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PCR Mutagenesis and Recombination In Vivo

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Site-directed mutagenesis is an underpinning of the recombinant DNA revolution. For example, site-directed mutagenesis is used to modify protein-coding domains and to characterize regulatory DNA elements. Even the routine subcloning of an insert into a plasmid is a site-directed insertional mutagenesis, and the creation of a recombinant construct such as a gene chimera is a site-directed mutagenesis where one DNA segment replaces another. Technology for the site-directed mutagenesis of DNA is vital for genetic engineering.

PCR is best known as a method for the retrieval and detection of a specific DNA sequence. More recently, PCR has also become a popular method for the in vitro modification of a DNA sequence. Modification of DNA can occur because the primers are incorporated into the ends of the amplification product, permitting primer-directed modification of such ends. Alteration of a DNA sequence can be accomplished by incorporating a nucleotide mismatch within a primer annealing domain or by using a primer whose 5′ end is not determined by the original template.[1] The variety of PCR-based methods that have been developed for the site-directed mutagenesis of DNA, including the generation of recombinant constructs, attests to the continuing search for better or simpler methods.[2]

Previous investigators have shown that DNA ends containing short regions of homology undergo intramolecular recombination in vivo in *Escherichia coli*, including RecA-minus *E. coli* strains used routinely for cloning,[3–5] and that *E. coli* can mediate intermolecular recombination between a short, single-stranded oligonucleotide and a restriction endonuclease-digested plasmid.[6] Recombination PCR is a method for making DNA joints in vivo by the recombination of PCR-generated homologous DNA ends in *E. coli*.[7,8] This section details two recombination PCR protocols in which intermolecular recombination in vivo of PCR-generated DNA ends mutates a plasmid.

In brief, each protocol involves generation of two products in two separate PCR amplifications. Each end of one PCR product is designed to be homologous to a different end of the other PCR product. These two unpurified PCR products are combined, and this single sample is used to transform *E. coli*. Transformation is accomplished by recombination in vivo between the PCR-generated homologous ends, resulting in recombinant circles. If these recombinant circles contain plasmid sequences that permit replication and a selectable marker such as an antibiotic resistance gene, *E. coli* can be transformed by the recombinant. Two protocols are detailed, one for the point mutagenesis of a plasmid and another for the generation of a recombinant construct in which a DNA segment from one plasmid is seamlessly and directionally inserted into a specific locus of another plasmid. In addition, a modification of the second protocol is described for subcloning any PCR product.

REAGENTS

AmpliTaq polymerase 5 U/μl (Cetus, Norwalk, CT). Routine 10× *Taq* polymerase buffer (500 mM KCl, 100 mM Tris-Cl at pH 8.3, 15 mM $MgCl_2$, 0.1% gelatin.

10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP (Boehringer Mannheim, Indianapolis, IN). Primers: (1) Two pairs of primers (four altogether), each pair used in a separate amplification. Each primer in one pair has 24 nucleotides of complementarity to a different primer in the other pair. (2) Sequencing primers that flank the insert or mutated region, used to characterize the resulting construct.

Agarose. Ethidium bromide. TAE buffer.[9] Long thin micropipette tips (gel loader tips T-010; Phenix Research Products, Hayward, CA). MAX efficiency-competent DH5α *E. coli* (BRL Life Technologies, Gaithersburg, MD). SOC

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media.[10] Top agar.[11] LB plates with 100 mg/ml of ampicillin.[11] Luria-Bertani medium (LB broth).[12] Qiagen midi-columns (Qiagen Inc., Chatsworth, CA). Sequenase 2.0 sequencing kit (U.S. Biochemical, Cleveland, OH).

