

# Site-Directed Mutagenesis

By Neal Cosby and Scott Lesley  
Promega Corporation

*Site-directed mutagenesis: "Any of various techniques by which defined mutations can be made in vitro in a cloned DNA (1)."*

*Site-directed mutagenesis has become one of the most commonly used methods in molecular biology. Here we trace its origins, provide information about resources that are available to facilitate oligonucleotide design and provide a brief analysis of some of the commonly used mutagenesis approaches.*

The multitude of investigators who want to perform site-directed mutagenesis are fueled by a need to manipulate genes and evaluate the effect of the proposed mutation. This need encompasses manipulation of structural and regulatory elements as well as the gene products themselves. Advances in recombinant DNA technology have greatly improved the ability of researchers to manipulate DNA, thereby allowing the manipulation of regulatory elements and gene products. The advances in recombinant DNA technology, and site-directed mutagenesis in particular, have resulted in corresponding advances in protein structure/function studies.

## Background

Numerous methods have been developed or exploited to mutate DNA. Initially all approaches focused on the generation of random mutations in chromosomal DNA such as those induced by X-rays (2) and chemicals (3). While these methods of random mutagenesis provided a valuable tool for classical genetic studies, they were limited by their inability to target the mutation to a specific gene or genetic element. Techniques for randomly mutagenizing a genome required screening or selection from massive numbers of mutants to obtain the desired mutation (4,5). The ability to manipulate DNA *in vitro*, through the use of plasmid vectors, became a driving force for newer technologies, which allowed precise changes in discrete, manageable segments of the genome with relatively little effort.

## Advent of recombinant DNA technology

Recombinant DNA technology removes the limitations imposed by biological systems and allows a variety of *in vitro* techniques to be used to create these alterations and, in many cases, alterations beyond those that could be made by a biological system. Strictly speaking, the common procedure of subcloning could be considered a mutagenesis technique. We will limit our discussion to those techniques designed to alter DNA for the sake of studying the effect of that alteration on a regulatory element or gene product.

Site-directed mutagenesis methods first benefited from recombinant DNA technology in the 1970s when isolated genes were exposed to conditions such as nucleotide analog incorporation or chemical agents to localize their mutagenic effects. During this time, the use of plasmid vectors for DNA replication greatly enhanced the study of mutations. Mutagenesis targeted to a defined region of DNA includes many techniques, some more popular than others.

*In vitro* approaches to site-directed mutagenesis can be grouped generally into three categories (5): i) methods that restructure fragments of DNA, such as cassette mutagenesis (6); ii) localized random mutagenesis; and iii) oligonucleotide-directed mutagenesis. Of these methods, oligonucleotide-directed mutagenesis is by far the most commonly used method.

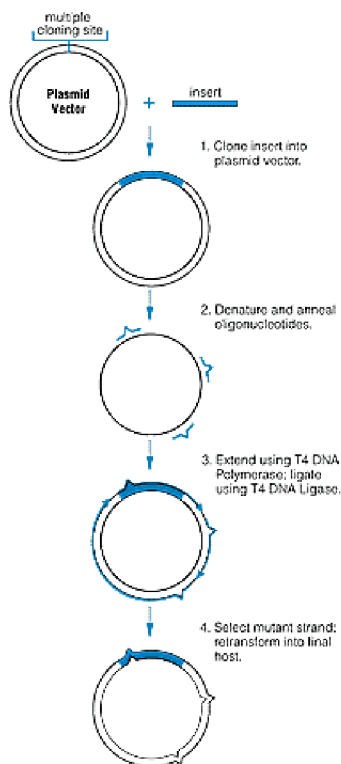
## Oligonucleotide-directed mutagenesis

All oligonucleotide-directed mutagenesis is based on the same concept - an oligonucleotide encoding the desired mutation(s) is annealed to one strand of the DNA of interest and serves as a primer for initiation of DNA synthesis. In this manner, the mutagenic oligonucleotide is incorporated into the newly synthesized strand. Mutagenic oligonucleotides incorporate at least one base change but can be designed to generate multiple substitutions, insertions or deletions. Examples include all PCR-based methods and practically all of the non-PCR-based methods in use today. [Table 1](#) describes the more commonly used approaches to site-directed mutagenesis.

