

Design and Use of Mismatched and Degenerate Primers

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INTRODUCTION

Although simple in concept, PCR requires a myriad of complex interactions between template, primers, deoxynucleoside triphosphates, and DNA polymerase to accomplish targeted amplification successfully. Primer design in conjunction with changes in the concentrations of reaction components and thermal cycling parameters provides a versatility available in no other molecular technique. For example, PCR can be employed to accommodate mismatches in the primer-template duplex, thereby permitting amplification not only of related sequences, but also of uncharacterized sequences. It can also be designed to amplify a small number of mutant genes with single base alterations selectively in a vast background of normal genes.

The flexibility of PCR has greatly simplified molecular manipulations. Procedures for altering a particular template sequence, which in the past required several steps, can now be performed with only a few manipulations. Because 3' and internal mismatches between primer-template duplexes are tolerated under appropriate conditions, point mutations and desired restriction endonuclease sites can be introduced directly into the primers. Likewise, nucleotide insertions and deletions can be similarly introduced into the amplified product via the primers.

Degenerate primers have made it possible to amplify related but distinct nucleic acid sequences as well as to amplify targets for which only amino acid sequences are available. The range of possible applications precludes providing detailed protocols for each. Instead, the critical parameters and a guide for representative use are summarized. Depending on the application, a subset of these recommendations or suggestions may suffice.

Mismatch Discrimination

Primers can be tailored to amplify selectively targets that vary by a single nucleotide (for review, see Ugozzoli and Wallace 1991). Genetic diseases commonly arise from single base-pair mutations. Sickle cell anemia, for example, results from A to T transversion in codon 6 of the human β -globin sequence (Marotta et al. 1977). Single point mutations at codons 12, 13, or 61 of the *ras* genes result in activation of the proto-oncogene that has been associated with a high frequency of human cancers (Bos 1989). Resistance to HIV antivirals such as zidovudine, ddI, ddC, and pyridinone reverse transcriptase inhibitors is conferred by multiple single-base mutations (Larder et al. 1989; Nunberg et al. 1991; St. Clair et al. 1991; Larder and Boucher 1991).

The discrimination of different PCR targets is based on the fact that *Taq* DNA polymerase lacks a 3' \rightarrow 5' exonuclease activity (Lawyer et al. 1989) and that mismatched 3' termini are extended at a lower rate than matched termini. Various mismatches are extended at different efficiencies (Creighton et al. 1992; Huang et al. 1992). Multiple acronyms have been used to describe sequence-specific amplification including ASPCR (allele-specific PCR) (Ugozzoli and Wallace 1991), ARMS (amplification refractory mutation system) (Newton et al. 1989), MAMA (mismatch amplification mutation assay) (Cha et al. 1992), and PASA (PCR amplification of specific alleles) (Dutton and Sommer 1991). The appellation "allele-specific" is somewhat of a misnomer because rare somatic mutations can also be identified using this approach.

In designing primer pair systems to discriminate mutant from wild-type sequences, one needs first to examine the mutations involved. The placement of a mismatch at the 3' terminus of a primer-template duplex is more detrimental to PCR than internal mismatches. However, not all 3'-terminal mismatches affect PCR equally. Kwok et al. (1990) demonstrated that even in a system designed to tolerate mismatches, 3'-terminal mismatches involving A:G, G:A, C:C, and G:G reduced product yield by 100-fold and A:A by 20-fold, whereas all other mismatches had little effect (Table 1). Because G:T mismatches are more stable than other mismatches (Kidd et al. 1983), G:T should be avoided when designing primers, although this mismatch has been used successfully (Li et al. 1990). On the other hand, one can still take advantage of this mismatch by targeting the complementary strand that forms an A:C mismatch. It is important to note that sequence context can significantly alter the properties of these mismatches, and as much as a 10-fold difference among sites has been reported (Mendelman et al. 1989; Huang et al. 1992).

Occasionally, a single-base mismatch at the 3' terminus is insufficient to achieve the desired level of discrimination, particularly when the ratio of mutant to wild-type sequences is low. A 3'-terminal mis-

Table 1 Relative Amplification Efficiencies of 3'-Terminal Mismatches in the Presence of 800 μ M dNTP

		Primer 3' base				
		T	C	G	A	
Corresponding template base	T	1.0	1.0	1.0	1.0	
	C	1.0	≤ 0.1	1.0	1.0	
	G	1.0	1.0	1.0	≤ 0.1	
	A	1.0	1.0	≤ 0.1	0.5	

Product yields were normalized to be the perfect matches (1.0).

