TA Instruments – Application Note

Isothermal Titration Calorimetry (ITC) with Reduced Cell Volumes: A Comparison of the TA Instruments Nano ITC-Low Volume with the GE Healthcare Auto-iTC$_{200}$.

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This study directly compares the performance of the Nano ITC-LV from TA Instruments to the Auto-iTC$_{200}$ of GE Healthcare and evaluates the overall performance and data reproducibility for each instrument. The evaluation reaches beyond simple numerical specification comparisons by completing multiple identical titrations with identical chemicals on each instrument. The accuracy of the instruments was determined via an acid-base titration (Figure 1), followed by a biological test system. All the data were fit using the NanoAnalyze™ data analysis software package from TA Instruments. In addition, the quality of the data and the goodness of the fit to the data were statistically evaluated for its precision.

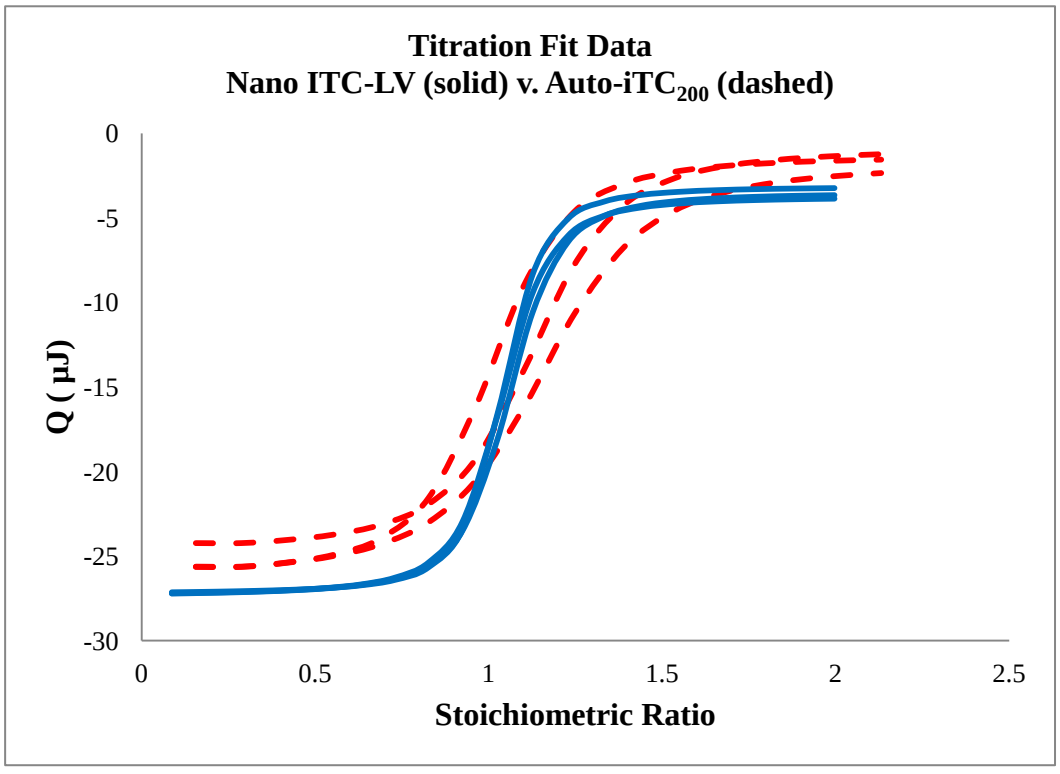


Figure 1. The fit data for titrations of HCl into $KHCO_3$ performed in triplicate. The blue solid lines are data collected on the Nano ITC-LV and the red dashed lines are data collected on the Auto-iTC$_{200}$.

Background

The accurateness of data collected on each instrument, was validated with an acid-base titration of hydrochloric acid (HCl) and potassium bicarbonate ($KHCO_3$). This acid-base titration enables calculation of the effective cell volume as well as the accuracy of the heat calibration of each instrument. The effective cell volume should be lower than the total cell volume as it takes into consideration the displacement of solution from the injection syringe.

