Avoiding accuracy-limiting pitfalls in the study of protein-ligand interactions with isothermal titration calorimetry

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Abstract

Isothermal titration calorimetry (ITC) can yield precise (±3%) estimates of the thermodynamic parameters describing biomolecular association (affinity, enthalpy, and entropy), making it an indispensable tool for biochemistry and drug discovery. Surprisingly, interlaboratory comparisons suggest that errors of ∼20% are common and widely underreported. Here, we show how to reduce precision- and accuracy-limiting errors while obtaining good estimates while minimizing material and time consumed by an experiment. We provide a simple spreadsheet that allows practitioners to identify precision-limiting operations during protocol design, track precision during the experiment, and propagate error to yield realistic final uncertainties.
Keywords: isothermal titration calorimetry (ITC), propagation of error, entropy-enthalpy compensation

Isothermal titration calorimetry (ITC) [1] is a popular technique for probing phenomena of biological interest, including protein-ligand interactions. While the method consumes more reagents than optical or spectroscopic techniques, it does not require specific labeling of the system under study, and a single experiment can yield estimates of all thermodynamic parameters[2] characterizing a reaction—the association constant K (and hence standard binding free energy $\Delta G = RT \ln K$), the enthalpy change ΔH, and the reaction standard entropy ΔS (henceforth omitting superscripts). With careful work on a well-behaved system, relative standard errors (RSEs) of 1-3% are regularly achievable [2]. However, in a large-scale survey (ABRF-MIRG'02) in which 14 core ITC facilities studied the association of carboxybenzenesulfonamide (CBS) with bovine carbonic anhydrase II (CAII), the variation among reported binding constants and enthalpies was more than an order of magnitude larger than the standard errors reported by the participants [3]. This unexpectedly large variation has been attributed mainly to errors in titrant (syringe reagent) concentration, which is treated as exact in standard analysis procedures [4]. Failure to propagate these errors into reported results can lead practitioners astray in the interpretation of their data, especially if differences in ΔG or ΔH are of interest—for example, within a structure-activity relationship (SAR) series or in interpretation of enthalpic (ΔH) and entropic ($-T\Delta S$) contributions to binding [5, 6].

We therefore strongly advocate that practitioners report the method by which the titrant is prepared, the uncertainty in titrant concentration, and the resulting total error in thermodynamic parameters including titrant uncertainty in all reports of calorimetric measurements. Otherwise, it *must* be assumed that the reported K, ΔH, and ΔS are contaminated by errors up to 20%, the best estimate of this unreported error available to date [4]. To aid practitioners in reducing and reporting error, we discuss accuracy-limiting steps in solution preparation and provide a spreadsheet[3] for automatically tracking uncertainties and propagating their contributions to produce realistic error estimates.

For illustration, we consider the target reaction from the ABRF-MIRG'02 survey [3], the 1:1 association of CBS and bovine CAII, which can be written,

$$M + X \overset{K}{\rightleftharpoons} MX, \tag{1}$$

where M denotes macromolecule and X ligand. This reaction has a $K_a \sim 10^6$M and $\Delta H \sim -10$ kcal/mol [3, 4].

As both protein and ligand may be precious, there is a desire to minimize material use in protein-ligand studies. Using concentrations only as large as necessary also minimizes the need for buffer additives such as DMSO to enhance solubility, reducing agents to prevent crosslinking, and detergents to inhibit aggregation. These additives pose additional experimental challenges, as calorimetrically-measured heats can be sensitive to even small composition mismatches between cell and syringe solutions. Minimizing these effects requires dialysis of the macromolecule by buffer followed by preparation of the ligand in the dialysate. If the ligand is already in solution (e.g. in DMSO stock), it may not be possible to fully eliminate excipients, leading to potential heat effects due to buffer mismatch even if attempts are made to match compositions [7].

In the ABRF-MIRG'02 survey [3], participants employed titrand (cell reagent) concentrations $[\mathrm{M}]_0$ in the range 7–71 μM.

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[2] All thermodynamic quantities refer to their standard forms.

[3] Available online at http://simtk.org/home/itc-worksheet.

