by default in an overlay plot.

Select specific rows in the cycle list to display the corresponding solvent correction curves. Clear the checkmark in the ***Included*** box to exclude cycles from the correction calculation. You must include at least one solvent correction cycle for each curve. Sample responses are corrected according to the curve obtained from the nearest preceding correction cycle in the run. If there is no preceding correction cycle, the nearest following cycle is used.

Examine the curves for fitting to the experimental points. Right-click on outlying points to exclude either the single point from the curve fit or the whole correction cycle from the correction process. Statistical fitting parameters (chi- square) are shown for each correction curve in the cycles list. Right-click on a point or curve in the right panel and choose ***Show sensorgrams*** if you want to examine the sensorgrams from solvent correction cycles as an aid in judging the quality of the data.

The solvent correction curve is fitted to the experimental points using a second- degree equation. Beware of applying solvent correction if the correction curve does not fit the experimental points closely. Scatter in the correction points indicates that the measurements are not reliable, and applying correction derived from such curves can distort the measured responses unnecessarily. For reliable solvent correction, the chi-square value should be less than 2 RU.

Note: *In judging the quality of the solvent correction data, take note of the y-axis scale in the display. The curves are automatically scaled to fit the window. If the range of solvent correction is small (as in the illustration above), points may appear to scatter around the fitted curve without necessarily indicating poor curve quality.*

The shape and slope of the solvent correction curve (even the direction of slope) may vary between measurements on different occasions. This is normal and the shape of the curve should not be taken as an indicator of curve quality.

The range of report point values that are candidates for solvent correction in the assay data is indicated by vertical red lines in the window. The vertical span between the red lines gives an indication of the magnitude of solvent correction for report points. If report points lie outside the correction range, these values cannot be properly corrected. Some small extrapolation of the correction plots may be permissible. Use the ***Extrapolate*** button to extend the correction range. The shape of the solvent correction plots is however not fully predictable, and extrapolation over more than about 10% of the range of the reference values is dangerous.

Click ***OK*** to apply the solvent correction. Correction will be applied to the sample and carry-over injection phase(s) of all sensorgrams. Any data points that lie outside the correction range will be discarded and the corresponding sensorgram will contain gaps corresponding to the invalid data.

Note: *In assays where the temperature is varied during the run, such as thermodynamics assays, you should make sure that there is a solvent correction curve included at the start of each temperature series. Correction will only be applied to sample measurements made at the same temperature as the correction curve.*

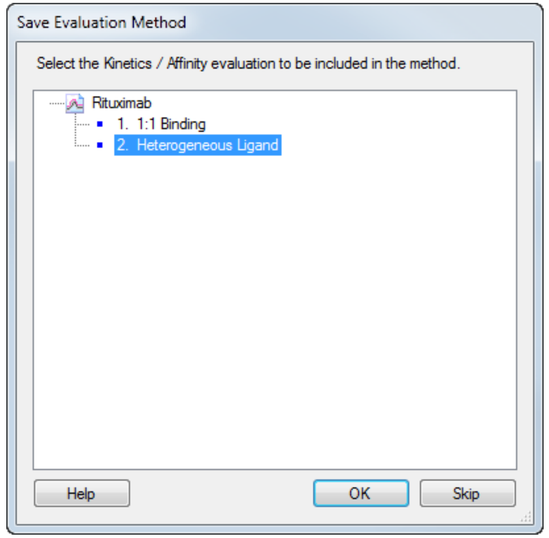
6.8 Evaluation methods

Evaluation methods allow you to save the definitions of evaluation items (with exceptions as listed below) in an evaluation session, and apply them automatically to the same or different result files or evaluation sessions. Use this feature to apply standardized evaluation procedures to different result files, or to re-create an evaluation session after operations that delete user-defined items such as appending a result file or changing the keyword table.

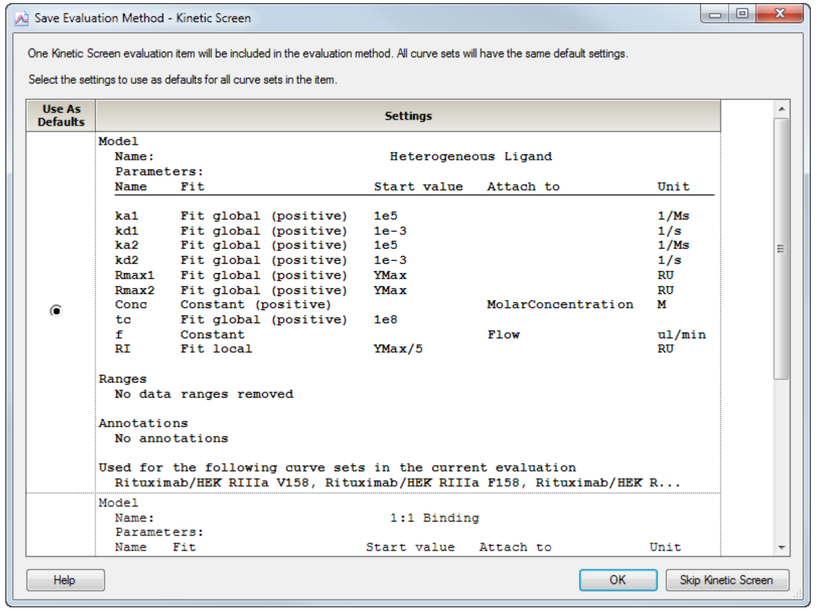
6.8.1 Creating evaluation methods

To create an evaluation method, choose ***File:Save Evaluation Method As*** and specify a file name and location. The method is saved with file extension ***.evalmethod***. Selection dialogs are displayed in the following cases. Otherwise the method is saved directly:

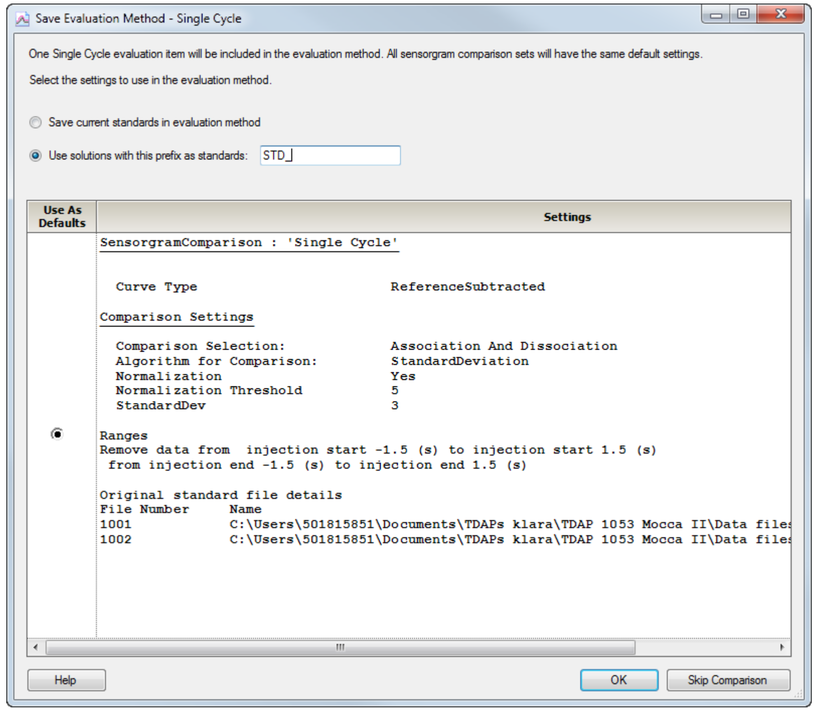
- For kinetics and affinity items, only one fitting model can be saved in the method. If the evaluation session contains multiple fittings, select the fitting model to save.



- For kinetic and affinty screen items, only one setting can be saved. Choose the setting to save.



- For sensorgram comparison items, choose whether to save standard sensorgrams in the method or to identify standards by a naming prefix. See Section 11.9 for more details of sensorgram comparison in evaluation methods.



6.8.2 Evaluation method restrictions

Data adjustments applied in result plots (Section 8.3) are saved in the evaluation method with the exception of median filter settings. Since the optimal settings for median filter vary according to the data, the filter cannot be automated and must be applied separately to each data set.

The following items and actions cannot be included in evaluation methods:

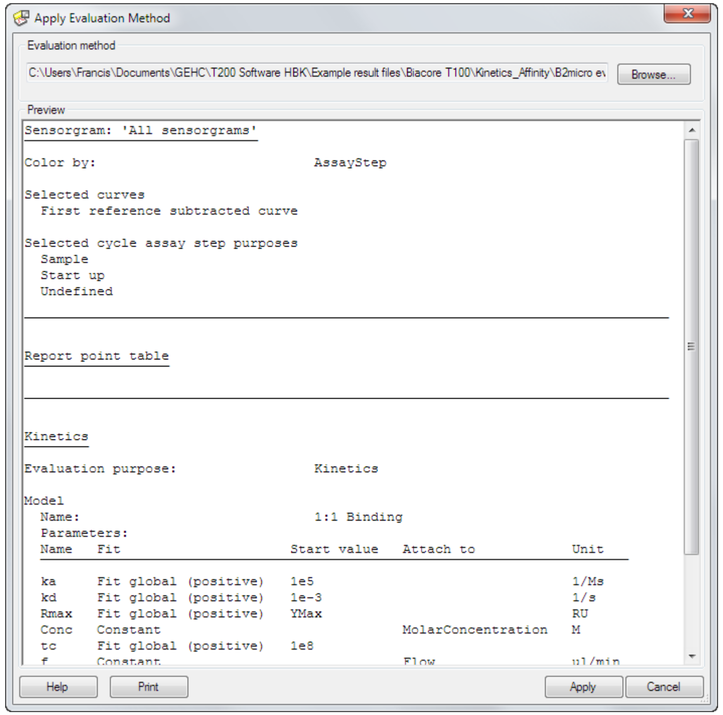
- Median filtering in result plots
- Kinetic/affinity items that use data sets with multiple R_{max} (Section 14.1.2)
- Thermodynamics items

There may only be one instance each of kinetics/affinity analysis, kinetic screen, affinity screen, concentration analysis, CFCA, and sensorgram comparison. If the evaluation session from which the method is created includes multiple instances of any of these items, choose which instance to save in the method.

For CFCA items that include samples with different fitting ranges, the default ranges will be used. If fitting ranges have been adjusted equally for all samples, the adjusted ranges will be saved in the method. Only one temperature can be saved for CFCA items.

6.8.3 Applying evaluation methods

To apply an evaluation method to the contents of an evaluation session, choose ***File:Apply Evaluation Method*** and choose the method. A preview of the method is shown so that you can check the method contents.



Click ***Apply*** to apply the method. Items in the method will be created where possible. Items that cannot be created will be reported in a dialog box.

For result plots, data adjustments are applied as far as possible.

For kinetic/affinity analysis items evaluation is applied in batch mode to all sample series in the result file. The evaluation process is completed automatically.

CFCA items can only be applied to data obtained at the same temperature as that saved in the method.

Screening items are applied as follows:

- The item is created with the fit settings from the method applied to all data series. The fit is not performed automatically.
- To complete the fitting, edit the item and click ***Fit***. For affinity screen using a constant $R_{max,}$ you will need to choose ***Fit Settings*** and provide the R_{max} value. You can if desired change the fit settings before performing the fit. For custom models that require parameter input, choose ***Fit Settings*** and click ***Parameters*** to complete the parameter table before performing the fit.

Sensorgram comparison items are applied and completed as far as possible using the settings saved in the method. Standards are either saved in the method or identified by a naming prefix (defined in the method) in the data set to which the method is applied. The comparison is applied to all sensorgrams in the evaluation session.

07

Data presentation

This chapter describes the tools available for presentation and examination of the data in a result set. These tools comprise:

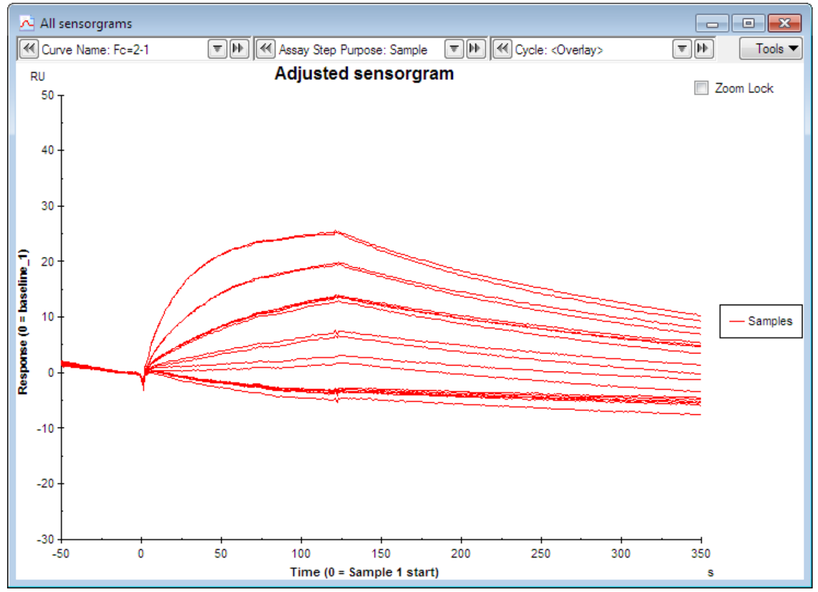
- Sensorgram display, with facilities for aligning sensorgrams in overlay plots.
- QC plot tool for displaying response values and eliminating unwanted outliers.
- Bar chart for displaying response values.
- A report point table for listing numerical values associated with report points.

Note: *The QC plot tool is intended primarily for simple graphical presentation of report point values and for excluding unwanted data points such as outliers. Result plots (Chapter 8) provide more functions for evaluating plot data.*

7.1 Sensorgram items

Sensorgram items display sensorgrams from one or more cycles in the results.

A sensorgram item containing all sensorgrams in the result file is created automatically in the Evaluation Explorer when the file is opened. You can change the display settings in this item, or create additional sensorgram items if required. Click ***Sensorgram*** in the toolbar or choose ***Add sensorgram*** from the ***Evaluation*** menu to add a new sensorgram item.

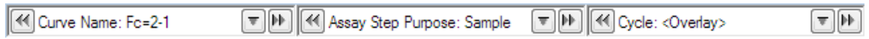


Hold the cursor over a sensorgram to display a tool tip identifying that particular curve. The sensorgram coloring can be changed if desired with the ***Tools:Color by*** option.

The following sections describe display functions specific to sensorgram items. General display functions are described in Section 6.3.

7.1.1 Selecting sensorgrams for display

The selector bar at the top of the window controls which sensorgrams will be displayed.



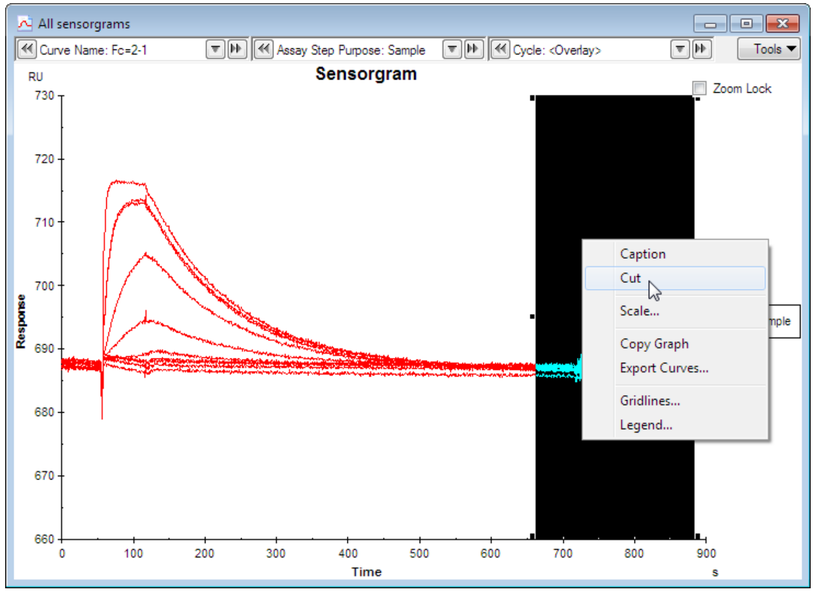
- ***Curve name*** lists the flow cell and type of sensorgram (active, reference, reference subtracted and solvent corrected where applicable).
- ***Assay Step Purpose*** filters the sensorgram according to the assay step purpose.
- ***Cycle*** lists all the cycles in the result set. When multiple files are open, cycles are identified with two numbers, one for the file in the result set and one for the cycle within the file (thus cycle 1-10 is the 10th cycle in the first file added to the result set; cycle 2-4 is the 4th cycle in the second file and so on).

For each display controller, click the browse buttons ( ) to browse backwards or forwards through the list, one item at a time.

Click the selector button ( ) to open the list for selecting one or more items. Drag with the mouse or use shift-click to select contiguous multiple items. Use control-click to select non-contiguous multiple items. To accept a selection, click anywhere outside the list or press Enter.

7.1.2 Removing data

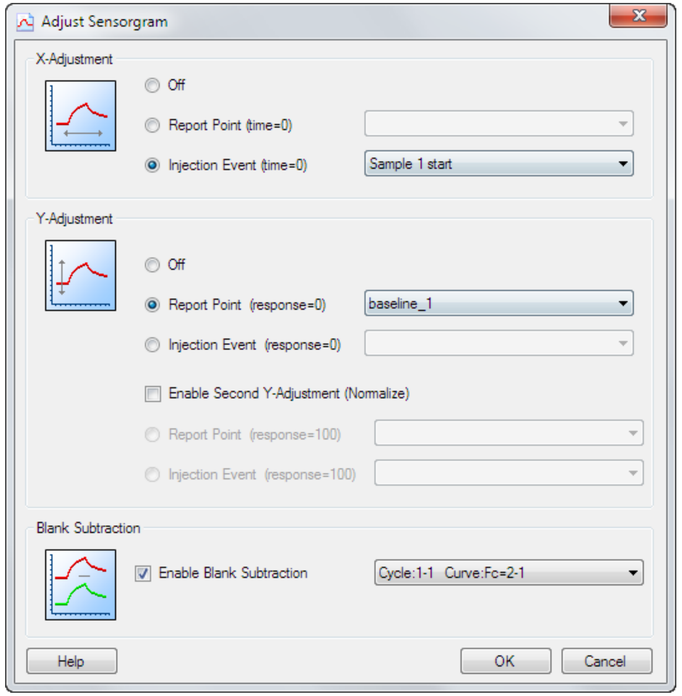
To remove data from the display, mark the section to be removed by dragging with the right mouse button, then choose ***Cut*** from the right-click menu. The data will be removed from the current sensorgram display item only. No other windows or evaluation items will be affected. This function can be useful for removing injections with high bulk contributions (such as regeneration injections) or other visual disturbances from the display.



Choose ***Undo Cut*** from the right-click menu to restore the removed data.

7.1.3 Sensorgram adjustment

Choose ***Sensorgram adjustment*** under the ***Tools*** button for options for aligning and adjusting the sensorgram display. For curve alignment, sensorgrams that do not include the chosen reference point for alignment will not be shown. Sensorgram adjustment only affects the display in the current sensorgram item.



X-adjustment

Choose to set the zero time point to either a report point or an injection event. If this setting is ***Off***, the zero time point will be at the beginning of the cycle.

Y-adjustment

Choose to set the zero response point to either a report point or an injection event. If this setting is ***Off***, the actual response values will be shown.

If you check ***Enable Second Y-Adjustment***, you can select a report point or injection event where the response value will be set to 100. Each sensorgram will then be normalized separately to the first and second adjustment point, so that all sensorgrams will have values of 0 and 100 at these points regardless of the original response levels. This can help in comparing the shapes of sensorgrams independently of their response levels, or in adjusting response levels that are dependent on others (e.g. adjusting analyte response for varying capture levels, by adjusting the baseline to 0 and the capture level to 100).

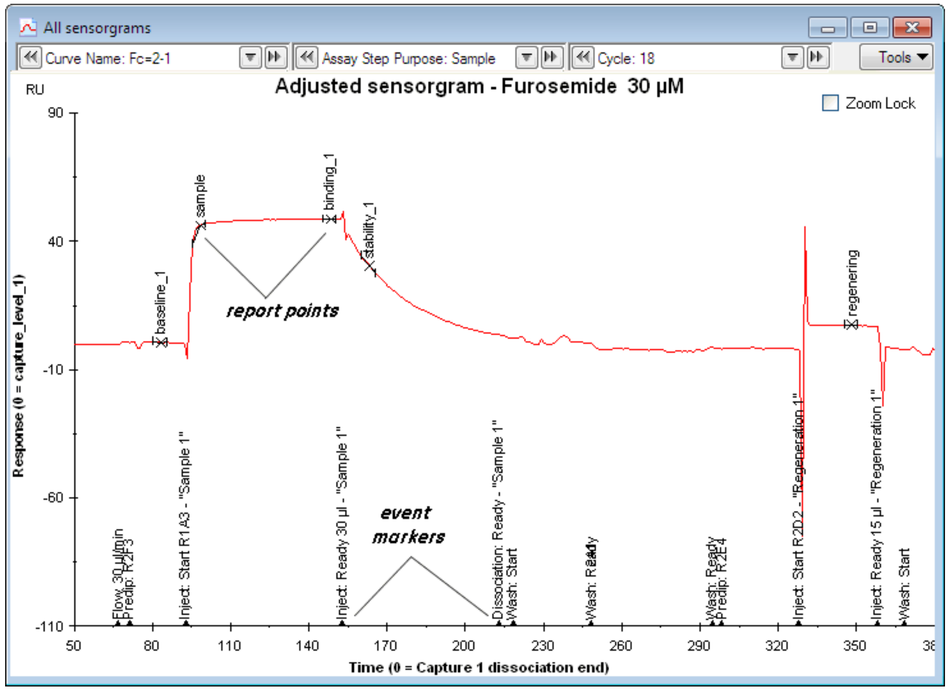
Blank subtraction

Check ***Enable Blank Subtraction*** and choose a curve to be used as the blank to subtract one sensorgram from all others in the display. Use this feature to eliminate systematic disturbances in sensorgrams that are not removed by reference subtraction. Blank subtraction only affects the current sensorgram window: other evaluation items are not affected. Blank subtraction is also available in several other evaluation items.

Note: *Subtracting a blank sensorgram is not the same as using reference- subtracted data. Reference subtraction gives the difference between active and reference values for each cycle separately for each curve, whereas blank subtraction subtracts one curve from all others in the result set.*

7.1.4 Markers

You can choose to display markers and/or labels for report points and events in the cycle with the ***Report points*** and ***Event markers*** options respectively under the ***Tools*** button. Report points are displayed on the curve and event markers on the x-axis.



7.2 QC plots

7.2.1 Introduction

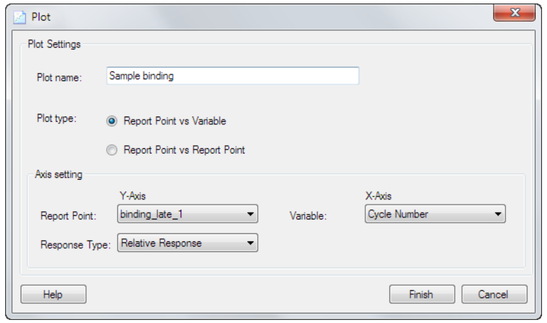
QC plots are intended primarily for presentation and quality control of data. Cycles that are excluded in QC plots are excluded automatically from all other subsequently created or edited evaluation items. Adjustment of response values for analyte molecular weight and ligand capture level (where appropriate) can be performed in user-defined QC plots: these adjustments affect only the current plot. Additional report point evaluation facilities are provided in result plots (see Chapter 8).

Note: *Pre-defined QC plots cannot be edited or adjusted.*

7.2.2 Creating QC plots

QC plots display report point values plotted against either variables or other report point values in the same cycle.

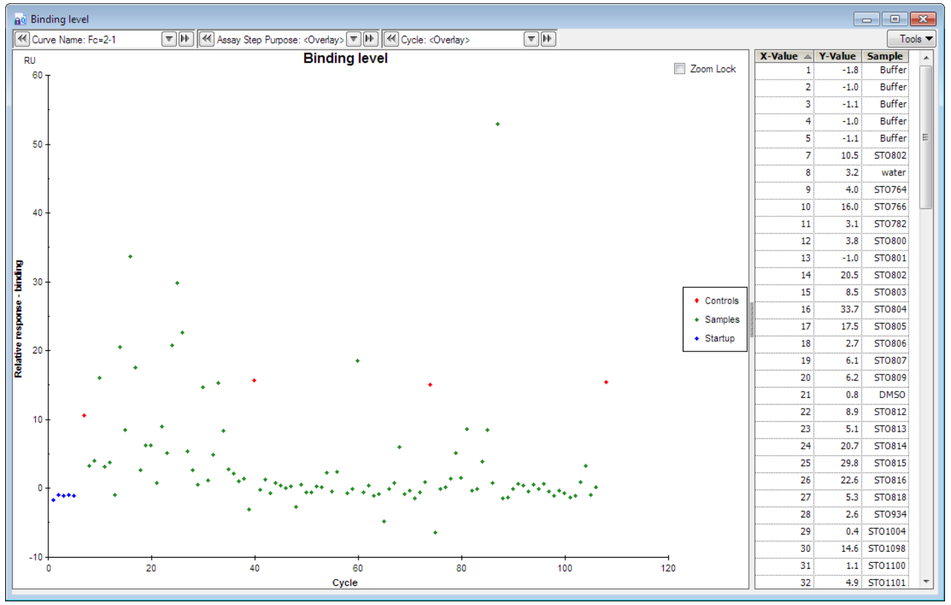
To create a QC plot item, click ***Plot*** in the toolbar and choose ***QC Plot*** or choose ***Add Plot:QC Plot*** from the ***Evaluation*** menu. Enter a name for the plot, choose the parameters that define the plot and click ***Finish***. Cycles that do not contain the selected report point(s) will not be represented in the plot.



Response type may be response (absolute or relative) or sensorgram slope at the selected report point.

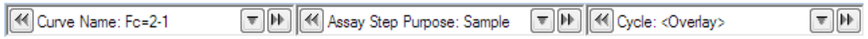
Variables may be numerical (e.g. molecular weight or concentration) or non- numerical (e.g. sample name or assay step purpose). Plots with non-numerical variables on the x-axis are grouped by the selected variable.

The plot will be created with default display settings, with a graphical representation at the left and a table of selected data at the right. Tool tips identify the data points (place the cursor on a point for a couple of seconds to display the tool tip).



7.2.3 Selector functions

The selector bar at the top of the window controls which points will be displayed.



- ***Curve name*** lists the type of sensorgram from which the points are taken (active, reference, reference subtracted and solvent corrected where applicable).
- ***Assay Step Purpose*** filters the points according to the assay step purpose.
- The third selector lists the variable values represented on the x-axis. This option is not available for plots of report point against report point.

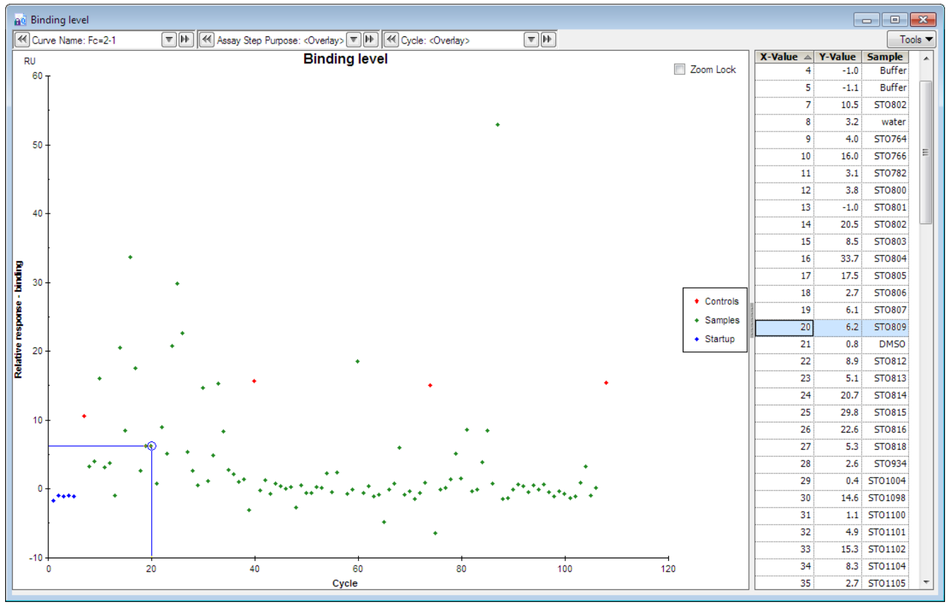
Selection operates in the same way as in the sensorgram window (Section 7.1.1).

Other general display functions are described in Section 6.3.

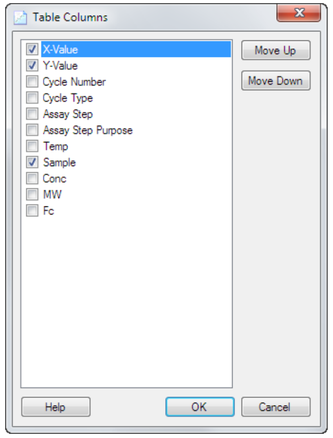
7.2.4 Table functions

The table to the right of the plot area lists values and sample names for the points in the plot. You can display sensorgrams and exclude or include cycles from the right-click menu in the table area, in the same way as from the right- click menu in the plot. The table also allows you to exclude or include multiple cycles in a single operation. Excluded points are shown struck out in red text.

Select rows in the table to highlight the corresponding points in the plot. If you select a single row, the highlight is augmented with lines drawn to the plot axes:



By default, the table shows x- and y-values sample name and cycle information and is sorted in ascending order of x-values. Click on a column header to sort the table by that column and to change the sort order. Sorting the table does not have any effect on the plot display.



Choose ***Tools:Table columns*** to select columns that will be displayed in the table. You can also change the order in which columns will be displayed using the ***Move up*** and ***Move down*** buttons (the top of the column list represents the left-hand column in the table).

7.2.5 Coloring points in the plot

Select a parameter in the ***Tools:Color by*** list to color the points in the plot according to the parameter value. Colors are identified in the plot legend.

7.2.6 Sorting the plot

QC plots of report point values against variables can be sorted in order of ascending or descending y-axis value, regardless of the variable chosen for the x-axis. A sorted plot can be useful for example in visualizing the frequency of different levels of response, which may be more difficult to see if the levels are scattered more or less randomly with respect to the variable parameter defined for the x-axis. Choose ***Sort:Ascending*** or ***Descending*** under the ***Tools*** button to sort the plot. Sorting the plot also sorts the rows in the table, although the table can be sorted independently of the plot by clicking in the table column header (Section 7.2.4).

Note: *By default, the table associated with a sorted plot retains a column labeled **X-Value**. This is the value of the variable originally defined for the plot, and does not correspond to the x-axis as displayed in the sorted plot.*

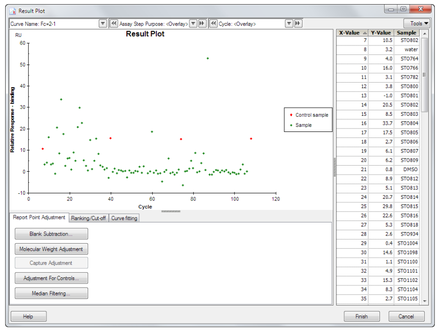
Choose ***Sort:As Defined*** to restore the x-axis to the originally defined variable value.

Plots of one report point against another cannot be sorted.

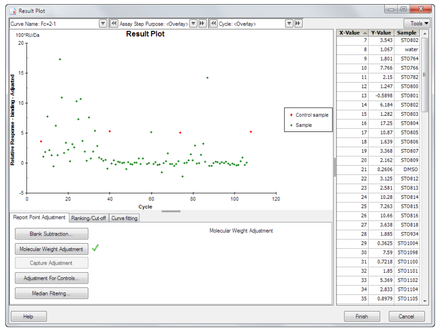
7.2.7 Adjusting for molecular weight

In some situations, quality control of the data may be easier if the results are adjusted for the molecular weight of the analyte, so that the displayed response levels are molar rather than weight-based. To apply this adjustment, click the ***Tools*** button and choose ***Molecular weight adjustment***. The adjustment is performed by dividing the response in RU by the molecular weight and multiplying the result by 100 (units 100*RU/Da). Points for which the molecular weight value is zero or missing are omitted from the adjusted plot.

Molecular weight adjustment cannot be applied to pre-defined QC plots.



Before molecular weight adjustment



After molecular weight adjustment

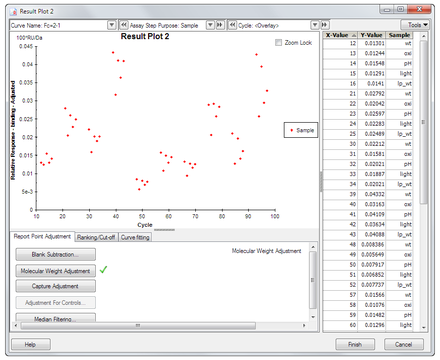
Notes: *Adjustment for molecular weight applies only to the QC plot is which it is selected, and does not affect report points displayed in other plot items. A corresponding adjustment function is provided in result plots (Chapter 8).*

Editing the definition of a molecular weight-adjusted plot will cancel the adjustment.

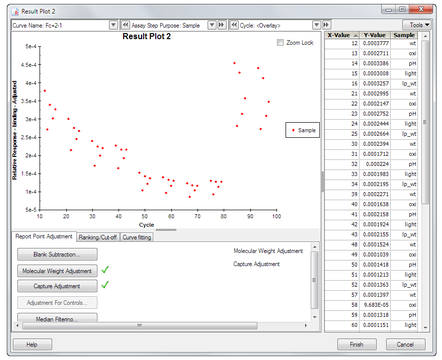
7.2.8 Adjusting for capture levels

Capture adjustment corrects sample responses for variations in the levels of captured ligand between cycles by dividing the sample response with the response for captured ligand. To apply this adjustment, choose ***Tools:Capture Adjustment***. When capture adjustment has been applied, adjusted response levels will be expressed as sample response divided by capture level. Both axes can be adjusted independently in a plot of report point against report point.

Capture adjustment cannot be applied to pre-defined QC plots.



Before capture adjustment



After capture adjustment

Notes: *Adjustment for capture should only be applied to report points that represent analyte response (either direct or enhanced). The adjustment is not appropriate for report points placed before the sample injection.*

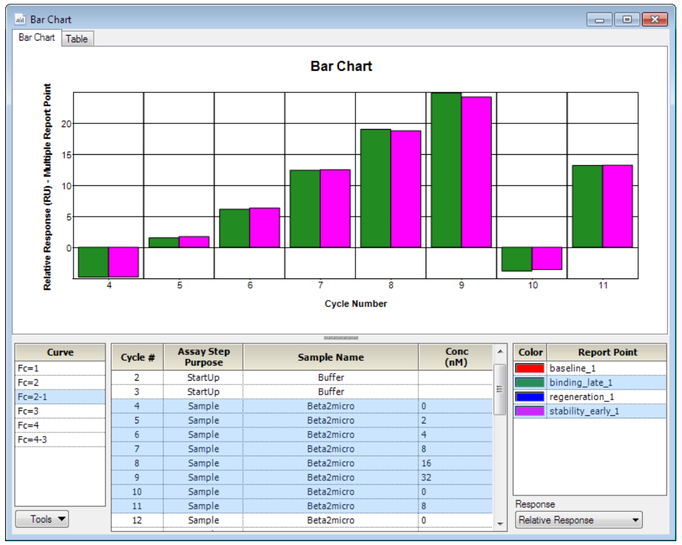
Beware of applying adjustment for capture to report points with significant bulk response contribution. The bulk component will be included in the adjustment and will distort the results.

Capture adjustment relies on response values from the report points ***capture_baseline*** *and* ***baseline****. The adjustment cannot be applied if these report points are not present. Editing the definition of a capture-adjusted plot will cancel the adjustment.*

7.3 Bar chart items

Bar charts display report point data plotted against cycle number. Unlike plots, bar charts can display multiple report points in the same chart, and can group the display in various ways.

To create a bar chart, click ***Bar Chart*** in the toolbar or choose ***Add Bar Chart*** from the ***Evaluation*** menu. The chart is created directly. Numerical values on which the chart is based are listed on the ***Table*** tab.



7.3.1 Selector functions

Select the curves to display in the lower left hand panel, cycles in the center panel and report points in the right-hand panel. In each panel you can select multiple rows by dragging with the mouse or using Shift-click for contiguous rows or Control-click for non-contiguous rows. Click on the column header in the table of cycles in the center panel to sort the table by the contents of the column, to simplify selection of rows according to the required criteria.

Select whether to display relative or absolute response values in the ***Response*** field. This selection applies to all report points shown in the chart. Points for which the selection is invalid are not shown (for example, the report point ***baseline*** will normally not be shown on a bar chart of relative response).

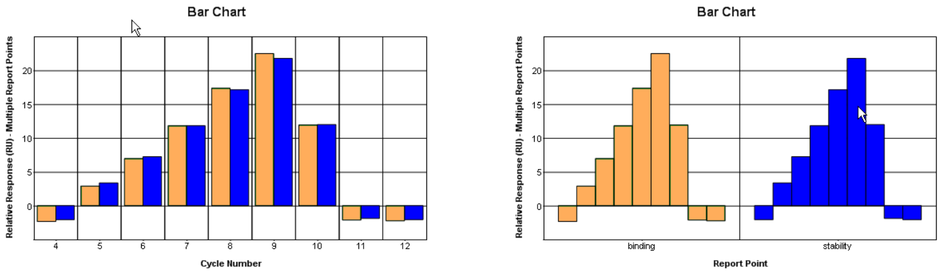
7.3.2 Display options

Drag with the mouse to zoom the bar chart display. Double-click to restore the original scaling.

Use the ***Tools*** menu to determine how the bar chart is organized and displayed:

Group by

You can group bars in the chart by various parameters such as curve, cycle, report point or sample name. Groups are separated by vertical lines in the chart. Grouping is most useful when you have multiple selections in more than one selection panel: the examples below illustrate bar charts showing two report points, grouped by cycle (left) and by report point (right).



Color by

Select a parameter in the ***Color by*** list to color the bars in the chart according to the parameter value. A ***Color*** key will be added to the table in the appropriate panel.

Show column labels

Use this option to display labels identifying the bars in the chart. The bars are also identified by tool tips, regardless of whether labels are displayed or not.

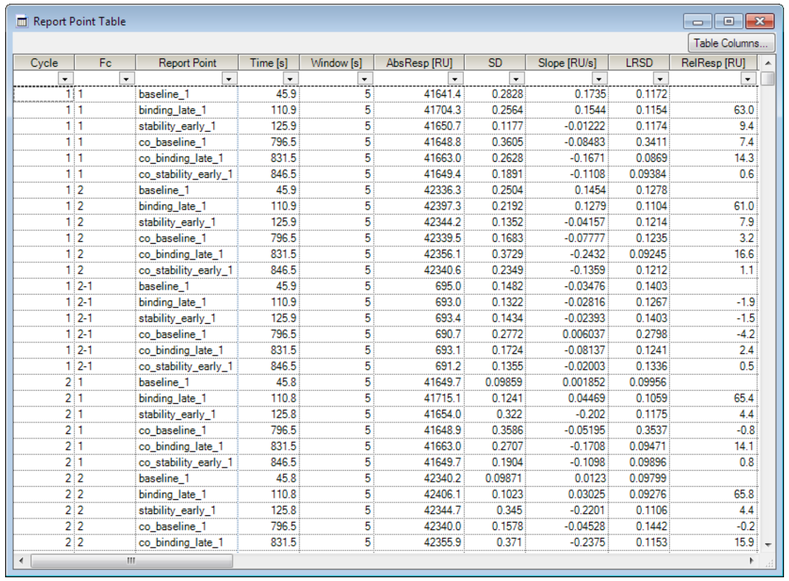
7.4 Report point table

Report points are automatically created for all wizard- and method-based runs, placed at strategic positions in relation to injections. See Section 6.5 for details of how to add custom report points.

Note: *The report point table does not list response values adjusted for molecular weight or capture level.*

7.4.1 Displaying the report point table

The report point table lists numerical values for all report points in the current result set. The report point table is created automatically as an evaluation item. Each evaluation session can only have one report point table item: the table is updated automatically if you add custom report points or apply solvent correction.



Cycle	Fc	Report Point	Time [s]	Window [s]	AbsResp [RU]	SD	Slope [RU/s]	LRSD	RelResp [RU]
1	1	baseline_1	45.9	5	41641.4	0.2828	0.1735	0.1172	
1	1	binding_late_1	110.9	5	41704.3	0.2564	0.1544	0.1154	63.0
1	1	stability_early_1	125.9	5	41650.7	0.1177	-0.01222	0.1174	9.4
1	1	co_baseline_1	796.5	5	41648.8	0.3605	-0.08483	0.3411	7.4
1	1	co_binding_late_1	831.5	5	41663.0	0.2628	-0.1671	0.0869	14.3
1	1	co_stability_early_1	846.5	5	41649.4	0.1891	-0.1108	0.09384	0.6
1	2	baseline_1	45.9	5	42336.3	0.2504	0.1454	0.1278	
1	2	binding_late_1	110.9	5	42397.3	0.2192	0.1279	0.1104	61.0
1	2	stability_early_1	125.9	5	42344.2	0.1352	-0.04157	0.1214	7.9
1	2	co_baseline_1	796.5	5	42339.5	0.1683	-0.07777	0.1235	3.2
1	2	co_binding_late_1	831.5	5	42356.1	0.3729	-0.2432	0.09245	16.6
1	2	co_stability_early_1	846.5	5	42340.6	0.2349	-0.1359	0.1212	1.1
1	2-1	baseline_1	45.9	5	695.0	0.1482	-0.03476	0.1403	
1	2-1	binding_late_1	110.9	5	693.0	0.1322	-0.02816	0.1267	-1.9
1	2-1	stability_early_1	125.9	5	693.4	0.1434	-0.02393	0.1403	-1.5
1	2-1	co_baseline_1	796.5	5	690.7	0.2772	0.006037	0.2798	-4.2
1	2-1	co_binding_late_1	831.5	5	693.1	0.1724	-0.08137	0.1241	2.4
1	2-1	co_stability_early_1	846.5	5	691.2	0.1355	-0.02003	0.1336	0.5
2	1	baseline_1	45.8	5	41649.7	0.09859	0.001852	0.09956	
2	1	binding_late_1	110.8	5	41715.1	0.1241	0.04469	0.1059	65.4
2	1	stability_early_1	125.8	5	41654.0	0.322	-0.202	0.1175	4.4
2	1	co_baseline_1	796.5	5	41648.9	0.3586	-0.05195	0.3537	-0.8
2	1	co_binding_late_1	831.5	5	41663.0	0.2707	-0.1708	0.09471	14.1
2	1	co_stability_early_1	846.5	5	41649.7	0.1904	-0.1098	0.09896	0.8
2	2	baseline_1	45.8	5	42340.2	0.09871	0.0123	0.09799	
2	2	binding_late_1	110.8	5	42406.1	0.1023	0.03025	0.09276	65.8
2	2	stability_early_1	125.8	5	42344.7	0.345	-0.2201	0.1106	4.4
2	2	co_baseline_1	796.5	5	42340.0	0.1578	-0.04528	0.1442	-0.2
2	2	co_binding_late_1	831.5	5	42355.9	0.371	-0.2375	0.1153	15.9

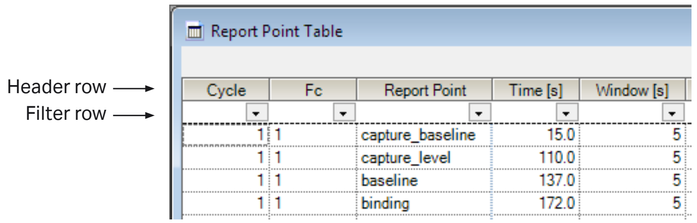
Values in the repoort point table cannot be edited, but the contents can be customized by sorting, filtering and selecting columns. Sorting, filtering and column selection are applied to the report table as exported to Excel or XML, but not to export in tab-separated text format (see Section A.1.1).

- Click on a column header to sort the table by the contents of that column.
- Click in the filter row (directly below the column header) and select a value to filter the table contents.
- Click the ***Table Columns*** button to choose which columns to display. The columns that can be included in the report point table are described below.

File	File number. This column is only shown when the evaluation session includes more than one file, the cycle number is prefixed with a file number. Choose ***File:Properties*** to display the mapping of source files to file numbers.
Cycle	Cycle number within the file.
Fc	The curve to which the report point applies, identified as the flow cell.
Report point	Report point id.
Time (s)	Report point time in seconds from the start of the cycle.
Window (s)	Report point time window in seconds.
AbsResp (RU)	Absolute response in RU, calculated as the mean value over the time window.
SD	Standard deviation of data points in the time window, calculated as $SD = \sqrt{\frac{1}{(n-1)}\sum(y-\bar{y})^2}$ where n = number of points and y = response in RU
Slope (RU/s)	Slope during time window in RU s^{-1}, calculated as $slope = \frac{\sum(y-\bar{y})(x-\bar{x})}{\sum(x-\bar{x})^2}$
LRSD	Alignment of slope to a straight line (regression coefficient), calculated as $LRSD = \sqrt{\frac{Q_0}{(n-2)}}$ where $Q_0 = \sum(y-\bar{y})^2 - \frac{(\sum(y-\bar{y})(x-\bar{x}))^2}{\sum(x-\bar{x})^2}$
Baseline	***Yes*** for report points defined as baseline. Otherwise ***No***.
RelResp (RU)	Relative response (difference in absolute response from the baseline) in RU. #N/A if no baseline has been set.
AssayStep Purpose ***CycleType***	Identifiers for the cycles, set explicitly in methods (see Chapter 5) and automatically in wizards.
Keywords	One column is created for each keyword in the data.

Sorting and filtering the report point table

The report point table can be sorted by any column in ascending or descending order for any column. Click in the header for a column to sort the table by that column. Each click in the same header toggles the sort order.



The second row in the report point table contains a filter setting for each column. All values will be included if the filter setting is blank. To apply a filter, click on the setting and select a value from the list. The value will be shown in the filter setting and only rows in the table that contain the value in the selected column will be displayed. You can apply multiple filters to the table at the same time. To remove a filter, choose ***All*** from the list of column values in the filter setting.

Table column selection and filter settings are saved in evaluation methods, and are applied as far as possible when the method is applied.

Copying report point table contents

To copy selected contents of the report point table, select cells by dragging with the mouse and press Ctrl-C or choose ***Copy*** from the right-click menu. Choose ***Copy Table*** to copy the whole table. The contents will be copied in tab- separated text format to the Windows clipboard, and can be pasted from there into other programs. All selected cells will be copied, including header cells and filter settings.

Exporting report point table contents

To export the report point table to a tab-separated text file, choose ***File:Export:Report Point Table***. The entire table will be exported, regardless of sorting or filtering.

08

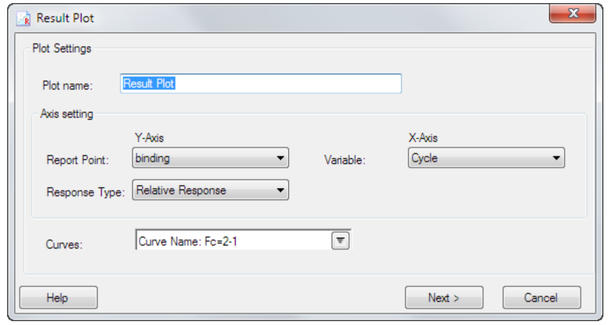
Result plots

This chapter describes result plot items. These plots differ from QC plots in the following respects:

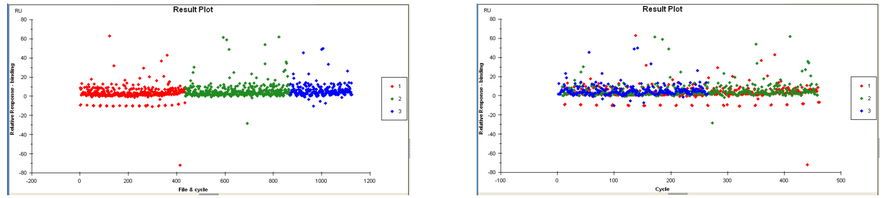
- Result plots support a range of data adjustment and evaluation functions, described in this chapter.
- Excluding points from a result plot affects only the current plot. Other items are not affected.

8.1 Creating and editing result plots

To create a result plot item, click ***Plot*** in the toolbar and choose ***Result Plot*** or choose ***Add Plot:Result Plot*** from the ***Evaluation*** menu. Enter a name for the plot, choose the parameters that define the plot and click ***Next***. Result plots display report point values plotted against a variable: plots of report point against report point are not supported. You can select multiple curves by dragging or using Ctrl-click in the curve list to create an overlay plot of points from more than one curve. Cycles that do not contain the selected report point will not be represented in the plot.



For evaluation sessions that include multiple result files, the default variable setting is ***File & Cycle***. This will plot the data grouped by file. Choose ***Cycle*** to plot the data by cycle number regardless of file (see illustration below).



