QuikChange XL Site-Directed Mutagenesis Kit

Instruction Manual

Catalog # 200516 (30 reactions) and 200517 (10 reactions)

Revision F.0

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

200516-12

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

Email

techservices@agilent.com

World Wide Web

www.genomics.agilent.com

Telephone

Location	Telephone
United States and Canada	800 227 9770
Austria	01 25125 6800
Benelux	02 404 92 22
Denmark	45 70 13 00 30
Finland	010 802 220
France	0810 446 446
Germany	0800 603 1000
Italy	800 012575
Netherlands	020 547 2600
Spain	901 11 68 90
Sweden	08 506 4 8960
Switzerland	0848 8035 60
UK/Ireland	0845 712 5292
All Other Countries	Please visit *www.agilent.com/genomics/contactus*

QuikChange XL Site-Directed Mutagenesis Kit

CONTENTS

QuikChange XL Site-Directed Mutagenesis Kit

MATERIALS PROVIDED

Materials provided	Quantity Catalog #200516[a] 30 reactions	Quantity Catalog #200517[b] 10 reactions
PfuTurbo DNA polymerase (2.5 U/µl)	80 U	25 U
10× reaction buffer[c]	500 µl	500 µl
Dpn I restriction enzyme (10 U/µl)	300 U	100 U
Oligonucleotide control primer #1 [34-mer (100 ng/µl)] 5′ CCA TGA TTA CGC CAA GCG CGC AAT TAA CCC TCA C 3′	750 ng	750 ng
Oligonucleotide control primer #2 [34-mer (100 ng/µl)] 5′ GTG AGG GTT AAT TGC GCG CTT GGC GTA ATC ATG G 3′	750 ng	750 ng
pWhitescript 4.5-kb control plasmid (5 ng/ µl)	50 ng	50 ng
QuikSolution	500 µl	500 µl
dNTP mix[d,e]	30 µl	10 µl
XL10-Gold ultracompetent cells[f] (yellow tubes)	10 × 135 µl	4 × 135 µl
XL10-Gold β-mercaptoethanol mix (β-ME)	2 × 50 µl	50 µl
pUC18 control plasmid (0.1 ng/µl in TE buffer[c])	10 µl	10 µl

[a] The QuikChange XL Site-Directed Mutagenesis Kit (Catalog #200516) contains enough reagents for 30 total reactions, which includes 5 control reactions.

[b] The QuikChange XL Site-Directed Mutagenesis Kit (Catalog #200517) contains enough reagents for 10 total reactions, which includes 5 control reactions.

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

[d] 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.**

[e] **The composition of the dNTP mix is proprietary.** This reagent has been optimized for the QuikChange XL site-directed mutagenesis protocols and has been qualified for use in conjunction with the other kit components. Do not substitute with dNTP mixes provided with other Agilent kits.

[f] 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]

STORAGE CONDITIONS

XL10-Gold Ultracompetent cells, XL10-Gold β-ME, and pUC18 Control Plasmid: –80°C
All Other Components: –20°C

ADDITIONAL MATERIALS REQUIRED

14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)
5-Bromo-4-chloro-3-indloyl-β-D-galactopyranoside (X-gal)
Isopropyl-1-thio-β-D-galactopyranoside (IPTG)

Revision F.0

NOTICES TO PURCHASER

Use of this product is licensed under one or more of the following U.S. Patent Nos. 5,789,166, 5,932,419, 6,391,548, 6,713,285, 7,132,265, and 7,176,004.

Notice to Purchaser: Limited License

Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 6,258,569, 6,171,785, 6,127,155, 6,030,787, 5,994,056, 5,876,930, 5,804,375, 5,789,224, 5,773,258 (claims 1 and 6 only), 5,723,591, 5,677,152 (claims 1 to 23 only), 5,618,711, 5,538,848, and claims outside the US corresponding to expired US Patent No. 5,079,352. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

INTRODUCTION

In vitro site-directed mutagenesis is an invaluable technique for studying protein structure-function relationships and gene expression, and for carrying out vector modification. Several approaches to this technique have been published, but these methods generally require single-stranded DNA (ssDNA) as the template[1-4] and are labor intensive or technically difficult. The QuikChange XL Site-Directed Mutagenesis Kit* allows site-specific mutation in virtually any double-stranded plasmid, thus eliminating the need for subcloning into M13-based bacteriophage vectors and for ssDNA rescue.[5] In addition, the QuikChange XL system requires no specialized vectors, unique restriction sites, or multiple transformations. This rapid four-step procedure generates mutants with greater than 80% efficiency. The protocol is simple and uses either miniprep plasmid DNA or cesium-chloride-purified DNA.