We used an ITC protocol design program [8], which indicated ~3% relative squared error (RSE) in K and ~1% for ΔH was possible with our instrument (a GE/MicroCal VP-ITC) using $[\mathrm{M}]_0 = 10\ \mu$M (consuming ~0.5 mg protein per experiment). While this gives $c = K[M]_0 \approx 10$, a key ITC parameter [1], in the low range of that recommended by standard protocols, high measurement precision can still be obtained at this c value by titrating to an optimal titrant:titrand ratio R_m given by,

$$R_m = \frac{6.4}{c^{0.2}} + \frac{13}{c}, \tag{2}$$

a heuristic expression[4] obtained from a comprehensive study of precision as a function of R_m [9]. The suggested $R_m = 5.3$ is significantly greater than the $R_m = 2$ that is widely used in standard protocols for ITC; with decreasing c, use of $R_m = 2$ progressively limits the fractional conversion of M to MX and thus limits the precision of estimation for both K and ΔH [8]. In the present case, use of $R_m = 2$ would cause significant precision loss, almost doubling the achievable RSEs for K and ΔH.

The same optimization study [9] demonstrated that the experimental precision depended only weakly on the number of injections m, recommending $m = 10$ for processes confidently known to involve 1:1 complexation. This is in sharp contrast to ~30 injections often recommended by standard protocols in order to visualize a full sigmoidal (S-shaped) curve in the enthalpogram, which would unnecessarily limit precision here by reducing the heat per injection (increasing RSEs to 19% and 4%, respectively), as well as increasing the duration of the titration experiment nearly three-fold [9]. Using 10 injections, each of volume $v = 10\ \mu$L, we can compute the approximate syringe concentration $[X]_s$ required from the relevant volumes. For perfusion-type instruments, assuming instantaneous mixing (appropriate for most reactions [10]), this is given by,

$$[X]_s = R_m[M]_0\left[1 - \exp\left(-\frac{mv}{V_0}\right)\right]^{-1} \approx R_m[M]_0\frac{V_0}{mv} \tag{3}$$

where V_0 is the cell active volume (~1.4 mL for the VP-ITC) and the approximate equality follows if the total titrant injected is small compared to the cell volume ($mv \ll V_0$). For our experiment, Eq. 3 suggests we should use a purity-corrected titrant concentration $[X]_s \sim 720\ \mu$M.

Our GE/MicroCal VP-ITC instrument has a syringe assembly that utilizes a worm gear which, after the recommended purge-refill process, will cause a titrant shortfall in the first injection unless a "down syringe" command is issued prior to loading the syringe into the sample cell [11]; we therefore executed a 10 μL "down syringe" command immediately after the purge-refill cycle. Because the instrument can take a substantial period of time to stabilize at the desired experimental temperature after loading the syringe, significant (> 0.1 μL) diffusive loss can also contribute to a first injection shortfall. We therefore programmed an initial 1 μL "throwaway injection" to avoid the need to correct for diffusive titrant loss during the first 10 μL injection. The contribution from this initial 1 μL "throwaway" injection was excluded from the fitting procedure during analysis.

The titrand solution, bovine CAII (Sigma-Aldrich, cat no. C2522, ~29 kDa[JDC: Check MW.], Lot No. 071M6261) in PBS buffer, was prepared following the assay conditions outlined by Myszka et al. [3]. Briefly, the contents of the glass vial containing ~ 5 mg of lyophilized CAII were resuspended in 750 μL filtered buffer and dialyzed overnight in 1 L buffer using a Novagen D-Tube Dialyzer MWCO 3.5 kDa (Cat No. 71506-3, Lot D00131446). The recovered protein was spun for 30 min at 16300 RPM with no visible precipitate observed. The dialysate was filtered again and used to prepare both titrant and tetrad to minimize buffer mismatch heats during the ITC experiment.

The protein concentration was determined spectrophotometrically via absorbance at 280 nm on a NanoDrop ND-1000. The NanoDrop (and similar instruments) utilize small sample volumes (3 μL was used here) and a 1 mm path length such that even high protein concentrations can be quickly read without the need to perform intermediate dilutions to ensure the absorbance falls in the linear range of Beer's law on standard 10 mm path-length spectrometers. The measured absorbance of 11.77±0.03 indicated a protein concentration of 235.0±0.6 μM (using the molar extension coefficient ($\epsilon_{280\,\mathrm{nm}} = 50070\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$ [3]). [JDC: Should we include the error in ϵ here? Or perhaps we should drop the error bars since high precision isn't required?] The sample was then diluted to $[\mathrm{M}]_0 \approx 10\ \mu$M using the purity-corrected post-dialysis concentration. Note that, because the site parameter n absorbs errors in $[\mathrm{M}]_0$ in standard least-squares data analysis [8], high precision is not required for these steps unless the stoichiometry is unknown.

