

## Supplementary Information

### Artificial restriction DNA cutter for site-selective gene insertion in human cells

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## Experimental Details

### Materials

The plasmid pBFP-Amp<sup>r</sup> was prepared from pBFP-N1<sup>18</sup> by exchanging the Kan<sup>r</sup>/Neo<sup>r</sup> gene to an ampicillin-resistance gene Amp<sup>r</sup>. The pcPNA strands were synthesized and characterized as described before.<sup>16,18</sup> In place of conventional nucleobases A and T, pseudo-complementary bases, 2,6-diaminopurine (D) and 2-thiouracil (U<sub>s</sub>), were used to facilitate the double-duplex invasion. The Ce(IV)/EDTA solution was prepared by mixing an aqueous solution of Ce(NH<sub>4</sub>)<sub>2</sub>(NO<sub>3</sub>)<sub>6</sub> and EDTA·4Na in HEPES buffer and then adjusting the pH to 7.0 with a small amount of NaOH. The human cells were maintained in DMEM supplemented with 10% fetal bovine serum at 37°C with 5% CO<sub>2</sub>.

The donor used for the insertion of IRES-Neo<sup>r</sup> was prepared by PCR from the corresponding vector, kindly provided by Prof. Hiroshi Ueda of the Tokyo Institute of Technology. In order to obtain the donor for the insertion of 2A-DsRed2, pDsRed2-C1 plasmid (Clontech) was used as the template for PCR.

### Site-selective scission of plasmid DNA by ARCUT

In order to form the invasion complex, pBFP-Amp<sup>r</sup> plasmid and pcPNAs were incubated in 5 mM pH 7.0 HEPES buffer at 50 °C for 1 h. The ARCUT reactions were started by adding aqueous solution of Ce(IV)/EDTA and carried out at 37°C and pH 7.0. The reactions were stopped by adding ethylenediaminetetramethylenephosphonic acid, and the scission product (4.3 kbp) was purified by 1 % SeaKem GTG agarose gel electrophoresis.

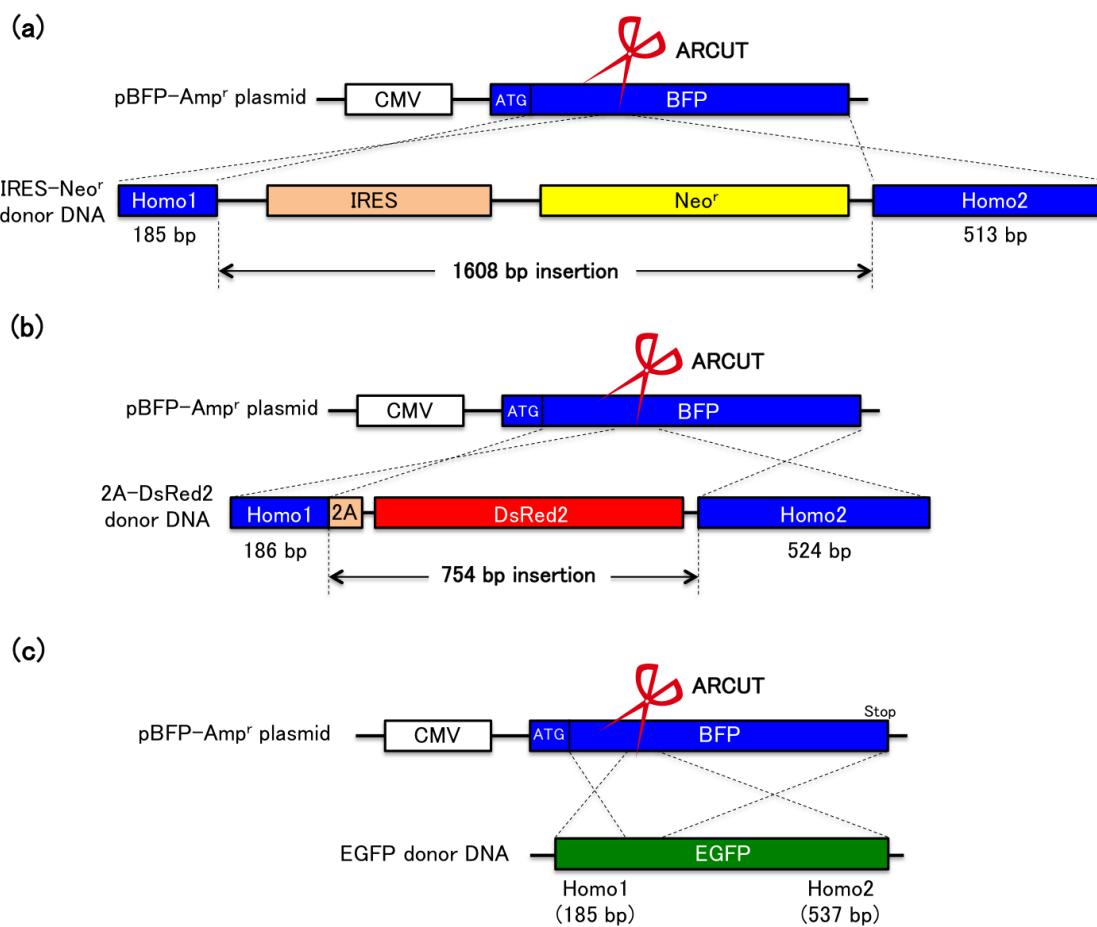
### Targeted insertion of IRES-Neo<sup>r</sup> gene cassette and antibiotic selection

