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Dynamic Genome Editing Using In Vivo Synthesized Donor ssDNA in *Escherichia coli*

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Abstract: As a key element of genome editing, donor DNA introduces the desired exogenous sequence while working with other crucial machinery such as CRISPR-Cas or recombinases. However, current methods for the delivery of donor DNA into cells are both inefficient and complicated. Here, we developed a new methodology that utilizes rolling circle replication and Cas9 mediated (RC-Cas-mediated) in vivo single strand DNA (ssDNA) synthesis. A single-gene rolling circle DNA replication system from Gram-negative bacteria was engineered to produce circular ssDNA from a Gram-positive parent plasmid at a designed sequence in *Escherichia coli*. Furthermore, it was demonstrated that the desired linear ssDNA fragment could be cut out using CRISPR-associated protein 9 (CRISPR-Cas9) nuclease and combined with lambda Red recombinase as donor for precise genome engineering. Various donor ssDNA fragments from hundreds to thousands of nucleotides in length were synthesized in *E. coli* cells, allowing successive genome editing in growing cells. We hope that this RC-Cas-mediated in vivo ssDNA on-site synthesis system will be widely adopted as a useful new tool for dynamic genome editing.

Keywords: rolling circle replication; rolling circle origin; single-strand DNA; guide RNA; PAM-independent; SpyCas9

1. Introduction

After several decades of development, genome engineering has become a highly developed and indispensable tool for biological study and bioengineering applications [1]. A large toolbox has been developed using different molecular machinery, including recombinases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) systems (CRISPR/Cas9 system) [2–4]. Homologous recombination can introduce precise deletions or insertions into the genome. The donor DNA template, either single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA), is a crucial part for homology-directed genome editing [5,6]. Recombineering mediated by linear dsDNA fragments or circular plasmids requires long (1–5 kb) homology arms flanking the insertion sequence [7,8]. By contrast, commercial ssDNA oligos can only provide short homology arms (<200 nts) [9,10]. Experiments with single-stranded donor DNA yielded between 10 and 100% recombination frequency for point mutations [7]. Oligo-mediated recombination can be used to induce allele substitution, small (20–30 base) insertions [11,12] or deletions of up to ~45 kb [13].

Although short ssDNA oligo are useful for the modification of multiple loci, they cannot be readily used for large knock-in applications [14]. It is known that longer sequences better accommodate the extended spacing at editing sites [15]. Long linear ssDNAs synthesized in vitro have been used for homology-directed genome editing [16,17]. For genomic knock-ins in human cell lines, long (~2 kb) ssDNA templates have unique advantages in terms of repair specificity, while dsDNA templates of a similar size (polymerase chain reaction (PCR) products or plasmids) suffer from high rates of off-target integration [18]. Furthermore, the editing efficiency for the integration of larger sequences into the genome is heavily dependent on the concentration of introduced donor DNA and the transformation efficiency, which often require laborious and outdated genetic engineering techniques [7,19]. Although there are still many unknown elements in the mechanism of homologous recombination, it is well established that ssDNA is often more favorable than dsDNA as donor template with regard to biological function. [15,18]. However, the synthesis of ssDNA fragments several kilobases in length requires very expensive and complicated synthesis processes. [20–22]

Theoretically, in-vivo synthesis of an ssDNA donor template in the cell could be of great advantage for genome editing, especially for knock-ins of exogenous sequences. Fortunately, in-vivo ssDNA synthesis is known to occur in nature under specific circumstances. Notably, bacteriophages such as M13, fd and f1 have ssDNA genomes, and phagemids based on their backbones can replicate as plasmids inside the cell [23–25]. In fact, phage systems were some of the first tools for the production of ssDNA. Alternatively, rolling-cycle replication (RCR) of plasmids has been shown to produce intermediates in the form of circular ssDNA. A number of small, multi-copy rolling-circle plasmids from *Staphylococcus aureus* and *Bacillus subtilis* have been studied in detail [26–31]. The replication of these RCR plasmids requires only three elements—a gene encoding the initiator protein (Rep), a double strand origin (dso), and a single strand origin (sso) [32], greatly simplifying their genetic engineering. In particular, the RCR plasmid pC194 has been demonstrated to replicate effectively in *Escherichia coli* and generate a circular ssDNA in this host [33,34]. Furthermore, there is evidence that a sequence of only 55 bp is required to initiate leading-chain replication, which can be terminated perfectly at the 36 bp sequence position [35]. It has been shown that ssDNA can be produced using a reverse transcriptase and directly applied for genome editing in vivo [36]. However, only short ssDNAs were synthesized using this system, while the production and application of longer ssDNAs was not reported.

Here, a rolling-circle replication system driven by a single gene from Gram-positive bacteria was engineered to generated large circular ssDNAs from its parent Gram-negative plasmid vector in a highly controlled fashion in *E. coli*. A specific sequence cassette was designed to tightly control the stable generation of circular ssDNA, from which a desired fragment was cut out using the SpyCas9 nuclease in a protospacer adjacent motif (PAM)-independent manner. Therefore, only circular ssDNA could be cleaved but not the dsDNA plasmid vector, while both of them carry the same target sequence. In *E. coli*, it was demonstrated that circular ssDNAs ranging from 581 to 4960 nts can be produced in vivo, and linearized ssDNA fragments were able to function as donor templates for lambda-Red mediated homologous recombination at target loci on the *E. coli* genome. Using a linear ssDNA fragment with a length of 1208 nts, a 1011 bp sequence was successfully inserted in the middle of the *lacZ* gene in place of an original 11bp sequence. To the best of our knowledge, this is the largest ssDNA fragment generated inside a cell for genome editing. Moreover, this rolling circle replication and Cas9 mediated(RC-Cas-mediated) ssDNA synthesis system can remain functional in the cell for long periods and even be passed on to the next generation. Accordingly, serial subculture significantly improved the genome editing efficiency. These novel features offer distinct advantages compared to transformation with DNA fragments from the outside of a cell, and more biological functions can potentially be coupled together with desired timing. Because the system comprises only two enzymes, we believe that it can be applied to other organisms. Therefore, this in vivo RC-Cas linear ssDNA synthesis system offers a new method for dynamic multiplex genome editing.

2. Materials and Methods

2.1. Strains and Culture Conditions

Wild-type *E. coli* strain DH5α, BL21, DH10β and MG1655 were used in this study. Those cells were cultured in lysogeny broth (LB; 1% (*w*/*v*) tryptone, 0.5% (*w*/*v*) yeast extract, 1% (*w*/*v*) NaCl) with shaking at 220 rpm or on LB agar plates. For ssDNA expression, overnight culture of *E. coli* cells harboring corresponding plasmid were inoculated to fresh LB medium with appropriate antibiotic in 1:100 dilution, then it was cultured at 37 °C with shaking 220 rpm. To induce RepH protein expression, L^+Rha was added to the culture when the OD_{600} reached 0.5. Cells were harvested at mid-log phase (OD_{600}~1.0), then purified the plasmids by plasmid Mini-preparation Kit (DP103, TIANGEN (Beijing) or lysed by cell lysis solution. For genome editing, *E. coli* MG1655 competent cells harboring pRedCas9 which was induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) for bacteriophage λ Red protein (λ-RED) proteins expressed. The cells with or without plasmids were grown in LB medium supplemented with the appropriate antibiotics at 30 °C. Bio-Rad Micro Pulser (0.1 cm cuvette, 1.80 kV) was used for electroporation. 1 μg of plasmid DNA or 1 μM of the editing cassette was mixed with 50 μL competent cells for electroporation. Cells after electroporation were immediately added into 2 mL LB and recovered for 2 h at 30 °C, and inoculated to fresh LB medium in 1:100 dilution or spread on LB agar plates with the appropriate antibiotics.

2.2. Determination and Separation of the Circular ssDNA in Vivo

For verify the activity of RepH protein and RCORI cassette, overnight culture of DH5α harboring plasmid pRC02 was inoculated to fresh LB medium with 100 μg/mL ampicillin in 1:100 dilution and harvested after 6 h induction at 37 °C with shaking. The detail of plasmids construction was shown in Supplementary Materials and Table S1. The purified plasmid DNA was detected on an agarose gel and the appropriate pRC03 band was cut and purified. The resulting purified pRC03 plasmids were then transformed into DH5α competent cells. A single colony was inoculated overnight into 5 mL of LB medium, containing 100 μg/mL ampicillin. The overnight culture was diluted 1:100 into fresh LB broth with appropriate resistance and grown until stationary phase. The plasmid was purified and assessed through the restriction analysis (ScaI).

For circular ssDNA verification analysis, PCR and enzyme digestion analysis was introduced to detect the existence of circular ssDNA. The pRC08 plasmids were purified and used as templates for PCR. Unless otherwise stated, 50 μL PCR reactions were performed using EasyTaq®DNA Polymerases (TransGen Biotech, Beijing, China) and the primer set PCR-2K-F/R (Table A1). Thermocycled reactions were initiated for 5 min at 94 °C and cycled 30 times, involving denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C and primer extension for 30 s per kilobase at 72 °C, finally, the reaction was terminated at 72 °C for 5 min. The PCR product was recovered using plus DNA extraction kit and sequenced by GENEWIG. For enzyme digestion analysis, 10 μL (1 μg) of purified DNA was digested at 37 °C in a solution containing each BamHI and HindIII of 1 μL (= 5 U), 5 μL of restriction buffer (10× Flycut buffer) and 33 μL of ddH2O. Thereafter, the digested sample serves as a template for PCR and the PCR was performed as above.

For circular ssDNA determination analysis, fluorochromes probe binding assays was performed to determine circular ssDNA in the cell lysate. Three kinds of fluorescent probe were designed, which were specifically combined with the corresponding target sequence (Supplementary Materials and Table A2). Overnight culture of DH10β cells harboring plasmid pRC17 were inoculated to fresh LB medium with 100 μg/mL ampicillin in 1:100 dilution and harvested at mid-log phase (OD600~1.0). After incubation, the circular ssDNAs were purified using cell lysis protocol (Supplementary Method). The hybridization assay took place in the same buffer as pre-experiments, the probe concentration was 10 nmol. The solutions were incubated at 30 °C for 1 h. The hybridization solution was then run on a 1.2% agarose gel and the appropriate band was judged by control.

2.3. Iterative Genome Editing Procedure

Genome editing efficiency with linear ssDNA was characterized that produced premature stop codon in the chromosomal lacZ gene. In general, 100 µL 10^{-6} diluted bacterial solution was plated on LB agar plates containing IPTG and 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and grown overnight. Efficiency of allelic replacement was calculated by taking the ratio of the number of white colonies to the total number of colonies on plates. A similar strategy was used in insertion 1011 bp sequences experiments to determine the edit capacity of linear ssDNA. Correct editing colonies were amplified and sequenced by colony PCR with primer set PCR-MG1655-F/R for 11 bp substitution and primer set PCR-1011-F/R for 1011 bp cassette insertion. All experiments were conducted in triplicate and the data were presented as mean values ± standard deviation.

The genome editing efficiency was characterized by transforming the two plasmids into MG1655 cells. The linear donor DNA was provided by plasmid in vivo. The circle ssDNA with specific cleavage site was produced and cut by Cas9 protein. This approach could produce any linear ssDNA of designed length and sequence. To verify the genome editing was performed by linear ssDNA, three plasmids were constructed and characterized that produced four premature stop codons in the chromosomal *lacZ* gene. The pRC22, pRC20 or pRC11 was added to electrocompetent cells (harboring plasmid pRedCas9) for each 50 µL electroporation reaction, respectively. After plasmid transformation, 500 µL cells were inoculated into fresh LB media until OD reached 1.0. Then, the cells were diluted (1:10) into fresh LB media (1:100) for subculture with appropriate selective condition. Sequencing was performed to confirm that the four stop codons was introducing in *lacZ* gene. A similar strategy was used in the integration experiments that inserted 1011 bp sequence on *lacZ* gene to determine the genome editing capacity for linear DNA cassettes in vivo.

2.4. Quantitative and Statistical Analysis

All experiments were conducted in triplicate and the data were presented as mean values ± standard deviation.

