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# Optimizing isothermal titration calorimetry protocols for the study of 1:1 binding: Keeping it simple

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ABSTRACT

*Background:* Successful ITC experiments require conversion of cell reagent (titrand M) to product and production or consumption of heat. These conditions are quantified for 1:1 binding, M + X ⇔ MX.
*Methods:* Nonlinear least squares is used in error-propagation mode to predict the precisions with which the key quantities — binding constant $K$, reaction enthalpy $\Delta H°$, and stoichiometry number $n$ — can be estimated over a wide range of the dimensionless quantity that governs isotherm shape, $c = K[M]_0$. The measurement precision $\sigma_q$ is estimated from analysis of water–water blanks.
*Results:* When the product conversion exceeds 90%, the parameter relative standard errors are proportional to $\sigma_q/q_{tot}$, where the total heat $q_{tot} \approx \Delta H° [M]_0 V_0$. Specifically, $\sigma_K/K \times q_{tot}/\sigma_q \approx 25$ for $c = 10^{-3} - 10$, $\approx 11\ c^{1/3}$ for $c = 10 - 10^4$. For $c > 1$, $n$ and $\Delta H°$ are more precise than $K$; this holds also at smaller $c$ for the product $n \times \Delta H°$ and for $\Delta H°$ when $n$ can be held fixed. Use of as few as 10 titrant injections can outperform the customary 20–40 while also improving productivity.
*Conclusion:* These principles are illustrated in experiment design using the program ITC-PLANNER15.
*General significance:* Simple quantitative guidelines replace the "$c$ rules" that have dominated the literature for decades.



Of all the methods for studying chemical binding in solution, only calorimetry can yield estimates of all three key thermodynamic properties for the binding process — $\Delta G°$, $\Delta H°$, and $\Delta S°$ — from experiments done at a single temperature. For this reason, isothermal titration calorimetry (ITC)[1] has become a preferred method for studying binding of moderate strength in a wide range of applications in a comparably wide number of disciplines. For illustration, a topic search of the Science Citation Index for "ITC OR isothermal titration calorimetry" turned up over 900 published papers in about 300 journals for the year 2014; these figures are about three times those for a similar search I did a decade ago [1].

In an ITC experiment for 1:1 binding, M + X ⇔ MX, one reagent (titrant X) is injected sequentially, with stirring, from a precision syringe into a cell containing the other reagent (titrand M) [2]. Commonly 10–40 such injections are programmed, spaced 4–10 min apart, to permit the instrument to return to baseline after each injection, in which there is incremental conversion of M to MX, producing the signal (usually compensation power) that is integrated to obtain the heat (Fig. 1).[2] By the conclusion of the experiment, the cell should contain an excess of titrant X large enough to ensure conversion of most of the original M to product. The data are analyzed by nonlinear least squares to obtain estimates of the binding constant $K$, $\Delta H°$, and the stoichiometry number $n$ (= 1 for 1:1 binding when concentrations and cell volume are well known).

From this description, a successful ITC experiment requires (1) production or consumption of heat, and (2) conversion of the cell reagent to product. The worker planning the experiment must deal with a number of questions, most important of which is, "Will this work for my reaction?" If the answer is "yes," then one must decide on concentrations for reagents and the number $m$ and volumes for the injections, keeping in mind practical considerations such as reagent expense, solubility, and aggregation. ITC experiment design has been addressed frequently in the literature, usually in the context of getting a suitable value for "$c$," a parameter ($= K[M]_0$, where $[M]_0$ is the initial titrand concentration in the cell) introduced in [2] that governs the shape of the titration profile (heat $q$ vs. X:M ratio). In a recent contribution, I challenged these "$c$" prescriptions as too limiting and addressed a number of misconceptions

*Abbreviations:* ITC, isothermal titration calorimetry; LS, least squares; NLS, nonlinear least squares; SE, standard error; RSE, relative standard error; SD, standard derviation; MC, Monte Carlo.

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[2] This figure also illustrates a systematic error that can occur without warning, with devastating consequences. The great exothermicity of the reaction has driven the compensation power below zero, producing invalid data that appear to be normal.

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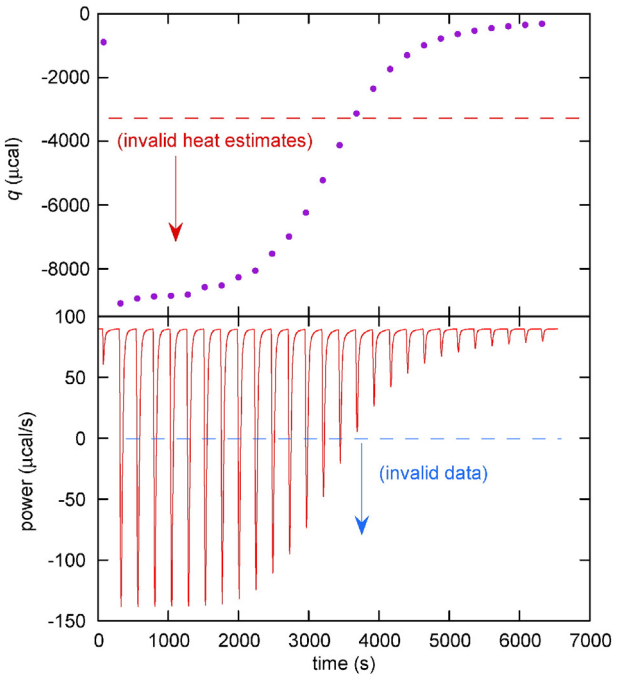


**Fig. 1.** Thermogram (lower) and integrated heats obtained for injecting 0.1 M $BaCl_2$ into 0.01 M 18-crown-6 ether on a MicroCal VP-ITC instrument with cell volume 1.37 ml. The program called for 1 2-µl and 26 9-µl injections. Note that most of the peaks drop below zero compensation power, making these data invalid.

