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Cross-platform comparison of nucleic acid hybridization: toward quantitative reference standards^a

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Abstract

Measuring interactions between biological molecules is vitally important to both basic and applied research, as well as development of pharmaceuticals. While a wide and growing range of techniques are available to measure various kinetic and thermodynamic properties of interacting biomolecules, it can be difficult to compare data across techniques of different laboratories and personnel, or even across different instruments using the same technique. Here we evaluate relevant biological interactions based on complementary DNA and RNA oligonucleotides that could be used as reference standards for many experimental systems. We measured thermodynamics of duplex formation using Isothermal Titration Calorimetry, Differential Scanning Calorimetry, and UV-Vis monitored denaturation/renaturation. These standards can be used to validate results, compare data from disparate techniques, act as a teaching tool for laboratory classes, or potentially to calibrate instruments. The RNA and DNA standards have many attractive features including low cost, high purity, easily measureable concentrations, and minimal handling concerns, making them ideal for use as a reference material.

Keywords

Isothermal Titration Calorimetry; DNA; RNA; oligonucleotides; biomolecular interaction analysis; Differential Scanning Calorimetry

Introduction

Characterizing interactions between and within biomolecules can be critical to understanding biology and to devising pharmaceuticals to intervene where normal biological pathways fail [1], [2]. A small arsenal of techniques is available to measure various aspects

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of biomolecular interactions, including Surface Plasmon Resonance (SPR) [3], Nuclear Magnetic Resonance (NMR) [4], Isothermal Titration Calorimetry (ITC) and Differential Scanning Calorimetry (DSC) [5], Polyacrylamide Gel Electrophoresis (PAGE) [6], fluorescence [7] and the Scintillation Proximity Assay (SPA) [8]. Considering the wide range of techniques, it can be challenging to interpret different results across various platforms. This is especially true in quantifying nucleic acid interactions with other nucleic acids, protein/peptides and small molecules.

To this end, it would be useful to have a well-characterized cross-platform biological reference standard. While many instruments use internal calibration standards (e.g. acid-base titrations in ITC [9], [10]), these standards are often specific for a single instrument and not transferable to other techniques, and may have little to no relevance to biological macromolecular interactions. We believe that nucleic acids could be an ideal cross-platform reference for several reasons: 1) Nucleic acid base pairing interactions are “tunable” based on the base content and length, 2) Oligonucleotides are readily available at low cost and high purity, 3) Oligonucleotides can be readily modified with a wide array of labels, and 4) they can be stored and handled without degradation.

We designed and evaluated a set of nucleic acids to be used as references for measuring biological interactions. We designed complementary 11-mer oligonucleotides in both DNA and RNA containing negligible secondary structure, as well as truncated versions consisting of 7 nucleotides. We chose to evaluate both DNA and RNA because each has practical advantages depending on the usage, with DNA being cheaper and more stable, and RNA being useful where RNase contamination is of concern. We measured the thermodynamic properties of the oligo duplexes with ITC, DSC, and UV-vis monitored denaturation/renaturation (melting) experiments. Our results provide useful reference points for researchers measuring biomolecular interactions with these or other methods.

Materials and Methods

Materials

Eight oligonucleotides were purchased from Integrated DNA Technologies (IDT) with standard desalting and deprotection (Table 1). IDT claims an average coupling efficiency of 99.2% per reaction, giving an approximate full length yield of 92% for 11-mers and ~95% for 7-mers. We verified the purity of the oligos using UPLC (Waters Corporation) and gel electrophoresis, and found a dominant single peak consistent with published purities. Phosphate buffered saline, PBS, buffer was made from a 10x concentrated solution (Fisher Scientific BP, with the 1x solution consisting of 11.9 mM Phosphates, 137 mM Sodium Chloride, and 2.7 mM Potassium Chloride at pH 7.4).

Extinction coefficient measurements

Extinction coefficients for the eight oligos were determined by monitoring UV absorbance of individual oligo solutions during digestion by Phosphodiesterase I as described previously [11]. Disposable semi-micro UV transparent cuvettes (Brand Scientific) were filled with 1.6 mL of PBS and individually zeroed. Concentrated oligos were added to each cuvette except

for one blank (20 μL for DNA and 30 μL for RNA - to give a final absorbance at 260 nm between 0.1 and 0.25 $\text{OD}_{260}/1.6 \text{ mL}$) and mixed before measuring the absorbances again. Phosphodiesterase I (10 μL) from snake venom (Sigma) was added to each cuvette. The extinction coefficient at room temperature was calculated by applying the sum of nucleotide absorbances to the hydrolyzed oligos, and extrapolating to the room temperature absorbances [11], [12].

