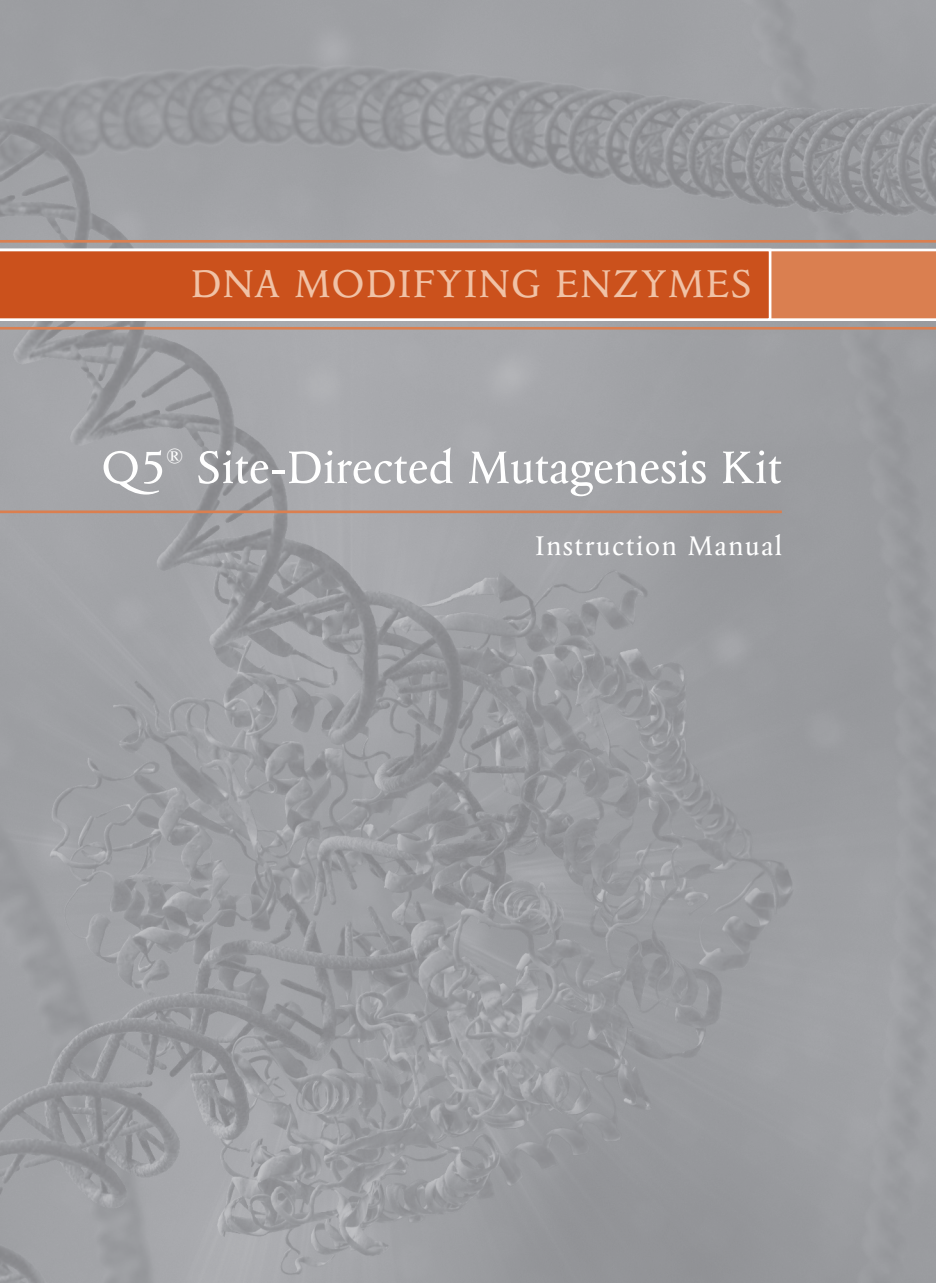
DNA MODIFYING ENZYMES

Q5® Site-Directed Mutagenesis Kit

Instruction Manual



NEB #E0554S
10 reactions
Version 1.0 1/13

NEW ENGLAND BioLabs® Inc.

