

A closed tube format for amplification and detection of DNA based on energy transfer

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ABSTRACT

A new method for the direct detection of PCR-amplified DNA in a closed system is described. The method is based on the incorporation of energy transfer-labeled primers into the amplification product. The PCR primers contain hairpin structures on their 5' ends with donor and acceptor moieties located in close proximity on the hairpin stem. The primers are designed in such a way that a fluorescent signal is generated only when the primers are incorporated into an amplification product. A signal to background ratio of 35:1 was obtained using the hairpin primers labeled with fluorescein as a donor and 4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL) as a quencher. The modified hairpin-primers do not interfere with the activity of DNA polymerase, and both thermostable *Pfu* and *Taq* polymerase can be used. This method was applied to the detection of cDNA for prostate specific antigen. The results demonstrate that the fluorescent intensity of the amplified product correlates with the amount of incorporated primers, and as few as 10 molecules of the initial template can be detected. This technology eliminates the risk of carry-over contamination, simplifies the amplification assay and opens up new possibilities for the real-time quantification of the amplified DNA over an extremely wide dynamic range.

INTRODUCTION

Polymerase chain reaction (PCR) and other nucleic acid amplification techniques provide a tool for the geometric amplification of minute amounts of initial target sequences (reviewed in 1,2). The extreme sensitivity of DNA/RNA amplification methods has encouraged the development of diagnostics for the early detection of cancer and infectious agents. However, drawbacks to the clinical use of nucleic acid amplification include the possibility of false-positive results due to carry-over contamination, and false-negative results caused by unsuccessful reactions and/or non-standardized reaction conditions (3). Amplification products from previous amplification reactions are a major source of carry-over contamination. Due to the extreme sensitivity of PCR, even minimal contamination can generate false-positive results, and accordingly, several approaches have been devised to deal

with this problem. These include incorporation of dUTP with subsequent treatment with uracil N-glycosylase (4), incorporation of ribonucleotides into the PCR primers followed by base treatment (5) or the use of isopsoralen derivatives which undergo a cycloaddition reaction with thymidine residues upon exposure to UV light (6). However, a simpler and more certain solution to the problem would be a closed system, where both the amplification reaction and the detection step take place in the same vessel, so that the reaction tube is never opened after amplification. In addition, the 'closed tube' format significantly simplifies the detection process, eliminating the need for post-amplification analysis by such methods as gel-electrophoresis or dot blot analysis.

Most reported attempts to create amplification systems in which detection of the amplification product does not require separation from non-reacted primers are based on the phenomenon of fluorescence resonance energy transfer (FRET). FRET is a process in which the energy from an excited fluorophore is transferred to an acceptor moiety at distances up to 70–100 Å (7,8). As a result, the emission of the fluorophore is quenched. Some methods require a post-PCR probing step (9–11), others could be performed in a 'closed-tube' format. One method that uses FRET to monitor the amplification process in a closed system is the 5'-nuclease PCR assay marketed by Perkin Elmer, also known as the TaqMan™ assay (12,13). This method uses a hybridization probe labeled with a fluorescent reporter and a quencher. During PCR, this probe is cleaved by the 5'-exonuclease activity of DNA polymerase if, and only if it hybridizes to the DNA segment being amplified. Cleavage of the probe between the donor and the quencher generates an increase in the fluorescence intensity of the donor. In the TaqMan™ assay, the donor and quencher are preferably located on the 3' and 5' ends of the probe, because the requirement that 5'–3' hydrolysis be performed between the fluorophore and quencher can be met only when these two moieties are not too close to each other (14). However, this requirement is a serious drawback of the assay since the efficiency of energy transfer decreases with the inverse sixth power of the distance between the reporter and quencher (8). As a consequence, the background emission from unhybridized probe can be quite high. Another method of detection of amplification products which relies on the use of energy transfer is the 'molecular beacon probe' method described by Tyagi and Kramer (15). This method employs oligonucleotide hybridization probes which can form hairpin structures. A donor fluorophore and a quencher are located on the 5' and 3' ends of the

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Table 1. Fluorescence intensity of the DNA amplified with different hairpin-primers labeled with fluorescein (f) and DABCYL (d)

