Mutagenesis: Site-Specific

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Mutagenesis: Site-specific

M Raafat El-Gewely, *University of Tromsø, Tromsø, Norway*

Chris Fenton, *University of Tromsø, Tromsø, Norway*

Elisabeth Kjeldsen, *University of Tromsø, Tromsø, Norway*

Hao Xu, *University of Tromsø, Tromsø, Norway*

Site-specific mutagenesis techniques are aimed at the precise substitution, insertion or deletion of any coding sequence *in vitro*. More recently, however, such precise alterations are also being developed for *in vivo* gene/genome modifications. These techniques are revolutionizing our understanding of the genetic and molecular mechanisms in several biological systems, which could lead to the development of new enzymes, therapeutics as well as improved agricultural applications.

Advanced article

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Introduction

Site-specific mutagenesis, also known as site-directed mutagenesis, is the method used to create specific mutations *in vitro* or *in vivo*, in a given sequence. This technique, which is now a fundamental tool in molecular biology, was first introduced by Michael Smith and co-workers (Hutchison *et al*., 1978). The late Michael Smith was awarded the Nobel Prize for his work in this area in 1993. The method has evolved significantly due to the recent advancements in polymerase chain reaction (PCR) techniques that facilitate making these specific deoxyribonucleic acid (DNA) alterations *in vitro* at ease. Alterations in the DNA sequence can be made at a single site or multiple sites within a defined sequence segment. Base pairs may be substituted, inserted or deleted. These alterations are often made to affect the encoded amino acids. Saturation mutagenesis is the process of substituting several nucleotides at a given site. Each base in any targeted coding triplet could be substituted by any nucleotide, which would give rise to a site-specific repertoire of all possible amino acids encoded by the mutated triplet. Random mutagenesis, on the other hand, helps to create molecular diversity that can be used to select desired activities of the encoded protein (Xu *et al*., 1999). Random mutagenesis can be compared to *in vitro* directed molecular evolution as it mimics the natural process of evolution on an accelerated time scale.

Designing Mutations

The two major concerns in designing a site-directed mutagenesis protocol are how to introduce the mutations into the gene and how to select the mutated gene from the background.

Making mutations

Site-specific mutations are most commonly designed in a synthetic oligonucleotide referred to as the mutagenic oligonucleotide. The mutagenic oligonucleotide sequence is identical to the parental DNA sequence with the exception of one or more base pairs. Given the correct conditions, the mutagenic primer can anneal to the parental DNA template. For best results, mutations should be placed in the middle of the oligonucleotide sequence (**Figure 1a,b**). Mutations at gene termini are possible by attaching additional base pairs on the distal ends of the oligonucleotide sequence (**Figure 1c**). For scattered mutations, sets of mutagenic oligonucleotides can be designed, with one oligonucleotide for each one or several nearby mutations using overlapping PCR (Ho *et al*., 1989) (**Figure 1d**). Another approach for scattered mutations is to design oligonucleotides, one for each mutagenic site. All the oligonucleotides anneal to the same strand of DNA and single-stranded-mutated-DNA is generated by PCR. The single-stranded DNA (ssDNA) is converted to double-stranded DNA (dsDNA) *in vivo* (Hogrefe *et al*., 2002) (**Figure 1e**). **See also**: Nucleotides: uncommon, modified and synthetic

The choice of DNA polymerase is an important factor in site-specific mutagenesis. A proofreading DNA polymerase without strand displacement is best (Kunkel *et al*., 1991; Su and El-Gewely, 1988).

Any amino acid in a protein can be replaced by any of the other 19 amino acids by site-specific mutagenesis. Some mutations will lead to inactivation of the protein, and can be used in protein domain screening, while some amino acid substitutions may retain activity as was demonstrated in studies of homologous protein evolution by Plapp (1995). **See also**: Evolutionary developmental biology: homologous regulatory genes and processes; Mutagenesis mechanisms

