Efficient Method for Genomic DNA Mutagenesis in *E. coli*

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Abstract

Objectives: Mutagenesis is a process used to generate the modified DNA sequences either by mutating, insertion, substitution, and/or deletion of codons. Mutagenesis is an effective means to introduce the changes to a protein, which is important for its mechanistic and functional studies. A variety of methods have been developed to introduce specific base changes at expected sites into target DNA sequences. However, a simple, quick, and effective method is still eluding. In present work, we have described a rapid and efficient method to perform site directed mutagenesis, multiple-site fragment deletion, insertion, and substitution mutagenesis based on a modified version of overlap extension by polymerase chain reaction (PCR). Results: For our modified overlap extension PCR method, we divided target gene into several fragments based on the site of mutagenesis, and then amplified the DNA fragments. These fragments were then annealed together with their complementary overhanging,

followed by extension and amplification by PCR to get full length gene with expected mutation. The full-length gene was placed into a vector, and the plasmid carrying the target gene was screened by colony PCR. By using this method, we have successfully generated three single-site mutations, replaced/ deleted a 200bp DNA fragment into/ from a target gene, and engineered a cysteine-free protein. Conclusions: The method yields various mutants rapidly, reliably and with high fidelity. It provides an efficient choice, especially for multiple-site or large DNA fragment modification mutagenesis. Therefore, this method can be utilized to generate desirable mutants.

Introduction

Site-specific mutagenesis of DNA, which allows deleting, inserting, or substituting multiple-site DNA fragment, is a very important tool in molecular biology, genetic engineering, biochemistry and protein engineering. A variety of methods have been applied to introduce specific base/bases changes at expected sites into target DNA sequences [1-9]. The QuikChange™ Site-Directed Mutagenesis System developed by Stratagene (La Jolla, CA), which works by using a pair of complementary primers with a mutation, has been shown to be a powerful tool for site-directed mutagenesis. However, the presence of parent template has shown to give higher false positives. As the primers completely overlap, self annealing may lead to the formation of "primer dimers" by partial annealing of a primer with the second primer in reaction, and

formation of tandem repeats of primers, thus, reducing the yield of successful transformants [10, 11]. For QuikChange™ site-directed mutagenesis, DNA sequencing is required to confirm the mutants. As originally developed QuikChange™ cannot introduce multiple mutations as well as long DNA fragment deletion, insertion or replacement mutations, a modified version of the kit (QuikChange™ Multi Site-Directed Mutagenesis kit) has been released and some other adaptations have been reported [12-18]. However, these procedures always require special designing of primers and/or can only be used for certain specific kind of mutagenesis. So far there is no simple, yet high efficient method that allows deleting, inserting, or substituting multiple-site DNA fragments. These conundrums prompted us to consider a new method of mutagenesis that would follow the rule of simplicity but have promising efficiency and applicability. Overlap extension by polymerase chain reaction (OE-PCR), described by [1, 19], has been shown the most powerful tools to generate mutagenesis. In our present work, we reported a modified overlap extension PCR method that allows us to get almost any kind of site-specific mutagenesis. For our modified method, first of all the target gene was divided into several fragments based on the site of mutation, and then amplified these DNA fragments, which were annealed together with their complementary overhanging, cohesive ends, and extended and amplified by following PCR. As we only amplified small DNA sequences, it is easy to get a high yield of these DNA fragments, which are always high fidelity [20]. The modified gene was

placed into a vector. The plasmids containing the desired insert can be screened by colony PCR directly from bacterial colonies. For this method, primers of special design were not required. In the present work, we have achieved various mutations, including single-site mutation, multiple-site mutation (13 mutants), insertion (200 bp) and deletion (200 bp) in *ERCC8* (Excision Repair Cross-Complementation Group 8) gene in humans by using this method. This modified procedure has proven to be simple but high in efficiency and application [21, 22].

Materials

Human cDNAs was purchased from Clontech. KOD hot start DNA polymerase was purchased from Novagen, restriction endonucleases, DNA marker, Taq DNA polymerase, and T4 DNA ligase from New England Biolabs, cloning kits from Qiagen. Vector pET30a, host strain *Escherichia coli* DH5α were obtained from Invitrogen Corp. Oligonucleotide primers were purchased from Invitrogen Corp. The PCR purification kit and gel extraction kit were purchased from Qiagen. The plasmids were isolated using a QIAprep Spin Miniprep Kit (Qiagen). Mutations were checked by DNA sequencing.

Methods and Results

Vector

In order to generate a parent vector necessary for annealing of mutated gene of interest, either of two methods was used. As shown in Fig. 1A, vector was gel purified upon restriction digestion and removal of already existing insert but

with restriction sites intact. Or use PCR method to generate a linear vector with two restriction recognition sites at each end (Fig. 1B). After purification by gel electrophoresis, PCR products were digested by restriction enzymes. The purified product was then digested by Dpn1 and allowed to go through another purification step using QIAquick® PCR purification kit to eliminate the residual contaminations of template.

Single-site mutagenesis

In this study, we used *ERCC8* gene (NCBI Reference Sequence: NM_000082.3, 1191 bp) which encodes DNA excision repair protein ERCC-8 in humans (NCBI Reference Sequence: NP_000073.1, 396 aa) as an example (see Supplementary Figure S1). *ERCC8* gene was amplified from a cDNA library (Clontech) and inserted into pET30a between EcoRI and HindIII restriction recognition sites. Figure 2 shows the scheme of the PCR amplification processes (A) and the primers design (B) used to generate single-site mutation. To generate single-site mutation (Figure 2A, filled black triangle), we designed four primers: primer 1, forward primer, which has a ~25 bp homologous sequence to the positive strand and an additional restriction enzyme cut site at 5' end; primer 2, reverse primer, which has a ~25 bp sequence that is homologous to the negative strand and an additional restriction enzyme cut site at 3' end; primer 3, which is a ~30 bp homologous sequence to the positive strand with mutated bases in the center primer; primer 4, which is a ~25 bp sequence and complementary to the primer 3 at 5' end

(Figure 2B). First, we used two pairs of primers, primer 1/ primer 4 and primer 3/ primer 2 to generate two DNA fragments using target gene or a vector that has been previously subcloned by a target gene as template. The PCR products were purified by agarose gel electrophoresis followed by gel extraction. These two DNA fragments have cohesive end at 5' or 3', which are therefore able to anneal together with their complementary overhanging cohesive ends, which are then extended and amplified by PCR using the primer pair 1/2 to get a full length gene with expected mutant (Figure 2A).