be INSPIRED
drive DISCOVERY
stay GENUINE

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

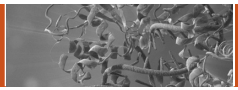


Table of Contents:

Kit Components:

The Q5 Site-Directed Mutagenesis Kit is stable at –80°C for one year. For convenience, the Q5 Hot Start High-Fidelity 2X Master Mix, KLD Enzyme Mix, KLD Reaction Buffer, Control Primers and Template DNA are packaged together in a separate box that can be removed and stored at –20°C for two years with no loss of activity. The SOC can be removed and stored at room temperature. It is important to store the NEB 5-alpha Competent E. coli at –80°C, and avoid repeated freeze-thaw cycles.

Q5 Hot Start High-Fidelity 2X Master Mix (2X)

KLD Enzyme Mix (10X)

KLD Reaction Buffer (2X)

Control SDM Primer Mix (10 µM each)

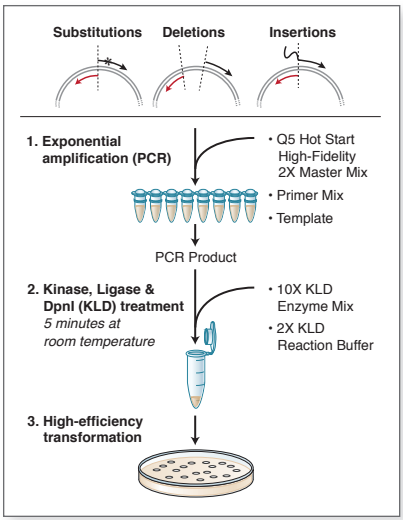
Control SDM Plasmid (5 µg/ml)

NEB 5-alpha Competent *E. coli* (High Efficiency)

pUC19 Transformation Control Plasmid (50 pg/µl)

SOC Outgrowth Medium

Q5 Site-Directed Mutagenesis Kit Quick Protocol:



Primers should be designed with 5´ ends annealing back-to-back. We recommend using the NEB online design software, NEBaseChanger™, which can be found at: NEBaseChanger.neb.com.

NEBaseChanger™

Step 1: Exponential Amplification

	25 µl RXN	FINAL CONC.
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl	1X
10 µM Forward Primer	1.25 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	0.5 µM
Template DNA (1–25 ng/µl)	1 µl	1-25 ng
Nuclease-free water	9.0 µl	

Cycling Conditions:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25 Cycles	98°C	10 seconds
	50–72°C*	10–30 seconds
	72°C	20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	

*For mutagenic primers, please use the Ta provided by the online NEB primer design software (NEBaseChanger.neb.com).

Step 2: KLD Reaction

	VOLUME	FINAL CONC.
PCR Product	1 µl	
2X KLD Reaction Buffer	5 µl	1X
10X KLD Enzyme Mix	1 µl	1X
Nuclease-free Water	3 µl	

Incubate for 5 minutes at room temperature.

Step 3: Transformation

1. Add 5 µl of KLD mix to 50 µl of chemically-competent cells.
2. Incubate on ice for 30 minutes.
3. Heat shock at 42°C for 30 seconds.
4. Incubate on ice for 5 minutes.
5. Add 950 µl SOC, gently shake at 37°C for 1 hour.
6. Spread 40–100 µl onto appropriate selection plate, incubate overnight at 37°C.

