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Short PNA molecular beacons for real-time PCR allelic discrimination of single nucleotide polymorphisms

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Abstract

The typing of a single nucleotide polymorphism with DNA probes is sometimes problematic because of the limited discriminating power of long DNA probes. As an alternative to existing assays, we have developed a real-time PCR assay for the genotyping of single nucleotide polymorphisms using short peptide nucleic acid (PNA) molecular beacons. A single nucleotide polymorphism in exon 6 of the *XPD* gene was chosen as the model system. The genotyping experiments were performed in the ABI 7700 using beacons labeled with either fluorescein or JOE, and in the Lightcycler using a fluorescein labeled beacon. QSY-7 was used as the quencher in all the beacons. The result of the genotyping was the same on both instruments and was in agreement with a previously performed RFLP genotyping of 79 samples. The length of PNA molecular beacons is significantly shorter than that of TaqMan or Lightcycler probes, making probe design and genotype discrimination easier.

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1. Introduction

Single nucleotide polymorphisms (SNPs) are single nucleotide variations that occur in more than 1% of the population. On the average, one out of every 1000 nucleotides in the human genome is expected to be a SNP site [1]. The majority of all SNPs are probably without biological significance. However, a few SNPs are associated with multifactorial diseases, such as cancer or cardiovascular diseases. Others may be associated with non-disease traits, such as drug responses. One of the many methods for SNP typing available today is real-time PCR [1,2]. Monitoring of the PCR reaction and genotyping of the amplicon rely on the addition of specially designed probes that are specific for the amplicon. Examples of probes that can be used in real-time PCR include: Lightcycler probes [3,4], Light-up probes [5], Molecular beacons [6], Scorpion probes [7], Q-PNA [8] and TaqMan probes [9,10].

Molecular beacons are probes carrying a fluorophore at one terminus and a quencher at the other terminus. The middle section of the probe is a sequence complementary to the target. Traditionally, this sequence is flanked on either side by short DNA sequences, which are complementary to each other. Consequently, the molecular beacon will adopt a stem-loop structure in solution, with the two flanking sequences hybridizing together. When the complementary target is present, the molecular beacon opens due to the hybridization of the central DNA sequence to its complement. The resulting large increase in the fluorescent signal can then be easily detected.

A unique property of the peptide nucleic acid (PNA) molecular beacon is that the stem-loop structure is not required for quenching. If a PNA probe is labeled with a fluorophore at one terminus and a quencher at the other terminus it will function as a beacon [11,12]. We have chosen to use PNA oligomers in our study because they show an improved discriminatory power compared to DNA probes. This is important for the Lightcycler assay where

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the use of probes labeled with different fluorophores is not possible. The discriminatory advantage of PNA probes is further enhanced by their increased affinity for DNA. This allows the use of short PNA probes. We have developed a real-time PCR assay using PNA molecular beacons to discriminate between the *A*-allele and *C*-allele of a SNP in exon 6 of the *XPD* gene.

2. Materials and methods

HPLC was performed on a C-18 column using a linear gradient of acetonitrile in water at 50 °C. Both solvents contained 0.1% trifluoroacetic acid (TFA). All chemicals were purchased from Sigma Aldrich, Applied Biosystems or Molecular Probes.

2.1. PNA synthesis

All PNA oligomers were synthesized on an ABI 433A using Boc chemistry [15]. The fluorescein was attached to the PNA by activating a 0.1 mol/l 5,(6)-carboxyfluorescein solution with 0.095 mol/l HATU and 0.1 mol/l diisopropylethylamine. After 1 min the activated 5,(6)-carboxyfluorescein was transferred to the resin (total volume 500 μl) and allowed to react for 30 min. This was repeated once. The resin was washed for 5 min with 20% piperidine in dimethylformamide followed by dimethylformamide (3 ×) and dichloromethane (3 ×) washes. The PNA was cleaved from the resin and purified by reverse phase HPLC.

The fluorescent PNA (20 OD_{260}) was dissolved in 5 μl water and 50 μl *N*-methyl-2-pyrrolidone. Then, 50 μl QSY 7-NHS (10 mg/ml) and 10 μl diisopropylethylamine were added. After 1 h, 2 ml of 0.1% TFA was added followed by injection onto the HPLC for purification. The identity of the PNA was confirmed using MALDI-TOF MS.

