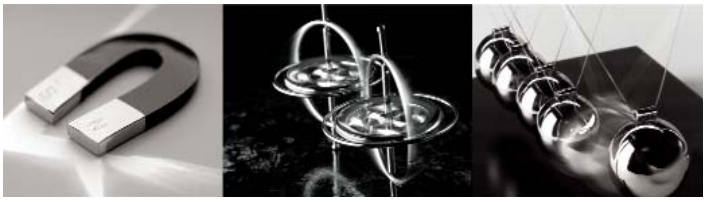

Binding Stability Kinetics

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AutoITC
Experimental Guide

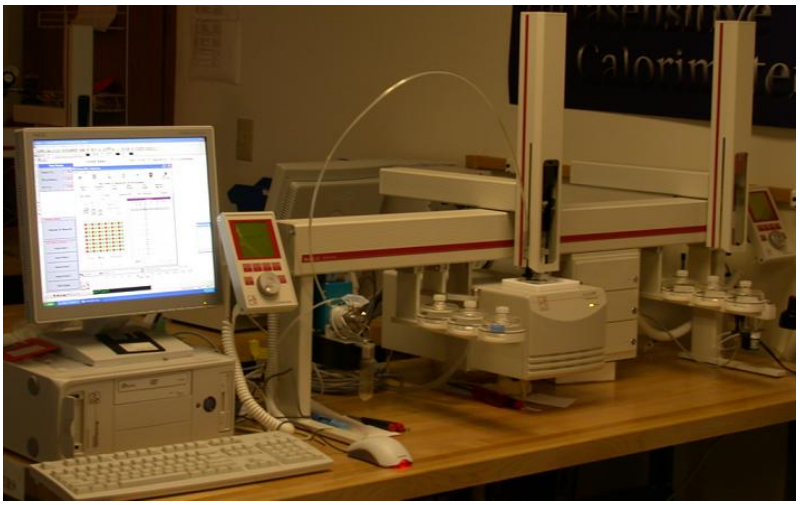
MAU230010 Rev C-2

Table of Contents

Important Points:

1. All solutions should be freed of visible particles by centrifuging or filtering before being used in ITC experiments. Some buffer solutions in the reservoir bottle could grow bacteria when sitting at room temperature for several days. Users should inspect the solution in the reservoir bottle frequently.
2. To prevent bacterial growth when the system is not to be used immediately, the closing operations should always be a couple of titrations where the "macromolecule" and "titrant" solutions are both .01 % sodium azide solution (If both bottle are buffer solutions, replace one of the bottles at the fast washing station with de-ionized water before doing sodium azide titration).
3. It is never good practice to leave the instrument with protein or other macromolecule solution in the cell or injection syringe for a long period of time, and the reaction cell should never be left dry.
4. It should be noted that the cell is empty after the detergent cleaning. If no sample run to be performed immediately, users should manually fill the cell with sodium azide (0.01 %) solution as closing operation (i.e. don't let the cell to be dry).
5. Never refill reference cell with buffer solution, since this may accelerate bacterial growth. The reference cell requires to be refilled manually by users with freshly degassed DI water or 0.01 % sodium azide on monthly or biweekly basis.
6. If autosampler ceases operation during rinsing and loading, the instrument needs to be re-set. First, click the "stop" button in VPViewer. Re-setup the titrations schedule starting from Section C (4) of this manual.

Experimental Guide for Auto ITC



This guide is intended to elaborate on certain aspects of the operation of the Auto ITC2 Platform. More detailed description of instrumentation, functions, and controls are found in two other manuals: ***MicroCal AutoITC User's Manual*** and ***MicroCal Autosampler User's Manual.***

Since proper sample preparation is critical for successful ITC experiments, general guidelines for sample preparation will be discussed here. These guidelines will be referenced in terms of binding experiments using biological samples, but may be readily modified to be appropriate for other types of samples.

A: Sample Preparation

The isothermal titration calorimeter (ITC) is designed to measure the heat of binding when the titrant solution (any solution placed in the syringe, containing either small molecule or macromolecule, will be referred to as titrant solution or ligand solution) is injected into the test solution (any solution placed in the reaction cell will be referred to as the test solution or macromolecule solution, even though in some cases the test solution may contain a small molecule) in the reaction cell and mixed at constant temperature by continuous stirring. When ligand solution is injected into the test solution and mixed, some heat effects (called the "control heat") other than the binding heat are evolved or absorbed simultaneously. The key for successful ITC experiments is to minimize the control heat, thereby allowing the binding heat to be measured more accurately. The control heat will include (1) the heat of mixing, which normally will be small unless there is a large mismatch in conditions (pH, salt concentration, additives such as glycerol or DMSO, temperature) between the two solutions, (2) the heat of dilution when high concentration of ligand solution from the syringe is injected into the

test solution. The heat of dilution will also normally be small, but may become large for ligands that form aggregates at the high concentration in the syringe.

The control heat, including contributions from both mixing and dilution, can be determined in one parallel control experiment. This involves injections of the ligand solution into the buffer solution or dialysate. The buffer solution should be identical in all respects to the test solution except containing none of the macromolecule or other test compound necessary to produce binding. If the heat peaks seen during the control experiment are small (relative to the binding heats) and constant for successive injections, then the control experiment can be terminated after 5-6 injections and an average heat subtracted from the sample heats during data analysis. If the heats seen in the control experiment are large and variable from injection-to-injection, then the control experiment should be carried out exactly as the sample experiment and control heats should be subtracted injection-by-injection from the sample heats during data analysis.

Anomalous heats caused by solution mismatch are the main culprit that leads to failed ITC experiments. The most common mismatch is produced by pH differences between the ligand solution and the macromolecule solution but mismatch also could involve salt concentration, buffer concentration, or additives such as dioxane, DMSO, glycerol, etc. Note that all three solutions used (i.e., the ligand solution, the macromolecule solution, and the buffer solution for the control experiment) should be matched as closely as possible using procedures indicated in the next sections.

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

(1) Preparation of Small Molecule Solutions

Most small molecule ligands (such as drugs and inhibitors) are supplied in solid form. Solutions can then be prepared by dissolving a known mass of these compounds directly into the buffer solution. After dissolution, the pH of the solution should be rechecked using an accurate pH meter. If the pH of the solution is found to differ from the pH of the buffer solution by more than 0.05 units the solution pH should be adjusted with a small amount of HCl or NaOH solution to within 0.05 unit of the buffer solution. Usually, 1 N HCl or 1 N NaOH can be prepared with the buffer and used for back titration. Precautions should be taken to prevent over titrating (use 2 or 3 μl at a time and check pH again before adding more). If the concentration of 1 N acid or alkali solution is too high for back titration it should be diluted out with buffer before use. 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 (i.e. 100 mM or higher, if possible) and then diluted 50-100 fold with the buffer (and hope it won't precipitate). The concentration of organic additives, such as DMSO, in the final ligand solution should be kept as low as possible (to 1 to 2 %, if possible; but no more than 5 %) since the macromolecule solution (usually protein) as the titration partner requires addition of the same additive at the same concentration in order to minimize the mismatch heats. While most proteins tolerate moderate amounts of organic additives, some proteins are not stable even in the presence of 2 % DMSO for long periods of time. This should be

checked before using organic solvent in the macromolecule solution. Matching the organic solvent (or any other additive) in both the macromolecule and ligand solutions and in the control buffer is extremely crucial. Even 0.1 % difference in DMSO between the titrating pairs (e.g., ligand solution at 5 % DMSO and macromolecule solution at 4.9 % DMSO) can generate a significant heat of mixing. Dialysis of solutions containing organic additives (such as glycerol) is often slower than in their absence, so sufficient time must be allowed (see below).

The pH of all three solutions should be checked (and adjusted if necessary) after all manipulations and concentration adjustments are carried out.

(2) Preparation of Macromolecule solutions

Macromolecule solutions should normally be dialyzed against the buffer solution using dialysis membrane having the proper molecular weight cut off. However, a lyophilized macromolecule sample devoid of salts or additives may be dissolved directly into the buffer, and used without dialysis. The pH of the solution after dissolution should be checked and adjusted back to the pH of the buffer solution, if necessary. Solid macromolecule samples containing salts and additives, after dissolution, should be dialyzed against the experimental buffer. Dialysis should normally be carried out at 4 ^{o}C using a relative large volume of buffer solution and at least two changes of buffer. The necessary duration of dialysis depends on the sample and buffer as well as the membrane used. For example, if glycerol at 10 % is added to aqueous buffer solution and a 6000-8000 MWCO membrane used, it requires at least one overnight dialysis for glycerol to reach concentration equilibrium in the macromolecule solution. The concentration of macromolecule should be determined after dialysis, and excessive particles in the solution removed by filtration or centrifugation. Accurate values for binding parameters depend on precise concentration measurements of ligand and macromolecule in the final solutions.

