3276–3277 *Nucleic Acids Research, 1996, Vol. 24, No. 16* © *1996 Oxford University Press*

An efficient PCR mutagenesis strategy without gel purificiation step that is amenable to automation

Bertrand Séraphin* and Stefanie Kandels-Lewis

EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

Received March 11, 1996; Revised and Accepted June 21, 1996

ABSTRACT

We describe here an improved megaprimer PCR mutagenesis strategy. The cumbersome gel purification step that is usually used can be omitted by appropriately cleaving the first and second DNA templates with restriction enzymes and enzymatically removing remaining primers from the first PCR reaction. We show that this improved procedure is reproducible and highly efficient. Furthermore this method is suitable for automation because all the steps are now carried out in reaction tubes.

Among the many methods for site directed mutagenesis, the PCR-based megaprimer strategy (reviewed in 1) is currently widely used because of its flexibility and the yield of mutants approaches 100%. This method is relatively cheap, as only a single mutagenic primer and two flanking primers are used per mutant. A limitation of the megaprimer strategy is that the products of the first PCR reaction need to be fractionated by gel electrophoresis and purified to remove left-over primers, before being used in the second round of PCR. This prevents the amplification of the wild-type template during the second PCR reaction that would dramatically decrease the mutant yield. This step is time consuming, expensive and error-prone, especially when multiple mutants are made in parallel. This step is also a potential barrier to automation. A second problem associated with the megaprimer strategy is the non-templated addition of nucleotide, most frequently but not always an A, at the 3′ end of the first PCR product. This can result in the presence of additional unwanted mutations. Various strategies have been described to eliminate these unwanted mutations (2–4) but they are time consuming and expensive, only applicable under particular conditions or sometime reduce but do not eliminate these mutations.

Here we describe an improved megaprimer mutagenesis strategy that circumvents the gel electrophoresis step (Fig. 1). This method is highly efficient with a mutant yield approaching 100%. All the steps are now carried out in reaction tubes, making the method well suited for automation. Our strategy relies on the observation that the gel purification step used in the megaprimer strategy is only required to remove the primer used during the first PCR reaction. The two modifications of the classical protocol described below eliminate the amplification of wild-type DNA template during the second PCR reaction rendering the gel purification step unnecessary. First, the templates used for PCR were cleaved with restriction enzymes to eliminate full length template. In this way, wild-type DNA should not be amplified by the two external primers (Fig. 1). Although the only requirement for choice of the restriction enzymes to be used is that they should cut the fragment to be amplified once or multiple times on only one side of the mutagenic primer, they are most conveniently chosen as the enzyme that will subsequently be used for the cloning of the mutated PCR fragment (Fig. 1). A second modification was the treatment of the product from the first PCR reaction with Klenow enzyme. The proofreading activity of the enzyme is used to reduce the concentration of the primers used during the first PCR reaction as well as reducing the level of non-templated nucleotides added by the *Taq* polymerase at the 3′ end of the PCR product (Fig. 1, step V). Using these modifications, the product of the first PCR reaction could be used directly as a megaprimer in the second reaction (Fig. 2). Both the cleavage of the template DNA and the reduction of the primer concentration after the treatment with the Klenow enzyme are required to prevent amplification of the wild-type sequence. Indeed, trace of full length DNA remaining after the digestion could still be amplified by standard amounts of primers 1 and 3 while the reduced levels of primers 1 and 3 obtained after the Klenow treatment could still produce low amount of product on a non-cleaved template. Experimentally, only mutant clones (36/36) were recovered in six independent mutagenesis experiments (Table 1). A few clones contained an additional unwanted mutation (T→C) one nucleotide 5′ to the mutagenic primer, suggesting that the proofreading activity of the Klenow enzyme did not remove all non-templated nucleotides added by the *Taq* polymerase. Further study will resolve whether other enzymes (e.g. T4 DNA polymerase) are better suited for this step. We and others (5) also noticed that in a few cases the megaprimer was not priming efficiently enough to produce suitable amounts of the second PCR product. This is an inherent limitation of the megaprimer strategy that is not affected by the modifications described here. In summary, our modification of the megaprimer PCR mutagenesis strategy makes it an efficient method amenable to automation.

The reaction conditions were as follows. PCR templates and plasmid DNA were cut with appropriate restriction enzymes following the manufacturer's recommendation (New England Biolabs). The first PCR reaction was carried out in a final volume of 100 µl with 1 ng template DNA cleaved with enzyme B, 100 pmol each of primers 1 and 2, 5 nmol of each dNTP, 1 U *Taq* polymerase (AmpliTaq, Perkin Elmer Cetus) using the buffer supplied by the manufacturer. The template DNA and primers were denatured for 5 min at 94°C before the addition of the enzyme and nucleotides. The reaction was overlaid with 50 µl

