Fluorescence measurement of hybridization between quencher (DABCYL) labelled PNA probes and a fluoresceine labelled DNA using the Fluorescence BioMelt Package

Application Note

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Introduction

In living cells, the process of reversible hybridization is a critical requirement to the process of replication, transcription and translation. Reversible hybridization is the ability for nucleic acids to anneal, denature and re-anneal once again. A nucleic acids ability to anneal and denature usually relies on the temperature and pH of the environment[1]. This naturally occurring phenomenon is increasingly being used by scientists to develop assays for detecting specific polynucleotide sequences. It is also being used as a tool in a variety of different ways, for example in the Polymerase Chain Reaction (PCR) to make more DNA (*Hoffman La Roche*) as well as in Peptide Nucleic Acid (PNA) technology[2].

PNA probes are increasingly being used in industry for the detection of micro-organisms in foods, beverages, water, and the environment[2]. In pharmaceutical and research areas, sequence specific PNA probes (when the sequence is known) are being created to undertake hybridization assays for specific DNA they would like to identify[2]. PNA probes are also being used as delivery vectors for antisense oligonucleotide analysis[3] and in triple helical DNA analysis for stability studies[4,5]. PNA is a DNA mimic with respect to Watson-Crick hybridization properties, with the difference being that the backbone is made up of repeating N-2-aminoethyl glycine units linked by amine bonds instead of the sugar phosphate backbone which is characteristic of DNA[6]. The unique structure of PNA molecules not only allows for normal base pair formation, but it is achiral, and has no electric

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charge in the backbone of the probe which results in very fast and strong hybridization[6]. Consequently, this results in higher Thermal Melt values than is observed for DNA. Furthermore, PNA probes are particularly resistant to protease and nuclease degradation in a cell environment which assists in minimizing any enzymatic attack during the hybridization process[6]. All of the above factors make the use of PNA probes in hybridization assays more robust than normal DNA probe hybridization.

Thermodynamic data is increasingly being used in biomedical research, pharmaceutical and diagnostic research areas as a means of optimizing specific assays. The Cary fluorescence or UV-Vis thermal melt application automatically performs thermodynamic calculations such as hybridization enthalpy (ΔH), entropy (ΔS), free energy (ΔG) and rate constants (k)[1,7,8]. This information can then be used to establish the optimal experimental conditions for the nucleic acid sample and the dye of interest. Specifically, temperature, equilibration times and experimental volumes can be established without having to perform several different experiments[9]. The Cary calculations employ a modified version of the Van't Hoff and Marky method[10].

There are other methods by which nucleic acid hybridization can be measured which do not involve dyes. For example, absorbance spectroscopy techniques such as circular dichroism[11] and hyperchromicity[8,1], calorimetric methods[12] and nuclear magnetic resonance[13] can also be used. These methods do not require fluorescence labelling, but do require larger amounts of sample. Although fluorescence does require labelling, it is a more sensitive technique and therefore allows for the use of lower concentrations. It also allows for multi-labelling capabilities (i.e., the ability to monitor fluorescence using several different fluorescent probes for each DNA type in the one experiment). In addition, fluorophores are sensitive to their environment and can therefore be used as a means of detecting hybridization/non-hybridization without having to separate the two species in a separation assay. FRET (Fluorescence Resonance Energy Transfer) is a good example of how fluorophore sensitivity to its environment can be used as a means of indicating the state of a nucleic acid (denatured/annealed)[14].

