



Published in final edited form as:

Methods Enzymol. 2009 ; 468: 371–387. doi:10.1016/S0076-6879(09)68017-4.

Optical Melting Measurements of Nucleic Acid Thermodynamics

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Abstract

Optical melting experiments provide measurements of thermodynamic parameters for nucleic acids. These thermodynamic parameters are widely used in RNA structure prediction programs and DNA primer design software. This review briefly summarizes the theory and underlying assumptions of the method and provides practical details for instrument calibration, experimental design, and data interpretation.

A theory is the more impressive the greater the simplicity of its premises is, the more different [*sic*] kinds of things it relates, and the more extended is its area of applicability. Therefore the deep impression which classical thermodynamics made upon me. It is the only physical theory of universal content concerning which I am convinced that, within the framework of the applicability of its basic concepts, it will never be overthrown. Albert Einstein (Einstein, 1970).

Nucleic acid folding is one area where the basic concepts of thermodynamics have found wide ranging applicability. RNA thermodynamic parameters have applications to diverse areas of study such as rhinovirus evolution and recombination (Palmenberg, 2009); antisense therapeutics, e.g. Vitravene, which is the first FDA-approved nucleic acid therapeutic and which targets cytomegalovirus in the human eye (Anderson, 1996); models of the HIV-1 RNA structural elements (Parisien, 2008; Wilkinson, 2008); cancer microRNA target specificity (Doench, 2004); the mechanisms of RNA interference (Ameres, 2007); the mechanism of group I introns (Bevilacqua, 1991; Narlikar, 1997; Pyle, 1994); the discovery of non-coding RNAs in genomes (Uzilov, 2006; Washietl, 2005); and tRNA codon recognition in protein translation (Ogle, 2002). In principle, thermodynamics can predict the populations of structures that would be present at equilibrium, although the current knowledge of the sequence dependence of nucleic acid thermodynamics limits the accuracy of such predictions. Much of the known thermodynamics has been measured by optical melting, which has several advantages over the more accurate calorimetric methods. Relatively small quantities of sample are required; the experiments are fast; and the

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instrumentation is relatively inexpensive. For example, if two 8-mer RNA oligonucleotides with internal loops are predicted to have different stabilities, with only approximately 1 μmol of each RNA and one day of optical melting experiments by a hard-working student, one can determine which internal loop is more thermodynamically stable. (Very few bets in the RNA world can be resolved so quickly!) This chapter provides details on the optical melting methods used most often, and includes both technical aspects and a discussion of the assumptions in interpretation.

Instrumentation

UV spectrometers suitable for optical melting experiments are commercially available from Beckman, Cary, and Shimadzu corporations. The primary requirements in a UV spectrometer are good optics; accurate, variable temperature control; and a cell holder for several small cuvettes. This article will discuss details of the Beckman DU800 spectrometer, but the general principles apply to all UV spectrometers. The Beckman DU800 spectrometer specifications for temperature are ± 1 °C from 20–60 °C with the DU800 high performance temperature controller unit, although the instrument range is 13–95 °C. A customized cell holder with chilled water circulation to remove heat from the peltier-controlled cell holder allows accurate ± 1 °C temperature control to 0 °C. Dry air or nitrogen gas flowing through the cell chamber prevents condensation on the cells at low temperatures. The microcell holder contains places for 6 cuvettes and uses the cell transporter unit. Standard Beckman cells have a 1 cm pathlength and a 400 μL volume. Custom quartz cells with pathlengths of 0.1 cm, 0.5 cm, 1.0 cm and volumes of 40 μL , 200 μL , and 400 μL , respectively, in dimensions that fit into the Beckman cell holder can be obtained from Hellma, Inc. and NSG Precision Cells.

Calibrations

The Beckman DU800 spectrometer software automatically runs several initialization calibration tests when the instrument is turned on. These tests are run with no samples in the instrument and the lid closed. The initialization tests check the gain, the visible lamp, the light path, the shutter, the filter, the wavelength drive, and the detector performance. Turn the instrument power off when not in use, so that these calibrations are automatically checked every time the instrument is used. In addition, the performance validation tests following the manufacturer's instructions should be run monthly to insure reliable instrument performance. The performance validation checks the wavelength accuracy (± 0.2 nm); wavelength repeatability (± 0.1 nm); resolution (< 1.80 nm); baseline flatness (< 0.0010 A); noise at 500 nm (< 0.000200 A); and stability at 340.0 nm for 60 minutes (< 0.0030 A drift). ("A" is a unit of absorbance defined by the NIST 930D solid filter at 546 nm.) Additional checks for temperature, absorbance, pathlength, and cell holder alignment can be done manually at installation and as necessary during use.