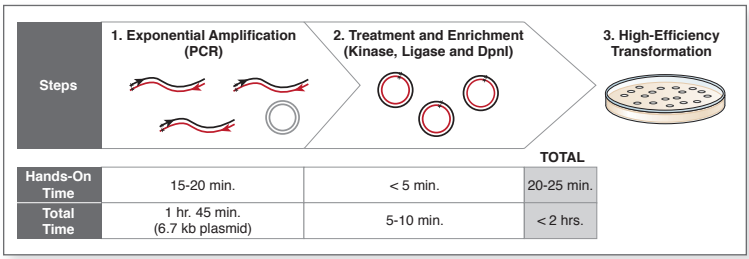
Introduction:

The Q5 Site-Directed Mutagenesis Kit enables rapid, site-specific mutagenesis of double-stranded plasmid DNA in less than 2 hours (Figure 1). The kit utilizes the robust Q5 Hot Start High-Fidelity DNA Polymerase along with custom mutagenic primers to create insertions, deletions and substitutions in a wide variety of plasmids. After PCR, the amplified material is added directly to a unique Kinase-Ligase-DpnI (KLD) enzyme mix for rapid (5 minutes), room temperature circularization and template removal (Figure 2). Transformation into high-efficiency NEB 5-alpha Competent *E. coli,* provided with the kit, ensures robust results with plasmids up to at least 14 kb in length.

Advantages:

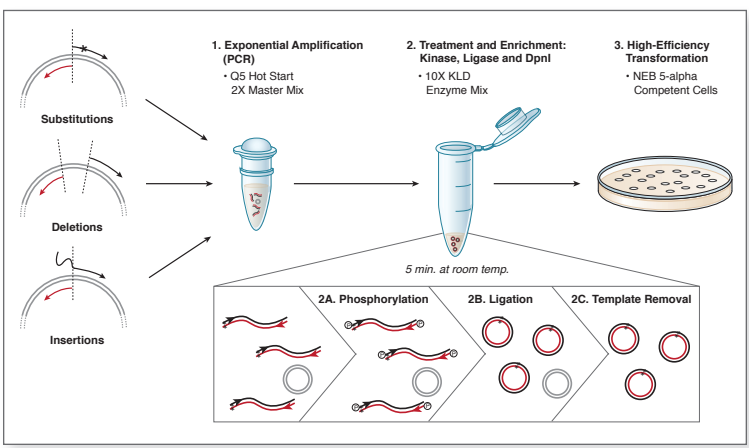
- Non-overlapping primer design ensures robust, exponential amplification, generating a high percentage of desired mutations from a wide range of templates
- Intramolecular ligation and transformation into NEB high-efficiency competent cells results in high colony yield
- Extremely low error rate of Q5 Hot Start High-Fidelity DNA Polymerase reduces screening time
- Hot start polymerase enables room temperature reaction set-up
- DpnI background reduction permits a wide range of starting template concentrations
- Use of standard primers eliminates additional expenses from phosphorylated or purified oligos
- Easy-to-use PCR master mix and unique multi-enzyme KLD mix offer convenience and quality
- Rapid and direct treatment step proceeds at room temperature in 5 minutes

Figure 1: Site-specific mutagenesis proceeds in less than 2 hours.



The use of a master mix, a unique multi-enzyme KLD enzyme mix, and a fast polymerase ensures that, for most plasmids, the mutagenesis reaction is complete in less than two hours.

Figure 2: Q5 Site-Directed Mutagenesis Kit Overview.



This kit is designed for rapid and efficient incorporation of insertions, deletions and substitutions into double-stranded plasmid DNA. The first step is an exponential amplification using standard primers and a master mix fomulation of Q5 Hot Start High-Fidelity DNA Polymerase. The second step involves incubation with a unique enzyme mix containing a kinase, a ligase and DpnI. Together, these enzymes allow for rapid circularization of the PCR product and removal of the template DNA. The last step is a high-efficiency transformation into chemically-competent cells (provided).

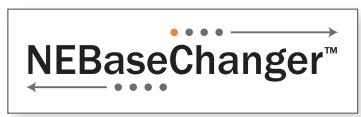
General Guidelines:

Primer Quality/Modification

Standard desalting of the oligonucleotides is recommended. Phosphorylation and purification are not required.

Primer Design

Although general recommendations are presented here, for best results, primers should be designed using NEBaseChanger™, the NEB online primer design software, which can be found at: NEBaseChanger.neb.com.