In this experiment, two different lengths of a DABCYL (4-((-4-(dimethylamino)-phenyl)-azo)-benzoic acid) labelled PNA probe (9mer and 13mer) bound to a 5- and 6-carboxyfluoresceine labelled DNA were analyzed.

a) 9mer

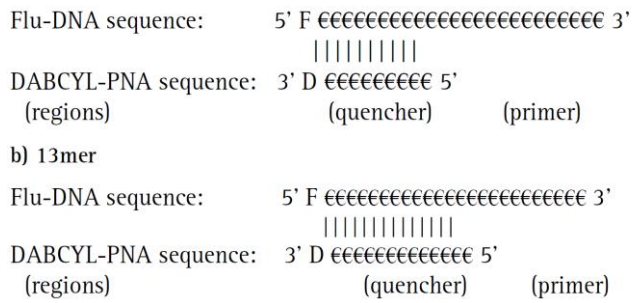

Figure 1. A representation of the 5′ 6-carboxyfluorescein labelled DNA hybridized to the DABCYL labelled PNA probe. Part a) represents the 9mer PNA probe and b) represents the 13mer PNA probe. The longer PNA probe binds more strongly to this region of the DNA than the 9mer and therefore a higher overall thermal melt (T_m) is expected because more energy is required to break the bonds and separate the strands

DABCYL is a non-fluorescent quencher, which, under proper conditions, can absorb energy from the excited state of the Fluorescein molecule, through the process of fluorescent resonance energy transfer (FRET)[14,15].

The system used in this experiment is designed in a way that the DNA primer is non-fluorescent until it is incorporated into a double stranded molecule during PCR[16]. Once incorporated, it becomes fluorescent because the DABCYL labelled PNA is displaced, and quenching can no longer occur. Using thermal melt (T_m) profiles, the stability of a 9mer PNA probe bound to DNA was analyzed and compared to a 13mer PNA probe bound to the same species of DNA.

Materials and methods

(For part numbers please see Reference 7.)

Fluorescence BioMelt system, comprising:

- Cary Eclipse Fluorescence Spectrophotometer
- Multicell Peltier
- Temperature controller
- Quartz 10 mm w/stopper
- Eclipse Thermal Software (Bio software package)

Sample preparation

Creating the strand: A non-fluorescent DNA primer is incorporated into a double stranded molecule during PCR[16].

The dye labels used are:

DNA: 5'6-carboxyfluorescein (mixed isomers)
PNA: DABCYL (4-((-4-(dimethylamino)-phenyl)-azo)-benzoic acid) (refer to figure 1 above).

Both the PNA and DNA were at 50 nM in a volume of 1 mL buffer solution; the buffer is 100 mM NaCl, 10 mM KPO_4 pH 7.1.

Instrument and software parameters

The Cary Eclipse 'Thermal' software application operating parameters illustrated in Figures 2–4 were set as follows:

Cary tab:

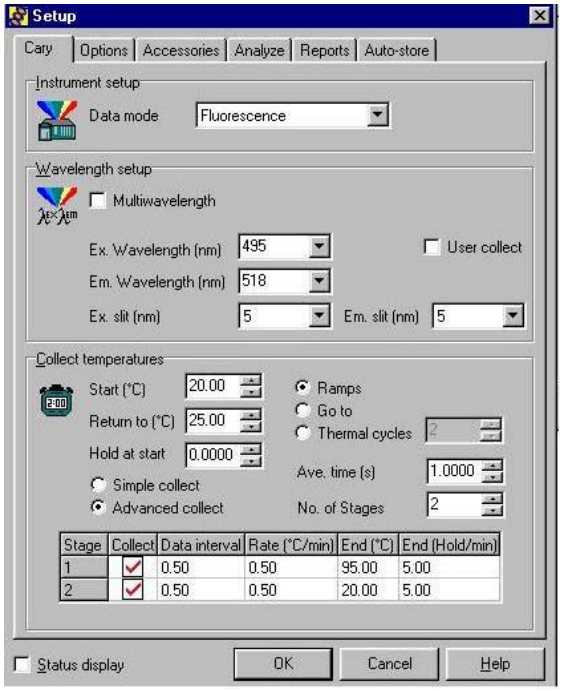

Figure 2. Excitation 495 nm Emission 518 nm, ramps 20–95 °C and 95–20 °C (a positive and negative ramp). Hold time 5 mins before ramping to 95 °C and then another 5 mins before ramping down to 20 °C again

