Protocol for DNA Duplex T_m Measurements

Contents

Introduction	• A set of written rules and procedures for performing Melting Temperature (T_m) experiment on DNA duplexes using the Cary 100 UV-Vis Spectrophotometer. A printed version of this document can be found in a labeled drawer under the UV-Vis spectrophotometer in the lab D20.
Buffer Preparation and Storage	• Melting buffer composition: 0.25M NaCl, 0.2mM EDTA, 20mM Sodium Phosphate (pH 7.0) • Stock melting buffer solution is often made in 1L or 500mL volume. • Make a 100mL stock solution of 1M Sodium Phosphate buffer by adding together 57.7 mL of 1M disodium hydrogen phosphate (Na_2HPO4) and 42.3 mL of 1M sodium dihydrogen phosphate (NaH_2PO4). This stock solution is used for preparation of melting buffer. • Add together calculated amounts of NaCl, EDTA and the sodium phosphate buffer stock solution to make the final melting buffer solution. • Store the melting buffer in a refrigerator for longer periods. For short term usage (1-2 weeks), the buffer can be kept at room temperature. • Inspect the buffer for any particulate matter (may be caused by microbial growth) before using.
Cuvettes	• Generally Quartz cuvettes are used. Cuvettes used for UV measurements should be spotlessly clean. Check for any scratches or spots, especially on the smooth side of the cuvette. • Rinse and wash the cuvettes and stoppers with the cleaning alkaline solution and air dry them. • Normally, cuvettes with 10mm path length are used for UV measurements. • While taking measurements, place the cuvette in the slot such that the rough side faces the user and the smooth side faces the beam path.
Instrument start-up	• It is assumed that you have used the specific Google Calendar to sign up for a slot on the spectrophotometer. • Log on to the instrument computer using the following information: User ID: let-ethz Password: abcd.1234 • The UV lamp should be switched on for at least 15 minutes before the start of measurements, for warm up. • The temperature controller should be switched on for duplex T_m measurements. • Select the appropriate program from the list under the Cary folder on the desktop screen.
Experimental Protocol	**A. Annealing Experiment:** • The oligonucleotides used for the preparation of stock solutions should be lyophilized or vacuum dried. • Prepare stock solutions of single strand oligonucleotides in the melting buffer. The UV absorbance of oligo solutions is measured by a program called *Simple Reads* (found under the Cary folder). • The measurement wavelength is 260nm for oligonucleotides. Calculate the concentration of stock oligo solutions from the absorbance values and using known extinction coefficients for the oligonucleotides. • Equation for calculation of oligo concentration: $C = A/\varepsilon . L$ Where, A: Absorbance at 260nm ε: Extinction coefficient of the oligonucleotide ($M^{-1}cm^{-1}$) L: Path length of the cuvette (cm) • Notes: Make sure that the absorbance reading for the oligo solution is never beyond 4 as it is the limit of accurate absorbance measurement on the instrument (i.e. Linear

absorbance range for the instrument is 4).

- For modified oligonucleotides, please use the extinction coefficients provided with the oligos.
- Check the volume capacity of the cuvettes. Depending on the volume capacity of the cuvette, one can measure absorbance values for 200µL, 400µL or 1mL solutions. The volume used regularly is 200µL.
- The default cuvette position for absorbance measurement is position 1. In the set up for Simple Reads program, choose the wavelength 260nm.
- Measure a blank (melting buffer) absorbance at 260nm by clicking read. Zero this absorbance to subtract the background absorbance. Then measure the sample absorbance at 260 nm.
- Save the measurements as a data file in your designated folder in My Documents directory.
- For T_m measurements, duplex solutions are needed. Prepare the duplex solutions from stock solutions of the complementary single stranded oligonucleotides. Please use the following equation to calculate the volume of stock solution of each oligonucleotide to prepare duplex solution of the required concentration.

$$C_1V_1=C_2V_2$$

Where, C1: concentration of stock solution of oligonucleotide (µM)
C2: concentration of duplex solution (xM)
V1: volume of stock solution of oligonucleotide needed to make the duplex solution of xM (µL)
V2: volume of duplex solution (µL)

- Notes: Make sure that the solutions are mixed properly. This can be done by pipetting or by having a stir bar in the cuvette.
- Make sure that no droplets are sticking to the wall of the cuvette and everything is in solution.
- To compensate for pipetting error, 0.2µL extra solutions should be pipetted.
- Upon mixing together the complementary oligonucleotide solutions, melting buffer is added to make up the required volume for the duplex solution.
- To prepare the duplex solution, the temperature of the mix is elevated from 25°Cto 90°C and then it is slowly brought back to 25°C. To do this, a program called Thermal is used. In the set-up of the Thermal program, please specify the following parameters. Other parameters are kept as default.

  Start temp.: 25°C; End temp.: 90°C; Return to: 25°C; Data Interval: 1
  Rate: 1°C/ min.; Hold: 1 min.

- When using multiple sample cuvettes, please select the cells in the accessories tab. Check the box for multi zero. Under the temperature display option, check the box for block.
- When one needs to run overnight experiments, please select the auto lamps off under the options tab.
- Make sure the temperature controller is running. The display on the main screen of the Thermal program will indicate the cell temperature. Click OK to complete the set-up.
- Once the temperature has reached 25°C as indicated on the display, the start tab will get highlighted. Please click on the start tab to start the absorbance measurements. Follow the prompts and designate the file name and folder for saving the data.
- Upon the end of measurements, please take out the cuvettes and keep them at room temperature for at least 30 minutes.
- Note: Often times, droplets accumulate on the inner surface of the cuvettes because of evaporation and condensation of solution. To ensure accurate measurements, please tap the cuvettes gently on a thick slab of paper with the caps on. This would remove the droplets.
- Check the duplex formation by measuring absorbance of the solutions at 260nm using the Simple Reads software. This absorbance value should be lower than the initial absorbance value of single stranded oligonucleotides.

	B. T_m Measurement: • After ensuring the duplex formation, put the cuvettes back into the spectrophotometer. Select the *Thermal* program from the list under the Cary folder. • Use the following parameters to run the Thermal software for T_m measurements. Start temp.: 20°C; End temp.: 80°C; Return to: 20°C; Data Interval: 1 Rate: 1°C/ min.; Hold: 1 min. • Note: For multiple measurements, select the Advanced collect option under the Cary tab. There, one can indicate the number of thermal stages you need to run e.g. for triplicate measurements, the number of stages would be 6. The parameters for first 2 steps would be: Stage 1: End temp.: 80°C; Data Interval: 1; Rate: 1°C/ min.; Hold: 1 min. Stage 2: End temp.: 20°C; Data Interval: 1; Rate: 1°C/ min.; Hold: 45 min. • Repeat these parameters for the next 4 stages. • Note: One can combine the annealing experiment and the T_m measurements by using the Advanced collect option. Then one would not have to keep the cuvettes outside the instrument for cooling and that way one would save time.
Instrument shut-down	• After the Thermal software has completed the measurements, please switch off the UV lamp and the temperature controller. Take the cuvettes out and discard the solutions. Rinse and clean the cuvettes by using an alkaline wash solution and dry them. • Log off the instrument computer.
Data Processing	• In the set-up window of the Thermal program, select the analyze option. Under analyze, check the box for Derivative option if not selected by default. Click OK to analyze the data. • The analysis would generate graphs for the thermal analysis. Once the graphs are generated, click on the command tab on the menu bar. Under the command tab, select the recalculate option. This would generate the T_m values for the selected graph. • One can easily copy and paste the graphs and T_m values in a word document.