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POLYMERASE CHAIN REACTION (PCR) TECHNIQUES FOR SITE-DIRECTED MUTAGENESIS

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ABSTRACT

Polymerase chain reaction (PCR) technology has revolutionized the process of isolating and amplifying segments of DNA. One powerful application of PCR is its use in precise site-directed mutagenesis (SDM). SDM provides an elegant tool for scientists and engineers to explore biocatalytic mechanisms and processes to understand the structural-functional relationships of enzymes and other proteins. This article reviews techniques and methodology used in site-directed mutagenesis of genes by PCR.

KEYWORDS: polymerase chain reaction, PCR, mutagenesis

INTRODUCTION

One of the most important industrial goals of biotechnology is understanding structural and functional relationships of enzymes/biocatalysts. Similar to the technical revolution caused by inorganic catalysis in the petroleum and chemical industries half a century ago, the understanding and design of new biocatalysts promises remarkable opportunities for novel products and processes.

By altering gene structures to create mutations in existing enzymes, we can probe the importance of structural features and determine their effect on biocatalytic behavior. One of the most powerful tools available for precise recombinant genetic mutations is the polymerase chain reaction (PCR). PCR provides a rapid, inexpensive means to isolate genes and create precise mutations. This article reviews techniques and methodology used in site-directed mutagenesis of genes by PCR.

POLYMERASE CHAIN REACTION

PCR (Saiki *et al.* 1985; Saiki, 1988) involves creating short sequences of synthetic DNA (primers) that are complementary to sections of the gene of interest (template DNA). The primers are designed to flank the gene sequence of interest. Following binding, a polymerase enzyme (*Taq* polymerase) is used to extend the primers by adding on base pairs in the 3' direction. After extension, the duplicated DNA strands are separated by heating, and fresh primers bind for another round of amplification (see Fig. 1). The newly created strands serve as templates. This process is repeated until the desired amount of DNA is obtained. Since duplicated DNA strands serve as templates, after several rounds of amplification only the DNA between the original primer sequences will be obtained. Therefore, PCR allows both isolation and amplification of specific DNA sequences.

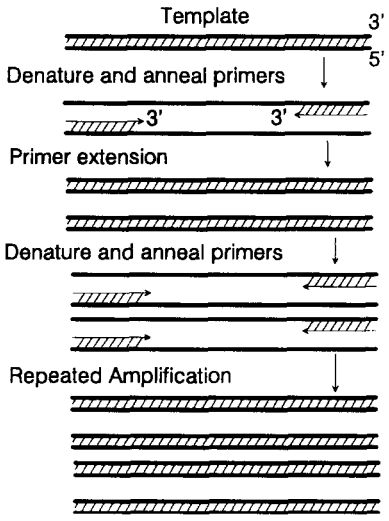

Figure 1. PCR Amplification of DNA

MUTAGENESIS BY PCR

PCR primers must be sufficiently long to allow unique binding to desired template locations. If longer primers, in excess of the length needed simply to locate and bind are used, it is possible to introduce new base pairs into the primer. This process is called mis-matching (Fig. 2). Mis-matching has been used by several researchers to introduce single amino acid changes in proteins (Caffrey *et al.*, 1991; Friedman *et al.*, 1991; Li and Rhode, 1990; Nassal and Rieger, 1990; Kadowaki *et al.*, 1989). Using primers with specifically mis-matched sequences to the template DNA, precise mutations can be introduced into the gene. The primary constraint of this method is that mutations are limited to the primer DNA sequences. Therefore, the mutagenesis is limited to the terminal

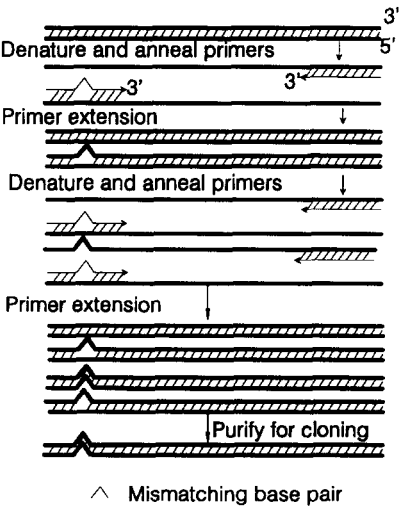

Figure 2. Terminal end PCR mutation

ends of the gene. To overcome this limitation, PCR site-directed mutagenesis methods have been devised for internal sequences.

