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Manipulation of single-stranded DNA using artificial site-selective DNA cutter composed of Ce(IV)/EDTA and phosphonate-oligonucleotide conjugates

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Dedication ((optional))

Abstract: An artificial site-selective DNA cutter to hydrolyze single-stranded DNA at a desired site was prepared from Ce(IV)/EDTA and two ethylenediamine-*N,N,N',N'*-tetrakis(methylenephosphonic acid)-oligonucleotide conjugates. By using this cutter, the sense strand of blue fluorescent protein (BFP) gene was selectively cut at a predetermined site in the chromophore-coding region. The upstream fragment obtained by the site-selective scission was ligated with the downstream fragment of closely related green fluorescent protein (GFP) gene so that the 5'-side and 3'-side portions of the chromophore came from the BFP fragment and the GFP fragment, respectively. The recombinant gene was successfully expressed in *E. coli* and the chimeric chromophore emitted green fluorescence as expected.

Keywords: Cerium • DNA cleavage • Hydrolysis • gene manipulation • GFP

Introduction

Man-made tools that are capable of site-selective DNA hydrolysis have been the subject of intensive study,[1] mainly because (1) the site-specificity of naturally occurring restriction enzymes is too low to manipulate huge DNA and (2) there are only a limited number of recognition sites of these enzymes (mostly palindrome sequences). In other words, new tools whose scission-sequence and site-specificity can be freely modulated are necessary for future biotechnology and molecular biology. We have already developed an artificial DNA cutter (ARCUT) which can cut double-stranded DNA site-selectively[2] and applied this system to manipulations of a variety of double-stranded DNA targets including human genomes.[3] These technologies to manipulate DNA have been developed with a focus on double-stranded DNA, however, the genomes of some organisms (e.g. viruses) are single-stranded. If new tools for site-selective scission of single-stranded DNA were also available, the scope of molecular biology and related fields would be further expanded. For example, the single-stranded genome of adeno-associated virus, which is currently used as a vector for clinical purposes,[4] could be easily and precisely manipulated for improved properties. Desired single-stranded DNAs could be clipped from single-stranded genomes and used for DNA nanotechnology (DNA nanostructures, DNA origami, molecular machines and so on).[5,6] Furthermore, these tools could even be utilized for the preparation of DNA containing modified nucleobases (e.g. methylated or non-natural bases), leading to many other applications.

In a previous paper,[7] we succeeded in site-selective hydrolysis of single-stranded DNA by combining Ce(IV)/EDTA complex[8] and two oligonucleotides (ODNs) complementary to sequences flanking the desired site of cleavage.[9] When a gap structure was formed in single-stranded DNA substrate by hybridizing it with these two ODNs, the scission by Ce(IV)/EDTA selectively occurred at the gap site because of the substrate specificity of this complex (Ce(IV)/EDTA can hydrolyze single-stranded DNA far more rapidly than double-stranded DNA; **Fig. 1a**, left). This site-selective DNA scission was greatly promoted by introducing monophosphates to the termini of the ODNs to recruit Ce(IV)/EDTA near the target site.[10] Furthermore, it was found that ligands bearing multiple phosphonate groups are still more effective than the monophosphate ligand for this purpose (**Fig.**

