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Detection of specific polymerase chain reaction product by utilizing the 5′ → 3′ exonuclease activity of *Thermus aquaticus* DNA polymerase

(oligonucleotide probe/human immunodeficiency virus)

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ABSTRACT The 5′ → 3′ exonuclease activity of the thermostable enzyme *Thermus aquaticus* DNA polymerase may be employed in a polymerase chain reaction product detection system to generate a specific detectable signal concomitantly with amplification. An oligonucleotide probe, nonextendable at the 3′ end, labeled at the 5′ end, and designed to hybridize within the target sequence, is introduced into the polymerase chain reaction assay. Annealing of probe to one of the polymerase chain reaction product strands during the course of amplification generates a substrate suitable for exonuclease activity. During amplification, the 5′ → 3′ exonuclease activity of *T. aquaticus* DNA polymerase degrades the probe into smaller fragments that can be differentiated from undegraded probe. The assay is sensitive and specific and is a significant improvement over more cumbersome detection methods.

The polymerase chain reaction (PCR) method of DNA amplification is a powerful and sensitive technique, with broad applications in fields such as molecular biology, diagnostics, and forensic analysis. Since it was initially described (1, 2), the PCR technique has undergone significant improvements in methodology. The substitution of a heat-stable protein for the originally described PCR enzyme, the Klenow fragment of *Escherichia coli* DNA polymerase I, has eliminated the need to replenish enzyme after each amplification cycle (3). The use of *Thermus aquaticus* (*Taq*) DNA polymerase has improved yield and specificity of product and facilitated automation. The product detection process, however, still remains a labor-intensive procedure to obtain high levels of specificity, sensitivity, and reproducibility. Assays involving various probing and/or blotting techniques require additional handling of sample, leading to a greater risk of subsequent carryover contamination. By utilizing the inherent 5′ → 3′ exonuclease activity of *Taq* DNA polymerase (4), we have devised a method whereby simultaneous target amplification and generation of target-specific signal are achieved. An assay system in which product detection occurs concurrently with target amplification, and requires little or no handling of the sample postamplification, would be ideal.

Similar to the 5′ → 3′ exonuclease activity of *E. coli* DNA polymerase I (5, 6), the 5′ → 3′ exonuclease activity of *Taq* DNA polymerase cleaves 5′ terminal nucleotides of double-stranded DNA and releases mono- and oligonucleotides. The preferred substrate for cleavage is displaced single-stranded DNA, a fork-like structure, with hydrolysis occurring at the phosphodiester bond joining the displaced region with the base-paired portion of the strand (unpublished data). We have created a substrate suitable for exonuclease activity in a PCR by the introduction of a labeled oligonucleotide probe designed to hybridize within the target sequence. During amplification, the 5′ → 3′ exonuclease activity of *Taq* DNA polymerase degrades the probe in a nick-translation reaction and releases the label on smaller fragments that are differentiated subsequently from undegraded probe (Fig. 1). We describe the method of PCR product detection utilizing the 5′ → 3′ exonuclease activity of *Taq* DNA polymerase and discuss the advantages of this system over current detection procedures.

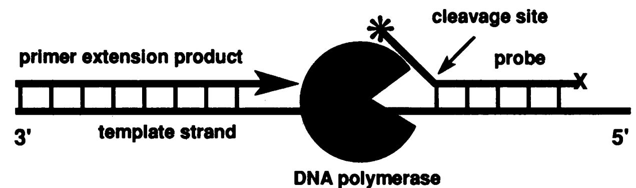

FIG. 1. Diagram of 5′ → 3′ exonuclease cleavage of 5′-labeled oligonucleotide probe to generate labeled fragments in a PCR. *, The 5′ label on the probe; X, a 3′-phosphate block on the probe.

MATERIALS AND METHODS

Enzymes. T4 polynucleotide kinase was purchased from New England Biolabs. AmpliTaq DNA polymerase was obtained from Perkin–Elmer/Cetus.

