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The Use of Phosphorothioate Primers and Exonuclease Hydrolysis for the Preparation of Single-stranded PCR Products and their Detection by Solid-phase Hybridization

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The effect of phosphorothioate bonds on the hydrolytic activity of the 5′ → 3′ double-strand-specific T7 gene 6 exonuclease was studied. Double-stranded DNA substrates containing one phosphorothioate residue at the 5′ end were found to be hydrolyzed by this enzyme as efficiently as unmodified ones. The enzyme activity was, however, completely inhibited by the presence of four phosphorothioates. On the basis of these results, a method for the conversion of double-stranded PCR products into full-length, single-stranded DNA fragments was developed. In this method, one of the PCR primers contains four phosphorothioates at its 5′ end, and the opposite strand primer is unmodified. Following the amplification, the double-stranded product is treated with T7 gene 6 exonuclease. The phosphorothioated strand is protected from the action of this enzyme, whereas the opposite strand is hydrolyzed. When the phosphorothioated PCR primer is 5′ biotinylated, the single-stranded PCR product can be easily detected colorimetrically after hybridization to an oligonucleotide probe immobilized on a microtiter plate. We also describe a simple and efficient method for the immobilization of relatively short oligonucleotides to microtiter plates with a hydrophilic surface in the presence of salt.

PCR[1] is currently the most widely used DNA amplification method. It serves as the first step in many genetic analysis methods. PCR normally produces double-stranded products. For a number of applications the double-stranded PCR product must be converted to the single-stranded form. Such applications include the sequencing of PCR products or their use as hybridization probes. In this paper we show that if one of the 5′ ends of a double-stranded PCR product is selectively protected from the hydrolytic action of T7 gene 6 exonuclease, the opposite strand can be hydrolyzed completely by this enzyme. Single-stranded PCR product is thereby generated with high efficiency. We have applied this approach to the development of a new, nonelectrophoretic, ELISA-type detection method for PCR products.

There are a number of analytical methods available for the detection of PCR products. The most specific of these methods, and at the same time one of the most labor-intensive, requires the polyacrylamide or agarose gel electrophoresis of the PCR product, followed by blotting onto a membrane and hybridization to a detectably labeled PCR product-specific probe. For more routine applications, the blotting and hybridization steps are omitted, but the need for gel electrophoresis still presents a bottleneck when a large number of PCR products have to be analyzed. There is a need for more efficient, rapid, nonelectrophoretic, nonradioactive methods for the detection of PCR products.

Currently, 96-well polystyrene plates are widely used in solid-phase immunoassays, and several PCR product detection methods that use plates as a solid support have been described.[2–12] The most specific of these methods require the immobilization of a suitable oligonucleotide probe into the microtiter wells followed by the capture of the PCR product by hybridization and colorimetric detection of a suitable hapten.

The method described in this paper also employs the widely used 96-well plate format. Thus, it is very well suited for automation and large-scale application.

MATERIALS AND METHODS

Oligonucleotide Synthesis

All oligonucleotides were synthesized using standard phosphoramidite chemistry on an Applied Biosystems 392/394 DNA synthesizer. For the synthesis of phosphorothioate primers, tetraethylthiuram disulfide (TETD, Applied Biosystems) was used as recommended by the manufacturer. Briefly, the TETD solution was placed at the iodine port of the synthesizer, and the regular oxidation step was replaced by a 15-min sulfurization with TETD. All oligonucleotides were deprotected with concentrated ammonia and desalted using NAP 5 (0.2 μmole scale synthesis) or NAP 25 (1 μmole) gel filtration columns (Pharmacia). The purity was checked by reverse-phase HPLC (5 μ APEX-I octadecyl, Jones Chromatography, Lakewood, CO). Because each phosphorothioate bond creates two diastereoisomers, oligonucleotides containing more than one such bond elute in

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one broad or several individual peaks. The two diastereoisomers of products with one phosphorothioate only were usually well separated by this method and the two isomers could be isolated individually.

Biotinylated PCR primers were prepared using a biotin phosphoramidite (DMT-biotin-C6-PA), obtained from Cambridge Research Biochemicals, Inc. (Wilmington, DE). The coupling time of this phosphoramidite was extended to 2 min. When phosphorothioate-modified PCR primers were labeled at the 5′ end with biotin, the phosphodiester bond between the 5′-terminal base and the biotin residue was also sulfurized to a phosphorothioate bond. For the synthesis of 3′ biotinylated oligonucleotides, biotin-modified CPG (BioTEG CPG, available from Glen Research, Sterling, VA) was used.