In our example, we will use EcoRI and HindIII to ligate target gene into the recipient plasmid. We designed primer 1 (ERCC8EcoRIfw), forward primer, which will use the sequence 5'- ATGCTGGGGTTTTTGTCCGCAC-3' for the region that binds the ORF and we will add the EcoRI restriction site (GAATTC) plus three bases (CCG) flanking that site to the 5' end of this primer [23], making our forward primer 5'-CCGGAATTCATGTGGCATATCTCGAAGTAC-3'. Also we designed primer 2 (ERCC8HindIIIrv), reserve primer, which will use the sequence 5'-TCATCCTTCTTCATCACTGCTGC-3' for the region that binds the ORF and we will add the HindIII restriction site (AAGCTT) plus three bases (CCC) flanking that site to the 5' end of this primer, making our reserve primer 5'- CCCAAGCTTTCATCCTTCTTCATCACTGCTGC-3'. To help the mutagenic oligonucleotide primers design, we used an automated web site (http://bioinformatics.org/primerx). In this method, melting temperature, GC content, the length, and complementary of primers are not severely limited. In

general, the mutation site can be placed as close as ten bases away from the 5'-terminus or 3'-terminus, and at least one G or C should be placed at the end of each terminus. To facilitate the primer design, we always design two mutagenic oligonucleotide primers: one has mutation in the middle of the primer with ~10–15 bases of correct sequence on both sides and at least one G or C at the end of each terminus; The other one has ~25 bp primer-primer complementary (overlapping) sequences at the 5' end. The schematic presentation of our new primer design is shown in Figure 2B. To evaluate the efficiency of this method for the generation of mutation, three residues at different position of ERCC-8 protein, i.e. S23, K212, and Y350 were selected for cysteine-replacement mutagenesis. The properties of designed primers are shown in Table 1 and Figure S2.

Two step PCR reactions were performed to introduce a mutation at a specific point in a final volume of 50 ul using High fidelity DNA polymerase, KOD hot start DNA polymerase (Novagen) by using the primer pair of ERCC8EcoRIfw/ ERCC8S23Crv, ERCC8S23Cfw/ ERCC8HindIIIrv, ERCC8EcoRIfw/ ERCC8K212Crv, ERCC8K212Cfw/ ERCC8HindIIIrv, ERCC8EcoRIfw/ ERCC8Y350Crv, ERCC8Y350Cfw/ ERCC8HindIIIrv (Table 1). After the initial denaturation step at 98℃ for 5 min, the PCR was conducted for 20 cycles with denaturation at 98℃ for 20s, primer annealing from 60℃ to 50℃ with a step of -0.5℃ each cycle for 20℃ and 72℃ for 30s, following by 10 cycles by fixing anneal temperature at 52℃. When all cycles completed, the samples were kept

at 72℃ for 10 min to finish all of DNA synthesis as indicated in Table 2. After the PCR, DNA products were purified by agarose gel electrophoresis followed by gel extraction. The two PCR products have cohesive end at 5' or 3', which were therefore able to anneal together with their complementary overhanging, cohesive ends, which were then extended and amplified by PCR as indicated in Table 2 using the primer pair ERCC8EcoRIfw/ ERCC8HindIIIrv to get a full length gene with desire mutation. Agarose gel electrophoresis of the synthesis of the DNA fragments, the primer pairs used to synthesize DNA fragments of desire, and the expect length of the DNA products were shown at Figure 2C.

DNA products were purified by agarose gel electrophoresis followed gel extraction and digested by EcoRI/HindIII restriction enzymes (NEB). The genes, containing the desire mutations were ligated into a backbone vector, which was digested by the same restriction enzymes using T4 ligase, and then transformed to DH5α competent cells by incubating for 15 min on ice, followed by heat-shocking at 42℃ for 90 s and then transferring to ice for 5 min. After adding 1 ml LB (Lysogeny broth), the cells were allowed to recover by incubating in a shaker at 37℃ for 60 min. Then cells were pelleted by centrifuging at 13000 rpm for 1 min. Pellets were respended in 200 ul LB, and spread onto LB plates containing 0.1 mg/ml ampicillin. After incubating the plates overnight at 37℃, for each transformation we selected at least 8 colonies at random and performed colony PCR for determining the presence or absence of insert DNA in plasmid constructs, with 5 units of Taq DNA polymerase (NEB)

and 1×ThermoPol® Buffer (NEB) in the presence of 200 μM dNTP, 1 mmol of a primer from vector, T7 promoter, and a primer from the insert gene, ERCC8HindIIIrv (Table 1) and small amount cells picked from the colony in a final volume of 20 μl. The colony PCR reaction programs were optimized as follows: 95℃ for 2 min, then 25 cycles of 95℃ for 30 s for denaturation, 50℃ for 30 s and 68℃ for 1.5 min, followed by 68℃ for 10 min for final extension as shown in Table 2. The plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen). Mutations were checked by DNA sequencing.

In comparison, completely overlapping primers designed as recommended in the QuikChange™ manual and another designed as described in [24] were also tested in same positions as described before (S23C, K212C, and Y350C) using primer pairs S23Cfw/ S23Crv, K212Cfw/ K212Crv, and Y350Cfw/ Y350Crv (Table 1). All of the reactions failed to produce any amplification product (Figure 2C, right panel), even though these primers were designed according to the protocols of the standard QuikChange™ mutagenesis protocol. For our developed method, we only amplified short DNA sequence, DNA quantification showed that amplifications of the DNA fragments were very high and high fidelity. The next step in the experiment was to identify the vector subcloned with the modified gene. The presence or absence of insert DNA in plasmid constructs was determined by colony PCR method (Table 2). DNA sequencing showed that in each mutagenesis reaction all eight transformants contained the desire mutations.