The temperature can be manually tested with a microprobe, such as the Ertco-eutechnics digital thermometer model 4400. Test the accuracy of the microprobe thermometer in a water bath and compare with an accurate mercury thermometer. Fill six 1-cm cuvettes with double distilled water and seal 5 cuvettes with Teflon tape and stoppers. Insert the

temperature probe into one of the cuvettes and seal with the small stopper around the probe. Check that the probe is directly upright in the cell and does not touch the sides of the cuvette. Keep the lid closed as much as possible while measuring temperature. The temperature of the cell holder can be manually set, and the actual temperature recorded by the software appears in the lower right hand corner of the screen. Check that the temperature is within ± 1 °C at all cell positions at several temperatures, for example 25, 35, 45, and 55 °C. Allow approximately 5 minutes for equilibration at each temperature. When checking the temperature at higher than 60 °C, take care to note any water evaporation. The temperature measurement will not be accurate if the cell is not full of water. Then check the temperature as if a melting experiment were being run with a heating rate of 0.5 or 1 °C per minute from 0–90 °C. Check the temperature in one of the first three and in one of the last three cell positions to insure that the peltier devices embedded in the bottom of the cell holder are accurately matched. The nitrogen flow, the chilled water flow, and the rate of heating can be adjusted so that the actual temperature of the cell matches the recorded temperature. Alternatively, temperature offsets can be included in the data analysis if there is a reproducible difference in temperature between cells, although this is not recommended.

The wavelength accuracy can be measured using a holmium oxide filter in place of the cell holder and scanning wavelength from 200–800 nm at a rate of 1200 nm/min. The shape and position of the peaks are the important features of the spectrum rather than the exact intensities. There should be three strong distinct peaks between 440 and 460 nm. Peaks should be clearly distinguished at 241.5, 279.3, 287.6, 333.8, 360.8, 385.8, 418.5, 453.4, 459.9, 536.4, and 637.5 ± 0.2 nm (Allen, 2007). Any peaks appearing below 225 nm indicate stray light in the instrument. If this scan does not show the appropriate peaks, then replace the UV bulb following the manufacturer's instructions or troubleshoot other possible problems in the optics.

The absorbance may be checked by measuring the absorbance of a known stock solution, such as 0.0400 g/L of K_2CrO_4 in 0.05 M KOH, referenced to a 0.05 M KOH solution at 25 °C. (Table 1)(Gordon, 1972). Measure the same sample in the same cell in each cell position to check the cell holder alignment. If the absorbance varies more than ± 0.0005 A at different cell positions, then rerun the transporter alignment with no samples in the cell holder. The service diagnostics calibrations can be run by a Beckman service technician to correct the alignment. Use the same solution with a known absorbance in cells of each pathlength and verify the pathlength accuracy using Beer's Law:

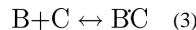
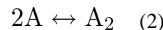
$$A = \epsilon cl \quad (1)$$

where A is absorbance; ϵ is the extinction coefficient; c is concentration; and l is the pathlength.

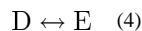
Brief Theory of Optical Melting Experiments

In principle, optical melting curves could be analyzed by a partition function approach in which every base pair is considered separately. This approach, however, would require a global fit of melting data for many sequences and refitting the data when additional sequences are added. Therefore data are typically analyzed with a two-state model, which

assumes that each strand is either completely paired or unpaired. The equilibrium for duplex formation is represented as either a self-complementary or non-self-complementary association (Cantor, 1980; Turner, 2000):



The equilibrium for a unimolecular transition, e.g. a hairpin, is represented as:



