



Short communication

Chimera construction using multiple-template-based sequential PCRs

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ABSTRACT

Chimera construction between different proteins is a useful method for investigating protein structure and function relationships. However, this technique, by its traditional application, has been daunting because of its complex procedure and low success rate. Here we describe a protocol for constructing chimeras between proteins that does not require the existence of restriction sites, or the purification of intermediate PCR products, which are essential in the traditional protocols. By introducing the “multiple-template” concept, this protocol only requires the use of two or three simple PCRs followed by general subcloning steps. Most importantly, the success rate is nearly 100%.

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1. Introduction

Chimera construction between different proteins is a useful method for investigating protein structure and function relationships. In the ion channel field, which we are working in, investigating the functions of chimeras between ion channel subunits of the same family, which usually have similar yet distinct functions, has revealed tremendous information on the structural basis of many channel functions (Erreger et al., 2007; Kelley et al., 2003; Kuhn et al., 2007; Young et al., 2008).

Traditional protocols used to construct chimeras rely on restriction sites, either naturally existing or artificially introduced by silent mutagenesis, so that protein domains can be swapped with each other. However, such protocols are limited since the restriction sites are not always available. Therefore, a few alternative protocols which avoid the involvement of restriction sites have been developed. For example, Padgett and Sorge took advantage of the restriction enzyme Eam1104I, which cleaves any DNA sequence located at a defined distance from its recognition sequence, to create chimeras by ligating together the insert cDNA and the remaining host cDNA and vector DNA (Padgett and Sorge, 1996). However, their protocol requires a long PCR when amplifying the host cDNA and vector DNA. The long PCRs are notorious for being difficult to achieve and they also need quite a few sequencing reactions for verifying the final sequences. Another protocol utilizes sequential PCRs by using primers overlapping the junction between two

parental cDNAs (Grandori et al., 1997), however, the primers used in the final PCR to amplify the full-length chimeric cDNA, can also anneal to one of the parental cDNA templates transmitted from the preceding steps. Thus, the final products are often contaminated with parental cDNAs, even if all the intermediate products are gel-purified. This contamination usually causes a low success rate. A similar situation also exists in a third protocol, which combines the sequential PCR and QuickChange™ techniques. In this protocol, even though the gel-purification of intermediate PCR products is performed, the success rate is very low. This might be due to the inefficiency of the QuickChange™ technique in such an application (Kirsch and Joly, 1998).

To overcome the obstacles described above, we have developed a new protocol which uses multiple-template-based sequential PCRs. In this protocol, the possibility of primers annealing to any off-target template introduced in the preceding steps has been eliminated. Therefore, even if gel-purification of intermediate products is skipped, the final PCR products are free from contamination by any parental cDNA. This protocol has been used in our lab to successfully construct over 20 chimeras between the $\alpha 1$ and the β subunits of the glycine receptor chloride channel (GlyR $\alpha 1$ and GlyR β) (Lynch, 2004), and subsequent electrophysiological analysis of these chimeras has revealed a huge body of information, yet to be published, on the structure and function relationship of this receptor.

2. Materials and methods

The basic principle of this protocol is illustrated in Fig. 1. There are two cases to consider when making a chimera. One is that the

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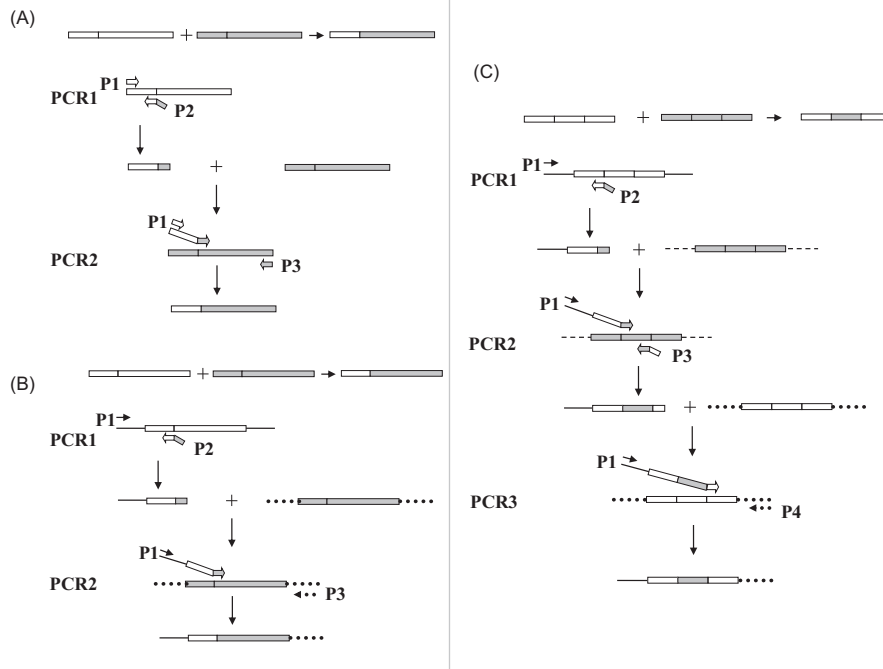


