Polymerase chain reaction (PCR)-mediated overlap extension is an important tool for the introduction of site-specific mutations and for the generation of recombinant gene constructs. The standard method used for overlap extension (4,8) demands addition of primers to a multiple-cycle PCR to obtain a sufficient amount of overlap extension product. The large number of cycles required for the overall procedure may cause problems of reduced fidelity, and the multiple rounds of the overlap extension reaction may result in loss of information and introduce a bias when the procedure is used for making genetic libraries. We describe a method that may reduce such problems by enabling high overlap extension efficiency after mixing two DNA fragments with complementary bases without addition of primers.

Among the thermostable DNA polymerases, Taq is commonly used for PCR amplification of DNA fragments because of its high efficiency. However, the low fidelity of Taq results in a high risk of introducing mutations when using the standard method for PCR-mediated overlap extension (1). Using few rounds in the amplification PCR and only one cycle in the overlap extension reaction will minimize this risk. Moreover, a portion of fragments amplified by Taq have nontemplated nucleotide addition (2). These extra nucleotides cause a reduced efficiency in blunt-end ligations and in site-directed mutagenesis of double-stranded DNA. The reduced efficiency can partly be overcome by “end polishing” the Taq-generated DNA fragments with polymerases possessing 3′–5′ exonuclease activity (3) such as Klenow, T4 or Pfu.

DNA polymerases before overlap extension or by mixing Taq with Pfu in both the amplification and overlap extension reactions. We take advantage of this knowledge to generate PCR fragments without extending bases to allow efficient overlap extension.

In our test system (Figure 1), we PCR-amplified DNA fragments from two different plasmids (tubes A and B), giving rise to an internal ribosome entry site (IRES) sequence and an hph gene sequence. The overlap extension was performed as a PCR with 5 cycles but without addition of primers, which thereby gives the potential for a single round of extension. The cycling was done 5 times to increase the probability of overlap annealing.

Figure 2 shows the effect of end polishing on the overlap extension reaction. Protocols of non-PCR-mediated overlap extension of PCR fragments by exonuclease end polishing are described in the legend of Figure 2. The DNA fragments (IRES: 664 bp and...
Hph: 1069 bp) were PCR-amplified with Taq and, after purification, treated for 30 min with either Klenow, T4, Pfu or Taq DNA polymerase. For each enzyme, the reaction was either not supplemented with nucleotides, supplemented with dGTP, or supplemented with dNTP. After treatment, the reaction mixture was immediately used in an overlap extension reaction with Taq. The overlap extension efficiency of IRES-hph (1716 bp) is low when Taq alone is used. When the fragments are not supplemented with nucleotides or are supplemented with dGTP or dNTP in the Klenow and T4 reactions, there is an improvement in the overlap extension efficiency. The same improvement is seen for the fragments supplemented with dGTP or dNTP in the Pfu reaction. This improvement is probably due to the 3′→5′ exonuclease activity of the enzymes that cleaves off the extended bases generated by Taq and thereby improves the ability of the complementary regions to prime the other fragment. With no nucleotides added to the Pfu reaction, there is little overlap extension, probably because of 3′→5′ exonuclease activity of Pfu cleaving off bases in the complementary region. After the first cycle in the overlap extension reaction, the gap is probably filled out by Taq, but then a high frequency of non-templated nucleotide addition reduces the overlap extension efficiency.

Figure 3A displays the effect on PCR amplification, with a mixture of Taq and Pfu for IRES (A) and hph (B). Taq buffer (50 mM KCl, 1.5 mM MgCl₂ and 10 mM Tris-HCl, pH 9.0 at room temperature) was used because we found that Pfu is as efficient in Taq buffer as in Pfu buffer. When using only Pfu (A8, B8), the efficiency of the PCR amplification is low compared to that using Taq (A1, B1) (6). If a high or medium amount of Taq is mixed with a low or medium amount of Pfu, the efficiencies are comparable to reactions with only Taq (A1 to A2–A4 and B1 to B2–B4). If a low amount of Taq is mixed with a high amount of Pfu, the efficiencies are decreasing but are still higher than if only Pfu is used (A8 to A5–A7 and B8 to B5–B7). Even if only 0.1 U Taq and 2.5 U Pfu are used, the efficiency is much higher than if only Pfu is used.

