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A High-Throughput Biological Calorimetry Core – Steps to Startup, Run, and Maintain a Multi-user Facility

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Abstract

Many labs have conventional calorimeters where denaturation and binding experiments are setup and run one at a time. While these systems are highly informative to biopolymer folding and ligand interaction, they require considerable manual intervention for cleaning and setup. As such, the throughput for such setups is limited typically to a few runs a day. With a large number of experimental parameters to explore including different buffers, macromolecule concentrations, temperatures, ligands, mutants, controls, replicates and instrument tests, the need for high-throughput automated calorimeters is on the rise. Lower sample volume requirements and reduced user intervention time compared to the manual instruments has improved turnover of calorimetry experiments in a high-throughput format where 25 or more runs can be conducted per day. The cost and efforts to maintain high-throughput equipment typically demands that these instruments be housed in a multi-user core facility. We describe here the steps taken to successfully start and run an automated biological calorimetry facility at Pennsylvania State University. Scientists from various departments at Penn State including Chemistry, Biochemistry and Molecular Biology, Bioengineering, Biology, Food Science and Chemical Engineering are benefitting from this core facility. Samples studied include proteins, nucleic acids, sugars, lipids, synthetic polymers, small molecules, natural products, and virus capsids. This facility has led to higher throughput of data, which has been leveraged into grant support, attracting new faculty hire and has led to some exciting publications.

Keywords

ITC; DSC; High-throughput; calorimetry; facility

1. Introduction

Conventional manual single-usage calorimeters have been used extensively in research labs for decades and provided accurate thermodynamic data of many interesting biological systems. Excellent calorimetric equipment from Microcal (now with Malvern instruments) and TA instruments for both isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) have seen a wide range of use for a variety of applications. Microcal offers several different ITC instruments, including the VP ITC and ITC200. The manual

MicroCal VP ITC, while providing excellent signal to noise, has a sample cell volume of 1400 μL , seven times greater than both the semiautomated MicroCal ITC200 and fully automated Auto-iTC200 models. The tradeoff with the MicroCal ITC200 is slightly higher noise (0.2 ncal/s versus 0.15 ncal/s for the Auto-iTC200 versus the VP ITC) however less time is spent on manual cleaning between experiments with the Auto-iTC200. With the many experimental parameters and variations in binding partners that can be studied in any biological or biological-like system, there is a need for low sample consumption, good sensitivity and high-throughput, as from any automated instruments. While pharmaceutical companies have applied automated calorimeters to screen for small molecule drugs for some time now, the academic community has only started to use high-throughput calorimeters and we anticipate this usage will continue to grow.

The Automated Biological Calorimetry core facility at the Pennsylvania State University is a one-of-a-kind fee-for-service facility in the north-east region of the US. This high-throughput state-of-the-art capability was envisioned in September 2011 and has been ably serving the needs of the scientific community at Penn State. It was set up with funds made available through a successful multi-user research instrumentation grant (MRI) from the National Science Foundation. The cost of establishing the facility is approximately \$400,000–\$500,000 direct costs, including costs of instruments and service contracts. Any costs of staff are in addition to this total. In recent years, service contracts have been dropped in favor of an annual preventative maintenance (PM). This dollar amount is an ideal range for funding from both NSF and NIH instrumental programs. In our case, administrative support including salaries for the facility staff and the five hundred square foot laboratory space for housing all the instrumentation were generously provided by the Huck Institutes of the Life Sciences at the Penn State (University Park campus). Dr. Neela Yennawar was appointed as the director and Julia Fecko as research technologist at the facility. An advisory committee was formed including Penn State faculty comprised of Profs. Philip Bevilacqua (Chair), Sarah Assman, Kenneth Keiler, and Scott Showalter as well as Dr. Nigel Deighton (Director of research instrumentation at the Huck Institutes of the Life Sciences). We placed the instruments within an existing facility for X-ray crystallography, since this facility is already adept at handling large amounts of pure protein and RNA. With these players in place the core facility was launched. We present here a model of starting and running a high-throughput biological calorimetry facility. Demand for such a high-throughput core facility currently exists in many large research institutions, and availability of such technologies could lead to higher success rates in obtaining biological research grants.

2. Instruments at the Facility

A MicroCal Auto-iTC200 ITC instrument and a Microcal VP-cap DSC instrument were purchased from General Electric (Microcal wing of GE, which is now with Malvern instruments) each of which has 96-well formats. At the time, these were the only automated high-throughput calorimetric instrumentation in the market. TA-instruments has also added both an automated Affinity ITC Auto and the 96-well plate format Nano DSC Auto-sampler in recent years. Here we provide an introduction to the calorimetry equipment at our core facility.