2.2. Lightcycler

The forward primer was 5′-aga cca ggg ttt gaa gag tg-3′ and the reverse primer 5′-cca tgg gca tca aat tcc tgg ga-3′. The reaction contained 2 μl Lightcycler DNA Master Hybridization Mix (Roche), 5 nmol $MgCl_2$, 40 pmol forward primer, 10 pmol reverse primer, 4 pmol OK-1670 and 1 μl DNA (approximately 20 ng) in a total volume of 20 μl. The PCR program was 95 °C for 30 s, then 45 cycles of 96 °C for 2 s, 58 °C for 15 s, and 72 °C for 40 s. To read the melting curves, we denatured at 95 °C for 30 s, cooled at 35 °C for 120 s, followed by a temperature ramp from 35 to 75 °C with a slope of 0.1 °C/s.

2.3. ABI7700

TaqMan assay: 10 μl reactions contained 1 × master mix, 100 nM of each TaqMan probe, 600 nM primers (TAGC Copenhagen, Denmark), and ca. 30 ng genomic DNA. Reactions were incubated in an ABI7700 at 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles of 15 s at 94 °C and 60 s at 63 °C.

The primers used were the same as those used with the Lightcycler. TaqMan probes: *A*-allele specific: 5′-VIC-ccc cac tgc cga ttc tat gag gtt-TAMRA-3′. *C*-allele specific: 5′-FAM-ccc cac tgc cgc ttc tat gag gt-TAMRA-3′. The TaqMan probes had a T_m of 65 and 66 °C, respectively. PNA probes: OK-1664: AcEELys(Flu)a tag aag cgg ca Lys(QSY7)E-NH_2; OK-1670: AcEELys(Flu)tca tag aat cgg ca Lys(QSY7)E-NH_2; OK-1675: AcEELys(JOE)tca tag aat cgg ca Lys(QSY7)E-NH_2.

3. Results

The PNA molecular beacons were designed to fulfill the criterion that the primers for the PCR reaction had an annealing temperature of 60 °C. Also, the T_m of the PNA should preferably be at least 5 °C higher than that, i.e. 65 °C, when hybridized to its complement (one of the alleles), and the T_m for the mismatch hybridization of the PNA probe (in this case, to the other allele) should preferably be 5° lower than the annealing temperature of the PCR primers, i.e. 55°. These requirements were to ensure an adequate discrimination between the two alleles and, also, to ensure maximal hybridization of the complementary PCR product to the PNA molecular beacon.

To determine the length of a PNA probe that met the above criteria, we synthesized a series of PNA oligomers of different lengths. PNA oligomers complementary to the *A*-allele and their melting points, when hybridized to a DNA oligomer with a sequence corresponding to either the *A*- or the *C*-allele, are shown in Table 1. The T_m of the matched 13-mer was 63 °C and the mismatched 15-mer had a T_m of 60 °C. Both melting points are too close to the annealing temperature. We conclude that 14 bases is the optimal length for a PNA beacon targeting the *A*-allele of this SNP. A similar approach to the design of a beacon complementary to the *C*-allele resulted in a 12-mer PNA oligomer with optimal T_ms (Data not shown).

We used QSY-7 as the quenching moiety, which we found to be a more efficient quencher than Tetramethylrhodamine or Dabcyl (Data not shown). To avoid potential solubility problems a total of three solubility enhancers (*E*), one at the C-terminus and two at the N-terminus, were added to the molecular beacon sequences [13].

In the Lightcycler assay, we used a fluorescein labeled PNA molecular beacon for the typing of the SNP. Discrimination between the two alleles was done using analysis of the melting curves at the end of the PCR reaction. The fluorescence signal during the PCR reaction of the three possible genotypes: AA, AC and CC are shown in Fig. 1A.

In the heterozygous samples, only half of the PCR product is complementary to the PNA molecular beacon.

