BIAevaluation Software Handbook

version 3.0

"Things should be made as simple as possible, but no simpler"

Einstein

Edition July 1997

BIAevaluation 3.0 Software Handbook, edition July 1997 (version AC)

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BIAevaluation Software Handbook

1.   Introduction

BIAevaluation is a stand-alone software package for presentation and evaluation of sensorgram data from real-time BIA analyses. The software supports

- evaluation of kinetic constants from sensorgram data using numerical integration and global fitting methods

- pre-defined and user-defined interaction models

- simulation of sensorgrams from specified models and kinetic parameters

- determination of affinity constants for both surface and solution interactions

- analysis of concentration measurements from either binding level or rate data.

1.1   Differences from version 2

BIAevaluation version 3.0 differs in several significant respects from the previous versions 2.x, both in terms of the functionality of the program and the user interface.

1.1.1   New functionality

The approach used for fitting experimental data to an interaction model has been revised and improved, with the result that the software is both easier to use and more powerful. Major new features include

- Simultaneous fitting to all curves in the working set, which improves the robustness of the fitting procedure. When one or more variables are in addition constrained to have the same value for each curve (e.g. association or dissociation rate constants), this is referred to as *global fitting*. Global fitting contributes greatly to the stability of the fitting procedure for complex models with many variables, and also reflects many physical interaction systems more faithfully (a set of sensorgrams for the same interaction at different analyte concentrations should *a priori* have identical rate constants).

- Simultaneous fitting to association and dissociation phases of the interaction, which improves the data throughput in the evaluation process. Separate fitting of the association and dissociation phases is also supported.

- The use of numerical integration algorithms allows evaluation according to complex interaction models, where analytical integration of the rate equations is difficult or impossible. This removes a serious limitation in previous versions of BIAevaluation, which were unable to handle non-integratable rate equations.

- A model editor based on reaction schemes instead of rate equations makes it easier to defined interaction models. The rate equations for the model as defined are displayed as an aid in checking the correctness of the model.

- Combined kinetic and concentration evaluation modules with integrated spreadsheet functions make it easier to perform concentration measurements using sensorgram data (binding rates or steady state levels). Report point tables can be opened directly in the spreadsheet from BIA result files for direct concentration evaluation.

- An improved 4-parameter fitting equation is introduced for more reliable creation of standard curves for concentration measurement.

1.1.2   New interface features

BIAevaluation version 3.0 is fully compatible with the Microsoft® Windows® 95 and Windows NT (version 4.0 or later) graphical user interfaces. New interface features introduced since version 2.x include:

- Long file names are fully supported.

- *Tool tips* provide assistance in using the toolbar buttons and in understanding dialogue boxes and data presentation. To display a tool tip, place the mouse cursor on the item for which you want help and wait a few seconds. The tip appears automatically (see illustration below).
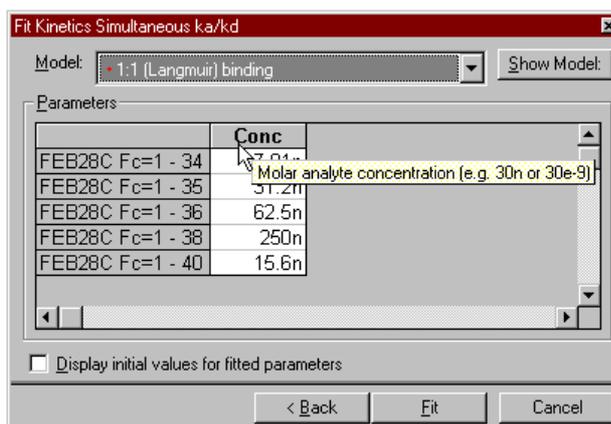
*Tool tip for the parameter **Conc** in kinetic data evaluation.*

- *Software wizards* guide you through procedures such as fitting kinetic data, opening text files and simulating sensorgrams. Wizards are sequences of dialogue boxes, often with text instructions, where **Next** and **Back** buttons allow you to step through the procedure and to return to previous steps without restarting the whole procedure. See Section 4.1.2 for a full description of a wizard example.
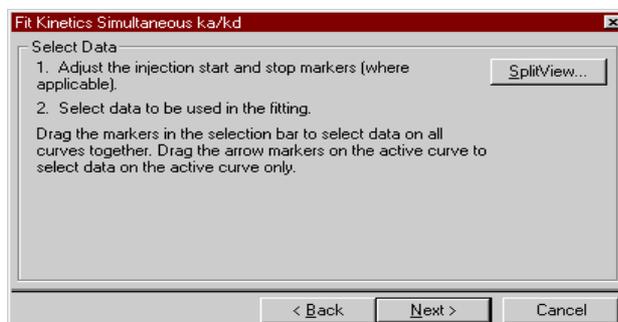
Example of a wizard dialogue box (from kinetic evaluation).

- *Right mouse button menus* or context menus are implemented in all graphical display windows. These are pop-up menus that appear when you press the right mouse button (left mouse button if you are using a left-handed mouse setting). The menu for plot windows offers commands for copying the plot window to the clipboard, exporting the data from the plot window, and setting presentation properties for the window. The right mouse button menu can be used at any time, even when the main menu commands are not available (e.g. when a wizard dialogue box is displayed).

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1.2   The BIAevaluation workspace

The BIAevaluation workspace can display the following windows:

- One (and only one) *project window*. The project window contains a list of the curves currently available for presentation and evaluation. Curves may be imported into the project window from several different source files including BIA result files and text files, or may be derived by transformation of source curves within BIAevaluation.

A project window with original and transformed curves.

- Any number of ***plot windows***. A plot window is a display of one or more curves from the project window. The curves included in any given plot window are collectively termed the ***working set*** for the window. The same curve can be displayed simultaneously in several plot windows: however, changes made to a curve in one plot window (by data transformation or changes in display properties) will affect the curve in all windows.
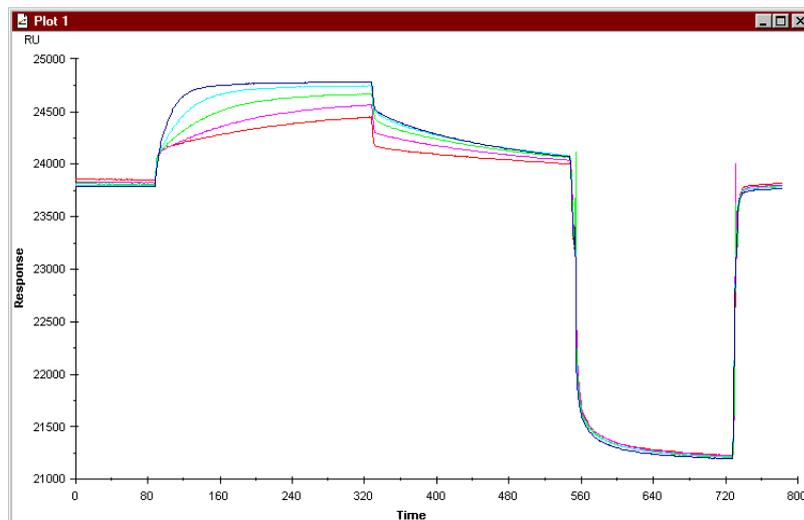
A plot window.

- Any number of ***fit windows***. A fit window is obtained from a plot window by fitting the curves in the plot window to a model equation. The fit window replaces the plot window, and display the results of the fitting both graphically and numerically. One fit window can contain any number of fits to the same curves.
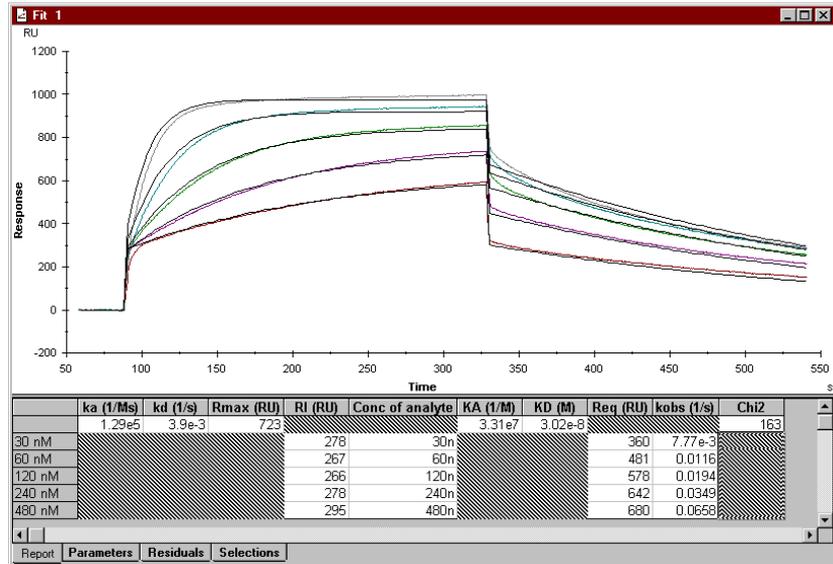
A fit window.

- Any number of *sheet windows*. A sheet window displays data numerically in a spreadsheet, which supports generation of calibration curves for concentration measurement as well as several common spreadsheet operations (filters, arithmetical operations and graphing functions).

A sheet window.

- Any number of *calibration windows*. A calibration window displays a calibration curve generated from spreadsheet data for concentration measurement, and is used for calculating concentrations in unknown samples.
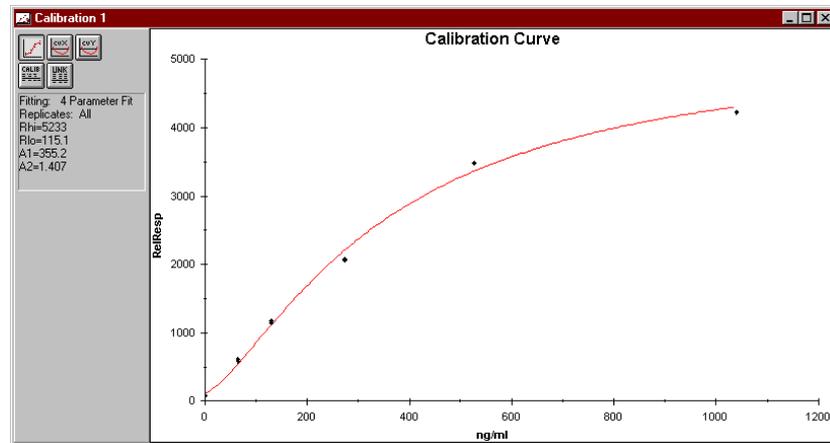
A calibration window.

BIAevaluation projects are saved to disk in *project files* (filename *.ble). Saving a project file saves the current state of the workspace including all open windows. Saving transformed curves in a project file does not affect the original curves in the source file.

2.   Installation

2.1   System requirements

Recommended system requirements for BIAevaluation are

Processor	Pentium 166 MHz or faster
RAM	At least 16 Mb
Windows version	Windows 95 or Windows NT version 4.0
Printer	Colour printer

2.2   Installation procedure

Close all running programs before installing BIAevaluation.

The software is delivered on five diskettes. To install the software:

1.   Insert diskette 1 in the floppy disk drive.

2.   Choose **Add/Remove Programs** in the Windows Control Panel. In the program installation wizard, choose to install software from diskettes. The installation program is **a:\setup.exe**.
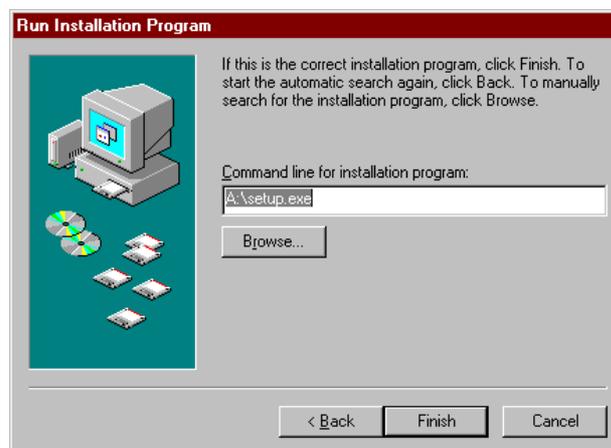
3.   Click on **Finish** and follow the instructions on the screen. The default installation folder is **C:\Program Files\Biaeval**. You will be required to enter your site name and software serial number during the installation procedure. The serial number is printed on the label on diskette 2.

Additional information on BIAevaluation Software may be found in the **Read.me** file. A shortcut for opening this file in Wordpad is placed under **BIA Programs** in the Windows start menu.
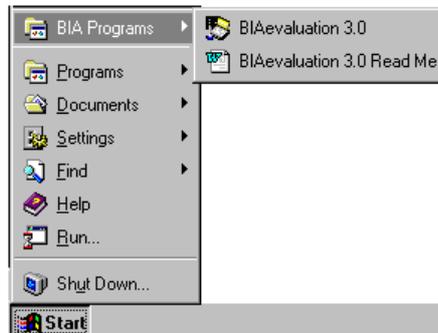
Notes: If you are installing BIAevaluation 3.0 over a previous version, models from the previous version will be saved in a file called **Bia_old.mdl**. If you want to use models from your previous installation (e.g. custom-written models), start BIAevaluation and use **File:Import models** to import chosen models from the **Bia_old.mdl** file. See Section 7A.2.7 for details of importing models.

If BIACORE X Control Software version 2.0.x is installed with BIAevaluation 3.0 present on the computer, BIAevaluation 3.0 must be reinstalled in order to work correctly.

Example data in text files provided with BIAevaluation uses a stop (.) as a decimal separator. If your Windows settings use a different separator, you may either change the setting temporarily in order to open example text files, or use the search-and-replace function in a word processor or text editor to change the decimal separator in the text files. Remember to save the files as text files if you use a word processor to make the changes.

3.   Data presentation

For information on how to start BIAevaluation, see Section 7.1.

3.1   Opening data files

To load data from source files into the project window, choose **File:Open** or press the **Open** button on the toolbar.
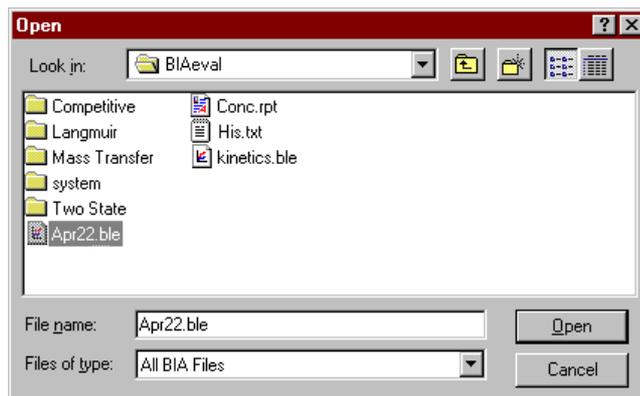
You can open the following types of files in BIAevaluation:

- previously saved BIAevaluation projects, including BIAevaluation version 2.x (extension .ble)

- result files from BIACORE instruments and from BIAlite (extensions .blr, .blc, .bls, .blf)

- report point tables from BIACORE (extension .rpt) or concentration evaluation files from BIAevaluation version 2

- text files containing tab-separated data (default extension .txt)

One project can include curves derived from several source files of the same or different types. The curve data used in the evaluation project is a copy of the data in the source file, and changes made to the curve in BIAevaluation do not affect the contents of the source file.

3.1.1 Previously saved projects

If the current project is empty when you open a previously saved project (extension .ble), the project is opened in the state in which it was saved, including any plot windows and fitting windows that were open at the time the project was saved.

If the current project is not empty when you open a previously saved project (extension .ble), you may choose to replace the current project or to add the curves to the current project:
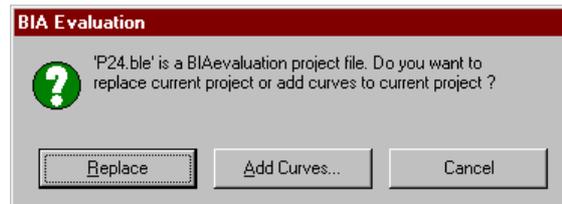
If you choose to add curves to the current project, select the curves to add from the dialogue box:
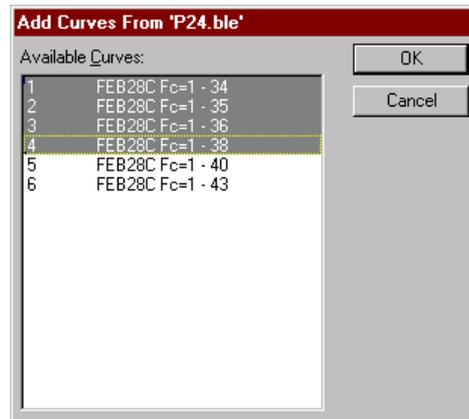
Note: If you choose to add curves from the saved project to the current project, only the curves are loaded into the project window for the current project. Any plot windows and fitting windows in the saved project are not opened.

3.1.2 BIACORE and BIAlite files

Data can be loaded from result files from BIACORE instruments (extension .blr, blc and bls) and BIAlite (extension .blf) as curves (sensorgrams), report point tables or both. To simplify selection of curves, you can choose to display only curves with a specified value for a chosen report point keyword. Some options in the dialogue box may not be available for older result files.

The dialogue box displays file description information where available, including date, control software version and instrument ID.

Curves from BIACORE and BIAlite result files are always added to the current project window. Report point tables are opened in a spreadsheet window (see Section 3.5).
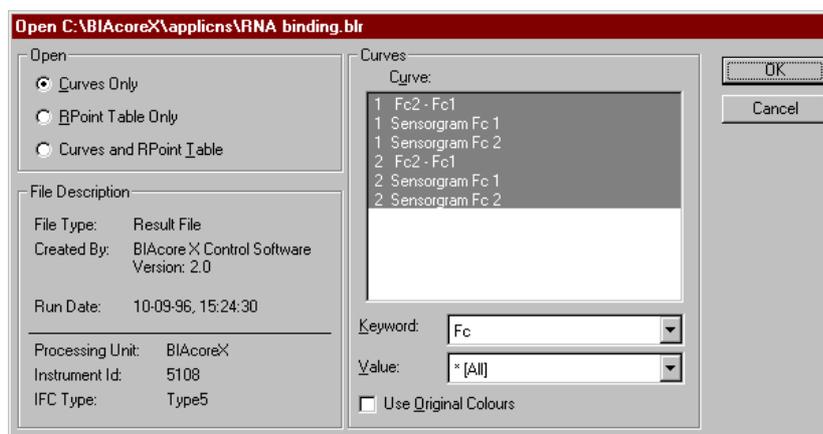
Curves Only	Sensorgrams will be added to the current project window. The report point table is ignored.
RPoint Table Only	The report point table will be opened in a spreadsheet. Sensorgram data is ignored.
Curves and RPoint Table	Sensorgrams will be added to the current project window and the report point table will be opened in a spreadsheet.
Curve list	Select the curves to be opened. Shift-click to select multiple adjacent curves: Ctrl-click to select multiple non-adjacent curves.
Keyword/Value	The curve list shows only curves that fit the selection criteria in the **Keyword** and **Value** fields. The keywords and their possible values are taken from the report point table in the result file. (Note however that selection of keyword and value does not affect report point data that will be loaded into the spreadsheet.) You can use this selection to restrict

the number of curves listed and make selection easier. For example, to list only curves from flow cell 1, choose **Fc** in **Keyword** and **1** in **Value**.

Use Original Colours Check this box to keep the original curve colours from the result file. If this box is not checked, BIAevaluation will assign colours to the curves.

3.1.3 Text files
BIAevaluation can load data from text files containing tab-separated columns of data values, either as curves or as a spreadsheet. For curves, the first entry in each column is read as the name of the data set in the column. Curves can be created from any combination of pairs of data sets.

Opening a text file first presents a preview of the file contents in spreadsheet format:
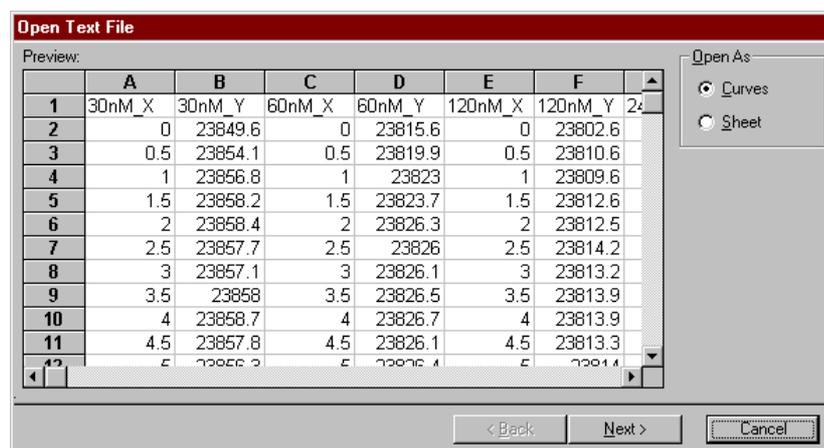
To open the file as a spreadsheet, select **Sheet** under **Open As** and click **Finish**.

To open the file as curves, select **Curves** under **Open As** and click **Next>**. In the next dialogue box, select the data sets you want to use for x- and y-values and then click **Finish**.
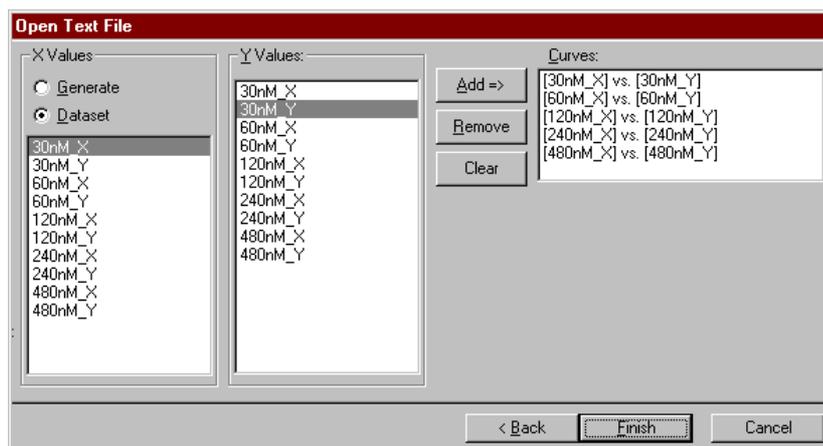
By default, curves are suggested from pairs of data sets with the same name and with suffixes x and y if such labels exist in the text file:

Curve	X-axis	Y-axis
1	Data set 1_x	Data set 1_y
2	Data set 2_x	Data set 2_y
3	Data set 3_x	Data set 3_y
...	...	...

To add a curve which is not in the default list, click on the required data sets in the **X-Values** and **Y-Values** lists, then press **Add=>**.

You can also choose to form a curve from generated x-values together with a chosen data set for y-values. To do this, choose **Generate** under **X-Values** and enter the starting value and step value in the dialogue box that follows. Choose the required data set under **Y-Values** and press **Add=>**. The curve will be generated by incrementing the x-value with the specified step for each value in the chosen data set.

Notes: When you open a text file, you have full freedom to choose any pair of data sets to create a curve. Choosing the correct data sets is easier if the first item in each column in the text file is an intelligible identification of the data in the column. Text files created from BIA software automatically have a column identifier at the top of each column.

Exported report point tables (extension .rpt) are opened as text files.

3.2   Presenting the data

The examples that follow are taken from the BIACORE result file
kinetics.blr, included with BIAevaluation software. Open the file and
load all the curves into the project window.

3.2.1   Displaying curves

The project contains five curves as displayed in the project window.
Curves to be displayed in a plot window are selected from the list in
the project window. To select one curve, click on the curve name. To
select several adjacent curves, drag the cursor through the group while
holding down the left mouse button, or hold down the Shift key while
you select the first and last curves in the group. To select several non-
adjacent curves, hold down the Ctrl key while you select each curve.
The curves you select define the working set for the plot window. In
the example, select curves 1-5 in the project window.
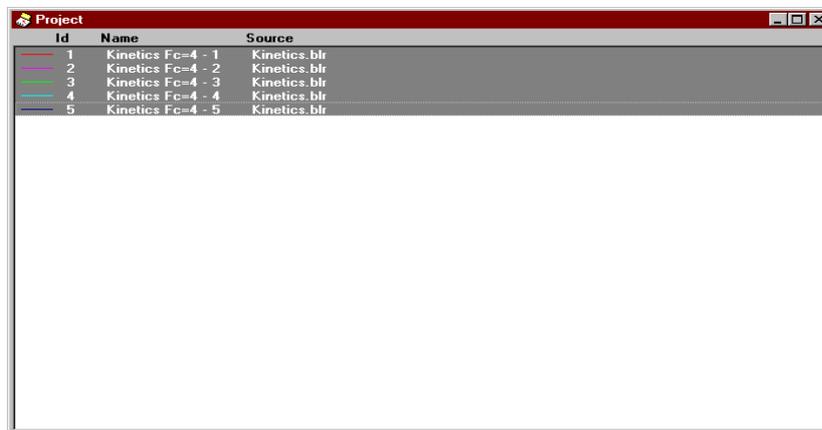
Id	Name	Source
1	Kinetics Fc=4 - 1	Kinetics.blr
2	Kinetics Fc=4 - 2	Kinetics.blr
3	Kinetics Fc=4 - 3	Kinetics.blr
4	Kinetics Fc=4 - 4	Kinetics.blr
5	Kinetics Fc=4 - 5	Kinetics.blr

Press the **Overlay plot** button or choose **View:Overlay plot**. This opens
a plot window with the selected curves in an overlay plot.
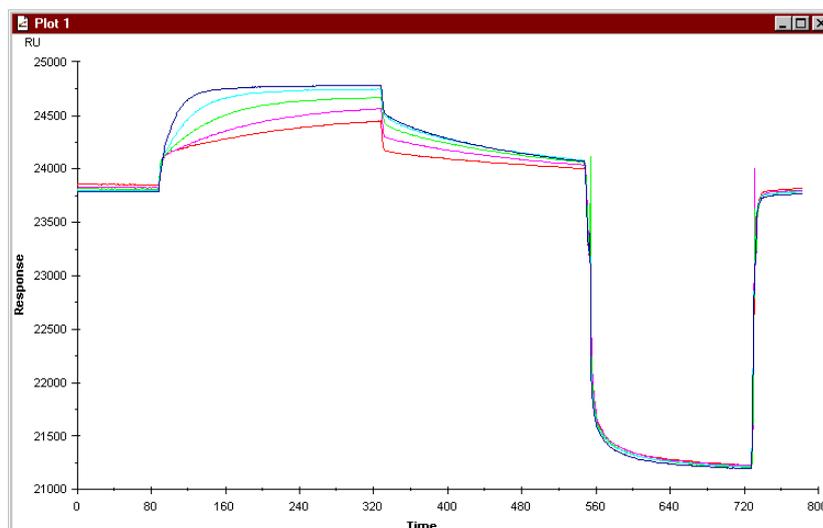
The sensorgrams in **kinetics.blr** show association and dissociation phases followed by a regeneration pulse.

With a plot window active, the **Overlay plot** button toggles between overlay and single plot mode. In single plot mode, only one curve is displayed at a time: you can choose the curve to display from the **Curve** list in the toolbar. Note that the display mode does not affect the number of curves in the working set. You can check which curves are in the current working set by expanding the **Curve** list in the toolbar.
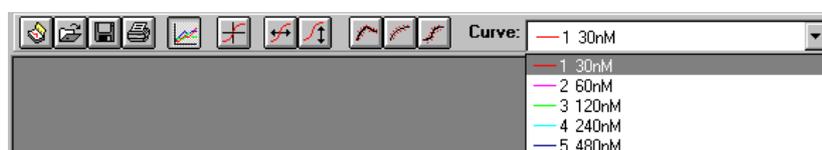
3.2.2   Changing curve properties

For presentation purposes, the appearance of curves can be set with the **Edit:Curve Properties** command. This command is available when either a project or plot window is active. Choose the command to change the properties of the active curve or double-click on the curve you wish to change.

Curve Properties

Name: 30nM

Curve Attributes

Line: Solid

Colour: ◼ Light Red

Marker: None

Size: ■ 1.0 mm

OK

Cancel

Sample
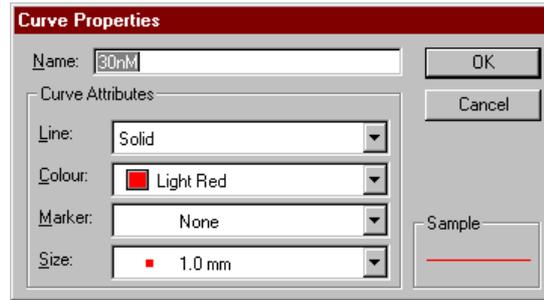
3.2.3   Scaling the display

You can scale the display either by setting scale values or by zooming.

To set scale values, choose **View:Scale** from the main menu or **Scale** from the right button menu, or double-click on an axis in the display.

Scale

X Scale
☐ Auto
☐ Logarithmic
Min: 0
Max: 800

Y Scale
☐ Auto
☐ Logarithmic
Min: 21000
Max: 25000

OK   Cancel
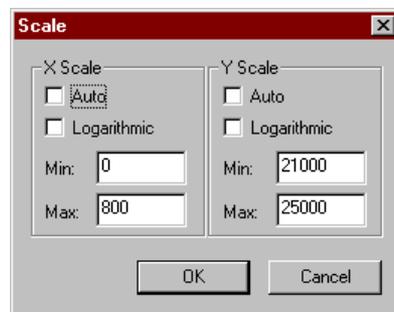
If **Auto** is checked for either x- or y-scale, the display will be scaled to show the whole curve(s) in the respective direction. To define a display area, uncheck **Auto** and enter values for the minimum and maximum values on the scale(s). To return to display of the whole curve(s), either choose **View:Scale** and check **Auto** in the dialogue box, or choose **View:Adjust Scale**.

To scale by zooming, drag with the left mouse button over the area to be displayed. To return to the previous zoom level, either choose **View:Unzoom** or double-click anywhere in the display area except on a curve or axis.

3.2.4   Displaying gridlines

You can display gridlines for x- and y-axes in plot and fitting windows with the **View:Gridlines** command from the main menu or **Gridlines** from the right button menu,. Major gridlines are drawn on numbered tick-marks on the axis, and minor gridlines on unnumbered tick-marks. Tick-marks are set automatically by BIAevaluation.
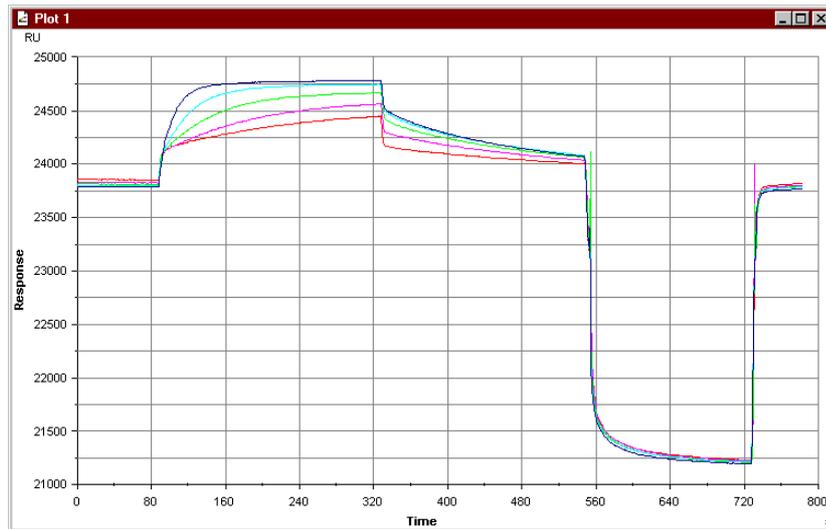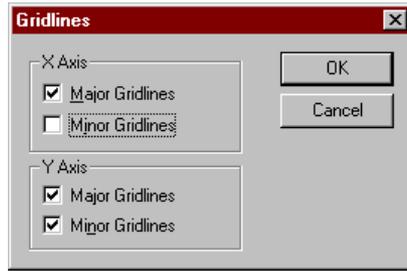
3.2.5   Labels and legends

You can add a title to the plot and change the labels on the x- and y-axes with the **View:Labels** command from the main menu or **Labels** from the right button menu,. The default labels are imported from the curve, and for sensorgrams are **Response** for the y-axis and **Time** for the x-axis.

You can display a legend in a plot window with the **View:Legend**
command from the main menu or **Legend** from the right button
menu,. The legend shows the line type and colour and curve name as it
appears in the project window for each curve in the plot, and can be
placed at the top, bottom or sides of the window.
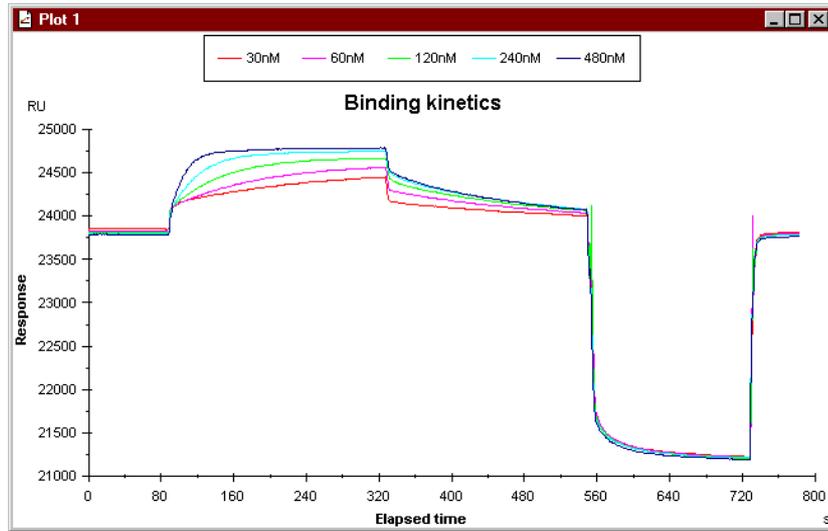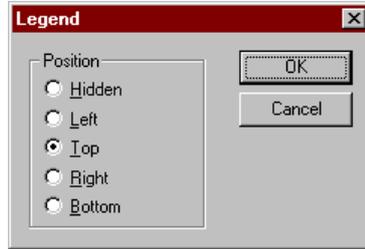
Plot window with legend and title.

3.3   Data transformation

There is a range of curve transformations that can be performed in BIAevaluation in both x- and y-directions. A full description of transformation facilities may be found in Sections 7B.5.1 and 7B.5.2. The sections below illustrate four examples: removing unwanted parts of the sensorgram, adjusting the curve baseline, aligning injection points and normalizing curves. Removing unwanted data and adjusting the baseline are normally the first steps in kinetic evaluation (see Section 4.1.2). Aligning injection points is helpful for files where one sensorgram includes several injection cycles (BIAlite and BIACORE X), and also for multi-channel injections (BIACORE X and BIACORE 2000) where there might be a slight displacement between curves.

3.3.1   Removing unwanted data

The example sensorgrams in **kinetics.blr** include a regeneration pulse where the response falls well below the pre-injection baseline. For presentation and/or analysis purposes, you may wish to delete this part of the sensorgrams so that only the interaction phases remain.

1. Select the section of the curves to be cut. Drag the cursor with the *right* mouse button to select the curve section. In the example, select data from just before the regeneration pulse to the end of the sensorgram. Alternatively, you can choose **Edit:Select** and enter the start and end times for the section to be selected.
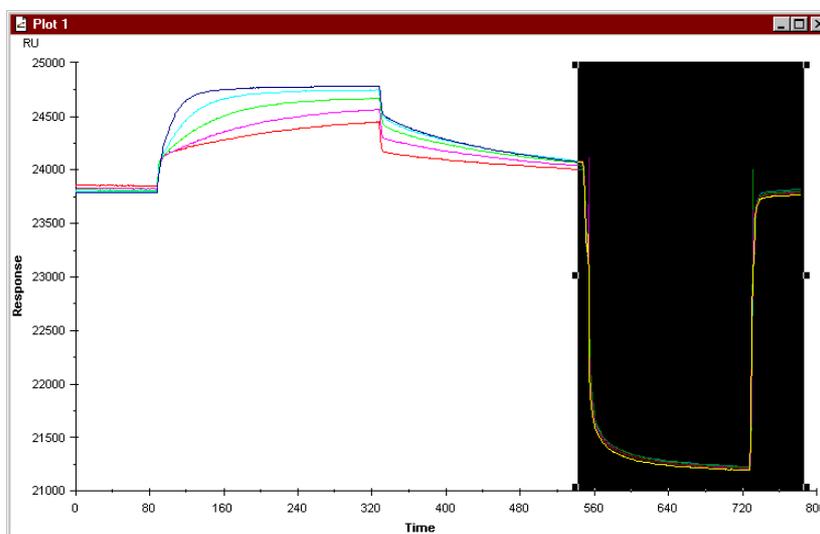
Once a section of data has been selected, you can drag the handles on the selection area using the left mouse button to adjust the selection limits. Make sure that the upper limit of the selection area extends beyond the end of the sensorgram.

2. Choose **Edit:Cut** (keyboard shortcut Ctrl-X). The selected data will be deleted from all curves displayed in the plot window. The display will be rescaled automatically. (If the display is not rescaled, you have probably not selected data all the way up to the end of the sensorgram. This removes a section of data from within each curve, leaving a set of discontinuous curves.)
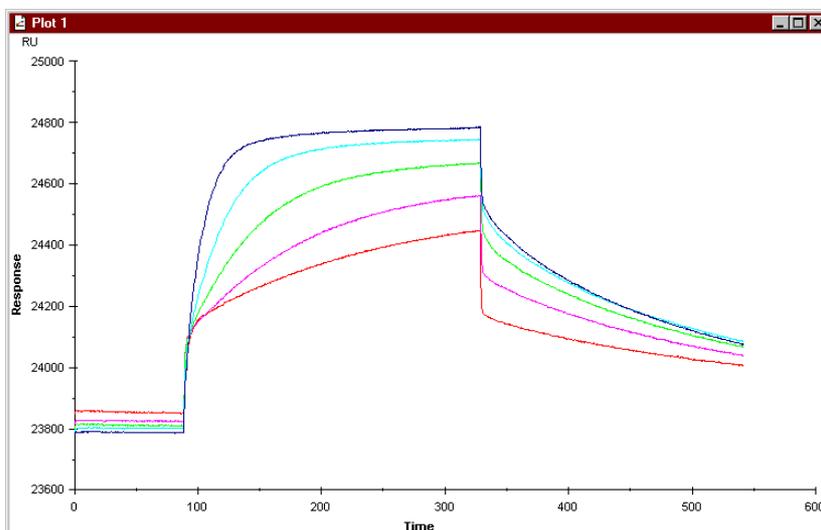
Notes: In single plot mode, data will only be deleted from the currently displayed curve. Other curves in the working set will not be affected. You can delete data from a single curve in overlay mode by using **Edit:Select** to select the data and unchecking **Apply to all curves in working set**.

The data is deleted from the curves in the project window, and any new plot windows which use the same curves will display the data without the regeneration pulses. The data in the source file is however not affected.

You can recover the deleted data by choosing **Edit:Undo Cut** before performing any other operation. Do not use **Edit:Paste Curve** to recover the deleted section: this will insert the section as a separate curve. If you have performed another operation and **Undo Cut** is not available, you must re-open the source file(s) to restore the original curve.

3.3.2 Adjusting the baseline

Baseline adjustment is one of a set of y-transformations available in BIAevaluation. Most kinetic evaluation models require a zero baseline for fitting, so that this step is almost always a preparatory step in kinetic evaluation.

1. Select a section of the curves to be treated as baseline using the right mouse button. Make sure that the selection does not extend outside the baseline region for any curve.
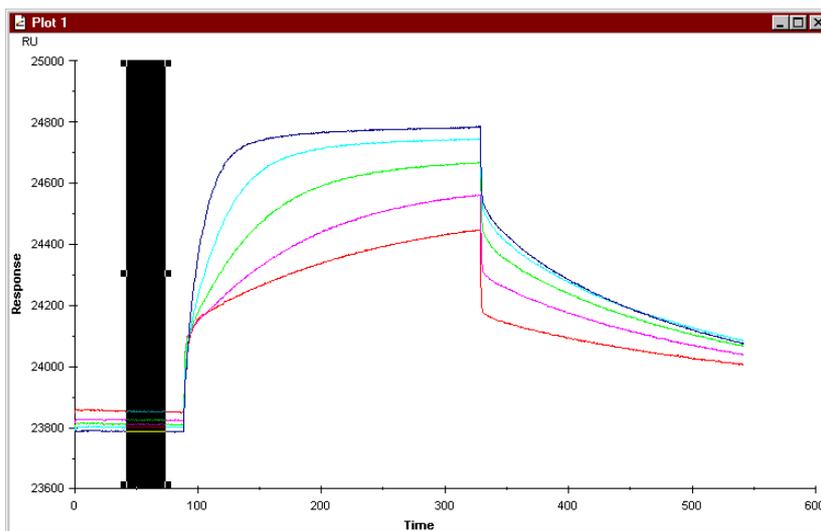
2. Press the **Y-Transform** button or choose **Calculate:Y-Transform**. In the dialogue box, choose **Zero at Average of Selection**, and make sure that **Apply to Entire Working Set** is checked.