For self-complementary (eq.2) or non-self-complementary equilibria (eq. 3 with equal concentrations of B and C), the equilibrium constant is given by:

$$K=(\alpha/2)/(C_T/a)(1-\alpha)^2 \quad (5)$$

C_T is the total strand concentration:

$$C_T=[A]+2[A_2]_{\text{self-complementary}} \quad (6)$$

$$C_T=[B]+[C]+2[BC]_{\text{non-self-complementary}} \quad (7)$$

“a” has a value of 1 for self-complementary duplexes and 4 for non-self-complementary duplexes. α is the fraction of strands in a duplex. For a unimolecular transition,

$$K=\alpha/(1-\alpha)=[E]/[D] \quad (8)$$

$$\text{Where } \alpha=[E]/([D]+[E]) \quad (9)$$

Figure 1 shows data for the non-self-complementary duplex, 5'GAGCGACGAC3' / 3'CUCGAAGGCUG5'. At low temperatures, the strands are in a duplex and the absorbance is low. As the temperature is increased, the strands dissociate into single strands. The total concentration of strands can be measured using the absorbance at 80 °C and the extinction coefficient for that sequence. The difference in absorbance between duplex and single strands is the hyperchromicity. For self-complementary or non-self-complementary duplexes with equal concentrations of each strand, the melting temperature, T_M , in kelvins or T_m in degrees Celsius, is the point at which the concentrations of strands in duplex and in single strands are equal. The steepness of the transition indicates the cooperativity of the transition. The width and maximum of first derivative of the melting curve can also indicate the cooperativity and melting temperature, although the peak of the derivative curve only °C occurs at the T_M when the transition is unimolecular (Gralla, 1973; Marky, 1987). T_M is most accurately measured by fitting the lower and upper baselines (see below). The melting temperature is measured at several concentrations over a 100-fold range. The melting temperature is then plotted versus the concentration in a van't Hoff plot. The van't Hoff

equation relates the melting temperature in kelvins (T_M), strand concentration (C_T), enthalpy (ΔH°), and entropy (ΔS°):

$$1/T_M = (R/\Delta H^\circ) \ln(C_T/a) + \Delta S^\circ/\Delta H^\circ \quad (10)$$

where R is the ideal gas constant, $1.987 \text{ cal K}^{-1} \text{ mol}^{-1}$ or $8.3145 \text{ J K}^{-1} \text{ mol}^{-1}$. The slope of the van't Hoff plot gives the enthalpy change, and the y-intercept gives the ratio of entropy change to enthalpy change. The free energy and equilibrium constant at any temperature can then be calculated using Gibb's relation:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad K = e^{-\Delta G^\circ/RT} \quad (11)$$

The errors in enthalpy and entropy changes are typically around 10%. Because these errors are correlated, the errors in free energy change are typically 2%.

The melting curve can be fit using Meltwin software (McDowell, 1996), although other software is available to perform the same mathematical analysis (Draper, 2001; Siegfried, 2009). The Meltwin software uses seven parameters to fit each curve (Figure 1): the concentration, enthalpy, and entropy changes of the transition, the slope and intercept of the lower baseline (double-stranded region), and the slope and intercept of the upper baseline (single-stranded region). The concentration is the only non-floating parameter and is determined at a high-temperature absorbance point. A Marquardt-Levenberg fitting routine is applied to each curve to find the best parameter values. The parameters and fitting follow an Ising model:

$$A_T = (1 - \alpha)A_{RC} + \alpha A_{ST} \quad (12)$$

where A_T is the total absorbance, A_{RC} is the absorbance of the random coil or single strands, A_{ST} is the absorbance of the stacked duplex, and α is the fraction of strands in the duplex conformation. The truncation points for the fitting can be selected by the user. It is important to have enough points in the upper and lower baselines to find a good fit, although with a low T_m the points in the lower baseline may be few in practice. At higher temperatures it is important to avoid evaporation effects, which cause the absorbance to appear higher than true values, and to exclude such points from the fit. The position of the truncation points of the fit can have a significant impact on the values of the enthalpy parameter.

Two-State Assumption

An important assumption in the analysis described above is that only two states exist for the RNA: single strands or duplex. Any intermediates between these two conformations are assumed to be at very low concentration and not contribute significantly to the absorbance. Also, the duplex must fold into a single conformation with no alternative folds. If the values for the enthalpy change calculated according to the fit of the melting curves and the van't Hoff plot agree within 15%, then the results are consistent with the two-state assumption. Although the exact cutoff value for consistent two-state behavior is a matter of debate and interpretation, if the enthalpy values calculated with the two different fitting methods are not consistent, then the analysis does not provide valid thermodynamic parameters.

