Q5® Site-Directed Mutagenesis (E0554)

New England Biolabs

Abstract

This is the protocol for the Q5® Site-Directed Mutagenesis Kit (E0554)

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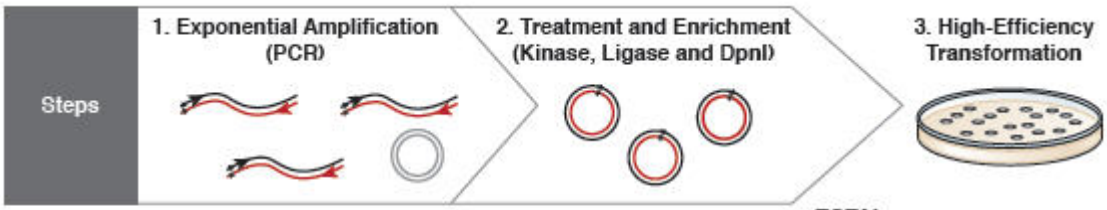
Guidelines

DESCRIPTION

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 20 kb in length.



			TOTAL
Hands-On Time	15-20 min.	< 5 min.	20-25 min.
Total Time	1 hr. 45 min. (6.7 kb plasmid)	5-10 min.	< 2 hrs.

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.

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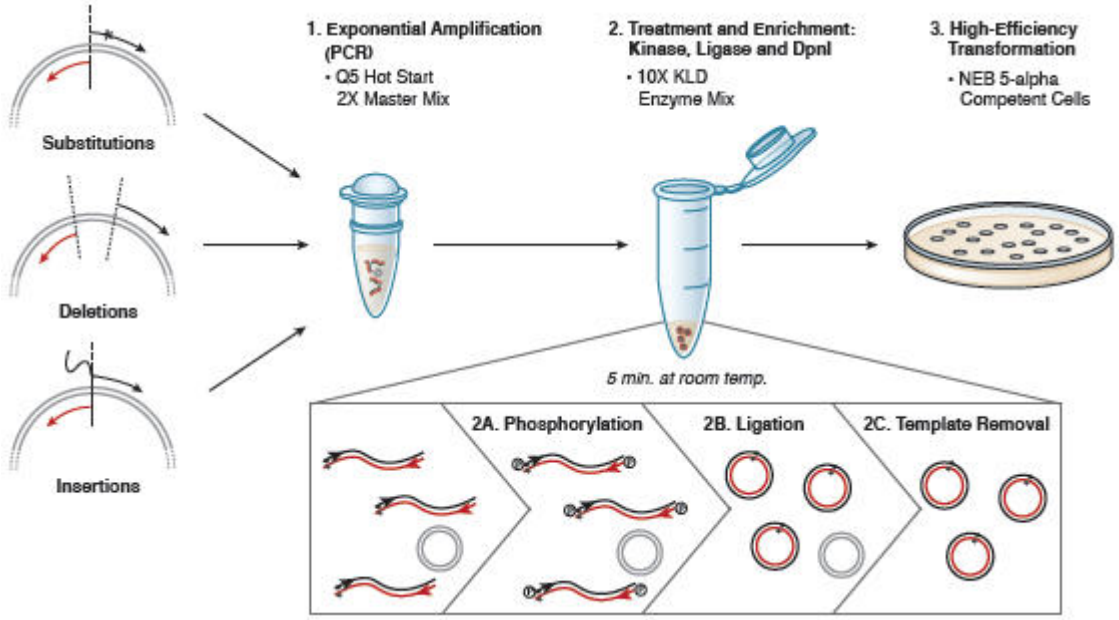

Figure 2: Q5 Site-Directed Mutagenesis Kit Overview.
This kit is designed for rapid and efficient incorporation of insertions, deletions and substitutions into doublestranded plasmid DNA. The first step is an exponential amplification using standard primers and a master mix fomulation of Q5 Hot Start 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 chemicallycompetent cells (provided).