3. Press **Add As New** if you want to have access to both the adjusted and original curves in the project, otherwise press **Replace Original**. The baseline for each curve will be set to zero at the average y-value within the selection for the curve.
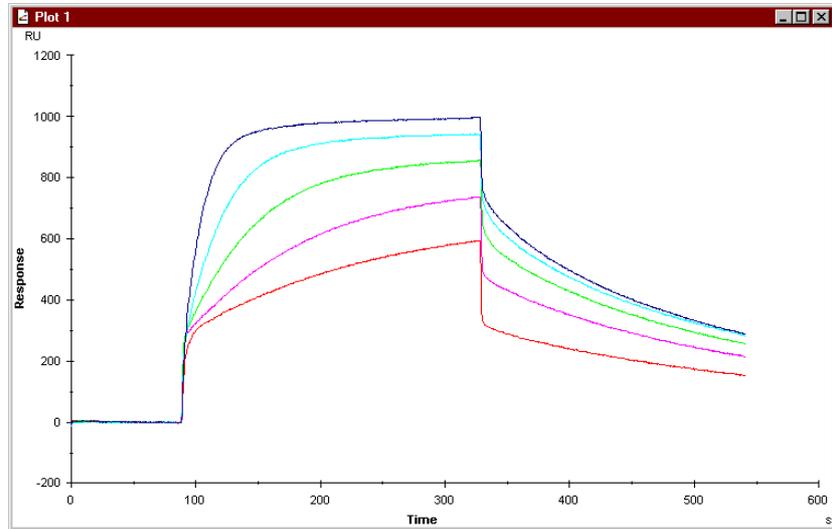
Notes: **Replace Original** is generally recommended after a simple transformation of this kind, to avoid accumulating large numbers of curves in the project window. However, if you press **Replace Original**, the transformation will affect all plots in all working sets which use the original curves. You are warned if any of the curves affected is used in more than one working set.

3.3.3   *Aligning curves*

X-transformations include a function for aligning curves to a defined time point. This is valuable for multi-channel applications, where the injection points on the sensorgrams from different flow cells may be slightly displaced, and for results files where one sensorgram includes several injection cycles (typically BIAlite and BIACORE X result files).

To align curves:

1. Press the **X-Transform** button or choose **Calculate:X-Transform**. In the dialogue box, choose **Curve alignment**. Uncheck **Replace Original** if you want to keep the original non-aligned curves. Press **Next>** for the next step in the alignment procedure.
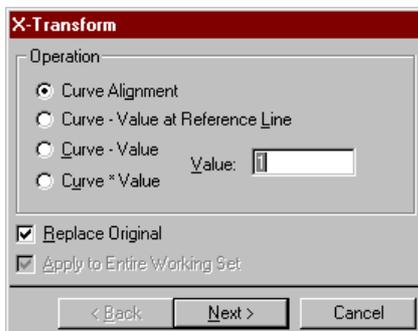
2. This will maximize the plot window for best resolution, and display the curves with arrow markings on each curve. The arrows have the same colour as the curves for ease of identification.
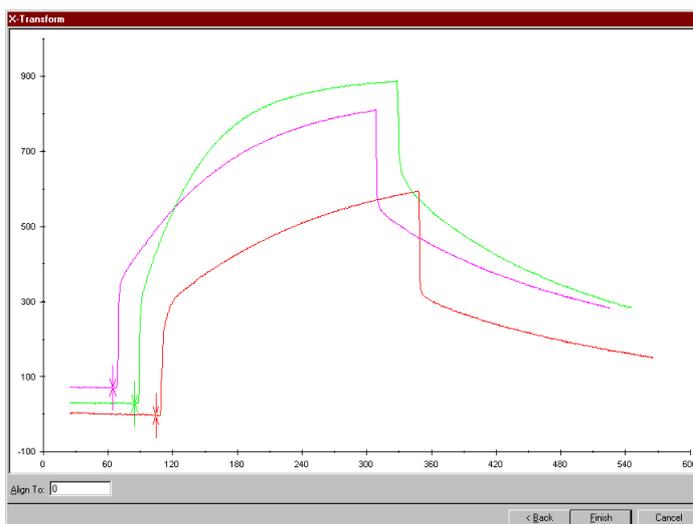
3. Move the arrows on each curves to the point to be aligned (typically an injection start). Enter a time value in the box at the bottom of the window if you want the alignment point to be set to a time other than zero.

4. Press **Finish**. The curves will be aligned in the x-direction.
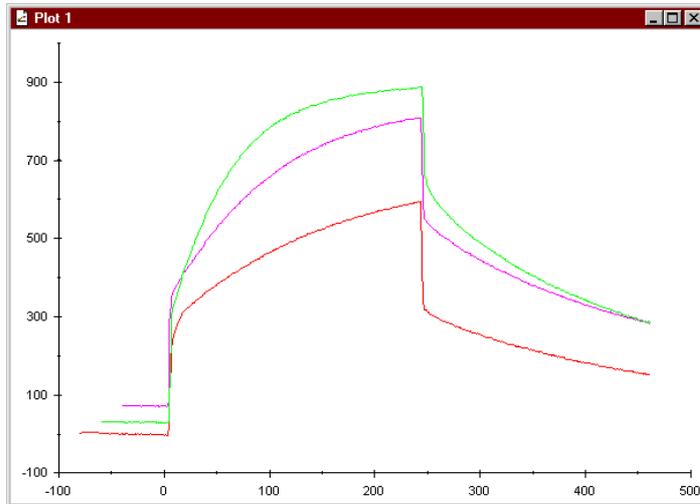
3.3.4 Normalizing curves

You can normalize curves using either the lowest and highest points in the curve or the lowest and highest points in a selected region. To normalize using a selected region, select the region of the curves using the right mouse button before choosing **Normalize**.

Choose **Calculate:Normalize.**
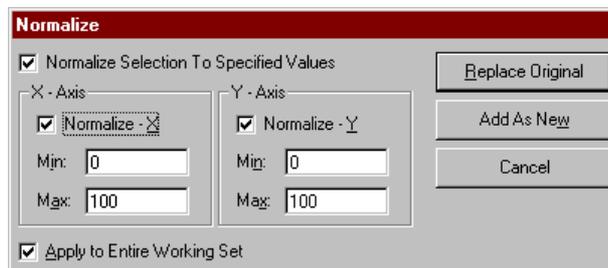
Check the boxes as appropriate:

Normalize Selection To Specified Values specifies that the lowest and highest points in the selected region will be used for normalization. If this box is not checked, the lowest and highest points in the entire curve will be used. Use this function to avoid normalizing to the top and bottom of response spikes. This box is not available if no data is selected in the curves.

Normalize-X, **Normalize-Y** selectes normalization in the x-and y- directions respectively. The **Max** and **Min** values set the scaling for normalization.

Apply to Entire Working Set specifies that all curves will be normalized, using the lowest and highest values in all curves taken together. If this box is not checked, normalization applies only to the active curve.

Normalization applied to the entire working set compares the curves according to values. Normalization applied to individual curves compares the curves according to shape. Shape comparisons can be important in cases where the interaction model predicts different sensorgram shapes (e.g. changes in the shape of the dissociation phase as a result of varying contact times may indicate heterogeneous interactions or conformational changes in the surface-bound complex[1]).
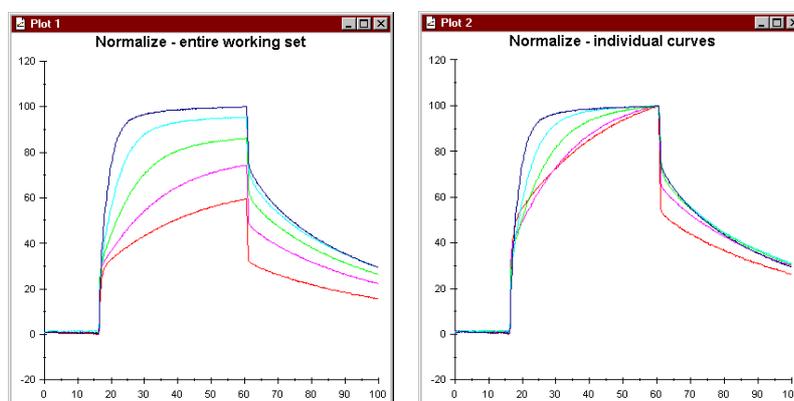
Normalization applied to (left) the entire working set and (right) individual curves.

_____

[1] Karlsson, R. and Fält, A. (1997) J. Immunol. Methods **200**, 121-133.

3.4   Creating an overlay plot from consecutive injections

In some cases (particularly data files from BIAlite and BIACORE X
and from interactive method development on all instruments), binding
experiments may be recorded sequentially in a single data file. This
section describes how to prepare separate aligned sensorgrams from
files of this type.

The text file **his.txt** supplied with BIAevaluation contains three
sensorgrams in sequence, each consisting of two injections. Follow the
procedure below to construct an overlay plot of the three sensorgrams.

1.   Choose **File:New** to start a new project.

2.   Open the text file **his.txt** and load the default curve [his.txt 1_X]
     vs [his.txt 1_Y].
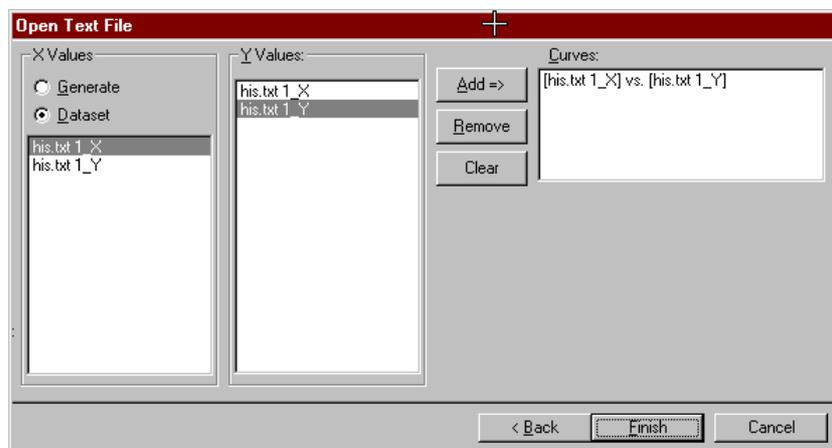
3.   Select the curve in the project window and press the **Overlay plot**
     button.
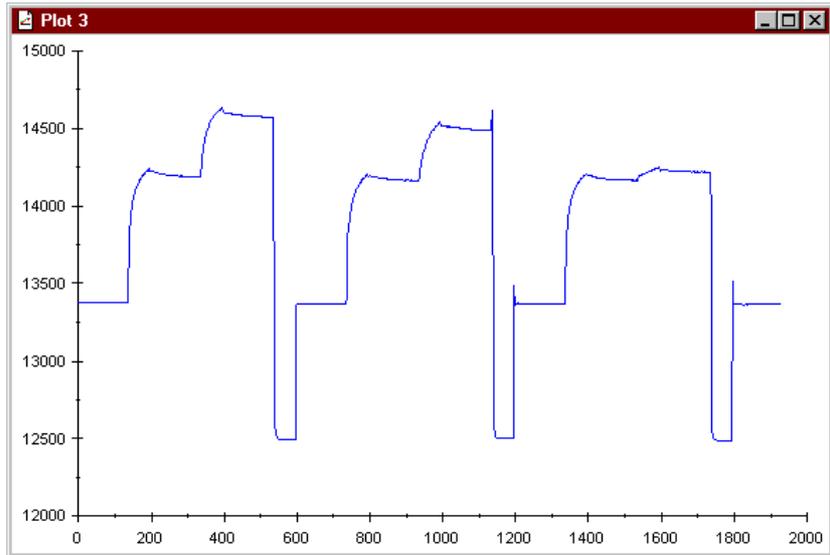
4. Using the right mouse button, select the third cycle. The left edge of the selection is not critical: you should however make sure that the selection extends past the end of the curve.
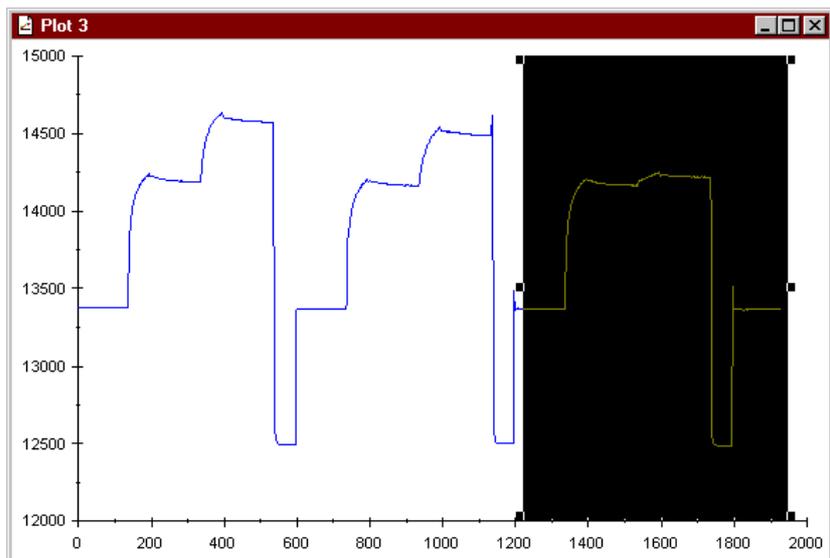
5. Choose **Edit:Cut** (keyboard shortcut Ctrl-X) to remove the data, then choose **Edit:Paste Curve** (Ctrl-V) to paste the data back into the window. In the **Paste Curve** dialogue box, choose **Original Position** and check the **New Colour** box.

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Paste Curves

Paste At
Original Position
Specified X
Specified Y
Specified X & Y

OK

Cancel

Paste: At X: [0]   At Y: [0]   ☑ New Colour
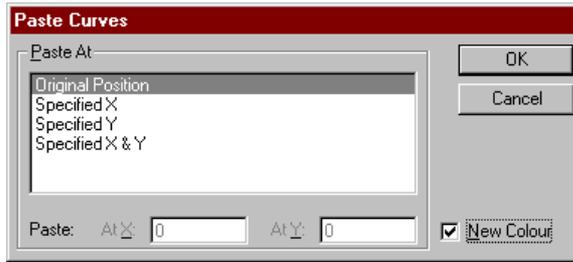
6. Repeat steps 4 and 5 for the second cycle. You will now have a
    plot window where the total curve shape is the same as the
    original, but the 3 cycles are identified in different colours
    (indicating that the window actually contains 3 separate curves).

7. Choose **Calculate:X-Transform.**

X-Transform

Operation
◉ Curve Alignment
○ Curve - Value at Reference Line
○ Curve - Value   Value: [1]
○ Curve * Value

☑ Replace Original
☑ Apply to Entire Working Set

< Back   Next >   Cancel
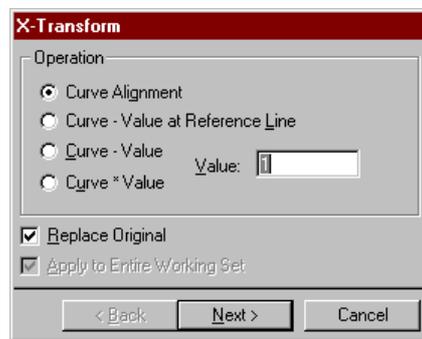
Select **Curve alignment** and press **Next>**. The curves are displayed
with an alignment marker in the same colour as the curve. Move
the marker to the beginning of the first injection for each curve.
Zoom the display for each curve to set the alignment point with
precision.
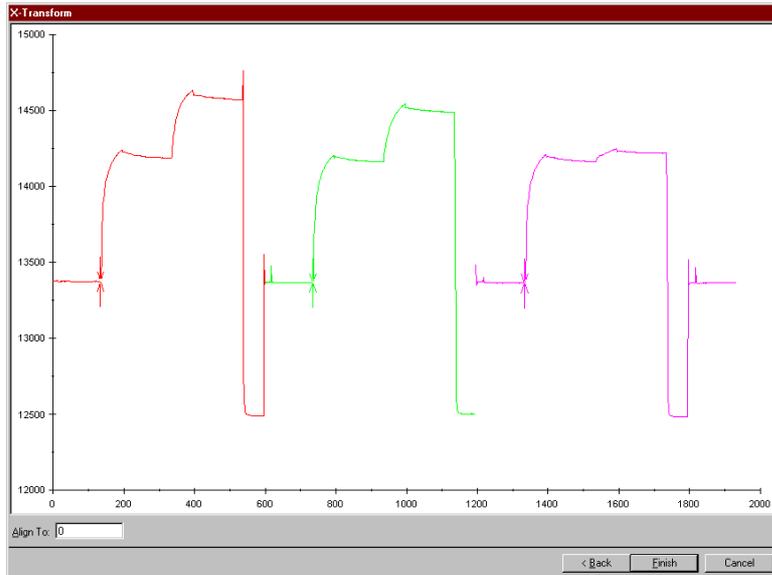
8.   Press **Finish** to complete the curve alignments.
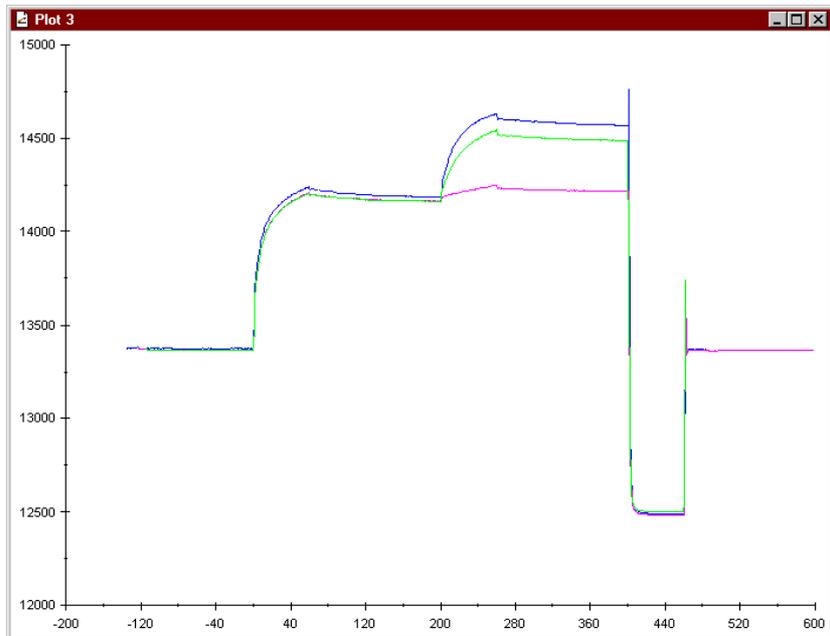
3.5   Spreadsheet functions

Sheet windows are used for several purposes in BIAevaluation:

- opening report point tables for display and evaluation of numerical result data (e.g. plotting baseline levels against cycle number to assess regeneration performance, see Section 3.6)

- numerical manipulation of the results obtained from curve fitting (e.g. calculations performed on fitted constants)

- evaluating data from concentration measurements (Chapter 5) and from solution affinity determination (Section 6.3)

The spreadsheet supports basic arithmetical operations (+ - / * ^) as well as a range of functions common to most commercial spreadsheet programs, like SUM, AVERAGE, COUNT, MAX and MIN. To perform calculations in the spreadsheet, enter an equals sign (=) followed by the appropriate expression in a cell. In expressions, cells are referred to by letter-number coordinates: ranges are specified by two cell references separated by a colon (:). Use parentheses to specify priorities in complex expressions. For example:

Expression	gives
=A2+B5	the sum of the values in A2 and B5
=A2*B5	the product of the values in A2 and B5
=(A2+B5)/C2	the result of dividing the sum of the values in A2 and B5 by the value in C2
=A2^2	the square of the value in A2
=SUM(A1:A45)	the sum of the values in cells A1 to A45 inclusive
=MAX(A1:C45)	the highest value in the region defined by A1 (upper left) and C45 (lower right)

Note: The functions supported in the spreadsheet represent a subset of those in Microsoft®Excel, and use a syntax compatible with Excel. If you require a special function, try entering the corresponding Excel formula.

Spreadsheet operations are illustrated here using data from the report point table **conc.rpt**, included as an example with BIAevaluation Software.

3.5.1   Opening data files

To open data files in a spreadsheet window, choose **File:Open** (see Section 3.1). For BIA result files containing report point information, choose to open the report point table. For saved report point tables and text files, choose to open the data as a sheet.

Open Text File							Open As
Preview of Conc.rpt							○ Curves
	A	B	C	D	E	F	S	● Sheet
1	Cycle	APROG	FC	Time	Window	AbsResp	S
2	1	immob	2	284	5	10127	
3	1	immob	2	2035	5	23052	
4	2	b2micro	2	65	5	23051	
5	2	b2micro	2	185	5	23666	
6	2	b2micro	2	388	5	27671	
7	3	b2micro	2	65	5	23025	
8	3	b2micro	2	185	5	23621	
9	3	b2micro	2	388	5	27589	
10	4	b2micro	2	65.5	5	22986	
11	4	b2micro	2	185.5	5	23326	
12	4	b2micro	2	388	5	25408	

< Back   Finish   Cancel

Note: Spreadsheets are part of the current project, although they are not listed in the project window. Saving the project will save any open spreadsheets in their current state.

3.5.2   Sorting and filtering data

BIAevaluation spreadsheet operations follow the same principles as operations in Microsoft Excel with respect to selecting and sorting data, with the following important exceptions:

- Many operations in BIAevaluation can only be applied to entire rows of data since each row represents one measurement (report point). For example, sorting data sorts entire rows even if only certain columns are selected. This ensures that integrity of experimental data is maintained.

- Dragging marked cells to a new location in the spreadsheet will overwrite the destination cells without warning. If you move cells in this way be accident. Choose **Edit:Undo** before performing any other operation.

Sorting data
It is often convenient (although not essential) to sort data in the spreadsheet. Choose **Data:Sort**. You can sort data using up to three sort values in one operation.
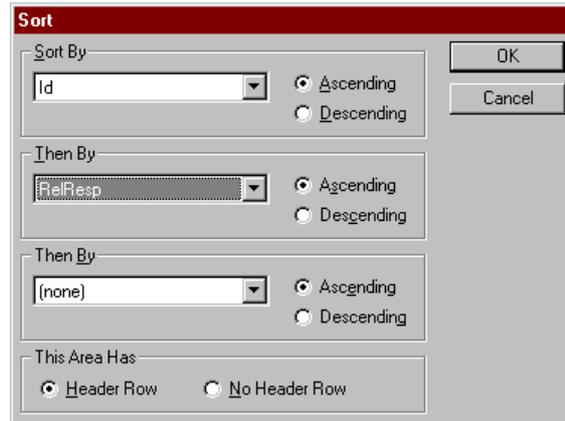
Normally, report point tables have a header row (the first row in the table contains the column name). With **Header Row** checked, columns for sort values are identified by the column name and the header row is not moved in the sorting process. With **No Header Row** checked, columns are identified by their column letters and the contents of the first row in the table are sorted together with the other rows.

Filtering data

Report point tables frequently contain more points than are required for concentration evaluation. Thus the file **conc.rpt**, representing sandwich assay data for determination of β2-microglobulin, includes report points for baseline, primary response and secondary response. You can choose to filter the spreadsheet so that you only see and work with rows that fulfil the filter conditions. For example, for concentration evaluation from **conc.rpt**, you may only want to work with rows showing the secondary response.

Note: When you filter the data in a spreadsheet, all data remains in the spreadsheet even though only a subset is displayed. You can display all data again by removing the filter conditions.

To filter data, choose **Data:Filter**. Choose whether the sheet has a header row or not: if you choose **Header Row**, the first row in the sheet will be treated as a header. If you choose **No Header Row**, the first row will be treated as data. Report point tables always have a header row.

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A filter box appears at the head of each column, with a pull-down menu including the unique values in the column together with the options **All** and **Custom**. For example, to display only secondary response values, choose **second_AB** from the filter options at the top of the **id** column.

The filter option **Custom** allows you to specify a selection condition for the column, e.g. to eliminate outliers by displaying only response values that are less than a certain value. For example, to select only rows with relative response between 100 and 3000, choose **Custom** for the filter for column **RelResp** and enter the following conditions in the dialogue box:

If a custom filter is applied, the option **Edit Custom** appears in the pull-down list of filter options at the top of the column. Use this option to edit the custom filter settings.

Removing filters
To remove the filter from one column in a filtered spreadsheet, set the filter condition for the column to **All**. To remove all filters, choose **Data:Filter**.

Scope of operations on filtered data
Most operations performed on a filtered spreadsheet affect only the
displayed rows. The exception is sorting data, which sorts both the
displayed and the hidden rows together.

3.5.3   Using data in a spreadsheet
You can use the data in a spreadsheet for a variety of purposes:

- Choose **Data:Generate Calibration** to create a calibration curve for
  concentration measurements (see Section 5.2).

- Choose **Data:Calculate Values** to calculate new values based on an
  open calibration curve and the values in an existing column (see
  Section 5.4.2). This option is only available if one or more
  calibration curve windows is open.

- Choose **Data:Create Curve** to plot values in one column against those
  in another. The plot appears in a new plot window and is entered as
  a curve in the project window. An example is given below.

Creating curves from spreadsheet data
BIAconcentration evaluation includes a function for plotting values
from any two columns in a report point table against each other. The
example below illustrates how to assess regeneration performance by
plotting absolute baseline response against cycle number.

1.   Open the report point table **conc.rpt** and filter the data to select
     the value **baseline** in the **id** column.

A1	Cycle									
	D	E	F	G	H	I	J	K	L	M
1	[All]	[All]	[All]	[All]	[All]	[All]	[All]	[All]	baseline	[All]
	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id	ng/ml
4	65	5	23051	0.66613	-0.30586	0.45517	YES	0	baseline	high
7	65	5	23025	0.55899	0.05558	0.58117	YES	0	baseline	high
10	65.5	5	22986	0.75142	0.25732	0.65196	YES	0	baseline	273
13	65.5	5	22986	0.62964	0.05369	0.65703	YES	0	baseline	273
16	65	5	22965	0.72404	0.09957	0.7431	YES	0	baseline	low
19	65.5	5	22915	0.69844	0.08928	0.71949	YES	0	baseline	0
22	65	5	22887	0.59952	0.07695	0.61746	YES	0	baseline	1040
25	65.5	5	22892	0.3323	0.06165	0.33329	YES	0	baseline	130
28	65	5	22863	0.89681	0.35234	0.71715	YES	0	baseline	high
31	65.5	5	22858	0.61305	0.13331	0.60273	YES	0	baseline	1040
34	65.5	5	22850	0.62793	0.17124	0.59035	YES	0	baseline	65
37	65.5	5	22813	0.55772	0.24165	0.40887	YES	0	baseline	0
40	65.5	5	22782	0.49606	0.09709	0.49459	YES	0	baseline	65
43	65	5	22761	0.45022	0.15234	0.39282	YES	0	baseline	high
46	65	5	22762	0.40326	0.12152	0.36819	YES	0	baseline	high
49	65.5	5	22753	0.46227	-0.01417	0.48665	YES	0	baseline	130
52	65	5	22729	0.40734	-0.08011	0.4059	YES	0	baseline	low
55	65.5	5	22705	0.33621	0.03697	0.34845	YES	0	baseline	527

2. Choose **Data:Create Curve** and select **Cycle** for the x-keyword and
    AbsResp for the y-keyword.
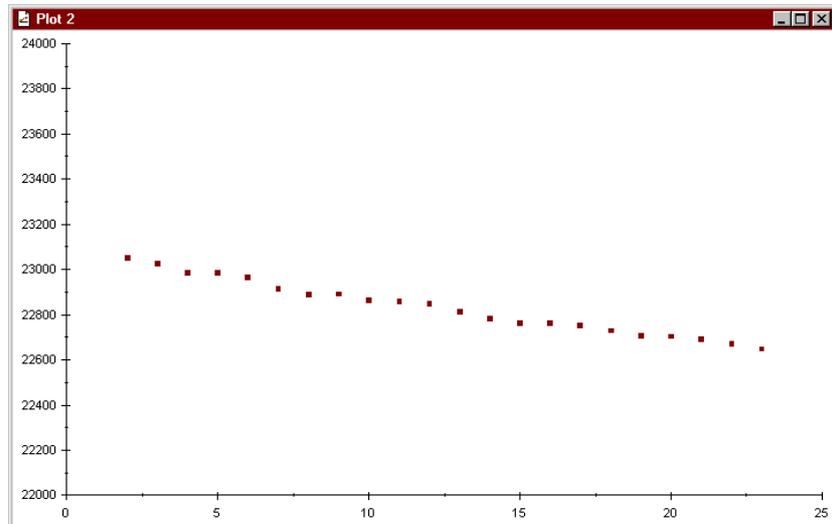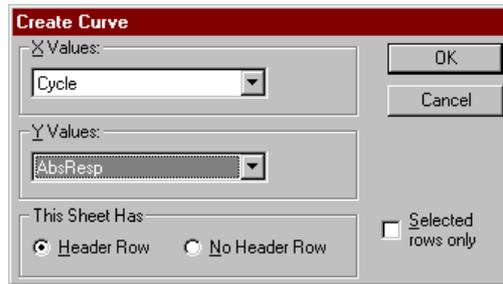
The plot is added to the project window. Perform a linear fit on the
plot if you want to draw a straight line through the points.

4. Evaluating kinetic data

This chapter describes the software tools for evaluating experimental sensorgram data in terms of a kinetic model. Evaluation procedures are illustrated by evaluation of the sensorgrams in the example file **kinetics.blr** using a simple model for 1:1 (Langmuir) binding. Analysis using other models follows the same principles. Details of the pre-defined models supplied with BIAevaluation are given in Appendix C.

4.1   Fitting sensorgram data

4.1.1   Opening the project and preparing curves

Open the project **kinetics.blr** as a new project (see Section 3.2.1). Select curves 1-5.
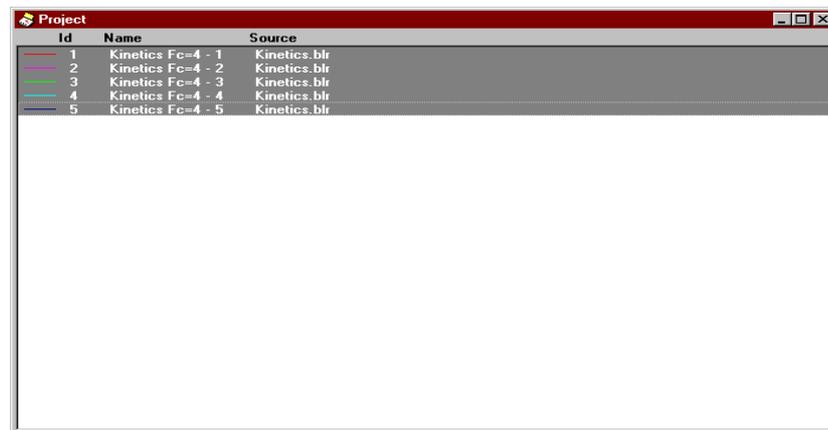
Click on the **Overlay plot** button to display the curves in an overlay plot (see Section 3.2.1).
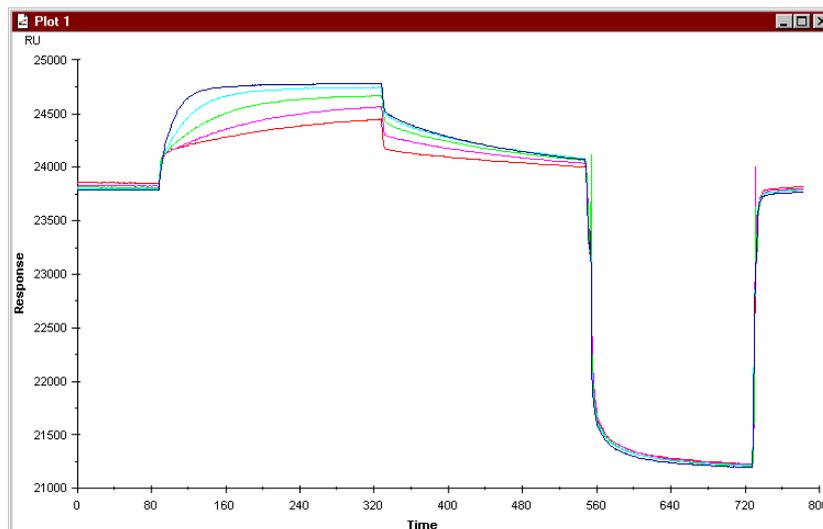
4.1.2 Fitting the data

To fit all curves in the working set, make sure that the display is in overlay mode. If you perform a fit in single plot mode, only the displayed curve will be fitted.

To simplify evaluation, make sure that the curves are aligned to the start of injection (see Section 3.3.3). Non-aligned curves can be evaluated, but selection of data to be fitted is much easier with aligned curves.

Choose **Fit:Kinetics Simultaneous ka/kd** or click on the corresponding toolbar button. This starts the kinetic evaluation wizard which leads you through the fitting procedure. The plot window is automatically maximized for optimal curve display at the beginning of a fitting procedure.

Evaluation wizard, step 1 – curve adjustments
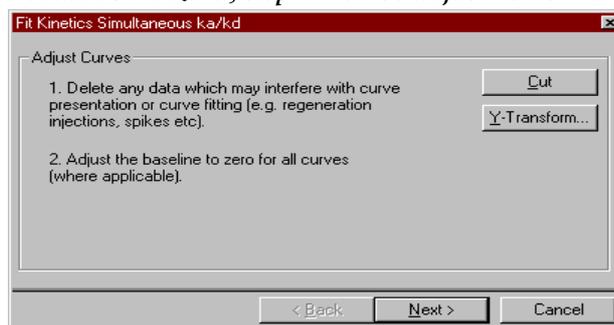
This panel reminds you of two prerequisites for successful fitting:

- unwanted data such as regeneration pulses, outlying points and disturbances in the curves (e.g. air segments) should first be removed. Disturbances outside the range of data to be fitted should be removed if they interfere with automatic scaling of the sensorgram display.

- for almost all kinetic models, the baseline prior to injection should be set to zero.

Both of these operations can be performed without closing the wizard window. Move the panel to the edge of the screen so that it does not obscure the curves.

To remove unwanted data, zoom into the area of interest, select the unwanted data with the right mouse button and click on **Cut** in the wizard panel or press Ctrl-X (the keyboard shortcut for **Cut**). See Section 3.3.1 for more details about removing data. Removing data points or segments from the curves will not affect the fitting procedure, although it might affect the results.

To adjust the baseline to zero, select the region to be used as baseline using the right mouse button and click on **Y-Transform** in the wizard panel. Choose **Zero at Average Of Selection**, make sure **Apply to Entire Working Set** is checked and click on either **Replace Original** or **Add as New** as required. See Section 3.3.2 for more details about adjusting the baseline.
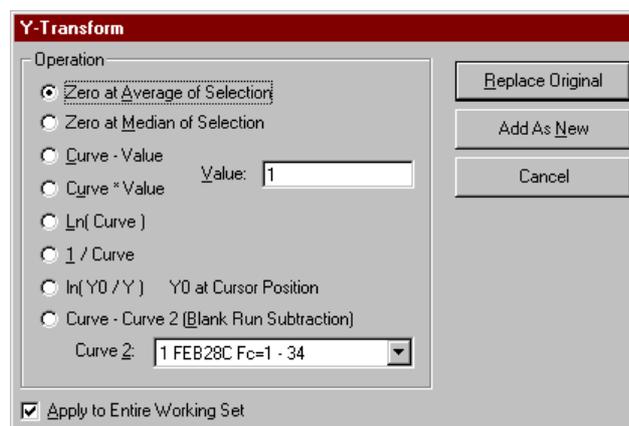
Click on **Next>** when the curve adjustments have been made.

Evaluation wizard, step 2 – data selection
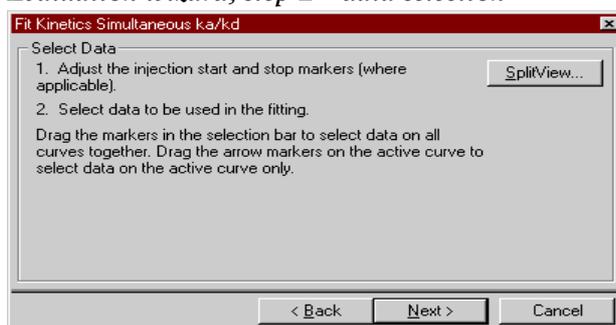
From this panel you can set the start and stop times for the injection and select the data ranges to be used in the fitting. BIAevaluation suggests start and stop times and data ranges based on the form of the curves: commonly, these suggestions will need to be adjusted. ***Do not continue with the evaluation without inspecting the suggested data selections and making adjustments where necessary.***

Selections are marked on a selection bar at the top of the plot window, with black lines for injection start and stop and grey bars for selected data. On all except the currently active curve, injection start and stop are marked with thin vertical black lines and selected data is marked with a thicker line. Arrows mark injection start and stop and the beginning and end of selected data on the currently active curve. Adjust the injection start and stop points before you adjust the selected data.
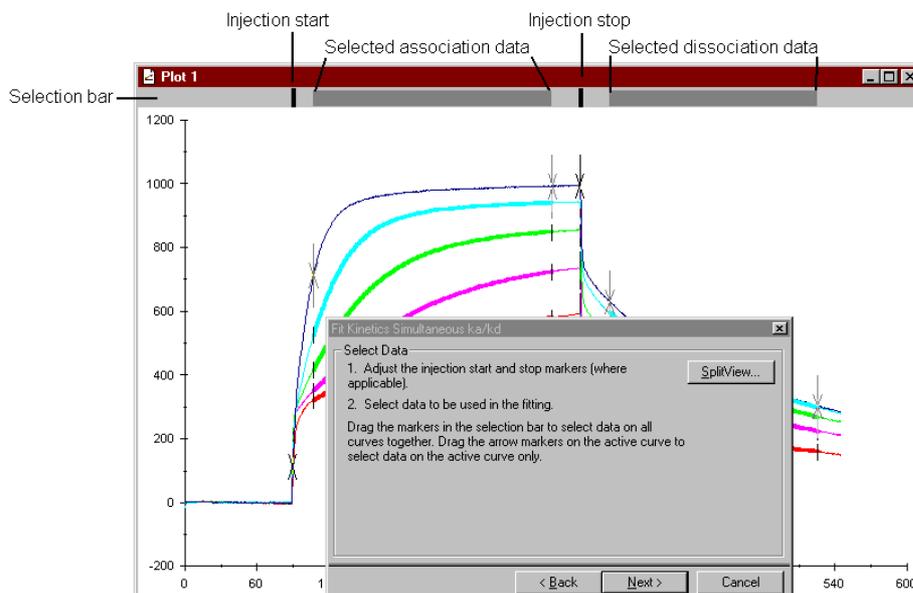
To change the selections for all curves together, drag the appropriate marking on the selection bar. To change selections for one curve only,

click on the curve to make it active (or select from the curve list in the toolbar) and drag the appropriate arrows. You may zoom the curve display to aid in selecting data.

Since BIAevaluation 3.0 can fit data to complex models including mass transfer-limited binding, it is not necessary to restrict the data selection to the parts of the sensorgram where binding is kinetically determined. In general, a short period after the injection start and stop should be excluded from the fitting to avoid sample dispersion effects. Otherwise, all of the data may be selected for fitting provided that there are no obvious disturbances. If desired, separate fits may be performed using different regions of the association and dissociation phases as a check that the calculated constants are consistent throughout the sensorgram.

