

Creation of Chimeric Junctions, Deletions, and Insertions by PCR

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1. Introduction

Recombinant polymerase chain reaction (PCR) (1) is the method of choice if one wants to modify a cloned DNA. It is a versatile technique that allows operations as different as creation of deletions, addition of small insertions, site-directed mutagenesis, and construction of chimeric molecules at any chosen location in the molecule of interest (*see Note 1*). This chapter describes in detail a simplification of the original recombinant PCR method. This fast and efficient method has been successful in fusing two different sequences with precision (2–4). It can also be used to create deletions or insert small fragments of DNA.

The method (*see Fig. 1*) relies on a “chimeric primer” (C) and two outside primers (A and B). The final product can be obtained in one or two rounds of PCR. The figure illustrates the construction of a chimeric molecule in which two different templates are joined. The creation of a deletion, or the introduction of a small insertion within a given template, would follow the same pathway (*see Note 1*). In all cases, the new junction is designed in the chimeric primer; the 3' half of the chimeric primer pairs with one of the templates (or one side of the deletion/insertion point), and its 5' half has homology with the other template (or the other side of the deletion/insertion point).

Both templates and the three primers (A–C) are placed in a reaction tube (**step 1**) and one PCR is performed. During the first cycles, only the primers A and C can prime exponential amplification (**step 2**). This amplification reaction gives rise to an “intermediate fragment” (**step 3**), that can itself act as a primer. One of its 3'-ending strands anneals to the second template and is extended (**step 4**). This extension provides a template (**step 5**) for exponential amplification of the final product using the primers A and B (**step 6**). To limit the amplification of the intermediate fragment to the first cycles of PCR, the chimeric primer is used at a lower concentration than the two outside primers (3,4).

2. Materials

1. Primers (*see Subheading 3.1.*)
2. DNA templates, linearized at a site outside the region to be amplified.

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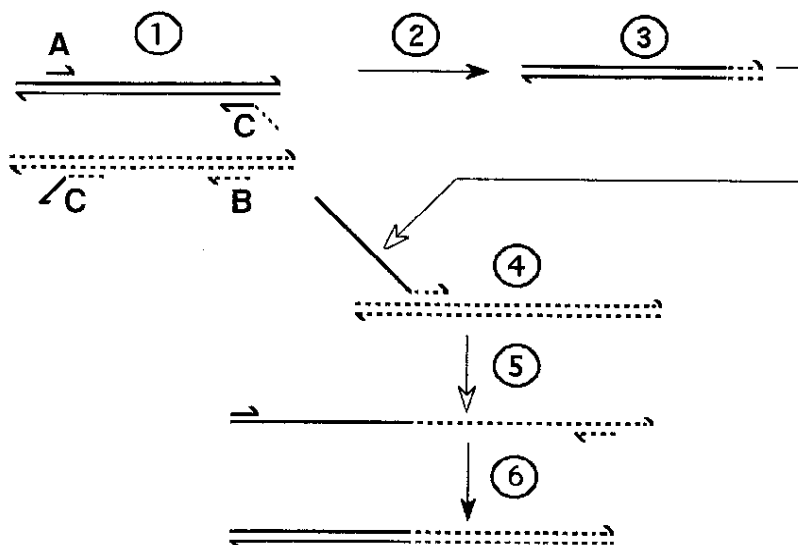


Fig. 1. Construction of a chimeric product. See text for explanation of **steps 1–6**. The dsDNA templates are the plain and dotted double lines. The outside primers are short, plain (**A**), or dotted (**B**) single lines. The drawing of the chimeric primer (half plain, half dotted) reflects its homologies to the templates. Half arrowheads indicate 3' ends. Closed arrows indicate amplification **steps (2 and 6)**.

3. GeneAmp PCR Core Reagent Kit (Perkin-Elmer, Foster City, CA) containing: AmpliTaq DNA polymerase (5 U/ μ L), Gene Amp dNTPs (10 mM solutions of dATP, dGTP, dTTP, and dCTP), GeneAMP 10 \times PCR buffer II (100 mM Tris-HCl, pH 8.3; 500 mM KCl), and 25 mM MgCl₂ solution.
4. Mineral oil.
5. Restriction endonucleases and buffers.
6. Agarose gel electrophoresis reagents and equipment.
7. Phenol:CHCl₃:isoamyl alcohol (25:24:1, v:v:v).
8. Ammonium acetate (7.5 M).
9. 100% Ethanol.
10. 70% Ethanol.

3. Methods

3.1. Design of Chimeric Primer

The chimeric primer is crucial because it contains the new junction and because sequences on each side of the junction serve as primers in different **steps** (i.e., **2** and **4** in **Fig. 1**) of the reaction. To allow priming by the nucleotides found on each side of the junction, the new junction should be placed in the middle of an oligonucleotide of sufficient length. Otherwise, the design of a chimeric primer should follow the classical rules of primer-design (**5**). Our chimeric primer was a 34-mer, with 17 bases homologous to one template and 17 bases homologous to the other. Some slightly longer chimeric primers (36- and 40-mer) have been used (**3,4**). See **Note 2** for more information on the design of the chimeric primer.