Approach	Comments on Use	References
<b>Positive Antibiotic Selection</b>	Ease of use; multiple mutation reactions are possible; no need to isolate or manipulate ssDNA; robust selection enhancement.	(10-13)
<b>Unique Restriction Site (USE)</b>	Can use any plasmid vector that contains a unique, nonessential restriction site; need to optimize restriction digestion; mutation efficiency is linked to restriction digest efficiency.	(14)
<b>Uracil Incorporation (i.e., Kunkel Method)</b>	Thorough, <i>in vivo</i> degradation of uracil-containing template strand; no selection oligonucleotide is necessary; can use any vector that produces ssDNA; need to isolate and manipulate ssDNA; need <i>dut-</i> , <i>ung-E. coli</i> .	(15,16)
<b>Phosphorothioate Incorporation</b>	Resistance to degradation of dNTP analog-containing template strand; need dNTP $\alpha$ S and multiple modifying enzymes (T5 exonuclease, Exonuclease III, DNA Polymerase I, T4 DNA Ligase); need to isolate and manipulate ssDNA.	(17,18)
<b>PCR-based Approaches</b>	Universality of thermal cycler; efficiency of incorporating the mutant oligonucleotide; increased risk of secondary mutations as many thermostable DNA polymerases are prone to nucleotide misincorporation; multiple amplification reactions are often required; need to subclone the mutagenesis product.	(19-23)

Oligonucleotides can also encode a library of mutations by randomizing the base composition at sites during chemical synthesis resulting in degenerate or "doped" oligonucleotides. (Doped oligonucleotides usually refers to the use of unequal amounts of each of the four standard dNTPs in oligonucleotide synthesis.) The ability to localize and specify mutations is greatly enhanced by the use of synthetic oligonucleotides hybridized to the DNA insert-containing plasmid vector. Primary limitations include the inability to synthesize large oligonucleotides and the efficiency of hybridization.

The general format for site-directed mutagenesis, outlined in [Figure 1](#), is as follows: Plasmid DNA containing the template of interest (cDNA, promoter, etc.) is denatured to produce single-stranded regions, a synthetic mutant oligonucleotide is annealed to the target strand, T4 DNA Polymerase is used to synthesize a new complementary strand, and finally T4 DNA Ligase is used to seal the resulting nick between the end of the new strand and the oligonucleotide. T4 DNA Polymerase is the optimal enzyme for use in site-directed mutagenesis as it has no 5'  $\rightarrow$  3' exonuclease activity or strand displacement activity. The polymerase will not displace the mutagenic primer when the mutant strand synthesis is completed (7,8). T4 DNA Polymerase also exhibits 1,000-fold higher fidelity compared to other polymerases that lack proofreading activity; therefore, it is useful in reducing undesirable secondary mutations which can arise in strand synthesis. (The overall fidelity of T4 DNA Polymerase is estimated at one misincorporation per  $10^7$  residues [9] making it the enzyme of choice for site-directed mutagenesis.)



**Figure 1. General schematic of oligonucleotide-directed mutagenesis using plasmid (double-stranded) DNA.** This diagram is meant to illustrate generally how site-directed mutagenesis can be accomplished using duplex DNA and does not provide specific details. The DNA fragment of interest is recovered and cloned into the plasmid vector. The dsDNA is denatured to make the template strand available for hybridization with the mutagenic and selection oligonucleotides. The mutant strand is synthesized by primer extension, using T4 DNA Polymerase, from the hybridized oligonucleotides. T4

DNA Ligase is used then to seal the resulting nicks at the 5'-ends of the primers. The heteroduplex is propagated and selected by transforming a mutation-deficient strain of *E. coli*, (i.e., *mutS*). Next, the plasmid DNA is recovered, purified and used to transform the final *E. coli* host strain.

