

MicroCal iTC₂₀₀ System

Getting Started Booklet



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Introduction

MicroCal iTC₂₀₀ System Getting Started is designed to introduce you to the basic operations of MicroCal iTC₂₀₀ system and software¹. This booklet provides guidance through the basic steps in a MicroCal iTC₂₀₀ experiment using reagents supplied in the MicroCal iTC₂₀₀ Test Kit.

The reagents in the kit are for training purposes only and are supplied in quantities sufficient for at least five repeated exercise sessions, following the instructions in this booklet. Malvern Instruments can accept no responsibility for results obtained with these reagents in any other context. The reagents should be stored at 2–8°C and should be used within one week after opening.

Requirements

The following are required for completing the Getting Started exercises:

- Time: approximately 1 day
- Familiarity with PC and Windows™
- MicroCal iTC₂₀₀ system
- MicroCal iTC₂₀₀ Test Kit
- MicroCal iTC₂₀₀ System Getting Started Booklet
- Micropipettes (20–200 µl and 100–1000 µl) and tips
- 200 µl Eppendorf microcentrifuge tubes
- Wash solutions (distilled water, methanol², Contrad™ 70 or Decon™ 90)

Contents of the MicroCal iTC₂₀₀ Test Kit

The contents of the MicroCal iTC₂₀₀ Test Kit are listed in Table 1. All solutions are ready for use. Instructions for using the kit are given in this booklet.

For further information, please visit www.malvern.com/microcal or contact your local Malvern Instruments representative.

¹ Applies to MicroCal iTC₂₀₀ Control Software v1.26 and MicroCal iTC₂₀₀ Analysis Software v7.20 or later.

² Methanol is classed as a hazardous chemical in some countries. Observe the applicable regulations for handling methanol.

Table 1. Contents of the MicroCal iTC₂₀₀ Test Kit. For *in vitro* use only. All solutions should be stored at 2–8°C.

Reagent/Item, quantity
0.4 mM EDTA in MES buffer, pH 5.6 (10 ml)
5 mM CaCl ₂ in MES buffer, pH 5.6 (1.3 ml)
10 mM MES buffer, pH 5.6 (7 ml)

Ordering information

EDTA Test Kit MicroCal iTC ₂₀₀	KIT020988
MicroCal iTC ₂₀₀ System Getting Started Booklet	MAN0559-01

References

For further information on the topics discussed in this booklet, refer to:

MicroCal iTC ₂₀₀ System User Manual	MAN0560-01
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Background information

This section provides the basis for a more detailed understanding of the main steps in a MicroCal iTC₂₀₀ experiment. The information goes beyond what is explicitly covered in the exercises and is, therefore, a complement to the exercises.

Principles of isothermal titration calorimetry (ITC)

Isothermal titration calorimeters measure the heat change that occurs when two molecules interact. Heat is liberated or absorbed as a result of the redistribution of non-covalent bonds when the interacting molecules go from the free to the bound state. ITC monitors these heat changes by measuring the differential power, applied to the cell heaters, required to maintain zero temperature difference between the reference and sample cells as the binding partners are mixed.

The reference cell usually contains water, while the sample cell contains one of the binding partners (the sample, often but not necessarily a macromolecule) and a stirring syringe which holds the other binding partner (the ligand). The ligand is injected into the sample cell, typically in 0.5 to 2 μl aliquots, until the ligand concentration is two- to three-fold greater than the sample. Each ligand injection results in a heat pulse that is integrated with respect to time and normalized for concentration to generate a titration curve of kcal/mol vs molar ratio (ligand/sample). The resulting isotherm is fitted to a binding model to obtain the affinity (K_A), stoichiometry (N) and enthalpy of interaction (ΔH).

A schematic representation of an ITC system such as MicroCal iTC₂₀₀ is shown in Figure 1.

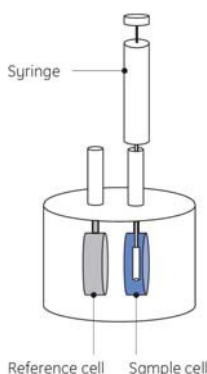


Figure 1. Schematic representation of the essential components of an ITC system such as MicroCal iTC₂₀₀.

General description

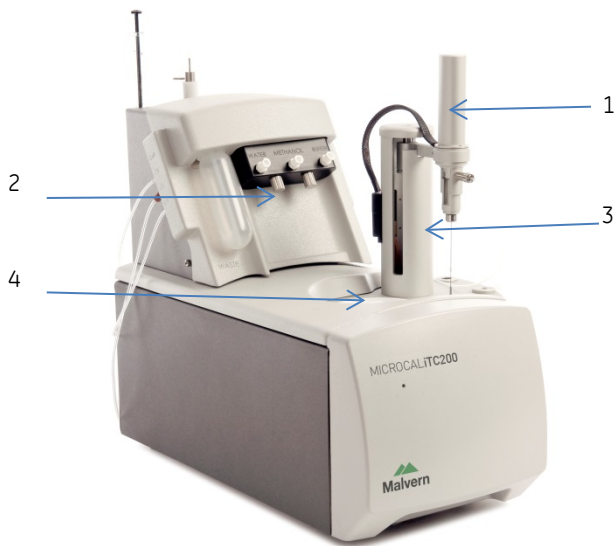


Figure 2. MicroCal iTC₂₀₀ system.

Part	Function
1	Pipette
2	Washing module
3	Tower
4	Cell unit

MicroCal iTC₂₀₀ isothermal titration calorimeter from Malvern Instruments 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 in liquid samples.

The sample and reference cells are made from Hastelloy™ alloy, a highly inert material. The cells are fixed in place providing reproducible, ultrasensitive performance with low maintenance requirements. The cells are accessible for filling through the top of the unit, which also includes a washing module for thorough cleaning.

Data analysis is performed with Origin™ software using fitting models to calculate the stoichiometry (N), the binding constant (K_A), enthalpy (ΔH), and entropy (ΔS) of the interaction.

Sample preparation

MicroCal iTC₂₀₀ is designed to measure the heat of binding when the ligand solution is injected into the sample (often a protein) solution in the reaction cell and mixed at constant temperature. When the ligand solution is injected into the sample solution there will be a heat change resulting from:

- 1 Interaction between the molecules of interest
- 2 Heats of dilution related to the interactant molecules
- 3 Heats of mixing and dilution resulting from concentration differences (mismatches) in other solvent and solute components

The key to successful ITC experiments is to minimize the heat changes arising from mismatches between the solutions that are mixed. The most common mismatch is produced by pH differences between the ligand solution and the sample solution but differences in salt concentration, buffer concentration or additives such as DMSO may also be involved. To minimize these differences, the interactants should be prepared as far as possible in identical solutions. If one interactant is a macromolecule and the other a pure solid, the macromolecule can be dialyzed or prepared using buffer exchange columns, and the other component dissolved directly in the dialysate or exchange buffer. Heat changes arising from buffer differences can then be determined in a separate control experiment by injecting the ligand solution into the dialysate or exchange buffer. Beware however of dissolving solid preparations that contains additives or residual components such as salt.

The presence of some reducing agents can cause a drift in the baseline. If a reducing agent is required for protein stability, β -mercaptoethanol (< 5 mM) or TCEP (Tris [2-carboxyethylphosphine] hydrochloride, < 2 mM) are recommended.

