Chapter 6

MultiSite Gateway Technology Is Useful for Donor DNA Plasmid Construction in CRISPR/Cas9-Mediated Knock-In System

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.80775

Abstract

The clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 method is a powerful tool for genome editing, by introducing a DNA double-strand break (DSB) at the specific site. The gene knock-out can be achieved by the deletion or insertion at the CRISPR/Cas9-mediated DSB site by error-prone nonhomologous end joining repair in targeted cells. However, the gene knock-in is still difficult as compared to the knock-out, because of the low efficiency of homology directed repair with donor DNA in cells. Therefore, to efficiently select the knock-in cells, we developed a complicated donor DNA plasmid containing an antibiotic-resistance gene, in addition to the knock-in sequence and the two homology arms. MultiSite Gateway technology is a useful tool for constructing this complicated plasmid. We describe the MultiSite Gateway technology and provide an overview of the DSB repair pathways to clarify the knock-out and knock-in methods by the CRISPR/Cas9 system.

Keywords: knock-in, homology directed repair (HDR), MultiSite Gateway, donor DNA plasmid, CRISPR/Cas9, DSB repair

1. Introduction

Genome editing has been an important technique to investigate gene function in biology since before the development of the clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 system. In microorganisms such as *Escherichia coli (E. coli)* and yeast, gene disruption can be achieved by simply introducing a donor DNA into the cells without inducing a DSB in the targeted gene [1–3]. With regards to gene disruption in vertebrate cells, chicken B lymphocyte

line DT 40 cells have been widely used because of their high efficiency of homologous DNA recombination [4–6]. Similar to *E. coli* and yeast cells, gene disruption in DT 40 cells can also be achieved by introducing a DNA vector for gene targeting into the cells. In contrast, gene disruption by this method in human cells is difficult, because of the inefficient homologous recombination in the cells. Therefore, gene knockdown by RNA interference (RNAi) has usually been used in human cells, in order to examine the functions of human genes [7]. However, the targeted protein cannot be completely eliminated from the cells by RNAi. Therefore, an efficient method for targeted gene disruption in human cells has been keenly desired.

The CRISPR/Cas9 method is an innovative genome editing technology. The history of this technology originated from the finding of unusual, functionally unknown repeated sequences in *E. coli* [8, 9]. In the repeated sequences, highly homologous repeats are separated by nonrepetitive nucleotides as spacers. Repeated sequences, composed of repeats and spacers, were also found in numerous genomes of other bacteria and archaea [10] and were named CRISPR [11]. In addition, well-conserved genes were identified adjacent to the CRISPR loci and were named CRISPR-associated (Cas) genes [11]. Some of the Cas protein families were found to share sequence homology with proteins involved in DNA metabolism, such as helicases and exonucleases [11], and the purified Cas proteins exhibited endonuclease activity [12–14]. Sequences identical to the CRISPR spacers were found among bacterial mobile genetic elements, such as plasmids and phages, suggesting that the CRISPR spacers in the bacterial genome are derived from DNA fragments of the invading foreign genetic elements [15, 16]. In bacteria, CRISPR/Cas serves as a defense system against the invasion of mobile genetic elements [17]. The molecular mechanism of the defense system was elucidated by biochemical experiments [13, 14, 18, 19]. Cas proteins bind to RNA transcribed from the CRISPR spacer sequence and cleave the precursor CRISPR RNA (pre-crRNA) [18]. Cas proteins exist in the complex with the cleaved mature crRNA [18] and cleave invading foreign genetic elements mediated by the crRNA guide, containing the complementary sequence with the targeted genetic elements [13, 14, 19]. Among the Cas family proteins, a single Cas9 protein complexed with a crRNA can introduce a specific DSB at the desired site in the target DNA [13, 14]. Therefore, the CRISPR/Cas9 system is being applied for genome engineering [20, 21]. The biology and technology of the CRISPR/Cas system are described in detail in excellent reviews [22–27].

A variety of CRISPR/Cas9-mediated genome editing tools is now commercially available. A gene knock-out can be accomplished simply by using the CRISPR/Cas9 tool. However, for a gene knock-in or replacement, a donor DNA must also be prepared individually. Here, we describe the usefulness of the MultiSite Gateway technology [28, 29] for the construction of the donor DNA plasmid.

2. DSB repair pathways involved in genome editing

The CRISPR/Cas9-mediated gene knock-out or knock-in is based on the mechanisms of DSB repair. To better understand the genome editing by the CRISPR/Cas9 method, we first provide an overview of the DSB repair mechanisms.

DSBs are repaired by multiple mechanisms [30] (**Figure 1**). One of the major DSB repair mechanisms is nonhomologous end joining (NHEJ). NHEJ is the simplest method for DSB repair,