The resulting heteroduplex is propagated by transformation in *E. coli*. In theory, this should result in a 50% efficiency of mutagenesis due to the semi-conservative nature of replication. Observed efficiencies, however, are much lower (if no selection is used) due to such factors as incomplete strand synthesis, random priming of DNA synthesis, oligonucleotide primer displacement and host repair mechanisms. Selection and enrichment methods have been incorporated into mutagenesis methods to greatly improve the efficiency of mutant strand recovery and rates approaching 80-90% are possible. Numerous methods exist to generate different types of mutations and to enhance for the selection of the mutant. Examples of methods to enhance for the selection of the mutant include positive antibiotic selection of the mutant strand, using a uracil-containing DNA strand which can be selectively degraded *in vivo*, and dNTP analog incorporation, which can render one strand of heteroduplex DNA impervious to digestion. Some approaches can be combined, such as cassette mutagenesis and the use of "doped" oligonucleotides to create a library of random mutations in a small, defined region.

An extension of the so-called "standard" methods of site-directed mutagenesis includes those that rely on DNA amplification, specifically the polymerase chain reaction (PCR). Although convenient, amplification methods do present some difficulties. The required thermostable polymerases typically misincorporate nucleotides resulting in secondary mutations. In addition, incorporating multiple mutagenic oligonucleotides can be problematic and cloning the PCR product is usually required.

The major commonality in site-directed mutagenesis is the use of a mutagenic oligonucleotide. And, as the oligonucleotide is essential in all of these procedures, it is important to understand what constitutes an effective mutagenizing oligonucleotide.

## Oligonucleotide primer design

Proper design of mutagenic oligonucleotides is critical to the efficiency of the reaction. Each oligonucleotide must be evaluated individually, but certain guidelines can be applied to their design. [Table 2](#) lists some important guidelines for the design of mutagenic oligonucleotides. The mutagenic oligonucleotide must hybridize efficiently to the template. For efficient hybridization, there needs to be 100% base pairing at either end of the target sequence without secondary structure formation. Many of the same design parameters optimized for PCR are relevant here. For small substitutions, 10-15 bases hybridizing on either side of the mismatch are usually sufficient. The composition of the 3'-end of the primer is particularly important as polymerases do not typically extend from a mismatched or poorly hybridized 3'-end. Potential secondary structure is best evaluated using a computer program, many of which exist for this purpose. For further information on the design of oligonucleotide primers for site-directed mutagenesis, refer to [Table 3](#) for helpful Internet sites.

**Table 2. Design of Mutagenic Oligonucleotide Primers for Site-Directed Mutagenesis.**

Oligonucleotide Parameter	Requirement	Explanation
<b>Length</b>	≥20 bases total for a single base mismatch; longer flanking sequences for additional mismatched bases.	The oligonucleotide must be long enough to hybridize to the target DNA at a unique loci. Also of importance, sufficient length depends upon the number of mismatched bases in the oligonucleotide. The calculated melting temperature (T <sub>m</sub> ) for hybridization is a useful tool when designing the oligonucleotide.
<b>Degree of Similarity</b>	100% over 10-15 bases at both the 5'- and 3'-ends.	It is important that the oligonucleotide 'sit' on the target DNA by binding both stretches of sequence surrounding the mismatch. Especially important for adequate extension by T4 DNA Polymerase is that the 3'-end of the oligonucleotide and the template match 100%.
<b>Sequence</b>	Lack of secondary structure.	The sequence should not generate secondary structure that would inhibit hybridization to the target.

