Optical Methods

Nucleic acids participate in a variety of processes in which nucleic acid complexes are formed. The stabilization of the DNA duplex, its replication and recombination, its translation into RNA, and the folding of RNA structures all depend on specific interactions between nucleic acids. To understand these processes and to take advantage of the exquisite recognition capabilities of nucleic acids requires an understanding of the thermodynamics of nucleic acid complex formation. Methods for extraction of thermodynamic parameters from equilibrium optical melting curves are described in this unit. Additional procedures are presented for making important preliminary determinations of molar extinction coefficients and the number of oligonucleotides that combine to form a complex.

Basic Protocol 1 presents a simple method for accurately determining the extinction coefficients of oligonucleotides and polynucleotides, while Basic Protocol 2 presents a method for simultaneously determining the extinction coefficient for a nucleic acid complex and the number of strands in the complex. Basic Protocol 3 describes a method for determining equilibrium melting curves of nucleic acids by monitoring the temperature dependence of UV absorbance. Methods for analysis of the equilibrium melting curves to extract values for $\Delta H°$ and $\Delta G°$ are described in Support Protocols 1 to 5.

DETERMINATION OF OLIGONUCLEOTIDE MOLAR EXTINCTION COEFFICIENTS

The molar extinction coefficient (ε_{260}) of an oligonucleotide is determined by means of a colorimetric phosphate assay (Snell and Snell, 1949; Griswald et al., 1951), whereby phosphate is released from the oligonucleotide enzymatically. A standard curve of color intensity (absorbance) produced versus phosphate concentration is used to quantify the released phosphate. Given that information, the extinction coefficient is calculated easily. Poly(rU) is used as a positive control to monitor completeness of the enzymatic digestion.

To avoid contamination that would compromise the molar extinction coefficient determination, phosphate should be scrupulously avoided in every step of the preparation of the oligonucleotide. In addition, the oligonucleotide should be desalted subsequent to purification and prior to determination of the extinction coefficient.

Materials

10 mM cacodylate buffer solution (see recipe)
1 mg/mL nuclease P1 solution (see recipe)
100 U/mL alkaline phosphatase solution (see recipe)
Oligonucleotide to be analyzed
53 μM poly(rU) (Amersham Pharmacia Biotech) in 10 mM cacodylate buffer solution
Standard phosphate solution (see recipe)
ANS solution (see recipe)
Molybdate solution (see recipe)

1-dram screw-capped glass vials
95°C water bath
Single- or double-beam UV/visible spectrophotometer
Matched quartz semimicro 10-mm spectrophotometer cuvettes

NOTE: In all steps, samples should be prepared in 1-dram screw-capped glass vials. Plastic microcentrifuge tubes should not be used.

1. Prepare 1 mL oligonucleotide solution with an A_{260} of ~0.4 to 0.5 in 10 mM cacodylate buffer solution and place in a quartz semimicro 10-mm cuvette. Fill another cuvette with 10 mM cacodylate buffer solution.

2a. *For a single-beam instrument:* Place the buffer-only cuvette in the instrument and zero the instrument at 260 nm. Replace cuvette with the oligonucleotide-containing cuvette and record absorbance at 260 nm.

2b. *For a double-beam instrument:* Place the buffer-only cuvette in the reference beam and the oligonucleotide-containing cuvette in the sample beam. Record the absorbance at 260 nm.

3. Repeat this procedure using a 1-mL sample of poly(rU) solution in place of the oligonucleotide.

    The selection of 260 nm is by convention, as it is at or near the absorbance maximum of most oligonucleoties. If the spectrophotometer has wavelength scanning capabilities, it is desirable to scan between 200 and 350 nm. If the oligonucleotide displays significant self-structure, as seen in a temperature-dependent absorbance, the absorbance measurement should be made at elevated temperature.

4. To each oligonucleotide and poly(rU) sample and to 1 mL of cacodylate buffer, add 3 µL of 1 mg/mL nuclease P1 solution and 10 µL of 100 U/mL alkaline phosphatase solution. Allow digestion to proceed overnight at room temperature.

    Nuclease P1 cleaves the phosphodiester backbone of the oligonucleotide, and alkaline phosphatase cleaves the phosphate group from the released nucleotides.

5. In separate tubes, prepare the following samples, then dilute each to 800 µL with cacodylate buffer solution:

    a. Phosphate standard curve: Prepare thirteen samples containing 0 through 120 µL standard phosphate solution at 10-µL intervals.

    b. Test samples: Prepare four parallel samples containing 50, 100, 150, and 200 µL enzyme-digested oligonucleotide solution.

    c. Positive control: Prepare four samples containing 50, 100, 150, and 200 µL enzyme-digested poly(rU) solution.

    d. Negative control: Prepare four samples containing 50, 100, 150, and 200 µL enzyme-containing negative control sample.

    The standard curve and controls (positive and negative) must be run with each assay as a quality control measure. If desired, additional oligonucleotides may be analyzed in parallel using the same standard curve and controls, provided that the same solutions are used and all samples are analyzed simultaneously.

6. Add 100 µL ANS solution and 100 µL molybdate solution to each sample. Heat for 10 min in a 95°C bath, then cool to 25°C.

    Upon heating, formation of a phosphomolybdate complex results in a blue tint to the solution that is proportional to the concentration of phosphate.

7. Set the spectrophotometer to read absorbance at 820 nm. Fill a cuvette with cacodylate buffer solution. Measure and record A_{820} for each sample, using the cacodylate buffer solution for a reference as described in steps 2a and 2b. Thoroughly rinse and dry the cuvette and change pipet tips between samples.
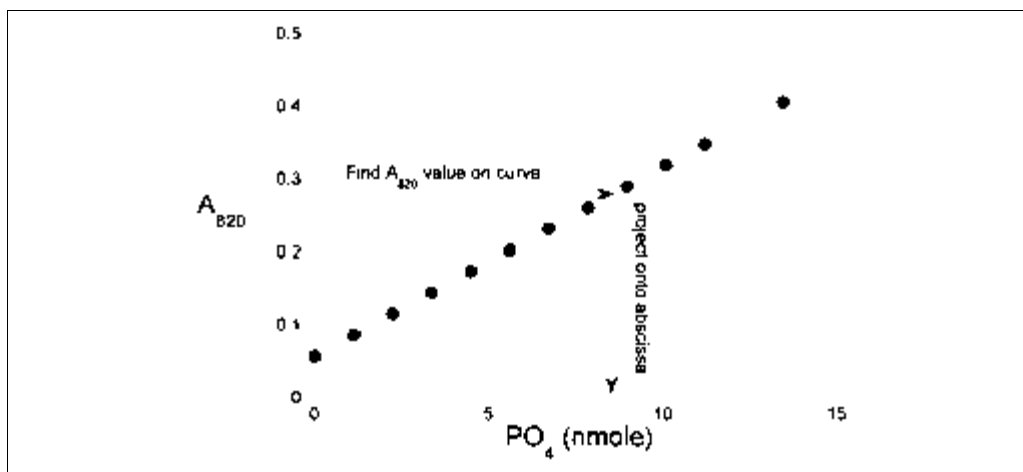
Figure 7.3.1 Standard curve for phosphate analysis.