http://dx.doi.org/10.5772/intechopen.80775

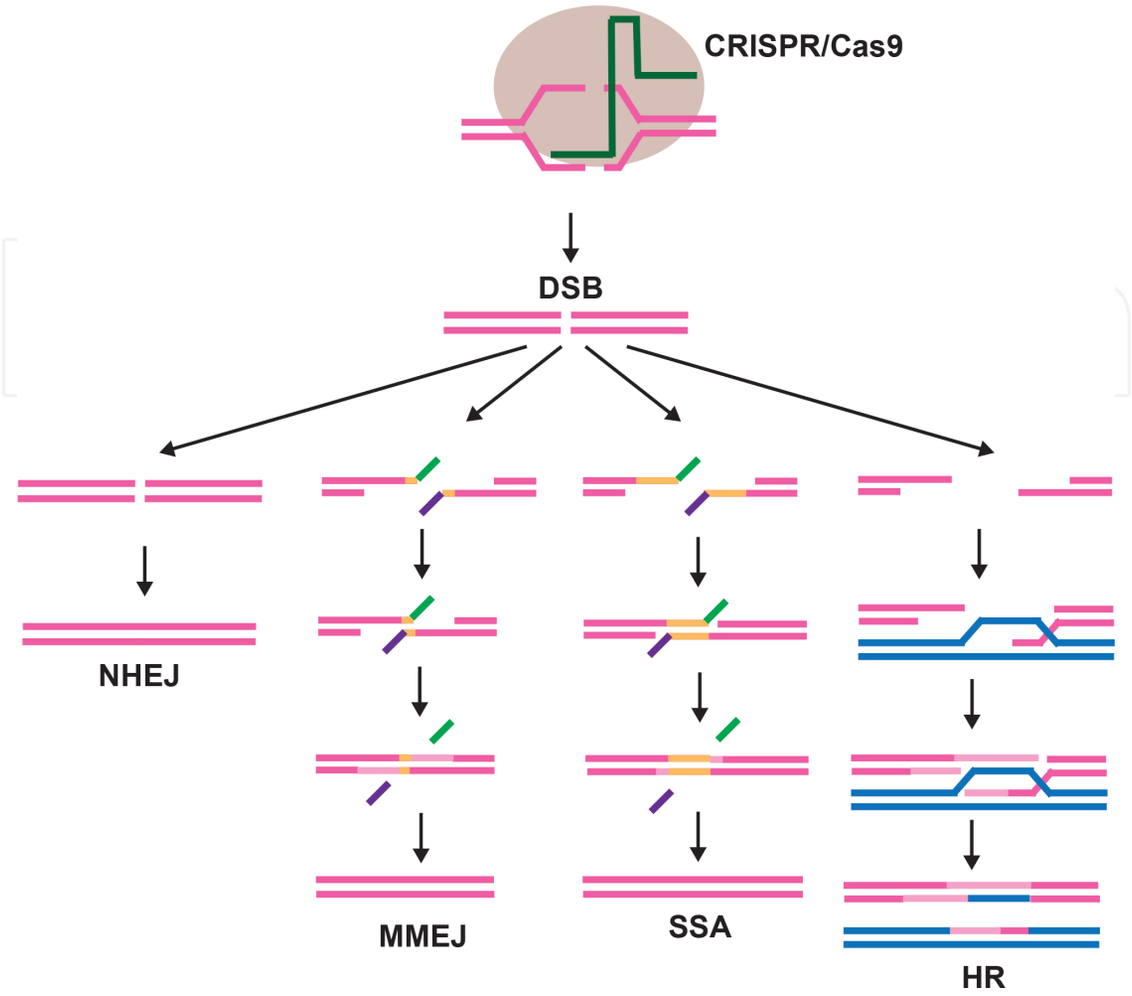

Figure 1. Multiple DSB repair pathways. The targeted DSB induced by CRISPR/Cas9 is repaired by NHEJ, MMEJ, SSA, or HR.

in which the broken ends of the DNA are rejoined, and is a rapid and predominant DSB repair pathway in mammalian cells [31]. The DSB is accurately repaired to its normal state when the broken ends are protected during repair. However, if the broken ends are digested before rejoining, the DNA information at or around the DSB site is lost. Thus, NHEJ is an error-prone DSB repair pathway [32]. CRISPR/Cas9-mediated gene knock-out technology utilizes this mutagenic aspect of NHEJ, and thus requires only DSB induction in the targeted gene. Most of the DSBs are repaired accurately by NHEJ [33]. However, if the DSB site is repaired accurately, then the target DNA site seems to be attacked repeatedly by CRISPR/Cas9, until the site is broken and thus insensitive to the hybridization with the crRNA (**Figure 2**). This apparently enhances the knock-out efficiency by CRISPR/Cas9.

The second major DSB repair pathway is homologous DNA recombination (HR) [34, 35] (**Figure 1**). In HR, DSBs are repaired by DNA strand exchange with the undamaged homologous DNA strand. In *E. coli* or yeast, HR is the predominant mechanism for DSB repair. In the first step of HR, the DSB ends are resected by a nuclease to generate 3′ single-strand (ss) DNA overhangs. Then, the ssDNA overhangs invade and anneal with the undamaged homologous DNA strand. New DNA is synthesized from the 3′ end of the invaded DNA as a primer, according to the sequence information of the undamaged DNA template, thereby

