Benchmarks

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Benchmarks are brief communications that describe helpful hints, shortcuts, techniques or substantive modifications of existing methods.

Sequencing of Genomic DNA

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Disease-causing gene mutations can produce gross alterations in DNA structure. In many cases however, mutations can be extremely difficult to detect structurally, as in the case of small deletions, insertions, inversions, duplications and missense mutations. While strategies, which allow for the identification of mutations that grossly alter gene structure, are well established, difficult and often laborious techniques are required for the identification of point mutations among the many thousands of nucleotides that comprise most genes. Strategies such as ribonuclease A protection and chemical cleavage can detect point mutations if they involve cleavage of heteroduplex molecules at the site of mismatched base pairs. Related methods that have been developed include heteroduplex and single-strand conformation polymorphism (SSCP) analyses, which rely on the altered electrophoretic mobility of mutated DNA, and denaturing gradient gel electrophoresis (DGGE), which assesses the melting point of heteroduplex molecules along a denaturing gradient. While these methods are useful for the screening of long stretches of DNA,

they are technically demanding and do not provide sufficient information to allow for the identification of mutations at the level of a particular amino acid or single base pair. Traditional sequencing techniques (5,8) will identify single base changes, however, these methods are laborious and require large amounts of template, which is typically obtained by the insertion of the target DNA into bacterial or viral vectors and the propagation of these vectors in bacterial host cells. Polymerase chain reaction (PCR) amplification of DNA templates before sequencing can bypass the tedious steps of subcloning and generally provides rapid access to accurate sequence information from complex DNA mixtures. Since the development of thermostable DNA polymerases, several strategies for the sequencing of PCR-amplified DNA have emerged (3,6,7). Nevertheless, it has remained difficult to obtain consistent and reproducible sequence information from these methods. We have developed several improvements over the existing protocols for the sequencing of genomic DNA. The method described here is easily reproducible and yields high-quality DNA sequence over 400–600 bp from as little as 100 ng of genomic DNA (ca. 10^5 copies of a single copy gene) and in <24 h. This technique is based on a three-step procedure and uses Platinum™ *Taq* DNA Polymerase for the initial amplification of double-stranded (ds)DNA, followed by size-exclusion chromatography and subsequent cycle sequencing using dichlororhodamine dye terminators (dRhodamine). This strategy is fast, reliable and simple to perform and may prove to be a powerful tool for the study of mutations, allelic variations and the expression of genes associated with cancer and other diseases.

Plasmid and genomic DNAs were prepared according to published protocols (1). The DNA was extracted three times with phenol/chloroform (1:1), three times with chloroform/isoamyl alcohol (24:1) and precipitated with ethanol (100%). It then was washed with 70% ethanol and resuspended in 1 mL double-distilled (dd)H₂O. DNA concentration was estimated by measuring the absorbance at 260 nm. DNA sequencing reactions were performed on a GeneAmp® PCR System 9600

Thermal Cycler and run on a Model 377 DNA Sequencer (both from PE Biosystems, Foster City, CA, USA). The detection system used 10-nm filters, which were centered at 540, 570, 595 and 625 nm (10). Sequencing reactions using 600 ng pcDNA3.1, 1 µg P1-DNA (clone 311E6) or 300 ng human genomic DNA were performed using 20–25-mer sequencing primers, which were selected to hybridize to different exon regions within the human *HDAC3* gene and were synthesized commercially (GENSET, La Jolla, CA, USA). The oligonucleotide primers were designed to have an equivalent GC-AT distribution and no internal complementarity or potential to form stable secondary structures. The sequencing strategy for genomic DNA comprised three steps. Twenty-seven amplification cycles were necessary before performing sequencing of genomic DNA. The sequencing reactions were performed in a volume of 20 µL containing 10 pmol of the sequencing primer, 8 µL of dRhodamine Terminator Ready Reaction Mix (PE Biosystems), DNA as indicated and ddH₂O to a final volume of 20 µL. The thermal cycling profile for the direct sequencing of pcDNA3.1 was as follows: denaturation at 95°C for 30 s, annealing at 50°C for 15 s, extension at 60°C for 4 min (25 cycles) and storage at 4°C. After setup for PCR amplification as described below, the thermal cycling profile for the direct sequencing of P1 DNA and the amplified, double-stranded genomic DNA was: denaturation for 10 min at 95°C (1 cycle) followed by 40 cycles alternating between 30 s at 60°C and 30 s at 95°C and final storage of the samples at 4°C. The following oligonucleotides were used for the amplification of the double-stranded genomic DNA template: 5'-GACAATGACAAGGAAAGCGATGTGGAGATTTAAGAGTGGC-3' and 5'-ACTTCCCATCATCTGGGATTCA-GGTGTTAGGGAGCCAGAG-3'. The amplification reactions were performed in a volume of 50 µL in a GeneAmp PCR System 9600 using thin-walled, 0.2-mL MicroAmp® reaction tubes (PE Biosystems) and a three-temperature thermal cycling profile. Three hundred nanograms of genomic DNA and 2.5 U of Platinum *Taq* DNA Polymerase (Life Technologies, Gaithersburg, MD, USA)