PCR Amplification

Horse genomic DNA was the source of DNA in all PCR amplifications reactions. PCR reactions were carried out in a total volume of 50 μl. The final concentration of the PCR primers was 0.5 μM. Following an initial 2-min denaturation step at 95°C, 35 cycles were carried out, each consisting of denaturation (1 min at 95°C), annealing (2 min at 60°C), and extension (3 min at 72°C). *Taq* DNA polymerase was obtained from Perkin-Elmer and used at a concentration of 0.025 U/μl. The final composition of the PCR buffer was 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 200 μg/ml of BSA.

Preparation of Single-stranded PCR Fragments

To protect one of the strands of the double-stranded PCR product from exonuclease hydrolysis, four phosphorothioate bonds were introduced during synthesis at the 5′ end of one of each pair of the PCR primers. For generation of single-stranded PCR products, following the PCR amplification, T7 gene 6 exonuclease was added to a final concentration of 2 U/μl of PCR reaction. Incubation was for 1 hr at room temperature. The T7 gene 6 exonuclease was purchased from U.S. Biochemical and diluted in a buffer recommended by the manufacturer.

Immobilization of Oligonucleotides onto 96-well ELISA Plates

Immulon 4 plates (Dynatech Laboratories, Chantilly, VA) were used. Aliquots (50μl) of a 0.2 μM oligonucleotide solution in a freshly prepared 30 mM solution of 1-ethyl-3-(3-dimethylaminopropyl)-1,3-carbodiimide hydrochloride (EDC) in water were added to individual wells of a 96-well plate and incubated overnight at room temperature. The plates were then washed with a solution of TNTw [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20]. In other experiments, EDC was replaced by the reagents listed in Table 1.

Hybridization of Single-stranded PCR Fragments to Oligonucleotides Immobilized onto ELISA Plates

After the exonuclease treatment, an equal volume of 3 M NaCl and 20 mM EDTA was added to the reaction mixture and 20-μl aliquots of the resulting solution transfered to individual wells containing the appropriate immobilized oligonucleotide molecule. Hybridization was carried out for 30 min at room temperature and was followed by washing with TNTw.

Colorimetric Detection

After hybridization, the plate was incubated with a 1:1200 dilution of anti-biotin horseradish peroxidase (HRP) conjugate (Vector Laboratories, Burlingame, CA) in 1% BSA in TNTw, for 30 min at room temperature. The plate was then washed six times with TNTw, and a solution of 1 mg/ml of *o*-phenylenediamine (OPD) in 0.1 M citrate buffer (pH 4.5) containing 0.012% H_2O_2, was added. The plate was immediately placed in a plate reader (v_{max}, Molecular Devices), and the development of color was followed at 450 nm for 2 min. The results were expressed as mOD_{450}/min.

RESULTS

Immobilization of Oligonucleotides in 96-well Polystyrene Plates

We were interested in the development of a simple method for the attachment of relatively short (15–40 bases), unmodified oligodeoxynucleotides in 96-well

TABLE 1 Immobilization of Oligonucleotides on 96-well Polystyrene Plates

A. Immobilization of oligonucleotide 1

Conditions of immobilization	No NaOH treatment of plate	After NaOH treatment of plate
A. H_2O	35	40
B. 50 mM NaCl	240	240
C. 250 mM NaCl	300	320
D. 500 mM NaCl	275	290
E. 50 mM octyldimethylamine hydrochloride (pH 7.0)	260	240
F. 30 mM EDC	290	260
G. 10 mM Tris-HCl, 150 mM NaCl (pH 7.5)	230	250
H. TNTw	1	1

B. Hybridization of oligonucleotide 2 (500 fmoles per well) to oligonucleotide 1

Conditions of immobilization of oligonucleotide 1	No NaOH treatment of plate	After NaOH treatment of plate
A. H_2O	3	1
B. 50 mM NaCl	80	70
C. 250 mM NaCl	120	95
D. 500 mM NaCl	130	100
E. 50 mM octyldimethylamine hydrochloride (pH 7.0)	130	95
F. 30 mM EDC	110	100
G. 10 mM Tris-HCl, 150 mM NaCl (pH 7.5)	85	55
H. TNTw	1	1

