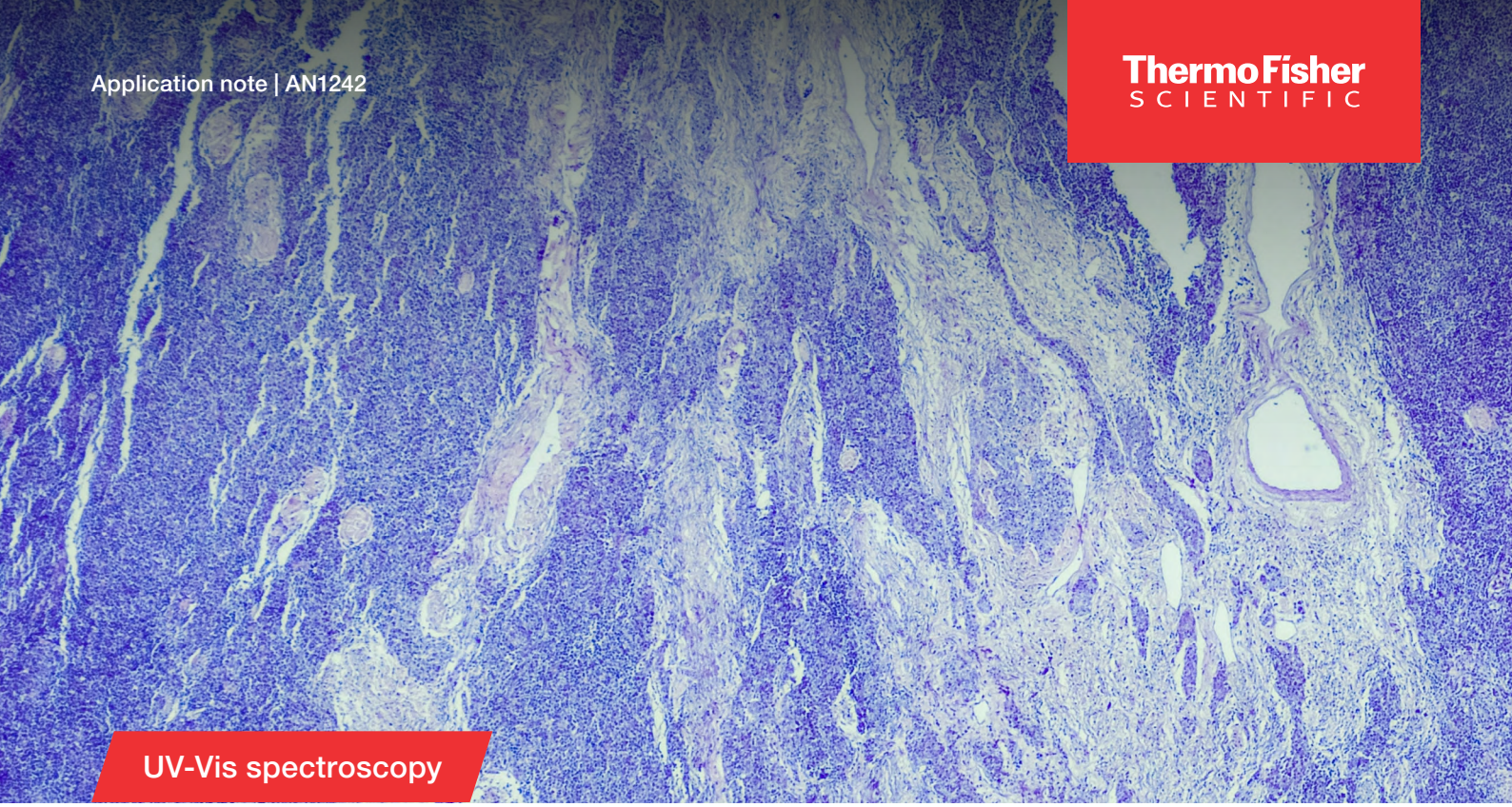
Application note | AN1242



UV-Vis spectroscopy

High-throughput DNA melting measurements using a multi-cell changer

Best practices

Introduction

Deoxyribose nucleic acid (DNA) is a biomolecule used frequently and in a wide variety of workflows, including gene therapy.[1-5] This molecule consists of a series of nucleic acids (adenine, thymine, guanine and cytosine) commonly known as "bases" connected through a phosphate backbone, forming a single strand. Two single strands can associate with one another through hydrogen-bonding of the base pairs (adenine-thymine (AT) or guanine-cytosine (GC)), forming the well-characterized double-helix structure. While hydrogen-bonding is strong, increasing the temperature of the solution can disrupt these intermolecular forces, leading to denaturation, also referred to as "melting," of the double-stranded DNA into its single-stranded components.