For small molecule ligands that are supplied in solid form, solutions can be prepared by dissolving the compounds directly in the buffer solution. After dissolving, the pH of the solution should be checked using an accurate pH meter. If the pH of the ligand solution differs by more than 0.05 units from the pH of the buffer solution, the ligand solution should be adjusted with a small amount of HCl or NaOH solution to within 0.05 unit of the buffer solution. The heat changes caused by the small difference in salt concentration will be less than those caused by the pH difference in the unadjusted solution.

Some ligands, which cannot be directly dissolved in the buffer due to low solubility, may be dissolved in DMSO or other organic solvents first in high concentration (10 to 100 mM or higher, if possible) and then diluted with buffer. The concentration of organic additives such as DMSO, in the final ligand solution should be kept as low as possible (preferably 1-2%, typically no more than 5%). The additive should then be added to the sample solution at the same concentration to avoid a large heat change due to solvent mismatch.

Designing an ITC experiment

Concentration of sample molecule for the reaction cell

ITC is designed to detect the heat that is absorbed (endothermic) or liberated (exothermic) when two solutions containing the interactants are mixed. The appropriate concentration of the component in the cell, usually a macromolecule, will depend on the binding affinity, number of binding sites, and heat of binding ΔH . These factors are discussed in detail by Wiseman *et al.* (*Anal. Biochem.* **179**, 131 (1989)), who derived the following equation to help in the design of ITC experiments:

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

where c is an arbitrary number between 1 and 1000 (preferably between 10 and 500 when solubility or availability of material is not limiting)

N is the binding stoichiometry (the number of ligand binding sites on the sample molecule)

M_{tot} is molar concentration of sample molecule in the cell

K_A and K_D are the association and dissociation equilibrium constants respectively for binding to a single site (K_D is the reciprocal of K_A)

There may be practical limitations that affect the choice of sample molecule concentration:

- High affinity interactions (low K_D) should be studied at low concentrations: however, 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) should be studied at high concentrations, but the concentration that can be used may be limited by availability or solubility of the sample molecule.

Techniques such as competition experiments and working at low c numbers can help to alleviate these limitations. These techniques are outside the scope of this Getting Started Booklet.

The **Experimental Design** tab in the MicroCal iTC₂₀₀ software can be used to simulate binding curves for systems with different affinities and sample concentrations. Use this tool to optimize experimental design and assess the likelihood that any given experimental set up will generate good quality data.

In practice, typical concentrations of sample molecule for the sample cell are 10 to 50 μM . If information about the molecules of interest is scant (for example

absence of prior knowledge of the binding affinity), a concentration of about 20 μM is recommended for a first attempt.

Concentration of ligand for the injection syringe

For a 1:1 binding reaction, the molar concentration of ligand in the injection syringe is typically 10–20 times higher than the molar concentration of sample molecule in the cell (for example, use 200 μM of ligand for 20 μM of sample molecule). This will ensure that the cell material will become saturated or close to saturation by the end of the titration experiment.

Injection volume and number of injections

A typical experiment in MicroCal iTC₂₀₀ will involve the injection of 18 injections of 2 μl each, with an initial injection of 0.4 μl to minimize the impact of equilibration artifacts sometimes seen with the first injection. The data point from this initial injection is discarded before data analysis. The pipette holds approximately 38 μl of ligand solution, sufficient for one typical experiment.

Control experiment

As discussed above, a control experiment is required to determine the heat associated with the dilution of the ligand as 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 “machine 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 doing 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 on Sample preparation above). 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.

Experimental temperature

It is most convenient to perform ITC experiments at 25–30°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 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 which 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 with

stirring, and in such cases it may be desirable to work at lower temperatures. For determination of the change in heat capacity ΔC_p associated with binding, experiments must be performed over a range of temperatures (e.g. 10-40°C) to obtain the temperature dependence of the heat of binding.

Exercises

The exercises described in this Getting Started Booklet are divided in two parts: the first part includes hands-on lab exercises (Exercises 1-4) and the second part one software exercise. On completion of the Getting Started exercises, you should have a basic understanding of the main steps in a MicroCal iTC₂₀₀ experiment and of how to handle the system and the software. You should also be ready to incorporate your own reagents into similar protocols.

Hands-on exercises

The hands-on experiment described in this booklet exploits the binding of Ca^{2+} to EDTA. (MicroCal iTC₂₀₀ is commonly used for studying interactions involving macromolecules, but the principles are equally well illustrated with the simple interaction between Ca^{2+} and EDTA.) The following steps are described:

- Exercise 1: System start-up and preparation
- Exercise 2: Hands-on experimental setup for measuring the affinity and thermodynamics of the interaction between Ca^{2+} and EDTA
- Exercise 3: Control experiment
- Exercise 4: Evaluation of the results

Note: *In the exercise below, distilled water can be used in place of CaCl_2 and EDTA for hands-on practice. There will be no heats of interaction involved so that this is an excellent diagnostic test for system performance.*

Exercise 1: System start-up and preparation

- 1 Take out the MicroCal iTC₂₀₀ Test Kit from the refrigerator and equilibrate to room temperature.
- 2 Switch on the PC and MicroCal iTC₂₀₀. The power switch on the instrument is located at the rear on the left hand side.
- 3 Start the MicroCal iTC₂₀₀ software.
- 4 After the system initialization process, the MicroCal iTC₂₀₀ setup software will open. Verify that the red light at the front of the instrument is on.
- 5 Make sure you have the wash station bottles filled to at least 50% with the appropriate solutions. Connect bottles with distilled water and methanol respectively to the marked ports on the wash station. For this exercise, connect a second bottle with distilled water to the buffer port. Figure 3 shows the wash station.



Figure 3. The wash station in MicroCal ITC₂₀₀.

- 6 The reference cell is normally filled with distilled water and only changed weekly. For this exercise you will change the content of the reference cell during the procedure for filling the sample cell. This is described later in the protocol.

Exercise 2: Ca²⁺/EDTA binding experiment

- 1 Go to **Options:Washing module** in the menu bar and make sure that **Advanced User Mode** is not checked, so that on-screen guidance for washing and setting up the analysis is turned on.
- 2 Click on the **Advanced Experimental Design** tab.
- 3 You will setup a method that has one initial small injection to minimize the impact of equilibration artifacts sometimes seen with the first injection, followed by 18 injections of 2.0 µl with 150 seconds between injections. The first small injection will be disregarded during evaluation of the data.

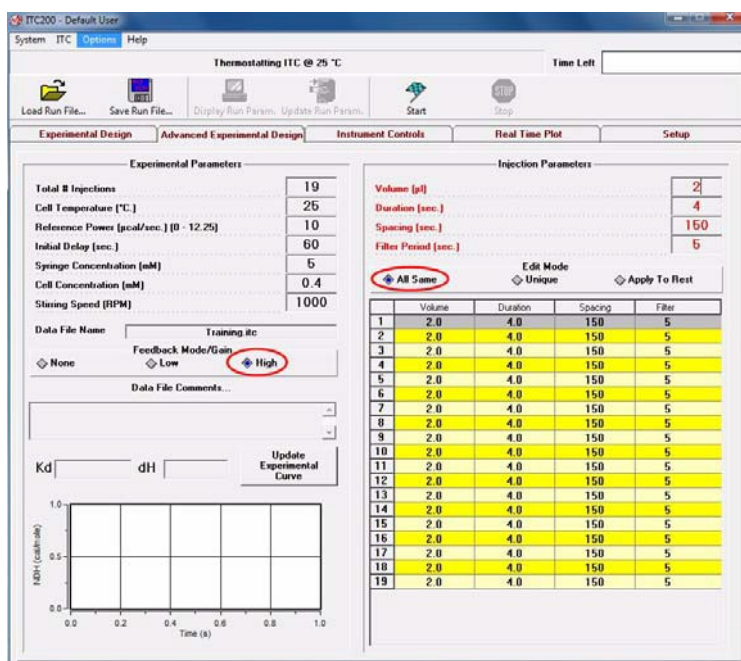
Set the **Edit Mode** to **All Same** and enter the following parameters:

