MicroCal Auto-iTC$_{200}$ System

User Manual

MAN0552-02-EN-00 May 2015

MicroCal Auto-iTC$_{200}$ System

This document is an addendum to the information in the MicroCal Auto-iTC$_{200}$ System User Manual MAN0552
 for the sections indicated below:

Section 3
Note: Step 2 should read: Click the *Autosampler Software* button from the menu.
If the CD does not start automatically, run Auto200Setup.exe from the CD. Do <u>not</u> run Setup_AutoItc200.exe.

Note: Step 1 should read: Click the *Autosampler Software* button from the menu.
If the CD does not start automatically, run Auto200Setup.exe from the CD. Do <u>not</u> run Setup_AutoItc200.exe.

Section 4 Control Software
Note: Stirring Speed (RPM) in the Method Parameters chart should read **750**.

Note: In the table the row labeled Stirring Speed should read: Enter the sample cell stirring speed in rpm (recommended value is 750 rpm). Faster stirring may be necessary if the sample cell contains suspended particles, for example, agarose beads.

Section 5 Performing a Run
Note: The row labeled Stirring Speed (RPM) found in the chart in Step 2 should read **750**.

Note: See note below for section 9.6.

Section 9 Trouble shooting
Note: Nitrogen source should be regulated to at least ˉ 20 and < 30 psi

Note: The *Plate* and *Plate2* cell load scripts' pre-equilibration schemes are erroneously described. The default, recommended cell load script, *Plate*, pre-equilibrates the sample in the tubing, to ambient temperature. An alternative cell load script, *Plate2*, pre-equilibrates the sample in the cell, to experimental temperature. *Plate* is the default cell loading script and was found to be the most robust at loading the cell, but *Plate2* can be more effective when operating at temperatures much higher than ambient.

The automation method using *Plate2* cell loading has been removed. To use *Plate2* cell loading, create a custom automation method that uses the *Plate2* cell loading script. We have added an additional cell load script called *Plate 30 min*, which equilibrates the cell material in the tubing for 30 minutes, instead of the

default 5 minutes used in **Plate**. To minimize outgassing effects, this script may be necessary in some cases, but the trade-off is adding time to the experiment. Similarly to the **Plate2** script, **Plate 30 min** can only be used if made part of a custom automation method.

Section 10 Reference information

Note: See below for updated <u>Automation Method</u> and <u>Cell Load Script</u> tables. The **Plate2** Automation Method has been removed. The **Plate 30 min, Plate Prerinse Quick, Plate2 Prerinse, Tube 30 min, Tube Prerinse Quick,** and **Tube2 Prerinse** cell load scripts have been added but can only be used if made part of a custom automation method. The **Plate Syringe Clean** and **Tube Syringe Clean** cell loading scripts are unnecessary and have been removed. Additionally, and as described previously, the descriptions of the **Plate** & **Plate2** cell load scripts have been corrected. All other script groups (Cell Clean, Pipette Load, Pipette Clean, Titrant Transfer, and Transfer Clean) remain the same as described in the user manual.

The Site requirements table has been updated (see table below labeled <u>Site requirements</u>).

Automation methods

Setup File	Cell Load	Cell Clean	Syringe Clean	Titrant Transfer Clean	Pre-rinse	Pre-equilibrate sample
Plates	P	D	W	W	N	Y (in tubing)
Plates Clean	P	D (extra)	W	W	N	Y (in tubing)
Plates Syringe Clean	P	D	D	D	N	Y (in tubing)
Plates Prerinse	P	D	W	W	Y	Y (in tubing)
Plates Prerinse Syringe Clean	P	D	D	D	Y	Y (in tubing)
Plates Quick	P	W	W	W	N	N
Tubes	T	D	W	W	N	Y (in tubing)
Tubes Clean	T	D (extra)	W	W	N	Y (in tubing)
Tubes Syringe Clean	T	D	D	D	N	Y (in tubing)
Tubes Prerinse	T	D	W	W	Y	Y (in tubing)
Tubes Prerinse Syringe Clean	T	D	D	D	Y	Y (in tubing)
Tubes Quick	T	W	W	W	N	N
Continue Injections	N/A	N/A	W	W	N	N

P:Plate; T:Tube; D:Detergent; W:Water; Y:Yes; N:No

Addendum

Automation script files

ScriptType	ScriptName	Description
Cell Load	*Plate*	Load from plate, pre-equilibrate sample for 5 min (in tubing)
	Plate 30 min	"*Plate*" with a 30 min sample equilibration (in tubing) – must be added to a custom automation method
	Plate2	Load from plate, pre-equilibrate sample for 5 min (in cell); This can be useful when experiments are run at temperatures higher than ambient. – must be added to a custom automation method
	Plate Quick	Minimal plates cell load script; no sample pre-equilibration
	Plate Prerinse	"Plate" plus a cell pre-rinse
	Plate Prerinse Quick	"*Plate Quick*" plus a cell pre-rinse – must be added to a custom automation method
	Plate2 Prerinse	"*Plate2*" plus a cell pre-rinse – must be added to a custom automation method
	Remove From Cell	Used to concatenate experiments
	Tube	Load from tube, pre-equilibrate sample for 5 min (in tubing)
	Tube 30 min	"*Tube*" with a 30 minute sample equilibration (in tubing) – must be added to a custom automation method
	Tube2	Load from tube, pre-equilibrate sample for 5 min (in cell); This can be useful when experiments are run at temperatures higher than ambient. – must be added to a custom automation method
	Tube Quick	Minimal tubes cell load script; no sample pre-equilibration
	Tube Prerinse	"*Tube*" plus a cell pre-rinse
	Tube Prerinse Quick	"*Tube Quick*" plus a cell pre-rinse – must be added to a custom automation method
	Tube2 Prerinse	"*Tube2*" plus a cell pre-rinse – must be added to a custom automation method

Site requirements

Part	Function
Bench space	˜ 4 ft. (125 cm) of lab bench, ˜ 30 in. (80 cm) in depth, - No obstructions for at least 32 in. (80 cm) above bench, - Rated for at least 250 lbs. (115 kg), These include the clearances. Service functions will require an additional 12 in. (131 cm) overhead clearance.
Clearance	˜ 6 in. (15 cm) behind the Autosampler, ˜ 15 in. (40 cm) in front.
Pressure	˜ 20 and < 30 psi laboratory nitrogen (if using tank, tank must not infringe on Autosampler clearances). Connection is made with a ¼ in. NPT fitting.

Table of Contents

1 Introduction

Purpose of the user manual

This user manual provides instructions needed to run MicroCal Auto-iTC$_{200}$ and to analyze isothermal titration calorimetry (ITC) data. This user manual is a complement to *MicroCal Auto-iTC$_{200}$ System Operating Instructions*.

Prerequisites

In order to operate the system 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.

* You must read and understand the Safety Instructions as outlined in Chapter 2 of *MicroCal Auto-iTC$_{200}$ System Operating Instructions*.

* The instrument and software must be installed, configured and calibrated as outlined in Chapter 3 of *MicroCal Auto-iTC$_{200}$ System Operating Instructions* by Malvern Instruments personnel.

In this chapter

This chapter contains the following sections:

1.1 Important user information

Read this before using MicroCal Auto-iTC$_{200}$

All users must read the Safety Instructions described in Chapter 2 of *MicroCal Auto-iTC$_{200}$ System Operating Instructions* to fully understand the safe use of MicroCal Auto-iTC$_{200}$ before using or maintaining the system.

Do not operate MicroCal Auto-iTC$_{200}$ in any other way than described in the user documentation. If you do so, you may be exposed to hazards that can lead to personal injury, and you may cause damage to the equipment.

Intended use

MicroCal Auto-iTC$_{200}$ is an isothermal titration calorimeter designed for biomolecular interaction studies in research applications.

MicroCal Auto-iTC$_{200}$ is intended for research use only, and shall not be used in any clinical procedures, or for diagnostic purposes.

Safety notices

This user documentation contains WARNINGS, CAUTIONS and NOTICES concerning the safe use of the product with meanings as defined below.

Warnings

> ⚠️ **WARNING**
>
> **WARNING** indicates a hazardous situation which, if not avoided, could result in death or serious injury. It is important not to proceed until all stated conditions are met and clearly understood.

Cautions

> **CAUTION**
>
> **CAUTION** indicates a hazardous situation which, if not avoided, could result in minor or moderate injury. It is important not to proceed until all stated conditions are met and clearly understood.

Notices

> **NOTICE**
>
> **NOTICE** indicates instructions that must be followed to avoid damage to the product or other equipment.

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.*

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 Auto-iTC$_{200}$ software generates or that the user must type are represented by a monotype typeface (e.g., `C:\Origin70\Samples`).

1.2   Regulatory information

For regulatory information regarding MicroCal Auto-iTC$_{200}$, refer to section 1.2 of *MicroCal Auto-iTC$_{200}$ System Operating Instructions.*

1.3   User documentation

Introduction

This section lists user documentation that is delivered with the MicroCal Auto-iTC$_{200}$.

User documentation

The user documentation for MicroCal Auto-iTC$_{200}$ consists of:

- *MicroCal Auto-iTC$_{200}$ System Operating Instructions*
- *MicroCal Auto-iTC$_{200}$ System User Manual* (this manual)

1.4   Associated documentation

Introduction

This section describes how to find related literature that can be downloaded or ordered from Malvern Instruments.

Downloadable content

Additional downloadable material can be found at:

- www.malvern.com/microcal

2   MicroCal Auto-iTC$_{200}$

Introduction

This section gives an overview of ITC and the MicroCal Auto-iTC$_{200}$ system.

In this chapter

This chapter contains the following sections:

2.1 Overview of an isothermal titration calorimeter

Introduction

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 noncovalent bonds, for example, when the interacting molecules go from the free to the bound state. An ITC mixes the binding partners and monitors these heat changes by measuring the power required to maintain zero temperature difference between the reference and sample cells (see *Main components of an ITC, on page 15*).

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).

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). An example experimental curve is depicted below.
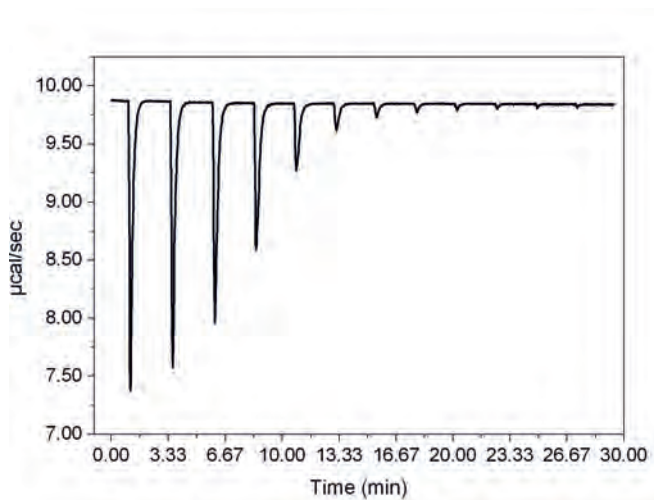
Notice that the first injection results in a larger deflection from the baseline, denoting a larger heat and nearly 100% binding. At the conclusion of the experiment, very little of the injected substance binds, resulting in little or no deflection from baseline (heat). Also, notice that the value on the y-axis decreases upon binding. In other words, 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 main components of an ITC system are illustrated below:
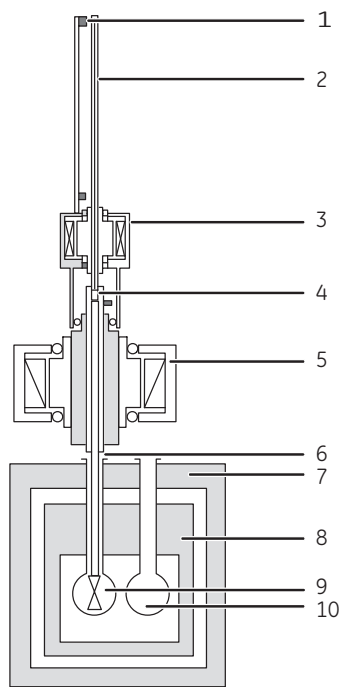
Part	Description
1	Sensor
2	Lead screw
3	Injector

Part	Description
4	Plunger
5	Stirring syringe
6	Syringe
7	Outer shield
8	Inner shield
9	Sample cell
10	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 depicted below. Each spike, followed by a return to the baseline, is an injection.
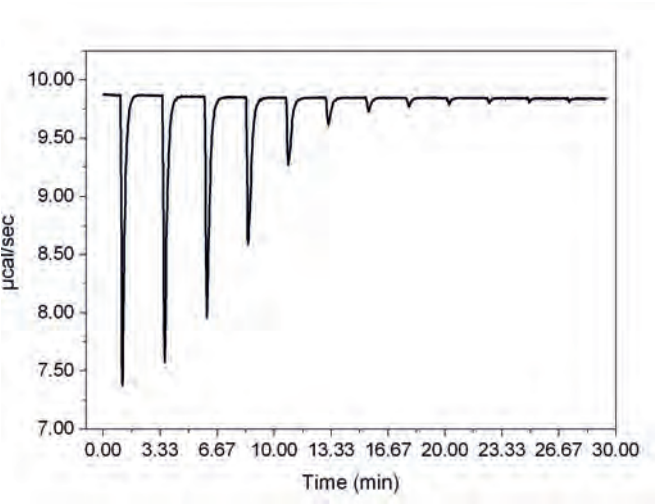
Injection heat

The individual injection heats are calculated by integrating the raw data (power) from each injection over time. The figure below depicts 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.
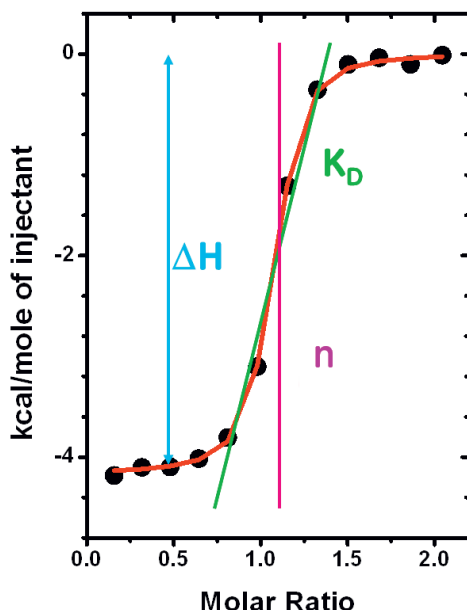
In the case of this simple 1:1 binding experiment, the enthalpy is directly measured/fitted as the heat of 100% binding. The stoichiometry is intuitively denoted by the midpoint of the titration, between 100% binding and 0% binding. The steepness of the rise to saturation is related to binding affinity. For any given system, the steepness of this region is also directly related to the sample concentration.

Data analysis will be explained in more detail in *Chapter 6 Data analysis using MicroCal Auto-iTC$_{200}$ software, on page 136* and *Chapter 7 Data analysis using Origin, on page 143*.

2.2   Description of MicroCal Auto-iTC$_{200}$
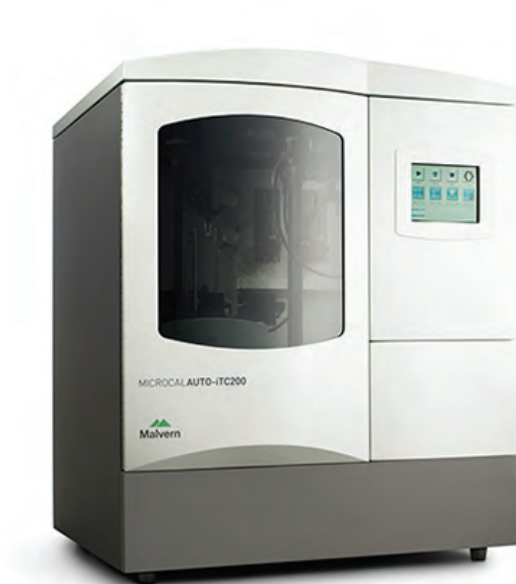
Introduction

MicroCal Auto-iTC$_{200}$ provides detailed insight into binding energetics.

The system has a 200 µl sample cell and provides direct measurement of the heat absorbed or evolved as a result of mixing precise amounts of reactants. The sample and reference cells are made from Hastelloy™, a highly inert material. The automated system cleans and loads the cell, cleans and loads the pipette, and keeps the samples at a user-specified temperature.

Automated data analysis is performed by the MicroCal Auto-iTC$_{200}$ software™, wherein the user obtains the stoichiometry (n), dissociation constant (K$_D$), and enthalpy (ΔH) of the interaction. Origin™ software can still be used to fit more complicated models.

Autosampler

The illustration below shows the MicroCal iTC$_{200}$ system inside the Autosampler.

MicroCal Auto-iTC$_{200}$ is composed of the calorimeter, a fluidic system, autosampler, and temperature-controlled tray for storing samples. A computer controller is provided with the instrument and contains proprietary software and calibration constants specific to the automation and MicroCal iTC$_{200}$. A set of reagent (water, detergent, and methanol) bottles, a waste bottle, and a nitrogen tank are plumbed to the rear of the Autosampler.

Primary components of MicroCal Auto-iTC$_{200}$

The primary components of MicroCal Auto-iTC$_{200}$ are illustrated below.

Part	Description
1	Titrant transfer arm
2	Titration pipette arm
3	Cell arm
4	Valve
5	Cannula
6	Pipette
7	Tube rack drawer (can hold five 30 ml tubes)
8	96-well plate tray drawer (can hold four 96-well plates)

MicroCal Auto-iTC$_{200}$ sits in the lower left corner of the automated box. Three robotic arms, one holding the titration pipette, and two holding the cannulas for fluid transfer, move within the box. A tube rack drawer at the right side of the box allows the user to insert samples in 30 ml centrifuge tubes. The sample tray at the bottom can hold up to four 96-well plates.

⚠ **CAUTION**

The tray drawer and tube drawer both have the potential to cause injury by pinching a body part between the face plate and the instrument's frame. Keep hands out of the drawers before instructing the instrument to close the drawers.

Connections on the rear panel

Connections on the lower left of the rear panel are provided for pressurized nitrogen, USB data connection to the controller, power connection, and connections to reagent and waste bottles.
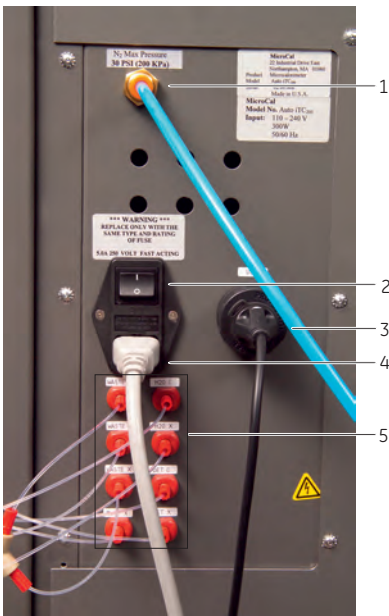
Part	Function
1	Connection for pressurized nitrogen
2	Power switch

Part	Function
3	USB data connection to the controller
4	Power connection
5	Connections to reagent and waste bottles

3 Getting started

Introduction

This chapter describes the installation of the MicroCal Auto-iTC$_{200}$ software and settings for Windows 7.

Information about Networking and TCP Port Conflicts, see *Section 10.2 Networking, on page 326* and *Section 10.3 TCP Port Conflicts, on page 333*.

In this chapter

This chapter contains the following sections:

Section	See page
3.1 Installing MicroCal Auto-iTC$_{200}$ software	24
3.2 Settings for Windows 7	45

3.1 Installing MicroCal Auto-iTC$_{200}$ software

Introduction

If a previous version of software is installed on the controller, then follow the software update instructions described in *Section 3.1.1 Updating the software, on page 25* otherwise follow the instructions for a full installation in *Section 3.1.2 Complete installation of the software, on page 33*.

In this section

This section contains the following topics:

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.1 Updating the software

3.1.1 Updating the software

Note: *Installation of the control software requires administrative privileges.*

Removing previous versions of MicroCal Auto-iTC$_{200}$ or OriginAddOn applications

To remove previous versions of MicroCal Auto-iTC$_{200}$ or OriginAddOn applications, follow the steps described below:

Step	Action
1	Navigate to *Start*:*Control Panel*.
2	Select *Add/Remove Programs* (Windows XP) or *Programs and Features* (Windows 7).
3	Select *Auto-iTC$_{200}$* and click *Remove/Uninstall*.
4	Select *OriginAddOn* and click *Remove/Uninstall*.

Updating the control software

The control software CD contains the following applications:

- MicroCal iTC$_{200}$ software
- Autosampler software
- USB driver for injector
- USB driver for data aquisition
- InitDT service
- .Net runtime

To update the control software, follow the steps described below:

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.1 Updating the software

Step	Action
1	Insert the CD into the CD-ROM drive of the PC.

The CD runs automatically and the **MicroCal Setup** window appears.

Note:
*If the CD does not start automatically, run **Auto200Setup.exe** from the CD.*

2	Click the **Autosampler Software** button from the menu or navigate to the folder **AutoITC200 Installer** on the CD and run the **Setup_AutoItc200.exe** file.

The **Welcome to the AutoItc200 Setup Wizard** window opens.

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.1 Updating the software

Step	Action
3	Click **Next**.
	The **Select Installation Folder** window appears.

4	Click **Next** to install in the default path.
	Note:
	Make sure that the radio button **Everyone** *is selected.*
	The **Confirm Installation** window appears.

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.1 Updating the software
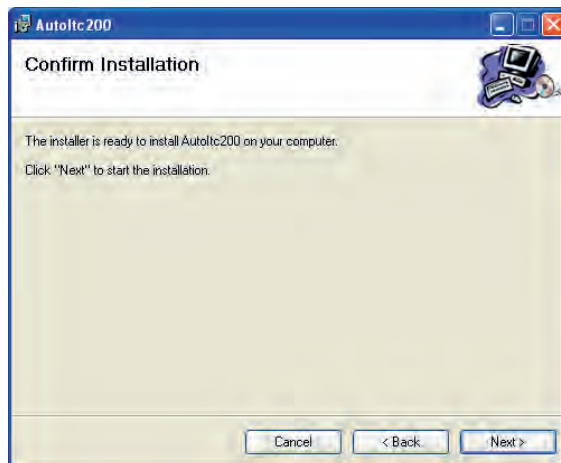
Step	Action
5	Click **Next** to start the installation.
	The **Installing AutoItc200** window appears.
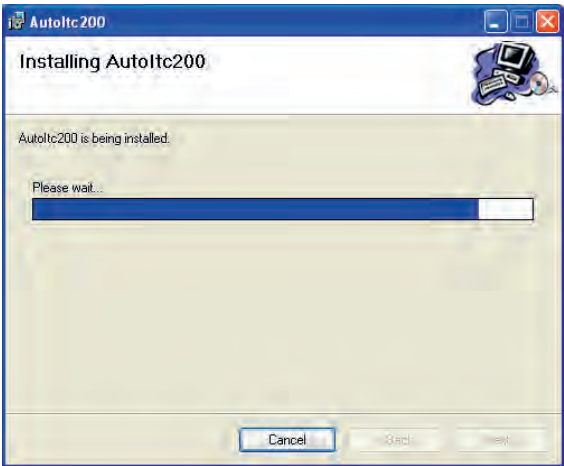
Once the installation is complete, the **Installation Complete** window appears.
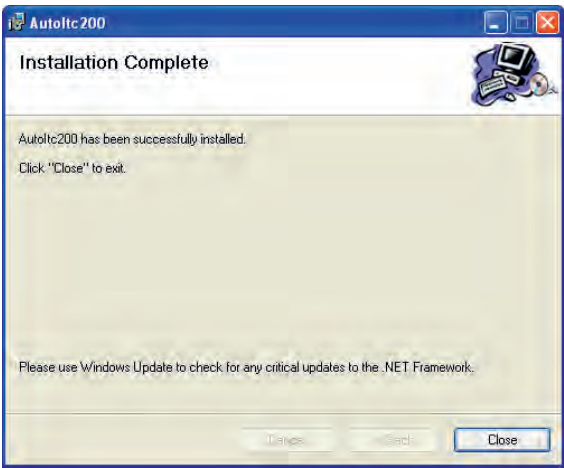
6	Click **Close** to exit the application.

To update the MicroCal iTC$_{200}$ control software, follow the steps below:

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.1 Updating the software

Step	Action
1	Click on the **iTC200 Software Update** button for the installation to start automatically.
2	Follow the on-screen instructions and click **Finish** when the installation is complete.

Be sure to select the option to restart the computer. Shortcut icons are created on the desktop automatically.
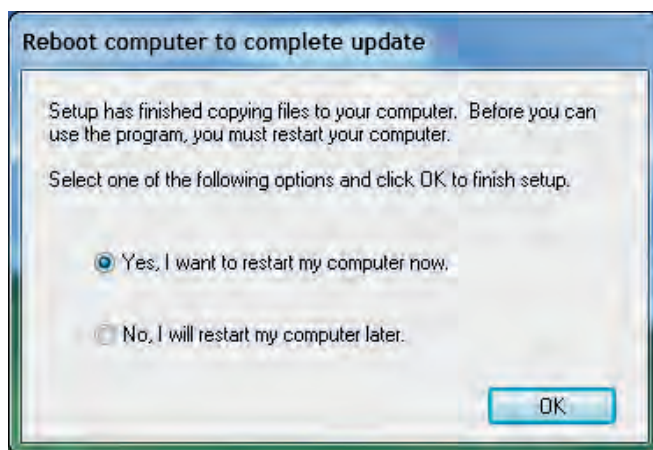
After installing the applications as described, exit the main menu, remove the CD and keep it in a safe place.

Updating analysis software

The MicroCal iTC$_{200}$ Analysis Software and License CD (formerly called the Origin analysis software installation CD) contains the following applications:

- Origin 7.0 (for scientific graphing and analysis)
- Origin Service Pack 4
- MicroCal AddOn for Origin 7.0 (for data analysis specific to MicroCal iTC$_{200}$ applications)

To update the Origin Data Analysis AddOn follow the steps described below:

3 Getting started

3.1 Installing MicroCal Auto-iTC$_{200}$ software

3.1.1 Updating the software

Step	Action
1	Insert the CD into the CD-ROM drive of the PC.
	The CD runs automatically and the **MicroCal's Setup** window appears.

Step	Action
2	Install only the Origin Data Analysis AddOn.
3	Click the **Install Origin Data Analysis Add-On** button.
	Note:
	*Ensure that the **Yes, I wish to install an Add-On disk now** option is checked in the pop-up window.*
4	Click **Next**.
5	The destination directory path will be automatically loaded.
	Click **Next**.

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.1 Updating the software
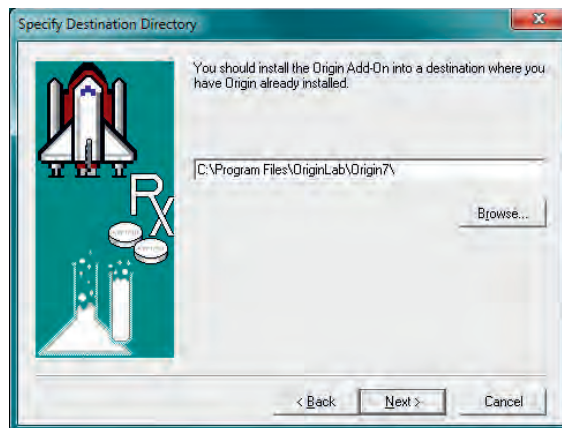
Step	Action
6	The software prompts for the add-on disc.

Note:

All add-on software is located on the analysis software installation disc and there is no need to insert any additional disc.

7	Specify the path for the disc by clicking the **Browse** button.
8	Select the CD drive that has the analysis software installation disc in it.

Tip:

This is usually the D:\ drive.

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.1 Updating the software

Step	Action
9	Double-click on the custom folder, **addon_disk_7.20** and click **OK**.
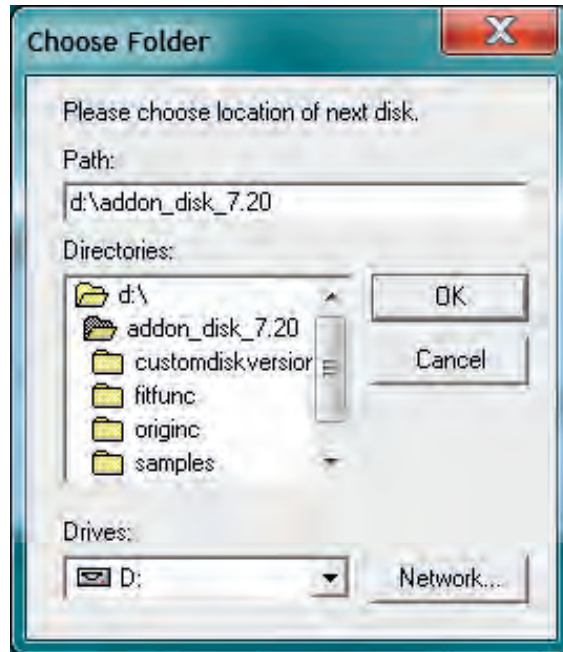
The path is now specified.

10	Click **OK** to continue.
	It may take a few minutes for the files to be installed.
11	Once the files have been installed, follow the on-screen instructions.
12	Click **Finish** to complete the installation.

After installing all the applications, exit the main menu, remove the CD and keep it in a safe place.

Restart the PC to complete the setup.

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.2 Complete installation of the software

3.1.2 Complete installation of the software

Note: *Installation of software requires administrative privileges.*

Installing the control software

The control software CD contains the following applications:

- MicroCal iTC$_{200}$ software
- Autosampler software
- USB driver for injector
- USB driver for data aquisition
- InitDT service
- .Net runtime

To install the control software, follow the steps described below:

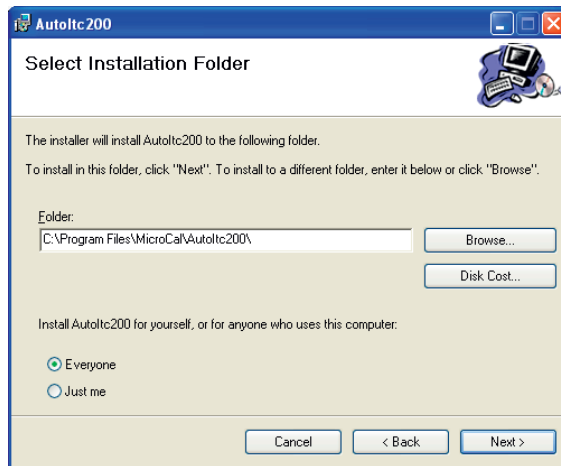| Step	Action
1	Insert the CD into the CD-ROM drive of the PC.
	The CD runs automatically and the ***MicroCal Setup*** window appears.
	Note:
	*If the CD does not start automatically, run **Auto200Setup.exe** from the CD.*

Installing the MicroCal iTC$_{200}$ software

To install the MicroCal iTC$_{200}$ software, follow the steps described below:

Step	Action
1	Click on the ***iTC200 Software Full Install*** button for the installation to start automatically.

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.2 Complete installation of the software

Step	Action
2	Follow the on-screen instructions and click **Finish** when the installation is complete.
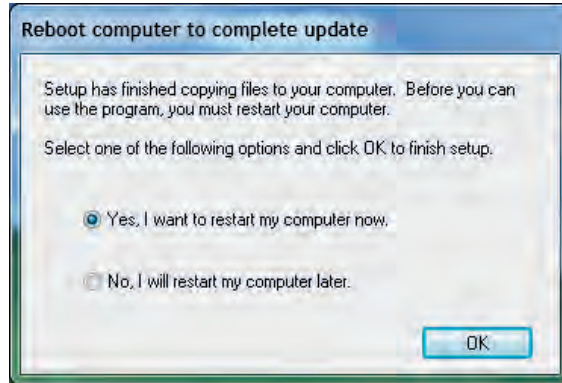
Be sure to select the option to restart the computer. Shortcut icons are created on the desktop automatically.

Installing the Autosampler software

To install the Autosampler software, follow the steps described below:

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.2 Complete installation of the software

Step	Action
1	Click the **Autosampler Software** button from the menu or navigate to the folder **AutoITC200 Installer** on the CD and run the **Setup_AutoItc200.exe** file.

The **Welcome to the AutoItc200 Setup Wizard** window opens.

2	Click **Next**.

The **Select Installation Folder** window appears.

3 Getting started

3.1 Installing MicroCal Auto-iTC$_{200}$ software

3.1.2 Complete installation of the software

Step	Action
3	Click **Next** to install in the default path.
	Note:
	*Make sure that the radio button **Everyone** is selected.*
	The **Confirm Installation** window appears.

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.2 Complete installation of the software

Step	Action
4	Click **Next** to start the installation.

The **Installing AutoItc200** window appears.
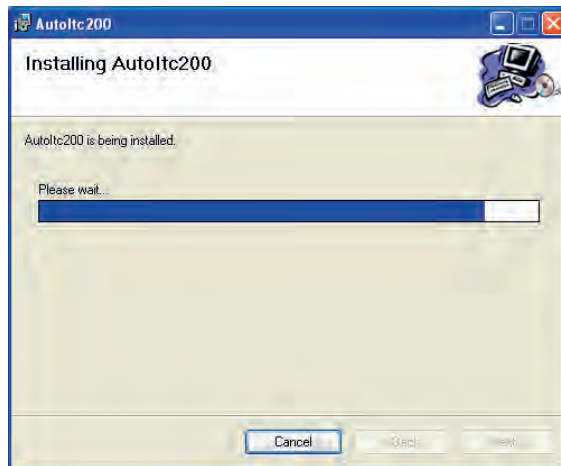
Once the installation is complete, the **Installation Complete** window appears.
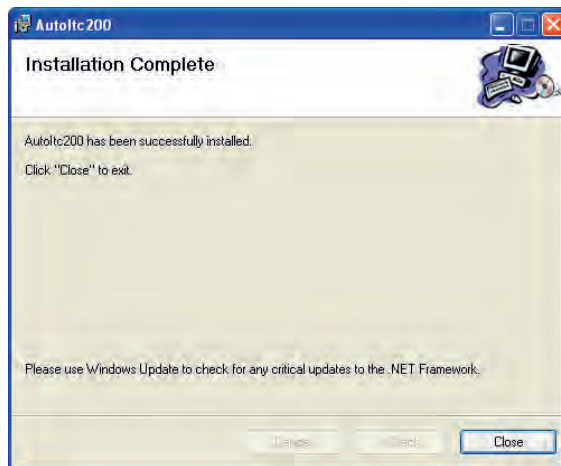
5	Click **Close** to exit the application.

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.2 Complete installation of the software

Installing the driver for the injector USB

To install the USB driver for the injector, click the **Injector USB Driver** button in the main menu to start the installation. A command window opens while the driver is being installed. This window closes automatically when the driver installation is complete.

Installing the driver for data aquisition USB

To install the USB driver for data aquisition, follow the steps described below:

Step	Action
1	Click the **Data Acquisition USB driver** button in the main menu.
2	Follow the on-screen instructions.
3	Click **Finish** when the installation is complete.

Installing .Net runtime

Note: *Installation of .Net runtime is necessary before installing InitDT service.*

To install the .Net runtime application, follow the steps described below:

Step	Action
1	Click the **Install .Net Runtime** button.
2	Follow the on-screen instructions.
3	Click **Finish** to complete the setup.

Installing InitDT service

Note: *The InitDT service is a low level service that runs in the background of the MicroCal iTC$_{200}$ software. This service operates only in the Windows administrator mode.*

To install the InitDT service, follow the steps described below:

Step	Action
1	Click the **Install InitDT Service** button.

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.2 Complete installation of the software

Step	Action
2	Follow the on-screen instructions. Use the default settings.
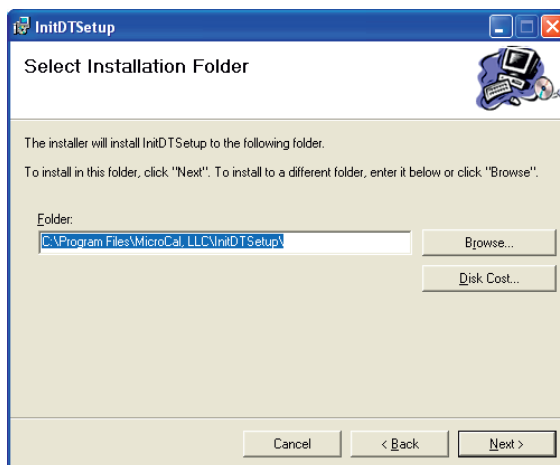
3	Click **Next** to continue with the installation.
4	Click **Close** to exit the setup after the installation is complete.

After installing the applications, exit the main menu, remove the CD and keep it in a safe place.

Restart the PC to complete the setup.

Installing analysis software

The MicroCal iTC$_{200}$ Analysis Software and License CD (formerly called the Origin analysis software installation CD) contains the following applications:

- Origin 7.0 (for scientific graphing and analysis)

- Origin Service Pack 4

- MicroCal AddOn for Origin 7.0 (for data analysis specific to MicroCal iTC$_{200}$ applications)

To install the analysis software follow the steps described below:

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.2 Complete installation of the software

Step	Action
1	Insert the CD into the CD-ROM drive of the PC.

The CD runs automatically and the **MicroCal's Setup** window appears.

Step	Action
2	Install each application in the main menu as described later in this section.

Installing Origin 7.0
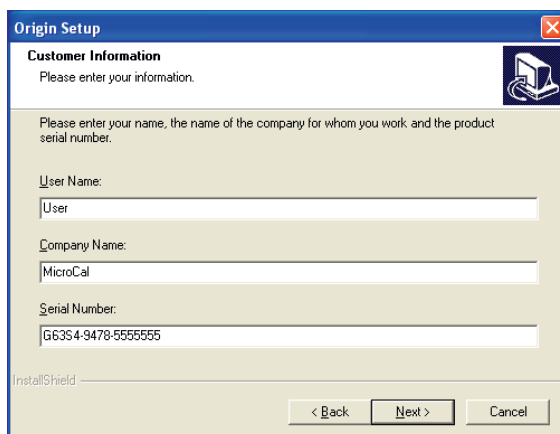
To install Origin 7.0, follow the steps described below:

Step	Action
1	Click the **1. Install Origin 7.0** button to start the installation.
2	Click on **Origin 7.0** in the pop-up window.

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.2 Complete installation of the software
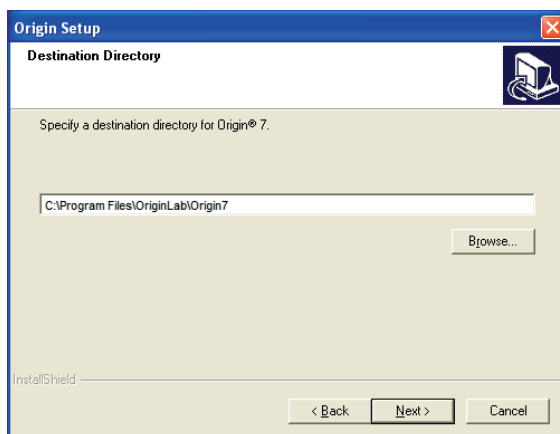
Step	Action
3	Follow the on-screen instructions to continue.

The **Origin Setup** window for **Customer Information** appears.

4	Enter the **User Name** and **Company Name**.
5	Locate the serial number on the front of the CD case or on the Origin box. Enter this number including the dashes, in the **Serial Number** text box.
6	Click **Next**.

The **Origin Setup** window for **Destination Directory** appears.

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.2 Complete installation of the software

Step	Action
7	The destination directory path will be automatically loaded. Click **Next**.
8	Follow the on-screen instructions. It is recommended to use the default settings.
9	Click **Finish** to complete the installation.
10	Exit the Origin 7.0 setup.

Installing Origin Service Pack 4

To install Origin Service Pack 4, follow the steps described below:

Step	Action
1	Click the **2. Install Origin Service Pack 4** button.
2	Follow the on-screen instructions.
3	Click **OK** to acknowledge the **Patch Warning** pop-up.
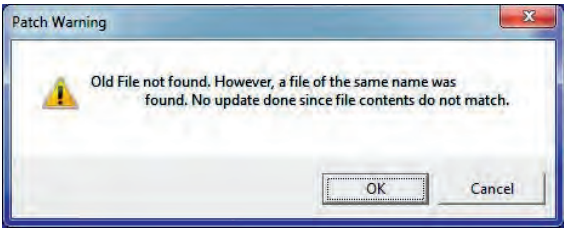
Installing Origin Data Analysis AddOn

To install the Origin Data Analysis AddOn, follow the steps described below:

Step	Action
1	Click the **Install Origin Data Analysis Add-On** button. *Note:* *Ensure that the **Yes, I wish to install an Add-On disk now** option is checked in the pop-up window.*
2	Click **Next**.
3	The destination directory path will be automatically loaded. Click **Next**.

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.2 Complete installation of the software

Step	Action
4	The software prompts for the addon disc.

Note:

All addon software is located on the analysis software installation disc and there is no need to insert any additional disc.

5	Specify the path for the disc by clicking the **Browse** button.
6	Select the CD drive that has the analysis software installation disc in it.

Tip:

Tip: This is usually the D:\ drive.

7	Double click on the custom folder, ***custom_d_itc_200*** and click ***OK***.
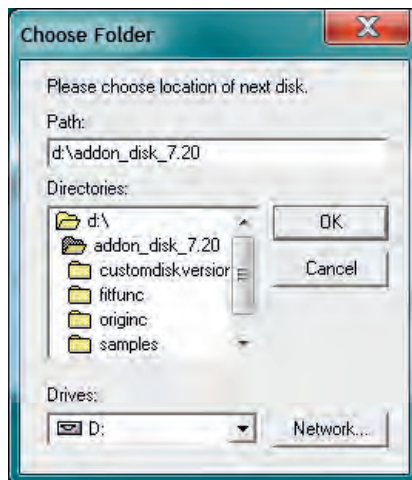
The path is now specified.

8	Click ***OK*** to continue.

It may take a few minutes for the files to be installed.

3 Getting started
3.1 Installing MicroCal Auto-iTC$_{200}$ software
3.1.2 Complete installation of the software

Step	Action
9	Once the files have been installed, follow the on-screen instructions.
10	Click **Finish** to complete the installation.

After installing all the applications, exit the main menu, remove the CD and keep it in a safe place.

Restart the PC to complete the setup.

3.2   Settings for Windows 7

After a full installation on a Windows 7 operating system running computer, the configuration described in section *Section 3.2.1 Modify the Origin 7 configuration for Windows 7, on page 46*, *Section 3.2.2 Modify the MicroCal iTC$_{200}$ software configuration for Windows 7, on page 48*, and *Section 3.2.3 Modify the user account control settings for Windows 7, on page 50* must be made.

3.2.1   Modify the Origin 7 configuration for Windows 7

Note: *This procedure is only required if you are installing software on a PC with a Windows 7 operating system. If the operating system is Windows XP, skip this procedure and move to the next procedure.*

Step	Action
1	Click the **Start** button on the Windows 7 operating computer, select **Computer**, and then navigate to the Origin installation folder, `C:\Program Files\OriginLab\Origin7.`
2	Locate and right-click the Origin 7 application file, **Origin70.exe** and select **Properties**.
3	In the Origin70 Properties dialog box, select the **Compatibility** tab and then click **Change settings for all users**.
4	In the Compatibility for all users dialog box, make the following modifications:

- Under Compatibility mode, select **Run this program in compatibility mode for:**, and then select **Windows XP (Service Pack 3)**.

- Under Privilege Level, select **Run this program as an administrator**.

- Click **OK**.

Step	Action
5	In the *Origin70 Properties* dialog box, click **OK**.

In the next image, all five steps required to make Origin compatible with Windows 7 are displayed.

3.2.2   Modify the MicroCal iTC$_{200}$ software configuration for Windows 7

Note: *This procedure is only required if you are installing software on a PC with a Windows 7 operating system. If the operating system is Windows XP, skip this procedure and move to the next procedure.*

Step	Action
1	Click the **Start** button on the Windows 7 operating computer, select **Computer**, and then navigate to the ITC$_{200}$ installation folder, `C:\ITC200`.
2	Locate and right-click the ITC$_{200}$ application file, **ITC200.exe** and select **Properties**.
3	In the ITC$_{200}$ Properties dialog box, select the **Compatibility** tab and then click **Change settings for all users**.
4	In the Compatibility for all users dialog box, make the following modifications:

- Under Compatibility mode, select **Run this program in compatibility mode for:**, and then select **Windows XP (Service Pack 3)**.

- Under Privilege Level, select **Run this program as an administrator**.

- Click **OK**.

Step	Action
5	In the **ITC200 Properties** dialog box, click **OK**.
	In the next image, all five steps required to make ITC$_{200}$ compatible with Windows 7 are displayed.
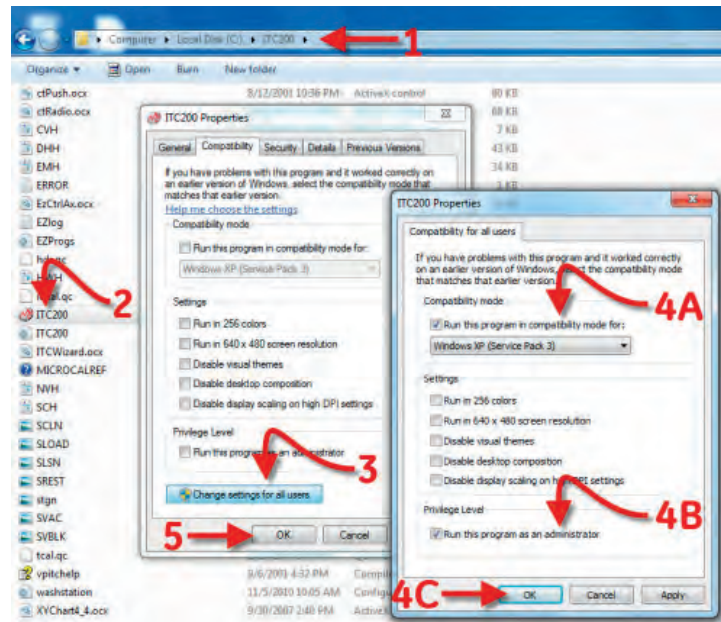
3.2.3   Modify the user account control settings for Windows 7

Note: *This procedure is only required if you are installing software on a PC with a Windows 7 operating system. If the operating system is Windows XP, do not perform this procedure.*

Windows 7 operating systems ship with the user account control settings modified to prevent the following warning message from displaying:
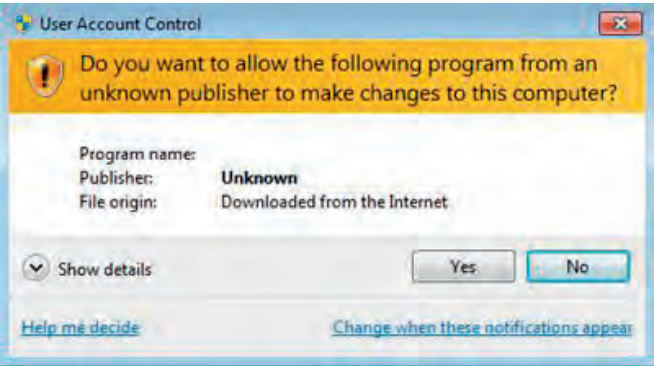
This message can occur if the regional settings are modified and potentially every time the user double-clicks the *ITC$_{200}$* software icon. Although harmless, you should disable the mechanism that causes this to display.

Step	Action
1	Click the **Start** button on the Windows 7 operating computer, select **Control Panel**, and then select **Action Center**.
2	In the left pane of the **Action Center** window, click **Change User Account Control settings**.

Step	Action
3	Drag the notification bar to *Never notify*, and click *OK*.
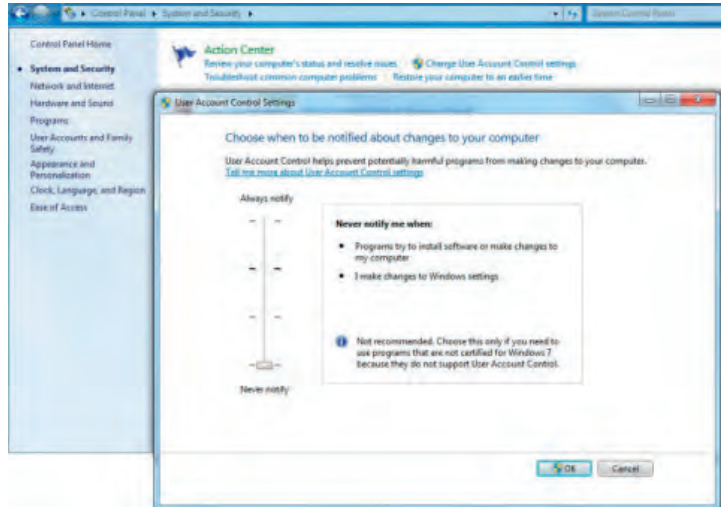
4	Restart the system.

4 Control software

Introduction

This chapter describes the automation control and data acquisition software that is delivered with MicroCal Auto-iTC$_{200}$. The user interfaces are also described in detail. See *Chapter 5 Performing a run, on page 113* for instructions on how to operate MicroCal Auto-iTC$_{200}$.

In this chapter

This chapter contains the following sections:

4.1 Overview

The MicroCal Auto-iTC$_{200}$ is delivered with three software components as outlined in the table below.

Software component	Icon	Description
MicroCal Auto-iTC$_{200}$ software		This is used to operate MicroCal iTC$_{200}$ together with the Autosampler. Automated data analysis is performed after each experimental run completes. This is described in *Chapter 6 Data analysis using MicroCal Auto-iTC$_{200}$ software, on page 136*. Also see *Section 4.2 MicroCal Auto-iTC$_{200}$ software, on page 54*.
MicroCal iTC$_{200}$ software		This software is used to control MicroCal iTC$_{200}$. See *Section 4.3 MicroCal iTC$_{200}$ software, on page 89*.
Origin		Origin is supplied for manual data analysis. See *Chapter 7 Data analysis using Origin, on page 143*. It is mentioned here, because an instance of Origin may be opened during data collection for real time display, though is not necessary.

4.2   MicroCal Auto-iTC$_{200}$ software

Introduction

The MicroCal Auto-iTC$_{200}$ software controls the Autosampler and communicates with the MicroCal iTC$_{200}$ software to control the MicroCal iTC$_{200}$ instrument.

Automated data analysis is performed by the MicroCal Auto-iTC$_{200}$ software, as described below and in *Chapter 6 Data analysis using MicroCal Auto-iTC$_{200}$ software, on page 136*. More advanced data analysis tools are available through Origin for data analysis, see *Chapter 7 Data analysis using Origin, on page 143*.

This section describes the user interface for the MicroCal Auto-iTC$_{200}$ software. Installation of the MicroCal Auto-iTC$_{200}$ software is described in *Section 3.1 Installing MicroCal Auto-iTC$_{200}$ software, on page 24*.

Note: *Installing any program not supplied by Malvern Instruments, changing the configuration settings of the Microsoft Windows operating system or making any changes to the computer's settings may adversely affect the performance of the MicroCal Auto-iTC200 software.*

In this section

This section contains the following topics:

4 Control software
4.2 MicroCal Auto-iTC$_{200}$ software
4.2.1 Starting MicroCal Auto-iTC$_{200}$ software

4.2.1   Starting MicroCal Auto-iTC$_{200}$ software

The MicroCal Auto-iTC$_{200}$ software controls the Autosampler and communicates with the MicroCal iTC$_{200}$ software to control the MicroCal iTC$_{200}$ instrument. The software and hardware should be started in sequence or errors may occur requiring that the system and software be restarted.

To start the MicroCal Auto-iTC$_{200}$ software, follow the steps described below:

Step	Action
1	Start the computer and log in to Windows.
2	Turn on MicroCal Auto-iTC$_{200}$ using the **Power** switch at the rear of the unit.
3	Start the MicroCal iTC$_{200}$ software. A description of this software can be found in *Section 4.3 MicroCal iTC$_{200}$ software, on page 89*.
4	Once the MicroCal iTC$_{200}$ software has initialized, start the MicroCal Auto-iTC$_{200}$ software.
5	To open an instance of Origin for real-time data display, select ***System:Establish DDE Link To Origin*** in the MicroCal iTC$_{200}$ software. *Note:* *It is normally not necessary to start Origin for real-time data display, since real time data can be viewed directly in the MicroCal Auto-iTC$_{200}$ software.*

4 Control software
4.2 MicroCal Auto-iTC$_{200}$ software
4.2.2 MicroCal Auto-iTC$_{200}$ software main screen
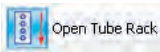
4.2.2　MicroCal Auto-iTC$_{200}$ software main screen

The MicroCal Auto-iTC$_{200}$ software screen is illustrated and described below.

The **Experiments** tab is displayed by default.
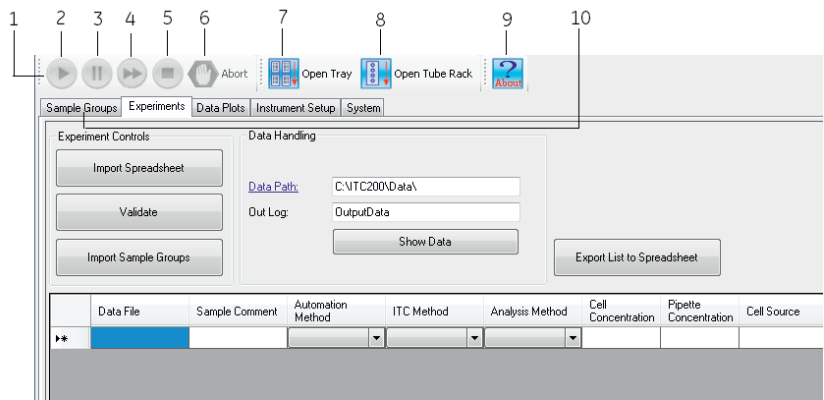
Part	Function
1 Main control buttons	Overview of all buttons
2 Start button	Starts an experiment that has been loaded and validated. Also resumes the experimental series after a pause command.
3 Pause button	Pauses the automation and allows the user to make changes in run parameters or check on syringes.
4 Skip button	Stops the current experiment and continues to the next in the current series.
5 Stop button	Stops the instrument in an orderly fashion. The current experiment will complete, post-run cleaning will be performed and the instrument will return to the idle state.

