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Direct sequencing of PCR products in agarose gel slices

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Direct DNA sequencing of amplified polymerase chain reaction (PCR) products offers several advantages over cloning of amplified DNA products. It is faster (1 day versus 3–5 days) and in DNA samples containing sequence polymorphisms both the normal and mutated sequence can be detected in the same sequencing reaction. The major problems encountered in direct sequencing of amplified DNA have been contamination by PCR primers and deoxynucleotides, and overcoming fast template renaturation. Techniques which have been used to purify the amplified product include: differential precipitation, electrophoretic purification, absorption of DNA to specific matrices, or differential filtration (i.e. centricon) (1–6). Each of these techniques is capable of improving the quality of sequence data, but all are time consuming and subject to some performance variability. The problem of template reannealing has also been approached by several methods including: rapid cooling following denaturation, use of thermal stable polymerases, increased primer concentration and preparation of a single-stranded DNA templates by: asymmetric PCR, biotinylation of one primer or use of strand-specific nuclease (2,5,7,8). We have devised a strategy for direct sequencing of PCR products using a simple modification of the standard Sequenase@ protocol (U.S. Biochemicals, Cleveland, OH).

The major change in the protocol is that template denaturation, primer annealing and DNA sequencing are all performed in the presence of agarose. This technique simplifies purification, eliminates the necessity for elution of the DNA, and may act to prevent reannealing of the DNA template. The method described here was developed to allow rapid analysis of DNA samples from patients with multiple endocrine neoplasia type 2 (9). These patients have heterozygous mutations in one of six codons in the RET proto-oncogene (10,11). DNA sequence analysis permits rapid determination of specific mutations contained in one of three exons. PCR products (2–3 μg) were purified by electrophoresis on 0.8–1% SeaPlaque® (FMC, Rockland, ME) agarose gels. Electrophoresis was performed with either TrisAcetate or TrisBorate buffer, in the absence or presence of ethidium bromide. We used an Owl Scientific (model B1A) apparatus with a 6 well 1.5 mm comb. Following electrophoresis the DNA band of interest was excised, taking care to remove as much excess agarose as possible. The gel slice containing the PCR product was weighed, an equal volume of dH_2O was added, and melted at 75°C for 5–15 minutes. The annealing reaction was prepared by combining: 5–10 μl of melted gel containing template DNA, 1 μl of sequencing primer (10 A_{260}/ml), and dH_2O to 11 μl. This mixture was denatured by incubation at 100°C for 3 minutes and annealing was performed by immediately placing the tube in ice for 5 minutes. Dideoxy-DNA sequencing was performed on 10 μl of annealed template using Sequenase® Version 2.0 (U.S. Biochemicals, Cleveland, OH) with minor modification of the manufacturer's protocol; 2 μl of 5× reaction buffer was added to the labeling mix and 4 μl of labeling mix was added to the termination tubes. The final volume of the labeling mix was 17.5 μl (18.5 μl if Mn^{2+} was used).

This protocol has provided reliable sequence analysis under a variety of conditions. We have used PCR products as small as 172 bp to over 2 kb in size with excellent results (data submitted but not shown). Sequencing works equally well with the PCR primers or with internal primers. The Sequenase® protocol provides a proven familiar methodology with a flexible choice of isotope. This eliminates the need for primer endlabeling

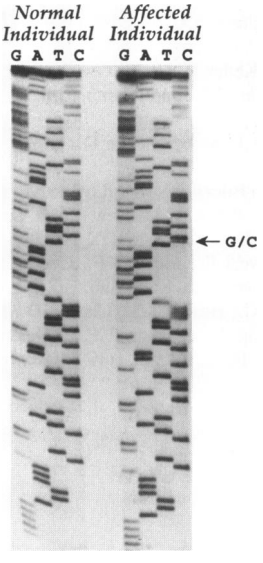

Figure 1. PCR product sequencing with ^{35}S-dATP of RET proto-oncogene exon 10. Genomic DNA isolated from peripheral blood leukocytes of a patient with documented MEN 2A and unaffected relative. PCR amplification of the RET proto-oncogene exon 10 was performed as previously described (10). PCR products were purified on 0.8% agarose gels buffered with TAE. ^{35}S-dATP DNA sequencing was performed using the forward PCR amplification primer. The arrow identifies a G-to-C missense mutation that occurs at codon 618 (10,11).

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required in many cycle sequencing protocols. The direct addition of agarose purified PCR template to the sequence reaction has several advantages. First, it is possible to directly assess the success of the PCR reaction. We routinely run a fixed volume of PCR reaction on SeaPlaque® gels and visualize the product bands with ethidium bromide. This provides an estimate of template amount, making it possible to optimize the sequencing reaction by varying the amount of template/gel used in the annealing reaction. Second, electrophoresis ensures isolation of single specific band. This reduces the sequencing background. If fact, we have been able to sequence multiple products of a single PCR reaction that migrated as distinct electrophoretic bands. Third, by varying the gel size or well number, several samples can be simultaneously purified without significant addition of time. DNA samples do not have to be purified from the agarose following electrophoresis. Fourth, if necessary samples can be stored in agarose for extended times, making it possible to resequence DNA samples with dITPs after observing sequence compression, or use a different primer to obtain additional sequence. Finally, performance of the annealing and sequencing in the presence of agarose appears to inhibit template reannealing. This, eliminates the need for snap cooling or production of single-stranded templates.

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