Isothermal Calorimetry (ITC)

All ITC experiments were performed using the low volume Nano ITC (TA Instruments) at 25 and 37 $^{\circ}\text{C}$. The instrument was factory calibrated using $\text{KHCO}_3\text{-HCL}$ titrations to determine the effective cell volume. The injection syringe was calibrated by mass of water [9], [13]. Experiments consisted of a solution of one oligo (the “B” strand) was titrated into a solution of its complement in the cell (the “A” strand) using twenty 2.5 μL injections spaced 200 s apart, with 350 rpm stirring. The burette was driven to 99% volume before the syringe was loaded to minimize the effects of mechanical backlash [14]. Experiments were performed in triplicate at various temperatures, using the same stock solutions where possible. Integration of the heat curves was performed using the NanoAnalyze software (TA Instruments), with automated baseline construction, integrating from the injection start to 150 s post injection. The curves were individually fit with two state binding models with a constant baseline adjustment to account for the mixing enthalpy contribution; these models been described in detail elsewhere [15]. This constant baseline was taken as a single value for all runs at a given temperature, determined by averaging the fit result from six runs comprising the DNA and RNA 11-mers, which gave well-defined baselines. The first data point was excluded from analysis as is the convention. Additionally, there was one other injection anomaly (representing 0.2% of the data), which was ignored in fitting but treated as a partial injection in determining the stoichiometry. Final values and error estimates for enthalpy, stoichiometry, and K_a were determined by using the mean and standard deviation of the fitted results for each of the triplicate experiments.

UV-vis Experiments

All UV melting experiments were performed in an Agilent Cary 100 system. Semi-micro quartz cuvettes held 1 mL DNA or RNA solution at various concentrations. The concentrations used were serially diluted by 2x from stocks with absorbances near 2.0, such that the range extended to absorbances as low as 0.05. The temperature was ramped between 10 $^{\circ}\text{C}$ and 90 $^{\circ}\text{C}$ at 2 $^{\circ}\text{C}/\text{min}$ for duplex melting experiments, and these conditions varied slightly for individual strand melting experiments (melting temperatures were unaffected by changing ramp speeds from 1 $^{\circ}\text{C}/\text{min}$ to 2 $^{\circ}\text{C}/\text{min}$). The temperature was monitored in a dummy cuvette at the height of the light path. Experiments were performed in triplicate and curves were averaged. Normalizing raw data traces by the high temperature absorbance collapsed all three curves together, suggesting that variation between curves is mostly due to evaporation. Forward and reverse curves were also superimposable in this way, suggesting that our ramp rate was slow enough to allow for equilibration at all temperatures. Averaged curves were converted to curves of fraction folded as described elsewhere [16]. Regions of full association and dissociation were chosen differently for each of the 4 duplexes (dependent on the melting temperature), but the same for various concentrations of each

duplex. Two types of melting analysis were performed: 1) Van't Hoff analysis and 2) analysis of the concentration dependence of the melting temperature. For Van't Hoff analysis, the highest concentration curve was used to determine the derivative of $\ln(K_a)$ with respect to $(1/T)$ at the melting temperature. This value was multiplied by the gas constant R to determine the H_{VH} for the interaction. Error was taken as 2% based on the magnitude of local variations in the value of the derivative. For the concentration dependence of melting temperature, $1/T_m$ was plotted against $\ln(C)$, where C is the concentration of oligo. This data, with error bars representing a 0.5°C in temperature and 10% in concentration, was fit with a line. Dividing the gas constant R by the slope of the line was used to determine H_{conc} . These analysis methods are presented in detail elsewhere [16].

