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Construction of recombinant DNA by exonuclease recession

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ABSTRACT

We describe a new exonuclease-based method for joining and/or constructing two or more DNA molecules. DNA fragments containing ends complementary to those of a vector or another independent molecules were generated by the polymerase chain reaction. The 3′ ends of these molecules as well as the vector DNA were then recessed by exonuclease activity and annealed in an orientation-determined manner via their complementary single-stranded regions. This recombinant DNA can be transformed directly into bacteria without a further ligase-dependent reaction. Using this approach, we have constructed recombinant DNA molecules rapidly, efficiently and directionally. This method can effectively replace conventional protocols for PCR cloning, PCR SOEing, DNA subcloning and site-directed mutagenesis.

INTRODUCTION

The use of the polymerase chain reaction (PCR) in nucleic acid research has provided a convenient way to amplify and construct genes. In most cases, subcloning of PCR products is required for further manipulation and generally involves the incorporation of restriction sites at the ends of PCR products (1,2), or blunt-ended ligation of PCR products into the vector (3). However, cloning of PCR products is often less straightforward than anticipated. The main problems which need to be solved in blunt-ended cloning of PCR products include the removal of extra nucleotides added to the 3′ ends by Taq DNA polymerase (3,4), the prevention of non-recombinant backgrounds, and the low efficiency of the blunt-end ligation reaction. The T/A cloning system (Invitrogen) has been used to overcome the extra nucleotide problem at the 3′ end but an extra dAMP is automatically inserted. This generates additional problems especially in expression studies as it will alter the reading frame. Cohesive end cloning (provided by the incorporation of restriction sites at the 5′ end of PCR primers) is a good alternative to blunt end cloning. Nonetheless, both methods require several steps of DNA fragment purification, ligase-dependent ligation and colony selection to determine the correct orientation of the insert and are labor intensive, time consuming and/or of low efficiency.

Strategies for ligase-free cloning of PCR products have been developed to overcome some of these problems. The recombinant circle PCR (RCPCR) technique generates circular DNA through heterologous annealing of sequence-overlapped ends on different PCR products (5,6). These circular DNA forms can be transformed directly into bacteria without a ligation procedure. However, this method requires either multiple sets of PCR primers or PCR reamplification of sequence-overlapped molecules to splice insert and vector DNA together (7). Also in these applications, both insert and vector DNA must be amplified. Vector DNA amplification adds to the limitation on the size of the DNA fragment that can be amplified by Taq DNA polymerase. An alternate strategy is to create sequence-specific, single-stranded ends on both PCR products of insert and vector ends (8–10), then splice them through a sequence homologous annealing process. In most applications, single-stranded ends are generated by the 3′ to 5′ exonuclease activity of T4 DNA polymerase (8,9) with the overlapped sequence specifically designed and incorporated into PCR primers for both insert as well as vector DNA amplification. A specified, unique length of 3′ recessed ends is then created in the presence of specific dNTP and T4 DNA polymerase and the circular form of DNA, assembled through sequence overlapped ends, is then ready for transformation. An alternative way to produce single-stranded ends employs uracil DNA glycosylase (UDG). This enzyme cleaves all dUMPs which are incorporated into the PCR primers (11). However, some of these methods, like RCPCR, require either multiple PCR primer sets, vector amplification, or vector end sequence modifications. Others require a double restriction enzyme cleavage of vector DNA followed by a ligation process for directional cloning (9, 10).

Recently we developed a simple exonuclease-based strategy to construct PCR products. This protocol has the advantages of the ligase-free PCR cloning technique but none of the disadvantages, such as vector amplification, enzymatic manipulation or lack of directional cloning. Here we document its application for constructing bacterial gene fusion mutants for an octamer binding protein (12).