Nucleotides, Oligonucleotides, and DNA. 2′-Deoxynucleoside 5′-triphosphates (dNTPs) were purchased from Pharmacia. Oligonucleotide primers and probes were synthesized on a Biosearch 8700 DNA synthesizer. Oligonucleotide probes were synthesized with a 3′-phosphate to prevent extension (7). [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. Single-stranded M13 DNA template (M13mp10) was prepared as described (8). Human immunodeficiency virus type 1 (HIV-1) DNA template (GeneAmplimer HIV-1 positive control DNA, Perkin–Elmer/Cetus) contained the HIVZ6 genome, which was rearranged to interrupt the *pol* gene region and block infectivity, and cloned into the plasmid pBR322 (9). Bacteriophage λ DNA template (GeneAmp positive control DNA) was obtained from Perkin–Elmer/Cetus. Human genomic DNA was prepared from the human cell line HL60 as described (10) and, where indicated, was degraded by hydrodynamic shearing in a French press (Aminco) to ≈800 base pairs (bp) in size.

Labeling Reaction. Oligonucleotide probes were 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (10) to a specific activity of 2–5 × 10^6 cpm/pmol.

PCRs. Amplifications consisted of 50-μl reaction mixtures containing 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 10 pmol of each oligonucleotide primer, 200 μM each of the four dNTPs,

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Abbreviations: *Taq*, *Thermus aquaticus*; HIV, human immunodeficiency virus.

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and 1.25 units of AmpliTaq DNA polymerase [diluted from 5 units/μl to 0.3125 unit/μl in 10 mM Tris·HCl (pH 8.0), 50 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 0.5% Tween 20, and 500 μg of gelatin per ml]. Reaction mixtures included either 2.5 mM $MgCl_2$, when HIV-1 target was used, or 3 mM $MgCl_2$, when M13mp10 or λ DNA target was used. The amount of HIV-1 DNA added was as indicated. M13mp10 single-stranded DNA was added at 1×10^{-3} pmol (6×10^8 copies) per reaction. λ DNA was at 1.6×10^{-5} pmol (9.6×10^6 copies) per reaction. Oligonucleotide probe concentrations were as indicated. Reaction mixtures were overlaid with 50 μl of mineral oil to prevent evaporation and subjected to the following amplification scheme: 95°C denaturation for 2 min in the first cycle, 95°C denaturation for 1 min and 60°C anneal/extension for 1 min for all subsequent cycles, followed by a 60°C extension for 10 min in the last cycle. Reactions were performed in a Perkin–Elmer/Cetus thermal cycler.

Analysis of PCR Products. After cycling, samples were mixed with 50 μl of high-purity chloroform and the aqueous phase containing the DNA product was extracted. Each sample (4 μl) was fractionated on a 5% polyacrylamide gel in Tris-borate buffer, stained with ethidium bromide, and visualized by UV fluorescence, as described (10). Sizes of PCR products were determined by using an *Msp* I digest of pBR322 as a size standard. For thin-layer chromatography (TLC) analysis, 1 μl of each sample was spotted onto a Polygram CEL 300 DEAE 20 × 20 cm cellulose thin-layer chromatogram plate (Macherey & Nagel), which was prespotted with 5 μl of sheared herring sperm DNA per sample (150 μg/ml). The plate was then placed in a glass development tank containing Homo-mix III (11), a solution of partially hydrolyzed RNA containing 7 M urea, in a 70°C oven. After homochromatography, the plate was dried, covered with plastic wrap, and analyzed by autoradiography. A series of short synthetic oligonucleotides having sequences identical to the 5′ ends of the probes were used as size standards. Where quantitation was performed, an AMBIS radioanalytic imaging system (AMBIS Systems, San Diego) was used.