Options tab:

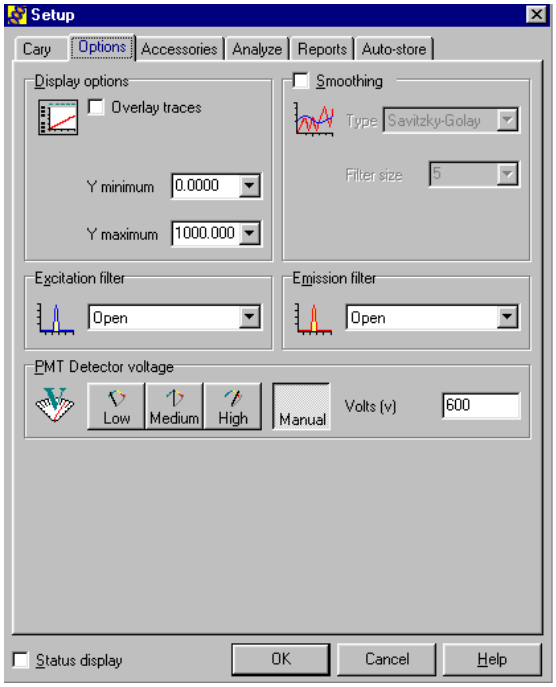

Figure 3. Filters were set on open for both the excitation and emission monochromators.The PMT voltage was set to 600 V

Accessories used:

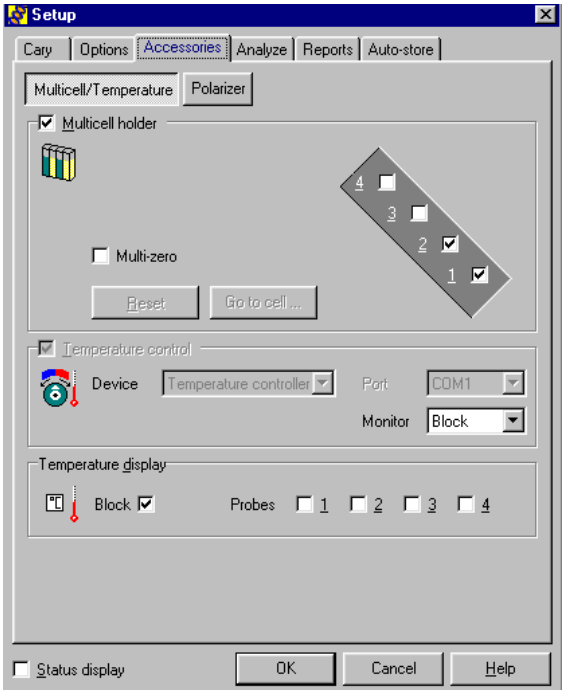

Figure 4. Two samples were measured using the Eclipse Multicell Peltier. The block was monitored and displayed, rather than the solution probe

Analyze Tab:

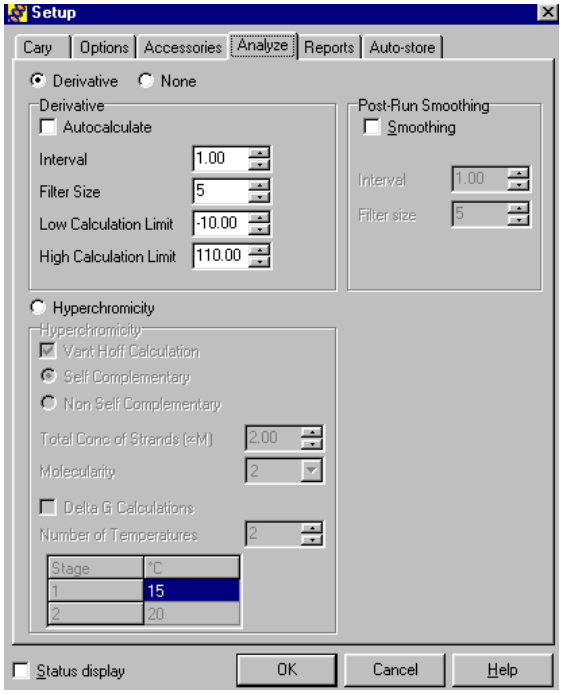

Figure 5. Calculations determined using a first derivative function set in the Thermal software