Substitutions:
Substitutions are created by designing a mismatch in the center of the mutagenic primer (Figure 3A). Include at least 10 nucleotides that are complementary to your plasmid at the 3´ end of the primer. To accomodate many mutations (from 7 to 50 per primer), changes should be incorporated at the 5´ end of the mutagenic primer.

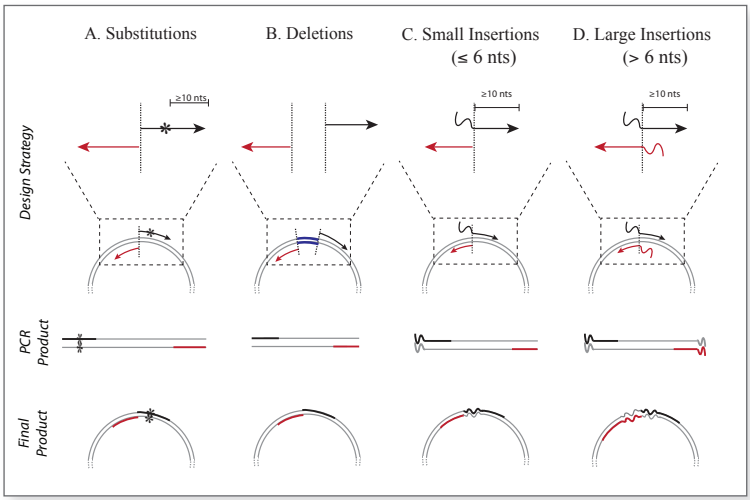
The 5´ end of the second primer will begin at the base next to the 5´ end of the first primer, and proceed in the opposite direction on the complementary strand. This second primer can be 100% complementary to the plasmid sequence or can contain mismatches, if desired. The absence of any overlap ensures that exponential (rather than linear) amplification will take place. The maximum size of the substitution is largely dictated by oligo synthesis limitations.

Deletions:
Deletions are created by designing primers that flank the region to be deleted (Figure 3B). The two primers should be designed in opposite directions with their 5´ ends adjacent to the area to be deleted. The primers can be 100% complementary to the plasmid sequence or can contain mismatches and/or insertions if desired.

Insertions:
The sequence to be inserted should be added to the 5´ end of the mutagenic primer (Figure 3C). For insertions of more than 6 nucleotides, the insertion sequence can be split between the two primers. Half of the new sequence should be added to the 5´ end of the forward primer and the other half should be added to the 5´ end of the reverse primer (Figure 3D). As described for substitutions, there should be at least 10 nucleotides that are complementary to your plasmid on the 3´ end of each primer.

The maximum size of the insertion is largely dictated by oligo synthesis limitations. Insertions of up to 100 nucleotides (50 nucleotides at the 5´ end of each primer) can routinely be accomodated using this kit.

Figure 3: Primer Design for the Q5 Site-Directed Mutagenesis Kit



Substitutions, deletions and insertions are incorporated into plasmid DNA through the use of specifically designed forward (black) and reverse (red) primers. ***Unlike kits that rely on linear amplification, primers designed for the Q5 Site-Directed Mutagenesis Kit should not overlap to ensure that the benefits of exponential amplification are realized.*** *A) Substitutions are created by incorporating the desired nucleotide change(s) (denoted by ∗) in the center of the forward primer, including at least 10 complementary nucleotides on the 3´side of the mutation(s). The reverse primer is designed so that the 5´ends of the two primers anneal back-to-back. B) Deletions are engineered by designing standard, non-mutagenic forward and reverse primers that flank the region to be deleted. C) Insertions less than or equal to 6 nucleotides are incorporated into the 5´end of the forward primer while the reverse primer anneals back-to-back with the 5´end of the complementary region of the forward primer. D) Larger insertions can be created by incorporating half of the desired insertion into the 5´ends of both primers. The maximum size of the insertion is largely dictated by oligonucleotide synthesis limitations.*

PCR Master Mix:

Q5 Hot Start High-Fidelity 2X Master Mix contains the polymerase buffer, dNTPs, Mg^{++} and Q5 Hot Start High-Fidelity DNA Polymerase.