By contrast, care must be taken to minimize inaccuracies in preparing titrant solutions, because the standard data analysis algorithms treat $[\mathrm{X}]_s$ as exactly known. Thus, a 1% error in $[\mathrm{X}]_s$ produces 1% errors in the estimates of K and ΔH [9, 4]. Our titrant (CBS, Sigma-Aldrich, lot #MKBF3323V, 97% purity, MW 201.2) comes as a powder, from which we aim to prepare a solution of purity-corrected[5] concentration $[X]_s \sim 720\ \mu$M using the dialysate. Uncertainties in the true $[\mathrm{X}]_s$ come from at least two sources: the mass of CBS and volume of buffer used in preparing this solution, each of which will be imprecise due to measurement error. Further dilution steps will introduce additional error.

To load the VP-ITC syringe, we require ~2.1 mL of our titrant[6]. For our chosen $[\mathrm{X}]_s \sim 720$, this requires only 0.3 mg of CBS, but given our analytical balance precision (±0.1 mg), this would yield 33% uncertainty in $[\mathrm{X}]_s$, and hence the final relative errors in K and ΔH would be *at least* this large. [JDC: Which model balance was used for this?] To reduce the mass uncertainty to 1%, we must weigh out at least 10 mg. Since

[4] While use of this expression requires a rough estimate of the reaction K and an $[M]_0$ that will produce observable heats, this is currently unavoidable in the practice of calorimetry. In the worst case, a pilot experiment using minimal material can be used to crudely estimate these quantities and Eq. 2 used to determine optimal conditions for a second experiment.

[5] Recall that 97% purity denotes 1 g of powder should contain 0.97 g CBS.

[6] A smaller 700 μL filling tube is also available.

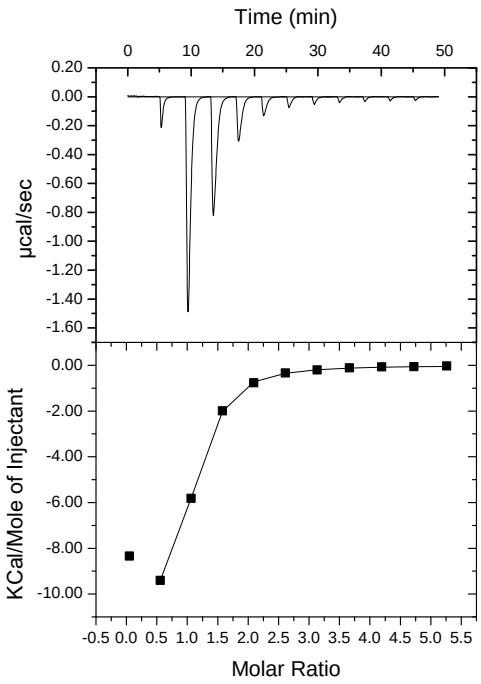

Figure 1: **Titration of CAII by CBS at 25 C.** *Top:* Differential power *vs.* time after blank subtraction and baseline correction in Origin. *Bottom:* Peak integration and 1:1 binding model fit in Origin. Note that no initial injections were discarded. Least-squares fit with Origin gives the following thermodynamic parameters and fit uncertainties: $K_a = 1.20(3) \times 10^6$ M^{-1}, $\Delta H = -11.27(6)$ kcal/mol, $n = 0.915(3)$. Propagation of titrant error with the provided spreadsheet gives updated parameters with realistic uncertainties: $K_a = 1.20(3) \times 10^6$ M^{-1}, $\Delta H = -11.3(2)$ kcal/mol, and $n = 0.915(4)$. See Supplementary Material for complete experimental details and links to download raw data.

the solubility of CBS in water is only 453 mg/L (which corresponds to a 2 250 μM solution), we need a volume of at least 22 mL to dissolve 10 mg. Using a 25 mL Class A volumetric flask or pipette (rated ±0.05 mL) would allow us to attain the desired 1% precision. On the other hand, graduated cylinders and serological pipettes with 25 mL capacity often possess a precision of only ±0.5 ml, which would raise the uncertainty in $[X]_s$ to 2%. Here, we found it convenient to employ multiple liquid transfers with a Gilson P5000 5 mL pipette, which has a stated reliability of ±0.03 mL at full capacity[7].

