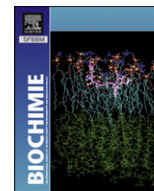
Biochimie 92 (2010) 1081–1084

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi



Short communication

# A convenient and robust method for construction of combinatorial and random mutant libraries

Yingfeng An, Wenfang Wu*, Anguo Lv

*Institute of Applied Ecology, Chinese Academy of Sciences, No.72 Wenhua Road. Shenyang 110016, China*

A R T I C L E I N F O

*Article history:*
Received 9 February 2010
Accepted 23 March 2010
Available online 27 March 2010

*Keywords:*
Mutant library
DNA shuffling
Error-prone PCR
Ligase-independent cloning

A B S T R A C T

Here we describe a convenient and robust ligase-independent method for construction of combinatorial and random mutant libraries. The homologous genes flanked by plasmid-derived DNA sequences are fragmented, and the random fragments are reassembled in a self-priming polymerase reaction to obtain chimeric genes. The product is then mixed with linearized vector and two pairs of flanking primers, followed by assembly of the chimeric genes and linearized vector by PCR to introduce recombinant plasmids of a combinatorial library. Commonly, it is difficult to find proper restriction sites during the construction of recombinant plasmids after DNA shuffling with multiple homologous genes. However, this disadvantage can be overcome by using the ligase-independent method because the steps of DNA digestion and ligation can be avoided during library construction. Similarly, DNA sequences with random mutations introduced by error-prone PCR can be used to construct recombinant plasmids of a random mutant library with this method. Additionally, this method can meet the needs of large and comprehensive DNA library construction.

© 2010 Elsevier Masson SAS. All rights reserved.

## 1. Introduction

DNA shuffling is a widely used method to rapidly generate gene diversity by homologous recombination [1–3]. The shuffling of homologous sequences for exploring protein sequence space is very useful for directed molecular evolution [3]. Commonly, combinatorial libraries can be generated by random fragmentation of homologous genes, followed by reassembly of the fragment mixture in a primerless polymerase reaction. Template switching causes crossovers in regions of sequence homology. DNA shuffling can also be used for the improvement of a single gene by recombination of DNA random mutations during DNA shuffling [2]. Until now, some modified methods have been developed to improve the efficiency of DNA shuffling. For example, DNAs are fragmented with restriction enzymes [4] or endonuclease V [5] instead of DNaseI, or single-stranded DNA sequences instead of double-stranded DNA sequences are used as templates [6]. In addition, the "semi-synthetic" DNA shuffling has been reported [7]. Although these methods can introduce high-quality DNA libraries, they are inconvenient in some cases. For example, after DNA shuffling, digestion and ligation steps are needed for combinatorial library construction. However, in some cases it is difficult to find proper restriction sites for cloning purpose, especially when multiple sequences are used for DNA shuffling. Furthermore, digestion and ligation should also increase the workload during DNA library construction, and may affect the complexity of the library in some cases. To overcome these disadvantages, here we present a PCR-based, ligase-independent method for construction of combinatorial or random mutant libraries by convenient reassembly of shuffled or randomly mutated sequences with vector.

## 2. Materials and methods

The plasmid pUC19 was linearized by digestion with *Bam*HI and *Eco*RI. The pUC19-sam1 and pUC19-sam2 plasmids containing sam1 and sam2 genes, respectively, were used as templates for PCR amplification using primers pUC19-For (5′-GTTTTCCCAGTCACGAC GTTGTAAAACGACGGCC-3′) and pUC19-Rev (5′-CGCCAAGCTTGCA TGCCTGCAGGTCGACTCTAGAG-3′), both of which anneal to the pUC19 vector. After purification, the amplified aim genes were mixed (1:1) and fragmented by DNaseI digestion. Fragments of 10–50 bp were purified from 0.8% agarose gel after electrophoresis. Primerless PCR was performed in mixture containing 5 U *Taq* DNA Polymerase, 1× *Taq* buffer, 10 mg/L of the fragment mixture, 0.2 mmol/L dNTP with a total volume of 50 μl. The reactions were carried out under the conditions of 94 °C for 2 min, 25 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min + 5 s/cycle, and a final incubation at 72 °C for 10 min. Then 1 μl of the primerless

* Corresponding author. Tel.: +86 24 83970383.
*E-mail address:* wshr100@sina.com.cn (W. Wu).