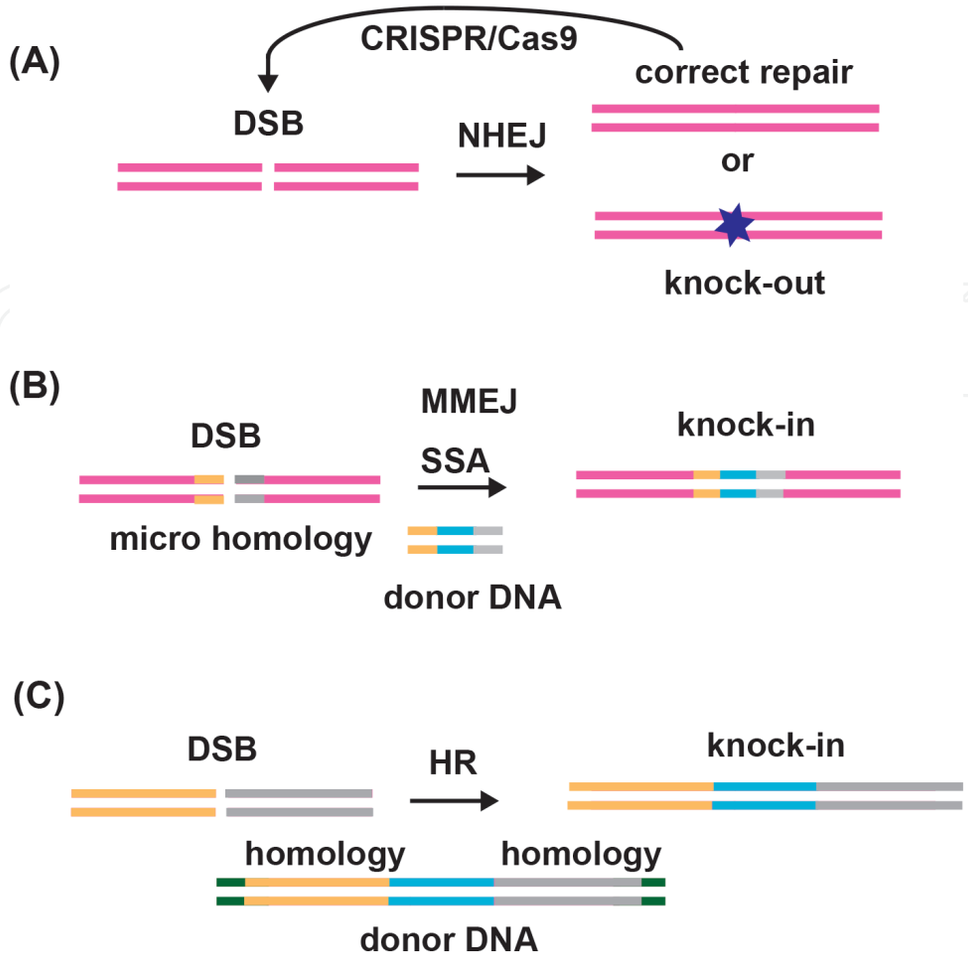

Figure 2. Gene knock-in or knock-out by DSB repair pathways. (A) Targeted gene disruption induced by NHEJ. (B) Targeted short DNA fragment insertion mediated by MMEJ or SSA. (C) Targeted long DNA fragment insertion mediated by HR.

restoring the lost sequence information at the damaged sites. The branch point in the crossed DNA strands moves during the repair process. Finally, the crossed DNA strands are resolved by cutting and rejoining. Thus, the DNA information can be restored by HR repair even if the broken DNA ends are digested, such as by nucleases. Therefore, HR is a more precise DSB repair mechanism, as compared to NHEJ. If the homologous DNA strand is available in the donor DNA, then the donor DNA is integrated into the damaged site by HR (**Figure 2**). Thus, HR is an important mechanism for a gene knock-in.

DSBs are also repaired by other mechanisms, including microhomology-mediated end joining (MMEJ) and single-strand annealing (SSA) (**Figure 1**). Although MMEJ and SSA are mechanistically similar, they are distinct pathways with different repair proteins. Both MMEJ and SSA use relatively short internal homologous sequences flanking both sides of the DNA break. The length of the homologous sequence required for MMEJ is shorter than that for SSA. The first step of MMEJ or SSA is the resection of the DSB site, and thereby the homologous dsDNA region becomes ssDNA. In the next step, the ssDNA regions with the homologous sequence are annealed, and the 3′ flaps of the nonhomologous region are removed. Finally, the gapped

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DNA regions are filled by DNA synthesis, and the resulting nicks are rejoined [36–39]. Both repair pathways induce a DNA deletion at the damaged site, and therefore could contribute to the CRISPR-Cas9-mediated gene knock-out in the absence of a DNA donor [40, 41]. However, when the donor DNA is provided, the repair pathways can be used for gene knock-in [42–44] (**Figure 2**).

3. Donor DNA plasmid for homology directed gene knock-in

The knock-in of a relatively short gene fragment (up to ~1.5 kb) can be achieved by MMEJ or SSA with a linearized donor DNA fragment containing short (about 20–60 bp) homologous DNA regions, called homology arms [42, 43, 45–47]. The knock-in efficiency decreases as the size of the insert DNA increases [48]. In the case of the insertion of a long DNA fragment (more than 1.5 kb), the knock-in efficiency increased as the length of the homology arms increased, up to about 1500 bp [48]. Thus, long homology arms (more than 500 bp) are usually used for the knock-in of such a long DNA fragment [45, 49–52]. In this case, the knock-in is mediated by HR. The knock-in efficiency by HR is low [53], and accordingly, a selectable marker is introduced into the donor DNA plasmid for screening the knock-in clones in some cases [49–52]. Therefore, the selectable donor DNA plasmid for the HR-mediated gene knock-in contains left and right arms, the inserted gene of interest, and a selectable marker gene with promoter and transcription terminator regions. An example of a donor DNA plasmid is shown in **Figure 3** [52]. In our donor DNA plasmid, the left and right arms contain the promoter and transcription terminator regions of the inserted gene of interest, respectively. However, the native transcription terminator region is separated from the gene of interest by the neighboring selectable

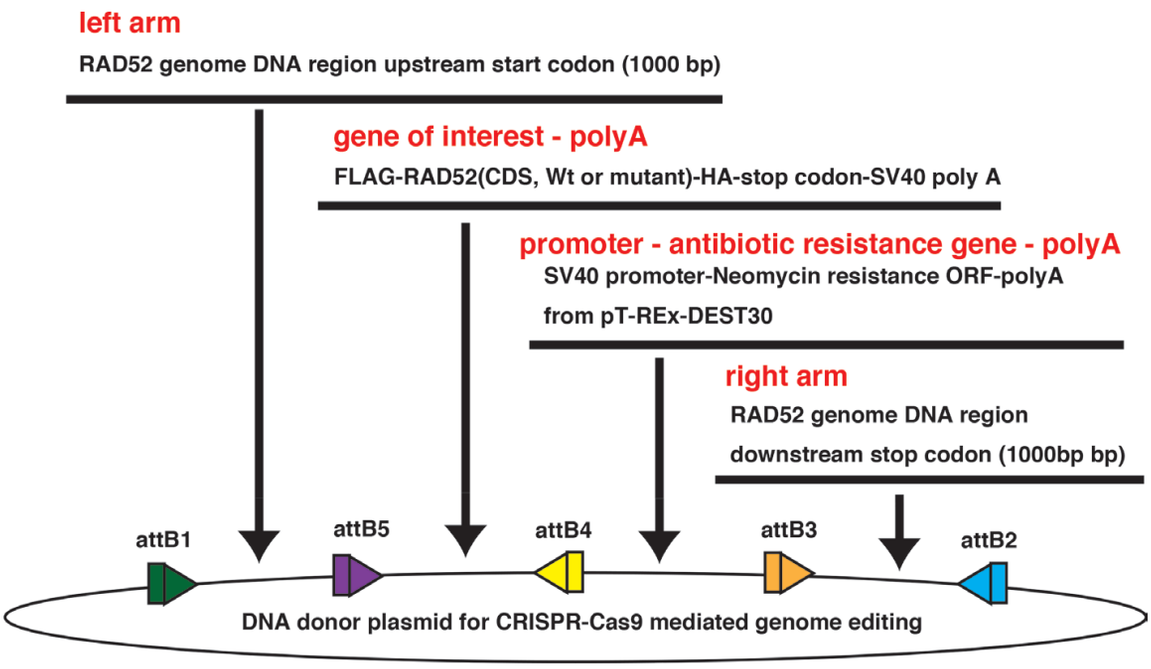

Figure 3. A donor DNA plasmid for HR-mediated gene knock-in.

