Malvern Instruments

# MICROCAL PEAQ-ITC SYSTEM

## Getting Started Booklet


Malvern

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# TABLE OF CONTENTS

# INTRODUCTION

**Note**: Before using the Getting Started Booklet all users must read the MicroCal PEAQ-ITC Operating Instructions which gives Health & Safety, maintenance, troubleshooting and other vital information.

MicroCal PEAQ-ITC System Getting Started booklet is designed to introduce you to the basic operations of MicroCal PEAQ-ITC system and software[1]. This booklet provides guidance through the basic steps in a MicroCal PEAQ-ITC experiment using reagents supplied in the EDTA Test Kit MicroCal PEAQ-ITC.

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 PEAQ-ITC System
- EDTA Test Kit MicroCal ITC
- MicroCal PEAQ-ITC System Getting Started Booklet
- Micropipettes (20-200 µL and 100-1000 µL) and tips
- 200 µL Eppendorf microcentrifuge tubes
- Wash solutions (distilled water, methanol[2], Contrad™ 70 or Decon™ 90)

## Contents of the MicroCal PEAQ-ITC Test Kit

The contents of the MicroCal PEAQ-ITC 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.

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

| Reagent/Item, quantity |
|---|
| 0.1 mM EDTA in MES buffer, pH 5.6 (10 mL) |
| 1 mM CaCl2 in MES buffer, pH 5.6 (1.3 mL) |
| 10 mM MES buffer, pH 5.6 (7 mL) |

[1] Applies to MicroCal ITC Control Software ver1.0 and MicroCal ITC Analysis Software version 1.0, or later.
[2] Methanol is classed as a hazardous chemical in some countries. Observe the applicable regulations for handling methanol.

# Ordering information

| | |
|---|---|
| EDTA Test Kit MicroCal PEAQ-ITC | 28-4290-63 |
| MicroCal PEAQ-ITC System Getting Started Booklet | |

# References

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

MicroCal PEAQ-ITC System User Manual
MicroCal PEAQ-ITC Analysis Software User Manual

# BACKGROUND INFORMATION

This section provides the basis for a more detailed understanding of the main steps in a MicroCal PEAQ-ITC experiment. The information goes beyond what is explicitly covered in the exercises and intended as a complement to them.

## 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 (titration) syringe which holds the other binding partner (the ligand). The ligand is injected into the sample cell, typically in 0.5 to 3 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 (KD), stoichiometry (N) and enthalpy of interaction (ΔH). A schematic representation of an ITC system such as MicroCal PEAQ-ITC is shown in Figure 1.

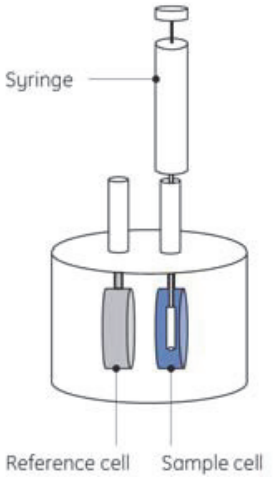



**Figure 1. Schematic representation of the essential components of an ITC system such as MicroCal ITC.**

## General description

MicroCal PEAQ-ITC 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 MicroCal PEAQ-ITC Analysis software using fitting models to calculate the stoichiometry (N), the binding constant (KD), enthalpy (ΔH), and entropy (ΔS) of the interaction.

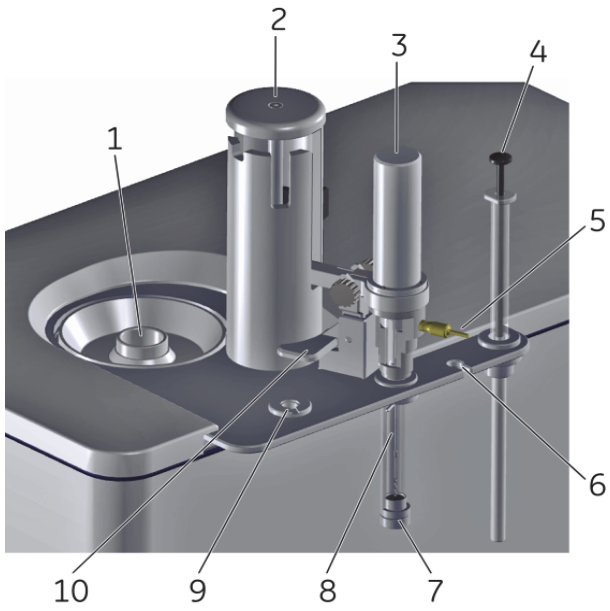



**Figure 2. Illustration showing the MicroCal PEAQ-ITC system cell unit.**

| Part | Description |
|---|---|
| 1 | Sample cell |
| 2 | Injector tower |
| 3 | Pipette |
| 4 | Loading syringe |
| 5 | Fill Port Adapter (FPA) |
| 6 | FPA storage location |
| 7 | Wash/load station |
| 8 | Titration syringe |
| 9 | Titration loading station |
| 10 | Clamp |

# Sample preparation

MicroCal PEAQ-ITC 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:

- Interaction between the molecules of interest
- Heats of dilution related to the interacting molecules
- 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 interacting species should be prepared as much as possible in identical solutions. If one solution contains 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/sample cell

ITC is designed to detect the heat that is absorbed (endothermic) or liberated (exothermic) when two solutions containing the interacting species 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. 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:

$$c = \frac{N.M_{tot}}{K_D}$$

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
- *KD* is the dissociation equilibrium constant for single site complex M:L.

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

- High affinity interactions (low KD) should be studied at low concentrations: however, the minimum concentration that will typically produce a confidently measurable heat change for a 1:1 interaction is about 10 µM.
- Low affinity interactions (high KD) 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 ***Design Experiment*** workspace in the MicroCal PEAQ-ITC Control 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 titration syringe

For a 1:1 binding reaction, the molar concentration of ligand in the titration 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 PEAQ-ITC system will involve a series of 18 injections of 2 µL each, plus an initial injection of 0.4 µL (total of 19 injections) 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.

## Subtracting control data / Control experiments

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 Sample preparation). 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.

In the data analysis software, you can select between different control subtraction methods:

- **None**
  No control subtraction will be applied to the selected experiment.
- **Fitted Offset**
  A constant control heat (Offset) will be assumed and fitted to the integrated heats along with the chosen model's fitting parameters.
- **Single**
  Control heats of a single dataset (control experiment) will be subtracted from the experiment's integrated heats.
- **Composite**
  Control heats of multiple datasets (control experiments) will be used to operate on the experiment's integrated heats.

These are described in more detail in the MicroCal PEAQ-ITC Analysis Software User Manual and will be further discussed in Software exercise 6.

## 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, and in such cases it may be desirable to work at lower temperatures. For determination of the change in heat capacity ΔCp 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 sections. Section one contains four hands-on lab exercises, and Section two contains two software exercises. On completion of the Getting Started exercises, you should have a basic understanding of the main steps in a MicroCal PEAQ-ITC 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

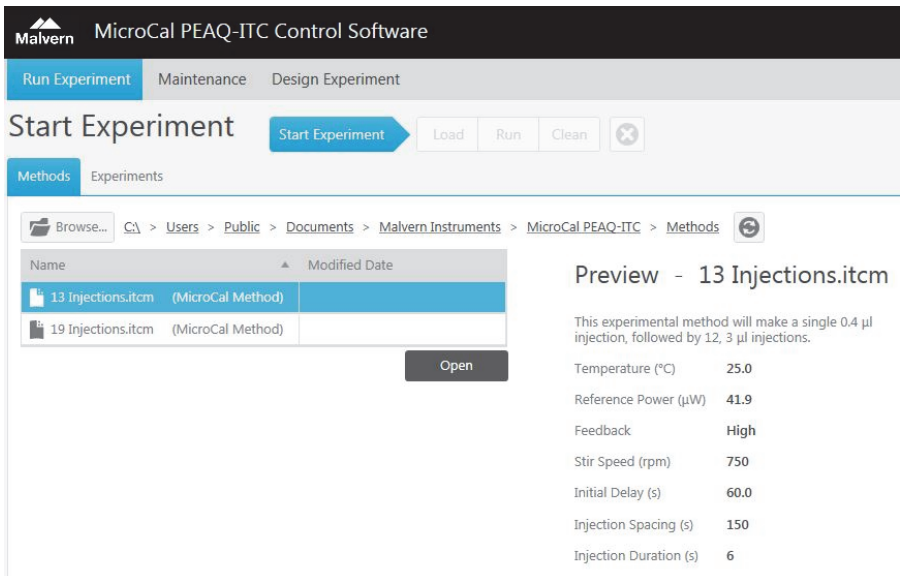
The hands-on exercises described in this booklet exploit the binding of CaCl2 to EDTA. MicroCal PEAQ-ITC is commonly used for studying interactions involving macromolecules, but the principles are equally well illustrated with the simple interaction between CaCl2 and EDTA. The following steps are described:

- **Exercise 1**: System start-up and preparation
- **Exercise 2**: CaCl2/EDTA titration experiment
- **Exercise 3**: Control experiment
- **Exercise 4**: Evaluation of the results

**Note**: For hands-on practice, distilled water can be used in place of CaCl2 and EDTA in the exercise 2 below.

## Exercise 1: System start-up and preparation

1. Take out the MicroCal PEAQ-ITC Test Kit from the refrigerator and equilibrate to room temperature.
2. Power up the MicroCal PEAQ-ITC Controller PC and the MicroCal ITC, doing the following steps in order.
    a. Turn on the controller PC.
    b. Once the controller PC is on and Windows is running, turn on the power switch at the rear on the right hand side of the MicroCal ITC.
    c. Launch the ***MicroCal PEAQ-ITC Control Software*** by double clicking the start icon ITC on the desktop. After the system initialization process, the ***MicroCal PEAQ-ITC Control Software*** will open. Verify that the green light at the front of the instrument is on.



3. Make sure the wash station bottles are filled to at least 50% with the appropriate solutions (distilled water, methanol and detergent solution, i.e. 20% Contrad 70 or 14% Decon 90) and that the waste and overflow bottles are empty (see Figure 3 below).

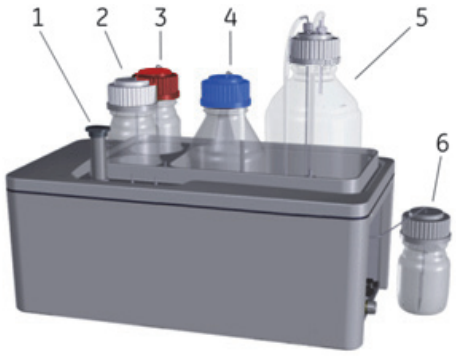



**Figure 3. The wash module in MicroCal PEAQ-ITC system.**

| Part | Description |
|---|---|
| 1 | Cell Cleaning Tool |
| 2 | Detergent bottle |
| 3 | Methanol bottle |
| 4 | Water bottle |
| 5 | Waste bottle |
| 6 | Overflow bottle |

4. The reference cell is normally filled with distilled water and changed weekly (to avoid bacterial growth and to ensure that it is full). 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 booklet (see Exercises 2, step 15).

## Exercise 2: CaCl2/EDTA titration experiment

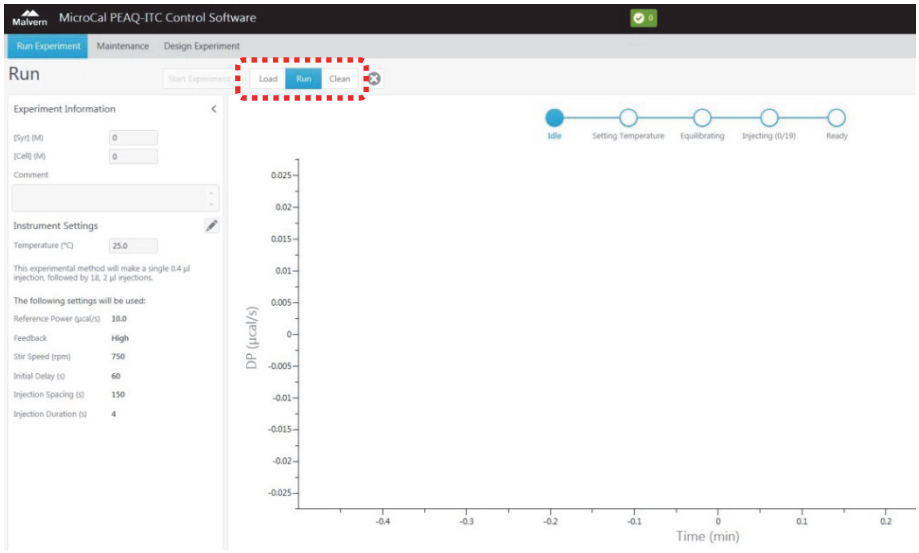
1. You will setup an experiment to study the binding of CaCl2 to EDTA. The method should have one initial small injection (0.4 µL) to minimize the impact of equilibration artifacts sometimes seen with the first injection, followed by 18 injections of 2.0 µL with 150 seconds between injections. The first small injection will be disregarded during evaluation of the data.

