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Creating Randomized Amino Acid Libraries with the QuikChange® Multi Site-Directed Mutagenesis Kit

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ABSTRACT

The QuikChange® Multi Site-Directed Mutagenesis Kit is a simple and efficient method for introducing point mutations at up to five sites simultaneously in plasmid DNA templates. Here we used the QuikChange Multi kit with degenerate (one codon) primers to introduce all possible amino acids at selected sites in the lac*Z gene. In reactions employing two or three degenerate primers, diverse libraries (10^4–10^5 mutants/reaction) are created consisting of random combinations of mutations at two or three different sites. This method provides a one-day procedure for performing site-directed saturation mutagenesis and, when coupled with a suitable screening assay, should greatly facilitate the process of evaluating alternative amino acid chain substitutions at key residues and evolving protein function.*

INTRODUCTION

Site-directed mutagenesis is a very powerful tool for studying gene expression and protein structure/function relationships. A number of oligonucleotide-directed site-directed mutagenesis techniques have been described (2), including PCR-based methods (6,7,15,19) and procedures employing plasmid DNA templates and mutant DNA selection (4,5,12,13). One of the most widely used methods is the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), which consistently produces mutation efficiencies of greater than 80% and uses a simple, one-day protocol. In the QuikChange method, point mutations are introduced by annealing two complementary oligonucleotides to a plasmid DNA template and extending the mutant primers in a linear cyclic amplification reaction with robust, high-fidelity *PfuTurbo*® DNA polymerase (Stratagene) (3,8). Extension products are digested with *Dpn*I to selectively eliminate methylated (parental plasmid) and hemi-methylated (parental/mutant hybrids) DNAs, and upon transformation, the majority of clones contains the desired mutation(s).

Introducing two or more mutations (>5 codons apart; Reference 10) with the QuikChange kit is less than ideal, since two or more primer pairs and rounds of mutagenesis (including DNA preparation and sequencing between rounds) are needed. Quicker methods for introducing multiple mutations simultaneously have been described (10,11,16–18). Although efficient (generally >60%), these procedures exhibit some disadvantages, including laboriousness (10), limitations on the number of sites (two sites; Reference 11) or distance between mutations (≤17 nucleotides shown; References 18), and the requirement for ssDNA templates (17). One multi-site mutagenesis method of particular note was described by Sawano and Miyawaki (16). This modified version of the QuikChange protocol employs one phosphorylated primer per mutation site, and, like other multiple-primer site-directed mutagenesis procedures (1,4,5,17), two or more primers are annealed to the same DNA template strand and incorporated into circular ssDNA with DNA polymerase and ligase. In addition to multi-site mutagenesis, this method has been used with degenerate primers to perform site-directed saturation mutagenesis (16). However, in our hands, this method

Table 1. Mutagenesis Systems

Plasmid	Mutation Primers	Mutation (bp)
	(*lacZ*$^-$→*lacZ*$^+$) (5′→3′)	
pWS-1	QC1 (sense) or QC2 (antisense) QC1: pCCATGATTACGCCAAGCGCG**C**AATTAACCCTCAC QC2: pGTGAGGGTTAATT**G**CGCGCTTGGCGTAATCATGG	792
pWS-2	QC1 + K2 (sense) or QC2 + K1 (antisense) K2: pTCGAGGGGGGGCCCG**GT**ACCCAATTCGCCCTAT K1: pATAGGGCGAATTGGGT**AC**CGGGCCCCCCCTCGA	792, 656/657
pWS-3	QC1 + K2 + H2 (sense) H2: pTCGATATCAAGC**TT**ATCGATACCGTCGACC	792, 656/657, 689
	(*KpnI*$^-$→*KpnI*$^+$) (5′→3′)	
pWS-1, -2, or -3	K2R (sense) K2R: pGCTCACTCATTAGG**T**ACCCCAGGCTTTACA	900
	K3R (sense) K3R: pCTGATTAAGCATTGGTA**C**CTGTCAGACCAAG	1973

Bold text, mutations that convert TAA→wild-type sequences or introduce a *Kpn*I site. Degenerate versions of QC1, K2, and H2 were synthesized with 25% each G, C, A, T (nucleotides 1 and 2) and 50% G and T (nucleotide 3) in the underlined codon.

proved to be less efficient than desired, generating mutation efficiencies of 10.1% (16 mutants/159 total clones; two primers) and 39.5% (215 mutants/544 total clones; three primers) (pWS system in Table 1), and its use in constructing diverse mutant libraries was limited by the low number of transformants produced.