0300-9084/$ – see front matter © 2010 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2010.03.016

PCR product was mixed with 5 mg/L linearized vector pUC19, 0.06 μmol/L primers (pUC19-For and pUC19-Rev), 0.2 mmol/L each dNTP, 1× *Pfu* PCR buffer and 0.1 U/μl *Pfu* DNA polymerase (50 μl final volume) to carry out a second PCR amplification. The PCR was performed with a program of 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 8 min. Then the newly assembled plasmid obtained by PCR was transformed into *Escherichia coli* JM109. Transformed bacteria were grown and selected by standard methods. Ten plasmids were extracted from randomly selected transformants and performed DNA sequencing. Plasmid mixture was extracted from pooled transformants and digested with *Eco*RI and *Bam*HI followed by agarose gel electrophoresis analysis. As a control, the primerless PCR product was subjected to agarose gel electrophoresis, and the fragments with size ranges of about 100–200 bp, 400–500 bp and 700–800 bp were extracted from DNA smear on agarose gel (Fig. 2-a), and used as templates to assemble recombinant plasmids by PCR as discribed above. The PCR products were transformed into *E. coli* and transformants were grown and selected by standard methods. A plasmid mixture was extracted from pooled transformants after each transformation and digested with *Eco*RI and *Bam*HI. The products were analyzed by agarose gel electrophoresis.

The sam1 and sam2 random mutant libraries were generated by error-prone PCRs using primers pUC19-For and pUC19-Rev. Each PCR was performed in mixture containing 2 ng of template DNA (pUC19-sam1 or pUC19-sam2), 0.6 μmol/L both primers, 1 mmol/L d(C/T)TP, 0.2 mmol/L d(A/G)TP, 20 nmol/L $MgCl_2$, 1× *Taq* polymerase buffer and 5 U *Taq* polymerase with a total volume of 50 μl. The reaction mixtures were heated at 94 °C for 2 min, followed by 30 cycles of incubation at 94 °C for 1 min, 49 °C for 40 s, and 72 °C for 3 min, and a final incubation at 72 °C for 10 min. After purification, each PCR product was used as template to assemble recombinant plasmid by PCR as described above. Then the PCR products were transformed into *E. coli* and transformants were grown and selected by standard methods. After each transformation, twenty plasmids were extracted from randomly selected transformants and performed DNA sequencing. In addition, a plasmid mixture was extracted from pooled transformants after each transformation and digested with *Eco*RI and *Bam*HI. The products were analyzed by agarose gel electrophoresis.

## 3. Results and discussion

The ligase-independent method for construction of combinatorial and random mutant libraries is illustrated in Fig. 1. After DNA shuffling (Fig. 1-a) or error-prone PCR (Fig. 1-b), the PCR product with reassembled sequences or randomly mutagenized gene (flanked by plasmid-derived DNA sequences) is mixed with linearized vector and low concentrations of the flanking primers to perform a PCR. This step is used to assemble the aim DNA fragments and the linearized vector to introduce nicked recombinant plasmids. Then the PCR product is transformed into *E. coli* and the nicked recombinant plasmids can be repaired in vivo (Step 4). With this method, we have constructed a combinatorial library and random mutant libraries with the genes sam1 and sam2.

