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Short running title

Exciplexes and Scorpion oligonucleotides

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Abbreviations

LES, locally excited state; I_E/I_M , emission intensity ratio between exciplex band at 480 nm and LES at 380 nm; TFE, trifluoroethanol.

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*** Manuscript**

Target-assembled exciplexes based on Scorpion oligonucleotides

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Abstract

Scorpion probes, specific DNA probe sequences held in a hairpin loop can be modified to carry the components of an exciplex, for use as a novel fluorescence-based method for specific detection of DNA. The exci-partners (5'-pyrenyl and 3'-naphthalenyl) were attached to oligonucleotides *via* phosphoramidate links to terminal phosphate groups. Hybridisation of the probe to a complementary target in a buffer containing trifluoroethanol led to an obvious fluorescence change from blue (pyrene locally excited state emission) to green (exciplex emission).

Keywords

DNA detection; exciplex; fluorescence; Scorpion probes; SNP.

Abbreviations

LES, locally excited state; I_E/I_M , emission intensity ratio between exciplex band at 480 nm and LES at 380 nm; TFE, trifluoroethanol.

1. Introduction

Molecular Beacons (Tyagi and Kramer 1996), Scorpion primers (Whitcombe et al 1999), HyBeacons (French et al 2001) and TaqMan probes (Holland et al 1991; Livak et al 1995) are generally single-stranded oligonucleotides that utilise a fluorophore and quencher. In their dark, quenched form they adopt a conformation with the quencher close to the fluorescent dye. On hybridisation to target, they undergo a conformational change (Molecular Beacons, Scorpions), an environmental change for the fluorophore (HyBeacons) or enzymatic cleavage (TaqMan) causing a fluorescence change.

Scorpion uni-probes, extremely sensitive and sequence-specific, consist of a single-stranded fluorescent probe sequence held in a hairpin-loop by complementary stem sequences at each end (Figure 1). The probe contains a 5'-reporter dye and a nearby internal quencher directly linked to the 5'-end of a PCR primer *via* a blocker, which prevents Taq DNA polymerase from extending the primer. Taq DNA polymerase extends the PCR primer making the complementary strand of the target. During the next cycle, the hairpin-loop unfolds and the loop-region hybridises intramolecularly to the newly synthesized target, giving increased fluorescence intensity (Hart et al 2001).

A recent (Bichenkova et al 2005; Bichenkova et al 2006; Bichenkova et al 2007) approach to diminish background fluorescence uses exciplexes assembled from components hybridising at the target sequence. This end-point exciplex method uses two components non- or weakly fluorescent at the detection wavelength, giving backgrounds <1 %. For exciplex (or excimer) emission the exci-partners must approach closely, ~3.5 Å for pyrene excimer (Birks 1970; Birks 1973). Therefore, the potential resolution of the exciplex is order of the thickness of a base pair. FRET systems work over 10–100 Å; ~ 3 bp. We now describe exciplex emission for Scorpion-based nucleic acid detectors (Figure 2), consisting of a Scorpion target (34-mer) bearing one exciplex-partner (exci-partner) on the 5'-terminus, plus a second probe oligonucleotide (8-mer) bearing the other exci-partner on its 3'-terminus (Figure 2). Exci-partners (5'-pyrenyl and 3'-N'-methyl-N'-naphthalenyl) and structures are in Figure 2C.

The 5'-naphthalenyl labeled Scorpion is called Scorpion-5'-N: the 3'-pyrenyl labeled short oligonucleotide is Exciprobe-3'-PY. Scorpion 5'-labeled with pyrene is called Scorpion-5'-PY, and 3'-naphthalenyl labeled Exciprobe is Exciprobe-3'-N. Control experiments used Exciprobe 3'-labeled with phosphate (Exciprobe-3'-p).

2. Materials and Methods

Scorpion oligonucleotides were from Biosearch Technologies, Inc (Novato CA, USA), DNA probes, and DNA targets from Sigma-Proligo Paris, deuterium oxide from Goss-Scientific Instruments Ltd, UK. N-Methyl-N-naphthalen-1-yl-ethane-1,2-diamine dihydrochloride exci-partner, 1-pyrenemethylamine and protocols for attachment to oligonucleotides and Scorpions were as described (Bichenkova et al 2005). Water was distilled and purified by ion exchange and charcoal (MilliQ, Millipore Ltd, UK). Tris buffer was prepared from analytical reagent grade materials.