4 Control software
4.2 MicroCal Auto-iTC$_{200}$ software
4.2.2 MicroCal Auto-iTC$_{200}$ software main screen

Part	Function
6 Abort button	Aborts the automation. The calorimeter will complete the running experiment, but both post-run cleaning and post-run data analysis will not be executed.
7 *Open Tray* button	Opens and closes the sample tray.
8 *Open Tube Rack* button	Opens and closes the tube rack.
9 *About* button	Displays software version information.
10 Control tabs	The functions available in these tabs are explained below.

4.2.3   *Sample Groups* tab

Introduction

The **Sample Groups** tab is used to populate the experiments list that controls the automation. Samples can be divided into up to ten sample groups. Defining multiple sample groups can be a convenient way of defining different series of runs that share the same run parameters. Each sample group would then be assigned a separate subtab within the **Sample Groups** tab.

Sample Groups tab overview

The following illustration shows the **Sample Groups** tab. Individual subtabs for each sample group are described below.

Part	Function
1	Select the number of sample groups using the **Number of Sample Groups** drop-down list. Up to ten sample groups may be selected.
2	Each sample group has a separate **Group** tab, numbered from 1 to 10.

Part	Function
3	Parameters for each sample group are entered in the subtabs for each group, **Dataset Naming**, **Methods**, **Concentrations** and **Plate Setup**. These subtabs are described below.

Dataset Naming subtab

The **Dataset Naming** subtab specifies the naming of the runs in the group. This is illustrated below.

Part	Function
Number of Samples	Enter the number of samples in this sample group.
Prefix	Enter a prefix that will be used for the file name.
Starting Number	Enter the number at which file numbering will start.
Suffix	Enter a suffix for the file name, if desired.
Name	Displays the file name for the first file in the group. The file name consists of the **Prefix**, followed by a number that is incremented sequentially for each sample in the group starting from **Starting Number**, and ends with the **Suffix**.

Part	Function
Sample Comment	Enter an optional comment that will be attached to each run in the sample group.

File naming recommendation

When reading multiple datasets into Origin, the last 10 characters of the file name (excluding the file extension and underscore characters) must be unique and not start with a numeric value. Incorrect naming can be corrected by renaming the files manually. This only affects the simultaneous analysis of multiple datasets. Single datasets can always be read into Origin.

Examples of good file names:

File Name	Reason name is good	Resulting Origin worksheet name
011511_1.itc 011511_2.itc	Although name starts with a number, the name is under ten characters and thus the last digit is not truncated. The resulting Origin worksheet names are unique.	a0115111 a0115112
Test_011511_1.itc Test_011511_2.itc	The tenth character is not numeric and thus the last digit is not truncated. The resulting Origin worksheet names are unique.	est0115111 est0115112

Examples of bad file names:

File Name	Reason name is bad	Resulting Origin work-sheet name
011511_test1.itc 011511_test2.itc	Because a number was the tenth character, a letter is added as a prefix and the last character is truncated. Since the last character was the unique character, the worksheet names are now identical and will not load properly in Origin.	a11511test a11511test
My EDTA Test 01Sept2011_1.itc My EDTA Test 01Sept2011_2.itc		a1Sept2011 a1Sept2011

Methods subtab

The **Methods** subtab specifies the run method for the sample group. This is illustrated below.

Part	Function
Automation Method	Choose a method for automated cleaning and loading from the drop-down list. Refer to *Section 10.4 Standard setup files, on page 338* for a list and description of the standard setup files.
Save Sample	Check the checkbox to save the material remaining in the cell at the end of an experiment. Specify the location in the **Plate Setup** subtab as described below in this section.

Part	Function
ITC Run Method	Choose a previously saved *.inj MicroCal iTC$_{200}$ run file from the drop-down list. These run files contain parameters that control the calorimeter. Refer to *Section 10.4 Standard setup files, on page 338* for a list and description of the standard setup files.
Analysis Method	Choose the post-run data analysis method from the drop-down list. The options are **Control**, **OneSite**, and **OneSiteFixedN**. Briefly, the Control analysis method calculates heats and fits no model; the OneSite method fits a 1:1 binding model; the OneSiteFixedN method fits a 1:1 binding model, while fixing the stoichiometry, n.
Load Cell from Tube	This is automatically set to **Yes** when loading from the tube rack, or **No** when loading from the 96-well plate.
Pre-Rinse	This is automatically set to **Yes** when including a pre-rinse routine, or **No** when not including a pre-rinse routine.

Concentrations **subtab**

The **Concentrations** subtab allows you to specify the concentrations of reagents in both the syringe and sample cell for the sample group. This is illustrated below.

If the concentrations are not same for all the runs in the sample group, manually edit the spreadsheet to reflect the actual concentrations, see *Section 4.2.4* **Experiments** *tab, on page 66*.

Note: *The cell and syringe concentrations will be used for the automated post-run analysis. Incorrect concentrations may result in incorrect interpretation of the data.*

Part	Function
Cell Concentration (mM)	Enter the concentration of sample for the cell.
Syringe Concentration (mM)	Enter the concentration of titrant for the syringe.

Plate Setup subtab

The *Plate Setup* subtab allows you to specify the source for the cell and pipette. The appearance of the subtab varies depending on whether the samples are to be loaded from a 96-well plate, whether the samples are to be saved, or whether the cell is to be loaded from the tube rack.

The following illustration shows the *Plate Setup* tab when loading from a 96-well plate and saving the sample is not required.
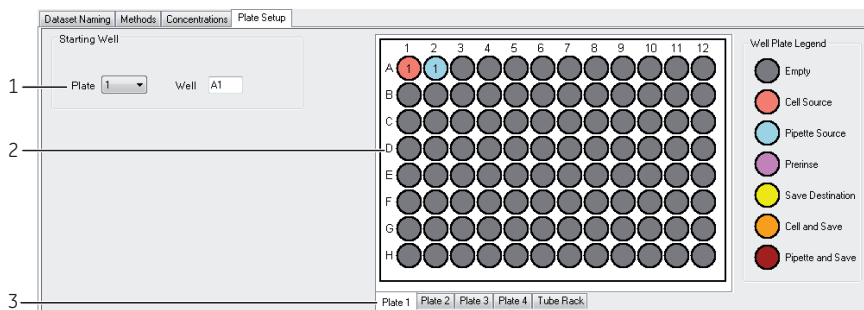
Part	Function
1	Select a starting well by choosing a plate from the *Plate* drop-down list, and typing a well coordinate in the *Well* text box. This may also be chosen by clicking on the well location in the graphical display on the right side of the screen.
	The starting well defaults to the first available well.
2	The graphical display shows the use of wells in the chosen 96-well plate.
3	The tabs display each of the four 96-well plates and the tube rack in the graphical display.

The following illustration shows the *Plate Setup* tab when loading both the cell and the pipette from a 96-well plate with the option of saving the sample.

Part	Function
1	This is only visible if the **Save Sample** checkbox is checked in the **Methods** subtab described above in this section.Select the desired option for saving the sample from the following:
	• **Save to Empty Well** saves the sample to a separate well (yellow well in the graphical display)
	• **Save to Cell Loading Well** saves the sample to the well from which the cell is loaded (orange well in the graphical display)
	• **Save to Syringe Loading Well** saves the sample to the well from which the syringe is loaded (red well in the graphical display)
	These locations, along with all the other experimental parameters may be changed manually in the **Experiments** tab, which is described in the next section.
2	Save destination legend: locations as defined above in 1.

The following illustration shows the graphical display within the **Plate Setup** tab when loading the cell from the tube rack.

Step	Action
1	Click on the **Tube Rack** tab to open the graphical display for the tube rack.

Note:
The pipette cannot be loaded from the tube rack.

Step	Action
2	Click on a tube position in the graphical display to select a different tube.
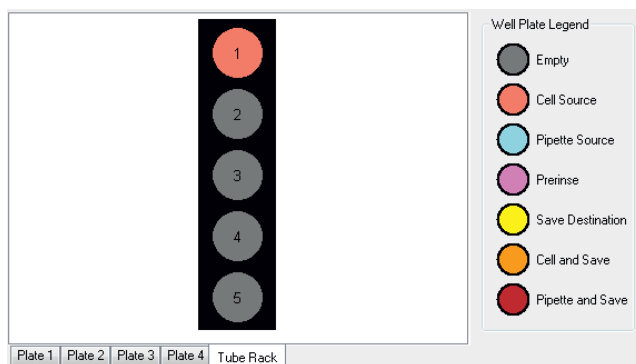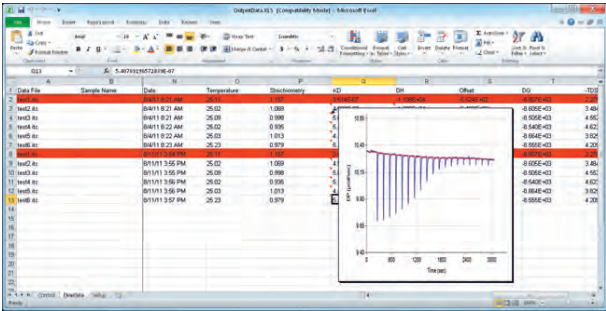
4.2.4 *Experiments* tab

The **Experiments** tab contains a spreadsheet with the details of each run. Once the experiments are loaded into this tab, each individual parameter can be edited.

The **Experiments** tab is illustrated below.

Part	Function
Import Spreadsheet	Imports the experimental run parameters from a previously saved .xls file.
Validate	Validates the run parameters before starting the experiments. A validation report opens and displays the amount of reagents required for the entire series. The start button will not be activated until the parameters have been validated. Any changes made to the ongoing list of experiments that are yet to complete will not take effect unless the validate button is pressed again (i.e., **ITC Methods** or **Sample Comments**, etc.). Depending on the timing, the next experiment in the list may not permit modification. Check to see if the validation report is altered to make sure (i.e., switch between detergent/no detergent).

Part	Function
Import Sample Groups	Imports information from the **Sample Groups** tab.
Data Path	Enter the full path to the directory where the output data should be stored.
Out Log	Enter the file name of the Microsoft Excel™ spreadsheet where the experimental parameters and automated data analysis results should be stored. See *Chapter 6 Data analysis using MicroCal Auto-iTC$_{200}$ software, on page 136.* *Note:* *Out log files are not backward compatible with prior versions of software. A new out log filename must be used.*
Show Data	Opens the Microsoft Excel spreadsheet containing the out log at any time during the series of experiments. This spreadsheet contains the run parameter information for each run, the calculated n, ΔH and K$_D$ and embedded graphs that show the raw data and normalized ΔH when the cursor is moved over the **Stoichiometry** or **KD** columns, respectively.
Export List to Spreadsheet	Exports the current experiment run parameter list to a .xls file for later use. The saved file can be read back in by clicking the **Import Spreadsheet** button.

4.2.5   *Data Plots* tab

Introduction

The **Data Plots** tab displays data being generated by the current series of titration runs. Results can be viewed in real time, or various plots can be generated for the runs in the series that have already completed.

Data Plots tab overview

The tab contains three subtabs, as described in the following table. Each subtab is described in more detail below.

Subtab	Description
Real Time Display	The raw DP (differential power) signal from the current titration run, see **Real Time Display** *subtab, on page 69*.
Results Plot	The results for the completed runs in the series currently running, see **Results Plot** *subtab, on page 70*.
Individual Results Plot	Data from completed runs in the currently running series, plotted as raw data, integrated injection heats or concentration-normalized injection heats, see **Individual Results Plot** *subtab, on page 72*.

Real Time Display subtab

The **Real Time Display** subtab shows the raw DP signal from the current titration run. This is illustrated below.
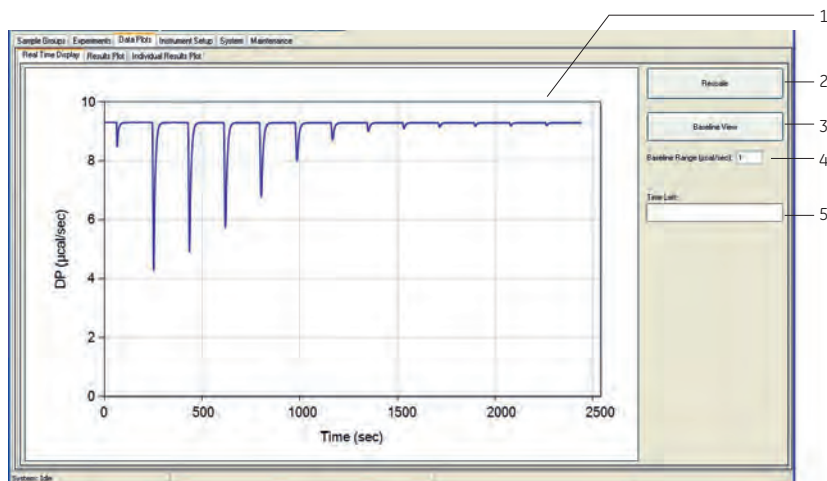
Part	Function
1	The graphical display area shows a graph of measured DP against time. Clicking and dragging in the area zooms into the selected area.
2	The **Rescale** button rescales the plot to fit the entire range of the data set.
3	The **Baseline View** button rescales the baseline to the range specified in the **Baseline Range** text box.
4	Enter a value for the baseline range in the **Baseline Range** text box. This value is applied when the **Baseline View** button is clicked.
5	The remaining time for the current experiment is shown in the **Time Left** box.

Results Plot subtab

The *Results Plot* subtab displays the following depending on the chosen analysis method subtab.

Subtab	Description
One Site	The fitted results for completed 1:1 binding experiments in the series currently running.
Control	Mean and standard deviation values for completed control experiments.

One Site subtab

The *One Site* subtab displays the analysis results as illustrated below.

Part	Function
1	Select a parameter to plot from the drop-down menu. Possible choices are *Stoichiometry*, *KD* (K$_D$), *ΔH*, *Offset*, *ΔG* and *-TΔS*. For a more detailed description, please see *Chapter 6 Data analysis using MicroCal Auto-iTC$_{200}$ software, on page 136*, *Chapter 7 Data analysis using Origin, on page 143* and *Appendix A Equations used for fitting ITC data, on page 349*.
2	The table displays the analyzed results for each completed run.
3	The graphical display area shows a scatter plot of the analyzed results versus experiment number. The value plotted is chosen using the *Plotted Data* drop-down menu.

Part	Function
4	Use the **Page Setup** and **Print** buttons to print a copy of the displayed plot, or **Save Picture** to save a copy of the plot.

Control subtab

The **Control** subtab is used for the analysis of control experiments, as illustrated below.
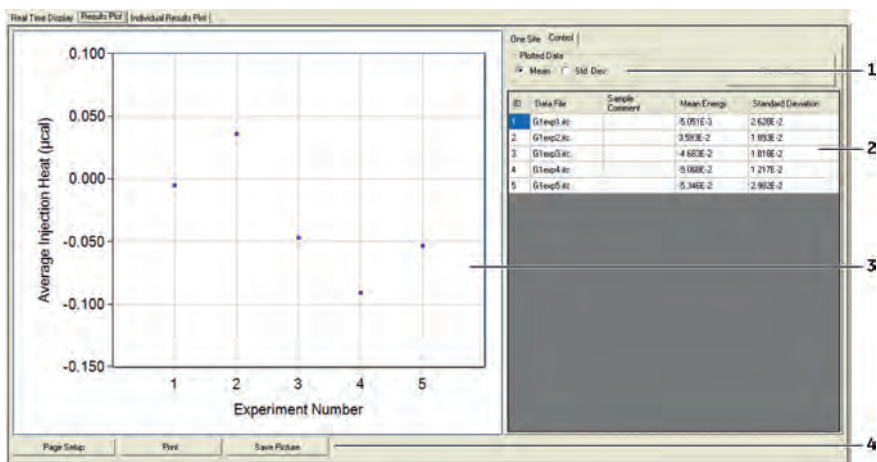
Part	Function
1	Select whether to plot either Mean or Std. Dev. (standard deviation) using the radio buttons.
2	The table displays the analyzed statistics for each completed run.
3	The graphical display area shows a scatter plot of the chosen statistic versus experiment number. The value plotted is chosen using the **Plotted Data** radio buttons.
4	Use the **Page Setup** and **Print** buttons to print a copy of the displayed plot, or **Save Picture** to save a copy of the plot.

Individual Results Plot **subtab**

The **Individual Results Plot** subtab displays data from completed runs in the currently running series. This is illustrated below.
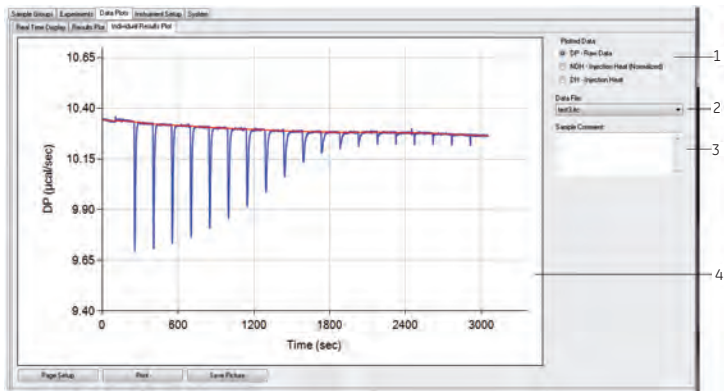
Part	Function
1	Select the appropriate **Plotted Data** radio button to display a plot of the raw data (**DP - Raw Data**), concentration-normalized injection heats (**NDH - Injection Heat (Normalized)**) or integrated injection heats (**DH - Injection Heat**).
2	To display the previously completed run, select the run using the **Data File** drop down list.
3	The sample comment, if one exists, is displayed in the **Sample Comment** text box.
4	The graphical display area shows the raw data, the integrated injection heats or the concentration-normalized injection heats for previously completed runs in the current list of experiments. The type of plot is chosen using the **Plotted Data** radio buttons.

4.2.6   *Instrument Setup* tab

Introduction

The parameters that control both MicroCal iTC$_{200}$ and the Autosampler are changed in the *Instrument Setup* tab.

This tab contains two subtabs, *ITC Method* and *Automation Method*.

The *ITC Method* subtab is used to generate the .inj run parameter file that controls the calorimeter. For an overview of this subtab, refer to *ITC Method subtab overview, on page 73*. Much of this subtab's functionality mirrors that of the *Advanced Experimental Design* tab in the MicroCal iTC$_{200}$ software, described in *Section 4.3.5 Advanced Experimental Design tab, on page 96*.

The *Automation Method* subtab accesses the routines used by the Autosampler. These are intended for advanced users who want to edit the scripts themselves. Editing is complicated and not necessary for basic operation. For a description of the contents of this subtab, refer to *Automation Method subtab overview, on page 78*.

ITC Method subtab overview

The *ITC Method* subtab is shown in the following illustration. The various parts of the subtab are described in more detail below.
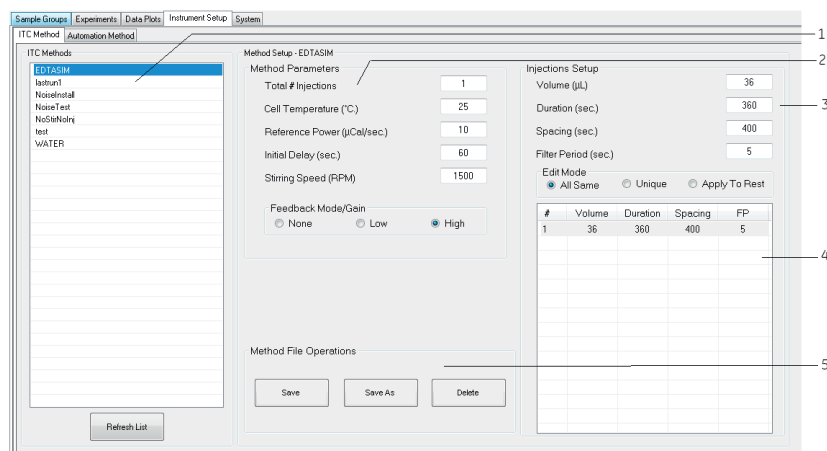
Part	Function
1	Methods list
2	*Method Parameters* controls, see *Method Parameters, on page 75*

Part	Function
3	**Injections Setup** controls, see **Injections Setup**, *on page 77*
4	Injection list
5	**Method File Operations** buttons, see **ITC Methods** *list and* **Method File Operations** *buttons, on page 74*

ITC Methods list and *Method File Operations* buttons
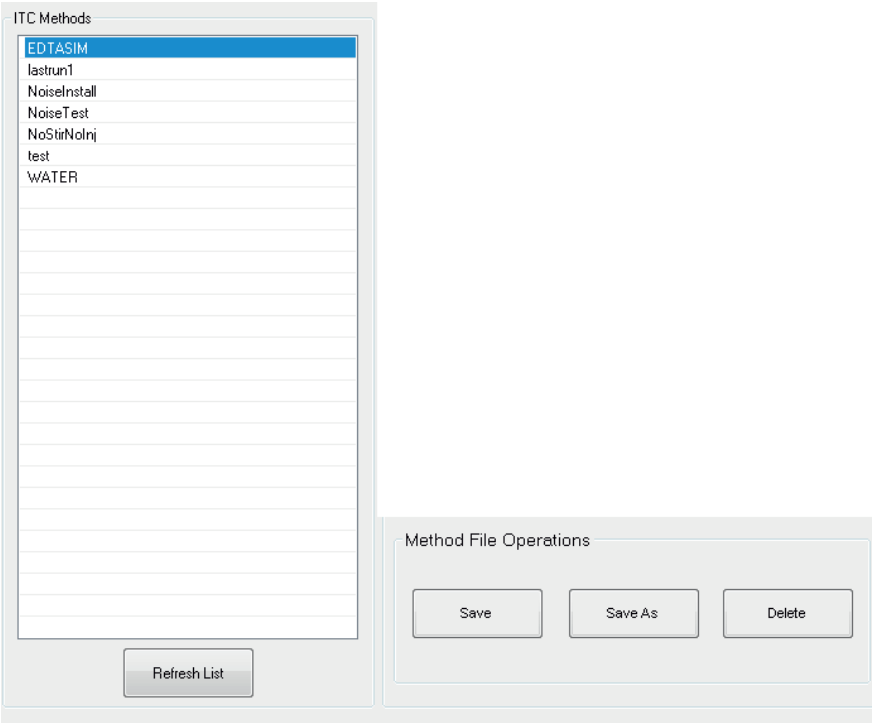
Part	Function
ITC Methods	Select a method to view or edit from the method list.
Save	Saves any changes made to the selected method. Altering a run file will not affect the current titration run but will affect all following runs using that file.

Part	Function
Save As	Saves the current parameters to a new file and creates a new method in the method list.
Delete	Deletes the selected method.

Method Parameters

Part	Function
Total # 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.
Cell Temperature	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).

Part	Function
Reference Power	Enter a value for reference power. The DP baseline will equilibrate near this value.
	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 table below gives some guidelines.
	<table><tr><th>Expected reaction type</th><th>Suggested reference power</th></tr><tr><td>Large exothermic</td><td>Large value (~10 µcal/s using high feedback)</td></tr><tr><td>Large endothermic</td><td>Small value (~0.5 µcal/s)</td></tr><tr><td>Unknown</td><td>Intermediate value (5 µcal/s using high feedback)</td></tr></table>
	Note:
	The titration heats must not cause the DP to exceed the usable range (0 to 5 - passive feeback, 12 to 25 - high feedback, etc.).
Initial Delay	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.
Stirring Speed	Enter the sample cell stirring speed in rpm (recommended value is 1000 rpm). Faster stirring may be necessary if the sample cell contains suspended particles, for example, agarose beads.
Feedback Mode/Gain	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.

Injections Setup

Part	Function
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 (sec.)	Enter the time (s) that the instrument should take to inject the titrant into the sample cell for the injection(s) selected in the injection list.
	The default value is twice the value entered in the **Volume** text box for the multiple injection method, and ten times the value entered in the **Volume** text box for the single injection method.

Part	Function
Spacing (sec.)	Enter the time (s) 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 s, depending on the feedback mode, temperature and reaction kinetics.
	Note:
	For the single injection method, the spacing should be at least 90 s greater than the duration.
Filter Period (sec.)	Enter the time period (s) over which data channel conversions are averaged to smooth the data. A longer filter period will result in smoother data, at the cost of time resolution. Data will be read out at a 1 s interval.
Edit Mode	Select the edit mode for the injection list.

Edit Mode	Description
All Same	All injections will have the same parameters
Unique	Only the selected injection(s) will be altered
Apply To Rest	Applies the parameters for the currently selected injection to all subsequent injections

Part	Function
Injection list	Select the injection(s) to edit in the injection list.

Automation Method subtab overview

The **Automation Method** subtab contains two subtabs, the **Setups** subtab and the **Scripts** subtab.

The **Setups** subtab is used to create and edit methods, which are a series of linked scripts. See **Setups** subtab, on page 79 for a description.

The **Scripts** subtab is used to create and edit individual scripts that correspond to distinct stages within methods. See **Scripts** *subtab, on page 80* for a description.

Setups subtab

The **Setups** subtab is illustrated below. Scripts for the various stages of the method are selected from the appropriate drop-down lists.

The instrument comes with a preloaded set of automation methods. See *Section 10.4 Standard setup files, on page 338* for a description of these methods. Not all possible method configurations are covered. For example, to clean the titrant transfer arm with detergent but not the syringe, a new method can be created by selecting the **Plates Syringe Clean** method and changing the **Pipette Clean** script selection from **Pipette Clean with Detergent** to **Pipette Clean**.Scripts may be created or edited in the **Scripts** subtab but should not be necessary for normal operation (see **Scripts** *subtab, on page 80*).

Module	Function
Automation Methods	Select a method to view or edit from the list.
Description	Enter an optional description to be stored in the method file.
Script Selections: Each automation method must have a script selected from these six categories.	
Cell Load	Select a script for loading the cell from the tray or tube rack.

Module	Function
Cell Clean	Select a script for emptying, cleaning and rinsing the cell.
Pipette Load	Select a script for loading the pipette from the wash/load station.
Pipette Clean	Select a script for cleaning, rinsing and drying the pipette.
Titrant Transfer	Select a script for transferring the titrant from the tray to the wash/load station, in order to load the pipette.
Titrant Transfer Clean	Select a script for cleaning the titrant transfer arm cannula.
Refresh Script Lists	Restores the current scripts to those stored in the file for the selected method. This button also updates the drop-down lists of scripts with any newly created scripts.
New	Creates a new method from scratch.
Save	Saves the currently selected method to a method file.
Save As	Saves the current method parameters to a new method file.
Delete	Deletes the currently selected method.
Script Summary	Displays a summary of the script selected in the drop-down list.

Scripts subtab

Individual scripts are edited in the **Scripts** subtab. There are six tabs with similar layout, which correspond to the six stages of a method. The **Scripts** subtab is illustrated and described below. Script modification is an advanced subject and is not discussed here in detail. Contact a Malvern Instruments service representative to modify the scripts or for more information.

Note: *Scripts supplied with the instrument are uneditable and are colored blue.*
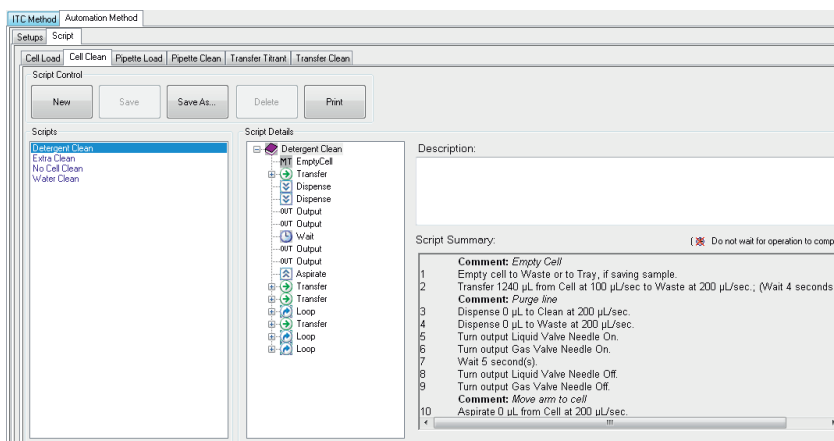
Part	Function
Cell Load	View/edit scripts for taking sample from the tray or tube rack, and loading the cell.
Cell Clean	View/edit scripts for emptying, cleaning and rinsing the sample cell. The number of iterations and reagent sources can be changed to create more or less vigorous and time-consuming cleaning scripts.
Pipette Load	View/edit scripts for loading a sample from the wash/load station into the pipette. *Note:* *The syringe can be loaded partially also.*
Pipette Clean	View/edit scripts for docking the pipette in the wash/load station, cleaning, rinsing with methanol and drying the pipette with nitrogen. The number of iterations and reagent sources can be changed to create more or less vigorous cleaning scripts of different durations.
Titrant Transfer	View/edit scripts for transferring the sample from the tray to the wash/load station, in order to load the pipette using a pipette load script.
Titrant Transfer Clean	View/edit scripts for cleaning the titrant transfer arm cannula. The number of iterations and reagent sources can be changed to create more or less vigorous and time-consuming cleaning scripts.

Part	Function
New	Creates a new script from scratch.
Save	Saves changes made to the currently selected script.
Save As	Saves changes made to a new script file.
Delete	Deletes the currently selected script.
Print	Prints the current script. A printer must be installed and connected.
Scripts	Select a script to view or edit from the list.
Script Details	Allows the script to be edited. Scripts should not normally be edited. Please contact Malvern Instruments for advice if you really need to edit a script.
Description	Enter an optional description that will be stored in the script file.
Script Summary	Displays a summary of the current script.

4.2.7   *System* tab

Introduction

The **System** tab provides access to various system functions and settings, including routine inspection and maintenance.

System tab overview

The **System** tab is illustrated below.
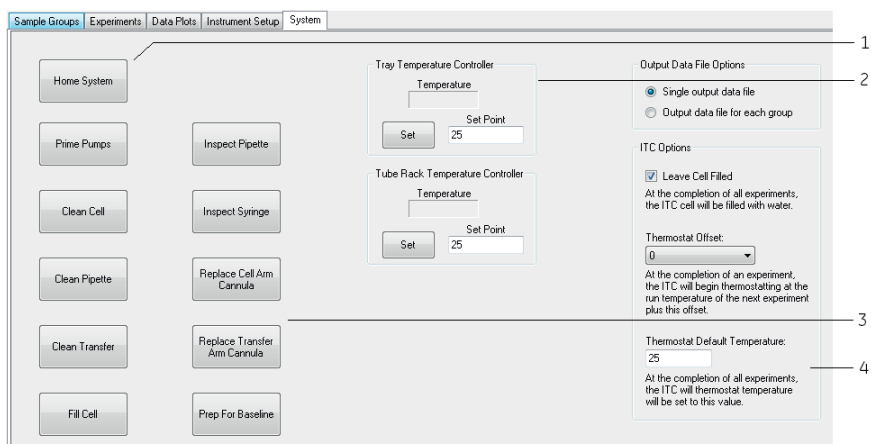
Part	Function
1	Maintenance buttons, see *Maintenance buttons, on page 84*
2	Temperature controls, see *Temperature controls, on page 86*
3	Part inspection and replacement buttons, see *Part inspection and replacement buttons, on page 85*
4	Miscellaneous controls, see *Miscellaneous controls, on page 87*

Maintenance buttons
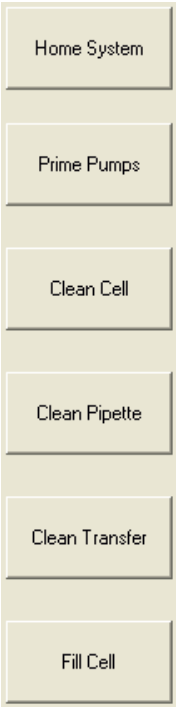
Part	Function
Home System	Homes the Autosampler components.
Prime Pumps	Pulls liquid through the reagent lines. Air must be removed from the lines when the system is first set up after the reagent bottles are filled and newly connected.
Clean Cell	Runs a cleaning script for the sample cell. This script empties the cell and fills it with detergent. It then heats the cell to 60°C and soaks it for 1 h before rinsing the cell.
Clean Pipette	Runs a cleaning script for the pipette. This script cleans with detergent.
Clean Transfer	Runs a cleaning script for the transfer arm cannula. This script cleans the transfer arm cannula with detergent.
Fill Cell	Fills the cell with water from the reagent bottle.

Part inspection and replacement buttons
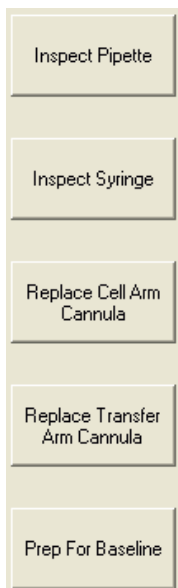
Part	Function
Inspect Pipette	Moves the pipette to the front of the instrument and down several cm for easy inspection and/or replacement.
Inspect Syringe	Moves the pipette to a suitable position and spins the syringe to allow easy inspection of the syringe.
Replace Cell Arm Cannula	Moves the cell arm to a convenient location for replacement of the cannula.
Replace Transfer Arm Cannula	Moves the cell arm out of the way and moves the transfer arm downwards for easy replacement of the cannula.
Prep For Baseline	Loads the cell and pipette with water and moves the pipette into the cell for a manual run. This can be a water/water titration run or a noise run.

Temperature controls

The *Tray Temperature Controller* and the *Tube Rack Temperature Controller* control the tray and tube racks, respectively. Both sets of controls behave in the same manner as outlined in the table below.

Part	Function
Temperature	Displays the current temperature of the tray or tube rack.
Set	Commands the temperature controller to go to the set point specified in the *Set Point* text box.
Set Point	Enter the desired temperature, from 4°C to ambient temperature for the tray or tube rack.

Miscellaneous controls

Part	Function
Output Data File Options	Select the desired output option using the radio buttons.
	Select **Single output data file** to produce one single out log for all runs in a series. See *Section 4.2.4 **Experiments** tab, on page 66*.
	Select **Output data file for each group** to write to a separate file for each sample group.
Leave Cell Filled	Checking this box leaves the cell filled with water at the conclusion of the series of experiments.
Thermostat Offset	Select a temperature offset for the MicroCal iTC$_{200}$ (default 0°C).
	When a run finishes, and before the next starts, the MicroCal iTC$_{200}$ thermostats at the temperature of the next run plus the temperature offset. Setting a value for the temperature offset may reduce the time required to reach the run temperature. However, this carries the risk of denaturing samples, since samples are run very close to their denaturation points.

Part	Function
Thermostat Default Temperature	Enter the temperature at which MicroCal iTC$_{200}$ should thermostat after all the runs have completed.

4.3 MicroCal iTC$_{200}$ software

Introduction

The MicroCal iTC$_{200}$ software controls the calorimeter. Normally, MicroCal Auto-iTC$_{200}$ can be controlled using only the MicroCal Auto-iTC$_{200}$ software while keeping the MicroCal iTC$_{200}$ software minimized and running in the background. See *Section 4.2 MicroCal Auto-iTC$_{200}$ software, on page 54*.

Note: *Since most of the functionality of the MicroCal iTC$_{200}$ software is mirrored in the MicroCal Auto-iTC$_{200}$ software, only the relevant functionality is described here.*

The MicroCal iTC$_{200}$ software is able to start an instance of Origin that can be used for real-time data display, see *Section 4.4 Origin for real-time data display, on page 110*. For manual data analysis, a separate instance of Origin should be used, see *Chapter 7 Data analysis using Origin, on page 143*.

This section describes the user interface for the MicroCal iTC$_{200}$ software.

Note: *It is not recommended to use the MicroCal iTC$_{200}$ software to manually perform a run.*

In this section

This section contains the following topics:

Section	See page
4.3.9 MicroCal iTC$_{200}$ software menus	106

4 Control software
4.3 MicroCal iTC$_{200}$ software
4.3.1 Starting MicroCal iTC$_{200}$ software

4.3.1   Starting MicroCal iTC$_{200}$ software

The MicroCal iTC$_{200}$ software is used to control the MicroCal iTC$_{200}$ instrument directly. The software and hardware need to be started in sequence for correct initialization.

To start the MicroCal iTC$_{200}$ software, follow the steps described below.

Step	Action
1	Start the computer and log in to Windows.
2	Turn on the MicroCal Auto-iTC$_{200}$ instrument using the **Power** switch at the rear of the unit.
3	Start the MicroCal iTC$_{200}$ software. *Result*: The MicroCal iTC$_{200}$ software is launched.
4	To open an instance of Origin for real-time data display, select **System:Establish DDE Link To Origin**. *Note:* *It is normally not necessary to start Origin for real-time data display, since real time data can be viewed directly in the MicroCal iTC$_{200}$ software.*

4 Control software
4.3 MicroCal iTC$_{200}$ software
4.3.2 MicroCal iTC$_{200}$ software interface overview

4.3.2   MicroCal iTC$_{200}$ software interface overview

Part	Function
1	Displays the time left until the end of the run when an experiment is in progress.
2	Menus, see *Section 4.3.9 MicroCal iTC$_{200}$ software menus, on page 106.*
3	Current MicroCal iTC$_{200}$ status. On start up, the status `System Initiation - Please Wait is displayed`. After a few seconds, the system heats or cools to the preset temperature. Once the instrument reaches the set temperature, it thermostats at that temperature.
4	Control buttons, see *Section 4.3.3 MicroCal iTC$_{200}$ software control buttons, on page 93.*
5	Control tabs: • **Experimental Design**, see *Section 4.3.4 **Experimental Design** tab, on page 94.* • **Advanced Experimental Design**, see *Section 4.3.5 **Advanced Experimental Design** tab, on page 96.* • **Instrument Controls**, see *Section 4.3.6 **Instrument Controls** tab, on page 100.* • **Real Time Plot**, see *Section 4.3.7 **Real Time Plot** tab, on page 103.* • **Setup**, see *Section 4.3.8 **Setup** tab, on page 104.*

4 Control software
4.3 MicroCal iTC$_{200}$ software
4.3.3 MicroCal iTC$_{200}$ software control buttons

4.3.3 MicroCal iTC$_{200}$ software control buttons

The control buttons are used to save and load experimental run parameters, view and update current run parameters and to start and stop a run. These buttons are not normally used during the operation of the MicroCal Auto-iTC$_{200}$ software except for the **Update Run Param.** button.

Part	Function
Load Run File...	Loads previously saved parameters. The parameters are loaded into the **Advanced Experimental Design** tab.
	Run parameters can be loaded from two types of files:
	• A data file from a previous experiment (*.itc)
	• A setup file (*.inj)
Save Run File...	Saves the currently displayed run parameters to a setup file (*.inj)
Display Run Param.	Displays the current run parameters for a run in progress. This button is active when MicroCal iTC$_{200}$ is in a non-idle state.
Update Run Param.	Updates the run parameters for a run in progress. Most commonly, this would include changing injection parameters. In some instances, experimental parameters may be changed while a run is in progress, but it is not advised. This button must be clicked for run parameter changes to take effect.
Start	Starts the run using the current parameters present in the **Experimental Design** or **Advanced Experimental Design** tabs.
	Check that all parameters are correct and that a valid, unique data file name has been entered before clicking this button. The system prompts for confirmation if any files will be overwritten. This button should not be used during normal operation of the MicroCal iTC$_{200}$ system.
Stop	Aborts the run immediately. The MicroCal iTC$_{200}$ system proceeds to the next experiment in the series as if the run completed itself.

4.3.4   *Experimental Design* tab

The **Experimental Design** tab permits the user to simulate basic experimental runs. Two experimental modes are available with different recommended protocols.

For greater control over injection protocols, the **Advanced Experimental Design** tab is used. See *Section 4.3.5 **Advanced Experimental Design** tab, on page 96.*

Part	Function
Experimental Mode	Choose an experimental mode. The two modes available are: • **Highest Quality** This uses 20 injections and a c-value of 100. These parameters produce data that is clear and easy to fit. • **Minimum Protein** This uses 10 injections and a c-value of 5, resulting in the use of the least amount of sample necessary for a successful titration. More information about c-value and calculating cell concentration can be found under *Section 5.1.4 Calculating cell concentrations, on page 119.*
N	Enter the number of binding sites, n, if this is known. Press **enter** to move on to K$_D$.

Part	Function
Kd	Enter the estimated binding constant, K_D, if known. Click the **Help** button for guidance depending on sample and titrant. Press **enter** to move on to ΔH.
ΔH	Enter the estimated heat of binding, ΔH, if known.
Temperature	Enter the desired run temperature.
Update Experimental Curve	Calculates a simulated result that is displayed in the plot in the center of the tab area.
Use Ka (1/M)	Selecting this option uses a binding constant (K_A) instead of a dissociation constant (K_D).
Plot	Select whether to view the simulation plot using raw heat per injection (**DH**) or the heat normalized to the molar ratio (**NDH**).
Results	This area displays values for sample concentration in the cell, titrant concentration in the syringe and a c-value that predicts the sigmoidicity of the curve. The values may be changed by clicking the corresponding **Change** button. Also, click the **Help** button for help.
	The c-value box is color coded as specified in the **C Value Legend** box. **Optimal** values will provide the best results. Values that are **Outside of Optimal Range** will not yield the best results. Values that are **Extremely Outside of Optimal Range** will probably not yield usable data.

Tip: *Any warnings, such as,* `Heats too high for the instrument to measure`*, appear in the status bar near the top of the tab. Carefully look at the simulated curve and ensure that the shape and values are reasonable before commencing a run.*

4.3.5 *Advanced Experimental Design* tab

Overview

The *Advanced Experimental Design* tab permits detailed specification of the run parameters. Most of the functionality is the same as described in *Section 4.2.6 Instrument Setup tab, on page 73* for the MicroCal Auto-iTC$_{200}$ software and will not be described here.
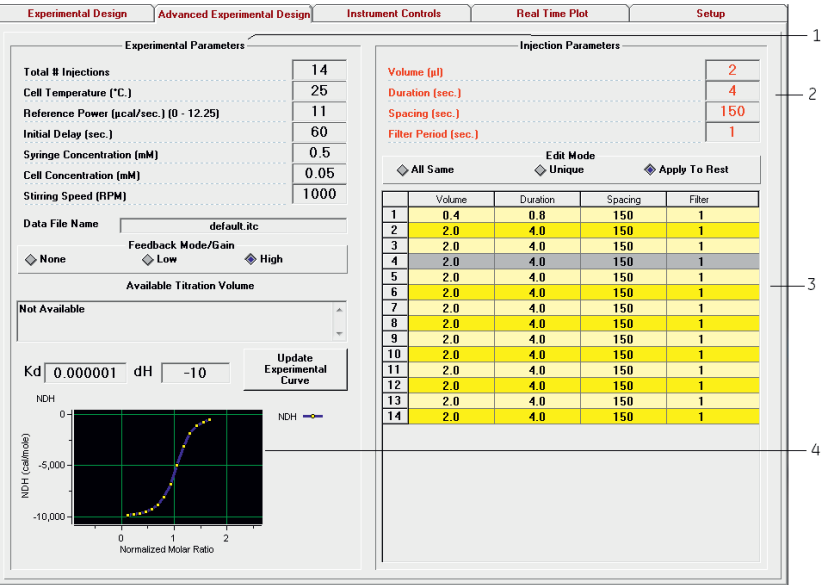
Part	Function
1	The controls in the **Experimental Parameters** area are used to change general parameters for the experimental run. See *Experimental parameters, on page 97*.
2	The controls in the **Injection Parameters** area are used to change injection parameters. The current parameters are displayed in the injection list. See *Injection parameters, on page 99*.
3	The injection list shows the parameters for each injection that will be performed during the run.
4	The simulated graph is calculated based on values from the **Experimental Design** tab but can be altered here based on the entries in the **Experimental Parameters** area. See *Experimental parameters, on page 97*.

Experimental parameters

The ***Experimental Parameters*** controls and simulated experimental curve are described below.

Note: *Most of the functionality is the same as described in Section 4.2.6 **Instrument Setup** tab, on page 73 for the MicroCal Auto-iTC$_{200}$ software and will not be repeated here.*

The MicroCal Auto-iTC$_{200}$ software populates the fields in this tab with the corresponding ***ITC Run Method*** parameters (see *Section 4.2.3 **Sample Groups** tab, on page 58*).

Though it is recommended that the MicroCal iTC$_{200}$ software be primarily ignored when operating the MicroCal Auto-iTC$_{200}$ instrument, the user should also remember that:

1   Injection parameters and some experimental parameters can be changed during a run or just before a run starts, respectively. Clicking the ***Update Run Param.*** control button will apply those changes. This option is not available in the MicroCal Auto-iTC$_{200}$ software.

2   Also, the plot on the bottom left corner is tied to the values displayed in both the experimental and injection parameters areas. Changing cell and syringe concentrations, and the injection parameters and clicking the ***Update Experimental Curve*** button affects this plot.

Part	Function
Kd	Displays the value entered in *Section 4.3.4* **Experimental Design** *tab, on page 94.*
dH	Displays the value entered in *Section 4.3.4* **Experimental Design** *tab, on page 94.*
Update Experimental Curve	Updates the simulated experimental curve, based on the parameters displayed above in the **Experimental Parameters** area.

Injection parameters

The ***Injection Parameters*** and injection list are described below. Most of the functionality is the same as described in *Section 4.2.6 **Instrument Setup** tab, on page 73* for the MicroCal Auto-iTC$_{200}$ software and will not be repeated here. The MicroCal Auto-iTC$_{200}$ software populates the fields in this tab with the corresponding ***ITC Run Method*** parameters (see *Section 4.2.3 **Sample Groups** tab, on page 58*).

Note: *The injection parameters can be changed during a run but will be applied only when the **Update Run Param.** button is clicked*

		Injection Parameters	
Volume (µl)			2
Duration (sec.)			4
Spacing (sec.)			150
Filter Period (sec.)			1

Edit Mode
◇ All Same   ◇ Unique   ◈ Apply To Rest

	Volume	Duration	Spacing	Filter
1	0.4	0.8	150	1
2	2.0	4.0	150	1
3	2.0	4.0	150	1
4	2.0	4.0	150	1
5	2.0	4.0	150	1
6	2.0	4.0	150	1
7	2.0	4.0	150	1
8	2.0	4.0	150	1
9	2.0	4.0	150	1
10	2.0	4.0	150	1
11	2.0	4.0	150	1
12	2.0	4.0	150	1
13	2.0	4.0	150	1
14	2.0	4.0	150	1

4.3.6   *Instrument Controls* **tab**

Overview

The **Instrument Controls** tab contains controls for direct operation and basic mainte-nance of the MicroCal iTC$_{200}$ instrument. The tab is illustrated below.

Note: *Only the functionality required for normal operation will be described here.*
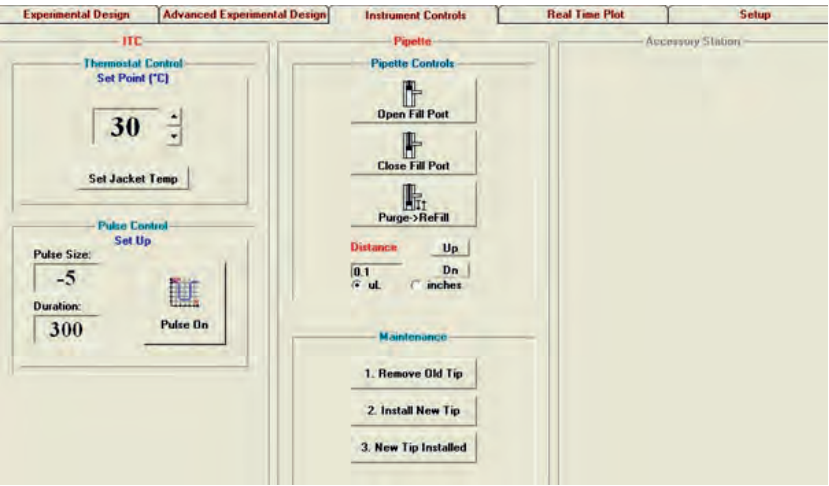
Part	Function
1	The **ITC** control pane contains controls for thermostat control and for ad-ministration of a calibration pulse. See **ITC** *control pane, on page 100*.
2	The **Pipette** control pane helps in cleaning the syringe and changing the plunger tip, which should be done when wear is visible (see *Chapter 8 Maintenance, on page 287*). See **Pipette** *control pane, on page 102*.

ITC **control pane**

The thermostat and pulse controls are described below.

The **Thermostat Control** is used to manually set the temperature of the MicroCal iTC$_{200}$ instrument.

Note: *The MicroCal Auto-iTC$_{200}$ software will take control if a run is set to perform.*

The **Pulse Control** is used to administer a DP calibration pulse. This is not intended for normal operation.

Part	Function
Set Point	Use the arrow buttons to set the desired thermostat temperature. Click the **Set Jacket Temp** button to apply the change.
Set Jacket Temp	Starts thermostatting MicroCal iTC$_{200}$ at the temperature specified in the **Set Point** box.
Pulse Size	Enter the size of the calibration pulse, in units of µcal/s.
Duration	Enter the duration of the calibration pulse in seconds.
Pulse On	Starts the calibration pulse.

Pipette control pane

The pipette controls are described below.
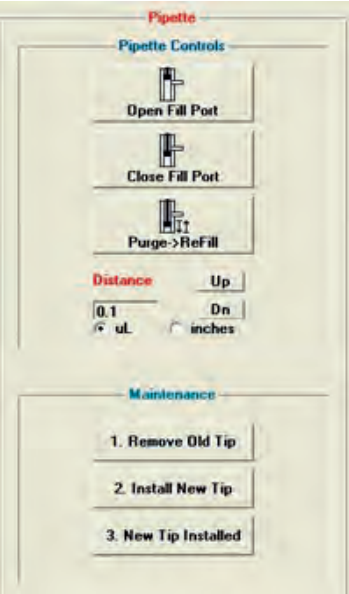
Part	Function
Open Fill Port	Moves the plunger tip to a position above the fill port on the side of the syringe.
Close Fill Port	Moves the plunger tip down so that it blocks the fill port.
Purge->Refill	Pushes the pipette tip all the way down and back up again, to dislodge bubbles, if any, on the sides of the syringe.
Distance	Enter the distance (by volume, µl or in inches) to move the pipette tip up or down.
Up	Moves the pipette tip up by the distance specified in the *Distance* text box.
Dn	Moves the pipette tip down by the distance specified in the *Distance* text box.
Remove Old Tip	Positions the plunger appropriately for tip removal.
Install New Tip	Positions the plunger appropriately to install the tip.
New Tip Installed	Positions the plunger appropriately to install the syringe.

4.3.7   *Real Time Plot* tab

The **Real Time Plot** tab displays the current data. A real time plot is also accessible in the MicroCal Auto-iTC$_{200}$ software. If no run has been started, the graph will be blank. The contents of the tab are described below.
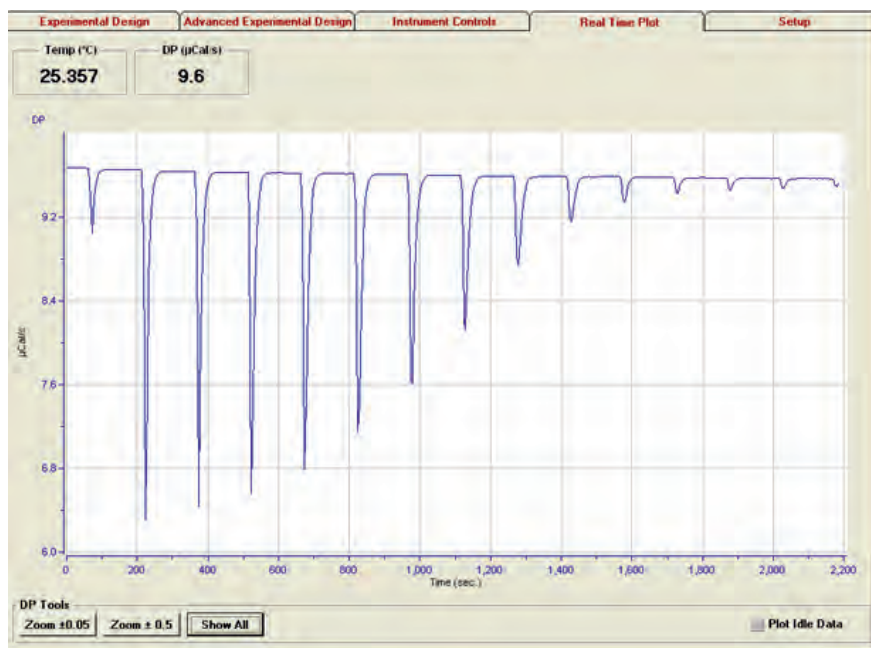
Part	Function
Temp (°C)	Displays the current temperature of the MicroCal iTC$_{200}$ instrument.
DP (µCal/s)	Displays the current MicroCal iTC$_{200}$ DP measurement.
Zoom ±0.05	Changes the vertical range to the last data point, plus or minus 0.05 µCal/s.
Zoom ± 0.5	Changes the vertical range to the last data point plus or minus 0.5 µCal/s.
Show All	Rescales the y-axis so that all the data points are visible.
Plot Idle Data	Selecting this option plots the current DP readings even if no run is currently being performed. This is helpful while troubleshooting.