marker gene. Therefore, the SV40 transcription terminator sequence is also placed just downstream of the gene of interest. Thus, the structure of the donor DNA plasmid is complicated, as it contains multiple inserted DNA regions. It is difficult to construct such a complicated plasmid by using classical restriction enzyme-mediated cloning methods. In some cases, the donor DNA plasmids were constructed by a site-specific recombinational cloning method named In-Fusion technology [54], provided by Clontech [42, 55].

4. Gateway technology for DNA cloning

Gateway technology is another site-specific recombinational cloning method [54, 56] (**Figure 4**). This technology is based on the site-specific recombination system of *E. coli* bacteriophage λ [57]. Bacteriophage λ integrates into the *E. coli* chromosome by site-specific recombination between the attachment (*att*) sites on the *b*acterial chromosome (attB) and the *att* sites on the *p*hage chromosome (attP), to generate left (attL) and right (attR) att sites. This recombination

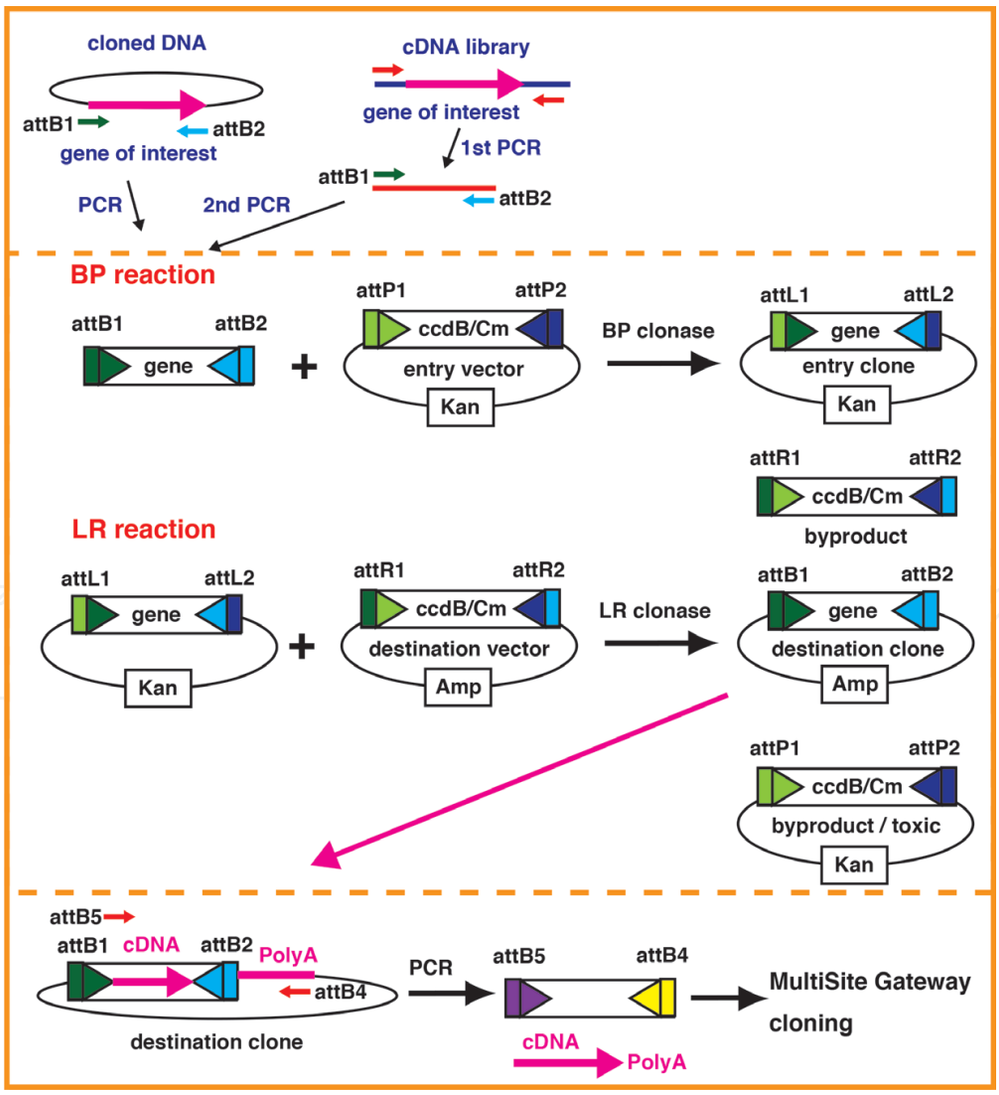

Figure 4. Standard Gateway method for single-fragment cloning. The DNA fragment of a gene of interest is amplified by PCR with a cloned DNA or cDNA library as the PCR template. The destination clone containing a polyadenylation (polyA) tail region downstream from the cDNA can be used as a PCR template for the next MultiSite Gateway cloning. Abbreviations: Kan, kanamycin resistance gene; Amp, ampicillin resistance gene; Cm, chloramphenicol resistance gene.

reaction is mediated by the integrase (Int) enzyme of bacteriophage λ and the integration host factor (IHF) of *E. coli.* The excision reaction requires another host factor, excisionase (Xis), in addition to Int and IHF. The Gateway technology has developed mutant att sites (such as attB1, attB2, attP1, and attP2) [56]. The attB1 site specifically recombines with the attP1 site, but not attP2, to generate the attL1 and attR1 sites. Similarly, the attB2 site specifically recombines with the attP2 site to generate the attL2 and attR2 sites.

