

GeneMorph™ PCR mutagenesis kit produces a unique mutational spectrum

Randomize Gene Sequences with New PCR Mutagenesis Kit

Janice Cline • Holly Hogrefe
Stratagene

The GeneMorph™ PCR mutagenesis kit¹ simplifies the process of constructing mutant libraries for protein evolution and structure-function studies. Errors are introduced during PCR at low, moderate, or high mutation frequencies using a single set of optimal reaction conditions. The kit features Mutazyme™ DNA polymerase, a novel error-prone DNA polymerase mutant developed at Stratagene. Mutazyme DNA polymerase produces all possible transition and transversion mutations with minimal bias, and its mutational spectrum is distinct from that of Taq DNA polymerase. Unlike current Taq-based protocols, the GeneMorph method is robust and can be applied to mutagenesis of long targets (1-10 kb).

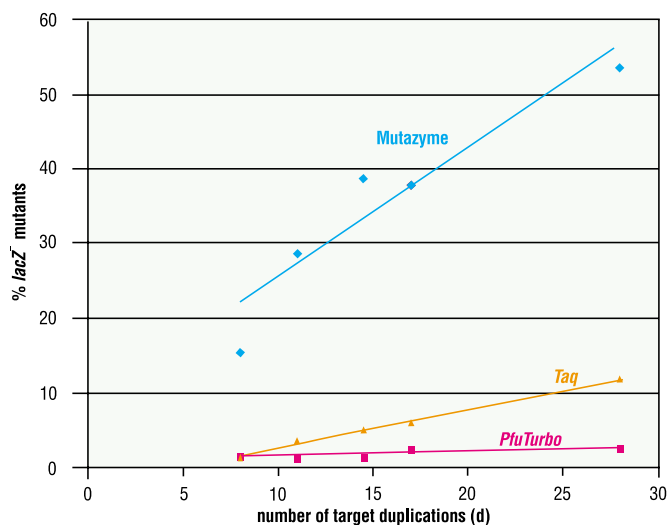
Error-prone PCR is a random mutagenesis technique for introducing amino acid changes into proteins. Mutations are deliberately introduced during PCR through the use of error-prone DNA polymerases and reaction conditions.^{1,2} Randomized DNA sequences are cloned into expression vectors, and the resulting mutant libraries are screened for altered or improved protein activity. To analyze structure-function relationships, mutation frequencies of one amino acid change (1-2 nucleotide changes) per gene are desired, while directed evolution studies typically employ mutation frequencies of one to four amino acid changes (2-7 nucleotide changes) per gene.^{3,6} Proteins with improved activities have also been isolated from highly mutagenized libraries exhibiting 20 point mutations per gene.⁷ Error-prone PCR methods commonly employ Taq DNA polymerase, as it lacks proofreading activity. To achieve useful mutation frequencies, the error rate of Taq DNA polymerase is further increased by employing PCR reaction buffers containing Mn²⁺ and unbalanced dNTP concentrations.^{1,2} These changes, however, can lead to lower PCR product yields⁴ and a strong bias in the types of mutations produced (mutational spectra).²

The GeneMorph PCR mutagenesis kit overcomes the limitations of Taq-based random mutagenesis methods by using an inherently error-prone PCR enzyme and varying DNA template concentration to achieve the desired mutation frequency.

Mutazyme™ DNA Polymerase

A key component of the GeneMorph kit is Mutazyme DNA polymerase, a novel DNA polymerase mutant developed at Stratagene. As shown in Figure 1, Mutazyme DNA polymerase introduces mutations much more frequently than Taq DNA polymerase. Moreover, high mutation frequencies are achieved under optimal PCR reaction conditions for the enzyme (MgCl₂, balanced nucleotides). Despite its inherent high error rate,

