

QuikChange Lightning Multi Site-Directed Mutagenesis Kit

Instruction Manual

Catalog # 210513 and #210515

Revision D1

For Research Use Only. Not for use in diagnostic procedures.

210513-12



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QuikChange Lightning Multi Site-Directed Mutagenesis Kit

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QuikChange Lightning Multi Site-Directed Mutagenesis Kit

MATERIALS PROVIDED

Materials provided	Quantity	
	Catalog #210513 ^a	Catalog #210515 ^b
QuikChange Lightning Multi enzyme blend	30 reactions	10 reactions
10× QuikChange Lightning Multi buffer	200 µl	200 µl
dNTP Mix ^{c,d}	30 µl	10 µl
<i>Dpn</i> I restriction enzyme ^d	30 reactions	10 reactions
QuikSolution	500 µl	500 µl
QuikChange Multi control template (50 ng/ µl)	5 µl	5 µl
QuikChange Multi control primer mix (100 ng/µl of each of three primers)	5 µl	5 µl
XL10-Gold ultracompetent cells ^e	10 × 135 µl	4 × 135 µl
XL10-Gold β-mercaptoethanol mix	2 × 50 µl	50 µl
pUC18 control plasmid (0.1 ng/µl in TE buffer ^f)	10 µl	10 µl

- ^a The QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Catalog #210513) contains enough reagents for 30 reactions total (control and experimental reactions combined).
- ^b The QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Catalog #210515) contains enough reagents for 10 reactions total (control and experimental reactions combined).
- ^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 **The composition of the dNTP mix and *Dpn* I enzyme are proprietary.** These reagents have been optimized for the QuikChange Lightning Multi protocols and have been qualified for use in conjunction with the other kit components. Do not substitute with dNTP mixes or *Dpn* I enzyme formulations provided with other Agilent kits or from other sources.
- ^e Genotype: Tet^rΔ (*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lacZΔM15 Tn10* (Tet^r) *Amy Cam*^r]. Available separately as catalog # 200314 and # 200315.
- ^f 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

14-ml Falcon round-bottom polypropylene tubes (Thermo Fisher Scientific p/n 352059)

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)

Isopropyl-1-thio-β-D-galactopyranoside (IPTG)

NOTICES TO PURCHASER

Use of the QuikChange Lightning Multi Site-Directed Mutagenesis Kit, catalog #210513 and catalog #210515, by commercial entities requires a commercial license Agilent Technologies.

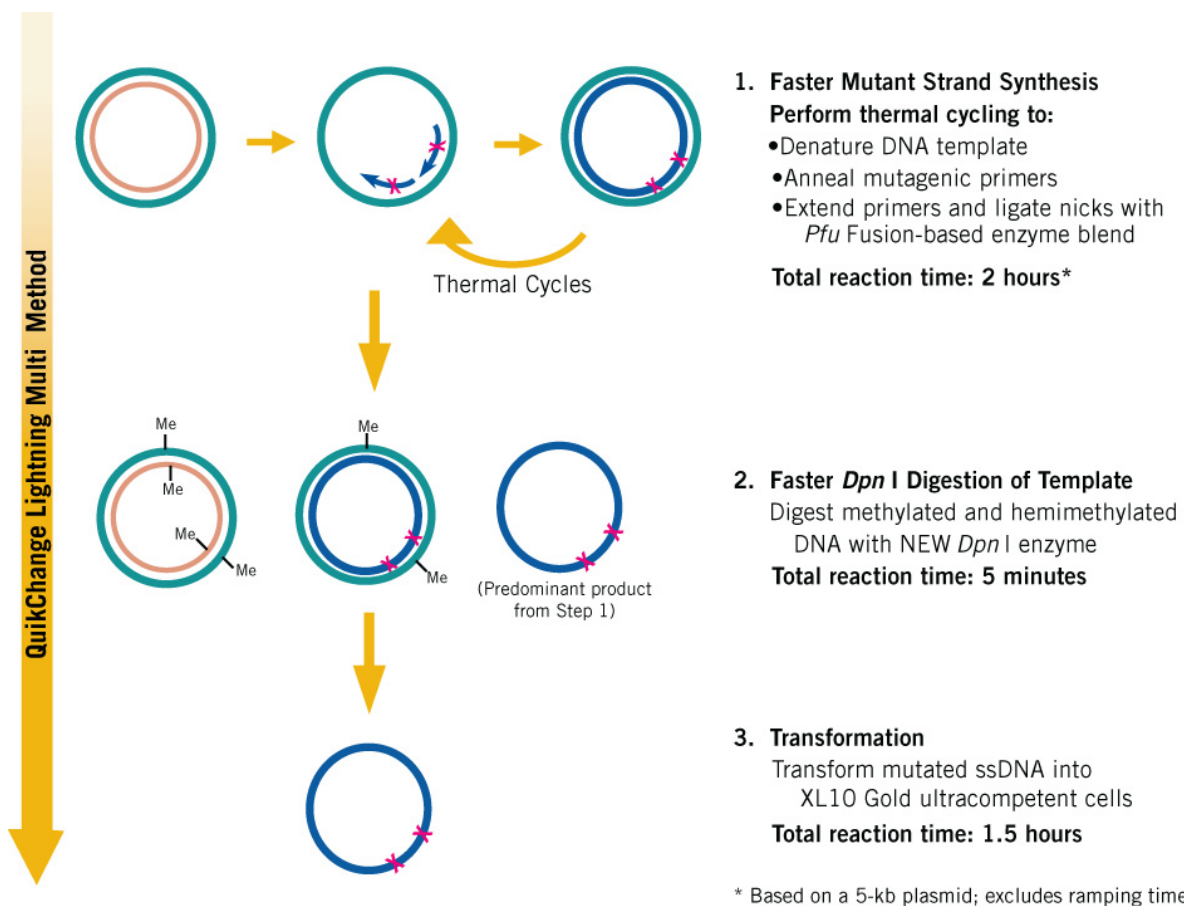
The QuikChange Lightning Multi Site-Directed Mutagenesis Kit, catalog #210514, is presently offered for sale to commercial entities with a limited use license.