Following the acid-base titrations a set of ten experiments were completed on each instrument with two well-defined thermodynamic systems, bovine carbonic anhydrase ii (bCAii) and acetazolamide (ACTAZ) and bCAii and p-aminomethylbenzylsulfonamide (PAMBS) (1). The thermodynamic properties of these systems, like many other protein–ligand interactions, is dependent on variables such pH, ionic strength, buffer, and temperature. The sample preparation and titration parameters used in this study were chosen so that the bCAii and ACTAZ would have a K_a of approximately 10^6 to 10^7 and the bCAii and PAMBS would have a K_a of approx 10^4 to 10^5. The samples used in these series of titrations were prepared by the same lab as a single lot of samples.

Experimental Design

For all titration experiments, the stirring speed for each instrument was set at the manufacturer's recommended value, 300 rpm (Nano ITC-LV) or 1000 rpm (Auto-iTC_{200}) at 25 ˚C. Each titration on each instrument was also programmed through the manufacturer's software "auto-equilibrate" function to start automatically when the pre-set equilibration conditions were met.

To calculate the active cell volume of both instruments, titration experiments consisted of 15 or 20, 2.5 µL incremental aliquots of 1.036 mM HCl (Sigma-Aldrich) injected into either 0.179 mM $KHCO_3$ or 0.0895 mM $KHCO_3$ (Malinnckrodt). The stock solutions were made gravimetrically with water that had been boiled in order to remove the majority of dissolved CO_2, which can be a concern when completing chemical calibrations (2). Each experiment was performed in triplicate and the data underwent iterative refinement of the apparent cell volume until convergence stoichiometric ratio (n) equal to one.

For the bCAii study, the instruments were set to deliver 19, 2 µL injections with a first injection of 0.5 µL. ACTAZ, PAMBS and bCAii were purchased from Sigma Aldrich and prepared by dissolving each in 100 mM NaPi, where the pH of the solutions was adjusted to 7.00 ± 0.03 as needed. The protein concentration was determined via UV-Vis using its absorbance at 280 nm. All solutions, 100 mM NaPi pH 7.0, 3 mM PAMBS, 30 µM ACTAZ, 0.222 mM bCAii and 2.7 µM bCAii were prepared in one lab and dispensed to the partner lab to minimize variability due to sample preparation inconsistencies.

Before comparing the data, each set was standardized. Auto-iTC_{200} raw data were converted to µW (µJ/sec). Because raw data collected on the Auto-iTC_{200} is collected every 5

seconds and the Nano ITC-LV data was collected every 1 second (user defined), Nano ITC-LV data was averaged and then decimated so that only 1 data point every 5 seconds was used. Also, since the first injection volume of 0.5 µL for the Auto-iTC$_{200}$ could not be changed, the first injection for the Nano ITC-LV was set to deliver 0.5 µL. This first injection is not displayed in the thermogram of the Auto-iTC$_{200}$ and was not in the raw data file that was imported into NanoAnalyze from Origin ®.

The data was analyzed with a one-site model: $K_a = \frac{[ML]}{[M][L]}$, where K_a is the association constant, ML is the complex and M and L are the respective free macromolecule and free ligand concentrations. The background was subtracted by averaging the last 3-5 injections that correspond to heat of dilution for the reactants at the end of the titration experiment.

Results

The Nano ITC-LV effective cell volume was calculated to be 170 µL, which is consistent with the syringe displacing 20 µL from the 190 µL total active cell volume. The effective cell volume of the Auto-iTC$_{200}$ was calculated to be 225 µL, which is substantially larger than the manufacturer published instrument cell volume of 200 µL. (Table 1). For this study it was critical to determine an accurate active cell volume as this value is used in the data fitting algorithms and would have a large effect on the stoichiometric values (n) calculated for each titration and the association constant (K_a). An inaccurate cell volume would lead to inaccurate fit values throughout the experiments in this comparison.

