UV Melting of G-Quadruplexes

Jean-Louis Mergny and Laurent Lacroix

1Laboratoire de Biophysique, Inserm U565, Muséum National d'Histoire Naturelle, Paris, France

ABSTRACT
Absorbance versus temperature curves can provide information on the thermal stability of DNA or RNA quadruplexes. Quadruplex denaturation or renaturation can be followed by recording absorbance at 295 nm rather than 260 nm, the wavelength used to monitor duplex denaturation. This unit describes the use of absorbance versus temperature curves to determine melting temperatures ($T_m$ values) and model-dependent thermodynamic parameters. Curr. Protoc. Nucleic Acid Chem. 37:17.1.1-17.1.15. © 2009 by John Wiley & Sons, Inc.

Keywords: quartets • thermodynamics • van’t Hoff analysis • thermal melting

INTRODUCTION
Guanine quadruplexes are a family of unusual nucleic acid structures adopted by G-rich sequences and based on planar guanine quartet assembly. Such sequences are found in key locations in genomes and could affect different biological processes. Therefore, knowledge regarding their stability is required to understand or design quadruplexes-interacting molecules (small molecules, proteins).

A UV melting experiment (i.e., UV spectroscopy in conjunction with thermal denaturation) can provide information about the stability of DNA or RNA quadruplexes. Thermal denaturation experiments on quadruplexes are performed using a UV-visible spectrophotometer to record absorbance at 295 nm as a function of temperature. This data can be used to determine $T_m$ values (for temperature of melting, or more accurately, temperature of mid-transition) and model-dependent thermodynamic parameters. UV melting experiments can also be used to characterize nonequilibrium melting processes, although data analysis requires caution in these cases. General rules for nucleic acids melting have been presented in UNIT 7.3.

This unit presents protocols for performing UV melting experiments (see Basic Protocol 1), analyzing the melting curves generated by these experiments (see Basic Protocol 2), and using alternative methods in conjunction with the basic UV melting experiments (see Alternate Protocol).

MELTING G-QUADRUPLEXES AND MEASURING UV ABSORBANCE
For the melting experiments described in this protocol, using cacodylate buffer ($pK_a = 6.14$) as the reference buffer for melting experiments is strongly recommended. It is important to choose a buffer that (1) does not absorb light in the far UV-region, (2) has a $pK_a$ close to the desired pH, (3) is relatively temperature-independent (phosphate and PIPES are commonly used for this reason), and (4) does not interact with other components of the solution (e.g., phosphate buffer should be avoided if working in the presence of Mg$^{2+}$ or Ca$^{2+}$). When an acidic pH is desired, acetate ($pK_a = 4.62$) is acceptable. One should be aware that the $pK_a$ of other buffers such as MES, BES, TES, Tris, Tricine, HEPES, MOPS, and TAPS change significantly with temperature (in the range of 2 pH units between 0° and 100°C; Fukada and Takahashi, 1998). Therefore,
these buffers are not appropriate for \( T_{m} \) experiments, even if the process studied is not pH dependent, as pH changes will also affect ionic strength of the buffer.

**Materials**

Oligonucleotide stock solutions (store at \(-20^\circ C\) between experiments)

2× stock buffer, e.g., 20 mM lithium cacodylate (see recipe)

10× salt solution, e.g., 1 M NaCl (filter sterilize using a 0.2-μm filter and store up to several weeks at 4°C)

MgCl\(_2\) or other ligand solution, optional

Mineral oil, optional

Pure ethanol

Quartz cuvettes and caps

UV-visible spectrophotometer equipped with a cell changer device

Temperature control device (e.g., water bath or Peltier effect heater)

1.5-mL microcentrifuge tubes, optional

Hair dryer, optional

External temperature probe

Computer attached to spectrophotometer

Additional reagents and equipment for determining molar extinction coefficients (e.g., see UNIT 7.3)

**NOTE:** It is recommended that oligonucleotide purity and concentration be determined before any thermal analysis begins. Do not depend on indicated quantities provided by the oligonucleotide manufacturer. Ensure that all cuvettes are clean and samples are transparent. Even weak diffusion (resulting from aggregation or precipitation) will ruin the experiment. Use UV-grade reagents of highest purity for all experiments.

**CAUTION:** Cacodylate buffer contains arsenic and should not be discarded into the drain system; rather the solution should be disposed of as toxic waste.

Prepare oligonucleotide stock solutions and determine sample concentration.

1. Resuspend the nucleic acid in a known volume of double-distilled water (300 to 500 μL for 40- to 200-nmol synthesis scale).

2. Determine the concentration of the stock solution by measuring the absorbance at 260 nm of an appropriate dilution (typically 1/100).

   *It is recommended that oligonucleotide purity be determined before beginning any thermal analysis for every new nucleic acid sample. See Critical Parameters.*

3. Analyze the purity of the sample by HPLC, mass spectrometry, and/or denaturing gel electrophoresis, depending upon available equipment.

   *The extinction coefficients provided by Cantor et al. (1970) are not completely accurate, with errors up to 20%. This error is generally acceptable for the reactions studied here, which do not involve mixing different strands in equimolar amounts.*

   *Another protocol for determining the molar extinction coefficient is suggested in UNIT 7.3.*

Prepare spectrophotometer

4. Turn on the spectrophotometer 15 to 60 min in advance of the experiment to provide stable measurements.

5. Turn on the Peltier effect heater or water bath in advance so that the desired temperature is reached in the sample compartment.
6. Prior to the thermal melting experiment, obtain a standard baseline measurement. Analysis of the baseline before starting the experiment may reveal evidence of instrumental problems.

