Simple one-week method to construct gene-targeting vectors: application to production of human knockout cell lines

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Targeted gene disruption is a powerful tool for studying gene function in cells and animals. In addition, this technology includes a potential to correct disease-causing mutations. However, constructing targeting vectors is a laborious step in the gene-targeting strategy, even apart from the low efficiency of homologous recombination in mammals. Here, we introduce a quick and simplified method to construct targeting vectors. This method is based on the commercially available MultiSite Gateway® technology. The sole critical step is to design primers to PCR amplify genomic fragments for homologous DNA arms, after which neither ligation reaction nor extensive restriction mapping is necessary at all. The method therefore is readily applicable to embryonic stem (ES) cell studies as well as all organisms whose genome has been sequenced. Recently, we and others have shown that the human pre-B cell line Nalm-6 allows for high-efficiency gene targeting. The combination of the simplified vector construction system and the high-efficiency gene targeting in the Nalm-6 cell line has enabled rapid disruption of virtually any locus of the human genome within one month, and homozygous knockout clones lacking a human gene of interest can be created within 2–3 months. Thus, our system greatly facilitates reverse genetic studies of mammalian—particularly human—genes.

INTRODUCTION

Targeted gene disruption provides a powerful means for studying gene function by a reverse genetic approach (1,2). This technology depends on homologous recombination reactions that occur, albeit rarely, between transfected DNA (i.e., targeting vector) and the host genome. In mice, a large number of genes have been knocked out thus far using embryonic stem (ES) cells, and their physiological functions have been elucidated. More recently, reverse genetic studies using chicken DT40 cells have made a significant contribution to our understanding of cellular functions of a variety of genes (3). It is important to note, however, that the findings in model organisms are not always the case in human cells. Hence, reverse genetic analysis using human somatic cells would be of greater importance in the postgenome era when one attempts to reliably

analyze the function of human genes. In human cultured cells, however, the frequency of gene targeting is typically too low for these approaches to be feasible (2,4). In this regard, it is noteworthy that we and others have shown that Nalm-6, a human pre-B acute lymphoblastic leukemia (ALL) cell line, is highly proficient in gene-targeting experiments (5–8).

A rate-limiting step in gene-targeting experiments, apart from the low efficiency of homologous recombination, is constructing targeting vectors. Vector construction typically involves cloning and mapping of genomic DNA fragments, which is highly time-consuming. Fortunately, the completion of the human genome sequence has greatly simplified those tedious processes, since virtually all DNA fragments can be readily PCR-amplified with specific primers (which must be designed not to include repetitive sequences such as Alu, however).

Nevertheless, the subsequent step—namely assembly of a genomic DNA fragment(s) with selectable markers—can still be a time-consuming and complicated process, partly due to the necessity of searching for appropriate restriction sites for ligation reactions as well as those for vector linearization. Thus, the success of vector construction depends crucially on the skill of the researcher. In this paper, we report a quick and simplified method for vector construction that we believe is simpler and quicker than any other methods reported thus far. We also show that virtually any genomic locus can be disrupted within one month in the human Nalm-6 cell line.

MATERIALS AND METHODS

Vector Construction Using the MultiSite Gateway® System

To construct the pDEST DTA-MLS plasmid, a diphtheria toxin A (DT-A) gene cassette, which was excised from pMC1DT-ApA (Kurabo, Osaka, Japan) and a 58-mer multiple-linearization site (MLS) fragment carrying *PacI*, *SwaI*, *I-SceI*, *AscI*, and *PmeI* sites were sequentially inserted at the *NdeI* and *AatII* sites of pDEST™ R4-R3, respectively (Invitrogen, Carlsbad, CA, USA; see Supplementary Figure S1 available online at www.BioTechniques.com). The 58-mer DNA fragment was made by annealing oligonucleotides MLS-A and MLS-B (Table 1).