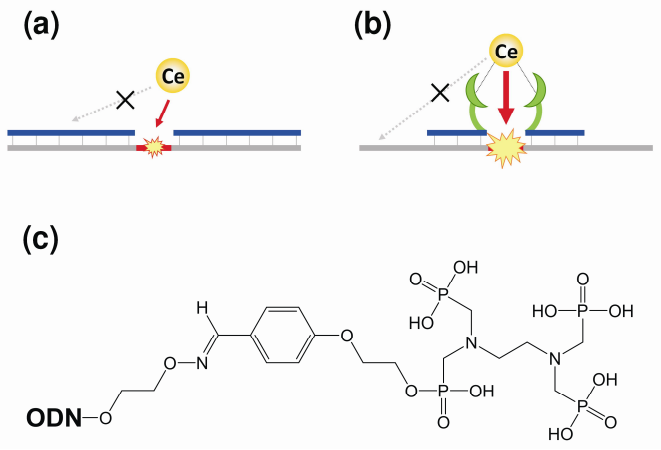

Figure 1. (a) Selective single-stranded DNA cleavage at a gap site by Ce(IV)/EDTA, which hydrolyzes single-stranded DNA preferentially. (b) Promotion of Ce(IV)/EDTA-induced site-selective DNA scission by the ethylenediamine-*N,N,N',N'*-tetrakis(methylenephosphonic acid) (EDTP) bound to ODNs. Note that the single-stranded portions formed in both sides of the additives are kept intact, and thus the desired DNA fragments are successfully obtained. (c) Introduction of EDTP ligand to ODN through an oxime linkage.

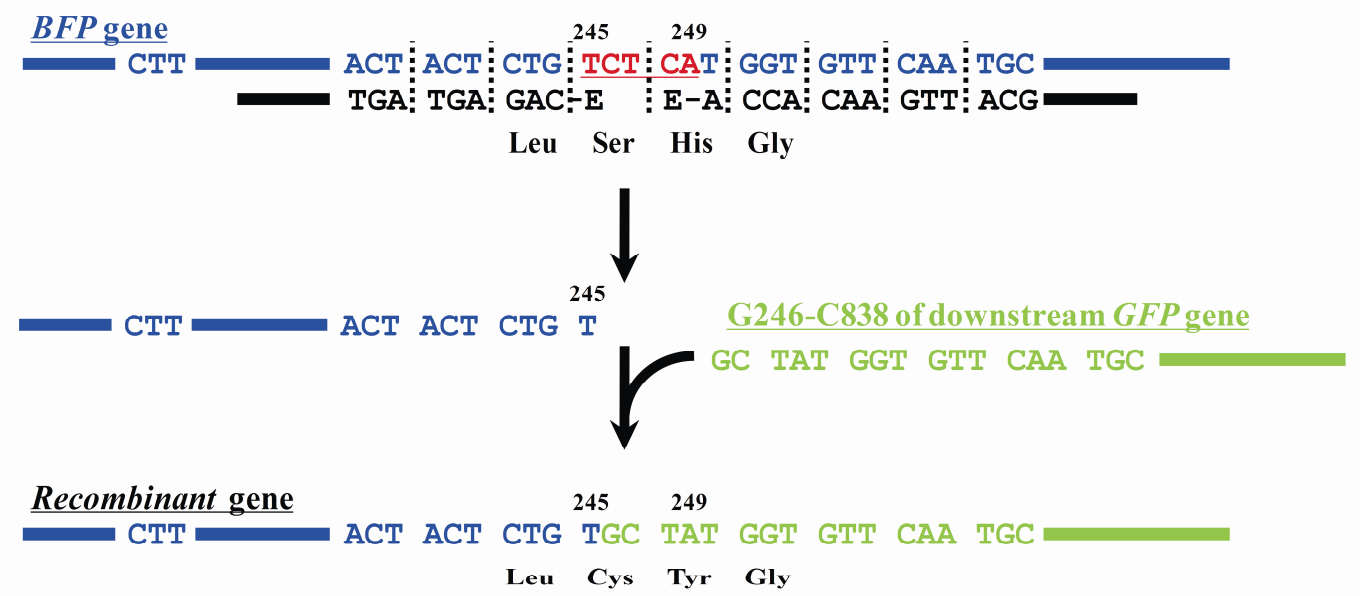

Figure 2. Conversion of *BFP* gene to *GFP* gene by the artificial DNA cutter composed of Ce(IV)/EDTA and EDTP-ODNs (E stands for EDTP ligand)