Prepare sample
7. For a total volume of 600 μL for a standard microcuvette, combine the following:

- 300 μL 2× buffer
- 60 μL 10× salt solution
- 5 to 50 μL oligonucleotide stock solution
- In some cases, MgCl$_2$ or other ligand solution
- Water to a final volume of 600 μL

Mix components in a 1.5-mL microcentrifuge tube or directly in the cuvette.

8. Overlay mineral oil if the cap does not fit perfectly on the cuvette. A well-fitting cap is preferred, as this facilitates cleaning of the cuvette. Use of cuvettes with a different volume would require adapting this protocol but be aware that using smaller volumes may lead to evaporation.

Perform melting experiment
9. For a typical experiment, record absorbance at 295 nm, 245 nm, and 405 nm (the wavelength choices are discussed in the Commentary). Use an integration time of 2 sec and a temperature gradient of 0.2°C/min.

Ensure that all cuvettes are clean and samples are transparent. Even weak diffusion (resulting from aggregation or precipitation) will ruin the experiment.

Once all parameters are set, data acquisition is automatic and the presence of the experimenter is no longer necessary. However, it is often useful to follow the first cycles to ensure that the temperature is changing according to the chosen gradient and that the instrument is working correctly.

The samples used in an overnight $T_m$ experiment may be used for some complementary measurements (see Alternate Protocol).

Wash cuvettes
10. Discard cuvette contents, unless the nucleic acid sample is extremely precious.

CAUTION: Cacodylate buffer contains arsenic and should not be discarded into the drain system; rather the solution should be disposed of as toxic waste.

Repurification of a sample after a denaturation experiment is generally a waste of time. The sample may have degraded upon incubation at a high temperature in certain buffer conditions (e.g., due to depurination).

11a. If mineral oil was not added, wash cuvettes several times with double-distilled water then with pure ethanol, and again with double-distilled water, then dry under hot air (provided by a hair dryer) or air dry.

11b. If mineral oil is used to minimize evaporation, use a more complex procedure, as this oil is only soluble in dichloromethane or chloroform. First briefly wash the cuvettes with water, then rinse one or two times with each of the following: acetone, dichloromethane or chloroform, and acetone followed by the water/ethanol/water procedure described in step 11a.

Solvents should be of decent quality (HPLC- or Pro-analysis grade) and should be discarded appropriately.
**Analyze data**

12. Represent the absorbance profile as a function of the temperature and then check the quality of the data.

Numerical analysis of unsound data could lead to flawed conclusions. In some cases, the software provided with the spectrophotometer can be used for direct calculation of the $T_m$ and while it is tempting to go directly to the “result,” this is not recommended. This automatic data analysis is most often of questionable value and one should remember that the result of the melting experiment is the absorbance profile per se. Numerical data analysis is often model dependent, and any hypotheses have to be clearly expressed to avoid dangerous over interpretation of data.

Figure 17.1.1A represents a reversible melting profile of a modified vertebrate telomeric G strand obtained at 295 nm. This profile clearly indicates that the reaction observed is fast relative to the steps of the temperature gradient, and thus most likely the quadruplex formed is intramolecular. The reversibility of this profile indicates that all the data points are equilibrium values, and thus they can be used for further thermodynamic analysis. A small evaporation artifact is visible for the heating profile above 80°C (Figure 17.1.1A, arrow). Using the same sample cuvette, the TDS and the CD profiles of this oligonucleotide were also recorded. These spectra, shown in Figures 17.1.2A and 17.1.2B, respectively, can also be used to demonstrate G-quadruplex formation (for more details regarding evidence for G-quadruplex formation, see Commentary). The hysteresis phenomenon was observed in the spectrum shown in Figure 17.1.1B. The hysteresis indicates that the structural transition is slow relative to the steps of the temperature gradient. In this case, the oligonucleotide used d(GGGUUUUGGGG) (where U is deoxyuracil) only contains two blocks of guanines and intramolecular folding is excluded; thus this hysteresis is not unexpected. Nevertheless, even the absorbance versus temperature plot of intramolecular quadruplex samples may sometimes show hysteresis (see below on analysis of non-reversible melting profiles) and thus stoichiometry should be determined before analysis is attempted (see Commentary). For the plot shown in

![Figure 17.1.1 Example of $T_m$ analyses. (A) A reversible thermal denaturation curve. Cooling-heating profiles at 295 nm of a 3-μM sample of the modified vertebrate telomeric G-strand oligonucleotide, d(AGGGTGGGTTAGGGTTAGGG) (where underscored G residues indicate ribose sugar and other residues are deoxy) in 20 mM lithium cacodylate (pH 7.2) in the presence of 100 mM NaCl. Data was recorded with a temperature gradient of 0.2°C/min. The heating and cooling profiles are superimposable. This spectrum indicates that most typical problems (e.g., bubbles, evaporation, degradation) were avoided, although some evaporation is visible above 80°C (indicated by the arrow) and that the transition was kinetically reversible. (B) Example of a hysteresis phenomenon. Cooling-heating profiles at 295 nm of a 5-μM sample of the oligodeoxynucleotide d(GGGUUUUGGGG) (where U is deoxyuracyl) in 10 mM sodium cacodylate buffer (pH 7.0) containing 100 mM NaCl. Filled circles indicate the cooling profile; open circles indicate the heating profile.](image-url)
Figure 17.1.1A, a true $T_m$, corresponding to the temperature at which half of the sample is folded and half is unfolded ($\theta = 0.5$), can be determined.