The QuikChange XL system is used to make point mutations, switch amino acids, and delete or insert single or multiple amino acids. The QuikChange XL method is performed using *PfuTurbo* DNA Polymerase** and a thermal temperature cycler. *PfuTurbo* DNA polymerase replicates both plasmid strands with high fidelity[‖] and without displacing the mutant oligonucleotide primers. The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation (see Figure 1). The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by using *PfuTurbo* DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *Dpn* I. The *Dpn* I endonuclease (target sequence: 5´-Gm^{6}ATC-3´) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA.[6] DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to *Dpn* I digestion. The nicked vector DNA incorporating the desired mutations is then transformed into XL10-Gold*** Ultracompetent Cells. The small amount of starting DNA template required to perform this method, the high-fidelity of the *PfuTurbo* DNA polymerase, and the low number of thermal cycles all contribute to the high mutation efficiency and decreased potential for random 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. 5,789,166, 5,932,419, 6,391,548, 6,713,285, 7,132,265, and 7,176,004.

** U.S. Patent Nos. 5,948,663, 6,183,997, 6,444,428, and 6,734,293.

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

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

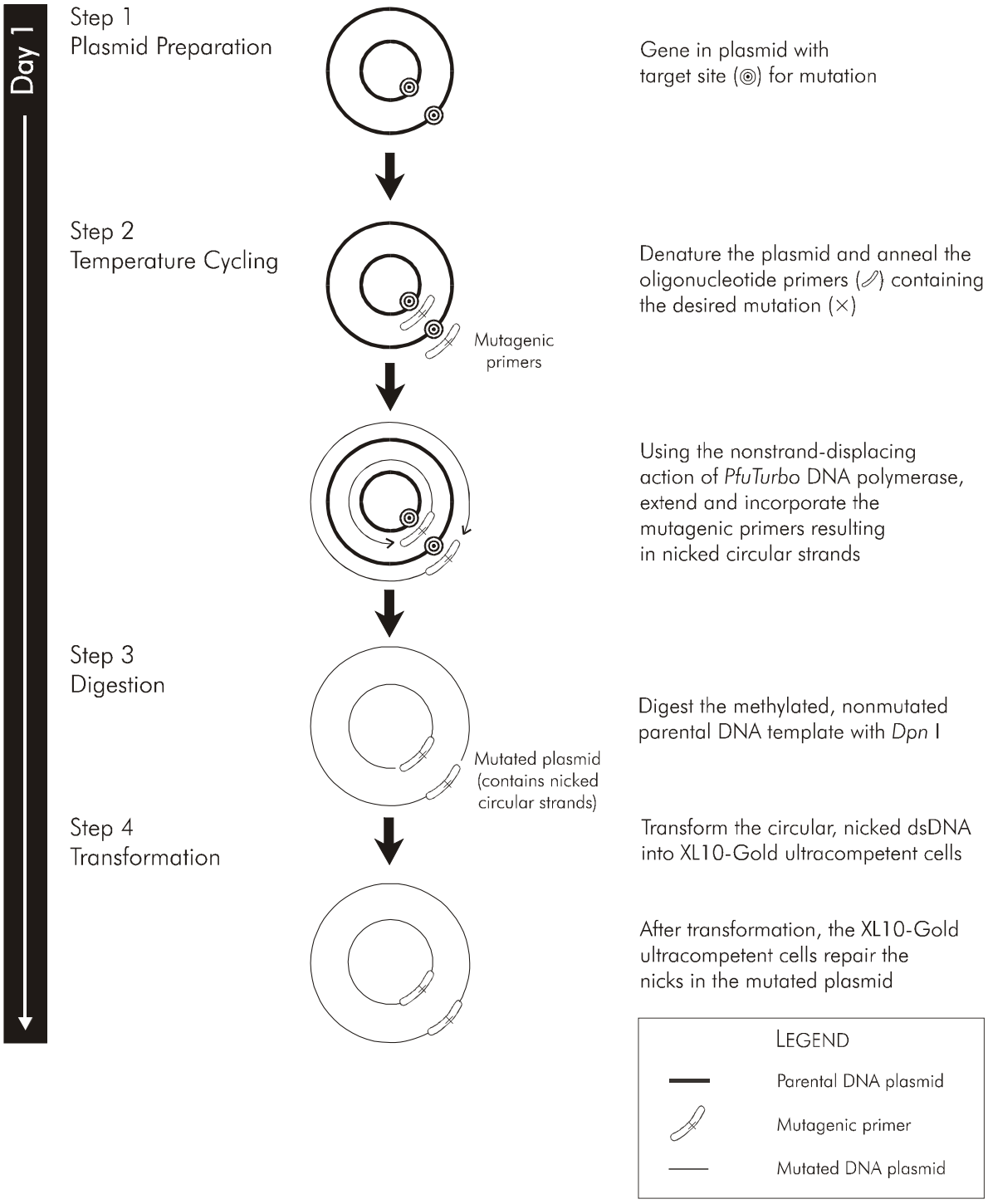

FIGURE 1 Overview of the QuikChange XL site-directed mutagenesis method.

The QuikChange XL site-directed mutagenesis kit is a specialized version of our popular QuikChange site-directed mutagenesis kit, created for efficient mutagenesis of large or otherwise difficult-to-mutagenize plasmid templates. The QuikChange XL kit features components specifically designed for more efficient DNA replication and bacterial transformation. The QuikChange solution is provided to facilitate replication of large plasmids, while XL10-Gold ultracompetent cells have been included to ensure the highest transformation efficiencies possible. The transformation efficiency of XL10-Gold cells is 5-fold higher than the transformation efficiency of XL1-Blue cells employed in the original QuikChange kit.[7] Moreover, XL10-Gold cells contain the Hte phenotype, which increases the transformation efficiency of larger DNA plasmids.