2.1 Isothermal titration calorimeter - Microcal Auto-iTC200

The MicroCal Auto-iTC200 ITC instrument allows high-throughput direct and label-free measurement of binding affinity and thermodynamics (Fig. 1). It measures the heat absorbed or generated when molecules interact. All filling, injection, and cell cleaning functions are fully automated, controlled, and operated through Origin® software that includes integrated experiment design wizards to assist in selecting experimental parameters. Up to four 96 well trays can be loaded and programmed to run without user intervention. The raw data obtained in this experiment are heat released or absorbed as a function of injection volume. The cell volume is 200 μ L (sample volume to be loaded in the 96-well tray is 400 μ L) and total injection volume is 40 μ L, with each injection typically in the range of 0.7 to 2 μ L (titration volume to be loaded in the 96-well tray is 120 μ L). This is as opposed to a cell volume of 1400 μ L and sample volume of 300 μ L in the manual MicroCal VP-ITC. Data analysis is also performed with Origin® software using several options of fitting models to calculate reaction stoichiometry, equilibrium association constant (inverted to derive equilibrium dissociation constant value) and enthalpy. Entropy is not a fitting parameter in the software, but rather a derived parameter calculated using the Gibbs free energy equation dependent on the known temperature and conversion of the equilibrium association constant to a Gibbs free energy.

2.2 Differential scanning calorimeter - Microcal VP-cap DSC

The Microcal VP-capillary DSC instrument allows high-throughput measurement of the thermal stability of macromolecules through an automated cell loading and cleaning system (Fig. 2). It measures differences in heat required to raise the temperature of the sample cell versus the reference cell. Like the Auto-iTC, all filling, injection, and cell cleaning functions are fully automated, controlled, and operated through Origin® software that includes options to select experimental parameters. Up to six 96 well trays can be loaded and programmed to run without user intervention. The raw data obtained in this experiment are a plot of heat capacity difference as a function of temperature. The MicroCal VP-Capillary DSC system has a sample cell and reference cell volume of 130 μ L each (need to fill in 400 μ L in the 96 well tray) as opposed to 500 μ L in the manual MicroCal VP-DSC. Sample concentrations typically range from 0.1 to 10 mg/ml in both instrument setups. Data analysis is performed with the Origin® software and yields the macromolecule's melting transition midpoint temperature (T_m) as well as the enthalpy of folding, ΔH . The T_m is useful for characterizing the folding behavior of proteins, nucleic acids, micelles, and many other macromolecular systems, alone or as complexes. Furthermore, this information can optimize shelf life, improve purification strategies, and evaluate protein constructs. In addition, affinities of small molecule to a protein target in a drug discovery pipeline can be ranked. Overall, differential scanning calorimetry is very sensitive, easy-to-use, fast, and accurate for screening solution thermal behavior and interactions.

2.3 FT-IR quantitation system

In order for users to obtain accurate figures for binding affinity, stoichiometry, enthalpy and entropy, concentrations of the binding partners must be known precisely. To assist users to quantify their samples, the facility acquired a Millipore Direct Detect biomolecular

quantitation system. Operation of the Direct Detect is based on infrared spectrometry and measures amide bond absorbance rather than aromatic residue absorbance for higher reproducibility across all proteins and peptides, with improved speed and accuracy over traditional biochemical assays. Because of the unique method of detection, this method also works in the presence of detergents and reducing agents commonly found in biological samples. As an added benefit, the method requires 2 μ L of sample per analysis. The Direct Detect can also be used with nucleic acids, although optical spectroscopy is typically used, and has proven to be a complementary instrument in the facility for both ITC and DSC runs. Alternately nano-drop or nano-vue instruments can also be used to determine biomolecular concentration.

2.4 Dynamic light scattering

Dynamic light scattering is a technique in which a beam of monochromatic laser light is directed through a biomolecule solution and fluctuations in scattered light intensity are analyzed. The experiment is non-invasive, requires only 12 μ L of sample and can in a matter of minutes provide information about size and homogeneity of biomolecules. Sample characteristics like aggregation, folding, or conformation can be monitored as a function of various preparations, solvent conditions, temperature or time. The facility houses a Viscotek 802 dynamic light scattering instrument. The system is being used for prescreening samples under conditions in which the calorimetry experiment is being planned. The instrument can be operated between 4 and 60°C. It is equipped with the OmniSIZE software that outputs information about the hydrodynamic radius (in the sample size range of 0.5 nm to 1 micrometer) along with percentage distribution of various components in the sample. Small traces of aggregation or oligomerization can be checked and corrected for by improving the buffer conditions or choosing the optimal temperature for the calorimetry experiment.

3. Running of the Facility

In the first year of its operation the calorimetry facility had users from ten Penn State research labs across four departments – Profs. Graham Thomas (Biology/BMB), Stephen Benkovic (Chemistry), Philip Bevilacqua (Chemistry), Scott Showalter (Chemistry), Joshua Lambert (Food Science), Sarah Ades (BMB), Kenneth Keiler (BMB), Craig Cameron (BMB), Daniel Cosgrove (Biology) and Katsuhiko Murakami (BMB) and two outside (Boston college, Lebanon valley college) users as well. The operation has been smooth with an established work-flow. A gradual growth in facility usage has been seen over the past three years, with more students receiving training annually. After initial training with sample preparation, instrument setup and data analysis, students are able to directly operate and run the machine independently with minimal support from the staff. The advisory committee has received positive feedback from the faculty regarding the usefulness of the facility. Having several faculty members keenly interested in the development of the facility is of paramount importance, especially in the first two years of set up and operation. The staff at GE (now Malvern) were knowledgeable and helpful with instrument maintenance issues, difficult-to-interpret experiments, and data analysis.

