GeneMorph II Random Mutagenesis Kit

INSTRUCTION MANUAL

Catalog #200550

Revision A.01

For In Vitro Use Only

200550-12

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GeneMorph II Random Mutagenesis Kit

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MATERIALS PROVIDED

Materials provided[a]	Concentration	Quantity
Mutazyme II DNA polymerase[b]	2.5 U/µl	30 µl
10× Mutazyme II reaction buffer	10×	150 µl
40 mM dNTP mix	10 mM each dNTP	30 µl
1.1-kb Gel standard	20 ng/µl	150 µl

[a] Sufficient reagents are provided for 30 reactions.

[b] Mutazyme II DNA polymerase is not sold separately.

STORAGE CONDITIONS

All Materials: –20°C

ADDITIONAL MATERIALS REQUIRED

Temperature cycler
PCR tubes[II]
PCR primers

[II] Thin-walled PCR tubes are highly recommended for use with Stratagene thermal cyclers. These PCR tubes ensure ideal contact with the multiblock design to permit more efficient heat transfer and to maximize thermal-cycling performance.

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Revision A.01

INTRODUCTION

Random mutagenesis is a powerful tool for elucidating protein structure-function relationships and for modifying proteins to improve or alter their characteristics. Error prone PCR is a random mutagenesis technique for generating amino acid substitutions in proteins by introducing mutations into a gene during PCR. Mutations are deliberately introduced through the use of error prone DNA polymerases and/or reaction conditions. The mutated PCR products are then cloned into an expression vector and the resulting mutant library can be screened for changes in protein activity. Random mutagenesis allows researchers to identify beneficial mutations in the absence of structural information, or when such mutations are difficult to predict from protein structure.[1]

Random Mutagenesis with the GeneMorph II Random Mutagenesis Kit

The mutational bias exhibited by error prone PCR enzymes undoubtedly skews representation of random mutant libraries, diminishing the effective size of the collection produced by error prone PCR. Mutazyme II DNA polymerase is a novel error prone PCR enzyme blend, formulated to provide useful mutation rates with minimal mutational bias. Mutazyme II is a blend of two error prone DNA polymerases—Mutazyme I DNA polymerase (from the original GeneMorph Random Mutagenesis Kit) and a novel *Taq* DNA polymerase mutant that exhibits increased misinsertion and misextension frequencies compared to wild type *Taq*. For the Mutazyme II polymerase formulation, the Mutazyme I polymerase and the *Taq* polymerase mutant have been combined to produce a less biased mutational spectrum with equivalent mutation rates at A's and T's vs. G's and C's. Therefore, libraries created with Mutazyme II should exhibit greater mutant representation compared to libraries generated with other enzymes. However, the original GeneMorph I kit favors mutations at G's and C's which in some cases may be desirable.

With the GeneMorph II random mutagenesis kit*, mutation rates of 1–16 mutations per kb can be achieved using a single set of buffer conditions ($MgCl_2$, balanced dNTPs) optimized for high product yield. The desired mutation rate can be controlled simply by varying the initial amount of target DNA in the reaction or the number of amplification cycles performed.

* U.S. Patent Nos. 7,045,328; 6,803,216; 6,734,293; 6,489,150; 6,444,428; 6,183,997; 5,489,523, and patent pending.

How Mutation Frequency is Controlled

Mutation frequency is the product of DNA polymerase error rate and number of duplications (see *Appendix*). In the GeneMorph II kit, a sufficiently high error rate is achieved through use of Mutazyme II DNA polymerase. A low, medium or high mutation frequency is produced by adjusting the initial target DNA amounts in the amplification reactions. For the same PCR yield, targets amplified from low amounts of target DNA undergo more duplications than targets amplified from high concentrations of DNA. 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 to limit the number of target duplications. Mutation rates can also be decreased by lowering the number of cycles to achieve fewer target duplications. For targets that produce high product yields after 30 cycles, lower mutation rates can be achieved by amplifying lower target amounts for 20–25 cycles.

