Multiple site-directed mutagenesis via simple cloning by prolonged overlap extension

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ABSTRACT

We describe the application of simple cloning by prolonged overlap extension for multiple site-directed mutagenesis in the same plasmid. We show that it is possible to use this technique with very short PCR templates. The technique is ideally suited for the generation of longer donor DNA sequences for CRISPR/Cas9-mediated homologous repair.

METHOD SUMMARY

Simple cloning by prolonged overlap extension utilizes overlapping PCR fragments to create large repetitive multimers, which can be used to directly transform bacteria. Internal circularization of these long concatemers generates the desired plasmid.

KEYWORDS:

donor template generation • gene editing • multiple site-directed mutagenesis • prolonged overlap extension • seamless cloning • seamless plasmid assembly

Classic subcloning in plasmids uses restriction enzymes and ligases. However, a method known as ‘simple cloning’ using direct transformation of a prolonged overlap extension (POE) PCR product has been used to generate seamless subcloning [1]. Overlapping PCR products are joined into long repetitive multimers by POE-PCR and directly transformed into bacteria, which circularize them into plasmids. This technique has been used for applications as different as large-size random gene mutagenesis [2], assembly of up to four fragments in a single reaction [3] and multi-site saturation mutagenesis [4].

Here we show the introduction of three specific mutations into an existing plasmid, utilizing mismatch primers that were assembled in a single reaction (Figure 1), without the need for DpnI digestion and several rounds of transformation and plasmid isolation, which is required in most commercially available directed mutagenesis kits. We optimized the method for the use of fragments as small as 70 bp; the shortest fragments reported previously have been 200 bp [1].

In order to create three mutations in the *RelA* insert of pBSII-SK-*RelA*, a combination of primer mutagenesis and simple cloning by POE was used. Insert 1 (I1), insert 2 (I2) and the vector backbone (VB) were generated using the plasmid as template. Each of the three primer sets introduced a one- or two-base-pair mutation by mismatch. A POE-PCR was performed with the three fragments, creating a large multimeric POE-PCR product which was then transformed directly into competent DH5α *Escherichia coli* with a transformation efficiency (TE) of 1.4×10^5 colony forming units (cfu)/μg POE-PCR product, compared with a TE of 9.18×10^7 cfu/μg using supercoiled pUC18. All colonies tested by PCR (25/25) showed the expected fragment size for a plasmid containing I1 and I2, while the presence of the functional VB was tested by ampicillin resistance. Sanger sequencing confirmed the predicted sequence, including all mutations, in 3/3 plasmids. The three purified fragments (I1, I2 and VB), the POE-PCR product, Cfr10I-digested POE-PCR product, purified plasmid and the same plasmid digested with Cfr10I were analyzed on a 1% (w/v) agarose gel (Figure 2). The smallest insert size previously reported to be joined by POE was 200 bp [1]. Here we show the joining of an insert of only 70 bp with two other fragments.

We also used POE-PCR to assemble a four-fragment plasmid, in which the shortest fragment was 149 bp. Direct transformation gave a TE of 6×10^4 cfu/μg POE-PCR product with 22/66 colonies (33%) tested by PCR showing the expected fragment sizes for a plasmid containing the three inserts. Sanger sequencing confirmed the presence of all four fragments in 3/3 plasmids, although two of these plasmids carried a duplication of the VB.

Different approaches for mutagenesis have been described. Most commercial site-directed mutagenesis kits introduce mutations by amplifying entire plasmids using mismatch primers. The mutagenized PCR product can be isolated because it escapes the methylation-sensitive DpnI restriction digest that removes the original plasmid [5]. To introduce three mutations, this method requires three rounds of mutagenesis followed by transformation and plasmid isolation, making this approach time-consuming and expensive.

In contrast, simple cloning by POE only needs relatively short (in our case 26–33 bases) oligonucleotides as PCR primers and a high-fidelity polymerase, making it a simple and inexpensive alternative.

Benchmarks

Figure 1. Multiple site-directed mutagenesis using simple cloning by prolonged overlap extension and mismatch primers. (A) The template, in this case a plasmid carrying a gene of interest, but possibly also individual DNA fragments, is used for PCR amplification with mismatch primers creating overlapping regions between the amplicons. Yellow, green and blue sections represent the three amplicons; the mismatches introducing mutations are red. (B) The three PCR products are purified; POE-PCR extends the products, creating long repetitive multimers. (C) The multimeric POE-PCR product is directly transformed into a bacterial host, where it is circularized. (D) The mutagenized plasmid is then purified from the bacterial host. Black arrows indicate the position of the sequencing primers used; the stippled lines mark the borders of the gene of interest. The two Cfr10I restriction sites were used for diagnostic digestion.

