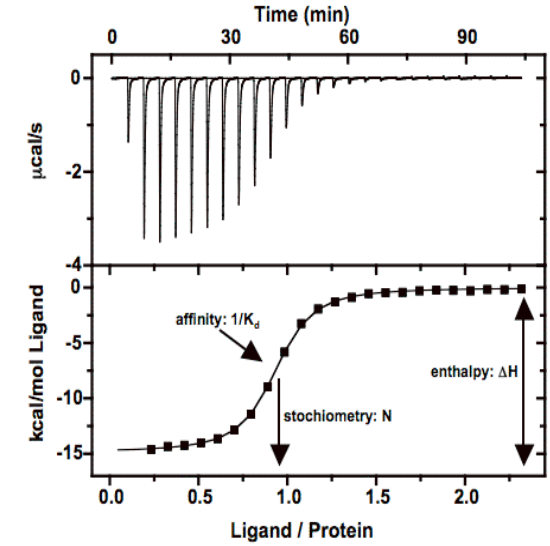
Isothermal Titration Calorimetry (ITC)

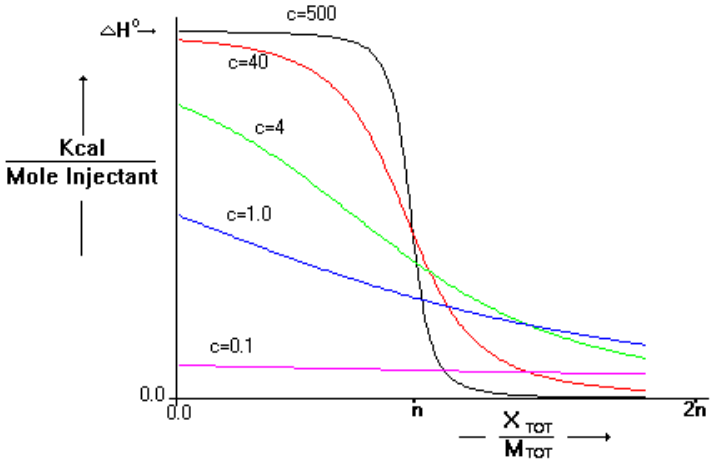


Estimation of Required Concentrations for ITC

The critical parameter which determines the shape of the binding isotherm is the unitless constant *c*, which is the product of the binding (association) constant K, the total macromolecule concentration in the cell at the start of the experiment, M_{tot}, and the stoichiometry parameter, *n*:

$$\mathbf{c = KM_{tot}n}$$

The graph below shows the effect of *c* on the binding isotherm:



Higher c-values result in titrations curves that are too steep to resolve *K* accurately (although *n* and Δ*H* are well resolved) because the cell reactant concentration is too high relative to *K*, whereas lower c-values result in shallow titration curves from which all three parameters (*n*, *K*, and Δ*H*) are poorly resolved. Most papers will say to shoot for a *c*-value between 10 and 100. However, in the following paper: D. Myszkra, Y. Abdiche, F. Arisaka, O. Byron, E. Eisenstein, P. Hensley, J.

Thomson, C. Lombardo, F. Schwarz, W. Stafford, M. Doyle. J. Biomol. Technol. 14, 247 (2003), the authors had multiple groups examine the same binding reaction by ITC. Participants were supplied with sample, but they were free to choose their own experimental conditions & parameters. They found that the binding constants were poorly resolved from experiments performed where c < 20 and therefore recommend that: 20 < c < 100.
The authors also noted that a significant part of the variability in the reported *n*, *K*, and Δ*H* values from the different groups was due to the variability in determining the concentration of the ligand. The accuracy of all three fitting parameters (*n*, *K*, and Δ*H*) is directly proportional to the accuracy with which the syringe reactant concentration is determined. In contrast, the accuracy of the cell reactant concentration only affects the molar binding ratio *(n)*. This observation reinforces the importance of accurately determining the concentration of the syringe reactant.

What concentrations should I use if I don't know the binding constant?

MicroCal suggests using macromolecule concentration (the species that goes in the sample cell) of 10 to 50 * $\mathbf{K}_d$ where $\mathbf{K}_d$ is the dissociation constant (i.e. the reciprocal of K).
This website: http://www.endocytosis.org/techniqs/ITC.html says that ideally the macromolecule concentration is 30 * $\mathbf{K}_d$ and that if the concentration is more than 100-fold above the dissociation constant the curve gets too steep and the fit is inaccurate. If $\mathbf{K}_d$ is unknown, a best guess will have to suffice.
The molar concentration of the ligand in the titration syringe can be estimated as:

$$15 \text{ to } 20 * \mathbf{n} * \mathbf{M}_{tot}$$ where **n** is the stoichiometry of the reaction

This is different from what the MicroCal User Manual says to estimate (7 * $\mathbf{M}_{tot}$ if n=1). If binding is very weak, making the ligand concentration >>> than the protein concentration is important to drive the equilibrium toward binding. MicroCal warns against stopping the experiment too early, before the binding curve has fully plateaued, as the data are harder to fit. The above website recommends that the ligand in the syringe should be titrated to a molar ratio of 4:1 compared to the binding partner in the sample cell, and that the volume of the individual injections should be chosen such that the ligand and the binding partner reach a molar ratio of 1:1 after about 10 injections.