HPLC was performed on an Agilent 1100 Series system, with both diode-array and fluorescence detection for on-line acquisition of spectra. Columns were: Zorbax Eclipse X DB-C8 column (125 x 4.6 mm, 5µm), or Luna C18 (2) (25 cm x 4.6 mm, 5 µm) using 0–50 % acetonitrile-water gradient.

NMR spectra were recorded on a Bruker 300 MHz (7.05 T) spectrometer (Avance-300) at 300 MHz for ¹H NMR and 121 MHz for ³¹P. Chemical shifts (δ) are in parts per million (ppm), relative to Me₄Si (0.00) for ¹H spectra, and the intramolecular nucleotide phosphate (0.00) for ³¹P spectra.

UV-visible absorption spectra were measured at 20 °C on a Peltier-thermostatted Cary-Varian 1E UV-visible spectrophotometer. Quantification of oligonucleotide components used millimolar extinction coefficients (ε₂₆₀) of 79.9 for ExciProbe-3'-phosphate, and 312.3 for Scorpion target. Extinction coefficients were calculated by the nearest neighbour method (Cantor et al 1970); the contribution of the exci-partners was small and could be neglected. T_m values (first derivative method) based on A₂₆₀ were determined in Peltier-thermostatted quartz cuvettes using a Cary 4000 UV-visible spectrophotometer.

Fluorescence emission and excitation spectra were recorded in 2 mL 4-sided quartz thermostatted cuvettes using a Peltier-controlled Cary-Eclipse, or a Shimadzu RF-5301PC spectrofluorophotometer with the temperature controlled by circulating water from a Haake GH water cooler. Spectra were recorded in Tris buffer (10 mM Tris, 0.1 M NaCl, pH 8.40) containing various percentages of trifluoroethanol (TFE). Excitation wavelengths for both Locally Excited State (LES) and exciplex emission were optimized in each experiment. Spectra were corrected for any TFE, buffer or naphthalene background emission under the optimized experimental conditions as appropriate. A working solution (700 μ L) was prepared, for example, by taking an aliquot (11 μ L of 0.224 mM stock) of Scorpion-5'-PY (final cuvette concentration, 3.52 μ M), 0.7 μ L Exciprobe-3'-N (final cuvette concentration, 3.80 μ M), Tris buffer (70 μ L, 10 mM Tris, 0.1 M NaCl at pH 8.40) and Milli-Q water (618.3 μ L). The emission spectrum was recorded, and TFE sequentially added (to give 19.6 %, 32 % and 41 %, v/v) with emission spectra sequentially taken.

After attachment of 1-pyrenemethylamine (giving Scorpion-5'-PY), free Scorpion oligonucleotide eluted early (26 min), followed by 5'-pyrene oligonucleotide-conjugate (30 min), and un-conjugated 1-pyrenemethylamine (33 min). Eluted peaks were detected at multiple wavelengths (260, 280, 340 and 480 nm): typical yield \sim 23 %. The 348 nm absorbance band is due to pyrene (also affects 260 nm band). The ratio of absorbance at 260 nm ($A = 0.303$) to 345 nm ($A = 0.026$) was \sim 11.6. Attachments of 1-pyrenemethylamine to 8-mer probe showed A_{260}/A_{345} 3.0 (Bichenkova et al 1997; Bichenkova et al 2005). Appropriate fractions were combined, lyophilised and the modified oligonucleotides characterised by ^{31}P NMR spectroscopy in D_2O . ^{31}P NMR spectroscopy of the unmodified Scorpion provided a control with free phosphate group having $\delta \sim 1.6$ ppm and the phosphodiester ~ 0 ppm. The free phosphate signal in the modified Scorpion shifted downfield to ~ 10.2 ppm, confirming the phosphoramidate bond (Gorenstein 1981).

After attachment of 1-naphthalenemethylamine to give Scorpion-5'-N, HPLC showed elution of 3'-naphthalene oligonucleotide-conjugate (29 min), and unconjugated N'-methyl-N'-naphthalen-1-yl-ethane-1, 2-diamine (34 min): typical yield \sim 83 %. UV/visible absorption spectra of unmodified Scorpion-5'-phosphate and Scorpion-5'-N were similar to the literature (Bichenkova et al 2005). The shoulder at 310 nm on the 260 nm absorption band substantiated the presence of attached naphthalene. In addition to the phosphodiester signal at ~ 0 ppm, a new ^{31}P NMR signal at ~ 10.2 ppm is attributable to phosphoramidate in Scorpion-5'-N (Gorenstein 1981).