PROTOCOLS

Protocol 1: Point Mutagenesis

Point mutagenesis using recombination PCR is illustrated in Figure 1. The plasmid carrying the insert that is to be mutated is linearized by restriction

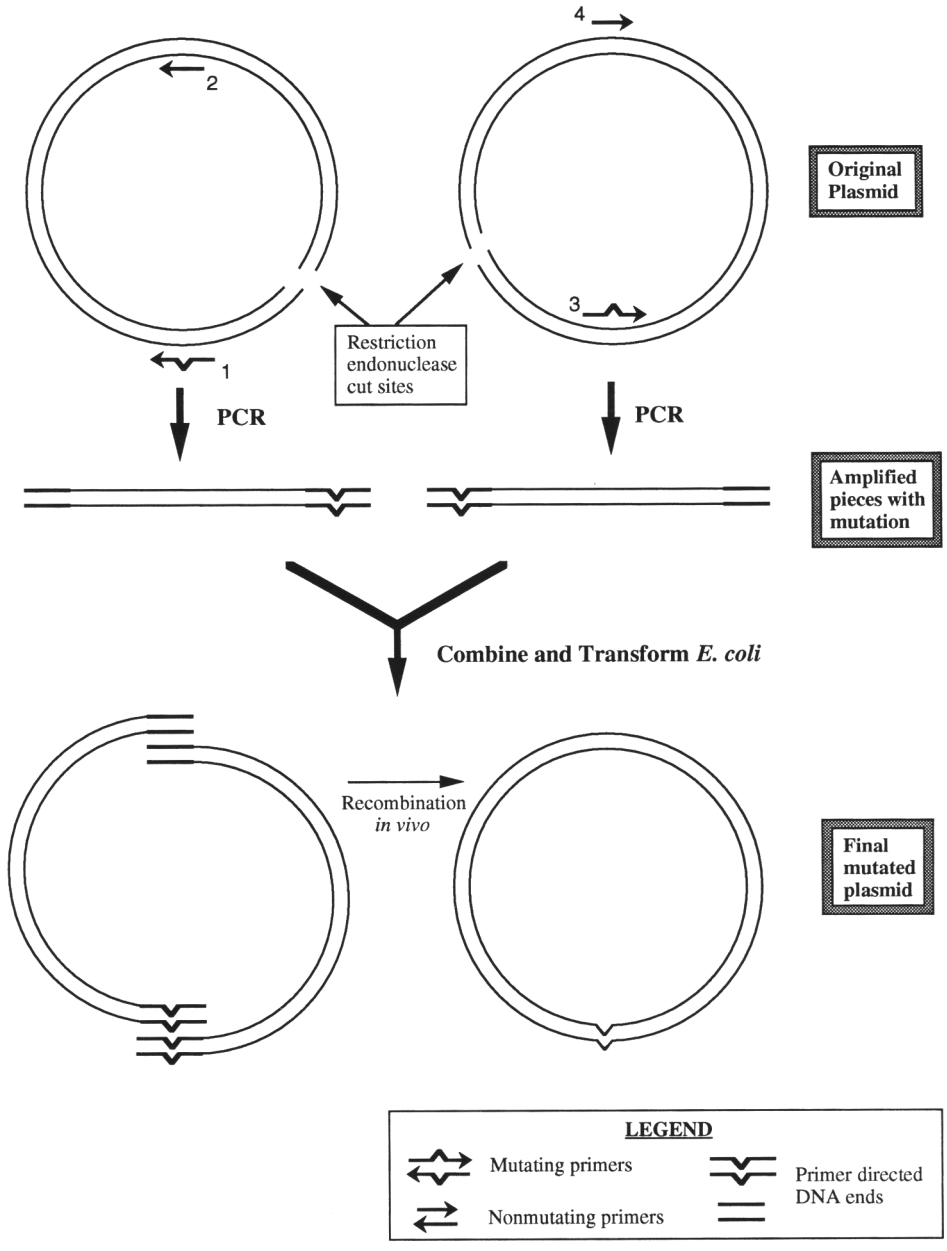

FIGURE 1 Point mutagenesis by recombination in vivo of two PCR products. The primers are arrows numbered 1–4. Notches designate point mismatches in the primers and resulting mutations in the PCR products. Primer 1 is complementary to primer 3, and primer 2 is complementary to primer 4. Two unique restriction enzyme recognition sites used to linearize the plasmid bracket the insert. There is no purification of the PCR products. For each additional single site-specific mutagenesis reaction, only new primers 1 and 3 need to be synthesized, and the same cut templates can be used.