QUIKCHANGE XL MUTAGENESIS CONTROL

To demonstrate the effectiveness of the QuikChange XL method, the pWhitescript 4.5-kb control plasmid is used to test the efficiency of mutant plasmid generation. The pWhitescript 4.5-kb control plasmid contains a stop codon (TAA) at the position where a glutamine codon (CAA) would normally appear in the β-galactosidase gene of the pBluescript II SK(–) phagemid (corresponding to amino acid 9 of the protein). XL10-Gold ultracompetent cells transformed with this control plasmid appear white on LB–ampicillin agar plates (see *Preparation of Media and Reagents*), containing IPTG and X-gal, because β-galactosidase activity has been obliterated. The oligonucleotide control primers create a point mutation that reverts the T residue of the stop codon (TAA) in the β-galactosidase gene encoded on the pWhitescript 4.5-kb control template to a C residue to produce a glutamine codon (Gln, CAA). Following transformation, colonies can be screened for β-galactosidase production (β-gal$^+$) by virtue of a blue colony phenotype.

MUTAGENIC PRIMER DESIGN

Note *Mutagenic primers can be designed using our web-based QuikChange Primer Design Program available online at www.agilent.com/genomics/qcpd.*

Primer Design Guidelines

The mutagenic oligonucleotide primers for used with this protocol must be designed individually according to the desired mutation. The following considerations should be made for designing mutagenic primers:

- Both mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.

- Primers should be between 25 and 45 bases in length, with a melting temperature (T_m) of ≥78°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. 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

  For calculating T_m for primers intended to introduce insertions or deletions, use this modified version of the above formula:

$$T_m = 81.5 + 0.41(\%GC) - (675/N)$$

  where N does not include the bases which are being inserted or deleted.