4.3.8   *Setup* tab

The **Setup** tab contains some miscellaneous functions that are illustrated and described below. The functionality described below is available only if the DDE link is established with Origin. (**System**:**Establish DDE Link to Origin**).
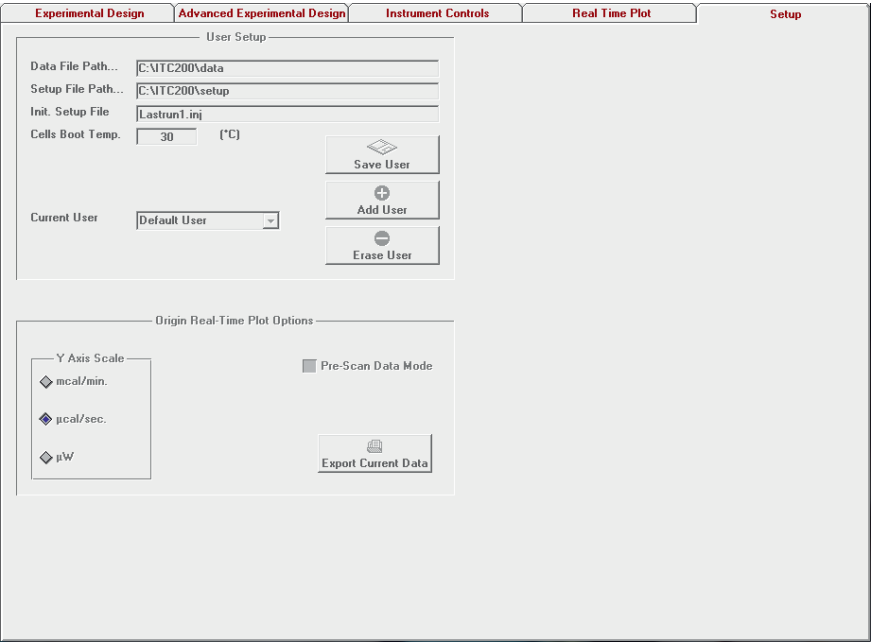
Part	Function
Y Axis Scale	Select the units in which the data should be displayed. Only the display will be affected, the data is always stored in µcal/s.
	The three choices are:
	• mcal/min
	• µcal/sec (Default)
	• µWatt

Part	Function
Pre-Scan Data Mode	Checking this box saves all data, including measurements that are taken before the run is started. Each scan will provide an additional *.dat file with the same name as the *.itc file. This data provides service personnel with extra data for the purpose of troubleshooting. *Note:* *This option requires Origin for real-time data display to be open. Select **System:Establish DDE Link To Origin** to open it.*
Export Current Data	Click this button to export and save the data that is currently displayed in the **Real Time Plot** tab to a file. The system prompts for a file name.

4 Control software
4.3 MicroCal iTC$_{200}$ software
4.3.9 MicroCal iTC$_{200}$ software menus

4.3.9   MicroCal iTC$_{200}$ software menus

Introduction

The main menus in the MicroCal iTC$_{200}$ software provide access to some of the less frequently used features of the application.

The four available menus are:

- *System*, see *System menu, on page 106*

- *ITC*, see *ITC menu, on page 107*

- *Options*, see *Options menu, on page 107*

- *Help*, see *Help menu, on page 109*

System menu

The *System* menu is illustrated below.
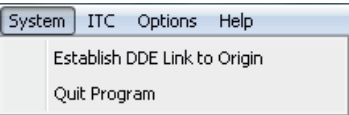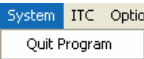
Part	Function
Quit Program	Terminates the application. The system prompts for a confirmation to quit the program. All ITC run data will be saved to disk. After approximately 1 minute, the power to the MicroCal iTC$_{200}$ instrument will also be shut down. The application needs to be restarted to restart the system.
Establish DDE Link to Origin	Opens a real time instance of Origin.

4 Control software
4.3 MicroCal iTC$_{200}$ software
4.3.9 MicroCal iTC$_{200}$ software menus

ITC menu

The **ITC** menu is illustrated and described below.

Part	Function
Print/Save As Text Run Parameters...	Prints or saves the run parameters currently loaded in the MicroCal iTC$_{200}$ software to a file. Run parameters are always stored in data file headers. Files generated using this option provide a formatted list of run parameters that may be useful for data presentation or general record keeping.
Pipette Tools	Opens a dialog where syringe calibrations can be adjusted.

Options menu

The **Options** menu is illustrated and described below.

Part	Function
System Coefficients	Opens the **System Coefficients** dialog, see **System Coefficients** *dialog, on page 108*. The values in this dialog should not be changed without direct instruction from a Malvern Instruments service engineer. **Tip:** The instrument serial number is displayed at the top left of the **System Coefficients** dialog.
Start in Advanced Mode	Displays the **Advanced Experimental Design** tab by default when the software is started.

4 Control software
4.3 MicroCal iTC$_{200}$ software
4.3.9 MicroCal iTC$_{200}$ software menus

Part	Function
ITC Equilibration Options	This option has two selectable suboptions that are selected by default. Generally, these options do not need to be deselected unless troubleshooting.
	• *Fast Equil.*
	When this option is disabled, the system goes through an additional pre-run, a non-stirring equilibration period before stirring starts. This can be useful if stirring-related noise problems are suspected.
	• *Auto Mode*
	When this option is disabled, the system will not progress automatically from one state to another during the pre-run equilibration period. To move to the next state, double-click on the *DP* data box in the *Real Time Plot* tab.
	When the option is enabled, no user interaction is required once a run has been started.

System Coefficients dialog

The **System Coefficients** dialog is illustrated below. It contains the most critical of the calibration constants. The dialog is password protected so that no changes are made by accident. None of these parameters should be changed without direct instruction from a Malvern Instruments service personnel.

4 Control software
4.3 MicroCal iTC$_{200}$ software
4.3.9 MicroCal iTC$_{200}$ software menus
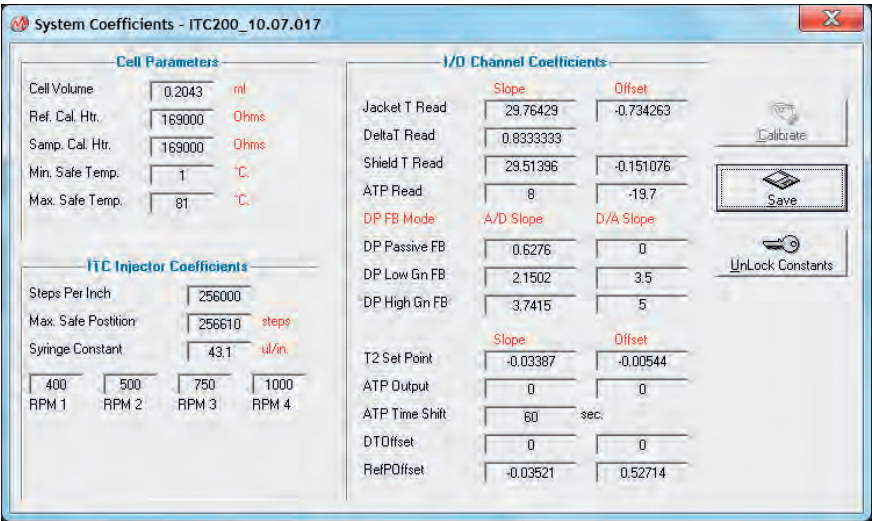
Help menu

The *Help* menu is illustrated below.

Help
About
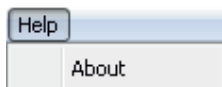
4.4   Origin for real-time data display

Introduction

An instance of Origin for real-time data display can be opened by selecting **System:Establish DDE Link to Origin** in the MicroCal iTC$_{200}$ software. This instance is customized for and dedicated to the real-time display of data acquired from the MicroCal Auto-iTC$_{200}$ instrument.

This section describes the features unique to this customized instance of Origin for real-time data display. For information about data analysis using Origin, refer to *Chapter 7 Data analysis using Origin, on page 143.*

Note: *It is normally not necessary to start the Origin for real-time data display, since real time data can be viewed directly either in the MicroCal iTC$_{200}$ software or MicroCal Auto-iTC$_{200}$ software.*

Origin for real-time data display main window

The main components of Origin for real-time data display main window are illustrated below:

Note: *Origin for real-time data display will always display the file name **VPITC-PLOT.OPJ** in the window border.*
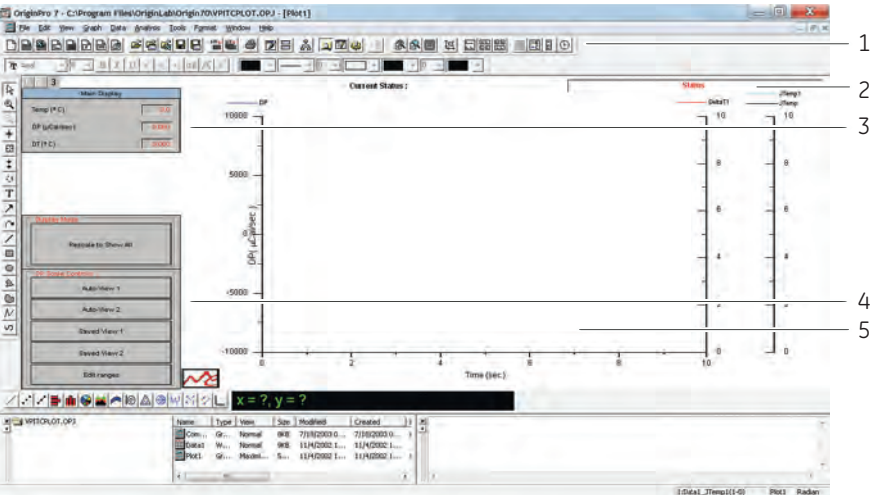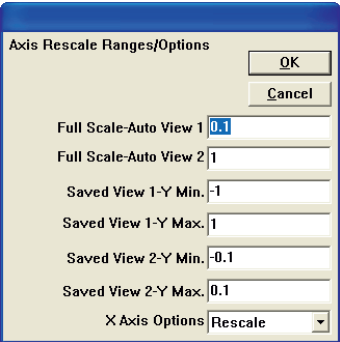
Part	Function
1	Origin main menus, refer to the *Origin User Manual*.
2	***Current Status*** Displays the current state of the instrument.
3	***Main Display*** Displays the current temperature, DP and temperature difference readings from the instrument.
4	***Display Mode*** and ***DP Scale Controls*** Controls the display of MicroCal Auto-iTC$_{200}$ data, see below.
5	Real time plot area.

Display Mode and *DP Scale Controls* buttons

The following table describes the function of the ***Display Mode*** and ***DP Scale Controls*** buttons.

Button	Description
Rescale To Show All	Automatically rescales the x- and y- axes range so that all data fits within the plot area.
Auto-View 1 *and* ***Auto-View 2***	Rescales the y-axis so that the most recent DP data point is centered. The range of the y-axis scale is determined by the value entered in the corresponding box in the ***Edit Ranges*** dialog.

Button	Description
Saved View 1 and *Saved View 2*	Rescales the y-axis to the preset values entered in the corresponding boxes in the **Edit Ranges** dialog.
Edit Ranges	Opens the **Axis Rescale Ranges/Options** dialog.

<div align="center">

Axis Rescale Ranges/Options

OK
Cancel

Full Scale-Auto View 1 | 0.1
Full Scale-Auto View 2 | 1
Saved View 1-Y Min. | -1
Saved View 1-Y Max. | 1
Saved View 2-Y Min. | -0.1
Saved View 2-Y Max. | 0.1
X Axis Options | Rescale

</div>

- **Full Scale-Auto View 1** and **Full Scale-Auto View 2**: Enter the range(s) to be used for the auto view functions

- **Saved View** text boxes : Enter the range(s) to be used for the save-view functions

- **X Axis Options**: Select the desired x-axis automatic scaling option from the drop-down list

  - **Disabled**: No x-axis rescaling is performed

  - **Rescale**: The x-axis will be rescaled by 25% when new data is acquired that exceeds the current range

  - **Scroll**: The x-axis range will scroll by 25% when new data is acquired that exceeds the current range

5   Performing a run

Introduction

This chapter describes the procedure for running MicroCal Auto-iTC$_{200}$.

The basic scheme of operation can be outlined as follows:

- **Preparing samples**. Refer to *Section 5.1 Preparing the samples, on page 114.*

- **Setting run parameters**. Refer to *Section 5.2 Setting run parameters, on page 126.*

- **Loading the samples and performing the experiment**. Refer to *Section 5.3 Loading the samples and performing the experiment, on page 134.*

In this chapter

This chapter contains the following sections:

5.1   Preparing the samples

Introduction

Since proper sample preparation is critical for successful ITC experiments, general guidelines for sample preparation will be discussed here. These guidelines use the terminology of binding experiments using biological samples, but may be readily used for other types of samples.

In this section

This section contains the following topics:

5 Performing a run
5.1 Preparing the samples
5.1.1 The importance of sample preparation

5.1.1   The importance of sample preparation

Introduction

Isothermal Titration Calorimetry (ITC) is designed to measure the heat of binding when the titrant, also referred to as the ligand, is injected into the sample cell containing the macromolecule sample material. ITC simultaneously determines all binding parameters (n, K, ΔH, ΔS) in a single experiment.

Minimizing control heat

When the titrant is injected into the cell material and mixed, some additional heat effects other than the binding heat are detectable. The key for successful ITC experiments is to minimize the control heat, thereby allowing the binding heat to be measured more accurately. This control heat will include both the heat of mixing and the heat of dilution. Two primary sources of large control heats are buffer mismatches between the titrant and the macromolecule sample in the sample cell, and a highly concentrated titrant.

Buffer mismatch

The most common mismatch occurs due to pH differences between the titrant and the macromolecule solution, but mismatch could also be a result of salt concentration, or additives such as dioxane, DMSO, glycerol, etc. and the heat of dilution when high concentration of ligand solution from the syringe is injected into the macromolecule solution. The heat of dilution will also be small, but may become large for ligands that form aggregates at higher concentration in the syringe. The most important step in preparing an assay is buffer exchange, which can be achieved by dialysis or by gel filtration.

Concentration determination

Accurate concentration determination is very important when running a calorimetric experiment. Errors will have direct impact on the thermodynamic results. Errors in cell concentration directly affect the stoichiometry, have little effect on enthalpy, and mildly affect affinity. Errors in titrant concentration, on the other hand, directly affect both the stoichiometry and enthalpy, and mildly affect affinity.

5 Performing a run
5.1 Preparing the samples
5.1.2 Preparing small molecule solutions

5.1.2   Preparing small molecule solutions

Introduction

Most small molecule ligands (such as drugs and inhibitors) are supplied in solid form. Solutions can be prepared by dissolving the compound in buffer solution or using organic solvents if the compund has low solubility in buffer solution.

Preparing samples using buffer solution

To prepare samples in buffer solution, follow the steps described below:

Step	Action
1	Prepare the buffer solution using distilled water.
2	Dissolve a known amount of the compound in the buffer solution.
3	Check the pH. If pH of the solution is found to differ from pH of the buffer solution by more than 0.05 units, the pH should be adjusted with a small amount of HCl or NaOH.

Preparing samples using organic solvents

To prepare samples using organic solvents, follow the steps described below:

Step	Action
1	Dissolve the the compound in DMSO or some other organic solvent (100 mM or higher).
2	Dilute 50 to 100 fold with buffer.
	Note:
	Care should be taken to keep the ligand from precipitating when diluted. The concentration of organic additives, such as DMSO, in the final ligand solution should be kept as low as possible (to 1% to 2%, if possible; but no more than 5%) since the macromolecule solution requires addition of the same additive at the same concentration in order to minimize the mismatch heats.

5 Performing a run
5.1 Preparing the samples
5.1.3 Preparing macromolecule solutions

5.1.3 Preparing macromolecule solutions

Introduction

Macromolecule solutions should normally be dialyzed against the buffer solution using a dialysis membrane having the proper molecular weight cut off (MWCO). However, a lyophilized macromolecule sample devoid of salts or additives may be dissolved directly into the buffer, and used without dialysis. The pH of the solution should be checked and adjusted, if necessary. Solid macromolecule samples containing salts and additives, should be dialyzed against the experimental buffer.

Preparing macromolecule solution by dialysis

To prepare a macromolecule solution by dialysis, follow the steps described below:

Step	Action
1	Dialyze the sample at 4°C against a relatively large volume of buffer solution and at least two changes of buffer. The duration of dialysis depends on the sample and buffer, as well as the membrane used.
	For example, if glycerol at 10% is added to aqueous buffer solution and a 6 000 to 8 000 MWCO membrane used, it requires at least one overnight dialysis for glycerol to reach concentration equilibrium in the macromolecule solution.
2	Determine the concentration of the macromolecule after dialysis, and remove excessive particles in the solution by filtration or centrifugation.
	Note:
	Accurate values for binding parameters depend on precise concentration measurements of ligand and macromolecule in the final solutions.

Alternatively, buffer exchange can also be performed using gel filtration. For more information, obtain the technical note, *Rapid sample preparation for MicroCal ITC and DSC experiments using PD MiniTrap G-25 columns* (28-9957-23 AA) at www.malvern.com/sample prep.

5 Performing a run
5.1 Preparing the samples
5.1.3 Preparing macromolecule solutions

Preparing macromolecule
solution with an additive

If one of the solutions (e.g., ligand solution) contains an additive such as DMSO, then the same additive at an identical concentration should be added to the other solution (e.g., protein solution) to minimize the heat of mixing. As indicated earlier, the stability of the macromolecule in the presence of the additive should be determined before proceeding. The pH of all final solutions should be checked after additives are added, and matched within 0.05 pH units.

5 Performing a run
5.1 Preparing the samples
5.1.4 Calculating cell concentrations

5.1.4   Calculating cell concentrations

c-value

ITC is designed to detect the heat that is absorbed (endothermic) or liberated (exothermic) when two solutions containing the binding partners are mixed. The appropriate concentration of the sample material in the sample cell, usually a macromolecule, will depend on the binding affinity, number of binding sites, and heat of binding, ΔH. The following equation (Wiseman et al., Anal. Biochem. 179, 131 (1989)) is used when designing ITC experiments to determine the appropriate sample concentration or c value.

$$c = n \cdot M_{tot} \cdot K_A = \frac{n \cdot M_{tot}}{K_D}$$

Parameter	Description
c	sample concentration, should lie between 1 and 1000 (preferably 10 to 500 when solubility, availability of material or the sensitivity of the instrument is not limiting)
n	binding stoichiometry (the number of ligand binding sites on the sample molecule)
M_{tot}	molar concentration of sample molecule in the cell
K_A	association equilibrium constant
K_D	dissociation equilibrium constant

5 Performing a run
5.1 Preparing the samples
5.1.4 Calculating cell concentrations

Sample concentration limitations

The figure below depicts simulated curves of the same macromolecular system run at different c-values.
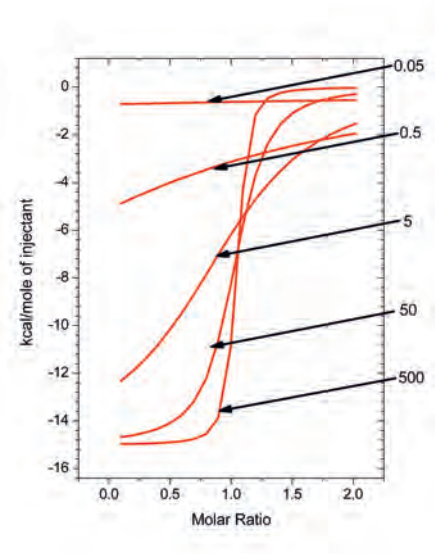
There may be practical limitations that affect the choice of sample concentration:

Experiments including...	should be studied at...
high affinity interactions (low K_D)	low concentrations. (The minimum concentration that will typically cause a confidently measurable heat change for a 1:1 interaction is about 10 µM.)
low affinity interactions (high K_D)	high concentrations. (The concentration that can be used may be limited by availability or solubility of the sample molecule.)

Note: *Techniques such as competition experiments and working at low c-values can help alleviate these limitations.*

5 Performing a run
5.1 Preparing the samples
5.1.4 Calculating cell concentrations

Affinity determination at different c-values

The affinity is poorly determined at high c-values. At low c-values, one may assume (and fix) a stoichiometry and inject enough titrant to attain a high molar ratio in order to extract both an affinity and a binding enthalpy.

At high c-values
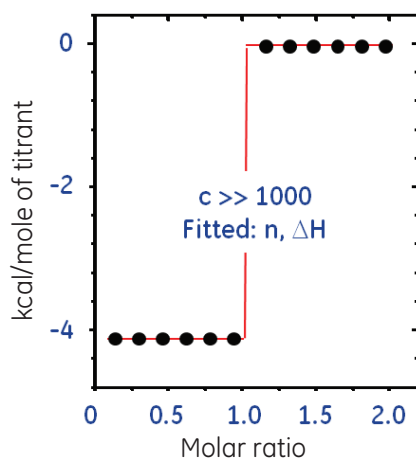
At medium c-values
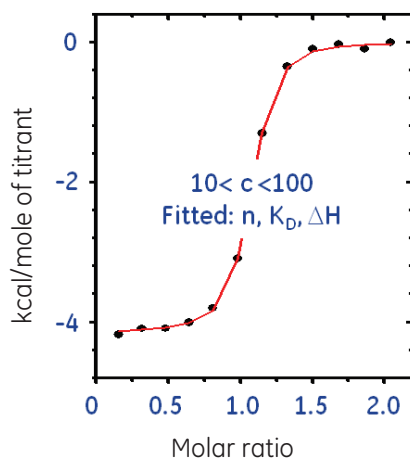
5 Performing a run
5.1 Preparing the samples
5.1.4 Calculating cell concentrations

At low c-values

5 Performing a run
5.1 Preparing the samples
5.1.5 Syringe concentration and number of injections
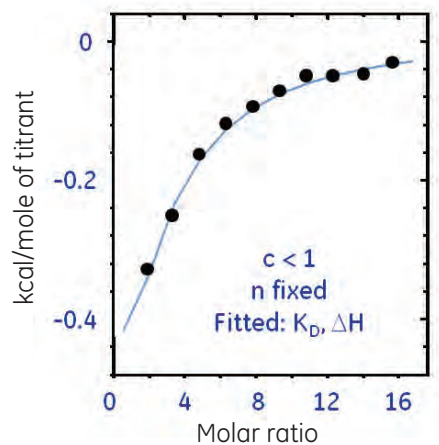
5.1.5   Syringe concentration and number of injections

Calculating syringe concentrations

For a 1:1 binding reaction, the molar concentration of ligand in the injection syringe is typically 10 to 20 times higher than the molar concentration of sample molecule in the cell. This will ensure that the cell material will become saturated or close to saturation by the end of the titration experiment.

Note: *Remember, errors in titrant concentration directly affect the stoichiometry, directly affect the enthalpy, and mildly affect affinity.*

Injection number and duration

The specifications for a typical experiment in MicroCal Auto-iTC$_{200}$ are presented below.

Parameter	Value
Number of injections	18
Injection volume	2 µl
Initial injection volume	0.4 µl (to minimize the impact of equilibration artifacts sometimes seen with the first injection) *Note:* *The data point from this initial injection is discarded before data analysis.*
Pipette volume	~38 µl of ligand solution (sufficient for one typical experiment)

5 Performing a run
5.1 Preparing the samples
5.1.6 Experimental temperature and control heat determination

5.1.6   Experimental temperature and control heat determination

Experimental temperature

It is most convenient to perform ITC experiments at 25°C to 30°C (i.e., slightly above room temperature) unless other factors dictate differently. Since the cells are passively cooled by heat exchange with the jacket, experiments at low temperature require a longer time for temperature to reach equilibrium before injections can begin.

At high temperatures (above 50°C), the baseline becomes noisier, which has an effect on the quality of data. Other factors that influence the choice of the experimental temperature are the binding affinity and the stability and/or solubility of the ligand or sample molecule. Some solutes, particularly proteins, are not stable above room temperature for long periods of time, and in such cases it may be desirable to work at lower temperatures.

To determe the change in heat capacity, ΔC_P, associated with binding, experiments must be performed over a range of temperatures (e.g., 10°C to 40°C) to obtain the temperature dependence of the heat of binding.

Control heat determination

As discussed above, a control experiment is required to determine the heat associated with the dilution of the ligand when it is injected from the syringe into the buffer. This experiment will also include contributions from the injection process itself and any other operational artifacts, which can collectively be thought of as the "instrument blank". If heat effects for the control run are small and constant, the average heat of injection can be subtracted from the results of the sample run before curve fitting to obtain binding parameters.

However, large heat effects for the control and heat effects that change as the titration proceeds may indicate mismatch between ligand and sample buffer (see *Section 5.1.1 The importance of sample preparation, on page 115*). Buffer matching should then be checked before proceeding with the experiment. If trends in the control results cannot be eliminated by careful buffer matching, they may result from ligand aggregation or self-association in the syringe. More complex evaluation algorithms should be considered in such cases.

5 Performing a run
5.1 Preparing the samples
5.1.7 Additional notes

5.1.7   Additional notes

Reducing agent

It has been found that the presence of DTT (1,4-dithiothreitol) in solution will often cause a drastic shift in ITC baseline as the experiment progresses. If the presence of a reducing agent is required for protein stability, then β-mercaptoethanol (less than 5 mM) or TCEP (Tris[2-carboxyethylphosphine] hydrochloride; less than 2 mM) should be used rather than DTT.

Reverse titration

Most titrations are carried out with the macromolecule solution in the cell and the ligand solution in the syringe. If both binding partners are macromolecules (or both are small molecules) normally the component with multiple binding sites is placed in the cell. However, there are instances where it might be advantageous or even necessary to switch the location of the two components and carry out the reverse titration. If the component, which normally goes in the syringe has low solubility, it may be easier to use that solution in the cell, where its concentration does not need to be nearly as high. If the macromolecule becomes unstable over time in the sample cell, either due to continuous stirring or a high experimental temperature, it may be more stable if placed in the syringe. The solution in the syringe is not stirred or thermostatted at experimental temperature until shortly before it is injected into the cell.

5.2   Setting run parameters

Introduction

This section describes how to set the run parameters in the MicroCal Auto-iTC$_{200}$ software.

In this section

This section contains the following subsections:

5 Performing a run
5.2 Setting run parameters
5.2.1 Creating a method

5.2.1   Creating a method

Introduction

This section describes how to create an ITC method in the MicroCal Auto-iTC$_{200}$ software. An example experiment is set up that has 18 injections of 2.0 µl each with 150 s between the injections and one initial, small injection, which is later disregarded to minimize any impact from diffusion during equilibrium of the instrument.

Creating the method

To create the example method, follow the steps described below:

Step	Action
1	Click on the **Instrument Setup** tab and the **ITC Method** subtab.
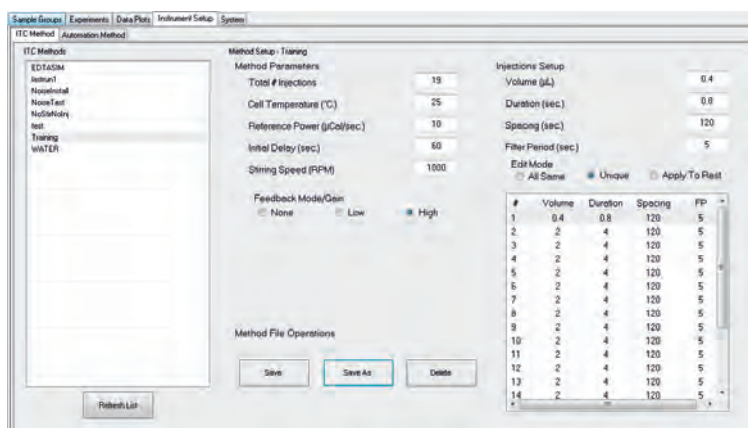
| 2 | Enter the following **Method Parameters**. |

Parameter	Value
Total # Injections	19
Cell Temperature (°C.)	25
Reference Power (µCal/sec.)	10
Initial Delay (sec.)	60
Stirring Speed (RPM)	1000

5 Performing a run
5.2 Setting run parameters
5.2.1 Creating a method

Step	Action
3	Set the *Feedback Mode/Gain* to *High*.
4	Enter the following parameters for *Injections Setup* with the *All Same* radio button clicked under *Edit Mode*.

Parameter	Value
Volume (µL)	2
Duration (sec.)	4
Spacing (sec.)	150
Filter Period (sec.)	5

Step	Action
5	Change the parameters of the first injection:

1 Select the first injection in the table to the right in the *Method Setup* workspace.

2 Select *Unique* under *Edit Mode*.

3 Change the following parameters under *Injections Setup*:

Parameter	Value
Volume (µL)	0.4
Duration (sec.)	0.8

Step	Action
6	Click the *Save As* button and save the method as `Training`. The method will appear on the *ITC Methods* list to the left.

5 Performing a run
5.2 Setting run parameters
5.2.2 Changing the sample group settings

5.2.2   Changing the sample group settings

Introduction

This section describes how to change the sample group settings in the MicroCal Auto-iTC$_{200}$ software.

Changing the sample group settings

To change the sample group settings, follow the steps described below:

Step	Action
1	Select the **Sample Groups** tab and select **1** in the **Number of Sample Groups** drop-down menu.
2	Select the **Dataset Naming** subtab under the **Group 1** tab and enter 2 in the **Number of Samples** field. One of these samples will be used for a control experiment.

- Enter `Training` as **Prefix** in the **Sample Naming** workspace.

- Set **Starting Number** to 1.

- Leave the **Suffix** field empty.

5 Performing a run
5.2 Setting run parameters
5.2.2 Changing the sample group settings

Step	Action
3	Select the **Methods** subtab and choose **Plates** from **Automation Method** drop-down menu. The **Automation Method** drop-down list consists of several pre-defined plate setups. For more information, see *Section 10.4 Standard setup files, on page 338.*

Plates cleans the cell with detergent and pre-equilibrates the sample in the cell to minimize experimental artifacts originating from loading cold samples. Some samples (i.e., high protein concentration) have responded more favorably when pre-equilibrated in the tubing, as opposed to in the cell. When using the **Plates2** automation method, which uses the **Plate2** cell load script, the sample will be pre-equilibrated to the ambient temperature, rather than the experimental temperature, but this may be suitable depending on the application.

Step	Action
4	Leave the **Save Sample** checkbox unchecked. If this is checked the sample from the sample cell may be saved in a separate well position once the run is finished.
5	In the **ITC Run Method** drop-down menu, choose the **Training** method created previously.
6	From **Analysis Method** drop-down menu choose **OneSite**.
7	For this experiment, all **Automation Description** properties should be set to **No**.

5 Performing a run
5.2 Setting run parameters
5.2.2 Changing the sample group settings

Step	Action

8   Enter the appropriate **Cell Concentration** and **Syringe Concentration** (i.e., `10` µM in the cell and `100` µM in the syringe) in the **Concentration** subtab.
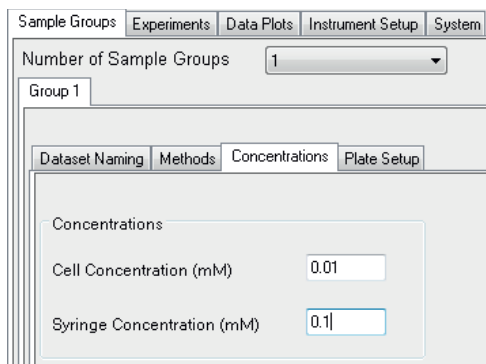
9   Select the **Plate Setup** subtab and check that four wells (two Cell Source and two Pipette Source) are active on Plate 1. Refer to the **Well Plate Legend** on the right side of the illustrated 96-well plate.

5 Performing a run
5.2 Setting run parameters
5.2.3 Changing the experiment settings
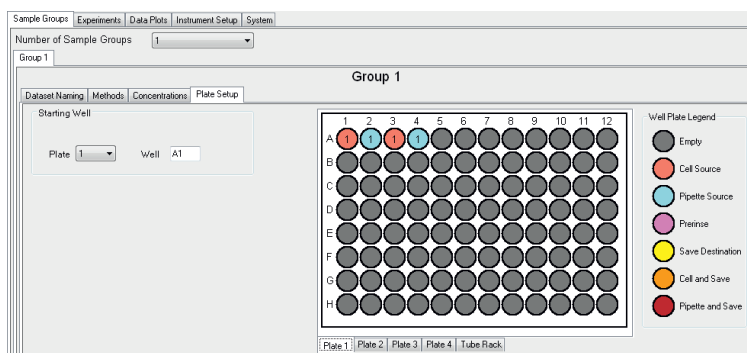
5.2.3   Changing the experiment settings

Introduction

This section describes how to change the experiment settings in the MicroCal Auto-iTC$_{200}$ software.

Changing the experiment settings

To change the experiment settings, follow the steps described below:

Step	Action
1	Select the **Experiments** tab and click the **Import Sample Groups** button to insert the settings in the spreadsheet.
2	In order to change experiment 2 to a control experiment, change the **Analysis Method** for experiment 2 by highlighting the table cell (now marked **OneSite**) and select **Control** from the drop-down menu. Also change the **Automation Method** for experiment 1 to **Plates Clean** using the same procedure. This change will introduce a more thorough cleaning of the system at the start of the experimental series.
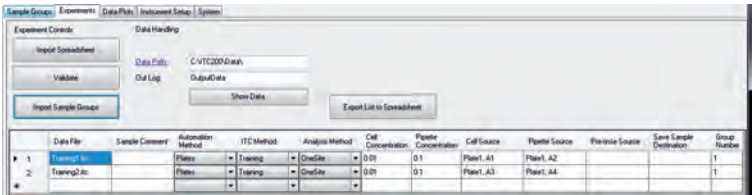
| 3 | Click **Validate** to confirm the run parameters. |
| 4 | The **Log already exists!** window appears and prompts if the data has to be appended to an existing log. For this exercise, create a new log to save data. Click **No**. |

5 Performing a run
5.2 Setting run parameters
5.2.3 Changing the experiment settings
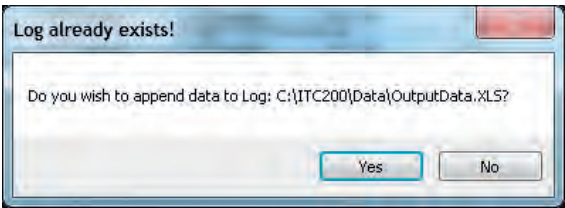
Step	Action
5	In the **Update Log Name** window enter Training to create a new log with this name. Click **OK**. This log will be saved to the path specified in **Data Path**.
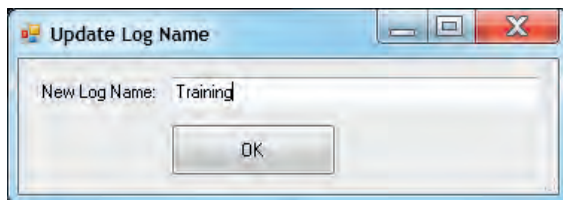
6	The **Validation Report** window opens and displays the total reagent consumption.

Note:

Make sure that the required volumes are available in the appropriate bottles.

Click **OK**.
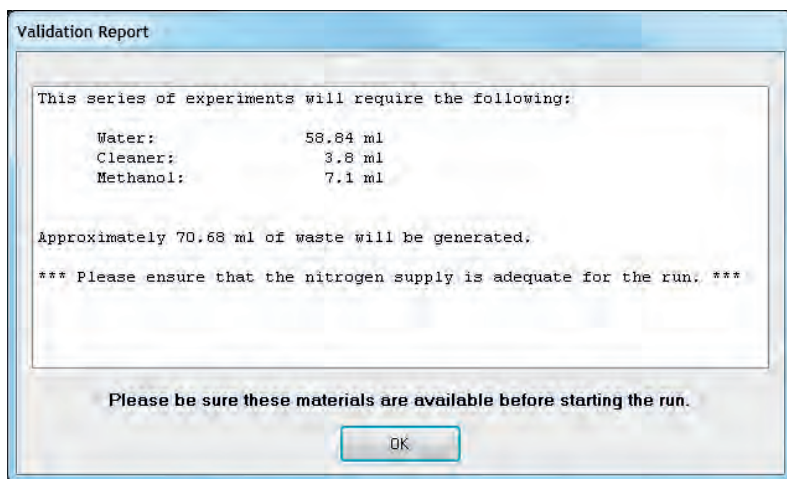
5.3 Loading the samples and performing the experiment

To load the samples and perform the experiment, follow the instructions below:

Step	Action
1	In a 96-deep well microtiter plate, load 370 µl of the sample solution that is to be transferred to the sample cell in the A1 well position (Cell Source) and 110 µl of the titrant into the A2 well position (Pipette Source). For the control experiment, load 370 µl of the sample buffer in the A3 well position (Cell Source) and 110 µl of the titrant into the A4 well position (Pipette Source).

Tip:

The easiest way to see if the plate is loaded properly is to hold it up to an overhead light.

The example plate below is loaded with alternating 370 µl for the cell and 110 µl for the syringe.
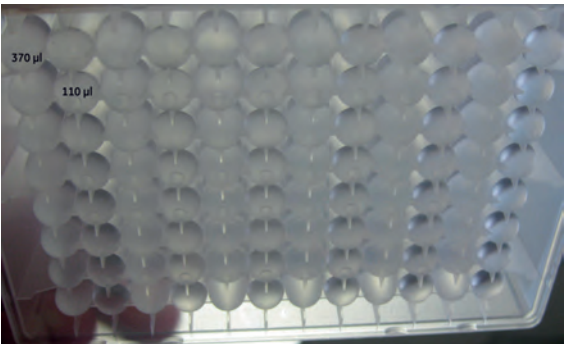
Step	Action
2	Cover the microtiter plate with a ZoneFree cover (Part No. 28429092) or a Biacore 96-well Microplate Foil (Part No. 28975816). Be careful to center the holes in the cover over the plate wells.
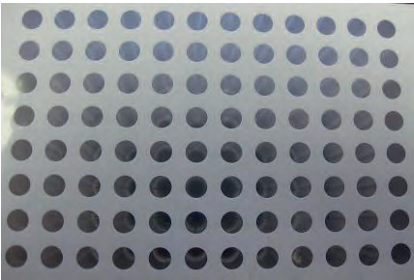
Step	Action
3	Tap the **Open Tray** button on the MicroCal Auto-iTC$_{200}$ touch screen and insert the plate into tray position 1. Tap **Close Tray** to close the tray. The software will be unresponsive until the tray homes.

4	Tap **Start** on the touch screen of the MicroCal Auto-iTC$_{200}$ system or the start button in the upper left corner of the software to begin the analysis. The analysis will take approximately 2 h including the initial washing of the system.

Once a titration run starts, the real-time data is displayed in the **Data Plots** tab, in the **Real Time Display** subtab. After each scan, an automatic data analysis is run and the results appear in the **Results Plot** subtab, showing a scatterplot of n, K_D, and ΔH. In addition, clicking the **Show Data** button in the **Experiments** tab brings up a Microsoft Excel spreadsheet where the results are tabulated, as well as graphs of the raw data and normalized ΔH curve that appear when the cursor moves over the **n** and **KD** columns respectively.

5	It is recommended that the instrument be shut down when not in use (**Power** switch in the rear).

6   Data analysis using MicroCal Auto-iTC$_{200}$ software

Introduction

This chapter describes the automated data analysis process using the MicroCal Auto-iTC$_{200}$ software.

In this chapter

This chapter contains the following sections:

6.1   Description

Data display

Once a titration run starts, the real-time data will be displayed in the Data Plots tab, in the Real Time Display subtab. After each scan, the specified analysis method will be performed and the results will appear in the Results Plot subtab. The Individual Results Plot subtab will permit the user to plot the raw data (DP), the integrated injection heats (DH), or the injection heats, normalized by the titrant concentration (NDH).

Results presented in Microsoft Excel

In addition, clicking the Show Data button in the Experiments tab will bring up a Microsoft Excel spreadsheet with the tabulated results for n, KD, and ΔH, as well as graphs of the raw data and normalized ΔH curve that appear when the cursor hovers over the Stoichiometry and KD columns, respectively.

Automated data analysis

Automated analysis of ITC data is performed in the MicroCal Auto-iTC$_{200}$ software. Origin is still available for data analysis and includes models for more complex data. Data analysis using Origin, however, requires user input. For a description of the MicroCal Auto-iTC$_{200}$ software user interface, see *Section 4.2 MicroCal Auto-iTC$_{200}$ software, on page 54*. For a description of data analysis using Origin, see *Chapter 7 Data analysis using Origin, on page 143*.

In this section, the procedure for automated data analysis is described, as well as the algorithm that is used for automatic baseline subtraction for titration data.

6.2   MicroCal Auto-iTC$_{200}$ software

This software drives the automation and communicates with the calorimeter to run experiments. After each experiment is completed, it executes the specified analysis method and delivers the result in a convenient format.

The table below lists some of the basic functionality available for data analysis using the MicroCal Auto-iTC$_{200}$ software.

Step	Action
Sample Groups: Methods	Allows user to specify the appropriate analysis method. See *Section 4.2.3* **Sample Groups** *tab, on page 58*.
Data Plots: Real Time Display	Displays the raw output of an experiment while it is still running. See *Section 4.2.5* **Data Plots** *tab, on page 68*.
Data Plots: Results Plot	Tabulates and displays the results of the experiment, organizing them according to the analysis method. See *Section 4.2.5* **Data Plots** *tab, on page 68* .
Data Plots: Individual Results Plot	Displays stored experimental results for completed experimental runs in the current series. See *Section 4.2.5* **Data Plots** *tab, on page 68*.
Experiments: Show Data	Opens a Microsoft Excel spreadsheet containing results of the analysis methods and embedded graphs.

6.3 Automated data analysis procedure

The automated data analysis procedure involves the following steps:

1 The portion of the raw data that does not represent injection heat is identified and defined as a baseline.

2 A baseline is fit to these inter-injection baseline regions.

3 This baseline is subtracted from the entire data set.

4 Each peak is integrated, and the heat is normalized to moles of titrant injected.

Note: *Proper sample preparation will be very evident here.*

5 The software ignores the first peak and automatically fits the specified analysis method to the normalized heat (NDH). The first peak is ignored because its heat may be inaccurate due to diffusion between the syringe tip and the cell during the equilibration process. Though the first injection's heat may be incorrect, it is important to note that the correct amount of titrant made it into the cell. The available analysis methods are the **Control**, **OneSite** and **OneSiteFixedN** models. The **Control** analysis method only integrates the injection heats and does not fit a model.

Analysis method	Procedure
OneSite	1:1 binding (extracts n, K$_D$, ΔH)
OneSiteFixedN	1:1 binding, n=1 (extracts K$_D$, ΔH), useful for weak binders
Control	Calculates mean control heat (and variability)

Note: *The **OneSite** and **OneSiteFixedN** models automatically fit an offset, assuming the control heat is a fixed value. This value is also stored in the Microsoft Excel out log.*

The figure below depicts each injection heat, normalized by the amount of titrant injected, as a function of the molar ratio of titrant/sample in the sample cell. The fitted parameters of a 1:1 binding model are overlaid (**OneSite**). Data analysis will be explained in more detail later in this chapter.
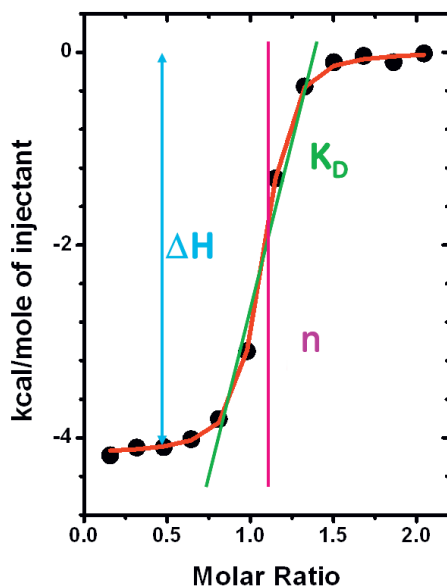
6   The n, K$_D$ (**KD**), and ΔH generated by this fit are displayed in **Data Plots: Results Plot** (see *Section 4.2.5 **Data Plots** tab, on page 68*) and also in the Microsoft Excel out log spreadsheet, see *Section 4.2.4 **Experiments** tab, on page 66*. The result is displayed and stored as:

- one file per titration run: the raw ***.itc** data file

- one general output file: the Microsoft Excel spreadsheet

Note: *To refit the data using a model not available in the automation, to subtract a control, or to adjust the data fitting, open Origin- MicroCal iTC$_{200}$ Analysis and read in the raw **.itc** file.*

6.4 Microsoft Excel spreadsheet

The Microsoft Excel out log spreadsheet contains the run parameters and data analysis results, for each experiment. It can be found in the same destination as the corresponding *.itc* files and can be opened at any time by pressing the **Show Data** button in the **Experiments** tab.

The two different sheets in the Microsoft Excel workbook contain the runs analyzed by the **Control** and **OneSite** analysis methods and contain specific setup parameters.

Control sheet:

1 The control run parameters are stored in the **Control** tab of the Microsoft Excel out log spreadsheet. It displays columns with the mean energy and standard deviation of the peak areas. This provides an analysis of the consistency of the peak sizes, as well as a measure of the blank heat of injection.

2 An embedded graph displaying the raw data pops up when the mouse hovers over the **Mean Energy** column.

3 Each graph is displayed when the mouse hovers over each cell in the column.

4 Scatterplots of the area under each peak are displayed in the **Standard Deviation** column.

OneSite sheet:

1 The run parameters and analyzed data of **OneSite** model runs are stored in this tab.

2 The fitted **Stoichiometry** (n), **KD** (K$_D$), **DH** (ΔH) and calculated fit errors for each variable and the overall Chi-squared value are also displayed.

3 A graph of the raw data appears when the mouse hovers over the **Stoichiometry** column. The automatically generated baseline is shown in red.
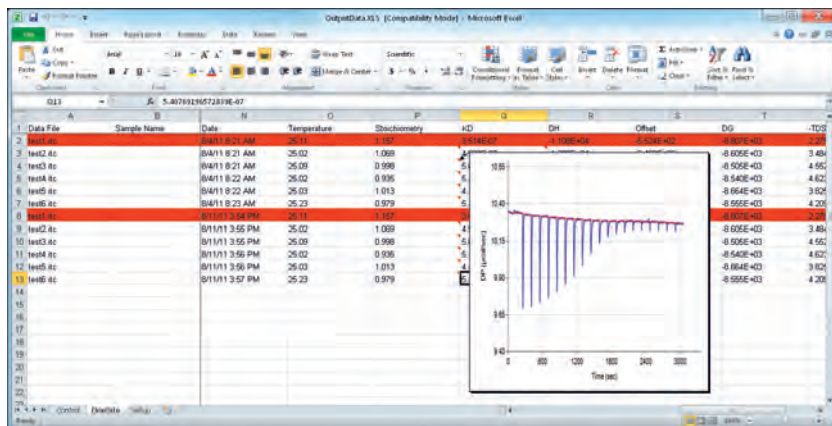
4 An embedded graph of the normalized ΔH plot with the fitted curve in red is displayed when the cursor hovers over the **KD** (K$_D$) column.
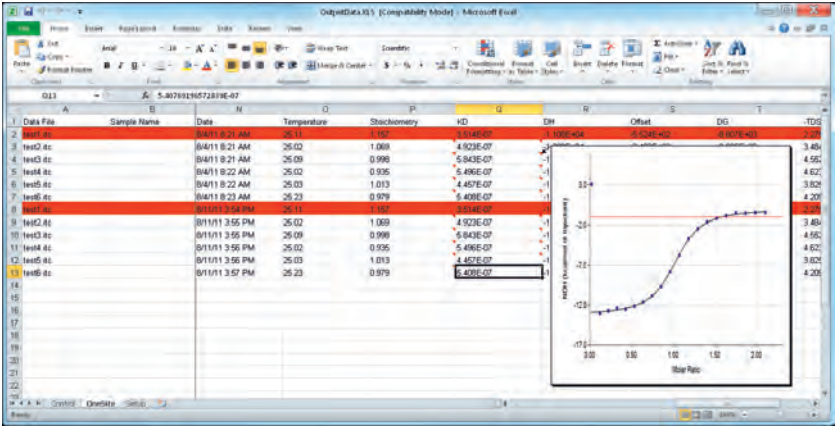
Setup sheet:

1   This tab displays certain setup parameters of the run.

2   If a fitted variable is outside the range set in the **Setup** tab, the entire row in the spreadsheet is highlighted in red.

3   This range can be changed by editing the **Setup** tab in the output spreadsheet template file, located at `C:\Documents and Settings\All Users\Application Data\MicroCal\ResultsLogTemplate.xls` (Windows XP); `C:\ProgramData\MicroCal\ResultsLogTemplate.xls` (Windows 7). This change affects all the subsequent data runs.

7   Data analysis using Origin

Introduction

This chapter describes the automated data analysis process using Origin software.

Origin, from OriginLabs, is a general purpose, scientific and technical data analysis and plotting tool.

The Origin software used with MicroCal Auto-iTC$_{200}$ is further enabled and includes routines designed to analyze the ITC data. Most of the ITC routines are implemented as buttons in plot window templates designed specifically for the ITC data analysis software. Some routines are located in the ITC menu in the Origin menu display bar. This tutorial explains how to use all of these ITC routines.

In this chapter

This section includes the following topics:

7.1   Basic ITC data analysis and fitting

Introduction

This section describes how to start Origin and its basic menu options. Installation of Origin is described in *Section 3.1 Installing MicroCal Auto-iTC$_{200}$ software, on page 24*.

This section also describes how to perform routine analysis of ITC data. Origin automatically determines the baseline, the range to integrate the injection peaks, and the initialization of the fitting parameters. These factors can be adjusted manually, as described in the following sections, if the automated data analysis is not adequate. Due to the automated data analysis functionality built into the MicroCal Auto-iTC$_{200}$ control software, the MicroCal iTC$_{200}$ Origin software can be used for most applications where Origin functionality is deemed necessary. In the case where a single control experiment applies to several runs, the MicroCal Auto-iTC$_{200}$ Origin software is advantageous.

Note:　　　*A series of sample ITC files are included with the MicroCal Auto-iTC$_{200}$ Analysis software. A typical file is designated* **RNAHHH.ITC**. *This file contains data from a single experiment of 20 injections. It is located at* `Origin70\Samples`.

Note:　　　*The* **.1** *file extension indicates an ITC file generated with the MicroCal data acquisition software. The* **.ITC** *extension indicates an OMEGA, MCS ITC, VP-ITC, or iTC$_{200}$ file generated with the Windows-based version of the MicroCal data acquisition software. The two file types are identical, except that the procedure for opening them differs slightly, as described below.*

Starting Origin

Double-click the **MicroCal Analysis Launcher** icon found on the desktop.
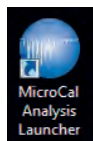
To access the non-automated version of the MicroCal iTC$_{200}$ Origin software select the **ITC$_{200}$** button. For batch processing the MicroCal iTC$_{200}$ data, which is described in *Section 7.7 Batch-processing data with Origin, on page 272*, select the **Auto-iTC$_{200}$** button.

7 Data analysis using Origin
7.1 Basic ITC data analysis and fitting
7.1.1 Routine ITC data analysis
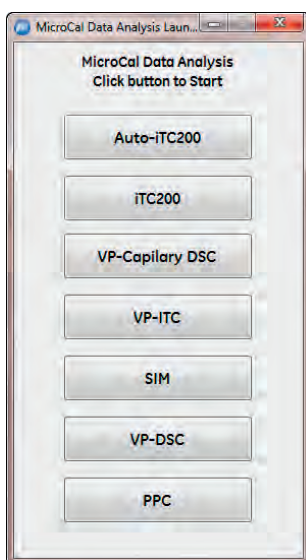
7.1.1   Routine ITC data analysis

Opening the *RNAHHH.ITC* file

To open the **RNAHHH.ITC** file, follow the steps described below:

Step	Action
1	Start Origin for ITC as described in *Starting Origin, on page 144* The next few sections describe the MicroCal iTC$_{200}$ Origin software. The MicroCal Auto-iTC$_{200}$ Origin software, which batch-processes data but lacks some of the model fitting, is described in *Section 7.7 Batch-processing data with Origin, on page 272*. The MicroCal Auto-iTC$_{200}$ Origin software is specifically meant to work on multiple data sets and their respective control data sets, which are specified during post run data analysis. The automated, constant offset subtraction that is performed in the MicroCal Auto-iTC$_{200}$ control software (see *Chapter 6 Data analysis using MicroCal Auto-iTC$_{200}$ software, on page 136*) is not done in Origin. This must still be done manually in Origin (see *Section 7.3 Analyzing multiple runs and subtracting reference, on page 167*).

The program opens and automatically displays the **RawITC** plot window. There are several buttons along the left side of the window, which give access to many of the ITC routines.

2	Click **Read Data**. The **Open** dialog box opens, with the **ITC Data (*.it?)** selected as the **Files of type:**.

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7.1.1 Routine ITC data analysis

Step	Action
3	Select `C:\Origin70\Samples\RNAHHH.ITC` from the files list.
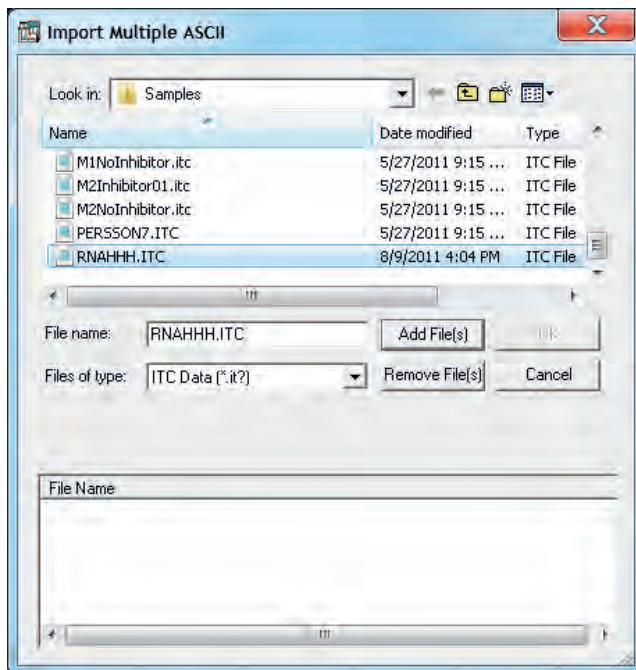
Note:

Data file names should not contain any hyphens, periods or spaces.