*Data from three appended files, plotted against **File & Cycle** (left) and against **Cycle** (right). Points are colored by file.*

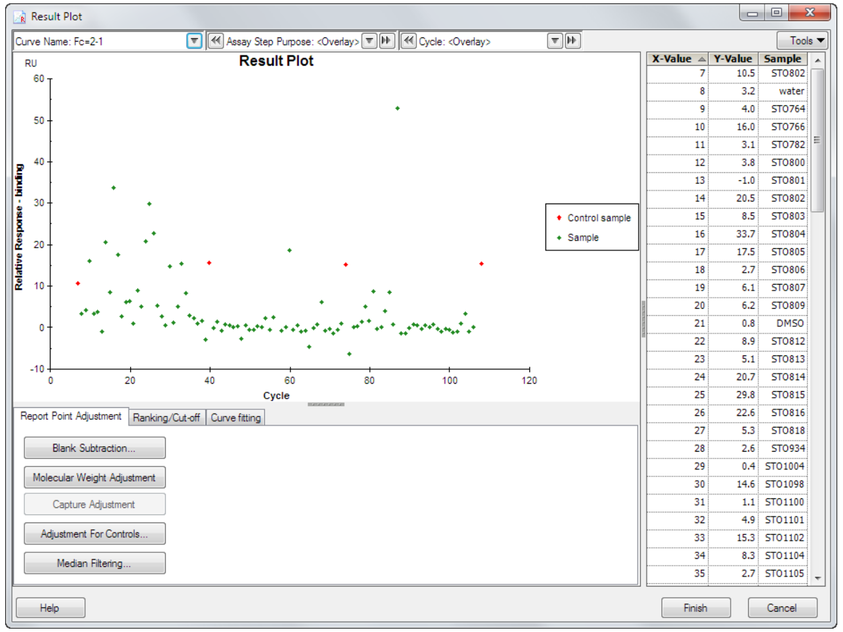
To edit a finished plot, right-click on the item name in the Evaluation Explorer and choose ***Edit*** from the menu.

Notes: *Unlike QC plots, the sensorgram curve(s) for result plots is chosen when the plot is created and cannot be changed except by editing the plot definition.*

Editing the plot definition will cancel any adjustments that have been applied.

8.2 Plot display functions

Result plots are displayed with a panel below the plot providing options for data adjustment (see Section 8.3). Numerical values for the plotted points are shown in the table to the right.



Selector functions

Selector functions operate as described for QC plots (Section 7.2.3), with the exception that the curve selection (defined when the item is created) cannot be changed.

Table functions

Table functions operate as described for QC plots (Section 7.2.4).

Excluding and including points

Points can be excluded from the plot by right-clicking on a point or on one or more selected rows in the table and choosing ***Exclude cycle*** from the right-click menu. Values for excluded points are shown struck out in red text in the table. Use the right-click menu in the table to re-include points.

Note: *Excluding points in a result plot affects the current result plot only. Points that have been excluded in a sensorgram window or QC plot are not shown in result plots and can only be accessed by re-including the points in a sensorgram or QC plot item.*

Viewing sensorgrams

To display sensorgrams corresponding to a point, right-click on the point and choose ***Show Sensorgram(s)*** from the right click menu. You can also select multiple rows in the table and right-click on the selection to show multiple sensorgrams in an overlay plot. The sensorgrams are adjusted to zero at the start of the sample injection.

Plot tools

The plot tools ***Color by***, ***Sort*** and ***Table columns*** are available in result plots in the same way as in QC plots (Section 7.2). In addition, the ***Tools*** menu includes ***Edit Annotations*** (see Section 8.6).

Ranking and cut-off as well as curve fitting functions are provided in a tab on the bottom panel of the plot display (Sections 8.4 and 8.5).

8.3 Data adjustment functions

Result plots support the following data adjustment functions:

- Blank subtraction
- Molecular weight adjustment
- Capture adjustment
- Adjustment for controls
- Median filtering

Each of these adjustments is described in detail below. Adjustments must be re- applied if the plot definition is edited.

Note: *Data adjustments in result plots are applied only to the plot points. Choosing* ***View Sensorgram*** *from the right-click menu shows the original sensorgram for the point regardless of adjustments applied in the plot.*

8.3.1 Adjustment dependencies

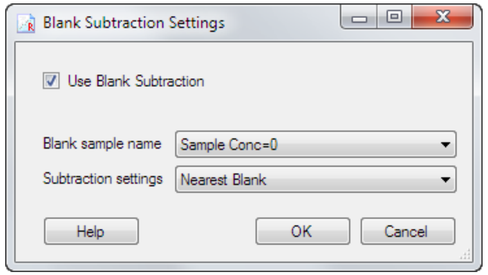
Data adjustments are applied in a the order listed above. For plots that display report points from several curves, adjustments are determined independently for each curve.

The effect of each adjustment may be dependent on the results of adjustments earlier in the sequence (e.g. adjustment for controls will be different according to whether adjustment for molecular weight has been applied or not). Individual adjustments can be skipped: however, if an earlier adjustment is changed adjustments later in the sequence will be canceled and must be re-applied. Similarly, if changes are made to the data set in the plot by excluding or including points so that an adjustment is affected, the affected and all dependent adjustments will be canceled.

Editing the plot definition will cancel all adjustments and ranking/cut-off boundaries that have been applied.

8.3.2 Blank subtraction

Click ***Blank Subtraction*** to subtract blanks from report point values.



Check ***Use Blank Subtraction*** to apply blank subtraction. Choose the sample to be used as blank and the subtraction setting. Blank samples are either controls or sample cycles with zero concentration. Subtraction settings are listed in the table below). Blank cycles themselves are always set to zero response.

Nearest Blank	Nearest blank in the cycle sequence.
Average Nearest Blanks	Average of the nearest preceding and nearest following blanks. If there is no preceding or following blank, the nearest blank is used.
Preceding Blank	Nearest preceding blank in the cycle sequence. If there is no preceding blank, the nearest blank is used.
Following Blank	Nearest following blank in the cycle sequence. If there is no following blank, the nearest blank is used.

Blank values are subtracted from all points in the plot as far as possible, using the same curve and report point settings as the plotted points. For evaluations using appended result files, the blank setting is applied within each file separately. Blank subtraction affects only the item where it is applied.

To remove blank subtraction, click ***Blank Subtraction*** and uncheck ***Use Blank Subtraction***.

8.3.3 Molecular weight adjustment

Adjustment for molecular weight divides the response by the molecular weight of the analyte and displays the result multiplied by 100. Use this function to normalize the response from differently sized analytes so that the values reflect molar binding levels rather than weight-based levels.

Any points for which analyte molecular weight is missing from the keyword table or is entered as zero will be excluded from the adjusted plot.

8.3.4 Capture adjustment

Capture adjustment corrects sample responses for variations in the levels of captured ligand between cycles by dividing the sample response with the response for captured ligand. When capture adjustment has been applied, adjusted response levels will be expressed as sample response divided by capture level.

Notes: *Capture adjustment should only be applied to report points that represent analyte response (either direct or enhanced). The adjustment is not appropriate for report points placed before the sample injection.*

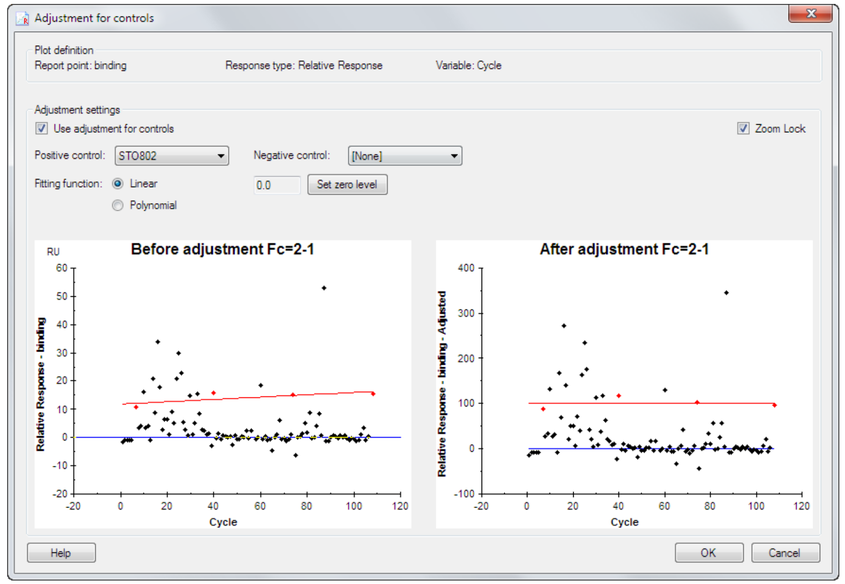
Capture adjustment relies on response values from the report points ***capture_baseline*** *and* ***baseline****. The adjustment cannot be applied if these report points are not present.*

8.3.5 Adjustment for controls

Adjustment for controls compensates for systematic changes in response during the course of the assay, such as progressive loss of binding capacity. Adjustment for controls can also be used to normalize response levels from multiple files that are evaluated together, provided that the same controls are used in all files. Adjustment is calculated from the response values obtained for control samples analyzed at intervals during the assay.

To apply this adjustment, choose ***Adjustment for controls***. Check ***Use adjustment for controls*** and select the sample to use as a positive control. You can also select a sample or specify a response level for the negative control: if no negative control is chosen, the specified zero level will be used as the negative control level. Select whether the adjustment should be

made using a linear or polynomial (second degree) fitting function. The display panels in the dialog show a plot of the response against cycle number before and after adjustment. Click ***OK*** to apply the adjustment.



Adjustment normalizes the sample responses relative to the positive and negative control levels as follows. Start-up cycles are automatically excluded from the adjustment calculations.

- Curves are fitted to the control sample responses for positive and (if used) negative controls. The ***Linear*** option fits the points to a function with the form **y= ax +b** (where **a** and **b** are constants). ***Polynomial*** fits the points to a second-degree function with the form **y= ax² + bx+c** (where **a**, **b** and **c** are constants).
- The fitted line(s) are transformed to straight horizontal lines with values 100 for the positive control and 0 for the negative control.
- The transformation used to create straight horizontal lines for the control points is applied to all points in the plot (including the actual control sample responses) so that each point retains the same position relative to the positive and negative controls before and after adjustment.
- For evaluation sessions that include multiple result files, adjustment for controls is applied separately to each file, regardless of whether the data is plotted against ***Cycle*** or ***File & Cycle***.

Adjustment for controls cannot be applied in regions where the positive control curve lies below the negative control curve (or below the specified zero level if no negative control is selected). Any points that lie in such regions will be excluded from the adjusted plot.

Note: *Beware of using a polynomial fitting function with less than 4 control samples. The parabolic curve created by the function can deviate greatly from the points, leading to adjustment that does not reflect the drift in the control responses (Figure 8-1).*

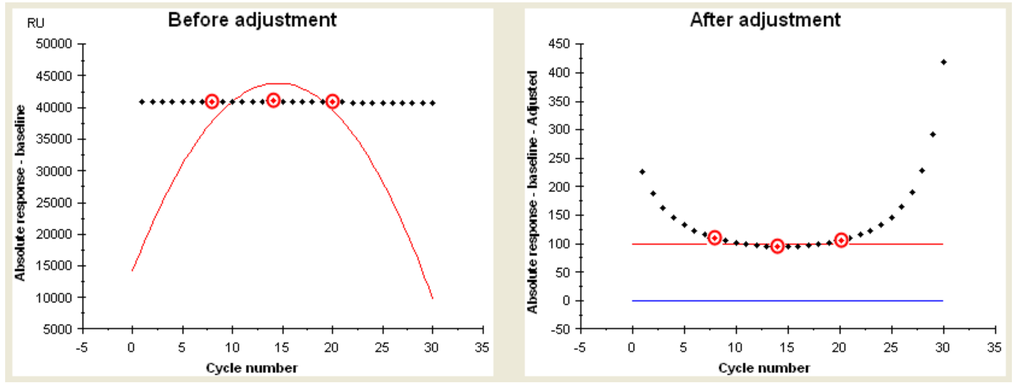
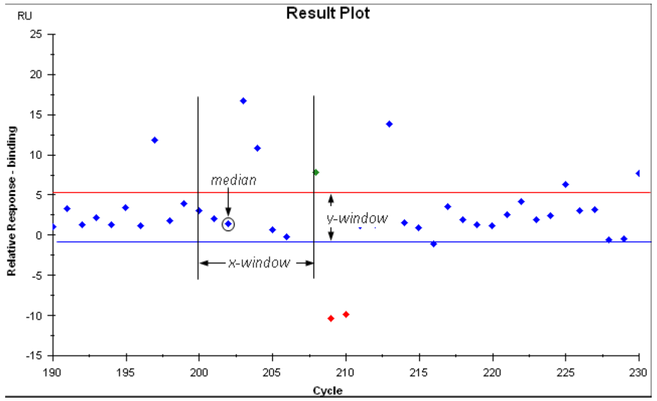


Figure 8-1. Polynomial function fitted to 3 control points (highlighted) with the resultant adjustment for controls.

8.3.6 Median filtering

Applying a median filter to a plot can markedly reduce noise and eliminate drift without obscuring binding responses. The filter is most useful for plots with drift or periodic variation in the baseline, and a horizontal y-axis window can be set so that the window includes presumed non-binders and excludes most of the potential binders. The effect of the filter is to align points within the y-axis window to a new zero baseline. Positive control samples should be included at regular intervals in the run, to provide a check on the suitability of the median filter settings. The filter is applied using the set y-axis (response) window and a sliding x-axis (cycle) window as follows. Start-up cycles are automatically excluded from the median filtering.

1. The median value for each x-coordinate is calculated from the points in the region defined by the x- and y-axis windows. Where points fall outside the y-axis window, more cycles are included so that the number of points within the windows is constant.
2. Each point in the plot (including those that lie outside the y-axis window) is adjusted by subtracting the calculated median value. A new baseline is thereby defined with response value 0.



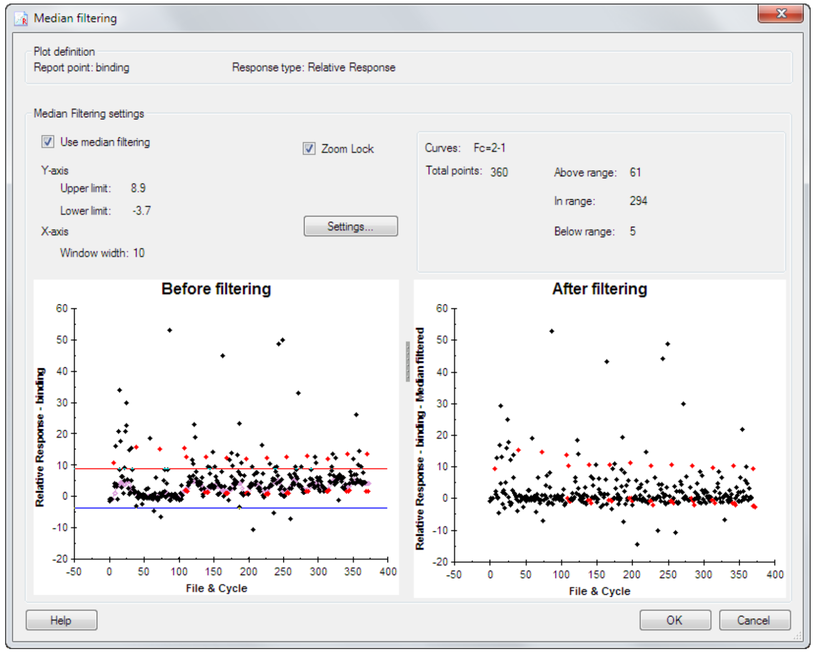
Principle of median filtering. The median response value of the points within the x- and y-windows is used as a new baseline for the current cycle.

For evaluation sessions that include multiple result files, median filter is applied to data grouped by file, regardless of whether the data is plotted against ***Cycle*** or ***File & Cycle***.

Note: *When adjacent files in a multi-file session have significantly different response levels, the order of the files will affect the details of the border effects. This can often be avoided by using adjustment for controls to normalize the response values (Section 8.3.5). To ensure that files are appended in the required order, append the files one by one to the evaluation session. You can see the order of the files in* ***File:Properties****.*

Applying median filter

Click ***Median Filter*** and choose ***Use median filtering*** to set the filter parameters.



To set the *y-axis window*, drag the horizontal lines or click ***Settings*** and enter the upper and lower response limits. The window should be wide enough to cover noise variations in negative (non-binding) samples, but narrow enough to exclude the response from binders. as far as possible.

To set the *x-axis window*, click ***Settings*** and enter the number of points in the window (minimum 5).

Judge the appropriateness of the median filter settings with reference to the positive control responses: filtering should not introduce new trends or significantly increase scatter among the positive control points.

Note: *Choosing appropriate settings can be critical to the value of median filtering. It may be necessary to experiment with the window settings to obtain the best results for a given plot. Using inappropriate settings or applying median filter to plots for which it is not suited can introduce artefacts in the evaluation.*

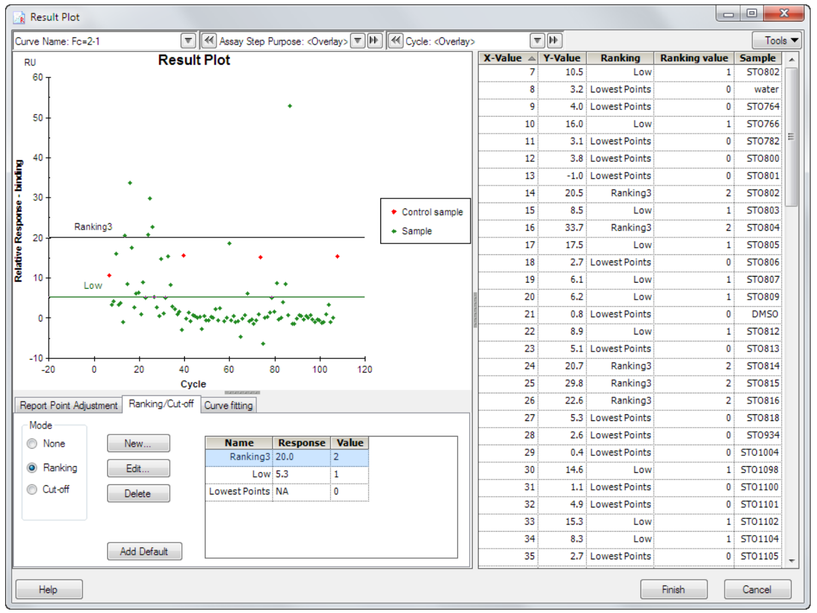
8.4 Ranking and Cut-off

Use the ***Ranking/Cut-off*** tab to add ranking or cut-off boundaries to the plot.

8.4.1 Ranking boundaries

Ranking boundaries divide the plot into a user-defined number of horizontal regions. Ranking boundaries are set automatically on the basis of average responses for control samples and may be edited freely. Boundaries cannot be set automatically if the run does not include control samples.

Choose ***Ranking*** in the ***Ranking/Cut-off*** tab.



Points are ranked in the plot table according to the name value of the nearest lower boundary. Points below the lowest boundary are ranked as ***Lowest points***.

To add a new boundary, click ***New*** and enter the settings for the boundary. Boundaries are assigned a numerical value that identifies the boundary and can be useful in data processing in third-party software.

To edit a boundary, drag the boundary line in the plot or click ***Edit*** and enter new settings. The actual boundary level is shown when the boundary is moved by dragging.

Select a boundary and click ***Delete*** to delete the boundary.

Click ***Add Default*** to add boundaries at average response levels for control samples. Existing boundaries will not be affected.

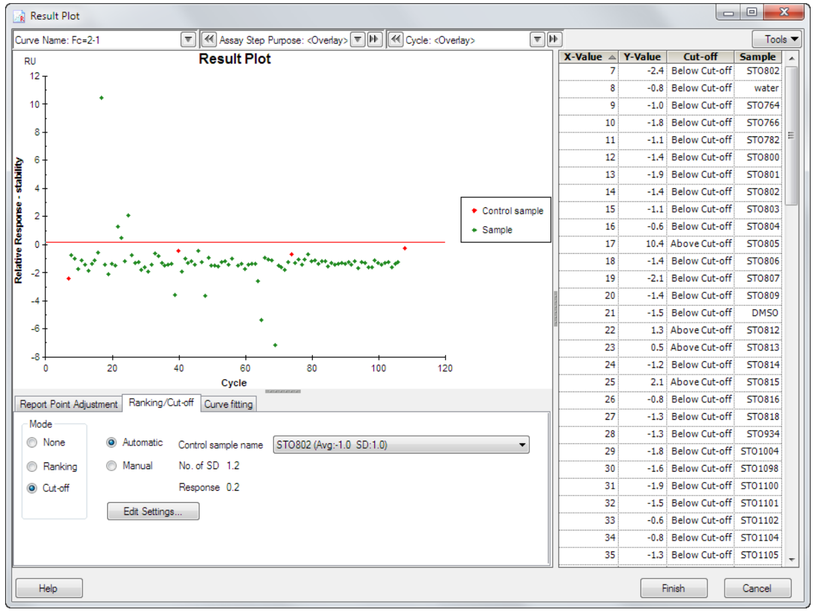
Note: *Ranking will be turned off if the plot definition is edited.*

8.4.2 Cut-off boundaries

Cut-off boundaries divide the plot into two horizontal regions, above and below a cut-off line.

Choose ***Cut-off*** in the ***Ranking/Cut-off*** tab. The cut-off boundary is set by default at the average response for the selected control sample plus 3 standard deviations (SD). If the data includes more than one control sample, the default setting is calculated from the first control in alphabetical order.

In ***Automatic*** mode, the boundary is set on the basis of the average response of a control sample plus a specified number of SD (default 3). Choose the control sample series to use for the boundary. Click ***Edit Settings*** to change the number of SD. You can also change the response level by dragging the boundary line or entering a new level in the ***Edit Settings*** dialog. The number of SD displayed is updated to reflect the changed setting relative to the selected control sample series.



X-Value	Y-Value	Cut-off	Sample
7	-2.4	Below Cut-off	STO802
8	-0.8	Below Cut-off	water
9	-1.0	Below Cut-off	STO764
10	-1.8	Below Cut-off	STO766
11	-1.1	Below Cut-off	STO782
12	-1.4	Below Cut-off	STO800
13	-1.9	Below Cut-off	STO801
14	-1.4	Below Cut-off	STO802
15	-1.1	Below Cut-off	STO803
16	-0.6	Below Cut-off	STO804
17	10.4	Above Cut-off	STO805
18	-1.4	Below Cut-off	STO806
19	-2.1	Below Cut-off	STO807
20	-1.4	Below Cut-off	STO809
21	-1.5	Below Cut-off	DMSO
22	1.3	Above Cut-off	STO812
23	0.5	Above Cut-off	STO813
24	-1.2	Below Cut-off	STO814
25	2.1	Above Cut-off	STO815
26	-0.8	Below Cut-off	STO816
27	-1.3	Below Cut-off	STO818
28	-1.3	Below Cut-off	STO934
29	-1.8	Below Cut-off	STO1004
30	-1.6	Below Cut-off	STO1098
31	-1.9	Below Cut-off	STO1100
32	-1.5	Below Cut-off	STO1101
33	-0.6	Below Cut-off	STO1102
34	-0.8	Below Cut-off	STO1104
35	-1.3	Below Cut-off	STO1105

In ***Manual*** mode, the boundary is set by dragging the boundary in the plot or entering the response level in the ***Edit Settings*** dialog.

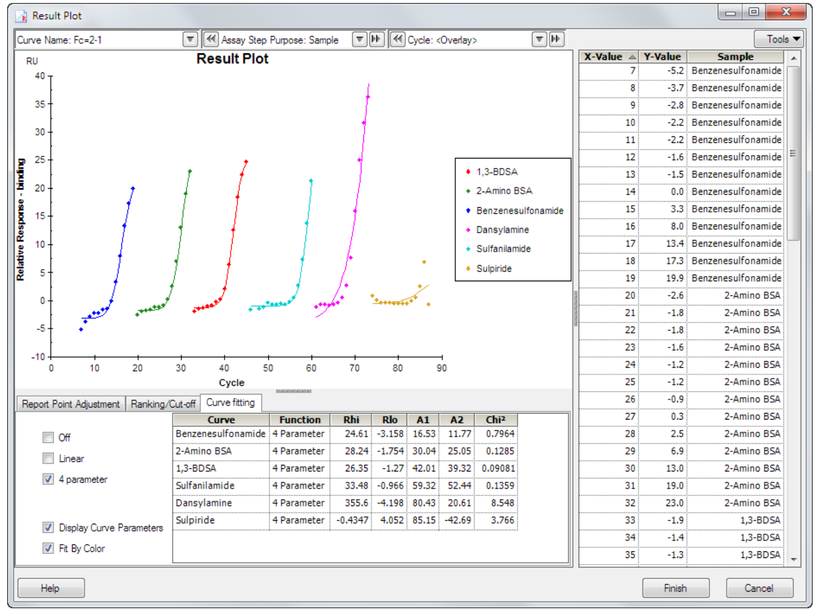
Points are classed as either ***Above Cut-off*** or ***Below Cut-off*** depending on the exact response value, at a precision higher than that displayed in the table.

Note: *Points that appear to lie precisely on the cut-off boundary may be classed as either* ***Above Cut-off*** *or* ***Below Cut-off*** *depending on the exact response value at a precision higher than that displayed in the software.*

8.5 Curve fitting

The ***Curve Fitting*** tab allows you to fit lines to the points in the plot. using either linear or curved (4-parameter) fitting functions. If ***Fit by color*** is checked, each color will be fitted to an independent line. If this option is not checked, all points derived from the same curve will be fitted to a single line. Check ***Display Curve Parameters*** to show the parameter values for the fitted curves.

Curve fitting can only be applied to plots that are sorted ***As defined***.



For linear fitting, the points are fitted to the equation

$$y = \text{slope} * x + \text{intercept}$$

The equation for a 4-parameter fit is

$$y = R_{hi} - \frac{R_{hi} - R_{lo}}{1 + \left(\frac{x}{A_1}\right)^{A_2}}$$

where ***y*** and ***x*** are the plot coordinates

R_{hi} and R_{lo} are fitting parameters that correspond to the maximum and minimum response levels respectively

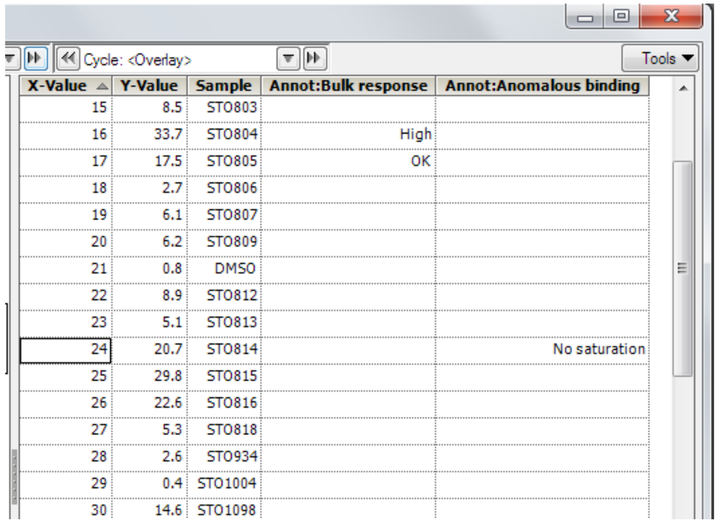
A_1 and A_2 are additional fitting parameters

The closeness of fit is reported for linear fitting as the coefficient of determination R^2 and for 4-parameter fitting as the chi-square value (see Section 12.2.1).

8.6 Annotations and comments

Samples of particular interest can be marked with annotations that are listed in the result summary table and included in printouts and exported data. Annotations are associated with one or more explanatory comments: in summary tables, each annotation is presented as a separate column with the comment as row values.

Note: *These annotations are not related to the* ***Annotations*** *option in* ***Sensorgram Comparison*** *(see Section 11.6.3).*



X-Value	Y-Value	Sample	Annot:Bulk response	Annot:Anomalous binding
15	8.5	STO803		
16	33.7	STO804	High	
17	17.5	STO805	OK	
18	2.7	STO806		
19	6.1	STO807		
20	6.2	STO809		
21	0.8	DMSO		
22	8.9	STO812		
23	5.1	STO813		
24	20.7	STO814		No saturation
25	29.8	STO815		
26	22.6	STO816		
27	5.3	STO818		
28	2.6	STO934		
29	0.4	STO1004		
30	14.6	STO1098		

8.6.1 Scope of annotations

Annotations are available in ***Result Plot***, ***Affinity Screen*** and ***Kinetic Screen*** items. The list of available annotations is common to the three item types, although usage of annotations is specific to individual items (so that for example a data point marked with an annotation in one result plot will not automatically be annotated in another result plot). Annotations are applied to data points in result plots and to data series in affinity and kinetic screen.

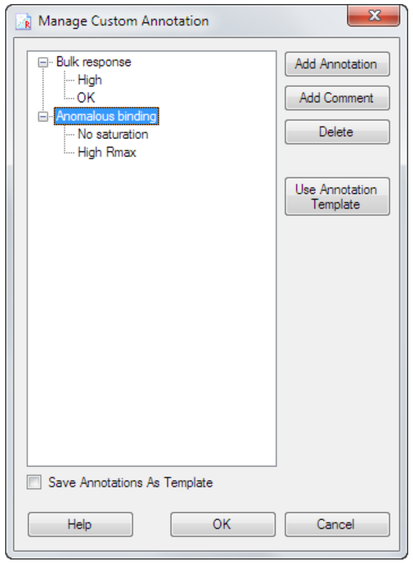
Annotations are saved either locally with the evaluation session or in a global template for use in other evaluation sessions.

- An evaluation session started by opening a result file (.blr) has access to annotations that have been saved as a template.
- A newly created item has access to all annotations in the current evaluation session.
- An item that is edited has access to the annotations existing at the time the item was finished, but not to annotations that were created later.
- A new evaluation session started by opening an evaluation file (.bme) has access to the annotations in the saved session.

8.6.2 Creating and editing annotations

Creating annotations

To create annotations, choose ***Edit Annotations*** from the ***Tools*** menu. Click ***Add Annotation*** and enter the text for the annotation. To add a comment, select the annotation then click ***Add Comment*** and enter a comment text.



Note: *An annotation must have at least one comment, since the comment text is listed in the annotations column in summary tables.*

Check ***Save Annotations As Template*** to save the annotations list in the software so that it is available for all result files opened on that specific computer. The current annotations list will replace any previously saved template. If this box is not checked, the list will only be saved with the evaluation file and will not be available for evaluation of other result files.

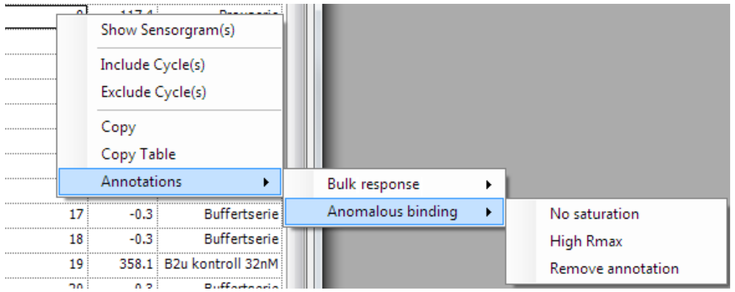
Editing annotations

To edit the text in an annotation or comment, hold the cursor over the text until it is highlighted and then type the new text. Editing annotations and comments will not affect the text in finished items where the annotation is used.

Note: *If you change the text of an annotation that has been applied to data points or data series, the column listed for the previous annotation text will be removed from the summary table. Re-display the annotation in the table using the* ***Table columns*** *option.*

8.6.3 Applying and removing annotations

To apply an annotation to a point in a result plot, right-click on the corresponding row in the table and choose ***Annotations*** from the menu. Select multiple rows to apply the same annotation to multiple points. Select the desired annotation and comment from the menu list. Select ***Remove Annotation*** to remove the annotation from the point.



Note: *Annotations are local to the result plot in which they are used and are not transferred to other evaluation items.*

Multiple annotations may be assigned to a single point, but only one comment may be applied for each annotation.

09

Calibrated concentration analysis

Calibrated concentration analysis determines the concentration in unknown samples by comparing the response with a calibration curve determined by measurement of known samples. An alternative approach (calibration-free concentration analysis (CFCA) does not require a calibration curve.

This chapter describes evaluation of calibrated measurements. Evaluation of calibration-free concentration analysis is described in Chapter 10.

General principles and experimental practice for concentration measurements are described in the Application Guide Concentration Measurement with Biacore Systems available at cytiva.com/biacoregetstarted.

9.1 Requirements for calibrated concentration measurements

In order to evaluate concentration analysis using a calibration curve, the run must include at least one calibration curve and unknown sample injections must have the appropriate properties and keywords. For wizard-based runs, the conditions are met automatically when the ***Concentration Analysis*** wizard is used. For method-based runs, the method must be constructed as described in Section 5.10.1. If necessary, the keyword table can be edited so that the conditions are met in full (see Section 6.6). Note however that the command name for the sample injection cannot be edited in the keyword table. Refer to Chapter 5 for details of how to construct methods in Method Builder.

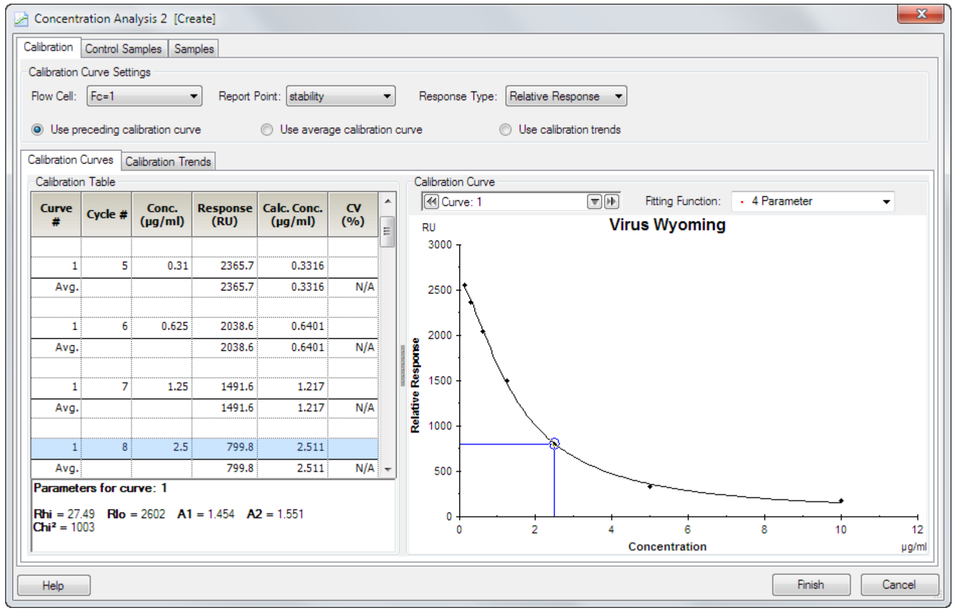
Unknown samples may be evaluated in three ways:

- Based on the nearest preceding measured calibration curve, or the nearest following curve if there is none preceding.
- Based on a single calibration curve constructed as an average of all measured curves.
- Based on individual calibration curves constructed for each separate cycle, using a calibration trend plot to interpolate calibration data between the measured points. This option is useful if the calibration responses show a steady drift during the course of the assay.

9.2 Evaluating calibrated concentration analyses

To evaluate a concentration analysis run, open the run and choose ***Concentration Analysis:Using calibration*** from the evaluation toolbar, or choose ***Add Concentration Analysis:Using calibration*** from the ***Evaluation*** menu. The evaluation dialog is divided into three tabs, for calibration curves, control samples and unknown samples respectively. Choose the appropriate settings for the calibration curves and click ***Finish*** to complete the evaluation.

9.2.1 Calibration curves



A calibration curve is constructed from the cycles in each calibration step. If two calibration steps are run in direct succession so that there are no other cycles between the steps, they will be combined in a single calibration curve.

Settings on the ***Calibration*** tab define the report point and fitting function that are used to create the calibration curve. The settings apply to all calibration curves in the evaluation. Choose the settings appropriate for your analysis:

Flow cell	Concentration analysis may be performed without a reference cell, since the unknown samples are determined by direct reference to a calibration curve obtained under the same conditions. If a reference cell is included in the flow path for the run, the reference-subtracted curve may however be selected if desired.
Report point	Response levels for concentration analysis (relative response) are normally taken from a report point shortly after the end of the sample injection, to avoid contributions from the bulk refractive index of the sample. For analyses based on the rate of binding (report point slope), a report point early in the sample injection is normally used.
Response type	Choose between relative response and slope.

Check ***Use average calibration curve*** to use one average curve constructed from all calibration points in the run.

Check ***Use calibration trends*** to create individual calibration curves for each cycle in the assay by interpolation between the actual measured calibration curves. See Section 9.2.2 for details of calibration trends.

The left-hand panel lists the details of the calibration curve data, with concentration and response or slope values for all calibration points. The ***Calc. Conc.*** column lists the concentration corresponding to the actual response value as determined from the fitted calibration curve. Select a row in the table to highlight the corresponding point on the calibration curve or *vice versa*. Fitting parameters are shown at the bottom of the left-hand panel when a single calibration curve is displayed.

The calibration curves are shown in the right-hand panel. Each calibration curve is constructed from the cycles in an assay step with the purpose ***Calibration***. Right-click on a curve or calibration point for options for excluding calibration curves or single cycles from the evaluation, and for displaying sensorgrams corresponding to individual calibration cycles. Excluded cycles are shown as open symbols and are marked in the left-hand panel with red strikethrough text. If you exclude a calibration curve and then re-include sufficient points to allow a new curve to be fitted, the curve will still be excluded but will be shown as a broken line.

You can choose the curve to display from the list at the top of the panel. The options differ according to how the calibration curves are used:

- For ***Use preceding calibration curve***, the panel shows one calibration curve by default. Individual curves or multiple curves can be selected from the list.
- For ***Use average calibration curves***, the panel shows all calibration points with a single average curve.
- For ***Use calibration trends***, the panel shows all measured calibration curves in an overlay plot.

Note: *The sample name entered for the calibration samples is used only as a title for the calibration curve. For wizard-based runs, the sample name will be the same for all calibration cycles, but different names can be introduced either in method-based runs or by editing the keyword table. If the points in a calibration curve have different sample names, all points will still be used and the title will be shown as* ***Mixed analytes****.*

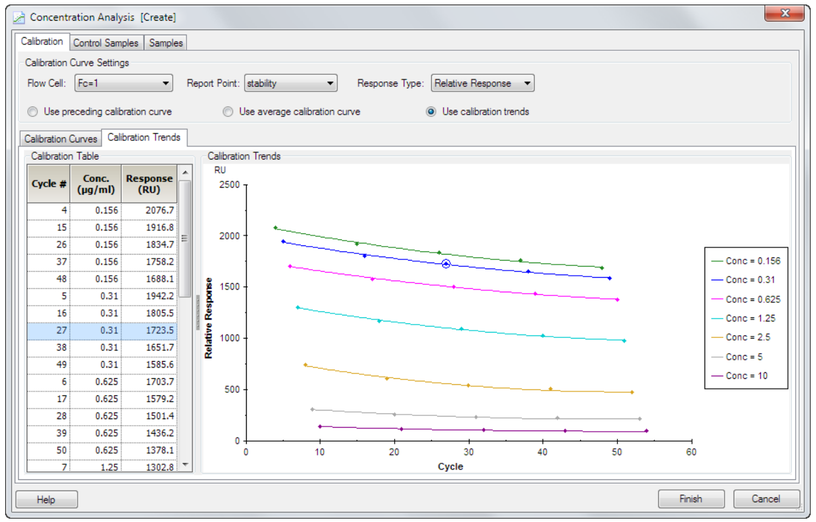
Choose the ***Fitting Function*** for the calibration curve from the option above the curve panel. Linear and 4-parameter functions and provided with the software, and custom models can be defined if required (Section 9.4). The 4-parameter function is a general fitting function for continuous curves, and is recommended for most purposes. Use a linear function only if you have good reason to expect the calibration curve to be a straight line. See Section 8.5 for the equations for fitting functions.

9.2.2 Calibration trends

Calibration trends represent the stability or otherwise of the calibration curves during the course of the assay, and can be used to compensate for drift in the calibration responses by constructing an interpolated calibration curve for each individual cycle.

Examining calibration trends

Open the ***Calibration trends*** tab to display the trends as a plot of calibration points against cycle number. Trend lines are fitted through each set of points with the same calibrant concentration, using a linear function for trends with two points and second-degree polynomial function for trends with three points or more. Actual measured points are listed in the table at the left.

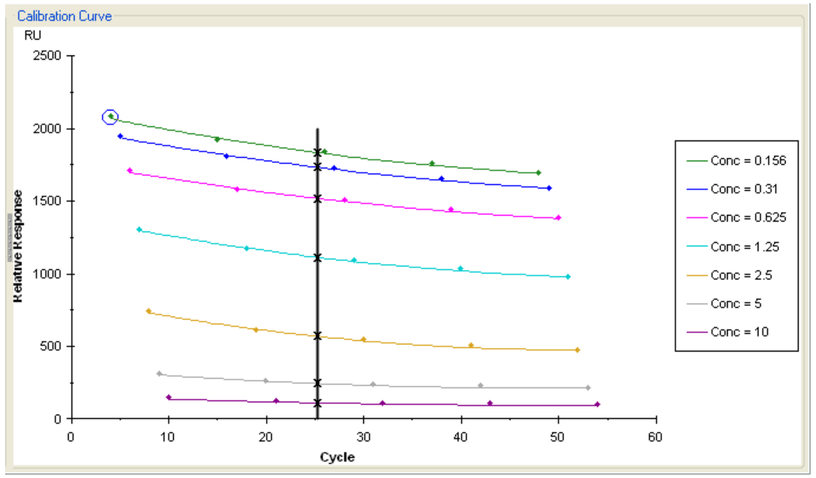


If calibration curves are run in replicate (i.e. using the same concentration in two successive calibration curves with no intervening cycles of another type), the trends are fitted to points representing the average of replicate measurements.

Right-click on points in the plot or rows in the table for options relating to the specific calibration sample.

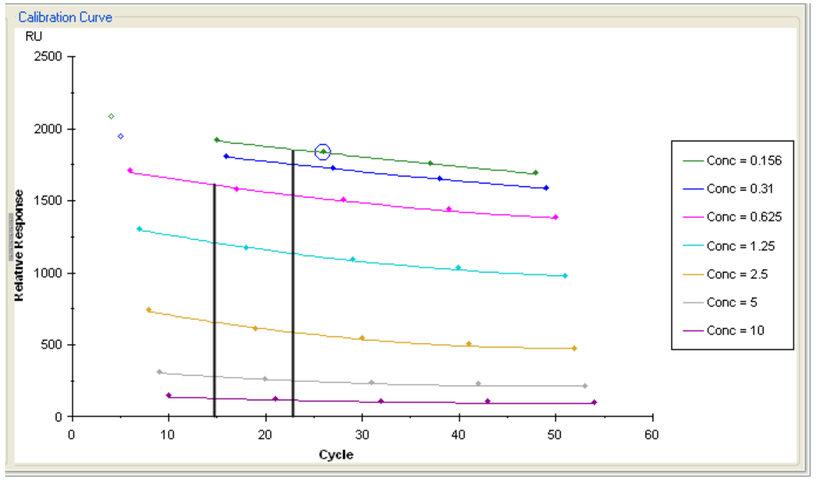
Using calibration trends

Check ***Use calibration trends*** on the ***Calibration*** tab to create an individual calibration curve for each cycle in the assay by using interpolated calibration points from the calibration trend plot. The illustration below shows how the calibration curve is constructed for cycle 25.



When calibration trends are used, the ***Calibration*** tab shows an overlay plot of the measured calibration curves. Note however that none of these measured curves is used directly for calibration: an individual (virtual) calibration curve is constructed for every cycle including the calibration cycles themselves.

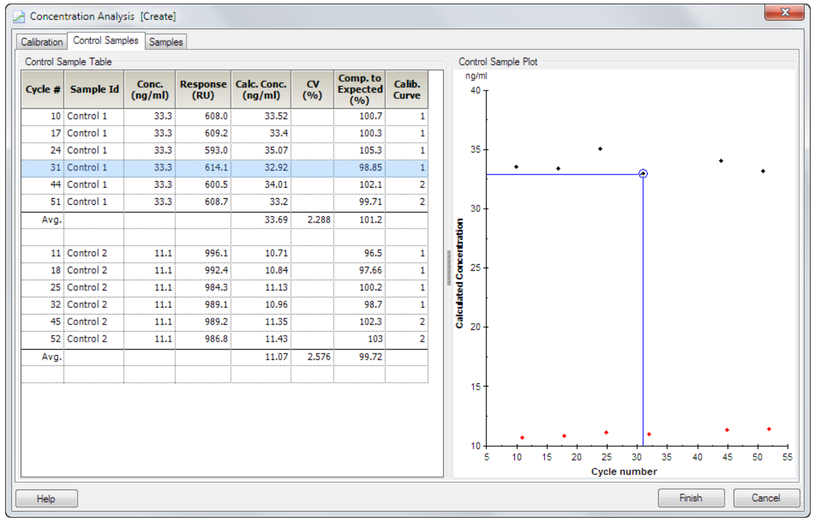
The trend lines are extrapolated to cover the cycles in the first and last calibration cycles so that every cycle has a complete virtual curve. However, if you exclude calibration points at the ends of the trend lines, the trend lines will not be extrapolated and calibration curves in the regions outside the excluded range may be incomplete (see illustration below).



Note: *When **Use calibration trends** is selected, results are recalculated whenever points are excluded from or included in the trend lines, which can take some time for large runs. For best performance, establish the trend lines before choosing **Use calibration trends**.*

9.2.3 Control samples

The ***Control Samples*** tab displays the measured concentration for control samples as a plot of response against cycle number.



Cycle #	Sample Id	Conc. (ng/ml)	Response (RU)	Calc. Conc. (ng/ml)	CV (%)	Comp. to Expected (%)	Calib. Curve
10	Control 1	33.3	608.0	33.52		100.7	1
17	Control 1	33.3	609.2	33.4		100.3	1
24	Control 1	33.3	593.0	35.07		105.3	1
31	Control 1	33.3	614.1	32.92		98.85	1
44	Control 1	33.3	600.5	34.01		102.1	2
51	Control 1	33.3	608.7	33.2		99.71	2
Avg.				33.69	2.288	101.2	
11	Control 2	11.1	996.1	10.71		96.5	1
18	Control 2	11.1	992.4	10.84		97.66	1
25	Control 2	11.1	984.3	11.13		100.2	1
32	Control 2	11.1	989.1	10.96		98.7	1
45	Control 2	11.1	989.2	11.35		102.3	2
52	Control 2	11.1	986.8	11.43		103	2
Avg.				11.07	2.576	99.72	

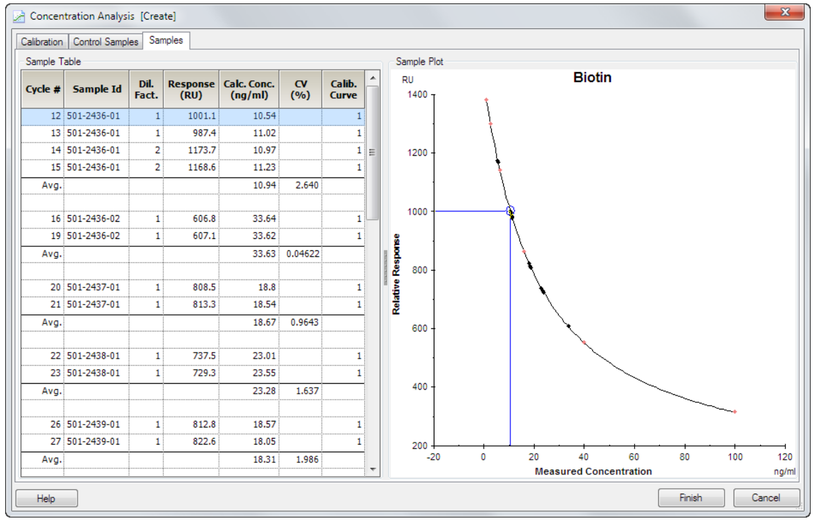
Numerical results are presented in the table at the left, and plotted as calculated concentration against cycle number on the right. Select a row in the table to highlight the corresponding point on the plot.

The table lists the expected concentration as entered for the control samples, the response and calculated concentration, the calculated concentration as a percentage of expected and the calibration curve used to calculate the concentration. Replicate control samples are summarized with average values and coefficient of variation (CV%) for the response and calculated concentration.

Right-click on a sample row in the table or a sample point in the plot and choose ***Exclude Cycle*** to exclude that sample from the sample evaluation. Excluded cycles are shown as open symbols and are marked in the table with red strikethrough text.

9.2.4 Samples

The ***Samples*** tab displays the measured concentrations for samples.



The left-hand panel lists the results sorted by sample ID, with averages and CV values for each sample ID. The column ***Calc.Conc.*** gives the concentration calculated for the original sample, obtained as the measured concentration multiplied by the dilution factor. Concentrations for samples that give a response outside the range of the calibration curve are listed as below or above the limits of the calibration.

Note: *The limits of calibration are defined by the concentrations corresponding to the response values on the fitted curve for the highest and lowest calibration samples. Depending on how well the curve fits the experimental points, these limits may not coincide exactly with the actual concentrations in the highest and lowest samples.*

The right-hand panel shows the calibration curve for the currently selected sample. All sample points calculated from the curve are shown in black: calibration points are shown in orange. Select a row in the table to highlight the corresponding point on the plot and *vice versa*.

Note: *If **Use calibration trends** is selected in the Calibration tab, each sample is calculated from its own individual calibration curve, shown in the right- hand panel with a single sample point.*

Right-click on a sample row in the table or a sample point on the curve and choose ***Exclude Cycle*** to exclude that sample from the average sample calculation. Excluded cycles are shown as open symbols and are marked in the table with red strikethrough text.

