QuikChange® Multi Site-Directed Mutagenesis Kit

INSTRUCTION MANUAL

Catalog #200514

Revision #113002e

For In Vitro Use Only

200514-12 113002E/

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QuikChange® Multi Site-Directed Mutagenesis Kit

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QuikChange® Multi Site-Directed Mutagenesis Kit

MATERIALS PROVIDED

Materials provided[a]	Quantity
QuikChange® Multi enzyme blend (2.5 U/μl)	80 U
10× QuikChange® Multi reaction buffer[b,c]	200 μl
QuikSolution	500 μl
dNTP Mix[b,c]	30 μl
Dpn I restriction enzyme (10 U/μl)	300 U
QuikChange® Multi control template (50 ng/ μl)	5 μl
QuikChange® Multi control primer mix (100 ng/μl of each of three primers)	5 μl
XL10-Gold® ultracompetent cells[d]	10 × 135 μl
XL10-Gold® β-mercaptoethanol mix	2 ×50 μl
pUC18 control plasmid (0.1 ng/μl in TE buffer[e])	10 μl

[a] Kits contain enough control template and primer mix for 5 control reactions, and enough reagents for 30 reactions total (control and experimental reactions combined).

[b] The reaction buffer and dNTP mix have been optimized for the QuikChange Multi site-directed mutagenesis protocols. Do not substitute with buffers or dNTP mixes provided with other Stratagene kits.

[c] Thaw the dNTP mix and reaction buffer 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}*Z*Δ*M15* Tn*10* (Tet^R) Amy Cam^R]

[e] See *Preparation of Media and Reagents*.

STORAGE CONDITIONS

XL10-Gold® Ultracompetent Cells and pUC18 Control Plasmid: –80°C
All Other Components: –20°C

ADDITIONAL MATERIALS REQUIRED

Falcon® 2059 polypropylene tubes (15 ml)
5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal)
Isopropyl-1-thio-β-D-galactopyranoside (IPTG)

NOTICE TO PURCHASER

Use of the QuikChange® Multi Site-Directed Mutagenesis Kit, catalog #200514, by commercial entities requires a commercial license from Stratagene.

The QuikChange® Multi Site-Directed Mutagenesis Kit, catalog #200513, is presently offered for sale to commercial entities with a limited use license.

Revision #113002e

INTRODUCTION

The QuikChange® Multi site-directed mutagenesis kit* offers a rapid and reliable method for site-directed mutagenesis of plasmid DNA at up to five different sites simultaneously. A single mutagenic oligonucleotide is required to mutagenize each site, using a double-stranded DNA template and following a one-day, three-step procedure.

Site-Directed Mutagenesis Overview

In vitro site-directed mutagenesis is an invaluable technique for studying gene and protein structure-function relationships and for modifying vector sequences to facilitate cloning and expression strategies. Several approaches to this technique have been published, but these methods generally require the use of single-stranded DNA (ss-DNA) template,[1–4] generally allow mutagenesis at only one site per round, and are labor intensive and technically difficult. Stratagene's original QuikChange site-directed mutagenesis kit** eliminated the need for subcloning into M13-based bacteriophage vectors and for ss-DNA rescue, making site directed mutagenesis studies simple and reliable, allowing oligo-mediated introduction of site-specific mutations into virtually any double-stranded plasmid DNA.[5]

Novel Technology of the QuikChange® Multi System Allows for Complex Protein Function Analysis

The QuikChange Multi site-directed mutagenesis system offers the same benefits of speed, simplicity and reliability afforded by the original QuikChange kit, but is based on a completely novel technology with distinct advantages. The novel technology of the QuikChange Multi system allows mutagenesis at multiple sites in a single round, using a single oligonucleotide per site. The QuikChange Multi site-directed mutagenesis system also makes it easy to randomize key amino acids using oligos containing degenerate codons. No specialized vectors or unique restriction sites are needed to use the QuikChange Multi kit—virtually any plasmid of up to 8 kb is a suitable template. The rapid three-step procedure introduces mutations at three different sites simultaneously in the 4-kb QuikChange Multi control plasmid with greater than 50% efficiency.