1a, right).[11,12] Ethylenediamine-*N*,*N*,*N*',*N*'-tetrakis(methylenephosphonic acid) (EDTP) was especially eminent.[13] In this paper, this artificial site-selective cutter of single-stranded DNA (combination of Ce(IV)/EDTA and two EDTP-ODNs conjugates) is used to manipulate single-stranded DNA. The following three points are evidenced; (1) the scission occurs exactly at the target site and provides the desired single-stranded DNA fragments, (2) no DNA damage occurs during the manipulation, and (3) the reading frames of the recombinant DNA can be precisely adjusted. As an example, blue fluorescent protein (BFP) gene is selectively cut at its chromophore-coding region and converted to the closely related green fluorescent protein (GFP) gene.[14] The recombinant construct is expressed in *E. coli*. and emits clear fluorescence. It should be noted that there was no clone having a mutation or deletion other than the chromophore-coding site, and this result strongly indicates that our single-stranded DNA cutter induces no critical side reactions on target DNA throughout the manipulation.

Results and Discussion

Site-selective hydrolysis of the sense strand of *BFP* gene by the DNA cutter. The target DNA for site-selective scission is an 838-mer single-stranded DNA which includes the whole *BFP* gene (the sense strand). As shown in **Fig. 2**, *BFP* gene has almost the same sequence as *GFP* gene, and only a few nucleotides in the chromophore regions are different from each other. In the sense strand of *BFP* there is T^{245}CT CAT GGT (Ser-His-Gly), whereas the corresponding sequence in *GFP* is T^{245}GC TAT GGT (Cys-Tyr-Gly). In the present DNA manipulation, the phosphodiester linkage between T^{245} and C^{246} in the sense strand of *BFP* was cut by using the artificial DNA cutter, and the upstream-side scission fragment (G^{1}-T^{245}), obtained by this scission, was ligated with the downstream fragment of the sense strand of *GFP* (G^{246}-C^{838}). Precise site-selective scission and the resultant successful gene recombination were confirmed by the green fluorescence from the expressed protein.

To the 5'-terminus of a 20-mer ODN and the 3'-terminus of a 21-mer ODN, EDTP was attached by an oxime linkage according to previous report[13] (see **Fig. 1b**; the sequences of ODNs are presented in supplementary material). These EDTP-ODN conjugates were hybridized with the C^{225}-G^{244} and T^{250}-G^{270} sequences of the 838-mer *BFP* gene, respectively. Thus, a gap structure was formed at the T^{245}-A^{249} portion of the target DNA, and one EDTP group was bound to each side of this gap site. This system was treated with Ce(IV)/EDTA, which preferentially hydrolyzes single-stranded DNA, and the products were analyzed by polyacrylamide gel electrophoresis (PAGE). As shown in lane 3 of **Fig. 3**, only two scission bands (corresponding to about 250 and 590-mer fragments) were formed exactly as expected from the site-selective scission at the gap site. It is noteworthy that the long flanking portions in both the 5'- and 3'-sides (G^{1}-C^{224} and A^{271}-C^{838}) were kept intact during the hydrolytic scission by Ce(IV)/EDTA although they were single-stranded. Apparently, the Ce(IV)/EDTA complex is so strongly attracted to the target scission site by the two EDTP ligands that undesired scission is effectively suppressed.[13] When either