INTRODUCTION

The QuikChange Lightning Multi Site-Directed Mutagenesis Kit offers the most rapid and reliable method for site-directed mutagenesis of plasmid DNA at up to five different sites simultaneously. Using the most advanced high fidelity enzyme technology, the protocols have been accelerated while maintaining the highest accuracy for site-directed mutagenesis. The QuikChange Lightning Multi Site-Directed Mutagenesis Kit delivers results up to three times faster than our original multi-site mutagenesis kit.

The rapid, simple three-step method is outlined in Figure 1. As with our original QuikChange Multi kit, a single mutagenic oligonucleotide is required to mutagenize each site, using a double-stranded DNA template. The kit's exclusive enzyme blend features a unique *Pfu* Fusion-based DNA polymerase, providing high fidelity DNA synthesis. The rapid PCR cycling parameters of the unique enzyme blend, together with our newly optimized *Dpn* I enzyme, allow for multi-site mutagenesis in approximately two hours, plus an overnight transformation.

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



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. The 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.

The Agilent QuikChange Multi site-directed mutagenesis kits offer the same benefits of simplicity and reliability afforded by the original QuikChange kit, but are based on a novel technology that allows mutagenesis at multiple sites in a single round, using a single oligonucleotide per site. No specialized vectors or unique restriction sites are needed—virtually any plasmid of up to 8 kb is a suitable template. The rapid three-step procedure used for the QuikChange Lightning Multi kit introduces mutations at three different sites simultaneously in the 4-kb control plasmid with greater than 55% efficiency. The QuikChange Lightning Multi site-directed mutagenesis system also makes it easy to randomize key amino acids using oligos containing degenerate codons.

Note *The QuikChange Lightning Multi site-directed mutagenesis system is recommended for mutating base pairs. To perform rapid insertion- or deletion-mutagenesis, use the single-site QuikChange Lightning kit (Catalog #210518).*

Outline of the Three-Step Protocol

The three-step QuikChange Lightning Multi site-directed mutagenesis method is outlined in Figure 1. Step 1 uses an accelerated 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 oligonucleotide primers containing the desired mutations, and the kit-provided enzyme blend featuring a *Pfu* Fusion DNA polymerase. 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.) The *Pfu*-based 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'-Gm⁶ATC-3') is specific for methylated and hemimethylated DNA⁵ 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, which is highly enriched for multiply mutated single stranded DNA, is transformed into XL10-Gold ultracompetent cells. 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.*

Engineered Mutant Clone Collections Applications

The QuikChange Lightning Multi site-directed mutagenesis system is well suited for constructing a diverse collection of engineered mutant clones to allow detailed protein structure-function analysis.⁶ The mutant clone collection may be constructed by performing site-specific saturation mutagenesis using primers containing degenerate nucleotides at 1–3 specific sites. For example, the system may be used to create mutant collections containing all possible amino acids 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 Lightning Multi kit reaction and then screened for mutants with improved activity using the appropriate functional assay. The QuikChange Lightning 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. See *Appendix: Guidelines for Creating Engineered Mutant Clone Collections* for additional information on using the kit for this application.