HCl titrated into $KHCO_3$			
	Enthalpy, ΔH (kJ/mol)	n	Effective Cell Volume
Auto-iTC$_{200}$	-9.4 ± 0.1	1.04 ± 0.04	225 µL
Nano ITC-LV	-9.17 ±0.06	1.002 ± 0.007	170 µL
Literature (3)	-9.15	1.00	N/A

Table 1. Average best-fit values associated with fitting ITC data to an independent model, where HCl was titrated into $KHCO_3$ at 25 ˚C.

When the acid-base titrations were performed on the Auto-iTC$_{200}$, the resulting cell volumes calculated for the Auto-iTC$_{200}$ gave an unexpected large range, which can be visually seen in the inflection of the binding curves in Figure 1. Even though the difference in the calculated cell volume was almost 10% greater than the volume reported by the manufacturer, when the cell volume of 225 µL was used in the data analysis in this study it did not translate to any apparent problems later on with the data analysis of the biological systems. The stoichiometries reported in later tables (Appendix A) are consistent with what was expected for the system, which is 1.00.

The enthalpy (ΔH) from the fit for the Nano ITC-LV data was -9.14 ± 0.06 kJ/mol which is consistent with the literature value of -9.15 kJ/mol (3) (Table 1). Both instruments agreed with this ΔH value within ≤ 3% than the actual value and with this data it was decided that each instrument is producing valid data with their current calibration factors. However, the enthalpy for the Auto-iTC$_{200}$ was significantly different from the published value.

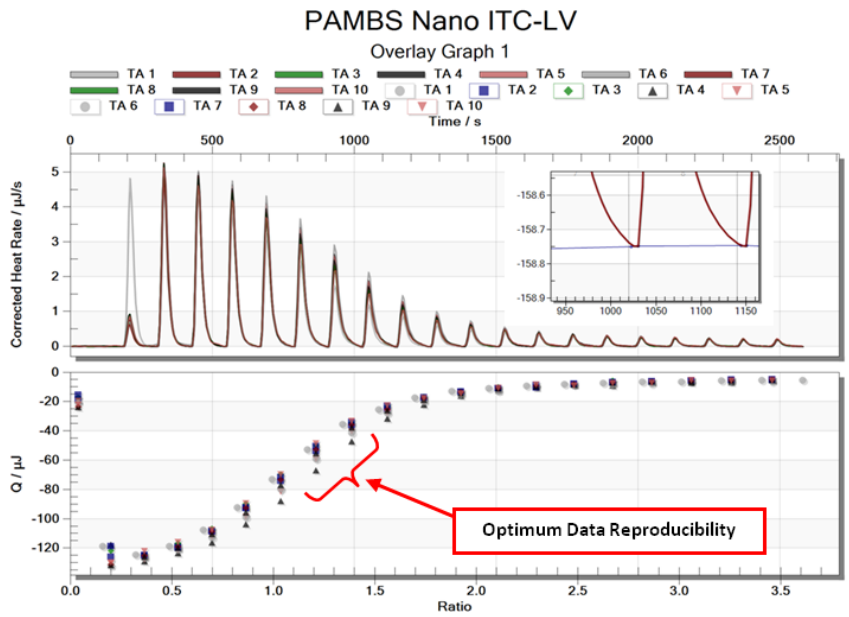
The titration thermograms for PAMBS generated from both instruments are shown in Figure 2 and the fit of each data is in Appendix A. For each thermogram, the injection interval of 120 seconds was included in the analysis and the data was successfully fit without any individual modifications to the fit or the baseline.