9.3 Evaluating combined result sets

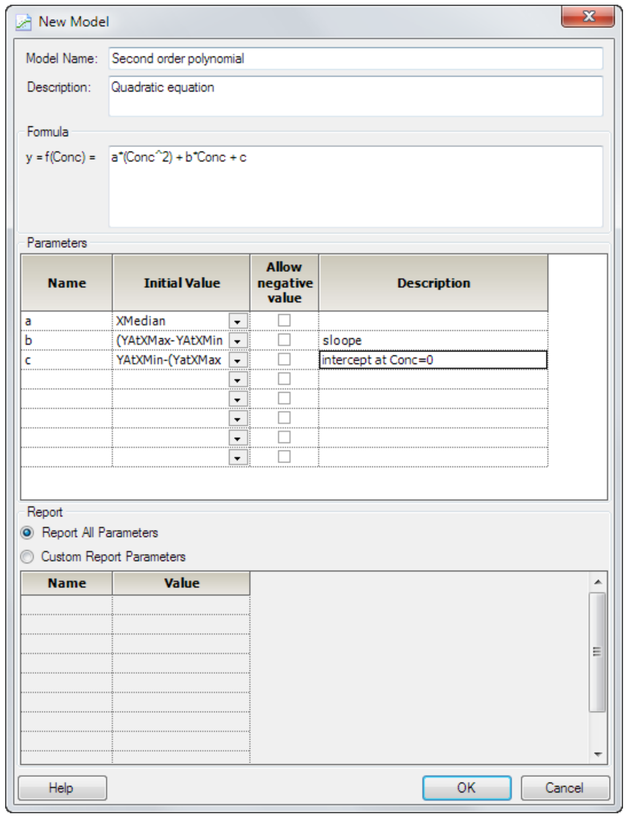
If you use the ***Append File*** function to combine result sets from separate runs, concentration analysis can be evaluated provided that the conditions specified in Section 5.10.1 are fulfilled in the combined set. The software does not check the validity of any evaluation applied to a combined result set, so that it is your responsibility to determine that the evaluation results are meaningful. It is for example in principle possible to append a kinetic analysis result file to a concentration analysis, and then apply concentration analysis evaluation. Provided that the report point used for the calibration curve also exists in the cycles from the kinetic run, calculated concentrations will be reported for these samples.

In order to ensure that concentration analysis is correctly evaluated in combined result sets, make sure that all files that contribute to the combined result set are derived from concentration analysis runs. Provided that each file starts with a calibration curve that is not excluded from the evaluation, the results will be calculated within the respective files even in the combined result set. However, if calibration curves at the beginning of files are missing or excluded, there will be overlap between the individual file sets and some samples from one file will be evaluated on the basis of calibration curves from another file. In such cases, it is important to ensure that the calibration curves and sample analyses in the different files refer to the same analyte and are performed under as far as possible identical condition.

Note: *Beware of using calibration trends with appended result files. If the appended files use the same calibration concentrations, trends will be fitted over the whole data set and will be valid only if the response levels from the different files form a continuous function. The trends will differ according to the order in which the result files are appended.*

9.4 Custom models for calibration curves

You can define your own fitting models for calibration curves in concentration analysis, for example to support validated assay procedures that do not use linear or 4-parameter calibration fitting. Choose ***Tools:Models:Concentration*** to define or edit fitting models. Models for calibration curves are defined using similar principles as equation models for kinetics and affinity (see Section 12.8.3).



10

Calibration-free concentration analysis

Calibration-free concentration analysis (CFCA) determines the analyte concentration in unknown samples from the diffusion properties of the analyte, measured as the rate of binding to the sensor surface under conditions of mass transport limitation, without reference to a calibration curve.

This chapter describes evaluation of calibration-free measurements. Evaluation of calibrated concentration assays is described in Chapter 9.

Note: *CFCA items created and saved in earlier versions of the Biacore T200 Evaluation Software can be opened and printed in the current version, but cannot be edited.*

10.1 Background

10.1.1 Principles

CFCA calculates the analyte concentration from the measured mass transport properties and values for the diffusion coefficient and molecular weight, provided as evaluation variables when the assay is run (Section B.2). The evaluation is based on fitting the sensorgram data to a model of 1:1 interaction kinetics, with mass transport parameters calculated from the diffusion coefficient and flow cell dimensions, and with the undiluted analyte concentration set as a globally fitted variable.

More detailed description of the principles of CFCA may be found in the *Application guide concentration measurement with Biacore systems*.

10.1.2 Quality control

Concentration measurement using CFCA requires that analyte binding is sufficiently limited by mass transport so that the diffusion properties of the analyte can be reliably determined from the sensorgrams. This is indicated by the separation between the sensorgrams at the lowest and highest flow rates (recommended 5 and 100 µL/min respectively). Binding rates that are limited entirely by kinetic properties of the interaction are independent of flow rate, while binding rates that are limited entirely by diffusion are proportional to the cube root of the flow rate. Many sensorgrams exhibit intermediate binding rate limitation, with contributions from both kinetics and diffusion. If the sensorgrams at different flow rates are close together, diffusion limitation may not be sufficient for reliable concentration measurement. The parameter ***QC ratio*** provides a guidance value for assessing whether there is sufficient mass transport limitation.

The QC ratio is calculated as follows from the quotient Q which reflects the degree of mass transport limitation:

$$\text{quotient Q} = \frac{\text{initial rate at high flow rate}}{\text{initial rate at low flow rate}} \times \sqrt[3]{\frac{\text{low flow rate}}{\text{high flow rate}}}$$

Under conditions of complete mass transport limitation, the binding rate is proportional to the cube root of the flow rate, so the quotient Q has a value of 1. When binding is on the other hand limited by kinetics, the binding rate is independent of the flow rate so Q has a value equal to the cube root of the flow rate ratio. The range of possible theoretical values for Q will thus depend on the flow rates used: for the recommended flow rates of 5 and 100 µL/min, the value is 0.37. The QC ratio is calculated from the measured value for Q relative to the possible range of values:

$$Q_{max} = 1$$

$$Q_{min} = \sqrt[3]{\frac{\text{low flow rate}}{\text{high flow rate}}}$$

$$\text{QC ratio} = \frac{Q_{measured} - Q_{min}}{Q_{max} - Q_{min}}$$

Ideal mass transport-limited binding has a QC ratio of 1. When binding rates are completely limited by interaction kinetics, the QC ratio approaches zero. Negative values for the QC ratio can occasionally be obtained as a result of experimental variation or disturbances in the sensorgram data. Sufficient mass transport limitation is indicated by a value for the QC ratio of about 0.2 or higher.

The QC ratio is presented as a preliminary value based on estimated initial binding rates to aid in selecting suitable data for evaluation, and as a final value after fitting based on the fitted binding rates to aid in assessing the calculated concentrations.

10.2 Requirements for CFCA evaluation

10.2.1 Assay requirements

CFCA requires a minimum of two cycles for each sample, run at different flow rates. Blank cycles for each flow rate are recommended. In the assay method, both samples and blanks must use a ***Sample*** command of type ***High performance*** in an assay step with ***Assay Step Purpose*** either ***Sample*** or ***Control Sample*** (see Chapter 5). No calibration standards are required.

10.2.2 Sample requirements

The useful range of the assay is limited by the requirement for mass transport limited binding. For typical protein analytes, this translates in practice to a measured concentration range of about 0.5 to 50 nM. If the original concentration is higher than this range, the samples should be diluted. In most cases, a series of 10-fold dilutions of the sample will cover sufficient range for determination of the concentration in unknown samples, provided that the original concentration is not below the useful range of the assay.

10.2.3 Sample grouping

For samples, cycles with the same analyte, ligand, flow cell and analysis temperature within the same file are evaluated together, regardless of sample dilution and run order. The fitting procedure takes account of the dilution factor so that one single concentration value for the undiluted sample is obtained. Individual dilutions can be excluded from the evaluated curve set if desired.

Control samples are handled differently. Each set of consecutive control sample cycles is evaluated separately, to provide a check on the consistency of measurement throughout the run. A simple case is illustrated below.

Run order | Evaluation

Cycle	Assay step purpose	Sample	Blank	Dilution	Evaluation
1	Startup	buffer			
2	Startup	buffer			
3	Control sample	C		250	Control sample
4	Control sample	C		250	
5	Control sample	C		250	
6	Sample	buffer	y		Blanks
7	Sample	buffer	y		
8	Sample	buffer	y		
9	Sample	A	n	1000	Sample A
10	Sample	A	n	1000	
11	Sample	A	n	1000	
12	Sample	A	n	300	
13	Sample	A	n	300	
14	Sample	A	n	300	
15	Sample	B	n	1200	Sample B
16	Sample	B	n	1200	
17	Sample	B	n	1200	
18	Sample	B	n	400	
19	Sample	B	n	400	
20	Sample	B	n	400	
21	Sample	buffer	y		Blanks
22	Sample	buffer	y		
23	Sample	buffer	y		
24	Control sample	C	n	250	Control sample
25	Control sample	C	n	250	
26	Control sample	C	n	250	
27	Sample	A	n	1000	Sample A
28	Sample	A	n	1000	
29	Sample	A	n	1000	
30	Sample	B	n	3600	Sample B
31	Sample	B	n	3600	
32	Sample	B	n	3600	
33	Sample	B	n	1200	
34	Sample	B	n	1200	
35	Sample	B	n	1200	
36	Control sample	C	n	250	Control sample
37	Control sample	C	n	250	
38	Control sample	C	n	250	

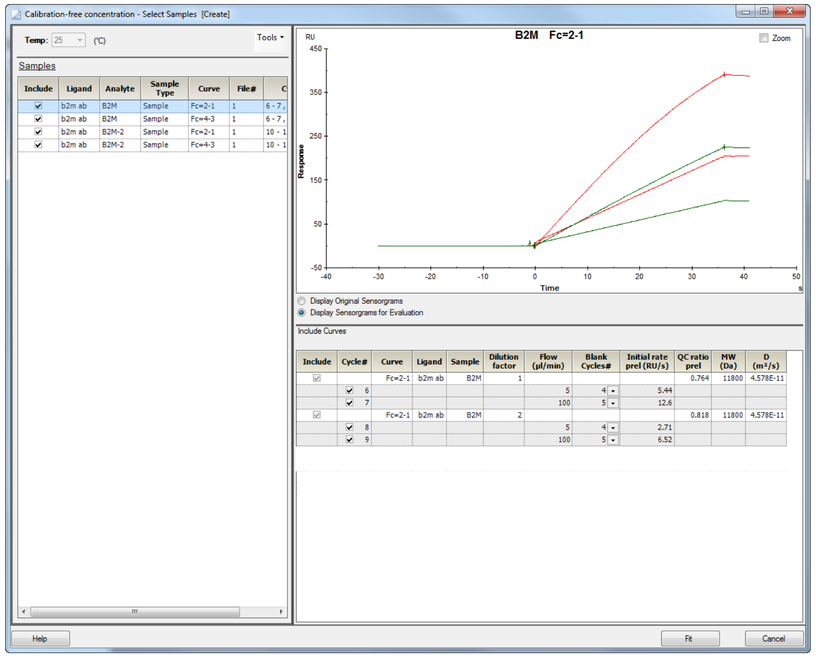
Sample properties can be edited if necessary in the keyword table (see Section 6.6).

Values for the diffusion coefficient D at 20°C and the molecular weight MW must be provided. These values are normally provided when the run is set up in the Control Software, but can be added or edited in the keyword table in the Evaluation Software. A tool for calculating diffusion coefficients from molecular size and shape is accessible through the ***Tools:Diffusion Coefficient Calculator*** option.

10.3 CFCA evaluation workflow

To evaluate CFCA, choose ***Concentration Analysis:Calibration-free*** from the evaluation toolbar, or choose ***Add Concentration Analysis:Calibration-free*** from the ***Evaluation*** menu.

10.3.1 Sample presentation and selection



If the result file includes runs at different temperatures, choose the desired temperature at the top of the left-hand panel. Only one temperature can be evaluated in one CFCA evaluation item.

Sample table

The left-hand panel lists the samples available for evaluation. Sample type (***Sample*** or ***Control Sample***) is determined by the assay step purpose in the method. For type ***Sample***, one row in the table represents all cycles that share the same values for file number, flow cell, ligand name and analyte name, regardless of the dilution factor or run order. For samples of type ***Control Sample***, one row represents all *consecutive* cycles that share these parameter values (see illustration in Section 10.2.3). In this way, repeated measurements of control samples are evaluated separately to provide a check on the stability of the assay throughout a run, while replicates and different dilutions of samples are evaluated to provide a single concentration value within the run.

Choose the run temperature (***Temp***) and ***Curve type*** at the top of the left-hand panel. These options are not shown if the run only includes one value for the respective option.

Use the check-box in the ***Include*** column to include or exclude samples from the evaluation. To exclude or include multiple samples, select the samples and use the appropriate option from the right-click menu.

The sample table can be sorted by clicking on the column header.

Sample details

The right-hand panel shows the details of the sample row currently selected in the sample table. Sensorgrams are aligned to zero time and response at the start of the injection. In the sensorgram display, choose whether to show blank- subtracted sensorgrams (***Display Sensorgrams for Evaluation***) or unsubtracted sensorgrams including blanks (***Display Original Sensorgrams***). This choice does not affect the results: evaluation always uses blank-subtracted data unless otherwise specified in the cycle details (see below).

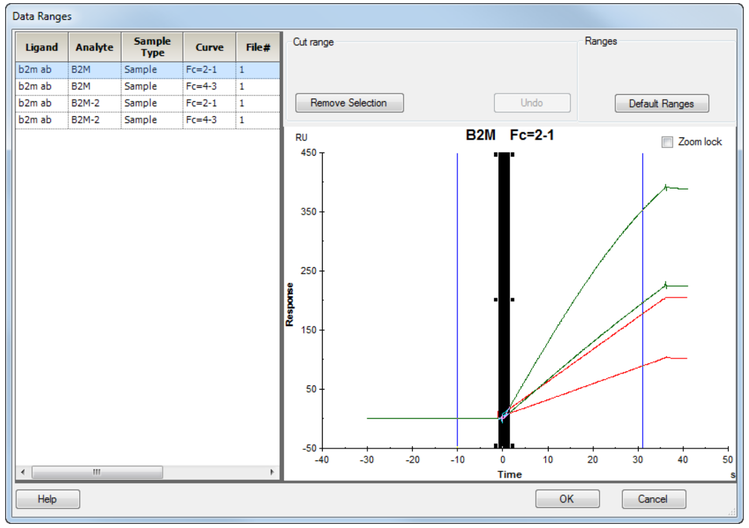
Details of the cycles for the sample are listed in the ***Include Curves*** table, separated according to dilution factor. Each dilution factor is represented by a white table row with parameters common to all cycles for that dilution: separate cycles are listed in gray rows with cycle-specific parameters. Use ***Expand all cycles*** from the right-click menu in the ***Included Curves*** table to expand or collapse the cycle details for each dilution. Columns that are not self- explanatory are described below:

Parameter	Description
Blank Cycles	By default, blank subtraction uses the nearest preceding blank cycle at the same flow rate, or the nearest following blank if there is none preceding. Other blanks from the same file, flow cell and flow rate can be selected in this column. Change the blank cycle if the default cycle is disturbed or otherwise unsuitable. Blank cycles may be either ***Sample*** or ***Control Sample***.
Initial rate prel	The preliminary initial binding rate is estimated for each cycle from the sensorgram data from 7.5 to 12.5 s after injection start. The initial binding rate should be clearly dependent on the flow rate.
QC ratio prel	The preliminary QC ratio is estimated for each dilution from the sensorgram data as described in Section 10.1.2.
D	The value for the diffusion coefficient D is adjusted to the analysis temperature, and may differ from the value at 20°C entered when the assay method was set up.

Remove the check-mark from the ***Include*** column to exclude a dilution from the evaluation, or from the ***Cycle#*** column to exclude individual cycles. Remember that each sample must be represented by at least two cycles with different flow rates.

10.3.2 Selecting data ranges

Choose ***Tools:Data Ranges*** if you want to adjust the range of data that will be used in the evaluation and/or remove any disturbances from the data.



Select samples to which the changes should be applied in the table in the left- hand panel of the dialog box. You can select multiple samples if you want to apply the same changes to several samples. The right-hand panel shows an overlay plot of the blank-subtracted sensorgrams for the selected samples.

To adjust the fitting range, drag the vertical blue lines that mark the limits of the range. The default range is from 10 seconds before the start of the injection to 5 seconds before the end. (Baseline data before the start of the injection is included to help to ensure robust evaluation of the data.) Click ***Default Ranges*** to restore the original range settings.

Note: *If you adjust the fitting range differently for different sample series, the range indicators will not be shown when you display* ***All sample series****. Different range settings within the same evaluation item cannot be saved in evaluation methods (Section 6.8).*

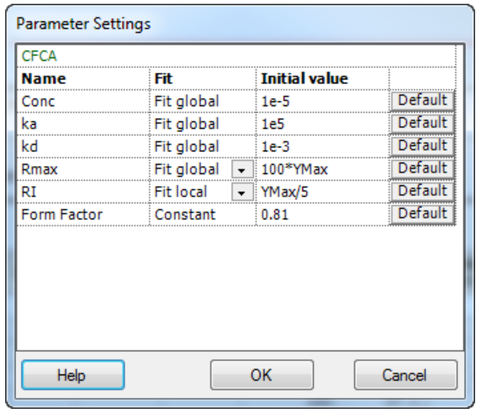
To remove disturbed data, drag with the right mouse button to select the data, then click ***Remove Selection***. Click ***Undo*** to restore deleted data.

10.3.3 Fitting parameters

Evaluation of CFCA uses the same curve fitting principles as kinetic evaluation (see Section 12.2). Initial values and status of the fitting parameters can be modified to a limited extent if required.

Note: *The fitting model is based on a 1:1 interaction. The model cannot be edited.*

Choose ***Tools:Parameter Settings*** to access the starting values for the fitting parameters. Do not change the parameter settings unless you have a good reason to do so. The default settings are appropriate for most CFCA evaluations.

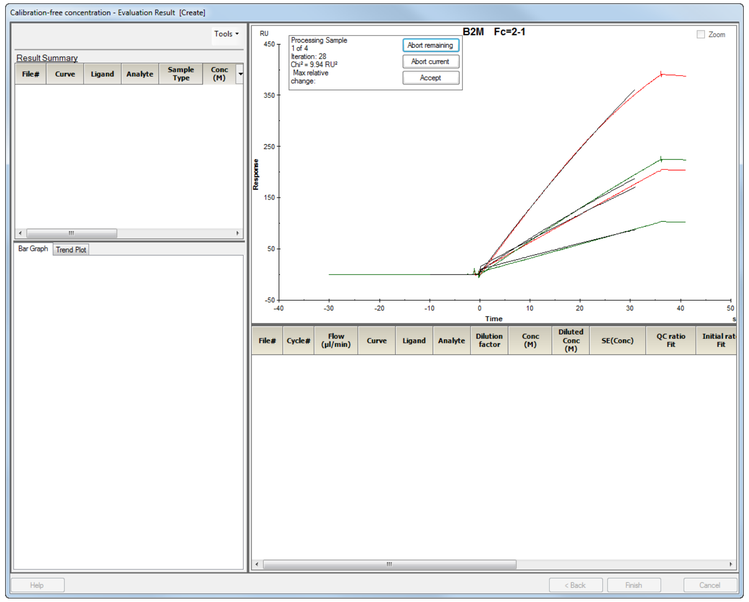


Parameter	Description	Status	Comment
Conc[1]	Undiluted analyte concentradtion.	Fitted globally.	Try changing the intial value by several orders ofmagnitude if the curve fitting fails.
ka	Association rate constant.	Fitted globally.	
kd	Dissociation rate constant.	Fitted globally.	
Rmax	Maximum analyte binding capacity of the surface.	May be fitted locally orglobally or set to a constant.	Change to local fitting if you capture ligand on the sensor surface.
RI	Bulk refractive index contribution.	May be fitted locally orset to a constant.	Set to constant = 0 if the fitting generates spuriousbulk refractive index contributions.
Form factor	Empirical factor related tothe flow cell dimensions and the sensor surface properties.	Constant	The default value is 0.81. Do not change this factor unless advised to do so by a Cytiva representative.

[1] *The parameter* ***Conc*** *refers to the stock sample concentration before dilution for analysis. If you are using dilution factors of about* 10^6 *or more in order to obtain usable data, you may need to increase the starting value of the* ***Conc*** *parameter. A general rule of thumb is to use the highest dilution factor multiplied by* 10^{-9} *as the initial value for* ***Conc****.*

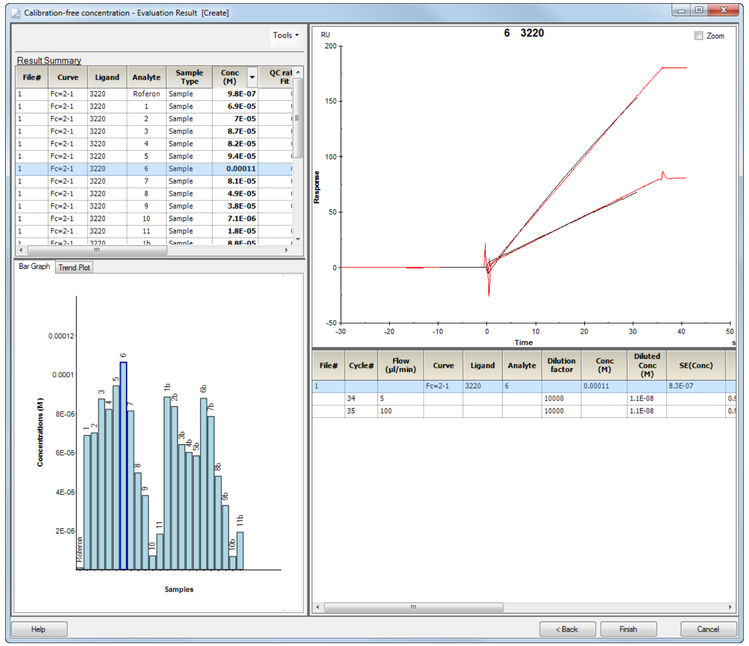
10.4 Result presentation

When you are satisfied with the data selection, click ***Fit*** to start the evaluation. Results appear in the table as each sample series is completed. The evaluation progress is shown in the sensorgram panel.



The fitting procedure is normally fast. If fitting appears to hang for a particular sample series, you can click ***Accept*** to accept the current fitting results for that series or ***Abort current*** to abandon the series. Click ***Abort remaining*** if you want to abandon all remaining series in the evaluation. The fit status is recorded in the table of results.

The finished results are presented with summary information in the left-hand panel and details in the right. Select a sample in the summary to show the details for that sample.



The results summary shows the evaluation results both as a table and as a bar chart and trend plot.

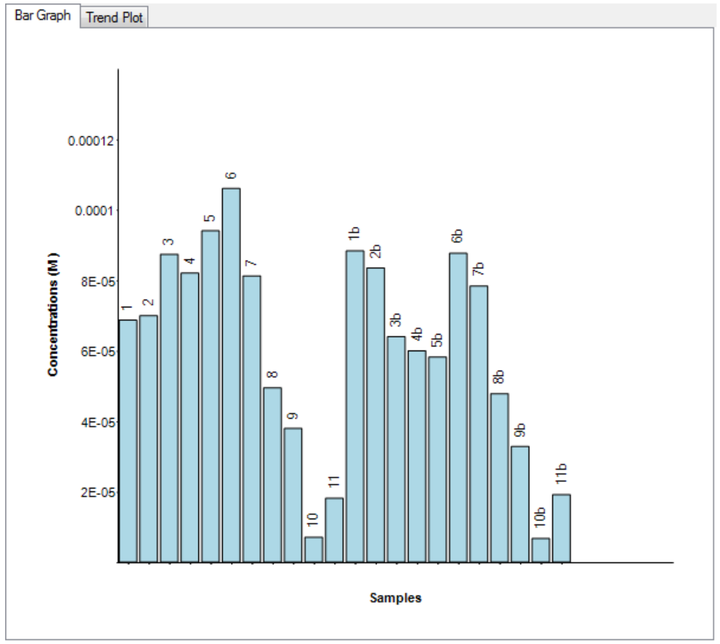
- the sample currently selected in the table is highlighted in the chart and trend plot: correspondingly, clicking on a sample in the chart or plot selects the corresponding row in the table,
- sorting the table (by clicking on a column header) also sorts the bar chart but not the trend plot.

Details of the currently selected sample are shown on the right, with QC ratio and cycle information listed separately for each sample dilution.

The value reported for ***Conc*** represents the concentration in the original sample before dilution, obtained by multiplying the fitted value by the dilution factor. You can change the units for the reported concentration in the header for the ***Conc*** column.

Note: *The Initial rate and QC ratio in the finished evaluation are calculated from the fitted curves and may differ from the preliminary values presented in the previous evaluation step.*

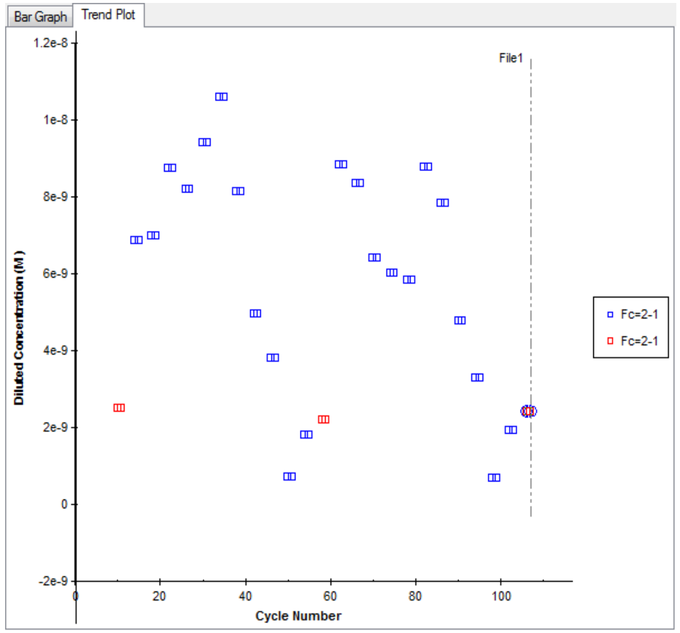
Bar chart



The bar chart shows the undiluted concentration in each sample, plotted against sample and sorted in the same way as the summary table. Control samples are not shown in the bar chart.

Trend plot

The ***Trend plot*** shows a plot of concentration in the diluted samples against cycle number. Samples are shown in blue and control samples in red. Multiple concentration values for the same cycle (e.g., when different ligands are immobilized in different flow cells) are distinguished by different symbols. Results from multiple files in the same session are separated by vertical lines.



Use this plot to examine the consistency of control sample measurements. If the concentration in control samples varies significantly during the course of the run, review the assay conditions and investigate the cause of the instability. You cannot use trends in the control sample results to correct for drift in the assay.

Note: *The sample measurements shown in the trend plot illustrate the range of concentrations in the diluted samples. These values are obtained by dividing the globally fitted original sample concentration by the dilution factor, so that all values for a given dilution are identical. Do not judge the stability of the assay from these concentration values.*

10.5 Assessing the results

10.5.1 General

Consider the following aspects of CFCA when interpreting the results:

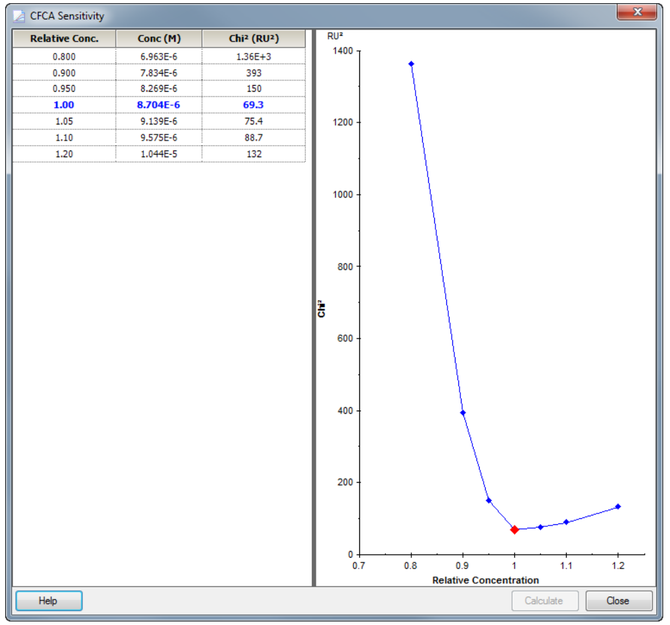
- Check the visual appearance of the sensorgrams and fitted curves. Reject samples where the fit is poor at one or both of the flow rates. Use the ***Chi2*** (chi-square) value as a guideline for fitting quality. As a rough guideline, acceptable chi-square values should be less than about 5% of the response reached at the end of the sample injection at the lowest flow rate (disregarding the difference in units between chi-square and response).
- If the fit is generally poor and sensorgrams at the highest concentrations (lowest dilutions) show significant curvature, try excluding these dilutions from the evaluation.
- Check the ***QC ratio***. Treat the results with caution if the value is lower than about 0.2.
- Examine the significance of the calculated concentration using the
- ***Sensitivity check*** tool (see below).
- Check the value for ***SE (Conc)*** or ***T (Conc)***. This value represents the statistical significance of the calculated concentration, and is shown as standard error (SE) or T-value (see Section 12.4.2) according to the setting in ***Tools:Preferences***. Reject samples where the standard error is more than about 20% of the calculated concentration (or correspondingly the T-value is lower than about 5).

10.5.2 Sensitivity check

The ***Sensitivity Check*** tool visualizes the reliability of the concentration measurements by examining the dependence of the closeness of fit on the concentration value. This is done by re-fitting the experimental data with fixed concentration values set to +/- 5%, 10% and 20% from the original fitted value. The results are presented as a plot of the statistical fitting parameter chi2 against relative concentration.

Click the ***Sensitivity Check*** button to apply this check procedure to the currently selected sample. Running the sensitivity check is recommended whenever the fitting is not ideal.

Note: *Calculating the sensitivity check results may take a significant time.*



The steepness of the curve on either side of the minimum indicates the sensitivity of the fitting to variations in concentration. If the curve is steep, the concentration is well-defined and the measurement is robust. On the other hand, a shallow curve on one or both sides of the minimum indicates that the concentration can be varied without greatly affecting the quality of fit, so that the reported concentration value is less securely determined.

If the sensitivity check curve is steep on one side of the measured concentration and shallow in the other, this means that the concentration is reliably determined as greater or less than the reported value. In the example illustrated above, the curve is steep at lower concentrations, so the sample concentration is reliably determined as the reported value or higher.

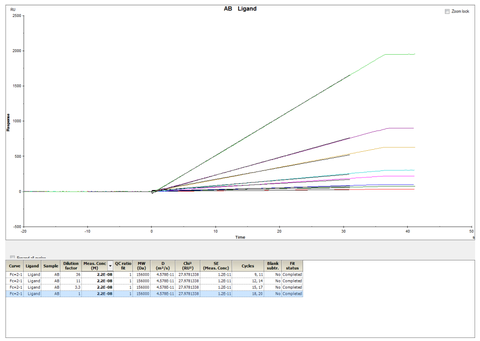
Occasionally, the sensitivity check may generate a curve where one or more simulated values give a lower chi2 than the reported value. This indicates that the fitting procedure has stuck in a local minimum for chi2 that does not represent the true best fit. In such cases, try repeating the evaluation with different values for the initial fitting parameters (Section 10.3.3).

10.5.3 Troubleshooting evaluation results

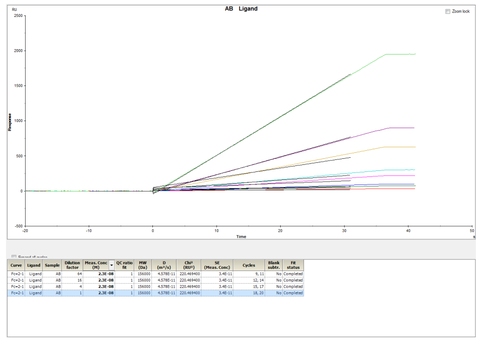
Fitting is poor at one or more dilutions

As a check that the dilution factors are entered correctly, the initial rate should be inversely proportional to the dilution factor at each flow rate. If the product of initial rate and dilution factor for a given flow rate varies significantly between dilutions, this may be an indication that the dilution factors are incorrect.

This does not necessarily mean that the reported concentration is incorrect. Using simulation of dilution errors by changing the dilution factors in the keyword table, a serial dilution error (using a serial factor of 4 when the correct value is 3) has only about 10% error in the reported concentration. The effect of the incorrect serial dilution is that fitting quality becomes progressively worse as the dilution increases, as illustrated below.



3-fold dilution

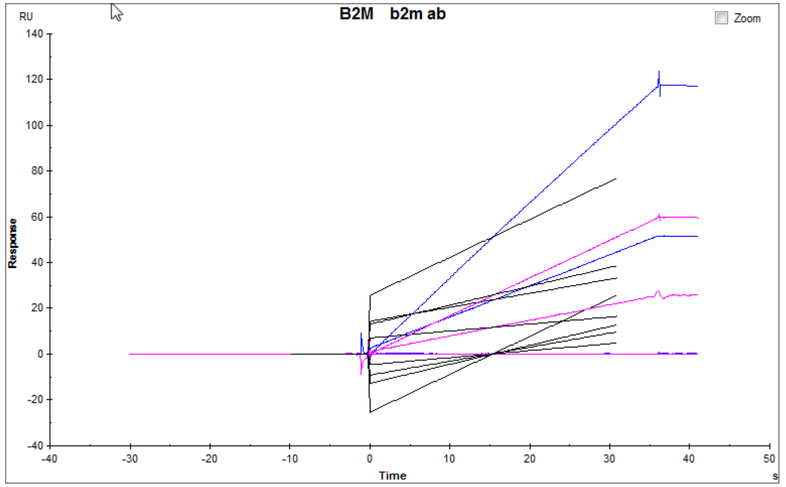


4-fold dilution

Isolated dilution errors similarly have only minor effects on the reported concentration. However, for reliable determinations it is important to use carefully designed and executed dilution protocols during sample preparation.

10.5.4 Fitting fails dramatically

If you have set up your assay so that sensorgrams are recorded from all four flow cells (***Detection*** set to ***Multi*** in the method, see Section 5.4), and have then injected samples over either Fc1-2 or Fc3-4, nonsense data will be recorded from Fc1 and Fc2 for samples injected over Fc3-4 and ***vice versa***. The nonsense data will be included in the evaluation and will result in dramatically failed fitting. An example of such a failed fit is illustrated below.



The cycles with nonsense data are readily identified in the table of included curves by initial binding rates close to zero. Exclude such cycles before completing the evaluation.

The recommended assay setup for CFCA uses ***Detection*** set to ***Dual***, recording data from either Fc1-2 or Fc3-4 but not both in the same run. The issue of nonsense data is avoided if this recommendation is followed.

10.6 Technical aspects of calibration-free assays

Calibration-free assays rely on calculating the analyte concentration from the measured diffusion rate, using a known value for the diffusion coefficient of the analyte. In Biacore systems where this measurement approach is explicitly supported, this involves fitting observed binding data to a mass transport- limited 1:1 interaction model with a known value for the mass transport coefficient and an unknown variable for the analyte concentration.

The overall interaction process can be represented by the scheme

$$A_{bulk} \underset{k_m}{\overset{k_m}{\rightleftharpoons}} A_{surface} + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

where k_m is the mass transport coefficient and k_a and k_d are the interaction rate constants. The rate of mass transport from bulk solution to the surface is given by

$$\frac{d[A_{surface}]}{dt} = k_m[A_{bulk}]$$

In kinetic analyses, this relationship is used to determine k_m from the observed binding behavior at known values of $[A_{bulk}]$. For calibration-free concentration measurements, a value is provided for k_m and the analysis calculates A_{bulk}.

As a general recommendation, measurements should be made at two or more widely separated flow rates (for example 5 and 100 µL/min). The data is then fitted to a model with a global variable for analyte concentration (so that the model is constrained to find a single concentration value that best fits both curves simultaneously).

10.6.1 Mass transport in laminar flow systems

In the laminar flow conditions that apply in Biacore, the mass transport coefficient k_m is related to the analyte diffusion coefficient D by the expression

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

where D is the diffusion coefficient of the analyte in m^2/s

f is the volumetric flow rate of liquid through the flow cell in m^3/s

h, w, l are the flow cell dimensions (height, width, length in m), assuming a rectangular flow cell

The mass transport coefficient k_m has units of m/s. Adjusting for the molecular weight of the analyte and the conversion from measured RU to concentration units gives the Biacore-specific *mass transfer constant* k_t:

$$k_t = k_m \cdot MW \cdot 10^9$$

Note: *The conversion constant 10^9 is approximate and is only valid for protein analytes on Sensor Chip CM5.*

10.6.2 Diffusion coefficients for protein analytes

Values for the diffusion coefficient of many proteins may be found in the literature. The diffusion coefficient is determined by the size and shape of the molecule, so that values for a physically similar molecule may be used if the specific analyte is not listed (for example, the diffusion coefficient for all antibodies of IgG class will be practically identical since the molecules are essentially constant in size and shape).

Diffusion coefficients are directly proportional to the absolute temperature and inversely proportional to the relative viscosity (η) of the solution, so that if values can be found for one set of conditions, corresponding values can easily be calculated for the experimental conditions:

$$D = D_{ref} \times \frac{T}{T_{ref}} \times \frac{\eta_{ref}}{\eta}$$

where D is the diffusion coefficient of the analyte

T is the absolute temperature in Kelvin (20°C = 293.15K)

η is the viscosity of the solvent

subscript $_{ref}$ indicates reference conditions

Relative viscosity values should always be corrected for temperature if the experimental temperature differs from the reference value. The viscosity of common physiological buffer solutions (containing 0.15 M salt and no major additives such as glycerol) may however be considered equal to that of water, and correction for buffer composition is seldom necessary.

10.6.3 Estimating diffusion coefficients from molecular properties

If there is no value available in the literature for the diffusion coefficient of the analyte being studied, a value may be estimated from the molecular weight and shape factor, or *frictional ratio* according to the equation below (this is a semi-empirical relationship based on Stokes low and the Einstein-Sutherland equation for molecular diffusion). The frictional ratio describes the extent of deviation of the molecule from a sphere. A perfect sphere has a frictional ratio of 1.0. Globular proteins such as antibodies typically have values around 1.2. Moderately elongated proteins such as fibronectin and plasminogen typically have values in the range 1.6 to 1.9. For rigid elongated molecules like fibrinogen and tropomyosin, values are usually in the range 2 to 3.

$$D = 342.3 \times \frac{1}{M^{1/3} \times f \times \eta_{rel}} \times 10^{-11}$$

where D is the diffusion coefficient in m^2/s

M is the molecular weight in daltons

f is the frictional ratio

η_{rel} is viscosity of the solvent relative to water at 20°C (η_{rel} for water or buffer at 25° = 0.89)

Values for globular proteins with molecular weight around 100,000 daltons are typically of the order of 6×10^{-11} m^2/s at 25°C.

10.6.4 Reliability of calibration-free concentration measurements

From the discussion above, it is evident that errors in the diffusion coefficient provided for evaluation of calibration-free concentration measurements will be transferred to corresponding errors in the measured concentration. Underestimation of the diffusion coefficient results in overestimation of the concentration by the error factor raised to the power of 2/3 (this follows from the relationship between k_m and D, Section 10.6.1). Thus for example underestimation of the diffusion coefficient by a factor of 2 will result in concentration values that are too high by a factor of about 1.6.

The reliability of measured diffusion coefficients reported in the literature must be assessed from case to case, on the basis of the validity of the experimental measurements.

Estimates of the diffusion coefficient from the molecular properties may be incorrect by a significant factor if the molecule is not globular and the frictional factor is unknown. Elongated and inflexible protein molecules may have frictional ratios as high as 10 or 20, so that some knowledge of the molecular shape is important for correct estimation.

Other factors such as viscosity and temperature have a relatively small effect on the diffusion coefficient. In general, errors in the value provided for the diffusion coefficient will often be less significant than other sources of experimental error such as less than perfect fitting of the binding data to the model or binding of mixed components to the sensor surface.

11

Sensorgram comparison

11.1 Principles of sensorgram comparison

General principles

Sensorgram comparison performs statistical comparison of sensorgrams in terms of sensorgram shape, to determine the degree of similarity between samples and a standard. The standard is derived from at least two (preferably more) replicate runs with the same material, and provides a window of variation for the sensorgrams. Samples may be grouped into *curve sets* (see Section 11.3) that represent repeated measurements of the same interaction. Comparison is performed on the sensorgram data without reference to the kinetics or mechanism of the interaction, and gives numerical values for the extent of similarity. It does not provide any further characterization of observed differences.

Comparison is best performed on analyses run under kinetic conditions, so that the sensorgrams reflect the binding behavior as comprehensively as possible.

The results of sensorgram comparison are shown in graphical and tabular form in terms of the similarity between samples and standards. Results are reported for the individual sensorgrams in a curve set as well as for the set as a whole.

Applications

Sensorgram comparison is generally used to reveal differences in binding behavior without performing detailed kinetic analysis. Some examples are:

- Investigating the effect of different conditions such as buffer or temperature, or different variants of the interactants. Such comparisons may be performed on the results of a few runs or even a single run that covers the conditions being investigated. One set of conditions is chosen as the standard.
- Investigating the consistency of assay performance or monitoring batch- to-batch variation or product stability over time, by comparison with an accepted reference standard. Such studies invariably use data obtained from several files. The standard sensorgrams can be saved in an evaluation method for repeated application to different sample sets.
- Screening experiments where the goal is to select binders with a binding profile that is sufficiently similar to a reference standard, or with interaction parameters within a specified range. For the latter applications, a standard representing the acceptable range can be established.

Many applications compare data from multiple runs, combined either by selecting multiple result files in the ***Open*** dialog or using ***Append Result File*** (see Section 6.2.1). Remember that any evaluation items in the current session will be deleted if a new result file is appended. Open or append all relevant files before applying sensorgram comparison.

11.2 Running analyses for comparison

Comparison can be applied in principle to any kind of sensorgrams, although sensorgrams obtained under kinetic analysis conditions are generally recommended.

General requirements and recommendations are summarized below. Many of the requirements are met automatically if the same method is used for different runs, and the same cycle type for different samples within a run.

11.2.1 Requirements

Requirement	Description
Data collection rate	All sensorgrams used in the comparison must be run at the same data collection rate. This requirement includes standard sensorgrams saved in evaluation methods.
Assay step purpose	The assay step purpose must be ***Sample*** or ***Control Sample***. Cycles with any other assay step purpose will be ignored.
Ligand and analyte names	All sensorgrams within sample and control curve sets must have the same ligand and analyte names. Sensorgrams with different names will be assigned to different curve sets. Ligand and analyte names can vary within the curve set for standard curves.
Analyte concentration	For comparison of single- or multi-cycle kinetic data, samples and controls must have the same concentration series as the standard. For comparison of single concentration data, the concentration must be the same within the standard sensorgrams but may vary for samples and controls. For comparison of multiple injection sensorgrams, the concentration must be provided for the first injection. The concentration must be the same within the standard sensorgrams but may vary for samples and controls.

11.2.2 Recommendations

Recommendation	Description
Assay step purpose	Run control samples in assay steps with the purpose ***Control sample***. Controls may be freely assigned in sensorgram comparison items that are created interactively, but are assigned from the assay step purpose and cannot be changed for comparison items created by application of an evaluation method.
Cycle definition	Use the same cycle definition for standards, controls and samples, to ensure that the cycle conditions are the same.
Number of cycles for standards	Run at least 2 cycles (preferably more) for standards. Two cycles (in the same or different runs) is a requirement: using more cycles gives a better representation of the variability of the sensorgram data.

11.3 Sensorgrams and curve sets

11.3.1 Comparison types

Four types of comparison are available, selected from the sub-menu under ***Sensorgram Comparison*** when the item is created. Only sensorgrams conforming to the selected type will be included in the comparison item.

Comparison type	Description
Single cycle	Compares curve sets from runs in single-cycle kinetics format.
Multi cycle	Compares curve sets from runs in multi-cycle kinetics format.
Single concentration	Compares curve sets that consist of sensorgrams run at a single analyte concentration. The concentration must be the same for standards, but may vary for samples and controls.
Multiple injections	Compares curve sets that consist of sensorgrams with multiple injections (for example, analyte followed by enhancement). Similarity scores are reported for the whole injection sequence as well as separately for each injection.

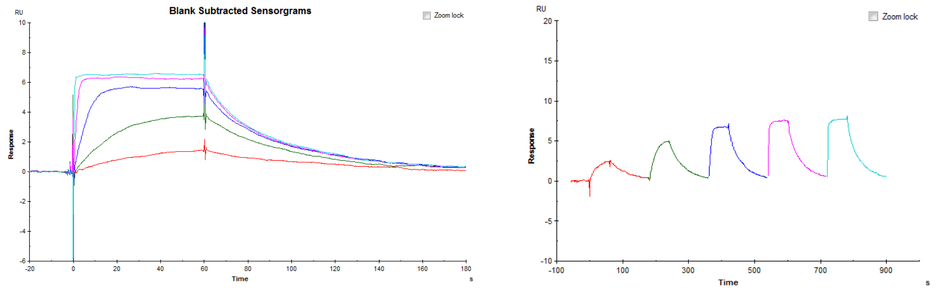
11.3.2 Cycles included in sensorgram comparison

In addition to conformance with the selected comparison type (Section 11.3.1), sensorgrams must be obtained from cycles with the following properties in Method Builder or the equivalent in application wizards:

- The assay step purpose must be ***Sample*** or ***Control Sample***. Cycles with any other assay step purpose will be ignored.
- For all types except ***Multiple injection***, the injection command must be ***Sample***.
- For ***Multiple injection*** comparisons, injection commands ***Dual*** and ***Enhancement*** are included in the comparison in addition to ***Sample***.
- The injection type must be ***High performance***, ***Low sample consumption*** or ***Single cycle kinetics.*** Injections of type ***Merged injection*** and ***Double mix*** are ignored.
- The predefined evaluation variable ***Conc*** must be included.

Cycles from multi-cycle kinetic experiments are first converted to a single-cycle format by concatenating cycles in order of increasing sample concentration before being compared (see illustration below).

Note: *Even though multi-cycle runs are converted to a single-cycle format for comparison, single- and multi-cycle sensorgrams cannot be included together in the same comparison item.*



Multi-cycle kinetic sensorgrams (left) are converted to a single-cycle format (right) for sensorgram comparison.

11.3.3 Curve sets

For the purposes of sensorgram comparison, cycles are combined into *curve sets*. All cycles in a curve set share the following properties:

- source file
- analysis temperature
- curve type (e.g., active, reference subtracted, corrected)
- sample concentrations and injection parameters
- flow rate
- ligand and analyte names (does not apply to standards)
- injection sequence (for ***Multiple injection*** comparisons)

Selected sensorgrams in a curve set can be excluded from a comparison if required.

11.3.4 Standards

At least two sensorgrams are required to define a standard. The sensorgrams may be taken from the same or different curve sets. The standard curve is the average or median of the included sensorgrams, according to the comparison approach used (see Section 11.4).

The sensorgrams included in the standard must:

- have the same sample contact times and dissociation times
- use the same sample concentrations in all corresponding injections

Sensorgrams from different files, flow cells and curve sets may be included in the standard as required, subject to the restrictions above.

11.3.5 Samples and controls

The sensorgrams in each curve set are compared separately with the standard. Sample and control sensorgrams must correspond to the standards in the following respects:

- For concentration series run as single-cycle (see Section 5.6.1), samples must correspond exactly to the standards with respect to injection times, dissociation times and sample concentrations.
- For series run as multi-cycle, only sample concentrations that correspond to those in the standard will be compared. Any cycles which do not have an equivalent cycle in the standard will be ignored.

All control sensorgram sets are combined into a single group for comparison with the standard. The ligand and analyte names must be the same for all sensorgrams in the control group, but do not have to be the same as those in the standard.

Sample sensorgram sets are compared individually with the standard and cannot be combined. Ligand and analyte names do not have to be the same in sample sets as in the standard.

11.4 Adjustments for sensorgram comparison

This section describes adjustments that can be applied in the sensorgram comparison items.

11.4.1 Sensorgram adjustment

Sensorgrams to be compared are automatically adjusted so that the injection start is always aligned to the same point on the time-axis. For ***Multiple injection*** comparisons, the start of the first injection is set to zero on the time axis. Subsequent injections are adjusted if necessary with slight time displacement to maintain alignment. The start of the first injection is always adjusted to zero response.

This adjustment cannot be changed.

11.4.2 Blank subtraction

Blank subtracted sensorgrams are normally used for sensorgram comparison. Blank cycles are cycles with zero-concentration sample injections. By default, the nearest preceding blank is used. If there is no preceding blank, the nearest following blank is used.

Note: *In general, blank-subtracted and non-subtracted sensorgrams should not be mixed in the same comparison.*

For comparison of curves derived from multi-cycle kinetic runs, the same blank cycle is subtracted from all cycles.

Blank subtraction settings can be changed in the ***Thumbnails*** section of the sensorgram comparison item (see Section 11.7.4).

11.4.3 Normalization

For comparison with the standard deviation or max/min approach, sensorgrams are by default normalized in order to compare sensorgram shape without reference to response levels. Normalization sets the highest response value during the injection to 100 and scales the remainder of the sensorgram accordingly. Since the start of the injection is already set to zero (Section 11.4.1), this normalizes the sensorgrams on a scale of 0 to 100.

For ***Multiple injection*** comparisons, the highest response in the last injection of the series is normalized to 100.

Set the ***Normalization threshold*** to avoid performing comparisons (and in particular creating reference standards) using sensorgrams with low response values, where signal noise can represent disproportionately high contributions to the comparison results. Sensorgrams where the highest response is below the specified ***Normalization threshold*** will be omitted from the comparison.