	Hairpin-primer	3'-single-stranded sequence (nucl)	Stem (bp)	Loop (nucl)	Fluorescence rel. (%)
A	<pre> T C d C A G A A G G T G A C C A A G T T C A T 3' C T C T T C C A C A f </pre>	12	7	6	100
B	<pre> C C T d C C A G A A G G T G A C C A A G T T C A T 3' A G T C T T C C A C T T f </pre>	12	8	8	58
C	<pre> C T C C C A d C G A A G G T G A C C A A G T T C A T 3' A C T T C C A C G f T T A </pre>	12	6	12	67
D	<pre> C T d C C A G A A G G T G A C C A A G T T C A T 3' C G T C T T C C A A T f </pre>	12	8	6	83
E	<pre> C T C d C A G A A G G T G A C C A A G T T C A T 3' C T C T T C C A A T A f </pre>	12	7	8	104
F	<pre> T C A d C G A A G G T G A C C A A G T T C A T 3' C C T T C C A C C A f </pre>	11	7	7	108
G	<pre> T C d C A G A A G G T G A C C A A A 3' C T C T T C C A C A f </pre>	6	7	6	5

Sequences complementary to the target DNA are shown in bold.

hybridization probe. Thus, when the molecular beacon probe is in the hairpin conformation, the fluorescence of the donor fluorophore is quenched. When employed in PCR, the molecular beacon probe, which hybridizes to one of the strands of the PCR product, is in 'open conformation', and fluorescence is detected (15). However, similar to the TaqMan assay, this method does not detect the amplified DNA directly, and therefore, the generated signal will be affected by the efficiency of the probe hybridization.

The method described in this paper is designed for the direct measurement of the amplified DNA by incorporation of labeled primers into the reaction product. It is based on the method of Tyagi and Kramer which utilizes the conformational transition of the oligonucleotide as a switch of energy transfer between two labels. In our method, the donor and acceptor (quencher) moieties are both attached to a hairpin structure on the 5' end of the amplification primer. Oligonucleotide primers are designed in such a way that the fluorescent signal is generated only when the labeled oligonucleotides are incorporated into the double-stranded amplification product. This highly sensitive method may be used to obtain quantitative or qualitative results. Applications for this system to the detection of a specific DNA sequence are shown here.

MATERIALS AND METHODS

Oligonucleotide primers

The following oligodeoxynucleotides complementary to the 172 bp segment of human prostate specific antigen (PSA) cDNA were chemically synthesized: d(CCCTCAGAAGGTGACCAAGTTCAT), as an upstream primer, and d(GGTGTACAGGGAAGGCCTTTTCGGGAC), as a downstream primer. The structures of the upstream hairpin-primers with energy transfer labels are shown in Table 1. 6-Fluorescein was incorporated into 5' end of hairpin-primers by using 6-fluorescein phosphoramidite in the last step of the chemical synthesis. A modified T-base was introduced into a designated position by the use of AminoModifier C6 dT (Glen Research), and the 4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL) was attached to the primary amino group as described by J. Ju *et al.* (16). Labeled oligonucleotides were purified by HPLC.

Preparation of PSA cDNA

The human PSA-expressing LNCaP cell line (American Type Culture Collection) was used in the experiments. LNCaP cells were

diluted with lymphocytes isolated from whole blood at ratios ranging from 1 LNCaP cell to 10^2 lymphocytes to 1 LNCaP cell to 10^6 lymphocytes. Messenger RNA was isolated using the Dynal purification kit. cDNA was synthesized from the isolated mRNA using reverse transcriptase (Appligene) and oligodT₁₂₋₁₈ primers (Pharmacia) according to the recommended protocol.

PCR conditions

Amplification of the PSA cDNA was performed in 100 μ l of 20 mM Tris-HCl (pH 8.5), 50 mM KCl, 2 mM MgCl₂, 200 μ M each dNTP, 500 nM upstream and downstream primers (each), and 5 U *Pfu*^{exo-} DNA polymerase (polymerase without 3'-5' exonuclease activity; Stratagene). Thermal cycling was performed with 5 min denaturation at 94°C, followed by 20-40 cycles: 30 s at 95°C, 45 s at 60°C and 1.5 min at 72°C, and completed with a final 5 min extension at 72°C.