2. In the Methods pane, double click on the pre-defined method 19 Injections.itcm file. Table 2 shows a summary of the parameters used in this method.

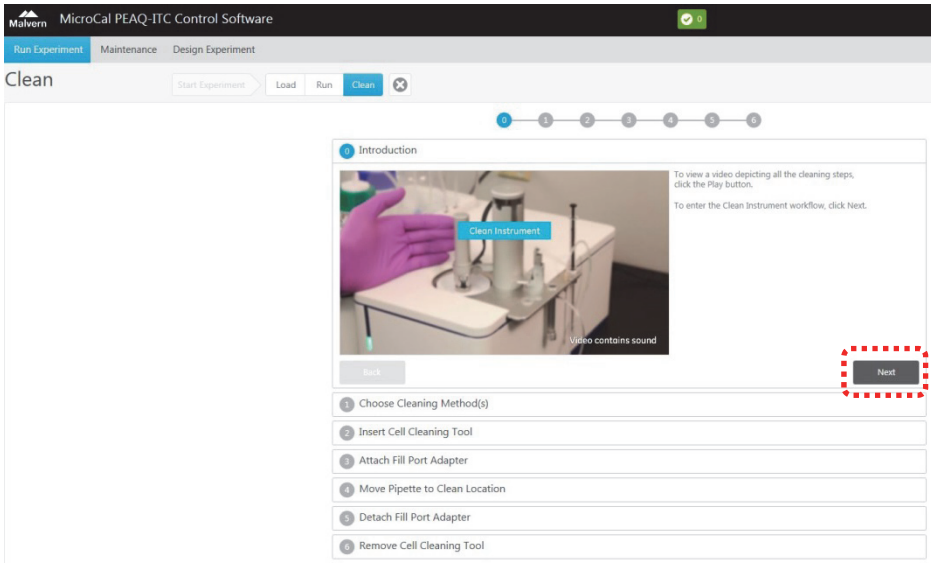
| **Run parameters** | |
|---|---|
| Temperature ( C) | 25 |
| Reference power (µcal/s) | 5 |
| Feedback | High |
| Stir speed (rpm) | 750 |
| Initial delay (s) | 60 |
| **Injection parameters** | |
| # of injections | 19 |
| Volume (µl) | 2 |
| Duration (s) | 4 |
| Spacing (s) | 150 |

**Table 2. Summary of the pre-defined method *19 Injections.itcm* that will be used in this exercise.**

3. MicroCal PEAQ-ITC Control Software then opens the ***Run*** workspace. Here you can work with the ***Load***, ***Run*** or ***Clean*** workspaces in any order as you like. In this exercise we will start with the ***Clean*** workspace, to ensure the instrument is clean before performing an experiment. Generally, you perform the cleaning as a last step before leaving the system (when you have completed your experiment).




4. Click on ***Clean*** to clean both the cell and the syringe. Follow the step-by-step video instructions and use the predefined settings. Click ***Next*** in the Introduction step.

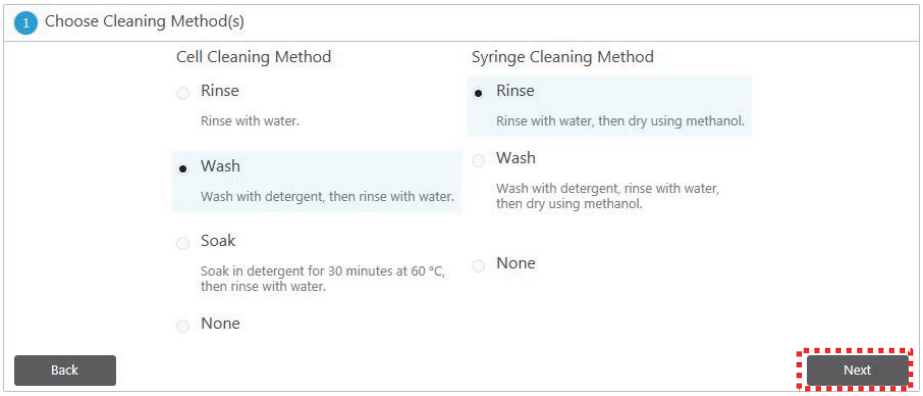



5. In step 1, you choose how to perform the cleaning. Use the default cleaning methods, which are described in the software:

   - Cell Cleaning Method – Wash

   - Syringe Cleaning Method – Rinse

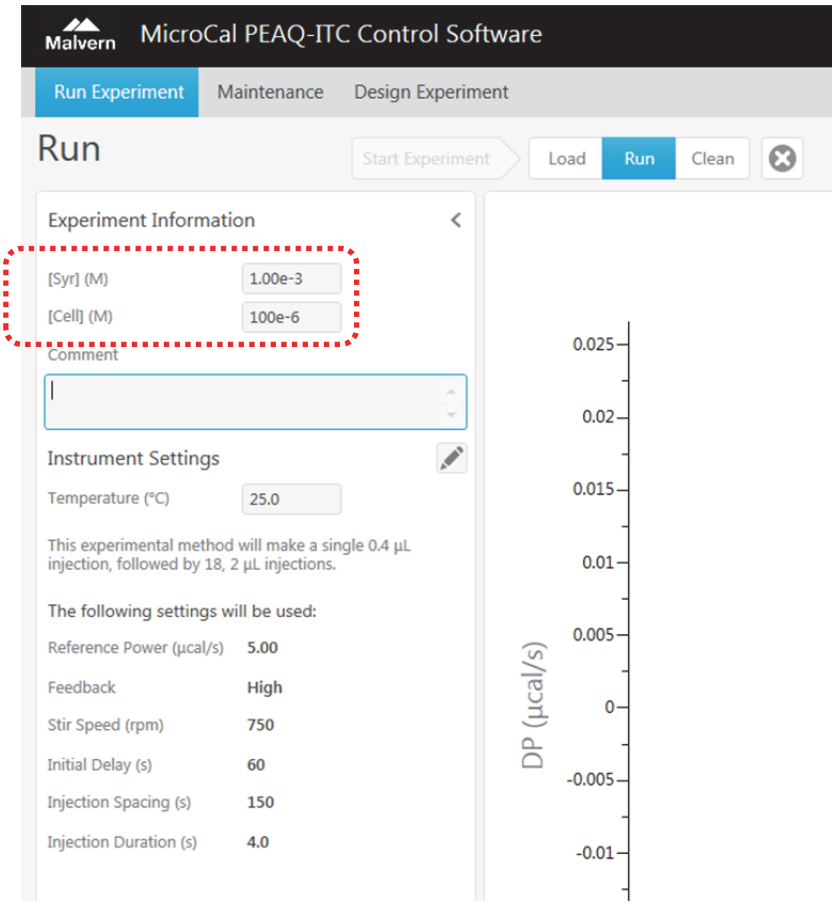
   Click ***Next*** to proceed through the steps (1-6) of the cleaning procedure.



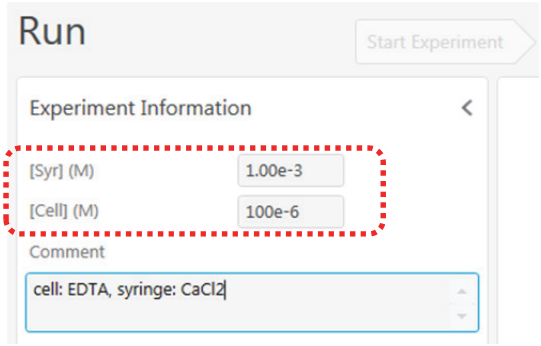

6. When the last step "Remove Cell Cleaning Tool" is performed, click Done.

7. Click Run to return to the Run workspace.

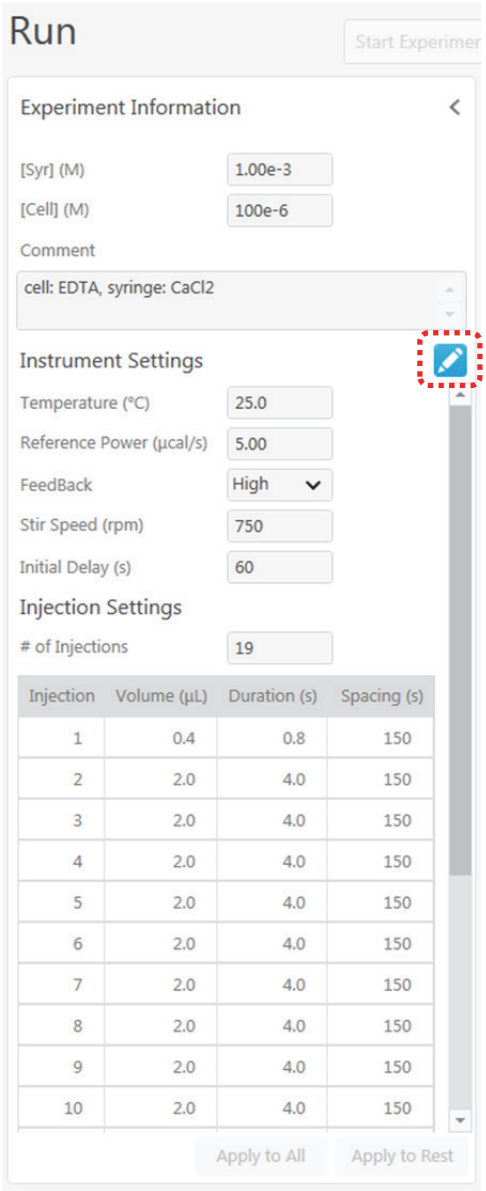
8. The ***Experiment*** pane (to the left in the following screenshot) shows the run parameters for the pre-defined method ***19 Injections.itcm***. The default settings are suitable for the $CaCl_2$/EDTA titration experiment. Enter the concentrations for the syringe Syr (M) = 1e-3 ($CaCl_2$) and the Cell (M) = 100e-6 (EDTA).



9. In the **Comment** box, enter the description of the samples, e.g. *cell: EDTA, syringe: CaCl2*.

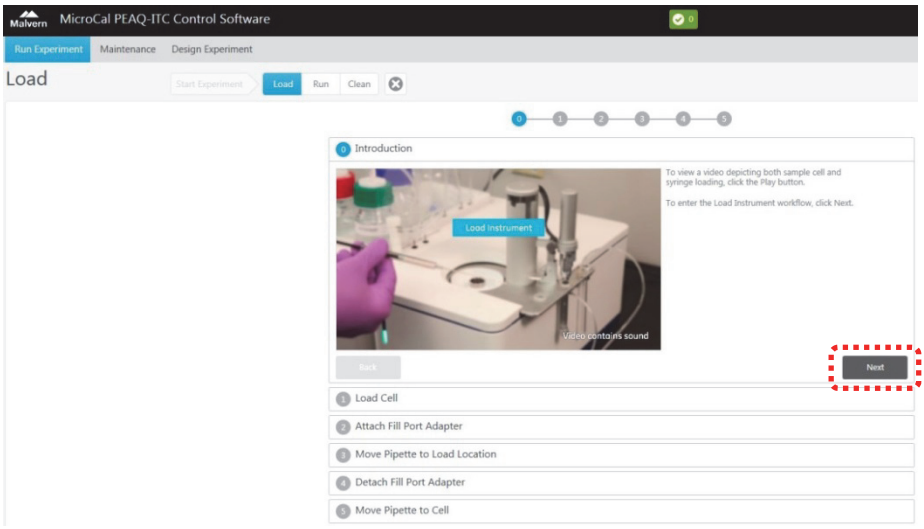



**Note:** To make changes in a method (e.g change the volume of a single or multiple injections), click the Edit Settings icon Click it again to accept the modification. In this exercise, no modifications are required.



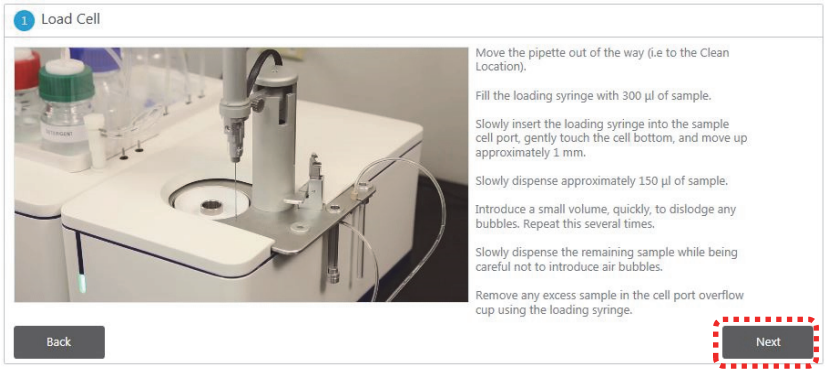
10. Click on ***Save As Method*** to save the settings as a new method for later use. Enter a name e.g. "***EDTA method.itcm***" and click ***Save***. This method is now saved in the pre-defined Methods folder.