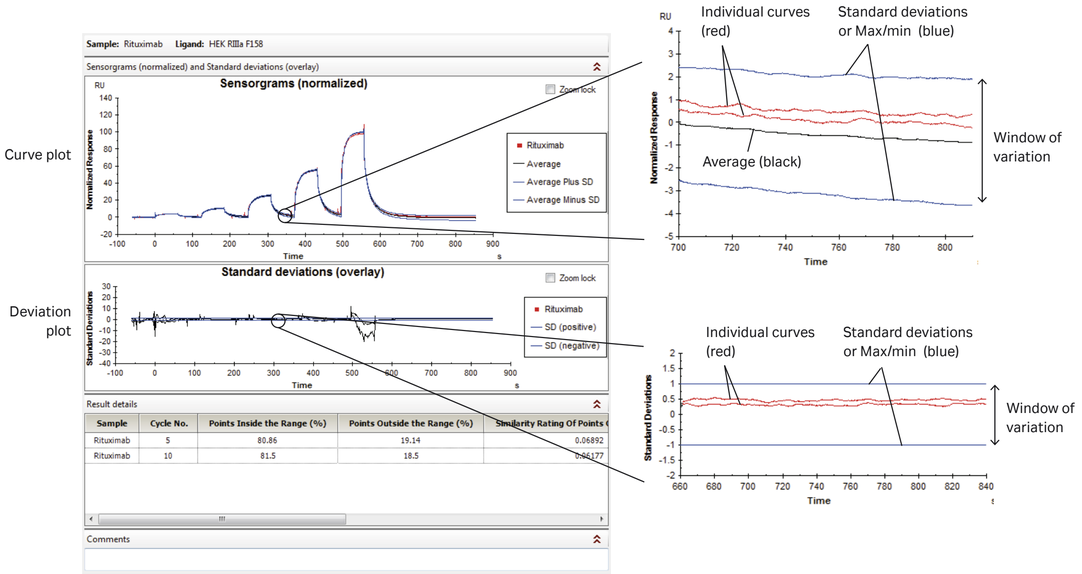
Sensorgrams compared using the percentage deviation approach cannot be normalized.

Normalization settings may be adjusted in the ***Comparison settings*** section of the sensorgram comparison item (see Section 11.7.5).

11.5 Comparison algorithms

11.5.1 Data comparison terminology

The diagram below illustrates the terms used in comparison, with reference to the presentation of the results (see Section 11.7.6).



11.5.2 Comparison approaches

The standard curve and window of variation is created from two or more individual sensorgrams using one of the following approaches:

Approach	Uses
Standard deviation	Useful when for establishing assay performance or comparing product/reagent stability over time (for example, quality control of production batches).
Max/min	Useful in screening applications where it can be used to precisely describe a window of variation.
Percentage deviation	Useful for establishing a reasonable window of variation with limited runs, when standard sensorgrams may show too little variation for the other approaches.

For comparison using the standard deviation or max/min approach, sensorgrams may be normalized before comparison. Comparing normalized data provides results based on sensorgram shape, independent of response levels. Differences in relative binding levels may be detected with the ***Apparent stoichiometry*** tool (see Section 11.6). Comparing data which has not been normalized places more emphasis on differences in response levels, which may obscure differences in sensorgram shape. Normalization is not available for the percentage deviation approach.

Standard deviation approach

In the standard deviation approach, the standard curve is the average of the individual standard sensorgrams at each point. The window of variation is based on a specified number of standard deviations (SD) above and below the average.

Deviation of sample curves from the standard (presented in a deviation plot, see Section 11.7.6) is calculated for each point as:

$$\text{Deviation} = \frac{\text{Sample} - \text{Average}}{\text{SD}}$$

Note that the deviation represents the number of SDs separating the sample and the average standard, not the absolute response separation. When the individual standard curves lie close to each other, the SD will be small and deviations in the sample curves will be magnified. Conversely, widely spread standard curves will create a large SD value and the deviations in the sample curves will be reduced.

Note: *Comparison cannot be performed if the standard curves are identical, since the SD will be zero. Isolated points where the curves coincide are however ignored in the comparison results.*

Max/min approach

In the max/min approach, the standard curve is the median of the individual standard sensorgrams at each point. The window of variation is defined by the maximum and minimum response values at each point, scaled by an optional curve factor.

Note: *For an odd number of curves, the median at each data point is the middle value in the set. For an even number of curves, the median is calculated as the average of the two middle values.*

The window of variation is presented in the deviation plot as follows:

$$\text{Upper window limit} = \frac{(\text{Max response} - \text{Median}) \times \text{Curve factor}}{\text{Tolerance}}$$

and

$$\text{Lower window limit} = \frac{(\text{Min response} - \text{Median}) \times \text{Curve factor}}{\text{Tolerance}}$$

where *Tolerance* is defined for each point as *(Min response - median)*. Thus the lower limit on the deviation plot is always a straight line at the curve factor value below the median, while the upper limit can vary according to the variation in response above the median.

For each sample curve, the deviation plot shows the deviation calculated at each point in the curve as:

$$\text{Deviation} = \frac{\text{Sample} - \text{Median}}{\text{Tolerance}}$$

Percentage deviation approach

In the percentage deviation approach, the standard curve is the average of the individual standard sensorgrams at each point. The window of variation is given by a specified percentage of the standard response or an absolute response value in RU, whichever is the larger. Setting one of the percentage or response values to zero will result in a window of variation calculated from the other parameter only. The window is applied both above and below the reference standard.

For each point on a sample curve, the deviation plot shows the deviation in RU:

$$\text{Deviation} = \text{Sample} - \text{Average}$$

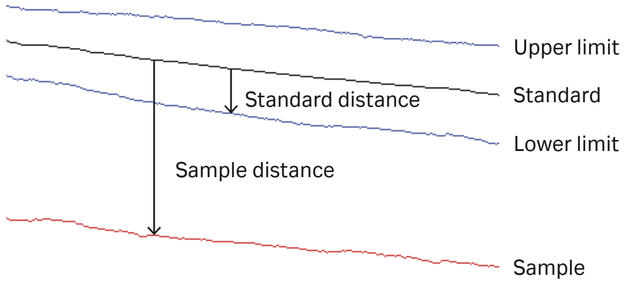
11.5.3 Result parameters

Similarity rating

For each curve, a similarity rating is calculated from all points that fall outside the window of variation as follows. Points inside the window or on the window limits do not contribute to the similarity rating.

$$\text{Similarity rating} = \frac{\sum (\text{standard distance})^2}{\sum (\text{sample distance})^2}$$

where *standard distance* is the distance of the window limit from the standard curve, and *sample distance* is the distance of the individual sample curve from the standard (see illustration below). For sample points that lie above or below the standard, the similarity rating is calculated using the upper or lower window limit respectively. The similarity rating can have a value between 0 and 1.



The same similarity rating may represent a few points with large deviations or many points with small deviations. These situations are distinguished by the similarity score (see below).

Similarity score

The similarity score for a sample sensorgram is derived from the percentage of points inside the window of variation, plus the percentage of points outside the window weighted with the similarity rating:

Similarity score = %inside + (%outside × Similarity rating)

One value for the similarity score is reported for the whole curve set, as the average and standard deviation of the values for the curves in the set. For ***Multiple injection*** comparisons, similarity scores are also reported for each separate injection.

The similarity score can have a value between 0 and 100, where 100 indicates that all points are inside the window of variation.

The similarity score helps to distinguish between frequent small deviations from the standard and a few larger deviations, which can give identical similarity ratings. This is illustrated in the table below.

Points inside window	Similarity rating	Similarity score
80%	0.5	90
20%	0.5	60

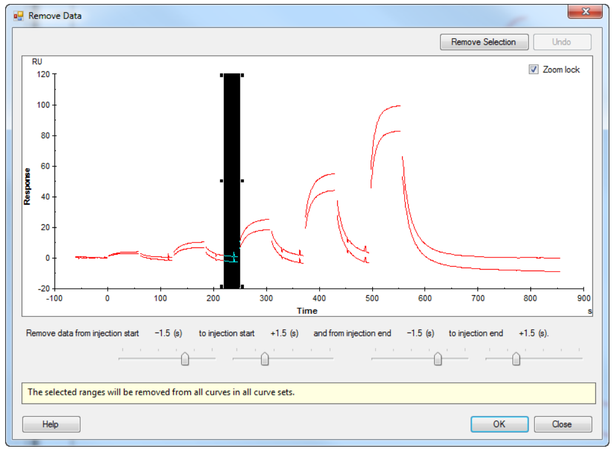
11.6 Tools menu

The ***Tools*** menu at the top of the left-hand panel in the comparison window offers a set of functions (described below) that are useful at different stages of the comparison workflow. The tools are available at all steps in the comparison. Results and screen displays are updated as required whenever tools are used.

11.6.1 Remove Data

Use this tool to remove selected data from the curves, to exclude artefacts that can appear at the beginning and end of injections, and to remove any other significantly disturbed data.

This operation is best performed from the thumbnail tab to give an overview of all curve sets, since data is removed from all curves in all curve sets.



Use the sliders to remove disturbed data at the beginning and end of the injections. The default setting removes ±1.5 s at injection start and stop: you can remove up to ±5 s.

To remove other regions of disturbed data, drag with the right mouse button over the region to be deleted and click ***Remove Selection***. Click ***Undo*** to restore the deleted data.

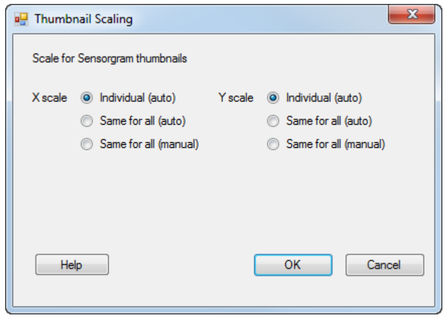
Any data that is removed, by using either the sliders or the ***Remove Selection*** option, will be removed from all curves in all curve sets in the current comparison.

Note: *The **Remove Data** dialog shows the sensorgrams in the currently selected curve set. After removing ranges, check that the settings are appropriate for the other curve sets.*

For ***Multiple injections*** comparisons, click ***Remove Wash*** to remove data collected during the automatic wash between injections. Sensorgrams may exhibit disturbances during the wash procedure that can distort the comparison results. This option is only available for ***Multiple injections*** comparisons.

11.6.2 Thumbnail scaling

Use this tool to set how the thumbnails are scaled.



Individual (auto) scales each thumbnail separately according to the range of data in the thumbnail.

Same for all (auto) scales all thumbnails to the same setting according to the range of data in the complete data set.

Note: *If you exclude or include curves so that the complete data range changes, thumbnails will not be rescaled until you reapply **Thumbnail scaling**.*

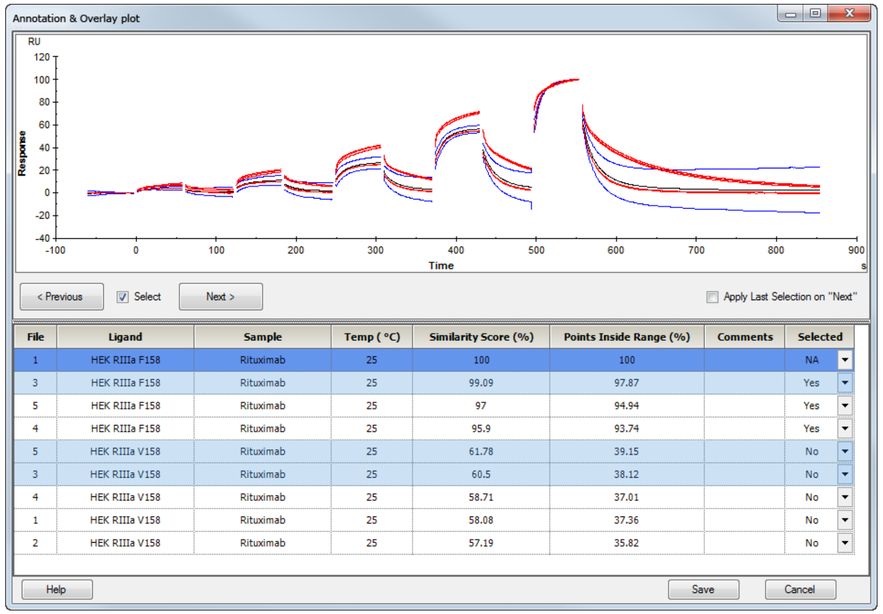
Same for all (manual) scales all thumbnails to the same manual setting. Specify maximum and minimum values for the axis.

11.6.3 Annotations & Overlay plots

This tool presents a summary of the sensorgram comparison with the current comparison settings. Select multiple rows in the table to display an overlay plot of the selected curve sets.

The table includes a ***Selected*** column where a quick assessment of the comparison can be recorded by setting the status to ***Yes*** or ***No***. The status for standards cannot be set. The status will be reset to ***NA*** if any changes are made to the comparison settings.

Note: *These annotations are not related to the* ***Annotations*** *option in result plots (see Section 8.6).*



File	Ligand	Sample	Temp (°C)	Similarity Score (%)	Points Inside Range (%)	Comments	Selected
1	HEK RIIIa F158	Rituximab	25	100	100		NA
3	HEK RIIIa F158	Rituximab	25	99.09	97.87		Yes
5	HEK RIIIa F158	Rituximab	25	97	94.94		Yes
4	HEK RIIIa F158	Rituximab	25	95.9	93.74		Yes
5	HEK RIIIa V158	Rituximab	25	61.78	39.15		No
3	HEK RIIIa V158	Rituximab	25	60.5	38.12		No
4	HEK RIIIa V158	Rituximab	25	58.71	37.01		No
1	HEK RIIIa V158	Rituximab	25	58.08	37.36		No
2	HEK RIIIa V158	Rituximab	25	57.19	35.82		No

The ***Selected*** status for curve sets is recorded in the comparison results. The status does not affect the comparison in any other way.

11.6.4 Apparent Stoichiometry

This tool calculates the apparent maximum binding stoichiometry reached during the interaction from the highest analyte binding response together with the ligand response and the molecular weights of ligand and analyte:

$$\frac{\text{Analyte response}}{\text{Analyte MW}} \times \frac{\text{Ligand MW}}{\text{Ligand response}}$$

Parameter values are obtained as listed in the table below. All parameters can be entered or edited in the table if required.

Parameter	Source
Analyte response	Highest response during sample injection, relative to the report point ***baseline***[1].
Analyte MW	Taken from the keyword table (see Section 6.6). The molecular weight may be provided as a variable in the run method or added to the keyword table during evaluation.
Ligand response	*For immobilized ligands:* Taken from the immobilization information recorded in ***File:Properties***. *For captured ligands:* Response at ***capture_level*** relative to ***capture_baseline***[1].
Ligand MW	This value must be entered manually.

[1] The apparent stoichiometry cannot be calculated if these report points are not present.

Note: *Parameters for apparent stoichiometry calculation are not saved in evaluation methods.*

Apparent stoichiometry values are listed on the ***Results*** tab, with values for individual cycles in the detail panel and average values for the curve set in the main ***Results*** table.

The apparent stoichiometry tool can help to reveal differences in relative binding levels that are obscured by normalization.

Note: *The apparent stoichiometry represents the maximum stoichiometry reached during the sensorgram, and may be significantly less than the true saturation stoichiometry for analyte-ligand binding.*

11.6.5 Hide Rejected

Select ths option to hide rejected sensorgram sets on the thumbnail and results tabs.

Note: *This option affects sets that are rejected (marked with) on the thumbnail tab (see Section 11.7.4). Series that are set to* ***Selected=No*** *in* ***Annotations*** *(Section 11.6.3) are not hidden.*

Note: *Rejected cycles are not hidden in the* ***Apparent Stoichiometry*** *table.*

11.6.6 Print

Prints the content of the sensorgram comparison item.

11.7 Applying sensorgram comparison

This section describes how to apply sensorgram comparison interactively. Applying sensorgram comparison from an evaluation method is described in Section 11.9.2. Recommendations for running analyses intended for comparison are considered in Section 11.2.

11.7.1 Starting sensorgram comparison

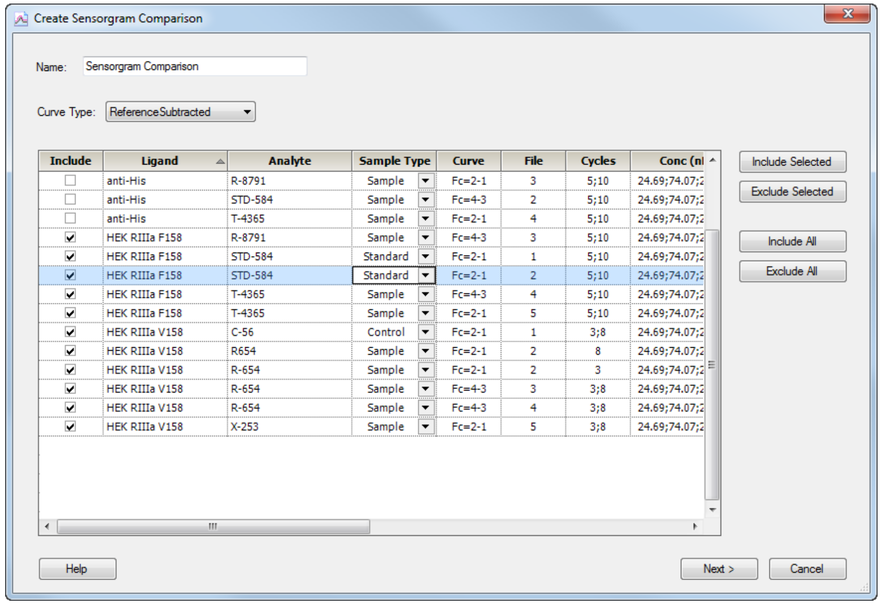
To start a sensorgram comparison, open the result files containing all the sensorgrams you want to compare in a single evaluation session. To open multiple files, you can either choose ***File:Open*** and select the files in the ***Open*** dialog, or use ***File:Append Result File*** to add result files to the current session.

Notes: *Appending result files to an existing session will delete all user-defined evaluation items in the session. Open or append all result files before starting the sensorgram comparison.*

Once all result files have been opened or appended, choose the appropriate option (see Section 11.3.1) from the ***Sensorgram Comparison*** button or the ***Evaluation*** menu. The resulting dialog lists all data in the current session with the specified curve type and analysis temperature.

Enter a name if required for the sensorgram comparison item. This name is used to identify the item in the ***Evaluation Explorer*** and in printed and exported documentation.

Choose the ***Curve Type*** for the comparison.



Include	Ligand	Analyte	Sample Type	Curve	File	Cycles	Conc (nl
☐	anti-His	R-8791	Sample	Fc=2-1	3	5;10	24.69;74.07;2
☐	anti-His	STD-584	Sample	Fc=4-3	2	5;10	24.69;74.07;2
☐	anti-His	T-4365	Sample	Fc=2-1	4	5;10	24.69;74.07;2
☑	HEK RIIIa F158	R-8791	Sample	Fc=4-3	3	5;10	24.69;74.07;2
☑	HEK RIIIa F158	STD-584	Standard	Fc=2-1	1	5;10	24.69;74.07;2
☑	HEK RIIIa F158	STD-584	Standard	Fc=2-1	2	5;10	24.69;74.07;2
☑	HEK RIIIa F158	T-4365	Sample	Fc=4-3	4	5;10	24.69;74.07;2
☑	HEK RIIIa F158	T-4365	Sample	Fc=2-1	5	5;10	24.69;74.07;2
☑	HEK RIIIa V158	C-56	Control	Fc=2-1	1	3;8	24.69;74.07;2
☑	HEK RIIIa V158	R654	Sample	Fc=2-1	2	8	24.69;74.07;2
☑	HEK RIIIa V158	R-654	Sample	Fc=2-1	2	3	24.69;74.07;2
☑	HEK RIIIa V158	R-654	Sample	Fc=4-3	3	3;8	24.69;74.07;2
☑	HEK RIIIa V158	R-654	Sample	Fc=4-3	4	3;8	24.69;74.07;2
☑	HEK RIIIa V158	X-253	Sample	Fc=2-1	5	3;8	24.69;74.07;2

Each row in the table represents one *curve set (see Section 11.3).*

Note: *If you want to change the way curves are assigned to curve sets, cancel the* ***Sensorgram Comparison*** *item and use* ***Tools:Keyword Table*** *to adjust parameter(s) as required.*

11.7.2 Selecting curve sets

Remove the checkmark from the ***Include*** box for curve sets that you do not wish to use in the comparison. To include or exclude several sets in one operation, select the appropriate rows and use the ***Include/Exclude Selected/All*** buttons. Click on a column header to sort the table contents by the contents of the column.

11.7.3 Setting sample type

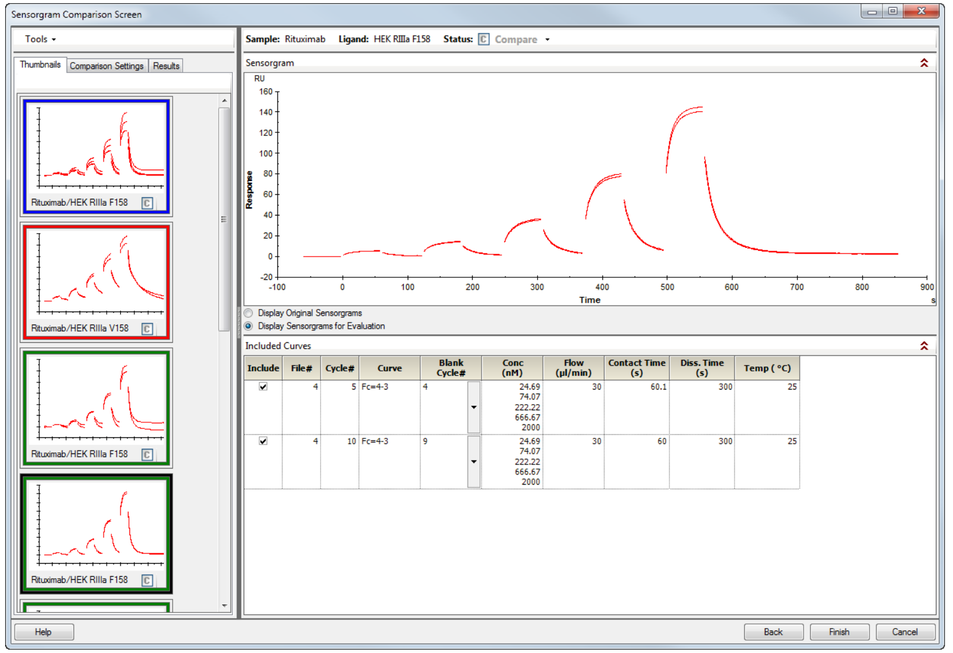
Sensorgram comparison requires that at least two sensorgrams (from the same or different curve sets) are defined as standards. Select ***Standard*** in the ***Sample Type*** column for the sets to be used as standards. All curves in the selected set(s) will be included in the standard by default: you can exclude individual curves if desired in the next step.

All other sensorgram sets are defined as either ***Control*** or ***Sample***. These are handled identically in the sensorgram comparison except for identification by color-coding in the thumbnails and results (see below). Typically, controls are known samples included each time new samples are run for comparison, to check the consistency of the interaction behavior between different run occasions.

Note: *Curve sets from cycles with assay step purposes* ***Sample*** *and* ***Control Sample*** *will be set by default to type* ***Sample*** *and* ***Control*** *respectively.* ***Standard*** *curve sets are not selected by default.*

All sensorgrams set as ***Control*** are combined into one curve set. Sensorgram sets of type ***Sample*** are compared separately with the standard. ***Control*** and ***Sample*** sets cannot be compared directly with each other.

Click ***Next*** to continue to the comparison window when curve sets have been selected and sample types defined.



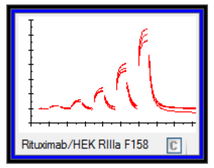
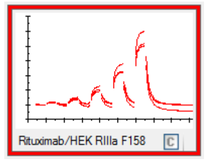
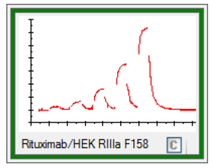
The comparison window contains three tabs:

- ***Thumbnails***, for examining and adjusting sensorgram sets (shown in the illustration above),
- ***Comparison settings***, for setting comparison approach and parameters,
- ***Results***, for displaying the comparison results.

11.7.4 Thumbnails

The left-hand panel of the ***Thumbnails*** tab shows a thumbnail for each curve set. The right-hand panel shows the detailed view of the currently selected thumbnail, with sub-panels for ***Sensorgram*** and ***Included Curves***. Use the ⏫ and ⏬ buttons to show and hide the sub-panels.

The color of the thumbnail frame identifies the curve set type:

	**Blue**	***Standard*** (derived from one or more curve sets)
	**Red**	***Control*** (derived from one or more curve sets)
	**Green**	***Sample*** (derived from a single curve set)

An additional black frame identifies the currently selected thumbnail.

Select ***Tools:Thumbnail scaling*** to set how the thumbnails are scaled (see Section 11.6).

Rejecting unwanted sensorgram sets

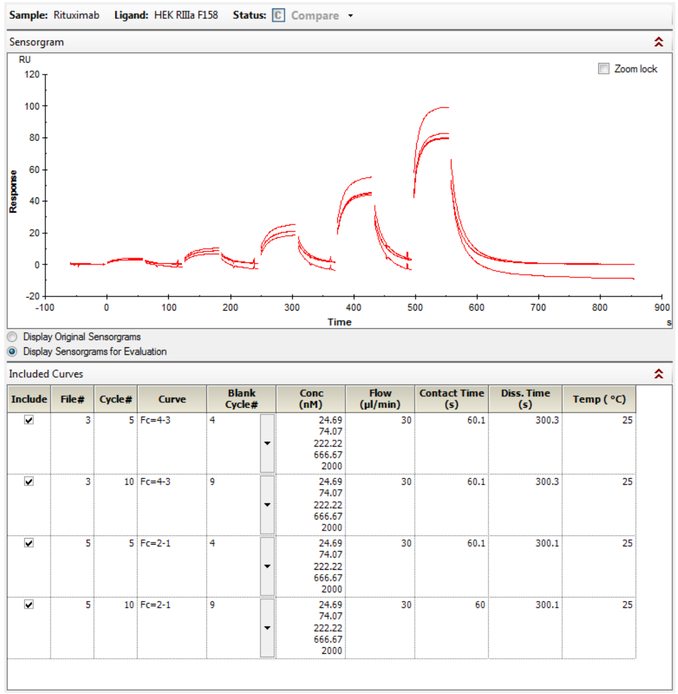
Scan through the thumbnails and reject any sensorgram sets where sensorgrams are clearly disturbed or unsuitable for comparison. To reject a set, either click on the status icon in the thumbnail and choose ❌, or select the thumbnail then choose ***Rejected*** in the ***Status*** field at the top of the detail panel. Rejected thumbnails can be hidden by choosing ***Tools:Hide Rejected***, and can be reinstated by setting the status to ***Compare*** (©).

Note: *In some circumstances, sensorgram sets with mismatched data such as injection times or sample concentrations that differ from the standard may be created but automatically rejected. Such sets cannot be reset to* ***Compare*** *status.*

For closer examination of the data, select a thumbnail to display a detailed view of the curve set in the right-hand panel. You can choose to show original sensorgrams with blanks (zero-concentration cycles) or blank-subtracted samples in the ***Sensorgram*** sub-panel: this is for inspection purposes only and does not affect the sensorgram data that will be compared.

Excluding individual curves

To exclude individual curves from a curve set, select the thumbnail for the set and expand the ***Included Curves*** sub-panel on the right. Remove the checkmark from the ***Include*** column for sensorgrams you want to exclude. The standard curve set must have at least two curves.



Include	File#	Cycle#	Curve	Blank Cycle#	Conc (nM)	Flow (µl/min)	Contact Time (s)	Diss. Time (s)	Temp (°C)
☑	3	5	Fc=4-3	4	24.69 74.07 222.22 666.67 2000	30	60.1	300.3	25
☑	3	10	Fc=4-3	9	24.69 74.07 222.22 666.67 2000	30	60.1	300.3	25
☑	5	5	Fc=2-1	4	24.69 74.07 222.22 666.67 2000	30	60.1	300.1	25
☑	5	10	Fc=2-1	9	24.69 74.07 222.22 666.67 2000	30	60	300.1	25

Blank subtraction

Sensorgrams for comparison are blank-subtracted by default, using the nearest preceding blank cycle (or nearest blank if there is none preceding). Alternative blank cycles can be selected in the ***Blank Cycle#*** column for the included curves. To compare data without blank subtraction, choose No blank in the ***Blank Cycle#*** list.

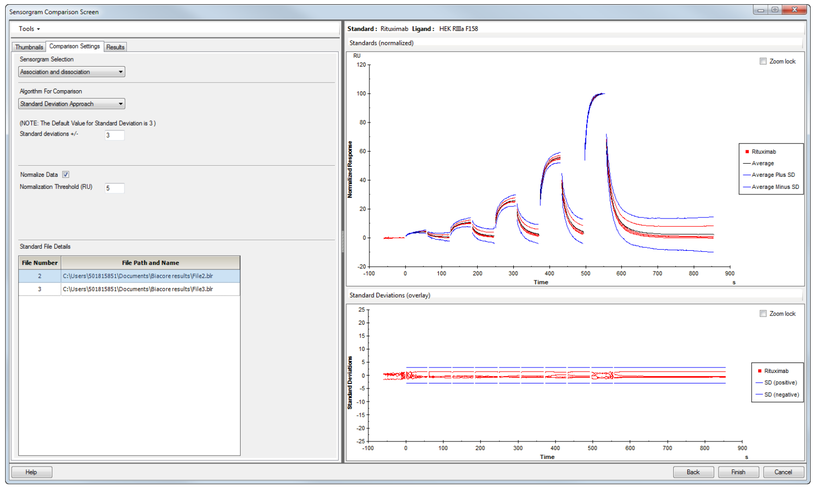
11.7.5 Comparison settings

The ***Comparison settings*** tab defines the approach and parameters for the sensorgram comparison. The right hand panel shows the standard curves and the deviations in separate panels. Curves and curve sets cannot be edited on this tab.

The following colors are used for curves in the curve and deviation plots:

Color	Used for
Red	Individual standard sensorgram curves
Blue	Window of variation
Black	Standard curve (average or median)

Note: *Sample sensorgrams are not displayed on the* ***Comparison settings*** *tab.*



File names and locations for sensorgrams used as standards are saved in the item and displayed on the ***Comparison settings*** tab.

Sensorgram selection

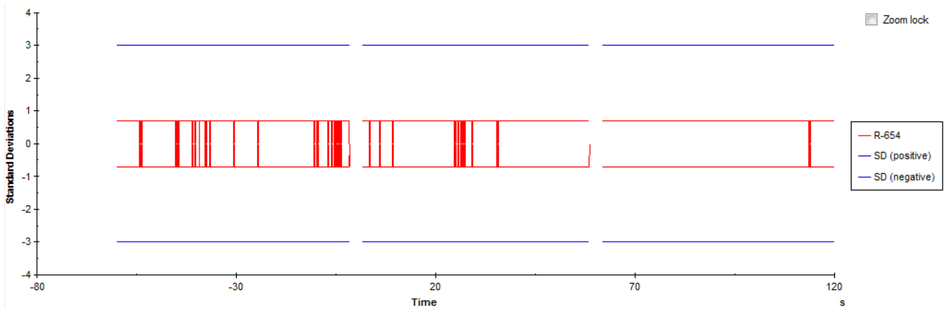
You can choose whether to compare either the association or dissociation phase of the sensorgrams or both phases together. The display in the right- hand panels shows the data that will be compared.

Algorithm for comparison

Choose the algorithm and settings for the comparison (see Section 11.5.1). Details of the reference standard are shown as sensorgrams and deviation plots in the right-hand panel. See Section 11.5 for details of how deviations are calculated.

- the sample data is shown in red
- the standard curve (average or median) is shown in black in the upper panel: it is omitted from the lower panel since the standard curve deviations are by definition zero
- the limits of the window of varation are shown in blue

Note: *When the standard is derived from only two sensorgrams, the window of variation in the deviation plot for the standard deviation or max/min approach will be constant. Deviations for samples may oscillate randomly between the limits, giving the kind of appearance illustrated below:*



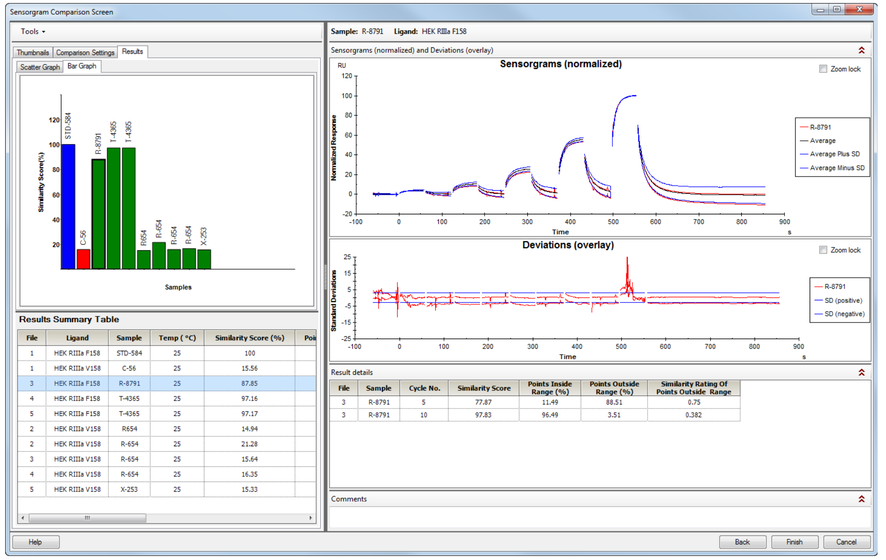
Normalize data

Check the ***Normalize data*** option to compare curves normalized as described in Section 11.4.3. This will focus the comparison on sensorgram shape without regard to response levels. Differences in response levels may be revealed by the ***Apparent Stoichiometry*** values (see Section 11.6). Uncheck the ***Normalize data*** option to compare curves without normalization.

Curves where the maximum response does not exceed the ***Normalization Threshold*** will be excluded from normalized comparison. The ***Normalization Threshold*** setting is only shown when ***Normalize data*** is selected.

11.7.6 Results

The results of the comparison are presented in the ***Results*** tab. The tab shows a graphical and tabular summary in the left-hand panel and comparison details in the right-hand panel.



Results summary

The results are displayed as a bar graph showing the similarity score for each curve set or a scatter graph of similarity score against the number of points inside the window of variation. Points and bars are colored according to the sample type (***Standard***, ***Sample*** or ***Control***). Select a column in the bar chart or point in the scatter graph to highlight the corresponding row in the summary table and *vice versa*.

Color usage in the results summary corresponds to that for thumbnail borders:

Color	Used for
Red	Controls
Blue	Standards
Green	Samples

The summary table lists the numerical results of the comparison for each curve set, sorted by default by descending similarity score. Click on a column header in the table to sort the table by the content of that column. Sorting the table sorts the bar graph correspondingy (but not the scatter graph).

The table also lists any ***Annotations*** (Section 11.6.3) and the average value for the ***Apparent Stoichiometry*** if this has been calculated (Section 11.6). The apparent stoichiometry for each sensorgram in the set is listed in the detailed result table (see below).

Select a point in the scatter graph, bar in the bar graph or row in the results summary table to display the results details for the selected curve set in the right-hand panel.

Result details

The top two sub-panels in the right-hand panel show the curves and deviation plots for the currently selected curve set. The presentation is equivalent to that described for the standard curve in the ***Comparison Settings*** tab (Section 11.7.5).

Color usage in the results summary is the same as in ***Comparison settings***:

Color	Used for
Red	Individual sensorgram curves
Blue	Window of variation
Black	Standard curve (average or median)

The details table lists the results separately for each curve in the set, and includes the parameters presented in the summary table together with the percentage of points outside the window of variation and a similarity rating for points outside the window.

Commenting results

To enter a comment for the currently selected comparison, expand the ***Comments*** subpanel at the bottom of the details panel and enter the comment text. Avoid using line-breaks in comments. Comments appear in the ***Results Summary*** table on the left-hand side, but may be truncated in this table depending on the column width.

Comments can also be typed directly in the ***Comments*** column in the ***Results summary*** table. However, comments cannot be edited in the summary table: typing new text directly in the table replaces any previous comment.

Note: *Only one comment line is shown for each row when the results are printed. As a general recommendation, avoid entering comments that exceed 100 characters.*

11.7.7 Saving the comparison

Click ***Finish*** to save the comparison as an evaluation item.

11.8 Assessing the results

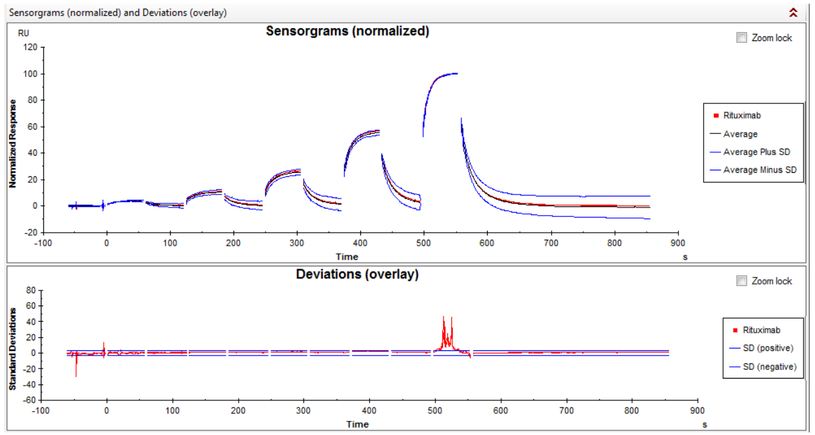
This section gives some guidelines for assessing the results of sensorgram comparison.

Use ***Tools:Anotations*** to record the assessment as ***Selected:Yes*** or ***No*** in the ***Results summary*** table.

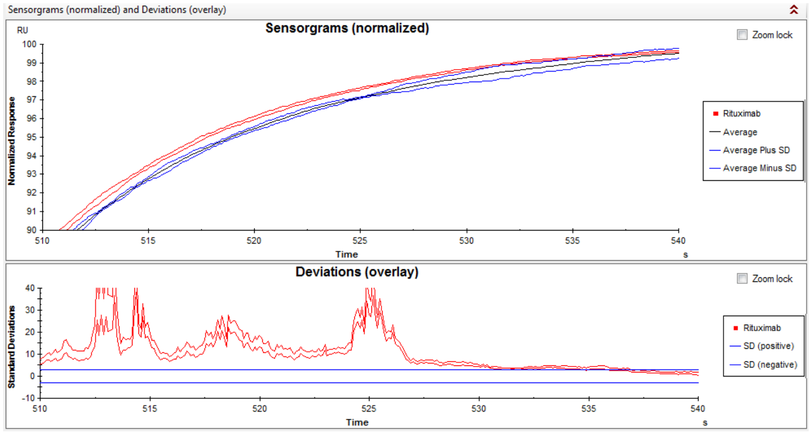
11.8.1 Curve appearance

Visual inspection of the compared curves often provides a good assessment of the comparison but is difficult to document objectively. Essentially, the curves are acceptably similar to the reference standard if a sufficient proportion of the sample sensorgram (red) lies within the window of variation (blue). The ***Comments*** field can be used to describe visual conclusions drawn for a given comparison.

In assessing the deviation plot, bear in mind that the deviation for standard deviation and max/min approaches reflects the difference between the sample curve and the reference standard in relation to the size of the window of variation. Regions where the standard sensorgrams happen to lie closer together will have a narrower window, and deviations in the sample sensorgrams will be correspondingly magnified. In the example illustrated below, the deviaton plot seems to indicate a considerable deviation in the region between 500 and 550 seconds:



However, zooming in to this region shows that this is entirely due to local narrowing of the window of variation in this region. The sample sensorgrams are essentially parallel to the reference standard. It can thus be misleading to assess the comparison results from the deviation plot alone.



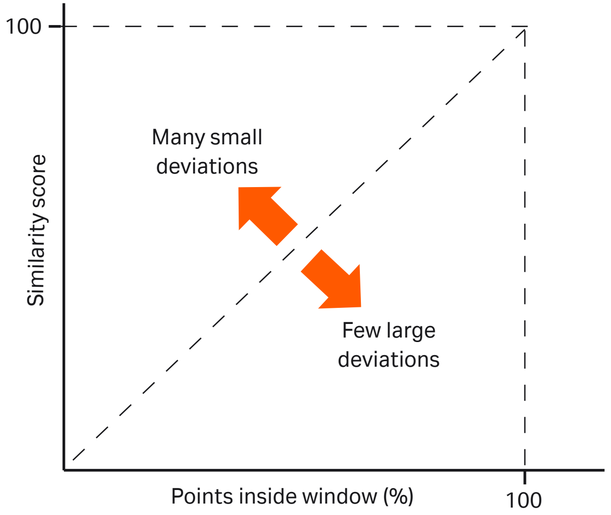
This effect is likely to be more pronounced when fewer curves are used as standards.

11.8.2 Similarity score

The similarity score provides a combined indication of how much of the sample curve lies inside the window of variation and how far outside the window deviating points lie. Higher values indicate more overall similarity to the standard. Note that the same similarity score may reflect curves that are largely outside the window but close to the limit or curves that are largely inside the window but with a few points deviate strongly (for instance, spikes and similar large but short-lived disturbances).

11.8.3 Points inside the window

The percentage of points inside the window can help to resolve the difference between many small deviations and a few large ones. Thus a relatively low similarity score combined with a high percentage of points inside the window indicates a few relatively large deviations. This is visualized in the scatter plot of similarity score against points inside the window (see illustration below).



When using the standard deviation approach, both the similarity score and the percentage of points inside the window should ideally be 100 for the standard curve. Any significant deviation from this value indicates either that the window of variation is too narrow or that the curves selected for the standard are too dissimilar to provide good reference data.

For individual curves, the similarity rating of points outside the window (listed in the detailed results table) also provides an indication of the type of deviation from the standard. A high similarity rating (close to 1) indicates that deviating points lie close to the window.

Acceptance criteria for the numerical results will depend on the particular application and the variability of replicate analyses, and should be determined by experience.

11.9 Sensorgram comparison in evaluation methods

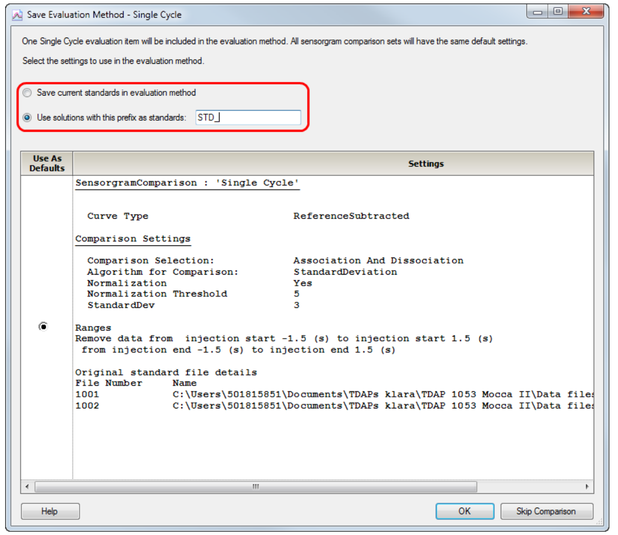
11.9.1 Settings saved in a method

The following parameters from sensorgram comparison items are saved in evaluation methods:

- All comparison settings (Section 11.7.5).
- Sensorgram data, blanks and data removal settings for standard sensorgrams.
- Apparent stoichiometry values for the standard sensorgrams, if defined. Values for sample and control sensorgrams are not saved in the method.
- Slider settings and data removal settings in the ***Remove data*** dialog (Section 11.7.4).

Note: *Slider settings applied from a method can be adjusted, but data removed by the method cannot be restored.*

Standard sensorgrams are saved in the method in one of two ways:



- Standards used in the evaluation session are saved in the method, independently of the location of the original result or evaluation files. Saved standards are used when the method is applied and cannot be changed.
- Standards are taken from the current data set, identified by a solution naming prefix that is defined when the evaluation method is created. Prefixes may be up to 10 characters long and are case-sensitive. Leading and trailing spaces in the prefix are ignored. Any sensorgram where either the analyte or ligand name starts with the prefix will be used as a standard. Standard assignment can be changed by editing the solution names in the keyword table before applying the evaluation method.

An evaluation method may only contain one sensorgram comparison item. If there are several comparison items in the evaluation session, the item to include in the evaluation method is selected when the method is created.

11.9.2 Applying sensorgram comparison from an evaluation method

When an evaluation method containing a sensorgram comparison item is applied, standard sensorgrams saved in the evaluation method are appended to the sensorgrams from the opened result file(s) and used as standards for the comparison. Alternatively, standards are identified by the solution name prefix as defined in the method. The comparison items are then created and completed if possible.

Note: *The choice of how standards are handled is made when the evaluation method is created, and cannot be changed except by creating a new method.*

A message is displayed if the sensorgrams in the opened result files cannot be compared with the standards (for example, multiple injection sensorgrams cannot be compared with single-cycle standards).

The ***Create sensorgram comparison*** dialog (Section 11.7.1) is not accessible for comparison items created from an evaluation method. All sensorgrams in the evaluation session will be included in the sensorgram comparison. Curve sets from assay steps with purpose ***Sample*** that are not identified as standards by a naming prefix are compared as samples. Those from assay steps with purpose ***Control sample*** are compared as controls.

12

Principles of kinetics and affinity analysis

Biacore T200 offers several functions for evaluating kinetic and affinity data:

- Screening tools, focused on dealing with a large number of interactions in one evaluation session (Chapter 13).
- Detailed kinetic and affinity analysis on the sensor surface. In both screening and detailed analysis, kinetic parameters are evaluated from the association and dissociation phases of the sensorgram, and affinity either from the kinetic parameters or from plots of steady-state analyte binding levels (R_{eq}) against concentration (Chapter 14).
- Thermodynamic analysis, which analyzes the dependence of either kinetics or affinity on variations in temperature (Chapter 15).
- Affinity in solution, where the interactants are mixed in known concentrations in solution and allowed to reach equilibrium. Biacore T200 is then used to determine the concentration of free interactant in equilibrium with the complex (Chapter 16).

This chapter describes the principles and features of kinetic and affinity evaluation that are common to screening and detailed analysis.

Kinetic constants derived from screening or detailed analysis in multiple separate experiments can be collated and summarized in the separate Biacore T200 Kinetics Summary software (Section 12.6).

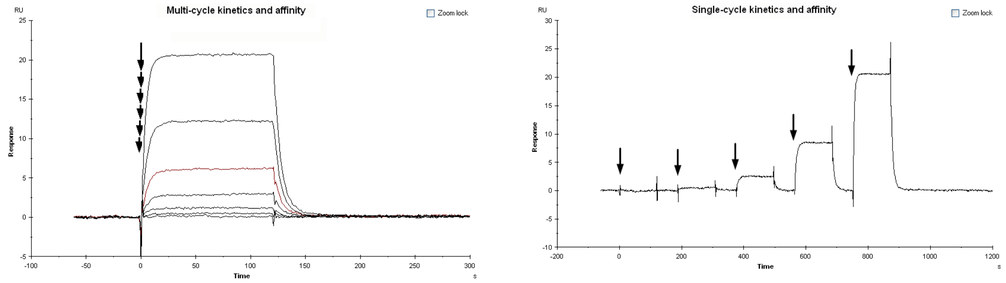


Figure 12-1. In multi-cycle kinetics and affinity determinations, each sample is injected in a separate cycle. The concentration series is presented as an overlay plot aligned at the start of the injection in the evaluation software. In single-cycle determinations, the samples are injected sequentially in the same cycle. Arrows in the illustrations mark the start of sample injections.

Kinetics and affinity are normally determined from the binding characteristics of a series of analyte concentrations. These 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 in Figure 12-1. Results from these two approaches are evaluated in the same way, using the same tools and fitting models, and may even be evaluated together in a single evaluation.

12.1 Requirements for kinetics and affinity evaluation

The absolute minimum requirements for evaluation of kinetics or affinity are one cycle with a ***Sample*** injection in an assay step with purpose ***Sample***, and with the sample concentration in the keyword ***Conc***. If the concentration is not given in molar-based units, the keyword ***MW*** must also be included with a value for the molecular weight. For method-based runs, the method must be correctly constructed as described in Section 5.10.3: if necessary, the keyword table can be edited after the run is completed so that the conditions are met in full (see Section 6.6). Note however that the command type cannot be edited in the keyword table. Refer to Chapter 5 for a description of how to construct methods in Method Builder.

The recommended minimum conditions for detailed kinetic and affinity analysis are:

- a concentration series of analyte with at least four non-zero concentrations
- at least one blank cycle consisting of zero concentration sample (for single-cycle kinetics the blank cycle must replicate the sequence of injections in the analysis cycle)
- for multi-cycle kinetics, duplicate determinations for at least one non-zero concentration.

These conditions are recommended but not mandatory in the ***Kinetics/Affinity*** wizard.

Kinetic screening is often performed using fewer analyte concentrations (typically 2 or 3) and without duplicates. Affinity screening, like detailed affinity analysis, requires at least 3 (recommended 4) analyte concentrations to provide sufficient data for evaluation. Blank cycles are recommended, although the same cycle may be used as a blank for several different analytes.

12.2 Curve fitting principles

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 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.

12.2.1 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. Equations may be created automatically from the definition of the interaction model or entered as mathematical expressions. 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 (see Section 12.4.2) for the fitting.