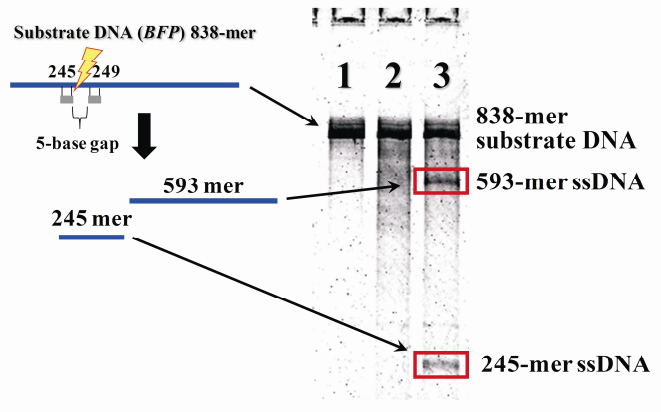

Figure 3. Site-selective scission of *BFP* gene by combination of Ce(IV)/EDTA and two EDTP-ODNs. Lane 1, DNA (single-stranded 838 mer) only; Lane 2, DNA + Ce(IV)/EDTA (without EDTP-ODNs); Lane 3, DNA + Ce(IV)/EDTA + EDTP-ODNs. Reaction conditions: [DNA] = 0.1 μM, [EDTP-ODN] = 0.5 μM, [HEPES (pH 7.0)] = 5 mM, [NaCl] = 100 mM, [Ce(IV)/EDTA] = 5 μM at 50 °C for 17 h. The whole sequences of EDTP-ODNs are presented in supplementary material.

