Short Technical Reports

Two-Stage PCR Protocol Allowing Introduction of Multiple Mutations, Deletions and Insertions Using QuikChange™ Site-Directed Mutagenesis

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ABSTRACT

We developed a two-stage procedure, based on the QuikChange™ Site-Directed Mutagenesis Protocol, that significantly expands its application to a variety of gene modification experiments. A pre-PCR, single-primer extension stage before the standard protocol allows the efficient introduction of not only point mutation but also multiple mutations and deletions and insertions to a sequence of interest.

INTRODUCTION

The invention of the polymerase chain reaction (PCR) (3,4) provided a powerful tool to modify DNA sequences in genetic engineering. With numerous mutagenesis methods available, such as traditional sequential PCR (1), "megaprimer PCR" (2), marker-coupled PCR (6) and so on, introducing changes to DNA sequences has become less tedious and more efficient. Recently introduced site-directed mutagenesis (SDM) kits, such as the Transformer™ Site-Directed Mutagenesis Kit (CLONTECH Laboratories, Palo Alto, CA, USA) and the Altered Site® II *in vitro* Site-Directed Mutagenesis Systems (Promega, Madison, WI, USA), even eliminate the necessity of subcloning the amplified fragment.

One of the most broadly applicable protocols currently available is the QuikChange™ Site-Directed Mutagenesis System (QCM) developed by Stratagene (La Jolla, CA, USA). This method allows the rapid introduction of point mutations into sequences of interest using a pair of complementary mutagenesis primers to amplify the entire plasmid in a single PCR. Destruction of the parental template plasmid by *Dpn*I digestion followed by direct transformation into *Escherichia coli* cells allows introduction of the desired mutation with high efficiency (70%–90%) in as little as 24 h.

However, the approach is basically limited to primer pairs of 25–45 bases (with melting temperatures [T_m] between the primer and the template no lower than 78°C, as indicated in the accompanying literature and confirmed in these studies). When multiple mutations spanning longer sequences (e.g., 5 amino acid residues) are needed, two or more primer pairs and rounds of mutagenesis are needed. This leads to higher cost and longer completion time. When longer primers are attempted, the mutagenesis efficiency is drastically decreased, largely due to the more favorable primer dimer formation (100% complementary) compared to the primer-template annealing (i.e., due to the mutiple mismatches).

In this study, a simple modification of the mutagenesis protocol is described that not only overcomes the limitation of the primer length, but also provides a convenient and efficient approach to gene modifications previously not possible with the standard QCM protocol, such as multiple and cassette mutagenesis, insertions and deletions.

MATERIALS AND METHODS

Plasmids PUC4K (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and pFastBac™ HTa (Life Technologies, Gaithersburg, MD, USA) were used as the mutagenesis templates. Five primer pairs designed to disrupt the *bla* gene (Table 1) were synthesized and polyacrylamide gel electrophoresis (PAGE)-purified by Genosys Biotechnologies (The Woodlands, TX, USA). XL-1-Blue competent cells, *Pfu* DNA Polymerase and the QuikChange Site-Directed Mutagenesis Kit (QCM) were all purchased from Stratagene.

The procedure consists of two stages. In stage one, two extension reactions are performed in separate tubes; one containing the forward primer and the other containing the reverse primer. Subsequently, the two reactions are mixed, and the standard QCM procedure is carried out on the mixture. In the single primer extension reaction, 50–200 ng template PUC4K plasmid along with 10 pmol of the particular primer were extended using 1 U of *Pfu* DNA polymerase in the QCM PCR buffer/dNTP cocktail in a 50-µL reaction. The extension reaction was initiated by preheating the reaction mixture to 95°C for 30 s: 1, 3 or 10 cycles of 95°C for 30 s, 55°C for 1 min and 68°C for 8 min. Following the completion of the extension reactions, 25 µL of each pair were mixed in one tube along with 1 U of *Pfu* DNA polymerase and subjected to the standard QCM PCR procedure. Following the PCR, 10 U of *Dpn*I were added (as per the QCM protocol), mixed well and incubated at 37°C for another hour before transformation or agarose gel electrophoresis. Five microliters of the final PCR products were transformed into 50 µL of XL-1-Blue cells, and appropriate volumes were spread on LB agar plates containing either ampicillin or kanamycin. The number of ampicillin- and kanamycin-resistant colonies was then used to calculate the mutagenesis efficiency.