  Note *When using primer design software for QuikChange site-directed mutagenesis applications, be aware that the T_m calculated by the primer design software may differ from the T_m value calculated using the formula presented above. We recommend verifying primer T_m's using the formula above or by using the QuikChange T_m calculator, available online at www.genomics.agilent.com.*

- The desired mutation (deletion or insertion) should be in the middle of the primer with ~10–15 bases of correct sequence on both sides.

- The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

Additional Primer Considerations

- The mutagenesis protocol uses 125 ng of each oligonucleotide primer. To convert nanograms to picomoles of oligo, use the following equation:

$$X \text{ pmoles of oligo} = \frac{\text{ng of oligo}}{330 \times \#\text{of bases in oligo}} \times 1000$$

For example, for 125 ng of a 25-mer:

$$\frac{125 \text{ ng of oligo}}{330 \times 25 \text{ bases}} \times 1000 = 15 \text{ pmole}$$

- Primers need not be 5´ phosphorylated but **must** be purified either by fast polynucleotide liquid chromatography (FPLC) or by polyacrylamide gel electrophoresis (PAGE). Failure to purify the primers results in a significant decrease in mutation efficiency.

- It is important to keep primer concentration in excess. We suggest that you vary the amount of template while keeping the concentration of the primer constantly in excess.

XL10-GOLD ULTRACOMPETENT CELLS

XL10-Gold ultracompetent cells are derived from the highest-efficiency Agilent competent cell line, XL2-Blue MRF´. These strains possess the Hte phenotype, which increases transformation efficiency of ligated DNA.[7] XL10-Gold cells are both endonuclease deficient (*endA1*) and recombination deficient (*recA*). The *endA1* mutation greatly improves the quality of plasmid miniprep DNA,[8] 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.[9, 10, 11] 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.[12] 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	7, 13, 14	Tet^{R} Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173* *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB lacI*^{q}ZΔ*M15* 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.

PROTOCOL

QuikSolution

QuikSolution has been shown to improve linear amplification. Enhanced amplification efficiencies are observed when using between 2.5–3.5 µl QuikSolution/50 µl reaction, with 3 µl being optimal for most targets.

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, ***we strongly recommend 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. Synthesize two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence. Purify these oligonucleotide primers prior to use in the following steps (see *Mutagenic Primer Design*).

2. Prepare the control reaction as indicated below:

   5 µl of 10× reaction buffer (see *Preparation of Media and Reagents*)
   2 µl (10 ng) of pWhitescript 4.5-kb control plasmid (5 ng/µl)
   1.25 µl (125 ng) of oligonucleotide control primer #1 [34-mer (100 ng/µl)]
   1.25 µl (125 ng) of oligonucleotide control primer #2 [34-mer (100 ng/µl)]
   1 µl of dNTP mix
   3 µl of QuikSolution
   36.5 µl of double-distilled water (ddH_2O) to a final volume of 50 µl

   Then add

   1 µl of *PfuTurbo* DNA polymerase (2.5 U/µl)

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

   Note *Set up an initial sample reaction using 10 ng of dsDNA template. If this initial reaction is unsuccessful, set up a series of sample reactions using various concentrations of dsDNA template ranging from 5 to 50 ng (e.g., 5, 10, 20, and 50 ng of dsDNA template) while keeping the primer concentration constant.*

   5 µl of 10× reaction buffer
   X µl (10 ng) of dsDNA template
   X µl (125 ng) of oligonucleotide primer #1
   X µl (125 ng) of oligonucleotide primer #2
   1 µl of dNTP mix
   3 µl of QuikSolution
   ddH_2O to a final volume of 50 µl

   Then add

   1 µl of *PfuTurbo* DNA polymerase (2.5 U/µl)

4. Cycle each reaction using the cycling parameters outlined in Table I.

   Note *It is important to adhere to the 18-cycle limit when cycling the mutagenesis reactions. More that 18 cycles can have deleterious effects on the reaction efficiency.*

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

Note *If desired, amplification may be checked by electrophoresis of 10 µl of the product on a 1% agarose gel. A band may or may not be visualized at this stage. In either case proceed with* Dpn *I digestion and transformation.*

TABLE I

Cycling Parameters for the QuikChange XL Method

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	18	95°C	50 seconds
		60°C	50 seconds
		68°C	1 minute/kb of plasmid length
3	1	68°C	7 minutes

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

Dpn I Digestion of the Amplification Products

1. Add 1 µl of the *Dpn* I restriction enzyme (10 U/µl) to each amplification reaction.

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 the reactions at 37°C for 1 hour 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 tetracycline and chloramphenicol. If the mutagenized plasmid contains only the tet^R *or* cam^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 β-ME mix 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 2 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 another 45-µl aliquot of cells.

   Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 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 *Preparing the Agar Plates for Color Screening*).

Transformation reaction plating volumes

Reaction Type	Volume to Plate
pWhitescript mutagenesis control	250 µl
pUC18 transformation control	5 µl (in 200 µl of NZY+ broth)*
Sample mutagenesis	250 µl on each of two plates (entire transformation reaction)

* 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 for the Control Transformations

The expected colony number from the transformation of the pWhitescript 4.5 kb control mutagenesis reaction is between 50 and 800 colonies. Greater than 80% of the colonies should contain the mutation and appear as blue colonies on agar plates containing IPTG and X-gal.

Note *The mutagenesis efficiency (ME) for the pWhitescript 4.5-kb control plasmid is calculated by the following formula:*

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

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 Sample Transformations

The expected colony number is between 10 and 1000 colonies, depending upon the base composition and length of the DNA template employed. 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 BD 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 BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059) 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.

Quantity of DNA Added

Greatest efficiencies are observed when adding 2 µl of the ligation mixture. A greater number of colonies will be obtained when adding up to 50 ng, although the overall efficiency may be lower.

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 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

When used according to the guidelines outlined in this instruction manual, this kit provides a reliable means to conduct site-directed mutagenesis using dsDNA templates. Variations in the base composition and length of the DNA template and in thermal cycler performance may contribute to differences in mutagenesis efficiency. We provide the following guidelines for troubleshooting these variations.

Observation	Suggestion(s)
Low transformation efficiency or low colony number	Ensure that sufficient DNA template is used in the reaction. Visualize the DNA template on a gel to verify the quantity and quality. Repeat reaction using higher amounts of plasmid DNA (100 ng, 200 ng, 500 ng).
	Ensure that sufficient mutant DNA is synthesized in the reaction. • Titrate QuikSolution in 1-µl increments from 0 to 5 µl • Increase the amount of the *Dpn* I-treated DNA used in the transformation reaction to 4 µl • Increase the extension time to 2.5 min/kb • Precipitate the entire reaction and use all of it in the transformation
	Ensure sufficient mutant DNA is synthesized by adjusting the cycling parameters for the sample reaction to overcome differences in ramping efficiencies of thermal cyclers. Increase initial denaturation step (segment 1) to 1–2 minutes and denaturation cycles (segment 2) to 1 minute.
	Ethanol precipitate the *Dpn* I digested PCR product, and resuspend in a decreased volume of water before transformation.
Low mutagenesis efficiency or low colony number with the control reaction	Different thermal cyclers contribute to variations in cycling efficiencies. Optimize the cycling parameters (including ramp rates) for the control reaction then repeat the protocol for the sample reactions using the optimized conditions.
	Ensure that ultracompetent cells are stored at the bottom of a –80°C freezer immediately upon arrival; use XL10-Gold β-ME in the transformation reactions (see also *Transformation Guidelines*).
	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. Do not subject the dNTP mix to multiple freeze-thaw cycles.
Low mutagenesis efficiency with the sample reaction(s)	Allow sufficient time for the *Dpn* I to completely digest the parental template; repeat the digestion if too much DNA template was present. Increase digestion time to 1.5–2.0 hours.
	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.
	The formation of secondary structures may be inhibiting the mutagenesis reaction. Increasing the annealing temperature up to 68°C may help to alleviate secondary structure formation and improve mutagenesis efficiency.
False positives	Poor quality primers can lead to false positives. Radiolabel the primers and check for degradation on an acrylamide gel or resynthesize the primers.
	False priming can lead to false positives. Increase the stringency of the reaction by increasing the annealing temperature up to 68°C.
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 Agilent's 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.

