

A general and fast method to generate multiple site directed mutations

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Many methods have been described for site directed mutagenesis. One of the most powerful ones is the overlap extension method described by Higuchi *et al.* (1, 2), but this method requires two new primers for each mutagenesis and for this reason is not adapted for an extensive mutagenesis in the same DNA sequence. We have developed a rapid and efficient method derived from the overlap extension method which allows one to generate multiple site directed mutations (deletion, insertion or substitution) in a given DNA fragment. It requires three universal primers chosen in the vector and only one specific primer for each mutation.

As shown in the figure, the method consists of two successive rounds of PCR. The first round consists of two simultaneous PCR reactions. The first one was done with primer 1 and 2. Primer 1 and 2 are homologous to the vector sequence, but primer 2 also contains a mismatched 3′ end. The second PCR was done with primer 3 and primer M which contains the mutation (deletion, substitution or insertion). Amplified fragments from each PCR were purified, mixed and subjected to another round of PCR with external primers 1 and 3. During this second PCR, parental fragments and hybrid B cannot be amplified. Solely hybrid A is amplified and because the 3′ end of primer 2 is not complementary to the DNA fragment, only the mutated strand is amplified. The amplified fragment can be then digested with restriction enzymes and ligated into the appropriate vector.

We used this method to generate 10 different mutants (deletions and substitutions) in the Epstein-Barr Virus transcription factor EB1 (or Z). In our hands, the efficiency of this procedure reached routinely 90%. Each of the mutants has been sequenced across the entire PCR amplified fragment, and we never observed additional sequence modifications than the one programmed.

The first PCR was carried out with Taq DNA polymerase (Promega) and the buffer provided by the manufacturer in the presence of 200 μM dNTP, 1 mM of each primer, 20 ng of template DNA and 1U of enzyme in a final volume of 100 μl for 30 cycles (94°C 20 sec, 72°C 30 sec, 50°C 40 sec). Amplified products were loaded on a 1.5% agarose gel and purified by squeeze freeze. 20 to 50 ng of each purified fragment were mixed and subjected to 30 cycles of PCR in the conditions described above with external primers 1 and 3. Primers 1 and 3 are 20 nt long. Primer 2 is 25 nt long and contains 8 mismatched nt at its 3′ end. Primers M have been chosen as for M13 mutagenesis with 15 nt feet on each ends. Furthermore, in order to avoid problems with template-independent incorporation at 3′ termini of amplified products, we have designed the mutagenic oligonucleotides as described by Kuipers *et al.* (3), such as the first 5′ nucleotide of the primer follows a T residue in the template sequence.

REFERENCES

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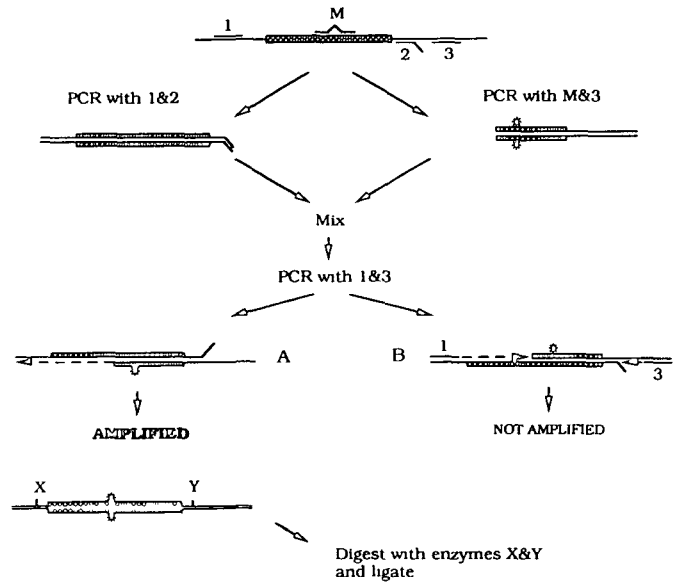

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