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Detection of known mutation by proof-reading PCR

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ABSTRACT

Proof-reading PCR (PR-PCR) is designed to detect known mutations within genomic DNA. It differs from standard PCR approaches in that one of the two primers has its 3′ end aligned with a putative mutation site, and has its 3′-OH replaced by a blocking group. Distinguishing a mutant gene from wild-type depends upon preferential removal of the blocked 3′ terminal nucleotide by the polymerase proof-reading activity when it is mismatched with the template. Preferential removal of the blocked nucleotide allows subsequent extension and selective amplification, and provides the basis for distinguishing mutant from normal genes. This method has been used here to detect a transition mutation within the *P53* gene of HaCaT cells with verification by direct sequencing of the selectively amplified DNA.

An ever increasing number of genes that cause inherited and acquired diseases continue to be cloned. Many of these diseases are a consequence of defined mutations where rapid detection for DNA-based diagnosis is becoming increasingly important for monitoring disease, for better defining informed therapeutic options, for counseling and for establishing prevention strategies. Several approaches including restriction enzyme analysis of PCR products (1), allele-specific PCR (AS-PCR) and its derivatives (2,3) and combined chain reaction (CCR) (4) have been developed to detect known mutations in genomic DNA. Here we describe a specialized modification of PCR, proof-reading PCR (PR-PCR), for detecting known mutations.

Fidelity of DNA replication *in vivo* is achieved, in part, by the proof-reading activity of the DNA polymerase. When an errant nucleotide is incorporated and forms a mismatch with the template, it is removed by a 3′ to 5′ exonuclease proof-reading activity associated with the polymerase. Some thermostable DNA polymerases have proof-reading activity, a characteristic desirable for accurate DNA amplification and for PCR amplification of long DNA sequences (5,6). The thermostable DNA polymerase most widely used for PCR is Taq polymerase, which lacks proof-reading activity.

Efficient proof-reading by the thermostable DNA polymerase is an essential element for PR-PCR (Fig. 1). After denaturation of the target, the two primers are annealed with target sequences. One of the two primers used for amplification is designed to have its 3′ terminal nucleotide aligned with the putative mutation site; however, the terminal 3′-OH group, which is required for the formation of the next phosphodiester bond, is replaced by a blocking group that prevents oligonucleotide extension unless it is removed. If the blocked nucleotide is mispaired, it will be removed by the proof-reading activity, permitting primer extension and subsequent target DNA amplification. In contrast, if the 3′ terminal nucleotide is faithfully paired with the template DNA, the blocked nucleotide will be removed inefficiently, if at all, and target DNA amplification will be minimal. Thus, differential amplification is achieved as a consequence of the varying efficiencies of removing the blocked nucleotide. A small difference in amplification efficiency during in a single cycle is greatly magnified over 35 cycles, such that the overall difference in amplification can be visualized by ethidium bromide staining after agarose gel electrophoresis.

A known mutation in *P53* of HaCaT cells was targeted for detection by PR-PCR. The HaCaT cell line was established from a human squamous carcinoma (7) and is a compound heterozygote with two different mutant *P53* alleles (8). The mutation targeted for detection was a dinucleotide substitution CC to TT at codon 281–282 in exon 8. There are no mutations in exon 5 through 9 of *P53* of human fibrosarcoma HT1080 cells (9). Since PR-PCR selectively amplifies alleles with a mismatch at the 3′ blocked nucleotide, the targeted mutant allele in HaCaT cells should be amplified with the reverse primer ‘W’ that hybridizes perfectly with the wild-type allele but not with the mutant. In contrast, the normal allele should be amplified with reverse primer M that is a perfect match with the mutant but not wild-type allele. Our prediction was that PR-PCR should amplify *P53* of HaCaT cells with either primer W or primer M because each of these primers forms a mismatch with one of the two alleles because of the compound heterozygosity at this locus. However, *P53* in genomic DNA of HT1080 cells should be amplified only with primer M because the blocked 3′ end of this primer forms a mismatch with the template.

Since Taq DNA polymerase does not have the required proof-reading activity for PR-PCR, it cannot excise the 3′ blocking group, thereby preventing amplification. As shown in Figure 2a, Taq DNA polymerase can only amplify *P53* of HaCaT cells with an unmodified wild-type but not 3′ blocked primer, indicating the requirement for proof-reading activity for PR-PCR.