An additional aid in selecting data ranges is split view. This displays the curves simultaneously with a chosen function of the curve in synchronized panels, so that zoom and selection functions are applied automatically to both panels. To enter split view mode, click the **SplitView** button in the wizard panel and select the curve function to display. See Section 7B.4.9 for a description of the split view functions and their uses. It may be easier to judge curves in split view if you click on the **Overlay** button to turn off overlay mode. Split view may be particularly useful in judging whether a simple 1:1 interaction model is appropriate for the data. The functions $\ln(dR/dt)$ and $\ln(R_0/R)$ are linear for a 1:1 interaction, constant for mass-transfer limited interaction and curved for more complex systems.
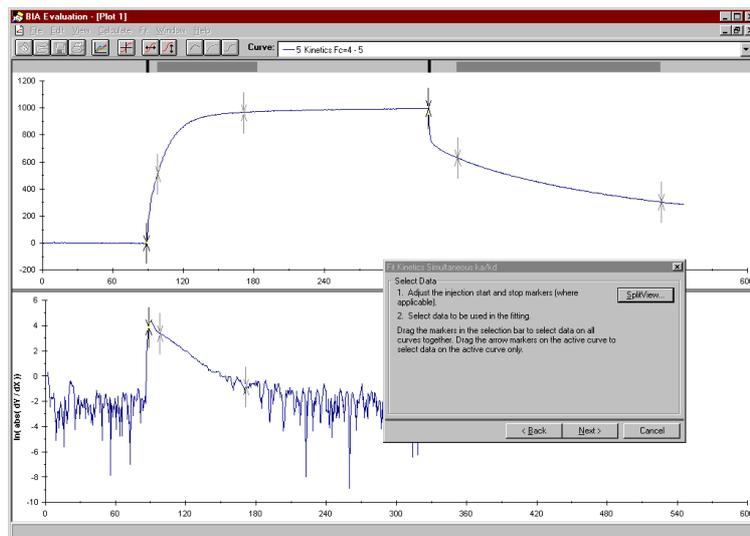
If you use single plot mode to make interpretation of split view plots easier, remember to click on the overlay plot button to display all curves again before continuing.

Click on **Next>** when data selection is complete.

Evaluation wizard, step 3 –model selection
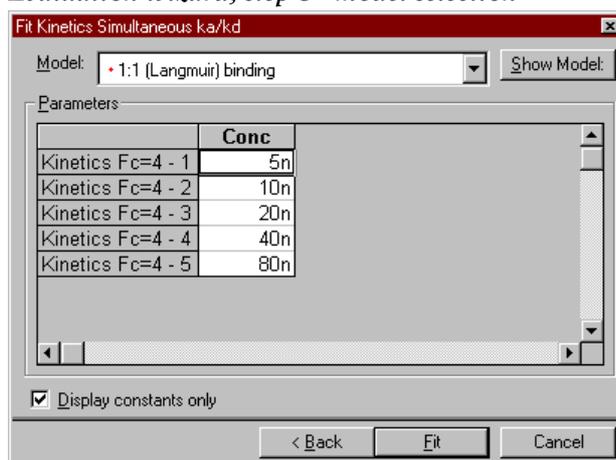
In this panel, you select the model to be used for the fitting and enter values where necessary for the constants. Values for constants are taken from keyword values if the constants are defined as keyword-related in the model (see Appendix B for details of keyword functions). Concentration is a typical example of such a constant, where analyte concentration may be included as a user-defined keyword in the report point table from a BIA analysis. These values are fetched for convenience only and may be changed if required. For **kinetics.blr**, enter the concentrations as shown in the illustration above.

By default, this panel of the wizard displays only constants which require input values for the fitting procedure. You may choose to display all parameters in the model with their initial values by unchecking the **Display constants only** box. Parameters which will be fitted globally (i.e. with a single value for all curves) are listed as a single value at the top of the table: cells for the separate curves are shaded. The reverse applies for constants and parameters defined to be fitted locally (i.e. with a different value for each curve). In the default settings for the 1:1 binding model, **Conc** is a constant, **RI** is a local parameter, **Rmax, ka** and **kd** are global.
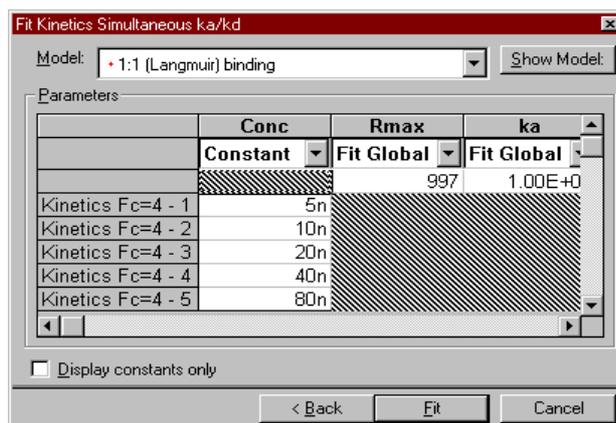
You can change the status (constant/local fit/global fit) of parameters in the table using the box under the parameter name. This provides flexibility in model fitting without requiring definition of multiple alternative models. Changes you make in this way apply only to the current fitting and do not affect the model definition.

You can also change the initial value for fitted parameters by entering a new value in the table. You should normally use the initial values as defined in the model, although changing the initial values can sometimes help to achieve a fit for difficult data sets (see Appendix A).

Click on **Fit** when you have chosen the model and entered the required constant values.

4.1.3   Following the progress of fitting

During fitting
During fitting, a small window indicates the progress of fitting both graphically (with the fitted curves in black) and numerically with the following information:
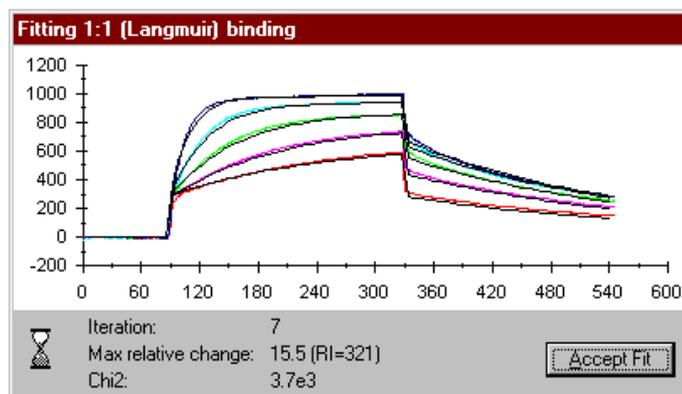
Iteration	The number of iterations performed. The number of iterations required to fit data varies with the data and the model chosen. The display is updated at a constant time interval, which may cover a variable number of iterations.
	Depending on the model chosen, the speed of iteration may vary considerably. Where possible, data is fitted to integrated rate equations, which is a rapid process. Where numerical integration is used (See Section A.3), fitting is significantly slower. In such cases, the first few iterations use only every tenth point in the selected data to speed up the approach to fitting. After the first few iterations, all points are used and the fitting may become significantly slower.
Max relative change	This figure shows the relative change (change/previous value) in the fitting parameter which varied most during the most recent iteration, with the name of the parameter and its current value in parentheses. This information can help you to judge the progress of the fit.
Chi2	The chi2 (χ^2) value is a standard statistical measure of the closeness of fit (see Section A.5). For good fitting to ideal data, χ^2 is of the same order of magnitude as the noise in RU (typically <2). However, a few points which deviate widely from the fitted curve (e.g. air spikes in the sensorgram) may have a marked effect on χ^2.

If the model is simple, fitting may be accomplished so rapidly that it is difficult to see the contents of the fitting progress box. However, this box can be useful for following the progress of slower fits.

The fitting process is terminated automatically when a minimum value is found for χ^2. You may terminate a fitting prematurely by clicking on **Accept Fit**.

4.1.4 Examining the completed fit

When fitting is complete, the plot window is replaced by a fit window which displays the results both graphically and numerically:

Fitted sensorgrams
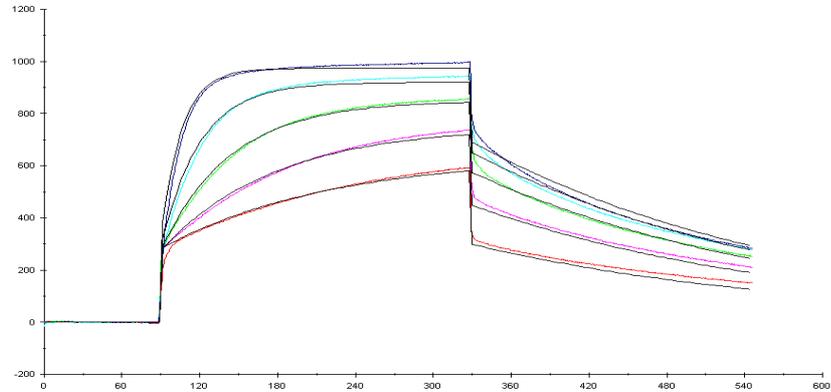
The top panel in the fit window displays the fitted curves (in black) overlaid on the original sensorgram curves. Visual assessment of the deviations between experimental and fitted data is often a good indicator of the validity of the fitting. In this example, there are clear systematic deviations in both association and dissociation phases, indicating that the model is not optimal for the experimental data. (A closer fit may be obtained by using a mass transfer limited 1:1 binding model.)

Residual plots
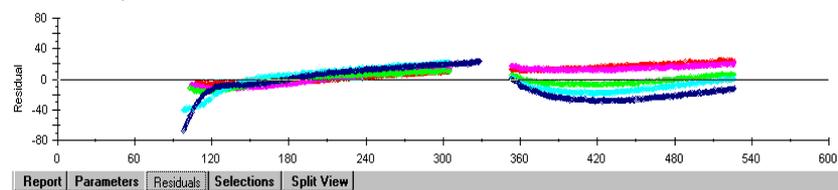
Residual plots are plots of the difference between experimental and fitted data for each curve. Click on the **Residuals** tab at the bottom of the fit window to display residual plots. An option under **Fit:Preferences** allows you to choose to display residuals as either absolute values (in RU) or relative values (the residual as a fraction of the experimental value at each point).

For a perfect fit, the *scatter* in the residual plots is a measure of the noise in the signal. Normally, noise levels should be of the order of ± 2 RU, which appears as a scatter in the residual values in this range.

The *form* of the residual plot reveals whether there are systematic deviations between the experimental and the fitted data. In the example here, the deviations are very clear, particularly in the dissociation phase. It is easier to detect small systematic deviations from the residual plots than from the sensorgrams. Systematic deviations indicate that the model is more or less inappropriate for the experimental data.

If systematic deviations are pronounced, this indicates that the chosen model may be inadequate or incorrect. Whether refitting the data to an alternative model is worthwhile depends on the extent of the deviations and the purpose of the experiment. In general:

- Systematic deviations that are comparable with the noise level may not represent significant deviations from the chosen model.

- Asymmetrical systematic deviations (as in the illustration above) may indicate more heterogeneity in the system than in the model. Symmetrical deviations may often have alternative explanations (e.g. fitting homogeneous dissociation data to the 1:1 dissociation model without adjusting the baseline to zero will give a symmetrical "hammock-shaped" residual plot). Mass transport-limited kinetics also give rise to symmetrical deviations.

Note: Global fitting procedures generally give higher residual values than local fitting (Section 4.1.5).

Numerical results

The **Report** tab at the bottom of the fit window displays the results of the fitting in terms of selected parameter values. Parameters that will be included in the report are defined in the model, and may include functions of calculated values, e.g. K_A and K_D derived from the fitted rate constants (see Appendix B).

	ka (1/Ms)	kd (1/s)	Rmax (RU)	RI (RU)	Conc of analyte	KA (1/M)	KD (M)	Req (RU)	kobs (1/s)	Chi2
	7.4e5	3.99e-3	742			1.85e8	5.4e-9			1.62E+04
Kinetics Fc=4 - 1				281	5n			357	7.69e-3	
Kinetics Fc=4 - 2				271	10n			482	0.0114	
Kinetics Fc=4 - 3				265	20n			584	0.0188	
Kinetics Fc=4 - 4				265	40n			654	0.0336	
Kinetics Fc=4 - 5				278	80n			695	0.0632	

Report | Parameters | Residuals | Selections | Split View

The **Parameters** tab displays the fitted values for the parameters defined in the model together with statistical information. Depending on the option chosen in **Fit:Preferences**, the statistical value may be either standard error or T-value (a relative measure of the standard error defined by dividing the value of the parameter by the standard error).

	Conc	Rmax	T(Rmax)	ka	T(ka)	kd	T(kd)	RI	T(RI)	
		742	1.07E+02	7.40E+05	2.82E+01	3.99E-03	4.26E+01			
Kinetics Fc=4 - 1	5n							281	3.53E+1	
Kinetics Fc=4 - 2	10n							271	3.05E+1	
Kinetics Fc=4 - 3	20n							265	3.00E+1	
Kinetics Fc=4 - 4	40n							265	3.25E+1	
Kinetics Fc=4 - 5	80n							278	7.63E+3	

Report | Parameters | Residuals | Selections | Split View

The **Selections** tab displays the start and stop times for injection and the selected data ranges used for the fitting. When the **Selections** tab is displayed, the graphical area of the fit window shows the experimental curves with the selected regions marked.

	Inject Start	Fit Start	Fit End	Inject End	Fit Start	Fit End	
30 nM	88.5	111	305	329	351	523	
60 nM	88.5	106	305	329	351	523	
120 nM	88.5	103	305	329	351	523	
240 nM	88.5	99.5	305	329	351	523	
480 nM	88.5	99.5	305	329	351	523	

Report | Parameters | Residuals | Selections
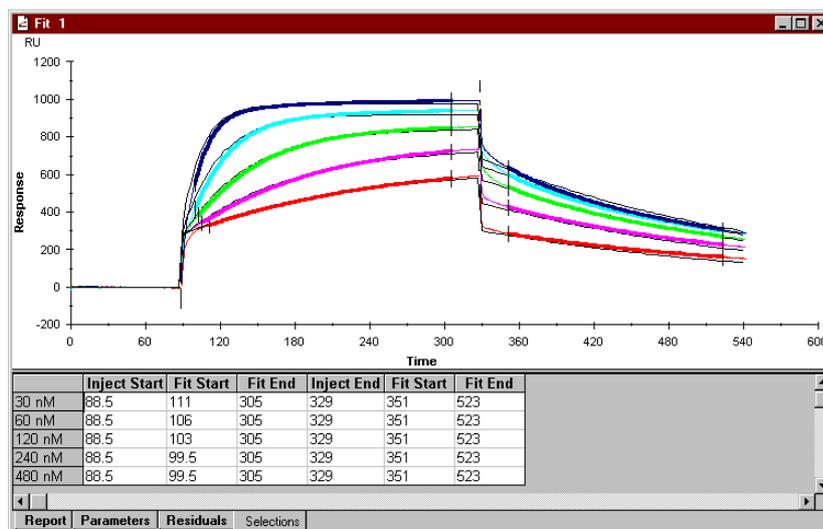
4.1.5   Assessing the fit

In assessing the results of a fitting, always check the numerical results for:

- reasonableness – for example, a model fitting which produces dissociation constants of the order of several s^{-1} should be regarded with suspicion, since current BIA technology cannot measure such rapid dissociation with any confidence.

- consistency – for example if locally fitted constants vary with the analyte concentration, the model may be inadequate or incorrect (or the concentration values may include a systematic error).

Visual inspection

Frequently, visual inspection of the experimental curves with overlaid fitted curves gives a good indication of the goodness of fit. Use the zoom function to look particularly at the beginning and end of the

injection to see that the injection start and stop times are correctly chosen. The illustration below shows the result of fitting the data in **kinetics.blr** with the injection start set a few seconds too early: the systematic deviation in the fitted curves clearly indicates the source of the error:
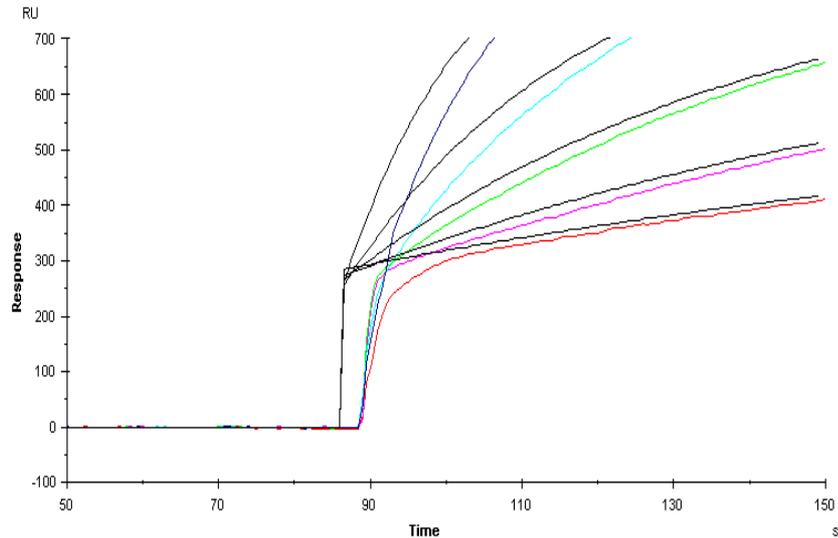
Residual plots give a graphical indication of how the experimental data deviate from the fitted curve. If you use residuals to assess the goodness of fit, remember that global fitting procedures generally give higher residual values than local fitting. This is because global fitting constrains the fit to the same values for each curve, while local fitting allows scatter in the fitted values (arising for example from dilution errors in a concentration series). The figures below compare the results of fitting the data in **kinetics.blr** using global and local fitting of k_a and k_d.
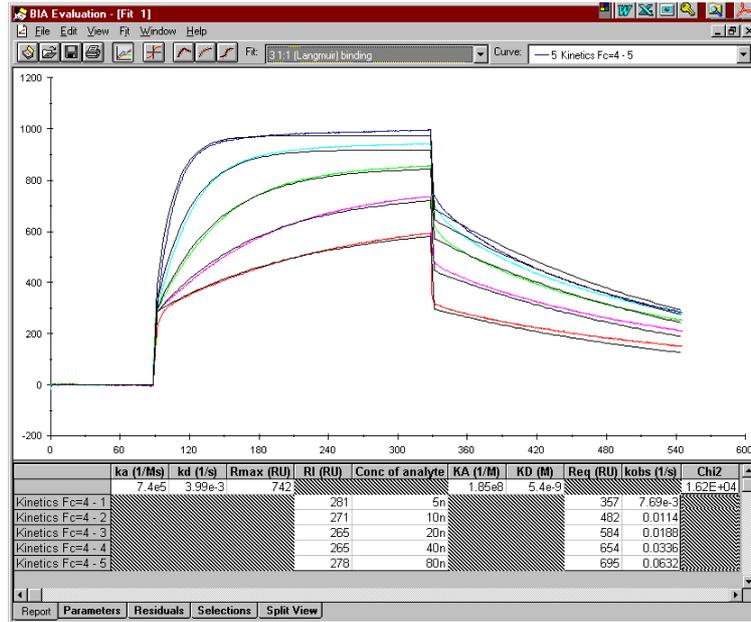
*Fitting **kinetics.blr** to a 1:1 interaction model using global settings for k_a and k_d. The fit is poor, with marked deviations during the dissociation phase and at the end of the association phase.*
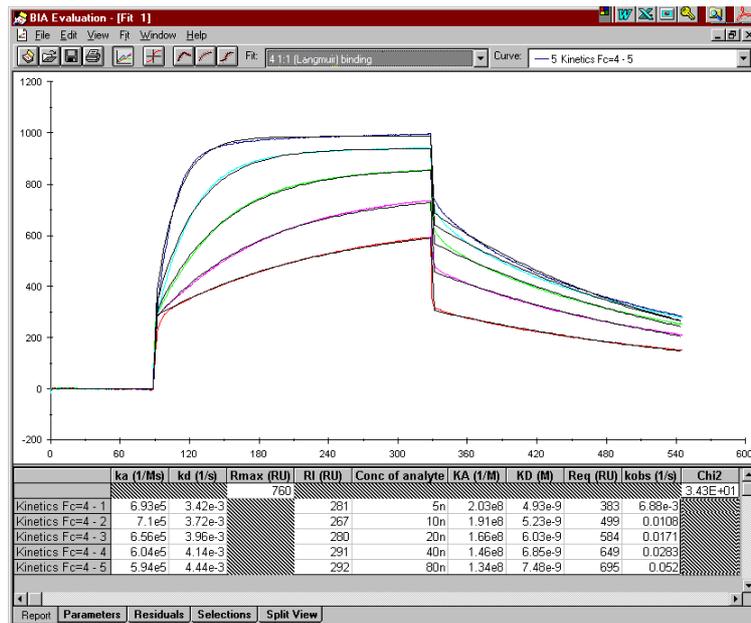
*Fitting **kinetics.blr** to a 1:1 interaction model using local settings for k_a and k_d. The fit is apparently much better, but this at the expense of considerable scatter in the values for k_a and k_d.*

In general, global fitting is preferable for parameters that should be the same for all curves (like k_a and k_d for a simple interaction). You may however want to perform a local fitting of these parameters if you suspect a systematic variation in the parameter values. Remember, however that certain parameters (such as the calculated steady state binding level R_{eq} and bulk refractive index contribution RI) are *expected* to vary with analyte concentration.

Fitting statistics
With the large number of data points in BIA sensorgrams, standard goodness-of-fit statistical parameters are fairly insensitive to the accuracy of fitting. For this reason, only the χ^2 value is provided: even this is of restricted use in judging the accuracy of fitting. Statistics of fitting are discussed more fully in Appendix A.

The standard error or T-value for fitted parameters is ***not*** an indication of the goodness of fit. This value indicates how sensitive the fitting is to changes in the respective parameters: a high standard error (low T-value) means that the parameter can be varied over a wider range without significantly affecting the goodness of fit. Use this value therefore to judge the significance of differences in a parameter between different fittings. For example, if the standard error for k_a is relatively high, the confidence that can be placed in small differences in fitted k_a values is low. See Section A.5 for a fuller discussion of this point.

4.2   Refitting to a different or modified model

If you are not satisfied with the fit, you can refit the same curves to a different model, or refit the curves to the same model with different data selections.

4.2.1   Refitting data

With the fit window active, choose **Fit:Kinetics Simultaneous ka/kd** or click on the corresponding toolbar button. The fitting procedure is repeated with the omission of step 1 (adjusting the data) since the data has already been adjusted.

Note: If you want to perform new data adjustment (baseline adjustment or removal of unwanted data), go back to the project window and open a new plot window containing the original curves. Only the first fitting operation on a plot window includes the curve adjustment step.

The steps in the evaluation wizard for refitting data are thus:

Data selection
By default, the selections used for the currently displayed fitting are used. You may however change these selections freely.

Model selection
By default, the model used for the most recent fitting is used (parameter status and initial values are however as defined in the model: any local changes made for the previous fitting are cancelled). You may change the status and initial values for the existing model or choose a new model.

When refitting is completed, the results are displayed in the same fit window. The previous fit is kept in the fit window and can be redisplayed by choosing it from the **Fit** list in the toolbar. One fit window can contain any number of fits for the same set of curves.
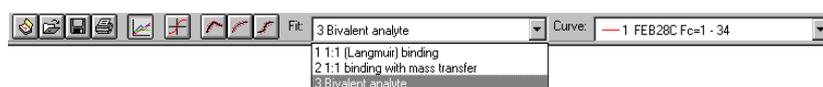
Note: Fits are identified in the **Fit** list by a serial number and the name of the model. Fits performed to the same model with different parameter status or data selection have identical names. The parameter status is apparent from the layout of the **Report** or **Parameters** tab; the data selection is shown on the **Selections** tab.

4.2.2   Removing a fit from the fit window
To remove a fit from the fit window, display the fit and choose **Edit:Remove Fit**. The fit will be deleted from the project.

4.3   Fitting mass transfer-limited binding data

Mass transfer limitations are among the most common complications in kinetic binding data. The observed binding rate is limited by mass transfer if the rate of transfer of analyte to the surface is significantly slower than the kinetic binding rate. The effect of mass transfer limitation is to slow down both the association phase (because analyte does not reach the surface fast enough) and the dissociation phase (because analyte is not transported fast enough away from the surface and is thus available for rebinding).The rate of transfer of analyte to the

surface can be increased by increasing the flow rate, and the rate of kinetic binding can be decreased by reducing the surface binding capacity. High flow rates and low surface binding capacities are thus recommended for kinetic work.

Even with these precautions, however, it may be difficult to obtain binding data with no mass transfer limitations, especially if the analyte is large (and thus diffuses slowly to the surface) and the association rate constant is high (so that the kinetic binding rate is high even at low binding capacities). In general, mass transfer limitations will be most significant at the beginning of the association phase and towards the end of the dissociation phase. As the binding proceeds during sample injection, the concentration of free binding sites on the surface decreases and kinetic interaction takes over the rate-limiting role. Conversely, the concentration of free binding sites is lowest at the beginning of the dissociation phase, minimizing rebinding effects.

BIAevaluation can fit data to models which include mass transfer limitations: a model for 1:1 interaction with mass transfer is provided (see Section C.2.3), and mass transfer terms can easily be added to other models (Section B.1.2). Interpreting the results of fitting data to mass transfer-limited models requires some care, however. If mass transfer is the dominant rate-limiting process, the kinetic interaction is obscured and the values for the association and dissociation rate constants are practically irrelevant. The fitting procedure will report values for these constants, but the significance of the values is low. By the same argument, if the observed binding is limited by kinetic interaction, the significance of the reported mass transfer coefficient will be low.

It is therefore important to determine whether the fitted sensorgrams include significant kinetic data or not. There are several indicators for this:

- Inspect the T-value (Section A.5.2) for the fitted parameters. This value is displayed in the **Parameters** tab of the fitting results. As a rule of thumb, if the T-value is less than 10, the significance of the reported parameter value is low. For fitting to predominantly mass-transfer-limited data, the T-value for the association and dissociation rate constants will be low.

- Choose **View:Split View** and display the **ln(abs(dY/dX))** function. During the association phase, this function is a straight line with a negative slope for fully kinetic-limited data. If the function shows a downward curvature throughout the association phase, there is significant mass transfer limitation.

- Repeat the fitting using local parameters for k_a, k_d and R_{max}. If the data is mass transfer limited, these parameters will probably show a significant scatter (over a several-fold range). This is because fitting to mass transfer-limited data is largely independent of the values for k_a

and k_d, and the value obtained for R_{max} depends on the values found for the rate constants.

- Repeat the experiment at varying flow rates. If the values obtained for the rate constants are consistent over a 5-fold range of flow rates (15-75 μl/min), mass transfer limitations are not serious and the reported rate constants are significant. If there is a significant variation with flow rate, the values from the highest flow rate are most likely (although not certain) to be reliable.

4.4 Other fitting facilities

Fitting to simultaneous k_a/k_d is the recommended method for most kinetic analyses. The validity of this approach relies however on the use of identical experimental conditions for both association and dissociation (if the experimental conditions differ, the rate constants obtained from one phase may not be valid for the other), and on the availability of usable experimental data in both phases. Other fitting methods are available for special circumstances and for fitting non-kinetic data.

4.4.1 Fitting k_a and k_d separately

You may choose to fit the association and dissociation phases separately. This can be necessary if you do not have access to valid data for one of the phases (e.g. the association phase may be obscured by a high bulk refractive index pulse) or if different conditions apply during association and dissociation. Separate fitting of the association phase requires values for the dissociation rate constant.

Fitting association and dissociation phases separately can use either local or global settings for parameters. The default setting is local fitting. Regardless of whether parameters are local or global, the fit is performed over all displayed sensorgrams simultaneously.

Dissociation phase

Choose **Fit:Separate ka/kd** or click on the separate k_a/k_d toolbar button. Adjust the curves from the first evaluation wizard panel:
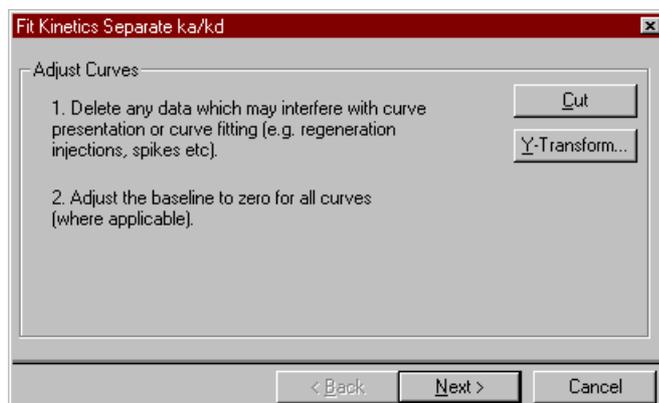
In the second panel, select the data and choose **Dissociation** under **Type**. (The selection bar is by default placed in the association phase: move it to the dissociation phase.)

The third panel allows you to choose the model from the list of models defined for separate dissociation. Only 1:1 (Langmuir) dissociation is included as a pre-defined model. Click on **Fit** to perform the fitting.

The fitting results are presented graphically with the fitted curves overlaid on the experimental curves over the region of data used for fitting:
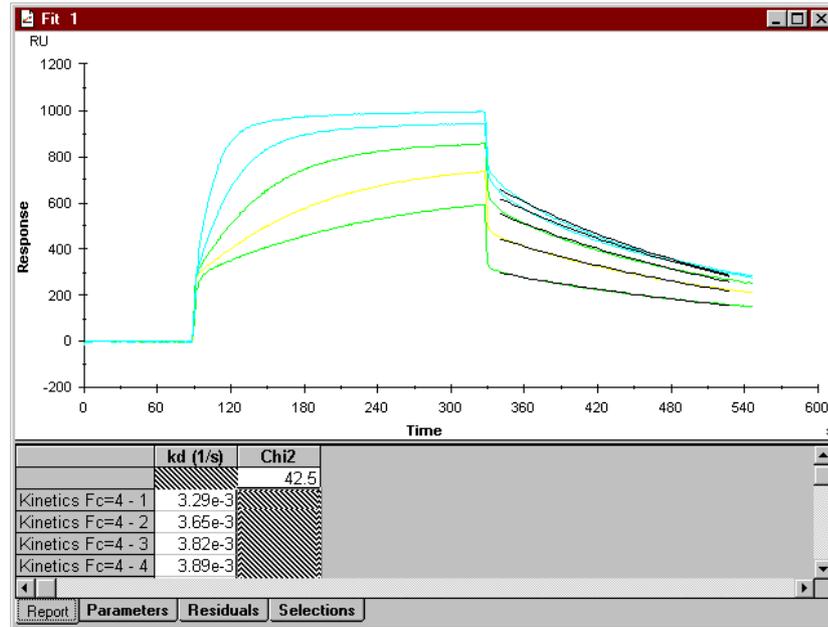
Numerical presentation of the results is similar to that described for simultaneous k_a/k_d fitting (Section 4.1.4).

Association phase
Repeat the procedure as described for the dissociation phase, but choose **Association** under **Type** in the second wizard panel. In the third panel, choose the model (only 1:1 (Langmuir) association is included as a pre-defined model), and enter values for analyte concentration and k_d. Concentration values may be fetched from the result file if report points include an appropriate keyword (see Section B.2.2). Dissociation rate constants are fetched from the most recent fit for separate k_d.

4.4.2   General fitting
Models for general fitting are designed for fitting non-kinetic curves. The following models are included:

• 4-parameter equation for curve fitting

• Linear fit (least-squares) for straight line fitting

• Solution affinity (see Chapter 5)

• Steady state affinity (see Chapter 5)

To perform a general fit, choose **Fit:General** or click on the general fit toolbar button. Curve fitting to a 4-parameter equation requires initial values for the 4 constants used in the model (see Section C.4.1).

4.5   Examining sensorgram components

For curves fitted to simultaneous ka/kd models with several components (e.g. multiple interacting species, or a simple interaction with bulk refractive index contribution), curves for the separate components can be displayed using **View:Components** from the fitting window. The separated components are shown for one sensorgram at once. The example below is taken from the example file for heterogeneous analyte **zkomp.ble**:
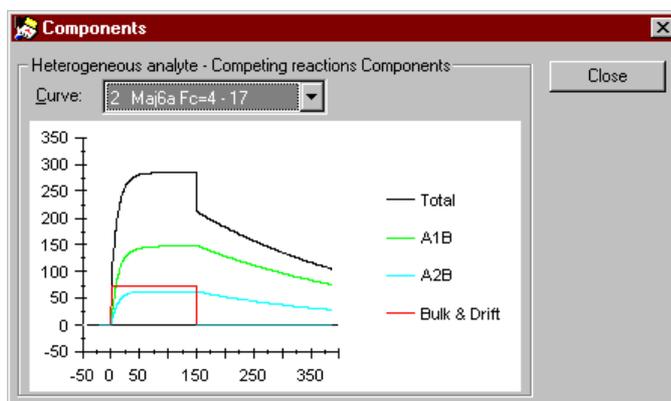
The right mouse button menu (see Section 1.1.2) can be used on the **Components** display, allowing you to export the fitted component curve data to a text file. The data sets in the text file are identified according to the component names in the model.

By opening the exported text file as curves, you can import the component curves back into the evaluation project. This can be useful if you for example want to subtract the fitted bulk refractive index contribution from the experimental data for presentation purposes:

1.   Fit the sensorgrams and choose **View:Components**.

2.   Using the right mouse button menu, export the component curves for each sensorgram.

3.   Open the exported text files as curves in the same project.

4.   For each sensorgram, choose **Calculate:Y-Transform** and subtract the appropriate **Bulk & Drift** curve.

4.6 Creating secondary plots

You can create secondary plots from data in the fitting results. Choose **Fit:Plot Parameters** and select the parameters to be used for the x- and y-axes of the plot. The parameters available are those displayed in the current fitting results tab (report or parameters). The created plot is added to the current project window.

For example, to plot values of R_{eq} against concentration, choose the report tab in the fitting window (so that R_{eq} is displayed) and choose **Fit:Plot Parameters**. Choose **Conc of analyte** for the x-axis and **Req** for the y-axis, then click on **OK**.

Note that the parameters available for plotting are those in the currently displayed tab in the fit window.

You can also copy the contents of the current fitting results tab (report or parameters) to a spreadsheet in BIAevaluation for further processing as follows:

1. Click in the cell at the top left of the displayed results table (to the left of the parameter name row) to select the whole table.

2. Choose **Edit:Copy** (keyboard shortcut Ctrl-C).

3. Activate the destination spreadsheet (or open a new sheet) and click in the cell at the top left of the area where you want to paste the data.

4. Choose **Edit:Paste** (keyboard shortcut Ctrl-V). Note that any existing values in the paste area will be overwritten.

4.7   Simulating kinetic data

BIAevaluation includes a simulator for generating theoretical sensorgram data from the interaction models used for kinetic analysis. This can be valuable for investigating how changes in kinetic constants or other parameters affect the appearance of sensorgrams, and can thus provide useful information for design of experimental conditions. For example, if you find that your observed interaction rates are limited by mass transfer, you can use the simulator to generate a set of curves representing different ligand immobilization levels (Rmax values) to see if mass transfer limitations can be avoided by reducing the ligand level.

To simulate kinetic data, choose **Fit:Simulate Curve(s)**. This starts the simulation wizard which leads you through the simulation procedure.

Simulation wizard, step 1 – define curve properties

Choose the number of curves to simulate, the preinjection delay (representing baseline) and the association and dissociation times.

Click on **Next>** when you have entered curve properties.

*Simulation wizard, step 2 – model selection*

Choose the model to be used for simulation and enter values for the parameters in the table. If you start the simulation wizard from a fit window, the values obtained for the fitted curves will be suggested by

default. Otherwise, the simulation wizard starts with blank values. You can change the status (local/global) of parameters in the table using the box under the parameter name. Changes you make in this way apply only to the current simulation and do not affect the model definition.

Click on **Simulate** when you have chosen the model and entered parameter values. The simulated curves are added to the current project and displayed in a new plot window. The curves are named according to the model from which they were simulated.
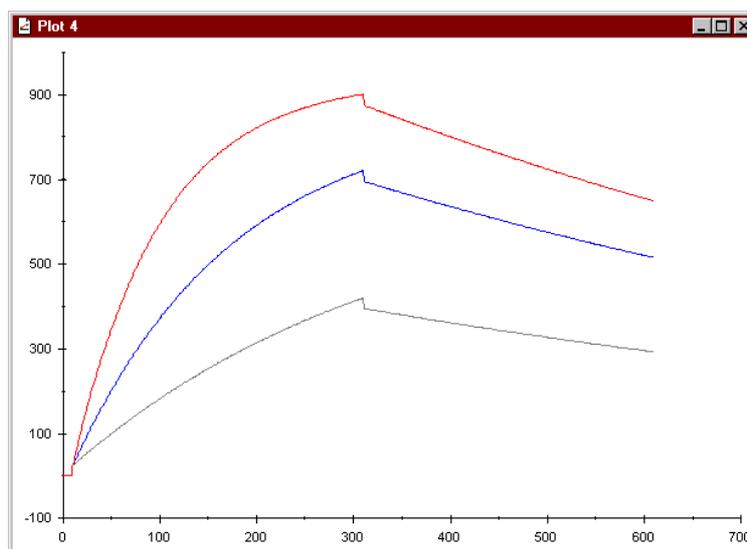
To compare simulated curves with experimental data, choose both sets of curves in the project window and create an overlay plot.

4.8 If the data do not fit the model

4.8.1 Experimental considerations

Obtaining reliable kinetic values from sensorgram data makes considerable demands on careful experimental design and execution[1]. Sophisticated evaluation software can never be a substitute for careful experimentation. Some of the most common causes of inadequate data are summarized below:

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[1] Karlsson, R. and Fält, A. (1997) J. Immunol. Methods **200**, 121-133.

Cause	Effect	Remedy
Impure reagents	Heterogeneous kinetics	Purify reagents rigorously
Immobilization chemistry	Multiple attachment sites give heterogeneous ligand	Use directed immobilization methods
Non-specific binding	Heterogeneous kinetics	Use a reference cell to check specificity
Immobilization level	High R_{max} favours mass transfer limitations	Keep R_{max} as low as possible
Analyte concentration	Low or high concentrations reduce the amount of kinetic information obtained	Use analyte concentrations in the range 0.1-10 K_D
Dilution errors	Systematic errors in k_a	Use calibrated pipettes
Flow rate	Low flow rates favour mass transfer limitations	Run at flow rates >20 µl/min
Sample dispersion	Obscures rapid events at injection start and stop	Use high flow rates and **Kinject** command. Load sample carefully (BIACORE X)
Bulk effects	Obscure rapid events at injection start and stop	Match refractive index in sample and running buffer

4.8.2   If the fitting fails

In some cases, the fitting procedure fails mathematically, with either an error message or clearly incorrect fitted curves as a result. The most common causes for this behaviour are:

- Incorrect data selection. You cannot for example fit a decreasing response to the association phase of a pre-defined model, since the models do not allow negative association rate constants. Make sure that you have selected appropriate parts of the sensorgram for association and dissociation phases.

- Unsuitable initial values for parameters. In some cases, the initial values for the parameters may be so far wrong that the fitting fails.

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This is often readily apparent from the fitting progress display (Section 4.1.3): the first attempts at a fitted curve show either zero response or an immediate off-scale increase, and the fitting process never "recovers" from the situation. To correct this, repeat the fit with a large change (several orders of magnitude) in one or more selected parameters. The appearance of the fitting progress can often give an indication of which parameter to adjust: for example, if the first attempts show extremely high initial binding rates, reduce the initial value for the association rate constant.

4.8.3   Evaluation as an experimental tool

If in spite of careful experimental design the data do not fit the chosen model adequately, fitting results can give suggestions for further experimental directions to investigate the nature of the interaction. The series of fitting attempts described below illustrates the way BIAevaluation can generate ideas about the nature of the interaction. See Section 4.2 for a description of how data is fitted to alternative or modified models.

The sensorgrams were obtained in BIACORE X using a 41,000 dalton analyte with an amine-coupled peptide ligand. The experiments were performed using carefully purified analyte. A 1:1 interaction was expected. The ligand level was low (about 120 RU maximum binding capacity estimated by injection of 1000 nM analyte) to minimize mass transfer limitations. Analyses were performed at 5 analyte concentrations from 5 to 100 nM. The second flow cell was used as a reference channel and subtracted from the sample channel during the run: bulk refractive index contributions were therefore expected to be zero (and were not significant in the reference-subtracted sensorgram). All fits were performed with the bulk contribution **RI** set to a constant value of zero.

