PCR-Based Random Mutagenesis Using Manganese and Reduced dNTP Concentration

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Procedures to introduce point mutations into specific DNA fragments are important tools to study gene function (2,10). UV light and chemical mutagens have been used in vivo to increase the frequency of random mutagenesis for specific DNA targets carried on plasmid or phage vectors being propagated in growing cells (2,11). Both methods induced mutations in the target DNA as well as in vector and host cell DNA. Chemical mutagenesis in vivo also resulted in a limited spectrum of substitutions at a limited set of hot spots on the DNA (2). Chemical mutagenesis has also been done in vitro for target DNA carried on plasmids (10). While a complete spectrum of substitutions was obtained, the procedure also produced vector mutations and required multiple cloning steps.

The polymerase chain reaction (PCR) process produces missense errors at a low rate. Others have reported on PCR modifications to induce mutations in vitro (5–7). This paper describes a simple modification of PCR amplification to enhance the production of mutant clones, the majority of which carry only a single base substitution in a specific target DNA sequence. The procedure relies on manganese-induced mis-insertion of nucleotides by AmpliTaq® DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) (1,4,6,7) as well as reduced concentration of each dNTP and increased number of PCR cycles to decrease the fidelity of PCR amplification. Over 50% of the mutated molecules contained only one mutation per clone. This mutagenesis method operates only on the region of interest (the PCR product) and uses only one cloning step to generate a library of mutations in the targeted region. This method is time-efficient, cost-effective and relatively effortless when compared to other random mutagenesis methods.

Table 1. Modified PCR Mutagenesis Rate across the Exons of hMSH2

Exon Number	Insert Size (bp)	White Colonies			Mutagenesis Rate (%)
		Total	Correct Insert	Mutants	
1	316	40	23	12	52.2
2	320	23	22	15	68.2
4	346	23	20	7	35.0
5	314	23	20	10	50.0
7	358	23	21	9	42.9
8	254	46	17	10	58.8
9	250	23	15	11	73.3
10	292	30	22	13	59.1
11	231	63	14	9	64.3
12	360	70	17	12	70.6
13	385	70	17	4	23.5
14	386	70	29	14	48.3
15	293	70	20	9	45.0
16	262	70	26	12	46.2
Totals		644	283	147	51.9

To prepare wild-type templates for mutagenesis, the PCR amplifications for the 16 exons of the wild-type hMSH2 (8) gene were performed on the GeneAmp® PCR System 9600 (Perkin-Elmer) in a 50-µL volume containing 1× PCR Buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl; Perkin-Elmer), 3 mM $MgCl_2$ (except for exon 1, which used 1.5 mM $MgCl_2$), 0.2 mM of each dNTP, 10 pmol of each primer, 1.25 U AmpliTaq DNA Polymerase and 10 ng of genomic DNA. Samples were subjected to heating at 94°C for 3 min followed by 35 PCR cycles (20 s at 94°C, 20 s at 55°C, 40 s at 72°C) and finished at 72°C for 10 min. The sequences of these wild-type PCR products (hMSH2 exons) were verified by direct sequencing on a PRISM™ Ready Reaction Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase FS (PE Applied Biosystems, Foster City, CA, USA). These wild-type PCR products were then used as DNA templates for PCR mutagenesis.

A library of mutants for each exon of the hMSH2 gene was generated by the PCR mutagenesis procedure using 10 ng wild-type PCR product as template in a 50-µL volume of mutagenesis buffer containing: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.25 mM $MnCl_2$ (added immediately prior to the PCR), 20 µM of each dNTP, 10 pmol of each primer and 1.25 U AmpliTaq. The PCR mutagenesis conditions were heating at 94°C for 4 min followed by 40 PCR cycles (15 s at 94°C, 15 s at 55°C, 45 s at 72°C) and finished at 72°C for 10 min. This extra extension time promoted the yield of a full-length product.

After PCR mutagenesis, a small aliquot of PCR product was ligated into TA Cloning® vector (Invitrogen, San Diego, CA, USA) without any further purification. The 10-µL ligation reaction mixture contained 1× ligation buffer, 50 ng pCR™ II vector (both from Invitrogen), 1 µL T4 DNA ligase and 2–4 µL of fresh PCR product and was incubated for 5–14 h at 14°C. Then this ligation mixture was used to transform One Shot™ cells (Invitrogen) following the protocol supplied by the manufacturer. Those cells were spread onto LB plates containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) (9), then grown at 37°C overnight for white colony selection. All white colonies were restreaked on fresh LB/ampicillin/X-gal/IPTG plates to confirm the white colony.