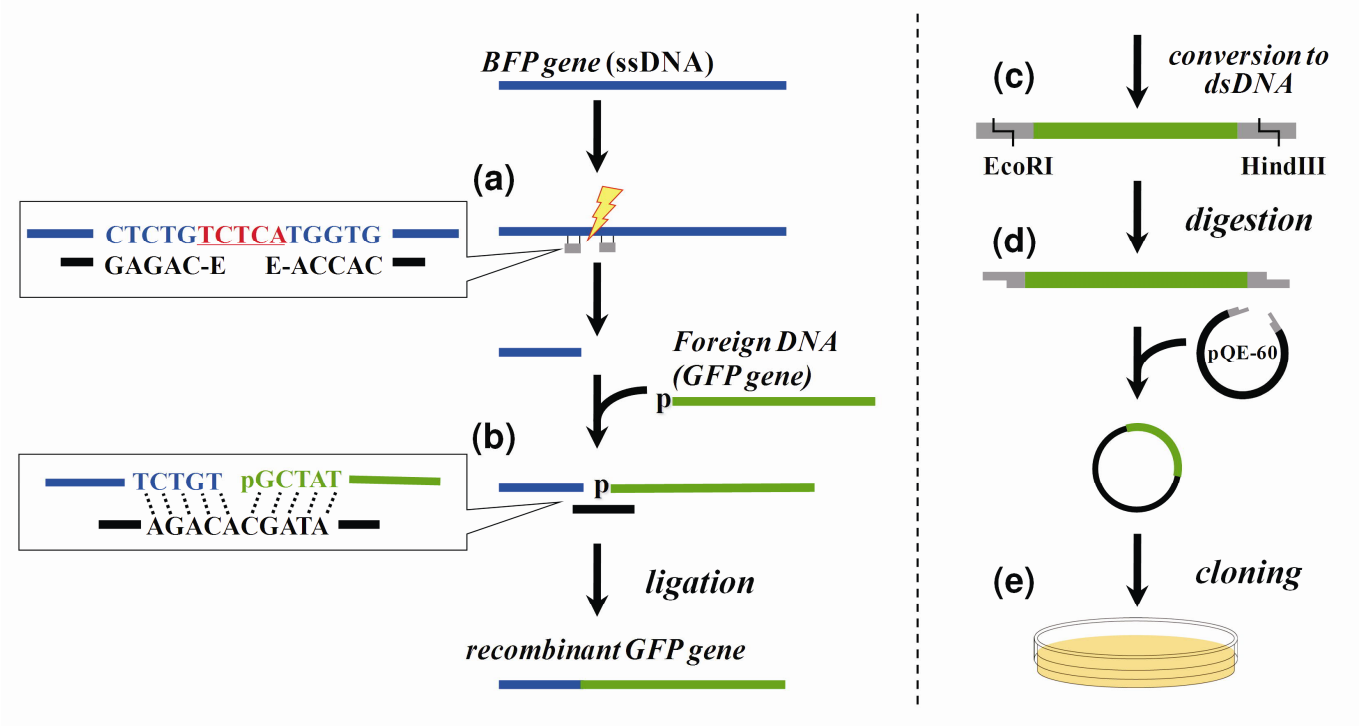

Figure 4. Scheme for the construction of recombinant *GFP* gene through site-selective scission of single-stranded DNA using the present DNA cutter. (a) Site-selective scission of the sense strand of *BFP* gene by Ce(IV)/EDTA and two short EDTP-ODNs. (b) Selective ligation of scission fragment and downstream of *GFP* gene. (c) Conversion of recombinant *GFP* gene to double-stranded DNA by PCR. (d) Incorporation of recombinant DNA to pQE-60 plasmid vector. (e) Transformation of *E. coli* and cloning.

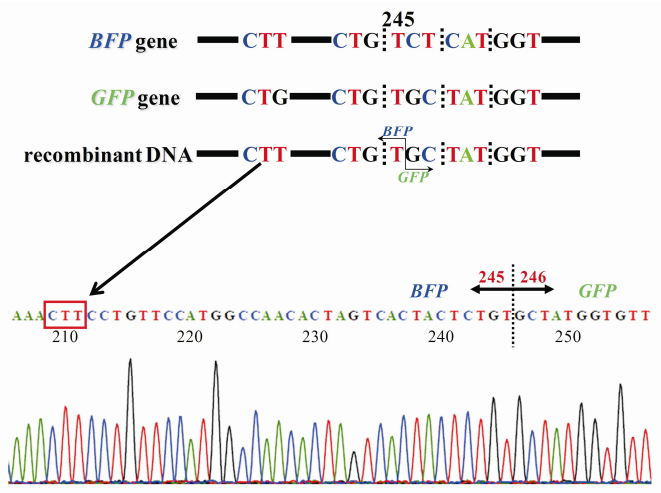

Figure 5. The sequencing result of recombinant DNA. The sequences of the sense strands of *BFP* and *GFP* are also shown. The codon of silent mutation at 209-211 is emphasized by red rectangle.

unmodified ODNs or ODN-monophosphate conjugates are used, however, flanking single-stranded portions in the 3'- and 5'-sides are notably cleaved by Ce(IV)/EDTA.

Enzymatic ligation of the upstream fragment of the *BFP* sense strand with the downstream fragment of *GFP* gene. The scission fragments of about 250 nucleotides were extracted from the polyacrylamide gel. This mixture contains several fragments formed by the scission of different phosphodiester linkages in the gap-site (these linkages are hydrolyzed at almost the same rate). In order to pick up only the G^{1}-T^{245} fragment of *BFP* from the scission mixture and ligate this fragment with the G^{246}-C^{838} of *GFP*, a ligation-guide ODN (20-mer; **Fig. 4b**) was used.[3] This ODN is complementary to both A^{236}-T^{245} in the G^{1}-T^{245} fragment of *BFP* and G^{246}-T^{255} in the G^{246}-C^{838} of the *GFP*, binding these two fragments together. Except for the addition of this ligation-guide ODN, the procedure was exactly the same as that in conventional ligation. By this simple method, only the G^{1}-T^{245} fragment of the sense strand of *BFP* gene was selectively ligated with the downstream portion of *GFP*, providing the desired recombinant single-stranded DNA with precise reading frame (see below).

With the use of another ligation-guide ODN, the corresponding scission fragment can be selectively picked up from the reaction mixture and incorporated into the recombinant DNA. Accordingly, any scission fragment can be obtained by using an appropriate ligation-guide ODN, although these recombinant DNAs involve deletion or insertion of nucleotide(s), and thus are of no use to prepare a fluorescent protein in the present manipulation.[3] This result confirms the versatility of the present ligation method.

