

# High efficiency of site-directed mutagenesis mediated by a single PCR product

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## ABSTRACT

**We describe a highly efficient procedure for site-specific mutagenesis of double-stranded plasmids. The method relies on a single PCR primer which incorporates both the mutations at the selection site and the desired single base substitutions at the mutant site. This primer is annealed to the denatured plasmid and directs the synthesis of the mutant strand. After digestion with selection enzyme, the plasmid DNA is amplified into *Escherichia coli* strain BMH71-18 and subjected to a second digestion and amplification into the bacterial strain DH5 $\alpha$ . A mutagenesis efficiency >80% was consistently achieved in the case of two unrelated plasmids.**

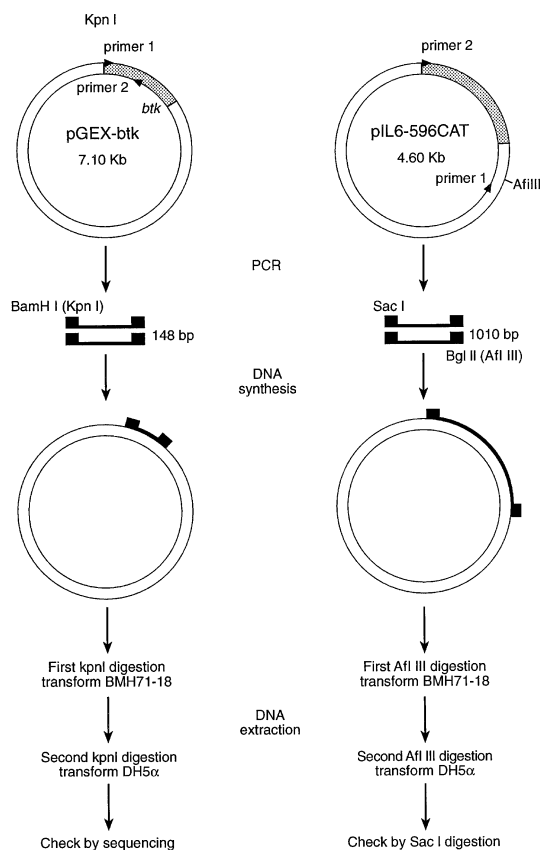
Site-directed mutagenesis by unique restriction site elimination introduced by Deng and Nickoloff allows a site-specific mutagenesis of a plasmid DNA without any subcloning step (1). This procedure uses two mutagenic primers: one carries the desired mutation, the second, acting as a selection primer, carries a mutation in a unique, non-essential restriction site in the target plasmid. The mutagenesis method relies on the simultaneous annealing of two primers (mutagenic and selection primers) to one strand of the denatured double-stranded plasmid. After DNA elongation and ligation, the selection for plasmids lacking the selection restriction site will also encode the desired second site mutation. However we have found that in the case of some plasmids, such as pGEX-BTK, the recovery of the desired mutants is much lower than that of the selection restriction site selection mutants, yielding the desired mutant products at a frequency of  $\leq 10\%$ . The reason for this low efficiency may be due to the sequence of the target DNA, which may acquire stable secondary structures, such as stem-loops. These structures may interfere with the annealing of the mutagenic primers and could result in low recovery of mutant plasmids. In order to circumvent this limitation, we developed a method of PCR-fragment directed DNA synthesis. This protocol relies on the use of selection and mutagenic primers to amplify a region of DNA lying between the annealed selection and mutagenic primers, generating the desired mutant DNA fragment. This mutant DNA fragment is then used as a primer for the subsequent production of heteroduplex DNA. Since the amplified DNA fragment contains both the desired and the selection mutations, this method results in a high efficiency of site-directed mutagenesis.

This methodology was utilized to introduce single base substitutions into the pGEX-BTK and pIL6-596 plasmids. pGEX-BTK

contains a stop codon (TAA) corresponding to codon amino acid (aa) 17 of the human BTK cDNA. Reversion of this codon to CAA enables the production of a GST-BTK fusion protein. pIL6-596 plasmid carries the region of -596/+15 of the IL-6 gene, which contains a binding motif for HIV-1 Tat (2). The introduction of point mutations into this motif allows the identification of single nucleotides essential for the binding of HIV-1 Tat.

