Module 1: Measuring DNA Melting Curves

Contents

1   Objectives and Learning Goals

- Implement a temperature sensor using the well-known Wheatstone bridge scheme.

- Build an instrument for recording DNA melting curves.

- Use your instrument to study the melting behavior of several DNA samples under different conditions.

- Understand the factors affecting DNA melting behavior, and what type of information can be usefully obtained from such measurements.

2 Roadmap and Milestones

1. Build and test the temperature-sensing circuit.

2. Calibrate the circuit for accurate temperature measurement.

3. Build an amplification/offset circuit for the DNA fluorescence signal.

4. Assemble an optics setup that will enable you to observe the light output of a DNA sample to be studied.

5. Combine the subsystems you have built to generate a DNA melting curve; troubleshoot and optimize your system.

6. Obtain DNA melting data for several sequences, and identify a single-base mismatch (SNP) sequence.

3 Background

You will learn in lecture about the importance and utility of measuring of DNA melting temperatures. A common application takes advantage of the length-dependence of DNA melting temperatures to examine PCR products, and determine whether the desired sequence was successfully amplified.

The goal of this lab is to build the hardware to be used for double-stranded DNA (dsDNA) concentration measurement with the common dsDNA-binding dye SYBR Green I. This setup, together with a temperature sensing circuit, enables DNA length and complementarity analysis via melting curve measurement. The tools developed in this lab will also be capable of analyzing other fluorescent dyes with similar excitation and emission wavelengths.

A typical plot of temperature-dependent fluorescence from a solution of DNA and SYBR Green I is shown in Fig. 1. The "melting temperature" T_m is defined as the temperature at which 50% of the DNA remains hybridized. Sometimes, the transition is not particularly sharp, or other factors in the measurement may create offsets or drifts in the signal (evident below 80°C in Fig. 1(a)), in which case the derivative of this
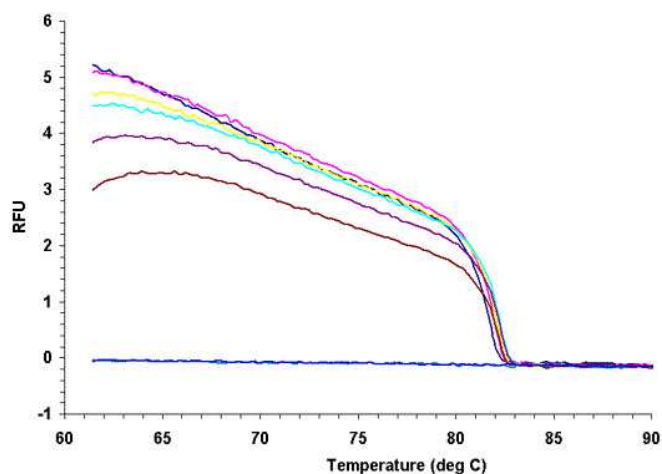
(a)
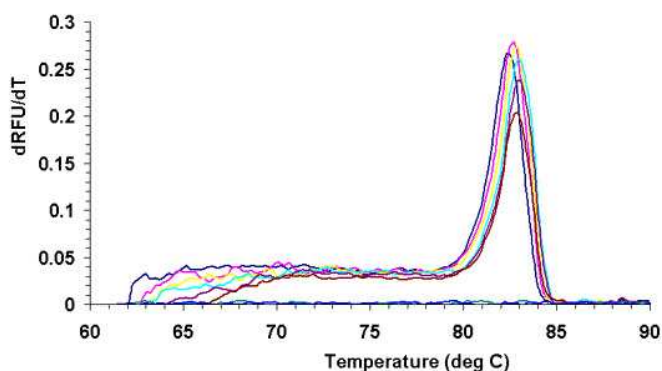
(b)

Figure 1: Typical melting curves using SYBR Green I.

curve is plotted (Fig. 1(b)), and the lo-
cation of its peak value gives T_m more clearly. More about this in Section 4.2.3.