What is a measurable amount of heat?

MicroCal recommends that you aim for at least 10ucal/injection for the first few injections and have an average of at least 5ucal/injection over the course of the titration. This roughly corresponds to a peak height of 0.5ucal/sec. (See also next section on temperature dependence of binding heats.) Volume of injections are usually 3-15uL (see section choosing run parameters for further details.)
Keep in mind that these are "rules of thumb" and not absolute. If $\mathbf{K}_d$ and **n** are unknown, a best guess and perhaps a range-finding experiment should quickly allow the investigator to determine the correct experimental conditions.

Temperature Dependence of Binding Heats

Excerpted from the ITC Expert Manual

The temperature dependence of binding heats is a phenomenon often overlooked by users of ITC. Consequently, experimental studies may be abandoned when investigators measure the binding enthalpy of a reaction at a temperature where the heat of binding is near zero. In such a case, the signal to noise ratio is poor, the binding isotherm is not well defined, and the heats of dilution are comparable to the reaction heats. The investigator may conclude that ITC is not suitable to study the interaction of interest. On the contrary, the temperature dependence of the heat of reaction is a valuable tool for optimizing the study of any binding event.

The heat of binding for a given reaction and the enthalpy change (ΔH) is typically temperature dependent. As you change the temperature of an experiment, the raw injection heat and therefore ΔH, change as well. The temperature dependence is due to the heat capacity change of the event ($\Delta Cp = \Delta H/T$). The value of ΔCp for biological interactions is almost always negative and ranges from approximately 0.3 to 2 kcal/degree/mole. If you collect a series of experiments using the same binding partners at different temperatures and plot the fitted values of ΔH vs. temperature, then the slope of a straight line through the data is the ΔCp. The ΔCp may be used to obtain higher heats of reaction and therefore better data. For example, suppose that you are currently working at 30°C and have obtained a fitted value for ΔH = -4 kcal/mol. Because binding enthalpy is temperature dependent, every degree that you increase the temperature of an experiment will increase the binding enthalpy 0.3 to 2 kcal/mole. The raw heats will increase as well. In this case, if you increase your experimental temperature to 37°C then you should obtain a binding enthalpy between -6.4 and -18 kcal/mole, higher raw heats, greater signal to noise, and a better defined binding curve. This is accomplished simply by changing the temperature of your experiment. You do not need to change the concentrations of your reactants. Alternatively, you could conduct the experiment at a lower temperature. Consider the above example. If the same experiment were performed at 5°C, then the binding enthalpy and raw heats will become more endothermic. If the ΔCp is -1 kcal/deg/mol, then reducing the experimental temperature to 5 degrees will yield a binding enthalpy of +21 kcal/mol. The raw injection heats will be endothermic as well and of greater magnitude than the original exothermic heats observed at 30°C. Since the ΔCp is usually linear and negative, then a measurement of binding enthalpy at two different temperatures will allow prediction of binding enthalpy at any temperature by fitting the data to a straight line.

Heat capacity change of binding is not a new concept. Long time users of MicroCal instruments have used the temperature dependence of binding heats to optimize experiments and obtain additional structural information associated with binding reactions. Most binding reactions have a temperature at which $\Delta H = 0$. If you collect data within 5 to 10 degrees of this temperature, then the heats are almost always going to be low. If you change the temperature of the experiment, then you will increase the heat of the reaction and obtain better signal to noise. Interestingly, many systems have values for ΔH that go through zero between 20 and 30 degrees.

From Alan Cooper (Glasgow):

Why "N" might not turn out as you expect…

"N" is the average number of binding sites per mole of protein in your solution, assuming:

a. all binding sites are identical and independent
b. you have pure protein (and ligand)
c. you have given the correct protein and ligand concentrations
d. all your protein is correctly folded and active…

This is rarely true in practice! Protein (and ligand) concentration determinations depend on the accuracy of the methods used. Protein extinction coefficients, for example, are rarely known better than ±5%, and are usually worse. Poor measurement techniques, incorrect UV baseline corrections, attempts to conserve material using "micro" cuvettes for example can lead to serious errors. Even if all your measurements are dead accurate, not all the protein may be correctly folded (a common experience with recombinant proteins).