Selecting the Appropriate Mutation Frequency

The GeneMorph II kit allows researchers to choose the mutation frequency that is most appropriate for a particular application. For analyzing protein structure-function relationships, the desired mutation frequency is one amino acid change (1–2 nucleotide changes) per gene.[2] In directed evolution studies, mutation frequencies of 1–4 amino acid changes (2–7 nucleotide changes) per gene are commonly employed.[3–6] Proteins with improved activities have also been isolated from highly mutagenized libraries exhibiting 20 mutations per gene.[1]

Achieving the Desired Mutation Frequency

Table I presents the initial amount of target DNA required to produce low, medium, or high mutation frequencies. An initial target amount of 500–1000 ng is recommended to achieve low mutation frequencies of 0–4.5 mutations/kb. Low mutation frequencies can also be achieved by using 100–500 ng of target DNA with a lower number of PCR cycles (see *Cycle Number* in *Preprotocol Considerations*). Initial target amounts ranging from 100–500 ng are recommended for producing mutation frequencies of 4.5–9 mutations/kb (medium mutation frequency range). High mutation frequencies (>9 mutations/kb) are obtained by using 0.1–100 ng of input target DNA, where the highest mutation rates can be achieved using the lowest recommended target amounts. Mutation rates up to 16 mutations per kb have been achieved using 0.01 ng of target DNA, although PCR product yields tend to decrease at amounts below 0.1 ng. The predicted mutation frequencies shown in Table I are accurate for amplification reactions producing the indicated approximate fold amplification. The actual number of mutations in individual clones may differ as the values in Table I represent the average mutation frequency for the entire pool of clones.

TABLE I

Mutation Frequency vs. Initial Target Quantity

Mutation rate	Mutation frequency (mutations/kb)[a]	Initial target amount (ng)[b,c]	Recommended fold amplification
Low	0–4.5	500–1000	1.5–10
Medium	4.5–9	100–500	10–100
High	9–16	0.1–100	100–10,000

[a] These values are accurate for reactions achieving the approximate fold amplification (total yield/input DNA) indicated. The actual number of mutations in each clone may differ as these values represent the average frequency for all clones.

[b] The amount of template indicated is the amount of target DNA to be amplified, not the total amount of DNA template to add to the reaction. See *Initial Amount of Target* in *Preprotocol Considerations* for an example on how to calculate initial target amount.

[c] The recommended DNA target amounts are higher for Mutazyme II compared to Mutazyme I since Mutazyme II exhibits a ~3-fold higher error rate compared to Mutazyme I.

Mutational Spectrum of the GeneMorph II Kit

The mutational spectra of Mutazyme II DNA polymerase, Mutazyme I DNA polymerase, and *Taq* DNA polymerase (with Mn^{2+}-containing buffer and unbalanced dNTP concentrations) are compared in Table II. These error prone PCR enzymes introduce all possible nucleotide substitutions, however, Mutazyme II DNA polymerase exhibits less mutational bias compared to Mutazyme I and *Taq* DNA polymerases.

There are several ways to assess bias in an enzyme's mutational spectra. Bias can be examined by analyzing the ratio of transition (Ts) to transversion (Tv) mutations produced. Transition mutations are purine (A and G) to purine changes and pyrimidine (C and T) to pyrimidine changes, while transversions are purine to pyrimidine and pyrimidine to purine changes. There are eight possible transversions and four possible transitions, and an enzyme completely lacking bias would exhibit a Ts/Tv ratio of 0.5. Secondly, mutational bias has been assessed by calculating the ratio of AT→GC to GC→AT transition mutations (AT→GC/GC→AT ratio), which would equal 1 for a completely unbiased enzyme. Thirdly, mutational bias can be assessed by comparing the frequency of mutating A's and T's vs. the frequency of mutating G's and C's (AT→NN/GC→NN ratio), which should be equal for an unbiased DNA polymerase.

[ll] The *Taq* DNA polymerase was used in the PCR with Mn^{2+}-containing buffer and unbalanced deoxynucleotide concentrations, which are mutagenic conditions for *Taq* DNA polymerase.

TABLE II

Mutational Spectra of Mutazyme and Taq DNA Polymerases

Type(s) of mutations	Mutazyme II DNA polymerase[a]	Mutazyme I DNA polymerase[a]	*Taq* DNA polymerase (Reference 6)[b]
Bias Indicators			
Ts/Tv	0.9	1.2	0.8
AT→GC/GC→AT	0.6	0.2	1.9
A→N, T→N	50.7%	25.6%	75.9%
G→N, C→N	43.8%	72.5%	19.6%
Transitions			
A→G, T→C	17.5%	10.3%	27.6%
G→A, C→T	25.5%	43.7%	13.6%
Transversions			
A→T, T→A	28.5%	11.1%	40.9%
A→C, T→G	4.7%	4.2%	7.3%
G→C, C→G	4.1%	8.8%	1.4%
G→T, C→A	14.1%	20.0%	4.5%
Insertions and Deletions			
Insertions	0.7%	0.8%	0.3%
Deletions	4.8%	1.1%	4.2%
Mutation Frequency			
Mutations/kb (per PCR)[c]	3–16 (per PCR)	<1 to 7 (per PCR)	4.9 (per PCR)

[a] The Mutazyme DNA polymerases were used with the corresponding GeneMorph random mutagenesis kits.