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)

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)

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)

10× Reaction Buffer

100 mM KCl
100 mM$(NH_4)_2SO_4$
200 mM Tris-HCl (pH 8.8)
20 mM $MgSO_4$
1% Triton® X-100
1 mg/ml nuclease-free bovine serum albumin (BSA)

TE Buffer

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

REFERENCES

1. Kunkel, T. A. (1985) *Proc Natl Acad Sci U S A* 82(2):488-92.
2. Sugimoto, M., Esaki, N., Tanaka, H. and Soda, K. (1989) *Anal Biochem* 179(2):309-11.
3. Taylor, J. W., Ott, J. and Eckstein, F. (1985) *Nucleic Acids Res* 13(24):8765-85.
4. Vandeyar, M. A., Weiner, M. P., Hutton, C. J. and Batt, C. A. (1988) *Gene* 65(1):129-33.
5. Papworth, C., Bauer, J. C., Braman, J. and Wright, D. A. (1996) *Strategies* 9(3):3–4.
6. Nelson, M. and McClelland, M. (1992) *Methods Enzymol* 216:279-303.
7. Jerpseth, B., Callahan, M. and Greener, A. (1997) *Strategies* 10(2):37–38.
8. Wnendt, S. (1994) *Biotechniques* 17(2):270, 272.
9. Kohler, S. W., Provost, G. S., Kretz, P. L., Dycaico, M. J., Sorge, J. A. *et al.* (1990) *Nucleic Acids Res* 18(10):3007-13.
10. Kretz, P. L., Kohler, S. W. and Short, J. M. (1991) *J Bacteriol* 173(15):4707-16.
11. Raleigh, E. A. and Wilson, G. (1986) *Proc Natl Acad Sci U S A* 83(23):9070-4.
12. Jerpseth, B., Greener, A., Short, J. M., Viola, J. and Kretz, P. L. (1992) *Strategies* 5(3):81–83.
13. Bullock, W. O., Fernandez, J. M. and Short, J. M. (1987) *Biotechniques* 5(4):376–378.
14. Greener, A. and Jerpseth, B. (1993) *Strategies* 6(2):57.

ENDNOTES

Triton® is a registered trademark of Rohm and Haas Co.

MSDS INFORMATION

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

QuikChange XL Site-Directed Mutagenesis Kit

Catalog #200516 and #200517

QUICK-REFERENCE PROTOCOL

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

Note *Set up an initial sample reaction using 10 ng of dsDNA template. If this initial sample reaction is unsuccessful, set up a series of reactions using various concentrations of dsDNA template ranging from 5 to 50 ng (e.g., 5, 10, 20, and 50 ng of dsDNA template) while keeping the primer concentration constant.*

Control Reaction

5 µl of 10× reaction buffer
2 µl (10 ng) of pWhitescript 4.5-kb control template (5 ng/µl)
1.25 µl (125 ng) of oligonucleotide control primer #1 [34-mer (100 ng/µl)]
1.25 µl (125 ng) of oligonucleotide control primer #2 [34-mer (100 ng/µl)]
1 µl of dNTP mix
3 µl of QuikSolution
36.5 µl ddH_2O to a final volume of 50 µl

Sample Reaction

5 µl of 10× reaction buffer
X µl (10 ng) of dsDNA template
X µl (125 ng) of oligonucleotide primer #1
X µl (125 ng) of oligonucleotide primer #2
1 µl of dNTP mix
3 µl of QuikSolution
ddH_2O to a final volume of 50 µl

- Then add 1 µl of *PfuTurbo* DNA polymerase (2.5 U/µl) to each control and sample reaction.
- Cycle each reaction using the cycling parameters outlined in the following table:

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	18	95°C	50 seconds
		60°C	50 seconds
		68°C	1 minute/kb of plasmid length
3	1	68°C	7 minutes

- Add 1 µl of *Dpn* I restriction enzyme (10 U/µl).
- 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 the parental supercoiled dsDNA.
- Transform 2 µl of the *Dpn* I-treated DNA from each control and sample reaction into separate 45-µl aliquots of XL10-Gold ultracompetent cells (see *Transformation of XL10-Gold Ultracompetent Cells* in the instruction manual).