3. Results

3.1. Engineering Rolling Cycle Replication for in Vivo Circular ssDNA Synthesis

The pRC02 plasmid was constructed to test the activity of the RepH protein and rolling circle origin (RCORI) in *E. coli*. The gene RCORI105 contains a 105 bp sequence which is necessary for the function of small plasmid replication origins, including the minimal replication origin of pC194 [35]. Efficient termination occurred at a 36 bp sequence (RCORI36) derived from pC194, which lacks a larger palindrome (14 bp) and was reported to have maximal termination activity (Figure 1A) [35]. RepH was placed under the control of prhaBAD so that it can be induced with L^{+}Rha. The RCR starts when RepH binds to the RCORI105 sequence of the pRC02 plasmids and creates a 3′ end as the primer for replication. RepH remains covalently attached to the 5′-end of the nick site. Thus, by designing a RCORI36 sequence at the end of the RCR replicon, the small circular plasmid pRC03 was generated in vivo (Figure 1B). The small plasmid continues to replicate in *E. coli* by the θ mechanism in the presence of ColE1, and is selected via ampicillin resistance selection. Electrophoresis of plasmid DNA extracted from pRC02-DH5α cells showed that the yield of pRC03 was sufficient to observe a slight band of the correct length on the agar gel (Figure 1C). The recovered plasmid was used to transform DH5α competent cells, which were cultured in LB medium. Thereafter, the plasmid DNA was extracted and verified using *Sca*I restriction analysis. The band on the agarose gel was in agreement with the size of pRC03 (Figure 1D), and the recovered band was sequenced by sanger sequencing. The sequence analysis revealed that the isolated small plasmid pRC03 was identical with the prediction (Table A2). This result confirmed that circular ssDNA synthesis is functional in vivo.

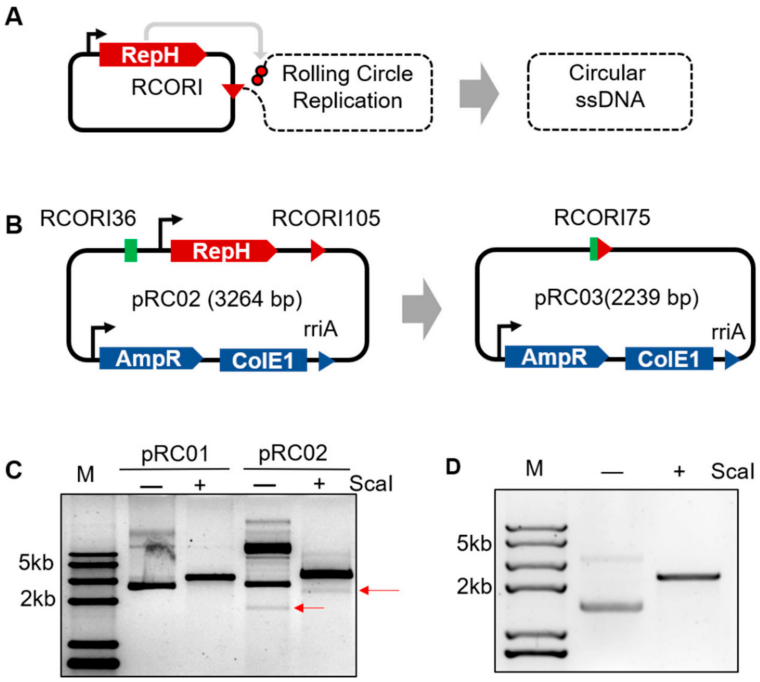

Figure 1. The mechanism for rolling circle origin (RCORI)-mediated DNA synthesis in *Escherichia coli*. (**A**) Schematic of circular single strand DNA (ssDNA) production in vivo. The RCR initiator protein RepH, which acts as a dimer, binds to the RCORI through a sequence-specific interaction. This is followed by nicking of the RCORI by initiator protein (RepH), unwinding of the DNA by helicase, and binding of the single-strand DNA-binding protein (SSB) to the displaced leading strand. Once the replication fork reaches the termination site, the free monomer of RepH cleaves the displaced ssDNA. A series of regulation and cleavage events follow, resulting in the release of a circular ssDNA (displaced leading strand). Solid lines represent double-stranded DNA and dashed lines represent single-stranded DNA. (**B**) Construction of the small plasmid. RCORI105, the RCR origin, sequence consist of 105 bp from pC194; RCORI36, the 36 bp RCR termination sequence from pC194; AmpR, ampicillin resistance cassette; ColEI, high-copy-number ColE1 origin of replication; RCORI75, the newly formed RCORI after circular ssDNA release. (**C**) Generation of the small plasmid pRC03. pRC01 is a control group which not including RCORI36 to verify small plasmid construction. The arrows show the band of the small plasmid. *Sca*I was applied to verify plasmid length. Lane M, DNA Marker. (**D**) Validation of the small plasmid after recovery. -, without ScaI; +, with ScaI. The full sequence of the small plasmid is shown in Table A2.

Next, a series of plasmids were designed to demonstrate the concept and test the ability of our system for circular ssDNA production in vivo (Figure 2A). First, the plasmid pRC08 was constructed to produce a circular ssDNA of 1959 nts (approximately 2 kb; Figure S1C). In addition to the RCORI and *repH* gene that were unchanged from pRC02, a 791 bp fragment of M13 was added into the plasmid. The fragment contained gene *V* and gene *X*, preceded by a strong promoter that was reported to increase circular ssDNA yield in vivo [34]. The plasmids were harvested and digested by *Bam*HI and *Hind*III. An assay was developed based on the PCR amplification using EasyTaq®DNA Polymerase. The plasmids and digested plasmid samples were used as templates to amplify the target products in the same PCR system. Comparative analysis of the PCR products clearly showed that circular ssDNA was generated in vivo (Figure S1C). The size of the target band was 1872 bp, and treatment with restriction enzymes revealed a strong band. Sequencing of the band by sanger sequencing confirmed that the ssDNA was circular (Figure S2B). Next, different plasmid vectors were tested to verify the yield of circular ssDNA. We introduced the plasmid pRC07, which was constructed based on pET19b, to express the RepH protein using the strong T7 promoter. After incubation under the same culture

conditions, PCR was performed to compare the circular ssDNA yield (581 nts circular ssDNA) of pRC06 and pRC07 (Figure S1A). The results showed that the pMVvector was a better choice for circular ssDNA production. After optimizing the vector, pRC09 was constructed to produce a circular ssDNA of 4974 nts (5 kb) in *E. coli* (Figure S2D). The cells of *DH10β* and *DH5α* harboring pRC09 were disrupted using cell lysis solution (CLS), and the target sequence amplified to verify the circular ssDNA yield. This result showed that a longer ssDNA could be produced in vivo with a similar yield. The relative yield and ratio of circular ssDNA were evaluated by comparing the PCR products of the circular ssDNA, plasmid and the 16S rRNA standard. An assay was developed based on the capability of pRC06 and pRC08 to produce circular ssDNAs of different length. We quantified the concentrations of PCR products using the gray-scale intensity of scanned bands (Figure S1B). The PCR yield of the circular ssDNA was only about 3–4 percent of the yields of the plasmid and 16S PCR products.

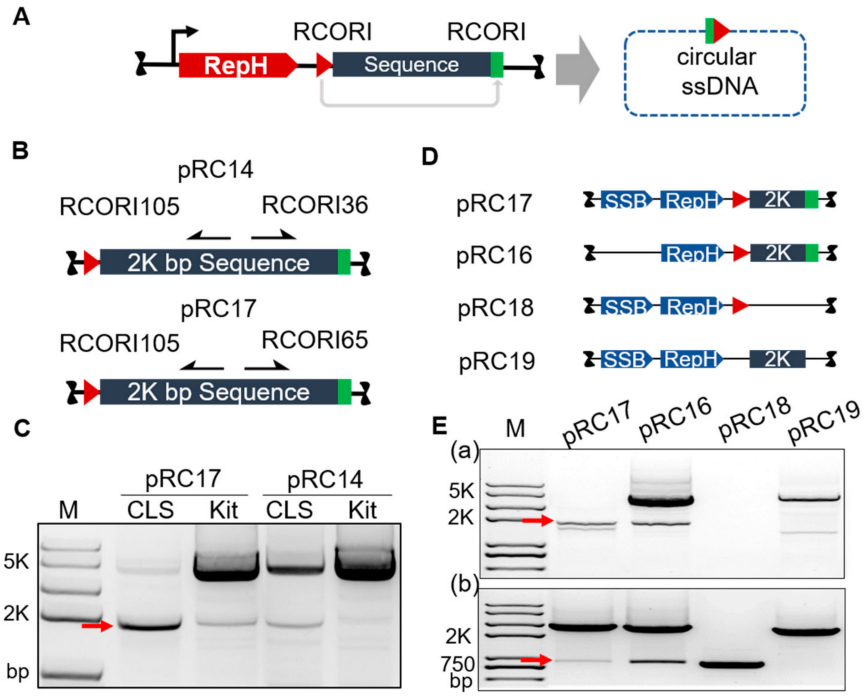

Figure 2. Design of genetic parts for circular ssDNA production in vivo (**A**) Schematic for the conversion of the customized gene to ssDNA. The main RCR sequence contains the *repH* gene, the desired ssDNA sequence and the RCORI gene. Solid lines represent double-stranded DNA and dashed lines represent single-stranded DNA. (**B**) The structure of pRC14 and pRC17 used to produce circular ssDNA in vivo. The only difference between these two plasmids is the RCR terminator, which is RCORI36 in pRC14 and RCORI65 in pRC17. The arrows represent the designed amplification primers of the circular ssDNA. (**C**) The terminator strengths of RCORI36 and RCORI65. The circular ssDNA yield was measured by comparing the abundance of the corresponding PCR products. CLS, cells lysed using a lytic buffer; Kit, plasmids extracted using a commercial miniprep kit; the arrow shows the PCR product (1978 bp) of the circular ssDNA. (**D**) Different combinations of the RCR system. Lane M, DNA Marker (**E**) The impact of different combinations on the circular ssDNA yield and the effect of RCORI65 as an RCR origin. (**a**) The expected PCR product for testing the necessary components to produce ssDNA is 1978 bp. (**b**) The expected PCR product for testing RCORI65 as an RCR origin is 778 bp. The plasmid pRC17 is a control containing all of the RCR components.

Based on these results and a pC194 model, we designed a new plasmid which included a 65 bp terminator (RCORI65) and *EcSSB* gene to increase the capacity of circular ssDNA production in *E. coli*. The RCORI65 sequence contains the replication origin of the ssDNA plasmid pC194 [37]. We developed an analytical protocol to amplify circular ssDNA products from cell lysates using PCR,

which can be visualized using agarose gel electrophoresis. Two primer pairs were introduced to confirm the presence of RCORI105, and if the RepH protein preferentially identified it rather than RCORI65 as the replication origin. The experimental results were in agreement with the expectations. When RICORD105 was used as the RCR replication origin with RICORD65 as the terminator, the yield of circular ssDNA was higher than with RICORD36 as the terminator (Figure 2B), and its initiation activity was negligible. This was further confirmed by passaging experiments (Figure S1F). To verify the sequence, the PCR band was separated by gel electrophoresis, purified and sequenced using a corresponding primer (Figure S2C). A comparison of the quantities of PCR products from cell lysates obtained using different cell disruption methods showed that the newly designed plasmid had a better ssDNA production capacity. Although the cell disruption methods were different, the results were consistent (Figure S1F). The advantage of using EcSSB as a single-strand binding protein for the circular ssDNA is that it is the endogenous ssDNA-binding protein of *E. coli.*

Taken together, the results indicated that the circular ssDNA yield could be increased by using pRC17. We next sought to determine the requirements for ssDNA synthesis using RCORI and RepH in *E. coli*, and constructed a series of plasmids with deletions of each element (Figure 2D). PCR was performed to verify the yield of circular ssDNA and confirm the sequence-specificity. The expression of RCORI105 or RepH protein alone was unable to produce the circular ssDNA because of the specificity of RepH protein for RCORI. By contrast, strong target band was observed in the presence of both the RCORI and RepH protein. This further confirmed that circular ssDNA can be produced only when all replication elements are present (Figure 2E). The expression of all three genes (RCORI, RepH protein and EcSSB protein) enhanced the production of the circular ssDNA eightfold compared to the combination of RCORI and RepH protein. The plasmid pRC17 was then used to produce circular ssDNA in *E. coli* DH10β, which lacks the intracellular exonuclease and SOS response. This plasmid included RCORI105, RCORI65, RepH protein and a strep-tagged EcSSB protein. The simple cell disruption methods of freeze-thawing and enzymatic lysis by lysozyme were used to prepare the cell extract. To better characterize the circular ssDNA, we used a fluorescent probe binding assay to demonstrate that the circular ssDNA was indeed present in the cell lysate (Figure 3A). The binding of the fluorescence probes to DNA in the cell lysate was analyzed using the electrophoretic mobility shift assay (EMSA) in agarose gels. We firstly used the 90 nt oligo to test the specific binding between the probe and the target sequence (Figure S3B). The oligo contained sequences complementary to probes 1 and 2, but identical with probe 3. After the binding assays, we validated the specific binding of the probe to the targeted DNA sequence in the presence of probes 1 and 2. It was clear that probe 3 could not bind to the 90 nt oligo, because the oligo has no complementary sequence with probe 3. Under different binding conditions, the binding efficiency was similar. Thus, the sample was incubated at 37 °C for 90 min.