that have persisted over the years [3]. However, many of the latter points are of minor practical significance, and in this work I focus on just the two essentials enumerated above: getting enough heat and sufficient conversion of titrand to product. Regarding the first of these, I showed that over a wide range of $c$ values, one can achieve 5% relative standard error (RSE) in $K$ by obtaining enough heat to give a value of ~700 for the ratio of total heat to measurement precision, $q_{tot}/\sigma_q$. For $c > 1$, $\Delta H^\circ$ is estimated with better precision than $K$, and for smaller $c$, the product $n \times \Delta H^\circ$ is similarly precise. To achieve adequate conversion of M to MX, I earlier obtained the empirical expression [1],

$$R_m = \frac{6.4}{c^{0.2}} + \frac{13}{c}, \text{but at least } 1.1, \qquad (1)$$

for setting the ratio $[X]_0/[M]_0$ of total X to total M in the cell after the final ($m$th) injection.

5% precision is a reasonable practical goal in most ITC work. While much better precision is achievable, in principle, from instrumentation available today, in practice much routine work may have reliability less than 5% from systematic errors. For example, in an interlaboratory comparison study of an enzyme-inhibitor process, 14 experienced laboratories obtained results showing overall standard deviations (SDs) ten times larger than expected from the individual precision estimates [4]. Chodera and I showed that most of this excess dispersion could be explained by concentration errors of order 10% in the preparation of the reagents by the participating laboratories, with additional contributions from baseline errors likely in some cases [5].

Besides heat and product conversion, there is also the question of number of injections. I have shown that the notion, "more points is better" for data analysis, is wrong for ITC in the low-heat limit [1,6,7]. This is because a fixed amount of heat is being subdivided through the $m$ injections, making the relative error in each $q$ value go up as $m$ while the statistical gain goes up only as $m^{1/2}$ [3]. For high-heat processes, this can reverse, because the nature of the data error changes for large $q$ [6–8]; but then the predicted precisions are often unrealistically good when systematic effects are acknowledged. Accordingly, I have recommended the use of 10 injections in most work. This result enhances productivity in studies where many similar reactions are to be run, since 10-injection experiments can be completed much faster than 30-injection ones. Still, in high-heat situations (as in Fig. 1) it may be necessary to use more than 10 injections to stay within the instrument's range limitations. Similar considerations apply to very low-$c$ processes, where variable volumes — small initially, increasing with $m$ — better distribute the heat and yield better parameter precision [3,9].

To plan an ITC experiment, one must have some idea of likely values for $K$ and $\Delta H^\circ$, *e.g.*, from other work on similar processes. Consider a reaction having dissociation constant $K_d = 10$ nM, giving $K = 10^8\ M^{-1}$. This is at the upper limit of ITC's capabilities, so conversion to product will not be an issue. If we use a relatively small $[M]_0 = 1$ µM, we have $c = 100$. Suppose $\Delta H^\circ = 10$ kcal/mol. Then $h\ (\equiv \Delta H^\circ \times [M]_0) = 0.01$ cal/l, and for the widely used MicroCal VP-ITC instrument (from Malvern Instruments since 2014), where the active cell volume is 1.4 ml, $q_{tot} = h \times V_0 = 14$ µcal. Using $\sigma_q = 0.2$ µcal [3], we obtain $q_{tot}/\sigma_q = 70$, and we can anticipate only crude estimates of the binding parameters from this experiment. On the other hand, if we can up $[M]_0$ to 10 µM ($c = 1000$), we will increase $q_{tot}/\sigma_q$ to 700, which is our target for 5% estimation. However, for $c$ this large, somewhat larger ratios are required for this precision, so we may need to settle for ~10% precision. At the other extreme, suppose we have $K = 100\ M^{-1}$. If we can set $[M]_0 = 1$ mM, we have $c = 0.1$, and Eq. (1) calls for a final concentration ratio of 140, which means a titrant concentration of $\sim 5 \times 140 \times 0.001\ M = 0.7$ M, since the total syringe volume is about a factor of 5 smaller than the cell volume. Even a modest $\Delta H^\circ$ will indicate adequate heat in this case, but syringe concentrations this high are impossible in many biological processes, from solubility and other practical considerations. So we need to ask how well we can do with less complete titration, say, with 100 mM titrant concentration.

To facilitate such estimation, I provided in Ref. [3] a program that prompts the user for the kind of information used in these two examples and produces sample results with estimates of parameter precisions. This program utilizes the method of least squares (LS) in error propagation mode [10] — exactly fitting data with known uncertainty. For linear LS on data with Gaussian random error, the parameter standard errors (SE) are exact, meaning that, *e.g.*, Monte Carlo simulations will yield normally distributed parameter estimates having SD within statistical error of these predictions [11]. This result holds also for nonlinear LS (NLS), but only in the limit of small data error. With increasing data error, the parameter distributions deviate increasingly from Gaussian, and these deviations mean that confidence limits may not be simply obtained from the stated SEs. As is discussed below, in ITC this concern is significant for estimates of $K$ at very high $c$, where its reciprocal $K_d$ is the nearly normal parameter, and for $\Delta H^\circ$ at low $c$, where the high correlation between $n$ and $\Delta H^\circ$ makes the product $n \times \Delta H^\circ$ well determined and $\Delta H^{\circ -1}$ approximately normal [1].