QUIKCHANGE LIGHTNING MULTI MUTAGENESIS CONTROL

The QuikChange Multi control template and control primer mix, provided with this kit, 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.

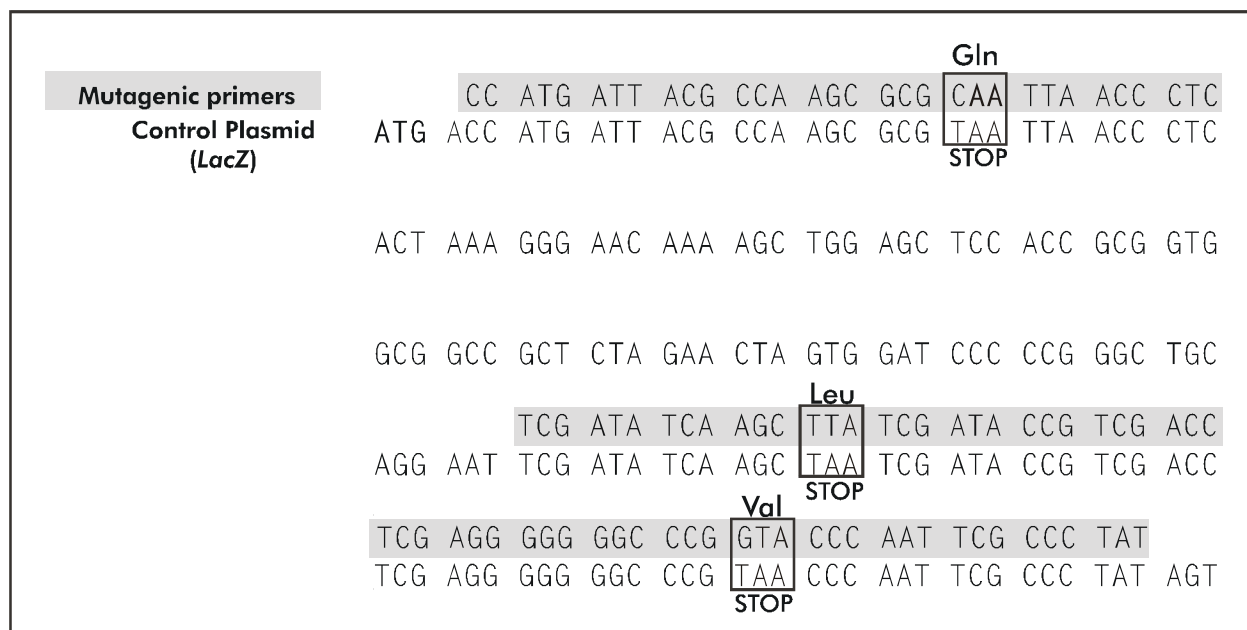


FIGURE 2 QuikChange Multi control plasmid and control primer sequences. A partial sequence of the *LacZ* coding region is shown, beginning with the ATG used for translation initiation. The QuikChange Multi control primer sequences are shown above their corresponding sites in the control plasmid, with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

MUTAGENIC PRIMER DESIGN

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

Consider the following guidelines when designing mutagenic primers:

1. Agilent recommends the use of our web-based primer design tool (<https://www.agilent.com/store/primerDesignProgram.jsp>) which was optimized to design mutagenic primer sequences specifically for the QuikChange kits.

If the primer sequences need to be modified (e.g., due to predicted secondary structure formation), then you may need to use an alternate primer design tool or manually manipulate the sequences.