Note:

Origin truncates the file names to the first 15 characters. Therefore, when reading in multiple files, the first 15 characters of the file name must be a unique combination to prevent overwriting the data.

When reading multiple datasets into Origin, the last 10 characters of the file name (excluding the file extension and underscore characters) must be unique and not start with a numeric value. Incorrect naming can be corrected by re-naming the files manually. This only affects the simultaneous analysis of multiple datasets. Single datasets can always be read into Origin. (Examples of good and bad file names, see Examples of good file names:, on page 60 and Examples of bad file names:, on page 61.)

Tip:

A default folder for Origin can be selected by navigating to **File:Set Default Folder** and entering the default path (for example, for this tutorial the path can be `C:\Origin70\Samples`). More information about the files can be viewed by clicking **Details**.

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Step	Action
4	Click **Add File(s)** and then click **OK**. **Tip:** The files can also be added by double-clicking on the file name. The default plot window depicts the normalized injection heat as a function of molar ratio in the cell (titrant conc./sample material conc.) in the **DeltaH** window.

Data windows

The available windows (i.e., **DeltaH**, **RawITC**, etc.) can be viewed by selecting the **Window** menu list. Alternatively, **ctrl**+**tab** cycles through the windows.

Data set worksheets

Origin creates three worksheets to hold the data sets mentioned in the previous section. To open these worksheets refer to *Section 7.4 ITC data handling, on page 185*, which describes how to open worksheets from plotted data, copy and paste data, and export data to an ASCII file.

Origin data sets

Each time an ITC raw data file series is opened, Origin creates eight data sets. These eight data sets follow a definite naming convention that includes the name of the ITC source file followed by an identifying suffix (injection number is indicated by the row number *i*). Double-click on the layer icon `1` to view the available data sets :

Data set	Description
Rnahhh_DH	Experimental heat change resulting from injection i, in µcal/injection (not displayed).
Rnahhh_MT	Concentration of macromolecule or sample in the sample cell before each injection i, after correction for volume displacement (not displayed).
Rnahhh_XT	Concentration of injected solute in the sample cell before each injection (not displayed).
Rnahhh_INJV	Volume of injectant added for the injection i.

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Data set	Description
Rnahhh_NDH	Normalized heat change for injection i, in calories per mole of injectant added (displayed in *DeltaH* window).
Rnahhh_XMT	Molar ratio of ligand to macromolecule after injection i.
Rnahhhbase	Baseline for the injection data (displayed in red in the *RawITC* window).
Rnahhhraw_CP	All of the original injection data (displayed in black in the *RawITC* window).

Note: *Two temporary data sets are also created; **Rnahhhbegin** that contains the indices (row numbers) of the beginning of an injection, and **Rnahhhrange** contains the indices of the integration range for the injection.*

Editing concentration values

Note: *Before fitting a curve to the data, it is recommended to check the current concentration values for the experiment.*

To edit concentration values, follow the steps described below:

Step	Action
1	Click the *Concentration* button located in the *Data Control* box to the left of the graph, in the *DeltaH* window.

A dialog box *For Data RNAHHH* opens and displays the concentration values for the current experiment.

The concentration (*C in Syringe (mM)* and *C in Cell (mM)*) values displayed are those entered by the operator before the experiment starts. Cell volume (*Cell Vol. (ml)*) is constant, which is stored in the data collection software. This value is read by Origin whenever an ITC data file is opened.

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Step	Action
2	Click **OK** or **Cancel** to close the dialog box.

Note:

Always check that the concentration values are correct for each experiment. Incorrect values will negate the fitting results. The concentration values can be edited by simply entering a new value in the appropriate text box.

At this point in the analysis, Origin has performed all the operations listed in *Chapter 6 Data analysis using MicroCal Auto-iTC$_{200}$ software, on page 136* up through step 4. Data can now be fit to a model.

Model fitting

This section describes the basic procedure for fitting a theoretical curve to the data. See *Section 7.6 Advanced curve fitting, on page 205*, and *Appendix A Equations used for fitting ITC data, on page 349* for a discussion of fitting the equations.

MicroCal iTC$_{200}$ Origin provides six built-in curve fitting models, namely :

- *One Set of Sites*,

- *Two Sets of Sites*,

- *Sequential Binding Sites*,

- *Competitive Binding*,

- *Dissociation*, and

- *Enzyme Assays*.

Note: *MicroCal Auto-iTC$_{200}$ software Origin software only contains the **One Set of Sites** and **Two Set of Sites** models.*

Fitting the peak area data to the *One Set of Sites* model

To fit the area data to the **One Set of Sites** model, follow the steps described below:

Step	Action
1	Click anywhere on the **DeltaH** plot window to make it the active window or select **DeltaH** from the **Window** menu.

Note:

It is a good practice to make the first injection of 0.4 µl and then remove the first data point from data fitting. This is because during the initial baseline equilibration, the titrant can be diluted by the cell material.

The material makes it into the cell but the heat is attenuated to an immeasurable degree, so the first integrated injection heat can be inaccurate.

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Step	Action
2	After the injection data are integrated, the integration results are displayed in the **DeltaH** plot window. Bad data points can be deleted from the **DeltaH** window before starting the fitting session.
	Let's remove the first data point. Notice that the normalized heat is less than expected. This is due to the reason described in Step 1, the heat of the first injection is attenuated to an immeasurable degree.

Step	Action
1	Click the **Remove Bad Data** button.
	The pointer becomes a cross-hair.
2	Click on the first point.
	A small red cross appears on the selected data point. The XY co-ordinates, index number, and data set name for the selected point are displayed immediately in the **Data Display Tool** (floating).
3	Press **enter**.
	The selected data point is deleted. Alternatively, after clicking on **Remove Bad Data**, double-click on a data point to delete it.

Note:

There is no undo command available with which to un-delete a data point.

It is possible to recover a mistakenly deleted data point by clicking the **Integrate All Peaks** button in the **RawITC** window. All of the injection peaks will re-integrate, and the area data, including the deleted data point, will replot in the **DeltaH** window.

Alternatively, click on the **Concentration** button and then click **OK**.

Even if the concentration has not been changed in the dialog box, Origin goes back to the worksheet and normalizes on the concentration again, which then restores the deleted point.

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7.1.1 Routine ITC data analysis

Step	Action
4	Click the **One Set of Sites** button.
	The **NonLinear Curve Fitting: Fitting Session** dialog box opens and displays the initial values for the three fitting parameters for this model - **N**, **K**, and **H**.
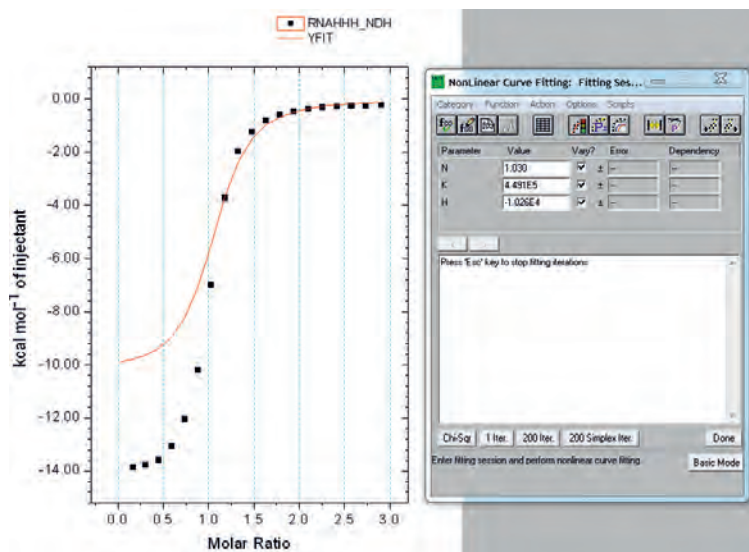
Note:

*There are two modes of the **NonLinear Curve Fitting: Fitting Session** dialog box, basic and advanced. See Section 7.6 Advanced curve fitting, on page 205, for more information.*

Origin initializes the fitting parameters, and plots an initial fit curve (as a straight line, in red)) in the *DeltaH* window. Please see *Appendix A Equations used for fitting ITC data, on page 349.*

5	Click *1 Iter.* or *200 Iter.* button in the **NonLinear Curve Fitting: Fitting Session** dialog box to control the iteration of the fitting cycles.
	1 Iter. performs a single iteration while *200 Iter.* performs up to 200 iterations.
	Note:
	*It may be necessary that the **200 Iter.** command be used more than once before a good fit is achieved.*
6	Repeat step 3 until a satisfactory fit is obtained, and Chi^2 is no longer decreasing.
	The fitting parameters in the dialog box update to reflect the current fit.

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Fitting parameter constraints

Each fitting model has a unique set of fitting parameters. For the *One Set of Sites* model, these are *N* (number of sites), *K* (binding constant in M^{-1}), and *ΔH* (heat change in cal/mole). A fourth parameter, *ΔS* (entropy change in cal/mole/deg), is calculated from *ΔH* and *K* and displayed after fitting. The *NonLinear Curve Fitting: Fitting Session* dialog box can be used to apply mathematical constraints to the fitting parameters. See *Controlling the fitting procedure, on page 209* and *Appendix A Equations used for fitting ITC data, on page 349* for more information.

Holding a parameter constant

The *Vary?* column in the *NonLinear Curve Fitting: Fitting Session* dialog box contains three checkboxes, one associated with each fitting parameter. If a box is check marked, Origin will vary that parameter during the fitting process in order to achieve a better fit. To hold a parameter constant during iterations, uncheck the checkbox.

Fitting parameters textbox

This section describes how to format and move the textbox and also how to view the results log.

Copying and pasting the fitting parameters to the *DeltaH* window

To copy and paste the fitting parameters to the *DeltaH* window, follow the steps described below:

Step	Action
1	Click on the *Done* button in the *NonLinear Curve Fitting:Fitting Session* dialog box once a good fit is achieved.

The fitting parameters will be automatically pasted into a text window named *Results Log* and to the *DeltaH* window in a text label. This label is a named object (called *Fit.P*) that is linked to the fitting process through Origin's label control feature.

```
Data: RNAHHH_NDH
Model: OneSites
Chi^2/DoF = 1.164E4
N       0.973   ±0.00346 Sites
K       8.34E5 ±3.78E4 M⁻¹
ΔH      -1.433E4        ±71.75 cal/mol
ΔS      -21.0 cal/mol/deg
```

For more information see *Origin User's Manual* or for online help, right-click anywhere in the text label, select *Label Control* and then press the **F1** key.

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7.1 Basic ITC data analysis and fitting
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Step	Action
2	Position and format this label to achieve the desirable look.
	The fitting parameters label replaces the **Fit Parameters** label, but retains its position and style when the fitting parameters are pasted. Origin will use any text label named **Fit.P** to display the fitting parameters.
3	Click on the label once to select it, select **Format:Label Control**, and enter a name in the **Object Name** text box in the **Label Control** dialog box to re-name the label.
4	Right-click anywhere in the text box and select **Properties** item from the drop-down menu.
	The **Text Control** dialog box appears, which allows formatting the fitting parameters text.
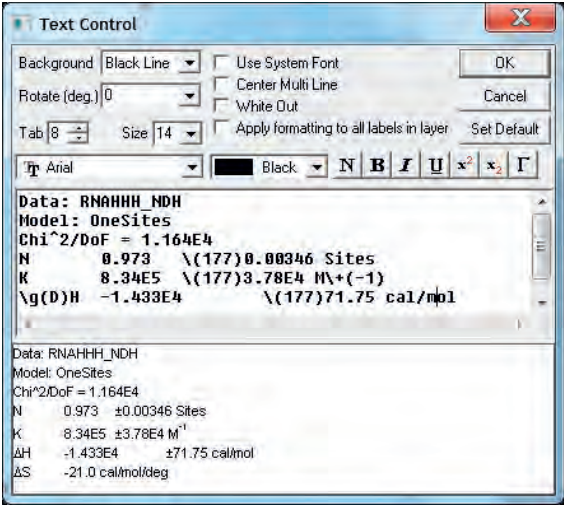
Text Control dialog box

The **Text Control** dialog box contains three sections as described below:

Section	Description
Upper section	This section contains various formatting options.
Middle section	This section contains the text box where the desired text, with formatting options, is entered.
Lower section	The lower view box provides a WYSIWYG (What You See Is What You Get) display of the text entered into the middle text box.

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7.1 Basic ITC data analysis and fitting
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Tip: *Press the **F1** key while the **Text Control** dialog box is open for online help and a thorough description of the text formatting options.*

Note: *Formatting changes can be saved as part of the **DeltaH** plot window template file. See Section 7.5 Modifying templates, on page 196 for more details.*

Viewing the *Results Log*

Introduction

Origin automatically routes most analysis and fitting results to the **Results Log** (a sub window of Origin's **Project Explorer**). In most cases, when results are output to the **Results Log**, it opens automatically (although it may be positioned out of view, docked to the lower edge of the workspace). However, it may be necessary to manually open the **Results Log** sometimes.

Opening the *Results Log* manually

To manually open (and close) the **Results Log**, navigate to **View:Results Log**.

Note: *Opening and closing the **Results Log** only controls its view state and does not result in data loss on closing it.*

Docking the *Results Log*

The **Results Log** is docked to the lower edge of the workspace. It can be docked to any other edge or displayed as a window in the workspace. To prevent the **Results Log** from docking when positioning it as a window, press **ctrl** while dragging.

Components of the *Results Log*

Each entry in the **Results Log** includes a date/time stamp, the window name, time stamp, the type of analysis performed, and the results.

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7.1.2 Creating a final figure for publication

7.1.2 Creating a final figure for publication

Introduction

This section describes how to edit or modify data to generate a final figure for publication.

Creating a final figure

To create a final figure for publication, select **Final Figure** from the **Analysis** menu.

The **ITCFINAL** plot window opens, which contains two related graphs.

Graph	Description
Top graph	The top graph displays raw data in terms of **µcal/sec** plotted against **Time (min)**, after the integration baseline has been subtracted.
Bottom graph	The bottom graph displays the normalized integration data in terms of **kcal/mole of injectant** plotted against **Molar Ratio**.

Note: *The two x-axes are linked, so that the integrated area for each peak appears directly below the corresponding peak in the raw data.*
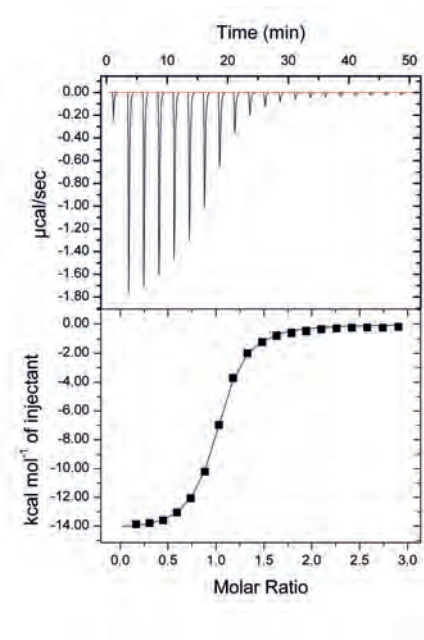
Updating the final figure

If the integration data or the fit curve in the **DeltaH** window, or the raw data in the **RawITC** window has been modified, select **Final Figure** again to update the **ITCFINAL** window with the changes.

7 Data analysis using Origin
7.1 Basic ITC data analysis and fitting
7.1.2 Creating a final figure for publication

Removing the baseline from the raw data

The top graph in the **ITCFINAL** window still includes the integration baseline at Y = 0. The baseline can be removed before printing the graph.

To remove the baseline from the raw data, follow the steps described below:

Step	Action
1	Double-click on the baseline. The **Plot Details** dialog box opens.
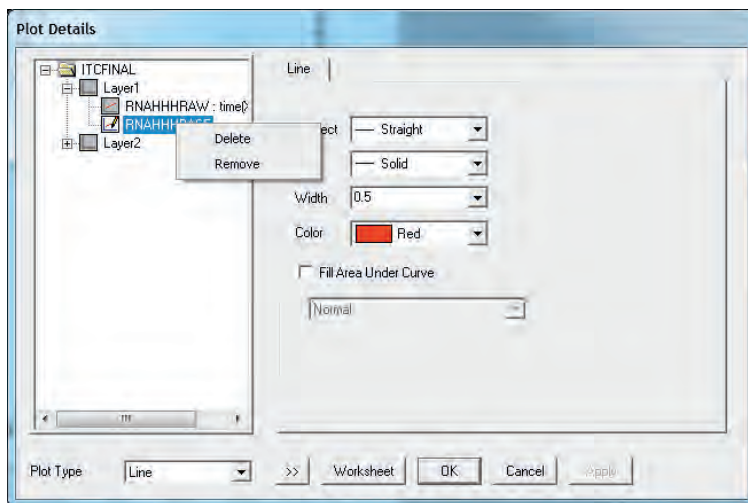
Step	Action
2	Right-click on the **RNAHHHBASE** data name in the **Layer 1** folder and click **Remove**.

The baseline data are removed from the project.

Note:

*The baseline can also be removed from the plotted data, by double-clicking on the **Layer Control** button in the upper left corner of the **ITCFINAL** window, and then moving the **RNAHHHBASE** data from the **Layer Contents** list to the **Available Data** list by first highlighting it and then selecting the left-pointing arrow.*

Pasting the fitting parameters to the ITCFINAL window

To paste the fitting parameters to the **ITCFINAL** window, follow the steps described below:

Step	Action
1	Click on the **DeltaH** window, or select **DeltaH** from the **Window** menu. **DeltaH** becomes the active window.

7 Data analysis using Origin

7.1 Basic ITC data analysis and fitting

7.1.2 Creating a final figure for publication

Step	Action
2	Click on the fitting parameters text label in the upper-left corner of the window. A colored selection square surrounds the text.
3	Select the *Edit:Copy* command.
4	Click on the *ITCFINAL* window, or select *ITCFINAL* from the *Window* menu. *ITCFINAL* becomes the active window. Click once on a position in the graph where the parameter box should appear.
5	Select the *Edit:Paste* command. The fitting parameters are pasted to the *ITCFINAL* window.
6	To position the text label next to the integration data, size of the label needs to be reduced first. Right-click inside the text box then select *Properties…* from the drop-down menu to open the *Text Control* dialog box. Select *10* (or type `10`) in the *Size* drop-down list to reduce the point size to *8*. Click *OK* to close the dialog box.
7	Click and drag the label to position it next to the integration data, as shown below.

7 Data analysis using Origin
7.1 Basic ITC data analysis and fitting
7.1.2 Creating a final figure for publication
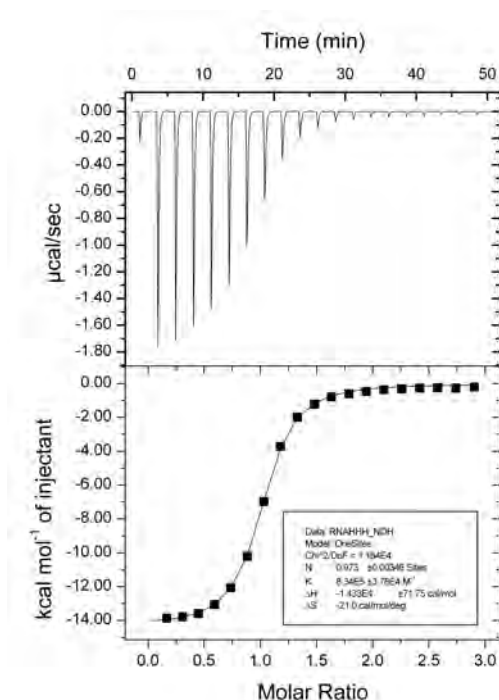
Printing the final figure

To print the page in the **ITCFINAL** window, follow the steps described below:

Step	Action
1	Ensure that **ITCFINAL** window is the active window.
2	Select **File:Print**.

Saving the project and exit

To save the project and exit, follow the steps described below:

Step	Action
1	Choose **File**: **Save Project As...**.
	The file **Save As** dialog box opens.
2	Enter a name for the project, for example `Lesson 1`, in the **File Name** text box.
	Note:
	The name for the project may contain up to 255 characters and include spaces.
3	Click the **Save** button.
	The entire contents of this project (including all data sets and plot windows) are saved into a file called **Lesson 1.OPJ**.
4	Select **File**:**Exit** to close Origin.

7.2   Adjusting baseline and integration range

Introduction

In routine data analysis, integration details (baselines and integration ranges) are determined automatically. However, sometimes the automatically determined values are not sufficiently accurate, and 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 the integration details.

Entering the *Adjust Integrations* session

To enter the **Adjust Integrations** session, follow the steps described below:

Step	Action
1	Start Origin as described in *Starting Origin, on page 144.*
2	Open the **RNAHHH.ITC** file as described in *Section 7.1 Basic ITC data analysis and fitting, on page 144.* Raw data are plotted in the **RawITC** window. Normalized area data are plotted in the **DeltaH** window.
3	Navigate to **Window:File:RawITC**. **Note:** *The **RawITC** window or any active window can lose its formatting instructions if in **Draft View** mode. Change to **Page View** mode to restore the formatting instructions.*
4	Click the **Adjust Integrations** button in the **RawITC** window. The cursor changes into a cross hair.

Step	Action
5	Move the cursor into the **RawITC** plot window and click on or near the injection peak to be adjusted.
	For example, click on peak **19** (second peak from the right). The window zooms in on the baseline region of peaks **18**, **19** and **20**.
	Note:
	Origin will show the injection peak before and after the chosen injection, but any manipulations will only affect the integrated area between the center injection.
	A new set of buttons appears along the top edge of the window. Also, Two blue lines appear denoting the integration range.

Adjusting the integration details

The baseline adjustment tool displays the raw data as a black line, the fitted baseline as a red line, and two vertical blue lines. The leftmost blue line denotes the start of the region to be integrated. The rightmost blue line denotes the end of the region to be integrated. Everything to the right of the rightmost blue line is defined as baseline. Each injection has a range defined as baseline. These regions are fit, as a whole, to yield the red baseline. Moving the rightmost blue line affects how well the baseline fits the raw data, and/or which data are integrated as heat.

To adjust integration details, follow these steps, which will also be described later in detail below (see *Adjusting the baseline, on page 163*):

Note: *Some steps negate the other steps, so the order in which these steps are followed is important.*

Step	Action
1	Adjust all the baseline ranges of applicable peaks using the vertical blue lines.
2	Click the **Recalculate Baseline** button.
3	Adjust all the integration ranges of applicable peaks using the vertical blue lines, again.
	Note:
	*Do not click the **Recalculate Baseline** button.*
	Also, move individual baseline points, if necessary, after selecting an injection peak by clicking the **Move Baseline Points** button.
4	Quit the session and click the **Integrate All Peaks** button.

The figure below shows the expanded view of the screen:
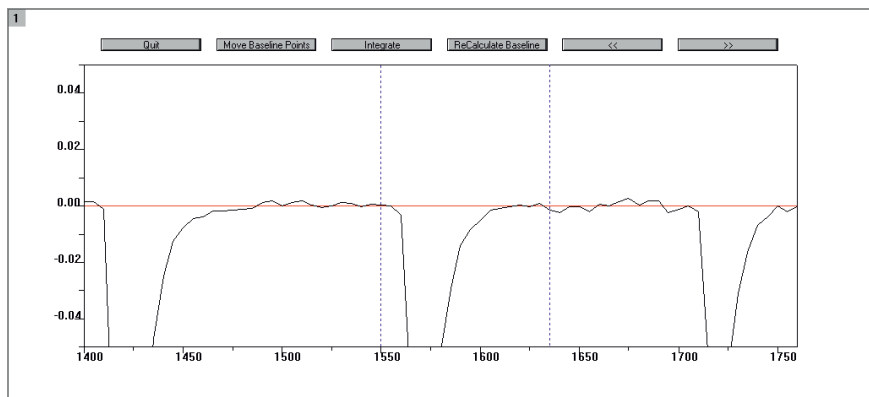
Integrating the selected peak

To integrate the selected peak, click on the **Integrate** button.

This integrates the peak, using the current baseline and integration range. The curve in the **DeltaH** window is updated accordingly. The integration results are also updated on the worksheet containing the injection data.

Selecting another peak

To select another peak, click on the `<<` and `>>` buttons to move to the previous or next peak respectively.

Note: *The current peak number is always displayed in the window title bar.*

Ending the *Adjust Integrations* session

To end the **Adjust Integrations** session, click the **Quit** button.

The **RawITC** window is restored to show all of the injection peaks. The area data in the **DeltaH** window is also updated to reflect any changes that have been made.

Integrating all peaks

Click the **Integrate All Peaks button** in the **RawITC** template to subtract the current baseline, integrate all injection peaks and replot the area data.

Note: *Clicking the **Auto Baseline** button will revert to the original baseline, negating any baseline adjustments.*

Adjusting the baseline

Altering the baseline range

To alter the baseline range, follow the steps described below:

Step	Action
1	Select a peak.
	The vertical blue lines only apply to one injection. The region between the blue lines is integrated. The remaining portion of the injection, to the right of the rightmost blue line is defined as a baseline. Because the red, fitted baseline is fit to the baseline region of each injection, the fit can be defined by simply dragging the rightmost blue line to either the right or the left. For example, if an experimental artifact (i.e., bubble spike) is confined to the baseline region, the baseline fit may be adversely affected. Reposition the rightmost blue line to the right of the spike.
2	Click the **Recalculate Baseline** button to improve the baseline fit.
	The spike will now be integrated, but that will taken care of in a later step.
	Note:
	*It is recommended that these baseline range adjustments be completed before moving on, because clicking the **Recalculate Baseline** button will negate the later steps.*

Altering the integration range

Note: *Altering the integration range and making fine adjustments using the **Move Baseline Points** button may be done at the same time. However, clicking the **Recalculate Baseline** button negates these steps.*

To alter the integration range, follow the steps described below:

Step	Action
1	Move the rightmost blue line so that it includes only what is to be integrated. For example, as described in step 2 above, to avoid integrating the bubble spike, simply reposition the blue line to the left of the spike.
2	Click the **Integrate** button. This depicts the integrated region.

Step	Action
3	Repeat for all applicable injections.

Making fine adjustments to the baseline

To make finer adjustments to the baseline, follow the steps described below:

Step	Action
1	Click the **Move Baseline Points** button in the **Adjust - Peak 11** window.
	The automatically generated points for this baseline are displayed.
	For the baseline, Origin displays 15 points, which include the central peak and each neighboring peak. In most cases adjusting only the central five points for the central peak of interest is sufficient. The outermost points are usually more closely associated with the neighboring peaks.
2	Click on a point, then drag the mouse or use the ↑ and ↓ keys to move the point (note that baseline points can only move vertically). Use the ← and → keys (or the mouse) to select the next point to the right or left. Repeat for each point to be moved.
	Note:
	When any point on the baseline is moved, the position of the moved point automatically becomes part of the baseline and any future integration will be calculated from this new baseline.
3	Press the **Esc** (escape) key (or the **enter** key) to set the baseline.
	The data points will disappear and the cursor will change from the cross hair to the pointer tool so that the integration range can be adjusted.
	If the integration range is already set, click the **Integrate** button and click on an arrow key to show an adjacent peak.
	Note:
	*Clicking the **Recalculate Baseline** button after making fine adjustments will negate the fine adjustments and will simply refit according to the position of the vertical blue lines.*

Expanding the baseline portion

To expand the flat baseline portion of the data, follow the steps described below:

Step	Action
1	Click on the magnifying glass icon in the **Toolbox**. Drag to zoom to a user-specified region.

Step	Action
2	Double-click on the magnifying glass icon to return to the original non-expanded display or proceed to integrate the next peak.
3	Double-click on the y-axis to bring up the **Y Axis** dialog box to maintain the same expanded y-axis limits for integrating other peaks.
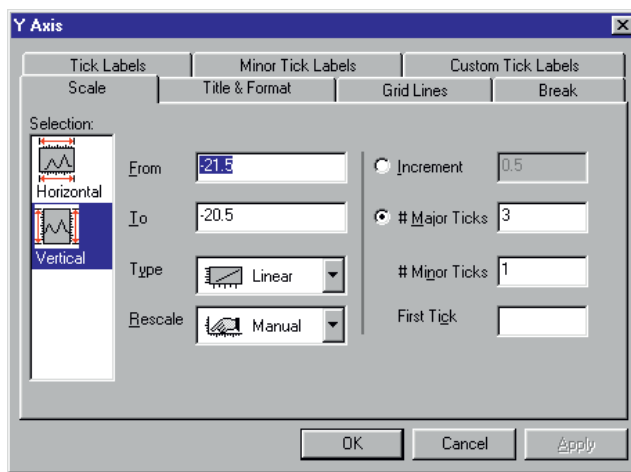
4	Click on the **Scale** tab in the lower left corner of the dialog box, change the **Rescale** option from **Normal** to **Manual** and click **OK**.
	The y-axis maintains these limits and does not rescale when adjusting integration for other peaks.

Viewing the worksheet data

To view the worksheet data, follow the steps described below:

Step	Action
1	Select the pointer tool by clicking on it in the toolbox.

Step	Action
2	Double-click anywhere on the trace of the **RawITC** data plot in the plot window or select **Format:Plot**. The **Plot Details** dialog box opens for this data plot.
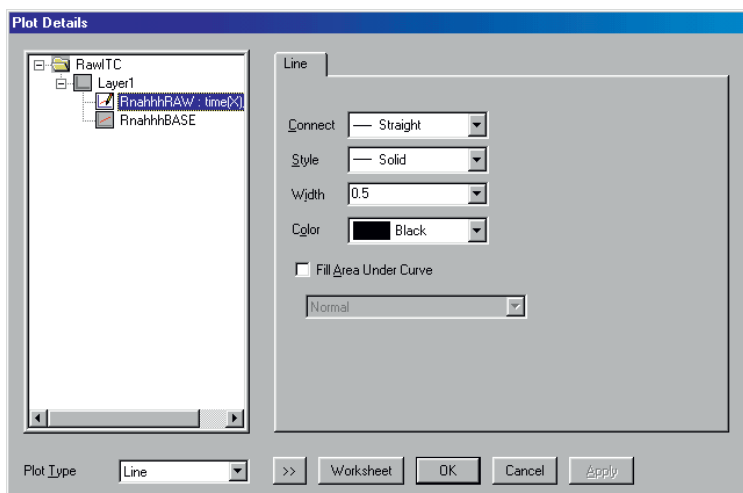
3	Click on the **Worksheet** button. The worksheet containing the injection data opens. ***Note:*** *The worksheet x-axis values are in seconds, while the plotted data is shown in minutes. This is because the x-axis has been factored, as described in Section 7.5 Modifying templates, on page 196.*

After adjusting integrations

After adjusting the integrations, proceed to fit the data as described in *Section 7.1 Basic ITC data analysis and fitting, on page 144*.

7.3   Analyzing multiple runs and subtracting reference

Introduction

Origin allows multiple runs of ITC data to be opened into the same project. The heat of ligand dilution into buffer can thus be subtracted from the reaction heat by performing the control experiment and subtracting this reference data from the reaction heat data.

In order to subtract the reference injections, both the sample and reference area data should be available in the controller memory. This section describes how to read two data files into Origin and to subtract one from the other. It also illustrates some helpful procedures for dealing with difficult data.

Note: *Before beginning this section, open a new project by selecting* **File:New:Project**, *to clear any old data that may be in memory.*

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.1 Opening multiple data files

7.3.1 Opening multiple data files

Introduction

In the following example, two area (*.itc*) data files will be opened and subtracted from one another.

Reading sample data into memory

To read the sample data into the controller memory, follow the steps described below:

Step	Action
1	Click on the **Read Data...** button in the **RawITC** plot window.
	The **Import Multiple ASCII** dialog box opens.
2	Click on the down arrow in the **Files of type:** drop-down list box and select **ITC Data (*.it?)**.
3	Navigate to `C:\Origin70\Samples`.

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.1 Opening multiple data files
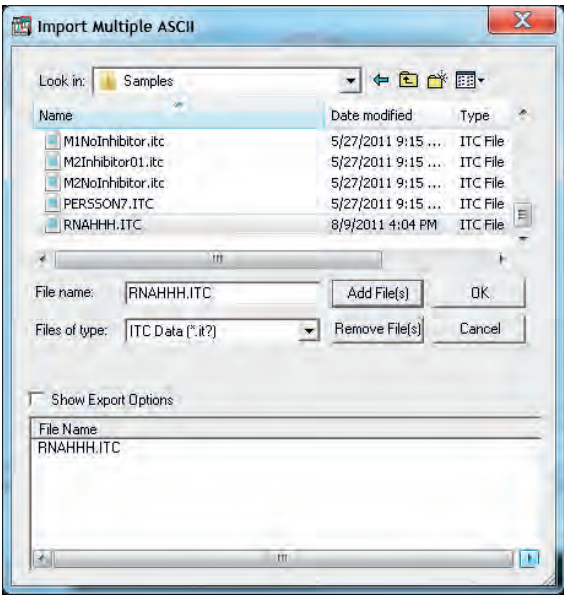
Step	Action
4	Double-click on **RNAHHH.ITC**. Alternatively, single click on **RNAHHH.ITC** and click the **Add File(s)** button. Click **OK**.
	The RNAHHH.ITC file opens and the data are normalized on concentration. This data is then plotted in the **DeltaH** window, as a scatter plot called **RNAHHH_NDH**. **RNAHHH_NDH** shows area data as kcal/mole of injectant plotted against Molar Ratio. Remove the first data point by clicking on the **Remove Bad Data** button, selecting the first data point, and pressing **enter**.
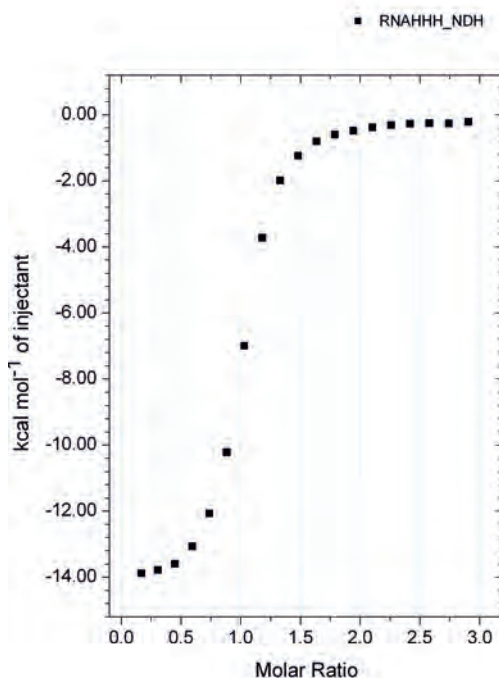
7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.1 Opening multiple data files

Step	Action
5	Return to the *RawITC* template and repeat the above steps to open the reference data file *BUFFER.ITC*. *BUFFER.ITC* is also located in the *Samples* subfolder. A new plot, *BUFFER_NDH*, replaces *RNAHHH_NDH* in the *DeltaH* window.
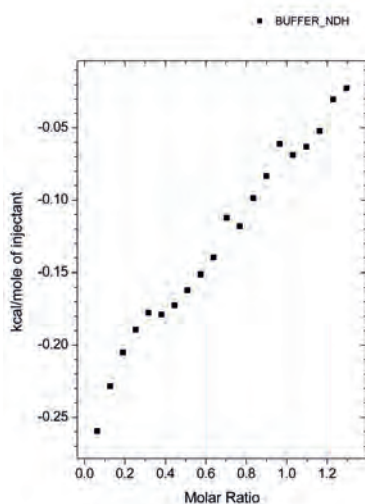
Note:

When the second ITC data file BUFFER_NDH is opened, the RNAHHH_NDH data are cleared from the DeltaH plot window. The RNAHHH_NDH data are not deleted from the project, but are simply removed from the window display.

Showing both the sample and reference area data

To show both the sample and the reference area data, follow the steps described below:

Step	Action
1	Double-click on the layer 1 icon 1 , at the top left corner of the *DeltaH* window. The *Layer Control* dialog box opens.

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.1 Opening multiple data files

Step	Action
2	Click on **rnahhh_ndh** in the **Available Data** list, then click on the **=>** button.

rnahhh_ndh is copied to the **Layer Contents** list. Be sure to have **Rescale on OK** checked.

Note:
It is not checked by default.

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.1 Opening multiple data files
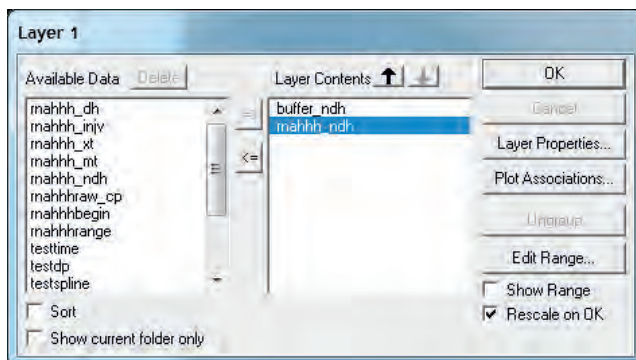
Step	Action
3	Click **OK**.

RNAHHH_NDH plots into the **DeltaH** window. The axes automatically rescale to show all of the data.
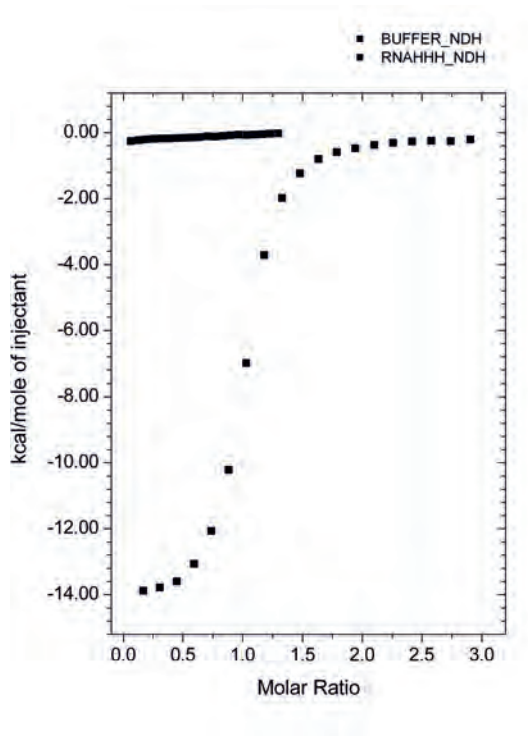
Note:

*The **Available Data** list in the **Layer Control** dialog box shows all data sets currently available for plotting in this project. The **Layer Contents** list shows all data sets currently plotted in the active layer. See Origin User's Manual or Origin's online **Help** menu item (or press **F1**) for more information on handling Origin data.*

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.1 Opening multiple data files

Editing data files

Any number of data files can be read into the same *DeltaH* window. When multiple data plots appear in the same window, the active data plot can be set by clicking on the plot

■ BUFFER_NDH
■ RNAHHH_NDH

type (line/symbol) icons next to the data set name in the legend:

A red border around the line/symbol icon indicates the currently active data plot. Editing, fitting, and other operations can only be carried out on the active plot.

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.2 Adjusting the molar ratio

7.3.2   Adjusting the molar ratio

Introduction

Origin automatically assigns a concentration of 1.0 mM in order to obtain non-infinite values for the molar ratio to allow plotting of the **BUFFER_NDH** points. Before subtracting the reference data, check that the molar ratio is identical for both data sets. This will ensure that the final result is accurate, and that the two data sets plot in register (that is, injection #1 of the control experiment plots at the same molar ratio as injection #1 of the sample experiment, and so on).

Note: *The **BUFFER_NDH** data plots from molar ratio 0 to ca. 1.3, while the **RNAH-HH_NDH** data plots from 0 to ca. 2.0. In the case of the **BUFFER_NDH** data, the molar ratio is, in fact, infinity since injections of 21.16 mM ligand solution were made into a cell, which contained only buffer and no macromolecule (i.e., in order to determine heats of dilution of ligand into buffer).*

Adjusting the molar ratio

To adjust the molar ratio, follow the steps described below:

Step	Action
1	Click on the **Data** menu, and check that **RNAHHH: XMt(X), NDH(Y)** is check-marked. If not, select **RNAHHH: XMt(X), NDH(Y)** from the menu.
	Alternatively, click on the **RNAHHH_NDH** listing in the plot type icon in the legend.
	RNAHHH is set as the active data set.
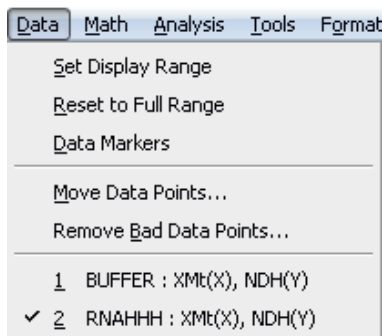
2	Click the **Concentration** button in the **DeltaH** window.
	Note the value in the **C in Cell (mM)** field (it should be 0.057) in the dialog box that opens.

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.2 Adjusting the molar ratio

Step	Action
3	Click **Cancel** to close the dialog box.
4	Repeat step 1, but this time set the **Buffer: XMt(X), NDH(Y)** data set as active.
5	In the **DeltaH** window, click the **Concentration** button again. A dialog box displays the concentration values for buffer.
6	Enter `0.057` in the **C in Cell (mM)** field.
7	Click **OK**. The two data sets will now plot in register, as shown below:

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.3 Subtracting reference data
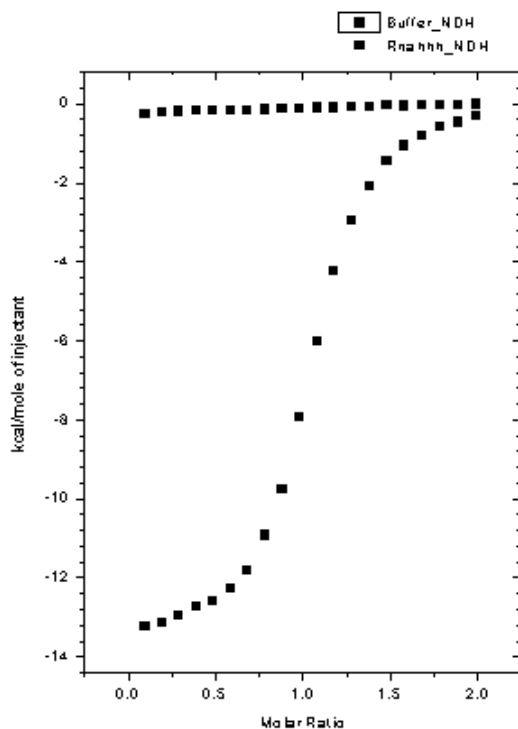
7.3.3   Subtracting reference data

Subtracting reference data

There are several ways to subtract the control heat of **BUFFER_NDH** from that of **RNAHHH_NDH**.

Note: *Select the method that best suits the application.*

Option1: Subtracting a constant

To subtract a constant from **RNAHHH_NDH**, for example, the mean value of **BUFFER_NDH**, follow the steps described below:

Step	Action
1	Open the area data worksheet by double-clicking on any data point of the **BUFFER.ITC** data set while in the **DeltaH** window. This opens the **Plot Details** window. Click the **Worksheet** button.
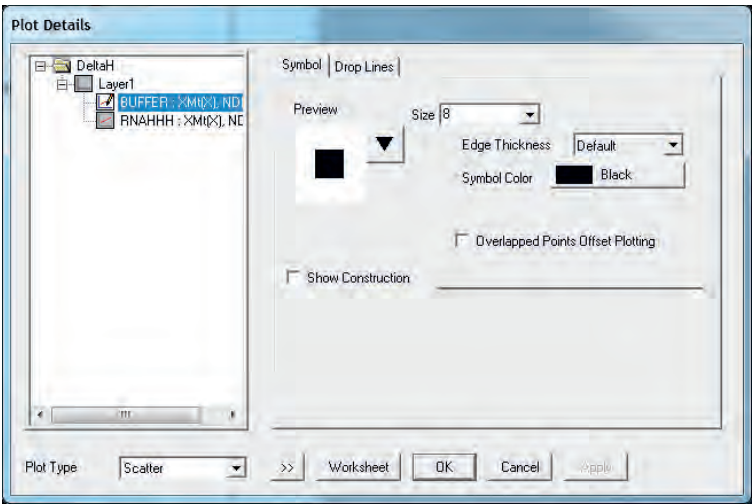
2	Select the **NDH** column by clicking on the column heading.
	All the cells of the column will be highlighted.

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.3 Subtracting reference data

Step	Action

3

Navigate to the **Statistics on Column(s)** button .
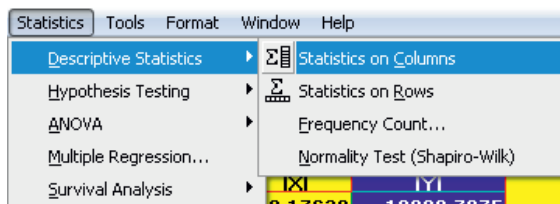
A new worksheet appears with the mean, standard deviation, standard error of the mean, the sum of the data and the number of data points of the **NDH** dataset column.

sd(yEr±)	se(yEr±)	Min(Y)	Imin(Y)	Max(Y)	Imax(Y)	Range(Y)	Sum(Y)	N(Y)
42.75861	12.34335	277.41502	10	428.41935	8	151.00433	4217.97962	

4

Select **Math:Simple Math**.

The **Math on/between Data Set** dialog box opens.

5

Select **RNAHHH_NDH** from the **Available Data** list, then click on the upper-most => button.

RNAHHH_NDH is copied to the **Y1** text box. **RNAHHH_NDH** also appears next to **Y:. Y:** indicates the name of the data set into which the resulting data will be copied.

6

Click in the **operator** box, and type – in the text box.
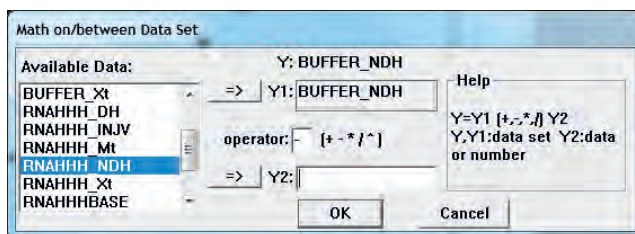
7

Click in the **Y2** text box and enter the mean value from step 3 above, at the insertion point.

8

Click **OK**.

The constant is subtracted from each value in the **RNAHHH_NDH** data set. The result is plotted as **RNAHHH_NDH** in the **DeltaH** window.

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.3 Subtracting reference data

Option2: Fitting a constant

This option does not utilize an actual control dataset. Instead, during the fitting process described in *Section 7.1 Basic ITC data analysis and fitting, on page 144*, navigate to **Math**:**Simple Math** and subtract constants from **RNAHHH.NDH** until the discrepancy between the fitted model and the dataset is minimized.

This manual, iterative process is similar to the automated control subtraction procedure in the MicroCal Auto-iTC$_{200}$ software control software.

To fit a constant, follow the steps described below:

Step	Action
1	Once the data set to be fit is active, click the **One Set of Sites** button. Click **200 Iter.** until the desirable solution is obtained.
2	Select **Math: Simple Math** from the menu bar.
3	Subtract (or add) a constant as described above in Option 1.
4	Click the **200 Iter.** button again and observe whether the Chi-sqr increases or decreases.
5	Repeat step 2 until the Chi-sqr is adequately minimized.

Option3: Subtracting a line

To subtract a straight line from **RNAHHH_NDH**, follow the steps described below:

Step	Action
1	Click on the pointer tool to deselect the screen reader tool.
2	Check the **Data** menu to see that **BUFFER : XMt(X), NDH(Y)** is the active data set (the active data set will be checkmarked). All editing, and fitting operations are carried out on the active data set. Select **BUFFER : XMt(X), NDH(Y)** if it is not active.

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.3 Subtracting reference data
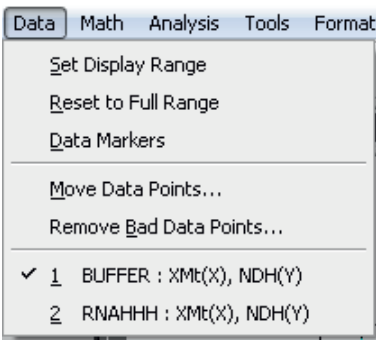
Step	Action
3	Select **Linear Regression** from the **Math** menu.

A straight line is fit to the **Buffer** data. Origin assigns the name **LinearFit1_bufferNDH** to the data set for this line.
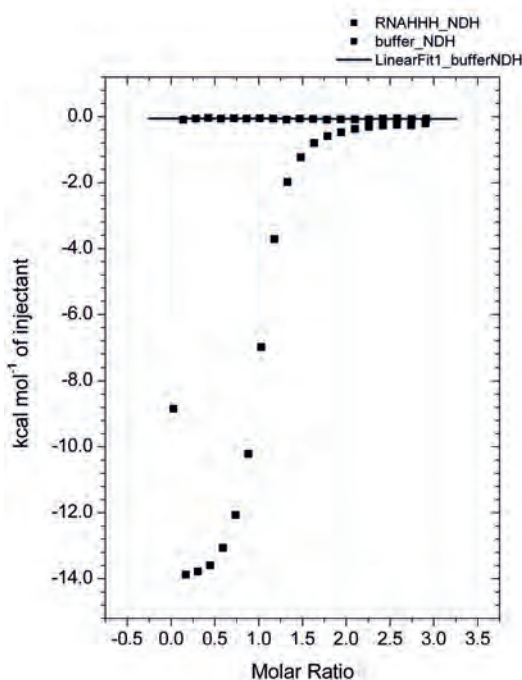
| 4 | Select **Simple Math** from the **Math** menu. |

The **Math** dialog box opens.

| 5 | Select **RNAHHH_NDH** from the **Available Data** list, then click on the uppermost => button. |

RNAHHH_NDH is copied to the **Y1** text box.

| 6 | Select **LinearFit1_bufferNDH** from the **Available Data** list, then click on the lowermost => button. |

LinearFit1_bufferNDH is copied to the **Y2** text box.

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.3 Subtracting reference data

Step	Action
7	Click in the **operator** box and type –.
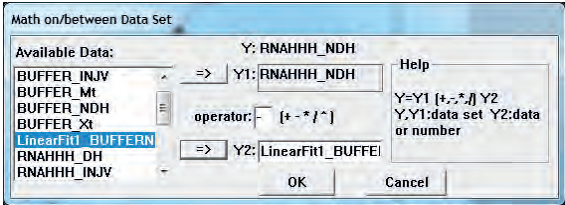
Step	Action
8	Click **OK**.

Every point in **LinearFit1_bufferNDH** is subtracted from the corresponding point in **RNAHHH_NDH**. The resulting data set is plotted as **RNAHHH_NDH** in the **DeltaH** plot window.

Note:

*The **BUFFER_NDH** reference data plot (the original twelve injection points) is not affected.*

Tip: *To make the difference in injection time spacing between **RNAHHHRAW_CP** and **BUFFERRAW_CP** more apparent, plot both raw data sets in the same plot window.*

Option 4: Point-by-point

Step	Action
1	Click on the **Subtract Reference Data..** button in the **DeltaH** window.

The **Subtract Reference Data** dialog box opens. The most recent file opened, in this case **Buffer_NDH**, appears in both the **Data** and **Reference** drop-down list box.

Note:

*The data set in the **Reference** box is subtracted from the data set in the **Data** box.*

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.3 Subtracting reference data

Step	Action
2	Select **Rnahhh_NDH** from the **Data** drop down list.
	Rnahhh_NDH is highlighted and entered as the data.
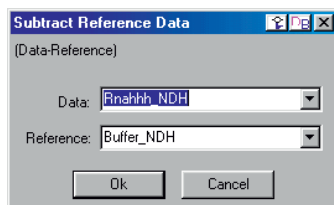
Step	Action
3	Click **OK**.
	Every point in **Buffer_NDH** is subtracted from the corresponding point in **Rnahhh_NDH**. The result is plotted as **Rnahhh_NDH** in the active layer, in this case layer 1, in the **DeltaH** plot window.
	Note:
	*BUFFER_NDH is not affected by this operation. It is cleared from the **DeltaH** window, but is still listed as available data in the **Layer Control** dialog box. The original **RNAHHH_NDH** data could be recovered by selecting **Math : Simple Math** and adding the **BUFFER_NDH** data set to the new **RNAH-HH_NDH** data set.*

Saving the project and all related data files

To save the project and all related data files, follow the steps described below:

Step	Action
1	Select the **File:Save Project As** command from the Origin menu bar.
	The **Save As** dialog box opens, with **untitled** selected as the file name.

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.3 Subtracting reference data

Step	Action
2	Enter a new name for the project, navigate to the folder in which to save the file, and click **OK**.

Tip:

Origin7.0 accepts long file names.

Note:

It is not necessary to enter the `.opj` *file extension. This will be added automatically. The file can now be accessed by selecting* **File:Save Project** *command.*

Tip:

Delete the original injection data to save some memory space. This may be useful when reading a large number of data sets into the same Origin project.

Deleting a data set from a project

To delete a data set from a project, follow the steps described below:

Step	Action
1	Double-click on any layer icon ![1] in the plot window .
2	Select a data set from the **Available Data** list, then click the **Delete** button.

