QuikChange II Site-Directed Mutagenesis Kit

Instruction Manual

Catalog #200523 (10 reactions) and #200524 (30 reactions)

Revision E1

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

200523-12

Agilent Technologies

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

Please visit www.agilent.com.

TECHNICAL SERVICES

United States and Canada

Email: genomics@agilent.com
Telephone: 800 227 9770 (option 3,4,3)

All Other Locations

Please visit www.agilent.com/en/contact-us/page.

QuikChange II Site-Directed Mutagenesis Kit

CONTENTS

QuikChange II Site-Directed Mutagenesis Kit

MATERIALS PROVIDED

Materials provided	Quantity Catalog #200524[a] 30 reactions	Quantity Catalog #200523[b] 10 reactions
PfuUltra High-Fidelity DNA polymerase (2.5 U/ µl)	80 U	25 U
10× reaction buffer	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
dNTP mix[c,d]	30 µl	10 µl
XL1-Blue supercompetent cells[e] (blue tubes)	8 × 200 µl	3 × 200 µl
pUC18 control plasmid (0.1 ng/µl in TE buffer[f])	10 µl	10 µl

[a] The QuikChange II Site-Directed Mutagenesis Kit (Catalog #200524) contains enough reagents for 30 reactions total (control and experimental reactions combined).

[b] The QuikChange II Site-Directed Mutagenesis Kit (Catalog #200523) 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 is proprietary.** This reagent has been optimized for the QuikChange II 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.

[e] Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔ*M15* Tn*10* (Tet^{r})]

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

STORAGE CONDITIONS

XL1-Blue Supercompetent 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)

Revision E1

INTRODUCTION

In vitro site-directed mutagenesis is an invaluable technique for characterizing the dynamic, complex relationships between protein structure and function, for studying gene expression elements, 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. Our QuikChange II Site-Directed Mutagenesis Kit allows site-specific mutation in virtually any double-stranded plasmid, thus eliminating the need for subcloning and for ssDNA rescue.[5] In addition, the QuikChange II site-directed mutagenesis kit does not require specialized vectors, unique restriction sites, multiple transformations or in vitro methylation treatment steps. The rapid three-step procedure generates mutants with greater than 80% efficiency in a single reaction (see Figure 1). The protocol is simple and uses either miniprep plasmid DNA or cesium-chloride-purified DNA. For long (~8 kb) or difficult targets, we offer the QuikChange II XL Site Directed Mutagenesis Kits (Catalog #200521 and #200522).

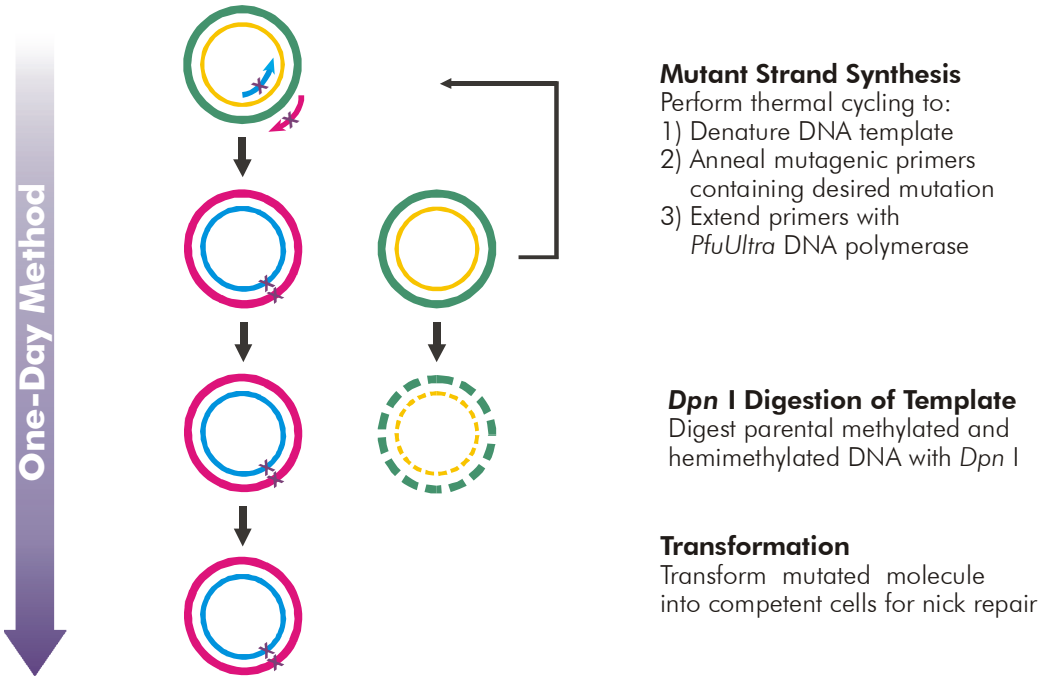

FIGURE 1 Overview of the QuikChange II site-directed mutagenesis method.