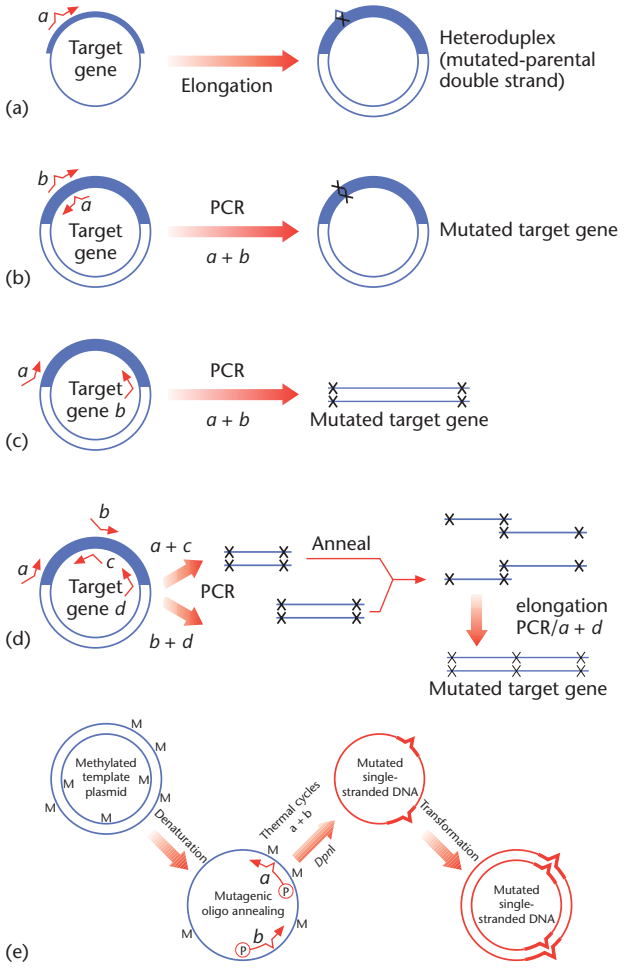

Figure 1 Mutagenic oligonucleotide design. The blue box represents the target gene. The short arrowhead line represents the oligonucleotide. The cross represents desired mutation. P, phosphate group at 5′-end of DNA, m, methyl groups. (a, b) Single or nearby multiple mutations can be designed into the middle area of one or a pair of complementary mutagenic oligos, which will lead to the synthesis of the whole plasmid. (c) Mutations at the terminal regions of a gene can be designed into a pair of mutagenic oligos, which will lead to the synthesis of the target gene. (d) Scattered multiple mutations can be designed into a pair of outside mutagenic oligos (*a* and *d*) and a pair of middle mutagenic oligos (*b* and *c*). Mutations in *b* and *c* oligos sit in the flanking areas that cannot anneal with the template but are complementary to each other. Polymerase chain reaction (PCR) products from oligos *a* and *c* and oligos *b* and *d* can anneal to each other and be elongated to form dsDNA. Thus, the mutated gene can be generated by a final PCR with oligos *a* and *d*. (e) Multiple mutations separated by long distance can be made in one PCR, using one oligo per mutation. All the oligos anneal to the same strand of DNA. The gaps between the oligos are filled and ligated to generate ssDNA. The ssDNA is then converted into dsDNA *in vivo* in the host cell (see Figure 10 for a more detailed description).

Screening for mutants

Following mutagenesis, the target gene must be cloned and transformed in the appropriate host strain for sequence analysis as well as gene expression of the new variant(s). DNA sequencing is commonly used to verify the selection of the desired mutants. In order to enrich for the desired mutants over the original DNA sequence, both biochemical and genetic approaches have been developed (**Table 1**). The higher the efficiency of site-directed mutagenesis, the easier it is to obtain the mutants with the desired sequence.
See also: DNA sequencing

Mutagenic Oligonucleotide

Despite the potential of the site-directed mutagenesis technique, the success of the oligonucleotide-based mutagenesis is often dependent on the design of the oligonucleotide primer. For successful mutagenesis, the following five factors should be optimized: primer length, melting point (primer and product), specificity (intra-, inter-, template homology), GC content and the 3′-terminal end. The use of a primer design software program may aid in the selection of optimal primers, especially for difficult reactions. A selection of primer design programs is available (**Table 2**); many are commercial, but others are free and net-based. Primer3 is an example of a free net-based primer design program that allows the user to optimize primer selection for all of the aforementioned factors.

There are some simple rules to keep in mind when designing oligonucleotide primers for site-directed mutagenesis. The greater the expected alterations in sequence, the longer the complementary region of the oligonucleotide primer should be. Deletions or insertions require primers with complementary sequences of 12–15 base pairs or more on either side of the target.

The melting point should be between 53 and 65°C. In some cases, a higher melting point may be required, e.g. 78°C or higher in the QuikChange™ method. An accurate estimate of T_m for primers between 18 and 24 base pairs in length can be calculated using the equation $T_m = 2(AT) + 4(GC)$ (Thein and Wallace, 1986). The melting point of the PCR product can be calculated under standard PCR conditions of 50 mmol L^{-1} KCl as $T_m = 59.9 + 0.41(\%G + C) - 675/\text{length}$ (Dieffenbach *et al.*, 1995).

The specificity of the oligonucleotide should prevent the primer from annealing to itself, to the other primer or to other sequences within the template. Therefore, primers should not contain any intra- or inter-primer homology greater than 3 base pairs structure, especially at the 3′-terminus of the oligonucleotide (Kwok *et al.*, 1990).

The GC content of primers should be between 40 and 55% whenever possible. Additionally, sequences with long polyG or polyC stretches can promote nonspecific annealing. Long stretches of polyA or polyT can dissociate (break) from the template, opening up the primer–template complex and interfering with amplification (Innis and Gelfand, 1994).

The 3′-terminal position of an oligonucleotide primer is essential for the control of mispriming. Therefore, primers

Table 1 Mutation selection methods in site-specific mutagenesis

Method	Mutation enrichment mechanism	References
Genetic approach		
Deoxyuridine incorporation	Parental DNA strand prepared from dut$^-$ung$^-$ *E. coli* strain incorporates dUTP into DNA, while mutated strand synthesized *in vitro* has no uracil residue. Thus, the parental strand will be degraded by uracil *N*-glycosylase after transformation of the heteroduplex into wild-type host strain	Kunkel *et al.* (1991), Su and El-Gewely (1988)
Antibiotic resistance	Simultaneous site-directed mutations alter the antibiotic resistance gene of the plasmid along with the target gene by specific oligonucleotides. Enrichment of the desired mutants is by antibiotic resistance	Ishii *et al.* (1998)
Biochemical approach		
Methylation and *Dpn*I	*Dpn*I recognizes methylated DNA sequence. Therefore, any *in vivo* synthesized methylated parental DNA strand will be degraded by treatment of *Dpn*I but not the newly *in vitro* synthesized mutant DNA strand	Weiner *et al.* (1994)
Methylation and *Mcr*Br	Before the mutagenesis reaction, DNA methylase methylates the DNA template *in vitro*. When the DNA is transformed into the host cell, the parental methylated DNA is digested by the *Mcr*Br endonuclease, leaving only the newly *in vitro* synthesized unmethylated mutated DNA	Kelleher and Raleigh (1991), Waite-Rees *et al.* (1991)
Thionucleotide selection	Phosphorothioated nucleotides are incorporated into mutated strand during *in vitro* DNA synthesis. The incorporation of phosphorothioated nucleotides protects the DNA from a special endonuclease. Parental DNA will be nicked and degraded by the exonuclease	
Unique site elimination	Simultaneous site-directed mutations alter a unique restriction site of the plasmid along with the target gene by specific oligonucleotides. Enrichment of the desired mutant by endonuclease restriction before transformation	Zhu (1996)
Biotinylation	Biotinylation of the mutagenic oligonucleotides can help the purification of mutated DNA from parental template	Wang *et al.* (1996)