The PCR product was purified using QIAquick Spin PCR Purification Kit (Qiagen) and cloned into pUC19 plasmid.

Fluorescence detection

A Shimadzu RF-5000 spectrofluorophotometer was used to measure the fluorescence spectra. The 100 μ l reaction mixture was diluted to 500 μ l with 20 mM Tris-HCl, pH 8.5, 50 mM NaCl, 2 mM MgCl₂ and placed into the 10 \times 3 cuvette (NSG Precision Cells, Inc.) at room temperature. For the fluorescein / DABCYL energy transfer pair, a 488 nm excitation wavelength was used and a spectrum was taken between 500 and 650 nm.

RESULTS

Experimental design of PCR with hairpin-primers

In this method, a hairpin structure is present on the 5' end of one (or both) of the PCR primers (Fig. 1). The sequence of the hairpin stem and loop may be partially complementary to the target DNA sequence, but it is not necessary. There are two moieties attached to the stem sequence of the hairpin: a fluorophore on the 5' end of the hairpin and a quencher on the opposite side of the hairpin stem. The positions of a fluorophore and a quencher could be switched, depending on the availability of the commercial precursors of these moieties and their effect on the PCR reaction. The fluorescein/DABCYL pair was suggested by Tyagi and Kramer (personal communication). DABCYL is a non-fluorescent chromophore whose absorption spectrum overlaps the emission spectrum of fluorescein. When stimulated by light of peak wavelength 488 nm, fluorescein emits fluorescence of peak wavelength 516 nm. However, when DABCYL is located sufficiently close to the donor fluorophore, the energy can be transferred to DABCYL and dissipated as heat. Therefore, when the modified primer is in a 'closed' configuration (hairpin), the fluorescein and DABCYL are in close proximity, and the emission of the fluorescein is quenched by DABCYL.

During the first cycle of PCR (Fig. 1) the primers are extended and become a template during the second cycle. Since the hairpin structures are very stable (17), we do not expect the stem to be melted during the annealing step of the PCR on every target molecule. In this case, when the DNA polymerase lacking 5'-3' exonuclease activity reaches the 5' end of the hairpin stem, it will displace it and copy the sequence. Thus, the hairpin-primer will be linearized by incorporation into the double-stranded helical

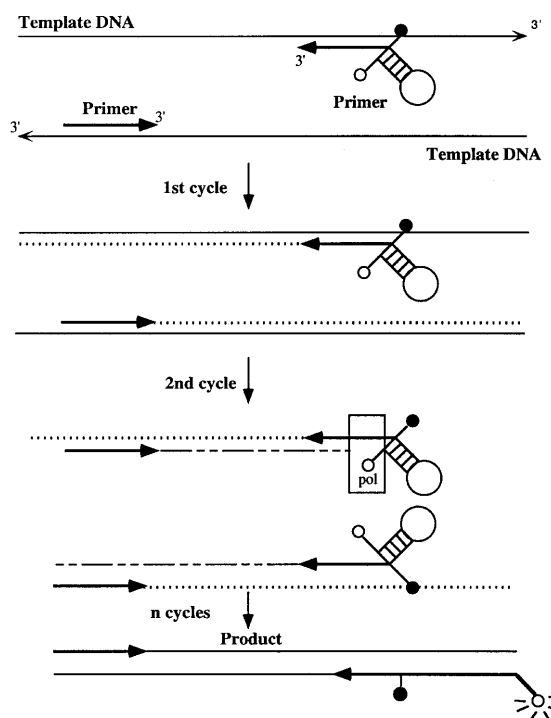


Figure 1. Generation of the energy transfer signal upon the incorporation of the hairpin-primers into the double-stranded PCR products. (○) donor fluorophore; (●) quencher.

structure during PCR, the donor and acceptor will be ~20 nucleotides (nt) (~70 Å) apart, resulting in no significant energy transfer between them (8), and the fluorescence from the fluorescein will be markedly enhanced.

