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L G Puskás and S Bottka

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METHODS AND APPLICATIONS

Reduction of Mispriming in Amplification Reactions with Restricted PCR

László G. Puskás and Sándor Bottka[1]

Biological Research Centre, Institute of Plant Biology, Hungarian Academy of Sciences, H-6701 Szeged, Hungary

A simple method is described for reducing nonspecific background, which is caused by mispriming during PCR. Besides the standard pair of primers, 3′-dideoxy-terminated competitor oligonucleotides were added to the amplification. Sequences to those of the primers which had identical base. In this way enhanced specificity was achieved. The competitor oligonucleotides may act by masking possible sites of nonspecific primer–template interaction, thus excluding undesired chain extensions. This technique is generally applicable when highly degenerate primers are used and therefore expands the potential of "restricted" PCR.

Failure of amplification of a specific sequence, or nonspecific amplification products caused by mispriming during PCR, represents the most commonly occurring problems in many kinds of applications. The purity and yield of the reaction products depend on several parameters, such as annealing temperature, Mg^{2+} concentration, pH, template purity, and concentration. Methods have been developed to optimize these factors. The specificity can be increased in almost all cases,[1,2] though the process is laborious and time-consuming. Optimization is especially important when degenerate primers are used or when total genomic DNA is the substrate for the amplification. Despite such efforts for optimization, specific amplification may not be achieved with some template/primer combinations. In these cases, only new sets of primers can lead to success.

Recently, we have developed the restricted PCR method for amplifying a specific sequence flanked by a highly repetitive element from total human DNA.[3,4] In this method the amplification is performed with only one specific primer in conjunction with two consensus Alu primers bearing the same sequence. One of the Alu primers terminates with a dideoxynucleotide at the 3′ end to prevent extension by the polymerase. The undesired, concomitant Alu–Alu amplification is suppressed by adding the 3′-terminated competitor primer to the reaction mixture in the appropriate ratio, because one Alu primer is necessary for specific extension, and two primers are necessary for nonspecific extension.

The method described here utilizes 3′-terminated oligonucleotides as well. It can be applied in combination with any freely chosen primer pair. The competitors can potentially inhibit the nonspecific side reactions originating from unwanted annealing of specific primers in any standard polymerase reaction, therefore significantly broadening the applicability of restricted PCR.

MATERIALS AND METHODS

Competitor Oligonucleotides

3′-Terminated oligonucleotides were prepared from normal deoxyoligonucleotides [synthesized with Expedite DNA Synthesizer (PerSeptive Biosystems)] with the following protocol. Primer oligonucleotide (200 pmoles), was treated with a 10-fold molar excess of the appropriate 2′,3′-dideoxynucleoside 5′-triphosphates (ddNTPs) and 10 units of terminal nucleotidyl transferase (Pharmacia). Two equivalents of the appropriate 2′-deoxynucleoside 5′-triphosphates (dNTPs) were also added to the reaction mixture. After 2 hr of incubation at 37°C the reaction was heat-inactivated and purified with Sephadex G-25 (Pharmacia).

PCR

Amplification of the intrinsic region of exons 21 and 22 of the retinoblastoma (RB) gene was performed with 50 pmoles of both a specific primer (Rb1, 5′-TCAAAACACGTTTGAATGTC-3′) and a degenerate primer (Rb2, 5′-ATGATGT-GYTCIATGTAYGG-3′), with or without mixed competitor Rb2 primers (3 or 6 pmoles), with 50 ng of human genomic DNA, 200 mM dNTP, and 1 unit of *Taq* polymerase (Amersham) in the presence of 1.5 or 2.5 mM $MgCl_2$ for 30 cycles at temperatures of 94°C for denaturation (30 sec), 47°C for annealing (30 sec), and 72°C for extension (90 sec) in a total volume of 50 μl.

Amplification of the same region of the RB gene was performed with Rb2 and Rb3 (5′-TCAAIACACGITTGAIT-GTC-3′) primers (50 pmoles of each) with or without 2 pmoles of competitor Rb2 and/or 2 pmoles of competitor Rb3 primer, with 50 ng of human genomic DNA, 200 mM dNTP, 0.5 unit of *Tfl* polymerase (Epicentre Technologies), and 1.5 mM $MgCl_2$ for 25, 30, or 35 cycles at temperatures of 94°C for denaturation (30 sec), 45°C for annealing (30 sec), and 72°C for extension (90 sec) in a total volume of 50 μl.