For added convenience, Q5 Hot Start High-Fidelity DNA Polymerase is inactive at room temperature, allowing room temperature reaction set up. Due to the unique aptamer-based hot start format, there is no requirement for a separate activation step in the cycling protocol.

Note: After thawing Q5 Hot Start High-Fidelity 2X Master Mix completely, a precipitate (most noticeable after the first 1–2 freeze/thaw cycles) is not uncommon. To ensure optimal performance, the master mix should be thawed completely and resuspended prior to use. Stability testing using up to 15 freeze/thaw cycles has shown no negative effect on master mix performance.

Template:

We recommend starting with 1-25 ng of plasmid DNA; however, due to the robust nature of Q5 DNA Polymerase, experiments have also been successful using as little as 10 pg and as much as 100 ng of starting plasmid.

Volume:

The kit is designed for 25 µl PCR final reaction volumes, however, 50 µl reaction volumes work equally well. For either reaction volume, 1 µl of PCR product should be added to Step II (see page 8).

Enzyme Mix:

The 10X Kinase-Ligase-DpnI Mix (10X KLD) contains a blend of kinase, ligase and DpnI enzymes in a buffer that preserves the activity of the enzymes. This formulation allows efficient phosphorylation, intramolecular ligation/circularization and template removal in a single 5 minute reaction step at room temperature.

Competent Cells:

The Q5 Site-Directed Mutagenesis Kit protocols are optimized for use with the supplied high-efficiency NEB 5-alpha Competent *E. coli* cells. Other chemically-competent *E. coli* strains suitable for cloning may be substituted, but results will vary depending upon the quality and efficiency of the cells.

Protocol:

Step I: Exponential Amplification (PCR)

1. Assemble the following reagents in a thin-walled PCR tube.

	25 µl RXN	FINAL CONC.
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl	1X
10 µM Forward Primer	1.25 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	0.5 µM
Template DNA (1–25 ng/µl)	1 µl	1-25 ng
Nuclease-free water	9.0 µl	

2. Mix reagents completely, then transfer to a thermocycler.

3. Perform the following cycling conditions:

Thermocycling Conditions for a Routine PCR:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25 Cycles	98°C	10 seconds
	50–72°C*	10–30 seconds
	72°C	20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	

*For a Q5-optimized annealing temperature of mutagenic primers, please use NEBaseChanger™, the online NEB primer design software (NEBaseChanger.neb.com). For pre-designed, back-to-back primer sets, a Ta = Tm + 3 rule can be applied, but optimization may be necessary.

Step II: Kinase, Ligase & DpnI (KLD) Treatment

1. Assemble the following reagents:

	VOLUME	FINAL CONC.
PCR Product	1 µl	
2X KLD Reaction Buffer	5 µl	1X
10X KLD Enzyme Mix	1 µl	1X
Nuclease-free Water	3 µl	

2. Mix well by pipetting up and down and incubate at room temperature for 5 minutes.

Step III: Transformation

1. Thaw a tube of NEB 5-alpha Competent *E. coli* cells on ice.
2. Add 5 µl of the KLD mix from Step II to the tube of thawed cells. Carefully flick the tube 4-5 times to mix. Do not vortex.
3. Place the mixture on ice for 30 minutes.
4. Heat shock at 42°C for 30 seconds.
5. Place on ice for 5 minutes.
6. Pipette 950 µl of room temperature SOC into the mixture.
7. Incubate at 37°C for 60 minutes with shaking (250 rpm).
8. Mix the cells thoroughly by flicking the tube and inverting, then spread 50-100 µl onto a selection plate and incubate overnight at 37°C. It may be necessary (particularly for simple substitution and deletion experiments) to make a 10- to 40-fold dilution of the transformation mix in SOC prior to plating, to avoid a lawn of colonies.