In some situations, the fitting algorithm may be unable to find a fit for the experimental data with the initial parameter values as specified in the model. This may happen typically if the concentration unit is incorrect: for example if the unit is set to mM instead of nM in the keyword table. On occasion, however, it can be necessary to adjust the starting values for fitting parameters, accessed through the ***Parameters*** button.

12.2.2 Local and global parameters

Parameters in the fitting equations are treated as either ***local*** or ***global variables*** or ***constants***:

- Local parameters are assigned an independent value for each curve in the data set (or sample injection in single-cycle kinetics). Typical local parameters are concentration (which is different for different curves) and bulk refractive index contribution (which may be expected to vary between curves).
- Global parameters have one single value that applies to the whole data set. Typical global parameters are the rate constants for the interaction, which should normally have the same value for all curves in the data set.
- Constants have a fixed value that is not changed in the fitting procedure. An example is the analyte concentration. Constants may also be local (separate values for each curve) or global (one value for the whole data set).

The local/global status of parameters can be changed through the ***Parameters*** button, without making changes to the model definition.

Evaluating kinetics or affinity 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.

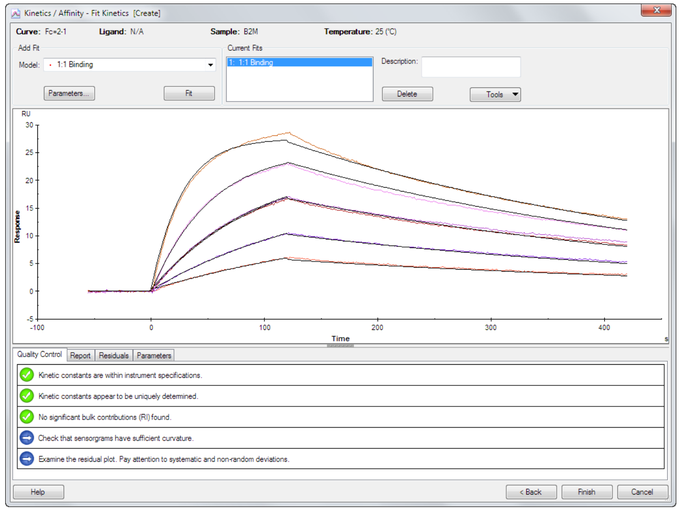
In general, rate constants should be fitted as global parameters and bulk refractive index contribution as a local parameter. The analyte binding capacity of the surface R_{max} is a global parameter by default in the predefined models: this assumes that the ligand activity is unchanged between cycles in the assay. It is however justified to use a local R_{max} if there is reason to believe that the ligand activity may vary between cycles (e.g. in a capture assay, if the capture level varies between cycles).

The local/global status is not relevant for affinity determination, since this evaluation fits the model to a single curve of response against analyte concentration.

12.3 Presentation of results

12.3.1 Kinetics results

Results for kinetics are displayed as fitted curves overlaid in black on the experimental data, with details in the panel below the curves:

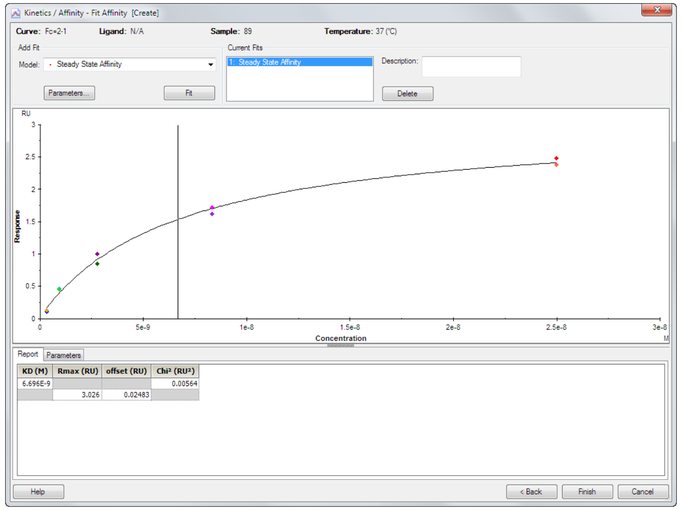


The detailed results are presented on four tabs:

- ***Quality control*** summarizes important aspects of the quality of the fitting, as an aid to judging the reliability of the reported results. The quality control criteria are discussed in Section 12.4.1. This tab is only shown for kinetic evaluations using the predefined 1:1 model.
- ***Report*** shows selected parameters and calculated values. The contents of the ***Report*** tab are defined in the model. Global parameters are listed on a single row at the top of the table, and local parameters are listed on a separate row for each curve.
- ***Residuals*** plots the difference between the experimental and fitted curves for each point in the curves. Use this display as an aid in judging how closely the results fit the experimental data.
- ***Parameters*** shows the values for all parameters in the fitting equations.

12.3.2 Affinity results

For affinity determination using a 1:1 interaction model, the equilibrium constant K_D is the same as the analyte concentration at a response equal to half R_{max}. The reported K_D value is marked on the plot as a vertical line. If the reported value is higher than half the highest concentration used, this line will be shown broken in red as a warning that the value may be unreliable because the plot does not flatten out sufficiently.



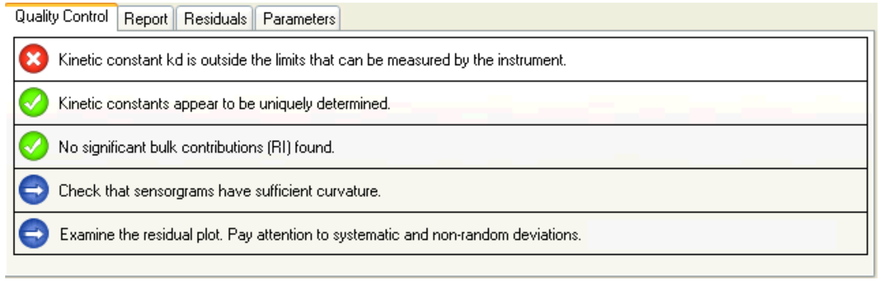
The detailed results are presented on two tabs:

- ***Report*** shows selected parameters and calculated values. The contents of the ***Report*** tab are defined in the model. Global parameters are listed on a single row at the top of the table, and local parameters are listed on one row for each curve.
- ***Parameters*** shows the values for all parameters in the fitting equations.

12.4 Quality assessment for kinetics evaluation

12.4.1 The Quality Control tab

The ***Quality Control*** tab in the kinetic evaluation results (for evaluations using the predefined 1:1 model only) gives a brief overview of the reliability of the results. If you prefer, you can hide the quality control tab by setting the appropriate option in ***Tools:Preferences*** on the main menu.



The symbols used on this tab have the following meanings:

	(Green)	Pass: quality assessment acceptable.
	(Yellow)	Warning: quality assessment close to the limits of acceptability
	(Red)	Fail: quality assessment unacceptable
	(Blue)	User assessment recommendations

The quality of the fitting is assessed in five areas:

Magnitude of kinetic constants

If either association or dissociation rate constants are close to or outside the limits that can be determined in the instrument, this will be reported. For values close to the limit, judge the validity of the results on other assessment criteria as described in this chapter.

Parameter uniqueness

In some situations, it may be possible to determine a value for two or more parameters in combination without being able to determine unique values for the individual parameters. Such parameters are said to be *correlated*. One example is the pair of kinetic rate constants k_a and k_d, that are related through the affinity constant K_D ($K_D = k_d/k_a$): it may be possible to determine the affinity constant reliably without being able to resolve the individual rate constants.

Parameter uniqueness is assessed by testing correlation between pairs of the parameters k_a, k_d and R_{max}. If significant correlation is found, this will be reported as a warning that parameters cannot be uniquely determined.

Note: *This test does not explore all possible parameter correlations. A Pass status for this test is not a fail-safe indication that parameters are uniquely determined.*

The ***Check Kinetic Data*** tool (Section 12.4.4) provides a visualization of potential correlation between k_a and k_d.

Bulk refractive index

After reference subtraction and blank subtraction, sensorgrams for kinetic evaluation should not in principle contain any bulk refractive index shifts (parameter ***RI*** in predefined models). However, there may be some circumstances where small bulk refractive index shifts may remain in reference- and blank-subtracted data. On the other hand, the fitting algorithm tends to interpret rapid interaction events (incorrectly) as bulk shifts. If the fitting returns significant values for ***RI***, a warning will be issued in the quality control tab.

Examine the sensorgrams and fitted curves to determine whether bulk shifts as reported by the fitting are true or false. In cases where reported bulk shifts are unreasonably large, you may want to set ***RI*** to a constant value of zero in the ***Parameters*** setting for the fitting. If you do this, the bulk contributions component in the quality control tab will be reported as neutral since the ***RI*** parameter was not evaluated.

Sensorgram curvature

You should check that the sensorgrams have sufficient curvature for kinetic determination. Ideally, the sensorgrams for at least the one or two highest concentrations should show measurable binding rates at the beginning of the sample injection and approach a steady state towards the end of the injection. Sensorgrams that approximate to "square-wave" pulses (indicating rapid association and dissociation) and those that do not flatten out during the injection generally do not contain sufficient kinetic information for reliable evaluation. Ideally, the dissociation phase should be long enough to monitor a fall in response of at least 10-15% of the starting value.

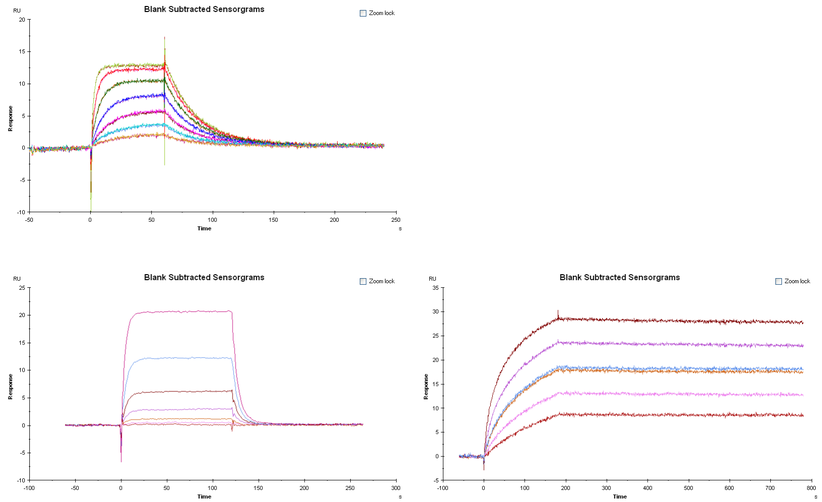


Figure 12-2. Examples of sufficient and insufficient sensorgram curvature. Top: Ideal sensorgrams approaching steady state during sample injection and returning to baseline during dissociation. Bottom left: Rapid interaction approaching "square wave" appearance. These sensorgrams return rate constants close to the limit of measurement for the instrument. Bottom right: Slow association and dissociation, giving insufficient curvature in both association and dissociation phases. Evaluation is possible but will not be very reliable.

If the interaction is too fast to provide kinetic information, you may only be able to determine affinity constants. Interactions that do not flatten out sufficiently during the injection or dissociate sufficiently during the dissociation phase may sometimes be analyzed by prolonging the association or dissociation phase respectively.

Residuals

You should check that the residuals (the difference between experimental and fitted value for each data point in the sensorgrams) lie within reasonable limits. For a perfect fit, the residuals reflect the short-term noise in the sensorgrams and scatter around zero (typically ±1-2 RU). Systematic deviations, seen as a definite shape in the residual plot, indicate that the interaction model is to a greater or lesser extent unsuitable for the interaction. As an aid in judging the residuals, guidelines are drawn on the residual plot to indicate the range of acceptability. Most of the residuals should be within the inner (green) limits.

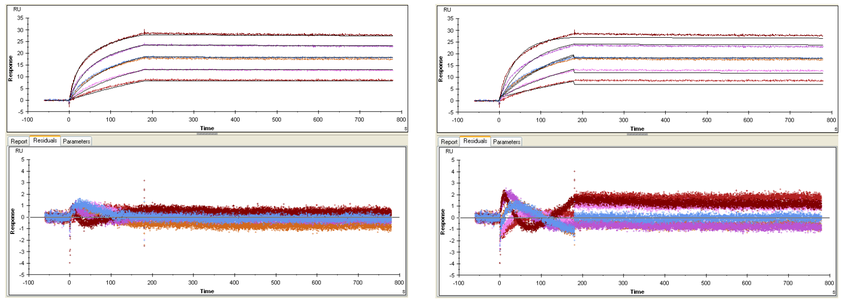


Figure 12-3. The residuals for a good fit (left) scatter around 0, ideally in a random distribution representing the noise in the sensorgrams. For a poor fit (right) the residual curves show a definite shape and deviate farther from 0.

The guideline positions are calculated in relation to the response range of the sensorgrams. The guidelines are only shown for evaluations using the predefined 1:1 model (i.e. when the quality control tab is included).

IMPORTANT!

Use the ***Quality Control*** assessment as a help in making your own judgement of the results. ***Pass*** status in the quality control parameters does not necessarily indicate that the fit is acceptable or that the results are biologically relevant. On the other hand, ***Fail*** status in any of the parameters is a reliable warning indicator.

Base your assessment on the overall quality of the results and the fitting, taking all quality control parameters into account.

12.4.2 Statistical parameters

Three statistical parameters are provided to help in assessing the results:

- *chi-square* is an indicator of how closely the fitted curves agree with the experimental data. One chi-square value is reported for the whole fitting.
- *Standard error* or *T-value* is an indicator of parameter significance, and is reported separately for each fitted parameter.
- *U-value* (calculated for the predefined 1:1 model only) is an overall indication of the uniqueness of the parameter values.

Chi-square

Chi-square is a measure of the average squared residual (the difference between the experimental data and the fitted curve), calculated as:

$$\text{chi-square} = \frac{\sum_{1}^{n} (r_f - r_x)^2}{n - p}$$

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

For sensorgram data, the number of data points is very much larger than the number of fitted parameters in the model, so

$$n - p \approx n$$

and chi-square reduces to the average squared residual per data point. If the model fits the experimental data precisely, chi-square represents the mean square of the signal noise.

Chi-square is listed on the ***Report*** tab.

Note: *Chi-square cannot be calculated for affinity determinations with three concentrations and are reported as* ***N/A****.*

Standard error and T-value

The significance of parameter values is indicated by the standard error (SE) or T- value listed on the ***Parameters*** tab in the fitting results. Lower standard error values indicate higher significance: if the standard error represents less than 10% of the parameter value, the parameter is significant for the experimental data.

For ease of comparison between parameters with widely different absolute values (e.g. k_a and k_d), the standard error may be expressed as a ***T-value***, which is obtained by dividing the value of the parameter by the standard error. A high T-value corresponds to a low standard error. As a general guideline, parameters with a T-value greater than about 10 should be regarded as significant.

The choice of whether to display parameter significance as standard error or T- value is made on the ***Fit*** tab of the ***Tools:Preferences*** dialog.

The significance of a parameter is a measure of how much 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. Typically (but not always), parameters with a low significance have unreasonable values: for example typical values for the mass transfer constant for proteins are around 10^8 $RU \cdot M^{-1}s^{-1}$, but evaluation of data with no mass transfer limitation might return a value of 10^{12} or higher. Similarly, rate constants that lack significance are often assigned values outside the reasonable range for biomolecular interactions, or outside the range that can be measured with the instrument.

Notes: *Even if parameters with low significance can have a wide range of values without affecting the fit, repeated evaluation of the same data set will always return the same value. Consistency of a value between repeated evaluations is* ***not*** *a test of significance.*

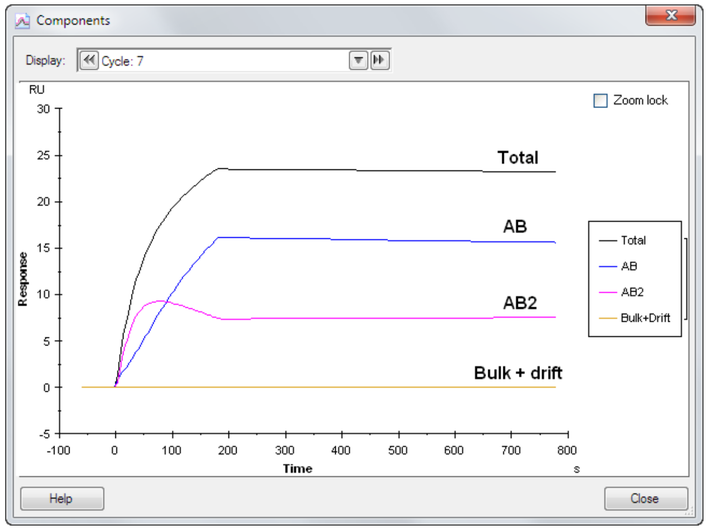
The standard error and the ***Check Kinetic Data*** *tool assess parameter significance in different ways, even if the results of the assessment may sometimes be related.* ***Check Kinetic Data*** *tests the contribution of a group of parameters (rate constants for the interaction and mass transport processes) to the closeness of fit by examining the results of correlated changes, whereas the standard error is a mathematical assessment of the significance of a single parameter. If the* ***Check Kinetic Data*** *tool indicates that the rate constants are not significant, the standard error for the constants may be expected to be high. However, the converse is not always true (a high standard error will not always be reflected in the behavior of the* ***Check Kinetic Data*** *tool).*

U-value

The U-value is an estimate of the *uniqueness* of the calculated values for rate constants and R_{max}. If parameters are correlated (see Section 12.4.1), 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 on the ***Report*** tab as a single value for the whole fitting. U-values above about 25 indicate that absolute values for two or more of the parameters (rate constants and R_{max}) are correlated and cannot be determined. If the U-value is below about 15 the parameter values are not significantly correlated.

12.4.3 Components of the fit

Choose ***Tools:Components*** from the fitted results window to display a plot showing the contribution of components in the interaction model to the fitted curve. Choose which cycle to display in the selector bar.



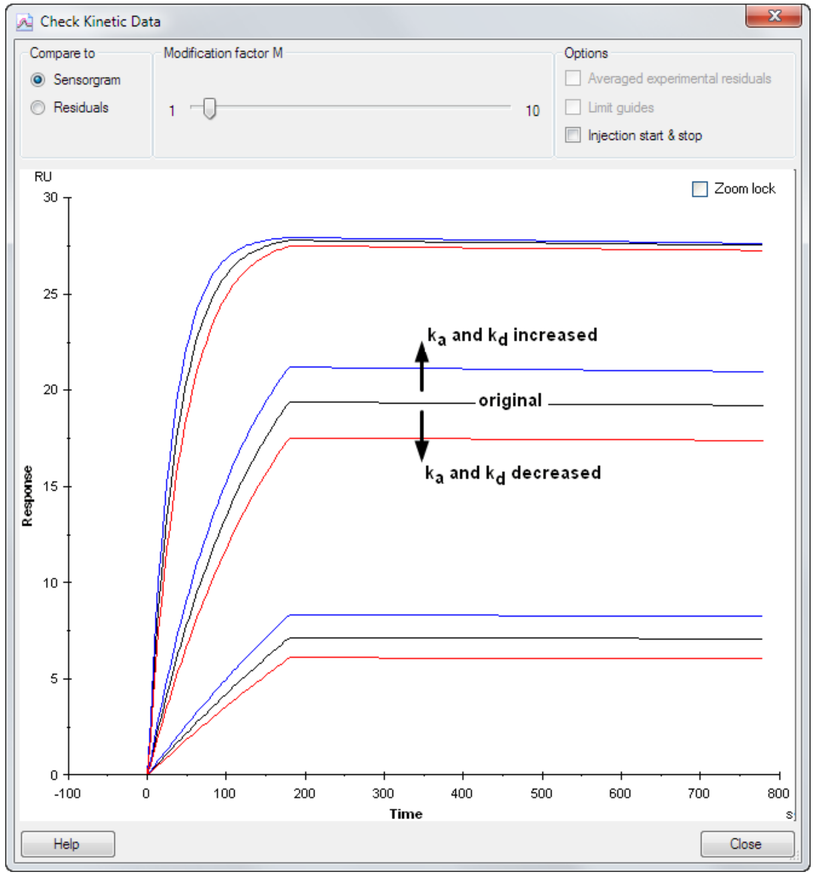
The example illustrated here is taken from a fitting to a bivalent analyte model (see Section 12.7.2), and shows clearly how the component AB2 (analyte attached to the surface through both binding sites) is displaced by AB as the interaction progresses.

12.4.4 Check kinetic data

Kinetic constants obtained from the fitting procedure are only significant if the observed binding is not seriously limited by mass transport of analyte to the surface (see Section 12.7). For 1:1 fitting results, you can check whether mass transport is limiting or not using the ***Check Kinetic Data*** function.

Choose ***Tools:Check Kinetic Data*** to open a dialog that displays simulated sensorgrams based on the fitting results, with the interaction rate constants k_a and k_d varied in parallel (so that the affinity constant, remains unchanged). If curves do not shift as values for k_a and k_d are changed, this means that the actual values are not important for the fitting, and the curves do not contain kinetic information. Conversely, if the simulated curve shape changes as the values of k_a and k_d are varied, the fitting is dependent on the actual values and the curves do contain kinetic information.

This tool is only available for results obtained with the 1:1 fitting model.



(This example illustrates a fitting with only three concentrations for clarity.)

To use the tool, drag the slider for the modification factor ***M*** and observe the behavior of the curve display. The original curves (which remain unchanged as you drag the slider) are shown in black: blue curves show the simulation for k_a and k_d multiplied by ***M***, while red curves show the simulation for k_a and k_d divided by ***M***. If the red (reduced rate constants) and blue (increased rate constants) curves clearly diverge from the original curves, the fitting is sensitive to changes in the rate constants and the curves probably contain significant kinetic information. If on the other hand the divergence is negligible, the values of the rate constants do not matter because the binding is fully limited by mass transfer. Mass transfer places an upper limit on the rate constants that can be measured: on the borderline, the fitting is sensitive to a reduction in rate constants but not to an increase.

Choose the ***Residuals*** option in the ***Compare to*** frame to examine the effect of varying the modification factor on the difference between the original and modified curves in relation to the experimental residuals. The tool display allows the simulated difference curves to be compared to the experimental residuals or to residuals averaged over a moving time window. The latter option smooths the experimental residual display, making it easier to observe the general shape of the residual curves. Movable horizontal ***Limit guides*** can be displayed to mark the extent of the residual variation and aid visual interpretation. (Note that the limit guides are not related to the guidelines shown on the residual tab for QC purposes (Section 12.4.1), and do not in any way imply acceptance limits.)

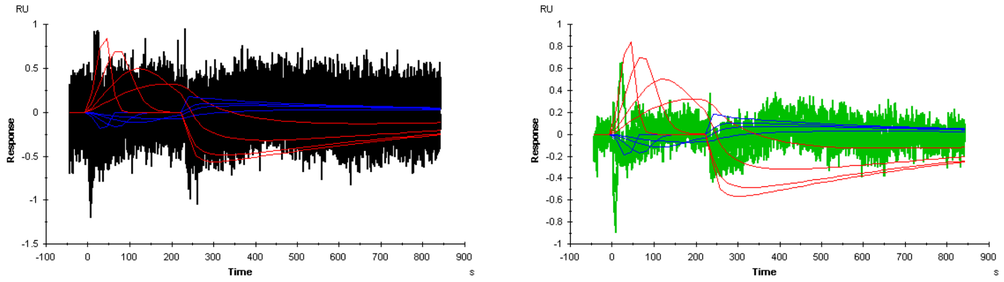


Figure 12-4. Kinetic data check comparison to residuals (left) and averaged residuals (right).

12.5 Quality assessment for affinity evaluation

Steady state affinity evaluations are performed by fitting a plot of R_{eq} against concentration C to a model representing equilibrium 1:1 binding. The closeness of fit is reported as a chi-square value, calculated in the same way as for kinetics. Note however that the number of points in the steady state affinity plot is very much lower than for kinetic evaluation, so that chi-square is a more sensitive indicator of fitting quality.

The plot of R_{eq} against C approaches a limiting value (equivalent to R_{max}) at very high concentrations. Robust evaluation of the data requires either that the plot shows sufficient curvature for reliable estimation of R_{max} or that a valid constant value is provided for R_{max}. As a rule of thumb, evaluation with variable R_{max} is acceptable only if the calculated K_D value is less than half the highest analyte concentration used. (For a 1:1 interaction, the K_D value is equal to the analyte concentration that gives 50% saturation of the binding sites, so that R_{eq}=0.5R_{max}. In other words, reliable evaluation is only obtained if the surface is more than 50% saturated at the highest analyte concentration.)

To help in this assessment, the calculated K_D value is indicated as a vertical line at the corresponding analyte concentration. The line is red and broken if the value is greater than half the highest concentration.

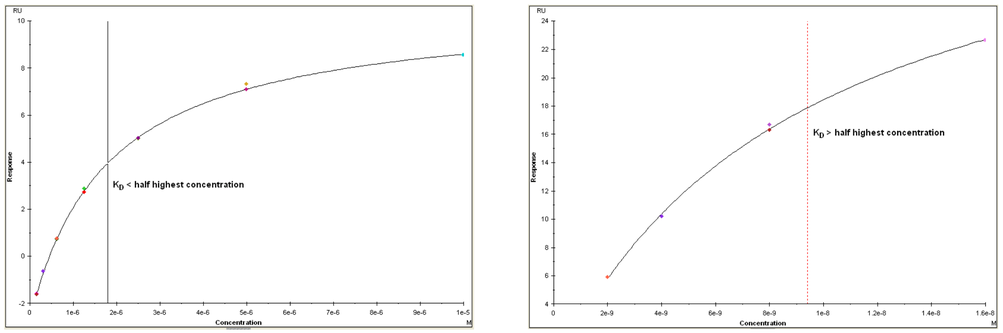


Figure 12-5. For reliable evaluation, the calculated K_D value should be less than half the highest analyte concentration used. When this condition is met, the K_D value is indicated with a full black line in the result plot (left). When the condition is not met, the K_D value is indicated with a broken red line.

For weak interactions where it is not possible to use concentrations approaching 0.5R_{max}, evaluation using a model with constant R_{max} (Section 12.7.6) may give more reliable results. This approach requires that R_{max} is determined separately, for example using high concentrations of a known binder.

12.6 Summarizing kinetics and affinity results

You can summarize the kinetics and affinity evaluation results from several saved evaluation files or from several evaluation items from the same file using the separate Biacore T200 Kinetics Summary software (this is installed automatically together with the Evaluation Software).

12.6.1 Creating kinetics summaries

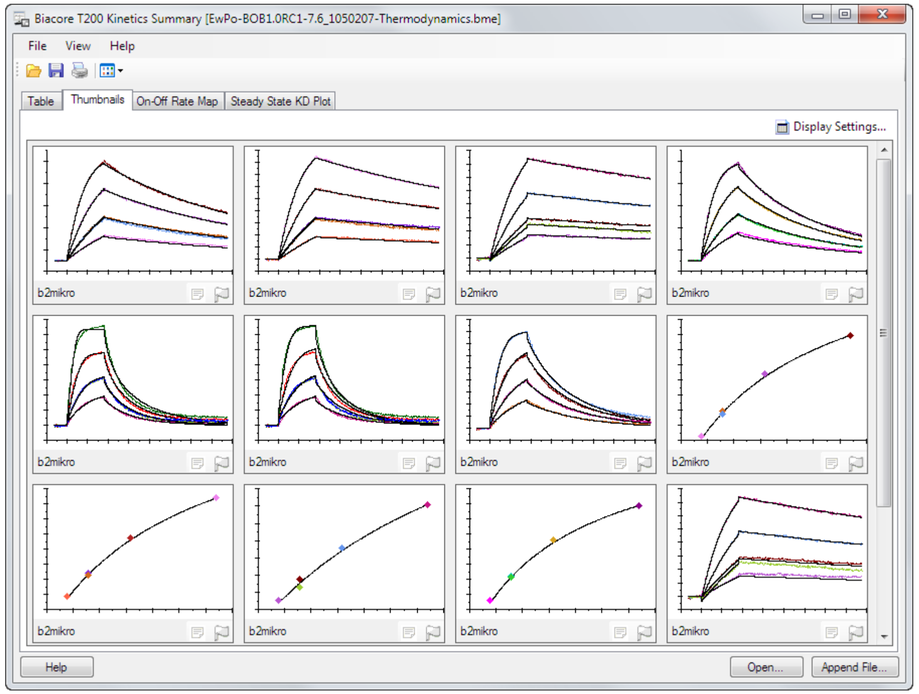
To create a kinetics summary of the results in a single evaluation file, simply open the evaluation file (file type **.bme**) in the Kinetics Summary software. The Kinetics Summary software can be started from the Windows start menu or from the ***Tools*** menu in the Evaluation Software.

Note: *Most of the functions in Kinetics Summary are duplicated in the kinetics and affinity screening tools.* ***Kinetics Summary*** *can however be useful for presenting the combined results of several screening evaluations.*

To create a summary of the results in multiple files, you can either select multiple files within the same folder in the ***Open*** dialog box using **Ctrl-click** and/or **Shift- click**, or use ***File:Append File*** to add results to an existing summary. You can open saved summary files (file type **.bks)** in addition to evaluation files.

12.6.2 Basic summary presentation

The summarized kinetic and affinity results are presented as thumbnail plots of the kinetics and evaluation items and as a table of result data. Multiple fits in the same item in the Evaluation Software are presented as separate fits in the summary.



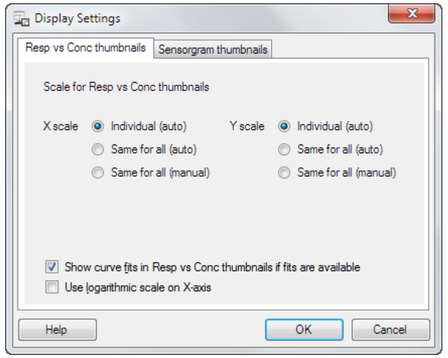
Thumbnail types

Thumbnails can be displayed as small, standard or extended diagrams, selected from the ***View*** menu.

Small	Provides an overview of many thumbnails for general comparison. Details will not be legible in the thumbnails. Individual thumbnails are identified in a tool tip.
Standard	Shows the thumbnails with identification of the interaction.
Extended	Shows more detailed identification of each thumbnail with fitting model and rate or affinity constants.

Thumbnail display settings

Choose ***Display Settings*** from the ***View*** menu or click the ***Display Settings*** button for display settings for the thumbnails. Settings for affinity (Resp vs Conc) and kinetic (sensorgram) thumbnails are set independently.



If you choose to use the same x- and/or y-axis scales, the scale(s) will be chosen to include the widest range in the currently included set of thumbnails. If scaling is set to ***Individual***, each thumbnail will be scaled according to the range of data in the item.

Check ***Show curve fits...*** to display the fitted curves overlaid on the experimental data in the thumbnails. This may not have any significant effect on the appearance of small thumbnails for kinetic evaluation items, but will in general be readily visible for affinity items.

Note: *Thumbnails retain any annotations and status assigned in the original kinetic and affinity screen items. Annotations and status are not supported for detailed kinetic and affinity evaluation.*

Sorting items

Choose ***Arrange by*** from the right-click menu in the thumbnail display to sort the items. The list of sorting parameters corresponds to the columns on the ***Table*** tab.

Sorting the thumbnail display also sorts the rows in the ***Table*** tab and *vice versa*. You can sort the rows directly in the table according to the contents of a column by clicking on the column header. Clicking repeatedly on the column header toggles the sort order.

Note: *The **Arrange by** option on the **Thumbnails** tab sorts the thumbnails and table rows initially in ascending order. Options for Ascending and Descending order appear in the menu once the thumbnails have been sorted.*

Hiding items

To exclude an evaluation from the summary, remove the checkmark from the ***Show*** column on the ***Table*** tab. You can also hide kinetic evaluation items using the right-click menu in the on-off rate map (see Section 12.6.3). Use the ***Show*** checkbox in the table or the right-click menu in graphical presentations to restore hidden evaluations.

Evaluation item details

Double-click on a thumbnail or table row or choose ***Evaluation details*** from the right-click menu to display the details of the evaluation results (see Section 12.3). You can also display evaluation details for a selected point in the on-off rate map using the right-click menu (see Section 12.6.3).

Note: *You can only display the evaluation details for one item at a time.*

Copying and exporting summaries

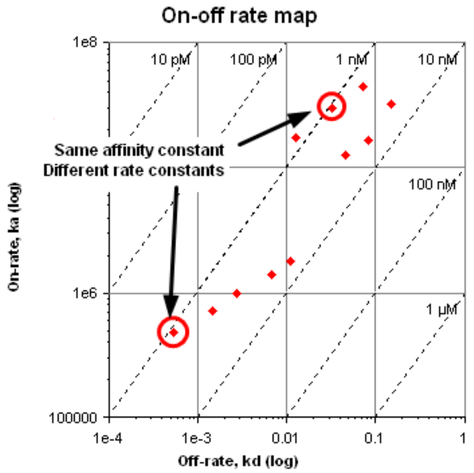
From the right-click menu on the ***Thumbnail*** tab, you can copy the single series or all graphs or thumbnails to the Windows clipboard as a graphic object for pasting into other programs. Copying a graph copies only the graph itself: copying a thumbnail copies the graph together with the additional information as shown on the screen. Copying all items copies all the included items in the summary (regardless of how many are currently visible on the tab) as a single graphic object, arranged as in the display.

You can also export the curve data from the currently selected item to a tab- separated text file for import into third-party software. The exported file includes data for the fitted curves if they are displayed in the summary.

From the Table tab, you can copy the table data for single rows or the complete table as text to the Windows clipboard.

Note: *The header row is not included when single rows are copied.*

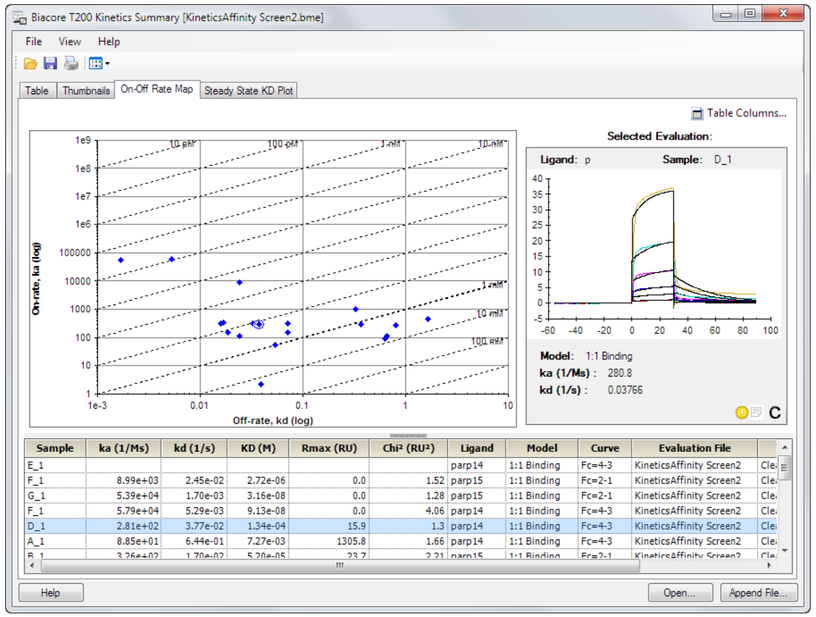
12.6.3 On-off rate maps



On-off rate maps (also called *ka/kd plots*) provide an overview of kinetic and affinity properties for the interactions in the summary by plotting the association rate constant k_a against the dissociation rate constant k_d, both on logarithmic scales. Since the affinity constant K_D is the ratio of k_d to k_a, interactions that have the same affinity will appear on diagonal lines representing the K_D value. The diagonals are shown as broken lines on the plot with the K_D value indicated. Points that lie widely separated on the same diagonal represent interactions with the same affinity but different kinetics.

Choose the ***On-Off Rate Map*** tab to view the rate map for the current kinetics summary. Only items with single sets of values for kinetic rate constants are represented in the plot. The plot does not therefore include steady state affinity items or kinetic evaluation items with multiple sets of kinetic constants, such as evaluation with heterogeneous models.

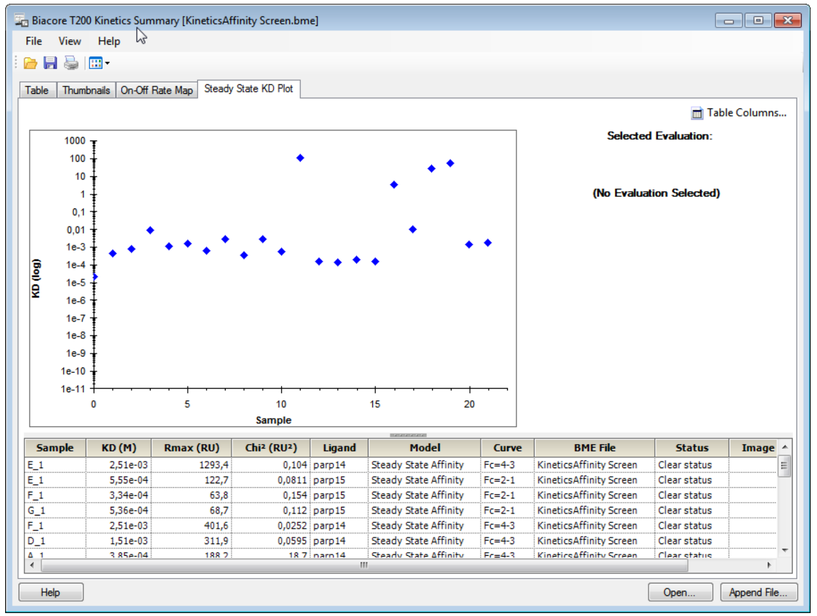
Click on a point in the on-off rate map or select a row in the table below the map to display the thumbnail for the evaluation.



Right-click in the on-off rate map for display options and copy and export functions.

12.6.4 Steady state KD plots

Steady state KD plots show the equilibrium dissociation constant K_D plotted against sample for steady state affinity screen and evaluation data. K_D values obtained from kinetics are not included. Duplicate sample series are plotted as separate samples.



12.7 Predefined models

A set of predefined models for kinetics and steady state affinity is provided with Biacore T200 Evaluation Software. These models are marked in the model selection list with a red dot, and cannot be removed or modified.

Mass transfer parameters

All kinetic models include a term for mass transfer of analyte to the surface. If transport is slow compared with binding of analyte to the ligand, the transport process will limit the observed binding rate, at least partially. All models take account of this potential limitation and can extract rate constants from the data provided that mass transfer is not totally limiting (see Section 12.7).

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

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

where D is the diffusion coefficient of the analyte ($m^2 \cdot s^{-1}$)

f is the volume flow rate of solution through the flow cell ($m^3 \cdot s^{-1}$)

h, w, l are the flow cell dimensions (height, width, length in m)

One form used in fitting models in Biacore T200 is referred to as the ***mass transfer constant*** k_t (units $RU \cdot M^{-1} \cdot s^{-1}$), obtained by adjusting the mass transfer coefficient approximately for the molecular weight of the analyte and for the conversion of surface concentration to RU:

$$k_t = k_m \times MW \times G$$

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.

A further modification of this expression gives the ***flow rate-independent component*** of the mass transfer constant (units $RU \cdot M^{-1} s^{-2/3} m^{-1}$), referred to as ***tc*** in the models:

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

12.7.1 Kinetics – 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:

$$A + B = AB$$

Model parameters		Obtained from
k_a	Association rate constant ($M^{-1}s^{-1}$)	Fitted
k_d	Dissociation rate constant (s^{-1})	Fitted
R_{max}	Analyte binding capacity of the surface (RU)	Fitted
Conc	Analyte concentration (M)	Input
t_c	Flow rate-independent component of the mass transfer constant	Fitted
f	Flow rate (µLmin)	Input
tOn	Sample injection start time (s)	Input
tOff	Sample injection end time (s)	Input
RI	Bulk refractive index contribution in the sample	Fitted

Report parameters		Calculated as
k_a	Association rate constant ($M^{-1}s^{-1}$)	k_a
k_d	Dissociation rate constant (s^{-1})	k_d
K_D	Equilibrium dissociation constant (M)	k_d/k_a
R_{max}	Analyte binding capacity of the surface (RU)	R_{max}
Conc	Analyte concentration (M)	Conc
t_c	Flow rate-independent component of the mass transfer constant	t_c
Flow	Flow rate (µL/min)	f
k_t	Mass transfer constant	$t_c \times f^{1/3}$
RI	Bulk refractive index contribution in the sample	RI

12.7.2 Kinetics – 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.

$$A + B = AB$$
$$AB + B = AB_2$$

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 conversion factor between RU and M is available. Similarly, a value for the overall affinity or avidity constant is not reported.*

Model parameters		Obtained from
k_{a1}	Association rate constant for the first site ($M^{-1}s^{-1}$)	Fitted
k_{a2}	Association rate constant for the second site ($RU^{-1}s^{-1}$)	Fitted
k_{d1} k_{d2}	Dissociation rate constant for the first and second sites (s^{-1})	Fitted
R_{max}	Analyte binding capacity of the surface (RU)	Fitted
Conc	Analyte concentration (M)	Input
t_c	Flow rate-independent component of the mass transfer constant	Fitted
f	Flow rate (µL/min)	Input
tOn	Sample injection start time (s)	Input
tOff	Sample injection end time (s)	Input
RI	Bulk refractive index contribution in the sample	Fitted

Report parameters		Calculated as
k_{a1}	Association rate constant for the first site ($M^{-1}s^{-1}$)	k_{a1}
k_{a2}	Association rate constant for the second site ($RU^{-1}s^{-1}$)	k_{a2}
k_{d1} k_{d2}	Dissociation rate constant for the first and second sites (s^{-1})	k_{d1} k_{d2}
R_{max}	Analyte binding capacity of the surface (RU)	R_{max}
Conc	Analyte concentration (M)	Conc
t_c	Flow rate-independent component of the mass transfer constant	t_c
Flow	Flow rate (µL/min)	f
k_t	Mass transfer constant	$t_c \times f^{1/3}$
RI	Bulk refractive index contribution in the sample	RI

12.7.3 Kinetics – Heterogeneous Analyte

This model is intended for analysis of the kinetics of interaction of mixtures of two analytes that compete for the same ligand site. Experiments of this kind can be used to deduce kinetic parameters for a low molecular weight analyte that gives a small response from measurements of binding of a competing high molecular weight analyte. Response contributions from both analytes are taken into account, although the high molecular weight analyte is responsible for the dominant component in the observed sensorgrams.

Concentrations and molecular weights are required for both analytes. If absolute molecular weights are not known, relative values can be entered without affecting the outcome of the fitting. The model cannot evaluate interactions where the proportions and relative sizes of the analytes are unknown.

A1 + B = A1B
A2 + B = A2B

Model parameters		Obtained from
k_{a1} k_{a2}	Association rate constant for the first and second analytes ($M^{-1}s^{-1}$)	Fitted
k_{d1} k_{d2}	Dissociation rate constant for the first and second analytes (s^{-1})	Fitted
Conc1 Conc2	Concentration of the first and second analytes (M)	Input
mw1 mw2	Molecular weights of the first and second analytes	Input
t_{c1} t_{c2}	Flow rate-independent component of the mass transfer constant for the first and second analytes	Fitted
R_{max1} R_{max2}	Analyte binding capacity of the surface for the first and second analytes (RU)	Fitted
rcf	Response correction factor, allowing for different refractive index contributions for the two analytes. This factor is defined as ($R_{max}1/R_{max}2$)/ (MW1/MW2).	Fitted
f	Flow rate (µL/min)	Input
tOn	Sample injection start time (s)	Input
tOff	Sample injection end time (s)	Input
RI	Bulk refractive index contribution in the sample	Fitted

Report parameters		Calculated as
k_{a1} k_{a2}	Association rate constant for the first and second analytes ($M^{-1}s^{-1}$)	k_{a1} k_{a2}
k_{d1} k_{d2}	Dissociation rate constant for the first and second analytes (s^{-1})	k_{d1} k_{d2}
K_{D1} K_{D2}	Equilibrium dissociation constant for the first and second analytes (M)	k_{d1}/k_{a1} k_{d2}/k_{a2}
R_{max1}	Analyte binding capacity of the surface for the first analyte (RU)	$R_{max2} \times rcf$
R_{max2}	Analyte binding capacity of the surface for the second analyte (RU)	R_{max2}
Conc1 Conc2	Analyte concentration (M)	Conc1 Conc2
t_{c1} t_{c2}	Flow rate-independent component of the mass transfer constant for the first and second analytes	t_{c1} t_{c2}
Flow	Flow rate (µL/min)	f
k_{t1} k_{t2}	Mass transfer constants for the first and second analytes	$t_{c1} \times f^{1/3}$ $t_{c2} \times f^{1/3}$
RI	Bulk refractive index contribution in the sample	RI

12.7.4 Kinetics – 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. Unlike the case of heterogeneous analyte, the relative amounts of the two ligands does not have to be known in advance.

Heterogeneous ligand situations frequently arise in practice through heterogeneous immobilization of ligand (e.g. amine coupling of proteins, where the ligand has multiple attachment points), as well as through heterogeneity in the ligand preparation itself. In cases where the heterogeneous ligand model is found to give the best fit to the observed sensorgrams, further experimental efforts to reduce the heterogeneity are recommended where possible.

A + B1 = AB1
A + B2 = AB2

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.*

Model parameters		Obtained from
k_{a1} k_{a2}	Association rate constant for the first and second ligands ($M^{-1}s^{-1}$)	Fitted
k_{d1} k_{d2}	Dissociation rate constant for the first and second ligands (s^{-1})	Fitted
R_{max1} R_{max2}	Analyte binding capacity of the first and second ligands (RU)	Fitted
Conc	Analyte concentration (M)	Input
t_c	Flow rate-independent component of the mass transfer constant	Fitted
f	Flow rate (µL/min)	Input
tOn	Sample injection start time (s)	Input
tOff	Sample injection end time (s)	Input
RI	Bulk refractive index contribution in the sample	Fitted

Report parameters		Calculated as
k_{a1} k_{a2}	Association rate constant for the first and second ligands ($M^{-1}s^{-1}$)	k_{a1} k_{a2}
k_{d1} k_{d2}	Dissociation rate constant for the first and second ligands (s^{-1})	k_{d1} k_{d2}
K_{D1} K_{D2}	Equilibrium dissociation constants (M)	k_{d1}/k_{a1} k_{d2}/k_{a2}
R_{max1} R_{max2}	Analyte binding capacity of the first and second ligands (RU)	R_{max1}
Conc	Analyte concentration (M)	Conc
t_c	Flow rate-independent component of the mass transfer constant	t_c
Flow	Flow rate (µL/min)	f
k_t	Mass transfer constant	$t_c \times f^{1/3}$
RI	Bulk refractive index contribution in the sample	RI

12.7.5 Kinetics – Two State Reaction

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

$$A + B = AB = AB^*$$

Note that conformational changes in ligand or complex do not normally give a response in Biacore. 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.

Model parameters		Obtained from
k_{a1}	Association rate constant for analyte binding ($M^{-1}s^{-1}$)	Fitted
k_{d1}	Dissociation rate constant for analyte from the complex (s^{-1})	Fitted
k_{a2}	Forward rate constant for the conformational change (s^{-1})	Fitted
k_{d2}	Reverse rate constant for the conformational change (s^{-1})	Fitted
R_{max}	Analyte binding capacity of the surface (RU)	Fitted
Conc	Analyte concentration (M)	Input
t_c	Flow rate-independent component of the mass transfer constant	Fitted
f	Flow rate (µL/min)	Input
tOn	Sample injection start time (s)	Input
tOff	Sample injection end time (s)	Input
RI	Bulk refractive index contribution in the sample	Fitted

Report parameters		Calculated as
k_{a1}	Association rate constant for analyte binding ($M^{-1}s^{-1}$)	k_{a1}
k_{d1}	Dissociation rate constant for analyte from the complex (s^{-1})	k_{d1}
k_{a2}	Forward rate constant for the conformational change (s^{-1})	k_{a2}
k_{d2}	Reverse rate constant for the conformational change (s^{-1})	k_{d2}
K_D	Overall equilibrium dissociation constant (M)	$k_{d1}/k_{a1} \times (k_{d2}/(k_{d2}+k_{a2}))$
R_{max}	Analyte binding capacity of the surface (RU)	R_{max}
Conc	Analyte concentration (M)	Conc
t_c	Flow rate-independent component of the mass transfer constant	t_c
Flow	Flow rate (µL/min)	f
k_t	Mass transfer constant	$t_c \times f^{1/3}$
RI	Bulk refractive index contribution in the sample	RI

12.7.6 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.

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

Model parameters and reported results are:

Parameters		Obtained from
K_D	Equilibrium dissociation constant (M)	Fitted
R_{max}	Analyte binding capacity of the surface (RU)	Fitted
offset	Bulk refractive index contribution in the sample	Fitted

Notes: *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. The reported K_D value is marked as a vertical line on the fitting plot (see Section 12.3).*

Steady state affinity with constant R_{max}

This model uses the same equation as above, but sets the R_{max} parameter to a constant. When the model is used for affinity screen evaluation (Section 13.2.4), the value for R_{max} is obtained for each analyte from the entered value for molecular-weight adjusted R_{max} for a positive control:

$$R_{max\,analyte} = R_{max\,control} \times \frac{MW_{analyte}}{MW_{control}}$$

Note: *If this model is used for evaluation of steady state affinity with the **Kinetics/Affinity** tool, the value for the analyte R_{max} must be provided directly.*

Steady state affinity with constant R_{max} (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 a n apparent value with undefined stoichiometry.