To create entry clones for floxed positive selectable markers hygromycin-resistance gene (*Hyg^r*), puromycin-resistance gene (*Puro^r*), and histidinol-resistance gene (*His^r*), each marker gene was subcloned into the donor vector pDONR™221, as illustrated in Supplementary Figure S2. Briefly, an entry clone plasmid, pENTR loxP, was first created by utilizing a DNA fragment generated by PCR with *attB1*- and *attB2*-containing primers (246-B1 and 246-B2, respectively; Table 1) using pBS246 (Gibco®; Invitrogen) as template. Floxed marker gene plasmids were constructed as depicted in Supplementary Figure S2, and each marker gene was subcloned into pENTR loxP at the *NotI* sites,

Table 1. Oligonucleotides

Name	Sequence	Use
MLS-A	5'-GTTTAAACGGCGCGCCTAGGGATAACAGGGTAATATTAAATTTAATTAAACGT-3'	Annealed to MLS-B to yield the MLS fragment ^a
MLS-B	5'-TTAATTAAATTTAAATATTACCCTGTTATCCCTAGGCGCGCCGTTTAAACACGT-3'	Annealed to MLS-A to yield the MLS fragment ^a
246-B1	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGGCCGCACGTCTAAGAAACCATT-3'	Forward primer with <i>attB1</i> sequence for construction of pENTR loxP ^b
246-B2	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTAAGCGGCCGCAATGGCCAGTACTA-3'	Reverse primer with <i>attB2</i> sequence for construction of pENTR loxP ^b
L4-1	5'-GGGGACAACCTTTGTATAGAAAAGTTGGTTGCTTGGATCAGCAAGCATTAT-3'	Forward primer with <i>attB4</i> sequence for 5' arm of <i>LIG4</i>
L4-2	5'-GGGGACTGCTTTTTTGTACAACTTGTCCCTGTCAGACTACCCATAGCA-3'	Reverse primer with <i>attB1</i> sequence for 5' arm of <i>LIG4</i>
L4-3	5'-GGGGACAGCTTTCTTGTACAAAGTGGCAGAGATCGTACCCAGTGATATGT-3'	Forward primer with <i>attB2</i> sequence for 3' arm of <i>LIG4</i>
L4-4	5'-GGGGACAACCTTTGTATAATAAAGTTGGCACACATAGTATCGCATGGATCA-3'	Reverse primer with <i>attB3</i> sequence for 3' arm of <i>LIG4</i> ^c
L4-N	5'-TAGCATAGCACTTAATCACCTTCC-3'	Primer for screening for <i>LIG4</i> targeting events
Universal primer A	5'-AATAATGGTTTCTTAGACGTGCG-3'	Universal primer for screening targeting events ^{c,d}
Universal primer B	5'-AGGTTCACTAGTACTGGCCATTG-3'	Universal primer for screening targeting events ^{c,d}
L4-F	5'-CACTGGAACCTCATGGAGATGCTGG-3'	Forward primer in <i>LIG4</i> for confirmation of <i>LIG4</i> knockout cells ^c
L4-R	5'-AGAACCTTCAGTAGGAGAAGCACC-3'	Reverse primer in <i>LIG4</i> for confirmation of <i>LIG4</i> knockout cells ^c

MLS, multiple-linearization site.
^aSee Supplementary Figure S1.
^bSee Supplementary Figure S2.
^cSee Figure 2.
^dSee Figure 1.

yielding pENTR lox-Hyg, pENTR lox-Puro, and pENTR lox-His.

To generate targeting vectors for the human DNA ligase IV gene (*LIG4*), 2.2- and 2.0-kb *LIG4* genomic fragments were obtained by PCR with ExTaq™ DNA polymerase (Takara Bio, Otsu, Japan) using Nalm-6 genomic DNA as template and were used as 5' and 3' arms, respectively. The primers used were L4-1 and L4-2 for the 5' arm and L4-3 and L4-4 for the 3' arm (see Table 1). By using the MultiSite Gateway system (more specifically, pENTR lox-Hyg, pENTR lox-Puro, and pDEST DTA-MLS), a floxed *Hyg^r* or *Puro^r* gene was inserted between the 5' and 3' arms on a plasmid carrying a DT-A gene, thus yielding targeting vectors pLIG4-Hyg and pLIG4-Puro.