In the case of Figure 17.1.1B, only the $T_{1/2}$ can be determined. The $T_{1/2}$ will be different for the heating and the cooling profiles (by $\sim 8^\circ C$, in this case) and will depend upon the temperature gradient used. For certain oligonucleotides, e.g., $d$(GGGGUUGGGG), the $T_{1/2}$ will also depend upon oligonucleotide concentration. For both reversible and non-reversible melting curves, these determinations imply that the absorbance properties of the folded and unfolded forms as a function of temperature are known.

Two baselines (upper and lower) are needed: these correspond to the folded and unfolded forms, respectively, as the transition is inverted. These baselines are usually chosen as straight lines (with a positive or negative slope). More complicated baseline models may be chosen but introduce a large number of variables and do not lead to a significant improvement of accuracy.

ANALYSIS OF REVERSIBLE MELTING PROFILES

Data analysis will often be performed on a computer separate from the one that interfaces with the spectrophotometer with a program such as Kaleidagraph (Synergy Software) or Origin (MicroCal). A sophisticated program is not necessary, provided that data files may be exported in a suitable format such as ASCII. The data is first plotted as absorbance versus temperature. Several graphs may be compared for the same sample if absorbance was recorded at different wavelengths.

Figure 17.1.3A shows the melting of $d$(AGGGTTAGGGTTAGGGTTAGGG) at 295 nm. This reversible profile indicates that the system is at equilibrium at each temperature and is in agreement with an intramolecular folding of a G-quadruplex. Normalization should be avoided as it results in a loss of information or could be based on invalid assumptions. If normalization is required to graphically compare and represent data, the high temperature value corresponding to the unfolded state should be preferred as normalization value.

The determination of upper and lower baselines for the transition (as shown in Fig. 17.1.3A) is subjective. It is valuable to have several investigators analyze the same graph and compare the $T_m$ values deduced by each person. In general, the accuracy should be within $1^\circ C$ if the melting curve is of sufficient quality.
**Figure 17.1.3**  
*Tm* determination and thermodynamic analysis. (A) Baseline and *Tm* determination. In this experiment, a 3-μM sample of the human telomeric repeat oligonucleotide d(AGGGTTAGGGTTAGGGTTAGGG) was melted in 10 mM lithium cacodylate (pH 7.2) in the presence of 10 mM NaCl. Absorbance was monitored at 295 nm. The upper and lower baselines (A underscores F and A underscores U, respectively) were manually chosen in Kaleidagraph; equations were calculated by a 2-points method. The median was drawn as the algebraic mean of the two baselines (median = (A underscores U + A underscores F)/2) and the intercept with the absorbance axis corresponds to the *Tm*. (B) Folded fraction calculation. The folded fraction (θ) was calculated using the first equation in Basic Protocol 2 and the baselines determined as described in Figure 17.1.2A. In this representation, the *Tm* corresponds to θ = 0.5. (C) A van’t Hoff representation. Assuming an intramolecular equilibrium, the association constant (K_a) was calculated using the second equation in Basic Protocol 2. Then ln(K_a) as a function of the inverse of the temperature (in K) was plotted. A linear fit using the fourth equation in Basic Protocol 2 was used to determine ΔH° and ΔS° (χ² and R values of the fitting process are represented). Note that as the values are in calories, the perfect gas constant, R, used is 1.987 cal·mol⁻¹·K⁻¹. (D) Global fit of the melting curve. Data from Figure 17.1.3A were also fitted to the ninth equation describing the two-state melting process with two linear baselines and a temperature independent ΔH°. The upper and lower baselines were drawn using the baseline parameters fitted within the equation. Note the agreement between the baselines in Figure 17.1.3A and D and between the fitted data shown in the table in Figure 17.1.3D (ΔH° and *Tm*) and the values indicated in Figures 17.1.3A,B,C.
After the choice of baselines, the next step is to draw a median line between the two baselines (shown by a dotted line in Fig. 17.1.3A). This median line may be obtained by observing the Y-intercept values of the two baselines at two different temperatures. The median line will be drawn between the middle of the two segments between the baseline Y-intercepts. This median can also be computed using the linear equations of both baselines using the formula: median \((T) = (A_U(T) + A_F(T))/2\), where \(A_U(T)\) and \(A_F(T)\) correspond to the baseline values of the unfolded and folded species, respectively. The \(T_m\) corresponds to the crossing point of the experimental curve and the median line. In this experiment, the \(T_m\) was 37°C.

A popular alternative to this baseline method (sometimes referred as the sloppy baseline approach) is to determine the maximum of the first derivative of the absorbance signal \((T_{\text{max}})\). Several data acquisition programs can be used to perform this calculation, often claiming a precision of \(\pm 0.1°C\). Unfortunately, this value does not exactly correspond to the true \(T_m\), as has been known for decades (Gralla and Crothers, 1973; Cantor and Schimmel, 1980b). In practice, the difference between \(T_{\text{max}}\) and \(T_m\) is often limited, but significant (≥2°C) especially when dealing with broad transitions (Cantor and Schimmel, 1980b). One should be aware that \(T_{\text{max}}\) determination using the derivative methods quite often requires smoothing of the data, which may change the result if the data acquisition frequency is not adapted.

Once the upper and lower baselines are determined, one can convert an absorbance versus temperature plot into a “fraction folded” versus temperature plot. The fraction folded, θ, should be a number between 0 and 1: \(θ = 0\) for \(T > > T_m\), \(θ = 1\) for \(T < < T_m\), and \(θ = 0.5\) for \(T = T_m\). The conversion of absorbance \((A_T)\) to \(θ_T\) at a given temperature is as follows:

\[
θ_T = \frac{[A(T) - A_U(T)]/[A_F(T) - A_U(T)]}
\]

Figure 17.1.3B illustrates such a conversion. Although this is another way to graphically represent \(T_m\), it is not an independent \(T_m\) determination. This representation could be useful when comparing melting profiles obtained in different conditions, but the information regarding the amplitude of the transition will be lost.