1:1 (Langmuir) binding model

Evaluation starts with the simplest model, 1:1 (Langmuir) binding. The fit is very poor, in both the association and dissociation phases. In addition, the reported R_{max} value is lower than that found experimentally:
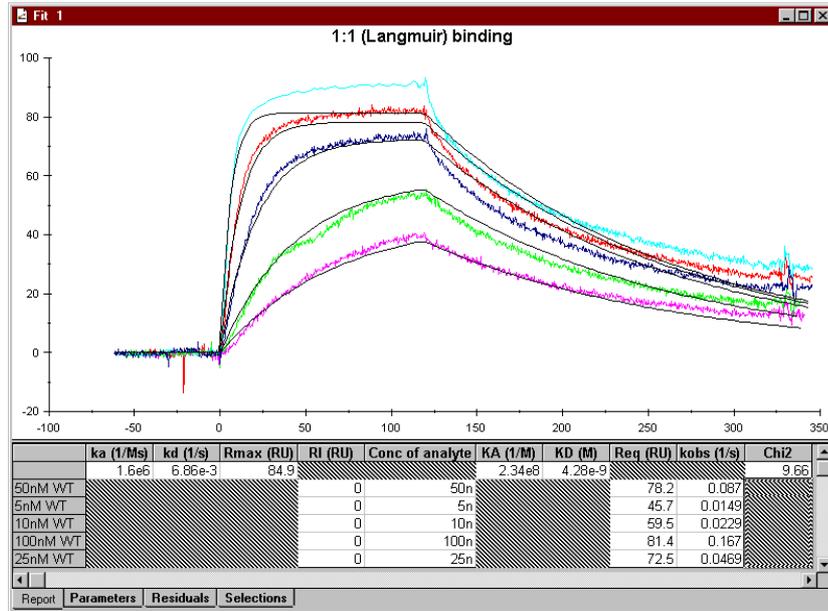
In order to check whether there is a systematic error in the concentrations, the fit is repeated with local fitting for k_a (the k_d value is independent of concentration and is left as a global parameter). Plotting k_a against concentration with **Fit:Plot Parameters** (Section 4.6) visualizes any correlation between these parameters:
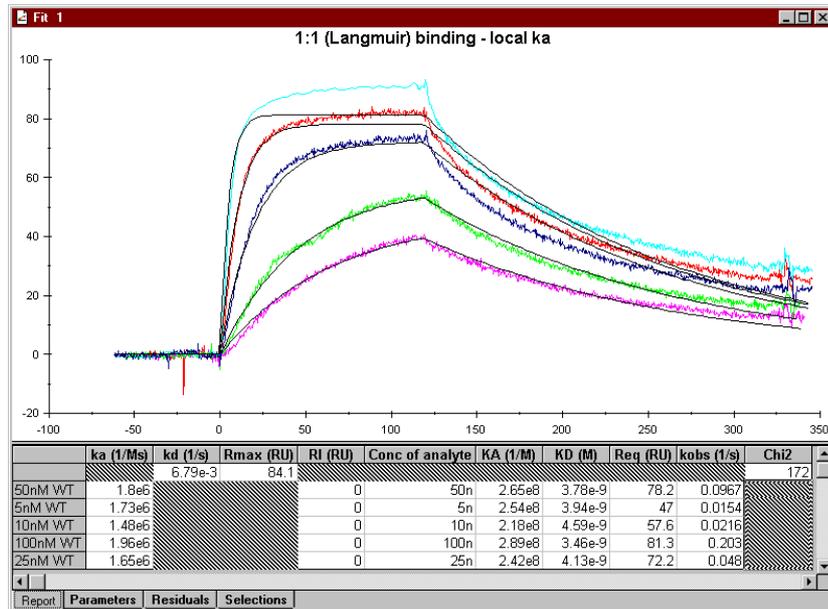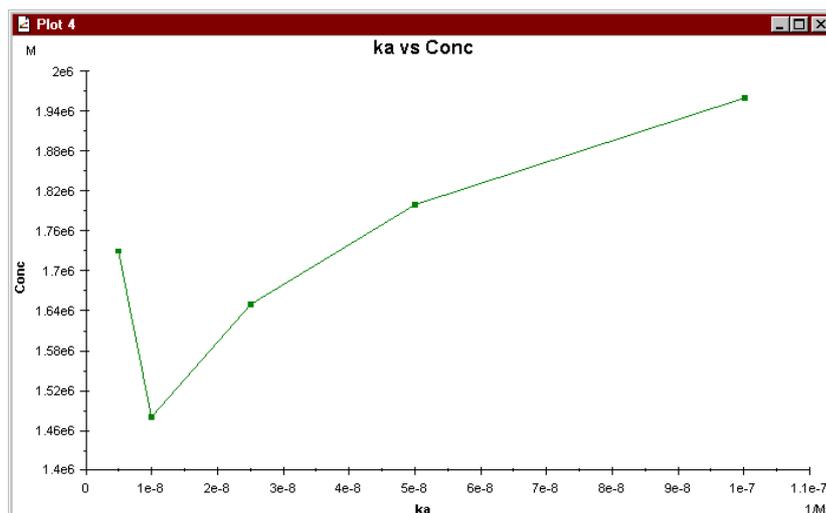
Although there may be some correlation between k_a and concentration (indicating a possible dilution error in preparing the samples), the variation in k_a values is slight and is not sufficient to explain the poor fit.

### *1:1 binding with mass transfer model*
The inadequacy of the fit in the dissociation phase is striking – the experimental curves flatten out much more rapidly than the calculated curves, indicating that dissociation slows down with time in comparison with a simple 1:1 interaction. A possible cause of this is rebinding of analyte during the dissociation phase, which is a result of mass transfer limitations preventing clearance of free analyte from the surface. Fitting to a 1:1 interaction with mass transfer improves the appearance of the dissociation phase slightly, but not nearly enough to give an acceptable fit:
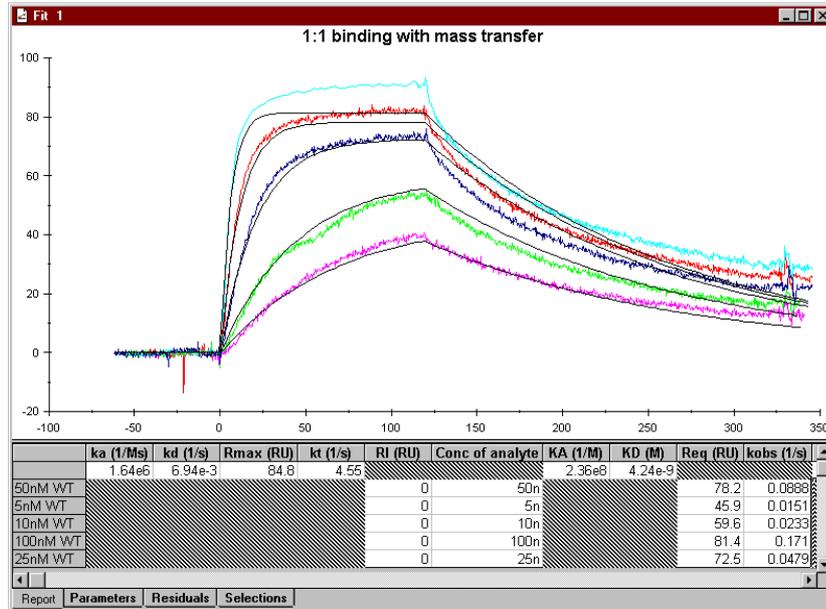
Including mass transfer limitations has improved the fit slightly in the dissociation phase, but by no means enough to be acceptable. The association phase is hardly improved at all.

Heterogeneous ligand (parallel reactions) model

The shape of the experimental dissociation phase suggests that there might be more than one process involved. Rebinding (modelled by mass transfer limitations) is not an adequate explanation. Fitting to a model for heterogeneous ligand (parallel reactions) was therefore tested:
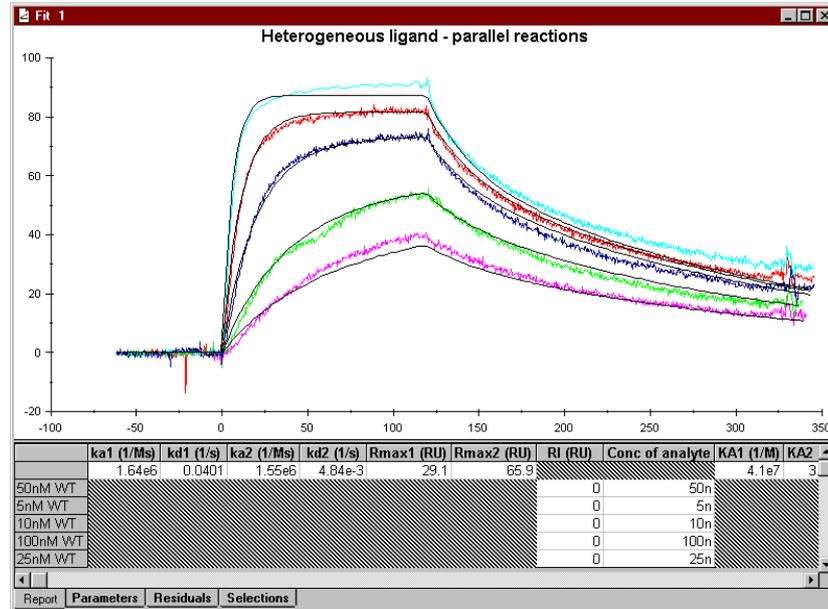
The shape of the calculated curves is significantly improved here, although deviations from the experimental curves are still significant. The reported parameters show closely similar k_a values for the two ligand components, but the k_d values differ by a factor of 10. The total R_{max} value is now 95, closer to the experimentally estimated value.

Examining the residuals for this fitting reveals a general upward trend in both association and dissociation phases:
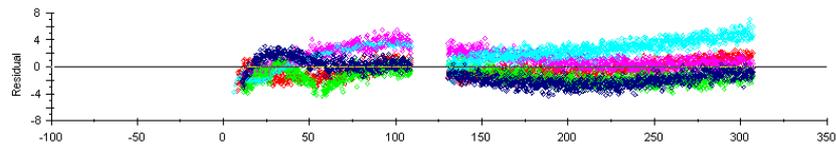

Using the model editor, therefore (see Section B.1.3), a linear drift term was added to the model for parallel reactions. The result is a reasonably close fit to all of the curves. Note in particular the excellent fit to the shape of the dissociation phase for all curves:
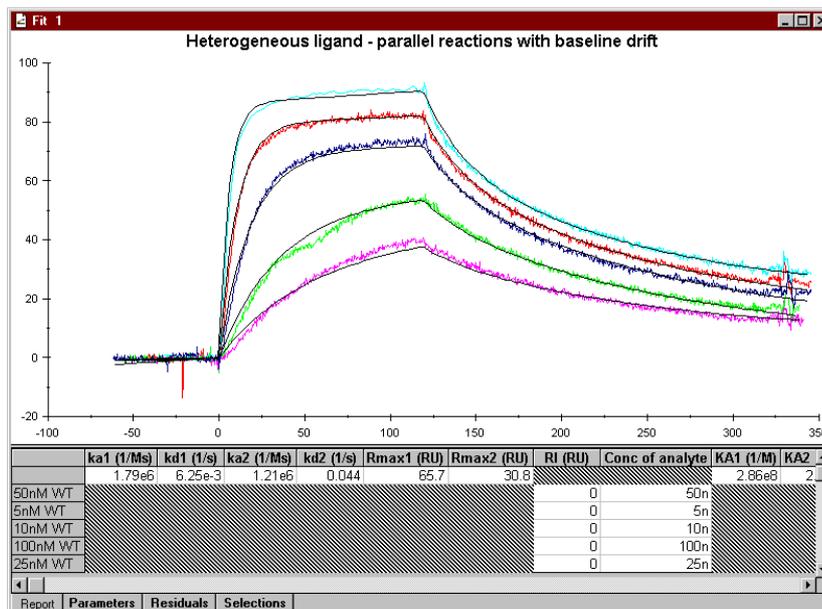
Conclusions

Having found a model which fits the experimental data well, the next step is to ask if the parameters for the model are reasonable and if so what experiments may be suggested to test the model.

- Is there reason to accept a heterogeneous ligand model? The peptide ligand was immobilized with amine coupling with potentially multiple attachment sites. It would be valuable to investigate the possibility of immobilizing the ligand using a more uniquely directed method.

- Are the two components reported in the model physically and biochemically reasonable? Values for k_a and k_d for both components are within a reasonable range for this kind of interactant, and also within the measurement range of BIACORE X. The two components represent about 66% and 33% of the total analyte binding capacity, so that contributions from both components are significant. Choosing **View:Components** shows the way the measured response is distributed between the components of the model:
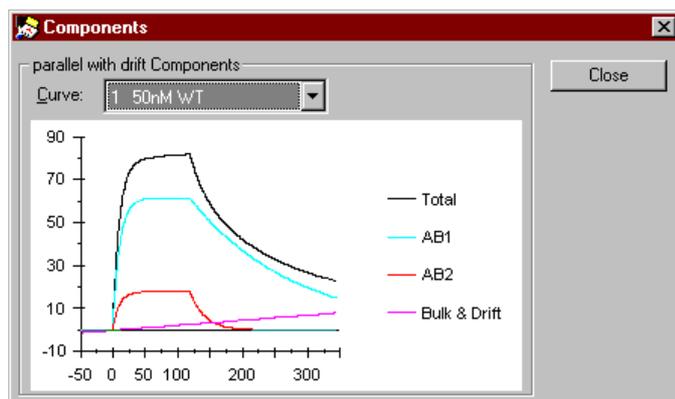
- Is the baseline drift reasonable? Values for the fitted drift parameter range from about 0.5 to 2 RU/min, which not very serious although it is fairly high for instrument-related drift in BIACORE X. It would be worthwhile investigating possible sources of drift in the experimental setup.

- As mentioned above, there was low but significant binding of analyte in the reference channel, so that the baseline drift in the reference-subtracted channel may be related to interaction events on the blank surface.

In conclusion, fitting attempts in BIAevaluation have suggested that the ligand may be heterogeneous and that there may be a drift introduced by subtraction of a response in the reference cell. This clearly indicates possible directions for future experiments, assuming that the goal of the experiment is kinetic characterization of the interaction. Note that the fitting results are not in themselves *evidence* of ligand heterogeneity, but generate a hypothesis that may be tested experimentally.

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5. Evaluating concentration data

BIAevaluation includes functions for evaluating concentration data from report point tables:

- generate calibration curves from standard samples

- calculate unknown concentrations from the calibration curve

- display CV_{dose} and $CV_{response}$ profiles

- create curves from data, e.g. baseline response plotted against cycle number for assessing regeneration performance.

5.1   Data for concentration evaluation

Concentration measurements with BIACORE may be based on binding level or binding rate determinations. Under conditions of limiting mass transfer of analyte to the surface, the initial binding rate is independent of the ligand density and the interaction characteristics, and depends only on the analyte concentration and mass transfer properties[1]. If mass transfer is only partially limiting, concentration can be determined independently of the binding characteristics from measurements over a range of flow rates[2]. Using this latter approach, a calibration curve is not needed provided that the diffusion coefficient of the analyte is known.

Binding levels are usually obtained from report points set at a fixed time after sample injection (usually after the injection is complete to avoid bulk refractive index contributions). For interactions which are amenable to kinetic analysis, it is also possible to use kinetic evaluation to estimate steady state (R_{eq}) values. Binding rates may be obtained from the slope at report points or from the binding level at specified time intervals. For mass transfer limited binding, the initial rate can also be obtained from a straight line fit to the early portion of the association phase, using the general fitting model **Linear fit**.

However the data is obtained, concentration evaluation is performed from a spreadsheet in BIAevaluation. To open the data in a spreadsheet:

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[1] U.S. patent pending. See Karlsson, R. (1993) J. Immunol. Methods **166**, 75-84.

[2] Richalet-Sécordel, P. M. *et al.* (1997) Analyt. Biochem. *in press.*

- for report point tables in result files, open the result files and choose **Rpoint Table** in the **Open** dialogue box (see Section 3.1.2)

- for exported report point tables and text files, open the file and choose **Open as Sheet** in the **Open** dialogue box (see Section 3.1.3)

- for data from kinetic fitting, select the contents of the report tab, press Ctrl-C to copy the data to the clipboard, open a new sheet and press Ctrl-V to paste the data into the sheet

Once the data is in the sheet, steps in concentration evaluation are as follows.

1. Filter the data so that only the standard and unknown values are shown.

2. Generate the calibration curve.

3. Calculate the unknown values. If the unknowns are included in the data set used to generate the calibration curve, this can be done at the same time as the calibration curve is generated. Unknowns can also be calculated at a later time on the basis of the calibration curve from data in the same or a different sheet.

These steps are described in detail below. The example data is taken from the example file **conc.rpt**, which is a report point table from determination of β2-microglobulin concentration using a sandwich assay protocol. The primary response obtained from binding β2-microglobulin to immobilized antibody is amplified by injection of a second antibody. The second antibody response is used to calculate concentration.

5.2   Generating a calibration curve

To prepare the spreadsheet from **conc.rpt** for evaluating concentration based on the secondary antibody response, filter the data to show only **second_AB** in the **Id** column.

Note: Concentration values for standard points are required for generating a calibration curve. Normally, these should be included as a custom keyword in the report point table in the result file. If concentration values are not included, type them directly into the spreadsheet before generating the calibration curve.

Unknown values are identified by non-numerical values (including blanks) in the concentration value column. Do not use numerical identifiers for your unknowns, since these will be interpreted as concentration values. If the unknowns do

have numerical identifiers, either change them to non-numerical or filter the unknown rows out of the spreadsheet before generating the calibration curve.

Choose **Data:Generate Calibration** to generate a calibration curve. If there is more than one keyword in the Id column, you will be warned that the report points have not been filtered. You may choose to continue, but be aware that your calibration curve will probably not be correct.

Choose the columns to be used for the calibration curve in the **X-Keyword** and **Y-Keyword** panels. By default, BIAevaluation suggests RelResp for the y-axis of the calibration curve and concentration-related data (labelled e.g. ng/ml or mg/ml) for the x-axis if such can be found.

Choose the type of fitting to use for the calibration curve. By default, BIAevaluation uses a 4-parameter equation which gives a robust fitting for practically all calibration curves. Other fitting functions available are spline, qubic spline, line and point to point, with or without

smoothing (see Section 7D.4.4 for details). Use these alternatives only if you have good reason not to use the 4-parameter equation.

By default, the calibration curve is generated by fitting to all calibration points, treating replicates as separate points (**All** is selected under **Replicates**). If you select **Median** under **Replicates**, the calibration curve will be generated using the median of replicate values (the middle point in an odd number of replicates or the average of the two middle points in an even number) for each standard concentration. In general, **All** is preferable to **Median**.

If you check **Calculate Unk.**, BIAevaluation will calculate concentration values for unknown points using the generated calibration curve. You can calculate values for unknowns and insert the results in the original sheet at a later stage, regardless of whether you calculate unknowns at this stage or not.

You can save the calibration curve in a BIAevaluation project file by choosing **File:Save**.
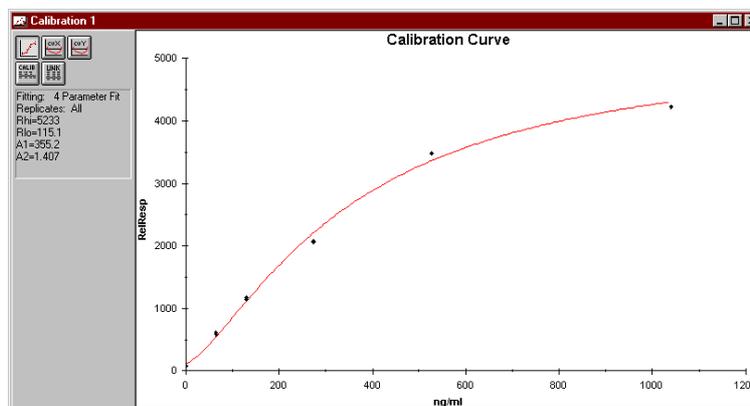
5.3   Examining the calibration curve

5.3.1   Calibration window components

The calibration curve is displayed in a calibration curve window. The fitting algorithm used is shown in the panel at the left of the curve window. There are five buttons in this panel for different display modes:
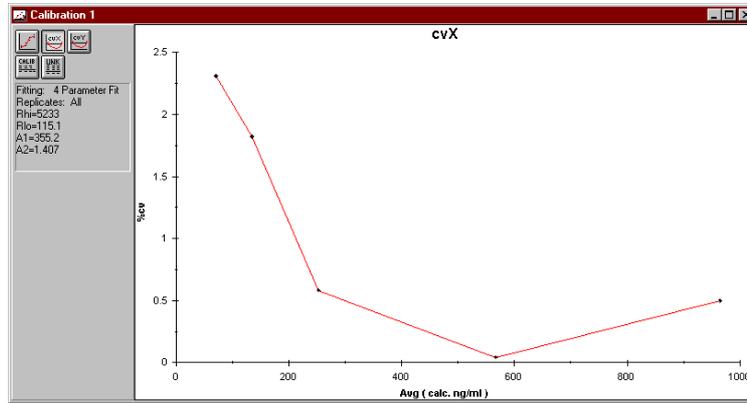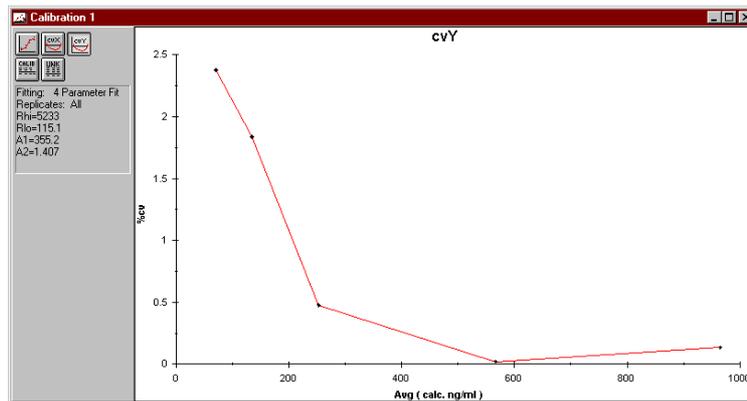
Displays the calibration curve.

If the calibration curve is generated using the 4-parameter equation, values for the parameters used in the fitting (R_{hi}, R_{lo}, A_1 and A_2; see Section C.4.1) are shown beside the curve.

Displays cvX, i.e. a plot of the precision in measured concentration as a function of the average concentration. cvX values are dependent on how well the standard curve fits the data.

Displays cvY, i.e. a plot of the precision in measured response as a function of the average concentration. cvY values are independent of the curve fitting.

Displays the calibration data in tabular form. The format for numerical display can be changed if desired (Section 7E.4.7).


Displays the calculated results for unknown points (only available when unknowns have been calculated). Results are grouped according to the value in the concentration column, and averages and CV values are presented for each group. Values outside the range of the calibration curve are displayed as **#N/A**.

5.3.2 Comparing calibrations
If two or more calibration windows are open at the same time, choose **Data:Compare Calibrations** to create an overlay plot of different calibration curves. The example below compares calibration curves for the primary response (**Id** keyword **b2micro**) and secondary response (**second_Ab**) in **conc.rpt**.
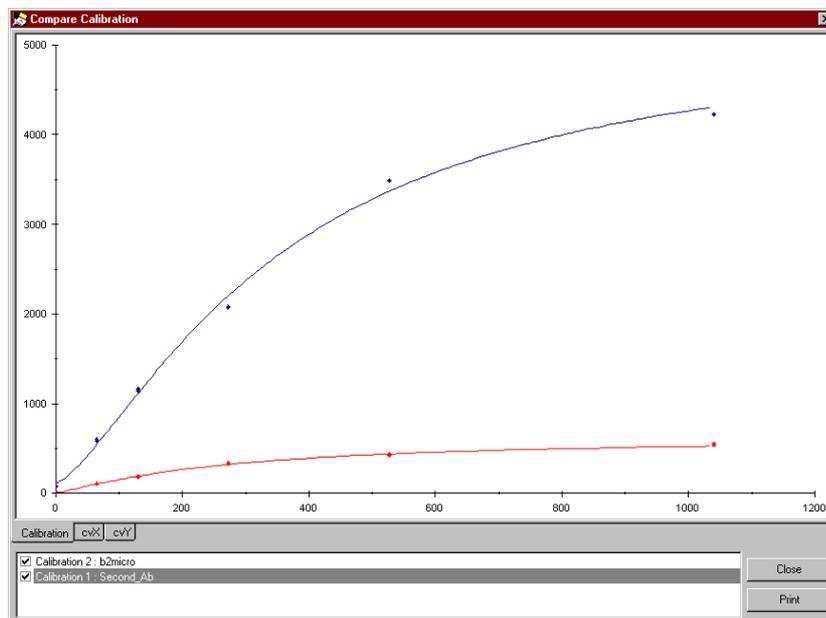
The area at the bottom of the window lists the available calibration curves by window name and title. (Choose **Labels** from the **View** menu or the right mouse button menu in the calibration window to set the title.) You can double-click on the name to change it: changes however will be lost when you close the window. Check the box beside a window name to display that calibration curve in the overlay window. Use the right mouse button menu (Section 1.1.2) if you want to copy the overlay plot to the clipboard or export the data to a text file, or to set labels or a legend in the overlay plot window for printing. The legend uses the names as listed at the bottom of the window. Changes made to labels and legend are lost when the window is closed.

5.4   Calculating unknown values

By checking the **Calculate Unk.** box in the dialogue box for generating a calibration curve, you can calculate values based on the calibration curve for any unknown points (non-numerical concentration values) in the data set used for calibration.

You can also calculate values separately, using a calibration curve prepared on a previous occasion. This is useful if you want to apply

the same calibration curve to concentration measurement data in several data sets. The presentation of calculated unknowns differs according to whether the operation is requested with a calibration window or a spreadsheet window active.

5.4.1   Calculating from a calibration window
If a calibration window is active when the unknowns are calculated, the results will be entered in the unknowns section of the calibration window. New results will be added to any previously existing results providing a cumulative report. The spreadsheet window containing the unknowns must be open.

To calculate new unknown values from the calibration window:

1.   With the calibration window active, choose **Data:Calculate Unknowns.**

2.   Choose the spreadsheet window containing the unknown data.
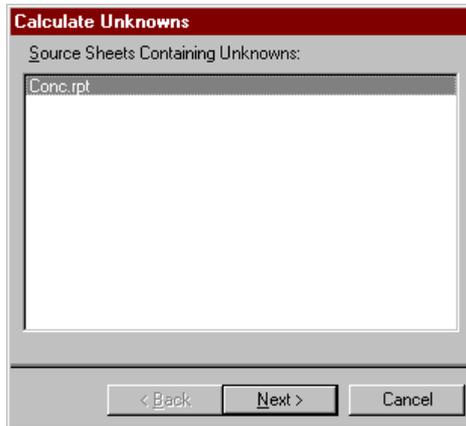
3.   Choose the columns in the spreadsheet containing the concentration (x-axis) and response (y-axis) values.
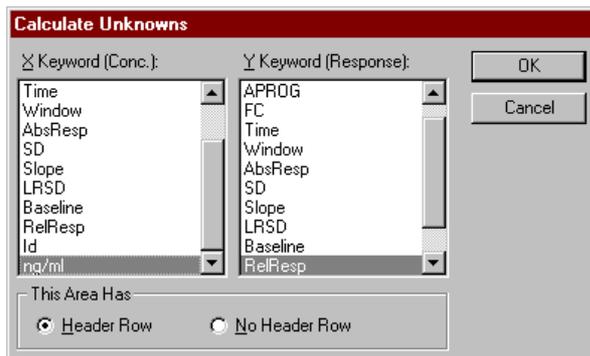
4. Press **OK**. Unknowns (identified by non-blank, non-numerical values in the concentration column) will be added to the unknowns section of the calibration window.

This approach groups the unknown values automatically by identifier and calculates average and CV values within each group. However, values are only calculated for points with non-blank, non-numerical identifiers in the concentration column.

5.4.2 Calculating from a sheet window

Values can also be calculated using a calibration curve by choosing **Calculate Values** from a spreadsheet window. The results will be entered in the first empty column of the spreadsheet. The calibration window containing the standard curve must be open.

To calculate values from the spreadsheet window:

1. With the spreadsheet window active, choose **Data:Calculate Values.**

2. Choose the calibration window to use for calculating the values.
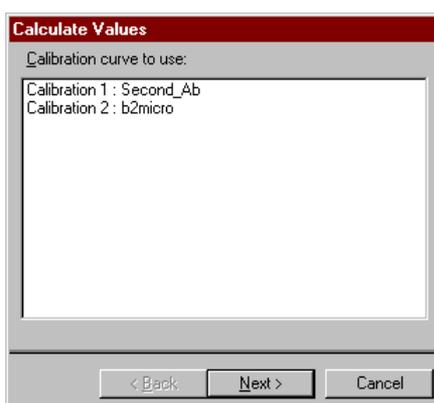
3. Choose the column in the spreadsheet containing the response (y-axis) values.
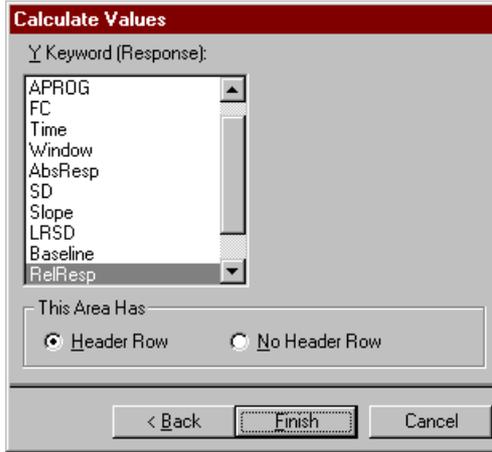
4. Press **Finish**. The calculated values will be inserted in the first empty column in the spreadsheet. Values will be calculated for all rows displayed in the sheet. Rows where the response value is out of range for the calibration curve will be marked as **#N/A**.

Use this approach when you want to calculate values for further processing, or when you want values for rows which have numerical identifiers in the concentration column (remember that calculating unknowns from a calibration window only gives values for points identified as unknown in the concentration column).

6.   Evaluating affinity data

BIAevaluation supports determination of affinity constants in three ways:

• calculation from the ratio of fitted rate constants.

• calculation from measured steady state binding levels

• calculation of affinity in solution by using BIACORE to measure the free concentration of one interactant in an equilibrium mixture

These approaches are described in this chapter.

6.1   Calculating affinity from rate constants

For a simple interaction, the affinity constant is defined as the ratio of the forward and reverse rate constants.

$$K_A = \frac{k_a}{k_d} \quad \text{and} \quad K_D = \frac{k_d}{k_a}$$

For the pre-defined kinetic evaluation models based on separate 1:1 interactions (1:1 (Langmuir) binding, heterogeneous analyte and heterogeneous ligand), affinity constants are included in the report definition in the model, so that kinetic evaluation automatically provides affinity constants. Bear in mind however that the reliability of the calculated affinity constants is directly dependent on the reliability of the kinetic constants (i.e. on the appropriateness of the model and the quality of the experimental data). Relatively small changes in k_a and k_d in opposite directions have a much larger effect on the calculated affinity constants.

For the predefined models for bivalent analyte and two-state interactions, the meaning of the affinity constants is less clear. For the two-state model, an apparent affinity constant is calculated based on the rate constants for the initial binding and the conformational change. Calculated constants for the individual steps are easily derived from the separate fitted rate constants.

6.2   Evaluating steady state affinity data

The steady state binding level is related to concentration according to the equation

$$R_{eq} = \frac{K_A C R_{max}}{1 + K_A C n}$$

where n is a steric interference factor specifying how many binding sites are on average blocked by binding one analyte molecule (default value 1). Rmax is the theoretical binding capacity, which will differ from an experimentally measured value if n is not equal to 1. This equation is used in the predefined general fitting model for surface affinity, which may be used to derive affinity constants from R_{eq} values.

Values for R_{eq} may be obtained in three ways:

- from report points set on the sensorgrams in the steady state region of the curve

- from "fitting" a horizontal straight line to a chosen section of the sensorgrams. A general fitting model for this purpose is included in the additional model file provided with BIAevaluation, and is described in Section C.4.4.

- from values calculated from kinetic evaluation of the sensorgrams. Note that this approach requires that the sensorgrams are amenable to kinetic evaluation and that the kinetic constants are fitted locally for each curve. With globally fitted constants, the plot of R_{eq} against C will give a perfect fit by definition.

Proceed as follows to determine affinity constants from measured steady state binding levels:

1. Obtain steady state binding levels (R_{eq}) as described above.

2. Create a plot of R_{eq} against C, using **Data:Create Curve** if the values are in a spreadsheet (report point values) or **Fit:Plot Parameters** if the values are in a fitting report.

3. Fit this plot to the general fitting model **Steady state affinity**. (Note that you will need to extend the suggested data selection area to include all points on the curve.)
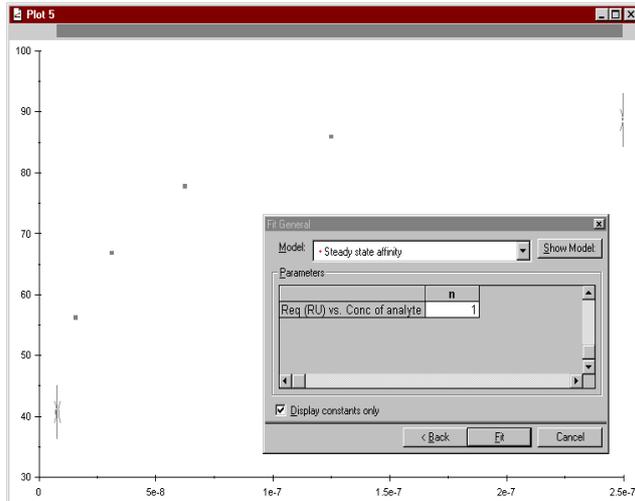
4.   The affinity constant is reported in the fitting results.
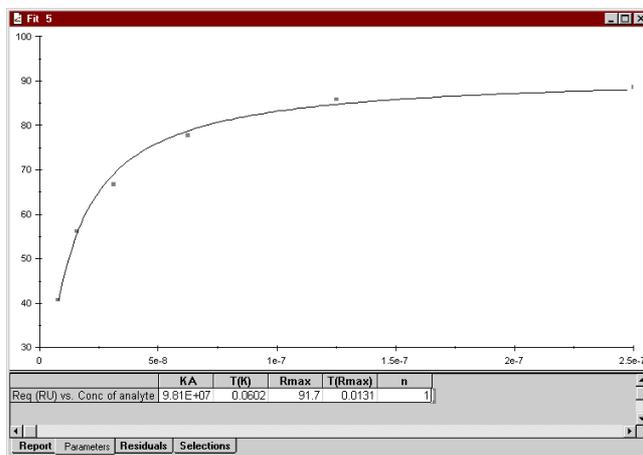
6.3   Evaluating solution affinity data

Measurement of affinity in solution with BIA is based on determination of the free concentration of one of the interactants in equ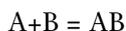ilibrium mixtures. Measurements have to be made on known concentrations of the free interactant for a standard curve as well as on the equilibrium mixtures for determination of affinity.

If the reaction in solution is written as

A+B = AB

the experiment is designed so that a constant concentration of B is mixed with a series of known concentrations of A. BIA is used to measure the remaining free concentration of interactant B. The affinity constant is calculated using the general fitting model **Solution affinity** according to the equation

$$B_{free} = \frac{B - A - K_D}{2} + \sqrt{\frac{(A + B + K_D)^2}{4} - A \cdot B}$$

where B_{free} is the free concentration of component B

            A and B are the total concentrations of A and B respectively

            K_D is the equilibrium dissociation constant for A+B=AB

Follow the procedure below to evaluate solution affinity data. The illustrations are taken from the example data file **SolutionAffinity.ble**:

1.   Obtain the binding data in a spreadsheet window for concentration analysis (see Section 5.1). If the data is derived from curve fitting to sensorgrams, copy the report tab from the fitting window to a sheet. In this example, the standard samples are in the same sheet as the unknowns. Cycles 1 to 11 are measurements on analyte B alone for a calibration curve; cycles 12 to 20 are measurements on 50 nM B mixed with the specified concentrations of A. The data in the file are simulated.

	A	B	C	D	E	F	G	H	I	J	K	L
1	Cycle	Fc	APROG	Time	Window	AbsResp	Slope	Baseline	Id	Conc B(M)	ConcA (M)	
2	1	1	SOLUTION AFFINITY	79.5	20	13437.9	0.00999	No	InitBindRate	1.00E-10	0	
3	2	1	SOLUTION AFFINITY	79.5	20	13437.9	0.01998	No	InitBindRate	2E-010	0	
4	3	1	SOLUTION AFFINITY	79.5	20	13433.5	0.03996	No	InitBindRate	4E-010	0	
5	4	1	SOLUTION AFFINITY	79.5	20	13431.3	0.07992	No	InitBindRate	8E-010	0	
6	5	1	SOLUTION AFFINITY	79.5	20	13427.5	0.15984	No	InitBindRate	1.6E-009	0	
7	6	1	SOLUTION AFFINITY	79.5	20	13427.7	0.31968	No	InitBindRate	3.2E-009	0	
8	7	1	SOLUTION AFFINITY	79.5	20	13424.1	0.63936	No	InitBindRate	6.4E-009	0	
9	8	1	SOLUTION AFFINITY	79.5	20	13425.6	1.27872	No	InitBindRate	1.28E-008	0	
10	9	1	SOLUTION AFFINITY	79.5	20	13422.1	2.55744	No	InitBindRate	2.56E-008	0	
11	10	1	SOLUTION AFFINITY	79.5	20	13423.5	5.11488	No	InitBindRate	5.12E-008	0	
12	11	1	SOLUTION AFFINITY	79.5	20	13420.1	10.22976	No	InitBindRate	1.024E-007	0	
13	12	1	SOLUTION AFFINITY	79.5	20	13422.3	4.83428015	No	InitBindRate	a	2E-009	
14	13	1	SOLUTION AFFINITY	79.5	20	13419.3	4.670540019	No	InitBindRate	b	4E-009	
15	14	1	SOLUTION AFFINITY	79.5	20	13421.3	4.349545417	No	InitBindRate	c	8E-009	
16	15	1	SOLUTION AFFINITY	79.5	20	13418.1	3.737715508	No	InitBindRate	d	1.6E-008	
17	16	1	SOLUTION AFFINITY	79.5	20	13425.6	2.671563338	No	InitBindRate	e	3.2E-008	
18	17	1	SOLUTION AFFINITY	79.5	20	13422.1	1.337715508	No	InitBindRate	f	6.4E-008	
19	18	1	SOLUTION AFFINITY	79.5	20	13423.5	0.535585072	No	InitBindRate	g	1.28E-007	
20	19	1	SOLUTION AFFINITY	79.5	20	13420.1	0.229052543	No	InitBindRate	h	2.56E-007	
21	20	1	SOLUTION AFFINITY	79.5	20	13420.1	0.105695518	No	InitBindRate	i	5.12E-007	

2. Filter the data if necessary and choose **Data:Generate Calibration**. In the example, the calibration curve is a plot of slope against the concentration of B. You do not need to calculate unknowns at this stage.
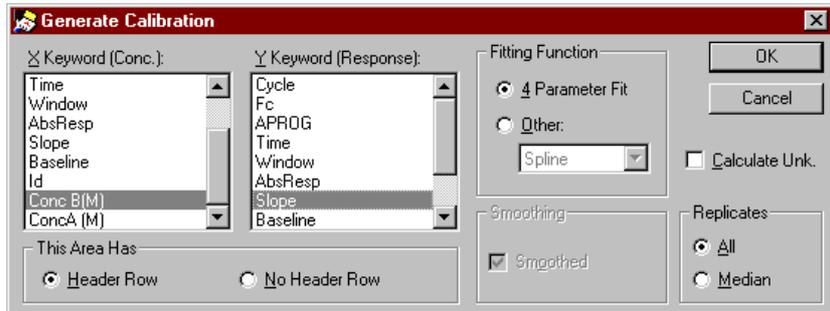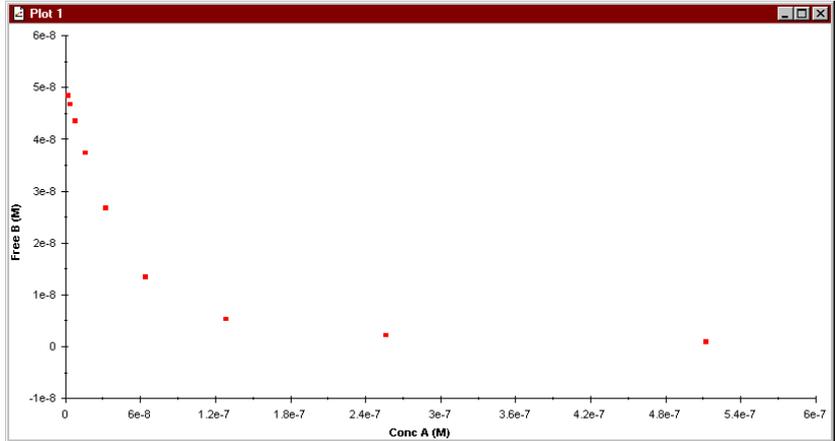

3. Activate the sheet. Choose **Data:Calculate Values** to calculate concentrations of free B in cycles 12-20 using the calibration curve and the values in the **Slope** column. The calculated values will be inserted in the first free column in the sheet. Enter a name for this column in the header row (e.g. **Free B**).