endonuclease digestion prior to each PCR amplification. This plasmid is amplified and mutated in two separate PCR amplifications. In each of the two amplifications, the identical base pair is mutated so that the mutated ends of each product are homologous to each other. The nonmutating primers are also designed to produce ends that are homologous to each other. Both unpurified PCR products are combined to transform *E. coli*, resulting in clones containing the mutation of interest.

1. PCR Mutagenesis: In each PCR amplification, start with a template plasmid that has been digested by restriction endonuclease outside the region to be amplified. Use 2 ng of linearized plasmid, 25 pmoles of each primer, 200 μM each dNTP, 1× PCR buffer, and 1.25 units of *Taq* polymerase in a total volume of 50 μl. Pipette 50 μl of mineral oil on top of each reaction mix prior to amplification. Amplification parameters are as follows: initial denaturation at 94°C for 1 min, 14–20 amplification cycles (94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min per kb of PCR product), and a final extension step (72°C for 7 min). **Note:** Each amplification uses a plasmid template that has undergone restriction endonuclease digestion at a different "side" of the plasmid, relative to the site targeted for mutagenesis. Usually, unique restriction endonuclease recognition sites in the original vector are used, so that the two linearized templates can be used to mutate any site in the insert. Each primer is designed to generate 15–45 bp of homology between each end of one PCR product relative to the other PCR product. Twenty-four nucleotides of homology work very well. Decreasing the length homology from 25 to 12 bp in an early protocol that entails a single recombination event decreases the transformation efficiency four- to fivefold. Single point mismatches lie no closer than 6 nucleotides from the 3′ end of a primer and are frequently placed toward the middle. Placing point mutations near the 5′ end of each mutating primer will generate two PCR products whose mutated ends have <24 bp of homology. Primers that generate point mismatches are typically 25–35 nucleotides long. Multiple point mismatches should be placed in the middle or toward the 5′ end of a primer, with primer lengths long enough to create 24 bp of homology between the mutated ends of the two PCR products. Primers that are nonmutating (primers 2 and 4 in Fig. 1) are generally 20–30 nucleotides long. The nonmutating primers can be designed to anneal to the β-lactamase gene, so that they can be used with many different plasmids.

The mutating and nonmutating primers are frequently designed to be perfect complements to each other. Alternatively, their 3′ ends can be offset relative to each other (recognizing that they participate in separate amplifications), so that the homologous ends generated are longer. It is clear that the nonmutating primers can anneal at positions that generate PCR product ends with long homology, whereas the mutating primers cannot, because each anneals to the mutagenesis site. Use of significantly longer homology between the nonmutated ends is not necessary and has, surprisingly, decreased the transformation efficiency using recombination PCR.[8]

2. PCR Product Detection: Visualize each product by electrophoresis on an agarose minigel. **Note:** If 5 μl of the PCR product can be clearly viewed following ethidium bromide staining (≥15 ng/5 μl), there is enough product.

3. Transformation of *E. coli*: Insert a long thin micropipette tip through the mineral oil, withdraw 2.5 μl from each PCR tube (typically 10–60 ng/2.5 μl; maintaining an even ratio of one product to another is not necessary), combine the two samples, and transform MAX efficiency-competent DH5α *E. coli* (transfection efficiency >1× 10^9 transformants/μg of monomer pUC19) with the 5 μl. **Note:** Transformation is done following the manufacturer's (BRL) protocol with the following modifications: (1) Use 50 μl of *E. coli* for each transformation, as this is effective and less expensive than the 100 μl

recommended. After incubation at 37°C in a shaker for 1 hr, add 2 ml of top agar, prewarmed to 42°C, to each sample immediately prior to pouring it onto an LB plate containing 100 mg/ml of ampicillin. Once an aliquot of bacteria is thawed, it is not used subsequently.

