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A rapid and efficient method for site-directed mutagenesis using one-step overlap extension PCR

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ABSTRACT

A rapid method is described to efficiently perform site-directed mutagenesis based on overlap extension polymerase chain reaction (OE-PCR). Two template DNA molecules in different orientations relative to only one universal primer were amplified in parallel. By choosing a high dilution of mutagenic primers it was possible to run an overlap extension PCR in only one reaction without purification of intermediate products. This method which we have named one-step overlap extension PCR (OOE-PCR) can in principle be applied to every DNA fragment which can be cloned into a multiple cloning site of any common cloning vector.

Mutagenesis by overlap extension has previously been described as a method for both site-directed mutagenesis to create base substitutions, insertions or deletions (1–3) and production of chimeric genes by combining two DNA fragments without a need for restriction sites (4,5). In separate PCR reactions, two fragments of a target sequence are amplified by using, for each reaction, one universal and one mutagenic primer. The two intermediate products with terminal complementarity form a new template DNA by duplexing in a second reaction. During this so-called overlap extension the fused product is amplified with the help of the two universal primers. To obtain a high mutant yield, it is necessary to fractionate and purify the products of the first PCR reaction by gel electrophoresis (2,5).

Here we describe an overlap extension strategy which is reduced to only one PCR reaction (Fig. 1) and is therefore named one-step overlap extension PCR (OOE-PCR). The target DNA for mutagenesis must be present in both orientations relative to one universal primer. Therefore, it has to be cloned into two vectors which only differ with respect to the orientation of their multiple cloning site with pBluescript II KS and SK or pUC18 and 19 as prominent examples. This cloning strategy has previously been used in a study to optimize the widespread megaprimer PCR method (6). The DNA fragments cloned into these plasmids serve as templates in a single PCR reaction in the presence of one universal and two mutagenic primers. Both mutagenic primers B and C are used in a 100-fold dilution as compared to the universal primer A. During the first PCR cycles, the intermediate product AB is amplified exponentially with primers A and B which bind to the template T1. In the same way, the product CA is amplified by binding of primers C and A to template T2. Since neither template exhibits terminal complementarity, wild-type DNA is not amplified. The hybridization of primer A to template T2 and primer C to T1, respectively, should also not lead to an amplification of the wild-type DNA sequence. Due to their high dilution, the mutagenic primers are used up during the early PCR cycles resulting in an asymmetric amplification of the intermediate products with those strands accumulating which form the duplex. The terminal complementarity of the mutagenic primers then leads to formation of the dimer BC thereby further withdrawing primers from the reaction and supporting the asymmetry of the amplification reaction. In this way, a competition between overlapping of the intermediate products and renaturation of these double strands favour the formation of the duplex DNA ABCA. A self-annealing of intermediate products can reduce the final product yield and has been observed for both the overlap extension and the megaprimer method (7,8). Introduction of an additional step of asymmetric synthesis was proposed to solve this problem (9,10). The fused mutant product AA contains one universal primer-binding site each at both ends and is therefore amplified as soon as it is formed in the late cycles of the PCR reaction. We tried to further simplify the OOE-PCR by using only one mutagenic and one universal primer which would also make this method less expensive. Although we obtained detectable products, the overall yield of mutant DNA was very low.

We have successfully applied the OOE-PCR method to introduce mutations into DNA of both prokaryotic and eukaryotic origin: a gene from the Gram-negative bacterium *Pseudomonas aeruginosa* encoding a lipase and a gene from the jellyfish *Aequorea victoria* encoding the green fluorescent protein. Unique restriction sites for *Afl*II and *Kpn*I, respectively, were created with an estimated yield of at least 95% of mutant DNA (see Fig. 2A and B). Dependent on the selection of mutagenic primers, this method can also be used to create insertions, deletions and chimeric genes as described for the original overlap extension strategy (1,2,4).

A restriction site for *Afl*II was introduced into *P.aeruginosa* DNA by first cloning a 689 nt *Eco*RI/*Sal*I fragment containing the 3′-end of the *lipA* gene into pBluescript II KS and SK. The PCR reaction was carried out in a final volume of 50 µl with 10 ng each of linearized template DNAs, 30 pmol of a modified T3 primer A (5′-CGCAATTAACCCTCACTAAAGGGAACAA-3′) and 300 fmol of both mutagenic primers B (5′-GGCGTTCTT***A***AGGCGG-TTGGCGTGC-3′) and C (5′-CAACCGCCT***T***AAGAACGCC-AGCCTGT-3′) which contain an overlapping region of 19 nt, 2.5 nmol of each dNTP and 2.5 U polymerase. Polymerases tested were five different batches of *Taq* (obtained from Angewandte

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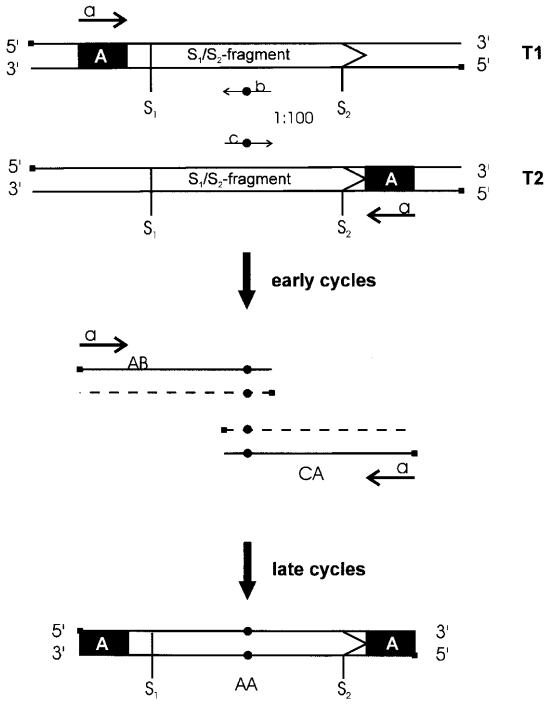