Sequence and spectroscopic properties of the hairpin-primer

The hairpin-primer for the amplification of cDNA for prostate specific antigen (PSA) consists of a 12 nt long single-stranded priming sequence, a 7 bp stem, and a 6 nt loop (Table 1A). The fluorescent moiety (fluorescein) is located on the 5' end of the primer and a quencher (DABCYL) is across from fluorescein on the opposite strand of the stem sequence. With no quencher present, fluorescein excited at 488 nm wavelength emits a peak at 516 wavelength. When the same oligonucleotide is also labeled with DABCYL, the fluorescence energy is transferred to the quencher and <3% peak is detected at 516 nm. The residual fluorescence of the Flu/DABCYL-labeled oligonucleotide is caused by non-quantitative quenching as well as by the presence of small quantities of oligonucleotides labeled with fluorescein alone.

Similar results were obtained with rhodamine as a quencher (data not shown). However, as a quencher DABCYL has an advantage of being a non-fluorescent chromophore: it absorbs the energy of the fluorescein without emitting light itself. As a result, the emission of the fluorescein may be detected more precisely, without interference from the emission of the acceptor.

The utility of hairpin-oligonucleotides as PCR primers

PCR of PSA cDNA was performed using total cDNA or cloned PSA cDNA as described in Materials and Methods. The efficiency

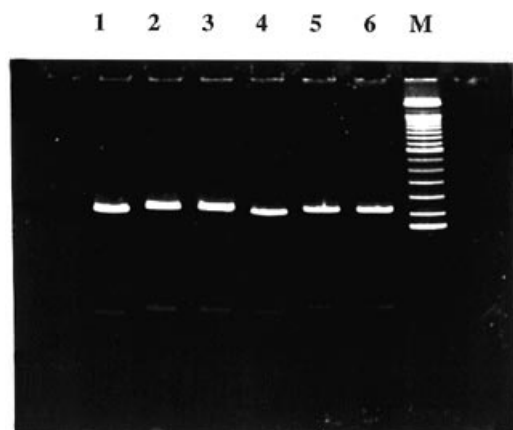
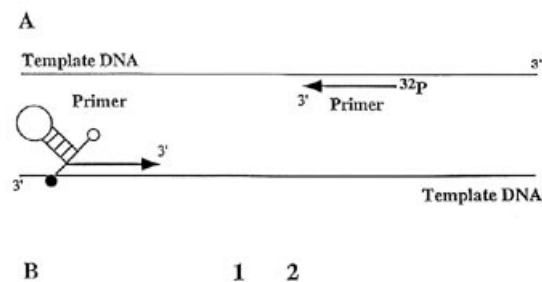


Figure 2. Efficiency of amplification with the hairpin-primers. Products of amplification of 10^{-9} M PSA cDNA with: control primer (1), Flu-hairpin-primer (2), Flu/DABCYL-hairpin-primer (3); 4–6 same as 1–3 but with 10^{-11} DNA template; M, 100 bp marker (Gibco-BRL) on the MDE™ gels (FMC BioProducts).

of amplification in the presence of the hairpin-primer was compared with that for the control primer, which lacks the hairpin structure and modifications. Figure 2 shows that the amount of amplified product was similar for the control primer, the hairpin-primer containing fluorescein alone and the hairpin-primer labeled with the fluorescein/DABCYL FRET pair.

A crucial requirement for the method is the linearization of the hairpin-primer during amplification. Therefore DNA polymerase must be able to synthesize the strand complementary to the hairpin-primer all the way through the hairpin to its 5' end. The following experiment was conducted to determine if modifications and/or the structure of the hairpin-primer affect the synthesis of the full-length PCR product. PCR amplification of PSA cDNA was performed with two primers: an upstream hairpin-primer labeled with DABCYL and fluorescein and a downstream primer labeled with ^{32}P on its 5' end (Fig. 3A). An upstream primer without the hairpin structure was used as a control. If the structure and/or the modifications of the hairpin-primer create an obstacle for DNA polymerase, this primer will not be copied all the way to its 5' end, and the ^{32}P -labeled strand will be shorter than the corresponding strand synthesized in the presence of the control primer. To estimate the length of the individual strands, denaturing gel electrophoresis was performed. As illustrated by the results in Figure 3B, the ^{32}P -labeled strand that was synthesized in the presence of hairpin-primer is longer than the corresponding strand made with the control primer, indicating that DNA polymerase was able to read through the hairpin structure and synthesize a full-length product.