[b] The *Taq* DNA polymerase was used with Mn^{2+}-containing buffer and unbalanced dNTP concentrations, which are mutagenic conditions for *Taq* DNA polymerase.

[c] Initial target amounts of 16 pg to 1 µg (Mutazyme II DNA polymerase), 1 pg to 100 ng (Mutazyme I DNA polymerase), and 0.01 nM template (*Taq* DNA polymerase) were used to generate data.

As shown in Table II, all error prone enzymes favor transitions over transversions, as shown by Ts/Tv ratios greater than 0.5, with Mutazyme II and *Taq* exhibiting a somewhat higher tendency to create transversions over transitions and Mutazyme I exhibiting a greater tendency for introducing transitions over transversions. Examining transition mutation frequencies shows that Mutazyme II produces AT→GC and GC→AT mutations with similar rates (AT→GC/GC→AT ratio = 0.6), while Mutazyme I is 4 times more likely to generate GC→AT transitions over AT→GC transitions, and *Taq* is 2 times more likely to introduce AT→GC transitions over GC→AT transitions. In addition, Mutazyme II DNA polymerase introduces mutations at A's and T's only slightly more frequently than G's and C's. In contrast, Mutazyme I is nearly 3 times more likely to mutate G's and C's, while *Taq* under error prone conditions is 4 times more likely to mutate A's and T's than G's and C's (see *Summary* in Table II).

The spectrum of mutations produced by the GeneMorph II kit is the same at all mutation frequencies. With the GeneMorph II kit, low, medium, and high mutation frequencies are achieved using a single set of buffer conditions ($MgCl_2$, balanced dNTPs) optimized for high product yield. The only parameter varied is the initial amount of target DNA in the reaction or the number of cycles employed. In contrast, *Taq* DNA polymerase–based mutagenesis methods typically employ different sets of reaction conditions to vary mutation levels. Varying the buffer conditions (e.g., different Mn^{2+} concentrations) and/or the concentrations of one or more nucleotides to alter mutation frequency can lead to changes in *Taq*'s mutational spectrum and increased mutational bias.

Furthermore, mutational hotspots have not been observed in any of the mutagenized genes generated by Mutazyme II DNA polymerase that have been sequenced. [7]

PREPROTOCOL CONSIDERATIONS

Amplification Targets

The GeneMorph II kit has been used to mutagenize plasmid DNA targets up to 6 kb in length.

Genomic DNA templates are not generally recommended for error prone PCR as researchers are limited to medium-to-high mutation levels due to the low copy number of genomic DNA targets. If genomic DNA is the only source of the target gene, we recommend amplifying the target with a high-fidelity DNA polymerase, such as *PfuUltra* high-fidelity DNA polymerase, followed by re-amplification of the diluted PCR product with Mutazyme II DNA polymerase.

Initial Amount of Target DNA

The mutation frequency depends upon the initial amount of target DNA employed in the reaction. The amount of target to add to a reaction can be determined using Table I.

The initial amount of target DNA required to achieve a particular mutation frequency refers to the amount of target DNA to amplify, not the total amount of plasmid DNA template to add to the reaction. As an example, to mutagenize a 1.0-kb target gene at a low mutation frequency, an initial target amount of 500 ng is recommended. For a 1.0-kb target gene that is an insert in a 3.0-kb plasmid (the total construct is 4.0 kb), 2 µg of the plasmid construct should be added to the reaction to provide 500 ng of target DNA.

Cycle Number

In addition to using higher target DNA amounts, mutation rates can also be lowered by decreasing the number of cycles employed to achieve fewer target duplications. For targets that produce high product yields after 30 cycles, lower mutation rates can be achieved by amplifying lower target amounts for 20 to 25 cycles (see Table III).

TABLE III

Achieving Low Mutation Frequency Using Fewer Cycle Numbers

Mutation frequency (mutations/kb)[a]	Cycle Number	Initial target amount[b]
0–4.5 (low range)	20–25	100 ng
	30	500 ng–1000 ng

[a] These values are accurate for reactions achieving the approximate 1.5–10 fold amplification (total yield/input DNA). The actual number of mutations in each clone may differ as these values represent the average frequency for all clones.