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MATERIALS AND METHODS

Vector DNA, pGEX-KG, was linearized at the Eco RI site and gel purified. Approximately 100 ng of template DNA was amplified using a standard polymerase chain reaction for 30 cycles and the PCR products chloroform extracted and salt precipitated. Approximately 0.5 μg of linearized vector DNA and PCR amplified DNA(s) were mixed with 1 unit of T4 DNA polymerase (Promega) in the absence of dNTPs at 37°C for 2 min in 20 μl of 33 mM Tris-acetate (pH 7.9) containing 66 mM KOAc, 10 mM Mg $(OAc)_2$, 0.5 mM DTT and 100 μg/ml BSA. Following enzyme inactivation at 70°C for 10 min, the mixture was cooled to annealing temperature (determined by the Tm value of each overlapped sequence region, generally we chose the temperature at about 30°C to 37°C) and held at that temperature for at least 2 hrs. One μl of 2 mM dNTPs, 1 μl of 10 mM DTT and another 2 unit of T4 DNA polymerase were added and the reaction mixture kept at the annealing temperature for an additional 30 min. Then without a ligation step, the circularized DNA was directly transformed into competent XL1-blue cells without further manipulation. Individual colonies were randomly selected from each construct for the following expression studies. The fusion proteins were induced with 0.2 mM IPTG for 1 hr. before the cells were harvested and the proteins purified by binding to glutathione agarose beads as described (13). SDS-PAGE and Western blot analyses were as described previously (12).

RESULTS

Basic strategy

All applications of our method involve two steps. The *first* utilizes bipartite oligonucleotide primer/adapters. In the most common application, one portion primes the PCR extension of one DNA molecule or has sequence homology to the ends of a DNA fragment, and the other portion is complementary to a second DNA molecule. Unlike previously reported protocols, no extra specific sequences are incorporated at the ends of the insert and vector DNA fragments (8,9,11). The *second* step utilizes exonuclease to generate unique ends. The DNA segments are recessed at the ends by a controlled, strand-specific exonuclease reaction (e.g. the 3′ exonuclease activity of T4 DNA polymerase manifested in the absence of dNTP incorporation). After single-stranded overhang ends are created, such DNA molecules of insert and vector are annealed via the complementary bases to form recombinant circular DNA. The remaining gaps are filled-in and the closed circles are transformed into a bacterial host.

Below we provide examples of how the method can be applied to PCR subcloning and deletion mutant construction. In both cases, the target molecule is a 2.7 kb cDNA of nonO, an octamer DNA binding protein cloned from mouse B cells (12). The vector molecule in both cases is pGEX-KG, a glutathione-S-transferase encoding bacterial expression vector (13).

PCR subcloning to generate truncations

Figure 1A summarizes the steps involved in the protocol. Primers 1 and 2 are for PCR amplification. The length chosen for primers 1 and 2 depends on the GC content in the sequences used for priming. Generally we chose a primer length with a Tm value of about 60°C. The 3′ end of each primer is complementary to the end of the target DNA and the most 5′ 12 nucleotides are complementary to sequences on each side of the Eco RI cloning site of pGEX-KG. The total length of primer 1 and 2 are 33 and 34 oligonucleotides. A detailed view of the primer-substrate complementarity is given in Figure 1B. An additional dAMP was introduced into primer 2 following the vector sequence to create

