A Novel Method for Site-directed Mutagenesis Using PCR and Uracil DNA Glycosylase

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A novel method for site-directed mutagenesis of DNA sequences based on the use of the PCR is described. The method uses two oligonucleotide primers that contain the desired sequence change and overlap at their 5' ends. In addition, the thymine residues in the overlap region have been substituted with deoxyuracil. Amplification of the template plasmid by PCR results in incorporation of the primers and the desired mutation into the PCR product. Excision of the deoxyuracil residues in the PCR products by uracil DNA glycosylase (UDG) destabilizes base-pairing at the ends of DNA molecules and thus generates 3' protruding ends in the opposite strand. Due to overlapping nature of the primers, the resulting 3' protruding ends are complementary and can anneal rapidly after treatment with UDG. When the entire plasmid is amplified, a linear mutant PCR product is generated that circularizes after treatment with UDG. Circularized molecules can then be transformed into competent cells without ligation, generating transformants with the mutant genotype. Alternatively, the gene of interest is amplified in two segments using overlapping mutant primers and cloned in the desired orientation into pAMP1 by UDG cloning. Application of this method to site-specific mutagenesis of the *lacZα* gene and the human *c-raf* oncogene was demonstrated. The accuracy of the mutations was confirmed by nucleotide sequence analysis as well as phenotypic assays. The method is rapid, highly efficient (>99%), and applicable to genes

cloned in any vector as well as to genomic DNA or RNA. The versatility of the method allows single base mutagenesis as well as insertions and deletions. UDG mutagenesis should prove to be a general, rapid, and high-fidelity method for site-directed mutagenesis.

The deliberate alteration of DNA sequences by site-specific mutagenesis is a widely used approach for studying the structure and function of genes and their products. Several approaches are now available for site-directed mutagenesis.⁽¹⁻⁴⁾ Although all of these methods are effective, the frequency of generation of desired mutation varies. The efficiency of mutagenesis is of particular importance when the sequence change(s) result in silent, unknown, and nonselectable phenotypes. The PCR has recently been used to create site-specific mutations in DNA.⁽⁵⁾ Although this method is reliable compared to other methods, it has been difficult to apply to DNA sequences greater than about 500 bp in length.⁽⁶⁾ In addition, it requires multiple sequential amplifications that increase the risk of introducing random and undesirable mutations in DNA due to the low fidelity of *Taq* DNA polymerase.⁽⁷⁾

Recently, we have developed a novel method for efficient cloning of PCR-amplified DNA using uracil DNA glycosylase (UDG).^(8,9) In this procedure, the desired sequences are amplified using primers in which the dT residues have been substituted with dU. Excision of the

dU residues in the PCR-amplified DNA fragments destabilizes base-pairing at the ends of molecules, producing 3' protruding ends in the opposite strand. Annealing of such fragments to complementary sequences on an appropriate vector and transformation of competent *Escherichia coli* cells has provided an efficient and rapid method for cloning PCR products. In this paper, we report a modification of the UDG cloning method for generation of site-specific mutations by PCR. We have applied this method to site-directed mutagenesis of the *lacZα* gene in plasmid pUC19 and mutagenesis of the human *raf* oncogene. The method can be used to generate single base mutations as well as deletions and insertions.

MATERIALS AND METHODS

Oligonucleotides

All oligonucleotide primers were synthesized using a model 380 A DNA synthesizer from Applied Biosystems, Inc. (Foster City, California). The deoxyuracil-CE-phosphoramidite for synthesis of dU-containing oligonucleotides was from Life Technologies Inc. (GIBCO/BRL). The sequences of the oligonucleotides for mutagenesis of *lacZα* were as shown below. The mutant bases are underlined.

Mutant primer(1241): 5'-AAC GUC GUG ACU GAG AAA ACC CTG G-3'

Wild type primer(1242): 5'-AAC GUC GUG ACU GGG AAA ACC CTG G-3'

Amplification primer(1319): 5'-AGU CAC GAC GUU GUA AAA CGA CGG C-3'

The following primers were used to introduce a single amino acid change in the human *raf* oncogene.