PR-PCR was tested for its ability to distinguish the normal *P53* allele from a mutant allele containing a CC to TT transition in one of the HaCaT chromosomes. The HT1080 *P53*, which has two

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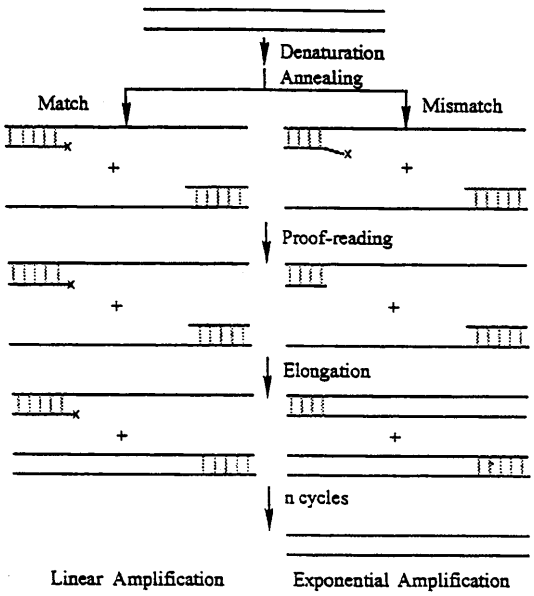

Figure 1. Schematic diagram of PR-PCR. As with standard PCR, two primers are used for amplification; however, with PR-PCR the terminal 3′-OH of one of the primers is replaced with a blocking group (x) which prevents exponential amplification in the absence of removal of the blocked terminal nucleotide. To effect excision of the blocked nucleotide, PR-PCR requires use of a DNA polymerase with proof-reading activity. If the blocked 3′ nucleotide forms a mismatch with the template, the blocked nucleotide will be removed by the proof-reading activity allowing primer extension and amplification of the target sequence. If the blocked 3′ nucleotide hybridizes faithfully with the template, the blocked nucleotide will be removed inefficiently, impeding exponential amplification.

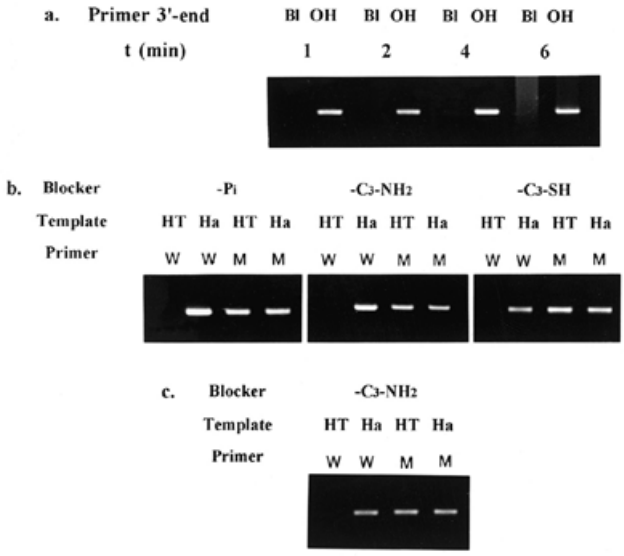

Figure 2. Detection of known *P53* mutations by PR-PCR. (**a**) Inability of Taq DNA polymerase (GIBCO-BRL) to extend a primer with a blocked 3′ nucleotide. PCR was carried out with Taq DNA polymerase with HaCaT *P53* as a target sequence. Bl and OH designate identical primers that differ only in their -C_3-NH_2 blocked (Bl) and normal (OH) 3′ ends. All reactions were performed in 50 µl containing 1.25 U Taq enzyme in 20 mM Tris-HCl (pH 8.4), 2.0 mM $MgCl_2$, 100 µg/ml acetylated BSA, 25 mM KCl and 0.2 mM each of NTP. Denaturation was carried out at 94°C for 30 s and annealing and elongation was performed at 65°C for 1, 2, 4 or 6 min. Forward primer: CGTTCACCGAGGACTGGACC; Reverse primer: AGATTCTCTTCCTCT-GTGCGCCGG. (**b**) PR-PCR detection of a genomic dinucleotide substitution in *P53* using Pfu polymerase (Stratagene, La Jolla, CA). Three blocking groups, -Pi (Perkin-Elmer), -C_3-NH_2 and -C_3-SH (Glenn Research), were compared for efficacy for detection of a CC→TT mutation in genomic *P53* of HaCaT cells. Template HT and Ha represent pre-amplified DNA containing *P53* from HT1080 and HaCaT cells respectively. External primers p53 Pi and p53 P2 are complementary to nucleotides 14 086–14 106 and 14 696–14 950 in the *P53* gene, respectively. W and M designate the diagnostic primers that match faithfully with wild-type and mutant alleles, respectively. The W primer is the wild-type reverse primer presented in the (a) legend above. The mutant reverse primer, M, is identical but with the 3′ GG replaced with AA. The reaction conditions were as above except that Taq polymerase was replaced with 2.0 U Pfu. As predicted, HT1080 *P53* amplification is prevented using wild-type primer blocked with any of the three blocking groups. (**c**) Identification of a single nucleotide transition (C→T) by PR-PCR. Wild-type (W) or mutant (M) primers blocked with a -C_3-NH 3′ blocking group were used to amplify *P53* DNA of HT1080 cells (HT) or HaCaT cells (HA) using Pfu polymerase. The HT1080 *P53* was amplified with blocked mutant primer, but not with blocked wild-type primer, whereas HaCaT *P53* was amplified with both due to heterozygosity at this site. Forward primer: CGTTCACCGAGGACTGGAC; Reverse primer: AGATTCTCTTCCTTGTGCTGCCG.

normal alleles at the site targeted, was used as a control. Three blocking chemistries, -3-NH_2, -C_3-SH and -P_i, were tested for efficacy. Selective amplification was achieved with each of these blocking groups (Fig. 2b).

