Robust one-Tube $\Omega$-PCR Strategy Accelerates Precise Sequence Modification of Plasmids for Functional Genomics

Letian Chen$^{1,2,3,*}$, Fengpin Wang$^{1,2,3}$, Xiaoyu Wang$^{1,2}$ and Yao-Guang Liu$^1$

$^1$State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, College of Life Sciences, South China Agricultural University, Guangzhou 510642, China

$^2$Guangdong Provincial Key Laboratory of Protein Function and Regulation in Agricultural Organisms, College of Life Sciences, South China Agricultural University, Guangzhou 510642, China

$^3$These authors contributed equally to this work.

*Corresponding author: E-mail, lotichen@scau.edu.cn; Fax, +86-20-85282180.

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Functional genomics requires vector construction for protein expression and functional characterization of target genes; therefore, a simple, flexible and low-cost molecular manipulation strategy will be highly advantageous for genomics approaches. Here, we describe a $\Omega$-PCR strategy that enables multiple types of sequence modification, including precise insertion, deletion and substitution, in any position of a circular plasmid. $\Omega$-PCR is based on an overlap extension site-directed mutagenesis technique, and is named for its characteristic $\Omega$-shaped secondary structure during PCR. $\Omega$-PCR can be performed either in two steps, or in one tube in combination with exonuclease I treatment. These strategies have wide applications for protein engineering, gene function analysis and in vitro gene splicing.

Keywords: Gene cloning • In vitro gene splicing • Molecular manipulation • $\Omega$-PCR.

Abbreviations: Den, Dendra2 fluorescent protein; gDNA, genomic DNA; GFP, green fluorescent protein; $\Omega$-PCR, omega-PCR; TAC, transformation-competent artificial chromosome.

Introduction

In the post-genomic era, examination of protein properties and functions of specific genes in transient and stable systems requires specific, nucleotide-level modification of large numbers of vector constructs carrying target genes. Therefore, a simple, flexible, low-cost and high-fidelity method for sequence modification is highly desirable for these processes. Sequence modification requires three types of manipulation: insertion, substitution and deletion. In conventional strategies, these sequence modifications are achieved in a cut-and-paste manner based on restriction endonucleases and modification enzymes such as ligases, phosphatases, kinases and others. This is tedious, time consuming and therefore expensive. In addition, finding suitable restriction site(s) in a specific vector and/or a target sequence can be problematic. These obstacles can be partially overcome by in vitro site-specific recombinational cloning (Hartley et al. 2000) and by In-Fusion$^\text{TM}$ assembly (Zhu et al. 2007), because of their fast reaction speeds and restriction enzyme-free nature. Although these technologies are commercially available as Invitrogen TOPO Gateway system and Clontech In-Fusion$^\text{R}$ kits, the high cost of the kits restricts their utility for routine vector construction in most molecular biology labs. Alternatively, numerous PCR-based cloning strategies and site-directed mutagenesis methods have been developed for their technical simplicity, low cost and high efficiency (Kunkel 1985, Kammann et al. 1989, Marchuk et al. 1991, Datta 1995, Ke and Madison 1997, Bryksin and Matsumura 2010), but most of these methods preferentially allow insertional sequence modification.

In 1989, Ho et al. reported development of the overlap extension site-directed mutagenesis technique, which enabled all three sequence modifications (Ho et al. 1989) and was adopted in the Stratagene QuikChange$^\text{TM}$ Site-Directed Mutagenesis Kit. This method requires supercoiled double-stranded DNA plasmid as template, two synthetic complementary oligonucleotides containing the desired point mutations as primers and the methylation-specific endonuclease DpnI to remove the parental DNA template. Kammann et al. (1989) first reported the use of double-stranded PCR products as PCR primers. A similar concept, using ‘megaprimers’, was developed and modified in a number of PCR-based mutagenesis protocols to introduce a mutation into a gene of interest (Giebel and Spritz 1990, Sarkar and Sommer 1990, Datta 1995, Ke and Madison 1997, Tyagi et al. 2004). However, these protocols incorporate the mutation, particularly insertions or substitutions, into synthetic primers, which limits the capacity of PCR-based modification of larger sequences. To by-pass the primer length limitation, here we introduce a special design for chimeric primers and incorporate use of PCR products as megaprimers into the site-directed mutagenesis protocol, establishing a set of