RESULTS

Probe Degradation in a PCR. A PCR amplification of a 350-bp product from the bacteriophage M13mp10 directed by primers BW36 and BW42 (see Table 1), and containing one of three different 5′ ^{32}P-labeled probes, was carried out. The probes (BW31, BW33, BW35) contained the same 30-base sequence complementary to M13mp10 but differed in the lengths of their noncomplementary 5′ "tail" regions (see Table 1). Probes contained a 3′-phosphate in order to block extension by *Taq* DNA polymerase and were added as indicated. The PAGE analysis (Fig. 2*A*) showed that the presence of each of the three probes at concentrations varying from 0.04 to 0.4 μM had no effect on the amount of amplified product generated. Samples with and without probe showed a discrete intensely stained 350-bp product. The TLC analysis indicated that a significant fraction of the probes was degraded into smaller fragments (Fig. 2*B*). With probe BW31, which was fully complementary to target sequence, the 5′-degraded fragments were primarily 1 and 2 bases in length (lanes 1–3). Undegraded probe remained at the origin. Probe BW33, having a 3-base 5′ noncomplementary tail, was cleaved to generate 5′ fragments primarily 4 and 5 bases long (lanes 4–6). Probe BW35 had a 10-base noncomplementary tail and released 5′ products primarily 11 and 12 bases long (lanes 7–9). Lanes 10–12 are reactions in which template was not added and illustrate that probe was not degraded in the absence of specific target sequence. The 5′-released fragments were specific in size, depending upon the length of the 5′ noncomplementary tail of each probe. This suggests that during polymerization the enzyme displaces the first one or two paired bases it encounters and then cleaves at that site, independent of the length of the 5′ noncomplementary tail.

In a separate experiment, we used an HIV-1 template to study the effects of starting target number and amplification cycles on probe degradation. The region to be amplified was a 142-bp segment of the HIV-1 *gag* gene (12). The PAGE analysis (data not shown) indicated that intended product was made in all cases. Product accumulated with increasing amplification cycles and initial target number, as expected, without probe interference. The amount of released, ^{32}P-labeled 5′ fragments was measured, and the results are shown in Table 2. By comparing the amounts of degraded probe, it is clear that more probe is cleaved when more starting target is available and more amplification cycles are performed. In addition, the amount of degraded probe is dependent on probe concentration. For example, at 30 cycles and 10^2 starting copies of HIV-1, 0.4 pmol (40%) of probe has been degraded when 1 pmol was added; when 10 pmol of probe was added, 2.9 pmol (29%) of probe has been degraded.

Specificity of Probe Degradation. The specificity of probe degradation was examined by performing a PCR amplification using bacteriophage λ DNA and a noncomplementary labeled probe. Probe BW31 (Table 1), which would not be expected to bind to a λ template, was added as described.

Table 1. Primers and probes for DNA amplification by PCR

Oligonucleotide	Sequence (5′ → 3′)	Location
M13mp10 DNA		
BW36 primer	CCGATAGTTTGAGTTCTTCTACTCAGGC	5241–5268
BW42 primer	GAAGAAAGCGAAAGGAGCGGGCGCTAGGGC	5591–5562
BW31 probe	*CGCTGCGCGTAACCACCACACCCGCCGCGCp	5541–5512
BW33 probe	*gatCGCTGCGCGTAACCACCACACCCGCCGCGCp	5541–5512
BW35 probe	*cgtcaccgatCGCTGCGCGTAACCACCACACCCGCCGCGCp	5541–5512
BW50 probe	*tatCCCGCCGCGCTTAATGCGCCGCTACAp	5521–5496
BW51 probe	*gcaTTAATGCGCCGCTACAGGGCGCGTACTATGGp	5511–5481
λ DNA		
PCR01 primer	GATGAGTTCGTGTCCGTACAACTGG	7131–7155
PCR02 primer	GGTTATCGAAATCAGCCACAGCGCC	7630–7606
HIV-1 DNA		
SK145† primer	AGTGGGGGGACATCAAGCAGCCATGCAAAT	1366–1395
SK431† primer	TGCTATGTCAGTTCCCCTTGGTTCTCT	1507–1481
SK102† probe	*GAGACCATCAATGAGGAAGCTGCAGAATGGGATp	1403–1435

Lower case a, t, g, and c = bases noncomplementary to template strand; p = 3′-phosphate.
*5′ ^{32}P label.
†HIV-1, isolate SF2, GenBank accession no. K02007.