As predicted, HaCaT *P53* was amplified with either blocked wild-type or blocked mutant primer because *P53* is heterozygous at this site. In contrast, HT1080 *P53* was amplified only with blocked mutant primer because HT1080 cells have two normal alleles at the targeted site (Fig. 2b). Although each of the 3′ blocked primers tested gave similar amplification patterns, they did show differences, most significantly in ease of excision by 3′→5′ exonuclease activity. Of the three blockers tested, -C_3-SH was the most easily removed, -P_i was the most resistant, and -C_3-NH_2 was intermediate (data not shown). Chemical structure, charge or interaction with template and DNA polymerase may all contribute to these differences.

To establish that PR-PCR can detect single nucleotide mutations, PR-PCR was used to identify the C→T transition at codon 282 (nucleotide 14513) at *P53* of HaCaT cells (Fig. 2c). As expected, *P53* from both HaCaT and HT1080 DNAs was amplified when the blocked mutant primer was complementary to the HaCaT mutant allele. However, only HaCaT *P53* was amplified when the blocked primer was wild-type. Again, HaCaT *P53* amplification with blocked mutant primer was expected since HaCaT *P53* is heterozygous, with one of the alleles having wild-type sequence at the targeted site. To verify selectivity of DNA amplification by PR-PCR, HaCaT genomic DNA was amplified with either wild-type or mutant *P53* primer blocked at its respective 3′ end. The PR-PCR products were purified and subjected to automated sequencing. The DNA sequence confirmed that the amplification was selective (Fig. 3). Only the mutant allele was amplified with the 3′ blocked wild-type primer, while the wild-type allele was amplified with the 3′ blocked mutant primer. Thus, PR-PCR can detect a known mutation in genomic DNA efficiently and with high precision.

The introduction of PCR has made possible the selective amplification and analysis of specific DNA sequences from very small amounts of DNA. An additional challenge has been the ability to selectively amplify a mutant sequence from a mixed population of mutant and wild-type DNAs. One approach that has

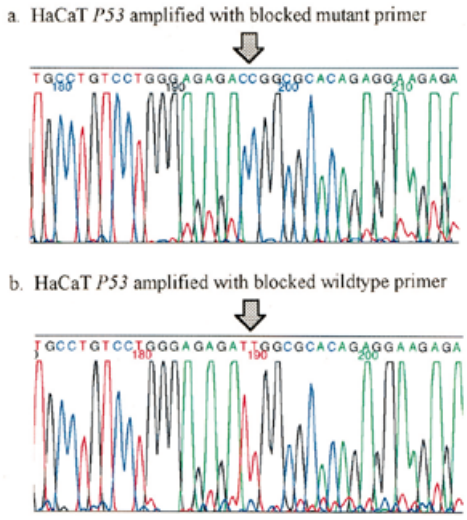

Figure 3. Sequence verification of PR-PCR selective amplification. Genomic *P53* from HaCaT cells was amplified by PR-PCR. The products were purified by Wizard Magic miniprep kit (Promega) and sequenced using an ABI DNA automated sequencer. (**a**) Selective amplification of the wild-type allele with mutant primer blocked with -C_3-NH_2. (**b**) Selective amplification of the mutant allele with blocked wild-type primer. The arrows identify the target nucleotides.

shown some success is AS-PCR, which can differentiate some mutant genes from wild-type in mixed populations. However, AS-PCR also has its limitations since four out of the 12 potential 3′ mismatched ends cannot be elongated effectively (10), and errant elongation leads to false negative or positive results. AS-PCR may extend mismatched primer and produce artifactual templates. In contrast, PR-PCR is based on a different premise and offers an alternative way for achieving selective amplification. In order to achieve substantial amplification with PR-PCR, the blocked 3′ nucleotide must be efficiently excised. The preferential removal of the blocked 3′ nucleotide at a mismatched end compared with a matched end occurs during each cycle, so that the accumulative effect over multiple cycles is very large. An additional difference is that in PR-PCR, the actual primer elongation starts at the potential mutation site instead of the neighboring nucleotide as in AS-PCR. Furthermore, the proof-reading activity of Pfu DNA polymerase makes it less likely for unwanted mutations to be introduced than with Taq or other thermostable polymerases that lack proof-reading activity. Given the increasing demands for DNA diagnostics for research, forensics and the clinical setting, development of reliable DNA testing methods that are accurate, sensitive, rapid, non-toxic and amenable to automation remains a continuing challenge. To this end, the strategy employed by PR-PCR provides a promising approach because amplification can be designed to be highly selective, and because procedures and manipulations are simple.

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