
Design and Synthesis of Hairpin Probe for Specific Mis-match Discrimination

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ABSTRACT

A single stranded hairpin probe labeled with fluorescein at its 5'-end and terminates with deoxyguanosine nucleotide at 3'- end, as quencher, has been designed and synthesized in an automated DNA synthesizer. The system has been used as an alternative to molecular beacon. The deoxyguanosine residues have been kept at the 3'-end of the complementary arm strand to quench the fluorescence intensity of the fluorophore, making the hairpin probe behave like a conventional molecular beacon. The proposed probe has been used to find a correlation between fluorescence and thermal behaviour on hybridizing it with several mismatched target oligonucleotides. The designed probe has shown greater degree of specificity with perfectly matched target oligonucleotide, while it shows a variable degree of destabilization with mismatched (A/C) target complementary oligonucleotides.

INTRODUCTION

Recently, hairpin oligonucleotide probes, each of which consists of a fluorophore at one of the termini and a quencher at the other end, have proven to be effective in conventional molecular beacons¹. The molecular beacon possesses a stem-loop-like structure and generally is dual-labeled, with a fluorophore (a donor dye) at the 5' end and another dye (an acceptor dye that acts as a quencher) at the 3' end of the stem part that may or may not be fluorescent. The presence of the specific target nucleic acid sequences, complementary to the loop part, is detected by a strong increase in the fluorescence intensity on hybridization, thereby making the homogeneous assay more sensitive and reliable. Thus, the strategy requires labeling at both ends of a single-stranded oligonucleotide probe with specific fluorescent dyes that suffer in overall yield and are expensive. To avoid the limitations that molecular beacons face, hairpin probes have been designed. This kind of probe uses the fluorescent label at one end and a nonfluorescent molecule (e.g., nucleobase) at the other end and is able to work as an acceptor as well as to quench the fluorescence intensity of the fluorophore in the closed-state conformation. For this purpose, a number of unique (rather than traditional) nonfluorescent quenchers, ranging from

DNA nucleotides (e.g., guanosine, deazaguanosine) to gold nanoparticles, have already been introduced successfully. Furthermore, instead of using interactions between the two extrinsic probes, interactions of fluorophores with DNA nucleobases or amino acids have potential for the specific detection of DNA or RNA sequences and antibodies at the single molecular level².

In the current study, in contrast to molecular beacons, only the 5' end-labeled hairpin probe (viz. FAM-C₃-catcAAAAAAAAAAAAAAAAAAAAAAAAgatggg-3') was synthesized and tested for mismatch discrimination in different target strands.

RESULTS AND DISCUSSION

All of the oligomers were synthesized at the 0.2 μM level on an ABI 3800 high-throughput DNA synthesizer using the standard phosphoramidite approach. For the synthesis of hairpin probe, the last coupling was done with amino modifier (viz. 3-(trifluoroacetyl-amino)-propyl-2-(cyanoethyl)-N,N'-diisopropyl phosphoramidite [Glen Research]) to get amino functionality at the 5' end as reported previously³. Two extra "Gs" have been introduced as an overhang in the dangling arm to utilize guanosine's characteristic inherent quenching ability-as a quencher, for homogeneous detection assays that have potential for detection at single molecule level. The quenching efficiency of guanosine is due to its low oxidation potential and also because of hydrophobic interaction between the dyes and DNA nucleotide⁴. Another reasonable possibility for fluorescence quenching is due to formation of non fluorescent ground state within close proximity of stacked arrangement of nucleobase G.

T_m values and the change in relative fluorescence intensity with fully matched and mismatched targets of base A/C in Tris-HCl buffer containing 5.0mM MgCl₂ and 0.1M NaCl, at pH 8.0 on Perkin-Elmer Lambda Bio 20 UV-visible spectrophotometer attached with PTP-I Peltier Temperature programmer and Fluoromax-3 (SPEX) spectrofluorometer (Jobin Yvon) respectively. First the denaturation and change in relative fluorescence intensity of the hairpin probe (70.0nM) was recorded in the absence of targets at different temperatures. The temperature was increased from 5°C to 80°C (increment of 10°C) with each step lasting for 10 min prior to the measurements of fluorescence. At lower

temperature there was no fluorescence confirming the presence of fluorophore and quencher in a close proximity to each other – closed state. However, on increasing the temperature the helical structure of the stem disrupts to a random coil conformation, separating the fluorophore and quencher apart from each other thereby restoring the fluorescence (56°C) of hairpin probe. Similarly, on repeating the experiment in the presence of excess of the perfectly matched target strand (175nM) complementary to