Multiple-site mutagenesis

Figure 3 shows the flow chart of the generation of multiple-site mutations. ERCC-8 protein has thirteen cysteines, i.e., C84, C88, C157, C171, C178, C222, C252, C288, C301, C303, C339, C340, and C356 (Figure 3). In this study, thirteen cysteines will be replaced by serine to get a cysteine free protein. As C84 and C88, C157, C171, and C178, C288, C301, and C303, C339, C340, and C356 are too close to amplify by regular PCR, each of them can be grouped. The whole gene can be divided into seven fragments by six cysteine-groups, i.e., C84/C88, C157/C171/C178, C222, C252, C288/C301/C303, and C339/C340/C356 (Figure 3). The primer pairs used to mutate cysteine to serine were designed as standard QuikChange™ Mutagenesis protocol (Table 1). Seven parallel PCR reactions were performed to amplify each DNA fragment by using the primers as showed in Table 1 and Figure 3. Amplified products were separated by 1% agarose gel electrophoresis and purified by gel extraction. DNA fragments ① and ② were annealed together with their complementary overhanging, cohesive ends, and extended and amplified by PCR using the primer pairs as showed in Figure 3 and Table 1 to generate DNA ⑧ following purification by 1% agarose electrophoresis gel and DNA extraction. By using the same way, DNA fragments ⑨ and ⑩ can be obtained (Figure 3). Next, DNA fragments ⑧ and ⑨ were annealed and extended to generate DNA fragment ⑪, and DNA fragments ⑩ and ⑦ were used to generate DNA fragment ⑫. Final, DNA fragment ⑪ and DNA fragment ⑫ were used

to engineer a full length gene with desire multiple-site mutations. Agarose gel electrophoresis of the synthesis of the DNA fragments, the primer pairs used to synthesize DNA fragments of desire, and the expect length of the DNA products were shown at Figure 4. The gene with multiple-site mutations was digested by EcoRI/HindIII restriction enzymes (NEB), was subcloned into a backbone vector, which was digested by the same restriction enzymes, and then transformed to DH5α competent cells. The presence or absence of insert DNA in plasmid constructs was determined by colony PCR method (see Table 3).

Replacement, Insertion, and Deletion Mutagenesis

Figure 5 shows the scheme that can be used to generate replacement, insertion, or deletion mutations. In this study, we plan to replace a 200 bp DNA fragment from *ERCC8* gene by other DNA fragment, or insert a 200 bp DNA fragment into *ERCC8* gene, or remove a 200 bp DNA fragment from *ERCC8* gene (Figure S3).

As showing in Figure 5, to replace a 200 bp DNA fragment (between 1 and 2, Figure S3) from *ERCC8* gene with another one (RE), three parallel PCR reaction were performed to amplify each DNA fragment by using the primer pairs, ERCC8EcoRIfw/Re1rv, Refw/Rerv, and Re2fw/ERCC8HindIIIrv as showed in Table 1 and Figure 5 to generate DNA fragment “1”, which has EcoRI recognition site at 5’ end and a complementary overhanging to RE DNA fragment at 3’ end, “2”, which has HindIII recognition site at 3’ end and a complementary overhanging to Re DNA fragment at 5’ end, and “RE”, which

has a complementary overhanging to DNA fragment “1” at 5’ end and a complementary overhanging to DNA fragment “2” at 3’ end (Figure 5, replacement). Amplified products were separated by 1% agarose gel electrophoresis and purified by gel extraction. DNA fragments “1” and “RE” were annealed together with their complementary overhanging, cohesive ends, and extended and amplified by PCR using the primer pair EcoRIfw/Rerv to generate DNA fragment “1+RE”, which has EcoRI recognition site at 5’ end and a complementary overhanging to “2” DNA fragment at 3’ end, “2”, following purification by 1% agarose electrophoresis gel and DNA extraction. Next, DNA fragments “1+RE” and “2” were annealed together with their complementary overhanging, cohesive ends, and extended and amplified by PCR using the primer pair ERCC8EcoRIfw/ERCC8HindIIIrv to generate DNA fragment “1+RE+2”, which has EcoRI recognition site at 5’ end, HindIII recognition site at 3’ end, and the DNA sequence between 1 and 2 has been replaced by RE DNA fragment. Amplified products were separated by 1% agarose gel electrophoresis and purified by gel extraction. Agarose gel electrophoresis of the amplified DNA is shown in Figure 6.

To insert a 200bp DNA fragment (IN) into *ERCC8* gene, three parallel PCR reaction were performed to amplify each DNA fragment by using the primer pairs, ERCC8EcoRIfw/IN1rv, INfw/INrv, and IN2fw/ERCC8HindIIIrv as showed in Table 1, Figure 5, and Figure S3 to generate DNA fragment “1”, which has EcoRI recognition site at 5’ end and a complementary overhanging to IN DNA

fragment at 3' end, "2", which has HindIII recognition site at 3' end and a complementary overhanging to IN DNA fragment at 5' end, and "IN", which has a complementary overhanging to DNA fragment "1" at 5' end and a complementary overhanging to DNA fragment "2" at 3' end (Figure 5, insertion). Amplified products were separated by 1% agarose gel electrophoresis and purified by gel extraction. DNA fragments "1" and "IN" were annealed together with their complementary overhanging, cohesive ends, and extended and amplified by following PCR using the primer pair ERCC8EcoRIfw/INrv to generate DNA fragment "1+IN", which has EcoRI recognition site at 5' end and a complementary overhanging to "2" DNA fragment at 3' end, "2", following purification by 1% agarose gel electrophoresis and DNA extraction. Next, DNA fragments "1+IN" and "2" were annealed together with their complementary overhanging, cohesive ends, and extended and amplified by PCR using the primer pair ERCC8EcoRIfw/ERCC8HindIIIrv to generate DNA fragment "1+IN+2", which has EcoRI recognition site at 5' end, HindIII recongnition site at 3' end, and the IN DNA fragment has been inserted into the target gene. Amplified products were separated by 1% agarose gel electrophoresis and purified by gel extraction. Agarose gel electrophoresis of the amplified DNA is shown in Figure 6.

To delete a 200 bp DNA fragment (DE) from the *ERCC8* gene between 1 and 2 (Figure 5, deletion, Figure S3), two parallel PCR reactions were performed to amplify each DNA fragment by using the primer pairs, ERCC8EcoRIfw/De1rv

and De2fw/ERCC8HindIIIrv to generate DNA fragment “1”, which has EcoRI recognition site at 5’ end and a complementary overhanging to “2” DNA fragment at 3’ end, “2”, which has HindIII recognition site at 3’ end and a complementary overhanging to “1” DNA fragment at 5’ end (Figure 5, Deletion). Amplified products were separated by 1% agarose gel electrophoresis and purified by gel extraction. DNA fragments “1” and “2” were annealed together with their complementary overhanging, cohesive ends, and extended and amplified by following PCR using the primer pair ERCC8EcoRIfw/ERCC8HindIIIrv to generate a modified gene, which has EcoRI recognition site at 5’ end, HindIII recognition site at 3’ end with DNA fragment deletion in target location. Amplified products were separated by 1% agarose gel electrophoresis and purified by gel extraction. Agarose gel electrophoresis of the amplified DNA is shown in Figure 6.