Figure 1
High Error Rate of Mutazyme™ DNA Polymerase



Mutation frequencies produced by various DNA polymerases were measured using the *lacZ* mutational target gene. PCR amplifications were performed with Mutazyme™, PfuTurbo™, or Taq DNA polymerase in each enzyme's recommended PCR buffer. For each enzyme, four reactions consisting of a single round of PCR and one reaction consisting of two sequential PCRs were performed. Primary PCRs were carried out with the following amounts of DNA template: 100 ng (d=8), 10 ng (d=11), 1 ng (d=14.5), 100 pg (d=17). For the sequential PCRs, 0.001 μl of a primary PCR reaction (d = 14.5) was reamplified with the appropriate PCR enzyme in a second PCR reaction. PCR products were purified and cloned (Methods). Transformants were plated on Xgal/IPTG/ampicillin plates, and *lacZ*⁻ mutants (white colonies) were scored in a color screening assay. Percent mutants were calculated as follows: (#*lacZ*⁻ mutants)/(total # clones).

Figure 2
Formula for Calculating PCR Mutation Frequency

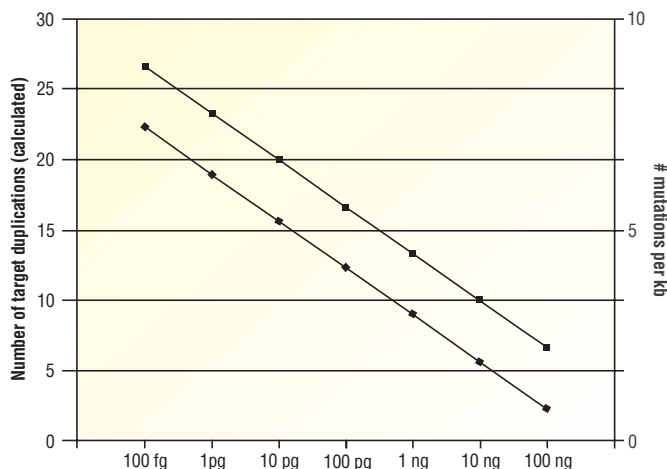
$$\text{Mutation frequency} = (\text{DNA polymerase error rate}) \times (\text{target duplication } (d))$$

$$\text{where } 2^d = \frac{\text{total amplicon yield}}{\text{initial amount of target DNA}}$$

Mutation frequency is related to DNA polymerase error rate and number of target duplications (d), which is a function of amplicon yield and initial amount of target DNA.

Figure 3

Target Duplications and Mutation Frequency Vary with Input DNA Concentration



We calculated *d* (Figure 2) using the indicated amounts of initial target DNA and amplicon yields of either 0.5 μ g or 10 μ g (per 50- μ l PCR). The number of target duplications (left Y axis) is shown as a function of initial target DNA amount. The mutation frequencies produced were determined using the formula in the Figure 5 legend and plotted (right Y axis) as a function of initial target DNA amount.

frequently than *Taq* DNA polymerase. Moreover, high mutation frequencies are achieved under optimal PCR reaction conditions for the enzyme (MgCl₂, balanced nucleotides). Despite its inherent high error rate, Mutazyme DNA polymerase produces high PCR product yields over a broad range of DNA template concentrations.

PCR Mutation Frequency

Mutation frequency (mutations/kb) is the product of DNA polymerase error rate and number of target duplications (Figure 2). In the GeneMorph kit, a sufficiently high error rate is achieved through use of Mutazyme DNA polymerase. Low, medium, or high mutation frequencies are obtained simply by varying the initial target DNA concentration in the PCR reaction. For the same PCR yield, targets amplified from low amounts of target DNA undergo more duplications than targets amplified from high concentrations of DNA. This principle is illustrated in Figure 3, which shows the number of times an amplicon is copied to produce theoretical yields of 0.5 μ g or 10 μ g. The more times a target is replicated, the more errors accumulate. Therefore, higher mutation frequencies are achieved simply by lowering input DNA template concentration. Conversely, lower PCR mutation frequencies can be achieved by using higher DNA template concentrations or fewer PCR cycles to limit the number of target duplications.