[b] The amount of template indicated is the amount of target DNA to be amplified, not the total amount of DNA template to add to the reaction. See *Initial Amount of Target* in *Preprotocol Considerations* for an example on how to calculate initial target amount.

Primer Design

For best results, PCR primers should be designed with similar melting temperatures ranging from 55 to 72°C. The use of primers with melting temperatures within this range reduces false priming and ensures complete denaturation of unextended primers at 94–95°C.

PCR Product Yield

The PCR product yield should be within the recommended range to obtain the predicted mutation frequencies listed in Tables I and III. To ensure sufficient product yield, sample PCR reactions are electrophoresed adjacent to a DNA standard provided in the kit. PCR product yields are quantified by comparing the staining intensity of PCR product bands to the DNA standard.

Achieving High Mutation Frequencies

The highest mutation frequency that can be achieved in one round of PCR is limited by the minimum amount of target DNA that can be amplified in high product yield. In the GeneMorph II kit, we recommend using 0.1–100 ng of target DNA, which is sufficient to produce high product yields after 30 cycles and mutation frequencies up to 9–16 mutations per kb of target. Higher mutation frequencies can be achieved by amplifying from <0.1 ng target DNA, although product yields may be noticeably lower. Alternatively, mutation frequencies > 20 mutations per kb can be achieved by performing sequential PCRs, in which a small aliquot of the first PCR reaction is re-amplified in a second PCR reaction.

PROTOCOL

Note *Gently mix and centrifuge each component before use. Prepare all reactions on ice.*

1. Refer to Table I to determine the initial amount of target to use in each reaction.

   Note *Target DNA refers to the DNA sequence to be amplified, not the total amount of plasmid DNA in the reaction (see* Initial Amount of Target *in* Preprotocol Considerations*).*

2. Prepare 50-µl reactions as follows:

   41.5 µl of water
   5 µl of 10× Mutazyme II reaction buffer
   1 µl of 40 mM dNTP mix (200 µM each final)
   0.5 µl of primer mix (250 ng/µl of each primer)
   1 µl of Mutazyme II DNA polymerase (2.5 U/µl)
   1 µl template (see Table I for recommended amount)

3. Centrifuge each reaction briefly.

4. If the thermal cycler does not have a heated lid, overlay each reaction with a few drops of mineral oil.

5. Place each reaction in a temperature cycler. Run either of the following suggested PCR programs or your own PCR program.

Suggested PCR Program for RoboCycler Temperature Cyclers

Segment	Number of cycles	Temperature	Duration
1	1	95°C[a]	2 minutes
2	30[b]	95°C	1 minute
		Primer T_m – 5°C[c]	1 minute
		72°C	1 minute (≤1-kb targets) or 1 minute/kb (>1-kb targets)
3	1	72°C	10 minutes

[a] Denaturing temperatures above 95°C are recommended only for GC-rich templates.

[b] Low mutation frequencies (0–4.5 mutations per kb) can be achieved by reducing the cycle number (see *Cycle Number* in *Preprotocol Considerations*).

[c] The annealing temperature may be lowered further if necessary to obtain optimal results. Typically annealing temperatures will range between 55° and 72°C.[8]

Suggested PCR Program for Single-Block Temperature Cyclers

Segment	Number of cycles	Temperature	Duration
1[a]	1	95°C[b]	2 minutes
2	30[c]	95°C	30 seconds
		Primer T_m – 5°C[d]	30 seconds
		72°C	1 minute (≤1-kb targets) or 1 minute/kb (>1-kb targets)
3	1	72°C	10 minutes

[a] Certain thermocyclers may require the removal of segment 1. Optimized cycling parameters are not necessarily transferable between thermal cyclers designed by different manufacturers; therefore, each manufacturer's recommendations for optimal cycling parameters should be consulted.

[b] Denaturing temperatures above 95°C are recommended only for GC-rich templates.

[c] Low mutation frequencies (0–4.5 mutations per kb) can be achieved by reducing the cycle number (see *Cycle Number* in *Preprotocol Considerations*).

[d] The annealing temperature may be lowered further if necessary to obtain optimal results. Typically annealing temperatures will range between 55° and 72°C.[8]

6. Quantitate the PCR product yield. Electrophorese 1–10 µl of each amplification reaction along with 5 µl (100 ng) of the 1.1-kb gel standard on a 1% agarose gel. Estimate the PCR product yield by comparing the intensities of the PCR product bands with the 1.1-kb gel standard. Comparisons can made either by visual inspection, or for a more accurate estimate, by using an imaging system. The PCR product yield should be within the range that provides the recommended fold amplification to achieve the expected mutation frequencies listed in Table I.