2. The QuikChange Lightning Multi Site-Directed Mutagenesis method requires only one primer per mutation site, but Agilent's primer design tool designs a pair of primers (one forward primer and one reverse primer) for each mutation site. All of the primers containing the desired mutations must have the same orientation and anneal to the same strand of the template plasmid.
3. Also note that Agilent's primer design tool may design overlapping primers without alerting you. Before placing an order, make sure you are ordering only the primers that anneal to the same strand and that they do not overlap.
4. Primers should be between 25 and 45 bases in length with a melting temperature (T_m) of $\geq 75^\circ\text{C}$. Optimal primer sets for simultaneous mutagenesis should have similar melting temperatures. If necessary, primers can be longer than 45 bases to achieve a $T_m \geq 75^\circ\text{C}$. However, please be aware that using longer primers increases the likelihood of secondary structure formation, which may affect the efficiency of the mutagenesis reaction. Use the following formula to estimate 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 between 0 and 100.
5. The desired mutation should be located near the center of the primer, at least 10 bases from either end. The maximum number of mismatches is 3 bases in a row or 2 bases with a maximum of 9 bases in between.

6. Ideally, the primers have a minimum GC content of 40% and terminate in one or more C or G bases at the 3' end.
7. An online oligo sequence analysis tool can be used to check the primer sequences for potential secondary structure (i.e., hairpin) formation at or above the annealing temperature of 55°C. Primers predicted to form stable hairpin structures at 55°C or above may need to be redesigned or modified (manually or using a primer design tool). If redesign or modification is not possible, then QuikSolution may be added to the mutant strand synthesis reaction (see page 10 for titration recommendations).
8. To avoid primer dimer formation, the ΔG of the primer must be greater (i.e., closer to zero) than -9 kcal/mole (-9 kJ). The formation of primer dimers (hetero dimers and self dimers) can affect the efficiency of linear amplification during the mutant strand synthesis reaction.
9. Similarly, binding between the primer and off-target sites on the plasmid can also affect the mutant strand synthesis reaction. The NCBI blastn alignment tool (or similar alignment tool) can identify potential off-target binding between the primer and plasmid.
10. Primers should not be methylated.
11. Primers do not require 5' phosphorylation, but they must be purified by HPLC, FPLC, or PAGE. Failure to adequately purify the primers significantly decreases mutation efficiency.

PROTOCOLS

Note For suggestions on adapting the QuikChange Lightning Multi kit to create collections of engineered mutant clones, see Appendix: 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, use thin-walled tubes, which ensure ideal contact with the temperature cycler's heat blocks. The following protocols were optimized using thin-walled tubes.

Mutagenic primers should be added in approximately equimolar amounts. If primer lengths differ significantly (>20% difference), adjust the amounts of individual primers added in step 2 accordingly.

1. Prepare the ds-DNA template either by standard miniprep protocols (e.g. StrataPrep Plasmid Miniprep Kit, catalog #400761) or by cesium chloride gradient purification.
2. Prepare the mutant strand synthesis reactions for thermal cycling as indicated below. Add the components in the order listed then mix gently by pipetting or tapping the side of the reaction tube.

Reaction Component	Templates ≤ 5 kb	Templates >5 kb	Control Template
10 \times QuikChange Lightning Multi reaction buffer	2.5 μ l	2.5 μ l	2.5 μ l
double-distilled H ₂ O	X μ l to final volume of 25 μ l	X μ l to final volume of 25 μ l	18.5 μ l
QuikSolution	–	0–0.75 μ l (titrate for each template)	–
ds-DNA template	X μ l (50 ng)	X μ l (100 ng)	1 μ l QuikChange Multi control template
mutagenic primers	X μ l (100 ng each primer for 1–3 primers; 50 ng each primer for 4–5 primers)	X μ l (100 ng each primer for 1–3 primers; 50 ng each primer for 4–5 primers)	1 μ l QuikChange Multi control primer mix
dNTP mix	1 μ l	1 μ l	1 μ l
QuikChange Lightning Multi enzyme blend	1 μ l	1 μ l	1 μ l

- Cycle the reactions using the cycling parameters outlined in the table below. (For the control reaction, use a 2.5-minute extension time.)

Cycling Parameters

Segment	Cycles	Temperature	Time
1	1	95°C	2 minutes
2	30	95°C	20 seconds
		55°C	30 seconds
		65°C	30 seconds/kb of plasmid length
3	1	65°C	5 minutes

- Following temperature cycling, place the reaction on ice for 2 minutes to cool the reaction to $\leq 37^{\circ}\text{C}$.

Dpn I Digestion of the Amplification Products

- Add 1 μl of *Dpn* I restriction enzyme directly to each amplification reaction.
- 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 5 minutes 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 and chloramphenicol. If the mutagenized plasmid contains only the tet^R or the cam^R resistance marker, an alternative strain of competent cells must be used.

