Chapter 10

Site-Directed Mutagenesis by Polymerase Chain Reaction

Fabiola Castorena-Torres,
Katia Peñuelas-Urquides and
Mario Bermúdez de León

Additional information is available at the end of the chapter

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Abstract

Since genomic data are widely available, many strategies have been implemented to reveal the function of specific nucleotides or amino acids in promoter regions or proteins, respectively. One of the methods most commonly used to determine the impact of mutations is the site-directed mutagenesis using the polymerase chain reaction (PCR). There are different published protocols to develop single or multiple site-directed mutagenesis. In this chapter, we reviewed the enzymes commonly used in site-directed mutagenesis, the methods for simple and multiple site-directed mutagenesis in large constructs, mediated by insertion of restriction sites. Other methods reviewed include high-throughput site-directed mutagenesis using oligonucleotides synthesized on DNA chips, and those based on multi-site-directed mutagenesis, based on recombination. Software tools to design site-directed mutagenesis primers are also presented.

Keywords: site-directed mutagenesis, polymerase chain reaction, plasmids

1. Introduction

With the advent of new technologies during the last decade, an important amount of genomic data has been provided by next-generation sequencing. With the information provided by genomic sequences, many gene polymorphisms, insertions, or deletions have been significantly associated with disorders that include mono- and polygenetic diseases to cancer. However, to demonstrate that these mutations, located either in coding or uncoding regions, are involved in

the illness, it is necessary to evaluate them in a simpler context using other molecular strategies. Taking the advantage of direct manipulation of DNA as well the availability of sequences, site-directed mutagenesis using polymerase chain reaction (PCR) has become an essential tool for studies of key sequences in regulatory regions and/or the relationship between the structure and function of proteins. Many strategies have been developed to simplify the method and increase its efficiency. Commonly, site-directed mutagenesis is used to introduce mutations in a DNA fragment, genome or plasmid, either by PCR or restriction endonucleases digestion. In this chapter, we summarized different strategies to perform the site-directed mutagenesis using PCR.

2. Site-directed mutagenesis

Mutagenesis is usually employed to understand the regulatory regions of genes and the relationship between the protein structure and its function [1]. Depending on the number of sites to be mutated, site-directed mutagenesis can be divided into two types: simple or multiple mutations [2]. For single mutations, methods are based on the amplification of double-stranded DNA from plasmids using complementary oligonucleotides carrying the mutation of interest [3]. Due to its simplicity, the low number of hours spent, and high efficiency, this is one of the most common strategies to introduce mutations in DNA fragments. For multiple mutations, methods incorporate the desired mutations simultaneously in the same reaction or they are obtained after several rounds of mutations.

There are a number of commercial kits for simple mutagenesis. These kits are easy to use but regularly have trouble getting large deletions [4]. With the intention of overcoming the limitations of commercial kit, other methods have been developed for other applications [5].

2.1. Enzymes used in site-directed mutagenesis

To ensure an accurate amplification by PCR, versions of the high-fidelity DNA polymerases are usually available for site mutagenesis. The common trait of this kind of polymerases is their low error rate. High-fidelity DNA polymerases contain a proofreading domain consisting of polymerase activity 5′-3′ and exonuclease activity 3′-5′ to remove wrong incorporated nucleotides. **Table 1** shows the most representative enzymes to amplify DNA by PCR.

The DNA polymerases *Pfu*Turbo and KOD are very useful to amplify products with complementary primers, however, Phusion DNA polymerase and others cannot. The failure probably is due that Phusion requires high annealing temperatures, and it is able to promote the formation of a perfect matching of complementary primers more than the formation of the primer-template duplex containing mismatches [6].

Another important part of site-directed mutagenesis is eliminating the template with a methylation-recognizing-nuclease, as *Dpn*I. Although digestion of *Dpn*I can eliminate fully methylated parental DNA, around 20–30% of hemimethylated molecules (parental strand combined with PCR-generated strand) could not be removed due to hemimethylated DNA, and the PCR product would be more resistant to *Dpn*I [7].

Enzyme	Published error rate (errors/bp/duplication)	Fidelity relative to *Taq*
Taq	$1–20 \times 10^{-5}$	1×
AccuPrime- *Taq*, HF	N/A	9× better
KOD	N/A	4× better, 50× better
Pfu	$1–2 \times 10^{-6}$	6–10× better
Phusion hot start	4×10^{-7} (HF buffer), 9.5×10^{-7} (GC buffer)	>50× better (HF buffer), 24× better (GC buffer)

Source: Taken from McInerney et al. (2014) [8].

Table 1. Published fidelity (error rate) values for DNA polymerases.

2.1.1. Phusion™ high-fidelity DNA polymerase

This enzyme is manufactured by New England Biolabs, Phusion high-fidelity DNA polymerase is recommended for DNA amplification with high fidelity and robust performance. This DNA polymerase has a unique structure obtained from a fusion of the dsDNA-binding domain to a *Pyrococcus*-like proofreading polymerase. The error rate reported by the provider webpage is 50-fold lower than that of *Taq* DNA polymerase and sixfold lower than that of *Pyrococcus furiosus* DNA polymerase [9]. This polymerase generates blunt-ended products and can amplify from GC-rich templates.

