Polymerase chain reaction (PCR) methodology (1) has become a routine method for selectively amplifying segments of DNA from a wide variety of sources. Amplification of specific sequences is dependent upon an exact match between the template DNA and the oligonucleotide primers. Mismatches at the 3' terminus lead to greatly reduced amplification, with no detectable product when amplified under the appropriate conditions (2,3). We demonstrate here that an oligonucleotide which can be extended by Taq DNA polymerase is able to block the amplification of a PCR product when situated between two flanking PCR primers. An oligonucleotide mismatched at its 3' terminus, however, does not demonstrate this ability. This allows the development of a method for the differential amplification of molecules with identical 5' and 3' ends.

Unless otherwise indicated, PCRs were performed in a programmable heating block using 20 rounds of temperature cycling (94°C for 1 min, 55°C for 2 min and 72°C for 3 min) followed by a final 10 min step at 72°C. One microgram of each primer, 5 μg of 'blocking' oligonucleotide, 10 ng of template and 2.5 units of Taq DNA polymerase (Perkin-Elmer Cetus) were used in a final volume of 100 μl with the reaction buffer as recommended by the manufacturer. The 'blocking' oligonucleotides, designed to block PCR amplification of the DX48 template, are indicated in Table 1. The templates used were a CAMPATH-1H humanised monoclonal antibody (mAb) heavy (H) chain (4) and DX48 humanised mAb H chain (unpublished), both in cDNA context in the plasmid vector pUC18 (GIBCO/BRL). The 5' PCR primer, X (5'-GATCAAGCTTTACAGTTACTCAGCACACAG), was designed to anneal to both templates such that the 3' end was at a position 12 nucleotides (nt) upstream of the ATG initiation codon in each case, and the 3' primer, Y (5'-GATCAAGCTTTACAGTTACTCAGACAG), was designed to anneal with the 3' end 20 nt upstream of the termination codon. The products of PCRs carried out on both templates using these primers would be expected to be 1462 base pairs (bp) in length.

Oligonucleotides A–N were able to anneal to the negative strand of the CDR3 region within the DX48 PCR, and possessed combinations of mismatches and modifications at their 5' and 3' termini (Table 1). They were added to the DX48 PCR to investigate whether such modifications would allow the oligonucleotides to block the PCR amplification. Taq DNA polymerase possesses a 5'-3' polymerase-dependent exonuclease activity (5). 5 μg of 'blocking' oligonucleotide was used in each reaction, this being an approximately 10^4-fold molar excess of 'blocking' oligonucleotide compared to template. The products of the PCR reactions were run on an agarose gel, as shown in Figure 1. The oligonucleotides with mismatches and modifications at their 3' termini were all unable to block the amplification of the 1462 bp PCR product, suggesting that either the 5' oligonucleotide modifications were unable to prevent degradation by the 5'-3' exonuclease activity of the extending Taq DNA polymerase (5), or that the polymerase possesses strand

Table 1. Sequences and modifications of oligonucleotides used to try and block PCR amplification

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>5' Modification</th>
<th>3' Modification</th>
<th>Nucleoside Linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Phosphothioate</td>
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<tr>
<td>B</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>C</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>D</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>E</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>F</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>G</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>H</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>I</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>J</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>K</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>L</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>M</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>N</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>O</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
<tr>
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<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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</tr>
<tr>
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<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>S</td>
<td>5'-ATATCAGAGCTCTTGTTACCTGGTTCGAGAACG</td>
<td>-</td>
<td>-</td>
<td>Fluorescein</td>
</tr>
</tbody>
</table>

*Target sequences of oligonucleotides A–O correspond to the full length (33 nt) positive strand of DX48 H chain complementary-determining region (CDR) 3, whilst oligonucleotides P–S correspond to positive strand sequences with 3' termini 30, 80, 180 and 380 nt upstream of the 3' end of PCR primer Y.

*Nucleosides that are mismatched with the target sequence are underlined.