8. Prepare a standard curve by plotting A_{820} versus phosphate concentration, $[PO_4]$, for the thirteen samples of standard phosphate solution. Confirm that A_{820} for the blank samples falls on the y intercept of a line through the data points.

   The amount of phosphate in each of the samples is simply the initial standard solution concentration multiplied by the volume of standard solution used: PO_4 (mol) = $[PO_4]_{std}$ (M) × V_{std} (L). Because $[PO_4]_{std} \approx 100\ \mu M$ and the volumes range from 10 to 120 μL, the amount of PO_4 will be in the 1 to 12 nmol range. See Figure 7.3.1 for an example.

9. Determine the amount (in mol) of phosphate present in each oligonucleotide and poly(rU) sample by finding the measured A_{820} on the standard curve and projecting a line onto the abscissa.

10. Calculate the molar extinction coefficient of the oligonucleotide using the equation

$$\varepsilon_{260} = \frac{A_{260}}{b\left(\dfrac{mol\,PO_4}{V}\right)\left(\dfrac{mol\,oligo}{mol\,PO_4}\right)}\ cm^{-1}M^{-1}$$

Equation 7.3.1

where b is the pathlength (1 cm), mol PO_4 is from step 9, A_{260} is from step 2a or 2b, and the mole unit of the final value refers to the oligonucleotide. Obtain the final value of the denominator from the length of the oligonucleotide, defined by

$$\frac{mol\,oligo}{mol\,PO_4} = (no.\ bases - 1)^{-1}$$

Equation 7.3.2

assuming the oligonucleotide is not end phosphorylated.

11. Confirm that the correct values for the extinction coefficient for poly(rU) positive controls are obtained.

   Because the polymer is of indeterminate length, a different mole unit must be used for the extinction coefficient, specifically, moles nucleotide/liter. The equation for ε_{260} reduces to the equation below because there is one phosphate per nucleotide. There are a number of slightly varying values for the poly(rU) extinction coefficient; a value of 9450 $cm^{-1}M^{-1}$ can be considered reliable (J. Völker, pers. comm.).

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$$\varepsilon_{260} = \frac{A_{260}}{b\left(\dfrac{\text{mol PO}_4}{V}\right)} \text{cm}^{-1}\text{M}^{-1}$$

Equation 7.3.3

DETERMINATION OF MOLECULARITY AND EXTINCTION COEFFICIENTS OF OLIGONUCLEOTIDE COMPLEXES

The same differences in UV absorbance between isolated oligonucleotides and the complexes they form that are exploited to produce melting profiles are used to determine the number of oligonucleotides in the complex. The method presented is based on careful preparation of mixing curves and measurement of absorbance as a function of the relative concentration of oligonucelotides. It is known as the method of continuous fractions (Felsenfeld et al., 1957). The resultant data are presented as a so-called Job plot. Multiple wavelengths are used because some wavelengths cannot detect formation of some complexes.

Materials

Two oligonucleotides, A and B, which are expected to form a complex
Buffer
Single- or double-beam UV/visible spectrophotometer
Quartz semimicro spectrophotometer cuvette (1-cm pathlength)

Additional reagents and equipment for determining molar extinction coefficients
 (see Basic Protocol 1)

1. Determine extinction coefficients for oligonucleotides A and B using Basic Protocol 1.

2. Prior to beginning the experiment, determine the minimum sample volume required for an accurate absorbance measurement in the particular spectrophotometer and cuvettes being used.

   The mounting of the cell holder in the spectrophotometer relative to the beam varies with the manufacturer and model. A volume of 600 µL is typically sufficient so that the meniscus does not impinge on the beam, but this should be confirmed. The measurement is easily done and need not be repeated, assuming the equipment used does not change.

   To determine the appropriate volume, prepare a solution with absorbance between 0.5 to 1.0 at 260 nm. It may be convenient to use a nucleic acid solution, although any solution with absorbance at the desired wavelength may be used. Begin with 500 µL of solution in the cell and measure the absorbance. Add 25-µL aliquots of the solution, each time noting the absorbance. The volume at which the absorbance becomes constant is the minimum usable volume. It is advisable to begin the experiment with a slightly larger volume.

3. Prepare 1.5 mL each of two oligonucleotide solutions, one with oligonucleotide A and another with oligonucleotide B, at identical concentrations. Use a concentration that gives an A_{260} of ~0.5 in a 1-cm-pathlength cuvette.

   A good choice of concentration is $c = 0.5/\varepsilon_{avg}$, where ε_{avg} is the average of the extinction coefficients for the two oligonucleotides.

   Because the solution conditions (salt concentration, pH, and temperature) determine the relative stabilities of complexes of various stoichiometries, the oligonucleotide solutions should be prepared in the buffer in which further experiments will be performed.

4. Fill the cuvette with buffer and record the absorbance at 220, 240, 260, 280, and 300 nm.

5. Place 600 μL (or the experimentally determined minimum volume) of oligonucleotide A solution in the cuvette and place it in the spectrophotometer. Measure the absorbance at 220, 240, 260, 280, and 300 nm.

6. Add 100 mL oligonucleotide B solution to the cuvette. Mix thoroughly.

   Because semimicro 1-cm-pathlength cuvettes are used, below-cell magnetic stirring does not work well. The best alternative is an immersible mixing device, although care must be taken that the immersed stirrer does not scratch the cuvette and does not impinge on the light beam when absorbance measurements are made. Alternatively, mixing can be accomplished by repeated drawing and dispensing of the solution with a transfer pipet or by capping and sealing of the cell followed by inversion. In either case, great care must be taken to avoid a loss of solution. If standard 1-cm-pathlength cuvettes and a spectrophotometer equipped with a built-in magnetic stirrer in the cell holder are used, adequate stirring should be possible with a 7 × 2–mm Teflon-coated magnetic stir bar. The amount of each solution required increases by a factor of ~3 when standard 1-cm cuvettes are used.

7. Measure the absorbance at 220, 240, 260, 280, and 300 nm. Repeat the absorbance measurements at 5-min intervals until constant values are observed.