In subsequent sections, I review the basis for using LS in error propagation mode, also called experiment design; and I discuss how Monte Carlo simulations support such results for ITC. I then refine and generalize the earlier guidelines, providing a result that permits reliable estimation of the precisions for determining $K$, $\Delta H^\circ$, and $n$ in all low-heat 1:1 binding situations having $c$ in the range $10^{-3} - 10^4$. I briefly discuss the issue of dilution blanks and show how the simplest blank — water into water — can be used to estimate the data error $\sigma_q$ needed for the ratio $q_{tot}/\sigma_q$ that determines the parameter precisions. Finally, I illustrate the program provided for experiment design in [3] on examples from the recent literature. The program itself has been modified to include provision for a fourth parameter in the form of a constant background and is available in this new version in the supplementary material.

Since the present work follows directly from my 2012 paper [3], with a focus on just the most important results discussed there for 1–1 binding, I do not consider here the many earlier contributions to the topic of ITC experiment design, unless their results are specifically

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relevant to the present discussion. Those works have much useful practical advice on the execution of ITC experiments, but on the specific matter of setting key parameters for optimal results, they are often incorrect. The interested reader can find an enumeration of these issues in ref. [3].

## 1. Least squares for experiment design

The great power of the method of least squares for data analysis is that it provides estimates of parameters in the fit model *and* their uncertainties. The most widely used form of LS, sometimes called "ordinary LS" and known to practically all scientists, is unweighted fitting of data to simple linear models, like low-order polynomials. This is also linear LS, not because the fit model is a straight line, but because the model is algebraically linear in the adjustable parameters. By using unweighted LS, the analyst is tacitly assuming that the data have constant error of unknown magnitude; then this data error (SD,σ) is estimated from the fit results using

$$\sigma^2 = S/\nu = \left(\sum w_i \delta_i^2\right)/(m\text{-}p), \tag{2}$$

where $\delta_i$ is the (calculated − observed) residual, $w_i$ is the weight (= 1 for unweighted LS), and the degrees of statistical freedom $\nu$ equals the number of data points $m$ minus the number of adjustable parameters $p$. The estimate of σ then serves as a scale factor in the estimation of the parameter SEs from the covariance matrix **V**, which is normally obtained in solving the LS equations [11]. For correct fit models and valid data, this σ estimate should agree statistically with the measurement precision when this is known (for which we use $\sigma_q$ here for ITC).

But suppose the data error is known in advance. This is always the case in Monte Carlo (MC) simulations, because the computationalist must specify the magnitude for the random error to be added to the true $y$ values for each of the $m$ $x$ values to obtain each simulated data set.[3] It can also be approximately true in experimental situations if the measurement precision has been determined reliably, for example, from abundant data for earlier similar work [8]. Then if we take $w_i = 1/\sigma_i^2$ (allowing for variation of $\sigma_i$ with $x_i$), S becomes an estimate of $\chi^2$ that can be used to check the reasonableness of the analysis; and the **V** matrix (unscaled) yields parameter SEs in linear LS that are *exact* for MC simulations, and reliable to the extent that the $\sigma_i$ are known in the case of experimentally based $\sigma_i$. Further, the parameter distributions are normal (Gaussian), making the estimation of confidence limits straightforward. In NLS, these properties hold only for true models in the limit of small data error. With increasing $\sigma_i$, the parameter distributions become increasingly nonnormal, complicating the estimation of confidence limits.[4] Still, the **V**-based estimates are an appropriate starting point, and these can be obtained by just fitting the true (exactly fitting) data [10]. This is what I have called LS in error-propagation mode, and I used it to generate many of the figures in my earlier works for RSE of $\Delta H°$, $K$, and $n$ from analysis of ITC data for 1:1 binding.

A key such figure is Fig. 6 from [3], the lower portion of which I reproduce here as Fig. 2, for a smaller $[M]_0$ value of 10 μM, as might be more suitable for many biological processes. I also use a more optimistic

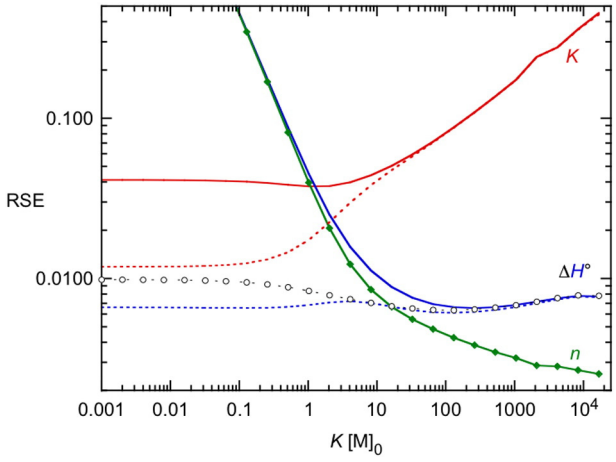


**Fig. 2.** RSE (*e.g.*, $\sigma_K/K$ for $K$) vs. $c$ for $\Delta H° = 10$ kcal/mol, $[M]_0 = 10$ μM, $m = 25$, and $R_m$ set by Eq. (1). The curve with open points is for the product of $n$ and $\Delta H°$ when this replaces $\Delta H°$ in the 3-parameter fits, while the dashed curves represent results from 2-parameter fits with $n$ fixed at 1.0.

value of 0.2 μcal (*cf* 0.8 μcal) for the constant contribution to the data error (see below). The net result of these changes is an upward shift by the factor 2.5, from the 10-fold reduction in heat and the 4-fold increase in sensitivity. This figure includes results for analysis using $n \times \Delta H°$ in place of $\Delta H°$ as the third fit parameter, also results obtained by taking $n = 1$ as known and fitting just the other two parameters. These steps may be necessary to obtain reliable fits for low $c$, because as $c$ decreases below 1, $n$ and $\Delta H°$ become increasingly correlated, making them uncertain and hard to estimate for some data sets. The problem here is the extreme nonnormality of $\Delta H°$, its reciprocal being the nearly normal quantity in this $c$ region [1].