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The principle of substitution Ω-PCR is illustrated in Fig. 1A. We designed a pair of chimeric primers for replacement of GFP with the gene encoding the photoactivatable fluorescent protein Dendra2 (Den) (Lippincott-Schwartz and Patterson 2009). The 5’ sequences of the forward chimeric primer (Vec-Den-F) and the reverse chimeric primer (Vec-Den-R) were identical to the flanking sequences of the starting plasmid, while the 3’ parts of these primers were identical to the 5’ end and 3’ end of the Den coding sequence, respectively (Fig. 1A; Supplementary Table S1). In the first PCR, the target Den fragment was amplified from a Den-containing template with the chimeric primers. Two tails identical to the flanking sequence were integrated in the resultant PCR product (Fig. 1A, B, first lane). In the second PCR, the destination vector pBI-GFP:OsRac3 served as the template and the denatured strands of the Den-containing PCR product served as megaprimer annealing to the complementary sequence of the destination vector through the two flanking tails. The Ω-shaped structure in the megaprimer was then extended by high-fidelity DNA polymerase along the vector during thermocycling. Thereby, the GFP fragment was replaced by the target Den fragment in the de novo circular plasmid with two staggered nicks at the end (Fig. 1A, B, second lane). The template plasmids isolated from Escherichia coli are usually methylated and can be digested by the restriction endonuclease DpnI, whereas in vitro synthesized DNA (PCR products) is resistant to this enzyme (Weiner and Costa 1994). After treatment with DpnI to remove the original template plasmids, the PCR product was transferred into E. coli. The pBI-Den:OsRac3 transformants were screened with a forward primer (F1) on the vector and a reverse primer (R1-2) within the target Den (Fig. 1A), and the rate of positive colonies was about 95% (Table 1; Supplementary Fig. S1A).

Deletion Ω-PCR

The principle of deletion Ω-PCR is illustrated in Fig. 2A. The pBI-Den:OsRac3 vector resulting from the substitution Ω-PCR was used as template to test the deletion mode of Ω-PCR. In this case, only complementary chimeric primers were applied. When the two portions of the chimeric primers annealed to their complementary sites on the target construct, the OsRac3 coding region in the template formed a Ω-shaped structure, and the OsRac3-containing loop region of the Ω-shaped structure was removed in the de novo PCR product (Fig. 2A, B). After treatment with DpnI, the PCR product was transformed into E. coli competent cells. The resultant pBI-Den transformants were screened using a pair of primers, F1/R2, flanking the deletion site (Fig. 2A; Supplementary Fig. S1B), and the positive rate was about 100% (Table 1).

Insertion Ω-PCR

The principle of insertion Ω-PCR is illustrated in Fig. 3A. The pBI-Den vector resulting from deletion Ω-PCR was further modified using the insertion mode. Similar to substitution, the target Rer1B being inserted was amplified from a Rer1B-containing template with chimeric primers in the first PCR (Fig. 3B). In the second PCR, the denatured strands of the Rer1B-containing PCR products served as megaprimer annealing to the flanking sequences of the insertion site on the plasmid to form a Ω-shaped structure. Thereby, Rer1B was integrated into the target constructs. After treatment with DpnI, the PCR product was transformed into E. coli competent cells. The pBI-Den:Rer1B transformants were screened by a pair of primers, F3/R2 (Fig. 3A; Supplementary Fig. S1C), and the positive rate was about 93% (Table 1).

One-tube Ω-PCR

To simplify the procedures for substitution and insertion modes, we applied exonuclease I to the Ω-PCR, so that these modes could be executed using a one-tube method. In the two-step protocol, the first PCR was performed with 28–30 cycles, and 2–3 μl of the first PCR product was supplied as megaprimer for the second reaction (20 μl). We found that the amount of the megaprimer used in the second reaction was equivalent to that generated by about 12–15 cycles of the first-round PCR (Fig. 4A). Since exonuclease I digests only single-stranded DNA without affecting double-stranded DNA, after 12–15
cycles of amplification we added this enzyme to the PCR to remove the remaining chimeric primers, and added 5–10 ng of the plasmid to be modified for further amplification. In this way we obtained the expected constructs with substituted or inserted target fragments in one-tube reactions (Fig. 4B).