During the analysis of the data files, it became apparent that although the same injection interval of 120 seconds had been set in the instrument control software for each instrument, the actual amount of time executed during the titrations for the injection interval on each instrument was different. For a Nano ITC-LV the injection interval is defined as the time from the beginning of one injection to the beginning of the subsequent injection. When the same 120 second injection interval time was set in the data acquisition software of the Auto-iTC$_{200}$, the time interval reflected in the raw data file was 150 seconds. Although the interval for the Nano ITC-LV was in effect shorter by 30 seconds than that for the Auto-iTC$_{200}$, the data collected on the Nano ITC-LV still appears baseline resolved in both instruments (Figure 2, inset). The resolution of the baseline is further addressed by Figure 3, where the time interval was increased to 200 seconds. This data with the increased time interval was fit with the entire 200 second interval. If the K_a and ΔH fit of this data did not agree with the data collected with a 120 second interval, then it could be assumed that the data from the 120 second interval was not fully baseline resolved. This was not the case as the fit of this data provided a $K_a = 8.6 \times 10^4$ and a ΔH = -21.8 kJ/mol, values that are essentially identical with those listed in Table 2 for the 120 second injection interval data.

The bottom graph on both Figure 2A and 2B display the integrated areas of the injection peaks for each titration on each instrument. Prior to any fitting function being applied to the data sets, the display indicates there is some obvious undesirable variability run-to-run for the Auto-iTC$_{200}$ data that is not seen in the data sets for the Nano ITC-LV.

For each instrument, the fit parameters, K_a, n and ΔH, from the ten titrations were averaged and the results compared (Table 2). Although the overall the quality of data was good, the Auto-iTC$_{200}$ data had two experimental K_a values that fell outside of the standard error, meaning that only 80 % of the data collected provided consistent results. This is even when the concentration of the chemicals were high and the K_a value in the optimal operating range of the instrument and the error was 10%, not 2% like the Nano ITC-LV. Although it is not shown in Table 2, there was a clear trend seen with the n fit value of each subsequent data set collected on the Auto-iTC$_{200}$ (see Appendix A). The n value gradually became smaller for each subsequent titration, with the first titration exhibiting an "n" value of 1.1 and the tenth titration exhibiting an "n" value of 0.8. The ten Nano ITC-LV data sets did not exhibit trends in the "n" values.

A.



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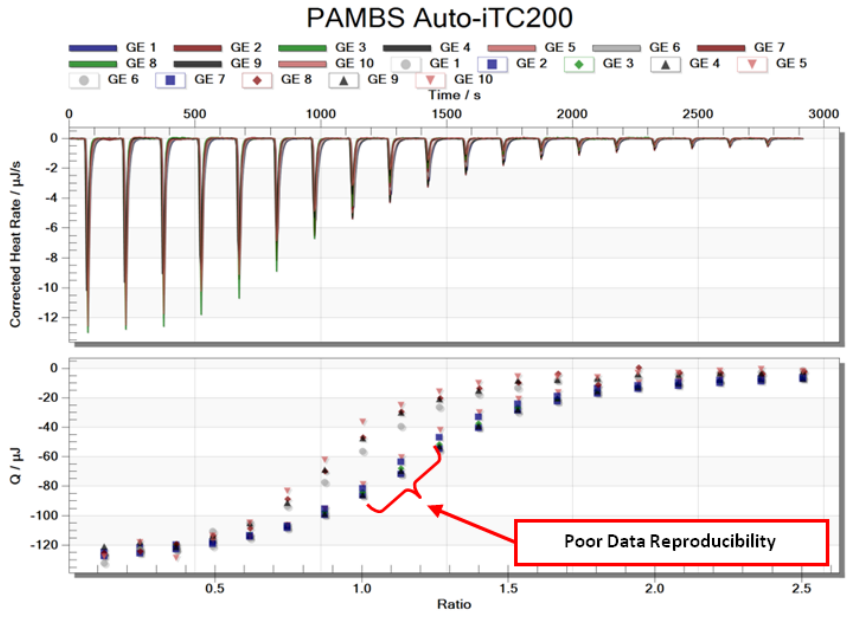

Figure 2. 3 mM PAMBS titrations into 0.222 mM bCAii in 100 mM NaPi, pH 7.0. The top thermogram of each is the raw data and the bottom is the integrated data. A. Nano ITC-LV data. Inset: zooming in between injection 7 and 8. B. Auto-iTC$_{200}$.

