Gene 524 (2013) 347–354

Contents lists available at SciVerse ScienceDirect

Gene

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

A simple, universal, efficient PCR-based gene synthesis method: Sequential OE-PCR gene synthesis☆

Pingping Zhang [a,b], Yingying Ding [a], Wenting Liao [a], Qiuli Chen [a], Huaqun Zhang [a], Peipei Qi [a], Ting He [a], Jinhong Wang [a], Songhua Deng [b], Tianyue Pan [c], Hao Ren [a,**], Wei Pan [a,*]

[a] Department of Medical Microbiology, Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, China
[b] Department of Physiology, Anhui Medical University, Hefei 230032, China
[c] Shanghai Medical College, Fudan University, Shanghai 200032, China

ARTICLE INFO

Article history:
Accepted 27 March 2013
Available online 15 April 2013

Keywords:
Gene synthesis
Sequential OE-PCR
Oligonucleotides
Error correction

ABSTRACT

Herein we present a simple, universal, efficient gene synthesis method based on sequential overlap extension polymerase chain reactions (OE-PCRs). This method involves four key steps: (i) the design of paired complementary 54-mer oligonucleotides with 18 bp overlaps, (ii) the utilisation of sequential OE-PCR to synthesise full-length genes, (iii) the cloning and sequencing of four positive T-clones of the synthesised genes and (iv) the resynthesis of target genes by OE-PCR with correct templates. Mispriming and secondary structure were found to be the principal obstacles preventing successful gene synthesis and were easily identified and solved in this method. Compensating for the disadvantages of being laborious and time-consuming, this method has many attractive advantages, such as the ability to guarantee successful gene synthesis in most cases and good allowance for Taq polymerase, oligonucleotides, PCR conditions and a high error rate. Thus, this method provides an alternative tool for individual gene synthesis without strict needs of the high-specialised experience.

1. Introduction

The synthesis of genes with engineered DNA sequences has become an increasingly common requirement in research involving engineered proteins (Xiong et al., 2004, 2008), artificial gene networks (Ye et al., 2009) and synthetic genomes (Cheong et al., 2010; Ely et al., 1998; Wu et al., 2006). Several methods for the synthesis and assembly of DNA based on oligonucleotides ("oligos"), including a method for the enzymatic ligation of oligos reported in the 1980s (Edge et al., 1983; Smith et al., 1982; Sproat and Gait, 1985), the FokI method published in 1988 (Mandecki and Bolling, 1988), de novo PCR (Cello et al., 2002; Kodumal et al., 2004), and ligase chain reaction (LCR)-based methods (Au et al., 1998; Smith et al., 2003), have been described previously. Among these methods, the best and most widely reported methods are based on PCR and rely on the use of overlapping oligos to construct genes. These methods include TopDown (TD) gene synthesis (Ye et al., 2009), the thermodynamically balanced inside-out (TBIO) method (Gao et al., 2003), successive PCR (Xiong et al., 2004), dual asymmetrical PCR (DA-PCR) (Sandhu et al., 1992), overlap extension PCR (OE-PCR) (Prodromou and Pearl, 1992; Young and Dong, 2004), PCR-based two-step DNA synthesis (Stemmer et al., 1995; Xiong et al., 2004, 2006), one-step gene synthesis (Wu et al., 2006; Ye et al., 2009) and automatic kinetics switch (AKS) one-step gene synthesis (Cheong et al., 2010).