Once the molecularity of the equilibrium is established, model-dependent thermodynamic parameters can be extracted from the melting curve (Cantor and Schimmel, 1980a). The \(T_m\) value gives only a gross estimation of the predominant form: at a temperature above the \(T_m\), the structure is predominantly unfolded and at a temperature below the \(T_m\), the structure is predominantly folded. The thermodynamic parameters of standard enthalpy \((\Delta H^o)\) and standard entropy \((\Delta S^o)\) of the reaction, provided that the model used is correct, allow determination of the association constant \((K_a)\) or free Gibbs enthalpy \((\Delta G^o)\) within, and in the vicinity of, the temperature interval corresponding to the transition. These parameters may then be used to estimate the thermodynamic stability of a given quadruplex under physiological conditions.

To determine thermodynamic parameters, one uses a two-state equilibrium analysis. Use of this analysis assumes that only two species are present in solution (fully associated or folded and fully dissociated or unfolded); this assumption is not always justifiable. A single-value decomposition (SVD) analysis can be used to confirm this assumption, where differences in melting profiles at different wavelength would invalidate such a hypothesis. The molecularity of the reaction must be known. In the case of an intramolecular equilibrium, \(K_a\) is related to the folded fraction according to the following equation:

\[
K_a = θ_T/(1 - θ_T)
\]
The next step in the analysis is to plot the natural logarithm of the affinity constant (ln($K_a$)) as a function of the reciprocal of the temperature (1/$T$) in K$^{-1}$; Mills et al., 1999). This analysis should be restricted to the temperature range for which $0.03 < \theta < 0.97$. Outside this range, it is difficult to evaluate the affinity constant. Others have suggested that the analysis interval be further restricted to $0.15 < \theta < 0.85$, keeping data only from the region where $K_a$ values are most precise (Puglisi and Tinoco, 1989). The interval choice depends on the experimenter’s confidence in the baseline determination.

By definition, the free Gibbs enthalpy may be written as:

$$\Delta G^\circ = -RT \ln(K_a) = \Delta H^\circ - (T \times \Delta S^\circ)$$

where, $T$ is the temperature in Kelvin, $\Delta H^\circ$ the standard enthalpy of the reaction, and $\Delta S^\circ$ the standard entropy and $R$ the perfect gas constant. $\Delta H^\circ$ describes the amount of heat produced or taken up at a constant pressure. For a reversible reaction, $\Delta H^\circ$ also describes the temperature dependence of the equilibrium constant. In most cases, $\Delta G^\circ$ is small and negative whereas $\Delta H^\circ$ may be large and negative (i.e., highly favorable). The $\Delta H^\circ$ term is partially compensated for by an entropic factor $T \times \Delta S^\circ$, which is also negative.

It can be deduced that

$$\ln(K_a) = [-\Delta H^\circ/R] \times [(1/T) + \Delta S^\circ/R]$$

provided that $\Delta H^\circ$ and $\Delta S^\circ$ are independent of temperature (see below), the so-called van’t Hoff representation (ln($K_a$) versus 1/$T$) gives a straight line with a slope of $-\Delta H^\circ/R$ and a Y-axis intercept of $\Delta S^\circ/R$. The $\Delta H^\circ$ of a reversible reaction is called the van’t Hoff enthalpy ($\Delta H^\circ_{vH}$) and is defined by:

$$\Delta H^\circ_{vH} = -R \, d\ln(K_a)/dT^{-1}$$

The plot of ln($K_a$) versus 1/$T$ shown in Figure 17.1.3C is adequately fitted by a straight line, thus allowing the determination of $\Delta H^\circ$ and $\Delta S^\circ$ ($-35.9$ kcal-mol$^{-1}$ and $-116$ cal-mol$^{-1}$-K$^{-1}$, respectively) for the melting of d(AGGGTAGGTTAGGGTTAGGG).

$\Delta H^\circ$ and $\Delta S^\circ$ are said to be “model-dependent”—the determination described relies on the validity of the two-state equilibrium hypothesis. The values for $\Delta H^\circ$ and $\Delta S^\circ$ calculated from absorbance versus temperature data are, therefore, less robust than the “model-independent” thermodynamic values provided by calorimetry (Haq et al., 2001; Völker et al., 2002). It is often instructive to compare van’t Hoff enthalpy and calorimetry enthalpy ($\Delta H^\circ_{cal}$) (Haq et al., 2001) as differences provide insight into the mechanism of the melting transition.

As mentioned above, this analysis assumes that $\Delta H^\circ$ is independent of temperature, which in turn means that the heat capacity change during the reaction ($\Delta C_p^\circ$) is zero. This $\Delta C_p^\circ = 0$ hypothesis is clearly not always valid for melting of nucleic acid structures, including quadruplexes. In most cases (Amrane et al., 2005), one cannot unambiguously determine whether or not the $\Delta H^\circ$ of the reaction is temperature independent (Chaires, 1997). If the $T_m$, $\Delta H^\circ$, and $\Delta C_p^\circ$ values are known, it is possible to extrapolate $\Delta G^\circ$ to other temperatures. However, if the classical assumption that $\Delta C_p^\circ = 0$ is untrue, there will be a large error in extrapolation of $\Delta G^\circ$ to temperatures far from the $T_m$.