11. Before starting the experiment, you need to load both the sample cell and the syringe. Click ***Load*** and follow the step-by-step video instructions to load the EDTA solution in the sample cell and the $CaCl_2$ solution in the syringe. You can choose to view a video depicting all steps in the Introduction step (click Play button in the video window) or enter the Load workflow directly. Click ***Next*** to proceed through the modules of the loading procedure.




12. **Step 1, Load cell**: In a typical experiment, rinse the sample cell with buffer, before loading the cell with real sample. In this exercise, rinse the sample cell with EDTA solution instead, since it is inexpensive and this practice will result in a stoichiometry closer to one.

13. Rinse the sample cell with EDTA solution as follows:




    a. Insert the Loading syringe needle gently into the sample cell until it touches the bottom and remove any remaining liquid from the cell.

    b. Load the Loading syringe with 300 µL EDTA solution.

    c. 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.

    d. 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 into the cell (approximately 250 µL). Gently wash the cell with EDTA solution by aspirating and dispensing 50 µL solution at least twice to mix the cell contents.

    e. Remove as much EDTA solution from the cell as possible.

14. Fill the sample cell with sample solution EDTA as described in the SW (or follow the instruction video):

15. To ensure there is fresh water in the reference cell, follow the steps below using distilled water.

a. Be sure to remove the reference cell lid over the reference cell port, before loading (Figure 4).

**Figure 4. Removing the reference cell lid over the reference cell port.**

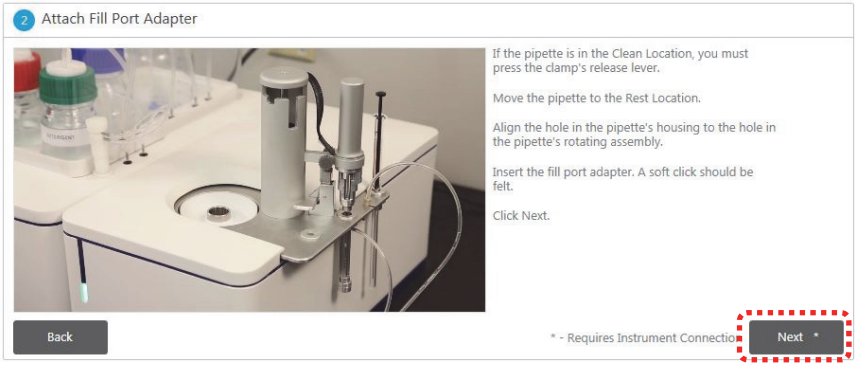
b. Remove as much water from the reference cell as possible.

c. Follow the same cell loading procedure as detailed for the sample cell.

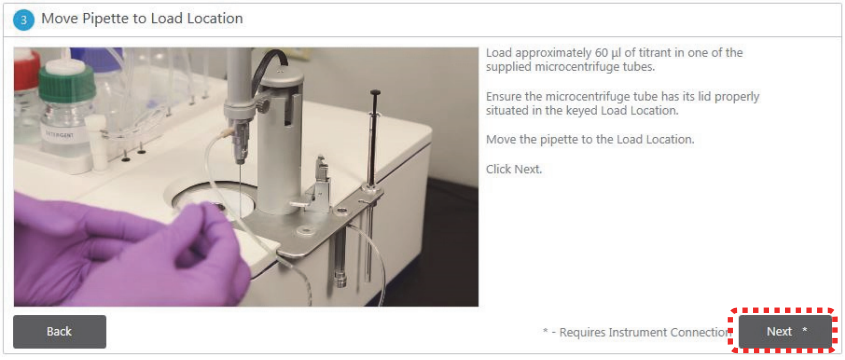
16. To prevent evaporation from the reference cell, be sure to replace the reference cell lid over the reference cell port.

17. Click ***Next***.

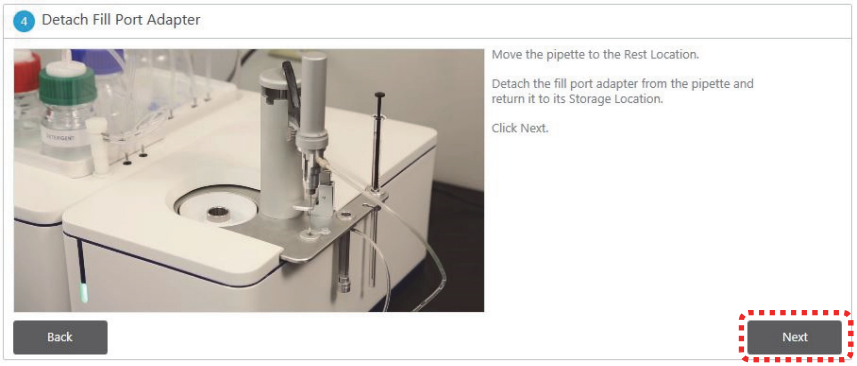
18. **Step 2, Attach Fill Port Adapter**: Follow the instructions in the software. Click ***Next***. The plunger will move down and status window appear.



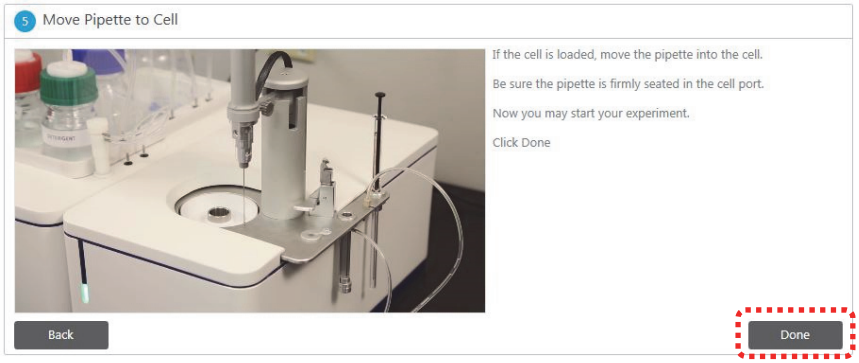

19. **Step 3, Move Pipette to Load Location**: Follow the instructions in the software. Place a microcentrifuge tube (PCR tube) containing 60 µL of the ligand solution ($CaCl_2$) in the tube holder. Check that there are no air bubbles at the base of the tube. Be sure to push the tube to the bottom of the holder and sitting in the correct orientation. Be careful not leave any part of the tube in the path of the syringe needle. Click ***Next***.



20. **Step 4, Detach Fill Port Adapter**: Follow the instructions in the software. Move the pipette to the rest location. Then detach the FPA from the pipette and return it to its storage location. Click ***Next***.

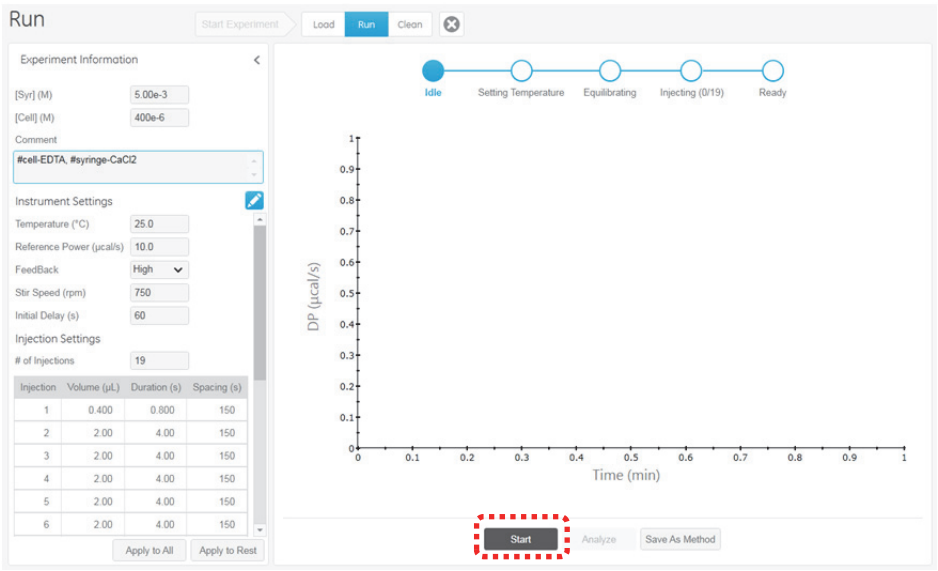



21. Step **5, Move Pipette to Cell**: Follow the instructions in the software. The sample cell is loaded, so move the Pipette into the sample cell. Be sure the pipette is firmly seated in the cell port. When this last step is performed, click ***Done***. You may now start the experiment.




22. Click ***Done*** you are returned to the ***Run*** workspace.

23. Select the ***Start*** button to start the experiment.




24. Enter a name for the result file, e.g. "***CaEDTAGetStart.itc***" and ***Save*** in the pre-defined folder *Experiments*.

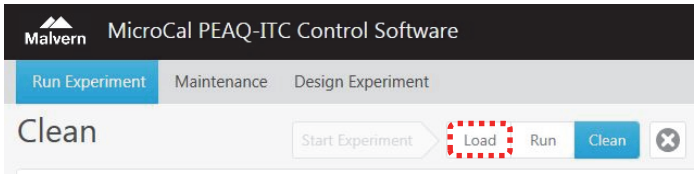
25. The experiment will take approximately 50 min (initial equilibration time not included).

# Exercise 3: Control experiment

1. For a control experiment, load MES buffer into the sample cell instead of EDTA and keep the same concentration of $CaCl_2$ (0.1 mM) as the ligand solution (in the syringe).

**Note:** 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 titration experiment during data analysis.

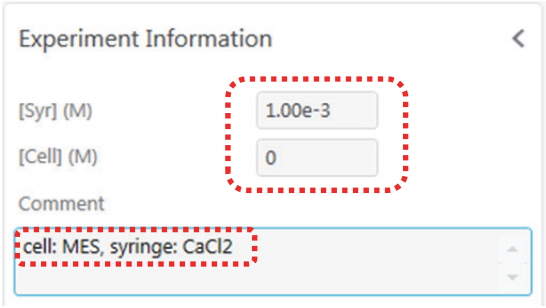
2. When the experiment in exercise 2 is complete, click ***Clean*** to enter the ***Clean*** workspace. ***Note:*** *Always perform Clean after each experiment!*




3. Repeat steps 4-6 in Exercise 2, to clean both sample cell and syringe. Follow the instructions in the software guided workflows.

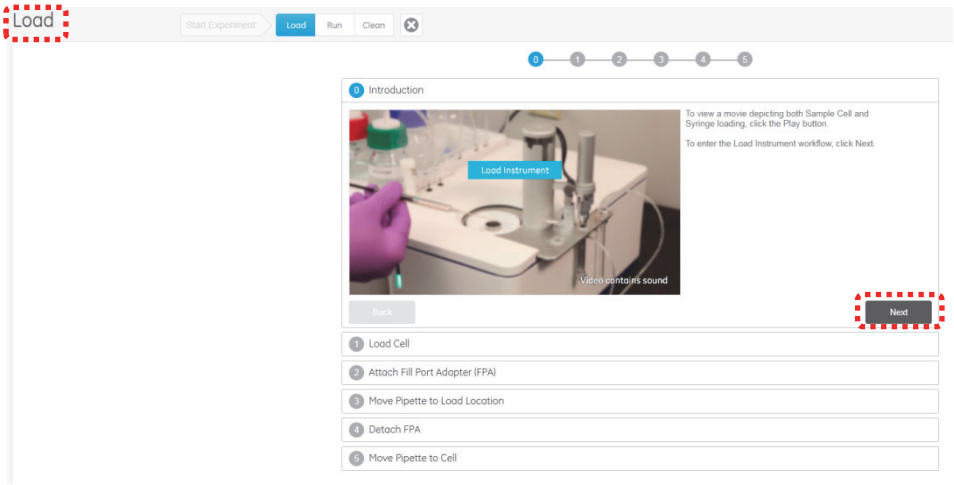
4. Click ***Run*** to return to the ***Run*** workspace.

5. Use the same method as in Exercise 2 (**EDTA method.itcm**, 19 injections). Make sure the concentration for the syringe is: ***Syr (M)*** = 1e-3 (CaCl2) and change the concentration in the cell to: ***Cell (M)*** = 0 (MES buffer).
In the Comment box, enter the description of the samples, e.g. cell: MES, syringe: $CaCl_2$.




**Note:** For experiments with a file name ending with "_ctrl" the concentration in the cell can be set to 0; [Cell]=0. These experiments (ending with "_ctrl") will automatically be designated as a Control when added to the analysis in the MicroCal PEAQ-ITC Analysis Software.

6. Click ***Load*** and follow the step-by-step video instructions to load the MES buffer solution in the sample cell and the $CaCl_2$ solution in the syringe. Click ***Next*** to proceed through the modules of the loading procedure.