For this analysis T_m values were calculated using the derivative method (refer to Figures 6 and 7 below). If required, further thermodynamic calculations can be undertaken (enthalpy (ΔH), entropy (ΔS), free energy (ΔG) and rate constants (k)) using the Thermal software calculations which employs a modified version of the Marky *et al* method[10].

Results

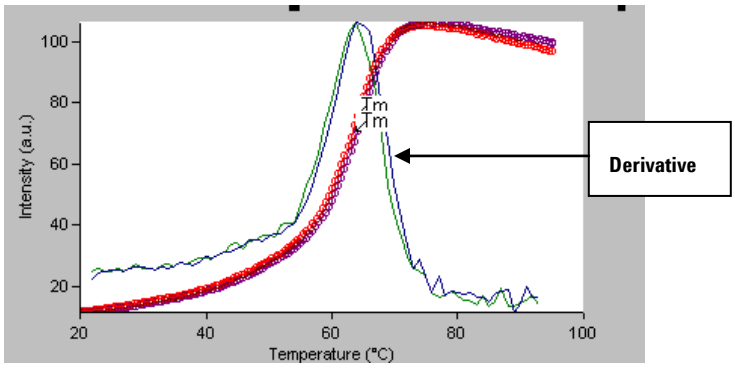

Figure 6: Tm value using 13mer DABCYL labelled PNA probe with 5′ 6-carboxyfluorescein labelled DNA. Tm was calculated by performing a derivative curve using the Cary Eclipse thermal software. It was calculated to be 63.9 °C

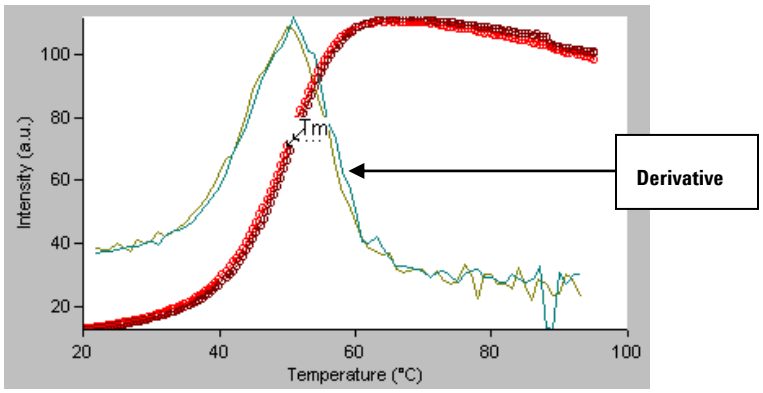

Figure 7: Tm value using 9mer DABCYL labelled PNA probe with 5′ 6-carboxyfluorescein labelled DNA. Tm was calculated by performing a derivative curve using the Cary Eclipse thermal software. It was calculated to be 49.94 °C

Figures 6 and 7 show the changes in the intensity of fluorescein as a function of temperature for both the 13mer and 9mer PNA sample respectively. Both samples show low fluorescence during hybridization (annealing between quencher (DABCYL) labelled PNA probes, and a 5′ 6-carboxyfluorescein labelled DNA).

As the temperature increases, the strands melt apart, the DABCYL is displaced from the fluorescein and there is no longer energy transfer resulting in an increase in fluorescence intensity of fluorescein. The 9mer PNA probe has a T_m value of 49.94 °C and the 13mer PNA probe has a T_m value of 63.9 °C.

