Malvern

LABEL-FREE BINDING ANALYSIS

MICROCALORIMETRY

MICROCAL **PEAQ-ITC** ANALYSIS SOFTWARE

USER MANUAL

MICROCAL PEAQ-ITC ANALYSIS SOFTWARE USER MANUAL

MAN0576-01-EN-00

Head office:

Malvern Instruments Ltd.
Enigma Business Park,
Grovewood Road,
Malvern,
Worcestershire WR14 1XZ
United Kingdom.

Tel + [44] (0)1684-892456

Fax + [44] (0)1684-892789

CONTENTS

1 INTRODUCTION

The following topics are covered in this section:

About MicroCal PEAQ-ITC Analysis Software

Introduction

This section describes the software and the software installation.

MicroCal PEAQ-ITC Analysis Software analyzes calorimetric data imported from ITC experiments. Data can be processed from both MicroCal PEAQ-ITC and MicroCal PEAQ-ITC Automated. With the software you can easily

- import ITC experiments,
- assign control experiments,
- adjust baselines and integration ranges,
- fit binding models, and
- export the results as images and tables.

Analysis workflow

Workflow step	Description
Select fitting model	Raw experimental data are processed and fitted to a mathematical model of the binding. When starting a new analysis, the fitting model **One Set of Sites** is selected as default. All valid experiments in the analysis are evaluated using the chosen fitting model. If needed, select another fitting model in the **Fitting Model** menu in the **Overview** workspace.
Adjust fitted baseline and integration region.	MicroCal PEAQ-ITC Analysis Software automatically determines the baseline and the range over which to integrate the injection peaks. If needed, adjust the integration baseline and integration range for each peak in the **Adjust Baseline** workspace (the workspace name and function may differ with selected fitting model).
Assign control experiments	In the **Assign Controls** workspace you can assign control experiments to your results. This workspace is not available for all fitting models.

Workflow step	Description
Adjust fitting parameters	It is possible to adjust parameters for the selected fitting model in the **Adjust Fit** workspace.
Export the analysis	In the **Presentation** workspace you can export figures and tables of the analysis.

MicroCal PEAQ-ITC Analysis Software installation

To install MicroCal PEAQ-ITC Analysis Software:

Step	Action
1	Download the program.
	Double-click the installation file. *Result:* The software will be installed automatically and a start icon will appear on the desktop.
2	

Note: MicroCal PEAQ-ITC Analysis Software is also preinstalled on the instrument controller PC for MicroCal PEAQ-ITC and MicroCal PEAQ-ITC Automated together with their respective control software.

About this manual

Purpose of the user manual

This user manual provides instructions needed to run MicroCal PEAQ-ITC Analysis Software and perform analysis on isothermal titration calorimetry (ITC) results.

Typographical conventions

Software items are identified in the text by **bold italic** text. A colon separates menu levels, thus **File:Open** refers to the **Open** command in the **File** menu.

Hardware items are identified in the text by **bold** text (e.g., **Power** switch).

Text entries that MicroCal PEAQ-ITC Analysis Software generates or that the user must type are represented by a monospaced typeface (e.g., **C:\MicrocalITC**).

Intended use

MicroCal PEAQ-ITC Analysis Software is intended for analysis of calorimetric data.

Prerequisites

In order to operate the software in the way it is intended, the following prerequisites must be fulfilled:

- You should have a general understanding of the use of a personal computer running Microsoft® Windows® in the version provided with your product.
- You should understand the concepts of isothermal titration calorimetry.
- The software must be installed.

Notes and tips

Note: A Note is used to indicate information that is important for trouble-free and optimal use of the product.

Tip: A tip contains useful information that can improve or optimize your procedures.

Associated documentation

User documentation

The user documentation associated with MicroCal PEAQ-ITC Analysis Software consists of:

Title	Description
MicroCal PEAQ-ITC Analysis Software User Manual (this manual)	In depth documentation of MicroCal PEAQ-ITC Analysis Software.
MicroCal PEAQ-ITC User Manual	In depth documentation of MicroCal PEAQ-ITC.
MicroCal PEAQ-ITC Operating Instructions	Instructions on how to operate MicroCal PEAQ-ITC in a safe way.
MicroCal PEAQ-ITC Automated User Manual	In depth documentation of MicroCal PEAQ-ITC Automated.
MicroCal PEAQ-ITC Automated Operating Instructions	Instructions on how to operate MicroCal PEAQ-ITC Automated in a safe way.

The user documentation can be downloaded or ordered from Malvern Instruments.

2

ISOTHERMAL TITRATION CALORIMETRY

The following topics are covered:

Introduction to ITC

Isothermal titration calorimeters (ITC) measure the heat change that occurs when two substances interact. Heat is liberated or absorbed as a result of the redistribution of non-covalent bonds, for example, when the interacting molecules go from the free to the bound state.

An ITC mixes the binding partners and monitors the heat changes by measuring the power required to maintain zero temperature difference between the reference and sample cells.

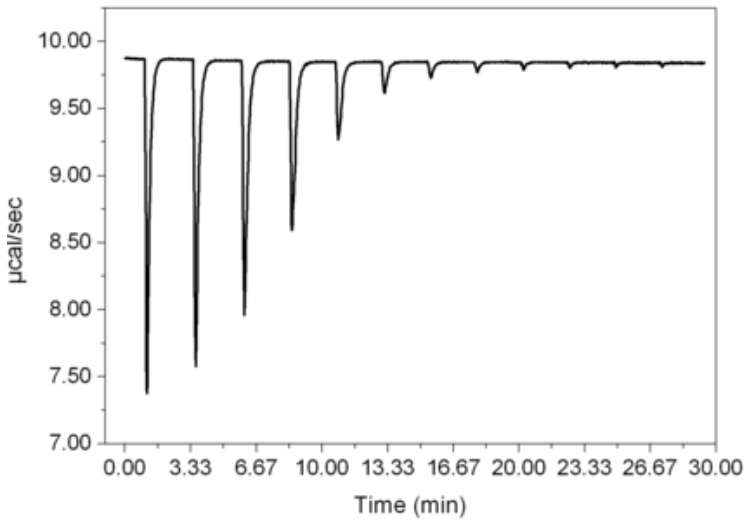
The reference cell usually contains water, which has the same heat capacity as most of the sample buffers. The sample cell contains:

- one of the binding partners (often, but not necessarily a macromolecule), and
- a stirring syringe, which holds the other binding partner (often, but not necessarily a ligand).

Experimental procedure

Typically, the ligand is injected into the sample cell, in 2 to 3 µl aliquots, until its concentration is two- to three-fold greater than that of the sample cell material. Each injection of the ligand results in a heat signature that is first integrated with respect to time and then normalized for concentration. This titration curve is fitted to a binding model to extract the affinity (K_D), stoichiometry (n) and the enthalpy of interaction (ΔH) (also called heat of reaction).

The following illustration shows an example experimental curve.



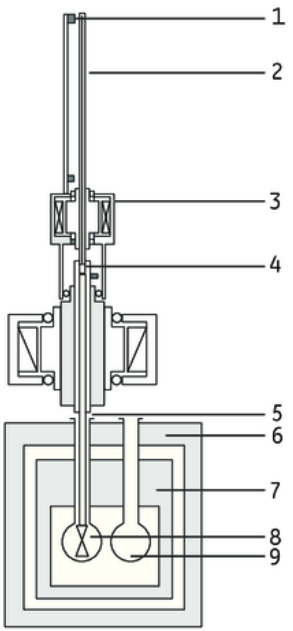
Notice that the first injection results in a larger deflection from the baseline, denoting a larger heat and nearly 100% binding. At the conclusion of the experiment, very little of the injected substance binds, resulting in little or no deflection (heat change) from the baseline.

Also, notice that the value on the y-axis decreases upon binding. This is the power needed to keep the sample cell at the same temperature as the reference cell.

Heat is given off during the reaction, therefore less power is required to compensate the temperature differences. This is characteristic of an exothermic reaction. In contrast, an endothermic reaction results in spikes rising from the baseline and hence, more power is required to compensate the temperature differences.

Main components of an ITC

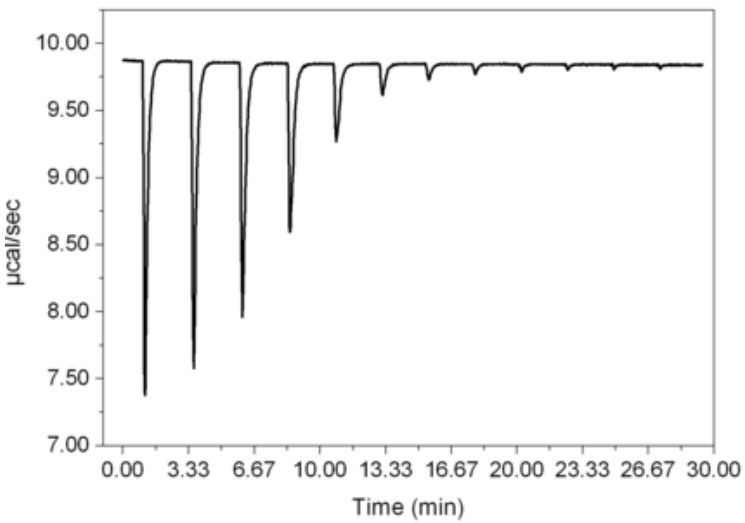
The following illustration shows the main components of an ITC instrument.



Part	Description
1	Sensor
2	Lead screw
3	Injector
4	Plunger
5	Syringe
6	Outer shield
7	Inner shield
8	Sample cell
9	Reference cell

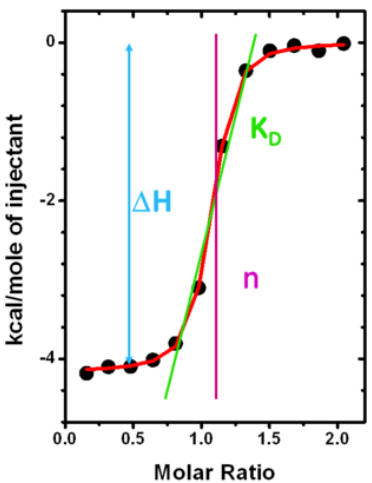
Raw data

The temperature difference between the sample cell and the reference cell is converted to power and directly read out as raw data. An example of this is shown in the following illustration. Each spike, followed by a return to the baseline, is an injection.



Injection heat

The individual injection heat is calculated by integrating the raw data (power) from each injection over time. The following figure shows each individual injection heat, normalized by the amount of titrant injected, as a function of the molar ratio of titrant/cell material in the sample cell. The fitted curve of a 1:1 binding model is overlaid in red. A general illustration of how the thermodynamic parameters n, K_D, and ΔH are related to the titration curve is also overlaid.



In the case of this simple 1:1 binding experiment, the enthalpy is directly measured/fitted as the heat of 100% binding. The stoichiometry is intuitively denoted by the midpoint of the titration, between 100% binding and 0% binding. The steepness of the rise to saturation is related to binding affinity. For any given system, the steepness of this region is also directly related to the sample concentration.

3

USING THE SYSTEM

This section describes how to start MicroCal PEAQ-ITC Analysis Software and how to open a new analysis, including the initial view: the **Start Analysis** workspace.

The following topics are covered in this section:

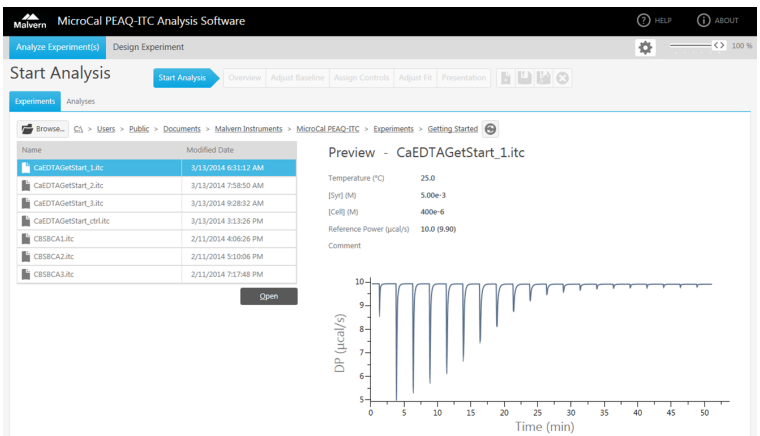
Start analysis

Start the software

To start MicroCal PEAQ-ITC Analysis Software:

- Double-click the **MicroCal PEAQ-ITC Analysis Software** icon found on the desktop.

Result: MicroCal PEAQ-ITC Analysis Software opens in the **Start Analysis** workspace showing a list of experiments.



Start Analysis workspace

The **Start Analysis** workspace contains two panes.

- The left file list pane, where you select experiments and analyses.
- The right **Preview** pane, where a preview of the selected experiments or analysis are displayed.

Start a new analysis

In the **Start Analysis** workspace, start a new analysis by opening one or several experiment files or open a previously created analysis containing one or several experiment files.

- Click **Experiments**, select one or several experiment files in the list and then click **Open**

  Result: A new analysis containing the selected experiments opens in the **Overview** workspace.

  or

- Click **Analyses**, select a analysis file in the list and then click **Open**.

  Result: The analysis opens in the **Overview** workspace.

Note: Recently opened and saved analyses are tagged with the word **(Recent)**.

Default folders in the file list pane

The default folders in the **Experiment** and **Analyses** tabs are set in the **Settings** workspace. See Common controls on page 31.

The path to the current folder is visible above the file list. To change the current folder:

- Click folder names in the path or in the file list to navigate.
  or

- Click the **Browse** button to open the **Browse For Folder** window.

Note: The **Browse For Folder** windows will browse for folders only.

- To refresh the file list, click the **Refresh** button.

Preview pane

If you have selected an analysis, the **Preview** pane will show information on the **Fitting model** chosen and the experiments added to the analysis. If you have selected an experiment, the **Preview** pane will show a list of experiment parameters and a preview of the differential power as a function of time.

Note: If more than one experiment is selected, a preview of the first selected experiment will be shown in the **Preview** pane.

Experiment and analysis files

MicroCal PEAQ-ITC Analysis Software can open calorimetry data files (experiment files) or analysis files. Several data files can be analyzed and saved in a single analysis file. Data from the experiment files is automatically imported into and saved with the analysis. The experiment files are never edited, all subsequent data edits are made on data in the analysis file.

Type of file	File name extension	Description
Experiment file	itc	Calorimetric data from MicroCal ITC.
Analysis file	apj	Analysis of calorimetric data. Can contain data from several experiments.