Differential Scanning Calorimetry (DSC)

All DSC experiments were performed on a Nano DSC (TA Instruments) with a 300 μL cell volume. Duplexes were formed by mixing the complementary DNA or RNA strands in equimolar concentrations from various stock concentrations (between 95 and 142 μM). The duplexes were dialyzed against 500 mL of PBS buffer for 18 hours at 4°C with a 1 kDa cutoff to ensure matching conditions between sample and reference cells. The 260 nm absorbances of the dialyzed duplexes were measured before the experiment. Final duplex concentrations were as follows: 47.6 μM for RNA 11-mer, 63.1 μM for DNA 11-mer, 65.5 μM for RNA 7-mer, and 65.8 μM for DNA 7-mer. Both the sample and matching reference buffer were extensively degassed under vacuum for at least 20 minutes to prevent bubbles. Scans were performed from 10°C to 100°C at $2^\circ\text{C}/\text{min}$, at a pressure of 3 atmospheres. The first heating and cooling cycle of each experiment was discarded due to its unique thermal history. Three additional heating and cooling scans were performed to ensure reproducibility. A reference scan was made under the same conditions using reference buffer in both cells. Data analysis was performed using the Nanoanalyze software (TA Instruments) and Origin 9.1 (Originlabs). Two representative reference scans (one each for heating and cooling) were subtracted from all duplex scans, and a partial specific volume for RNA was assumed to be $0.5\text{ cm}^3/\text{g}$ based on previous reports [17]. We used spline interpolation between pre-transition and post-transition regions to define the baseline that was used for integration and determination of H and S [18]. Specifically, a spline was fit to 6 data points consisting of 3 low temperature points and 3 high temperature points. These temperatures were chosen consistently for each duplex, but varied slightly between the 7- and 11-mers to accommodate shifting melting temperatures. For each duplex, the three heating scans and three cooling scans were individually analyzed and the results were averaged.

Gel Electrophoresis

Single and double stranded oligomers were analyzed with a 15% polyacrylamide gel (Bio-Rad Mini-Protean precast gel) in 0.5x Tris borate EDTA (45 mM Tris base; 45 mM boric acid; 1 mM EDTA, TBE) buffer. A Ficoll based loading buffer was used for the samples. The gel was run at 150 V for 60 min, and post-stained for 20 min in a 1x SYBR Gold solution (Life Technologies). The stained gel was imaged using a Bio-Rad Gel Doc XR+ system.

Results

We designed and evaluated a set of RNA and DNA oligos to be used as reference standards for measurements of biomolecular interactions. An 11-mer RNA duplex was designed to have properties similar to the Dickerson dodecamer [19], [20]. The length was chosen to be one full A-form helical turn. The sequences of the two individual 11-mer strands were designed to avoid all possible self-complementariness so that they would not self-interact, or fold on oneself (Table 1). Predictions in Mfold [21] show that the sequences exhibit no secondary structure, and a search in BLAST [22] reveals no such genomic sequence. The shorter sequences are taken from the 11-mers (Table 1). Electrophoretic gel migrations of the individual strands and their duplexes were consistent with their lengths and whether or not they were duplexes. Varying the ratio of RNA 11-mer “A” strand with its complementary “B” strand demonstrated stoichiometric 1/1 duplex formation when the constructs were run on a gel (Figure 1). In all cases, the concentration-limited strand appears to be present only in the duplex, indicating a dissociation constant significantly smaller than the micromolar level concentrations used in the gel.

Room temperature extinction coefficients of the oligos at 260 nm were determined to ensure accurate concentrations requisite for extracting thermodynamic parameters. DNA and RNA oligos were fully hydrolyzed using phosphodiesterase I from snake venom and room temperature extinction coefficients were calculated using base extinction coefficients and the ratio of intact vs. hydrolyzed oligos (Table 1) [11], [12]. The errors are estimated to be less than 2% based on the primary source of measurement error – concentration changes due to evaporation from the cuvettes. Consistent with previous work [11], we found that our measured values were significantly lower than calculated extinction coefficients for these sequences. For example, the manufacturer (Integrated DNA Technologies) reported extinction coefficients for the RNA 11-mers of 113000 L/mol-cm and 96700 L/mol-cm for strands A and B, overestimating the measured values by 11% and 10%, respectively. We additionally measured the change in absorbance of the individual strands for a temperature ramp between 20°C and 90°C (Supplemental Figure 1). We found that the RNA “A” strands have changes in absorbance near room temperature, but none of the other strands show this behavior. We attribute this behavior to a tendency of the “A” strands to form homodimers, which is supported by predictions in Mfold and supported by other experimental evidence from Isothermal Titration Calorimetry (ITC) data at different temperatures (see below). It is likely not seen in the DNA “A” strands due to the lower stability of the homodimers in DNA than in RNA. The changes in absorbance for the individual strands during temperature ramping do not recapitulate the changes seen from the digestion. This suggests that high temperature should not be substituted for digestion in determining accurate extinction coefficients for oligos, as is sometimes done [23], [24].

