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PCR
METHODS · AND · APPLICATIONS

A Highly Efficient Procedure for Site-specific Mutagenesis of Full-length Plasmids Using *Vent* DNA Polymerase

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Careful titration of *Vent* polymerase activity allows efficient amplification of full-length plasmids (12 kb). The high processivity and fidelity of this enzyme made oligonucleotide-directed site-specific mutagenesis of plasmids a straight-forward process. Using only two primers, a mutagenic and a complementary, single-base mutants of recombinant plasmids were obtained consistently with >90% efficiency from a single round of PCR. This procedure also made site-specific deletion, insertion, and several bases mutagenesis facile and efficient.

Site-specific mutants[1] have played a crucial role in defining structure–function relationships of proteins and in deciphering the significance of gene regulatory elements. The recombinant DNA technology for generating site-specific mutants has developed rapidly in the last decade. Several novel strategies and commercial kits have been developed to generate oligonucleotide-directed site-specific mutants at a faster pace. In general, the various strategies fall into two broad categories. In one, a mutagenic primer is annealed to a single-stranded plasmid DNA template to prime synthesis of the mutant strand. The mutant strand can then be enriched by one of the several biochemical or biological means to obtain the desired mutants.(1,2) However, obtaining single-stranded DNA is a cumbersome process. Alternatively, site-specific mutants can also be generated by amplifying mutant DNA employing two sequential rounds of PCR from a double-stranded DNA template using 2 to 4 primers.(3–5) However, in both cases, several experimental steps are required and the frequency of obtaining mutants can vary from as low as 0.1 to as high as 90%. Although we have efficiently created site-specific mutants using single-strand DNA templates,(2) we consistently had problems in obtaining mutants using the currently available PCR mutagenesis strategies.

Here, we present a simple, rapid, and inexpensive PCR scheme to generate mutants with a minimum number of steps. With only a single round of PCR, single-base mutants were obtained at a frequency >90%. Our strategy is based on utilization of *Vent* polymerase, a highly processive and high-fidelity thermophilic DNA polymerase from *Thermococcus litoralis*.(6–8) High processivity of the polymerase allows high-yield synthesis of DNA fragments longer than 10 kb with no requirements for additional constituents in the reaction mixture. As most recombinant plasmids are <10 kb in size, *Vent* polymerase allows efficient amplification of an entire plasmid. The high fidelity of *Vent* polymerase is primarily attributable to the potent integral 3′ → 5′ exonuclease activity.(6,8) However, high level of this activity necessitates an optimization of reaction conditions with each new batch of the polymerase. Once standardized, the procedure works consistently well. An additional advantage to using *Vent* polymerase is that it generates blunt-ended products, ready for blunt-end ligation and cloning. In contrast, *Taq* polymerase generates products with 3′ overhangs requiring especially created vectors for cloning.

MATERIALS AND METHODS

Recombinant Plasmids

All DNA manipulations were carried out following the standard protocols.(9) The Sendai virus P/C gene (~1.9 kb)(2) was cloned into pBC12/CMV/IL-2,(10) into pcDNAI/amp (Invitrogen), and into pg1f1(2) yielding recombinant molecules pBCPC (6.6 kb), pcPC (6.7 kb), and pgfPC (5.5 kb), respectively. Plasmid DNA from minipreparations was used for PCR amplifications as well as for transfections in mammalian cells.

Primers

Primers (18-mer to 21-mer) were custom synthesized by Integrated DNA Technologies, Inc. Primers were deprotected, dried, resuspended in water, and quantitated by UV spectrometry. Each primer (1000 pmoles) was phosphorylated at its 5′ end in a 40-μl reaction mixture [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 nmoles of ATP, 15 units of polynucleotide kinase (U.S. Biochemical)] at 37° for 30 min. At the end of incubation, 40 μl of water was

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Table 1 Characteristics of PCR Primers Used for Site-specific Point Mutagenesis

Primer	Sequence	Length	T_m	GC (%)	Mutants[a]	Frequency
P120	GGA CCG GGT GCC AGG CAC	18	79	77	4/4	100
P121	**G**CA CCG CTG AAT CGT TAC AAC	21	74	52		
P123	AAG GCA TCT TGA TCC ATG GT	20	70	45	6/6	100
P122	CAT TCT **A**AA AGA AGA TTC TG	20	60	35		
P124	CCG GGT GCC AGG CAC GGT	18	82	77	6/8	75
P125	**GA**C CCA CCG CTG AAT CGT TAC	21	75	57		

Bold and underlined nucleotides represent mutagenized sites.