We chose to prepare a 1 500 μM CBS stock solution as a compromise between ensuring complete solubility of CBS (solubility 2 250 μM in water) and minimizing buffer use (preparing a solution of ~720 μM directly with 10 μg CBS would have doubled the quantity of buffer required). To do this, we added 10.0±0.1 mg [henceforth written 10.1(1) mg] CBS to 32.1(2) mL PBS dialysate and vortexed to ensure the compound was completely dissolved, yielding 32.2(2) mL of a 1 500(20) μM CBS stock solution.

To ensure sufficient ~718.29 μM titrant to allow for a ligand-into-buffer blank titration and additional experimental replications if needed, we planned to prepare 9 mL of titrant solution. This is more than necessary, as minimum of 700 μL/experiment is required for the VP-ITC if the low-volume syringe loading tube is utilized. Using the Gilson P5000, we then added 4.309(12) mL CBS stock to 4.691(12) mL PBS to obtain a 719(9) μM CBS titrant (1% RSE). Error propagation was performed automatically by the spreadsheet (Figure A.2) using standard techniques (discussed below). Note that gravimetric solution preparation—in which the mass of both compound and solvent is used to determine the final concentration—could have been used instead, eliminating the need to ensure all solutions are at the appropriate temperature for volumetric glassware or pipettes. [JDC: Can we say something about volume variation with temperature and how much of a problem this is expected to be?] Automated systems for gravimetric solution preparation and concentration error determination are available, though more commonly used in industrial settings.

Alternatively, we could have determined the titrant concentration $[X]_s$ spectrophotometrically using the known extinction coefficient of CBS at 272 nm (reported as $\epsilon_{272\,\mathrm{nm}} = 1.31(13) \times 10^3$ M^{-1} cm^{-1} [3]). However, since the uncertainty in the absorbance measurement is 1%, the uncertainty in the extinction coefficient ϵ (10%) would dominate the concentration error, resulting in a spectrophotometrically-determined concentration that is uncertain by 10%. Indeed, the concentration we measure in this manner—700(70) μM [JDC: I have to re-measure the absorbance of the latest titrant solution.]—is consistent with that determined by mass and volume, but is an order of magnitude more uncertain; had we chosen to use this spectrophotometrically-determined concentration for $[X]_s$, our final uncertainties in K and ΔH would be at least 10%.

The titration dataset (Figure 1) was analyzed using Origin 7.0 (OriginLab Corp.) after subtracting heats obtained from a separate ligand-into-buffer blank titration utilizing the same protocol. The least-squares (LS) fit of the thermodynamic parameters to the integrated injection heats are shown in the caption. Note that the site parameter n absorbs errors in $[M]_0$ and the cell volume V_0; if the actual concentration of active macromolecule is of interest, these quantities will require more precision [13]. While ΔH is rather insensitive to errors in the stated cell volume V_0 as a result, those errors can have a substantial effect on K, so careful calibration of V_0 using standard reactions (e.g. [2, 10]) is advised if highly accurate quantitation K is necessary [13]. [JDC: Joel, is this statement not sufficient, or do you think we really need to include the V_0 calibration results?]

The error reported by the LS fit only represents the error in model fitting assuming the specified concentration for titrant is *exact*—we must now include the uncertainty in the titrant concentration to obtain an estimate of the true error. The general rule for random error propagation for a quantity $f(x, y, z, \ldots)$ dependent on *independent* measurements

[7] For pipettes, the stated systematic error δ is generally the dominant source of error, and can be considered random error if each pipette is used once. However, if the same pipette is used to deliver *multiple* aliquots, the random error contribution for the total volume transferred should be estimated from summing the systematic error δ for each transfer. For example, if 25 mL are transferred in five transfers of 5 mL using a P5000 ($\delta = 30\,\mu$L), the random error contribution is (5)(0.030) = 0.15 mL.