were used. All four dNTPs were at 0.2 mM, the final MgCl_2 (both from Life Technologies) concentration was 1.5 mM, the 5' and 3' primers were at a final concentration of 0.1 μM , and the 10 $\times$ PCR buffer (Life Technologies) was at 10% of the total reaction volume. The dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) concentration was between 2%–10% of the total reaction volume, and ddH₂O was added to a total volume of 50 μL . After variation of the initial denaturation between 1–10 min at 95°C, adjustment of the annealing temperature for the first two cycles between 45°–70°C and for the subsequent cycles between 60°–72°C, we had also varied the number of cycles at high stringency between 15–35 cycles. As a result, our best thermal cycling profile for the amplification step was found to be: denaturation at 95°C for 10 min, annealing at 48°C for

30 s and extension at 72°C for 2 min (2 cycles), followed by denaturation at 95°C for 2 min, annealing at 65°C for 30 s and extension at 72°C for 2 min (25 cycles). The samples were then kept at 4°C. PCR-amplified dsDNA was separated from unincorporated dNTPs, salts, primers and the polymerase by using MicroSpin® Sephacryl S-400 HR Columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. The concentration of recovered DNA template was estimated by agarose gel electrophoresis and ethidium bromide staining of a small aliquot of the purified DNA. The intensity of the stained band was compared to that of a *Hind*III-digested λ DNA standard of known concentration (Life Technologies). Ten microliters of the filtrate, which were usually equal to approximately 0.5–1 μg of amplified DNA, were typically

used for cycle sequencing.

Traditionally, templates for DNA sequencing have been generated by inserting target DNA into vectors of bacterial or viral origin for later multiplication in bacterial host cells. Even though such approaches greatly enhance the quality of sequencing results, they are still subject to multiple problems. Individual PCR products can vary from the sequence to be amplified by point mutations or PCR-associated in vitro recombination events. In addition, living host cells as the source for vector DNA might be associated with de novo mutations in both the vector and the host cell genome. Accordingly, the sequencing of PCR products, rather than sequencing following subcloning (9), is preferable in most cases.

Using an improved approach for the sequencing of genomic DNA, we compared sequencing data obtained from

Figure 1. Sequencing from genomic DNA after PCR amplification of the region of interest compared to sequencing from bacterial expression vectors (P1 DNA and pcDNA3.1) in which no previous amplification of the template was necessary. Sequencing reactions from 600 ng pcDNA3.1 (upper panel), 1 μg P1 DNA (middle panel) or 300 ng genomic DNA (lower panel) were performed using 5'-AggAAAGcGATgTggAg-3' as the sequencing primer.

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DNA templates of increasing structural complexity—pcDNA3.1, P1 DNA and genomic DNA. All three strategies that were used, yielded sequencing results of very similar readability, suggesting our strategy to be highly specific (Figure 1). The strategy that we have used is highly efficient and rapid. It is easy to perform and has been reproduced also on other genes (MIF and IPFK2) with similar results. Therefore, it represents a significant improvement over existing protocols for the sequencing of genomic DNA (3,6,7). It is based on a three-step procedure using Platinum *Taq* DNA polymerase for the initial amplification of dsDNA, followed by sephacryl column chromatography of the template and subsequent cycle sequencing using dichlororhodamine dye terminators (dRhodamine). Unfortunately, the application of the predecessor of the dRhodamine dye terminators, the ABI PRISM® Dye Terminator Cycle

Sequencing Ready Reaction Kit (PE Biosystems) for the sequencing of amplified genomic DNA did not yield satisfactory sequencing results. We had also tried to use normal *Taq* DNA polymerase from different companies instead of Platinum *Taq* DNA polymerase for the amplification of genomic DNA. The results, however, were very poor. The approach with Platinum *Taq* DNA polymerase apparently has the advantage that the enzyme is blocked by a proprietary antibody that inhibits its activity at room temperature. Enzyme activity is regained during denaturation at 94°C, thus providing an automatic “hot start”. Hot starts are typically used to increase both the sensitivity and specificity of the reaction (2). Subsequent purification of the PCR template from primers and other contaminants is achieved by sephacryl-column chromatography. This yields a highly pure double-stranded template, which is suitable for DNA sequencing. Sequencing was performed with energy transfer dyes that comprised the 5- and 6-carboxy isomers of 4'-aminomethyl-fluorescein as the donor dyes and four 4,7-dichloro-substituted rhodamine acceptor dyes that have narrower emission spectra than the standard unsubstituted dyes (4). These reagents increase significantly the signal-to-noise ratio and provide an improvement in both the sensitivity and the reproducibility of the sequencing data obtained with difficult templates (4).

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