Alternatively, a data set from a project can also be deleted as described below:

Step	Action
1	Double-click on the trace of the data plot to be deleted in the plot window. The **Plot Details** dialog box opens. The name of the data set appears in the **File List** box under the layer icon.
2	Right-click on the file name to be deleted and then click **Delete**.
Note:	*In either case, the data set along with any related data plots is deleted from the project. However, any data set saved to disk will not be affected.*

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.3 Subtracting reference data

Displacing overlapping data sets

Whenever multiple data sets are included in the same plot, data points from the different data sets may overlap. There are two ways to eliminate this overlap by displacing one or more of the curves on the y-axis.

Displacing data sets

A data set can be displaced by selecting **Math:Simple Math** and adding or subtracting a constant from all points in one data set to displace it.

Alternatively, a data set can be displaced by following the steps described below:

Step	Action
1	Make the appropriate data set active by selecting it in the list for plot type icons.
2	Select **Math:Y Translate**.
3	Use the resulting cross-hair icon to select one data point in the active set, click on it, and press **enter** (or double-click on a data point).
4	Move the icon to the **Y position** on the graph where the point should be after displacement, click on it and press **enter**.
	The entire data set will be translated on the y-axis by that amount.

Plotting both *rnahhhraw_cp* and *bufferraw_cp* in the *RawITC* plot window

To plot both **rnahhhraw_cp** and **bufferraw_cp** in the **RawITC** plot window, follow the steps described below:

Step	Action
1	Click on the **RawITC** window to make it active (or select **RawITC** from the **Window** menu).
2	Double-click on the layer 1 icon ⬚**1** in the **RawITC** window.
	The **Layer 1** dialog box opens.

7 Data analysis using Origin
7.3 Analyzing multiple runs and subtracting reference
7.3.3 Subtracting reference data

Step	Action
3	Select **rnahhhraw_cp** in the **Available Data** list, then click the => button.
	rnahhhraw_cp is added to the **Layer Contents** list.
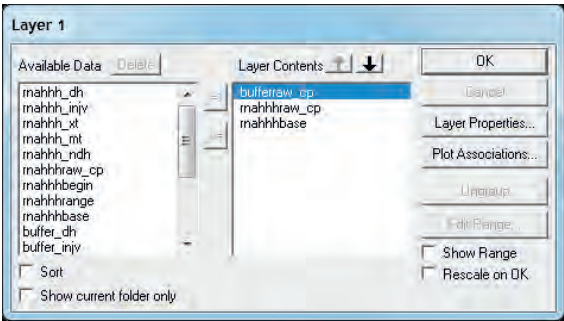
Step	Action
4	Click **OK**.
	Both RNAHHHRAW_cp and bufferRAW_cp are now plotted in the **RawITC** window. Note the difference in the time spacing of the injections.
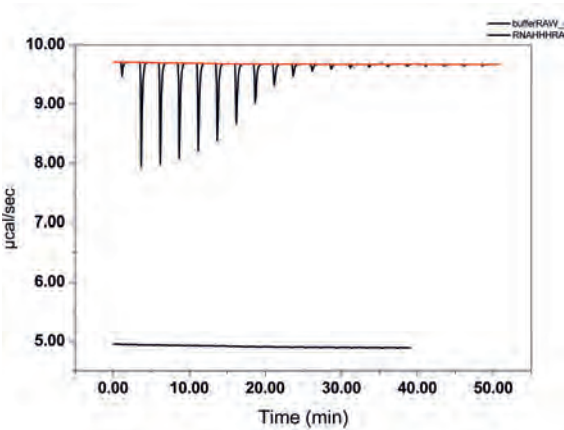
Note:

The difference in peak spacing is not a problem when subtracting reference data. Data files having different time spacing can still be accessed, since only the integration area data for each peak is important.

7.4   ITC data handling

Introduction

Every data plot in Origin has an associated worksheet. The worksheet contains the X, Y and, if appropriate, the error bar values for the plot. A worksheet can contain values for more than one data plot.

It is always possible to view the worksheet from which data were plotted. This section describes how to open the worksheet associated with a particular data plot, copy/paste the data, export the data to an ASCII file, and import ASCII data.

7 Data analysis using Origin
7.4 ITC data handling
7.4.1 Reading worksheet values from plotted data

7.4.1 Reading worksheet values from plotted data

To read worksheet values from plotted data, follow the steps described below:

Step	Action
1	Select **File:New:Project**. A new Origin project opens to display the **RawITC** plot window.
2	Click on the **Read Data...** button. The **File Open** dialog box opens, with the **ITC Data (*.it?)** file name extension selected. **Note:** *If a default folder has not been set previously, navigate to the* `C:\Origin70\Samples` *folder.*
3	Select **Rnahhh** from the file name list, and click **OK**. Origin plots the **Rnahhh** data as a line graph in the **RawITC** plot window, automatically creates a baseline, integrates the peaks, normalizes the integration data, and plots the normalized data in the **DeltaH** plot window. As a result, the eight data sets are created.

Data sets

The eight data sets created by Origin are described below:

Data set	Description
Rnahhh_DH	Experimental heat change resulting from injection i, in µcal/injection (not displayed).
Rnahhh_MT	Concentration of macromolecule in the cell before each injection i, after correction for volume displacement (not displayed).
Rnahhh_XT	Concentration of injected solute in the cell before each injection (not displayed).
Rnahhh_INJV	Volume of injectant added for the injection i.
Rnahhh_NDH	Normalized heat change for injection i, in calories per mole of injectant added (displayed in **DeltaH** window).
Rnahhh_XMT	Molar ratio of ligand to macromolecule after injection i (X value of data point).

7 Data analysis using Origin
7.4 ITC data handling
7.4.1 Reading worksheet values from plotted data

Data set	Description
Rnahhhbase	Baseline for the injection data (displayed in red in the *RawITC* window).
Rnahhhraw_CP	All of the original injection data (displayed in black in the *RawITC* window).

Note: *An Origin data set is named after its worksheet and worksheet column, usually separated by an underscore. Thus, the first six data sets above will all be found on the same worksheet (RNAHHH), in columns named DH, INJV, Xt, Mt, XMt and NDH, respectively.*

Temporary data sets

In addition to the above eight data sets, Origin also creates the following two temporary data sets:

Temporary data set	Description
Rnahhhbegin	Contains the indices (row numbers) of the start of an injection.
Rnahhhrange	Contains the indices of the integration range for the injections.

Note: *The two temporary data sets are located on separate worksheets, named rnahhhbase (an Origin created baseline) and RnahhhRAW (the experimental data). The temporary data sets are indices created by Origin and do not have a worksheet created.*

Saving area data to a separate file

To save area data to a separate file, follow the steps described below:

Step	Action
1	Select *Window:DeltaH*. Alternatively, press and hold the **ctrl** key and press the **tab** key to scroll through Origin's open windows, until *DeltaH* window is active.

7 Data analysis using Origin
7.4 ITC data handling
7.4.1 Reading worksheet values from plotted data

Step	Action
2	Click the **Save Area Data** button located in the **Data Control** box to the left of the graph.
	Origin opens the **File Save As** dialog box, with **Rnahhh.DH** selected in the **File name** text box.
3	Select a folder for the file and click **OK**.

Opening the *RNAHHH* worksheet

To open the **RNAHHH** worksheet, follow the steps described below:

Step	Action
1	Select the **Plot...** command from the **Format** menu.
	The **Plot Details** dialog box opens for the **RNAHHH_NDH** data plot (if the **DeltaH** window is active).

7 Data analysis using Origin
7.4 ITC data handling
7.4.1 Reading worksheet values from plotted data
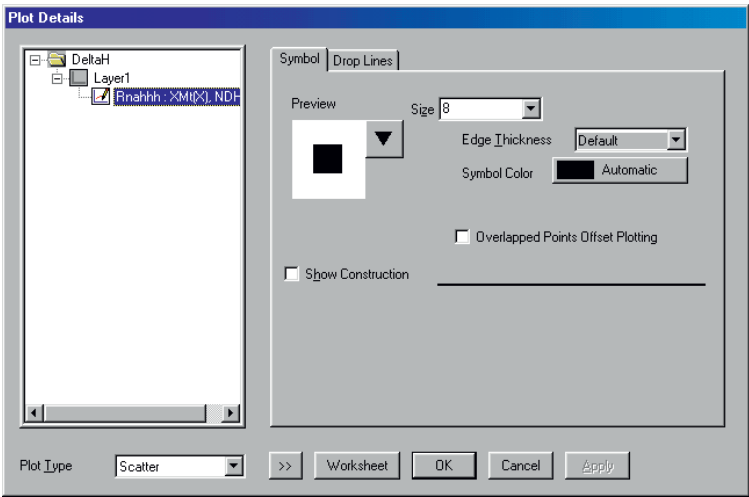
Step	Action
2	Click on the **Worksheet** button.

The **RNAHHH** worksheet opens.

Note:

*If a worksheet cell is not wide enough to display the entire number, Origin fills the cell with ###### signs. To view the full number, increase the column width, by placing the cursor on the left or right border of the column name, waiting till the cursor changes to a double headed arrow, then moving the column edge to the right to increase the column width. Alternatively, right-click the column name select **Properties** from the drop-down list and increase the value in the* **Column Width** *text box.*

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	DH (Y)	INJV (Y)	Xt (Y)	Mt (Y)	XMt (X)	NDH (Y)
1	-2.81295	0.4	0	0.057	0.02766	-8781.48457
2	-22.06078	2	0.00157	0.05689	0.16678	-13810.23052
3	-21.9174	2	0.00939	0.05633	0.30727	-13719.83842
4	-21.61066	2	0.01714	0.05577	0.44912	-13526.70782
5	-20.77464	2	0.0248	0.05522	0.59235	-13000.69854
6	-19.1915	2	0.03239	0.05468	0.73694	-12004.79162
7	-16.23101	2	0.0399	0.05414	0.8829	-10142.63231
8	-11.10665	2	0.04733	0.0536	1.03023	-6919.54202
9	-5.90742	2	0.05468	0.05307	1.17893	-3649.36542
10	-3.15596	2	0.06195	0.05255	1.329	-1918.66421
11	-1.9587	2	0.06915	0.05203	1.48044	-1165.43904
12	-1.27078	2	0.07627	0.05152	1.63324	-732.55785
13	-0.9473	2	0.08331	0.05101	1.78742	-528.8822
14	-0.75312	2	0.09027	0.0505	1.94296	-406.52075
15	-0.60205	2	0.09716	0.05	2.09987	-311.27525
16	-0.48763	2	0.10396	0.04951	2.25815	-239.07266
17	-0.42211	2	0.11069	0.04902	2.4178	-197.62411
18	-0.396	2	0.11734	0.04853	2.57882	-180.96459
19	-0.41661	2	0.12391	0.04805	2.7412	-193.68106
20	-0.32861	2	0.13041	0.04757	2.90496	-138.09007
21		—	0.13682	0.0471		—
22						

7 Data analysis using Origin
7.4 ITC data handling
7.4.2 Copy and paste worksheet data

7.4.2   Copy and paste worksheet data

Introduction

Data can be copied from a worksheet to the clipboard, then pasted from the clipboard into another Origin worksheet, a plot window, or another Windows-based application.

Selecting a range of worksheet values

To select a range of worksheet values, follow the steps described below:

Selection object	Action
A cell	Click on the cell.
An entire row	Click on the row number.
An entire column	Click on the column heading.
A contiguous portion of worksheet values	Click on the first cell, row or column, keep the mouse button depressed, drag to the final cell, row, or column that should be included in the selection range, then release the mouse button. *Note:* *To select a range of cells where the initial cell but not the final cell is in view, click on the first cell and scroll to the final cell, press and hold the **shift** key and then click the final cell.*

Copying the selected values to the clipboard

To copy the selected values to the clipboard, select **Copy** from the **Edit** menu.

Alternatively, right-click inside the highlighted text and select **Copy** from the menu.

7 Data analysis using Origin
7.4 ITC data handling
7.4.2 Copy and paste worksheet data

Selecting a destination for the copied values

To select a destination for the copied values, follow one of the steps described below:

Destination	Action
A plot window	Click on the plot window to make it active.
A worksheet	Click on the worksheet (or select **File:New:Worksheet** to open a new worksheet), then click to select a single cell. This cell will be in the upper left corner of the destination range.
Another Windows-based application	Switch to the target application, then follow the pasting procedure for that application.

Pasting the copied values from the clipboard to the destination

To paste the copied values from the clipboard to the destination, select **Paste** from the **Edit** menu.

Alternatively, right-click and select **Paste**.

	A(X)	B(Y)
1	0.09681	#########
2	0.19391	#########
3	0.29129	-12967.3443
4	0.38896	#########
5	0.48692	#########
6	0.58517	#########
7	0.6837	#########
8	0.78252	#########
9	0.88163	-9748.91693
10	0.98103	-7922.79781
11	1.08072	-6013.36964
12	1.18069	-4256.64162

Note: *It may happen that the worksheet does not show the data, but only displays pound signs. The data is available for manipulations but is not displayed because the column is not wide enough. Increase the column width by placing the cursor at the right edge of the column header (the cursor changes into a double headed arrow) then clicking and dragging the cursor to the right. Alternatively right-click the column heading, select **Properties**, then increase the number for the column width.*

7 Data analysis using Origin
7.4 ITC data handling
7.4.3 Exporting worksheet data

7.4.3   Exporting worksheet data

Introduction

The contents of any worksheet can be saved into an ASCII file. This section describes how to open the worksheet for the **RnahhhBASE** baseline data plotted in the **RawITC** window, and export the X and Y data to an ASCII file.

Opening the *RnahhhBASE* worksheet

To open the **RnahhhBASE** worksheet, follow the steps described below:

Step	Action
1	Click on the **RawITC** window (or choose **RawITC** from the **Window** menu) to make it the active window.
2	Select **2 RnahhhBASE** from the **Data** menu.
	2 RnahhhBASE is checkmarked to show it is selected.
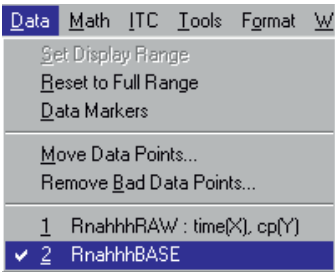
Step	Action
3	Select **Plot...** from the **Format** menu.
	The **Plot Details** dialog box opens.

7 Data analysis using Origin
7.4 ITC data handling
7.4.3 Exporting worksheet data

Step	Action
4	Click the **Worksheet** button.
	The **RnahhhBASE** worksheet opens.

7 Data analysis using Origin
7.4 ITC data handling
7.4.3 Exporting worksheet data

Exporting the worksheet data as an ASCII file

To export the worksheet data as an ASCII file, follow the steps described below:

Step	Action
1	Select **Export ASCII...** from the **File** menu.

The **Export ASCII** dialog box opens, with **RnahhhBASE.DAT** selected as the file name.
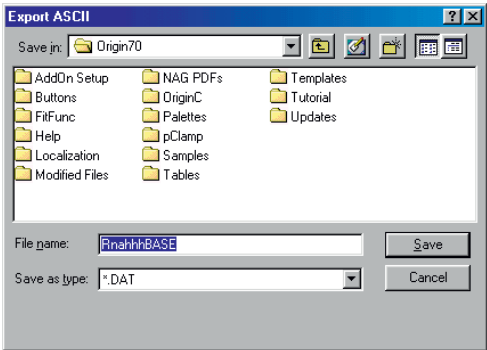
| 2 | Click **Save**. |

The **ASCII Export into RnahhhBASE.DAT** dialog box opens.
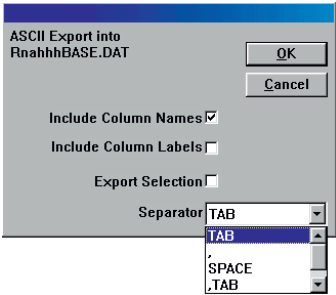
Note:

The output of this ASCII file can be formatted (Please refer to the Origin User's Manual for more information about exporting worksheet data). This file may then be opened into any application that recognizes ASCII text files.

7 Data analysis using Origin
7.4 ITC data handling
7.4.4 Importing worksheet data

7.4.4   Importing worksheet data

Introduction

ASCII files can be imported directly into an Origin worksheet or plot window. Origin menu supports a number of additional file formats for importing data (Lotus, Microsoft Excel, dBASE, LabTech, etc.) while the menus for ITC or DSC data analysis support routine ASCII files.

Importing an ASCII file into a new worksheet

To import an ASCII file into a new worksheet, follow the steps described below:

Step	Action
1	Navigate to *File:New:Worksheet*. A new Origin worksheet, *Data1*, opens.
2	Select the *File:Import:ASCII* command. Alternatively, *File:ASCII:Options* can also be selected to set ASCII file import options. The *Import ASCII* dialog box opens, set to open a data file with a *.DAT* extension.
3	Double-click on a file in the *File Name* list (for example, the *RnahhhBASE.DAT* file that has just been exported). The *RnahhhBASE* data is imported into the worksheet.

Importing an ASCII data file into a plot window

To import an ASCII data file into a plot window, follow the steps described below:

Step	Action
1	Navigate to *File:New:Graph*.
2	Select *Import ASCII:Single File* from the *File* menu.
3	Select the *RnahhhBASE.dat* ASCII file from the *Files* list. Enter the appropriate *Initial X Value* (0 for *RnahhhBASE.dat*) and *Increment in X* (28.25287).
4	Click *OK*.

7.5   Modifying templates

Introduction

The template files of Origin can be changed. This section describes how to edit both, the **DeltaH** and **ITCFinal** plot windows and save the changes into the corresponding template file. Though the changes made will be minor, any property of a template can be changed. For more information about customizing templates, refer to the *Origin User's Manual* or press the **F1** key for online help.

> ⚠️ **CAUTION**
>
> In this section, modifications in the plot window templates that are basic to Origin's operation will be performed. In the unlikely event that a mistake is made, which cannot be corrected, simply copy the original template file from the **Custom** folder of the installation CD-ROM. This will correct any problem that may arise.

Template files in Origin

The **RawITC**, **DeltaH**, and **ITCFinal** plot windows (and all other plot windows in Origin) are created from template files (***.OTP** file extension). A template file contains all of the attributes of a plot window (or a worksheet) except the data. The important thing about template files is that a plot window can be changed, and the changes saved into the template file for that window. The next time this window is opened, it will include the changes. Thus, template files allow customization of plot windows to meet the specifications.

7 Data analysis using Origin
7.5 Modifying templates
7.5.1 Modifying the *DeltaH* template

7.5.1   Modifying the *DeltaH* template

DeltaH template

The **DeltaH** template shows units of **kcal/mole of injectant** along the left y-axis. The scale for this axis is actually defined in terms of cal/mole of injectant, but the axis is factored by 1000 to yield units of kcal/mole.

The right y-axis labels for the **DeltaH** template are hidden from view. In the following example, the template will be modified so that the right y-axis labels are visible. These labels will then be factored by 1000 so that they are identical to the left y-axis labels, and the changes saved into the **DeltaH** template file.

Opening the *DeltaH* plot window

To open the **DeltaH** plot window, follow the steps described below:

Step	Action
1	Click on the **New Project** button from the **Standard** toolbar or navigate to **File:New:Project** to create a new project.
2	Click on the **Read Data...** button in the **RawITC** window.
	The **File Open** dialog box opens, with the **ITC Data (*.ITC)** file extension selected.

7 Data analysis using Origin
7.5 Modifying templates
7.5.1 Modifying the *DeltaH* template

Step	Action
3	Navigate to the `C:\Origin70\Samples` folder and open any ITC data file (for example, *Buffer.ITC*). The *DeltaH* template opens to show the normalized area data.

7 Data analysis using Origin
7.5 Modifying templates
7.5.1 Modifying the **DeltaH** template
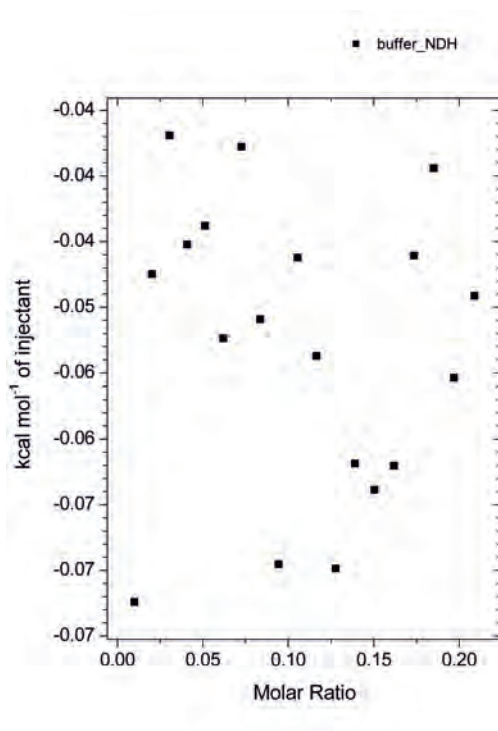
Changing the axes properties

To change the axes properties, follow the steps described below:

Step	Action
1	Double-click either axes' labels in the **DeltaH** window. Alternatively, select **Format:Axes:Y Axis**.

The **Y Axis - Layer 1** dialog box opens.
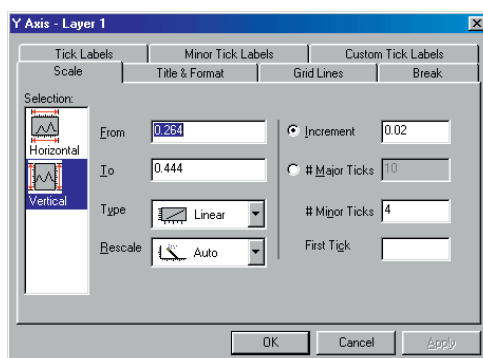
2	Click on the **Tick Labels** tab.
3	Select **Left** from the **Selection:** list box.

4	Change the font value from 22 to 20. Do the same for the x-axis (bottom).
5	Click **OK**.

The dialog box closes. The **DeltaH** window redraws.

7 Data analysis using Origin
7.5 Modifying templates
7.5.1 Modifying the *DeltaH* template

Saving changes into the *DeltaH* template file

To save the changes into the **DeltaH** template file, follow the steps described below:

Step	Action
1	Select **File:SaveTemplate As...**.
	Origin opens a dialog box asking if the file should be saved as **DELTAH.OTP** (the DeltaH template file).
2	Click **Cancel** to cancel changing the original DeltaH template. Click **OK** to save the modified **DeltaH** window as **DELTAH.OTP**.

| *Tip:* | *If the modified template has been saved, navigating now to **File:Read Data...** will open the the modified **DeltaH** window.* |
| *Note:* | *The plotted data cannot be saved to a template file, so there is no need to delete the plotted area data before saving the **DeltaH** window.* |

Reverting to the original *DeltaH* template

To revert to the original **DeltaH** template, reverse the steps used to create the modified template as described below:

Step	Action
1	Open the **DeltaH** window.
2	Open the **Y-Axis - Layer 1** dialog box.
3	Click on the **Tick Labels** tab.
4	Remove the check mark from the **Show Major Labels** check box.
5	Select **File:Save Template As...**.

7 Data analysis using Origin
7.5 Modifying templates
7.5.2 Modifying the *RawITC* template

7.5.2   Modifying the *RawITC* template

Introduction

The **RawITC** plot window shows bottom x-axis tick labels in units of minutes. This section describes how to change this axis scale so that the tick labels are in units of hours rather than minutes.
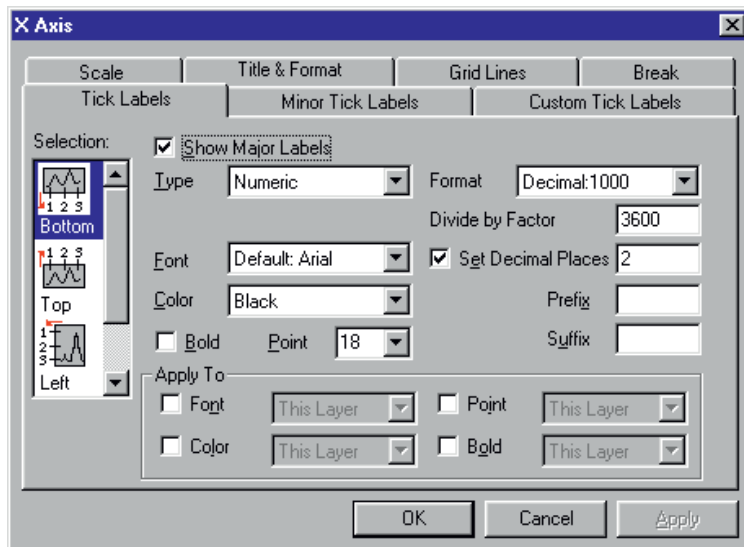
Factoring the *RawITC* X axis tick labels by 3600

To factor the **RawITC** x-axis tick labels by 3600, follow the steps described below:

Step	Action
1	Set the **RawITC** window as the active window (by either pressing and holding the **ctrl** key then pressing the **tab** key, or selecting **RawITC** from the **Window** menu).
2	Double-click on the bottom x-axis tick labels or select **Format:Axis:X Axis**. The **X Axis** dialog box opens.
3	Click on the **Tick Labels** tab.
4	Select **Bottom** from the **Selection:** list box.
5	Enter 3600 in the **Divide by Factor** text box. Since the worksheet X values for raw ITC data are in terms of seconds, a factor of 3600 displays axis tick label values in units of hours for this axis.

7 Data analysis using Origin
7.5 Modifying templates
7.5.2 Modifying the *RawITC* template

Step	Action
6	Enter 2 in **Set Decimal Places** box.

Step	Action
7	Click **OK** to close the dialog box.
8	Double-click on the x-axis title (it reads **Time (min)**) to open the **Text Control** dialog box, and edit the text to read **Time (hrs)**).

Saving changes into the *RawITC* template file

To save the changes into the **RawITC** template file, follow the steps described below:

Step	Action
1	Select **File:Save Template As...**.
2	Click **Cancel** in the **Attention** dialog box to cancel changing the original **RawITC** template. Click **OK** to save the modified **RawITC** window as **Raw-ITC.OTP**.

7 Data analysis using Origin
7.5 Modifying templates
7.5.3 Units notation in Origin

7.5.3   Units notation in Origin

RawITC data files

Raw data in ITC files are stored in terms of µcal/s vs s. The integrated area under the peaks data are stored (in the worksheet column *DH*) in units of µcal per injection. This is apparent if a worksheet containing integrated data is opened.

Curve fitting and better publication presentation

For curve fitting and better publication presentation, both the *DeltaH* and *ITCFINAL* plot windows present the integrated heat data as *H'* (kcal per mole of ligand injected), which is more closely related to the fitting parameter *H* (calories per mole of ligand bound). That is, H' will be nearly equal to H (except for the factor of 1000) in early injections when nearly all of the ligand added is bound. The factor of 1000 is achieved by entering that factor to the y-axis tick labels, as discussed earlier in this section.

x-axis values

Both the *RawITC* plot window and the upper graph in the *ITCFINAL* plot window display x-axis values in minutes, while the stored values are in seconds. In this case, the x-axis labels are factored by 60, as discussed (for the *RawITC* window) earlier in this section. Double-clicking on the top x-axis labels in the *ITCFINAL* window, will display a factor of 60 in the *Divide by Factor* text box, just as there was with the *RawITC* window. Again, this factor setting is saved as part of the *ITCFINAL* template.

y-axis values

Note: *The y-axis data plotted in the **DeltaH** and lower **ITCFINAL** templates (i.e., data with **.NDH** extension) are normalized on moles of injectant.*

Viewing the experimental integrated heats

To view the experimental integrated heats in µcal per injection, follow the steps described below:

Step	Action
1	Double-click on the *Layer* dialog box.
2	Move the *_NDH* file out of the *Active* data and move the *_DH* file into the *Active* data.

7 Data analysis using Origin
7.5 Modifying templates
7.5.3 Units notation in Origin

Step	Action
3	Double-click on the y-axis tick labels and remove the factor of 1000.

7.6   Advanced curve fitting

Introduction

The model for one set of sites discussed in *Section 7.1 Basic ITC data analysis and fitting, on page 144* will work for any number of sites, n, if all sites have the same K and ΔH. If a macromolecule has sites with two different values of K and/or ΔH, then the model with two sets of sites must be used.

Whenever there are two sets of sites, the automatic initialization procedure is rarely effective. If the initialization parameters are extremely far away from best values, then convergence to the best values cannot take place as iterations proceed. In fact, the fit often gets worse rather than better with successive iterations. Therefore, the user must arrive at initialization parameters before the iterations can be started. An indication of poor initialization occurs when values for the K parameter become negative during the fitting procedure.

In this section

This section contains the following topics:

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.1 Nonlinear curve fitting

7.6.1 Nonlinear curve fitting

Introduction

Origin offers two modes of its nonlinear least squares fitting tool, basic and advanced. The two modes differ substantially in the options they provide as well as in the degree of complexity. When the *NonLinear Curve Fitting: Fitting Session* is started by selecting the *One Set of Sites* ITC curve fitting model, by default Origin's nonlinear least squares fitting tool starts in the mode most recently used.

Basic mode

This mode allows iterative curve fitting to the built-in functions and results plotting to the graph.
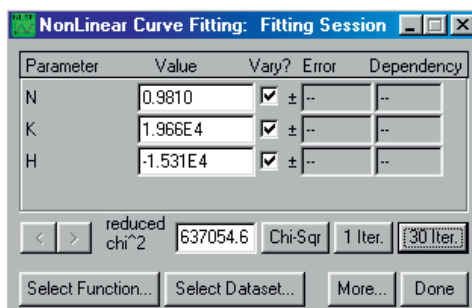
Tip: *Click on the **More...** button to enter the advanced mode.*

Advanced mode

In addition to the basic mode features, the advanced mode allows defining linear constraints, adjusting the configuration of the fitting parameters, simulating data and defining the fitting function.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.1 Nonlinear curve fitting

Tip: Click on the **Basic Mode** button to return to the basic mode.

Aborting the *NonLinear Curve Fitting: Fitting Session*

To exit the **NonLinear Curve Fitting: Fitting Session** without printing the fitting parameters to the **Results** window or the graph text box, follow the steps described below:

Step	Action
1	Click on the **NonLinear Curve Fitting: Fitting Session** dialog box close ✕ button. A pop up window asks "**Do you want to end the current fitting session?**".
2	Click **No** in the pop up window.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.1 Nonlinear curve fitting

Controlling the fitting procedure

To control the fitting procedure, follow the steps described below:

Step	Action
1	Enter the **NonLinear Curve Fitting: Fitting Session**.
2	From the **NonLinear Curve Fitting: Fitting Session** window, select **Options:Control** to open the **Control Parameters** dialog box.
3	Edit this dialog box to specify several quantitative properties of the fitting procedure. These properties directly affect the way the fitting software performs iterations.
	Refer to the next section for more details.

The *Control Parameters* dialog box

NonLinear Curve Fitting: Control Parameters dialog box
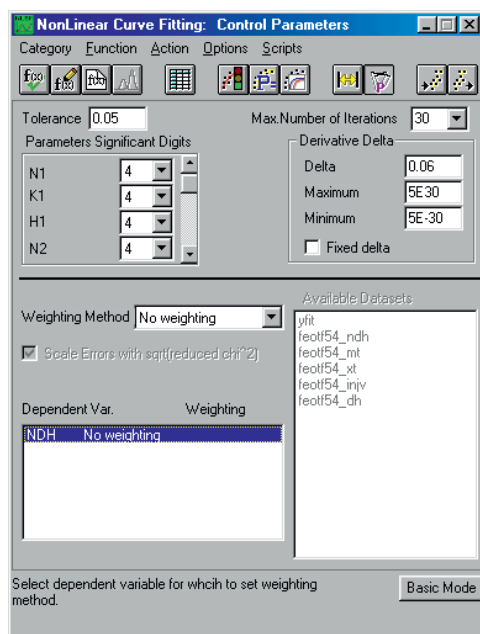
7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.1 Nonlinear curve fitting

The *Tolerance* text box

Note: Depending on how the fitting session was initialized, this value is preset to 0.05, but a new value can be entered for the tolerance in this text box.

The value in the **Tolerance** text box determines the number of iterations to be performed, as described below:

- Clicking on **n Iter** in the **NonLinear Curve Fitting: Fitting Session** dialog box causes the fitting software to try to perform, at most, n Levenberg-Marquardt (LM) iterations.

- If the relative change of the value of Chi-square between two successive iterations is less than the value in the **Tolerance** text box, less than n iterations are performed.

- Clicking on either the **n Iter** or the **1 Iter** button in the **NonLinear Curve Fitting: Fitting Session** dialog box causes the fitter to perform more than n iterations.

Note: The value, 100, is specified as 'n' in the **Max. Number of Iterations** text box.

The *Max. Number of Iterations* drop-down list

Note: This value is preset to 30, but this number can be changed to be effective during a session of Origin by entering a new value in the text box. However, the value is reset to 30 after exiting Origin.

The **Max. Number of Iterations** drop-down list allows the user to specify the value for the maximum number of iterations performed when the **n Iter** button is clicked in the **NonLinear Curve Fitting: Fitting Session** dialog box.

The *Derivative Delta* group

This group determines how the fitting software will compute the partial derivatives with respect to parameters for ITC fitting functions during the iterative procedure, as described below:

Note: The **Delta** value is preset to 0.06 with the **Maximum** as $5 \times 10^{+30}$ and the **Minimum** as 5×10^{-30}.

Component	Description
Fixed Delta check box	Unchecking this check box (recommended for ITC users), sets the actual value of Delta (derivative step size) for a particular parameter equal to the current value of the parameter times the value specified in the **Delta** text box.
Maximum and **Minimum** text boxes	These boxes specify the limiting values of the actual Delta, in case a parameter value becomes too large or too small.

Note: If the fit curve is not converging well, try a different value for the **Delta.** For ITC users, this is typically a larger value (e.g., 0.07, 0.08). The new value is valid for the current session of Origin, but will default back to 0.01 the next time Origin ITC is opened.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.1 Nonlinear curve fitting

The *Parameters Significant Digits* group

Note: The significant digits value is preset to 4 for all parameters.

The **Parameters Significant Digits** group allows the user to select values for the significant digits for each parameter from the associated drop-down list. Selecting **Free** from the drop-down list uses the current Origin setting.

Note: This will only effect the text box display in the **NonLinear Curve Fitting: Fitting Sessions** dialog box.

The *Weighting Method* drop-down list

The **Weighting Method** drop-down list allows the user to select how different dataset points are to be weighted when computing Chi-square during the iterative procedure. The selections are:

- *No weighting*

- *Instrumental*

- *Statistical*

- *Arbitrary dataset*

- *Direct weighting*

Note: It is recommended that the default option of **No weighting** be used for all ITC data unless there is a strong reason to choose a more appropriate for a particular data set. No weighting assumes that each data point has the same absolute error probability.

Returning to the *NonLinear Curve Fitting: Fitting Session* dialog box

To return to the **NonLinear Curve Fitting: Fitting Session** dialog box, click on the button or select **Action:Fit**.

Using macromolecule concentration, rather than n, as a fitting parameter

Introduction

Even though the value for the stoichiometric parameter, n, can be distinguished from independent studies, an accurate estimate for macromolecule concentration M_t may be used (Sigurskjold, Altman & Bundle (1991) Eur. J. Biochem. 197, 239-246.). Using Origin, M_t (along with the correct binding constant and heat of binding) can be determined from curve-fitting.

Determining M_t from curve fitting

To determine M_t from curve fitting, follow the steps described below:

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.1 Nonlinear curve fitting

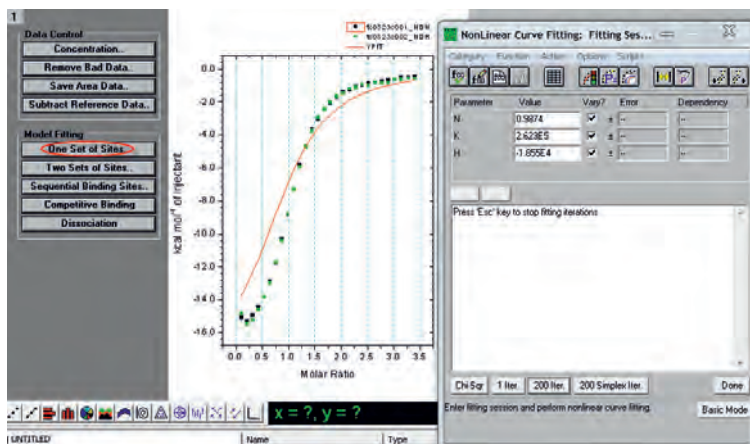| Step	Action
1	Enter an estimated macromolecular concentration, M_t*, into the **Concentrations** dialog box.
2	Select the model for curve-fitting and proceed to find the best fit in the usual way.
	The values obtained for the binding constant and heat of binding will be correct since these depend only on the accuracy of the ligand concentration. However, the best value for the stochiometric parameter, n*, will be incorrect since this is assigned manually by the operator and, after making the correct assignment n, determines the actual M_t.
3	Once curve-fitting is completed, calculate the correct M_t, which is equal to the incorrect concentration M_t* times the ratio n*/n.
4	Check if the above procedure is correct by calling the **RNAHHH.ITC** data into Origin, performing curve-fitting using the correct concentration, and recording the best values of parameters n, K and H as the correct values.
5	Change the concentration by multiplying the correct concentration in the cell by 2. Enter that incorrect value into the **Concentrations** dialog box.
6	Perform curve-fitting again.
	The new, incorrect value of n is exactly 50% of the correct value obtained using the correct concentration. The values for binding constant and heat of binding should be the same in both the cases.

Global Fitting

Origin permits global fitting to be performed on multiple datasets.

Step	Action
1	Start the manual version of the MicroCal iTC$_{200}$ Origin software as described in *Starting Origin, on page 144*.
2	Click Read Data. The Open dialog box opens, with the ITC Data (*.it?) selected as the Files of type:. Select `C:\Origin70\Samples\ itc0523c001.itc` and `itc0523c002.itc` from the files list.

7 Data analysis using Origin
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Step	Action
3	Click on the **One Set of Sites** Model Fitting button.

4	Click the spreadsheet icon in the **Fitting Session** Window.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.1 Nonlinear curve fitting
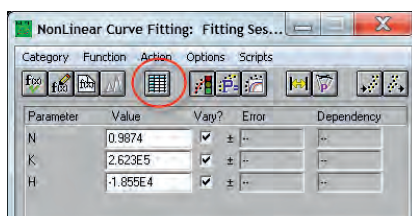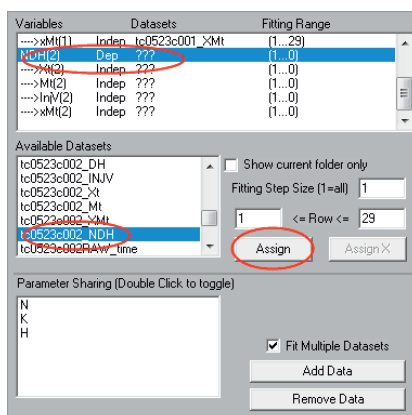
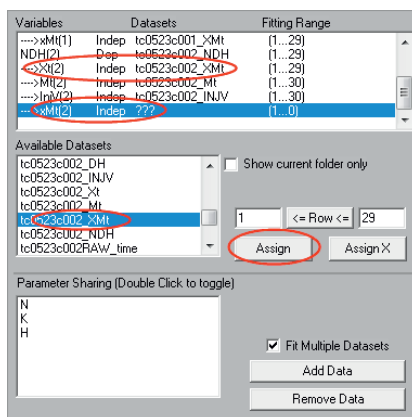| Step	Action
5	This opens the **Select Dataset** window.
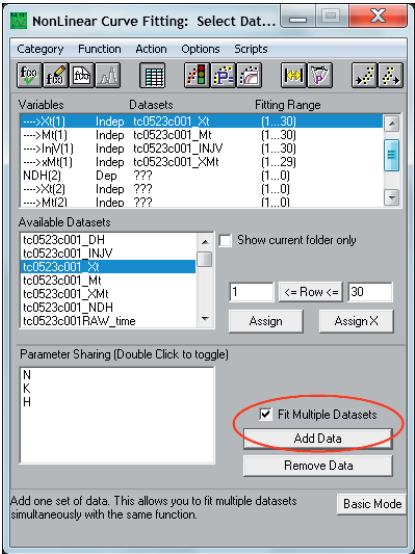
Click the **Fit Multiple Datasets** checkbox as depicted above, which enables the Add Data button. Pressing this button will add datasets to the list above. Notice the questions marks. The user must assign the data manually, from the Available Datasets list. The following steps must be followed:

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.1 Nonlinear curve fitting

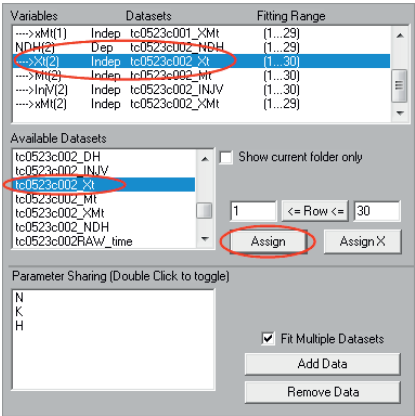| Step	Action
1	Click **NDH(2) Dep ???** in the topmost window as shown below. Click **tc0523c002_NDH** in the middle window. Then press **assign**. Notice Origin mistakes the Xt vector for the XMt vector. Do not correct this yet.

| 2 | Assign the other 3 variables: Mt, InjV, and xMt, leaving the second variable uncorrected. |

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.1 Nonlinear curve fitting

Step	Action
3	Finally, assign the second variable, Xt, to the corresponding dataset's Xt vector.

| 4 | Double-click parameters in the **Parameter Sharing** box to have them fit globally to the 2 datasets. |

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.1 Nonlinear curve fitting
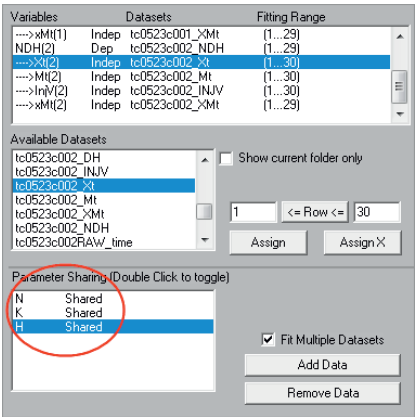
Step	Action
5	Click the button depicted below first and then secondly check the **Generate Fit Curve** checkbox.
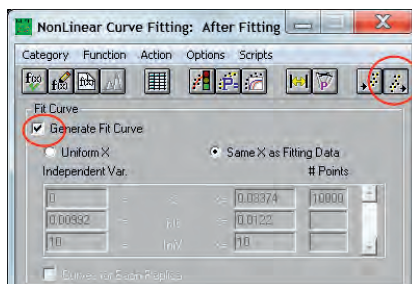
| Step	Action
6	Click the stoplight button to return to the original **Fitting Session** window.
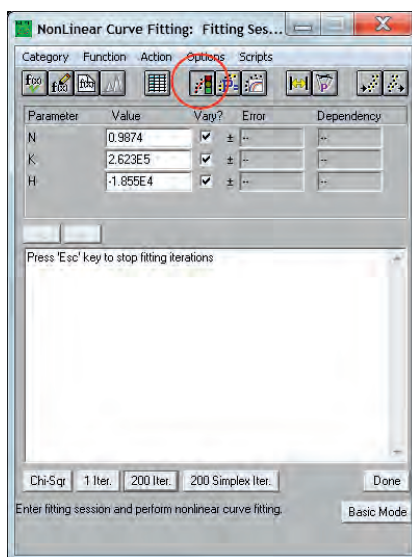
7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.1 Nonlinear curve fitting

Step	Action
7	Perform curve fitting as normal, acknowledge the warning after pressing **Done**, and you are left with a curve fitting both datasets, with the same variables.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.2 Fitting with the *two sets of sites* model
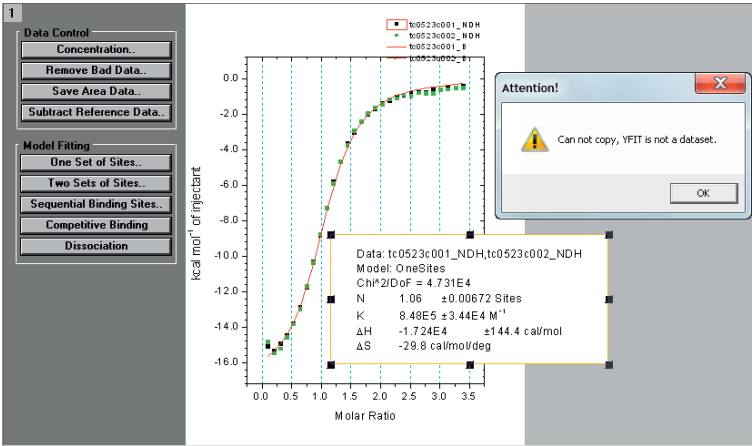
7.6.2   Fitting with the *two sets of sites* model

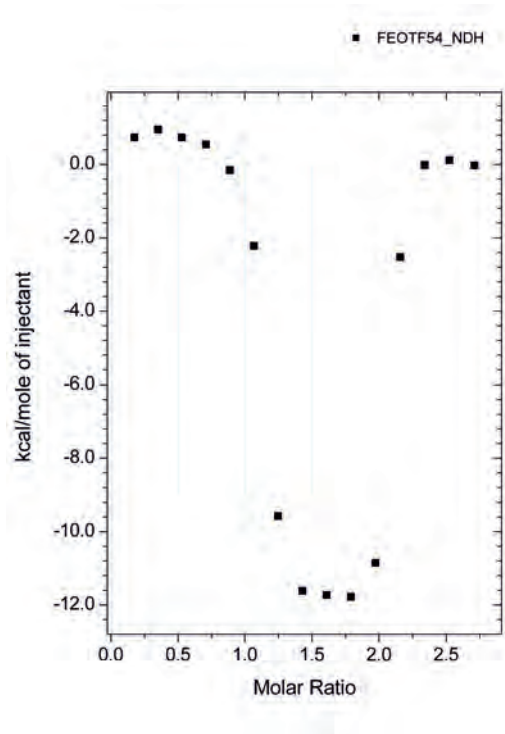### Titration experiments with ovotransferrin

The protein, ovotransferrin, has two very tight, non-identical sites for binding ferric ions; one located in the N domain and one in the C domain. The Origin area data **FeOTF54.NDH** shown below were obtained by titrating ovotransferrin with ferric ion. Injections 1 to 5 titrate primarily the stronger N site, injections 7 to 11 primarily the C site, while injections 13 to 15 result in no binding since both the sites are already saturated.

Fitting ovotransferrin titrations with the two sets of sites model

To fit ovotransferrin titrations with the two sets of sites model, follow the steps described below:

| Step	Action
1	Select **File:New:Project** (or click on the **New Project** button) to create a new project.
2	Click on the **Read Data...** button in the **RawITC** window and select **Area Data (*.DH)** from the **File of type** drop-down list.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.2 Fitting with the *two sets of sites* model

Step	Action
3	Go to the `C:\Origin70\Samples` folder, and open *FeOTF54.DH*.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.2 Fitting with the *two sets of sites* model
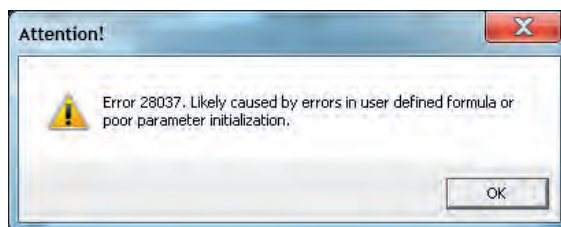
Step	Action
4	Click the **Two sets of Sites** button in the **DeltaH** window.
	Origin opens the **NonLinear Curve Fitting: Fitting Session** dialog box, but produces an attention dialog box and, upon clicking **200 Iter.** a very poor initial fit to the curve.
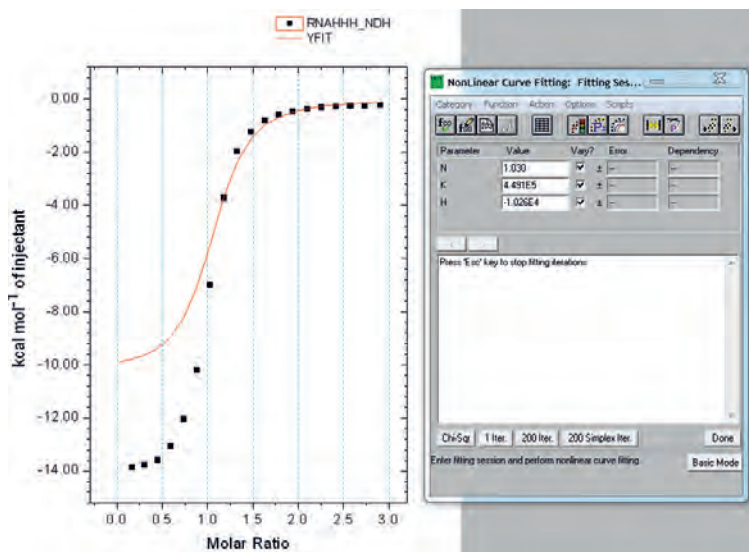
| 5 | Click **OK** in the warning dialog to proceed. |

Note:

The auto initialization produces a curve, which represents the data very poorly. If iterations are started from this, the fit will not converge. With experience, a satisfactory initialization that leads to convergence can be obtained.

7 Data analysis using Origin
7.6 Advanced curve fitting
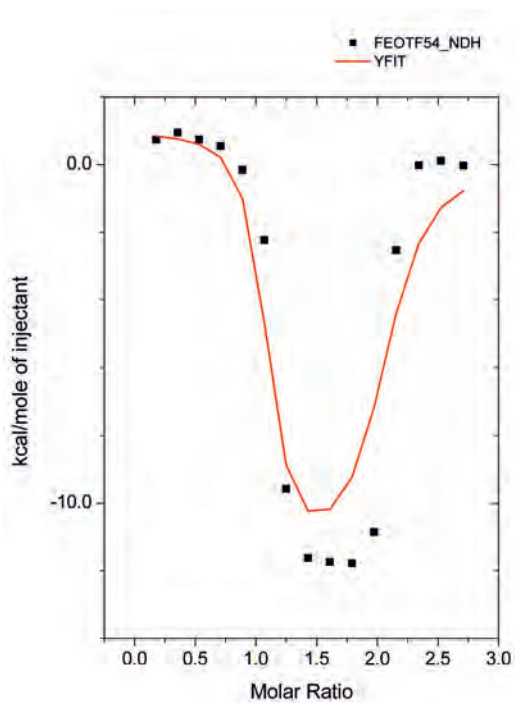7.6.2 Fitting with the **two sets of sites** model

Manual initialization

Examination of the experimental points shows that the first few injections at a molar ratio below 1 produce ~1 kcal per mole of injectant, changing to ~-12 kcal for molar ratio 1 to 2 and finally changing to 0 at molar ratios larger than 2.

To begin manual initialization, follow the steps described below:

Step	Action
1	Enter 1 into both the **N1** and **N2** parameter boxes in the **NonLinear Curve Fitting: Fitting Session** dialog box.
	Note: *H1 must be near +1000 and H2 close to -12,000.*
2	Enter **H1** as +1000 and **H2** as −12,000 into the appropriate parameter boxes.
	Note: *Since the experimental heats fall off quickly from the **H1** value to the **H2** value, it is clear that **K1** must be much larger than **K2**, and because the heat changes abruptly from the H2 value to zero (i.e., beginning with the eleventh injection) it is also clear that K2 itself must be large (i.e., even though it is smaller than K1).*
3	Enter 1e8 into the **K1** parameter box, and 1e6 into the **K2** parameter box.
	Note: *Do not insert a space before or after the e when using exponential notation, or Origin will not accept the value.*

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.2 Fitting with the *two sets of sites* model

| Step	Action
4	Click on the *Chi-Sqr* button in the dialog box. Origin draws a new fit curve using the entered parameters, which is a much better representation of the data.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.2 Fitting with the **two sets of sites** model

Step	Action
5	Select the **200 Iter.** button a few times, and convergence occurs with a final Chi^2 of about 33,000.
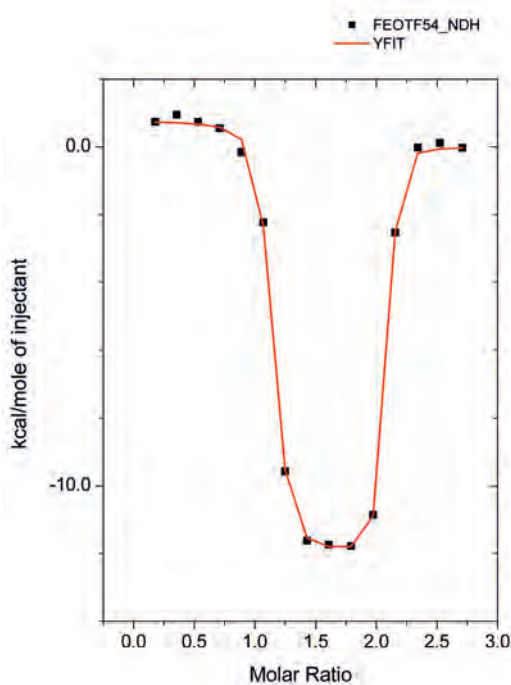
Note:

N1 and N2 are nearly the same magnitude, but not quite. It would be interesting to see if a fit of nearly equal quality could be obtained with N1 and N2 exactly equal to each other, although theoretically they should each be 1.0.

6	Enter the value `1.0` into the **N1** and **N2** parameter value box.
7	Click the **N1** and **N2** checkboxes to remove the checkmark, and continue the iterations. This fit is not as good, but this could also mean that the concentrations were incorrect.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.3 Reverse titrations

7.6.3 Reverse titrations

Introduction

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).