C_p° Assumption

The above equations neglect the temperature dependence of H° and S° , which for a constant heat capacity, C_p° , is given by:

$$\Delta H^\circ = \Delta H^\circ_{T_o} + \Delta C_p^\circ (T - T_o) \quad (13)$$

$$\Delta S^\circ = \Delta S^\circ_{T_o} + \Delta C_p^\circ \ln(T/T_o) \quad (14)$$

Usually, C_p° will not be zero because stacking in the single strands is temperature dependent (Holbrook, 1999). The best way to determine C_p° is by isothermal titration calorimetry at different temperatures (Diamond, 2001; Mikulecky, 2006). It is sometimes possible, however, to obtain estimates from optical melting data by plotting the fitted values of H° from individual melting curves versus T_M and the fitted values of S° versus $\ln T_M$ (Diamond, 2001; Petersheim, 1983). This is best attempted when the H° is not large so that there is a large concentration-dependence of T_M . In general, however, the experimental errors in optical melting data do not allow accurate determination of C_p° (Chaires, 1997). Fortunately, the systematic errors in fitting H° and S° due to neglecting C_p° compensate each other so that the G° is a reliable parameter.

Experimental Design

When designing sequences to study the thermodynamic stabilities of different noncanonical RNA motifs, consider carefully the stems' stabilities, the expected T_m , the advantages of non-self-complementary or self-complementary systems, and possible alternate folds. When choosing stem duplexes for internal loops or multibranch loop motifs, the predicted thermodynamic stabilities of each stem should be close so that the duplexes unfold in a two-state manner. The predicted T_m of the folded forms should be as close to 37 °C as possible to minimize the extrapolation for calculating G°_{37} . Self-complementary systems have the advantages of simplicity and not requiring determination of oligonucleotide concentrations prior to the melt because mixing of equimolar amounts of two different strands is not required. A self-complementary system also enables incorporation of two motifs in one duplex, thus doubling the effect of the motif. This is particularly useful when the free energy increment of the noncanonical motif is small, such as for dangling ends. While non-self-complementary duplexes require accurately mixing equimolar amounts of two strands, they have the potential to "mix and match" different strands and thus enable a wider sequence diversity within a motif using fewer different sequences.

The possibilities of alternate folds should be considered even for short sequences. Sometimes changing a Watson-Crick base pair distant from the intended noncanonical pair can change the propensity for alternate duplex formation. Prediction programs such as mfold (Zuker, 1989) and RNAstructure (Mathews, 2004) can generate some suboptimal folds for both unimolecular and bimolecular folds. A simple graphical method for finding alternate folds is to create a grid as shown in Figure 2. Every possible Watson-Crick pairing is assigned an X, potentially stable noncanonical pairs such as GU can be assigned an O. Possible helices appear as a diagonal line of X's and O's. For example, in the sequence on

the left in Figure 2, the intended duplex has two GU pairs at the ends of the helix; the thermodynamic stability of GU is often idiosyncratic and depends on the helix position. The only alternative duplexes have positive predicted free energy and are thus unlikely to form. In contrast, the sequence on the right differs only in the order of the two middle GC pairs, but this slight change creates the possibility for several more stable alternative duplexes. The middle GC pairs are unlikely to affect the measurement of the thermodynamic stability of the terminal GU pairs. Keep in mind that these are predicted free energy values; and the idiosyncratic nature of non-Watson-Crick pairs, such as GU pair stabilities, is the reason to continue measuring thermodynamic parameters with optical melting experiments.

One-dimensional proton NMR of 0.2–1 mM RNA in 90% H₂O and 10% D₂O, 100 mM NaCl, 10 mM phosphate, and 0.5 mM Na₂EDTA in a 500 MHz spectrometer is a quick way to check that the expected duplex forms. The extensive dialysis and sample preparation necessary for high quality 2D NMR spectra is often not necessary for a quick 1D proton spectrum. The correct number and chemical shift of imino protons can verify that the proton spectrum is consistent with the intended duplex design. The imino protons in the pairs at the terminus of a duplex may be missing or weak due to exchange with water and fraying at the duplex ends. Too many imino protons implies that more than one conformation of the RNA is stable. The imino protons in GC and AU Watson-Crick pairs resonate between 11–13 ppm and 13–15 ppm, respectively. Imino protons in stable mismatches may be protected from exchange with water and then resonate anywhere between 9.5–15 ppm depending on the conformation and protonation of the mismatch (Santa Lucia, 1991; Schroeder, 2000). These resonances for non-Watson-Crick pairs can provide strong supporting evidence that different hydrogen-bonded conformations are the basis for the sequence dependence of the thermodynamic stability. For example, wobble GU pairs show imino proton resonances between 10–11.5 ppm and a strong NOE between the two imino protons (Schroeder, 2001).