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

It is a good practice to check the stability of a protein or other macromolecule at the ITC experimental temperature before setting up for the ITC experiment. This can be easily done by placing a small amount of the solution in a test tube at the experimental temperature and engaging a small stir bar. If the solution becomes cloudy or looses its activity after 1-2 hours of stirring, then the macromolecule is not stable. In this case, the experimental temperature should be adjusted to a lower temperature and the stability re-tested.

B: Designing an ITC Experiment

(1) Concentration of Macromolecule for the Reaction Cell.

ITC is designed to detect the heat (or enthalpy, ΔH) which is absorbed (endothermic) or liberated (exothermic) when two solutions containing the titrating pair are mixed. The necessary concentration of the component in the cell, usually macromolecule, will depend on the binding affinity, number of binding sites, and heat of binding ΔH. These factors have been discussed in detail by Wiseman et.al. (*Anal. BioChem. 179*, 131 1989), and the following equation was derived,

$$\boldsymbol{c} = n \bullet M \bullet K_a = n \bullet M / K_d \qquad (1)$$

where c is a unitless parameter which is equal to the ratio (bound ligand)/(free ligand) at the beginning of the titration, and where n is the number of ligand binding sites on each macromolecule in the cell, M is the molar concentration of macromolecule (mole/liter) in the cell, K_a is the binding constant (liter/mole), and K_d is the dissociation constant (mole/liter). For 1:1 binding, the ***c*** value is simply $M \bullet K_a$ or M/K_d.

In designing ITC experiments, the starting macromolecule concentration M should be selected so that the ***c*** value is between ca. 1 and 1000 if it is desired to determine all three binding parameters (stoichiometry (N), binding constant (K_b), and binding enthalpy (ΔH)). When not limited by solubility or available material (see below), it is best to select the c value between 10 and 400. In attempting to satisfy these general criteria on c values, some possible limitations and other observations are discussed below:

(1) For high affinity interactions, which must be studied at low concentrations, limitations arise due to sensitivity of heat detection. The minimum concentration of macromolecule (assuming 1:1 stoichiometry) which can be used (if sufficient heat is to be available) is in the range 1-3 μM, depending on ΔH. Referring back to eq. (1), this means that the maximum binding constant K_b which can be measured (i.e., $c \leq 1000$) is in the range 0.3-1.0 x 10^9 M^{-1} (K_d of 1-3 nM). It is still possible, however, to directly determine both stoichiometry N and heat of binding ΔH when c exceeds 1000 so these parameters are available by ITC no matter how tight the binding.

(2) Binding constants tighter than 10^9 M^{-1} may also be determined using ITC by carrying out competition experiments whereby a second competing ligand is added to the macromolecule solution prior to carrying out the titration with the tight-binding ligand. The general experimental method and theory for competition experiments is discussed by Sigurskjold (2002) in *Anal. Biochem.* **277,** 260-266. Also see the discussion of competition experiments included in the Tutorial Manual, ***ITC Data analysis in Origin, Tutorial Guide****,* supplied with your instrument.

(3) At the other end of the affinity spectrum, weak interactions must be studied at high concentration (i.e., $c \geq 1$) which can present problems if solubility of either component is low or if insufficient quantity is available. For example, for a system with binding constant of 10^2 M^{-1} (K_d of 10 mM) the concentration of the component in the cell should be at least 10 mM while the component in the syringe (see below) must then be near 100 mM. Carrying out an experiment at such high concentrations may not be

possible if one or both components are sparingly soluble or available only in small quantities.

In some cases, it is possible to avoid extremely high concentrations and work at c values much less than 1.0 if the binding stoichiometry is known beforehand. In such a case, the stoichiometry parameter N is set as a constant (1.0 for 1:1 stoichiometry, 2.0 for 2:1 stoichiometry) during data fitting to the binding model. Accurate values for K_b and ΔH may then be obtained on that basis. When experiments at low c are carried out, it is best to use higher-than-normal ligand concentrations so the final molar ratio of (ligand)/(macromolecule) is at least 5 times the stoichiometry. This ensures that the titration progresses to the point where a significant fraction of the sites are saturated which in turn makes the fitting more precise.

(4) When carrying out a titration where the binding affinity is unknown, then it is best to use a high concentration of macromolecule and ligand, consistent with their availability (e.g., macromolecule at 0.1 mM, ligand at 2.0 mM). Even for weak interactions, these high concentrations will give some indication of binding constant and then the second experiment can be designed knowing this information. If concentrations are too low to detect binding, then nothing is learned from the first experiment.

(2) Concentration of Titrant for the Injection Syringe.

After determining the macromolecule concentration of the solution to be placed in the reaction cell, then the appropriate concentration of ligand solution in the syringe can be determined. The ligand concentration is selected so that, whenever possible, all or nearly all binding sites on the macromolecule will be saturated at completion of the titration. Thus, the concentration and injection volume (i.e. injection number times the volume per injection) of titrant to be used for titration will depend not only on the concentration of the component in the cell, but also on the binding affinity and number of binding sites. The reaction cell volume is ca. 1.4 ml for the ITC while the maximum delivery volume from the syringe is ca. 300 ul. For a normal ITC experiment, the concentration of titrant to be placed in the syringe usually is 10 to 50 fold higher than the concentration of binding sites in the reaction cell. Due to the limitation of the instrument sensitivity, the titrant concentration in the syringe may not be lower than 25 μM (using 10 μl injections; also depending on ΔH).

An empirical equation which may be used to estimate the concentration of titrant required for ITC is:

$$R = n\,(1.2 + 6/c^{1/2}) \qquad (2)$$

Here, R is the final molar ratio of ligand-to-macromolecule at the end of the titration, and n is number of macromolecule binding sites for ligand. From the above equation, a final molar ratio R of the ligand-to-macromolecule (for 1:1 complex) of ca. 2.2 is sufficient for an ITC experiment with ***c*** value of 36. However, if the ***c*** value for an experiment is only 1, the final molar ratio of ligand-to-macromolecule should be 7.2 or more. For an ITC experiment, R can also be approximately expressed as

$$R = (v_i \bullet n_i \bullet M_i)/(1400M) \quad (3)$$

Here, v_i is volume (µl) per injection; n_i is the desired number of injections; M_i (mM) is the concentration of ligand in the titrant solution; 1400 (µl) is the approximate reaction cell volume; M (mM) is the macromolecule concentration to be placed in the reaction cell.

Combining eq.'s (2) and (3) then leads to the following expression for determining the concentration of ligand M_i in the titrant solution.

$$M_i = 1400 \cdot M \cdot n\,(1.2 + 6/c^{1/2})/(v_i \cdot n_i) \quad (4)$$

For example, if macromolecule at 0.1 mM (M) is in the reaction cell for a 1:1 binding experiment (n = 1) with an estimated c value of 36, then the ligand concentration required to be loaded into the syringe is 1.54 mM (M_i) when the titration is designed to have twenty 10 µl ($n_i \cdot v_i$ = 20 x 10) injections. However, if the estimated ***c*** value is only 1 the titrant concentration required should be ca. 5.04 mM for the experiment with the same injection number and injection volume.

(3) Injection Volume and Injection Number

The optimum injection volume for the Auto ITC instrument is 3-15 µl per injection while the maximum injection volume per titration experiment is 300 µl. For a well designed ITC experiment, 10 injections per experiment may be sufficient to determine the binding parameters although more precise parameters might be obtained with more injections. On the other hand, fewer injections per experiment result in higher sample throughput. Titrant remaining in the syringe after an experiment will automatically be discarded with the AutoITC instrument.

(4) Control Experiment

As discussed above, the control experiment is essential for ITC since heat effects other than the binding heat are always evolved or absorbed simultaneously with the binding heat and these must be quantified and subtracted before data analysis. The control run usually is performed by titrating the buffer solution (or dialysate) with the same titrant used in the sample run using the same injection parameters. It is best to perform the control run immediately before the sample run. If heat effects for the control run are small and constant the control experiment can be terminated after 5 or 6 injections and a constant value (or the averaged value) of these heat effects can be subtracted from those of the sample run before doing curve fitting to obtain binding parameters. However, if the heat effects for the control are large and the size of the peak is changing as the titration proceeds, the control run should be carried out identically to the sample run and the control heats subtracted point-by-point from the sample heats. If very large heats are observed for the control run and there is reason to believe these might result from mismatch between solutions (see sections on Sample Preparation), then that possibility should be tracked down before proceeding with the experiment.

(5) Experimental Temperature

It is most convenient to perform auto ITC experiments at 25-30 ^{o}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 much 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 macromolecule. Some solutes, particularly proteins and other biomolecules, are not stable above room temperature for long periods of time with stirring so it is desirable to work at lower temperatures with such labile compounds. Finally, to determine the change in heat capacity ΔC_P for binding, experiments must be carried over a range of different temperatures (e.g., 10-40 °C) to obtain the temperature dependence of the heat of binding.