Capacity and efficiency of the $\Omega$-PCR strategy

To test the capacity and efficiency of the $\Omega$-PCR strategy, we used different modes of $\Omega$-PCR for sequence modification of two other gene fragments: OsGEN-L (1.89 kb) (Moritoh et al. 2005) and Pi-ta cDNA (2.787 kb) (Bryan et al. 2000), and two vector backbones: pENTR (2.6 kb) (Invitrogen) and pYLTAC747 (15.8 kb) (Lin et al. 2003). The results showed that $\Omega$-PCR can handle large plasmids with sizes ranging from 5.4 to 16.5 kb (Fig. 5, Table 1).

Sequence and functional validation of constructs modified by $\Omega$-PCR strategies

We sequenced the junction regions in the three vector constructs modified in succession by the $\Omega$-PCR strategies. A diagram and the sequences of the three junction regions in the original vector pBI-GFP:OsRac3 are shown in Fig. 6A and B, and these regions were re-sequenced for verification after modification (Fig. 6C–E). We found that all three resultant plasmids had been correctly modified. These plasmids were transiently expressed in rice protoplasts to confirm the subcellular localizations of the resultant proteins in vivo (Fig. 7). Indeed, Den:OsRac3 (pBI-Den:OsRac3) was localized in the plasma membrane like the original GFP:OsRac3 (pBI-GFP:OsRac3). The Den (pBI-Den) fluorescent protein alone was distributed in the cytoplasm and Den:Rer1B (pBI-Den:Rer1B) was localized in the Golgi, as expected. All of the Den proteins in the different constructs were able to be photoactivated by a 405 nm laser and properly converted from green to red (Fig. 7).

Discussion

Although a number of PCR-based cloning and mutagenesis approaches have been reported, most of them are useful for

Fig. 1 Continued

3' junction of the modification site 2 (ms2) and the 3' portion (brown) corresponds to the 3' region of the Den fragment. The denatured strands of the first PCR product containing Den (brown) serve as de novo ‘megaprimer’ for substitution $\Omega$-PCR in the following reaction. To simplify the figure, only the forward megaprimer and one strand of the plasmid template are shown in the preceding steps and figures. When the ‘megaprimer’ anneals to the template, the sequences of GFP (green) and Den (brown) form individual $\Omega$-shaped structures. (B) The GFP sequence in the pBI-GFP:OsRac3 construct was replaced by Den through substitution $\Omega$-PCR, resulting in a new construct, pBI-Den:OsRac3. Lane 1, the first run PCR product Den; lane 2, the second run PCR product pBI-Den:OsRac3; lane 3, the starting plasmid pBI-GFP:OsRac3 digested with BamHI; * indicates target product bands; Marker, Trans15K DNA ladder (TransGen Biotech).
only one type of sequence modification; those that can be used for insertion or substitution can handle only very short sequences. The Ω-PCR methods described herein can be used to manipulate large sequences, and therefore enable all types of sequence modification. The key principle of our Ω-PCR sequence modification strategies is the formation of the characteristic secondary structure between the megaprimers (target PCR products) and templates (plasmids) during the PCR. The formation of this secondary structure is attributable to the specific design of the chimeric primers. Several overlapping extension cloning protocols which allow only insertion sequence modification have been reported (Chen et al. 2000, Bryksin and Matsumura 2010, Bond and Naus 2012), However, our Ω-PCR methods enable all three types of modification in one-tube reactions.

In the two-step reactions, the chimeric primers carried into the second PCR are highly diluted, and thus do not affect the Ω-PCR. However, for the one-tube Ω-PCR strategy without exonuclease I treatment, the first PCR product was preferentially amplified in the second PCR phase, leading to failure of amplification of the target plasmid (data not shown). However, reducing the concentration of the chimeric primers often resulted in low amplification efficiency. One of the novel features of the one-tube Ω-PCR strategy is the utilization of exonuclease I to remove the extra chimeric primers in the second round of PCR to avoid these problems. Therefore, no gel purification step of the target fragment is required in either of the strategies. In addition, Ω-PCRs do not require post-PCR procedures such as restriction enzyme digestions and ligation.

