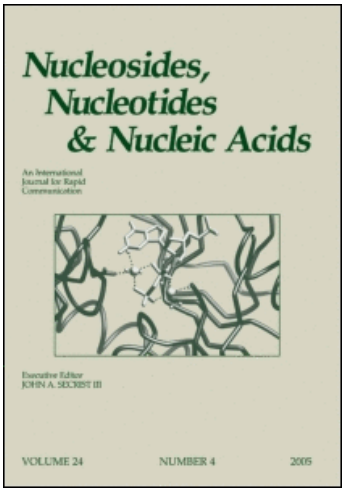

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Phosphorothioate-Based Site-Directed Mutagenesis: Extending the Basic Methodology

Jon R. Sayers[a]; Walter Schmidt[a]; Fritz Eckstein[a]
[a] Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, Göttingen, FRG

To cite this Article Sayers, Jon R. , Schmidt, Walter and Eckstein, Fritz(1988) 'Phosphorothioate-Based Site-Directed Mutagenesis: Extending the Basic Methodology', Nucleosides, Nucleotides and Nucleic Acids, 7: 5, 625 — 628
To link to this Article: DOI: 10.1080/07328318808056298
URL: http://dx.doi.org/10.1080/07328318808056298

NUCLEOSIDES & NUCLEOTIDES, 7(5&6), 625-628 (1988)

PHOSPHOROTHIOATE-BASED SITE-DIRECTED MUTAGENESIS: EXTENDING THE BASIC METHODOLOGY

Jon R. Sayers, Walter Schmidt and Fritz Eckstein*.
Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, Hermann-Rein-Strasse 3, D-3400 Göttingen, FRG

ABSTRACT: The versatility of the phosphorothioate-based site-directed mutagenesis method has been extended by employing 5′-3′ exonucleases in the gapping reaction. Mutational frequencies of 70-95% were routinely achieved.

INTRODUCTION

Site-directed mutagenesis has emerged as a powerful tool in molecular biology, not only for the elucidation of enzyme structure-function relationships, but also in the manipulation of clones for easier sub-cloning. The basic phosphorothioate-based site-directed mutagenesis method involves annealing an oligonucleotide primer to the gene fragment of interest, cloned as the (+)strand of a single-stranded phage derivative. The oligonucleotide carries the desired mismatch. The template-annealed DNA is then subjected to a polymerisation reaction in which deoxycytidine triphosphate is replaced by deoxycytidine 5′-O-(1-thiotriphosphate), dCTPαS, its phosphorothioate analogue. The polymerisation is performed in the presence of T4 DNA ligase and thus yields double-stranded closed-circular DNA (RF IV DNA) containing phosphorothioate groups in the (-)strand only[1-3].

A few restriction enzymes are incapable of completely hydrolysing DNA containing a phosphorothioate linkage at the site of cleavage[4]. Thus, the wild type (+)strand may be nicked by a restriction enzyme e.g. Nci I, and then degraded by exonuclease III,

a 3′-5′ exonuclease. The procedure generates a gapped DNA species containing the mutated sequence in single-stranded form. Repolymerisation of this gapped DNA gives the mutant sequence as a fully complementary double-stranded species. Transfection of this homoduplex RF IV DNA gives mutational efficiencies of between 45 and 89% [2,3].

The procedure is limited by two factors. Firstly, only a few restriction enzymes generate nicked DNA efficiently from phosphorothioate-containing DNA. Nci I, Hind II, Pvu I and Pst I have been used in the procedure. The second factor limiting the general applicability of the method is that the enzyme used to gap the nicked DNA, exonuclease III, does so only in the 3′-5′ direction and requires a nick close to the 3′ side of the mismatch on the (+)strand. This exonuclease is capable of hydrolysing long tracts of DNA but commercial samples are often contaminated with substances which destroy the (-)strand. Exonuclease III is also quite sensitive to NaCl concentration, removing approx. 100 nucleotides per minute under optimal conditions[5]. In order to extend the usefulness of the method we decided to investigate the use of 5′-3′ exonucleases instead of exonuclease III to perform the gapping reaction. We have also extended the number of restriction enzymes suitable for use in the procedure, thus enzymes which normally linearise RF IV phosphorothioate-containing DNA may be induced to nick the non-substituted (+)strand by performing the reaction in the presence of ethidium bromide.

RESULTS AND DISCUSSION

All experimental details are as described previously[5,6]. Two enzymes, λ exonuclease and T7 exonuclease, proved to be suited to the task of gapping nicked DNA. T7 exonuclease proved to be particularly useful as it rapidly hydrolyses almost all of the (+)strand and appears to function efficiently in the buffers used in the nicking step. The restriction enzymes Ava I and Ban II nick RF IV DNA prepared with dCTPαS and were used to introduce nicks 400-600 nucleotides upstream from the site of the mismatch. The (+)strand of M13 RF II DNA prepared with oligonucleotide primers introducing single or double base mismatches was gapped by T7

exonuclease (approx. 5 units per μg DNA) in less than 30 min. Transfection of a repolymerised sample in SMH50 cells afforded mutational efficiencies in the range 70-92%. Similar mutational efficiencies were obtained using λ exonuclease in the gapping step.

We also wanted to test the suitability of the method for the creation of deletion and insertion mutations. Mismatch oligonucleotides designed to insert or delete 16 bases were employed in the mutagenesis method using the 5′-3′ exonucleases in the gapping reaction. Repolymerisation and transfection gave mutational efficiencies in the range 72-96%[5].

We wanted to be able to employ a wider range of restriction enzymes in the nicking reaction in order to extend the versatility of the method even further. Frequently used enzymes such as BamHI, Bgl I, Eco RI, Hind III, Hpa II, Pvu II and Sac I are all capable of linearising phosphorothioate-containing DNA under normal reaction conditions. However, by performing the nicking reaction in the presence of ethidium bromide all of the enzymes we tested (11 in total) could be induced to convert RF IV phosphorothioate-containing DNA to the nicked (RF II) form with a minimum of linearisation. The concentration of ethidium bromide required to prevent linearisation varied for each enzyme but was in the range of 5 μg-80 μg per ml. We then employed such nicking reactions in our mutagenesis protocols. The mutational efficiencies obtained were as follows: Bgl I, 84%; Hind III, 89%; Hpa II, 64%; Pvu II 79%[6].

High efficiency site-directed mutagenesis procedures allow direct sequencing of a few putative mutant clones, obviating the need for time consuming hybridisation assays and the screening of large numbers of plaques associated with standard methods. The procedures developed here do not require special cell lines and any single-stranded vector may be used as a cloning vehicle.

REFERENCES

1) Sayers, J. R. and Eckstein, F. In "Genetic Engineering: Principles and Methods" Vol 10. Setlow, J. K. and Holleander, A (eds). Plenum Press, New York and London, in press.
2) Taylor, J. W., Ott, J. and Eckstein, F. (1985) Nucleic Acids Res. **13**, 8765-8785.
3) Nakamaye, K. L, and Eckstein, F. (1986) Nucleic Acids Res. **14**, 9679-9698.

4) Taylor, J. W., Schmidt, W., Cosstick, R., Okruszek, A. and Eckstein, F. (1985) Nucleic Acids Res. **13**, 8749-8764.
5) Sayers, J. R., Schmidt, W. and Eckstein, F. (1988) Nucleic Acids Res. **16**, 791-802.
6) Sayers, J. R., Wendler, A., Schmidt, W. and Eckstein, F. (1988) Nucleic Acids Res. **16**, 803-814.