2.1.2. Q5® High-fidelity DNA polymerase

This recombinant enzyme is also produced by New England Biolabs, and described with both, fidelity and robust performance. According to providers webpage, Q5 DNA polymerase is composed of a novel polymerase fused to Sso7d DNA binding domain. Its error rate is >100-

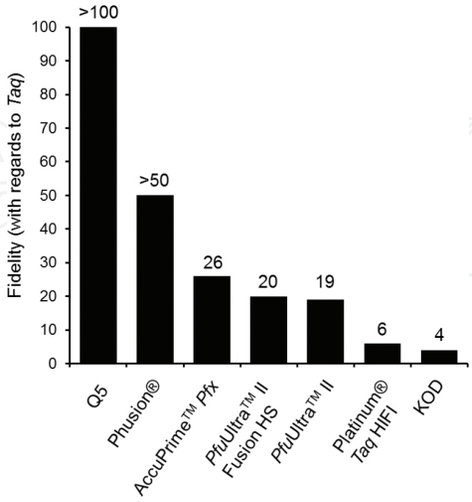

Figure 1. Comparison of fidelity rate among high fidelity DNA polymerases. Modified from: https://www.neb.com/products/pcr-polymerases-and-amplification-technologies/q5-high-fidelity-dna-polymerases/q5-high-fidelity-dna-polymerases.

fold lower than that of *Taq* DNA polymerase and 12-fold lower than that *Pyrococcus furiosus* (*Pfu*) DNA polymerase (**Figure 1**). This polymerase has been optimized with a buffer system to allow a robust amplification in GC-rich regions. Moreover, New England Biolabs webpage has included a Tm calculator to determine the appropriate annealing temperature for primers in GC-rich regions.

2.1.3. AccuPrime™ Pfx

This product is a preparation of DNA polymerase obtained from *Thermococcus* species strain KOD [10, 11]. ThermoFisher Scientific mentions that this polymerase is highly processive and possesses a fast chain extension capability. The preparation contains an antibody bound to the inactive polymerase at ambient temperatures, but it dissociates after the initial denaturation step at 94°C.

2.1.4. PfuUltra high-fidelity DNA polymerase

The *Pfu*Ultra high-fidelity DNA polymerase (Agilent Technologies, La Jolla, CA, USA) is a formulation of a genetically engineered mutant of *Pfu* DNA polymerase and the ArchaeMaxx polymerase-enhancing factor. According to the manufacturer, this enzyme exhibits an average error rate threefold lower than *Pfu*Turbo DNA polymerase and 18-fold lower than *Taq* DNA polymerase. *Pfu*Ultra high-fidelity DNA polymerase is described and it provides a robust amplification of long and complex genomic targets. The ArchaeMaxx factor eliminates dUTP, a PCR inhibitor, and promotes shorter extension times, higher yield, and greater target length capabilities. In fact, the ArchaeMaxx factor functions as a dUTPase.

2.2. Endonuclease *Dpn*I

The use of *Dpn*I endonuclease, which is able to cut methylated DNA, has enabled progress on site-directed mutagenesis. Plasmid DNA extracted from bacteria contains methylated DNA, which makes it susceptible to *Dpn*I enzyme [12], whereas the DNA amplified by PCR does not contain methylated DNA. Based on this principle, the PCR using plasmid DNA as template is useful for the *in vitro* replication and mutagenesis and *Dpn*I digestion. PCR products digested by *Dpn*I have been widely used in mutagenesis.

2.3. Site-directed mutagenesis in large constructs

The QuikChange mutagenesis kits (Agilent Technologies) have become the standard to develop site-directed mutagenesis due to the simplicity of their protocols and their high efficiency. The single-site mutagenesis approach use a pair of complementary mutagenic primers to amplify a target plasmid [2]. The main problem in mutagenesis with large inserts (up to 2100 bp) is due to the efficiency, which is lower than small and medium inserts. In this case, one limitation for site-directed mutagenesis is the size of the target plasmid. The factors that may affect the efficiency of the method are focused on the quality and efficacy of the polymerases and primers used [13].

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Wang et al. [14] have described a method to generate site-directed mutagenesis in large genes. The method consists of two PCR products with four primers (containing mutations and restriction enzyme sites). Fragments are transiently ligated into TA cloning vectors, and, after cutting with appropriate enzymes, fragments are ligated into a final vector (**Figure 2**).