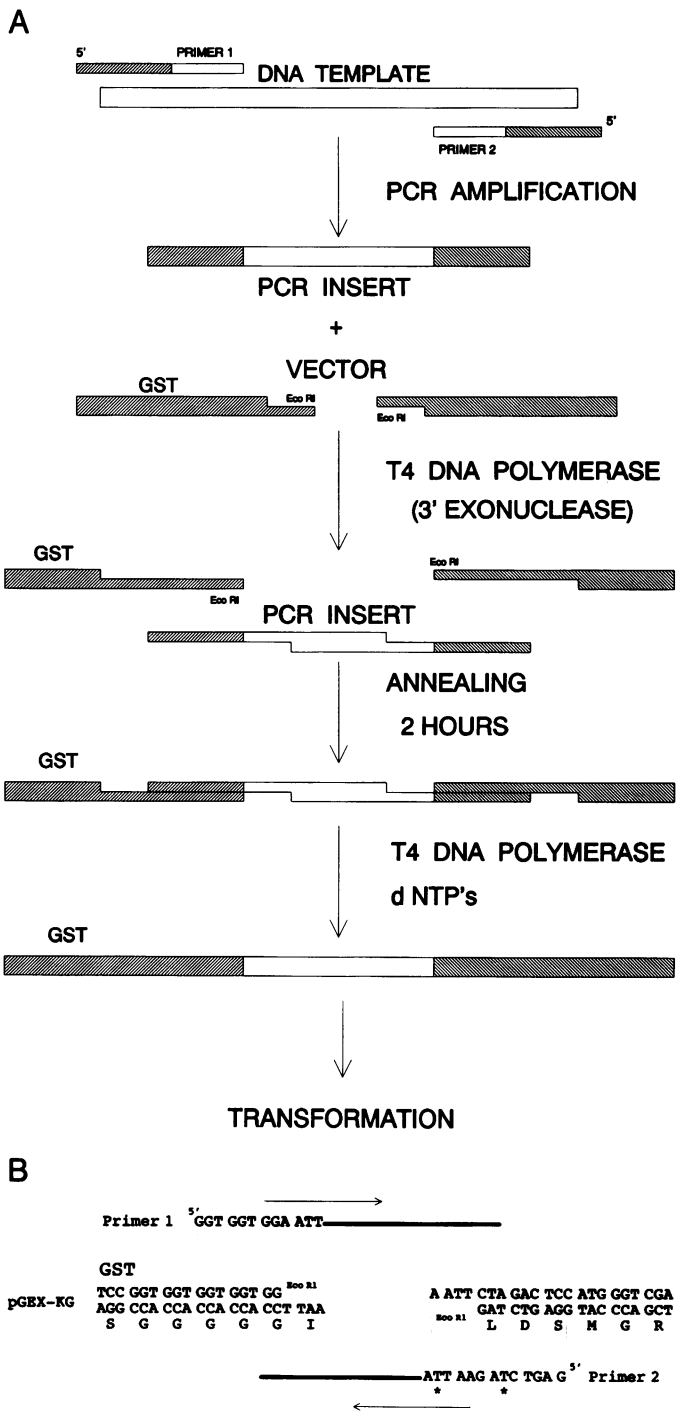

Figure 1. Construction of recombinant DNA by exonuclease recession. **A.** Strategy for PCR subcloning. The hatched boxes represent vector sequences. Identical hatch directions within boxes indicates sequence complementarity. The narrow part on the box represents single-stranded DNA, created initially for the vector by restriction enzyme cleavage, and then for both vector and insert, by T4 DNA polymerase recession. Details of cloning and other methods are described in the text. **B.** Complementary sequences of PCR primers 1 and 2. The 5′ end bases complementary to the vector ends are marked. Solid bars represent the PCR priming portion and arrows indicate the direction of DNA polymerization. Stars in primer 2 represent the stop codons incorporated.

two in-frame stop codons for the constructed genes. Thus the PCR product obtained from these primers incorporates unique vector end sequences on each of its defined ends. Thirty cycles of standard PCR were carried out, then a controlled strand-specific exonuclease reaction (T4 DNA polymerase, 3′ to 5′ exonuclease) was applied to both PCR product and vector DNA. With a DNA to enzyme ratio recommended by the manufacturer (Promega) and a short reaction time of 2 minutes, the T4 DNA polymerase recessed the 3′ ends beyond the overlapped sequence regions but did not degrade the DNA fragments extensively (Figure 1A). After the creation of single-stranded complementary ends, recombinant circular DNA molecules were generated by annealing together the insert and vector DNAs for two hours. The remaining single-stranded gaps were filled-in by T4 DNA polymerase (alternatively, Klenow could be used) by adding dNTPs to the reaction. It has been reported that single-stranded gaps of up to 19 nucleotides do not alter transformation efficiency (6). Therefore, it is theoretically possible to eliminate this step. However, the fill-in steps serve an additional purpose of blunt-ending the non-annealed vector molecules. This eliminates their recircularization by self-annealing and reduces transformation background.