Noteworthy points: (1) In the 3-parameter fits, $n$ and $\Delta H°$ are better determined than $K$ for $c > 1$, while the product $n \times \Delta H°$ is more precise for all $c$; similarly, with $n$ frozen, $\Delta H°$ has smaller RSE for all $c$. (2) 5% determination of $K$ is achievable up to $c = 15$ ($K = 1.5 \times 10^6$ M$^{-1}$), and 10% can be obtained up to $c = 200$ ($K = 2 \times 10^7$ M$^{-1}$); (3) The total heat $q_{tot}$ (not shown) is between 124 μcal and 134 μcal across the full $c$ range of this display, giving $q_{tot}/\sigma_q$ in the range 620–670. (4) These results are for $\Delta H° = 10$ kcal/mol; for larger or smaller $\Delta H°$, just slide the curves vertically by the factor (10 kcal/mol)/$\Delta H°$ to obtain results valid to a good approximation.[5] (5) Changes in $[M]_0$ can be handled similarly; thus, doubling this to 20 μM doubles the heat and lowers the curves by the factor 2 (also altering the relation between $c$ and $K$). (6) The small-$c$ limit assumes moderate-to-high solubility for the titrant; for example, the syringe concentration $[X]_s = 0.66$ M for the results at $c = 0.001$. Reducing the titrant concentration lowers $q_{tot}$ and reduces the precision in $K$; for example (from the program ITC-PLANNER15 illustrated below), 0.22 M increases the % SE from 4% to 7%. (7) Though obtained for $m = 25$ injections, these results remain approximately valid for $m =$ 10–40; see Fig. 2 in [3]. (8) These results are for constant injection volume; for heat-starved reactions at low $c$, variable-volume protocols can reduce the RSE by ~30% (Fig. 9 in [3]).

The scaling properties noted in points (4) and (5) above follow from the dependence of the RSE on just the ratio $q_{tot}/\sigma_q$, so we can convert these results to a completely general form, valid for constant $\sigma_q$ when product conversion exceeds 90%. Fig. 3 shows the product of the

[3] It follows that correct use of MC simulations for prediction of parameter precisions and distributions must employ realistic estimates of the data error σ. Users of MC simulation programs for ITC should be suspicious of data error inconsistency if the MC SDs differ much from the LS fit SEs.

[4] If MC simulations are used for this, one can expect the MC SDs to fall within 10% of the least-squares SE predictions unless the parameter RSEs significantly exceed 1/10, and then only in certain $c$ regimes [6,7]. The need to go beyond the **V** matrix for confidence limits depends also on what confidence limits are desired. For NLS parameters, deviations from the normal distribution are most significant in the wings of the distribution, so they may be important in specifying 90–99% confidence limits but insignificant for 68% (1 σ) limits.

[5] Such shifts would be exact corrections if the data error were taken as constant, but I have used the full variance function [3,8], $\sigma_i^2 = \sigma_q^2 + (\sigma_p q_i)^2 + \sigma_v (q_i/v_i)^2$, where $\sigma_v$ represents the syringe delivery volume uncertainty and $\sigma_p$ is the coefficient of the proportional error. The 2nd and 3rd terms increase in significance with increasing heat $q_i$ and decreasing volume $v_i$, respectively.

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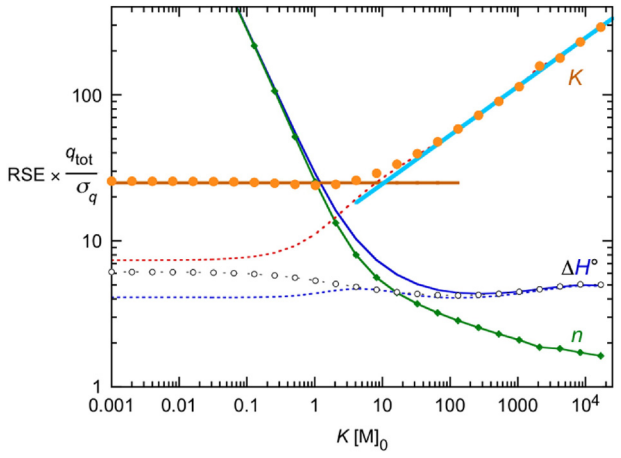


**Fig. 3.** Dimensionless quantities RSE×$q_{tot}/\sigma_q$ as a function of $c$. The straight lines on the $K$ results illustrate the approximately constant behavior at small $c$ (=25) and the cube root dependence for $c > 10$ ($11.4\,c^{1/3}$). Other quantities identified as in Fig. 2.

parameter RSEs and this ratio as a function of $c$, from which we can now predict precision for any conditions. Returning to the example in the Introduction, with 10 μM titrand and $c = 1000$, the $y$ value in Fig. 3 is 114; the heat ratio of 700 yields $\sigma_K/K = 0.16$. For $\Delta H°$ and $n$, the RSEs are <0.01.