4

OVERVIEW WORKSPACE

The following topics are covered in this section:

Workspace presentation

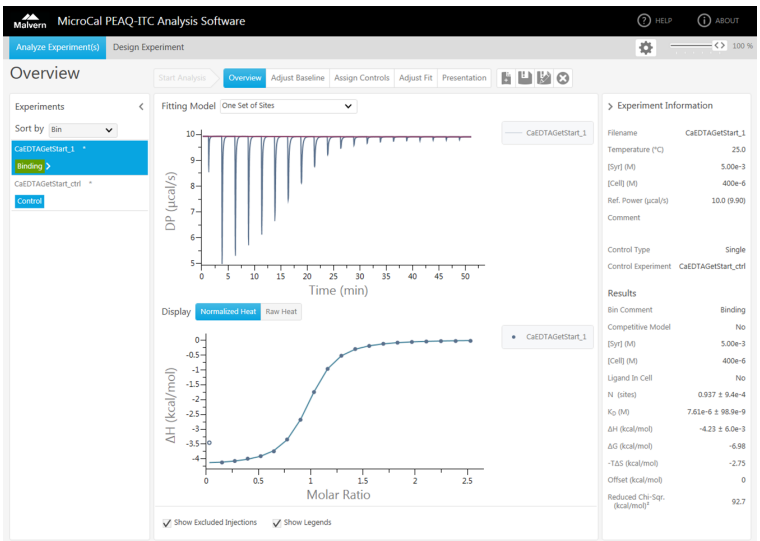
Introduction

The **Overview** workspace contains three panes.

- **Experiments** pane, where the experiments used in the analysis are displayed.
- Charts pane, where the differential power (DP) as a function of time, and graphs of fitted data are displayed.
- **Experiment Information** pane, where both information about the experiment and the analysis results are displayed.

Pane descriptions

When starting a new analysis the fitting model **One Set of Binding Sites** is selected and all fitting model parameters are calculated for added experiments. The calculated fitting model parameters are displayed under the heading **Results** in the **Experiment Information** pane.



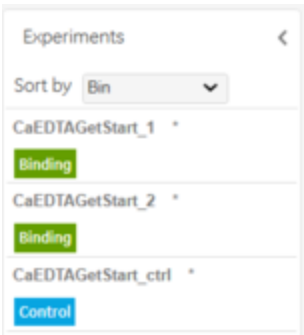
Pane	Function
1 (left pane)	Experiments pane The files that are used in the analysis and their **Bin** are displayed in this pane.
2 (middle pane)	Charts pane The differential power (DP) as a function of time, and graphs of fitted data are displayed in this pane.
3 (right pane)	Experiment Information pane Information about the experiment and the analysis results are displayed in this pane.

Experiments pane

Introduction

The **Experiments** pane lists all experiments added to the current analysis.

- Click the left arrow ‹ to hide the **Experiments** pane.
- Click the right arrow › to show the **Experiments** pane.



Bin

Bin is a categorization of an experiment. When an experiment is added the program automatically sets a **Bin** to the experiment. Whenever adjustments to the dataset are made (baseline adjustment or data point exclusion/inclusion), the **Bin** is reassessed.

Note: The **Bin** of an experiment can be manually set in all workspaces except **Presentation**.

The following table describes the different **Bin** options.

Bin	Description
OK	Used for all fitting models except **One Set of Sites** and **One Set of Sites - SIM** models.
Binding	Binding event detected. Used for **One Set of Sites** and **One Set of Sites - SIM** fitting models.
No binding	No binding event detected. Used for **One Set of Sites** and **One Set of Sites - SIM** fitting models.
Control	Based upon file name, the experiment is automatically assigned as a control to one or several open experiments when opened. Used for all fitting models, except **Enzyme Kinetics - Multiple Injections**, **Enzyme Kinetics - Single Injection** and **One Set of Sites - SIM**. Only experiments binned as **Control** are available to assign as controls in the **Assign Controls** workspace. See Assign controls workspace on page 44.
Check data	One or several values exceeds a predefined threshold. Used for all fitting models. An explanation of the **Bin** will be noted in the **Experiment Information** pane under **Bin**.

The **Bin** comment attempts to explain the **Bin**. An explanation for the **Bin** is displayed as **Bin Comment** in the ScreenTip for the experiments in the Experiment pane.

The following table describes **Bin Comment** options for different **Bin**.

Bin Comment	Description
OK	Passes all data quality criteria.
Binding	Shows signs of binding (total heat and difference in start/finish heat). Only included injections are considered.
No binding	Shows no signs of binding. Only included injections are considered.
Binning Overridden	User has overridden the **Bin**.
Check Baseline	Baseline deviates from raw data. Only included injections are considered.
Check Baseline Position	Baseline near zero.
Too Little Heat	Too little total heat to be **Binding**. Only included injections are considered.
Undefined	Default until defined otherwise.
Low Initial Baseline	Initial baseline unacceptably below reference power.
High Initial Baseline	Initial baseline unacceptably above reference power.
Concentrations = 0.0	Required concentrations = 0.
Data Below Minimum	Raw data < 0.
Check Baseline Drift	Too much difference in start/finish baseline.
Data Exceeds Maximum	Raw data exceeds maximum.
Possible Poor Load	Baseline stepping was detected. This could also be remedied in **Adjust Baseline** workspace.
Possible Buffer Mismatch	A possible buffer mismatch was detected.
Injection Spacing Too Short	Too many injections appear to not return to baseline.
Check Baseline Fit	Baseline failed to fit.
Too Many Injections	Analyzing a data set with multiple injections using the **One Set of Sites – SIM** fitting model.
Too Few Injections	No injections using SIM methods or fewer than five injections using MIM methods.

Change Bin

The **Bin** of the experiment is shown below the name of the experiment (e.g. **Binding**).

Step	Action
1	To show a menu of available **Bin** options, click the right arrow ›.
2	Click a new **Bin** option to override the experiment **Bin**.

Note: If the **Bin** is manually overridden, data manipulations that would normally reassess the **Bin** will not trigger that reassessment.

Select experiments

Charts of selected experiments are displayed in the middle pane.

- Click an experiment to select it.
- Select multiple experiments by holding down the **Ctrl** key while you click on other experiments that you want to selector
- select the first experiment and then click the last experiment you want to select while holding down the **Shift** key.*Result:* All experiments between the first and last experiment will be selected.

Remove experiments

- To remove an experiment from the analysis, point to the experiment in the list and then click the **Remove Experiment** button.

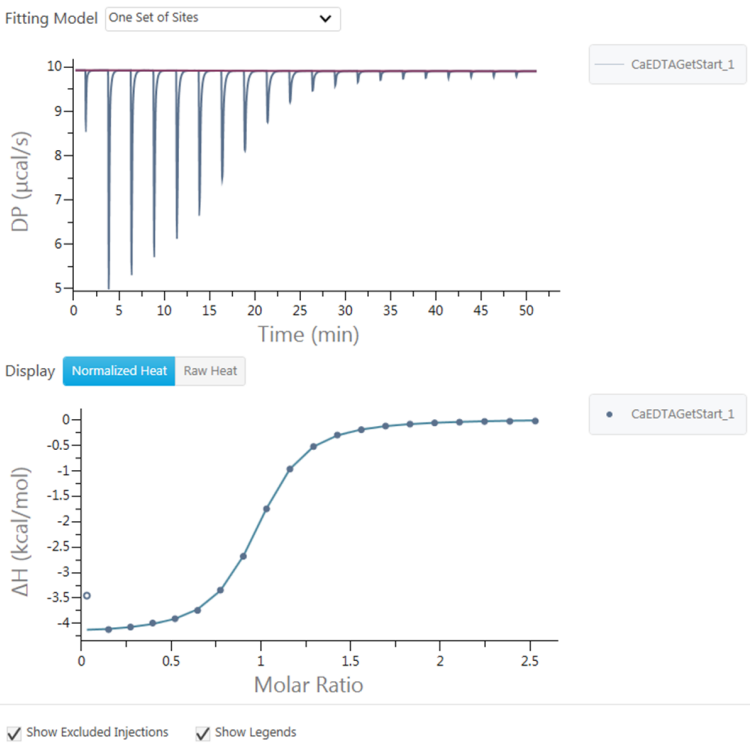
Note: All changes to an experiment will be lost when it is removed from an analysis.

Charts pane of the Overview workspace

Introduction

In the charts pane you can select the fitting model. Graphs of differential power as a function of time and other model specific graphs are displayed. You can also remove data points that you do not want to include in the fitting model.

You can not remove data points when using the **One Set of Sites - SIM** or **Enzyme Kinetics - Single Injection** fitting models.



Show legends

It is possible to select multiple experiments. The axes of the graphs will automatically scale to fit all selected experiments.

- To show a legend of selected experiments in the charts, select the **Show Legends** check box.

Show Excluded Injections

Data points represented by empty circles (as shown to the left) are excluded from the fitting model. See Adjust baseline on page 36.

Note: The first injection in a titration series is automatically removed from the fitting model calculation.

- To show excluded data points, select the **Show Excluded Injections** check box. (Show excluded data points is the default settings).
- To hide excluded data points, clear the **Show Excluded Injections** check box. (This can be helpful if the excluded data points are skewing the chart's y-scale).

Fitting models

The upper section of the charts pane shows the **Fitting Model** menu. When adding an experiment to a new analysis, **One Set of Sites** is the default model. To change the model used for curve fitting click the down arrow next to **Fitting Model** and then click the model that you want.

MicroCal PEAQ-ITC Analysis Software provides seven curve fitting models:

- **One Set of Sites**. For more information see Adjust Fit - Fitting Model: One Set of Sites on page 54.
- **Two Sets of Sites**. For more information see Adjust Fit - Fitting Model: Two Sets of Sites on page 58.
- **Dissociation**. For more information see Adjust Fit - Fitting Model: Dissociation on page 60.

- **Sequential Binding Sites**. For more information see Adjust Fit - Fitting Model: Sequential Binding Sites on page 62.
- **One Set of Sites - SIM**. For more information see Adjust Fit - Fitting Model: One Set of Sites - SIM on page 65.
- **Enzyme Kinetics - Multiple Injections**. For more information see Adjust Fit - Fitting Model: Enzyme Kinetics - Multiple Injections on page 67.
- **Enzyme Kinetics - Single Injection**. For more information see Adjust Fit - Fitting Model: Enzyme Kinetics - Single Injection on page 69.

When selecting a new fitting model for one or several experiments:

- The new curve fit is automatically calculated and plotted.
- The **Experiment Information** pane is updated with the new calculations of corresponding fitting model parameters.

Differential power graph

Below the **Fitting Model** menu there is a raw injection data graph of the measured differential power over time. The baseline is also visible in this graph.

Fitted data plots

Below the differential power plot there is a chart of fitted data. The type of graph(s) shown depends on the selected fitting model.

If the fitting model is ...	Then...
One Set of Sites, **Two Sets of Sites**, **Dissociation**, **Sequential Binding Sites** or **One Set of Sites - SIM**	• Click **Normalized Heat** to view concentration-normalized injection heats. • Click **Raw Heat** to view injection heats.
Enzyme Kinetics - Multiple Injections or **Enzyme Kinetics - Single Injection**	The graph will show substrate conversion rate against substrate concentration.

Zoom in graphs

To zoom in any graph:

- Hold down the mouse button in graphs that permit zooming and draw a zoom rectangle. Then release the mouse button.
  Result: The graph displays the area within the zoom rectangle.
- Double-click in the graph to reset the zoom level.

Remove bad data points

It is possible to manually remove data points from the fitting model. When data points are excluded (or included), the fitting model is automatically re-calculated using the new set of data points. Data points can be removed (and re-added) from all workspaces except the **Presentation** workspace.

Note: Single injection fitting models (**One Set of Sites - SIM** and **Enzyme Kinetics - Single Injection**) does not support removal of data points.

Data points that can be removed from the current fitting model are represented by a filled circle

To remove a data point:

- Right-click the data point, then click **Exclude**.

  Result: The filled circle will be replaced by an empty circle and the fitting model is re-calculated.

To re-add a data point:

- Right-click the data point, then click **Include**.

  Result: The empty circle will be replaced by a filled circle and the fitting model is re-calculated.

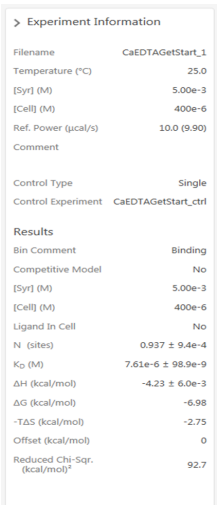
Note: The first injection in a titration series is automatically removed from the fitting model calculation. It is displayed as an empty circle.

Note: When a data point is removed and the **Show Excluded Injections** check box is cleared, the graph will rescale to fit the displayed data points.

Experiment Information pane

The upper part of the **Experiment Information** pane displays experiment parameters and conditions. Under the heading **Results** all the calculated fitting parameters for the selected fitting model are displayed. The parameters displayed will depend upon the currently selected fitting model. If multiple experiments are selected, the **Experiment Information** pane will only display experimental parameters of the experiment that is first clicked (selected) when the multiple experiment selection is done.

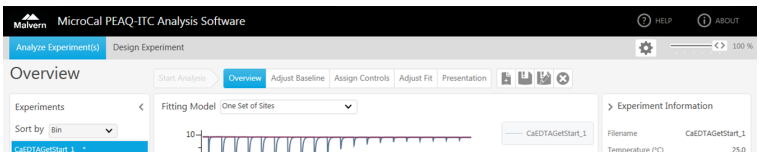
- To hide the **Experiment Information** pane, click the right arrow ›.
- To show the **Experiment Information** pane, click the left arrow ‹.



Common controls

Introduction

This section describes buttons and labels available in all work spaces.



Help and About

Click the **Help** button to open the software documentation.

Click the **About** button to display information on software and license agreement.

Settings

Click the **Settings** button to enter the **Settings** workspace.

Setting	Description
Analysis folder	Set what folder to initially browse in the **Analyses** tab of the **Start** workspace. • Type the path to the folder in the **Analyses Folder** box or • click the **Browse for folder** button and then navigate to the folder in the **Browse For Folder** dialog.

