Quick Start: Isothermal Titration Calorimetry (ITC)

Keywords: Isothermal titration calorimetry, experimental design

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INTRODUCTION

Isothermal titration calorimetry (ITC) is a technique used to determine the thermodynamic properties of a chemical or physical equilibria. While the most abundant applications lie in the field of biochemical characterization, its use has increased in other fields because of minimal sample and buffer restrictions. The foundation of this technique is based on the simple measurement of heat, q. Because of the relationship between the change in enthalpy and the change in internal energy (ΔE) of a system, the heat (q) absorbed or released is equal to ΔH when the system is at constant pressure (P) (eqns. 1-3, ΔV = change in volume and w = work).

$$\Delta H = \Delta E + P\Delta V \quad [1]$$

$$\Delta E = q + w = q - P\Delta V \quad [2]$$

$$\Delta H = q_p - P\Delta V + P\Delta V = q_p \quad [3]$$

Heat exchange is present in essentially all physiological processes, making this technique particularly relevant in understanding binding equilibria and other molecular processes. The values extracted under isothermal and isobaric conditions include the stability constant (K), binding stoichiometry (n), changes in free energy (ΔG˚), enthalpy (ΔH˚) and entropy (ΔS˚). This quick start document focuses on the theory of ITC, as well as experimental, instrument, and sample considerations.

THEORY BEHIND ITC

A typical ITC instrument consists of a reference cell and a reaction cell, both made of an inert highly conductive metal (Figure 1) . The cells are located in an adiabatic jacket and are connected by a thermoelectric device (TED) or Peltier device that is sensitive to relative changes in the cells' temperatures and is connected to a feedback power supply.

The temperature difference between the reference and the sample cell is measured and calibrated to a power level displayed in μW. When plotted as exo up, a negative value indicates that this amount of power is removed to maintain steady state. This "feedback" power is used to maintain constant temperature and is somtimes referred to as the differential power (DP) or cell feedback power (CFB). When a constant power is supplied to the sample cell heater, and the thermocouples detect a difference in temperature (ΔT) between the two cells, power is reduced or increased to the sample cell feedback heater proportionally to ΔT, thereby returning to the equilibrium state. When heat is given off in an exothermic reaction, the heater decreases its power to the

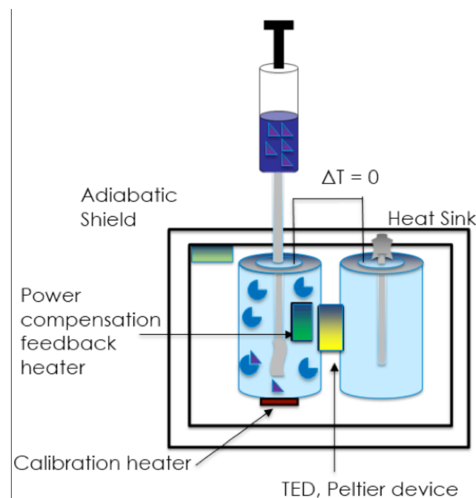


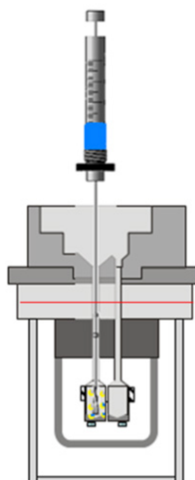
Figure 1: Drawing of an ITC instrument with some control elements

cell to maintain a constant temperature and, depending on the data collection preference, this event can be exo down indicating that the plot is following the heater turning off, or plotted exo up indicating that the plot is following the events in the cell with heat released (up). The opposite, of course, is true for an endothermic event.

INSTRUMENT EXPERIMENTAL DESIGN

SAMPLE CELL

The instrument is designed to run in an overfill mode for optimal control of the signal and pre-equilibration of the titrant. The red line below in Figure 2 shows the approximate fill level of the cell when filled correctly.



At the overfill level, the liquid overlaps with a zone where many signals are collected and regulated, and the connection between the liquid and the large block also enables the block to serve as a heat sink. Under-filling the cells results in a noisy signal as instrument control is not optimal.

Pre-equilibration of the titrant is another reason to overfill and is particularly evident when working at non-ambient temperatures (Figure 3). The overfilled

Figure 2: Drawing of ITC instrument. Red line represents approximate fill volume.