3.2. Design of Outside Primers

Fewer constraints apply to the design of the two outside primers, and therefore following the general rules of primer design should be adequate. In principle, the outside primers can be kilobase pairs away (within the limit of PCR feasibility) from the new junction. However, because their location determines the size of the product that will be cloned, the choice of their position is important. For several reasons, it is advantageous to plan the cloning of a fragment of few hundred nucleotides instead of a longer one: First, the smaller the fragment is, the less chance there is to find PCR-induced mutations in the final clone. Second, a smaller piece of DNA has to be sequenced to verify the integrity of the newly cloned DNA. This can be achieved by using primers that anneal close to the junction and have "built in" restriction sites at their 5' ends. Another approach is to choose primers that anneal further from the junction and clone with restriction sites that closely surround the chimeric junction in the final product. The choice between these two possibilities depends on the cloning strategy and the availability of cloning sites in the amplification product. If the chimeric fragment has to be cloned into a new vector, restriction sites unique in both vector and chimeric fragment can be engineered at the 5' ends of the two outside primers. If the chimeric junction has to be replaced by cloning in one of the original templates, restriction sites that exist in this template and in the chimeric junction have to be used.

The size of the outside primers can be different than the size of the chimeric primer. We have been successful with a 34-mer chimeric primer, and two outside primers of 23 and 20 nucleotides, respectively. If the size of the two outside primers is very different than half the size of the chimeric primer, series of cycles with different annealing temperatures can be performed (*see Note 3*).

3.3. Procedure

1. Assemble the components in one 50- μ L reaction containing: both templates (10 fmol each), both outside primers (25 pmol each), chimeric primer (1 pmol), 50 μ M each dNTP (*see Note 4*), 1 \times AmpliTaq DNA polymerase buffer, 1.5 mM MgCl₂ (*see Note 5*), and 2.5 U of AmpliTaq DNA polymerase.
2. Top PCR mix with 50 μ L of mineral oil.
3. Perform PCR as follows (*see Note 3*):
 - a. Three to five initial cycles to allow initiation and amplification (**steps 2–4 in Fig. 1**) using the chimeric primer: 95°C for 30 s, T1 (*see Note 3*) for 30 s, and 72°C for 1 min for each kilobase of intermediate fragment.
 - b. Twenty to thirty cycles to amplify the final chimeric product: 95°C for 30 s, T2 (*see Note 3*) for 30 s and 72°C for 1 min for each kilobase of chimeric product.
4. Directly analyze 10 μ L of the amplified DNA by standard procedures (restriction enzyme digests and electrophoresis, *see Note 6*).
5. To clone the new junction, clean the PCR product by extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1).
6. Transfer the aqueous phase to a sterile microcentrifuge tube. Remove the excess dNTP (it can inhibit T4 ligase) by ethanol precipitation. Add 1/2 vol of 7.5 M ammonium acetate and 2.5 vol of 100% ethanol.
7. Mix well and incubate at room temperature for 10 min.
8. Spin in a microfuge at maximum speed for 5 min.

9. Invert tube and allow to drain.
10. Wash pellet with 70% ethanol.
11. Resuspend the DNA in 20–50 μL of TE. Quantify the DNA by UV spectrophotometry and use for cloning.

4. Notes

1. The applications of this method are diverse; it allows the creation of:
 - a. Chimeric molecules: This method is well suited to cases in which the two molecules to be joined are unrelated. In the case where the two templates are fused in a region of homology, another PCR technique (6) might be preferred.
 - b. Deletions: A set of different deletions can be easily obtained from the same template by using a set of different chimeric primers and only one set of outside primers.
 - c. Insertions: The size of potential insertion using this technique is limited to the size of the chimeric primer. A restriction site sequence, sandwiched into a chimeric primer, could be introduced at will into any DNA.
2. As in the Megaprimer method (7), the technique described here uses a PCR product as a primer. It has been mentioned for the Megaprimer method (8) that mutations can be found in the final product because of the tendency of *Taq* polymerase to add nontemplated nucleotides at the 3' end of newly synthesized DNA strands. The frequency of these mutations should be low since 3'-ending DNA strands carrying nontemplated nucleotides should not prime well for the synthesis of the final product. Although we did not observe such mutations in the two final clones that we obtained and sequenced, this phenomenon could apply here, and we encourage the reader to refer to **Note 1** in **ref. 8** for a complete discussion.
3. In this technique, each half of the chimeric primer must anneal with its target. We have limited the size of our chimeric primer to 34 nucleotides, giving us 17 nucleotides for each half. Because our outside primers are 20 and 23 nucleotides in length, we felt that it was necessary to plan a first set of cycles with a lower annealing temperature to allow the stable annealing of each half of the chimeric primer in the **steps 2** and **4**. This precaution is not necessary if all the sequences with a "priming" role (each half of the chimeric primer and both outside primers) are close in length and in G+C content.

The temperature T1 is the approximate "annealing temperature" ($[\text{number of G} + \text{C} \times 4^\circ\text{C}] + [\text{number of A} + \text{T} \times 2^\circ\text{C}] - 10^\circ\text{C}$) of the less stable half of the chimeric primer. The temperature T2 is the approximate annealing temperature of the less stable of the two outside primers.
4. To limit the number of PCR-induced mutations in the final chimeric product, a low concentration of each dNTP (50 μM) is used.
5. The optimal MgCl_2 concentration can vary among different pairs of primers. It is wise to define the best MgCl_2 concentration in a test experiment (9). In fact, it is possible that the two consecutive PCRs that occur in the tube have incompatible requirements for MgCl_2 . If this is the case, the chimeric product can be obtained by a two-step method (2). In this alternative, the intermediate fragment obtained during the first reaction is extracted with phenol:chloroform:isoamyl alcohol and purified from excess primers by precipitation with isopropanol from 2 *M* NH_4OAc . The purified DNA is then used as a primer in a second reaction that contains the second template and the two outside primers.
6. It is often stated that the oil that tops the PCR has to be removed to properly load the DNA into the well of an electrophoresis gel. We found that this step is not needed if the pipet tip is cleaned with tissue paper (Kimwipe) just after it has been filled with a DNA sample. In fact we found that the oil left in the tube allows for longer conservation of the sample at 4°C.

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