Generation of Deletion and Point Mutations with One Primer in a Single Cloning Step

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Site-directed mutagenesis is an important technique for studying DNA and protein function. With the advent of PCR, several new approaches have been successfully developed (1,3,5–9). Early methods involved two-step PCR followed by subcloning, which is time consuming and error prone. Recently introduced site-directed mutagenesis kits such as QuikChange™ Site-Directed Mutagenesis Kit (QCS) developed by Stratagene (La Jolla, CA, USA) is a widely used PCR-based system (4) that eliminates the necessity to subclone the amplified DNA fragment. It requires two complementary mutagenic oligonucleotide primers and uses a plasmid to amplify the desired product by high-fidelity *Pfu* DNA polymerase (Stratagene). Following temperature cycling, the product is treated by the restriction endonuclease *Dpn*I (Stratagene) that will cut only fully or hemimethylated 5′-G^{m6}ATG-3′ DNA sequences. Thus, only the original parental methylated plasmid DNA will be digested. The remaining unmethylated and nicked double-stranded plasmid incorporating the desired mutations is then transformed into Epicurian Coli® XL-1Blue supercompetent cells (Stratagene). The yield of clones harboring mutations is very high and varies, depending on the template length, from 80% to more than 90% for 2.9-kb plasmids (4).

Methods for generating internal deletions have been described by using inverse PCR, but these involved two steps (9). A simplified method for deletion mutagenesis is used in the Ex-Site™ mutagenesis kit by Stratagene, but this method also involves the use of phosphorylated primers and a ligation step. The use of the QuikChange mutagenesis kit has been reported for introducing deletions or insertions of only 12 bp (4). Here, we present a significantly improved method based on the QuikChange system for generating deletion and point mutants. This method allows the use of a single primer to generate deletions exceeding 3000 bp and point mutations in a single linear amplification reaction.

In our laboratory, we routinely use the QuikChange kit for point mutations. However, it was not clear how this protocol could be applied for deletions of more than 12 bp (4). We used a plasmid containing Pals1 cDNA (total length 6.7 kb) as a template to make several deletion and point mutants (2). The PCR mutagenesis reaction was performed in a reaction volume of 25 µL that included 1 µL plasmid DNA (in TE or water) from Wizard® Plus SV Minipreps DNA Purification System by Promega (Madison, WI, USA) or from QIAprep® Spin miniprep kit by Qiagen (Valencia, CA, USA) miniprep (100–300 ng/µL) template DNA, 200 nM each primer, 200 µM dNTPs and 1.25 U *Pfu* enzyme in 1× *Pfu* DNA polymerase reaction buffer. A preliminary step of denaturation at 95°C for 3 min was followed by 18 cycles of PCR. These PCR cycles consisted of 15 s of denaturation at 95°C, 1 min of annealing at a temperature 2°C higher than the melting temperature (T_m) of the head of the primer to increase specificity of amplification (see below) and 12 min of extension at 68°C using a PTC-200™ thermal cycler (MJ Research, Watertown, MA, USA). In cases when the T_m exceeded the extension temperature, we applied a two-step PCR annealing and extending of 68°C. Finally, the PCR product was treated with 1 µL *Dpn*I endonuclease for 2 h at 37°C and 2 µL *Dpn*I-digested DNA was transformed into 50 µL Epicurean Coli XL1 Blue supercompetent cells. The number of kanamycin-resistant clones varied from 100 to 2000.

We experimented with primer pairs that would result in the highest yield of the desired deletion. We routinely analyzed clones by PCR using primers flanking the deletions and obtained efficiencies of between 25% and 70%. Selected clones were sequenced and confirmed the PCR results. Table 1 summarizes different examples of primer pairs or single primers used in the mutagenesis experiments. For simplicity, the 5′ part of the primers complementary to the region upstream of the introduced deletion we called the tail, and the 3′ portion of the primer complementary to the region downstream of the deletion we called the head.

As shown in Table 1, when using primer pairs of different design, deletions of up to 3 kb could be introduced into the plasmid by this simple one-step protocol. We believe that the same approach could be applied to any sequence because we successfully deleted several regions of the coding

