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An improved PCR-mutagenesis strategy for two-site mutagenesis or sequence swapping between related genes

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ABSTRACT

The QuikChange™ protocol is one of the simplest and fastest methods for site-directed mutagenesis, but introduces mutations at only one site at a time, and requires two HPLC-purified complementary oligonucleotides. Here, we describe that this method can be used with non-overlapping oligonucleotides. By doing this, two separate sites can be mutagenised simultaneously, or money can be saved by using a second 'standard' oligonucleotide. By a further modification, we have also used the QuikChange™ approach to exchange DNA sequences between closely related genes.

The QuikChange™ protocol (Stratagene) relies on the fact that DNA synthesized *in vitro* is not methylated, and therefore resistant to digestion by the enzyme *Dpn*I (which cuts at G^{m}ATC sequences in methylated or hemi-methylated DNA). The whole plasmid is copied and amplified *in vitro* to incorporate the mutagenic oligonucleotides by a high-fidelity thermostable DNA polymerase, and the original template is subsequently digested by *Dpn*I (for which, statistically, a site is found every 256 bp). The resulting mutated plasmid DNA is recovered directly by transformation into competent bacteria.

In the new approach (Fig. 1), the *in vitro* DNA synthesis takes place in two successive phases. During the first phase, a DNA fragment is amplified in a typical PCR reaction (1) between two oligonucleotides (Fig. 1, B). During the second phase, the two strands of the newly amplified DNA fragment serve as 'mega-primers' (2) to complete the synthesis of the remainder of the plasmid. To favour this, annealing during the second phase is performed at the elongation temperature of the thermostable DNA polymerase (i.e. 68°C for Pfu).

Both these modified protocols are still applicable to any sequence provided in any plasmid vector without requiring any specific restriction site.

Performing QuikChange™ mutagenesis with non-overlapping oligonucleotides. Via this approach, mutations can be introduced in a plasmid at two different sites simultaneously (left part of Fig. 1). For this, the oligonucleotides used in the initial PCR reaction both carry mutagenic substitutions (Fig. 1, C). If only one mutation is desired, a 'standard' second oligonucleotide annealing in the plasmid outside the cloning site can be used repeatedly (Fig. 1, A3), thereby saving money on chemically synthesized, HPLC-purified, oligonucleotides.

This approach was used successfully to perform site directed mutagenesis of Clontech's EGFP version of green fluorescent protein (accession no. U55761), cloned into the pCATCH-NLS expression plasmid (3). The 50 µl reaction mixes were assembled as directed in the QuikChange™ kit's instructions, using 50 ng of template plasmid. The oligonucleotides (12.5 pmol/reaction of each) were always chosen so as to yield a short PCR fragment during the first phase of the amplification reaction (Fig. 1, B; Table 1). The thermal cycler was then programmed as follows: 9× (95°C for 30 s, 55°C for 1 min, 68°C for 1 min) followed by 9× (95°C for 30 s, 68°C for 6 min). In the final cycle, extension time was increased to 16 min, followed by digestion with 10 U *Dpn*I at 37°C for 1 h. One microlitre of this was then used to transform 10–20 µl of competent XL1blue bacteria (provided with the kit), and consistently produced between 10 and 200 colonies (Table 1). This was ~5-fold lower than expected from the original QuikChange™ protocol, but still ample to recover mutagenised plasmids. All the mutations introduced in EGFP influenced its fluorescence properties (E.J., manuscript in preparation). The efficiency of mutagenesis was therefore scored by flow cytometry of transiently transfected COS 7 cells. Of the plasmids recovered and analysed, >85% had the expected structure and still expressed a fluorescent protein (FP).

For the first three rows of Table 1, two mutagenic oligonucleotides were used simultaneously. Of the plasmids recovered, 60–100% had incorporated both mutations, and 10–20% had either one mutation or none. On the following three rows, only one of the oligonucleotides used was mutagenic, and the efficiency of mutagenesis was also in the range of 60–100%. Apart from gross structure alterations detected by restriction digest analysis in <10% of plasmids, no undesired mutation was found outside the mutagenic oligonucleotides in all the sequences that were checked by automated sequencing (i.e. >8 kb in total).