Another important aspect of this method is the thermostability of the hairpin-primer. If oligonucleotide phosphodiester bonds or the linker arms through which donor and/or acceptor are tethered to the oligonucleotide are cleaved as a result of high temperature, the quencher will be separated from the fluorophore and the background will increase. Indeed, when 50 pmol of the hairpin-primer was incubated in 100 μl reaction for 40 cycles, the background signal increased from 3.8 to 8 U of fluorescence intensity. However, the observed background is still very low: it comprises only 4% of the fluorescence emitted by 50 pmol of fluorescein-labeled oligonucleotides (200 U), which is the amount used in the assays.



B 1 2



Figure 3. Size of the PCR product synthesized in the presence of hairpin-primer. PCR amplification of PSA cDNA was performed with two primers: an upstream hairpin-primer labeled with DABCYL and fluorescein and a downstream primer labeled with ^{32}P on its 5' end. An upstream primer without the hairpin structure was used as a control. The structure of the hairpin-primer is presented in Table 1A and the sequences of the regular primers are presented in Materials and Methods. (A) Scheme of the experiment. (B) ^{32}P -Labeled strands of the PCR products synthesized in the presence of the control primer (1) or hairpin-primer (2) analyzed on a 6% denaturing polyacrylamide gel.

Monitoring of PCR with hairpin-primers

To demonstrate that the fluorescence of the PCR product can be used to monitor the reaction, total cDNA from the $1:10^4$ mixture of human PSA-expressing LNCaP cells and lymphocytes was amplified with the hairpin-primers labeled with DABCYL/fluorescein. The fluorescence intensity of the amplified product was determined after different numbers of cycles using a spectrofluorophotometer (Fig. 4). The results show that after only 20 cycles, the fluorescence intensity increases five times compared to the non-amplified reaction mixture, and a 35-fold increase is detected after 40 cycles of amplification. The same samples were also analyzed by denaturing gel electrophoresis with subsequent quantification on the PhosphorImager to determine the fraction of ^{32}P -labeled primers incorporated into the product. The results demonstrate that the fluorescence intensity of the reaction mixture correlates with the amount of primers incorporated into the product.

In another experiment, the sensitivity of this method was explored. For quantification purposes cloned PSA cDNA was used as a template. Forty cycles of PCR were performed with 0, 10, 10^2 , 10^3 , 10^4 , 10^5 , 10^6 molecules of DNA target per reaction. The results in Figure 5 demonstrate that the method is sensitive enough to detect 10 molecules of the initial DNA template with a spectrofluorophotometer. We have also visualized the fluorescent

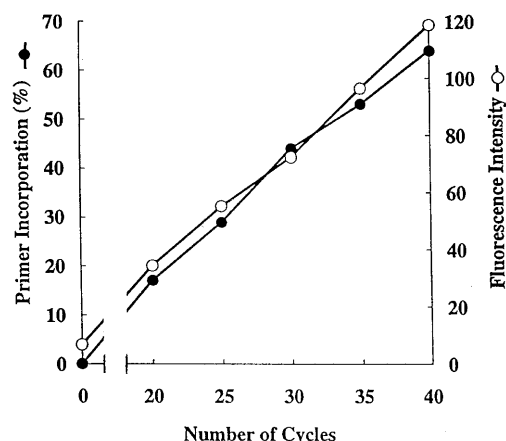


Figure 4. The fluorescence intensity of the amplified PSA cDNA after 0 (1), 20 (2), 25 (3), 30 (4), 35 (5) or 40 (6) cycles. The structure of the hairpin-primer labeled with fluorescein and DABCYL is presented in Table 1A and the sequence of the regular downstream primer is presented in Materials and Methods. Fluorescence intensity of the amplification reaction mixtures and the fraction of the ^{32}P -labeled primers incorporated into the PCR products were plotted against the number of cycles. The incorporation of the ^{32}P -labeled primers into the PCR products was determined by electrophoresis on 6% denaturing gel and quantitated using the PhosphorImager-SP (Molecular Dynamics).

