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Laboratory Experiment

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A DNA Melting Exercise for a Large Laboratory Class

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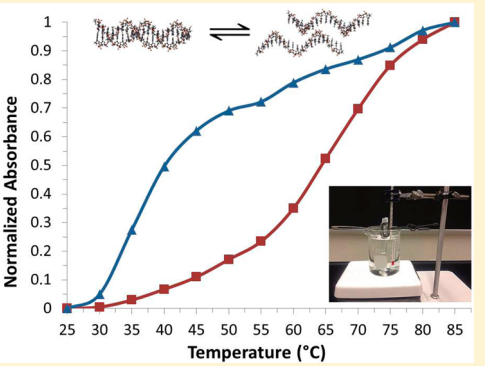
Supporting Information

ABSTRACT: A simple and economical experimental setup is described that enables multiple individuals or groups within a laboratory class to measure the thermal melting of double stranded DNA simultaneously. The setup utilizes a basic spectrophotometer capable of measuring absorbance at 260 nm, UV plastic cuvettes, and a stirring hot plate. Students measure melting curves of short matched and mismatched DNA duplexes, and use quantitative analysis and first derivative curves to determine each duplex's transition melting temperature, T_m. Student data are highly reproducible among groups and from year to year, and give distinct transition melting temperatures for the different duplexes. The protocol could easily be adapted to investigate the effects of other parameters on DNA melting and stability, such as changes in base composition, chain length, and buffer ionic strength.



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In living cells, DNA is predominantly double stranded with two antiparallel strands held together by hydrogen bonds between complementary bases. These strands can reversibly dissociate into single strands (denature) and reassociate to a duplex (anneal), a property that is exploited both in nature and in the laboratory. Denaturation and annealing are critical in many important biological processes including DNA replication, transcription, and DNA repair.[1] It is also key to many common laboratory techniques, including Northern and Southern blotting and polymerase chain reaction (PCR). This makes DNA denaturation and annealing an important concept in biochemistry courses.

This laboratory experiment introduces students to DNA denaturation and annealing using an experimental setup that easily accommodates a large laboratory class (20 students in our case). Denaturation and annealing are carried out by heating to "melt" the DNA and cooling to re-form the duplex. Double stranded DNA exhibits hypochromism (a lower absorbance compared with the absorbance of the corresponding single strands) so that denaturation occurs with an increase in absorbance.[2] Transitions between single and double stranded DNA can thus be tracked by absorbance changes.

Previously published laboratory experiments have used a similar approach to explore DNA denaturation, but differ in that they employed sophisticated instrumentation that, from a practical standpoint, limits the number of students that can simultaneously perform the experiment.[3] These experiments utilized UV−visible spectrophotometers equipped with stirred temperature controlled cells and quartz cuvettes, and employed methods that require either slow heating or longer incubation times for rigorous temperature equilibration, but that are very time-consuming.[3] In contrast, this experiment employs a simple experimental setup containing a water bath, a basic UV spectrophotometer, and UV-transmissible plastic cuvettes. The system generates melting curves that are highly reproducible, can be analyzed by derivative plots, and yield transition melting temperature (T_m) values (temperatures for 50% denaturation) that are less exact but comparable to those measured for the same duplexes using traditional sophisticated instrumentation and methods. Using multiple basic spectrophotometers as found in many departments, a large group of students can simultaneously carry out the melting and annealing of two DNA duplexes (double stranded complexes of DNA) in a single laboratory period.

In the protocol described here, students compare relative stabilities and T_m values of two different DNA duplexes, each formed from pairs of oligonucleotides that are only 20 bases long. Students relate the differences in T_m to the duplexes' sequences as one duplex contains six mismatched base pairs while the other has no mismatches. Duplex annealing after cooling is also measured to confirm the reversibility of denaturation. Upon completion of this experiment, students should be able to (i) determine a transition melting temperature; (ii) justify that melting is reversible; (iii) explain how mismatches affect stability of DNA duplexes; and (iv) explain how equilibria between single and double stranded DNA can be exploited in more complex biochemical protocols such as PCR and Northern and Southern blotting.