Engineered Mutant Collections Applications

The QuikChange Multi side-directed mutagenesis system is well suited for constructing diverse Engineered mutant clone™ collections from random combinations of point mutations or by performing site-specific saturation mutagenesis using degenerate codons. This allows for detailed protein structure-function analysis. Using one degenerate codon-containing primer per site, the system is useful for creating mutant collections containing all possible amino acid side chains at one site as well as combinations of different amino acids at multiple sites. A collection of mutants can be created in a single QuikChange Multi kit reaction and then assayed for mutants with improved activity using any appropriate functional screen for

* Patent pending.

** U.S. Patent Nos. 5,789,166, 5,923,419, 6,391,548 and patents pending.

your gene. The QuikChange Multi kit overcomes issues associated with mutagenesis methods that employ mutagenic primers corresponding to both strands of DNA, where representation of mutants in the collection may be limited by the preferential binding of complementary strands of mutagenic primers to each other.

Outline of the Three-Step Protocol

The three-step QuikChange Multi site-directed mutagenesis method is outlined in Figure 1. Step 1 uses a thermal cycling procedure to achieve multiple rounds of mutant strand synthesis. Components of the thermal cycling reaction include a supercoiled double-stranded DNA template, two or more synthetic phosphorylated oligonucleotide primers containing the desired mutations, and the kit-provided enzyme blend featuring *PfuTurbo*® DNA polymerase.[6***] First the mutagenic primers are annealed to denatured template DNA. (Note that all oligonucleotides are designed to bind the same strand of the template DNA.) *PfuTurbo* DNA polymerase then extends the mutagenic primers with high fidelity[||] and without primer displacement, generating ds-DNA molecules with one strand bearing multiple mutations and containing nicks. The nicks are sealed by components in the enzyme blend.

In Step 2 of the procedure, the thermal cycling reaction products are treated with the restriction endonuclease *Dpn* I. The *Dpn* I endonuclease (target sequence: 5´-Gm^{6}ATC-3´) is specific for methylated and hemimethylated DNA[7] and is used to digest the parental DNA template. DNA isolated from almost all *Escherichia coli* strains is dam methylated and therefore susceptible to digestion.

In Step 3, the reaction mixture, enriched for multiply mutated single stranded DNA, is transformed into XL10-Gold® ultracompetent cells, where the mutant closed circle ss-DNA is converted into duplex form in vivo. Double stranded plasmid DNA may then be prepared from the transformants and analyzed by appropriate methods to identify clones bearing each of the desired mutations.

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.*

***U.S. Patent Nos. 6,183,997, 6,333,165, 6,379,553, 5,948,663, 5,866,395, 5,545,552 and patents pending.

|| *PfuTurbo* DNA polymerase has 6-fold higher fidelity in DNA synthesis than *Taq* DNA polymerase.

