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Short Communication

Faster and cheaper PCR on a standard thermocycler*

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The PCR conditions have been optimized to make the process faster and more economical. When short DNA fragments are to be amplified, the time of denaturation, annealing and extension steps can be as short as 1 s each, and the yield of PCR product is still high, sufficient for many types of analysis. The PCR can be done even in a reaction volume as low as 1 µl. The recommended volume, 2.5 µl or 5 µl, allows significant savings in the laboratory budget especially for laboratories which use PCR frequently and on a large scale.

Many procedures of molecular genetics call for repeatable running of numerous PCR reactions. This applies, for example, to genetic [1] and physical [2] mapping and searching for mutations in disease genes by SSCP analysis [3, 4]. Large scale of analysis makes it important to consider such factors as length of the PCR process and its costs. Rapid cycle DNA amplification in capillary tubes has been described [5, 6] but it requires special instrumentation. Here we present some simple ways to increase the throughput of PCR on a typical Perkin Elmer DNA thermal cycler, and to make the analysis significantly cheaper. For many applications this can be done as shown below by shortening, to the necessary minimum, all repeated incubation steps and by substantially reducing the reaction volume. As an extreme example we show the result of the so called "4 × 1 PCR" in which the time of denaturation, annealing and extension steps is one second each and the reaction volume is 1 µl. The amount of PCR product obtained in this way is sufficient to be visualized on EtBr-agarose gel and to be used for several runs of SSCP analysis.

MATERIALS AND METHODS

DNA templates. The PCR cycling parameters have been optimized using λgt10 clones from human breast cancer cDNA library (Clontech). Fifty random clones were picked from LB-agar plate and DNA was eluted to 1 ml of water. Aliquots of the solutions were used as phage DNA templates for PCR. Four clones with inserts of appropriate length were selected for further experiments. Genomic DNAs from breast cancer tissues were isolated using a standard procedure involving proteinase K-digestion and phenol extraction, and brought to the concentration of 50 ng/µl.

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Abbreviations used: kbp, kilobase pairs; OPC, Oligonucleotide Purification Cartridge; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism.

PCR. PCR primers were synthesized on model 392 DNA/RNA Synthesizer ABI, and purified on OPC columns (ABI) according to manufacturer's recommendations. The λgt10 primers flanked the *Eco*RI site of the vector. The p53 primers were designed to amplify exon 7 of the gene. DNA amplifications were done on Perkin Elmer DNA thermal cycler type 480. All reagents used for PCR were purchased from Perkin Elmer. For SSCP analysis PCR was done with primers labelled at the 5′ end with [γ^{32}P] ATP 5000 Ci/mmol (Amersham) and T4 polynucleotide kinase (BRL).

SSCP analysis. The labelled PCR products were diluted with 5 volumes of the denaturing solution composed of: 95% formamide, 20 mM EDTA, 0.05% Bromophenol blue and 0.05% Xylene cyanol. That mixture was heated at 85°C for 5 min. Then the denatured DNA samples were loaded on 5% native polyacrylamide gel (49:1) containing 10% glycerol. Electrophoresis was performed at constant power of 0.5 W/cm until Bromophenol blue marker reached the bottom of the gel. The gel was dried and subjected to autoradiography at –80°C with intensifying screen.

RESULTS AND DISCUSSION

The PCR optimization experiments have been done using λgt10 clones with insert lengths of 0.3, 0.6, 0.9 and 1.3 kbp. Two primers corresponding to flanking vector sequences were 5′-GCT GGG TAG TCC CCA CCT TT-3′ (forward) and 5′-CTT ATG AGT ATT TCT TCC AGG GTA-3′ (reverse). Starting from the basic PCR protocol (initial denaturation 94°C for 4 min, then 35 cycles: 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min followed by final extension 72°C for 5 min) we first shortened the denaturation time to 30, 15, 7, and 2 s respectively, leaving all other parameters unchanged. It was observed that at shorter denaturation times the yield of PCR product was even somewhat higher (Fig. 1A). This was most likely due to lower decomposition of Taq polymerase during cycling. The shortening of the annealing step from 2 min to 2 s with 2 s denaturation and 3 min extension did not influence the yield of PCR products (Fig. 1B). However, when the extension step was cut from 3 min to 2 s at 2 s denaturation and annealing, the amounts of 1.3 and 0.9 kbp products were significantly re-