1303 5'-CUU UAG GAU CCA UAC UGC
AAC ATC TCC GTG-3'
1304 5'-GUA UGG AUC CUA AAG GUU
GTC GAC CCA A-3'
1301 5'-CUA CUA CUA CUA GGT TTT
CCC AGT CAC GAC G-3'
1302 5'-CAU CAU CAU CAU TAA CAA
TTT CAC ACA GGA AAC-3'

Amplification of the mutant genes in pAMP1 was carried out using the M13 23-base forward primer (5' CCC AGT CAC GAC GTT GTA AAA CG-3') and the T7 promoter primer (5'-TAA TAC GAC TCA CTA TAG GG 3').

Enzymes and Reagents

Taq DNA polymerase was purchased from Perkin-Elmer Cetus (Emeryville, California). Competent cells, restriction enzymes, uracil DNA glycosylase, and plasmid pAMP1 were obtained from Life Technologies Inc. (GIBCO/BRL; Gaithersburg, Maryland).

PCR Amplification

All PCR amplifications were performed in 50 μ l of 1 $\times$ PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM MgCl₂). The reaction also contained 0.05–0.2 mM of each dNTP, 1–2 units of *Taq* DNA polymerase, and 1.0 μ M of each primer unless otherwise stated. Thermocycling of PCR reactions was performed using a DNA Thermal Cycler from Perkin-Elmer Cetus. Unless otherwise noted, after an initial 5-min denaturation at 94°C, 30 cycles of 30 sec at 94°C, 1 min at 55°C, and 2 min at 72°C were performed. An additional 5-min extension at 72°C was used following the last cycle. When amplifying pUC19, 500 pg was used for amplification. The procedure used for amplification of DNA from bacterial lysates was as described previously.⁽⁹⁾ Products of the PCR reactions were analyzed by agarose gel electrophoresis and visualized after ethidium bromide staining.

Mutagenesis

For mutagenesis of the *lacZ* α gene in plasmid pUC19, supercoiled plasmid DNA was amplified using overlapping oligonucleotide primers. To improve the fidelity of DNA polymerization, all PCR reactions for mutagenesis were performed with 0.05 mM of each dNTP. Plasmid DNA (500 pg) was diluted into 25 μ l of 1 $\times$ PCR buffer and overlaid with

mineral oil. The tube was then placed in the thermocycler and heated to 100°C for 10 min followed by rapid cooling on ice. Then 24 μ l of 1 $\times$ PCR buffer containing nucleotides, the appropriate primers, and *Taq* DNA polymerase were added and amplified by PCR as described above. A 4- μ l aliquot (25 ng) of the PCR products was treated with 1 unit of UDG for 30 min at 37°C in a final volume of 20 μ l (in 1 $\times$ PCR buffer). A 2- μ l aliquot of the UDG-treated products was used directly to transform *E. coli* DH5 α or DH10B competent cells (Life Technologies, Inc., Gaithersburg, Maryland).

Plasmid pCMVBXB was used for mutagenesis of the human *c-raf-1* gene. This plasmid contains the activated BXB version of a *c-raf-1* cDNA clone⁽¹⁰⁾ preceded by the immediate early cytomegalovirus (CMV) promoter and followed by the SV40 poly(A) signal. The plasmid was linearized by digestion with *Xmn*I, and 15 ng of linearized plasmid was used for amplification. Two separate amplifications were performed as described above except that only 18 cycles of PCR were performed. Four microliters (25 ng) of each PCR and 4 μ l (80 ng) of plasmid pAMP1 were mixed in an Eppendorf tube, and the reaction volume was increased to 19 μ l with 1 $\times$ PCR buffer. One unit of UDG was then added and the reaction (annealing mix) was incubated at 37°C for 30 min. A sample (1–5 μ l) of the UDG-treated annealing mix was used directly to transform competent *E. coli* DH10B or DH5 α cells following the manufacturer's recommendations. Transformants were plated onto LB plates containing ampicillin (100 μ g/ml), X-Gal, and IPTG.

