GeneMorph II EZClone Domain Mutagenesis Kit

Instruction Manual

Catalog #200552

Revision E.0

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GeneMorph II EZClone Domain Mutagenesis Kit

CONTENTS

Materials Provided **1**

Storage Conditions **1**

Additional Materials Required **1**

Notice to Purchaser **2**

Introduction **3**

Eliminate Bias and Easily Control Mutation Rate 5

EZClone Reaction 9

GeneMorph II EZClone Domain Mutagenesis Control **10**

XL10-Gold Ultracompetent Cells **10**

Preprotocol Considerations **11**

Mutant Megaprimer Synthesis Considerations 11

EZClone Reaction Considerations 13

Protocol **14**

Mutant Megaprimer Synthesis 14

EZClone Reaction 17

Expected Results 20

Transformation Guidelines **21**

Troubleshooting **22**

Preparation of Media and Reagents **24**

Appendix: How to Calculate Mutation Frequency **25**

References **27**

MSDS Information **27**

Quick-Reference Protocol **28**

GeneMorph II EZClone Domain Mutagenesis Kit

MATERIALS PROVIDED

Materials provided[a]	Concentration	Quantity
Mutazyme II DNA polymerase[b]	2.5 U/µl	25 U
10× Mutazyme II reaction buffer	10×	150 µl
40 mM dNTP mix[c]	10 mM each dNTP	10 µl
2× EZClone enzyme mix	2×	250 µl
Dpn I restriction enzyme	10 U/µl	100 U
EZClone solution	—	30 µl
Positive control plasmid	10 ng/µl	210 ng
Positive control primer mix	250 ng/µl	750 ng
1.1-kb gel standard	20 ng/µl	50 µl
XL10-Gold ultracompetent cells[d,e] (yellow tubes)	—	4 × 135 µl
XL10-Gold β-mercaptoethanol mix (β-ME)	—	50 µl
pUC18 control plasmid (0.1 ng/µl in TE buffer[f])	0.1 ng/µl	10 µl

[a] Sufficient reagents are provided for 10 reactions, which includes 3 control reactions.

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

[c] Thaw the dNTP mix once, prepare single-use aliquots, and store the aliquots at –20°C. Do not subject the dNTP mix to multiple freeze-thaw cycles.

[d] Genotype: Tet^{r}Δ (*mcrA*)*183 Δ*(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ proAB *lacI*^{q}*ZΔM15* Tn*10* (Tet^r) Amy Cam^r]

[e] The XL10-Gold ultracompetent cells must be stored at the bottom of a –80°C freezer immediately on receipt. The ultracompetent cells are very sensitive to small variations in temperature. Transferring tubes from one freezer to another may result in a loss of efficiency.

[f] The pUC18 control plasmid is stored in TE buffer (see *Preparation of Media and Reagents*).

STORAGE CONDITIONS

XL10-Gold ultracompetent cells: Store the cells immediately at –80°C.

Do not place the cells in liquid nitrogen.

All other components: Store at –20°C upon receipt. Store the 2× EZClone enzyme mix at 4°C after thawing. Once thawed, full activity is guaranteed for 3 months.

ADDITIONAL MATERIALS REQUIRED

Temperature cycler
PCR tubes
PCR primers
14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)

Revision E.0

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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, domains or promoter elements by introducing mutations during PCR. However, cloning amplicons generated by error-prone PCR can be difficult and labor-intensive due to low product yields, mutations at the ends which interfere with restriction-based cloning, and/or inefficient synthesis of 3´ dA overhangs or blunt ends which reduces the efficiency of TA- or blunt-end cloning strategies. In addition, targeting specific functional domains for random mutagenesis can also be difficult and inefficient using current methods. To address the need for efficient and flexible cloning methods, the GeneMorph II EZClone domain mutagenesis kit* offers an easy and fast cloning method to perform targeted random mutagenesis on protein domains and promoter elements, while delivering a uniform mutational spectrum. Utilizing a unique cloning method, the GeneMorph II EZClone kit allows you to target specific protein domains or promoter elements without the need for restriction sites or sub-cloning (see Figure 1).[1, 2]