The buffer used for optical melting experiments is typically 1 M NaCl, 10 or 20 mM sodium phosphate or sodium cacodylate at pH 7, and 0.5 mM Na₂EDTA. One M NaCl was initially chosen to stabilize short RNA sequences when RNA synthesis was difficult and time-consuming and has become the standard salt concentration. The phosphate or cacodylate buffers maintain a constant pH over a wide temperature range. Because cacodylate anion has an arsenic atom, the buffer can be stored without concerns about bacterial growth. The buffering range of cacodylate is 5.0–7.4 with a pK_a of 6.3. Noncanonical pairs such as A⁺·C or C⁺·C are more stable at low pH, and increased stability at low pH provides evidence for the formation of protonated pairs (Santa Lucia, 1991). The Na₂EDTA chelates any divalent cations that promote RNA hydrolysis, especially at high temperatures. Other buffers may be used to test the effects of salt on thermodynamic stabilities. A typical buffer that may resemble more physiological salts is 0.15 M KCl, 10 mM MgCl₂ and 10 mM sodium cacodylate pH 7. When using buffers that contain magnesium, however, the samples cannot be diluted and used again for a melt at another concentration because magnesium facilitates hydrolysis at high temperatures.

If a transition is truly two-state, then the thermodynamics should be the same when measured at any wavelength at which the folded and unfolded states have different absorbances (Cantor, 1980). Typically, 260 or 280 nm light is used. For sequences with a

high fraction of GC or AU pairs, 280 or 260 nm, respectively, are preferred in order to maximize the hyperchromicity (Fresco, 1963). Sometimes local information can be deduced from optical melting curves at other wavelengths. One case is when a nucleotide absorbs outside the region where the rest of the nucleotides absorb. Measurements at 296 nm provide information on global conformation, and the absorption is due to an n to π^* transition (Testa, 1993). DNA duplexes with Hoogsteen pairs also have a signature melting profile at 295 nm (Mergny, 2005; Miyoshi, 2009).

The purity of oligonucleotides for optical melting experiments should be at least 90% as measured by HPLC or by gel electrophoresis on a denaturing gel. The RNA can be diluted to provide 10 concentrations over a 100-fold range. The maximum and minimum accurate absorbance of the Beckman spectrometer is 2.5 and 0.2, approximately a 10-fold range. The use of cuvettes with 0.1 cm and 1.0 cm pathlengths enables another 10-fold range in concentration. A typical RNA duplex concentration range is from 2.5×10^{-4} M to 2.5×10^{-6} M, and concentration ranges from 60-fold to 150-fold are common. Thus, use Beer's Law and the sequence dependent extinction coefficient to calculate the amount of RNA necessary to dilute the RNA samples with optical melting buffer (Cantor, 1980). Unless magnesium is used in the buffer, 5 sample concentrations can be prepared, run in an optical melting experiment, and then diluted and used again in the next optical melting experiment. Signal-to-noise is usually not a consideration with modern spectrophotometers, but if it is, then the optimum absorbance is 0.434 if the noise is not due to statistical fluctuations in the number of photons hitting the detector (shot noise) (Hammes, 1974; Turner, 1986).

Data Interpretation

Analysis of optical melting experiments provides thermodynamic parameters for enthalpy, entropy, and free energy changes for duplex formation. Optical melting experiments, however, do not provide definitive information about the duplex structure, hydrogen bonding, or heat capacity. Although pairing patterns are sometimes inferred from the sequence dependence of RNA motifs, NMR or crystallography are required to provide definitive information about the structure of the RNA duplex. For example, hydrogen-bonded GA and GG pairs are relatively stable thermodynamically in certain contexts (Burkard, 2000; Schroeder, 2000; Walter, 1994; Wu, 1996); however a combination of optical melting experiments and NMR spectroscopy was necessary to determine this. As a consequence of these results, when GA or GG pairs have the potential to form in loops, the possibility of forming a GA or GG pair is often invoked to rationalize empirical rules for thermodynamic stabilities of unmeasured sequences.