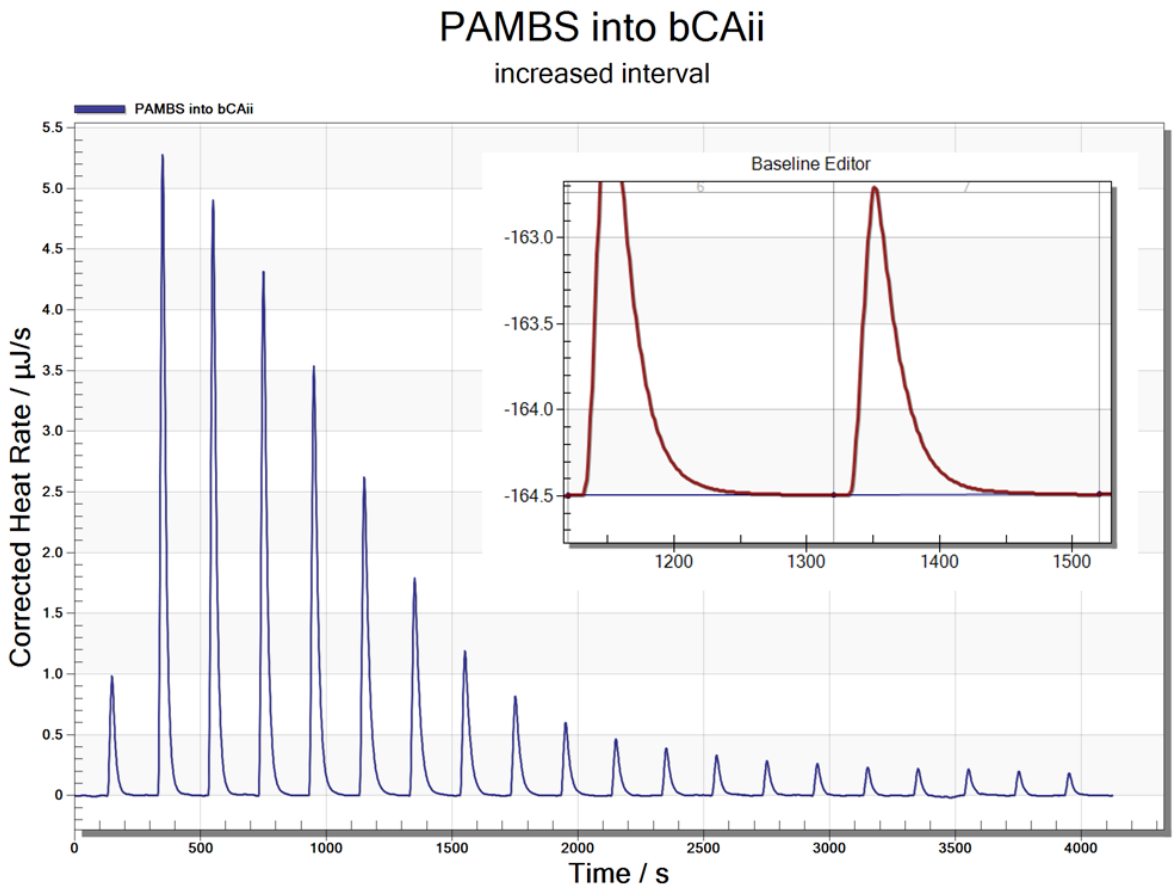

Figure 3. 3 mM PAMBS titrations into 0.222 mM bCAii in 100 mM NaPi, pH 7.0 collected on a Nano ITC-LV. Inset: zooming in between injection 6 and 7 the baseline is resolved after 150 s; inset is plotted as differential power (µW) v. time (s).