*The requirements listed above are given as guidelines only. The performance of a given oligonucleotide primer depends upon intrinsic characteristics and the target sequence. See Table 3 for additional information on oligonucleotide design.*

**Table 3. Useful Web Sites for Site-Directed Mutagenesis.**

General Information	
Title and web address of sites (last modified)	Comments
<b>Biology WWW Resources (11/95)</b> http://ba-itumac1.lib.unimelb.edu.au/VivsBioinformatics.html	*Mega*-site including links to software programs, protocols, data bases, etc. See links to <a href="#">Software Sites</a> and <a href="#">Molecular Biology Protocols</a> .
<b>The National Center for Biotechnology Information (1/97)</b> http://www.ncbi.nlm.nih.gov/	Links to GenBank® and other sequence programs.
<b>Pedro's BioMolecular Research Tools (3/96)</b> http://www.public.iastate.edu/~pedro/research_tools.html	*A Collection of WWW Links to Information and Services Useful to Molecular Biologists.*
Specific Information	
Title and web address of sites (last modified)	Comments
<b>Java-based Molecular Biology Work Bench (1/97)</b> http://www.embl-heidelberg.de/~toldo/JaMBW.html	Multiple links to sequence analyses programs including <a href="#">PCR Primer Design</a> by Luca Toldo, and <a href="#">Oligonucleotide Calculator</a> by Eugen Buehler.
<b>The EBI Molecular Biology Software Archive</b> http://www.ebi.ac.uk/software/software.html	Free software for molecular biologists.
<b>Biological Software</b> http://pantheon.cis.yale.edu/~huckaby/bi_csoft.html	Links to sites for various software for biology including multiple primer design programs at <a href="#">PCR Software</a> .
<b>Virtual Genome Center (6/96)</b> http://alices.med.umn.edu/VGC.html	See <a href="#">Oligonucleotide T<sub>m</sub></a> to calculate the melting temperature of an oligonucleotide based on Breslauer <i>et al.</i> (1986) <i>Proc. Natl. Acad. Sci. USA</i> <b>83</b> , 3746. Also, see protein <a href="#">Coding Regions</a> .
<b>Primer3 (primer3.cgi v 0.11)*</b> http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi	Primer picking program.
<b>Gopher Menu</b> gopher://ly.bio.indiana.edu:7011/#UBio-Software+Data/molbio	See <a href="#">Primer</a> .
<i>The above Internet sites, which are categorized as "General" and "Specific", contain helpful information on the design of oligonucleotide primers and links to sites for molecular techniques used in site-directed mutagenesis.</i>	
<i>* Copyright © 1996 Whitehead Institute for Biomedical Research. All rights reserved. This product includes software developed by the Whitehead Institute for Biomedical Research.</i>	

## Common approaches to oligonucleotide-directed mutagenesis

As the efficiency of any site-directed mutagenesis method relies on the nature of the DNA being mutated, in addition to other parameters, it is not helpful to list reported efficiencies here. They can differ widely, however, in their ease of use and hands-on time required. There are a number of caveats to consider for different aspects of the procedures. Most of these can be addressed for the particular site-directed mutagenesis method.

The following *in vitro* approaches to site-directed mutagenesis (summarized in [Table 1](#)) include the use of a mutagenizing oligonucleotide, and in some cases, a selection oligonucleotide. These methods represent the most popular methods of the day. With all methods of site-directed mutagenesis, sequencing of the mutant product(s) is recommended to confirm the nature of the identity of the mutation.

### Positive antibiotic selection

The basis for site-directed mutagenesis by positive antibiotic selection is that a selection oligonucleotide or oligonucleotides are simultaneously annealed, with the mutagenic oligonucleotide, to repair an antibiotic resistance gene (10-13). Selection for the mutant strand is enabled by antibiotic resistance of the mutated DNA and sensitivity of the nonmutated strand. This approach offers a very efficient means to generate an indefinite number of the desired mutations with little hands-on time. Transformation following mutagenesis is performed in a mismatch repair-deficient strain of *E. coli* (such as ES1301 *mutS*) followed by transformation into a final host (13).