$x, y, z, \ldots$ gives squared standard error s_f in f as [12],

$$s_f^2 = \left(\frac{\partial f}{\partial x}\right)^2 s_x^2 + \left(\frac{\partial f}{\partial y}\right)^2 s_y^2 + \left(\frac{\partial f}{\partial z}\right)^2 s_z^2 + \cdots \tag{4}$$

where s_x, s_y, and s_z are the standard errors of the corresponding measurements. This form, based on a Taylor expansion of the function f, can be extended to any number of contributing terms (e.g., additional volume contributions if multiple dilutions are used) [12]. If f has a simple form, so that it can be written $f(x, y, z, \ldots) = x^i y^j z^k \cdots$, then Eq. 4 assumes the simple form,

$$\left(\frac{s_f}{f}\right)^2 = i^2\left(\frac{s_x}{x}\right)^2 + j^2\left(\frac{s_y}{y}\right)^2 + k^2\left(\frac{s_y}{z}\right)^2 + \cdots \tag{5}$$

where i, j, and k are the powers to which the independent measurements x, y, and z are raised, and (s_f/f) is the relative error in f. Often, a single term in Eq. 5 will dominate, and the relative error is essentially identical to this contribution. For example, if $f = x/y$ with $(s_x/x) = 4\%$ and $(s_y/y) = 1\%$, then $(s_f/f) = 4.1\% \approx 4\%$.

Provided the relative errors in concentration $[X]_s$ are sufficiently small (<20%) for the Taylor expansion above to be accurate, we can use Eq. 5 to estimate the relative error in the thermodynamic parameters K and ΔH given corresponding uncertainties from the least-squares fit $s_{\mathrm{K,LS}}$ and $s_{\Delta\mathrm{H,LS}}$ and the uncertainty in the titrant concentration $s_{[\mathrm{X}]_s}$ [4],

$$\left(\frac{s_K}{K}\right)^2 = \left(\frac{s_{\mathrm{K,LS}}}{K}\right)^2 + \left(\frac{s_{[\mathrm{X}]_s}}{[X]_s}\right)^2 \;;\; \left(\frac{s_{\Delta H}}{\Delta H}\right)^2 = \left(\frac{s_{\Delta\mathrm{H,LS}}}{\Delta H}\right)^2 + \left(\frac{s_{[\mathrm{X}]_s}}{[X]_s}\right)^2. \tag{6}$$

Since the uncertainty in our $[\mathrm{X}]_s$ is only 1%, the 3% LS fit uncertainty dominates for K; but for ΔH the titrant uncertainty is more important, increasing the RSE from 0.7% to 1.2%. These computations are automatically handled by the spreadsheet, which also computes ΔG and ΔS and their uncertainties.

Since ΔG logarithmically depends on K through the relation $\Delta G = RT \ln K$, the uncertainty in ΔG computed using Eq. 6 (0.02 kcal/mol) is much smaller than that in ΔH (0.15 kcal/mol). If the entropic contribution to binding, $-T\Delta S = \Delta G - \Delta H$ is of interest, its uncertainty can similarly be obtained from Eq. 4, and found to be of the same magnitude as that in ΔH (0.15 kcal/mol)[8].

Comparing our results including the titrant error propagated by the spreadsheet [$K = 1.20(3)\times10^{-6}$ M^{-1} and $\Delta H = -11.3(2)$ kcal/mol] with the best-fit to the ABRF-MIRG'02 results [$K = 1.08(4) \times 10^6$ M^{-1} and $\Delta H = -11.11(4)$ kcal/mol] [4], we see that the agreement with the best-fit values is quite close, differing by only 2.4 and 1 standard errors. The RSEs of our results are quite good—3% in K and 1% in ΔH—and in line with the predicted errors from our initial experimental modeling step.

Notably, had we instead inadvertently made a 10% error in the titrant concentration $[\mathrm{X}]_s$ during its preparation through a precision-degrading step (such as massing out less than 10 mg or using low-precision liquid transfer devices such as serological pipettes or graduated cylinders), this could have easily led to RSEs in K and ΔH of 10% or greater. Although the absolute error in ΔG would remain small (~0.04 kcal/mol), the absolute error in ΔH would be large (~1.1 kcal/mol), making the error in $-T\Delta S$ comparable in magnitude but opposite in sign. This can have important consequences in trying to ascribe significance to differences in entropy-enthalpy compensation within a congeneric series, especially when differences in ΔG are small [14, 5, 6]. We note, for example, that the reported errors in ΔH (and hence $T\Delta S$) for the ABRF-MIRG'02 study were as much as two orders of magnitude smaller than the actual error deduced from variation among independent measurements, and that simply repeating the experiment using the same titrant solution or ligand stock solution (from which the titrant is prepared) would not reveal this variation [4].