Results are given in mOD_{450}/min.

polystyrene plates and their use in hybridization-based assays. Previous work has shown that various small biomolecules can be immobilized to polystyrene plates by incubation with EDC.[13] No examples of oligonucleotide immobilization were given in that work, and it is generally believed that short, single-stranded oligonucleotide molecules are immobilized only very inefficiently to this type of solid phase. Initially, we concentrated on the use of 5′ amino-modified oligonucleotides and attempted their covalent attachment in the presence of the water-soluble carbodiimide EDC. In the course of these experiments, we found that neither the 5′ amino function nor EDC are required for the desired immobilization of these oligonucleotide probes, which proceeds efficiently even in solutions containing as little as 50 mM NaCl. In a typical experiment, the immobilization of oligonucleotide 1, a 5′ biotinylated 25-mer, was attempted using several different solutions. After overnight incubation, some of the wells were treated with a 0.1 N NaOH solution for 1 hr at room temperature, and the plates were then assayed for the presence of biotin. In a parallel experiment, unlabeled oligonucleotide 2, which is complementary to oligonucleotide 1, was attached under the same set of conditions. This was then followed by hybridization to oligonucleotide 1, with and without a previous NaOH treatment of the plate. The results are summarized in Table 1. Oligonucleotide 1 was 5′-B-ACCAGGCACCACGCGGTCTGAGGCT, and oligonucleotide 2 was 5′-AGCCTCAGACCGCGTGGTGCCTGGT.

The results from this experiment show that the immobilization of the oligonucleotides requires the presence of salt. The immobilization is very inefficient in salt-free aqueous solutions. Also, in the presence of Tween 20, no immobilization takes place. This is probably attributable to an "inactivation" of the plate surface by the preferential adsorbance of this detergent molecule. Similarly, no immobilization can be achieved if the plate is washed with a solution containing Tween 20 before the attachment step, even if the attachment solution does not contain the detergent. The use of 250 mM NaCl in the attachment step results in slightly higher signals in the subsequent hybridization assay.

We have tested a number of different commercially available 96-well plates for their suitability for oligonucleotide immobilization. In general, plates that are described as having a more hydrophilic surface gave good results, whereas those with a hydrophobic surface were found unsuitable. Examples of suitable plates include Immulon 4 (Dynatech), Maxisorp (Nunc), and ImmunoWare (Pierce). No attachment could be achieved on Immulon 1 (Dynatech) and Polysorp (Nunc) plates. All experiments described below have been carried out with Immulon 4 plates.

Stability of Phosphorothioate Bonds to Hydrolysis by T7 Gene 6 Exonuclease

The bacteriophage T7 gene 6 exonuclease hydrolyzes double-stranded DNA in the 5′ → 3′ direction.[14] To study the effect on the enzyme activity of the substitution of regular phosphodiester bonds with phosphorothioates, we synthesized the following 3′ biotinylated, self-complementary oligonucleotides (45-mers; the bonds between the highlighted bases are phosphorothioates; B denotes a biotin residue): oligonucleotide 3, 5′-CCGCGTGGTGCCTGGTGCCCTTTTTGGGCACCAGGCACCACGCGG-B; oligonucleotide 4, 5′-**CC**GCGTGGTGCCTGGTGCCCTTTTTGGGCACCAGGCACCACGCGG-B; oligonucleotide 5, 5′-**CCGCG**TGGTGCCTGGTGCCCTTTTTGGGCACCAGGCACCACGCGG-B.

Oligonucleotides 3–5 were synthesized trityl-on, purified by reverse-phase HPLC, detritylated by treatment with 80% acetic acid, and desalted. Oligonucleotide 4 contains one phosphorothioate bond at the 5′ end and is therefore a mixture of two diastereoisomers, Rp and Sp. These two diastereoisomers were well separated by reverse-phase HPLC at the trityl-on level and obtained in pure form after detritylation. The two individual diastereoisomers of oligonucleotide 4 thus obtained are referred to as peak A (eluting earlier) and peak B (eluting later) in the text.