### Unique restriction site elimination (USE)

Site-directed mutagenesis by the use of a unique restriction site is based on the methods of Deng and Nickoloff (14). In this approach, a selection oligonucleotide containing a mutated sequence for a unique restriction site is annealed simultaneously with the mutagenic oligonucleotide. The selection oligonucleotide renders the nonessential site immune to restriction by the corresponding enzyme. Selection for the mutant strand is enhanced by digesting the resulting pool of plasmids with the unique restriction enzyme. The digestion linearizes the parental plasmid thereby effectively decreasing its ability to transform bacteria. This approach requires that a unique restriction site be available, and that the subsequent digestion be optimal, so as to linearize the majority of the template strand while leaving the targeted strand intact. Post-mutation transformation is performed in a mismatch repair-deficient strain of *E. coli*.

### Deoxyuridine incorporation

Commonly referred to as the Kunkel Method, site-directed mutagenesis by deoxyuridine incorporation relies on the ability of a host strain to degrade template DNA that contains uracil (U) in place of thymidine (T) (15,16). A small number of dUTPs are incorporated into the template strand in place of dTTP in a host that lacks dUTPase (*dut*-) and uracil *N*-glycosidase (*ung*-) activities. (Uracil *per se*

is not mutagenic and it base pairs with adenine.) Normally, dUTPase degrades deoxyuridine and uracil *N*-deglycosidase removes any incorporated uracil. Post-mutation replication in a *dut<sup>+</sup> ung<sup>+</sup> E. coli* strain is used then to degrade nontarget strand DNA. This approach requires that single-stranded DNA be used so that only one strand contains the Us which are susceptible to degradation.

## Phosphorothioate incorporation

This approach to site-directed mutagenesis rests on the ability of a dNTP analog containing a thiol group to render heteroduplex DNA resistant to restriction enzyme digestion. Based on the work of Eckstein and colleagues (17,18), the mutant strand is extended from the mutagenic oligonucleotide and synthesized in the presence of dCTP $\alpha$ S. Unused template DNA is removed by digestion with T5 exonuclease. (Nicked, dsDNA is also a template for T5 exonuclease.) Theoretically, only circular, heteroduplex DNA remains. The heteroduplex is then nicked, but not cut, at the restriction site(s). Exonuclease III is used to digest the nicked strand (the nonmutant strand) of the heteroduplex to approximately 800 bases in length. This fragment then acts as a primer for repolymerization with DNA Polymerase I and T4 DNA Ligase creating a mutant homoduplex. This approach requires the use of ssDNA and a dNTP $\alpha$ S nucleotide analog in addition to the mutagenic oligonucleotide, transformations and restriction enzymes and digestions.

## PCR-based approaches

Many variations on a theme exist in which the polymerase chain reaction (PCR) is used to generate a mutation in DNA. For the most part, the PCR approach to site-directed mutagenesis is based on the methods of Higuchi *et al.* (19). Like traditional PCR, a template is amplified using a set of gene-specific oligonucleotide primers except that one oligonucleotide, or more in protocols that use multiple amplifications (20), contains the desired mutation. Variations include altering the hybridization site of the oligonucleotides to produce multiple, overlapping PCR fragments with the mutation in the overlap (21,22) and the "megaprimer" approach (23), which uses three oligonucleotides and two rounds of amplification wherein a product strand from the first amplification serves as a primer in the second amplification. Many other variations too numerous to be covered here also exist. PCR-based methods offer the advantages of speed and convenience for simple substitutions. These methods, however, typically are dependent on the properties of the thermostable DNA polymerases being used. The high error rate of these polymerases, due to a lack of 3'  $\rightarrow$  5' proofreading activity, can pose a significant risk of introducing secondary mutations when amplifying large segments of DNA. Therefore, the lack of proofreading activity of many purified, thermostable DNA polymerases, which is exploited in some approaches to random mutagenesis, is disadvantageous in PCR-based site-directed mutagenesis.