In the first step of the Gateway cloning, the DNA fragment for cloning is amplified by PCR with primer sets containing attB1 or attB2 sequences at the 5′ ends (**Figure 4**). Gateway cloning vectors (pDONR) contain attP1 and attP2 sites for cloning. Therefore, the PCR product containing the attB1 or attB2 sites at both ends can be specifically inserted in between the attP1 and attP2 sites of the pDONR vector by site-specific recombination, to generate the entry clone of the Gateway system. A protein mixture (BP clonase) containing Int and IHF is used for this *in vitro* site-specific recombination reaction (BP reaction). In the pDONR vector, the *ccdB* gene from F factor of *E. coli* is located between the attP1 and attP2 sites. The CcdB protein is a toxin for *E. coli* cells that lack the antitoxin, the CcdA protein, which is also produced from F factor of *E. coli* [58–61]. Therefore, *E. coli* cells without F factor or the *ccdA* gene cannot grow, due to the production of the CcdB protein from the pDONR vector. For example, *E. coli* DH5α cells lack F factor and the *ccdA* gene. Therefore, the cell growth of the DH5α strain is inhibited in the presence of the pDONR vector. The Gateway cloning method applies this cell killing mechanism mediated by the *ccdB* gene for efficient DNA cloning. If the PCR product is successfully cloned between the attP1 and attP2 sites of the pDONR vector, then the *ccdB* gene is removed from the vector by the site-specific recombination. Therefore, when the *in vitro* site-specific reaction mixture is transformed into DH5α cells, only the cells containing the generated entry clone can grow. Thus, all of the transformed colonies contain the successfully cloned plasmid DNA. This is an excellent point of the Gateway cloning method. In the constructed entry clone, the cloned gene is present between the generated attL1 and attL2 sites.

The Gateway cloning system provides destination vectors for numerous purposes, such as for expressing the cloned gene in a variety of organisms. Each destination vector contains attR sites for cloning on both sides of the *ccdB* gene (**Figure 4**). The cloned gene in the entry clone can be transferred to destination vectors by an *in vitro* site-specific recombination reaction (LR reaction) between the attL and attR sites with a protein mixture (LR clonase) containing Int, IHF, and Xis, to generate the destination clone. As a result of the reaction, the *ccdB* gene of the destination vector is replaced with the gene of interest and is transferred to the pDONR vector as a by-product. Therefore, when the LR reaction mixture is transformed into DH5α cells, the growth of the cells containing the by-product plasmid is inhibited by the expression of the toxic *ccdB* gene. The antibiotic resistance gene of the destination vector is different from that of the pDONR vector. Therefore, only the desired destination clone is selected in the presence of the appropriate antibiotics. Thus, once the DNA fragment of interest is cloned into the pDONR vector, the DNA fragment can be transferred to a variety of destination vectors quite easily.

5. MultiSite Gateway technology for donor DNA plasmid construction

The original Gateway cloning system was further improved for cloning multiple DNA fragments into a single vector [28, 62]. The improved cloning method is called MultiSite Gateway

[29, 63] (**Figure 5**). In the MultiSite Gateway cloning method, numerous attB, attP, attL, and attR variant sites were developed. Each attB site is specifically recombined with the corresponding attP site by the BP reaction, to generate the corresponding attL and attR sites. Similarly, each attL

