

QuikChange[®] Site-Directed Mutagenesis Kit

INSTRUCTION MANUAL

Catalog #200518 (30 reactions) and #200519 (10 reactions)

Revision #102008i

For In Vitro Use Only



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QuikChange® Site-Directed Mutagenesis Kit

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QuikChange[®] Site-Directed Mutagenesis Kit

MATERIALS PROVIDED

Material provided ^a	Quantity	
	Catalog #200518 30 reactions	Catalog #200519 10 reactions
<i>PfuTurbo</i> [®] DNA polymerase (2.5 U/ μ l)	80 U	25 U
10 \times reaction buffer ^b	500 μ l	500 μ l
<i>Dpn</i> I restriction enzyme (10 U/ μ l)	300 U	100 U
Oligonucleotide control primer #1 [34-mer (100 ng/ μ l)] 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/ μ l)] 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/ μ l)	50 ng	50 ng
dNTP mix ^c	30 μ l	10 μ l
XL1-Blue supercompetent cells ^d (blue tubes)	8 \times 200 μ l	3 \times 200 μ l
pUC18 control plasmid (0.1 ng/ μ l in TE buffer ^b)	10 μ l	10 μ l

^a The QuikChange Site-Directed Mutagenesis Kits contain enough reagents for 5 control reactions.

^b See *Preparation of Media and Reagents*.

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

^d Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^qZ Δ M15 Tn10* (Tet^r)]

STORAGE CONDITIONS

XL1-Blue Supercompetent 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)

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 template¹⁻⁴ and are labor intensive or technically difficult. Stratagene's QuikChange[®] 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 site-directed mutagenesis 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. For long (~8 kb) or difficult targets, Stratagene offers the QuikChange[®] XL site directed mutagenesis kit (Catalog #200516).

The QuikChange site-directed mutagenesis kit is used to make point mutations, switch amino acids, and delete or insert single or multiple amino acids. The QuikChange site-directed mutagenesis method is performed using *PfuTurbo*[®] DNA polymerase** and a 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 *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'-Gm⁶ATC-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 containing the desired mutations is then transformed into XL1-Blue supercompetent 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 generating random mutations during the reaction.

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, 5,923,419, 6,391,548 and patents pending.

