Troubleshooting guide for Isothermal Titration Calorimetry

M.R. Kasimova

This is a very short summary of common problems encountered with ITC equipment and experiments. It is based on my personal experience of working with Omega and VP-ITC from MicroCal, as well as Nano ITC from TA Instruments. The first one, although a very reliable machine, is probably extinct, so the following troubleshooting guide is intended mostly for the users of VP-ITC and Nano ITC.

This guide is neither complete, nor intended as a substitution for the manuals provided with the ITC machines. It' to help those students, who, after wasting a whole day on trying to make an experiment work, still cannot figure out what's going on with the baseline and why. Also, I often encountered insufficiency of the assistance available through the producers of calorimetry equipment, because the technical personnel is not particularly trained to help with practical problems, simply because these people have never done a real-life ITC experiment themselves. So instead of wasting your time discussing bubbles and the importance of degassing your sample with the producer, look through the subtitles below and see if you can find the one describing your specific case.

Experimental setup: equilibration time, stirring rate, etc.

Most thermodynamically controlled reactions under typical experimental conditions occur rather fast. This means that allowing 5 minutes between injections should be sufficient in most cases and an output of your experiment should look like the one presented in Fig. 1A. It is very important that the level of the baseline after each injection is the same as before. If this condition is not fulfilled, and the onset of the following injection occurs before the previous one is over (Fig. 1B), you should check the following:

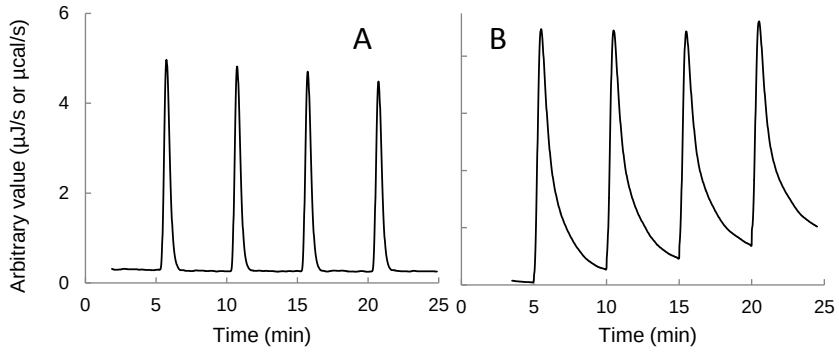

Figure 1. Illustration of sufficient equilibration time (A) and insufficient one (B).

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Is the stirring on?

The stirring of the injection cell is not the most crucial parameter of the experimental setup. However, it defines how fast the titrant diffuses through the volume of the cell and reaches the titrate, consequently enabling the reaction. The stirring rate will not affect the total heat measured over the course of an injection[1], but it can affect the injection shape, as slower diffusion will prolong the reaction period and thus make the injection profile wider. Therefore, this parameter is more important when the titrant is a bulky macromolecule or particles, for which the diffusion rate is slower. A typical stirring rate for protein/peptide solutions is between 200 and 300 rpm.

Sometimes, it is desirable to avoid the stirring all together. In that case, try to increase the interval between the injections to 10-15 min. If it still does not help improving the baseline between two consecutive injections, your problem is most likely due to something else (see Sample-related problems).

Is the heat produced by an injection too large?

It is possible that the concentrations of the reactants are too high and it takes longer than 5 minutes for the system to return to equilibrium. This is not so easy to check, because most of the manuals do not specify the upper limit of the injection heat. However, I usually avoid having the injection heat above 500 µJ. This will also save you some of your precious reactant material. Sometimes, this problem can also be identified by the cut-off tops of the reaction peaks (see "Instrument-specific problems").

Experiment is running, but no heat is visible, not even the dilution heat

Check if the syringe is clogged or stopped. It can happen if you waited too long between loading it and inserting it into the injection chamber. Sometimes, the liquid on the tip dries out, forming a hard-to-break film. Clogging can also happen when the syringe is loaded with aggregating protein or synthetic particles, which increase the friction between the plunger and the inner wall of the syringe. Try to push the plunger by hand to make sure it's moving freely.