3.1 Steps used in setting up the core facility

The following summarizes the steps and approximate time frame needed in setting up the ITC and DSC capabilities at Penn State

- Grouped around eight faculty interested in calorimetry research and willing to participate and write a multi-user instrument and training grant – 2 months
- Gathered administrative support from Penn State for housing the instruments and staffing the core facility – 1 month
- Prepared shared instrumentation grant for NSF – 2 months
- Placed purchase orders to GE for the fully automated calorimeter instrumentation, Auto-iTC200 and VP-capDSC – 2 months
- Formed advisory committee consisting of four faculty members from the pool of interested faculty – 2 weeks
- Hired staff scientist at 50% effort to help run facility.
- Organized training workshops for staff and core facility lab users and advertised capabilities of the newly formed “Automated Biological Calorimetry Core” via open house, newsletters, website and training opportunities– 2 years
- Currently operating the machine successfully without the annual service contract. Annual preventive maintenance is mandatory - ongoing.
- Providing user training every 3 months for all Penn State users - ongoing

3.2 Role of advisory committee –

The advisory committee meets with facility staff annually to discuss operations of the calorimetry facility. They have been active in helping run the facility smoothly and are in contact with the director on a monthly basis. The advisory committee plays an important role in setting policies and providing guidance for the facility. For example, it was the committee that considered the proposal to drop instrument service contracts and ultimately approved the decision. The advisory committee has also been actively involved in collaboration with the director to establish education, training, and outreach activities. The Showalter and Bevilacqua labs have also been involved in facility protocol establishment and periodically run tests, providing protein and RNA samples. Even after leaving Penn State, some of our students have continued to contribute ideas and suggestions that have helped to improve the usage of the calorimetry instrumentation.

3.3 Role of facility staff –

The staff, which consists of the director and a staff scientist, train users, help design experiments and prepare samples, and address project-specific challenges. To increase awareness of the calorimetry equipment, the facility staff have met with faculty from various departments and presented in their group meetings. In order to highlight Penn State’s new capability to a larger audience, they have also presented posters and talks at several national and international meetings, and have advertised the facility through newsletters, open houses and training sessions. Core facility usage has grown steadily over the past four years, with

the Auto-iTC200 running ~90% of the time including weekends, and the VP-cap DSC used fifty percent of the time.

3.4 Workflow –

Users interested in micro-calorimetry meet with the facility staff to discuss individual projects and optimal strategies for obtaining good quality calorimetric data. The staff become familiar with published literature on the project and the users are made aware of review articles and the basics of sample preparation [Feig, 2009; Buurma & Haq, 2007; Baranauskiene, Petrikaite, Matulienė & Matulis, 2009; Tellinghuisen, 2005; Zhao, Piszczek, & Schuck, 2015; Ghai, Falconer, & Collins, 2012; Cooper, 1998; Velázquez-Campoy, Ohtaka, Nezami, Muzammil & Freire, 2004; Chaires, Hansen, Keller, Brautigam, Zhao & Schuck, 2015]. Users can also learn more about the principles of calorimetry by taking a course in Biophysical Chemistry, Chem 540 offered at Penn State. The facility staff combines research literature with “the design of experiment” tool in the Origin® software to optimize starting conditions. AutoITC parameters include macromolecule and ligand concentrations, titration volumes, temperature, stirrer speed, and buffer choice, while cap-DSC parameters include temperature range, scan rate, and sample concentration. Once initial parameters are selected, users are trained on setting up their experiment using the 96 well plates, which are used in the fully automated equipment. In a 24-hour period, depending on the chosen run parameters, ~ 8–10 titrations can be acquired on the Auto-iTC200 and 20–24 thermograms on the VP-cap DSC. This throughput enables users to optimize their run parameters and analyze their system quickly and efficiently. Some users find it useful to perform a dynamic light scattering experiment to determine sample homogeneity. Few others run a DSC experiment prior to an ITC run to determine the thermal denaturation midpoint so that the temperature-dependent ITC series can be designed with rational upper limits. Often the Auto-iTC200 is used during the pilot phase of experiments to rapidly screen multiple conditions and hone in on optimum experimental parameters. The staff assists in setting up the equipment and running the initial experiment as well as analyzing the data. After the results of the first set of runs, recommendations are made to improve data for ensuing experiments. Once a user is trained and feels comfortable running the equipment, they can schedule time to use the instrumentation independently and have more ownership and control of the outcome of the runs. Training in data analyses using Origin® and SEDPHAT software is also provided [Zhao *et al.*, 2015, Chaires *et al.*, 2015].

