Introducing undesired mutations during the preparation of the vector, can negatively affect the frequency of gene targeting in ES cells (13).

In summary, the method described here offers an attractive alternative for the preparation of replacement vectors for gene targeting. This protocol can be used to perform site-directed mutagenesis in long genomic sequences cloned into plasmids with the highest degree of fidelity of DNA replication. This method provides also an approach for correcting mutations on long PCR products.

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We thank Douglas Davidson for his technical help and James Hardin and Karen D’Arezzo for critical reading of the manuscript. Address correspondence to Pietro De Togni, Arkansas Cancer Research Center, 4301 West Markham, Slot 753, Little Rock, AR 72205, USA. Internet: ppdetogni@acrc.uams.edu

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Method of Site-Directed Mutagenesis Using Long Primer-Unique Site Elimination and Exonuclease III

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Site-directed mutagenesis of DNA is a widely used approach for studying gene regulation and protein structure and function (2). In the last few years, numerous methods have been developed using polymerase chain reaction (PCR), generating a mutant fragment from a double-stranded DNA template and mismatched oligonucleotides.

Among these recently developed strategies, the method called long primer-unique site elimination (LP-USE) mutagenesis (4) provides rapid and efficient means to introduce specific mutations into any double-stranded plasmid that contains a unique nonessential restriction site and the desired cloned gene. The principle of this method consists first of generating long primer (LP) by PCR with two mutated synthetic oligonucleotides: (i) the selection mutagenic primer is directed to a unique nonessential restriction site of the plasmid and introduces a mutation, which eliminates the enzyme recognition sequence; (ii) the target mutagenic primer is complementary to the cloned gene and carries the desired mutation. The PCR products are annealed to single-stranded target DNA and used as primers for second-strand synthesis. Thus, PCR products function as long primers with linked mutations. Following transformation in E. coli strain BMH 71-18 mutS (a mismatch repair deficient strain, which increases the probability of co-segregation of both mutations) (7) and growth, plasmids are purified and digested with the restriction enzyme, the site of which has been eliminated by the selection mutagenic primer. As reported by Conley and Saunders (1), linearized plasmids transform 100–1000 times less than circular plasmids. So, plasmids with a mutation in this unique restriction site are preferentially recovered by this procedure since they are not linearized by enzymatic digestion and therefore
transform *E. coli* more efficiently than linear nonmutated plasmids. However, the frequency of nonmutant plasmid recovery can be high. It could be related to either an incomplete digestion by the selection restriction enzyme or transformation of linear wild-type plasmids, especially if they are in high proportion compared to mutated circular plasmids. According to the protocol, there will be at least 50% of wild-type plasmids after the first transformation in *E. coli* strain BMH 71-18 mutS. This proportion may be higher than 50% because not all plasmid molecules anneal with LPs even with a high LP-to-plasmid ratio. So, to be very efficient, this method may require two rounds of selection with plasmid preparation, digestion and transformation (requiring at least 24 h). To avoid a second round of selection, we have introduced a simple step, which can eliminate the wild-type plasmids by treatment with exonuclease III. This enzyme catalyzes the stepwise removal of mononucleotides from recessed or blunt 3′ hydroxy termini of double-stranded DNA. However, protruding 3′ termini are resistant to the activity of the exonuclease III (6). Thus, the chosen restriction enzyme must generate blunt or recessed 3′ termini. The exonuclease III step is introduced just after plasmid digestion by the selection restriction enzyme. The exonuclease III step has been tested in our studies on the effects of mutations on spectrin self-association.