Setting	Description
	Note: It is only possible to select a folder that the current Windows user has access to.
Experiment folder	Set what folder to initially browse in the **Experiment** tab of the **Start** workspace. • Type the path to the folder in the **Experiments Folder** box or • click the **Browse for folder** button and then navigate to the folder in the **Browse For Folder** dialog.
Unit	MicroCal PEAQ-ITC Analysis Software will display measured and calculated energy in either Joules or Calories. To select displayed energy units, click the down arrow next to **Unit** and then click **Calorie** or **Joule**.

When a setting has been changed, an asterisk * will appear next to the name of the setting (e.g. **Display Unit***).

- Click **Apply** to save all changes.
- Click a workspace name to leave the **Settings** workspace.

Font scale slider

Use the slider to change the size of text and icons in the workspace. Move the slider left to decrease the size of text and icons, move the slider right to increase the size of text and icons. Graphs in the workspace will automatically change size to fill available space. That is, decreasing the font and icon size will make graphs larger, increasing the font and icon size will make graphs smaller.

File management buttons

Icon	Description
	Click the **Add Experiment** button to add one or several experiment(s) to the current analysis. The newly added experiment(s) will be listed in the **Experiments** pane.
	Click the **Save** button to save the current analysis.
	Click the **Save As** button to save the current analysis under a new name.
	Click the **Close** button to close the current analysis and return to the **Start** workspace.

5

ADJUST BASELINE AND INTEGRATION RANGE

In routine data analysis, integration values (baselines and integration ranges) are determined automatically. Sometimes these are not sufficient, so the integration details need to be set manually. This is especially true when working with very small injection peaks. This section describes how to manually set these ranges.

The following topics are covered in this section:

Adjust baseline

Introduction

The baseline and the integration range can be manually adjusted in the **Adjust Baseline** workspace, if necessary. This workspace is not available for the **One Set of Sites - SIM**, **Enzyme Kinetics - Single Injection** or **Enzyme Kinetics - Multiple Injections** fitting models. To adjust SIM markers for the the **One Set of Sites - SIM** and **Enzyme Kinetics - Single Injection** fitting models, see Adjust SIM Markers on page 41.

Adjust baseline workspace

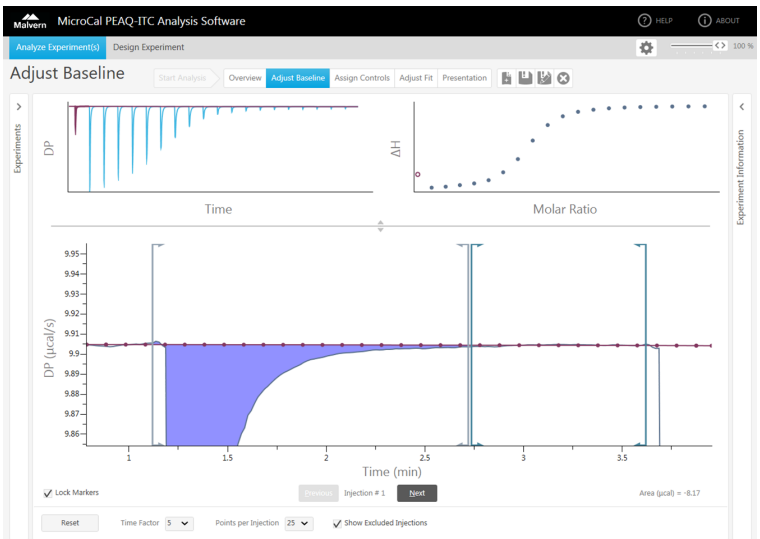
The **Adjust Baseline** workspace contains three panes.

- **Experiments** pane, where the files that are used in the analysis and their **Bin** are displayed.
- Charts pane, where graphs and parameter settings necessary for baseline and integration range adjustments are displayed. This pane is the work area for adjustment of the baseline and integration range.

  Experiment Information pane, where information about the experiment and the analysis results are displayed.

Charts pane

The charts pane is divided into three subsections as described in the following illustration.



Section	Function
1 (upper section)	Curves representing the entire experiment. This section displays graphical presentations of the raw data as a function of time and the normalized heat, respectively.
2 (middle section)	Injection peak open for baseline and integration peak adjustment. This section displays the injection peak to be adjusted.
3 (lower section)	Commands, parameters and check boxes. This section displays commands, parameters and check boxes that may facilitate the adjustment procedure.

Upper section of charts pane

The two graphs in this section display the raw data (DP) as a function of time and the normalized heat (ΔH).

- To remove bad data points in the normalized heat graph, right-click the data point and then click **Exclude**. See Adjust baseline on page 36
- You can select peaks that you want to adjust by clicking them.

Middle section of charts pane

The middle section displays the raw data (DP) as a function of time for only the selected injection. You can adjust the integration range and the baseline in this section. The injection number is shown in the area between the **Previous** and **Next** buttons, and the corresponding injection is also highlighted in the raw data graph in the upper section.

The left-hand gray marker pair define the integration area and the blue right-hand marker pair define the baseline. The outermost markers are fixed and span the time period from the start of one injection peak to the start of the next injection peak. The inner markers define the selected integration range and baseline.

To adjust the baseline and integration range markers individually, clear the **Lock Markers** check box. This may be helpful if you are trying to avoid both integrating and fitting the baseline to a problem region in the current injection.

If the current injection does not meeting quality standards, the following icon will appear:

Adjusting this injection's baseline or excluding this injection may affect the experiment's bin assignment.

Adjust integration range

To adjust the integration range, perform the following steps.

Step	Action
1	Select an injection peak to be adjusted.
2	Clear the **Lock Markers** check box, if necessary.
3	Drag the right-hand integration marker to the desired position. *Result*: Data between the gray integration markers is integrated.
4	If more injection peaks need to be adjusted, continue to the next peak, either by • clicking the desired peak in the raw data graph in the upper section or • by clicking **Previous** or **Next** in the middle section.
5	Repeat step 1 to 3.

Adjust baseline

To adjust the baseline, perform the following steps.

Step	Action
1	Select an injection peak to be adjusted.
2	Clear the **Lock Markers** check box, if necessary.
3	Drag the left-hand baseline marker to the desired position. *Result*: Data between the blue baseline markers is fit as baseline.
4	If more injection peaks need to be adjusted, continue to the next peak, either by • clicking the desired peak in the raw data graph in the upper section or • by clicking **Previous** or **Next** in the middle section.
5	Repeat step 1 to 3.

It is also possible to do fine adjustments of the integrated area by dragging the red data points, but note that moving the baseline marker will automatically refit the entire baseline, thus negating any fine adjustments.

Lower section of charts pane

- Select the **Show Excluded Injections** check box to show excluded data points in the normalized heat plot in the upper section.
- Change **Points per injection** to set the number of dragable red data points between injections.
- Change **Time Factor** to affect the automatic placement of baseline markers and integration markers. A larger time factor will generally place the markers further along the injection.
- To undo adjustments, click **Reset Baseline**.

Note: Changing **Time Factor** or **Points per injection** restores the default positions of the baseline and integration markers. This negates any changes for the current experiment in this workspace.

Adjust SIM Markers

Introduction

When using the **One Set of Sites - SIM** or the **Enzyme Kinetics - Single injection** fitting models, manual data adjustments can be made in the **Adjust SIM Markers** or the **Adjust Enzyme Markers** workspace, respectively.

Adjust SIM Markers and **Adjust Enzyme Markers** workspaces

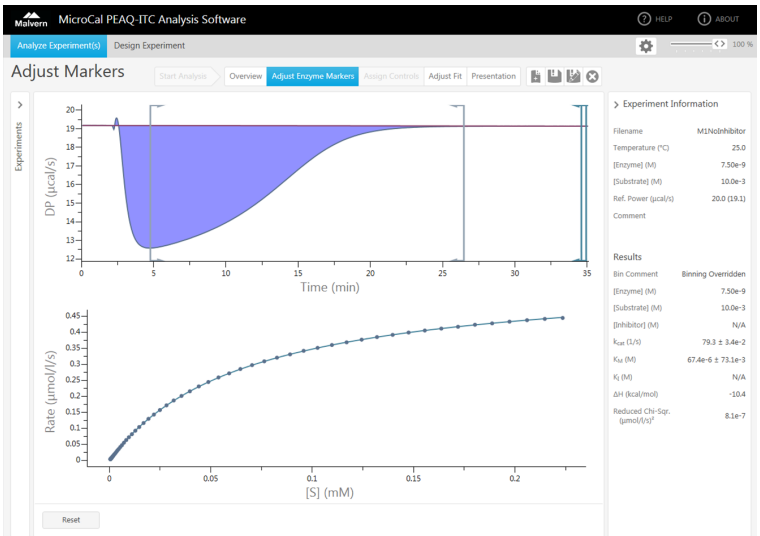
The **Adjust SIM markers** and **Adjust Enzyme Markers** workspaces contains three panes.

- **Experiments** pane, where the files that are used in the analysis and their **Bin** are displayed.
- Charts pane, where graphs and parameter settings necessary for baseline and integration range adjustments are displayed. This pane is the work area for adjustment of the baseline and integration range.

  Experiment Information pane, where information about the experiment and the analysis results are displayed.

Charts pane

The middle pane is further divided into three subsections as described in the following illustration:



Section	Function
1 (upper section)	Graph displaying the raw injection data. This section displays graphical presentations of the raw injection data as a function of time. The left-hand gray truncation marker pair selects data to be fitted, and the blue right-hand marker pair define the region fit as baseline. In the case of **Enzyme Kinetics - Single Injection**, you may select which injection to analyze. Only one may be analyzed at a time. In this case, markers will be placed on that injection.
2 (middle section)	Graph displaying the fitted data.
3 (lower section)	Commands To undo adjustments, click **Reset Markers**. In the case of **Enzyme Kinetics - Single Injection**, you may select which injection to analyze. If applicable, buttons will appear, allowing the user to select this injection.

6

ASSIGN CONTROLS

This section describes how to assign controls and the available control subtraction methods.

The following topics are covered in this section:

Assign controls workspace

Introduction

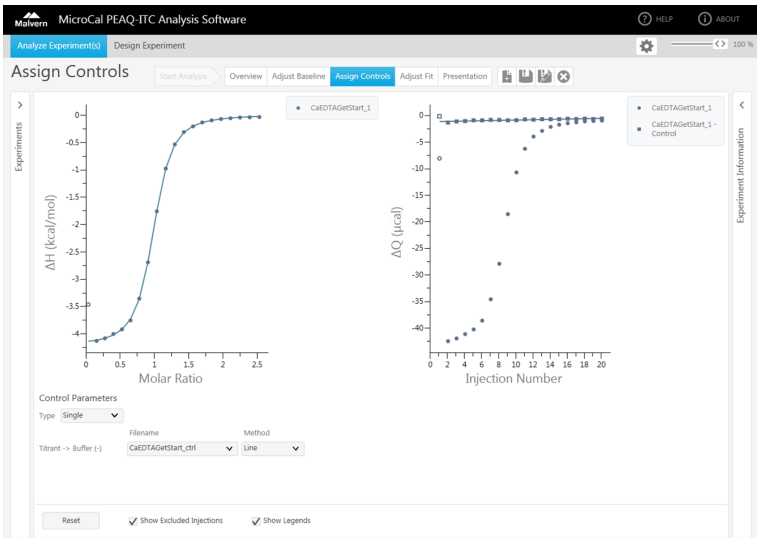
The **Assign Controls** workspace is available for the **One Set of Sites**, **Two Sets of Sites**, **Dissociation** and **Sequential Binding Sites** fitting models.

The workspace contains three panes.

- **Experiments** pane, where the experiments used in the analysis and their individual **Bin** are displayed.
- Charts pane, where graphs of integrated heats as a function of injections are displayed and controls can be assigned to experiments.
- **Experiment Information** pane, where both information about the experiment and the analysis results are displayed.

Charts pane

The charts pane displays two graphs of the experiments selected in the **Experiments** pane. The left graph shows concentration-normalized integrated injection heats as a function of molar ratio. The right graph shows raw integrated heats as a function of injection number. If a control data file is assigned to the experiments displayed, the control data is also plotted in the graph.



Common commands

In the lowest section of the middle pane of the **Assign Controls** workspace, there are one button and two check boxes that are common for all control subtraction types. These are described in the following table.

Command	Function
Reset Controls	Click this button to reset all controls for the selected experiment(s) to the default assignments.
Show Excluded Injections	Select this check box to show excluded injections. This check box is selected by default.
Show Legend	Clear this check box to hide all graph legends. This check box is selected by default.

Reset Controls button

Clicking **Reset Controls** will reassign all selected control experiment(s) and then initialize a new fit. However, the current fitting parameters will be used as initial values for this fit. This means that the new fit will not necessarily settle on the same fitting parameters as when initially loading the experiment.

To fit the model using the same initial values as when initially loading the experiment:

- In the **Adjust Fit** workspace, click **Reset Parameters** and then click **Fit**.

Subtract control data

Introduction

Integrated heats from control experiments can be used as references. The control heats will be subtracted from the reaction heats.

Note: If an assigned control's baseline is modified or if one of its data points is excluded/included, its injection heats will be newly integrated. Any dataset that references that control will automatically be refit.

Depending on the control subtraction method selected you can assign one or more controls to an experiment. Select the desired control subtraction method on the **Type** menu below the heading **Control Subtraction**. The selected control method is listed as **Control Method** in the **Experiment Information** pane.

- Controls may not be subtracted from controls.
- The **Bin** of a control may not be changed if any datasets are referencing that control.
- You may not assign the same control to an experiment and its referenced competitor.

Available control methods are:

- **None**
- **Fitted Offset**
- **Single** (only available if experiments where the **Bin** is set to **Control** have been added to the analysis)
- **Composite** (only available if experiments where the **Bin** is set to **Control** have been added to the analysis)

Note: If the fitted offset option is selected (or deselected), it is assumed the user does not wish to fit for an offset. Thus, the **Offset fitting** parameters is automatically set to 0 and varied (or not varied).

Constant control

This is the default control method when opening an experiment without an assigned control experiment.

- On the **Type** menu, click **Fitted Offset**.

  Result: A constant control heat will be assumed and fitted to the integrated heats along with the chosen model's fitting parameters. The constant control heat is listed as **Offset** under the **Results** heading in the **Experiment Information** pane.

No control subtraction

- On the **Type** menu, click **None**.

  Result: No control subtraction will be applied to the selected experiment.

Automatic assignment of control experiments

Only experiments where the **Bin** is set to **Control** can be assigned as controls to another experiment. Experiments with a file name ending with "**_ctrl**" will automatically be set as **Control** when added to the analysis.