The genes with multiple-site mutations, which were digested by EcoRI/HindIII restriction enzymes (NEB), were ligated into a backbone vector, which was digested by the same restriction enzymes using T4 ligase, and then transformed to DH5α competent cells. The presence or absence of insert DNA in plasmid constructs was determined by colony PCR method.

Discussions

QuickChange™ site-directed mutagenesis, which employs complementary primer pairs designed with mismatching nucleotides at the center of the primers in the same PCR reaction, has been widely used to generate DNA sequences

with mutated codons, insertions or deletions. However, the use of complementary primer pairs may lead to the formation of “primer dimers” and formation of tandem repeats of primers, which would reduce the yield of successful transformants [10] and the primer design needs care [17]. For this method, the parental DNA templates (methylated DNA molecules) much be removed completely by DpnI digestion [25]. Incomplete digestion results in recovery of non-mutated DNA. The originally developed QuikChange™ cannot introduce multiple mutations as well as long DNA fragment deletion, insertion or replacement mutations. Through many modified versions QuikChange™ Site-Directed Mutagenesis have been developed, a simple, but high efficient method that allows deleting, inserting, or substituting multiple-site DNA fragment is required. We therefore develop a new method for rapid and efficient multiple-site fragment deletion, insertion, and substitution mutagenesis with a modified overlap extension PCR. For this method, all of the mutagenesis was operated on the target gene, and then the gene containing desired mutants with restriction enzyme cutting sites at 5’ and 3’ would be replaced into a vector, which was digested with same restriction enzyme. For this method, the primers are designed as the suggestion for QuikChange™ and therefore no special design of primers are required. As only short DNA fragments were amplified, the PCR amplification using these primers showed high efficiency. It requires none of the plasmid as the parental template, which eliminates the potential of the recovery of the parental DNA. To reduce the probability of the contamination

from the backbone vector, which was not digested completely, the plasmid, which has been subcloned by a gene, was digested with restriction enzyme, followed by gel purification. In this way, it would be much easy to separate the vectors, which have been digested, to those vectors that have not been completely digested. The plasmid contained desired gene was screened by colony PCR using a primer from vector and a primer from the insert gene. In our experience, once the colony PCR gives a positive result, the sequence is always correct. Under our PCR conditions, no parental plasmid contaminations were detected. So for this method, no DNA sequencing is required.

This method also can be used to rapidly generate multiple-site mutagenesis, for example, cysteine free mutants. In the present work, we used this method to mutate 13 cysteine of ERCC8 protein to serine to get a cysteine free mutant protein in totally nine and half hours (one day). For QuickChange™ site-directed mutagenesis method, someone has to mutate cysteine to serine one by one. So for this method, it may need at least 13 days to get the same desire mutant. The important thing is that DNA sequencing is required for each mutant to make sure the mutant has been successfully induced into the target gene. It normally takes longer time to get multiple-site mutagenesis by using QuickChange™ site-directed mutagenesis method. For our method, multiple-site mutagenesis can be achieved in a short time by dividing target gene to DNA fragments, and then these DNA fragments will be ligated and extended by PCR (Figure 3). The advantage of this method over other methods is its

simplicity and saving of time since no DNA sequencing is required for each step.

This method also can be used to engineer insertion and deletion mutagenesis. Protein engineering is a technique to change the amino acid sequence of proteins in order to improve their specific properties. In the present work, we have successfully inserted or deleted a 200bp DNA to or from the target gene. To insert a DNA fragment, PCR amplification of three fragments were performed by using the primers as shown in Figure 5. To delete a DNA fragment, PCR amplification of two fragments were performed by using the primers as shown in Figure 5. As we never used complementary primer pair to amplify DNA, this design would eliminate the problems associated with primer pair self-annealing, and Tm values are not strict limit. DNA fragment that is going to be deleted, replaced, can be at anywhere of target gene. The length of the DNA fragments for replacement, deletion, or insertion, is not specially required.

Our results demonstrated that the modified protocol is a high efficient method for single site mutagenesis and can be extended to multiple site-directed insertion and/or deletion mutagenesis protocol.

Conclusions

As a result of the present work, we have developed a new method for rapid and efficient multiple-site fragment deletion, insertion, and substitution mutagenesis with a modified overlap extension PCR. This method utilizes a new DNA fragment-designing scheme, which facilitated the design of primer and PCR procedure, but enhanced the overall efficiency and reliability.

By using this method, we have successfully generated single/ multiple-site mutations, deletions, insertion and substitution mutations. The results demonstrated that this new protocol would not increase any reagent costs but increased the overall success rates. It provided an efficient choice, especially for multiple-site, or large DNA fragment modification mutagenesis of DNAs

Ethics

Not applicable

Financial competing interests

The authors declare no competing financial interests.

Authors' contributions

AP and FL designed the experiments, carried out the practical work and drafted the manuscript. All authors read and approved the final manuscript.

Availability of supporting data

The data sets supporting the results of this article are included within the article and its additional file.

Abbreviation

PCR, polymerase chain reaction; OE-PCR, overlap extension by polymerase chain reaction; ERCC8, Excision Repair Cross-Complementation Group 8; RE,

DNA fragment for replacement mutagenesis; IN, DNA fragment for insertion mutagenesis; DE, DNA fragment for deletion mutagenesis.

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FIGURE LEGENDS

FIGURE 1. Schematic presentations of parent vector generation processes. The parent vector was generated by restriction digestion of a vector which is already existing insert but with restriction sites intact (A), or by PCR method (B). For detail, see text.

FIGURE 2. Schematic presentations of single-site mutagenesis PCR amplification processes. (A) The scheme of method used to generate single-site mutation. (B) Schematic diagram of the primer design for site-directed mutagenesis. Black filled triangles show the location of mutations. Red filled rectangles show the restriction digestion sites. (C) Agarose gel electrophoresis of the PCR reactions for single-site mutagenesis. The primers used for DNA synthesis and the expect length of the DNA products are shown on the top of each lane of

agarose gel. DNA samples were electrophoresed in 2% agarose gel for short fragments (left panel) or 1% agarose gel for longer fragments (right panel).

FIGURE 3. Flow chart of the multiple-site mutagenesis method. Black filled triangles show the location of mutations. Red filled rectangles show the restriction digestion sites. The gray lines with arrowhead show the primers used for DNA synthesis. The time required for each step was shown on the right.

FIGURE 4. Agarose gel electrophoresis of the PCR reactions for multiple-site mutagenesis following the processes as described in Figure 3. The primers used for DNA synthesis and the expect length of the DNA products are shown on the top of each lane of agarose gel. DNA samples were electrophoresed in 2% agarose gel.