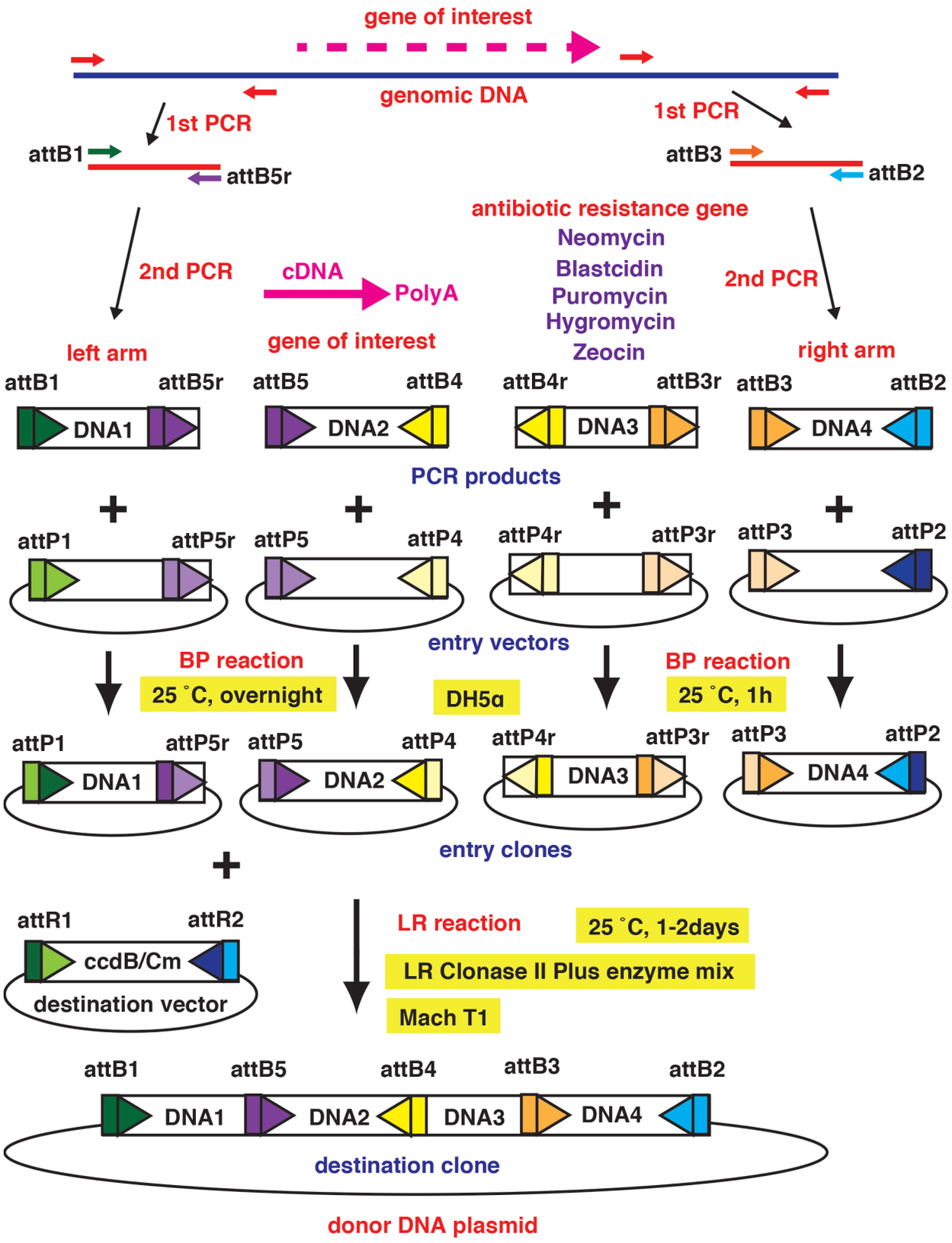

Figure 5. Cloning four-fragments by the MultiSite Gateway method. The strategy for the construction of the donor DNA plasmid is shown.

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site is specifically recombined with the corresponding attR site by the LR reaction. At present, up to four DNA fragments can be cloned into a single vector. The 4-fragment MultiSite Gateway cloning technology is suitable for the construction of a donor DNA plasmid for CRISPR/Cas9 and HR-mediated gene knock-in (**Figure 5**). In the first step of the 4-fragment cloning, each PCR-amplified DNA fragment is cloned into the corresponding pDONR vectors. As shown in **Figure 5**, the orientation of the att site sequence is important. The attB3r, attB4r, and attB5r sequences are reversely oriented relative to the attB3, attB4, and attB5 sequences, respectively. In the second cloning step, the four entry clones are mixed with an appropriate destination vector and are subjected to the LR reaction. Thus, the four DNA fragments are simultaneously assembled and inserted into a single destination vector, in the desired order and orientation. The outline of our example is described below [52].

We used a CRISPR/Cas9-mediated gene knock-in, in order to express the wild type or mutant gene of interest (human RAD52 gene in our case) with its own native promoter in the genome and to examine the cellular effects of the mutant protein expressed at the endogenous level. The 1000 bp genomic DNA region upstream from the start codon of the gene of interest was amplified by PCR with primer sets containing attB1 or attB5r sequences at the 5′ ends and was cloned between the corresponding attP1 and attP5r sites of the first pDONR vector. The 1000 bp genomic DNA region downstream from the stop codon of the gene was cloned between the attP3 and attP2 sites of the fourth pDONR vector (**Figures 3** and **5**). The cDNA of the gene (wild type or mutant) was first cloned via standard Gateway cloning into a destination vector (pT-Rex-DEST30), for gene expression with the CMV promoter of the vector (**Figure 4**). In the destination clone, the SV40 polyadenylation region required for transcriptional termination is present downstream from the stop codon of the cloned gene. We amplified the DNA region encoding the cDNA of the gene and the SV40 polyadenylation region by PCR, using the destination clone as the PCR template. The PCR product was cloned between the attP5 and attP4 sites of the second pDONR vector (**Figure 5**). The DNA region containing the SV40 promoter, neomycin resistance gene, and polyadenylation region was amplified by PCR with pT-Rex-DEST30 as the PCR template and was cloned between the attP4r and attP3r sites of the third pDONR vector (**Figure 5**). The DNA fragments in each of the entry clones were verified by DNA sequencing. Then, the LR reaction was performed with the confirmed four types of entry clones and the destination vector (a simple vector, pDEST14, in our case). Thus, the complicated donor DNA plasmids could be constructed relatively easily, by using the MultiSite Gateway technology.

6. Advantages of MultiSite Gateway technology for donor DNA plasmid construction

MultiSite Gateway technology is suitable for the construction of a complicated donor DNA plasmid, for several reasons. In this method, all parts of the complicated donor DNA are first cloned into entry vectors, before their assembly. The cloned parts are easily verified by DNA sequencing. The verified entry clones can be used as parts for constructing other donor DNAs. For example, the entry clone containing the required part for expressing the neomycin resistance gene can be reused for constructing donor DNA plasmids targeting the other genes. For generating knock-in cells, other antibiotic (such as blasticidin, puromycin, hygromycin, and zeocin) resistance genes are sometimes required as the selection markers. In this case, if the

DNA fragments required for expressing the other antibiotic resistance genes are cloned into the entry vector, we can substitute the neomycin resistance gene of the donor DNA plasmid in **Figure 3** with the other antibiotic genes very easily (**Figure 5**). One of the purposes of generating knock-in cells is to elucidate the function and regulation of the gene product of interest, by expressing the mutants of the gene related to genetic diseases or altered protein modification sites. By using the MultiSite Gateway cloning method, the donor DNA plasmids containing a variety of mutant genes can be easily constructed by simply substituting only the entry clones containing the mutant genes of interest (**Figure 5**). For these reasons, MultiSite Gateway cloning is a convenient and useful method for constructing the complicated donor DNA plasmids.