Do Figs. 2 and 3 give the best possible precisions for the given $[M]_0$? No, but they represent a reasonable target in most experiments. For example, in Fig. 2 of [3], the standard protocol ($R_m = 2$) outperforms Eq. (1) in setting the titration range for $c > 300$. If we examine the precision as a function of $R_m$ (achieved by increasing $[X]_0$), we get results similar to those found for $c = 5000$ in Fig. 5 of [3], but present already at $c$ values an order of magnitude smaller: a strong oscillatory dependence with RSE varying by a factor of 4 (see Fig. 4). The inset titragram shows what is happening: As the titrant concentration is increased, the titration is completed at progressively smaller numbers of injections — 6 in the case of the minimum near $R_m = 9$; this means that the same precision can be achieved by titrating just to $R_m = 2.1$ in 6 injections. In other words, the results are demonstrating a preference for fewer injections. At the same time they are showing a sensitivity to just where these aliquots fall on the stoichiometry axis. Eq. (1) gives a median precision with minimal titrant demand ($R_m \approx 2$), without the risk of hitting

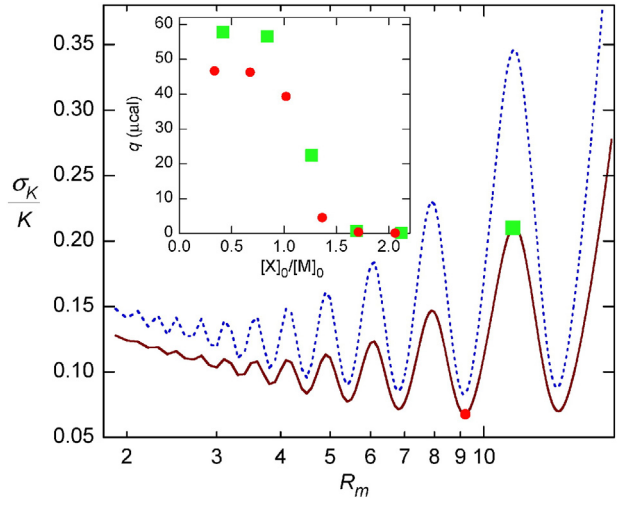


**Fig. 4.** RSE in $K$ as a function of $R_m$ for $c = 400$ (solid) and 700 (dashed). The inset shows the heats as a functon of titration stoichiometry for the two marked points on the solid curve. Other parameters as in Fig. 2.

a peak in Fig. 4. On the other hand, the similarity in the curves for $c = 400$ and 700 shows that one does not need precise information about $K$ to design a small-$m$ protocol that can lower the RSE in $K$ by a factor of ~2 at large $c$.

When Fig. 3 predicts precisions of order ~1%, those estimates will likely be optimistic, for several reasons. Such precision can come only from "heat-rich" experiments, and for those, the data error will include significant contributions from proportional error (*i.e.*, error proportional to $q$; see note 5). More importantly, realizing such precision requires great care in preparing solutions to known concentrations — a task that can be difficult for many biological systems [5]. In any event, predictions of high precision mean that Fig. 3 has achieved its purpose: The experiment should succeed easily, and the data analysis will provide the actual precision estimates.

Since Fig. 3 is based on getting ~90% product conversion, its predictions will be optimistic for low-$c$ processes when titrant properties limit $[X]_0$ to values that cannot achieve this conversion. Also, Figs. 2 and 3 cover just the standard 3-parameter fit model. It is common practice in some labs to include a 4th parameter in the form of a constant background, to account for dilution heat and instrumental effects. This must invariably reduce the precisions of the other parameters, but the precision losses depend on the magnitude of the background, so they cannot be accommodated in a general way. This complication is discussed further below, including in the illustration of the revised version of the program ITC-PLANNER.

When the data have been collected, they will still need to be analyzed with an appropriate NLS code to obtain best estimates of the parameters and their uncertainties. For the latter, there is also the matter of confidence limits and the question whether MC simulations are needed (as are offered in some analysis packages [12,13]). I have previously used MC simulations to examine the parameter distributions from analyses like those leading to the results in Fig. 2. In simplest terms, at very large $c$, $\Delta H°$ and $n$ are close to normal, while $K$ becomes biased and nonnormal, with its reciprocal ($K_d$) being close to normal. At the other extreme, $K$ and $n$ (and $n \times \Delta H°$) are near-normal but $\Delta H°$ is strongly nonnormal, with its reciprocal now being the near-normal variate. Specifically, distribution results for $K$ and $K_d$ were shown for $c = 1000$ in [6,7] and for $\Delta H°$ and its reciprocal at $c = 0.1$ in [1]. For most applications, 1-σ confidence limits obtained from the parameter SEs should suffice, but should be applied symmetrically to $K_d$ at very large $c$ and to $\Delta H°^{-1}$ below $c = 1$ (using the same RSEs for the reciprocals, from error propagation). This will yield asymmetric confidence limits, the degree of asymmetry being greater the greater the RSE. For example, if the analysis yields $K = 1.00\,(16) \times 10^7\ M^{-1}$ (16% SE) at large $c$, take $K_d = 100\ (16)$ nM and invert 84 nM and 116 nM to obtain $-0.14 \times 10^7\ M^{-1}$ and $+0.19 \times 10^7\ M^{-1}$ as the 1-σ confidence limits on $K$.

## 2. Product conversion and titration range

The main drawback to what I called the "standard protocol" in [3] is the failure to add enough titrant to the cell to achieve adequate conversion of titrand (M) to product. The default titration in much work has been to two equivalents of titrant, likely stemming from displays of $q$ vs extent of titration that show the familiar sigmoidal shape, with a clear inflection point at the stoichiometry ratio $R \equiv [X]_0/[M]_0 \approx 1$. Such reactions are clearly "complete" at $R = 2$. But as $c$ decreases, this shape changes, with the inflection point moving to smaller $R$ and eventually disappearing altogether at $c = 1$ (see below). Correspondingly, the titration range must be increased to achieve sufficient conversion. This need is incorporated in Eq. (1), which I obtained in [1] by conducting computations like those behind Fig. 2 for a wide range of values of the key parameters and the reagent concentrations. But similar results can be obtained more easily, just by examining the relation between $R$ and the product conversion fraction $f \equiv [MX]/[M]_0$ as a function of $c$.