7. Rinse the sample cell with 10 mM MES buffer as follows:

    a. Insert the Loading syringe needle gently into the sample cell until it touches the bottom and remove any remaining liquid from the cell.

    b. Load the Loading syringe with 300 µL sample buffer (10 mM MES).

    c. Invert the syringe and tap it to free any air bubbles, and then depress the plunger until all air is dispelled and a drop of liquid appears at the needle tip.

    d. 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 µL). Gently wash the cell with buffer by aspirating and dispensing 50 µL buffer at least twice to mix the cell contents.

    e. Remove as much buffer from the cell as possible.

8. Fill the sample cell with MES buffer solution as follows (or follow the video):

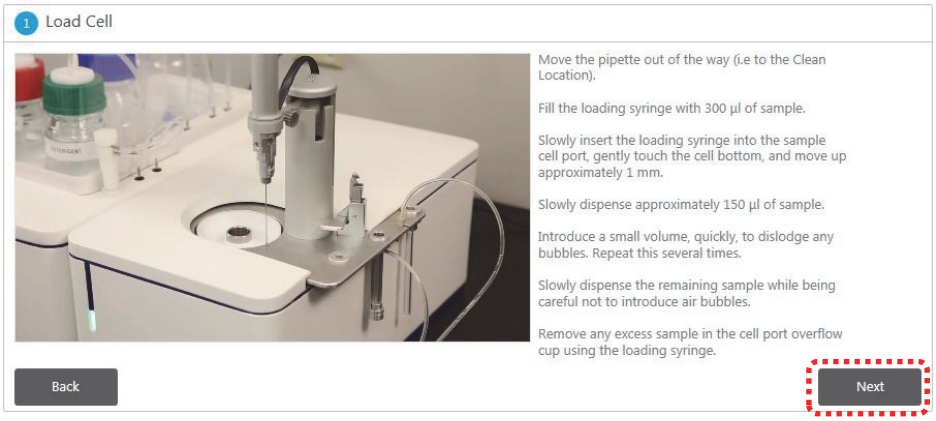
    a. Fill the Loading syringe with 300 µL EDTA solution (at room temperature). Invert the syringe and tap it to free any air bubbles, next depress the plunger until all air is dispelled and a drop of liquid appears at the needle tip.

    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.

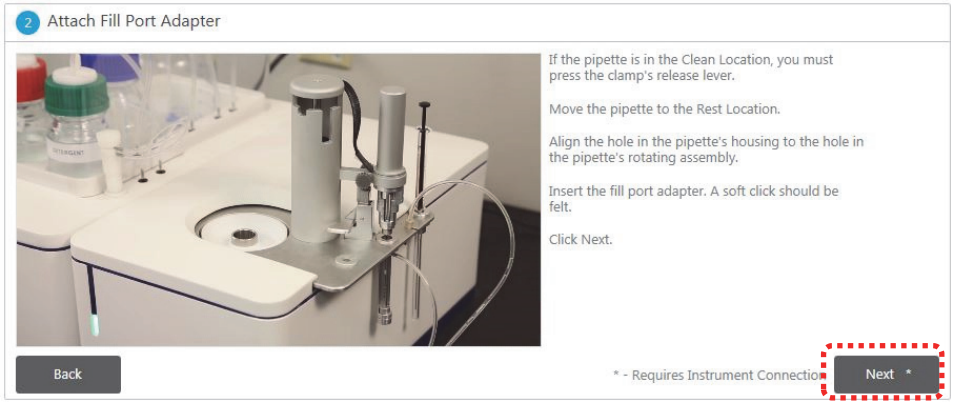
    c. Agitate the solution with the syringe by stirring or gently tapping the bottom of the cell.

    d. Lift the tip of the syringe to the ledge at the top of the metal cell stem and remove the excess solution.

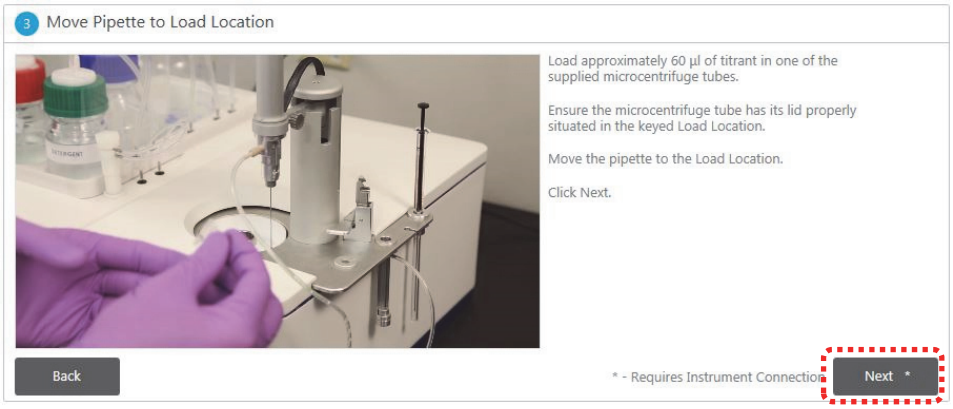
9. Click ***Next***.



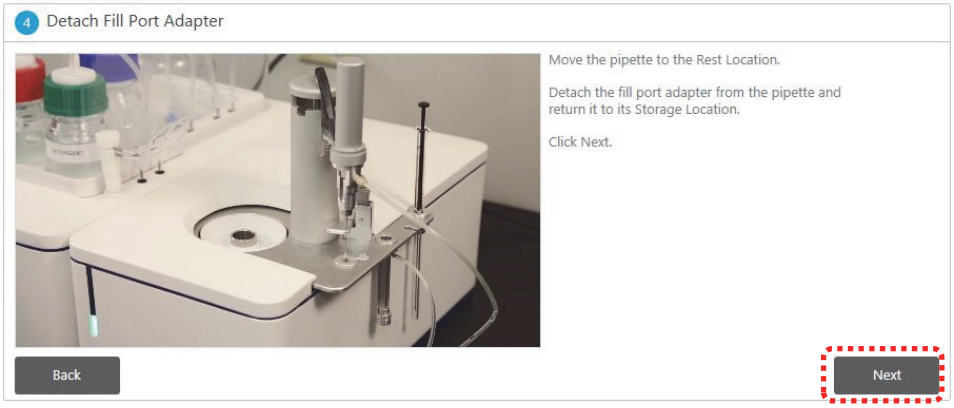
10. **Step 2, Attach Fill Port Adapter**: Follow the instructions in the software.
    Click ***Next***.



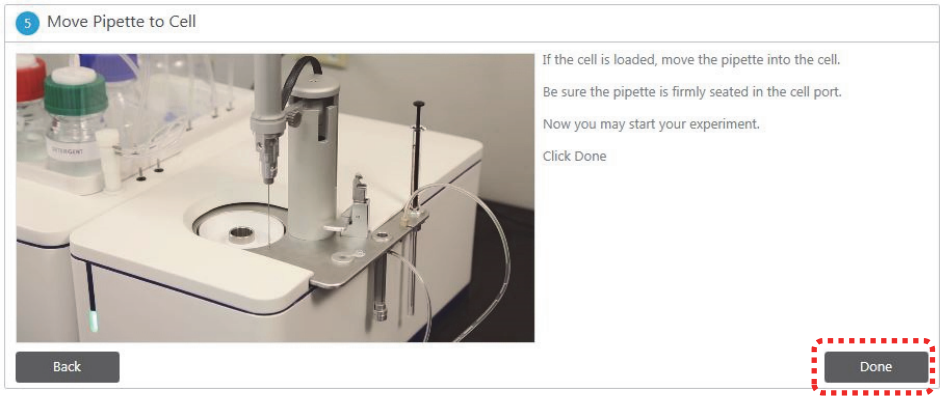

11. **Step 3, Move pipette to Load Location**: Follow the instructions in the software. Click ***Next***.




12. **Step 4, Detach Fill Port Adapter**: Follow the instructions in the software. Click ***Next***.



13. **Step 5, Move pipette to Cell**: Follow the instructions in the software. When this last step is performed, click ***Done***.




14. Click ***Run*** to return to the ***Run*** workspace.

15. Select the ***Start*** button to start the control experiment.

16. Enter a name for the result file, e.g. "**CaEDTAGetStart_ctrl.itc**" and ***Save*** in the pre-defined folder *Experiments*. Experiments with a file name ending with "_ctrl" will automatically be set as Control when added to the analysis in the MicroCal PEAQ-ITC Analysis Software.

17. The experiment will take approximately 50 minutes (initial equilibration time not included).

18. The system should be cleaned after each run. Click ***Clean*** (in the Run experiment workspace) to perform cell and syringe washing. Follow the step-by-step video instructions and use the predefined settings. Click ***Next*** to proceed through the modules of the washing procedure.

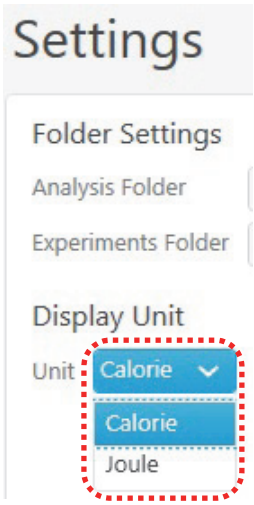
## Exercise 4: Evaluation of the results

This section describes how to perform analysis on the data generated in the CaCl2/EDTA titration experiment.

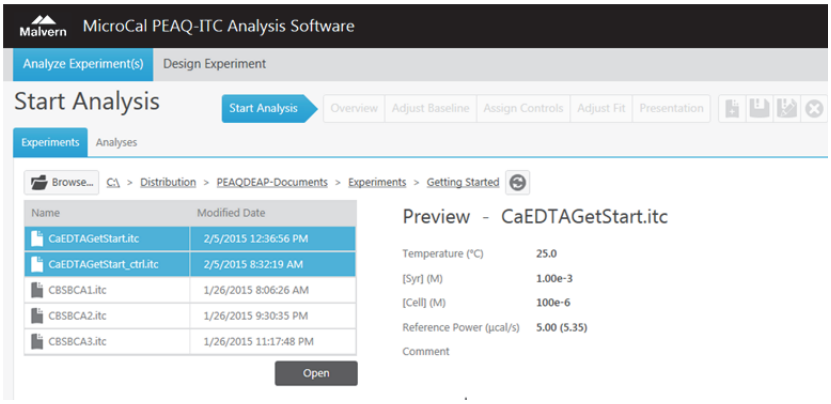
1. Open the ***MicroCal PEAQ-ITC Analysis Software*** by clicking the Analyze button in the MicroCal PEAQ-ITC ***Control Software*** or by double-clicking the start icon  on the desktop.

2. In the ***Start Analysis*** workspace/***Experiments*** tab, data available in the ***Experiments*** folder will be shown by default. Here you can browse to any folder of interest.

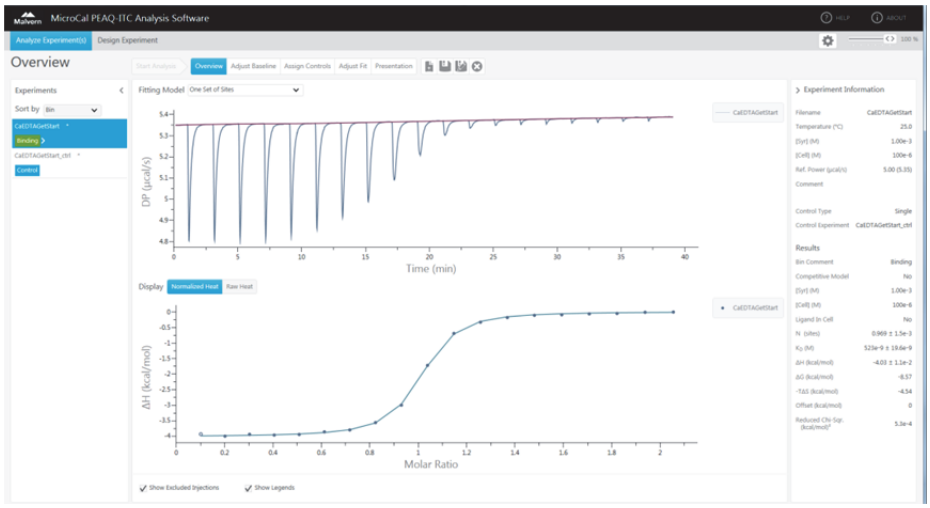
3. MicroCal PEAQ-ITC will display measured and calculated data in either English or SI units. To select English (Calorie) or SI (Joule) units, click the ***Settings*** icon  and make a selection in the drop down menu. Calorie will be used in this tutorial. Click on ***Analyze Experiment(s)*** to go back to the previous workspace.



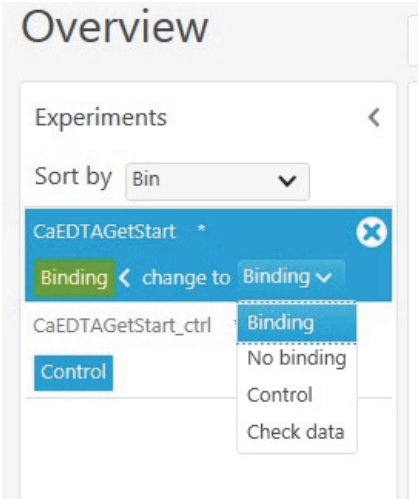
4. Select the CaEDTAGetStart.itc and the CaEDTAGetStart _ctrl.itc files located in the Experiments folder (highlight both data files). Click ***Open*** to create a new analysis. The data from exercise 2 and 3 will now be loaded.




