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Efficient random mutagenesis method with adjustable mutation frequency by use of PCR and dITP

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Two general methods for random mutagenesis on a specific DNA sequence by use of PCR have been described. One method (1) is based on the intrinsic error frequency of *Taq* DNA polymerase using standard conditions, resulting in about 5.5×10^{-4} mutations per basepair. This frequency is adequate for mutagenizing relatively large genes, but not for small DNA fragments (<500 bp). In the other method (2) the infidelity of *Taq* DNA polymerase is increased by the addition of Mn^{2+} and by use of relatively high concentrations of dNTP and Mg^{2+}, resulting in more than 30×10^{-4} mutations per basepair. We have developed an alternative method that allows the adjustment of the mutation frequency to the size of the target DNA, offers the opportunity of influencing the types of mutation, and does not require a high dNTP concentration.

The method is based on the following principles: (a) one of the four dNTPs is present in limiting amounts in each of four separate PCR reactions; (b) when the pool of the limiting dNTP has been depleted it is possible that misincorporation of one of the other dNTPs is favored; (c) misincorporation might be stimulated when dITP is present, since it is known to be incorporated by *Taq* DNA polymerase, although with a four times reduced efficiency (3); in the next cycle this would result in the incorporation of any of the three natural nucleotides left, as a complement to dITP.

Random mutagenesis experiments on the 171 bp *Lactococcus lactis nisZ* gene (4) were performed using the conditions described previously (5), except for the concentration of Mg^{2+} which was increased from 2 mM to 5 mM. In a mutagenesis experiment four PCR reactions were performed, each of which contained limiting amounts of one of the four dNTPs. In some cases 0.2 mM dITP was added. PCR fragments were pooled and cloned in *Escherichia coli* using a suitable vector. To assess the mutation frequency, the DNA sequence of a 200 bp fragment, including the *nisZ* gene, was determined for each of 24 independent clones. When the concentration of the limiting nucleotide was adjusted to 20 μM the yield of PCR product was reduced to 80%, whereas a concentration of 14 μM reduced the yield to 10–30%. The effects of several different PCR conditions on the mutation frequency are given in Table 1.

The use of a reduced amount of one dNTP without the addition of dITP resulted only in a small increase in mutation frequency, which shows that *Taq* DNA polymerase cannot efficiently replace the depleted dNTP by any of the other dNTPs. However, when simultaneously 0.2 mM dITP was added the mutation frequency increased considerably, indicating that dITP is indeed incorporated, generating a point mutation in two out of three cases in the next cycle. When the experiment was repeated with dITP and 14 μM of the limiting dNTP, the mutation frequency increased still further. As a consequence, the concentration of the limiting dNTP is directly related to the number of mutations that will occur. With dITP and 40 μM of the limiting nucleotide, which gives a normal yield of PCR product, the number of mutations was low again, confirming the need for a limiting amount of one dNTP in each PCR reaction. Analyzing the nature of the point mutations, we found 60% of AG or TC mutations, 26% of GA or CT mutations and 14% of transversions. A low frequency of insertions or deletions was found (Table 1). These results show that it is possible to modulate the number of mutations in a certain DNA fragment by varying the percentage of the limiting dNTP and by adding dITP, simultaneously. In principle one can vary the types of mutation, by selectively using only one or more of the limited PCR reactions or using different amounts of each PCR reaction when pooling the amplified DNA. Another possibility to get more evenly distributed mutations is to include 0.5 mM Mn^{2+} in the PCR reactions, which will randomize the types of mutation (2). If one wishes to raise the mutation frequency further one might take into account the AT/GC ratio of the target DNA in determining the concentration of the limiting dNTP in each of the four PCR reactions and/or include Mn^{2+} in the reaction to increase the infidelity of *Taq* DNA polymerase (2). The relationship between the concentration

Table 1. Effect of PCR conditions on the frequencies of point mutations and insertions/deletions.

Condition	mutations per 10^4 basepairs	deletions/insertions per 10^4 basepairs
Standard PCR (200 μM each dNTP, 5 mM Mg^{2+})	6.3	<2.2
20 μM of one dNTP	8.3	<2.2
40 μM of one dNTP + 200 μM dITP	4.2	<2.2
20 μM of one dNTP + 200 μM dITP	27.1	<2.2
14 μM of one dNTP + 200 μM dITP	37.5	<2.2

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of the limiting dNTP and the mutation frequency will depend on the specific parameters of each individual experiment and must be determined empirically.

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