Protocol for Control Reaction:

A control mutagenesis reaction is included in this kit, allowing blue/white screening of mutagenesis efficiency. The Control SDM Plasmid is 6.7 kb and contains a premature stop-codon at amino acid #23 in the *lacZ*α open-reading frame (1), as well as two BglII sites at positions #1948 and #2841. The Control SDM Primer Mix contains one primer that is 100% complementary to the Control SDM Plasmid and a second mutagenic primer. The mutagenic primer is designed to revert the aforementioned stop-codon to glutamine, thus allowing *lacZ*α expression and blue colony formation upon transformation and plating on media containing X-gal and IPTG. The mutagenic primer also removes the BglII site at position #2841, allowing for restriction endonuclease screening of positive colonies. A BglII digestion reaction of the control plasmid will yield two DNA fragments of 893 bp and 5.8 kb. After a successful mutagenesis reaction, only a single DNA fragment of 6.7 kb will be present after BglII digestion.

CONTROL SDM PRIMER MIX	
Forward	AACCCTGGCGttAcCcAACTTAATCG
Reverse	TTCCCAGTCACGACGTTGTAAAA

Step 1: Exponential Amplification (PCR)

For the control mutagenesis reaction, assemble PCR components as described in the following table:

	25 µl RXN	FINAL CONC.
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl	1X
Control SDM Primer Mix	1.25 µl	0.5 µM
Control SDM Plasmid	2 µl	10 ng
Nuclease-free water	9.25 µl	

Thermocycling Conditions for the Control PCR:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25 Cycles	98°C	10 seconds
	60°C*	20 seconds
	72°C	1 min 45 sec
Final Extension	72°C	2 minutes
Hold	4–10°C	

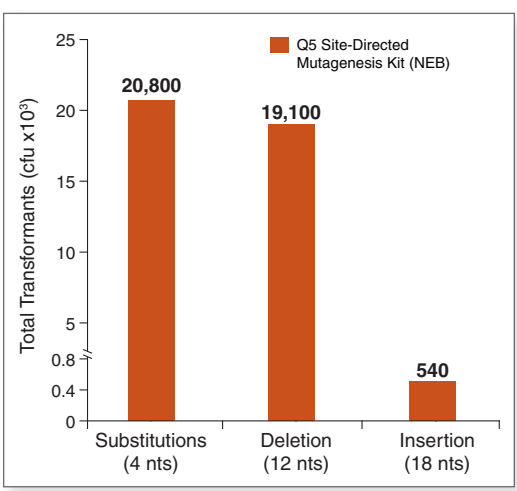
*although the control reaction works optimally with a 60°C annealing temperature, it can be successful with an annealing temperature from 54-68°C, allowing the control reaction to be cycled with similar experimental samples.

For Step II (KLD reaction) and Step III (Transformation), follow the protocol found on page 8. Prior to plating, make a 40-fold dilution of cells in room temperature SOC (25 µl of cells and 975 µl of SOC) to avoid a lawn of colonies. For blue/white screening, spread cells on room temperature LB plates containing 100 µg/ml of ampicillin, 40 µg/ml X-gal and 50 µg/ml IPTG (stock solutions of X-gal and IPTG are made in dimethylformamide to 40 mg/ml and 50 mg/ml, respectively). Allow the plates to grow overnight at 37°C. For optimal color development, let plates sit at room temperature or 30°C for an additional 24 hours.

Expected Results:

Exponential amplification with Q5 Hot Start High-Fidelity DNA Polymerase, combined with the intramolecular ligation reaction that precedes high-efficiency transformation, enables high numbers of transformants for simple mutagenesis experiments (substitutions and deletions), and more complex strategies (insertions). In contrast, linear amplification methods typically generate fewer colonies in substitution experiments and are unable to perform large deletions or insertions. Typical results from three different mutagenesis experiments with three different Q5 amplified site-directed templates are shown in Figure 4. For substitutions and deletions, thousands of colonies were observed with > 90% of the colonies having incorporated the desired mutation. An insertion of 18 nucleotides (6 amino acids for the addition of a 6X His tag) at the end of an open-reading frame still generated over 500 colonies. All isolates screened for the proper insertion sequences were found to be correct. As with all PCR experiments, success rate is critically dependent on proper primer design. We highly recommend designing primers using NEBaseChanger™, the NEB online primer design software, which can be found at: NEBaseChanger.neb.com.