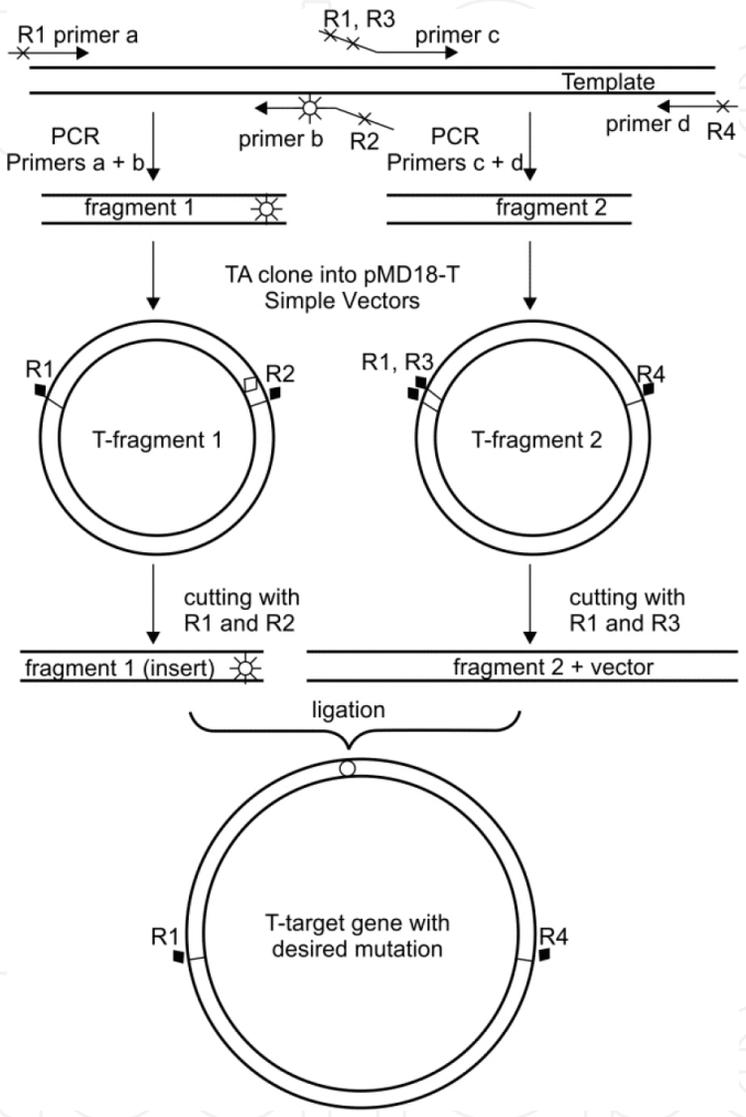

Figure 2. Schematic diagram for site-directed mutagenesis in large constructs. Two target gene segments are amplified from template DNA by PCR using four primers, including two flanking primers (a and d). Restriction enzyme sites (R1 and R4) are incorporated. The mutation of interest (indicated by a star) and/or a same-sense mutation are introduced to create restriction enzyme sites (R2 and R3). Next, PCR fragments are TA-subcloned into T-vectors, and one fragment is cut (R1 and R2) and inserted into the T-vector (predigested by R1 and R3) containing another fragment.

Munteanu et al. [13] used the KOD Hot Start polymerase in combination with high performance liquid chromatography purified primers to achieve site-directed mutagenesis in big plasmid (up to 16 kb). The procedure allowed the incorporation of single or multiple base changes using 6 cycles of PCR instead of 18.

2.4. Site-directed mutagenesis mediated by insertion of restriction sites

The method described by Rouached et al. [15] takes the advantage of the plasticity of the genetic code and the use of compatible restriction sites (**Figure 3**). Method is developed in two steps. First, target DNA is subcloned in a vector, which is the template for the next two PCRs. One reaction amplifies from the start codon to mutagenized site that contains the new introduced restriction site. The other reaction amplifies from the mutagenized site con-