Controls will automatically be assigned to experiments based on filenames. String matching in experiment and control filenames will determine what control will be automatically assigned to what experiment, but try to keep **_ctrl** will be compared to all non-control experiment names. If that key string is found anywhere in a filename, that file will be assigned that control. See the following example.

You can also manually change the **Bin** of an experiment to **Control** in the **Experiments** pane. See Experiments pane on page 22.

Example

Experiment	Possible controls
test_1.itc	**1_ctrl.itc** **test_ctrl.itc**
test2.itc	**2_ctrl.itc** **test_ctrl.itc**
Monday_test.itc	**test_ctrl.itc** **nda_ctrl.itc**

Note: Be careful with your naming scheme. It is possible to load more than one control dataset to the experiments, and the automatic control assignment will simply pick the first valid match in the list.

The **Method** menu

The **Method** menu is displayed when the control methods **Single** or **Composite** is selected. For each control experiment select **Line**, **Mean** or **Point to Point**.

Option	Description
Line	A line fitted to the control dataset's injection heats will be subtracted from the experiment's injection heats.
Mean	The mean of the control dataset's injection heats will be subtracted from the experiment's injection heats.
Point to Point	The control dataset's injection heats will be subtracted from the experiment's corresponding injection heats.

Subtract single control

Control heats of a single dataset will be subtracted from the experiment's integrated heats.

Step	Action
1	On the **Type** menu, click **Single**. *Result:* The **Filename** and **Method** menus will be shown below the **Type** menu.
2	On the **Filename** menu, click the experiment you want to assign as control to the selected experiment(s). **Note:** Only experiments with a **Bin** of **Control** are listed in the **Filename** menu.
3	On the **Method** menu, click the subtraction method you want to use. *Result:* The chosen control will be displayed in the graph of integrated heats as a function of injections. Parameters below the **Results** heading in the **Experiment Information** pane will also be updated.

Types of composite control experiments

When the **Composite** control method is selected, three rows of **Filename** and **Method** menus will be shown below the **Type** menu. The names given are suggestions on what type of control experiments that you may want to run.

Name	Description
Titrant -> Buffer (-)	A dataset of a control experiment where the titrant is injected into the sample cell containing buffer solution only.
Buffer -> Cell Material (-)	A dataset of a control experiment where buffer solution is injected into the sample cell containing macromolecules in buffer solution.
Buffer -> Buffer (+)	A dataset of a control experiment where buffer solution is injected into the sample cell containing buffer solution only. **Note:** This experiment's dataset will be added, not subtracted, to the controlled experiment's dataset.

Subtract composite controls

Control heats of multiple datasets will be subtracted from the experiment's integrated heats.

Step	Action
1	On the **Type** menu, click **Composite**. *Result:* Three rows of **Filename** and **Method** menus will be shown below the **Type** menu.
2	On the **Filename** menu, click the experiment you want to assign as control to the selected experiment(s). You can assign up to three different controls. **Note:** Only experiments with a **Bin** of **Control** are listed in the **Filename** menu.
3	On the **Method** menu, click the subtraction method you want to use. *Result:* The chosen control will be displayed in the graph of integrated heats as a function of injections. Parameters below the **Results** heading in the **Experiment Information** pane will also be updated.

7

ADJUST CURVE FIT

If the automatically generated analysis data is not satisfactory, it is possible to adjust the fitting parameters manually in order to obtain good analysis results. This can be the case when: too many fitting parameters are varied, using the more complex models or erroneous concentrations have been entered.

This section describes advanced curve fitting adjustment for all fitting models and covers:

Adjust Fit workspace

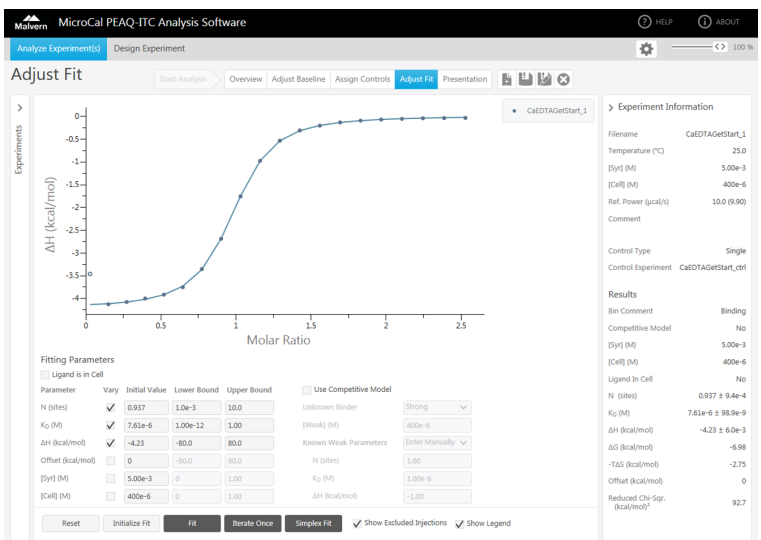
Introduction

The **Adjust Fit** workspace contains three panes.

- **Experiments** pane, where the experiments used in the analysis and their individual **Bin** are displayed.
- Charts pane, where graphs of integrated heats as a function of injections are displayed and controls can be assigned to experiments.
- **Experiment Information** pane, where both information about the experiment and the analysis results are displayed.

Charts pane

The charts pane is divided into three subsections as described in the following illustration.



Section	Function
1 (upper section)	Charts view This section displays a graphical presentation of the injection heat calculated from integrated raw data.
2 (middle section)	Fitting controls This section displays the fitting parameters that can be adjusted before starting the fitting iteration procedure.
3 (lower section)	Common commands and check boxes This section displays commands and check boxes that are common for all fitting models.

Common commands

At the lower section of the charts pane in the **Adjust Fit** workspace, there are three commands and two check boxes that are common for all fitting models. These commands and check boxes are described in the following table.

Feature	Function
Reset	Command that will reset all parameters to the initial values.
Initialize Fit	Command that will overlay a curve calculated using the manually entered parameter values. This can be helpful when searching for good initial values for the fit, which can be helpful for the more complicated fitting models.
Fit	Command that will perform a maximum of 200 iterations using the Levenberg-Marquardt algorithm.
Show Excluded Injections	Selecting this check box will show any excluded injections. This check box is selected by default.
Show Legend	Clearing this check box will hide all figure legends. This check box is selected by default.
Iterate Once	Command that will perform one iteration using the Levenberg-Marquardt algorithm. This can be helpful when you are trying to converge using a complex model.
Simplex Fit	Command that will perform one iteration using the Simplex algorithm. This can be helpful when the Levenberg-Marquardt algorithm fails to converge. **Note:** Fitting parameter errors and prediction bands are not calculated unless iterations of the Levenberg-Marquardt algorithm are performed.

Adjust Fit - Fitting Model: One Set of Sites

Introduction

The one site fitting model is the most commonly used fitting model for calorimetry experiments, and are set as the default fitting model. The model for one set of sites will work for any number of sites, n, if all sites have the same K_D and ΔH.

If a macromolecule has sites with two different values of K_D and/or ΔH, then the model with two sets of sites must be used.

You may need to adjust the fitting parameters to get a good fit if:

- the one site binding is a weak binding interaction
- there is not enough information in the curve fitting for all four parameter (N, K_D, ΔH and Offset)
- you want to vary the concentrations
- competitive binding needs to be modeled

Competitive binding

Competitive binding experiments are carried out by injecting a strongly binding ligand into a solution that contains both the macromolecule and the weaker, competing ligand. The strong ligand will appear to bind more weakly to the macromolecule in the presence of the competing ligand than when present alone.

In order to perform curve-fitting on results from a competitive binding experiment, a second non-competitive experiment must first be carried out in the conventional way to determine those binding parameters. These known parameters are used as input to the competitive experiment.

Some rules:

- You may not reference a dataset as a competitor if it shares a control.
- You may not reference a dataset as a competitor if it is also fitted to the competitive model.

- You may not use the competitive model if you are also using the ligand is in cell option.
- You may not reference a dataset as a competitor if it is using the ligand is in cell option.

  If a competitive reference has its baseline modified or if one of its data points is excluded/included, its results will automatically change. Any dataset that references that competitor will automatically be refit as well.

Reverse titrations

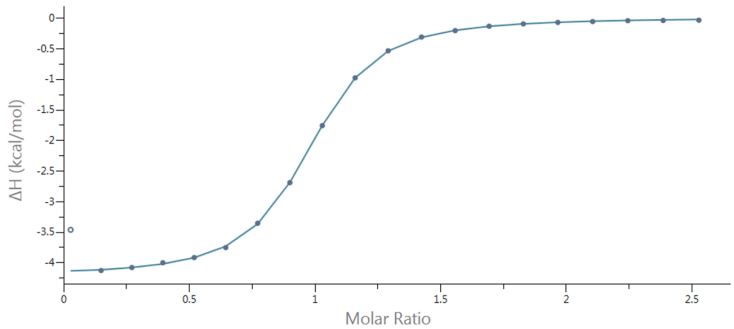
Whenever the ligand and macromolecule each have only one site for interaction with the other, then the system is symmetrical, and it does not matter which of the two is loaded into the sample cell and which into the injection syringe. It is important to carefully record the proper concentration of the species in the syringe and cell.

In cases where the ligand is sparingly soluble and the macromolecule is not, it may be useful to load the ligand into the sample cell since the starting concentration then does not need to be so high. Cases where the ligand is loaded in the sample cell and the macromolecule in the syringe are often called reverse titrations. The situation is more complicated if the macromolecule has more than one site (even if there is only one set of sites).

Some rules:

- You may not use this option if you are already using the competitive model.
- You may not use this option on a datset if it is referenced as a competitor.

Fitted model plot



Fitting Parameters

Ligand is in Cell

Parameter	Vary	Initial Value	Lower Bound	Upper Bound
N (sites)	✓	0.937	1.0e-3	10.0
K_D (M)	✓	7.61e-6	1.00e-12	1.00
ΔH (kcal/mol)	✓	-4.23	-80.0	80.0
Offset (kcal/mol)		0	-80.0	80.0
[Syr] (M)		5.00e-3	0	1.00
[Cell] (M)		400e-6	0	1.00

Use Competitive Model

Unknown Binder	Strong
[Weak] (M)	400e-6
Known Weak Parameters	Enter Manually
N (sites)	1.00
K_D (M)	1.00e-6
ΔH (kcal/mol)	-1.00

The charts pane displays the curve for each individual injection heat, calculated from integrated raw data using the **One Set of Sites** fitting model. The graph shows the estimated heat of binding (ΔH) as a function of molar ratio.

Fitting parameters

The fitting model parameters that can be adjusted for the **One Set of Sites** fitting model are displayed below the fitted model plot. All values, such as **Initial Value**, **Lower Bound** and **Upper Bound**, can be changed if the **Vary** check box is selected. If the **Vary** check box is cleared, the **Lower Bound** and **Upper Bound** cannot be edited.

The parameters that can be adjusted in this fitting model are:

- the number of binding sites (N),
- the estimated binding constant (K_D),
- the estimated heat of binding (ΔH),
- the offset, and
- the concentrations in the syringe and the cell.

If the **Fitted Offset** control subtraction option is selected (or deselected), it is assumed the user does not wish to fit for an offset: Thus, the **Offset** fitting parameters is automatically set to 0 and varied (or not varied). This can be manually overridden. For example, no control option may be specified, but the offset parameter may still be varied.

Select the **Ligand is in Cell** check box if you would like the enthalpy data to be reported per mol of the material in the cell rather than the syringe. This option is useful when comparing the fitted parameters of a titration with another with the same binding partners but loaded with the reverse configuration.

If a ligand is present in the cell, the **Ligand In Cell** check box has to be selected.

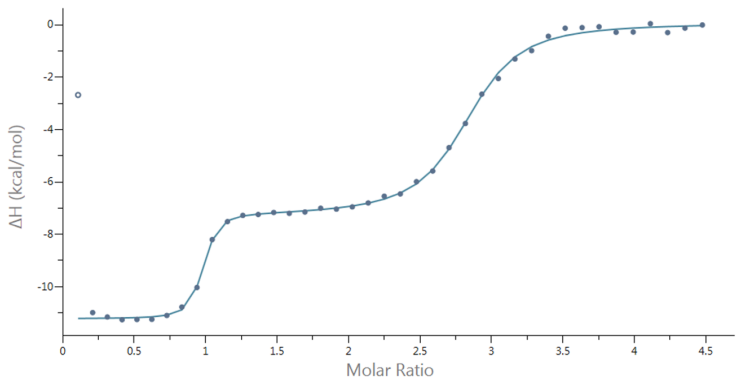
If a competitor is included, the **Use Competitive Model** check box has to be selected. The parameters for the competing molecule are enabled for adjustments by selecting this check box. Apart from adjusting the number of binding sites (N), the estimated binding constant (K_D), the estimated heat of binding ΔH, and the cell concentration, it is also possible to define the properties of the unknown binder and parameter source.

Adjust Fit - Fitting Model: Two Sets of Sites

Introduction

This fitting model is used for proteins with two non-identical sites for binding.

Fitted model plot



Fitting Parameters

Ligand is in Cell [Syr] (M) 400e-6 [Cell] (M) 20.0e-6

Parameter	Vary	Initial Value	Lower Bound	Upper Bound	Parameter	Vary	Initial Value	Lower Bound	Upper Bound
N_1 (sites)	✓	0.929	1.0e-3	10.0	N_2 (sites)	✓	1.89	1.0e-3	10.0
K_{D1} (M)	✓	4.69e-10	1.00e-12	1.00	K_{D2} (M)	✓	355e-9	1.00e-12	1.00
ΔH_1(kcal/mol)	✓	-11.3	-80.0	80.0	ΔH_2(kcal/mol)	✓	-7.28	-80.0	80.0
Offset (kcal/mol)	✓	3.0e-2	-80.0	80.0					

The charts pane displays the curve for each individual injection heat, calculated from integrated raw data using the **Two Sets of Sites** fitting model. The graph shows the estimated heat of binding (ΔH) as a function of molar ratio.

Fitting parameters

The fitting parameters that can be adjusted for the **Two Sets of Sites** fitting model are displayed below the fitting model plot. All values, such as **Initial Value**, **Lower Bound** and **Upper Bound**, can be changed if the **Vary** check box is selected. If the **Vary** check box is cleared, the **Lower Bound** and **Upper Bound** cannot be edited.