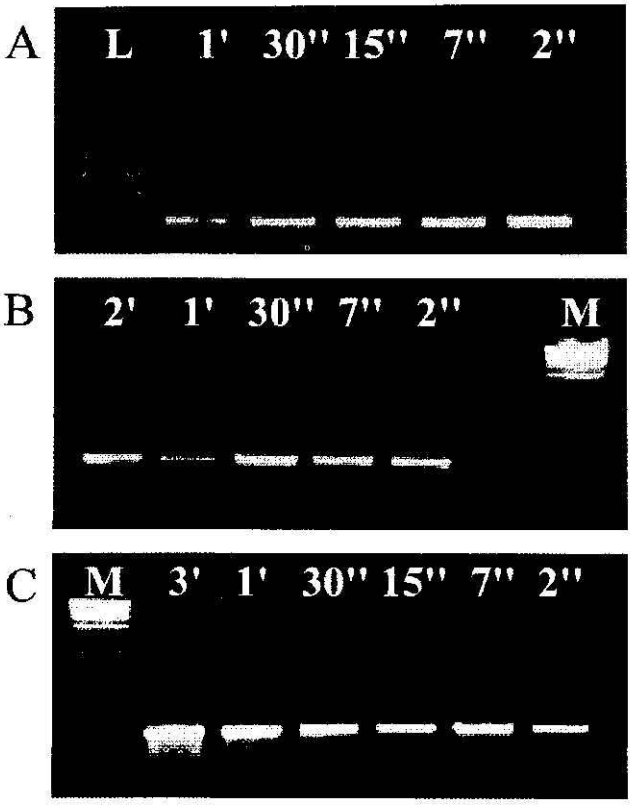

Fig. 1. EtBr-agarose gel electrophoresis of PCR products obtained in optimization experiments for A, denaturation, B, annealing and C, extension steps.
The length of PCR products was 0.3 kbp (A, C) and 0.6 kbp (B). Above each line the time length of the analyzed step is indicated. L, Φ x174/*Hae*III marker; M, λ/*Hin*dIII marker. All reactions were run in 20 μl, 10^4 phages in each, 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatin, 200 μM each dNTP, 1 μM each primer, 0.5 U Ampli Taq DNA polymerase.

duced. The yield of shorter products was also decreased but still remained high (Fig. 1C).

To lower the costs of PCR analysis we took advantage of the experience of the laboratory in which the SSCP analysis has been invented [7, 8]. We cut down the volume of the reaction mixture further to 2.5 µl, in which we run most reactions, or even to 1 µl. This is an important factor as in many PCR analytical applications the amount of product formed is much higher than required for analysis. Usually an individual reaction mixture is prepared by the adding DNA template solution to an aliquot of PCR master mix. It is easy to prepare the 2.5 µl mixture in a 0.5 ml tube by mixing 0.5 µl of DNA with 2 µl of master mix prepared according to the standard Perkin Elmer Cetus DNA amplification protocol. This is covered with 15 µl mineral oil. After thermocycling is finished, the product, which is of small volume, is diluted with H_2O to make a total of 10 µl and removed from the tube after adding 20 µl of chloroform. The results of PCR carried out in different volumes are shown in Fig. 2. It is clearly seen that, on decreasing the reaction volume from 100 to 2.5 µl, there is no drop of the reaction yield.