LP-USE mutagenesis was mainly performed as described by Ray and Nickoloff (4) and in the Transformer™ Site-Directed Mutagenesis Kit (CLONTECH Laboratories, Palo Alto, CA, USA). The mutant long-primer was obtained by PCR using as target template the plasmid pGEX-KG (3), in which an insert coding for residues 1899–2137 of the spectrin β chain had been introduced. This plasmid was amplified using two kinds of mutant primers: (i) the selection primer (forward primer), GG-TTCCGCGTGGTACC CCCGGGAATT-TCC, eliminated the unique *Bam*HI site present in the pGEX-KG plasmid by converting it into a *Kpn*I site (underlying sequence); (ii) the other primers (backward primers) contained the desired mutations that we wanted to introduce into spectrin peptides. Oligonucleotide primers were synthesized, purified and phosphorylated at the 5′ termini by Genset (Paris, France). Long primers containing both mutations were obtained in 30 cycles PCR with *Taq* DNA Polymerase (Appligene, Illkirch, France) using standard conditions recommended by the manufacturer. Approximately 1–5 pmol of PCR products, purified with the JETpure™ Kit (Genomed/Bioprobe® Systems, Montreuil-sous-Bois, France), were mixed with about 0.020–0.025 pmol of target plasmid, purified with the JETprep™ Kit (Genomed/Bioprobe Systems), in a solution containing 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂ and 50 mM NaCl, in a final volume of 20 µL. Samples were boiled to 100°C for 3 min in order to denature the plasmid and PCR products. Then, samples were quickly cooled to 4°C for 5 min. Annealing of PCR products with plasmid was performed at room temperature for 30 min. Second-strand synthesis and ligation were performed simultaneously by adding the following components: 3 µL of a solution containing 5 mM of each deoxynucleotide triphosphate (dNTP) in Tris-HCl, pH 7.5, 3 U of T4 DNA Polymerase (New England Biolabs/Ozyme, Montigny le Bretonneux, France), 1000 U of T4 DNA ligase (2000 U/µL; New England Biolabs/Ozyme) and 5.5 µL of water. Synthesis was performed for 30 min at 37°C.

### Table 1. Effect of Exonuclease III on Transformation Efficiency of Circular and Linearized Plasmids

<table>
<thead>
<tr>
<th>Exonuclease III Digestion</th>
<th>BanHI Digestion No</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>no</td>
<td>(100%)</td>
<td>(0.8%–1.7%)</td>
</tr>
<tr>
<td>yes</td>
<td>(100%)</td>
<td>(0.2%–0.4%)</td>
</tr>
</tbody>
</table>

Transformation have been performed using 50 ng of plasmids. 100% corresponds to an efficiency transformation of 2–4 × 10⁶ transformants per µg of plasmids.
Reactions were stopped by adding 3 µL of 0.25% sodium dodecyl sulfate and 5 mM EDTA, pH 8.0 and incubating at 65°C for 5 min. The newly synthesized plasmids were digested with BamHI (Life Technologies, Cergy Pontoise, France) and used to transform *E. coli* strain BMH 71-18 mutS (CLONTECH) bacteria in presence of CaCl$_2$ (5). After overnight growth in 20 mL of Terrific Broth (Difco Laboratories, Detroit, MI, USA) with 0.1 mg/L ampicillin, plasmids were purified by the JETprep Kit from 2 mL of the culture. Two micrograms of plasmids were completely digested with BamHI using conditions recommended by Life Technologies. Exonuclease III (Life Technologies) digestion was performed with 65 U of enzyme at 37°C in a solution containing 50 mM Tris-HCl, pH 8.0, 10 mM MgCl$_2$ and 100 mM NaCl in a final volume of 41 µL for 15 min before second transformation in *E. coli* strain DH5α™ (Life Technologies) or JM109 (Stratagene/Ozyme, Montigny le Bretonneux, France). The effect of exonuclease III on the yield of transformation was first tested on wild-type plasmids. We observed that linearized plasmids after BamHI digestion were 60–125 times less efficient than circular plasmids (Table 1). After treatment of linearized plasmids by exonuclease.