Possible cases:

N < 1

- protein concentration is lower than you think, or…
- protein is impure, or…
- protein (polypeptide) is pure but not all correctly folded, or…
- ligand concentration is higher than you think, or…
- simple non-cooperative binding model is inappropriate, or…
- all of the above

N > 1

- your protein has multiple binding sites, or…
- ligand concentration is lower than you think, or…
- simple non-cooperative binding model is inappropriate, or…
- all of the above

And again from: http://www.endocytosis.org/techniqs/ITC.html

Stoichiometry and multiple sites

One of the powerful features of microcalorimetry is the fact that the stoichiometry (N) of binding can be determined directly and simultaneously. On the other hand any error in determining the concentration of the binding partner or the ligand becomes apparent after fitting traces as one expects the molar ratio of the reaction to be an integral multiple of 1. If N deviates by more that 20% of the expected value, one should carefully repeat the measurement of the concentration, ideally with an independent assay (Bradford, OD280, BCA,…). If both concentrations are correct, one can start to think about the fraction of active protein in the sample or about alternative binding models. If only 80% of a protein are active and take part in the reaction, the concentrations can be adjusted before fitting the isotherm to the data. For 2:1 complexes it is advisable to have the binding partner with two binding sites in the sample cell. If the two ligands bind with different affinities, this will be much easier to recognize than in the opposite scenario where the N is 0.5:1.

Choosing ITC Run Parameters

From VP-ITC Users Manual. The comments in red are tidbits gleaned from various MicroCal scientists.

Total Number of Injections will in part be determined by your experimental design and sample concentrations. You will need a minimum of 10-15 injections to define a binding isotherm. *NOTE: Don't stop the experiment until your binding is saturated, otherwise the data are harder to fit.*

Cell Temperature is determined by your experimental requirements. Typically, the binding constant and heat of binding will be temperature dependent, but the stoichiometry of binding will not be. The ITC's operating range is 2-80ºC. *NOTE: It's a good idea to thermostat at the desired temperature while you prepare for your run. Adjust the temperature setpoint under the "Thermostat/Calib" tab. Also remember that your ligand needs to equilibrate to the temperature of the cell before injection (see 'Volume' section below).*

Reference Power setting determines the approximate value that the baseline will settle at when the system is equilibrated. Measuring large exothermic reactions will require a 'large' reference power of about 30 µCal/sec., while large endothermic reactions will require a low reference power setting of about 2 µCal/sec. If you have little information about the expected heats it will be best to use a reference power setting of 10-15 µCal/sec. *NOTE: These values assume that the Analog Input Range = +/- 1.25 volts which is the default value and is adequate for almost all ITC applications. (The A.I. Range is set under the "Setup/Maintenance" tab.) Set the Reference Power so that your Differential Power (DP) remains positive throughout the run. A higher reference power gives more baseline noise, so for optimum sensitivity choose the lowest Reference Power that keeps your data on scale.*

Stirring Speed is typically set at 300 RPM. Faster stir speeds will increase the baseline noise level but may be necessary if solutions are more viscous than water. Also, when binding is extremely tight, a significant error is encountered near the equivalence point if the injected ligand solution does not mix completely throughout the entire volume of the sample cell, so in those cases you may obtain better values using a stir speed of 580 RPM. Finally, when you are studying particulate suspensions, which tend to settle from gravity, more stirring will be needed to keep a uniform suspension.

Volume of injection is generally between 3-15µl. This range ensures high volumetric accuracy while allowing enough time for the injectant to equilibrate to the temperature of the cell before injection. The injectant equilibrates to the cell temperature in the stem prior to reaching the cell. Injections ≥ 15µl may result in a reduction in the repeatability of the *injection blank* heat. The injection blank heat is the thermal energy associated with force of the injection and any temperature differences between the cell volume and the injection volume. A water/water titration will show the injection blank heat. *NOTE: The limiting VP-ITC sensitivity is ~0.1 µcal, so for precise measurement, each injection should have an average of at least 3-5 µCal of heat absorbed or evolved. If the heats are too small, then either the concentration of the reactants or injection volume should be increased.*

Duration of the injection is usually left at the default value, which yields an injection rate of 0.5 µl/sec. Very subtle changes in control peak shapes and sizes can be obtained by varying the injection duration, and is not usually beneficial. *NOTE: 0.5 µl/sec is the slowest rate at which you can inject.*

Spacing between injections is usually 240-360 seconds to allow for reaction and equilibration. *NOTE: The spacing needs to be long enough for your baseline to reestablish before the next injection. Sometimes it takes longer for the ligand to find unoccupied binding sites after some (or most) have been filled, so the equilibration time is longer in the middle of an experiment than at the start. Waiting up to 600 sec. in between injections is unusual, but not unheard of.*