Sequencing

DNA sequencing of the *lacZ* α mutants was carried out using the dsDNA Cycle Sequencing System (Life Technologies, Inc.) directly from colony lysates as described by Young and Blakesley.⁽¹¹⁾ The primer used for sequencing was the ³²P-labeled M13/pUC forward 23-base primer (Life Technologies, Inc.). The mutations in human *c-raf-1* oncogene were confirmed by sequencing plasmid minipreps with the Sequenase system from United States Biochemicals (Cleveland, Ohio) using an internal primer that hybridizes between nucleotides 1050 and 1065 of the *c-raf-1* cDNA sequence.⁽¹²⁾

Biological assay of pCMVBXB301

Five micrograms of linear plasmid DNA were cotransfected with 0.5 μ g of linear pSV2neo plasmid DNA into NIH-3T3 cells using the calcium phosphate method as previously described.⁽¹³⁾ Neomycin-resistant clones were selected using G418 at a final concentration of 800 μ g/ml of media. After scoring for phenotype, 10 clones were expanded for protein analysis. Cells from 1 T25 were lysed in 1 ml of Tris-buffered saline with 1% Triton X-100 and 1 mM PMSF. After clearing the lysate, anti-SP63 antibody⁽¹⁴⁾ and Protein A-Sepharose (Boehringer-Mannheim Biochemicals) were used to collect Raf proteins. Immunoprecipitates were separated by gel electrophoresis and transferred to nitrocellulose. Blots were probed with anti-SP63 antibody and bands were visualized by alkaline phosphatase according to the manufacturer's instructions (Life Technologies, Inc.).

RESULTS

Principle of the Method

We have developed a method for site-directed mutagenesis of DNA sequences based on amplification with PCR and use of UDG. The schematic representation of the method is shown in Figure 1A. The method is based on synthesis of two overlapping primers containing the desired nucleotide change(s) in which some or all of the dT residues have been substituted with deoxyuracil. The primers are designed such that during the PCR the entire plasmid is amplified as a linear DNA fragment with the desired base change(s) in the primer and its complementary sequence in the opposite strand. Incorporation of the dU-containing primers into PCR product renders the 5' end of the fragments susceptible to UDG. Treatment of such PCR products with UDG results in excision of dU residues, producing 3' sticky ends. Due to the overlapping nature of the primers used, the 3' protruding ends are complementary and result in circularization of the PCR products. Transformation and in vivo repair of the circularized plasmids result in generation of new plasmid molecules that are identical to the wild-type parental plasmid except for the desired mutation(s).

In cases where the gene of interest is not in a plasmid or the plasmid is too

FIGURE 1 Schematic representation of the strategy used for mutagenesis of *lacZα* (A) and mutagenesis of *c-ras* oncogene (B). The stippled regions on primers indicate the sequences where T residues have been substituted with dU. The mutant primers in B have been labeled MP1 and MP2. The arrows denote the 3' ends of DNA strands.

large to be amplified in its entirety, the gene of interest can be amplified in two segments and subsequently cloned using UDG. This alternative method is depicted in Figure 1B. In this procedure, mutagenesis is performed by designing two dU-substituted overlapping primers containing the appropriate mutation(s). Each of the primers is used in separate PCR amplifications with an appropriate 5' or 3' primer containing dU sequences to enable UDG cloning into pAMP1 or other appropriate vectors.^(8,9) The resulting amplification products are mixed in a single tube containing the pAMP1 vector and UDG, generating chimeric molecules with the desired mutations. The

resulting circular chimeric plasmids are used directly for transformation of competent *E. coli* cells. Clones carrying the chimeric plasmid contain the desired mutation.

Mutagenesis of *LacZα*

To demonstrate the application of the UDG mutagenesis method, the *lacZα* complementation gene in plasmid pUC19 was used. Normally, *E. coli* strains harboring this plasmid with the wild-type gene grow as dark blue colonies on agar plates containing X-Gal and IPTG. However, Kunkel et al.⁽¹⁵⁾ have shown that a single guanine-to-adenine substi-

tution at position 89 of this gene (where +1 is the first transcribed base of the *lacZα* gene) creates an opal codon, yielding white colonies (*lac*⁻ phenotype). A set of synthetic oligonucleotides containing dU residues was synthesized to introduce the single-base guanine-to-adenine substitution at this position. PCR amplifications were performed using circular pUC19 DNA as the amplification target, with the mutant primer 1241 and amplification primer 1319. A separate amplification was also performed with the wild-type primer 1242 and amplification primer 1319 as control. Both amplifications resulted in a PCR fragment corresponding in size to linearized