Table 1
Melting point of PNA oligomers targeted to the A-allele in exon 6 of the XPD gene

PNA sequence	Length	T_m A-allele[a] (°C)	T_m C-allele[b] (°C)	ΔT_m (°C)
ACC TCA TAG AA*T* CGG CA OOLys(Flu)	17-mer	75	65	10
C TCA TAG AA*T* CGG CA OOLys(Flu)	15-mer	72	60	12
CA TAG AA*T* CGG CA OOLys(Flu)	13-mer	63	49	14
TAG AA*T* CGG CA OOLys(Flu)	11-mer	57	42	15

Buffer: 20 mmol/l Tris–HCl, 50 mmol/l KCl, 1.0 mmol/l $MgCl_2$, pH 8.4 (PCR buffer). ΔT/min = 0.5 °C. Oligo concentration: 1.7 μmol/l. Abbreviations: Flu is used to indicate labelling with 5,6 carboxy-fluorescein, and O is used to indicate that [2-(2-amino ethoxy)ethoxy] acetic acid is used as a linker.

[a] Complementary DNA: 5′-ca ctg ccg att cta tga ggt tac-3′.

[b] Complementary DNA: 5′-ca ctg ccg ctt cta tga ggt tac-3′.

This was the reason for the lower signal intensity of the PNA molecular beacon in the heterozygous samples (Fig. 1A). The homozygous and heterozygous samples gave the expected results, when a single fluorescein labeled beacon was used to type the SNP. The profile of the melting curves for these samples confirmed the genotyping (Fig. 1B).

In the ABI 7700 we used a set of PNA beacons one was labeled with fluorescein (for the *C*-allele) and the other was labeled with JOE (for the *A*-allele). These were compared to two standard TaqMan probes labeled with fluorescein and Vic. Fig. 2 shows the typing of the three possible genotypes and, as expected, the heterozygous sample produced signals with both beacons while the homozygous samples only

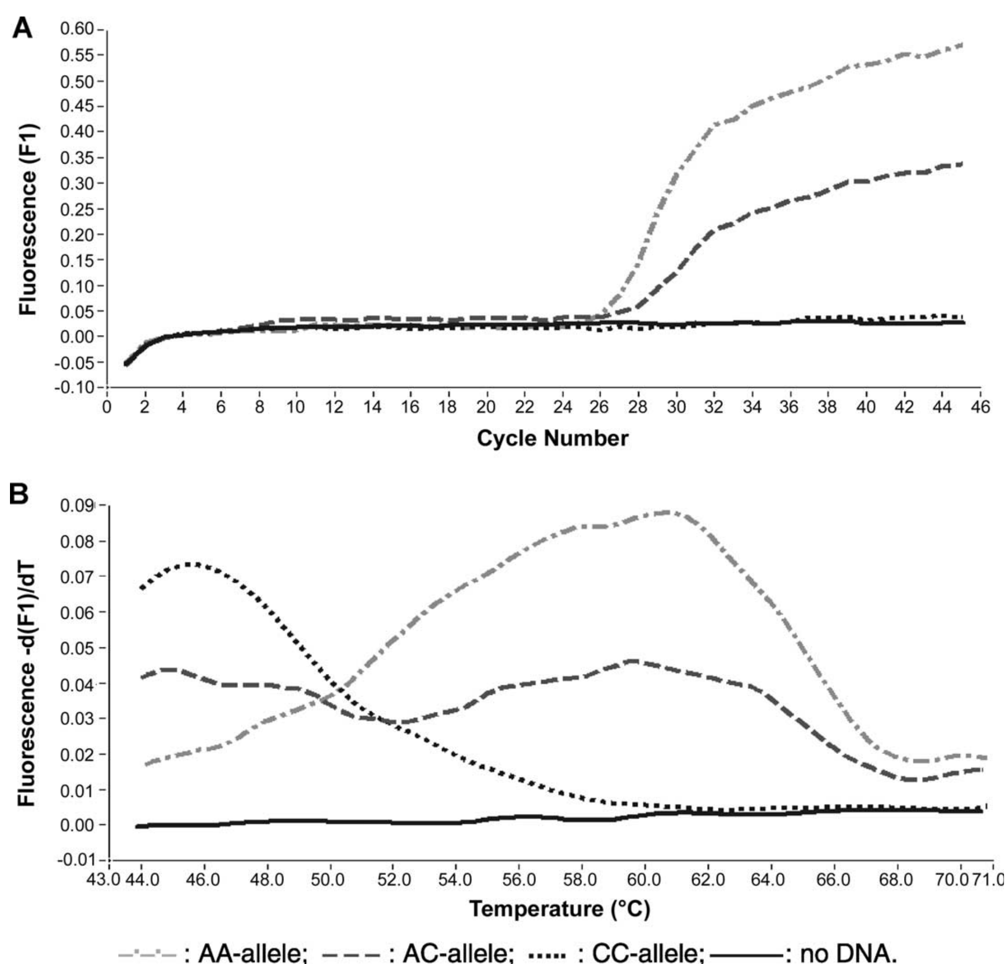

Fig. 1. Lightcycler signal during real-time PCR of XPD exon 6 using the PNA probe OK1670. (A) Fluorescence as a function of the number of PCR cycles. (B) Melting point analysis at the end of the PCR reaction.