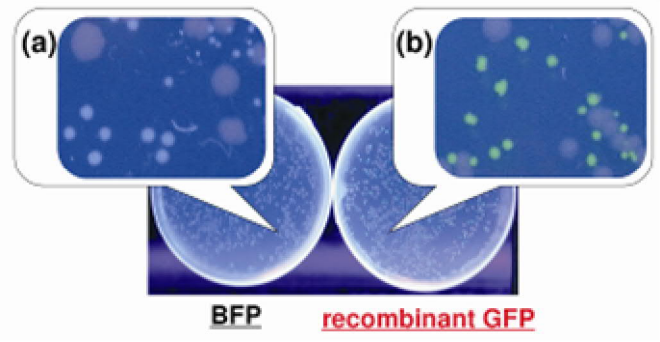

Figure 6. Expression in *E. coli* of the recombinant gene which was constructed by using the artificial DNA cutter composed of Ce(IV)/EDTA and EDTP-ODNs (b). In (a), the plasmid coding BFP was transformed as a control.

Protein expression from the recombinant gene in *E. coli*. Successful preparation of the desired single-stranded DNA fragment with the use of the present DNA cutter, as well as formation of the corresponding recombinant DNA, was confirmed as follows. The single-stranded recombinant DNA was first converted to a double-stranded form by PCR (**Fig. 4c**), integrated into plasmid DNA (pQE-60 vector; **Fig. 4d**), and cloned (**Fig. 4e**). The sequencing results are completely consistent with the arguments (**Fig. 5**). Note that the nucleotide 211 in the recombinant DNA was T, showing that this portion really came from the *BFP* fragment. The corresponding nucleotide in *GFP* is G, making a silent mutation (both CTT^{211} and CTG^{211} code Leu). This result completely rules out the possibility that this obtained clone was the result of *GFP* contamination. As expected, the protein expressed in *E. coli* clearly emitted green fluorescence (**Fig. 6b**). It has been concluded that no critical side reactions (e.g., elimination, oxidative damage of nucleobases or off-target scission) occurred during the preparation of the single-stranded DNA fragment and its manipulation. Otherwise no clone could be obtained or several mutations should be induced at sites not limited to the chromophore-coding region.

Conclusion

By combining Ce(IV)/EDTA and ethylenediamine-*N,N,N',N'*-tetrakis(methylenephosphonic acid)-oligonucleotide conjugates, a new tool to manipulate long single-stranded DNA has been developed. With the use of the multi-phosphonate ligands, undesired off-target scission is sufficiently suppressed to allow site-selective scission of long single-stranded DNA even with the use of short oligonucleotides (around 20-mers). When the previous artificial DNA cutter without EDTP ligands was used, the gene manipulation was achievable only by adding two long complementary DNA strands which form duplexes with target DNA and protect it from undesired scission. Consequently our previous system can only deal with DNA of limited size. The new system, on the other hand, can cover even long single-stranded DNA, such as viral genomes. Furthermore, it should be noted that EDTP-ODNs (only about 20 mer) are very short compared to the 838-mer *BFP* gene and more than 95% of the target DNA is single-stranded. In addition, the target gap site is only 0.6% of total single-stranded portion, and thus the high site-selectivity of our system was also clearly evidenced here.

This tool is easy to prepare and the scission site can be completely freely chosen. The resultant scission fragment can be successfully ligated with foreign DNA, and the recombinant DNA is satisfactorily expressed to produce the desired proteins. To our best knowledge, this is the sole man-made tool that can manipulate single-stranded DNA by using short ODN conjugates and achieve DNA recombination. This present new tool should be useful to manipulate various single-stranded DNAs (e.g., viral genomes) and these attempts are currently under way in our laboratory.