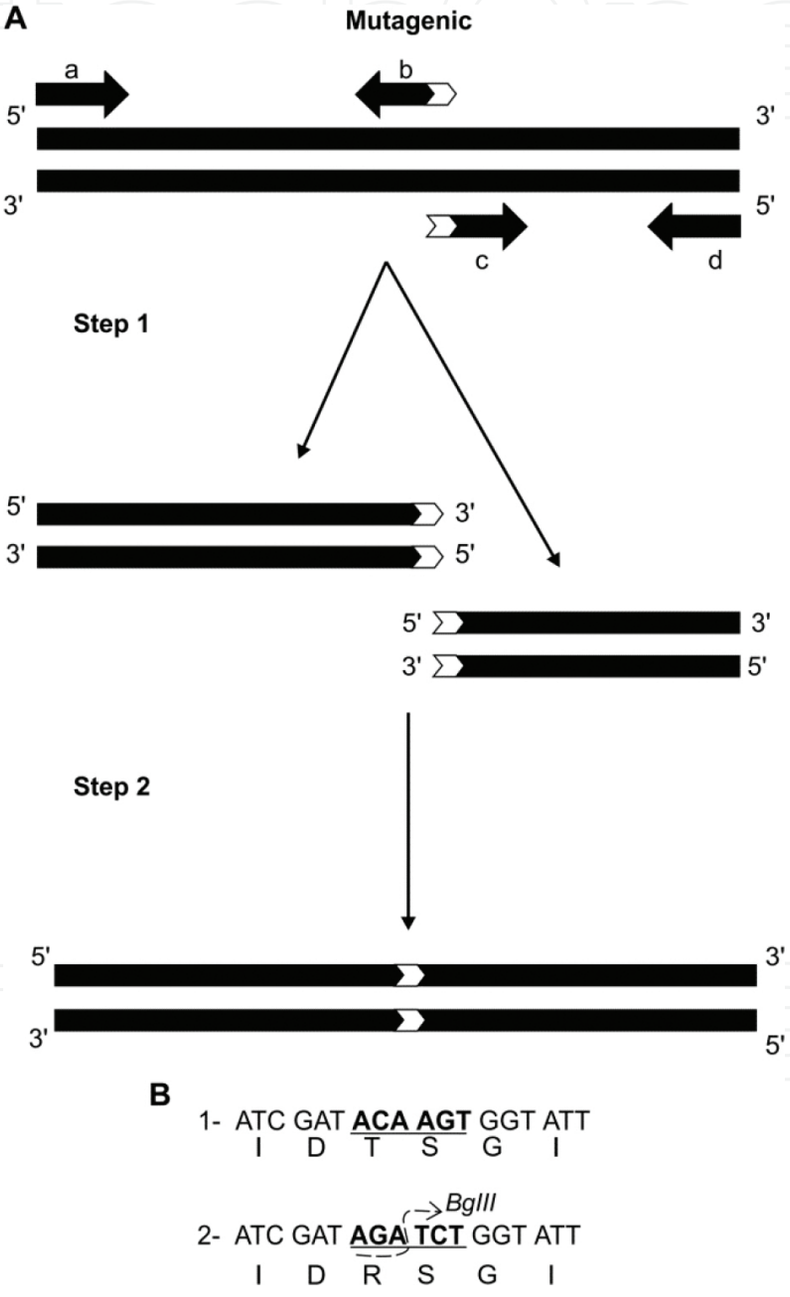

Figure 3. Mutagenesis strategy by restriction enzyme site insertion. Panel A, Two PCR products are obtained from the full length coding sequence. Mutations, including the restriction enzyme site, are contained in primers b and c. PCR product are subcloned by separate in cloning vectors. Next, two fragments are digested with the appropriate enzyme and ligated to reassemble by in-frame coding sequence. Panel B, example of amino acid substitution after introduction of *Bgl*II restriction site.

taining the restriction site to the end of the coding sequence. After amplification, PCR products are digested with the appropriate enzyme and ligated. Primers containing the restriction site are partially overlapped to allow an in-frame assembly of the whole coding sequence [15].

Zhang et al. [16] reported a method of site-directed mutagenesis where they introduced restriction enzyme sites to facilitate the mutant screening (**Figure 4**). The method uses a dsDNA plasmid as a template. In order to select the restriction enzyme sites to be introduced, authors translate the DNA sequence into amino acid sequence, and afterward the amino acid sequence is reversely translated into DNA sequence again with degenerate codons. This approach allows selection of a large number of sequences with silent mutations, which contains several restriction enzyme sites. The transformants are screened by digesting with the appropriate enzyme [16].

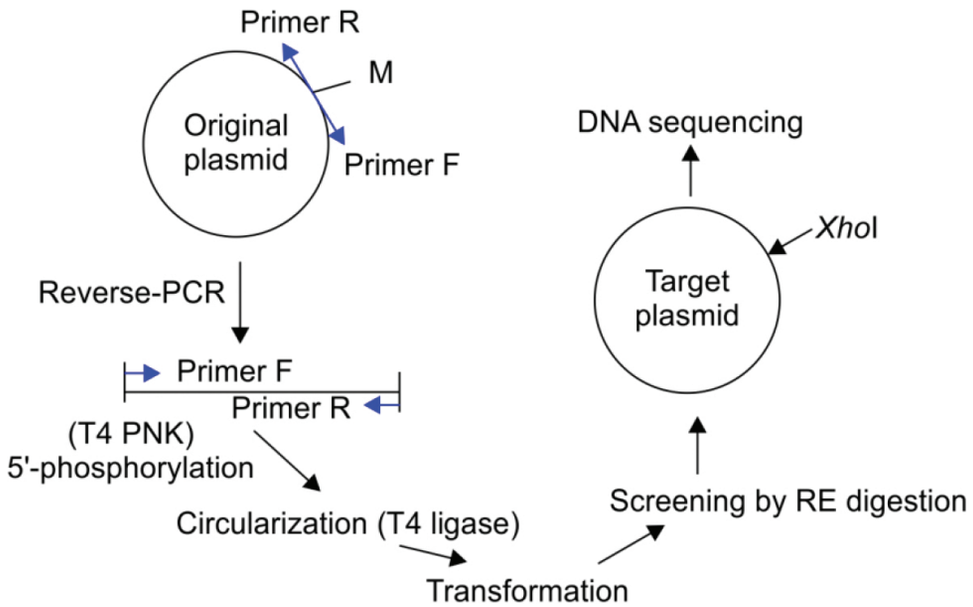

Figure 4. Schematic diagram of the site-directed mutagenesis introducing a restriction enzyme site. Primers contain a new restriction enzyme site. Next to PCR assay, product is phosphorylated and ligated to be transformated in a *E. coli* strain. After restriction enzyme digestion (*Xho*I), plasmid is selected to be sequenced. PNK, polynucleotide kinase; RE, restriction endonuclease.

2.5. High-throughput site-directed mutagenesis using oligonucleotides synthesized on DNA chips

In order to generate a series of constructs with multiple mutants unlimited by the cost of oligonucleotides, Saboulard et al. [17] described the first generation of a library of single and multiple site-directed mutants using a mixture of oligonucleotides synthesized on DNA chips (**Figure 5**). They used the human interleukin15 gene as a model. The library produced 96 different clones in 37 different codons using pools of oligonucleotides. Authors described this approach as straightforward and flexible way to address resolve the problem of massive mutagenesis after successive rounds [17].