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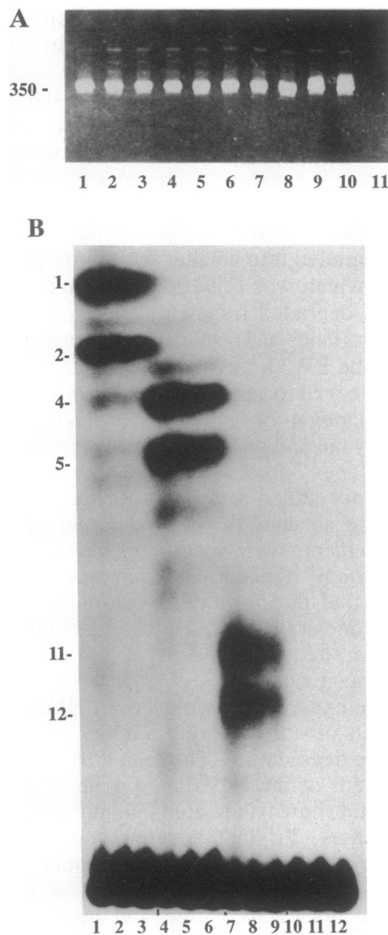

FIG. 2. PCR product accumulation and nucleolytic cleavage of probe. (*A*) PAGE analysis of 15-cycle amplification of 350-bp product from M13mp10 using primers BW36 and BW42. Lane 1, PCR with no probe present; lanes 2–4, 2, 10, or 20 pmol of probe BW31, respectively, which is fully complementary to target; lanes 5–7, 2, 10, or 20 pmol of probe BW33, respectively, containing a 3-base 5′ noncomplementary tail; lanes 8–10, 2, 10, or 20 pmol of probe BW35, respectively, containing a 10-base 5′ noncomplementary tail; lane 11, control reaction containing no template. (*B*) Autoradiogram of TLC analysis of samples from PCR depicted in *A*. Samples were applied at the origin and migrate upward. Lanes 1–3, probe BW31 at 2, 10, and 20 pmol, respectively; lanes 4–6, probe BW33 at 2, 10, and 20 pmol, respectively; lanes 7–9, probe BW35 at 2, 10, and 20 pmol, respectively; lanes 10–12, 10 pmol of probes BW31, BW33, and BW35, respectively, with no template added.

Results indicated that the presence of the probe at various concentrations had no influence on the amount of amplified product generated (Fig. 3*A*). A sample control reaction containing no probe and all reactions containing probe showed a comparable level of specific 500-bp product. The TLC analysis provided in Fig. 3*B* shows reactions in which degradation of the probe was not observed. This illustrates that probe is not degraded unless specifically bound to target and is able to withstand the PCR cycling conditions. Identical results were also obtained with probes BW33 and BW35 (data not shown).

To determine if the presence of high-complexity DNA would interfere with probe hybridization and degradation, we added 1 μg of sheared or untreated human genomic DNA to a PCR assay. The sheared DNA was intended to generate a large number of potential enzyme binding sites, which might increase the probability of nonspecific interactions. Additionally, sheared DNA might provide greater nonspecific probe binding sites. The PAGE analysis indicated that PCR product yield varied with the amount of starting template and the number of amplification cycles (data not shown). The presence of either sheared or untreated human genomic DNA showed no effect on product formation. In addition, the presence of a high-complexity DNA did not interfere with probe hybridization and degradation, as is illustrated by Fig. 4. Low or high molecular weight genomic DNA with various concentrations of M13mp10 DNA target (lanes 5–8) generated the same amount of released product as when genomic DNA was absent (lanes 3 and 4). These results confirm the specificity of the 5′ → 3′ exonuclease activity of *Taq* DNA polymerase even in the presence of a complex DNA background.