Fig. 1. Schematic illustration of chimera construction protocols. Unfilled and grey bars represent different parental cDNAs. Solid, dashed and dotted lines represent sequences from pUC19, pGEMHE and pcDNA3.1zeo+ vectors, respectively. P1, P2, P3 and P4 represent primers. Panels A, B and C illustrate different protocols as shown diagrammatically in the top row of each panel.

segment to be taken from each parental protein occupies either its N- or C-terminal, in which case one junction is required (Fig. 1A and B). The other is that the segment from one parental protein is placed in the middle of another parental protein, in which case two junctions are required (Fig. 1C).

To construct a chimera with one junction, three primers, P1, P2 and P3, are required (Fig. 1A and B). P1 and P3 are the primers used to anneal to the 5' and 3' ends, respectively, of the resultant chimeric cDNA. P2 is a 35–45 base long primer overlapping the junction of two parental cDNAs. This primer is a merge of two simple primers, either of which can anneal to one side of the cDNAs flanking the resultant chimeric junction (see examples in Table S1, Supplementary material). As described next, only that part corresponding to the simple primer anneals to the template in any PCR, therefore both simple primers should be designed following the general primer-designing rules such as appropriate melting temperature, G/C at the 3' end, etc. As shown in Fig. 1A and B, P1 and P2 are used first to amplify the N-terminal cDNA (PCR1), the product of which contains the sequences flanking the desired chimeric junction. The plus strand of the PCR1 product then acts as a mega-primer in the second PCR (PCR2), in which this mega-primer together with P3 is used to amplify the C-terminal cDNA. Since the mega-primer has a much lower concentration than optimal, P1 is also added to achieve enough yield. An issue to consider is that if both P1 and P3 in PCR2 could anneal to the same parental cDNA, one or both of the parental cDNAs would be amplified. This would cause the PCR2 product to be contaminated with parental cDNAs. To avoid this possibility, P1 and P3 can be designed to anneal to the very beginning and the very end, respectively, of the parental cDNAs (Fig. 1A). In such a case, no cross-reaction could occur as long as the cDNA sequence homology of the N- and C- terminals between the two parental cDNAs is not high. If the homology is high, P1 or P3, or both, would have to be designed to anneal to the vector sequence flanking the parental cDNAs (Fig. 1B). In this case, P1 and P3 would usually be the 5' and 3' sequencing primers, respectively. In order to avoid cross-template reaction, the two parental cDNAs must be constructed in different vectors, otherwise,

P1 and P3 in PCR2 would amplify the full-length parental cDNAs, either present in PCR2 or transmitted from PCR1. This would cause the PCR2 product to be composed mainly of both parental cDNAs.

Single-junction chimeras are needed only when studying either the N- or C-terminal domains of a protein (Fig. 1A and B), however, more often, a domain in the middle of a protein needs to be investigated. In such a case, this domain is usually placed into the middle of another protein and two junctions are required to create the chimera (Fig. 1C). This can be achieved by using the protocol for introducing one junction, described above, except that it is done in two steps, i.e. one junction is introduced each step. However, this is time-consuming. Here we have developed a more efficient protocol for introducing two junctions by using three-template-based sequential PCRs. As shown in Fig. 1C, similar to the case of introducing a single-junction, the first PCR (PCR1) uses the 5' primer P1 and the junction primer P2 to amplify the N-terminal of the host cDNA. The P2 overlaps the first junction and therefore the PCR1 product can be used as a mega-primer for the second PCR (PCR2). In PCR2, this mega-primer and another junction primer (P3) are used to amplify the insert cDNA, the product of which is maximized by the addition of the P1. Again, since P3 contains the sequence overlapping the second junction, the PCR2 product can be used as a mega-primer in the next PCR (PCR3). In PCR3, through the same mechanism as in PCR2, P1, the PCR2 product and the 3' primer P4 are used to amplify the C-terminal of host cDNA, which produces the desired chimeric cDNA. The 5' and 3' primers, unlike in the case of introducing one junction, must anneal to the vector rather than the N- or C-terminal cDNA sequences, otherwise, in PCR3, P1 and P4 would anneal to the host cDNA directly and eventually contaminate the PCR3 product. Therefore, to avoid cross-template reaction, the host cDNA needs to be subcloned into two different plasmid vectors and the insert cDNA needs to be constructed into a third one (Fig. 1C).