should be designed to have 100% match with the template, especially towards the 3′-end. Two or three G or C nucleotides at the 3′-terminal will increase the chances of a successful reaction because the stronger hydrogen bonding between GC nucleotides provides a 'clamp' from which the reaction can proceed (Sharrocks, 1994).

Methods for Site-specific Mutagenesis

Methods for site-specific mutagenesis can be classified into two groups: non-PCR-based and PCR-based.

Non-PCR methods

Deoxyuridine incorporation-based method

This biological selection-based method (Kunkel, 1985) is based on the deoxyuridine incorporation mutant screening method (**Table 1**). The target gene is cloned in M13, for example, and then propagated to form an ssDNA template in a *dut*$^-$ *ung*$^-$ *Escherichia coli* strain. The mutagenic oligonucleotide is then annealed to the uracil-containing parental ssDNA (**Figure 2**). The elongation and ligation of the mutated strand are carried out by T4 DNA polymerase and ligase (Kunkel *et al.*, 1991). The generated heteroduplex is subsequently transformed to wild-type *E. coli*

Table 2 Selection of some of the available software used to aid in oligonucleotide primer design (listed alphabetically)

Name	Description	Address
CODEHOP	COnsensus-DEgenerate Hybrid Oligonucleotide Primers. PCR primers designed from protein multiple sequence alignments	http://www.blocks.fhcrc.org/codehop.html
Gene Walker	The program is based on the philosophy of ease to use. GeneWalker allows you to work with two primer sequences simultaneously	http://www.cybergene.se/primerdesign/
NetPrimer	(PREMIER Biosoft International). NetPrimer combines the latest primer design algorithms with a web-based interface, allowing the user to analyse the primer over the internet	http://www.premierbiosoft.com/netprimer.html
Primer3	An on-line service to pick PCR primers from nucleotide sequences	http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
PrimerDesign 1.10	PrimerDesign is a DOS-program to choose primer for PCR or oligonucleotide probes	http://www.chemie.uni-marburg.de/~becker/#Primer
Rawprimer	The program is designed for selection of sets of primers along very large queries, all with a relatively narrow Tm range. It is also useful in more traditional PCR applications	http://alces.med.umn.edu/rawprimer.html
The Primer Generator	The Primer Generator is an automated generator of primers for site-directed mutagenesis. The program analyses the original nucleotide sequence and desired amino acid sequence and designs a primer that either has a new restriction enzyme site or is missing an old one This allows for faster sorting out of mutated and nonmutated sequences	http://www.med.jhu.edu/medcenter/primer/primer.cgi
Web Primer	An application that designs primers for PCR or sequencing purposes	http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer

strains selecting against uracil-containing DNA, resulting in mutation frequencies of 50% or greater. The use of T7 DNA polymerase or Sequenase™ instead of T4 DNA polymerase improved the efficiency and complexity of the generated mutations, by faster polymerization of DNA and without strand displacement (Su and El-Gewely, 1988), and increased the usefulness of the method by cloning the gene in phagemids instead of M13. The basic method is commercially available as a kit (e.g. Muta-Gene® Phagemid *in vitro* Mutagenesis Kit version 2, Bio-Rad, Hercules, CA, USA).

Altered sites® II *In vitro* mutagenesis systems

In the Altered Sites II *In vitro* Mutagenesis System (Promega, Madison, WI, USA), a set of dual primers is used to introduce a site-specific mutation as well as simultaneously restoring an antibiotic resistance marker in a dsDNA template/plasmid (**Table 1**). The mismatch repair-deficient *E. coli* host strain (*mut*S) will not restrict the resulting heteroduplex DNA. Subsequent rounds of mutagenesis and selection can be performed on the same plasmid without subcloning (**Figure 3**).

Thionucleotide selection-based method

In the thionucleotide selection-based method, the mutagenic oligonucleotides are annealed to a circular ssDNA template containing the desired gene. The primer is extended by T7 DNA polymerase using a mixture of three deoxynucleotide triphosphates (dNTPs) and one dNTPαS such as deoxycytidine triphosphate αS (dCTPαS) to form a mutated strand with phosphorothioate groups (**Figure 4**). The parental strand, which does not contain phosphorothioate groups, is then degraded by endonuclease and exonucleases treatment (**Table 1**). Double-stranded plasmid DNA template can also be used (Olsen *et al*., 1993).