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METHODS

Reagents and Equipment

All chemicals are ACS grade. The cuvettes are plastic, semimicro, UV-transparent, disposable cuvettes with a 1.0 cm path length. Single stranded DNA is purchased from Integrated DNA Technologies. DNA duplex solutions are prepared in advance for students and contained 1.3 μM double stranded DNA (1.3 μM each strand) in 5 mM phosphate buffer with 50 mM sodium chloride, pH 7.0. Two 20mer DNA duplexes are used (Figure 1). Duplex A contains two fully complementary strands while duplex B differs in the sequence of one strand to give six mismatched base pairs at one end (underlined).

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Duplex A:  5′ CACACGGACTTATAGATCTC 3′
           3′ GTGTGCCTGAATATCTAGAG 5′
Duplex B:  5′ CACACGGACTTATAGATCTC 3′
           3′ CGTCCACTGAATATCTAGAG 5′
                                   80
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Figure 1. Sequences of DNA sequences (duplex A and duplex B) used in melting experiment. Mismatched base pairs are underlined.

Absorbance measurements are performed using a UV spectrophotometer capable of measuring absorbance at 260 nm without a temperature-controlled cell.

Experiment

Students work in groups of two or three. The experiment requires 2 h to complete. Spectrometers are blanked with buffer at 260 nm. A sample (1 mL) of DNA duplex (A or B) is transferred into a labeled, plastic cuvette, and the absorbance is measured at room temperature. The sample is transferred from the cuvette to a labeled culture tube suspended with two test tube clamps in a water bath containing a thermometer on a stirring hot plate (Figure 2). The culture tubes are covered with

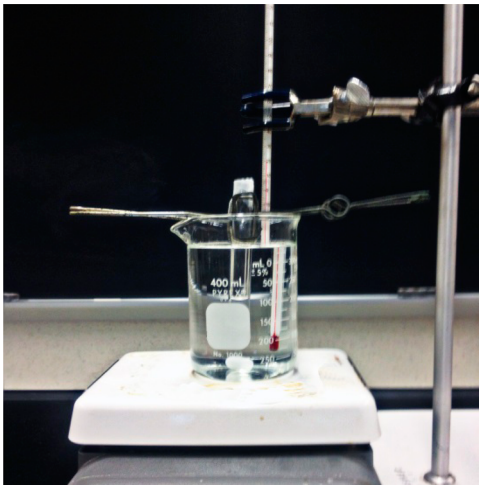

Figure 2. Experimental setup for DNA thermal melt.

Parafilm to minimize evaporation. The heat is turned on, and, every five degrees, the DNA sample is transferred quickly by pipet from the culture tube to the labeled cuvette kept in the spectrophotometer to measure the absorbance, after which the sample is transferred back to the tube in the water bath. Both the temperature and the absorbance are recorded. This is repeated every five degrees up to a maximum of 85 °C. After the data are collected, the duplexes are allowed to cool to room temperature and the absorbance is recorded to test for renaturation. The experiment is repeated for the other duplex, allowing each group to collect data for duplexes A and B.

During the experiment, students plot absorbance vs temperature. For lab reports done after class, students normalize the absorbance data so that the melting curve for each sequence can be overlaid on the same plot using eq 1,

$$\frac{(\mathrm{Abs}_{\mathrm{temp}} - \mathrm{Abs}_{\mathrm{rt}})}{(\mathrm{Abs}_{85^\circ\mathrm{C}} - \mathrm{Abs}_{\mathrm{rt}})} = \mathrm{Abs}_{\mathrm{norm}} \quad (1)$$

where $\mathrm{Abs}_{\mathrm{temp}}$ is the absorbance at a specific temperature, $\mathrm{Abs}_{\mathrm{rt}}$ is the absorbance at room temperature, and $\mathrm{Abs}_{\mathrm{norm}}$ is the normalized absorbance change for the specific temperature. Normalized melting curves are plotted with duplexes A and B on the same graph. The derivative of each melting curve is plotted to determine the T_m.