To perform a DNA melting experiment, the temperature of the DNA sample is usually ramped up at a controlled rate, and carefully monitored, while the concentration of dsDNA is recorded, most often using a fluorescent dye. To avoid the need for precise temperature control, we will ramp the temperature downward by heating the DNA sample to above its melting temperature, and letting it cool naturally.

4   Building the apparatus

Your apparatus will consist of two major subsystems for (a) temperature measurement, and (b) quantification of dsDNA in your sample. The outputs of the subsystems give you the two data sets that constitute a DNA melting curve: temperature and relative fluorescence intensity.

Note: as you build the setup, keep stability in mind. This is a sensitive high-gain system, and it will not perform well if it is constantly getting bumped and wires are being moved or disconnected. This means making solid electrical connections, keeping wires and cables clamped or taped down, and setting things up to move as little as possible when you connect and disconnect the sample.

4.1   Temperature sensing

4.1.1   Thermistor

A thermistor is a resistor whose resistance varies with temperature. Thermistors come in two "flavors:" positive temperature coefficient (PTC – R increases with higher T) and negative temperature coefficients (NTC – R decreases with higher T). To build and test your temperature-sensing circuit, you can use a 5 kΩ NTC thermistor (black with red leads, available in our usual small parts drawers – part number RL0503-2890-95-MS). Its temperature-resistance characteristic is nonlinear, and the datasheet is available in the lab.

Once you have a good feel for the temperature sensing circuit (see Sec. 4.1.2), you will actually use a thermistor called an RTD (resistance temperature device) for the melting experiment (see 4.2.6. It is a *positive* temperature coefficient platinum thermistor with a nominal value of 1 kΩ that has a very linear temperature response. This one has part number PPG102A1, and the datasheet can be likewise found in the lab.

4.1.2   Wheatstone bridge

Though very simple, the voltage divider is a very powerful concept in electronics – divider-based circuits contribute to many types of sensor and measurement systems. Here we will use a well-known circuit called a Wheatstone bridge to make temperature measurements.

The Wheatstone bridge was invented in the 1800's in order to measure the resistance of an unknown value R_x. The circuit consists of four resistors in the configuration shown in Figure 2. Typically, two have known values, R_1 and R_2, and a third resistor R_3 is variable. The unknown resistance R_x takes the fourth position, and the circuit is wired with a voltage source V_{in}. The bridge is "balanced" when the ratio of R_1 to R_3 is exactly equal to the ratio of R_2 to R_4, in which case the voltages at nodes a and b are equal. Otherwise a voltage difference develops, and current will flow if a connection is made across the bridge.

Wire up a Wheatstone bridge on your breadboard, as shown in Figure 2, using a 1-10 kΩ potentiometer for R_3 (blue rectangular, not the round multiturn type) and values for R_1 and R_2
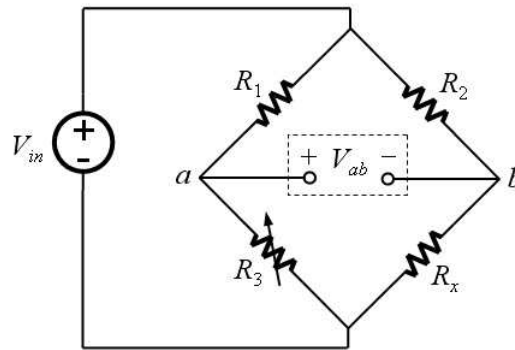
Figure 2: A Wheatstone bridge circuit.

that will give good sensitivity if R_x is in the 1 kΩ range. **Note that the RTD should have no more than 1 mA of current passed through it, so build your circuit accordingly.**

One way to measure the unknown resistance R_x is by monitoring the voltage difference $V_a - V_b$, and adjusting the value of R_3 until the bridge is balanced. Another method is to perform a calibration to derive the relationship between the voltage across the bridge to the change in resistance. Our approach will be a combination of these methods – you will first adjust R_3 to balance the bridge, then use your knowledge of how V_{ab} depends on R_x to calculate resistances from voltages.

