PCR-Generated Cross-over Linkers for Site-Directed Mutagenesis

BioTechniques 23:827-830 (November 1997)

In the crossover linker method (11,16) for site-directed mutagenesis, a synthetic oligonucleotide DNA duplex containing the mutation (a substitution, deletion or small insertion) adjacent to a small region of appropriate homologous DNA is ligated to a linearized target plasmid. On transformation into E. coli, linear molecules so formed have the potential to be circularized by homology-mediated recombination attributable to double-strand break repair, thus incorporating the mutation. The method is limited by the maximum practical length of synthetic crossover linkers (approximately 200 bp).

We have increased the versatility of this method by replacing the short linker with one generated by polymerase chain reaction (PCR) (up to 5 kb commonly attainable) followed by restriction cleavage. This has several advan-

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Figure 1. Schematic representation of PCR-generated crossover linker mutagenesis. The target is a plasmid containing the DNA to be mutagenized, shown as a boxed XABY region. Z represents the target-unique restriction site, \( f \) a mutagenic forward primer and \( r \) a wild-type reverse primer. The mutation to be introduced is shown as an asterisk. The inner boxed region AB is eventually partially replaced by the crossover linker. The PCR product and its derivatives are shown shaded and in broken-edged boxes. '?' designates any alternative source of mutant DNA.
The utility of our method is that it allows facile PCR-mediated site-directed mutagenesis to be carried out on plasmids containing a single nearby restriction site (Figure 1). The mutated linker precursor fragment is generated by one of two routes: PCR is carried out either on the target itself using a mutagenic forward primer \( f \) and wild-type reverse primer \( r \), or using wild-type primers on the unique site \( Z \) and deletion were encoded by the reverse primer.

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1.4-kb region comprising the 6a/6b intron and flanking exon sequences from a human genomic P-1-derived artificial chromosome (PAC) template CF3 (180 kb) that contains part of the human CFTR gene (unpublished). EcoRI-digested PAC DNA (250 pg) was amplified in three identical 100-µL reaction mixtures, each comprising 25 pmol of S1U and S1L, 1.25 µL enzyme mixture and 75 µM each dNTP in the manufacturer’s magnesium-free buffer (Boehringer Mannheim GmbH) plus 2.75 mM MgCl₂. The PCR was carried out on a TRIO Thermoblock™ (Biometra, Göttingen, Germany) for 25 cycles using these conditions: denaturation at 94°C for 2 min; 25X at 94°C for 10 s, 60°C for 1 min and 68°C for 2 min; and a final extension at 72°C for 5 min. Residual polymerase was then removed by proteinase K digestion (4). The amplified DNA was digested with AflIII, the resulting 1327-bp linker gel-purified and approximately 40 ng ligated to approximately 200 ng AflIII-linearized, dephosphorylated (with calf intestinal phosphatase [CIP]; Boehringer Mannheim GmbH) pCMV-CFTR in a 16-µL reaction mixture containing 15% PEG 6000 (Merck Ltd., Leicester, England, UK) as described (2). Aliquots (25 µL) of electro-competent JC8679 cells prepared as described (7) were transformed using a Gene Pulser® (Bio-Rad, Hercules, CA, USA) at 2.5 kV, 25 µF, 200 Ω with 1–2 µL of drop-dialyzed (12) ligation mixture and recombinants selected on ampicillin plates. The final yield was equivalent to over 10⁴ ampicillin-resistant (Ap⁺) colonies per microgram target DNA, tenfold more than that obtained from control ligations omitting insert DNA. This is comparable to that expected in a conventional cloning and demonstrates the high efficiency of double-strand break repair in JC8679 (13). Plasmid DNA was isolated (8) from 12 colonies and examined by restriction analysis. Five out of ten clones were found to contain the intron, 2/10 were regenerated target, and 3/10 appeared to be deletion derivatives. The structures of four of the intron-containing plasmids were further investigated by sequencing (using the PRISM™ Cycle Sequencing Kit [PE Applied Biosystems, Foster City, CA, USA] with S1U, S1L and other primers) on a Model 373 DNA Sequencer (PE Applied Biosystems). This confirmed that double-strand break repair had faithfully recombined the linear DNA within the shared homologous regions. In only one case was an undesired mutation detected; this occurred within the intron and probably arose during PCR.