RESULTS AND DISCUSSION

The plasmid PUC4K contains two antibiotic-resistant genes: *bla* conferring ampicillin resistance and *Tn903* yielding kanamycin resistance. The mutagenesis primer pairs were designed to disrupt the *bla* gene by introducing nonsense mutations through point mutations, frameshifts, insertions or deletions, so that the mutagenesis could be easily scored by antibiotic selection. The knockout strategy of the oligonucleotide primer pairs is summarized below:

P1: B01F/B02R, 34-mer with 2 point mutations, typical QCM primers; P2: B03F/B04R, 60-mer with the same 2-base replacements as P1; P3: B05F/B06R, 60-mer with 9 point mutations spanning 6 consecutive codons; P4: B07F/B08R, 43-mer, with a 31-base deletion; and P5: B09F/B10R, 73-mer, with a 31-base insertion.

If the failure to generate mutants using longer primers is the result of primer dimerization, distinguishable differences in mutagenesis efficiency would be expected with the P1 and P2

Table 1. Oligonucleotide Primers Used in the Mutagenesis Experiments

Primer	Pairs	Primer Sequences
P1[a]	B01F	GGCGCGGTATTATCC**T**G**A**GTTGACGCCGGGCAAG
	B02R	CTTGCCCGGCGTCAAC**T**C**A**GGATAATACCGCGCC
P2[a]	B03F	GTTCTGCTATGTGGCGCGGTATTATCC**T**G**A**GTTGACGCCGGGCAAGAGCAACTCGGTCGC
	B04R	GCGACCGAGTTGCTCTTGCCCGGCGTCAAC**T**C**A**GGATAATACCGCGCCACATAGCAGAAC
P3[a]	B05F	GTTCTGCTATGTGGCGCGGTA**GC**AT**GGTAG**G**C**TGACG**A**GGGGCAAGAGCAACTCGGTCGC
	B06R	GCGACCGAGTTGCTCTTGCCCC**T**CGTCA**GC****CTACC**AT**GC**TACCGCGCCACATAGCAGAAC
P4[b]	B07F	GTTCTGCTATGTGGCGCGGTA(**TTATCCCGTGTTGACGCCGGGCAAGAGCAAC**)TCGGTCG CCGCATACACTATTC
	B08R	GAATAGTGTATGCGGCGACCGA(**GTTGCTCTTGCCCGGCGTCAACACGGGATAA**)TACCGC GCCACATAGCAGAAC
P5[c]	B09F	GTTCTGCTATGTGGCGCGGTA**GCCTAACCAAACGACGAGCGTGACACCACGG**TTACCCGTG TTGACGCCGGGC
	B10R	GCCCGGCGTCAACACGGGTAA**CCGTGGTGTCACGCTCGTCGTTTGGTTAGGC**TACCGCG CCACATAGCAGAAC

[a]Bases that are bold and underlined are designed mutations in the *bla* sequence.
[b]Bases that are marked in the parentheses are the designed deletions in the *bla* sequence.
[c]Bases that are bold and underlined are the expected insertions in the *bla* sequence.

Table 2. Generation of Mutant Clones[a]

Mutagenesis Methods	Primer Pair	Colonies on Kanamycin Plates	Colonies on Ampicillin Plates	Mutagenesis Efficiency
QCM	P1	232	24	89.7%
	P2	0	0	N/A[c]
	P3	0	0	N/A
	P4	0	0	N/A
	P5	0	0	N/A
S1-QCM[b]	P1	1432	72	95.0%
	P2	336	45	86.6%
	P3	680	128	81.2%
	P4	358	60	83.2%
	P5	528	186	64.8%
S3-QCM[b]	P1	640	62	90.3%
	P2	220	28	87.3%
	P3	110	31	71.8%
	P4	347	31	91.0%
	P5	291	83	71.5%
S10-QCM[b]	P1	580	52	91.0%
	P2	333	27	91.9%
	P3	313	93	70.3%
	P4	132	35	73.5%
	P5	276	82	70.3%

[a]Transformation efficiency using PUC4K and XL-1-Blue cells was 10^7/µg. After *Dpn*I digestion and transformation, no colony was observed on the ampicillin plate.
[b]The number indicates the cycle number in the single-primer PCR step.
[c]N/A is not applicable.

sets using the standard QCM protocol. Indeed, the results indicated that the P1 pair introduced the mutation with the expected efficiency (70%–90%), while the P2 pair failed to produce any mutants under the same PCR conditions. Using the QCM procedure, no amplification for either the P3, P4 or P5 primer pairs was detectable by either agarose gel electrophoresis or transformation (Figure 1, lanes 5–8).