Table 2. Amount of probe degradation in a PCR

Probe added, pmol	Probe fragment released, pmol			
	10^2 HIV copies		10^4 HIV copies	
	Cycle 30	Cycle 40	Cycle 30	Cycle 40
1	0.4	0.6	0.5	0.7
10	2.9	3.8	3.2	4.5

Data depict the amount of detected probe degradation products from a PCR assay for HIV-1. Primers were SK145 and SK431, at 10 pmol each. HIV-1 target was added at 10^2 or 10^4 copies per reaction; ^{32}P-labeled probe SK102 was present at 1 or 10 pmol. Amplifications were performed for 30 or 40 cycles.

Sizes of Released Products Are Sequence Specific. To assess the effects of base pairing at the 5′ fork region of a probe on the size of released product, we designed two M13mp10 probes, BW50 and BW51, to contain either a (G+C)- or (A+T)-rich region at their 5′ complementary end. Probes BW50 and BW51 were compared to M13mp10 probe BW33 (see Table 1). The PAGE analysis determined that probe presence did not interfere with PCR product formation (data not shown). The TLC analysis (Fig. 5) revealed that the sizes of labeled, hydrolyzed, 5′ oligonucleotide fragments varied depending on base composition at the 5′ fork region. Probe BW50, which was (G+C)-rich, released primarily a 3-base product. Probe BW51, which was (A+T)-rich, released primarily a 6-base product, and probe BW33 released primarily 4- and 5-base products. These results suggest that the nature

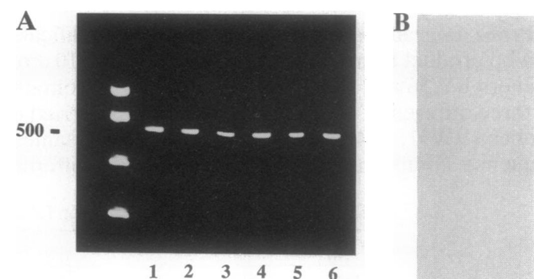

FIG. 3. PCR with noncomplementary probes. (*A*) PAGE analysis of λ DNA PCR in the presence of noncomplementary probes. Region amplified was a 500-bp sequence flanked by primers PCR01 and PCR02. Labeled probe BW31 was added at 2, 10, or 20 pmol per reaction. Amplifications were for 15 cycles. Lane 1, no probe in the reaction; lanes 2 and 3, 2 pmol of probe; lanes 4 and 5, 10 pmol of probe; lane 6, 20 pmol of probe. (*B*) Autoradiogram of TLC analysis of λ DNA amplification containing noncomplementary probes. Lanes 1–3, probe BW31 at 2, 10, and 20 pmol, respectively; lane 4, 10 pmol of probe BW31 with no template present in the reaction.

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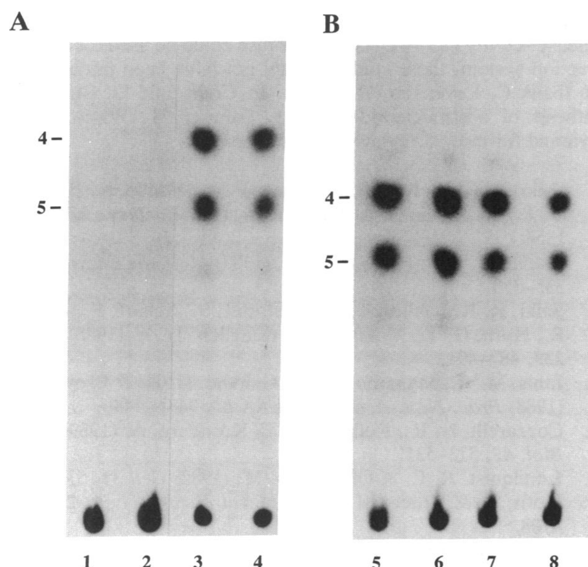