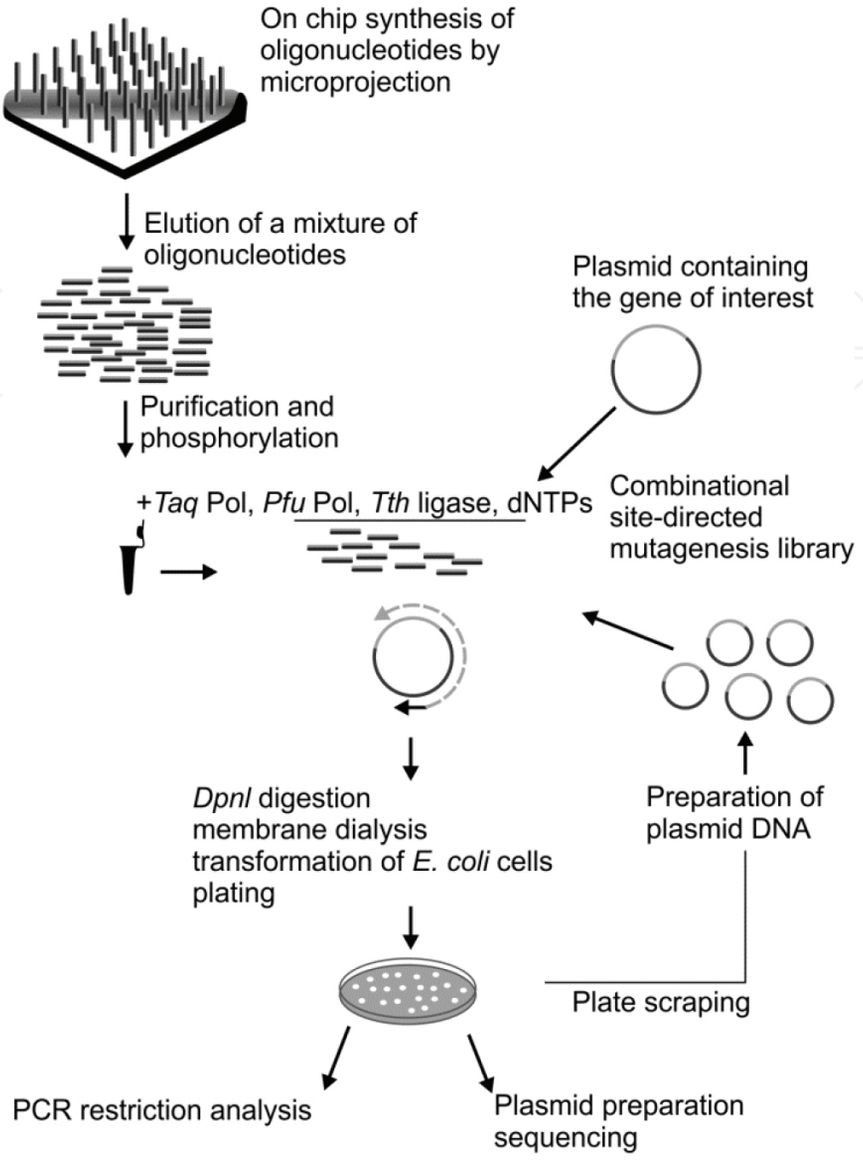

Figure 5. Strategy for massive mutagenesis using oligonucleotides synthesized on DNA chips. The oligonucleotides have been synthesized *in situ* by microprojection on chips. These oligonucleotides, eluted as a mix, are purified and phosphorylated. Next, target plasmid is amplified in a single-strand with the mutagenic oligonucleotides. The amplified product is digested using *Dpn*I, dialyzed and transformed in *E. coli*. Several successive rounds can be performed. Analysis of the clones is made by sequencing and/or by PCR and restriction analysis. Taken from Saboulard et al. [17].

2.6. Methods for multiple site-directed mutagenesis

Fushan et al. [18] developed a method to introduce multiple and complex mutations on plasmids without intermediate subcloning. The procedure is depicted in **Figure 6**. By sequential rounds, each one with PCR amplification with two nonoverlapping pair of primers, the mutation is introduced at the 5′ end of one or both internal primers. Next, PCR products are mixed and ligated to be amplified with external primers. These external primers are suppression adapters to limit the amplification only to target DNA. In order to generate other mutations, an aliquot of the previous reaction is used as a template [18].