Oligonucleotides 3–5 were designed to form stable hairpin-type self-complementary secondary structures, with a single-stranded loop of five thymidine residues. Upon treatment with T7 gene 6 exonuclease, these oligonucleotides should be hydrolyzed from the 5′ end up to the thymidine loop, and would thereby be converted to single-stranded molecules. To capture these resulting 3′ biotinylated single-stranded oligonucleotides onto a solid phase by hybridization, oligonucleotide 2 was immobilized in 96-well plates. The 16 3′-terminal bases of this oligonucleotide are complementary to the 3′ ends of biotinylated oligonucleotides 3–5.

Approximately 60 pmoles of the purified oligonucleotides 3–5 were treated with 0 or 4 U/μl of T7 gene 6 exonuclease, at 37°C in a total volume of 100 μl. Following this treatment, aliquots were removed at intervals and mixed with an equal volume of 3 M NaCl, 20 mM EDTA. After an additional dilution step in 1.5 M NaCl, 10 mM EDTA, aliquots containing ~1 pmole of oligonucleotide were added to the wells of a 96-well plate containing the immobilized oligonucleotide 2. The presence or absence of biotin was then detected in a colorimetric assay. The results of this assay are summarized in Table 2. The signals given in Table 2 are those obtained after a 15-min incubation with exonuclease. No in-

TABLE 2 Effect of Phosphorothioate Residues on the Activity of T7 Gene 6 Exonuclease

Oligonucleotide	Number of 5′ phosphorothioate residues	Signal without exonuclease treatment	Signal after treatment with 4 U/μl of exonuclease
3	0	4	196
4 peak A	1	4	220
4 peak B	1	4	180
5	4	5	6

Self-complementary oligonucleotides 3–5 were treated with 0 or 4 U/μl of exonuclease and hybridized to an oligonucleotide complementary to the 3′ region of the hairpin. Removal of the 5′ double-stranded portion of the self-complementary molecule allowed hybridization. Detection of biotin at the 3′ end was by the colorimetric assay. The results are shown in mOD_{450}/min.

crease in signal was seen upon longer incubation.

Several important results emerged from these experiments. As expected, none of the self-complementary, double-stranded oligonucleotides was able to hybridize to the solid-phase immobilized oligonucleotide. Hybridization only took place if a single-stranded, biotinylated oligonucleotide was obtained by treatment with T7 gene 6 exonuclease. In this assay, oligonucleotide 3, as well as both diastereoisomers of oligonucleotide 4, were found to be equally good substrates for the exonuclease. Thus, the presence of only one phosphorothioate residue does not provide sufficient protection. In contrast, four phosphorothioate residues at the 5′ end of oligonucleotide 5 provide complete protection from the hydrolytic activity of T7 gene 6 exonuclease. Most likely, the enzyme is capable of bypassing one 5′-terminal phosphorothioate bond and starting the hydrolysis from the next phosphodiester. Similar behavior was reported for DNA polymerase I and 5′ phosphorothioated primers.[15] We have not studied oligonucleotides containing two and three phosphorothioates.

Colorimetric Detection of PCR Products in 96-well Plates

Having established that phosphorothioate bonds can provide protection from the hydrolytic action of T7 gene 6 exonuclease, we then prepared PCR primers containing four internucleotidic phosphorothioate bonds at their 5′ ends. A fifth phosphorothioate bond links the 5′-terminal nucleotides of these primers to a biotin residue, which allows the nonradioactive detection of the PCR products. These labeled primers were used together with unmodified opposite strand primers to amplify fragments from horse genomic DNA. The sequences of the PCR primers used are listed in Table 3. For all PCR reactions, negative controls were carried out that contained all reactions components with the exception of the horse genomic DNA. A positive result of such a reaction would indicate contamination of one of the reaction components by a previously obtained PCR product.

Following the PCR amplification, aliquots of the reaction mixtures were withdrawn and saved as double-stranded PCR controls, whereas the rest of the mixtures was treated with T7 gene 6 exonuclease as described in Materials and Methods. Analysis was then carried out using polyacrylamide gel electrophoresis and also by hybridization of the single-stranded products of the exonuclease reaction to oligonucleotide probes immobilized in 96-well plates. These capture oligonucleotides were designed to hybridize to internal regions of the PCR products, thereby eliminating the possible capture of primer–dimers. The sequences of these capture oligonucleotides are listed in Table 3. Following the hybridization step, the presence or absence of biotin was determined with a colorimetric reaction using an anti-biotin HRP conjugate. The polyacrylamide gel electrophoresis of the PCR products is shown in Figure 1, and the results of the microtiter plate hybridization assay are summarized in Table 4.