3.5 Outreach and training –

The facility has seen an increase in its user base through various advertising efforts. An annual open house is well attended by faculty, graduate students, and staff from various departments. Free user training sessions are offered on a quarterly basis where anyone from a diverse cross section of research fields can learn about the theory of micro-calorimetry and its applications, as well as gain practical hands-on experience. The facility also distributes a semi-annual newsletter highlighting the latest news, publications and on-going research, which are posted on the website. Acknowledgements to the facility are made in publications resulting from core facility usage [Khanna, Mattie, Browder, Radyk, Harper, Speicher, & Thomas 2015; Warui, Pandelia, Rajakovich, Krebs, Bollinger & Booker, 2015; Bastidas & Showalter, 2013; Liu, Hanoian, French, Pringle, Hammes-Schifferand & Benkovic, 2013;

Toroney, Hull, Sokoloski, & Bevilacqua, 2012; Patel, Blose, Sokoloski, Pollack, & Bevilacqua, 2012; Mukherjee, Yakhnin, Kysela, Sokoloski, Babitzke & Kearns, 2011], and highlighting these on the facility website is the best advertising possible.

The facility's website under the Huck Institutes of the Life Sciences details all the instrumentation and also features guidelines for successful sample preparation, applications and testimonials (<http://www.huck.psu.edu/content/instrumentation-facilities/automated-biological-calorimetry-facility>). The website has been successful in reaching researchers outside the University and has recently generated use by industry.

The Automated Biological Calorimetry facility hosted a workshop featuring guest speakers including Prof. Andrew Feig from Wayne State University, technical talks from GE healthcare, and Penn State faculty and student research presentations. The workshop offered informative presentations and technical discussions on automated ITC and DSC. It was well attended with participants from Penn State's College of Agricultural Sciences, and the Departments of Biochemistry and Molecular Biology, Chemistry, Biology, Chemical Engineering, and Physics. Many attendees provided positive feedback. The facility staff has presented poster sessions at several local, national and international meetings. The director was invited to talk at the international calorimetry conference in Buzios, Brazil where she highlighted the collaborative crystallography and calorimetry work done with the Cosgrove lab [Georgelis, Yennawar & Cosgrove, 2012].

3.6 Sample preparation –

Proper sample preparation is critical for achieving good microcalorimetry data for both the auto-iTC200 and the VP-cap DSC. Details of sample preparation and experimental parameters are not very different for the automated and manual calorimeters and have been reviewed in detail in several earlier publications [Feig, 2009; Buurma *et al.*, 2007; Baranauskiene, *et al.*, 2009; Tellinghuisen, 2005; Zhao, *et al.*, 2015; Ghai *et al.*, 2012; Cooper, 1998; Velázquez-Campoy *et al.*, 2004; Chaires, *et al.*, 2015]. We provide a brief overview of these steps here.

1. Sample purification
2. Buffer Matching
3. Design of experiments
4. Concentration Determination
5. Buffer choice

1. Samples must be pure and free of degradation and it is recommended that all molecules be gel or column purified before use. It is good practice to test macromolecular samples using dynamic light scattering in order to confirm homogeneity. The macromolecule should be tested for stability under the full range of experimental conditions. Precipitation will make the data unusable and could potentially damage the instruments via the fine tubing that is part of the liquid handling. Filtration may be necessary and degassing is recommended.

2. Buffer matching is crucial when preparing samples for both Auto-iTC200 and VP-cap DSC. Both syringe and cell contents must be very precisely buffer matched including all solvents and additives. Macromolecules should be dialyzed against their buffer to ensure exact salt matching of the sample. Small molecule ligands should be dissolved into the dialysis flow-through, checked for pH changes and matched. The dialysis buffer should also be used for the reference run in which ligand in buffer is titrated into buffer alone. The auto ITC equipment is so sensitive that even a slight mismatch of the buffer components between the macromolecule in the cell and the ligand in the syringe, even as low as 1%, can contribute substantial heat and mask the binding signal. Any difference in pH between the buffer in the cell and syringe can have a similar detrimental effect. Particular attention should be given to lyophilized samples as there may be salts present prior to lyophilization that may not be accounted during the buffer matching. Some small molecule ligands may need small percentage organic solvents like DMSO to dissolve. In this scenario, the same percentage of organic solvent should be added to the macromolecule as well.

3. An invaluable feature included in Auto-ITC200 software is the design of experiments simulation program written in Origin®, which allows users to optimize their starting concentrations. Through insights derived from prior research, using similar molecules or data from a previous run, one can estimate a stoichiometry (n), binding affinity (K_d) and enthalpy (ΔH) and enter them into the “design of experiment” aspect of the program. The software simulates the binding curve for the given concentrations of macromolecule and ligand. Initial experiments are performed by estimating the required sample concentration in the cell as $\sim 10 * K_d$ and concentration in syringe: $\sim \text{cell concentration} * \text{no. of binding sites} * 10$.

4. Concentrations of the protein, DNA, RNA, or small molecule should be accurately known in order to obtain the best quantitative results. The core facility is equipped with the Millipore direct detect IR based quantitation system as described above.