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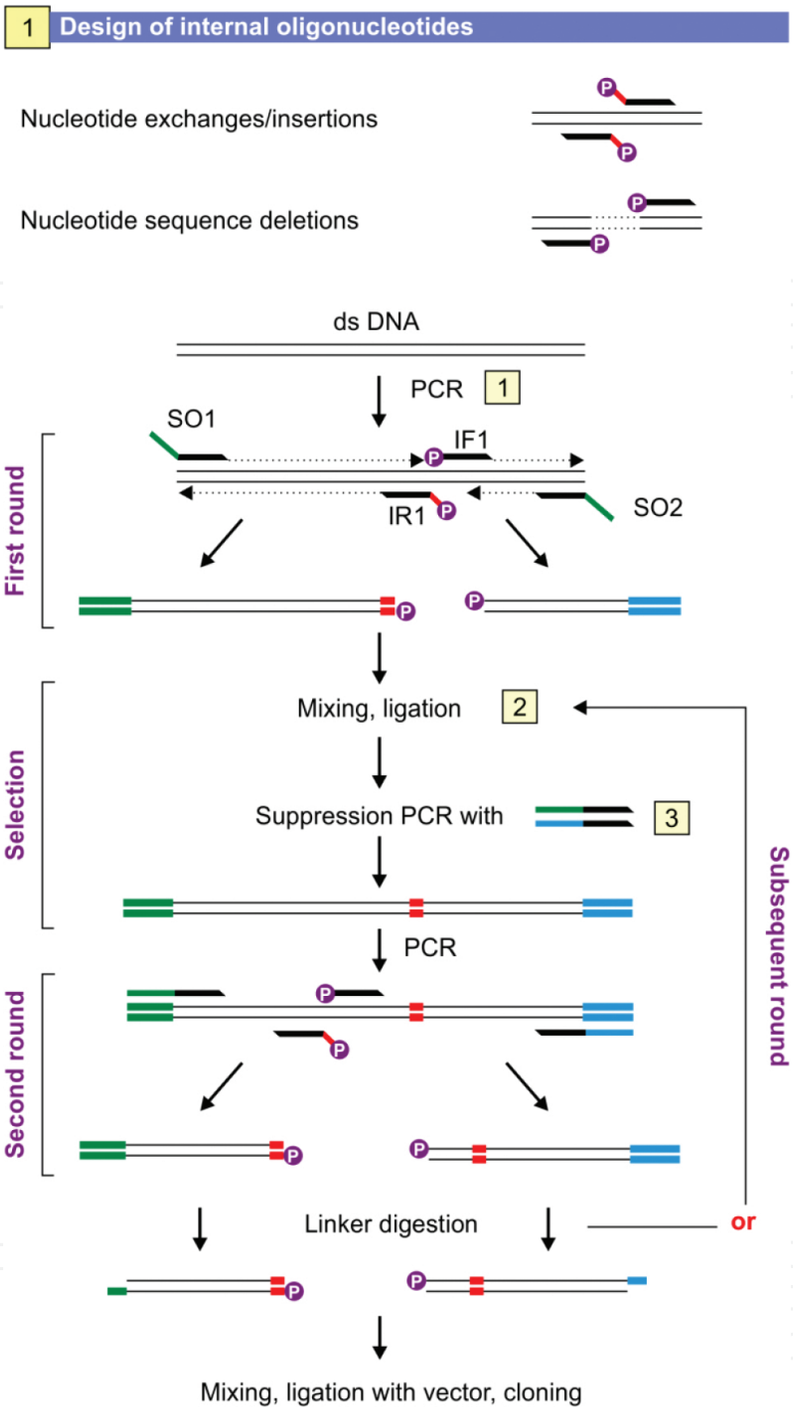

Figure 6. Schematic representation of site-directed mutagenesis by amplification, ligation and suppression PCR. Target DNA is amplified by using two pairs of primers (SO1, IR1, SO2 and IF1). Mutations are on the 5′ end of IR1. After amplification, DNA fragments are ligated generating different types of products (right panel). Then, DNA is amplified with suppression primers SO1 and SO2 where predominant products are type C molecules. After first round of mutagenesis, an aliquot of product is used to the next round to introduce a new mutation. After multiples rounds, the final product is digested with restriction enzymes to be subcloned in a vector.

Another method to generate multiple site-directed mutagenesis is that developed by Holland et al. [19]. They named their method as AXM mutagenesis. Scheme of Holland's method is

shown in **Figure 7**. By PCR, using a prone-error polymerase, a large and mutated DNA fragment is generated with a modified primer containing phosphorothioate linkage at 5′ end. After amplification, a bacteriophage T7-exonuclease treatment allows the removal of strand synthesized with the nonmodified primer. The resulting PCR product is a megaprimer, which is used in a subsequent mutagenesis reaction. The DNA base excision repair pathway in *Escherichia coli* favors the nucleotide base-change to DNA synthesized by megaprimer instead of the complementary uracilated DNA sequence [3, 19]. The method facilitates a rapid generation of multiple mutagenic sites in parallel. A recently modified method of this procedure has been published, where the Eco29k I enzyme is incorporated to selectively degrade the original DNA [3].