Thereafter, a control group was introduced to confirm the size of the hybrid product containing the probe and the circular ssDNA. The fluorescence hybridization was intense and highly specific to the target gene. Three of the probes are visible in Figure 3B. The DNA in the cell lysate hybridized with probe 1 and showed a clear band (1984 nt circular ssDNA-probe) on the 1.2% agarose gel. Hybridization probe 2, which was hybridized with the plasmid and had no fluorescence band, confirming that the fluorescence band is a circular ssDNA product. These results visualized and further confirmed the nature of the circular ssDNA product via fluorescence probe binding.

The purification protocol was based on the specific binding between the strep-tagged EcSSB and Magrose Strep-Tactin beads, which were used to isolate circular ssDNA products from lysed cells (Figure 3C). The concentration of the recovered samples was sufficient to observe a slight band of the correct size (Figure 3D). To confirm that the band contained circular ssDNA, we recovered it using a gel extraction kit and amplified it by PCR (Figure 3E). The results clearly showed there was only circular ssDNA in the eluate. To verify the sequence, the 1897 bp band was gel purified and sequenced by sanger sequencing.

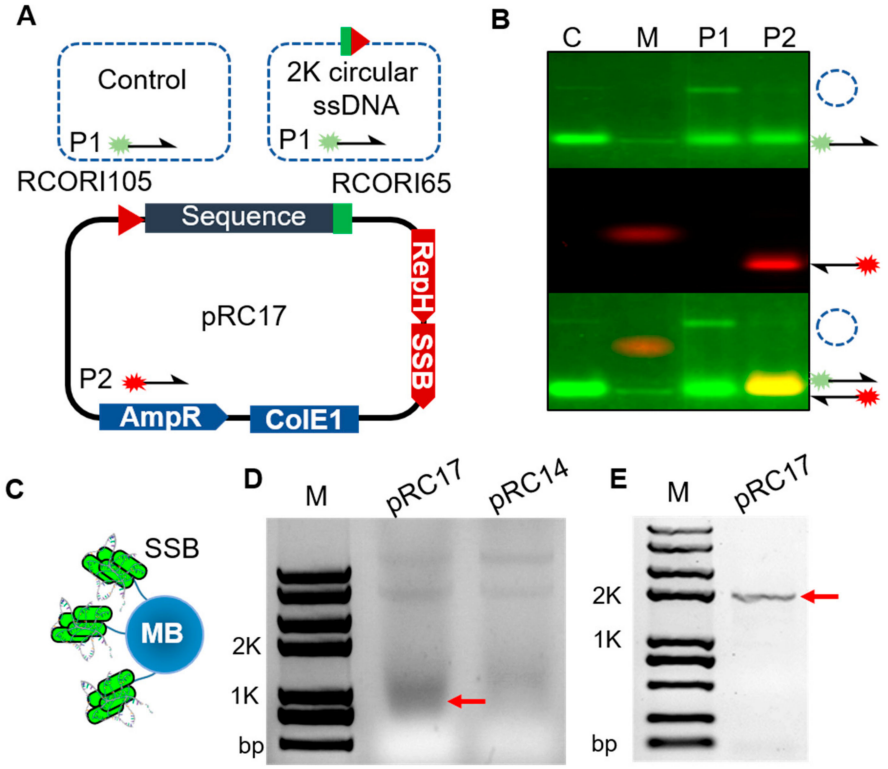

Figure 3. Verification and separation of circular ssDNA. (**A**) The mechanism of the probe binding with the target sequence. Probe 1 is complementary to the circular ssDNA and the control group; probe 2 is complementary to the plasmid. (**B**) Fluorescence images of the probe binding with DNA from the cell lysate. Lane C, probe 1 + control; Lane P1, probe 1 + cell lysate; Lane P2, probe 2 + cell lysate; Lane M, DNA Marker (**C**) Schematic of the ssDNA recovery using Magrose Strep-Tactin beads. (**D**) Comparison of the circular ssDNA recovery yield from cell lysate. Data are shown for the production of circular ssDNA under culture conditions for plasmid extraction. The arrow indicates the band of the circular ssDNA. (**E**) PCR analysis of the recovered circular ssDNA. The arrow shows the PCR products (1978 bp) of the circular ssDNA.

3.2. Homologous Recombination Using in Vivo Synthesized ssDNA Donor

Since the initial data strongly suggested that circular ssDNA could indeed be produced in vivo, we converted the circular ssDNA to linear ssDNA in vivo and used it directly for genome editing (Figure 4A). For this objective, Spy Cas9 protein and a corresponding guide RNA (gRNA) were introduced. It has been demonstrated that the Cas9 protein can recognize and cleave single-stranded DNA (ssDNA) by an RNA-guided, PAM-independent recognition mechanism [38]. Furthermore, the Spy Cas9 enzyme is highly selective for its cognate guide RNA and only supports dsDNA or ssDNA cleavage when its own guide RNA is used in the reaction [39]. The results suggested that Cas9 protein nicked the circular ssDNA under the guide RNA without a PAM site (Figure S4A), and converted it into linear ssDNA. The guide RNA was carefully designed to avoid recognizing homologous sequences within the genome of *E. coli* MG1655. To appropriately design the sequences of our guide RNA (Figure S4A), we used the nucleic acid structure prediction tool Nucleic Acid Package (NUPACK) [40].

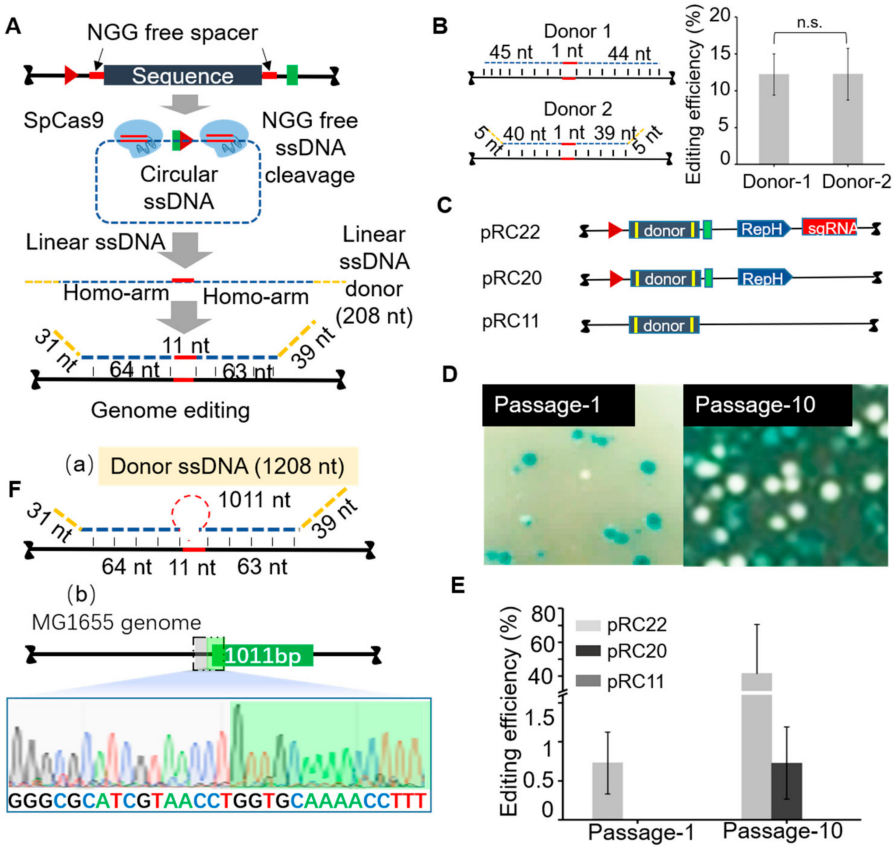

Figure 4. RC-Cas-mediated genome editing in vivo. (**A**) The mechanism of the production of linear ssDNA. SpCas9-mediated DNA cleavage of the targeted circular ssDNA, in the absence of a protospacer adjacent motif (PAM) sequence. (**B**) Verification of the allelic replacement efficiency. There was no significant difference (p = 0.23) in the replacement efficiency between donor 1 and 2 (around 12%) (**C**) The plasmids with different components used for gene editing. pRC22 includes the RCR system, gRNA and donor DNA; pRC20 includes the RCR system and donor DNA; pRC11 only includes the donor DNA (**D**) Photograph of the plates showing the effect of linear ssDNA synthesis for gene editing using pRC22. Passage 1, the first round of culture for introducing the allele substitution (11 bp substitution in the *lacZ* gene); Passage 10, the tenth round of culture for introducing the allele substitution (11bp substitution in the *LacZ* gene). (**E**) The efficiency of substituting 11 bp in the *LacZ* gene using the linear ssDNA donor (**F**) Verification of the insertion efficiency of 1011 bp. (**a**) The mechanism of the 1011 bp insertion (**b**) Sanger sequencing of the 1011 bp insertion.

Firstly, we used 90 nt oligos to test the efficiency of genome editing, whereby one donor was not completely complementary to the target sequence at the 3′ and 5′ ends (Figure 4B). The two 90 nt oligos were introduced to produce mismatch changes in a targeted region of the *lacZ* gene in two distinct cell populations. Blue-white colony counting was used for preliminary statistical analysis of the editing efficiency. Through successive cycles of MAGE, the chromosomal sequence of the *lacZ* region increasingly diverged away from the wild type. The editing efficiency was quantified after MAGE cycle 4, which showed no significant difference (p = 0.23) in editing efficiency (around 12%) between the two groups (Figure 4B). Then, the targeted genomic region of the white colonies was amplified by PCR and sequenced to confirm the validity of genome editing.

Genome editing using the linear ssDNA system is determined by a combination of three factors: the guide RNA for Cas9 protein, the RCR system for the production of the donor circular ssDNA, and the λ-Red homologous recombination system. Under the tested experimental conditions, the genome editing was effective. Three different plasmids (Figure 4C) were separately introduced into MG1655

cells harboring pRedCas9. In passage-1, pRC22 produced a white colony on the plate, which indicates the generation of a correctly edited colony within 12 h (Figure 4D). Through successive passaging, the chromosomal sequence of the *lacZ* region increasingly diverged away from the wild type (Figure 4D, Figure S4C). The rate at which pRC22 induced genomic changes progressively increased from 0.73% (passage 1) to 41.45% (passage 10) (Figure 4E). By contrast, pRC11 showed 0% editing efficiency even at the 10th passage. The pRC20 plasmid led to a gradual increase of correct editing from 0% to 0.73%. Because our screening is not selective, the calculation of editing efficiency here is based on the total number of bacterial cells. The sequencing results from amplified products in passage-1 and passage-10 indicated that the correct editing rate was 100% in passage 1, but passage-10 had only around 60–80% correctly edited colonies. However, the incorrect colonies were accounted for by 2–4 false positives or colonies that yielded no PCR products. Given the scalable nature of our approach, further increase of the insert at the identical genome editing site to 1011 bp did not significantly affect the number of correctly edited colonies (Figure 4F). We detected insertions by amplifying the target region followed by Sanger sequencing. These results collectively confirmed that the RC-Cas mediated genome editing system allows the efficient integration of DNA as large as 1 kb.

4. Discussion and Conclusions

In this study, we developed a scalable platform for genome editing based on in vivo synthesis of linear ssDNA in living cells. The system utilizes a modular rolling circle replication structure, which is converted into linear ssDNA via cleavage by the PAM-independent SpyCas9 nuclease. By comparing the ratios of blue/white colonies obtained after targeting the chromosomal *lacZ* gene with three different plasmids, we confirmed that the RC-Cas-mediated linear ssDNA integration system could effectively improve the efficiency of genome editing. Notably, the editing efficiency was calculated based on the entire population of bacteria, rather than using a survival-based selection step. Correct editing was only observed in the experimental group in passage 1, and the efficiency was 0.73%. For verification of the editing capability, a 1011 bp sequence was inserted into the genome and sequencing was used to confirm that the sequence was inserted in the correct location. It is noteworthy that the ssDNAs in our system have been used directly for genome editing without additional preparation steps.