Methylation-based method

In the methylation-based method, the mutagenic oligonucleotide is annealed to one strand of the heat-denatured target plasmid DNA prepared using a *dam*$^+$ *E. coli* strain. Subsequent extension with T4 DNA polymerase generates a hemimethylated, double-stranded, 'half-mutant' DNA molecule. *Dpn*I digestions restrict the nonmutant methylated parental strand. Subsequent transformation is made using an *E. coli mut*S strain deficient in DNA repair

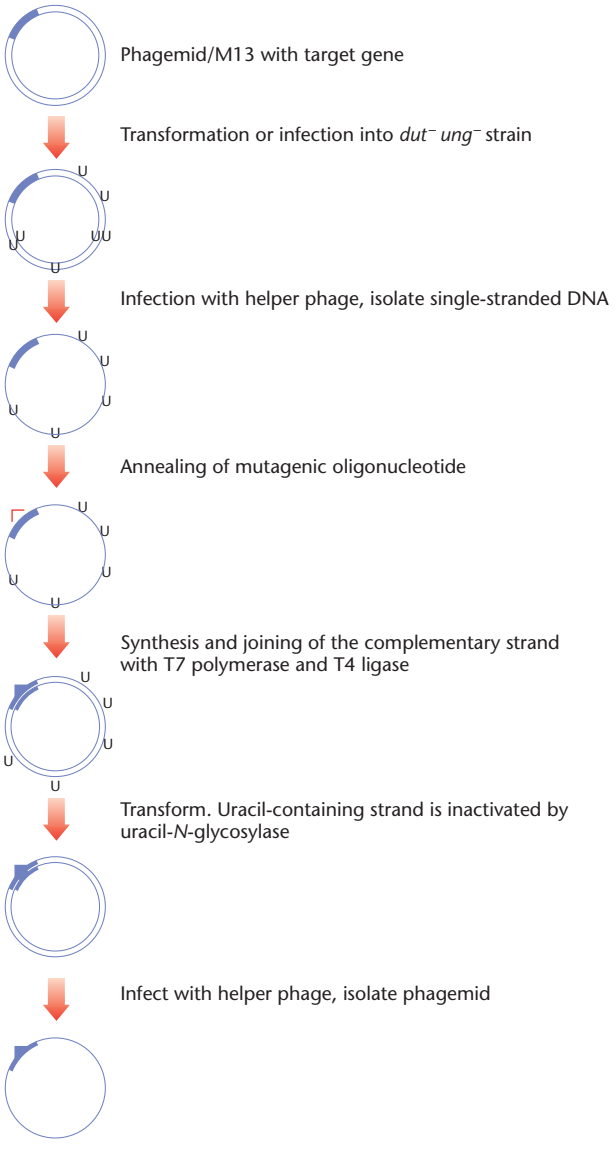

Figure 2 *In vitro* mutagenesis using *dut*⁻ *ung*⁻ genetic selection method.

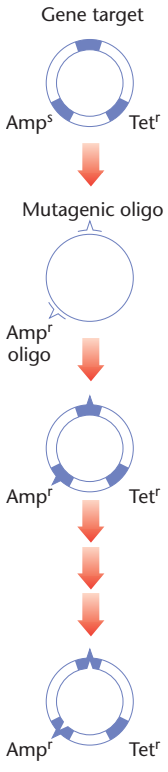

Figure 3 Schematic diagram of *in vitro* mutagenesis procedure using the pALTER®-1 vector as an example (Promega protocol). Target gene was cloned in a plasmid with both tetracycline-resistant gene and point-mutated ampicillin-resistant gene (Amp^S). Bacterial cells transformed with the plasmid are tetracycline resistant, but ampicillin sensitive. Mutagenic oligo is made containing the desired mutation for the target gene. Meanwhile, Amp^r oligo is made containing the mutation, which can repair the point-mutated ampicillin-resistant gene back to its wild-type coding sequence. Alkaline-denatured plasmid, ssDNA can anneal to both mutagenic and Amp^r oligo. The mutant DNA strand can be synthesized with T4 DNA polymerase and T4 DNA ligase. The generated plasmid was first transformed to mismatch-repair-deficient cell ES1301 *mut*S allowing the formation of double-stranded mutant plasmid that can be selected with ampicillin plates. Purified plasmid DNA from surviving ES1301 *mut*S was then transformed to JM109 grown on ampicillin plates to select and amplify mutant plasmid.

(**Table 1**), with 50% mutation efficiency (Amato *et al.*, 1996).

Unique site elimination-based method

In the unique site elimination-based method (e.g. Transformer™ Site-Directed Mutagenesis Kit, Clontech, Palo Alto, CA, USA), two primers are annealed simultaneously to the same strand of a heat-denatured dsDNA template. One primer is mutagenic and the other is a selection primer (**Table 1**). Elongation by T4 DNA polymerase results in the incorporation of both the desired and the selection mutations in the same newly synthesized strand (**Figure 5**). A mismatch repair-deficient *E. coli* strain (*mut*S) is used for propagation of the mutated plasmids. Two rounds of selection are performed, resulting in a very high mutagenesis frequency (Zhu, 1996).

PCR methods

QuikChange™ Site-Directed Mutagenesis Kit

The QuikChange protocol (Stratagene, La Jolla, CA, USA) is a simple and commercially available method for site-directed mutagenesis. A complementary pair of primers with one or more desired mutations anneals to the methylated ssDNA template prepared in a *dam E. coli*