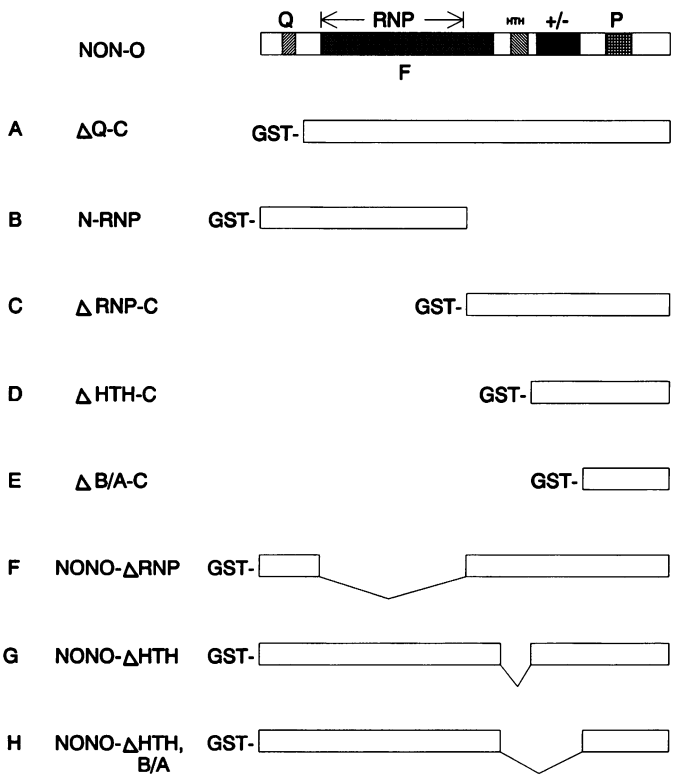

Figure 2. NonO truncation and deletion mutant constructs. Open boxes for all clones represent individual PCR products. The cloning strategies are illustrated in Figure 1 (clones A−E) and Figure 4 (clones F−H). Domains marked on the nonO protein are as described previously (12). The glutathione-S-transferase gene was linked at the 5′ end of each individual clone.

Examples of nonO truncation mutants generated by this method are shown in Figure 2 (A−E). All of these glutathione-S-transferase fusion proteins were expressed in *E. coli* and migrated at the predicted size on an SDS-PAGE gel (Figure 3A). Western blot analysis indicated that each mutant retained serologic epitopes, confirming that the appropriate translation frame was maintained (Figure 3B). DNA sequencing through the junction regions of these clones (data not shown) showed that no nucleotides were lost or gained during the joining process.

Internal deletion mutants

As with PCR SOEing, our protocol for generating internal mutations (e.g. fusions, insertions, or deletions) conceptually requires two target DNA molecules (Figure 4). For deletion and insertion mutants, target DNA 1 and 2 use the same template; for chimeric fusions, DNA 1 and 2 use different templates. The protocol shown in Figure 4 illustrates internal deletions or chimeric gene construction. Two sets of oligonucleotide primers were designed for each PCR amplification. Primers 1 and 4 were designed using the same strategy as for PCR subcloning. Primer 2 has a 12 nucleotide overlap with target DNA 2 at its 5′ end. The 3′ half of primer 2 and all of primer 3 are complementary to the boundary specified (in this case, at the breakpoints of the deletion). All of the primers were designed to maintain the original reading frame in deletion constructs. Following the PCR reaction that generated DNA fragments PCR1 and PCR2, 0.5 μg of Eco RI digested pGEX-KG vector DNA was added, and

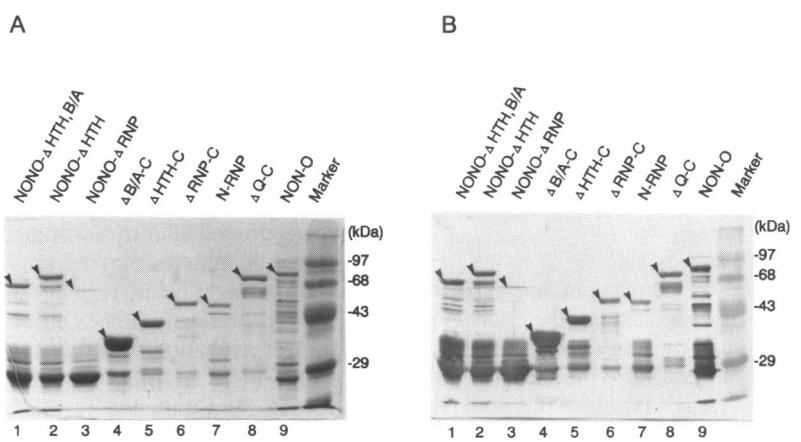

Figure 3. Verification of constructs by bacterial expression. **A.** Coomassie stain of bacterial fusion proteins. *E. coli* expressed fusion protein was affinity purified (13) and analyzed on a 10% SDS-PAGE gel. Labels over lanes correspond to the constructs shown in Figure 2. Arrowheads denote the predicted size of authentic fusion proteins. **B.** Western blot analysis of expressed proteins. A gel prepared identically to that in (A) was transferred to a PVDF membrane and probed with a rabbit polyclonal antibody specific for the nonO protein (12).