PAMBS titrated into bCAii						
	K_a	% error, K_a	ΔH (kJ/mol)	% error, ΔH	n	% error, n
Nano ITC-LV	8.67×10^4	2%	-21.55	1%	1.05	1%
Auto-iTC_{200}	1.38×10^5	10%	-20.44	1%	1.01	4%

Table 2. Average best-fit values associated fitting ITC data to an independent model, where 3 mM PAMBS was titrated into 0.222 mM bCAii at 25 ˚C. The error generated was the standard error of all ten sets of data.

The binding thermograms for the ACTAZ-bCAii are shown in Figure 4 and the fit of each data file is shown in Appendix A. The ACTAZ data were more difficult to analyze because the heat signal for each injection was smaller and the integration region contained fewer data points, which is due to the larger K_a value ($10^6 – 10^7$) for this ligand and protein. With fewer points through the inflection region the error associated with the fit becomes greater. Because of the increase in baseline noise due to the smaller heats, during the data analysis, for both instruments many of the baselines for the 120 second injection intervals were evaluated to make sure that the baseline pivot points that delineate the injection intervals had been appropriately placed. In some cases, several of these points were manually moved to ensure the inclusion of the entire injection region and to minimize the noise that had the potential of inaccurately being included with the signal, leading to inaccurate fit values. The final fit parameter calculated for ΔH over the ten titrations gave clear indication that any manual adjustments that had been made were most likely appropriately placed (Table 3).

ACTAZ titrated into bCAii						
	K_a	% error, K_a	ΔH (kJ/mol)	% error, ΔH	n	% error, n
Nano ITC-LV	6.6×10^7	28%	-60.3	3%	0.88	3%
AutoiTC_{200}	1.2×10^8	45%	-63.6	5%	0.91	2%

Table 3. Average best-fit values associated fitting ITC data to an independent model, where 30 µM ACTAZ was titrated into 2.7µM bCAii at 25 ˚C. Data were fit with the independent model in NanoAnalyze and the error generated was the standard error for all ten sets of data.

As was expected, the % error in the binding constant for the data collected for ACTAZ with both instruments was significantly larger than the error associated with the PAMBS data. The titrations with ACTAZ were designed to test the sensitivity of the instrument and the upper range of the binding constants that can be effectively measured. The ACTAZ binding data collected in this study on both low volume ITC instruments suggests that for ligands with binding constants at 10^7 or higher, experimental methods that utilize either a single continuous injection or a smaller injection volume, both of which can be executed in the instrument operational software, might decrease the variability. Since the heat signal for each injection for both instruments is well above the baseline noise level (Figure 4, inset) another way to reduce the % error would be to collect more data points in the integration region. Also, with regards to the baseline noise, both the Nano ITC-LV and the Auto-iTC_{200} have comparable values when using ACTAZ-bCAii. For the ACTAZ-bCAii titrations, the % error for the K_a was significantly better for the titrations performed on the Nano ITC-LV. The % error for ΔH and n values for the ACTAZ-bCAii were not significantly different for either instrument.

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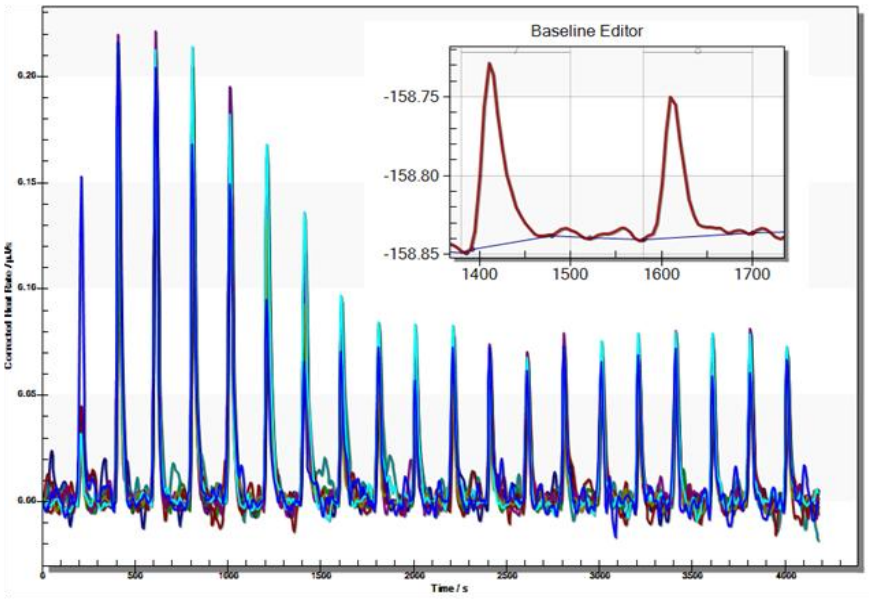