Test your temperature circuit using the black-with-red-leads thermistor, and small amounts of cold or warm water to vary its temperature. Observe the circuit output and make sure that the temperature reading makes sense to you. A thermometer is likely to be helpful here.

Once you are satisfied with this, connect your circuit to a the sample block with the attached RTD. Make sure you are able to convert V_{ab} to resistance, and use the datasheet to determine the calibration relationship between temperature and resistance.

You should now have a functional electronic thermometer, and a straightforward way to convert its output to temperature.

4.2 Fluorescence readout system

4.2.1 Amplification circuit

(If you need a brush-up on op-amps, refer to Section A.5 in the previous lab module).

To usefully measure and record the fluorescence signal you will need what's called a *transimpedance* amplifier (sometimes called a current-to-voltage converter) with a gain of approximately 10^8 V/A. Be sure you understand why this is the case, and explain it to your lab instructor.

The simplest transimpedance amplifier looks like Figure 3:

Determine the DC gain V_{out}/i_{in} of this configuration (in V/A) in terms of the resistance R_f. It should be clear that getting a large gain from this circuit requires the use of a very large resistor, which we'd prefer to avoid. (Optional: why not simply use a resistor, and omit the op-amp?)
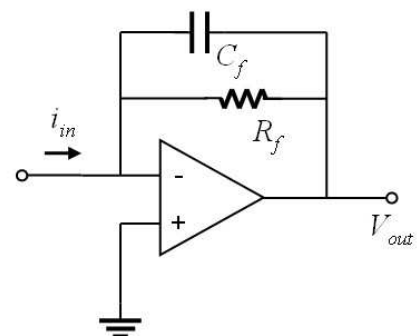

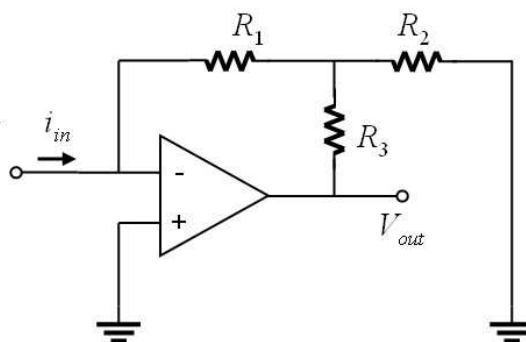Figure 3: Basic transimpedance amplifier circuit.

Figure 4: The high-gain version of the transimpedance amplifier circuit. Don't forget to add a capacitor for high-frequency noise rejection.

Instead, we can use the circuit from Homework Set 1,
which you have already analyzed, shown in Fig. 4. Since the signal you will measure will be very slowly-varying (near DC), you will also want to include a capacitor for rejecting high-frequency fluctuations (as discussed in HW1).

One final note about the type of op-amp to use. Though real op-amps don't behave exactly like ideal ones we use for analysis, we want an op-amp whose input current is as close to the ideal value of zero as possible (why?). Op-amps with this characteristic have JFET inputs, and in our lab, the LF411 or LF351 are both suitable.

4.2.2 Offset circuit

A final addition to the system that will greatly improve its usability is a knob that lets you control the level of the output signal. A simple way to do this is using an LM741 op-amp, with a 10kΩ potentiometer connected as shown in Fig. 5. Use one of the high-quality round pots to obtain smooth and precise control.

4.2.3 SYBR Green I

This is a common intercalating dye that binds highly preferentially to dsDNA (not ssDNA), and exhibits strong fluorescence when bound and nearly zero fluorescence when unbound. We therefore expect to see an decreasing signal (using an inverting configuration) as the temperature of the DNA sample drops, and more and more DNA duplexes are formed. SYBR Green I is excited by light at a wavelength of 498 nm (blue), and emits at 520 nm (green).
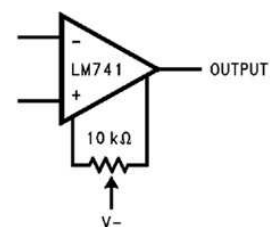

Figure 5: Offset circuit using the LM741. Note that all three of the pot's leads are used, and the wiper is connected to the negative supply voltage.

