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Megaprimer PCR

Application in Mutagenesis and Gene Fusion

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1. Introduction

Since the advent of the polymerase chain reaction (PCR), a variety of PCR-based procedures of mutagenesis have been developed through the use of synthetic primers encoding the mutation. Among these, the megaprimer method and related ones *(1–5)* remain some of the simplest and most versatile. Variations and improvements of the basic technique have been suggested over the past few years; these include a combination of magapriming and overlap extension, improvement of yield, use of single-stranded DNA, avoidance of unwanted mutations arising from nontemplated insertions by *Taq* polymerase, and the inclusion of various kinds of mutations, including multiple, nonadjacent ones *(2–11)*. The basic method (**Fig. 1**) requires three oligonucleotide primers and two PCRs (termed PCR-1 and -2 here) using the wild-type DNA as template *(1,2,8,10)*. The "mutant" primer is represented by M and the two "outside" primers by A and B. The M primer may encode a substitution, a deletion, an insertion, or a combination of these mutations, thus providing versatility while using the same basic strategy *(10)*. The first PCR (PCR-1) is performed using the mutant primer M and one of the outside primers, such as A (**Fig. 1**). The double-stranded product A-M is purified and used as a primer (hence the name megaprimer; **ref. *1***) in the second PCR (PCR-2) together with the other outside primer, B. Although both strands of the megaprimer may prime on the respective complementary strands of the template, the fundamental principles of PCR amplification ensure that only the one that extends to the other primer, that is, B in Fig. 1, will be exponentially amplified into the double stranded product in PCR-2. As mentioned, the wild-type DNA is used as template in both PCRs. This article describes the most optimized megaprimer method in our experience and has drawn freely on the improvements described by various authors *(3–28)*.

1.1. Improving the Yield of PCR-2

Poor yields from PCR-2 have sometimes been reported even when proper primer design (see above) was followed, especially when the megaprimer is large (0.8 kb and above). Although the exact reasons remain unclear, the most likely reasons are

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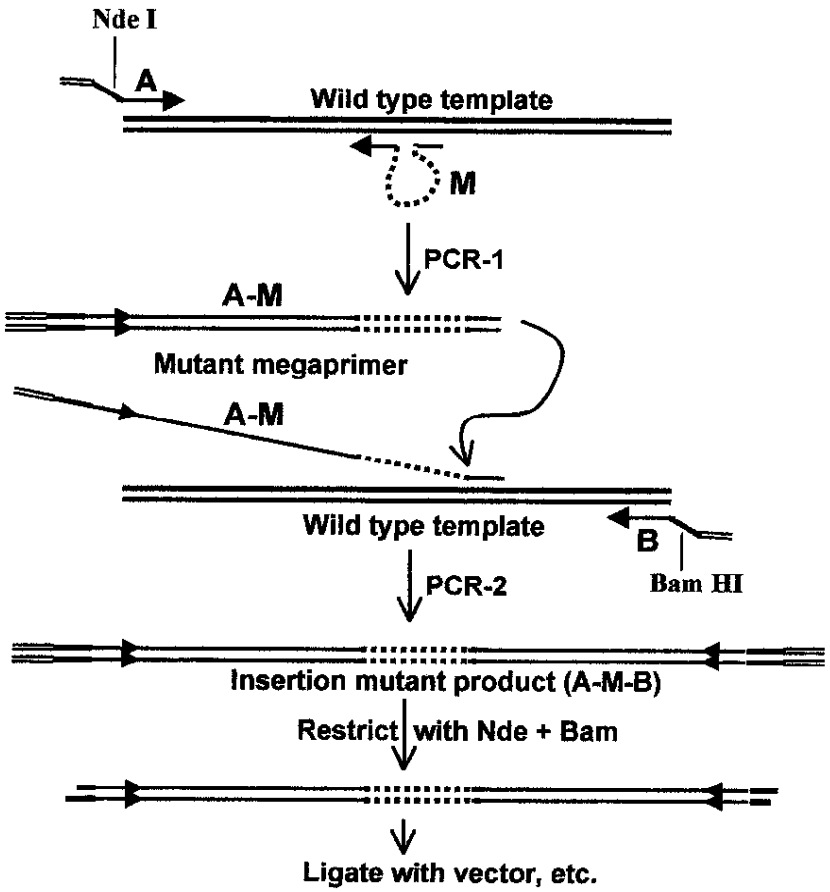