Another thing to check is whether the injector mechanism operates properly (see "Instrument-specific problems").

Large baseline noise, unsteady baseline

These problems can be caused by a bent syringe, which scraps the walls of the reaction cell upon rotation. Often, when you are under time pressure, it is easy to overlook both the

[1] provided that it is an equilibrium reaction and that the next injection is not initiated prematurely

elevated noise level and the unsteady baseline. If, in spite of your suspicion, you decide to ignore these indications and start the experiment anyway, you will observe some weird-looking injections. Sometimes, the signal will oscillate up and down, only slowly settling back to the pre-injection level. Other times, the baseline will drift strongly up or down, behaving unpredictably.

To check, if the syringe is straight, put it on a flat surface, *e.g.* at the edge of a table. Rotate it slowly along the surface watching the distance between the needle and the table. If syringe is bent, you will clearly see it. Then contact your ITC company.

Large shift of baseline after initiation of stirring

If the baseline is perfect before the stirring is on, and when you turn it on the baseline goes out of range or never returns to the usual level, you definitely got a bent syringe.

Instrument-specific problems

The baseline is completely flat (no noise)

The signal is out of dynamic range (VP-ITC).

Flat tops of peaks

This problem is illustrated in Fig. 2. It is specific to MicroCal Instruments (both VP-ITC and VP-DSC) and it indicates that either the injection heat is too large (for ITC) or the major equilibration baseline is shifted (both ITC and DSC). The former is easily resolved by decreasing concentrations of the reactants, while the latter is often caused by dirty cells. Clean the calorimeter once again and if the problem persists, check the reference cell. Sometimes, when the heat capacities of the experimental and the reference chambers differ considerably, it causes a shift in the equilibration baseline level and thus, affects the available dynamic range. This happens often, when the content of the reference cell dries out, gets contaminated or when you're working with concentrated solutions of denaturants.

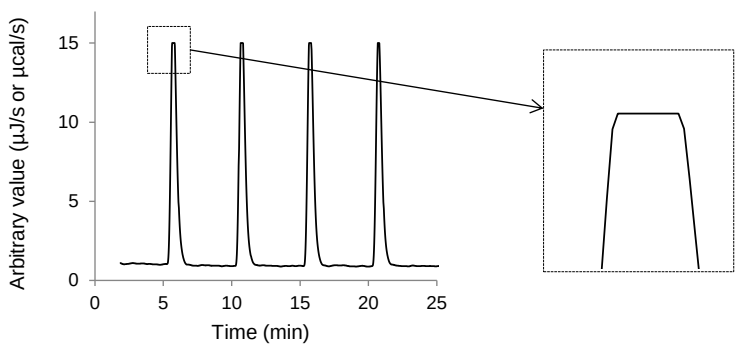


Figure 2. The signal is out of dynamic range.

Experiment won't start, even though the baseline is flat.

This problem occurs only on MicroCal machines with an automatic experiment initiation, where the instrument evaluates the baseline quality and starts an experiment by itself, without intervention from a user. I encountered this problem very often, when working at higher temperatures (above 37 ˚C). When tired of waiting, check the baseline quality (remember to zoom in) and if you think that the baseline is flat enough, go to VPViewer window. Find the button named "DP" and click twice on the red number, displayed beneath the "DP". This will force the program to the next stage in its schedule.

Experiment is running, but no heat is visible, not even the dilution heat

A problem specific to some Nano ITC instruments is a mechanical glitch associated with the automatic injection system. Sometimes, the plunger of the injector gets stuck and although it appears that the experiment is running, no heat is evolving – the baseline stays completely flat. If so, remove the syringe and check if the plunger is moving, when you click "up" or "down" buttons in the operation menu. (Also see a section of the same title in "Experimental setup" above)

Sample-related problems

There is an ocean of things that can go wrong, if you're working with a system, which has not been described before. In addition, since you do not know what to expect from your titration, it's hard to decide what is causing a problem: are the solutions too diluted? Is there binding at all? And so on. Although it is hard to describe each individual case, there are some problems that occur rather often and these are described below.