(Reprinted, with permission, from Kwok et al. 1990 [Oxford University Press].)

match coupled with an additional mismatch either 1, 2, or 3 bases from the 3' terminus can increase discrimination (Newton et al. 1989; Cha et al. 1992). Therefore, if necessary, the deliberate introduction of a second mutation 1-3 bases from the 3' terminus will destabilize the 3' end and provide even greater differentiation. To increase the ratio of mutant to wild-type sequences, an "enriched" PCR was employed by Kahn et al. (1991) to identify mutations at codon 12 of the *Ki-ras* gene. A restriction enzyme site was generated in the wild-type sequence using a mismatched primer in the first round of amplification. The wild-type sequences were eliminated by digesting the PCR products with the appropriate restriction enzyme, and mutant sequences were enriched with a second round of amplification using mutant-specific primers.

The use of pairs of sequence-specific primers for PCR facilitates direct haplotype determination (Lo et al. 1991; Sarkar and Sommer 1991). This procedure is particularly attractive for determining the haplotypes of individuals who are heterozygous at a number of polymorphic sites and/or in whom pedigree samples are not available. An example of this approach is illustrated in Figure 1. In this biallelic system, the polymorphic site at allele 1 is either T or C and the polymorphic site at allele 2 is either G or T. The four possible

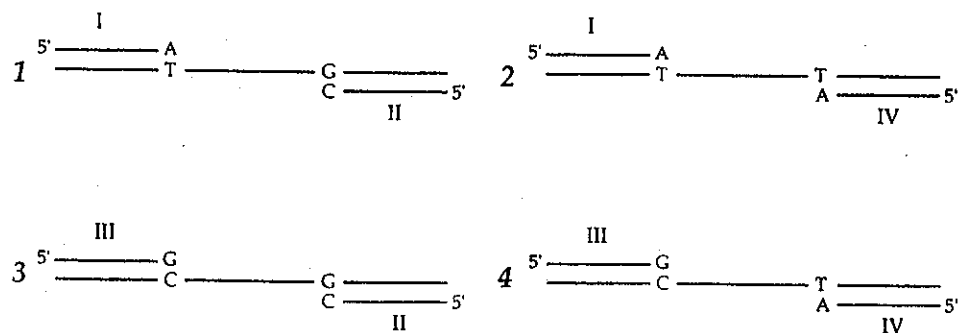


Figure 1 Schematic of haplotype determination with sequence-specific primers.

haplotypes (1, 2, 3, 4) can be differentially amplified by using pairwise combinations of primers specific to each allele. Specific primer pairs I and II would amplify haplotype 1; I/III, haplotype II/III, haplotype 3; and II/IV, haplotype 4. In addition, this procedure could be used to identify regions that contain as many as four mutations and would be available, for example, for the analysis of mutations in the HIV genome that confer zidovudine resistance.

In addition to 3'-terminal mismatches, other factors should also be considered. Shorter primer (< 20 bases, $T_m < 55^\circ\text{C}$); lower dNTP levels (Ehlen and Dubeau 1989); higher annealing temperatures; lower primer, MgCl_2 , and enzyme concentrations; and fewer cycles increase the stringency of the amplifications and can be used to skew amplifications to favor the target sequence (see Table 2). Tada et al. (1993) found that the Stoffel fragment, an amino-terminally truncated variant of *Taq* DNA polymerase, when used in combination with hot start (Chou et al. 1992), further enhanced discrimination. The detection of a small number of single-base mutant genes in a 10^4 - and 10^5 -fold excess of unaltered genes has been demonstrated successfully in the aforementioned studies.