SYBR Green I fluorescence is not only dependent on its binding state with dsDNA, but also has a dependence on temperature. Higher temperatures reduce its fluorescence, which introduces an approximately-linear drift into the signal as the temperature is ramped. This is one of the reasons for taking the derivative of the recorded data to determine T_m.
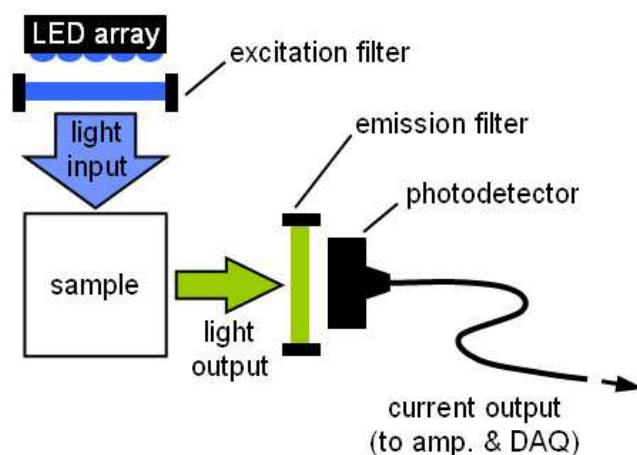
Figure 6: Recommended layout of the light source, filters, and detector for the optics setup.

4.2.4 Optical system

Figure 6 shows a schematic of the recommended optical configuration. The right-angle geometry helps to reduce any background signal that gets through the excitation filter. Build this on an optical breadboard, keeping the components close to the board for stability and simplicity of design. This will mostly involve short rails, rail clamps, and posts.

The filters used are a D470/40x bandpass filter (excitation), and a E515LPv2 long-pass filter (emission) from Chroma Technologies. Take care not to touch the optical surfaces when handling these components, as they have coatings that are easily damaged. Datasheets are available, as usual.

The LED array connects to the circuit just like a single LED, using two leads - anode and cathode. It can be powered directly from your power supply, and $\approx 8.8V$ is known to supply a good amount of light while dissipating manageable heat.

4.2.5 Sample handling

The DNA samples for these experiments are loaded inside a glass cuvette. You should use $500\mu L$ of DNA solution for each run. *Pipet (20μL) of mineral oil on top of the DNA solution to prevent evaporation; make sure the oil stays on top by always keeping the block vertical, especially when heating it.*

To change samples, pipette out the previous DNA solution, and discard it in the waste container provided. Discard the pipette tip. Rinse the cuvette with water, then, using a fresh pipette tip, fill it with $500\mu L$ of new DNA solution.

You should be able to use the same DNA sample for many heating/cooling cycles, so only replace it if you lose significant volume due to evaporation. If you need to leave the sample overnight, store it in the lab refrigerator, and clearly label it with your name.

4.2.6 Heating block

For better temperature stability and easier handling, the cuvette slips inside a machined aluminum block, also provided for you. The RTD thermistor for temperature measurement is hard-mounted onto this block. Use a hotplate to heat up the block to above the DNA melting temperature

($\approx 90°$C), then move the block to your setup for measurement, and connect the thermistor to the Wheatstone circuit.

You'll find that the speed at which the block cools down is likely too fast if it's placed directly in contact with the metal optical breadboard. You can control this speed by placing one or a few pieces of paper or layers of paper towel underneath the block. Experiment with this until the block takes between 5 and 10 minutes to cool down from $90°$C to $40°$C.

5 Experimental Protocol

5.1 Preparing the setup

1. (The system will generate the best data when both the amplifier circuit and LED have been on for at least 60 min. and all drifts have stabilized. However, you certainly don't need to wait idly while this happens, and can test the setup and try measurements in the meantime.)

2. (Do this first without the sample and the block.) Using a box and a piece of black cloth, make sure the entire optical setup is isolated from stray light. If you can see any blue light coming out at all, light is also getting in.