Linear Template Mutation

Most PCR methods use template DNA that exists in linear form, usually cleaved chromosomal DNA, due to their high efficiency.

Overlap Extension. The first method devised to mutate DNA, which was not at the end of the target sequence, is called Overlap Extension PCR (OE-PCR). OE-PCR utilizes four primers in two sequential PCR reactions to introduce a mutation into the target sequence (Higuchi, 1990; Ho *et al.*, 1989). As shown in the Fig. 3, two separate PCR reactions are run, one using primers 1 and 3 to amplify a portion of the target sequence and the other using primers 2 and 4 to amplify the remaining sequence. There is a short overlap between the PCR products, equal to the length of primers 2 and 3. The primers are removed by diafiltration and the DNA is denatured and reannealed. A small portion of the DNA will anneal to form heteroduplexes, and those with recessed 3' ends will be extended by *Taq* polymerase to give the full-length target sequence containing the mutation. PCR is then performed using primers 1 and 4 (the outer, conserved primers) to amplify the entire segment.

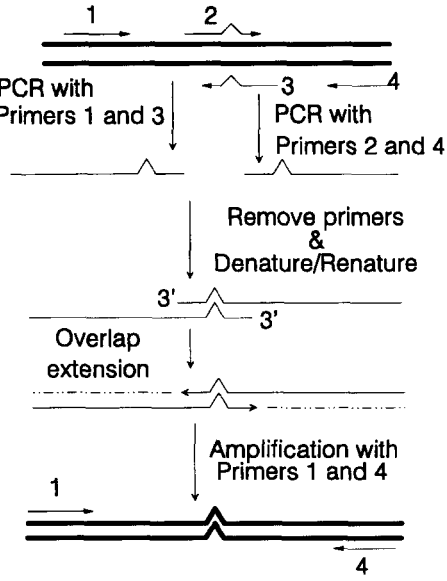

Figure 3. Overlap Extension PCR

With OE-PCR, since the region of overlap is quite short, most of the DNA reanneals to form the products of the first PCR, with very few heteroduplexes formed (Ito *et al.*, 1991). Therefore, yields of mutations are low and must be selectively recovered from the mixture. In addition, since the number of unwanted mutations increase as the PCR reaction occurs, the two sequential PCR reactions pose a greater risk for errors in polymerization (Sarker and Sommer, 1990). Also, for each new mutation two new primers are needed, increasing the cost. Lastly, it is cumbersome to use diafiltration to remove primers in between steps. An improved OE-PCR method has been developed that uses biotinylated universal primers and streptavidin-coated magnetic beads to simplify this purification step (Hall and Emery, 1991).

Modified Restriction Site. Modified restriction site PCR (MR-PCR) is similar to OE-PCR. This method utilizes three common primers outside of the DNA insert and a fourth primer containing the desired mutation. The DNA of interest is first inserted into the polylinker site of a vector. Two restriction sites are then chosen on either side of the DNA target sequence. Primer 2 covers one of these sites and contains a mutation that destroys the restriction site. Primers 1 and 3 flank the DNA insert and the two restriction sites. Primer 4 is specific for the desired mutation. Two DNA fragments are amplified in separate PCR reactions, one using primers 1 and 4 and the other using primers 2 and 3 (Fig. 4). The fragments produced are combined, denatured, reannealed, and then amplified using primers 1 and 3. Two restriction enzymes are used to digest the DNA, one of which cuts at the site which was destroyed by primer 2. Since only the DNA segments with both restriction sites functional contain the desired mutation, no wild type DNA should be recloned.