Selecting the Mutation Frequency of GeneMorph™ Reactions

Table I presents the initial amount of target DNA required to produce the desired mutation frequency using the GeneMorph kit. For simplicity, we divided the range

Table I

Mutation Levels Produced by GeneMorph™ Kit^a

Initial target amount ^b	# mutations per kb ^c (calculated)	Mutation level
100 ng	1.1-2.5	low
10 ng	2.1-3.5	
1 ng	3.2-4.5	medium
100 pg	4.2-5.6	
10 pg	5.2-6.6	
1 pg	6.3-7.6	high
Double PCR	5.7-12.8	
Triple PCR	8.8-17.5	

^aTo simplify mutation frequency calculations, the GeneMorph kit provides estimates of mutation frequencies (# mutations per kb) based upon starting amount of target DNA. These estimates assume product yields of 0.5-10 μ g/50 μ l reaction. These conditions are met when the staining intensity of product bands (10 μ l) run on gels (Methods) is intermediate between the staining intensities of 100 ng and 2 μ g of the DNA standard. Using Table 1, researchers can determine the appropriate amount of target DNA to amplify to achieve the mutation frequency desired. Alternatively, actual mutation frequencies can be calculated from experimentally determined *d* values (figure 2) using the equation in figure 5.

^bamount of mutational target is determined as: (size of mutational target/size of plasmid DNA) x (plasmid DNA template amount).

^cmutation frequencies were calculated using the equation M.F. = 0.31*d* + 0.41 (figure 5).

of mutation frequencies produced by Mutazyme DNA polymerase into three different levels: low (0-3 mutations/kb), medium (3-8 mutations/kb), and high (>8 mutations/kb). Reactions employing 10 ng to 100 ng of target DNA produced mutation frequencies up to three mutations per kb (0.3%), while PCRs carried out with 10 pg to 1 ng of DNA template introduced errors at a rate of three to seven mutations per kb (0.3%-0.7%). Mutation frequencies greater than 0.7% are achieved by performing sequential amplifications. By reamplifying a small portion of a PCR reaction, the number of target doublings can be substantially increased. Mutation frequencies of 0.6% to 1.3% or 0.9% to 1.8% were produced by performing two or three sequential PCR reactions, respectively. (Table 1)

Random Mutagenesis of Target Genes

The efficiency and ease of use of the GeneMorph kit was demonstrated in studies employing *lacZ* as the mutational target gene. A 650-bp DNA fragment containing a portion of the *lacZ* gene was amplified with Mutazyme DNA polymerase from varying amounts of plasmid DNA (Figure 4). In this study, the PCR primers were designed to introduce LIC (ligation independent cloning) sites for cloning into the Affinity LIC vector. Mutazyme produced high yields of the 650-bp amplicon from 10 pg up to 100 ng of DNA template (Figure 4). Product yields were quantified by comparison to a DNA standard, and total yields ranged from 1 μ g to 7.5 μ g per 50- μ l PCR reaction. In addition, a small portion of one reaction (Figure 4, Lane 5) was reamplified in two or three sequential PCR reactions (Figure 4, Lanes 8 and 9).

The frequency of mutations in the *lacZ* gene was determined for numerous PCR reactions employing various concentrations of DNA template. PCR products were gel purified and cloned into the Affinity vector (Methods). For each PCR reaction, 2 to 10 clones were randomly selected and sequenced. Figure 5 shows a plot of mutation frequency (number of mutations per kb) as a

Figure 4
Error-Prone Amplification of *lacZ* Gene



Amplification of a 650-bp target (Lanes 1-9) was performed with Mutazyme DNA polymerase (Methods). The following amounts of *lacZ* DNA were used as template: (Lane 1) 100 ng; (Lane 2) 10 ng; (Lane 3) 1 ng; (Lane 4) 100 pg; (Lane 5) 10 pg; (Lane 6) 1 pg; (Lane 7) 100 fg. Two rounds of PCR were performed by reamplifying 0.001 μ l of the reaction in Lane 5 in a second PCR (Lane 8). Three sequential PCRs were carried out by reamplifying 0.001 μ l of the reaction in Lane 8 in a third PCR (Lane 9). PCR product yields were determined (Methods) by comparison to a 3-kb DNA standard, used at the following amounts: (Lane a) 100 ng; (Lane b) 200 ng; (Lane c) 500 ng; (Lane d) 1 μ g; (Lane e) 2 μ g. Number of target duplications (d), calculated from the formula in figure 1, were 5.8-16.4 for one round of PCR (Lanes 1-5), 34.2 for two rounds of PCR (Lane 8), and 49.2 for three rounds of PCR (Lane 9).