5. It is important to choose a buffer that has a low H_{ion} (heat of ionization) thus not contributing excessive heat to the experiment. Examples of some buffers with low H_{ion} include phosphate, acetate, formate, citrate, sulfate, cacodylate and glycine [Feig, 2009; Buurma *et al.*, 2007; Baranauskiene, *et al.*, 2009; Tellinghuisen, 2005; Zhao, *et al.*, 2015; Ghai *et al.*, 2012; Cooper, 1998; Velázquez-Campoy *et al.*, 2004; Chaires, *et al.*, 2015]. Proper procedure for disposal of toxic and hazardous waste such as the cacodylate buffer should be followed. Tris buffer should be avoided for VP-Cap DSC experiments as it has a high temperature dependence of its pK_a . Dithiothreitol should also be avoided, and tris(2-carboxyethyl)phosphine (TCEP) or beta-mercapto ethanol (BME) can be substituted as reductants instead.

3.7 Experimental Setup

3.7.1 For auto-iTC200 —Origin® software is used to control the instrument and for choosing the run parameters in the micro-calorimeters. While the AutoITC can hold four 96-well trays, the VP-capDSC can hold six 96 well trays. We describe experimental setup of the Auto-iTC200, followed by the VP-capDSC.

1. 96-well plate setup
2. Volumes
3. Auto sample setup
4. Experimental parameters

1. Once the samples are prepared, they are ready to be dispensed into the 96-well plate. Each experiment is comprised of a reference run as well as a sample run. The reference run mixes the ligand against the buffer to test if there is a reaction. The reference run will be subtracted from the sample run during the analysis stage, so it is essential that there be minimal or no reaction between the ligand and the buffer.

Figure 3 illustrates a typical 96-well plate setup for the reference and sample runs. Four wells are utilized when setting up a run. The first well holds the sample, which will be dispensed into the cell. The second well holds the ligand, which will be titrated into the cell with the pipette. The third well holds buffer to be used as a pre-rinse. The pre-rinse will be dispensed into the cell prior to the macromolecule, held there for a minute and then discarded. This helps in reducing noise during the sample run. A fourth well, which is empty at the start, can be used to save the sample after the titration if desired. With this setup, a total of 24 experiments can be run using a single 96 well plate. Typically an entire plate is filled with replicates, as well as different samples, ligands, and buffers.

2. The volume of the sample needed to be loaded in the 96-well tray - 400 μ L for the calorimeter cell, 120 μ L for the titration syringe and 400 μ L for the pre-rinsing with matching buffer.
3. Once the plate has been filled without air bubbles and the adhesive lid has been secured, the plate is ready to be placed into the holding tray on the equipment. The Auto-iTC200 holds up to four plates and can be configured to run them in any desired order. The temperature of the tray can also be adjusted from 4–25°C. The Auto-iTC200 is fully automated and can be set up to run continuously overnight. The operator simply has to enter in the number of samples, method of run, concentrations, and details of the plate setup.
4. The experimental parameters can also be customized for each individual experiment. They include total number of injections, cell temperature (value between 4 to 40 °C can be chosen), reference power, injection volume, spacing, and stir speed. An automated cleaning procedure is used between each run, with a more stringent cleaning typically every 3–15 runs. These parameters can be fine-tuned in order to get a better-fitted binding curve.

3.7.2. For VP-cap DSC

1. 96-well plate setup
2. Volumes
3. Auto sample setup
4. Experimental parameters

1. The VP-cap DSC is fully automated so once the samples have been properly prepared they are ready to be dispensed into the 96 well plate. The VP-cap DSC is comprised of two cells, a reference cell and a sample cell. Each experiment consists of a reference run and a sample run. The reference run tests buffer against buffer to see if there is any heat, which will be subtracted from the sample run during the analysis stage. To set up the reference run, the first two wells of the plate will be used. The first well holds the buffer, which will be dispensed into the reference cell, while the second well also holds the buffer which will be dispensed into the sample cell. The sample run is similarly set up with the next two wells. The third well holds buffer for the reference cell, the fourth holds the sample for the sample cell.

2. The volume needed to be loaded in the 96-well tray for each of the reference cell and sample cell is 400 μ L. An additional 200 mL of buffer is required to be used as a rinse between experiments.

3. Once the plate has been filled and the semi-permeated lid has been secured to prevent evaporation, the plate is placed into the holding tray on the equipment. The DSC holds up to six plates and can be programmed to run the plates in any desired order. The temperature of the tray can be adjusted from 10–25°C. The instrument is fully automated and can be set up to run continuously overnight. The operator simply has to enter in the number of samples, cleaning method, concentrations and plate setup into the auto sampler.

4. The experimental scan parameters can also be customized for each individual experiment. They include starting temperature, final temperature (up to 120°C), scan rate, pre-scan thermostat, post-scan thermostat and feedback mode. These parameters can all be finely adjusted in the DSC control tab.

The analysis of ITC binding curves and DSC thermograms are described in detail in the examples discussed in the following sections.

3.8 Maintaining the facility and cost recovery –

Having all the micro-calorimetry equipment located at a central core facility is beneficial when it comes to maintaining the equipment. The research technologist takes ownership of the instrumentation and follows all cleaning protocols and oversees regular preventative maintenance. The research technologist also knows the equipment well, which is advantageous when it comes to troubleshooting equipment issues. The following maintenance steps are followed diligently:

- A water install test is conducted by using deionized, distilled water both in the cell as well as the syringe. Experimental parameters for the test usually are 19 injections, 2 μ L injection volume, 5 μ Cal/sec reference power, 120 seconds spacing, 750 rpm stir speed, high gain and 25°C cell temperature. Typically a series of 4–6 water tests are run in a row to ensure that the baseline does not drift appreciably, that the injections are good (i.e. have a typical smooth shape) and that there is very slight heat and noise in the water-water injections. A good quality water-water injection profile is shown in Figure 4. The mean energy per injection should be less than 1.5 μ cal and the standard deviation less than 0.25

µcal. It is good practice to also incorporate several water-water runs in between the experiment schedule as a crosscheck.