Discussion

In comparing the data in Figure 6 and Figure 7, we notice slightly different melting profiles. Considering the DNA component is the same for both samples, the difference observed could be attributed to the varying length of the PNA probe. It is clearly evident that the 9mer PNA (Figure 7) gives a lower melting temperature (T_m) than the 13mer PNA (Figure 6). As mentioned previously, the polyamine backbone of PNA probes is more stable than the sugar phosphate backbone of DNA. It is therefore not difficult to imagine that when there are more polyamine bonds (example 13mer PNA probe) you have stronger binding for this region of the DNA. This in turn requires higher temperatures to break the bonds between the two strands and the result is a higher overall T_m value. The higher T_m observed where the PNA component in the complex is longer supports the idea that the binding properties of PNA (polyamine bonds) are stronger than that of DNA (sugar phosphate bonds)[6]. It is therefore quite reasonable to imagine that higher stability and stronger hybridization properties of PNA probes may be more beneficial in some hybridization assays than the traditional DNA probes currently being used.

These results show how valuable thermal melt profiles can be in characterizing the stability of a probe and in doing so optimizing your hybridization assay. It is also easy to imagine how this technique could be valuable in characterizing the thermodynamic properties of other samples such as proteins, RNA and DNA. The Agilent Cary Eclipse instrument, multicell peltier and thermal software used in this experiment provide the flexibility to perform data collection and all the necessary calculations required for this type of hybridization assay[7]. Although a ramp rate of 0.5 °C/min was used for this particular thermal melt assay, it is possible to vary the ramp rate during certain stages in the melt curve. The software provides ramp rates as slow as 0.06 °C/min (to obtain more data during the temperature regions of interest) or as fast as 30 °C/min when you just want to get to a temperature quickly (refer to Figure 2). During the experiment, a hold time was programmed (refer to Figure 2) to ensure the instrumentation remained fixed at the extreme temperatures long enough to allow the sample to denature or re-anneal. Due to the efficient temperature transfer between the Eclipse multicell peltier block and the quartz cuvettes, a five minute hold time was sufficient. Since temperature accuracy and reproducibility is critical for optimizing conditions for this type of assay, very precise temperature control was required[7].

Conclusion

The results presented in this paper demonstrate how T_m values can assist in establishing which probe will give the best stability under specific conditions. It is easy to see how understanding the thermal melt characteristics of a sample in its environment can assist with establishing and optimizing the efficiency of a hybridization assay. From the results obtained in this experiment, it can clearly be concluded that the length of the PNA probe can affect the stability of the DNA complex.

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7. Part numbers Cary Eclipse:

Product	Part Number
Agilent Cary Eclipse Fluorescence Spectrophotometer	00 100752 00
Peltier Thermostatted Multicell holder	00 100755 00
Temperature Controller for Peltier	00 100386 00
Temperature Probe accessory	00 100408 00
Quartz cuvettes, rectangular stoppered	66 100012 00
Kit door for thermostatted accessories	99 101032 00
Eclipse Thermal Software (Bio software pack)	85 101774 00
Country kit (98 1002 XXXX) 9000 (AUS); 9100 (US); 9200 (Europe)	

8. Cary WinUV:

Product	Part Number
Agilent Cary 300 UV-Vis Spectrophotometer or:	00 100691 00
Agilent Cary 100 UV-Vis Spectrophotometer	00 100693 00
Extended sample compartment	00 100418 00
6x6 Peltier Multicell holder	00 100409 00
Temperature Controller for Peltier	00 100386 00
Temperature Probe accessory	00 100408 00
Quartz cuvettes, 0.9ml masked	66 100127 00
Cary WinUV Thermal Software (Bio software pack)	85 101585 00
Country kit (99 1002 XXXX) 6000 (AUS); 6100 (US); 6200 (Europe)	

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