The GeneMorph kit has been used to mutagenize other target genes, including a 2.5-kb polymerase gene and a 720-bp sequence encoding Green Fluorescent Protein (GFP). (Figure 5)

Mutational Spectrum of Mutazyme

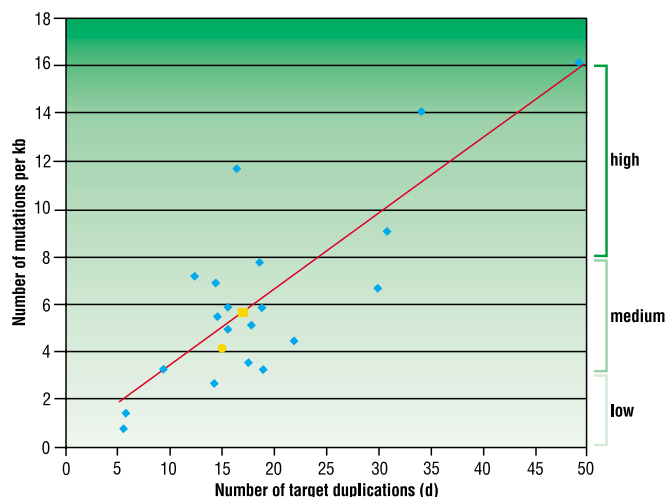
Table 2 shows the types of mutations introduced by Mutazyme DNA polymerase. These data were compiled from DNA sequence analysis (37.5-kb total) of 45 clones randomly selected from *lacZ* mutagenesis studies. Mutations were distributed uniformly throughout the *lacZ* gene, rather than localized in hotspots (data not shown). Mutazyme DNA polymerase introduced all possible transition (purine \rightarrow purine; pyrimidine \rightarrow pyrimidine) and transversion (purine \rightarrow pyrimidine; pyrimidine \rightarrow purine) mutations, while insertions and deletions were relatively rare (\sim 1%).

Mutational bias is most commonly analyzed by calculating either the ratio of transition (Ts) to transversion (Tv) mutations or the ratio of AT to GC changes (AT \rightarrow GC/GC \rightarrow AT). Since there are eight possible transversions and four possible transitions, a DNA polymerase completely lacking bias would exhibit a Ts/Tv ratio of 0.5. Mutazyme DNA polymerase produces a Ts/Tv ratio of 1.2, indicating that the enzyme preferentially introduces transition mutations. In addition, Mutazyme exhibits a AT \rightarrow GC/GC \rightarrow AT ratio of 0.2, denoting a tendency to replace G or C with A or T. In comparison, an enzyme that lacks bias would produce an AT \rightarrow GC/GC \rightarrow AT ratio of 1.0. Similar Ts/Tv and AT \rightarrow GC/GC \rightarrow AT ratios were determined in limited mutagenesis studies employing the polymerase and GFP genes (data not shown).

GeneMorph™ Kit Produces a Unique Mutational Spectrum

In the GeneMorph kit, low, medium, and high mutation frequencies are achieved using a single set of optimal reaction conditions (MgCl₂, balanced dNTPs). The only parameter varied is DNA template concentration. Therefore, the same spectrum of mutations is produced over a broad range of mutation frequencies (<0.1% to 0.7% per PCR). In contrast, *Taq*-based error-prone PCR methods typically employ different sets of