To demonstrate the specificity of hybridization, each of the same three PCR products after the exonuclease treatment was hybridized to wells that contain each of the three capture oligonucleotides. The results of this cross-hybridization experiment are also included in Table 4. Each of the three PCR products hybridized only to its specific capture oligonucleotide.

TABLE 3 Sequences of the PCR Primers and Oligonucleotide Capture Probes

A. (93 bp) PCR: 5′-**B-CCAAA**GGAGCTGGGTCTGAAACAAA;
5′-ATGGCTTCCCACCCTACCCATCCCG;
capture probe: 5′-TGTTCTGGGAAAGACCACATTATTT

B. (201 bp) PCR: 5′**B-ATGCT**CCCAGGTGATTCCAGTGTGC;
5′-GGTGCTGTGCGAGGTACACTTGACTG;
capture probe: 5′-AGAAACACAAGGCCCAAGAACAGGA

C. (547 bp) PCR: 5′-**B-GGATC**CAGATGAACAACCAGATGAA;
5′-CTGCAGCCCACTGGGCCTTCTTTGT;
capture probe: 5′-CCTTTGTGTAGAGTAGTTCAAGGAC

The lengths of the PCR products are given in parentheses. Phosphorothioate bonds are located between the highlighted nucleotides. Note that the 5′ terminal biotin residues are also attached through a phosphorothioate bond.

DISCUSSION

Immobilization of Capture Oligonucleotides in 96-well ELISA Plates

Several methods for the immobilization of relatively short oligonucleotides onto 96-well plates for subsequent hybridization-based assays have been described in the literature. Probably the most widely used method consists of the coating of the ELISA plates with avidin or streptavidin, followed by the immobilization of biotinylated oligonucleotides.[2] Another known method requires the precoating of polystyrene plates with poly(Lys, Phe), followed by the covalent attachment of amino- or sulfhydryl-modified oligonucleotides using bifunctional cross-linking reagents.[3,16] A method for the direct covalent attachment of short, 5′ phosphorylated primers to chemically modified polystyrene plates has also been published.[17]

In this work we demonstrate that relatively short, unmodified oligonucle-

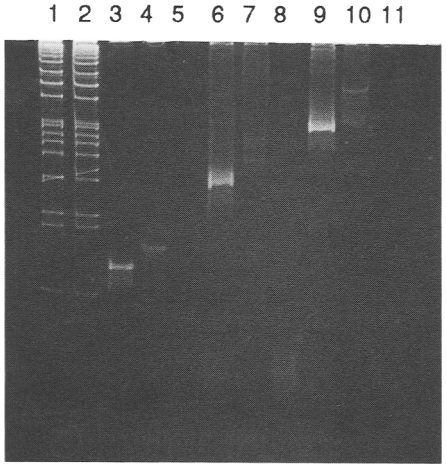

FIGURE 1 Gel analysis of PCR reactions A, B, and C. (Lanes *1,2*) One-kilobase marker; (lane *3* PCR reaction A, before exonuclease treatment; (lane *4*) PCR reaction A after the exonuclease treatment; (lane *5*) PCR reaction A, negative control (no input DNA); (lane *6*) PCR reaction B before exonuclease treatment; (lane *7*) PCR reaction B after exonuclease treatment; (lane *8*) negative control of PCR reaction B; (lane *9*) PCR reaction C before exonuclease treatment; (lane *10*) PCR reaction C after exonuclease treatment; (lane *11*) negative control of PCR reaction C.

TABLE 4 Microtiter Plate Hybridization Assay of PCR Reactions A, B, and C

PCR reaction	Hybridization to capture oligonucleotide for reaction	Signal without exonuclease treatment	Signal after exonuclease treatment (2 U/μl)
A	A	2	450
	B	N.T.	3
	C	N.T.	1
A (neg. control)	A	N.T.	1
B	A	N.T.	4
	B	1	630
	C	N.T.	1
B (neg. control)	B	N.T.	4
C	A	N.T.	3
	B	N.T.	1
	C	2	450
C (neg. control)	C	N.T.	4

The products of PCR reactions A, B, and C were rendered single-stranded by treatment with 2 U/μl of T7 gene 6 exonuclease and aliquots corresponding to 5 μl of the initial PCR reaction were added to the wells of a microtiter plate containing the appropriate capture oligonucleotides for hybridization. The capture oligonucleotides were immobilized to the plate using 500 mM NaCl. The results of the colorimetric assay are presented in mOD_{450}/min. All experiments were carried out in duplicate; the results shown are averages. (N.T.) Not tested.