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The equilibrium expression is

$$K = \frac{[MX]}{[M][X]} = \frac{[MX]}{([M]_0 - [MX])([X]_0 - [MX])} = \frac{f}{(1-f)([X]_0 - f[M]_0)} = \frac{f}{(1-f)(R-f)[M]_0} \quad (3)$$

Multiplying by $[M]_0$ gives $c$ on the left, and the resulting quadratic equation for $f$ can be solved for $f$ as a function of $R$ and $c$. I did this for Fig. 4 in [3], but it is instructive to solve for $R$, obtaining.

$$R = f + \frac{f}{(1-f)c}, \quad (4)$$

from which 90% product conversion requires $R \approx 1$ for $c = 100$, but $R \approx 10$ for $c = 1$ and $R \approx 9/c$ for $c = 0.1$ and smaller, where the 2nd term in Eq. (4) dominates. So, while the sigmoidal profile is not necessary for good results in ITC, significant product conversion is still required, and this can mean huge titration ranges as $c$ decreases below 0.1. As I have already noted, a titrant concentration of 0.66 M is required for $c = 0.001$ in Fig. 2; that would be unacceptably large for many biological processes. Note that increasing $c$ by increasing $[M]_0$ cannot solve this problem: The initial concentration is proportional to $R \times [M]_0$ and remains the same; thus the required $[X]_0$ becomes a function of just $K$, becoming $13/K$ when Eq. (1) is used [1].

Eq. (3) and its treatment suggest that displays of $q$ vs $R$ for constant-injection-volume protocols should be determined only by $c$ and $\Delta H°$, a result that was obtained early in the development of modern ITC instrumentation [2]. Although the data are properly analyzed as integral heat from stepwise changes in the concentrations with each injection [3,14,15], the interpretation of Eq. (3) as a continuous function of $R$ is useful in discussing the shapes of the profiles for constant-$v$ experiments. Then the production of heat is proportional to $df/dR$, the slope of which is $d^2f/dR^2$. This slope achieves its minimum (largest negative value) at the inflection point, obtained by solving $d^3f/dR^3 = 0$. The solution is $R = 1 - 1/c$, from which the inflection point falls close to the stoichiometric end point for large $c$ but shifts down with decreasing $c$, becoming $R = 0$ at $c = 1$, meaning there is no inflection point for $c < 1$.

## 3. The role of blanks

A key quantity needed to predict parameter precisions is the data error or measurement precision $\sigma_q$. Since the predictions are of most concern for low heat, we need only the constant contribution to the variance function [8], since this dominates at low signal. The simplest way to estimate this quantity is from a series of low-heat injections expected to have constant $q$, and an expedient choice is a water–water blank, since these are often recorded in multiuser labs to confirm that the cell and syringe have been adequately cleaned after a series of experiments. Fig. 5 shows results from 25 10-μl injections at 3-min intervals. The heats are shown below. The resulting $\sigma_q$ is 0.06 μcal, which is smaller than the 0.2 μcal used in the earlier predictions. The 0.06 μcal is commensurate with many other estimates from similar data; however, baseline estimation can be a greater source of uncertainty in heat estimation for the more energetic peaks from experiments like those modeled in Fig. 2, which is why I used the more conservative 0.2 μcal there.[6]

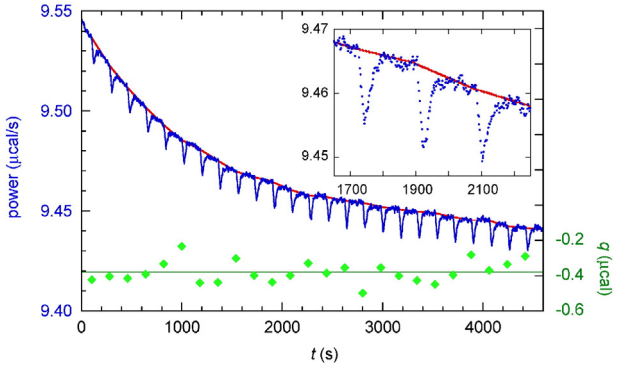


**Fig. 5.** Water–water blank obtained for 25 10-μl injections at rate 0.5 μl/s on a VP-ITC. The piecewise linear estimated baseline is included (see inset), as are the integrated heats (scale right). The latter have mean −0.380 μcal and standard deviation 0.061 μcal.

Keller and coworkers have presented a new algorithm for estimating baselines and peak areas and have demonstrated its performance on several 1:1 binding reactions [16]. Although they do not state actual $\sigma_q$ values, their results imply a value of 0.2 μJ, which is surprisingly close to my value from Fig. 5, even though their experiments employed the MicroCal ITC200, which is putatively more sensitive than the VP-ITC used for Fig. 5.

In [8] I estimated the data variance function directly from binding data for 35 different experiments on $Ba^{2+}$ + 18-crown-6 ether complexation [17]. Such estimates are sensitive to any systematic errors, including fit model inadequacies, so they represent upper bounds on the true data error. The constant contribution $\sigma_q$ obtained in [8] was 0.77 μcal; however the analyzed data were the differences between reaction peaks and titrant-into-water dilution peaks, so the $\sigma_q$ for these individually should be smaller by $\sqrt{2}$, giving 0.54 μcal. The data were also collected using the less sensitive scale of the VP-ITC, which is expected to give a larger data error.

Biological binding studies are commonly run in buffered solutions, matched in cell and syringe. If they are truly matched in ionic strength, the blanks — titrant into buffer, buffer into cell containing buffer and titrand, and buffer into buffer — should all resemble the water–water blank. However, buffer mismatches can yield significantly larger heats. As I noted earlier, some workers add a constant to the fit model to allow for such mismatch. But I would suggest also recording at least the titrant-into-buffer blank to confirm that this fit constant is reasonable. Adding a constant to the fit model must reduce the precision of the other parameters. For example, if this heat is taken as 1 μcal per injection in Fig. 2, where the total reaction heat is ~130 μcal, the RSE in $K$ rises by ~50% at small $c$; at large $c$, the precision loss can be offset by increasing the titration range so that late injections give essentially just this heat.