   If significant self-structure or self-association is observed, as detected by temperature-dependent transitions in the absorbance of either oligonucleotide, it is necessary to include an annealing step. The cuvette is heated above the melting temperature of the complex and cooled slowly. If this step is included, the cells must be securely capped to minimize evaporation.

8. Repeat steps 6 and 7 five times using 100-μL aliquots of oligonucleotide solution B.

9. Clean and dry the cuvette. Repeat steps 4 through 8, beginning with 600 μL oligonucleotide solution B and adding 100-μL aliquots of oligonucleotide solution A.

10. Prepare a plot of A_{260} versus the mole fraction of oligonucleotide B (X_B).

   Because oligonucleotide A and B solutions are at identical concentrations, $X_B = [V_B/(V_A + V_B)]$, where V_A and V_B are the total volumes of oligonucleotide solutions A and B in the
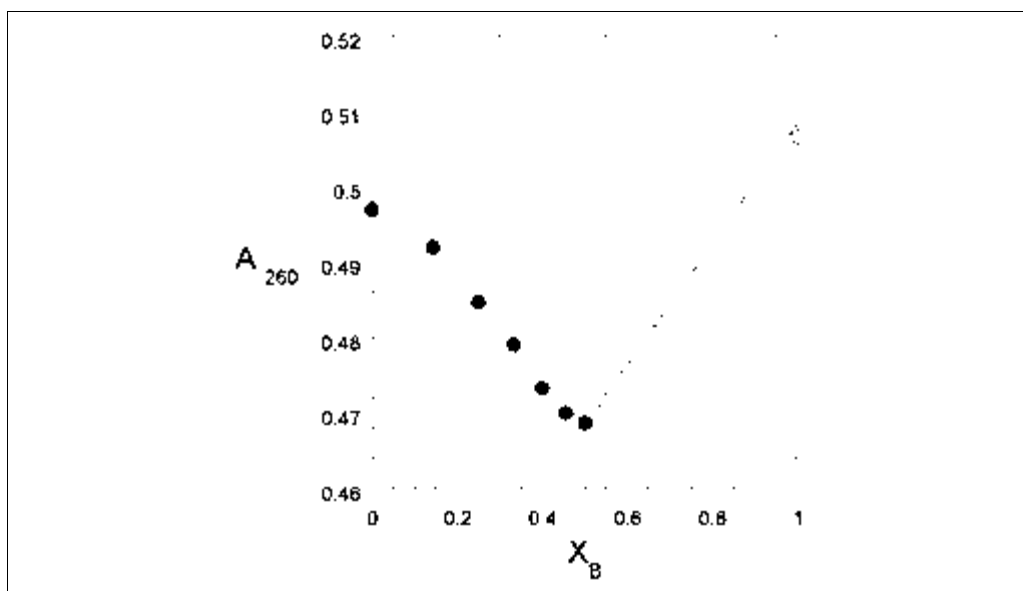

Figure 7.3.2 Example of an oligonucleotide mixing curve. The filled circles represent additions of oligonucleotide B to oligonucleotide A. Open circles represent additions of oligonucleotide A to oligonucleotide B.

cuvette. An example is shown in Figure 7.3.2. The inflection point at $X_B = 0.5$ in the example indicates the formation of an n:n complex. In the absence of contrary information, it is reasonable to assume that a 1:1 complex has formed. Because the concentration, c, of the complex is known from the concentrations of the oligonucleotide solutions, the extinction coefficient of the complex is easily determined from $\varepsilon_{260} = (A_{260}inf/bc)$, where $A_{260}inf$ is the absorbance at the inflection point and b is the pathlength. Failure of the two titration curves to meet indicates a probable error in one of the oligonucleotide extinction coefficients or in solution preparation. Excessive curvature may indicate failure to reach equilibrium or a very low association constant.

11. Repeat step 10 for data collected at remaining wavelengths to confirm that no other stoichiometries are observed.

PREPARATION OF EQUILIBRIUM MELTING CURVES

The absorbance of nucleic acids differs between the less structured isolated single strands observed at high temperature and the folded or complexed strands observed at low temperature. The thermal melting profiles obtained from a temperature-dependent absorbance experiment can be used to extract thermodynamic parameters. The concentration dependence of the melting temperature is particularly useful in determining the thermodynamic parameters (see Support Protocol 1) for complexes of molecularity >1; therefore, the experiment is described in terms of a concentration-dependent study.

Materials

Oligonucleotides in buffer solution (single folded chains or multimolecular complexes)

Double-beam UV/visible spectrophotometer with temperature-controlled (stepping and/or scanning) cell holder

Stoppered quartz spectrophotometer cuvettes (0.1-, 0.2-, 0.5-, and 1.0-cm pathlengths) with metal spacers (shorter pathlength cuvettes are useful, if available)

Additional reagents and equipment for determining molar extinction coefficients (see Basic Protocol 1) and determining molecularity and extinction coefficients of complexes (see Basic Protocol 2)

NOTE: Buffers with large heats of ionization, which includes most popular biochemical buffers, should be avoided, as the pH of solutions prepared with these buffers is temperature dependent. Tris solutions are particularly susceptible to this effect. Phosphate, cacodylate, and PIPES are acceptable choices.

1. Determine extinction coefficients for the individual oligonucleotides (see Basic Protocol 1).

2. If a multimolecular complex is to be examined, determine the molecularity of the complex and its extinction coefficient (see Basic Protocol 2).

3a. *For a single melting experiment:* Fill a stoppered, 1-cm-pathlength semimicro cuvette with 1300 μL of an oligonucleotide solution that gives an A_{260} of ~0.4 to 0.6.

3b. *For a concentration-dependence study:* Prepare eight to ten solutions of the oligonucleotide complex to cover as wide a range of concentrations as possible (≥200-fold). Calculate the concentration from $c = (A_{260}/\varepsilon_{260}b)$.

The oligonucleotide complex concentrations should be designed to span the A_{260} range of 0.1 in a 1.0-cm cuvette to 2.0 in a 0.1-cm cuvette. Depending on the quality of the spectrophotometer, this concentration range may be expanded.

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Table 7.3.1 Selection of Concentration and Cuvette Pathlength[a]

		$A = \varepsilon c b$[b]			
$\ln(\varepsilon c)$	εc	$b = 1$ mm	$b = 2$ mm	$b = 5$ mm	$b = 10$ mm
−4.61	0.01	0.01	0.02	0.05	**0.1**
−4.08	0.017	0.017	0.034	0.085	**0.17**
−3.55	0.029	0.029	0.057	**0.14**	**0.29**
−3.02	0.049	0.049	**0.10**	**0.24**	**0.49**
−2.49	0.083	0.083	**0.17**	**0.41**	**0.83**
−1.96	0.14	**0.14**	**0.28**	0.7	1.4
−1.43	0.24	**0.24**	**0.48**	1.2	2.4
−0.90	0.41	**0.41**	**0.81**	**2**	4.1
−0.37	0.69	**0.69**	1.4	3.5	6.9
0.16	1.2	**1.2**	2.3	5.9	12.0
0.69	2.0	**2.0**	4.0	10.0	20.0

[a]To use the table, select from each row a target absorbance (A) and a useable pathlength (b from colums 3, 4, 5, or 6). Use the known ε value to calculate c from the εc column.
[b]Boldface indicates absorbance values that are usable with the pathlength b.