The concentration of donor DNA is another crucial issue influencing the efficiency of genome editing, but this could be addressed by improving the efficiency of the RCR module and developing a more suitable method for the linearization of circular ssDNA in vivo. Previous studies revealed that many factors in *E. coli* play important roles during pC194 replication. For example, the production of ssDNA can be effectively increased (up to 70% of the total plasmid DNA) by the overexpression of a single-stranded DNA-binding protein (M13 gene *V*) in the cell [34]. Similarly, the deletion of rriB from plasmids can effectively prevent the transformation of circular ssDNA into the dsDNA form [34]. UvrD can act as a helicase during the replication of pC194 in *E. coli* [41]. Consequently, we examined several parameters to improve the efficiency for circular ssDNA production in *E. coli*. The highest ssDNA yield was achieved using stable modules (RCORI105 and RCORI65) combined with the expression of RepH and EcSSB proteins. Implementing this requires that the terminator ensures that the circular ssDNA ends precisely at RCORI65 after replication. We also confirmed that the production of circular ssDNA could be increased by overexpressing an ssDNA binding protein. The EcSSB protein is the endogenous ssDNA-binding protein of *E. coli* and is known to protect ssDNA during plasmid replication [42]. All ssDNA production experiments were performed in the *E. coli* strain DH10 beta, which lacks intracellular exonuclease activity and SOS response, thus preventing the ssDNA degradation [43]. To our best knowledge, this is the first study demonstrating that the rolling cycle replication of pC194 is functional in a commonly used *E. coli* strain, while other studies relied on specifically constructed laboratory strains [34,35]. However, the efficiency of circular ssDNA synthesis was strain-dependent, whereby DH10 beta was better compatible than MG6155.

In addition, we consider that the conversion efficiency of circular ssDNA to linear ssDNA is also important for genome editing in vivo. This might be solved by introduced other mechanisms

for DNA cleavage, such as DNAzymes [44,45] or CRISPR-Cas12a proteins [46,47]. Here, we used the RNA-guided, PAM-independent recognition mechanism of SpyCas9 to recognize and cleave circular ssDNA [38]. Due to this mechanism, the Cas9 nuclease complex only cleave the circular ssDNA in complex with the gRNA, while it does not damage the dsDNA plasmid vector. This is very convenient for engineering, since SpyCas9 can be combined with different gRNAs to specifically cleave genomic dsDNA as well as the in vivo synthesized ssDNA. The genetic modifications were successfully introduced in the cell population probably because of the RC-Cas-mediated platform directing synthesized ssDNA to the lagging strand of the replication fork during DNA replication. Further exploration of this platform is required to determine the exact mechanisms involved in ssDNA synthesis and integration into genomic DNA. Another potential benefit of our platform is that the desired ssDNA can be integrated into the cells and used directly in gene editing without additional expenses. The cost of commercially synthesized oligos currently ranges from $0.10 to 0.30 per bp ($100–300 for a 1kb gene) [48]. Consequently, approximately $30 of synthesized oligos are needed to introduce 27 bp of mutations at full degeneracy for a single genomic target [13]. Particularly, it demonstrated that circular ssDNA may be a suitable intermediate material in living cell for bioengineering, due to liner ssDNA generally being considered with important role as signal for cell damage and rising many molecular rescue reactions [49,50]. In view of replication intermediate ssDNA of many bacteria plasmids or phage was converted to dsDNA from specific loci [23,37], it may implicit that in nature the circular ssDNA may couple with more in vivo DNA metabolism pathways and genomic element transfer. Furthermore, because there were only two proteins, RepH and Cas9, necessary for this in vivo ssDNA generation, we believe that it is very possible to be applied on mammal cells; however, there will be more demand in bacteria cell engineering with large genome modification for bio-product synthesis in highly human designed way. To the best of our knowledge, this is the largest ssDNA structure synthesized in vivo for genome editing to date. We hope that this RC-Cas-mediated on-site ssDNA synthesis system will become a useful molecular tool that is compatible with most genome editing system as donor template, and pave the way for dynamic genome editing applications.

5. Patents

The initial filings were assigned Chinese patent application 201911382846.5.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/9/2/467/s1, Figure S1: Verification and optimization of circular ssDNA, Figure S2: Sequencing analysis, Figure S3. Confirmation of circular ssDNA in vivo, Figure S4. Design and Verify gene editing capability of ssDNA in *E. coli*, Table S1: List of the plasmids used in this study.

Author Contributions: Conceptualization, methodology, M.H.; validation, Z.W.; formal analysis, M.H. and H.Y.Q.; investigation, P.Y.; data curation, writing—original draft preparation, writing—review and editing, M.H.; visualization, Z.W.; supervision, H.Q. and J.Q. resources, project administration, funding acquisition, H.Q. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: H.Q. is the inventor of one patent application for the biochemical method described in this article. The initial filings were assigned Chinese patent application 201911382846.5. The remaining authors declare no competing financial interests.

Appendix A. Supplementary Method

Appendix A.1. Strains and Culture Conditions

Chemically competent cells of *E. coli* DH5α and BL21 were prepared as described previously [51]. Ampicillin (Amp) and spectinomycin (Spe) were added at final concentrations of 100 μg/mL. L (+)

rhamnose monohydrate (L+Rha; 1 mM final concentration) and Isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM final concentration) were used to induce protein expression. X-gal (40 μg/mL final concentration) was added to the solid LB plates for blue-white selection. For ssDNA recovery, cells were treated using a plasmid Mini-preparation Kit (DP103; TIANGEN (Beijing) or lysed using cell lysis solution (CLS; 10% Triton X-100, 2 mM Ethylene Diamine Tetraacetic Acid (EDTA), 20 mM Tris-HCl, pH 8.5) at 98 °C for 10 min. A sample comprising 1 mL of bacterial solution was centrifuged and suspended in 200 μL CLS. Unless indicated otherwise, 50 ng of plasmid DNA or 1 μL of cells treated with CLS was used as templates for PCR. For genome editing, electrocompetent cells of *E. coli* MG1655 were prepared as described previously [7,13] and cultured in LB at 30 °C.

Appendix A.2. Construction of Plasmids

The primers used for plasmid construction were synthesized by GENEWIZ (Suzhou, China) and are listed in Table A1. For the preparation DNA fragments, PCR was performed using 1X PrimeSTAR®Max DNA Polymerase (Takara Co., Ltd., Beijing, China) unless specified otherwise. The DNA cassettes were purified using the plus DNA clean/extraction kit (DP034P; GMbiolab Co, Ltd., China). The concentration of the recovered DNA cassettes was measured using a Nano-100 (Hangzhou Allsheng Instrument co., ltd.). All plasmids were constructed in DH5α competent cells. The plasmids were purified using a commercial plasmid miniprep Kit. For analytical purposes, restriction digestion and PCR were performed using restriction enzymes (TransGen Biotech Co., Ltd, China) and EasyTaq®DNA Polymerase (TransGen Biotech, Beijing, China), respectively. DNA samples were separated by electrophoresis on 1.2% agarose gels, visualized using ethidium bromide staining (EB), and photographed using an C300 gel imaging system (Azure Biosystems, US). Gel bands were analyzed and quantitated using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The gel background intensity was calculated and subtracted from the presented intensity values. The bands of the correct size were excised and purified using a commercial kit. All the images have been inverted. All plasmids were sequenced by GENEWIZ corporation (Suzhou, China). The plasmids and detailed construction methods are listed in Supplementary Table S1.

To confirm the activity of RepH protein and the RCORI cassette in *E. coli*, plasmid pRC02 was constructed to produce a small plasmid. The RCORI36 sequence was used as the terminator. It was synthesized by GENEWIE and ligated between the *Bgl*II and *Xho*I sites of the delivery plasmid. The plasmid backbone with pRC01 was amplified using the primer pair pMR5-F/R. These two parts were ligated together using T4 DNA ligase (Takara Biomedical Technology Co., Ltd., China), resulting in plasmid pRC02. The small plasmid was generated and named pRC03.

To verify the production capacity of circular ssDNA and optimize the circular ssDNA yield, a series of plasmids were constructed and listed in Supplementary Table S1. pRC06 was constructed for the production of a 581 nts circular ssDNA based on pMV (The plasmid vector from pUC19 which was not included *rriB* and MCS cassette). pRC07 was constructed for the production of a 581 base circular ssDNA based on pET19b. pRC08 was constructed for the production of a 1973 nts circular ssDNA based on pMV. pRC09 was constructed for the production of a 4974 nts circular ssDNA based on pMV. pRC14 was constructed for the production of a 1975 nts circular ssDNA based on pUC (The plasmid vector from pUC19 which was not included *rriA*, *rriB* and MCS cassette). pRC17 was constructed for the production of a 1984 nts circular ssDNA based on pUC. The sequence information is available in Table A2.

To construct the ssDNA-mediated genome editing system, we introduced the pRedCas9 plasmid to express Cas9 protein and the λ Red recombineering system. Oligos were synthesized by GENEWIZ and used as donor DNA of 90 nts, containing four phosphorothioate linkages at the 5′ terminus. pRC12 was constructed to produce ssDNA, which could insert four consecutive termination codons into the *lacZ* gene on the *MG1655* genome. To generate a linear ssDNA template for genome editing in vivo, five modularized parts were needed, which included Cas9 protein, λ Red proteins, a left homologous arm (L) with a CRISPR/Cas9 recognition region (N20), ssDNA producing structure with RepH protein,

RCORI cassette and the substitution or insertion fragment, and a right homologous arm (R) also with a CRISPR/Cas9 recognition region (N20). Cas9 protein and the λ Red recombineering system were expressed from the plasmid pRedCas9, which contained a p15A replication origin and a Spe resistance gene. pRedCas9 [52] was a gift from the Tao Chen lab (Tianjin University). The 20 bp spacer sequence (5′-GTTTGAGGGGACGACGACAG-3′, named gRNA2) with the scaffold was designed to have no sequence homology with the *E. coli* genome using NUPACK (http://www.nupack.org) and incorporated a pJ23119 promoter which was synthesized by the Beijing Genomics Institute (BGI; Beijing, China). The gRNA2 was used as a guide RNA for Cas9 protein to cut the ssDNA target without a PAM sequence [38]. A series of plasmids were constructed to verify the capacity of genome editing in vivo (Supplementary Table S1). The plasmid pRC22 was constructed to produce a 208 nt linear donor DNA for genome editing, while pRC20 and pRC11 acted as controls. The plasmid pRC22 contained the complete structure encompassing RCORI, RepH and target-gRNA, which could produce a linear ssDNA for genome editing. pRC20 contained RCORI and RepH but no target-gRNA, and could produce only a circular ssDNA. pRC11 only had the donor sequence but no structural of RCORI, *repH* gene or target-gRNA, and therefore, could not produce any ssDNA. Thereafter, pRC24 was constructed for genome editing with a curing system to afford the 1208 linear ssDNA for 1101 bp sequence insertion. The details of plasmid construction are listed in Table S1.

Appendix A.3. Quantification of Circular ssDNA

Overnight cultures of DH5α harboring plasmid pRC06 or pRC08 were used to inoculate 5 mL of fresh LB medium with 100 μg/mL ampicillin at a 1:100 dilution and harvested at mid-log phase (OD_{600}~1.0). The cells were lysed using cell lysis solution (CLS; 10% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.5) for 10 min at 98 °C. A sample comprising 1 mL of the bacterial solution was centrifuged and resuspended in 200 μL CLS, and used as templates for PCR. Using the same template, 16S, plasmid and circular ssDNA cassette were amplified using the same system with different primer pairs (16S-F1/R1, plasmid-F1/R1, PCR-2K-F/R or PCR-LacZ-F/R). The gray-scale values of corresponding PCR products were quantified using ImageJ.

Appendix A.4. Optimization the Cassette for Circular ssDNA Yield

In order to compare the ssDNA production capacity of different vectors, the plasmid pRC06 was introduced into DH5α and pRC07 was introduced into JM109 (DE3). The JM109 strain harboring the plasmid pET28a-g5p and JM109 were used as control groups. When the OD_{600} reached 0.5, the expression of RepH protein was induced by the addition of L^{+}Rha (pRC06) or IPTG (pRC07). Cells were harvested by centrifugation during the mid-log phase (OD_{600}~1.0) and lysed with CLS. After the PCR (EasyTaq® DNA Polymerase, 1 μL CLS-lysed cell suspension was used as template, primer pair PCR-LacZ-F/R). The circular ssDNA yield was compared densitometrically.

To verify the production capacity of long circular ssDNA, plasmid pRC09 was constructed to produce a circular ssDNA of 4975 nts. After incubation (OD_{600} ~1.0) and lysis by CLS, the resulting CLS products were then amplified using the primer pair PCR-2K-F/R with EasyTaq polymerase and an extension time of 5 min.