3. Results

Exciplex emission of Scorpion-5'-PY plus Exciprobe-3'-N was not seen in Tris buffer (10 mM Tris, 0.1 M NaCl, pH 8.4 at 5 $^{\circ}\text{C}$) in the absence of TFE. Sequentially adding (19.6 %, 32 % and 41 %, v/v) led to increased fluorescence intensity at 466 nm, largest for 32 % TFE (Figure 3). No exciplex signal was seen for 47 % - 90 % TFE.

No exciplex band at 466 nm in the corresponding control system in 32% TFE/ Tris (pH 8.4) formed when the naphthalenyl group on Exciprobe-3'-N was replaced by a phosphate (Exci-probe-3'-phosphate), even after heating the system to 60 $^{\circ}\text{C}$ and re-annealing by cooling back to 5 $^{\circ}\text{C}$.

T_m for the Scorpion-5'-phosphate alone in 41 % v/v TFE / Tris buffer (10 mM Tris, 0.1 M NaCl, pH 8.4) was 50.0 ± 1.0 $^{\circ}\text{C}$ (Figure 4), and for the (full system) of Exciprobe-3'-N and Scorpion-5'-PY together in 41 % TFE v/v /Tris buffer (10 mM Tris, 0.1 M NaCl, pH 8.4, Figure 4) values were 16.0 ± 0.5 and 52.0 ± 0.5 $^{\circ}\text{C}$, respectively.

No exciplex signal was detected at any TFE concentration for the system composed of Scorpion-5'-N and Exci-probe-3'-PY.

4. Discussion

Sigmoidal melting curves on the tandem duplex Scorpion systems provided strong evidence of duplex formation (Figure 4). The single melting transition of Scorpion-5'-phosphate alone (T_m 50.0 ± 1.0 °C) corresponds to opening the hairpin of the Scorpion (inset to Figure 4), consistent with literature findings (Cuesta-Lopez et al 2005; Goddard et al 2000; May et al 2005). For the full exciplex system of Exciprobe-3'-N and Scorpion-5'-PY, the two transitions (16.0 ± 0.5 and 52.0 ± 0.5 °C) probably correspond to the melting transitions of the short, 8-mer Exciprobe-3'-N and Scorpion hairpin loop, respectively (Kim and Kim 2003), the value of the higher T_m being close to that of the parent Scorpion-5'-phosphate under the same conditions (Figure 4).

Additional evidence of duplex formation comes from the emission spectra. The Scorpion target alone showed no exciplex peak at 466 nm in the absence of Exciprobe-3'-N. Addition of Exciprobe-3'-N to the Scorpion target caused a 2-4 nm red shift of the pyrene LES emission, accompanied by its quenching. A new structureless band appeared at ~ 466 nm (Figure 3), characteristic of a naphthalene-pyrene exciplex (Bichenkova et al 2005; Bichenkova et al 2006; Bichenkova et al 2007). Exciplex emission is achievable due to interaction of the exci-partners on duplex formation if they are perfectly located close to one another. As pyrene can form exciplexes with guanine (especially) (Geacintov et al 1993; Manoharan et al 1995), it was important to establish whether the apparent exciplex emission seen in the present system is the result of such background effects. Experiments with Scorpion-5'-PY and Exciprobe-3'-p, bearing only a phosphate group at its "nick terminus" and no naphthalenyl group, showed no exciplex emission (data not shown). Thus, the second exci-partner is needed for exciplex emission, and the emission detected results from interaction between the exci-partners. Emission typical of pyrene LES was observed for Scorpion-5'-PY in the absence of Exciprobe-3'-N, with little or no background exciplex emission due to Scorpion-5'-PY oligonucleotide (Figure 3).

The new band at 466 nm was attributed to a pyrene-naphthalene exciplex (Figure 3) because) the band shape (broad) and position (466 nm) is typical of pyrene-naphthalene exciplex emission, and the intensities of the monomer bands are quenched and the 466 nm band concomitantly increased in intensity, *i.e.* LES emission is maximal at 376 nm, characteristic of pyrene in the non-hybridised pyrene-bearing probe, but on addition of Exciprobe-3'-N, λ_{max} of LES emission shifts to 380 nm, accompanied by its quenching. The excitation maximum of the unbound probe is 340 nm, and when Exciprobe-3'-N is bound to Scorpion-5'-PY the excitation spectrum shifts to 349 nm, indicating hybridisation.