(6) Reverse Titrations

Most titrations are carried out with the macromolecule solution in the cell and the ligand solution in the syringe. If both binding partners are macromolecules (or both are small molecules) normally the component with multiple binding sites is placed in the cell. However, there are instances where it might be advantageous or even necessary to switch the location of the two components and carry out the *reverse titration*. 1) If the component which normally goes in the syringe has low solubility, it may be advantageous to place the solution containing that component into the cell where its concentration will be lower than it would be if it were in the syringe. 2) If the macromolecule slowly deactivates in the cell, either due to continuous stirring or to a high experimental temperature, it may do better if placed in the syringe. The solution in the syringe is not stirred nor thermostatted at experimental temperature until the point at which it is injected into the cell.

C. Setting up a Titration Experiment on the AutoITC

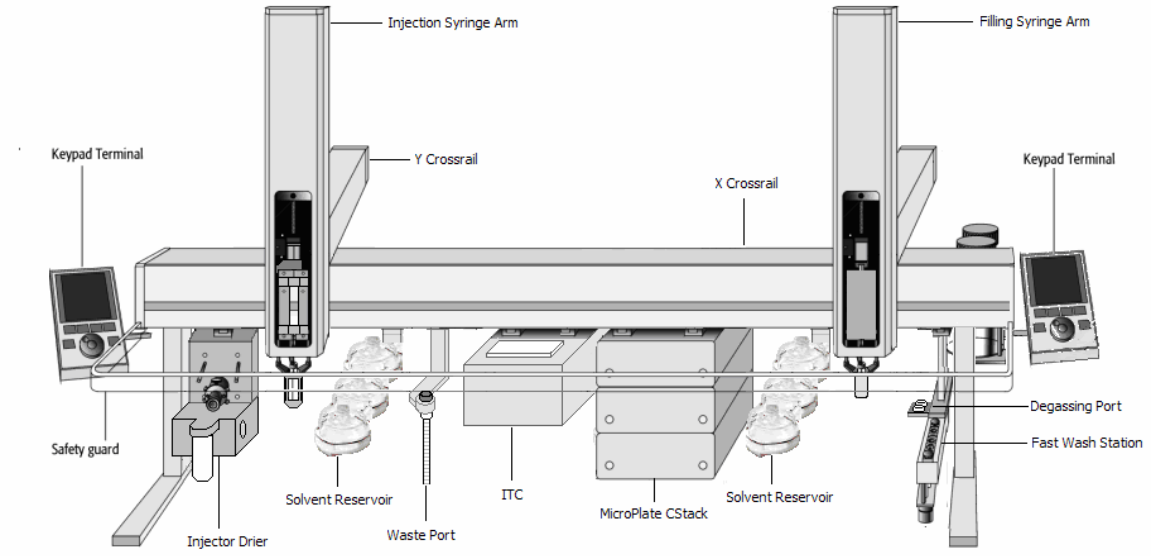


Figure 1

The AutoITC Platform consists of an autosampler, an ITC calorimeter, and a computer controller. A schematic diagram of the instrument platform is shown in Figure 1. The autosampler itself has two injector arms. The right-arm injector, equipped with a

regular 2.5 ml Hamilton gas-tight syringe (filling syringe) surround by a heating unit is mainly for the reaction cell rinsing & macromolecule solution filling. The filling syringe also serves for both ligand & macromolecule degassing. The left-arm injector, equipped with a precision syringe (injection syringe), served as ITC injector. Besides the two autosampler arms, there is one switching valve connecting to a vacuum pump (for titrant rinsing, drying, & loading), one titration syringe solvent reservoir (left hand side), one cell solvent reservoir (right hand side), & one fast washing station with two injection ports (one for solution degassing & one for loading titrant to the titration syringe). Users should refer to ***MicroCal AutoITC User's Manual*** & ***MicroCal Autosampler, User's Manual*** for further details. This document contains only the detailed procedures for setting up and carrying out ITC experiments.

(1) Loading Sample Solutions into Microtiter Plate Wells

Deep, square-well, 2 ml, 96-well polypropylene plates with pyramid bottoms (Innovative Microplate; cat. # S30009) are used for storing the macromolecule and titrant solutions in the microplate stack (three temperature-controlled drawers) of the autosampler. Each drawer holds two microplates for loading titrant & macromolecule. A total of 288 titrations can be set up in a single multiple-sample experiment if no recovery of the titrated solution is required. Only 144 titrations can be set up for the experiment if users need to recover the titrated solution. In such case, the front microplate in each drawer is used for loading titrant & macromolecule to be tested, and the back one (should be empty) is used for recovering the titrated solutions. The cover mat for microplate is a 7 mm, 96-well pre-slit Masterblock MicroMat CLR (Sun International, cat. # 300 013). The mat is autoclavable, made of pure silicone and compatible with methanol and DMSO. According to the manufacturer, the cover mat will reseal up to 100 injections for each well. For an ITC experiment, 0.70 ml of titrant solution and 1.9 ml of macromolecule solution must be placed in appropriate wells in order to properly fill the injection syringe and reaction cell, respectively. The

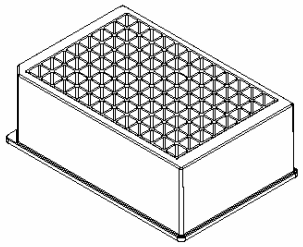


Figure 2

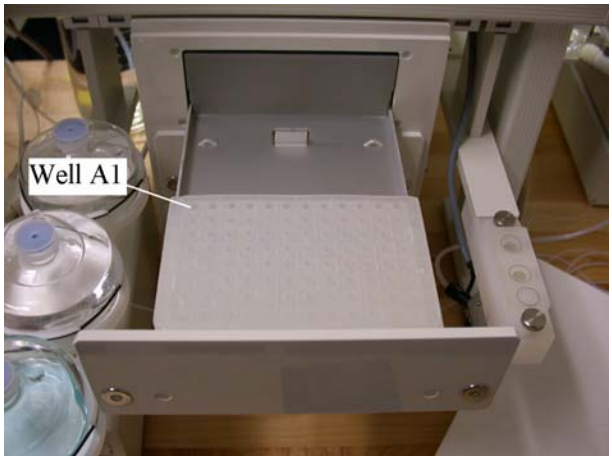


Figure 3

actual volume of macromolecule solution transferred from the wells to the reaction cell is ca. 1.8 ml while the volume of titrant loading to the injector will depend on injection volume set up for the titration. A 5 ml pipette with adjustable volume may be used to transfer the titrant and macromolecule solutions to the wells, taking precautions not to contaminate adjacent wells during the transfer. A pair of adjacent wells (for example, A1 and A2; A3 and A4, etc; see microplate image in Figure 2) are used for each titration *(note: only A1 well is marked on the microplate)*.

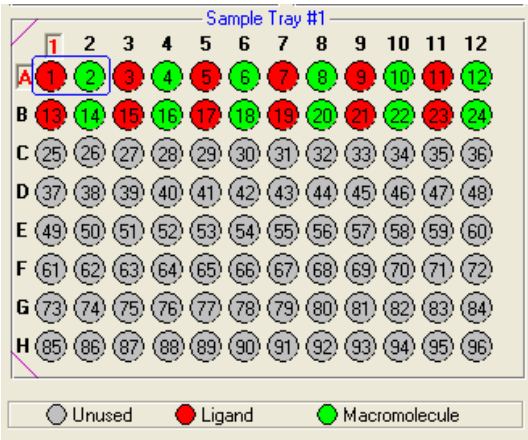


Figure 4

Titrant solution should be placed in the first well of the pair (i.e. A1) to be loaded into the titration syringe while the macromolecule solution should be placed in the second well of the pair (i.e. A2) to be loaded into the sample cell. After loading all wells and attaching the cover mat, the plate should be placed into the appropriate drawer with the proper orientation as shown in Figure 2 (i.e. the A1 well should be positioned at the left inside corner).

Note: If samples are to be stored at 4 ^{o}C in the storage drawers, the temperature of the storage chamber should be set at least 2 hours in advance (to adjust the temperature setting: 1. Press "P" briefly (less than 2 seconds) until "SP1" is displayed . 2. Use the "up" and "down" keys to select the desired temperature setting. 3. Press "P" again).

Figure 5

(2) Setting up Rinsing & Cleaning Solutions

Two one-liter aspirator bottles are provided, and should be connected to the fast washing stations # 1 and # 2, respectively. The solution from these two bottles is used mainly for rinsing the loading syringe & the reaction cell. To reduce any solution

mismatch, the bottles must be filled with the buffer solutions (or dialyzate) used to prepare the macromolecule and ligand solutions. The two bottles can be filled with the same buffer, two different buffers, or one for the buffer & one for de-ionized water (DI water). If additives (such as DMSO, glycerol, detergent, etc) are present in the sample solutions the same amounts of the additives should also be present in the buffer solution (or dialyzates).