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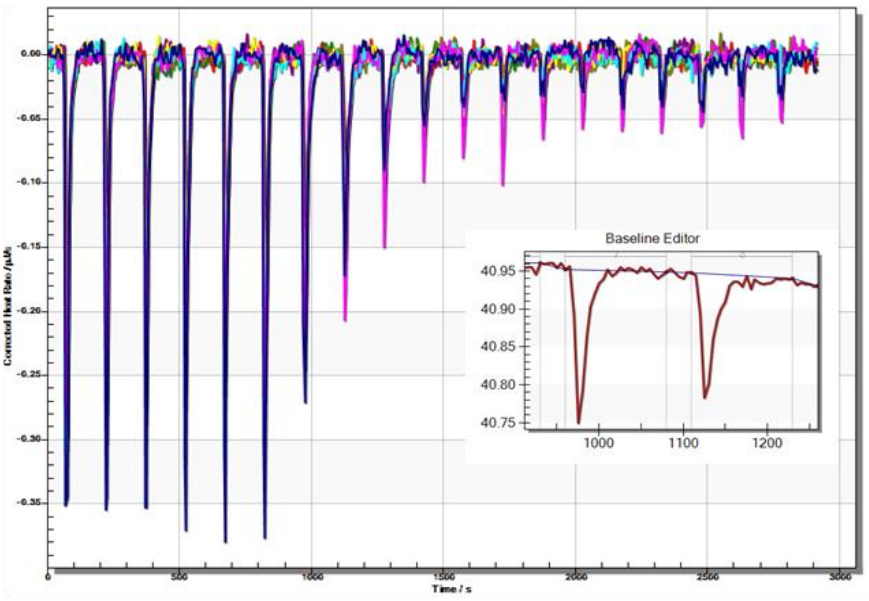

Figure 4. Baseline resolved ITC thermogram for 30 µM ACTAZ titrations into 2.7 µM bCAii in 100 mM NaPi, pH 7.0. A. Nano ITC-LV data. B. Auto-iTC$_{200}$ data. The inset in both A and B is an enhanced view between injection 7 and 8, showing the difference between the signal and noise.

Summary

Recent advances in ITC instrumentation that have decreased the amount of sample required to perform an experiment have thereby enabled the effective measurement of thermodynamics to many biological applications. The Nano ITC-LV with a cell volume of 190 µL from TA Instruments and the Auto-iTC$_{200}$ with a cell volume of 200 µL from GE Healthcare are the only low volume ITC instruments currently available. This comparative study was undertaken to determine any performance differences that might exist when completing standard incremental titration experiments in these two low volume ITC instruments.

The acid-base titrations used in this study revealed that the Nano ITC-LV has a significantly lower effective cell volume at 170 µL than the Auto-iTC$_{200}$ instrument at 225 µL, which was somewhat higher than the manufacturer published volumetric cell volume of 200 µL. It was also noted that the precision and the accuracy of the data collected for the acid-base calibration on the Nano ITC-LV were more consistent with the published literature values than the data collected on the Auto-iTC$_{200}$.