Fig. 1. The basic megaprimer method. Primers A, B, M, and the priming strand of the megaprimer AM are indicated by thinner lines with arrowhead, while the thicker double lines represent the wild type template (usually part of a plasmid clone, not shown). Primers A and B contain restriction sites (e.g., *Nde*I and *Bam*HI) indicated as thicker regions, and extra "clamp" sequence at the 5′ end indicated by double lines. The sequence to be inserted is shown as the dotted region in primer M and the subsequent PCR products. The final product containing the insertion is restricted and cloned.

the unique features of the megaprimer, viz., its double-stranded nature and large size. Strand separation of the double-stranded megaprimer is essentially achieved in the denaturation steps of the PCR cycle. Under some conditions, however, self-annealing of the megaprimer apparently tends to reduce the yield of the product *(4)*.

Various solutions to this problem have been suggested. In one approach, a biotin tag is added to the 5′ end of primer A, which would generate a biotin-labeled megaprimer in PCR-1. After denaturation, the biotinylated strand of the megaprimer is purified on avidin attached to magnetic beads *(26)*. In another method *(27)*, the use of two parallel templates allowed the inclusion of two outside primers as well as the megaprimer in PCR-2, resulting in a direct amplification of the final product. Use of a "one-tube" method (described above), when properly optimized, should eliminate loss of megaprimer during the purification step. Other strategies for increasing the yield of PCR-2 involve optimizing the concentrations of both template and megaprimer. In

some instances, the use of higher amounts of template (in the microgram range, as opposed to nanogram quantities used in standard PCR) in PCR-2 has been shown to dramatically increase the product yield *(4)*. Unfortunately, higher concentrations of template also tend to increase mispriming by a megaprimer with a mismatched 3′ end (our unpublished results). Thus, a more effective strategy may be to increase the amount of the megaprimer. A method that we have found useful is to perform the first several cycles of PCR-2 with the megaprimer only. After this initial asymmetric PCR, the small primer is added *(11)*. In an optimization of this strategy *(28)*, the starting concentration of megaprimer is increased to 6 µg (from 25 ng) per 100 µL of PCR-2. We have adopted a combination of the last two approaches in this article.

2. Materials

2.1. Template

About 100 ng of DNA template to be mutated (e.g., a gene cloned in a plasmid).

2.2. Primers

100 pg of oligonucleotide primers A and B, and 50 ng of mutant primer M; one primer, say A, in the opposite sense, and other primer, B, in the same sense as the mutant primer M (**Fig. 1**). Include restriction sites, preferably unique, in these primers so that the final product can be efficiently digested with restriction enzymes and cloned. The mutant primer may be designed to contain a point mutation, or insertion, or deletion, as desired (*see* **Notes 1** and **2**).

2.3. PCR Buffer

10× PCR buffer for *Pfu* polymerase (Stratagene Cloning Systems, La Jolla, CA) is: 200 m*M* Tris-HCl (pH 8.0–8.3); 100 m*M* KCl; 20 m*M* $MgCl_2$; 60 m*M* ammonium sulfate; 1% Triton X-100 100 µg/mL nuclease-free BSA; the buffer is usually supplied with the enzyme by most manufacturers.

2.4. Deoxyribonucleotides

The final dNTP concentration is generally 200 µ*M* for each nucleotide. Make a stock dNTP mix containing 2 m*M* of each dNTP (dATP, dCTP, dGTP, dTTP); we make it by adding 50 µL of 10 m*M* stock solutions of each nucleotide, available commercially, into 50 µL H_2O, to produce 250 µL of the mix.

2.5. Analysis and Purification of DNA

A system for purifying the PCR products, such as gel electrophoresis, followed by recovery of the appropriate DNA band in the excised agarose fragment *(8)*.

Wherever needed in this procedure, use deionized (e.g., Millipore) autoclaved water.