Amplification of the intrinsic region of exon 1 and 2 of Nms-25, a nodule-specific gene of *Medicago sativa*, was performed with Nm1 (5′-CATGTIYTIGCI-TATAAYATGC-3′) and Nm2 (5′-GCT-CTTITGGTCRTCIARTG-3′) primers (50

[1]Corresponding author.
E-MAIL bottka@everex.szbk.u-szeged.hu; FAX (36)62433434.

pmoles of each), with or without 2 pmoles of competitor Nm1 and/or 2 pmoles of competitor Nm2 primer, with 40 ng of *M. sativa* genomic DNA, 200 mM dNTP, 1 unit of *Tfl* polymerase, 3% dimethylsulfoxide (DMSO), and 1.5 mM $MgCl_2$ for 35 cycles at temperatures of 94°C for denaturation (40 sec), 46°C for annealing (40 sec), and 72°C for extension (75 sec) in a total volume of 50 µl.

RESULTS

To reveal the power and scope of this technique, we performed several PCRs with different primer pairs and template DNAs of diverse origin. Besides the standard pair of primers, we also added 3′ end-terminated oligomers having identical sequences to those of the primers. End-terminated oligonucleotides were synthesized by enzymatic linking of all four dideoxynucleotides to the primers except the first coupled nucleotide of the specific extension in the 3′ direction.

In the first example, we chose the human RB susceptibility gene as a target. To model a real case, degenerate- and deoxyinosine-containing primers were applied. We amplified the intrinsic region of exon 21 and 22 of the *RB* gene with a specific primer, Rb1, and a degenerate primer, Rb2, which contains one deoxyinosine, and 2 mixed bases at two other positions. Without optimization, amplification was very weak (Fig. 1, lane 1). By increasing the Mg^{2+} concentration, the desired product was obtained, although an additional nonspecific band appeared (Fig. 1, lane 4) as the main amplification product. This band was not seen in separate reactions with Rb1 and Rb2. This nonspecific amplification was suppressed by the addition of terminated Rb2 primers to the reaction mixture. The first nucleotide added to the 3′ end of the Rb2 primer by the polymerase is C during the specific reaction.[5] Accordingly, we extended the Rb2 primer with ddATP, ddGTP, and ddTTP. Two equivalents of dATP, dGTP, and dTTP were also added to the reaction mixture to generate terminated molecules of greater length, which can hybridize more efficiently to their targets and at higher temperature, than unmodified ones. dCTP and ddCTP were omitted to avoid blocking the expected process. As shown in Figure 1, by using these competitor primers, a considerably greater amount of specific product was obtained under normal amplification conditions.

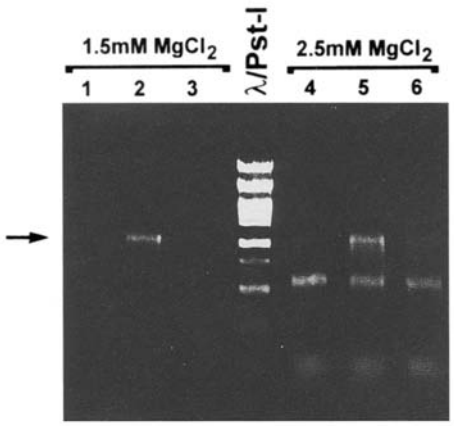

FIGURE 1 PCR products (1200 bp) are indicated by an arrow, after separation by agarose gel (0.8%) electrophoresis and ethidium bromide staining. Reactions contained mixed Rb2 competitor primers. (Lanes *2,5*) 3 pmoles; (lanes *3,6*) 6 pmoles; (lanes *1,4*) no competitor primer. The reaction mixtures contained either 1.5 or 2.5 mM $MgCl_2$.