Slow non-equilibrium processes

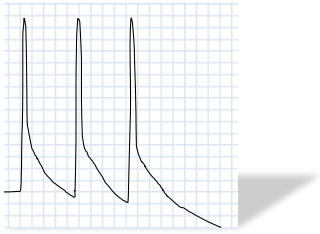


Figure 3. An example of injection profiles affected by slow aggregation processes.

Experimental heat measured by a calorimeter is not necessarily produced by the reaction under study. Other processes, such as degradation of reactants, aggregation/fibrillation and so on can contribute as well. These processes are rather slow on the time-scale of typical binding reactions and if this is the problem, the post-injectional baseline will have a long sloping tail, which can go on for tens of minutes without leveling out (Fig. 3). In that case, check for degradation products or measure the size distribution of your sample (DLS, HPLC, Spectrophotometry) before and after the titration. Aggregation of the sample can sometimes be avoided by changing the solution conditions (pH or ionic strength) or by decreasing concentrations of the reactants. If you're working with liposomes, the "tail" can be also due to liposome fusion or leakage.

Why do I not get anything that looks remotely like a titration curve?

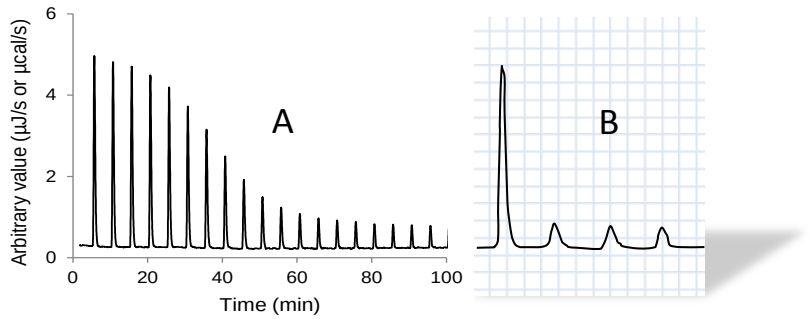


Figure 4. Comparison of a perfect titration (A) to an unfortunate one (B).

If, instead of a classic titration (Fig. 4A), you get something like the drawing on the right-hand side (Fig. 4B) you should re-examine your experimental design or check the concentrations. In this particular example, all of the cell content is saturated in the first injection (Fig. 4B), which means that you either have to increase the concentration in the cell or decrease the one in the syringe. It is useful to analyze the heat produced by the first injection to evaluate the enthalpy of binding. This will help you to decide which way the concentrations should be adjusted to receive a reasonable heat effect. The same explanation is also valid for the case when all injections are of the same size, but not the same as the dilution heat.

Shifts of baseline

When the post-injectional baseline is flat and horizontal, but does not reach the same level as before (see Fig. 5), there can be several reasons for that. Most probably the binding reaction is associated with the dramatic changes in the heat capacity of the system. For

example, the baseline shift can be due to the occurrence of an enzymatic reaction (see for example Todd & Gomez (*Analytical Biochemistry* 2001, **296**, 179-187). Another example describe by Myers *et al* (*Biochemistry* 1987, **26**, 4309-4315) is the baseline shift associated with liposome fusion.

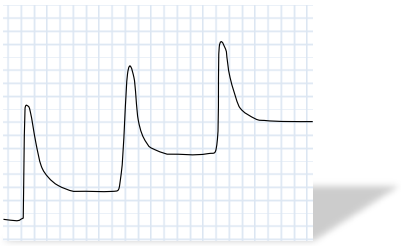


Figure 5. Illustration of the baseline shift upon injection.