- Gently thaw the XL10-Gold ultracompetent cells on ice. For each mutagenesis reaction, aliquot 45 μl of the ultracompetent cells to a *prechilled* 14-ml Falcon round-bottom polypropylene tube.
- Add 2 μl of the β -ME mix provided with the kit to the 45 μl of cells. Using an alternative source of β -ME may reduce transformation efficiency.
- Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.

- Transfer 1.5 μl of the *Dpn* I-treated DNA from each mutagenesis reaction to a separate aliquot of the ultracompetent cells.

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.*

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

- 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 Falcon round-bottom polypropylene tubes.*

- Incubate the tubes on ice for 2 minutes.
- 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.
- 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 $\mu\text{g}/\text{ml}$ 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.

- 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 55% 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 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 (>10⁹ cfu/μg pUC18) 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. XL10-Gold cells are both endonuclease deficient (*endA1*) and recombination deficient (*recA*). The *endA1* mutation greatly improves the quality of plasmid miniprep DNA,⁷ 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	Genotype
XL10-Gold Ultracompetent Cells	Tet ^R $\Delta(mcrA)183$ $\Delta(mcrCB-hsdSMR-mrr)173$ <i>endA1 supE44</i> <i>thi-1 recA1 gyrA96 relA1 lac</i> Hte [F' <i>proAB lacI^qZ</i> Δ M15 Tn10 (Tet ^R) Amy Cam ^R]

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 polypropylene tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into the prechilled tubes.

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

It is important that 14-ml Falcon round-bottom polypropylene tubes (Thermo Fisher Scientific p/n 352059) 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 these 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 $80\ \mu\text{g}/\text{ml}$ of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), $20\ \text{mM}$ isopropyl-1-thio- β -D-galactopyranoside (IPTG), and the appropriate antibiotic to the LB agar. Alternatively, $100\ \mu\text{l}$ of $10\ \text{mM}$ IPTG and $100\ \mu\text{l}$ 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: GUIDELINES FOR CREATING ENGINEERED MUTANT CLONE COLLECTIONS

Optimizing Degenerate Primer Design

When designing primers containing degenerate codons, it is important to match the T_m s of the two primer sequences flanking the degenerate codon. If one side has a lower T_m due to a lower GC content, extend the primer length on that side to increase the T_m . Sequences flanking the degenerate codon may differ in length by up to about 5 nucleotides. In addition to these considerations, follow the general primer design guidelines described in *Mutagenic Primer Design*.

Increasing Collection Representation and Size

In order to identify the best amino acid substitution(s) for a desired protein function, a mutant clone collection should contain all possible amino acid sequence permutations. The QuikChange Lightning Multi kit can be adapted to increase mutant collection representation and size by applying the suggestions listed in the table below.

QuikChange Lightning Multi kit condition	Standard reaction specification	Suggested reaction specification for generating mutant clone collections
Mutagenesis reaction volume	25 μ l	50–100 μ l
QuikSolution reagent concentration	0–3%	0–10%
DNA template amount	50–100 ng	100–500 ng
Dpn I digestion time	5 minutes	1 hour

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. Start with 100 ng of each primer for 1–3 primers or 50 ng of each primer for 4–5 primers, and vary 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, this 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	Verify the quality and quantity of the DNA template by visualizing the DNA on a gel. We have observed reduced colony numbers and mutagenesis efficiencies from poor-quality DNA preparations. Produce a fresh preparation of template DNA.
	Ensure that competent cells are stored at the bottom of a -80°C freezer immediately upon arrival. Test the efficiency of the competent cells using the pUC18 control plasmid.
	Increase the amount of the <i>Dpn</i> I-treated DNA used in the transformation reaction to 4 μl . Alternatively, ethanol precipitate the <i>Dpn</i> I digestion reaction, then resuspend and transform with the entire sample.
Low mutagenesis efficiency with the experimental mutagenesis reaction(s)	Verify the quality and quantity of the DNA template by visualizing the DNA on a gel. We have observed reduced mutagenesis efficiencies and reduced colony numbers from poor-quality DNA preparations. Produce a fresh preparation of template DNA.
	Allow sufficient time for the <i>Dpn</i> I to completely digest the parental template; repeat the digestion if too much DNA template was present.
	Titrate the amounts of primer and template added to the thermal cycling reaction.
	Avoid subjecting the dNTP mix and primer solutions to multiple freeze-thaw cycles. Thaw the dNTP mix once, prepare single-use aliquots, and store the aliquots at -20°C .
	Verify that the template DNA was isolated from a <i>dam</i> ⁺ <i>E. coli</i> strain. Plasmid DNA isolated from <i>dam</i> ⁻ strains, such as JM110 or SCS100, lacks methylation and is not digested by <i>Dpn</i> 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 per 25- μl mutant strand synthesis reaction. For most long templates, up to 0.75 μl of QuikSolution is optimal, but for certain long templates, including up to 1.5 μl of QuikSolution may increase recovery of the desired mutants.
	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 4 μl of the mutagenesis reaction, and plate a larger proportion of the transformation reaction.