Optical melting studies provide estimates of H° and S° and therefore G° on the basis of the two-state model. The most reliable values for G° are those near the melting temperatures of the experiment. Relative stabilities depend not only on melting temperature but also on the enthalpy change. For example, if duplex A and duplex B have melting temperatures of 70 °C and 60 °C, respectively, then duplex A is more stable at 70 °C if both RNA concentrations are the same. If the enthalpy of duplex A is larger than the enthalpy of duplex B, however, duplex B may be the more stable duplex at 37 °C. The G° at the temperature of interest is the true measure of relative stability.

Error Analysis

There are many possible sources of error in optical melting studies. SantaLucia and Turner (SantaLucia, 1997) list two sources of random error: (1) signal-to-noise ratio of the absorbance measurements and (2) variations in sample preparation, and four sources of systematic error: (1) incorrect calibration, (2) a non-two-state transition, (3) incorrect choice of baselines, and (4) neglect of C_P° .

Sampling errors in $1/T_M$ vs $\ln C_T$ plots and in fitted data provide measures of the random errors. Equations for calculating sampling error are given by SantaLucia and Turner (SantaLucia, 1997) and Xia et al. (Xia, 1998). The random errors from $1/T_M$ vs $\ln C_T$ plots are typically on the order of 3%, 3%, and 1% for H° , S° and G° near the T_M , respectively (Xia, 1998). Random errors from averaging values from fitting curves are typically two- to three-fold larger. In a study of 51 Watson-Crick complementary duplexes, the errors in S° were about 13% larger than in H° because the uncertainty in S° depends on more terms (Xia, 1998). The error in G° is smaller than that in H° and S° because the errors in H° and S° are highly correlated in a compensating manner. Random errors in T_m are typically 1 to 2 °C.

The magnitudes of systematic errors are difficult to estimate. Optical melting results from different laboratories provide one measure. For three DNA sequences reported by separate laboratories (Breslauer, 1986; SantaLucia, 1996; Sugimoto, 1996), differences were 6%, 6%, 3%, and 1 °C for H° , S° , G_{37}° , and T_M , respectively (SantaLucia, 1997). Values of G_{50}° for four sequences measured by substrate inhibition in a group I ribozyme reaction (Narlikar, 1997) differed an average of 8% from those measured by optical melting (Pyle, 1994).

When comparing the free energies of two RNA duplexes, 0.5 kcal/mol is a reasonable rule of thumb for estimating a significant difference in thermodynamic stabilities. The calculated error in duplex free energy is typically ± 0.2 kcal/mol when using Meltwin software. Non-nearest neighbor effects due to the length of the duplex stems or the position of the mismatch in a helix can be approximately 0.5 kcal/mol (Kierzek, 1999; Schroeder, 2000). The rules for predicting thermodynamic stabilities of duplexes include terms with values less than 0.5 kcal/mol, however. These terms are calculated from linear regression analysis of typically 50–200 duplex free energies, and the terms are justified by statistical significance.

Thus, optical melting experiments can provide a large number of thermodynamic measurements from which generalized rules for predicting nucleic acid stabilities can be derived. These thermodynamic measurements and rules form the core of most RNA and DNA structure prediction algorithms. Contrafold (Do, 2006), a new algorithm based on computational conditional training methods and databases of known RNA structures (Griffiths-Jones, 2003; Griffiths-Jones, 2005), calculated base stacking energies with the same rank order as the nearest neighbor parameters measured by optical melting experiments. This result supports the use of thermodynamic parameters and free energy minimization to predict the structure of functional RNA conformations. Thus,

thermodynamic analysis and optical melting experiments are useful tools for exploring the unknown landscapes of the RNA world amidst a flood of sequencing information, low free energy valleys, and peaks of activation energies in ribozymes.

Acknowledgments

The authors thank Koree Clanton-Arrowood, Nic Hammond, Biao Liu, and Mai-Thao Nguyen for critical reading of the manuscript. We thank all our students who remind us that teaching and learning are a dynamic equilibrium with arrows that go both ways. D.H. Turner is supported by NIH grant #GM22939. S.J. Schroeder is supported by grants from the Oklahoma Center for the Advancement of Science and Technology Plant Science Research Program, the Pharmaceutical Research and Manufacturers of America Foundation, and the American Cancer Society Institutional Research Grant to the Oklahoma Health Science Center.