FIGURE 5. Schematic presentations of deletion, replacement, and insertion mutagenesis PCR amplification processes. Black filled triangles show the location of mutations. Red filled rectangles show the restriction digestion sites. The lines with arrowhead show the primers used for DNA synthesis. RE indicates the DNA fragment that uses to replace other DNA fragment. IN indicates the DNA fragment that will be inserted into other gene. DE indicates the DNA fragment that will be deleted.

FIGURE 6. Agarose gel electrophoresis of the PCR reactions for deletion, replacement, and insertion mutagenesis following the processes as described in Figure 5. The primers used for DNA synthesis and the expect length of the DNA products are shown on the top of each lane of agarose gel. DNA samples were electrophoresed in 2% agarose gel.

Table 1: Primers used for mutagenesis.
Table 2: Thermocycling conditions for a touchdown PCR.
Table 3: Thermocycling conditions for a colony PCR.

Figure 1

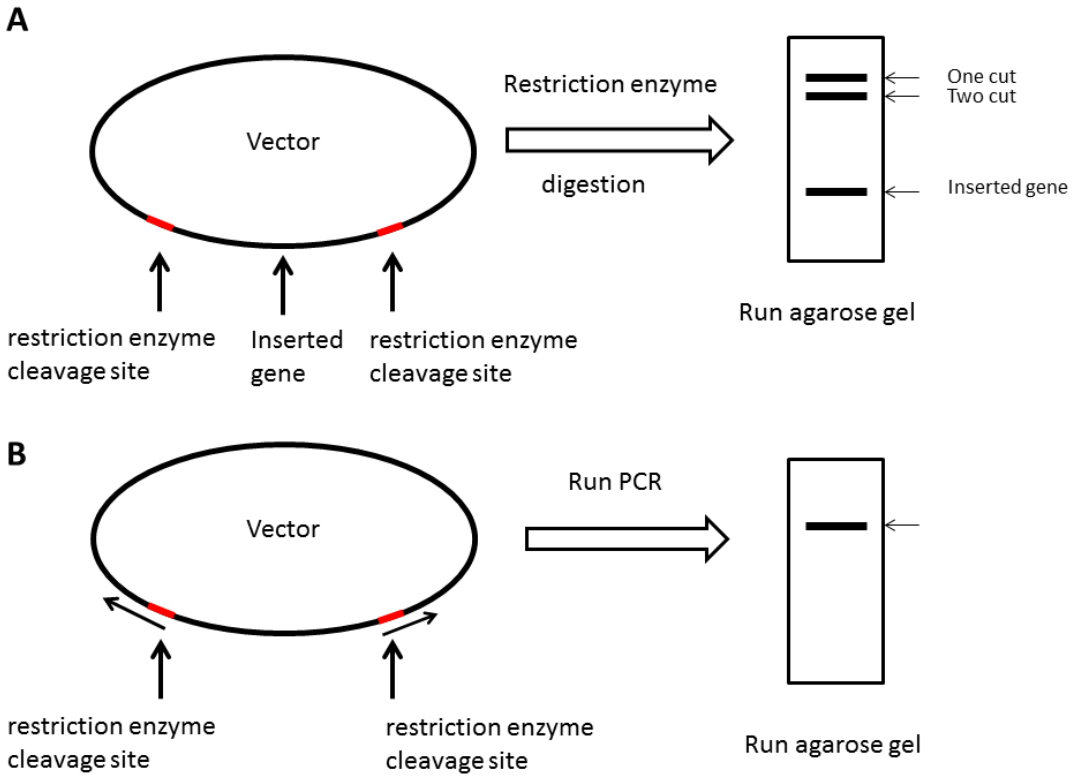

A
Vector
Restriction enzyme
digestion
One cut
Two cut
Inserted gene
Run agarose gel
restriction enzyme cleavage site
Inserted gene
restriction enzyme cleavage site
B
Vector
Run PCR
Run agarose gel
restriction enzyme cleavage site
restriction enzyme cleavage site