The parameters that can be adjusted in this fitting model are:

- the value for binding site 1 (N_1) and binding site 2 (N_2),
- the estimated binding constant for binding site 1 (K_{D1}) and binding site 2 (K_{D2}),
- the estimated heat of binding for binding site 1 (ΔH_1) and for binding site 2 (ΔH_2), and
- the offset.

If the **Fitted Offset** control subtraction option is selected (or deselected), it is assumed the user does not wish to fit for an offset. Thus, the **Offset** fitting parameters is automatically set to 0 and varied (or not varied). This can be manually overridden. For example, no control option may be specified, but the offset parameter may still be varied.

If a ligand is present in the cell, the **Ligand In Cell** check box has to be selected.

Adjust Fit - Fitting Model: Dissociation

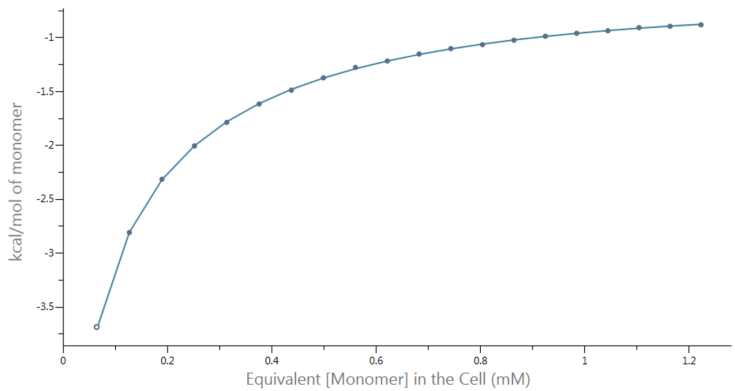
Introduction

This model is intended for the analysis of heats of dilution data where the sample compound in the syringe has a tendency to form dimers.

Multiple injections are made from the syringe and the resulting heats analyzed to give best values for the dissociation constant K_D, and the heat of dissociation ΔH.

It is assumed in this model that the stoichiometry is well-defined, that is, no aggregates with stoichiometry higher than 2 are present. By measuring heats for a series of injections it is then possible, using curve-fitting, to determine the dissociation constant K_D, and heat of dissociation.

Fitted model plot



Fitting Parameters

[Syr] (M) 21.2e-3

Parameter	Vary	Initial Value	Lower Bound	Upper Bound
K_D (M)	✓	600e-6	1.00e-12	1.00
ΔH (kcal/mol)	✓	-10.1	-80.0	80.0
Offset (kcal/mol)	✓	-6.8e-3	-80.0	80.0

The charts pane displays the curve for each individual injection heat of the monomer, calculated from integrated raw data using the **Dissociation** fitting model. The graph shows the estimated energy per mol of monomer as a function of equivalent monomer concentration in the cell.

Fitting parameters

The fitting parameters that can be adjusted for the **Dissociation** fitting model are displayed below the fitted model plot. All values, such as **Initial Value**, **Lower Bound** and **Upper Bound**, can be changed if the **Vary** check box is selected. If the **Vary** check box is cleared, the **Lower Bound** and **Upper Bound** cannot be edited.

The parameters that can be adjusted in this fitting model are:

- the estimated binding constant (K_D),
- the estimated heat of binding (ΔH),
- the offset, and
- the concentrations in the syringe and the cell.

If the **Fitted Offset** control subtraction option is selected (or deselected), it is assumed the user does not wish to fit for an offset. Thus, the **Offset** fitting parameters is automatically set to 0 and varied (or not varied). This can be manually overridden. For example, no control option may be specified, but the offset parameter may still be varied.

Adjust Fit - Fitting Model: Sequential Binding Sites

Introduction

The models discussed previously applies to independent sites. In biological systems the binding of a ligand to one site is often influenced by whether or not ligands are bound to any of the other sites. The influence phenomenon is called cooperativity.

If the sites are non-identical, then binding studies alone cannot determine whether the sites are independent or interacting.

On the other hand, if the sites within a molecule are known to be identical, then it may be possible to determine if they are interacting.

Cooperativity case description

Consider the simplest case, that of a macromolecule with two identical sites, for example, a homodimeric protein.

If the sites are identical, then it is not possible to distinguish between binding at the first site and binding at the second site, but there is a sequential saturation since the first ligand (K_{D1}, H_1) has more available empty sites to bind to than the second ligand (K_{D2}, H_2).

Cooperativity can be determined at half saturation when the dominant molecular forms are the macromolecules with either two or no ligands attached, with very little of the singly-liganded form.

Positive cooperativity

A system displays positive cooperativity if $K_{D2}>K_{D1}$.

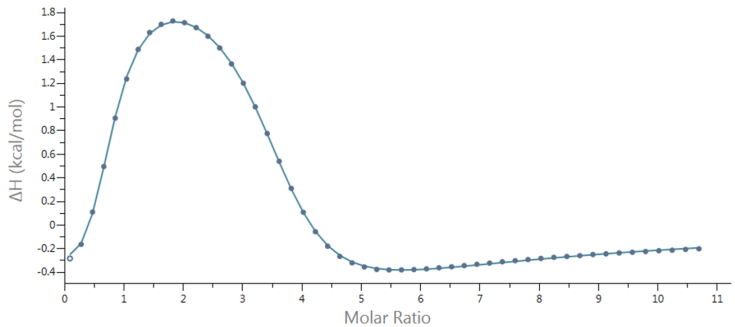
Positive cooperativity is generally more difficult to distinguish from binding studies alone, since the tendency is for both sites on any single molecule to saturate together with heat change $H_1 + H_2$, so that only one "phase" is seen in the titration curve.

Negative cooperativity

A system displays negative cooperativity if $K_{D1}>K_{D2}$.

Negative cooperativity can be more easily detected from binding studies, since there will be two different "phases" occurring: the strong binding of the first ligand and weaker binding of the second.

Fitted model plot



Fitting Parameters

Number of Sites 4 | Ligand is in Cell | [Syr] (M) 315e-3 | [Cell] (M) 6.36e-3

Parameter	Vary	Initial Value	Lower Bound	Upper Bound	Parameter	Vary	Initial Value	Lower Bound	Upper Bound
Offset (kcal/mol)	✓	-3.7e-3	-80.0	80.0					
K_{D1} (M)	✓	416e-6	1.00e-12	1.00	ΔH_1(kcal/mol)	✓	-0.288	-80.0	80.0
K_{D2} (M)	✓	10.3e-3	1.00e-12	1.00	ΔH_2(kcal/mol)	✓	4.89	-80.0	80.0
K_{D3} (M)	✓	384e-6	1.00e-12	1.00	ΔH_3(kcal/mol)	✓	0.733	-80.0	80.0
K_{D4} (M)	✓	41.8e-3	1.00e-12	1.00	ΔH_4(kcal/mol)	✓	-6.69	-80.0	80.0

The charts pane displays the curve for each individual injection heat, calculated from integrated raw data using the **Sequential Binding Sites** fitting model. The graph shows the estimated heat of binding (ΔH) as a function of molar ratio.

Fitting parameters

The fitting parameters that can be adjusted for the **Sequential Binding Sites** fitting model are displayed below the fitted model plot. All values, such as **Initial Value**, **Lower Bound** and **Upper Bound**, can be changed if the **Vary** check box is selected. If the **Vary** check box is cleared, the **Lower Bound** and **Upper Bound** cannot be edited.

The parameters that can be adjusted in this fitting model are:

- the estimated binding constant for binding site 1 (K_{D1}) and for up to 5 binding sites (K_{D2} to K_{D5}),
- the estimated heat of binding for binding site 1 (ΔH_1) and for up to 5 binding sites (ΔH_2 to ΔH_5), and
- the offset.

If the **Fitted Offset** control subtraction option is selected (or deselected), it is assumed the user does not wish to fit for an offset. Thus, the **Offset** fitting parameters is automatically set to 0 and varied (or not varied). This can be manually overridden. For example, no control option may be specified, but the offset parameter may still be varied.

If a ligand is present in the cell, the **Ligand In Cell** check box has to be selected.

The values for the number of sites (**Number of sites**), the concentration in the syringe (**[Syr] (M)**), and the concentration in the cell (**[Cell] (M)**) can also be adjusted.

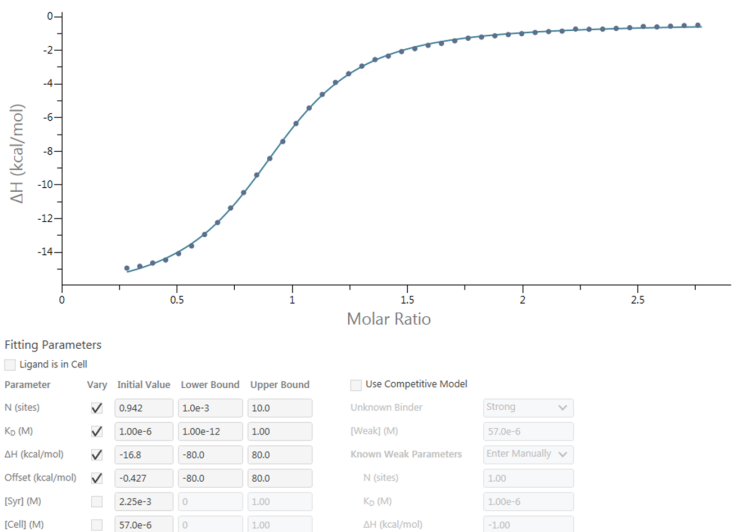
Adjust Fit - Fitting Model: One Set of Sites - SIM

Introduction

MicroCal PEAQ-ITC Analysis Software is also capable of analyzing a complete binding experiment using only a single, continuous injection, as opposed to the normal procedure that requires multiple injections. In this single injection procedure, only one slow, continuous injection of titrant solution is made into the cell material.

The binding parameters obtained from a well designed multiple injection experiment usually have a higher degree of accuracy than a single injection experiment. If the sample turnover rate is not a prime concern, perform the multiple injection experiment for more precise binding parameters.

Fitted model plot



The charts pane displays the curve for each individual injection heat, calculated from integrated raw data using the **One Set of Sites - SIM** fitting model. The graph shows the estimated heat of binding (ΔH) as a function of molar ratio.

Fitting parameters

The fitting parameters that can be adjusted for the **One Set of Sites - SIM** fitting model are displayed below the fitted model plot. All values, such as **Initial Value**, **Lower Bound** and **Upper Bound**, can be changed if the **Vary** check box is selected. If the **Vary** check box is cleared, the **Lower Bound** and **Upper Bound** cannot be edited.

The parameters that can be adjusted in this fitting model are:

- the number of binding sites (N),
- the estimated binding constant (K_D),
- the estimated heat of binding (ΔH),
- the offset, and
- the concentrations in the syringe and the cell.

If the **Fitted Offset** control subtraction option is selected (or deselected), it is assumed the user does not wish to fit for an offset. Thus, the **Offset** fitting parameters is automatically set to 0 and varied (or not varied). This can be manually overridden. For example, no control option may be specified, but the offset parameter may still be varied.

If a competitor is included, the **Use Competitive Model** check box has to be selected. The parameters for the competing molecule are enabled for adjustments by selecting this check box. Apart from adjusting the number of binding sites (N), the estimated binding constant (K_D), the estimated heat of binding ΔH, and the cell concentration, it is also possible to define the properties of the unknown binder and parameter source.

Adjust Fit - Fitting Model: Enzyme Kinetics - Multiple Injections

Introduction

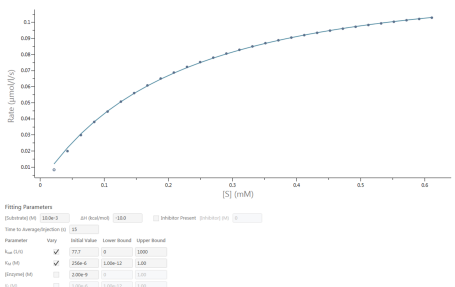
Using this approach, multiple injections of substrate solution from the syringe are made into the reaction cell containing enzyme solution (with or without inhibitor). After each injection, a sufficient time is allowed for the instrument to equilibrate at the new power level resulting from the increased substrate concentration.

Measurements are carried out quickly enough, however, so that little conversion of substrate takes place relative to the total substrate contained in the cell. That is, substrate concentration [S] is calculated directly from the total added substrate assuming no significant conversion.

The DP signal is automatically corrected for the instrument's response time to remove any effect on the experimental data.

To get a good fit you must enter the heat of substrate conversion (ΔH) in the fitting model.

Fitted model plot



The charts pane displays the curve for the rate of the reaction, calculated from integrated raw data using the **Enzyme Kinetics - Multiple Injections** fitting model. The graph shows the rate of substrate conversion as a function of substrate concentration.

Fitting parameters

The fitting parameters that can be adjusted for the **Enzyme Kinetics - Multiple Injections** fitting model are displayed below the fitted model plot. All values, such as **Initial Value**, **Lower Bound** and **Upper Bound**, can be changed if the **Vary** check box is selected. If the **Vary** check box is cleared, the **Lower Bound** and **Upper Bound** cannot be edited.

The heat of substrate conversion (ΔH) must be entered for the model to fit properly. ΔH must be obtained from a separate experiment. This separate experiment can be opened together with the multiple injection file. First, set the fitting model to Enzyme Kinetics - Single Injection and obtain the ΔH for as many single injections as desired. This can be found in the bottom right corner of the Adjust Enzyme Markers workspace. Second, change the fitting model to Enzyme Kinetics - Multiple Injections and enter the ΔH value in this workspace.

The parameters that can be adjusted in this fitting model are:

- the catalytic rate constant for substrate conversion (k_{cat}),
- Michaelis constant (K_M),
- the enzyme concentration,
- the inhibition constant (K_I),
- the inhibitor concentration, and
- Time to Average/Injection (s) - This specifies the time interval, counting backward from the next injection, used for averaging the DP signal.

If an inhibitor is present, the **Inhibitor Present** check box must be selected.

Adjust Fit - Fitting Model: Enzyme Kinetics - Single Injection

Introduction

Assaying enzymes, inhibitors or substrates by calorimetric activity has the major advantage that it works well for any enzyme/substrate/inhibitor system with no prior chemical modification of any participants in the reaction.