Because of the logarithmic dependence of the melting temperature on oligonucleotide complex concentration, it is desirable to distribute the concentrations evenly on a logarithmic scale. Selection of concentrations and cuvette pathlengths to produce solutions giving appropriate absorbance values is nontrivial. Table 7.3.1 provides a guide for solution preparation and pathlength selection. The desired 200-fold concentration range (from εc = 0.01 to 2.0) is covered with even steps on a logarithmic scale.

Solutions should be prepared in sufficient volume to fill the selected cuvette. Table 7.3.2 provides the volume necessary to fill commonly available cuvettes.

4. Fill a cuvette (or cuvettes, if spectrophotometer is equipped with a multiple-cell holder) and securely seal with a stopper. Place cuvette in spectrophotometer cell holder. Use metal spacers to ensure proper alignment of the cuvette and good thermal contact with the thermostatically controlled cell holder.

   If stoppered cuvettes are not available, a small amount of silicon oil may be layered on top of the sample.

   It is good practice to heat the sample above T_m and slowly cool it to assure proper annealing of the complex. This annealing step need not be performed in the spectrophotometer.

5. Equilibrate the instrument at the starting temperature, usually 1° to 5°C.

6a. *For scanning temperature:* Increase the temperature at a rate of 0.5°C/min or less. Collect absorbance data at intervals such that four or more absorbance measurements are recorded for each sample for each degree increase in temperature. Collect data

Table 7.3.2 Approximate Filling Volumes for Common Cuvettes

Pathlength (cm)	Total volume (μL)
1.0[a]	1300
0.5[a]	600
0.2	600
0.1	300

[a]Semimicro cuvette.

until the temperature has increased well beyond the apparent melting temperature of the complex, usually to 95° to 100°C. Cool and remove the cuvette.

6b. *For stepping temperature (preferred):* Collect data at 0.25°C or smaller temperature intervals. Collect data until the temperature has increased well beyond the apparent melting temperature of the complex, usually to 95° to 100°C. Cool and remove the cuvette.

7. If conducting a concentration dependent study, repeat steps 4 to 6 until all samples have been heated in the spectrophotometer.

8. Analyze data (see Support Protocol 1 for concentration-dependent data or one of Support Protocols 2 through 4 and Support Protocol 5 for a single-melting study).

ANALYSIS OF EQUILIBRIUM MELTING CURVES

A number of methods for analyzing single or multiple equilibrium melting curves to extract thermodynamic parameters are described below. All of the calculations described are easily implemented in a spreadsheet. All of the thermodynamic parameters determined using Support Protocols 1 to 5 describe the association equilibrium. The preferred method of analysis for equilibrium melting curves is described in Support Protocol 1, which allows determination of the enthalpy change ($\Delta H°$), the entropy change ($\Delta S°$), and the free energy change ($\Delta G°$). If concentration-dependent curves are not available or if the process is monomolecular (hairpin or other folded single-chain structure) or pseudomonomolecular (polynucleotide), Support Protocols 2 through 4 may be used to extract $\Delta H°$ and Support Protocol 5 may be used to determine $\Delta G°$. For pseudomonomolecular processes, which formally are bimolecular but show no concentration dependence, a value of 1 can be used for the molecularity in the following equations.

In all of the equations that follow, the concentration C_T refers to total oligonucleotide concentration.

$$C_T = \sum_{i=1}^{n} [A_i]$$

Equation 7.3.4

All methods of analysis described below depend on the experimental setup to ensure that

$$[A_1] = \ldots [A_i] \ldots = [A_n], \text{thus} [A_i] = \frac{C_T}{n}$$

Equation 7.3.5

Due to statistical effects, the equations for the association constant differ for complexes comprised of non-self-complementary strands and for complexes comprised of self-complementary strands. When appropriate, two forms of the equations are presented.

Although it is convenient to present data as a function of temperature in degrees Celsius, all calculations of thermodynamic parameters must be computed using temperature in Kelvin units.
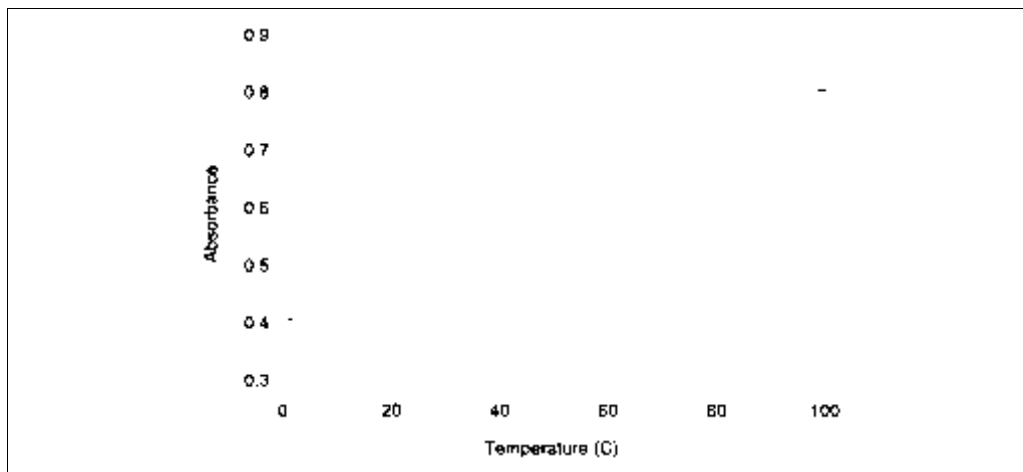
Figure 7.3.3 Simulated UV-monitored oligonucleotide melting curve. The dashed lines represent the upper and lower baselines that are used to calculate the $\alpha(T)$ versus T curve.

CALCULATION OF $\Delta H°$ FROM CONCENTRATION-DEPENDENT MELTING CURVES

In this protocol, thermodynamic parameters are determined for short oligonucleotide complexes (i.e., duplexes with <12 bp) from a plot of $1/T_m$ versus $\ln C_T$ as described by Marky and Breslauer (1987).