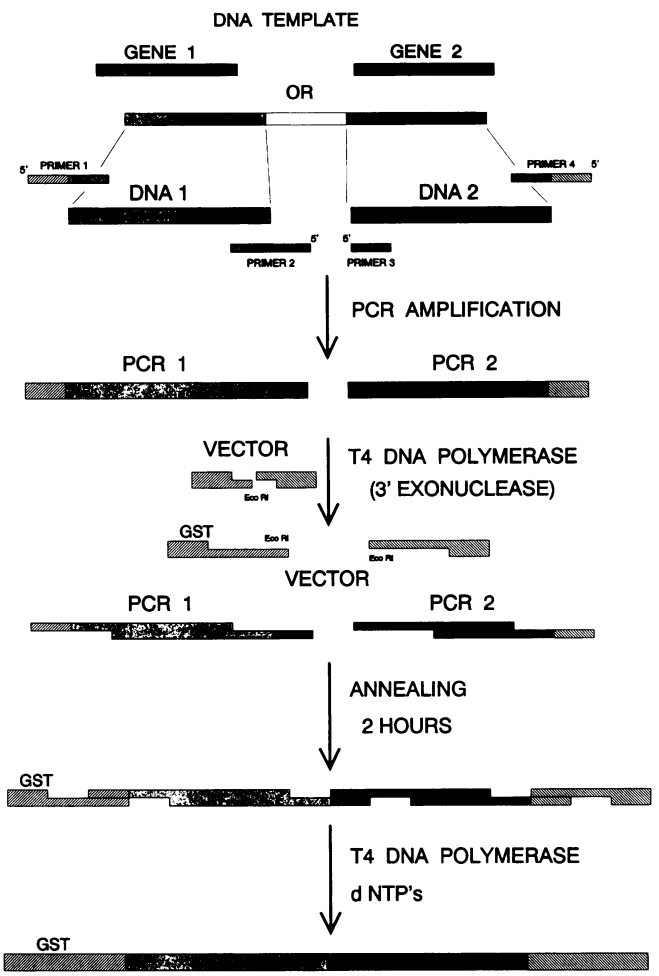

Figure 4. Construction of internal deletions or chimeric genes by exonuclease recession. Primers were designed as discussed in the text. All symbols correspond to those in Figure 1A.

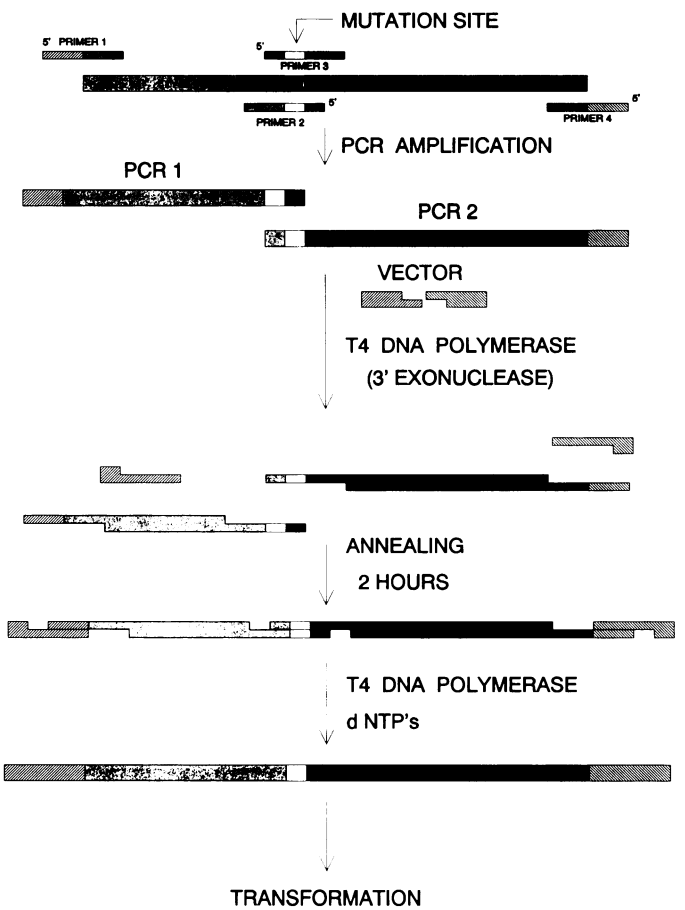

Figure 5. Construction of site-directed mutations by exonuclease recession. A designated mutated site (open box) can be incorporated into primers 2 and 3. All other symbols are as in Figure 1A.

then all three DNA fragments were recessed with T4 DNA polymerase as described above. The recessed heteroduplexes were assembled via annealing of homologous overlapped end sequences. Through this process, recombinant circular molecules of defined orientation were ready for transformation without further manipulation or purification.