Mismatch Tolerance

Although the effects of primer-template mismatches aid in the design of primers for sequence or allele-specific amplification, these effects can also be used to design primers to maximize detection of variant sequences. Most notably, retroviruses and RNA viruses replicate with less fidelity than many DNA viruses, and therefore viral genomes within and among patients may vary. False negatives due to mis-

Table 2 Flexibility of PCR Amplification

	Single nucleotide discrimination	Mismatch tolerance
[enzyme]	↓	↑
[MgCl_2]	↓	↑
[dNTPs]	↓	↑
[primers]	↓	↑
Nature of mismatch	short/↓ purine:purine pyrimidine:pyrimidine> purine:pyrimidine avoid G:T	long/↑ 3' terminal T
Annealing temperature/time	↓/↑	↑/↓
extension step (time)	↓ or eliminate	↑

Parameters affecting mismatch discrimination and mismatch tolerance of PCR amplification. Brackets represent the concentration of the parameter.

matches at the 3' terminus can be minimized by designing primers with a 3'-terminal T. Primers that terminate in T were efficiently extended even when mismatched with T, G, or C (Kwok et al. 1990). Our unpublished studies likewise demonstrate that 3'-terminal dU primers also tolerate mismatches. However, as a rule, 3'-terminal mismatches should be avoided if at all possible to maximize efficient extension by *Taq* DNA polymerase.

For amplification of closely related, yet distinct, sequences, chimeric primers can be designed. This approach is particularly attractive when the number of base differences between two sequences in the primer-binding region is small. The primer used, then, is not homologous to either sequence but has a limited number of mismatches to both. By designing the primers so that the mismatches are in the middle or at the 5' terminus, amplification of both target sequences can occur without compromise. Kwok et al. (1988) have described this approach for amplification of HTLV-I and -II.

The use of primers that are at least 25 bases long ($T_m > 70^\circ\text{C}$), high dNTP concentrations (800 μM), and annealing temperatures below the T_m of the primers will better accommodate mismatches (Table 2). The increase in specificity conferred by hot start PCR may not be suitable if accommodation of mismatches is desired.

Because single-base mismatches can significantly lower the melting temperature of a probe-target duplex, modified bases such as 5-bromodeoxyuracil and 5-methyldeoxycytosine have been used to increase duplex stability (Hoheisel et al. 1990). These bases have enabled the use of short oligonucleotides such as the *NotI* octadeoxynucleotide to probe genomic libraries (Hoheisel et al. 1990). The use of 2,6-diaminopurine, an adenine analog, may similarly enhance the stability of duplexes (Cheong et al. 1988). The stability of these nucleotide duplexes is influenced by base stacking and also by sequence context.

Recently, Nichols et al. (1994) described the use of a nondiscriminatory base analog, 1-(2'-deoxy- β -D-ribofuranosyl-3-nitropyrrole) or M, at ambiguous sites in DNA primers. Oligonucleotides that contained M at several sites were successfully used as primers for sequencing and PCR. The incorporation of M at the 3' terminus may improve mismatch tolerance.

Degenerate Primers

Some PCR applications require that primers not only tolerate mismatches, but also accommodate primer binding to an unknown sequence. Because of the degenerate nature of the genetic code, a given amino acid may be encoded by different triplets. The number of triplets that encode each amino acid is listed in Table 3. The degenerate

Table 3 Codon Degeneracy

Number of codons	Amino acids
1	Met, Trp
2	Phe, Tyr, His, Gln, Asn, Lys, Asp, Glu, Cys
3	Ile
4	Val, Pro, Thr, Ala, Gly
6	Leu, Ser, Arg

primers are designed as a pool of all the possible combinations of nucleotides that could code for a given amino acid sequence. Degenerate oligonucleotides can either be synthesized individually and then pooled or multiple bases can be programmed at one position in the DNA synthesizer. Because of the different stability of the blocked bases, care should be taken to use fresh reagents to ensure reproducible and equivalent synthesis of degenerate primers (Bartlett and Szostak 1993). The degeneracy of a given primer pool can be determined by multiplying the number of possible nucleotides at each position. For example, the degree of degeneracy of the primer pool for "AlaAsnIleLysMet" is $4 \times 2 \times 3 \times 2 \times 1 = 64$, where the codon degeneracy for Ala=4, Asn=2, Ile=3, Lys=2, and Met=1. Mixed oligonucleotide primers derived from an amino acid sequence can be used to amplify a specific sequence from genomic DNA or cDNA that in turn, can then be used as a probe to screen a genomic/cDNA library for the corresponding clones (Girgis et al. 1988; Lee et al. 1988). This is a powerful technique for obtaining a DNA probe when only a limited portion of a protein sequence is available for a sought-after gene. Degenerate primers based on the amino acid sequence of conserved regions were also used to search for members of a gene family (Wilks et al. 1989), homologous genes from different species (Kopin et al. 1990), or related viruses (Mack and Sninsky 1988; Manos et al. 1989; Shih et al. 1989).