Typically, we set up the following five plates and perform transformations using the following PCR samples and controls: Plate A, 2.5 μl of PCR #1 + 2.5 μl of PCR #2; Plate B, 2.5 μl of PCR #1 + 2.5 μl of TE; Plate C, 2.5 μl of PCR #2 + 2.5 μl of TE; Plate D, 0.5 ng of a supercoiled template in 5 μl of TE; and Plate E, 5 μl of TE.

Only 25 μl of *E. coli* are used for the control plates D and E, so that only one BRL tube, which contains 200 μl of bacteria, needs to be used per mutagenesis.

The yield of colonies from plate A will be >2× that from plate B + C, confirming a high percentage of recombinants in plate A. Plate D is a transformation control and should yield a thick lawn of colonies. Plate E is an antibiotic control and should yield no colonies because the bacteria that have not been transformed are sensitive to ampicillin.

4. Plasmid Screening: (a) Place individual colonies in 2 ml of LB broth containing 100 mg/ml of ampicillin and grow at 37°C for 6–24 hr. The plasmids are screened using PCR by a modification of a method described previously[13] as follows: Remove 2 μl of the LB broth, place directly in a PCR tube, and amplify for 25 cycles using primers that flank the mutated site or insert (e.g., M13 primers). Usually, the site mutated can be designed to either create or remove a restriction endonuclease site into the amplified product. For example, the degenerate amino acid code can be used to make the desired amino acid change and, at the same time, create a restriction endonuclease recognition site to facilitate screening. (b) Screen for the mutation by adding 3 units of the restriction endonuclease and 1 μl of the appropriate 10× restriction buffer directly to 5 μl of the unpurified PCR product in a total volume of 10 μl. Creation or elimination of the restriction endonuclease recognition site can then be directly assessed by minigel analysis. (c) Purify the plasmid and sequence the mutated region. Qiagen columns can be used for plasmid purification and Sequenase 2.0 for sequencing following the manufacturers' instructions.

Note: (1) Plasmids can be screened immediately by placing a colony in 5 μl of LB broth, vortexing, and then amplifying 2 μl as described. The remaining LB sample can later be grown up for isolation of the plasmid and storage of the colony. (2) If the mutagenesis cannot be designed to eliminate or create a restriction enzyme recognition site, the PCR screening primers can be designed to amplify the mutant preferentially by creating a perfect match between the 3′ end of one of the primers and the mutated site, such that plasmids containing the original, nonmutated template sequence are not amplified in sufficient quantity to be detected by ethidium bromide staining.[14] Alternatively, the nonmutating primers that anneal to the original vector (primers 2 and 4 in Fig. 1) can be designed to mutate a nucleotide that creates or eliminates a restriction endonuclease recognition site. Screening for mutagenesis of this site will effectively screen for the site of interest, because both sites will be mutated concurrently.

Protocol 2: *Recombining DNA Segments*

A protocol for amplifying a portion of a donor plasmid and placing it in a recipient plasmid at a defined location and orientation, with the simultaneous removal of a DNA segment in the recipient plasmid, is illustrated in Figure 2. The conditions for PCR amplification and transformation are identical to those detailed above and are not restated. In Figure 2, the donor