	A	B	C	D	E	F	G	H	I	J	K	L
1	Cycle	Fc	APROG	Time	Window	AbsResp	Slope	Baseline	Id	Conc B(M)	ConcA (M)	Free B (M)
2	1	1	SOLUTION AFFINITY	79.5	20	13437.9	0.00999	No	InitBindRate	1.00E-10	0	1E-010
3	2	1	SOLUTION AFFINITY	79.5	20	13437.9	0.01998	No	InitBindRate	2E-010	0	2E-010
4	3	1	SOLUTION AFFINITY	79.5	20	13433.5	0.03996	No	InitBindRate	4E-010	0	4E-010
5	4	1	SOLUTION AFFINITY	79.5	20	13431.3	0.07992	No	InitBindRate	8E-010	0	8E-010
6	5	1	SOLUTION AFFINITY	79.5	20	13427.5	0.15984	No	InitBindRate	1.6E-009	0	1.6E-009
7	6	1	SOLUTION AFFINITY	79.5	20	13427.7	0.31968	No	InitBindRate	3.2E-009	0	3.2E-009
8	7	1	SOLUTION AFFINITY	79.5	20	13424.1	0.63936	No	InitBindRate	6.4E-009	0	6.4E-009
9	8	1	SOLUTION AFFINITY	79.5	20	13425.6	1.27872	No	InitBindRate	1.28E-008	0	1.28E-008
10	9	1	SOLUTION AFFINITY	79.5	20	13422.5	2.55744	No	InitBindRate	2.56E-008	0	2.56E-008
11	10	1	SOLUTION AFFINITY	79.5	20	13423.5	5.11488	No	InitBindRate	5.12E-008	0	5.12E-008
12	11	1	SOLUTION AFFINITY	79.5	20	13420.1	10.22976	No	InitBindRate	1.024E-007	0	1.02E-007
13	12	1	SOLUTION AFFINITY	79.5	20	13422.3	4.83428015	No	InitBindRate a		2E-009	4.84E-008
14	13	1	SOLUTION AFFINITY	79.5	20	13419.3	4.670540019	No	InitBindRate b		4E-009	4.68E-008
15	14	1	SOLUTION AFFINITY	79.5	20	13421.3	4.349545417	No	InitBindRate c		8E-009	4.35E-008
16	15	1	SOLUTION AFFINITY	79.5	20	13418.1	3.737715508	No	InitBindRate d		1.6E-008	3.74E-008
17	16	1	SOLUTION AFFINITY	79.5	20	13425.6	2.671563338	No	InitBindRate e		3.2E-008	2.67E-008
18	17	1	SOLUTION AFFINITY	79.5	20	13422.1	1.337715508	No	InitBindRate f		6.4E-008	1.34E-008
19	18	1	SOLUTION AFFINITY	79.5	20	13423.5	0.535585072	No	InitBindRate g		1.28E-007	5.36E-009
20	19	1	SOLUTION AFFINITY	79.5	20	13420.1	0.229052543	No	InitBindRate h		2.56E-007	2.29E-009
21	20	1	SOLUTION AFFINITY	79.5	20	13420.1	0.105695518	No	InitBindRate i		5.12E-007	1.06E-009
22												
23												

4. Select cycles 12-20 in the sheet and choose **Data:Create Curve**. Use **Free B** as the Y-axis and **Conc A** as the X-axis. Check **Selected rows only** so that the points from the calibration curve are not included. (This is an alternative to filtering the data so that only the desired rows are displayed.)

5. Click on the **Fit:General** button and fit the curve to the solution affinity model. Enter a value of 50n for parameter B (the total concentration of B in the mixtures). The calculated affinity constant is reported in the fitting results.
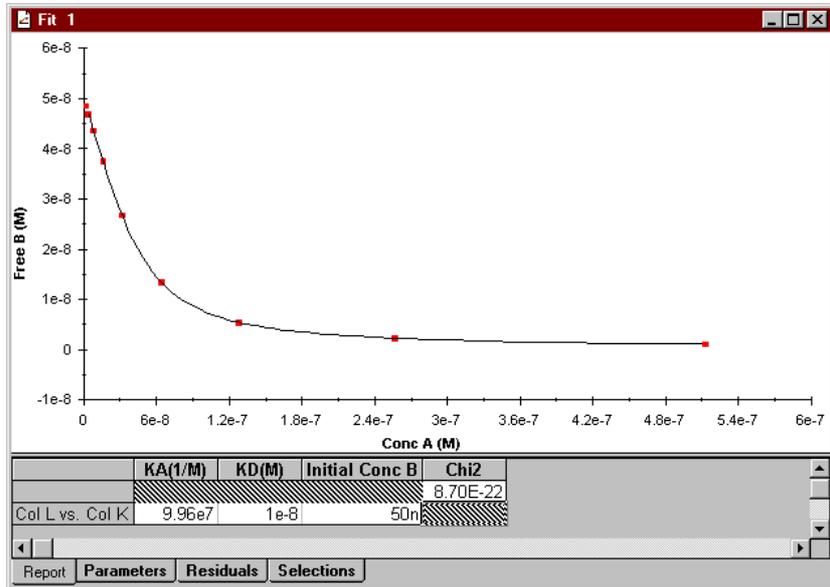
7. BIAevaluation Software reference

This chapter provides a full reference to BIAevaluation software. Since the menu options vary according to the currently active window, the menu descriptions are organized by window type:

- project window   Section 7A
- plot window   Section 7B
- fit window   Section 7C
- sheet window   Section 7D
- calibration window   Section 7E

7.1   Starting BIAevaluation

BIAevaluation 3.0 is installed as a shortcut in the **BIA Programs** section of the Windows 95 Start menu.
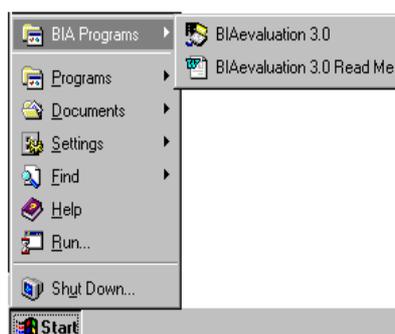
Starting from this shortcut opens BIAevaluation with an empty project window.

BIAevaluation can also be started from a toolbar button in Windows 95-compatible BIACORE Control Software. See the respective BIACORE Instrument Manual for details.

7.2   User interface

BIAevaluation uses the standard Windows 95 graphical environment
with enhanced mouse functionality.

7.2.1   *Mouse functions*

The following tables summarize the mouse functions in the various
windows in the BIAevaluation workspace:

Project window

Left mouse button	Click to select one curve. Drag or shift-click to select multiple adjacent curves. Ctrl-click to select multiple non-adjacent curves. Double-click to set curve properties.

All graph windows

Right mouse button	Click for context menu.

Plot window

Left mouse button	Click on a curve to activate the curve. Double-click on a curve to change curve properties. Double-click on an axis to change scale. Double-click on labels or legend to change. Double-click on a blank area to unzoom. Drag to zoom. Drag on reference line to move reference line.
Right mouse button	Click for context menu. Drag to select data.

Fitting window

Left mouse button	Click on a curve to activate the curve.
	Double-click on a curve to change curve properties. Double-click on an axis to change scale. Double-click on labels or legend to change. Double-click on a blank area to unzoom. Drag to zoom. Drag on reference line to move reference line.
Right mouse button	Click for context menu.

Sheet window

Left mouse button	Click to select one cell. Drag or shift-click to select multiple cells.

Calibration window

Left mouse button	Double-click on an axis to change scale. Double-click on labels or legend to change. Double-click on a blank area to unzoom. Drag to zoom.
Right mouse button	Click for context menu.

7.2.2 Entering numerical data

In fitting data to models, numerical input is frequently required for concentrations, constant values etc. The following shorthand may be used for numerical input:

Suffix	Meaning	Normally entered as
a	10^{-18}	e-18
f	10^{-15}	e-15
p	10^{-12}	e-12
n	10^{-9}	e-9
u	10^{-6}	e-6
m	10^{-3}	e-3
k	10^{3}	e3
M	10^{6}	e6
G	10^{9}	e9
T	10^{12}	e12

The shorthand letter must follow the value directly, with no separating space. The case of shorthand letters is significant (note particularly that m means milli and M means mega!).

Values can also be entered in standard exponential format. For example, a concentration of 30 nM may be entered as 3e-8 or as 30n.

This shorthand for numerical values does not apply to the sheet window.

7A The project window

7A.1 Toolbar buttons

The following toolbar buttons are available when a project window is active. To display a tool tip with a reminder of the button function, place the cursor on the button and wait for a short time.

[icon]	**File:Open**	Section 7A.2.3
[icon]	**File:Save**	Section 7A.2.4
[icon]	**View:Overlay Plot**	Section 7A.4.2 (Only available when curves are selected.)

## 7A.2 The File menu (project window)
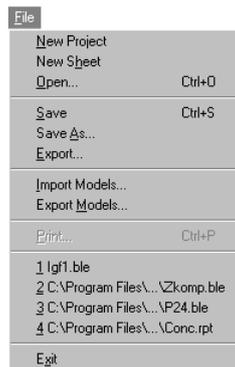
7A.2.1 *New project*

Closes the current project and starts a new project. Before closing, you will be asked if you want to save changes in the current project.

7A.2.2 *New sheet*

Opens a new empty sheet window.

7A.2.3 *Open (Ctrl-O)* [icon]

Opens a file, either replacing the current project or adding curves, sheets or calibration windows to the current project.
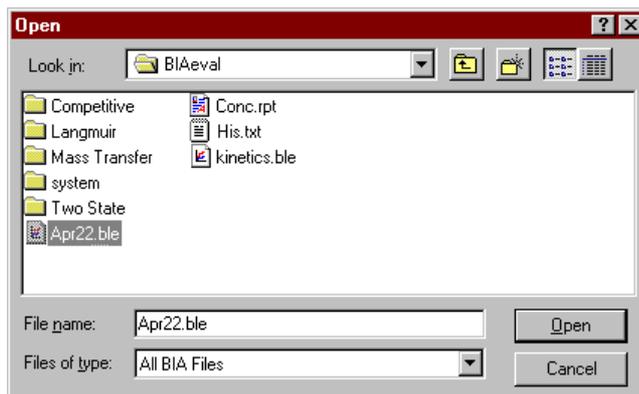
You can open the following types of files in BIAevaluation:

• previously saved BIAevaluation projects, including BIAevaluation version 2.x (extension .ble)

• result files from BIACORE and BIAlite (extensions .blr, .blc, .bls, .blf)

• report point tables from BIACORE (extension .rpt) or concentration evaluation files from BIAevaluation version 2.x (extension .clb)

• text files containing tab-separated data (default extension .txt)

See Section Section 3.1 for details of opening different types of files.

7A.2.4   Save (Ctrl-S) 🖫
Saves the current evaluation project. If the project has previously been saved, the previous file is overwritten. If the project is untitled (i.e. has not previously been saved), the **Save** command is equivalent to **Save As** (see below).

7A.2.5   Save As
Saves the current evaluation project under a new file name. Enter a file name in the **File name** field and change the directory if required in the **Directories** list (see the Windows User Guide for more information on saving application files).
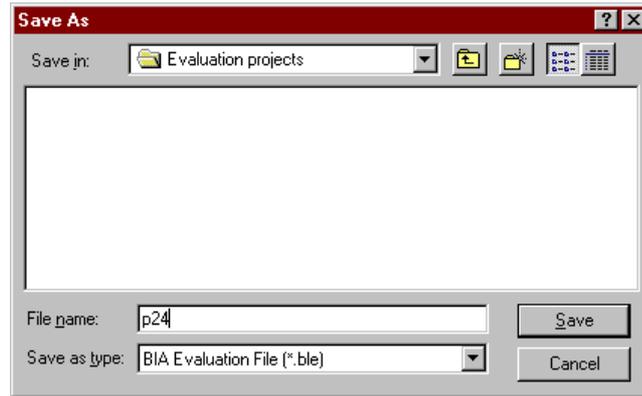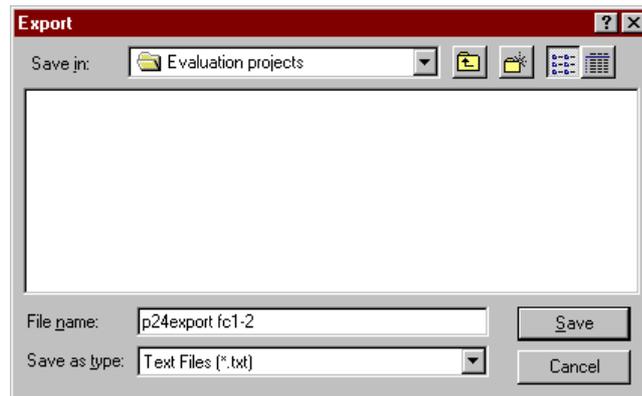
Do not specify a file name extension. The default extension will be used (*.ble).

Projects can only be saved in BIAevaluation file format. Use **File:Export** to save files in other formats.

7A.2.6   Export
Exports selected curves to text files. Specify a file name (default extension .txt) and change the directory if required.


Data is exported to tab-separated text files which may be opened in BIAevaluation or in all standard spreadsheet and presentation software packages.

7A.2.7 Import Models

Imports evaluation models from BIAevaluation model files (extension
.mdl). This function is only available from the project window.
Choose the file from which to import models:
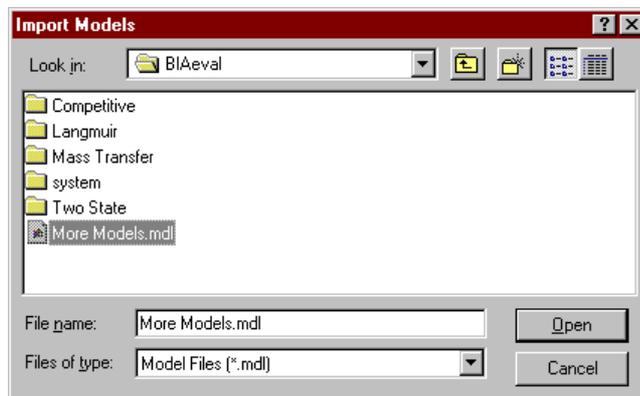
In the dialogue box, mark the models you wish to import in the
Models list and click on **Add=>**. To remove models from the list which
will be imported, mark models in the **Import** list and click on **Remove**.
The **Clear** button removes all models from the **Import** list. Click on the
Import button to import models in the **Import** list.
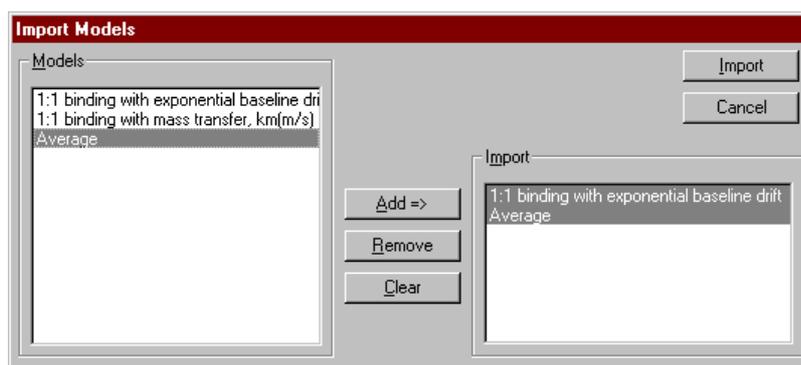
Note: Models are stored in proprietary format in BIAevaluation
        model files. The main use of **Import Models** is to move
        customized models between installations and to restore
        customized models after installation of software upgrades (see
        Chapter 2).

        Models can be imported to BIAevaluation version 3.0 from
        BIAevaluation version 2.2 but not from earlier versions.

7A.2.8   Export Models

Exports model definitions to a proprietary file format (extension .mdl). This function is only available from the project window. Use this function together with **File:Import Models** to transfer customized models between BIAevaluation installations.

Select the models you wish to export in the dialogue box. Mark the models you wish to export in the **Models** list and click on **Add=>**. To remove models from the list which will be exported, mark models in the **Export** list and click on **Remove**. The **Clear** button removes all models from the **Export** list. Click on the **Export** button to export models in the **Export** list, and specify the file name for the exported models in the dialogue box that follows.
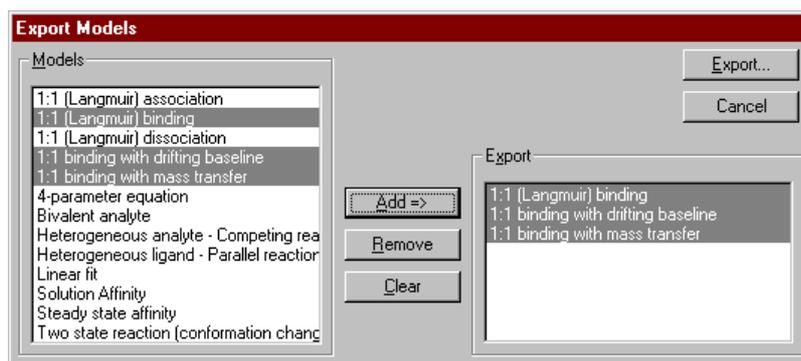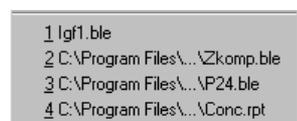

Note: This dialogue box lists all models currently loaded in BIAevaluation, regardless of type.

7A.2.9   Print (Ctrl-P)

Print is not available in the project window.

7A2.10   Recent file list

The **File** menu lists the four most recently opened files. Click on a file to open it.

7A.2.11 Exit

Closes BIAevaluation. If the current project has not been saved since the last change, you will be asked if you want to save it.

7A.3 The Edit menu (project window)

7A.3.1 Cut (Ctrl-X)

Cuts selected curve(s) from the current project and places them on the BIAevaluation clipboard.

Curves which have been cut from a project window can be pasted from the clipboard into other project or plot windows.

Note: Curves placed on the BIAevaluation clipboard can only be pasted into BIAevaluation project, plot or sheet windows. Use **Copy Graph** from the **Edit** menu or the right mouse button menu to place curves on the Windows clipboard for pasting as graphic objects into other programs.

7A.3.2 Copy (Ctrl-C)

Copy has the same function as **Cut** with the exception that the selected curve or data is not removed from the window from which the operation is performed.

7A.3.3 Paste (Ctrl-V)

Pastes the contents of the BIAevaluation clipboard into the current project as a new curve. The command is only available after curve data has been copied or cut from a project or plot window.

Data remains in the BIAevaluation clipboard after a **Paste** operation and can be pasted as many times as required into new windows.

7A.3.4 Remove (Del)

Deletes selected curve(s) from the project. Select the curves to delete, then choose **Edit:Remove**. Confirm the deletion in the dialogue box.

The removed curves are not placed in the BIAevaluation clipboard (compare **Edit:Cut**).

7A.3.5   Curve Properties

Sets the properties of a selected curve for display in the plot and fitting windows. The properties are:

- curve name
- line type (solid or none)
- colour
- marker type
- marker size
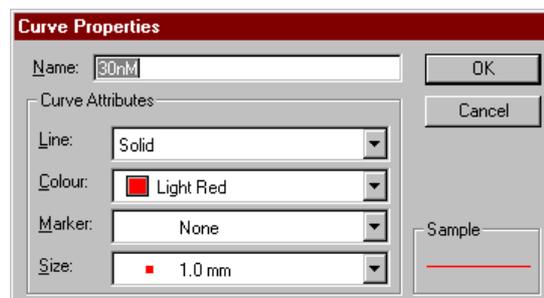
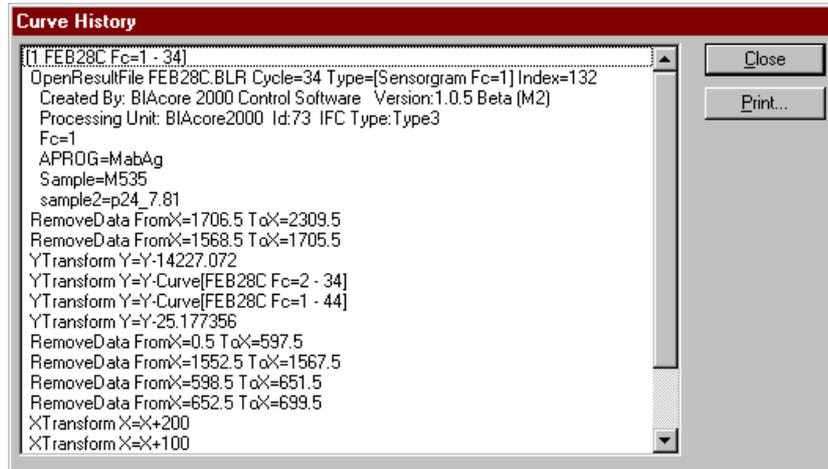The command operates on the currently selected curve, or on the first selected curve if more than one is selected.

Double-click on a curve as a short-cut to **Edit:Curve Properties**.

7A.4 The View menu (project window)

7A.4.1   Curve History
Displays the history of selected curve(s), listing all manipulations that have been performed on the curve.

```
Curve History
┌──────────────────────────────────────────────────────────┐
│ [1 FEB28C Fc=1 - 34]                                    ▲ │   ┌─────────┐
│ OpenResultFile FEB28C.BLR Cycle=34 Type=[Sensorgram Fc=1]  │   │  Close  │
│   Index=132                                                │   └─────────┘
│   Created By: BIAcore 2000 Control Software  Version:1.0.5 │   ┌─────────┐
│     Beta (M2)                                              │   │  Print..│
│   Processing Unit: BIAcore2000  Id:73  IFC Type:Type3      │   └─────────┘
│   Fc=1                                                     │
│   APROG=MabAg                                              │
│   Sample=M535                                              │
│   sample2=p24_7.81                                         │
│ RemoveData FromX=1706.5 ToX=2309.5                         │
│ RemoveData FromX=1568.5 ToX=1705.5                         │
│ YTransform Y=Y-14227.072                                   │
│ YTransform Y=Y-Curve[FEB28C Fc=2 - 34]                     │
│ YTransform Y=Y-Curve[FEB28C Fc=1 - 44]                     │
│ YTransform Y=Y-25.177356                                   │
│ RemoveData FromX=0.5 ToX=597.5                             │
│ RemoveData FromX=1552.5 ToX=1567.5                         │
│ RemoveData FromX=598.5 ToX=651.5                           │
│ RemoveData FromX=652.5 ToX=699.5                           │
│ XTransform X=X+200                                         │
│ XTransform X=X+100                                       ▼ │
└──────────────────────────────────────────────────────────┘
```

Click on **Print** to print a copy of the curve history.

7A.4.2   Overlay plot
Opens a plot window containing the selected curves.

7A.5 The Fit menu (project window)
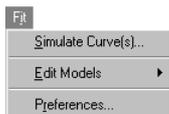
7A.5.1   Simulate Curve(s)
Simulates curves according to a chosen model with specified parameter values. See Section 4.7 for a description of curve simulation.

When curves are simulated from the project window, default values are suggested for the number and duration of the curves. The kinetic parameters for simulation are blank. To simulate curves using values obtained from sensorgram fitting, choose **Fit:Simulate Curve(s)** from the fit window.
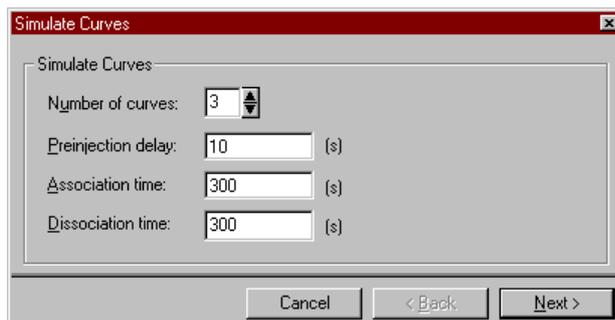
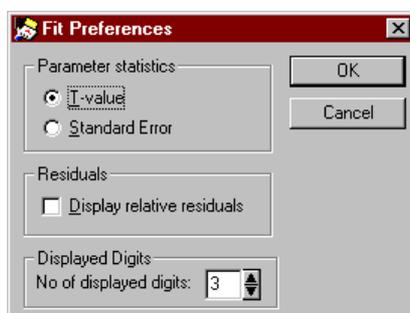*Default number and duration of curves for simulation.*

7A.5.2 Edit Models

Opens the model editor for editing and creating models. Choose the type of model from the cascade menu:

See Appendix B for a full description of the model editor.

7A.5.3 Preferences

Sets preferences for display of fitting results.

Parameter statistics

Select **T-value** or **Standard Error**. The value for a given parameter provides an indication of how sensitive the curve fit is to changes in the parameter (see Section A.5.2). The standard error is expressed in the same units as the parameter. The T-value is a relative measure of

the standard error obtained by dividing the value of the parameter by the standard error. The default setting is **T-value**.

Residuals
Check the **Display relative residuals** box to display residuals as a fraction of the response value at each point. If this box is not checked, residuals are displayed in RU. The default setting is unchecked (i.e. display absolute residuals).

Displayed digits
Choose the number of digits (maximum 9) to display for numerical fitting results. Results are displayed in exponential format (e.g. 1.2E4 for 12,000) if the number of digits required for the number exceed the number selected for display. The default setting is 3.

7A.6   The Window menu (project window)

7A.6.1   Cascade
Arranges the currently open windows in an overlapping stack with the active window at the top of the stack:

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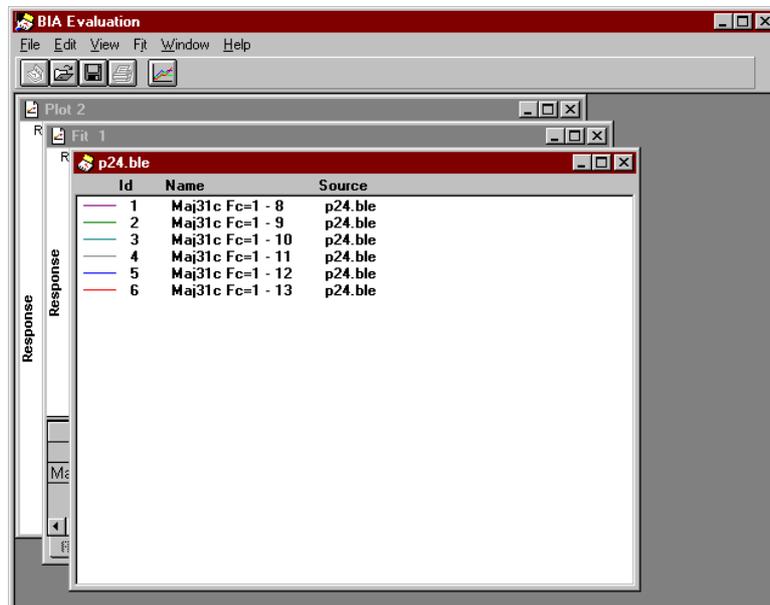
7A.6.2   Tile Horizontal
Arranges the currently open windows horizontally:
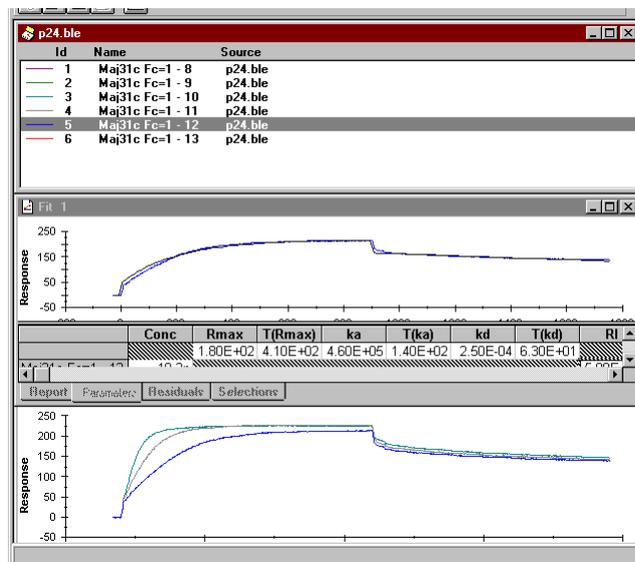
If there are more than three windows open, the windows are tiled both horizontally and vertically to make the best use of the workspace area.

7A.6.3   Tile Vertical
Arranges the currently open windows vertically:
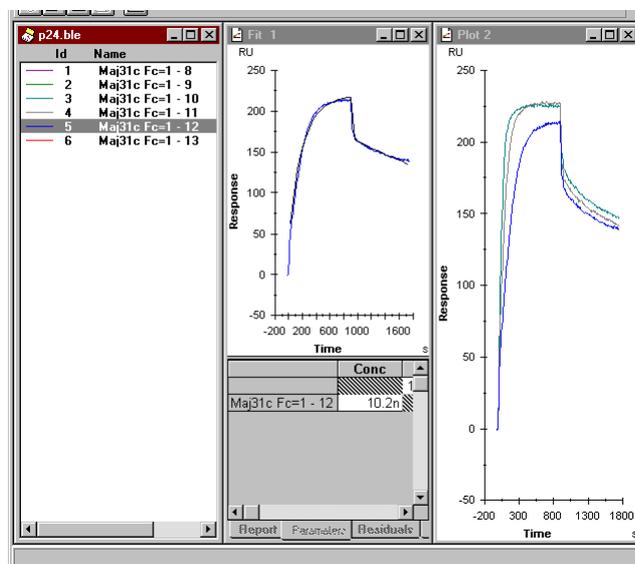
If there are more than three windows open, the windows are tiled both vertically and horizontally to make the best use of the workspace.
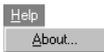
7A.6.4   Arrange icons

Arranges the icons for any minimized windows at the bottom of the workspace. This command has no effect on windows that are not minimized.

7A.6.5   Open window list

The lower part of the **Window** menu is a numbered list of all open windows. Click on a window in this list to activate it.

7A.7   The Help menu (project window)

Help
About...

7A.7.1   About

Displays an information box with the current version of BIAevaluation and registration details:

About BIAevaluation

BIAevaluation
Version: 3.0

Copyright © 1994-1997
Biacore AB

This software is licensed to:
BIACORE AB

BIACORE   Serial number: EVCO-0001-B2

OK

7B The plot window

7B.1 Toolbar buttons

The following toolbar buttons are available when a plot window is active. To display a tool tip with a reminder of the button function, place the cursor on the button and wait for a short time.

	–	Go to the project window
	File:Open	Section 7A.2.3
	File:Save	Section 7A.2.4
	File:Print	Section 7B.2.7
	View:Overlay	Section 7B.4.10
	View:Reference Line	Section 7B.4.8
	Fit:Kinetics Simultaneous ka/kd	Section 7B.6.2
	Fit:Kinetics Separate ka/kd	Section 7B.6.3
	Fit:General	Section 7B.6.4

In addition, the toolbar includes the following selection boxes:

Curve list	Curve: ── 4 FEB28C Fc=1 - 38	Selects the active curve

7B.2   The File menu (plot window)
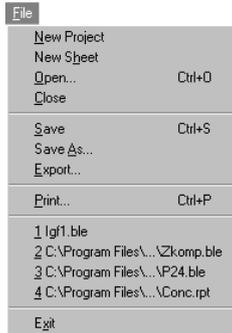
7B.2.1   New project
Closes the current project and starts a new project. Before closing, you will be asked if you want to save changes in the current project.

7B.2.2   New sheet
Opens a new empty sheet window.

7B.2.3   Open (Ctrl-O)
Opens a file. See Section 7A.2.3.

7B.2.4   Save (Ctrl-S)
Saves the current evaluation project. See Section 7A.2.4.

7B.2.5   Save As
Saves the current evaluation project under a new file name. See Section 7A.2.5.

7B.2.6   Export
Exports all curves in the current plot window to text files. If parts of curves are selected, the selected data is exported for all curves.

7B.2.7   Print (Ctrl-P)
Prints the contents of the current plot window. Choose printing options from the dialogue box:
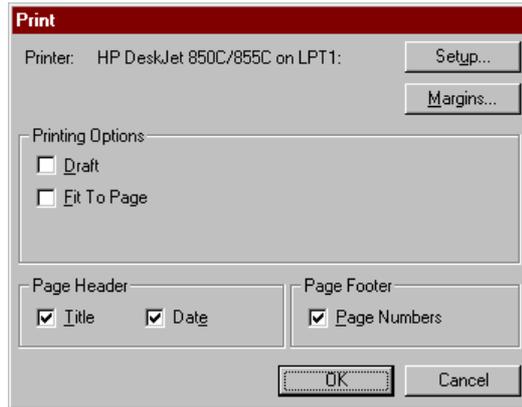
Check **Draft** for faster printing. On some printers this may produce a lower quality printout.

Check **Fit To Page** to fit the graph to within the page margins. If this box is not checked, the graph will be printed with the same proportions as the screen display.

In the **Page Header**, the name of the currently active curve (as displayed in the curve list box) is used as the title. The date refers to the date of printing.

Click on **Setup** to choose the printer and page orientation. This opens the standard Windows printer setup dialogue, which differs according to the type of printer connected. The printer settings defined in the Windows Control Panel apply if settings are not changed in BIAevaluation.

Click on **Margins** to set the page margins.

7B.2.8   Recent file list
The **File** menu lists the four most recently opened files. Click on a file to open it.

```
1 lgf1.ble
2 C:\Program Files\...\Zkomp.ble
3 C:\Program Files\...\P24.ble
4 C:\Program Files\...\Conc.rpt
```
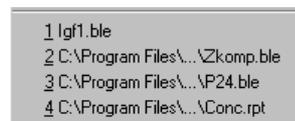
7B.2.9   Exit
Closes BIAevaluation.

7B.3 The Edit menu (plot window)
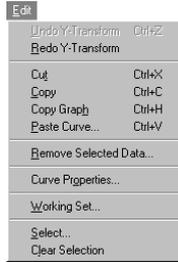
7B.3.1   Undo <command> (Ctrl-Z)

Reverses the most recent editing operation in the current session. The operation that will be undone is identified in the menu (e.g. after a **Y-Transform** operation the command appears as **Undo Y-Transform**).

7B.3.2   Redo <command>

Reverses the effect of an **Undo** command. The operation which will be redone is identified in the command (e.g. after a **Y-Transform** operation the command appears as **Redo Y-Transform**). **Redo** is available until the next editing operation is performed.

7B.3.3   Cut (Ctrl-X)

Cuts selected data from the curves in the plot window and places the data in the BIAevaluation clipboard. All curves currently displayed in the window are affected. To cut data from one curve only, press the **Overlay** button to switch to single plot mode.

The data can be pasted into the same or a different plot window or a project window as curves and into a sheet window as numerical data.

Note: The BIAevaluation clipboard is distinct from the general Windows clipboard, and its contents can only be pasted into BIAevaluation project or plot windows.

7B.3.4   Copy (Ctrl-C)

Copy has the same function as **Cut** with the exception that the selected curve or data is not removed from the window from which the operation is performed.

7B.3.5   Copy Graph (Ctrl-H)

Copies the currently displayed plot to the Windows clipboard, from which it can be pasted as a graphic into other applications such as word processing or presentation software. The graph is copied in Windows clipboard format.

Note: Do not confuse **Copy Graph**, which copies the whole graph to the Windows clipboard, with **Copy**, which copies selected data to the internal BIAevaluation clipboard.

7B.3.6   *Paste Curve (Ctrl-V)*

Pastes the contents of the BIAevaluation clipboard into the current plot window as a new curve. The command is only available after curve data has been copied or cut from a project or plot window.

Select the position of the curve to be pasted from the dialogue box:
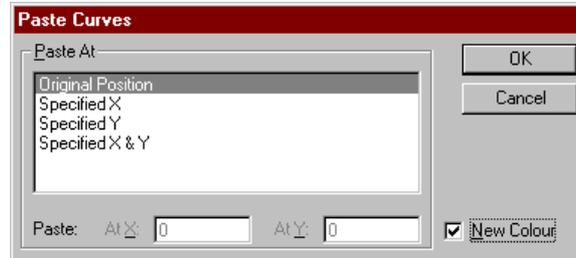
Original position

The curve will be pasted at the x- and y-coordinates from which it was cut or copied.

Specified position

The curve will be pasted with the first data point at the coordinates specified. If **SpecifiedX** or **SpecifiedY** is chosen, the original position will be used for the unspecified coordinate.

If the **New Colour** box is checked, the pasted curve will be assigned a new colour. Otherwise, the original colour will be used. Checking this box makes it easier to distinguish the pasted curve in an overlay plot window.

Note: The curve is always pasted as a separate curve. If you cut a section from a curve and then paste the section at the original position in the same window, you will get an apparently continuous curve (particularly if **New Colour** is not checked). The curve consists however of two sections.

7B.3.7   **Remove Selected Data**

Deletes selected data from one or more curves in the plot window. Choose whether to fill the removed section with an interpolated straight line or to make the data points invalid:

Remove Selected Data

Type
- ○ Remove Data Points
- ● Make Data Points Invalid

☑ Apply to Entire Working Set

[OK]
[Cancel]
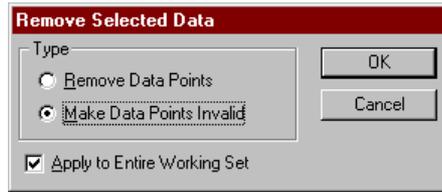
If you choose to make the data points invalid, the curve will appear discontinuous. It is however still a single curve, and you can include part or all of the invalid data in a selection for fitting purposes.

Choosing **Edit:Remove Selected Data** and **Make Data Points Invalid** is analogous to **Edit:Cut** in the plot window, with the exception that the removed data is not placed in the BIAevaluation clipboard.

7B.3.8   Curve properties
Sets the properties of a selected curve for display in the plot and fitting windows. See Section 3.2.2.

7B.3.9   Working Set
Edits the contents of the current working set, allowing you to add and remove curves. Curves that can be added are those contained in the current project but not included in the working set. Removing curves from the working set does not remove them from the project.

Edit Working Set

Available Curves in Project:
```
1  Maj31c Fc=1 - 8
2  Maj31c Fc=1 - 9
6  Maj31c Fc=1 - 13
7  Maj31c Fc=1 - 10
8  Maj31c Fc=1 - 11
9  Maj31c Fc=1 - 12
10 Maj31c Fc=1 - 10
11 Maj31c Fc=1 - 11
12 Maj31c Fc=1 - 12
```

[Add =>]
[<= Remove]
[Clear]

Curves in Working Set
```
3  Maj31c Fc=1 - 10
4  Maj31c Fc=1 - 11
5  Maj31c Fc=1 - 12
```

[OK]
[Cancel]
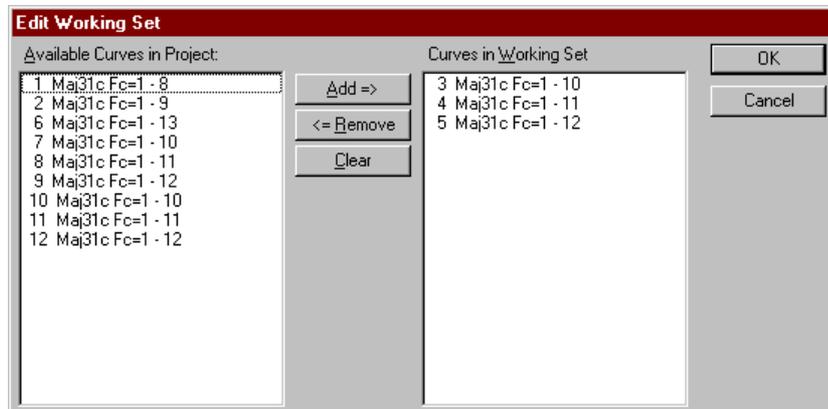
To add curves, select the required curves in the **Available Curves in Project** panel and click on **Add->**. To remove curves, select curves in the **Curves in Working Set** panel and click on **<-Remove**. Click on **Clear** to remove all curves from the working set.

7B.3.10   Select
Selects a section of either the current curve or all curves in the working set, specified by coordinates on the x-axis.

Enter the limits of the required selection in the **From** and **To** fields. Check **Apply to Entire Working Set** if you want the selection to apply to all curves in the current working set. If this box is not checked, the selection will only apply to the currently active curve (as identified in the curve list on the toolbar). Click on **OK** to make the selection.

You can also select data by dragging with the right mouse button. Use **Edit:Select** if you want to select between known x-coordinates. Drag with the mouse if you want to set or adjust the selection limits visually.

If a section of one or more curves is currently selected, the selection limits for the active curve are entered by default in the dialogue box. Click on **Remove** to clear the selection.

Notes: If **Apply to Entire Working Set** is checked when you click on **Remove**, the selections will be cleared from all curves regardless of the values entered in the **From** and **To** boxes.

