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The "Evolution" of Mutagenesis

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Abstract

DNA cloning and mutagenesis are widespread tools in biology. Understanding the structural/functional relationship of the proteins is of great interest, with the aim to clarify the relationships often the researchers used the mutagenic approach. In the last 20 years, several mutagenesis systems have been developed, analyzing them it's possible to observe the "evolution" of mutagenesis. Here, a short description and differences among three methods widely used of mutagenesis are shown.

Keywords: Mutagenesis; Kunkel; Quick change method; RF system

Introduction

One of the most powerful techniques developed to study the structure/function relationships of proteins has been to mutate a gene to verify the effect of the mutation, both in vitro and in vivo. In general, any DNA fragment, showing regulatory functions or not, can be studied by mutagenesis approach. Early attempts of DNA mutagenesis were non-site-specific and made using radiation or chemical mutagens [1]. Analogs of nucleotides and other chemicals were later used to generate localized point mutations [2], but they were not specific point mutations. Site-directed mutagenesis is the method that allows to make specific and intentional changes to the DNA sequence, it is used for investigating the structure and biological activity of DNA, RNA, and protein molecules. In 1978 Prof. Michael Smith described the first technique of site-directed mutagenesis, which was developed in collaboration with Prof. Clyde Hutchinson [3]. They used as primer a 12-nucleotide oligomer with single nucleotide mismatch, as template φX174 DNA, and the *E. coli* DNA polymerase I to synthesize DNA, to construct a closed circular double-stranded DNA with the mutagenic oligonucleotide in one strand [3,4]. This technic must be considered as revolutionary and starting from this idea several protocols has been developed to obtain DNA mutations in vitro. Here we analyze in detail three methods: the mutagenesis by Kunkel, by PCR and by quick change. In particular, we focused on the timing of realization, type of mutations, rate of mutants and costs. Through the analysis of these three methods it can be highlighted an "evolution" of the in vitro mutagenesis technics.

Mutagenesis by Kunkel

This method was designed to increase the rate of mutagenesis respect to the method developed by Smith. The main feature of the method is obtain the vector containing the gene of interest with uracil in place of thymidine. The single strand DNA template is obtained by transforming the vector, having the origin of replication of the M13 phage, into an *E. coli* dut- ung- strain [5]. The infection of recombinant colonies with M13 phage determines the production of viral particles constituted by single strand uracil-containing DNA vector (Figure 1A). After the single strand uracil-containing vector DNA extraction by phenol/chloroform, the site-specific mutagenesis protocol described by Smith is applied. In detail, starting from the single strand uracil-containing vector as template, it is synthesized in vitro the second strand thymidine-containing vector, using as primer the desired mutagenic oligonucleotide. The DNA newly synthesized in vitro is a double strand heterogeneous/mutagenic vector (Figure 1B), containing the desired mutation but not uracil, this give a selective advantage when an *E. coli* ung+ strain is transformed with the synthesized DNA, because the uracil-containing strand is degraded and only the mutated strand is amplified (Figure 1B). The rate of mutation obtained is about 80% [3]. Regarding the timing of realization, this method is quite long, because it takes about eight days excluding the final sequencing of the clones, but it does not provide the use of restriction enzymes (Table 1).

Mutagenesis by PCR

In 1989 a new system of mutagenesis, based on overlap extension by PCR, was developed [6]. In this method mutagenic complementary oligonucleotide primers (oligonucleotides 2 and 4 in Figure 2A), two external oligonucleotides (1 and 3 in Figure 2A) and the polymerase chain reaction are used to generate two DNA fragments of the gene of interest having both the desired mutation and overlapping ends. After purification, these fragments are combined in a subsequent 'fusion' reaction in which the overlapping ends anneal, allowing the 3' overlap of each strand to serve as a primer for the 3' extension of the complementary strand. The resulting fusion product is amplified further by PCR using the external oligonucleotides (1 and 3 in Figure 2A). At this point the desired mutation is present in all the amplified products. The second phase of the mutagenesis consist in the cloning of the mutated gene in a specific vector. In Figure 2B is reported the scheme of a general procedure to clone a gene in a vector by using of specific restriction enzymes to digest both, the gene and vector, DNA ligation of the fragments, and finally transformation into an *E. coli* recA- strain (Figure 2B). Regarding the timing of realization, about 4 days are requested, excluding the final sequencing of the clones, but in this case it's necessary the use of restriction enzymes and DNA-ligase (Table 1).