Using QuikChange™ for exchanging DNA sequences. The same principle of using two successive amplification phases can be applied to exchange DNA sequences between closely related genes (right-hand side of Fig. 1). For this, oligonucleotides are designed on either side of the region to be exchanged so that they can anneal and prime on both gene versions. This region is first amplified by PCR on one sequence (Fig. 1, B). This PCR-amplified DNA fragment is then purified (1F), and a second amplification performed on the other sequence (1G), using both strands of the

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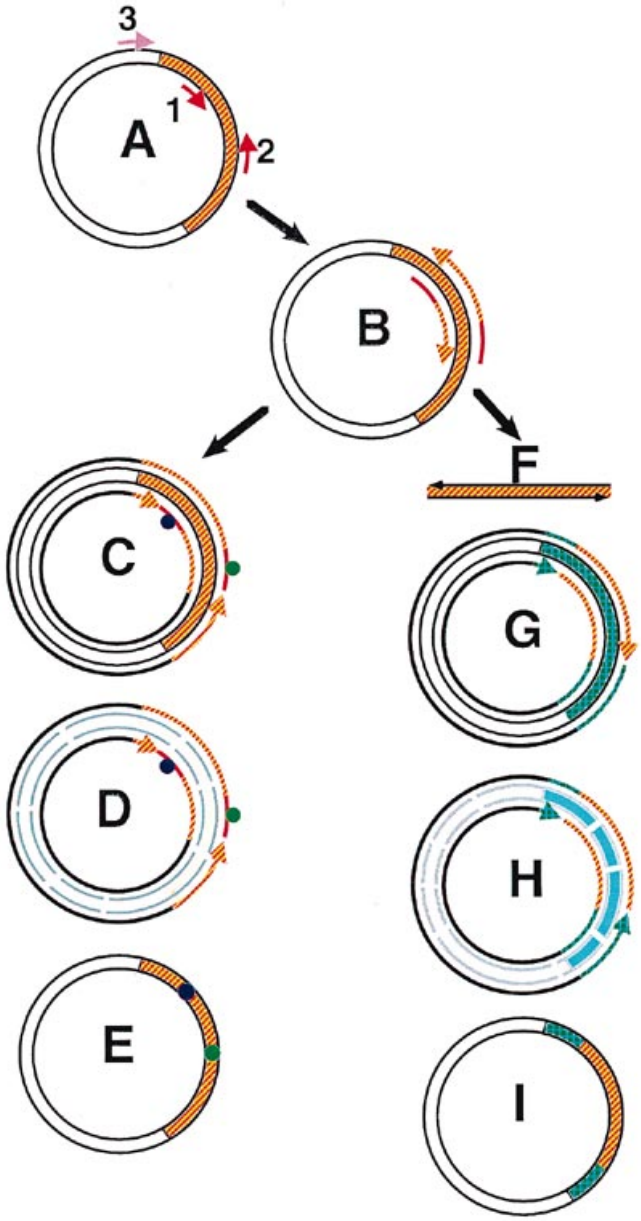

Figure 1. Using the Quikchange™ approach with non-overlapping primers. Oligonucleotides are designed as PCR primers (A). Both can anneal to the cloned sequence (A1, A2). Alternatively, a second 'standard' primer can be used, that anneals to the plasmid (A2, A3). In the first phase, a typical PCR reaction (1) is carried out between these two primers (B). The DNA fragments resulting from this PCR reaction will then serve as megaprimers during the second phase of amplification. For simple site-directed mutagenesis, in the second phase of amplification, the annealing temperature is raised to match the elongation temperature, thereby favouring the use of the megaprimers (C). For swapping of sequences, the PCR-amplified DNA is purified (F), and both strands used as megaprimers on the other sequence (G). At the end of the second phase, template plasmids are digested with *Dpn*I (D,H), and mutagenised products recovered by transformation in bacteria (E,I).

purified fragment as megaprimers (2). After digestion with *Dpn*I to eliminate the parental plasmid DNA (1H), the plasmid carrying the hybrid DNA molecules are recovered directly by transformation of competent bacteria (1I).

When closely related genes differ in their particular function, comparison of their respective sequences can often assist in mapping that function to a specific portion of the gene, or even to a single point mutation. In most cases, however, such sequences will differ at several positions along the gene sequence, and swapping of DNA sequences is usually the quickest way to initiate the mapping of the function under study.

We have used this new approach successfully to create two different hybrid sequences between two rat MHC class I molecules, RT1-A^{u} (4) (accession no. X82106) and RT1-A1^{c} (5) (accession no. X90370). The two cDNAs are >90% homologous, but the MHC class I molecules they encode are recognised differently by natural killer cells (NK cells) (6). We therefore hope to map the part of the MHC class I molecules recognised by NK cells by swapping portions of one molecule with that found in the other allele.

To achieve this, we designed 'reverse' oligonucleotides which annealed on regions of the DNA sequence that were identical in the two sequences in the regions encoding for aa 33–26 (F33 back: 5′-GAACTCCGTGTCGTCCACGTAGCC-3′) and aa 61–54 (E61 back: 5′-CTCCCAATACTCCGGCCCCTCCCG-3′). A single upstream oligonucleotide, pCMU5′, was chosen in the vector outside of the cloning site (5′-CCGCGCCCCAAGCATA-AACCCTGG-3′).