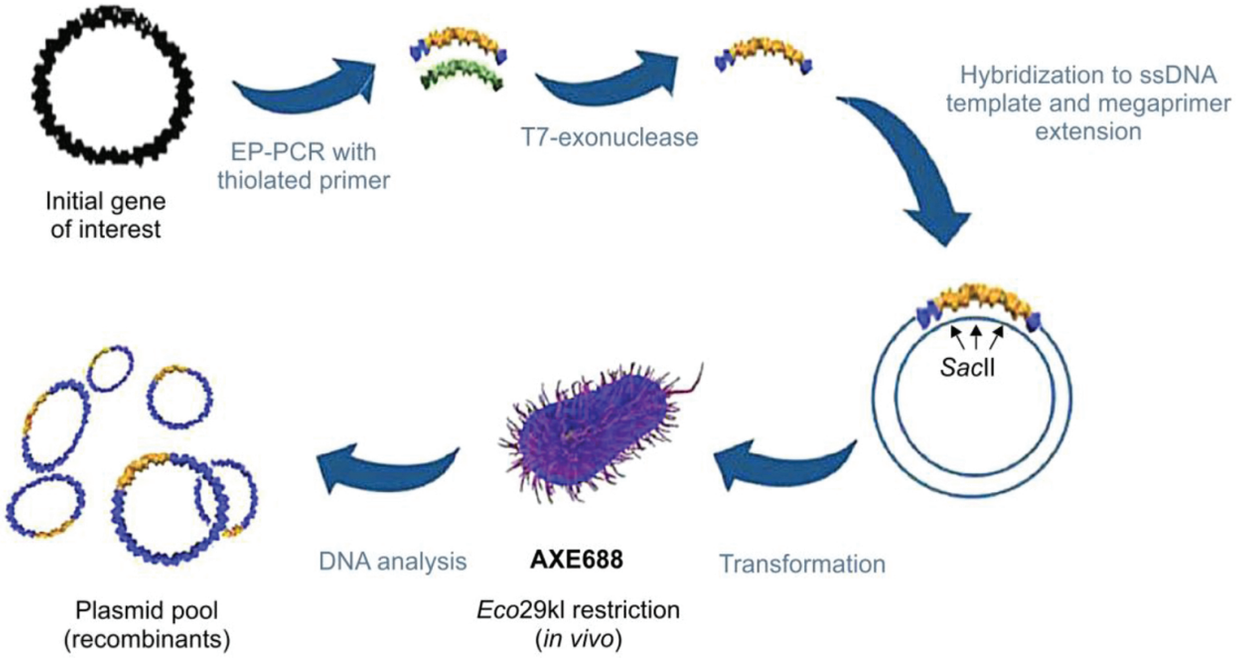

Figure 7. Schematic procedure of AXM mutagenesis. An error-prone PCR (EP-PCR) reaction using a reverse primer containing phosphorothioate linkages on its 5′ end is carried out. The double-stranded DNA is treated with T7 exonuclease to selectively degrade the unmodified strand of the dsDNA molecule. The resulting megaprimer is then annealed to the uracilated, circular, single-stranded phagemid DNA and used to prime *in vitro* synthesis by DNA polymerase. The final product is transformed into *E. coli* AXE688 cells, where the uracilated strand is removed by the uracil N-glycosylase, allowing the survival of the newly synthesized, recombinant strand generated by the megaprimer. The *Eco*29k I enzyme selectively degrades the original DNA retaining *Eco*29k I recognition sites.

2.7. Methods for multi-site-directed mutagenesis based on recombination

Trehan et al. [20] reported a method named REPLACR-mutagenesis (recombineering of Ends of linearized PLAsmids after PCR), which is able to create mutations (substitutions, deletions and insertions) in plasmids by *in vivo* recombineering (**Figure 8**). REPLACR-mutagenesis requires the transformation of PCR products in bacteria expressing Red/ET recombineering proteins. This method can used a variety of plasmids up to bacterial artificial chromosomes [20].

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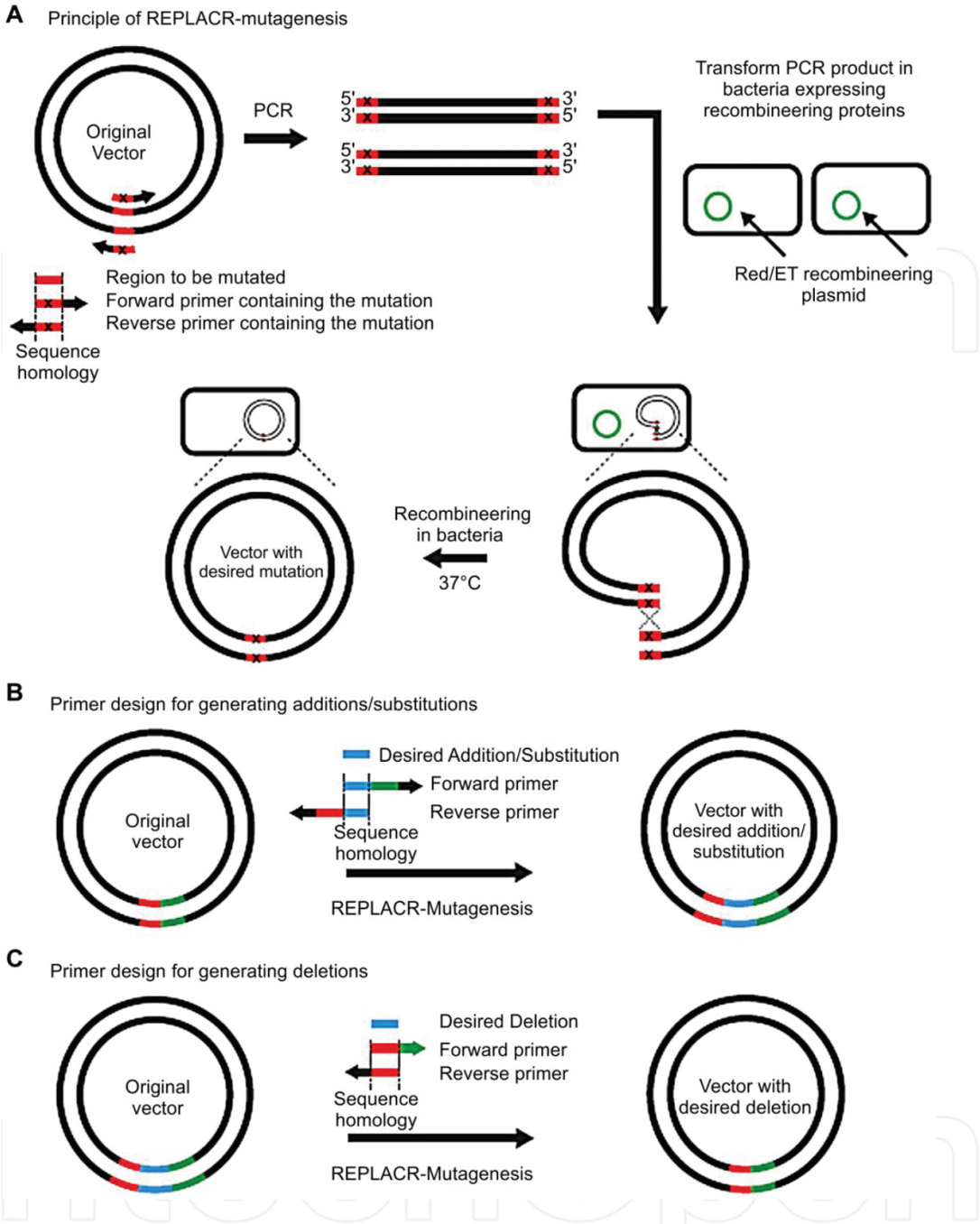