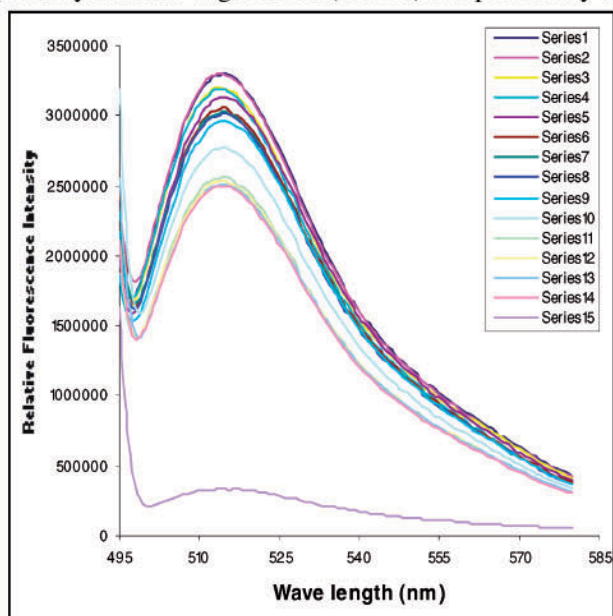


Fig.1 Change in Fluorescence intensity of different oligonucleotide target strands with hairpin probe.

the loop domain of the probe (wild type) there were 10-11 fold enhancements in fluorescence signal at lower temperature. As the temperature is slowly raised further, fluorescence started diminishing. The transition from the helical conformation to a random coil configuration in the absence of target strand occurred at 56°C, while in the case of the perfectly matched duplex, a transition to the quenched hairpin stem occurred at 51°C. This is because stem part of the hairpin probe is less stable than the full matched duplex. As the temperature raised further, the hairpin probe in the absence and presence of the target strands again started melting into a fluorescent random coil, thereby again showing small enhancement in the relative fluorescence intensity (fig not shown). The results clearly demonstrated the quenching efficiency of guanosine residue however, the percentage of signal generated was found small in comparison to molecular beacons and taqman probes thereby confirming that in the present case the quenching or energy transfer is contact mediated not via FRET mode.

For the mismatch studies targets ON2-15 were synthesized and the T_m values (Table-1) and change in relative fluorescence intensity (Fig. 1) of the respective duplexes were recorded at constant concentration in same

hybridization buffer. According to the T_m & ΔT_m values there was appreciable increase in degree of destabilization of duplexes with the introduction of A/C mismatch from 5'-end to middle but after that towards the 3'-end destabilization factor decreases. On analysing the fluorescence data it has been observed that as the destabilization varies there was also similar variation in the relative fluorescence intensities. The fluorescence signal generated by the different duplexes were found more than the hairpin probe but the were less than the wild-type probe-target duplex (ON2). The difference in the fluorescence intensity generated by the ON3-5 from 5'-end and ON13-15 from 3'-end on hybridization with hairpin probe was relatively less. On moving towards the middle region as in case of ON6-12 there was distinctive decrease in the relative fluorescence intensities.

S. No.	Oligonucleotides	T_m (°C)	ΔT_m (°C)
1	3'-TT TTT TTT T TT TTT TTT TTT-5' (ON2)	49.111	---
2	5'-CTT TTT TTT TTT TTT TT-3' (ON3)	48.44	(-) 0.67
3	5'-TTC TTT TTT TTT TTT TT-3' (ON4)	46.14	(-) 2.97
4	5'-TTT CTT TTT TTT TTT TT-3' (ON5)	43.44	(-) 5.67
5	5'-TTT TTC TTT TTT TTT TT-3' (ON6)	41.75	(-) 7.36
6	5'-TTT TTT CTT TTT TTT TT-3' (ON7)	40.02	(-) 9.09
7	5'-TTT TTT TTC TTT TTT TT-3' (ON8)	40.35	(-) 8.76
8	5'-TTT TTT TTT CTT TTT TT-3' (ON9)	39.52	(-) 9.59
9	5'-TTT TTT TTT TTC TTT TT-3' (ON10)	40.67	(-) 8.44
10	5'-TTT TTT TTT TTT CTT TT-3' (ON11)	39.50	(-) 9.61
11	5'-TTT TTT TTT TTT TTC TT-3' (ON12)	41.14	(-) 7.97
12	5'-TTT TTT TTT TTT CTT TT-3' (ON13)	41.75	(-) 7.36
13	5'-TTT TTT TTT TTT TTC TT-3' (ON14)	45.10	(-) 4.01
14	5'-TTT TTT TTT TTT TTT TC-3' (ON15)	48.18	(-) 0.93

Table-1 Complementary Oligonucleotide targets used for mismatch studies.

CONCLUSION

Conclusively, a labeled hairpin probe has been designed and synthesized using inherent quenching property of DNA nucleotide deoxyguanosine to study the fluorescence and thermal behaviour which was found sensitive to variations and identities of mismatches in the complementary targets. In contrast to dual labeled molecular beacon, the synthesis of such kind of probes is highly simple and economical in the sense it does not required engineered polymer support and stringent purification. The high specificity of the structured probe suggests their use in a variety of practical applications like detection of single nucleotide polymorphism.

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