The temperature at which half the double-stranded DNA has denatured to its single-stranded components is commonly referred to as the melting temperature (T_m) of the sequence.[6] This temperature is highly dependent on the sequence itself, as well as the presence of mismatched bases, intercalating species, and salt content. As such, T_m is unique to not only the substance studied, but the solvent environment as well, leading to the analysis of this value as a means of DNA characterization.

One commonly used technique for determining T_m is temperature-dependent UV-Visible absorption spectrophotometry. A more detailed description of this analysis can be found elsewhere.[7] Briefly, by taking advantage of the difference in molar absorptivity between single-stranded and double-stranded DNA, changes in the absorbance monitored at 260 nm as a function of solution temperature are used.[6,8] This technique is often used for both research purposes and quality testing in production settings. In these spaces, high-throughput measurements can often be beneficial as this method provides a speedier analysis time. Consequently, a multi-cell holder is often used to allow for the measurement of multiple DNA samples at once. While this allows for the faster assessment of T_m for multiple samples, there are considerations related to the use of a multi-cell holder which must be accounted for to ensure an accurate measurement.

Herein, DNA melting experiments were carried out using the Thermo Scientific™ Evolution™ One UV-Visible Spectrophotometer equipped with the Thermo Scientific™ 6-cell Rotary Peltier. Multiple calf thymus DNA samples of the same concentration were analyzed to demonstrate best lab practices when using a multi-cell changer to ensure consistent and accurate results across different cell positions. Descriptions of techniques for overcoming common pitfalls associated with multi-cell changers are also outlined.

Experimental

First, a stock solution of 200 ng/µL calf thymus DNA was prepared by diluting 0.1 mL 10 mg/mL calf thymus DNA stock with 4.9 mL tris-EDTA buffer (pH 7.6). A 10 ng/ mL calf thymus DNA solution was then prepared by diluting 0.6 mL of the 200 ng/mL with 11.4 mL tris-EDTA buffer. Aliquots of this solution were used for all subsequent UV-Visible measurements.

For the DNA melting experiments, a Thermo Scientific Evolution One UV-Visible Spectrophotometer equipped with a Thermo Scientific 6-cell Rotary Peltier was used. Absorbance measurements as a function of temperature were collected at 260 nm using a 1.0 nm bandwidth, 1.0 s integration time and 1.0 s dwell time. The blank was established using the same tris-EDTA buffer used to dilute the calf thymus stock solution. Table 1 includes the temperature ramp parameters used for all melting experiments described herein. For samples which were stirred, a small stir bar was placed in each cuvette and the stirring rate was set to 400 rpm. Note that the melting experiments described herein were collected using the "block" or Peltier temperature.

Stage	Final temperature (˚c)	Ramp rate (˚c/s)	Hold time (s)	Data interval (s)
1	40	0.0833	2	30
2	95	0.0167	15	30
3	25	0.0833	0	N/A

Table 1: Experimental temperature ramp parameters set within the Thermo Scientific™ Insight™ Pro Software.

Results and discussion

Baseline collection:
Single blank vs position-dependent blanks

Figure 1a includes the resulting DNA melting curves for three calf thymus DNA samples, all with 10 ng/µL concentration. Using the inflection point method for determining T_m, described elsewhere,[6] the average melting temperature was found to be 67.5 ± 0.1 ˚C. The little variation in observed T_m implies the Thermo Scientific 6-cell Rotary Peltier uniformly heats across the full accessory, leading to accurate and comparable results.

However, note that an offset can be observed between each melting profile in Figure 1a, though each sample is from the same batch. This type of variation can often arise when the baseline is established using a cuvette other than the cuvette used during the sample measurement. While quartz cuvettes can transmit a substantial amount of UV light, there can be small variations in the light allowed to pass through the windows. This inherently leads to the inclusion of an offset in the measured absorbance of a material. Additionally, imperfections in the cuvette (e.g., scratches, fingerprints, etc.) can also lead to a similar offset.

This issue is typically mitigated by using the same cuvette when establishing the baseline and when measuring the sample. However, when using cell-changers, a single blank solution in one cell position is often used to measure the applicable baseline, leading to the aforementioned issues. Figure 1b includes the same melting profiles as depicted in Figure 1a adjusted to match one another through the addition of an arbitrary offset value. Given the little variation in the calculated T_m, the close matching of the adjusted melting curves across the full experimental temperature range meets expectations. This suggests the use of a single blank is not ideal and can lead to changes in the perceived absorbance, and therefore apparent concentration, of the single and double stranded DNA per each sample.