Experimental Parameters

<i>Total # Injections</i>	19
<i>Cell Temperature (°C)</i>	25
<i>Reference Power (µcal/sec)</i>	10
<i>Initial Delay (sec)</i>	60
<i>Syringe Concentration (mM)</i>	5
<i>Cell Concentration (mM)</i>	0.4
<i>Stirring Speed (RPM)</i>	1000

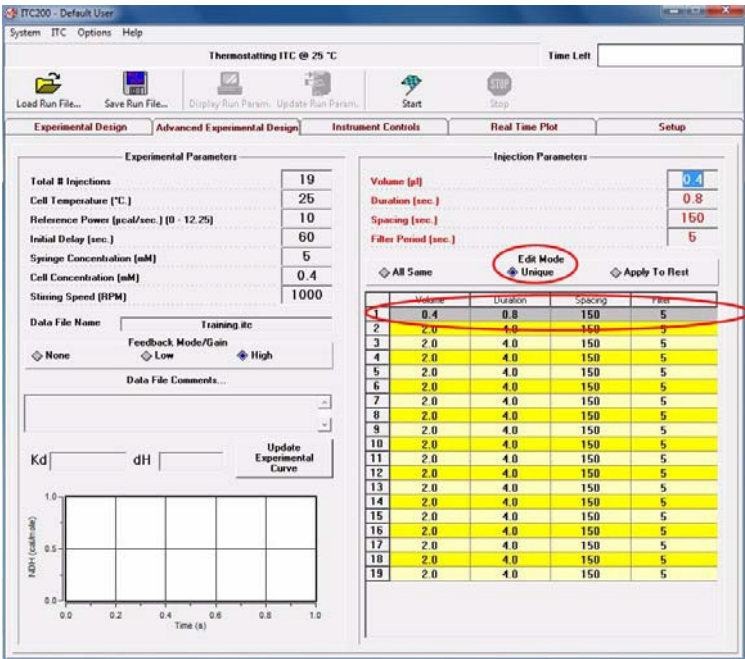
Injection Parameters

Volume (μl)	2
Duration (sec)	4
Spacing (sec)	150
Filter Period (sec)	5



Set the **Feedback Mode/Gain** to **High**.

- Enter a file name for the results (e.g. *Training.itc*) in the **Data File Name** field.
- Change the parameters of the first injection: select the injection in the table on the right and then click **Unique** under **Edit Mode**, then change the settings under **Injection Parameters** to **Volume**: 0.4 and **Duration**: 0.8.

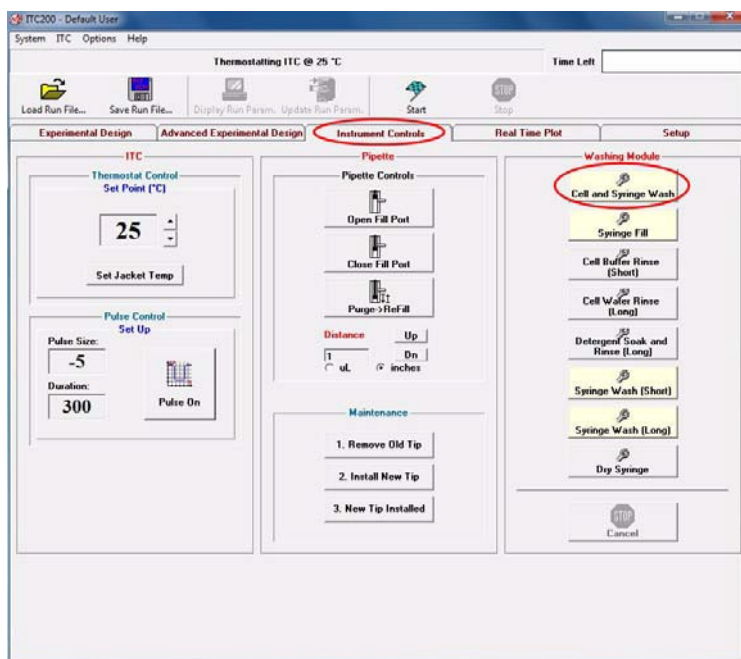


- 6 Inspect the tubing on the washing module to get familiar with the different tubing connections (Figure 4):
- a) C1 to Fill port adaptor storage position (right side)
 - b) C2 to needle wash port (right side)
 - c) C3 to top of wash tool (left side)
 - d) C4 to side of wash tool (left side)
 - e) C5 to waste (left side)

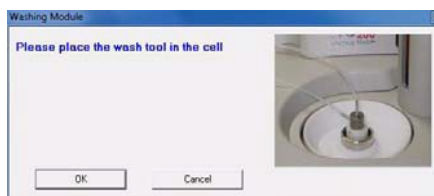


Figure 4. Tubing connections on the washing module.

- 7 Select the **Instrument Controls** tab and click the **Cell and Syringe Wash** button in the **Washing Module** panel to clean the cell and syringe. This procedure should be performed before every experiment.



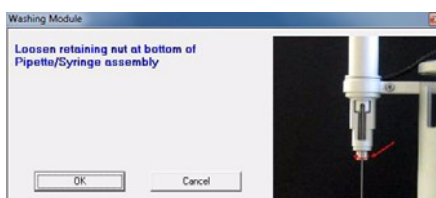
- 8 Follow the step-by-step screen instructions for the washing procedure:
- Place the wash tool into the cell. Click **OK**.



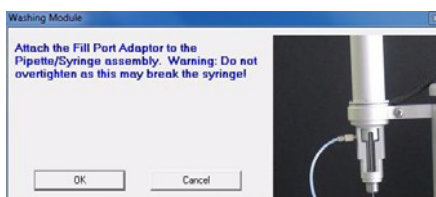
- Place the pipette in the rest position. Click **OK**.



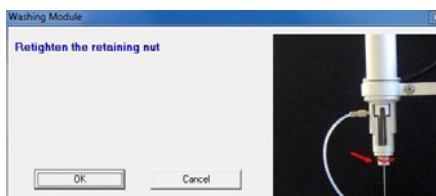
- c) Loosen the retaining nut at the bottom of the pipette by holding the armature and turning the retaining nut a half-turn counter-clockwise. Click **OK**.



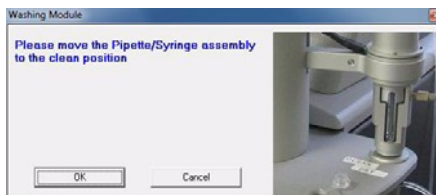
- d) Attach the fill port adaptor to the pipette. You might have to turn the syringe in order to align the two holes on the outer casing of the pipette and the inner syringe holder, respectively. Click **OK**.



- e) Make sure to re-tighten the retaining nut by gently turning it clockwise. Click **OK**.



- f) Move the pipette to the cleaning position and make sure it is correctly seated. Click **OK**. The pipette and sample cell washing procedure will now start.

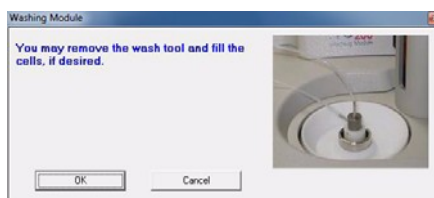


- 9 While the washing procedure is running, place a microcentrifuge tube containing 100 μ l of the ligand solution (CaCl_2) in the tube holder. Check that there are no air bubbles at the base of the tube. Be sure to push the tube to the bottom of the holder and fit the lid into the slot provided (see Figure 5). Be careful not leave any part of the tube in the path of the syringe needle.