The QuikChange II site-directed mutagenesis kit is used to make point mutations, replace amino acids, and delete or insert single or multiple adjacent amino acids. The QuikChange II site-directed mutagenesis method is performed using *PfuUltra* high-fidelity (HF) DNA polymerase for mutagenic primer-directed replication of both plasmid strands with the highest fidelity. The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers, both containing the desired mutation (see Figure 1). The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by *PfuUltra* HF DNA polymerase, without primer displacement. Extension 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 containing the desired mutations is then transformed into XL1-Blue supercompetent cells.

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.*

Unwanted second-site errors are virtually eliminated and high mutation efficiencies are obtained using this method due to the high fidelity of the *PfuUltra* HF DNA polymerase, the use of a small amount of starting DNA template and the use of a low number of thermal cycles.

QUIKCHANGE II MUTAGENESIS CONTROL

The pWhitescript 4.5-kb control plasmid is used to test the efficiency of mutant plasmid generation using the QuikChange II 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$^+$) phenotype of blue color on media containing IPTG and X-gal.

MUTAGENIC PRIMER DESIGN

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 was optimized to design mutagenic primer sequences specifically for the QuikChange kits.

   Please note that the tool only designs one pair of primers. 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. Both mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.

3. Primers should be between 25 and 45 bases in length with a melting temperature (T_m) of ≥78°C. Optimum primer sets for simultaneous mutagenesis should have similar melting temperatures. If necessary, primers can be longer than 45 bases to achieve a T_m ≥78°C. However, 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

   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.

4. 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.

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

6. 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).

7. To avoid primer dimer formation, the ΔG of the primer must be greater (i.e., closer to zero) than –9 kcal/mole (–9kJ). The formation of primer dimers (hetero dimers and self dimers) can affect the efficiency of linear amplification during the mutant strand synthesis reaction.

8. Similary, 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.

9. Primers should not be methylated.

10. 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.

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, ***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
   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
   38.5 µl ddH_2O (to bring the final reaction volume to 50 µl)

   Then add

   1 µl of *PfuUltra* HF DNA polymerase (2.5 U/µl)

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

   Note *Set up a series of sample reactions using various amounts 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
   ddH_2O to a final volume of 50 µl

   Then add

   1 µl of *PfuUltra* HF DNA polymerase (2.5 U/µl)

4. Cycle each reaction using the cycling parameters outlined in Table I. (For the control reaction, use a 5-minute extension time and run the reaction for 18 cycles.)

TABLE I

Cycling Parameters for the QuikChange II 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.

5. Adjust segment 2 of the cycling parameters according to 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

6. Following temperature cycling, place the reaction on ice for 2 minutes to cool the reaction 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.*

Dpn I Digestion of the Amplification Products

1. Add 1 µl of the *Dpn* I restriction enzyme (10 U/µl) directly 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 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 µl of the supercompetent cells to a *prechilled* 14-ml Falcon round-bottom polypropylene tube.

2. Transfer 1 µl of the *Dpn* I-treated DNA from each control and sample reaction to separate aliquots of the supercompetent cells.

   As an optional control, verify the transformation efficiency of the XL1-Blue supercompetent cells by adding 1 µl of the pUC18 control plasmid (0.1 ng/µl) to a 50-µl 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 14-ml Falcon round-bottom polypropylene tubes.*

4. 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.

5. 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.

6. 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:*

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

If transformation of the pUC18 control plasmid was performed, >250 colonies should be observed (transformation efficiency >10^8 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 cells should be placed at –80°C directly from the dry ice shipping container.

Aliquoting Cells

When aliquoting, keep the XL1-Blue supercompetent 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 Falcon Round-Bottom Polypropylene Tubes

It is important that 14-ml Falcon round-bottom polypropylene tubes (Thermo Fisher Scientific p/n 352059) 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 these tubes.

Length of the Heat Pulse

There is a defined "window" of highest efficiency for transformation 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 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 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, 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 mutant DNA is synthesized in the reaction. Increase the amount of the *Dpn* I-treated DNA used in the transformation reaction to 4 µl.
	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.
	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 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 *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.
	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)

(Use for reduced satellite colony formation)
1 liter of LB agar
Autoclave
Cool to 55°C
Add 100 mg of 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)

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. Qi, R., & Otting, G. (2019) *PloS one*, 14(1), e0211065. https://doi.org/10.1371/journal.pone.0211065
8. Zhu, M., Yao, X., Wu, M., Qian, H., Wu, Y., & Chen, Y. (2016) *Molecular medicine reports*, 13(2), 1909–1913. https://doi.org/10.3892/mmr.2015.4688

MSDS INFORMATION

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

QuikChange II Site-Directed Mutagenesis Kit

Catalog #200523 and #200524

QUICK-REFERENCE PROTOCOL

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

  Note *Set up a series of sample reactions using various amounts of dsDNA template (e.g., 5, 10, 20, and 50 ng of dsDNA template).*

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
38.5 µl ddH_2O (for a final volume of 50 µl)

Sample Reaction

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
ddH_2O to a final volume of 50 µl

- Then add 1 µl of *PfuUltra* HF 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	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 µl of the *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 1 µl of the *Dpn* I-treated DNA from each control and sample reaction into separate 50-µl aliquots of XL1-Blue supercompetent cells (see *Transformation of XL1-Blue Supercompetent Cells* in the instruction manual)