It is possible to add the $\Delta C_p^\circ$ parameter to the fit of the van’t Hoff representation:

$$\ln(K_a) = -(\Delta H^\circ - \Delta C_p^\circ \times T)/R \times 1/T + \Delta C_p^\circ/R \times \ln(T) + [\Delta S^\circ - \Delta C_p^\circ \times \ln(T)]/R - \Delta C_p^\circ/R + \ln(K_a')$$
where $\Delta H^r$, $\Delta S^r$, and $K_a^r$ are the thermodynamics parameters at a reference temperature $T^r$. Thus, for intramolecular equilibrium, if one chooses $T^r = T_m$, the equation becomes:

$$\ln(K_a) = -(\Delta H^{T_m} - \Delta C_p^\circ T_m)/R \times 1/T + \Delta C_p^\circ /R \times \ln(T_m) + [\Delta S^{T_m} - \Delta C_p^\circ \ln(T_m)]/R - \Delta C_p^\circ /R$$

If one hypothesizes a linear response of the folded and unfolded states, a two-state equilibrium, and $\Delta C_p^\circ = 0$, it is possible to analytically describe and fit a melting curve of an intramolecular quadruplex with the following equations:

$$A(T) = A_u^0 + m_u T + \frac{A_f^0 - A_u^0 + (m_f - m_u)T}{1 + e^{\left[\frac{\Delta H^r (1/ (T/T_m))}{R}ight]}}$$

or

$$A(T) = A_u^0 + m_u T + \frac{A_f^0 - A_u^0 + (m_f - m_u)T}{1 + e^{\left[\frac{\Delta H^r (1/ (T/T_m))}{R}ight]}}$$

Some programs allow the direct nonlinear square fitting of the melting curve to extract these numbers (Petersheim and Turner, 1983). Such an approach is not recommended as the fitting process might yield aberrant results if all parameters have similar degrees of freedom. It is possible to carefully fit the data as described by Bishop et al. (2007), but the user loses the quality control present in the analytical scheme. It is recommended that the baselines chosen by the fitting process be visually inspected to check their adequacy. The linearity of the van’t Hoff representation should also be determined as the global fitting process assumes $\Delta C_p^\circ = 0$. Adding $\Delta C_p^\circ$ to this fitting process appears unrealistic.

In the analysis of non-reversible melting profiles, even intramolecular quadruplexes may lead to a hysteresis phenomenon if the temperature is stepped rapidly (on the order of degrees per second; Merkina and Fox, 2005). Gradients stepped this rapidly may be obtained only with real-time PCR techniques and fluorescence measurements. Most UV absorbance melting experiments have temperature gradients that allow equilibrium to be reached for intramolecular folding processes. On the other hand, bimolecular (Saccà et al., 2005) and tetramolecular (Mergny et al., 2005a) quadruplexes seldom lead to reversible melting profiles, even with very slow temperature gradients (on the order of degrees per minute or slower). The step-by-step analysis of these profiles, which is somewhat complicated, is outside the scope of this unit but has been addressed previously (Mergny and Lacroix, 2003).

**USING OTHER APPROACHES IN CONJUNCTION WITH UV MELTING**

UV melting may be used in conjunction with other approaches as evidence for quadruplex formation and stoichiometry.

**Circular dichroism spectrum (CD)**

If available, a CD spectrophotometer may be used to record the ellipticity versus wavelength of the sample in the 220- to 400-nm range. Spectra are usually recorded at $\sim 20^\circ$C. This spectrum may confirm that the folded form(s) that gave a melting transition in fact corresponds to quadruplex species. The analysis of melting data provided by circular dichroism is a powerful method for evaluating quadruplex formation and an excellent alternative to absorbance for the analysis of thermal melting. Once the ellipticity versus temperature signal is transformed into (un)folded fraction versus temperature, the analysis is identical to that for absorbance melting curves.
Thermal difference spectrum (TDS)
A thermal difference spectrum (TDS) is obtained by the mathematical subtraction of two spectra recorded at a temperature above the $T_m$ when the oligonucleotide is fully dissociated (the fraction folded, $\theta = 0$) and at low temperature when the oligonucleotide is fully associated ($\theta = 1$). The shape of this difference spectrum is different for all types of nucleic acids structures studied thus far and provides a signature for quadruplexes.

Analysis of melting data provided by fluorescence
Provided that the G4-forming oligonucleotide is labeled with a fluorophore and quencher (typically at opposite ends of the sequence), one can follow the opening of the quadruplex through a temperature-dependent increase in donor emission and, in some examples, a concomitant decrease in acceptor-sensitized emission (Mergny and Maurizot, 2001). The fluorescence versus temperature signal is transformed into (un)folded fraction versus temperature and data analysis proceeds as for absorbance melting curves.

Three-dimensional (3-D) melting curves
More and more instruments allow for the acquisition of 3-D melting curves or, in other words, these instruments record absorbance spectra at each chosen temperature. The 3-D melting curve corresponds to absorbance, temperature, and wavelength or, if using circular dichroism, ellipticity, temperature, and wavelength. The 3-D curves are not only aesthetic; these curves provide much additional information compared to a traditional single wavelength analysis. First, the need to determine first the optimal wavelengths is alleviated—one can simply extract 2-D melting data at convenient wavelengths from the data file. But the main advantage of this analysis is obvious when one performs a single value determination of the 3-D file (Haq et al., 1997; Gray and Chaires, 2008). Nevertheless, in the authors’ experience, variations of the absorbance properties of the folded and unfolded species significantly complicate the analysis. Analysis requires good statistical evaluation to decipher true non two-state equilibrium from artifacts of the data analysis.