Figure 5. Sample tube in place in the holder. The lid of the sample tube fits into the groove in the holder (marked with an arrow in the inset).

- 10 When prompted in the on screen instructions, move the wash tool back to the wash tool storage position. Click **OK** to continue the syringe washing procedure.



- 11 While the syringe washing procedure is running, rinse the sample cell with 10 mM MES buffer and EDTA solution as follows (see Figure 6):
 - a) Insert the Hamilton syringe needle gently into the sample cell until it touches the bottom and remove any remaining liquid from the cell.
 - b) Load the Hamilton syringe with 350 μ l sample buffer (10 mM MES). Invert the syringe and tap it to free any air bubbles, then depress the plunger until all air is dispelled and a drop of liquid appears at the needle tip.
 - c) Insert the syringe needle gently in the sample cell until it touches the bottom, then lift the syringe 1-2 mm above the bottom and slowly inject buffer into the cell (approximately 250 μ l). Gently wash the cell with buffer by aspirating and dispensing 50 μ l buffer at least twice to mix the cell contents.
 - d) Remove as much buffer from the cell as possible.
 - e) Repeat the above procedure using sample solution (EDTA). This procedure may be skipped for runs with valuable sample, but rinsing the cell with sample solution minimizes dilution effects.

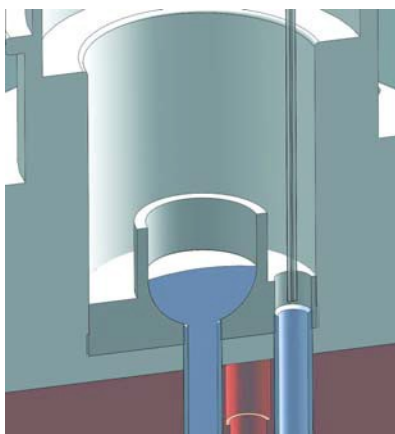


Figure 6. Cross-section of the injection well seen from the front of the instrument, showing the injection ports for the sample cell (left) and the reference cell (right). The illustration shows a Hamilton syringe needle in the reference cell port.

- 12 Fill the sample cell with sample solution (EDTA):
 - a) Fill the Hamilton syringe with 350 μl EDTA solution (at room temperature). Invert the syringe and tap it to free any air bubbles, then depress the plunger until all air is expelled and a drop of liquid appears at the needle tip.
 - b) Insert the syringe needle gently in the sample cell until it touches the bottom, then lift the syringe 1-2 mm above the bottom and slowly inject EDTA solution into the cell until it flows out of the top of the cell stem. Finish by gently aspirating and dispensing 50 μl several times to dislodge any bubbles that may have formed. Be careful not to introduce air when doing this.
 - c) Agitate the solution with the syringe by stirring or gently tapping the bottom of the cell.
 - d) Lift the tip of the syringe to the ledge at the top of the metal cell stem and remove the excess solution.
- 13 Repeat steps 11 and 12 for the reference cell, using distilled water instead of buffer and EDTA. To prevent evaporation from the reference cell, be sure to replace the reference cell lid over the reference cell port (Figure 7).

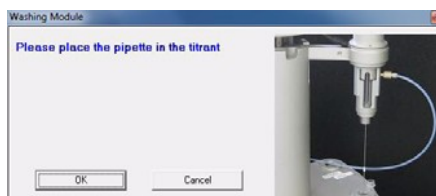


Figure 7. Replacing the reference cell lid over the reference cell port.

- 14 Replace the Hamilton syringe in the syringe holder.
- 15 When the cleaning procedure is finished click on the **Syringe Fill** button in the **Washing module** panel and follow the step-by-step instructions on screen (described in detail below) to load the syringe with CaCl_2 solution.
 - a) Place the pipette in the rest position (the shallow slot facing the user). Click **OK**. You will hear the plunger being driven to the bottom of the syringe.

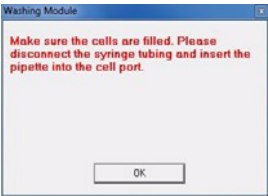


- b) You will be asked to attach the fill port adaptor to the pipette. Since this has already been done in during the washing procedure (steps 8c-8e), you can skip this step by clicking **OK** three times.
- c) When prompted, place the pipette in the microcentrifuge tube containing the CaCl_2 solution (titrant). Click **OK** to fill the syringe. Wait for the filling procedure to complete (approximately 1 minute).

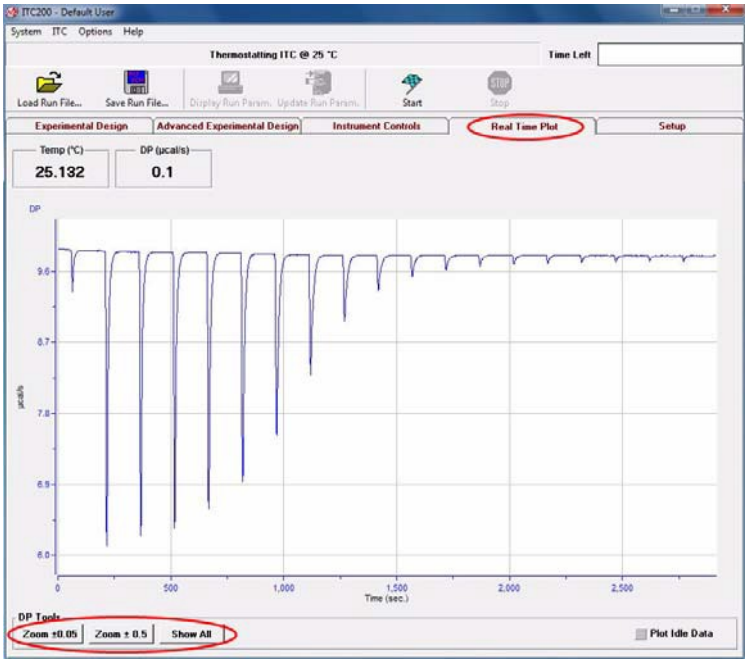


- d) Disconnect the fill port adaptor (syringe tubing) when the syringe loading is completed (the dialogue box shown below will be displayed). Return the fill port adaptor to the fill port adaptor storage position.

- e) Insert the pipette into the cell port. Apply light pressure on the top to make sure it is correctly seated. Click **OK**.



- 16 Click **Start** to begin the assay. After approximately 1 to 5 minutes the Differential Power (DP) signal (shown in red) should stabilize at a value above 9 $\mu\text{cal/sec}$. This is the first indication that the experiment has been set up successfully. If it is lower than this (probably as a result of air bubbles), click **Stop** and reload the cell with the EDTA solution. The run time will be approximately 1 hour.
- 17 Click on the **Real Time Plot** tab to view the raw data. Click the **Zoom ± 0.5** or the **Zoom ± 0.05** button to zoom. Click **Show All** to zoom out.