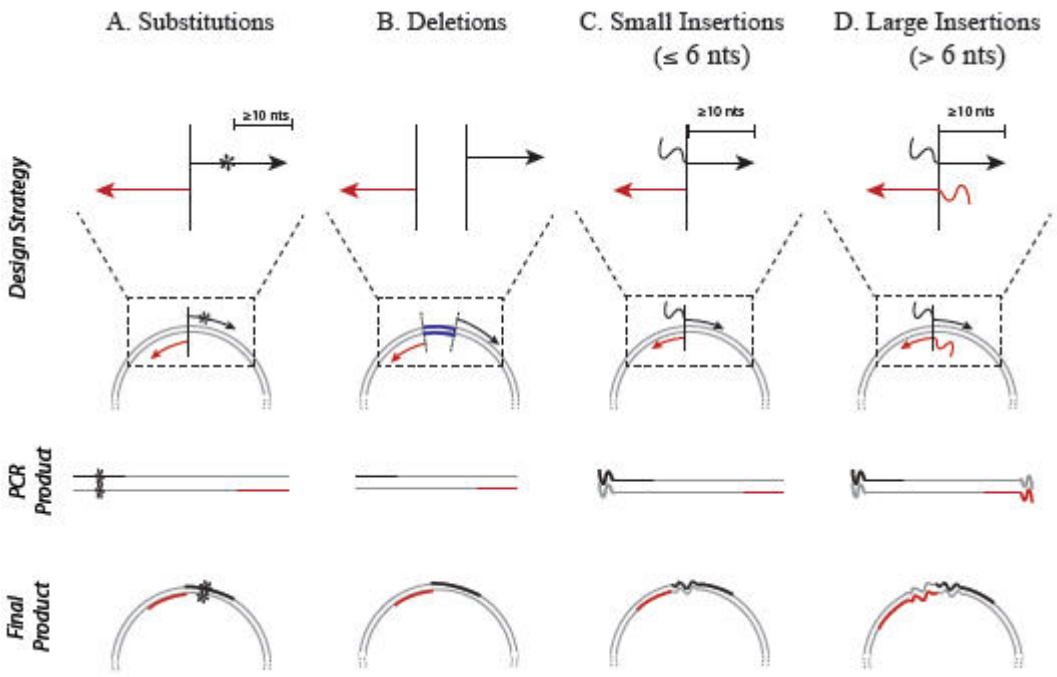

Figure 3: Primer Design for the Q5 Site-Directed Mutagenesis Kit
Substitutions, deletions and insertions are incorporated into plasmid DNA through the use of specifically designed forward (black) and reverse (red) primers. ***Unlike kits that rely on linear amplification, primers designed for the Q5 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*

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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.

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.
- 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 109 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 109 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. 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 10–20 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.

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NOTES
Storage Note:
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.

REFERENCES
1. Kalnins et al., (1983). The EMBO Journal. 2, 593-597.
2. Dickinson DJ, Ward JD, Reiner DJ, Goldstein B. (2013). Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination.. Nat Methods. Sep 1, PubMedID: 23995389

Materials

Q5 Site-Directed Mutagenesis Kit - 10 rxns E0554S by New England Biolabs

Protocol

Exponential Amplification (PCR)

Step 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	

PROTOCOL

. E0552 Q5 PCR Mixture

CONTACT: New England Biolabs
Step 1.1.
Q5 Hot Start High-Fidelity 2X Master Mix **12.5 ul**

Step 1.2.
10 µM Forward Primer **1.25ul**

Step 1.3.
10 µM Reverse Primer **1.25ul**

Step 1.4.
Template DNA (1–25 ng/µl) **1ul**

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Step 1.5.
Nuclease-free water **9ul**

Exponential Amplification (PCR)

Step 2.
Mix reagents completely.

Exponential Amplification (PCR)

Step 3.
Transfer to a thermocycler and 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	

ANNOTATIONS

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

Kinase, Ligase & DpnI (KLD) Treatment

Step 4.
Assemble the following reagents:

PROTOCOL

. E0552 KLD Mixure

CONTACT: New England Biolabs

Step 4.1.
PCR Product **1ul**

Step 4.2.
2X KLD Reaction Buffer **5ul**

Step 4.3.
10X KLD Enzyme Mix **1ul**

Step 4.4.
Nuclease-free Water **3ul**

Kinase, Ligase & DpnI (KLD) Treatment

Step 5.
Mix well by pipetting up and down.

Kinase, Ligase & DpnI (KLD) Treatment

Step 6.
Incubate at room temperature for 5 minutes.

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DURATION
00:05:00

Transformation

Step 7.
Thaw a tube of NEB 5-alpha Competent *E. coli* cells on ice.

Transformation

Step 8.
Add **5 µl** of the KLD mix from the "KLD Section" above to the tube of thawed cells.

Transformation

Step 9.
Carefully flick the tube 4-5 times to mix. **Do not vortex.**

Transformation

Step 10.
Place the mixture on ice for 30 minutes.

DURATION
00:30:00

Transformation

Step 11.
Heat shock at 42°C for 30 seconds.

DURATION
00:00:30

Transformation

Step 12.
Place on ice for 5 minutes.

DURATION
00:05:00

Transformation

Step 13.
Pipette 950 µl of room temperature SOC into the mixture.

AMOUNT
950 µl Additional info:

REAGENTS
SOC Outgrowth Medium - 100 ml B9020S by New England Biolabs

Transformation

Step 14.
Incubate at 37°C for 60 minutes with shaking (250 rpm).

DURATION
01:00:00

Transformation

Step 15.
Mix the cells thoroughly by flicking the tube and inverting.

ANNOTATIONS
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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

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Transformation

Step 16.

Spread 50-100 µl onto a selection plate.

Transformation

Step 17.

Incubate overnight at 37°C

DURATION

15:00:00

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