HAZARDS

Reagents used in this laboratory exercise are not hazardous substances. Students should take care when using hot plates.

RESULTS AND DISCUSSION

The experimental protocol was optimized to provide reproducible quantitative results while minimizing cost and complexity of instrumentation. Plastic UV disposable cuvettes were substituted for more expensive quartz cuvettes (~$0.85 per plastic cuvette vs ~ $80 per quartz cuvette) allowing for a large number of simultaneous experimental setups. The nominal absorbance of the plastic cuvette at 260 nm vs air is ~0.4, low enough to allow for ample signal for the DNA after blanking. The DNA duplexes were formed from 20 base oligonucleotides, comparable to lengths typically used in PCR and providing duplex melting transitions easily measured by the experimental setup. For the duplex that contained mismatches, the mismatches were incorporated at the end of the sequence since this provided a sharper and more distinct melting transition than incorporating the mismatches into the middle of the sequence (creating a single stranded "bubble") or interspersing the mismatches within the sequence. Heating for this experiment is limited to 85 °C. Temperatures above 85 °C caused distortions to the plastic cuvettes, and distortions also occurred if samples were heated continuously in the cuvettes in the water bath instead of in the glass culture tube.

A representative student data set for melting duplexes A and B in Figure 3A shows the significant increase in absorbance observed with the melting of each duplex. Duplex B exhibited a higher initial absorbance than duplex A due to a reduced hypochromism from having fewer complementary base pairs. Figure 3B shows the same data normalized for the change in absorbance, which allows students to visualize more easily the dramatic difference in melting curves between duplexes A and B. Inspection of the curves in Figure 3B gave a T_m of ~65 °C for duplex A and a much lower T_m of ~40 °C for duplex B. To determine more accurate T_m values, the first derivatives of the normalized curves were plotted (Figure 3C). Even with the relatively low temperature resolution of 5 °C increments, distinct maxima were observed to give T_m values of 65 and 35 °C for duplexes A and B, respectively.

Figure 4 shows averaged data with standard deviations for six laboratory sections over two years (n = 41 groups for duplex A and n = 39 groups for duplex B); nine groups' data were not included in the average data because the data were collected

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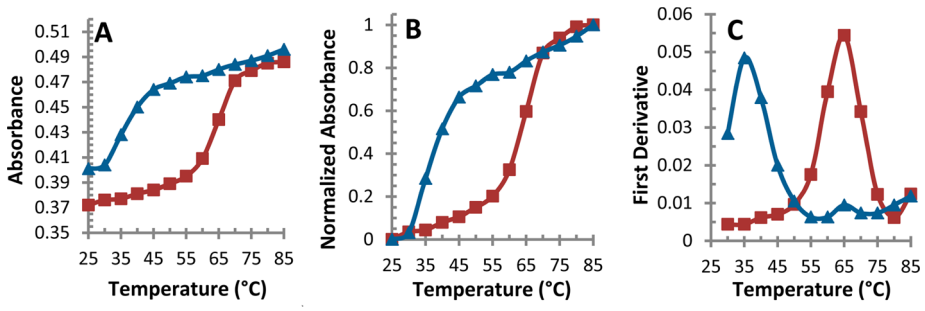

Figure 3. Representative student data for duplex A (red squares) and duplex B (blue triangles). Heating was done at a rate of about 1 °C per minute. (A) Melting curve with raw absorbance data. (B) Melting curve with normalized absorbance data. (C) First derivative of normalized absorbance data.