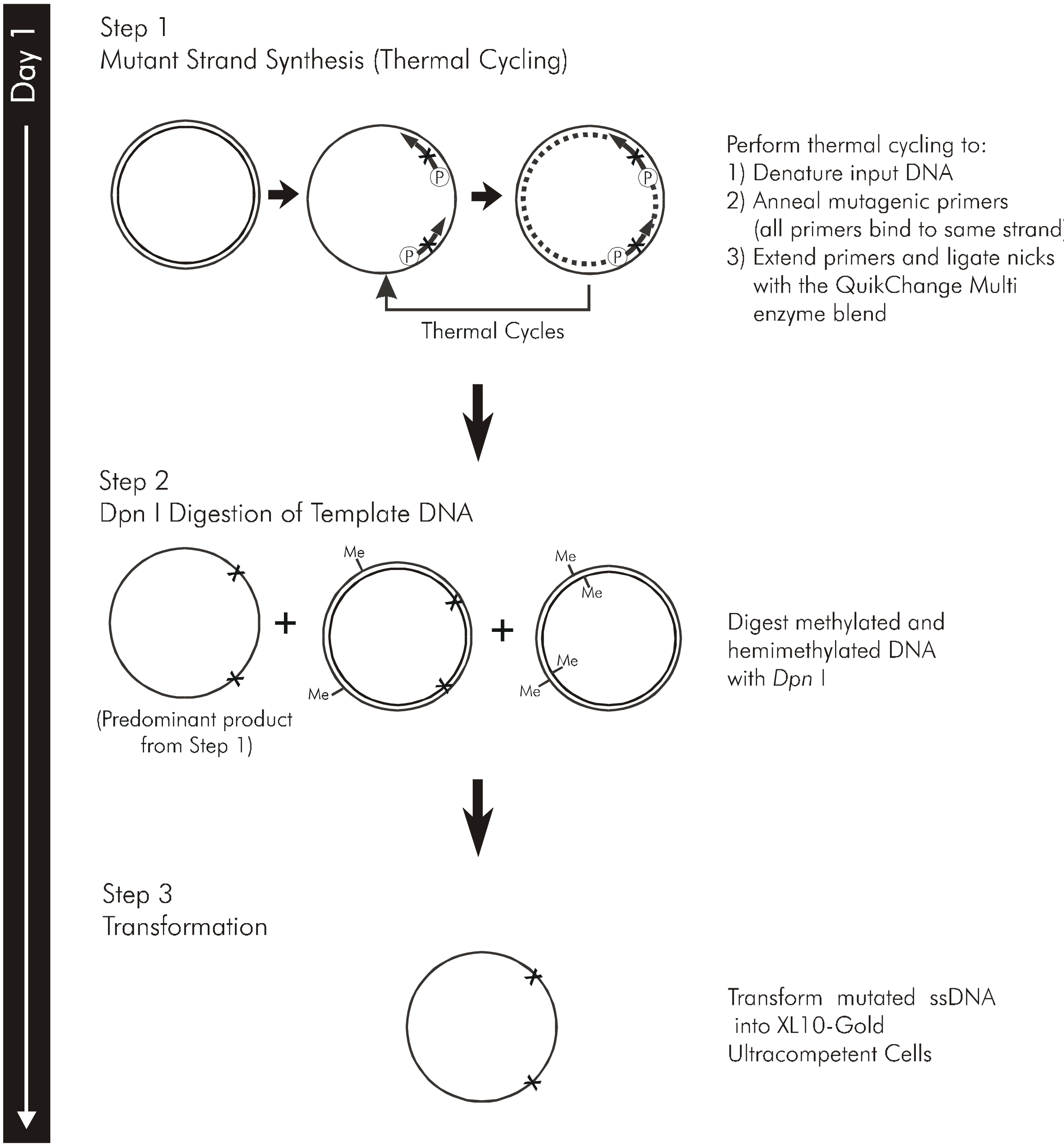

FIGURE 1 Overview of the QuikChange Multi Site-Directed Mutagenesis method.

QUIKCHANGE® MULTI MUTAGENESIS CONTROL

The QuikChange Multi control template and control primer mix are used to test the efficiency of simultaneous site-directed mutagenesis at three independent sites. The 4-kb control template is derived from the pBluescript® II SK(–) phagemid that encodes the first 146 amino acids of β-galactosidase (encoded by the *LacZ* gene). The *LacZ* gene product is responsible for the production of blue colonies when appropriate *E. coli* transformants are grown on media containing X-gal (a chromogenic substrate of β-galactosidase) in the presence of the inducer IPTG. The QuikChange Multi control template was modified to contain stop codons at three positions in the *LacZ* coding sequence (see Figure 2). XL10-Gold cells transformed with the control template appear white on LB–ampicillin agar plates containing IPTG and X-gal, because each of the three mutations prevents the production of active β-galactosidase. The QuikChange Multi control primer mix contains three primers, each of which reverts one of these stop codons to the codon found in the original *LacZ* gene from pBluescript II SK(–) (see Figure 2). Restoration of active β-galactosidase requires that all three reversion events occur in the same molecule. Following transformation, colonies can be scored for the β-galactosidase (β-gal⁺, blue) phenotype, where a blue colony indicates the production of a triple-mutant plasmid.