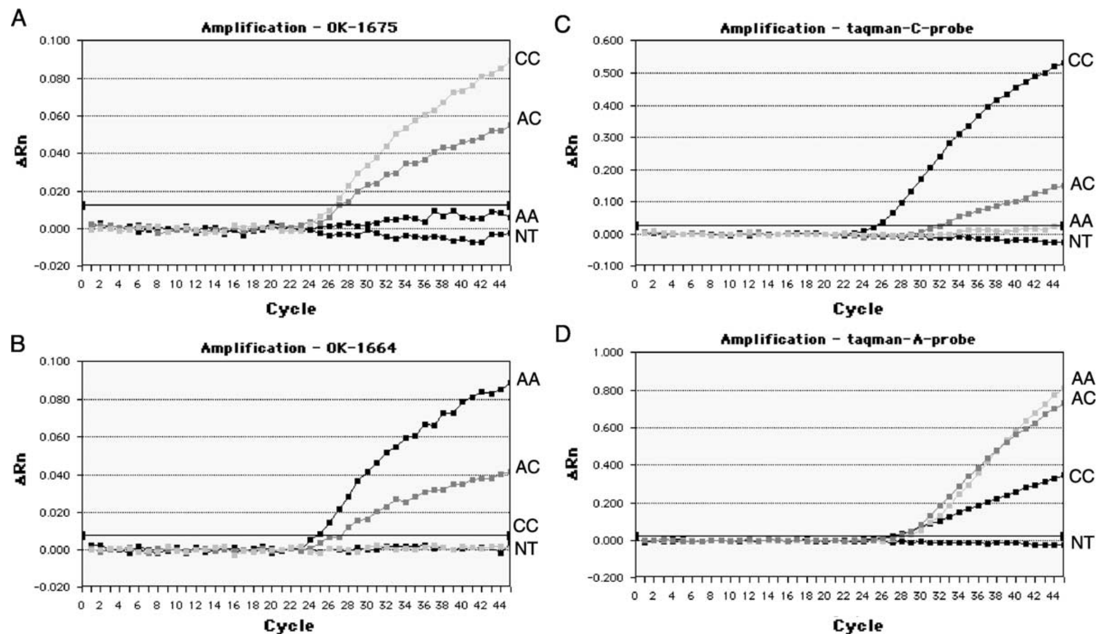

Fig. 2. Real-time PCR of three samples on the ABI 7700 using PNA molecular beacons (A + B) or TaqMan probes (C + D). The genotype of the different samples is indicated at the right of the figures (NT is the no template control).

produced a signal with either the fluorescein or the JOE labeled beacon. The signal intensity of the PNA beacons was up to 10 times lower than that obtained with TaqMan probes.

To evaluate the reliability of the PNA molecular beacon we genotyped 79 human DNA samples, which had previously been genotyped for the SNP in question using restriction fragment length analysis [14]. We achieved complete agreement between the Lightcycler genotyping, the ABI 7700 genotyping and the restriction fragment length analysis.

4. Discussion and conclusions

The short length of the PNA molecular beacons used here is advantageous compared to the longer TaqMan and Lightcycler probes. The latter are usually at least 23 nucleotides in length. The shorter length of the PNA molecular beacons increases the discriminating power of the PNA and, combined with the high specificity of PNAs, results in a very efficient discrimination of SNPs. This is illustrated in our experiments where the TaqMan probe for the *A*-allele produced a very high signal for both the heterozygous *AC* and the homozygous *CC* genotypes (Fig. 2D). The PNA molecular beacons discriminated perfectly between the two samples (Fig. 2B). In situations where the SNP is close to an intron, the decreased length of the PNA probe is an advantage because the decreased probe length makes it easier to avoid the inclusion of intron sequences in the probe. Similarly, the use of short beacons can be advantageous in situations where multiple polymorphisms are closely spaced.