7. Strategies and improvements for generating knock-in cells

To replace the whole endogenous gene with the donor gene, the targeted gene was cleaved around both the start and stop codons by the CRISPR/Cas9 method [52]. To avoid the cleavage of the donor vector, a specific cleavage sequence that does not exist within the donor DNA must be selected in the endogenous gene. Therefore, the targeted sequence for CRISPR/Cas9-mediated cleavage was designed within introns around both the start and stop codons of the gene. In our case, the cDNA of the donor gene contains FLAG and HA tag sequences at the amino (N)- and carboxyl (C)-terminal ends, respectively. Therefore, the DNA sequences around the start and stop codons are different and allow discrimination between the endogenous gene and the donor gene. By selecting the sequence containing the start or stop codon as the CRISPR/Cas9-targeted region, the endogenous gene can be cleaved selectively. For CRISPR/Cas9-mediated cleavage, a 5′-NGG-3′ sequence named the proto-spacer adjacent motif (PAM) is required on the 3′ end of the target sequence. In our case, PAM sequences were found in the vicinities of the start and stop codons of the target gene. Therefore, we could design the CRISPR RNAs to specifically guide Cas9 to the vicinity of the start or stop codon of the targeted endogenous gene. We used the commercially available GeneArt CRISPR Nuclease Vector from Life Technologies for the production of the CRISPR RNA and the Cas9 protein in cells. To generate the knock-in cells, the donor DNA plasmid was transfected into the cells with the two plasmids expressing the CRISPR RNAs targeting the vicinities of the start codon and the stop codon of the targeted gene. The transfected cells were initially cultured in the absence of selectable antibiotics for a few days and subsequently cultured in the presence of appropriate concentrations of the antibiotics for the selection of the knock-in cells. Most of the cells died after the selection. However, the antibiotic-resistant cells were observed after a long cultivation in the same culture dish. The antibiotic-resistant clones were isolated, the genomic DNA from each clone was purified, and the desired knock-in cells were subsequently verified by PCR and sequencing analyses. Thus, we obtained the desired knock-in cells by our method. However, we think that there is still room for improvements of our method.

In our method, we used circular plasmid DNA as the donor DNA (**Figure 3**). However, the knock-in efficiency is reportedly enhanced when the circular DNA is linearized [53]. Therefore, our strategy might be improved by using linearized donor DNA. According to the report, the knock-in efficiency of the donor DNA fragment is decreased when nonhomologous terminal DNA regions are present adjacent to the two homology arms [53]. The inhibitory effect of

the nonhomologous DNA regions is increased in accordance with the length. Therefore, the most suitable donor DNA is a linearized DNA fragment containing homology arms at both terminal ends, without nonhomologous regions at the ends. When restriction enzyme sites are introduced at the terminal ends of the homology arms of our donor DNA plasmid, this linearized donor DNA can be produced by restriction enzyme cleavage. This modification of our strategy will improve the knock-in efficiency.

The DSB repair pathway choice is an important consideration to improve the knock-in efficiency. Among the several DSB repair pathways, the HR pathway choice is enhanced when the NHEJ pathway does not work [64]. Therefore, in order to increase the knock-in efficiency by HR, the inhibition of the NHEJ pathway was attempted in CRISPR/Cas9-mediated genome editing [65–68]. A chemical compound, SCR7, inhibits DNA ligase IV, which is an essential protein for NHEJ [69]. The CRISPR/Cas9-mediated homology-directed genome editing was enhanced by treating the cells with SCR7 [65, 66, 68]. In addition, a high throughput chemical screen identified small molecules that modulate CRISPR/Cas9-mediated genome editing [70]. The HR-mediated knock-in efficiency was improved by two chemical compounds, L755507 and BrefeldinA, which are a β3-adrenergic receptor agonist and an inhibitor of protein transport from the ER to the Golgi apparatus, respectively [70]. In contrast, the HR-mediated knock-in efficiency was decreased by the chemical compounds azidothymidine (AZT) and Trifluridine (TFT), which are anti-HIV and anti-herpes virus drugs, respectively [70]. In contrast to their effects on the HR-mediated knock-in, L755507 inhibits the NHEJ-mediated knock-out, whereas AZT enhanced it [70]. Therefore, in addition to their known activities, these chemical compounds could modulate the DSB repair pathway choice. The effectiveness of L755507 for the HR-mediated knock-in was also demonstrated in another study [68]. Therefore, the knock-in strategy could be further improved in combination with the usage of these chemical compounds.

8. Experimental procedure for donor DNA plasmid construction by MultiSite Gateway technology

Here, we describe our protocol for the donor DNA plasmid construction by the MultiSite Gateway technology.

8.1. PCR amplification of arm DNA fragments (1000 bp)

8.1.1. The first-round PCR

Primers (forward and reverse): arm DNA-specific oligonucleotides (35 mer)

Primer stocks (50 or 100 μM) are diluted to 10 μM prior to use.

Template: human genomic DNA, purified with a Blood & Cell Culture DNA Mini Kit (QIAGEN).

Template stock is diluted to 50 ng/μl prior to use.

PCR amplification is performed with PrimeSTAR GXL DNA Polymerase kit (Takara).

5X PrimeSTAR GXL buffer: 10 μl

dNTP Mixture (2.5 mM each): 4 μl

Forward primer (10 μM): 1 μl

Reverse primer (10 μM): 1 μl

Template (50 ng/μl): 1 μl

Sterile distilled water: 32 μl

PrimeSTAR GXL DNA Polymerase: 1 μl

PCR conditions

Initial step: 3 s at 98°C (denaturation)

25 cycles:

10 s at 98°C (denaturation)

15 s at 55°C (annealing)

1–2 min/kb at 68°C (extension)

Hold: 4°C (storage)

The PCR reaction mixture is purified with a QIAquick PCR Purification Kit (QIAGEN) to remove the PCR primers.

8.1.2. The second-round PCR

Forward primer for the left arm:

5′-GGGG-attB1 (ACAAGTTTGTACAAAAAAGCAGGCT)-(NN)-(template-specific sequence)-3′

Reverse primer for the left arm:

5′-GGGG-attB5r (ACAACTTTTGTATACAAAGTTG)T-(template-specific sequence)-3′

Forward primer for the right arm:

5′-GGGG-attB3 (ACAACTTTGTATAATAAAGTTG)-(NN)-(template-specific sequence)-3′

Reverse primer for the right arm:

5′-GGGG-attB2 (ACCACTTTGTACAAGAAAGCTGGGT)A-(template-specific sequence)-3′

The first-round primer sequences are used as each template-specific sequence.

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If AA, AG, or GA is present 5′ of the template-specific sequence, then NN (except for AA, AG, or GA) is added in order to avoid the generation of a stop codon, as described in the MultiSite Gateway User Manual.