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

Parameters		Obtained from
K_{D1}	Equilibrium dissociation constant (M) for the high affinity site	Fitted
R_{max1}	Analyte binding capacity (RU) of the defined stoichiometry	Constant
K_{D2}	Equilibrium dissociation constant (M) representing the low affinity site(s)	Fitted
R_{max2}	Analyte binding capacity (RU) corresponding to the stoichiometry from free fitting	Fitted
C	Analyte concentration (M)	Input
offset	Bulk refractive index contribution in the sample	Fitted

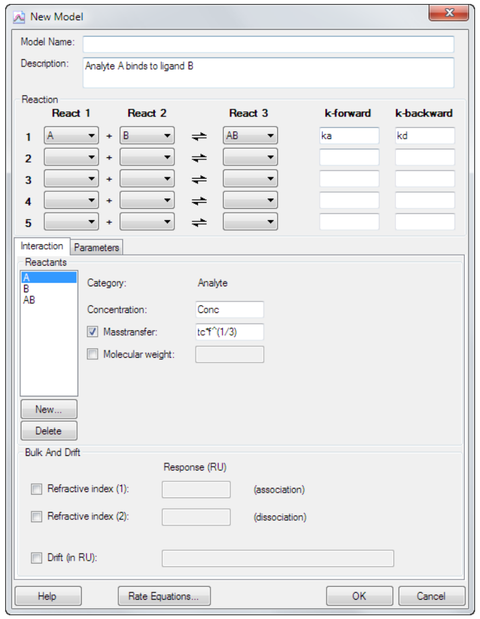
When the model is used for affinity screen evaluation (Section 13.2.4), the value for R_{max} is obtained for each analyte from the entered value for molecular- weight adjusted R_{max} for a positive control as described above.

12.8 Creating and editing models

To create your own models for kinetics of affinity evaluation, choose ***Tools:Models*** from the main menu and select the type of model you want to work with. You can use existing models as templates. Choose an existing model from the list and click ***New***: answer ***Yes*** in the following dialog to create a new model based on the chosen template or ***No*** to create a blank model. For kinetic models, you can define a new model either as a reaction scheme describing the interaction or as an equation defining response as a function of time. Interaction models are described in Section 12.8.1 and equation models in Section 12.8.2.

Predefined models cannot be edited or removed. If you want to modify a predefined model, create a new model using the predefined model as a template.

12.8.1 Interaction models for kinetics



The reaction scheme for an interaction model supports up to five component reactions. Follow the steps below to define a new model or edit an existing definition.

1. On the ***Interaction*** tab, click ***New*** to add new reactants. For each reactant, choose whether it is analyte, ligand or complex (see below) and enter an identifier for the reactant. Enter parameter names or expressions for the reactant properties.

Note: *Numbers are used as part of the identifier, not in the conventional chemical sense of stoichiometry. Thus a complex named AB2 does not imply two molecules of B binding to one of A.*

Analyte

The analyte is injected in solution at a constant concentration, and has the properties listed below. Analyte is usually denoted by the letter A.

Concentration	Injected concentration in molar units.
Mass transfer	Check this box to include a mass transfer term in the fitting, and enter a parameter name or expression for the mass transfer constant.
Molecular weight	Check this box and enter a molecular weight if required. This information is used to calculate relative response contributions for heterogeneous analyte models (it is not used for conversion of weight-based to molar concentration units: this conversion is performed if necessary in the sample table).

Ligand

The ligand is immobilized or captured on the surface, and has the properties listed below. Ligand is usually denoted by the letter B.

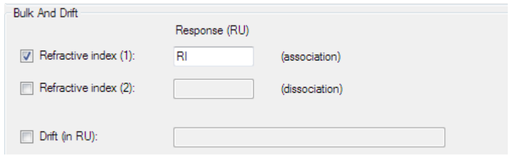
Binding capacity	Maximum analyte binding capacity of the surface in RU.
At molecular weight	This parameters is only used in heterogeneous analyte models. Check the box and enter the molecular weight parameter for the analyte to which the binding capacity parameter refers. Binding capacity for the other analyte will be calculated using the molecular weight values.

Complex

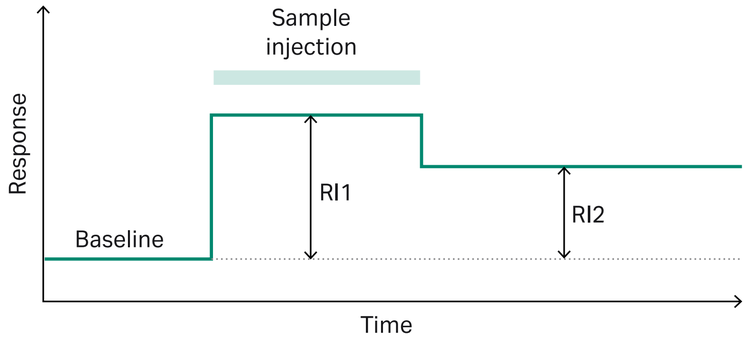
The complex is formed on the surface and generates response and has the properties listed below.

Generates response	Uncheck this box for complexes that form in solution and that do not contribute to the response.
Molecular weight	Check this box and specify a parameter for complexes that form in solution and then bind to the surface. Do not check this box if ***Generates response*** is also checked.

In the ***Bulk and Drift*** panel, enter details for bulk refractive index contribution. Normally, there will be one bulk refractive index term applicable during association (from the start to the end of the injection). A second term can be used if necessary during dissociation (after the end of the injection), for example to accommodate a permanent shift in baseline as a result of the sample injection:

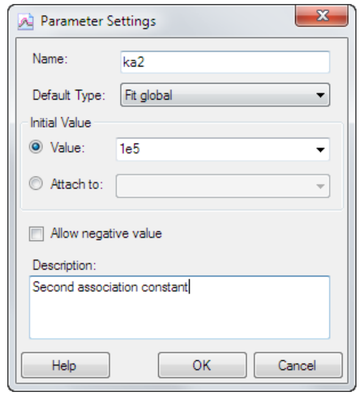


Check the ***Drift*** box and enter an expression describing the drift (most commonly a linear function of time) to account for baseline drift.



2. Enter the reaction scheme in the ***Reaction*** panel using the pull-down list for each reactant. Enter parameter names for the forward and backward rate constants for each line in the reaction scheme. (The terms ***k-forward*** and ***k-backward*** apply to the reaction as entered in the scheme, reading from left to right). You can also enter mathematical expressions or constant values for the rate constants.

3. Click the ***Parameters*** tab and define the parameters used in the reaction scheme. Click ***Add*** to add a new parameter, and define the parameter properties in the dialog:



Choose a default type for the parameter (***Fit global***, ***Fit local*** or ***Constant***).

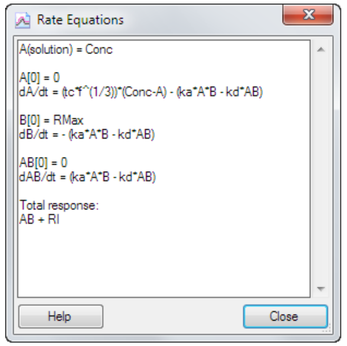
For the ***Initial value***, enter a numerical value or select a value expression from the pull-down list. The expressions represent functions evaluated within the current data set (e.g. ***Ymax*** is the maximum y-value in the data set). Alternatively, choose ***Attach to*** and select a parameter from the list. If you attach a parameter to ***Keyword***, the initial parameter value will be set to the value of the keyword with the same name as the parameter.

Check ***Allow negative value*** if the parameter can be below zero. Enter a description of the parameter for ease of identification.

If you have only used single parameter names (as opposed to expressions) for the rate constants and properties, you can click ***Rate equations*** or ***OK*** as a shortcut to defining parameters. The software will then enter suggested definitions for all undefined parameters. This shortcut cannot be used if you have entered expressions.

In the ***Report*** panel, define the parameters you want to appear in the ***Report*** tab of the results. Report parameters are defined by a name that may be chosen freely and a value that is entered as a parameter or expression containing parameters.

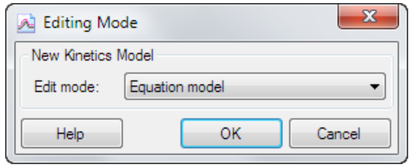
4. Click ***Rate Equations*** to display the equations generated by the software.



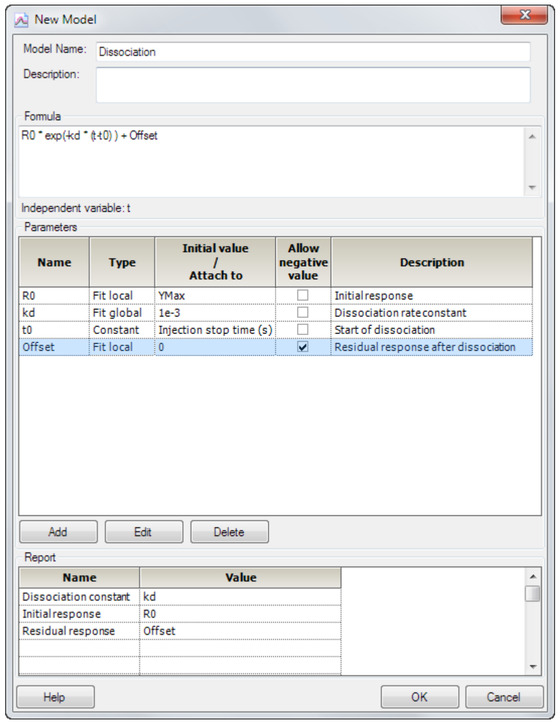
You can select the equations in the display and click ***Copy*** to copy the equations to the Windows clipboard. Use this function and paste the equations in to e.g. Wordpad to print a copy of the rate equations.

12.8.2 Equation models for kinetics

Models for kinetic evaluation can also be entered as an expression defining response as a function of time t. To create an equation model, choose ***New*** in the kinetics models dialog, then choose to create the new model without using the currently selected model as a template. Select ***Equation model*** in the subsequent dialog.



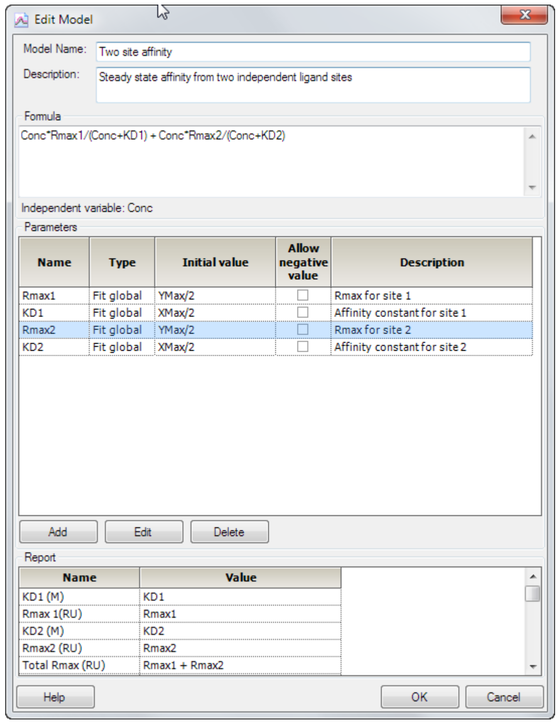
The example below shows a model for evaluation of the dissociation phase only.



Parameters and report parameters are defined in the same way as for interaction models.

12.8.3 Models for steady state affinity

Models for steady state affinity evaluation are entered as an expression defining R_{eq} as a function of concentration Conc. The example below shows a model for two-site affinity evaluation.



Parameters and report parameters are defined in the same way as for kinetic models.

Note: *Beware of trying to define and use complex models for steady state affinity. Because of the relatively few points available for fitting to steady state affinity models (typically about five concentrations in duplicate), complex models tend to give unstable fitting behavior.*

13

Kinetics and affinity screening

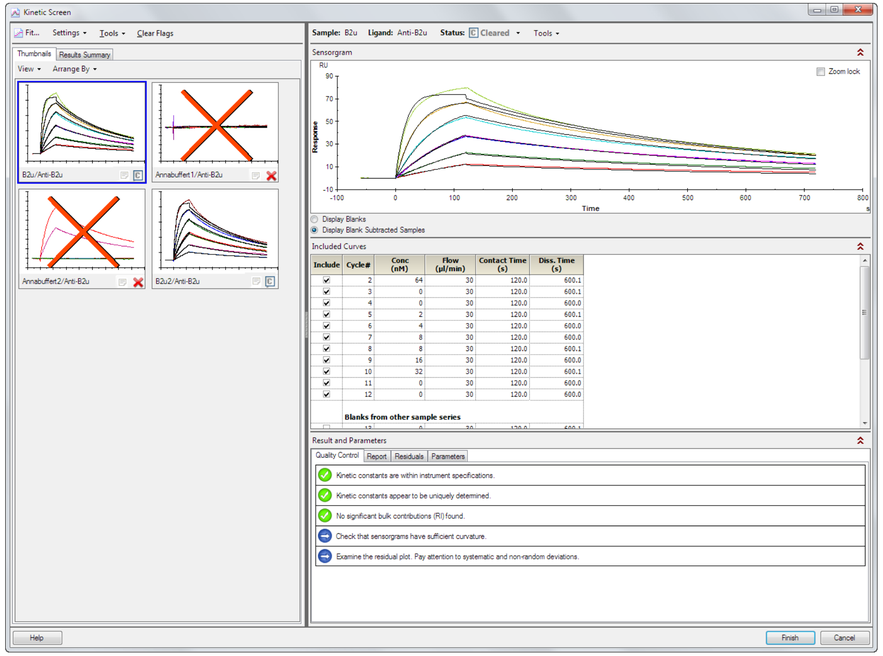
Tools for determination of interaction kinetics and/or affinity in screening contexts are designed to handle large numbers of samples. The kinetics and affinity screening tools provide an overview of the analysis of multiple samples as well as detailed presentation of the results for selected samples.

This chapter describes how to use the kinetic and affinity screening tools. More detailed analysis suitable for evaluation of one or a few interactions is supported by the kinetic and affinity analysis tools (Chapter 14).

Evaluations performed with the kinetics and affinity screening tools are not compatible with thermodynamic analysis (Chapter 15).

13.1 User interface

The screen for kinetics and affinity screen is divided into a number of areas:



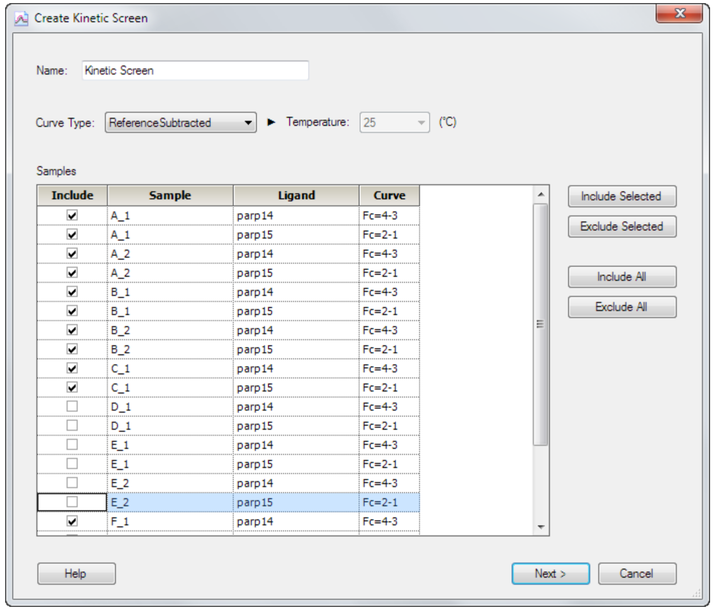
The *left-hand panel* contains tabs for *thumbnail display* and *results summary*. Menu options in this panel apply to all data series. By default, thumbnails show sensorgrams for kinetic screen and a plot of response against concentration for affinity screen. Sensorgrams are blank-subtracted and adjusted to t=0 at injection start. The display type may be changed under the ***View*** menu. Other options for thumbnail display are described in Section 12.6.2. Thumbnails can be sorted using the options in the ***Arrange By*** menu.

The right-hand panel holds a detail view of the currently selected data series with up to four sub-panels. Use the and buttons to show and hide the sub-panels.

- The top sub-panel displays the sensorgrams (for kinetic screening) or response plotted against concentration (for affinity screen). Sensorgrams are blank-subtracted by default using the average of blank sensorgrams for the same sample. Check selected ***Blanks from other sample series*** in the ***Included Curves*** sub-panel to include other blank cycles in the blank subtraction average.
- For affinity screen, sensorgrams are displayed in an additional panel.
- The ***Included Curves*** sub-panel lists the curves in the data series. Use the ***Include*** checkbox to include or exclude curves from the series.
- The ***Results and Parameters*** sub-panel lists the fitting report and the parameters used. Kinetic screen using the 1:1 model also generates a QC- report (see Section 12.4.1).

13.2 Evaluating a kinetic or affinity screen

To start a screening evaluation, choose ***Affinity Screen*** or ***Kinetic Screen*** from the ***Screening*** button or the ***Add Screening*** submenu in the ***Evaluation*** menu.



Choose the curve type and select the interactions to be included in the evaluation item. If the result file contains measurements at different temperatures, choose the temperature to use in the item. Provide a name for the item if desired, then click ***Next***.

A sample series in screening is defined as a set of cycles with the same analyte and ligand name in the same flow cell and the same analysis temperature. For co-evaluation of multiple result files, cycles in different files form separate series.

13.2.1 Series status

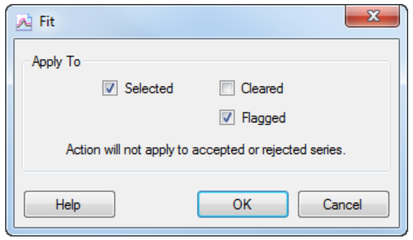
The status of each series can be set using the icons in standard or extended thumbnails or the status indicator at the top of the detail view panel:

	Rejected	Can be hidden. Cannot be addressed with fit settings or fitting.
	Cleared	Default setting. Can be addressed with fit settings and fitting.
	Flagged	Can be used to select series for fit settings and fitting.
	Accepted	Can only be set after fitting. Cannot be addressed with fit settings or fitting.

You can also select one or more thumbnails, right-click in the thumbnail panel and choose ***Set Selection to...***. Status can also be set from the right-click menu in the results summary table. Status settings remain in force until changed by the user. Click ***Clear flags*** to set all flagged series to ***Cleared***.

You can hide rejected series by turning on the ***Tools:Hide rejected*** option in the thumbnail panel.

Fit settings and fitting can be applied to series on the basis of selection and/or status.

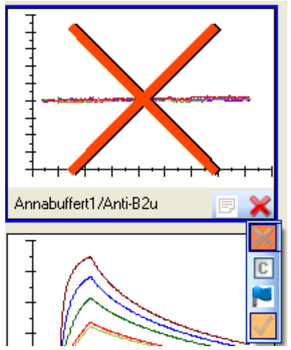


Checking for example both ***Selected*** and ***Flagged*** will apply the operation to series that are either selected or flagged (or both).

13.2.2 Preliminary examination

An overview of the screening is provided by the thumbnails in the left-hand panel, with one thumbnail for each data series.

Scan through the thumbnails and set the status to ***Rejected*** for series that are clearly disturbed or unsuitable for evaluation. For closer examination of the data, select a thumbnail to display the series in the detail view in the right-hand pane. Rejected series are marked with a red X on the thumbnail (as long as ***Hide rejected*** is not active).



Sample sensorgrams are displayed in the detail panel by default, corrected by subtracting an average of the blank sensorgrams. Choose ***Display blanks*** to examine the blank (zero-concentration) sensorgrams.

The ***Included Curves*** list shows both the sample series (included by default) and blanks from other sample series (excluded by default). Use the ***Include*** check- mark in this list to define which curves are included in the data series.

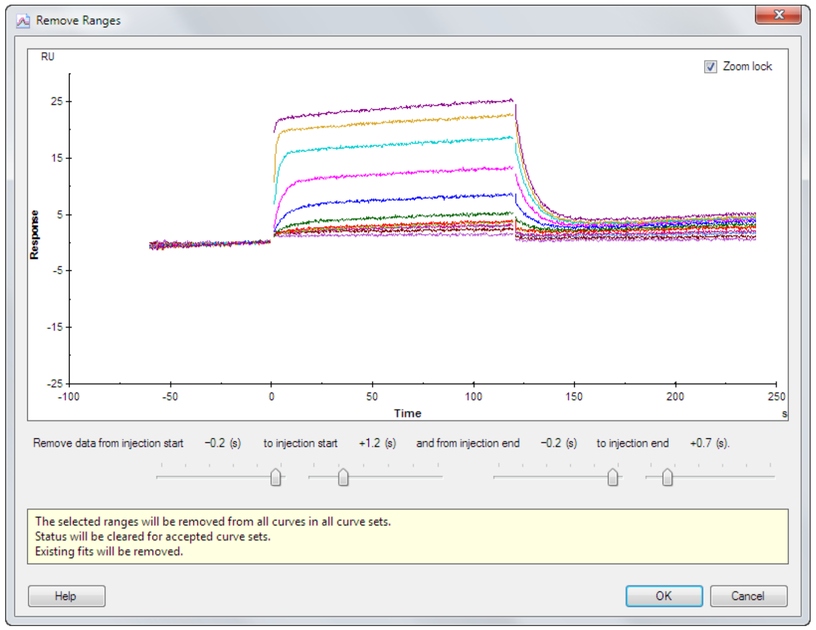
13.2.3 Avoiding disturbed data

If sensorgrams contain spikes at the beginning and end of the sample injection (reference subtraction spikes) or transient disturbances such as airspikes, these may be removed if desired. This can assist automatic scaling of thumbnails and detail views.

Note: *Brief transient disturbances usually have negligible effect on kinetic screening results, but you should beware of removing disturbances from the report point window for affinity screen. If the report point is affected by disturbances, move the report point instead (see below).*

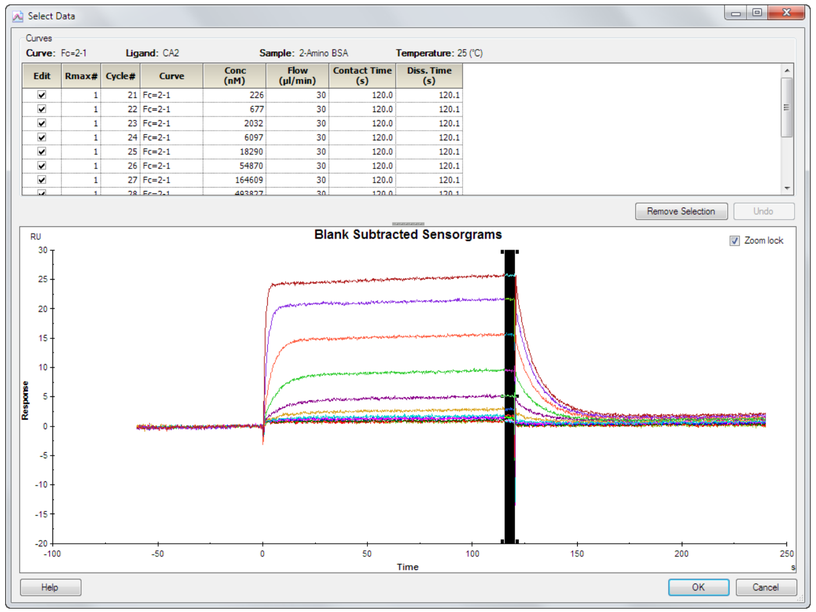
Removing data from all series

To remove spikes at the beginning and/or end of the sample injection (caused by slight mismatching of the active and reference sensorgrams on the time axis), choose ***Tools:Remove Ranges*** in the thumbnail panel. Adjust the sliders until the spikes are removed. You can remove up to ±5 s at the beginning and end of the injection. The ranges are removed from all sensorgrams in all series, and any existing fits will be removed.



Removing data from individual sensorgrams

To remove random spikes from one or more sensorgrams in a series, choose ***Tools:Select data*** in the detail panel. To delete a selected region from all curves in the series, drag with the right mouse button over the region to be deleted and click ***Remove Selection***. Click ***Undo*** to restore the deleted data.



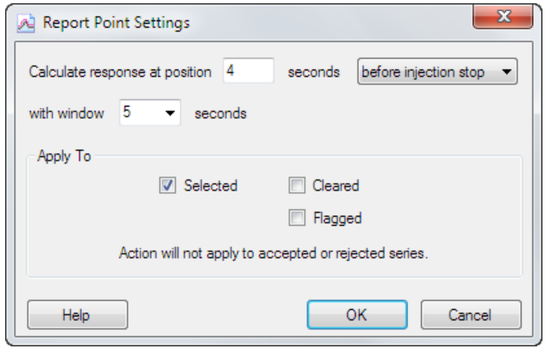
If you want to delete a region from only selected curves, remove the checkmark from the ***Edit*** column in the table for the curves that are to be left unchanged. All curves are selected by default and are shown in dark color. Curves that are not selected for editing are shown in light grey. Note that all curves will be evaluated, whether they are selected for editing or not: removing the ***Edit*** checkmark does not exclude a curve from the data set for evaluation.

Moving the report point for affinity screen

Steady state binding levels for affinity screen are calculated from a report point placed by default 4 seconds before the end of the sample injection.

Note: *This report point is marked on the sensorgrams in the detail view but is not listed in the report point table. You cannot use a report point from the report point table for this purpose.*

To move the report point, choose ***Settings:Report Point Settings*** in the thumbnail panel and enter the new position. Choose which series the change should be applied to and click ***OK***.



13.2.4 Fitting the data – affinity screen

Affinity screen evaluation is based on analysis of steady state binding levels (R_{eq}) as a function of analyte concentration (see Section 12.7.6). To evaluate an affinity screen:

- Choose the model as required from the ***Setting:Fit Settings*** menu in the thumbnail panel. Choose the series to which the model will be applied. The choice of model is listed in the ***Results Summary*** table in the left-hand panel and is also shown on extended thumbnails. Click ***Parameters*** if you need to edit any starting values for parameters or enter values that are missing in custom models.
- Click ***Fit*** in the thumbnail panel and choose the data series to fit according to either selection or series status.

Notes: *Applying a fit without changing the fit settings or data selection to data series that have already been evaluated will have no effect.*

The fitting will fail for a series if required parameters have not been provided. A warning is issued that fitting has failed for one or more series.

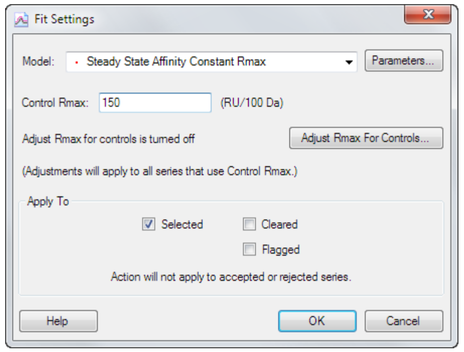
For low affinity interactions, fitting may be performed using a fixed value for R_{max} to provide robust fitting, with the model ***Steady State Affinity Constant Rmax***, To determine the value for constant R_{max}, inject high concentrations of a positive control and use the maximum response reached as R_{max}. Alternatively, run a kinetic or affinity analysis for the control to obtain a fitted R_{max}. Be aware that the value obtained in this way may be significantly incorrect if the chosen interaction model does not fit the data closely.

Note: *Fitting to a model with constant R_{max} requires that molecular weights are specified for all analytes, in order to adapt the control R_{max} value to the individual analytes.*

To use this method, choose the fitting model from the drop-down list and enter a value for the ***Control Rmax***. This is the R_{max} for the positive control, expressed as a molecular weight-adjusted response (100*RU Da). The R_{max} for a given analyte is calculated by adjusting for the molecular weight:

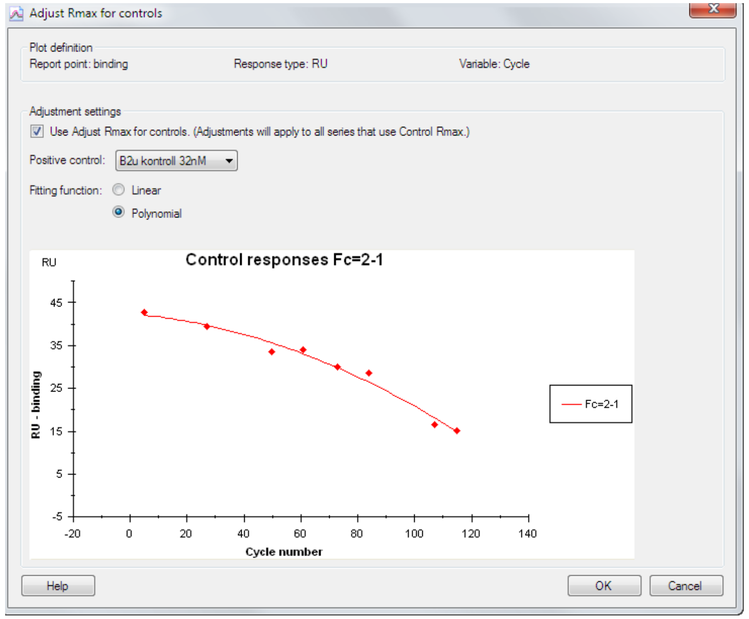
$$R_{max\,analyte} = \text{Control } R_{max} \times \frac{MW_{analyte}}{100}$$

where *ControlRmax* is the molecular weight-adjusted R_{max} value for the positive control.



The model for multi-site affinity with constant R_{max} handles substances that show binding to more than one site. For correct results it is important that the positive control substance binds only to one site. The constant R_{max} term applies to the site defined by the positive control binding.

The value for the constant R_{max} can be adjusted automatically to compensate for changes in surface activity during the course of the run. To do this, click ***Adjust Rmax for Controls*** and check ***Use Adjust Rmax...***. Select the positive control on which the adjustment should be based (this does not have to be the same substance as that used to determine the initial R_{max} value) and choose either linear or polynomial fitting.



This will adjust the R_{max} value for each data series according to the first cycle number in the series (see Figure 13-1). The R_{max} value remains constant within the series.

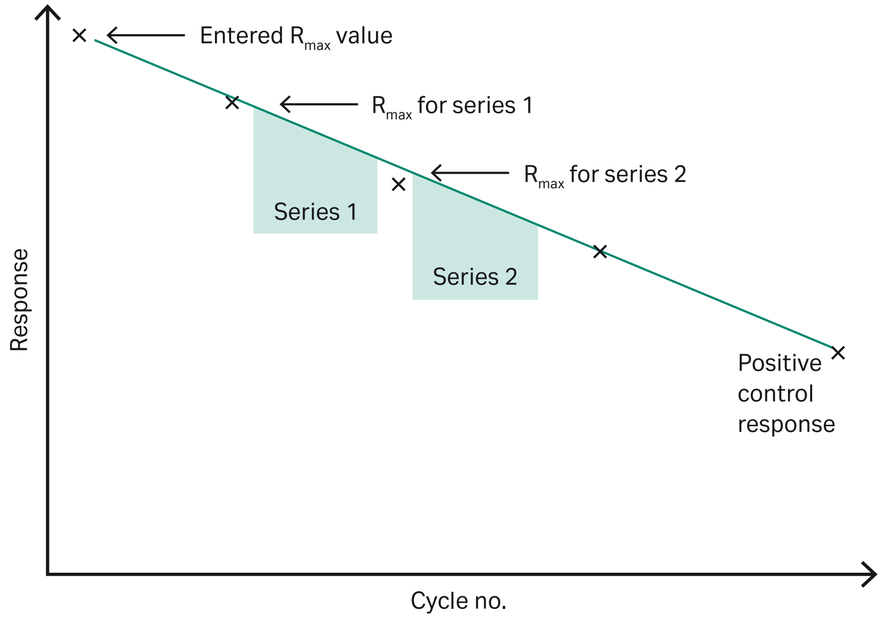


Figure 13-1. Principle of R_{max} adjustment. The entered value applies at the start of the run. For individual data series, the R_{max} value is adjusted to the start of the data series according to a line fitted through the positive control response points. The drift is exaggerated for illustration. Normally, the drift in control response during the course of one cycle should be negligible.

Note: *Custom models can be used with constant R_{max} functionality provided they are defined with a parameter **Rmax** that is set to **Constant** with initial value blank (see Section 12.8.1).*

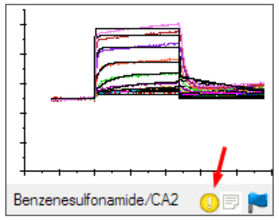
13.2.5 Fitting the data - kinetic screen

To evaluate a kinetic screen:

- Choose the model as required from the ***Setting:Fit Settings*** menu in the thumbnail panel. The default model is 1:1 binding. Choose the series to which the model will be applied in terms of series status (Section 13.2.1). The choice of model is listed in the ***Results Summary*** table in the left-hand panel and is also shown on extended thumbnails. Click ***Parameters*** if you need to edit any starting values for parameters or enter values that are missing in custom models.
- Click ***Fit*** in the thumbnail panel and choose the data series to fit according to either selection or series status.

Fitting progress is indicated by the iteration number and the current chi-square value. You can use the ***Abort*** or ***Accept*** buttons to cancel or accept the fitting. Clicking on ***Accept*** will stop the fitting at the end of the current iteration, which may take a few moments to complete.

For fitting to the 1:1 interaction model, automatic quality assessment is performed as described in 12.4. Series that do not pass the quality assessment on all counts are marked with a yellow warning icon below the thumbnail (indicated by the arrow in the illustration below).



Notes: *Fitting to a large number of sample series may take considerable time.*

Applying a fit without changing the fit settings or data selection to data series that have already been evaluated will have no effect.

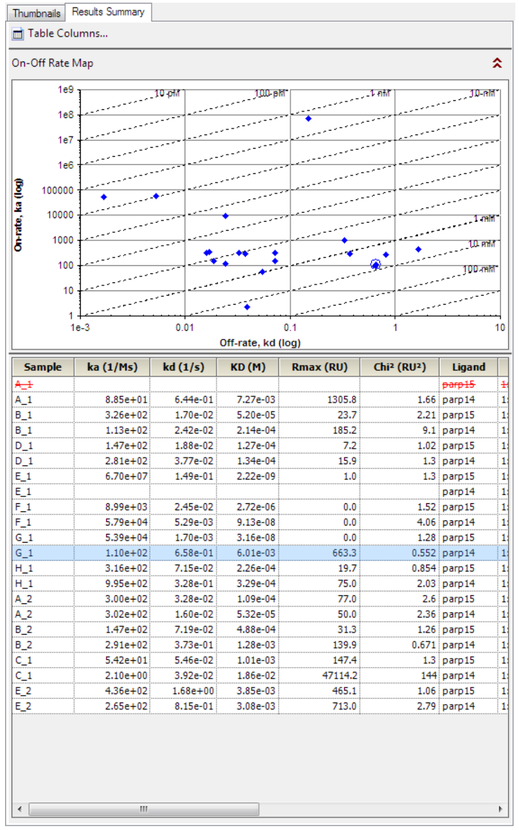
The fitting will fail for a series if required parameters have not been provided. A warning is issued that fitting has failed for one or more series.

To refit the data using a different model or parameter settings, change the settings under ***Settings:Fit settings*** and click ***Fit*** again.

13.2.6 Examining the results

When the fit is completed, the results for the currently selected thumbnail are displayed in the detail panel as fitted curves overlaid in black on the experimental data. Fitted curves may also be displayed in the thumbnails according to the option under ***View:Display Settings***.

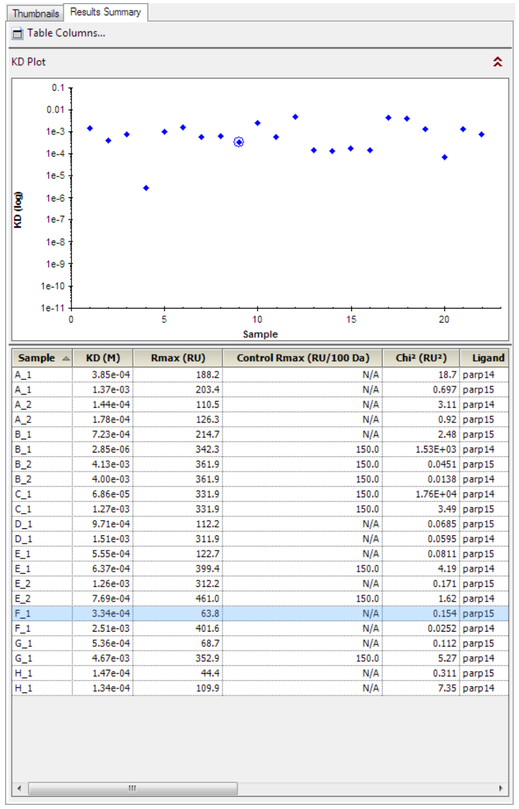
13.2.7 Results summary



Sample	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Chi² (RU²)	Ligand
~~A_1~~						~~parp15~~
A_1	8.85e+01	6.44e-01	7.27e-03	1305.8	1.66	parp14
B_1	3.26e+02	1.70e-02	5.20e-05	23.7	2.21	parp15
B_1	1.13e+02	2.42e-02	2.14e-04	185.2	9.1	parp14
D_1	1.47e+02	1.88e-02	1.27e-04	7.2	1.02	parp15
D_1	2.81e+02	3.77e-02	1.34e-04	15.9	1.3	parp14
E_1	6.70e+07	1.49e-01	2.22e-09	1.0	1.3	parp15
E_1						parp14
F_1	8.99e+03	2.45e-02	2.72e-06	0.0	1.52	parp15
F_1	5.79e+04	5.29e-03	9.13e-08	0.0	4.06	parp14
G_1	5.39e+04	1.70e-03	3.16e-08	0.0	1.28	parp15
G_1	1.10e+02	6.58e-01	6.01e-03	663.3	0.552	parp14
H_1	3.16e+02	7.15e-02	2.26e-04	19.7	0.854	parp15
H_1	9.95e+02	3.28e-01	3.29e-04	75.0	2.03	parp14
A_2	3.00e+02	3.28e-02	1.09e-04	77.0	2.6	parp15
A_2	3.02e+02	1.60e-02	5.32e-05	50.0	2.36	parp14
B_2	1.47e+02	7.19e-02	4.88e-04	31.3	1.26	parp15
B_2	2.91e+02	3.73e-01	1.28e-03	139.9	0.671	parp14
C_1	5.42e+01	5.46e-02	1.01e-03	147.4	1.3	parp15
C_1	2.10e+00	3.92e-02	1.86e-02	47114.2	144	parp14
E_2	4.36e+02	1.68e+00	3.85e-03	465.1	1.06	parp15
E_2	2.65e+02	8.15e-01	3.08e-03	713.0	2.79	parp14

The ***Results Summary*** tab in the left-hand panel presents a summary of the screening in graphical and tabular format.

- Before fitting, the table lists details of the data series, including the model selected for each series.
- After fitting for a kinetic screen, the results summary lists the fitting results for each series, and displays the results as an on-off rate map (Section 12.6.3).
- After fitting for an affinity screen, the results summary lists the fitting results for each series and displays the results as KD-plot (Section 12.6.4).

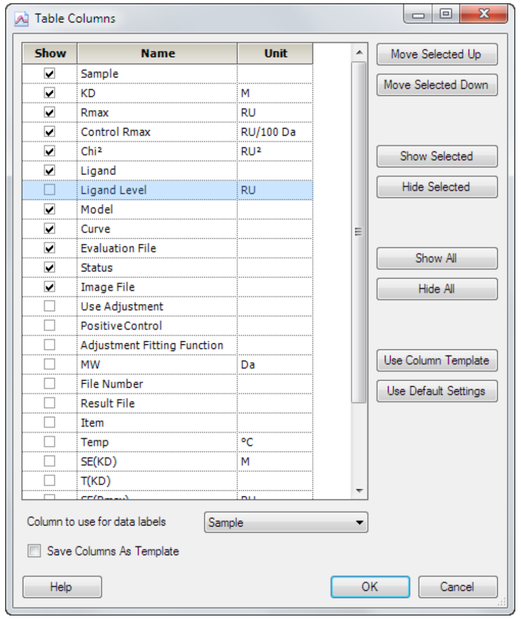


Sample	KD (M)	Rmax (RU)	Control Rmax (RU/100 Da)	Chi² (RU²)	Ligand
A_1	3.85e-04	188.2	N/A	18.7	parp14
A_1	1.37e-03	203.4	N/A	0.697	parp15
A_2	1.44e-04	110.5	N/A	3.11	parp14
A_2	1.78e-04	126.3	N/A	0.92	parp15
B_1	7.23e-04	214.7	N/A	2.48	parp15
B_1	2.85e-06	342.3	150.0	1.53E+03	parp14
B_2	4.13e-03	361.9	150.0	0.0451	parp15
B_2	4.00e-03	361.9	150.0	0.0138	parp14
C_1	6.86e-05	331.9	150.0	1.76E+04	parp14
C_1	1.27e-03	331.9	150.0	3.49	parp15
D_1	9.71e-04	112.2	N/A	0.0685	parp15
D_1	1.51e-03	311.9	N/A	0.0595	parp14
E_1	5.55e-04	122.7	N/A	0.0811	parp15
E_1	6.37e-04	399.4	150.0	4.19	parp14
E_2	1.26e-03	312.2	N/A	0.171	parp15
E_2	7.69e-04	461.0	150.0	1.62	parp14
F_1	3.34e-04	63.8	N/A	0.154	parp15
F_1	2.51e-03	401.6	N/A	0.0252	parp14
G_1	5.36e-04	68.7	N/A	0.112	parp15
G_1	4.67e-03	352.9	150.0	5.27	parp14
H_1	1.47e-04	44.4	N/A	0.311	parp15
H_1	1.34e-04	109.9	N/A	7.35	parp14

Choose ***Evaluation details*** from the right-click menu in the results summary to show the details of the evaluation results in a separate window. Choose ***Show data labels*** to show the sample name as a label on each point in the summary plot.

Table columns

Use the ***Table Columns*** button to select which columns should be included in the summary table.



Check ***Save Columns As Template*** to save the table column settings as a template on the local computer. A saved template will be used as default when a new result file is opened. If this box is not checked, the settings will only be saved with the evaluation file and will not be apply to evaluation of other result files. Separate templates are saved for kinetic screen, affinity screen and kinetics summary. Click ***Use Column Template*** to apply the saved template to the current evaluation. Click ***Use Default Settings*** to restore the column selection to that originally provided with the software.

13.3 Annotations and comments

Data series for kinetic and affinity screen can be annotated using the ***Annotations*** function as described for result plots in Section 8.6. The list of available annotations is common to result plots and screening items.

Notes: *Annotations cannot be applied to single data points or sensorgrams in screening items.*

Include annotation and comment columns in the result table if you want annotations to be included in data export and printing.

13.4 Exporting screening results

Exporting kinetic or affinity screen items to Excel or XML by using the ***File:Export*** option from the main menu exports the ***Results Summary*** table as well as the ***Report*** and ***Parameters*** tables for each fitting. Data exported from the ***Results Summary*** table can be customized using the ***Table Columns*** option (see Section 13.2.7).

Choosing ***Export All Graphs and Table*** from the right-click menu in the thumbnail panel of a saved evaluation file will create a folder with the same name as the evaluation file, containing all thumbnails as separate files in ***.png*** format and the results summary table in tab-separated text format. The file name for each exported thumbnail is listed in the Image file column of the results summary.

To export detail graphs or tables from the right-hand panel, use the ***Copy Graph***, ***Export curves*** and ***Copy Table*** functions from the right-click menu (Section 6.3.2).

14

Detailed kinetics and affinity analysis

This chapter describes how to perform detailed analysis of surface-bound kinetics and affinity. The tools for this are aimed at experiments where only a few samples are analyzed in each session. If there are multiple sample series in the same data set, evaluation can be performed either in *single mode* (where each sample series is evaluated separately with interactive control over several aspects of the evaluation), or in *batch mode* (where multiple series are evaluated automatically using default settings). Kinetic and affinity screening (Chapter 13) provides evaluation tools adapted to dealing with large numbers of samples.

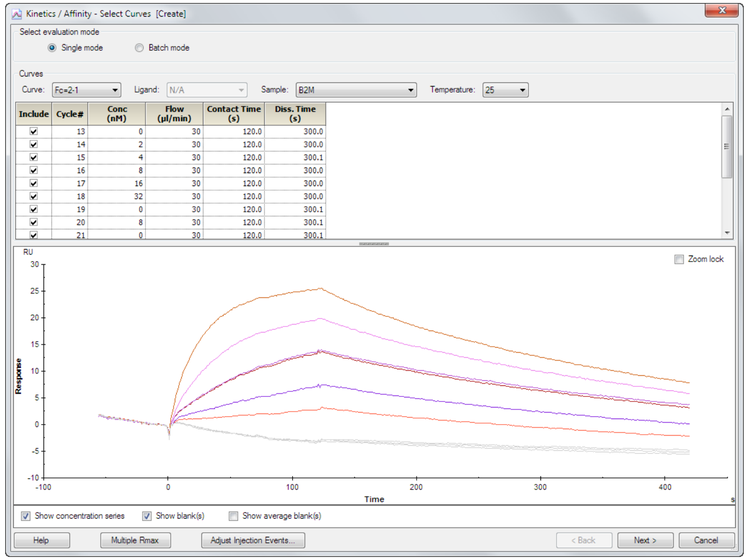
14.1 Evaluating kinetics and affinity in single mode

14.1.1 Basic procedure

To start a kinetics or affinity evaluation in single mode, choose ***Surface-bound kinetics/ Affinity*** from the ***Kinetics/Affinity*** button on the toolbar or the ***Add Kinetics/Affinity*** in the ***Evaluation*** menu. Check the ***Single mode*** option in the first dialog.

1. The first dialog presents the concentration series available in the current result set and allows you to choose the curves included in the evaluation.

Note: *A sample series in kinetics and affinity analysis defined as a set of sensorgrams with the same analyte and ligand name in the same flow cell, regardless of whether the cycles are consecutive or not.*



A concentration series is defined by a set of curves with the same sample name, analysis temperature and curve identity. Select the concentration series you want to work with in the respective pull-down lists.

Note: *For runs with immobilized ligand, there will be only one choice for **Ligand name**. Multiple choices may be available if the ligand is captured and varies between cycles.*

For single-cycle kinetics, the cycles table lists all concentrations injected in each cycle. You can however only include or exclude whole cycles at this stage.

Include	Cycle#	Conc (µM)	Flow (µl/min)	Contact Time (s)	Diss. Time (s)
☑	9	0 0 0 0 0	15	32.0	600.0
☑	10	0.062 0.185 0.556 1.667 5	15	32.0	600.1
☑	11	0	15	32.0	600.1

Note: *Sample names are case-sensitive, so that "Sample" and "sample" belong to different concentration series. Edit the sample names in the keyword table if you have unintentionally mixed upper- and lower- case letters.*

If the result set contains data from more than one file, curves with the same ligand, sample name, temperature and curve identity are grouped together in a single concentration series.

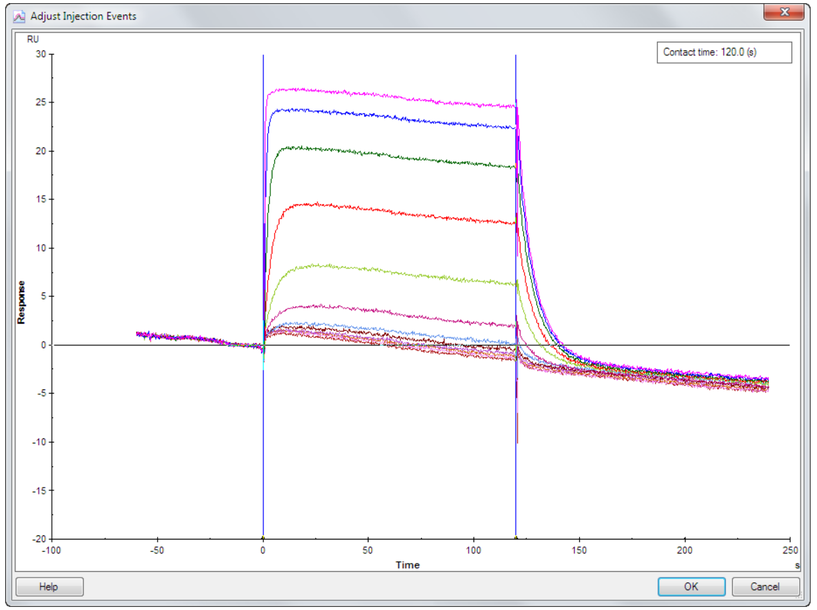
Use the ***Include*** column in the table of curves to choose which curves should be included in the data set to be evaluated. You can select several curves and use the right-click menu to exclude or include multiple curves in one operation. By default, all curves for the sample are included.

Sensorgrams for non-zero concentrations are shown in color, and those for blanks (zero concentrations) in light gray. The sensorgrams are adjusted to zero at the start of the sample injection on both the response and time axes. The average of the blank sensorgrams will be automatically subtracted from the other curves when you proceed to the next step. If you do not want to perform blank subtraction, exclude the zero concentration sensorgrams from the data set. You can also choose to use blanks from other concentration series for blank subtraction: these are listed at the bottom of the table, and are excluded by default. Only blank sensorgrams with the same contact and dissociation times as the samples are used.

The three check-boxes below the sensorgram panel control the type of curves shown in the display. You can use these check-boxes to examine the sample and blank curves without interference from each other, and to show the average blank that will be used for subtraction. Bear in mind however that these boxes control the display only and do not affect the data set that will be evaluated.

If you have multiple ligand densities represented in the result set, click ***Multiple Rmax*** to assign curves to the different sets (see Section 14.1.2).