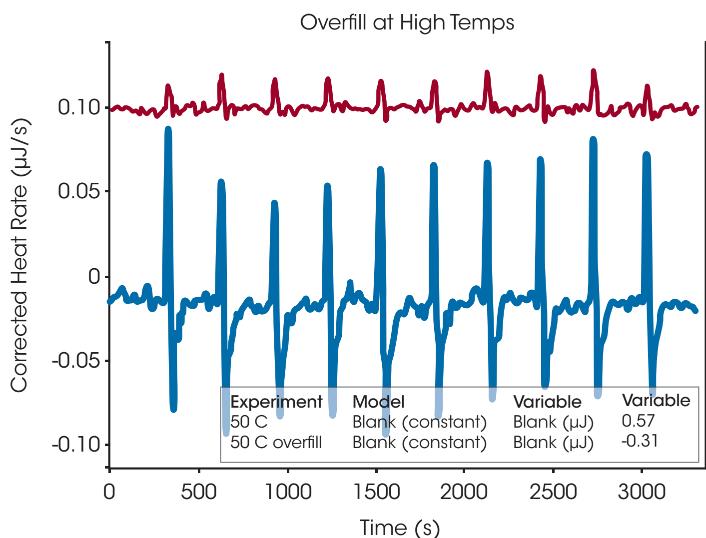


Figure 3: Thermograms showing importance of overfilling. Red data is for an overfilled system.

cell simply shows frictional heat and possibility pressure-volume work, both being exothermic. The under-filled cell shows an endotherm and exotherm, where the endotherm arising from the introduction of the colder solution from the injection syringe.

The overfilled volume, which is identical for the sample and reference, is not the active cell volume that is used in the calculations. If a user wants to accurately determine the active cell volume an analytically prepared chemical system can be used to adjust the volume of the cell until the appropriate stoichiometry is reached (Ref 1).

Instrument	Manual Cell load volume (µL)	Active cell volume (µL, approximate)
Nano SV	1265 - 1375	950
Affinity SV	1300 - 1375	965
Nano LV	300 - 410	170
Affinity LV	300 - 410	180

Table 1. Cell fill volumes and active cell volumes for TA ITC Instruments with the titration syringe in place.

REFERENCE CELL

For most titrations, filling the reference with water sufficiently balances the heat capacity of the sample cell. Loading with buffer in the reference is often avoided for cleaning reasons as well as instrument response being similar whether buffer or water is in this cell. An exception is when organics are used, and under these conditions, the same percent organic should be added to the reference cell. Reference solutions should be exchanged with fresh solution at least once a week. However, when operating at an elevated temperature or with volatile solvents, replacing the solution may be needed more frequently, such as once per day.

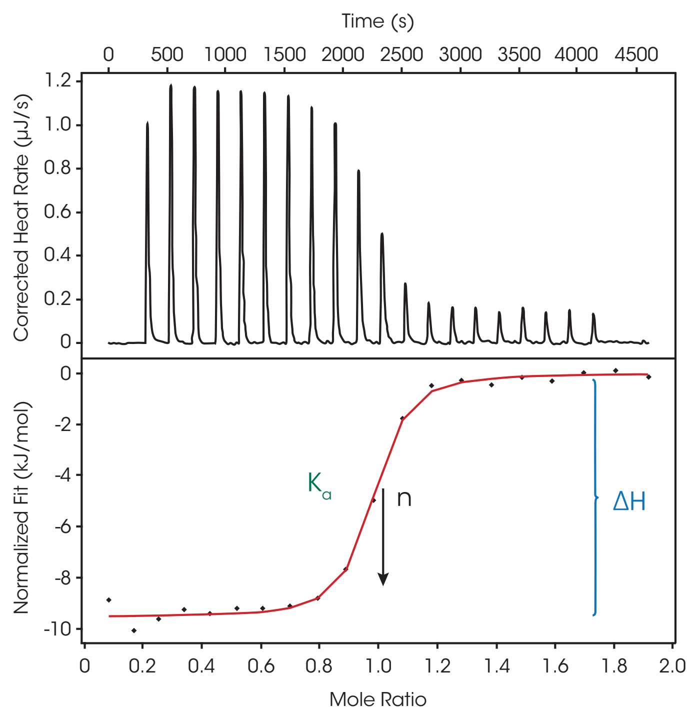


Figure 4: Typical ITC thermogram.

DESIGNING FOR THE INCREMENTAL THERMOGRAM

The most common method used in the ITC is the incremental titration. In the upper portion of Figure 4, the peaks associated with injections of titrant into the cell are shown. A baseline was established between each injection for accurate heat determination. In the lower portion the areas under the peaks were integrated and normalized using the moles of titrant delivered. This data is then fitted to an assumed model (red line).