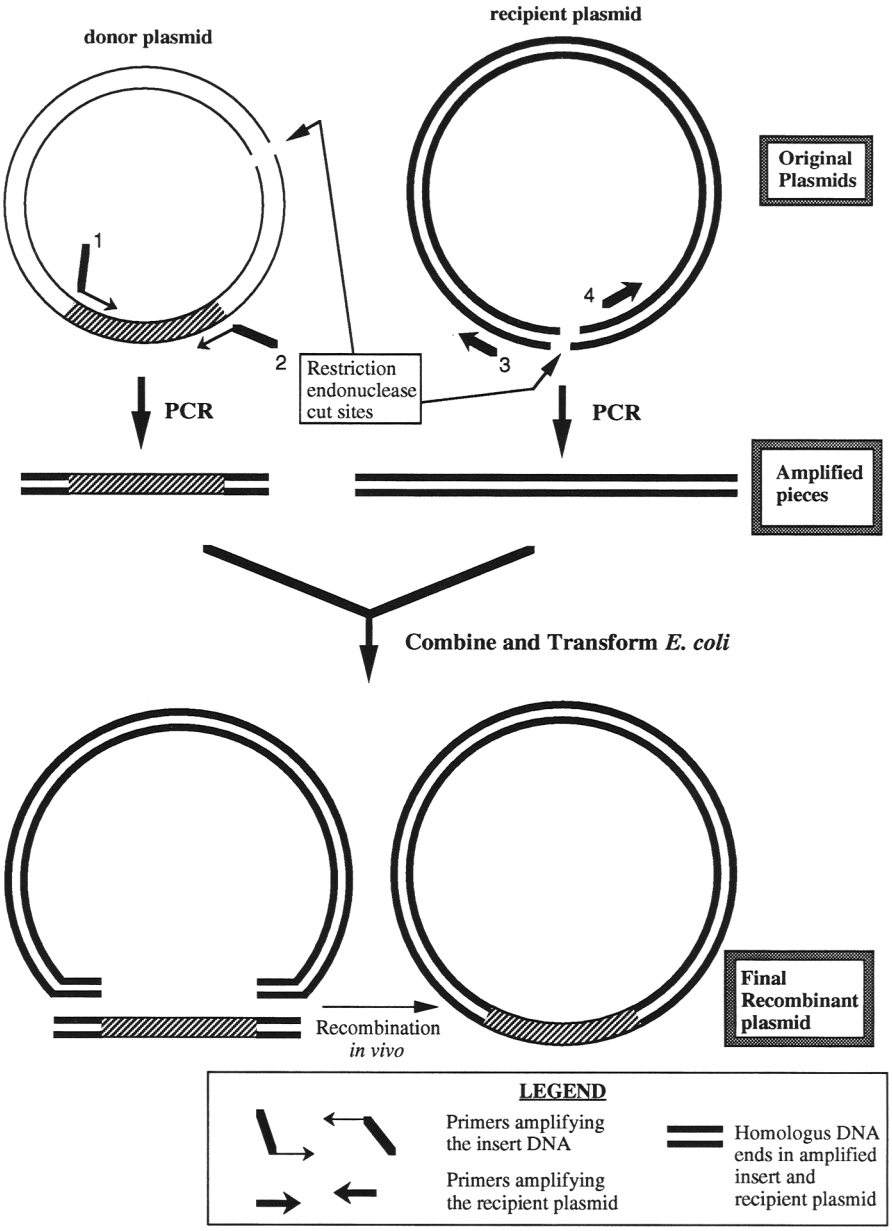

FIGURE 2 Generation of a recombinant construct by recombination in vivo of two PCR products. The insert is hatched. Thin circles represent the DNA strands of the donor plasmid, and thick circles represent the DNA strands of the recipient plasmid. The 5′ regions of primers 1 and 2 are complementary to primers 3 and 4.

plasmid DNA is shown on the left side and the recipient plasmid on the right side. The DNA segment that is to be inserted into the recipient construct is amplified from the donor plasmid using primers 1 and 2. In a separate PCR amplification, the recipient plasmid is amplified with primers 3 and 4. The 5′ regions of primers 1 and 2 are complementary to primers 3 and 4. Each plasmid is linearized by restriction enzyme digestion prior to PCR amplification. In Figure 2, the 5′ regions of primers 1 and 2 contain regions that are homologous to the recipient plasmid sequences to which primers 3 and 4 anneal. The only requirement for this method is that primers 1 and 2 must have regions of complementarity to primers 3 and 4. As in protocol 1, the homologous ends between the PCR products are ~24 bp long. A similar

strategy, in which the recipient vector is modified by PCR to contain ends that are homologous to a given PCR product, can be used for the rapid subcloning of any PCR product. This is illustrated in Figure 3.