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Five µg of oligonucleotide O (Table 1), containing nuclease-resistant phosphorothioate nucleotide linkages and a triple base mismatch at the 3' terminus, was added to the DX48 PCR. The oligonucleotide was unable to block the amplification of the 1462 bp PCR product (data not shown), suggesting that the Taq DNA polymerase is able to displace the 'blocking' oligonucleotide from the template.

PCRs were performed to investigate whether the oligonucleotide A was able to specifically block the amplification of a template sequence allowing the amplification of a 'mutated' sequence present in the same reaction. The templates used were humanised mAb H chain sequences DX48 and CAMPATH-1H which could both be amplified by primers X and Y, but which possessed different CDR sequences allowing the use of oligonucleotide A to specifically block the DX48 PCR. PCRs were carried out using primers X and Y, and 'blocking' oligonucleotide A, with titrations of CAMPATH-1H template either in the presence or absence of 10 ng of DX48. The resulting PCR products are indicated in Figure 2. A CAMPATH-1H PCR product was amplified in each of the reactions with a titration of CAMPATH-1H down to 10 pg. The addition of 10 ng of DX48 template to the reactions, and thus also to the 'blocking' PCR product, had no observable effect on these amplifications. One is thus able to specifically amplify a mutated sequence from a mixture of wild-type and mutated sequences where the mutated sequence is at a concentration 1000-fold less than the wild-type sequence.

PCR amplifications of the DX48 template using primers X and Y were performed with 'blocking' oligonucleotides P-S (Table 1), corresponding to sequences able to anneal to the positive strand of the template at various positions between the two PCR primers. The 72°C extension steps of the PCR cycles were carried out for either 3 or 6 minutes. The products were run on an agarose gel, as shown in Figure 3. Each of the 'blocking' oligonucleotides was able to inhibit the reaction when an extension time of 3 minutes was used. On increasing the extension time to 6 minutes, however, the oligonucleotides were unable to prevent the amplification of the PCR product XY. This observation suggests that at the shorter extension time the Taq DNA polymerase is not able to degrade or displace the extending 'blocking' oligonucleotide as far as the region corresponding to the 5' PCR primer Y, but on increasing this time to 6 minutes, appropriate extension of PCR primer X can occur, enabling amplification of XY.

We have demonstrated that oligonucleotides which can be extended by Taq DNA polymerase are able to block PCRs when situated between the two flanking PCR primers, whereas oligonucleotides that are mismatched or modified to prevent extension, are not able to block. Langley et al. (5) have described a 5'-3' degradative exonuclease activity for Taq DNA polymerase, but were not able to observe strand displacement. We found, however, that the addition of 5' modifications to an oligonucleotide or the use of exonuclease-resistant phosphorothioate nucleotide linkages, was not able to prevent the progress of the Taq DNA polymerase, thus suggesting a strand displacement activity for Taq DNA polymerase, perhaps in the absence of 5'-3' degradation.

It is possible to discriminate between single point mutations by using PCR primers mismatched at their 3' termini (2–3, 6–8). One use of this has been to detect point mutations in the HIV RT gene conferring zidovudine resistance (8). By using such
PCRs it is possible to identify which mutations overall are present in clinical samples, but not which combinations of mutations (without cloning out individual isolates). The use of ‘blocking’ oligonucleotides should allow this type of identification. Essentially, \(2^n\) PCRs, using \(n\) ‘blocking’ oligonucleotides in each reaction, would be required to identify each combination of possible mutations, where \(n\) is the number of mutation sites. This would allow the analysis of sequential clinical samples from infected individuals thus establishing the order of appearance of such mutations in different patient populations.

A second utility of this process is in mutagenesis protocols where the desired product is at a low percentage of a total mixture, for example, when simultaneously carrying out a number of different mutations with multiple oligonucleotides. Addition of wild-type oligonucleotides to the reaction will allow PCR amplification of the completely mutated product from the mixture by inhibiting amplification of any intermediate products that contain less than the desired number of mutations.

REFERENCES