Bubbles

These look like sharp spikes which can appear anywhere in the titration profile. If the bubbles are injected with the titrant from the syringe, the injection itself will be affected and the height of the neighboring injections can be strikingly different. However, this is easy to check by taking the syringe out for close inspection.

On the contrary, a visual inspection of the bubbles formed on the walls of the experimental cell is rather difficult. In this case, their release can be either initiated by injection or happen spontaneously at any time during the experiment.

Assuming that you do degas your sample, remember to pay attention to its temperature. The formation of the bubbles is often induced upon the contact of a cold sample (just from the fridge) and the warm cell (injector) walls. Also, if experiments are done at the temperatures much higher or lower than 25 ˚C, it is beneficial to equilibrate your sample at the experimental temperature before loading it into the cell or the injector.

Overshoot to the opposite side

Two simultaneous reactions with the opposite heat effect.

General advice

★ You can learn quite a lot from reproducing your experiment in a glass test tube, while observing the progress of the titration through the transparent wall.

Let's say you want to titrate a peptide solution with liposomes. The experiment must be performed without stirring, because the peptide fibrillates upon agitation. In the real ITC experiment the V_{cell}=1 mL and V_{inj}=10 µL. In your simulated experiment you can use the same volumes or you can scale

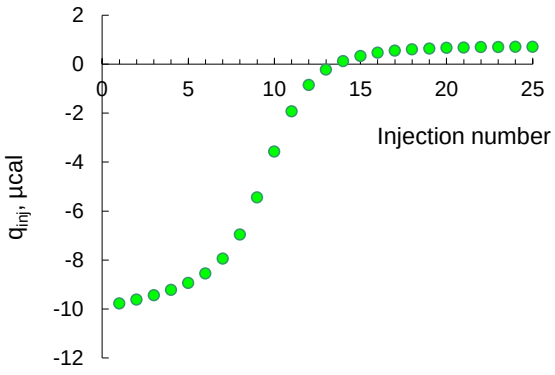
them up, according to your test tube size, for example V_{cell_test} = 5 mL and V_{inj_test} = 50 µL. Typically, a liposome suspension is opaque, thus allowing you to observe the progress of its diffusion through the test tube upon each addition. Remember that in the real ITC cell, the tip of the injection syringe is located close to the bottom of the cell. Now you can answer at least two questions:

a) how fast is the suspension mixed with the rest of the solution?

b) does addition of liposomes induce aggregation of the peptide?

★ It's always a good idea to make a reference titration at the beginning of the binding experiment or a batch of experiments. Make sure to repeat it again, if you change temperature or solution conditions. However, often, the heat produced in the reference titration is not the same as the one you obtain at the very end of your binding experiment. In other words, at the end of the titration, when the reaction is complete and only the dilution heat is expected, the obtained heats are different from those, measured in the reference titration. This phenomenon has two explanations:

a) Heat of dilution of a ligand in buffer solution is not the same as the dilution of this ligand in a solution of the macromolecule;
b) Upon each injection, some of the cell content is being pushed out, thus decreasing the concentration of the macromolecule originally loaded in the cell. Therefore at the end of the titration, one has a decreasing concentration of macromolecule, although the concentration of the ligand continues to increase. This leads to a shift in equilibrium between the components with the consequent dissociation of the ligand. Fig. 6 shows a simulated titration, where the reference heat is set to zero. Nevertheless, the heat at the end of the experiment is not zero and has a sign opposite to the binding heat (Fig. 6).



[M] cell =	0.01	mM
[L] syringe =	0.1	mM
Vcell =	1.0	mL
Vinj =	10	µL
ΔH =	-10000	cal/mol
n =	1.00	
k =	5	μM^{-1}

Figure 6. Simulated titration curve of macromolecule M with ligand L. Parameters used in the simulation are indicated in the figure. The dilution heat is set to 0. The non-zero heat at the end of experiment is due to the decreasing concentration of macromolecule in the cell.