We recently developed a new kit for introducing multiple mutations simultaneously. In the QuikChange Multi Site-Directed Mutagenesis Kit, point mutations are introduced by annealing one or more mutagenic primers to either the sense or antisense strand of a plasmid DNA template (Figure 1). During thermal cycling, the mutagenic primers are extended and nicks are sealed with the QuikChange Multi enzyme blend, containing *PfuTurbo* DNA polymerase and other enzymes. Reaction products are then digested with *Dpn*I and transformed into XL10-Gold ultracompetent cells. Finally, clones are selected and screened by DNA sequencing to identify the desired multiple mutants.

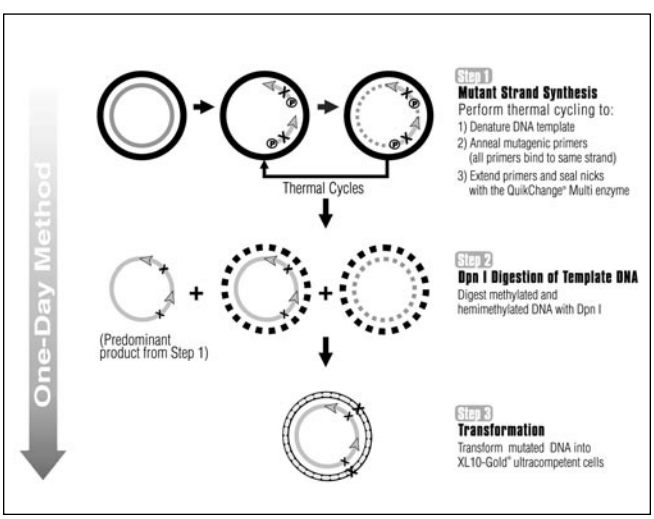

Figure 1. QuikChange Multi site-directed mutagenesis method.

Using one primer per mutation site in the QuikChange Multi kit, instead of two (QuikChange Kit), not only reduces primer costs but allows the use of degenerate primers to construct random amino acid libraries (16). Random mutagenesis methods such as error-prone PCR are designed to introduce single-base mutations; hence, only 5.7 amino acid substitutions on average are accessible from any given amino acid residue (resulting from one-base changes) (14). To access a larger fraction of protein sequence space, site-directed saturation mutagenesis is commonly used to introduce all 20 amino acid side chains at one or more site(s) known to confer the desired phenotype. Randomized amino acid libraries are then screened to identify the amino acid, or combination of amino acids, that provides the greatest improvement in activity. Site-directed saturation mutagenesis typically requires two rounds of PCR (to add degenerate primers and assemble fragments by splice overlap extension), followed by restriction digestion, purification, and ligation of inserts (14). In contrast, incorporating degenerate primers with the QuikChange Multi kit should provide a quicker and easier method for generating site-directed random libraries. Here we evaluate the utility of the QuikChange Multi kit for site-directed saturation mutagenesis by randomizing one, two, or three amino acids in β-galactosidase using degenerate primers.

MATERIALS AND METHODS

Plasmid DNA templates

Plasmid templates were derived from pWhitescript (pWS; 4 kb), the QuikChange kit control (Table 1). pWS-1 contains a stop codon (TAA) mutation in the *lac*Z gene (792 bp), which prevents β-galactosidase synthesis. Plasmids pWS-2 and pWS-3 contain one (mutations at 656 and 657 bp) or two (mutations at 656, 657, and 689 bp) additional stop codons in *lac*Z. Plasmid DNA was purified using the StrataPrep® Plasmid Miniprep kit (Stratagene).

Table 2. Mutation Efficiencies Achieved with the QuikChange Multi Site-Directed Mutagenesis Kit

No.	Primers	No. cfus[c]	Mutation Frequency (%)	Mean Mutation Frequency (%)
1	QC1	23 268[b]	93.1 (± 3.9)[b]	95
	QC2	20 600	97.2	
2	QC1, K2	5660[b]	85.2 (± 6.6)[b]	86
	QC2, K1	10 360	86.8	
3	QC1, K2, H2	2832[b]	51.9 (± 4.4)[b]	67
	QC1, K2, K2R	6880	65.5	
	QC1, K2, K3R	8600	82.2	
4	QC1, K2, H2, K2R	5780	39.0	45
	QC1, K2, H2, K3R	4960	53.0	
	QC1, K2, K2R, K3R[a]	5880	44.1	
5	QC1, K2, H2, K2R, K3R[a]	2820	32.4	32

[a]50 ng each primer/reaction
[b]Results averaged for three independent reactions
[c]Total number of colonies obtained after transforming 1.5 µL each reaction and plating the entire transformation (6% of QuikChange Multi reaction)

Mutagenic Primers

Oligonucleotides were synthesized with a 5′ phosphate moiety (Table 1). Standard primers were synthesized by Genset (La Jolla, CA, USA) and either ethanol-precipitated or PAGE-purified. Degenerate primers were synthesized by TriLink BioTechnologies (San Diego, CA, USA) and PAGE-purified. Degenerate codons (NNG/T) were positioned near the center of each primer and consisted of 25% each G, C, A, and T (nucleotides 1 and 2) and 50% G and T (nucleotide 3).