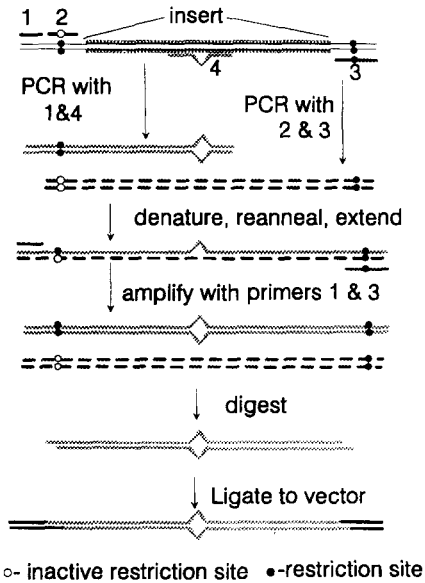

Figure 4. Modified Restriction Site PCR

This method is highly efficient (Ito *et al.*, 1991) and since the length of the overlap region of the two DNA fragments is relatively long, a greater number of heteroduplexes are formed. Contamination by wild type clones in final products is extremely low, since only the mutant products may be recloned. Additionally, subsequent mutations require only one additional primer to be produced (primer 4) while primers 1, 2, and 3 are conserved for all reactions. The main disadvantage to this method are that 2 adjacent endonuclease restriction sites must exist near the mutation site.

Introduction of New Restriction Sites. New restriction site PCR (NR-PCR) introduces new restriction site sequences into the target template, where mutations can be inserted (Fig. 5). Four primers are used for PCR in this technique: two outer conserved primers and two mutant primers containing restriction sites. Two PCR reactions are run, one with primers 1 and 3 and the other with primers 2 and 4. The two fragments are combined, digested with the appropriate restriction enzyme, ligated to form the complete target sequence, and then digested with a different restriction enzyme with sites outside the target sequence for recloning. This process can be repeated to create new restriction sites flanking the region of mutation. Mutations are created by synthesizing mutant sequences with matching terminal restriction sites and ligating mutations into the restriction sites.

Although this method would normally create a restriction site along with the desired mutation, this can be prevented with careful primer design. Tomic *et al.* (1990) designed mutagenic primers that contained Bsp MI sites that would be eliminated with digestion of Bsp MI. This restriction enzyme cut leaves a four base pair 3'-overhang. The primers were designed so that after digestion these cohesive ends were complementary to each other and corresponded to the original template DNA. Thus, only the desired mutation was created.

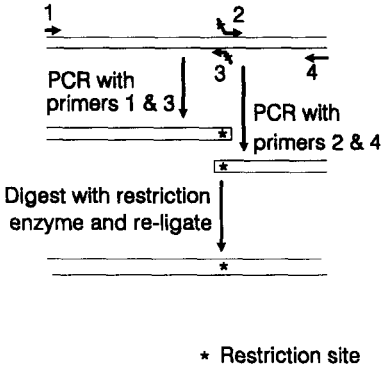

Figure 5. New Restriction Site PCR

This is a highly efficient process (Zaret *et al.*, 1990) and has an advantage over both previously described methods in that after introduction of the restriction sites, mutations can be introduced by simple restriction/ligation methods. The major limitation of NR-PCR is that mutations are restricted to sequences where new restriction sites can be introduced without disrupting the protein codon sequence (i.e. via codon wobble). This method is also requires more enzymatic reactions than just PCR, unlike some of the other methods. Also, addition of restriction sites to the primers may cause mis-priming.

Megaprimer. The Megaprimer method (Fig. 6) is a simple method which uses only three primers in two rounds of PCR. The first round of PCR uses only primers 2 and 3 to amplify a short segment of the DNA,with primer 2 creating the desired mutation (Landt *et al.*, 1990). This PCR product is then purified and used as a "megaprimer" for the second round of PCR in conjunction with primer 1. This method is less costly than the other methods since it uses only three primers, and each additional mutation requires only one new primer to be synthesized. Although two sequential PCR reactions are performed, the first PCR is only amplifying a short segment of the total sequence, so polymerization errors are less likely than with the overlap extension or MR methods. A major constraint of the megaprimer method is the non-specific, non-template addition of nucleotides (usually a single bp) to the 3' terminal site during PCR reaction (Clark, 1988). This can lead to unwanted mutations. Since nearly all of the

