Large Insertions: Two Simple Steps Using Quikchange® II Site-Directed Mutagenesis Kits

Abstract

This Technical Note describes a modified method to introduce large insertions in plasmids in two simple steps using the QuikChange® II Site-Directed Mutagenesis kit. Dr. Wenge Wang in Dr. Wafik S. El-Deiry's lab at the University of Pennsylvania has used the QuikChange II kit and a modification of the method described by Geiser et al. (2001)[1] to perform a large insertion of a PCR product into a target plasmid. He inserted the enhanced green fluorescent protein (EGFP) open reading frame (760 bp) to the C-terminus of death receptor TRAIL receptor 1/death receptor 4 (TRAIL-R1/DR4) right after the signal sequence, using the QuikChange II Site-Directed Mutagenesis kit with some modifications to the basic protocol. Dr. Wang transfected cells with the recombined plasmid and it generated a fusion protein with the correct molecular size (Figure 1).

BACKGROUND

Dr. Wafik S. El-Deiry's lab is focused on studying the mechanism of action of the tumor suppressor p53 and the contribution of its downstream target genes to control cell growth. Their lab has identified a number of genes that are directly regulated by p53 and which can inhibit cell cycle progression (p21WAF1), induce apoptosis (KILLER/DR5, Bid, caspase 6, Traf4 and others) or activate DNA repair (DDB2). The research has provided knowledge into the tissue specificity of the DNA damage response *in vivo* and into the mechanism by which wild-type p53 sensitizes cells to destruction by anti-cancer drugs. The lab is studying the regulation of p53 activity through control of its stability and its target gene activation. An area of focus in the lab that resulted from studies on p53 involves analysis of the cell death pathway and its activation by the death ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). The lab's work on the TRAIL pathway has involved studying the mechanisms of sensitivity and resistance of

cancer cells, studying the intracellular signaling events that control the activation of caspases and studies of how cell death occurs with respect to mitochondrial involvement. TRAIL may have potential as a new anti-cancer agent, as binding to the death receptors TRAIL receptor 1/death receptor 4 (TRAIL-R1/DR4) or TRAIL receptor 2/death receptor 5 (TRAIL-R2/DR5) can trigger apoptosis in cancer cells.

High-Efficiency Mutagenesis Method

Mutagenesis is a powerful tool in protein discovery and engineering. Our QuikChange Site-Directed Mutagenesis kits offer unparalleled advantages in rapid and accurate mutagenesis of your target gene. In addition to its quick and easy protocol, you have the advantage of altering your DNA sequence without the limitations of restriction enzyme-based sub-cloning methods.

Our QuikChange II and QuikChange II XL kits offer a one-day method to introduce point mutations, amino acid substitutions, small insertions, and deletions in virtually any double-stranded plasmid template at efficiencies up to 100% (Figure 2). These kits feature our highest fidelity *PfuUltra*® DNA polymerase and a linear amplification strategy. Together, these reduce the mutation frequency due to incorporation errors typically caused by using an exponential PCR amplification approach. The result is high-efficiency mutagenesis without unwanted errors.

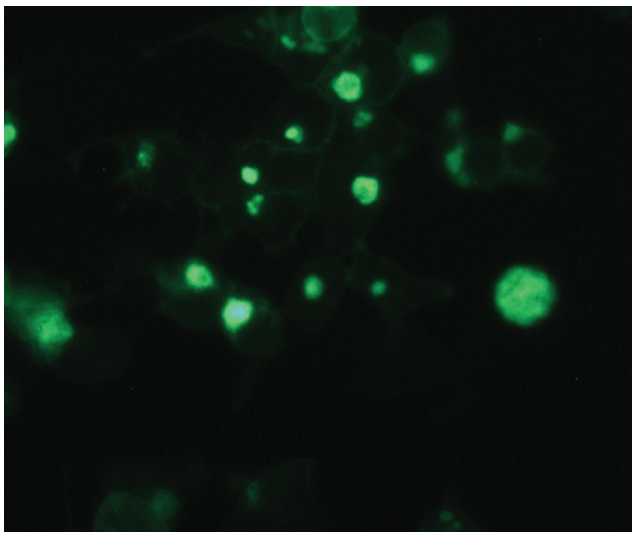

Figure 1
Enhanced green fluorescent protein (EGFP) was inserted in the open reading frame (760 bp) to the C-terminus of death receptor TRAIL receptor 1/death receptor 4 (TRAIL-R1/DR4) right after the signal sequence. Cell staining shows the member protein fused with EGFP.