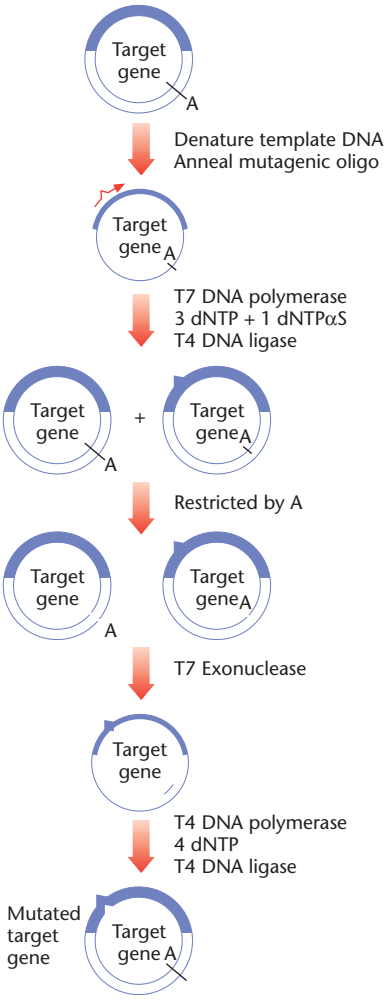

Figure 4 Thionucleotide selection-based mutagenesis. The blue box represents the target gene. The short arrowhead line represents the mutagenic oligo. The peak arch represents desired mutation. 'A' represents restriction endonuclease and its site. The incorporation of a certain type of deoxynucleotide triphosphate αS (dNTPαS) can prevent A restriction, resulting in a nick in the DNA strand without thionucleotide incorporation.

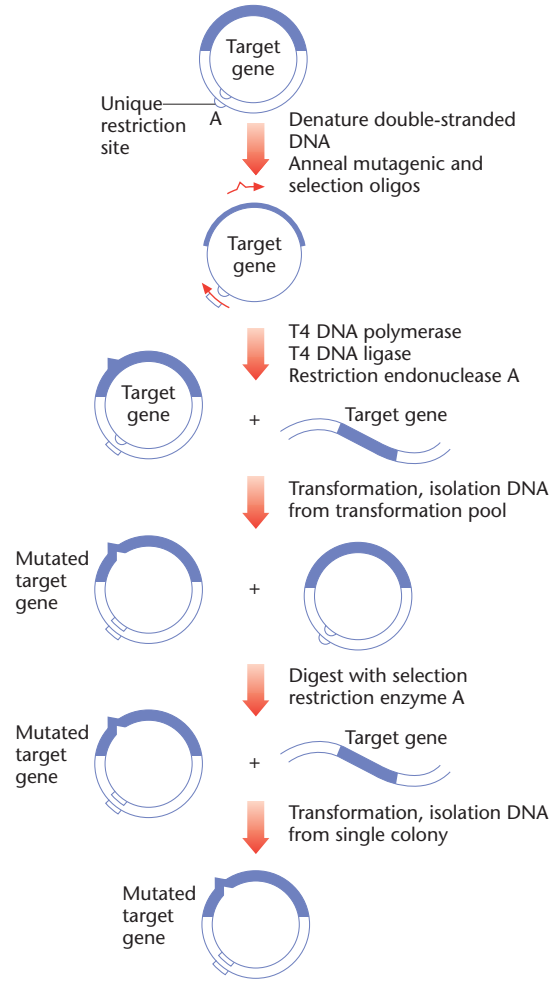

Figure 5 Unique site elimination-based mutagenesis. The blue box represents the target gene. The short arrowhead line represents the mutagenic oligo (with peak arch) or selection oligo (with square). The peak arch in the blue box represents desired mutation. 'A' represents unique restriction endonuclease, and the semicircle on the plasmid represents the sequence for unique restriction enzyme A.

strain. High-fidelity Pfu DNA polymerase is used to synthesize the remaining plasmid DNA without displacing the mutant oligonucleotide primers (**Figure 6**). Upon subsequent treatment with *Dpn*I restriction enzyme, which is specific for methylated DNA, *Dpn*I restricts the methylated parental DNA template, whereas the PCR-generated nonmethylated mutant strand remains intact (**Table 1**). Up to 80% mutagenesis efficiency can be achieved. A two-step procedure based on the QuikChange Site-Directed Mutagenesis Protocol was modified by Wang and Malcolm (1999). It increases the mutagenesis efficiency for large primers, introducing multiple site substitutions (60mer/9 substitutions), long deletion (43mer/31-base deletion) and insertion (73mer/31-base insertion). Improvement was made by adding a pre-PCR, single-primer extension step before the standard QuikChange protocol, to prevent the formation of primer-dimers. **See also**: Polymerase chain reaction (PCR)

GeneTailor™ Site-Directed mutagenesis system

GeneTailor Site-Directed mutagenesis system (Invitrogen, Paisley, UK) is another simple and commercially available method for site-directed mutagenesis. The target DNA is incubated with DNA methylase before the mutagenesis reaction (**Figure 7**). This methylase methylates cytosine residues within specific sequence in the dsDNA (**Table 1**). The DNA is then amplified with two overlapping oligonucleotides, one of which contains the target mutation, giving dsDNA containing the mutation. The mutagenesis mix is then transformed directly into *E. coli* cells. No *in vitro*