Besides the fast washing station, there are two other rinsing & cleaning stations (with three 120 ml bottles) installed at the front of autosampler. The left hand station is used for rinsing & drying the injection syringe. The two inside bottles of the station should be filled with DI water while the outside one should be filled with 100 % methanol for speeding up syringe drying under the vacuum. The right hand side station is mainly used for cell cleaning with detergents in the auto mode set up by users. The inside bottle on this station should be filled with the detergent (i.e. 20% Contrad 70) while the outside two bottles can be filled with other solvents or water at users' choice.

After the rinsing & cleaning solutions are properly installed, the ITC experiment can be set up by carrying out the following steps ***in sequence***.

(3) Powering up the Auto ITC Instrument and Autosampler

The AutoITC should be properly installed and interconnected using the cables provided, with all power cords connected to the proper source. The Autosampler has three control boxes, which are stacked together on the side of the platform. One of the boxes provides power to the autosampler itself. The 2nd one is for selecting the set temperature of the sample storage drawers. The 3rd one is for powering the valve. The switches for the two boxes are located on the rear of each box and each should be in the **on** position. In order to control the temperature of samples prior to beginning the ITC experiment, the set temperature of the storage drawers should be selected at least 2 hours before the experiment. The procedure for setting temperature is described in the section "***Setting up rinsing & cleaning solutions"***. The switch for the ITC instrument itself is also located on the rear of each unit and should be in the ***on*** position. Once powered up, all units can remain **on** continuously so long as the instrument is functioning properly.

Figure 6

Once all other units are **on**, the computer controller should also be **on** with the Windows operating system active.

Double click on the **VPViewer2000 Auto ITC** icon on the desktop display in order to load the instrument control program and the data plot program as well as to activate the ITC instrument. Two overlapping window displays will appear (Figure 4). One is for plotting real time data in Origin, the other is the VPViewer2000 ITC Controls window for setting up the titration experiments. VPViewer2000 main screen contains five *main* windows (i.e. **Auto Sampler; ITC Controls; Thermstat/Calib; Setup/maintenance; Constants**) which are described in details in ***MicroCal AutoITC User's Manual***. Only those pertinent to setting up ITC experiment are discussed below.

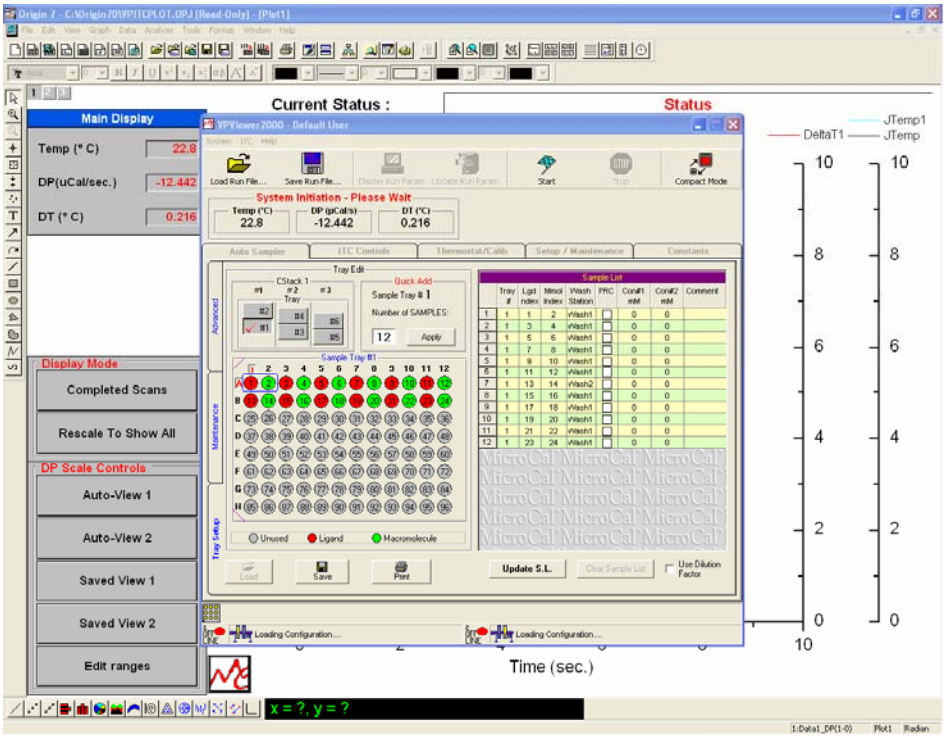


Figure 7

(4) Setting up an ITC Experiment.

(a) Autosampler options for rinsing cycles, solution dilution factors, post run cleaning, & sample recovery

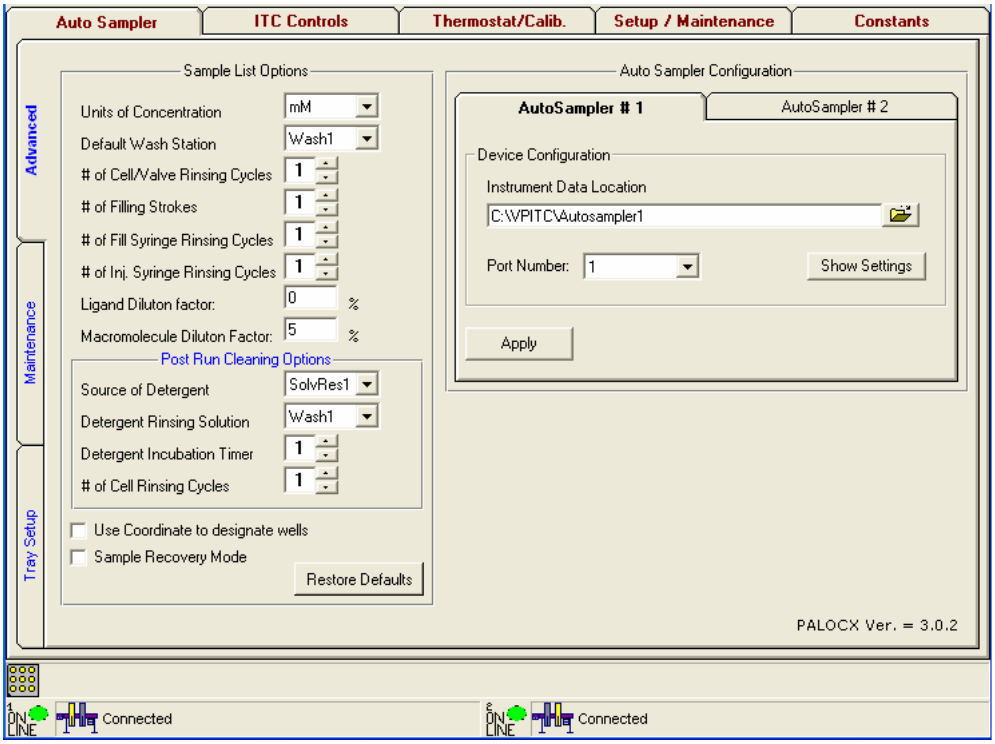


Figure 8

From VPViewer main screen, click on the **Auto Sampler** menu as shown above & then click the ***"advanced*** " tab (located on the left side of the window) to view the reaction cell, loading syringe, & titration syringe rinsing options. The default settings are two cycles of rinsing for the cell & filling syringe, & one cycle rinsing for the injector syringe. These default settings should be appropriate for most binding experiments. The default filling strokes is set at two. However, users should feel free to re-set these parameters to suit their experimental goal.

Just below rinsing cycle option, there are two lines with boxes for users to correct solution dilutions. User should be aware that the actual concentrations for the titration will be slightly lower than the concentrations originally measured, due to the dilution effect of the residual buffer solution left in the cell, & loading syringe during rinsing cycles. The percentage of dilution for the ligand and macromolecule varies slightly with the solution properties (i.e. surface tension, density, viscosity, etc.), temperature, and cleanliness of the syringe & cell (i.e. dirty surfaces have a greater adherence to solutions). MicroCal experiments (using 2'CMP and RNase A in 50 mM KAc, pH 5.5) have shown that the dilution factors, in a temperature range between 5 and 50 ºC, could range from 2 to 3 % for the ligand and up to 3-6 % for the macromolecule. In a temperature range between 20 and 30 ºC, the dilution factors were found to be ca. 2 % for the ligand and ca.

4 % for the macromolecule, & were set as default values at MicroCal. For initial experiment, users may the default values for ligand & macromolecule dilution correction. Both ligand & macromolecule concentrations will be automatically corrected for 2 & 4 % during data analysis. Users also have the option not to enter the dilution factors for ligand & macromolecule during the setup & do the correction later in the Origin software during the data analyses (see lesson 9 of the **ITC Data Analysis in Origin, Tutorial Guide).**

In the **"sample list options"** group, users also have options to set the default concentration unit as mM or µM, & set the default washing station as # 1 or # 2.