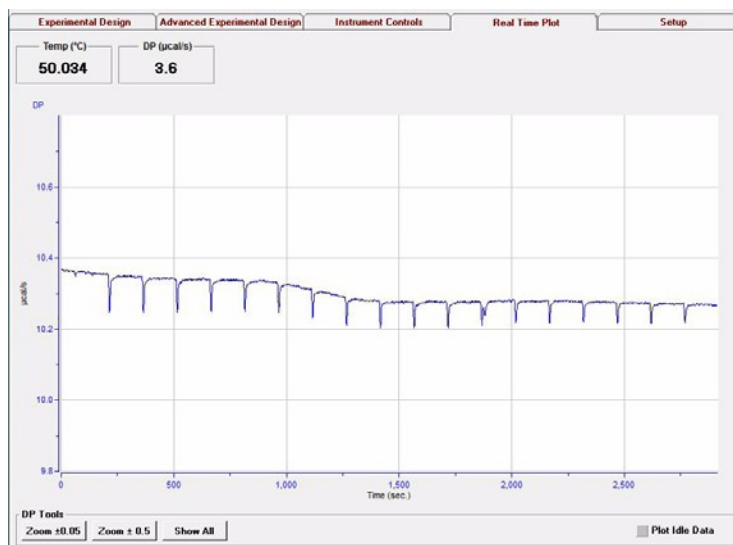


Exercise 3: Control experiment

As a control experiment, repeat the complete experimental procedure (Exercise 2, step 7-15: skip step 13, since is not needed here), using MES buffer instead of EDTA as the sample solution. Keep the same concentration of CaCl_2 (0.4 mM) as

the ligand solution. Remember to change the file name (enter *Control.itc* in the **Advanced experimental design** tab).

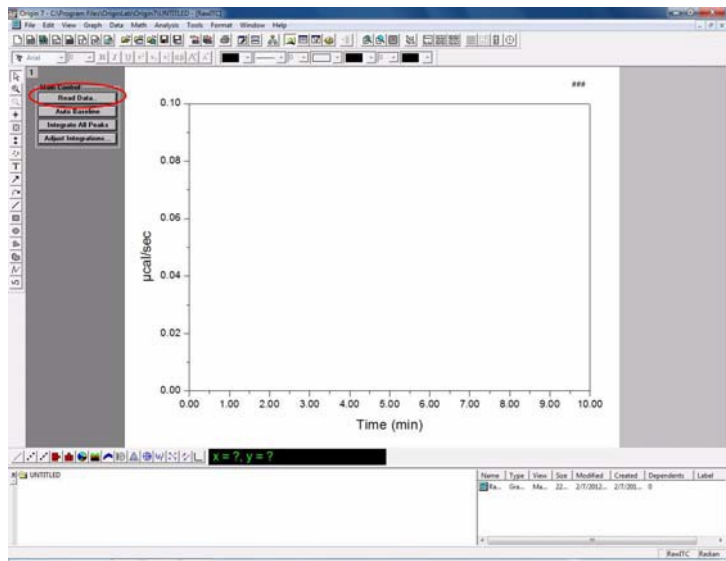
There will be no binding but small heats will be generated representing the heat of dilution of the CaCl_2 and the machine blank under these conditions. These will be subtracted from the results of the titration experiment prior to fitting the data.



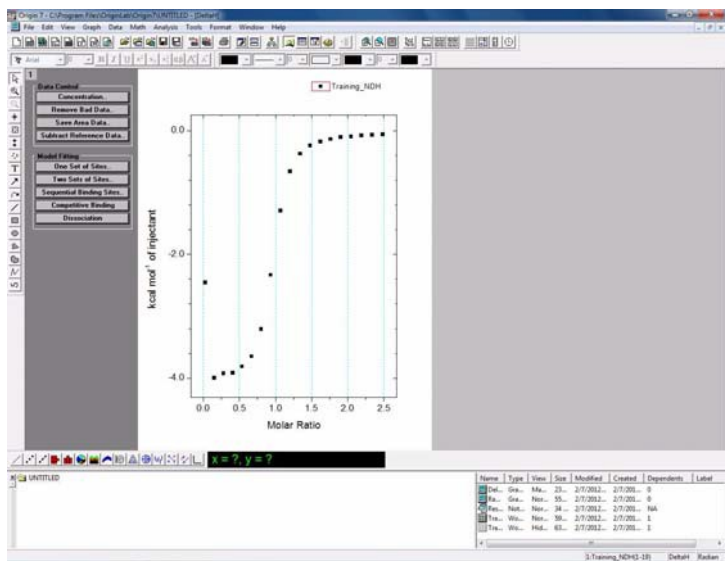
Exercise 4: Data analysis

This section describes how to analyze the data generated in the binding experiment.

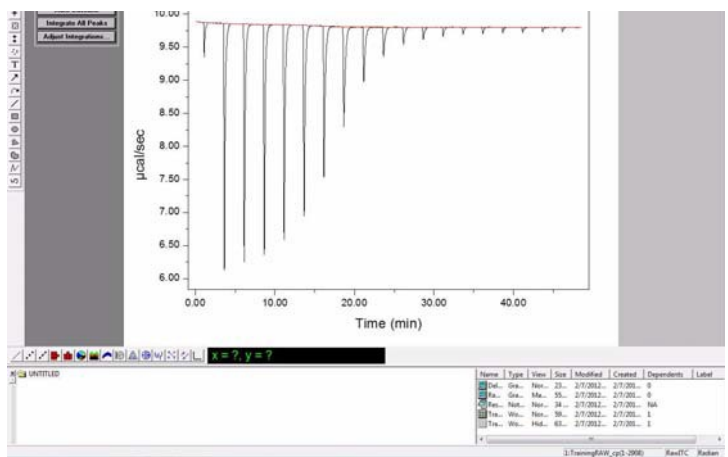
- 1 Double click on the **MicroCal Analysis Launcher** icon on the desktop.
- 2 Click on **iTC200**. This will open the analysis software for MicroCal iTC₂₀₀.
- 3 Click on **Read Data**. To open your titration experiment data file **Training.itc**, click on the data file, click on **Add File(s)** and then on **OK**.



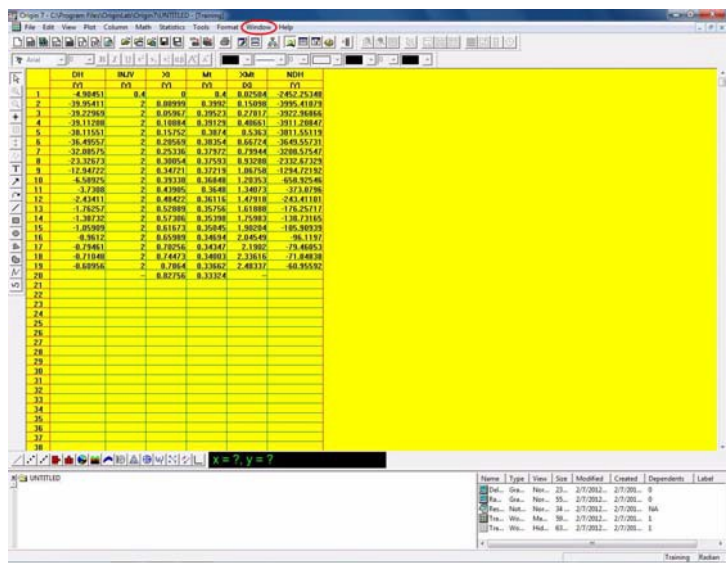
- 4 The software will automatically calculate the area of each peak and plot these values versus Molar Ratio as shown below. If the data points are significantly more scattered than those shown here you may need to run the experiment again. Poor quality data is most commonly caused by insufficient cleaning of the sample cell or by air bubbles in the cells. Following maintenance procedures and practicing the cell loading procedure will reduce the risk for poor quality data.