1. Define upper and lower absorbance baselines, $A_U(T)$ and $A_L(T)$, by fitting the linear portions of the absorbance versus temperature curve, $A(T)$ versus T.

   Figure 7.3.3 shows an example of a melting curve and the fitted baselines.

2. Define a parameter $\alpha(T)$ to represent the relative fraction of the complex that remains in the initial state at temperature T. Plot $\alpha(T)$ versus T using the slopes (m) and intercepts (b) from the fitted baselines as follows

$$\alpha(T) = \frac{A_U(T) - A(T)}{A_U(T) - A_L(T)} = \frac{m_U T + b_U - A(T)}{(m_U - m_L)T + b_U - b_L}$$

Equation 7.3.6

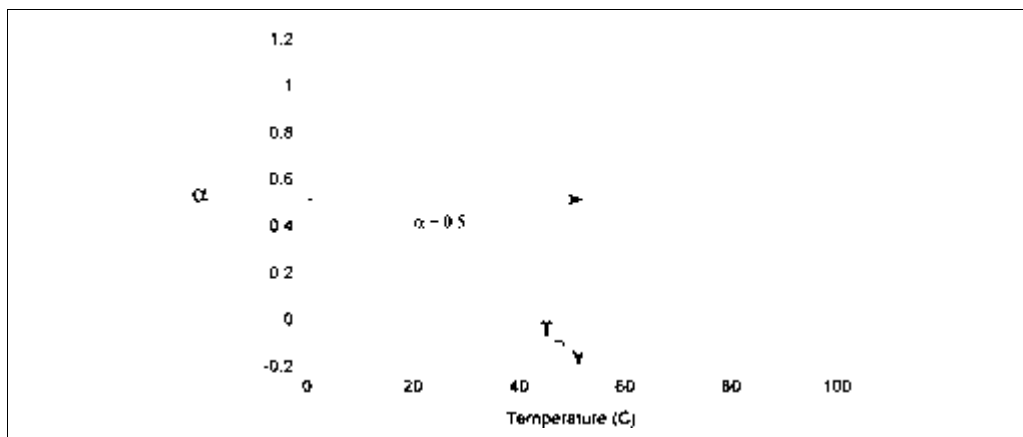where $A_U(T) = m_U T + b_U$ and $A_L(T) = m_L T + b_L$.

Figure 7.3.4 The α versus T curve. This curve shows the fraction of the initial complex as a function of temperature. Determination of T_m from the curve is shown.

3. Determine T_m, which is defined as the temperature at which $\alpha = 0.5$.

Figure 7.3.4 shows an α versus T curve and the determination of T_m.

The melting temperature T_m is of limited value in comparing the stability of two duplexes or other complexes. Unfortunately, this is common practice. The T_m value depends on nucleic acid concentration for duplexes and higher molecularity complexes. Also, T_m comparisons assume implicitly that $\Delta H°$ is identical for both processes. The solution conditions also influence T_m significantly. Because of the change in linear charge density associated with the transition from complex to single strands, the T_m depends on salt concentration. Dependence on pH is observed for C^+GC-containing triple helices and some lesion-containing duplexes. All complexes are affected by extremes of pH (<5 or >9). Before any useful T_m comparison can be made, the nucleic acid concentrations and solution conditions must be identical.

4. Plot $1/T_m$ versus $\ln C_T$ and determine $\Delta H°$ using the measured slope and the slope from the following equation

$$\frac{1}{T_m} = \frac{(n-1)R}{\Delta H°} \ln C_T + \frac{[\Delta S° - (n-1)R \ln 2n]}{\Delta H°}$$

Equation 7.3.7

for non-self-complementary oligonucleotide complexes or

$$\frac{1}{T_m} = \frac{(n-1)R}{\Delta H°} \ln C_T + \frac{[\Delta S° - (n-1)R \ln 2n + R \ln n]}{\Delta H°}$$

Equation 7.3.8

for self-complementary oligonucleotide complexes. In both cases, n is molecularity and R is the gas constant.

Note that in both cases, $1/T_m$ versus $\ln C_T$ is linear with a slope of $(n-1)R/\Delta H°$. Thus, $\Delta H°$ is readily determined from the measured slope. Further note that when $n = 1$ the slope = 0. This is consistent with the observed concentration independence of monomolecular processes, such as hairpin melting.

5. Determine values of $\Delta S°$ from the intercept of the appropriate equation shown above. Determine values of $\Delta G°$ using $\Delta G° = \Delta H° - T\Delta S°$.

Use caution in interpreting these values. The $\Delta S°$ and $\Delta G°$ values depend directly on the value of $\Delta H°$. This coupling of the $\Delta S°$ and $\Delta G°$ values to $\Delta H°$ may result in apparent entropy-enthalpy compensation when none exists.

For intermediate-length complexes, a concentration-dependent T_m is still observed. This dependence is reduced relative to that expected for complexes with the same $\Delta H°$ in a two-state equilibrium. Therefore, $\Delta H°$ is overestimated by the above equations. The length where this effect is observed depends on the molecularity, on the sequence, and possibly on the solution conditions. For DNA duplexes, the length is roughly between 12 and ~50 base pairs, where pseudomonomolecular behavior begins.

CALCULATION OF $\Delta H°$ FROM $\alpha(T)$ VERSUS T PLOTS

This method is from Marky and Breslauer (1987).

1. Calculate values for $\alpha(T)$ as described above (see Support Protocol 1, steps 1 and 2).

2. Plot $\alpha(T)$ as a function of $1/T$ as in Figure 7.3.5.

3. Examine the melting curves to get an indication of the enthalpy change associated with the transition.
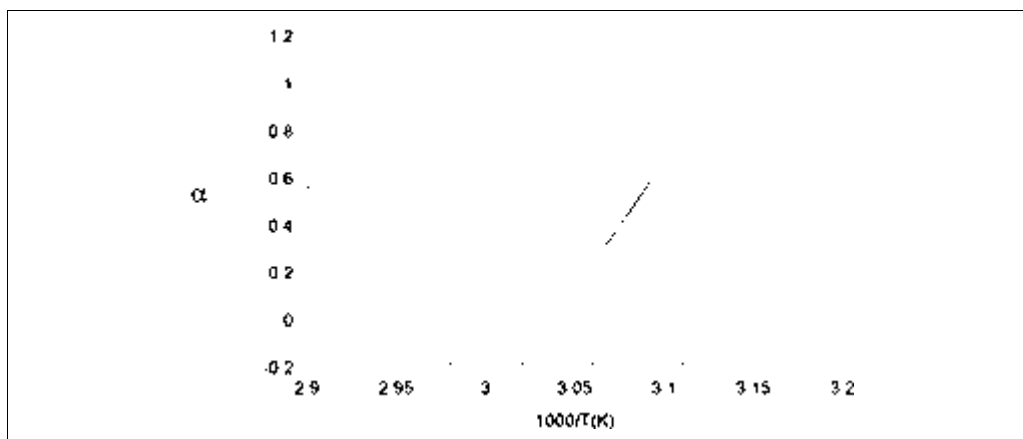
Figure 7.3.5 Determination of ΔH° from a plot of α versus $1/T$. The slope of the line shown is used to calculate ΔH°.