The kit contains Mutazyme II DNA polymerase, which deliberately introduces mutations during PCR with a more uniform mutational spectrum compared to other error-prone PCR enzymes. Additionally, this kit includes an optimized reaction buffer for the error-prone PCR step so that you need only vary the input DNA amount added to the reaction to produce the desired mutational frequency. The resulting purified mutated PCR products serve as megaprimers for the EZClone reaction** during which they are denatured and annealed to the original donor plasmid and extended with a specialized enzyme mix containing a high fidelity DNA polymerase. Using a high-fidelity polymerase minimizes unwanted secondary mutations during the cloning process, which can affect downstream results. The EZClone reaction is temperature cycled several times before being treated with a unique enzyme to remove parental DNA prior to transformation into competent *E. coli*. Our XL10-Gold ultracompetent cells are included to maximize library size and diversity. Screening libraries created by 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.[3]

* U.S. Patent No. 6,803,216.

** U.S. Patent Nos. 6,713,285, 6,391,548, 5,789,166 and 5,932,419.

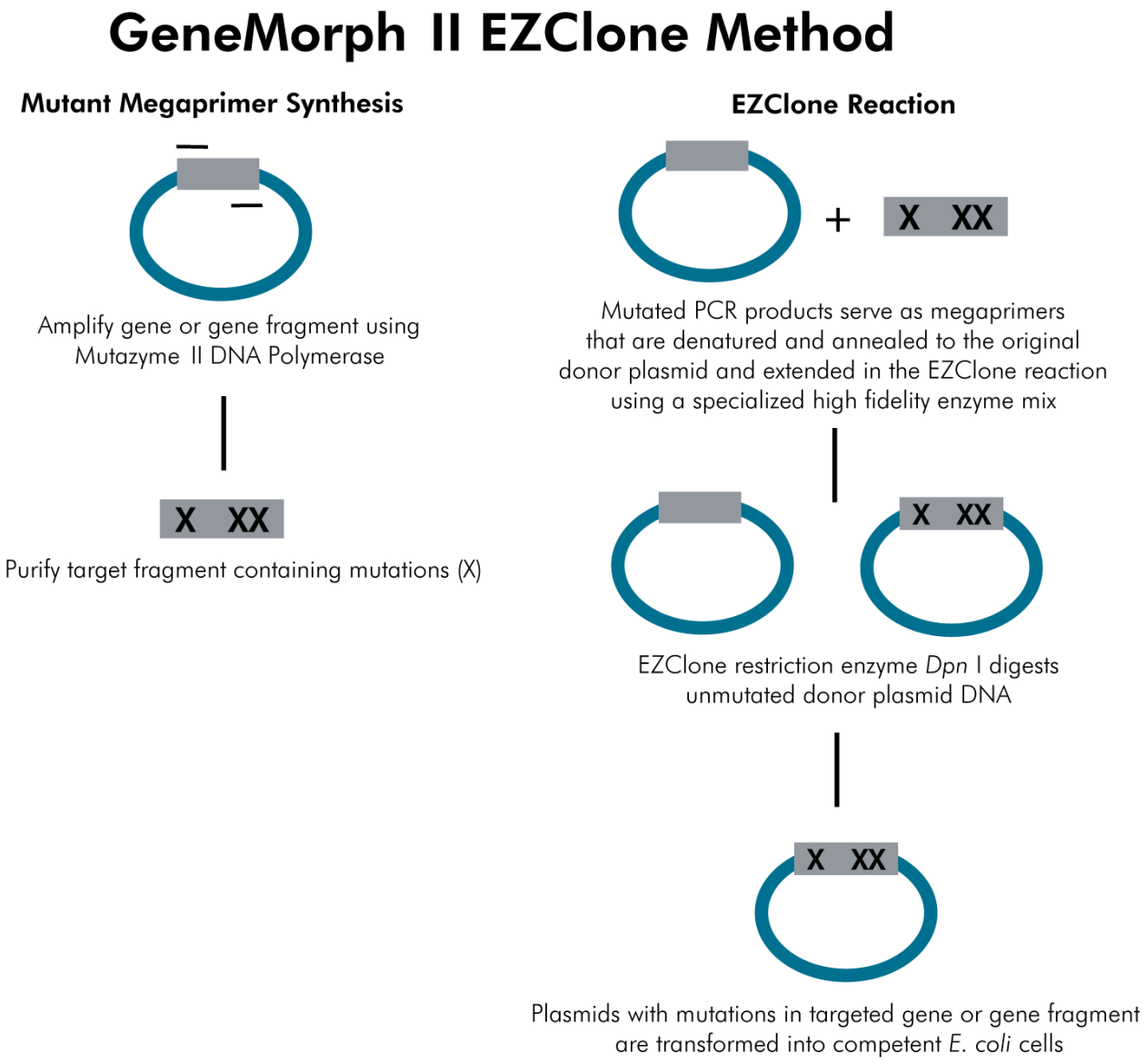

FIGURE 1 GeneMorph II EZClone domain mutagenesis kit method.

Eliminate Bias and Easily Control Mutation Rate

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 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 exhibit greater mutant representation compared to libraries generated with other enzymes.

With the GeneMorph II EZClone kit, mutation rates of 1–16 mutations per kb can be achieved using the provided buffer, which is 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.

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 EZClone 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 EZClone 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.[4] In directed evolution studies, mutation frequencies of 1–4 amino acid changes (2–7 nucleotide changes) per gene are commonly employed.[5, 6, 7, 8] Proteins with improved activities have also been isolated from highly mutagenized libraries exhibiting 20 mutations per gene.[3]

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 spectrum. 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 5)[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, error-prone enzymes generally 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.

The spectrum of mutations produced by the GeneMorph II EZClone kit is the same at all mutation frequencies. With the GeneMorph II EZClone 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.[9]

EZClone Reaction