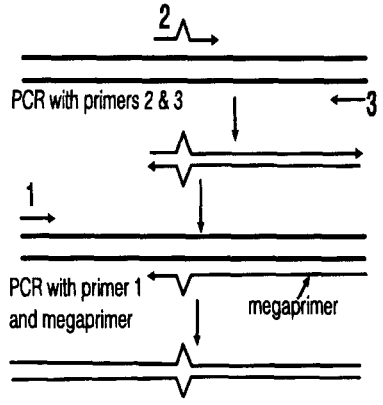

Figure 6. Megaprimer PCR

additions were single adenine residues, it was suggested (Kuipers *et al.*, 1991) that the inside primer (primer 2) should be designed so that the first 5' nucleotide follows a thymine residue in the same strand of the template sequence. When this was done, all the clones sequenced had no error. If the first 5' nucleotide did not follow a T residue, then only 17 percent of the clones sequenced had no error. Although this method is applicable in many cases, it may be difficult to find a T residue if a mutation involves a G-C rich stretch of DNA. In this case, it was suggested (Sharrocks and Shaw, 1992) that the primer be designed so that the 5' end immediately follows the wobble position of a codon. Thus, even if a mutation occurs in the genetic sequence, the amino acid sequence will not vary. This method also approaches 100 percent efficiency (Sharrocks and Shaw, 1992).

Non-Template Hybrid Primer. This method uses three general primers designed to specifically amplify the mutant segment (Fig. 7), eliminating purification steps. Four primers are used in all. Primer 1 contains the desired mutation, and primer 3 is the outer primer used for amplification of the entire segment. Primer 2 is a long hybrid primer with its 3' sequence complementary to the template and its 5' sequence noncomplementary. Primer 4 is identical to the 5' sequence of primer 2, which is the key to this method. The first PCR amplification is done with primers 1 and 2, producing only a small portion of the gene as with the megaprimer method. This segment is then mixed with additional template DNA, denatured, reannealed, and extended by the *Taq* polymerase. Primers 3 and 4 are then added for a second PCR amplification. Since primer 4 is identical to the non-template portion of primer 2, it will only bind to and amplify the mutant strands produced in the first PCR reaction, thus increasing the efficiency of this method. Using this method, ten of thirteen clones sequenced showed the desired mutation (Nelson and Long, 1989). There are several advantages to this method. Since the mutant is amplified exclusively, the purification steps can be simplified. As with the megaprimer method, only one new primer is needed for each additional mutation, and polymerization errors are reduced since only a short sequence is amplified in the first PCR. The disadvantage of this method is the difficulty in primer design. The 5' portion of primer 2 and primer 4 must be carefully designed so as not to be complementary to either strand anywhere in the gene sequence.

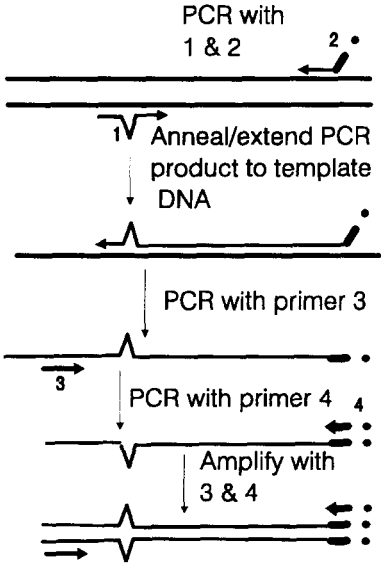

Figure 7. Non-Template Hybrid PCR

Whole Plasmid Mutation

To simplify the ligation/transformation process, methods have been developed to use PCR to mutate genes that already have been cloned into plasmid vectors. The main advantage of these methods is that mutated genes are already cloned into transformation vectors, avoiding ligation problems due to non-template additions inherent in PCR (Clark, 1988).

Inverted PCR. Inverted or "Counter" (Street *et al.*, 1991) PCR (Fig. 8) entails the replication of the entire plasmid containing the insert of interest. In this method the circular plasmid is amplified using two primers in a back-to-back position, one of which contains the desired mutation. An advantage to this method is that only one PCR amplification is performed, lowering the chance of random error by the polymerase. This method has been shown to yield mutants with 82% overall efficiency (Hemsley *et al.*, 1989).