For all TFE concentrations used, the emission spectra of the Scorpion-5'-PY showed λ_{max} 376 nm. On addition of Exciprobe-3'-N, the emission maximum red-shifted by a few nanometers, and its emission intensity decreased, indicating duplex formation. However, no exciplex emission was seen for this system in Tris buffer for any TFE concentration less than 19.6%. For the full Scorpion system, exciplex emission in the presence of TFE was strongest at 32% TFE. Thus, the precise structural nature of the (local) duplex formed in the Scorpion exciprobe stem loop differs from conventional linear duplex DNA, for which exciplex emission is optimal at 80% TFE (Bichenkova et al 2005; Bichenkova et al 2007). The role of TFE in allowing exciplex emission for nucleic acid systems is under investigation (Bichenkova et al 2005; Bichenkova et al 2007) but clearly the optimal conformation of the nucleic acid system (whether linear or Scorpion stem loop) is critical as the optimal percentage TFE varies with the system (Bichenkova et al 2005; Bichenkova et al 2007) and in organic model exciplexes TFE fully quenches exciplex emission (Bichenkova et al 2006).

Despite the close proximity of pyrene and naphthalene, no exciplex signal was detected at any TFE concentration for the system composed of Scorpion-5'-N and Exci-probe-3'-PY. It may be that the pyrene and naphthalene sites do not achieve the required exciplex geometry (Paris et al 1998). Another possibility regarding the absence of the exciplex signal is that the 3'-terminal pyrene is attached directly to a guanosine nucleotide (Figure 2). Nevertheless, no background exciplex signal was detected for Exci-probe-3'-PY. This implies that the linkage at the 3'-phosphate may prevent back interaction of pyrene with nucleobases.

In summary, we show that nucleic acid-mounted exciplexes can be used in combination with Scorpion systems to provide fluorescent emission, and that nucleic acid-mounted exciplexes serve as sensitive monitors of subtle conformational differences.

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Figure 1

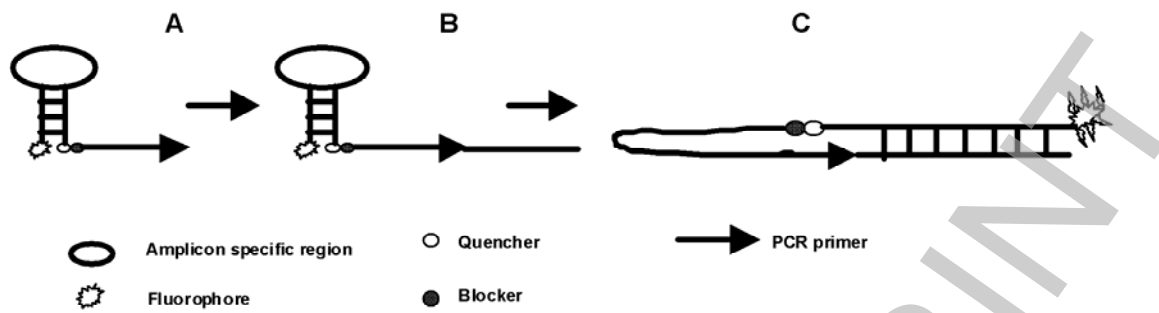


Figure 1. Detection of PCR products with Scorpion oligonucleotide probes (A) the Scorpion primer with a 5'-extension of a probe, a pair of self-complementary stem sequences and a fluorophore-quencher pair. (B) After PCR extension from the primer, the target region is attached to the same strand as the probe. (C) Following a second round of denaturation and annealing, when the probe hybridises to target, the fluorophore is no longer close to the quencher and fluorescence is emitted (Whitcombe et al 1999).