> *A steep slope indicates a large value of ΔH°, whereas a shallow one indicates a small value of ΔH°.*

4. Fit a line to the linear portion of the melting curve centered at T_m using the linear least squares technique. Calculate the slope of this line.

> *The linear region of the melting curve is typically found at α values between 0.3 and 0.7. Significant curvature in this region may indicate a complex dissociation process; that is, the two-state assumption is violated.*

5. Calculate ΔH° using the equation

$$\Delta H^\mathrm{o} = (2n + 2)RT_\mathrm{m}^2 \left(\frac{\partial \alpha(T)}{\partial T} \right)_{T=T_\mathrm{m}} = -(2n + 2)R \left(\frac{\partial \alpha(T)}{\partial (1/T)} \right)_{T=T_\mathrm{m}}$$

Equation 7.3.9

for non-self-complementary or self-complementary complexes.

CALCULATION OF ΔH° BY DIRECT APPLICATION OF THE VAN'T HOFF EQUATION

This method is from Puglisi and Tinoco (1989).

1. Calculate values for $\alpha(T)$ as described (see Support Protocol 1, steps 1 and 2).

2. Use the value of α at any temperature T to calculate a value for the association constant, $K(T)$, where the expressions for $K(T)$ depend upon the number of molecules (strands), n, in the equilibrium and the sum of their concentrations, C_T. For non-self-complementary complexes use the equation

$$K(T) = \frac{\alpha(T)}{\left(\dfrac{C_\mathrm{T}}{n} \right)^{n-1} [1 - \alpha(T)]^n}$$

Equation 7.3.10

and for non-self-complementary complexes use the equation

$$K(T) = \frac{\alpha(T)}{nC_\mathrm{T}^{n-1} [1 - \alpha(T)]^n}$$

Equation 7.3.11

3. Plot $\ln K(T)$ versus $1/T$ and use the slope of this line to determine $\Delta H°$ by direct application of the van't Hoff equation.

$$\Delta H° = -R \frac{\partial \ln K(T)}{\partial (1/T)}$$

Equation 7.3.12

CALCULATION OF $\Delta H°$ FROM DIFFERENTIAL CURVES

In this protocol, the shape of the curve defined by the derivative of α with respect to $1/T$ is used to make a robust determination of $\Delta H°$ (Marky and Breslauer, 1987).

1. Calculate values for $\alpha(T)$ as described (see Support Protocol 1, steps 1 and 2).

2. Construct the derivative curve, $\partial\alpha(T)/\partial(1/T)$ versus T, in a spreadsheet using the method of Savitsky and Golay (1964). Because the Savitsky-Golay method requires evenly spaced values on the temperature axis, compute $\partial\alpha(T)/\partial T$ and use the relation $\partial\alpha(T)/\partial(1/T) = -T^2[\partial\alpha(T)/\partial T]$ to get the requisite derivative.

3. Determine T_{max} as the temperature at which the $\alpha(T)$ versus T curve attains its maximum slope.

   T_{max} is easily found as the maximum of the derivative curve. It is important to note that T_m = T_{max} only for monomolecular transitions.

4. Define T_1 and T_2 as the temperatures at which $\partial\alpha(T)/\partial(1/T) = \frac{1}{2}[\partial\alpha(T)/\partial(1/T)]_{max}$.

   Figure 7.3.6 shows an example of the derivative curve with T_{max}, T_1, and T_2 indicated.

5. Calculate the value of $\Delta H°$ for the derived temperatures using the following relations

$$\Delta H° = \frac{B}{\left(\frac{1}{T_1} - \frac{1}{T_2}\right)} \text{ or } \Delta H° = \frac{B'}{\left(\frac{1}{T_{max}} - \frac{1}{T_2}\right)}$$

Equation 7.3.13

where values for B and B' depend on the molecularity, n, of the transition and are shown in Table 7.3.3.

This method is not recommended for analysis of repeating-sequence polynucleotide melting. These transitions are extremely sharp, making accurate differential curves difficult to calculate. Use Support Protocols 2 or 3 for these molecules.

Table 7.3.3 Values for the Constants B and B' Used in Calculating $\Delta H°$ Values from Derivative Curves

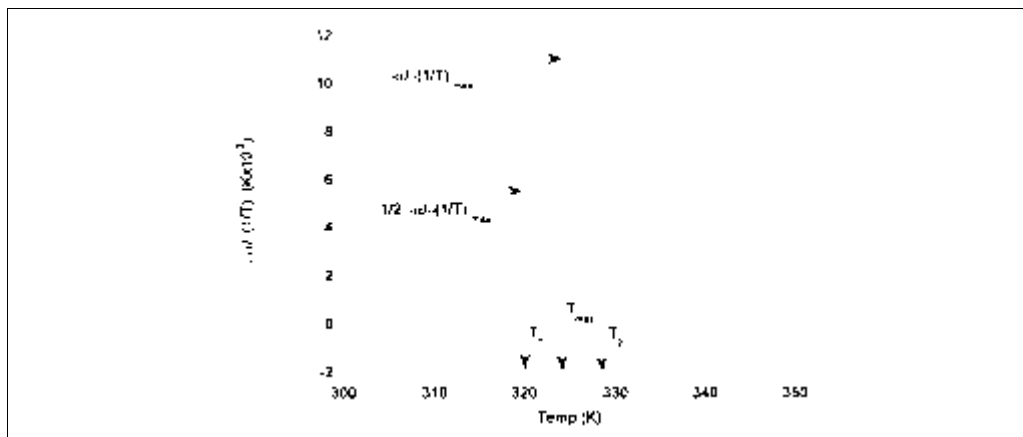| Molecularity, n	B	B'
1	−7.00	−3.50
2	−10.14	−4.38
3	−12.88	−5.06
4	−15.40	−5.63

Figure 7.3.6 Determination of $\Delta H°$ from a derivative curve. The value of T_{max}, T_1, and T_2 are easily found and used to calculate the value of $\Delta H°$.

CALCULATION OF $\Delta G°$ USING THE VAN'T HOFF EQUATION

This method is from Plum et al. (1999).