Mutagenesis by Quick Change

The most recent method developed for mutagenesis is called quick change (actually is available also as mutagenesis kit from Agilent Technologies, USA). Quick change is based on both methods above described, because it makes use of a pair of mutagenic and complementary oligonucleotides to insert the desired mutation and provides the amplification of the whole vector containing the gene of

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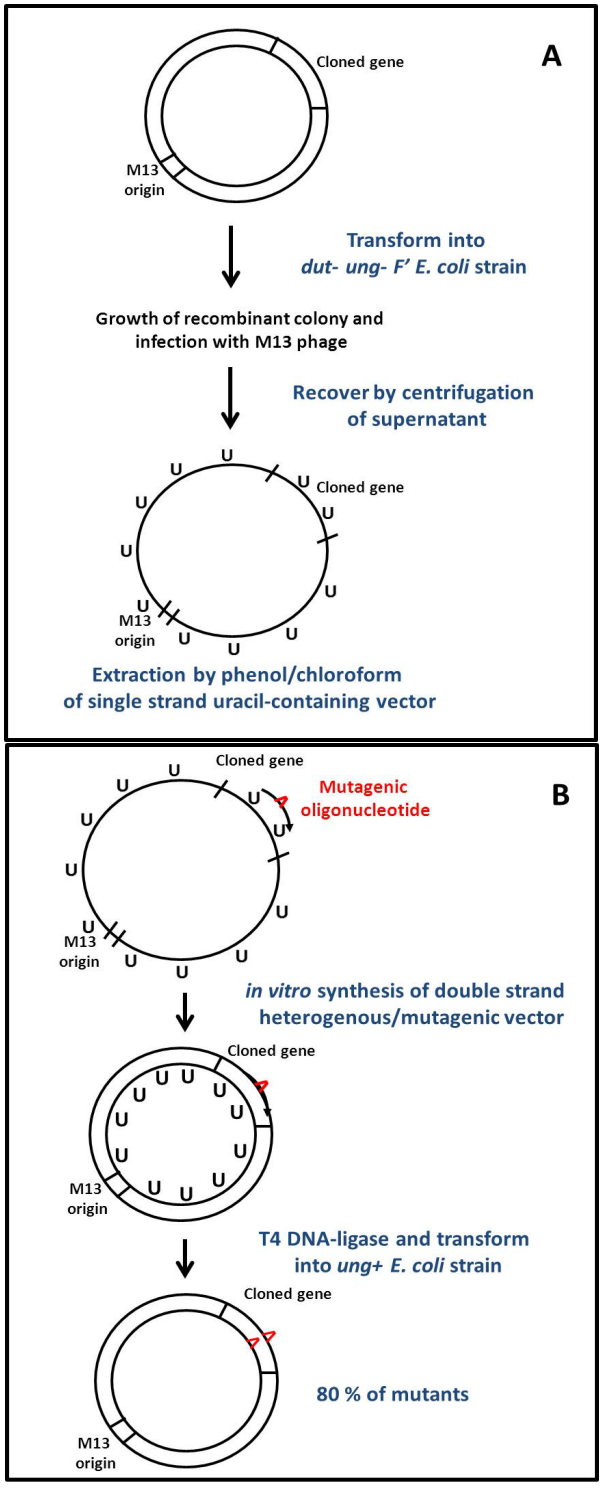

Figure 1: Schematic diagram of the mutagenesis by Kunkel. A) the main feature of this method is to obtain the vector containing the gene of interest with uracil in place of thymidine. For this reason the first step is the transformation in an *E. coli dut⁻ ung⁻* strain, followed by the infection with M13 phage. B) The second phase of the mutagenesis is the *in vitro* synthesis of heterogeneous mutated DNA. The mutagenic oligonucleotide is represented by line with arrow, and the point mutation as red triangle. The new heterogeneous double strand DNA is used to transform an *E. coli ung⁺* strain, in which the parental not mutated strand uracil-containing vector is degraded.