SYRINGE

An automated syringe is used to introduce microaliquots of a titrant (syringe material) into the titrand (sample cell solution). The syringe of the Nano ITC also serves as a stirring mechanism thereby aiding in even distribution of the ligand that is expelled from the syringe. For the Affinity ITC, the stirring paddle and syringe are separated and the titrant is delivered on top of the paddle for optimal mixing at a slower rate. Delivery volumes depend on the instrument cell volume and heat generated in the experiment. The proposed incremental design tries to balance a sufficient number of data points while still producing significant heats per injection. Initially it is recommended to deliver 5-10 µL on the standard volume (SV) instrument and 1-3 µL for the low volume (LV) instrument. A common titrant volume at completion of a run is 250 µl for an SV and 50 µl for an LV. For example, a typical SV run is 30 x 8 µL with each injection delivered every 300 s and a typical LV run is 25 x 2 µL delivered every 200 s. For both systems it is recommended to have a small initial injection: 4 µL for SV and 1 µL for LV. This point is typically not included in analysis due to diffusion during the equilibration period. Both instruments from TA Instruments have been designed to eliminate mechanical backlash that would also affect the first injection.

Even when overfilling, as in normal operation, the largest injections suggested for the LV instrument is 4 µL and for the SV is 18 µL. Larger incremental injection volumes could lead to less accurate measured heat under non-ambient conditions because the titrant was not pre-equilibrated.

SPACING AND STIRRING

When assaying a new chemical system the first two injections should be observed and the schedule table in the run software updated as needed to establish the baseline after each injection. A warning: for many systems the peaks tend to broaden in the inflection region and additional spacing (s) should be added to account for this phenomena. Spacing shouldn't be set too excessive in duration because there would then be a concern of diffusion during the baseline collection region between every injection.

The default stirring speed is instrument dependent. For the Affinity ITC, a rate of 75-150 rpm is recommended and for the Nano ITC, 300-400 rpm is ideal. A good starting point is in the middle of the acceptable range. However, if the signal-to-noise ratio is too small to differentiate between a binding event and noise, or excessive perturbation shears the material or induces aggregation, a slower stirring speed is recommended.

Instrument	1st Inj vol (µL)	Inj vol (µL)	# inj	Spacing (s)	Stir Rate (rpm)
Nano SV	4	8	30	300	350
Affinity SV	4	8	30	300	125
Nano LV	1	2	25	200	350
Affinity LV	1	2	25	200	75

Table 2: Initial instrument parameters.

Equilibration settings must also be considered when setting up the titration conditions. For simplicity and reproducibility, select an automated equilibration whereby the instrument's differential power signal is monitored prior to starting the run and starts automatically. When operating at 25 °C, an LV should be set to with a "timeout" start after 30 minutes and an SV, 60 minutes. If the instrument reaches this maximum limit it is likely that the syringe is bent, the reference cell solution is low, the sample cell is dirty, or the instrument is not controlling properly. Also, before the first injection a baseline (min 60 s) should be collected.

CONCENTRATION

Determining the concentrations appropriate for an ITC experiment depends on the relationship between the macromolecule and the ligand, as the amounts of each need to be adjusted for the magnitude of heat and the expected binding constant. These values should fit into a theoretical c-window in order to gather analyzable data (2). The c-window is described by equation 4, where K is the binding constant and n is the number of ligands that will bind the macromolecule:

$$c = nK_a[\text{titrand}] \text{ or } c = n[\text{titrand}]/K_d \qquad [4, \text{Ref } 2]$$

An acceptable range for the c window is $1 < c < 1000$, with ideal values falling between 5 and 500 (2). The relationship between the c-value and the shape of the binding curve is seen in Figure 5.

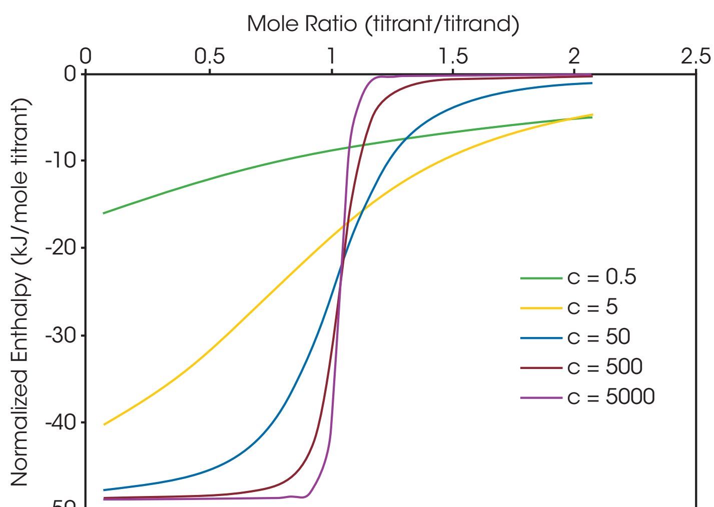


Figure 5: Influence of the c-value on the shape of the binding isotherm. K_d, ΔH, and n are identical for all isotherms show.

