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Genome Res. 1992 1: 171-174
Access the most recent version at doi:10.1101/gr.1.3.171

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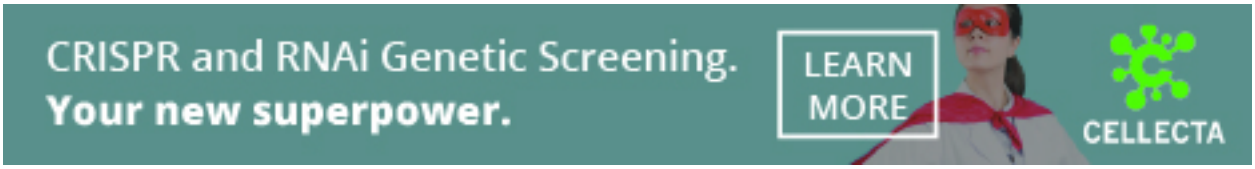

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Reliable and Efficient Direct Sequencing of PCR-amplified Double-stranded Genomic DNA Template

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Modified PCR amplification and direct sequencing procedures for the double-stranded genomic DNA template are described. Advantages of the approach we describe are: background artifact bands previously observed using high-molecular-weight DNA as a template were eliminated by this protocol; no gel purification or subcloning of the PCR-amplified double-stranded fragment was required prior to direct sequencing; and sequences of 300 nucleotides can be easily read even after a single loading. The successful use of the modified dideoxynucleotide chain-termination method for direct sequencing of both strands demonstrates the efficiency of this technique for removing sequencing artifacts and for producing reliable sequence data.

The polymerase chain reaction (PCR) has proven beneficial for biological and clinical studies because of its sensitivity and specificity and of the amplification of the target DNA in microgram amounts within a few hours. However, this method raises concerns about the fidelity of DNA synthesis due to the potential of nucleotide misincorporation, resulting in an artifactual mutational analysis.[1,2] In general, two methods—direct sequencing of the asymmetrically amplified PCR product and sequencing of amplified products in conjunction with subcloning of the single-stranded or double-stranded DNA template into a representative prokaryotic vector—have been used for sequence determination of the PCR product.[3,4] However, when individual PCR products are cloned and sequenced, the one molecule cloned may possibly contain a sequence generated by misincorporation in the PCR product. In addition, the asymmetric amplification procedure is error prone because of the necessary reamplification of the asymmetric product prior to sequencing. Furthermore, the sequence information is restricted to only one strand of DNA, thereby enhancing the chances of inaccurate data analysis. To overcome these and other problems, many modifications of the PCR and sequencing conditions have been applied.[5–7]

In this report, we present data to resolve the problem of misincorporation that is due possibly to an extended PCR and to sequence directly both strands of the PCR-amplified, double-stranded and high-molecular-weight DNA template. The major advantages of our system are: elimination of artifact fragments and base misincorporations, one-step amplification, direct sequencing of both strands of the double-stranded amplified DNA template, and the feasibility of simultaneous generation of multisample data. With these modified procedures of PCR and sequencing, we were able to confirm the previously reported[8] 1300-bp exonic sequence representative of the normal human c-*raf*-1 gene. Due to the relatively high incidence of tandem repeats present within certain introns of this gene, we were unable to subclone these fragments for subsequent sequencing. The development of this direct sequencing procedure has facilitated the sequence analysis of the various introns of c-*raf*-1 in normal DNA and in human tumor DNA samples.[9]

MATERIALS AND METHODS

Double-stranded DNA Amplification

High-molecular-weight DNA (0.5 μg) was amplified through 25 or 30 cycles of PCR in a DNA Thermal Cycler (Perkin–Elmer Cetus). All oligonucleotides were synthesized trityl-on by the solid-phase triester method on an Applied Bio-synthesizer. The reaction products were then cleaved and deprotected by standard protocols using Applied Biosystems Oligonucleotide Purification Cartridges. The oligonucleotide primers (20-mers) were designed based on the calculation of the melting point temperature: T_m (°C) = 4(G + C) + 2(A + T) – 5. The reaction mixture was maintained in ice (4°C) and contained: 0.5 μg of genomic DNA; 100 pmoles of each primer; 200 μM each of dATP, dCTP, dGTP, and dTTP (U.S. Biochemical); 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.01%

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(wt/vol) gelatin; 2 units of the polymerase (*Taq*, U.S. Biochemical); and ddH_20 to a final volume of 100 μl. The reaction sample was overlayed with 50 μl of mineral oil (U.S. Biochemical).