FIG. 4. Presence of high-complexity DNA in a PCR. Autoradiogram of TLC analysis from PCR assay. Ten picomoles each of primers BW36 and BW42 and probe BW33 were added with 10^{-2} or 10^{-3} pmol of M13mp10 target and amplified for 20 cycles. Lanes 1 and 2, probe and sheared or untreated genomic DNA, respectively, but no M13mp10 target; lanes 3 and 4, 10^{-2} or 10^{-3} pmol of M13mp10, respectively, but no genomic DNA; lanes 5 and 6, 10^{-2} pmol of M13mp10 with sheared or untreated genomic DNA, respectively; lanes 7 and 8, 10^{-3} pmol of M13mp10 with sheared or untreated DNA, respectively.

of probe label release is dependent on base composition at the 5′ end. The (A+T)-rich 5′ portion of probe BW51 may facilitate probe strand displacement prior to cleavage.

DISCUSSION

The advent of *in vitro* DNA amplification has facilitated the direct detection of rare DNA and RNA sequences as well as infectious agents present at extremely low levels. However, due to spurious extension of primers at partially complementary regions, it is often desirable to include a separate probe hybridization step to obtain specific product discrimination and provide increased sensitivity over ethidium bromide staining of gel electrophoresis products. By selection of unique probe sequences, it is possible to distinguish between very closely related organisms, such as human T-lymphotropic virus types I and II or HIV-1 and HIV-2 (13, 14). The 5′ → 3′ exonuclease-based detection assay described incorporates this added product discrimination directly into the PCR. Additionally, differentiation between allelic variations or small deletions may be achieved by modifying the probe region at the 5′ end to alter the sizes of released fragments.

We have demonstrated that when an oligonucleotide probe is included in a PCR, amplification of specific product is not inhibited. The 5′ → 3′ exonuclease activity of *Taq* DNA polymerase degrades the probe only when the probe is hybridized to specific target. Even in a high background of complex DNA, degradation occurs only in a target-specific fashion. The amount of probe degradation is influenced by the initial number of target molecules, probe concentration, and number of PCR cycles. Detectable probe release has been demonstrated from a starting target number of 100.

At the initial copy numbers, and number of amplification cycles used in the experiments described, the PCR has already entered what is known as plateau phase. Amplification plateau is that stage of a PCR when the desired amplification product stops accumulating exponentially. As shown in Table 2, probe degradation also appears to follow a plateau phase—that is, degradation is not occurring exponentially in late stages of amplification. An increase from 3.2 pmol of probe released after 30 cycles to 4.5 pmol of probe released after 40 cycles, when 10 pmol of probe was initially added to the reaction, illustrates that released label is not accumulating exponentially. Preliminary data suggest that the relationship between probe degradation and product accumulation may not be linear and that these two phenomena may enter their own plateau phases at different stages of the amplification reaction.

The relationship between probe degradation and PCR product formation varies somewhat among different probe and primer systems. One requirement for this detection method is that probe must bind to target before primer extension occludes the probe binding site. If the annealing temperature of the probe is close to or below the annealing temperature of the upstream primer, there is a greater chance that primer will be extended by enzyme before probe has bound to target. This would result in inefficient label release, where efficiency is calculated as the fraction of primer extensions that lead to successful probe degradations. This may be overcome by varying the relative amounts of primer and probe (as described above), by manipulating the sequence and length of the probe, or by using more stable base analogs. During a ramp from the denaturation temperature to the anneal/extend temperature, probe binding would thus be favored.

Although the data presented utilize a 5′ ^{32}P-labeled probe, various other labels can be substituted in this assay. Examples include other radioisotopes or other nonisotopic labels such as fluorescent dyes, chromophores, chemiluminescent labels, or ligands such as biotin. The detection limit of the label determines the sensitivity of the assay. An ideal label for this assay is one that (*i*) can be easily attached to DNA, (*ii*) is detectable at very low concentrations with simple instrumentation, (*iii*) is safe to use, (*iv*) is stable at elevated temperatures, and (*v*) does not interfere with the activities of the polymerase. We have performed this assay with some of the nonisotopic labels mentioned and have achieved similar results with regard to amount of detectable probe degradation. Probes can also be labeled at their 3′ end. We have found that a 3′-labeled probe yields a broad distribution of product sizes, consistent with the 5′ → 3′ exonuclease activity of *Taq* DNA polymerase degrading the probe until remaining probe–target duplex is no longer stable and probe dissociates from template (data not shown). Additionally, the desired sizes of labeled probe fragments may be manipulated by designing the probe to contain modified bases such as phosphorothioate,