To determine multi-site mutation frequencies, stop codons were converted to wild-type sequences using sense primers QC1, K2, and H2, while *Kpn*I sites were introduced using sense primers K2R and K3R (Table 1). Successful mutagenesis of one (pWS-1), two (pWS-2), or three (pWS-3) stop codons was monitored by plating transformants on X-gal/IPTG plates and scoring the percentage of blue colonies (*lac*Z+). Incorporation of K2R and K3R primers (in addition to *lac*Z primers) was monitored by determining the percentage of blue clones that contain extra *Kpn*I sites (900 and/or 1973 bp).

Mutagenesis Reaction Conditions

Mutagenesis was performed as described in the QuikChange Multi kit manual (Stratagene). Reactions (25 µL) contained 1× QuikChange Multi buffer, 1× QuikChange Multi dNTPs, 50 ng plasmid DNA, 50 ng (4–5 primers) or 100 ng (1–3 primers) each primer, and 1 µL QuikChange Multi enzyme blend. Reactions were thermocycled and digested with *Dpn*I as described in the kit manual. XL10-Gold® ultracompetent cells (50 µL; Stratagene) were transformed with 1.5 µL each *Dpn*I-digested sample, and *lac*Z+ revertants were scored by blue/white color screening as described in the kit manual. Clones (20–40) were randomly selected from each QuikChange Multi library and sequenced from plasmid DNA using the *lac*Z reverse primer (Sequetech, Mountain View, CA, USA).

RESULTS AND DISCUSSION

Multiple Site Mutagenesis Efficiency

The QuikChange Multi kit was tested with pWS derivatives using 1, 2, 3, 4, or 5 mutagenic primers simultaneously. Primers were 30–34 nucleotides in length, with melting temperatures ranging between 65°C and 80°C (Table 1). Primers H2 and QC1 introduce a single point mutation, while K2 incorporates two point mutations. Depending on the combination employed, primers anneal immediately adjacent (H2 and K2) or close (72–106 nucleotide gaps for H2-QC1, K2-QC1, and QC1-K2R) to each other, or they anneal at least 1 kb apart (1- and 2.7-kb gaps for K3R-K2R and K3R-K2, respectively). As shown in Table 2, greater than 85% of the clones produced from reactions employing one or two primers contained the desired mutations (*lac*Z+). Using three, four, or five primers, incorporation of all mutations was achieved with mean frequencies of 67%, 45%, and 32%, respectively.

The results in Table 2 indicate that primers located immediately adjacent to each other are incorporated nearly as efficiently as primers that anneal farther apart. For example, primer combinations QC1+K2+H2 (0- and 76-nucleotide gaps), QC1+K2+K2R (72- and 106-nucleotide gaps), and QC1+K2+K3R (106- and 1147-nucleotide gaps) produced mutation frequencies of 51.9%, 65.5%, and 82.2%, respectively. In other experiments, primers QC1+K2 (106-nucleotide gap), QC1+K2R (72-nucleotide gap), and QC1+K3R (1147-nucleotide gap) were incorporated with mutation frequencies of 85.2%, 94.6%, and 96.4%, respectively (Table 2 and data not shown). Additional experiments showed that, in general, comparable mutation efficiencies (<10% variation) are obtained with primer sets that anneal to the sense or the antisense strand (Table 2).