[a] The number of mutants obtained from the number of independent clones analyzed.

added and the mixture was heated to 90°C for 5 min. Primers were used directly from this mixture for PCR reactions. Primer sequences (Table 1) were determined by the requirements to generate particular mutants rather than on the guidelines used by many software programs to synthesize optimum primers. It is interesting to note that we had no problem using any primers irrespective of their sequences, melting temperatures, and/or matching parameters. However, wherever possible, we kept the melting temperature of the primer pairs within 10°C. The melting temperatures were determined using the Primer software (Scientific & Educational Software) with the algorithm developed by Freier et al.(11)

PCR Reactions

As *Vent* polymerase has an efficient proof-reading 3′ → 5′ exonuclease activity, it degrades the synthesized DNA rapidly presumably after the DNA synthesis precursors have been depleted. To overcome this problem, the reactions were terminated during the log phase of the reaction. A titration of enzyme amount and the number of cycles were performed each time a new enzyme preparation was used. In general, our titrations indicated that using 0.5 unit of *Vent* polymerase (New England Biolabs) in 50 μl of reaction yielded a good quantity of PCR product at 15 cycles. Thus, amplifications were carried out in a 50-μl reaction mixture [500 μM dNTPs, 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100] with 25 pmoles of each phosphorylated primer pair and 100 ng of plasmid DNA. At each PCR cycle, denaturation of DNA was carried out at 92°C for 30 sec. The annealing temperatures were generally 5° below the lower melting temperature of the primer pair. However, we observed that an annealing temperature of 50°C worked well with all the primers we used (Table 1). Extension time at 72°C was based on 1000 nucleotides/min as suggested by the *Vent* polymerase supplier and a previous report.(7) At the end of each PCR reaction, an additional extension reaction of 10 min was carried out to complete synthesis of any lagging chains.

To obtain a sufficient amount of DNA, three to five simultaneous reactions were carried out for each mutagenesis. The reactions were processed immediately at the end of the PCR cycles to minimize exonuclease activity. The products were resolved in 0.7%–0.8% agarose gels in Tris-acetate–EDTA (TAE) buffer. Appropriate bands were excised from the gel and purified using Geneclean (Bio 101). Purified DNA (100–200 ng) was self-ligated and used to transform *Escherichia coli* HB101. For each mutagenesis, four to eight colonies were picked, DNA was prepared by minipreparations (from 3-ml cultures) and sequenced using Sequenase 2.0 (U.S. Biochemical). Five sequencing primers distributed over the entire P/C gene were used to determine sequence errors as a result of amplification. One primer was used to authenticate the mutagenesis.

RESULTS AND DISCUSSION

Amplification of Plasmid DNA

To determine the appropriate number of cycles for PCR reactions, we amplified pcPC at 15, 20, 25, and 30 cycles using the reaction conditions described in Materials and Methods. Analysis of reaction products (5 μl) revealed that whereas 15 cycles gave a distinct band of the appropriate size DNA, at 20, 25, and 30 cycles the synthesized DNA was progressively degraded. Among the degradation products were distinct bands of lower molecular weight DNA. We have no idea about the basis of their origin. At the end of 30 cycles, a smear of heterogeneous size DNA was visible (Fig. 1). *Vent* polymerase was used previously for site-specific deletion mutagenesis.(12) However, when we used the recommended reaction conditions, the products were completely degraded. Clearly, inappropriate optimization of amplification conditions results in degraded or inappropriate sized products (Fig. 1). Analysis of chain extension time revealed that *Vent* polymerase under our reaction conditions synthesized ~1000 bp/min consistent with a previous report.(7) Thus, the vectors presented in this article required about 6 min of extension reaction for each PCR cycle. We have also used *Vent* polymerase to extend a 12-kb plasmid DNA without any problem (data not shown). Therefore, this procedure permits direct amplification of an entire plasmid of interest and its site-specific mutagenesis. Recent success in