** 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.

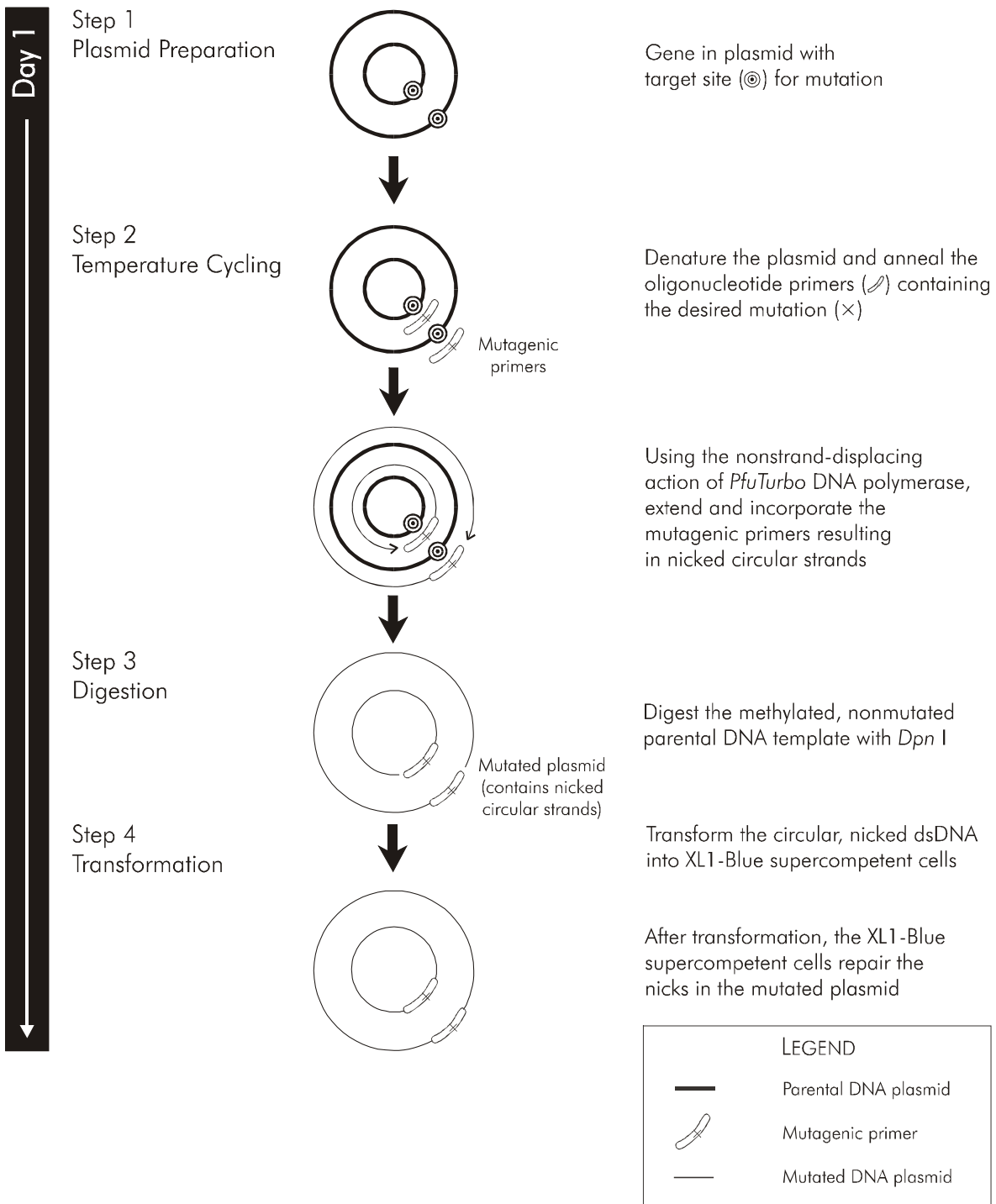


FIGURE 1 Overview of the QuikChange® site-directed mutagenesis method.

QUIKCHANGE® MUTAGENESIS CONTROL

The pWhitescript™ 4.5-kb control plasmid is used to test the efficiency of mutant plasmid generation using the QuikChange site-directed mutagenesis kit. 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). XL1-Blue supercompetent 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 on the pWhitescript 4.5-kb control plasmid that reverts the T residue of the stop codon (TAA) at amino acid 9 of the β -galactosidase gene to a C residue, to produce the glutamine codon (CAA) found in the wild-type sequence. Following transformation, colonies can be screened for the β -galactosidase (β -gal⁺, blue) phenotype.

MUTAGENIC PRIMER DESIGN

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

1. Both of the mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.
2. Primers should be between 25 and 45 bases in length, and the melting temperature (T_m) of the primers should be greater than or equal to 78°C. 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.

3. The desired mutation (deletion or insertion) should be in the middle of the primer with ~10–15 bases of correct sequence on both sides.
4. The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.
5. 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.
6. It is important to keep primer concentration in excess. Stratagene suggests varying the amount of template while keeping the concentration of the primer constantly in excess.

PROTOCOL

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. 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
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
Double-distilled water (ddH₂O) 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 *Stratagene recommends setting 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 (5–50 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
ddH₂O to a final volume of 50 µl

Then add

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

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

TABLE I

Cycling Parameters for the QuikChange Site-Directed Mutagenesis Method

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	12–18	95°C	30 seconds
		55°C	1 minute
		68°C	1 minute/kb of plasmid length*

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

- Cycle each reaction using the cycling parameters outlined in Table I. (For the control reaction, use a 12-minute extension time and run the reaction for 12 cycles.)
- Adjust segment 2 of the cycling parameters in accordance with the type of mutation desired (see the following table):

Type of mutation desired	Number of cycles
Point mutations	12
Single amino acid changes	16
Multiple amino acid deletions or insertions	18

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

Note *If desired, amplification may be checked by electrophoresis of 10 μ l 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.*

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 μ l of 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 μ l of the Dpn I restriction enzyme (10 U/ μ l) 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 and immediately incubate each reaction at 37°C for 1 hour to digest the parental (i.e., the nonmutated) supercoiled dsDNA.

Transformation of XL1-Blue Supercompetent Cells

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

XL1-Blue 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 XL1-Blue supercompetent cells on ice. For each control and sample reaction to be transformed, aliquot 50 μ l of the supercompetent cells to a *prechilled* Falcon® 2059 polypropylene tube.
2. Transfer 1 μ l of the Dpn I-treated DNA from each control and sample reaction to separate aliquots of the supercompetent cells.

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

As an optional control, verify the transformation efficiency of the XL1-Blue supercompetent cells by adding 1 μ l of the pUC18 control plasmid (0.1 ng/ μ l) to a 50- μ l aliquot of the supercompetent cells.

Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 minutes.

3. Heat pulse the transformation reactions for 45 seconds at 42°C and then place the reactions on ice for 2 minutes.

Note *This heat pulse has been optimized for transformation in Falcon 2059 polypropylene tubes.*

- Add 0.5 ml of NZY⁺ broth preheated to 42°C and incubate the transformation reactions 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 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.

- 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 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:*

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

If transformation of the pUC18 control plasmid was performed, >250 colonies should be observed (transformation efficiency >10⁸ cfu/µg) with >98% of the colonies 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

It is important to store the XL1-Blue supercompetent 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 XL1-Blue supercompetent 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 XL1-Blue supercompetent cells should be placed at -80°C directly from the dry ice shipping container. Cells stored in this manner should retain their guaranteed efficiency for 6 months.

Aliquoting Cells

When aliquoting, keep the XL1-Blue supercompetent 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 because the duration of the heat-pulse step is critical and has been optimized for the thickness and shape of the Falcon 2059 polypropylene tubes.

