Rolling circle transcription of ssDNA circles encoding self-processing ribozymes is a novel, simple and rapid means to obtain small, regularly spaced, RNA size marker ladders. To our knowledge, the combination of small-size RNA production represented as a regularly spaced ladder found in our system is currently not available from any single in vitro transcription system. The DNA circle synthesis is easily carried out, and the stability of these DNAs allows for a single synthesis to create ample template for long-term usage.

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Address correspondence to Dr. Eric Kool, University of Rochester, Department of Chemistry, College of Arts & Sciences, Rochester, NY 14627-9000, USA. Internet: etk@etk.chem.rochester.edu

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Amy M. Diegelman, Sarah L. Daubendiek and Eric T. Kool
University of Rochester
Rochester, NY, USA

Combined PCR/Gapped-Duplex Method for Site-Directed Mutagenesis


Here we present a new method for oligonucleotide-mediated, site-directed mutagenesis (SDM) of cloned DNA sequences that combines the high mutagenic efficiency of polymerase chain reaction (PCR)-based methods (2,4,9,10,13) with the cloning flexibility provided by the gapped-duplex method (5).

Mutagenesis techniques involving one mutagenic oligonucleotide and a single-stranded (ss) (8,14) or double-stranded (ds) (5) template have variable efficiencies of mutagenesis. These protocols, however, have the advantage of not requiring specifically located restriction sites. In the gapped-duplex method (5), any pair of restriction sites that flank the region to be mutated is sufficient.

PCR-based mutagenesis (4) has the advantage of using either ss- or dsDNA as template and of producing large quantities of amplified ds products containing the desired sequence. For further manipulation, it is often desirable or necessary to clone these mutant PCR products into plasmid or phage vectors. This cloning step requires appropriate unique restriction enzyme sites that are either present naturally in the cloning vector and in the amplified sequence or that were specifically designed into the primers used for amplification. On occasion, this seemingly trivial cloning step presents unforeseen difficulties for any one of a number of reasons, including: (i) absence of appropriate naturally occurring restriction sites in the amplified sequence, (ii) the use of amplification oligonucleotides with new restriction sites that may introduce unwanted mutations (however, see Reference 13) and (iii) inefficiency of ligation of the amplified PCR product into the parental vector after restriction enzyme digestion.

To overcome such difficulties, we have successfully used a variation of the gapped-duplex method for oligonucleotide-mediated mutagenesis (5) in which ds PCR product containing the desired mutation is used instead of a ss mutagenic oligonucleotide. Figure 1 shows the general scheme of the protocol.
In the first phase of the protocol, the ds plasmid or phage vector containing the template DNA undergoes three separate treatments. (i) PCR-based mutagenesis produces a ds product containing the mutation on both DNA strands. This product is then 5'-phosphorylated with polynucleotide kinase (Figure 1: First Phase, Steps 1 and 2). Alternatively, the PCR product can be digested with one or two restriction enzymes that cut on either side of the mutation. In this case, DNA fragments with 5'-phosphates are produced, and the phosphorylation step can be omitted. (ii) Digestion with two restriction enzymes (B and C in Figure 1) that cut the vector on either side of the target sequence amplified by PCR. The mixture is then phosphatased, and the B-C fragment that does not contain the PCR-amplified sequence is purified (Figure 1, First Phase, Steps 3–5). (iii) Digestion with a restriction enzyme (A in Figure 1) that linearizes the vector followed by phosphatase treatment to prevent recircularization in the subsequent ligation (Figure 1, First Phase, Steps 6 and 7).

In the second phase of the protocol, equimolar amounts of the three products from the first phase are mixed, heat-denatured and annealed (Figure 1, Second Phase, Steps 1 and 2). There were five major products formed—the three original ds reagents plus the two gapped duplexes (labeled I and II in Figure 1). Finally, the annealing mixture is treated with DNA polymerase (Klenow fragment) and T4 DNA ligase for extension and ligation steps (Figure 1, Second Phase, Step 3: Reference 11) to convert the gapped duplexes (I and II in Figure 1) into closed DNA circles (not shown). This mixture is then used to transform competent Escherichia coli (Figure 1, Second Phase, Step 4). Single colonies are picked and either plasmid or phage DNA mini-preps are made for mutation screening by sequencing. Alternatively, if the mutation created a new restriction site, then mutants can be discriminated by restriction enzyme digestion.

