Use of modified T7 DNA polymerase (sequenase version 2.0™) for oligonucleotide site-directed mutagenesis

Ashok R.Venkitaraman

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK
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Efficient oligonucleotide site-directed mutagenesis is critically dependent on successful primer extension from the mutagenic oligonucleotide. Modified T7 DNA polymerase (Sequenase) may be particularly suitable for this application because of its high processivity and lack of 3'-5' exonuclease activity. I describe here a procedure for oligonucleotide site-directed mutagenesis using this enzyme. This procedure was used to make a 90 bp insertion into a gene cloned into M13mp18 with an oligonucleotide 120 bp long, for which a conventional procedure using the Klenow enzyme (1) had been unsuccessful.

The method of Kunkel (2) was used to impose a strong selection for the mutant (-) DNA strand so that different polymerases could be directly compared. Single stranded (+) template DNA was prepared in the CJ236 (dut- ung-) strain of *E. coli*; the final double stranded product of the mutagenesis reaction was transfected into the TG1 strain (ung+). Mutagenesis reactions were performed in parallel by standard procedures using Klenow enzyme (1) and T4 DNA polymerase (2), or the Sequenase procedure described below. The first two reactions yielded very few (<10) phage plaques, suggesting that primer extension had been inefficient. None of the plaques contained the desired mutation. In contrast more than 500 phage plaques were obtained in the Sequenase procedure, of which 1 in 16 were hybridization positive (9/148). Two of these plaques were selected, and both were confirmed to have the desired mutation by DNA sequencing.

Annealing of the mutagenic primer to the template was performed under the conditions described by Kunkel (2) while primer extension and ligation were under the manufacturer's recommended conditions for Sequenase Version 2.0™. Briefly, 4 µl of kinased oligonucleotide (5 pmol/µl), 1 µl template (1 µg/µl), 0.6 µl 20x SSC and 5 µl water were mixed, heated to 80° C for 1 minute and slowly cooled to room temperature over 30 minutes. Two µl 10 x reaction buffer (1x=40 mM Tris, pH 7.4, 20 mM MgCl₂, 32 mM NaCl, 5 mM dithiothreitol), 2 µl 5mM dNTPs, 1 µl 5 mM rATP, 1 µl water, 2 µl T4 DNA ligase (400000 U/ml, New England BioLabs Inc) and 2 µl Sequenase Version 2.0™ (diluted 1:8 in manufacturer's dilution buffer, US Biochemical) were added in order, and primer extension/ligation performed for 20 minutes at 37° C. The reaction was stopped by adding 15 µl 0.1 M EDTA, and the DNA concentration adjusted to approximately 5 ng/µl by adding 165 µl of TE buffer. One, 10 and 100 ng of DNA were transfected into *E. coli* cells made competent by the Hanahan method (3).

The procedure described here is rapid, simple and of general applicability. It may be particularly useful for complex mutageneses where conventional procedures fail. I have used it for insertional mutagenesis, but see no objection to its use in deletional or point mutational procedures; with or without additional selection for the mutant (-) DNA strand.

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References

1. Carter P, H Bedouelle, MM Waye, G Winter. 1985. In : Oligonucleotide Site-directed Mutagenesis in M13. Anglian Biotechnology Limited, Colchester, Essex, UK. pp.18-20.
2. Kunkel TA. 1987. In : Current Protocols in Molecular Biology 1987-88. F Ausbel, R Brent, RE Kingston et al. [Eds]. John Wiley and Sons, New York. pp. 8.1.1-8.1.6.
3. Hanahan, D. 1983. *J. Mol. Biol.* 166:557-580.