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Multi-fragment site-directed mutagenic overlap extension polymerase chain reaction as a competitive alternative to the enzymatic assembly method ☆

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ABSTRACT

Methods for introducing multiple site-directed mutations are important experimental tools in molecular biology. Research areas that use these methods include the investigation of various protein modifications in cellular processes, modifying proteins for efficient recombinant expression, and the stabilization of mRNAs to allow for increased protein expression. Introducing multiple site-directed mutations is also an important tool in the field of synthetic biology. There are two main methods used in the assembling of fragments generated by mutagenic primers: enzymatic assembly and overlap extension polymerase chain reaction (OE–PCR). In this article, we present an improved OE–PCR method that can be used for the generation of large DNA fragments (up to 7.4 kb) where at least 13 changes can be introduced using a genomic template. The improved method is faster (due to fewer reaction steps) and more accurate (due to fewer PCR cycles), meaning that it can effectively compete with the enzymatic assembly method. Data presented here show that the site-directed mutations can be introduced anywhere between 50 and 1800 bp from each other. The method is highly reliable and predicted to be applicable to most DNA engineering when the introduction of multiple changes in a DNA sequence is required.

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Methods for introducing multiple site-directed mutations in a gene of interest are important fundamental tools in many experiments. These methods can potentially have multiple uses, including (but not limited to) mRNA stability experiments for efficient protein production [1], improvement of protein thermo stability [2], and removal or addition of phosphorylation [3] or SUMOylation [4] sites. The introduction of multiple site-directed mutations can also be an important tool in the field of synthetic biology [5]. Other potentially important applications are codon substitutions for recombinant protein expression of genes with alternative codon use. For example, many mitochondrial-encoded proteins have an alternative codon use [6]. In addition, nearly all of the pathogenic yeasts of the genus *Candida* belong to the CTG clade and use an alternative serine codon [7]. This means that these yeasts have an ambiguous mutated $tRNA_{Serine}$ that translates the leucine codon CTG to serine approximately 97% of the time [7]. As a consequence, all foreign genes to be expressed in these yeasts need to be adapted to allow for functional protein expression [8]. Moreover, recombinant expression of *Candida* proteins in any organism other than yeast within the CTG clade is impossible without first changing all CTG codons before expression.

The ability to introduce two or more site-directed mutations simultaneously in a gene of interest is primarily done by one of two different alternative approaches: overlap extension polymerase chain reaction (OE–PCR)[1] and enzymatic assembly [9–11]. Both methods can be used to assemble fragments with overlapping sequences that have been amplified with mutagenic oligos. For example, Lee and coworkers used OE–PCR in 2002 to perform site-directed mutagenesis of 17 CTG codons in the 541-bp LIP2 gene from *Candida rugosa* using a plasmid template [12]. The mutations were introduced in two intermediary steps. First, 10 CTG codons were mutated by amplifying the LIP2 gene into 7 fragments, with overlapping sequences, from a plasmid template. The assembled gene was cloned into a plasmid that was used as a template when mutating the remaining 7 CTG codons using the same method. Site-directed mutagenesis and OE–PCR using genomic template were shown to work for genes of up to 1100 bp by Luo and coworkers in 2012 [13].

The enzymatic assembly method uses an enzyme mix of exonuclease, DNA polymerase, and DNA ligase to assemble multiple gene

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[1] *Abbreviations used:* OE–PCR, overlap extension polymerase chain reaction; T_m, melting temperature; SNP, single-nucleotide polymorphism.

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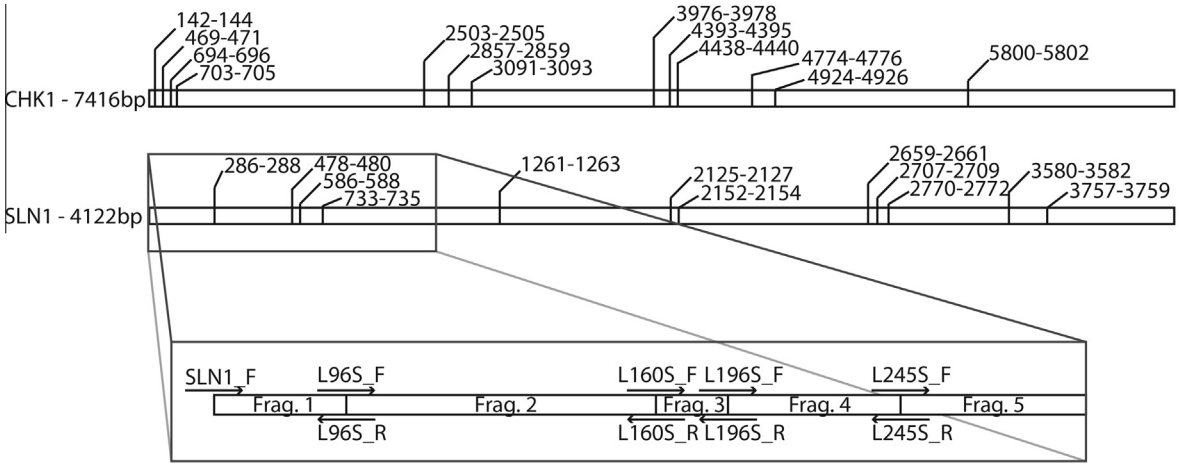