3. Method

3.1. PCR-1: Synthesis of the Megaprimer

1. It is assumed that the reader is familiar with standard PCR protocols. Use the following recipe for the first PCR. Make the following 100-µL reaction mix in an appropriate microcentrifuge tube (0.5 or 1.7 mL, dictated by the heating block of your thermal cycler): H_2O (75 µL); 10× PCR buffer (10 µL); 2 m*M* each of dNTP mix (10 UL; the final

concentration of each nucleotide is 200 μ*M*); Primer A (50 pmol); Primer M (50 pmol) (**Note 3**); DNA template (10–100 ng); and 2.5 U *Pfu* polymerase (0.5 μL);or 2.5 U *Taq* plus 0.1 U *Pfu* polymerase) for a total of 100 μL.

2. Vortex well to mix, then spin briefly in a microfuge. If the thermal cycler has a heated lid, then proceed to do PCR; otherwise, reopen the tube, overlay the reaction mixture with enough mineral oil to cover the reaction (~100 μL for a 0.5-mL microfuge tube), then close cap. The tube is now ready for thermal cycling.
3. Perform PCR-1 using the following cycle profiles. Initial denaturation: 94°C, 3 min; 30 to 35 main cycles: 94°C, 1 min (denaturation): T° (depending on the T_m of the primers), 2 min (annealing); 72°C, appropriate time, depending on product length (extension); and final extension 72°C, 1.5 × *N* min.

   After synthesis, the samples are maintained at 4°C (called "soak" file in older Perkin–Elmer programs) for a specified time. Some instruments lack an active cooling mechanism and keep samples at an ambient temperature of about 20°C by circulating tap water around the heat block, which appears to be adequate for overnight runs; others just shut off at the end of the final extension.
4. After PCR, proceed directly to the next step if there is no oil overlay. Otherwise, first remove the oil as follows. (If oil is not removed completely, the sample will float up when loaded in horizontal agarose gels!). Add 200 μL of chloroform to each tube. The mineral oil and chloroform will mix to form a single phase and sink to the bottom of the tube. Spin for 30 s in a microfuge. Carefully collect ~80 μL of top aqueous layer and transfer to a fresh Eppendorf tube.
5. Purify the megaprimer using any standard procedures such as gel purification (*see* **Chapter 18**) and use it in PCR-2 below.

3.2. PCR-2: Synthesis of the Mutant Using the Megaprimer

1. Reconstitute 100-μL PCR as follows: 10× PCR buffer (10 μL); 2 m*M* each of dNTP mix (10 μL; final concentration of each nucleotide is 200 μ*M*); All of the recovered megaprimer (A-M) from the previous step (20–50 μL); DNA template (0.2 μg); Make up volume to 100 μL with H_2O; and mix well.
2. Start reaction essentially as described for PCR-1, except that a "hot-start" is preferred (*see* **Note 4**) and is performed as follows. When the reaction is in the annealing step of the first cycle, open the cap briefly, quickly add 0.5 μL of *Pfu* polymerase (2.5 U, or 2.5 U *Taq* plus 0.1 U *Pfu* polymerase), and mix by pipetting. Close the cap and let PCR continue.
3. After five cycles, when the reaction is again at an annealing step, promptly add 50 pmol of primer B, mix well, and let PCR continue another 30 cycles. (The small amounts of primer B and *Pfu* polymerase do not contribute significantly to the total reaction volume and, therefore, have been ignored in the volume calculations).
4. Do another PCR in parallel, using primers A and B (but no megaprimer) and the same wild-type template; use an aliquot (5 μL) of this PCR as a size marker when analyzing PCR-2 by gel electrophoresis. This will also help in identifying the real product (in PCR-2) among the wrong ones that sometimes result from mispriming.
5. Gel purify the final mutant PCR product essentially as described earlier for the purification of the megaprimer (*see* **Notes 5** and **6**).