When designing degenerate primers, the degeneracy of the genetic code for the selected amino acids of the region targeted for amplification must be examined. Obviously, selection of amino acids with the least degeneracy is desirable because it provides the greatest specificity. Several approaches can be considered for increasing the specificity of the amplification using degenerate primers.

1. The pools may be synthesized as subsets such that one pool may contain either a G or C at a particular position, whereas the other contains either an A or T at the same position.
2. The degeneracy of the mixed primer may be reduced by using the codon bias for translation (Wada et al. 1991).

3. The size of the degenerate primers can be as short as 4–6 amino acid codons in length. Six to nine base extensions that contain restriction enzyme sites can be added to the 5' terminus to facilitate cloning. Mack and Sninsky (1988) showed that for short primers, the 5' extensions facilitated amplification.
4. Degeneracy at the 3' end of the primer should be avoided, because single-base mismatches may obviate extension. The last base of the terminal codon can be omitted unless the amino acid is Met or Trp.
5. The inclusion of deoxyinosine at some ambiguous positions may reduce the complexity of the primer pool (Table 4) (Ohtsuka et al. 1985; Sakanari et al. 1989).

Inosine occurs naturally in the wobble position of the anticodon of some transfer RNAs and is known to form base pairs with A, C, and U in the translation process. Several experiments have suggested that deoxyinosine might be an "inert" base; its presence in an oligonucleotide sequence seems neither to disturb DNA duplex formation nor to destabilize the duplex (Martin et al. 1985; Ohtsuka et al. 1985). Independent of sequence effects, the order of stabilities of base-pairing is I:C > I:A > I:T = I:G (Martin et al. 1985). The deoxyinosine-containing primers have been used successfully to amplify cDNA or genomic fragments for the generation of DNA probes from a peptide sequence (Schuchman et al. 1990; Aarts et al. 1991). Deoxyinosine was generally incorporated at base positions with three- to fourfold redundancy. This method may be useful for proteins with highly degenerate codons. Inosine usually directs the incorporation of C when in the template. Unfortunately, the studies to date do not discern whether inosine actually assists mismatched priming. Another approach, pioneered by Lin and Brown (1992), exploits the use of unconventional bases that are capable of base-pairing with either a purine or a pyrimidine. These unconventional bases are 6H, 8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one (P) and 2-amino-6-methoxyaminopurine (K), where P can base-pair with A and G, and K can base-pair with C and T. They demonstrated that oligonucleotides containing the degenerate bases P and K can be used as primers in PCR.

Table 4 Approaches in Designing Degenerate Primers

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1. Pools can be synthesized as subsets
 2. Use codon bias for translation
 3. Use 4–6 amino acids (12–18 bases) in length
 4. Avoid degenerate base at the 3' terminus; omit the last base of the terminal codon unless the amino acid is Met or Trp
 5. Consider using deoxyinosine for accommodating multiple components to reduce the degeneracy
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Similarly, the universal nucleoside 1-(2'-deoxy- β -D-ribofuranosyl)nitropyrrole (M) can also be used at ambiguous sites in DNA primers for amplification (Nichols et al. 1994).

It has been observed that the PCR thermal profile can dramatically alter the success rate of degenerate PCR (Compton 1990). The preferred degenerate PCR amplification profile starts with a non-stringent annealing temperature (35–45°C) for 2–5 cycles, followed by 25–40 cycles at a more stringent annealing temperature. The relaxed annealing conditions allow the short complementary primer portion to hybridize to the target. After the second cycle of amplification, the 5' extension becomes incorporated into the amplified product and serves as the template for subsequent rounds of amplification. By shifting to a more stringent annealing temperature, increased specificity can be achieved. Furthermore, a 4- or 5-minute ramp time between the annealing and extension temperatures may also result in greater specificity.