5. The analysis opens in the Overview workspace. Here you can choose to view single or multiple data sets. Select both files and click open.




The files that are used in the analysis and their assigned ***Bin*** are displayed in the left ***Experiments*** pane (to the left). The ***Bin*** is a categorization based on predefined data quality/evidence of binding criteria. The data files can be sorted by ***Bin***, ***Name***, ***Bin and Name***, or ***Modified Date***. The software automatically assigns a ***Bin*** to each experiment, however, the ***Bin*** can be manually set at any time by selecting one of the options listed in the drop down menu which appears by clicking the arrow to the right of the ***Bin*** assignment label.



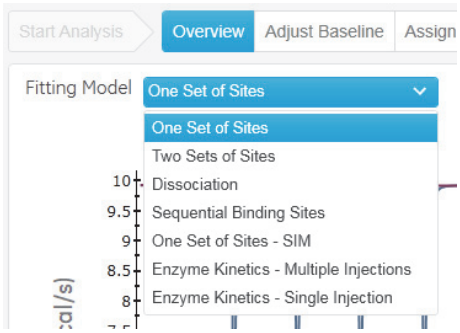

In this analysis, the titration of $CaCl_2$ into EDTA is labelled as ***Binding*** while the titration of $CaCl_2$ into MES buffer as ***Control***. Experiments with a file name ending with "*_ctrl*" are automatically designated as ***Control*** when added to

the analysis. For a more detailed description of the ***Bin*** *assignments*, see *Section 4.2 in the MicroCal PEAQ-ITC Analysis software User Manual.* For a more detailed description of the ***Control*** *assignments*, see *Section 6.2 in the MicroCal PEAQ-ITC Analysis software User Manual.*

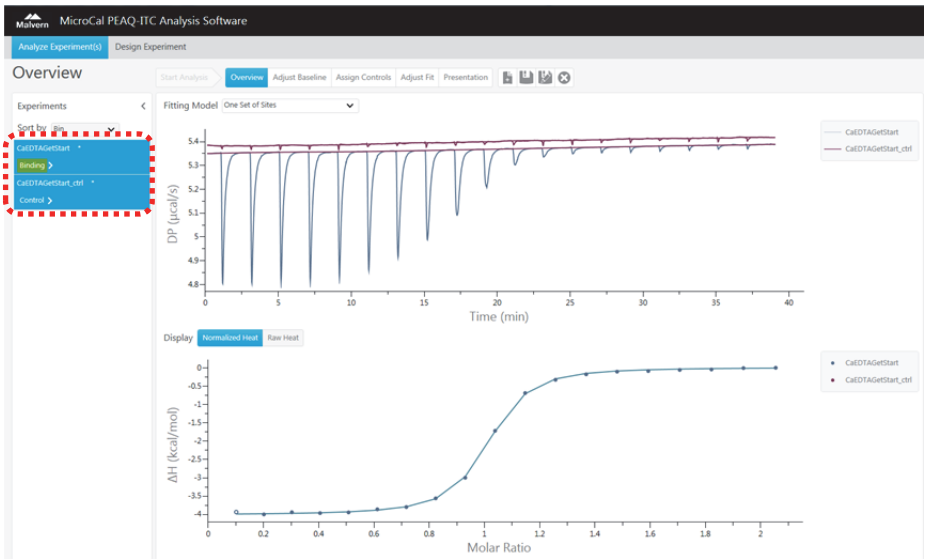
The raw data plot (shown as differential power *DP* as a function of time) and the corresponding integrated heats as a function of injection or molar ratio are displayed in the middle pane. It is possible to manually exclude ***Bad Data*** points from the integrated plot. To exclude a data point (*filled circle*), ***Right Click*** the data point and select ***Exclude***. To include a data point (*empty circle*) ***Right Click*** the data point and select ***Include.*** When data points are excluded (or included), the fitting model is automatically re-calculated using the current set of included data points. Notice the first injection has automatically been excluded.

Some experiment information and the analysis results are displayed in the ***Experiment Information*** pane. When starting a new analysis the fitting model ***One Set of Sites*** is selected and the calculated fitting model parameters are displayed under the ***Results*** heading.

6. Select **One Set of Sites** (default) among the available fitting models listed in the drop down menu next to the heading **Fitting Model**.

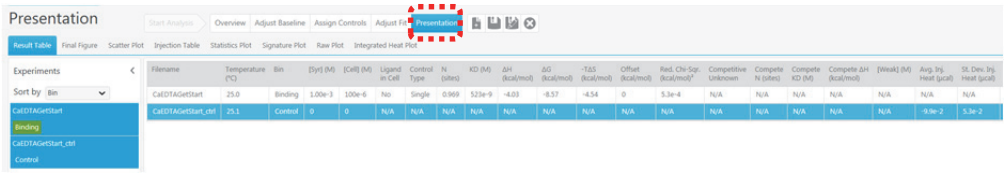



7. Select both data sets by clicking the ***CaEDTAGetStart*** and the ***CaEDTAGetStart _ctrl*** files, while holding down the ***CTRL*** key. Both Binding and Control experiments will be displayed. Note that no fitting will be generated for the control data set since the software only fits data that are labelled as **Binding**.

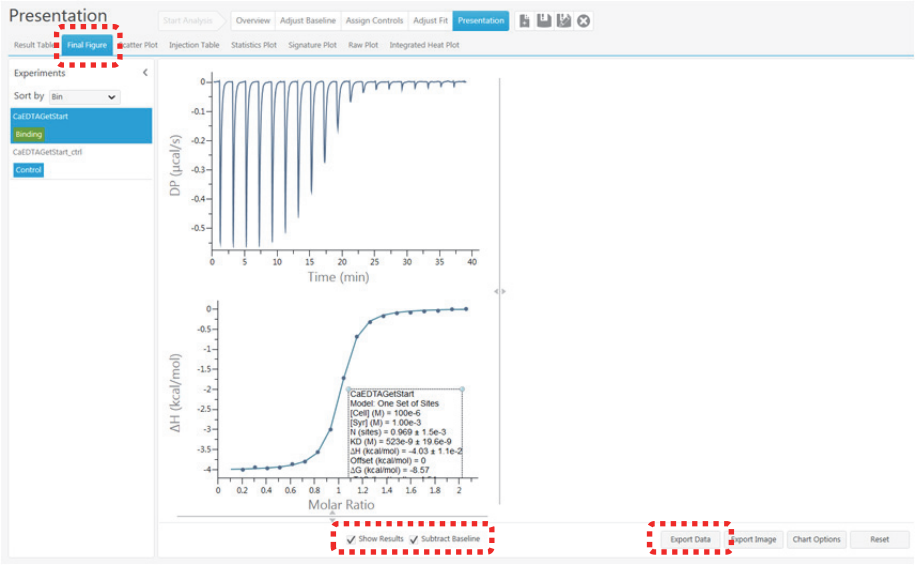



8. The data generated during this exercise should be of high quality and the control subtracted automatically (if the control was named correctly with "_ctrl" extension). In other words, the fitted values listed in the right-hand panel are the final result. Adjustments using the Adjust baseline, Assign controls, and Adjust Fit workspaces, which are unnecessary here, will be described in Software exercise 6.

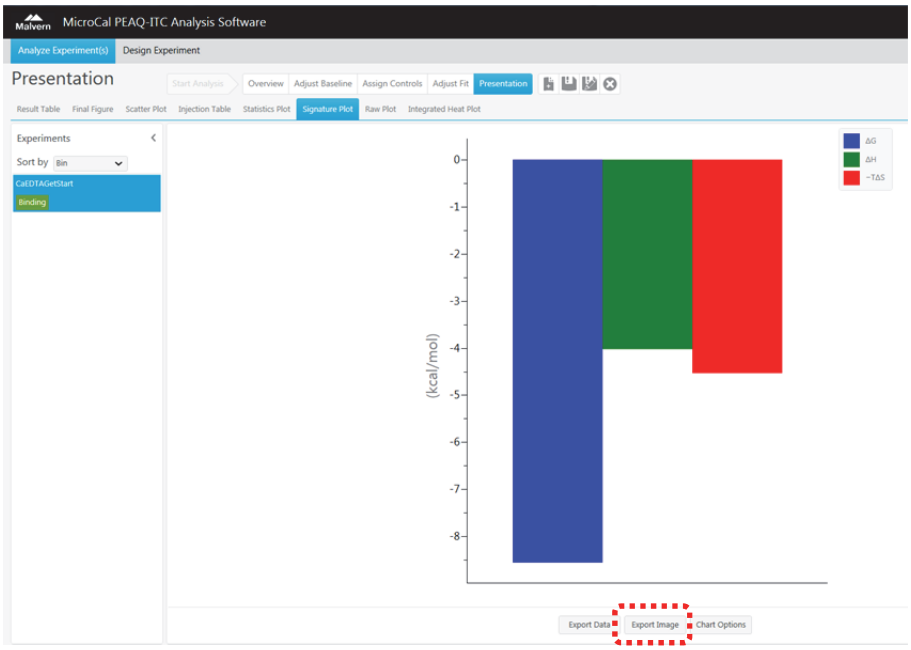
9. Click ***Presentation*** to access to the analysis results from the experiments. The workspace is divided into two panes. The top pane lists the available presentation items. The left pane lists the experiments available for the selected presentation item. The **Result Table** is selected by default and lists all parameters specific to the chosen fitting model.




10. Click ***Final Figure*** to create a final figure for publication. This window contains two related graphs. The top graph depicts raw data as a function of time, before the integration baseline has been subtracted. One can also choose to display the baseline subtracted-raw data by checking the ***Subtract Baseline*** option. The bottom graph depicts the fitted data. Click ***Show Results*** to display the analysis results in the figure. Click ***Export Image*** to export the experimental data in the desired file format. Alternatively, click ***Export Data*** to retrieve the raw data.

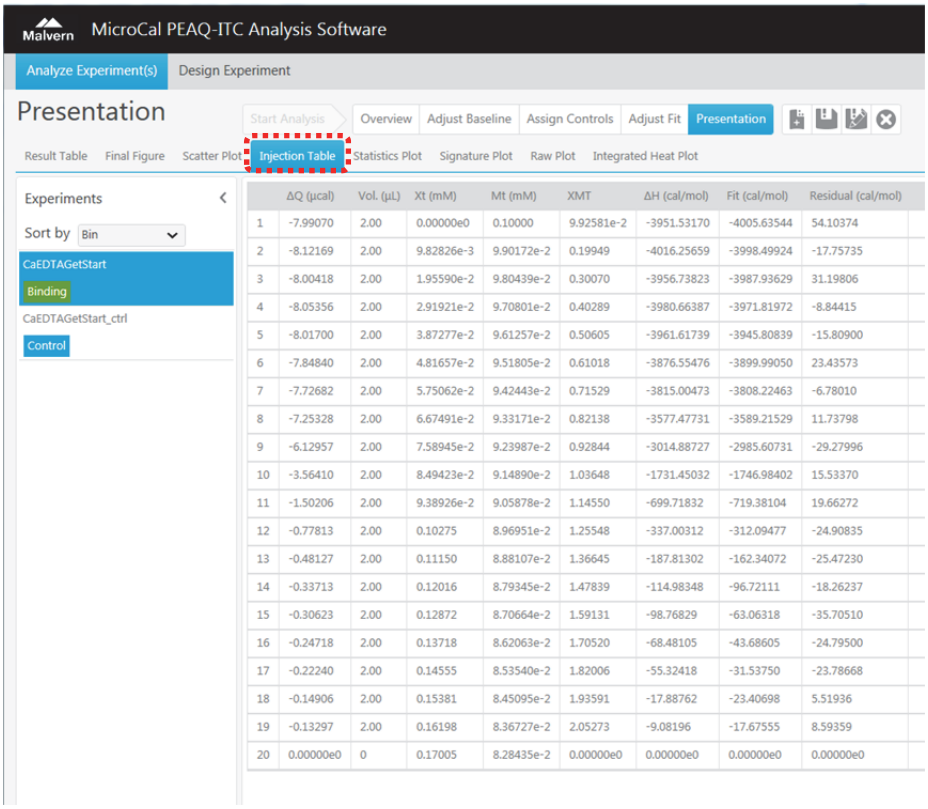



11. Click the ***Signature Plot*** in the ***Presentation*** workspace to display the distribution of *Gibbs free energy (ΔG)*, *enthalpy change (ΔH)*, and change in *entropy (ΔS)*. Click ***Export Image*** to export the plot in the desired file format.