Figure 8. Principle of REPLACR-mutagenesis and primer design strategy for sequence substitution, insertion or deletion. (A) Primers containing the desired mutation overlap for recombination. Bacteria expressing the recombineering proteins (Redγ, β, α and RecA) are transformed with the PCR product. Recombination takes places inside the bacteria and after extraction of plasmid, mutations are confirmed by PCR and sequencing. (B) Example of forward and reverse primers contains the desired addition/substitution. (C) Example for generating deletion mutants, where the forward primer contains the sequence adjoining the sequence to be deleted, and the reverse primer contains a sequence homologous to the forward primer and the adjoining sequence in the vector.

Liang et al. [2] developed a method for the simultaneous introduction of up to three mutations in a plasmid DNA via homologous recombination. The strategy is depicted in **Figure 9**, and it is compatible with a variety of mutations, including degenerate codons in plasmids of different

sizes [2]. The procedure consists of a single multiplex or three independent PCR assays. Each pair of primers contains the desired mutation. Final PCR products have homology at end-terminal to be recombinated. After PCR, a 15-min pulse of recombination activity is carried out and sample is transformed in *E. coli*.

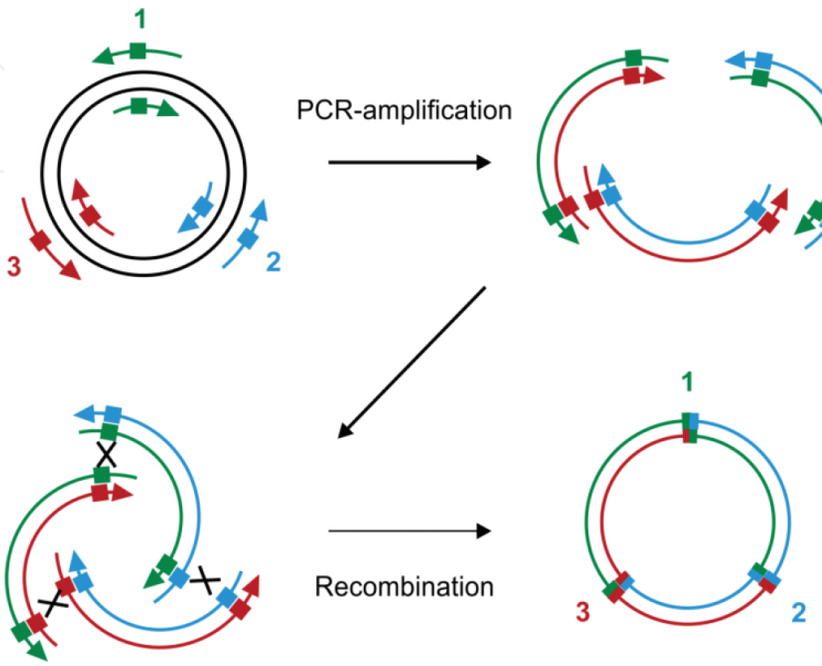

Figure 9. Strategy for multi-site-directed mutagenesis by homologous recombination. Numbers denote the mutation sites. To introduce three mutated sites, typically three pairs of overlapping forward and reverse primers are used unless a pair of long primers is used to cover the mutation sites that are in very close proximity.

2.8. Software tools to design site-directed mutagenesis primers introducing "silent" restriction sites

The critical points of site-directed mutagenesis are the primer design and the annealing temperature. Specific software programs, such as Primer Generator and SiteFind [21, 22], can be used for the design of a restriction enzyme site within the mutation primers without altering the translated amino acid sequence [12].

For example, SiteFind allows the introduction of a restriction site near to the point mutation in manner such that the restriction site has no effect on the peptide sequence. Based on the redundancy of genetic code, a peptide can be encoded by different DNA sequences. Then, the novel restriction site can be used as a marker to be easily screened [22]. The software can work with sequences up to 400 bp.

Another program developed is SDM-Assist, which creates primers to site-directed mutagenesis based on their thermodynamic characteristics. The primer contains the desired mutation and a restriction site for identification of mutant constructs. The algorithm consider factors such as Tm, GC content, and secondary structure [23].

2.9. Conclusion—key results

The site-directed mutagenesis using PCR has been used in molecular biology to modify gene sequences. Methods described here have allowed the introduction of single or multiple mutations into the same target. Despite the wide range of commercial kits for site-directed mutagenesis, there is a constant search to improve the efficiency and simplicity, with a concomitant reducing of costs.

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Author details

Fabiola Castorena-Torres[1], Katia Peñuelas-Urquides[2] and Mario Bermúdez de León[2*]

*Address all correspondence to: mario.bermudez@imss.gob.mx

1 School of Medicine, Tecnológico de Monterrey, Nuevo León, Mexico

2 Department of Molecular Biology, Northeast Biomedical Research Center, Instituto Mexicano del Seguro Social, Nuevo León, Mexico

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