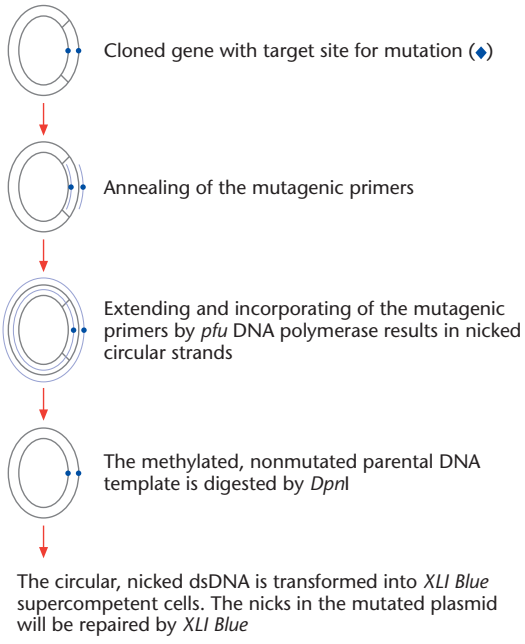

Figure 6 Site-directed mutagenesis method based on the QuikChange method. Two complementary primers with the desired mutation are used in a PCR to synthesize mutated DNA. The parental strands, methylated before PCR by *dam*$^{+}$ *E. coli* host, is digested by *Dpn*I (restriction endonuclease cut at G^{me}A$^{\downarrow}$ TC site) and the nicked vector with the mutation was transformed into *XLI Blue*. The mutation is verified by sequencing.

digestion or purification steps after the mutagenesis reaction are required. The McrBC endonuclease in the host cell digests specifically the methylated parental DNA template (Kelleher and Raleigh, 1991; Waite-Rees *et al*., 1991), leaving only an unmethylated, mutated product.

Mutagenesis at multiple sites

Mutagenesis at multiple sites can be made in one PCR , using a single oligonucleotide per mutagenic site, using for example QuikChange multi Site-Directed Mutagenesis, Stratagene. All of the mutagenic oligonucleotides are designed to anneal to the same strand of denaturated template DNA. The polymerase then extends the primers with high fidelity and without strand displacement, generating dsDNA molecules with one strand bearing multiple mutations and nicks (**Figure 8**). These nicks are sealed by the components in the enzyme blend. The methylated parental DNA and hemimethylated parental mutated heteroduplex DNA are then restricted by *Dpn*I (**Table 1**). The mutated ssDNA is transformed into competent cells where the mutant closed circle ssDNA is converted into duplex form *in vivo*. Mutation efficiencies ranging from 95 to 32% using 1–5 mutagenic primers have been reported (Hogrefe *et al*., 2002).

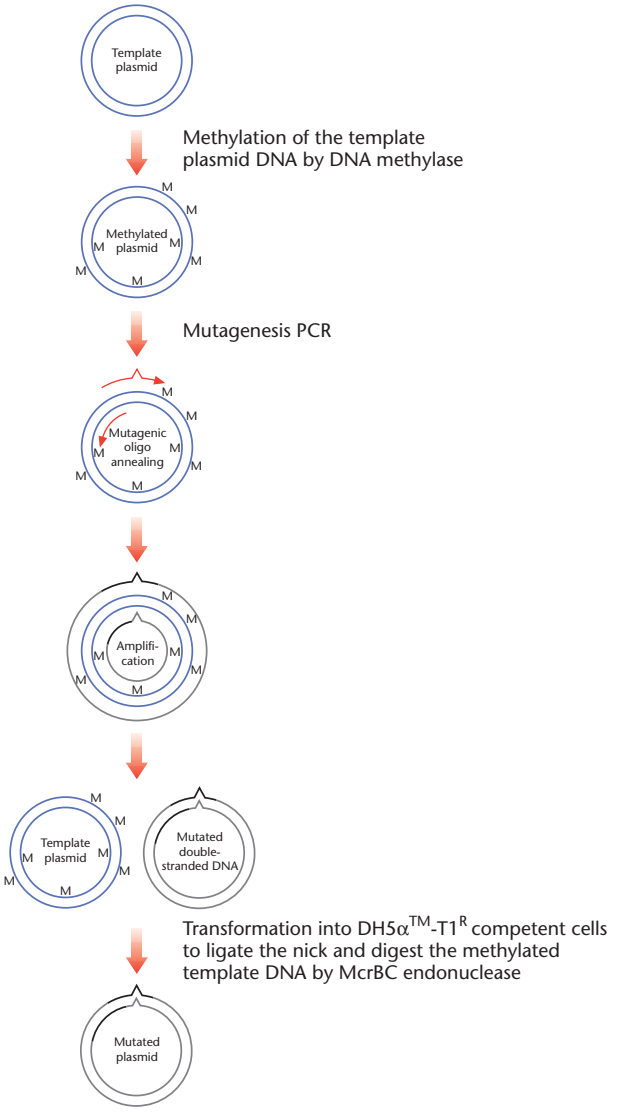

Figure 7 GeneTailor Site-Directed mutagenesis system. The short arrowhead line represents oligonucleotide, m, methyl group. The DNA is methylated *in vitro* by DNA methylase. In the following mutagenesis reaction, the DNA is amplified with two overlapping oligonucleotides, one that contains the mutation. The DNA is transformed into DH5α™-T1^{R} cells. These cells contain McrBC endonuclease, which digests the methylated parental DNA.

Inverse PCR method

Inverse PCR (e.g. ExSite™ PCR-Based Site-Directed Mutagenesis Kit, Stratagene) (Fisher and Pei, 1997) uses a pair of tail-to-tail primers (at least one is phosphorylated) to amplify the entire double-stranded plasmid containing the desired gene, which produces linear dsDNA with blunt ends. Selection of mutants was as described earlier employing *dam* host strain and *Dpn*I. After treatment with *Dpn*I restriction enzyme, the product is then circularized by