* To whom correspondence should be addressed

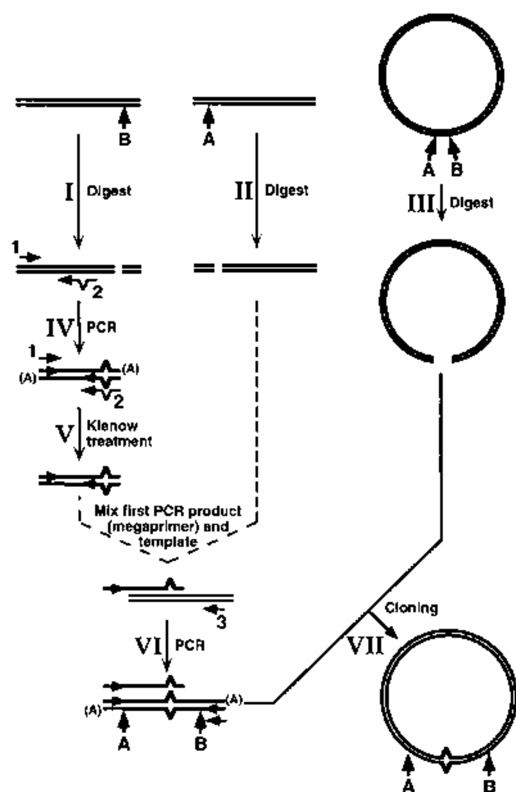

Figure 1. Gel-free PCR mutagenesis strategy. The various steps (arrows I–VII) used in our strategy are depicted with double-stranded DNA schematically represented as a double line, primers 1–3 as horizontal arrows, the mutation by a spike and non-templated nucleotides added by the *Taq* polymerase as (A). Step I: a fraction of the PCR template DNA is cleaved with a restriction enzyme B cutting once or multiple times in the region located between primers 2 and 3 but not cutting in the region located between primers 1 and 2. Step II: another fraction of the PCR template DNA is cleaved with restriction enzyme A cutting once or multiple times in the region located between primers 1 and 2 but not cutting in the region located between primers 2 and 3. For both of these steps, the starting DNA molecule can be linear (as shown) or circular (e.g., the template can be identical to the vector used for recloning depicted in step III). Restriction enzymes A and B can conveniently be selected as the enzymes that will be used for cloning the mutated DNA fragment (as shown here) but this is not a necessity. The DNA fragment that will be cloned should be kept as small as possible as it is advisable to sequence it entirely to check for the absence of unwanted mutations potentially introduced by the PCR reaction. Step III: the cloning vector is cleaved with restriction enzymes A and B. Note that steps I–III can conveniently be performed in parallel. Step IV: a first PCR amplification is performed using primers 1 and 2. Step V: Klenow enzyme is added to an aliquot of the first PCR reaction. The proofreading activity of the enzyme degrades the single-stranded primers and non-templated nucleotides. The second templated DNA (prepared at step I) is added to an aliquot of this reaction (dashed lines). Step VI: the second PCR amplification reaction is performed between the megaprimer and oligonucleotide 3. Step VII: the mutated DNA fragment is cleaved with enzymes A and B, ligated with the vector prepared at step III and introduced in *Escherichia coli*. The sequence of DNA located between sites A and B is determined to ensure the presence of the desired mutation and the absence of unwanted mutations.

paraffin oil. PCR was performed for 30 cycles consisting of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C. This was followed by a filling-in reaction of 5 min at 72°C. One microliter (5 U) of the Klenow fragment of DNA polymerase I (Boehringer) was added to 90 µl of the first PCR reaction and incubated at 37°C for 45 min. A second PCR reaction was carried out in a final volume of 50 µl

Figure 2. Klenow treatment and cleavage of the first template prevent efficient amplification of this wild-type template. Lane 1: megaprimer produced in the first PCR reaction after Klenow treatment (i.e., after step V of Fig. 1). Lanes 2 and 3: a substantial amount of the mutated fragment is formed after the second round of PCR (i.e. after step VI of Fig. 1). Lane 4: as a control, the second PCR reaction was done without the addition of the second template. The absence of product demonstrates that the Klenow treatment and the cleavage of the first template efficiently prevented the amplification of any remaining wild-type template.

with 1 ng template DNA cleaved with enzyme A, 100 pmol of oligonucleotide 3, 5 nmol of each dNTP, 25 µl of the first PCR reaction treated with Klenow enzyme (see above) and 1 U *Taq* polymerase (AmpliTaq, Perkin Elmer Cetus) using the buffer supplied by the manufacturer. The template DNA, oligonucleotide and Klenow-treated products of the first PCR reaction were denatured for 5 min at 94°C before the addition of the enzyme and nucleotides. The reaction was overlaid with 50 µl paraffin oil. PCR was performed for 30 cycles consisting of 1 min at 94°C, 10 min at 55°C (5) and 2 min at 72°C. DNA was extracted with phenol/chloroform/isoamylalcohol and precipitated with ethanol. It was then digested, cloned and sequenced.

Table 1. Summary of five mutagenesis experiments

Exp. no.	Sequence change from	to	Oligonucleotide length (nt)	No. mutants/no. clones analysed
1	GGTTCTG	GCAAGAG	22	3/3
2	AAAG	ATAG or ATCG[a]	18	6/6[b]
3	AAAA	AGTA	18	3/3
4	AAAG	ATCG	18	6/6
5	AAAG	ATAG or ATGG[a]	18	12/12[c]
6	AAG	AGG	18	6/6

[a]Mutagenesis with a degenerate oligonucleotide.
[b]Six clones contained the ATAG sequence and none the ATCG sequence.
[c]Four clones contained the ATAG sequence and eight the ATGG sequence.

ACKNOWLEDGEMENTS

We thank M. Luukkonen, P. Gadroy and K. Bohmann for testing our protocol. We are grateful to J. Lewis, M. Luukkonen and H. Voss for critical comments on the manuscript.

REFERENCES

1 Tao, B.Y. and Lee, K.C.P. (1994) In Griffin, H.G. and Griffin, A.M. (eds), *PCR Technology: Current Innovations.* CRC Press, Boca Raton, Florida. pp. 69–83.
2 Kuipers, O.P., Boot, H.J. and de Vos, V.W. (1991) *Nucleic Acids Res.* **19**, 4558.
3 Sharrocks, A.D. and Shaw, P.E. (1992) *Nucleic Acids Res.* **20**, 1147.
4 Armengaud, J. and Jouanneau, Y. (1993) *Nucleic Acids Res.* **21**, 4424–4425.
5 Good, L. and Nazar, R.N. (1992) *Nucleic Acids Res.* **20**, 4934.