   Note *The expected mutation frequencies are typically achieved when the PCR yield of a 10-µl sample is between 100 ng and 2 µg, which corresponds to a yield of between 500 ng and 10 µg for a 50-µl reaction. If the mutation frequency is to be calculated using the graphs in the* Appendix *rather than the guidelines in Table I, it is recommended that actual PCR product yield is determined from a DNA standard curve. To prepare a standard curve, customers use their own DNA standard, consisting of linear double-stranded DNA of known concentration and of similar size to the sample amplicon. Four known amounts (100 ng, 500 ng, 1000 ng and 2000 ng) of the DNA standard should be electrophoresed adjacent to 10 µl of the PCR product to be analyzed. A densitometry program should be used to quantify the DNA in each standard lane so that a calibration curve can be constructed. PCR product yield (per 10 µl) can then be determined from the density of the PCR product band by extrapolation from the DNA standard curve.*

The PCR product is now ready to be cloned into an expression vector and introduced into competent cells according to appropriate protocols.

TROUBLESHOOTING

Observation	Suggestion(s)
No product or low yield	Ensure that extension times are of sufficient length. Increase extension time to 2 minutes/kb of PCR target.
	Ensure that the annealing temperature is not too high. Lower the annealing temperature in 5°C increments.
	Consider the GC content or secondary structure. For high GC content or secondary structure, use higher denaturing temperatures (94–98°C) (see also Reference 9). Use cosolvents such as DMSO in a 1–10% (v/v) final concentration or glycerol in a 5–20% (v/v) final concentration.
	Ensure that the primer concentration is sufficient. Use primer concentrations between 0.1 and 0.5 μM (generally 100–250 ng for typical 18- to 25-mer oligonucleotide primers in a 100-μl reaction volume).
	Evaluate primers. Use high-quality primers. Check the melting temperature, purity, GC content, and length of the primers.
	Check the ionic strength of the reaction mixture. If ionic strength is high, remove extraneous salts from the PCR primers and DNA preparations.
	Ensure that the amount of Mutazyme II DNA polymerase* is sufficient. The amount of Mutazyme II DNA polymerase can be increased to 5 U/reaction.
	Ensure that there is adequate heat exchange between the reaction tubes and the thermal cycler. Use thin-wall PCR tubes for Stratagene thermal cyclers (i.e., RoboCycler temperature cyclers). These PCR tubes ensure ideal contact with the multiblock design to permit more efficient heat transfer and to maximize thermal-cycling performance.
	Increase the number of cycles to greater than 30.
Multiple bands	Ensure that the primer annealing temperature is sufficient. Increase the annealing temperature in 5°C increments.
	Multiple bands can be caused by nonspecific primer–template annealing. Use Perfect Match PCR enhancer to improve PCR product specificity.
Artifactual smears	Ensure that the amount of Mutazyme II DNA polymerase is not excessive. Decrease the amount of Mutazyme II DNA polymerase.
	Ensure that the extension time is not too long. Reduce the extension time.
	If smearing occurs in sequential PCR reactions, reduce the amount of PCR product used as template.

* Mutazyme II DNA polymerase is not sold separately.

APPENDIX: HOW TO CALCULATE MUTATION FREQUENCY

The mutation frequency is controlled by adjusting the initial amount of target DNA in an amplification reaction or the number of thermal cycles as explained by the following equations.

The mutation frequency of an amplification reaction is determined by the formula:

$$\textit{Mutation frequency} = \textit{error rate} \times d \quad (1)$$

where *mutation frequency* is expressed as mutations/kb, *error rate* is the error rate of the DNA polymerase in errors/(kb·duplication), and *d* is the number of duplications during PCR.

The variable *d* can be calculated from the following equation:

$$2^d = \text{PCR yield}/\textit{initial amount of target} \quad (2)$$

Note: In these calculations, initial amount of target refers to the amount of amplicon DNA present in the DNA template, and not the total amount of plasmid DNA added to the reaction. As an example, to mutagenize a 1.0-kb target gene at a low mutation frequency, an initial target amount of 500 ng is recommended. For a 1.0-kb target gene that is an insert in a 3.0-kb plasmid (the total construct is 4.0 kb), 2 µg of the plasmid construct should be added to the reaction to provide 500 ng of target DNA.