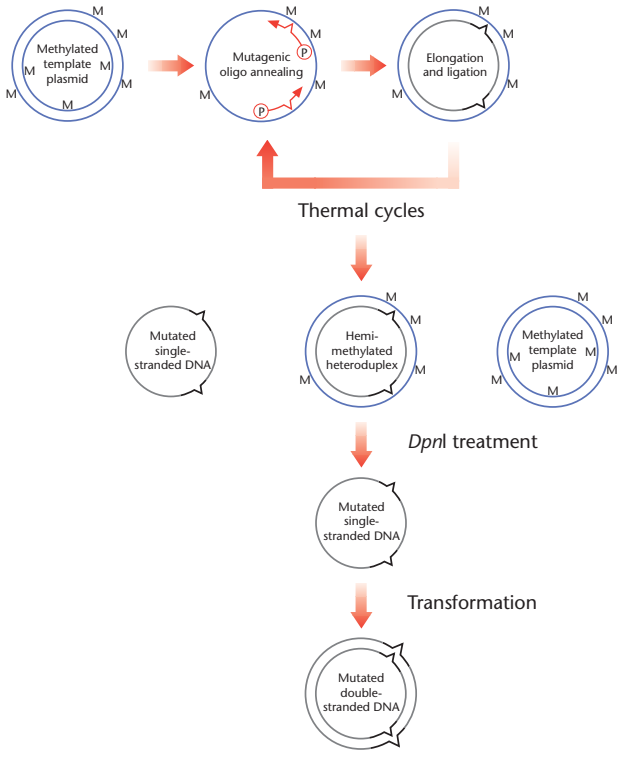

Figure 8 Outline of the mutagenesis strategy at multiple sites. The short arrowed line represents oligonucleotide, m, methyl group; P, phosphate group at 5′-end of DNA. All the oligos are designed to anneal to the same strand of DNA, one oligonucleotide per mutation. The polymerase extends the primers without strand displacement, and the nicks created are sealed by the components of the enzyme blend. The methylated parental DNA is restricted by *Dpn*I (restriction endonuclease cut at $G^{me}A^{\downarrow}$ TC site). The ss-mutated-DNA is converted to dsDNA *in vivo* in XL-10-Gold® cells.

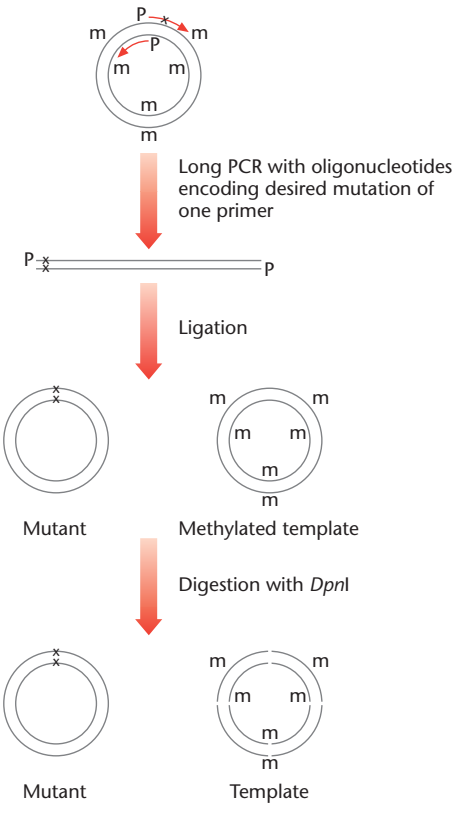

Figure 9 Overview of the inverse PCR-based site-directed mutagenesis protocol (Fisher and Pei, 1997), ExSite. This method requires phosphorylated primer(s). It permits insertions and deletions. *Dpn*I, restriction endonuclease cut at $G^{me}A^{\downarrow}TC$ site; m, methyl group; P, phosphate group at 5′-end of DNA.

ligation and transformed in the appropriate *E. coli* host (**Figure 9**). When inverse PCR is used for base-pair substitutions, mutations should be placed in the middle of one of the primers (Stemmer and Morris, 1992). Inverse PCR can also be used in insertion and deletion mutagenesis (Dorrell *et al.*, 1996).

Megaprimer method

In the megaprimer method (e.g. Mutan-Super Express Km, Pan Vera, Madison, WI, USA), two primers flanking the sequence region of interest/gene and one mutagenic primer in the middle are used in a two-step PCR procedure. In the first PCR, the mutagenic primer and one of the flanking primers are used, resulting in a dsDNA fragment containing the desired mutation. This is referred to as a megaprimer. Following fragment purification, a second PCR is performed with the second flanking primer and the megaprimer, generating a larger fragment for cloning into the appropriate vector. A mutagenesis efficiency of at least 85% has been reported (Brons-Poulsen *et al.*, 1998). It should be noted that the DNA polymerase used in this method should lack any 3′-terminal transferase activity. In the Mutan-Super Express Km protocol, only two primers are needed: one is the mutagenic primer and the other is an antibiotic selection primer. The first PCR produces the megaprimers that can be used on the plasmid template to produce the entire length of the plasmid.

Overlap extension method

The overlap extension method (e.g. LA PCR *In vitro* Mutagenesis Kit, Pan Vera) uses two flanking primers that introduce unique restriction sites for cloning; two mutagenic middle primers are designed with overlapping sequence at their 5′-ends (**Figure 1d**). Two PCR reactions are performed, each with one flanking primer and one middle primer, to generate two DNA fragments with overlapping ends containing the desired mutation(s). Subsequent fusion reaction is made so that the overlapping ends anneal. The fusion product is amplified in a second PCR with the two flanking primers and subsequently cloned into a proper vector (Ho *et al.*, 1989).