The EZClone reaction utilizes (1) a supercoiled double-stranded DNA (dsDNA) vector containing the same region targeted for mutagenesis in the mutagenesis reaction and (2) two megaprimers generated in the mutagenesis reaction containing random mutations. The EZClone enzyme mix is a 2× formulation containing a high fidelity DNA polymerase to minimize unwanted second site errors, an optimized PCR reaction buffer, magnesium, and dNTPs. The megaprimers, each complementary to opposite strands of the vector, are extended during temperature cycling by the EZClone enzyme mix, without primer displacement. Extension of the megaprimers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *Dpn* I restriction enzyme. The *Dpn* I endonuclease (target sequence: 5′-Gm^6ATC-3′) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template. Because DNA produced during PCR is not methylated, *Dpn* I selects for the mutation-containing synthesized DNA.[10] The nicked vector DNA incorporating the desired mutations is then transformed into competent cells (provided), where the nicks are repaired by endogenous enzymes in the cell.

Note *While plasmid DNA isolated from almost all of the commonly used* E. coli *strains (dam^+) is methylated and is a suitable template for mutagenesis, plasmid DNA isolated from the exceptional dam^-* E. coli *strains, including JM110 and SCS110, is not suitable.*

GeneMorph II EZClone Domain Mutagenesis Control

To demonstrate the effectiveness of the GeneMorph II EZClone method the 3.0-kb positive control plasmid, which contains the *lacZ* gene, is used to test the efficiency of mutant megaprimer synthesis and the EZClone reaction. XL10-Gold ultracompetent cells* transformed with this control plasmid appear blue on LB–ampicillin agar plates (see *Preparation of Media and Reagents*), containing IPTG and X-gal. Following random mutagenesis and EZCloning, >25% of the colonies plated appear white on LB–ampicillin plates containing IPTG and X-gal because β-galactosidase activity has been obliterated.

XL10-Gold Ultracompetent Cells

Agilent's XL10-Gold ultracompetent cells, a derivative of the highest-efficiency Agilent competent cell line, XL2-Blue MRF´, possess the Hte phenotype, which increases transformation efficiency of ligated DNA.[11] XL10-Gold cells are both endonuclease deficient (*endA1*) and recombination deficient (*recA*). The *endA1* mutation greatly improves the quality of plasmid miniprep DNA,[12] and the *recA* mutation helps ensure insert stability. In addition, the McrA, McrCB, McrF, Mrr, and HsdR systems have been removed from XL10-Gold ultracompetent cells. The *mcrA*, *mcrCB* and *mrr* mutations prevent cleavage of cloned DNA that carries cytosine and/or adenine methylation, which is often present in eukaryotic DNA and cDNA.[13, 14, 15] The McrA and McrCB systems recognize and restrict methylated cytosine DNA sequences, and the Mrr system recognizes and restricts methylated adenine DNA sequences. The Mrr system also restricts methylated cytosine DNA sequences with a specificity differing from that of McrA and McrCB. This activity has been named McrF. This McrF activity against methylated cytosines has been shown to be equal to or greater than the restriction activity of the McrA and McrCB systems.[16] The *hsdR* mutation prevents the cleavage of cloned DNA by the *Eco*K (*hsdR*) endonuclease system. XL10-Gold cells grow faster than XL1 or XL2-Blue cells, resulting in larger colonies. To permit blue-white color screening, the XL10-Gold ultracompetent cells contain the *lacI*^{q}*ZΔM15* gene on the F´ episome.