Figure 5
Mutagenesis of *lacZ* Gene with the GeneMorph™ Kit



PCR reactions were performed (Methods) using varying concentrations (1 pg-100 ng) of *lacZ* (diamond), polymerase (rectangle), or GFP (circle) target DNA. Number of target duplications was calculated for each PCR reaction using the formula shown in Figure 2 (Methods). Mutation frequencies (# mutations per kb) were determined by DNA sequencing. For each *lacZ* PCR reaction, a total of 0.8 kb-5.3 kb of DNA sequence from 2-10 random clones was analyzed. For the polymerase and GFP reactions, 3.2-kb (six clones) and 4.5-kb (six clones) of DNA sequence were analyzed. The equation of the line relating mutation frequency to d value is as follows: # mutations/kb = 0.31d + 0.41.

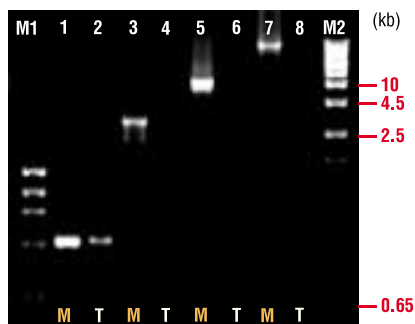
reaction conditions to vary mutation levels.^{1-3,9,10} The particular conditions employed (Mn²⁺ or dNTP ratios) can influence the types of mutations produced.^{2,9,10}

The mutational spectrum of Mutazyme DNA polymerase was compared to the spectra of mutations generated by *Taq* under typical error-prone conditions (0.5 mM MnCl₂; 0.2 mM dATP/dGTP; 1 mM dCTP/TTP).²³ In one published study,³ different mutation levels (0.1% -0.5% per PCR) were achieved by performing sequential PCRs and varying the MnCl₂ concentration. Under these conditions, *Taq* preferentially introduces A \rightarrow T, T \rightarrow A, A \rightarrow G, and T \rightarrow C mutations (66%-68% of total mutations) and rarely incorporates G \rightarrow C and C \rightarrow G transversions. In comparison, Mutazyme DNA polymerase tends to produce G \rightarrow A, C \rightarrow T, G \rightarrow T, and C \rightarrow A changes (64% total mutations), and all transversion mutations are represented. *Taq* is approximately three times more likely to mutate As and Ts than Gs and Cs (AT \rightarrow GC/GC \rightarrow AT ratio >1²³), while Mutazyme DNA polymerase is nearly three times more likely to mutate Gs and Cs (AT \rightarrow GC/GC \rightarrow AT ratio 0.2). Both enzymes prefer to incorporate transition mutations rather than transversion mutations (Ts/Tv = 0.8-1.2) (Table 2).

As shown in Table 2, Mutazyme DNA polymerase produces a broad and unique spectrum of mutations, thereby facilitating the production of representative mutant libraries. The distinct mutational spectra of Mutazyme and *Taq* DNA polymerases implies that different types of mutations will be present in mutant libraries constructed by these different error-prone methods. For example, the GeneMorph kit facilitates mutagenesis of GC-rich targets and synthesis of GC \rightarrow CG transversions, both of which are relatively difficult for *Taq* DNA

Figure 6

PCR Performance Comparisons



Error-prone PCR reactions were carried out with Mutazyme "M" (Lanes 1,3,5,7) or *Taq* "T" DNA polymerase (Lanes 2,4,6,8). Mutazyme DNA polymerase reactions employed 2.5 U of enzyme and 200 μ M each dNTP, while *Taq* reactions contained 5 U of enzyme, 0.5 mM $MnCl_2$, and an unbalanced dNTP pool (Methods). The following targets were amplified from 1 ng of plasmid or lambda phage DNA: (Lanes 1,2) 650-bp *lacZ* sequence; (Lanes 3,4) 2.5-kb polymerase gene; (Lanes 5,6) 4.5-kb archaeal polymerase gene; (Lanes 7,8) 10-kb fragment of lambda DNA. M1: ϕ X174/*Hae*III markers; M2: Kb DNA ladder.