Below the "sample list options" is the "post run cleaning options", which allow users to set up the detergent cleaning of the cell in the middle of series of titrations. Users can assign the source of the detergent (i.e. reservoir # 1 for example), & the duration of detergent cleaning, & the source & number of the rinsing solution (i.e. DI water or the buffer).

There are two other buttons at the bottom of the "***Advanced***" window (on left-side). One is for users to change the coordinate numbers of microplate from 1, 2, 3,… to A1, A2, A3,… etc. The other is for users to have option to recover the titrated solution. Users should mark the square & place an empty microplate in the back of the same drawer if the recovery of the titrated solution is required. The solution will be saved in the wells of the second microplate with the same well number as the first one (for example, the A1 & A2 wells of the back micrpolate are used for depositing the titrated solution of A1, A2 wells of the front microplate).

b) Default titration parameters

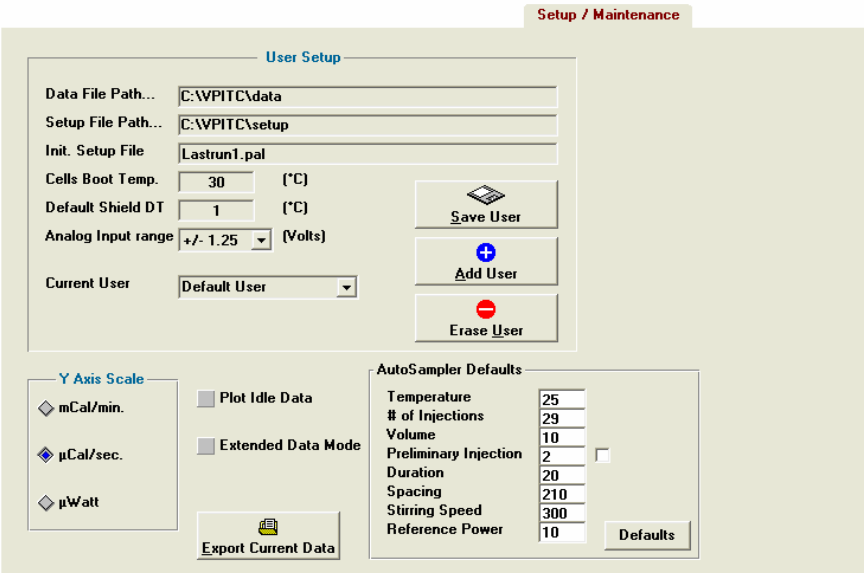


Figure 9

After setting up the rinsing cycle parameters etc, users may want to set up default titration parameters by clicking "**Setup/Maintenance"** window. It should be noted that the initial ITC running parameters shown on "***ITC controls***" window (see below) are reloaded from the default settings on this window. These parameters include the experimental temperatures, titration number, number of injections, volume per injection, preliminary injection (if yes, click the squares), injection duration, injection spacing,

stirring speed, and reference power. When VPViewer2000 is reloaded or the controller is rebooted, the initial parameters are those used in the last experiment. To set up an entirely new set of default parameters, first click on the **Setup/ Maintenance** tab. Then enter the new parameters in **Auto Sampler Defaults** group (see Figure 9), but do not click on the "default" button (if "default" button is clicked, then all parameters return to the original parameters set at MicroCal). Users also have option of not setting up the default parameters as described in this section, & do the titration parameters settings later in the sample list on ***"ITC controls***" window. It is for users' convenience to edit these parameters first before setting up the sample list.

c) Autosampler titration schedules (Auto Sampler window)

From VPViewer main screen, click on the **Auto Sampler** tab to open the tray setup window as shown in Diagram 1 (if this screen doesn't show up, click on the **Tray Setup** tab located at the left hand side of the screen). Users should click on "#1" in CStack1 area if the 1st microplate (filled with titrant & macromolecule solutions) will be placed in the outside position of the upper drawer. The microplate should be properly oriented with the A1 well of each microplate at the inside left corner (Figure 11). An empty microplate with cover mate should be placed in the inside position of the upper drawer if users want to recover the titrated solution after each titration experiment ***(i.e. "sample recovery mode" in "Advanced"*** window" is marked). In "Quick Add" area of the screen, users can then enter the number of samples to be run for the 1st microplates. For example, if all 96 wells of the 1st microplate situated in the outside position of the upper drawer are filled with macromolecule solutions and titrant solutions for the ITC experiments then the number 48 should be entered, followed by a mouse click on the "apply" button. A 2nd microplate filled with samples for titration can be placed in the inside position of the first drawer (or other positions) & followed by a click on " # 2" in CStack2 area if users have no desire to recover the titrated solution. However, a 2nd microplate filled with samples for

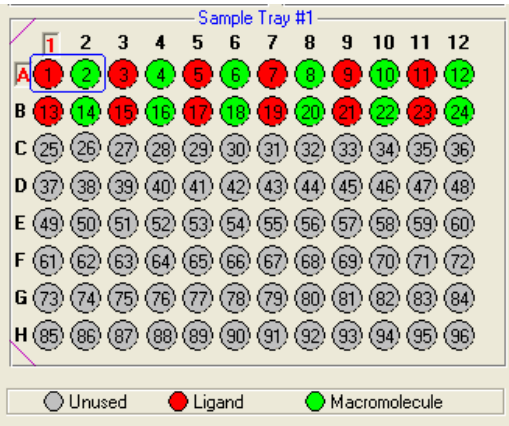


Figure 10

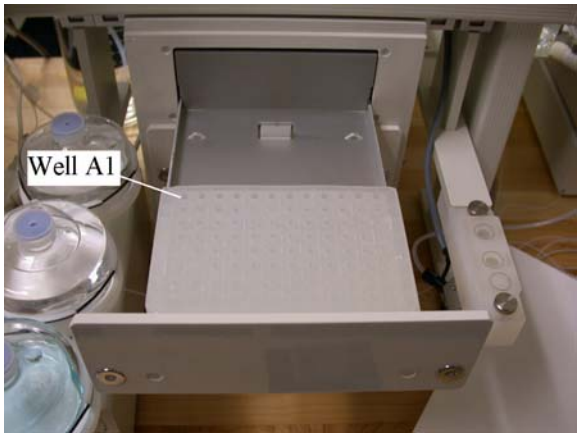


Figure 11

titration should be placed in the outside position of the middle drawer if users mark the "sample recovery mode" in '***Adanced***" window, then click on "# 3" in CStack3 area and enter the number of the sample to be run, followed by a click on "apply" in the "Quick Add" area.

In the microplate image (Figure 10), the titrant wells (odd number) turn red color while the macromolecule (even number) wells turn green color when they are selected. It is not necessary that all microplates to be used in an experiment are loaded with solutions and stored in the drawers *prior* to the start of the experiment. The sample solutions may be placed in the wells of the appropriate microplate and positioned in the drawer at any time before the execution of the experiment that will use samples from those wells (note: the titration can also be set up by selecting the well numbers by pressing left button the mouse, then click "update S L" button at the bottom of the window).

An inventory table now appears on the right hand side of the screen, showing the sequential titrations to be executed, the tray number and well numbers for each titration (odd number for titrant solution to be loaded into the injection syringe; even number for macromolecule solution to be loaded into the reaction cell), the number of the fast washing station to be used for rinsing the loading syringe, the PRC column (i.e. for **p**ost **r**un detergent **c**leaning option if it is set up in "***Advanced***" window as described in ***section 4(a)),*** the concentrations for titrant and macromolecule solutions (Con#1 & Con#2 columns, respectively; zero is default value), and a comment column. One may edit the washing station number (default is 1), and enter the appropriate concentrations of the titrant and macromolecule (in mM) for each titration experiment by selecting the cell (i.e. double click), enter the number, and click on the "apply" button (note: the same macromolecule concentration must be entered for the corresponding control run as that for the sample run). The cell in the last column is for any comments about each experiment, and can be accessed by double clicking on the cell, typing in the comments, and clicking on the "apply". *(Note: The concentrations and comments in the table can be entered/edited in VPViewer only before the execution of the titration. However, once results are called into Origin for data analysis, there is a second opportunity to modify the entries.)*

Entries into the inventory table may be done one cell at a time as described above, or as a whole column (i.e. when all experiments have the same parameter value) or as a group of experiments by either pressing down the "Ctrl" key (as a whole) or "shift" key (as a group) while clicking on the "apply". For example, if the titrant concentration for all experiments is identical and is 1.0 mM users should select the "Con#1" cell of any experiment number in the sample list by double clicking on it, enter 1.0, & then press down the "Ctrl" while clicking on "apply". All cells in column "Con#1" should change to 1.0 (default value is 0). To enter the same concentration for a group of experiments, the process begins from the top of screen (i.e. titrations # 1, # 2, # 3, etc). For example, if the macromolecule concentration of the first four titration experiments is 0.1 mM while the next four titrations (i.e. #5, 6, 7, 8) is 0.05 mM, double click on the macromolecule concentration cell of the 1st experiment (i.e. 1st cell of "Con # 2" column), enter 0.1, & press down the shift key while clicking on "apply". Users shall see that all the concentrations in the column changes to 0.1. Next, double click on the number 5 cell (i.e. the experiment # 5) in "Con#2" column, enter 0.05, and press the shift key while clicking on the "apply". It will be seen that the macromolecule concentration from experiment # 5 and down changes to 0.05. If the macromolecule concentrations for

experiment # 9 and and subsequent experiments are different from these of # 5 to 8, the procedure may be continued, etc.