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Figure 2

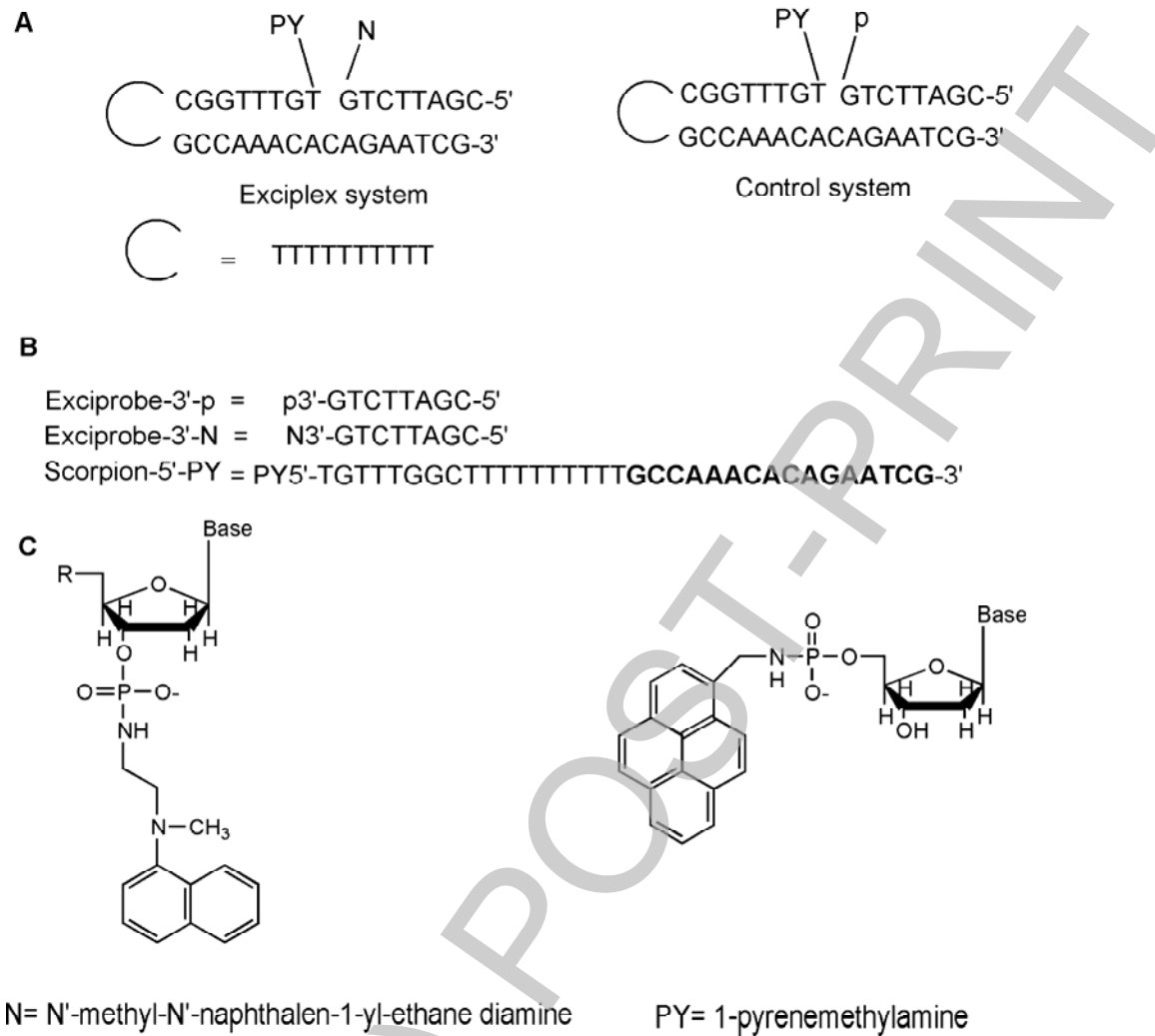


Figure 2. Scorpion sequence with chemical modifications and exciplex-partner bearing probe oligonucleotides used. (A) Diagram of constructs used (the region of sequence complementary to the sequences modified with potential exciplex partners is in bold). (B) Sequences of control probe and exci-probe oligonucleotide components of the detection system. Exciprobe-3'-p indicates that the 3' position bears a free phosphate group instead of an exciplex partner. (C) Chemical structures of exciplex partners shown with 5'-pyrenyl and 3'-naphthalenyl modifications as examples.

