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Oligonucleotide-directed mutagenesis using plasmid DNA: a screening step to confirm the first successful step to positive mutagenesis

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The technique of site-directed mutagenesis (SDM) is widely used in molecular biology to introduce mutations into DNA (Ref. [1]). This article discusses a quick screening step that can be incorporated into mutagenesis systems such as the GeneEditor *in vitro* site-directed mutagenesis system (Promega, Madison, WI, USA), which manipulates target DNA cloned into an *Escherichia coli* plasmid and uses antibiotic selection to select for mutants.

1. GeneEditor

▼Selection oligonucleotides provided with the GeneEditor system encode mutations that alter the ampicillin resistance gene in the pGEM plasmid, creating a new, additional resistance to the GeneEditor antibiotic selection mix. In the GeneEditor protocol, the selection oligonucleotide is annealed to the denatured double-stranded DNA template at the same time as the mutagenic oligonucleotide. Subsequent synthesis and ligation of the mutant strand links the two oligonucleotides. The resistance to the GeneEditor antibiotic selection mix encoded by this mutant DNA strand facilitates selection of the desired mutation.

After mutant-strand synthesis, the reaction mix is transformed into *E. coli* BMH 71-18 (a mismatch repair deficient strain) and cultured overnight in GeneEditor antibiotic selection mix, which selects bacteria containing plasmid derived from the mutant strand. Plasmid DNA from this overnight culture is isolated and then used to transform *E. coli* JM109, followed by plating on ampicillin agar containing the GeneEditor antibiotic selection mix, which ensures proper segregation of mutant and wild-type plasmids. Bacterial colonies are then picked and screened for positive mutagenesis.

The weakest links in this mutagenesis system are annealing the mutagenic oligonucleotide to the template DNA and synthesis of the mutant strand. If all the correct oligonucleotide primer design techniques are followed then the annealing and synthesis reactions usually proceed without problems. Annealing problems can, however, occur for a number of reasons. If it is suspected that a mutagenic oligonucleotide could have annealing problems, it would be useful to have a screening step after the annealing and synthesis reactions that would screen for success or failure.

2. Novel screening step

We have used such a screening step on the plasmid DNA isolated from the overnight culture of *E. coli* BMH 71-18 that is transformed with the synthesis reaction mix. The screening step saves valuable time when a negative result is obtained because it eliminates the second round of transformation, overnight culture of bacteria on selective agar and screening of potential mutant colonies. A requirement of this screening step is that the mutagenic oligonucleotide introduces a restriction enzyme site.

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Our rapid screening step is as follows. The template for our SDM was a β-lactam-antibiotic resistance gene of *Streptococcus pneumoniae*, *pbp1A*, cloned into plasmid pGEM3zf(+). The mutagenic oligonucleotide included a GGTACC sequence, which created a *Kpn*I restriction enzyme site in the mutated DNA. SDM was performed using the methods described by Promega. After mutant-strand synthesis, *E. coli* BMH 71-18 was transformed with the reaction mix and cultured overnight in GeneEditor antibiotic selection mix. Plasmid DNA was isolated from the bacteria and 1 μg was digested with *Kpn*I (Promega), and the digested DNA was analysed by agarose gel electrophoresis (Fig. 1) together with a control reaction without the mutagenic oligonucleotide.

The control reaction reveals a single band of linear DNA (Fig. 1b), indicating a single cut on the plasmid DNA. In the experimental lane, however, successful annealing and synthesis have created an extra restriction enzyme site, which is shown by the appearance of two additional bands (Fig. 1a). Other information, such as the mutagenesis efficiency, can also be deduced from the DNA bands by comparing the intensity (concentration) of band 1 with the collective intensities of bands 2 and 3. By this method, the mutagenesis efficiency was found to be ~40%. Should this visual analysis indicate a very low level of mutagenesis efficiency then the proportion of mutant colonies finally isolated on selective agar will also be low.

Thus, this screening step can be used after the annealing and synthesis reactions of SDM in order to indicate the failure or the level of success of a mutagenesis reaction.

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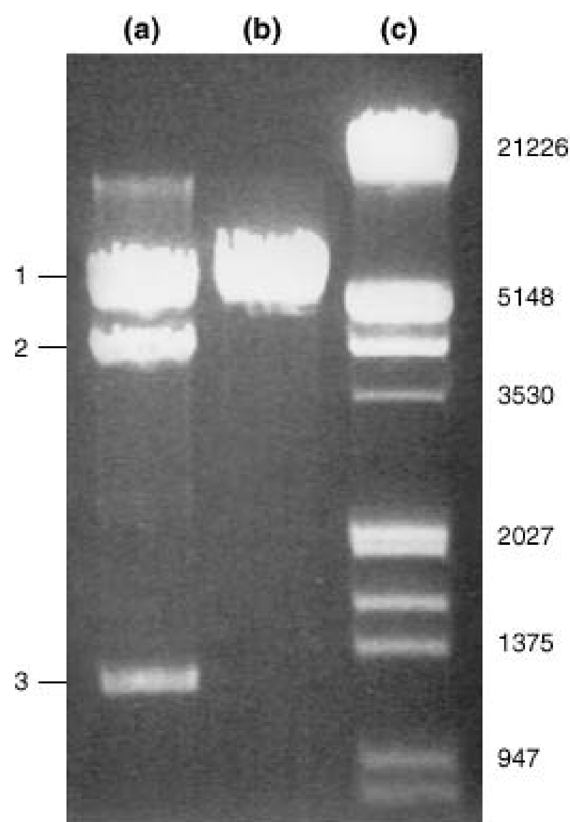

Fig. 1. *Kpn*I digestion patterns of plasmid pGEM3zf(+) with cloned *pbp1A* genes. Plasmid DNA was digested and electrophoresed on an ethidium-bromide-stained 1% agarose gel. (a) Digested plasmid DNA resulting from mutant strand synthesis incorporating the mutagenic oligonucleotide. (b) Digested plasmid DNA from a control reaction without the mutagenic oligonucleotide. (c) Molecular size markers (size in base pairs is indicated; Roche Molecular Biochemicals marker III). The numbered bands show the whole linear DNA (band 1) and the two fragments resulting from *Kpn*I digestion (bands 2 and 3).

Reference

1 Smith, M. (1985) *Annnu. Rev. Genet.* 19, 423–462.