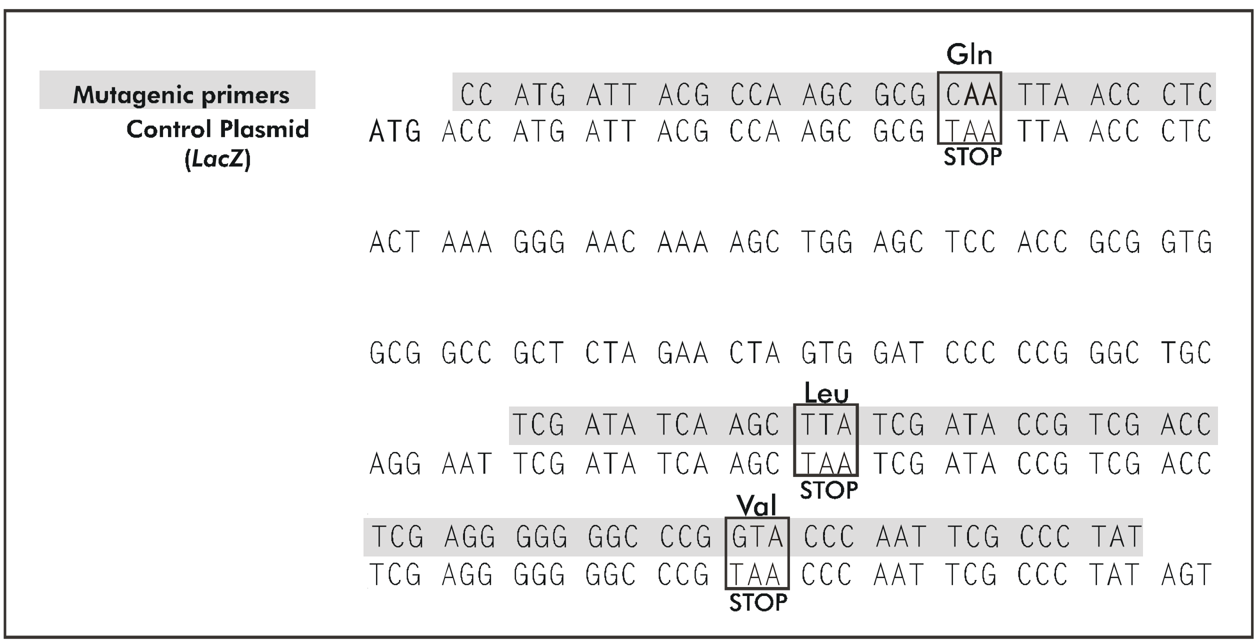

FIGURE 2 QuikChange Multi control plasmid and control primer sequences. A partial sequence of the *LacZ* coding region is shown, beginning with the ATG used for translation initiation. The QuikChange Multi control primer sequences are shown above their corresponding sites in the control plasmid, with the stop codons reverted by the control mutagenesis reaction boxed.

MUTAGENIC PRIMER DESIGN

Notes *Stratagene has developed a web-based primer design software program specifically for designing optimal mutagenic primers for use with the QuikChange Multi site-directed mutagenesis kit. This program incorporates the design guidelines listed below, as well as additional primer design parameters involving free energy, mismatches, and codon replacement strategy. Go to* ***http://labtools.stratagene.com/QC*** *to design template-specific mutagenic primers using this program.*

When creating Engineered mutant clone collections, see Appendix: General Guidelines for Creating Engineered Mutant Clone™ Collections *for additional mutagenic primer design suggestions.*

The mutagenic oligonucleotide primers must be designed individually to incorporate the desired point mutation or degenerate codon. The following considerations should be made when designing mutagenic primers:

1. Primers should be 5´-phosphorylated for maximum mutagenesis efficiency.

2. All of the primers used for simultaneous mutagenesis must anneal to the same strand of the template plasmid. In most cases, primers binding to either strand will be incorporated into mutant plasmids with equal efficiency. However, certain secondary structures or features may influence the efficiency of the mutagenesis reaction. If a low mutagenesis efficiency (<30%) is observed, synthesize primers corresponding to the opposite strand of the plasmid.

3. Primers may be designed to bind to adjacent sequences or to well-separated regions on the same strand of the template plasmid. Stratagene has not observed any primer spacing-dependent effects on multiple mutagenesis efficiency.