Figure 4: Typical Results from Substitution, Deletion and Insertion Mutagenesis Reactions using the Q5 Site-Directed Mutagenesis Kit



Typical results from a substitution reaction (4 nt) using the Control SDM Primer Mix and Control SDM Plasmid (6.7 kb) are shown. For comparison, results from a 12 nt deletion experiment (5.8 kb plasmid) and an 18 nt insertion experiment (7.0 kb plasmid) are also shown. In all three cases, over 90% of the resultant colonies contained the desired mutation(s). Results are normalized to the total number of transformants if cells were not diluted prior to plating.

Troubleshooting

No/Low Colonies

- Ensure that your primers are designed properly. To take advantage of the exponential nature of the amplification reaction, the 5´ ends of the two primers should align back-to-back unless deletions are being made (see Figure 3). For best results, primers should be designed and annealing temperatures calculated using NEBaseChanger™, the NEB online primer design software, which can be found at: NEBaseChanger.neb.com.
- Ensure there is a clean PCR product by visualizing 2–5 µl of the reaction on an agarose gel. Follow the suggestions below for low or impure PCR products.
- Only use 1 µl of PCR product in the KLD reaction. Carrying too much PCR product forward can decrease transformation efficiency. If the PCR yield is low, more product can be added to the KLD reaction, however a buffer exchange step, such as PCR purification, must be included prior to transformation.
- Only use 5 µl of the KLD reaction in the transformation. If more KLD reaction is added, a buffer exchange step, such as PCR purification, should be included prior to transformation.
- Ensure that the selectable marker in the plasmid matches the selection agent used in the plates
- Ensure the NEB 5-alpha Competent *E. coli* cells have been stored at –80° C.
- Check that the transformation efficiency of the competent cells is ~1 x 10^9 colony forming units (cfu) per µg. To calculate transformation efficiency, transform 2 µl of the provided control pUC19 DNA (100 pg) into 50 µl of cells. Follow the transformation protocol on page 8. Prior to plating, dilute 10 µl of cells up to 1 ml in SOC. Plate 100 µl of this dilution. In this case, 150 colonies will yield a transformation efficiency of 1.5 x 10^9 cfu/µg (µg DNA=0.0001, dilution=10/1000 x 100/1000).

No/Low PCR Product

- Ensure that the optimal annealing temperature (Ta) is used. High-Fidelity polymerases benefit from a Tm+3 annealing temp. Use NEBaseChanger™, the NEB online primer design software, to calculate Ta (NEBaseChanger.neb.com). Alternatively, the optimal annealing temperature could be determined using a gradient PCR followed by agarose gel analysis.
- Ensure that the elongation time is adequate for the plasmid length. We recommend 20–30 seconds per kb of plasmid.
- Ensure that the final concentration of each primer is 0.5 µm.
- Purify the primers with polyacrylamide gel electrophoresis (PAGE).

Resulting Plasmids Do Not Contain the Desired Mutation

- Ensure proper design of the mutagenic primers
- Optimize the PCR conditions (see above)
- Use 1–25 ng of template in the PCR step. A small increase in the number of clones with no/incorrect mutation incorporated can occur if less than 1 ng or more than 25 ng of template is used.

Quality Controls

Product specifications for individual components in the Q5 Site-Directed Mutagenesis Kit are available separately. The Q5 Site-Directed Mutagenesis Kit has been tested using the control plasmid and primer mix provided in the kit.

Reference:

(1) Kalnins et al., (1983) *The EMBO Journal* 2, 593-597.

Ordering Information

PRODUCT	NEB #	SIZE
Q5 Site-Directed Mutagenesis Kit	E0554S	10 reactions
COMPANION PRODUCTS		
Q5 Hot Start High-Fidelity 2X Master Mix	M0494S/L	100/500 rxns
NEB 5-alpha Competent *E. coli* (High Efficiency)	C2987I/H	6 x 0.2 ml/ 20 x 0.05 ml
SOC Outgrowth Medium	B9020S	4 x 25 ml

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