In addition to pWS derivatives, the QuikChange Multi kit has been used to eliminate two *Eco*RI sites from a 4.6-kb plasmid construct (two primers; 83% efficiency), introduce

Table 3. QuikChange Multi Libraries

No. Primers	Degenerate Primers	Library Size[a]	Mutation Frequency (% Blue)	Clone Analysis: No. Mutants (Non-TAA Codons)	Clone Analysis: No. Wild-Type (wt)	Clone Analysis: (No. Selected) No. Unique β-Galactosidase Mutants
1	QC1	2.5×10^5	82.5	18 mutants/18 blue colonies	2 wt/	(20) 14:
		9.6×10^4	91.8	(16 unique codons)	2 white	[Q9]-VGDFAYLKEQIYHR
	K2	1.0×10^4	73.7[b]	14 mutants/19 white colonies[c]	5 wt/	(19) 9:
		5.9×10^4	nd	(11 unique codons)	19 white	[V54]-GQLTRHSPY
		4.1×10^4	nd			
	H2	5.2×10^4	nd	—	—	—
		1.1×10^5	nd			
2	QC1, K2	5.3×10^4	81.2	32 double mutants/32 blue	2 wt/	(40) 33 unique double mutants
		2.3×10^4	80.4	colonies (32 unique codon	8 white	[Q9/V54]-IS,TF,GE,SC,YS PR,RD,
		1.2×10^5	80.1	combinations); *of 8 white*		EM,DI,RA,IL,LL,IV,SV,ML,WM,PG,
			(**95.3%** for	*colonies*: 5 single mutants and		RS,SA,CR,LH,LA,VT,YR,IR,PY,GG,
			1–2 mutants)	1 double mutant (inactive?)		FC,IS,LV,LF,FL,WV
3	QC1, K2, H2	2.8×10^4	52.6	20 triple mutants/20 blue colonies	7 wt/	(40) 20 unique triple mutants
		1.6×10^4	46.2	(20 unique codon combinations);	20 white	[Q9/L43/V54]-NTT,CPY,AAS,AVG,
		7.2×10^4	66.5	*of 20 white colonies*: 9 double		TYG,AVL,VMT,VGT,NCS,FSL,SDI,
			(**84.3%** for	and 4 single mutants		HHP,GHA,QGC,RQT,RMI,VHY,IHT,
			1–3 mutants)			DPP,PXM

[a]Results of independent experiments; library size calculated as mean cfus/μL tranformation × 550 μL tranformation × 25 μL reaction/1.5 μL transformed; mean cfus/μL tranformation determined from 2–3 platings/transformation

[b]Determined by DNA sequencing

[c]Blue/white color screening not applicable to K2/pWS-1 system

nd, not determined

two point mutations in a DNA polymerase gene (5.2-kb plasmid; two primers; 50% efficiency), and create five point mutations simultaneously in a gene encoding GFP (4.3-kb plasmid; four primers; 10% efficiency) (data not shown). In the DNA polymerase and GFP studies, there were no unintended mutations identified in any of the clones sequenced. Additionally, successful mutagenesis of larger plasmid templates (5.7-kb versions of pWS-1, -2, and -3) has also been demonstrated (data not shown).

Site-Directed Saturation Mutagenesis

The QuikChange Multi enzyme blend, reaction buffer, and cycling conditions have been specifically optimized to maximize the number of transformants produced. As shown in Table 2, approximately 300 000 colonies is generated in reactions employing one primer, while a total of 40 000–170 000 colonies is produced in reactions employing two or three primers (total theoretically possible from 25-μL reactions, assuming multiple transformations of 1.5-μL portions). When preparing specific mutants, obtaining greater than 10–20 transformants is generally unnecessary. However, high yields are desired when constructing mutant libraries, since the number of clones needed to ensure adequate representation increases exponentially with the number of amino acids randomized.

In these studies, each amino acid was randomized by incorporating a primer with a degenerate NNG/T codon (N = 25% each GCTA; G/T = 50% GT) using the QuikChange Multi kit protocol. The minimum number of clones containing all possible single mutants is dictated by the frequency of the least represented mutants (e.g., encoded by only one codon; N, D, C, E, Q, H, I, K, M, F, W, Y, and W mutants for NNG/T codons), the efficiency of QuikChange Multi mutagenesis, and the statistical distribution of mutation types. For NNG/T codons, the frequency of the least-represented mutants can be calculated as (¼ × ¼ × ½) = 1/32. Assuming 100% mutation efficiency, there is a greater than 95% likelihood of observing all possible mutants in a random sampling of approximately 100 clones [0.95 = 1-(1-f)^n, where f = frequency of the least represented mutants and n = number of clones screened;

Reference 9]. When incorporating two random NNG/T codons, the frequency of the least represented double mutants is 1/1024 [(¼ × ¼ × ½)^2]; to ensure representation of all possible double mutants, approximately 3100 clones should be screened (>95% confidence). Similarly, when randomizing three amino acids, there is a greater than 95% likelihood of observing all possible triple mutants in a random sampling of approximately 10^5 clones (f = 1/32 768).