4. Primers should be between 25 and 45 bases in length, with a melting temperature (T_m) of ≥75°C. Primers longer than 45 bases may be used, but using longer primers increases the likelihood of secondary structure formation, which may affect the efficiency of the mutagenesis reaction. Optimum primer sets for simultaneous mutagenesis should have similar melting temperatures. The following formula is commonly used for estimating the T_m of primers:

   $$T_m = 81.5 + 0.41(\%GC) - 675/N - \%\text{ mismatch}$$

   For calculating T_m:

   - N is the primer length in bases
   - values for **%GC** and **% mismatch** are whole numbers

5. Primers for simultaneous mutagenesis should be added to the mutagenesis reaction in approximately equimolar amounts. The mutagenesis reaction protocol in *Protocols* assumes that all primers used together are of similar length. If primers are >20% different in length, amounts of each primer added should be adjusted accordingly. For example, if primer 1 is 25 bases and primer 2 is 35 bases (a length ratio of 1:1.4), use 100 ng of primer 1 and 140 ng of primer 2.

6. The desired point mutation or degenerate codon should be close to the middle of the primer with ~10–15 bases of template-complementary sequence on both sides.

7. Optimum primers have a minimum GC content of 40% and terminate in one or more C or G bases at the 3'-end.

8. PAGE purification of primers is not necessary in all cases; however, incomplete primer synthesis may result in a low mutagenesis efficiency and PAGE purification of the full-length primers may be beneficial.

PROTOCOLS

Note *For suggestions on adapting the QuikChange Multi kit to create Engineered mutant clone collections, see* Appendix: General Guidelines for Creating Engineered Mutant Clone™ Collections.

Mutant Strand Synthesis Reaction (Thermal Cycling)

Notes *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, ***Stratagene strongly recommends using thin-walled tubes****, which ensure ideal contact with the temperature cycler's heat blocks. The following protocols were optimized using thin-walled tubes.*

1. Prepare the ds-DNA template either by standard miniprep protocols (e.g. Stratagene's StrataPrep® Plasmid Miniprep Kit, catalog #400761) or by cesium chloride gradient purification.

2. Prepare the mutant strand synthesis reactions for thermal cycling as indicated in the tables below, according to the template size and number of mutagenic primers. Add the components in the order listed then mix gently by pipetting or tapping the reaction tube.

Mutagenesis Reactions

	Template Size	
Component	**≤ 5 kb**	**> 5 kb**
10× QuikChange® Multi reaction buffer	2.5 μl	2.5 μl
Double-distilled H_2O to a final volume of 25 μl	X μl	X μl
QuikSolution	—	0.75 μl
ds-DNA template	X μl (50 ng)	X μl (100 ng)
Mutagenic primers: If using 1–3 primers, add 100 ng of each primer* If using 4–5 primers, add 50 ng of each primer*	X μl	X μl
dNTP mix	1 μl	1 μl
QuikChange® Multi enzyme blend	1 μl	1 μl

* Mutagenic primers should be added in approximately equimolar amounts. If primer lengths differ significantly (>20% difference), adjust the amounts of individual primers accordingly.

Control Reaction

Component	Volume
10× QuikChange® Multi reaction buffer	2.5 µl
Double-distilled H_20	18.5 µl
QuikChange® Multi control template (50 ng/µl)	1 µl
QuikChange® Multi control primer mix (100 ng/µl for each primer)	1 µl
dNTP mix	1 µl
QuikChange® Multi enzyme blend	1 µl

3. If the thermal cycler to be used does not have a hot-top assembly, overlay each reaction with ~30 µl of mineral oil.

4. Cycle the reactions using the cycling parameters outlined in the table below. (For the control reaction, use an 8-minute extension time.)

Cycling Parameters

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	30	95°C	1 minute
		55°C	1 minute
		65°C	2 minutes/kb of plasmid length

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

Dpn I Digestion of the Amplification Products

Note *It is important to insert the pipet tip below the mineral oil overlay when adding the* Dpn *I restriction enzyme to the reaction tubes during the digestion step or when transferring the 1.5 µl of the* Dpn *I-treated DNA to the transformation reaction. Stratagene suggests using specialized aerosol-resistant pipet tips, which are small and pointed, to facilitate this process.*

1. Add 1 µl of *Dpn* I restriction enzyme (10 U/µl) directly to each amplification reaction below the mineral oil overlay 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, then immediately incubate each reaction at 37°C for 1 hour to digest the parental (nonmutated) ds-DNA.