We tested two different PCR conditions as follows. In the first cycle, the samples were denatured at 94°C for 10 min, followed by 2 min annealing at 55°C and 2 min polymerization at 75°C, as described by Saiki et al..[2] Under these conditions, each step of the next 20 cycles lasted for 2 min and each step of the last 9 cycles for 3 min followed by the last extension for 10 min. In our modified assay, the first temperature cycle consisted of an initial denaturation step for 5 min at 94°C followed by 30 sec of annealing at 55°C and 30 sec of extension at 72°C. Each of the additional 24 cycles was performed as follows: 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C followed by 5 min for the last extension. The products were analyzed on a 4% agarose gel [3% Nusieve GTG (FMC) and 1% Seakem GTG (FMC) agarose].

Direct Sequencing Analysis

The PCR-amplified genomic tumor DNA samples were extracted once with chloroform to remove mineral oil and purified by using the Qiagen PCR purification kit. According to an earlier report,[7] the annealing mixture contained 2 μl of the [γ-^{32}P]ATP (3000 Ci/mole, New England Nuclear) 5′-end-labeled oligonucleotide primer (>10^4 cpm/reaction) using T4 polynucleotide kinase (BioLab) in 5x Sequenase reaction buffer [200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl]. The samples were first heat-denatured (100°C) for 5 min and transferred to ice for 5 min. This was followed by the standard Sanger dideoxynucleotide sequencing method,[10] with the labeling step omitted. In our modified method of direct sequencing, the purified PCR samples generated by 30 or 25 cycles were combined from two independent reactions for each template. One-third of this purified PCR sample was mixed with 2 μl (0.5 μg) of the same primer used for PCR amplification and 2 μl of the modified Sequenase 5x reaction buffer containing 200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl, and 2% Nonidet P-40 (NP-40) in a final volume of 10 μl. The reaction mixture was then heat-denatured (100°C, 3 min), followed by a quick chill in powdered dry ice. To the 10-μl annealing reaction mixture, we added 1 μl of 0.1 M DTT, 2 μl of labeling mix (1.5 μM each of dGTP, dCTP, dTTP), 1 μl of 10 μM [α-^{35}S]dATP (1000 Ci/mmole, New England Nuclear), 1 μl of Mn^{2+} buffer (0.15 M sodium isocitrate, 0.1 M $MnCl_2$), and 1.5 units of Sequenase 2.0 (U.S. Biochemical) in ice (4°C). Then 3.5 μl of the mixture was aliquoted into each of the four tubes containing 2.5 μl of respective deoxy/dideoxy nucleotide triphosphate followed by incubation at 37°C for 10 min. The reactions were terminated with stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), heated at 85°C for 3 min, and chilled on ice (4°C). The reactions were analyzed on a 6% polyacrylamide/8 M urea gel. The gel was dried (1.5 hr at 80°C) and exposed overnight to the Kodak XAR-5 film.

RESULTS AND DISCUSSION

Nucleotide base mutations have been implicated in the etiology of a number of human genetic disorders as well as in the activation of some oncogenes[11,12]; therefore, it is critical to verify that the parent DNA contains these diagnostic mutations. The most general application of the PCR amplification method is the investigation of the presence or absence of a particular nucleic acid sequence. However, there are some drawbacks concerning the fidelity of the sequence information obtained. In general, asymmetric PCR or recombination is used for the analysis of the target sequence followed by direct sequencing or subcloning and sequencing of the single- or double-stranded PCR product.