For confirm the optimal cassette, plasmids pRC10, pRC08, pRC14 or pRC17 were introduced into DH10β, DH10B and DH5α, respectively. After PCR (EasyTaq® DNA Polymerase, 1 μL CLS products as template, primer pair PCR-2K-F1/R1), the circular ssDNA yield was compared densitometrically. Furthermore, the pRC14 and pRC17 plasmids were purified and used as templates for PCR. Here, RCORI65 was also available as a circular ssDNA replication origin. Thus, another primer pair was used to judge the reverse circular ssDNA yield, which was named primer 2 (PCR-PU-F/R). The PCR was performed using EasyTaq® DNA Polymerase and the product corresponding to the reverse circular ssDNA was 778 bp. To verify that the RCORI cassette was necessary for circular ssDNA production, three control plasmids (pRC16, pRC18, pRC19) were constructed to produce circular ssDNA in vivo.

After culture, PCR was performed (EasyTaq® DNA Polymerase, 1 µL CLS products as template, primer pair PCR-2K-F1/R1 or PCR-PU-F/R) to compare the circular ssDNA yield via the gray-scale levels.

For verify the stability of pRC17, a single colony was used to inoculate 5 mL of LB medium, and cultivated at 37 °C under shaking at 220 rpm. After 12 h, 50 µL of the bacterial suspension was transferred into 5 mL of fresh LB medium and cultured under continuous shaking for another 12 h. The culture was passaged every 8 h, and the plasmid was purified using a commercial DNA extraction kit. For the PCR, 50 ng of plasmid DNA was used as template, using EasyTaq® DNA Polymerase, and the primer set 1 (PCR-2K-F1/R1) or 2 (PCR-PU-F/R) (Table A1) for 30 cycles with an annealing temperature of 55 °C and an extension time of 1 min.

Appendix A.5. Cell Lysis

After incubation, 100 mL of cultured cells were harvested by centrifugation at 12,000× *g* at 4 °C for 10 min and washed two times with binding buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0), then resuspended in 20 mL binding buffer in a 50 mL centrifuge tube. Resuspended cells were treated with lysozyme to weaken the cell wall and then lysed completely by freezing and thawing. Lysozyme was added to the thawed cell suspension at a concentration of 0.1 mg/mL and incubated at 37 °C under shaking (220 rpm) for 1 h, as reported previously [53]. Then, lysates were incubated at 55 °C for 20 min to inactivate the lysozyme. Two freeze-thaw cycles were performed by freezing the cell suspensions in liquid nitrogen for 15 min and thawing for 30 min in a heating block at 37 °C [54]. The lysates were centrifuged at 12,000× *g* for 10 min at 4 °C, and the pellet was discarded. The extract was flash-frozen in liquid nitrogen and stored at −80 °C.

Appendix A.6. Fluorochromes Probe Binding Assays

Three different fluorescent probes were designed to specifically bind to the corresponding target sequences (Table A2). Probe 1 was complementary with the circular ssDNA and modified with FAM (Abs, 495 nm, green) linkers attached at the 5′ end. Probe 2 was complementary with the Amp gene and modified with ROX (Abs, 576 nm, red) linkers attached at the 5′ end. Probe 3 was complementary with probe 1, and had exactly same sequence as the circular ssDNA, and was modified with ROX (Abs, 576 nm, red) linkers attached at the 5′ end.

Preliminary experiments were performed to verify the binding ability and specificity of the probes. A 90 nt oligo was synthesized by GENEWIZ. The hybridization assay was conducted in 20 mM Tris-HCl pH 8.3, containing 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$. The oligo and probe concentration were 10 nM. The solutions were incubated under different conditions for 1 h, then separated on a 2% agarose gel to confirm the binding specificity.

An overnight culture of DH10β cells harboring the plasmid pRC17 was used to inoculate 100 mL of fresh LB medium with 100 µg/mL ampicillin at a 1:100 dilution and harvested at the mid-log phase (OD_{600}~1.0). The circular ssDNA (pCSHI-PB) of the control group was produced using HmuI and ExoIII. The extracted plasmid DNA of pCSHI-PB was incubated with HmuI and ExoIII (Mat. M0303S; 4 U, New England BioLabs, USA) at 30 °C for 4 h. Then, the samples were purified using the plus DNA clean/extraction kit.

Appendix A.7. Purification of Circular ssDNA From Cell Lysis

For 2 mL cell lysis solution, 50 µL of Magrose Strep-Tactin beads (Beaverbio Co., Ltd, Suzhou, China) was washed twice with binding buffer on a magnetic stand. The bead pellet was then resuspended in cell lysis solution and incubated for 30 min at room temperature (RT, 25 °C) to immobilize the SSB-ssDNA complexes on the surface of the magnetic beads. After binding, the bead-bound circular ssDNA was isolated using a magnet and washed three times with binding buffer. Finally, the circular ssDNA was resuspended in 50 µL of elution buffer (containing 2.5 mM desthiobiotin) and the magnetic beads were washed three times with 1 mL ddH_2O, three times with 1 mL 0.5 M NaOH, and then washed with ddH_2O to neutralize the base. The magnetic beads were

stored at 4 °C in 5 µL regeneration buffer. After recovery, 1 µL of the recovered product was used as template for PCR using EasyTaq® DNA Polymerase and the primer pair PCR-2K-F1/R1 (Table A1) for 30 cycles with an annealing temperature of 55 °C and an extension time of 1 min. The products were then separated on a 1.2% agarose gel and the appropriate band was purified to sequence the resulting PCR product.

Appendix A.8. Genome Editing Using a DNA Oligo

The genome editing efficiency of donor DNA with imperfect complementarity was characterized by performing the MAGE protocol [13]. Two different 90 nt oligos were used to produce a substitution in the *lacZ* gene in EcNR2 cells which produces a premature stop codon. These oligos contained two phosphorothioate bonds at the 5′-end, and their sequence is shown in Table A2. The difference between the two oligos is that one (donor 1) is completely complementary to the target chain while the other (donor 2) is not. Donor 2 contained 5 bases at both the 5′ and 3′ termini which were non-complementary with the *lacZ* gene (5′-GGGGG, 3′-TTTTT). For the genome editing cycles, 1 µM oligos were used to transform electrocompetent cells, which were regenerated in 1 mL of SOB medium. Each stage of MAGE was done by plating on selection plates containing IPTG and X-gal. The efficiency of allelic replacement was calculated via the ratio of the number of white colonies to the total number of colonies on the plates. The targeted *lacZ* region was amplified by PCR and sequenced after 4 cycles of MAGE.

Appendix A.9. Genomic DNA Extraction

Genomic DNA was extracted from *E. coli* MG1655 using the Easy Pure Genomic DNA Kit (TRANS, Beijing, China) according to the manufacturer's instructions. The crude lysate was stored at −20 °C.

Appendix B.

Table A1. List of the primer sequence in this study.

Primer	Sequence (5′ to 3′)
pMR5-F	CCGCTCGAGTGTGAACATCATC
pMR5-R	GGAAGATCTAGAGACGCAAGACACTGC
2K-F	ACGCGTCGACTCAAGTCCTCAGCTGCAAG
2K-R	AAGGAAAAAAGCGGCCGCCCTTACCTGGGTCTGTTGTAT
G5P-F	TCGATCCGCAGTGTCTTGCGTCTCTCTCGAGAAATCCGCGACCTGCT
G5P-R	AAAGATGAACGTGATGATGTTCACACTCGAGCGAATTAAAACGCGATATTTGAAG
ty-pET-F	TGTTTAACTTTAAGAAGGAGATATACCATGGGCTGTTATAACATGGAAAAGT
ty-pET-R	TATCACGAGGCCCTTTCGTCTTCAAGAATTCAAGCTTGGCAATAGTTAC
BglII36-F	GGAAGATCTTTAATTGCGTTGCGCTCAC
BglII36-R	GGAAGATCTGGCAATAGTTACCCTTATTATCAAGATAAGAAAGAATGTGAGTTAGCTCACTCAT
Lac36-F	CCCTTAAGTTATTGGTATGACTGGTGAGCTCAGACAAGCCCGTCAGG
Lac36-R	CTCGGTTGGCAGTGACTCCGTCTCTAAGCTTGGCAATAGTTACCCTTATTAT
pMRG53-F	AAGGAAAAAAGCGGCCGCTGTAAAGCCTGGGGTGC
pMRG53-R	ACGCGTCGACCATCCAAATCTACAACGTCG
5K-F	ACGCGTCGACTGGAGTGGGCCTACCTC
5K-R	AAGGAAAAAAGCGGCCGCCCTTACCTGGGTCTGTTGTAT
Assembly-a	TACTGAGAGTGCACCATATGGTTGGTGTTGTTATGTTGAGAGATCTTAAAGGATTTGAGC
Assembly-b	TAGTTACCCTTATTATCAAGATAAGAAAGAAAAGGATTTTTCGCTACGCTCAAATCCTTTAAGATCTCTCAACATA
Assembly-c	CTTTTCTTTCTTATCTTGATAATAAGGGTAACTATTGCCGGCGAGGCTAGTTACCCTTAAGTTATTGGTATGACTG
Assembly-d	AGGTTAAGGGCAAATCAACACCACCAAACTACCAGTCATACCAATAACTTAAGGGTAAC
Assembly-e	TGTTGATTTGCCCTTAACCTCACATACCACCTCGAGGAGCTCGGTACCGTCG
Assembly-f	GTTACCCTTATTATCAAGATAAGAAAGAAAAGGATTTTTAAGCTTCTGCAGGTCGACGGTACCGAGCTC
Assembly-g	CCTTTTCTTTCTTATCTTGATAATAAGGGTAACTATTGCCGGCGAGGCTAGTTACCCTTAGATCTGTTTACTTT
Assembly-h	TGACCATGATTACGCCAAGCTTATGTTCACAATAAAGTAAACAGATCTAAGGGTAACTAGC
Assembly-e1	TGTTGATTTGCCCTTAACCTCACATACCACCTCGAGGAGCTCGGTAC
Assembly-f1	TTATCAAGATAAGAAAGAAAAGCTTGCATGCCTGCAGGTCGACGGTACCGAGCTCCTCGAG
Assembly-g1	CATGCAAGCTTTTCTTTCTTATCTTGATAATAAGGGTAACTATTGCCAGATCTGTTTACTTTATTGTG
Assembly-h1	GACCATGATTACGCCAAGCTTATGTTCACAATAAAGTAAACAGATCTGGCAATAG