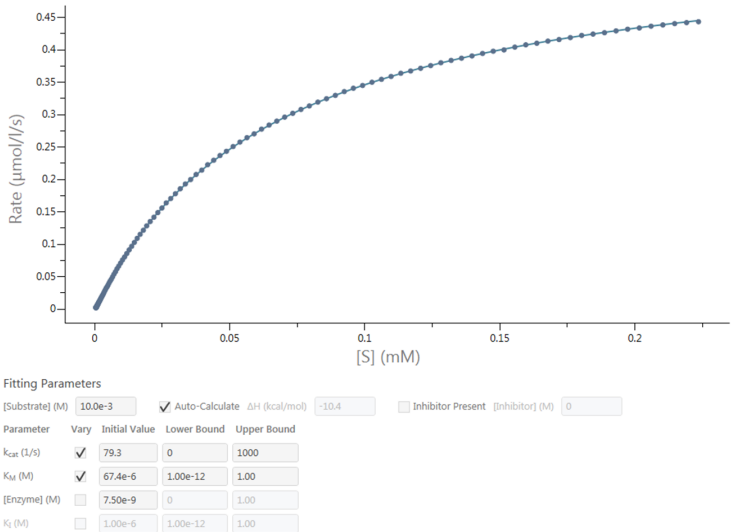
Using this approach, the reaction is initiated by the injecting enzyme solution from the syringe into the sample cell containing substrate solution, or vice versa. If desired, a competitive inhibitor may also be included in one solution or the other.

The reaction is allowed to go to completion in the calorimeter cell, and the rate of substrate conversion is recorded as a function of time.

The DP signal is automatically corrected for the instrument's response time to remove any effect on the experimental data.

You can enter the heat of substrate conversion (ΔH) in the fitting model or let it be automatically calculated (default).

Fitted model plot



Fitting Parameters

[Substrate] (M) 10.0e-3 ✓ Auto-Calculate ΔH (kcal/mol) -10.4 Inhibitor Present [Inhibitor] (M) 0

Parameter	Vary	Initial Value	Lower Bound	Upper Bound
k_{cat} (1/s)	✓	79.3	0	1000
K_M (M)	✓	67.4e-6	1.00e-12	1.00
[Enzyme] (M)		7.50e-9	0	1.00
K_I (M)		1.00e-6	1.00e-12	1.00

The charts pane displays the curve for the rate of the reaction, calculated from integrated raw data using the **Enzyme Kinetics - Single Injection** fitting model. The graph shows the rate of substrate conversion as a function of substrate concentration.

Fitting parameters

The fitting parameters that can be adjusted for the **Enzyme Kinetics - Single Injection** fitting model are displayed below the fitted model plot. All values, such as **Initial Value**, **Lower Bound** and **Upper Bound**, can be changed if the **Vary** check box is selected. If the **Vary** check box is cleared, the **Lower Bound** and **Upper Bound** cannot be edited.

The parameters that can be adjusted in this fitting model are:

- the heat of substrate conversion (ΔH),
- the catalytic rate constant for substrate conversion (k_{cat}),
- Michaelis constant (K_M),
- the enzyme concentration,
- the inhibition constant (K_I), and
- the inhibitor concentration.

If an inhibitor is present, the **Inhibitor Present** check box must be selected.

If the **Auto-calculate** check box is selected, the estimated heat of substrate conversion will be calculated automatically.

8 PRESENTATION

This section describes the available analysis results and presentation options in the **Presentation** workspace.

The following topics are covered in this section:

The analysis presentation workspace

Introduction

Analysis results are displayed in the **Presentation** workspace. The workspace is divided into two panes. The left pane displays the experiments included in the analysis and their **Bin**, and the right analysis result pane presents the results from the analysis.

Analysis result pane

In total nine analysis result panes are available.

- Result table
- Final figure
- Scatter plot
- Injection table
- Statistics plot
- Signature plot
- Raw plot
- Injection plot
- Rate plot

The number of displayed analysis result panes vary depending on which fitting model is used. Furthermore, the displayed analysis result parameters are dependent on the selected fitting model.

Result table

Introduction

The analysis results from the experiments can be summarized in a table that is displayed under the **Result Table** tab in the **Presentation** workspace. All parameters specific for the fitting model used are presented in the table.

The result table is available for all fitting models.

Table parameters and display options

The result table displays information about the experiment and the analysis results. These results are also displayed under the **Experiment Information** pane in the other work-spaces. Only the parameters that are specific for the fitting model are shown in the table.

Selected experiments are displayed in the **Experiments** pane on the left hand side. It is possible to select both a single experiment or multiple experiments.

Error values can be added if the **Display Errors** check box is selected.

Average injection heat and standard deviation is tabulated for an experiment's injection heats if the experiment is not **Bin** categorized as **OK** or **Binding**. This tabulation will be done for all fitting models except for **Enzyme Kinetics - Multiple Injections**, **Enzyme Kinetics - Single Injection**, and **One Set of Sites - SIM**.

Export of data

To export data from a table or from a curve, click the **Export Data** button.

Final figure

Introduction

The final figure from the analysis is displayed under the **Final Figure** tab in the **Presentation** workspace. Both raw data and integrated data are displayed in two graphs, respectively.

The final figure is available for **One Set of Sites**, **Two Sets of Sites**, **Dissociation** and **Sequential Binding Sites** fitting models.

Figure parameters and display options

The **Final Figure** displays raw data (upper curve) and integrated data (lower curve) from the analysis. The units on the vertical and horizontal axis are adapted according to the fitting model used.

Selected experiments are displayed in the **Experiments** pane on the left hand side. It is possible to select both a single experiment or multiple experiments. It is possible to select both a single data file or multiple data files.

- Select the **Subtract Baseline** check box to subtract the baseline from the curves.
- Select the **Show Results** check box to display the analysis results in the charts.
- To change the settings of the chart, click **Chart Options**.
  Result: The **Edit Chart And Curve Settings** dialog opens.

Chart options

If the currently selected tab depicts a chart, you may click the **Chart Option** button to adjust the chart and curve settings.

Export of data

To export data from a table or from a curve, click the **Export Data** button.

Note: Export Data can only be performed for one data file at a time.

Export of image

To export a chart as an image, click the **Export Image** button.

Note: Export Data can only be performed for one data file at a time.

Note: The entire image including both the raw data curve and the integrated curve will be saved.

Scatter plot

Introduction

A scatter plot from the analysis is displayed under the **Scatter Plot** tab in the **Presentation** workspace.

The scatter plot is available for all fitting models.

Plot parameters and display options

The **Scatter Plot** displays results for one specific analysis parameter at a time for a single or multiple experiments from the analysis.

Selected experiments are displayed in the **Experiments** pane on the left hand side. It is possible to select both a single experiment or multiple experiments.

The X axis displays the experiment with one experiment index for each experiment displayed.

The Y axis displays the result for the selected parameter .

- Select what parameter to plot in the **Plotted Parameter** menu. Available are dependent on the fitting model chosen.

  To change the settings of the chart, click **Chart Options**.
  Result: The **Edit Chart And Curve Settings** dialog opens.

Chart options

If the currently selected tab depicts a chart, you may click the **Chart Option** button to adjust the chart and curve settings.

Export of data

To export data from a table or from a curve, click the **Export Data** button.

Export of image

To export a chart as an image, click the **Export Image** button.

Injection table

Introduction

The analysis results from the experiments can be summarized in a table that is displayed under the **Injection Table** tab in the **Presentation** workspace. All parameters specific for the fitting model used are presented in the table.

The injection table is available for all fitting models.

Table parameters and display options

The injection table displays information about the experiment and the analysis results. Only the parameters that are specific for the fitting model are shown in the table.

The experiment to display can be selected in the **Experiments** pane on the left hand side. It is only possible to select one experiment at a time.

Export of data

To export data from a table or from a curve, click the **Export Data** button.

Statistics plot

Introduction

A statistics plot from the analysis is displayed under the **Statistics Plot** tab in the **Presentation** workspace.

The statistics plot is available for all fitting models and can be instructive on how sensitive a model's results may be to scatter in the data or to how many unknowns are varied.

Plot parameters and display options

The **Statistics Plot** displays a curve with the statistical deviations of integrated data from the analysis (upper curve). The units on the Y and X axis are adapted after the fitting model used.

Prediction bands are only available if the fitted result was a product of the Levenberg-Marquardt algorithm. These prediction bands are not saved with the analysis project, so to view them in a loaded analysis project requires you to perform additional iteratios using the L-M algorithm.

The experiment to display can be selected in the **Experiments** pane on the left hand side. It is only possible to select one experiment at a time.

The lower curve display residuals for the same integrated data analysis as the upper curve.

- Adjust the prediction band (in percent) by typing in the **Prediction Band (%)** box (or with the percentage scale slider) at the bottom of the pane.

  To change the settings of the chart, click **Chart Options**.
  Result: The **Edit Chart And Curve Settings** dialog opens.

Chart options

If the currently selected tab depicts a chart, you may click the **Chart Option** button to adjust the chart and curve settings.

Export of data

To export data from a table or from a curve, click the **Export Data** button.

Note: Export Data can only be performed for one data file at a time.

Export of image

To export a chart as an image, click the **Export Image** button.

Note: Export Image can only be performed for one data file at a time.

Note: The entire image including the integrated curve and the residual curve will be saved.

Signature plot

Introduction

The signature plot from the analysis is displayed under the **Signature Plot** tab in the **Presentation** workspace. The signature plot presents how much of the change in Gibbs free energy (ΔG) is dependent on enthalpy heat change (ΔH) and change in entropy (ΔS) according to the equation ΔG = ΔH - TΔS (where T is the experiment's average run temperature).

The signature plot is available for **One Set of Sites** and **One Set of Sites - SIM** fitting models only.

Plot parameters and display options

The **Signature Plot** displays the distribution of change in Gibbs free energy (ΔG), enthalpy heat change (ΔH), and change in entropy (TΔS) in energy per mol.

Selected experiments are displayed in the **Experiments** pane on the left hand side. It is possible to select both a single experiment or multiple experiments.

- To change the settings of the chart, click **Chart Options**.
  Result: The **Edit Chart And Curve Settings** dialog opens.

Chart options

If the currently selected tab depicts a chart, you may click the **Chart Option** button to adjust the chart and curve settings.

Export of data

To export data from a table or from a curve, click the **Export Data** button.

Note:Export Data can only be performed for one data file at a time.

Export of image

To export a chart as an image, click the **Export Image** button.

Note: Export Image can only be performed for one data file at a time.

Raw plot

Introduction

A raw plot from the analysis is displayed under the **Raw Plot** tab in the **Presentation** workspace.

The raw plot is available for all fitting models.

Plot parameters and display options

The **Raw Plot** displays a detailed view of the raw data for a single or multiple experiments, which is also displayed in the upper graph under the **Overview** workspace (see Workspace presentation on page 20).

Selected experiments are displayed in the **Experiments** pane on the left hand side. It is possible to select both a single experiment or multiple experiments.

The X axis displays the time.

The Y axis displays the raw signal (Differential Power). Y axis offset can be selected by selecting the **Y-offset** check box, and an offset value for the Y axis can be entered in the **Y-offset** text box.

- To change the settings of the chart, click **Chart Options**.
  Result: The **Edit Chart And Curve Settings** dialog opens.

Chart options

If the currently selected tab depicts a chart, you may click the **Chart Option** button to adjust the chart and curve settings.

Export of data

To export data from a table or from a curve, click the **Export Data** button.

Export of image

To export a chart as an image, click the **Export Image** button.

Integrated Heat Plot

Introduction

The injection plot from the analysis is displayed under the **Integrated Heat Plot** tab in the **Presentation** workspace.

This figure is available for **One Set of Sites**, **Two Sets of Sites**, **Dissociation**, **Sequential Binding Sites**, and **One Set of Sites - SIM** fitting models.

Plot parameters and display options

The **Integrated Heat Plot** displays a detailed view of integrated heat for each injection in a single or multiple experiments.

- Click **Raw Heat** to view a chart of raw heat plotted against injections.
- Click **Normalized Heat** to view a chart of normalized heat as a function of molar ratio.
- To change the settings of the chart, click **Chart Options**.
  Result: The **Edit Chart And Curve Settings** dialog opens.

Selected experiments are displayed in the **Experiments** pane on the left hand side. It is possible to select both a single experiment or multiple experiments.

Chart options

If the currently selected tab depicts a chart, you may click the **Chart Option** button to adjust the chart and curve settings.

Export of data

To export data from a table or from a curve, click the **Export Data** button.

Export of image

To export a chart as an image, click the **Export Image** button.

Rate plot

Introduction

The rate plot from the analysis is displayed under the **Rate Plot** tab in the **Presentation** workspace.

The rate plot is available for **Enzyme Kinetics - Multiple Injections** and **Enzyme Kinetics - Single Injection** fitting models only.

Plot parameters and display options

The **Rate Plot** displays the rate of substrate conversion as a function of substrate concentration.

Selected experiments are displayed in the **Experiments** pane on the left hand side. It is possible to select both a single experiment or multiple experiments.

Settings can be changed in the **Edit Chart And Curve Settings** dialog, which is accessed through the **Chart Option** button.

Chart options

If the currently selected tab depicts a chart, you may click the **Chart Option** button to adjust the chart and curve settings.

Export of data

To export data from a table or from a curve, click the **Export Data** button.

Export of image

To export a chart as an image, click the **Export Image** button.

9

EXPERIMENTAL DESIGN

This section provides guidelines on how to design an ITC experiment. The MicroCal PEAQ-ITC Analysis Software has preinstalled default method parameter settings for the different fitting models. The **Design Experiment** workspace can be used to create and simulate experiments with alternative method parameter settings.

The following topics are covered in this section:

Guided experimental design

Introduction

The **Guided** mode of the **Experimental design** view helps you design one-to-one binding experiments and simulate basic runs.

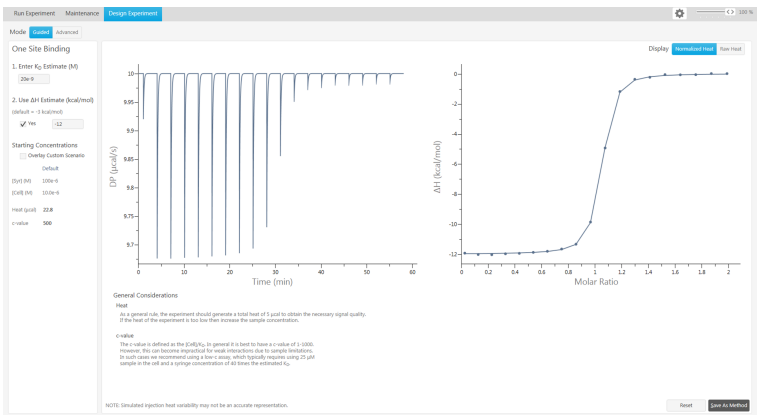
- Click the **Guided** tab to enter **Guided** mode.