One of the most common and useful measures of biomolecular interactions is the association constant K_a (or its inverse K_d), which reports the affinity of the two molecules at equilibrium. ITC is becoming a useful tool by the biophysical community of scientists as a means to assess oligonucleotide interactions as well as to understand the thermodynamics of these interactions [25]–[27]. Using ITC, we measured the exothermic heat of reacting one strand with the other as the duplex was formed (Figure 2a,b). Experiments were performed

in triplicate from a single set of working solutions whose concentrations were determined by a Nanodrop spectrophotometer using the extinction coefficients previously determined. The data from experiments in PBS at 25 °C and 37 °C were analyzed with and fit a two-state model (Table 2 and Figure 2c).

As expected, the stoichiometry for all experiments was near 1.0 and typically within a few percent of 1.0, indicating a 1:1 reaction between strands. For all duplexes, the stoichiometry decreased as the temperature was increased from 25 °C to 37 °C, even when the same stock solutions were used for both temperatures. This suggests that at lower temperatures, some fraction of strands is unavailable to form duplex during the injection due to self-interaction or dimerization. This deviation was especially dramatic for the shorter strands, which is likely due to higher uncertainty in concentration measurement at the higher concentrations used due to homodimer formation. It could also be partially attributed to the greater difficulty in accurately determining the stoichiometry as the association constant decreases.

At 37 °C, where self-interactions and homodimers are minimized, association constants and enthalpies behaved as expected, increasing with longer strands and increasing from DNA to RNA. Somewhat counter intuitively, the enthalpy of formation of all four duplexes becomes more favorable as the temperature increases from 25 °C to 37 °C. This again suggests that the oligos have some structure at 25 °C, but that much of this structure is removed once the temperature is increased to 37 °C. This is consistent with the tendency of the “A” strands to form homodimers. The ITC data suggests that nearly all of the material is forming duplex at these conditions (within 150 seconds), but that there is a small enthalpic penalty for removing structure from the isolated oligo solutions before forming the enthalpic favored duplex.

Complementary experiments were performed by heat denaturing the duplexes using temperature ramps. These were performed using both DSC, which measures the power required to maintain the reference cell at the same temperature as the sample cell, and UV melting at 260 nm, which measures changes in absorbance due to base stacking. The DSC experiments, performed by ramping the temperature from 10 °C and 100 °C at 2 °C/minute, were conducted with the pressure held at 3 atmospheres, enough to prevent evaporation but not to significantly affect thermodynamics of duplex formation [28]. Data between heating and cooling cycles was repeatable, showing only a fraction of a degree shift in melting temperature in the most extreme cases (Figure 3a).

The integral between the molar heat capacity curve and baseline for all four duplexes is equal to ΔH between the folded and unfolded states (Figure 3b; Table 3). These data follow the same trend as did the ITC data, and for three of the four duplexes the enthalpies are within about 10% of those determined from ITC at 37 °C. The errors listed from the table are statistical errors from the automated integration routine, but we noticed that choice of baseline endpoints and shape (e.g. linear, cubic, spline, etc.) could result in changes up to 10% as well. The DSC results from the 7-mer DNA differed substantially from that of the ITC experiments, underestimating ΔH by almost 40%. We believe this is likely due to the difficulty in establishing a reliable baseline since this duplex uniquely shows a steep pre-transition phase and a low melting temperature. The entropy values were determined by

dividing the curve by absolute temperature and integrating the area between the curves using the same spline parameters. We found entropies that were within error of RNA values calculated from ITC values at 37 °C, and within 30% for those of DNA. It is worth noting that the DSC curves all exhibit some asymmetry with a more gradual rise at low temperatures than the fall at high temperatures. This behavior is most pronounced for the 11-mers, and for the RNA in particular, suggesting less than perfect two-state behavior.

For UV melt experiments, similar protocols were employed, where the 260 nm absorbance of duplexes measured the change in base stacking. Again we found repeatable data between subsequent heating and cooling cycles, with only slight shifts in absorbance at high temperature due to evaporation (Figure 4a). We measured the melting behavior at a variety of different concentrations, and found increasing melting temperatures as concentrations were increased (Figure 4b). Enthalpies of formation were determined from the data by two methods: van't Hoff analysis (Figure 4c) and concentration dependence of melting temperature (Figure 4d). Van't Hoff analysis of the data (Table 3) shows some non-linearity for all duplexes, while the concentration dependence of the melting temperature shows near perfect linearity over the concentrations probed. Enthalpies determined from these methods were typically more negative than those determined from the other methods, sometimes dramatically so. This likely arises since both analysis methods are valid only for two state reactions, and we have already shown deviation from that behavior in the previous experiments.