Transformation of XL10-Gold® Ultracompetent Cells

Note *Please see* Transformation Guidelines *for detailed information about parameters that affect transformation of XL10-Gold ultracompetent cells.*

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

1. Gently thaw the XL10-Gold ultracompetent cells on ice. For each mutagenesis reaction to be transformed, aliquot 45 μl of the ultracompetent cells to a *prechilled* Falcon® 2059 polypropylene tube.

2. Add 2 μl of the β-ME mix provided with the kit to the 45 μl of cells. (Stratagene cannot guarantee highest efficiencies with β-ME from other sources.)

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 mutagenesis reaction to a separate aliquot of the ultracompetent cells.

   Note *Carefully remove any residual mineral oil from the pipet tip before transferring the* Dpn *I-treated DNA to each reaction.*

Optional *Verify the transformation efficiency of the XL10-Gold ultracompetent cells by adding 1 μl of 0.01 ng/μl pUC18 control plasmid (dilute the provided pUC18 DNA 1:10) to another 45-μl aliquot of cells.*

5. Swirl the transformation reactions gently to mix, then incubate the reactions on ice for 30 minutes.

6. Preheat NZY$^+$ broth (see *Preparation of Media and Reagents*) in a 42°C water bath for use in step 9.

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

7. 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 Falcon 2059 polypropylene tubes.*

8. Incubate the tubes on ice for 2 minutes.

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

10. 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 (see *Preparation of Media and Reagents*) that have been prepared with 80 μg/ml X-gal and 20 mM IPTG (see *Preparing the Agar Plates for Color Screening*).

Transformation Reaction Plating Volumes

Reaction Type	Volume to Plate*
Experimental mutagenesis	1 μl, 10 μl, and 100 μl
Mutagenesis control	10 μl
Transformation control (pUC 18)	5 μl

* When plating less than 100 μl from the transformation reaction, place a 100-μl pool of NZY^+ broth on the agar plate, pipet the cells into the pool, then spread the mixture.

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

Expected Results for the Control Transformations

The expected colony number from the mutagenesis control transformation is between 50 and 800 colonies. Greater than 50% of colonies from the control mutagenesis transformation should contain all three mutations and appear as blue colonies on agar plates containing IPTG and X-gal.

Note *The mutagenesis efficiency (ME) for the 4-kb QuikChange Multi control plasmid is calculated by the following formula:*

$$ME = \frac{\text{Number of blue colony forming units (cfu)}}{\text{Total number of colony forming units (cfu)}} \times 100\%$$

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

Expected Results for Transformation of the Experimental Mutagenesis

Expect between 10 and 1000 colonies, depending upon the number and nature of primers and the length and base composition of the DNA template used. For suggestions on increasing the colony number or mutagenesis efficiency, see *Troubleshooting*.

XL10-GOLD® ULTRACOMPETENT CELLS

XL10-Gold ultracompetent cells feature the Hte phenotype, exhibiting increased transformation efficiencies with ligated DNA and large DNA molecules compared to most *E. coli* host strains.[8] XL10-Gold cells are both endonuclease deficient (*endA1*) and recombination deficient (*recA*). The *endA1* mutation greatly improves the quality of plasmid miniprep DNA,[9] and the *recA* mutation helps ensure insert stability. XL10-Gold cells grow faster than XL1- or XL2-Blue cells, resulting in larger colonies.

Host strain	References	Genotype
XL10-Gold Ultracompetent Cells	8, 10, 11	Tet^R Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173* *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*^{q}*Z*Δ*M15* Tn*10* (Tet^R) Amy Cam^R]^a

[a] Chloramphenicol resistant (Cam^R) at concentrations of <40 µg/ml, but chloramphenicol sensitive (Cam^s) at concentrations of 100 µg/ml.

TRANSFORMATION GUIDELINES

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.

Storage Conditions

The XL10-Gold ultracompetent cells are very 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. The XL10-Gold ultracompetent cells should be placed at –80°C directly from the dry ice shipping container.

Aliquoting Cells

When aliquoting, keep the XL10-Gold ultracompetent cells on ice at all times. It is essential that the Falcon 2059 polypropylene tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into the prechilled tubes.