In the overlap extension reactions in Figure 3B, the same mixture of Taq and Pfu has been used as in Figure 3A. In A + B1, fragments from A1 and B1 are mixed in an overlap extension reaction with only Taq, and this gives a low overlap extension efficiency. In A + B2–A + B7, different mixtures of Taq and Pfu are used, as in Figure 2A, and all the reactions show high overlap extension efficiencies. For A + B8, the efficiency is as low as for A + B1, and this might be because of the low progressivity of Pfu. The results from Figures 2 and 3 show that mixing of Pfu and Taq in the reaction gives a more efficient overlap extension than end polishing, which is probably because of the presence of 3′→5′ exonuclease activity in the reaction.

This method demonstrates a way of improving overlap extension of DNA fragments. This is done in one cycle of elongation in the overlap extension.

Benchmarks

Figure 3. PCR amplification with mixed enzymes and the effect on overlap extension. Gel electrophoresis was accomplished with 1% SeaKem agarose gels stained with ethidium bromide. The numbers correspond to the enzyme amount of Taq and Pfu DNA polymerases in the reactions. Lane 1 (number 1 from A [IRE5 fragment], B [hph fragment] and A+B [overlap extension]): 3 U of Taq and no Pfu; lane 2: 3 U Taq, 0.5 U Pfu; lane 3: 2 U Taq, 1 U Pfu; lane 4: 1 U Taq, 2 U Pfu; lane 5: 0.5 U Taq, 2.5 U Pfu; lane 6: 0.25 U Taq, 2.5 U Pfu; lane 7: 0.1 U Taq, 2.5 U Pfu; and lane 8: no Taq, 2.5 U Pfu. All reactions were performed in Taq buffer. (A) PCRs were done with 0.2 mM dNTP and 50 pmol of each primer in a 100-µL solution. The PCR amplification of IRES (A) consisted of 15 cycles at 95.5°C for 1.3 min, 45°C for 1.3 min and 72°C for 2 min, preceded by 3 min at 95.5°C and followed by 5 min at 72°C. PCR conditions were adjusted to the presence of a highly GC-rich area in the IRES sequence. The PCR amplification of hph was done as with IRES but in only a 12-cycle PCR and with a denaturation temperature of 94°C. (B) Overlap extension with different amounts of Taq and Pfu DNA polymerases carried out without use of primers. Fragments shown in Figure 3A were purified and used in overlap extension reactions as described in Figure 2.
reaction without addition of primers. By lowering the total number of cycles in the two reactions, the risk of introducing mutations in the DNA sequence is reduced. The improvement of the method may be important for generation of genetic libraries where reduction of the overlap extension to one cycle, together with high assembly efficiency, may be crucial for preserving the diversity and maintaining a balanced representation.

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Combination of Direct DNA Sequencing with Degenerate Primer-Mediated PCR and 5′/-3′-RACE to Screen Novel cDNA Sequences

Cloning of new DNA sequences using amino acid sequence data is a cumbersome and time-consuming procedure that usually involves screening of the cDNA or genomic libraries with 32P-labeled probes or antibodies, subcloning the positive clones, purification of plasmid DNA and, finally, sequencing of inserted DNA fragments. Polymerase chain reaction (PCR)-mediated amplification of cDNA with degenerate oligonucleotide primers allows isolation of the corresponding cDNA without screening libraries by traditional hybridization. It is still, however, necessary to subclone several PCR fragments before sequencing because the frequently used T7 Sequenase® Version 2.0 DNA Sequencing Kit (Amersham, Arlington Heights, IL, USA) is not designed for use with degenerate primers. Another option is the direct sequencing of the purified PCR products with degenerate primers either by a fairly complicated cycle sequencing method (7) or by automated DNA sequencer (2,6). The rapid amplification of cDNA ends (RACE) technique has been very useful in extending the sequence towards 5′ and 3′ ends, although this method also requires subcloning of many PCR products or their purification by commercial kits before direct sequencing. Taking advantage of existing protocols for direct detergent-enhanced sequencing with specific primers (3,4,8) and the Marathon™ cDNA Amplification Kit (CLONTECH Laboratories, Palo Alto, CA, USA), we have developed a simple, reliable and reproducible method that combines the modified direct DNA sequencing with the degenerate primer-mediated PCR and the RACE protocol. We believe that this protocol will significantly reduce the time and expenses required for cloning compared with the traditional