REAGENTS AND SOLUTIONS
Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Lithium cacodylate buffer (pH 6 to 7.2), 20 mM
To prepare a 200-mL volume of 20 mM lithium cacodylate at pH 6 to 7.2, dissolve 0.55 g of cacodylic acid in 190 mL of double-distilled water. Adjust the pH of the solution using a 1 M LiOH solution starting with the volume indicated in Table 17.1.1 and then add drop-by-drop until the desired pH is reached. Adjust the volume to 200 mL with double-distilled water, filter the solution (0.2-μm filter), and store up to several weeks in a glass bottle at 4°C. Check the pH of the buffer solution at regular intervals, and make sure the solution is clear.

CAUTION: The cacodylic acid powder contains arsenic. Use gloves, goggles, and mask or hood.

UV-grade reagents of high purity should be chosen for all experiments.

Lithium cacodylate is preferred over the commercially available sodium cacodylate, as the presence of sodium may modulate the stability or topology of quadruplex. Lithium has very little effect on quadruplex stability and may therefore be considered a “neutral” cation.

A lithium cacodylate buffer with a final concentration of 10 or 20 mM should be used; concentration will depend on the pH and the buffering power required in the experiment. Prepare buffer solutions in advance (2× to 10× stock solutions) and keep for several weeks at 4°C.
**Table 17.1.1** Volume of LiOH to Grossly Adjust the pH of a 20 mM Lithium Cacodylate Solution

<table>
<thead>
<tr>
<th>Desired pH</th>
<th>Volume of 1 M LiOH to add (mL)</th>
<th>Theoretical volume of 1 M LiOH to add (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>1.5</td>
<td>1.68</td>
</tr>
<tr>
<td>6.4</td>
<td>2.4</td>
<td>2.58</td>
</tr>
<tr>
<td>6.8</td>
<td>3.1</td>
<td>3.28</td>
</tr>
<tr>
<td>7</td>
<td>3.3</td>
<td>3.51</td>
</tr>
<tr>
<td>7.2</td>
<td>3.4</td>
<td>3.68</td>
</tr>
</tbody>
</table>

*The final lithium concentration will be slightly below 20 mM.*

**COMMENTARY**

**Background Information**

Although UV melting per se cannot demonstrate quadruplex formation, it can provide strong evidence for the presence of the quadruplex structure. Preparation of the sample is simple and the absorbance melting experiment itself requires an inexpensive apparatus. Furthermore, reasonably low amounts of sample are required; a single classical 0.2-μmol oligonucleotide synthesis is usually sufficient to perform dozens of experiments.

A classical hallmark of quadruplex formation is the cation nature dependency; this is not observed for most other nucleic acid structures. A quadruplex is more stable in potassium/sodium than in lithium salt. This feature is also useful when dealing with quadruplexes that are “too stable” (i.e., with a $T_m$ or $T_{1/2}$ higher than 85°C). For such quadruplexes, precise determination of the melting parameters is hindered by the lack of a higher temperature plateau. It is nevertheless possible to tune the quadruplex stability without changing the total salt concentration by substituting a lithium salt for all or part of the potassium or sodium. For example, while no transition would be observed in 100 mM KCl, an experiment in 10 mM KCl and 90 mM LiCl might allow characterization of quadruplex melting. Whereas a K$^+$ to Na$^+$ switch might alter the quadruplex folding and thus its stability, such a caveat is not expected with a change to Li$^+$.

Another approach that may provide evidence of quadruplex formation in a given system is comparison of thermal stability of the parent oligonucleotide and oligonucleotides with base substitutions. G-quadruplex formation requires G and thus G to T or G to A mutations can be used to demonstrate the involvement of G in the structure. The mutated sequence should not give rise to any transition or should greatly destabilize the system. G to C mutations are usually not recommended because these could result in the formation of G:C base pairs in G rich sequences. One also needs to be careful when working with RNA quadruplexes. In oligoribonucleotides, the G to U mutation might result in a G:U base pair. G:C and G:U base pairing might allow for the formation of alternative duplex structures. These alternative structures can be distinguished from quadruplexes as there should not be a transition observed at 295 nm and duplex stability is not dependent on the nature of the cation.

**Choice of the wavelength(s)**

The wavelength of 260 nm chosen for most nucleic acid UV-melting studies is suboptimal for analysis of quadruplex structures. In fact, evaluation of quadruplex dissociation at 260 nm may lead to a completely inaccurate determination of thermodynamic parameters as quadruplex denaturation does not lead to large variation in absorbance at this wavelength (Mergny et al., 1998; Rachwal and Fox, 2007). Each structure has a characteristic differential absorbance signature (Mergny et al., 1998, 2005b; Alberti et al., 2002), meaning that a simple absorbance analysis at high and low temperatures will help to determine the proper wavelengths for melting analysis and will also give indications on the nature of the folded conformation.

In the case of quadruplexes, it is possible to follow denaturation at 295 nm (Puglisi et al., 1989; Testa and Gilham, 1993; Mergny et al., 1998). But, in the absence of other data, the existence of an inverted transition at 295 nm does not demonstrate that a quadruplex is formed as other structures, such as i-motif, pyrimidine triplexes, and Hoogsteen duplexes, may also exhibit similar behavior (Mergny et al.,...
On the other hand, the absence of any cooperative transition at 295 nm provides evidence that the structure formed is not a G-quadruplex. The exception is in the case of very stable G-quadruplexes, which may not melt even in boiling water. This limitation could be alleviated by using higher pressure or by altering cation conditions (vide supra). One should record absorbance at a higher wavelength (e.g., 405 nm) to detect potential artifacts (see Troubleshooting). The analysis of melting profiles at several wavelengths (e.g., 295 and 245 nm) is useful in cases where the two-state assumption is not valid. The two-state hypothesis implies that at every wavelength at which a transition is observed, the temperature response must be the same, meaning same \( T_m \) and also the same thermodynamic parameters. Any deviation from this is a clear demonstration of the presence of a more complex melting process.