Optimal amplification was reached with 3 pmoles of the terminated oligonucleotide mixture. Increasing the Mg^{2+} concentration resulted in a higher ratio of nonspecific products. The isolated fragment was cloned into Bluescript vector (Stratagene). The nucleotide sequences of the two flanking regions of the amplification product were identical to the published one.[5]

The same sequence was also amplified with two degenerate primers, Rb2 and Rb3. The latter 20-mer contained three deoxyinosine residues. Reactions were performed with four different compositions of primers: without the addition of terminator primers (Fig. 2, lane 1), with addition of terminated Rb2 (lane 2), terminated Rb3 (lane 3), or both terminated Rb2 and Rb3 (lane 4). With the standard composition, the amount of the expected product just exceeded the level of detection. The second and third combinations gave significantly higher yields compared with that of the first one, whereas the fourth combination provided only a modest yield. The product was found to be identical to the one amplified previously, as determined by sequencing (data not shown). The effect of the number of cycles with the Rb2/Rb3 primer pair was also examined. Using both competitor primers, the specific product appeared even after 25 cycles (Fig. 3, lane 1), whereas a very faint band could be observed only after 30 cycles of PCR without competitor primers (lane 2). A sharp difference in the yield of the specific product could be detected after 35 cycles without or with competitor Rb2 and Rb3 primers (lanes 4,5).

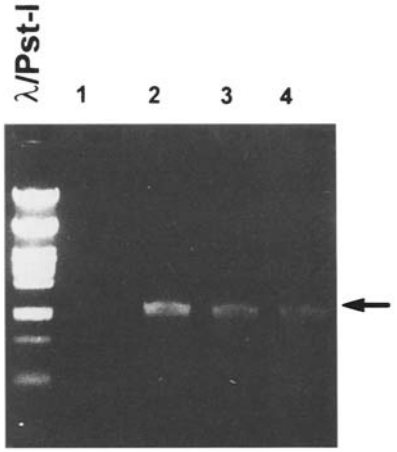

FIGURE 2 Amplification of the intrinsic region of exons 21 and 22 of the *RB* gene with Rb2 and Rb3 primers (50 pmoles of each). (Lane *1*) No competitor; (lane *2*) 2 pmoles of competitor Rb2; (lane *3*) 2 pmoles of competitor Rb3; (lane *4*) 2 pmoles from both of the competitor primers. Each lane contains 12 µl of the PCR mixture.

In another example, we amplified the 950-bp fragment of Nms-25, a nodule specific gene from total genomic DNA of *M. sativa*. Simulating a real case, we designed our primers on the basis of the amino acid sequence corresponding to the region of exons 1 and 2. In almost every ambiguous position, mixed bases or deoxyinosine were used. Nm1 primer,

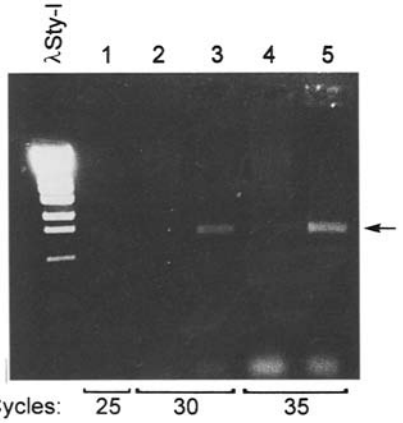

FIGURE 3 Amplification of the intrinsic region of exons 21 and 22 of the *RB* gene with Rb2 and Rb3 primers (50 pmoles of each). (Lanes *2,4*) No competitor; (lanes *1,3,5*) 2–2 pmoles of competitor Rb2 and Rb3 primers for 25, 30, or 35 cycles. Each lane contains 11 µl of the PCR mixture.

complementary to a region of exon 1, contains 3 deoxyinosine and 2 mixed bases, whereas Nm2 primer, complementary to a region of exon 2, contains 2 deoxyinosine and 3 mixed bases. With this degenerate primer pair and standard cycling protocol, amplification of the target region failed. Further efforts to optimize the reaction (annealing temperature of 46°C, 48°C, or 50°C; Mg^{2+} concentration of 1.5 mM or 2.5 mM) were also unsuccessful. In the preparation of Nm1 competitor primer it was considered that the last amino acid encoded by Nm1 is arginine. The second base in the codon of arginine is G, so we terminated Nm1 with ddA, ddC, and ddT to block the nonspecific reactions where the first base is not G during the elongation of the polymerase. In the case of Nm2, we mixed ddATP, ddCTP, ddGTP and dATP, dCTP, and dGTP to the termination reaction. The last amino acid encoded by Nm2 is threonine, and its first complementary base is T; therefore, during the specific reaction the first nucleotide in the 3′-end on Nm2 is T. We could detect the 950-bp fragment by using both of the competitor primers (Fig. 4, lane 4), but the fragment could not be detected when a competitor was not used (lane 1) or when a competitor was used from only one side (lanes 2,3).