To perform successful gene synthesis based on PCR, numerous important influencing factors, such as the concentration of the outer primers (Cheong et al., 2010; Peng et al., 2003; Wu et al., 2006; Xiong et al., 2004; Ye et al., 2009), the design of the primers (Cheong et al., 2010; Wu et al., 2006; Ye et al., 2009), the concentration of the oligos (Cheong et al., 2010; Wu et al., 2006; Xiong et al., 2004; Ye et al., 2009), the length of the oligos (Binkowski et al., 2005; Jayaraj et al., 2005; Prodromou and Pearl, 1992; Smith et al., 2003; Xiong et al., 2004, 2008), the quality and purity of the oligos (Edge et al., 1983; Smith et al., 1982; Stemmer et al., 1995; Xiong et al., 2004, 2006), the annealing time (Cheong et al., 2010; Ye et al., 2009), the annealing temperature (Cheong et al., 2010; Ye et al., 2009), the concentration and type of Taq polymerase (Cheong et al., 2010; Cline et al., 1996; Wu et al., 2006; Xiong et al., 2004; Ye et al., 2009), the number of PCR cycles (Cheong et al., 2010; Ye et al., 2009), the Mg^{2+} ion concentration, the dNTP concentration (Cheong et al., 2010; Ely et al., 1998; Ye et al., 2009), and the melting temperature (Tm) had to be optimised. In addition, individual optimisation for each gene sequence (Xiong et al., 2006), specialised oligonucleotide design programmes (Au et al., 1998; Cello et al., 2002; Gao et al., 2003; Kodumal et al., 2004; Mandecki and Bolling, 1988; Sandhu et al., 1992; Smith et al., 2003), and special error correction approaches (Carr et al., 2004; Fuhrmann et al., 2005; Heckman and Pease, 2007; Huang et al., 2002; Peng et al., 2006; Smith and Modrich, 1997; Xiong et al., 2004,

Abbreviations: OE-PCR, overlap extension polymerase chain reaction; Tm, melting temperature; oligos, oligonucleotides; nt, nucleotide.

☆ The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint first authors.

* Corresponding author. Tel./fax: +86 21 81870989.
** Corresponding author. Tel./fax: +86 21 81870990.
E-mail addresses: hmren@yahoo.com (H. Ren), pwpanwei@yahoo.com.cn (W. Pan).

0378-1119/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2013.03.126

2006, 2008) were necessary. Therefore, well-trained experimenters and highly specialised software are critical for successful gene synthesis, and many laboratories that need to synthesise designed genes do not have these resources. However, all of these improvements do not always guarantee successful synthesis. The complexity of the target genes, the complexity of the multiple polymerase chain assembly (PCA) reactions and multiple PCR, the complex structures of DNA, the poor quality of oligos, and some unknown factors negatively influence gene synthesis. Herein we present a simple, universal, efficient synthesis method based on sequential overlap extension polymerase chain reactions (OE-PCRs) that can guarantee the successful synthesis of most designed genes without strict needs of the high-specialised experience.

2. Materials and methods

2.1. Chemicals, enzymes, vectors and strains

Taq DNA polymerase, dNTPs, the high-efficiency TA cloning vector, pMD18-T, and the DL2000 DNA marker were purchased from Takara Co. Ltd. (Dalian, People's Republic of China). The DNA purification kit was obtained from Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. (Shanghai, People's Republic of China). Biowest regular agarose G-10 was purchased from Gene Company (Hong Kong, People's Republic of China).

2.2. Gene sequences and oligonucleotide design

The original purpose of the study was to synthesise seven *Escherichia coli* codon-optimised genes for prokaryotic expression. The amino acid sequences of EV71-VP0, EV71-VP1, EV71-VP3 (*Human enterovirus 71 capsid proteins* VP0, VP1 and VP3) and CA16-VP1 (*Human coxsackievirus A16 capsid protein* VP1) were obtained from GenBank (GenBank accession no.: ADC53098.1 and ADC53098.1). The amino acid sequence of IGDL (IgG derived linker peptide) is presented in Supplementary Fig. S1. The sequences of CHIKV (*Chikungunya virus*) *structural polyprotein* C and E2 were obtained from GenBank (AF369024.2). The *E. coli* codon-optimised coding sequences of these proteins were derived from their amino acid sequences using the DNASTAR software package. Each whole DNA sequence was divided into a number of oligonucleotides (oligos) that were 54 nt long with 18 nt overlaps at both the 5′ and 3′ ends, leaving an 18 nt gap between the overlapping regions. The sequences of all oligonucleotide sets are provided in Supplementary Fig. S2.

2.3. Oligonucleotides

The oligonucleotides were purchased from Shanghai Sangon. All oligonucleotides were purified by HPLC. The oligonucleotides were diluted to 10 μM with TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) before use.