Host strain	References	Genotype
XL10-Gold ultracompetent cells	11, 17, 18	Tet^{R} Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173* *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{R}) Amy Cam^{R}]

It is important to store the XL10-Gold ultracompetent cells at –80°C to prevent a loss of efficiency. For best results, please follow the directions outlined in the following sections.

* U.S. Patent No. 6,706,525 and equivalent foreign patents.

PREPROTOCOL CONSIDERATIONS

Mutant Megaprimer Synthesis Considerations

Amplification Targets

The GeneMorph II EZClone kit has been used to mutagenize targets up to 3.5 kb in length from plasmid DNA.

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

Standard PCR primers flanking the region targeted for mutagenesis are used in the mutant megaprimer synthesis reaction. 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 <0.1ng 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.

EZClone Reaction Considerations

Required Host Strain

Ensure that the plasmid DNA template is isolated from a dam+ *E. coli* strain. The majority of the commonly used *E. coli* strains are dam+. Plasmid DNA isolated from dam– strains (e.g. JM110 and SCS110) is not suitable.

Plasmid DNA Template Guidelines

The plasmid DNA template used in the EZClone reaction may be the same or different than the original plasmid DNA used as template in the mutant megaprimer synthesis reaction, provided that the region targeted in the mutagenesis reaction is present in the plasmid.

The plasmid DNA template used in the EZClone reaction should be less than 10 kb in length to ensure full extension during temperature cycling.

PROTOCOL

Mutant Megaprimer Synthesis

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 the control reaction as indicated below:

   5 µl of 10× Mutazyme II reaction buffer
   2 µl of 0.5 ng/µl positive control plasmid (dilute the control provided 1:20 in high-quality water for a final concentration of 0.5 ng/µl)
   1 µl of positive control primer mix (125 ng/µl of each primer)
   1 µl of 40 mM dNTP mix (200 µM each final)
   40 µl of ddH_2O
   1 µl of Mutazyme II DNA polymerase (2.5 U/µl)

3. Prepare the sample reaction(s) as indicated below:

   5 µl of 10× Mutazyme II reaction buffer
   x µl template (see Table I for recommended amount)
   1 µl of sample primers (125 ng/µl of each primer)
   1 µl of 40 mM dNTP mix (200 µM each final)
   x µl of ddH_2O for a final reaction volume of 50 µl
   1 µl of Mutazyme II DNA polymerase (2.5 U/µl)

4. Centrifuge each reaction briefly.

5. Place each reaction in a temperature cycler. Run the following suggested PCR program. For the control reaction (600-bp target), use an annealing temperature of 55°C and an extension duration of 1 minute.

Suggested PCR Program

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.[19]

6. Quantitate the PCR product yield. Electrophorese 1–10 µl of each amplification reaction along with 2.5 µl (50 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 be 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.*

7. Purify the PCR product.

   If the amount of the initial DNA template is less than 50 ng per 50 µl reaction and the same plasmid DNA is used in the following EZClone reaction, use a commercial PCR purification kit (e.g., StrataPrep PCR Purification Kit, Catalog #400771 or #400773).

   If the amount of the initial DNA template is more than 50 ng or if a different plasmid DNA will be used in the following EZClone reaction, use a commercial DNA gel extraction kit (e.g., StrataPrep DNA Gel Extraction Kit, Catalog #400766 or #400768).