A binding curve with a c =1 has a shallow slope with less than 50% of the reaction occurring per injection (3). On the other hand a c-value larger than 10000 corresponds to a step function where either all (before mole stoichiometric equivalence of tirant and titrand) or none (after stoichiometric equivalence) of the species are reacting in the thermogram (3). Although enthalpy can be determined with accuracy at a "c" value larger than 1000, the binding constants reported contain significant uncertainty.

In simple terms, for a 1:1 interaction the concentration in the cell should be between 5^*K_d to 500^*K_d. If a K_d is unknown via ITC or another binding technique, similar systems can provide a good starting point for the unknown chemistry. Anecdotely, if the IC_{50} if known, this value can be used in place of the Kd in equation 4 after it is multplied by 10. This relationship between K_d and IC_{50} is not a "hard" rule but has worked as reasonable first estimation.

When working at a small "c", less than 5, a syringe concentration 20x greater than the cell is recommeded. When working at a "c" between 5 to 50 a ratio of 10:1 (syringe:cell) for syringe: cell is a good starting point and when working at "c" greater than 50, 8:1 is a better ratio. If the K_d is unknown and "c" can't be calculated, 10:1 is recommended for a 1:1 interaction. Finally if the K_d is an absolute unknown, try 10-50 µM for the cell concentration and 10:1. Experimental design programs can be used to better design experiments and it is suggested to model the data prior to starting the first experiment.

BACKGROUND EXPERIMENTS

A background titration helps to distinguish between heat resulting from the ligand binding to the macromolecule and heat associated with dilution of the titrant. There are

three background titrations that can be subtracted from the binding isotherm: buffer into buffer, buffer into macromolecule, and ligand into buffer. Generally, only the heat from titrant into buffer is subtracted from the binding isotherm because the heat from the other two backgrounds typically arise from frictional heat only, with this heat accounted for in the titrant into buffer titration. However, this is not true for all cases and considerations of heat origin are completed on a case by case basis. To simplify post collection analysis it is best to have the small molecule or compound with the consistent and small background heat as the titrand. Many proteins can oligomerize and biomolecules that have this tendency are best loaded into the cell to minimize the secondary chemistries occurring during the binding experiment. Typically the contents of the cell are diluted by less than 25% whereas the syringe concentration at the end of a titration is 25% of its initial concentration.

To minimize heat of dilution of buffer 'contaminants' in protein and ligand solution: 1) dialyze protein and ligand 2) use the dialysate to dissolve the ligand. This ideal preparation is not always possible and other methods such as buffer exchange spin columns can often be substituted for a dialysis preparation.

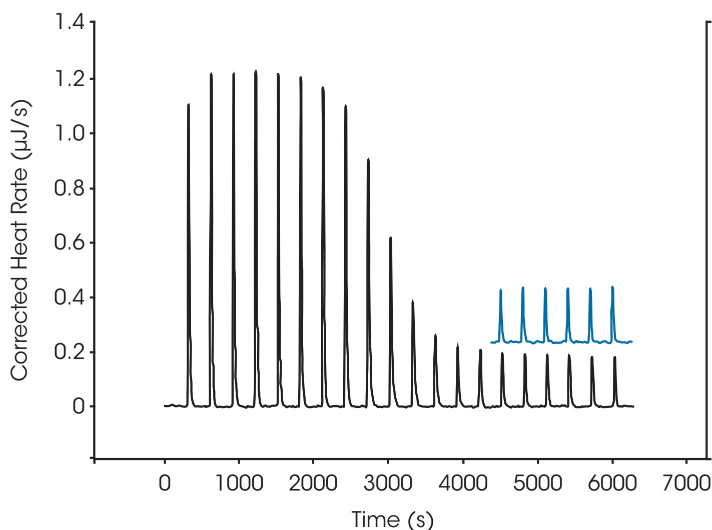


Figure 6: Binding isotherm of titrant into titrand (black) and background titration of titrant into buffer (blue).