Figure 2

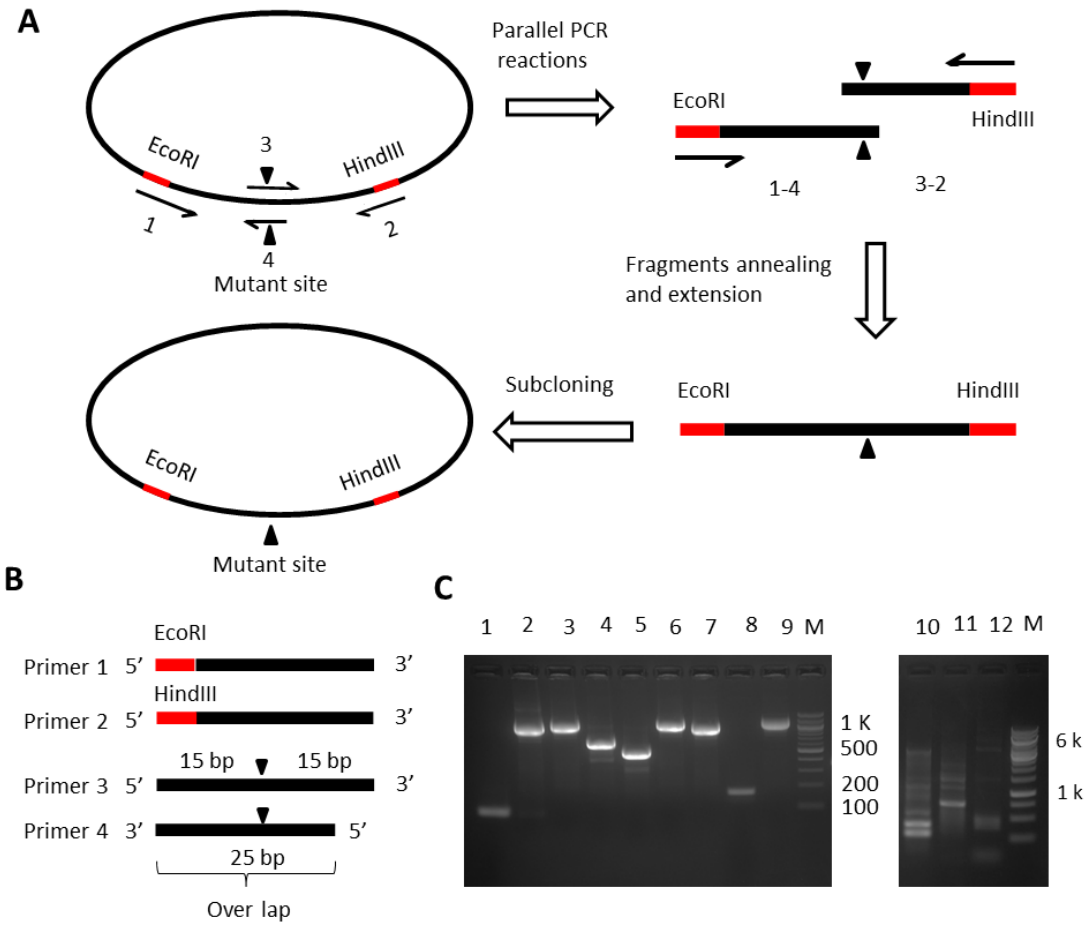

A
EcoRI
3
HindIII
1
2
4
Mutant site
Parallel PCR reactions
EcoRI
HindIII
1-4
3-2
Fragments annealing and extension
EcoRI
HindIII
Subcloning
EcoRI
HindIII
Mutant site
B
EcoRI
Primer 1 5' 3'
HindIII
Primer 2 5' 3'
15 bp
15 bp
Primer 3 5' 3'
Primer 4 3' 5'
25 bp
Over lap
C
1 2 3 4 5 6 7 8 9 M
1 K
500
200
100
10 11 12 M
6 k
1 k

Figure 3

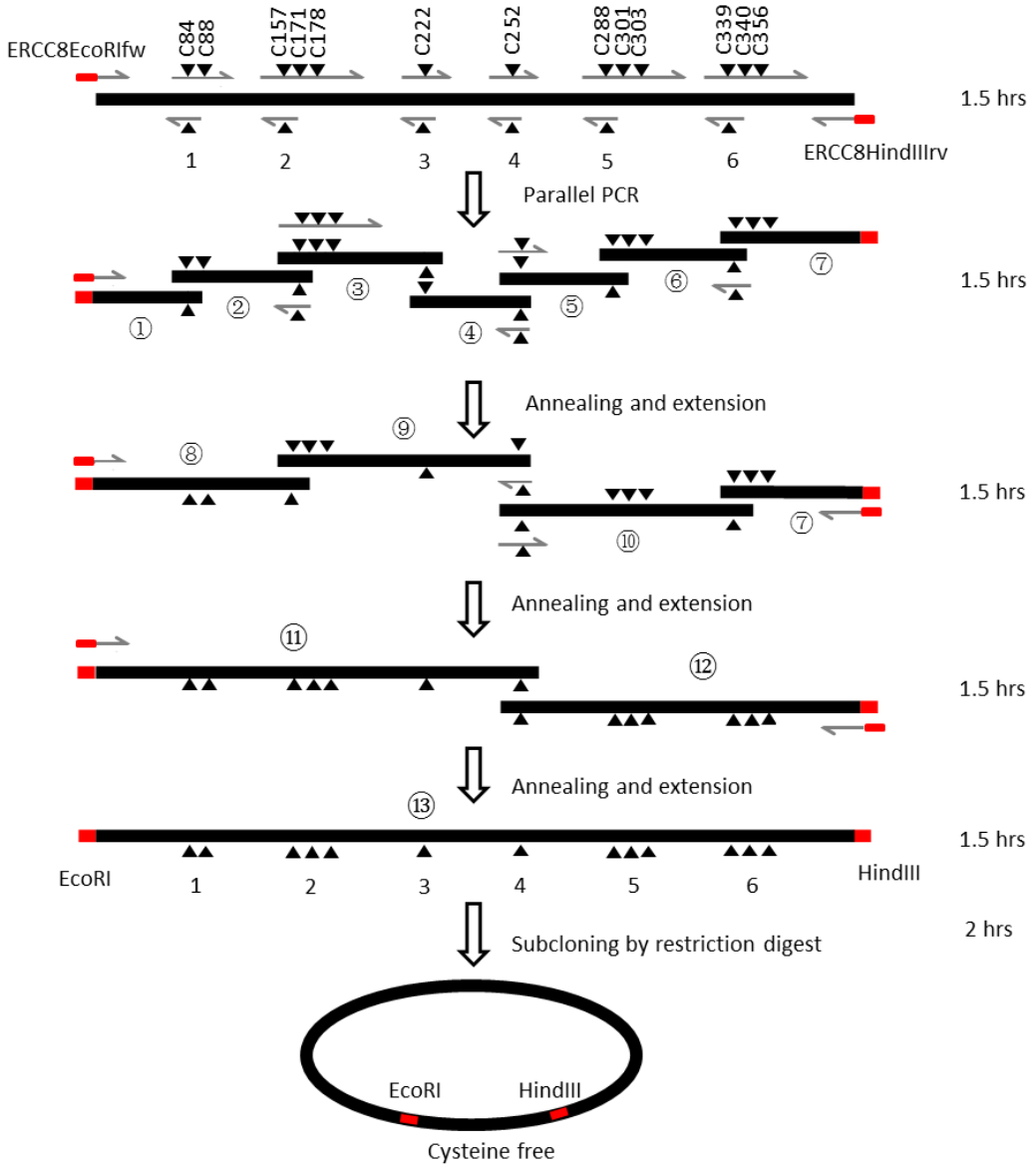

ERCC8EcoRIfw
C84
C88
C157
C171
C178
C222
C252
C288
C301
C303
C339
C340
C356
1.5 hrs
1
2
3
4
5
6
ERCC8HindIIIrv
Parallel PCR
1.5 hrs
Annealing and extension
1.5 hrs
Annealing and extension
1.5 hrs
Annealing and extension
1.5 hrs
EcoRI
HindIII
2 hrs
Subcloning by restriction digest
EcoRI
HindIII
Cysteine free