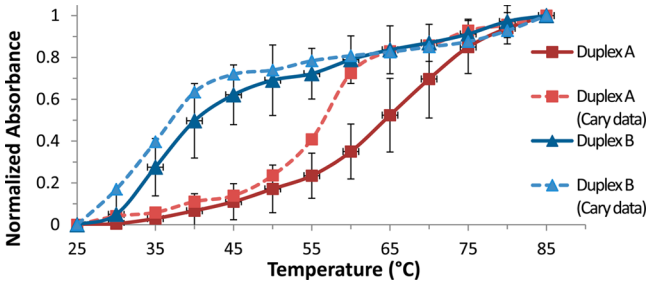

Figure 4. Data for duplex A (red squares) and duplex B (blue triangles). Solid lines represent average class data including standard deviation. Dashed lines represent data collected on a Cary 50 spectrophotometer using a temperature-controlled cell.

using different temperature intervals or the data sets were incomplete. The two averaged melting curves are clearly offset in temperature while the relatively small standard deviations demonstrate the reproducibility of the melting curves, even from year to year. Using the first derivative curves for each student group, the average T_m was 67 ± 5 °C for duplex A and 36 ± 3 °C for duplex B (Figure S1 shows first derivative curves for averaged class data). Figure S2 shows the high degree of consistency in T_m values among different lab sections.

The accuracy of the simplistic experimental setup was assessed by repeating the thermal melting experiments using the "traditional method"[3a,c] (a Cary 50 spectrophotometer with a stirred, temperature-controlled, single cell Peltier accessory). Absorbance measurements were taken every 5 °C with 5 min incubation at each temperature for equilibration. The thermal melting curves were shifted to slightly lower temperatures compared with those obtained with the simplistic setup (Figure 4). From first derivative analyses, the T_m was 55 °C for duplex A and 35 °C for duplex B. Oligo Calc software[4] was used to calculate T_m values of 56.4 °C for duplex A and 36.0 °C for duplex B; the T_m for duplex B was calculated using only the matched portion of the sequence. The higher melting temperatures obtained with the simplistic system likely resulted from the samples not having time to equilibrate with the water bath temperature and from cooling during the transfers and absorbance measurements. However, the simplistic method still showed a distinct difference in melting curves that was consistent and reproducible, allowing a large class of students to distinguish the stability of the two duplexes successfully.

To confirm the reversibility of the duplex denaturation, students measured absorbance values at room temperature before and after the melt experiment. On average, cooled duplexes returned to within 9.8% and 11.6% of the original absorbance for duplexes A and B, respectively, compared to 7.7% and 17.8% of the original absorbance of duplexes A and B for the CARY experiment, indicating that the duplexes had mostly reannealed in both experiments.

Student achievement of the learning objectives of this exercise was assessed in questions students answered in their lab report (see student handout in Supporting Information). Students were asked to justify their experimental determination of the T_m values and the degree of reversibility in melting the two duplexes. Students were also asked to correlate the differences in duplex melting temperatures with the differences in extent of base pairing. For error analysis, students compared their measured T_m values with those predicted by the online Oligo Calc software and were asked to provide explanations for the differences that could arise from the experimental design. On the graded lab reports, 82% of students achieved a score of 80% or better (n = 96) with an average of 87 ± 10%, indicating that the vast majority of students had mastered the main concepts of the experiment.

The DNA melting experiment is followed in subsequent weeks by a PCR laboratory experiment so students can apply their new expertise to understanding the melting and annealing process at the heart of PCR, including the relationship between the annealing temperature and the primer T_m. The robust nature of this melting experiment allows for many variations, including exploring the effects of DNA length, G/C vs A/T base content, and salt concentration. The advantage of the experimental protocol described here is that these experiments can now be done in a large laboratory class setting.

CONCLUSION

Data show that the simplistic laboratory setup illustrates the differences in stability between matched and mismatched DNA sequences. This widely applicable experimental design allows for entire laboratory sections to collect data on transition melting temperatures simultaneously versus previous experimental designs. Furthermore, there is flexibility to experiment with factors that affect DNA stability, which allows customization of the experimental design toward lecture material.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.5b00049.

First derivative plots of averaged student data, average T_m data by laboratory section, a student handout, and instructor notes (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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