Another titration parameter that is important in ensuring the maximum data definition for ITC measurements is the data point collection frequency. Methods that include setting data collection time intervals at multi-second intervals or averaging multiple short time intervals have a tendency to smooth the data which may have a negative influence on the accuracy. The Nano ITC-LV data collection frequency of 1 second gave more data definition than the 5 second data collection frequency default setting on the Auto-iTC$_{200}$.

The analysis of the titration data collected on both instruments indicated the following:

a) Both instruments performed well and provided comparable precision (≤ 10% error) for ΔH, and n for both the PAMBS-bCAii and the ACTAZ-bCAii binding reactions.

b) The precision for the binding constant (K_a) for both PAMBS and ACTAZ data generated on a Nano ITC-LV was better than that determined from the data generated on the Auto-iTC$_{200}$. The PAMBS data had a 2% error for Nano ITC-LV vs 10% error for Auto-iTC$_{200}$ and the ACTAZ data had a 28% error for Nano ITC-LV vs 45% error for Auto-iTC$_{200}$.

c) Seven out of the eight % error values reported in this study were smaller for the data generated in the Nano ITC-LV than the data generated in the Auto-iTC$_{200}$.

d) In this direct comparison the Nano ITC-LV demonstrated itself to be a powerful low volume instrument that provides the lowest sample cell volume available, produces superior data precision and is the most flexible low volume ITC available.

References

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2. Wadsö, R.N. Goldberg, Standards in isothermal microcalorimetry, Pure & Applied Chem. 73(10) (2001) 1625-1639.

3. R.N. Goldberg, N. Kishore, R.M. Lennen, Thermodynamic quantities for the ionization reactions of buffers. J. Phys. Chem. Ref. Data 31 (2002) 231-370.

Appendix A. Fit data

PAMBS - bCAii Nano ITC-LV			
run	K_a	ΔH	n
1	7.92E+04	-21.59	1.00
2	8.38E+04	-21.48	1.05
3	9.05E+04	-20.94	1.02
4	9.63E+04	-21.51	1.14
5	8.72E+04	-21.84	1.08
6	8.65E+04	-21.76	1.08
7	9.84E+04	-20.85	1.04
8	7.98E+04	-21.97	1.03
9	8.34E+04	-21.91	1.05
10	8.13E+04	-21.63	1.00

PAMBS - bCAii Auto-iTC$_{200}$			
run	K_a	ΔH	n
1	1.14E+05	-20.41	1.13
2	8.55E+04	-20.51	1.12
3	1.18E+05	-20.05	1.12
4	1.27E+05	-19.75	1.14
5	1.26E+05	-20.76	1.06
6	1.04E+05	-21.87	0.91
7	1.14E+05	-20.76	1.08
8	1.86E+05	-20.27	0.85
9	1.89E+05	-19.76	0.86
10	2.20E+05	-20.29	0.80

ACTAZ - bCAii Nano ITC-LV			
run	K_a	ΔH	n
1	1.86E+07	-55.39	0.97
2	1.81E+08	-52.75	0.90
3	2.52E+07	-69.18	0.89
4	3.52E+07	-64.77	0.95
5	7.38E+07	-55.23	0.91
6	5.00E+07	-66.41	0.79
7	3.21E+07	-60.64	0.85
8	4.86E+07	-53.35	0.84
9	3.07E+07	-62.24	0.98
10	1.62E+08	-62.63	0.75

ACTAZ - bCAii Auto-iTC$_{200}$			
run	K_a	ΔH	n
1	1.25E+07	-80.82	0.97
2	2.28E+07	-67.46	0.85
3	7.36E+07	-55.13	0.92
4	5.29E+07	-53.40	0.92
5	5.58E+08	-51.63	0.92
6	2.45E+07	-60.41	0.93
7	1.56E+08	-58.35	0.90
8	4.78E+07	-61.94	0.96
9	3.12E+07	-63.93	0.91
10	1.83E+08	-83.41	0.81