12. Click the ***Injection Table*** tab in the ***Presentation*** workspace to view tabulated injection data. The first column ***ΔQ*** lists the raw injection heats. The heat of the first injection is often lower than expected due to some interfacial mixing of the liquids in the cell and the pipette prior to injection. As a general rule, the minimum heat of the second injection should typically be larger 1.5 µcals. If the heats are too small, it may be useful to increase the injection volumes and/or the concentration of the cell and syringe material and repeat the experiment. The second column ***Vol*** lists the

injection volumes in µl. ***Xt*** and ***Mt*** list the concentration of the titrant and cell material, in the cell, respectively, before the injection. ***XMT*** is the ratio of concentration of the materials in the cell after the injection. ***ΔH*** lists the injection heat, normalized per mole of *X* (added titrant or sample in the syringe). If a fit has been successful, a **Fit** column will list the values of that fitted curve. A **Residual** column will list the deviation of that fit from the ***ΔH*** values.




| | ΔQ (µcal) | Vol. (µL) | Xt (mM) | Mt (mM) | XMT | ΔH (cal/mol) | Fit (cal/mol) | Residual (cal/mol) |
|---|---|---|---|---|---|---|---|---|
| 1 | -7.99070 | 2.00 | 0.00000e0 | 0.10000 | 9.92581e-2 | -3951.53170 | -4005.63544 | 54.10374 |
| 2 | -8.12169 | 2.00 | 9.82826e-3 | 9.90172e-2 | 0.19949 | -4016.25659 | -3998.49924 | -17.75735 |
| 3 | -8.00418 | 2.00 | 1.95590e-2 | 9.80439e-2 | 0.30070 | -3956.73823 | -3987.93629 | 31.19806 |
| 4 | -8.05356 | 2.00 | 2.91921e-2 | 9.70801e-2 | 0.40289 | -3980.66387 | -3971.81972 | -8.84415 |
| 5 | -8.01700 | 2.00 | 3.87277e-2 | 9.61257e-2 | 0.50605 | -3961.61739 | -3945.80839 | -15.80900 |
| 6 | -7.84840 | 2.00 | 4.81657e-2 | 9.51805e-2 | 0.61018 | -3876.55476 | -3899.99050 | 23.43573 |
| 7 | -7.72682 | 2.00 | 5.75062e-2 | 9.42443e-2 | 0.71529 | -3815.00473 | -3808.22463 | -6.78010 |
| 8 | -7.25328 | 2.00 | 6.67491e-2 | 9.33171e-2 | 0.82138 | -3577.47731 | -3589.21529 | 11.73798 |
| 9 | -6.12957 | 2.00 | 7.58945e-2 | 9.23987e-2 | 0.92844 | -3014.88727 | -2985.60731 | -29.27996 |
| 10 | -3.56410 | 2.00 | 8.49423e-2 | 9.14890e-2 | 1.03648 | -1731.45032 | -1746.98402 | 15.53370 |
| 11 | -1.50206 | 2.00 | 9.38926e-2 | 9.05878e-2 | 1.14550 | -699.71832 | -719.38104 | 19.66272 |
| 12 | -0.77813 | 2.00 | 0.10275 | 8.96951e-2 | 1.25548 | -337.00312 | -312.09477 | -24.90835 |
| 13 | -0.48127 | 2.00 | 0.11150 | 8.88107e-2 | 1.36645 | -187.81302 | -162.34072 | -25.47230 |
| 14 | -0.33713 | 2.00 | 0.12016 | 8.79345e-2 | 1.47839 | -114.98348 | -96.72111 | -18.26237 |
| 15 | -0.30623 | 2.00 | 0.12872 | 8.70664e-2 | 1.59131 | -98.76829 | -63.06318 | -35.70510 |
| 16 | -0.24718 | 2.00 | 0.13718 | 8.62063e-2 | 1.70520 | -68.48105 | -43.68605 | -24.79500 |
| 17 | -0.22240 | 2.00 | 0.14555 | 8.53540e-2 | 1.82006 | -55.32418 | -31.53750 | -23.78668 |
| 18 | -0.14906 | 2.00 | 0.15381 | 8.45095e-2 | 1.93591 | -17.88762 | -23.40698 | 5.51936 |
| 19 | -0.13297 | 2.00 | 0.16198 | 8.36727e-2 | 2.05273 | -9.08196 | -17.67555 | 8.59359 |
| 20 | 0.00000e0 | 0 | 0.17005 | 8.28435e-2 | 0.00000e0 | 0.00000e0 | 0.00000e0 | 0.00000e0 |

13. Click on the ***Save*** button  to save the entire content of the analysis into a file called ***CaEDTA.apj***.

# Software Exercises

This section of the booklet will help you gain more experience with experimental design and data analysis.

- Exercise 5: Experimental design
- Exercise 6: Evaluation of the 1:1 binding of a 4-carboxybenzenesulfonamide (CBS) to bovine carbonic anhydrase (BCAII)

## Exercise 5: Experimental design

The ***Design Experiment*** workspace can be used to create and simulate experiments with different parameter settings and/or fitting models. Two modes of experimental design are available: ***Guided*** and ***Advanced***.

1. Open the ***MicroCal PEAQ-ITC Analysis Software*** by clicking the ***Analyze*** button in the ***MicroCal PEAQ-ITC Control Software*** or by double-clicking the  start icon on the desktop.

2. Click the ***Guided*** tab to enter the Guided mode. This mode is available only for One Site Binding interactions.

   We will simulate experiments for macromolecule/ligand interaction with:

   - **High** affinity
   - **Moderate** affinity
   - **Low** affinity

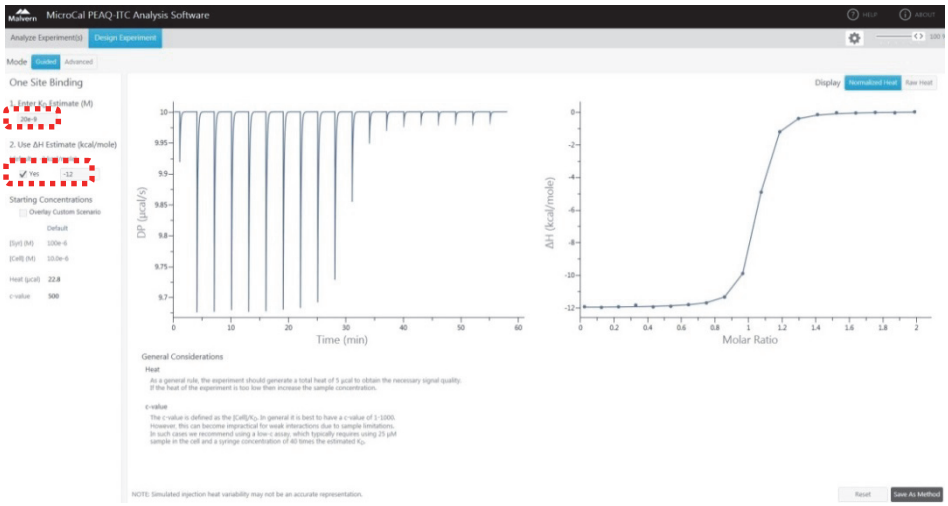
### Macromolecule/ligand interaction with high affinity

KD>10-8 M, e.g. titration of carbonic anhydrase with acetazolamide

3. In the left pane, enter a dissociation constant (20e-9) in the ***Enter $K_D$ estimate (M)*** box.

4. Select the ***Yes*** checkbox and enter an estimated enthalpy value (-12) in the ***Use ΔH Estimate (kcal/mole)***. In many cases you may not know the enthalpy, then use the default value -3 (a typical value).

5. The software will suggest the recommended concentrations (as a starting point) for the currently set estimates. The simulation pane on the right displays simulations of raw injection data in the left chart and a curve of the simulated integrated injections data in the right chart.




6. Below the heading ***General considerations*** there are comments and recommendations on how to design the experiment.

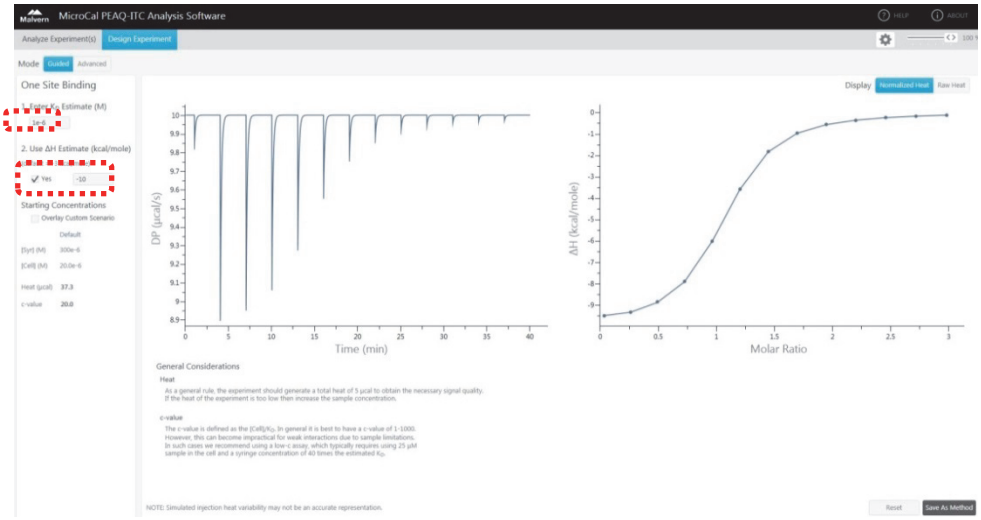
7. Click ***Save as Method*** and enter a name to save the simulation parameters as *.itcm* method file. This method can then be used in the control software.

### Macromolecule/ligand interaction with moderate affinity

10-8 M <KD<10-4 M, e.g. titration of carbonic anhydrase with CBS

8. Repeat steps 3-6 using $K_D$ = 1e-6 (M) and $\Delta H$ = -10 (kcal/mole).

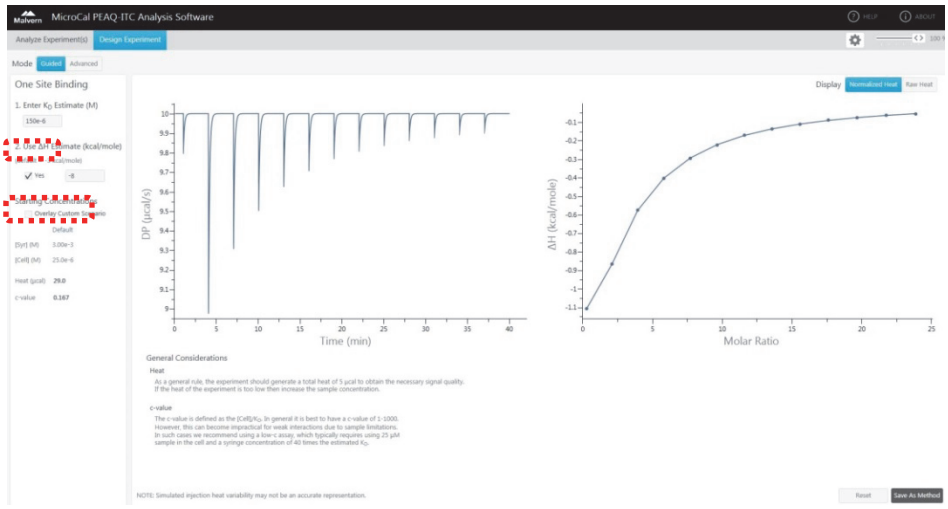
9. Notice that the recommended sample requirement and the [Syr]/[Cell] is higher than for the tighter interaction in the previous example. Also notice, more injections are recommended for studying tighter interactions.



Macromolecule/ligand interaction with low affinity
KD<10-4 M, e.g. titration of human carbonic anhydrase with sulfanilamide

10. Repeat steps 3-6 using $K_D$ = 150e-6 (M) and $\Delta H$ = -8 (kcal/mole).

11. You will notice that, unlike the other scenarios, the recommended concentration in the cell is lower than the $K_D$. These types of measurements are known as low C measurements. The binding isotherms generated are non-sigmoidal. These types of experiments yield useful $K_D$ values without using the high sample concentrations that would be required of a more typical ITC measurement. The stoichiometry cannot be fitted for and should be fixed in the fitting process.




12. If you tick the option ***Overlay Custom Scenario*** in the ***Starting concentrations***, you can enter any custom concentrations (both cell and syringe), then see how the data will look like based on these. The simulated data can then advise you if you need adopt/change the concentrations before you start the real experiment.