8. Quantitate the purified PCR product by electrophoresing 1–10 µl of each amplification reaction on a 1% agarose gel. In a nearby lane, load 2.5 µl (50 ng) of the 1.1-kb gel standard. Estimate the purified PCR product yield by comparing the intensities of the purified PCR product band with the 1.1-kb gel standard. Comparisons can be made either by visual inspection, or for a more accurate estimate, by using an imaging system.

EZClone Reaction

Notes *The plasmid DNA template used in the EZClone reaction may be the same or different than the original plasmid DNA used as template in the mutant megaprimer synthesis reaction, provided that the region targeted in the mutagenesis reaction is present in the plasmid.*

Ensure that the plasmid DNA template is isolated from a dam+ E. coli *strain. The majority of the commonly used* E. coli *strains are dam+. Plasmid DNA isolated from dam– strains (e.g. JM110 and SCS110) is not suitable.*

To maximize temperature-cycling performance, use thin-walled tubes, which ensure ideal contact with the temperature cycler's heat blocks. The following protocols were optimized using thin-walled tubes.

The EZClone solution has been shown to improve linear amplification, specifically of long, GC-rich, or difficult targets. Enhanced amplification efficiencies are observed when using between 2.5–3.5 µl EZClone solution per 50-µl reaction, with 3 µl being optimal for most targets.

1. Prepare the control reaction as indicated below:

   25 µl of the 2× EZClone enzyme mix
   5 µl of the positive control plasmid (10 ng/µl)
   250 ng megaprimer
   3 µl of EZClone solution
   ddH_2O to a final volume of 50 µl

2. Prepare the sample reaction(s) as indicated below:

   25 µl of the 2× EZClone enzyme mix
   50 ng of template plasmid
   250 ng megaprimer (<1kb), or 500 ng for ≥1 kb megaprimer
   3 µl of EZClone solution
   ddH_2O to a final volume of 50 µl

3. Place each reaction in a temperature cycler and run the following PCR program. For the control reaction, use an extension time of 6 minutes.

Cycling Parameters for the Mutagenesis Method

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	25	95°C	50 seconds
		60°C	50 seconds
		68°C	2 minute/kb of plasmid length[a]

[a] For example, a 5-kb plasmid requires 10 minutes at 68°C per cycle.

4. Following temperature cycling, place the reaction on ice for 2 minutes to cool the reaction to ≤37°C.

Digestion of the Amplification Products

1. Add 1 µl of *Dpn* I restriction enzyme (10 U/µl) directly to each amplification reaction using a small, pointed pipet tip.

2. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 2 hours to digest the parental (i.e., the nonmutated) supercoiled dsDNA.

Transformation of XL10-Gold Ultracompetent Cells

Notes *Please read the* Transformation Guidelines *before proceeding with the transformation protocol.*

XL10-Gold cells are resistant to chloramphenicol and tetracycline. If the mutagenized plasmid contains only the Cam^R or Tet^R resistance marker, an alternative strain of competent cells must be used.

1. Gently thaw the XL10-Gold ultracompetent cells on ice. For each control and sample reaction to be transformed, aliquot 45 µl of the ultracompetent cells to a *prechilled* 14-ml BD Falcon polypropylene round-bottom tube.

2. Add 2 µl of the XL10-Gold β-mercaptoethanol mix (β-ME) provided with the kit to the 45 µl of cells. (Using an alternative source of β-ME may reduce transformation efficiency.)

3. Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.

4. Transfer 1.5 µl of the *Dpn* I-treated DNA from each control and sample reaction to separate aliquots of the ultracompetent cells.

   As an optional control, verify the transformation efficiency of the XL10-Gold ultracompetent cells by adding 1 µl of 0.01 ng/µl pUC18 control plasmid (dilute the control provided 1:10 in high-quality water) to a separate 45-µl aliquot of the ultracompetent cells.

   Swirl the transformation reactions gently to mix and incubate the reactions on ice for 10 minutes.

5. Preheat NZY^+ broth (see *Preparation of Media and Reagents*) in a 42°C water bath for use in step 8.

   Note *Transformation of XL10-Gold ultracompetent cells has been optimized using NZY+ broth.*

6. Heat-pulse the tubes in a 42°C water bath for 30 seconds. The duration of the heat pulse is *critical* for obtaining the highest efficiencies. Do not exceed 42°C.