**Choice of temperature gradient and equipment**

In contrast to proteins, the thermal denaturation of short nucleic acids is generally reversible (i.e., cooling the sample leads to renaturation of the structure). For quadruplexes, two different situations will be mentioned here. In most cases, the folding of intramolecular quadruplexes is fast enough to appear reversible in a denaturing/renaturing experiment when using classical temperature gradients (temperature steps of 0.1° to 0.5°C/min). If the sample is chemically resistant to heat degradation, a denaturation/renaturation cycle should be performed. The experiment should begin at a high temperature (80° to 95°C) and the absorbance should be recorded during cooling (to 20°C or 0°C depending on the expected stability of the sample) followed by heating (to 80° to 95°C). At high temperatures, the samples should be denatured and the dissociated state should be well defined. In contrast, starting from a low temperature implies that the initial state corresponds to a thermodynamic equilibrium and it is often difficult to estimate the incubation time required to achieve this state. Temperature gradients in the 0.2° to 0.4°C/min range (i.e., 12° to 24°C per hr) is usually preferred. One full cycle (cooling and heating) may then be performed overnight.

In the second case, thermal denaturation may appear as an irreversible phenomenon. This is observed for some intermolecular (and especially tetramolecular) complexes. Once dissociated, no reformation of the complex is observed. This makes the renaturation/denaturation cycle described above uninformative as no reformation of the complex is expected. For this type of system, observation over a simple heating gradient (from 0° to 20°C to 80° to 95°C) is recommended.

The water bath–based and Peltier effect heater-based temperature control systems are not equivalent and the latter might not be the better for all applications. A Peltier effect heater appears to be cleaner as no fluid is involved in the spectrophotometer and the temperature control is faster and more robust with such a device. Nevertheless, a temperature-controlled water bath is recommended for the following reasons. Even though the slower to equilibrate water bath might seem to waste time at the beginning of an experiment, for most of the experiment, temperature change speed is not an issue. More important is the homogeneity of the temperature gradient especially when dealing with non-equilibrium transitions. Slow ramping with a Peltier effect heater usually requires setting up small temperature increments resulting in a stairs-like temperature ramp rather than a linear one. Finally, fluidic systems in water bath–based temperature-controlled cell holders are prone to clogging and thus might result in a heterogeneous temperature in the cell holder. But for Peltier effect heaters that go below room temperature, water cooling is also involved and if this becomes clogged, the Peltier system might not be cooled properly at a high temperature and may burn itself out; this is not a problem with a water bath.

**Spectrophotometer choice and data acquisition**

Routinely, a dual-beam UV-visible spectrophotometer equipped with photomultipliers is used for detection but others use a single-beam apparatus and/or photodiode detection system. The most important parameter is the thermal and temporal stability of the apparatus; it is critical that temperature changes in the sample chamber do not affect the optics and electronics of the spectrophotometer. Visible light is only required for the control wavelength or when working with fluorescent oligonucleotides or with certain ligands.

The spectrophotometer chosen should be able to record temperature and absorbance, which is not always the case. Each spectrophotometer has its own software for data acquisition; it is therefore impossible to provide
here a step-by-step protocol for setting the parameters concerning data collection. Nevertheless, the following general comments apply for most applications.

Absorbance values at discrete wavelengths (or, if possible, full spectra in the range 220 to 400 nm) should be collected at regular time or temperature intervals. In most cases, collecting values every 1°C or 0.5°C is sufficient.

It may be advantageous to increase the averaging time from the typical default setting (defaults are usually at ≤1 sec; the authors recommend 2- to 3-sec measurements at each wavelength for each cell).

Temperature measurements should be as accurate as possible. Recording the absorbance of a “blank” sample using an external temperature probe is recommended. Many experimental settings use the controller temperature and simply assume that it is equivalent to the temperature of the sample. The authors have observed that this assumption is often inaccurate: the temperature of the sample may differ by ≥6°C from that or the water bath or Peltier effect heater especially at very high or very low temperatures or when a fast temperature gradient is employed. Transmission of heat to the cuvette (to reach thermal equilibrium) is not instantaneous. With temperature gradients >0.5°C/min, the sample temperature may not reach equilibrium. One simple experiment to determine the maximum gradient applicable to a given spectrophotometer is to record the heating and cooling profile of a known simple nucleic acid duplex at 0.1 M to 1 M NaCl. Formation and denaturation of this structure is known to occur quickly and the $T_m$ determined with the heating curve should be identical to the $T_m$ determined with the cooling experiment.

Determination of molecularity
When dealing with an intramolecular quadruplex, the $T_m$ should be concentration independent. Testing each quadruplex sequence at different concentrations is therefore an important control. In contrast, when molecularity is >1, an increase in the concentration of some or all of the partners should favor the association reaction, which is translated into an increase in the melting temperature. For bimolecular and tetramolecular quadruplexes, the slow kinetics of association and dissociation generally lead to a hysteresis phenomenon, which complicates the analysis. For these quadruplexes, one cannot simply determine a van’t Hoff enthalpy from the concentration dependency of the $T_{1/2}$ deduced from the heating curve. One should notice that melting of some tetramolecular quadruplexes is concentration independent, but these are not equilibrium profiles and thus cannot be used to deduce that the system is intramolecular.

