Directed termination PCR: a one-step approach to mutation detection

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Received November 11, 1997; Revised and Accepted January 26, 1998

ABSTRACT

We describe a novel PCR-based method that allows the generation of nested termination fragments by integrating both selective DNA amplification and directed chain termination into a single PCR reaction. These termination fragments can be examined for sequence variation in either denaturing or non-denaturing polyacrylamide gels. This method provides a one-step and highly effective approach for the detection of both insertions/deletions and single base pair substitutions in sequences up to 1 kb in length.

Surveys of DNA sequence diversity are the basis of investigations in a broad range of biological disciplines. There have been ongoing efforts to develop methods that allow rapid scanning for sequence variation (1). We describe a new PCR-based method for mutation detection that integrates both selective DNA amplification and directed chain termination into a single PCR reaction. Because it exploits unbalanced nucleotide concentrations to induce the polymerase chain reaction to terminate at specific nucleotide sites, we call the method directed termination PCR (DT-PCR).

DT-PCR is performed when 1 nt is provided at a concentration five to ten times lower than the other 3 nt that are typically supplied at their $K_m$ value (10–15 $\mu$M) (2). Because the nucleotide in low concentration plays a rate-limiting step in DNA synthesis, DT-PCR has a biphasic nature (Fig. 1). The first phase is characterized by the selective amplification of full-length DNA fragments. However, once the time required to replicate the DNA fragment exceeds the extension time in the PCR protocol (typically after 10–15 cycles), DNA synthesis halts at one of the rate-limiting sites. In later cycles of replication, the continuing decline in concentration of the limiting nucleotide further slows the progress of chain synthesis and leads to the generation of termination fragments of ever diminishing length. After 20–25 additional cycles, the full length DNA strands amplify during the first phase of the reaction have served as templates for the generation of two sets of nested termination fragments. These sets of fragments (one originating from each primer) identify all of the limiting nucleotide.

Visualization of the complete array of termination fragments can be achieved through the inclusion of a $^{35}$S-labeled analog of the limiting nucleotide. Typically, 1.5–3 $\mu$Ci of $[\alpha-^{35}$S]dCTP (or dATP) (10 mCi/ml, 1000 Ci/mmol) is used in a 20 $\mu$l reaction. Because Taq DNA polymerase shows >10-fold differential incorporation of the normal, rather than the thiol nucleotide, the proportion of the radiolabeled form of the limiting nucleotide rises in later cycles, leading to its preferential incorporation into smaller fragments.

Interestingly, the incorporation of $[\alpha-^{35}$S]dCTP usually produces better results in DNA fragments with high G-C content. Alternatively, one of the PCR primers in a reaction carried out without a thiol radionucleotide can be end-labeled with $[\gamma-^{32}$P]ATP, an approach which enables just one set of the termination fragments to be visualized. Hence, based on the labelling approach, DT-PCR can be classified as a bidirectional or unidirectional reaction.

As DT-PCR requires the sequential occurrence of amplification and termination reactions, it is important that neither processes be truncated prematurely. Although several factors have effects on the DT-PCR dynamics, the optimization of the DT-PCR protocol for a new sequence fragment is readily achieved through a two-step procedure. The first step involves the optimization of parameters such as template copy number, $\text{MgCl}_2$ concentration and PCR buffer in a 20 $\mu$l reaction using balanced dNTP concentrations (10–15 $\mu$M), so that a clean PCR product is visible on an agarose gel. The second step involves optimization of the unbalanced dNTP ratio (the limiting nucleotide/three others) and incorporation of the radioactive thiol nucleotide to generate full sets of termination fragments. Following these steps, the DT-PCR products for a broad range of genes with differing G-C content (35–59%) have been generated from both mitochondrial and nuclear genomes.