- The noise test is run by using deionized, distilled water both in the cell and the syringe. There are no injections during the test and it runs for 20 minutes. The parameters for the test are 5 µcal/sec reference power, 750 rpm stir speed, high gain and 25 °C cell temperature. A good noise test run that “passes” the test will have only a slight drift in the baseline that is a small fraction of the offset power and uniform. The average RMS is not more than 1.5 ncal/sec and the injections approach the baseline without significant shoulders or other protracted secondary features (Fig. 5). Excessive noise can indicate a dirty cell or a bent syringe. The noise test also will ensure the proper functioning of all electronics. Excessive noise during a sample run may indicate that the sample has some aggregation and needs to be filtered.
- An EDTA-CaCl₂ standard sample test is run to ensure accurate heats and injections. 400µl of 0.4mM EDTA is used to fill the cell and 120µl of 5mM CaCl₂ is used in the titration syringe. The buffer used is 10mM MES at pH 5.6. The test parameters are 19 injections, 2 µl injection volume, 150 second spacing, high gain, 750 rpm stir speed, 10 µcal/sec reference power, 25°C cell temperature. Successful sample test results will have stoichiometry, binding affinity, and enthalpy within the limits listed for the standard [Griko, 1999] (Fig. 6). In this instance, these values are 0.95, $1.26 \times 10^5 \text{ M}^{-1}$, and -4.27 kcal/mol ($= -17.9 \text{ kJ/mol}$), respectively.
- The auto-iTC200 is set up to automatically clean the cell, cannulas and syringe with 20% contrad70 between each sample run. An extensive cleaning protocol includes 30 minutes 20% contrad70 soak of the cell at 60°C and is to be done between users.
- Cleaning cannula, syringe and cell with 20% contrad70, rinsing with water, methanol and nitrogen air blast between each experimental run
- Changing water in the reference cell every two weeks
- Maintenance log
- Usage log

A maintenance log is an essential tool to prevent downtime. Regular tests are routinely performed to ensure that the instrument is in the best working condition. Users book instrument time in advance and prepare samples accordingly. No service contract has been purchased in the year after the three-year service contract expired and annual preventive maintenance has so far been sufficient for upkeep. Trained users are allowed to operate the machine and take ownership of their runs. Users also have key access to the instrument 24 hours of the day, 7 days of the week.

The facility service fees (Table below) collected have been sufficient to recover the cost of the preventive maintenance. No staff salaries are currently being recovered. The

maintenance contract if purchased would increase the service fee to a level that may have an impact on usage. The cost recovery of the facility in the year 2014 is found in Table 1.

4. Science Facilitated

The Automated Biological Calorimetry Facility is employed by a wide spectrum of departments at the Penn State University Park campus including Agricultural Science, Biology, Biochemistry and Molecular Biology, Chemistry, Chemical Engineering, Food Science, and Engineering Science and Mechanics. In addition, Penn State Hershey College of Medicine, as well as some external research labs and universities, have used the facility. A number of exciting publications have ensued as a result of the capability [Khanna *et al.*, 2015; Warui *et al.*, 2015; Bastidas *et al.*, 2013; Liu *et al.*, 2013; Toroney *et al.*, 2012; Patel *et al.*, 2012; Mukherjee *et al.*, 2011]. The speed of the Automated ITC and DSC combined with access to additional supporting instrumentation helps streamline research data collection considerably. The fully automated equipment enables users to set up a series of data acquisitions and also run experiments unattended overnight and during weekends for maximum efficiency. In addition, some of the Penn State faculty (Profs. Bevilacqua, Murakami, Showalter, Cameron) have included the facility as part of successful NIH and NSF grant applications.

4.1 Examples of applications on the Auto-iTC200

The Bevilacqua lab from the Chemistry department has employed the high through-put and low sample requirements of the Auto-iTC200 in order to screen for NTP interactions with the protein kinase PKR showing that only ATP binds to the protein [Toroney *et al.*, 2012] (Fig. 7). These features also were essential in determination of the binding heat capacity for magnesium ion interactions with ATP and tRNA by rapidly performing a series of repeated titrations at different temperatures [Sokoloski, 2011]. Both of these studies benefited from the inherent advantages of the Auto-iTC200 as these series of measurements could be accomplished using the same amount of sample as a single titration in a traditional calorimeter and each series was carried out in a single day compared to a week or longer timeframe for acquiring the same data using a traditional calorimeter.