A sufficiently large concentration range should be scanned to confirm concentration dependence. The minimal acceptable range is ten-fold, with at least three to six different concentrations (for example: 1, 3, and 10 μM, or 1, 2, 3, 5, 7, and 10 μM). If possible, a wider concentration range should be chosen, e.g., 1 to 40 μM. To record the spectra of the samples at a high concentration, one may use microcuvettes with a path length of 0.2 cm. For typical applications, using square section (10 × 10-mm) quartz cuvettes is recommended. If quadruplex formation has previously been established and 295 nm is the only wavelength used in one experiment, linear response is not an issue and a very high concentration can be used, but TDS, CD, and other wavelength melting profiles are then excluded.

Critical Parameters

**Purity of the oligonucleotide.** An extremely high level of purity (e.g., no detectable n–mers) is unnecessary. Decent melting profiles may be obtained with samples with 80% to 90% purity.

**Stability of the instrument (versus temperature and versus time).** A typical melting experiment takes 6 to 15 hr. No significant drift should occur during that time with the chosen instrument. Ideally, constant values should be obtained (1) for absorbance of a known stable DNA structure is recorded at a constant temperature over the time frame of a typical experiment and (2) for absorbance of a “blank” sample (buffer only) over the temperature gradient of a typical experiment.

**Correct temperature measurements.** Interpreting absorbance versus temperature curves require that both Y- and X-axis values are as accurate as possible. Many users pay attention to absorbance accuracy and neglect the precision of the temperature measurement. The temperature recorded must correspond to the sample temperature.

Troubleshooting

**Nucleic acid purity.** Most experiments will be performed with chemically synthesized oligonucleotides. Although now routine, this synthesis may fail spectacularly; a small fraction of the commercially synthesized sequences may be of very low purity. Quality
control by HPLC, mass spectrometry, capillary, or regular electrophoresis is convenient for most applications.

**Evaporation of the sample.** Partial evaporation is difficult to avoid when the sample is incubated at a high temperature for long periods of time. The most efficient way to prevent this problem is to manually check that each cap fits tightly in each cuvette. One may also seal the cuvettes with commercial nail polish or add a thin layer of mineral oil to the top of the solution. In any case, the sample should spend as little time as possible at very high temperatures.

**Formation of air bubbles at high temperature.** This problem is due to the reduced gas solubility at high temperatures, which leads to the formation of air bubbles in the sample if working with non-degassed samples. These bubbles can be removed by preincubating the sample or the buffer before adding DNA or RNA, if one worries about degradation of the sample or needs to perform a heating denaturation experiment, at the high temperature.

**Condensation at low temperature.** At low temperatures (≤20°C) condensation may occur. One can solve this problem by constantly blowing a stream of dry air through the sample compartment.

**Precipitation.** Aggregation or precipitation of the sample ruins an experiment. Whenever diffusion is visible during the recording of an absorbance spectrum, the corresponding melting profile is likely to be unusable. There are few solutions to a precipitation problem. Changing buffer conditions or reducing sample concentration is a possibility, but does not guarantee success.

**Extreme melting temperatures.** If the melting temperature is too high or too low, it will not be possible to record a sufficient number of experimental points below and above the melting temperature. This will complicate baseline determination and T_m determination will be less precise. If possible and compatible with the study, lowering the Na^+ or K^+ content of the buffer might allow a more precise T_m determination.

**Noisy signal.** The Y-signal variation is important both in absolute terms (ΔA) and relative terms (ΔA/A). One may improve the quality of the melting profile by increasing the sampling averaging time and increasing sample concentration. One should use a maximum absorbance value in the linearity range of the machine. In the worst case scenario, this value is around an absorbance of 1, but most spectrophotometers have a linear response with an absorbance value of up to ≥2 even in the UV region. This can be experimentally determined by checking Beer-Lambert’s law.

**Detection of artifacts.** Many artifacts can be detected by recording absorbance at a control wavelength, at which no constituent absorbs light (e.g., ~400 nm in the presence of nucleic acids only). Absorbance at this wavelength should be low and vary with temperature in a monotonic manner (or even better, not at all). For example, a sudden variation at this wavelength may indicate (1) the formation or release of an air bubble; (2) temperature-dependent aggregation of the sample, or (3) misalignment of the cuvette.

**Anticipated Results**

A simple melting experiment provides a wealth of information on the stability of quadruplexes. It is possible to extract thermodynamic values, and sometimes kinetic values, from an absorbance melting profile. This usually allows us to determine a ΔG° at 37°C, a value that is directly related to the association constant and therefore has more biological relevance than a simple T_m value. A single melting experiment allows for the determination of various thermodynamic parameters over a wide temperature range. Nevertheless, even if information is obtained on the stability of a complex, a melting experiment does not directly provide information about the structure of the complex. However, as discussed above, nucleic acid structures have characteristic absorbance profiles that can be used to infer structure. One can determine molecularity from a comprehensive absorbance melting study. It is also useful to compare these “model-dependent” thermodynamic parameters with model-independent calorimetric data (Albergo et al., 1981). Microcalorimetry should give reliable data, but is more expensive and less sensitive.

**Time Considerations**

A typical UV melting experiment is performed overnight. Provided that a sample changer is available, 5 to 18 samples (depending on the experimental setting and manufacturer of the machine) may be tested in parallel. Automated data analysis is more or less accurate, depending on the software provided, but is generally fast. Manual baseline determination and data analysis takes longer (a few hours for an experiment) but is often more accurate and this is the method recommended.
Literature Cited


Internet Resources
www.quadruplex.org
A Website to serve the quadruplex community.