Principle

For this discussion, assume that the macromolecule has two fairly strong sites with differing affinity for the ligand. The measured heat change will depend on where the ligand and the macromolecule are loaded, syringe or sample cell.

If the macromolecule is loaded in the sample cell and the ligand in the syringe, then the tightest of the two sites will titrate in the early injections with heat change H1 and the weakest of the two will titrate in subsequent injections with heat change H2 until both sites are saturated, whereupon the heat change goes to zero.

If the ligand is loaded into the sample cell and the macromolecule into the syringe, then the ligand will be in excess in the early injections and both the sites will titrate with a heat change of H1 + H2.

Once sufficient macromolecule (i.e., molar ratio of macromolecule/ligand of 0.5) has been added to bind all of the ligand as a 2-to-1 complex, further injections of the macromolecule will result in some of the ligand being removed from the weaker site in the 2-to-1 complex, so that it can bind to the stronger site on the newly-injected macromolecule. The heat change for this second phase of the titration will then be H1 - H2, assuming that site 1 is sufficiently strong. In such a case, all of the ligand will be in the 1-to-1 complex when the molar ratio reaches 1.0, and further injections of macromolecule will give zero heats.

Example

Fitting by the *Two sets of Sites* model
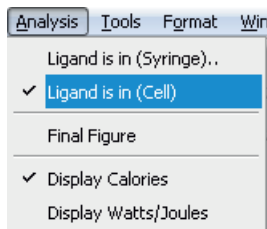
To open the **OTFFE3.DH** file, follow the steps described below:

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.3 Reverse titrations

Step	Action
1	Select *File:New:Project* (or click on the *New Project* button) to create a new project.
2	Click on the *Read Data...* button in the *RawITC* window.
3	Select *Files of type:Area Data (*.DH)*.
4	Navigate to the `C:\Origin70\Samples` folder.
5	Double-click on *OTFFE3* in the *File Name* list.
	The normalized (*.NDH*) data are populated in the plot window.
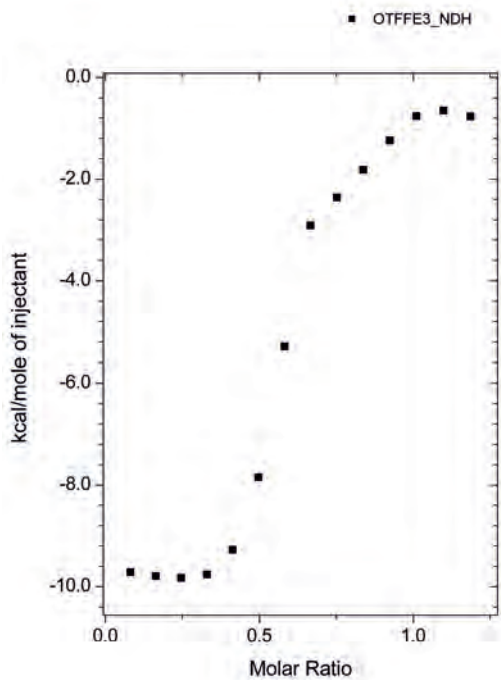
The data in file *OTFFE3.DH* were obtained with the macromolecule (ovotransferrin) in the syringe and the ligand (a chelated form of ferric ion) in the sample cell. Injections 1 to 5 correspond to formation of the diferric form of ovotransferrin with heat change H1 + H2. Injections 8 to 14 involve conversion of the diferric form into the mono ferric form with heat change H1 - H2.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.3 Reverse titrations

Step	Action

6   Select **Ligand is in (Cell)** from the **Analysis** menu before fitting to this data.

This switches the settings, letting Origin know that the ligand is now in the cell. Confirm this by clicking on the **Analysis** menu again and noting that the checkmark is next to **Ligand is in (Cell)**.

Note:

Origin defines the "macromolecule" as the species with n greater than 1.0 and the "ligand" as the species with only one site, irrespective of their molecular weights.
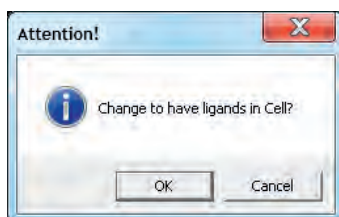
7   Click **OK**.

8   Click on the **Two sets of Sites** button to select the appropriate fitting model.

              Note:

              The default fitting parameters will lead to a satisfactory convergence in this case but can be improved before beginning interations.

Changing values in the *Parameters Significant Digits* group

The first several injections indicate that H1 + H2 equals about -10,000 cal/mole. Change the values in the **Parameters Significant Digits** group as described below:

Step	Action

1   Start off with values of `-7000` for **H1** and `-3000` for **H2**.

2   Set **n1** and **n2** equal to `1.0`.

3   Uncheck the **N1** and **N2** checkboxes.
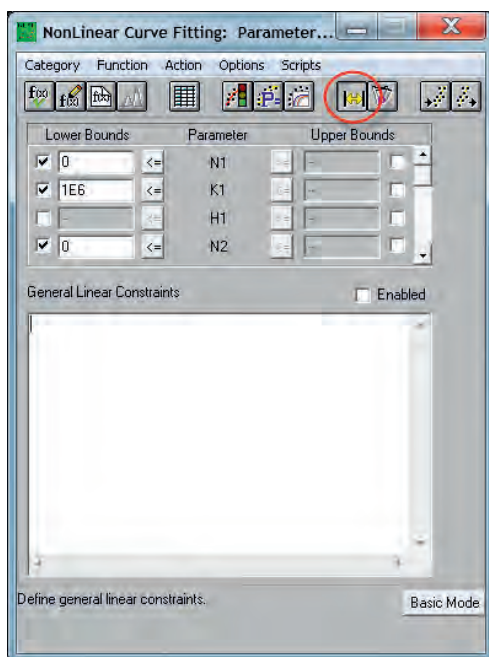
4   Enter `1e8` for **K1** and `1e6` for **K2**.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.3 Reverse titrations

| Step	Action
5	Select **Chi-Sqr**, and use the **1 Iter.** command to iterate once. Iterating a second time generates an error. Complex models like this one can be heavily dependent on initial parameters.
6	Constrain **K1** by pressing the icon circled below.

Check that the **Lower Bounds** checkbox is checked for **K1**. Enter 1e6 and press the stoplight icon to return to the fitting session.

| 7 | Click **200 Iter.** to converge on a solution. |

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.3 Reverse titrations

Running experiments with poor fitting parameters

This section describes how experiments with poorly defined fitting parameters can be run. This situation will most likely occur with two sets of sites, where K1 and K2 values are less than 10-fold different. It is even possible that the set of "best fit" parameters may be quite different depending on the initialization parameters, which are used to start the fit; i.e., the curve-fitting routine can become trapped in a local minimum for Chi-square and be unable to find the global minimum. This can be detected by starting with several different sets of initialization parameters to see if the same final minimum with nearly the same fitting parameters is achieved.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.4 The *Sequential Binding Sites* model
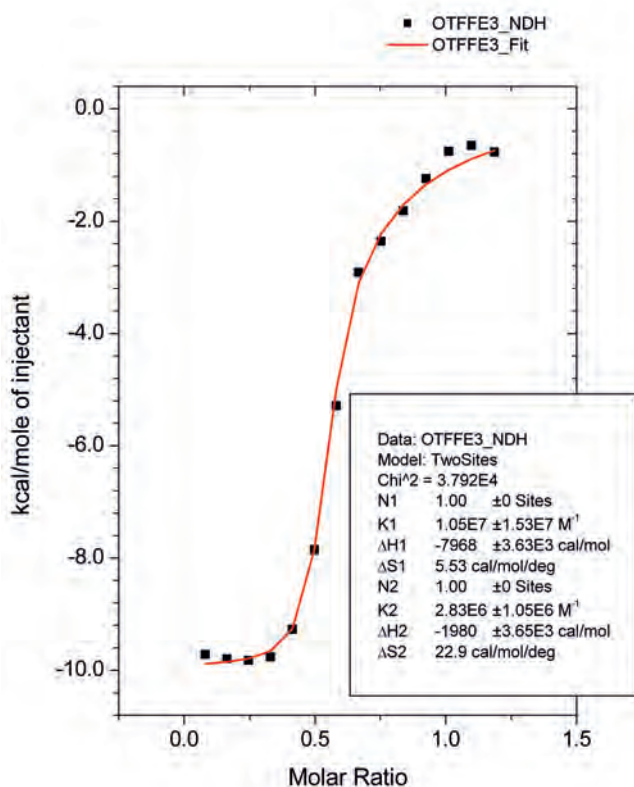
7.6.4   The *Sequential Binding Sites* model

Introduction

The models discussed previously have been concerned with independent sites. It often occurs in biological systems that the binding of a ligand to one site will be influenced by whether or not ligands are bound to any of the other sites. 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

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 (K1, H1) to bind has more empty sites to choose from than does the second ligand (K2, H2), as described in *Appendix A Equations used for fitting ITC data, on page 349*. 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 with positive cooperativity means K2>K1. 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 H1 + H2, so that only one "phase" is seen in the titration curve.
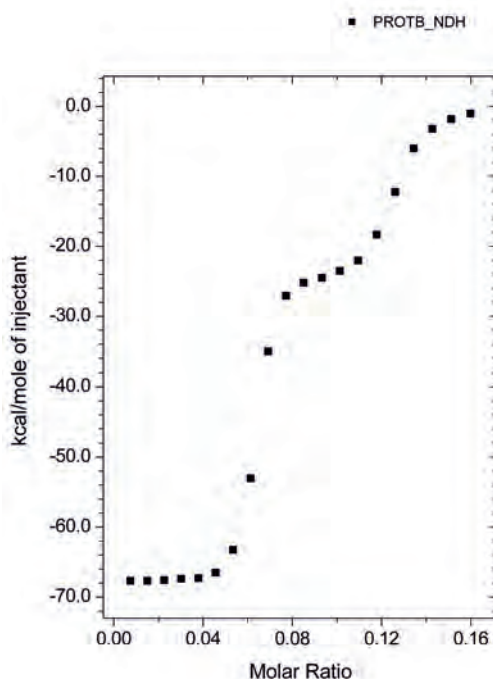
Negative cooperativity

A system with negative cooperativity means K1>K2. 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.

Fitting data with *Sequential Binding Sites* model

To fit the data with **Sequential Binding Sites** model, follow the steps described below:

Step	Action
1	Select **File:New:Project** (or click on the **New Project** button) to open a new project.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.4 The *Sequential Binding Sites* model

Step	Action
2	Click on the **Read Data...** button in the **RawITC** window, then select **Area Data (*.dh)** from the **File of Type:** drop-down box.
3	Go to the C:\Origin70\Samples sub-folder, and double-click on **protb.dh**.

Since there are clearly two "phases" to this binding isotherm, it exhibits negative cooperativity.

4	Click the **Concentration..** button in the **DeltaH** window to edit the concentrations for this data before fitting.
5	Enter the following values in the dialog box: 20.7 mM ligand in the syringe; 0.494 mM macromolecule in the cell; 4 μl injection volume; 1.32 ml cell volume.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.4 The *Sequential Binding Sites* model

Step	Action
6	Click **OK**.
	The y-axis automatically rescales according to the changes made.
	Note:
	*Ensure that **Ligand is in (Syringe)** has the check mark next to it in the **ITC** menu indicating it is the active mode. If the check mark is next to the **Ligand is in (Cell)**, select the menu item **Ligand is in (Syringe)**. This causes the mode to switch to having the ligand in the syringe.*
7	Click the **Sequential Binding Sites** button in the **DeltaH** window to fit the data to the interacting sites model.
8	Click on the **Chi-Sqr** button and enter 2 for the number of sites. Enter guesses of `1e8, -8000, 1e6, -3000` for the parameters **K1**, **H1**, **K2**, and **H2**, respectively.
9	Click the **200 Iter.** button several times, until a satisfactory convergence is obtained.

Conclusion

The above data can be deconvoluted with the default initialization parameters based on the following observations:

- The binding constant for the second ligand is about 70 times weaker than the binding constant for the first ligand.

- The heat of binding is also less exothermic.

- Stoichiometric parameters n1 and n2 are not included as floating parameters with the model of interacting sites. This would allow a non-integral number of ligand molecules to bind in each step, which is a physical impossibility.

- Accurate concentrations of ligand and macromolecule are more important here since concentration errors cannot be overcome by non-integral values of n1 and n2 as is the case with the model of two independent sites.

Systems with identical binding sites

Systems with identical binding sites have statistical degeneracy that influences the saturation profile.

For example, in a system with two identical sites the first ligand has two empty sites at which to bind while the second ligand has only one. The binding constants reported in the parameter box are phenomenological binding constants, which include effects from degeneracy. To remove these effects and compare intrinisic binding constants K^0 at each site, refer to eq (19) in *Appendix A Equations used for fitting ITC data, on page 349*.
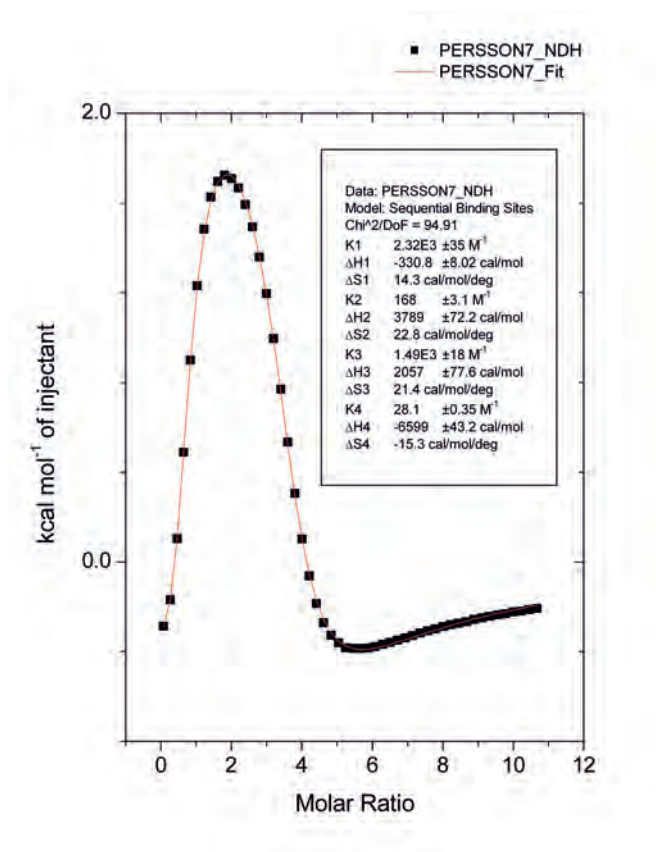
7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.5 Binding of multiple ligands to transition metal ions

7.6.5   Binding of multiple ligands to transition metal ions

Introduction

The binding of multiple ligands to transition metal ions is another example where the sequential binding model is appropriate, and where all sites are identical in the apo-metal ion.

Practice example

Practice fitting data for binding of multiple ligands to transition metal ions using the **Sequential Binding Sites** model on the sample file **Persson7.ITC** (contains data on the binding of four Br^- to Cd^{++} to form $CdBr_4^{--}$) as described below:

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.5 Binding of multiple ligands to transition metal ions

1 Open the file and perform curve fitting to obtain binding parameters for each of the four Br⁻ using the **Sequential Binding Sites** model.

   Note: *The concentrations of both, Br⁻ and Cd⁺⁺ are correct as contained in the file.*

2 Click the **200 Iter.** button several times to obtain a satisfactory convergence without selecting the initial parameters manually.

3 Practice and try to improve the initialization.

Using the *Sequential Binding Sites* model with non-identical sites

Difference between *Two sets of Sites* model and *Sequential Binding Sites* model

The Sequential Binding Sites model can also be applied to systems with non-identical sites.

The **Two sets of Sites** model, considers:

- the saturation of individual sites on the same molecule

- assumes they saturate independently of one another

- uses three fitting parameters for each site; N, K and H.

The **Sequential Binding Sites** model assumes:

- a fixed sequence of binding, i.e., the first ligand, which binds to an individual molecule always binds to site 1, the second ligand, which binds to an individual molecule always binds to site 2, etc.

- the number of sequential sites must be exactly integral (1,2,3,...) so there is no fitting parameter equivalent to N

- best-fit is determined by only two parameters, K and H, at each site once the total number of sites has been selected by the operator

Note: *For a molecule which has 2 sites with quite different affinity (e.g., K values different by a factor of five or more), the two models tend to give equivalent values of K and H since thermodynamics will dictate binding to the site of highest affinity first. However, when K values at two independent sites are more or less nearly equivalent, sequential binding will not be strictly followed.*

Advantages of *Sequential Binding Sites* model over *Two sets of Sites* model

The following basic advantages of **Sequential Binding Sites** model over **Two sets of Sites** model make it the only choice available for providing a unique phenomenal characterization of binding parameters for some multi-site systems:

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.5 Binding of multiple ligands to transition metal ions

- the smaller number of fitting parameters used for each site

- ability to provide a unique fit even for systems with four binding sites (if the K and/or H values are sufficiently different for each site)

Note: *Using a model for independent sites, it would be extremely difficult to obtain a unique fit for more than two sets of sites, which is why no fitting model for three sets of independent sites has been included in this software.*

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.6 Enzyme/substrate/inhibitor assay

7.6.6   Enzyme/substrate/inhibitor assay

Introduction

There are two different methods for carrying out an enzyme assay. These methods are discussed in *Enzyme/substrate/inhibitor assay, on page 355*, where the appropriate equations are also included. Both methods assume that no significant product inhibition occurs.

The methods are summarized in the table below and are explained in detail in the following four sections.

Method	Enzyme Assay
Method 1	A: Enzyme assay- substrate only (*Section 7.6.7 Method 1A: Enzyme assay- substrate only, on page 237*)
	B: Enzyme assay- substrate plus inhibitor (*Section 7.6.7 Method 1A: Enzyme assay- substrate only, on page 237*)
Method 2	A: Enzyme assay- substrate only (*Section 7.6.7 Method 1A: Enzyme assay- substrate only, on page 237*)
	B: Enzyme assay- substrate plus inhibitor (*Section 7.6.7 Method 1A: Enzyme assay- substrate only, on page 237*)

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7.6 Advanced curve fitting
7.6.7 Method 1A: Enzyme assay- substrate only

7.6.7   Method 1A: Enzyme assay- substrate only

Principle

The basic principle of this method is described below:

Step	Action
1	An enzyme solution is in the sample cell and the experiment involves a single injection of substrate solution into the sample cell.
2	Immediately after the injection, the calorimeter baseline shifts prominently to reflect heat effects that occur due to the decomposition of substrate as it comes into contact with the enzyme.
	Note:
	Because of the finite response time of the instrument, it takes a few minutes before the calorimetric signal becomes equilibrated with the actual heat from substrate turnover.
3	After all the substrate has reacted, the baseline returns to its original position prior to the next injection of substrate.

Analysis

Analysis of the decay resulting from the substrate decomposition curve allows determination of:

1   the Michaelis parameters, K_M (mM) and K_{cat} (s^{-1})

2   the heat of substrate decomposition, ΔH

If a second similar experiment is carried out with an inhibitor in the sample cell along with the enzyme, then analysis of the resulting decay curve will:

1   use parameters determined in the first experiment (K_M, K_{cat} and ΔH) as input parameters and use only K_I as a fitting parameter

2   determine the Michaelis inhibitor constant, K_I (mM)

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.7 Method 1A: Enzyme assay- substrate only

Procedure

To employ Method 1: Enzyme assay- substrate only, follow the steps described below:

Step	Action
1	Select *File:New:Project*.
	A new Origin project opens to display the **RawITC** plot window.
2	Click the **Read Data..** button.
3	Click the drop-down arrow of the **Files of type** text box and select **Enzyme Assay (*.it?)** file type.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.7 Method 1A: Enzyme assay- substrate only
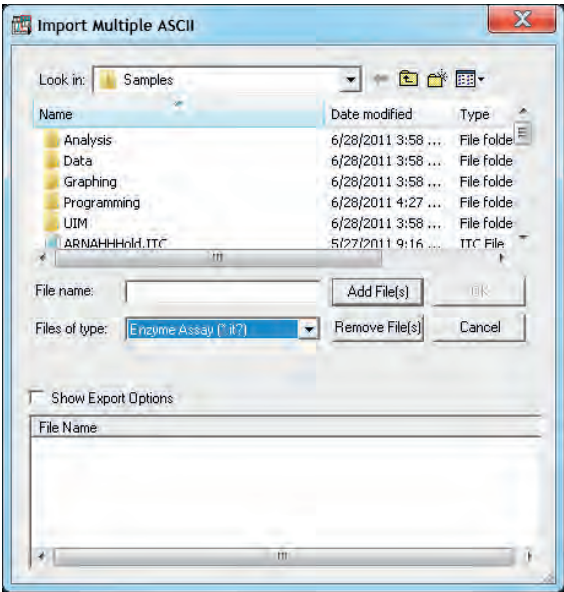
Step	Action
4	Navigate to the `C:\Origin70\Samples` folder and select **M1NoInhibitor.itc** from the **File Name** list, and click **OK**.
	The **Enzyme Assay** dialog box opens, allowing the selection of one of the four models.
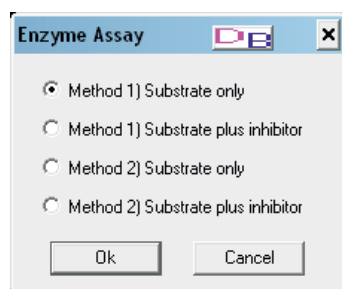
5	Select **Method 1) Substrate only** and click **OK**.
	The **Method 1) Substrate only** dialog box opens up. If no value is entered for ΔH, the program calculates ΔH (using the formulae in *Enzyme/substrate/inhibitor assay, on page 355*).

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.7 Method 1A: Enzyme assay- substrate only
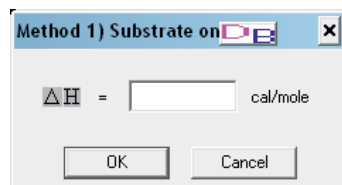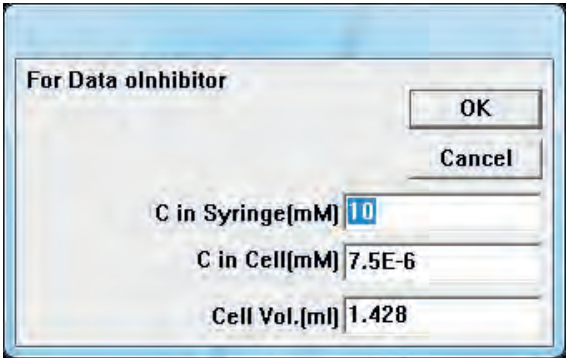
Step	Action
6	Click **Cancel**.

The data file is read in and plotted in a new window.
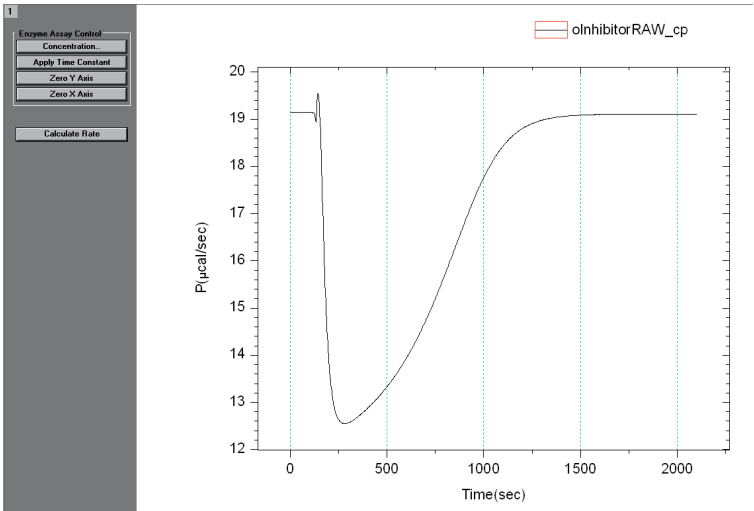
Concentration

Note: *The concentration and injection volume values which displayed initially are those entered manually before the experiment starts. The cell volume is a constant, which is stored in the data collection software. This value is read by Origin whenever an ITC data file is called. Always check that the concentration values are correct for each experiment. Incorrect values will negate the fitting results.*

To edit the concentration values, follow the steps described below:

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.7 Method 1A: Enzyme assay- substrate only

Step	Action
1	Click the **Concentration** button.
	The concentration dialog box, **For Data oInhibitor**, opens showing the concentration values and the cell volume.

2	Enter a new value in the appropriate text box.
3	Click **OK** to save the new values or **Cancel** to use the default values displayed.

Applying time constant

Response time of the instrument

The response time of the instrument is dependant on the feedback gain mode used during the experiment. Typical values for the relaxation time are ~18.5 s for high gain, 51 s for low gain and 72 s for no active feedback (passive) gain mode. The actual values are measured for the instrument and stored in the **VPViewer.ini** file.

Methods to reduce the effect of instrument response time on final parameters

When a substrate is injected into the enzyme solution, it decomposes immediately. However, it takes approximately one minute after the injection before the baseline has reached the position where it reflects the full amount of heat being released in the cell because of the finite response time of the instrument.

There are two software procedures designed to reduce the effect that the instrument response time exerts on final parameters obtained from the data, as described below:

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.7 Method 1A: Enzyme assay- substrate only

Procedure	Action
1	The first procedure is activated from the **Apply Time Constant** button. Knowing the actual time constant for the instrument (determined by Malvern Instruments before shipment, and stored in Origin), the experimental data are mathematically "corrected" to remove the response time effect on the experimental data. When this operation is carried out, the old data is transferred out of the active window and the corrected data is displayed in the active window.
2	The second procedure is activated by clicking the **Truncate Data** button. Remove that portion of the data immediately after the injection where distortion remains even after correcting the time constant.

Applying time constant

To apply time constant, follow the steps described below:

Step	Action
1	Click the **Apply Time Constant** button.
	The time constant dialog box opens. The value of 18.5 is correct for high gain feedback mode.

2	Click **OK** or **Cancel**.
	Note:
	Once the time constant correction has been applied, the original data is replaced in the active window by the corrected data.

Zeroing the axes

Zeroing the y-axis

To zero the y-axis, follow the steps described below:

Step	Action
1	Select the **Zero Y Axis** button.
	The cursor will turn to a cross hair.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.7 Method 1A: Enzyme assay- substrate only

Step	Action
2	Double-click a point to place it at y=0. **Tip:** Choose a point on the flat part of the baseline before the injection is made.

Zeroing the x-axis

To zero the x-axis, follow the steps described below:

Step	Action
1	Select the ***Zero X Axis*** button. The cursor will turn to a cross hair.
2	Click a point, then use the arrow keys to move the point and then press **enter** to select that point. *Note:* *Zero the x-axis at the point where the injection is made (where the first small deflection in the baseline is observed).*

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.7 Method 1A: Enzyme assay- substrate only

Calculating the rate

To calculate the rate, follow the steps described below:

Step	Action
1	Click the **Calculate Rate** button.
	The rate is calculated and plotted in a new window, in a graph of the Rate (mM/s) vs [S] (mM) where [S] is the concentration of the unreacted substrate in the cell.
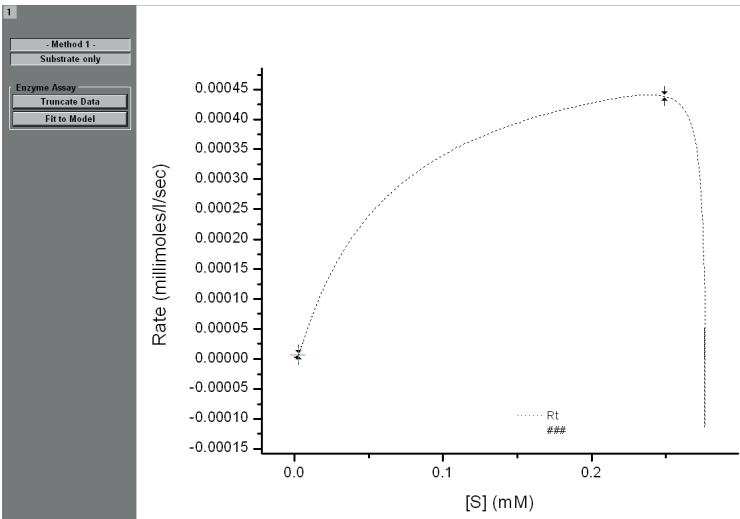
Step	Action
2	Click the **Truncate Data** button to eliminate the artifact at the start of the experiment or click **Fit to Model** to open Origin's nonlinear least squares curve fitting to perform the fitting iterations.

Truncating data

To truncate data, follow the steps described below:

Step	Action
1	Click the **Truncate Data** button.
2	Move the data markers to the positions shown.
3	Double-click on one of the markers or press **enter**.
	This will eliminate the data obtained immediately after the injection of the substrate, before the calorimeter equilibrates with the ongoing reaction.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.7 Method 1A: Enzyme assay- substrate only

Fitting to model

To fit to model, follow the steps described below:

Step	Action
1	Click the **Fit to Model** button. The **Fitting Sessions** dialog box opens.
2	Click the **200 Iter.** button, two or three times, to ensure that the Chi-square value is no longer decreasing.
3	Click **Done** to end the fitting session. K_{cat} and K_m are used as the variable parameters during the iterative fitting and the best values, along with ΔH, are reported in the output parameter box.

Note:

ΔH is determined from the total area of the negative peak of the raw data, but is not used as a fitting parameter.
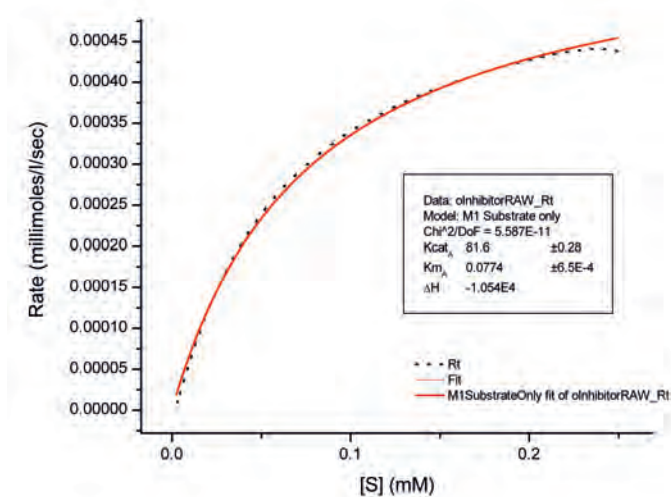
7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.8 Method 1B: Enzyme assay- substrate plus inhibitor

7.6.8   Method 1B: Enzyme assay- substrate plus inhibitor

Open the ITC data file, *M1Inhibitor0175.itc*, as follows:

Note: *In the presence of an inhibitor I, it is necessary to enter previously determined values of K_{cat}, K_M and ΔH (as determined in the previous example) and use K_I as the only fitting variable.*

Step	Action
1	Select **File:New:Project**.
	A new Origin project opens to display the **RawITC** plot window.
2	Click the **Read Data..** button.
3	Click the drop-down arrow of the **Files of type** text box and select **Enzyme Assay (*.it?)** file type.
4	Navigate to the `C:\Origin70\Samples` folder and select **M1Inhibitor0175.itc** from the **File Name** list, and click **OK**.
	The **Enzyme Assay** dialog box opens, which allows selection of one of the four models.

Step	Action
5	Select **Method 1) Substrate plus inhibitor** and click **OK**.
	The **Method 1) Substrate plus inhibitor** dialog box opens.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.8 Method 1B: Enzyme assay- substrate plus inhibitor

Step	Action
6	Enter `.0175` (*mM*) for *[I]* inhibitor concentration, `-10500` (*cal/mole*) for *ΔH*, as determined in the previous example. Enter `81.9` (*sec⁻¹*) for K^O_{cat} and `.076` (*mM*) for K^O_M. Click *OK*. *Note:* *The values for K^O_M and K^O_{cat} may be slightly different depending on where the data is truncated.*
7	Click the **Concentration** button. The **Concentration** dialog box opens. Verify or edit the concentrations.
8	Click the **Zero Y Axis** button. The cursor turns to a cross hair. Double-click a point, to place at y=0.
9	Click the **Apply Time Constant** button. The time constant dialog box opens. Verify or edit the time constant for the data.
10	Click the **Calculate Rate** button. As illustrated in the previous example, the rate is calculated and plotted in a new window versus the concentration of the injectant in the cell.
11	Select the **Truncate Data** button.
12	Move the data marker to remove the artifact on the right side of the data display, then double-click on one of the markers or press **enter** to set the point to truncate the curve.
13	Click the **Fit to Model** button. The **Fitting Sessions** dialog box opens.
14	Click the **200 Iter.** button, two or three times, then click **Done** to end the fitting session. The inhibition constant K_I, is used as the variable parameter during the iterative fitting and reported in the output parameter box. The values for the three entered parameters (K^O_{cat}, K^O_M and *ΔH*) are also displayed in the parameter box.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.9 Method 2A: Enzyme assay- substrate only

7.6.9 Method 2A: Enzyme assay- substrate only

Procedure

To employ Method 2A: Enzyme assay-substrate only, follow the steps described below:

Step	Action
1	Select *File:New:Project*.
	A new Origin project opens to display the *RawITC* plot window.
2	Click the *Read Data..* button.
3	Click the drop-down arrow of the *Files of type* text box and select *Enzyme Assay (*.it?)* file type.
4	Navigate to the `C:\Origin70\Samples` folder and select *M2NoInhibitor* from the *File Name* list, and click *OK*.
	The *Enzyme Assay* dialog box opens allowing selection of one of the four models.
5	Select *Method 2) Substrate only* and click *OK*.
	The *Method 2) Substrate only* dialog box opens.
6	Enter `-10500` (cal/mole) for ΔH.
	Note:
	In Method 2, the ΔH must be independently determined in a separate single-injection experiment (Method 1) and that value should be entered here.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.9 Method 2A: Enzyme assay- substrate only
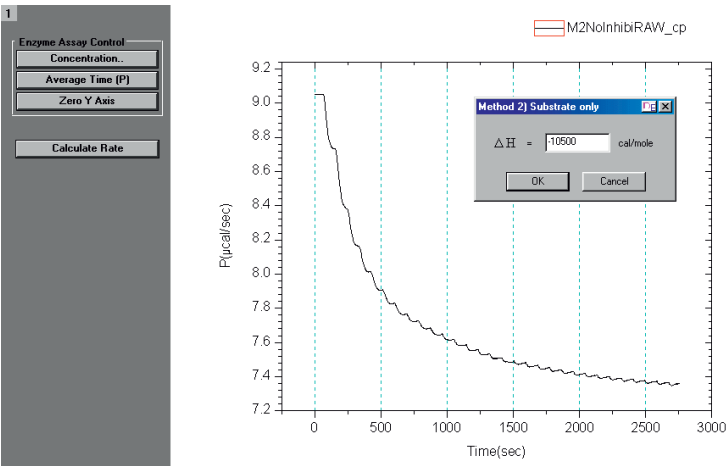
Step	Action
7	Click the **Concentration** button. The **Concentration** dialog box opens allowing to verify or edit the concentrations.

Average time (P)

Power level

The rate of substrate decomposition reactions are determined by measuring the change in the power output in the calorimeter cell that results after each addition of the substrate. The new power level is determined by averaging the power level for a specified time prior to the next injection. After each injection, allow enough time for the instrument to equilibrate at the new power level, but not so much time that significant hydrolysis of substrate occurs. A default value of 15 s is entered for the time period to average the power signal before each injection. However, this default value can be changed as required by a particular substrate.

Changing the time period to average the power signal

To change the time period to average the power signal before each injection, follow the steps described below:

Step	Action
1	Click the **Average Time (P)** button. A dialog box opens that allows the user to change or accept the default value of 15 s.
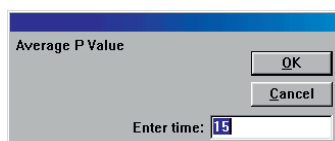
Step	Action
2	Click **OK** to accept 15 s for average the power level.
3	Click the **Zero Y Axis** button. The cursor will turn to a cross hair.
4	Double-click a point, to place at y=0.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.9 Method 2A: Enzyme assay- substrate only

Step	Action
5	Click the **Calculate Rate** button.

As illustrated in the previous examples, the rate will be calculated and plotted in a new window versus the concentration of the injectant in the cell.

Note:

*This example does not need the **Truncate Data** nor the **Apply Time Constant** buttons.*
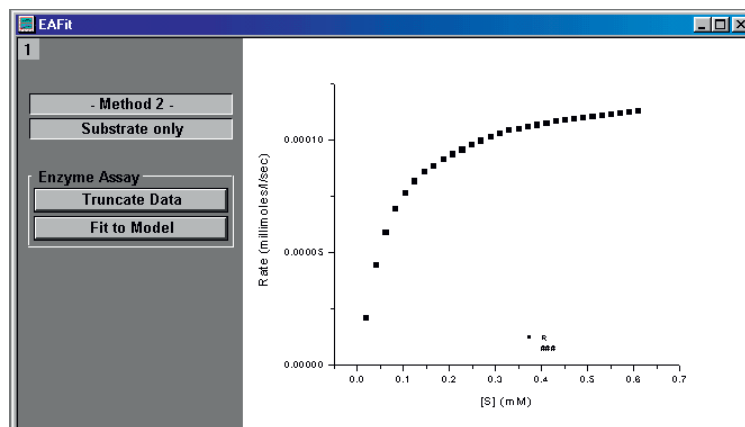
6	Click the **Fit to Model** button.
	The **Fitting Sessions** dialog box opens.
7	Click the **200 Iter.** button, one or two times.
8	Click **Done** to end the fitting session.

K_{cat} and K_m are used as the variable parameters during the iterative fitting process and, along with the entered ΔH, are reported in the output parameter box.

```
Data: M2NoInhibi_R
Model: M2SubstrateOnly
Chi^2/DoF = 2.129E-12
Kcat     67.8     ±0.423
Km       0.084    ±0.00219
ΔH       -1.050E4
```

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.10 Method 2B: Enzyme assay- substrate plus inhibitor

7.6.10   Method 2B: Enzyme assay- substrate plus inhibitor

To employ Method 2B: Enzyme assay-substrate plus inhibitor, follow the steps described below:

Step	Action
1	Select **File:New:Project**.
	A new Origin project opens and displays the **RawITC** plot window.
2	Click the **Read Data..** button.
3	Click the drop-down arrow of the **Files of type** text box and select **Enzyme Assay (*.it?)** file type.
4	Navigate to the `C:\Origin70\Samples` folder and select **M2NoInhibitor** from the **File Name** list, and click **OK**.
	The **Enzyme Assay** dialog box opens and allows selection of one of the four models.
5	Select **Method 2) Substrate plus inhibitor** and click **OK**.
	The **Method 2) Substrate plus inhibitor** dialog box opens.
6	Enter `0.01` (mM) for **[I]** inhibitor concentration, `-10500` (cal/mole) for **ΔH**, as determined in the previous example. Enter `67.8` (sec^{-1}) for K_{cat} and `0.084` mM for K_M.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.10 Method 2B: Enzyme assay- substrate plus inhibitor

Step	Action
7	Click the **Zero Y Axis** button. The cursor turns to a cross hair.
8	Double-click a point, to place at y=0.
9	Click the **Calculate Rate** button. As illustrated in the previous example, the rate is calculated and plotted in a new window versus the concentration of the injectant in the cell. *Note:* *Method 2 does not need to use the **Truncate Data** button.*
10	Click the **Fit to Model** button. The **Fitting Sessions** dialog box will open.
11	Click the **200 Iter.** button, one or two times.
12	Click **Done** to end the fitting session. K_I is used as the variable parameter during the iterative fitting and reported in the output parameter box and should have a value near 0.0076 mM.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.11 Dimer dissociation model

7.6.11   Dimer dissociation model

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, i.e.,

$$P_2 \overset{\Delta H}{\rightleftharpoons} 2P$$

$$K = \frac{[P]^2}{[P_2]}$$

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

Fitting data using the dimer dissociation model

To fit data using the dimer dissociation model, follow the steps described below:

Step	Action
1	Select *File:New:Project*.
	A new Origin project opens and displays the *RawITC* plot window.
2	Click the *Read Data..* button.
3	Click the drop-down arrow of the *Files of type* text box and select *Dissociation (*.it?)* file type.
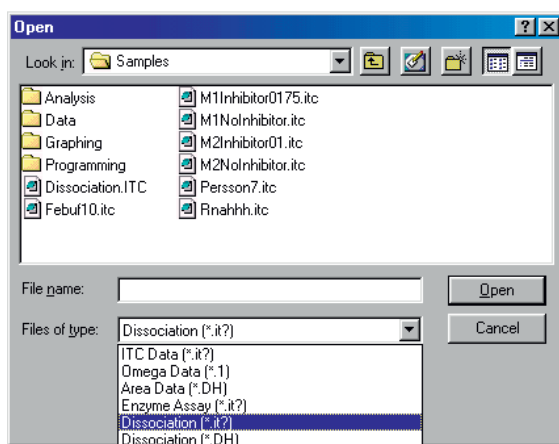
7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.11 Dimer dissociation model

Step	Action
4	Navigate to the `C:\Origin70\Samples` folder and select **Dissociation.ITC** from the **File Name** list, and click **OK**.

Similar to the normal ITC files, the dissociation file is read and plotted as a line graph in the **RawITC** window, in units of μcal/second versus minutes. Origin then automatically performs the following operations:

1. Selects **Auto Baseline** routine. Each injection peak is analyzed and a baseline is created.

2. Selects **Integrate All Peaks** routine. The peaks are integrated and the area (μcal) under each peak is obtained.

3. Opens the **DeltaH** window. The difference for this model is that Origin then plots the normalized area (kcal/mole of monomer) versus equivalent monomer concentration (mM).

Note:

Equivalent monomer concentration represents the total monomer units in the cell. It is not the free monomer concentration.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.11 Dimer dissociation model
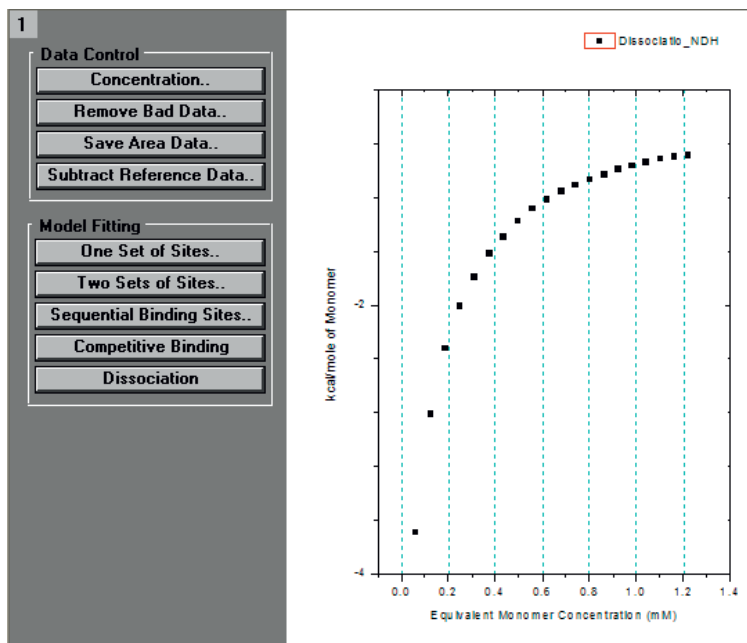
Step	Action
5	Check the concentration in the syringe.

This concentration must always be entered as equivalent monomer concentration. In this case, the concentration is correctly entered and stored in the data file. Click **OK** or **Cancel**.
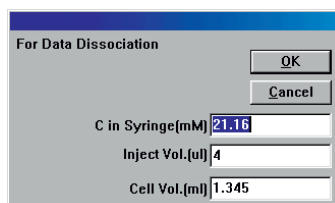
Step	Action
6	Click the **Concentration** button.

In this case, the concentration is correctly entered and stored in the data file.

Step	Action
7	Click **OK** or **Cancel**.

Note:
Unlike typical ITC files, concentration of macromolecule need not be entered, since it is 0.

Step	Action
8	Click the **Dissociation** button.

The **NonLinear Curve Fitting: Fitting Session** dialog box for the dissociation model opens.

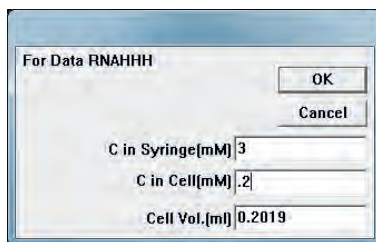| Step	Action
9	Click the **200 Iter.** button one or two times to ensure that Chi-square is no longer decreasing and then click **OK**.

The fitting parameters should be similar to those displayed below.

Data: Dissociatio_NDH
Model: Dissociation
Chi^2 = 0.1881
DH (cal/mole)   -9994   ±16.7
K (mM)   0.623   ±0.0037

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.12 Competitive ligand binding

7.6.12 Competitive ligand binding

Introduction

Competitive binding experiments are carried out by injecting a strongly binding ligand A into a solution that contains both the macromolecule and the competing ligand B. The ligand A appears to bind more weakly to the macromolecule in the presence of the competing ligand B 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 the binding parameters for ligand B (N_B, K_B and ΔH_B) itself. These three parameters are used as input allowing N_A, K_A and ΔH_A to be determined from results of the competitive experiment.

Designing a competitive experiment

When designing a competitive experiment, the total concentration of the competing ligand, $[B]_{tot}$, should be selected so that

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

where "K_A" is the estimated value of K_A. This insures that the apparent binding constant in the competitive experiment will be in the best "window", 10^5 to 10^8 M^{-1}, to be easily measured by ITC.

Fitting data using the competitive ligand binding model

To fit data using the competitive ligand binding model, follow the steps described below:

Note: *In the following example, results from a conventional, non-competitive experiment have already been analyzed to obtain the parameters N_B=0.993, K_B=21600 M^{-1} and ΔH_B= -11700 cal/mole. The data from the competitive experiment have been saved in an area data file named **Competitive.DH**, which will be analyzed below.*

Step	Action
1	Select **File:New:Project**.
	A new Origin project opens to display the **RawITC** plot window.
2	Click the **Read Data..** button.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.12 Competitive ligand binding
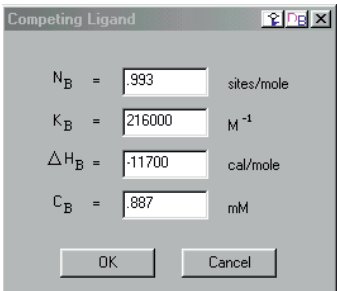
Step	Action
3	Click the drop-down arrow of the **Files of type** text box and select **Area Data (*.DH)** file type.
4	Navigate to the `C:\Origin70\Samples` folder and select **Competitive.DH** from the **File Name** list, and click **OK**.

The **Competitive.DH** file opens, the data are normalized on concentration and plotted in the **DeltaH** window.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.12 Competitive ligand binding
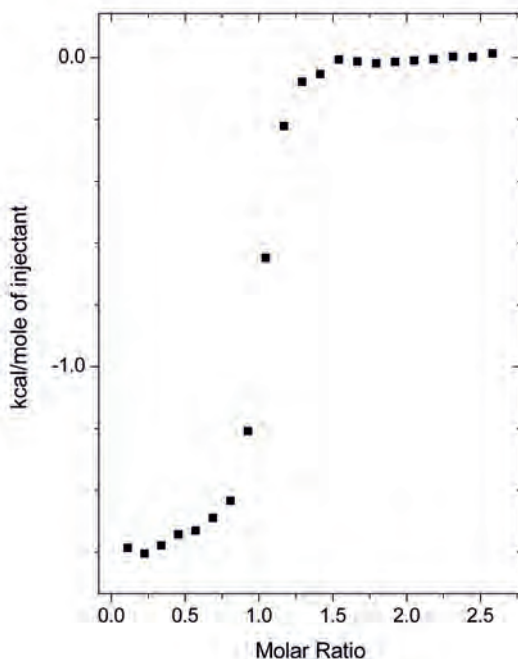
Step	Action
5	Click the **Competitive Binding** button.
	The **Competing Ligand** dialog box opens.

Step	Action
6	Enter the following values obtained from the first experiment $N_B = 0.993$, $K_B = 216000$, $\Delta H_B = -11700$ and $C_B = 0.887$ and then click **OK**.
	The **NonLinear Curve Fitting: Fitting Sessions** dialog box for the competitive binding model opens.
7	Click the **200 Iter.** button one or two times to ensure that Chi-square is no longer decreasing and then click **OK**.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.13 Simulating curves
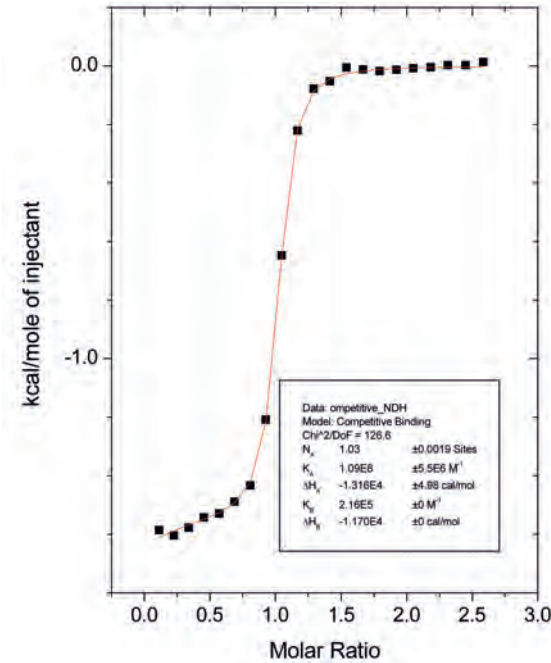
7.6.13   Simulating curves

Introduction

Titration experiments can be simulated without actually going through the fitting routine. The simulated curve may or may not be related to actual data obtained. To simulate data, there must be some ITC results in computer memory (either raw data called up, or an Origin project that contains data) but these results need not be related to the simulations carried out. The data in memory should contain at least as many data points (or number of injections) as the curve to be simulated.

Note: *For proper simulation, use a data file that has all injections of the same volume. Do not use a file that has a preliminary first injection of a different size.*

Simulating a fit curve

To simulate a fit curve, follow the steps described below:

Step	Action
1	Exit the fitting session and start a new project by selecting **File:New:Project** (or click on the **New Project** button) from the menu.
2	Click the **Read Data..** button in the **RawITC** window.
3	Select **ITC Data (*.ITC)** from the **List Files As** type box.
4	Open the **Rnahhh.ITC** data file located in the C:\Origin70\Samples folder. The **DeltaH** window becomes the active window.
5	Click the **Concentration..** button in the **DeltaH** window.
6	Change the concentrations and injection volume values to those desired for the simulation. For this example, set concentration in syringe to 3, concentration in cell to .2.

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7.6 Advanced curve fitting
7.6.13 Simulating curves

Step	Action
7	Click **OK**.
8	Click the **Two sets of Sites** button from the model fitting box.
	The fitting session dialog box opens with the **Two Sets of Sites** model selected.
9	Enter the following parameters in the parameters text boxes:
	n1 = 1, **K1** = 1e7, **H1** = 10000, **n2** = 1, **K2** = 1e5, **H2** = −10000.
10	Click the **Chi-Sqr** button.
	The simulated curve **YFIT** appears in the **DeltaH** window.
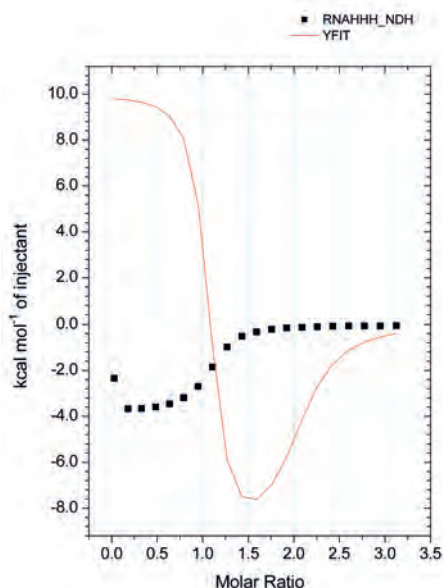
Note:

*Do not to click on the **1 Iter.** or **200 Iter.** buttons or the parameters will be changed.*

Tip:

The original data subtracts from the simulated data, but this data is required to be in memory for simulated data. This data cannot be deleted but can be hidden from view. Right-click directly on any of the square data points of the RNAHHH_NDH curve and select **Hide**.

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7.6.13 Simulating curves

Step	Action
11	Right-click on the simulated data trace and select **Change to Line + Symbol**. *Note:* *The simulated data has twenty data points just as the original Rnahhh curve. Also it appears that the simulated curve has not leveled due to complete binding. This can be corrected by clicking the **Concentration..** button to increase the concentration in the syringe, decrease the concentration in the cell or increase the volume of the injection. Alternatively, start over and read in a data set with more data points (or injections).*
12	Select **Window:DeltaH** and click the **Concentrations** button. Enter 0.2 mM for the concentration in the cell. The graph rescales on the x-axis, but the simulated curve will not be affected until the **Chi-Sqr** button is clicked again.
13	Click the **Chi-Sqr** button. The curve is simulated using the new concentration.

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7.6.14 Single injection method (SIM)

7.6.14 Single injection method (SIM)

Introduction

The MicroCal Auto-iTC$_{200}$ is also capable of carrying out 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.

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

Automated steps performed before analysis

1 Data is corrected using the instrument's time constant.

2 The corrected data set is filtered using the standard Fourier transform filter in Origin 7.0 and a bandwidth of 15 data points.

Perform the following actions:

Step	Action
1	Zero the baseline from which the experimental data is to be subtracted (see*Zeroing the baseline, on page 265*).
2	Exclude distorted or extraneous data points prior to subsequent analysis.