Examples of nonO deletion mutants constructed in this way are shown in Figure 2 (F–H). Western blot analysis (Figure 3A and B) and DNA sequencing (data not shown) confirmed that the hybrid proteins were authentic.

DISCUSSION

PCR cloning technology provides a convenient approach to construct recombinant DNA. In many applications, the orientation of the DNA fragment in the construct is crucial for gene expression. The incorporation of restriction enzyme sites into both ends of PCR products is a common way to achieve this goal (1). However, restriction enzyme cleavage at the ends of PCR products is often inefficient, and in some cases compromised by the presence of internal sites within the insert. Here we describe a straightforward solution to this problem. The idea is simple: Different DNA fragments can be linked by annealing sequence complementary single-stranded ends. Since the end sequence of each DNA fragment is unique, two or more DNA fragments can be spliced in a defined orientation without manipulating restriction enzyme sites. Endonuclease cleavage is only required to linearize the vector DNA. In our protocol, vector DNA does not require a second restriction enzyme cleavage or dephosphorylation of the ends. After annealing the DNA inserts with vector, the recombinant circular DNA is transformed into a bacterial host without further ligation.

The efficiency of this method varys widely. Generally for 0.5 μg of vector DNA and PCR fragments 50 to 200 colonies grew in the selection medium. A particular advantage of the method is that T4 DNA polymerase treated vector DNA gives a very low background after transformation into host cells (occasionally one or two colonies may be present). The variable efficiency, therefore, might be the result of intrinsic properties of the insert DNA. Theoretically higher efficiencies could be achieved with extended incubation times during annealing.

While PCR SOEing (14,15) is a powerful technique to construct gene mutants, fusion genes and chimeric genes, it requires at least two sequential PCR amplifications. By using the protocol described here, only one PCR amplification is required to synthesize appropriate DNA fragments. Neither PCR reamplification of the first set of PCR products nor restriction enzyme cleavage for cohesive end-cloning of the final DNA

products is required. Any mutation created by Taq DNA polymerase (16) is therefore limited to the first amplification, and a theoretical reduction of the error by at least 50% provides a significant advantage over PCR SOEing. It is noteworthy that the overlapped, annealing sequences incorporated into the 5′ end of PCR primer 2 (Figure 4) naturally exist in target DNA 2. Therefore, no additional nucleotides are introduced into the spliced constructs and there are neither changed nor additional amino acid(s) at the junctions. For PCR amplification conditions we suggest using fewer cycles (e.g. 20) and high concentrations of templates (100 to 200 ng) to further reduce the potential mutations incorporated by Taq DNA polymerase. Using these conditions, we found no sequence errors in eight of our clones.

This same strategy can be applied to site-directed mutagenesis (Figure 5). A pair of oligomers, primer 2 and primer 3, containing the mutated sequence and 5′ end overlapped sequence to the other target DNA molecule can be used as PCR amplification primers. The mutant gene can be assembled as described for the other applications.

Recently several protocols utilizing ligase-free ligation of PCR products have been published (5−8,11). All of these protocols avoid the problems associated with restriction sites and low ligation efficiency, but still have at least one or more of the following drawbacks: vector amplification, multiple primer sets, sequential PCR reamplification(s) and non-directional cloning.

While preparing this manuscript, Kaluz et al. (10) reported directional cloning of PCR products without restriction enzyme cleavage of insert DNA fragments. They used a strategy similar to that of Stoker (9) to create cohesive termini for subcloning. However, both protocols require a ligase-dependent ligation procedure. Other protocols with a ligase-independent procedure require vector amplification to incorporate a specific sequence for annealing (7,8,11). In our method the recombinant DNA is created by annealing single-stranded cohesive termini, without the requirement of a ligase-dependent ligation procedure to attain directional cloning and also no vector amplification. More importantly, the cohesive ends created are not restricted to any defined sequence. Therefore, we can splice two independent PCR fragments together without actually 'SOEing' them by the second PCR reamplification (14,15). The Stoker (9), Kaluz (10), RCPCR (5−7) and ligation-independent cloning methods (8,11) do not allow the construction of chimeric products of the PCR 'SOEing' variety. None of the previously published protocols can achieve this objective without PCR reamplification. Currently we are attempting to apply this approach to subclone any DNA fragment with known sequence by using properly designed oligonucleotide adapters.

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