For the “ARCUT” experiment (red, in Fig. 2), both 234 ng of ARCUT product of pBFP-Amp<sup>r</sup> and 766 ng of IRES-Neo<sup>r</sup> donor were introduced to human Flp-in 293<sup>TM</sup> cells by 6 µl of FuGENE HD transfection reagent (Promega). Two days after the transfection (Day 1), G418 antibiotic was added to the medium at 1.2 mg/ml. From Day 1 through Day 7, the numbers of living cells were counted. Blue line in Fig. 2 is for the experiments where the plasmid was not treated with ARCUT and directly transfected into the cells together with the corresponding donor. In the purple or green line, only the plasmid (without the donor) or the donor (without the plasmid) was respectively transfected into the cells. The orange line was for the treatment with only transfection reagent.

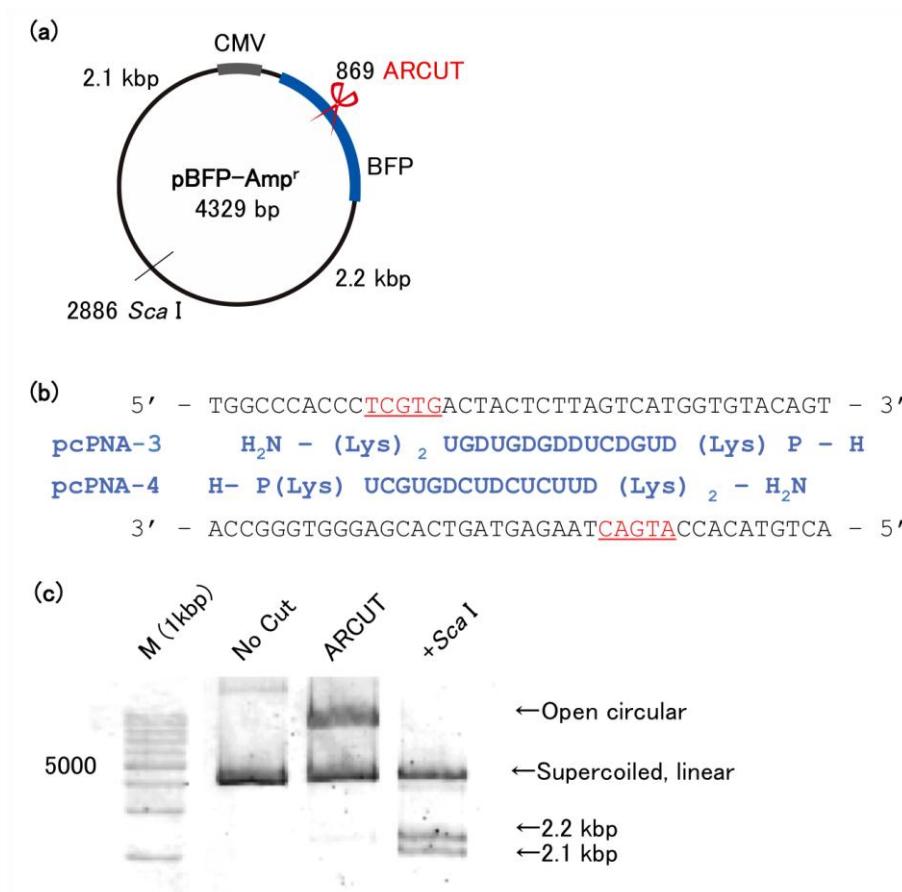
### 2A-DsRed2 gene cassette insertion, BFP-EGFP gene mutation and fluorometric evaluation of the insertion efficiency