We have used this protocol to produce a site-directed mutation in the 5431-bp cDNA of human complement component C4 in which the Cys codon at position 1010 was changed to an Ala codon (C1010A). The plasmid carrying this cDNA, pSVC4 (9.8 kb), contains the β-lactamase gene and has been described elsewhere (1). For this discussion, restriction site A is a SalI site at position 8 of the cDNA, and restriction sites B and C are both SmaI sites at positions 1438 and 4625, respectively, of the cDNA.

First Phase. A fragment containing the desired mutation in both strands was obtained by the method of Higuchi (4). This protocol consists of two PCR amplifications as described below. First, a 354-bp fragment was amplified from the template pSVC4 using the oligonucleotide primers: C4OL1 5’-GCCTGTTGCTTCTC-3’ and the mutant C4OL3 5’-TTGCTCCCCGGCGCTCG-3’ (mutated codon underlined). Similarly, a 1572-bp fragment was obtained using C4OL2 5’-GGTGGGGACCGAGTC-3’ and the mutant primer C4OL4 5’-CGAGGGCGCGGGAGCA-3’ (mutated codon underlined). For both reactions, 2 pmol of template pSVC4 were denatured for 5 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s annealing at 48°C and 60 s extension at 72°C, followed by an extra 5 min of extension at 72°C. The 354- and 1572-bp fragments were purified from a preparative 0.8% low-melting agarose gel (Life Technologies, Gaithersburg, MD, USA). A second round of amplifications was performed using these last fragments as templates. Equimolar concentrations of both PCR fragments were denatured at 94°C for 5 min and annealed for 5 min at room temperature. Extension with 2.5 U of Taq DNA Polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden) was for 7 min at 72°C. The oligonucleotide primers C4OL1 and C4OL4 were then added, and a new PCR was initiated using the same cycle times and temperatures as described above. The PCR product was then treated with T4 polynucleotide kinase (11). pSVC4 was digested with either SalI or SmalI and treated with calf intestine phosphatase. The larger of the two SmaI fragments (6.6 kb) was gel-purified.

Second Phase. The kinased PCR product and phosphorylated pSVC4 fragments were combined in equimolar amounts (50 fmol), denatured at 100°C for 3 min and slowly renatured by cooling to room temperature in a 300-mL water bath over a 30-min period. The gaps were filled in using dNTPs, DNA polymerase (Klenow fragment) and T4 DNA ligase (11). The resulting mixture was used to transform E. coli strain HB101 cells. DNA from ampicillin-resistant colonies was purified and screened for a newly created NarI site present in the C1010A mutant. Twenty

**Figure 1. New protocol for SDM.** A, B and C are restriction enzyme sites. The small open circle and half-circle represent the wild-type sequence to be mutated, whereas the small dark circle and half-circle represent the mutated sequence. P-ase indicates treatment with alkaline phosphatase. See text for details.
percent of the colonies possessed the newly created NarI site. The C1010A mutation was confirmed by dideoxy-se-quecing (12).

The wild-type and mutant C4 cDNAs were used to transfect mammalian COS-1 cell cultures, and after metabolic labeling with [35S]methionine, the recombinant proteins were immunoprecipitated with anti-human C4 antibodies from rabbit serum plus Staphylococcus aureus - Protein A. The immunoprecipitated proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), analyzed by autoradiography, and the results were published elsewhere (6). We detected equivalent pre-cursor form synthesis rates for both C1010A and wild-type C4 proteins. The integrity of the mutated cDNA was further confirmed by the fact that the C1010A C4 protein was secreted at the same rate, exhibited the same stability and was processed into α, β and γ chains in a manner similar to that of recombinant wild-type C4 (6).

In conclusion, we have developed a variation of the gapped-duplex method for oligonucleotide-mediated mutagenesis in which a ds PCR product containing the desired mutation is used instead of a mutagenic ss oligonucleotide. The mutagenic frequency of our protocol is sufficiently high for screening by sequencing or restriction digest analysis of a relatively small number of clones, and it derives from the fact that the relatively short oligonucleotide in the original method (5) has been replaced by a pair of much larger PCR-derived fragments. These mutagenic fragments can compete more successfully for their complementary sites during annealing and are more resistant to the 3′→5′ exonuclease activity of some commercially available DNA polymerases. Full-plasmid PCR protocols (10) present a simple and efficient means for SDM. However, all PCR-based methods are susceptible to unwanted additional muta-tions (3,7), and the efficiency and fidelity of amplification decrease with the increasing size of the PCR product. Therefore, the combined PCR/gapped-duplex method described here can be a useful alternative to full-plasmid PCR methods when the template for mutage-nesis is a relatively large plasmid.

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Lourdes Isaac and Chuck S. Farah
Universidade de São Paulo
São Paulo, Brazil