   Note *This heat pulse has been optimized for transformation in 14-ml BD Falcon polypropylene round-bottom tubes.*

7. Incubate the tubes on ice for 2 minutes.

8. Add 0.5 ml of preheated (42°C) NZY$^+$ broth to each tube, then incubate the tubes at 37°C for 1 hour with shaking at 225–250 rpm.

9. Plate the appropriate volume of each transformation reaction, as indicated in the table below, on agar plates containing the appropriate antibiotic for the plasmid vector.

   For the mutagenesis and transformation controls, spread cells on LB–ampicillin agar plates containing 80 µg/ml X-gal and 20 mM IPTG (see *Preparation of Media and Reagents*).

Transformation reaction plating volumes

Reaction Type	Volume to Plate
Positive mutagenesis control plasmid	10 µl (in 200 µl of NZY$^+$ broth)*
pUC18 transformation control	5 µl (in 200 µl of NZY$^+$ broth)*
Sample mutagenesis	20 and 200 µl on each of two plates

* Place a 200-µl pool of NZY$^+$ broth on the agar plate, pipet the 5 µl of the transformation reaction into the pool, then spread the mixture.

10. Incubate the transformation plates at 37°C for >16 hours.

Expected Results

Expected Results for the Control Transformations

The positive mutagenesis control reaction monitors the introduction of mutations into the *lacZ* target gene. The expected colony number from the transformation of the positive mutagenesis control reaction is >50 colonies. Under highly mutagenic PCR conditions (i.e., 30 PCR cycles using 1 ng of positive control template), >25% of the transformants appear white (phenotypic mutants) as compared to <1% white colonies for the non-mutagenized transformed control plasmid, when plated on agar plates containing IPTG and X-gal. White colonies indicate that random mutations made within the *lacZ* gene have destroyed β-galactosidase activity, and that the EZClone reaction was successful. Colonies that appear blue indicate that either the random mutations made to the megaprimer do not result in a phenotypic change from blue- to white-colored colonies, or that the *Dpn* I digestion was not 100% efficient.

Notes *Both true blue and light blue colonies are counted as blue colonies.*

White colonies appearing on the control transformation plate indicate that random mutations were made in the portion of the lacZ *gene that results in a phenotypic change from blue to white colonies. The expected percentage of white colonies does not represent the overall genotypic mutation rate, which typically can be much higher than the phenotypic rate.*

If transformation of the pUC18 control plasmid was performed, >100 colonies should be observed (transformation efficiency $>10^9$ cfu/μg) with >98% of the colonies having the blue phenotype.

Expected Results for Sample Transformations

The colony number will vary depending on experimental conditions, including the volume plated and plasmid DNA size. For suggestions on increasing colony number, see *Troubleshooting*. The insert of interest should be sequenced to verify that selected clones contain the desired mutation(s).

TRANSFORMATION GUIDELINES

Storage Conditions

Ultracompetent cells are sensitive to even small variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency. Ultracompetent cells should be placed at –80°C directly from the dry ice shipping container.

Aliquoting Cells

When aliquoting, keep ultracompetent cells on ice at all times. It is essential that the 14-ml BD Falcon polypropylene round-bottom tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into the chilled tubes. It is also important to use at least 40 µl of ultracompetent cells/transformation.

Use of 14-ml BD Falcon Polypropylene Round-Bottom Tubes

It is important that 14-ml BD Falcon polypropylene round-bottom tubes are used for the transformation protocol, since other tubes may be degraded by the β-mercaptoethanol used in the transformation protocol. In addition, the duration of the heat-pulse step is critical and has been optimized specifically for the thickness and shape of these tubes.

Use of β-Mercaptoethanol

β-mercaptoethanol (β-ME) has been shown to increase transformation efficiency. The XL10-Gold β-mercaptoethanol mix provided in this kit is diluted and ready to use. For optimum efficiency, use 2 µl of the provided β-ME mix. (Using an alternative source of β-ME may reduce transformation efficiency.)

Length and Temperature of the Heat Pulse

There is a defined window of highest efficiency resulting from the heat pulse during transformation. Optimal efficiencies are observed when cells are heat pulsed for 30 seconds. Heat pulsing for at least 30 seconds is recommended to allow for slight variations in the length of incubation. Efficiencies decrease when the duration of the heat pulse is <30 seconds or >40 seconds. Do not exceed 42°C.