COMMENTS

1. In protocols 1 and 2, specificity for the construct of interest is high but the transformation efficiency is low, averaging 10 colonies with the mutation per nanogram of total DNA transfected. Therefore, highly competent bacteria must be used. Only highly competent *E. coli* (transfection efficiency $>1\times10^9$ transformants/μg of monomer pUC19) are used. The proportion of clones containing the recombinant of interest is ⩾50%.

2. If a plasmid cannot be linearized outside the region to be amplified by PCR (i.e., in the right side portion of Fig. 2), the PCR product must be purified from the plasmid by agarose gel electrophoresis, because supercoiled plas-

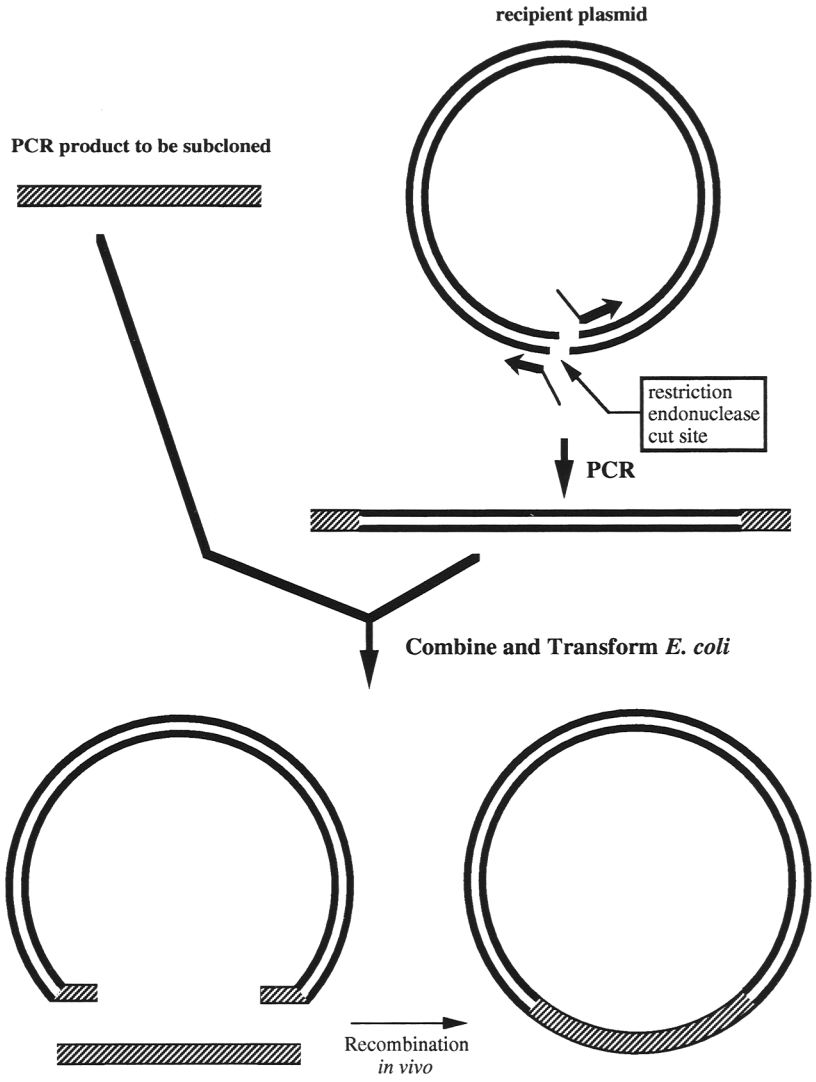

FIGURE 3 Subcloning of any PCR product by recombination in vivo of two PCR products. The PCR product that is to be subcloned is hatched, and the primers that amplified this product are not shown. The circles represent the DNA strands of the recipient plasmid. The 5′ regions of the primers that amplify the recipient plasmid are complementary to the primers used to amplify the PCR product to be subcloned.

