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Preparation of chimeric genes without subcloning

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Introduction

PCR methods are widely used for the modification of proteins, including point mutations, deletions, and insertions, as well as for gene ligation. Sub-cloning of PCR-generated gene product into the appropriate expression vector often decelerates this process; therefore, methods that avoid the need for subcloning, such as the QuikChange® mutagenesis approach, are much faster (1,2). Chimeric genes represent a very useful tool for the study of protein function. They allow for the addition of different functionalities, such as various labels (e.g., fluorescent, enzymatic activities resulting in luminescence or colored products, and immuno-tags), domains for protein oligomerization, and for combinations of functional fragments of different proteins. Chimeric genes can be produced by engineering appropriate restriction sites between the gene fragments or, alternatively, by gene splicing by extension of the overlap, where the PCR-generated product needs to be subcloned into the vector of choice (3).

Here we describe an approach for the generation of chimeric genes based on back-to-back PCR, without the need for subcloning. The only requirement of this approach is that the original genes are available in the same or in a related vector, as outlined in (Figure 1). The principle of the method is that two primers are selected and positioned at the edges of the genes, which will be ligated. Primers are oriented in opposite directions with their 5′ at the site of the junction between both proteins. Before PCR, each plasmid is cleaved with a different enzyme, at a site near the 5′ of each primer, in the region common to both vector templates. Polymerization from primers in the first PCR extension step results in two single-stranded products based on two different templates. Because each plasmid template is cut with a different enzyme, extension of the primer ends at a different position, providing a long complementary region that is common to the vector in both templates ((Figure 1)). Single-stranded PCR products are annealed and extended in the next PCR cycle, resulting in a final product that is amplified in the subsequent cycles. The final PCR products are circularized by ligation, which brings together the two extreme ends of the product, corresponding to the ligated gene fragments ((Figure 1)). For the purpose of ligation, primers have to be phosphorylated or, alternatively, PCR product can be phosphorylated after the amplification and before ligation. As a precaution, we have incorporated additional digestion of the original template plasmid with *Dpn*I, which cleaves the methylated template plasmid isolated from bacteria but not the PCR product. Linear PCR product might also be able to transform bacteria at low efficiency, as previously described (4).

Figure 1.

Diagram of the preparation of chimeric genes without subcloning. Both genes to be ligated have to be available in the same type of vector (e.g., expression plasmid) of a pET series. The whole procedure consists of four steps: (*i*) cleavage of the template plasmids with different enzymes, (*ii*) PCR, (*iii*) purification of products with the removal of the remaining template plasmids, and (*iv*) ligation. Two phosphorylated primers oriented back-to-back are designed, each complementary to the selected region of the genes to be ligated, with the possibility of adding a linker sequence to their 5′ ends. Template plasmids are cut prior to PCR with different enzymes (in this case, *Hin*dIII and *Sal*I) 5′ of each primer so that polymerization products of the first PCR cycle contain a long stretch of complementary sequence. In the next PCR cycle, products of the first cycle are annealed, extended to a final length, and amplified in subsequent cycles. Remaining template plasmids originating from bacteria are digested with *Dpn*I, and purified reaction products are circularized by blunt end ligation.