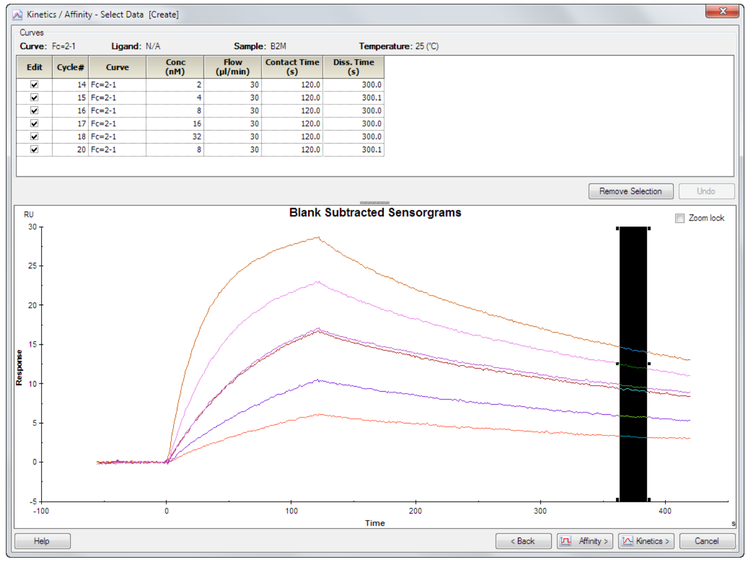
Click ***Adjust Injection Events*** if you want to adjust the injection start and end positions for the evaluation. These positions are set automatically from the event markers in the run, but may need slight adjustment for best fitting to fast interaction processes. The adjustment compensates for small systematic discrepancies in the interval between the event as recorded in the event log and the time that the sample actually reaches the detection spot on the sensor surface. The difference is most apparent at low flow rates.



The event markers for injection start and end in the result file are shown on the x-axis. Drag the vertical reference lines to adjust the injection start or end point for evaluation. You can adjust the events by ±10 s from the original position. The same adjustment is applied to all curves in the data set, whether they are currently included for evaluation or not. This function cannot be used for single-cycle kinetics, for evaluations with mixed single- and multi-cycle analyses, or for multi-cycle analyses with different contact times in different cycles.

Note: *If you adjust the injection start, the time axis in the evaluated data is adjusted correspondingly so that the start of the injection is always at time zero. The display in the* ***Adjust injection events*** *dialog however always shows the original time axis, with zero at the event marker in the run data.*

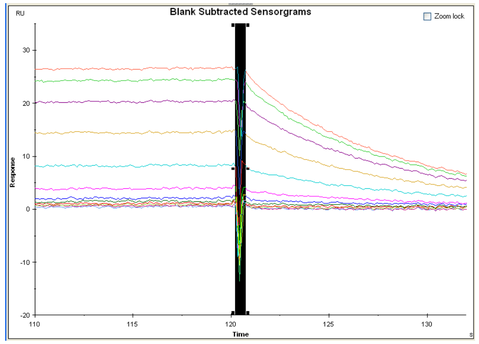
2. The second dialog shows the blank subtracted curve set and allows you to delete selected regions from all or selected curves, for example to eliminate spikes or other disturbances.



To delete a selected region from all curves, drag with the right mouse button over the region to be deleted and click ***Remove Selection***. Click ***Undo*** to restore the deleted data.

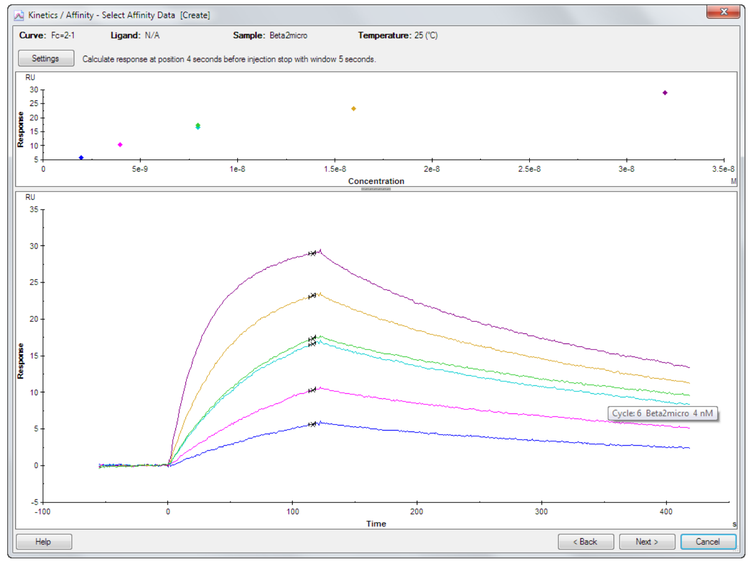
If you want to delete a region from only selected curves, remove the checkmark from the ***Edit*** column in the table for the curves that are to be left unchanged. All curves are selected by default and are shown in dark color. Curves that are not selected for editing are shown in light color. Note that all curves will be evaluated, whether they are selected for editing or not: removing the ***Edit*** checkmark does not exclude a curve from the data set for evaluation.

Note: *For best resolution of fast kinetics, you should delete any disturbances in connection with injection start and stop (see illustration below). These are commonly caused by small misalignments in reference and blank subtraction, leading to spikes at the beginning and end of the injection. The effect of subtraction spikes on the calculated kinetic constants is usually negligible except for very fast interactions.*

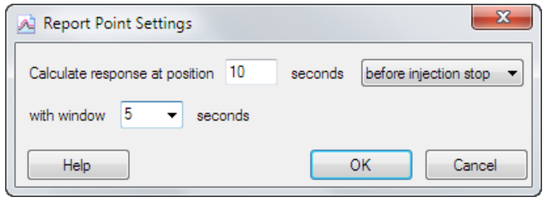


Click ***Affinity*** for steady state affinity evaluation or ***Kinetics*** for kinetics evaluation when you are satisfied with the curves.

3. (***Affinity*** only). If you choose to evaluate steady state affinity, the next dialog gives a preview of the plot of steady state response against concentration, with the option to adjust the selection of data used to calculate response values.



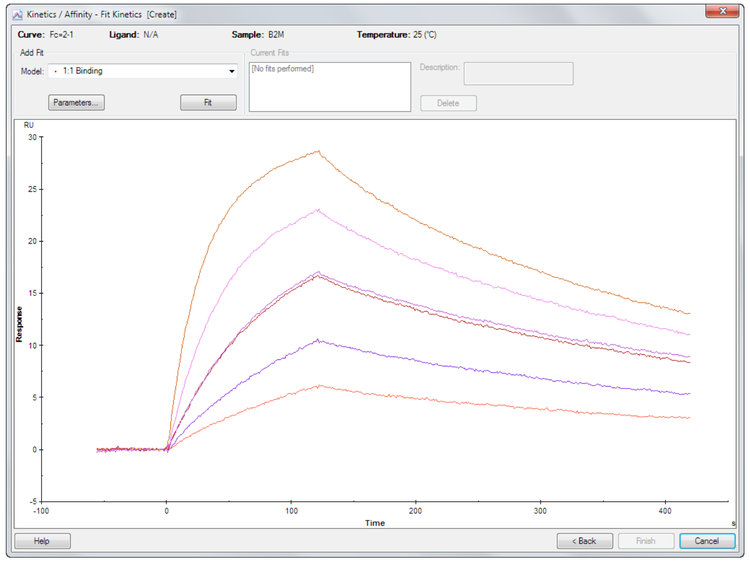
The top panel shows the plot of R_{eq} against C, based on average response values over the region marked on the sensorgrams. Click ***Settings*** to adjust the region used for calculation of R_{eq} values.



For single-cycle affinity runs, the same settings are applied to each injection in the cycle.

Click ***Next>*** when you are satisfied with the data selection.

4. In the next dialog (applicable to both ***Kinetics*** and ***Affinity***), you select the fitting model and perform the fit. The same data can be fitted repeatedly to different models or to the same model with different settings.



Select the model from the pull-down list. Available models are described in Section 12.7. Click ***Parameters*** if you want to change the starting values or scope of any of the parameters (see Section 12.2.2 for details), then click ***Fit*** to perform the fitting.

During the fitting procedure, the fitted curves are shown in black overlaid on the experimental data. Fitting progress is indicated in the sensorgram window by display of the iteration number, the current chi-squared value and the relative change in the parameter that was changed most from the previous iteration. You can use the ***Abort*** or ***Accept*** buttons to cancel the fitting or accept the fitting after the current iteration. You may want to cancel the fitting if it is clear that a fit cannot be found, or to accept the fitting if the chi-squared value and/or maximum relative change indicate that an acceptable fit has been achieved. Clicking on ***Accept*** will stop the fitting at the end of the current iteration, which may take a few moments to complete.

You can enter a short description for the fit in the ***Description*** box. This can be useful for example to distinguish different fits for thermodynamic analysis (Section 15.2).

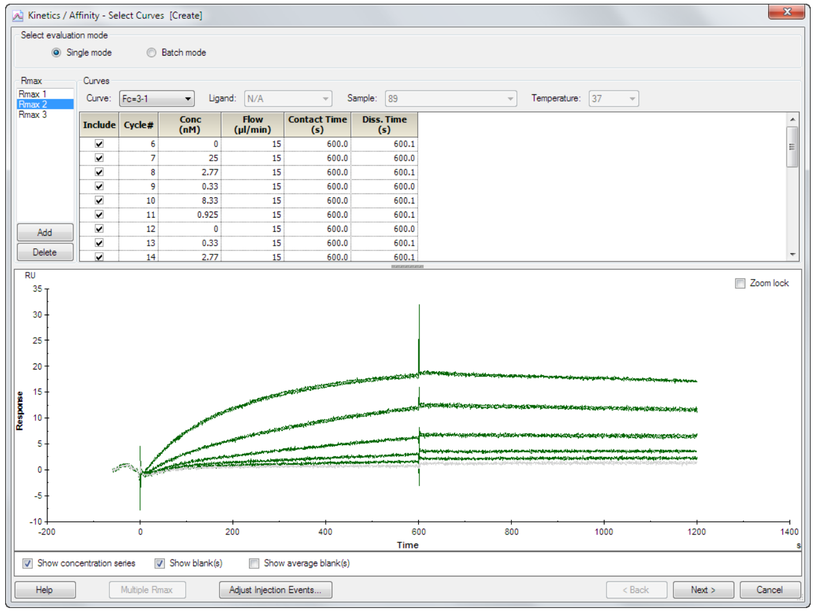
To perform additional fits on the same data, choose a new model or new parameter settings and click ***Fit***. To remove a fit from the evaluation item, select the fit in the list of current fits and click ***Delete***.

Click ***Finish*** to complete the evaluation and place the item in the evaluation explorer panel. You can click ***Back*** to review the choice of data for the evaluation: however, if you make any changes to the data (e.g. remove additional sections from a curve or exclude a curve from the set), all current fits will be deleted. Current fits are also deleted if you switch between kinetics and affinity evaluation.

14.1.2 Multiple ligand densities

Analysis of the same analyte concentration series over multiple ligand densities can provide more robust fitting than a single ligand density. The kinetic and steady-state affinity fitting functions support simultaneous analysis of up to five sets of data with independent values for R_{max}, returning a single set of rate constants for the whole combined data set. Analyses over multiple ligand densities may be performed in separate runs that are combined with the ***Append file*** function, but should preferably be performed on multiple flow cells in the same run to ensure that the experimental conditions are comparable as far as possible.

To set up evaluation of the same sample series over multiple ligand densities, click ***Multiple Rmax*** in the first dialog box for kinetics and affinity evaluation. A panel for data subsets representing different R_{max} values opens to the left of the curve table.



Click ***Add*** to add a new data subset. If you have multiple sets of the currently chosen curve type in the evaluation session (e.g. multiple reference subtracted curves), the next curve in the list is assigned to the new data subset. If there are no more curves of the same type available, the new set will be a copy of the most recently created subset. You can add up to five data subsets, representing five ligand densities. The same sample name and analysis temperature apply to all subsets.

Click on a subset to manage the contents of the subset. You cannot mix subsets that use different samples or different temperatures.

IMPORTANT!

Make sure that the same curve numbers are not assigned to more than one data subset. If a curve is assigned to two data sets, the software will try to evaluate the same curve with two different R_{max} values, and the fitting may be distorted.

Do not evaluate multiple copies of the same subset for multiple ligand densities. Subsets that are duplicated will be weighted more than those that are not duplicated in the fitting procedure.

Do not attempt to use subsets for any purpose other than multiple ligand densities. The data will be evaluated in terms of multiple ligand densities regardless of how you have assigned curves to the subsets.

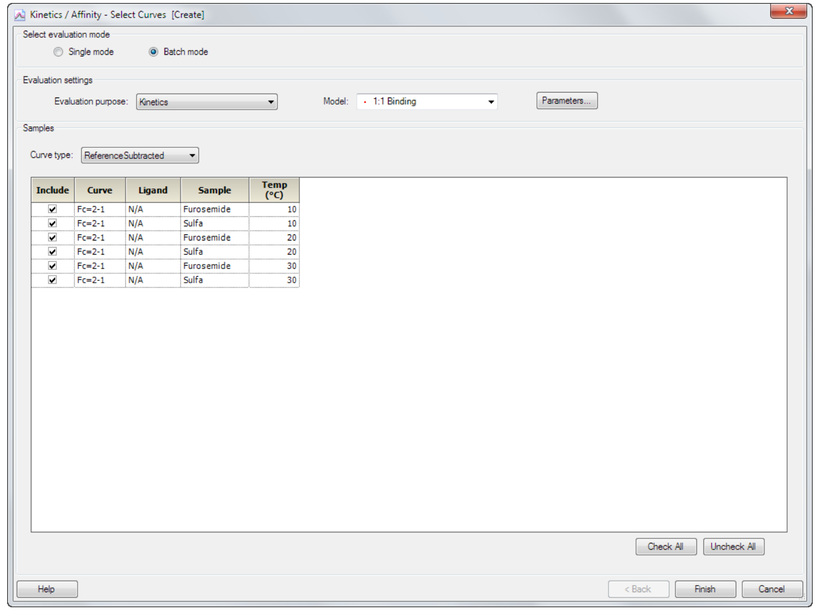
The subsets will be evaluated together, with rate or affinity constants that are global for the whole data set and R_{max} values that are global within each subset but can differ between subsets.

Note: *The **Parameters** tab in evaluation of kinetics with a global R_{max} parameter within subsets lists a parameter $\boldsymbol{i_n}$ which is set to 1 for the n^{th} subset and 0 for all others. This is used to control R_{max} values for each subset: values are fitted as $R_{max} \times i_n$, which returns a non-zero value for the n^{th} subset only. For affinity evaluations, each subset is represented by one curve and R_{max} is simply evaluated as a local parameter.*

14.2 Batch mode evaluation

Batch mode allows you to evaluate multiple sample series (e.g. different analytes interacting with a common ligand, or measurement of the same interaction at different temperatures) automatically. You select the series to be evaluated and the fitting model to be used, and evaluation is carried to completion without further intervention.

To perform evaluation in batch mode, start the kinetics/affinity evaluation (Section 14.1.1) and check the ***Batch mode*** option in the first dialog.



Choose the ***Evaluation purpose*** as kinetics or affinity, and choose the fitting model. Click the ***Parameters*** button if you want to make adjustments to the fitting parameters (see Section 14.1.1). The same model and parameter settings will be used for all evaluations in the batch.

Choose the curve type to use (you should normally evaluate reference- subtracted curves) and check the data sets you want to evaluate in the table. Click ***Finish*** to perform the evaluation. One evaluation item will be created for each sample series.

Notes: *A data set for batch evaluation is defined as a set of cycles with the same ligand, sample and analysis temperature. Blank cycles will be subtracted within each data set.*

Evaluation in batch mode does not support multiple ligand densities, adjustment of injection start, selection of blank cycles or removal of selected data. Individual evaluation items created during batch evaluation can however be edited separately.

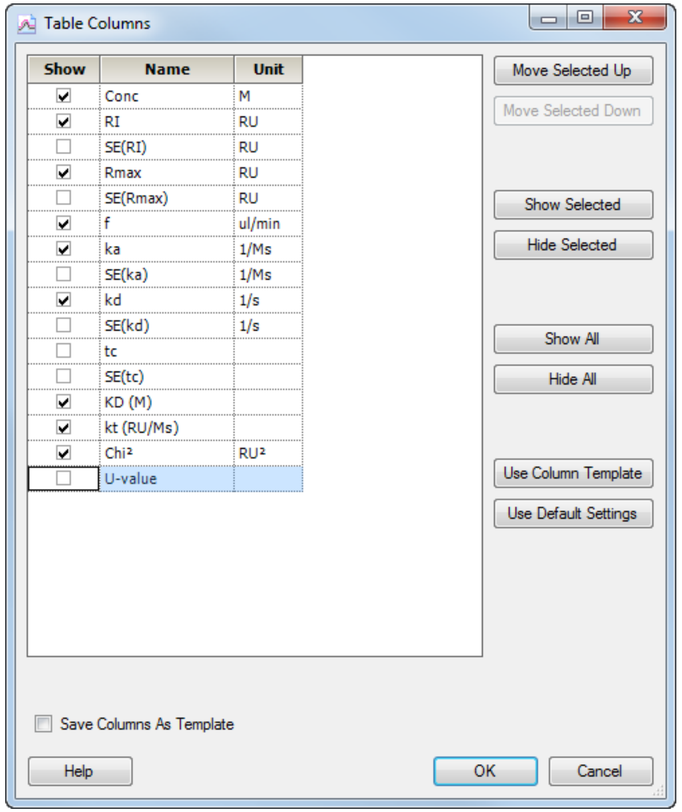
14.3 Presentation and assessment of results

Presentation and assessment of results are closely similar in kinetic/affinity screening and detailed analysis, and are described in Sections 12.3 through 12.5.

14.4 Exporting results

Data that is exported to Excel and XML may be customized if desired. Choose ***Tools:Customize Export*** in the evaluation results (in ***Edit*** mode) to customize the exported data. Customized settings are saved in evaluation methods.

Note: *The **Customize Export** option is not available for evaluation results that use **Multiple Rmax**.*



Select the table columns to include in the exported data. The available columns are taken from the ***Results*** and ***Parameters*** tabs. Use the ***Move Selected Up/Down*** buttons to change the column order.

The table column settings can be saved as a template and reused as described for kinetic/affinity screening in Section 13.2.7.

A new tab labeled ***Data for Export*** will appear in the results presentation. The contents of this tab will be exported when ***File:Export to Excel*** or ***File:Export to XML*** is chosen from the main menu.

Quality Control | Report | Residuals | Parameters | Data for Export

Curve	Conc (M)	RI (RU)	Rmax (RU)	f (ul/min)	ka (1/Ms)	kd (1/s)	KD (M)	kt (RU/Ms)	Chi² (RU²)
			6.829		7.343E+6	0.08180	1.114E-8		0.0880
Cycle: 16 0.0039 µM	3.900E-9	-0.01122		30				1.592E+7	
Cycle: 17 0.0156 µM	1.560E-8	-0.1696		30				1.592E+7	
Cycle: 18 0.0625 µM	6.250E-8	-0.2158		30				1.592E+7	
Cycle: 19 0.25 µM	2.500E-7	-0.2917		30				1.592E+7	
Cycle: 20 1 µM	1.000E-6	-0.2434		30				1.592E+7	

If the export operation has not been customized, the contents of the ***Report*** and ***Parameters*** tabs are included as separate tables in the exported data.

15

Thermodynamic analysis

Biacore T200 supports automated measurement of kinetics or affinity at a series of temperatures using the ***Thermodynamics*** wizard (Section 4.12). In addition to displaying the variation of kinetic and affinity constants with temperature, the evaluation software extracts standard thermodynamic parameters from the data.

15.1 Background

15.1.1 Equilibrium thermodynamics

For equilibrium thermodynamics, the *van't Hoff equation* states:

$$\Delta G^\circ = -RT\ln\frac{1}{K_D} = RT\ln K_D$$

where ΔG° is the standard Gibbs free energy change
R is the universal gas constant
T is the absolute temperature (K)
K_D is the equilibrium dissociation constant

Substituting in the expression

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

and rearranging gives:

$$\ln K_D = \frac{\Delta H^\circ}{RT} - \frac{\Delta S^\circ}{R}$$

where ΔH° is the standard enthalpy change
ΔS° is the standard entropy change

A plot of $\ln K_D$ against $1/T$ should thus be a straight line, with slope $\Delta H^\circ/R$ and intercept on the y-axis $\Delta S^\circ/R$.

This simplified relationship does not hold if the heat capacities of reagents and products differ, since different amounts of energy will be required to raise the temperature by the same amount on the two sides of the reaction. In such cases, the plot of $\ln K_D$ against $1/T$ is not linear, and the relationship becomes

$$RT\ln K_D = \Delta H^\circ_{T_0} - T\Delta S^\circ_{T_0} + \Delta C_P^\circ(T - T_0) - T\Delta C_P^\circ \ln\left(\frac{T}{T_0}\right)$$

where ΔC_P° is the heat capacity change under standard conditions
T_0 is the reference temperature (25°C = 298.15 K for standard conditions)

A value for the standard heat capacity change ΔC_P° can thus be obtained in addition to ΔH° and ΔS° from non-linear fitting of the data to this extended equation.

15.1.2 Transition state thermodynamics

Transition state theory holds that the equilibrium constant for formation of the transition state in a reaction can be related to the rate constant for the overall reaction by the *Eyring equation*:

$$K^\ddagger = \frac{k\hbar}{k_B T}$$

where $K^\ddagger$ is the equilibrium constant for formation of the transition state for the forward or back reaction
k is the kinetic rate constant for the interaction in the corresponding direction (k_a or k_d)
$\hbar$ is Planck's constant
k_B is Boltzmann's constant

Applying a similar rearrangement of the thermodynamic equations for the transition state gives:

$$\ln\frac{k\hbar}{k_B T} = -\frac{\Delta H^{o\ddagger}}{RT} + \frac{\Delta S^{o\ddagger}}{R}$$

so that the thermodynamic transition state constants for the forward and backward reactions can be obtained from plots of $\ln(k_a/T)$ and $\ln(k_d/T)$ respectively against $1/T$.

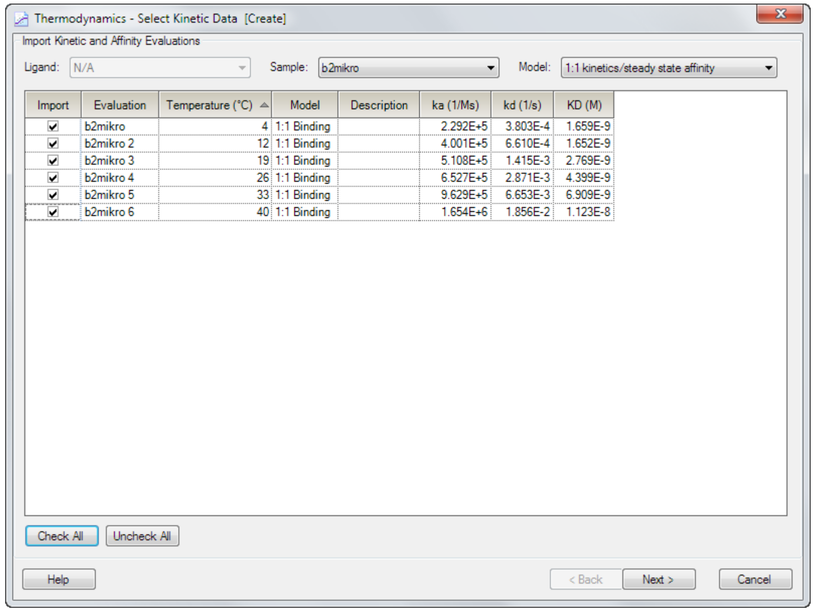
Note that the Eyring equation does not have a corresponding non-linear form that takes account of the heat capacity change for transition state formation. Non-linear fitting to obtain values for ΔC_P° can only be applied to equilibrium thermodynamic analysis.

15.2 Performing thermodynamic analysis

Before thermodynamic analysis can be performed for a set of data, the kinetics and/or affinity must be evaluated at each temperature. Create a separate ***Kinetics/Affinity*** evaluation item at each temperature used in the run, using the same fitting model for each item (see Section 14.1).

Note: *Thermodynamic analysis is not supported for data obtained with the kinetics and affinity screening tools (Chapter 13).*

When all required kinetic and affinity evaluation items have been created, click ***Thermodynamics*** on the toolbar.

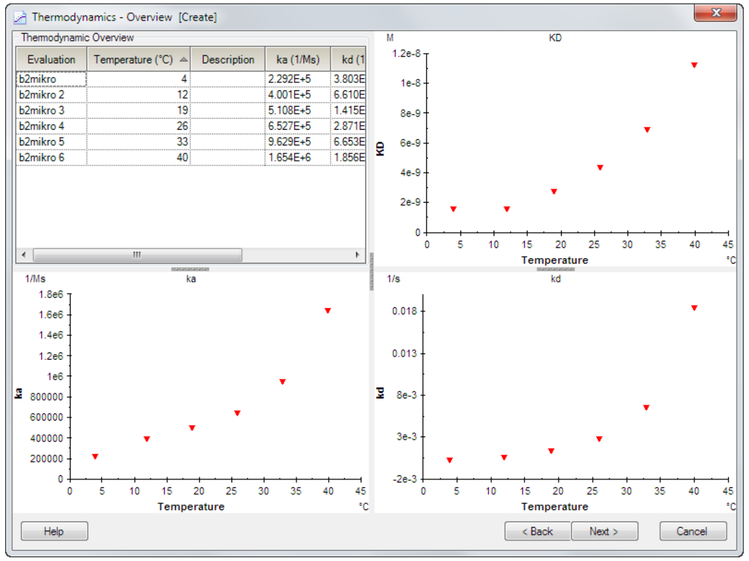


Choose the sample and the fitting model in the pull-down lists. You may only choose one sample, and you should only choose one fitting model. Options for the model are ***1:1 kinetics/steady state affinity*** (recommended) or ***All***. If you choose ***All*** it is possible to combine data from different fitting models in the same evaluation: however, values for thermodynamic constants are in all likelihood meaningless if the data is obtained from a mixture of different models.

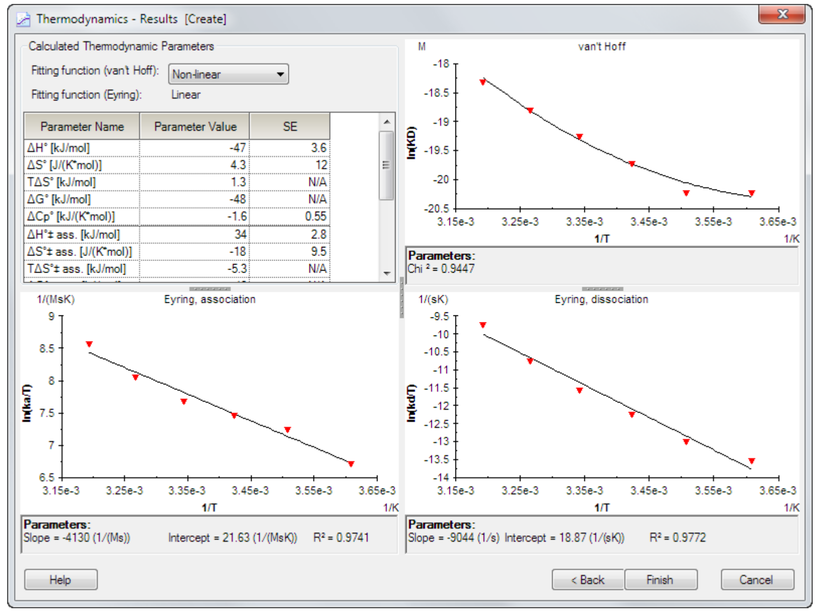
Check the rows for data that you want to use in the thermodynamic evaluation. Use the ***Check All*** and ***Uncheck All*** as quick options to select and deselect the whole list.

Note: *If you use data from fitting models that include multiple rate or affinity constants, be sure to select the correct rows so that equivalent constants are included from each fit. In some cases it may be necessary to examine the kinetic or affinity evaluation items to determine which constants belong together.*

Click ***Next>*** when you have selected the data to be included. The results are displayed first as plots of affinity and rate constants against temperature.



Click ***Next>*** to display the van't Hoff and Eyring plots together with a table of thermodynamic constants for the equilibrium and transition state formation. In any of the plots, right-click on a point to exclude the point from the line fitting.



Choose whether to use a linear or non-linear fitting function for the van't Hoff plot (see Section 15.1.1). If you choose non-linear fitting, a value for ΔCp will be included in the reported parameters. Energies of activation (E_a), derived from the Eyring plots, are also listed for the transition states. All thermodynamic parameters are calculated for a temperature of 25°C.

In any of the plots, right click on a point to exclude it from the evaluation.

Notes: *Regardless of the setting for the van't Hoff plot, the Eyring plots are always fitted to a linear function. Calculation of ΔC_p by non-linear fitting is not valid for transition state data (see Section 15.1.2).*

If you have combined kinetic and steady state affinity data in the thermodynamic evaluation, the van't Hoff plot will show all affinity values, but the Eyring plots will be empty because the steady state data lacks values for the rate constants.

Plots of kinetic and affinity constants against temperature show temperature values in °C, while van't Hoff and Eyring plots use absolute temperature values (K).

Click on ***Finish*** to finalize the thermodynamic analysis.

When assessing the validity of thermodynamic constants reported by this analysis procedure, pay particular attention to the kinetic analysis at different temperatures. With complex interactions involving macromolecules, there is a significant possibility that the characteristics of the interaction (including the role of mass transport limitations in the observed interaction) change with temperature, resulting in different fitting quality at different temperatures. This may be evident from direct comparison of the kinetic fits, but will not be immediately apparent in the thermodynamic analysis.

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Affinity in solution

Determination of affinity in solution provides an alternative to steady state affinity measurements (see Chapter 14) for interactions that take a long time to reach equilibrium or for any other reason are difficult to determine with a direct binding assay. In principle, the affinity in solution approach uses Biacore to determine the free concentration of one interactant in equilibrium mixtures containing known total interactant concentrations.

16.1 Conventions and background

16.1.1 Experimental setup

The interactants in affinity in solution determination are denoted A and B:

A + B = AB

Experiments are set up so that a fixed concentration of B is mixed with variable concentrations of A and allowed to reach equilibrium. The free concentration of B is then determined by injecting the sample over a ligand that binds B but not A or the complex AB (the interactant A or a derivative thereof is usually suitable as ligand). It is assumed that the measurement itself does not significantly disturb the equilibrium in the sample.

The experimental setup requires a calibration curve with known concentrations of B determined over the same sensor surface, in order to calculate the free B concentrations in the samples.

16.1.2 Evaluation principles

The equilibrium constant for a 1:1 interaction is given by

$$K_D = \frac{A_{free} \cdot B_{free}}{AB}$$

or

$$K_D = \frac{(A_{tot} - AB)(B_{tot} - AB)}{AB}$$

Rearranging gives

$$K_D \cdot AB = A_{tot} \cdot B_{tot} - AB(A_{tot} + B_{tot}) + AB^2$$

or

$$AB^2 - AB(A_{tot} + B_{tot} + K_D) + A_{tot} \cdot B_{tot} = 0$$

Solving for AB:

$$AB = \frac{(A_{tot} + B_{tot} + K_D)}{2} \pm \sqrt{\frac{(A_{tot} + B_{tot} + K_D)^2}{4} - A_{tot} \cdot B_{tot}}$$

Substituting in the relationship Bfree = Btot – AB gives

$$B_{free} = \frac{(B_{tot} - A_{tot} - K_D)}{2} \pm \sqrt{\frac{(A_{tot} + B_{tot} + K_D)^2}{4} - A_{tot} \cdot B_{tot}}$$

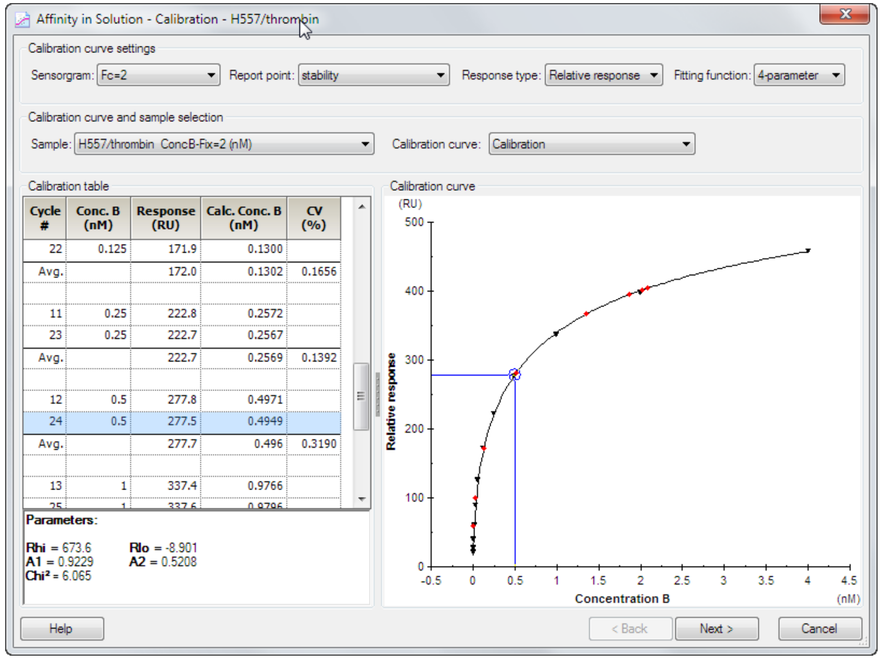
This equation can be fitted to a plot of B_{free} against A_{tot} to calculate a value for K_D. (Formally, the equation has two solutions, but one is always negative and is not meaningful in the context of an affinity determination.)

16.2 Requirements for affinity in solution

Affinity in solution experiments are run using a method. The method must be correctly constructed as described in Section 5.10.6: if necessary, the keyword table can be edited so that the conditions are met in full (see Section 6.6). Note however that the command type cannot be edited in the keyword table. Refer to Chapter 5 for details of how to construct methods in Method Builder. Determination of affinity in solution is not supported by a wizard.

16.3 Evaluation of affinity in solution

To evaluate affinity in solution measurements, open the result file and click ***Affinity in Solution*** on the toolbar. The first step displays the calibration curve for measurement of free B:



Choose the sensorgram, report point, response type and fitting function from the pull-down lists at the top of the dialog. See Section 9.2.1 for more details of these choices.

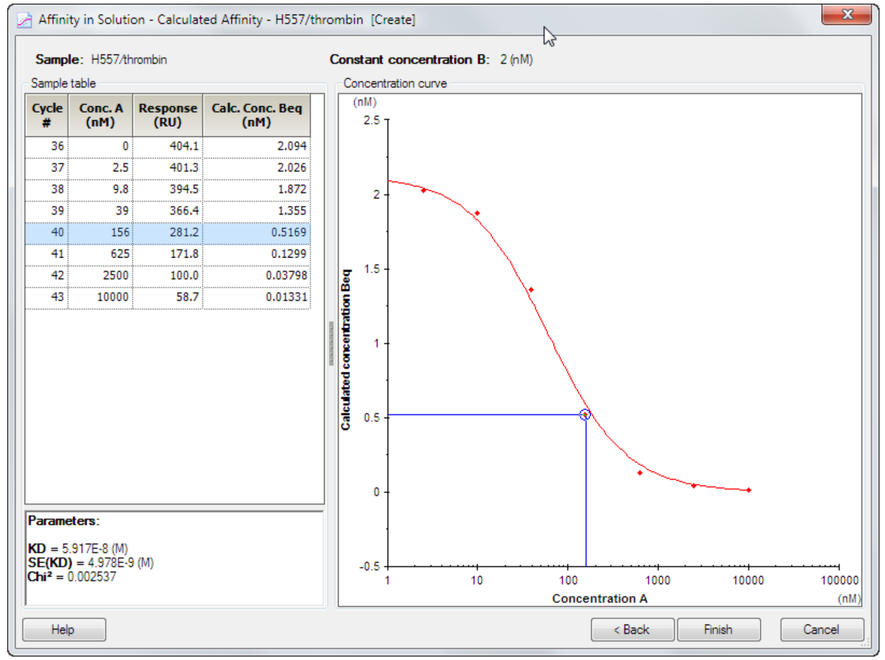
If you have run multiple sample series in the experiment, choose the sample to evaluate in the ***Sample*** list. A sample series is defined as all cycles with the same sample name in the assay step(s) with purpose ***Sample***.

If you have run multiple calibration curves in the experiment, choose the curve to use in the ***Calibration curve*** list. A calibration curve is defined as measurements from assay step(s) with purpose ***Calibration***, regardless of the sample name. If two or more ***Calibration*** assay steps are run contiguously with no intervening steps with a different purpose, they will be combined into a single calibration curve.

Note: *All samples in a series are evaluated against the chosen calibration curve. You cannot use different calibration curves for different samples in the same series.*

The table lists the data for the calibration curve. The plot panel shows the curve with calibration points as black inverted triangles and sample points as red squares. Samples that lie outside the range of the calibration curve are not shown. Right click on calibration points to exclude the points from the curve.

Click ***Next>*** to calculate the results.



The table shows the numerical results for the sample series. Samples that lie outside the range of the calibration curve are marked as ***N/A*** (not applicable) in the column for ***Calc. Conc. Beq***.

The plot panel shows the sample measurements with a line fitted according to the equation for 1:1 affinity (see Section 16.1.2). Right-click on a point to exclude it from the fitting.

Notes: *The plot of free B against total A is presented by default with a logarithmic scale on the x-axis.*

Zero values cannot be plotted on a logarithmic scale. If you have included a sample with zero concentration of A in the sample series and want to display this point on the plot, choose ***Scale*** *from the right-click menu in the plot panel and set a linear scale for the x-axis.*

The intercept of the fitted curve on the y-axis represents a fitted value for the parameter ***ConcB****. This value should be the same as or close to the value entered for the variable* ***ConcB*** *in the method.*

The calculated K_D value is shown in the panel below the table.

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Immunogenicity

Evaluation of immunogenicity experiments is supported by 4 application-specific tools in the evaluation software:

- ***Screening***, based on ranking plots for detection of antibody responses.
- ***Confirmation***, for evaluating antibody specificity as tested by inhibition of responses by added drug. Confirmation evaluation can be based either on the degree of inhibition by added drug or on a cut-off boundary to classify inhibition in yes/no terms.
- ***Isotyping***, for identifying positive responses from isotyping reagents. Isotyping results are presented as bar charts with cut-off boundaries to exclude negative responses from the chart.
- ***Stability***, which assesses the rate dissociation of detected antibody from the antigen using a two-site dissociation model.

Evaluation of immunogenicity studies is described in detail in the separate Biacore T200 Immunogenicity Handbook.

Appendix A

Data import and export

This appendix describes the functions and data format for data import and export.

A.1 Exporting data

A.1.1 Export functions

Several options are available for exporting data from Biacore T200 software to third-party programs. General options are summarized in the table below and described in more detail in the sections that follow.

Option	Context	Destination	Comments
Right click, ***Copy Table***	Most tables containing numerical data	Windows clipboard	Copied to tab-separated text as shown on the screen
Right click, ***Copy Graph***	Most sensorgram displays, plots, and charts	Windows clipboard	Copied as a graphical object
Right click, ***Export curves***	Most sensorgram displays and plots	Windows clipboard	Data point coordinates exported as tab-separated text
File:Export to Excel ***File:Export to XML***	Control and Evaluation Software	Excel or XML file	All session content exported (see below)
File:Export Report Point Table	Control and Evaluation Software	Text file	Entire report point table exported regardless of columns, sorting and filtering

There are also some export facilities restricted to specific functions, such as export of rack positions from the ***Rack Positions*** dialog in the Control Software and export of screening results with both graphical thumbnails and numerical data from finished kinetics and affinity screening evaluation items.

A.1.2 Export to Excel

To export data to an Excel file, choose ***File:Export:Results to Excel***.

Export from the Control Software creates an Excel spreadsheet file (extension ***.xls***) containing separate worksheets for the file properties and report point table. The audit trail is also exported to a separate worksheet if it is present when the GxP module is installed (see the separate Biacore T200 GxP Handbook).

Export from the Evaluation software creates an Excel spreadsheet file (extension ***.xls***) containing separate worksheets for the file properties and for tabulated data for all evaluation items where appropriate (i.e. plot data and evaluation results). The worksheets for each item are identified with the item name. For plots, only the columns shown in the plot window table are exported. Data from sensorgram items is not exported. The audit trail is also exported to a separate worksheet if it is present when the GxP module is installed (see the separate Biacore T200 GxP Handbook).

A.1.3 Export to XML

To export data to an XML file, choose ***File:Export:Results to XML***.

This option exports the same data as ***Results to Excel*** but creates a text file in XML format (file extension ***.xml***). Details of the XML format are most easily determined by exporting data from the Control or Evaluation Software and opening the exported file in an XML-compatible editor.

An example of the beginning of an XML export file is shown below.

```
<?xml version="1.0" encoding="utf-8"?>
<EvaluationFile>
  <FileProperties>
    <EvalFileProperties>
      <EvaluationFile>
      </EvaluationFile>
      <UserInformation>
        <PerformedBy>501815</PerformedBy>
        <CurrentUser>501815</CurrentUser>
      </UserInformation>
      <CreatedWithSoftware>
        <Name>Biacore T200 Evaluation Software</Name>
        <Version>3.0</Version>
      </CreatedWithSoftware>
      <Notebook><![CDATA[]]></Notebook>
    </EvalFileProperties>
    <ResultFiles>
      <ResultFile1>
        <ResultFile>
```

A.1.4 Report point table

To export the report point table to a tab-separated text file, choose ***File:Export:Report Point Table***. The exported file has the extension ***.rpt***. The entire report point table is exported regardless of settings for ***Table columns***, sorting, and filtering. Use ***Copy Table*** from the right-click menu to copy the report point table as displayed on the screen.

Note: *If you open an exported report point table in Microsoft Excel, make sure that the format for the **Fc** column is set to **Text** in the Excel import file wizard. The default setting of **General for** text file import may interpret the flow cell identification for reference-subtracted data as a date instead of a text string.*

A.1.5 Kinetics and affinity screening

To export both thumbnail graphs and numerical data from kinetic and affinity screening items, choose ***Export All Graphs and Table*** from the right-click menu in the thumbnail panel of the finished item. This will create a folder with the same name as the evaluation file, containing all thumbnails as separate files in ***.png*** format and the results summary table in tab-separated text format. The file name for each exported thumbnail is listed in the Image file column of the results summary.

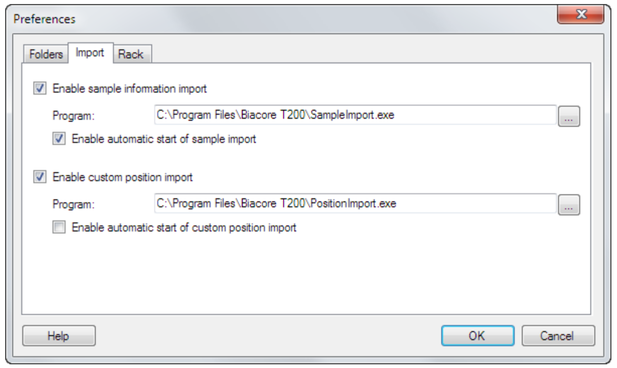
A.1.6 Rack positions

Rack positions can be exported from the Control Software to a tab-separated text file in either ASCII or Unicode format using the ***Menu:Export Positions*** function in the ***Rack Positions*** dialog (Section 4.2.6). The file contains two lines identifying the microplate and reagent rack settings followed by the contents of the rack positions table with the columns separated by tabs.

A.2 Importing data

A.2.1 Control Software

The Control Software supports data import to sample tables in assay wizard templates and in the ***Setup Run*** step of methods and to position information to the ***Rack Positions*** step in all runs. In order to use the import function, the option must be activated in ***Tools:Preferences*** and valid import programs must be specified. Import programs and data files for use with import are the responsibility of the user.



For each import function, a check box allows the import program to be started automatically without user intervention. If the respective box is not checked, the program will only be started when the user actively requests data import. If the box is checked:

- Data is imported automatically to the sample table only if the table is empty. The program is not started if the table already contains data.
- Data is imported to the ***Rack Positions*** dialog whenever automatic positioning is invoked. This happens when the dialog is first opened with new or modified sample data, and also when the user requests ***Automatic positioning*** from the dialog menu (Section 4.2.6). The program is not started when the user makes manual changes to the rack positioning or when the dialog is opened with no changes in previously positioned samples (for example when the user clicks ***Back*** and ***Next*** in the dialog sequence without changing sample information).

Sample table import

When the sample import function is invoked, the contents of the sample table are first exported in Extended Markup Language (XML) format to a temporary file that is submitted to the specified import program. The import program may append new sample data to the file or overwrite the file contents with new data as required. The modified file is then imported back into the sample table and the temporary file is deleted.

Development or choice of a suitable import program is the responsibility of the user. To document the detailed XML format of the import file, specify an XML- compatible text editor as the import program and save a copy of the import file from a suitable table.

```
<?xml version="1.0" encoding="iso8859-1" ?>
<!DOCTYPE MethodBuilderImport (View Source for full doctype...)>
- <MethodBuilderImport importFileVersion="1.0">
  - <AssaySteps>
    - <AssayStep name="Startup">
      - <DataTable row="1">
          <Data cmd="Sample 1" grp="" fld="Solution" val="sample 1/2" />
          <Data cmd="Sample 1" grp="" fld="Conc1" val="0" />
          <Data cmd="Sample 1" grp="" fld="Conc2" val="125" />
          <Data cmd="Sample 1" grp="" fld="MW1" val="200" />
          <Data cmd="Sample 1" grp="" fld="MW2" val="10000" />
        </DataTable>
      </AssayStep>
    - <AssayStep name="Sample">
      - <DataTable row="1">
          <Data cmd="Sample 1" grp="" fld="Solution" val="sample 1/2" />
          <Data cmd="Sample 1" grp="" fld="Conc1" val="0" />
          <Data cmd="Sample 1" grp="" fld="Conc2" val="0" />
          <Data cmd="Sample 1" grp="" fld="MW1" val="200" />
          <Data cmd="Sample 1" grp="" fld="MW2" val="10000" />
        </DataTable>
      - <DataTable row="2">
          <Data cmd="Sample 1" grp="" fld="Solution" val="sample 1/2" />
          <Data cmd="Sample 1" grp="" fld="Conc1" val="0" />
          <Data cmd="Sample 1" grp="" fld="Conc2" val="195" />
```

Figure A-1. Part of an XML import file from a method for heterogeneous analyte kinetics displayed in an XML-compatible editor.

Rack positions import

The ***Menu:Custom Position Import*** and ***Simple Position Import*** functions in the ***Rack Positions*** dialog (Section 4.2.6) import rack position data from an external file such as one from a laboratory robot used to prepare sample microplates. If you choose ***Custom Position Import***, the external file is first processed by the import program as specified in ***Files:Preferences***. Output from this program must be tab-separated text in either ASCII or Unicode format conforming to the specification below. The ***Simple Position Import*** option imports data from a file conforming to the specifications with no intervention from an external program.

- Two lines in the file specify the microplate and reagent rack settings, in the format

  Rack1=<microplate specification>

  Rack2=<reagent rack specification>

- Specifications are not case-sensitive, but microplate and reagent rack specifications must be given otherwise exactly as they appear in the selection lists in the ***Rack Positions*** dialog. If either specification is invalid, the corresponding definition will not be imported. The position of these two lines in the file does not matter.
- One line specifies the headers for table columns to be imported, separated by tabs. The headers should correspond to the column headers as they appear in the ***Rack Positions*** table, with the exception of the ***Volume*** column in the table which can be omitted from the import file (and is ignored if it is present). This line may not be preceded by any line other than the microplate and reagent rack specifications.
- A set of lines hold the content of the table columns separated by tabs. Each line must contain the same number of tab characters as the header line.

When import is requested, the contents of each table line in the import file are matched as far as possible to the contents of the ***Rack Positions*** table, with the exception of the ***Position*** and ***Volume*** column. For matched rows, the ***Position*** in the table is replaced by the value in the ***Position*** column from the import file. Rows for which a match cannot be found are not imported. Any rows in the ***Rack positions*** table which do not have a matching row in the import file are left without a ***Position*** specification and must be placed in the microplate or reagent rack before the run can be started.

Details of the required import file format can be investigated further by examining a file created with the ***Menu:Export Positions*** command.

```
<?xml version="1.0" encoding="utf-8"?>
<EvaluationFile>
  <FileProperties>
    <EvalFileProperties>
      <EvaluationFile>
      </EvaluationFile>
      <UserInformation>
        <PerformedBy>501815</PerformedBy>
        <CurrentUser>501815</CurrentUser>
      </UserInformation>
      <CreatedWithSoftware>
        <Name>Biacore T200 Evaluation Software</Name>
        <Version>3.0</Version>
      </CreatedWithSoftware>
      <Notebook><![CDATA[]]></Notebook>
    </EvalFileProperties>
    <ResultFiles>
      <ResultFile1>
        <ResultFile>
          <Name>LMW screen.blr</Name>
          <Path>C:\Users\501815\Documents\Test run</Path>
          <Size>1 215 488 bytes</Size>
        </ResultFile>
        <RunInformation>
          <Type>Method Builder</Type>
          <Method>(Untitled)</Method>
          <Cycles>24</Cycles>
          <Start>20/01/2014 10:15:20</Start>
          <End>20/01/2014 14:27:02</End>
        </RunInformation>
        <Instrument>
          <InstrumentType>BiacoreT200</InstrumentType>
          <InstrumentId>1358419</InstrumentId>
          <IFC>TYPE105</IFC>
        </Instrument>
        <UserInformation>
```

Figure A-2. Example of an exported file for rack positions, opened in Microsoft Excel.