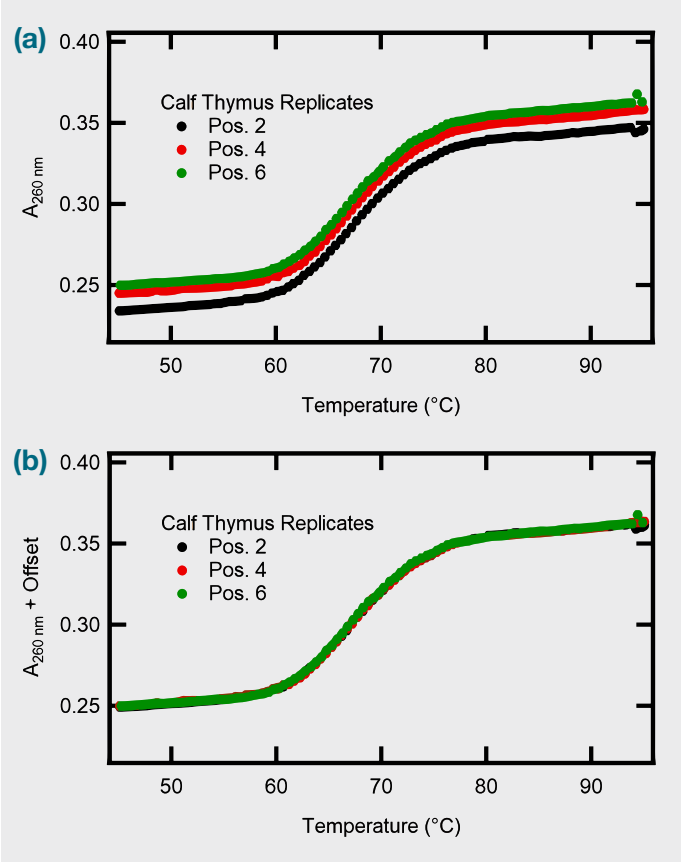


Figure 1: Calf thymus DNA melting profiles measured at 260 nm as a function of temperature. Shown are (a) raw and (b) adjusted absorbance values for three 10 ng/µL calf thymus measured in different cell positions. The adjusted melt curves include the addition of an arbitrary offset value to match the absorbance between 50 ˚C and 60 ˚C of the sample held in position 6. Samples were stirred at 200 rpm.

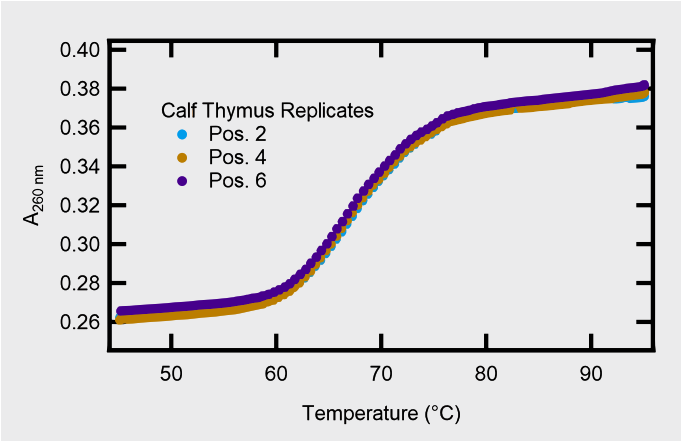


Figure 2: Raw DNA melting profiles for 10 ng/µL calf thymus DNA collected using the Multi-Zero capabilities within the Insight Pro Software.

Instead, using the "Multi-Zero" function in the Thermo Scientific Insight Pro Software allows for individual blanks to be applied to each cell position. In this way, the cuvette used to measure the DNA samples can also be used to measure the blank solution, removing the offset. Figure 2 includes 10 ng/µL calf thymus samples measured using the individual blanks per position. As is shown, there is much closer overlap between the melting curves obtained for these samples than the raw melting profiles in Figure 1a. Through these samples, T_m was found to be 67.3 ± 0.2 ˚C, similar to the values observed in the samples measured previously, once again confirming the heating uniformity across the different cell changer positions.

Temperature equilibration and monitoring

While equilibration time is built into the instrument procedure, it is best practice to ensure the samples are stirred. This aids in the even heating of the overall solution. Without stirring, the temperature differential across the cuvette can be varied enough to produce different melting profiles, as can be seen in Figure 3. For experiments conducted without stirring, the average T_m for the triplicate samples was 68.1 ± 0.4 ˚C, higher than the samples measured using stirring (T_m = 67.3 ± 0.2 ˚C). While this difference is not substantial, it highlights the need to ensure proper stirring to allow for speedy equilibration of the sample temperature.