[8] Because ΔH and K (hence ΔG are obtained from the same fit—and hence are correlated—cross-terms of the form $2(\partial f/\partial x)(\partial f/\partial y)s_{xy}$ with $x \equiv \Delta G$ and $y \equiv -\Delta H$ must be added to Eq. 4, but because the uncertainty in ΔH is an order of magnitude larger than that in ΔG, it still dominates the overall uncertainty even if these correlation terms are included.

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Appendix A. Supplementary Material

Appendix A.1. Experimental Details

Both the CBS-into-CAII and CBS-into-buffer titrations were conducted using the following protocol in Table A.1. An archive of the MicroCal VP-ITC data files (.itc) generated by these experiments are available as Supplementary Material.

Table A.1: **Experimental parameters for VP-ITC.**

initial "down syringe" following purge-refill	10 μL
number of injections	11
cell temperature	25 C
equilibration time	300 s
stir speed	307 RPM
reference power	10 μcal/s
feedback mode/gain	high
equilibration	fast
initial "throwaway" injection volume	1 μL
subsequent injection volume	10 μL
injection rate	2 s/μL
time between injections	240 s
filter time for power measurements	2 s

Appendix A.2. ITC Spreadsheet

Figure A.2 depicts the spreadsheet (available for for download from Supplementary Material in multiple formats, and online at `http://simtk.org/home/itc-worksheet`) with the details for the CBS-CAII titration experiment reported here filled in.

The spreadsheet is divided into sections corresponding to the different components of a typical ITC experiment. The first section (*Experimental Details*) contains general details of the experiment, the second section (*Ligand*) the details of ligand (titrant) solution preparation, the third section (*Protein*) the protein (titrand) preparation, and the final section (*Thermodynamic Parameters*) the details of the least-squares fit and overall error. Green cells indicate records the user is to fill in during the planning stages of the experiment, yellow cells are filled in by the user during the course of preparing solutions and executing the experiment, grey cells are automatically computed by the spreadsheet to aid the user in experimental design and analysis. Importantly, during both preparation of the titrant and titrate, a "typical error" sets the upper bound for the error the experimenter should be able to achieve. Exceeding this typical error is a clear indication that a precision-limiting step has crept into the workflow.

We stress that the "desired" grey fields specify target values that the experimenter is encouraged to meet as closely as possible, but the practicalities of experimental work often necessitate practical deviations from these goals. The spreadsheet is still able to allow the experimenter to track their actual measurements at each step and propagate error to the final results accordingly.

[JDC: Should we add step-by-step instructions for filling in and using the spreadsheet?]

[JDC: To propagate the error in n, I also used the rule of adding in quadrature:

$$\left(\frac{s_n}{n}\right)^2 = \left(\frac{s_{n,\mathrm{LS}}}{n}\right)^2 + \left(\frac{s_{[\mathrm{X}]_s}}{[X]_s}\right)^2 . \tag{A.1}$$

If this is inappropriate, I should change this—I haven't worked through this yet.]

Appendix A.3. Propagation of pipetting error

[JDC: This section should have more information on propagation of error for pipetting steps and a summary of common pipette errors. I'm still trying to figure out if there is a way to have this done automatically in the spreadsheet, such as having the experimenter list the number of volume transfers of a particular pipette class.]