Our goal was the preparation of a fusion protein between human MD-2 and 24 kDa fragment of *Escherichia coli* gyrase B to investigate the influence of MD-2 dimerization on signal transduction. Gyrase B dimerizes in the presence of double-headed inhibitor coumermycin and is therefore a suitable partner, where dimerization is induced upon the addition of coumermycin (5). Based on structural arguments, we decided to prepare fusion protein of MD-2 with gyrase B domain fused at its C terminus. We used *md*-2 cloned in pET14b and *gyrB* cloned in pET3a (Novagen, Dermstadt, Germany) plasmids (6,7), which were isolated with the GenElute™ Plasmid Miniprep Kit (Sigma, St. Louis, MO, USA). We have designed oligo-nucleotides 5′-ACCAGAACCATTTGAATTAGGTTGGTGAAGG-3′ and 5′-TCTGGTTCTTCGAATTCTTATGACTCCTCC-3′, where the underlined region corresponds to the region complementary to the end of *md*-2 and to the beginning of *gyrB*, respectively, while the rest of the oligonucleotide corresponds to the sequence coding for hydrophilic flexible linker SGSSGS. Primers were synthesized by Gen-Script (Scotch Plains, NJ, USA), phosphorylated at their 5′, and purified by polyacrylamide gel electrophoresis (PAGE). PCR was performed in a 20-µL final volume containing 100 ng each of the two plasmids. Plasmid pET14b carrying *md*-2 and plasmid pET3a carrying *gyrB* have been previously cleaved with *Hin*dIII and *Sal*I (each 10 U), respectively ((Figure 1)). Enzymes were heat-inactivated prior to addition to the reaction mixture, which also contained 20 pmol primers, 2.5 mM dNTP, 1.25 mM $MgCl_2$, and 1 U *Tth* and *Pfu* DNA polymerase mixture from a CERTAMP Long Amplification Kit (Biotools, Madrid, Spain). Buffer conditions were as recommended by the manufacturer (Biotools). The thermal cycler was programmed for an initial 3-min denaturation at 94°C, followed by 30 cycles of 1 min denaturation at 94°C, 30 s annealing at 61°C, and 10 min elongation at 72°C, concluding with a final 11-min elongation step at 72°C. In order to remove any remaining methylated template DNA, the PCR mixture was incubated with 10 U of *Dpn*I (New England Biolabs, Beverly, MA, USA) (8), which were added directly to the PCR mixture. After *Dpn*I digestion, the PCR products were fractionated with agarose gel electrophoresis ((Figure 2)) and gel-purified using a MiniElute™ Gel Extraction Kit (Qiagen, Hilden, Germany). One hundred nanograms of each column-purified DNA were further ligated with the Quick Ligation™ Kit (New England Biolabs) using 1 U of T4 DNA ligase and transformed into competent *E. coli* DH-5α cells with the efficiency of chimeric gene construction between 20% and 50%, depending on the plasmid cleavage by restriction endonuclease.

Figure 2.

Production of an amplified DNA fragment containing full-length vector with gene fragments from different genes at their ends. The *Dpn*I-treated PCR mixture prepared according to the diagram in (Figure 1) was purified by 1.2% agarose gel electrophoresis. Each lane represents the PCR mixture from differently treated templates prior to PCR. M, 1-kb ladder standard (BenchTop 1Kb DNA Ladder; Promega, Madison, WI, USA); lane 1, *Hin*dIII and *Sal*I cleaved *md*-2 and *gyrB* template plasmid, respectively; lane 2, *Hin*dIII cleaved *md*-2 template and untreated *gyrB* template plasmid; lane 3, untreated *md*-2 template and *Sal*I cleaved *gyrB* template; lane 4, both

md-2 and *gyrB* templates plasmid have not been cleaved.

It is also possible to cleave only one of the plasmids because the shorter product of polymerization will anneal to the complementary strand and result in the amplified product in the next PCR rounds ((Figure 2), lanes 2 and 3). We obtained the correct final product of the correct size even without prior cleavage with restriction enzymes ((Figure 2), lane 4). The successful polymerization probably results from the presence of shorter polymerization products, which can anneal to the complementary strand and are amplified in subsequent cycles. As expected, no product was obtained if only one of the plasmid templates was present in the PCR mixture. In this particular example, we have used two partners cloned in similar but not the same vectors (pET3a and pET14b), which are identical over most of the sequence. Therefore, this approach can be used even if the two vectors have only limited common regions (e.g., selection markers) provided that the plasmid is cleaved in this region.

Given the availability of a collection of genes in the same type of vector, which is usually the case in gene libraries, the described method probably enables the fastest generation of chimeric genes because it can be accomplished in a couple of hours. This method allows for the generation of chimeras of different orientation as well as the addition of linker sequences between the genes by the addition of desired extension at 5′ of primers. The number of possible chimeras that can thus be rapidly generated increases as the square of the library size.

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Competing Interests Statement

The authors declare no competing interests.

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