If either of the requested selection limits is invalid for any curve in the working set (e.g. lies outside the limits of the curve), the actual limit is adjusted to the nearest valid point on the curve. If both limits are invalid, no selection is made.

7B.3.11   Clear Selection
Clears selections from all curves in the plot window. Choosing this command has the same effect as choosing **Edit:Select**, checking the **Apply to Entire Working Set** box then clicking on **Remove.**

7B.4   The View menu (plot window)

7B.4.1   Scale

Sets the scale for the plot in the current window. If **Auto-X** or **Auto-Y** is checked, the scale for the respective axis will be determined automatically from the limits of the curve data. The default setting is **Auto**.
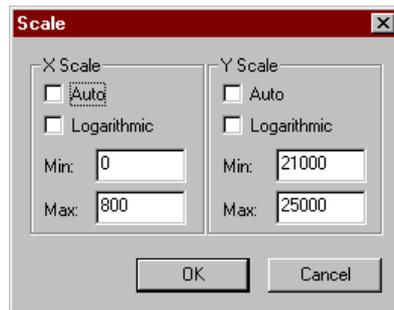
You can also choose **View:Scale** by double-clicking on the x- or y-axis in the window.

Note: Minimum and maximum values cannot be specified as negative or zero values for axes with a logarithmic scale.

7B.4.2   Adjust scale

Sets the scale of the x- and y-axis according to the limits of the data for curves in the window. This operation is equivalent to choosing **View:Scale** and checking the **Auto-X** or **Auto-Y** boxes.

7B.4.3   Unzoom (Ctrl-U)

Restores the previous display after a **Zoom** operation.

To zoom the display, drag with the left mouse button over the area to be enlarged. Consecutive **Zoom** operations are restored in reversed sequence by repeatedly choosing **View:Unzoom** or double-clicking with the left mouse button in the window.

7B.4.4   Gridlines

Displays gridlines in the current window. Major and minor gridlines can be set independently for the x- and y-axes.
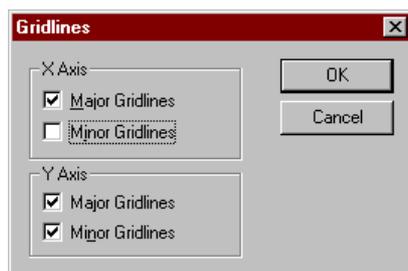
Major gridlines appear on numbered axis marks. Minor gridlines appear on intervening (unnumbered) axis marks. The frequency of axis marks is set automatically according to the scale of the display.

Note: Setting minor gridlines also displays major gridlines.

7B.4.5   Labels
Sets a title and axis labels for the plot or fitting window. Auto-labels for the axes are taken from the header in the data file, and are **Time (s)** and **Response (RU)** for BIA files. The default title is blank.
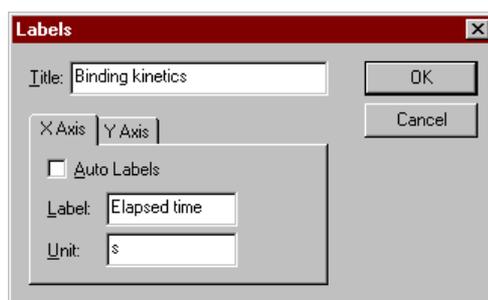
7B.4.6   Legend
Displays a legend identifying the curves in the plot window. Curves are identified by a coloured line with the name of the curve as it appears in the project window. The name can be changed with the **Edit:Curve properties** command.

Choose the placing of the legend from the dialogue box. The legend will be placed outside the plot area. Choose **Hidden** to remove the legend.
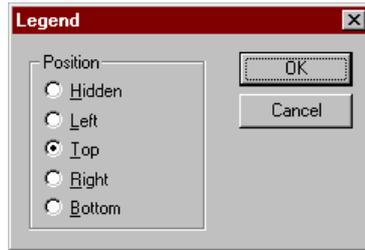
7B.4.7 Curve History

Displays the history of all the curves in the plot window. See Section 7A.4.1 for details.

7B.4.8 Reference Line

Displays horizontal and vertical reference lines, with their intersection on the currently active curve. The coordinates of the intersection are shown on the status line at the bottom of the screen. As you move the vertical reference line by dragging with the mouse, the horizontal line follows the y-coordinate of the current curve.

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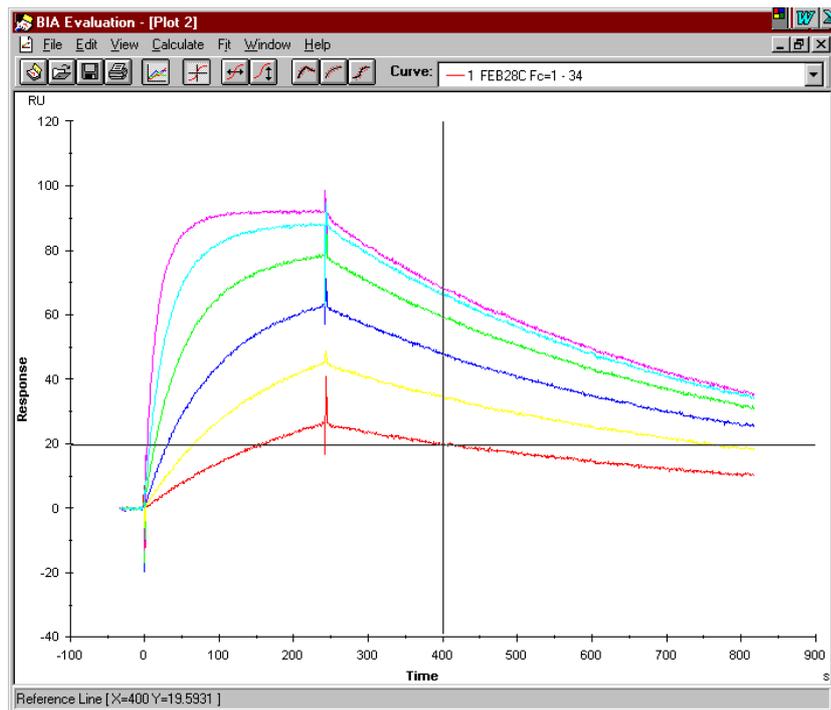
7B.4.9   Split View

Displays the current plot in a split view, with the curves in the upper panel and a selected function of the curves in the lower panel. The panels are coordinated so that zoom, select and reference line operations affect both panels simultaneously. It is usually easier to interpret split view display in single plot mode.
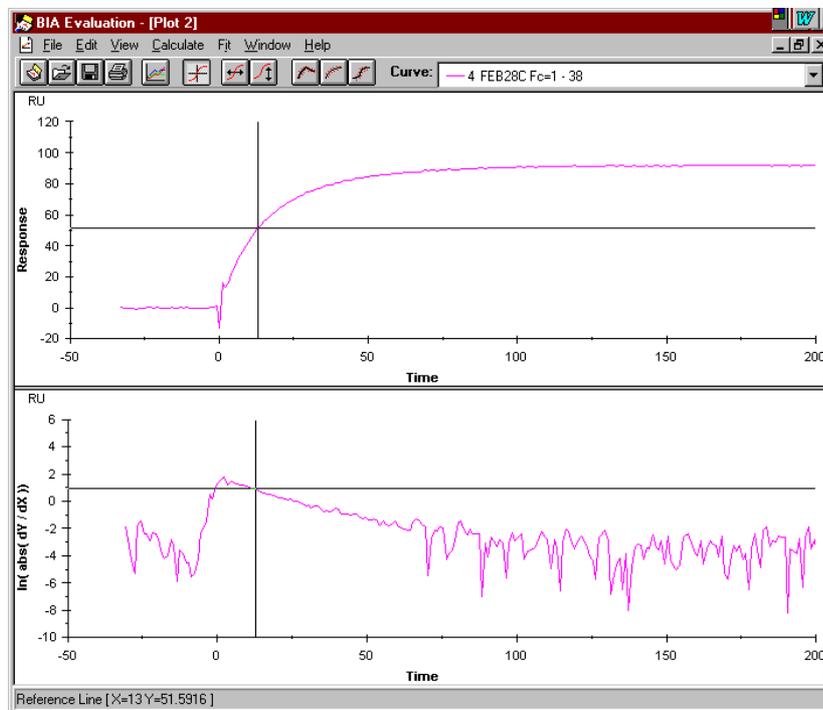
Choose the curve function to display in the lower panel from the dialogue box:
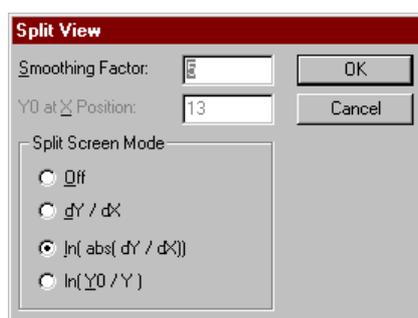
Off   Closes split view mode.

dY/dX   Derivative plot. Enter the time window over which the derivative is to be calculated in the **Smoothing Factor** field. A suitable value for BIA sensorgrams is usually *5*.

A value of 0 will simply plot the difference between each point and the preceding point.

ln(abs(dY/dX)) Logarithmic plot of the absolute value of the derivative. A logarithmic plot emphasises small values, so that signal noise is more readily apparent. Note that the logarithmic plot does not distinguish between positive and negative slopes in the curves. The smoothing factor applies as described for **dY/dX** above.

ln(Y0/Y) Logarithmic plot of the quotient of a chosen starting point (Y0) and the y-values (Y). Enter the x-coordinate of the starting point in the **Y0 at X position** field. If the reference line is displayed when split view is chosen, the coordinate of the reference line is entered by default in this field. Use this plot in assessing data for use in curve fitting (see Section 4.1).

Important: If the Y0/Y quotient is zero or negative, no data is plotted in the ln(Y0/Y) panel of the split view. Do not use a starting point with a negative y-value unless the section of interest in the curve also has negative y-values.

7B.4.10   Overlay
Toggles the display between overlay and single plot mode.

7B.5   The Calculate menu (plot window)

7B.5.1   Y-Transform
Transforms one or more plots in the y-direction. The transformation operates either on the selected curve or on the entire working set.

After selecting the transformation to perform, press **Replace Original** to replace the original curve in the project with the transformed curve, or press **Add As New** to add the transformed curve to the project.
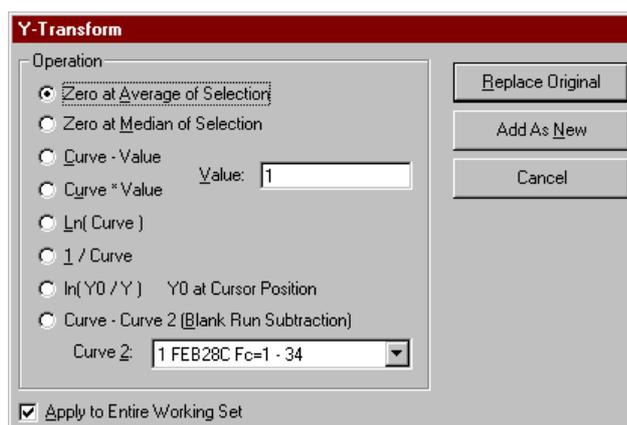
Zero at Average/Median of Selection
Sets the average or median respectively of the selected region to zero
and shifts the curve accordingly. This transformation is not available if
no data is selected. If **Apply to Entire Working Set** is checked, the zero
value is set separately for each curve. Use one of these functions to
correct for differences in absolute baseline values between different
curves (see Section 3.3.2).

Curve - Value
Shifts the curve(s) by subtracting a fixed number from the y-value at
each data point. Specify the amount of the shift in the **Value** field. To
add a fixed number, enter a negative number in **Value**.

Curve * Value
Scales the curve(s) by a fixed factor in the y-direction. Specify the
factor in the **Value** field. (Do not confuse this function with **View:Scale**
which scales the display without affecting the data.)

ln(Curve)
Creates a logarithmic plot by taking the natural logarithm of the
y-value at each data point.

1/Curve
Creates an inverse plot by taking the reciprocal of the y-value at each
data point.

ln(Y0/Y)
Transforms each data point to the value ln(Y0/Y), where Y0 is the
y-value at the reference line and Y is the y-value at the data point. If
the quotient Y0/Y is negative for a given data point, the corresponding

transformed point is omitted from the resulting plot. This transformation is only available if the reference line is displayed (see **View:Reference Line**).

This plot can also be displayed in a split view window (see **View:Split View**).

Curve - Curve 2
Creates a difference plot by subtracting the y-value of another specified curve from the y-value of the curve being transformed for each data point. Choose curve 2 from the list box. Any curve in the current project may be used as curve 2, even if it is not included in the current working set.

7B.5.2   X-Transform

Transforms one or more plots in the x-direction. The transformation operates either on the selected curve or on the entire working set.

After selecting the transformation to perform, press **Next** to go to the next step in the transformation (for **Curve Alignment**) or **Finish** to complete the transformation for other operations.
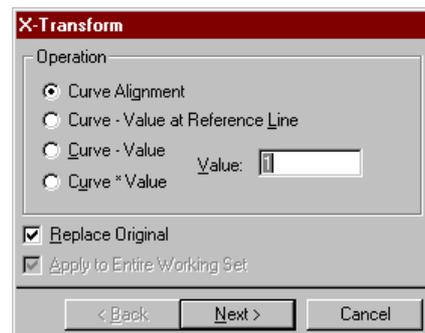
Curve Alignment
Aligns curves in the x-direction to selected alignment points. See Section 3.3.3 for details.

Curve - Value at Reference Line
Shifts the curve(s) by subtracting the x-value at the reference line from the x-value at each data point. This transformation is only available if the reference line is displayed (see **View:Reference Line**).

Curve - Value
Shifts the curve(s) by subtracting a fixed number from the x-value at each data point. Specify the amount of the shift in the **Value** field. To add a fixed number, enter a negative number in **Value**.

Curve * Value
Scales the curve(s) by a fixed factor in the x-direction. Specify the factor in the **Value** field. (Do not confuse this function with **View:Scale** which scales the display without affecting the data.)

7B.5.3 Derivatize
Creates a derivative curve. The transformation operates either on the selected curve or on the entire working set.

After selecting the transformation to perform, press **Replace Original** to replace the original curve in the project with the transformed curve, or press **Add As New** to add the transformed curve to the project.
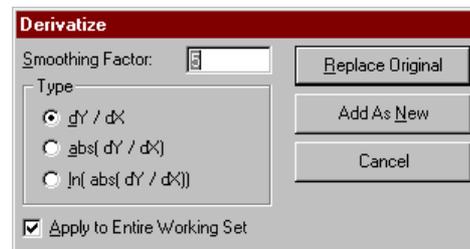
The **Smoothing factor** is the window in x-axis units over which the derivative will be calculated. A suitable value for BIA sensorgrams is usually 5. A value of 0 will simply plot the difference between each point and the preceding point.

dY/dX
Plots the first order derivative. This function can also be displayed in split view mode (Section 5.4.11).

abs(dY/dX)
Plots the absolute value of the first order derivative. This transformation does not distinguish between positive and negative slopes in the original curve.

ln(abs(dY/dX))
Plots the natural logarithm of the absolute value of the first order derivative. This transformation does not distinguish between positive and negative slopes in the original curve, and visually emphasises low slope values, so that the transformed plot tends to appear "noisier".

This function can also be displayed in split view mode (Section 5.4.11).

7B.5.4   *Normalize*

Normalizes the curve along one or both axes, so that the curve limits are adjusted to the specified maximum and minimum values.

After selecting the transformation to perform, press **Replace Original** to replace the original curve in the project with the transformed curve, or press **Add As New** to add the transformed curve to the project.
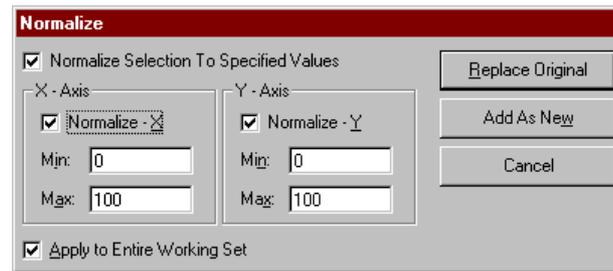
Check the direction(s) in which the normalization is to be performed and enter the required minimum and maximum values for the normalization in the respective fields.

If a region of the curve is selected, **Normalize Selection To Specified Values** may be checked so that the limits of the selected region are adjusted to the specified maximum and minimum values. The rest of the curve is also transformed accordingly.

If **Apply to Entire Working Set** is checked, all curves will be normalized using the maximum and minimum points in the whole data set. This is equivalent to scaling all curves. To normalize curves for comparison of curve shape (see Section 3.3.4), make sure **Apply to Entire Working Set** is not checked, or perform the normalization in single plot mode.

7B.6 The Fit menu (plot window)
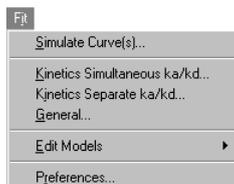
7B.6.1 Simulate Curve(s)
Simulates curves according to a chosen model with specified parameter values. See Section 4.7 for a description of curve simulation.

7B.6.2 Kinetics Simultaneous ka/kd
Starts the wizard for fitting sensorgram data using models for simultaneous ka/kd. See Section 4.1.2 for a description of the wizard.

7B.6.3 Kinetics Separate ka/kd
Starts the wizard for fitting sensorgram data using models for separate ka/kd. See Section 4.4.1 for a description of the wizard.

7B.6.4 General
Starts the wizard for fitting curve data using models for simultaneous ka/kd.

7B.6.5 Edit Models
Opens the model editor for editing and creating models. Choose the type of model from the cascade menu:

See Appendix B for a full description of the model editor.

7B.6.6 Preferences
Sets preferences for display of fitting results. See Section 7A.5.3.

7B.7   The Window menu (plot window)

Provides options for arranging windows on the workspace and activating windows. See Section 7A.6.

7B.8   The Help menu (plot window)

Provides information about BIAevaluation 3.0. See Section 7A.7.

7C The fit window

7C.1   Toolbar buttons

The following toolbar buttons are available when a fit window is active. To display a tool tip with a reminder of the button function, place the cursor on the button and wait for a short time.

	–	Go to project window
	File:Open	Section 7A.2.3
	File:Save	Section 7A.2.4
	File:Print	Section 7C.2.7
	View:Overlay	Section 7C.4.10
	View:Reference Line	Section 7B.4.8
	Fit:Kinetics Simultaneous ka/kd	Section 7B.6.2
	Fit:Kinetics Separate ka/kd	Section 7B.6.3
	Fit:General	Section 7B.6.4

In addition, the toolbar includes the following selection boxes:
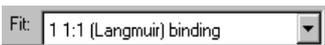
Fit list	Fit: [1 1:1 (Langmuir) binding]	Displays fitting results
Curve list	Curve: [— 4 FEB28C Fc=1 - 38]	Selects the active curve

7C.2   The File menu (fit window)
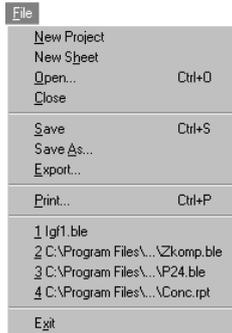
7C.2.1   New project
Closes the current project and starts a new project. Before closing, you will be asked if you want to save changes in the current project.

7C.2.2   New sheet
Opens a new empty sheet window.

7C.2.3   Open (Ctrl-O)
Opens a file. See Section 7A.2.3.

7C.2.4   Save (Ctrl-S)
Saves the current evaluation project. See Section 7A.2.4.

7C.2.5   Save As
Saves the current evaluation project under a new file name. See Section 7A.2.5.

7C.2.6   Export
Exports all curves in the current fit window to a text file, including both experimental and fitted curves.

7C.2.7   Print (Ctrl-P)
Prints the contents of the current fit window. Choose printing options from the dialogue box. See Section 7B2.7 for details.
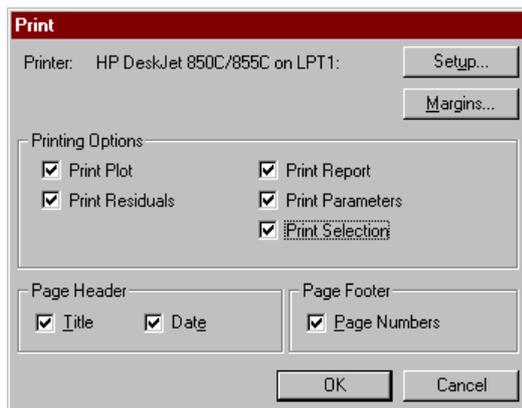
The name of the fit as displayed in the fit list on the toolbar is used as the title in **Page Header.**

7C.2.8   Recent file list
The **File** menu lists the four most recently opened files. Click on a file to open it.
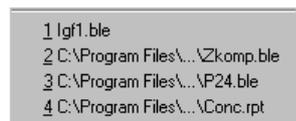
7C.2.9   Exit
Closes BIAevaluation.

7C.3   The Edit menu (fit window)

7C.3.1   Copy (Ctrl-C)
Copies selected data from the numerical display tabs to the Windows clipboard. Copied data can be pasted into a BIAevaluation sheet window or into other programs.

Copy cannot be used on graphical data from the fit window. To copy fitted curves as graphical objects, use **Copy Graph** from the **Edit** menu or from the right button menu. To copy fitted curves as numerical data, use **Export** from the **File** menu or from the right button menu.

7C.3.2   Copy Graph (Ctrl-H)
Copies the currently displayed plot to the Windows clipboard, from which it can be pasted as a graphic into other applications such as word processing or presentation software. The graph is copied in Windows clipboard format.

7C.3.3   Remove fit
Removes the currently displayed fit from the fit window.

7C.3.4   Curve properties
Sets the properties of a selected curve for display in the plot and fitting windows. See Section 3.2.2.

7C.4   The View menu (fit window)

7C.4.1   Scale
Sets the scale for the plot in the current window. See Section 3.2.3.

7C.4.2   Adjust scale
Sets the scale of the x- and y-axis according to the limits of the data for curves in the window. See Section 3.2.3.

7C.4.3 Unzoom (Ctrl-U)

Restores the previous display after a **Zoom** operation. See Section 3.2.3.

7C.4.4 Gridlines

Displays gridlines in the fit window. See Section 3.2.4.

7C.4.5 Labels

Sets a title and axis labels for the fit window. See Section 3.2.5.

7C.4.6 Legend

Displays a legend identifying the curves in the fit window. See Section 3.2.5.

7C.4.7 Curve History

Displays the history of all the curves in the fit window. See Section 7A.4.1.

7C.4.8 Reference Line

Displays horizontal and vertical reference lines, with their intersection on the currently active curve. See Section 7B.4.8.

7C.4.9 Components

Displays a breakdown of the fitted curve into components (total, complex(es) and bulk&drift).
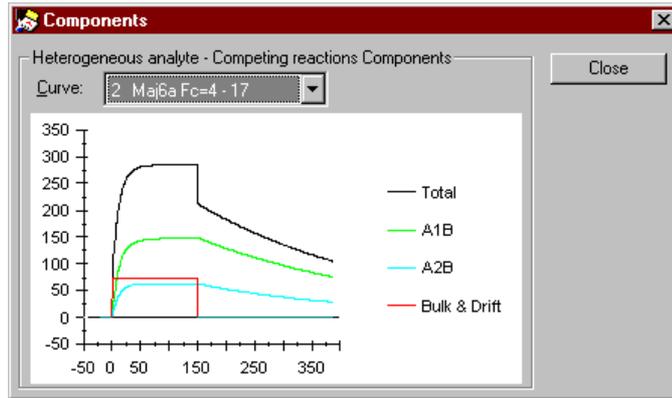
The right mouse button menu may be used on this window to export the component curves to text files. The exported files can then be imported back into BIAevaluation as curves to obtain the component curves in the project window. This may be used for example to obtain a curve for bulk and drift effects which may be subtracted from the experimental curve for presentation purposes with the curve subtraction function in **Calculate:Y-Transform.**

7C.4.10   Split View
Displays the current plot in a split view. See Section 7B.4.9.

7C.4.10   Overlay
Toggles the display between overlay and single plot mode.

7C.5 The Fit menu (fit window)
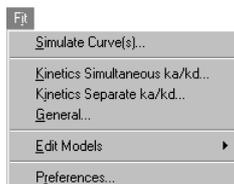
7C.5.1   *Simulate curve(s)*

Simulates curves according to a chosen model with specified parameter values. See Section 4.7 for a description of curve simulation.

When curves are simulated from the fit window, fitted values are suggested for all parameters in the simulation.

7C.5.2   *Plot Parameters*

Creates a curve by plotting two chosen parameters from the currently displayed results tab (report or parameters). This option is not available if the residuals or selections tab is displayed. See Section 4.6.

7C.5.2   *Kinetics Simultaneous ka/kd*

Starts the wizard for fitting sensorgram data using models for simultaneous ka/kd. See Section 4.1.2 for a description of the wizard.

7C.5.3   *Kinetics Separate ka/kd*

Starts the wizard for fitting sensorgram data using models for separate ka/kd. See Section 4.4.1 for a description of the wizard.

7C.5.4   *General*

Starts the wizard for fitting curve data using models for simultaneous ka/kd.

7C.5.5   *Edit models*

Opens the model editor for editing and creating models. Choose the type of model from the cascade menu:

See Appendix B for a full description of the model editor.

7C.5.6   Preferences

Sets preferences for display of fitting results. See Section 7A.5.3.

7C.6   The Window menu (fit window)
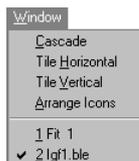
Provides options for arranging windows on the workspace and activating windows. See Section 7A.6.

7C.7   The Help menu (fit window)

Provides information about BIAevaluation 3.0. See Section 7A.7.

7D The sheet window

7D.1 Toolbar buttons

The following toolbar buttons are available when a sheet window is active. To display a tool tip with a reminder of the button function, place the cursor on the button and wait for a short time.

	–	Go to project window
	File:Open	Section 7A.2.3
	File:Save	Section 7A.2.4
	File:Print	Section 7B.2.7
	Data:Sort	Section 7D.4.1
	Data:Create Curve	Section 7D.4.3

7D.2 The File menu (sheet window)
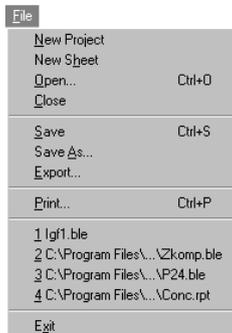
7D.2.1   New project
Closes the current project and starts a new project. Before closing, you will be asked if you want to save changes in the current project.

7D.2.2   New sheet
Opens a new empty sheet window.

7D.2.3   Open (Ctrl-O)
Opens a file. See Section 7A.2.3.

7D.2.4   Save (Ctrl-S)
Saves the current evaluation project. See Section 7A.2.4.

7D.2.5   Save As

Saves the current evaluation project under a new file name. See Section 7A.2.5.

7D.2.6   Export

Exports the data in the current sheet window to a text file. Use this function for saving report point tables from result files as text files.

7D.2.7   Print (Ctrl-P)

Prints the contents of the current sheet window. Choose printing options from the dialogue box.
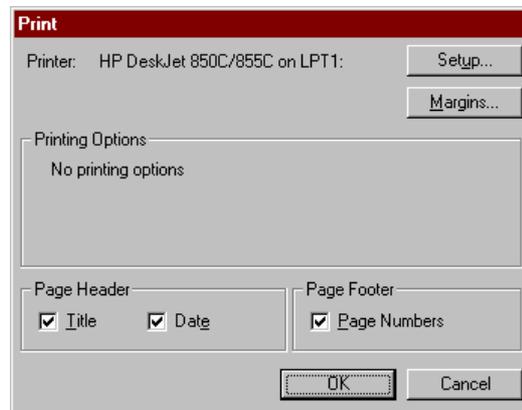
The name of the sheet window is used as the title in **Page Header**.

7D.2.8   Recent file list

The **File** menu lists the four most recently opened files. Click on a file to open it.
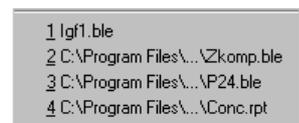
7D.2.9   Exit

Closes BIAevaluation.

7D.3   The Edit menu (sheet window)

Edit
Undo	Ctrl+Z
Cut	Ctrl+X
Copy	Ctrl+C
Paste	Ctrl+V
Insert Paste	
Clear	Del
Delete	

7D.3.1   Undo (Ctrl-Z)
Reverses the most recent editing operation in the current session.

7D.3.2   Cut (Ctrl-X)
Cuts the contents of the currently marked cells and places them on the Windows clipboard. Cut data can be pasted into a BIAevaluation sheet window or into other programs.

7D.3.3   Copy (Ctrl-C)
Copies the contents of the currently marked cells to the Windows clipboard. Copied data can be pasted into a BIAevaluation sheet window or into other programs.

7D.3.4   Paste (Ctrl-V)
Pastes sheet data from the Windows clipboard into the current sheet, with the currently selected cell at the top left of the pasted area. The current values in the target cells are replaced without warning.

If only a single cell is selected, all of the data in the clipboard is pasted. If a range of cells is selected when data is pasted, data is only pasted into the target area.

If the cells contain formulae with relative cell references (e.g. **=D2*C2**) are pasted into a BIAevaluation sheet, the relative references are adjusted according to the paste position. Prefix the column letter and/or row number with a dollar sign (**$**) in the formula to specify absolute references (e.g. **=D2*C2**) and prevent adjustment of the formula. If cells containing formulae are cut or copied from a BIAevaluation sheet and pasted into another program (including spreadsheet programs), the value of the formula is pasted.

7D.3.5   Insert Paste
Pastes sheet data from the Windows clipboard into the current sheet, inserting new rows to accommodate the pasted data. The clipboard data is pasted with the currently selected cell at the top left of the pasted area. No data in the target sheet is overwritten.

7D.3.6   Clear (Del)

Clears the currently selected cells. The contents of the cleared cells are not placed on the clipboard, and can only be recovered by choosing **Edit:Undo** (Ctrl-Z) before performing any other operation.

7D.3.7   Delete

Removes the contents of the currently selected cells from the sheet. Choose the mode of deletion from the dialogue box:
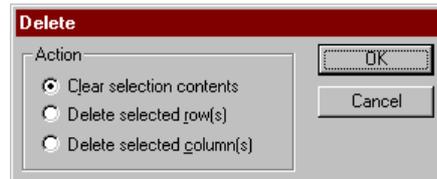
Clear selection contents
This option is equivalent to **Edit:Clear.**

Delete selected row(s)
Removes the row(s) containing the selected cells.

Delete selected column(s)
Removes the column(s) containing the selected cells.

Note: The option of shifting cells up or left to accommodate cell deletions that is found in commercial spreadsheet programs is not supported in BIAevaluation. This ensures that the integrity of row and column data is maintained.

7D.4   The Data menu (sheet window)
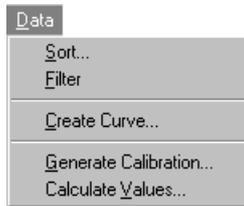
7D.4.1   Sort

Sorts data in the sheet by up to three sort criteria. Choose the column(s) on which to sort from the dialogue box:
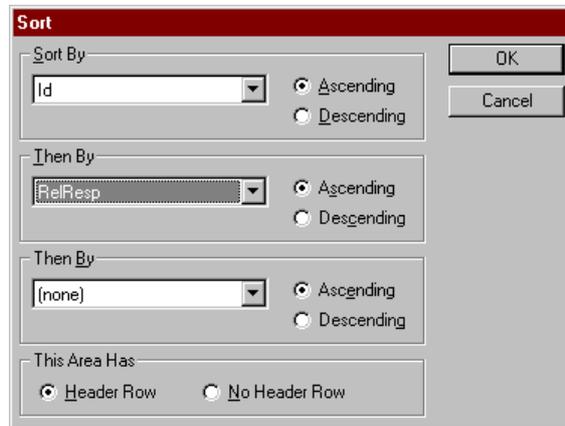
Columns are identified by the content of the first row if **Header Row** is checked. The header row is excluded from the sort. Otherwise columns are identified by column letter.

7D.4.2   Filter

Filters data in the sheet. When a filter is applied, only rows which fulfil the filter conditions are displayed. All operations on the sheet except **Sort** apply only to the filtered data: data which is not displayed in the filtered sheet is not affected by the operation.

Choosing **Data:Filter** requires you to specify whether the sheet has a header row or not:
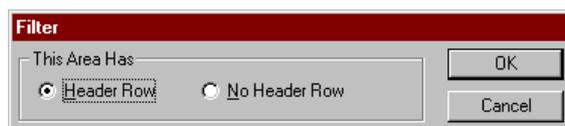
If a header row is specified, the first row in the sheet will be excluded from filter operations.

When **Filter** is chosen, a pull-down list of filter conditions is inserted at the top of each column. Each list contains all unique values in the column together with the entries **All** and **Custom**. Values in the list are case-sensitive. To display only rows containing a given value in a chosen column, select the value from the filter condition list at the top

of the column. The example below has been filtered to show only
second_Ab in the **Id** column (column L):

To display rows containing a range of values, choose **Custom** from the
filter condition list and enter the condition(s) in the dialogue box

If a custom filter is applied, the option **Edit Custom** is added to the
filter condition list. Choose this option to change the custom
conditions.

When a filter is applied so that some rows in the sheet are excluded,
the original row numbers are retained. The discontinuities in the row
numbering thus indicate how many rows have been excluded by the
filter.

To remove a filter from a column, choose **All** from the filter condition
list. To remove all filters, choose **Data:Filter:** this removes the row
holding the filter conditions and re-displays the unfiltered sheet.

7D.4.3 Create Curve

Creates a curve from the values in two columns in the sheet. Choose the columns to be used for y- and x-values from the dialogue box:
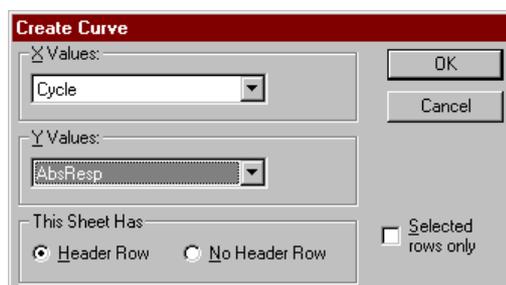
If **Header Row** is checked, the first row in the sheet will be excluded from the curve.

If cells are selected in the sheet, the **Selected rows only** option is available in the dialogue box. Checking this option creates the curve only from values in the selected rows. Note that it does not matter which columns are selected: all columns are available for curve data.

7D.4.4 Generate Calibration

Generates a calibration curve from data in the sheet window. If the sheet is not filtered, you will be warned of this. You can still generate a calibration curve, but in most situations involving concentration measurement with BIA the report points should be grouped by Id for correct generation of a calibration curve.
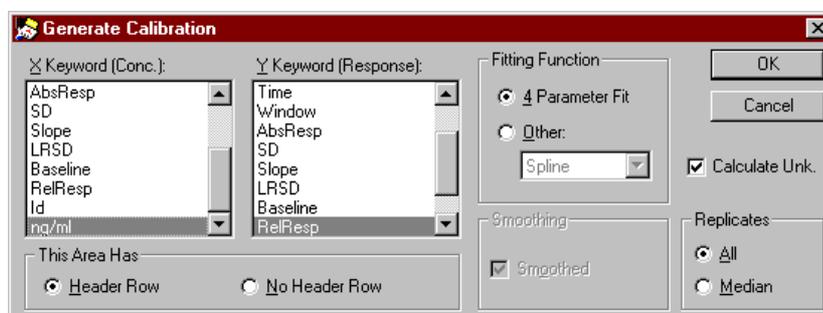
Choose the columns containing values for the x- and y-axes of the calibration curve from the respective keyword lists. BIAconcentration evaluation suggests a concentration keyword (identified as **Conc**, **ng/ml**, **mg/ml** or similar) for the x-axis and relative response (**RelResp**) for the y-axis if such keywords can be found.

Choose the fitting function from the **Fitting Function** options. The following options are available. The default choice is **4-Parameter Fit**.

Other functions may be chosen by checking **Other** and selecting the function from the list.

4-Parameter Fit	Fits the calibration curve to a 4-parameter equation (see Section C.3.1) which gives good fitting for most calibration curve data.
Spline	Fits the calibration curve to the data points using the spline fitting algorithm, with or without smoothing according to the **Smoothing** option.
Qubic spline	Fits the calibration curve to the data points using a cubic spline algorithm, with a degree of smoothing as specified in the **Smoothing** box (see Press *et al., Numerical Recipes in C* (Cambridge University Press 1988) for a description of this algorithm). A lower smoothing factor fits the curve closer to deviating data points.
Line	Fits a linear calibration curve to the data points using a least squares linear regression algorithm. Smoothing is not applicable to linear fitting.
Point to point	Constructs a calibration curve consisting of straight-line segments joining adjacent data points. Smoothing is not applicable to point-to-point fitting.

Replicate values are handled in different ways according to the fitting function chosen as listed below:

Option	Available for function	Description
All	4-parameter fit	Treats each replicate point separately.
Median	4-parameter fit Spline Qubic spline	Fits the curve to the middle point in the replicates (or the average of the two middle points if there is an even number of replicates).
Weighting	Spline	Uses a replicate weighting procedure included in the fitting algorithm.
Average	Qubic spline	Uses the average value of the replicates.

If the **Calculate Unk.** box is checked, results for any unknown data points in the table will be generated automatically. See Section 5.4 for more information about calculating unknowns.

7D.4.5 Calculate Values

Calculates new values based on a selected calibration curve and the data in a selected column.

Choose the calibration curve to use from the first dialogue box. Currently open calibration curves are listed:
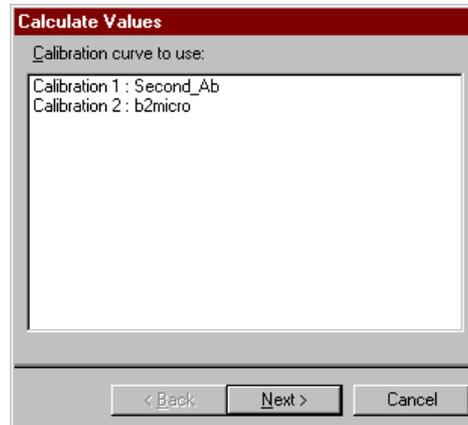
Choose the column containing the source values (corresponding to the y-axis values in the calibration curve) from the second dialogue box:
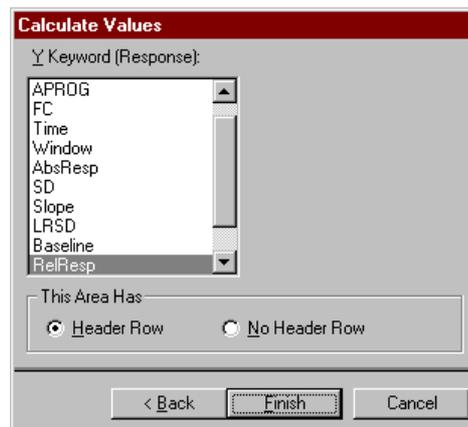
The new values (corresponding to the x-axis values on the calibration curve) are entered in the first empty column in the sheet.

	G	H	I	J	K	L	M	N	O
1	[All] ▼	[All] ▼	[All] ▼	[All] ▼	[All] ▼	second_/ ▼	[All] ▼		
	SD	Slope	LRSD	Baseline	RelResp	Id	ng/ml		
6	3.38347	-2.02958	0.3653	YES	4006	second_Ab	high	806.40544	
9	3.9672	-2.38374	0.35373	YES	3968	second_Ab	high	783.63562	
12	2.94728	-1.68242	1.00138	YES	2082	second_Ab	273	254.08019	
15	2.48228	-1.45732	0.59753	YES	2068	second_Ab	273	251.99849	
18	1.63845	-0.94279	0.51659	YES	997	second_Ab	low	116.43153	
21	0.66538	-0.04439	0.69707	YES	77	second_Ab	0	#N/A	
24	3.67097	-2.1945	0.50832	YES	4222	second_Ab	1040	961.53956	
27	2.06945	-1.12038	0.96071	YES	1139	second_Ab	130	132.64001	
30	4.11952	-2.44947	0.7232	YES	3983	second_Ab	high	792.48996	
33	3.62546	-2.15689	0.62408	YES	4230	second_Ab	1040	968.31943	
36	1.26398	-0.6744	0.62087	YES	605	second_Ab	65	71.994786	
39	0.62259	-0.15941	0.59418	YES	82	second_Ab	0	#N/A	
42	1.05334	-0.56584	0.50449	YES	585	second_Ab	65	69.679427	
45	3.59749	-2.14744	0.53777	YES	3993	second_Ab	high	798.48885	
48	4.10994	-2.43097	0.84344	YES	3993	second_Ab	high	798.48885	
51	1.82169	-1.03409	0.64796	YES	1169	second_Ab	130	136.09994	
54	1.57718	-0.84425	0.76553	YES	1068	second_Ab	low	124.50504	
57	3.64233	-2.16623	0.63426	YES	3489	second_Ab	527	567.62514	

7D.5   The Window menu (sheet window)
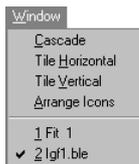
Provides options for arranging windows on the workspace and activating windows. See Section 7A.6.