To overcome the tendency of the perfectly complementary mutagenic primer pairs to dimerize with each other, rather than anneal to the target sequence, two separate single-primer reactions were introduced before the actual PCR amplification to generate mutated single-stranded copies of the plasmid. The newly generated hybrid plasmids (i.e., with one wild-type and a newly synthesized mutant strand) allow the subsequent annealing of the mutagenesis primers to the template strands to be more efficient, as the primers no longer must compete with a perfectly matched wild-type duplex in that area. Unlike primer titration strategies (5) and the use of partially overlapping primers (7) to optimize priming, this approach is universal, irrespective of primer length and sequence.

By introducing the single-primer PCR stage, coupled with the QCM protocol, a nicked double-stranded plasmid product was observed for all the mutagenesis reactions, as shown in Figure 1, lanes 9–23. Although *Dpn*I removes the template plasmid with almost 100% efficiency (Figure 1, compare lanes 2 and 3), it requires that the adenine bases in the recognition site on both strands be methylated. Consequently, hybrid plasmids (with one template strand and one newly synthesized mutant strand) can escape digestion, leading to ampicillin-resistant (but "non-mutant") transformants. The mutagenesis efficiency determined by antibiotic selection ranged from 65% to 95%, which was comparable to the efficiency of a standard mutagenesis using the QCM procedure. The number of rounds of extension before the QCM amplification procedure did not seem to affect the mutagenesis efficiency; only one cycle was required to produce comparable mutagenesis efficiency (Table 2). Similar mutagenesis results were observed using larger plasmids (5 and 7.5 kb; data not shown).

In conclusion, a simple modification of the QCM protocol has been described in which a single-primer extension reaction is carried out before the standard QCM amplification. One cycle of extension was sufficient for potentiating efficient mutagenesis using a wide variety of primer pairs that were ineffective using the standard QCM protocol. Unlike primer titration and partial overlap strategies, which require optimization on a case by case basis, this method retains the simplicity and mutation efficiency of the original protocol but expands its application to a much broader range of gene manipulation procedures, such as multiple and cassette mutations, and deletions and insertion of DNA sequences.

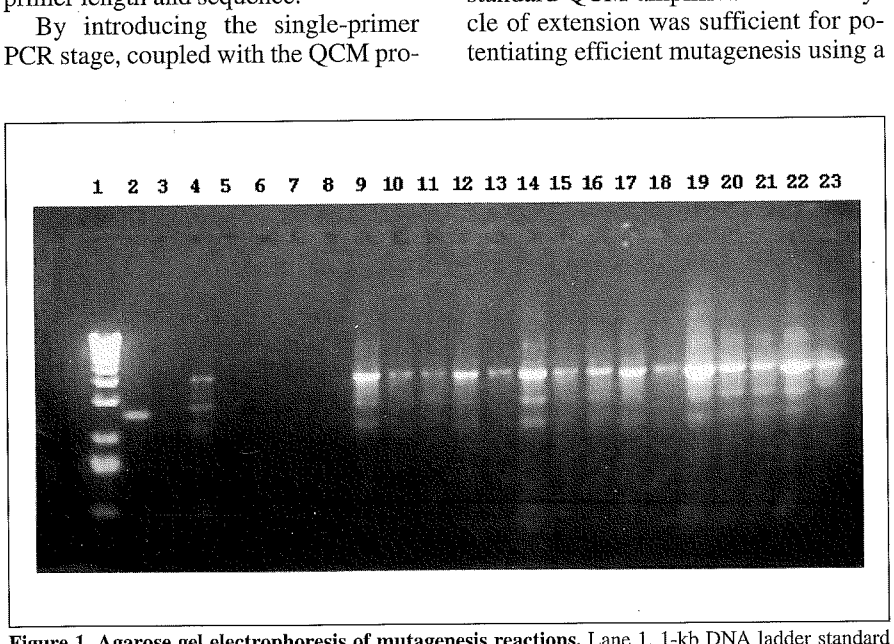

Figure 1. Agarose gel electrophoresis of mutagenesis reactions. Lane 1, 1-kb DNA ladder standard (Life Technologies); lane 2, template plasmid PUC4K; lane 3, template plasmid PUC4K after *Dpn*I digestion; lanes 4–8, mutagenesis reactions using standard QCM procedure with primer pairs P1, P2, P3, P4 and P5, respectively. Mutagenesis reactions using the modified protocol incorporating single primer extension reactions before QCM procedure: lanes 9–13, with 1 cycle of extension (primer pairs P1, P2, P3, P4 and P5, respectively); lanes 14–18, with 3 cycles of extension (primer pairs P1, P2, P3, P4 and P5, respectively); and lanes 19–23, with 10 cycles of extension (primer pairs P1, P2, P3, P4 and P5, respectively).

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