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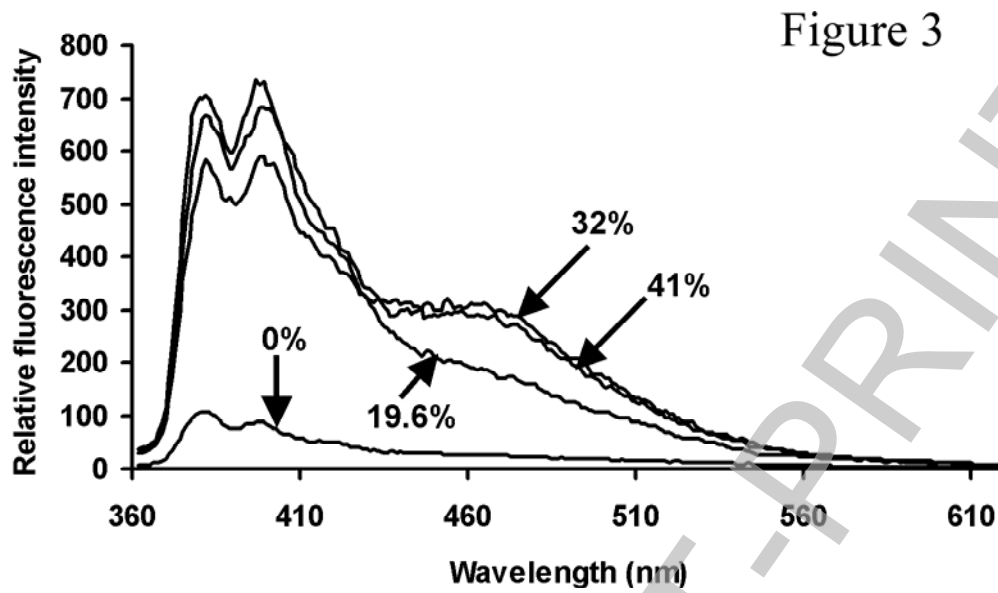


Figure 3

Figure 3. Emission spectra of Scorpion-5'-PY plus Exciprobe-3'-N after addition of different concentrations of TFE for the full system (TFE 0, 19.6, 32.0 and 41 %, v/v) to Tris buffer (10 mM Tris, 0.1 M NaCl, pH 8.4) at 5 °C: excitation 349 nm, slitwidth 5 nm.

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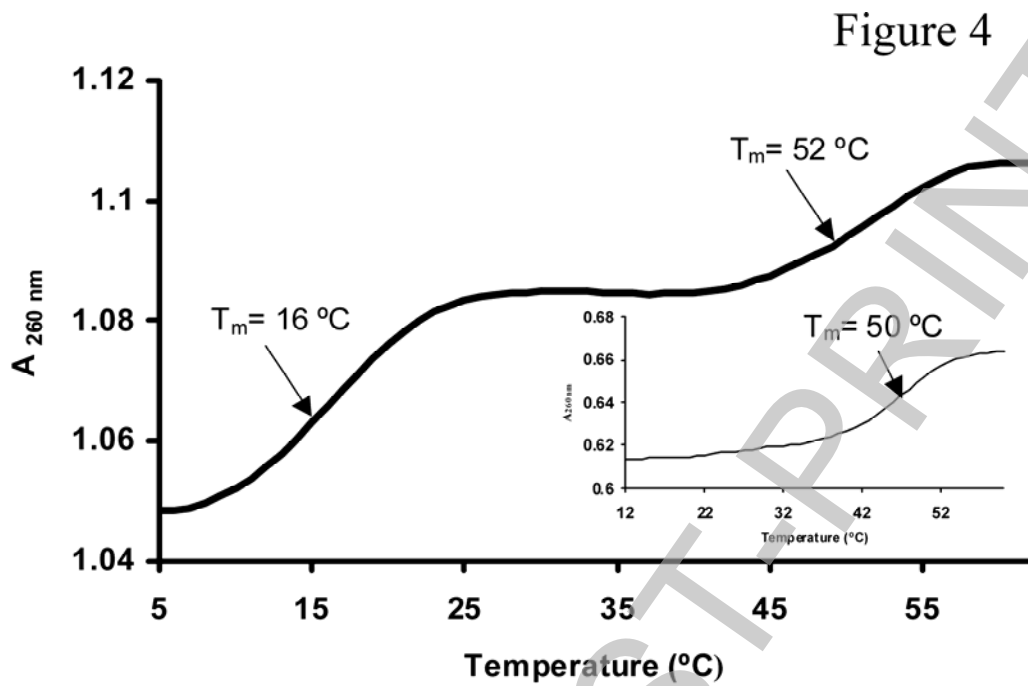


Figure 4. Melting curves of Scorpion-5'-PY (1.91 μ M) and Exciprobe-3'-N (2.10 μ M) in 10 mM Tris, 0.1 M NaCl, pH 8.40 containing 41 % v/v TFE, based on change in absorbance at 260 nm: temperature ramped at 0.25 °C/min. The inset shows melting curve of Scorpion-5'-phosphate (2.0 μ M) under the same conditions.

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