DT-PCR provides a rapid and highly effective method for the detection of DNA sequence changes. Because each mutation impacts the base composition of all fragments that terminate downstream of its site of occurrence, bidirectional DT-PCR generates two families of mutant molecules, which can be exploited in sequence characterization. Their examination in a denaturing polyacrylamide gel provides a sensitive technique for the detection of insertions/deletions (indels) in sequences of 1000 or more base pairs in length, because insertion or deletion events result in an upwards or downwards shift in the mobility of all fragments downstream from the indel. Moreover, the size of indels can be determined by the extent of the shift in mobility they induce in downstream bands, while the location of the changes can also be accurately positioned by the initiation of the band shifts.

Denaturation of the DT-PCR products and their subsequent examination in a non-denaturing gel allow the single-stranded termination fragments to segregate based on both size and conformation. This procedure, which we term DT-SSCP, provides the simplest approach for the recognition of nucleotide changes. In contrast to conventional SSCP analysis (3), the generation of two families of single-stranded termination fragments containing the same mutational event greatly enhances the probability of producing easily detectable conformational shifts. Tests on sixteen 1000 bp mitochondrial DNA (mtDNA) segments with known point mutations and 37 blind samples (Fig. 2) demonstrated 100%

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Figure 1. Autoradiograph of a denaturing 6% polyacrylamide gel showing the transition from amplification of a full length PCR product after 10 cycles to the generation of directed termination products of diminishing size in later cycles. A 245 bp fragment of the D-loop region of the mtDNA from three individuals of *Ameiurus nebulosus* was amplified in a DT-PCR reaction with limiting dATP. The DT-PCR products were labeled by 1.5 µCi of \([\alpha-^{35}S]\) dATP in the presence of 10 mM each of dTTP, dCTP, dGTP and of 2 µM dATP. The remaining cocktail consisted of 1× PCR buffer, 1.5 mM MgCl₂, 2 pmol of each primer, 1 U of Taq DNA polymerase and 30 ng of DNA template in a 20 µl reaction. Amplification conditions were 95°C (1 min) for initial denaturation, followed by 40 cycles of 1 min at 92°C for denaturation, 1 min at 54°C for annealing and 1 min at 72°C for extension. Amplified DNA was removed every 5 cycles over a 40 cycle reaction. The banding shifts indicated by arrows are a consequence of nucleotide diversity among the individuals. Individual 2 has a point mutation (G to A) at a site 54 bp from the forward primer, while individual 3 has substitutions (T to C and C to T) at sites 84 and 85 bp. Efficiency in detecting both small indels and single base pair substitutions in a single gel electrophoresis. The variable intensity of bands in each sample reflects differential termination of polymerase chain replication, likely caused by local sequence context effects. However, this effect is consistent among samples, so that each genotype has a stable phenotype in DT-SSCP. The multiple bands serve as internal standards that greatly aid the recognition of band changes and permit preliminary positioning of mutational events.

DT-PCR not only enables the detection of sequence diversity in any haploid genome including bacterial, plasmid and mtDNA as well as reporter genes in transgenic organisms, but is also useful in detecting mutations in nuclear genes. It is particularly effective in prescreening for variation which can defeat conventional sequencing analysis, such as studies examining individuals heterozygous for deletions or insertions. In a preliminary study, we have characterized and located length variation in both homozygous and heterozygous variants of different exons of the human BRCA1 gene using DT-PCR followed by denaturing gel electrophoresis. Further analysis of the DT-PCR products of these exons in a non-denaturing gel allowed the identification of different genotypes. In cases where the banding pattern is complex, such as heterozygous mutations, unidirectional DT-PCR reactions can be desirable as they reduce the band count by half.

In conclusion, DT-PCR provides a simple, high throughput methodology for screening large DNA molecules for both indels and single nucleotide substitutions. Equally important, the technique allows the recognition and positioning of sequence changes in a single-step reaction from a total DNA extraction.

ACKNOWLEDGEMENTS

We are grateful to Dr R Cotton for providing both DNA samples and PCR primers of the BRCA1 gene, and thank Dr T Crease for her comments on early versions of this manuscript. Funding for this research was provided by research grants from NSERC to P.D.N.H.

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