![Figure 1. Agarose (1% wt/vol) gel electrophoresis of circular and linear plasmids treated or not treated with exonuclease III.](image-url) Circular plasmid (lanes 1 and 2) was linearized with BamHI (lanes 3 and 4) and treated (lanes 2 and 4) or not treated (lanes 1 and 3) with exonuclease III as described in the text.
III for 15 min, the transformation efficiency went down and was roughly four times lower than that observed without exonuclease III treatment. Similar treatment of circular plasmid by exonuclease III did not affect the efficiency of transformation, since a similar number of clones was obtained with or without exonuclease III treatment (Table 1). Concurrently, the plasmid digestion by exonuclease III was monitored by electrophoresis on an agarose gel (Figure 1). Exonuclease III had no effect on circular plasmids, whereas BamHI-digested plasmids were completely degraded in 15 min. These data suggest that the exonuclease III is able to eliminate all the wild linearized plasmids after BamHI treatment, preserving the mutant circular plasmids resistant to BamHI digestion.

The effect of exonuclease III was tested in six different experiments designed to introduce mutations in spectrin peptides. Mutant plasmids were prepared as described above. After the first transformation in E. coli strain BMH 71-18 mutS, purified plasmids were treated with BamHI. While one half was directly used to transform E. coli strains such as DH5α or JM109, the other half was treated with exonuclease III before transformation. When plasmids were digested only with BamHI before the second transformation, between 1500 and 2500 clones were obtained in each experiment. When plasmids were submitted to exonuclease III after BamHI digestion, only between 25 to 800 clones were obtained. Three to eleven clones of each experiment were checked for the presence of either BamHI or KpnI site. Without exonuclease III treatment, 9 out of 43 (21%) were digested by KpnI (Life Technologies) and resistant to BamHI, whereas the other 34 clones were sensitive to BamHI and resistant to KpnI. These data indicate that 21% of the bacteria contain the mutant plasmid. After exonuclease III treatment, 35 clones out of 36 (97%) were digested by KpnI and were resistant to BamHI, demonstrating the efficiency of exonuclease III in the selection of mutant plasmids. According to the principle of the LP-USE mutagenesis method, both mutations must be associated with each other. Even if this association has already been demonstrated (4), we have verified the presence of the second mutation in 12 samples, either by sequencing or by digestion when this new mutation created or deleted a restriction site. In all tested cases, the desired mutation was present and associated with the BamHI→KpnI mutation.

So, the exonuclease III treatment introduced in the site-directed mutagenesis method called LP-USE mutagenesis is a single step requiring short time (only 15 min digestion). This step eliminates most wild-type plasmids and greatly enriches mutant plasmids to allow just one round of selection. Besides, exonuclease III treatment is not LP-USE-specific, but could be useful for standard USE as well.
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Scanning Mutagenesis to Localize DNA-Protein Interactions
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Transcription, which plays an important role in the control of gene expression during cellular differentiation, is regulated by specific interactions between DNA sequences and the proteins that bind to them. In recent years, useful techniques have been developed to characterize the interactions of transcription factors with their cognate DNA binding sites. We developed a novel technique, which we call scanning mutagenesis in the electrophoretic mobility shift assay (SMEMSA), that combines scanning mutagenesis with the electrophoretic mobility shift assay (EMSA). This approach can be used to localize sites of protein-DNA interactions and to correlate protein binding with functional assays of DNA elements.

CD18 (β2 leukocyte integrin) is transcriptionally regulated during myeloid differentiation (4). The 79 nucleotide (nt) CD18 minimal promoter, which regulates its leukocyte-specific and myeloid-inducible activity (3), contains three binding sites for ets-related transcription factors. Two ets factors, PU.1 and GABPα, bind to this region of the promoter and cooperate to increase CD18 transcription. Together, these ets factors are sufficient to activate CD18 in otherwise non-permissive, non-hematopoietic cells. Although PU.1 and GABPα functionally cooperate, conventional EMSA with

Figure 1. (A) Scanning mutants of the CD18 (-79) promoter. The sequence of the entire wild-type CD18 (-79) promoter and the individual mutagenized regions are indicated. Horizontal bars labeled A, B and C indicate the location of the three ets sites in the CD18 minimal promoter. (B) The strategy for PCR-based scanning mutagenesis of the CD18 minimal promoter is schematized. The orientation and length of the arrows represent the oligonucleotides that were used in the PCRs. The -50/-41, -40/-31 and -30/-21 constructs required two separate PCRs whose products were ligated by means of the common mutagenizing restriction site.