Use of Falcon® 2059 Polypropylene Tubes

It is important that Falcon 2059 polypropylene tubes are used for the transformation protocol, since other tubes may be degraded by the β-mercaptoethanol during transformation. In addition, the duration of the heat-pulse step is critical and has been optimized specifically for the thickness and shape of the Falcon 2059 polypropylene tubes.

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 incubating for <30 seconds or for >40 seconds. Do not exceed 42°C.

Preparing 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-inodlyl-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 (see *Preparation of Media and Reagents*) 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 the X-gal before pipetting them onto the plates because these chemicals may precipitate.

APPENDIX: GENERAL GUIDELINES FOR CREATING ENGINEERED MUTANT CLONE™ COLLECTIONS

Suggestions for Optimizing Degenerate Primer Design

When designing primers containing degenerate codons, in addition to following the primer design guidelines described in *Mutagenic Primer Design*, it is important that the T_m's of both primer sequences flanking the degenerate codon match. If one side has a lower T_m due to a lower GC content, extend the primer length to increase the T_m. The lengths of both sides can be slightly unequal if the difference in lengths does not exceed approximately 5 nucleotides.

Suggestions for Increasing Collection Representation and Size

In order to identify the best amino acid substitution or combination of point mutations or amino acid substitutions for a desired protein function, a mutant clone collection must contain all possible permutations. The QuikChange Multi kit can be adapted to increase mutant collection representation and size by applying the suggestions listed in the table below.

QuikChange® Multi kit condition	Standard reaction specification	Suggested reaction specification for generating mutant clone collections
Mutagenesis reaction volume	25 μl	50–100 μl
QuikSolution™ reagent concentration	3%	4–10%
DNA template amount	50–100 ng	300 ng
Dpn I digestion time	1 hour	2–3 hours
Competent cell type	XL10-Gold® ultracompetent cells	Electroporation competent cells [e.g., Stratagene's ElectroTen-Blue® electroporation competent cells (Catalog #200159)][a,b]

[a] The ElectroTen-Blue electroporation competent cells are specifically designed for transforming large, ligated DNA. Therefore, Stratagene recommends using ElectroTen-Blue competent cells for the transformation in order to increase mutant collection size and representation.

[b] Stratagene recommends adding a spin column purification step [e.g., Stratagene's StrataPrep® PCR purification kit (Catalog #400771 or 400773)] to remove excess salt from the DNA prior to transformation, as electroporation competent cells can be sensitive to salts.

Suggestions for Adjusting Individual Site Mutation Efficiencies

It is possible to adjust the mutation efficiency at each site by varying primer concentrations. The appropriate amounts of each primer required to achieve the desired mutation efficiencies need to be determined empirically for each primer-template system. Stratagene recommends starting with 100 ng of each primer for 1–3 primers or 50 ng of each primer for 4–7 primers, and varying the amount from 2- to 10-fold depending on the system and the desired result.

TROUBLESHOOTING

When used according to the guidelines outlined in this instruction manual, Stratagene's kit will provide a reliable means to conduct site-directed mutagenesis at multiple sites using ds-DNA templates. Variations in the base composition and length of primers, in length of the DNA template and in the thermal cycler may contribute to differences in mutagenesis efficiency. Refer to the following guidelines for troubleshooting the recovery of fewer than expected mutagenized plasmids.