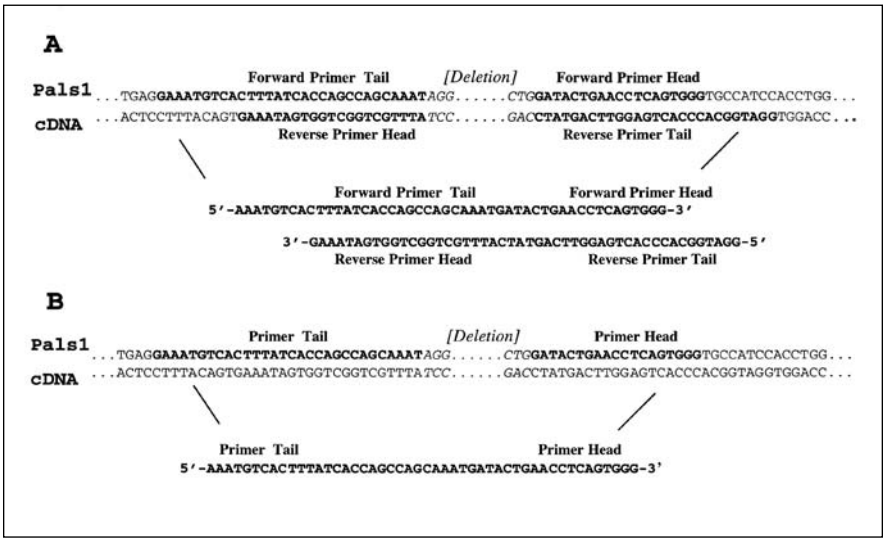

Figure 1. Deletion mutagenesis experiment using a primer pair or single primer. Deletion of a functional (555 bp) domain in Pals1 cDNA with a pair of mutagenic primers (A) or a single mutagenic primer (B). The primer sequences are indicated in bold, and the region of deletion is marked in italics.

Table 1. Summary of Deletion and Point Mutant Experiments Performed with Plasmid pEYFP-N1[a] Containing PALS1 cDNA (Total Length 6.7 kb)

	Primer Length Head and Tail	T_m of the Tail and the Head[b]	Observed Efficiency[c]	Length of Deletion
	Primer pairs			
1	Forward 27 + 27	59 + 72	25%	225
	Reverse 27 + 27	72 + 59		
2	Forward 24 + 24	69 + 64	50%	132
	Reverse 24 + 24	58 + 75		
3	Forward 30 + 19	67 + 49	60%	555
	Reverse 27 + 21	68 + 55		
4	Forward 12 + 23	26 + 63	30%	555
	Reverse 17 + 25	37 + 63		
5	Forward 30 + 23	65 + 54	60%	3184
	Reverse 27 + 25	64 + 56		
6	Forward 16 + 27	32 + 64	70%	3184
	Reverse 17+ 28	62 + 42		
7	Forward 21 + 21	55 + 54	25%	555
	Reverse 21 + 21	55 + 54		
8	Forward 15 + 19	28 + 50	70%	174
	Reverse 16 + 24	53 + 41		
	Single primer			
1	21 + 21	55 + 54	50%	555
2	22 + 16	57 + 57	50%	60
3	16 + 20	48 + 48	30%	183
4	18 + 17	45 + 46	50%	204
	Point mutation primer			
1	19 + 22	45 + 53	70%	
2	16 + 14	32 + 33	66%	

[a]Plasmid pEYFP-N1 is from Clontech Laboratories (Palo Alto, CA, USA).

[b]For definition of head and tail see Figure 1. T_m was calculated using the Primer Premier program (Biosoft International, Palo Alto, CA, USA).

[c]Efficiency was calculated by analyzing at least 10 clones by PCR with analytical primers flanking the mutation. Efficiency was calculated as percentage of the mutant clones versus the total number of colonies obtained. Selected clones were sequenced in duplicate.

sequence of the plasmid corresponding to different functional domains in the target protein. We made deletions of up to 3184 bp in length and did not observe any decrease in efficiency. This implies that there is probably no practical limitation to the size of deletion or truncation that can be generated.

Surprisingly, we found that the same method works well when we use just one primer. For example, we obtained similar results generating a 555-bp deletion using the primer pair no. 7 versus the single primer no. 1 (Table 1). The primer pair gave more colonies, 1430 colonies compared with 770, but the efficiency of generating this deletion was better using the single primer. During mutagenic PCR with two complementary primers, free primer concentration could be drastically reduced because of more favorable primer-dimer formation compared to primer-template annealing. A single primer could yield more efficient polymerization than two primers. At this point, we do not know the exact nature of the DNA species involved in transformation in our protocol. We speculate that linear single-stranded mutant DNA might survive within the cells following transformation. This protection could be due to fragments originating from the parental plasmid after degradation by *Dpn*I endonuclease. We also successfully applied a single-primer mutagenesis protocol to generate point mutations (Table 1).

We checked whether the same approach would work well for another *E. coli* strain such as DH5α™ (Life Technologies, Rockville, MD, USA). The *Dpn*I-digested DNA from a PCR with just one mutagenic primer was used for transformation of DH5α cells made competent using calcium chloride (transformation efficiency 10^7 cfu/µg). To 50 µL competent cells, we added 2 µL PCR product treated with *Dpn*I. We have obtained about three times fewer clones compared to Epicurean Coli XL-1Blue supercompetent cells, but the efficiency was the same as for the cells provided with the QuikChange system. This implies that the mechanism enabling us to work with just one primer is not restricted to one particular *E. coli* strain. Taken together, our data allows the use of this single-step mutagenic technique in a broad variety of applications and at the same time simplifies some of the published protocols for making large deletions. Cost is also reduced because only one mutagenic primer needs to be purchased. We have been successfully using the described single-primer protocol in our laboratory for the generation of multiple deletion and point mutants in several different constructs and plasmids.

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