Discussion

Knowledge of thermodynamics, stoichiometries and other biophysical parameters of RNA foldings and its interactions with other RNAs, proteins and small molecules is key to understanding of RNA biology, RNA as target, tool and possible therapeutic. However, the academic and biotechnology communities lack oligonucleotide standards for the many different types of instrumentation relevant to this research. RNA and DNA standards would be useful across various measurement technology platforms, especially for RNA, in order to maintain instrument reliability from day to day, and week to week, and to compare one's own data with published data. We have evaluated RNA and DNA oligonucleotide duplexes of different lengths using three methods to quantify thermodynamic properties. We found good agreement between calorimetric methods for most duplexes, but there were still some differences that warrant further discussion.

One issue is the comparison of enthalpy among the various techniques. Several recent papers have commented on the difficulty in comparing results from these experimental methods, specifically in comparing van't Hoff and calorimetric enthalpies [29]–[33]. It is noted that enthalpies determined at low temperature using ITC are not predictable from enthalpies measured over a narrow temperature range near T_m in melting experiments unless

C_p is known. Since C_p is not well known in this case, it adds additional uncertainty in comparison of the measured enthalpies. A second issue in comparing results is the analysis methods used. Both ITC data and UV-vis data require fitting of a two state model to extract thermodynamic data. For ITC, this is probably a valid approximation for the ensemble since there are vast energy differences between the single strands and the duplex at experimental

temperatures. Any intermediate states that may exist are likely to be rapidly populated and unpopulated within the reaction time of 150 s. The ITC data additionally supports the two state model, with near perfect correspondence between model and data for all experiments. For UV-vis data, the assumption of a two-state model is probably not valid, as evidenced by non-linear van't Hoff curves and asymmetrical curves from DSC experiments. The non-linearity in the van't Hoff curves may also be due in part to heat capacity effects. However, the slope changes in our van't Hoff curves are more dramatic (up to 2.6 kcal/mol/K) than would be expected from heat capacity changes for duplex formation, which are typically smaller [26]. We suspect that intermediate states may be significantly populated at elevated temperatures, but that these states are only transiently sampled in isothermal experiments. Analysis of UV-vis data can also be affected by subjectivity in choosing the baseline regions that define "fully associated" and "fully dissociated" states. DSC analysis is in principle model independent, but requires defining a baseline to perform integration, which can again introduce subjectivity. These issues can contribute to uncertainties that go beyond the errors reported from statistical variations of multiple experiments.

Additional uncertainty arises if the concentration of oligos is not well known, since the thermodynamic measures here all depend at least on relative concentrations (for UV-Vis) and sometimes on absolute concentrations (for calorimetry). We have mostly addressed this issue by measuring the extinction coefficients. However, the noted propensity of some of the "A" strands to form homodimers may complicate the ability to determine concentrations accurately. The primary effects of homodimer formation as they relate to these experiments are 1) to introduce additional uncertainty in the measurement of concentration for those strands, and 2) to introduce a "reference" enthalpy for the unpaired duplex. The first effect arises since homodimer formation is concentration dependent, and homodimerized oligos absorb more UV light than free oligos due to hyperchromic shift. Practically speaking, this results in the extinction coefficient being concentration dependent. The second effect is due to the highly favorable enthalpy of duplex formation being slightly offset by the unfavorable enthalpy of dissociating homodimers. This effect is mainly evident with ITC, where both strands are mixed at constant temperature, and the measured enthalpy for the reaction is less than the enthalpy for duplex formation from separated strands. This is further substantiated by the increase in measured enthalpy with increasing temperature for all of our ITC experiments, since higher temperatures cause fewer of the strands to be involved in homodimer formation. For melting experiments, the effect of homodimers is minimal if the experiment is done at equimolar concentrations or with an excess of "B" strands, since full duplex formation is highly energetically favored over homodimer formation. In the future, we hope to optimize the oligo sequences to minimize homodimer formation without compromising the negligible secondary structure of individual oligos or the deep free energy minimum of full duplex formation. This would necessarily involve significant computational effort to develop in silico "screening" to find those sequences that give the closest approximation to true two state behavior.