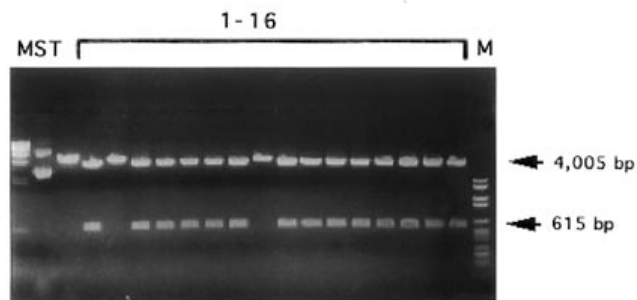
The outline of the procedure is shown in Figure 1. In the case of pGEX-BTK, the selection primer 1 was 5'-CATCTGGGAAGG-ATCCTGCATCAA-3' (the original *Kpn*I site is changed into a *Bam*HI site; the mutant bases are underlined), while the mutagenic primer 2 was 5'-GTTTTCTTTTTCTGTTGGGATCGC-3' (mutant base is underlined), with an expected PCR product of 148 bp. In the case of pIL6-596, the selection primer 1 was 5'-CTTTTGCTCA-GATCTTCTTTCCTG-3' (the original *Afl*III site is changed into a new *Bgl*III site), while the mutagenic primer 2 was 5'-CTGCGGTC-GAGCTCAGAATGAGCC-3' (a new *Sac*I site is created), with an expected PCR product of 1010 bp. Amplification of the fragments between primer 1 and 2 for each plasmid was performed in a 100  $\mu$ l reaction containing 3 fmol template DNA, 30 pM each primer, 200 mM each deoxynucleotide triphosphate, 5 U *Pfu* DNA polymerase (Stratagene, Heidelberg), 20 mM Tris-HCl pH 8, 10 mM KCl, 6 mM ammonium sulfate, 2 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 10  $\mu$ g/ml BSA. In the case of pGEX-BTK, PCR conditions were as follows: 25 cycles of 1.5 min at 94°C, 2 min at 50°C and 2 min at 72°C. To amplify pIL6-596, the elongation time was 4 min at 72°C. At the end of 25 cycles, the elongation time was extended to 10 min. *Pfu* DNA polymerase (Stratagene) was used to decrease potential second-site mutations during the PCR amplification. After PCR amplification, the fragments were purified by using PCR Prep DNA Purification Kit (Qiagen, Hilden, Germany), as specified by the manufacturer. Both PCR fragments were phosphorylated by T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis) in a buffer containing 3–10 pmol primers, 50 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 5 mM DTT, 1 mM ATP and 5 U T4 polynucleotide kinase in a volume of 10  $\mu$ l for 1 h at 37°C. The subsequent DNA elongation, ligation and selection were carried out as previously reported (1), with some modifications. Briefly, a 10  $\mu$ l solution containing each phosphorylated PCR primer (as detailed in Table 1), 50 ng double stranded pGEX-BTK or pIL6-596 plasmid, 20 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 1.5 U T4 DNA polymerase (New England Biolabs, Hitchin, UK) and 2.5 U T4 DNA ligase (Boehringer Mannheim) was heated to 100°C for 3 min and immediately placed on ice for 10 min. Primer-directed DNA

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**Figure 1.** Schematic representation of the methodology for site-directed mutagenesis. The primers are indicated by arrows. In the case of pGEX-BTK, primer 1 (selection primer) changes the *Kpn*I site into a new *Bam*HI site, while primer 2 (mutagenic primer) changes the TAA stop codon into CAA. The amplified PCR fragment is 148 bp (bold line). In the case of pIL6-596, primer 1 (selection primer) changes the *Afl*III site into a new *Bgl*III site, while primer 2 (mutagenic primer) creates a new *Sac*I site. The expected fragment is 1010 bp. Because an original *Sac*I existed in the template plasmid, after mutagenesis and digestion with *Sac*I, two bands were anticipated to migrate at 615 and 4005 bp, respectively. The PCR-directed DNA synthesis generates the second mutant strand. The duplex DNA proceeds with two cycles of digestion and transformation into the indicated bacterial strains. Transformants were checked by sequencing and by selected restriction enzymes.

synthesis was performed by incubating the reaction for 5 min on ice, followed by a 5 min incubation at room temperature and a 120 min incubation at 37°C. The reaction was stopped by heating at 68°C for 10 min. During the PCR product-directed DNA synthesis, we examined the mutagenesis efficiency versus the rate of PCR product to DNA template. We found that the optimal rate was dependent upon the size of the amplified fragment. In the case of the 146 bp fragment, a 50-fold molar excess over the wild-type template concentration was found to be optimal, while in the case of the 1010 bp fragment a 10-fold molar excess worked better (Table 1). In both plasmids we obtained a mutagenesis efficiency of >80% (Table 1; Fig. 2). To compare the mutagenesis efficiencies of the conventional (1) and the present procedure, we introduced single base substitutions in pGEX-BTK and in pIL6-596 by utilising the two methods in parallel experiments. As reported in Table 2, we consistently obtained a 70–80% mutagenesis efficiency, with an 8–12-fold increase over the standard methodology (1). Next, six randomly



**Figure 2.** Analysis of pIL6-596 transformants by *Sac*I digestion. pIL6-596 transformants were digested with *Sac*I and analyzed on agarose gel. Lane (M), DNA size markers; lane (S), undigested plasmid; lane (T), template plasmid digested with *Sac*I; lane (1–16), pIL6-596 transformants digested with *Sac*I. Mutant pIL6-596 carries two *Sac*I sites, and yields 615 and 4005 bp fragments.