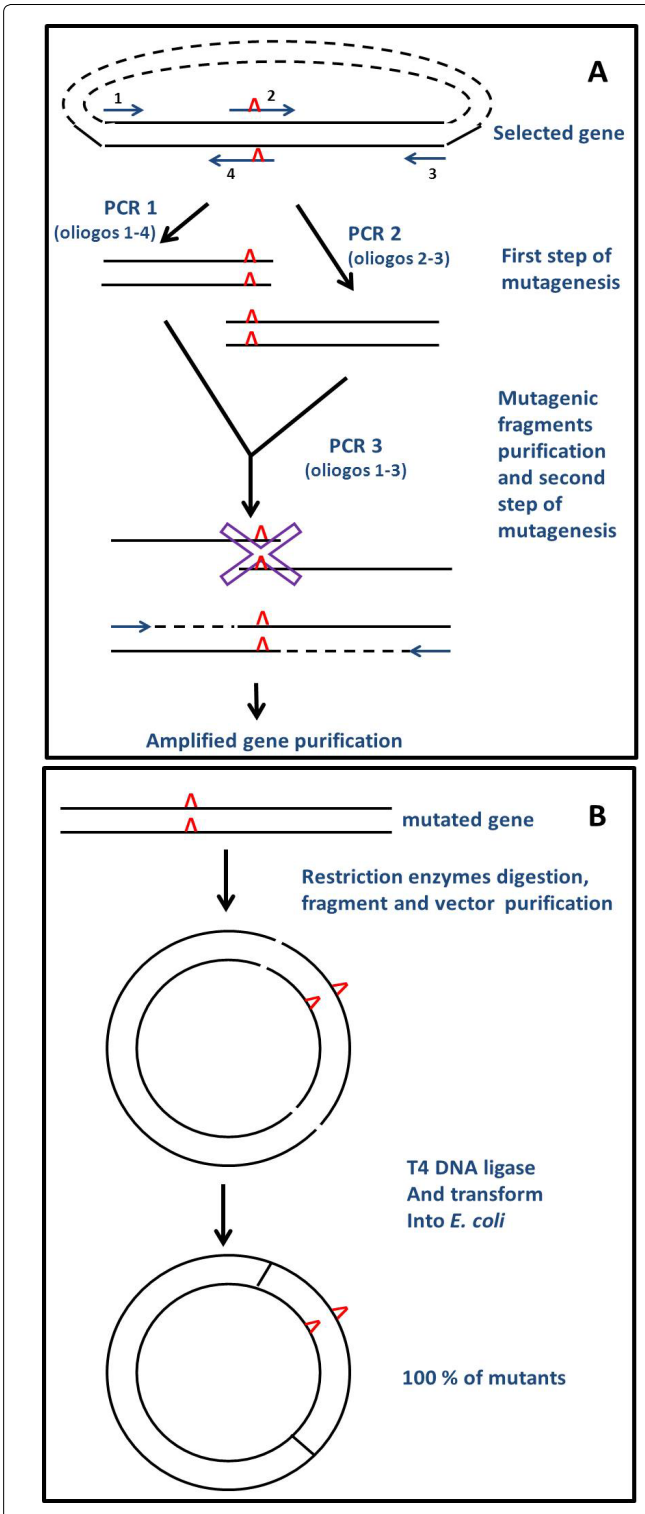

Figure 2: Schematic diagram of mutagenesis by overlap extension. The ds DNA and synthetic oligonucleotides are represented by lines with arrows indicating the 5'- to -3' orientation. The site of mutagenesis is indicated by red triangle. A) In the first step of mutagenesis are produced two fragment of the gene, both containing the mutation. In the second step of mutagenesis the denatured fragments anneal at the overlap and are extended 3' by DNA polymerase to form the mutant fusion product. By adding additional primers 1 and 4 the mutant fusion product is further amplified by PCR. B) schematic diagram of the cloning of the mutated gene in the desired vector by using restriction enzymes and DNA-ligase.

Main characteristics	Methods		
	Kunkel	PCR	Quick change#
% of mutations	80%	100%	90%
time of realization	8 days	4 days	2 days
point mutations	Y	Y	Y
saturation mutagenesis	Y	Y	Y
short deletion/insertion	Y	Y	Y
random mutagenesis	N	N	N
costs*	200-250 €	80-100 €	60-80 €

this methods is developed and commercialized by Agilent Technologies.

***** the costs include only disposable, enzymes and kits.

Table 1: Main characteristics and differences among the three methods of mutagenesis analyzed. The line related to the costs it has been obtained considering the current average costs of materials.