We have used this method for several diverse constructions including other modifications of the CFTR gene and obtained similarly high yields of Ap⁺ colonies (approximately 10³–10⁴ per µg input target DNA using electro-competent JC8679 cells with a transformation efficiency of 10⁶–10⁷ Ap⁺ colonies per µg uncut pBR322), significant proportions of which contained the desired mutation (Table 1). As expected, the proportion of mutant plasmid obtained is approximately equivalent to the ratio of flanking homologies A and B (Figure 1 and Table 1), although we have not attempted to demonstrate this rigorously. So far, we have generated crossover linkers in which the A/B ratio is greater than 0.3, but in many constructions, A will necessarily be much shorter than B, probably causing the mutant yield to be reduced correspondingly. Since the total colony yield is high, however, it should always be possible to identify mutant plasmids with a suitable screen. It must be stressed that because of the relatively high proportion of clones with gross deletions or rearrangements produced by this method, screening of clones by restriction analysis prior to sequencing is essential.

A disadvantage of other widely used PCR methods exploiting single sites (6,18), including inverse PCR mutagenesis (5) is that they are unsuitable for creating large insertions. Also, they require amplification of the entire target, with a higher probability of incorporating unwanted mutations because of the intrinsic infidelity of PCR (10). While such a drawback is not significant when the cloned region to be mutagenized is small (<500 bp) and readily sequenced, multiple sequencing rounds are required when the region is much larger, as is the case when mutagenizing most full-length genes. Another disadvantage is that large target plasmids may be impossible to amplify as a whole. In contrast, such plasmids may be mutag-
References


We thank Heather Davidson for the PAC CF3, and David Sherratt and David Leach for bacterial strains. This work was supported by a grant awarded by the Realising Our Potential Award (ROPA) scheme administered by the MRC. Address correspondence to A. Christopher Boyd, MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh, Scotland EH4 2XU, UK. Internet: chts@igum.mrc.ac.uk

Received 10 March 1997; accepted 19 May 1997.

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Site-Specific Mutagenesis of Immunoglobulin Domains by Multiple-Fragment Homologous Recombination

BioTechniques 23:830-834 (November 1997)

The polymerase chain reaction (PCR) is a widely used and versatile technique for the amplification and modification of immunoglobulin (Ig) genes. The rapidity and accuracy with which antibody genes can be modified in vitro has produced an assortment of novel antibodies. For example, PCR methods have been used for engineering Igs with increased affinity to antigen, for “humanizing” antibodies and for modulating effector function (7–9).

As part of a more comprehensive study, we desired to introduce various site-specific mutations into the CH2 constant domain of human IgG1. Six specific amino acid residues distributed throughout the CH2 domain previously identified to play a role in immune effector function were marked as targets for mutagenesis (1,8,10). Five of the six residues were grouped into two clusters; one cluster consisted of two residues, two amino acids apart (Location 1 or L1), and the second cluster consisted of three residues spanning a sequence of five amino acids (L2). The remaining amino acid position (L3) brought the total to six residues. We were interested in constructing a panel of mutant CH2 domain IgGs consisting of each L mutation by itself as well as in combination with other L mutants (e.g., L1; L1 and L2; L1, L2 and L3 etc.).

Various in vitro methods have been described in which PCR is used to simultaneously introduce multiple distally located site-specific mutations within a gene sequence (2,3). Alternatively, in vivo procedure termed recombination PCR has also successfully been used for rapidly and efficiently generating distally located site-specific mutations (4,5). Recombinant PCR uses E. coli’s recombination machinery to generate intact circular recombinant plasmids from a transfected mixture of linear PCR-generated product and...