Table A1. *Cont.*

Primer	Sequence (5′ to 3′)
P19-F	GGTTATCCACAGAATCAGG
P19-R	CCTCGTGATACGCCTATTT
RepH-F	GATCTGTTTACTTTATTGTGAACATTGTGAACATCATCACGTTC
RepH-R	TTATCCCCTGATTCTGTGGATAACCTTTCTTTTGGGTATAGCGTC
TY2k-F	GCCTGCAGGTCGACGGTACCGAGCTCCCTTACCTGGGTCTGTTGTAT
TY2k-R	ACCTCACATACCACCTCGAGGAGCTCTCAAGTCCTCAGCTGCAAG
P19-F1	GGTTATCCACAGAATCAGG
P19-R1	CCTCGTGATACGCCTATT
P_{laclq}-F	GGGTGGCTCCAGCTTGCCATATGGTACCTTTGTCCTCTT
P_{laclq}-R	CCCTGATTCTGTGGATAACCTTCGTCTTCAAGAATTCTGG
EcSSB-R	AAAGAGGACAAAGGTACCATATGGCAAGCTGGAGCCAC
EcSSB-F	GACGCTATACCCAAAAGAAACAAAGCCCGCCGAAAGGCGGGCTTTTCTGTTCAGAACGGAATGTCATCATC
lacZE1-F	TAATACTAGTCTGTCGTCGTCCCCTCAAACGTTTTAGAGCTAGAAATAGCAAG
lacZE1-R	GCTCTAAAACGTTTGAGGGGACGACGACAGACTAGTATTATACCTAGGACTG
Sc-gRNA-F	TAATACTAGTATCAACTTCAAACTCACAACGTTTTAGAGCTAGAAATAGCAAG
Sc-gRNA-R	GCTCTAAAACGTTGTGAGTTTGAAGTTGATACTAGTATTATACCTAGGACTG
Donor-F	ATTTGCCCTTAACCTCACATACCACCTCGAGTTACCTTCACATCCTACCTC
Donor-R	CAAGATAAGAAAGAAAAGGATTTTTAAGCTTGGTAAGGCAGGTAGTAGAG
gRNA2-F	GAATCAGGGGATAACGCAGGAAAGAACATGTTTGACAGCTAGCTCAGTCC
gRNA2-R	CTGGCCTTTTGCTGGCCTTTTGCTCACATGTGGTGCCACTTTTTCAAGTTG
PCR-2K-F	GCAGTCAGGCCACAATTAC
PCR-2K-R	AACTGTCCTCTGCAGGTG
plasmid-F1	CCAGTCACAGAAAAGCATC
plasmid-R1	ACCAATGCTTAATCAGTGAG
ssDNA-F1	GTAAAACGACGGCCAGTG
ssDNA-R1	GCTGGCGTAATAGCGAAG
16S-F1	GAAGCTTGCTTCTTTGCT
16S-R1	GAGCCCGGGGATTTCACAT
PCR-LacZ-F	ATGCGTAAGGAGAAAATACC
PCR-LacZ-R	ATATGGTGCACTCTCAGTAC
PCR-2K-F1	CATAAAGTGTAAAGCCTGG
PCR-2K-R1	CACCAGTCATACCAATAACT
plasmid-F	GGAAGCTAGAGTAAGTAGT
plasmid-R	GAATGAAGCCATACCAAAC
16S-F	CATCCACAGAACTTTCCAGAG
16S-R	CCAACATTTCACAACACGAG
PCR-PU-F	CAGAACTTTAAAAGTGCTCAT
PCR-PU-R	GATTCTCTCCAATATGACGT
PCR-vactor-F	GGTTACGATGCGCCCATC
PCR-vactor-R	AGTTTGAGGGGACGACGAC
TY-1011-F	GATACTGTCGTCGTCCCCTCAAACTATTTGTCCTACTCAGGAGAG
TY-1011-R	TGGTGTAGATGGGCGCATCGTAACCTGGTGCAAAACCTTTCGC
D-5+2K-F	CTGTTTACTTTATTGTGAACATTGTGAACATCATCACGTTCATCT
D-5+2K-R	ACAATGTTCACAATAAAGTAAACAGACCAGTCATACCAATAACTT
TY-D-dso-2K-F	TATAAAAATAGGCGTATCACGAGGTCAAGTCCTCAGCTGCAAG
TY-D-dso-2K-R	GATGATGTTCACAATGTTCACAATCCTTACCTGGGTCTGTTGTAT
TY-D-DSO-F	ATTGTGAACATTGTGAACATCAT
TY-D-DSO-R	CCTCGTGATACGCCTATT
PCR-MG1655-F	GGTTTATGCAGCAACGAGAC
PCR-MG1655-R	GATTCATTAATGCAGCTGG
PCR-1011-R	CTAATTCAACAGAATTGGGACAAC
TY-gR-F	GAATCAGGGGATAACGCAGGAAAGAACATGTTTGACAGCTAGCTCAGTCCT
TY-gR-R	CTGGCCTTTTGCTGGCCTTTTGCTCACATGTAAAAAAACACCGACTCGGTG
D-Rep-F	AACATGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGA
D-Rep-R	CCCTGATTCTGTGGATAACCATGTTCACAATAAAGTAAACAG
TY-RepH-F	TGCAAGCGAGCTTGGCGTAATCATGTGTGAACATCATCACGTTCATC
TY-gRNA2-R	TTGCTGGCCTTTTGCTCACATGTTCGGTGCCACTTTTTCAAGTTG
Phm01-F	GAACATGTGAGCAAAAGG
Phm01-R	CATGATTACGCCAAGCTC
Rep-F	CATGCCATGGGCTGTTATAACATGGAAAAGT
Rep-R	CCGGGATCCTCAAAGACTTTTTGATTTAT
G-F	TTAACTGTGATAAACTACCGCATTAAAGCTTAAATCCGCGACCTGCT
G-R	TCATGTTTGACAGCTTATCATCGATAAGCTTCGAATTAAAACGCGATATTTGAAG
RCR-F	ATCGATGATAAGCTGTCAAACATGAGAATTCAAAAATCCTTTTCTTTCTTATC
RCR-R	TATCACGAGGCCCTTTCGTCTTCAAGAATTCAAGCTTGGCAATAGTTAC

Table A2. List of the gene sequence in this study.

Name	Sequence (5′–3′)
RCORI 105	TAAAGGATTTGAGCGTAGCGAAAAATCCTTTTCTTTCTTATCTTGATAAT AAGGGTAACTATTGCCGGCGAGGCTAGTTACCCTTAAGTTATTGGTATGA CTGGT
RCORI 65	AAAAATCCTTTTCTTTCTTATCTTGATAATAAGGGTAACTATTGCCGGCG AGGCTAGTTACCCTT
RCORI 36	TTCTTTCTTATCTTGATAATAAGGGTAACTATTGCC
RCORI 75	TTCTTTCTTATCTTGATAATAAGGGTAACTATTGCCGGCGAGGCTAGTTA CCCTTAAGTTATTGGTATGACTGGT
RCORI 85	AAAAATCCTTTTCTTTCTTATCTTGATAATAAGGGTAACTATTGCCGGCG AGGCTAGTTACCCTTAAGTTATTGGTATGACTGGT
pRC03	AAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACC TGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGC GTATCACGAGGCCCTTTCGTTGTAAAACGACGGCCAGTCGAACCACGCAA TGCGTCTCGATCCGCAGTGTCTTGCGTCTCTAGATCTTTCTTTCTTATCT TGATAATAAGGGTAACTATTGCCGGCGAGGCTAGTTACCCTTAAGTTATT GGTATGACTGGTGAGCTCGGTACCCGGCCATGGTCTAGAGTCGACCTGCA GGCATGCAAGCTTAGAGACGGAGTCACTGCCAACCGAGACGGTCATAGCT GTTTCCTGTGTGCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTT CGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTACC CACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGC AAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGG CTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTG GCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCT CCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCC GCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAG GTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACG AACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTT GAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGG TAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGA AGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGC GCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATC CGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGC AGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCT ACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGT CATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAAT GAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGT TACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCG TTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGG AGGGCTTACCATCTGGCCCCAGTGCTGCAATAATACCGCGGGACCCACGC TCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGA GCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATT GTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAAC GTTGTTGCCATCGCTACAGGCATCGTGGTATCACGCTCGTCGTTTGGTAT GGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCC CCATGTTGCGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTC AGAAGTAAGTTGGCCGCCGTGTTATCACTCATGGTTATGGCAGCACTACA TAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTG AGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGC TCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTT AAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGA TCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAAC TGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAAC AGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTT GAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGT TATTGTCTCATGAGCGGATACATATTTGAATGTATTTAG

Table A2. *Cont.*

Name	Sequence (5′–3′)
pRC06- circular ssDNA	TTCTTTCTTATCTTGATAATAAGGGTAACTATTGCCGGCGAGGCTAGTTA CCCTTAAGTTATTGGTATGACTGGTGAGCTCAGATCTCCGACGTTGTAGA TTTGGATGAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGT CGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCA CCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCA TCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCG GTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGC AAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTA AAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTC GACCTGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG TGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGC ATAAAGTGTAAAGCCTGGGGTGCCTAATGAG
pRC08- circular ssDNA	TTCTTTCTTATCTTGATAATAAGGGTAACTATTGCCGGCGAGGCTAGTTA CCCTTAAGTTATTGGTATGACTGGTGAGCTCAGATCTCCGACGTTGTAGA TTTGGATGGTCGACTCAAGTCCTCAGCTGCAAGAATATCTGAATGTCTTT TGGAGTGTTAGAGTCCTCTGTGTCTTAGAAATTTTGAAAAGAAAAACAAA TCTCAATATTAATGTTGATTAGTTTCTCTGAGCCAATTGGGGAAAATAAA CGTCCTTCACCTCAAAGGTTTAAGTGACACCGAAGGGTAGCCACCAGTGT CTCGGCCACTGAAGCCTCATGCATGCTCTCACTACCAGTTTGATTTGCAG CCCCATAGTTGTGTTGTACTAAATATTCTTTCCTCTGGCCTTGTCCAGTG AACACGGTTCACATGGCTAACACCACTTCTTGAGATGCGAGCACCATGCA AAGCTGAGAACGGATTGGGTTTTGTGACCATTGTGCCTCCTCCTCACCTG AGAGGCCCATTTTTCCTGGTTGATTCATTAAGTGTATTGGTGCTGTCAGT CGCCTCTGGACAATTGAAATGACAAGTGGCTGTTGATTCATAAAGAAAAT GAAGGCTTTAGATGTGAAACCCTCGTTTTCTCTTGTCCTTCTCTTAGGTG AAAGATTTTATTTTTTTCAAAAGGCTACATACTGGTATCCCAGCAGGTGT AGTGTGAGAACTGGCATATGTTAGGCTATGGTGTCAGTGTGGATGGGCAA TTCTTCAAGATGGAAAACCAAGTCTCACTGAGTTGCTGGAGCCACACTGA CCTTTCTCCACATCCCCCACCATGGGCTTTCACTTTTATCCTGTGCTTGA ATTTTTTTCACATACAAATTCTTTATACACACACACAGACACACACACAC ATATCTCACTCTGTCAATGCAGTGGCTGAATCATGGGTCACTGCATCTTC AAATTCTTAGGCTCCAGTGATGCTTTCAAATCAGCCTCTCAAGTAGCTGG GACTACAGGCATGCAAAGCTACACCCAGACAATTTTTAAATATTTTTCTA GAGACTGAGCCTACTTATGTTGCTCAGACTCGTCTTGCACTCCTGGGATC AAACGATAATCCCACCTTGACCACCCAAAGTGTTTAGATTACAGGTGTGA GCTAGCACTCTCAGCAAAAATATATTTTAAAGAACCGTTACAACCAAATT ATGAGTTATCATTATGCCACTGCCCTCCACCCTGGGCACCAGAACAAGAC CTTGTATCCAAAAACTAAGCAAAACTAAACAAGAACAAAAAAAAAAAAAC TTATAAATAAACTTTGAAGATTGTGTCATCTGTGTCCTTCCCTGCCCTCC AAGCTATCAATGTTAAATATAATGGTTATTGAGAAAATGGTTAGATATTA TTAAGAAATTTCTATATATCTTCCAGCTGAGAATAGGTATTCTGTTGTGG CCCAAATATTTTCTCACCGCTACCTTCAGGGTCTAAACTAGCAAATCAGG ACACCTGCAGAGGACAGTTGGCCGTTTTCAAATAGAAAGAGAAATACCCC CGTTCATGAGAGTAATCCAGTGATTTTCAAAAAGACAAGTCAGACTGACA TGCAGCGCAGTCAGGCCACAATTACCCTGGAATAATCACTTCACACAGAA TGGTTGAGGAGACTTTCTAAGATGAGCAAATTTGGGCAGCATAATCCTTG CTTATTTATTCCCAGCCCCCACTGCCCGCCTGATTCCTAATGGCTACCCT ACAATGTGGTCAGCAGTGGGATGTAGCGTGGTGAGAGAGGGGCTCAGGGA CGGGATGAAGGTCTTTCCTGCATTATCAAAATGCAGGTTAAAAAGTTGTT AAAAAGATGTCCAAATGTTCTAATTCCTACTGTTAAATAGCTGCTAAGAT GCATTATACAACAGACCCAGGTAAGGGCGGCCGCTGTAAAGCCTGGGGTG CCTAATGAG