Ideal for Large Insertions and Deletions

Insertion and deletion studies offer the ability to map discrete functional areas of genes, to modify vectors, to introduce affinity tags, and to correct frame shifts. However, inserting or deleting large regions of DNA at specific locations can be problematic, as it typically relies on rare or non-existent restriction sites or multiple-step PCR protocols that are time-consuming and introduce unwanted errors. We have used the QuikChange kit method for small insertions and deletions (~12 bp) and observed greater than 80% efficiency[2]. Many of our customers have used the QuikChange method or have modified it to introduce deletions of 31 bp[3], 87 bp[4], and 3 kb[5] or insertions of 31 bp[3] and up to ~1 kb[5]. In order to achieve large insertions of up to 1 kb, megaprimers must be generated from an initial PCR reaction. To ensure the highest mutagenesis efficiency, we recommend gel purification with our StrataPrep® PCR Purification Kit. To create the megaprimers, a minimum of 20 bp upstream and downstream of the insertion sequence should be complementary to your template vector that will be used in the QuikChange kit reaction. Therefore, each oligo used in the initial PCR reaction will require 20 bp overhangs on the 5′ ends (Figure 3).

Using the QuikChange kit for large deletions is even easier since oligos can be synthesized, rendering the initial PCR reaction and fragment purification unnecessary. Again, 20 to 30 bp may be required to bind to your template DNA both upstream and downstream of the region that you want to delete. However, the largest oligo required for most deletions should not exceed 60 bp.

This Technical Note describes performing a large insertion that can be completed in two simple steps. First, PCR-amplify the megaprimer. Second, add your gel purified megaprimer to our QuikChange Site-Directed Mutagenesis kit (Figures 2 and 3). This strategy avoids tedious and time-consuming sub-cloning. With our QuikChange kits, you will have confidence in generating an error free clone while saving valuable time.

Materials & Methods

Part I. PCR reaction to generate the megaprimer

Please refer to the appendix section for the ***PfuUltra*® II Fusion HS DNA polymerase** reaction and cycling conditions.

***PfuUltra*® II Fusion HS DNA polymerase** (Stratagene)

Thermal Cycler used: TECHNE: Touchgene® Gradient

- The forward primer: GACTCCGAATCCCGGGAGC GCAGCG-GTGAGCAAGGGCGAGGAG, the first 25 bp overlaps the target vector, the remaining primer sequence is complementary to EGFP. The primers were resuspended in 50 mM NaCl.
- The reverse primer: CGCGGCTGCCTCTGTCCCACT-CTTGTACAGCTCGTCCATGCC, the first 22 bp targets the vector and the remaining primer sequence is complementary to EGFP. The primers were resuspended in 50 mM NaCl.
- Template for PCR reaction: 200 ng of plasmid DNA (pEGFP-N1; Clontech)
- PfuUltra® II Fusion HS DNA Polymerase (Stratagene)
- PCR product was purified with Qiagen's QIAEX® II Gel extraction kit
- The quantity of the PCR product was estimated on agarose gel with Ethidium Bromide

Note: The size of the PCR product is 760 bp, which includes the insert (EGFP) of 714 bp plus overlapping sequence of the target vector.

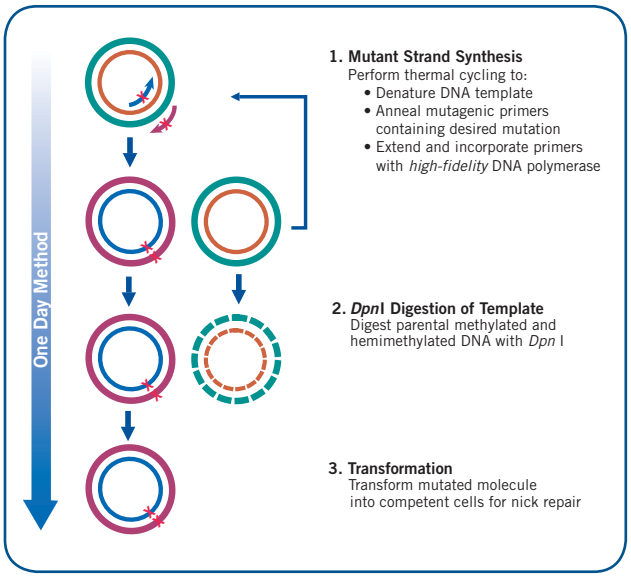

Figure 2
The QuikChange® II One-Day Site-Directed Mutagenesis Method
1. Mutant strand synthesis. 2. *Dpn* I Digestion of parental DNA template. 3. Transformation of the resulting annealed double-stranded nicked DNA molecules. After transformation, the XL-1 Blue *E. coli* cell repairs nicks in the plasmid.