In our experience with the sequence determination of the relatively long (>2-kb) fragments of high-molecular-weight genomic DNA, previous protocols for the above approach have yielded rather ambiguous results. In the process of reproducing our sequence data, we were certain that the point mutation(s) observed were artifacts of misincorporation associated with multiple amplifications. Due to the complexity of the genomic DNA, several artifactual bands were consistently amplifiable in the same reac-

TABLE 1 Templates and Primers Used for PCR and Sequencing

Template		Primers
size (bp)	% GC	
217	50	5′-GCTGCATCAATGGAGCACAT-3′ 5′-CTGTTCTTTGCTTGTTCGGC-3′
104	50	5′-TCAATGTGCGAAATGGAATG-3′ 5′-GTTCGTGGAGAAGTCTGAAC-3′
88	48	5′-GCACGCTTAGATTGGAATAC-3′ 5′-GATGTTACTCCAGTCCACAC-3′
148	48	5′-CTCGGAAGACGTTCCTGAAG-3′ 5′-GAGTTGTCTGATGTTACTCC-3′
600	43	5′-CATGTTCCCCTCACAACACA-3′ 5′-GAGTTGTCTGATGTTACTCC-3′
75	60	5′-GGTGATAGTGGAGTCCCAGC-3′ 5′-CAGGCATCCTGGAAACAGAC-3′
139	59	5′-CTACACCTCACGCCTTCACC-3′ 5′-TCAATCATCCAGCTGTCCAC-3′
650	48	5′-TGCAATTCGAAGTCACAGCG-3′ 5′-GAATCTCTCTGTCCACGAGG-3′
450	50	5′-CACCTTCAGCCCTGTCCAGT-3′ 5′-CTCCTGGGTCCCAGATACTG-3′

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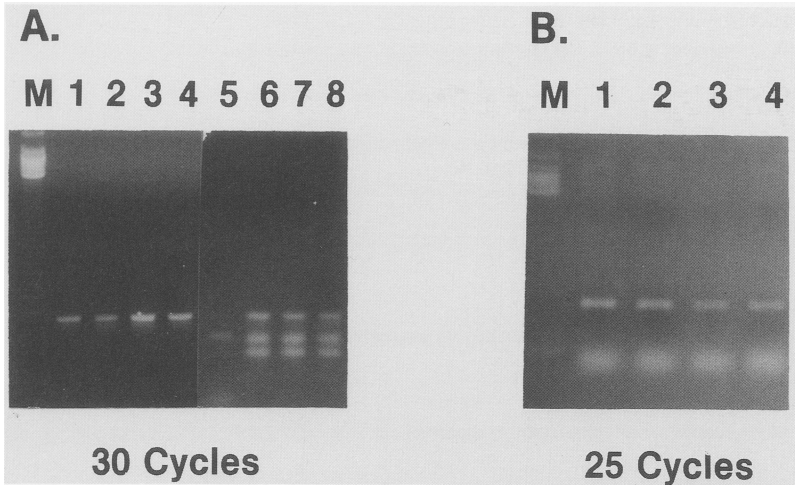

FIGURE 1 Comparison of PCR products (450 bp) generated under different conditions. The samples were amplified through 25 or 30 cycles, and then 5 μl of the sample (100 μl in a reaction) was electrophoresed in an agarose gel containing 0.5 μg/ml of ethidium bromide. (*A*, lanes *1*, *2*, *3*, and *4*) The specific region (exons 9–10) of c-*raf*-1 amplified for 30 cycles using genomic DNAs from four different human tumor cell lines and our modified PCR procedure. The products of asymmetric amplification followed by reamplification of the same region are shown for comparison, in lanes 5–8. (*B*) Twenty-five cycles and the modified incubation conditions of PCR were employed in the reaction mixture containing respective tumor DNA samples (lanes *1–4*).