Guided mode workspace

The workspace is divided into two panes.

In the left, design pane you can enter experiment estimates and read the recommended concentrations for the current estimates.

The right, simulation pane displays two graphs showing simulated raw injection data and integrated injection data.



Pane	Function
1 (left pane)	Design pane In this pane you can: • enter estimates for the dissociation constant

Pane	Function
	(K_D) and heat change (ΔH) • read the recommended concentrations for the current estimates.
2 (right pane)	Simulation pane This pane displays the simulation by two graphs showing: • the raw injection data, and • the integrated injection data. In this pane you can also read a discussion on how to design the experiment using the currently set estimates.

Enter an estimate of the dissociation constant

In the left pane, enter an estimation of the dissociation constant for the ligand-protein system used in the experiment in the **Enter K_D estimate (M)** box.

Enter an estimate of heat exchange

If you want to enter an estimate of heat change for the ligand-protein system:

Step	Action
1	In the left pane, select the **Yes** check box.
2	Enter the estimate in the **Use ΔH Estimate (kcal/mol)** box.

Simulate custom concentrations

If you want to simulate custom concentrations:

Step	Action
1	In the left pane, select the **Overlay Custom Scenario** check box.
2	Enter the concentrations in the **[Cell] (M)** and **[Syr] (M)** boxes. Result: The simulated runs are displayed in the right pane.

Starting concentrations

The starting concentrations for the currently set estimates are displayed below the heading **Starting Concentrations** in the left pane.

Default values for the concentration in the cell, the concentration in the syringe, the total heat of the experiment, and the c-value are shown, and can be adjusted by selecting the **Overlay Custom Scenario**.

If a less than ideal experiment is simulated, the **Heat** and **c-value** can be accompanied by warnings.

Simulated graphs

The right pane displays simulations of raw injection data in the left chart and a fitted curve of the simulated integrated injections data in the right chart.

There are two display options for the simulated integrated injections data plot, normalized heat and raw heat.

Reset button

Click **Reset** to reset all simulation and experimental parameters.

Save as Method button

You can save simulation and experimental parameters as method files. Save as the **ITC Method** file type for methods that are to be used in MicroCal PEAQ-ITC. Save as the **INJ** file type for methods that are to be used in MicroCal PEAQ-ITC Automated.

Step	Action
1	Click **Save as Method**. *Result:* The **Save As** dialog opens.
2	Type a file name in the **File name** box if necessary.
3	Select file type in the **Save as type** list.
4	Browse to select a destination to save the file.
5	Click **Save**. *Result*: The method will be saved in the file format selected above.

Advanced experimental design

Introduction

Advanced experimental design can be performed in the **Advanced** mode of the **Design Experiment** view.

It is possible to edit more experimental parameters in the **Advanced** mode than in the **Guided** mode, but you will not receive recommendations or comments as help text.

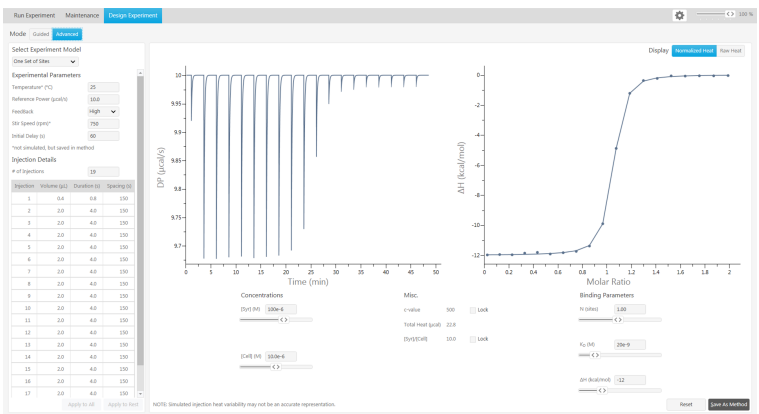
- Click the **Advanced** tab to enter **Advanced** mode.

Advanced mode workspace

The workspace is divided into two panes.

Select fitting model and experimental parameters in the left, design pane.

The right, simulation pane displays two graphs showing simulated raw injection data and integrated injection data.



Pane	Function
1 (left pane)	Design pane In this pane you can:

Pane	Function
	• select fitting model • edit the experimental parameters for the instrument • edit injection details.
2 (right pane)	Simulation pane This pane displays the simulation by two graphs showing: • the raw injection data, and • the integrated injection data. In this pane you can also adjust the experimental parameters for the simulation.

Experimental method

You can select fitting model for your simulation in the **Select Experiment Method** menu in the left, design pane.

The selectable models are:

- **One Set of Sites**
- **Competitive Binding**
- **Two Sets of Sites**
- **Dissociation**
- **Sequential Binding Sites**

Instrument settings

You can edit the settings for the instrument in the left, design pane.

Note: Not all settings reflect a change in the simulation, but they will be reflected in a saved method file.

Instrument setting	Description
Temp (°C)	Enter the desired run temperature for the experiment. The instrument's operating range is 2°C to 80°C. Most runs are performed at 25°C (room temperature).
Reference Power	Enter a value for reference power. The differential power (DP) baseline will equilibrate near this value.
FeedBack	The feedback mode affects both response time and sensitivity. High gain provides the fastest response time. No gain (passive mode, **None**) provides the highest sensitivity. Most ITC reactions require using the **High** setting. Monitoring long, slow thermal processes (for example, kinetics, metabolic rates) might benefit from using the **None** (passive) or **Low** settings.
Stir speed (rpm)	Enter the sample cell stirring speed in revolutions per minute. Faster stirring may be necessary if the sample cell contains suspended particles, for example agarose beads.
Initial Delay (s)	Enter the time (s) between the start of the run and the first injection (standard value 60 s). This is necessary to establish a baseline before the first injection.

Injection settings

You can edit the injection settings in the left, design pane.

Injection setting	Description
# of Injections	Enter the number of injections for the titration (ITC) experiment. The multiple injection method requires a minimum of 10 to 15 injections. The single injection method uses one injection.
Volume(µL)	Enter the volume (µl) of titrant to be injected from the pipette into the sample cell for the injection(s) selected in the injection list.
Duration(s)	Enter how long (seconds) each injection of titrant into the sample cell for will be.
Spacing(s)	Enter the time (seconds) between the beginning of the injection(s) selected in the injection list and the beginning of the next injection (or end of run). The injection spacing must allow enough time between injections to allow the DP signal to return to the baseline after an injection peak deflection. Typical values for this parameter range from 90 to 180 seconds, depending on the feedback mode, temperature and reaction kinetics.  For the single injection method, the spacing should be at least 90 seconds longer than the duration of the injection.

Edit injections

- Double-click a box in the injection list to edit an injection.
- Click **Apply to all** to copy the selected injection's settings to all other injections in the list.
- Click **Apply to Rest** to copy the selected injection's settings to all following injections in the list.

Reference power

The reference power is a small constant amount of power supplied to the offset heater of the reference cell. This causes the DP feedback system to supply compensating power to the sample cell to equilibrate the temperatures. The best choice for the reference power setting can be determined by the anticipated size and sign of the titration heats. The following table gives some guidelines.

Expected reaction type	Suggested reference power
Large exothermic	Large value (approx. 10 µcal/s using high feedback)
Large endothermic	Small value (approx. 0.5 µcal/s)
Unknown	Intermediate value (5 µcal/s using high feedback)

Simulated graphs

In the right, simulation pane, you can follow your simulation and adjust simulations parameter, such as concentration and binding parameters.

The upper section displays two graphs that are used to evaluate the simulation, one displaying raw injection data and one displaying integrated injection data.

There are two display options for the integrated injection data plot, normalized heat and raw heat.

The lower section provides options for adjusting the simulation parameters.

Note: All graphs and simulation parameters displayed in this pane are specific for the fitting model chosen.

Reset button

Click **Reset** to reset all simulation and experimental parameters.

Save as Method button

You can save simulation and experimental parameters as method files. Save as the **ITC Method** file type for methods that are to be used in MicroCal PEAQ-ITC. Save as the **INJ** file type for methods that are to be used in MicroCal PEAQ-ITC Automated.

Step	Action
1	Click **Save as Method**. *Result:* The **Save As** dialog opens.
2	Type a file name in the **File name** box if necessary.
3	Select file type in the **Save as type** list.
4	Browse to select a destination to save the file.
5	Click **Save**. *Result*: The method will be saved in the file format selected above.

10 REFERENCE INFORMATION

This section provides useful information about computer requirements, getting help, and equations used for fitting ITC data.

The following topics are covered in this section:

Computer specifications

Introduction

MicroCal PEAQ-ITC Analysis Software can be installed on a standard PC. The operating systems recommended for installation are Windows 7 and Windows 8.1.

Requirements

The following table describes the recommended computer specifications for MicroCal PEAQ-ITC Analysis Software.

Part	Requirements
Hard disk space	Minimum 10 GB
Internal memory space	Minimum 2 GB with at least 1 GB free
Operating system	Windows 7 and 8.1 , 32 or 64 bits versions, recommended
Operating system language	English (U.S.) Code 1033

Where to get help

This section gives information on the various channels in place to get help with your MicroCal PEAQ-ITC Analysis Software system.

Help desk

All queries about the system should be directed to your local Malvern Instruments representative, quoting the following information:

- **Model and serial number** of the instrument (usually located on the outside casing of the instrument).
- **Software version** (see **Help-About** in the software).
- **Firmware version** (the Helpdesk will inform you how to locate this information).

Contact the International Helpdesk if the local Malvern Instruments representative is not available:

Telephone: +44 (0) 1684 891800 or **email: helpdesk@malvern.com**.

Note: this help line is primarily English speaking.

Remote support

Malvern Instruments offers a remote support service, delivered by an Internet connection. Benefits include fast and efficient fault diagnosis, reducing downtime and costs.

Malvern website - www.malvern.com

The Malvern Instruments website offers a comprehensive range of particle characterization resources for use by customers 24 hours a day, seven days a week.

Please contact Malvern Instruments for any data analysis questions or issues you may have.

Equations used for fitting ITC data

This section describes the equations that are the basis for fitting ITC data with MicroCal PEAQ-ITC Analysis Software.

In this section:

- Displaced volume effects
- Single set of identical sites
- Two sets of independent sites
- Sequential binding sites
- Enzyme, substrate, inhibitor assay
- Dimer dissociation mode
- Competitive binding mode
- Chi-square (Chi2) minimization

Displaced volume effects

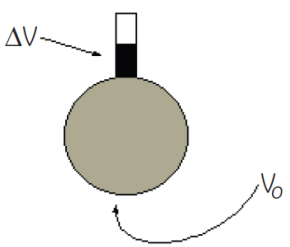
The table describes the parameters used in the following equations.

Parameter	Description
V_0	active cell volume
ΔV_i	i^{th} injection volume

Parameter	Description
ΔV	total volume injected
M^0_t	initial bulk concentration of cell material
M^0_t	bulk concentration of cell material in the active volume
X_t	bulk concentration of injectant in the active volume
[*syr*]	concentration of injectant in the syringe

Note: It will be assumed throughout that the macromolecule M is in the cell at an initial bulk concentration (moles/l) before the first injection, and the ligand X to be injected is initially at zero concentration in the cell.

The working volume (grey) of the lollipop-shaped cell is V_0, the size of the i^{th} injection is ΔV_i and the total liquid, which has been injected at any point during the experiment, ΔV is simply the sum of the individual ΔV_i for all injections.



Both the cell and the cell stem are filled with macromolecule solution, but only the active volume is monitored calorimetrically. Each injection drives liquid from the active volume into the cell stem (darkened portion representing ΔV). Consequently, in a typical experiment, M_t decreases slightly (~1 %) with each injection. We assume no mixing occurs between the active volume and the cell stem, so the average bulk concentration of macromolecule in ΔV is the computed to be the average of M^0_t and M^0_t. Taking this into account, conservation of mass requires that

$$M_t^0 V_0 = M_t V_0 + \frac{1}{2}(M_t + M_t^0)\Delta V$$

Equation (1)

so that

$$M_t = M_t^0 \left[\frac{1 - \frac{\Delta V}{2V_0}}{1 + \frac{\Delta V}{2V_0}}\right]$$

Equation (2)

Using similar reasoning, it is shown that X_t can be defined as follows:

$$[syr]\Delta V = X_t V_0 + \frac{1}{2} X_t \Delta V$$

Equation (3)

so that

$$X_t = [syr]\frac{\Delta V}{V_0}\left[\frac{1}{1 + \frac{\Delta V}{2V_0}}\right]$$

$$X_t = [syr]\frac{\Delta V}{V_0}\left[\frac{1 - \frac{\Delta V}{2V_0}}{1 - [\frac{\Delta V}{2V_0}]^2}\right]$$

$$X_t = [syr]\frac{\Delta V}{V_0}\left[1 - \frac{\Delta V}{2\Delta V_0}\right]$$

if we assume that $[\frac{\Delta V}{2V_0}]^2$ is approximately or equal to 0.

Equation (4)

The above expressions for M_t and X_t are used by MicroCal PEAQ-ITC Analysis Software to correct for displaced volume effects, which occur with each injection.

Single set of identical sites

The table describes the parameters used in the following equations.