Experimental Section

Materials

All the phosphoramidite monomers were purchased from Glen Research Co. and EDTP-ODNs were synthesized by using an automated DNA/RNA synthesizer (ABI). The attachment of EDTP to ODN was achieved according to a previous report.[11] Other ODNs for primer and ligation-guide ODN were obtained from Hokkaido System Science Co., Ltd. and were purified by usual methods. The Ce(IV)/EDTA complex was prepared from $Ce(NH_4)_2(NO_3)_6$ (Nacalai Tesque, Japan) and EDTA·4Na (TCI, Japan) as described perviously.[7]

The 838-mer single-stranded DNA of *BFP* gene and 593-mer single-stranded DNA of *GFP* gene were prepared by asymmetric PCR ([template DNA] = 10 ng, [Fw primer] = 1 μM, [Rev primer] = 0.1 μM, [dNTP] = 0.2 mM, [Ex Taq®] = 2.5 U in 50 μL of Ex Taq® buffer), and single-stranded DNA was separated by 5% non-denaturing polyacrylamide gel electrophoresis (PAGE). These DNAs were extracted from gel and purified by dialysis and ethanol precipitation. The template GFP plasmid was derived from pQBI T7-GFP (Wako), and the BFP plasmid was prepared by using QuikChange® Site-Directed Mutagenesis Method (STRATAGENE). When the mutation was introduced at chromophore-coding site (TCT CAT to TGC TAT), another silent mutation was also introduced (CTT to CTG). This silent mutation was used as a marker to distinguish the *BFP* and the *GFP* genes respectively as explained in the text in detail.

Site-selective scission of single-stranded DNA coding *BFP* gene

The substrate single-stranded DNA was mixed with 5 equivalents of two EDTP-ODN additives in HEPES buffer (pH 7.0). This solution was incubated at 50 °C for 30 min and then the reaction was started by adding the Ce(IV)/EDTA complex (reaction conditions: [substrate DNA] = 0.1 μM, [each of additive ODNs] = 0.5 μM, [HEPES (pH 7.0)] = 5 mM, [NaCl] = 100 mM and [Ce(IV)/EDTA] = 5 μM). This mixture was incubated at 50°C for 17 h and then the reaction was stopped by adding an excess of EDTP. The site-selective scission was assayed by 5% PAGE. The bands were stained by GelStar® (Lonza) and analyzed by a FLA-3000G imaging analyzer (Fujifilm). The desired scission fragments were extracted from the gel and purified as mentioned above.

Ligation of the sense strands of *BFP* (upstream fragment) and of *GFP* (downstream fragment) for the construction of desired recombinant DNA.

The ligation was accomplished by using DNA Ligation Kit ver. 2.1 (TaKaRa) including T4 DNA ligase. The upstream scission fragment of *BFP* was mixed with the downstream of *GFP* and a ligation-guide ODN (5'-ACACCATAGCACAGAGTAGT-3') which is complementary to both 3'-terminus of *BFP* and 5'-terminus of *GFP*. The mole ratio of *GFP* gene, the guide ODN, and *BFP* gene was 3:3:1. The mixture solution was evaporated to a final volume of 5 μL, to which 2.5 μL of Solution II in the kit was added. The ligation reaction was started by adding 7.5 μL of Solution I, and carried out at 16 °C overnight.

Cloning and sequencing of recombinant DNA

The single-stranded recombinant DNA prepared above was converted to double-stranded form by conventional PCR and purified by QIA quick PCR purification kit (QIAGEN). This double-stranded product contains the whole gene of fluorescent protein and has restriction-enzyme site at both ends (*Eco*RI and *Hind*III). After double digestion by these enzymes (from TaKaRa), the product was inserted into the pQE-60 vector and cloned by *E. coli* JM109 (Toyobo). The plasmid DNA was extracted by QIAprep Spin Miniprep Kit (QIAGEN), and the sequence of the recombinant DNA was determined by a sequencer (Applied Biosystems 3130*xl* Genetic Analyzer) using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

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