The PNA molecular beacon showed decreased signal intensity compared to the TaqMan probe. The observed difference between the TaqMan probes and the PNA molecular beacons in signal intensity is a reflection of how the two probes work. The TaqMan assay involves the degradation of the TaqMan probe by the DNA polymerase during the elongation step. This separates the fluorophore from the quencher, and fluorescence accumulates from cycle to cycle. The PNA probe is not degraded by the polymerase and, therefore, the signal from the PNA molecular beacon is equal to the amount of PCR product present. During real-time PCR the fluorescence is measured at the end of the elongation step at which point the PNA is hybridized to its complementary target and the TaqMan probe has been degraded by the polymerase. The signal from the part of the TaqMan probe that is degraded in the current cycle should be equal to the signal from the PNA probe, but, in the TaqMan assay, the fluorescent signal from all the previous cycles has accumulated giving a signal intensity almost twice that of the PNA molecular beacon. Another factor contributing to the decreased signal intensity of the PNA molecular beacon is the distance between the fluorophore and the quencher. While the fluorophore and the quencher becomes totally dissociated during the TaqMan procedure, the fluorophore and the quencher in PNA molecular beacons will be separated at a fixed distance when the probe is hybridized to the PCR

product. At this distance, partial quenching of the light from the fluorophore will still occur, resulting in lower intensity of the signal.

Some of the difference in signal intensity may also arise from the timing of the fluorescence measurement. In a TaqMan experiment, the fluorescence is measured at the end of the elongation step when the degradation of the TaqMan probe is complete. This may not be the best time to measure the signal from the PNA molecular beacons, since the beacons can be displaced by the DNA polymerase during the elongation step. We have tried measuring the fluorescent signal at the start of the elongation step and, as expected, a stronger signal was obtained. The stronger signal is due to the fact that more of the PNA beacon is bound at the beginning of the elongation step than at the end of the elongation step. There are two possible explanations for this difference. One explanation is that the DNA polymerase removes the PNA as it is moving along the DNA [16]. Another explanation is that the PNA is removed by the hybridization of the complementary DNA strand. This led us to investigate how stable the PNA/DNA duplex was in the PCR reaction after 40 cycles. The temperature was kept constant at 60 °C. This is the temperature used during the annealing/elongation step. During the first 10 min no significant change in signal intensity was observed. After 10 min the signal intensity started to decrease. This leads us to suggest that another contributing factor to the difference in signal intensity between the TaqMan probes and the PNA molecular beacons is that the polymerase can displace the PNA molecular beacon as it elongates the DNA strand. Overall, we find that the signal to threshold ratio is lower for the PNA molecular beacons we have used, compared to the TaqMan probes. The lower signal intensity of the PNA molecular beacons is, however, outweighed by the advantages of their increased specificity and shorter length.

The traditional Lightcycler probes are like the PNA beacons in that neither type of probe requires degradation. The quantity of probe hybridized to its target determines the signal intensity. Since the fluorescence signal from both the Lightcycler probes and the PNA molecular beacons are based on their hybridization to the target, their signal intensities were expected to be of the same magnitude. In our experiments we did not see any significant difference in the signal intensities and the signal to noise ratios when comparing the two types of probe.

Quencher labeled PNA probes have previously been used in real-time PCR assays [8]. However, this system can only be used for SNP typing in PCR reactions where the primer can discriminate between the two SNPs. The present use of the PNA molecular beacon is not dependent on the ability of a PCR primer to discriminate between the targets.

As for the TaqMan probes, the PNA beacons show a linear increase in signal for the first 4–5 cycles once the signal intensity has risen above the threshold. This should make quantitative analysis of the results possible.

We have designed two PNA molecular beacons directed to an A/T polymorphism in exon 6 of the *RAI* gene based on the design of the PNA molecular beacons we describe here. The two new PNA molecular beacons have a total of six G + C bases out of 14 bases. We have successfully developed an assay that works in both the Lightcycler and the ABI 7700 with these two PNA molecular beacons.

We believe that the PNA molecular beacons described here are a useful alternative to other real-time PCR probes, especially for the design of probes that target problematic sequences.

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