SAMPLE PREPATIONS

BUFFER

The ideal buffer for an ITC assay is the buffer of your choice. The most important criteria is that the buffers are matched. Ideally a system is dialyzed and if needed a solid is dissolved in the dialysate. If one molecule requires a small percentage of organic solvent for solubility this same amount is required in the other solution. Not matching buffers can mask the event of interest (Figure 7).

There are a few additives that should be used with caution - not for compatibility reasons but rather for minimizing secondary chemistries and ideal mixing. For example, viscous additives such as glycerol should be kept at a low concentration for ideal mixing.

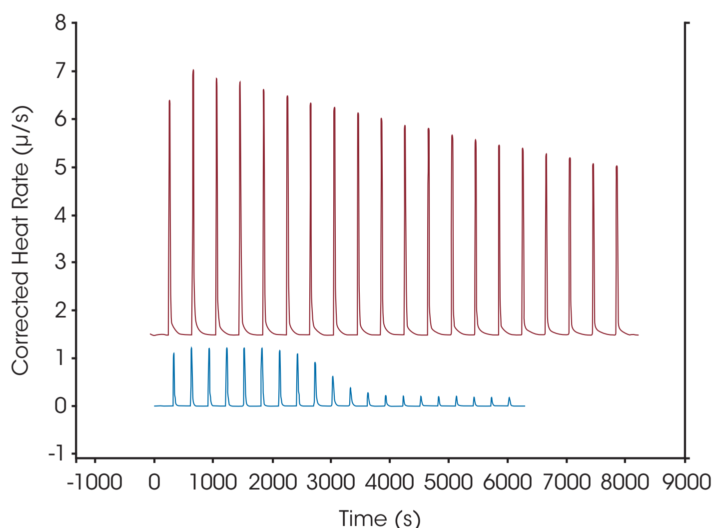


Figure 7: Red (top) 2% organic mismatch in cell and syringe. Blue (bottom) binding isotherm with matched solutions.

Reducing agents like diothiothreitol (DTT), β–mercaptoethanol or Tri-2-carboxyehtylphosphine hydrochloride (TCEP) are all compatible with the equipment. This being said, the concentration of a reducing agent is ideally kept below 1 mM with TCEP and β-mercaptoethanol being preferable. DTT can be used but its oxidation and concurrent ring-closing event has significant heat which manifests itself as a constant slope in the baseline until the reduced form is exhausted.

Not matching the pH of the buffer is a common issue and can be compounded when using buffers like Tris that have a large enthalpy of ionization. This doesn't mean that this buffer should be abandoned, on the other hand if there is a coupled protonation event an experiment in Tris buffer will yield a large signal. This buffer and two or more others can be part of a buffer assay to quantify the number of protons released or taken-up during the binding event (4).

CLEANING

A good cleaning routine can be as unique as the system studied in the instrument. A standard method includes rinsing twice with a 2% detergent followed by eight to ten rinses of water and a single buffer rinse to prepare the cell and syringe for the next titration. Chelating agents, organic solvents, and a peptidase may be required for systems more difficult to clean.

UNEXPECTED STOICHIOMETRY

Unexpectedly low stoichiometry could be due to: Titrand concentration lower than anticipated or titrant concentration higher than anticipated. Concentration complications could also come from contaminating proteins or a partially unfolded protein leading to a lower active concentration. Another common issue with unexpected stoichiometry is partially soluble drugs, and if loaded into the syringe would compromise the accuracy of the enthalpy as this concentration is used to normalize the heat value. This issue can be overcome in the fitting as long as the cell concentration and stoichiometry is known and the fit can

be adjusted by changing the syringe concentration to a value where the known stoichiometry is achieved. Insufficient curvature in data, when working at a low "c" can also lead to a lower stoichiometry then expected if the stoichiometry is known for these data with this feature the "n" should be fixed to a known value to avoid errors in ΔH.

CONCLUSION

ITC is valuable. It assigns quantitative numbers to physical and chemical events occurring during the reaction of interest. The experimental design and fitting are just the beginning. After values are assigned, rationalization of structural changes or inferences in the binding pockets can be made. With a few more well-designed experiments oligomer states can be determined, and coupled protonation or counter-ion release can be quantified. This label-free technique directly measures the thermodynamic driving forces of a system.

REFERENCES

1. Demarse, N., Quinn, C., Eggert, D., Russell, D., Hansen, L. Anal. Biochm. 417 (2011) 247-256.

2. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. (1989) Anal. Biochem. 179, 131-137.

3. Hansen, L.D., Fellingham, G.W., and Russell, D.J. (2011) Anal. Biochem. 409, 220-229.

4. Bradshaw and Waksman, Biochemistry 37, 15400-15407, 1999.

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