pUC19 plasmid. PCR products were treated with UDG and used to transform competent cells. After overnight growth, colonies were scored for the blue or white phenotype. An identical sample of the PCR product that had not been treated with UDG was used as a control. The results showed that >99% of colonies with the mutant primer had the mutant phenotype (Table 1). The small number of wild-type colonies observed are believed to have arisen from transformation with wild-type plasmid used as mutagenesis target. However, as expected, amplification with wild-type primers showed 95% of the colonies to be wild type. The 5% mutation rate that is observed with wild-type primers is believed to arise from random mutants generated during amplification. Control experiments in which the UDG treatment had been omitted showed only a very small number of colonies (0.5–1%) that resulted from transformation of cells with the amplification template.

Reverse Mutagenesis to Wild Type

The precision of the mutagenesis in the desired position with UDG cloning was examined by reverse mutagenesis of the G-to-A mutants to wild-type phenotype. Two independent mutant clones were randomly selected (MA101, MA102) and the plasmids were amplified using the wild-type primers. Transformation of the amplified DNA from both clones after UDG treatment showed 95% reversion to the wild-type phenotype. The remaining colonies with the mutant phenotype derived from the original templates, as well as from the random mutations introduced during PCR. The fact that two randomly selected mutants could be efficiently mutagenized back to the wild-type phenotype strongly suggests that the mutation giving rise to

white colony phenotype was indeed the intended G-to-A mutation at position 89.

Mutagenesis from Colony Lysates

To simplify the mutagenesis protocol further, we also tested mutagenesis amplification directly from bacterial colonies. A small amount of bacterial cells from mutant colonies MA101 and MA102 (white phenotype) were resuspended in 1× PCR buffer and amplified using wild-type primers, as described in Materials and Methods. UDG treatment and transformation showed effective mutagenesis (>97%) of the *lacZα* mutants to the wild-type phenotype. In other studies, we have observed that the frequency of mutagenesis varies from 95 to 99%. This variation is related to the amount of cells used for mutagenesis (i.e., amount of original wild-type template plasmid) and also the number of amplification cycles. We have found that generally 15–30 cycles of PCR are quite satisfactory. In some cases, we have analyzed the amplification products after 10–15 cycles of PCR by gel electrophoresis followed by UDG treatment and transformation. Even in cases where no DNA was visible by ethidium bromide staining, the transformation efficiency was high enough for isolation of mutants. These results are in concordance with our previous results regarding ligase-free cloning with UDG.⁽⁹⁾

DNA Sequence Analysis

The accuracy of mutagenesis and the precision of the in vivo repair of the mutagenesis overlap junctions were examined by nucleotide sequence analysis. The mutant plasmids from MA101 and MA102 were sequenced using ³²P-labeled M13/pUC forward primer. The desired G-to-A mutation at position 89 was

confirmed in both mutant plasmids. As expected, the plasmids recovered from amplification with wild-type primers showed no mutations. In all of the plasmids examined, the junctions of the overlapping 3' ends were repaired correctly, and no other changes were observed (Fig. 2).

Mutagenesis of Human *c-ras*-1 Oncogene

Plasmid pCMVBXB encodes the kinase domain of the serine/threonine protein kinase Raf-1 under the control of the CMV promoter.⁽¹⁰⁾ Cotransformation of this plasmid with pSV2neo into NIH-3T3 cells produces colonies with the transformed phenotype. Replacing the lysine with tryptophan at position 375 in full-length Raf-1 inactivates kinase activity, conferring a dominant negative phenotype⁽¹⁶⁾ and abolishing the transforming activity in activated versions of Raf-1.⁽¹³⁾ For mutagenesis of this site, two overlapping primers 1303 and 1304 were synthesized in which the codon for lysine had been changed to tryptophan. The gene was amplified in two segments us-

TABLE 1 Mutagenesis of the *lacZα*^a

Primers used in the PCR mix	+ UDG		– UDG	
	blue	white	blue	white
Mutant (1241, 1319)	5	610	19	23
Wild type (1242, 1319)	885	45	35	0

^aPlasmid pUC19 was amplified using the primer sets indicated above and the PCR products were incubated in the presence or absence of UDG at 37°C. The amplified DNA was then used to transform competent *E. coli* cells and transformants were scored for the Lac phenotype.