The Showalter lab from the Chemistry department applies biophysical chemistry techniques to understand the function of intrinsically disordered proteins and to define the features of protein-RNA interactions. They have had multiple users running hundreds of experiments on the Auto-iTC200 and have effectively utilized the automation for maximum throughput, which has enabled the investigation of DNA binding by the homeodomain transcription factor Pdx1, which elucidated the determinants of sequence selection that cause Pdx1 to select its target promoters against a non-specific DNA background. This study generated the first testable hypotheses regarding the mechanism whereby Pdx1 binds with differential affinity among its multiple validated targets [Bastidas *et al.*, 2013]. The Auto-iTC200 permitted the acquisition of up to 16 experimental titrations per 24 h period, even with water-water flushes included once per six experiments to provide extra cleaning. In a second study, binding between RNA and the protein TRBP2 was evaluated at different temperatures, in order to discover the thermodynamic driving forces behind target-RNA acquisition by

TRBP. Due to the added time needed for temperature equilibration in these experiments it was more typical to complete 10–12 experimental runs per 24 h period.

Other Auto-ITC applications include

- The Anantheswaran lab from the Food Science department studies the microwave processing and packaging of foods; as part of their study on diffusion of nisin through packaging materials they studied the binding interaction between nisin with food additives chitosen and alginate.
- The Benkovic Group from the Chemistry department is engaged in a variety of projects connected by the general theme of understanding enzyme catalysis at various levels. They used the Auto-iTC200 to study the binding between human PCNA and Pol η .
- The Boehr lab's research in the Chemistry department is directed towards understanding catalytic mechanisms through the biophysical study of enzymes. The Auto-iTC200 was used in a study to assess the binding between a peptide and four different types of proteins. The user was able to set up multiple runs over several days to optimize conditions for each protein.
- The Cameron lab in the Biochemistry and Molecular Biology department has research interests including the role of RNA polymerases and RNA-binding proteins in viral infection and mitochondrial disease. The Auto-iTC200 was used in studying the binding reaction between the protein Bin1-SH3 and the intrinsically disordered domain from the protein NS5A, as well as interactions involving full-length NS5A. Similar studies were conducted with the cSrc-SH3 domain for comparison to the Bin1 data. In a very systematic study, the user made use of over 80 ITC experiments and also used the method of concatenation with continuous injections [Grossoehme, Akilesh, Guerinot & Wilcox, 2006; Grossoehme, Spuches & Wilcox, 2010] to reach saturation when concentrations were limited.
- The Elias lab from the Food Science department aims to understand the mechanistic basis of chemical food stability, especially as it relates to both product quality and health consequences in consumers. The Auto-iTC200 was used to study the interaction between gliadin (a major protein in gluten) with gallic acid and catechin, which required running 33 experiments using three different pH levels.
- The Golbeck lab at the Biochemistry and Molecular Biology department conducts research with emphasis on photosynthesis. Binding of menadione and quinone with proteins of the photosystem I reaction center were studied. Three different buffer concentrations were used in the preliminary phase to quickly determine best buffer conditions.
- The Thomas lab from the department of Biochemistry and Molecular Biology used the Auto-iTC200 to quantify the oligomerization-interaction between the

tetramerization domains of α , β and β [heavy] spectrins and several variants from the fruit fly genus *Drosophila* [Khanna *et al.*, 2015].

- The Tien lab from the Biochemistry and Molecular Biology department focuses its research in three areas: characterization and biochemical analysis of cellulose synthesis in a variety of organisms, mechanism and regulation of fungal degradation of lignin and regulation of fungal degradation of lignin, and dissimilatory iron reduction. The Auto-iTC200 was used to study the interaction between the protein CCpax and cellulose.
- The Wood lab, which is affiliated with both the Biochemistry and Molecular Biology department and the Chemical Engineering department, studies the physiological relevance of bacterial toxin/antitoxin systems, the genetic basis of biofilm formation, and mechanisms to control biofilm formations for engineering applications. Binding affinity was studied between the protein Ygis and its small molecule target, deoxycholate. In a second project, the Auto-iTC200 was used to study the interaction of 5 different metals with the protein RaIR, and separately with DNA, using three different buffers. The user was able to perform more than 60 experiments over a four-day period to find the best binding conditions.
- The Zydney lab from the Chemical Engineering department has research interests including bioseparations, artificial organs, and membrane processes. ITC was used to study the binding between DNA and various amino acids.

4.2 Examples of applications on the VP-cap DSC

The Showalter lab from the Chemistry department utilizes the VP-cap DSC extensively in preparation for ITC, NMR spectroscopy, and many biochemical experiments. Critically, many of the Showalter laboratory's RNA binding experiments must be performed at high RNA concentrations, where hairpin structures are often less thermodynamically stable than biologically irrelevant homo-dimers. The VP-cap DSC is used to establish conditions under which RNA samples retain their biologically relevant hairpin structures, which have distinct melting profiles from the duplex artifacts (Fig. 8). Additionally, many of the Showalter laboratory's ITC experiments are performed in temperature-dependent series that can only be designed effectively if the thermal denaturation midpoint temperature is known for all involved biological macromolecules. The VP-cap DSC provides a robust and time-efficient mechanism to generate this essential data because ten runs or more can be collected in an overnight period, allowing many conditions to be efficiently screened. The screening capabilities of the VP-cap DSC thus make it a helpful companion to the Auto-iTC200.