Table A2. *Cont.*

Name	Sequence (5′–3′)
pRC09- circular ssDNA	ATAATAAGGGTAACTATTGCCGGCGAGGCTAGTTACCCTTAAGTTATTGG TATGACTGGTGAGCTCAGATCTCCGACGTTGTAGATTTGGATGGTCGACT GGAGTGGGCCTACCTCCCATCAAGCTGTGTCTCCACAGCTGACCCTTGTA ACCAGGAGGTGTTTTACAACATGTGCAAGGCAGTGAGCTCCATCAGCTGT GTGGCATTCAACACTCACTTCAACTCGGACATCTCACCAGAAAGCAGTGG GGACTGGCCAATGCAGAAGCCTGCAAAGTGGAACAGAGCGTCATGGGGTG GGGGATGTGGGGCCTGCCTGCTCATCTGAGCACTGCTCCCTGAGGGTGTG ATCTGCAGGCTTCCTGAAGGAGGGCTGTGAGCTCTTCTGCGAGGCCCTGA GCCTGTGGAACATAGCTGAGGCCAAGCCCATGGGGATTTGTGTCTACTTG CACCTCCTTGCTCATCTCAGTACACTACAGGTGACTGTGCCGAGGTGGGC CTTGAGCATCCCCTGGGCTGTGTCAGCAAATGGCTCTGGGCCTGGCCTGG CATTGAGGGATGGCAAAAAAGGAGCCTGGGGTTGCATTGTCATCCCCTAT GGTAGCATAAAATGAGAGAGTCCAGACCTGCAGGACTGGAACCCTAACAA AGGGGTTAGGAGACTGCTCACTTTCCCTCAGGAACCCATGTGGAGGAGCT GAGGGAGGTTAAGGAGACCCTAGGGACTCACTTGTTCTGTCTGGGCTTCC CCCTGCTCCATCGTTTGATGACCATTTTCTGGGAAGAGCTCAGGAACCTC CTGTGCTCTAGTGAGACGGGGCCTCCCCTCACAGGGTATTCTGAGACTGT GAGTGAGAAGCTAACACAGTGCCTTGCAATACTCATGGGAGCTGTCATCC TCTGTGACCATCACGTGGCCTTGTAGTGTTCAGACTGCCTGGCCTGCCTG GGGTTTGGTGAGGCTGTTTTGTGGTCAGGTGCTTTAGAAGCTCACTTTCT CTGCAATCAAACAGTGACTGTTTTCATGTCTGTTTATGGGTTTAAAAAAT CCTAATATTTCCTTTATAGTAGTTCACCTTGTATGTGTTTATTTGTATAA ATTTTATTAGAGTAAAGAGAGCTTAAGACAATAGCATTTTAAGGTCTTAA TGAGGCATAGACTTTCATGTCACAACAGCTAATGTTGACCTCCTTTTGCT GCCTTTGTGTAAATTACACATAAAAAGTGCAGCCAGAGGTGACTAGAGCT GAGCTGCTTGGGCTTGCTTGCTGGCCTGCAGTCAGGTGGACTCTGGCTGT GAGGCAGTGCCCACCCTGGATCTACATCCCCCACCCCCTCTCCTTAGTCC CTGAGTAACCAACAAGGCCGTGCTAATGAGAGGGCGAGTGATGGGCATCG GGCACCCCCATATTATCCGGGAAGATTTGAATGCCATCTGGGCTGGAGCT GTTGGGATTAGGGGCTGAGGCTGTCTTGGCTTGTCATGGTGCCACCCACA GATGTGCCTGCCCTGTGCTGCTTCTCCAGAAGCCGGCTGCCCATGGCCCT GAGCCTGTCACACCATGCTTGCTACCTCATGCTGCTTGTGTTTGAAAAAC CCATCCCGAGATGACGCTGCTGGATGTAAGTCCTGAAAAGGGGGCATCAC CTTTGTCCTGGGGGATTAGGAGCTGACCAGATTCCTCTTGACTCCCTCCC AGAACAAGTGGGGCAGGTGCTGCAATTAATGTTGCCCCCTAGAAGATGTG TTTGCACTGGCTGAGCAAATATACGATGCAGAGACCTAAATGAAGACACG TGAATGGGGTGTGTGGACATCAGTTAGTAGCTGGGAAACAGGTGCCTCTC AGGCGTCTCGTGTTCCAGCAAGTGTGGAATATGCCTGTGCCCATGAGTGT AGACATCTGAAGTGTATACATTTGGCTGCTGCTTTTGCTGCCACTATTCC CAGGCCCAACCTGGCTTAAAGTCCAGGTTTTAAGTAAAAAGTAGGAGGCT TTTTGCCATACAGCTACTTGAGAGGCTGAGGTGAAAGCATCACTGGAGCC TAAGAGATTGAGGCTGCAGTGACCCATGATTCAGCCACTGCACTGACACA GTGAGACCTGCGTGTGCCCTTCTACAGAGAATAGCTCTGGGGCATTTGGG GATCCCTACAGTCCCGGACCCTCCCTGTCCCCTGCTGCCTGTGCTCCTTT CCTTGCCTGCTGTCAGAGCCTAACATGGAGGCGGTTGCCACCCTGTGAGC CTGAGGGAGCTGTGTCTGACTGGAACTTCTGTCTGAGGTTTTGCGAAGTC TTACTTATGAATATGGTCTGTCCAGATACCTTGTTTCAAAGGAAGTGAGC ATGAGCTAGCAAGTGTAGCCACCCCACAGCTGATAAACAACTTTGTCTTG TTTTTAAATCATCAATCTTCATTTCACATTGGAATAAAGTAAGTGAAACC TGCTACCCGAGCCTCGCCCGTGTGTTCTGTAACCCAGACTCATGTGGTTG TGTGGGCTGTTGTCAGAAATGTTATAAAAAGGTTATGCATAAATTAGATC AAATATAAAATTATGCTTATAATGTCACTTGAGTGGGAGGTAAGAGGGTA GAGTCACAGGAAATCTGTTGGGGTTTACACCCCTGCTACTTACCAAGCTC ATGAGAGTGTGGCACTGGTGACCATCACCTGACATTGGTGACAGAAGAGA AAAGGTCGAAGTGAAGGCCAGGTAGGAGAGAGGTGCCAGGCTGTGGGGCC AGGCCCTGCGCATGCTGGGCCTGTTAGGTCACTGAACATCTAACTACCCG GGAACCAGCTCTTTTCACATCATTTGAGGTAAGACGATGGGGGAGCACTC TCCAGAAGTCACACTGCGCTGGGAGAATGGAGGAGAGTCTACATACCGCC

Table A2. *Cont.*

Name	Sequence (5′–3′)
pRC09- circular ssDNA	ATCTTAGGGTAGGTTTTAGATTGAGCTGAACTGTCTTGGAGAGCTAATGA GATGGGAGGAAGACAGTCCCCCAGGTGCACCTAACAGCCAGAGCCTATGA AGTTAGGGGGGTTGTGTGGGGGTGGCCTTTCCCTATAAGAGGAGGAGCTT AAAGCTCTTAAAGCTGGTGGCTGCTGCTCTGCCATCCCTCTACAGAGCAG TCAAGTCCTCAGCTGCAAGAATATCTGAATGTCTTTTGGAGTGTTAGAGT CCTCTGTGTCTTAGAAATTTTGAAAAGAAAAACAAATCTCAATATTAATG TTGATTAGTTTCTCTGAGCCAATTGGGGAAAATAAACGTCCTTCACCTCA AAGGTTTAAGTGACACCGAAGGGTAGCCACCAGTGTCTCGGCCACTGAAG CCTCATGCATGCTCTCACTACCAGTTTGATTTGCAGCCCCATAGTTGTGT TGTACTAAATATTCTTTCCTCTGGCCTTGTCCAGTGAACACGGTTCACAT GGCTAACACCACTTCTTGAGATGCGAGCACCATGCAAAGCTGAGAACGGA TTGGGTTTTGTGACCATTGTGCCTCCTCCTCACCTGAGAGGCCCATTTTT CCTGGTTGATTCATTAAGTGTATTGGTGCTGTCAGTCGCCTCTGGACAAT TGAAATGACAAGTGGCTGTTGATTCATAAAGAAAATGAAGGCTTTAGATG TGAAACCCTCGTTTTCTCTTGTCCTTCTCTTAGGTGAAAGATTTTATTTT TTTCAAAAGGCTACATACTGGTATCCCAGCAGGTGTAGTGTGAGAACTGG CATATGTTAGGCTATGGTGTCAGTGTGGATGGGCAATTCTTCAAGATGGA AAACCAAGTCTCACTGAGTTGCTGGAGCCACACTGACCTTTCTCCACATC CCCCACCATGGGCTTTCACTTTTATCCTGTGCTTGAATTTTTTTCACATA CAAATTCTTTATACACACACACAGACACACACACACATATCTCACTCTGT CAATGCAGTGGCTGAATCATGGGTCACTGCATCTTCAAATTCTTAGGCTC CAGTGATGCTTTCAAATCAGCCTCTCAAGTAGCTGGGACTACAGGCATGC AAAGCTACACCCAGACAATTTTTAAATATTTTTCTAGAGACTGAGCCTAC TTATGTTGCTCAGACTCGTCTTGCACTCCTGGGATCAAACGATAATCCCA CCTTGACCACCCAAAGTGTTTAGATTACAGGTGTGAGCTAGCACTCTCAG CAAAAATATATTTTAAAGAACCGTTACAACCAAATTATGAGTTATCATTA TGCCACTGCCCTCCACCCTGGGCACCAGAACAAGACCTTGTATCCAAAAA CTAAGCAAAACTAAACAAGAACAAAAAAAAAAAAAACTTATAAATAAACTT TGAAGATTGTGTCATCTGTGTCCTTCCCTGCCCTCCAAGCTATCAATGTT AAATATAATGGTTATTGAGAAAATGGTTAGATATTATTAAGAAATTTCTA TATATCTTCCAGCTGAGAATAGGTATTCTGTTGTGGCCCAAATATTTTCT CACCGCTACCTTCAGGGTCTAAACTAGCAAATCAGGACACCTGCAGAGGA CAGTTGGCCGTTTTCAAATAGAAAGAGAAATACCCCCGTTCATGAGAGTA ATCCAGTGATTTTCAAAAAGACAAGTCAGACTGACATGCAGCGCAGTCAG GCCACAATTACCCTGGAATAATCACTTCACACAGAATGGTTGAGGAGACT TTCTAAGATGAGCAAATTTGGGCAGCATAATCCTTGCTTATTTATTCCCA GCCCCCACTGCCCGCCTGATTCCTAATGGCTACCCTACAATGTGGTCAGC AGTGGGATGTAGCGTGGTGAGAGAGGGGCTCAGGGACGGGATGAAGGTCT TTCCTGCATTATCAAAATGCAGGTTAAAAAGTTGTTAAAAAGATGTCCAA ATGTTCTAATTCCTACTGTTAAATAGCTGCTAAGATGCATTATACAACAG ACCCAGGTAAGGGCGGCCGCTGTAAAGCCTGGGGTGCCTAATGAGTGAGC TAACTCACATTCTTTCTTATCTTG
pRC17- circular ssDNA	ATAATAAGGGTAACTATTGCCGGCGAGGCTAGTTACCCTTAAGTTATTGG TATGACTGGTAGTTTGGTGGTGTTGATTTGCCCTTAACCTCACATACCAC CTCGAGGAGCTCTCAAGTCCTCAGCTGCAAGAATATCTGAATGTCTTTTG GAGTGTTAGAGTCCTCTGTGTCTTAGAAATTTTGAAAAGAAAAACAAATC TCAATATTAATGTTGATTAGTTTCTCTGAGCCAATTGGGGAAAATAAACG TCCTTCACCTCAAAGGTTTAAGTGACACCGAAGGGTAGCCACCAGTGTCT CGGCCACTGAAGCCTCATGCATGCTCTCACTACCAGTTTGATTTGCAGCC CCATAGTTGTGTTGTACTAAATATTCTTTCCTCTGGCCTTGTCCAGTGAA CACGGTTCACATGGCTAACACCACTTCTTGAGATGCGAGCACCATGCAAA GCTGAGAACGGATTGGGTTTTGTGACCATTGTGCCTCCTCCTCACCTGAG AGGCCCATTTTTCCTGGTTGATTCATTAAGTGTATTGGTGCTGTCAGTCG CCTCTGGACAATTGAAATGACAAGTGGCTGTTGATTCATAAAGAAAATGA AGGCTTTAGATGTGAAACCCTCGTTTTCTCTTGTCCTTCTCTTAGGTGAA