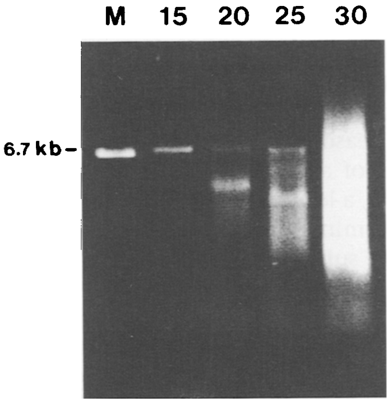

FIGURE 1 Amplification products of pcPC with *Vent* polymerase at indicated number of PCR cycles. Number of cycles are presented on the *top* of the respective lanes. Mock (M) presents the template plasmid linearized at a unique *Hin*dIII site. Amplified 6.7-kb product is clearly seen in the 15-cycle lane, but only faint bands are detected at this size in 20- and 25-cycle lanes. No distinct band is seen in the 30-cycle lane.

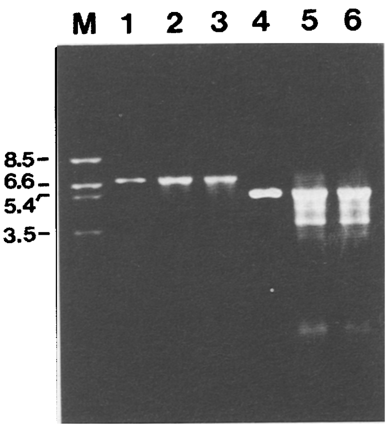

FIGURE 2 Amplification products of recombinant plasmids pcPC and pgfPC. Primer pairs P120/P121 and P124/P125 were used to amplify pcPC and pgfPC. (Lane *M*) The molecular weight markers in kb. (Lanes *1, 4*) Linearized pcPC, and pgfPC, respectively. (Lanes *2, 3*) The amplification products of pcPC with P120/P121 and P124/P125, respectively. (Lanes *5,6*) The amplification products of pgfPC with P120/P121 and P124/P125, respectively.

amplifying longer templates (>40 kb) using a mixture of thermostable DNA polymerases (13,14) indicates that mutagenesis of long templates will be possible with optimized conditions and appropriate polymerases.

Design of Primers

Mutagenic primers have been designed previously to carry the mismatch bases in the center of the primers flanked by complementary arms.(1–3,5) However, we reasoned that for stable hybridization of a mutagenic primer to its template, a longer complementary arm with 5′ terminal mismatch sequences may be more suited for site-specific mutagenesis by PCR. Therefore, we designed 21-mer primers (P121 and P125) such that they carried 1 or 2 base mismatches at their 5′ termini (Table 1). Alternatively, the 20-mer primer (P122) had a mismatch 6 nucleotides from the 5′ end (Table 1). As all the primers worked efficiently in mutagenesis, we believe that mismatches at the 5′ termini in mutagenic primers may be a better means of designing appropriate primers. In addition, because primer pairs with wide differences in their melting temperatures and GC content yielded appropriate products, we could choose any target site on the plasmid for mutagenesis.

Site-specific Mutagenesis

Using the procedure described in Materials and Methods, we created site-specific mutants in the P and C reading frames of the bicistronic P/C gene of Sendai virus. The P reading frame is 568 amino acids long and encodes the viral polymerase associated phosphoprotein, whereas the C reading frame is 204 amino acids long and encodes a nonstructural protein of unknown function. Our aim was to mutagenize Ser-249 (UCC) and Pro-250 (CCA) of the P reading frame to Asp (GAC) and Ala (GCA), respectively, and Leu-5 (UUA) in the C reading frame to a terminator codon (UAA). The first two mutations were carried out using recombinant pcPC, whereas the later mutagenesis was carried out using pgfPC.