5X PrimeSTAR GXL buffer: 10 μl

dNTP Mixture (2.5 mM each): 4 μl

Forward primer (100 μM): 0.5 μl

Reverse primer (100 μM): 0.5 μl

Template (5 ng/μl): 2 μl

Sterile distilled water: 32 μl

PrimeSTAR GXL DNA Polymerase: 1 μl

PCR conditions

Initial step: 3 s at 98°C (denaturation)

10 cycles:

10 s at 98°C (denaturation)

1 min/kb at 68°C (annealing/extension)

Hold: 4°C (storage)

8.2. PCR amplification of the gene of interest (cDNA of the gene and polyadenylation region) or the antibiotic resistance gene (promoter, antibiotic resistance gene, and polyadenylation region)

Template: plasmid DNA

Forward primer for the gene of interest:

5′-GGGG-attB5 (ACAACTTTGTATACAAAAGTTG)-(NN)-Kozak sequence and start codon (ACCATG)-template-specific sequence)-3′

Reverse primer for the gene of interest:

5′-GGGG-attB4 (ACAACTTTGTATAGAAAAGTTGGGT)G-(template-specific sequence)-3′

Forward primer for the antibiotic resistance gene:

5′-GGGG-attB4r (ACAACTTTTCTATACAAAGTTG)-(NN)-(template-specific sequence)-3′

Reverse primer for the antibiotic resistance gene:

5′-GGGG-attB3r (ACAACTTTATTATACAAAGTTG)T-(template-specific sequence)-3′

If AA, AG, or GA is present 5′ of the template-specific sequence, then NN is added as above-mentioned.

5X PrimeSTAR GXL buffer: 10 μl

dNTP Mixture (2.5 mM each): 4 μl

Forward primer (100 μM): 0.5 μl

Reverse primer (100 μM): 0.5 μl

Template: 10 ng

PrimeSTAR GXL DNA Polymerase: 1 μl

Sterile distilled water: to final reaction volume of 50 μl

PCR conditions

Initial step: 3 s at 98°C (denaturation)

30–35 cycles:

10 s at 98°C (denaturation)

min/kb at 68°C (annealing/extension)

Hold: 4°C (storage)

If the template plasmid DNA contains a kanamycin resistance gene, which also exists in the pDONR entry vector, then the PCR reaction mixture is treated with the *Dpn*I enzyme to digest the template DNA.

The PCR products are purified by phenol/chloroform extraction and ethanol precipitation and are resuspended in TE buffer (10–50 μl).

8.3. BP reaction

pDONR entry vector (150 ng/μl; Invitrogen): 0.25 μl

BP Clonase II enzyme mix (Invitrogen): 0.5 μl

PCR product: 1–1.75 μl (10–100 ng)

TE buffer: to final reaction volume of 2.5 μl

Incubate at 25°C for 60 min (for attB4r/attB3r and attB3/attB2 fragments) or overnight (for attB1/attB5r and attB5/attB4 fragments).

(Note: the cloning efficiencies of the attB1/attB5r and attB5/attB4 fragments were low. A longer incubation time in the BP reaction improved the cloning efficiency.)

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Add 0.25 μl Proteinase K solution (20 mg/ml; Invitrogen) to the reaction mixture. Incubate at 37°C for 10 min.

Transform the reaction mixture into DH5α competent cells by the heat-shock method. Spread the transformed cells on an LB agar plate containing 20 μg/ml of kanamycin and incubate it at 37°C overnight. The plasmids are purified from each colony on the LB agar plate and are verified by DNA sequencing.

8.4. MultiSite Gateway LR reaction

Destination vector (20 fmol/μl; Invitrogen): 0.5 μl

attB1/attB5r entry clone (10 fmol/μl): 0.5 μl

attB5/attB4 entry clone (10 fmol/μl): 0.5 μl

attB4r/attB3r entry clone (10 fmol/μl): 0.5 μl

attB3/attB2 entry clone (10 fmol/μl): 0.5 μl

TE buffer: 1.5 μl

LR Clonase II Plus enzyme mix (Invitrogen): 1 μl

(Note: LR Clonase enzyme and LR Clonase II enzyme mix, which are used for standard single fragment cloning, cannot be used for cloning multiple fragments.)

Incubate at 25°C for 1–2 days.

Add 0.5 μl Proteinase K solution (20 mg/ml; Invitrogen) to the reaction mixture. Incubate at 37°C for 10 min.

Transform the reaction mixture into Mach T1 competent cells (Invitrogen) by the heat-shock method. Spread the transformed cells on LB agar plates containing appropriate antibiotics and incubate them at 37°C overnight. The plasmid DNA is purified from the colonies on the LB agar plates and used as the donor DNA after verification.

(Notes: Mach T1 competent cells were more suitable for cloning multiple fragments than DH5α competent cells.)

9. Conclusion

The CRISPR/Cas9 technology has opened a new window to investigate gene functions by targeted knock-ins. By replacing the endogenous gene with the mutant gene, the cellular effects of the mutant can be examined under the most suitable native conditions of the gene expressed from the native promoter. The native expression level of the gene is also important for investigating the intracellular localization and behavior of the gene product, because over-expression of the gene by a nonnative promoter sometimes induces artifactual effects on the intracellular localization of the protein. The CRISPR/Cas9-mediated knock-in of specific tag

sequences into the endogenous gene allows the investigation of the intracellular localization of the protein at the native expression level, by monitoring the introduced tag sequences. The construction of a complicated donor DNA is required for gene knock-in mediated by HR. This is a bottleneck point for the CRISPR/Cas9-mediated targeted knock-in technology. Standard Gateway cloning is a popular method for constructing ordinary expression plasmids and is therefore more commonly used as compared to MultiSite Gateway cloning. However, MultiSite Gateway cloning is a quite useful method, especially for constructing the complicated donor DNA plasmid. Therefore, this technology will contribute to the spread of CRISPR/Cas9-mediated targeted knock-in methods.

Acknowledgements

This work was supported by Grants-in Aid for Scientific Research from Japan Society for the Promotion of Science. This work was also supported by the general fund of NIRS, QST.

Conflict of interest

The authors declare no conflict of interest associated with this manuscript.

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