Parameter	Description
K	binding constant
n	number of sites
[M]	free concentration of macromolecule in the active volume
[X]	free concentration of ligand in the active volume
Θ	fraction of sites occupied by the ligand **X**
Δ*H*	molar heat of ligand binding

$$K = \frac{\Theta}{(1 - \Theta)[X]}$$

Equation (5)

$$X_t = [X] + n\Theta M_t$$

Equation (6)

Combining (5) and (6) gives

$$\Theta^2 - \Theta\left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} + \frac{X_t}{nM_t}\right] = 0$$

Equation (7)

The total heat content Q of the solution contained in V_0 (determined relative to zero for the unliganded species) at fractional saturation Θ is

$$Q = n\Theta M_t \Delta H V_0$$

Equation (8)

Solving the quadratic Equation (7) for Θ and then substituting this into Equation (8) gives

$$Q = \frac{nM_t\Delta HV_0}{2}[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{(1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t})^2 - \frac{4X_t}{nM_t}}]$$

Equation (9)

The value of *Q* above can be calculated (for any designated values of *n*, *K*, and Δ*H*) at-the end of the i^{th} injection and designated *Q (i)*. The parameter of interest for comparison with experiment, however, is the change in heat content from the completion of the i^{-1} injection to completion of the *i* injection. The expression for *Q* in Equation (9) only applies to the liquid contained in volume V_0. Therefore, after completing an injection, it is obvious that a correction must be made for displaced volume (i.e., ΔV_i = injection volume) since some of the liquid in V_0 after the i^{-1} injection will no longer be in V_0 after the i^{th} injection, even though it will contribute to the heat effect (assuming the kinetics of reaction and mixing are fast) before it passes out of the working volume V_0. The liquid in the displaced volume contributes about 50% as much heat effect as an equivalent volume remaining in V_0. The correct expression for heat released, Δ*Q(i)*, from the i^{th} injection is

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0}[\frac{Q(i) + Q(i-1)}{2}] - Q(i-1)$$

Equation (10)

The process of fitting experimental data then involves:

1. initial guesses (which most often can be made accurately enough by MicroCal PEAQ-ITC Analysis Software) of *n*, *K*, and Δ*H*
2. calculation of Δ*Q(i)* for each injection and comparison of these values with the measured heat for the corresponding experimental injection
3. improvement in the initial values of *n*, *K*, and Δ*H* by standard Marquardt methods
4. iteration of the above procedure until no further significant improvement in fit occurs with continued iteration

Two sets of independent sites

Using the same definition of symbols as above for set 1 and set 2, we have

$$K_1 = \frac{\Theta_1}{(1 - \Theta_1)[X]}$$

$$K_2 = \frac{\Theta_2}{(1 - \Theta_2)[X]}$$

Equation (11)

$$X_t = [X] + M_t(n_1\Theta_1 + n_2\Theta_2)$$

Equation (12)

Solving Equation (11) for Θ_1 and Θ_2 and then substituting into Equation (12) gives

$$X_t = [X] + \frac{n_1 M_t [X] K_1}{1 + [X] K_1} + \frac{n_2 M_t [X] K_2}{1 + [X] K_2}$$

Equation (13)

Clearing Equation (13) of fractions and collecting like terms leads to a cubic equation of the form

$$[X^3] + p[X^2] + q[X] + r = 0$$

Equation (14)

where,

$$p = \frac{1}{K_1} + \frac{1}{K_2} + (n_1 + n_2) M_t - X_t$$

$$q = (\frac{n_1}{K_2} + \frac{n_2}{K_1}) M_t - (\frac{1}{K_1} + \frac{1}{K_2}) X_t + \frac{1}{K_1 K_2}$$

$$r = \frac{-X_t}{K_1 K_2}$$

Equation (15)

Equation 14 and Equation 15 can be solved for [*X*] either in closed form or (as done in MicroCal PEAQ-ITC Analysis Software) numerically by using Newton's Method if parameters n_1, n_2, K_1, and K_2 are assigned. Both Θ_1 and Θ_2 may then be obtained from Equation 11 above.

As discussed in Single set of identical sites, the heat content after any injection *i* is equal to

$$Q = M_t V_0 (n_1 \Theta_1 \Delta H_1 + n_2 \Theta_2 \Delta H_2)$$

Equation (16)

After a similar correction for the displaced volume, the pertinent calculated heat effect for the i^{th} injection is

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1)$$

Equation (17)

which may be used in the Marquardt algorithm to obtain best values for the six fitting parameters.

Sequential binding sites

For sequential binding, the binding constants K_1, K_2, ... K_n must be defined relative to the progress of saturation, so that

$$K_1 = \frac{[MX]}{[M][X]}$$

$$K_2 = \frac{[MX_2]}{[MX][X]}$$

$$K_3 = \frac{[MX_3]}{[MX_2][X]}$$

Equation (18)

In the sequential model, there is no distinction as to which sites are saturated, but only as to the total number of sites that are saturated. If the sites are K_i (defined by Equation (18)) and the intrinsic binding constants K^0_i where the effect of degeneracies has been removed. The relationship between the two binding constants is given by:

$$K_i = \frac{n - i + 1}{i} K_i^o$$

Equation (19)

All calculations given below, as well as parameters reported from curve-fitting, are in terms of K_i values but the operator may convert to K^0_i values, if desired, using Equation (19). Since concentrations of all liganded species $[ML_i]$ can be easily expressed in terms of the concentration of the non-liganded species, $[M]$, then the fraction of total macromolecule having i bound ligands, F_i, is simply

$$F_0 = \frac{1}{P}$$
$$F_1 = \frac{K_1[X]}{P}$$
$$F_2 = \frac{K_1 K_2[X]^2}{P}$$
$$F_n = \frac{K_1 K_2 \ldots K_n[X]^n}{P}$$

Equation (20)

where

$$P = 1 + K_1[X] + K_1 K_2[X]^2 + \ldots + K_1 K_2 \ldots K_n[X]^n$$

$$X_t = [X] + M_t \sum_{i=1}^{n} iF_i$$

Equation (21)

Once *n* and values of fitting parameters K_1 through K_n are assigned, then Equation (20) and Equation (21) may be solved for [*X*] by numerical methods (the Bisection method is used). After [*X*] is known, all F_i may be calculated from Equation (20) and the heat content after the i^{th} injection is determined from

$$Q = M_t V_0 (F_1 \Delta H_1 + F_2[\Delta H_1 + \Delta H_2] + \ldots + F_n[\Delta H_1 + \Delta H_2 + \Delta H_3 + \ldots + \Delta H_n])$$

Equation (22)

and, as before,

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1)$$

Equation (23)

which then leads into the Marquardt minimization routine.

Enzyme, substrate, inhibitor assay

Assaying enzymes, inhibitors or substrates by calorimetric activity has the major advantage that it works well for any enzyme/substrate/inhibitor system with no prior chemical modification of any participants in the reaction. The rate R_t of the substrate conversion reaction is directly proportional to the power output in the calorimeter cell, i.e.,

$$R_t = \frac{P}{\Delta H V_0}$$

Equation (24)

where,

Parameter	Description
P	power generated by the reaction
ΔH	heat of conversion of the substrate

The units of R_t will be moles/l/sec if P is expressed in µcal/sec, ΔH in µcal per mol of substrate, and V_0 in liters, for example.

If Michaelis-Menten kinetics are assumed then the experimental values for the rate R_t can be expressed as

$$R_t = \frac{k_{cat}[E]_{cat}[S]_t}{[S]_t + K_M\left(1 + \frac{[I]}{K_I}\right)}$$

Equation (25)

where,

Parameter	Description
k_{cat}	catalytic rate constant for substrate conversion
K_M	Michaelis constant
$[E]_{tot}$	total enzyme concentration
$[S]_t$	instantaneous concentration of substrate
$[I]$	concentration of competitive inhibitor
$[K]_1$	inhibition constant

The equation as written is valid both in the absence or presence of a $[I]$ and $[K]_1$.

The use of Equation (25) assumes no effects from product inhibition. This assumption has been discussed by Todd and Gomez (Todd, M. J. & Gomez, J. (2001) Analytical Biochemistry 296, 179-187.) and found to be quantitative in many cases. In those cases where product inhibition is significant, then Equation (25) can only be used to express initial rates of reaction prior to accumulation of product.

Todd and Gomez discussed in some detail the two methods by which assays can be carried out in a titration calorimeter, and these are summarized in the following topics.

Method 1: Single injection

Using this approach, the reaction is initiated by the injecting enzyme solution from the syringe into the sample cell containing substrate solution, or vice versa. If desired, a competitive inhibitor may also be included in one solution or the other. The reaction is allowed to go to completion in the calorimeter cell, and the power P is recorded as a function of time t.

Integration of the excess power P associated with the reaction enables ΔH to be determined, i.e.,

$$\Delta H = \frac{\int_0^\infty P\,dt}{[S]_{t=0} V_0}$$

Equation (26)

where $[S]_{t=0}$ is the starting substrate concentration. Knowing ΔH, the substrate concentration can be determined as a function of time from the equation:

$$[S]_t = [S]_{t=0} - \frac{\int_0^t P\,dt}{\Delta H V_0}$$

Equation (27)

After obtaining the time-dependent rate from Equation (24), these data can be equated to the Michaelis expression in Equation (25) so that the final equation can be fit by non-linear least squares. In the absence of inhibitor, k_{cat} and K_M are used as variable parameters during iterative fitting. In the presence inhibitor I, it is best to enter previously determined values of k_{cat} and K_M and use K_1 as the only variable fitting parameter.

Method 2: Multiple injections

In this method, multiple injections of substrate solution from the syringe are made into the reaction cell containing enzyme solution (with or without inhibitor). After each injection, a sufficient time is allowed for the instrument to equilibrate at the new power level resulting from the increased substrate concentration. Measurements are carried out quickly enough, however, so that little conversion of substrate takes place relative to the total substrate contained in the cell. That is, $[S]_t$ is calculated directly from the total added substrate assuming no significant conversion.

Equation (24) and Equation (25) are still valid for Method 2, except that R_t and $[S]_t$ now correspond to discrete values of the rate and substrate concentration after each injection, rather than time-dependent values. To determine ΔH from Equation (26), it is necessary to carry out another single-injection experiment where conversion is allowed to go to completion. Having done this, then discrete values of R_t at different $[S]_t$ Rt are calculated, so that Equation (25) can then be fit to obtain best values of k_{cat} and K_M (in the absence of inhibitor). In the presence of a competitive inhibitor, data are also fit to Equation (25) but using k_{cat} and K_M as fixed (results obtained from previous experiment with no inhibitor present) and treating K_1 as the only fitting parameter.

Dimer dissociation mode

A protein molecule *P*, may associate at high concentrations to form a dimer. The dilution of this concentrated protein solution by injection into the calorimeter cell containing buffer can then result in some heat effects from dissociation

$$P_2 \overset{\Delta H_{disc}}{\Leftrightarrow} 2P$$

$$K_D = \frac{(P)^2}{(P_2)}$$

where,

Parameter	Description
(P)	concentration of monomer
(P_2)	concentration of dimer
ΔH_{disc}	heat of dissociation of the dimer
K_D	dissociation constant

It is assumed in this model that the stoichiometry is well-defined, i.e., no aggregates with stoichiometry higher than 2 are present. By measuring heats for a series of injections it is then possible, using curve-fitting, to determine K_D and ΔH_{disc}.

The equivalent monomer concentration *C* is defined as

$$C = [P] + 2[P_2]$$

Equation (28)

Solving for *P* we have

$$[P] = \frac{\sqrt{1 + \frac{8}{K_D}C} - 1}{\frac{4}{K_D}}$$

Equation (29)

from which $[P_2]$ follows.

The heat released q_i when the i^{th} injection of volume dV_i is made into a fixed-volume (V_0) cell will be

$$q_i = \Delta H_{disc}\left(dV_i\left[P_{2_{syr}}\right] - V_0\left[P_{2_i}\right] + V_0\left[P_{2_{i-1}}\right] - dV_i\left[P_{2_{i-1}}\right]\right)$$

Equation (30)

The first term in Equation (30) is the heat content of the aggregate contained in the injection volume prior to injection while the second and third terms are the net heat content due to the difference in aggregate present in the cell before and after the injection.

Assuming experimental parameters V_0, dV_i, and C_{syr} are known, Equation (28), Equation (29), and Equation (30) are simultaneous equations, which can be solved for q_i whenever values are assigned to K_D and ΔH_{disc}.

Competitive binding mode

Using conventional ITC methods, binding constants from 10^3 M^{-1} to 10^8 M^{-1} can be measured most accurately. When binding constants significantly exceed 10^8 M^{-1}, instrument sensitivity becomes challenged as concentrations are lowered to the point where quantitative measurements of the binding constant would be possible. On the other hand, binding constants substantially in excess of 10^8 M^{-1} can be measured quantitatively if such strong-binding ligands are studied in competition with a second ligand, which binds competitively but more weakly to the same binding site.

Competitive binding studies are carried out using the strong-binding ligand A as the injectant, with the solution in the cell containing the second competitive ligand B as well as the binding protein P (or other target molecule). This system has two equilibria, which are displaced with each injection, i.e.,

$$A + P \overset{\Delta H_A}{\Leftrightarrow} PA \qquad K_A = \frac{[PA]}{[P][A]}$$

$$B + P \overset{\Delta H_B}{\Leftrightarrow} PA \qquad K_B = \frac{[PB]}{[P][B]}$$

The value of K_B and ΔH_B and for the competing ligand are first measured in a conventional ITC experiment, and these parameter values are entered as known parameters when determining K_A from results of the competition experiment. For the competition experiment, the total concentration of competing ligand, $[B]_{tot}$, should be selected such that

$$\frac{"K_A"}{K_B[B]_{tot}} \approx 10^5 - 10^8\ M^{-1}$$

where "K_A" is the estimated value of K_A.

The detailed equations used in the fitting model for competitive binding are found in a paper by Sigurskjold (Sigurskjold, B. W. (2000) Analytical Biochemistry 277, 260-266).

Chi-square (Chi^2) minimization

The aim of the fitting procedure is to find those values of the parameters that best describe the data. The standard way of defining the best fit is to choose the parameters so that the sum of the squares of the deviations of the theoretical curve(s) from the experimental points for a range of independent variables is at a minimum.

For ITC models where there is no weighting, the theoretical models can be represented by:

$$y = f(x; p_1, p_2, p_3, \ldots)$$

where:

P_i = the fitting parameters

Hence, the expression for X^2 simplifies to:

$$\chi^2 = \frac{1}{n^{eff} - p} \sum [y_i - f(x_i; p_1, p_2, \ldots)]^2$$

where:

Parameter	Description
n^{eff}	the total number of experimental points used in the fitting
p	total number of adjustable parameters
y_i	experimental data points
$f(x; p_1, p_2, p_3 \ldots)$	fitting function

Note: The difference $d = n^{eff} - p$ is usually referred to as the number of degrees of freedom.

The previous equation states that the Chi-squared value of the fit is equal to the sum of the squares of the deviations of the theoretical curve(s) from the experimental points divided by the number of degrees of freedom (DoF). Since there is no weighting, it can be seen that the calculated values are dependent on the magnitude of the scale and the number of data points. After fitting, this value is reported as Chi^2/DoF.

INDEX

F

G

H

I

M

N

O

Malvern Instruments Limited
Grovewood Road, Malvern
Worcestershire, WR14 1XZ, UK

Tel +44 1684 892456
Fax +44 1684 892789

www.malvern.com

MAN0576
Cover: MRK2161