Appropriate primer pairs (Table 1) were used to amplify the entire recombinant plasmid as described above. The full-length DNA product was purified, self-ligated, and transformed into bacterial cells as described above (Fig. 2). Figure 3 schematically represents the overall procedure of site-directed mutagenesis used. For each mutagenesis experiment, four to eight colonies were grown in minicultures and plasmid DNA was purified and sequenced by chain-terminating dideoxynucleotides using Sequenase. In both single-base mutagenesis, all six clones for Pro-250–Ala and Leu-5–Ter were authentic mutants. In the double-base mutagenesis of Ser-249–Asp, six out of eight clones were authentic mutants (Table 1). This mutagenesis was further authenticated by restriction enzyme digestion and expression of the

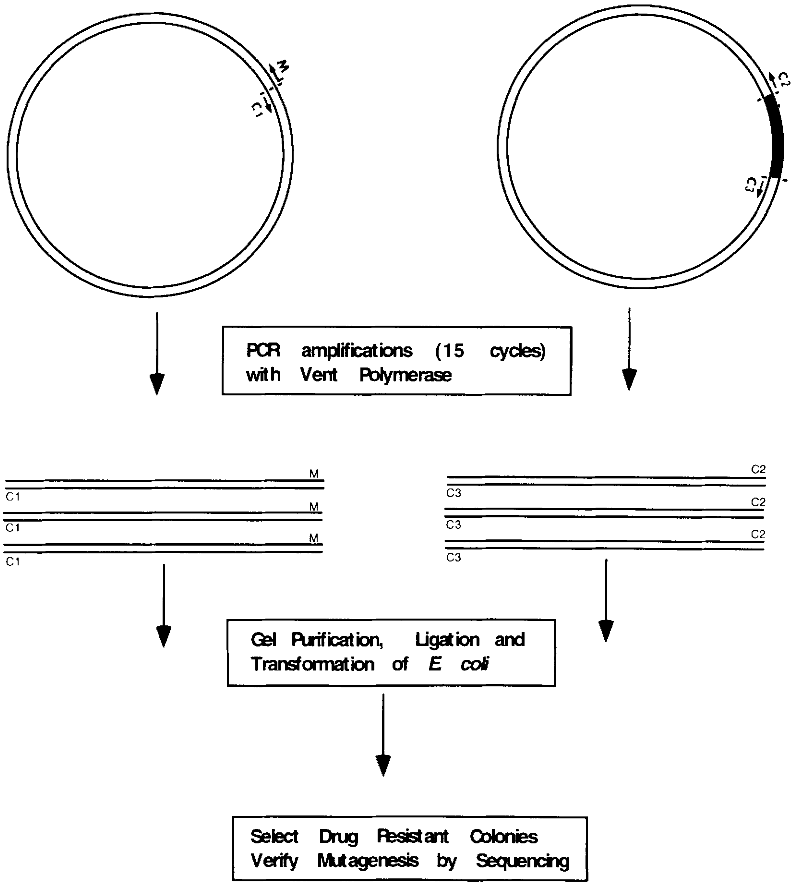

FIGURE 3 Schematic presentation of the protocol for the oligonucleotide-directed site-specific point mutagenesis (*left*) and deletion mutagenisis (*right*) of a plasmid. M1 and C1 represent a primer pair with no gap between them except that the M primer contained a mismatch at the 5′ end. C2 and C3 represent a primer pair designed to delete the filled-in region.

P and C proteins in CV1-transfected cells. In transfected cells the P and C proteins were expressed at the expected levels from mutants indicating that *Vent* polymerase amplified the plasmid with high fidelity (data not shown). Restriction digestions of the mutants revealed no detectable changes in the restriction fragment patterns, further indicating the high fidelity amplification by *Vent* polymerase (data not shown). To estimate polymerase error rate during plasmid amplification, we sequenced five different regions of the mutant P/C gene spanning ~1250 nucleotides. In total, we sequenced >10,000 nucleotides from several mutant plasmids and did not encounter any error. This result indicated that the *Vent* polymerase under our reaction conditions had a very low error rate ($<1 \times 10^5$) and amplified the plasmids with a high degree of fidelity. This is consistent with a previous report.(6) However, these results underscore the importance of reaction optimization; failing this, random undesirable mutations may occur in the amplified mutant plasmid.

As this procedure allowed efficient mutagenesis of three entirely different plasmid vectors (two presented here and one not presented), we believe that this procedure will be universally applicable to generate mutants directly in plasmids regardless of the plasmid origin. Following essentially the identical protocol, we also obtained several deletion and insertion mutants (not shown). The procedure presented in this paper is more rapid and efficient at obtaining site-specific point and deletion mutants than previously described methods.(1,2,4,5,15)

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