Figure 4

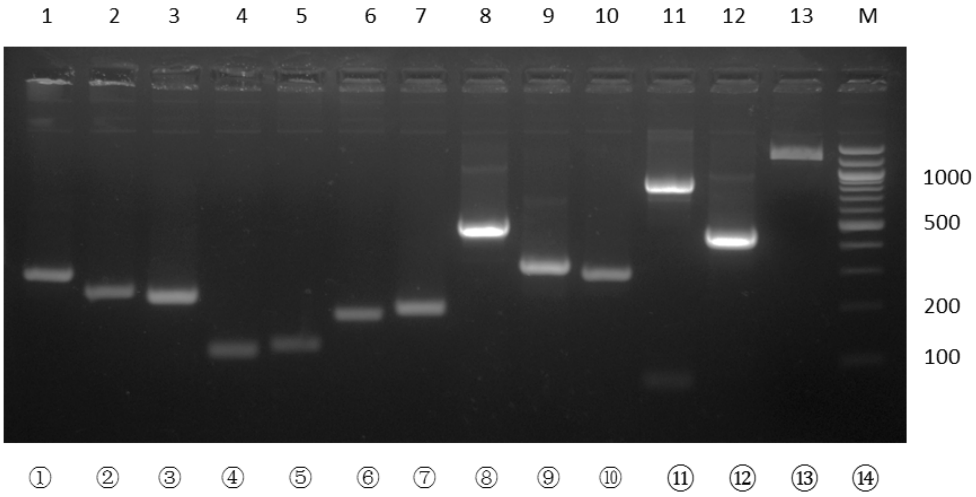


Figure 5

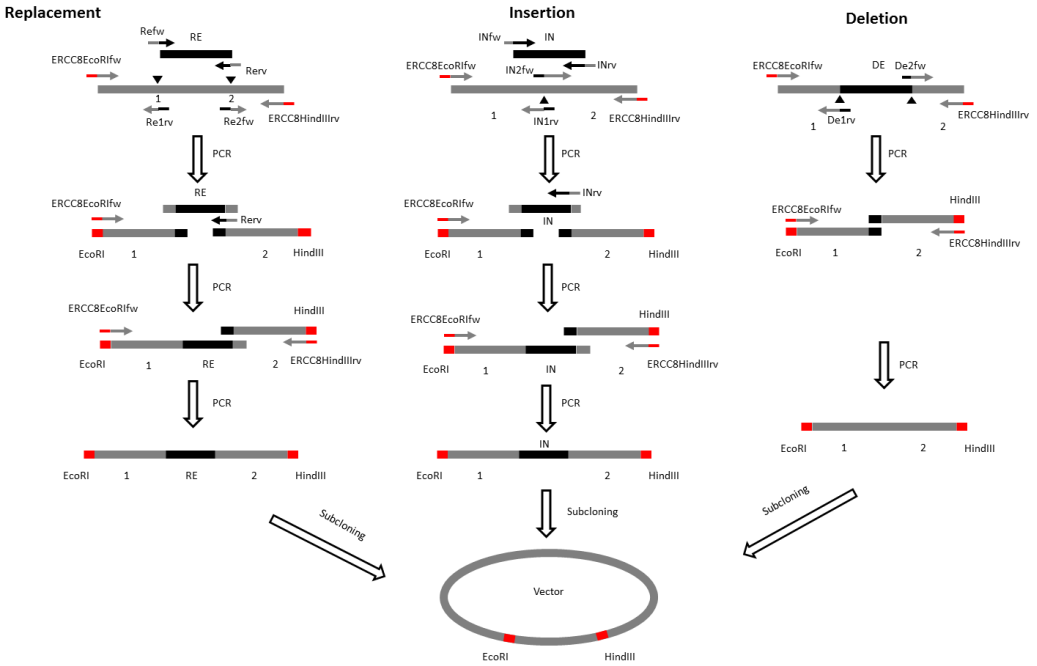
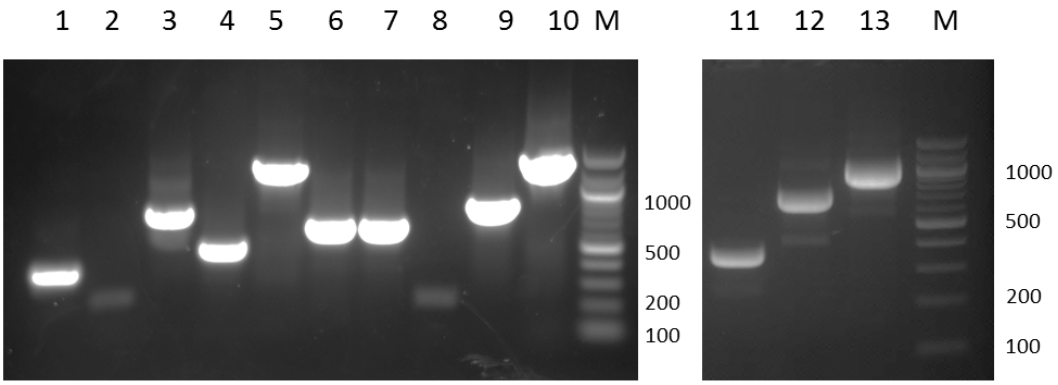

Replacement
Refw
RE
ERCC8EcoRIfw
Rerv
1
2
Re1rv
Re2fw
ERCC8HindIIIrv
PCR
RE
ERCC8EcoRIfw
Rerv
EcoRI
1
2
HindIII
PCR
ERCC8EcoRIfw
HindIII
EcoRI
1
RE
2
ERCC8HindIIIrv
PCR
EcoRI
1
RE
2
HindIII
Insertion
INfw
IN
ERCC8EcoRIfw
IN2fw
INrv
1
IN1rv
2
ERCC8HindIIIrv
PCR
INrv
ERCC8EcoRIfw
IN
EcoRI
1
2
HindIII
PCR
ERCC8EcoRIfw
HindIII
EcoRI
1
IN
2
ERCC8HindIIIrv
PCR
IN
EcoRI
1
2
HindIII
Subcloning
Vector
EcoRI
HindIII
Deletion
ERCC8EcoRIfw
DE
De2fw
1
De1rv
2
ERCC8HindIIIrv
PCR
HindIII
ERCC8EcoRIfw
EcoRI
1
2
ERCC8HindIIIrv
PCR
EcoRI
1
2
HindIII
Subcloning