When ionic reagents are reacted in water, the dilution heat can amount to a significant fraction of the net reaction heat, and it can vary strongly with injection number and temperature. Fig. 6 shows results for $Ba(NO_3)_2$ at several temperatures, of relevance to experiments done in [17]. With such behavior, one must correct the reaction heats peak by peak, a procedure that can be shown to properly account for most dilution heat effects. Significant dilution heats can come also from mismatches in the concentrations of additives like glycerol and DMSO, used to improve solubilities in some biological work. These behaviors reinforce the need to record dilution blanks rather than just add a constant to the fit model in analysis.

## 4. Minor issues

As I have noted, other matters addressed in [3] are less important for successful ITC than heat production and product conversion, but some are easily fixed so worthy of mention here. One of these is the "throw-away" first injection, which has long been incorporated in protocols to compensate for observed heat shortfall in the first injection. This effect is mainly due to backlash in the syringe drive screw, which amounts

[6] The $\sigma_q$ estimate decreases as more points are used to define the baseline, for example to <0.03 μcal in Fig. 5 when 50 points on each side of each peak are fitted to a straight line in place of the 10×2 used in Fig. 5. With energetic peaks, typically fewer points are at baseline level, or, if long injection intervals are used, are temporally extended, making the baseline function less reliable for the peak.

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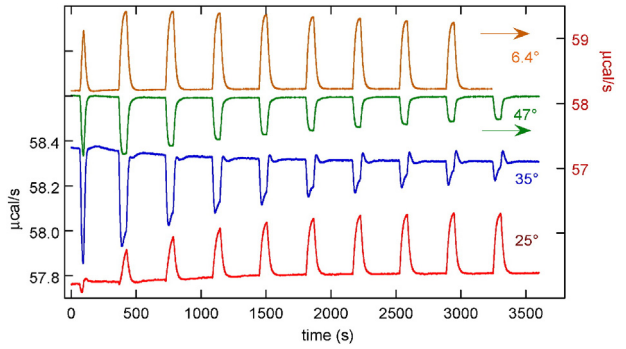


**Fig. 6.** Thermograms for injection of 19.2-mM $Ba(NO_3)_2$ into water at several temperatures (°C) on a VP-ITC. Each injection sequence involved one 10-µL and 9 20-µL injections, at a rate of 0.25 µL/s. Note that the ordinate scale for display of the upper two traces (to right) is compressed by a factor of 3.5 relative to that for the lower two.

to about 1 µl in the VP-ITC [18]. That, for example, could give a ~2% error in a high-$c$ process if the stoichiometric end point falls near 50 µl of injected titrant. The fix is easy: Execute a small downward motion of the screw before mounting the syringe in the instrument. There is still some loss of titrant — perhaps 0.1–0.2 µl — during the long stabilization period prior to the start of injections, so discarding the first point may still be wise. But the downward motion should anyway be included, to ensure that the calculated reagent concentrations are valid.

A second matter, requiring more effort, is the true volume $V_0$ of the active region of the cell. This is best checked using a reaction of moderate to high $c$ for which the reagents can be prepared to precisely known concentrations, like the $Ba^{2+}$ + crown ether complexation. In this way I found $V_0$ errors as large as −5% of the stated value [3,19]. Moreover, $V_0$ can change when syringes are replaced or remounted: In a recent reanalysis (unpublished) of the data recorded in [17], I found that the two sets of experiments performed 3 months apart had $V_0$ values differing by 1%, and with allowance for this and the temperature calibration error [15], the calorimetric and van't Hoff estimates of $\Delta H°$ now agree statistically over most of the 40° range of the experiments.

The just-mentioned temperature error was discovered in a check of the instrument's temperature calibration with a small thermistor. Such devices are easy and cheap to fabricate but require careful calibration. A recent repetition of the earlier measurements with a newly calibrated thermistor returned the same results, amounting to 2 K error in $\Delta T$ over the ~40 K range of the measurements (and hence giving 5% error in the van't Hoff treatment of the $T$ dependence of ln $K$).

## 5. Designing ITC experiments with ITC-PLANNER15

This program was provided in the supplementary material in [3], as .exe modules produced by Microsoft FORTRAN (usable only on 32-bit PC machines) and by GNU (for 32- and 64-bit PCs). However, that version incorporated only the 3-parameter fit model. I have now included provision for a 4th parameter in the form of an additive constant heat for each injection. The program is "clunky" by modern standards, in that it operates in a text window rather than a graphical user interface. However, it does have the advantage of being "linear," meaning the user is prompted for all input. I will illustrate it here on the CAII + TFMSA reaction in Fig. 4 of [16], using the concentrations and other parameters used there.