A.2.2 Evaluation Software

The Evaluation Software supports import of model definitions for kinetics and affinity evaluation. Model files for import should be obtained from GE or created by exporting models from another installation.

Appendix B

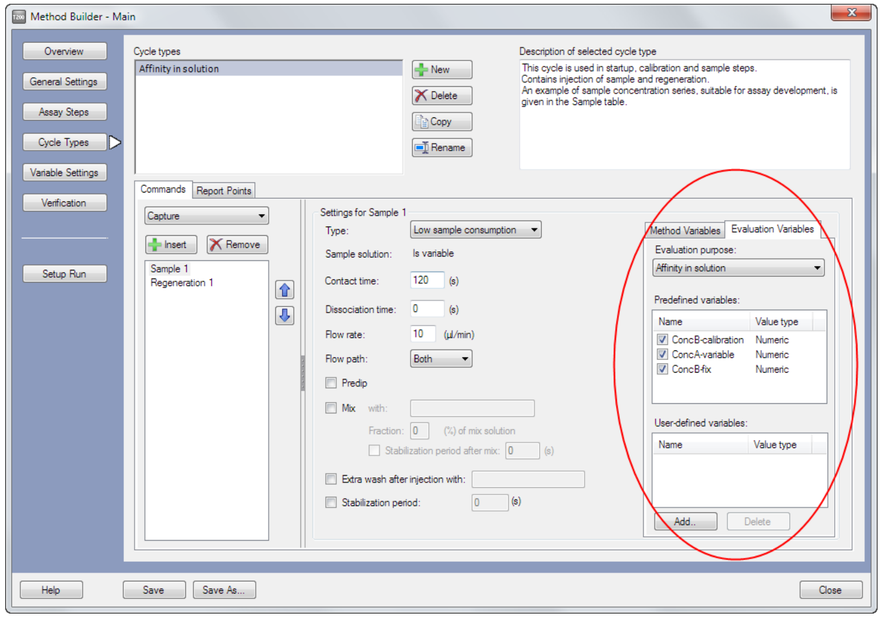
Method examples and recommendations

A selection of predefined methods covering common applications is provided in the ***Biacore Methods*** folder (see Section 5.1). Use these methods either directly or as starting points for your own method development. This section describes the essential features in each method that are not supported in wizards. Refer to these methods as guidelines in constructing your own methods that exploit similar features.

B.1 Affinity in solution

This method is designed for measurement of affinity in solution as described in Chapter 16. The method includes a ***Calibration*** assay step for measurement of component B and a ***Sample*** step for measurement of mixtures of components A and B. Both these assay steps are connected to the same cycle type.

Predefined evaluation variables are included for the evaluation purpose ***Affinity in solution***:



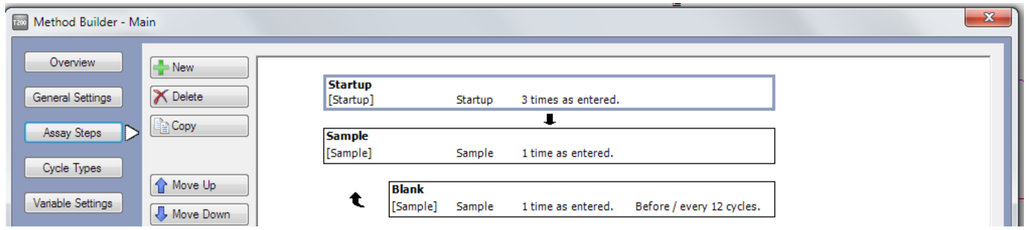
The ***Variable Settings*** are different for the two assay steps, so that only the relevant variables are entered at run-time for each assay step.

B.2 Calibration-free concentration analysis

Requirements and recommendations for running calibration-free concentration analysis are provided in this method.

B.2.1 Assay steps and general settings

Blank cycles are run before the samples and are repeated every 12 sample cycles. Assay steps for samples and blanks are connected to the same cycle types:

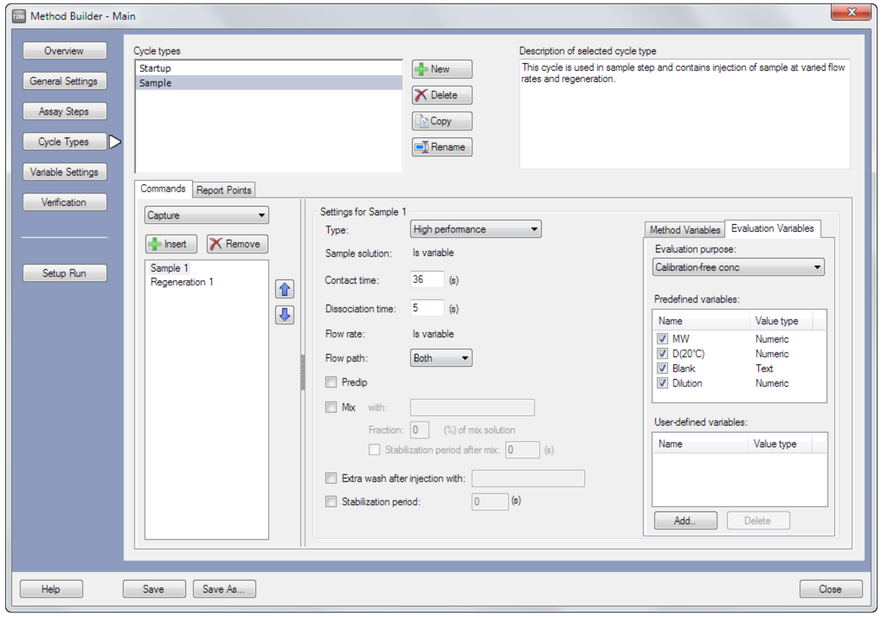


Analysis is performed at a data collection rate of 10 Hz (set in the ***General*** workspace). Do not change this setting.

B.2.2 Cycle types

The cycle type for sample analysis performs a ***High performance*** injection with a contact time of 36 seconds and a dissociation time of 5 seconds. The dissociation time is not critical: evaluation uses only data from the association phase. Do not use shorter contact times than 36 seconds, since the actual contact time (which is determined by the injected volume, rounded to the nearest µL) may differ significantly at the lowest flow rate.

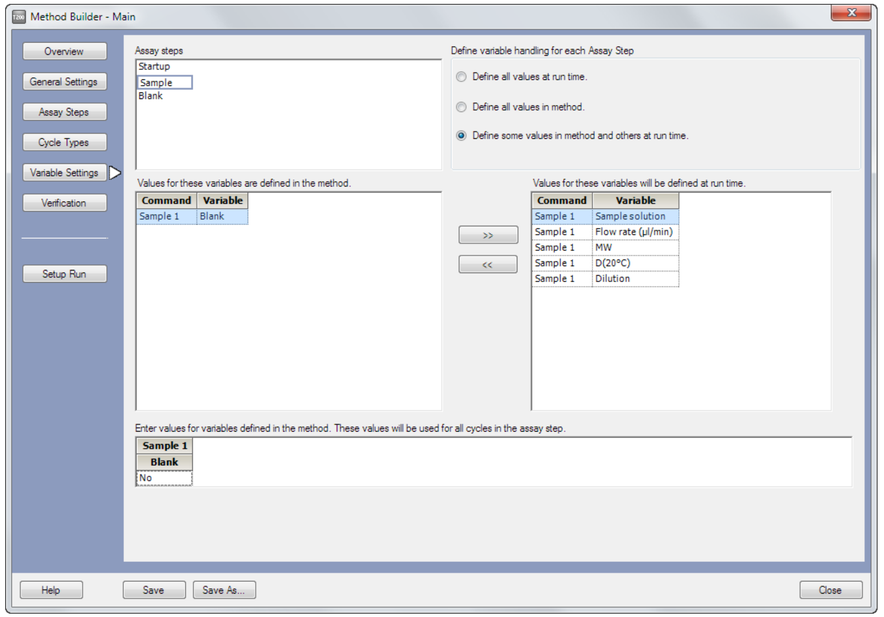
Sample solution and flow rate are set as method variables. For evaluation variables, the evaluation purpose is set to ***Calibration-free conc*** and predefined variables ***MW***, ***D(20°C)***, ***Blank*** and ***Dilution*** are required.



B.2.3 Variable settings

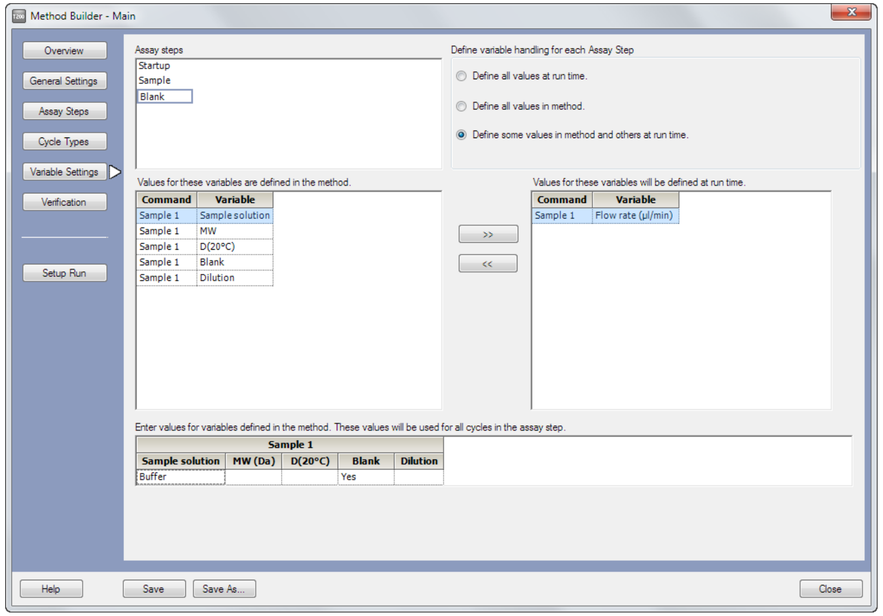
Sample cycles

All the variables for assay step ***Sample*** are set at run time, except for the method variable ***Blank*** which is set to ***No*** in the method.



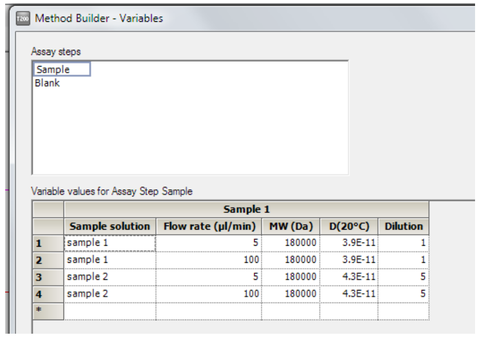
Blank cycles

For assay step ***Blank***, only the flow rate is set at run time. The method variable ***Blank*** is set to ***Yes*** in the method and ***Sample solution*** to ***Buffer***. The remaining variables are not used and may be left blank.

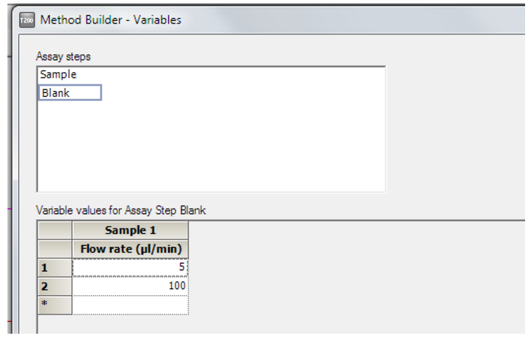


B.2.4 Setup Run

At run time, variables need to be assigned for assay steps ***Sample*** and ***Blank***. Each sample should be run at two or more flow rates: values of 5 and 100 µL/min are recommended and are entered for one sample. Use the same set of flow rates for each sample. Values are also required for analyte molecular weight (***MW***), diffusion coefficient at 20°C (***D(20°C)***) and dilution factor (***Dilution***).



	Sample 1				
	Sample solution	Flow rate (µl/min)	MW (Da)	D(20°C)	Dilution
1	sample 1	5	180000	3.9E-11	1
2	sample 1	100	180000	3.9E-11	1
3	sample 2	5	180000	4.3E-11	5
4	sample 2	100	180000	4.3E-11	5
*					



	Sample 1
	Flow rate (µl/min)
1	5
2	100
*	

Only flow rates are required for assay step ***Blank***: these values must be the same as the flow rates used for samples.

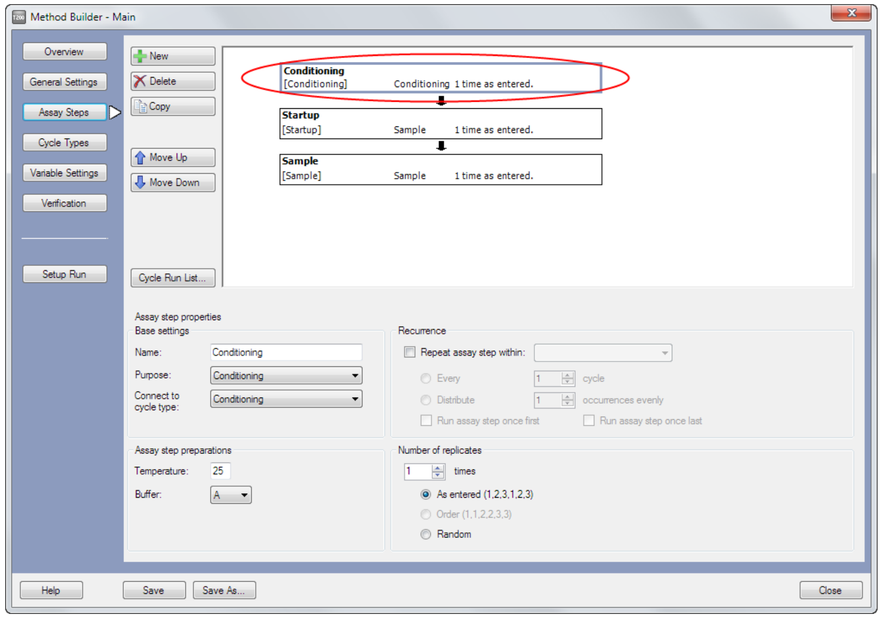
B.3 CAP single-cycle kinetics

This method implements single-cycle kinetics using reversible capture of biotinylated ligand on Sensor Chip CAP (supported by the Biotin CAPture Kit from Cytiva). This section focuses on the method aspects required for using the Biotin CAPture Kit. If you want to create a method for other applications using the Biotin CAPture Kit, modify the ***Sample*** cycle definition in the ***CAP single-cycle kinetics*** method. See Section B.14 for aspects of the method directly related to single-cycle kinetics.

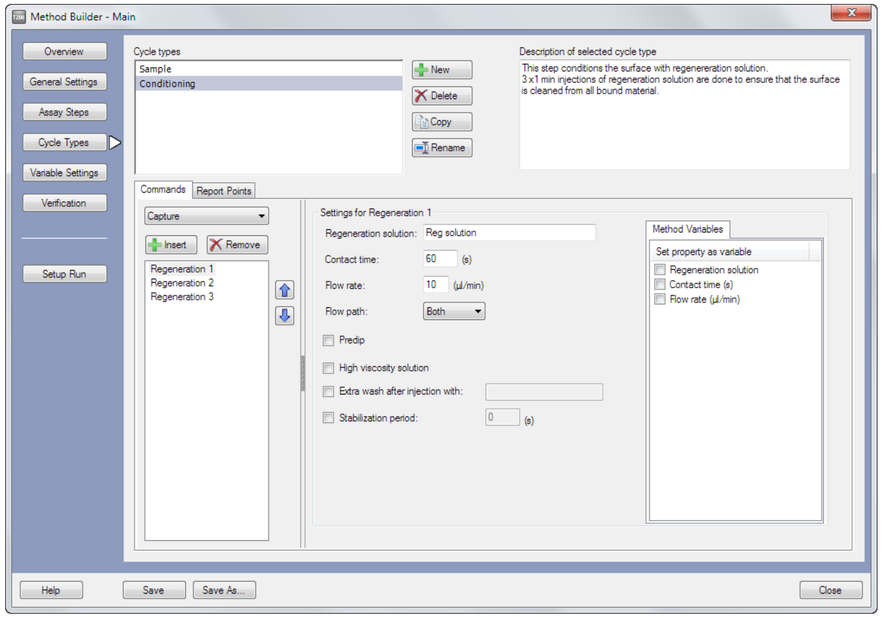
Follow the Instructions for Use supplied with the Biotin CAPture Kit before using a new Sensor Chip CAP.

B.3.1 Assay steps

The method includes a ***Conditioning*** step run once before the start-up cycles.



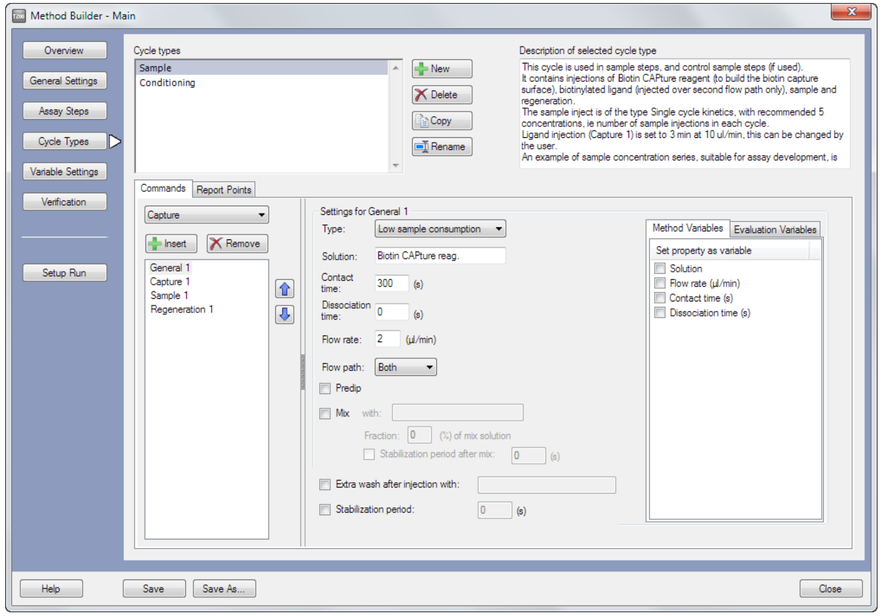
This step injects three 1-minute pulses of regeneration solution, and should always be included at the start of an assay using a new sensor chip. The step can be omitted if the sensor chip has been used previously.



B.3.2 Sample analysis cycle for Sensor Chip CAP

The cycle definition for sample analysis with Sensor Chip CAP includes 4 injection commands:

- ***General*** for injection of the Biatin CAPture reagent
- ***Capture*** for injection of the biotinylated ligand
- ***Sample*** for injection of analyte
- ***Regeneration*** to remove the Biotin CAPture reagent, ligand and bound analyte



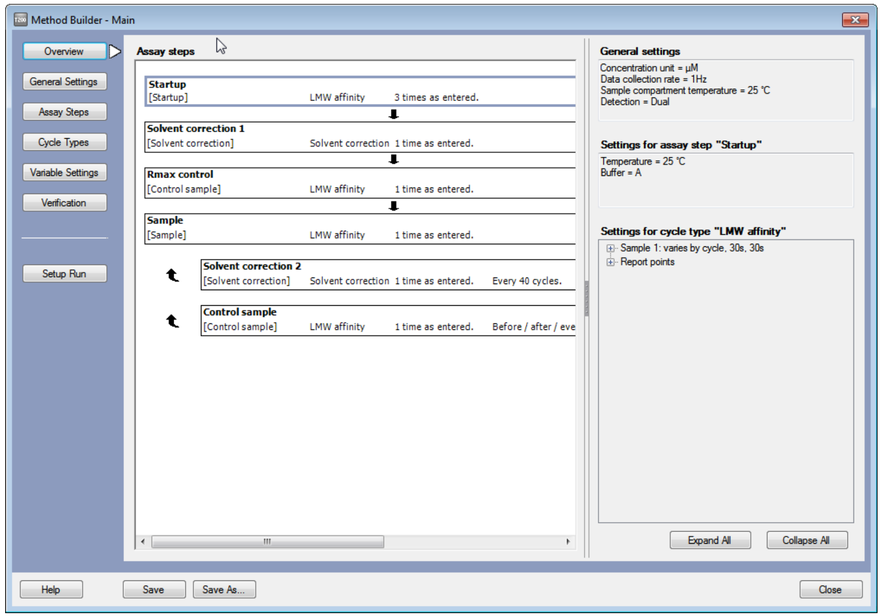
An extra wash with buffer after the injection is included in the ***Regeneration*** command.

B.4 Fragments – Affinity screen

In fragment screening contexts, ***Affinity screen*** is used to prioritize fragments according to their steady-state binding affinities. ***Affinity screen*** is typically used downstream of ***Binding level screen***, and provides confirmation of ***Binding level screen*** results as well as more comprehensive characterization.

The ***Affinity screen*** method may be used as a starting point for screening any molecule library on the basis of binding affinity. Adjustments to the injection time, use of regeneration and placement of the ***binding*** report point may be necessary to adapt the method to screening analytes that do not exhibit rapid binding and dissociaiton.

B.4.1 Assay steps



Solvent correction 1 provides the first solvent correction cycle, run before any of the sample or control sample cycles.

Rmax control uses a single cycle type that injects a positive control to determine the experimental R_{max}. This allows evaluation using a constant R_{max} model (Section 12.7.6) with the R_{max} value adjusted for drift in surface binding capacity.

The remaining assay steps provide analysis of samples, with solvent correction every 40 cycles and a control sample at the beginning and end and every 32 cycles.

B.4.2 Cycle types

The cycle type for sample analysis performs a ***High performance*** injection with a contact time and dissociation time of 30 seconds, which is long enough to reveal deviations from the typical "square wave" binding behavior of fragments. No regeneration is required, but an extra wash with 50% DMSO is included.

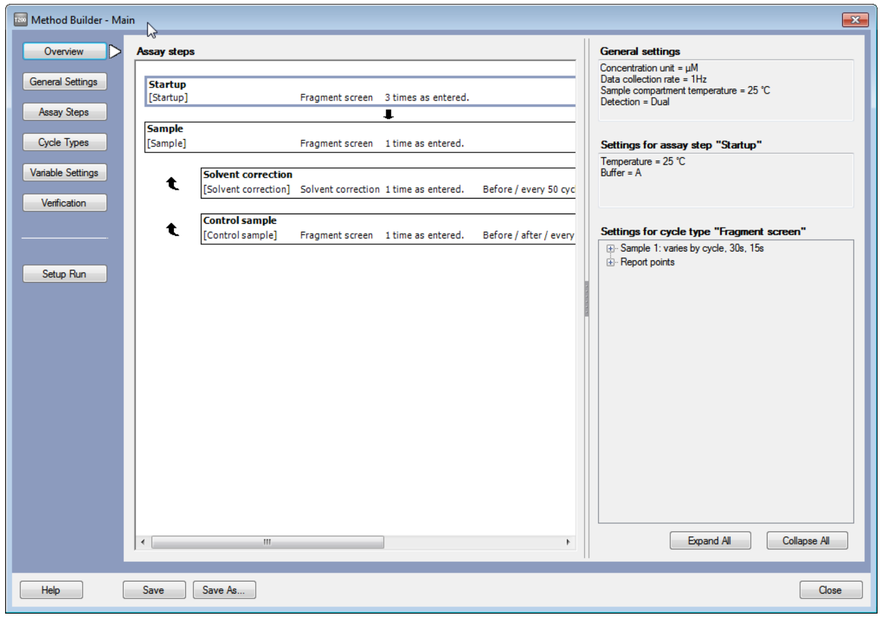
The report point ***Binding*** is set to 7 seconds after the start of the injection, which reduces the risk that promiscuous binding behavior may distort the affinity evaluation.

Sample solution is set as method variable. For evaluation variables, the evaluation purpose is set to ***Kinetics/Affinity*** and predefined variables ***Conc*** and ***MW*** are included. Molecular weight is required if a fitting model using constant R_{max} is to be used, even if concentrations are given in molar units.

B.5 Fragments – Binding level screen

Binding level screen is used to select fragments for downstream processing based on the binding response at single concentrations. The same concentration is used for all samples, chosen to be relevant for the expected affinities. While the ***Binding level screen*** method is designed primarily for fragments, it may be used as a starting point for any single-concentration screen.

B.5.1 Assay steps



Samples are analyzed with solvent correction run at the beginning and repeated every 50 cycles, and control samples run at the beginning and end and repeated every 30 cycles.

B.5.2 Cycle types

The cycle type for sample analysis performs a ***High performance*** injection with a contact time of 30 seconds and a dissociation time of 15 seconds, which is long enough to reveal deviations from the typical "square wave" binding behavior of fragments. No regeneration is required, but an extra wash with 50% DMSO is included.

The report point ***Binding*** is moved to 7 seconds after the start of the injection, which reduces the risk that promiscuous binding behavior may distort the affinity evaluation.

Sample solution is set as method variable. For evaluation variables, the evaluation purpose is set to ***General*** and predefined variables ***Conc*** and ***MW*** are required.

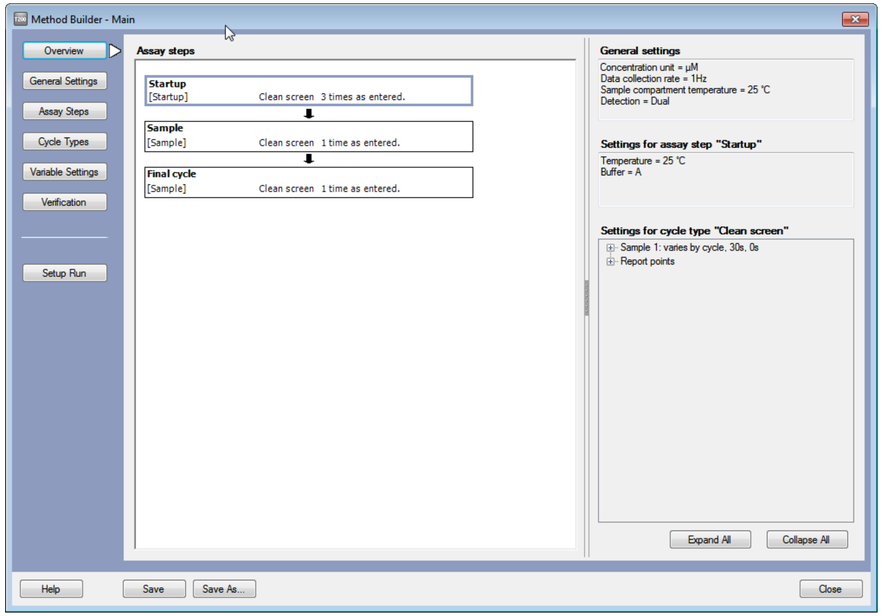
B.5.3 Evaluation

Evaluation is performed using the ranking or cut-off function in result plots.

B.6 Fragments – Clean screen

In fragment screening applications, the ***Clean screen*** method may be run as a first step in the screening procedure, to identify and exclude "sticky" compounds that affect subsequent cycles. Elevation of the baseline is the only criterion used to detect this behavior.

B.6.1 Method definition



Since evaluation of ***Clean screen*** is based on the baseline in each cycle, solvent correction and control samples are not required in a ***Clean screen***. The assay step ***Final cycle*** injects buffer or a dummy sample to provide a baseline for evaluating the last compound in the ***Sample*** assay step.

The cycle type for sample analysis and the final cycle performs a ***Low sample consumption*** injection with a contact time of 30 seconds, followed by an extra wash with 50% DMSO. The contact time should be adjusted to the value used in downstream screening analyses.

Note: *The extra wash with 50% DMSO will remove many "sticky" compounds. The purpose of* ***Clean screen*** *is however to identify compounds that show persistent binding to the surface even after normal washing procedures.*

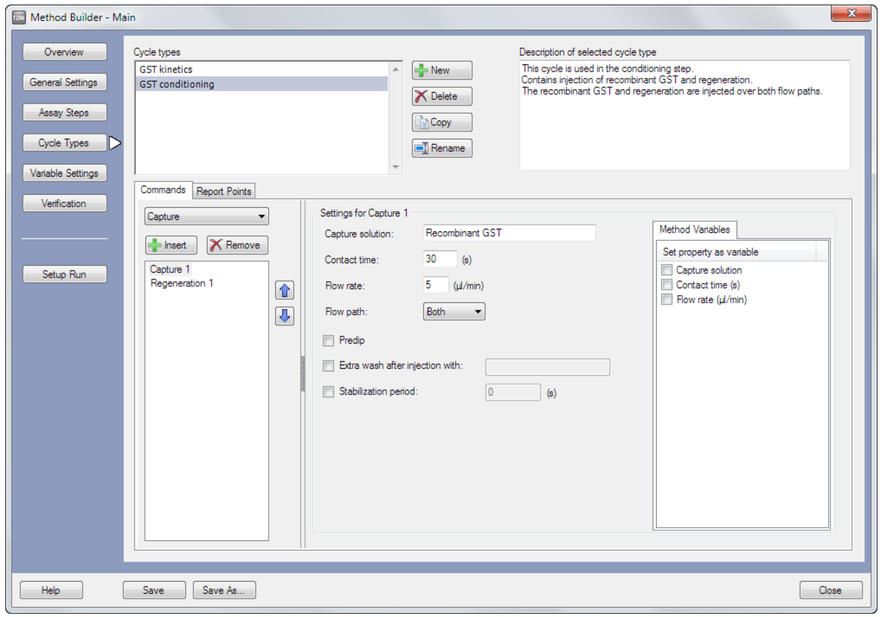
Molecular weight is included as an evaluation variable. This is not necessary for the clean screen itself, but may be useful if binding data is evaluated for samples not disturbed by persistent binding behavior.

B.6.2 Evaluation

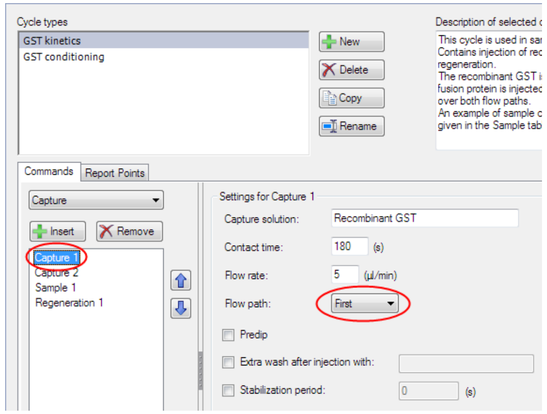
Evaluation is performed by examining the plot of Baseline against cycle number (provided as a pre-defined plot). Any cycles that show elevated baseline (particularly if the baseline increase persists over two or more cycles) indicate persistent binding of the sample analyzed immediately before the elevation.

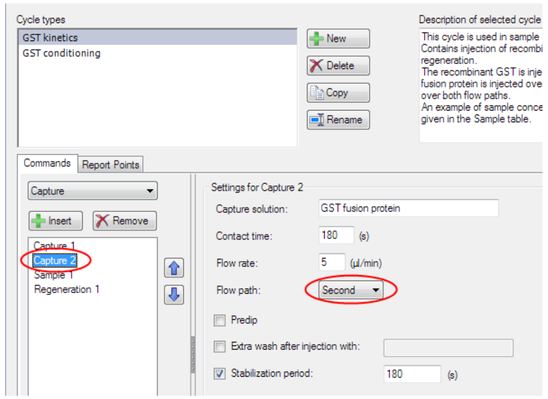
B.7 GST kinetics

The recommended method for using a GST-tagged ligand is set up with ***Dual*** detection in ***General Settings***. Detection settings ***Multi*** and ***Single*** are not recommended. The method involves 5 conditioning cycles with injection of recombinant GST (provided in the GST Capture Kit from Cytiva) over both flow cells followed by regeneration:



The sample analysis cycle uses two capture injections, one to capture recombinant GST on the reference surface only (flow path setting ***First***) and the second to capture GST-tagged ligand on the active surface only (flow path setting ***Second***). A stabilization time of 180 seconds is included after capturing the ligand on the active surface, to allow for dissociation of any weakly bound components. The sample is then injected over both surfaces. This approach provides a reference surface that mimics the active surface with respect to occupancy of the anti-GST capturing sites.



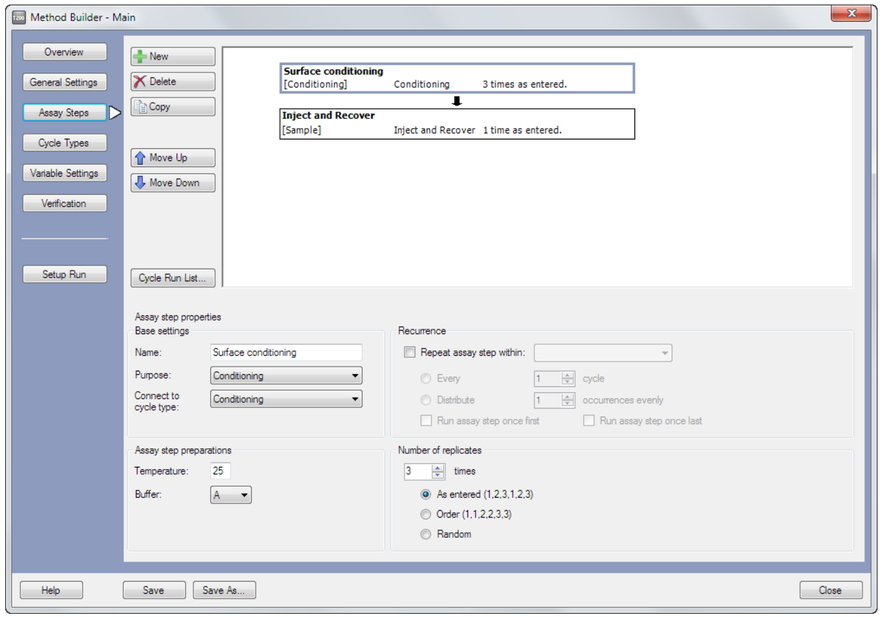


This method is set up for kinetic analysis but can be adapted for other assays.

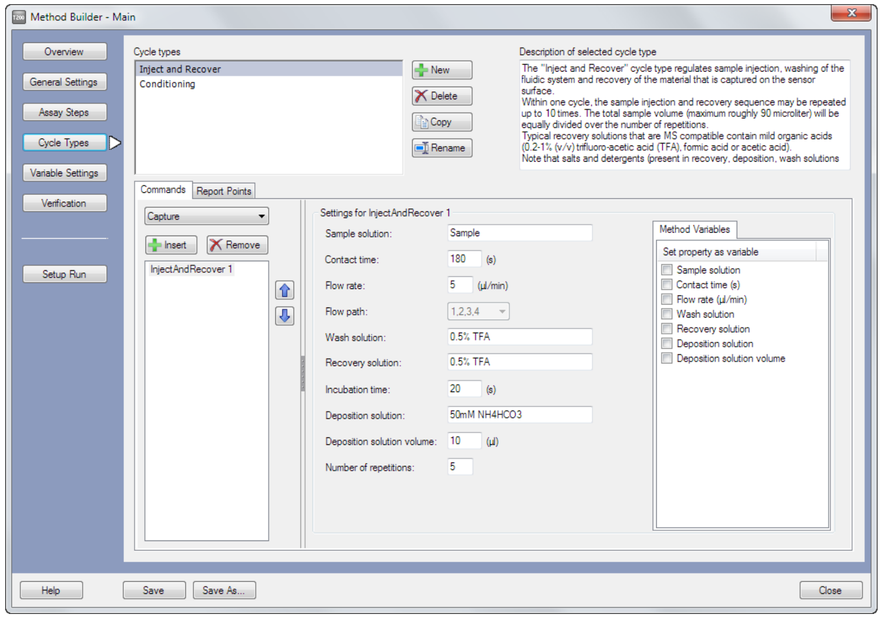
Note: *If you change the detection setting to* ***Multi****, you will need to include additional capture injections for the active surfaces in flow cells 3 and 4. Do not use detection setting* ***Single*** *without removing the first capture injection.*

B.8 Inject and Recover

The example method provided for using the ***InjectAndRecover*** command is targeted to recovery for mass spectrometry, and contains two assay steps. The first conditions the surface by washing three times with 0.5% trifluoroacetic acid, while the second performs the sample injection and recovery operation.



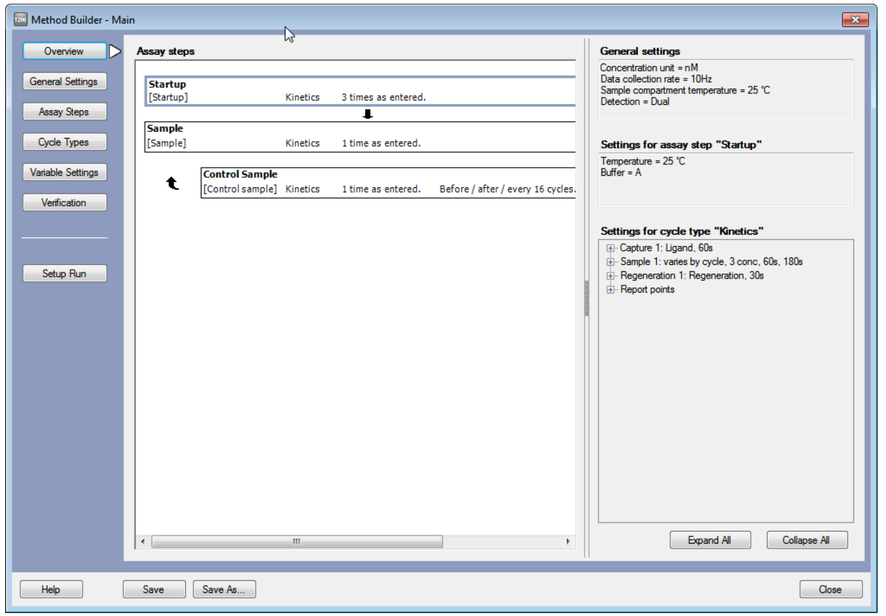
The cycle for recovery of bound analyte contains just one single ***InjectAndRecover*** command. Additional commands are usually not needed, since the recovery component of the ***InjectAndRecover*** command serves as a regeneration step (see Section 5.6.1).



All parameters in the ***InjectAndRecover*** command are fixed: check the appropriate boxes in the ***Method Variables*** list if you want to use variable parameters. There are no evaluation variables for this command.

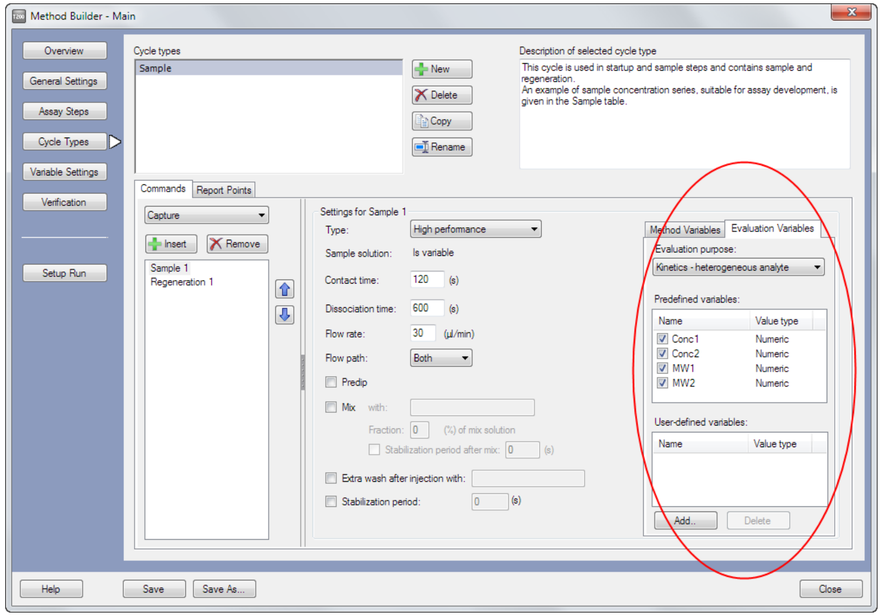
B.9 Kinetic screen

The ***Kinetic screen*** method provides an example of high-capacity kinetic analysis, based on single-cycle analysis of each sample at 3 concentrations. A zero-concentration blank is run for each sample. The method is set up as a capture assay to provide flexibility for screening against multiple ligands. The capture injection may be deleted if the ligand is directly immobilized on the surface.



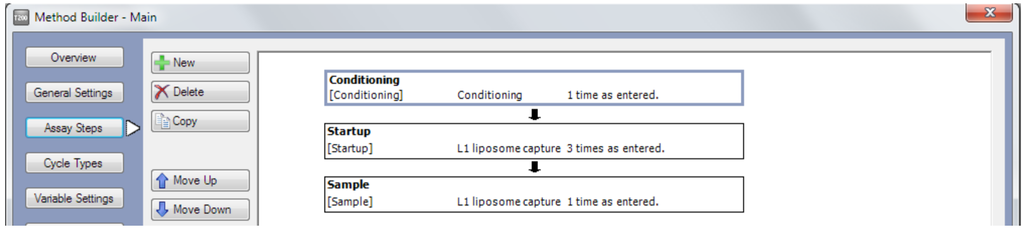
B.10 Kinetics heterogeneous analyte

This method is a straightforward kinetics determination with evaluation variables included for the evaluation purpose ***Kinetics – heterogeneous analyte***, providing separate concentration and molecular weight variables for two analytes.



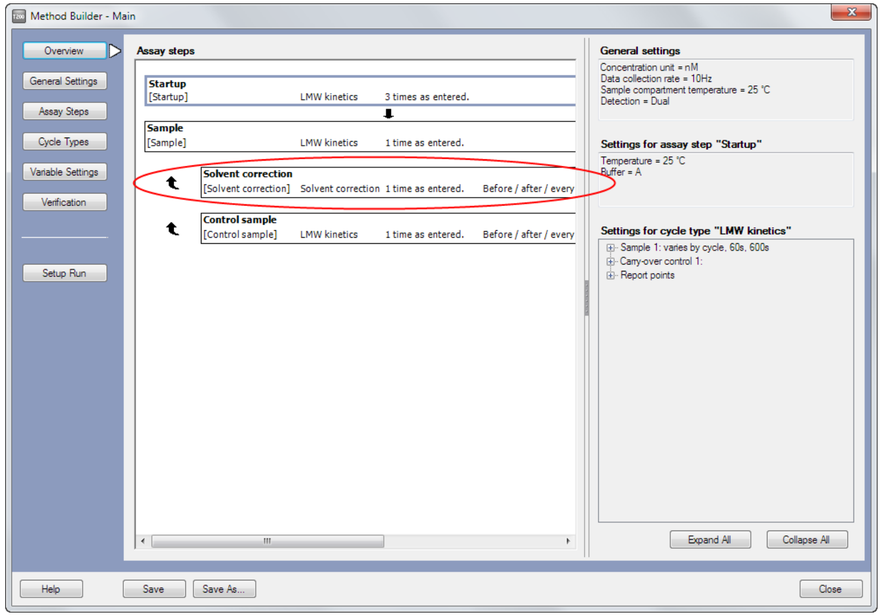
B.11 L1 liposome capture

The recommended method for liposome capture on Sensor Chip L1 uses one conditioning cycle consisting of two injections of 40 mM octylglucoside, followed by three start-up cycles using a dummy sample before the actual samples are run.

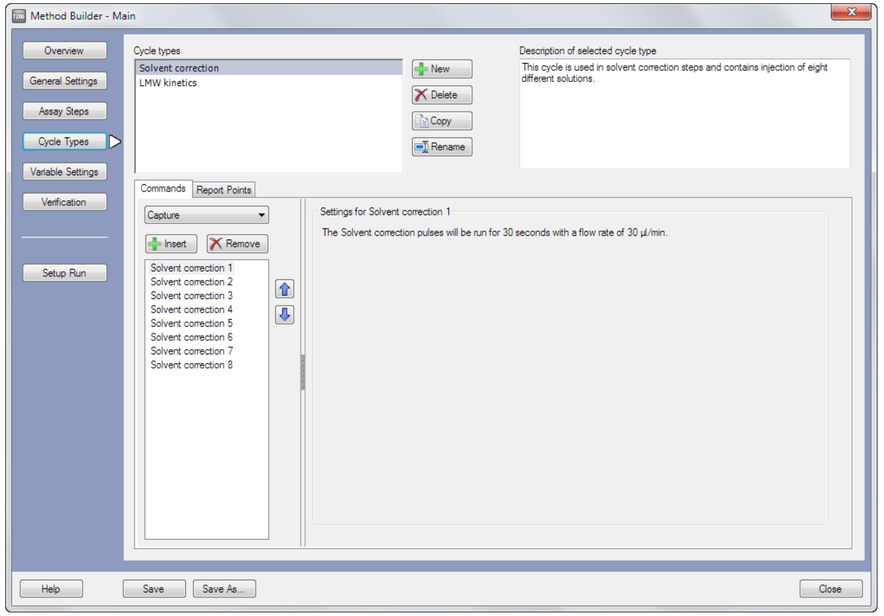


B.12 LMW kinetics and LMW Screen

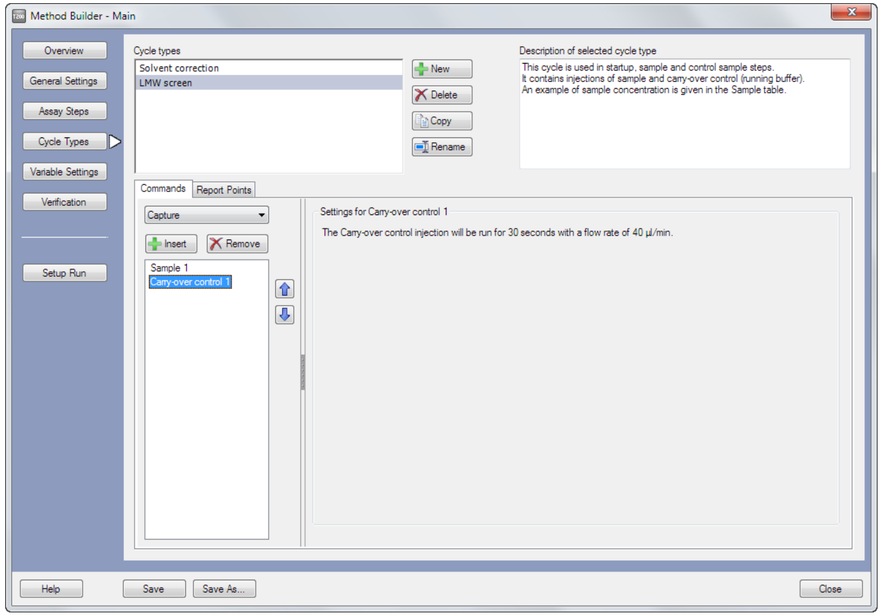
Methods are provided for both kinetics and screening of low molecular weight compounds. The essential addition to wizard-based counterparts is the inclusion of a ***Solvent Correction*** assay step.



This assay step is connected to a cycle type that includes 8 ***Solvent Correction*** commands for injection of 8 different solvent concentrations (see Section 6.7).



Screening applications suitably include a carry-over control injection, to identify potential carry-over problems from “sticky” compounds that may affect the response in subsequent injections. The Biacore method is designed for screening of low molecular weight compounds and includes a solvent correction step as described in Section B.12. Low molecular weight compounds frequently dissociate readily from their targets, and the example method does not include regeneration. If you require a regeneration step, it is advisable to include regeneration after both the sample and the carry-over injections.

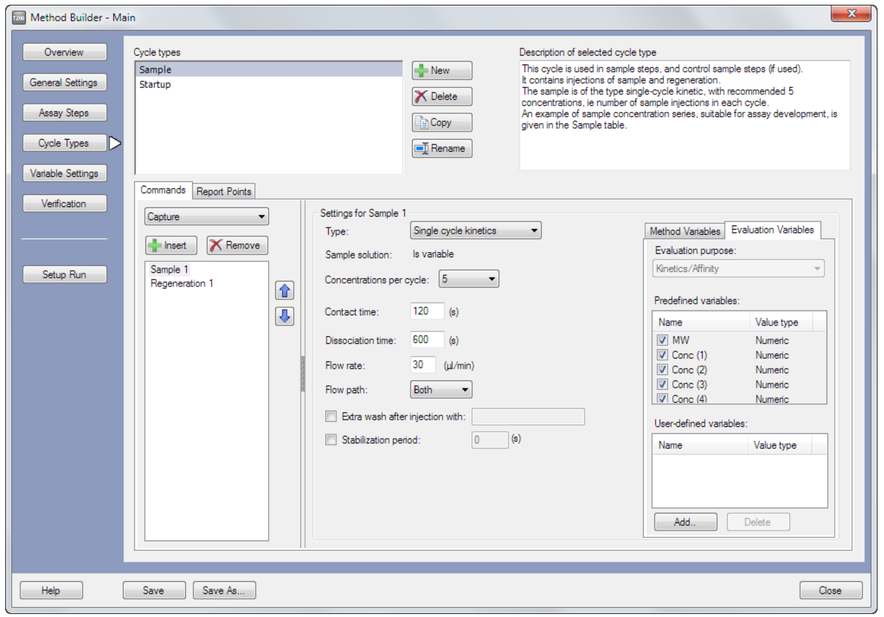


B.13 LMW single-cycle kinetics

This method combines functions for working with low molecular weight analytes (Section B.12) with the Sample command for single-cycle kinetics (Section B.14).

B.14 Single-cycle kinetics

The Biacore method for single-cycle kinetics uses the sample command type ***Single cycle kinetics*** with 5 sample concentrations per cycle. Note that with this command type, the evaluation purpose is fixed as ***Kinetics/Affinity*** and the number of predefined variables for sample concentration corresponds automatically to the number of sample concentrations per cycle. The predefined variables cannot be deselected.



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