7D.6   The Help menu (sheet window)

Provides information about BIAevaluation 3.0. See Section 7A.7.

7E The calibration window

7E.1 Toolbar buttons

The following toolbar buttons are available when a calibration window is active. To display a tool tip with a reminder of the button function, place the cursor on the button and wait for a short time.

	–	Go to project window
	File:Open	Section 7A.2.3
	File:Save	Section 7A.2.4
	File:Print	Section 7B.2.7

7E.2 The File menu (calibration window)
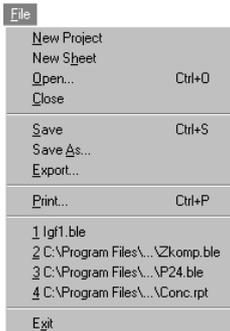
7E.2.1   *New project*
Closes the current project and starts a new project. Before closing, you will be asked if you want to save changes in the current project.

7E.2.2   *New sheet*
Opens a new empty sheet window.

7E.2.3   *Open (Ctrl-O)*
Opens a file. See Section 7A.2.3.

7E.2.4   *Save (Ctrl-S)*
Saves the current evaluation project. See Section 7A.2.4.

7E.2.5 Save As

Saves the current evaluation project under a new file name. See Section 7A.2.5.

7E.2.6 Export

Exports the data in the current calibration view to a text file. For numerical display (calibration and unknown points), values are exported with 12 decimal places regardless of the current display format.

7E.2.7 Print (Ctrl-P)

Prints the contents of the current calibration window. Choose printing options from the dialogue box.
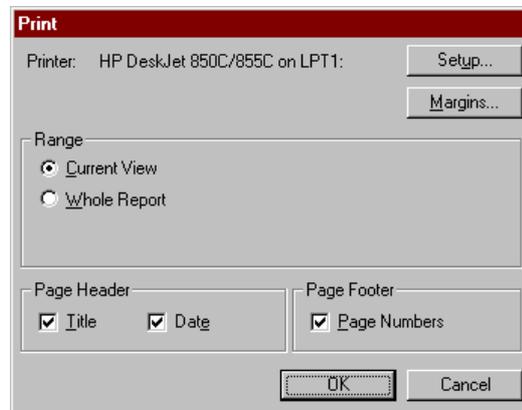
7E.2.8 Recent file list

The **File** menu lists the four most recently opened files. Click on a file to open it.
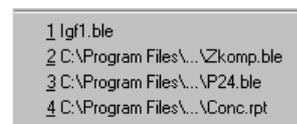
7E.2.9 Exit

Closes BIAevaluation.

7E.3   The Edit menu (calibration window)
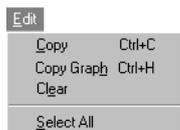
7E.3.1   Copy (Ctrl-C)
Copies the contents of the currently marked cells to the Windows clipboard. Copied data can be pasted into a BIAevaluation sheet window or into other programs. This option can only be used on the numerical displays (calibration data and unknown points).

7E.3.4   Copy Graph (Ctrl-H)
Copies the currently displayed plot to the Windows clipboard, from which it can be pasted as a graphic into other applications such as word processing or presentation software. The graph is copied in Windows clipboard format. This option can only be used on the graphical displays (calibration curve, CVx and CVy).

7E.3.6   Clear
Clears the whole unknown points table. This option can only be used on unknown points display.

7E.3.7   Select all
Selects all cells in the current display. This option can only be used on the numerical displays (calibration data and unknown points).

7E.4   The View menu (calibration window)
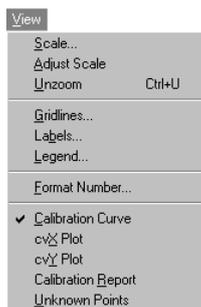
7E.4.1   Scale
Sets the scale for the plot in the current window. See Section 3.2.3. This option can only be used on the graphical displays (calibration curve, CVx and CVy).

7E.4.2   Adjust scale
Sets the scale of the x- and y-axis according to the limits of the data for curves in the window. See Section 3.2.3. This option can only be used on the graphical displays (calibration curve, CVx and CVy).

7E.4.3   Unzoom (Ctrl-U)

Restores the previous display after a **Zoom** operation. See Section 3.2.3. This option can only be used on the graphical displays (calibration curve, CVx and CVy).

7E.4.4   Gridlines

Displays gridlines in the current window. See Section 3.2.4. This option can only be used on the graphical displays (calibration curve, CVx and CVy).

7E.4.5   Labels

Sets a title and axis labels for the plot or fitting window. See Section 3.2.5. This option can only be used on the graphical displays (calibration curve, CVx and CVy).

7E.4.6   Legend

Displays a legend identifying the curves in the plot window. See Section 3.2.5. This option can only be used on the graphical displays (calibration curve, CVx and CVy).

7E.4.7   Format Number

Sets the display format for numbers in selected cells in the numerical displays (calibration data and unknown points). Non-selected cells are not affected. The format is selected from a pre-defined list:
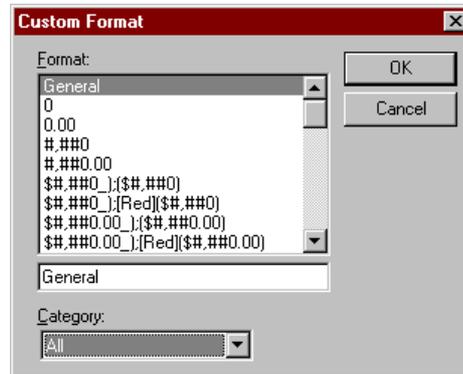
Choose a pre-defined format or enter a format definition in the box directly below the list.

Formats are coded using the conventions in Microsoft Excel. By default, numbers higher than 1 are formatted with two decimal places (format definition 0.00) and numbers below 1 are formatted in exponential notation (format definition 0.00E+00). Values which are invalid (e.g. outside the range of the calibration curve) are displayed as **#N/A**. These default settings are sufficient for most purposes.

7E.4.8   Display list
Selects which view of the calibration window to display. These options correspond to the buttons at the left of the calibration window. See Section 5.3.1 for details.

7E.5   The Data menu (calibration window)
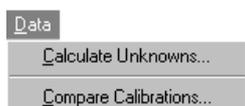
7E.5.1   Calculate Unknowns
Calculates unknown values based on the current calibration curve.

Choose the sheet to use from the first dialogue box. Currently open sheets are listed:
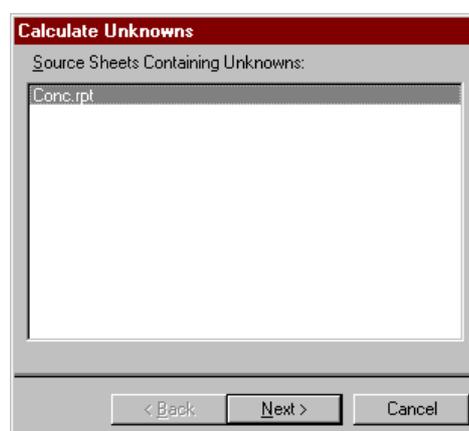
Choose the columns containing the source values (corresponding to the y- and x-axis values in the calibration curve) from the second dialogue box. Unknown values are identified by non-numerical values including blanks in the concentration (x-axis) value column. Note that you may choose any two columns, not only those used in the original calibration curve:
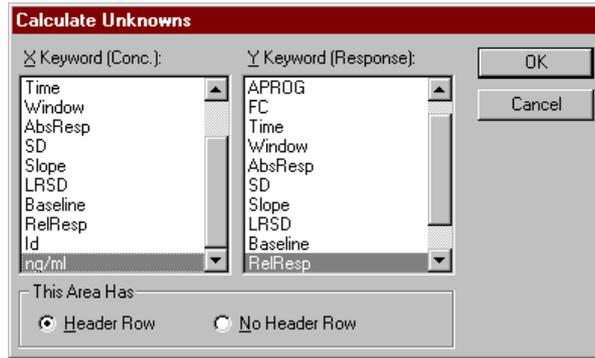
The calculated unknowns are added to the unknown display in the calibration window. New results will be added to any previously existing results providing a cumulative report.

7E.5.2   Compare Calibrations
Compares two or more calibrations in an overlay plot. See Section 5.3.2 for details.

7E.6   The Window menu (calibration window)

Provides options for arranging windows on the workspace and activating windows. See Section 7A.6.

7E.7   The Help menu (calibration window)

Provides information about BIAevaluation 3.0. See Section 7A.7.

Appendix A.
Principles of curve fitting

Fitting experimental data to simultaneous ka/kd models in BIAevaluation relies on two mathematical steps:

- generation of the rate equations for the model used (model definition)

- finding the values for parameters in the rate equation that best fit the experimental data (curve fitting)

Rate equations are generated when the model is defined (see Appendix B). This appendix considers the principles behind the curve fitting process and their consequences for evaluation of experimental data.

A.1 Curve fitting algorithms

Curve fitting in BIAevaluation relies on the so-called Marquardt-Levenberg algorithm[1], which optimizes parameter values by minimizing the sum of the squared residuals. The residuals are the difference between the calculated and experimental curve at each point: squared residuals are used so that deviations above and below the experimental curve are weighted equally:

_____
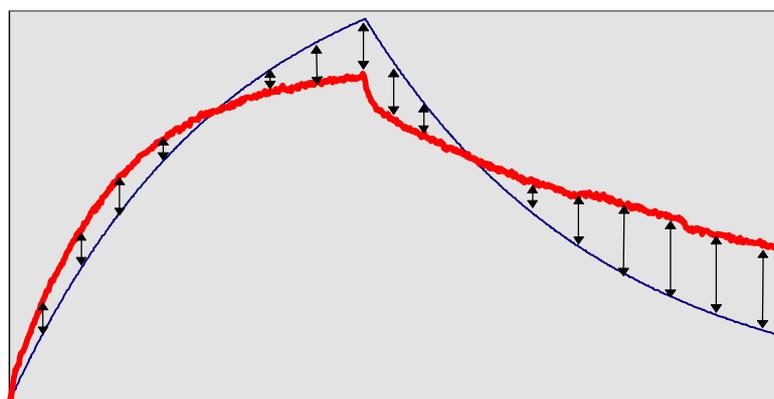
[1] J. W. Wells, in Receptor-Ligand Interactions, A Practical Approach, ed, E.C. Hulme.

$$S = \sum_{1}^{n} \left(r_f - r_x \right)^2$$

where   S is the sum of squared residuals
          r_f is the fitted value at a given point
          r_x is the experimental value at the same point

The algorithm is an iterative process that begins with an initial value for each parameter in the equation. These values are defined in the model (see Appendix B), either as constants or as values at defined points on the experimental curve (e.g. Ymax, Xmin). The initial values chosen can in some cases affect the outcome of the curve fitting as described below. While there is no guarantee that the initial values are appropriate, the values set in the predefined models in BIAevaluation give robust curve fitting for most experimental data sets.

It is important to realise that the fitting algorithm is a purely mathematical procedure that does not have any "knowledge" of the biological relevance of the values found. For example, the algorithm itself will not prevent negative values for rate constants, which would be meaningless in kinetic terms. The model definitions specify whether negative values are allowed for individual parameters, so that this particular unreasonable kind of result can be prevented. However, it is always important to examine the results of a fit for "reasonableness" of the parameter values.

A.2 Generating the fitted curve

The fitted curve is generated for each iteration of the fitting algorithm by calculation from the rate equations for the model (or the fitting equation for non-kinetic data) using the current set of parameter values. In kinetic systems, rate equations are fundamentally differential equations (the rate is the differential of response with respect to time dR/dt). For a number of models, the differential rate equations can be integrated to give equations from which the response against time can be calculated directly.

Other models such as interaction with mass transfer limitations or two-state processes are described by a set of rate equations which together are difficult or impossible to integrate mathematically to give a single expression describing response as a function of time. For these models, a mathematical tool called a numerical integrator is required to generate the calculated binding curves. A so-called "stiff" integrator is required to ensure that data involving rapid binding rates is handled correctly.

Analytical integration is used in BIAevaluation for models where the rate equations can readily be integrated, to give rapid fitting of the data. Numerical integration is much more time-consuming than calculation from integrated rate equations, but allows evaluation with models where the rate equations cannot be integrated analytically.

A.3   Finding the best fit

The "best fit" of experimental data to a model is influenced by two factors:

- the ability of the fitting algorithm to find the true minimum in the sum of squared residuals function (convergence)

- the number of parameters that can be varied in the model (complexity)

In finding the best fit, the fitting algorithm scans combinations of parameter values, searching for the set of values that gives the lowest sum of squared residuals.

For models with relatively few parameters, there will usually be a single set of parameter values which gives the best fit, and there will be no difficulty in finding this set. The fitting is fast and stable. For more complicated models, there may be more than one local minimum in the sum of squared residuals. The illustration below shows a three-dimensional plot of the sum of squared residuals as a function of the values for two parameters, with two local minima (**MIN A** and **MIN B**). A fit which starts with the initial values represented by point **A** will find the minimum **MIN A**, while a fit which starts at point **B** will find **MIN B**. Depending on the values for the initial parameters, therefore, this system could give two different "best fits".
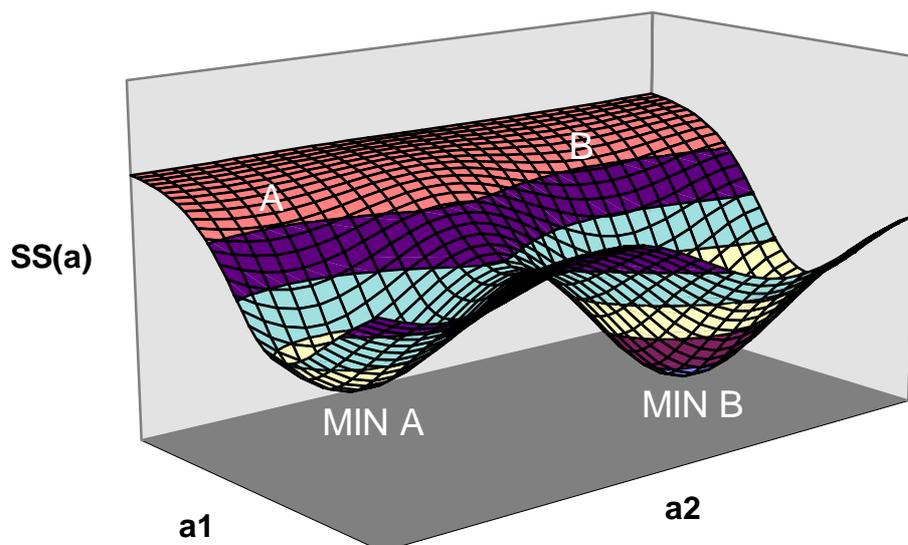
In real situations there are many more than two parameters in the fitting equations, and the potential for different local minima in the χ^2 function is greater. In practice the equations used in the predefined models usually find the minimum sum of squared residuals reliably provided that the initial values are biologically reasonable. "Incorrect" local minima are seen either as markedly poor fits or as unreasonable fitted values. For the majority of sensorgrams, there is considerable freedom in choice of initial values and there is no reason to change the values defined in the model. In some rare cases, however, it may be apparent that the fitting algorithm has difficulty in finding a good fit, and seems to "get stuck" with wildly wrong fitted curves. In such cases a large (usually orders of magnitude) change in the initial values for one or more parameters may help to push the fit towards the true minimum for the sum of squared residuals. It should be noted however that difficulty in fitting experimental data to a model is most commonly caused by poor data quality or a very inappropriate choice of model, and only rarely by failure of the fitting algorithm.

A.4 Model complexity

As the complexity of a model (represented by the number of parameters involved) increases, the ability to fit the equations to an experimental curve improves automatically. This is simply because there is more scope for varying parameters to generate a close fit. It is very important in evaluating kinetic data to accept the simplest model which fits the data within the limits of the experiment. A more complex model may fit the data better, but the crucial question is whether the improvement is biologically or experimentally significant.

At the same time, increasing the complexity of a model increases the potential for multiple local minima in the χ^2 function. This means that the fitting procedure may be less stable and more sensitive to the chosen initial values. The injection start and stop times and the range of data selected may also be critical for fitting to a complex model. It is important to realise that this decrease in stability of the fitting procedure as model complexity increases is a property of the mathematical algorithms used, and does not indicate a defect in either the experimental data or the evaluation software.

The number of parameters in a fitting is a function of the number of sensorgrams and the parameter status as well as the complexity of the model. For example, the simplest model (1:1 binding) has three global parameters and one local. Global parameters represent for the whole data set. Locally fitted parameters are treated separately for each sensorgram. Applying the model to 6 sensorgrams simultaneously gives the following situation:

Parameter	Status	No. of variables
Rmax	Fitted globally	1
ka	Fitted globally	1
kd	Fitted globally	1
RI	Fitted locally	6
	Total	9

If Rmax is also set to be a local parameter, the number of variables increases to 14.

Fitting several sensorgrams simultaneously to a complex model can result in a large number of fitted parameters, particularly if several of the parameters are fitted locally. There is a limit to the number of parameters that BIAevaluation can handle at the same time. If this limit is exceeded (as indicated by an error message when the fit is attempted), either reduce the number of sensorgrams or fit more of the parameters globally. However, if your experimental system requires such a complex model, you should seriously consider efforts to simplify the system. ***Complex systems are inevitably difficult to evaluate and interpret, and sophisticated evaluation procedures can never provide a substitute for careful experimental design.***

A.5 Statistical fitting parameters

A.5.1 χ^2 and correlation coefficient
The closeness of fit is described by the statistical value χ^2:

$$\chi^2 = \frac{\sum\limits_{1}^{n}\left(r_f - r_x\right)^2}{n - p}$$

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
and   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 χ^2 reduces to the average squared residual per data point.

If the model fits the experimental data precisely, χ^2 represents the mean square of the signal noise (since this is the only deviation from the fitted curve). In practice, values of χ^2 below 10 are frequently acceptable. The form of the fitted curve in relation to the experimental data (i.e. the shape of the residual plot) is often more valuable in assessing the goodness of fit than the absolute χ^2 value.

In many contexts, the statistical closeness of a curve fit is reported as a correlation coefficient instead of a χ^2 value. BIAevaluation does not report correlation coefficients because this is a very insensitive measure of the closeness of fit for a curve with a large number of points.

A.5.2 Standard error and T-value
The standard error reported for each fitted parameter is an estimate of how sensitive the fitting is to changes in the parameter. The standard error may be visualized on a χ^2 function with the help of the illustration below.

SS(a)

a1

a2
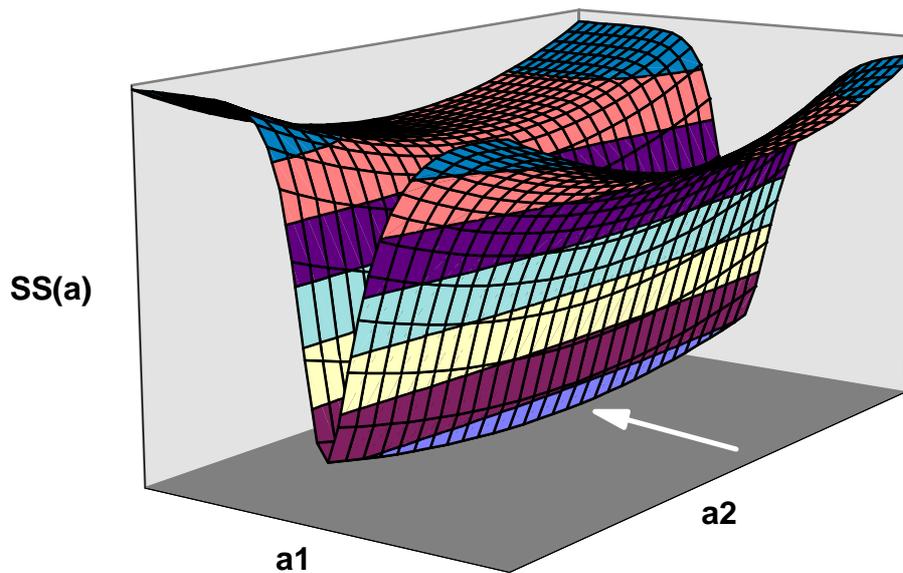
In the **a1** direction, the χ^2 minimum is sharply defined and the standard error for **a1** is low. Conversely, the minimum in the **a2** direction is poorly defined and the standard error for **a2** is high.

As an alternative under **Fit:Preferences**, the standard error may be expressed as a T-value, which is the value of the parameter divided by the standard error. This is analogous to a signal-to-noise value in a response. Using the T-value makes it easier to compare standard errors for parameters with widely different absolute values (e.g. k_a and k_d). As a general guide, T-values greater than 10 indicate that the value obtained for the parameter is significant.

Note that the standard error (or T-value) is not a measure of the goodness of fit for a given parameter. The goodness of fit is defined by the *depth* of the χ^2 minimum, while the standard error is a function of the *shape* of the minimum. Variations in a parameter with a large standard error will not affect the goodness of fit to any great extent.

A.5.3 Correlated and non-correlated parameters
Correlated parameters are parameters that are linked to each other in the set of equations for the model. When two parameters are tightly correlated, a change in one of the parameters matched by a compensating change in the other will have no effect on the closeness of fit. In such a case, it is impossible to decide on absolute values for the two parameters. For non-correlated parameters, the best fit gives unique parameter values.

An example of this behaviour may be found in the number of binding sites blocked by one analyte molecule (Section B.1.3), when this parameter is applied to a simple 1:1 binding model. The number of binding sites blocked is tightly correlated with the association rate constant and the maximum binding capacity, as shown by the results below (obtained by fitting the example data set P24.BLE to a 1:1 model with number of binding sites blocked = n. The closeness of fit is identical in all cases.

N	k_a	k_d	R_{max}	χ^2
1	2.2×10^5	1.6×10^{-3}	90	1.1
10	2.2×10^4	1.6×10^{-3}	900	1.1
50	4.4×10^3	1.6×10^{-3}	4500	1.1

Clearly, in this example the number of binding sites *actually* blocked by one analyte molecule must be known if a meaningful value is to obtained for k_a and R_{max}. The dissociation rate constant is however not correlated with n and gives the same value in all cases.

Parameters which are tightly correlated usually give high standard errors (low T-values).

A.5.4 Standard deviations for fitted parameters

Parameters which are calculated using global fitting are reported as a single value for the whole data set. Statistical evaluation of the results from a single fitting operation is thus neither possible nor meaningful from a single fitting operation. To obtain statistical values such as standard deviation for the fitted parameters, results must be calculated from independent replicate measurements, each evaluated separately.

Even if the fitting is performed with local parameters, it is incorrect to calculate statistics for the reported values based on evaluation of a single data set. The results of one concentration series should not be regarded as replicate measurements on which statistics can be based, rather as experimental variants designed to extend the data set and improved the consistency of the results.

Appendix B.
Editing models

BIAevaluation is provided with a set of pre-defined models for kinetic evaluation and curve fitting. New models can be created with the model editor using either an existing model or a blank form as template. The pre-defined models are write-protected and cannot be changed or deleted.

Recommendation
Defining models for evaluation of kinetic data requires a good understanding both of kinetic principles and of the way models are created in BIAevaluation. Unless you are experienced in kinetic theory, you are advised to use the model editor only for making minor changes to existing models, such as

- changing the properties of parameters (e.g. default fitting status)

- adding simple terms such as baseline drift

- changing the pre-defined report parameters

If you do intend to use the model editor to create your own models, read Section B.2 carefully.

B.1   Making simple changes to existing models

B.1.1   Opening models for editing
To change a pre-existing model definition, choose **Fit:Edit Models** and select appropriate group of models (**Simultaneous ka/kd, Separate ka/kd** or **General**).
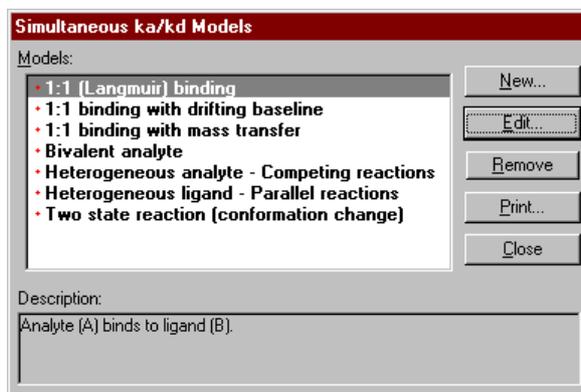
Double-click on the model you want to edit or select the model and choose **Edit**. In the model editor, change the model name so that you can save the edited model (you cannot save a model with the same name as a pre-defined model). You may also change the model description of the model: this text is shown for guidance in the model selection box (see for example the illustration above).
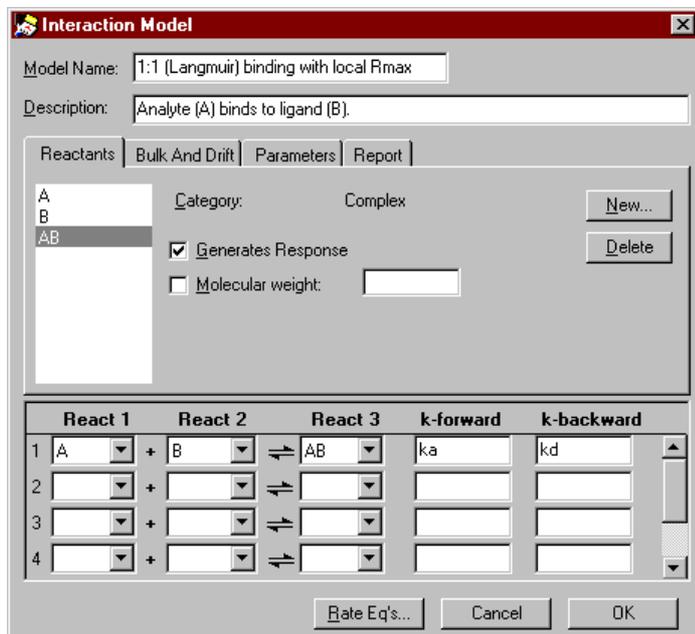
B.1.2 Changing parameter properties

You can change the status (constant/local fitting/global fitting) and the initial values for parameters during the fitting procedure (see Section 4.1.2). However, changes made in this way are not saved with the model, and must be made on every fitting occasion. If you repeatedly use a pre-defined model with one or more changed parameters, it may be convenient to define a new model with the required properties.

For example, to change the status of the Rmax parameter from global to local fitting:

1. Open the model for editing and change the name as described above (Section B.1.1).

2. Choose the **Parameters** tab in the model editor window. Select the parameter Rmax and set **Default Type** to **Fit locally**:
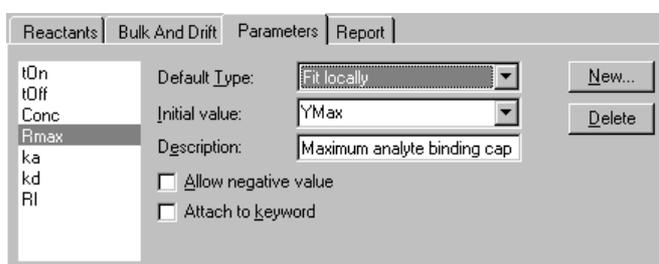
3. Press **OK** to save the changed model.

B.1.3 Adding simple terms

You can add simple terms like baseline drift, bulk refractive index changes and mass transfer limitations to existing models.

Baseline drift
Follow the procedure below to add a linear baseline drift to an existing model:

1. Open the model for editing and change the name as described above (Section B.1.1).

2. Choose the **Parameters** tab and click on **New** to add a parameter for the slope of the baseline drift. Enter a suitable name for the parameter:

3. Set the properties for this parameter to
   Default Type: Fit globally
   Initial value: 0.01 [suggested value]
   Description: [as appropriate: the description is used as a tool tip in fitting wizard and results display]

   Check the **Allow negative value** box.
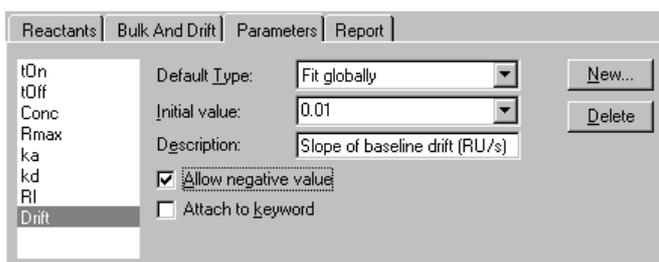
4. Choose the **Bulk And Drift** tab. Check the **Drift** box and enter the expression **Drift*(t-tOn)** in the field. The parameters **t** and **tOn** are reserved parameters for the time and injection start time in seconds respectively. This expression will therefore produce a baseline drift which intercepts the sensorgrams at the start of the injection.
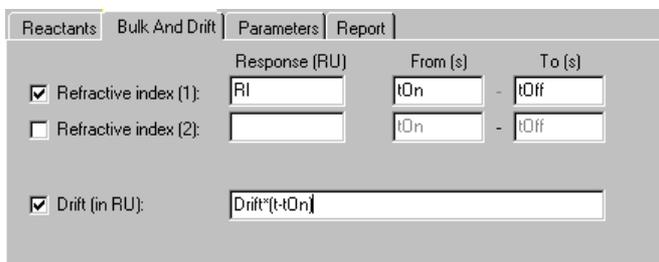
5. Press **OK** to save the changed model.

Changes in bulk refractive index
All predefined models include a parameter **RI** for the bulk refractive index contribution during sample injection. This parameter is fitted locally to each curve. BIAevaluation can also handle the situation where the bulk shifts are different at the beginning and end of the injection.

Follow the procedure below to define a model with this property:

1. Open the model for editing and change the name as described above (Section B.1.1).

2. Choose the **Parameters** tab and click on **New** to add a parameter for the refractive index contribution after the end of the injection. Enter a suitable name for the parameter:

3. Set the properties for this parameter to
    Default Type: Fit locally
    Initial value: 10 [suggested value]
    Description: [as appropriate: the description is used as a tool tip in fitting wizard and results display]

    Check the **Allow negative value** box.

4. Choose the **Bulk And Drift** tab. Check the **Refractive index (2)** box, and enter the name of your parameter in the **Response** field and **tOff** in the **From** field. Leave the **To** field empty. This specifies that the bulk contribution defined by the new parameter applies from the end of the injection to the end of the sensorgram (see illustration below).
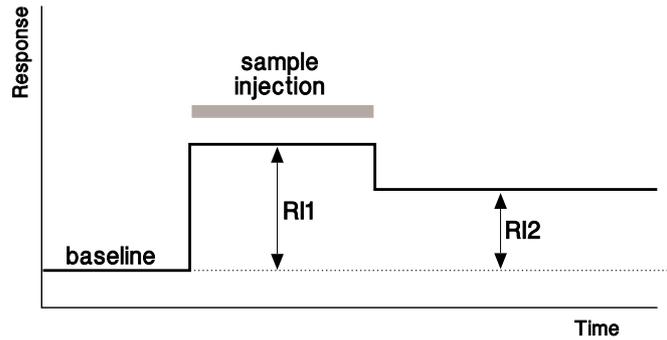
5. Press **OK** to save the changed model.

Note: If you want to define a model with no refractive index contribution, uncheck both the **Refractive index (1)** and **Refractive index (2)** boxes.

Mass transfer terms
You can include mass transfer of analyte to the surface in any model as follows:

1. Open the model for editing and change the name as described above (Section B.1.1).

2. Choose the **Parameters** tab and click on **New** to add a parameter for the rate constant for mass transfer of analyte to the surface. Enter a suitable name for the parameter (e.g. **kt**).

3. Set the properties for this parameter to
   Default Type: Fit globally
   Initial value: 0.1 [suggested value]
   Description: [as appropriate: the description is used as a tool tip in fitting wizard and results display]
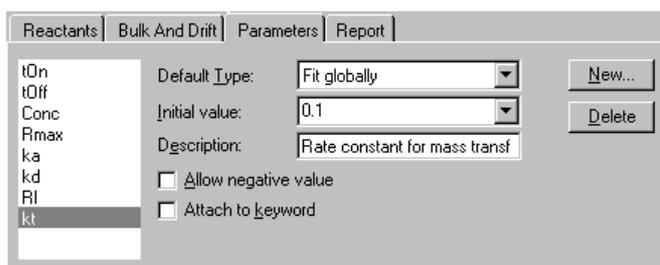
4. Choose the **Reactants** tab, select the analyte (A) and check the **Mass transfer** box. Enter the parameter name for the mass transfer constant in the field.

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5. Press **OK** to save the changed model.

Note: The mass transfer constant k_t obtained from this model is an empirical constant which is related to the generally accepted mass transfer coefficient k_m by the expression:
$$k_t \approx 10^9 \times MW \times k_m$$
Values for k_t are typically of the order of 10^8 for proteins of molecular weight 50-100 kilodaltons.

Number of blocked sites
You can allow for analytes which block more than one ligand site by steric interference (note that this is distinct from analytes *binding* to more than one ligand site, which is represented by the bivalent analyte model):

1. Open the model for editing and change the name as described above (Section B.1.1).

2. Choose the **Parameters** tab and click on **New** to add a parameter for the average number of ligand sites blocked by each analyte molecule. Enter a suitable name for the parameter:

3. Set the properties for this parameter to
Default Type: Fit globally
Initial value: 1 [suggested value]
Description: [as appropriate: the description is used as a tool tip in fitting wizard and results display]

4. Choose the **Reactants** tab, select the analyte (A) and check the **No of blocked sites** box. Enter the parameter name for the rate constant in the field.

Reactants	Bulk And Drift	Parameters	Report

A
B
AB

Category: Analyte   New...

Concentration: Conc   Delete

Injection (s):   tOn   -   tOff

☐ Masstransfer
☐ Molecular weight:
☑ No of blocked sites: n

5. Press **OK** to save the changed model.

Note: Unless the analyte is known from independent evidence to block more than one binding site, the number of blocked sites should only be used for models involving several analytes which block different numbers of sites (see the model for heterogeneous analyte as an example). Introduction of the number of blocked sites **n** in a model with only one analyte will scale the association rate constant by 1/**n** and the Rmax value by **n**, without affecting the closeness of fit.

B.1.4 Changing report parameters

The parameters displayed in the **Report** tab in the fitting window are defined in the model. You must change the model definition to change these parameters.

Follow the procedure below to change report parameters:

1. Open the model for editing and change the name as described above (Section B.1.1).

2. Choose the **Report** tab.

3. To delete a parameter, click on the row number to select the row containing the parameter and press **Del** on the keyboard. Choose **All** in the **Clear** dialogue box and click on **OK.**

> Note: Always choose **All** or **Values** in this dialogue box. Choosing **Formats** will not delete the entries.

4. To add a new parameter, enter a name and value in the first empty row in the list. The name will appear in the header row in the report tab. The value is an expression using the parameters defined in the model.

5. Press **OK** to save the changed model.

B.2 Creating new models

You can create new models with the method editor, using either a blank definition or an existing model as a template.

Caution!
Do not attempt to create models unless you understand the principles of kinetics. The only way you can check that your model is correct is to examine the rate equations generated by the model.

B.2.1 Conventions in model definition

For simultaneous k_a/k_d fitting, BIAevaluation uses a model editor based on a reaction scheme for ease of use. The reaction scheme is defined in terms of analyte (in solution), ligand (immobilized on the surface) or complex (formed on the surface) using the conventions listed below. Required properties must be entered for all species; other properties are either required in special cases or optional.

Species	Identification	Required properties	Other properties
Analyte	Injected in solution. Concentration constant during injection.	Concentration Injection start and stop	Mass transfer Molecular weight* No. of blocked sites
Ligand	Immobilized on the surface.	Binding capacity	At molecular weight*
Complex	Formed on the surface. Initial concentration = 0.	Generates response (yes/no)	Molecular weight*

Molecular weight information is used for heterogeneous analyte models only and should not be used in other circumstances.

The reaction scheme supports up to 5 first order (A = B) and second order (A + B = AB) reactions.

Note: The concentration of analyte is always constant, and models may not explicitly include expressions for changing the analyte concentration. If such a model is required (e.g for a situation where a solution reaction is occurring in parallel with the binding of analyte to the surface), a suggested work-around is to define the variable-concentration analyte as a complex which does not generate response (in the scheme below, A is the injected analyte, with constant concentration; A1 is defined as a complex which does not generate response):

B.2.2 *Creating models for simultaneous ka/kd*

Follow the steps below to create a new model (the illustrations show how the pre-defined model for heterogeneous ligand – parallel reactions is constructed):

1. Choose **Fit:Edit models** and choose the type of model you wish to create (**Simultaneous ka/kd**, **Separate ka/kd** or **General**). If you want to use an existing model as a template, select the model. Click on **New** and enter a name and description for the model.

    If you choose not to use a template, the model editor will be opened with a simple interaction for reactants A (analyte), B (ligand) and AB (complex) pre-defined. No parameters are defined. You may remove the predefined reactants if you want to use other names.
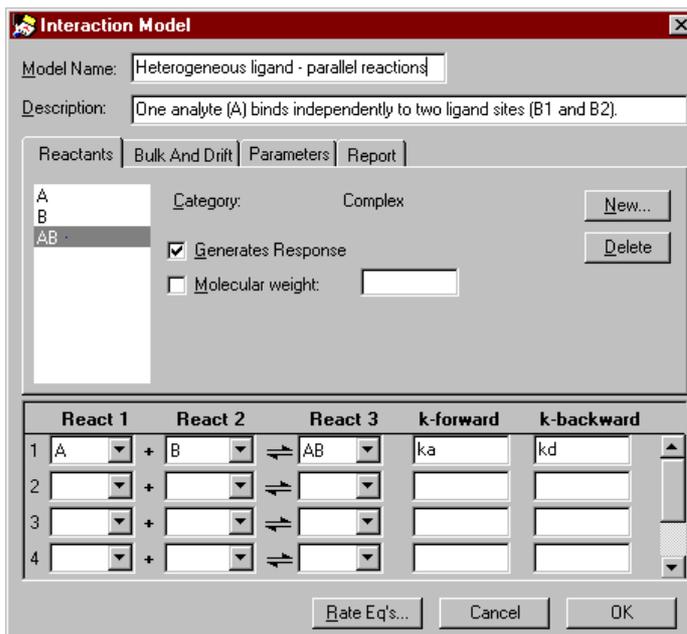
2. In the **Reactants** tab, click on **New** to add new reactants. For each
    reactant, choose whether it is analyte, ligand or complex (see
    Section B.2.1) and enter an identifier for the reactant. Enter
    parameter names or expressions for the reactant properties.
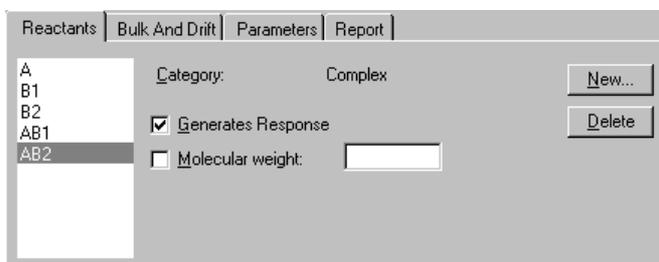
Note: Numbers are used as part of the identifier, not in the
        conventional chemical sense of stoichiometry. Thus a
        complex named AB2 does not imply two molecules of B
        binding to one of A.

3. Enter the reaction scheme using the pull-down list for each reactant. To remove a reactant from a box in the scheme, select the box and press **Del** on the keyboard. 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.

	React 1		React 2			React 3	k-forward	k-backward	
1	A ▼	+	B1 ▼	⇌		AB1 ▼	ka1	kd1	▲
2	A ▼	+	B2 ▼	⇌		AB2 ▼	ka2	kd2	
3	▼	+	▼	⇌		▼			
4	▼	+	▼	⇌		▼			▼

4. Click on the **Parameters** tab and define the parameters used in the reaction scheme. If you have only used single parameter names (as opposed to expressions) for the rate constants and properties, you can click on **Rate eq's** as a shortcut to defining parameters. BIAevaluation will then enter suggested definitions for all undefined parameters. This shortcut cannot be used if you have entered expressions.

