High-efficiency Scarless Genetic Modification in *Escherichia coli* Using Lambda-Red Recombination and I-SceI Cleavage

Method for Bacterial Genetic Modification

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ABSTRACT

Genetic modifications of bacterial chromosomes are important for both fundamental and applied research. Here, we developed an efficient, easy-to-use system for genetic modification of the *Escherichia coli* chromosome. The system uses two plasmids, lambda (λ)-Red recombination and I-SceI cleavage. An intermediate strain is generated by integration of a resistance marker gene(s) and I-SceI recognition sites in or near the target gene locus using λ-Red PCR targeting. The intermediate strain is transformed with a donor plasmid carrying the target gene fragment with the desired modification flanked by I-SceI recognition sites, together with a bifunctional helper plasmid for λ-Red recombination and I-SceI endonuclease. I-SceI cleavage of the chromosome and donor plasmid allows λ-Red recombination between chromosomal breaks and linear double strand DNA from the donor plasmid. Genetic modifications are introduced into the chromosome and the placement of the I-SceI sites determines the nature of the recombination and the modification. This method was successfully used for *cadA* knock-out, *gdhA* knock-in, *pepD* seamless deletion, site-directed mutagenesis of the essential *metK* gene, and replacement of *metK* with *Rickettsia* S-adenosylmethionine transporter gene. This effective method can be used with both essential and nonessential genes modification, and will benefit basic and applied genetic research.

**Key words:** I-SceI endonuclease; lambda Red recombination; genetic modification; markerless; *Escherichia coli*
INTRODUCTION

Lambda (λ)-Red recombination, which triggers recombination between a bacterial chromosome and foreign, linear double-stranded DNA (dsDNA), is a common technique in *Escherichia coli* for genetic modification (1-4). The λ-Red recombination method relies on three proteins: 5′→3′ exonuclease exo, the single-stranded DNA (ssDNA)-binding protein beta, and the degradation inhibiting protein gam (4). The λ-Red recombination replaces a specific chromosomal sequence with a selectable antibiotic resistance gene flanked by homologous arms. Homologous recombination is efficient for linear DNA with homologous arms of approximate 40 or more bp (2, 3). For markerless genetic modification, the antibiotic resistance cassette integrated into the chromosome must be removed. Common methods for markerless genetic modification are based on the Cre-loxP or Flp-FRT systems (3, 5). These methods leave a single loxP or FRT scar sequence at the chromosomal locus, which can have undesirable consequences such as native-locus gene replacement or mutagenesis. If a second modification site is close to a former site, recombination can occur between the loxP or FRT sequences.

Counterselection is effective for generating genetic modifications without leaving selectable markers or scars. Published protocols are usually based on two successive rounds of recombination: (1) integration of a positively selectable marker (e.g., an antibiotic resistance gene), and (2) selection for marker loss (counterselection). Counterselectable markers include *Bacillus subtilis* SacB (6), *E. coli* galactokinase (galK) (7) and thymidylate synthase A (thyA) (8).
9), streptomycin resistance (10) and the fusaric acid sensitivity system (11). These methods have several disadvantages including: (1) some counterselectable markers, such as SacB, must be combined with a positively selectable marker, which increases cassette length; and (2) some counterselectable markers as galK or thyA can be used only in host strains with a particular auxotrophy.

The yeast *Saccharomyces cerevisiae* endonuclease I-SceI is a novel tool for unmarked chromosomal manipulation. I-SceI is an intron-encoded endonuclease with an unusually long recognition sequence of 18 bp (12, 13). Bacterial genomes usually do not have natural I-SceI recognition sites. If an I-SceI recognition site is introduced into a bacterial genome, the expression of I-SceI endonuclease induces lethal double-strand breaks (DSBs) within the genome, inhibiting DNA propagation and cell growth. Several methods for site-directed mutagenesis of BACs or bacterial genomes that use I-SceI expression are reported (14-17). These methods are based on two successive rounds of recombination, similar to other counterselection systems. The approaches can be categorized into two types (18). The first type requires integration of a duplicated sequence with the I-SceI recognition site sequences in a first round of recombination. The duplicated sequence is as a substrate for recombination in the second round (16, 17, 19). This type of approach is efficient, but requires complex design and construction. The second type of approach includes two rounds of separated recombination. Blank, et al. reported a method for scarless mutagenesis of the *Salmonella enterica* chromosome (20). Resistance marker gene and the I-SceI recognition site(s) are integrated into genome, and then dsDNA fragment with modification replaces the resistance marker gene. Kuhlman and Cox reported another method that integrates large fragments into
the *E. coli* genome (14, 15). Resistance gene flanked by I-SceI recognition sites and 25bp exogenous sequences for “landing pads” are inserted into the chromosome. Then a fragment carrying insertion is excised by I-SceI from a donor plasmid and incorporated into the genome via recombination at the landing pads (14, 15).

Another technique, “Gene Gorging”, also uses two plasmids, λ-Red recombination and I-SceI cleavage. I-SceI cleavage of the donor plasmid creates a linear dsDNA substrate, which would replace the wild type chromosomal allele. “Gene Gorging” frequencies for precise mutations in the *E. coli* genome are not sufficiently high, only 1-15%, limiting its use (21).

In this study, a new, two-plasmid method for unmarked genetic modification of *E. coli* was established. The method uses λ-Red recombination and I-SceI cleavage. *E. coli* endogenous sequences were used as substrates for recombination and no scar was introduced into genome. The new method was successfully used for gene deletion and insertion, seamless gene deletion, site-directed mutagenesis, and gene replacement. The new method could be used to modify both nonessential and essential genes.