Figure 6



ERCC8 gene

>gi|189083847:12-1320 Homo sapiens excision repair cross-complementation group 8 (ERCC8), transcript variant 1, mRNA
CCCGCCCCTGAGCTCTGTCATGGCGACGTCCAGTGCTCCAGCCGGTGTGAGGACACGATATGCTGGGGTTT
TTGTCCGCACGCCAAACGGGTTTGGAGGACCCTCTTCGCCTTCGGAGAGCAGAGTCAACACGGAGAGTTT
TGGGACTGGAATTAAATAAAGACAGAGATGTTGAAAGAATCCACGGCGGTGGAATTAACACCCTTGACATT
GAACCTGTTGAAGGGAGATACATGTTATCAGGTGGTTCAGATGGTGTGATTGTACTTTATGACCTTGAGAAC
TCCAGCAGACAATCTTATTACACATGTAAAGCAGTGTGTTCCATTGGCAGAGATCATCCTGATGTTCACAGAT
ACAGTGTGGAGACTGTACAGTGGTATCCTCATGACACTGGCATGTTCACATCAAGCTCATTTGATAAAACTCT
GAAAGTATGGGATACAAATACATTACAAACTGCAGATGTATTTAATTTTGAGGAAACAGTTTATAGTCATCATA
TGTCTCCAGTCTCCACCAAGCACTGTTTGGTAGCAGTTGGTACTAGAGGACCCAAAGTACAACTTTGTGACT
TGAAGTCTGGATCCTGTTCTCACATTCTACAGGGTCACAGACAAGAAATATTAGCAGTTTCCTGGTCTCCACG
TTATGACTATATCTTGGCAACAGCAAGTGCTGACAGTAGAGTAAAATTATGGGATGTGAGAAGAGCATCAGG
ATGTTTGATTACTCTTGATCAACATAATGGGAAAAAGTCACAAGCTGTTGAATCAGCAAACACTGCTCATAAT
GGGAAAGTTAATGGCTTATGTTTTACAAGTGATGGACTTCACCTCCTCACTGTTGGTACAGATAATCGAATGA
GGCTCTGGAATAGTTCCAATGGAGAAAACACACTTGTGAACTATGGAAAAGTTTGTAATAACAGTAAAAAA
GGATTGAAATTCACTGTCTCCTGTGGCTGCAGTTCAGAATTTGTTTTTGTACCATATGGTAGCACCATTGCTGT
TTATACAGTTTACTCAGGAGAACAGATAACTATGCTTAAGGGACATTATAAAACTGTTGACTGCTGTGTATTTC
AGTCAAATTTCCAGGAACTTTATAGTGGTAGCAGAGACTGCAACATTCTGGCTTGGGTTCCATCCTTATATGA
ACCAGTTCCTGATGATGATGAGACTACAACAAAATCACAATTAAATCCGGCCTTTGAAGATGCCTGGAGCAG
CAGTGATGAAGAAGGATGAATATCATCTTTAGTACCTTTTTGTCTCTGCTGAAACTTTTTAAATGAGACTGTG
TTTTT

ERCC8 protein

>gi|4557467|ref|NP_000073.1| DNA excision repair protein ERCC-8 isoform 1 [Homo sapiens]
MLGFLSARQTGLEDPLRLRRAESTRRVLGLELNKDRDVERIHGGGINTLDIEPVEGRYMLSGGSDGVIVLYDLEN
SSRQSYYTCKAVCSIGRDHPDVHRYSVETVQWYPHDTGMFTSSSFDKTLKVWDTNTLQTADVFNFEETVYSHH
MSPVSTKHCLVAVGTRGPKVQLCDLKSGSCSHILQGHRQEILAVSWSPRYDYILATASADSRVKLWDVRRASGC
LITLDQHNGKKSQAVESANTAHNGKVNGLCFTSDGLHLLTVGTDNRMRLWNSSNGENTLVNYGKVCNNSKKG
LKFTVSCGCSSEFVFVPYGSTIAVYTVYSGEQITMLKGHYKTVDCCVFQSNFQELYSGSRDCNILAWVPSLYEPVP
DDDETTTKSQLNPAFEDAWSSSDEEG

Figure S1 The sequences of *ERCC8* gene and ERCC8 protein involved in this study. The highlight shows the coding sequence of *ERCC8* gene.

```
                                                  * *
ERCC8ES23Cfw     5' CTTCGG AGAGCAGAGTGCACACGGAGAGTTTTG 3'
                    || | | | | | | | | | | | | | | | | | | | | | | | | | |
ERCC8ES23Crv     3' GAAGCCT CTCGT CTCACGTGTGCC 5'
                                                  * *

                                                * * *
ERCC8EK212Cfw    5' GCTGACAGTAGAGTATGCTTA TGGGATGTGAG 3'
                    || | | | | | | | | | | | | | | | | | | |
```

1-300

atgctggggtttttgtccgcacgccaaacgggtttggaggaccctcttcgccttcggagagcagagtcaacacggagagttttgggactggaa
ttaaataaagacagagatgttgaaagaatccacggcggtggaattaacacccttgacattgaacctgttgaagggagatacatgttatcagg
tggttcagatggtgtgattgtactttatgaccttgagaactccagcagacaatcttattacacatgtaaagcagtgtgttccattggcagagatc
atcctgatgttcacagatac

301-500

agtgtggagactgtacagtggtatcctcatgacactggcatgttcacatcaagctcatttgataaaactctgaaagtatgggatacaaataca
ttacaaactgcagatgtatttaattttgaggaaacagtttatagtcatcatatgtctccagtctccaccaagcactgtttggtagcagttggtact
agaggacccaa

501-

agtacaactttgtgacttgaagtctggatcctgttctcacattctacagggtcacagacaagaaatattagcagtttcctggtctccacgttatg
actatatcttggcaacagcaagtgctgacagtagagtaaaattatgggatgtgagaagagcatcaggatgtttgattactcttgatcaacata
atgggaaaaagtcacaagctgttgaatcagcaaacactgctcataatgggaaagttaatggcttatgttttacaagtgatggacttcacctcc
tcactgttggtacagataatcgaatgaggctctggaatagttccaatggagaaaacacacttgtgaactatggaaaagtttgtaataacagta
aaaaaggattgaaattcactgtctcctgtggctgcagttcagaatttgtttttgtaccatatggtagcaccattgctgtttatacagtttactcag
gagaacagataactatgcttaagggacattataaaactgttgactgctgtgtatttcagtcaaatttccaggaactttatagtggtagcagaga
ctgcaacattctggcttgggttccatccttatatgaaccagttcctgatgatgatgagactacaacaaaatcacaattaaatccggcctttgaa
gatgcctggagcagcagtgatgaagaaggatga

DNA sequence using for insertion or replacement mutagenesis

TTCCTTATCCGCGTTTTTTTGCCAGTCTGGCAGGCTTTGTTACGGCAGGTGTGACGCTACCATTTGTTA
ATTATGTCGGCGGTGGCGATCGGGGATTTGGCTTTCAGATGTTCACTCTGGTACTGATCGCCTTTTTTA
TTGTTTCAACCATCATCACTCTGCGCAATGTGCATGAAGTCTTTTCGTCAGACAATCAACCG

Figure S3 The DNA sequences involving in replacement, insertion, and deletion mutagenesis studies. The highlight DNA sequence shows the location of the DNA that will be replaced by the other one DNA sequence which was marked in underline text or will be deleted completely. The red arrow shows the position where the other one DNA sequence (marked in underline text) will be inserted into.