4. Notes

1. Design of the mutant primer. Perhaps the most unique feature of the megaprimer method is that the product of one PCR becomes a primer in the next, which creates the following potential problem. *Taq* polymerase, as a result of its lack of proofreading activity, tends

to extend the product DNA beyond the template by adding one or two non-templated residues, predominantly As *(12)*. When the product is used as a primer in the next round of PCR (PCR-2), these nontemplated A residues may not match with the template and, therefore, will either abrogate amplification *(13–15)* or produce an undesired A-substitution. A variety of solutions to this problem have been recommended *(5,8,10,16)*. The first is to design the mutant primer such that there is at least one T residue beyond the 5′ end of the primer sequence in the template. Thus, when the complementary strand incorporates a non-templated A at the 3′ end, it will still be complementary to the other strand. If the template sequence does not permit this, a second solution is to use a mixture of *Taq* and *Pfu* DNA polymerases in 20:1 ratio in PCR-2 *(3)* or to use *Pfu* exclusively. This is what we have recommended in this chapter. The 3′ exonuclease activity of *Pfu* should remove any mismatch at the 3′ end of the megaprimer; however, this proofreading ability also necessitates the addition of at least 10 perfectly matched bases on both the 5′ and 3′ ends of the mutagenic primer *(8,10,13,17)*. Finally, one can use enzymes, such as mung bean nuclease, that will remove nontemplated nucleotides from the megaprimer *(16)*. In addition to these unique considerations, the general rules of primer design described below, should be followed.

2. Length of the megaprimer. Try to avoid making megaprimers (A-M) that approach the size of the final, full-length product (gene) A-B (*see* **Fig. 1**). Briefly, if M is too close to B, it will make separation of AB and AM (unincorporated, left-over megaprimer) difficult after PCR-2. When the mutation is to be created near B, one should make an M primer of the opposite polarity, and synthesize BM megaprimer (rather than AM), and then do PCR-2 with BM megaprimer and A primer. When the mutation is at or very near the 5′ or 3′ end of the gene (within 1–50 nucleotides), there is no need to use the megaprimer method; one can simply incorporate the mutation in either A or B primer and do a straightforward PCR using A and B primers! For borderline situations, such as when the mutation is, for example, 120 nucleotides away from the 5′ end of the gene, incorporation of the mutation in primer A may make the primer too big to synthesize; or else, it will make the megaprimer AM too short to purify away from primer B. In such a case, simply back up primer A to a few hundred bases further upstream to make the AM megaprimer longer. In general, realize that primers A and B can be located virtually anywhere on either side of the mutant primer M, and therefore, try to utilize this flexibility as an advantage when designing these primers.
3. Molar amount of megaprimer. Because the megaprimer is large, one needs to use a greater quantity of it to achieve the same number of moles as a smaller primer. Example: 50 pmol of a 20 nt-long single-stranded primer will equal 0.3 μg; however, 50 pmol of a 500 nt-long double-stranded megaprimer will equal 6 μg. A good yield and recovery of megaprimer is, therefore, important. If needed, do 2× 100 μL PCRs to generate the megaprimer. There is no need to remove the template DNA after PCR-1 because the same DNA will be used as template in PCR-2.
4. "Hot start" PCR-2. The hot-start technique used in PCR-2 works just as well as the more expensive commercial methods. Hot start tends to reduce false and nonspecific priming in PCR in general *(29)* and is particularly useful in PCR-2 of the megaprimer method (our unpublished observation).
5. Poor yield of mutant. If the final yield is poor, the surest strategy is to amplify a portion of the gel-purified mutant product in a third PCR (PCR-3) using primers A and B and hot start. This may also be necessary if PCR-2 produces nonspecific products in addition to the specific one. Before PCR-3 is conducted, however, it is very important to ensure that the mutant product of PCR-2 is well separated from the wild type template in the gel purification; otherwise, PCR-3 will amplify the wild-type DNA as well. The final gel-

purified mutant DNA (from either PCR-2 or PCR-3) is ready for a variety of applications, such as sequencing *(30–36)* or cloning *(33,34)*.

6. Single-tube methods. Recently, various investigators have reported successful modifications of the megaprimer method in which the purification step is not required. One involves cleavage of the template, coupled with enzymatic removal of PCR-1 primers, to ensure amplification of the correct product in PCR-2 *(24)*. A second possibility is to exploit the unusually high T_m of the megaprimer by designing a short, low Tm flanking primer for PCR-1, and a long flanking primer for PCR-2. This enables the use of a higher Tm for PCR-2 such that it will only allow annealing of the appropriate flanking primer *(25)*. A third method uses a limiting amount of the first flanking primer, such that when the second flanking primer is added, the principle product will be the mutant DNA *(17)*. Since we have not tested any of these modifications, the interested reader is advised to consult the original papers.

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