For primer nomenclature and sequences, see Table 1.

Both fragment generation and POE-PCR rely on 25–30 replication cycles. Before transformation, each DNA fragment will be amplified by 50–60 PCR cycles, which bears the risk of PCR-generated mutations. For a single site-directed mutagenesis a classical approach would require fewer PCR cycles. However, multiple mutagenesis steps will require a comparable number of (or more) PCR cycles. A high-fidelity polymerase should be used for POE-PCR to avoid undesired mutations. After unsuccessful attempts using the *Pfu* Polymerase (Thermo Scientific, MA, USA) for POE-PCR, we recommend using the Phusion polymerase, as supported by other literature describing the method [1,4,6,7]. We found very few PCR-generated mutations; however, in some cases, duplication of the VB was observed, suggesting that recombination in the transformed bacteria might result in unexpected events.

Protocols describing simple cloning by POE differ in their quantitative calculations for the POE-PCR. Some use 2 ng/μl of insert DNA fragment and an equimolar amount of VB [1,4], while others use 4–5 ng/μl VB DNA fragment and an equimolar amount of insert [6,7]. In the first approach, the length of the insert determines the total amount of PCR template and possibly the POE product which, in the second approach, depends on the length of the vector. It would appear more logical to mix the fragments in equimolar amounts by a defined molarity independent of VB and insert size. Using this approach, the POE-PCR reaction and the transformation could be optimized for the molarity of those fragments yielding the highest TE (as long as the total DNA concentration stayed below the product inhibition threshold of the given polymerase).

Figure 2. Agarose gel of PCR products and plasmid. M (100 bp): GeneRuler 100-bp DNA ladder. M (1 kb): GeneRuler 1-kb DNA ladder. Lanes 1–3: PCR products using pBSII-SK-RelA as template. 1: Insert 1, expected size 70 bp. The weak band at about 3.7 kb results from PCR amplification of the template plasmid. 2: Insert 2, expected size 433 bp. 3: The plasmid backbone, expected size 3129 bp. Lane 4: Prolonged overlap extension (POE)-PCR product. Note that the majority of the product is trapped in the slot due to its large size. Smear and lower bands most likely represent concatemers of different sizes. Lane 5: POE-PCR product digested with Cfr10I, a double cutter, which is expected to result in fragments of 2576 bp and 964 bp. Lanes 6 and 7: Analysis of the POE-generated plasmid. 6: Undigested. 7: Digested with Cfr10I.

Table 1. Sequence of the primers used for PCR and sequencing.

Primer name	Sequence (5'–3')	T _m (°C)	GC%
VR (Vector backbone reverse)	CCTCGGGGCTTCCCAACGGTCTCTCAGGAGATG	69.1	63.6
VF (Vector backbone forward)	GTGCCAAGGCCAGGGCTTGGTTTGAGATCTGCC	69.4	60.6
I2R (Insert 2 reverse)	GGCAGATCTCAAACCAAGCCCTGGCCTTGCCAC	69.4	60.6
I2F (Insert 2 forward)	GTGGGAGCTGGGCTAGGGGGCCTCTG	70.0	73.1
I1R (Insert 1 reverse)	CAGAGGCCCCCTAGCCAGCTCCAC	70.0	73.1
I1F (Insert 1 forward)	CATCTCCTGAGAGACCGTTGGGAAGCCCCGAGG	69.1	63.6
M13 forward	GTAACGACGGCCAG	50.7	56.2
Sequencing reverse	CACCTGTTCCAAAGAGCACC	56.3	55
Colony forward	AGCGCGCGTAATACGACTCACTATAGTTCGCTGTACAGCACCTTAG	69.0	53.3
Colony reverse	CTGCAGGAATTCGATATCAAGCTTACCTACACCTTCCAGCATC	66.2	47.7

Introduced mutations are bold/underlined. In the colony PCR primers, the insert-specific sequence is underlined. These primers are unnecessarily long but were available from the original cloning of the gene of interest into the pBSII-SK vector.
GC%: GC content; T_m: Theoretical primer melting temperature.

POE-PCR has previously been described to join fragments as small as 200 bp [1]. In our experiment, one of the three fragments was only 70 bp long, and the POE-PCR was first successful when high concentrations of fragments were added. A concentration of 8 ng/μl VB was needed, while the concentration originally recommended was 4–5 ng/μl [6,7]. This shows that it is possible to join small fragments, but the actual reaction conditions might need to be adjusted if the fragment sizes vary considerably.