The aforementioned techniques offer clues for designing or optimizing a mutagenesis protocol as needed, or may

Table 3 Site-directed mutagenesis kit (alphabetically by supplier)

Kit	Supplier	Address
Muta-Gene *in Vitro* Mutagenesis Kit	Bio-Rad	http://www.bio-rad.com/
Transformer Site Directed Mutagenesis Kit	Clontech Laboratories, Inc.	http://www.clontech.com/
GeneTailor Site-Directed Mutagenesis System	Invitrogen	http://www.invitrogen.com/
LA PCR *in vitro* Mutagenesis Kit	Pan Vera	http://www.panvera.com/
Mutan™-Express Km		
Altered Sites II *In Vitro* Mutagenesis System	Promega Corp.	http://www.promega.com/
GeneEditor™		
Tfu Direct™	Qbiogene	http://www.qbiogene.com/
Chameleon® Double-Stranded Site-Directed Mutagenesis Kit	Stratagene	http://www.stratagene.com/
QuikChange® Site-Directed Mutagenesis Kit		
QuikChange® XL Site-Directed Mutagenesis Kit		
QuikChange® Multi Site-Directed Mutagenesis Kit		
ExSite PCR-Based Site-Directed Mutagenesis Kit		

help in selecting a commercially available site-directed mutagenesis kit (**Table 3**)

Expression of Engineered Mutants

Since all site-specific techniques aim at introducing alterations in an encoded protein or a regulatory DNA sequence, the engineered mutants must be transformed into an appropriate host strain for testing the mutants.

Application of Site-specific Mutagenesis

Site-specific mutagenesis methods are performed for different objectives. Some examples of the most widely used applications are listed below.

Structure–function relationship

Perhaps the main application of site-specific mutagenesis is the study of the structure–function relationship of proteins. These studies are diverse in their objectives, being used, for example, to investigate the role of a specific residue in a protein, motif or domain (Bittova *et al*., 1999; Hagen *et al*., 1999), or the intragenic complementation between specific engineered mutants (Storbakk *et al*., 1996). The active sites of several proteins have been subjected to analysis by site-specific mutagenesis (Chan *et al*., 1999; Komori *et al*., 1999).

Regulatory sequences analysis and optimization by site-specific mutagenesis

Cis-acting DNA sequences such as operators and promoters are often subjected to site-specific mutagenesis, for example for the analysis of gene regulation and to verify the exact bases involved in DNA–protein interactions. The optimization of such an interaction can then be followed (Doan and Aymerich, 2003; Guillier *et al*., 2002; Papp *et al*., 2002).

Optimization of heterologous gene expression

Heterologous gene expression can be optimized by site-specific mutagenesis, for example, for codon optimization of the gene to be expressed in the new host (Hale and Thompson, 1998; Zimmerman *et al*., 1998). However, a modified *E. coli* host strain with adjusted codon bias is now commercially available (http://www.stratagene.com/). In several reports, attempts have been made to alter the primary structure of a protein by site-directed mutagenesis. The alterations could prevent the formation of inclusion bodies in heterologous gene expression without compromising the activity of the recombinant protein (Yoshikawa *et al*., 1999). Substituting amino acids that clash with the *N*-end rule (Varshavsky, 1996) by site-directed mutagenesis renders these mutants resistant to degradation by the ubiquitin-proteasome pathway in heterologous gene expression (Hammerle *et al*., 1998). **See also**: Codon usage in molecular evolution; Gene expression in *Escherichia coli*; Ubiquitin pathway

Synthetic mutations for human diseases

The synthetic mutation approach (El-Gewely, 1991; Xu and El-Gewely, 2003) enables scientists to make mutations *in vitro* that mimic mutations discovered only by molecular diagnostic and DNA sequence studies. The construction of mutants is simpler than the option of constructing a

complementary DNA (cDNA) library of the appropriate tissues from the new patient. This is especially helpful when the gene under investigation is highly mutated and present in a wide range of tissues. **See also**: Human genetics: online resources; Tumour suppressor genes

Site-specific mutations to facilitate drug development

In the process of drug development and following the discovery of lead compounds, mutations of key residues in the target protein are performed in order to study the interaction between the target and the screened ligand. Moreover, molecular modelling as well as the simulation of drug-resistant variants by site-specific mutagenesis may lead to further improvements in the structure of the lead compound and consequently may result in more efficient drug (Yao *et al*., 2003; Wang *et al*., 1999).

In vivo-specific genomic DNA mutagenesis

As an alternative to *in vitro* mutagenesis, we should also be aware that *in vivo*-specific genomic DNA mutagenesis is possible. Early work has focused on 'knockout' mutations, which eliminate gene function completely (Rossant and Nagy, 1995). Other more precise alterations for single-nucleotide exchange have been developed in order to gain further understanding in establishing human genetic syndrome models and as a guide for gene therapy. These methods include homologous recombination, site-specific recombinases (Rossant and Nagy, 1995) and the ribonucleic acid (RNA)/DNA chimaeric oligonucleotide method (Kochevenko and Willmitzer 2003; Beetham *et al*., 1999). A method for gene replacement without selection was recently developed using *E. coli* (Herring *et al*., 2003). **See also**: Knockout and knock-in animals

Summary

The need to change specific sequences in modern molecular biology is even more important than it was for the early geneticists to introduce mutation in the genome of a given organism at random. Techniques for making site-specific changes are becoming easier and the applications of site-specific alterations are almost unlimited. Specific amino acids can be substituted, deleted or inserted for various studies via changes in the DNA. Equally important is that the *cis*-acting elements in gene regulations can also be analysed and subsequently optimized by site-specific mutagenesis. In the modern drug discovery process, site-specific mutagenesis is a valuable tool used to modify the target protein in order to optimize the ligand potential as a therapeutic drug.

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