The QuikChange Multi kit was evaluated for performing site-directed saturation mutagenesis. The kit protocol was used with one, two, or three degenerate primers to randomize amino acids 9, 45, and/or 54 in β-galactosidase. Stop codons in pWS-1, -2, and -3 were randomly mutated using degenerate versions of the QC1, K2, and H2 primers, containing one random codon per primer (TAA→NNG/T) (Table 1). Incorporation of degenerate primers was monitored by blue/white color selection, where the percentage of blue colonies equals the frequency of incorporating a functional (*lac*Z$^+$) mutation and the percentage of white colonies equals the frequency of incorporating nonfunctional (*lac*Z$^-$) mutations plus background (% parental clones). As shown below, the percentage of blue colonies was very close to true mutation frequency, since the majority of *lac*Z mutants retained β-galactosidase activity (highly substitutable sites).

As summarized in Table 3, the mean number of transformants recovered using one, two, or three degenerate primers was 8.8×10^4 (three different primers), 6.5×10^4, or 3.9×10^4 colonies/25 μL reaction, respectively. Therefore, sufficient numbers of transformants can be obtained to ensure representation of all possible single and double mutants (approximately 10^2 and 3×10^3 clones required for 95% confidence). Libraries created to randomize three amino acids should contain many, but not all, triple mutants (need to screen 1.8×10^5 for 95% confidence, assuming 55.1% triple-mutation frequency). As shown in Table 3, the frequency of parental clones in one-, two-, and three-primer reactions was 13%–26%, 5%, and 16%, respectively.

In libraries constructed to randomize amino acid 9 (degenerate QC1), 87% of the clones were *lac*Z$^+$ mutants (% blues), and 14 different β-galactosidase mutants were represented among 20 clones randomly selected. Randomizing amino acid 54 (degenerate K2) produced a mutation frequency of 74%, and nine unique β-galactosidase variants were identified among 19 randomly selected clones. When amino acids 9 and 54 were randomized simultaneously (degenerate QC1 + degenerate K2), 95% of the 40 randomly selected clones contained at least one mutation, while 80% of the clones contained mutations at both sites. Among the 40 clones sequenced, 33 different double mutants and five different single mutants were recovered. The β-galactosidase double mutants represented random combinations of 15 different amino acids at each position (Table 3). When amino acids 9, 43, and 54 were randomized simultaneously (degenerate QC1 + degenerate K2 + degenerate H2), 84% of the 40 randomly se-

lected clones contained at least one mutation, while 55% of the clones on average contained mutations at all three sites. Of the 40 clones sequenced, 20 unique triple mutants were isolated (created by random combinations of 10–13 different amino acids at each position), in addition to nine different double mutants and four different single mutants.

CONCLUSIONS

The QuikChange Multi kit provides a quick and efficient method for carrying out site-directed mutagenesis at up to five sites simultaneously. In this study, mutation efficiencies ranging from 32% to 95% were obtained using 1–5 mutagenic primers with the 4-kb pWS vector. Mutation efficiencies achieved with other primer/template systems may vary depending on plasmid DNA size and primer/template sequence complexity (GC content, secondary structure, repeat sequences, etc.).

Lower mutation efficiencies obtained when constructing multi-site versus single-site mutants reflects the multiplicative effects of incorporating primers, which are introduced separately with less than 100% efficiency. Lower multi-site efficiency (e.g., 32%–45% for 4–5 primers vs. 93% for one primer) simply means that higher numbers of clones must be sequenced to ensure the identification of the desired multiple mutant. Certainly, the time saved by incorporating multiple mutations simultaneously more than makes up for the increased DNA sequencing requirement. For example, mutations can be introduced (and sequence verified) at 4–5 sites simultaneously in about three days with the QuikChange Multi kit, compared to 12–15 days with the original QuikChange kit. Sequencing 5–10 clones per reaction should be sufficient to identify clones containing all of the desired mutations.

In addition to multi-site mutagenesis, the QuikChange Multi kit can be readily applied to the construction of site-directed random libraries, with no modifications to the kit protocol. Site-specific saturation mutagenesis can be performed in a single day, and no additional steps are required to randomize two or three amino acids simultaneously. The QuikChange Multi method for constructing degenerate-primer libraries is significantly quicker and less laborious compared to conventional PCR/ligation-based techniques. In this report, diverse collections ($1–25 \times 10^4$ clones) of β-galactosidase mutants were created by randomly combining mutations at up to three different sites. QuikChange Multi mutant collections can be analyzed by DNA sequencing, to identify a panel of unique mutants, or by activity screening for altered function. The speed and simplicity of this saturation mutagenesis method should greatly facilitate comprehensive structure-function analyses and protein engineering efforts.

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