A possible application of this method is the generation of long donor DNA sequences for CRISPR/Cas9 mutagenesis. If the backbone plasmid contains an RNA polymerase promoter (e.g., T7) a single-strand donor for EasiCRISPR can easily be generated [8]. This is done by synthesizing RNA from the T7 promoter, followed by reverse transcription of the RNA.

Primers were designed with a 26- to 33-nucleotide overlap as described previously [1,4]. Each primer also contained a mutation that was flanked by the overlap. As much as possible, primers were designed with a GC content between 40 and 60% and a T_m of 65 ± 5°C (Table 1).

All fragments were amplified using Phusion high-fidelity DNA polymerase (Thermo Scientific #F530S) following the manufacturer's protocol. pBSII-SK-RelA, a modified version of the pBSII-SK (Stratagene) carrying most of exon 11 of the murine RelA, was used as template. I1 (70 bp) and I2 (433 bp) were amplified in a total reaction volume of 50 μl using primer pairs I1F/I1R and I2F/I2R, respectively, with the following program: 1 × 98°C for 30 s; 25 cycles of 10 s at 98°C, 30 s at 64°C, 30 s at 72°C; and a final elongation of 10 min at 72°C. The VB (3 kb) was amplified in a total reaction volume of 100 μl with primers VF and VR (forward and reverse) using the following program: 1 × 98°C for 30 s; 25 cycles of 10 s at 98°C, 1 min 30 s at 64°C, 30 s at 72°C; and a final elongation of 10 min at 72°C. I1 and I2

were purified using a GeneJet PCR Purification Kit (Thermo Scientific #K0702), and the VB was purified from 1% (w/v) agarose gel using the GeneJET Gel Extraction Kit (Thermo Scientific #K0691); yields were 0.4, 1.5 and 1.4 μg , respectively.

POE-PCR was performed in 25 μl 1 $\times$ Green HF buffer containing 4 nM (8 ng/ μl) purified plasmid backbone, equimolar amounts of I1 and I2, 0.2 mM dNTP and 1.25 U Phusion, with the following program: 1 $\times$ 98°C for 30 s; 30 cycles of 10 s at 98°C, 15 s at 60°C, 2 min 42 s at 72°C; and a final elongation of 10 min at 72°C.

The 5 μl of the POE-PCR product, 20 μl 5 $\times$ KCM buffer and 75 μl H₂O were precooled on ice for 2 min. The 100 μl were mixed with 10⁸–10⁹ competent DH5 α *E. coli* bacteria and put on ice for 20 min. Heat shock was carried out for 10 min at room temperature. Bacteria recovered in LB medium without antibiotics for 50 min with shaking (200 rpm) at 37°C. Transformed bacteria were selected overnight on LB-agar plates supplemented with 100 $\mu\text{g}/\text{ml}$ ampicillin. Single colonies were grown in 5 ml LB medium supplemented with 100 $\mu\text{g}/\text{ml}$ ampicillin overnight.

Colony PCR was performed using DreamTaq polymerase (ThermoScientific, #EP0701) in a final volume of 25 μl . Single colonies were transferred to 50 μl of LB medium and 1 μl of this was used as template for the PCR, with the Colony Forward and Colony Reverse primers and the following conditions: 1 $\times$ 95°C for 5 min; 20 cycles of 45 s at 94°C, 1 min at 61°C and 1 min 21 s. at 72°C. Selected positive colonies were Sanger sequenced (Eurofins Genomics) using the M13 Forward and the Sequencing Reverse primers (Table 1). Plasmids and the POE-PCR product were digested with Cfr10I (ThermoScientific, #FD0184) to verify the correct unit size. All DNA fragments were analyzed on a 1% (w/v) agarose gel (Figure 2).

We show that several mutations can be introduced in a plasmid simultaneously using simple cloning by POE, a fast and economic method to assemble different fragments seamlessly into one plasmid. Furthermore, we expanded the range of POE-PCR to an insert size of only 70 bp, considerably smaller than the 200 bp reported previously.

There is increasing demand for complex changes in donor DNA templates, used in CRISPR/Cas9-mediated mutagenesis. We believe that multiple site-directed mutagenesis and simple cloning by POE-PCR will be a favorable tool for this application.

Author contributions

R Hejlesen designed the study, performed the experiments and wrote the manuscript. E-M Füchtbauer designed the study and wrote the manuscript.

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