Preparing the Agar Plates for Color Screening

To prepare the LB agar plates for blue–white color screening, add 80 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 20 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), and the appropriate antibiotic to the LB agar. Alternatively, 100 µl of 10 mM IPTG and 100 µl of 2% X-gal can be spread on the LB agar plates 30 minutes prior to plating the transformations. Prepare the IPTG in sterile dH_2O; prepare the X-gal in dimethylformamide (DMF). Do not mix the IPTG and X-gal before pipetting them onto the plates because these chemicals may precipitate.

TROUBLESHOOTING

Mutagenesis Protocol Troubleshooting

Observation	Suggestion(s)
No product or low yield of megaprimer	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 20). 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.
	Increase the number of cycles to greater than 30.
	Avoid multiple freeze-thaw cycles for the dNTP mix. Thaw the dNTP mix once, prepare single-use aliquots, and store the aliquots at –20°C. Do not subject the dNTP mix to multiple freeze-thaw cycles.
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.

TROUBLESHOOTING CONTINUED

EZClone Reaction Troubleshooting

Observation	Suggestion(s)
Low transformation efficiency or low colony number	Ensure that sufficient mutant DNA is synthesized in the reaction. Increase the amount of the *Dpn* I-treated DNA used in the transformation reaction to 4 µl.
	Visualize the recipient DNA template on a gel to verify the quantity and quality. Nicked or linearized plasmid DNA will not generate complete circular product. Verify that the template DNA is at least 80% supercoiled.
	It is not uncommon to observe low numbers of colonies, depending on the size of the donor plasmid. Most of the colonies that do appear, however, will contain mutagenized plasmid.
	Different thermal cyclers may contribute to variations in ramping efficiencies. Adjust the cycling parameters and repeat the protocol for the sample reactions.
	Ensure that supercompetent cells are stored at the bottom of a –80°C freezer immediately upon arrival (see also *Transformation Guidelines*).
	For the control reaction, verify that the agar plates were prepared correctly. See *Preparation of Media and Reagents*, and follow the recommendations for IPTG and X-Gal concentrations carefully.
	For best visualization of the blue (β-gal$^+$) phenotype in the control reaction, plates must be incubated for at least 16 hours at 37°C.
Low mutagenesis efficiency	Allow sufficient time for the *Dpn* I to completely digest the parental template; repeat the digestion if too much DNA template was present.
	Set up an initial sample reaction using 250 ng of megaprimer for primers <1 kb and 500 ng of megaprimer for primers ≥1 kb. If this initial reaction is unsuccessful, set up a series of sample reactions using various concentrations of megaprimer while keeping the template concentration constant.
	Avoid subjecting the EZClone enzyme mix to multiple freeze-thaw cycles. Store at –20°C upon receipt. Store the 2× EZClone enzyme mix at 4°C after thawing. Once thawed, full activity is guaranteed for 3 months.

PREPARATION OF MEDIA AND REAGENTS

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

LB–Ampicillin Agar (per Liter)

(Use for reduced satellite colony formation)
1 liter of LB agar
Autoclave
Cool to 55°C
Add 100 mg of filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)

NZY+ Broth (per Liter)

10 g of NZ amine (casein hydrolysate)
5 g of yeast extract
5 g of NaCl
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.5 using NaOH
Autoclave
Add the following filer-sterilized supplements prior to use:

12.5 ml of 1 M $MgCl_2$
12.5 ml of 1 M $MgSO_4$
20 ml of 20% (w/v) glucose (or 10 ml of 2 M glucose)

TE Buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

Agar Plates for Blue-White Color Screening

Prepare LB agar as indicated above. When adding the antibiotic, also add 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to a final concentration of 80 µg/ml [prepared in dimethylformamide (DMF)] and isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 20 mM (prepared in sterile dH_2O). Alternatively, 100 µl of 10 mM IPTG and 100 µl of 2% X-gal may be spread on solidified LB agar plates 30 minutes prior to plating the transformations. (For consistent color development across the plate, pipet the X-gal and the IPTG into a 100-µl pool of SOC medium and then spread the mixture across the plate. Do not mix the IPTG and X-gal before pipetting them into the pool of medium because a precipitate may form.) Spreading the IPTG and X-gal on the plate surface produces darker blue colonies compared to the pale blue colonies produced when IPTG and X-gal are added to the medium prior to pouring).