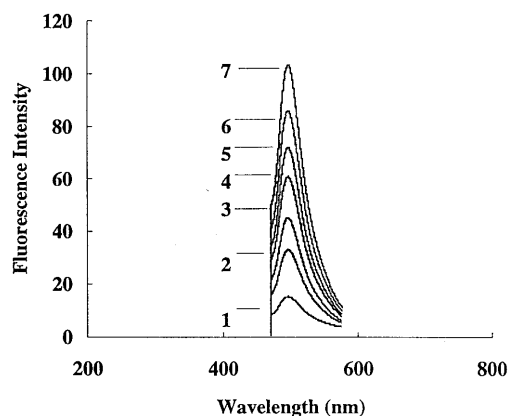


Figure 5. The sensitivity of PCR with hairpin-primers. 0 (1), 10^2 (2), 10^3 (3), 10^4 (4), 10^5 (5) or 10^6 (7) molecules of cloned PSA cDNA per reaction were used as a template DNA for the 40 cycles of PCR. The structure of the hairpin-primer labeled with fluorescein and DABCYL is presented in Table 1A and the sequence of the regular downstream primer is presented in Materials and Methods. The emission spectra were measured as described in Materials and Methods.

PCR product by placing the tube directly on a UV transilluminator (Fig. 6). The fluorescence of the PCR reaction performed with 10^4 , 10^5 and 10^6 molecules of the initial template could easily be detected by visual inspection of the tubes.

Effect of the structure of labeled hairpin-primer on amplification and detection

Several hairpin-primers with variant sizes of stem, loop and 3' single-stranded sequence were synthesized to estimate how these parameters may affect the efficiency of PCR and signal to background ratio. The structures and the relative fluorescence

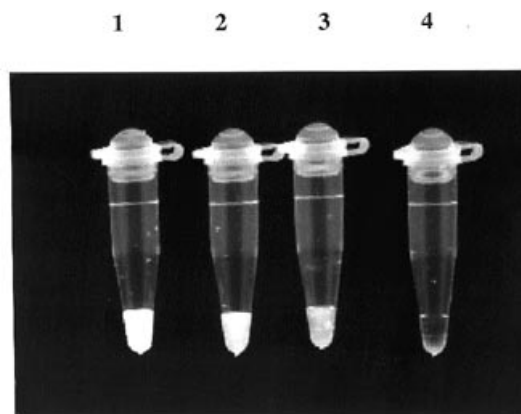


Figure 6. Visible fluorescence of PCR products synthesized with hairpin-primers. 10^6 (1), 10^4 (2), 10^3 (3) and 0 (4) molecules of the cloned PSA cDNA were used as a template DNA for the 40 cycles of PCR with the hairpin-primers labeled with fluorescein and DABCYL. DNA fluorescence was visualized in 0.2 ml thin-walled PCR tubes by placing them directly against a UV transilluminator image analysis system (Appligene), and photographed with a mounted camera using a D540/40 filter (Chroma Technology).

intensities are presented in Table 1. All primers tested had at least an 18 nt sequence complementary to the target, which comprised a 3' single-stranded priming sequence, a 3' stem sequence and part of the loop (highlighted in bold in Table 1). The length of the 3' single-stranded priming sequence was found to be important for the efficiency of the hairpin-primers in the PCR reaction. Almost no product was detected when its length was decreased from 12 nt in structure A to 6 nt in structure G. A possible explanation for this result is that the hairpin structure is the preferred conformation of this oligonucleotide even at the 60°C annealing temperature, and the nucleotides in the stem and loop of the hairpin are not available for hybridization to the target DNA. In this case, the only part of the molecule not involved in the secondary structure is the 3' single-stranded sequence; however, the 6 nt sequence on the 3' end of structure G is not long enough to be an efficient PCR primer.

Only minor variations in the amount of product generated were found when the sizes of stem and loop were changed slightly. The PCR was slightly less efficient when the length of the stem is >7 bp. Stabilization of the stem by replacement of an AT-base pair at the 3' end with GC increased the signal to background ratio by 10%.