tion. Furthermore, the subcloning of the PCR-amplified products was difficult for those fragments (mainly the introns) that contained a high incidence of tandem repeats. To eliminate these problems, we have attempted to establish reliable and optimal conditions for the PCR and nucleotide sequence analysis. Table 1 shows the sizes and GC contents of the genomic DNA templates and sequence of the various primers tested in this study. First, we found that the longer time for denaturation, annealing, and extension during each cycle was not necessary. By using 2 min or 30 sec for the denaturation or the annealing, the comparable amplified products up to 2 kb were produced through 30 cycles (data not shown). Therefore, 30 sec for each cycle was subsequently selected for the amplification. Second, two different cycles, 25 versus 30, were tested using 30 sec for each cycle (Fig. 1). The smaller fragments generated by asymmetric amplification (Fig. 1, lanes 5–8), contributed to a significant background in the sequence data (not shown). These fragments were reduced significantly by the symmetric amplification procedure using 30 cycles (Fig. 1A, lanes 1–4) or 25 cycles (Fig. 1B). However, the subsequent sequencing of the product of 30 cycles clearly demonstrated ambiguous bases compared to the product of 25 cycles of PCR amplification (see below).

Nucleotide sequence of the PCR-amplified product was analyzed by the direct double-strand sequencing procedure. In this method, 0.5 μg of the PCR product was denatured in the presence of 2% NP-40 for 3 min by boiling and then snap-cooled in powdered dry ice to avoid the fast reassociation of relatively short, linear, double-stranded templates (<600 bp). As described in the legend to Figure 2, three different conditions of sequencing reaction were compared. In Figure 2A, the template generated from 30 cycles of PCR was annealed to the 5′ end-labeled primer followed by the direct sequencing. Disadvantages of using this method were the following: poor resolution of sequences adjacent to the primer and of the sequences greater than 200 bp, β-emission and radioactive contamination, and blunted bands. Recently, Ruano and Kidd[13] reported an alternative method, called coupled amplification and sequencing of genomic DNA, which uses [γ-^{32}P]ATP end-labeled primer and 30 cycles of PCR amplification. This method appears to be suitable for short templates (<300 bp). In addition, the sequencing data revealed high background noise.

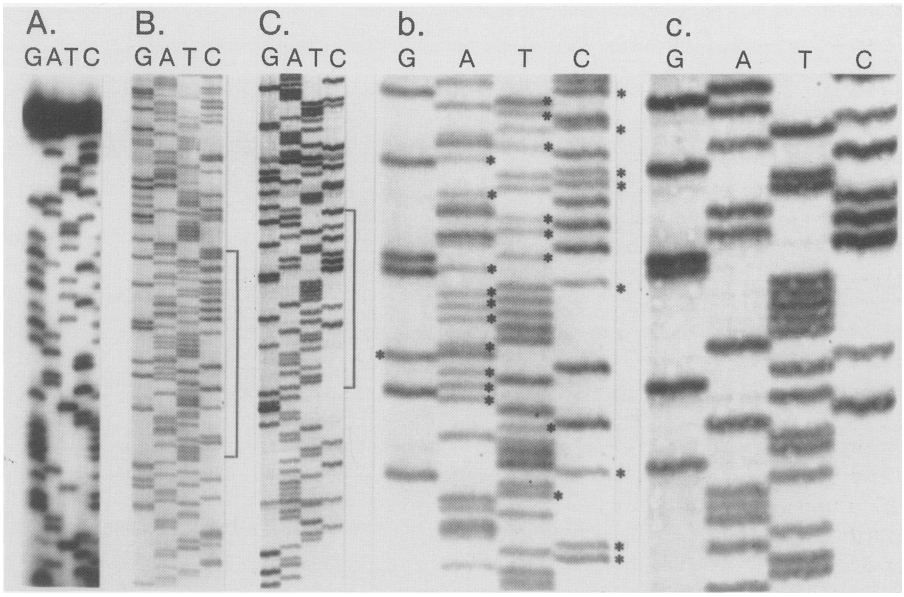

FIGURE 2 Autoradiographs showing the direct sequencing of the double-stranded DNA templates. (*A*) Sequence data from the DNA template (100 bp) was obtained using 5′-end-labeled [γ-^{32}P]ATP primer and the Sanger dideoxynucleotide sequencing procedure. (*B* and *C*) The templates (450 bp) were generated by amplification through the modified PCR procedure for 30 and 25 cycles, respectively. The expanded sequence data shown in *b* and *c* correspond to the bracketed segments in *B* and *C*, respectively. (*) Ambiguous bases observed with the 30-cycle PCR procedure. Sequencing was performed by the modified direct sequencing procedure using [α-^{35}S]dATP.