Other VP-cap DSC applications include

- Fujirebio diagnostics Inc. tested the stability of six different antibodies in six different buffer formulations varying sucrose concentrations and pH. All tests were done in duplicate and 96 tests were completed over a five-day period.
- The Cremer group in the Chemistry department works at the crossroads of biological interfaces, nanomaterials, spectroscopy, and microfluidics. DSC was used to study the effect of ions on polyethylene oxide at varying Na_3PO_4

concentrations. They also compared the effect of solvation in H₂O versus D₂O on this interaction. A second study was conducted to measure the thermal melting temperature and enthalpy of unfolding seen for various proteins, the polymer PNIPAM-ddi, and elastin-like peptides in varying concentrations of TMAO.

- The Demirel lab from the Engineering Science and Mechanics department conducts research focusing on theory-driven functional materials synthesis and fabrication for designing novel engineering materials to produce next generation materials for an array of fields including energy, biomedicine and security/defense. They used the VP-Cap DSC to determine the stability of a squid protein known to have some unusual material properties.
- The Harte lab from the Food Science department focuses on structure-function properties of milk proteins, with an emphasis on casein proteins. They used the VP-Cap DSC to study how increasing concentrations of ethanol affect the stability of various proteins in cow's milk. Seventy eight experiments in total were conducted uninterrupted over a week with one easy setup.
- The Nixon lab from the Biochemistry and Molecular Biology department focuses on the functional basis of signal transduction and gene regulation by AAA+ ATPases in bacteria, degradation of lignocellulose by cellulases, and the synthesis of cellulose. They used the VP-Cap DSC to study the folding and unfolding of several cellulases.

5. Conclusions

In this article we described our high-throughput biological calorimetry facility including the instruments it houses, best practices for running the facility, and examples of the science facilitated. Having these calorimeters has inspired new science and collaborations across campus, which in turn has facilitated the writing of new grants. It has been a positive factor in new faculty recruitment. Automated calorimeters are fairly unusual on most college campuses; however, they offer many benefits to the campus and regional research community. Although the initial cost of acquisition is high, the costs of opening such a facility fit well into the funding levels and objective of most multi-user instrumentation grant programs. We hope to see an increase of high-throughput calorimeters in academic research and look forward to the new science they will generate.

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Figure 1. Auto-iTC200 instrument from MicroCal. This instrument allows up to four 96 well trays to be loaded and programmed without user intervention. Figure used with permission from Malvern.

Figure 2. VP-cap DSC Instrument from MicroCal. This instrument allows up to six 96 well trays to be loaded and programmed without user intervention. Figure used with permission from Malvern.

Figure 3. Plate setup for Auto-iTC200 Experiment. Screen shot of the setup window for Auto-iTC200. Shown are four wells, which hold sample, ligand, and buffer, and collect sample, which comprise one experiment. All 96 wells can be filled to give 24 experiments.

Figure 4.

Water test result for Auto-iTC200. Test is done with titration of water against water and reference power set to $5\mu\text{cal/sec}$. Shown here is a series of 19 injections of $2\mu\text{l}$ each. An acceptable result is an upward deflection from heat of mixing of not more than $0.25\mu\text{cal/sec}$, drift of a small fraction of the offset power with uniform and sharp injection peaks.

Figure 5.
Noise test to ensure that the electronics in the instrument is working optimally.

Figure 6.

Titration of Ca^{2+} into EDTA to check if the thermodynamic parameters obtained after analysis of the binding curve passes the standard test. (A) Injections of Ca^{2+} . (B) Integrated binding curve. Successful sample test results will have stoichiometry N, binding affinity K_a and enthalpy ΔH within the limits listed for the standard, which are described in the text.

Figure 7.

Sample output from Auto-iTC200. ITC titration curves for ATP binding to K296R mutant of PKR. Titration of ATP into buffer is included (black trace). ATP titration curve is fitted to a two-site binding model. The major contribution is with site 1 ($n = 1.32 \pm 0.09$) and a K_d of $19.6 \pm 1.8 \mu\text{M}$. Figure adapted with permission from Toroney et al. (2012), copyright (2012) Cold Spring Harbor Press.

Figure 8.

Sample output from VP-cap DSC. Screening for conditions that favor retention of RNA hairpin structure at high nucleic acid concentration is facilitated by the VP-cap DSC. In this experiment, 20 μ M RNA was screened for hairpin-to-duplex transitions as a function of total sodium chloride concentration (increasing from 40 mM through 200 mM in 40 mM increments, progressing from black to light grey).

Table 1.

Cost recovery of calorimetry facility for year 2014.

Auto- iTC200 Expense Preventative Maintenance ^a	\$6700
ITC 106 days – internal (Penn State) experiments @ \$65 per day	\$6900
Total	\$200

^aThe PM covers travel cost of service engineer, replacement of all tubing, pumps, cannula, checking for alignment, testing of all electronics, cleaning of cell and titration syringe, checking for leaks and performing all standard tests such as temperature validation, differential power (Y axis) validation, water/water validation, noise validation and Ca-EDTA validation.