In our case, the sequences of RT1-A^{u} and RT1-A1^{c} were sufficiently similar that 24 nt long stretches of perfect identity could easily be identified. One could envisage, however, that this might not always be the case. In such instances, slightly longer oligonucleotides might be needed, and designed to be homologous to the first gene towards their 3′ end, and homologous to the second gene towards their 5′ end. Indeed, in the PCR-amplified megaprimers, the 3′ end will consist of what has been copied from the 5′ end of the original oligonucleotides. Such oligonucleotides, however, would not be suitable for reciprocal exchanges between the two sequences, whereas oligonucleotides picked in regions of perfect homology would. Conceivably, the QuikChange approach could even be adapted for splicing by overlap extention (gene SOEing) whereby fusion of unrelated genes is obtained by PCR using an oligonucleotide where the 3′ end is homologous to the first gene, and the 5′ end to the second gene (7,8).

The initial PCR reactions were performed in 50 µl using 2.5 U Pfu polymerase (Stratagene), the provided 10× Pfu buffer, 50 µM each dNTP, 50 ng of pCMU-A1^{c}, and 1 µM of each HPLC-purified oligonucleotide. PCR amplifications were performed using the following program: 95°C for 90 s, 20× (62°C for 1 min, 68°C for 1 min, 95°C for 30 s), 62°C for 1 min, 68°C for 11 min. The whole PCR reactions were loaded onto a 2% agarose TAE gel, revealing DNA fragments of the expected size (i.e. 290 bp for the pair pCMU5′/F33 back, and 390 bp for the pair pCMU5′/E61 back). These DNA fragments were purified using the QiaEx kit (Qiagen), and eluted in water, with final concentrations of 50 ng/µl (as estimated by analysis on another agarose gel).

For the second phase of the protocol, 350 ng of these fragments were used as megaprimers, with 50 ng of pCMU-Au as a template. As previously, the 50 µl amplification reactions were assembled with 2.5 U Pfu polymerase and 50 µM dNTPs, and the amplification program used was: 95°C for 90 s, 20× (68°C for 10 min, 95°C for 30 s), 68°C for 20 min. Following this amplification, digestion with 10 U of *Dpn*I was performed at 37°C for 90 min, before being precipitated with 100 µl ethanol. This was then used to transform XL1-blue supercompetent bacteria (Stratagene).

Table 1. Site-directed mutagenesis of EGFP

Template	US oligo	DS oligo	No. colonies obtained from 1 µl/50	No. plasmids expressing FP/total analysed	Single mutations	Properly mutagenised	Resulting plasmids
pCATCH-nls-EGFP	Y66H	Y145F	40	17/18	3	10	no. 5 (66H + 145F) + no. 3 (66H only)
no. 5	LTH>LSH	V163A	15	6/8	1	5	no. 1
no. 3	LTH>LSH	V163A	30	6/7	0	6	no. 10
no. 5	T7	V163A	30	8/8	N/A	6	no. 18
no. 3	T7	V163A	10	8/8	N/A	5	no. 28
no. 5	LTH>LSH	Y145F	200	10/10	N/A	10	no. 7

Mutagenesis was scored by FACS analysis of COS 7 cells transiently transfected with the plasmids recovered. The various oligonucleotides used had the following sequences:
T7: 5′-TAATACGACTCACTATAGGG-3′
Y66H: 5′-ACCACCCTGACCCACGGCGTGCAGT-3′
Y145F: 5′-TTGTGGCTGTTGAAGTTGTACTCCAG-3′
LTH>LSH: 5′-CTCGTGACCACCCTGTCCCACGGCGTGCAGTGC-3′
V163A: 5′-GGATCTTGAAGTTCGCCTTGATGCCG-3′
No unexpected mutations were found in any of the sequences coding for the fluorescent proteins listed in the last column.

Table 2. Results of sequence swapping experiments between two rat MHC class I cDNAs, RT1-A^u and RT1-A1^c

Primer pair	Size of PCR fragment	No. colonies	No. plasmids analysed	No. plasmids with altered structure	No. unmodified pCMU-A^u	No. hybrids
pCMU5′ + F33 back	290	20	6	1	3	2
pCMU5′ + E61 back	390	20	6	0	3	3
negative control	N/A	10	0	N/A	N/A	N/A

For the 11 plasmids in the last two columns, the whole 1.1 kb cDNAs were sequenced, and no unexpected mutations were found. For the negative control, no primers were added to pCMU-A^u in the second amplification.
cDNA inserts of all 11 plasmids in the last two columns were checked by automated sequencing, and no point mutations were identified.
N/A: not appropriate.

The results of the analysis of the plasmids recovered from this experiment are shown in Table 2. In both experiments, plasmids with duly swapped sequences were recovered, albeit at a relatively low frequency (33% for the F33 primer, and 50% for the E61 primer). We do feel that optimised conditions for amplification with the megaprimers (9–14) would probably improve the efficiency and frequency of recovery of the expected hybrid DNA molecules. Our results already show that this novel approach can rapidly yield hybrid DNA molecules between closely related genes without recourse to any specific restriction site.

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