otides can be efficiently immobilized onto the surface of hydrophilic polystyrene plates simply by incubation in a solution containing ~50 mM NaCl. Other reagents that were effective include the water-soluble carbodiimide EDC, as well as octyldimethylamine hydrochloride. The latter compound has a structure very similar to EDC but does not contain its reactive diimide functional group. Other oligonucleotides that were efficiently immobilized by this method include 5′ biotin T_{25} (results not shown). All of these results taken together strongly suggest that the immobilization is noncovalent. Rather, the oligonucleotides are probably immobilized by a combination of hydrophobic and ionic interactions to the polystyrene surface. The need for the presence of ⩾50 mM salt to achieve immobilization seems to support this, because hydrophobic interactions are stronger at higher salt concentrations.

Whatever the exact mechanism of immobilization, oligonucleotide molecules are attached to the surface with sufficient stability and are not removed by a relatively short (1 hr) treatment with 0.1 N NaOH. Moreover, the immobilized oligonucleotides can participate efficiently in hybridization reactions. Although shorter oligonucleotides can also be immobilized, a length of ⩾12 bases was found to be required to be able to hybridize efficiently to the single-stranded PCR templates. Thus, it is very likely that portions of the immobilized molecules are inaccessible to hybridization because they are involved with interactions with the polystyrene surface.

Preparation of Single-stranded DNA after PCR

Double-stranded PCR products do not hybridize to the immobilized capture oligonucleotides without prior denaturation by heat or alkali. However, in our laboratory the efficiency of hybridization was found to be relatively low even after such a denaturation step. Similar results have also been reported by others.[18] Also, these denaturation steps are difficult to control, especially when a large number of PCR reactions have to be analyzed simultaneously. Initially, we used asymmetric PCR [19] to produce the required single-stranded fragments. This method generates single-stranded PCR products only linearly, and we found the results to be variable.

Another known method for the generation of single-stranded PCR products requires the use of one 5′ phosphorylated PCR primer and exploits the preference of the bacteriophage λ exonuclease for phosphorylated over nonphosphorylated 5′ ends of double-stranded DNA.[20] A major operational disadvantage of this method was the need for an additional buffer exchange step after the PCR step, because the λ exonuclease has a relatively narrow activity optimum at a pH of ~9.4. Nonphosphorylated 5′ ends are also attacked by λ exonuclease, albeit at a slower rate, and thus are not completely protected from degradation.

We have developed a new, efficient approach for the preparation of full-length, single-stranded PCR products following a regular exponential amplification.[21] In this method, the PCR primer for the desired strand is modified at its 5′ end by the introduction of four phosphorothioate bonds. These phosphorothioates provide complete protection of the initial double-stranded PCR product from the hydrolytic action of the double-strand-specific, 5′ → 3′ exonuclease T7 gene 6 exonuclease. The opposite, nonprotected strand is hydrolyzed. Because this exonuclease has a high activity in PCR buffer conditions, it is simply added to the reaction mixture after amplification and the exonuclease reaction is allowed to proceed for 1 hr at room temperature or for 15–30 min at 37°C. No further purification steps are then required before the hybridization to the capture oligonucleotide, which now proceeds with high efficiency.

In phosphorothioate analogs of nucleic acids one of the nonbridging oxygen atoms at phosphorus is replaced by sulfur. Because of the well-known resistance to nuclease activity of oligonucleotides containing phosphorothioate bonds, such analogs are attracting significant interest as potential antisense inhibitors of gene expression.[22] This exonuclease resistance has also been exploited in a number of important experimental procedures such as site-directed mutagenesis[23] and the preparation of single-stranded restriction endonuclease fragments.[24] The use of oligonucleotides containing one phosphorothioate bond at their 3′ end as PCR primers has been described.[25] The presence of one single phosphorothioate bond in these primers inhibited the 3′ → 5′ exonuclease activity of the thermostable polymerases used and led to significantly improved outcome of the PCR amplifications using these enzymes. During the preparation of our paper a publication describing the use of PCR primers containing several phosphorothioate residues at the 5′ end followed by treatment of the double-stranded PCR

products with T7 gene 6 exonuclease appeared,[18] but no details were given on the number of phosphorothioate bonds required for adequate protection.