By electroporation using Neon<sup>TM</sup> Transfection System (Life Technologies), 4.8 nM of ARCUT product of pBFP-Amp<sup>r</sup> and 34 nM of 2A-DsRed donor DNA (or of EGFP donor) were introduced to human 293T cells. Details of the constructs are presented in Fig. S1. (“ARCUT” bars in Fig. 3a and 3b). Details of the constructs are presented in Fig. S1. The bars “No cut” are for the experiments where the plasmid was not treated with ARCUT and directly transfected into the cells together with the corresponding donor. In the bars “Donor”, only the donor (without the plasmid) was transfected into the cells. The conditions of electroporation were Square wave, Pulse Voltage 1150 V, Pulse Width 20 ms, Pulse Number 2, 150 ng of plasmid and 350 ng of donor DNA. After 36 h (for 2A-DsRed2) or 24 h (for EGFP) incubation which is sufficient for the folding of those fluorescent proteins at 37 °C in 5 % CO<sub>2</sub>, the cells were observed by the fluorescent microscopy DMI6000B (Leica Microsystems) and analyzed by flow cytometry (Guava EasyCyte Plus, Millipore).

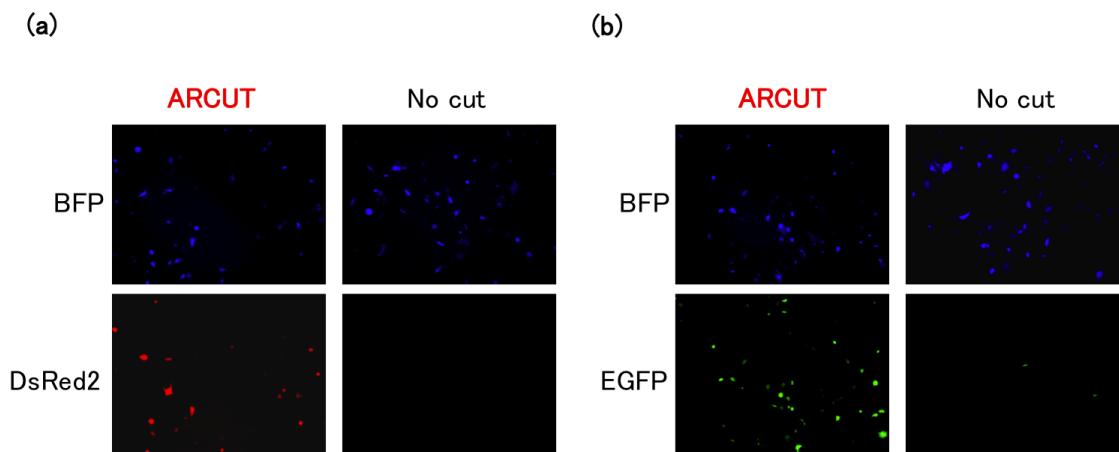
## Supplementary Figures



**Fig. S1** Plasmids and donor DNAs used in this study. (a) For IRES-Neo<sup>r</sup> cassette insertion, pBFP-Amp<sup>r</sup> plasmid DNA was chosen as a target DNA. As a donor DNA, a gene cassette (2306 bp) which includes two homologous sequences, Homo1 (upstream, 185 bp) and Homo2 (downstream, 513 bp), IRES region and neomycin-resistance gene (Neo<sup>r</sup>) was used. Precise homologous recombination results in insertion of a 1608 bp-gene cassette. (b) pBFP-Amp<sup>r</sup> plasmid and 2A-DsRed2 donor DNA (1464 bp) was used for DsRed2 gene insertion study. The donor DNA includes 186 bp and 524 bp of homologous sequences and 2A-DsRed2 gene cassette as 754 bp insertion. (c) For mutation study, ARCUT-cleaved pBFP-Amp<sup>r</sup> plasmid and 741 bp of EGFP donor DNA were electroporated. Further details are available in Refs. 18 and 19.