Mutagenesis Primers

With the advent of PCR, recombinant DNA procedures such as M13 phage site-directed mutagenesis and the construction of "fusion proteins" have been greatly simplified (Higuchi 1990; Scharf 1990). There are several advantages in generating a mutation with mismatched primers by PCR. First, PCR provides substantial flexibility in the types of mutations that can be introduced. For example, single-base substitutions, deletions, and insertions of different sizes can all be easily generated with one or two rounds of PCR. Second, the mutation(s) of interest located in the PCR product can be cloned into any desirable vector by a single cloning step. Third, almost all the clones harbor the expected mutation.

Conventional methods for the construction of sequences encoding a fusion protein require several manipulations. In addition, the sequence at the junction is generally not ideal because the restriction enzyme selected for the joining of the two sequences is usually not present in the native sequence. Therefore, it is difficult to construct a sequence encoding an authentic fusion protein. With PCR, the junction of two segments can easily be engineered into the primers, thereby eliminating extraneous bases.

The location of the mutated base(s) in the primers depends on the nature of the desired mutation. To introduce single-base substitutions and insertions at a location with a unique restriction enzyme site nearby, only two primers are needed (Fig. 2A). A mismatched mutagenesis primer (P1) with the desired mutation sequence and restriction enzyme site sequence (site X) is paired with another primer (P2) with the second restriction enzyme site (site Y) for PCR.

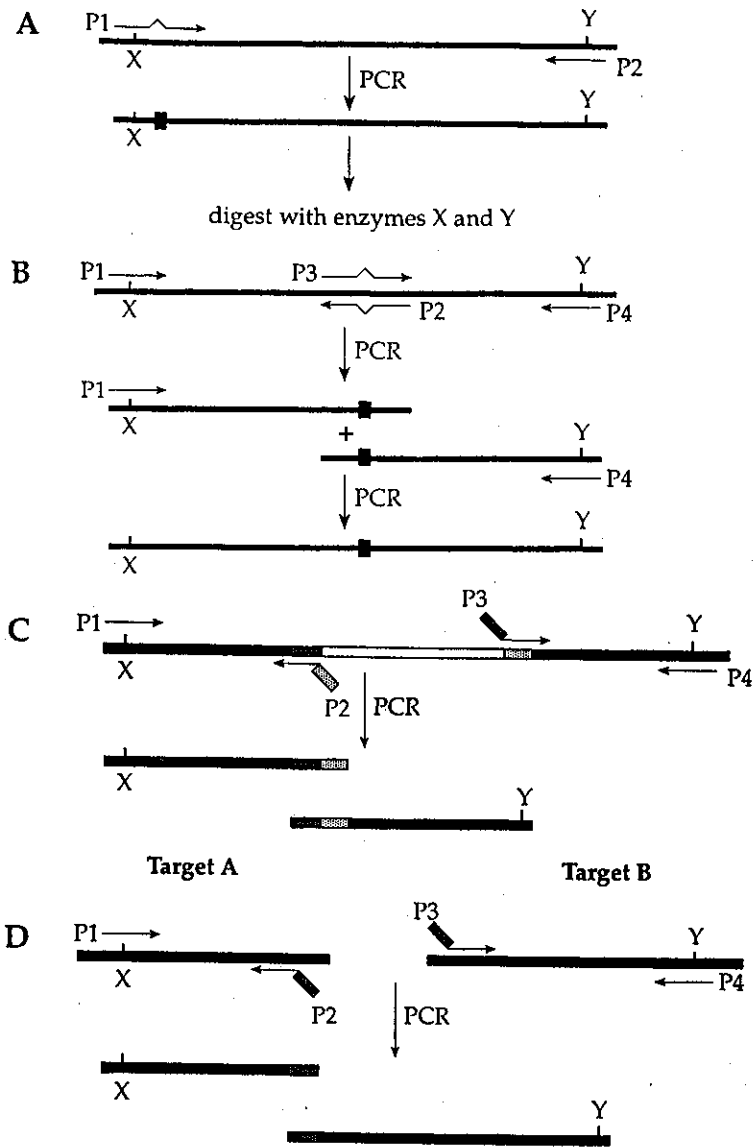


Figure 2 Strategies of using mismatched primers for generating mutations (A-C), and fusion of two fragments of DNA (D). Bends in arrows represent the mismatched sequence between primer and target. (Closed box) The mutation introduced by the mismatched primer. (X and Y) Two restriction enzyme sequences shown in the DNA sequence. The shaded areas with the same pattern have identical sequence.