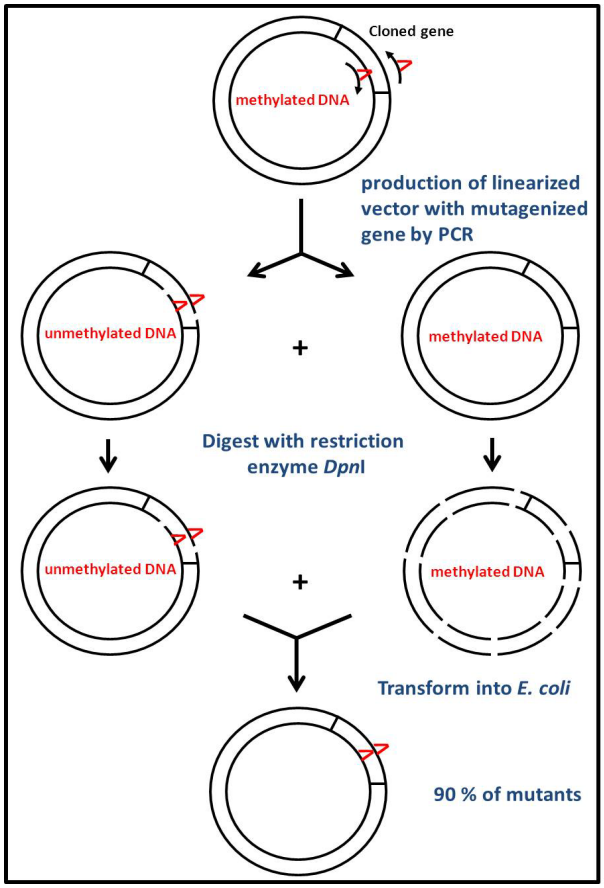

Figure 3: Schematic diagram of mutagenesis by quick change. In the first step of mutagenesis by an overlap extension using mutagenic complementary primers, both containing the desired mutation, a mutated plasmid containing staggered nicks are generated. In the second step by digestion with the restriction enzyme DpnI the methylated parental DNA template is removed. In the third step the nicked mutated vector is transformed into an *E. coli* recA- strain.

interest [7]. In this case using a high fidelity DNA-polymerase and a pair of oligonucleotides (mutagenic and complementary), in one step of amplification is obtained a linear double strand mutagenic DNA in mixture with the template DNA (Figure 3) [8,9]. The main difference between these two DNA is that the template DNA is methylated while the linearized (amplified and mutagenic) DNA is not methylated. By exploiting this difference, a simply digestion of the polymerase chain reaction mixture, with the restriction enzyme DpnI, is achieved the complete digestion of the parental DNA and transforming directly into an *E. coli* recA- strain will be obtained only colonies containing the mutant clones (Figure 3). The quick change method is very fast, in fact only 2 days are requested, excluding the final sequencing of the clones. The rate of mutation can reach 90% of mutated clones (Table 1).

Conclusion

In this brief editorial I wanted focus the attention on three methods of mutagenesis that have occurred and which have been widely used over the last twenty years. Personally, I got to use them and I could verify the real evolution among these methods [10-12]. The rate of mutants it's very high for all the three methods, reaching percentages exceeding 80%. In terms of time of realization, passing from the eight day of the method of Kunkel to two days of the quick change, and also from the economic point of view because the estimated costs are reduced by four times (Table 1). In research time and cost are critical factors, in this case both have been reduced, and this is the sense of the "evolution". Despite the evolution, these methods of mutagenesis have some limitations, for example, they cannot be used for random mutagenesis or for insertions and deletions of long DNA segments. In these cases it can be used classical methods by PCR, however, if are used external oligonucleotides complementary with the receiving vector, the amplified/mutated gene can be used as mega-primer in the Restriction Free (RF) cloning method [13]. The RF is a powerful method of DNA cloning and manipulation, developed for the introduction of foreign DNA into a plasmid at any position. The RF cloning method is based on PCR amplification of a DNA (gene or fragment), which serves as a mega-primer for the linear amplification of the vector and insert, followed by DpnI digestion and transform into an *E. coli* recA- strain [13]. Therefore the use of modern methods of mutagenesis with the RF system allows any type of DNA manipulation in a simple, fast and economical way.

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