FIGURE 2 Nucleotide sequence analysis of G-to-A mutation in position 89 of *lacZα*. Arrow indicates the mutated base.

ing the mutant primer 1303 and vector primer 1302, or the mutant primer 1304 and vector primer 1301.

Agarose gel electrophoresis of the PCR products showed the expected PCR-amplified fragments of 1379 bp and 1170 bp, respectively (Fig. 3). The two PCR products were mixed with plasmid pAMP1 and treated with UDG for 30 min at 37°C. A portion of this annealing mix was used to transform competent *E. coli* cells. The transformants were selected on ampicillin plates. An identical preparation that had not been treated with UDG was used as a control. These experiments showed the mutagenesis to be dependent on UDG treatment, as well as on the presence of the appropriate amplified segments with overlapping ends and plasmid pAMP1 (Table 2). Analysis of plasmid DNA in the transformants by PCR amplification directly from colonies with primers flanking the insert is shown in Figure 3. All tested transformants arising from UDG-treated PCR products showed a 2534-bp insert in plasmid pAMP1. The clones obtained from transformation of cells with the mixture without UDG, contained the original wild-type plasmid used as the PCR target for amplification (pCMVBXB containing the wild-type *c-raf* gene), and

FIGURE 3 Mutagenesis of the *c-raf* oncogene with UDG and dU containing mutant primers. Plasmid pCMVBXB was amplified in two separate PCR reactions using primers 1302 and 1303 (lane 1) or using primers 1301 and 1304 (lane 2). The two PCR products and plasmid pAMP1 were mixed, treated with UDG, and used for transformation of competent *E. coli* cells as described in Materials and Methods. Resulting colonies were screened for presence of full-length *c-raf* insert in plasmid pAMP1 (lanes 3–9) using vector-specific primers and PCR. Lane M is the 100-bp ladder from BRL. The bands in the marker lane are spaced 100 bp apart, the smallest fragment being 100 bp long.

TABLE 2 Mutagenesis of the Human *c-raf* Oncogene

Annealing mix	+ UDG		– UDG	
	clones	<i>c-raf</i> recombinants	clones	<i>c-raf</i> recombinants
Seg. 1, 2, pAMP1	155 ^a	11/11 ^b	7	0/2
Seg. 1, pAMP1	3	NT ^c	4	NT
Seg. 2, pAMP1	4	NT	5	NT
pAMP1	0		0	

^aEfficiency of cloning was 1.55×10^5 transformants per microgram of PCR products.

^bRecombinant clones were tested for presence of full-length *c-raf* insert in pAMP1 by PCR analysis as described in Materials and Methods.

^c(NT) Not tested.

did not amplify with primers specific for pAMP1 (data not shown).

Sequence analysis of 200 bp surrounding codon 375 in four independent mutant clones (pCMVBXB 301) showed no sequence divergence from the wild-type sequence except for the intended AA-to-TG exchanges resulting in the Lys-to-Trp substitution. These mutant plasmids were also assayed biologically for transforming activity.⁽¹³⁾ Cotransfection of plasmid DNA from two clones with pSV2neo into NIH-3T3 cells revealed that the transforming activity of the parental plasmid had been abolished without affecting the amount of the protein being produced in neomycin-resistant clones (data not shown).

DISCUSSION

We have described a rapid and efficient method for site-directed mutagenesis using PCR and UDG. Two alternative procedures were used and both were shown to be effective. In the first procedure, the entire plasmid containing the gene of interest is amplified using two overlapping primers containing deoxyuracil residues. In this case, a single amplification reaction yields amplified mutant DNA that can be used directly for UDG treatment and subsequent transformation. This method is applicable to cloned DNA in any plasmid vector as long as the entire plasmid can be amplified by PCR. Prior to testing this method, we had expected that amplification of long DNA sequences (2.5–4.0 kb), particularly using circular plasmid as template, would be problematic. Furthermore, we expected that primers with overlapping sequences at their 5' ends would exacerbate this potential problem. We have, however, found that the PCR can effectively am-

plify the supercoiled plasmid when an initial denaturation step at 100°C for 7–10 min is included prior to PCR. Although efficiency of PCR is better for amplification of smaller DNA fragments, plasmids in the range of 2.5–4.0 kb are amplified sufficiently for use in the UDG mutagenesis method. This is at least partly due to the high efficiency of cloning with the ligase-free method used in these experiments.⁽⁹⁾