The present method for the conversion of double-stranded PCR products into single-stranded should be useful for a number of applications. One obvious application is the preparation of sequencing templates following the amplification reaction. Also, the single-stranded products should be very well suited as labeled probes. A recent publication demonstrates the use and advantages of PCR-derived, single-stranded DNA probes for in situ hybridizations.[26] Other applications, such as the detection of mutations by single-strand conformation polymorphism (SSCP), could benefit from this method, as no thermal denaturation is required.

An intriguing potential application would involve the placement of phosphorothioate bonds at the 3′, rather than the 5′, end of the PCR primers. Upon treatment with T7 gene 6 exonuclease, the 5′ unmodified parts of the double-stranded PCR products will be degraded up to the phosphorothioate bonds. The resulting product can be either single-stranded or double-stranded, depending on whether only one PCR primer contained phosphorothioates, or both. In both cases, assuming a very high efficiency of the exonuclease reaction, the resulting products should not be reamplifiable in a subsequent PCR that uses the same primers, as the parts of the molecule where the primers should hybridize will have been destroyed. This could constitute an alternative method to preventing PCR cross-contamination.

Development of a Microtiter Plate PCR Product Detection Assay

PCR is widely used in a number of clinical applications.[27] There is significant interest in the development of nonradioactive approaches for the detection of PCR reaction products that will allow the rapid assessment of the outcome of a large number of amplification reactions. A number of such nonradioactive, microtiter plate-based detection methods have been developed recently.[2–12] Many of these methods use labeled PCR primers and, therefore, various PCR artifacts such as primer–dimers are not easily distinguished from the desired specific amplification product. The methods of Holmstrom et al.[2] and of Newman et al.[3] suffer from the same disadvantage. In the former method, digoxigenin is incorporated into the PCR product during elongation, and the product captured by the biotin residue at one of the primers. In the latter method, single-stranded 5′ tails are introduced in double-stranded PCR products by the synthetic incorporation of modified residues in the PCR primers that prevent the polymerase extension. One of the resulting single-stranded tails is then used to capture the PCR product by hybridization, and the other to hybridize to an alkaline phosphatase-labeled detection probe.

In more specific methods, the PCR products are hybridized to a detectably labeled probe that hybridizes to an internal region of the amplified product. False-positive results arising from the detection of primer-dimers are therefore eliminated. In one of these methods the hybridizing probe is covalently attached to the plate,[4] and used to capture the labeled complementary strand of the PCR product. The main disadvantage of this method is the cumbersome procedure for attachment of the probes to the plates, as well as the relatively high cost of the modified plates (sold as Covalink plates). In the method of Kohsaka et al., one of the strands of the PCR product is captured by covalent binding to carboxylate-modified plates using one 5′ amino-modified primer and subsequently detected after hybridization to a labeled probe.[5] Here, the post-PCR removal of excess primers is required, because otherwise they would compete in the binding step with the PCR product. In a conceptually similar approach, the biotinylated strand of the PCR product is affinity captured on a coated plate and detected following probe hybridization.[7] Here, too, excess biotinylated primer will compete with the PCR product. In addition, in many of these methods the denaturation of the double-stranded PCR products by heat or alkali is required prior to the hybridization. This decreases the efficiency of the latter and makes the processing of large number of samples difficult.

In our method, a separate denaturation step is not required. Rather, the initially double-stranded PCR product, protected at one 5′ end by four phosphorothioates, is converted to a single-stranded template by treatment with T7 gene 6 exonuclease. This conversion is conveniently carried out at room temperature. The phosphorothioated strand also contains a detectable biotin residue. No modifications of the PCR protocols and no purification of the PCR product are required. Thus, the processing of a large number of samples using commercially available liquid handlers is possible.

In our laboratory we are using this solid-phase capture of single-stranded PCR products as a first step in our method for nonradioactive typing of single nucleotide DNA polymorphisms. In this approach, using a DNA polymerase, the 3′ end of the capture oligonucleotide is enzymatically extended in a template-directed reaction by one labeled dideoxynucleoside triphosphate.[28] This not only demonstrates the accessibility of the immobilized oligonucleotides for hybridizations but also for enzyme-catalyzed reactions and broadens the applicability of the solid-phase immobilization/single-strand preparation techniques described in this paper.

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