DISCUSSION

The method for detection of amplification products in a 'closed tube' format is an important step towards a PCR-based automated diagnostic system, since it not only reduces the complexity of the reaction, but also eliminates the chances of carry-over contamination and, consequently minimizes the chances of false-positive results. The amplification primer contains a hairpin structure with two labels on its stem that can undergo fluorescence resonance energy transfer. One label is a fluorophore donor and another is a quencher that can absorb energy emitted by the fluorophore. A 35-fold quenching of the fluorescence was observed when the oligonucleotide primers were in the hairpin conformation, so that <3% of maximum fluorescence is detected when the primers are not incorporated into the product. The switch from the hairpin to linearized conformation occurs as a result of replication: the 5'

end of the stem is displaced by DNA polymerase, a complementary strand is synthesized and the hairpin can no longer be formed. In the incorporated primers the distance between the fluorophore and the quencher is ~20 bp, which is close to 70 Å, the distance at which energy transfer is negligible (8) and so the quantitative emission of the fluorophore can be detected.

The main advantage of this method is the generation of the fluorescent signal by the product itself, rather than by the hybridized probe, as in previous methods (12,13,15). This keeps background low and allows the real-time quantification of the amplified DNA over an extremely wide dynamic range. In addition, the detection does not require special buffer or temperature conditions that are necessary for methods involving hybridization. The discrimination between a long double-stranded DNA product and the short hairpin-primer is so efficient, that the signal to background ratio will be the same over a wide temperature range under a variety of reaction conditions.

This method can be applied to any amplification system in which a single-stranded oligonucleotide is incorporated into the double-stranded product, and is compatible with any thermostable DNA polymerase. Here we used *Pfu*^{exo-} DNA polymerase, an enzyme without 5'–3' and 3'–5' exonuclease activity. However, similar results were obtained with *Taq* polymerase which has 5'–3' exonuclease activity (data not shown). 5'–3' exonuclease activity is a part of the excision–repair function of some DNA polymerases, and it will not attack a free primer. However, if the extended hairpin-primer still maintains its hairpin conformation when annealed to the template DNA, then DNA polymerase will hydrolyse the 5' end of the hairpin stem and the 5' nucleotide with tethered donor or acceptor will be released into the solution. In either case, replication or hydrolysis, the donor fluorophore will be separated from the acceptor, quenching will be eliminated and the fluorescence signal from the amplification product will be detected, allowing any thermostable DNA polymerase to be used for the proposed amplification/detection method.

The 35-fold signal to background ratio presented in this paper can probably be increased even further. Published data suggest that when the fluorophore and the quencher are covalently linked to each other, 200-fold quenching may be achieved (18). This implies that locating energy transfer labels in closer proximity on the stem structure will increase the efficiency of quenching. This goal may be achieved by several approaches, such as variation of the linker arms, changing the positions of the labels, or using FRET pairs where the donor and acceptor have some affinity to each other. Another way to improve the system is to increase the thermostability of the FRET labeled oligonucleotides to prevent an increase in the background during amplification due to the spontaneous release of the labels into the solution.

The method described in this paper can be applied to any diagnostic procedure where the presence of the target nucleic acid is to be detected either qualitatively or quantitatively. It may be

applied to the detection of infectious disease agents, microorganism contamination of food or water as well as to the detection of some forms of cancer. An important step in the development of any application of this method is optimization of the structure of the primers and cycling conditions, since any side product can give a signal. However, optimization is facilitated by the fact that the size and purity of the product can be confirmed by gel electrophoresis, since the DNA amplified with the labeled hairpin-primers can be analyzed by any of the traditional methods. Here we demonstrate the application of this method for the detection of cDNA of prostate specific antigen. The results show that the specificity and the sensitivity of detection are comparable to that of other amplification based methods. This method can also be used for multiplex analysis, where several targets are to be amplified in the same reaction by using hairpin-primers labeled with different fluorophores. For clinical applications, when a large number of samples are to be tested, a fluorescence plate reader could be used separately or coupled with the PCR machine.

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