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

Application Note

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Abstract

This Application 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).

Introduction

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.

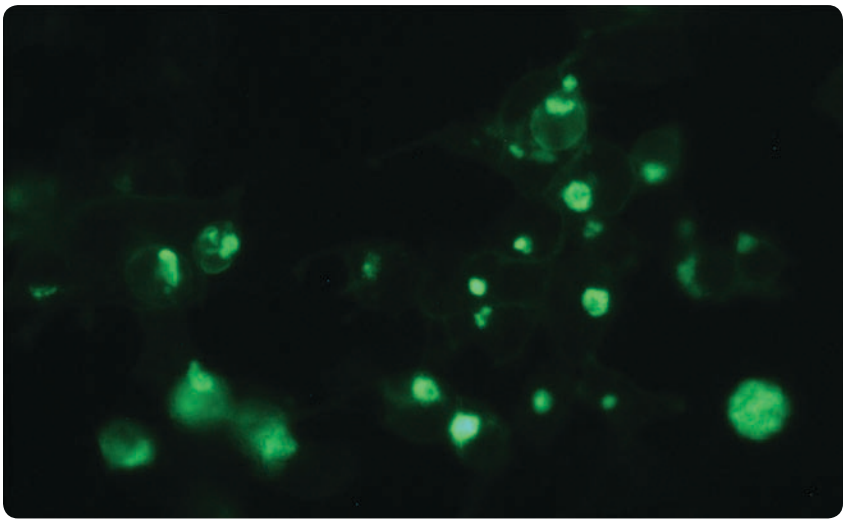

Figure 1

Enhanced Green Fluorescent Protein

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.

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 greater than 80% (Figure 2). These kits feature our high-fidelity *PfuUltra* DNA polymerase and a linear amplification strategy. Together, these features reduce unwanted secondary mutations that result from incorporation errors in exponential PCR amplification. The result is high-efficiency mutagenesis without unwanted errors.

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 reaction. Therefore, each oligo used in the initial PCR reaction will require 20 bp overhangs on the 5′ ends (Figure 3).

Using the QuikChange II kit for large deletions is even easier since oligos can be synthesized, rendering the initial PCR

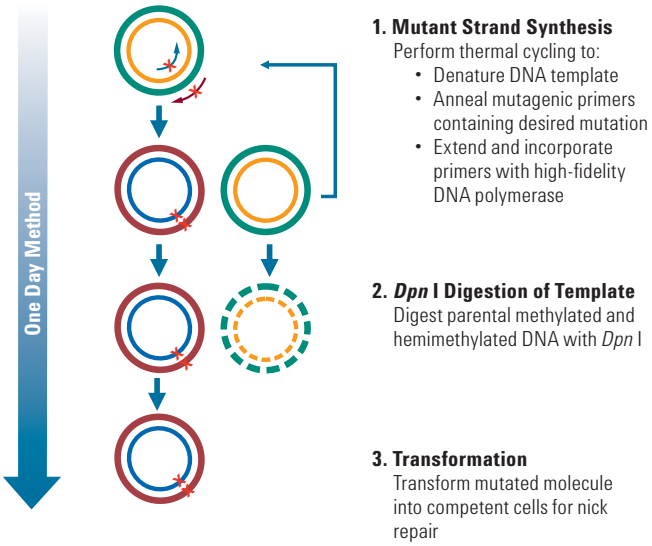

Figure 2
The QuikChange II One-Day Site-Directed Mutagenesis Method
1. Mutant strand synthesis. 2. *Dpn* I Digestion of parental DNA template. 3. Transformation of the resulting annealed double-stranded nicked DNA molecules. After transformation, the XL-1 Blue *E. coli* cell repairs nicks in the plasmid.

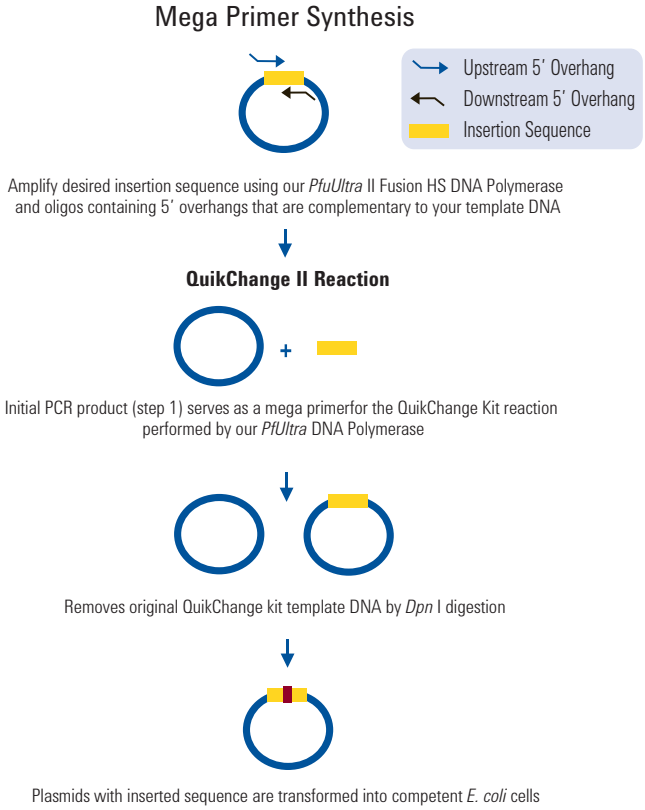

Figure 3
Generating Large Insertions
Mechanism for generating large insertions of a PCR product into a target plasmid DNA using the QuikChange II Mutagenesis Kit.

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 Application 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 II Kit (Figures 2 and 3). This strategy avoids tedious and time consuming subcloning. With our QuikChange II Kit, you will have confidence in generating an error free clone while saving valuable time.

Materials and Methods

Part I.
PCR Reaction to Generate the Megaprimer
Please refer to the *PfuUltra* II Fusion HS DNA Polymerase manual for reaction and cycling conditions.

***PfuUltra* II Fusion HS DNA Polymerase (Agilent)**
Thermal Cycler used: TECHNE Touchgene Gradient

- The forward primer: GACTCCGAATCCCGGGAGCGCAGCG-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: CGCGGCTGCCTCTGTCCCACTCTTGTACAGCTCGTCCATGCC, 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 (Agilent)
- 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

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.
- Follow reaction with *Dpn* I digestion and transformation per the QuikChange II manual.

Component	Amount per reaction
Distilled water (dH2O)	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 1
Reaction Set-up

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

Table 2
Cycling Method

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.

References

1. Geiser, M., et al. (2001) *Biotechniques 31*: 88-92.
2. Papworth, C., Braman, J., and Wright, D.A. (1996) *Strategies 9*: 3-4.
3. Wang, W. and Malcolm, B. (1999) *Biotechniques 26*: 680-682.
4. Burke, T., et al. (1998) *Oncogene 16*: 1031-1040.
5. Makarova, O., et al. (2000) *Biotechniques 29*: 970-972.

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