The mismatch(es) should be placed in the middle of a 24- or 36-base oligonucleotide. The PCR product can be digested with enzymes X and Y and cloned into a suitable vector. For generating mutations in a sequence that does not contain a convenient restriction enzyme site nearby, two sets of primers are needed for two rounds of PCR (Fig. 2B). Primer 1 (P1) paired with mutagenesis primer P2 and mutagenesis primer 3 (complementary to primer P2) paired with

primer 4 (P4) are used in two separate PCR assays in the first round of PCR. Following the first round of PCR, a small aliquot (1/20 to 1/50) of the two PCR products (or gel-purified PCR products) is mixed and amplified in the presence of primers 1 and 4 in the second round of PCR. Because the two PCR products from the first round of PCR have overlapping sequences at the "mutagenized end," one of the two partially matched duplexes formed after denaturation and annealing serves as the template in the second round of PCR. The PCR product from the second round of amplification is used for cloning.

The introduction of large deletions and the fusion of sequences can be accomplished in a similar manner. The mutagenesis primers for generating a large deletion should contain sequences flanking the deletion region (Fig. 2C). Both mutagenesis primers (P2 and P3) should have a region of more than 12 bases at the 3' terminus that matches the target sequence and a mismatched tail of more than 7 bases that matches the region flanking the other end of the deletion. The length of the overlapping sequences at the mutagenized end of two first-round PCR products is determined by the total number of bases in the tails of primers P2 and P3. For the joining of two target sequences, the length of the mismatched tail of mutagenesis primers should be more than 12 bases (Fig. 2D). Primers 1 and 4 illustrated in Figure 2B-D are in the regions with restriction enzyme sites for cloning of the PCR product. Alternatively, the restriction enzyme site for cloning can be introduced with the primer containing the sequence of the restriction site and a few extra bases to ensure enzyme digestion at the 5' terminus.

The length of the primer used in these experiments depends on the G/C content of the sequence and the type of desired mutation. In general, 12-14 bases of perfect complementarity ($T_m \sim 40^\circ\text{C}$) at the 3' terminus are sufficient for a primer to be annealed and extended, particularly if it is not especially AT-rich. The annealing temperature can also be controlled to achieve an efficient amplification.

If the nucleotide sequence of the mutated fragment is important for further analysis, the location of primer 2 in Figure 1A and primer pair P1/P4 in Figure 1B-D should be as close as possible to the mutagenesis primer(s) to minimize the number of nucleotides to be confirmed by sequencing. The conditions to minimize the misincorporation of *Taq* DNA polymerase should be observed. The parameters of the PCR reaction, such as the concentration of dNTP and MgCl_2 , and pH, all have effects on the fidelity of the enzyme (Eckert and Kunkel 1990; Cha and Thilly, this volume). The amount of template and the number of cycles of amplification should be properly controlled to minimize amplification at the plateau phase. Other proofreading DNA polymerases, such as those for *Thermotoga maritima* (*UITma*), *Thermococcus litoralis* (Vent), and *Pyrococcus furiosus* (*Pfu*), can be used

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as alternative enzymes for mutagenesis PCR. However, one should bear in mind that the proofreading activity of these enzymes also degrades primers, single-stranded PCR products, and template; therefore, their use may require careful optimization.

CONCLUSION

Although the published procedures noted were successful, very few comprehensive studies have been performed to explain fully the precise mechanisms involved. As a result, the amount of information available is perhaps less than what might have been expected given the large number of studies carried out. The procedures outlined above are intended to serve as guidelines for the design and use of mismatched and degenerate primers. Depending on the particular PCR application, a subset of these recommendations or suggestions may suffice. Familiarity with the accomplishments and limits of published studies, an understanding of the principles of enzymology and nucleic acid hybridization, and a willingness to reflect first on the goal before embarking on experimentation will increase an investigator's likelihood of success. PCR—like any good tool—can be productive with minimal familiarity. However, understanding the intricacies of how it works and the breadth of potential alterations permits greater exploration of its limits and its application to new areas.

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