3. Use the potentiometer to adjust the amplifier voltage offset until the baseline signal is approx. 0 V.

4. The baseline should be relatively flat – if it is not, generally it's because the setup is not well covered.

5. Now include the block with DNA solution in the setup. Adjust the block's placement and the angle of the LED source to maximize the ratio of DNA signal vs. background signal without the block. Use the plastic alignment jig if you need, or simply mark the position of the block in some way.

5.2 DNA melting curve experiment

1. Place the block on the hotplate (set to $95°$C) for at least 10 min. Measure the RTD thermistor to make sure the temperature is above $80°$C before removing the block

2. Move the block to your setup. Immediately connect the RTD to its Wheatstone bridge circuit. Cover the setup in a box and put a piece of black cloth over it.

3. Start the LabVIEW recording program (called `DNAmelting.vi`). Wait for the block to cool to below $40°$C, which should take about 10 min. if the block sits on a single piece of paper towel.

4. A monotonically decreasing fluorescence signal is expected (for an inverting amplifier setup). If there is any LED output fluctuation, or if the signal drifts upward, you'll need to restart the experiment. When you're satisfied with the melting run, save the data.

6   Report Requirements

This lab report is due by noon on Friday, Oct. 6.

6.1   Data to take

Once your instrument is running to your satisfaction, you should record the following melting curves:

- 40bp perfect match

- 19bp perfect match

- 19bp complete mismatch

- 19bp single-base mismatch (SNP)

- 19bp at 1-2 different ionic strengths

(Note: Other than the last one, the 19bp samples will only be identified as A, B, and C, and you will need to identify, based on your measurements, which is which.)

You will need to take the derivative of the recorded fluorescence data, and combine it with the temperature data to generate plots. Generally, the region of interest will fall between 40 and 65°C. It will be helpful to create a MATLAB script to convert raw data to a plot of dF/dT vs. temperature.

Having generated your melting profiles, you need to produce the following plots:

1. Comparison of perfect match 40bp and 19bp sequences.

2. Comparison of 19bp perfect match vs. SNP vs. complete mismatch sequences.

3. Comparison of 19bp sequences at different ionic strengths.

In each situation, discuss the melting temperatures and shapes of the melting curves for the samples relative to each other. Briefly explain the curves are the way they are in each case.

Compare your melting curves with those of other students in the class. You may find the quite different even under the same conditions. What might cause these variations? What factors affect the DNA melting temperature, and the "sharpness" of the melting transition?

6.2   Model vs. reality

In class, we derived an expression that relates the melting temperature to the enthalpy change ΔH° and entropy change ΔS° of the hybridization reaction:

$$T(f) = \frac{\Delta H^\circ}{\Delta S^\circ - R \ln(2f/C_T(1-f)^2)} \,, \tag{1}$$

Here, f is the fraction of DNA strands hybridized (dimerized) at a particular temperature (at T_m, this is 1/2), and C_T is the total concentration of single-strand oligonucleotides (or $2\times$ the dsDNA concentration when all strands are hybridized).

Choose one of the perfect-match sequences that you measured, and use MATLAB to fit the model to your measured data, which will allow you to extract the ΔH° and ΔS° parameters.

To perform the fit, you will need a MATLAB function that will evaluate $T(f)$ given an input `const` for the ΔH° and ΔS° parameters. The function will be something like this:

```
function Tf = melt(const, f)
R=8.3;
C_T=33e-6;
dH = const(1);
dS = const(2);
Tf = dH./(dS - R*log(2*f./(C_T*(1-f).^2)));
```

You can then invoke MATLAB's `lsqcurvefit` routine to do the fit, which will return the best values for ΔH° and ΔS°.

```
FitVals = lsqcurvefit(@melt, [dH_guess, dS_guess], frac_vector, temp_vector)
```

Bonus (optional):

1. Calculate ΔH° and ΔS° for this sequence using the nearest-neighbor model from class.

2. Compare these to the fit parameters, and speculate about why they might be different? What factors affect ΔH° and ΔS°?