There is significant utility in developing short DNA and RNA duplexes into reference standards, considering the lack of cross-platform standards for biomolecular nucleic acid interaction analysis by today's highly sensitive instrumentation. One of the most notable features of using nucleic acids for this purpose is that their interactions are predictable and

“tunable” depending on the number of interacting base pairs. Thus, they can be used both as absolute standards for quantitative instruments, or as relative standards for qualitative or semi-quantitative instruments. Their tunable nature also enables their use for instruments that may be able to study only a limited range of kinetics or equilibrium constants. This is a common problem with other interactions, which may be either “too weak” or “too strong” for certain instruments. For our experiments, we found data to be very repeatable, indicating that the DNA and RNA were stable for the duration of the experiments. Their tunable nature has been demonstrated in the use of various lengths as qualitative standards of size in gel electrophoresis. However, the compositions of these ladders differ from the various commercial sources and are not useful in standardizing other instruments. Characterized nucleic acids of tunable length could be useful in standardizing and calibrating separations from day-to-day, especially in use of ultrahigh performance liquid chromatography. Nucleic acid standards are attractive since oligos are commercially available at low cost and high purity, and their concentrations can be readily measured by spectrophotometry. Thus, DNA and RNA standards have the potential for use in a broad set of instrumentation, and could additionally be used as a training tool for laboratory staff or students.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Bongrand P. Ligand-receptor interactions. *Reports on Progress in Physics*. 1999; 62:921.
2. Bleicher KH, Böhm HJ, Müller K, Alanine AI. A guide to drug discovery: Hit and lead generation: beyond high-throughput screening. *Nat Rev Drug Discov*. May; 2003 2(5):369–378. [PubMed: 12750740]
3. Myszkka DG. Kinetic, equilibrium, and thermodynamic analysis of macromolecular interactions with BIACORE. *Meth Enzymol*. 2000; 323:325–340. [PubMed: 10944758]
4. Fielding L. NMR methods for the determination of protein-ligand dissociation constants. *Curr Top Med Chem*. 2003; 3(1):39–53. [PubMed: 12577990]
5. Jelesarov I, Bosshard HR. Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition. *J Mol Recognit*. Jan; 1999 12(1):3–18. [PubMed: 10398392]
6. Hellman LM, Fried MG. Electrophoretic mobility shift assay (EMSA) for detecting protein–nucleic acid interactions. *Nat Protoc*. Aug; 2007 2(8):1849–1861. [PubMed: 17703195]
7. Royer CA, Scarlata SF. Fluorescence approaches to quantifying biomolecular interactions. *Meth Enzymol*. 2008; 450:79–106. [PubMed: 19152857]
8. Bylund DB, Toews ML. Radioligand binding methods: practical guide and tips. *Am J Physiol*. Nov; 1993 265(5):L421–9. [PubMed: 8238529]
9. Tellinghuisen J. Calibration in isothermal titration calorimetry: Heat and cell volume from heat of dilution of NaCl(aq). *Analytical Biochemistry*. Jan; 2007 360(1):47–55. [PubMed: 17107650]
10. Sgarlata C, Zito V, Arena G. Conditions for calibration of an isothermal titration calorimeter using chemical reactions. *Anal Bioanal Chem*. Nov; 2012 405(2):1085–1094. [PubMed: 23196751]