The program starts with values for the data variance function from [8] and certain default values for $V_0$ (1.4 ml), $m$ (10), and the (constant) injection volume based on experience with the VP-ITC. The user may change any or all of these. These points are best appreciated by viewing the sample results in Fig. 7; the output files included in the Supplement provide additional information. Note that the energy units are cal, as used by the VP-ITC to produce the .itc file.

```
                              ...
Var fn params, sq(ucal), sp, sv(ul) =    .80   .0024   .015
Change ? (Y or N)  Y
Enter new sq, sp, sv:  .05,.0005,.005

Var fn params, sq(ucal), sp, sv(ul) =    .05   .0005   .005
Cell V0(ml) =  1.40
  10 Injections of v(ul) =   25.00
Change any of these ? (Y or N)  Y
Enter new V0:  .2
Enter # injections (max 50):  40
Stick with constant injection volume ? (Y/N)  Y
Enter injection v(ul):  1
Cell V0(ml) =  .20
  40 Injections of v(ul) =    1.00
Default fits K, DH, & n.  Add background ? (Y/N)  n
Freeze n ? (Y/N)  n
Fit n x DeltaH instead of DeltaH ? (Y/N)  n
 Enter title (max 80 characters).
Check Fig. 4 in Keller, et al. (AC 2012).
Enter concentration (mM) of titrand M:  .04
Enter values of n, K(M^-1), DeltaH (cal/mol):  1,5e7,10000
c (= K*[M]0) =  2.000E+03  giving Rm =  1.4 and [X]0,0 = .253
Change [X]0,0 ? (Y or N)  y
Enter new [X]0,0 (mM):  .443
  #      v        q       sig        wt
  1    1.00     4.43       .05    333.89
  2    1.00     4.43       .05    333.90
                              ...
 17    1.00     3.65       .05    352.49
 18    1.00     1.59       .05    389.98
 19    1.00      .35       .05    399.50
                              ...
 39    1.00      .00       .05    400.00
 40    1.00      .00       .05    400.00
    K(1/M)       DH(cal/mol)          n              back
  5.00000E+07   1.00000E+04   1.00000E+00   0.00000E+00
   Standard Errors
  4.95435E+06   3.23054E+01   1.23796E-03   0.00000E+00

c (= K*[M]0) =  2.000E+03
Actual titration range (Rm) =      2.46
Total q(ucal) =    76.35
Net rx q(ucal) =    76.35

Repeat but change concentrations ? (Y/N)  n
Repeat for new parameters ? (Y or N)  n
 Another process, same instrumental params ? (Y or N)
y
                              ...
```

**Fig. 7.** Text window for interactive input and output from the program ITC-PLANNER15 for the example of Fig. 4 in [16].

I have changed the variance function parameters to keep $\sigma_q$ nearly constant at 0.05 µcal and have altered $V_0$, $m$, and injection volume $v$ to match values in [16]. The options to freeze $n$ and to fit $n \times \Delta H°$ are pertinent only at low $c$ so are declined here. After the concentrations are entered, the program produces output for each injection (truncated in Fig. 7) and final results. We see that both $n$ and $\Delta H°$ are obtained with RSEs well below 1%, while $K$ is determined to 10% (1 σ), increasing to 10.5% when a 1 µJ background is added (not shown here; see supplement). In [16] the stated 95% confidence limits on $K_d$ (which is closer to Gaussian than is $K$) are about 35% of $K_d$, whereas from earlier MC simulations, I estimate <25% of $K_d$ from the present 4-parameter results. This discrepancy is greater than expected; its causes are being investigated.[7]

[7] My reanalysis of the data shown in Fig. 4 of [16] confirms the uncertainty estimates given there but yields an average data error of 0.075 µcal. This is 50% larger than assumed in the simulations and fully accounts for the discrepancy. More importantly, the data in question show a heat shortfall for injections 1 *and* 2. This is a clear indication of a backlash volume error, which is found to be 1.3 µl when the data are analyzed with a model that includes this correction. That amount is comparable to that found previously for a VP-ITC, but it is more significant for the much smaller cell of the ITC200, because the stoichiometric end point is typically reached with much smaller total titrant volume — about 16 µl in this case. The effect on the estimated values of $K$ and $n$ is about 8%, which is much greater than expected for typical VP-ITC experiments. Accordingly, the prior "down" motion of the syringe becomes a more important part of the procedures for experiments in the ITC200 and perhaps also in other small-$V_0$ instruments.

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Since this is a low-heat situation, it is interesting to see how we might do with fewer injections. Changing only $m$ to 14 and $v$ to 3 μl, we obtain a small increase in the $K$ RSE, to 11%. But if we follow Eq. (1) and set the syringe concentration to 0.24 mM, we get 9% RSE. Dropping both concentrations by a factor of 2 (roughly as in Fig. 5 of [16]), we find an increase to only 13%. Another factor of 2 dilution gives 19%. This ~2-fold loss of precision with a 4-fold reduction in $q_{tot}$ from dilution stems partly from the $c^{1/3}$ dependence at large $c$ in Fig. 3; but the sensitivity to titration range shown in Fig. 4 also plays a role. For example, I was able to reduce to 12% the RSE in $K$ for $[M]_0 = 0.01$ mM by using 7 5-μl injections. Compared with the original 40-injection program, the small-$m$ schemes give better results in ~1/3 the time. Results for these calculations are included in the supplement.

## 6. Conclusion

The hand-waving $c$-based suggestions for designing ITC experiments are replaced with simple guidelines that permit quantitative feasibility predictions for 1:1 binding. These predictions can be translated into detailed experiment design using the program ITC-PLANNER15, which permits the user to explore any combination of concentrations for titrant and titrand, with the stoichiometry parameter $n$ either fitted or frozen, and now with allowance for a 4th parameter for a constant background.

The simple role of $\sigma_q/q_{tot}$ in determining ITC parameter precisions has interesting consequences relative to instrument cell size. Since $q_{tot} \propto V_0$, if $\sigma_q$ doesn't track $V_0$, users will lose precision on turning to smaller cell size. For example, preliminary measurements of water–water blanks for the MicroCal auto-ITC200 indicate $\sigma_q$ comparable to that for the VP-ITC (Chodera, *et al.*, unpublished). Since $V_0$ changes by a factor of 7, this means loss of parameter precision with the ITC200. This can be true even for a fixed amount of titrand, since dilution preserves $q_{tot}$; but for $c > 10$ in Figs. 2 and 3, the move to lower $c$ on dilution also means better precision for $K$, the least precise parameter (but reduced precision for $\Delta H°$ and $n$).

## Conflict of interest

There are no conflicts of interest by the author in the preparation of this work.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2015.10.011.

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