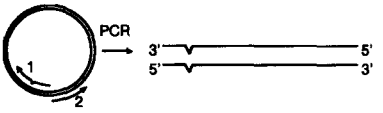

Figure 8. Inverted PCR

There are several disadvantages, however. There are no conserved primers used in Inverted PCR, therefore, each mutation requires two new primers to be synthesized. Also, there are numerous enzymatic reactions required with this method. The PCR products require treatment with the Klenow fragment and must also be phosphorylated. Using the entire plasmid may also produce problems. Segments and domains of the protein cannot be produced, only the entire protein. Also, amplification of the entire plasmid may lead to errors in the plasmid sequence resulting from polymerase errors.

Recombinant Circle PCR. Recombinant Circle PCR (RCPCR) is similar to the Inverted PCR method but simplified so that all steps may be performed in one day. RCPCR requires only one set of PCR and it accomplishes the cloning step simultaneously. The key to RCPCR is the positioning of the primers in two separate PCR reactions. Primers 1 and 3 introduce the mutation into the gene. Primer 2 is in a back-to-back position with primer 1, as primer 4 is with primer 3. The products of these PCR reactions are then purified, combined, denatured, and reannealed to recombine (Fig. 9). These recombinant circles can then be transfected directly into *E. coli*, without treatment with the Klenow fragment, restriction enzyme

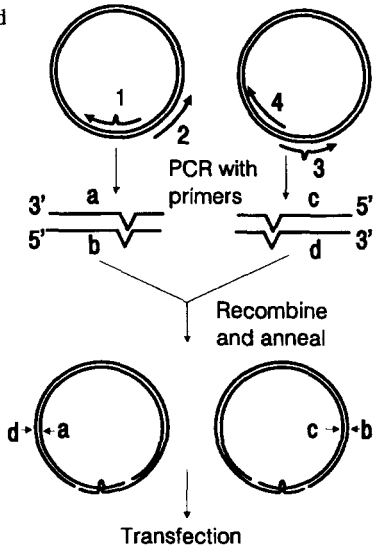

Figure 9. Recombinant Circle PCR

digestion, phosphorylation, or ligation. The gaps in the plasmid are then repaired *in vivo*. In the original experiments the gap sizes were approximately twenty nucleotides, but circles with gaps of over 1000 nucleotides have been produced with reduced efficiency (Jones *et al.*, 1992). With small gap sizes (up to 19 bp), efficiencies ranged between 83 and 100 percent (Jones and Howard, 1990). Plasmids up to 6.1 kb in size have been used in this technique with success (Jones *et al.*, 1990). RCPCR has the same disadvantages as Inverted PCR, except that no enzymatic treatments are necessary other than PCR. Also, primers 2 and 4 can be conserved for creating other mutations.

Recombination PCR. Recombination PCR (RPCR) further reduces the number of primers and steps required in the process of creating a mutant. Only one PCR amplification is performed with two overlapping primers (Fig. 10). The linear PCR product is then transfected directly into *E. coli* (Jones and Winistorfer, 1991). Since the DNA segment has homologous ends, recombination occurs *in vivo* to produce the mutated plasmid.

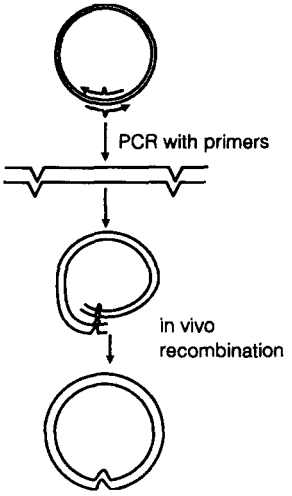

Figure 10. Recombination PCR

With this simplified protocol, the need to form staggered ends *in vitro* is eliminated, only two primers are required, and the denaturation and reannealing step is not necessary. However, the efficiency is much less than in RCPCR, at only 50 percent (Jones and Winistorfer, 1992). This results from an error in ligation in up to one in four clones. There is also a lower yield of clones with RPCR than RCPCR due to the overlap of the primers. Also, each new mutation requires novel primers. As with all methods that amplify the entire plasmid in PCR, only the entire protein can be produced and the plasmid may be mutated due to polymerase errors.