To verify the correct insert size, each white colony was subjected to PCR amplification (using the wild-type protocol above) followed by gel electrophoresis. All products of the correct size were purified with the QIAquick™ 96 PCR Purification Kit (Qiagen, Chatsworth, CA, USA), then subjected

Table 2. Frequency of Individual Substitutions Resulting from a Modified PCR Mutagenesis Procedure

	No.	Percent of Total (%)
A→G	91	32.2
T→C	85	31.0
T→A	24	8.8
A→T	20	7.3
C→T	14	5.1
G→A	12	4.4
T→G	8	2.9
A→C	3	1.1
C→A	5	1.8
G→T	4	1.5
C→G	2	0.7
G→C	2	0.7
Single Deletion	4	1.5
Total[a]	274	100

[a]147 mutant inserts yielded 274 individual mutations.

to cycle sequencing reactions using the PRISM kit with AmpliTaq. Sequencing reaction products were separated on a Model 373 Automated DNA Sequencer (PE Applied Biosystems), and the electropherograms of each clone were analyzed using Sequencher™ 3.0 software (Gene Codes, Ann Arbor, MI, USA) and compared to those of wild-type DNA for each exon.

A total of 644 white colonies were screened by colony PCR amplification (Table 1). Among them, 361 white colonies were revertants containing self-ligated TA vector without any insert; 283 colonies contained the correct-size insert and were analyzed further by sequencing. Of these 283, 147 clones contained one or more base substitutions. The average mutation rate was approximately 52%. This compares favorably to the 35% reported by Zhou et al. (12). Our procedures increased the mutation rate by lowering the fidelity of AmpliTaq with the addition of manganese chloride, a higher magnesium chloride concentration, lower dNTPs and greater cycle number during PCR mutagenesis.

The mutagenesis rate varied from exon to exon; for example, exon 9 of hMSH2 had the highest mutation rate at 73.3%, while exon 13 had only 23.5%. The differences do not appear

to be insert length-dependent: both exon 12 and exon 13 are about 370 bp in length, yet exon 12 had a mutation rate of 70.6% and exon 13 of only 23.5%.

Most of the hMSH2 mutations generated by our method were single substitutions including transversions and transitions. All twelve possible substitutions were obtained. The A→G and T→C mutation appeared to predominate (Table 2). The combination of these two totaled 63.2% of all mutations. The G→C and C→G mutation was the least seen and only comprised 1.4% of all mutations. Similar results were also reported (3,5). The A→G and T→C mutation was more frequent because of favored G:T wobble pairs and could be reduced by lowering the dATP and dGTP concentration during PCR mutagenesis.

A total of 278 independent single substitutions were distributed across 147 clones. The number of substitutions per clone did not follow a Poisson distribution. The majority (53.5%) of all mutants in this study contained a single substitution per clone. Next, 25.2% of them had two independent substitutions within the same cloned insert. Only 10.7% carried 3 independent substitutions, and less than 6.3% of them contained 4 or more independent substitutions. There were very few deletion mutations found (about 4.4%) with this procedure. Fromant et al. (5) have also reported an in vitro mutagenesis procedure using PCR and manganese. Our procedure differs significantly in that we used half the manganese ion concentration, one quarter the magnesium ion concentration, 1/170 the dNTP concentration and PCR cycles of significantly shorter duration. On the average, they reported multiple substitutions per target DNA, while our procedure resulted in only one substitution per clone in approximately half of the mutated clones. These mutants containing a single substitution are ideal for studying the effect of amino acid changes on gene function.

This effective random mutagenesis method has several advantages over existing methods: it uses a one-step PCR mutagenesis and a one-step cloning procedure, achieves a high mutation rate and generates a large number of mutants containing a single substitution per clone.

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Near-Zero Background Cloning of PCR Products

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Cloning of polymerase chain reaction (PCR)-amplified fragments has become a widely used technique in molecular biology. Common cloning strategies include: sticky-end cloning, blunt-end cloning, TA cloning and ligation-free cloning (1,5). Among these, sticky-end cloning is the most efficient and is preferred whenever possible, especially for subcloning.

In sticky-end cloning, restriction sites are designed into the two PCR primers (15). Often the two sites are different to allow directional cloning. After amplification, the PCR fragment and the vector plasmid are digested with restriction enzymes, purified, ligated and transformed into *E. coli* (13). Since self-ligated and uncut vector plasmid can also transform bacteria, many colonies with no insert (false-positives) are generated as background, and a large number of colonies have to be screened to find a colony with an insert (true-positive). For this reason, several strategies have been developed to reduce or eliminate the background caused by self-ligated or uncut vector. For example, the linear vector can be dephosphorylated before mixing with PCR product and ligation. However, this not only reduces the cloning efficiency (13) but also is tedious since the PCR product and the vector have to be digested and purified separately before mixing the PCR fragment and the dephosphorylated vector for ligation. Alternatively, positive-selection vectors have been developed to reduce false-positive colonies (2–4,6,7, 9,10,12,14,18,19), but these "zero-background" approaches require special vectors and, very often, special strains of bacteria (12,14,18).

Earlier studies have found that blunt-end PCR product cloning efficiency could be improved by digestion of the ligation product with appropriate restriction enzymes during (11) or after ligation (16). Moving beyond this strategy, we developed a simple and universal sticky-end cloning strategy in which we use a restriction digestion step between ligation and transformation,