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 2, 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.

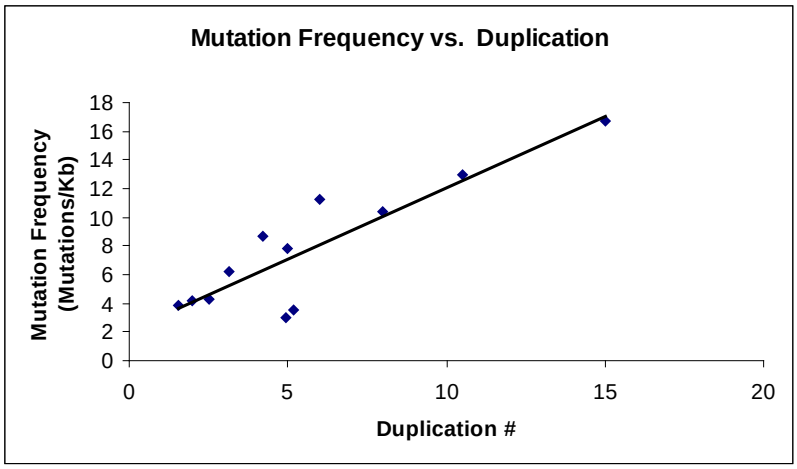

Figure 2 Relationship between mutation frequency and duplications.

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 2).

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MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

GeneMorph II EZClone Domain 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

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

Mutant Megaprimer Synthesis

Prepare the control and sample reaction(s) as indicated below:

Control Reaction	Sample Reaction
5 µl of 10× Mutazyme II reaction buffer 2 µl positive control plasmid (dilute the control provided 1:20 in high-quality water for a final concentration of 0.5 ng/µl) 1 µl of positive control primer mix (125 ng/µl of each primer) 1 µl of 40 mM dNTP mix (200 µM each final) 40 µl of ddH_2O 1 µl of Mutazyme II DNA polymerase (2.5 U/µl)	5 µl of 10× Mutazyme II reaction buffer x µl template (see Table above for recommended amount) 1 µl of sample primers (125 ng/µl of each primer) 1 µl of 40 mM dNTP mix (200 µM each final) x µl of ddH_2O for a final reaction volume of 50 µl 1 µl of Mutazyme II DNA polymerase (2.5 U/µl)

Cycle each reaction using the cycling parameters outlined in the following table:

Suggested PCR Program

Segment	Number of cycles	Temperature	Duration
1	1	95°C	2 minutes
2	30[a]	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

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

Quantitate the PCR yield by running 2.5 µl of the PCR product and 2.5 µl (50 ng) of the 1.1 kb gel standard on a 1% agarose gel and compare the two bands.

Purify the PCR product.

Quantitate the purified megaprimer using the 1.1 kb gel standard.

EZClone Reaction

Prepare the control and sample reaction(s) as indicated below:

Control Reaction	Sample Reaction
25 µl of 2× EZClone enzyme mix	25 µl of 2× EZClone enzyme mix
5 µl (50 ng) of the positive control plasmid (10 ng/µl)	50 ng of template plasmid
250 ng megaprimer	250 ng megaprimer (<1kb), or 500 ng for > 1 kb megaprimer
3 µl of EZClone solution	3 µl of EZClone solution
ddH_2O to a final volume of 50 µl	ddH_2O to a final volume of 50 µl

Cycle each reaction using the cycling parameters outlined in the following table:

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	25	95°C	50 seconds
		60°C	50 seconds
		68°C	2 minute/kb of plasmid length

Cool the reactions to ≤37°C by placing them on ice.

Add 1 µl of *Dpn* I restriction enzyme.

Gently and thoroughly mix each reaction, spin the tube in a microcentrifuge for 1 minute, and immediately incubate at 37°C for 2 hours to digest the parental supercoiled dsDNA.

Transform 1.5 µl of the *Dpn* I-treated DNA from each control and sample reaction into separate 45-µl aliquots of XL10-Gold ultracompetent cells.