Figure 11 shows site-directed mutagenesis of two distal sites using RPCR (double mutation RPCR). The procedure uses four primers and the plasmid is cut with a restriction enzyme before amplification. Two separate PCR amplifications are performed, the products are mixed, transfected into *E. coli*, and recombined *in vivo*.

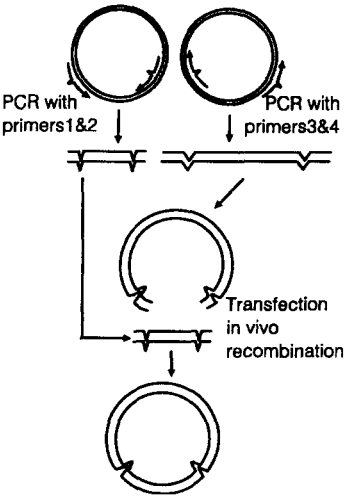

Figure 11. Double mutation RPCR

Marker-Coupled PCR. The Marker-Coupled method (Fig. 12) eliminates the need for screening colonies for the mutation. Two primers are used, one that contains the mutation and another that repairs a deletion in an inactivated marker gene (such as *tet* in pUT 18, Shen *et al.*, 1991) to restore its activity. Mutagenesis is accomplished in five steps. In step 1, mutant and marker primers are used in PCR with target DNA previously inserted into the vector. The PCR product fragments contain both the mutation and repaired marker. In step 2, the vector is cut with different restriction enzymes to produce a nicked vector and a large fragment that does not contain either the mutation or marker sites. This large fragment is used as a primer in later steps. Step 3 combines the PCR product with the restricted and nicked fragments to form a gapped duplex plasmid/target DNA template, which is extended and ligated *in vitro* to form a closed, circular plasmid with one of the strands mutated. In step 4, this uni-strand mutant is nicked and combined with the previous large restriction fragment to produce gapped duplexes, separating the mutant and non-mutant strands. In step 5, the gapped duplexes are transformed into *E. coli* cells and repaired *in vivo*, and spread on plates screening for the marker. Since the marker and the mutant are closely coupled, the only cells which grow contain both the active marker and the desired mutation. Although all of the colonies produced by this method are mutant, few colonies are usually obtained (Shen *et al.*, 1991). Complexity is the major disadvantage of this method with multiple restriction cuts and repeated low efficiency heteroduplex formation. While the extra steps may eliminate screening, the process is very tedious and has a low output efficiency.