# Exercise 6: Evaluation of the 1:1 binding of a 4-carboxybenzenesulfonamide (CBS) to bovine carbonic anhydrase (BCAII)

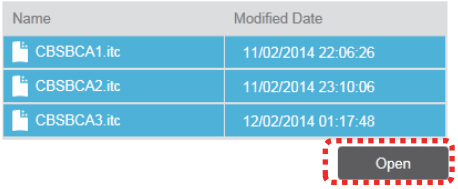
For this purpose three data sets are provided for the 1:1 binding of a 4-carboxybenzenesulfonamide (CBS) to bovine carbonic anhydrase (BCAII). The data refer to the following experiments:

| File | BCAII (µM) in the sample cell | CBS (µM) in the syringe |
|---|---|---|
| CBSBCA1 | 50 | 500 |
| CBSBCA2 | 20 | 200 |
| CBSBCA3 | 10 | 100 |

1. Open the ***MicroCal PEAQ-ITC Analysis Software*** by double-clicking the start icon on the desktop.

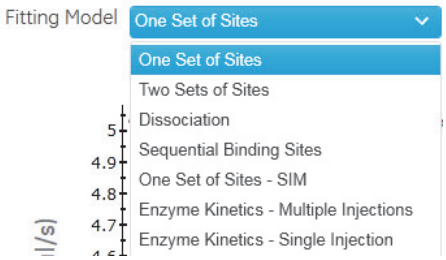
2. In the ***Start Analysis*** workspace, browse to the Getting Started folder (located in the Experiments folder) and select the CBSBCA1.itc, CBSBCA2.itc, and CBSBCA3.itc files.

3. Highlight all three data files. Click ***Open*** to create a new analysis and add the selected experiments.

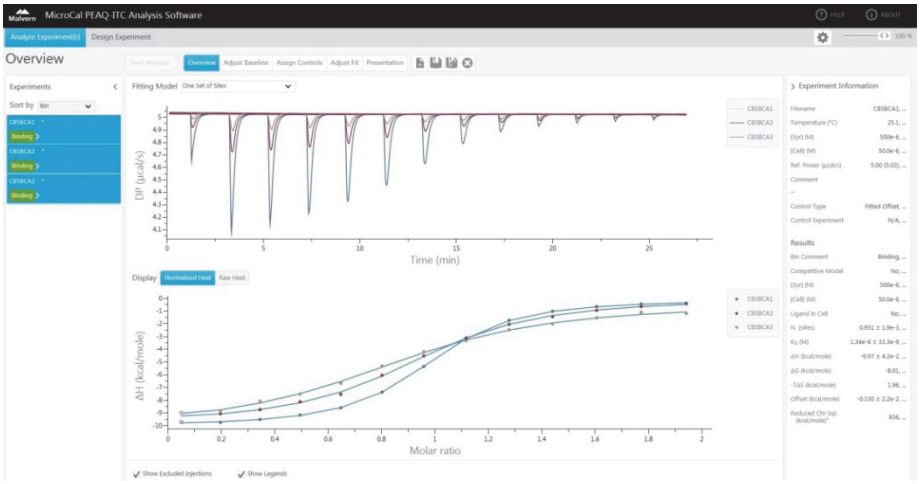


4. The analysis opens in the ***Overview*** workspace. This exercise uses 3 data sets, all containing binding data (indicated by **Bin** = Binding). Control subtraction will be performed using the **Fitted *Offset*** (explained later in this exercise).

5. Ensure that ***One Set of Sites*** is chosen (default) among the available fitting models listed in the drop down menu next to the heading ***Fitting Model***.

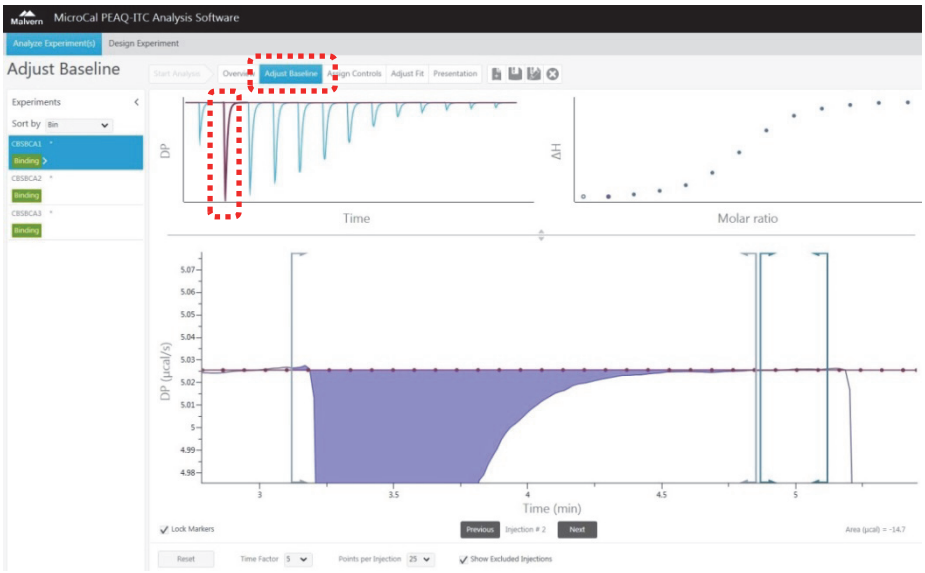



6. Overlay all data sets by selecting the 3 files while holding down the ***CTRL*** key on your keyboard. All Binding experiments will be displayed in the middle pane in the raw data as well as in the normalized heat plots.




7. Click ***Adjust Baseline*** to manually correct for the baseline and the integration range, if necessary (not required in this case). To adjust the integration range:

    a. Select in the raw data, or in the normalized heat plots (both located in the *upper section*), an injection peak to be adjusted, e.g. Injection #2. The peak is highlighted and opens for baseline and integration adjustments (*middle section*).

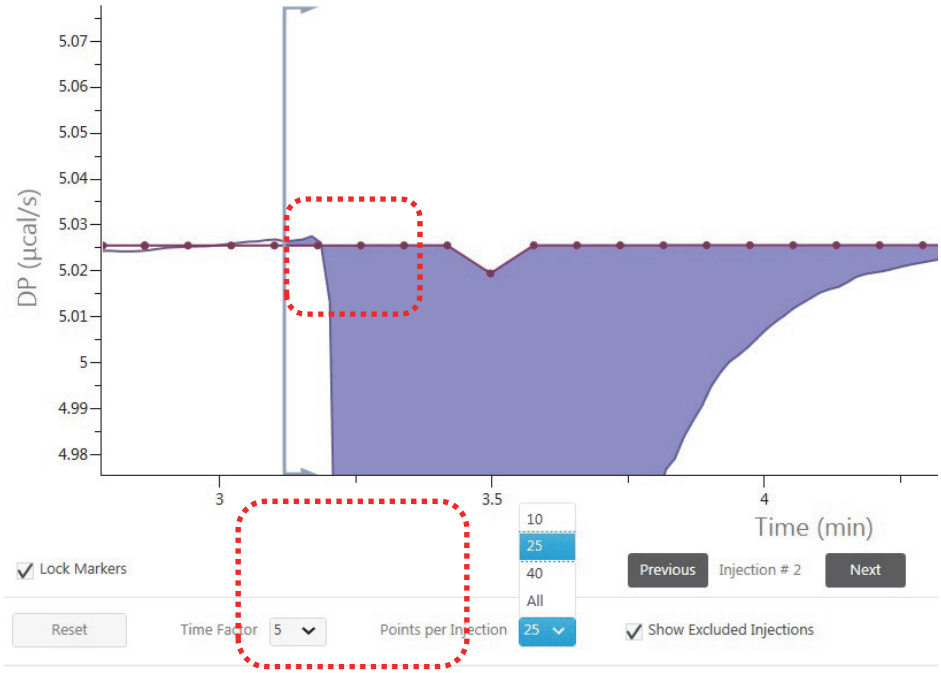


b. Drag the marker bar with arrows to the desired position. Data between the gray integration markers is integrated as the injection peak. Data between the blue baseline markers is fit as baseline.

   Moving a Baseline marker will negate any fine adjustments made in the next step, so if more injection peaks need to be adjusted, continue to the next peak either by clicking the desired peak or by clicking ***Previous*** or ***Next*** in the middle section.

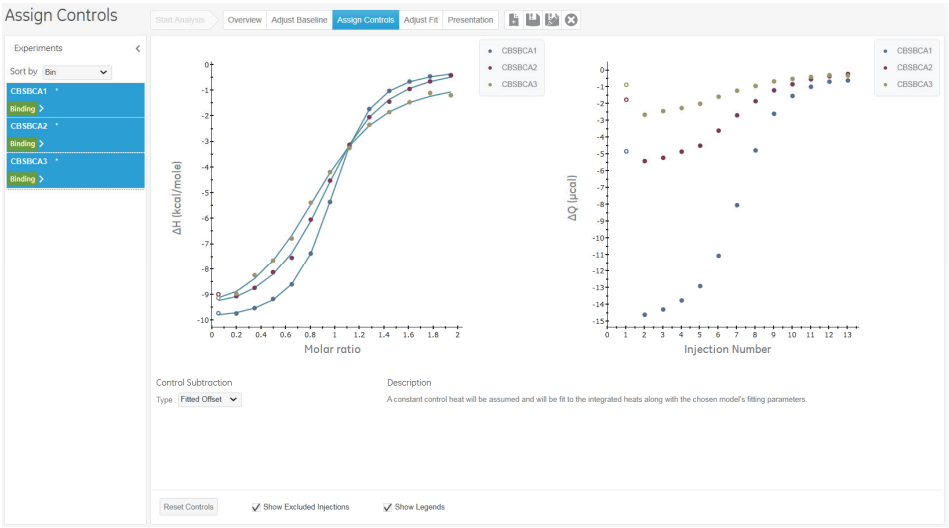
c. For fine adjustment, drag the red data point of interest in the graph to the desired position (indicated in the screen below, no correction required though).

d. The number of data points defining the baseline can be selected in the lower section among 10, 25, 40, or All. The Time Factor affects the default locations of the Integration and Baseline markers. Typically these settings do not need adjusting, and doing so will negate any prior baseline/integration manipulations.

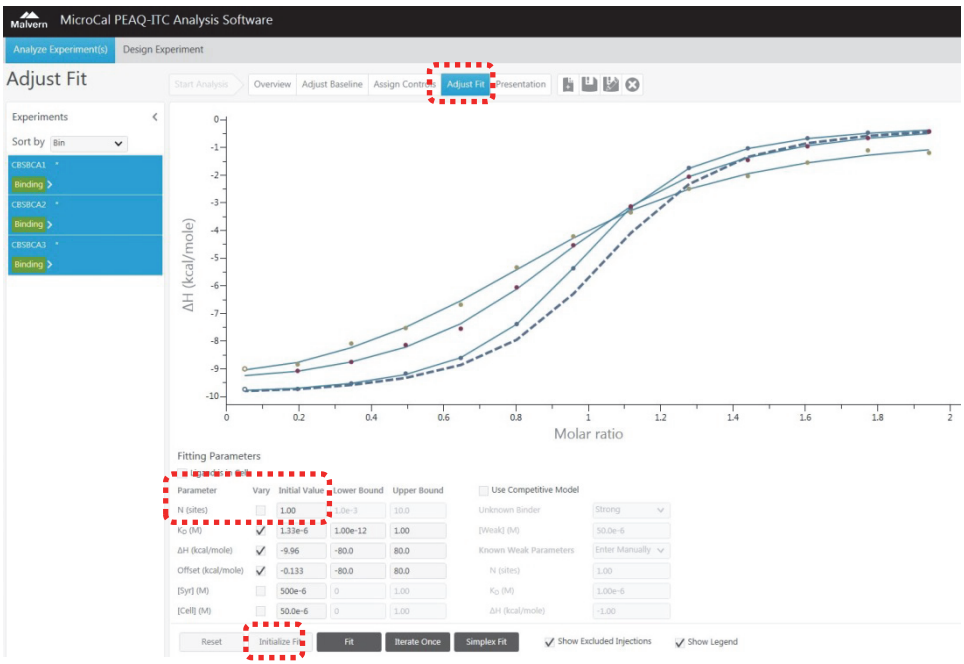



8. In this exercise the control heats will be estimated and subtracted automatically by the software. This ***Fitted Offset*** method is the default Control Subtraction type unless a control experiment is applied (manually or automatically as in Exercise 4).
   This method assumes a constant control heat and fits it to the integrated heats along with the chosen model's fitting parameters. The constant control heat is listed as ***Offset*** under the ***Results*** heading in the ***Experiment Information*** pane.

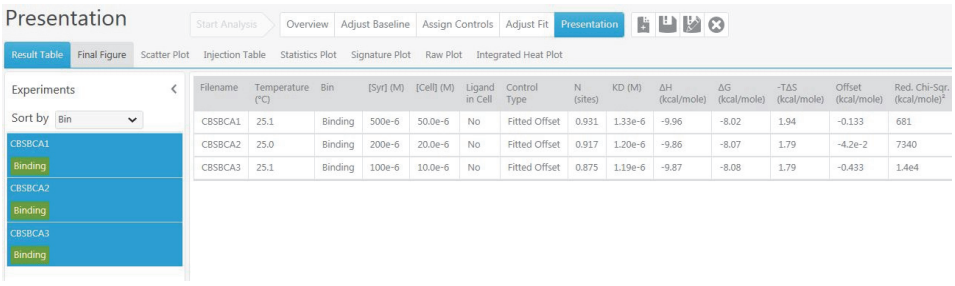
9. If the fitted offset is not satisfactory, click ***Assign Controls*** to change the control subtraction. On the ***Type*** menu below the heading ***Control Subtraction***, choose a suitable control subtraction type and then method. In this exercise, use the default setting ***Type: Fitted Offset***. For alternative ***Control Subtraction Types*** and ***Methods***, see *Section 6.2 in the MicroCal PEAQ-ITC Analysis Software User Manual*. The Single and Composite types are not available unless the current analysis contains a valid Control experiment.