1. Calculate values for $\alpha(T)$ as described (see Support Protocol 1, steps 1 and 2).

2. Determine the value of $\Delta H°$ by one of the methods described in the other Support Protocols and use it to extrapolate the free energy change ($\Delta G°$) associated with the duplex disruption to a reference temperature, typically 37° or 25°C (310° or 298°K). Use the equation

$$\Delta G° = \frac{\Delta H°}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right) + (n-1)\ln\left(\frac{C_T}{2n}\right)$$

Equation 7.3.14

for non-self-complementary oligonucleotide complexes and

$$\Delta G° = \frac{\Delta H°}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right) + (n-1)\ln\left(\frac{nC_T}{2}\right)$$

Equation 7.3.15

for self-complementary oligonucleotide complexes.

There is an assumption implicit in these equations that the heat capacity change ($\Delta C_p°$) for the transition is zero and that $\Delta H°$ is temperature invariant.

These equations allow one to calculate $\Delta G°$ at any temperature, although they are valid only for matched pairs of T_m and C_T values. Complementary expressions in terms of T_{max} also can be used (Plum et al., 1995a). These are particularly useful when either the upper or lower baseline is not well defined, thereby making T_m difficult to determine.

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.

Alkaline phosphatase solution, 100 U/mL

Dilute 25 µL of the enzyme (1000 U/mL; Calbiochem) with 225 µL of 10 mM cacodylate buffer solution (see recipe). Store at –20°C for up to 6 to 12 months.

1-Amino-2-naphthol-4-sulfonic acid (ANS) solution

Weigh 5.95 g sodium bisulfite and 0.2 g anhydrous sodium sulfite. Dissolve in 25 mL water. Add 0.1 g 1-amino-2-naphthol-4-sulfonic acid (ANS). Dilute to 100 mL final volume. Store for up to 1 to 2 weeks at room temperature, protected from air and light.

Cacodylate buffer solution, 10 mM

Dissolve 0.21 g $NaC_2H_6AsO_2 \cdot 3H_2O$ (sodium cacodylate trihydrate) in 800 mL water in a 1-liter beaker. Adjust the pH to 7.0 with 1 N HCl. Transfer to a 1-liter volumetric flask and add water to bring the final volume to 1 liter, giving a 10 mM cacodylate solution. Store at 4°C for up to 6 months.

CAUTION: *Be careful not to breath the dust or come into contact with solutions containing cacodylate as it is an arsenic-containing compound.*

Molybdate solution

Dissolve 2.5 g $Na_2MoO_4 \cdot 2H_2O$ (sodium molybdate dihydrate) in 100 mL of 10 N sulfuric acid (28 mL of concentrated H_2SO_4 plus 72 mL water). Store at room temperature for up to 12 months.

CAUTION: *Add acid slowly to water. Significant heat is produced upon mixing. Place the vessel in a bucket to prevent spills in the event of breakage.*

P1 nuclease solution, 1 mg/mL

Dissolve 1 mg in 1 mL water in vial provided by supplier (Calbiochem). Store at –20°C for up to 6 to 12 months.

Standard phosphate solution

Dry KH_2PO_4 in an oven overnight or under vacuum over P_2O_5 overnight. Dissolve 13.6 mg KH_2PO_4 in 1 liter of water using volumetric glassware to produce a 100 µM PO_4 solution. Store at room temperature for up to 1 to 2 weeks.

Prepare this solution with utmost care, as the entire procedure is dependent on its accuracy. If necessary, make a higher concentration solution and dilute accordingly. Preparation of a solution of exactly 100 µM PO_4 concentration is less important than knowing the concentration precisely.

COMMENTARY

Background Information

The well-known relation described by van't Hoff between the temperature dependence of the equilibrium constant and the enthalpy change associated with the equilibrium is shown in the eponymous equation:

$$\Delta H^o = RT^2 \frac{\partial \ln K(T)}{\partial T} = -R \frac{\partial \ln K(T)}{\partial (1/T)}$$

Equation 7.3.16

The applications of this equation described herein are predicated on several assumptions. (1) The system examined is at equilibrium. Most oligonucleotide duplexes equilibrate rapidly; however, longer duplexes and higher-molecularity complexes (especially quadruplexes) may require long equilibration times. (2) The equilibrium described must be two-state; that is, no thermodynamically significant intermediate states may be present. In the case

of nucleic acid melting transitions, only the fully formed complex (or structure if the process is monomolecular) or fully melted single strands may be present. (3) The enthalpy change, $\Delta H°$, is assumed to be independent of temperature; that is, the heat capacity change ($\Delta C_p°$) is zero. A large $\Delta C_p°$ results in curved van't Hoff plots, which typically are not observed for nucleic acid melting transitions. (4) The observable, here UV, absorbance must reflect linearly the global extent of the melting transition.

The shape of the melting curve is related to the transition enthalpy change, $\Delta H°$, for the thermodynamic cooperative unit. For short duplexes, the cooperative unit is comprised of the entire molecule; however, for polymeric duplexes, the cooperative unit may be only a small fraction of the entire duplex. In all cases, the mole unit of the van't Hoff enthalpy refers to the cooperative unit. The only method for determining the size of the cooperative unit is by comparison of the van't Hoff enthalpy to the model-independent enthalpy value determined by differential scanning calorimetry.

For complexes of molecularity >1, the association constant (K) depends on oligonucleotide concentration. Because of the relationship between K and T_m, the melting temperature also depends on oligonucleotide concentration. This leads to the preferred method (when $n > 1$; see Support Protocol 1) for extraction of thermodynamic data from equilibrium melting curves. For monomolecular processes, one or more of the alternate methods (see Support Protocols 2 to 5), which are based on analysis of single melting curves, must be used.

It is useful to consider the behavior of oligonucleotide complexes as the length of the constituent strands increases. As the oligonucleotides increase in length the transition curves become sharper. This increase in sharpness does not necessarily indicate an increase in cooperativity. Rather, it is a manifestation of the increase in $\Delta H°$ of the thermodynamic unit due to the increase in the number of base pairs. As long as the two-state assumption is valid, cooperativity is maximal, yet the curves become sharper as the length increases.

Eventually, the length increases to a point where the two-state assumption fails and thermodynamically significant intermediate states participate in the equilibrium. The general appearance of the melting curves is unaffected and, for $n \geq 2$, the T_m still depends strongly on concentration. Yet the assumptions underlying the methods of analysis described here are invalid, and the resultant thermodynamic data are compromised. In this length regime, the only recourse is to study the system by direct calorimetric methods (see *UNIT 7.4*).