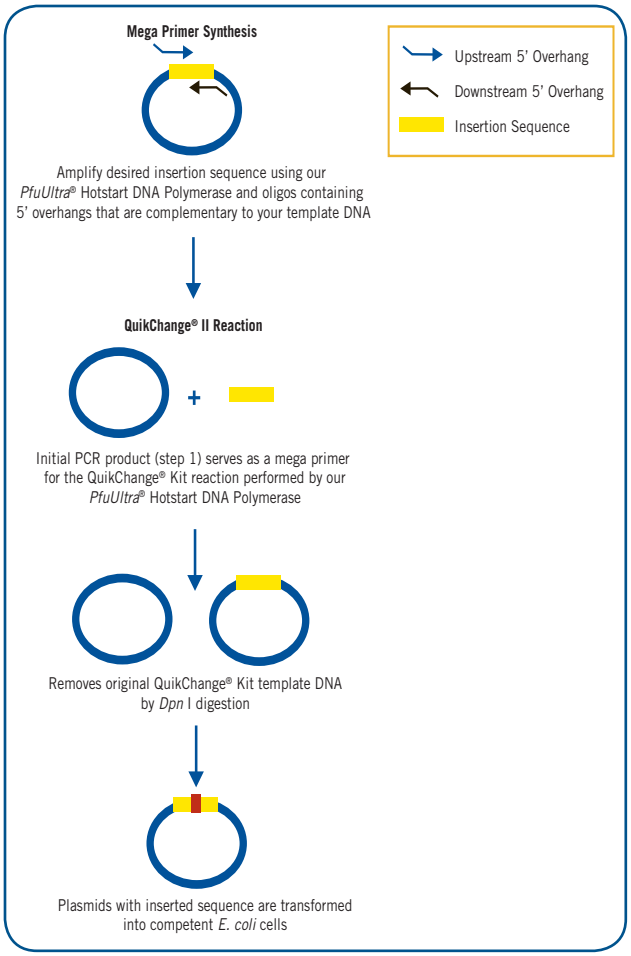

Figure 3
Mechanism for generating large insertions of a PCR product into a target plasmid DNA using the QuikChange® II Mutagenesis kit.

Part II. The QuikChange® II mutagenesis reaction can be set up using the following guidelines (Tables 1 and 2).

- Primer for the insertion reaction 300-500 ng of PCR product (760 bp EGFP) to serve as a megaprimer.

TABLE 1
REACTION SET-UP

Component	Amount per reaction
Distilled water (dH_2O)	X µl
10x QuikChange reaction buffer	5.0 µl
dNTP mix	1 µl
DNA template (50 ng)	X µl
Megaprimer (300 - 500 ng)	X µl
High Fidelity DNA polymerase (2.5 U/µl)	1.0 µl (2.5U)
Total reaction volume	**50 µl**

TABLE 2
CYCLING METHOD

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	5	95°C	30 seconds
		52°C	1 minute
		68°C	1 minute/kb of plasmid length* (7 min)
3	13	95°C	30 seconds
		55°C	1 minute
		68°C	1 minute/kb of plasmid length* (7 min)

* For example, a 7 kb plasmid would have a 7 minute extension time

Results

The results of this mutagenesis experiment demonstrate that large PCR fragments can be successfully inserted into the target plasmid DNA. The 760 bp PCR fragment had at each end only short regions of homology where the insertion occurred. There were approximately 100 colonies produced. About 20 colonies were selected for further analysis of mutagenesis efficiency, and they were 100% positive by sequencing.

Note: Follow reaction with *Dpn* I digestion and transformation per the QuikChange II manual. For *PfuUltra* II Fusion HS DNA polymerase information, please refer to the manual.

Acknowledgments
This Technical Note was written based on data provided by Wenge Wang, M.D., Ph.D. in Dr. Wafik El-Deiry's Lab, Division of Hematology and Oncology in the Department of Medicine at the University of Pennsylvania.

References
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