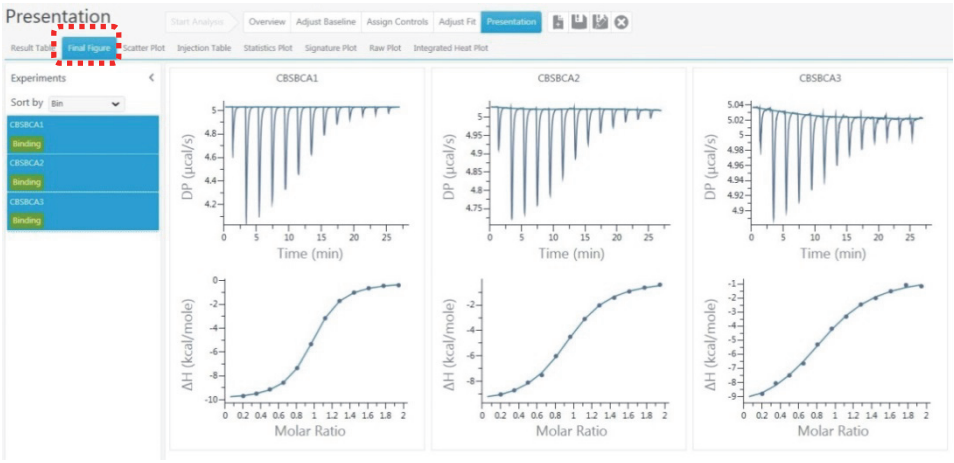

10. If the automatically generated fitted result is not satisfactory, click ***Adjust Fit*** to initialize the fitting parameters manually. Example: to explore the effect of syringe and cell concentrations on the stoichiometry for a 1:1 binding interaction, uncheck the ***Vary*** checkbox next to the ***Parameter N*** (***sites***). Enter 1 as its ***Initial Value*** and click ***Initialize Fit***. A dotted line will show the simulated model using the currently displayed Initial Values. Click ***Reset*** to restore the original parameters and then ***Fit***.



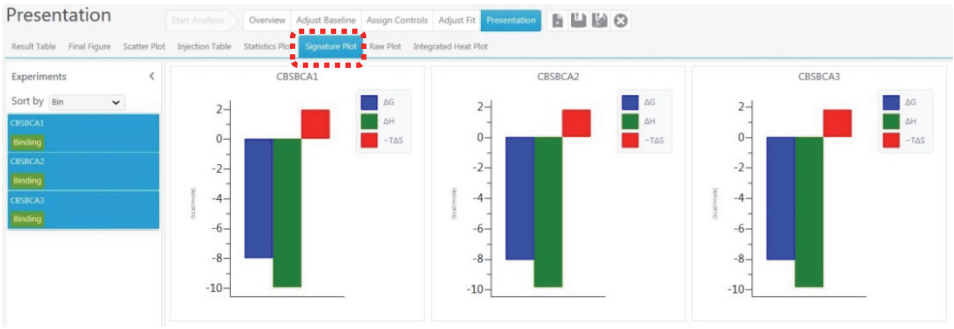

11. Click ***Presentation*** to access to the analysis results from the experiments. Parameters specific to the fitting model are summarized in the ***Result Table*** presentation item.




12. Click ***Final Figure*** to create a final figure for publication.




13. Click the ***Signature Plot*** in the ***Presentation*** workspace to display the distribution of *Gibbs free energy (ΔG), enthalpy change (ΔH)*, and the change in *entropy (ΔS)*.




14. Click on the ***Save*** button  to save the entire content of the analysis into a file called ***CBS.apj***.

# MAINTENANCE

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

## Daily maintenance

The system should be cleaned after each run. Click ***Clean*** (in the ***Run*** experiment workspace) to perform cell and syringe washing. Follow the step-by-step video instructions and use the predefined settings. Click ***Next*** to proceed through the modules for the washing procedure.

## Weekly maintenance

Replace the distilled water in the reference cell.

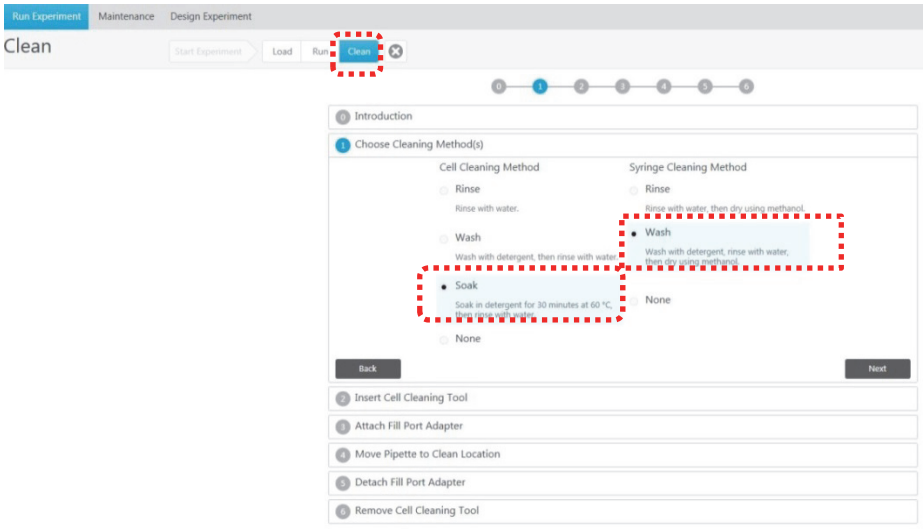
## Cleaning the cells and syringe

- Clean the cell(s) with detergent (20% Contrad™ 70 or 14% Decon™ 90) at elevated temperature.

  Choose the ***Soak*** method (Click ***Clean*** in the ***Run experiment*** workspace). Follow the instructions to load the cell with 20% Contrad 70 (14% Decon 90). The cell will be held for 30 minutes at 60 °C and then rinsed with water. This should be done whenever the standard cleaning procedure (***Wash***) of rinsing with detergent at room temperature is insufficient to clean the cell properly. The easiest away 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.

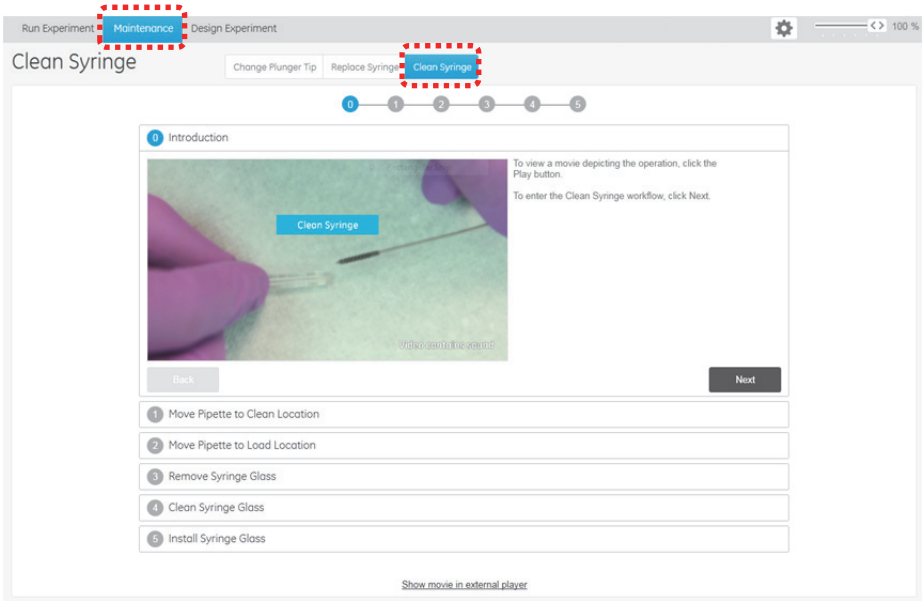
- Clean the syringe with detergent (20% Contrad™ 70 or 14% Decon™ 90).

  Choose the ***Wash*** syringe clean method (Click ***Clean*** in the ***Run experiment*** workspace). Follow the instructions. The syringe is washed with detergent and then rinsed with water.



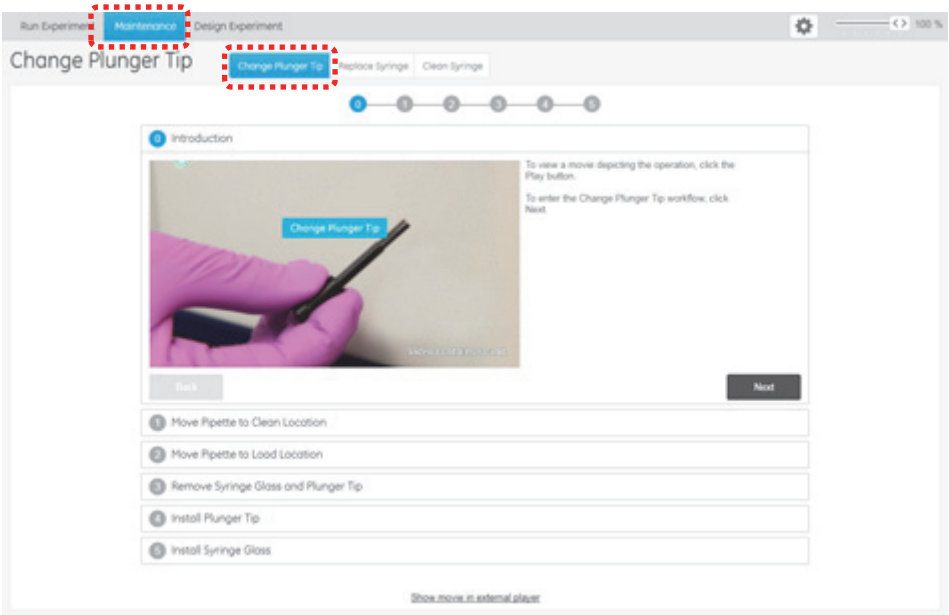
## Cleaning the syringe

A more thorough cleaning of the syringe can be achieved with the syringe brush. Click ***Maintenance*** to access the Maintenance workspace. Click ***Clean Syringe*** and follow the step-by-step video instructions. Click ***Next*** to proceed through the modules of the Clean Syringe procedure.



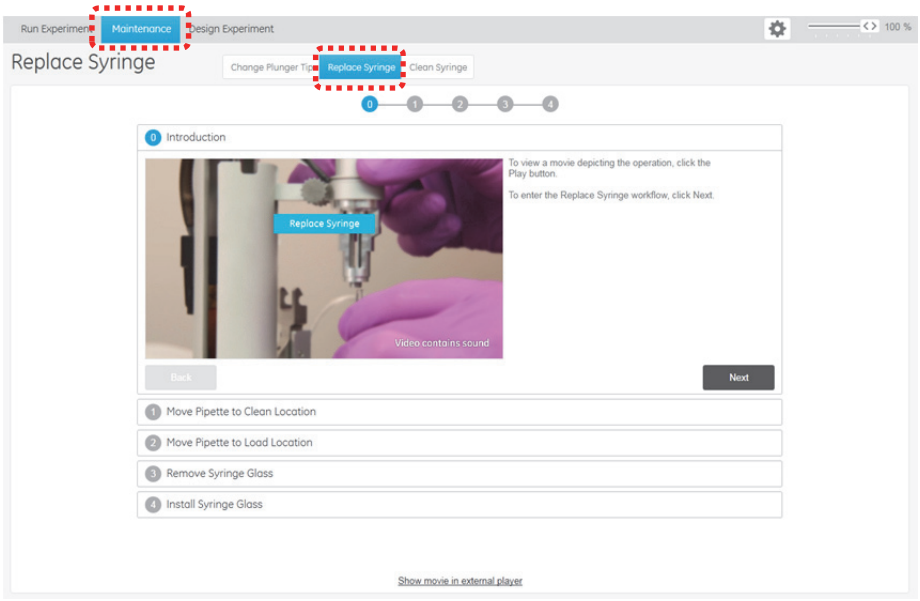

## Replacing the pipette plunger tip

It is recommended that when using the MicroCal PEAQ-ITC instrument on a daily basis, that the plunger tips of the pipette be replaced approximately once every month or two, or after 300 injections. Click Maintenance to access the ***Maintenance*** workspace. Click ***Change Plunger Tip*** and follow the step-by-step video instructions. Click ***Next*** to proceed through the modules of the Change Plunger Tip procedure.



# Replacing the titration syringe

Click ***Maintenance*** to access the Maintenance workspace. Click ***Replace Syringe*** and follow the step-by-step movie instructions. Click ***Next*** to proceed through the modules of the Replace Syringe procedure.



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