mids have a very high transformation efficiency. Extraction from the agarose gel may be done using GeneClean (Bio101, La Jolla, CA). When a supercoiled plasmid is used, more amplification cycles may be necessary, because PCR product yields are higher when using a linearized template than when using a supercoiled template.

3. In protocol 1, primers 2 and 4 can be reused for any mutagenesis of the insert, so that only two new primers need to be generated for each new site targeted for mutagenesis (primers 1 and 3). Furthermore, only approximately one-half of the length of the entire template needs to be amplified in each of the two PCR amplifications, facilitating the mutagenesis of large constructs. Recombination PCR has been used to mutate constructs up to 7.1 kb.[15]

4. Because the region of complementarity of one primer to another need not anneal to the original template sequence, a variety of DNA sequence modifications can be carried out during a single transformation. For instance, in protocol 2, as long as primers 1 and 2 contain regions that are complementary to regions of primers 3 and 4, the PCR products will contain ends that are homologous to each other, and these primer-determined DNA ends do not need to be determined by the original donor or recipient templates. Therefore, it is clear that point mutations can be placed in the recipient plasmid simultaneously with insertion of an amplified fragment.[16]

5. Difficulty may be encountered in protocol 2 when attempting to insert a DNA segment to generate a large direct repeat in a plasmid, as recombination between the large direct repeats could compete with recombination between PCR product ends.

6. A strategy similar to the original recombination PCR protocol for subcloning has been described that substitutes a restriction enzyme-digested plasmid for a PCR-amplified plasmid.[7,17,18] A modification of protocol 2, illustrated in Figure 3, is now used here to subclone PCR products.[19] The PCR product to be subcloned is not purified, and the molar ratio of insert to the amplified recipient plasmid can vary widely. Furthermore, the PCR product to be subcloned is not amplified by primers containing 5′-end extensions that do not anneal to the original template. This is desirable when optimizing primer lengths and T_m values to avoid spurious product in protocols for amplifying a rare sequence from a complex mixture.

7. In the original description of the recombination PCR method, 2 clones of 19 sequenced contained a base deletion in a primer sequence, and we speculated that this may have resulted from the recombination in vivo that generated the construct of interest.[7] Since that paper was published, we utilized recombination PCR and recombinant circle PCR multiple times to generate a new plasmid.[16] There were six sequence errors in the resulting plasmid, and all of these errors were base substitutions. Thirty-four primer sequences were incorporated into this new plasmid using recombination PCR protocol 1 or protocol 2 described above. These primer sequences constitute 36% of the resulting plasmid, and the cumulative regions of homology between the PCR product ends generated using these primers that recombined to generate this plasmid constitute 34% of this plasmid sequence. Two of the six sequence errors (33%) in the plasmid reside within these primer sequences as well as within the regions of homology generated by these primers. Therefore, the distribution of sequence errors did not cluster within the primer sequences or the short regions of homology generated by these primers, suggesting that sequence errors resulting from recombination in vivo between short regions of homology generated by primers are rare. The most likely cause for the majority of the errors that did occur in the final plasmid is nucleotide misincorporation during the 196 sequential polymerase extensions (11 recombination PCR and 3 recombinant circle PCR protocols with 14 cycles per PCR) used to generate this plasmid. Because there is always the

possibility of a sequence error in a single clone following PCR amplification, a mutated region should be sequenced, and one may choose to clone a restriction fragment containing the mutation into a construct that has not undergone PCR amplification.

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