As previous described in **the *section 4 (a)***, VPViewer will correct the dilution factor in the "***Advanced***" window using 2% for the ligand & 4 % for the macromolecule as default values, & mark the "use dilution factor" under the sample list. When the ITC data are loaded to Origin for data analyses the solution dilutions will be automatically corrected before data fit. Alternatively, users may choose not to correct the dilution factor during sample list setup, & do concentration correction later during data analyses. In this case, users should de-selects the "use dilution factor" square.

As a multi-sample experiment progresses, titrations that have not yet been carried out are shown in black in the inventory table while those that have been executed are shown in red. Entries may be edited (concentrations, comments, etc.) or deleted at any point before they turn red. To delete a run (before start of the experiment only), click on the row of the run to be deleted (the color of the whole row turns blue), press the delete key on the keyboard, and click on "yes". The next titration will move one run ahead (for example, titration # 15 becomes titration # 14 if # 14 is deleted) & the wells for that deleted run (i.e. # 27 & 28) will be skipped and not sampled.

To add a run (if the microplate hasn't been fully set up), select the pair of wells (or a group of wells, if more than one titration is to be added) to be sampled using the microplate image on the left hand side of the screen. This may be done by pressing the left button of the mouse, then dragging the mouse to select the appropriate new wells. The selected wells will then change color to red and blue depending on their odd or even number. After that, click on the **Update S. L**. button under the "sample list".

The newly selected wells for the titration experiment will be executed according to the new sequences shown in inventory table (If the titration parameters for the newly added runs are to be different from the default parameters users should go to **ITC Control** window to edit the parameters as described in the next section). The 'Clear sample list" button at the button right hand side of the screen allows users to clear all parameters and do a completely new setup before launching the experiment.

Figure 13

The Auto ITC was designed for high throughput operation. The reaction cell requires periodic cleaning with detergent since only rinsing solution is used between experiments during routine operation. The need to carry out detergent cleaning may be indicated by increased baseline noise, a steep baseline slope, or abnormal injection peaks. The frequency with which detergent cleaning should be carried out will depend on the properties of the macromolecule and titrant solutions at the experimental temperature. The "PRC" column is for users to set up automatic detergent cleaning by marking the square of the titration. The detergent cleaning routine set up in the "***Advanced"*** window will kick in after that particular titration.

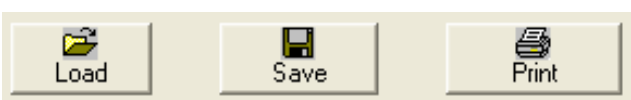


Figure 12

At the bottom of the "***Auto Sampler"*** window (left hand side), there are three buttons (Figure 12) that allow users to save, print, or reload the current autosampler parameters (i.e. "Sample List").

Besides the "**Tray Setup"** & "***Advanced***" windows described above, there is an additional sub menu (i.e. ***Maintenance*)** in the "*autosampler*" window (Figure 13). In the "***Maintenance"*** window, users may set up cleaning or rinsing for the reaction cell, the injector syringe, & the transfer syringe when the autosampler isn't in auto mode. There are other functions in the "**Maintenance"** window; users should refer to "***MicroCal AutoITC User's Manual***" for details.

d) ITC parameters (ITC Controls window) for multiple injection experiment.

After setting up the auto sampler titration schedules as described above, click on the "***ITC Controls"*** tab for editing ITC experimental parameters.

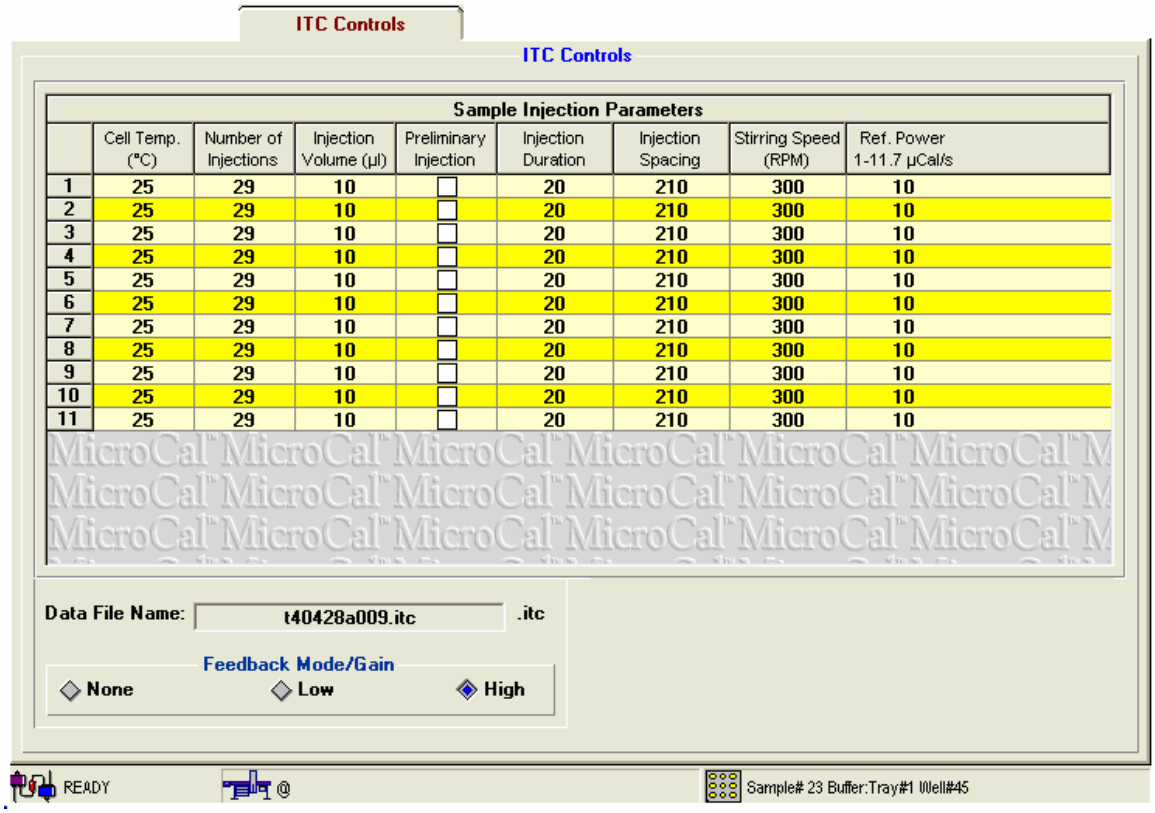


Figure 14: ITC Controls Window

These parameters include titration number (1st column), the experimental temperatures (2nd column), number of injections, volume per injection, preliminary injection (if yes, click the squares), injection duration, injection spacing, stirring speed, and reference power. The initial ITC running parameters shown on this screen are

reloaded from the default settings (as entered in the **Setup/Maintenance** window) when the new sample list is loaded. When VPViewer2000 is reloaded or the controller is rebooted, the initial parameters are those used in the last experiment. The details for setting up an entirely new set of default parameters have been described in section ***4 (b).***

In the "***ITC Controls"*** window (Figure 14), the parameters for an upcoming experiment can be edited one at a time, if desired. To do this, double click on the appropriate cell of the table, enter the new number, and click on "apply". Users can also edit the parameters as a whole column or as a group of titrations in the column. This has been previously described in detail for the setup of the titrant and macromolecule concentrations in the above section, "***Setting up Auto Sampler titration schedules"***. For example, if the temperature of 1st four titration experiments are to be a 10 °C while the next four titrations (i.e. #5, 6, 7, 8) are to be at 30 °C, then double click on the temperature cell of the 1st titration experiment, enter 10, and press down the shift key before clicking on the "apply". Users shall see that all the temperature in the column change to 10 °C. Then, double click on the temperature cell of experiment # 5, enter 30, and press the shift key before clicking on the "apply". All temperature readings for titration # 5 and beyond change to 30 °C. Using this method can sometimes save time relative to editing parameters one by one. Finally, users should enter the new filename (limited to 10 characters and digits combined; the computer will then append 001, 002, & 003 to the filename for titrations # 1, 2, & 3 etc). All parameters in the Autosampler and ITC setup menus should be rechecked for correctness before clicking on the "start" button to launch the experiment. As mentioned before, any editable parameters for a specific titration can be changed before that titration is executed. Following are some guidelines for selecting titration parameters:

1. Temperature: It is most convenient to perform auto 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, thereby reducing sample throughput. *If the ITC experiments are to be performed at several different temperatures, it should start from the lowest temperature in order to speed up the sample turnover rate during the temperature changes (i.e. from 10 to 20, 30, 40 °C etc.).* At high temperatures above 50 °C (Note: the maximum temperature for running an experiment is 70 °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 macromolecule. Some solutes, particularly proteins and other biomolecules, are not stable above room temperature for long periods of time with stirring so it is desirable to work at lower temperatures with such labile compounds. Finally, to determine the change in heat capacity ΔC_P for binding, experiments must be carried over a range of different temperatures (e.g., 10 -40 °C) to obtain the temperature dependence of the heat of binding.