Commonly, integrity of reassembled fragments is necessary when traditional DNA shuffling method is performed to construct combinatorial library, because these fragments need to be digested for cloning purpose. However, DNA shuffling is a skill-intensive protocol [8], and the integrity of the reassembled fragments is dependent on many important factors (e.g., the size range, concentration and purity of DNA fragments used for reassembly), and even a subtle change can affect the final result. Therefore, in some cases, enough full-length reassembled fragments can be obtained only after optimization of the PCR conditions. For example, in this work, only a DNA smear was obtained after agarose gel electrophoresis of the primerless PCR product, indicating that most of the reassembled fragments obtained were non-full-length sequences (Fig. 2-a). Fortunately, the ligase-independent method presented here is independent on the integrity of the reassembled fragments from primerless PCR, because digestion of the fragments for cloning purpose is not required, and the primerless PCR product is only used as template for the following amplification to directly assemble the aim DNA fragments and the linearized vector. This characteristic was verified by the fact that efficient amplifications of recombinant plasmids were all realized by using gel-selected reassembled fragments with different size ranges as templates. After transformation of *E. coli* with the PCR products, the plasmid mixtures were extracted from pooled transformants, digested with *Eco*RI and *Bam*HI and analyzed by agarose gel electrophoresis. As a result, the clear main DNA bands on agarose gel corresponding to the sizes of full-length DNA inserts and vector were obtained (Fig. 2-b), indicating that even the non-full-length reassembled fragments can also be used as templates for construction of the recombinant plasmids. Therefore, combinatorial library construction is independent on integrity of the reassembled fragments after primerless PCR, which should increase repeatability during the library construction. In addition, similar results were obtained from digestion of mixed recombinant plasmids after the random mutant

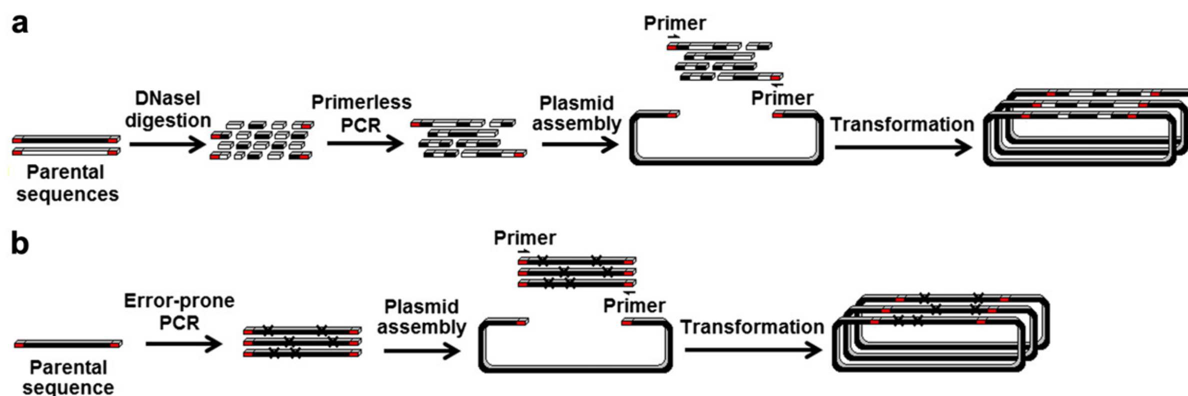



**Fig. 1.** Library construction with the ligase-independent method. (a) Combinatorial library construction. Parental genes are digested with DnaseI, and fragments of 10–50 bp are purified from agarose gel. Then the purified fragments are reassembled into full-length or non-full-length sequences in the absence of primers. The product is mixed with linearized vector and low concentrations of flanking primers to assemble recombinant plasmid with additional cycles of PCR. The PCR product is transformed into *E. coli*. (b) Random mutant library construction. Random mutations are introduced to aim gene by error-prone PCR. Then randomly mutated sequences are mixed with linearized vector and low concentrations of flanking primers to assemble recombinant plasmid with additional cycles of PCR. The PCR product is transformed into *E. coli*. Random mutations are indicated with "×".

Download English Version:

https://daneshyari.com/en/article/1952722

Download Persian Version:

https://daneshyari.com/article/1952722

Daneshyari.com