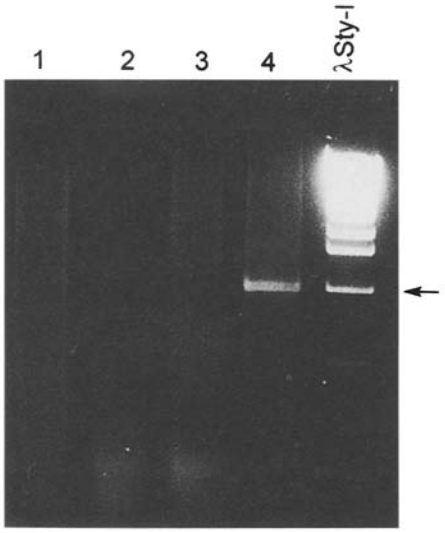

FIGURE 4 The PCR product (950-bp) of the Nms-25 gene of *M. sativa* is indicated by an arrow. Amplification was performed with Nm1 and Nm2 primers. (Lane *1*) No competitor; (lane *2*) 2 pmoles of competitor Nm1; (lane *3*) 2 pmoles of competitor Nm2; (lane *4*) 2 pmoles from both of the competitor primers.

DISCUSSION

Degenerate primers having mixed bases or deoxyinosine at ambiguous positions are useful when the DNA sequence that encodes a certain amino acid sequence is unknown. The background of these reactions is usually high, especially when genomic DNA or a complete cDNA library is the template. Thus, blocking nonspecific amplification should result in improved amplification. The primary goal of our work was to develop a new procedure to reduce nonspecific amplification. Accordingly, the targeted sequences for PCR were chosen from well-characterized genes instead of by making efforts to isolate new ones. In this way, the results could be controlled directly. The primer design was based on amino acid sequence to mimic real conditions.

The intrinsic region of exons 21 and 22 of the *RB* gene was amplified with two different primer pairs. We obtained the desired product in both cases with low yield and high background. Optimization resulted in only a modest increase in yield; a much higher yield was obtained using competitor primers. When we used Rb2 and Rb3 primers together with competitors from both sides, a smaller amount of product could be detected. These results can be explained in terms of the proposed mechanism of restricted PCR,[3,4] that is, the competitor primers inhibit the nonspecific amplification and thus raise the yield of specific product when restriction occurs from one side only. In the case of double-sided restriction, when two sets of competitors are used, selectivity is probably enhanced further; however, the formation of the specific product is also retarded. This problem can be circumvented simply by raising the number of the cycles or the volume of the reaction.

A further example was provided when we amplified part of the Nms-25 gene of *M. sativa*.[6] Amplification of the complex genome of this tetraploid organism as a template in PCR often represents a difficult task. The 20- and 22-nucleotide primers failed to produce the specific product, whereas the same oligonucleotides in combination with competitor primers succeeded without preliminary optimization. These results suggest that the diverse side reactions are blocked much more effectively by the competitors than the specific reaction. This may result in the overall enhancement of the specific reaction. The only prerequisite of using restricted PCR is the knowledge of the nucleotide following the primer at 3′ direction. It can be also used when only a partial information is known concerning this position. In such cases, only one or two dideoxynucleotides are applied to prepare the terminated oligomers. In these cases, partial inhibition will be achieved, and the restriction will be less effective.

Restricted PCR was originally limited to the amplification of specific sequences flanked by highly repetitive elements. The procedure described here largely broadens the scope of this method. Enhanced selectivity and/or higher specific yields can be expected also with highly degenerate primers. It can be suggested as a further possibility to convert an unsuccessful PCR into a successful one without new primers.

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