The targeting construct and knockout strategies for the *KU80* gene have been described earlier (9).

Cell Culture and Transfection

Nalm-6 cells were maintained in ES medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% calf serum (Hyclone, Logan, UT, USA) and 50 μM 2-mercaptoethanol at 37°C in a humidified atmosphere of 5% CO₂ in air. DNA transfection was performed as described previously (6,7,10). Briefly, 4 × 10⁶ cells were electroporated with 4 μg linearized targeting vector in a 40-μL chamber of Electro Gene Transfer Equipment (GTE-1; Shimadzu, Kyoto, Japan). After a 22- to 24-h incubation, the cells were then incubated for

2–3 weeks at 37°C in agarose medium containing 0.4 mg/mL hygromycin B (Wako Pure Chemical, Osaka, Japan) or 0.5 μg/mL puromycin (Wako). Genomic DNA was isolated from drug-resistant colonies and subjected to PCR and Southern blot analyses.

PCR Screening for *LIG4* Gene Targeting

The targeting vector pLIG4-Puro was introduced into Nalm-6 cells, and puromycin-resistant clones were subjected to PCR analysis using a *LIG4*-specific primer, L4-N, and the universal primer A, which corresponds to nucleotide positions 26–4 of pBS246 (Table 1). Subsequently, pLIG4-Hyg was introduced into *LIG4*^{+/–} cells,

Figure 1. Novel method to construct gene-targeting vectors. The method is based using the MultiSite Gateway system and consists of three steps: (1) genomic PCR with *attB*-containing primers; (2) BP recombination of each arm fragment; and (3) LR recombination of four DNA fragments. Because neither ligation reaction nor restriction mapping is necessary at all, one can make desired targeting vectors within 5–10 days after primers' arrival. Triangles represent *loxP* sequences. *Puro^r*, puromycin-resistance gene; *His^r*, histidinol-resistance gene; *Hyg^r*, hygromycin-resistance gene; *Km^r*, kanamycin-resistance gene; *Amp^r*, ampicillin-resistance gene; *DT-A*, a gene that codes for a diphtheria toxin A fragment.

and hygromycin-resistant clones were subjected to PCR analysis using the L4-N primer and the universal primer B, which corresponds to nucleotide positions 224–246 of pBS246 (Table 1). Two additional primers, L4-F and L4-R, which fail to anneal to the disrupted *LIG4* locus, were also used for PCR analysis (Table 1). To verify the correct targeting event, PCR-positive clones were subjected to Southern and Western blot analyses (6,9).

RESULTS AND DISCUSSION

Quick and Simple Method to Construct Targeting Vectors

As illustrated in Figure 1, the vector construction method is based on the MultiSite Gateway technology, which is commercially available from Invitrogen (MultiSite Gateway Three-Fragment Vector Construction kit). This system allows for one-step assembly of four DNA fragments (the backbone of a destination vector and three insert fragments subcloned

as entry clones), by virtue of three independent site-specific recombination (LR recombination) reactions at one time. In order to make the most of this system for targeting-vector construction, we made two major improvements. First, we inserted a DT-A gene cassette and an MLS sequence into the destination vector pDEST R4-R3, yielding the plasmid pDEST DTA-MLS (see Supplementary Figure S1). The DT-A gene, which codes for a diphtheria toxin A fragment, serves as a negative selectable marker that allows for selection against unwanted random integrants. The MLS sequence facilitates linearization of targeting vectors, as it contains the 18-bp *I-SceI* endonuclease cleavage site and restriction sites for four 8-base cutters (*PacI*, *SwaI*, *AscI*, and *PmeI*). Second, we prepared a series of floxed positive selectable markers (*Hyg^r*, *Puro^r*, and *His^r*), each being subcloned into the donor vector pDONR 221. Accordingly, these marker fragments are all ready to use as entry clones (Supplementary Figure S2). The use of floxed positive selectable markers is of particular importance

when one attempts to generate double- or triple-mutant cells, as a floxed region can easily be removed from the genome by transient expression of Cre recombinase in the cell.