Length of the Heat Pulse

There is a defined "window" of highest efficiency for the XL1-Blue supercompetent cells resulting from the heat pulse in step 3 of the transformation protocol. Optimal efficiencies are observed when cells are heat pulsed for 45 seconds. Heat pulsing for at least 45 seconds is recommended to allow for slight variations in the length of incubation. Efficiencies decrease sharply when pulsing for <30 seconds or for >45 seconds.

Preparing the 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 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 the 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, Stratagene's kit will provide a reliable means to conduct site-directed mutagenesis using dsDNA templates. Undoubtedly, there will be variations in the base composition and length of the DNA template and in the thermal cycler that may contribute to differences in mutagenesis efficiency. Stratagene provides the following guidelines for troubleshooting these variations.

Observation	Suggestion(s)
Low transformation efficiency or low colony number	Ensure that excess mineral oil is not transferred into the transformation reaction when pipetting the <i>Dpn</i> I-treated DNA. Using the smallest pipet tips available, insert the pipet tip completely below the mineral layer overlay and clear the pipet tip while submerged beneath the mineral oil overlay before collecting the sample
	Ensure that sufficient mutant DNA is synthesized in the reaction. Increase the amount of the <i>Dpn</i> I-treated DNA used in the transformation reaction to 4 μ l
	Visualize the DNA template on a gel to verify the quantity and quality. Nicked or linearized plasmid DNA will not generate complete circular product. Verify that the template DNA is at least 80% supercoiled
	It is not uncommon to observe low numbers of colonies, especially when generating large mutations. Most of the colonies that do appear, however, will contain mutagenized plasmid
Low mutagenesis efficiency or low colony number with the control reaction	Different thermal cyclers may contribute to variations in ramping efficiencies. Adjust the cycling parameters for the control reaction and repeat the protocol for the sample reactions
	Ensure that supercompetent cells are stored at the bottom of a -80°C freezer immediately upon arrival (see also <i>Transformation Guidelines</i>)
	Verify that the agar plates were prepared correctly. See <i>Preparing the Agar Plates for Color Screening</i> , 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
	Adjust the cycling parameters for the sample reaction to overcome differences in ramping efficiencies of thermal cyclers
Low mutagenesis efficiency with the sample reaction(s)	Add the <i>Dpn</i> I restriction enzyme below the mineral oil overlay in the digestion step and ensure proper mixing of all components in the reaction especially the <i>Dpn</i> I
	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
	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
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 to within 5°C of the melting temperature of the mutagenic primers

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>LB–Ampicillin Agar (per Liter) (Use for reduced satellite colony formation) 1 liter of LB agar Autoclave Cool to 55°C Add 50 mg of filter-sterilized ampicillin 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>10× Reaction Buffer 100 mM KCl 100 mM(NH₄)₂SO₄ 200 mM Tris-HCl (pH 8.8) 20 mM MgSO₄ 1% Triton[®] X-100 1 mg/ml nuclease-free bovine serum albumin (BSA)</p> <hr/> <p>TE Buffer 10 mM Tris-HCl (pH 7.5) 1 mM EDTA</p>

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ENDNOTES

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QuikChange® Site-Directed Mutagenesis Kit

Catalog #200518

QUICK-REFERENCE PROTOCOL

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

Note *Stratagene recommends setting up a series of sample reactions using various concentrations ranging from 5 to 50 ng of dsDNA template (e.g., 5, 10, 20, and 50 ng of dsDNA template).*

Control Reaction

5 μ l of 10 \times reaction buffer
 2 μ l (10 ng) of pWhitescript™ 4.5-kb control template (5 ng/ μ l)
 1.25 μ l (125 ng) of oligonucleotide control primer #1 [34-mer (100 ng/ μ l)]
 1.25 μ l (125 ng) of oligonucleotide control primer #2 [34-mer (100 ng/ μ l)]
 1 μ l of dNTP mix
 ddH₂O to a final volume of 50 μ l

Sample Reaction

5 μ l of 10 \times reaction buffer
 X μ l (5–50 ng) of dsDNA template
 X μ l (125 ng) of oligonucleotide primer #1
 X μ l (125 ng) of oligonucleotide primer #2
 1 μ l of dNTP mix
 ddH₂O to a final volume of 50 μ l

- ◆ Then add 1 μ l of *PfuTurbo* DNA polymerase (2.5 U/ μ l) to each control and sample reaction
- ◆ Overlay each reaction with 30 μ l of mineral oil
- ◆ Cycle each reaction using the cycling parameters outlined in the following table:

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	12–18	95°C	30 seconds
		55°C	1 minute
		68°C	1 minute/kb of plasmid length

- ◆ Adjust segment 2 of the cycling parameters in accordance with the type of mutation desired (see the table in step 6 of *Mutant Strand Synthesis Reaction (Thermal Cycling)* in the instruction manual)
- ◆ Add 1 μ l of the *Dpn* I restriction enzyme (10 U/ μ l) below the mineral oil overlay
- ◆ 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 1 μ l of the *Dpn* I-treated DNA from each control and sample reaction into separate 50- μ l aliquots of XL1-Blue supercompetent cells (see *Transformation of XL1-Blue Supercompetent Cells* in the instruction manual)