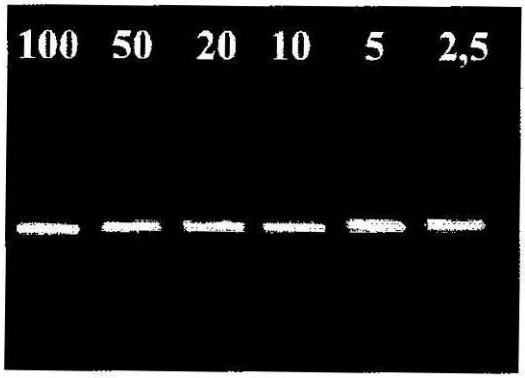

Fig. 2. Amplification of 0.3 kbp λgt10 insert in the decreasing reaction volume specified above each agarose gel line (in µl).
PCR was done with 2 s denaturation, 2 s annealing and 2 s extension. Other parameters were as described in the text. Reaction products were diluted with 5 volumes of H_2O and 5 µl of each was loaded on 0.7% EtBr-agarose gel.

The results shown above have prompted us to apply the above described conditions in our routine search for mutations in tumor suppressor genes by PCR-SSCP analysis. The faster and low volume PCR protocol is especially well suited for subsequent SSCP analysis as the highest mutant detection rate is observed for PCR products which are 150–250 bp long. Figure 3 shows the results of amplification of exon 7 of p53 gene from genomic DNA of a breast cancer patient and SSCP analysis of PCR products obtained from five 1 µl reactions. The cycling conditions were: initial denaturation at 94°C 4 min, 35 cycles of 1 s at 94°C, 1 s at 58°C and 1 s at 72°C and then 5 min at 72°C.

The modified PCR protocol shown here should be useful, especially in large scale PCR applications. With the use of this protocol the cost of all the reagents necessary for a single reaction becomes comparable to the cost of the tube in which the reaction is carried out.

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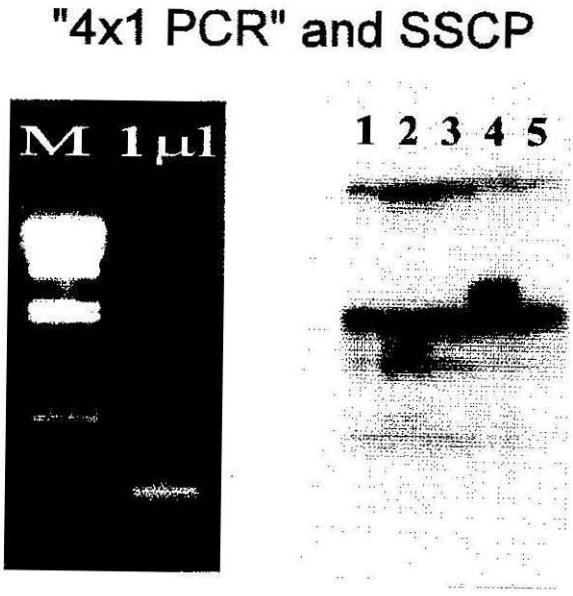

Fig. 3. Results of fast low volume "4 × 1 PCR" (left) and subsequent SSCP analysis (right).
The PCR mix contained 10 ng/µl of genomic DNA; primers used at 1 µM concentration were: 5'-GTG TTG CCT CCT AGG TTG GC-3' (forward) and 5'-CAA GTG GCT CCT GAC CTG GA-3' (reverse), that amplify exon 7 of p53 gene (139 bp). Other components of the PCR mixture were as described in legend to Fig. 1. For SSCP analysis ^{32}P-labelled primers were used. The PCR products were diluted with 9 µl of denaturing solution (95 % formamide, 20 mM EDTA, 0.005 % Bromophenol blue and 0.005 % Xylene cyanol). The mixture was heated at 80°C for 5 min before loading of 2 µl aliquots on 6% polyacrylamide gel containing 10% glycerol. Electrophoresis was run at constant power of 20 W for 3 cm × 30 cm × 0.4 cm gel. Mutations are visible in lanes 2 and 4.

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