Figure 1. Schematic diagram of the OOE-PCR method for site-directed mutagenesis. A nucleotide substitution (solid circle dot) is introduced into a template DNA which has to be cloned in two different orientations (T1 and T2) relative to one universal primer A (single line with arrowhead), e.g., into the multiple cloning sites of pBluescript KS and SK using two unique restriction sites S_1 and S_2. The concentration of two mutagenic primers B and C (single line with arrowhead and solid circle dot) is reduced by a factor of 100 as compared with the universal primer resulting in an asymmetric amplification of the intermediate products AB and CA during the early cycles. Due to their terminal complementarity, these products will overlap and will subsequently be extended. The mutant product AA possessing one binding site for the universal primer on each side (black boxes) will be amplified as soon as it is formed during the late cycles of the reaction. Finally, the recombinant PCR product can be recloned making use of restriction sites S_1 and S_2.

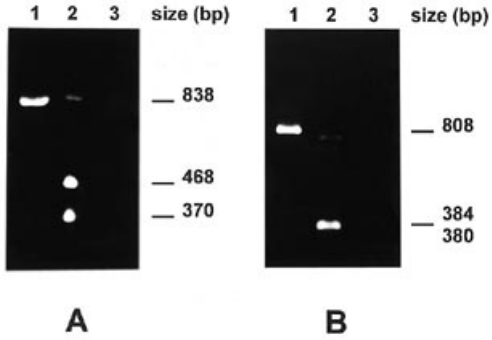

Figure 2. Application of OOE-PCR to generate unique restriction sites. (**A**) A pBluescript II KS/SK template system, the T3 primer and the *Taq* polymerase (Boehringer Mannheim) were used to create an *Afl*II site in the *lipA* gene of *P.aeruginosa* and (**B**) a *Kpn*I site in the *gfp* gene of *A.victoria* using pUC18/19 as the template system, the reverse primer and the *Taq* polymerase (Eurogentec, Seraing, Belgium). Samples were digested with 5 U of the appropriate restriction enzymes for 2 h at 37°C and loaded onto a 1.2 % agarose gel. Lanes 1, PCR products (A, 1/10 and B, 1/20 vol of the total PCR reaction). Lanes 2, PCR products digested with *Afl*II (A) and *Kpn*I (B), respectively. Lanes 3, PCR control reactions in the absence of mutagenic primers B and C.

Gentechnologie Systeme, Heidelberg; Boehringer Mannheim; Eurogentec, Seraing, Belgium; MBI Fermentas, St. Leon-Rot; and Life Technologies, Eggenstein), *Tth* (Biozym Diagnostik, Hessisch Oldendorf) and *Pfu* (Stratagene, Heidelberg) with proofreading activity to minimize unwanted PCR-generated mutations. With all polymerases used we obtained comparably high yields of mutant DNA. An exception was *Tfl* polymerase (Promega, Heidelberg) which did not give any detectable product. PCR was performed in a Robocycler (Stratagene, Heidelberg) at the following conditions: 35 cycles of 1 min at 94°C, 2 min at 50°C and 1 min at 72°C. The first denaturation step was carried out for 3 min and the last elongation step for 5 min. The PCR products were purified using the QiaGen PCR Purification Kit (QiaGen, Duesseldorf) and a 1/10 vol was loaded onto a 1.2% (w/v) agarose gel (Fig. 2A). An aliquot was digested with *Afl*III (1 U, 2 h) to determine the proportion of the recombinant product. The recombinant DNA was digested with *Eco*RI and *Sal*I to generate a 689 nt fragment which was ligated into the wildtype *lipA* gene from which the homologous 689 nt *Eco*RI/*Sal*I fragment had been removed.

A *Kpn*I site was created in the middle of the *A.victoria gfp* gene which was cloned as a 728 nt *Hin*dIII/*Xho*I fragment into a pUC18/19 template system. PCR conditions were as described above with mutagenic primers B (5′-TAACAAGGGTA***C***CACC-TTCAAACTTG-3′) and C (5′-GAAGGTG***G***TACCCTTGTTA-ATAGAATC-3′) containing an overlapping region of 19 nt and reverse primer A (5′-CAGGAAACAGCTATGAC-3′) as the flanking primer. Digestion of *A.victoria* DNA with *Kpn*I (Fig. 2B) generated two fragments (384 and 380 bp) which did not add up to the length of the mutant DNA fragment (808 bp) due to the presence of an additional *Kpn*I site in pUC generating a third 44 bp fragment which is not visible in lane 2 of Figure 2B.

The OOE-PCR method we describe here allows the rapid and efficient introduction of mutations into DNA. The results shown in Figure 2 led us to estimate an at least 95% efficiency in the generation of mutant DNA. The method is fast, simple, generally applicable, and therefore represents a significant improvement of the widely used OE-PCR method.

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