3. Results

Examples of the construction of chimeras with one and two junctions will now be described. First, by using 5'KpnI/3'EcoRI, the

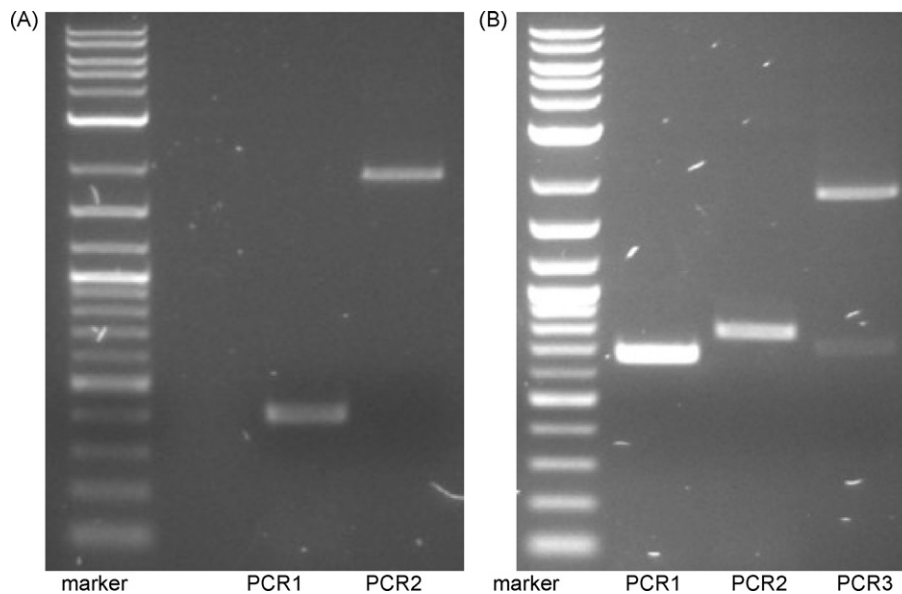


Fig. 2. Images of PCR products, run in 1.3% agarose gel, for creating the chimeras with one (A) or two junctions (B), as described in the text.

cDNAs of GlyR α 1 and GlyR β were subcloned into pUC19 (New England Biolabs), pGEMHE (Liman et al., 1992) and pcDNA3.1zeo+ (Invitrogen), which are *E. coli* cloning, *Xenopus* oocyte expression and mammalian expression vectors, respectively. These three vectors have mutually distinct sequences flanking their multiple cloning sites. The 5' sequencing primer UC5 of pUC19 and 3' sequencing primer CDNA3 of pcDNA3.1zeo+ (Table S1, Supplementary material) were used as the 5' and 3' primers, respectively, in the following sequential PCRs. All PCRs were set up as containing 10 \times Phusion enzyme buffer (New England Biolabs), 0.2 mM dNTP, 0.8 μ M of each primer, 0.4 ng/ μ l template and 0.02 U/ μ l Phusion enzyme (New England Biolabs) and run for 25 cycles following manufacturer's instructions. The final PCR products were subsequently subcloned into the pcDNA3.1zeo+ vector by 5'KpnI/3'EcoRI (New England Biolabs).

The first example describes the construction of a single-junction chimera with GlyR β amino acids 1–77 at the N-terminal and the GlyR α 1 amino acids 63–449 at the C-terminal. By following the procedure shown in Fig. 1B, we used primers UC5 (P1) and GlyRb63a (P2, Table S1, Supplementary material) to amplify GlyR β /pUC19 (PCR1). The size of the PCR1 product was verified by running it on an agarose gel (Fig. 2A). The PCR1 product was then diluted 100 times and 1 μ l was added to every 25 μ l of PCR2. The PCR2 also contained UC5 (P1) and CDNA3 (P3) as the primers and GlyR α 1/pcDNA3.1zeo+ as the template. The PCR2 product had the expected size (Fig. 2A) and was then subcloned into pcDNA3.1zeo+ by a general protocol. In brief, PCR2 was purified by using the QIAquick PCR Purification Kit (Qiagen) and, together with the pcDNA3.1zeo+ vector, was digested with KpnI and EcoRIHF (New England Biolabs) for 4 h. The digestion products were gel-purified by using the QIAquick Gel Extraction Kit (Qiagen) and ligated at room temperature for 1 h by using T4-DNA ligase (New England Biolabs). The ligation product was transformed into DH5 α competent cells (New England Biolabs) and single colonies were grown in LB medium containing ampicillin. The culture was miniprep using the QIAprep Spin Miniprep Kit (Qiagen) and recombination was identified by KpnI/EcoRIHF digestion. Six recombinant plasmids were sequenced, with five of them having the desired sequence. The exceptional one had one T to C base substitution, which was far from the junction though. This had apparently arisen from the limited fidelity of the Phusion polymerase.