selected non-mutant clones of pIL6-596 derived from the two procedures were analysed by *Sac*I digestion (not shown) and by sequencing the regions corresponding to the selection and to the mutagenic sites. In the case of the standard procedure, one clone showed a wild-type sequence in both the selection and in the mutagenic site, likely arising from a lack of cleavage of the selection restriction site. The remaining five clones all showed a wild-type configuration of the mutant site, while the selection site showed the desired mutations (Table 3). This suggests a lack of concomitant annealing of the mutagenic primer and the selection primer. The analysis of six non-mutants generated by our procedure revealed that one transformant had a wild-type sequence in both the selection and mutant site, again suggesting an incomplete cleavage of the wild-type plasmid. In five clones we observed the sequence 5'-AGATGT-3', corresponding to truncated selection primers, and resulting in the lack of a *Bgl*III restriction site (5'-AGATCT-3'). In these cases, contaminant primers with a truncated sequence could have generated a PCR fragment lacking a functional *Bgl*III site. As expected, the region corresponding to the mutant site showed a wild-type configuration (Table 3). Due to the low percentage of non-mutant clones obtained with our procedure, this finding does not appear to significantly decrease the efficiency of the procedure.

**Table 1.** Effect of different concentrations of PCR primers on mutagenic efficiency

PCR fragment (bp) <sup>a</sup>	Fold molar excess	Transformants analyzed	Mutagenesis efficiency (%)
pGEX-BTK (148)	10	6	50
	20	6	66
	50	6	82
pIL6-596 (1010)	10	20	85
	20	20	75
	50	20	75

<sup>a</sup>PCR fragments were mixed in a 10–50-fold molar excess with 50 ng template DNA in a 15 µl volume. After digestion and final transformation, the randomly chosen transformants were examined either by sequencing (pGEX-BTK) or by digesting with *Sac*I (pIL6-596). The mutagenesis efficiency was calculated as the number of mutants/number of analyzed transformants.

**Table 2.** Mutagenic efficiencies of the standard and the present procedures

Plasmid <sup>a</sup>		Standard procedure <sup>b</sup>	Present procedure <sup>b</sup>
pGEX-BTK	Exp. 1	6.8% (2/29)	83.3% (5/6)
	Exp. 2	4.1% (1/24)	75.0% (9/12)
	Exp. 3	8.3% (2/24)	83.3% (10/12)
pIL6-596	Exp. 1	6.6% (2/30)	85.0% (17/20)
	Exp. 2	8.3% (2/24)	75.0% (18/24)
	Exp. 3	4.1% (1/24)	79.1% (19/24)

<sup>a</sup>Randomly selected transformants from three independent experiments of mutagenesis were analysed by sequencing, in the case of pGEX-BTK, or by *SacI* digestions in the case of pIL6-596.

<sup>b</sup>The indicated plasmids were subjected to mutagenesis as reported by Deng and Nickoloff (1; standard procedure), or as described under Results and Discussion section (present procedure). Mutagenesis efficiency was evaluated as the percentage of clones carrying the desired mutations/analysed clones.

The results shown in Tables 1 and 2 indicate that site-directed mutagenesis mediated by a single PCR fragment is a valuable method. Since this method combines the selection and desired mutations into a single primer, it has great advantage when attempting to isolate 'difficult' mutants that are generated with low efficiency in the *in vitro* primer-extension reaction. In addition, a long PCR primer may overcome the inability of short primers to anneal to refractory templates resulting from secondary structures. This could contribute to the higher mutagenesis efficiency achieved by our procedure.

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**Table 3.** Analysis of non-mutant clones derived from pIL6-596 plasmid subjected to the standard or to the present mutagenesis procedures

Clones <sup>a</sup>	Standard procedure <sup>b</sup>		Present procedure <sup>b</sup>	
	Selection site	Mutant site	Selection site	Mutant site
1	5'-ACATGT-3' <sup>c</sup>	WT	5'-ACATGT-3' <sup>c</sup>	WT
2	5'-AGATCT-3' <sup>d</sup>	WT	5'-AGATGT-3' <sup>e</sup>	WT
3	5'-AGATCT-3' <sup>d</sup>	WT	5'-AGATGT-3' <sup>e</sup>	WT
4	5'-AGATCT-3' <sup>d</sup>	WT	5'-AGATGT-3' <sup>e</sup>	WT
5	5'-AGATCT-3' <sup>d</sup>	WT	5'-AGATGT-3' <sup>e</sup>	WT
6	5'-AGATCT-3' <sup>d</sup>	WT	5'-AGATGT-3' <sup>e</sup>	WT

<sup>a</sup>Six non-mutant clones were randomly selected from three independent experiments and analysed by sequencing the region corresponding to the selection restriction site, or to the desired mutations.

<sup>b</sup>pIL6-596 plasmid was subjected to mutagenesis as reported by Deng and Nickoloff (1, standard procedure), or as described under Results and Discussion section (present procedure).

<sup>c</sup>The sequence 5'-ACATGT-3' corresponds to the wild-type *AflIII* site in pIL6-596.

<sup>d</sup>The sequence 5'-AGATCT-3' corresponds to the mutated selection site where the *AflIII* site was changed to a *BglII* site (single base substitutions are underlined).

<sup>e</sup>The sequence 5'-AGATGT-3' corresponds to an incomplete *BglII* site (the single base substitution is underlined).

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