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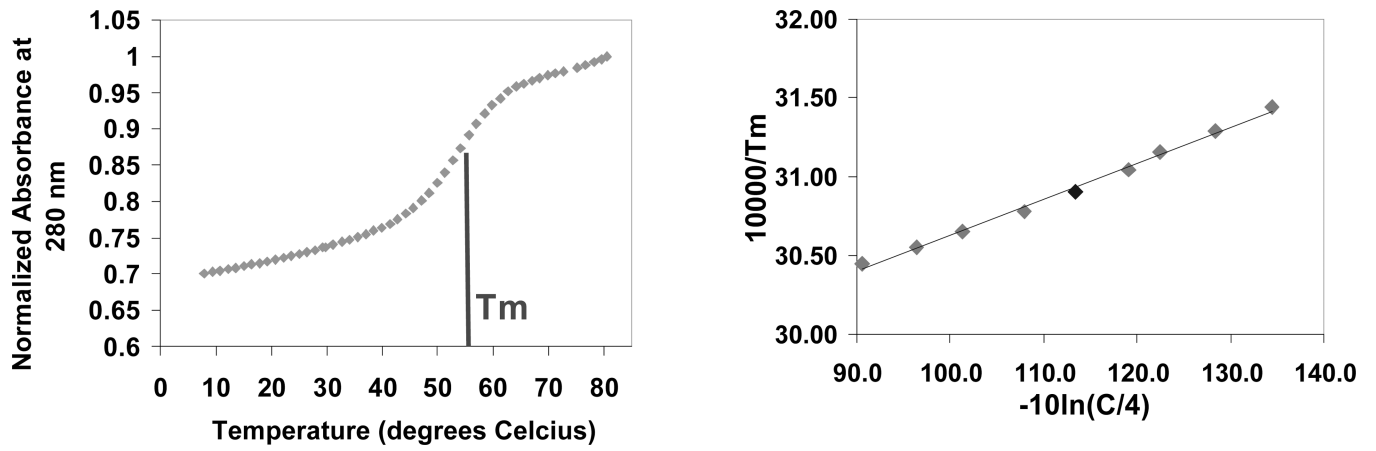


Figure 1. Optical Melting Data

The normalized UV absorbance (280 nm) versus temperature (°C) curve (left). Van't Hoff plot (right): $1/T_M$ versus $\ln(C_T/a)$.

	5'G	U	A	U	C	G	A	U	G	U
U	O		X			O	X		O	
G		O		O	X			O		O
U	O		X			O	X		O	
A		X		X				X		X
G		O		O	X			O		O
C	X					X			X	
U	O		X			O	X		O	
A		X		X				X		X
U	O		X			O	X		O	
5'G		O		O	X			O		O

	5'G	U	A	U	G	C	A	U	G	U
U	O		X		O		X		O	
G		O		O		X		O		O
U	O		X		O		X		O	
A		X		X				X		X
C	X				X				X	
G		O		O		X		O		O
U	O		X		O		X		O	
A		X		X				X		X
U	O		X		O		X		O	
5'G		O		O		X		O		O

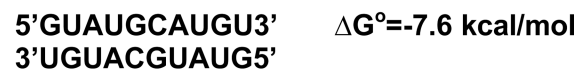
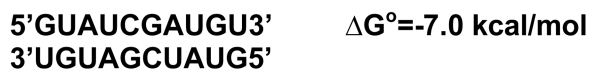


Figure 2. Plots to facilitate sequence design

The X's represent possible Watson-Crick pairs. The O's represent possible GU pairs. The intended duplex with terminal GU pairs is shown as the central diagonal. Possible alternative duplexes are shown as shorter diagonals. The sequence differences between the left and right examples are highlighted in bold. The predicted free energies at 37 °C of the possible duplexes are calculated according to (Clanton-Arrowood, 2008; He, 1991; Mathews, 2004; Mathews, 1999; Miller, 2008; O'Toole, 2006; O'Toole, 2005; Xia, 1998). Note that the nearest-neighbor parameter for 5'GU/3'UG is known to be context-dependent and shows non-nearest neighbor effects. In this example, the lowest energy possibility for the terminal mismatches was used.

Table 1

Absorbance for 0.0400 g/L of K_2CrO_4 in 0.05 M KOH ^a

λ (nm)	A	λ (nm)	A	λ (nm)	A
220	0.446	315	0.046	400	0.396
230	0.171	330	0.149	420	0.124
240	0.295	340	0.316	440	0.054
250	0.496	350	0.559	460	0.018
260	0.633	360	0.830	480	0.004
275	0.757	370	0.987	500	0.000
290	0.428	375	0.991		
300	0.149	390	0.695		

^aValues are from (Gordon, 1972).