Creating a new worksheet

The raw data (after time constant correction, Fourier filtering, baseline subtraction, and eliminating inappropriate data) can then be used to form a new worksheet, which is modeled after the existing worksheet used with multi-injection binding data.

7 Data analysis using Origin
7.6 Advanced curve fitting
7.6.14 Single injection method (SIM)

Creating *SIM ITC* icon on the desktop

To create a *SIM Analysis* icon on the desktop, follow the steps described below:

Step	Action
1	Right-click any Origin 7 icon on the desktop.
2	Select **Copy**, right-click on desktop and select **Paste** to create a copy of Origin 7 icon.
3	Right-click the copy of the icon, select **Rename**, and enter SIM ITC to rename the icon.
4	Right-click the **MicroCal SIM AnalysisC** icon, select **Properties**. In **Target** window, change the final number of target to 8, and click **OK** to change the target of the desktop icon to SIM.

Input SIM data

To input SIM data, follow the steps described below:

Step	Action
1	Double-click the **MicroCal SIM Analysis** icon on desktop.

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Step	Action
2	Click the **Read Data** button in the **Single Injection** group.

The import multiple ASCII **Open** dialog box opens. The only option for **Files of type** is **ITC Data (*.sim or *.itc)**.
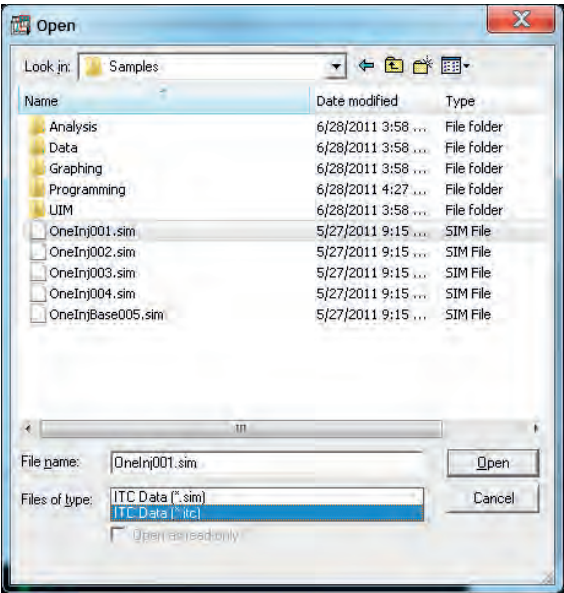
3	Navigate to the `C:\Origin70\Samples` folder and select **OneInj001.sim** from the files list.

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Step	Action
4	Click **Open**.
	The file is then read in and the following operations are executed on the data set:

1. The data is read into a worksheet that is created with the corresponding name and RAW appended (i.e., the worksheet is named **OneInj001RAW**).

2. The time before the injection starts (60 s) is subtracted from the x data so that the injection starts at t=0 and all data points are shifted to the left.

   Note:

   The x data before t=0 is removed from the worksheet, but the data is still plotted in the graph for use in baseline subtraction.

3. The data is corrected for the time constant of the instrument.

4. The noise introduced by the time constant correction is filtered using the standard Fourier transform filter of Origin and a bandwidth of 15 data points.

5. The corrected and filtered data is then plotted in the **ARawITCsi** window.

Zeroing the baseline

Subtract Options

Clicking on the **Subtract Options** button opens the **Control Baseline Subtraction** window, which displays the following options:

Button	Function
Input final numerical Y position	Clicking this button prompts for a final Y position (in µcal/sec). The end point of plotted data set is placed at that position and the rest of the data set is offset proportionately. Typically 0 is used as the final Y position. *Note:* *Use this button for fast data reduction.*
Subtract a constant	Clicking this button prompts for a constant (µcal/sec) that will be subtracted from all data sets plotted in the **ARawITCsi** graph.

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7.6.14 Single injection method (SIM)

Button	Function
Subtract reference data	Clicking this button allows the heats from the control experiment to be subtracted from the data set that is plotted in the **ARawITCsi** graph. **Note:** Data set from a control experiment is required.
Y axis shift	Clicking this button changes the cursor to the data reader tool. Click once to see the y-axis position of the data reader tool. Double-click or press **enter** to move the end point of the data set to that y position.
Straight line	Clicking this button changes the cursor to the data reader tool. Double-click at the point on the graph where the line should begin, and double-click again at the point where the line should end. A straight line is created between the two points and extrapolated to be subtracted from all data points. **Note:** Use this button when baseline is not horizontal.

The baseline can be set to zero by following either of the two recommended methods described below:

Method 1:

Step	Action
1	Click the **Subtract Options** button from the **Single Injection** group of buttons. The **Control Baseline Subtraction** dialog box pops up.
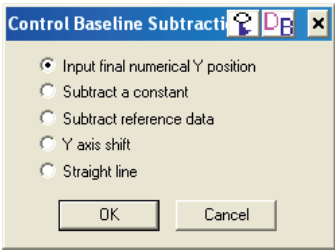
Step	Action
2	Select **Straight line** option. The cursor changes to the data reader tool.

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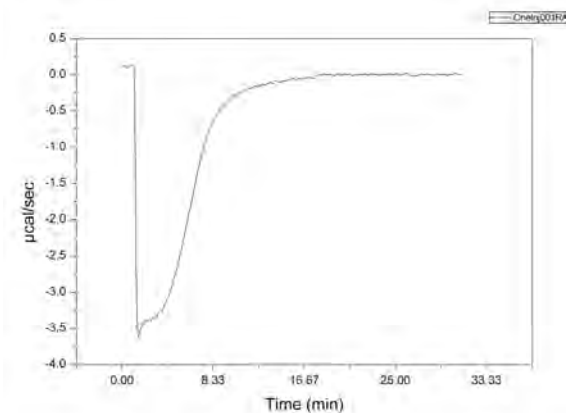
Step	Action
3	Double-click the data reader tool near the end of the curve (about 30 min) and double-click again on the curve at 0 min.
	Origin quickly creates a straight line, which is extrapolated and subtracted from all data points, as shown below.
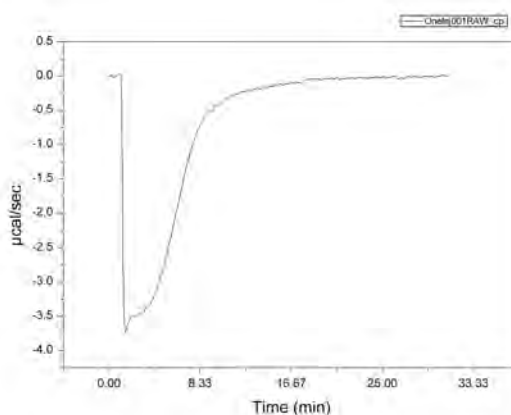
Method 2:

Step	Action
1	Click the **Read Data** button
2	Re-open the **OneInj001.sim** data file.
3	Click the **Subtract Options** button and select **Input final numerical Y position** option.
4	Enter 0 in **Value** window.

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Step	Action
5	Click **OK**.

A plot similar to the one shown below is displayed.

Removing bad data

Introduction

Data at the beginning of the experiment might be distorted when the time constant is corrected and there may be extraneous data points after the injection is complete. The **Remove Bad Data...** button simplifies the task of excluding these data points from subsequent analysis. At the start of the injection, typical experiments exhibit a high point (exothermic reaction) or a low point (endothermic reaction).

Options in the *Remove Bad Data* window

Clicking the **Remove Bad Data...** button opens the **Remove Bad Data** window. This window displays the following options:

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Option	Function
Remove data before	This option allows selection of either **High Point** or **Low Point**.
	Origin searches each data set and deletes all data before the corresponding high or low point in the data. The data is then plotted on the graph with the beginning data point removed.
Remove selected range	Each data set is sequentially plotted on the graph with two data markers displayed on the trace.
	Tip:
	Click and drag on a marker to move it to the desired point on the trace, then double-click or press **enter** to set the point. All data between the two markers will be removed from the graph and eliminated from future analysis.
	Tip:
	When moving a data marker, press the space bar to increase the size of the cross-hair.

Removing bad data

To remove bad data, follow the steps described below:

Step	Action
1	Click the **Remove Bad Data...** button from the **Single Injection** group.
2	Select the **Remove data before** option and checkmark the **Low Point** box.

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Step	Action
3	Click **OK**.

A graph similar to one shown below is plotted.
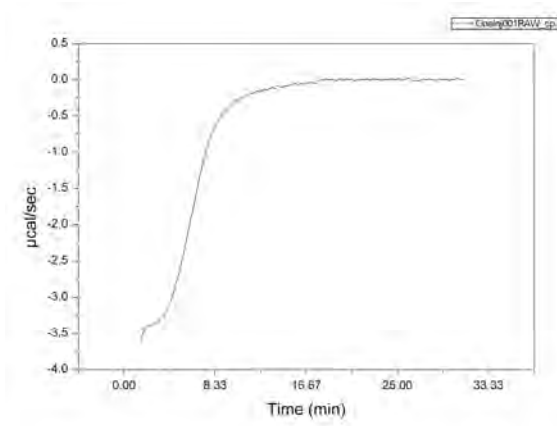
Normalizing data points

To normalize data points, click the **Normalize Data** button ![Normalize Data] from the **Single Injection** group.

The concentration is calculated and the normalized heat is plotted in a new window, named **DeltaH**. The data is now in the form of conventional ITC normalized data and may be fit with the methods described in previous sections (see *Using the **One Set of Sites** model with multiple data sets, on page 280*).

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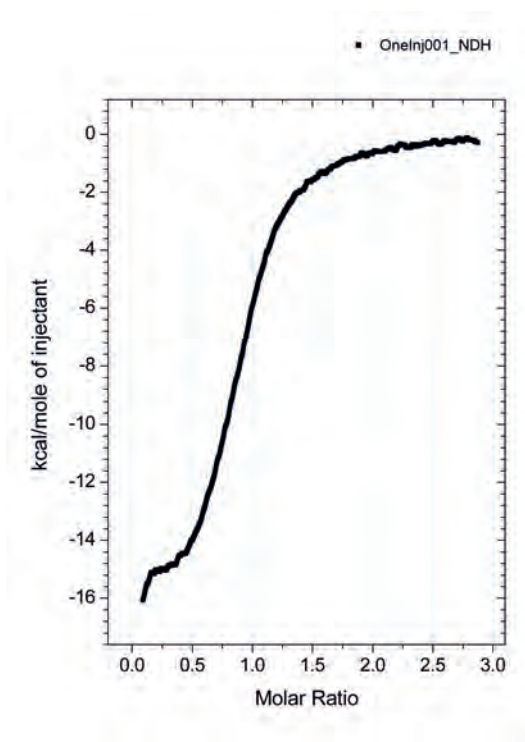
7.7　Batch-processing data with Origin

Introduction

The MicroCal Auto-iTC$_{200}$ Origin module contains curve fitting routines that operate on multiple data files.

Note:　*Review the following sections of this chapter, before starting this section, if not familiar with the curve fitting of standard ITC data:*

- *Section 7.1 Basic ITC data analysis and fitting, on page 144*

- *Section 7.2 Adjusting baseline and integration range, on page 160*

- *Section 7.3 Analyzing multiple runs and subtracting reference, on page 167*

Launching the MicroCal Auto-iTC$_{200}$ data session

To launch the batch-processing version of Origin, double-click on the **MicroCal Auto-iTC$_{200}$** icon on the desktop.
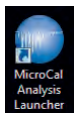
Select the **Auto-iTC$_{200}$** button.

Opening individual files

To open individual files or a selection of files, follow the steps described below:

Step	Action
1	Click the **Read Data...** button.
	The **Import Multiple ASCII** dialog box opens.
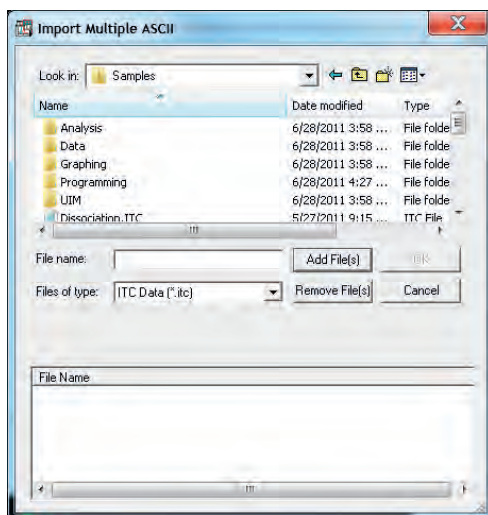
Step	Action
2	Select the **Files of type** to be **ITC Data (*.itc)**.
	All files with the .itc extension will be listed in the upper list box.
3	Manually select the file named **itc0523c001-006.itc** and add it to the lower list box to be read into Origin.

7 Data analysis using Origin
7.7 Batch-processing data with Origin
7.7.1 Post import updating

7.7.1   Post import updating

Changes to concentrations, dilution factors, and control runs can be carried out after import. These changes can be done easily in the ITC summary table. To activate this window, select the menu item **Window:MicroCal VP-ITC Autosampler Summary Table**.

Filename	Worksheet	Control Run	Tray #	Well #	Syr. Conc.	Cell Conc.	Syr. Dil. Factor	Cell Dil. Factor	Comments
File001.ITC	Data1	Control	1	4	0.1825	0.0122	2	4	
File002.ITC	Data2	File001.ITC	1	6	0.1825	0.0122	2	4	
File003.ITC	Data3	Control	1	8	0.1825	0.0122	2	4	
File004.ITC	Data4	File003.ITC	1	10	0.1825	0.0122	2	4	
File005.ITC	Data5	File003.ITC	1	12	0.1825	0.0122	2	4	
File006.ITC	Data6	File003.ITC	1	14	0.1825	0.0122	2	4	

Editing control runs

To edit control runs, follow the steps described below:

Step	Action
1	Select **Set Control Run** from the **Advanced** menu to designate a control run for an experiment. This opens the **Set Control File** dialog box (only if control runs are designated).
2	Select the control run from the **Control Run** drop-down list. Only designated control runs can be selected.
3	Select the **Data File** from the **Data File** drop-down list. • The **Apply to All Files** check box sets the control run for all non-control run files. • The **Apply to Rest** check box sets the control run for **Data File** and the non-control run files in the table below the **Data File** row.

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7.7 Batch-processing data with Origin
7.7.1 Post import updating
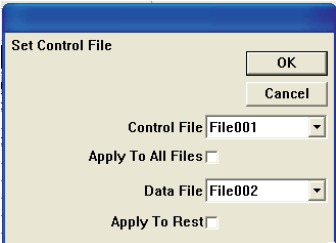
Step	Action

4   Select either **Subtract Control DP Data** or **Subtract Control NDH Data** to perform control run subtraction.
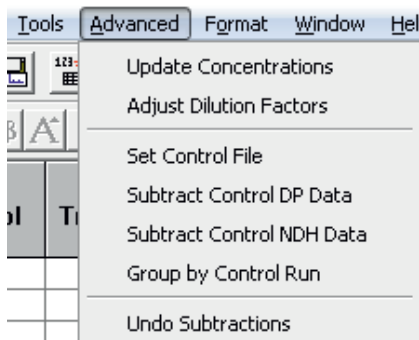
Note:

*The **Group by Control Run** option can be toggled to plot the subtracted data into **ADELTAHControlRunName** or **ADELTAHControlRunName** plots.*

5   Select the **Undo Subtractions** option from the **Advanced** menu to restore original DP and NDH data.

7 Data analysis using Origin
7.7 Batch-processing data with Origin
7.7.1 Post import updating

Updating dilution factors and concentrations

To update dilution factors and concentrations, follow the steps described below:

Step	Action
1	Select the **Adjust Dilution Factors** option in the **Advanced** menu.
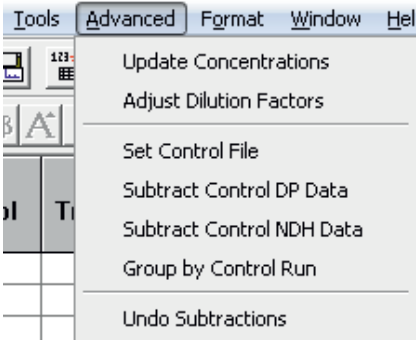
This opens the **Dilution Factors** dialog box.
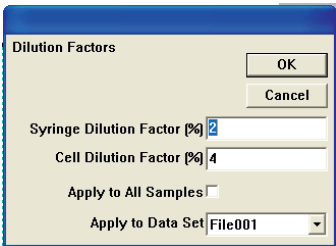
2	Edit the summary table with the desired concentrations to update concentrations.
3	Choose **Update Concentrations** from the **Advanced** menu.

Note:

The internal concentration used in calculating NDH is the product of the concentration and the dilution factor:

*Internal Concentration = Concentration * (100 – Dilution Factor) / 100*

7 Data analysis using Origin
7.7 Batch-processing data with Origin
7.7.2 Analysis control buttons

7.7.2   Analysis control buttons

Description of analysis control buttons

Apart from the buttons associated with standard ITC, there is a group of buttons labeled analysis control that perform data manipulations on multiple ITC files. These are visible only in the *DeltaH* window. Examples of the use of each button are presented in the following sections. The different options available in the analysis control buttons and their functions are described below:
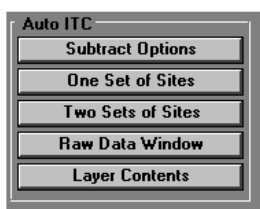
Button	Description
Subtract Options	This button opens the *Control Baseline Subtraction* dialog box, which provides four different means to adjust the experimental datasets to minimize the heats of dilution.
One set of Sites	This button applies the *One set of Sites* fitting model to all data sets plotted in the *AdeltaH* graph. The calculated fitting parameters will then be printed in a summary table.
Two sets of Sites	This button applies the *Two sets of Sites* fitting model to all data sets plotted in the active layer and prints the fitting parameters in a summary table.
Raw Data Window	This button provides a quick method to return to the *ARawITC* window.
Layer Contents	This button opens the *Layer Contents* dialog box. This box displays available data sets along with the data sets that are being displayed. Data sets can be moved in and out of the displayed layer contents list.

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7.7 Batch-processing data with Origin
7.7.2 Analysis control buttons

Subtracting the heats of dilution from area data manually

To subtract the heats of dilution effect from area data, click the **Subtract Options** button

Subtract Options .

The **Control Baseline Subtraction** dialog box opens, which displays multiple options.
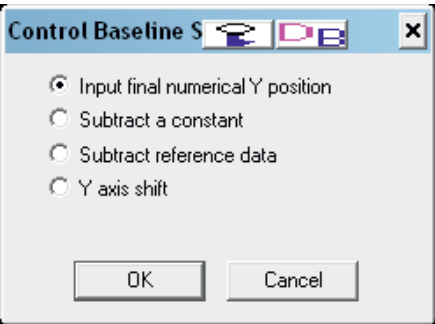
Options in the *Control Baseline Subtraction* window

The **Control Baseline Subtraction** window displays the following options:

Button	Description
Input final numerical Y position	This button prompts for a final Y position (in kcal/mole). The end point of each plotted data set is placed at that position and the rest of the data set is offset proportionately.
Subtract a constant	This button prompts for a constant (kcal/mole) to be subtracted from all data sets plotted in the **ADeltaH** graph.
Subtract reference data	This button subtracts the heats from a control experiment from all data sets that are plotted in the **ADeltaH** graph. The control experiment must have same or greater number of injections (data points) as any of the plotted data sets.

7 Data analysis using Origin
7.7 Batch-processing data with Origin
7.7.2 Analysis control buttons

Button	Description
Y axis shift	This button moves all data sets out of the graph (layer) and the cursor changes to the data reader tool. The first data set is moved back into the graph. Click once to see the y-axis position of the data reader tool. Double-click or press **enter** to move the end point of the data set to that y position. The data set is removed from the graph and the next file in the series is plotted in the graph. Repeat the process for all data sets that were originally plotted in the graph.

Auto control run subtraction

Subtracting control runs from datasets

When control runs are designated in **VPViewer Detection**, they will automatically populate the summary table with the control run designation. To view the summary table, select the **MicroCal Auto-iTC200 summary table** from the **Window** menu.

Experiments that are designated controls in the MicroCal Auto-iTC$_{200}$ control software will not have the summary table automatically populated. In the **Raw Data** window, use the **Control Run Detection Options** in the **Advanced** menu to control run designation and subtraction (see illustration below). Remember, not all control subtraction methods are the same. Subtracting control data point-by-point may not be appropriate for a given system. Fitting a line to control data, and then subtracting that line manually may be more appropriate (for example, see *Section 7.3 Analyzing multiple runs and subtracting reference, on page 167* for all control subtraction options).
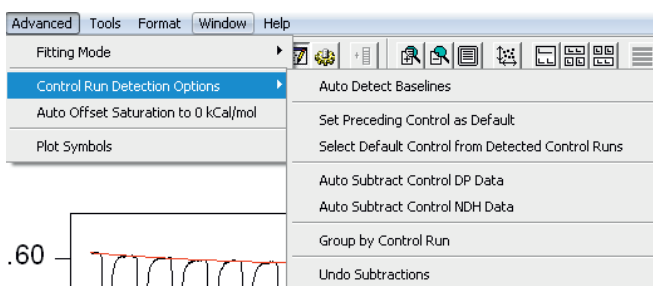
Control Run Options

The following table describes the functions of the **Control Run Options**:

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7.7 Batch-processing data with Origin
7.7.2 Analysis control buttons

Part	Function
Auto Detect Baselines	Activates control run detection. All designated control runs will be assigned as "Control" in the **Control Run** column of the AutoITC summary table.
Set Preceding Control as Default	This option sets the previously detected control run as the control of all following data runs, until another control run is detected.
Select Default Control from Detected Control Runs	After all files are imported, choose the default control run to apply to all data sets from all detected control runs.
Auto Subtract Control DP Data	Automatically subtracts control run DP from data DP.
Auto Subtract Control NDH Data	Automatically subtracts control NDH from data NDH.
Group by Control Run	All data sets with the same control run are plotted in the same window. If DP data is subtracted, data sets are plotted in a plot called **ARAWITCControlRun**. If NDH data is subtracted, data sets are plotted in a graph called **ADELTAHControlRun**.
Undo Subtractions	This option restores all data sets to original values before subtraction.

Using the *One Set of Sites* model with multiple data sets

To use the **One Set of Sites** model with multiple data sets, follow the steps described below:

Step	Action
1	Click the **One Set of Sites** button.
	Each data set that is plotted in the graph will be fitted to the **One Set of Sites** model and the parameters **n** (number of sites), **K** (binding constant), ΔH (enthalpy change) and ΔS (entropy change) are calculated. These parameters are then printed in the summary table.

7 Data analysis using Origin
7.7 Batch-processing data with Origin
7.7.2 Analysis control buttons

Step	Action

2

Select the menu item *Window:MicroCal Auto-iTC$_{200}$ Summary Table*.

The summary table displays the parameter values.

Using the *Two Sets of Sites* model with multiple data sets

To use the *Two Sets of Sites* model with multiple data sets, fit the data to the *Two Sets of Sites* model. Perform the same preliminary operations as described in the previous section and then click the *Two Sets of Sites* button [Two Sets of Sites] .

Each data set that is plotted in the graph will be fitted to the *Two Sets of Sites* model and the eight parameters *n1*, *n2* (number of sites), *K1*, *K2* (binding constants), *ΔH1*, *ΔH2* (enthalpy change) and *ΔS1*, *ΔS2* (entropy change) are calculated. These parameters are then printed in the summary table.

Returning to the *ARawITC* window

To return to the *ARawITC* window, click the *Raw Data Window* button [Raw Data Window] .
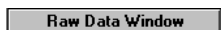
Editing the layer contents

To edit the layer contents, click the *Layer Contents* button to open the layer contents dialog box.

The available data column contains all the data sets in the current project. The displayed data column contains all the data sets to be plotted.

7.8   Other useful details

Chi-square (Chi²) 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 χ^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 above 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. 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 Chi²/DoF.

Line types for fit curves

Plot Details dialog box

To open the **Plot Details** dialog box, follow the steps described below:

Step	Action
1	Double-click on the data plot.
2	Right-click on the data plot and select **Plot Details** from the shortcut menu.
	Alternatively, select the desired data plot from the **Data** menu data list and select **Format:Plot**.

The line group

Select the desired line connection from the associated **Connect** drop-down list. The line connection type affects the interpolation results. The default line type for fit curves is straight line.

The most common methods of connecting the fit curve data points are described below:

Line type	Description
Straight	A straight line is displayed between the data points. This type of line connection will not give a smooth representation of the fit curve if there are only a few data points.
Spline	This option generates a cubic spline connection. To use the connection, the X values must be discrete and increasing. Furthermore, the number of data points cannot exceed 900. The operation fails if the data set exceeds this number.
	Since the curvature information is held in memory, the spline resolution remains the same regardless of the page magnification. The **SplineStep** variable in the **ORIGIN.INI** file controls the spline calculation increment. It is expressed in units of .1 point. This is usually the most satisfactory representation of the fit curve, but may exhibit an excursion from the actual fit curve if there is a sharp corner in the data.
B-Spline	The B-spline curve can be described by parametric equations. Unlike spline curves, which pass through the original data points, the B-spline curve winds around the original data points without passing through them. Thus, this curve may not produce a satisfactory representation of the fit curve. For a complete discussion of the B-spline connection, see *Origin User's Manual*.

View mode

Introduction

Each Origin plot window can be viewed in any of four different view modes:

- *Print View*,

- *Page View*,

- *Window View*, and

- *Draft View*.

These are available under the **View** menu option.

Print view

Print View is a true WYSIWYG (What You See is What You Get) view mode. This view mode displays a page that corresponds exactly to the page from the hard copy device. Exact font placement and size is guaranteed, but with some sacrifice to screen appearance, since the printer driver fonts must be scaled to fit their positions on the page (this will not harm the appearance of true vector fonts). This is a slow process, and screen refresh speed suffers as a result. Thus, reserve the **Print View** mode for previewing the work prior to printing.

Note: *Origin automatically changes to **Print View** mode when graphics are exported to another application and when printing. The view mode automatically returns to the selected view mode after the operation is complete.*

Page view

Page View provides faster screen updating than **Print View**, but does not guarantee exact text placement on the screen unless typeface scaling software (such as **Adobe Type Manager**) is being used. Use **Page View** mode until the application is ready for printing or copying to another application. Change to **Print View** mode to check object placement before exporting, copying, or printing.

Window view

Window View expands the page to fill up the entire graph window.

Note: *Labels, buttons, or other objects in a graph window that reside in the gray area of the page are not visible in **Window View** mode.*

Draft view

Draft View has the fastest screen update of the four view modes. In **Draft View**, the page automatically sizes to fill the graph window. This is a convenient mode to use when looking at on-screen data is the primary focus. **Draft View** is the fastest view mode, and is very useful when precise formatting is not required.

Note: *The type of view mode will not affect the print-outs, but only on-screen display will be affected.*

Inserting an Origin graph into Microsoft Word

There are two ways to include the Origin graph into Microsoft Word (or other applications), either import the graph into Word or link (share) the graph to Word. When importing the graph, Word displays the graph as an object and it cannot be edited by Origin tools (although it may be resized or repositioned in the Word document). When linking (share) the graph, Word displays the graph as an object, which can be edited by Origin and updated when the Origin graph changes.

Please refer to the Origin manual for more information about creating a graphical presentation.

Importing the graph into Microsoft Word

To import the graph into Word, follow the steps described below:

Step	Action
1	Create the graph in Origin and select **Edit:Copy Page**.
2	Open the Word document and click at the location where the graph should be placed.
3	Select **Edit:Paste Special**.
4	Select **Origin Graph Object** from the **As:** list box.
5	Select the **Paste Link** radio button.
6	Click **OK**.

Linking the graph to Microsoft Word

To link the graph to Word, follow the steps described below:

Step	Action
1	Create the graph in Origin and then save it as part of an Origin project (***.OPJ**).
2	Open the saved Origin project (if it is not already open) that includes the desired graph window.
3	Make the desired graph window active, and select **Edit:Copy Page**.
4	Open the Word document and click at the location where the graph needs to be inserted.
5	Select **Edit:Paste Special**.
6	Select **Origin Graph Object** from the **As:** list box
7	Select the **Paste Link** radio button.

Step	Action
8	Click **OK**.

After the Origin graph is linked to Word, return to the original Origin graph and make changes to the graph. These changes can be reflected in the Word document by selecting **Edit:Update Client** from the Origin menu.

Tip: *Start Origin and load the linked graph by simply double-clicking on the graph while in Word. Origin starts with the original document loaded, and the changes can be made by selecting* **Edit:Update Client***. The changes are automatically reflected in the Word document*.

8   Maintenance

Introduction

This chapter provides information about the maintenance of the instrument to ensure proper operation. Regular maintenance by the user of the MicroCal Auto-iTC$_{200}$ instrument is essential for quality experiments and results. The maintenance tasks described below are listed roughly in the order of their required frequency. Also, it is recommended that the instrument be shut down when not in use (**Power** switch in the rear).

In this chapter

This chapter contains the following sections:

8.1 Cleaning the cell

Introduction

Cleanliness of the cell has a significant impact on data quality. A dirty cell typically manifests itself in poor loading of the cell (a low baseline position – see *Section 9.4 Upward stepping baseline, on page 311* and *Section 9.7 Low baseline, on page 317*)

Basic cleaning

The most basic cleaning procedure using detergent (20% Contrad 70™ (or 14% Decon 90™) in deionized water) involves briefly soaking the cell with detergent and then flushing with water. The **Plates** automation method, for example, and any method using the **Detergent Clean** cell cleaning script performs this operation.

Extra Clean

The **Extra Clean** cell cleaning script loads the cell with detergent, raises the cell temperature to 60°C, soaks for one hour, and then rinses the cell with water. Clicking the **Clean Cell** button in the **System tab** in the MicroCal Auto-iTC$_{200}$ software also performs this operation.
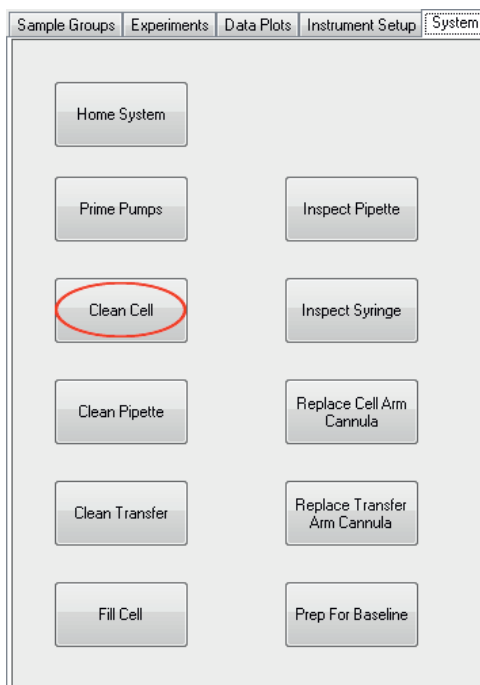
Recommended cleaning routines

Interval	Cleaning Instructions
After every run	Clean the cell with detergent (i.e., **Plates** automation method).
Weekly or If poor data is evident	Perform a rigorous cell cleaning by selecting the **Clean Cell** button in the **System** tab (see above) or by running an experiment that uses the **Plates Clean** automation method. Water rinses follow detergent rinses and are rigorous enough to remove all the detergent.

8.2   Refilling the reference cell

Introduction

The MicroCal Auto-iTC$_{200}$ has two cells, the sample cell and the reference cell. The Autosampler cleans and refills the sample cell for each run. However, the reference cell must be refilled manually, approximately once a week. An underfilled reference cell can manifest itself as a starting baseline position *greater* than specified in the ITC Method.

Procedure

To refill the reference cell, follow the steps described below:

Step	Action
1	Tap **Open Door** on the touchscreen.
2	Gently insert the glass Hamilton syringe into the right reference cell until it touches the bottom.
3	Suck out the liquid completely by pulling up the syringe plunger. ***Note:*** *Ensure that no bubbles are trapped in the cell.*
4	Remove and empty the syringe. Clean the syringe, if necessary.
5	Pull approximately 300 μl of degassed, distilled water into the syringe. Tap the syringe glass gently so that all the bubbles are at the top volume of the syringe.
6	Insert the syringe into the cell and gently touch the bottom of the cell with the tip of the syringe needle. Raise the needle tip about 1 mm off the bottom of the cell, and hold it there until finished filling. ***Note:*** *Make sure not to raise the syringe during the filling process.*
7	Inject the solution slowly into the cell until it spills out the top of the cell stem. Dislodge any trapped bubbles with several abrupt spurts of the solution. ***Note:*** *Ensure that no bubbles are transported into the reference cell while loading the solution.*
8	Lift the tip of the syringe to the cell port (just below the visible portion of the cell port) and remove the excess solution.

Step	Action
9	Remove the syringe. Install the reference cell plug to prevent evaporation. Close and latch the left door.

8.3 Replacing the syringe plunger tip

The plunger tip forms a seal with the syringe glass. Consequently, it spins along with the syringe glass while the metal plunger itself remains stationary. As the plunger drives titrant out of the syringe glass, wear on the plunger tip can occur. Too much wear can manifest itself as poor data. If left unreplaced, the plunger can drive itself through the tip. Best practice is to replace the tip at the first sign of wear (PTFE shavings along plunger shaft, above tip) or about every 300 experiments. Practice this a few times so as to become comfortable with the routine.

To replace the syringe plunger tip, follow the steps described below:

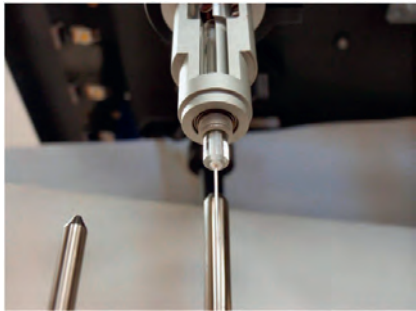| Step	Action
1	Cover the cell using a cell cap or any other means.
2	On the *System* tab of the MicroCal Auto-iTC$_{200}$ software, click the *Inspect Pipette* button.
	The pipette arm swings around to a position above the cell where the pipette and syringe can be easily accessed.
3	Loosen and remove the securing nut from bottom of the pipette.
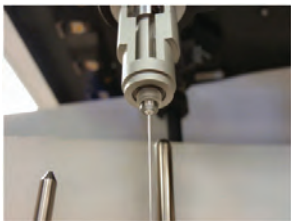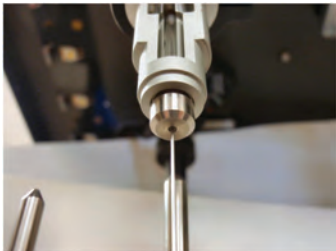
Step	Action
4	In the MicroCal iTC$_{200}$ software, click on the **Instrument Controls** tab and click **1. Remove Old Tip** in the **Maintenance** section.
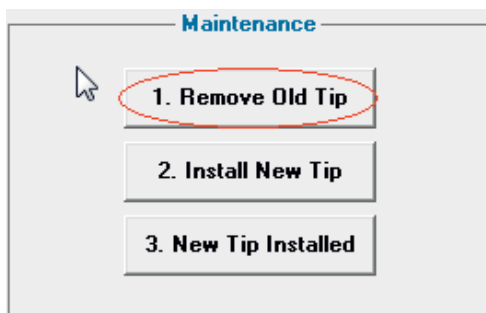
5	The software prompts to remove the old tip.

6	Click **OK**.
	Without the nut, the syringe moves down with the plunger.

| Step	Action
7	Grab the syringe bore firmly and pull straight down to remove the syringe from the pipette. The soft-grip tweezers can be used to help grip the syringe without damaging it. Set the syringe aside.

Step	Action
8	The tip of the tip puller uses a traction design that grabs the plunger tip and allows movement only in one direction.

Step	Action
9	Insert the tip puller into the pipette until the tip of the puller grabs the plunger tip. Then gently extract the tip puller (along with the pipette tip) from the pipette.

Note:
Make sure nothing falls into the cell.

Step	Action
10	In the MicroCal iTC$_{200}$ software, click on the **Instrument Controls** tab and click **2. Install New Tip** in the **Maintenance** section.
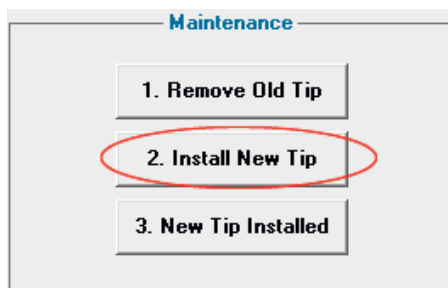
The plunger moves downward and displays the following message:

Step	Action
11	Click **OK**.
12	Insert a new plunger tip inside the tip pusher tool.
13	The tip pusher tool has a hole on one side. Insert the plunger tip inside that hole with the plunger tip hole facing outward.

Step	Action
14	Insert the tip pusher and new tip into the pipette and press the tip into place.

Note:

Do not push too hard. Resistance should be felt initially.
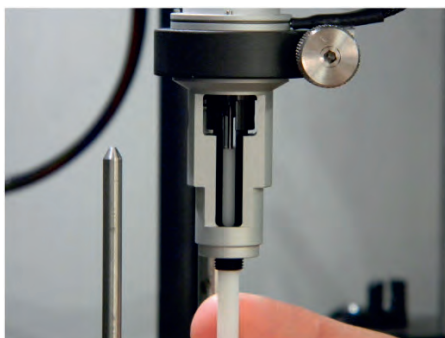
Once the tip slips over the barbed plunger, remove all pressure.

15	In the MicroCal iTC$_{200}$ software, click on the **Instrument Controls** tab and click **3. New Tip Installed** in the **Maintenance** section.
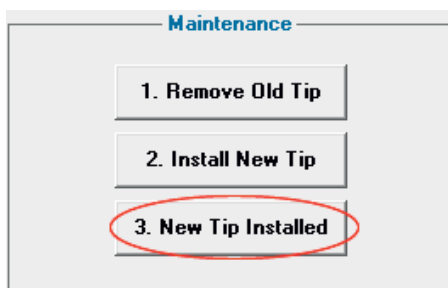
See *Section 8.4 Replacing and cleaning the titration syringe, on page 297* to reinstall the syringe.

8.4   Replacing and cleaning the titration syringe

Introduction

The syringe must be removed, cleaned and carefully inspected as a part of preventive maintenance. To inspect the upper section of the syringe, it must be removed from the pipette.

Step	Action
1	Check the upper (glass) section of the syringe extra carefully.
2	Replace the syringe if it shows any sign of damage.

A broken syringe will not operate in the wash/load station properly, will likely result in poor experimental results, and could contaminate the cell with broken glass. A dirty syringe is not nearly as common as a dirty cell but can also result in poor data.

Detergent cleaning of the syringe between runs is recommended if performing reverse titrations (protein is loaded into the syringe). If poor data persists after extensive cell cleaning, remove the syringe for cleaning.

> **WARNING**
>
> The syringe may be contaminated with hazardous residual compounds. Consult your completed Health and Safety Declarations Form to determine if any biologically or chemically hazardous substances have been used in the instrument. Use the appropriate personal protective equipment (PPE) as specified in the MSDS for those substances.

Note: *If the inspection of the syringe shows a break at or near the fill port, inspect the fill port adaptor, fill port plunger and the syringe-fill port plunger carefully.*

Removing the titration syringe

To remove the titration syringe, follow the steps described below:

Step	Action
1	On the **System** tab of the MicroCal Auto-iTC$_{200}$ software, click the **Inspect Pipette** button. The pipette arm swings around to a position above the cell where the pipette and syringe can be easily accessed.

Step	Action
2	Loosen and remove the securing nut from bottom of the pipette.
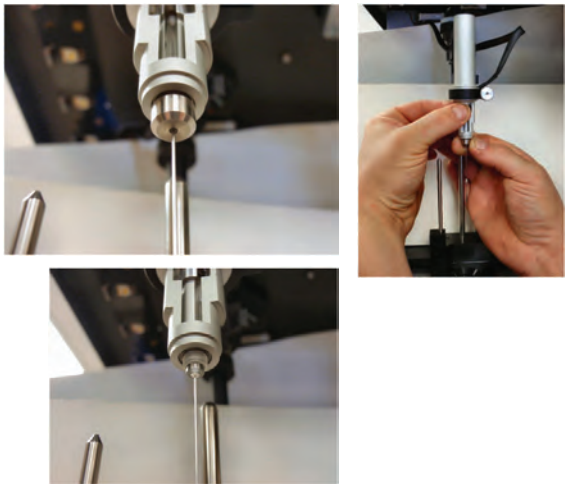
| 3 | In the MicroCal iTC$_{200}$ software, click on the **Instrument Controls** tab and click **1. Remove Old Tip** in the **Maintenance** section. |
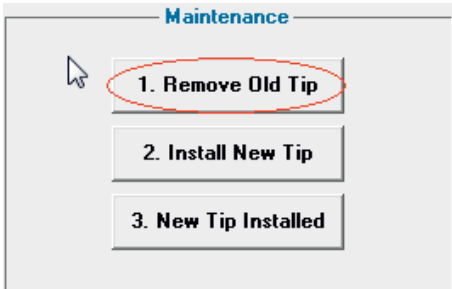
Without the nut, the syringe moves down with the plunger.

Step	Action
4	Grab the syringe bore firmly and pull straight down to remove the syringe from the pipette. The soft-grip tweezers can be used to help grip the syringe without damaging it. Set the syringe aside.
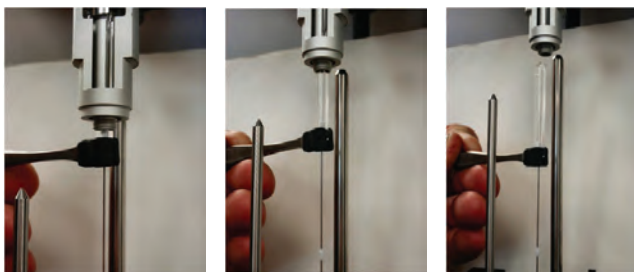
5	Once the syringe has been removed from the pipette, inspect it carefully for cracks, chips and breaks.

If the syringe shows any signs of damage, it must be replaced.

Note:

If the inspection of the syringe shows a discernible crack or break at or near the fill port adaptor (FPA) input, inspect the fill port adaptor, plunger and the alignment of the fill port plunger with the syringe carefully.

If the syringe will continue to be used, clean the top section carefully with the syringe brush.
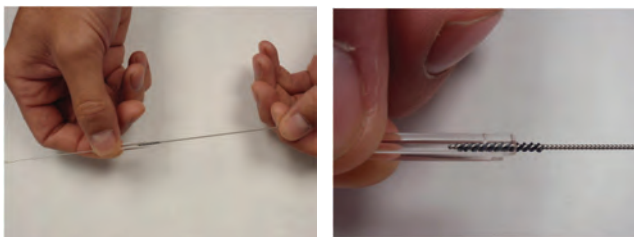
6	Slide the syringe brush in gently as far as it will go and then withdraw it. If the syringe is particularly dirty, use Contrad 70 detergent on the brush and repeat as many times as necessary until the syringe is completely clean.

Step	Action
7	Once the top section is clean, run the small piece of wire through the channel in the syringe while watching for exiting material at the bottom of the syringe. It may take a few attempts to thread the wire into the channel.
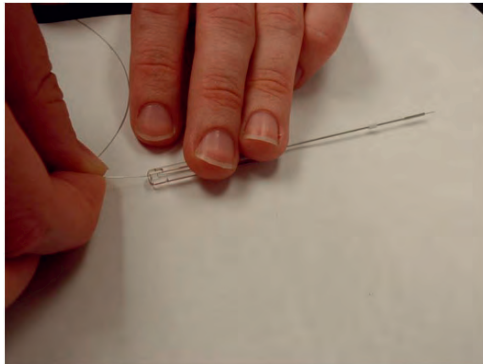
Step	Action
8	Rinse the syringe well. Replace the syringe if any material remains in the syringe that cannot be removed by this method.
9	Once the syringe is clean, carefully inspect the topmost section for evidence of cracks. Cracks tend to originate at the fill port, which is the horizontal hole where the fill port plunger slides into the syringe. Inspect that area carefully while slowly rotating the syringe to look for the cracks.

If inspection shows that the top section of the syringe has cracks or other damage, or if you are unable to pass the wire though the channel in the syringe, the syringe must be replaced.

Always examine the fill port adaptor closely following the identification of a damaged syringe.

Note: *A clogged or partially clogged syringe will result in filling problems that will show up as bad data when tests are conducted.*

Installing the titration syringe

The top portion of the syringe is keyed to slide into the pipette in only one way. The groove in the glass is keyed to slide into the small stop pin in the syringe holder.

Tip: *Syringes designed for MicroCal Auto-iTC$_{200}$ may be used in the MicroCal iTC$_{200}$ system. The pin is absent in the MicroCal iTC$_{200}$ system, so the opposite is not true.*
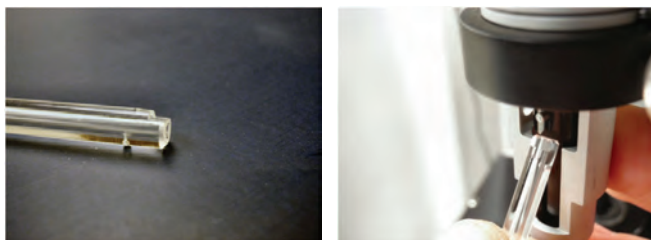
To insert a titration syringe, follow the steps described below:

Step	Action
1	In the MicroCal iTC$_{200}$ software, click on the **Instrument Controls** tab and click **3. New Tip Installed** in the **Maintenance** section.
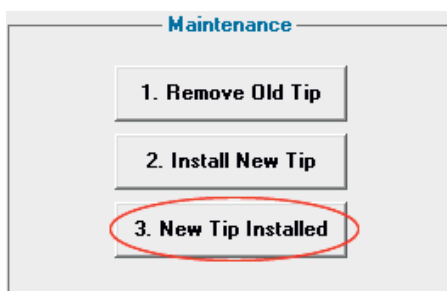
This ensures the plunger is in the topmost position.

2	Rotate the pin in the pipette forward and gently push the new syringe up into the pipette. Use the index finger of one hand to keep some pressure on the back side of the pipette to keep it from rotating. Keep the small metal pin facing forward and centered in the opening in the pipette while sliding the syringe up and into the pipette.

Step	Action
3	The syringe usually comes to a stop with about 4 mm of syringe glass exposed below the metal. (If it seats directly then there will be about 2 mm of glass exposed and the pipette and syringe can no longer be moved independently.)

Step	Action
4	Hold the pin with the index finger while rotating the syringe with the other hand. When the notch in the syringe aligns with the pin in the holder, slide the syringe up approximately another 2 mm.

When the syringe is properly seated, the syringe and the syringe holder in the pipette are locked together. The syringe is not properly seated if the syringe can spin without spinning the syringe holder.

Step	Action
5	Replace and tighten the bottom nut. Be careful not to bend or otherwise damage the paddle when reinstalling the bottom nut. Note that the tightening of the bottom nut onto the syringe affects the ultimate height of the syringe in the pipette. Over or under tightening the nut can cause a vertical misalignment of the connection between the syringe and the fill port plunger. The bottom nut should be snug but easily removed.
6	Home the system.

9 Troubleshooting

Introduction

This section contains tips and information for troubleshooting MicroCal Auto-iTC$_{200}$ System.

In this chapter

This chapter contains the following sections:

9.1   Peaks too large

Introduction

Baselines should always be within 1 µcal/s of the user-specified reference power. A normal baseline noise is visible between the tiny water into water injection peaks, if the titration syringe and cannulas are properly cleaned and completely dry so that there is no residual methanol.

The figure below came from an instrument with a broken fill port adaptor (FPA). If methanol is suspected of contaminating experiments, be sure to check the condition of the FPA.
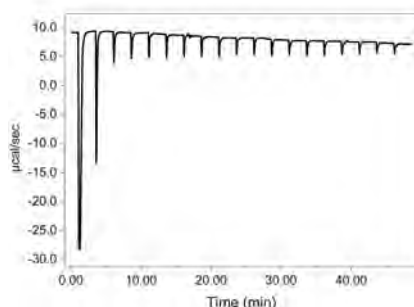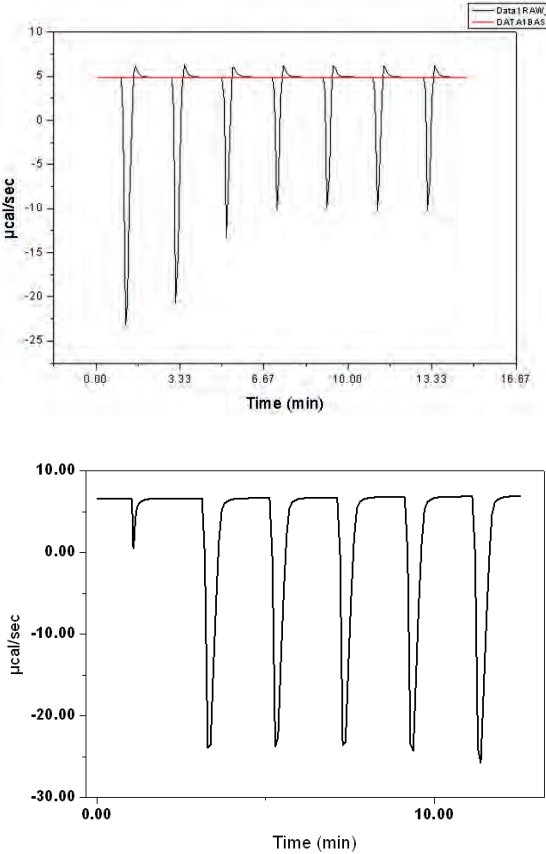
The figure below depicts a binding event before and after dialysis.
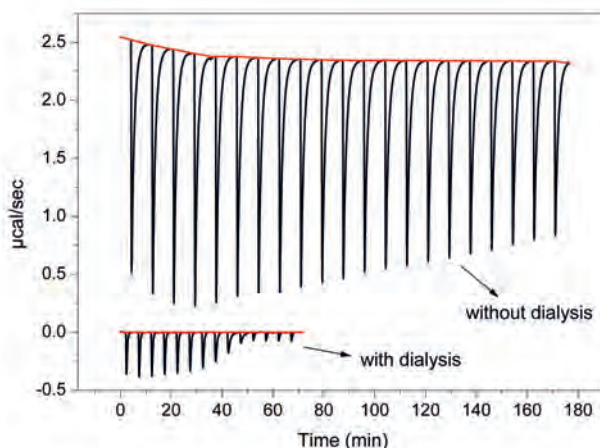
Problem causes

There are several causes of peaks being too large.

Cause	Corrective action
A buffer mismatch between the titrant and cell material.	Revisit sample preparation in *Section 5.1 Preparing the samples, on page 114.*
Methanol- All samples have consistently large peaks.	- Check if the nitrogen tank is turned on. In the figure to the left, the nitrogen tank was not turned on and the extreme heat from each injection caused the signal to negatively saturate. - Check for and fix any nitrogen leaks in the Autosampler cabinet. - The peaks will exhibit extreme heat signatures in the presence of any residual methanol. The "bounce" after each injection is typical when the signal goes below 0. *Note:* *Nitrogen source should be regulated to at least 20 psi.*

9.2   Broad peaks

Introduction

To measure the heat accurately, the spacing between injections should be sufficient to allow the signal to return to baseline.

Problem causes

Cause	Corrective action
Injection spacing is too short	Increase the injection spacing in the ITC Method (see *Section 4.2.6 Instrument Setup tab, on page 73*) or change the injection spacing "on-the-fly" (see *Section 4.3.5 Advanced Experimental Design tab, on page 96*).
Feedback mode is set to an unexpected value. This directly affects the response time of the instrument. Low feedback (or none) requires larger injection spacing than the high feedback setting.	Check the feedback setting and adjust it, or the injection spacing, accordingly.

Cause	Corrective action
The kinetics of the system can also affect the time required to return to baseline. If a given system routinely takes a long time, and the injection spacing is set to just return to baseline, on rare occasions the baseline fitting algorithm will not perform well.	Contact Malvern Instruments support for assistance.

9.3 Downward stepping baseline

Introduction

The baseline might start in the normal range, within 1 µcal/s of the reference power, but after each injection, the baseline steps down. The heat capacitance of the sample cell also decreases with each injection.
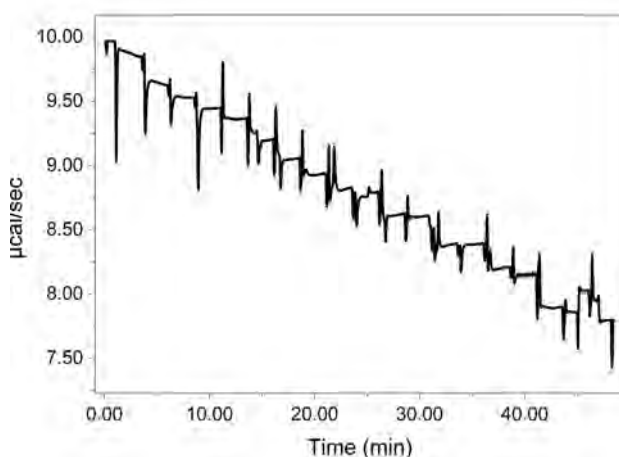
Problem causes

There are several causes of a downward stepping baseline.

Cause	Corrective action
The titration syringe is empty or underfilled.	The syringe injects air into the cell, which shifts the heat capacity of the sample cell and offsets the baseline.
An empty well location was accessed.	Check that the correct well in the 96-well plate was filled. A small amount of liquid should be left in the plate after loading, and should still be visible.
The lid above the well has not been punched.	• Check for problems with the titrant transfer arm. • Check if a recently replaced pipette is initialized properly.