Observation	Suggestion(s)
Low transformation efficiency or low colony number	Ensure that sufficient DNA template is used in the mutagenesis reaction. Visualize the DNA template on a gel to verify the quantity and quality
	Ensure that competent cells are stored at the bottom of a –80°C freezer immediately upon arrival (see also *Transformation Guidelines*). Test the efficiency of the competent cells using the pUC18 control plasmid
	Ensure that mineral oil is not transferred into the transformation reaction when pipetting the *Dpn* I-treated DNA. Using the smallest pipet tips available, insert the pipet tip completely below the mineral oil overlay and clear the pipet tip while it is submerged beneath the overlay before collecting the sample
	Increase the amount of the *Dpn* I-treated DNA used in the transformation reaction to 4 μl. Alternatively, ethanol precipitate the *Dpn* I digestion reaction, then resuspend and transform with the entire sample
Low mutagenesis efficiency or low colony number with the control reaction	Different thermal cyclers may vary in ramping efficiencies. Optimize the cycling parameters for the control reaction, then repeat the protocol for the mutagenesis reactions
	Verify that the agar plates were prepared correctly. See *Preparing the Agar Plates for Color Screening*, and follow the recommendations for IPTG and X-Gal concentrations carefully
	For best visualization of the blue (β-gal$^+$) phenotype, the control plates must be incubated for at least 16 hours at 37°C
	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
Low mutagenesis efficiency with the experimental mutagenesis reaction(s)	Add the *Dpn* I restriction enzyme below the mineral oil overlay and ensure proper mixing of all components (especially the *Dpn* I) in the reaction
	Allow sufficient time for the *Dpn* I to completely digest the parental template; repeat the digestion if too much DNA template was present
	Use 5′-phosphorylated primers for maximum mutagenesis efficiency
	Titrate the amounts of primer and template added to the thermal cycling reaction
	Verify that the template DNA was isolated from a *dam*$^+$ *E. coli* strain. Plasmid DNA isolated from *dam*$^-$ strains, such as JM110 or SCS100, lacks methylation and is not digested by *Dpn* I
	If mutagenesis efficiency remains low (<30%) after addressing other possibilities, redesign mutagenic primers to bind to the opposite strand of the plasmid
Low colony number or low mutagenesis efficiency for long templates (>5 kb)	Titrate the amount of QuikSolution added from 0 to 1.5 μl
	Mutagenesis of long templates may require optimization of the amounts of template and primers added to the thermal cycling reaction
	Transformation efficiency will decrease as plasmid size increases. Transform XL10-Gold Ultracompetent Cells with up to 4μl of the mutagenesis reaction, and plate a larger proportion of the transformation reaction

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)	**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)
LB–Ampicillin Agar (per Liter) 1 liter of LB agar, autoclaved Cool to 55°C Add 10 ml of 10-mg/ml filter-sterilized ampicillin Pour into petri dishes (~25 ml/100-mm plate)	**TE Buffer** 10 mM Tris-HCl (pH 7.5) 1 mM EDTA
10 mM IPTG (per 10 ml) 24 mg of isopropyl-1-thio-β-D-galactopyranoside (IPTG) 10 ml of sterile dH_2O Store at –20°C Spread 100 µl per LB-agar plate	**2% X-Gal (per 10 ml)** 0.2 g of 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside (X-Gal) 10 ml of dimethylformamide (DMF) Store at –20°C Spread 100 µl per LB-agar plate

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ENDNOTES

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MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on Stratagene's website 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.

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QuikChange® Multi Site-Directed Mutagenesis Kit

Catalog #200514

QUICK-REFERENCE PROTOCOL

- Prepare mutant strand synthesis reaction(s) in thin-walled tubes as indicated below:

Control Reaction

2.5 μl 10× Reaction Buffer
1 μl control template
1 μl control primer mix
1 μl dNTP mix
1 μl QuikChange Multi enzyme blend
18.5 μl ddH$_2$O

Mutagenesis Reactions

2.5 μl 10× Reaction Buffer
X μl ds-DNA template
(50ng for ≤5 kb, or
100 ng for >5 kb*)
X μl each primer
(100 ng each for 1–3 primers or
50 ng each for 4–5 primers)
1 μl dNTP mix
1 μl QuikChange Multi enzyme blend
ddH$_2$O to a final volume of 25 μl

*For templates >5 kb, also add
0.75 μl QuikSolution to the reaction

- If the thermal cycler to be used does not have a hot-top assembly, overlay each reaction with ~30 μl of mineral oil
- Cycle each reaction using the cycling parameters outlined in the following table:

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	30	95°C	1 minute
		55°C	1 minute
		65°C	2 minutes/kb of plasmid length

- Add 1 μl of *Dpn* I restriction enzyme below the mineral oil overlay (if used)
- Gently and thoroughly mix each reaction, spin down in a microcentrifuge for 1 minute, and immediately incubate at 37°C for 1 hour to digest ds-DNA containing parental strands
- Transform 1.5 μl of the *Dpn* I-treated DNA from each reaction into separate 45-μl aliquots of XL10-Gold ultracompetent cells (see *Transformation of XL10-Gold Ultracompetent Cells* in the instruction manual)