The point at which one must abandon van't Hoff methods for calorimetry is not well defined. One approach that, while not absolute, does help address this problem is a comparison of the results from several of the methods described here. If all of the assumptions underlying the van't Hoff methodology are valid, $\Delta H°$ values derived from the various methods should be in good agreement. The various methods (particularly the concentration-dependent versus single-curve methods) vary in sensitivity to deviations from the van't Hoff model. While small differences are expected, significant disparities among the data derived from the various methods provide a good indicator of equilibria that are not amenable to analysis by van't Hoff methods. The point where disparities become significant is a matter of debate; however, disparities greater than 5% to 10% call into question whether the two-state van't Hoff model has been applied appropriately. Typically, disparities among the methods begin to appear in duplexes >12 base pairs in length.

As the duplex length increases further, the concentration dependence of T_m vanishes. This is directly related to the mechanism of duplex disruption. Duplex formation can be described, at least qualitatively, by two parameters: initiation and propagation. Initiation involves the encounter of the two separate strands and the formation of a few base pairs. Initiation is a bimolecular process and, therefore, depends on concentration. Short duplex formation is dominated by initiation. In fact, the two-state assumption implies that initiation is the only thermodynamically significant event. Propagation is the extension of base pairing beyond the initiation complex. The process does not involve a change in the number of strands in the complex and is therefore concentration independent. Polymer formation is dominated by propagation, resulting in the failure of polymer melting temperatures to change with concentration. Intermediate-length duplexes form with significant contributions from initiation and propagation. They exhibit concentration-dependent melting due to the contribution from initiation, but significant populations of partially paired intermediate states contribute to the melting profile.

Rigorous treatment of complexes of intermediate length (that is, those displaying non-two-state behavior) requires application of par-

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7.3.15

tition function techniques in which all states are enumerated (Poland, 1974). A useful compromise can be achieved by combining the calorimetrically determined $\Delta H°_{cal}$ and the concentration dependence of T_m (or T_{max}) to obtain self-consistent $\Delta G°$ values (Plum et al., 1995a). The measured slope, m, of the $1/T_m$ versus $\ln C_T$ plot is substituted into the following expression to find a value for the effective molecularity, n_{eff}.

$$n_{eff} = \frac{m \Delta H^o_{cal}}{R} + 1$$

Equation 7.3.17

The thermal disruption of higher-order complexes is assumed to proceed by a mechanism similar to that of duplexes, although far fewer data address this issue for nucleic acid complexes composed of three or more strands. The length at which the van't Hoff treatment fails will depend on the molecularity of the complex. For DNA triple helices, there is evidence that the van't Hoff methods are not reliable, even for relatively short oligonucleotides (Plum et al., 1995b). Higher-order complexes have not yet received sufficient study to define even crudely the length range over which van't Hoff methods may be applied with confidence.

To ensure that reliable values for thermodynamic parameters are obtained for oligonucleotide duplexes >12 base pairs and for higher-order complexes, model-independent, calorimetric experiments are advisable in parallel to the optical characterizations described here.

Critical Parameters

Accurate knowledge of nucleic acid concentration is necessary for determining values of the thermodynamic parameters that describe the stability ($\Delta G°$) of a nucleic acid structure and the temperature dependence ($\Delta H°$) of that stability. Knowledge of the molecularity of the nucleic acid complex also is necessary. Determination of both concentration and molecularity is dependent on accurate values of extinction coefficients.

Accurate concentration measurements are necessary to prepare solutions so that oligonucleotides are present in the correct ratios for complex formation, and reliable molar extinction coefficients for oligonucleotides are necessary to ensure that solutions are prepared accurately. Estimation of the extinction coefficient based on the base sequence is an alternative to the phosphate analysis procedure described here (Puglisi and Tinoco, 1989). The

level of precision (±10%) is often not satisfactory for thermodynamic measurements, particularly if parallel calorimetric studies are contemplated.

Assuming that the concentration of the standard phosphate solution is known accurately, the most common error in the extinction coefficient determination is incomplete enzymatic digestion of the oligonucleotide. In this case, the amount of phosphate is underestimated and thus the extinction coefficient is overestimated. The use of the poly(rU) positive control should alert one to this problem. Failure of the color to develop properly indicates that fresh solutions should be prepared.

All of the methods for extracting thermodynamic data from equilibrium melting curves assume a model for the oligonucleotide complex. Therefore, it is vital that the molecularity (i.e., the number of oligonucleotides in the complex) be known. Mixing curves are used to confirm the molecularities of the complexes formed by oligonucleotides, to validate the single-strand molar extinction coefficient values, and to determine molar extinction coefficient values for the complexes.

The critical parameters in the determination of the molecularity of the complex are the individual extinction coefficients. Failure of the curves to meet indicates errors in the extinction coefficients or errors in solution preparation. If the points on the absorbance axis where $X = 0.0$ or $X = 1.0$ are not colinear with the data where $0.0 < X < 1.0$, an incorrect determination of the minimum necessary volume for the absorbance measurement is indicated. Time dependence of the measured absorbance may indicate the necessity of an annealing step.

The most important parameter in the determination of thermodynamic parameters by the several analysis protocols presented here is the length of the nucleic acid. As described in detail in Background Information, the methods presented here are valid only for nucleic acid structures that melt via a two-state process. This requirement restricts application of the methods to very short (≤12 base pairs) and very long (demonstrating no concentration dependence of T_m) nucleic acid complexes. Monomolecular processes may or may not be two state. The only method for evaluating the validity of the two-state approximation is comparison of the model-dependent values calculated as described here to the model-independent calorimetric values.

Anticipated Results

The determination of the extinction coefficient described in Basic Protocol 1 is dependent on the calibration curve as shown in Figure 7.3.1. Mixing curves typically will look like Figure 7.3.2. When like numbers of strands A and B form a complex, the inflection point of the curve will be at 0.5. When different numbers of strands A and B form a complex, the inflection point will shift. For example, formation of an A_2B complex is indicated by an inflection point at 0.33 and formation of AB_2 complex by an inflection point at 0.67. The melting curve of most nucleic acids will look like Figure 7.3.3. This appearance is in itself not proof of two-state behavior. If multiple transitions are observed, as is frequently seen for triple helices, two-state behavior clearly cannot be assumed. It may be possible, however, to dissect multiphasic transitions as a series of two-state transitions. In the absence of calorimetric data, any assumption of two-state behavior must be considered tentative.

Time Considerations

The determination of oligonucleotide extinction coefficients requires 4 to 5 hr divided over 2 days. The determination of complex molecularity and extinction coefficient by the method of continuous fractions requires ~4 hr. The melting experiments require ~2 hr to set up, while the running time can range from 3 to 12 hr (unattended spectrophotometer time) depending on the heating rate for each melting experiment. Temperature-programmable multiple-cell holders greatly accelerate data throughput.

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