Rapid site-directed mutagenesis by a method that selects for full length mutated DNA

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Received January 3, 1996; Revised and Accepted February 14, 1996

Site-directed mutagenesis is a powerful tool for exploring structure–function relationships of proteins and nucleic acids. Traditional methods of site-specific mutagenesis involve the hybridization of an oligonucleotide containing mismatched nucleotides to a region of single-stranded target DNA (1,2). DNA polymerization and ligation then complete the synthesis of the mutated strand. Following replication and selection of the double-stranded mutant DNA, mutagenized vectors can then be propagated in vivo.

Although classical methods for site-directed mutagenesis allow reliable production of mutated DNA, the necessary steps in the protocols are very laborious (1–3), since selection and isolation of single-stranded parent and mutated DNA constitute major rate-limiting steps. Here we report a novel method of site-directed mutagenesis that employs both T7 DNA polymerase for reliable polymerization of full length template DNA (4) and a biotinylated primer for high affinity selection of full length double-stranded mutated DNA (5). When employed in the scheme described below, this method eliminates time consuming single strand selection procedures.

Briefly, 10 pmol of plasmid DNA to be mutated is denatured by exposure to alkaline pH as described elsewhere (step 1) (6). An initial primer X (90 pmol) (in our case; 5′-biotin-TCTCAGCTGCAGGAGCTGGTGTAGGACGAA; PvuII site underlined; * site of mutation) is mixed with denatured DNA in 10 µl of 1× annealing buffer (40 mM Tris–HCl, pH 7.5, 20 mM MgCl2, 50 mM NaCl). DNA primer annealing (Fig. 1; step 2) is then accomplished by heating the mixture to 70°C for 5 min and cooling to 37°C at room temperature over a 45 min period. Primer extension for the synthesis of mutant strand (step 3) is achieved by adding 20 µl 2.5 mM dNTPs mixture, 9 µl 5× annealing buffer, 49 µl dH2O, 10 µl 1× synthesis buffer and 2 µl T7 DNA polymerase (10 U/µl, USB, Cleveland, OH). The total volume of primer extension solution is 100 µl, and the enzyme is added just before initiation of the reaction. The mutated DNA strand is synthesized by incubation at 25°C for 5 min followed by 37°C for ≥ 1 h before terminating the reaction by heating to 70°C for 10 min. In step 4, avidin coated beads (Pierce Biotech, Rockford, IL) are used to purify the newly-synthesized biotin-linked DNA strands by incubating the reaction mixture at 37°C for 30 min–1 h with 100 µl of the beads previously washed twice with 250 µl washing buffer (20 mM phosphate buffer, pH 7.5, 1.0 M NaCl). After incubation, the beads are pelleted and washed three times with 1.0 ml washing buffer. In order to isolate the mutated single strand for complementary DNA synthesis, the immobilized DNA is again denatured in alkaline solution (step 5) by suspending the coupled beads in 50 µl of 0.2 M NaOH plus 0.2 mM EDTA and incubating at room temperature for 15 min. The mixture is next neutralized by adding 10 µl 1 M Tris–HCl, pH 6.8 plus 5 µl 1 M HCl. The beads are pelleted and the supernatant is discarded. The beads are then washed three times with 500 µl PBS (10 mM phosphate buffer, pH 7.5, 150 mM NaCl) followed by three washes in 1× annealing buffer. Annealing of primer Y in step 6 (5′-CTCCACATAGACCTTG) and second strand synthesis (step 7) is accomplished as in steps 2 and 3. In order to isolate only the full length mutated DNA, the beads are pelleted and washed twice with 300 µl 1× restriction buffer and then suspended in 100 µl 1× restriction buffer containing 1 U PvuII (45 U/µl, New England Biolabs, Beverly, MA). The released full length strands are ethanol-precipitated and ligated and transformed into bacteria. The bacteria are expanded, and the plasmids

Figure 1. Reaction scheme for direct double-stranded DNA mutagenesis: A, avidin coated beads; B, biotin; X, initial biotinylated primer for mutant strand synthesis; Y, phosphorylated second strand synthesis primer. (Primers were purchased from Integrated DNA Technologies, Inc., USA.) For details see text.

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are purified and subjected to double-stranded DNA sequencing, as described in the USB sequenase 2.0 kit (USB, Cleveland, OH).

Using this procedure, we have rapidly mutated a single base pair in the KCDB3/T7-7 plasmid (7) from a C to a G residue (Fig. 2). In a separate experiment, we have mutated cysteine 201 of human band 3 protein to an alanine residue by changing the TG\textsuperscript{T} codon into a GCT codon in plasmid cdb3/T7-7 (8) (data submitted but not shown). We observed an overall efficiency of 40–45% in these experiments. Although the protocol can be followed as described with reproducible results, one step (design of primer X) may require careful attention. Starting from its 5' terminus, primer X is composed of a biotin cap for immobilization, a nucleotide spacer for access and flexibility, a unique restriction enzyme site for release from the avidin beads of only full length duplex strands of mutated DNA, and the complementary primer sequence containing the mutated bases. If the restriction site is designed for a blunt end endonuclease, the site of cleavage should allow for ligation to the opposite end of the strand without introduction of extra bases or deletion of bases found in the original plasmid. The same principle obviously applies to jagged end restriction enzymes following removal of the overhanging bases with a single strand exonuclease. With the number of restriction enzymes now available and the ability to screen a plasmid sequence rapidly for restriction sites, an appropriate restriction endonuclease should be readily identifiable that can cleave only at the site designed into primer X.

Our method is applicable to any DNA template including single-stranded, linear double-stranded and circularized double-stranded DNA, although mutation of nicked double-stranded plasmids may prove to be more efficient (9). While the process can be conducted with a Taq polymerase using PCR, the unreliability of most thermally stable polymerases renders this option less desirable (10). We have employed T7 DNA polymerase since this enzyme replicates with high fidelity and can incorporate thousands of nucleotide bases before dissociating from the template strand (11). Using T7 DNA polymerase, our mutagenesis method is both reliable and efficient, requiring usually only 1 day to generate a mutated plasmid.

ACKNOWLEDGEMENT

Supported in part by NIH grant GM24417.

REFERENCES