Recently, Jones and Winistorer⁽¹⁷⁾ have demonstrated that cotransformation of PCR products with homologous ends can result in *in vivo* recombination and generation of circular plasmids containing mutation(s). Since this method requires *in vivo* recombination, the frequency of transformation is very low, necessitating use of highly competent cells. In addition, the frequency of transformation is highly dependent on the length of homology between two PCR products; decreasing the length of the homology from 25 bp to 12 bp reduces the efficiency of transformation four- to five-fold.⁽¹⁷⁾ These problems have been solved in the UDG mutagenesis method. The efficiency of cloning with the UDG method is high (10^6 – 10^7 transformants per microgram⁽⁹⁾) and overlap of only 10–12 bases is needed. In the experiments with mutagenesis of *C-raf*, the efficiency of cloning was lower than previously reported due to simultaneous cloning of two PCR fragments into the pAMP1 vector (Table 2).

The method used to mutagenize the *c-raf-1* gene is most advantageous for mutagenesis of large genes, genes cloned into very large vectors such as cosmids and yeast artificial chromosomes, and uncloned genes (i.e., genomic DNA, cDNA, etc.). This method combines mutagenesis and cloning of the mutant

gene into a one-step procedure. The results show that efficiency of mutagenesis is high and all of the examined clones contain the desired mutation.

The UDG mutagenesis method described here combines many of the advantages of the existing mutagenesis procedures and circumvents their shortcomings. When compared to the phagemid method described by Kunkel,⁽⁴⁾ UDG mutagenesis offers a higher frequency of mutation because the use of PCR allows exponential amplification of the mutant molecules. The frequency of mutation is high (>99%), and a single-stranded dU template (phagemids grown in a special *dut⁻ ung⁻* strain) is not needed. The method is also applicable to DNA sequences present in any cloning vector, and does not require subcloning of genes into phagemids to isolate single-stranded DNA.

Although UDG mutagenesis is similar to the approach described by Higuchi et al.,⁽⁵⁾ it has features that make it more practical and advantageous. It has been shown that PCR amplification can introduce mutations in the amplified DNA due to low fidelity of *Taq* DNA polymerase.⁽⁷⁾ This problem can be further exacerbated by two sequential amplifications required using the method of Higuchi et al.⁽⁶⁾ This problem can be addressed by two approaches: (1) reduction in the total number of cycles used in PCR and (2) use of a thermostable DNA polymerase with proofreading activity.⁽¹⁸⁻²⁰⁾ In the UDG mutagenesis method, the use of overlapping mutant oligonucleotide primers containing dU allows two PCR products to be joined without the need for further amplification, thereby reducing the number of cycles needed. Furthermore, the high efficiency of mutagenesis combined with high efficiency of cloning (Table 2) allows cloning after as few as 10 cycles. Not only does this simplify the method, it also reduces the chance of introducing further unwanted mutations in the amplified DNA. Therefore, it is reasonable to propose that UDG mutagenesis in conjunction with use of a DNA polymerase with proofreading capability⁽¹⁸⁻²⁰⁾ will result in a very high-fidelity mutagenesis method. Another advantage of this method is that by using UDG the joining of the mutant fragments and cloning of the full-length fragment into an appropriate host are combined and performed simultaneously.

The use of deoxyuracil in PCR primers and the ability to generate 3' cohesive ends by UDG excision of dU residues offer a novel method for joining nucleic acid fragments. The method is versatile and can be used with essentially any DNA sequence containing dU. This method of joining DNA fragments allows researchers to assemble PCR products without being limited to the presence of restriction enzyme sites in the desired site.

In both of the procedures described for UDG mutagenesis, treatment of PCR products with UDG, annealing of complementary ends, and circularization of plasmids occur simultaneously within the incubation time. In addition, both procedures require only one PCR step (i.e., no overlap extension is needed), which can be as few as 10–15 cycles. This is simplified even further by using bacterial lysates from individual colonies as mutagenesis template. These features and the high efficiency of mutagenesis make UDG mutagenesis a rapid and generally applicable method for site-directed mutagenesis.