With the plasmid pDEST DTA-MLS and the entry clones for positive selectable markers, the remaining steps for vector construction are: (i) isolation of genomic DNA fragments for 5' and 3' arms; (ii) subcloning of these arm fragments using BP recombination reactions to create 5' and 3' entry clones; and (iii) one-step assembly of four DNA fragments, namely LR recombination reactions between the two entry clones, an entry clone for *Hyg^r*, *Puro^r*, or *His^r*, and the destination vector pDEST DTA-MLS (see Figure 1). The 5' arm should be PCR-amplified using a forward primer starting with an *attB4* sequence and a reverse primer with an *attB1* sequence, and the amplified PCR fragment must be subcloned into the donor vector pDONR P4-P1R. (It should be noted that in the MultiSite Gateway system, each arm fragment must be subcloned as an entry clone that contains an *attL*

Figure 2. Targeted disruption of the human *LIG4* gene. (A) *LIG4* disruption in Nalm-6 cells. The human *LIG4* gene is composed of three exons, located on chromosome 13q33.3 (genome.ucsc.edu). Arrows A–E stand for PCR primers: A, universal primer A; B, universal primer B; C, L4-N; D, L4-F; and E, L4-R. (B) PCR analysis. +/+, wild-type; +Puro, *LIG4*^{+/-}; Hyg/Puro, *LIG4*^{+/-}; -/-, *LIG4*^{-/-} (where selectable markers have been excised). The primers used are shown in panel A. (C) Southern blot analysis of *EcoRV*-digested genomic DNA. The probe used is shown in panel A. (D) Western blot analysis for LIG4 protein. Samples used contained 20 μ g total protein extracted from wild-type (+/+) and *LIG4*^{-/-} (-/-) cells. Actin served as a loading control. *Puro*^r, puromycin-resistance gene; *Hyg*^r, hygromycin-resistance gene; DT-A, a gene that codes for a diphtheria toxin A fragment.

or *attR* sequence, which is a prerequisite for LR recombination.) Likewise, the 3' arm should be amplified using a forward primer with an *attB2* sequence and a reverse primer with an *attB3* sequence, and the amplified fragment must be subcloned into pDONR P2R-P3. (In general, amplification of highly GC-rich regions should be avoided in these genomic PCRs, and PCR primers should not involve repetitive sequences such as Alu.) These processes, as well as the subsequent LR recombination reactions, can be quite easily performed according to the manufacturer's instructions. Additionally, owing to the absence of any ligation step and the presence of the MLS sequence in the vector backbone, it is unnecessary to search for appropriate restriction sites for vector construction/linearization. Moreover, as the BP and LR recombination reactions, unlike conventional ligation reactions, are highly efficient and specific, checking for approximately 10 *Escherichia coli* transformants is sufficient to obtain correctly recombined plasmids in each step. Hence, with four primers harboring an appropriate *attB* sequence (i.e., two

primers for each arm), one can create targeting vectors with different positive selectable markers within 5–10 days (see Figures 1 and 3). In fact, we have constructed targeting vectors for more than 10 genes, within 1 week after primers' arrival.

Considerable efforts have been made in recent years to simplify targeting-vector construction. For example, Kohli et al. (11) developed a facile method for generating recombinant adeno-associated virus-based targeting vectors using fusion PCR. Zhang et al. reported a simplified strategy for vector construction with the use of *E. coli* recombination cloning (12). Cottade-Almeida et al. (13) also utilized *E. coli* recombination cloning to generate mouse gene-targeting vectors. It should be noted, however, that these strategies still involve genomic library construction/screening, virus packaging, extensive restriction mapping, or DNA ligations. Gasperowicz et al. (14) recently reported a long-range PCR-based method that circumvented the need for unique restriction sites; however, this strategy still involved multiple DNA ligation steps. We would

like to emphasize, therefore, that our strategy described above is simpler and quicker than any other methods reported thus far and involves none of their tedious or inefficient processes (i.e., library construction/screening, virus packaging, or searching for restriction sites for vector construction/linearization).