11. Kallansrud G, Ward B. A comparison of measured and calculated single- and double-stranded oligodeoxynucleotide extinction coefficients. *Analytical Biochemistry*. Apr; 1996 236(1):134–138. [PubMed: 8619477]
12. Cavaluzzi MJ. Revised UV extinction coefficients for nucleoside-5'-monophosphates and unpaired DNA and RNA. *Nucleic Acids Research*. Jan; 2004 32(1):13e–13.
13. Demarse NA, Quinn CF, Eggett DL, Russell DJ, Hansen LD. Calibration of nanowatt isothermal titration calorimeters with overflow reaction vessels. *Analytical Biochemistry*. Oct; 2011 417(2): 247–255. [PubMed: 21741951]
14. Mizoue LS, Tellinghuisen J. The role of backlash in the 'first injection anomaly' in isothermal titration calorimetry. *Analytical Biochemistry*. Mar; 2004 326(1):125–127. [PubMed: 14769346]
15. Freire E, Mayorga OL, Straume M. Isothermal titration calorimetry. *Anal Chem*. 1990
16. Mergny JL, Lacroix L. Analysis of thermal melting curves. *Oligonucleotides*. 2003; 13(6):515–537. [PubMed: 15025917]
17. Durchschlag, H. *Thermodynamic data for biochemistry and biotechnology*. Springer; 1986. Specific volumes of biological macromolecules and some other molecules of biological interest; p. 45–128.
18. Spink, CH. *Methods in Cell Biology*. Vol. 84. Elsevier; 2008. *Differential Scanning Calorimetry*; p. 115–141.
19. Wing R, Drew H, Takano T, Broka C, Tanaka S, Itakura K, Dickerson RE. Crystal structure analysis of a complete turn of B-DNA. *Nature*. Oct; 1980 287(5784):755–758. [PubMed: 7432492]
20. Drew HR, Wing RM, Takano T, Broka C, Tanaka S, Itakura K, Dickerson RE. Structure of a B-DNA dodecamer: conformation and dynamics. *P Natl Acad Sci Usa*. Apr; 1981 78(4):2179–2183.
21. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research*. Jul; 2003 31(13):3406–3415. [PubMed: 12824337]
22. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. Oct; 1990 215(3):403–410. [PubMed: 2231712]
23. Pan T, Sosnick TR. Intermediates and kinetic traps in the folding of a large ribozyme revealed by circular dichroism and UV absorbance spectroscopies and catalytic activity. *Nat Struct Biol*. Nov; 1997 4(11):931–938. [PubMed: 9360610]
24. Hartmann, RK. *Handbook of RNA biochemistry*. Weinheim: Wiley-VCH; 2009.
25. Feig AL. Applications of isothermal titration calorimetry in RNA biochemistry and biophysics. *Biopolymers*. 2007; 87(5):293–301. [PubMed: 17671974]
26. Mikulecky PJ, Feig AL. Heat capacity changes associated with nucleic acid folding. *Biopolymers*. 2006; 82(1):38–58. [PubMed: 16429398]
27. Feig, AL. *Methods in Enzymology*. Vol. 468. Elsevier; 2009. *Studying RNA–RNA and RNA–Protein Interactions by Isothermal Titration Calorimetry*; p. 409–422.
28. Macgregor RB. Chain length and oligonucleotide stability at high pressure. *Biopolymers*. 1996; 38(3):321–328. [PubMed: 8906968]
29. Holbrook JA, Capp MW, Saecker RM, Record MT. Enthalpy and Heat Capacity Changes for Formation of an Oligomeric DNA Duplex: Interpretation in Terms of Coupled Processes of Formation and Association of Single-Stranded Helices †. *Biochemistry*. Jun; 1999 38(26):8409–8422. [PubMed: 10387087]
30. Liu Y, Sturtevant JM. Significant discrepancies between van't Hoff and calorimetric enthalpies. III. *Biophys Chem*. Feb; 1997 64(1):121–126. [PubMed: 17029832]
31. Chaires JB. Possible origin of differences between van't Hoff and calorimetric enthalpy estimates. *Biophys Chem*. Feb; 1997 64(1):15–23. [PubMed: 9127935]
32. Horn JR, Russell D, Lewis EA, Murphy KP. Van't Hoff and calorimetric enthalpies from isothermal titration calorimetry: are there significant discrepancies? *Biochemistry*. Feb; 2001 40(6):1774–1778. [PubMed: 11327839]
33. Naghibi H, Tamura A, Sturtevant JM. Significant discrepancies between van't Hoff and calorimetric enthalpies. *P Natl Acad Sci Usa*. Jun; 1995 92(12):5597–5599.

Figure 1. Electrophoresis of oligo standards

Increasing molar ratios of “B” strands to “A” strands of the RNA 11-mer oligos from left to right. Distinct bands are visible for single stranded “A” and “B” strands and the duplex “AB”. The center lane has only a slight excess of strand “B”, and is the first lane from the left to exhibit single-stranded “B” strand (black triangle) in addition to the duplex, consistent with a 1:1 stoichiometry.

Figure 2. Isothermal titration calorimetry of the RNA 11-mer duplex at 37°C

a) Representative raw data of the power required to maintain a constant temperature (exotherm up) for 2.45 μL injections of 18.9 μM RNA 11-mer strand “B” into 2.3 μM of RNA 11-mer strand “A” at 37°C. b) Total heat of each injection (squares), determined by integrating the area under each of the curves from (a) for the first 150 seconds following injection with a 2-state fit of the data overlaid (solid line). c) Summary of thermodynamic data from triplicate experiments for all 8 conditions. Error bars represent the standard deviation from the triplicate experiments.