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REFERENCES

- Smith, M., and S. Gillam. 1981. Constructed mutants using synthetic oligodeoxyribonucleotides as site specific mutagens. In *Genetic engineering: Principles and methods* (ed. J.K. Setlow and A. Hollaender) vol. 3, pp. 1–32. Plenum Press, New York.
- Shortle, D., D. Di Maio, and D. Nathans. 1981. Directed mutagenesis. *Annu. Rev. Genet.* **15**: 265–294.
- Smith, M. 1985. In vitro mutagenesis. *Annu. Rev. Genet.* **19**: 423–462.
- Kunkel, T.A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci.* **82**: 488–492.
- Higuchi, R., B. Krummel, and R.K. Saiki. 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions. *Nucleic Acids Res.* **16**: 7351–7367.
- Perrin, S. and G. Gililand. 1991. Site-specific mutagenesis using asymmetric polymerase chain reaction and a single mutant primer. *Nucleic Acids Res.* **18**: 7433–7438.
- Tindall, K.R. and T.A. Kunkel. 1988. Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry* **27**: 6008–6013.
- Nisson, P.C., A. Rashtchian, and P.C. Watkins. 1991. Rapid and efficient cloning of alu-PCR products using uracil DNA glycosylase. *PCR Methods Applic.* **1**: 120–123.
- Rashtchian, A., G. Buchman, D. Schuster, and M. Berninger. 1992. UDG-mediated cloning of PCR-amplified DNA: Application to genomic and cDNA cloning. *Anal. Biochem.* **206**: 91–97.
- Bruder, J.T., G. Heidecker, and U.R. Rapp. 1992. Serum, TPA, and ras-induced expression from Ap-1/Ets-driven promoters requires Raf-1 kinase. *Genes & Dev.* **6**: 545–556.
- Young, A. and R. Blakesly. 1991. Sequencing plasmids from single colonies with the dsDNA cycle sequencing system. *Focus* **13**: 137.
- Bonner, T.I., P. Oppermann, P. Seeburg, S.B. Kerby, M.A. Gunnell, A.C. Young, and U.R. Rapp. 1986. The complete coding sequence of human raf oncogene and the corresponding structure of the c-raf-1 gene. *Nucleic Acids Res.* **14**: 1009.
- Heidecker, G., M. Huleihel, J.L. Cleveland, W. Kolch, T.W. Beck, P. Lloyd, T. Pawson, and U.R. Rapp. 1990. Mutational activation of c-raf-1 and definition of the minimal transforming sequence. *Mol. Cell. Biol.* **10**: 2503–2512.
- Kolch, W., E. Weissinger, H. Mischak, J. Troppmair, S.D. Showalter, P. Lloyd, and G. Heidecker. 1990. Probing structure and function of the raf protein kinase domain with monoclonal antibodies. *Oncogene* **5**: 713–720.
- Kunkel, T.A., R.D. Sabatino, and R.A. Bambara. 1987. Exonucleolytic proofreading by calf thymus DNA polymerase δ . *Proc. Natl. Acad. Sci.* **84**: 4865–4869.
- Kolch, W., G. Heidecker, P. Lloyd, and U.R. Rapp. 1991. Raf-1 protein kinase is required for growth of NIH/3T3 cells induced by serum, TPA, and ras oncogenes. *Nature* **349**: 426–428.
- Jones, D.H. and S.C. Winistorer. 1990. Simultaneous site-specific mutagenesis of two distal sites by in vivo recombination of PCR products. *Technique* **2**: 273–278.
- Cariello, N.F., J.A. Swenberg, and T.R. Skopek. 1991. Fidelity of *Thermus litoralis* DNA polymerase (Vent) in PCR determined by denaturing gradient gel electrophoresis. *Nucleic Acids Res.* **19**: 4193–4198.
- Mattila, P., J. Korpela, T. Tenkanen, and K. Pitkanen. 1991. Fidelity of DNA synthesis by the *Thermus litoralis* DNA polymerase—an extremely heat-stable en-

- zyme with proofreading activity. *Nucleic Acids Res.* **19**: 4967–4973.
20. Lundberg, K.S., D.D. Shoemaker, M.W.W. Adams, J.A. Short, J.A. Sorge, and E.J. Mathur. 1991. High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. *Gene* **108**: 1–6.

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References

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