A Simple Method to Attach a Universal 50-bp GC-Clamp to PCR Fragments Used for Mutation Analysis by DGGE

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The search for sequence variations in genomic DNA becomes increasingly important in the study of inherited disease genes as well as of genes that play a role in the development of cancer. A wide variety of different methods to detect DNA sequence variations has been developed during the past few years.⁽¹⁾ One of these methods, denaturing gradient gel electrophoresis (DGGE),⁽²⁾ has been shown to be very sensitive⁽³⁾ and the method of choice in studying mutations in large genes.⁽⁴⁾

The detection of mutations by DGGE is based on the sequence-dependent electrophoretic mobility of double-stranded DNA fragments in a polyacrylamide gel that contains a linear denaturing gradient. A key requirement of DGGE is that the DNA fragment of interest is composed of at least two melting domains (blocks of sequence with a discrete melting temperature, or T_m). DGGE involves electrophoresis of double-stranded DNA fragments through a polyacrylamide gel containing a linear gradient of DNA-denaturing agents (e.g., a combination of formamide and urea) at a fixed temperature (usually 60°C). Initially, the migration rate of the fragment depends on its molecular weight. However, at a specific point in the gel, the combination of denaturant concentration and temperature equals the T_m of the lowest melting domain, resulting in a partially single-stranded fragment. The mobility of these branched fragments in the polyacrylamide gel is abruptly retarded. The fact that the T_m for a given domain is determined by its sequence and base composition means that two DNA fragments that differ by a single base change (and thus in T_m) in the lowest melting domain will be separated from each other at the end of the run.

The addition of an artificial GC-rich sequence, a so-called GC-clamp, to DNA fragments has been a major improvement in DGGE methodology.^(5,6) Such a GC-clamp serves as the most stable part of the fragment and allows the detection of sequence variation in the remainder of the fragment. In general, it should be possible to detect sequence variation in any fragment attached to the GC-clamp. This GC-clamp usually consists of a stretch of 40 nucleotides of G + C that is added to the DNA fragment of interest by the polymerase chain reaction (PCR),⁽⁷⁾ using primers in which one is extended with the GC sequence at its 5'

end.⁽⁵⁾ A practical drawback of this approach is that for each fragment to be analyzed, such a (GC-clamped) 60-mer primer has to be synthesized. To overcome the need for the synthesis of these GC-clamped primers, a simple method was developed that allows the incorporation of a universal 50-bp GC-clamp in any amplified DNA fragment during the PCR (Fig. 1).

The basic strategy of this method is a PCR carried out with three primers (referred to as A, B, and C). Primers A and B are complementary to the genomic DNA sequence to be amplified. In addition, primer B is extended at the 5' end with a 15-bp GC-rich linker sequence that is not complementary to the target sequence. Primer C is a 50-bp G + C-rich primer and also contains this 15-bp linker sequence, however, at the 3' end. Amplification with primers A and B generates DNA fragments that are extended with the 15-bp linker sequence. Since this linker sequence can serve as target sequence to which primer C can anneal, a second round of amplification with primers A and C will generate fragments that are further extended with 35 bp. As the linker sequence is also GC-rich, with two consecutive rounds of amplification, the target sequence originally defined by primers A and B will be extended with the 50-bp GC-rich sequence defined by primer C.

The feasibility of this approach was examined on two different genomic DNA fragments: (1) an 84-bp fragment comprising the membrane-spanning region of the human *c-erbB-2* proto-oncogene⁽⁸⁾ and (2) a 230-bp fragment comprising exon 6 of the human nuclear phosphoprotein p53 gene.⁽⁹⁾ For the amplification of these fragments, primer A was a 20-mer primer and primer B was a 30-mer primer composed of a 15-bp sequence complementary to the respective gene sequences and the 15-bp linker sequence (Table 1). Optimal conditions to amplify specifically the respective genomic fragments with primers A and B were first determined (Fig. 2, lanes 1 and 3). Using this condition, the GC-clamp sequence was attached to the fragments by two consecutive rounds of PCR. The first round of amplification was carried out on ~100 ng of genomic DNA using 25 pmoles of each PCR primer A and B in a volume of 50 μ l and consists of 20 cycles. After the amplification, 1 μ l of this reaction mixture was then transferred to

This procedure for adding GC-clamps to PCR fragments is now being used in our laboratory for the screening of p53 gene mutations by DGGE in non-small cell lung cancer (NSCLC). Prior to the screening, optimal denaturing gradient

In summary, an alternative method was developed to generate GC-clamped DNA fragments that are suitable for mutation analysis by DGGE. The advantages of this method are that the 50-bp GC-rich primer has to be synthesized only once and that it can be attached to any

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FIGURE 3 DGGE analysis of GC-clamped exon 6 fragments of the p53 gene. Electrophoresis was performed on an 8% polyacrylamide gel containing a 30–55% linear denaturing gradient at 150 V for 5.5 hr at 60°C. (Lane 1) wild type; (lanes 2 and 3) mutated fragments. Visualization of the DNA fragments was performed by ethidium bromide staining. DGGE equipment was purchased from CBS Scientific Inc. (Del Mar, California).

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