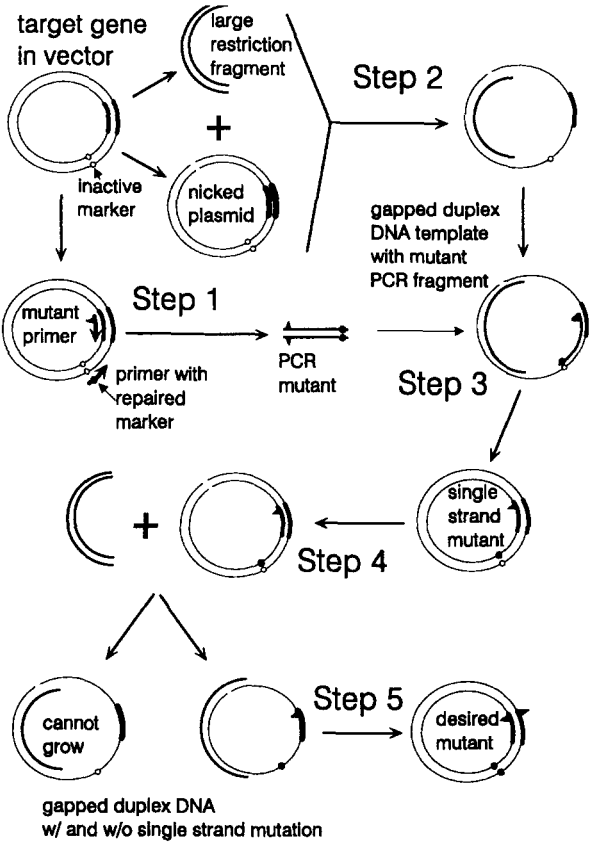

Figure 12. Marker-Coupled PCR

DISCUSSION

PCR provides a unique means to probe the structural-functional relationships of proteins and biocatalysts by permitting precisely controlled changes of existing genes. Through the use of both plasmid and linear PCR techniques, mutations can be introduced almost anywhere within a gene at high levels of efficiency. If mutations are desired at or near the termini of the gene, simple mis-match PCR amplification techniques can be used, provided sufficiently long primers are available. For internal mutations, the MR-PCR and NR-PCR are the most efficient techniques. Introduction of restriction endonuclease sites within the gene flanking the desired mutation site, provides a rapid means to directly insert mutations at high efficiency. New mutant segments can be created either synthetically or by mis-match PCR techniques. While this is a highly effective technique, it is dependent upon the existence of native sequences near the desired mutational site that can be easily modified to become restriction sites by using codon wobble.

All mutation methods rely on the fundamental mis-matching of base pairs within a complementary synthetic DNA primer. Therefore, primer length, the number of mis-matched base pairs and primer specificity are all critical factors in successful mutational PCR. Practical DNA oligomer primer synthesis is currently limited to something on the order of 50 bp. This restricts the size and number of mutations per primer, since mis-matchs are usually limited to 1 to 3 base pair changes per 20-30 oligomer primer. With large DNA templates, such as chromosomal DNA, the potential for non-specific primer binding also dramatically increases with decreasing primer length. Therefore, longer primers with a maximum complementarity are desirable for high efficiency. As primer length increases, it also may be necessary to modify PCR annealing times and temperatures to accommodate the more extensive uncoiling and re-association of long primers. Long initial denaturation steps have been successfully used to increase PCR efficiency, so called 'hot start' PCR (D'Aquila, *et al.*, 1991, Nuovo *et al.*, 1991).

Logistical differences between various PCR mutational methods primarily involve the number of primers needed, the number of PCR reactions needed, the need for heteroduplex formation, the use of restriction endonucleases and the need for product purification.

Linear methods, in general, have higher mutational efficiencies than whole plasmid methods. Linear techniques use shorter target DNA templates, minimizing polymerase fidelity problems and resulting in shorter denaturation and annealing times. Resulting advantages are that the efficiency of mutation may be very high (> 90%), DNA segment length is precisely controlled, simple purification methods are available and few by-products are created. However, ligation of linear PCR products into vectors may be difficult due to non-template additions at terminal ends (Clark, 1988). The use of

Taq polymerase results in a ragged 3' terminal addition of single nucleotides, usually A. This may cause problems if internal primers are used for mutation. Selection of primers that terminate next to an A residue can minimize the effects of this problem. While blunt end ligation may be used for inserting PCR mutations into host vectors, PCR ligation kits, such as Invitrogen's TA Cloning(c) kit, may be used with much higher efficiency.

Linear PCR methods also permit the mutation of short sequences of DNA, which can be inserted into larger genes by restriction cloning methods. This is the basis for highly efficient techniques such as MR-PCR methods.

Whole plasmid mutation techniques offer the advantage of rapid transformation into host cells. Due to *in vivo* gap repair or restriction endonuclease cleavage, problems with non-template terminal additions inherent in linear PCR methods are avoided. However, whole plasmid mutation techniques are significantly more complicated than linear techniques. Many whole plasmid techniques depend very low efficiency processes, such as heteroduplex formation. Some involve extensive, multiple restriction cuttings and *in vivo* gap repair. Care must be taken select primers to avoid non-template binding to vector DNA sequences. Finally, polymerase fidelity may become an issue if repeated PCR reactions are needed, since the entire plasmid and gene must be amplified. (Note: Other commercial polymerases (New Eng. Biolabs, 1990) have claimed higher levels of fidelity than *Taq*.)

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