Figure 3. Differential scanning calorimetry of nucleic acid duplexes

a) Heating and cooling curves for the RNA 11 bp duplex show minimal hysteresis ($\sim 0.5^\circ\text{C}$) at $2^\circ\text{C}/\text{min}$, and near identical peak areas. b) Representative heating traces for all 4 duplexes with arbitrary y-offsets. Grey lines show baselines used for determining integrated area to get H_{DSC} .

Figure 4. UV-vis spectrophotometry of the 11-mer RNA duplex melting

a) Representative raw traces of Absorbance as a function of temperature for heating and cooling ramps of 1 °C/min. Small divergence between curves occurs at higher temperatures as evaporation increases the concentration slightly. b) Normalized melt curves at 6 different concentrations (11.7 μM, 5.9 μM, 3.0 μM, 1.5 μM, 670 nM, and 320 nM) show the increase in melting temperature with concentration. c) Van't Hoff plot of curves from panel b, d) Linear plot showing concentration dependence of the melting temperature.

Table 1

Oligonucleotide properties

Designation	Sequence	MW (Da.)	Extinction Coefficient [*]
R11A	GACGUGCGAAG	3568.2	98529
R11B	CUUCGCACGUC	3402.1	88041
D11A	GACGTGCGAAG	3406.3	99081
D11B	CTTCGCACGTC	3268.2	84475
R7A	CGUGCGA	2219.4	58056
R7B	UCGCACG	2179.4	58251
D7A	CGTGCGA	2121.4	60685
D7B	TCGCACG	2081.4	57095

* Measured values at A260~0.2

Table 2

Measurements of duplex formation by Isothermal Titration Calorimetry

	RNA 11 mer		DNA 11 mer		RNA 7 mer		DNA 7 mer	
	25	37	25	37	25	37	25	37
Temperature (°C)								
[A strand] (μM)*	2.3		3.1		6.7	6.9	7.6	51.6
[B strand] (μM)*	18.2		20.1		34.9		52.4	221.4
Stoichiometry	1.02 ± .05	0.99 ± .01	1.03 ± .01	1.01 ± .02	0.98 ± .02	0.94 ± .02	1.07 ± .02	0.90 ± .01
Ka (1/μM)	205 ± 30	125 ± 41	268 ± 41	77 ± 20	102 ± 12	3.6 ± 0.8	5.0 ± 0.3	0.16 ± .01
H (kcal/mol)	-68.0 ± 2.6	-87.1 ± 0.9	-56.1 ± 1.8	-64.1 ± 1.7	-30.7 ± 0.3	-49.2 ± 1.6	-40.7 ± 0.7	-45.7 ± 0.3
S (cal/mol/K)§	-190.0 ± 8.4	-244.0 ± 0.5	-149.5 ± 6.4	-170.6 ± 5.3	-66.2 ± 1.3	-128.5 ± 5.6	-105.8 ± 2.5	-123.6 ± 0.8
G (kcal/mol)§	-11.3 ± 0.1	-11.5 ± 0.2	-11.5 ± 0.1	-11.2 ± 0.2	-10.9 ± 0.1	-9.3 ± 0.1	-9.14 ± 0.04	-7.38 ± 0.05

Note: errors represent only statistical variations between experiments

* Merged cells signify a common solution for multiple experiments

§ Calculated values from other parameters

Table 3

Measurement of duplex formation by DSC and UV melts

	RNA 11 mer	DNA 11 mer	RNA 7 mer	DNA 7 mer
H _{DSC} (kcal/mol)	-81.9 ± 2.6	-70.9 ± 1.3	-45.4 ± 3.0	-28.7 ± 2.0
S _{DSC} (cal/mol/K)	-240 ± 10	-212 ± 9	-139 ± 14	-87 ± 9
H _{VH} (kcal/mol)*	-85.9 ± 1.7	-79.3 ± 1.6	-63.4 ± 1.3	-51.5 ± 1.0
H _{Conc} (kcal/mol)*	-121.6 ± 3.7	-86.1 ± 2.0	-74.3 ± 1.0	-70.2 ± 0.8

* Assuming a two state system