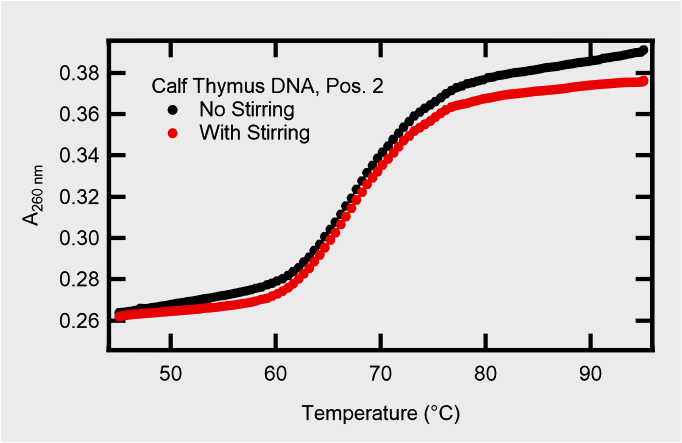


Figure 3: DNA melting curves for 10 ng/µL calf thymus samples measured with and without stirring. Both samples were measured in the same position in the Thermo Scientific 6-cell Rotary Peltier.

In addition to stirring, use of a temperature probe is also the best method for accurately recording the temperature of a solution. As mentioned previously, the block temperature does not necessarily reflect the true temperature of the solution. Consequently, the melting curve determined using the measured block temperature may be partially shifted along the temperature axis from a curve collected using the temperature probe. This can then lead to differences in the calculated melting temperature. By using the temperature probe, the solution's true temperature can be monitored, leading to a more accurate representation of the DNA melting profile. This can be especially important when using a cell changer, as separate cuvettes may transfer heat to the solution differently, resulting in slightly different temperatures per sample. When using a temperature probe, refer to the manufacturer's user guide for additional details about minimum probe submersion depths and compatible solvents.

Cuvette considerations

Often, temperature probes cannot be readily used with many types of microcells because the probe may, in the case of some smaller microcells, touch the cell walls. If this occurs, the temperature recorded may include the temperature of the cuvette instead of only measuring the solution temperature. Much like the other examples outlined herein, this can lead to an error in the recorded temperature and therefore the calculated melting temperature.

In general, microcells are not recommended for use with multi-cell changers. Unlike traditional cuvettes, where the full beam is able to interact with the sample, microcells typically include a masked aperture which will block or "clip" the light beam, thereby limiting the amount of light allowed to interact with the sample. Assuming the baseline is collected using the same microcell in the exact same position, this will not lead to errors in the reported absorbance; however, a noisier spectrum is to be expected.

Because multi-cell changers require the movement of cuvettes, an issue can arise: While traditional cuvettes as described previously do not show significant errors if its position is slightly shifted relative to the beam, masked or microcells are not as forgiving. A small shift of the aperture on the microcell can lead to a partially blocked or "clipped" light beam. Inherently, the position of the cuvette in one position will not be exactly the same in a separate position and can lead to significantly different intensity of light which is blocked by the microcell mask. This will manifest as an offset in the measured absorption spectrum. If a microcell absolutely must be used, it is vital that the "Multi-Zero" function be in use to establish the baseline. Much like what has been discussed previously, this will ensure that the same cuvette, and therefore the same aperture position, is accounted for in the blank measurement. However, keep in mind that some hysteresis may be present as a result of the iterative movement of the multi-cell changer during the experiment. This may cause the presence of an offset over the course of the experiment, especially for microcells with very small apertures. Given these issues, it is highly advised to avoid using microcells when performing any experiment using a multi-cell changer.

Additionally, ensuring the cuvette is well capped is also vital to temperature-based measurements, regardless of the use of a multi-cell changer. Without a proper cover, there is a high likelihood that the solvent will partially evaporate off at higher temperatures, leading to a change in the overall concentration. This can be a problem for samples that will be further characterized, or which need to maintain the same concentration for future use. For low volume samples, the changes can be much more drastic, further exemplifying why microcells are not recommended for use with temperature-based experiments.

Conclusions

As has been outlined herein, DNA melting measurements collected using a multi-cell changer can be highly accurate and useful for providing higher throughput measurements. By following the best lab practices described above, common errors inherent to temperature-controlled experiments and the use of a multi-cell changer can be avoided. In this way, an efficient and precise characterization of the DNA can be carried out.

References

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