Cause	Corrective action
The small fill port adaptor tip that fits into the fill port in the syringe is damaged.	With all the arms homed, open the doors and examine the tip. If it is damaged, please contact Malvern Instruments.
Failed communication from the fill port dock.	Check log files for any applicable error messages (see *Section 10.5 Zip support files, on page 342*).
If the above causes are not applicable, a hardware issue may need to be resolved. A damaged FPA tip could indicate a completely different problem.	Contact Malvern Instruments support.

After troubleshooting

After troubleshooting the cause of a downward stepping baseline, shut down the MicroCal Auto-iTC$_{200}$ system and all software and restart the system. This should reset the communications and resolve any remaining communications problems.

9.4   Upward stepping baseline

Introduction

The upward steps result from the sample cell getting more full with each injection. The heat capacitance of the sample cell also increases with each injection.
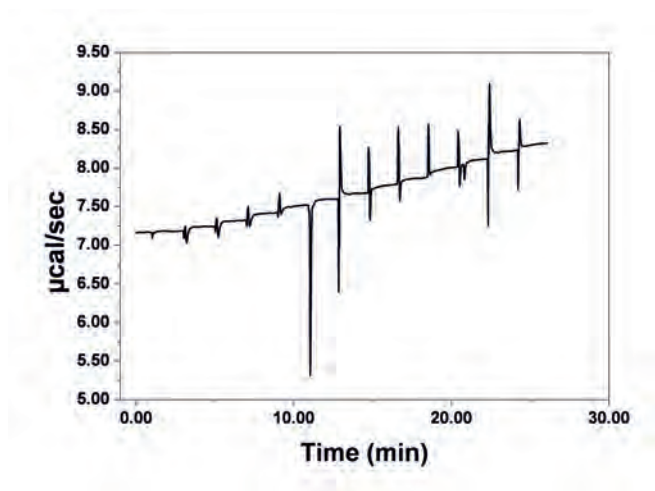
Problem causes

The causes of an upward stepping baseline are described below.

Cause	Corrective action
The cell is dirty.	Clean the cell using the **Maintenance** button in the **System** tab. See *Section 4.2.7* **System** *tab, on page 83*.
The sample cell is underfilled.	• Check that the correct well in the 96-well plate was filled. A small amount of liquid should be left in the plate after loading, and should still be visible. • Check that the appropriate well was loaded with enough sample (370 µl).

Cause	Corrective action
The cannula is dirty.	• Check if well plate lid was punctured within the area without the adhesive. If not, adhesive can adhere to the cannula and affect the cell loading (see *Section 5.3 Loading the samples and performing the experiment, on page 134*). Clean the cannula while being very careful to not bend it. • Clean the cell.
A fluid handling error has occurred.	If extensive cell cleaning does not help, carefully, without damaging cannulas, ensure the fittings on the rightmost of 3 valves (cell arm valve) are tight. Then, perform a water-into-water titration. If the problem still persists, contact Malvern Instruments service representative.

9.5 Reversed/oscillating peaks
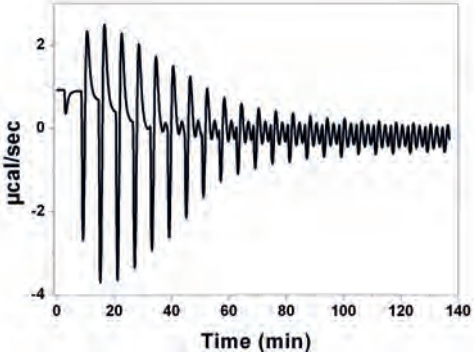
Introduction

Reversed peaks is a rather strange-looking condition in which the baseline starts flat and the peaks initially look normal, but start to shrink quickly midway through the run and then drift in the opposite direction. The baseline may start low, but begins to drift slightly as the peaks reverse their direction.

Problem causes

There are several causes of reversed/oscillating peaks.

Cause	Corrective action
The sample cell is underfilled.	• Check that the correct well in the 96-well plate was filled. A small amount of liquid should be left in the plate after loading, and should still be visible. • If the sample was taken from the tube rack, make sure that the proper tube still has sample in it. *Note:* *It is also possible for an endothermic reaction, with a large buffer mismatch, to have negative initial peaks and positive later peaks.*
The lid above the well has not been punched.	Check for problems with the cell arm.
The sample shifts in the tubing or drips during loading.	Watch cell loading for one autosampler cycle. Check that there is no air leaking into the lines.

Cause	Corrective action
	This oscillatory behavior is due to the differential power dropping below 0. Set the reference power higher.

9.6   Baseline spikes

Introduction

If there are spikes in the baseline, the automated baseline fitting may have difficulties.

Two types of bubble spikes are observed:

- Sharp isolated spikes typically occur when the samples are held at a temperature about 15°C lower than the experimental temperature. Greater gas solubility at lower temperatures can cause bubbles to come out of solution during experiments.

- Prolonged noise spikes are more likely to occur when the experimental temperature is about 15°C above the storage temperature.

All automation methods pre-equilibrate the sample, except **Plates Quick**. Use a method that pre-equilibrates when samples are kept at lower temperatures.

The recommended cell load script **Plate** pre-equilibrates the sample in the cell to minimize experimental artifacts originating from using cold samples. Some samples (i.e., high protein concentration) have responded more favorably when pre-equilibrated in the tubing, as opposed to in the cell. See section 8.3 for a description of the scripts provided with the instrument. When using the **Plate2** cell load script, the sample will be pre-equilibrated to the ambient temperature, rather than the experimental temperature, but this may be suitable depending on the application.

Sharp spikes

The graph below displays sharp isolated baseline spikes.
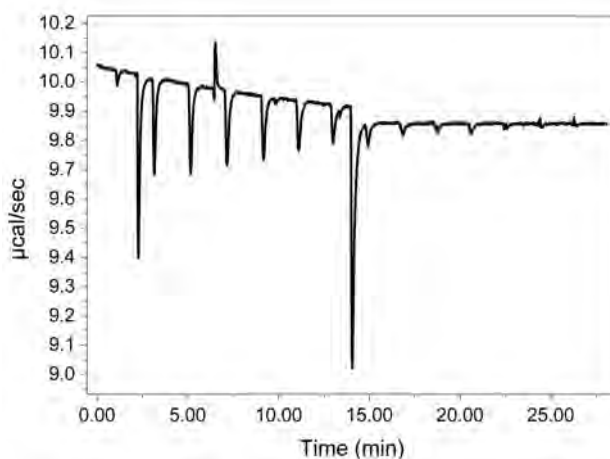
Prolonged noise spikes

The graph below exhibits prolonged noise baseline spikes (in black), as well as a sharp, isolated spike (in green).

Problem causes

Cause	Corrective action
Air bubbles are trapped in the cell.	• Degas the sample solution properly (see *Section 5.1 Preparing the samples, on page 114*). • Spikes are convoluted with an injection- discard that data point and manually fit using Origin. • Spikes are confined to an injection's baseline- save the data point by removing the spike in Origin.

9.7 Low baseline

Introduction

If the baseline settles at more than 1 µcal/s below the user-specified reference power, the results may be less than optimal. For example, the reference power was set to 10 in the example illustrated below. The data look fine, aside from the displaced baseline position. However, the stoichiometric result may be slightly affected.
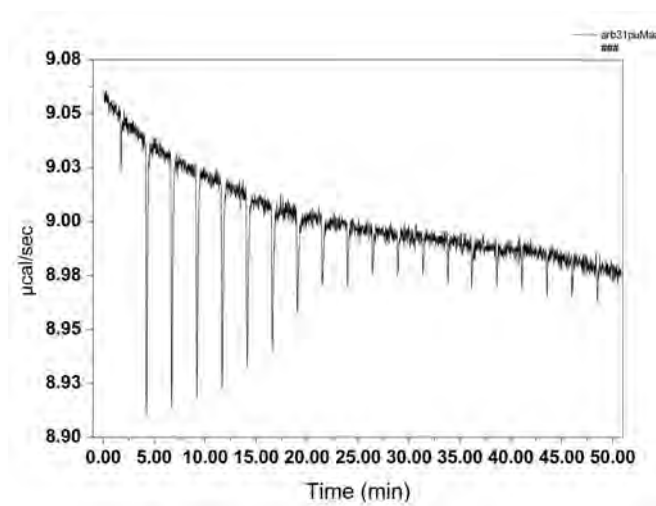
Problem causes

There are several causes of a low baseline, but they all center around an underfilled cell.

Cause	Corrective action
Dirty cell caused a poor load.	Clean the cell.
Air bubbles are trapped in the cell.	Degas the sample solution properly.
The sample cell is underfilled.	Check that the correct well in the 96-well plate (or tube) was filled. A small amount of liquid should be left in the plate after loading, and should still be visible.

Cause	Corrective action
Note: *A baseline position larger than the reference power might be due to an underfilled (or evaporated) reference cell.*	Fill the reference cell (see section 6.3)

9.8   Abnormal peaks

Introduction

A few examples of abnormal peaks are illustrated below.

Example 1

The repeatable trend displayed below implies that the sample cell needs cleaning.
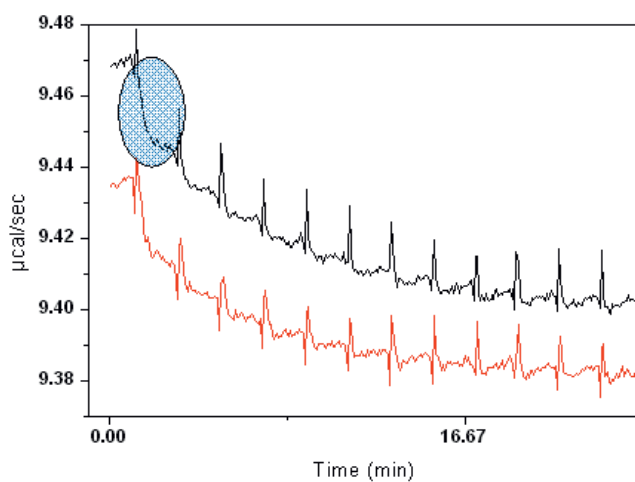
Example 2

In the example illustrated below, there was not enough time left between injections. Increase the spacing between injection and/or check the feedback settings. See *Section 9.1 Peaks too large, on page 305* for a similar discussion.
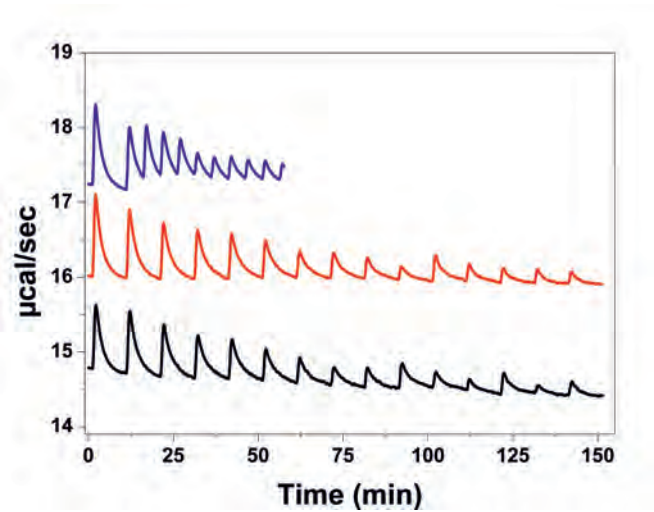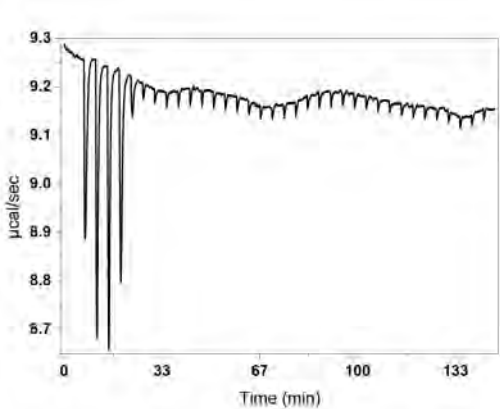
9.9   Unexpected thermodynamic results
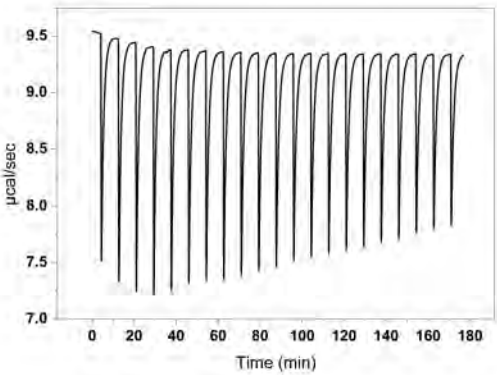
Introduction

Often results do not yield a "textbook" sigmoidal binding isotherm. This may be a result of the system itself, or sample preparation, or both. Several scenarios are described below to help diagnose the problem.

Problem causes

There are several causes of unexpected thermodynamic results. Keep the following scenarios in mind when troubleshooting results:

Observed result	Corrective action
Stoichiometry (n) varies with enthalpy (ΔH).	Check that the syringe concentration is correct.
Stoichiometry (n) varies alone.	Check that the cell concentration is correct.
Early saturation.	• Increase protein concentration or decrease ligand concentration. • Examine sample preparation (see *Section 5.1 Preparing the samples, on page 114*).
Experimental heats are same as control heats.	Change experimental temperature by at least 10°C and/or increase sample concentration.

Observed result	Corrective action
No saturation.	Weaker-than-expected binding or buffer mismatch.

10   Reference information

Introduction

This chapter provides reference information that may be useful when installing, operating, maintaining and troubleshooting the MicroCal Auto-iTC$_{200}$ system. It also contains ordering information.

In this chapter

This chapter contains the following sections:

10.1   How to get help

Contact information

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

For contact information for your local office, please visit: www.malvern.com/contact or for Microcal-specific information, please visit: www.malvern.com/microcal

Include data file

When e-mailing for technical assistance, if possible, please attach a recent data file(s) (*.itc raw ITC data file) that demonstrates the problem. Also, please include all details that may be relevant to the problem. Where the problem or question relates to post run data analysis, it is best to attach the raw data file (*.itc).

Problem categories

There are two general categories of troubleshooting for MicroCal Auto-iTC$_{200}$ and its operation.

Problem category	Description
1 (severe)	The system is not working at all.
	Problems that prevent the operation of the instrument require immediate consultation with a Malvern Instruments technician. Customers should not attempt to repair the hardware or software unless instructed to do so by a Malvern Instruments service representative.
2 (moderate)	The MicroCal Auto-iTC$_{200}$ instrument is functioning, but is not operating within its normal performance specifications.
	Large baseline drifting, non-repeatable control peaks (water/water) and/or an increase in short term noise level are examples of performance problems. These problems may be corrected by the operator in most cases. For these types of performance issues, Malvern Instruments recommends that customers a perform few diagnostic steps prior to requesting service.

Diagnosing the problem

Perform the following minimum diagnostic steps prior to requesting service:

Step	Action
1	Run a thorough cleaning routine.
2	Load one row in a 96-well plate with 400 µl of degassed distilled water.
3	Set up a run of 6 identical titrations.
4	Start the run. If possible, observe the cleaning and loading routines.

If, after completion of the steps listed above, the MicroCal Auto-iTC$_{200}$ performance is not corrected, please contact the service department for help. The water runs should be provided to the MicroCal service technician for evaluation. Following the evaluation, a representative from the service department will contact you with comments and recommendations.

10.2   Networking

Note: *If the instrument must be connected to a network, each subcomponent of this iTC$_{200}$ software installation must be performed with full ADMINISTRATIVE privileges.*

Networking requirements are operating system specific.

For Windows XP operating systems:

- If the instrument must be connected to a network, each subcomponent of the iTC$_{200}$ software installation must be performed with full administrative privileges.

- Once the system is operational, if users without administrative privileges will be operating the system, then the access rights of those users must be modified by the administrator in order to assure a proper operating environment for those users.

For Windows 7 operating systems:

Verify that the Windows 7-based configuration settings described below have been completed:

- *Section 3.2.1 Modify the Origin 7 configuration for Windows 7, on page 46*

- *Section 3.2.2 Modify the MicroCal iTC$_{200}$ software configuration for Windows 7, on page 48*

- *Section 3.2.3 Modify the user account control settings for Windows 7, on page 50*

Once the system is operational, if users without administrative privileges will be operating the system, then the access rights of those users must be modified by the administrator in order to assure a proper operating environment for those users.

Installing a Windows XP-based system onto a network

On a system using Windows XP, do the following:

- Before beginning the installation, make sure you are logged into the PC with full administrative privileges.

- If the software will be run by users without administrative privileges, do the following to assure proper operating environment for ITC$_{200}$ and Origin 7.0 software:

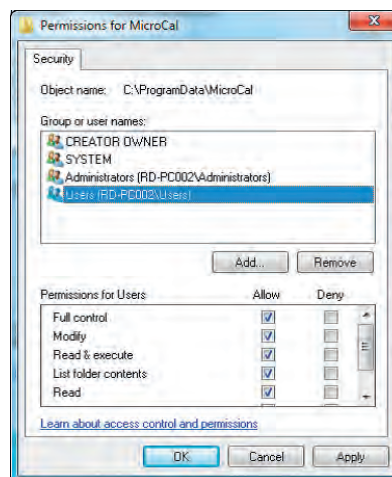| Step	Action
1	Make sure you are logged onto the PC with administrative privileges.
	Note:
	Please consult the local IT department for instructions regarding the changing of local security settings.
2	Go to *My Computer* and navigate to `C:\ITC200`.

Step	Action
3	Right-click the folder, `C:\ITC200` and select **Properties**.
4	Click the **Security** tab.

Note:

In Windows XP Home Edition and Windows XP Professional, if working in a workgroup, the Security tab is hidden by default. This behavior occurs because in Windows XP Home Edition and Windows XP Professional, guests are forced to log on to a workgroup. To resolve this, see the Microsoft knowledgebase article #290403: `http://support.microsoft.com/kb/290403/`.

*Article 290403 is also on the installation CD in the **Documents** folder.*

Important Resolving this problem requires you to modify the registry. However, serious problems might occur if you modif the registry incorrectly. For added protection, back up the registry before you modify it. Then, you can restore the registry if a problem occurs. For more information about how to back up and restore the registry, see the Microsoft knowledge base article # 322756: `http://support.microsoft.com/kb/322756/`

5	Specify **Full Control** for the user or the group, **Users**.

Step	Action
6	Navigate to `C:\Program Files\OriginLab\Origin7\` and repeat steps 3–5. Security properties for ITC$_{200}$ software:
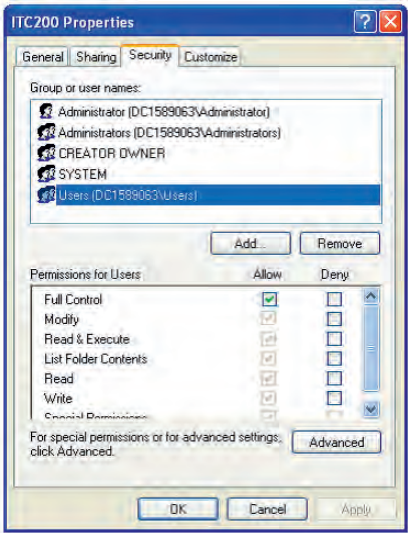
Security properties for Origin software:

In the images above, each user is a member of the Users group by default. Simply increase the permission level of the group by selecting **Full Control**.
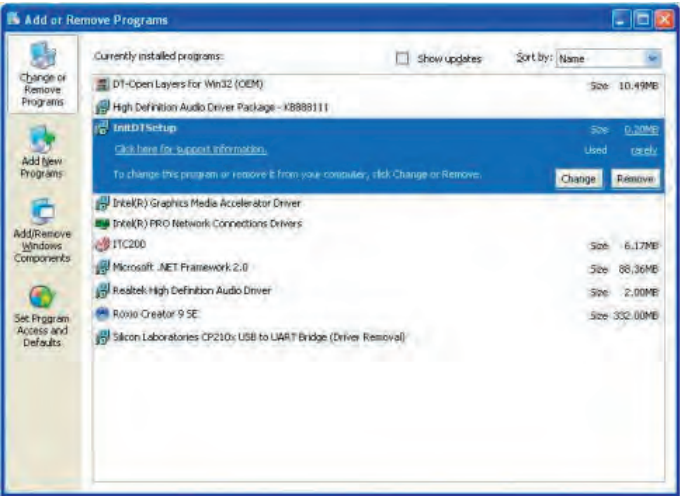
Step	Action
7	If InitDT service is installed, you must uninstall it.
	InitDT service is incompatible with users without administrative privileges. Failure to uninstall InitDT service in this case will result in instrument malfunction.
	Note:
	InitDT Service should not be installed on models 02.10.310 or higher or on an older system that has a newer version of the DT9836 board, firmware, and driver installed. (refer to service records if necessary.)
	To uninstall InitDT service, go to the **Control Panel>Add or Remove Programs**, navigate to InitDTSetup, and click **Remove**.

Installing a Windows 7-based system onto a network

On a system using Windows 7, do the following:

- Before beginning the installation, make sure you are logged into the PC with full administrative privileges.

- If the software will be run by users without administrative privileges, do the following to assure proper operating environment for Auto-iTC$_{200}$ and Origin 7.0 software:

• Step	Action
1	Make sure you are logged onto the PC with administrative privileges. *Note:* *Please consult the local IT department for instructions regarding the* *changing of local security settings.*
2	Go to *My Computer* and navigate to `C:\ProgramData\MicroCal`.
3	Right-click the folder, `C:\ProgramData\MicroCal` and select *Proper-ties*.
4	Click the *Security* tab.
5	Specify *Full Control* for the user or the group, *Users*.

Step	Action
6	Security properties for Auto-iTC$_{200}$ software:
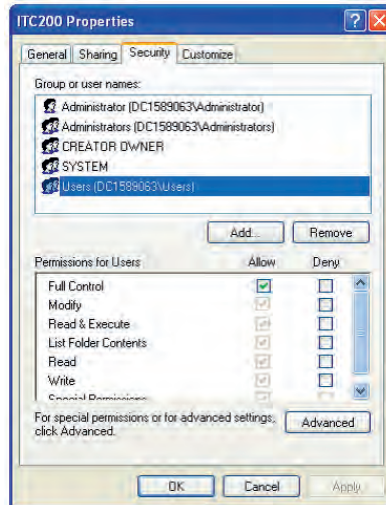
Full permissions for `C:\ProgramData\MicroCal` for Windows 7-based networking:

In the images above, each user is a member of the Users group by default. Simply increase the permission level of the group by selecting **Full Control**.

Step	Action
7	If InitDT service is installed, you must uninstall it.
	InitDT service is incompatible with users without administrative privileges. Failure to uninstall InitDT service in this case will result in instrument malfunction.

Note:

InitDT Service should not be installed on models 02.10.310 or higher or on an older system that has a newer version of the DT9836 board, firmware, and driver installed. (refer to service records if necessary.)

To uninstall InitDT service, go to the **Control Panel>Add or Remove Programs**, navigate to InitDTSetup, and click **Remove**.

10.3   TCP Port Conflicts

Updating to v1.1 and v1.26

Note: *For customers updating to v1.1 and v1.26 where the system has already been networked and a port conflict has occurred in the past.*

Since this is an update, running the v1.26 updater will leave the working TCP port number in the ITC200.ini file.

After installing the new software, start the iTC$_{200}$ application. Then start the Auto-iTC$_{200}$ application. If the following error is displayed upon startup of the Auto-iTC$_{200}$ application, this is an indication that the default TCP port number is not being used.
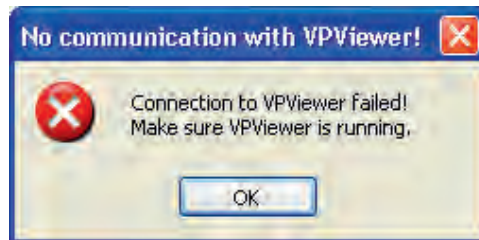
The TCP port number specified in the iTC$_{200}$ initialization file needs to transfered to the Auto-iTC$_{200}$ configuration file.

Open the file `C:\ITC200\ITC200.ini` – this file can be opened with notepad.
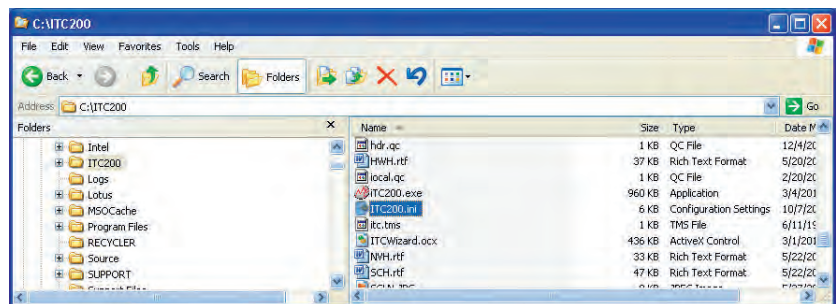
Search for the string TCPPort.
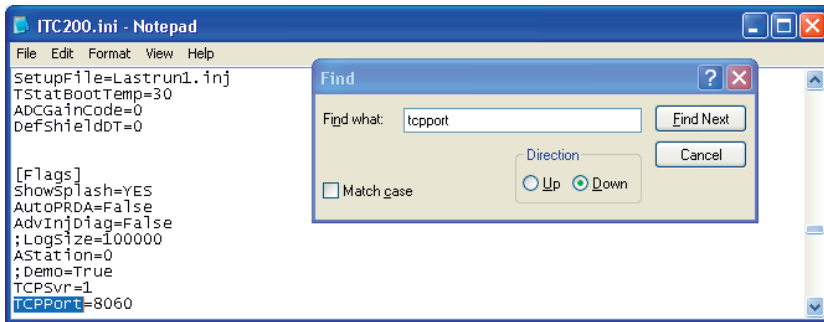
Record the value – in this case 8060.

Open the file `C:\Program Files\MicroCal\AutoItc200\MicroCal.AutoItc200.exe.config` – this file can be opened with notepad.
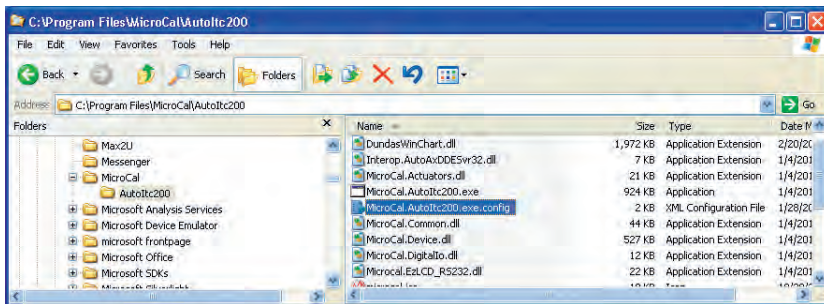
Search for the string VPVIEWER.PORT.
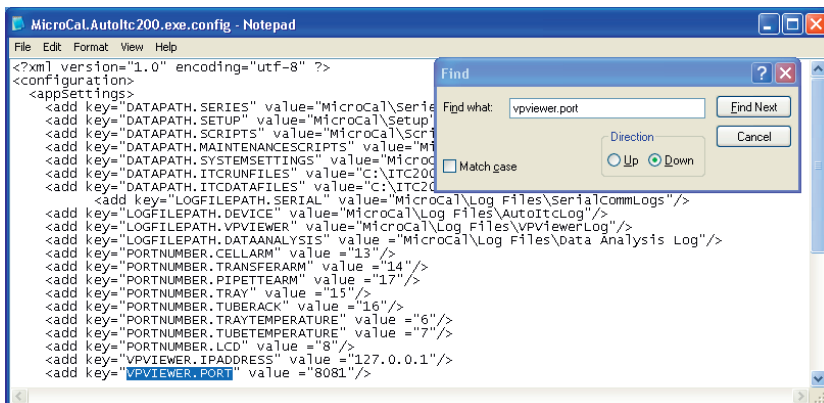
Edit this value to match the value previously recorded from the ITC200.ini file and save the change.

Restart the Auto-iTC$_{200}$ application.

Networking or installing other software

Note: *Possible issues from networking or installing other software such as anti-virus software.*

The iTC$_{200}$ and Auto-iTC$_{200}$ applications communicate over TCP using the local host address and the default port number of 8081.

After networking or installing other software, it is possible that some other program may be using the port that the iTC$_{200}$ and Auto-iTC$_{200}$ applications are trying to use. If this happens, the following message box will be displayed when starting the iTC$_{200}$ program:

If this error is encountered, close the iTC$_{200}$ program. Open the file `C:\ITC200\ITC200.ini`. This is a text file that can be opened with Notepad.exe. Be careful not to make any inadvertent changes to this file. Search for FLAGS to go to the [FLAGS] section of the ini file. The TCPPort keyword may or may not be present. If it is not present, then the default value of 8081 is being used and you will need to add the TCPPort keyword to the FLAGS section.

Below shows the FLAGS section of an ITC200.ini file without the TCPPort keyword present.
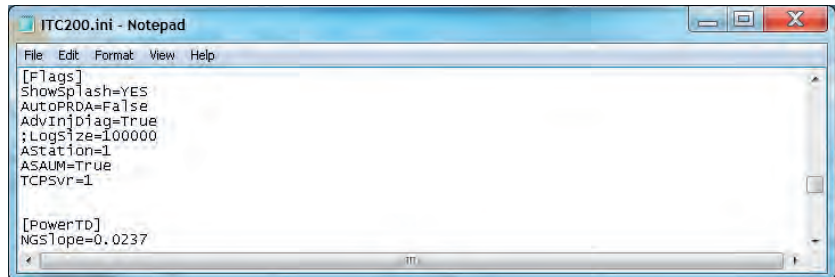
And here is the same file with the TCPPort keyword added and the value set to 8060.
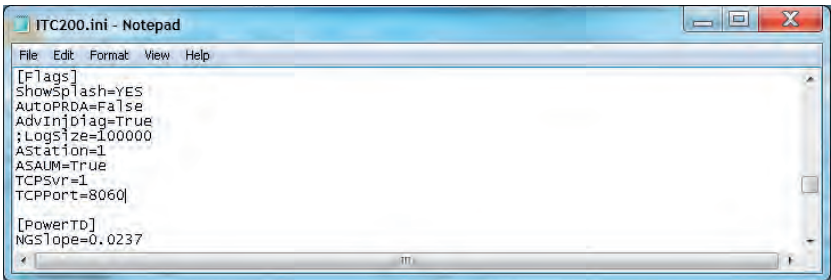
The problem is to find an unused TCP port for the programs to communicate on. It is recommended that you stay in the 8000 range. Enter a new value, save the change to the ITC200.ini file. Restart the iTC$_{200}$ application. If the 'Address in use' error is displayed again, close the application and repeat the process selecting a new value for the TCPPort setting. When a TCP port number is specified in the ITC200.ini file that is available, the iTC$_{200}$ application will start without the 'Address in use' message.

Once an available TCP port number if determined, that same number must be entered into the configuration file for the Auto-iTC$_{200}$ application.

Open the file `C:\Program Files\MicroCal\AutoItc200\MicroCal.AutoItc200.exe.config` – this file can be opened with notepad.
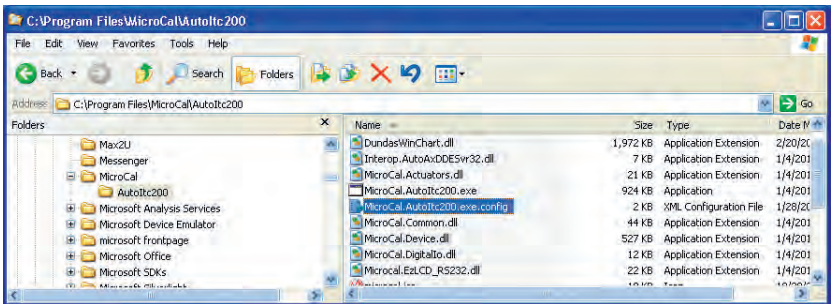
Search for the string VPVIEWER.PORT
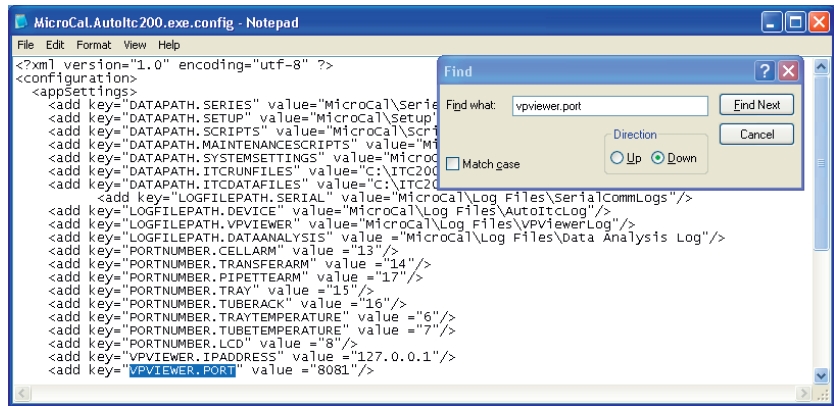
Edit this value to match the value previously recorded from the ITC200.ini file and save the change.

10.4   Standard setup files

Introduction

This section describes the setup files provided with the MicroCal Auto-iTC$_{200}$ software. These are located at the following location:

`C:\ProgramData\MicroCal\Setup` (Windows 7) or `C:\Documents and Settings\All Users\Application Data\MicroCal\Setup` (Windows XP).

Automation methods

Setup file	Cell load	Cell clean	Syringe clean	Titrant transfer clean	Pre-rinse	Pre-equilibrate sample
Plates	P	D	W	W	N	Y (in cell)
Plates2	P	D	W	W	N	Y (in tubing)
Plates Clean	P	D (extra)	W	W	N	Y (in cell)
Plates Syringe Clean	P	D	D	D	N	Y (in cell)
Plates Prerinse	P	D	W	W	Y	Y (in cell)
Plates Prerinse Syringe Clean	P	D	D	D	Y	Y (in cell)
Plates Quick	P	W	W	W	N	N
Tubes	T	D	W	W	N	Y (in cell)
Tubes2	T	D	W	W	N	Y (in tubing)
Tubes Clean	T	D (extra)	W	W	N	Y (in cell)
Tubes Syringe Clean	T	D	D	D	N	Y (in cell)
Tubes Prerinse	T	D	W	W	Y	Y (in cell)
Tubes Prerinse Syringe Clean	T	D	D	D	Y	Y (in cell)

Setup file	Cell load	Cell clean	Syringe clean	Titrant transfer clean	Pre-rinse	Pre-equilibrate sample
Tubes Quick	T	W	W	W	N	N
Continue Injections	N/A	N/A	W	W	N	N

P: Plate; T: Tube; D: Detergent; W: Water; Y: Yes; N: No

Automation script files

The extension of each file indicates the type of script. Some files have only one standard script; some contain multiple scripts with different options. These are located at the following location:

`C:\ProgramData\MicroCal\Scripts` (Windows 7) or `C:\Documents and Settings\All Users\Application Data\MicroCal\Scripts` (Windows XP).

Script type	Script name	Description
Cell Load	*Plate*	Load from plate, pre-equilibrate sample (in cell)
	Plate2	Load from plate, pre-equilibrate sample (in tubing)
	Plate Quick	Load from plate
	Plate Syringe Clean	Load from plate, pre-equilibrate sample
	Plate Prerinse	Pre-rinse, load from plate, pre-equilibrate sample
	Remove From Cell	Only remove sample
	Tube	Load from tube, pre-equilibrate sample
	Tube Quick	Load from tube
	Tube Syringe Clean	Load from tube, pre-equilibrate sample
	Tube Prerinse	Pre-rinse, load from tube, pre-equilibrate sample

Script type	Script name	Description
Cell Clean	*Detergent Clean*	Empty cell, briefly soak cell with detergent at post-experiment temperature
	Extra Clean	Empty cell, soak cell with detergent for 1 h at 60°C
	No Cell Clean	Do not empty or clean cell
	Water Clean	Empty cell, rinse with water
Pipette Load	*Load Pipette Quick*	Load pipette
	Load Pipette with Purge	Load pipette with one purge/refill cycle
	Load Pipette	Load pipette (timing different)
Pipette Clean	*Pipette Clean with Detergent*	Clean pipette with detergent, water, methanol, and then dry
	Pipette Clean	Clean pipette with water, methanol, and then dry
Transfer Titrant	*Transfer Titrant Quick*	Transfer titrant
	Transfer Titrant Syringe Clean	Transfer titrant (timing different)
	Transfer Titrant	Transfer titrant (timing different)
Transfer Clean	*Transfer Clean with Detergent*	Clean transfer arm with detergent, water, methanol, and then dry
	Transfer Clean	Clean transfer arm with water, methanol, and then dry

MicroCal iTC$_{200}$ ITC methods

These files have the same extension (.inj) and format as the manual MicroCal iTC$_{200}$ system setup files. These files can also be found in the same location (`C:\ITC200\Setup`) as the manual MicroCal iTC$_{200}$ system setup files.

The standard ITC methods provided with the controller are:

- **lastrun1.inj** is a special file created and used by the software, which contains the parameters of the last run.

- **EDTA.inj** contains the parameters for the CaCl$_2$/EDTA kit provided by Malvern, Instruments and may be used as the basis for sample titration setup files.

- **NoiseTest.inj** is a 0 injection run used for standard testing of instrument noise.

- **WATER.inj** is a 19 injection run, intended for water-into-water or buffer-into-buffer titrations.

10.5   Zip support files

Introduction

The **Zip Support Files** utility simplifies the process of preparing the MicroCal Auto-iTC$_{200}$ software system files for transfer to Malvern Instruments service. These may be helpful to a technician diagnosing a problem remotely. Each button prepares a different type of system file.

Creating support files for MicroCal Auto-iTC$_{200}$

If a problem cannot be solved by proper cleaning or maintenance as outlined in *Chapter 8 Maintenance, on page 287*, it may be necessary to send support files to your local service support representative for assistance in diagnosing the problem.

The MicroCal Auto-iTC$_{200}$ software automatically creates a log of activities, which helps in accurately diagnosing and resolving problems with the Autosampler. These log files are created on a regular basis and can become quite numerous and large. A utility program has been created to prepare a compressed package that can be easily sent by email for evaluation.

The program is generally installed on the instrument's PC and appears as a desktop icon, **Create Support Files**. Start the software by selecting this icon. If the software is not installed, it can be installed from the original CD and by navigating to the directory **CreateSupportFilesUtil**.

If the **Create Support Files** icon does not appear on the PC's desktop, it can be accessed directly at `C:\Support Files\ziplogfiles.exe`.

The software offers three options for creating a zip file in the `C:\SupportFiles` directory. It is generally recommended to create zip files for each option individually and send all three zip files to ensure that the widest range of issues can be addressed.

Types of zip support files

The table below describes the different zip file types that can be created.

Support File	Description
Zip AutoItc200 System Files	Zips the system settings files.

Support File	Description
Zip AutoItc200 Log Files	Zips the general system error logs.
Zip Serial Logs Files	Zips the low-level communication logs.
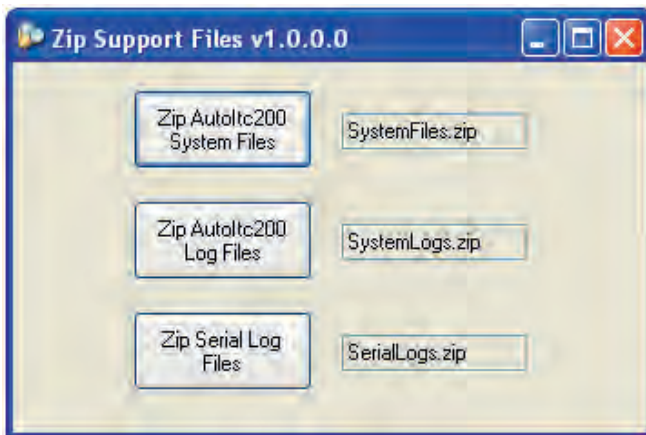
Files to be provided to the Malvern Instruments Instruments service technician

Depending on the type of problem, a service technician may ask for one or more of these files. All three buttons create a zip directory with the relevent files, using the file name that is entered in the textbox to the right of the button. The zipped directories are found at `C:\Support Files`, and may be e-mailed or otherwise transfered to the Malvern Instruments service technician.

10.6   MicroCal Auto-iTC$_{200}$ System specifications

Performance specifications

Property	Value
Operating Temperature Range	2°C to 80°C
Sample Storage Temperature Range	4°C to ambient
Response Time	10 seconds
Cell Design	200 µl, coin-shaped
Titration Syringe	40 µl
Maximum Usable Volume	38 µl
Smallest Injection Size	0.1 µl
Stirring Rate	500 to 1500 rpm

Physical specifications

Property	Value
Cell Material	Hastelloy Alloy C-276

Weight (fully assembled):
  Autosampler without calorimeter
  Autosampler with calorimeter
  Calorimeter
	
	84.4 kg
	90.7 kg
	6 kg

Dimensions:
  Fully assembled
  Calorimeter
  Autosampler
	
	62.5 × 57.2 × 76.8 cm
	17 × 16 × 36 cm
	61 × 76 × 58 cm

Electrical specifications

Electrical specifications are for the calorimeter and Autosampler only. Autosampler specifications, where different, are enclosed in parentheses.

Part	Function
Electrical Ratings:	
Voltage	100 to 240 V grounded
Frequency	50 / 60 Hz
Power	70 W (300 W)
Fuses (2)	4.0 A, 5.0 A, 250 V, Time delay (Fast acting)
Output	Secondary/Data connection only
Protective Earth Terminals	Internal/external marked (Internal marked)
Mode of Operation	Continuous
Classification	Class I

Site requirements

Part	Function
Benchspace	• ≥4 ft (125 cm) of lab bench, • ≥30 in. (80 cm) in depth, • no obstructions for at least 32 in. (80 cm) above bench, • rated for at least 250 lbs (115 kg). These include the clearances. Service functions will require an additional 12 in. (31 cm) overhead clearance.
Clearance	• ≥6 in. (15 cm) behind the Autosampler, • ≥15 in. (40 cm) in front.
Pressure	≥20 psi laboratory nitrogen (if using tank, tank must not infringe on Autosampler clearances). Connection is made with a 1/4" NPT fitting.

> **WARNING**
>
> Nitrogen is a colorless, odorless gas which can displace oxygen and present an asphyxiation hazard.
>
> - Compressed nitrogen should be stored in a cool, dry, well-ventilated area.
>
> - Containers of nitrogen should be protected from physical damage and heat.
>
> - Soapy water or other suitable gas detection equipment should be used to verify that there are no leaks in the gas transport system.
>
> - The unit should not be operated in tanks or other enclosed areas without proper ventilation.

Atmospheric specifications

Part	Function
Operating:	
Temperature	10°C to 28°C
Humidity	0% to 70% RH, non-condensing
Atmospheric Pressure	700 to 1060 HPa
Storage (no liquid in cells):	
Temperature	-25°C to 60°C (no liquid in cells)
Humidity	10% to 90%, non-condensing
Atmospheric Pressure	500 to 1060 HPa

10.7   Reagent and sample container replacements

Reagents

The Autosampler requires:

- distilled water

- ≥99% pure methanol ("HPLC Grade" is recommended)

> ⚠️ **WARNING**
>
> Methanol is highly volatile and can be hazardous to humans.
>
> - Storage containers should be kept tightly closed.
>
> - Methanol should always be transferred in a well-ventilated area with no ignition sources. The operator should have protective clothing and gloves.
>
> - Methanol can be absorbed through the skin. Do not allow methanol to be swallowed or to come in contact with skin or eyes. If accidental exposure occurs, flush the affected area with water. If methanol is swallowed, or there is significant skin or eye exposure, seek medical help.

- Detergent: 20% Contrad 70 from Decon Laboratories, Inc., King of Prussia, PA, USA (or 14% Decon 90 from Decon Laboratories Limited, Hove, East Sussex, UK) is the recommended detergent. Contrad 70 and Decon 90 contain dodecylbenzensulfonic acid, potassium hydroxide, sodium citrate and sodium laurel ether sulfate. It is biodegradable and can be rinsed off easily.

Sample containers

The Autosampler uses 96-well plates and 30 ml centrifuge tubes. Replacements may be purchased through Malvern Instruments, or directly from a supplier. Using supplies otherthan ones recommended here may lead to improper performance, potential Autosampler damage, and may void the warranty.

- Tube, Oak Ridge Centrifuge; Part No. 28428970

- 96-Well Plates: Nunc 96 DeepWell Plates; Mfr.#260252; Part No. 28429091

- Well Plate Covers: Excel Scientific EZ-Pierce Zone-Free Microplate Film; Cole-Parmer# R-13024-66; Part No. 28429092
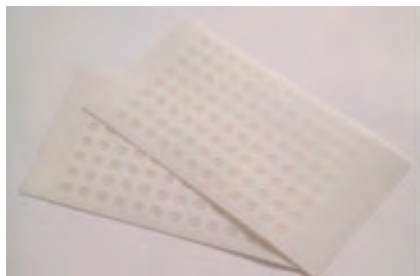
Appendix A Equations used for fitting ITC data

General considerations

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_o, the size of the ith 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.
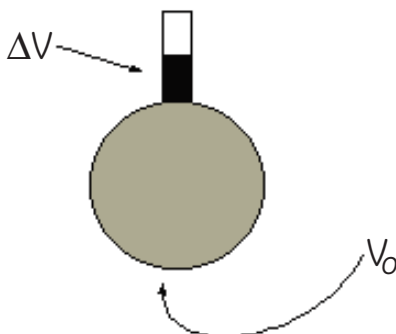
At the beginning of an experiment, both the cell and the long communication tube are filled with macromolecule solution, but it is only that contained within V_o that is sensed calorimetrically, Because of the total-fill nature of the cell, each injection acts to drive liquid out of the working volume and up into the inactive tube as shown by the darkened portion representing ΔV. Thus, the concentration of macromolecule in V changes a small amount with each injection since the total number of moles of macromolecule initially in V (i.e., times V_o) at the beginning of the experiment is later distributed in a larger volume, $V_o + \Delta V$. Since the average bulk concentration of macromolecule in ΔV is the mean of the beginning concentration and the present concentration M_t in the active volume, conservation of mass requires that

Equation 1:

$$M_t^o V_o = M_t V_o + \frac{1}{2}(M_t + M_t^o)\Delta V$$

so that

Equation 2:

$$M_t = M_t^o[\frac{1 - \frac{\Delta V}{2V_o}}{1 + \frac{\Delta V}{2V_o}}]$$

Using similar reasoning, it is easily shown that the actual bulk concentration of ligand in V_o, X_t, is related to the hypothetical bulk concentration (assuming that all of the injected ligand remained in V_o) as follows:

Equation 3:

$$X_t^o V_o = X_t V_o + \frac{1}{2}X_t \Delta V$$

Equation 4:

$$X_t = X_t^o[\frac{1}{1 + \frac{\Delta V}{2V_o}}]$$

The above expressions for M_t and X_t are used by Origin to correct for displaced volume effects, which occur with each injection.

Single set of identical sites

In the following equations,

Parameter	Description
K	binding constant
n	number of sites
V_o	active cell volume
M_t	bulk concentration of macromolecule in V_o
$[M]$	free concentration of macromolecule in V_o
X_t	bulk concentration of ligand
$[X]$	free concentration of ligand
Θ	fraction of sites occupied by the ligand X

Equation 5:

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

Equation 6:

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

Combining equations (5) and (6) above gives

Equation 7:

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

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

Equation 8:

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

where ΔH is the molar heat of ligand binding. Solving the quadratic equation (7) for Θ and then substituting this into equation (8) gives

Equation 9:

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

The value of Q above can be calculated (for any designated values of n, K, and ΔH) at the end of the ith 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_o. Therefore, after completing an injection, it is obvious that a correction must be made for displaced volume (i.e., $\Delta V_i =$ injection volume) since some of the liquid in V_o after the i^{-1} injection will no longer be in V_o after the ith 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_o. The liquid in the displaced volume contributes about 50% as much heat effect as an equivalent volume remaining in V_o. The correct expression for heat released, $\Delta Q(i)$, from the ith injection is

Equation 10:

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

The process of fitting experimental data then involves:

1   initial guesses (which most often can be made accurately enough by Origin) of n, K, and ΔH

2   calculation of $\Delta Q(i)$ 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 ΔH 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

Equation 11:

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

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

Equation 12:

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

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

Equation 13:

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

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

Equation 14:

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

where,

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

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

Equation 15:

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

Equations 14 and 15 can be solved for $[X]$ either in closed form or (as done in Origin) 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 earlier in section II, the heat content after any injection i is equal to

Equation 16:

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

After a similar correction for the displaced volume, the pertinent calculated heat effect for the ith injection is

Equation 17:

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

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

Equation 18:

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

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

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

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 identical, then there is a statistical degeneracy associated with the sequential saturation since the first ligand to bind has more empty sites of the same kind to choose from than does the second ligand, etc. For identical interacting sites then, we can distinguish between the phenomenological binding constants K_i (defined by equation (18)) and the intrinsic binding constants K_i^o where the effect of degeneracies has been removed. The relationship between the two binding constants is given by:

Equation 19:

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

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_i^o 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

Equation 20:

$$F_o = \frac{1}{P}$$

$$F_1 = \frac{K_1[X]}{P}$$

$$F_2 = \frac{K_1K_2[X]^2}{P}$$

$$F_n = \frac{K_1K_2...K_n[X]^n}{P}$$

where

Equation 21:

$$P = 1 + K_1[X] + K_1K_2[X]^2 + ... + K_1K_2...K_n[X]^n$$

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

Once n and values of fitting parameters K_1 through K_n are assigned, then equations (20) - (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 ith injection is determined from

Equation 22:

$$Q = M_tV_0(F_1\Delta H_1 + F_2[\Delta H_1 + \Delta H_2] + ... + F_n[\Delta H_1 + \Delta H_2 + \Delta H_3 + ... + \Delta H_n])$$

and, as before,

Equation 23:

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

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 decomposition reaction is directly proportional to the power output in the calorimeter cell, i.e.,

Equation 24:

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

where,

Parameter	Description
P	power generated by the reaction
ΔH	heat of decomposition of the substrate
V_o	cell volume

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

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

Equation 25

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

where,

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

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

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 below.

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.,

Equation 26

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

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:

Equation 27

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

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 nonlinear least squares. In the absence of inhibitor, k_{cat} and K_M are used as variable parameters during iterative fitting. In the presence of inhibitor I, it is best to enter previously determined values of k_{cat} and K_M and use K_I 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 hydrolysis 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 hydrolysis.

Equations (24)-(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 hydrolysis is allowed to go to completion. Having done this, then discrete values of R_t at different $[S]_t$ 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_I as the only fitting parameter.

Dimer dissociation model

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

$$P_2 \overset{\Delta H_{disc}}{\Leftrightarrow} 2P$$
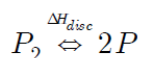
$$K_D = \frac{(P)^2}{(P_2)}$$

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 to K_D and ΔH_{disc}.

The equivalent monomer concentration C is defined as

Equation 28

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

Solving for P we have

Equation 29

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

from which $[P_2]$ follows.

The heat released q_i when the ith injection of volume dV_i is made into a fixed-volume (V_o) cell will be:

Equation 30

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

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_o, 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 model

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.,

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

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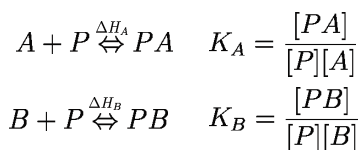
The value of K_B and ΔH_B for the competing ligand are first measured in a conventional ITC experiment, and these parameter values are entered as known parameters when determining K_A from results of the competition experiment. For the competition experiment, the total concentration of competing ligand,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

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

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).

Single injection method

Creating new worksheet

The raw data (after time constant correction, Fourier filtering, baseline subtraction, and eliminating inappropriate data) are used to form a new worksheet, which is modeled after the existing worksheet used with multi-injection binding data.

Input parameters

In addition to the raw data parameters (ΔP(μcal/sec) from the Y axis and time t (sec) from the X axis of the corrected raw data), the known parameters are the injection rate R (ml/sec, stored in header), total delivery volume V_{inj} (μl, stored in header), active cell volume V_{cell} (ml), the initial macromole concentration in the cell M_o (mM) before any dilution, the dilution factor dM for the macromolecule solution resulting from Autosampler loading, the initial ligand concentration in the syringe X_o before any dilution, the dilution factor dX for the ligand concentration resulting from loading. The approximate values are 0.95 for dM and 0.91 for dX, and the values are independent of the instrument used in the experiment.

Point numbering

In the existing Origin worksheet for multiple injections, the rows are numbered 1,2,3, ... according to the injection number. In the worksheet for single injection experiments, the numbering corresponds to the data point number. The data points will be spaced at one for each filter period (2 s).

DH and time t columns

The DH column corresponds to the column of the same name in the existing Origin ITC worksheet while the time t column is one, which does not exist in the existing worksheet and must be added. The DH and time t columns should be filled with the data points from the above data set (after TC correction, FT smoothing, control subtraction, and data trimming). DH is the y axis value ΔP(μcal/sec) and time t (sec) is the corresponding x axis value.

INJV column

All entries into this column should be identical and equal to the injection rate R (μl/sec) times the filter time (2 s).

X$_t$ column

$$X_t = (\frac{X^o d_X R t}{1000 V_{cell}})(1 - \frac{Rt}{2000 V_{cell}})$$

M$_t$ column

$$M_t = M^o d_M(\frac{1 - \frac{rt}{2000 V_{cell}}}{1 + \frac{rt}{2000 V_{cell}}})$$

XM$_t$ column

$$XM_t = \frac{X_t}{M_t}$$

Note: *Indexing for X$_t$, M$_t$, and INJV refer to values before the ith injection, while DH, XM$_t$, NDH refer to indexing after the ith injection (the new column time t is also indexed after the ith injection).*

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