High-Efficiency Gene Targeting in a Human Cell Line

To evaluate the usefulness of above-constructed targeting vectors for actual gene-targeting experiments, we used the Nalm-6 cell line to examine the efficiency of gene targeting. We found that the gene-targeting efficiencies at the *LIG4* (Figure 2) and *KU80* (9) loci were 3.4% and 8.3%, respectively. These results, together with previous reports by us and others (5–8), indicate that targeting vectors harboring positive and negative selectable markers are sufficient to disrupt virtually any genomic locus in Nalm-6 cells within 1 month. Previous work has suggested that the length of homologous DNA sequences (i.e., 5' and 3' arms) of the

Figure 3. Strategy to generate human knockout cell lines. The strategy involves 4 steps: (Step 1) targeting-vector construction (see Figure 1); (Step 2) first gene targeting with a targeting vector; (Step 3) second gene targeting with another targeting vector; and (Step 4) Cre-mediated excision of drug-selectable markers. Step 4 is only required when additional gene(s) will be disrupted.

targeting vector is a major determinant of gene-targeting efficiencies (15–19), although recent work by Lu et al. (20) argues against this notion. Notably, in Nalm-6 cells, high-efficiency gene targeting is feasible with vectors with relatively short arms. Because short-arm vectors are sufficient for Nalm-6 cell gene targeting, drug-resistant clones can be easily checked by genomic PCR, making screening processes simple and rapid. The PCR screening can be performed using a specific primer outside of a homologous arm region together with either of the two universal primers outside of the floxed markers (Figure 1). PCR-positive clones should then be subjected to Southern blot analysis to verify the correct targeting event.

In general, unlike the situation in animals, where homozygosity is achieved by breeding, both alleles must be disrupted to cause a loss-of-function phenotype for the study of

autosomal genes in a cell. Thus, we performed targeted gene disruption on the second allele of the *LIG4* gene (Figure 2), which allowed us to obtain homozygous mutant cells with the gene-targeting efficiency of 0.85%. We therefore conclude that with the use of our system described here, homozygous knockout clones lacking a human gene of interest can be created within 2 to 3 months. In order to generate double mutant cells deficient for an additional gene(s), positive selectable markers (*Hyg^r* and *Puro^r*) must be removed from the *LIG4*-null (*LIG4^{Hyg/Puro}*) cells. As we reported previously (6), marker excision was easily performed with high efficiency (approximately 20%) by transient expression of Cre recombinase, and the removal of both markers was verified by drug sensitivity as well as PCR and Southern blot analyses (data not shown). The outline of our strategy to generate human knockout cells is illustrated in Figure 3.

We have established a quick and simplified method for targeting vector construction. This method is readily applicable to gene-knockout experiments in ES cells as well as in virtually all sequenced organisms. We have also shown that Nalm-6 is highly proficient for gene-targeting experiments with thus created targeting vectors. Therefore, our system greatly facilitates gene-knockout experiments in human cells. The usefulness of Nalm-6 in gene targeting is quite intriguing and must be emphasized. Indeed, B-lymphocytes are reliable sources for analyzing gene functions via gene targeting, as exemplified by recent studies with the avian DT40 cell line. Additionally and more importantly, Nalm-6 is a human cell line that is karyotypically stable and expresses normal p53 (21). Hence, the results presented here underscore the usefulness of Nalm-6 in the postgenome era for reverse genetic studies of human genes.

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COMPETING INTEREST STATEMENT

The authors declare no competing interests.

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