2. Number of injections & injection volume. The optimum injection volume for the Auto ITC instrument is 3 to 15 µl per injection while the maximum injection volume per titration experiment is 280 µl. It should be noted that the actual injection volume for AutoITC is stepwise & not a whole number. For example, if users enter 3, 5 or 10 µl as the injection volume the actual injection volume shown in the table will be 2.81, 4.81 &

10.02 µl, respectively. Users should take notice that the injector will load just enough ligand into the titration syringe according the setup, instead of loading the full syringe (in order to save ligand solution). For example, the volume of ligand loading into injector syringe for a 20 x 3 µl titration schedule would be 140 µl less than for a 20 x 10 µl titration. For a well designed ITC experiment, 10 injections per experiment may be sufficient to determine the binding parameters although more precise parameters might be obtained with more injections. On the other hand, fewer injections per experiment result in higher sample throughput. Titrant remaining in the syringe after an experiment will automatically be discarded to waste. Whenever possible, a sufficient amount of titrant should be added to insure that a large fraction of the binding sites are saturated when injections are completed. *(Note: Good binding parameters can be obtained using injection volumes as small as 3 µl per injection. The smaller injection volume usually produces smaller control heat).*

3. Preliminary injection: For ITC experiments, the heat effect for the 1st injection peak is usually slightly smaller than expected relative to subsequent injections. This can result from a slow diffusive exchange of solution from the cell with solution in the syringe tip during the relatively long time which stirring takes place prior to the 1st injection. Because of this, it is usually desirable to delete the first data point before carrying out curve-fitting to obtain parameter values. Deleting the first data point becomes less significant in the overall analysis if a smaller injection volume is used for the first injection relative to subsequent injections. Users are encouraged therefore to use a smaller injection volume (2 to 3 ul) for the 1st injection and remove this data point later prior to curve-fitting in Origin. The volume for this preliminary injection can be assigned by clicking on the "***Setup & maintenance***" and entering the volume for the preliminary injection in the "Auto Sampler Defaults" listing. If a preliminary injection is desired for a particular titration experiment, then it is a simple matter to mark the appropriate square by clicking on it.

4. Injection duration: Enter the time, in seconds, for the titrant to be injected into the reaction cell. For most experiments, injection duration should be approximately twice the value of the injection volume. For example, if injection volume is 10 ul/inj the injection duration is ca. 20 sec.

5. Injection spacing: Injection spacing is the elapsed time between the beginning of one injection and the beginning of the next injection. For most binding experiments (i.e. if the binding reaction is fast relative to normal instrument equilibration time), this parameter may be set at 180 to 240 seconds, depending on the magnitude of the injection peaks. For slow binding kinetics, a longer time may be required so the entire response is recorded. The important requirement is to allow sufficient time to permit the differential power signal (i.e., Y axis in real time plots) to return to its resting baseline position seen before the injection was made. Note: For the fastest instrument response time, the Feedback Mode/Gain in **ITC Controls** window should be set at "High".

6. Stirring speed: For a dilute buffer solution, a stirring speed of 300 rpm is sufficient to achieve complete mixing in a short period of time (180 to 240 seconds).

Highly viscous solutions may require faster stirring or a longer injection spacing. The maximum stirring rate is ca. 1000 rpm, but speeds higher than 300 rpm usually produce higher noise in the baseline trace.

7***. Reference cell power:*** Reference cell power (which is nearly equal to the resting baseline position during an experiment) may be set from ca. 1 to ca. 35 ucal/sec. (exact range is indicated in the table, since it is a little different for each instrument) prior to starting an experiment. The purpose here is to select the reference power such that during an experiment the differential power reading (Y axis value) stays within the range from 0 to 35 ucal/sec to avoid saturation of the feedback circuit. For virtually all experiments, including both exothermic and endothermic binding, a reference power setting of ~25 ucal/sec is satisfactory. If the DP signal is saturated during an experiment with endothermic peaks (positive Y axis peaks that exceed 37 ucal/sec), the experiment should be repeated with a lower reference power setting. DP saturation will be identifiable as clipped peaks. If the DP signal crosses 0 uCal/sec, the experiment should be repeated using a higher setting of reference power. Crossing 0 uCal/sec may cause clipped peaks or a ringing in baseline. Other options which would correct saturation problems would be to reduce the injection volume or to lower the concentrations of binding components, or to increase input power (refer to "***Setup & maintenance" menu*).

There are other menus and controls for ITC instrument and users should refer to the ***MicroCal AutoITC User's Manual*** for details.

(5) Launching the ITC Experiment

The auto sampler will execute the following steps of rinsing and loading for the first titration after the "start" button is clicked:

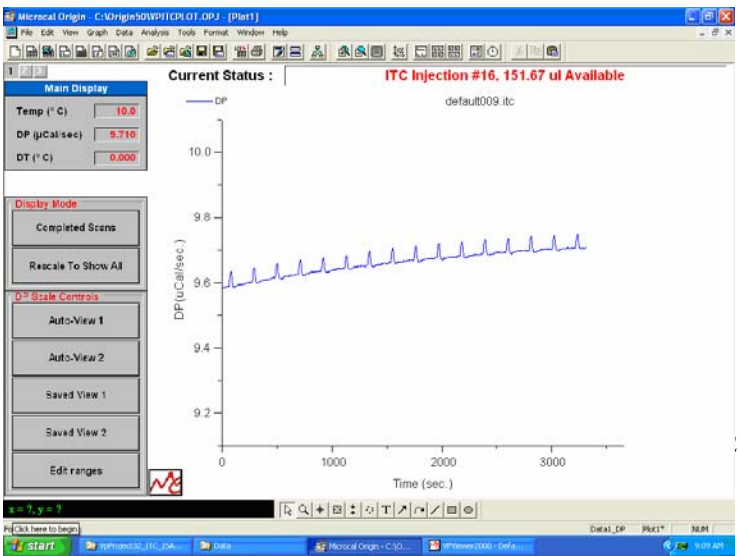
1. The titration syringe on the left-arm injector travels to the 1st bottle (DI water) on the left hand side rinsing station. At the same time, the vacuum pump is turned on. After aspirating DI water into the syringe, the injector travels back to the resting position & lifts up the plunger to let vacuum pump remove the rinsing water. Depending on rinsing cycle set up in "***Advanced***" menu, the rinsing can be performed up to 3 times in each bottle. After finishing the 1st DI water bottle rinsing, the syringe travel to the 2nd DI water bottles for more rinsing. Finally, the titration syringe travels to the 3rd bottle (i.e. outside one) to aspirate methanol, & goes to syringe drying place to be dried by the vacuum pump (for inside of the syringe) & by a fan (for outside of the syringe). The drying procedure takes ca. 5 minutes, & the vacuum pump automatically stops. The injector returns to the resting position waiting to load the lgand solution.
2. At the same time as titration syringe is being rinsing & drying, the loading syringe on the left-arm injector is preparing to rinse the cell & to load the macromolecule

solution. First, it travels to the reaction cell to remove previous run solution (if it is not saved) & disposes it at the fast washing station.

3. After rinsing with the buffer solution at the fast washing station, the loading syringe aspirates ca. 2.2 ml of the buffer solution & waits at the fast washing station for the solution to be warmed up to one degree below the experimental temperature by the heating unit (it should be noted that the loading syringe is surround by a heat block). Then, the injector travels to the reaction cell for rinsing. The default setting for the cell rinsing cycles is two.
4. The loading syringe then opens the sample drawer to aspirate 1.8 ml of the macromolecule solution, & goes to degassing port, which creating a vacuum by lifting up the plunger while the macromolecule solution is warming up. After ca. 1.5 minutes at the degassing station, the loading syringe opens the sample drawer, & re-deposits the macromolecule solution to the original well, & immediately re-aspirates 1.8 ml into the syringe for the 2nd degassing. The 2nd degassing takes ca. 5 minutes. The loading syringe goes to the empty reaction cell for filling cell with the macromolecule solution.
5. The loading syringe, after rinsing with the buffer solution at the fast washing station, opens the sample drawer to aspirate 0.6 ml the ligand solution, & then goes to degassing port to outgas the ligand solution in the identical way as described for the macromolecule solution.
6. After degassing, the loading syringe re-deposits the ligand solution into the original well. Then, the titration syringe moves to the ligand well for ligand loading. At the same time, the loading syringe is positioned at an injection port (on the fast washing station) to assistance the ligand loading by aspirating the air into the syringe through a plastic tubing connecting to the titration syringe (through the switching
7. After closing the sample drawer, the titration syringe travels to the reaction cell for the titration experiment to be started.