- 5 To view a graphical presentation of the data, select **Window** (in the menu bar) and then **RawITC**. The red line is generated automatically and is used as the baseline for peak integration. The heat of each injection calculated in this way is used to generate the concentration-normalized binding isotherm shown in step 4 above. The baseline can be adjusted manually if required as explained in the software exercise later in this booklet.

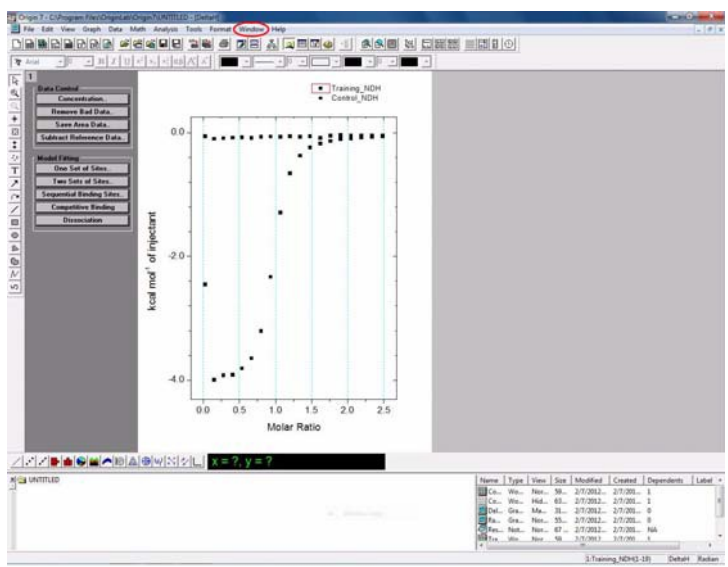


- 6 To view the data in numerical form, select **Window:Training**. The table contains all the information required for data analysis. There is no need for any user intervention at this point, but examining the data can be useful for some troubleshooting purposes. The first column shows the heat of each injection in μcal . The first (small volume) injection is discarded. As a general rule, the minimum absolute heat of the second injection should typically be larger than $\pm 3 \mu\text{cal}$. If the heats are smaller it may be useful to repeat the experiment and increase the concentration of the cell and syringe material. The second column displays the injection volumes. X_t and M_t refer to the concentration of the ligand and cell material in the cell, respectively, before the injection. X/M_t is the ratio of concentration of the materials in the cell after the injection and the NDH is the heat of injection normalized per mole of X_t .



	QIN	INJ	V	X	M	X/M	NDH
1	-4.00451	0.4	0	0	0.00504	-2.462	25340
2	-39.95411	2	0.00919	0.2992	0.15019	-3995.41873	
3	-29.22909	2	0.00917	0.29523	0.27017	-3922.90866	
4	-29.11298	2	0.10084	0.29129	0.40661	-3911.20847	
5	-38.11551	2	0.15152	0.3074	0.5363	-3811.55119	
6	-36.49557	2	0.26038	0.30264	0.66174	-3649.55731	
7	-32.88975	2	0.25136	0.37372	0.73944	-3288.97547	
8	-23.32673	2	0.30854	0.37531	0.83200	-2332.67329	
9	-12.94722	2	0.34721	0.37218	1.08758	-1294.72132	
10	-6.50925	2	0.39138	0.36848	1.28753	-650.92546	
11	-3.7208	2	0.42905	0.3649	1.4673	-372.0796	
12	-2.43411	2	0.48422	0.36116	1.47918	-243.41181	
13	-1.76257	2	0.52989	0.35796	1.61889	-176.25717	
14	-1.38732	2	0.57388	0.35398	1.75982	-138.73165	
15	-1.05099	2	0.61673	0.35045	1.90284	-105.09929	
16	-0.9612	2	0.65988	0.34634	2.04649	-96.1197	
17	-0.73461	2	0.70256	0.34347	2.1982	-73.46853	
18	-0.71946	2	0.74472	0.34051	2.33616	-71.94838	
19	-0.60956	2	0.7864	0.33652	2.48337	-60.95532	
20			0.82756	0.33324			
21							
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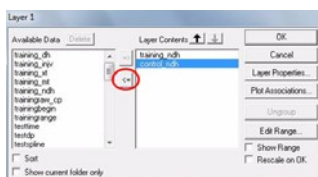
- 7 Select **Window:RawITC**. Click on **Read Data** and open your control experiment data file, *Control.itc*. The software generates a baseline and integrates each peak before displaying the concentration normalized isotherm. Select **Window:DeltaH**. The data should look similar to the figure below.



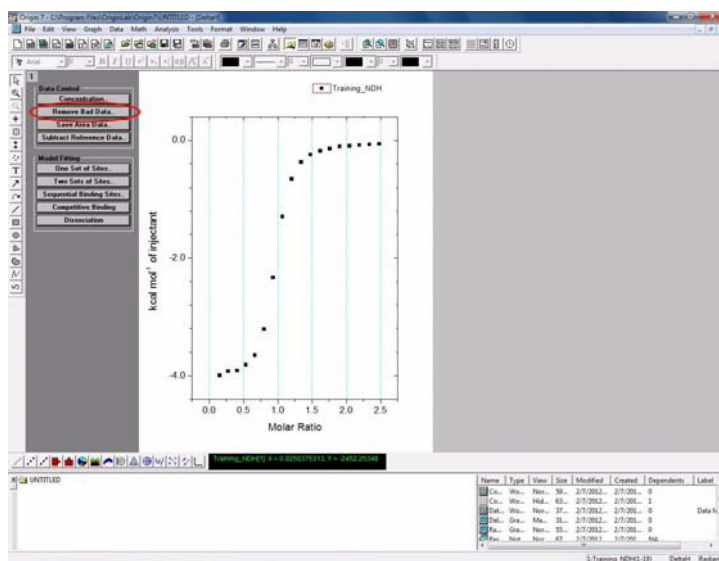
- 8 Select **Window:Control**. Highlight the values in the **NDH** column excluding the first injection. Select **Statistics:Descriptive Statistics** and then **Statistics on Columns**.



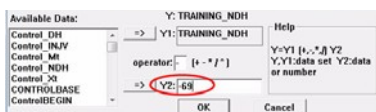
Then click on the left arrow between the columns. Click **OK** to remove the control data set from the plot.



- 11 In order to remove the first injection, click the **Remove Bad Data** button, and place the cross-hair over the first point and double click to remove the data point.



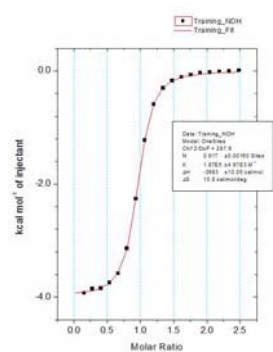
- 12 Select **Math** (in the menu bar) and then **Simple Math**. Enter a minus sign (-) into the space provided for the operator (to subtract the mean value of the control heat) and then enter the mean value for the control heat that was determined in step 9 into the **Y2** box.



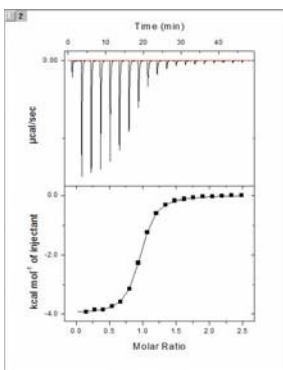
- 13 Click the **One Set of Sites** button (in the model fitting) to fit the data. This will open the fitting session dialogue box showing the initial fitting and values for stoichiometry (**N**), association equilibrium constant (**K**) and enthalpy change (**H**).