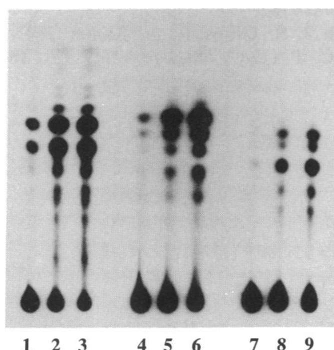

FIG. 5. Sizes of released products are sequence specific. Autoradiogram of TLC analysis from PCR assay. Using 10 pmol of primers BW36 and BW42, 1 pmol of each probe was added to a reaction mixture and amplified for 10, 15, or 20 cycles. Lanes 1–3, probe BW33 at 10, 15, or 20 cycles, respectively; lanes 4–6, probe BW50, which has a (G+C)-rich fork region, at 10, 15, or 20 cycles, respectively; lanes 7–9, probe BW51, which has an (A+T)-rich fork region, at 10, 15, or 20 cycles, respectively. The sizes of released products are as stated in the text.

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which may prevent enzyme cleavage at specific sites (15), or by targeting a particular sequence at the 5′ end.

It is also desirable to not have extension from the probe, as this serves to limit the amount of reaction components available for primer extension and subsequent probe degradation. Probe extension will also generate the formation of PCR products that cannot be utilized in 5′ → 3′ exonuclease reactions. Oligonucleotide probes were synthesized with a 3′-phosphate moiety and can be 5′ end-labeled using a 3′-phosphatase-free polynucleotide kinase (Boehringer Mannheim) to minimize probe extension. In addition, we have also synthesized probes that contain a two- to six-base mismatch at the 3′ end, which does not compromise probe hybridization but also efficiently prevents probe extension (16) (data not shown).

Other detection techniques such as Southern, dot, and "reverse" dot blotting (17–19) are well established, but the methodology is time consuming and labor intensive. Such methods require multiple handling steps of fixing and washing amplified sample, which may contribute to sample contamination. The recently described techniques of oligomer restriction and oligomer hybridization do not require fixing or washing but do require electrophoretic separation (12, 20). Other detection techniques employ the use of fluorescently labeled primers (21) but do not incorporate the additional level of specificity obtained from adding a probe to the PCR assay. In a 5′ → 3′ exonuclease-based assay, the hybridization step also occurs in solution but coincident with amplification, and additional probing steps are eliminated. The specificity of the assay allows for the introduction in the amplification reaction of one or more probes with different labels, directly allowing the simultaneous detection of more than one sequence, a feature of the assay that would be useful in multiplex PCR (22).

In this paper we present a convenient and effective protocol for detection of PCR product. The method employs the 5′ → 3′ exonuclease activity of *Taq* DNA polymerase to generate a signal for target detection during the amplification process. The technique described is sensitive, specific, quantifiable, and convenient for assaying large numbers of samples. The reduction of additional handling steps over current hybridization methods is timesaving and minimizes the chance of sample contamination. It is conceivable that such an assay may be incorporated into many PCR systems. One need only design a probe that will effectively hybridize within the target sequence and subsequently detect its specific cleavage. Since detectable label is generated simultaneously with product amplification, we envision the use of this technique to create a truly homogeneous assay, in which amplification and actual detection occur in one tube.

If it were not for the novel idea of R. K. Saiki that the inherent nuclease activity of *Taq* DNA polymerase could be used for a unique detection system, these studies would not have been initiated. We also thank C. Levenson, D. Spasic, L. Goda, and O. Budker for synthesis of oligonucleotides and J. Sninsky, T. White, and J. Raymond for critical review of the manuscript.

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