Table continues on the following page

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Unwanted deletion or recombination of plasmid DNA following mutagenesis and transformation	Transform the mutagenesis reaction into competent cells that are designed to prevent recombination events, such as SURE 2 Supercompetent Cells (Catalog #200152). Note that SURE 2 competent cells are not recommended for use with mutagenized plasmids greater than 10 kb in size; note also that SURE 2 cells are Kan ^r , Tet ^r , and Chl ^r , and are not compatible with plasmid selection using kanamycin, tetracycline, or chloramphenicol resistance markers.
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 subjecting the dNTP mix and primer mix to multiple freeze-thaw cycles. Thaw the dNTP mix once, prepare single-use aliquots, and store the aliquots at -20°C.

PREPARATION OF MEDIA AND REAGENTS

<p>LB Agar (per Liter) 10 g of NaCl 10 g of tryptone 5 g of yeast extract 20 g of agar Add deionized H₂O 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)</p>	<p>NZY⁺ Broth (per Liter) 10 g of NZ amine (casein hydrolysate) 5 g of yeast extract 5 g of NaCl Add deionized H₂O to a final volume of 1 liter Adjust to pH 7.5 using NaOH Autoclave Add the following filter-sterilized supplements prior to use: 12.5 ml of 1 M MgCl₂ 12.5 ml of 1 M MgSO₄ 20 ml of 20% (w/v) glucose (or 10 ml of 2 M glucose)</p>
<p>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)</p>	<p>TE Buffer 10 mM Tris-HCl (pH 7.5) 1 mM EDTA</p>
<p>10 mM IPTG (per 10 ml) 24 mg of isopropyl-1-thio-β-D-galactopyranoside (IPTG) 10 ml of sterile dH₂O Store at -20°C Spread 100 μl per LB-agar plate</p>	<p>2% X-Gal (per 10 ml) 0.2 g of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) 10 ml of dimethylformamide (DMF) Store at -20°C Spread 100 μl per LB-agar plate</p>

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MSDS INFORMATION

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

QuikChange Lightning Multi Site-Directed Mutagenesis Kit

Catalog #210513 and #210515

QUICK-REFERENCE PROTOCOL

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

Control Reaction

2.5 μ l 10 \times Reaction Buffer
1 μ l control template
1 μ l control primer mix
1 μ l dNTP mix
1 μ l QuikChange Lightning Multi enzyme blend
18.5 μ l ddH₂O

Mutagenesis Reactions

2.5 μ l 10 \times Reaction Buffer
X μ l ds-DNA template
(50ng for \leq 5 kb, or
100 ng for >5 kb*)
X μ l each primer
(100 ng each for 1–3 primers or
50 ng each for 4–5 primers)
1 μ l dNTP mix
1 μ l QuikChange Lightning Multi enzyme blend
ddH₂O to a final volume of 25 μ l

* For templates >5 kb, also add
0–0.75 μ l QuikSolution to the reaction
(titrate the QuikSolution to determine optimal
amount).

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

Segment	Cycles	Temperature	Time
1	1	95°C	2 minutes
2	30	95°C	20 seconds
		55°C	30 seconds
		65°C	30 seconds/kb of plasmid length
3	1	65°C	5 minutes

- ◆ Add 1 μ l of *Dpn* I restriction enzyme
- ◆ Gently and thoroughly mix each reaction, spin down in a microcentrifuge for 1 minute, and immediately incubate at 37°C for 5 minutes to digest parental DNA
- ◆ Transform 1.5 μ l of the *Dpn* I-treated DNA into 45- μ l aliquot of XL10-Gold ultracompetent cells (see *Transformation of XL10-Gold Ultracompetent Cells* in the instruction manual)