polymerase. Since mutation levels are varied without altering reaction conditions, the broad spectrum of mutations produced by the GeneMorph kit is maintained over a wide range of mutation frequencies, even at high mutation levels (6-13 mutations per kb/2 PCRs). In contrast, to achieve high mutation frequencies with *Taq* (>4.9-6.6 mutations per kb²³), it is typical to increase the dGTP concentration (final conditions: 0.5 mM $MnCl_2$; 0.2 mM dATP; 1 mM dCTP/TTP/dGTP), which leads to significant bias in *Taq*'s mutational spectrum (Ts/Tv ratio, 2.7; AT→GC/GC→AT ratio, 10).¹²

GeneMorph Kit Provides Superior Yields

The GeneMorph kit must employ a robust PCR enzyme to ensure high product yield from low DNA template concentrations (highly mutagenic conditions). Since Mutazyme DNA polymerase is inherently error prone, high mutation frequencies are achieved using optimal PCR reaction conditions. In contrast, employing Mn^{2+} and unbalanced dNTP pools to lower the fidelity of *Taq* leads to reduced product yield,⁸ and such conditions are generally only useful for amplifying targets up to 1 kb in length.¹

The superior performance of Mutazyme DNA polymerase is demonstrated in Figure 6. A series of targets, 0.65 kb to 10 kb in length, were amplified under error-prone conditions using Mutazyme and *Taq* DNA polymerases. For *Taq* reactions, the error-prone reaction conditions were employed,² except that the $MgCl_2$ concentration was decreased from 7 mM to 1.5 mM. PCRs were performed using identical cycling conditions and DNA template concentrations. Mutazyme DNA polymerase (2.5 U) produced high yields of all amplicons, while *Taq* DNA polymerase (5 U) successfully amplified only the shortest 650-bp product (Figure 6). The 650-bp product was not successfully amplified using 2.5 U *Taq* DNA polymerase or buffers containing 7 mM $MgCl_2$, as recommended² (data not shown).

Conclusions

Stratagene's GeneMorph PCR Mutagenesis kit is a superior alternative to *Taq*-based random mutagenesis methods. The benefits of this kit include the unique characteristics of the

error-prone Mutazyme DNA polymerase, synthesis of high product yields over a broad range of amplicon sizes (0.1-10 kb), efficient mutagenesis rates of one to seven bases per kb per PCR, incorporation of all mutation types with minimal bias, and a simplified protocol with one set of robust reaction conditions. The GeneMorph kit is a finely tuned system for achieving desired mutation frequencies and, producing a unique spectrum of mutations.

Methods

Error-prone PCR conditions: DNA sequences were amplified as described in the manual for the GeneMorph PCR mutagenesis kit. Briefly, an amount of plasmid DNA corresponding to 1 μ g to 100 ng of amplicon was amplified in PCR reactions (50 μ l) containing 1X Mutazyme reaction buffer, 200 μ M each dNTP, 125 ng of each primer, and 2.5 U Mutazyme DNA polymerase. PCR reactions were performed with *Taq* DNA polymerase using identical conditions, except that 1X *Taq* reaction buffer (1.5 mM $MgCl_2$) was employed. Error-prone PCR reactions contained 5 U of *Taq* DNA polymerase, 1X *Taq* reaction buffer (supplemented with 0.5 mM $MnCl_2$), and a nucleotide mixture of 200 mM dGTP, 200 mM dATP, 1 mM dCTP, and 1 mM TTP.²

Sequential PCR amplifications: To obtain high mutation levels (>0.7%), PCR products were reamplified in a second PCR reaction. A small portion of a PCR reaction (e.g., amplicon synthesized from 0.1 ng-1 ng of DNA target) was first diluted 1:1000 in TE buffer. One μ l of diluted amplicon was then reamplified as described above.

Quantitating PCR product yield: PCR products (10 μ l) were electrophoresed on 1% agarose gels along side the 1-kb double-stranded DNA standard provided in the kit. PCR product yields were quantified using the Eagle Eye[®] II still video system as described in the manual for the GeneMorph PCR mutagenesis kit.