- 14 Click on the **200 iter** button to start the fitting. Repeat this until the **Reduced Chi-sqr** value remains constant. Click **Done** to finish the fitting procedure.
- 15 The N, K (K_A), ΔH and ΔS values based on the fitting will be displayed next to the plot



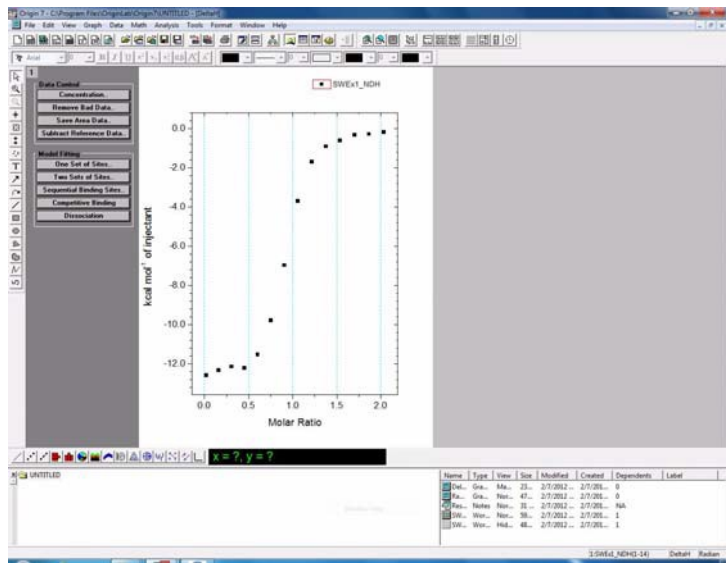
- 16 Select **File:Save Project As** to save the fitted results. Enter a name and click **Save**.
- 17 A final figure showing the baseline corrected raw data and the fitted data together can be generated by clicking **Analysis** in the menu bar and selecting **Final Figure**. These figures can be copied using the **Edit:Copy Page** command.



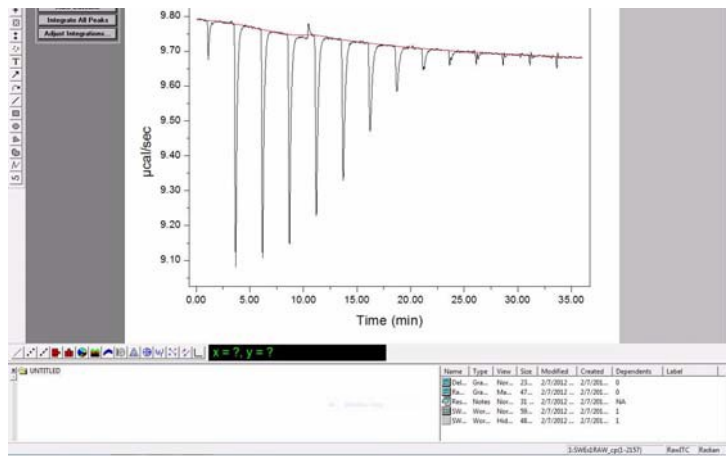
Software exercise

This section of the booklet will help you gain more experience with data analysis. For this purpose you have been provided with 2 data sets; one is for 1:1 binding of a small molecule ligand to a protein (**SW Ex1.itc**) and one is a control experiment (**SW Ex1 Control.itc**).

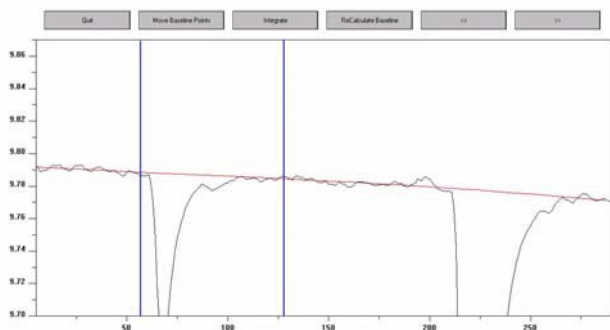
- 1 Double click on the **MicroCal Analysis Launcher** icon on the desktop.
- 2 Click on **iTC200**. This will open the analysis software for MicroCal iTC₂₀₀.
- 3 Click on **Read Data**. Open the data file **SW Ex1.itc**. It is located in the folder **Origin7** and subfolder **Samples**. Occasionally there will be an air-bubble in the sample cell, as in this data set (seen between peak 4 and 5). It is recommended to remove this experimental artifact as will be explained in the steps below.



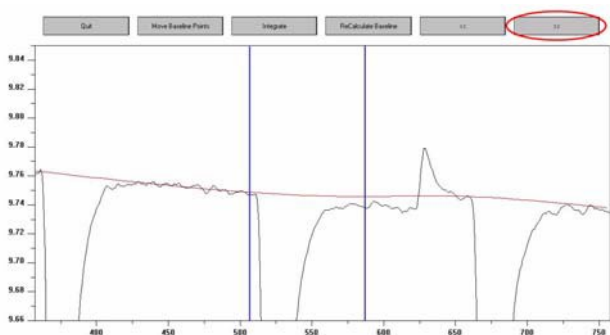
- 4 Select **Window:RawITC**. The red line is generated automatically and is used as the baseline for peak integration. The heat of each injection calculated in this way is used to generate the concentration-normalized binding isotherm. The baseline can be adjusted manually as explained in the following steps.



- 5 Click on **Adjust Integrations**. Place the cross-hair on the first peak and click the left mouse button. The display will appear as below. The data is integrated between the two blue vertical lines for each peak.

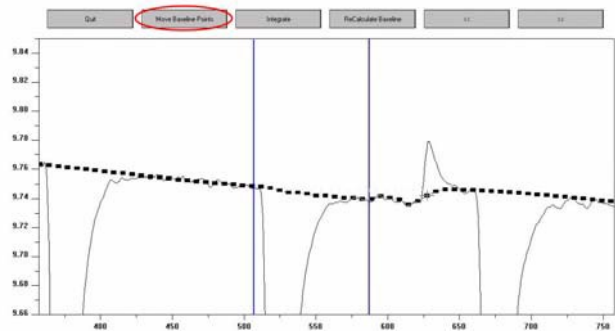


- 6 Click on the >> (forward) button to move to the next peak. Repeat three times to move to the fourth peak. The baseline fits well to the experimental data for the first three peaks. The air bubble between peak four and five has resulted in a poor fit of the baseline.

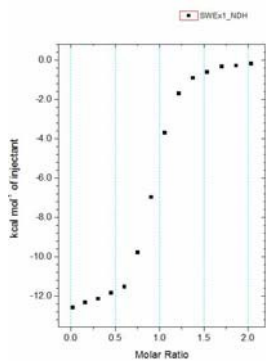


- 7 The baseline can be adjusted manually. Click on **Move Baseline points** to activate the baseline manipulation feature. Adjust the baseline by dragging the black handles so that you form a smooth line between the beginning and the end of the peak.

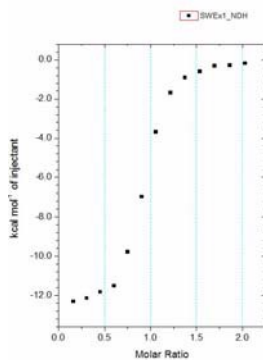
Note: This manual manipulation will be lost if you press **ReCalculate Baseline**.