Reactants	Bulk And Drift	Parameters	Report

tOff	Default Type:	Fit globally ▼	New...
Conc			
RMax1	Initial value:	1e3 ▼	Delete
RMax2			
ka1	Description:	Association rate constant for li	
kd1	☐ Allow negative value		
ka2	☐ Attach to keyword		
kd2			
RI			

For each parameter, define the properties as follows:

Default Type   Fit globally, fit locally or constant.

Initial value   Starting value for fitted parameters, suggested value for constants. Leave blank for constants which are attached to keywords.

Description   Text description, used as a tool tip in the fitting wizard.

Allow negative value   Check this box if negative values are allowed.

Attach to keyword　　BIAevaluation "remembers" values on a curve-by-curve basis for parameters that are attached to keywords within a session, and suggests these values for each fitting. In addition, if the parameter name is used as a keyword in a BIA result file, the values in the keyword column of the report point table will be assigned to the parameter in BIAevaluation when the result file is opened. A typical example is the parameter **Conc**, allowing concentration values entered in the method for the BIA analysis and imported automatically to BIAevaluation.

5.　Click on the **Bulk and Drift** tab and enter details for bulk refractive index contribution. See above (Section B.1.2) for a description of these parameters.

6.　Click on the **Report** tab and enter definitions for parameters to be displayed in the fitting report.

7.　Click on **Rate Eq's** to display the equations generated by BIAevaluation, and check these for consistency with the intended model (see Section B.2.3).

```
Heterogeneous ligand - Parallel reactions
A = Conc

B1[0] = RMax1
dB1/dt = - (ka1*A*B1 - kd1*AB1)

B2[0] = RMax2
dB2/dt = - (ka2*A*B2 - kd2*AB2)

AB1[0] = 0
dAB1/dt = (ka1*A*B1 - kd1*AB1)

AB2[0] = 0
dAB2/dt = (ka2*A*B2 - kd2*AB2)

Total response:

                              Copy        Close
```
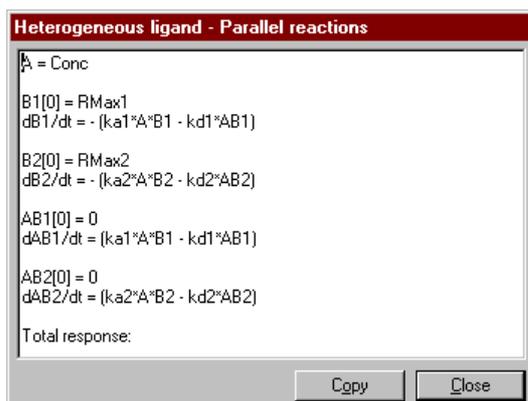
You can select the equations in the display and click on **Copy** to copy the equations to the Windows clipboard. Use this function and paste the equations in to e.g. Wordpad to print a copy of the rate equations.

8.   Click on **OK** to save the new model.

B.2.3   Checking the model
BIAevaluation will not let you save a model that is mathematically incorrect. If rate equations cannot be generated (e.g. because of a missing parameter definition), the model cannot be saved.

A model which is internally consistent is however not necessarily correct. There are two ways of examining a new model for correctness:

1.   In the model definition dialogue box, click on **Rate eq's** and examine the generated equations carefully. The equations are presented as differential equations for the rate of change of components in the interaction. For example, the rate equations for a simple 1:1 interaction are presented as:
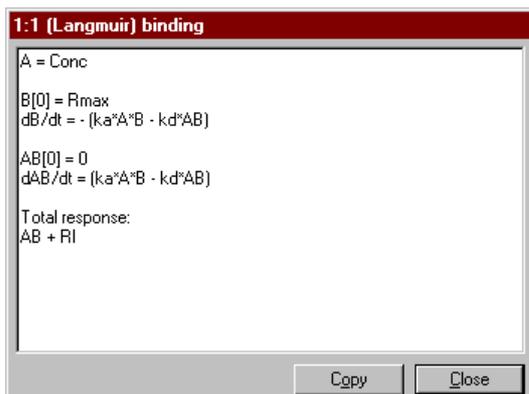
You should preferably derive your own rate equations from the model you intended to define and compare these with the equations generated by BIAevaluation. Pay particular attention to the units implied for the various parameters in the equation, remembering that the ultimate units for the total response must be in RU/s.

2. Use the simulator to generate sensorgrams for your model (see Section 4.7). Enter parameter values that are typical for your system and ensure that the simulator generates reasonable sensorgrams. This approach can be useful for checking that the parameters are written with correct units, since mistakes in this respect will often result in errors of several orders of magnitude.

Important!
Apart from careful attention to the model definition and the aids mentioned above, there is no way to check that the model as entered correctly describes the required interaction situation. A good understanding of kinetics is therefore essential for creation of new models.

B.2.4 Creating models for separate ka/kd and general fitting

For separate ka/kd and general fitting, models are defined in terms of integrated rate equations or general equations. The reaction scheme is not used.

Models of this type are defined using the **Parameters** and **Report** tabs only (see steps 4 and 6 in Section B.2.2). The editor window has a field for the fitting equation and an identification of the independent variable. The equation is written as a formula expressing the ordinate (y-values) as a function of the independent variable (x-values). For fitting sensorgram data to rate equations (kinetics with separate ka/kd), the formula must represent the integrated rate equation, and

the independent variable is always time (see illustration below). This kind of model cannot be used for rate equations that cannot be integrated.

For general fitting models, any expression may be used in the formula. The example below shows the model definition for a linear fit (y=kx+m):

Appendix C.
Predefined models

This appendix describes the predefined models and example data files provided with BIAevaluation. For details of how each model is defined, open the model in the model editor and examine the definition screens. For conventions in model definition, see Section B.2.1.

The models included in BIAevaluation are:

Simultaneous ka/kd
- 1:1 (Langmuir) binding
- 1:1 binding with drifting baseline
- 1:1 binding with mass transfer
- Bivalent analyte
- Heterogeneous analyte – competing reactions
- Heterogeneous ligand – parallel reactions
- Two-state reaction (conformation change)

Separate ka/kd
- 1:1 (Langmuir) association
- 1:1 (Langmuir) dissociation

General
- 4-parameter equation
- Linear fit
- Solution affinity
- Steady state affinity

Additional models that can be imported into BIAevaluation are provided in the file MORE MODELS.MDL. These are:
- 1:1 binding with exponential baseline drift
- 1:1 binding with mass transfer, km(m/s)
- 1:1 (Langmuir) association (kobs)
- Average

More models may be published as they are developed on the BIAweb Internet site (http://www.biacore.com).

Example data is provided for several of the models in the form of BIAevaluation project files. Values for all constants required for fitting to the respective models have been entered in the example data files. Unless otherwise stated, the example data is obtained from actual BIACORE runs.

C.1   Models for simultaneous ka/kd

C.1.1   1:1 (Langmuir) binding

This is the simplest model for 1:1 interaction between analyte and immobilized ligand, and is equivalent to the Langmuir isotherm for adsorption to a surface.

Reaction scheme and reactants

Species	Type	Comments
A	Analyte	–
B	Ligand	–
AB	Complex	–

Bulk and drift

Bulk refractive index contribution **RI** during sample injection.
No drift.

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Parameters

Parameter	Description	Fitting status	Comments
tOn	Starting time for sample injection (s)	Constant	
tOff	Ending time for sample injection (s)	Constant	
Conc	Analyte concentration (M)	Constant	Attached to keyword
Rmax	Maximum analyte binding capacity (RU)	Fit globally	
k_a	Association rate constant ($M^{-1}s^{-1}$)	Fit globally	
k_d	Dissociation rate constant (s^{-1})	Fit globally	
RI	Bulk refractive index contribution (RU)	Fit locally	Negative values allowed

Rate equations


```
1:1 (Langmuir) binding

A = Conc

B[0] = Rmax
dB/dt = - (ka*A*B - kd*AB)

AB[0] = 0
dAB/dt = (ka*A*B - kd*AB)

Total response:
AB + RI
```

Copy   Close

Calculated report parameters
The fitting report also includes values for selected parameters.

Parameter	Value	Description	Status
K_A (1/M)	k_a/k_d	Equilibrium binding (affinity) constant	Global
K_D (M)	k_d/k_a	Equilibrium dissociation constant	Global
R_{eq} (RU)	$k_a*Conc*R_{max}/(k_a*Conc+k_d)$	Steady state binding level	Local
k_{obs} (1/s)	$k_a*Conc+k_d$	Observed rate constant (slope of ln(dR/dt) against t)	Local

Example data
BIAevaluation project file **Langmuir\P24.ble**.

Note: The closeness of this fit for this example data can be improved
        slightly by setting Rmax to a local parameter. This is
        experimentally justified since the data was obtained from
        binding HIV p24 protein to monoclonal antibody captured on
        a rabbit anti-mouse Fc (RAM-Fc) surface, so that the level of
        captured antibody may vary slightly between sensorgrams.

C.1.2 1:1 binding with drifting baseline

This model describes a 1:1 interaction superimposed on a linear
baseline drift. Instrument drift is normally low in BIACORE systems
(typically less than 0.005RU/s) and can largely be eliminated by the
use of a reference cell, but the use of a model which includes linear
drift may be appropriate in experiments with low surface binding
capacity (Rmax 100 RU or less). A model with exponentially drifting
baseline, which may be used as an approximation to a capturing
situation with slow dissociation of capturing molecule, is provided in
the additional models file (see Section C.4).

Reaction scheme and reactants

	React 1		React 2			React 3		k-forward	k-backward	
1	A	▼	+	B	▼	⇌	AB	▼	ka	kd	▲
2		▼	+		▼	⇌		▼			
3		▼	+		▼	⇌		▼			
4		▼	+		▼	⇌		▼			▼

Species	Type	Comments
A	Analyte	–
B	Ligand	–
AB	Complex	–

Bulk and drift

Bulk refractive index contribution **RI** during sample injection.

Drift in RU = Drift*(t-tOn)
where   Drift is the rate constant for linear drift (RU/s)
           tOn is the injection start time

This expression produces a drift component which has a value of zero at the injection start. The drift may be positive or negative.

Parameters

Parameter	Description	Fitting status	Comments
tOn	Starting time for sample injection (s)	Constant	
tOff	Ending time for sample injection (s)	Constant	
Drift	Slope of the baseline drift (RU.s^{-1})	Fit locally	Negative values allowed
Conc	Analyte concentration (M)	Constant	Attached to keyword
Rmax	Maximum analyte binding capacity (RU)	Fit globally	
k_a	Association rate constant (M^{-1}s^{-1})	Fit globally	
k_d	Dissociation rate constant (s^{-1})	Fit globally	
RI	Bulk refractive index contribution (RU)	Fit locally	

Rate equations

```
1:1 binding with drifting baseline

A = Conc

B[0] = RMax
dB/dt = - (ka*A*B - kd*AB)

AB[0] = 0
dAB/dt = (ka*A*B - kd*AB)

Total response:
AB + RI + (Drift*(t-tOn))
```

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Calculated report parameters
The fitting report also includes values for selected parameters.

Parameter	Value	Description	Status
K_A (1/M)	k_a/k_d	Equilibrium binding (affinity) constant	Global
K_D (M)	k_d/k_a	Equilibrium dissociation constant	Global
R_{eq} (RU)	$k_a*Conc*R_{max}/(k_a*Conc+k_d)$	Steady state binding level	Local
k_{obs} (1/s)	$k_a*Conc+k_d$	Observed rate constant (slope of ln(dR/dt) against t)	Local

Example data
None provided.

C.1.3 1:1 binding with mass transfer

This model describes a 1:1 interaction with a simplified expression for mass transfer limitation. An alternative model for mass transfer-limited interactions is provided in the additional models file (Section C.4).

Reaction scheme and reactants

Species	Type	Comments
A	Analyte	Mass transfer limited, see note.
B	Ligand	–
AB	Complex	–

Note: Mass transfer limitation is included by checking the **Mass transfer** box in the analyte properties.

Bulk and drift

Bulk refractive index contribution **RI** during sample injection.
No drift.

Parameters

Parameter	Description	Fitting status	Comments
tOn	Starting time for sample injection (s)	Constant	
tOff	Ending time for sample injection (s)	Constant	
Conc	Analyte concentration (M)	Constant	Attached to keyword
Rmax	Maximum analyte binding capacity (RU)	Fit globally	
k_a	Association rate constant ($M^{-1}s^{-1}$)	Fit globally	
k_d	Dissociation rate constant (s^{-1})	Fit globally	
RI	Bulk refractive index contribution (RU)	Fit locally	
k_t	Rate constant for mass transfer (s^{-1})	Fit globally	See comment under rate equations

Rate equations

```
1:1 binding with mass transfer

A(solution) = Conc

A[0] = 0
dA/dt = kt*(Conc-A) - (ka*A*B - kd*AB)

B[0] = Rmax
dB/dt = - (ka*A*B - kd*AB)

AB[0] = 0
dAB/dt = (ka*A*B - kd*AB)

Total response:
AB + RI
```
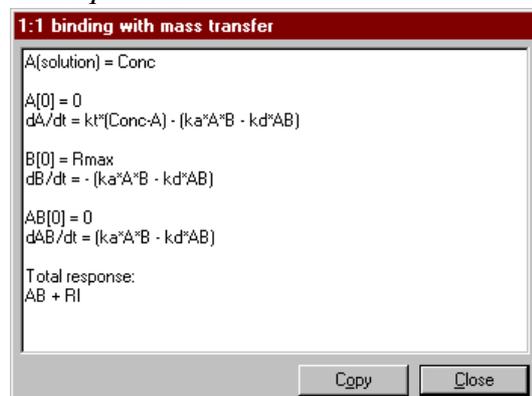
Notes: Introduction of mass transfer limitation (by checking the **Mass transfer** box) introduces an expression for **dA/dt,** the rate of change in concentration of analyte A. In the rate equations, **A** represents the analyte close to the surface which is capable of binding to ligand. The basic assumption of constant analyte concentration in BIAevaluation (see Section B.2.1) is

maintained insofar as the constant value **Conc** represents the bulk concentration of analyte.

The mass transfer constant k_t obtained from this model is an empirical constant which is related to the generally accepted mass transfer coefficient k_m by the expression:

$$k_t \approx 10^9 \times MW \times k_m$$

Values for k_t are typically of the order of 10^8 for proteins of molecular weight 50-100 kilodaltons.

Calculated report parameters
The fitting report also includes values for selected parameters.

Parameter	Value	Description	Status
K_A (1/M)	k_a/k_d	Equilibrium binding (affinity) constant	Global
K_D (M)	k_d/k_a	Equilibrium dissociation constant	Global
R_{eq} (RU)	$k_a*Conc*R_{max}/(ka*Conc+k_d)$	Steady state binding level	Local
k_{obs} (1/s)	$k_a*Conc+k_d$	Observed rate constant (slope of ln(dR/dt) against t)	Local

Example data
BIAevaluation project file **Mass Transfer\Igf1.ble**.

C.1.4 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. Binding to the first ligand molecule is described by a single set of rate constants, so that the two sites on the analyte are equivalent in the first binding step. Binding of the second ligand molecule is described by a second set of rate constants, allowing the model to take cooperative effects into account. If the two sets of rate constants are the same, there are no cooperative effects and the increased affinity resulting from bivalent binding is frequently referred to as avidity.

The model may be relevant to studies among others with signalling molecules binding to immobilized cell surface receptors (where dimerization of the receptor is common) and to studies using intact

antibodies binding to immobilized antigen. Bear in mind however that the association rate constant for the second site interaction is reported in the unconventional units of $RU^{-1}s^{-1}$ (see below).

If you fit experimental data to this model, use **View:Components** to examine the relative contributions of first and second site binding to the fitted curves.

Reaction scheme and reactants

	React 1		React 2			React 3		k-forward	k-backward	
1	A	▼	+	B	▼	⇌	AB	▼	ka1	kd1	▲
2	AB	▼	+	B	▼	⇌	AB2	▼	ka2	kd2	
3		▼	+		▼	⇌		▼			
4		▼	+		▼	⇌		▼			▼

Species	Type	Comments
A	Analyte	–
B	Ligand	–
AB	Complex	Formed by binding one A to B
AB2	Complex	Formed by binding one A to AB

Bulk and drift

Reactants	Bulk And Drift	Parameters	Report

	Response (RU)	From (s)	To (s)
☑ Refractive index (1):	RI	tOn	- tOff
☐ Refractive index (2):		tOff	-
☐ Drift (in RU):			

Bulk refractive index contribution **RI** during sample injection.
No drift.

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Parameters

Parameter	Description	Fitting status	Comments
tOn	Starting time for sample injection (s)	Constant	
tOff	Ending time for sample injection (s)	Constant	
Conc	Analyte concentration (M)	Constant	Attached to keyword
Rmax	Maximum analyte binding capacity (RU)	Fit globally	
ka1	Association rate constant for A+B=AB ($M^{-1}s^{-1}$)	Fit globally	
kd1	Dissociation rate constant for AB=A+B (s^{-1})	Fit globally	
ka2	Association rate constant for AB+B=AB2 ($RU^{-1}s^{-1}$)	Fit globally	See note
kd2	Dissociation rate constant for AB2=AB+B (s^{-1})	Fit globally	
RI	Bulk refractive index contribution (RU)	Fit locally	Negative values allowed

Note that the association rate constant for the second site binding is in $RU^{-1}s^{-1}$, not $M^{-1}s^{-1}$. This is because the reaction involves complex (in RU) binding to ligand (in RU).

Rate equations

Bivalent analyte

```
A = Conc

B[0] = RMax
dB/dt = - (ka1*A*B - kd1*AB) - (ka2*AB*B - kd2*AB2)

AB[0] = 0
dAB/dt = (ka1*A*B - kd1*AB) - (ka2*AB*B - kd2*AB2)

AB2[0] = 0
dAB2/dt = (ka2*AB*B - kd2*AB2)

Total response:
AB + AB2 + RI
```

Copy   Close

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Calculated report parameters
The fitting report includes values for selected parameters.

Example data
None provided.

C.1.5 Heterogeneous analyte (competing reactions)
This model is intended for analysis of the kinetics of interaction of
mixtures of two analytes. A common application is determination of
the kinetics for a low molecular weight analyte binding to immobilized
ligand, deduced 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.

If you fit experimental data to this model, use **View:Components** to
examine the relative contributions of the two analytes to the fitted
curves.

Reaction scheme and reactants

	React 1	React 2		React 3	k-forward	k-backward
1	A1 ▼	+	B ▼	⇌	A1B ▼	ka1	kd1
2	A2 ▼	+	B ▼	⇌	A2B ▼	ka2	kd2
3	▼	+	▼	⇌	▼		
4	▼	+	▼	⇌	▼		

Species	Type	Comments
A1	Analyte	–
A2	Analyte	
B	Ligand	–
A1B	Complex	Formed by binding A1 to B
A2B	Complex	Formed by binding A2 to B

Bulk and drift

Reactants	Bulk And Drift	Parameters	Report		
		Response (RU)	From (s)		To (s)
☑ Refractive index (1):		RI	tOn	-	tOff
☐ Refractive index (2):			tOff	-	
☐ Drift (in RU):					

Bulk refractive index contribution **RI** during sample injection.
No drift.

Parameters

Parameter	Description	Fitting status	Comments
tOn	Starting time for sample injection (s)	Constant	
tOff	Ending time for sample injection (s)	Constant	
Conc	Analyte concentration (M)	Constant	Attached to keyword
Rmax	Maximum analyte binding capacity (RU)	Fit globally	
ka1	Association rate constant for $A1+B=A1B$ ($M^{-1}s^{-1}$)	Fit globally	
kd1	Dissociation rate constant for $A1B=A1+B$ (s^{-1})	Fit globally	
mw1	(Relative) molecular weight of A1	Constant	
n1	Number of binding sites blocked by A1	Constant	Default value 1
ka2	Association rate constant for $A2+B=A2B$ ($M^{-1}s^{-1}$)	Fit globally	
kd2	Dissociation rate constant for $A2B=A2+B$ (s^{-1})	Fit globally	
mw2	(Relative) molecular weight of A2	Constant	
n2	Number of binding sites blocked by A2	Constant	Default value 1
RI	Bulk refractive index contribution (RU)	Fit locally	Negative values allowed

Rate equations

```
┌─────────────────────────────────────────────────────────┐
│ Heterogeneous analyte - Competing reactions             │
├─────────────────────────────────────────────────────────┤
│ A1 = Conc1                                              │
│ A2 = Conc2                                              │
│                                                         │
│ B[0] = Rmax/mw1                                         │
│ dB/dt = - (ka1*A1*mw1*B - kd1*A1B)/mw1*n1 - (ka2*A2*mw2*B - │
│ kd2*A2B)/mw2*n2                                         │
│                                                         │
│ A1B[0] = 0                                             │
│ dA1B/dt = (ka1*A1*mw1*B - kd1*A1B)                     │
│                                                         │
│ A2B[0] = 0                                             │
│ dA2B/dt = (ka2*A2*mw2*B - kd2*A2B)                     │
│                                                         │
│ Total response:                                        │
│ A1B + A2B + RI                                         │
│                                                         │
│                          [ Copy ]      [ Close ]       │
└─────────────────────────────────────────────────────────┘
```

Calculated report parameters
The fitting report also includes values for selected parameters.

Parameter	Value	Description	Status
KA1 (1/M)	k_a1/k_d1	Affinity constant for A1+B	Global
KA2 (1/M)	k_a2/k_d2	Affinity constant for A2+B	Global

Example data
BIAevaluation project file **Competitive\Zkomp.ble**. To fit this data
adequately, the higher molecular weight analyte is assumed to block
two ligand binding sites per analyte molecule.

C.1.6 Heterogeneous ligand (parallel reactions)

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.

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.

If you fit experimental data to this model, use **View:Components** to examine the relative contributions of binding to the two ligands to the fitted curves.

Reaction scheme and reactants

	React 1	React 2		React 3	k-forward	k-backward
1	A	+ B1	⇌	AB1	ka1	kd1
2	A	+ B2	⇌	AB2	ka2	kd2
3		+	⇌			
4		+	⇌			

Species	Type	Comments
A	Analyte	–
B1	Ligand	–
B2	Ligand	–
AB1	Complex	Formed by binding A to B1
AB2	Complex	Formed by binding A to B2

Bulk and drift

Reactants	Bulk And Drift	Parameters	Report

	Response (RU)	From (s)	To (s)
☑ Refractive index (1):	RI	tOn	- tOff
☐ Refractive index (2):		tOff	-
☐ Drift (in RU):			

Bulk refractive index contribution **RI** during sample injection.
No drift.

Parameters

Parameter	Description	Fitting status	Comments
tOn	Starting time for sample injection (s)	Constant	
tOff	Ending time for sample injection (s)	Constant	
Conc	Analyte concentration (M)	Constant	Attached to keyword
Rmax	Maximum analyte binding capacity (RU)	Fit globally	
ka1	Association rate constant for A+B1=AB1 ($M^{-1}s^{-1}$)	Fit globally	
kd1	Dissociation rate constant for AB1=A+B1 (s^{-1})	Fit globally	
ka2	Association rate constant for A+B2=AB2 ($M^{-1}s^{-1}$)	Fit globally	
kd2	Dissociation rate constant for AB2=A+B2 (s^{-1})	Fit globally	
RI	Bulk refractive index contribution (RU)	Fit locally	Negative values allowed

Rate equations

Heterogeneous ligand - Parallel reactions

```
A = Conc

B1[0] = RMax1
dB1/dt = - (ka1*A*B1 - kd1*AB1)

B2[0] = RMax2
dB2/dt = - (ka2*A*B2 - kd2*AB2)

AB1[0] = 0
dAB1/dt = (ka1*A*B1 - kd1*AB1)

AB2[0] = 0
dAB2/dt = (ka2*A*B2 - kd2*AB2)

Total response:
```

Calculated report parameters
The fitting report also includes values for selected parameters.

Parameter	Value	Description	Status
KA1 (1/M)	k_a1/k_d1	Affinity constant for A+B1	Global
KA2 (1/M)	k_a2/k_d2	Affinity constant for A+B2	Global

Example data
None provided.

C.1.7   Two-state (conformational change)
This model describes a 1:1 binding of analyte to immobilized ligand followed by a conformational change in 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 a multi-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.

Reaction scheme and reactants

	React 1		React 2		React 3	k-forward	k-backward	
1	A ▼	+	B ▼	⇌	AB ▼	ka1	kd1	▲
2	AB ▼	+	▼	⇌	ABx ▼	ka2	kd2	
3	▼	+	▼	⇌	▼			
4	▼	+	▼	⇌	▼			▼

Species	Type	Comments
A	Analyte	–
B	Ligand	–
AB	Complex	Formed by binding A to B
ABx	Complex	Formed by conformational change from AB

Note that in this model the conformational change only occurs in the complex AB, not in free ligand B. In addition, the only way ABx can dissociate to release free A is through prior conversion to the form AB. This is probably a considerable simplification of real systems involving conformational change.

Bulk and drift

Reactants | Bulk And Drift | Parameters | Report

	Response (RU)	From (s)	To (s)
☑ Refractive index (1):	RI	tOn	- tOff
☐ Refractive index (2):		tOff	-
☐ Drift (in RU):			

Bulk refractive index contribution **RI** during sample injection.
No drift.

Parameters

Parameter	Description	Fitting status	Comments
tOn	Starting time for sample injection (s)	Constant	
tOff	Ending time for sample injection (s)	Constant	
Conc	Analyte concentration (M)	Constant	Attached to keyword
Rmax	Maximum analyte binding capacity (RU)	Fit globally	
ka1	Association rate constant for A+B1=AB1 ($M^{-1}s^{-1}$)	Fit globally	
kd1	Dissociation rate constant for AB1=A+B1 (s^{-1})	Fit globally	
ka2	Forward rate constant for AB=ABx (s^{-1})	Fit globally	
kd2	Backward rate constant for AB=ABx (s^{-1})	Fit globally	
RI	Bulk refractive index contribution (RU)	Fit locally	Negative values allowed

Rate equations

```
Two state reaction (conformation change)

A = Conc

B[0] = Rmax
dB/dt = -(ka1*A*B - kd1*AB)

AB[0] = 0
dAB/dt = (ka1*A*B - kd1*AB) - (ka2*AB - kd2*ABx)

ABx[0] = 0
dABx/dt = (ka2*AB - kd2*ABx)

Total response:
AB + ABx + RI

                              Copy      Close
```

Calculated report parameters
The fitting report also includes values for selected parameters.

Parameter	Value	Description	Status
K (1/M)	$(k_a1/k_d1)*$ (k_a2/k_d2)	Apparent affinity constant	Global

Example data
BIAevaluation project file **Two State\Myo.ble**.

C.2 Models for separate ka/kd

Models for separate ka/kd fitting describe only the association or dissociation phase of the sensorgram, and are intended for use in situations where one of the phases cannot be used for evaluation. Wherever possible, simultaneous evaluation of ka and kd is preferable, both for convenience and robustness.

Models for separate ka/kd are defined in terms of an integrated rate equation rather than a reaction scheme. Systems where the rate equations cannot be integrated mathematically cannot be modelled with separate ka/kd. The model definition specifies the independent variable (x-axis parameter) for the equation, which in kinetic contexts is always time.

C.2.1   1:1 (Langmuir) association
This model fits the association phase of a 1:1 interaction to the integrated rate equation:

$$R = R_{eq}\left(1 - e^{-(k_aC + k_d)(t - t_0)}\right) + RI$$

where

$$R_{eq} = \frac{k_aC}{k_aC + k_d} \cdot R_{max}$$

The model requires values for k_d, which may be obtained by first evaluating the dissociation phase of the sensorgram. The association phase can be evaluated without knowledge of k_d by using the model **1:1 (Langmuir) association (kobs)** provided as an additional model (Section C.4.3).

Parameters

Parameter	Description	Fitting status	Comments
ka	Association rate constant ($M^{-1}s^{-1}$)	Fit locally	
Rmax	Maximum analyte binding capacity (RU)	Fit locally	
Conc	Analyte concentration (M)	Constant	
t0	Injection start time (s)	Constant	
kd	Dissociation rate constant (s^{-1})	Constant	
RI	Bulk refractive index contribution (RU)	Fit locally	Negative values allowed

Calculated report parameters
The fitting report also includes values for selected parameters.

Parameter	Value	Description	Status
K_A (1/M)	k_a/k_d	Equilibrium binding (affinity) constant	Global
K_D (M)	k_d/k_a	Equilibrium dissociation constant	Global
R_{eq} (RU)	$k_a*Conc*R_{max}/$ $(k_a*Conc+k_d)$	Steady state binding level	Local
k_{obs} (1/s)	$k_a*Conc+k_d$	Observed rate constant (slope of ln(dR/dt) against t)	Local

C.2.2 1:1 (Langmuir) dissociation
This model fits the dissociation phase of a 1:1 interaction to the integrated rate equation:

$$R = R_0 e^{-k_d(t-t_0)} + \text{Offset}$$

The **Offset** term allows the dissociation phase to approach a non-zero baseline (compare the use of the second bulk refractive index contribution in models for simultaneous ka/kd, Section B.1.3).

Parameters

Parameter	Description	Fitting status	Comments
kd	Dissociation rate constant (s^{-1})	Fit locally	
R0	Response at start of fitted data (RU)	Constant	
t0	Time at start of fitted data (s)	Constant	
Offset	Response at infinite time (RU)	Fit locally	

C.3 Models for general fitting

Models for general fitting are intended for fitting non-kinetic data. Applications of such fitting procedures in BIAevaluation include calculation of calibration curves using a 4-parameter equation (Section 5.2), calculation of affinity in solution from measurements of free interactant concentrations (Section 6.3) and calculation of surface interaction affinity from a plot of R_{eq} against C (Section 6.2).

Models for general fitting are defined in terms of an expression describing the ordinate (y-axis) value as a function of an independent variable (x-axis).

C.3.1 4-parameter equation

The 4-parameter equation is a general curve fitting model which is suitable for generating calibration curves for concentration determination (Section 5.2). The model fits a plot of the response against concentration to the equation:

$$\text{response} = R_{hi} - \frac{R_{hi} - R_{lo}}{1 + \left(\dfrac{\text{Conc}}{A_1}\right)^{A_2}}$$
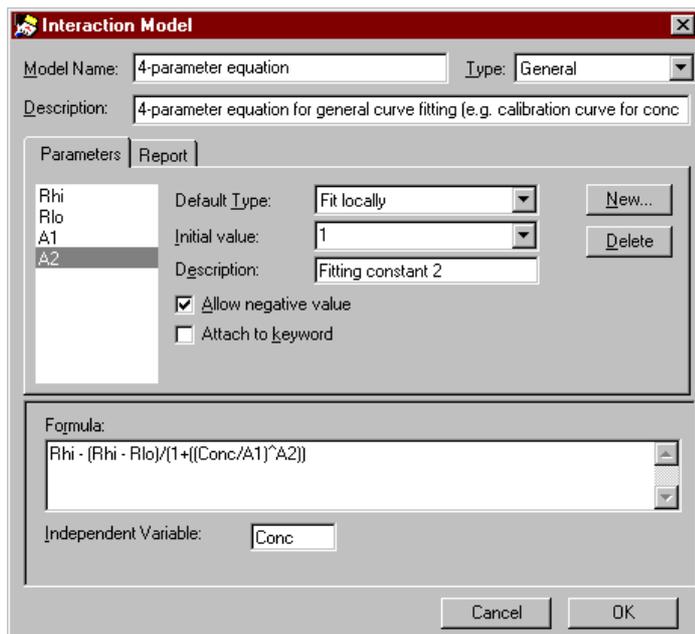
Parameters

Parameter	Description	Fitting status	Comments
R_{hi}	Response at infinite concentration	Fit locally	
R_{lo}	Response at zero concentration	Fit locally	
Conc	Analyte concentration (M)	Constant	Independent variable
A_1	Fitting constant	Fit locally	
A_2	Fitting constant	Fit locally	

The fitting constants A1 and A2 are mathematical parameters that do not have any direct physical interpretation.

C.3.2   Linear fit
This model fits data to a straight line:

$y=kx+m$

BIAevaluation Software Handbook
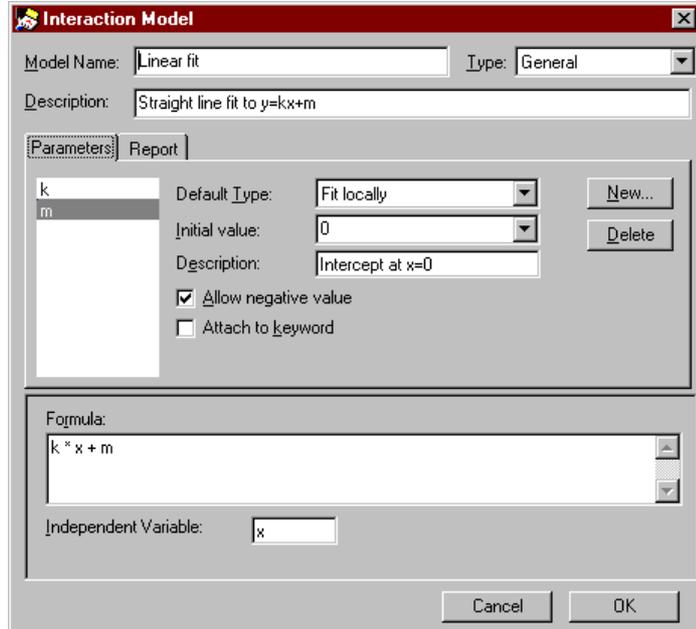
C.3.3 Solution affinity

This model derives the affinity constant in solution from measurements of the free concentration of one interactant in steady state mixtures with the other. In the steady state mixtures, the total concentration of component A is varied while that of B is kept constant. To use the model, prepare a plot of the free concentration of B against the total concentration of A and fit the curve to the model. The total concentration of B is entered as a constant in the fitting wizard. The affinity constant for the interaction of A and B is displayed in the report (see (Section 6.3).

C.3.4 Steady state affinity

This model calculates the affinity constant for the surface interaction from a plot of steady state binding level R_{eq} against analyte concentration (Section 6.2).

The steric interference factor **n** may be used to compensate for steric blocking of additional binding sites by a single analyte molecule. Note that this is not the same as multivalent analyte binding to more than one site, which gives an avidity effect.

C.4   Additional models

The models described below may be imported into BIAevaluation from the file **More Models.mdl**.

C.4.1   1:1 binding with exponential baseline drift

This model for simultaneous ka/kd evaluation describes 1:1 binding superimposed on an exponential decrease in baseline starting at a given time. By setting the start of the baseline drift to the start of the sensorgram (the default initial value), the model approximates a capturing situation with slight dissociation of the capturing molecule from the surface.

This model is not a completely correct description of a capturing situation with dissociation, since the baseline drift does not account for the loss in analyte binding capacity resulting from dissociation of the capturing molecule with attached ligand. Moreover, the loss in response is an approximation which does not take into account the difference in effect between dissociation of a capturing molecule with and without attached ligand. However, definition of an accurate model for the capturing situation is difficult, and the increased number of parameters required potentially reduces the stability of the fitting (see Section A.4). A situation where capturing molecule dissociates to a significant extent is not suitable for kinetic studies, and the model with exponential baseline drift is a fair approximation provided that the drift is small.

The baseline should not be set to zero when this model is used to fit to sensorgrams.

If you fit experimental data to this model, use **View:Components** to examine the relative contributions of analyte binding and baseline drift to the fitted curves.

The model is identical to that for 1:1 (Langmuir) interaction except for the addition of an exponential expression for drift:

$$\text{Drift} = R_0 e^{-k\_bg(t-tOn)}$$

where R_0 is the response at the start of drift
       k_bg is the rate constant for the drift in $RU.s^{-1}$

The drift may be positive or negative (k_bg is allowed to have a negative value). For evaluation of capturing situations with background dissociation, the value obtained for k_bg should always be positive (so that the drift is negative).

C.4.2   1:1 binding with mass transfer km(m/s)

This model for simultaneous ka/kd evaluation represents an alternative approach to modelling mass transfer limitations (compare Section C.1.3), using explicit mathematical expressions for the forward and backward rate equations instead of checking the **Mass transfer** box for the analyte.

BIAevaluation Software Handbook
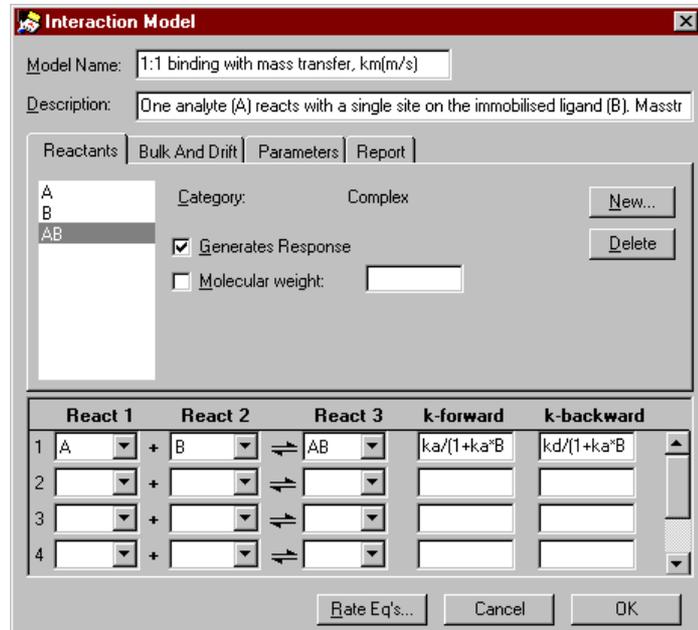
The forward and backward rate constants are given by

$$k_{forward} = \frac{k_a}{1 + \dfrac{k_a B}{10^9 \cdot mw \cdot k_m}} \qquad \text{entered as } \textbf{ka/(1+ka*B/(1e9*mw*km))}$$

and

$$k_{backward} = \frac{k_d}{1 + \dfrac{k_a B}{10^9 \cdot mw \cdot k_m}} \qquad \text{entered as } \textbf{kd/(1+ka*B/(1e9*mw*km))}$$

where k_a, k_d are the rate constants for the interaction
mw is the molecular weight of the analyte
k_m is the mass transport coefficient
10^9 is a conversion factor between molar concentrations and RU

This approach thus modifies the effective forward and backward rate constants directly with an expression that is related to both the rate of mass transfer and the concentration of available binding sites (i.e. the potential rate of interaction). At high mass transfer rates, the denominator in the expressions for $k_{forward}$ and $k_{backward}$ approaches 1 and the constants approach the interaction rate constants.

The units of k_m used in this model are $m.s^{-1}$, and the model includes analyte molecular weight as a parameter. When mass transfer is modelled using the **Mass transfer** box for analyte (Section C.1.3), the constant k_t is expressed in s^{-1} and the molecular weight is included in the magnitude of k_t. These two ways of expressing the mass transfer constant are related by the expression:

$$k_t \approx 10^9 \cdot mw \cdot k_m$$

Fitting mass transport-limited data to the two kinds of model gives equivalent results.

C.4.3 1:1 Langmuir association (kobs)

This model for separate ka/kd evaluation calculates the observed rate constant ($k_{obs}=k_aC+k_d$) and the steady state binding level R_{eq}. The association rate constant k_a may be obtained from a secondary plot of k_{obs} against C without knowledge of the dissociation rate constant k_d.

Parameters

Parameter	Description	Fitting status	Comments
kobs	Observed rate constant (s^{-1})	Fit locally	
Req	Steady state binding level (RU)	Fit locally	
Conc	Analyte concentration (M)	Constant	
t0	Injection start time (s)	Constant	
RI	Bulk refractive index contribution (RU)	Fit locally	Negative values allowed

Notes: The analyte concentration **Conc** is not used in fitting data to the model. It is however included as a parameter so that a secondary plot of k_{obs} against C can be constructed from the fitting data.

To obtain a correct estimate of the steady state response R_{eq}, the starting time for the association t_0 must be set to the actual time of injection. By default, t_0 is the starting time for the selected data. You may therefore need to adjust the suggested values for t_0 before performing the fit. This is particularly important if you intend to plot R_{eq} against C to estimate the affinity constant.

C.4.4 Average

This is a general fitting model designed to calculate the average value over a selected portion of a curve. It is useful for calculating steady state levels from sensorgrams where steady state has been reached.

The formula used for the fitting equation is simply a single parameter representing the average:

The initial value for this parameter is set to **YAverage**, which is the average ordinate (y-axis) value over the range of the selected data.

When steady-state binding data is fitted using this model, the fitting window displays the average value as a horizontal line over the range of selected data (this line is in fact the calculated "fitted curve"). The **Parameters** tab in the fit window displays values for **Req** and **Conc**, so that a plot of R_{eq} against C can be constructed directly using **Fit:Plot Parameters** (see Section 4.6).

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