Table A2. *Cont.*

Name	Sequence (5′–3′)
pRC17- circular ssDNA	AGATTTTATTTTTTTCAAAAGGCTACATACTGGTATCCCAGCAGGTGTAG TGTGAGAACTGGCATATGTTAGGCTATGGTGTCAGTGTGGATGGGCAATT CTTCAAGATGGAAAACCAAGTCTCACTGAGTTGCTGGAGCCACACTGACC TTTCTCCACATCCCCCACCATGGGCTTTCACTTTTATCCTGTGCTTGAAT TTTTTTCACATACAAATTCTTTATACACACACACAGACACACACACACAT ATCTCACTCTGTCAATGCAGTGGCTGAATCATGGGTCACTGCATCTTCAA ATTCTTAGGCTCCAGTGATGCTTTCAAATCAGCCTCTCAAGTAGCTGGGA CTACAGGCATGCAAAGCTACACCCAGACAATTTTTAAATATTTTTCTAGA GACTGAGCCTACTTATGTTGCTCAGACTCGTCTTGCACTCCTGGGATCAA ACGATAATCCCACCTTGACCACCCAAAGTGTTTAGATTACAGGTGTGAGC TAGCACTCTCAGCAAAAATATATTTTAAAGAACCGTTACAACCAAATTAT GAGTTATCATTATGCCACTGCCCTCCACCCTGGGCACCAGAACAAGACCT TGTATCCAAAAACTAAGCAAAACTAAACAAGAACAAAAAAAAAAAAACTT ATAAATAAATTAAACTTTGAAGATTGTGTCATCTGTGTCCTTCCCTGCCC TCCAAGCTATCAATGTTAAATATAATGGTTATTGAGAAAATGGTTAGATA TTATTAAGAAATTTCTATATATCTTCCAGCTGAGAATAGGTATTCTGTTG TGGCCCAAATATTTTCTCACCGCTACCTTCAGGGTCTAAACTAGCAAATC AGGACACCTGCAGAGGACAGTTGGCCGTTTTCAAATAGAAAGAGAAATAC CCCCGTTCATGAGAGTAATCCAGTGATTTTCAAAAAGACAAGTCAGACTG ACATGCAGCGCAGTCAGGCCACAATTACCCTGGAATAATCACTTCACACA GAATGGTTGAGGAGACTTTCTAAGATGAGCAAATTTGGGCAGCATAATCC TTGCTTATTTATTCCCAGCCCCCACTGCCCGCCTGATTCCTAATGGCTAC CCTACAATGTGGTCAGCAGTGGGATGTAGCGTGGTGAGAGAGGGGCTCAG GGACGGGATGAAGGTCTTTCCTGCATTATCAAAATGCAGGTTAAAAAGTT GTTAAAAAGATGTCCAAATGTTCTAATTCCTACTGTTAAATAGCTGCTAA GATGCATTATACAACAGACCCAGGTAAGGGAGCTCGGTACCGTCGACCTG CAGAAGCTTAAAAATCCTTTTCTTTCTTATCTTG
pCSHI-PB- circular ssDNA	CAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCC AGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGC GGATAACAATTTCACACAGGAAACAGCTCTGTTGAGATCCAGTTCGATGT AACCCACTTCTGCTCTGATGCCGCATAGCAGTCAGGCCACAATTACCCTG GAATAATCGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCA TGATAATAATGGTTTCTTATTAACAGAGTAACCTCCTCAAAGTAATGAGC CTAACGCTCAGCAATTCCCACTTAGACGTCAGGTGGCACTTTTCGGGGAA ATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATG TATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAA AAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTT TTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAA GTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACT GGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTT TTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCC CGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCA GAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATG GCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAAC ACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAAC CGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGG AACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATG CCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACT TACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAG TTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCT GATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACT GGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGA GTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCC TCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACT TTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGA

Table A2. *Cont.*

Name	Sequence (5′–3′)
pCSHI-PB-circular ssDNA	TCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTC CACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCC TTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTAC CAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAG GTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTA GCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACC TCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCG TGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCG GTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGA CCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACG CTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGG AACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTT ATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGA TGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTT TTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTG CGTTATCCCCTGATTCTGTGGATAACCG
P rhaBAD	TGTGAACATCATCACGTTCATCTTTCCCTGGTTGCCAATGGCCCATTTTC CTGTCAGTAACGAGAAGGTCGCGAATTCAGGCGCTTTTTAGACTGGTCGT A
PlacIq	TGGTGCAAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCA ATTCAGG
Probe 1	FAM- GATTATTCCAGGGTAATTGTGGCCTGACTG
Probe 2	ROX- AGTGGGTTACATCGAACTGGATCTCAACAG
Probe 3	ROX-CAGTCAGGCCACAATTACCCTGGAATAATC
Donor 1	A*T*GATTACGGATTCACTGGCCGTCGTTTTACAACGTCGTGACTGAGAAAACC CTGGCGTTACCCAACTTAATCGCCTTGCAGCACATC*C*C
Donor 2	G*G*GGGTACGGATTCACTGGCCGTCGTTTTACAACGTCGTGACTGAGAAAACC CTGGCGTTACCCAACTTAATCGCCTTGCAGCACTTT*T*T
gRNA2	GTTGTGAGTTTGAAGTTGATGTTTTAGAGCTAGAAATAGCAAGTTAAAAT AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTG
circular ssDNA for 11 nts substitution	AAAAATCCTTTTCTTTCTTATCTTGATAATAAGGGTAACTATTGCCGGCG AGGCTAGTTACCCTTAAGTTATTGGTATGACTGGTAGTTTGGTGGTGTTG ATTTGCCCTTAACCTCACATACCACCTCGAGTTACCTTCACATCCTACCT CGAATTCAAAAAAAAAAAATCAACTTCAAACTCACAACAAAAAAAAAAAGA TCTAAGCTTATCGATGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTGA GGCCGATACTGTCGTCGTCCCCTCAAACTAGTAGTAGTAGGGTTACGATG CGCCCATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTTT GTTTCTAGACTGCAGAAAAAAAAAAAATCAACTTCAAACTCACAACAAAAA AAAAAGAATTCACTCTACTACCTGCCTTACCAAGCTT
circular ssDNA for 1011 nts insertion	AAAAATCCTTTTCTTTCTTATCTTGATAATAAGGGTAACTATTGCCGGCG AGGCTAGTTACCCTTAAGTTATTGGTATGACTGGTAGTTTGGTGGTGTTG ATTTGCCCTTAACCTCACATACCACCTCGAGTTACCTTCACATCCTACCT CGAATTCAAAAAAAAAAAATCAACTTCAAACTCACAACAAAAAAAAAAAGA TCTAAGCTTATCGATGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTGA GGCCGATACTGTCGTCGTCCCCTCAAACTATTTGTCCTACTCAGGAGAGC GTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAG CCTTTCGTTTTATTTGATGCCTCTAGCACGCGTACCATGGGATCCCCCAT CAAGCTTATTAAACTCGTCTTCTGTTCGCCTGGCCCGCGTTCAGCATAAA GGTCGGCACTTCGTTGGTGTTTTCTTCGTTTTTGTTCGCCGCTTTGTATA GTTCATCCATGCCATGTGTAATCCCAGCAGCTGTTACAAACTCAAGAAGG ACCATGTGGTCTCTCTTTTCGTTGGGATCTTTCGAAAGGGCAGATTGTGT GGACAGGTAATGGTTGTCTGGTAAAAGGACAGGGCCATCGCCAATTGGAG TATTTTGTTGATAATGGTCTGCTAGTTGAACGCTTCCATCTTCAATGTTG

Table A2. *Cont.*

Name	Sequence (5′–3′)
circular ssDNA for 1011 nts insertion	TGTCTAATTTTGAAGTTAACTTTGATTCCATTCTTTTGTTTGTCTGCCAT GATGTATACATTGTGTGAGTTATAGTTGTATTCCAATTTGTGTCCAAGAA TGTTTCCATCTTCTTTAAAATCAATACCTTTTAACTCGATTCTATTAACA AGGGTATCACCTTCAAACTTGACTTCAGCACGTGTCTTGTAGTTCCCGTC ATCTTTGAAAAATATAGTTCTTTCCTGTACGTAACCTTCGGGCATGGCAC TCTTGAAAAAGTCATGCTGTTTCATATGATCTGGGTATCTCGCAAAGCAT TGAACACCATAACCGAAAGTAGTGACAAGTGTTGGCCATGGAACAGGTAG TTTTCCAGTAGTGCAAATAAATTTAAGGGTAAGTTTTCCGTATGTTGCAT CACCTTCACCCTCTCCACTGACAGAAAATTTGTGCCCATTAACATCACCA TCTAATTCAACAAGAATTGGGACAACTCCAGTGAAAAGTTCTTCTCCTTT ACGCATGGTACCTTTGTCCTCTTTAATGAATTCCCTGAATTGACTCTCTT CCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCACCAGGTTACGATG CGCCCATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTTT GTTTCTAGACTGCAGAAAAAAAAAAAATCAACTTCAAACTCACAACAAAAA AAAAAGAATTCACTCTACTACCTGCCTTACCAAGCTT
Donor ssDNA for 11 nts substitution	AACAAAAAAAAAAAAGATCTAAGCTTATCGATGTGCCGGAAAGCTGGCTGG AGTGCGATCTTCCTGAGGCCGATACTGTCGTCGTCCCCTCAAACTAGTAG TAGTAGGGTTACGATGCGCCCATCTACACCAACGTGACCTATCCCATTAC GGTCAATCCGCCGTTTGTTTCTAGACTGCAGAAAAAAAAAAAATCAACTTC AAACTCAC
Donor ssDNA for 1011 nts insertion	AACAAAAAAAAAAAAGATCTAAGCTTATCGATGTGCCGGAAAGCTGGCTGG AGTGCGATCTTCCTGAGGCCGATACTGTCGTCGTCCCCTCAAACTATTTG TCCTACTCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCC CAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTCTAGCACGCGTA CCATGGGATCCCCCATCAAGCTTATTAAACTCGTCTTCTGTTCGCCTGGC CCGCGTTCAGCATAAAGGTCGGCACTTCGTTGGTGTTTTCTTCGTTTTTG TTCGCCGCTTTGTATAGTTCATCCATGCCATGTGTAATCCCAGCAGCTGT TACAAACTCAAGAAGGACCATGTGGTCTCTCTTTTCGTTGGGATCTTTCG AAAGGGCAGATTGTGTGGACAGGTAATGGTTGTCTGGTAAAAGGACAGGG CCATCGCCAATTGGAGTATTTTGTTGATAATGGTCTGCTAGTTGAACGCT TCCATCTTCAATGTTGTGTCTAATTTTGAAGTTAACTTTGATTCCATTCT TTTGTTTGTCTGCCATGATGTATACATTGTGTGAGTTATAGTTGTATTCC AATTTGTGTCCAAGAATGTTTCCATCTTCTTTAAAATCAATACCTTTTAA CTCGATTCTATTAACAAGGGTATCACCTTCAAACTTGACTTCAGCACGTG TCTTGTAGTTCCCGTCATCTTTGAAAAATATAGTTCTTTCCTGTACGTAA CCTTCGGGCATGGCACTCTTGAAAAAGTCATGCTGTTTCATATGATCTGG GTATCTCGCAAAGCATTGAACACCATAACCGAAAGTAGTGACAAGTGTTG GCCATGGAACAGGTAGTTTTCCAGTAGTGCAAATAAATTTAAGGGTAAGT TTTCCGTATGTTGCATCACCTTCACCCTCTCCACTGACAGAAAATTTGTG CCCATTAACATCACCATCTAATTCAACAAGAATTGGGACAACTCCAGTGA AAAGTTCTTCTCCTTTACGCATGGTACCTTTGTCCTCTTTAATGAATTCC CTGAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTT GCACCAGGTTACGATGCGCCCATCTACACCAACGTGACCTATCCCATTAC GGTCAATCCGCCGTTTGTTTCTAGACTGCAGAAAAAAAAAAAATCAACTTC AAACTCAC
Seq-11 nts substitution	TCCGTGCGCTCTCCGCCACATATCCTGATCTTCCAGATAACTGCCGTCAC TCCAGCGCAGCACCATCACCGCGAGGCGGTTTTCTCCGGCGCGTAAAAAT GCGCTCAGGTCAAATTCAGACGGCAAACGACTGTCCTGGCCGTAACCGAC CCAGCGCCCGTTGCACCACAGATGAAACGCCGAGTTAACGCCATCAAAAA TAATTCGCGTCTGGCCTTCCTGTAGCCAGCTTTCATCAACATTAAATGTG AGCGAGTAACAACCCGTCGGATTCTCCGTGGGAACAAACGGCGGATTGAC CGTAATGGGATAGGTCACGTTGGTGTAGATGGGCGCATCGTAACCCTACT ACTACTAGTTTGAGGGGACGACGACAGTATCGGCCTCAGGAAGATCGCAC TCCAGCCAGCTTTCCGGCACCGCTTCTGGTGCCGGAAACCAGGCAAAGCG

Table A2. *Cont.*

Name	Sequence (5′–3′)
Seq-11 nts substitution	CCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGG CCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGA TTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGAC GGCCAGTGAATCCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGT TATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAA AGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCT CACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGTTATTATTG GAATCAAA
Seq-1011 nts insertion	CCGGACCGCCGCTTATCCGCCACTTATCCTGATCTTCCAGATAACTGCCG TCACTCCAGCGCAGCACCATCACCGCGAGGCGGTTTTCTCCGGCGCGTAA AAATGCGCTCAGGTCAAATTCAGACGGCAAACGACTGTCCTGGCCGTAAC CGACCCAGCGCCCGTTGCACCACAGATGAAACGCCGAGTTAACGCCATCA AAAATAATTCGCGTCTGGCCTTCCTGTAGCCAGCTTTCATCAACATTAAA TGTGAGCGAGTAACAACCCGTCGGATTCTCCGTGGGAACAAACGGCGGAT TGACCGTAATGGGATAGGTCACGTTGGTGTAGATGGGCGCATCGTAACCT GTTGCAAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAA TTCAGGGAATTCATTAAAGAGGACAAAGGTACCATGCGTAAAGGAGAAGA ACTTTTCACTGGAGTTGTCCCATTTCTTTGTTGAA

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