## Summary

The ability to introduce a mutation -- a deletion, an insertion or a single base change at a specific locus *in vitro* -- has benefited greatly from recombinant DNA technology. Site-directed mutagenesis possesses much power as a research tool and is the basis for a wealth of the molecular biology experimentation in protein structure and function studies today. Understanding what types of mutations are possible and what resources are necessary is an important first step in exploiting site-directed mutagenesis.

## References

1. Kendrew, J. (1994) In: *The Encyclopedia of Molecular Biology*, Blackwell Science, London.
2. Muller, H.J. (1927) *Science* **66**, 84.
3. Auerbach, C. and Robson, J.M. (1947) *Proc. R. Soc. Edinburgh B* **62**, 279.
4. Hong, J.S. and Ames, B.N. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 3158.
5. Botstein, D. and Shortle, D. (1985) *Science* **229**, 4719.
6. Lo, K.-M. *et al.* (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2285.
7. Lesley, S.A., Slater, M. and Nelson, L. (1995) *Promega Notes* **54**, 26.
8. Capson, T.L. *et al.* (1992) *Biochem.* **31**, 10984.
9. Kunkel, T.A., Loeb, L.A. and Goodman, M.F. (1984) *J. Biol. Chem.* **259**, 1539.
10. Lewis, M.K. and Thompson, D.V. (1990) *Nucl. Acids Res.* **18**, 3439.
11. Bohnsack, R.N. (1996) *Meth. Mol. Biol.* **57**, 1.
12. Vavra, S. and Brondyk, W.H. (1996) *Promega Notes* **58**, 30.
13. *Altered Sites<sup>®</sup> II in vitro Mutagenesis Systems Technical Manual #TM001*, Promega Corporation.
14. Deng, W.P. and Nickoloff, J.A. (1992) *Anal. Biochem.* **200**, 81.
15. Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 488.
16. Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) *Meth. Enzymol.* **154**, 367.
17. Taylor, J.W., Ott, J. and Eckstein, F. (1985) *Nucl. Acids Res.* **13**, 8764.
18. Nakamaye, K. and Eckstein, F. (1986) *Nucl. Acids Res.* **14**, 9679.
19. Higuchi, R., Krummel, B. and Saiki, R. (1988) *Nucl. Acids Res.* **16**, 7351.
20. Shimada, A. (1996) *Meth. Mol. Biol.* **57**, 157.
21. Ho, S.N. *et al.* (1989) *Gene* **77**, 51.
22. Horton, R.M. *et al.* (1989) *Gene* **77**, 61.
23. Sarkar, G. and Sommer, S.S. (1990) *BioTechniques* **8**, 404.

**Editor's note:** Promega offers the family of Altered Sites<sup>®</sup> II *in vitro* Mutagenesis Systems, and supporting reagents, for site-directed

*mutagenesis by positive antibiotic selection. For more information, please contact your Promega Representative, or access the 1997 Promega Catalog at: <http://www.promega.com> or in Europe at: <http://www.euro.promega.com>*

*Also, Promega will introduce the GeneEditor™ in vitro Site-Directed Mutagenesis System in June '97. This system can be used on any vector containing an ampicillin-resistance gene and will allow easy, high efficiency site-directed mutagenesis. For information on this new mutagenesis system, please contact Angela Ryan, Protein Biochemistry/Molecular Biology Product Manager, Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711-5399. E-mail: [aryan@promega.com](mailto:aryan@promega.com); Phone: 608-277-2690.*

---

*© 1997 Promega Corporation. All Rights Reserved.*

*Altered Sites is a trademark of Promega Corporation and is registered with the U.S. Patent and Trademark Office. GeneEditor is a trademark of Promega Corporation.*

*GenBank is a registered trademark of the U.S. Department of Health and Human Services.*