(6) Observing Live Data

Once the macromolecule solution and titrant are loaded, the equilibration (i.e. pre-titration) baseline as well as the actual

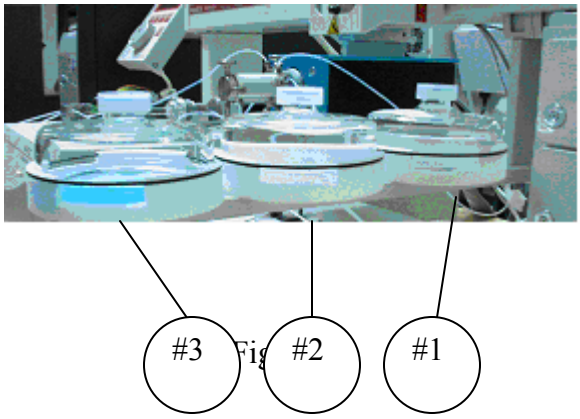


titration data in progress are automatically displayed in an Origin plot (Figure 15). During the "pre-stirring equilibration" several different variables are plotted using multiple Y axes (versus time on the X axis), including the differential power DP, jacket temperature, and differential temperature DT (refer to VP-ITC Manual for more details). When the cell temperature has nearly equilibrated with that of jacket (DT ~ 0) the stirrer is started and another round of thermal equilibration begins ("final baseline equilibration" shows up on the screen) and only one data plot (i.e. DP (µcal/sec) vs. time (sec)) is shown on the screen. Users may click on "rescale to show all" button if the signal goes off-scale. "Auto-View 1" or "Auto-Viewer 2" can be used to zoom in if desired (full-scale ranges of 0.1 and 1.0 ucal/sec, respectively). Once the experiment begins there is a 60 sec delay before the first injection is started so that the resting baseline is seen in the final data.

As mentioned earlier, if the DP signal ever goes negative during a sequence of injections, the data should be discarded and the experiment must be run again using a larger setting for the Reference Cell Power. A setting near 25 ucal/sec should be suitable for almost all experiments. Unusually large heats can result if the ligand solution and macromolecule solution are "mismatched" due to large differences in pH, additive concentrations, etc. The computer using the default path C:\VPITC\data saves continuously the experimental results plotted during an experiment. For data analysis procedures, refer to **ITC Data Analysis in Origin, Tutorial Guide**. Special procedures for the autosampling ITC are included in Chapter 8, but the more general chapters on data treatment and model fitting should also be studied.

D: Routine Care for the Auto ITC Instrument

(1) Cleaning Procedure



Fig

VPViewer has an automated cleaning routine, which will perform the detergent cleaning for the cell upon operator's request. The detergent cleaning station (Figure 16) has three 120 ml glass bottles, which may be filled with detergent solution, de-ionized water, and methanol (necessary if liposomes have been used in experiments), respectively. These three bottles are identified as "SolvRes1", "SolvRes2", and "SolvRes3" in the "***Advanced"*** tab of the "Auto Sampler" menu. If detergent cleaning is deemed necessary in the middle of series of titrations, users should set up the cleaning parameters in "***Advanced"*** window & mark the squares on the PRC column on the sample list table in the "***Tray Setup***" window. For example, if users want to clean the reaction cell with detergent for every ten titrations they mark the PRC column every 10 titrations (# 10, # 20, etc).

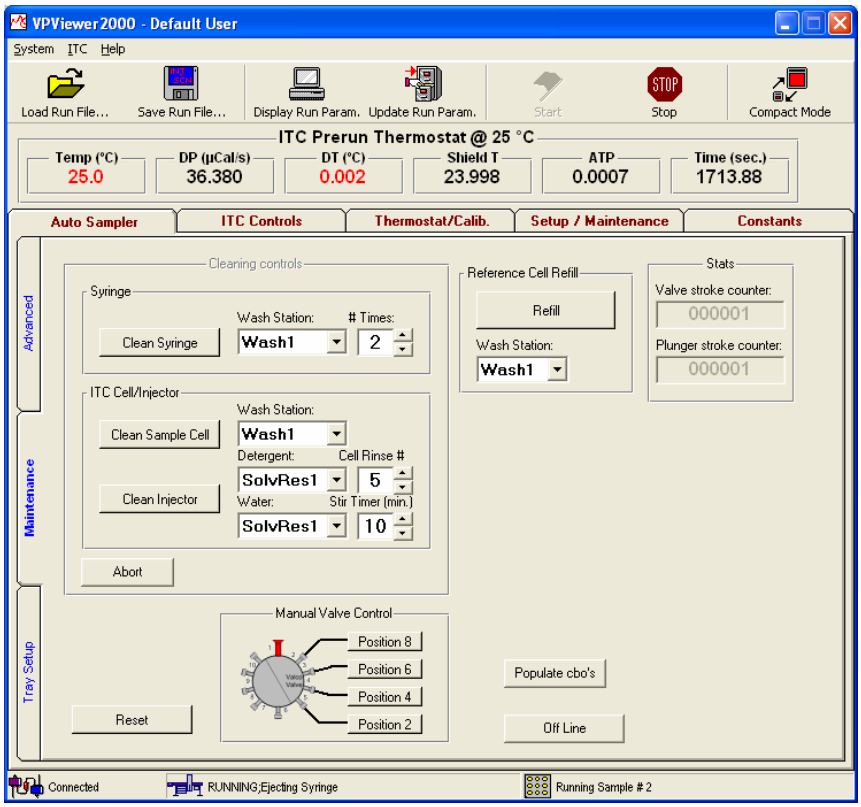


Figure 17: Auto Sampler – Maintenance Window

An excellent detergent solution to use for cleaning is a 20 % solution of Contrad 70 (cat # 1003, Decon Labs, Inc.) in deionized water. For regular cleaning, the reaction cell temperature may be at 30 ^{o}C, using a 10-30 minutes stirring time with the detergent (i.e. set "stir timer" at 10-30) and using five rinsing cycles (i.e. set "cell rinsing #" at 5). For a more thorough cleaning, a temperature of 60 ^{o}C may be used. Then, set the stirring time with the detergent at 60-90 minutes, and the number of de-ionized water rinsing cycles up to 10 or more. It should be noted that after detergent cleaning both the cell & syringe are empty. If no sample runs are to be performed immediately, users should set up a couple sodium azide vs. sodium azide (0.01 %) titrations as closing runs.

Alternatively, users may set up a regular ITC titration schedule at a temperature at 30 ^{o}C or 50 ^{o}C (2 or 3 runs), filling the tray wells with Contrad 70 (20%) for both the sample and reference cells followed by a rinse consisting of 4 to 5 de-ionized water Vs. water runs (if the instrument is not going to be used for awhile then rinse with 0.01% sodium azide). Since these runs are for cleaning only, a typical injection schedule might be Number of Injections = 3, Injection Volume = 100 , Injection Duration = 50 sec., Injection Spacing = 60 sec.. Note: before running this experiment makes sure that all rinsing solutions in the reservoir bottles contain only de-ionized water or 0.01% sodium azide.

(2) Refilling the Reference Cell

The reference cell will normally be filled with deionized water or 0.01 % sodium azide solution, and should not require cleaning with detergent. However, because of evaporation and/or bubble formation, it should be drained and refilled on a monthly or biweekly basis. One possible indication of evaporation in the reference cell is if the baseline is routinely lower than the reference power setting, but unusual baseline noise could also result from problems in the reference cell. The reference cell needs to be manually refilling.

E: Tips and Troubleshooting

(1) Autosampler doesn't start after clicking "start" button.

Check that the power line is active and the switches on all units are in on position with all power cords connected to the source. If the instrument platform has been relocated, check that the individual components are properly interconnected. Sometimes, VPViewer may need to be shut down and rebooted again.

(2) The autosampler ceases operation during rinsing, loading, etc.

If this happens, the instrument needs to be re-set. First, click the "stop" button on the VPViewer. Re-set up the titrations starting from the "Auto Sampling" menu as described in the ***Section (C)***.

(3) The instrument baseline is excessively noisy (i.e. peak to peak noise for de-ionized water vs. water titration larger than 0.005 µcal/sec)

A dirty or contaminated reaction cell is the most likely cause of excessive baseline noise and/or baseline drift. This can usually be remedied by carrying out several titrations using 0.01% sodium azide solution in both cell and syringe and/or cleaning the cell and syringe with detergent. Both procedures were described earlier. If the situation doesn't improve, contact MicroCal for further instructions.