**Fig. S2** ARCUT-cleavage experiment of pBFP-Amp<sup>r</sup> plasmid DNA. (a) The map of pBFP-Amp<sup>r</sup> plasmid. ARCUT scission site is located in the chromophore-forming site of BFP gene. Double-scission by ARCUT and restriction enzyme *Sca* I generates two DNA fragments (2.1 and 2.2 kbp). (b) Sequences of two pcPNAs (pcPNA-3 and pcPNA-4) used in this study. Each pcPNAs is complementary to sense and antisense strand, respectively. Five bases colored in red on each DNA strands are exposed as single-stranded and hydrolyzed by Ce(IV)/EDTA complex. (c) Agarose gel electrophoresis analysis of ARCUT-cleaved plasmids. Intact plasmid (Lane “No Cut”), ARCUT-treated plasmid (Lane “ARCUT”) and ARCUT/*Sca* I-treated plasmid (Lane “+*Sca* I”) were analyzed. Three forms of the plasmid were indicated by the arrows. With the formation of two DNA fragments (2.1 and 2.2 kbp) which were produced by double scission by ARCUT and *Sca* I (the “+*Sca* I” lane), site-selective scission on BFP gene by ARCUT was confirmed.



**Fig. S3** Fluorescence images of the electroporated cells. (a) 2A-DsRed2 insertion study. Left column shows blue and red fluorescence emitting cells, which were electroporated with ARCUT-cleaved pBFP-Amp<sup>r</sup> and 2A-DsRed donor DNA. With Neon<sup>TM</sup> Transfection System (Life Technologies), 4.8 nM of ARCUT product of pBFP-Amp<sup>r</sup> and 34 nM of 2A-DsRed donor DNA were introduced to human 293T cells (more details are described above in the Experimental Details section). Right column shows the control experiments in which intact pBFP-Amp<sup>r</sup> and the donor DNA were electroporated. (b) BFP-EGFP mutation study. The same amounts of ARCUT product of pBFP-Amp<sup>r</sup> (4.8 nM) and the donor involving the chromophore-forming part of EGFP gene (34 nM) were electroporated into the human cells.

## Sequences of pBFP-Amp<sup>r</sup> and donors

### pBFP-Amp<sup>r</sup>

TAGTTATTAATAGTAATCAATTACGGGGTCATTAGTCATAGCCCCATATATGGA  
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**IRES-Neo<sup>r</sup> donor**

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## 2A-DsRed donor

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**EGFP donor**

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