The second example describes the construction of a chimera with GlyR α 1 amino acids 1–162 and 201–449 at the N- and C-terminals, respectively, between which are inserted the GlyR β amino acids 180–217. This construct required the introduction of two junctions, so the protocol illustrated in Fig. 1C was employed. PCR1 and PCR2 were performed as in the case of the single-junction chimera described above, except that the P2 primer and the template in PCR1, and the P3 primer and the template in PCR2, were substituted with GlyRa158b-, GlyR α 1/pUC19, GlyRb195a- and GlyR β /pGEMHE, respectively (Table S1, Supplementary material). The PCR2 product was diluted 100 times and 1 μ l was added to every 25 μ l of PCR3, which used UC5 (P1) and CDNA3 (P4) as primers and the GlyR α 1/pcDNA3.1zeo+ as template. The sizes of the PCR1, PCR2 and PCR3 products were verified by running them on an agarose gel (Fig. 2B). The PCR3 product was then subcloned into the pcDNA3.1zeo+ vector as described above in the case of creating the single-junction chimera. Five of the six recombinant clones tested had the expected sequence, while the exceptional one had a one-base frame-shift around the first junction. Since this frame-shift existed within the sequence of the P2 primer, the quality of the oligo synthesis was to blame.

4. Discussion

Here we have developed a simple but efficient protocol to construct chimeras by using multiple-template-based sequential PCRs. A chimera can be made between any two proteins with extreme flexibility and its construction is not limited by the availability of restriction sites. This protocol can also be used to introduce an artificial motif into any protein. For example, we have introduced the FLAG and Myc tags into the middle of GlyR α 1 and GlyR β by using this protocol. This protocol avoids the need for the purification of any intermediate PCR product. Most importantly, the chance of introducing desired junctions is nearly 100%. The only disadvantage, compared with other protocols, might be the extra step of subcloning both parental cDNAs each into three vectors. However, this is usually not an issue since most labs, like ours, would have the parental cDNAs in different vectors in place already for other purposes. Nevertheless, any inconvenience caused by this step would be outweighed by the simplicity, flexibility and high success rate of this protocol, especially when many chimeras need to be created.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jneumeth.2010.08.033](https://doi.org/10.1016/j.jneumeth.2010.08.033).

References

- Erreger K, Geballe MT, Kristensen A, Chen PE, Hansen KB, Lee CJ, et al. Subunit-specific agonist activity at NR2A-, NR2B-, NR2C-, and NR2D-containing N-methyl-D-aspartate glutamate receptors. *Mol Pharmacol* 2007;72:907–20.
- Grandori R, Struck K, Giovanielli K, Carey J. A three-step PCR protocol for construction of chimeric proteins. *Protein Eng* 1997;10:1099–100.
- Kelley SP, Dunlop JJ, Kirkness EF, Lambert JJ, Peters JA. A cytoplasmic region determines single-channel conductance in 5-HT₃ receptors. *Nature* 2003;424:321–4.
- Kirsch RD, Joly E. An improved PCR-mutagenesis strategy for two-site mutagenesis or sequence swapping between related genes. *Nucleic Acids Res* 1998;26:1848–50.
- Kuhn FJ, Knop G, Luckhoff A. The transmembrane segment S6 determines cation versus anion selectivity of TRPM2 and TRPM8. *J Biol Chem* 2007;282:27598–609.
- Liman ER, Tytgat J, Hess P. Subunit stoichiometry of a mammalian K⁺ channel determined by construction of multimeric cDNAs. *Neuron* 1992;9:861–71.
- Lynch JW. Molecular structure and function of the glycine receptor chloride channel. *Physiol Rev* 2004;84:1051–95.
- Padgett KA, Sorge JA. Creating seamless junctions independent of restriction sites in PCR cloning. *Gene* 1996;168:31–5.
- Young GT, Zwart R, Walker AS, Sher E, Millar NS. Potentiation of alpha7 nicotinic acetylcholine receptors via an allosteric transmembrane site. *Proc Natl Acad Sci USA* 2008;105:14686–91.