The Ω-PCR technique can be used to insert, swap or remove any fragment at any position of a circular plasmid for functional analysis. The Ω-PCR insertion mode enables subcloning of a target gene directly into a destination plasmid vector by PCR alone. This strategy overcomes the limitation of conventional restriction–ligation-based cloning methods. The substitution mode of Ω-PCR can be used to exchange fluorescent protein genes, fusion tags or subcellular sorting signals, and the deletion mode can be used to generate a series of truncated gene fragments for deletion assays. It is also a very good choice for in vitro splicing to remove introns from genomic DNA (gDNA) without the need for reverse transcription to clone full-length cDNA for long genes with few introns. Using deletion Ω-PCR, we have successfully obtained a full length Pi-ta cDNA (2,787 bp) from gDNA (4,250 bp) (containing one 1.5 kb intron, data not shown). The only limitation for Ω-PCR is the capacity of the DNA polymerase for amplification of longer DNA fragments (target sequences and plasmid vectors). We highly recommend using a DNA polymerase of high performance and high fidelity, such as PrimeSTAR, Pfx, Pfu, KOD or Phusion, to increase the efficiency and fidelity of the Ω-PCR.

Using the PrimeSTAR Taq DNA polymerase, 1.89 and 2.787 kb fragments were successfully inserted or substituted into target plasmids by Ω-PCR (Fig. 5, Table 1). The Den fragment was inserted into a transformation-competent artificial chromosome (TAC) vector pYTAC747 with a final size of 16.5 kb.
Fig. 3 Insertion Ω-PCR. (A) Principle of insertion mode Ω-PCR. Coding sequence of a Golgi sorting protein Rer1B is to be inserted into the starting construct pBl-Den. The 5’ portion (brown) of the forward chimeric primer Rer1B-F for insertion mode Ω-PCR is identical to the 5’-flanking sequence of the modification site 2/3 (ms2/3) and the 3’ portion of the forward primer (purple) is identical to the 3’ end of insert Rer1B. The 5’ portion (cyan) of the reverse chimeric primer Rer1B-R corresponds to the 3’-flanking sequence of the ms2/3 in the vector and the 3’ portion (purple) of the reverse primer corresponds to the 3’ end of the insert Rer1B. A Rer1B-containing fragment is PCR-amplified using Rer1B-F and Rer1B-R and serves as de novo 'megaprimers' for insertion in the following Ω-PCR. (B) Use of one-tube Ω-PCR for insertion and substitution modifications in plasmid construction. Constructs pBl-Den:OsRac3 (left) and pBl-Den:Rer1B (right) were generated by substitution or insertion mode Ω-PCR using the one-tube method; Marker, Trans15K DNA ladder (TransGen Biotech).

Fig. 4 One-tube Ω-PCR strategy. (A) For the first phase of the one-tube Ω-PCR strategy, we determined that the amount of target sequence in 5 μl of product from a 15-cycle amplification was approximately equal to the amount in 0.5 μl of PCR product produced by 30 cycles of amplification. Therefore, the total amount of the target product in the 20 μl reaction from the 15-cycle amplification in the one-tube strategy was equal to approximately 2 μl of the fully amplified product added to the second PCR in the two-step strategy. (B) Use of one-tube Ω-PCR for insertion and substitution modifications in plasmid construction. Constructs pBl-Den:OsRac3 (left) and pBl-Den:Rer1B (right) were generated by substitution or insertion mode Ω-PCR using the one-tube method; Marker, Trans15K DNA ladder (TransGen Biotech).
Design of Ω-PCR primers

Primers for substitution of GFP with Den. Forward primer Vec-Den-F for substitution mode Ω-PCR consisted of a 21 base 5’ portion identical to the 5’-flanking sequence of the modification site 1 (ms1) on the vector, and a 21 base 3’ portion identical to the 5’ end of the insert Den. The 5’ portion (20 bases) of reverse primer Den-Rac3-R corresponded to the 5’ end of OsRac3, and the 3’ portion (21 bases) of Den-Rac3-R corresponded to the 3’ end of the insert Den (Supplementary Table S1).