2.4. Strategies to synthesise the gene fragments

Two types of methods, a modified two-step method and a sequential overlap extension polymerase chain reaction (sequential OE-PCR) method, were implemented in our gene synthesis. The modified two-step method was derived from the TopDown (TD) gene synthesis method (Ye et al., 2009). Figs. 1 and S3 show the sequential OE-PCR-based synthesis of the EV71-VP0 gene and other genes. The general information for the seven genes is presented in Table 1.

2.5. PCRs and assembly

The sequential OE-PCR-based synthesis of the EV71-VP0 gene involved five rounds of OE-PCR, as shown in Fig. 1. The first round involved the extension of 13 fragments (F1R1, F2R2 …, F13R13) of 90 bp in length with two corresponding oligos as primers. The first-round PCRs were performed in a 25 μl mixture containing 1 U Taq polymerase, 2 mM $MgSO_4$, 1 mM each dNTP, and 100 nM oligos. The PCRs were conducted as follows: a 5 min initial denaturation at 95 °C; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; and a final step of 10 min at 72 °C to ensure complete extension. Seven pairs of overlapping fragments from the 13 PCR products from the first round were utilised to extend the fragments in the second PCR, which generated seven fragments (F1R2, F3R4 …, F12R13) of 162 bp in length. The conditions used were the same as use for the first-round PCR.

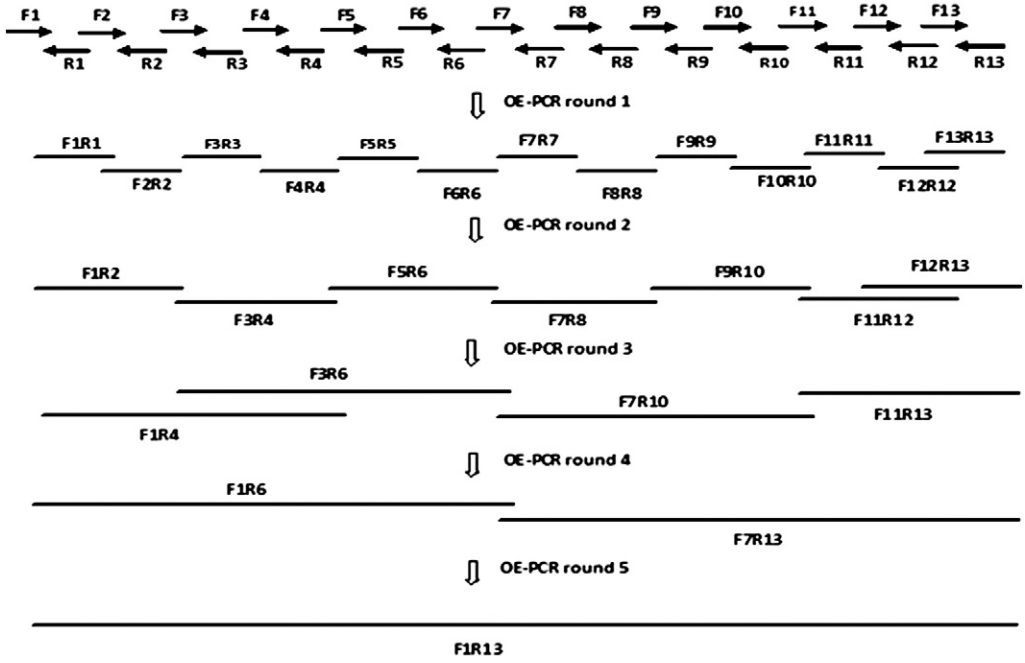

Fig. 1. Schematic diagram of the sequential OE-PCR gene synthesis strategy. The target DNA (EV71-VP0 gene) was divided into 26 oligos of 54 nt each. Each pair of oligos with 18 nt complementary sequences was used to perform the first-round OE-PCR; two adjacent PCR products were then mixed to perform each subsequent step. The full-length EV71-VP0 gene (969 bp) was synthesised by 5 rounds of sequential OE-PCR.