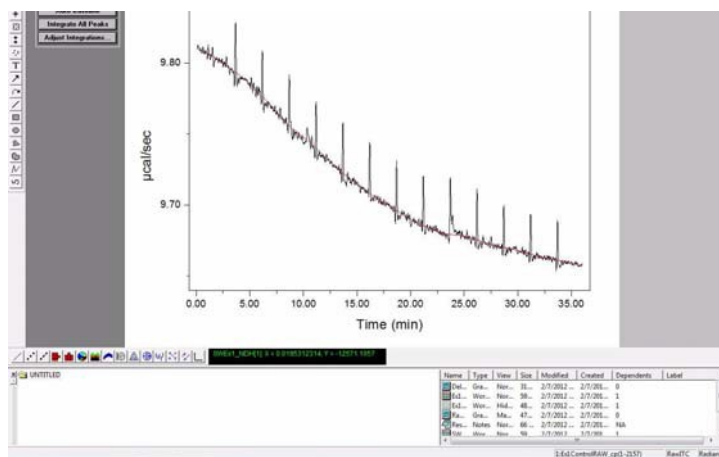
- 8 Click on the >> (forward) button and continue to go through all the peaks. In this exercise no more manipulations are necessary.
- 9 Click **Quit** to return to the raw data.
- 10 Click **Integrate all peaks** to reintegrate the data. The normalized ΔH plot will be generated.



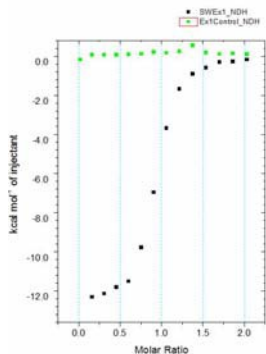
- 11 In order to remove the first injection, click the **Remove Bad Data** button, place the cross-hair over the first point and double click to remove the data point. The software will automatically calculate the area of each peak and plot these values versus Molar Ratio as shown below.



- 12 Repeat the evaluation for the control experiment (file name **SW Ex Control.itc**). Select **Window:RawITC**. Click on **Read Data** and open your control experiment data file.



- 13 Click **Integrate All Peaks**.



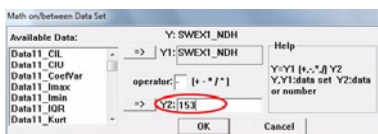
- 14 Select **Window:Ex1 Control**. Highlight the values in the **NDH** column excluding the first injection. Select **Statistics:Descriptive Statistics** and then **Statistics on Columns**.

	DH	INLV	XI	Mt	XMt	NDH
	Y1	Y1	Y1	Y1	DQ	Y1
1	-0.0181	0.4	0	0.025	0.01953	-101.00507
2	0.04121	2.9	0.00399	0.02495	0.16227	56.8466
3	0.04188	2.9	0.00399	0.0246	0.30701	57.76299
4	0.03922	2.9	0.00745	0.02426	0.45375	54.99316
5	0.0587	2.9	0.01085	0.02391	0.6025	80.96402
6	0.07561	2.9	0.01421	0.02358	0.75324	104.28421
7	0.15582	2.9	0.01751	0.02325	0.90599	214.92716
8	0.11803	2.9	0.02076	0.02292	1.06974	162.80252
9	0.16695	2.9	0.02397	0.0226	1.21748	229.04095
10	0.39297	2.9	0.02712	0.02228	1.37623	542.02799
11	0.12156	2.9	0.03023	0.02196	1.53699	167.66232
12	0.07503	2.9	0.03320	0.02165	1.69974	104.59949
13	0.09527	2.9	0.03629	0.02135	1.86449	131.40221
14	0.05906	2.9	0.03924	0.02105	2.03124	81.46465
15			0.04215	0.02075		
16						

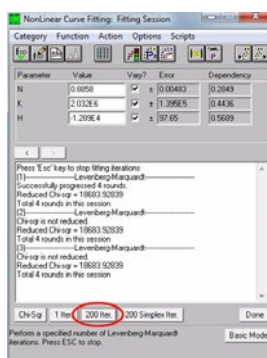
- 15 Some basic statistical information will be tabulated as shown below. Write down the **Mean(Y)** value for the control heats found in the third column. This value represents the heat associated with ligand dilution and the machine blank and will be subtracted from your data set later on.

Data from Ex1Control		Recalculate		Entire Dataset	
		Use Rows			
Advanced Statistics		Percentile		95.00	
Col(X)	Rows(Y)	Mean(Y)	sd(yf1)	sd(yf2)	sd(yf3)
1	NDH	2.14	152.91354	139.66816	36.22806
2					
3					
4					

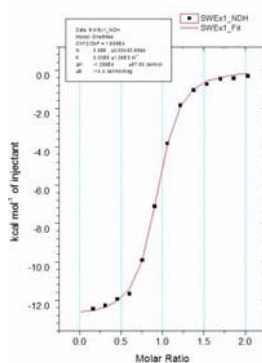
- 16 Select **Window:DeltaH**. Remove the control experiment from the plot by double clicking on the **1** in the top left hand corner of the display page and selecting **ex1control_ndh** in the **Layer Contents** column. Then click on the left arrow between the columns. Click **OK** to remove the control data set from the plot.
- 17 Select **Math:Simple Math**. Enter a minus sign (-) into the space provided for the operator and then enter the mean value for the control heats that was determined in step 15 into the **Y2** box. Click **OK**.



- 18 Click the **One Set of Sites** button to fit the data. This will open the fitting session dialogue box showing the initial fitting and values for stoichiometry (**N**), association equilibrium constant (**K**) and enthalpy change (**H**).
- 19 Click on the **200 iter** button to start the fitting. Repeat this until the **Reduced Chi-sqr** value remains constant. Click **Done** to finish the fitting procedure.

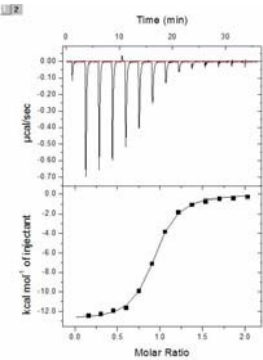


- 20 Click **Done**. The final fitted parameters will be inserted into the figure.



- 21 Select **File:Save Project As** to save the fitted data. Enter a name and click **Save**.

- 22 A final figure showing the baseline corrected raw data and the fitted data together, can be generated by clicking **Analysis** (in the menu bar) and selecting **Final Figure**. The figures can be copied to the Windows clipboard for pasting into other programs by using **Edit:Copy Page**.



Maintenance

Maintenance

Regular maintenance of MicroCal iTC₂₀₀ is of the utmost importance for reliable results. It is important to avoid contamination, such as microbial growth and adsorbed proteins in the system.

- The system should be cleaned after each run using the **Cell and Syringe Wash** command.
- Replace the distilled water in the reference cell every week.

When necessary

- Clean the cell(s) with 20% Contrad 70 (or 14% Decon 90). Use the **Detergent Soak and Rinse (long)** command and follow the instructions to load the cell with 20% Contrad 70 (or 14% Decon 90) and heat for 30 minutes at 60°C. This should be done whenever a water rinse is insufficient to clean the cell properly. The easiest way to check if the cell needs cleaning is to check that the baseline position is no more than 1 µcal/sec lower than the reference power setting in the ITC experiment set up.
- Clean the pipette with 20% Contrad 70 (or 14% Decon 90). Pull 2 x 100 µl of detergent into the pipette and then rinse with water using **Syringe rinse long** on the **Accessory Station**.
- Replace the plunger tip.
- If the system is to be left for long periods make sure the sample and reference cells are emptied.

For additional information on maintenance of MicroCal iTC₂₀₀, refer to:

- MicroCal iTC₂₀₀ System User Manual (code no. MAN0560-01)
- www.malvern/microcal

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