													Notes
experiment title	VP-ITC CBS-CAII												
date	10/25/2012												
user	John D. Chodera <jchodera@berkeley.edu>												
EXPERIMENTAL DETAILS													
temperature		25	C										
estimated Ka		1.00E+06	1/M										
sample cell volume (approximate)		1.4	mL										
number of injections		10											
injection volume		10	uL										
dilution factor for single injection (d)		0.992857142857143											
dilution factor after final injection (d^n)		0.930824157149801											
		desired						actual		error		percent error	
cell concentration		10.00	uM					10.01	uM	0.09	uM	0.87%	
c-value		10.00						12.01		0.03		0.26%	
optimal Rm		5.34						4.98					
syringe concentration		718.29	uM					718.23	uM	8.69	uM	1.21%	
LIGAND													
compound name	4-Carboxybenzenesulfonamide (CBS)												
description	4-sulfonylbenzoic acid at 97% purity												Color key
vendor	Sigma-Aldrich												fill in before experiment
product no.	000944798												fill in during experiment
lot no.	#MKBF3323V												automatically computed
purity		97.00%											
molecular weight		201.2	g/mol										
solubility		453	mg/L	2,251.49	uM								
Stock solution preparation													
		desired		typical error		typical percent error		actual		error		percent error	
target compound mass	(most balances need min 10 mg)	10	mg	0.1	mg	1.00%		10.01	mg	0.1	mg	1.00%	balance tared to 50 mL Falcon tube + holder near 0.1 mg uncertainty
buffer volume needed for target mass	(for planning buffer usage)	32.14	mL										
buffer volume needed for actual mass	(use this for actual preparation)	32.17	mL	0.64	mL	2.00%		32.17	mL	0.21	mL	0.65%	via P5000: 4x5 mL + 4.130 mL + 2x4.020 mL
purity-corrected stock solution concentration	(be careful not to exceed solubility)	1,500.00	uM	33.54	uM	2.24%		1,500.12	uM	17.90	uM	1.19%	
Syringe solution preparation													
		desired		typical error		typical percent error		actual		error		percent error	
dilution factor from stock to titrant solution		0.479		0.004		0.74%		0.47878		0.00094		0.20%	
stock solution volume		4.309	mL	0.04	mL	1.00%		4.309	mL	0.012	mL	0.28%	via P5000: 4.309 mL
buffer volume		4.691	mL	0.05	mL	1.00%		4.691	mL	0.012	mL	0.26%	via P5000: 4.691 mL
total volume for titrant	(need min 350 uL for VP-ITC)	9	mL	0.06	mL	0.71%		9	mL	0.017	mL	0.19%	
titrant concentration		718.29	uM	17	uM	2.35%		718	uM	9	uM	1.21%	(this error is included in the 'actual error' in thermodynamic parameters below)
PROTEIN													
protein name	carbonic anhydrase II (CAII)												
source	Sigma-Aldrich C2522-25mg												
lot no.													
molar extinction coefficient		50070	M-1 cm-1										
protein purity (or 100% if unknown)		100.00%											
Dialyzed stock solution concentration													
								actual		error			
absorbance measurement for 1 cm path length	(e.g. NanoDrop)							11.768	A	0.03	A	0.25%	used error from spec manual (for 200-350nm)
concentration of stock solution								235.03	uM	0.60	uM	0.25%	
purity-corrected concentration of stock solution								235.03	uM	0.60	uM	0.25%	
Cell solution preparation													Add a section for UV-VIS measurement of protein final dilution
		desired		typical error				actual		error		percent error	
dilution factor		0.04255		0.00058				0.04258		0.00036		0.83%	
stock solution volume		0.511	mL	0.005	mL			0.511	mL	0.004	mL	0.78%	via P1000
buffer volume		11.489	mL	0.11	mL			11.489	mL	0.044	mL	0.38%	via P5000: 2x5 + 1.489
total volume for titrate	(need min 2.1 mL for VP-ITC)	12.00	mL	0.12	mL			12.00	mL	0.044	mL	0.37%	
titrate concentration		10.00	uM	0.14	uM			10.01	uM	0.087	uM	0.87%	(this error is absorbed into the n parameter in the ITC fit)
THERMODYNAMIC PARAMETERS													
		reported from fit		error from fit		percent error from fit		actual		error		percent error	
n (stoichiometry, purity, and V0 correction)		0.915		0.003		0.37%		0.915		0.004		0.42%	Origin fit used purity-corrected protein concentration in cell
K (association constant)		1.20E+06	M-1	2.78E+04	M-1	2.32%		1.20E+06	M-1	3.14E+04	M-1	2.61%	
Kd (dissociation constant)		8.33E-07	M	1.93E-08	M	2.32%		8.33E-07	M	2.18E-08	M	2.61%	
DeltaH		-11.27	kcal/mol	0.05721	kcal/mol	0.51%		-11.27	kcal/mol	0.15	kcal/mol	1.31%	
TDeltaS		-2.97	kcal/mol	0.06	kcal/mol	1.98%		-2.97	kcal/mol	0.15	kcal/mol	5.00%	
DeltaG		-8.30	kcal/mol	0.01	kcal/mol	0.17%		-8.30	kcal/mol	0.02	kcal/mol	0.19%	

Figure A.2: **Spreadsheet for this experiment showing automated propagation of error.** This spreadsheet and blank templates is available for download in multiple formats at http://simtk.org/home/itc-worksheet.