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The sequencing data in Figure 2, B and C, were derived from the samples generated by 30 and 25 cycles of PCR, respectively, and our modified dideoxynucleotide chain-termination method as described in Materials and Methods. Figure 2, B and C, represent the expanded panels B and C, respectively. The sequence data using the template obtained from 30 cycles of PCR amplification contained a high frequency of ambiguous bases (marked with asterisks, Fig. 2B). The sequence data shown in Figure 2C, using the PCR products generated by 25 cycles, were reproducible and reliable, easy to read up to 300 nucleotides with a single loading, and of as good quality as that obtained from the plasmid DNA template. In most cases, we were able to read the nucleotide sequence 10 bases from the primer. The common stops observed in the sequence data may be either due to the incomplete extension of the primer in the sequencing reaction or, as proposed earlier, due to the selective preferential annealing of the complimentary strands, resulting in difficult readthrough for the polymerase.[13] On the basis of our data, we recommend 25 cycles for our modified PCR method with 30 sec for each step in the cycle and the modified direct sequencing using random primer labeling as detailed above. This procedure has also proven to be time effective because both strands can be sequenced simultaneously from the double-stranded template generated in one 25-cycle amplification step.

ACKNOWLEDGMENTS

This work was supported by the NIH grant to UK and by the Department of Radiation Medicine.

REFERENCES

1. Tindall, K.R. and T.A. Kunkel. 1988. Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry* **27:** 6008–6013.
2. Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487–491.
3. Mihovilovic, M. and J.E. Lee. 1989. An efficient method for sequencing PCR amplified DNA. *BioTechniques* **7:** 14–16.
4. Scharf, S.J., G.T. Horn, and H.A. Erlich. 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. *Science* **233:** 1076–1078.
5. Gyllensten, U.B. 1989. PCR and DNA sequencing. *BioTechniques* **7:** 700–708.
6. Dicker, A.P., M. Volkenandt, A. Adamo, C. Barreda, and J.R. Bertino. 1989. Sequence analysis of a human gene responsible for drug resistance: A rapid method for manual and automated direct sequencing of products generated by the polymerase chain reaction. *BioTechniques* **7:** 830–837.
7. Innis, M.A., K.B. Myambo, D.H. Gelfand, and A.D. Brow. 1988. DNA sequencing with Thermus aquaticus DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proc. Natl. Acad. Sci.* **85:** 9436–9440.
8. Bonner, T.I., H. Oppermann, P. Seeburg, S.B. Kerby, M.A. Gunnell, A.C. Young, and U.R. Rapp. 1986. The complete coding sequence of the human raf oncogene and the corresponding structure of the c-raf-1 gene. *Nucleic Acids Res.* **14:** 1009–1014.
9. Jung, M., A. Dritschilo, G. Mark, and U. Kasid, in preparation.
10. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* **74:** 5463–5467.
11. Gibbs, R.A., P. Nguyen, L.J. McBride, S.M. Koepf, and C.T. Caskey. 1989. Identification of mutations leading to Lesch-Nyhan syndrome by automated direct DNA sequencing of *in vitro* amplified DNA. *Proc. Natl. Acad. Sci.* **86:** 1919–1923.
12. Bos, J.L., J.H. Fearon, A.J. Van der Eb, and B. Vogelstein. 1987. Prevalence of ras gene mutations in human colorectal cancers. *Nature* **327:** 293–297.
13. Ruano, G. and K.K. Kidd. 1991. Coupled amplification and sequencing of genomic DNA. *Proc. Natl. Acad. Sci.* **88:** 2815–2819.

Received May 28, 1991; accepted in revised form October 18, 1991.