Fig.1. Graphic representation of the 25 CTG codons within the two genes, *CHK1* and *SLN1*, of *C. albicans* with their nucleotide positions in each gene. As an example, the mutagenesis of the first 4 CTG codons of the *SLN1* gene is shown with higher magnification, displaying which oligos were used and the fragments obtained.

fragments using overlapping sequences. With this method, multiple mutations can be introduced when mutagenic oligos are used for the amplification of the fragments [14]. An exonuclease chews up the 3′ end of the fragments that are to be assembled, resulting in single-stranded DNA (ssDNA) overhangs that are then annealed together using the overlapping complementary sequences. Subsequently, the DNA polymerase fills in the gap, and the DNA ligase repairs the nick in the DNA backbone [14]. The enzymatic assembly method is expedient but is more expensive than OE–PCR (Gibson Assembly Mastermix, New England Biolabs). The introduction of mutations using the enzymatic assembly is a two-step reaction, as compared with OE–PCR that includes multiple steps.

In this article, we present an improvement of the multi-fragment site-directed mutagenic OE–PCR method by reducing the number of reaction steps. This is done through omitting the use of nested primers to amplify each intermediate assembled DNA fragment. Moreover, we show that this improved OE–PCR method can efficiently join 13 fragments, whereas enzymatic assembly works best for a smaller number of fragments (Gibson Assembly Mastermix). Thus, this method is highly suitable for fast and efficient mutagenesis and can effectively compete with the enzymatic assembly method. We have used this improved OE–PCR technique for the introduction of 13 site-directed mutations in a 7.4-kb DNA fragment from genomic template, with mutation sites anywhere between 50 and 1800 bp from each other. In addition, 12 mutations in a 4.1-kb gene have also been introduced from genomic template. The result is two histidine kinase genes, *CHK1* and *SLN1*, from *Candida albicans* with universal codons to be used for recombinant protein expression and further studies.

Materials and methods

Oligo design

The positions of the 25 CTG codons in the *SLN1* and *CHK1* genes were first identified (Fig. 1). Subsequently, two mutagenic oligos were designed for each CTG codon: one oligo for the coding strand and one for the complementary strand. The serine codon, TCT, was chosen as the most suitable CTG codon replacement because it is the most conserved serine codon for both highly and lowly expressed genes in most used recombinant expression systems [15]. Each mutagenic oligo was designed following basic oligo design rules with an appropriate melting temperature (T_m) value of below 65 °C and, when possible, a C or G but no more than two C or G nucleotides at the 3′ end of the oligo. The mutation site of each oligo was positioned in the middle, taking care to have as close to an equal number of flanking nucleotides as possible on either side (Table 1). All oligos were designed in pairs, with their reverse complementary sequence for later use during fragment amplification (Table 1). In one region of the *CHK1* gene that contained two CTG codons only 6 nt from each other, oligo design was compromised in order to not exceed the desired T_m value and, thus, a mutagenic oligo with a mutation site close to the 5′ end of the oligo was designed (Table 1).

Fragment amplification via PCR

All fragment amplification PCRs were performed with Phusion high-fidelity DNA polymerase (Fermentas) and with a modified reagent and cycle protocol (Table 2). Genomic DNA from the sequenced *C. albicans* strain SC5314 (http://www.candidagenome.org), obtained from W.A. Fonzi (Georgetown University, laboratory stock), was used as template, and the annealing temperature used for each PCR was calculated using the manufacturer's own oligo Phusion T_m value calculator (http://www.thermoscientificbio.com/webtools/tmc). Using the mutagenic oligos, both of the genes were divided into 13 fragments, with each fragment consisting of the sequence between each mutation site (Fig. 1), and amplified using the modified PCR reagent and cycle protocol described in Table 2. The result was amplified fragments with a mutation site roughly 15 to 20 nt from its 3′ and 5′ ends. Thus, all fragments for both genes were amplified with PCR at the same time as the desired mutations were introduced into the sequence. Because of the proximity of two CTG codons in the sequences of both genes of interest, we designed a 120-bp oligo pair with coding and complementary sequence for *CHK1* and a 76-bp oligo pair with coding and complementary sequence for *SLN1*, both containing two mutation sites (Table 1). Both large oligos acted as individual fragments after the coding and complementary strands had been annealed together. The *CHK1* 120-bp oligo became fragment 9 during the later *CHK1* fragment assembly, and the *SLN1* 76-bp oligo became fragment 7 during the *SLN1* fragment assembly. The annealing of the coding and complementary strands for both of the two large oligo pairs was done by mixing 5 µl of each strand (1:1 molar ratio), followed by denaturing and slowly cooling the reaction down to 4 °C over 30 min. All PCR-amplified fragments were separated on a 1% agarose TAE buffer gel and extracted.

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