Primers for deletion of OsRac3. The primers for deletion mode Ω-PCR also consisted of two parts demarcating the ms2 and ms3. The 5’ portion of the forward primer Den-Vec-F consisted of 18 bases and was identical to the 5’-flanking sequence of the ms2, and the 3’ portion (25 bases) of Den-Vec-F was identical to the 3’-flanking sequence of the ms3. The reverse primer Den-Vec-R was reversed and complementary to the forward primer Den-Vec-F (Supplementary Table S1).

Primers for insertion of Rer1B. The 5’ portion (22 bases) of forward primer Rer1B-F for insertion mode Ω-PCR was identical to the 5’-flanking sequence of the ms2/3 in the plasmid pBI-Den, and the 3’ portion of the forward primer consisted of 21 bases and was identical to the 5’ end of insert Rer1B. The 5’ portion of the reverse primer Rer1B-R consisted of 21 bases and corresponded to the 3’-flanking sequence of the ms2/3 in the vector, and the 3’ portion of the reverse primer consisted of 21 bases and corresponded to the 3’ end of the insert Rer1B (Supplementary Table S1).

Two-step Ω-PCR

For Ω-PCR insertion and substitution modes, Ω-PCR could be conducted in a two-step reaction. In the first reaction, the PCR mixture contained 4.0 μl of S× PS buffer, 0.2 mM dNTPs, 0.5 μM each of the chimeric primers, and 1–5 ng of template DNA (such as plasmid DNA or gDNA) containing the target insertion sequence or substitution fragment, 0.3 U of PrimeSTAR Taq DNA polymerase (TAKARA) and deionized water to a final volume of 20 μl. A Taq DNA polymerase that produces blunt-end products must be used for this technique. The PCR mixture was subjected to 28–30 cycles of 96°C for 20 s, 60°C for 30 s, 72°C for 1–5 min (according to the size of the amplified fragment, approximately 45–60 s kb⁻¹). In the second-step PCRs, 2–3 μl of the PCR product resulting from the first reaction served as the megaprimer, and 5–10 ng of
Fig. 6 Validation of the modified constructs by sequencing. (A) Schematic diagram of the original construct pBI-GFP:OsRac3 used for manipulation. In substitution mode, GFP was replaced by Den, which encodes a photoconvertible fluorescent protein Den, resulting in pBI-Den:OsRac3. OsRac3 was then removed from pBI-Den:OsRac3, resulting in pBI-Den:Rer1B encoding a Golgi protein was inserted into the 3' region of Den using insertion mode, resulting in pBI-Den:Rer1B. Junctions 1–3 represent the flanking regions of the modification sites (ms). (B) Original sequences of the three junction regions of pBI-GFP:OsRac3 shown in (A). (C–E) Sequence confirmation of the junction regions in the modified constructs produced by different PCR modes. Primers F1–F4 indicated in (A) were used for sequencing analysis. The arrowheads indicate the modification sites.
One-tube Ω-PCR

Since current PCR machines possess very powerful programming capability, Ω-PCR steps can be performed in the same tube with one program using the link or insertion function of PCR machines such as ABI PE9700 or TAKARA TP650. In the one-tube Ω-PCR, the PCR mixture contained 4.0 μl of 5× PS buffer, 0.25 mM dNTPs, 0.5 μM each of the chimeric primers, 1–5 ng of template DNA containing the target insertion or substitution fragment, 0.5 U of PrimeSTAR Taq DNA polymerase (TAKARA) and deionized water to a final volume of 20 μl. Five cycles of 96°C for 30 s, 55°C for 1 min, 68°C for 5–10 min (according to the final size of the plasmid, approximately 1 in kb⁻¹), and 15 cycles of 96°C for 1 min, 68°C for 5–10 min were carried out. The final PCR products were treated at 37°C for 30 min with 5–10 U of DpnI to digest specifically the original template plasmids.

Supplementary data

Supplementary data are available at PCP online.

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