Solving for *d*, we obtain

$$d = \log_{10}(\text{PCR yield}/\textit{initial target amount})/\log_{10}2. \quad (3)$$

Equation (1) shows that mutation frequency is the product of DNA polymerase error rate and number of duplications. So, any change in *d* leads to a proportional change in the mutation frequency. An example of this relationship is given in Figure 1, which shows experimental data obtained using the GeneMorph II kit. Equation (3) shows that *d* depends on the ratio of total PCR product yield (per 50-µl reaction) to initial amount of target DNA. In the GeneMorph II kit, *d* (and hence mutation frequency) is varied by varying the initial amount of target DNA in the amplification reaction. For the same PCR yield, targets amplified from low amounts of target DNA undergo more duplications than targets amplified from high concentrations of target DNA. 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.

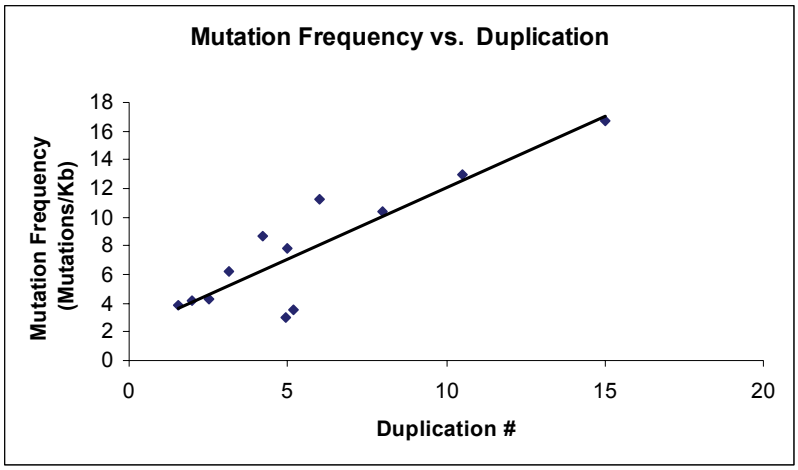

Figure 1 Relationship between mutation frequency and duplications.

The observed mutation frequencies produced by the GeneMorph II kit for varying amounts of lacZ input DNA is shown in Table 1. These target amounts have given a total PCR product yield (per 50-µl reaction) of 200 ng to 6 µg.

Customers interested in determining the mean mutation frequency achieved in their amplification reaction can do so by determining the *d* value; *d* values are calculated from equation (3) after quantifying PCR product yield using the recommendations (standard curve) described in step 6 in *Protocol* section. Expected average mutation frequency (mutations per kb) can then be determined by extrapolation from the above graph (see Figure 1).

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MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

STRATAGENE

An **Agilent Technologies** Division

GeneMorph II Random Mutagenesis Kit

QUICK-REFERENCE PROTOCOL

Mutation Frequency

Mutation frequency (mutations/kb)	Initial target quantity
0–4.5 (low range)	500–1000 ng
4.5–9 (medium range)	100–500 ng
9–16 (high range)	0.1–100 ng

Equation

$d = \log_{10}(\text{PCR yield}/\textit{initial target amount})/\log_{10}2$

PCR Protocol

41.5 µl of water
5 µl of 10× Mutazyme II reaction buffer
1 µl of 40 mM dNTP mix (200 µM each final)
0.5 µl of primer mix (250 ng/µl of each primer)
1 µl of Mutazyme II DNA polymerase (2.5 U/µl)
1 µl template (see Table I for recommendations)

Suggested PCR Program for RoboCycler Temperature Cyclers

Segment	Number of cycles	Temperature	Duration
1	1	95°C	2 minutes
2	30	95°C	1 minute
		Primer T_m - 5°C	1 minute
		72°C	1 minute (≤1-kb targets) or 1 minute/kb (>1 kb targets)
3	1	72°C	10 minutes

Suggested PCR Program for Single-Block Temperature Cyclers

Segment	Number of cycles	Temperature	Duration
1	1	95°C	2 minutes
2	30	95°C	30 seconds
		Primer T_m – 5°C	30 seconds
		72°C	1 minute (≤1-kb targets) or 1 minute/kb (>1 kb targets)
3	1	72°C	10 minutes

Quantitate the PCR yield by running 10 µl of the PCR product and 5 µl (100 ng) of the 1.1 kb gel standard on a 1% agarose gel and compare the two bands.