***lacZ* mutagenesis assay:** A 650-bp product, containing a 300-bp fragment of *lacZ*, was amplified from a chloramphenicol resistant *lacZ*/pBC SK⁺ plasmid construct. The PCR primers were designed to introduce LIC (ligation independent cloning) sequences at the 5' and 3' ends of the insert. The PCR products were gel purified using the StrataPrep[®] gel extraction kit and then cloned into the Affinity LIC vector as described in the cloning kit manual. SoloPack[®] competent cells were transformed and plated on ampicillin/LB plates. *lacZ*⁻ mutants were scored in a color screening assay by plating transformants on Xgal/IPTG/amp plates. Percent *lacZ*⁻ mutants was calculated as the number of colorless colonies (mutants lacking β -galactosidase activity) divided by the total number of colonies plated (blue plus colorless colonies).

DNA sequencing: Mutation frequencies and mutational spectra were analyzed by DNA sequencing. Individual colonies were randomly selected and resuspended in 200 μ l of TE. One μ l of each suspension was amplified with Herculase[™] enhanced DNA polymerase using a gene-specific primer and a vector-specific primer. PCR products were purified using the StrataPrep[®] PCR purification kit, and DNA sequencing was performed using a nested gene-specific primer (Sequetech).

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‡ See page 168.

GeneMorph[™] PCR Mutagenesis Kit

Table 2

Mutational Spectra of Mutazyme and Taq DNA Polymerases

	Mutazyme DNA polymerase [0.2mM dATP/dGTP/dCTP/TTP]		Taq DNA polymerase ³ [0.2mM dATP/dGTP; 1mM dCTP/TTP]	
	No MnCl ₂		No MnCl ₂	0.5mM MnCl ₂
Bias indicators				
Ts/Tv	1.2		1.1	0.8
AT→GC/GC→AT	0.2		1.4	1.9
Transitions				
A→G, T→C	10.3%	(3.8%, 6.5%)	30.0%	27.6%
G→A, C→T	43.7%	(23.4%, 20.3%)	20.0%	13.6%
Transversions				
A→T, T→A	11.1%	(6.1%, 5.0%)	33.3%	40.9%
A→C, T→G	4.2%	(0.8%, 3.4%)	6.7%	7.3%
G→C, C→G	8.8%	(4.6%, 4.2%)	0.0%	1.4%
G→T, C→A	20%	(12.3%, 7.7%)	6.7%	4.5%
Insertions and Deletions				
Insertions	0.8%		0.0%	0.3%
Deletions	1.1%		3.3%	4.2%
Summary				
# mutations per kb	<1 to 7 (per PCR)		1.1 (per PCR)	4.9 (per PCR)
A→N, T→N	25.6%		70.0%	75.9%
G→N, C→N	72.5%		26.7%	19.6%

30 rxns

#600550

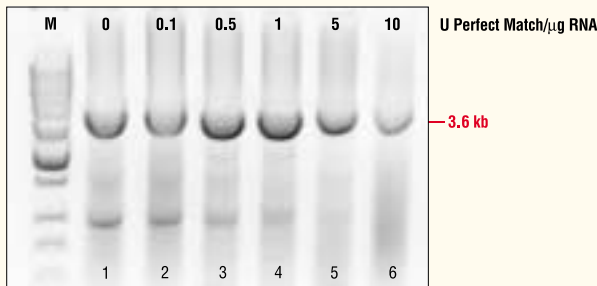
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Figure 1
Improve the Amplification of Difficult RNA Targets



A 3.6-kb fragment of mouse complement component C5S was amplified from 100 ng mouse liver total RNA. Perfect Match® enhancer was added to the reactions in varying amounts as indicated. Cycling parameters: one cycle of 42°C for 45 minutes, followed by 1 cycle of 95°C for 1 minute, 40 cycles of 95°C for 30 S; 60°C for 30 S; 68°C for 8-10 minutes (2 minutes/kb), and 1 cycle of 68°C for 10 minutes. "M" contains Kb DNA ladder.

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