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. One of these methods, denaturing gradient gel electrophoresis (DGGE), has been shown to be very sensitive and the method of choice in studying mutations in large genes.

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. Amplification with primers A and B generates DNA 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 and (2) a 230-bp fragment comprising exon 6 of the human nuclear phosphoprotein p53 gene. 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 μl and consists of 20 cycles. After the amplification, 1 μl of this reaction mixture was then transferred to
Primer A and B are complementary (filled box) to the target sequence to be amplified. Primer B contains a 15-bp linker sequence at the 5' end (open box). Primer C is a 50-mer primer that has the 15-bp linker sequence at its 3' end. The remaining 35 bp of this primer (hatched box) are random G or C. When the PCR is carried out with primers A and B, the linker sequence is incorporated into the amplified fragment and can serve as the specific target sequence with which primer C can anneal. Subsequent amplification of the fragment with primers A and C then results in the amplification of PCR fragments that comprise the original genomic DNA target sequence and the additional 50-bp GC-clamp.

A second PCR reaction mixture (50 μl volume) containing 25 pmoles of each PCR primer A and C, and a further amplification was done in 35 cycles. Analysis of the generated DNA fragments on a 2.5% agarose gel demonstrates that the second round of amplification yields a single DNA fragment of the expected size (Fig. 2, lanes 1 and 3). The GC-clamped c-erbB-2 gene fragment is 134 bp in size, whereas the GC-clamped exon 6 fragment of the p53 gene is 280 bp, indicating that the 50-bp GC-clamp is indeed attached to these fragments.

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 and electrophoresis conditions were determined by travel schedule experiments (not shown). Two examples of a mutation that have been found in exon 6 of the p53 gene are shown in Figure 3 (lanes 2 and 3). Genomic DNA isolated from NSCLC tissues was amplified as described above and the GC-clamped fragments were analyzed on a 30–55% denaturing gradient gel. This result proves that PCR fragments that are GC-clamped by this method are suitable for the detection of mutations.

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 DNA fragment that has been amplified by primers in which one contains the 15-bp linker sequence.

Table 1: PCR Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
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<tbody>
<tr>
<td>Aα-erbB-2</td>
<td>AGAGCCAGCCCCCTCTGAGTC</td>
</tr>
<tr>
<td>Bα-erbB-2</td>
<td>CGCCGCCCGCCGCCGCCGCCGAGATCCTCCAAAGAC</td>
</tr>
<tr>
<td>Aα-p53</td>
<td>GCCACTGACAACCACCTTA</td>
</tr>
<tr>
<td>Bα-p53</td>
<td>CGCCGCCCGCCGCCGAGACGACAGGGCTGG</td>
</tr>
<tr>
<td>C</td>
<td>CGCCGCCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGC</td>
</tr>
</tbody>
</table>

The linker sequence in primers B and C is underlined.

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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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