**MATERIALS AND METHODS**

**Strains, plasmids and growth conditions**

Strains and plasmids used in this study are in Table 1. *E. coli* strains MG1655 and BL21 (DE3) were used for genetic modification experiments; strain DH5 was used for recombinant DNA manipulations. All *E. coli* strains were cultured in LB (Luria-Bertani) medium [1% tryptone (Oxoid or Angel Yeast), 0.5% yeast extract (Oxoid or Angel Yeast), 1% NaCl] supplemented
with 100 mg/L ampicillin, 50 mg/L kanamycin, 100 mg/L spectinomycin, 100 mg/L apramycin or
1 mmol/L S-adenosylmethionine (SAM), as required.

Oligonucleotides used are in Table S1. PCR was performed using Taq (Fermentas), KOD-plus-neo or KOD-FX (Toyobo) polymerase. I-SceI endonuclease and restriction enzymes were purchased from Takara or Fermentas.

**Modular plasmids for donor and templates**

A 1.5 kb NotI fragment containing the kanamycin-resistance gene kanMX from pUG6 (22) was cloned into pBluescript II KS (-) (23) yielding pKS-K. A 1.7 kb fragment containing the kanMX gene was amplified from pKS-K using primer pairs T3_IsceI/T7_IsceI and digested with BssHII. The resulting fragment was used to replace a 0.2 kb BssHII region containing the multiple cloning sites (MCS) of pBluescript II KS (-). The resulting ampicillin-resistance and kanamycin-resistance plasmids were pKSKI-1 and pKSKI-2 and differed in insert direction. pKSKI-2 was digested with NotI to excise kanMX and the plasmid backbone was recircularized with T4 DNA ligase, yielding pKSI-1.

Apramycin-resistance or spectinomycin-resistance genes on 1.5 kb fragments were amplified from pIJ773 and pIJ778 (24), and T-A cloned into pMD18-T simple (Takara) for plasmids pMDSI and pMDSI.

**Bifunctional helper plasmids**

The rhaB promoter fragment and the I-SceI encoding region fragment from pUC19RP12 (19) were joined by overlapping PCR and cloned into pKD46 (3) at the Ncol site for plasmid pREDIA, which was similar to previous reported pREDI plasmids (16) with some differences in
the I-SceI-coding region.

The ampicillin-resistance gene of helper plasmids was replaced with the kanamycin-resistance gene from pPIC3.5K (Invitrogen). Bifunctional helper plasmids pREDTAI and pREDTKI with the trc promoter were constructed using the process used for the rhaB promoter. The trc promoter was amplified from pTRC99a (25). (See Supplementary Material for detail process of plasmid construction, available online)

**Modifications in nonessential genes**

A linear fragment with resistance genes flanked by I-SceI recognition sites was introduced by electroporation into MG1655 using conventional Red-mediated recombination (3) to obtain an intermediate strain. A bifunctional helper plasmid or the Red helper plasmid pKD46 were used for this step. Transformants were tested by colony PCR to confirm integration of the resistance gene and I-SceI recognition sites. After transformation, the bifunctional helper plasmid remained for further recombination and the conventional Red helper plasmid pKD46 was cured by 42°C growth (3).

Donor plasmid based on the pKSI-1 vector or conventional T-vectors (Takara) harboring modifications were constructed by traditional restriction cloning, overlapping PCR, or Gibson assembly (26). (See Supplementary Material for detail process of donor plasmid construction, available online) Donor plasmids were transformed into intermediate strains; or donor plasmids and bifunctional helper plasmids were co-transformed into helper-plasmid-free intermediate strains.

Resulting colonies were incubated in LB medium with 0.5% glucose and 50 μg/mL...
ampicillin and kanamycin (selection for both the donor and the helper plasmid) for 6–8 hours or overnight; 40 µl seed culture was used to inoculate a test tube with 4 mL LB with 10 mmol/L L-arabinose and 50 µg/mL ampicillin or kanamycin (selection for the helper plasmid only). After 30 °C and 200 rpm for ~2 h, L-rhamnose or IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to 20 mmol/L with cultivation at 30 °C for 6–8 hours or overnight. From this culture, 40 µl were used to inoculate 4 mL LB medium with 20 mmol/L L-rhamnose or IPTG, 10 mmol/L L-arabinose, and 50 µg/mL ampicillin or kanamycin (selection for the helper plasmid only), which was cultivated at 30 °C for 6–8 hours or overnight. Cells were harvested, suspended in sterile water, diluted (10⁻¹ to 10⁻⁴) and spread on LB plates with 20 mmol/L L-rhamnose or IPTG, 10 mmol/L L-arabinose and 50 µg/mL ampicillin or kanamycin (selection for the helper plasmid only). Controls were LB plates with apramycin or spectinomycin, L-rhamnose or IPTG, L-arabinose, and ampicillin or kanamycin spread at the same time. (Alternatively, after the first ~2 h L-arabinose inducing Red enzymes, arabinose-free medium with only L-rhamnose or IPTG for inducing I-SceI and antibiotic for maintaining helper plasmid could be used, to avoid the potential deleterious effects caused by the prolonged expression of the lambda Red functions; (27) but a slightly decreased efficiency would be obtained. ) After overnight at 30 °C, colonies were analyzed for apramycin or spectinomycin sensitivity and final clones were confirmed by colony PCR. (See Supplementary Material for a step-by-step protocol, available online)

**Modifications in metK**

Using Red-mediated recombination(3), an apramycin resistance gene and I-SceI
recognition sites (IsceI-aprR-IsceI cassette) was inserted into the yqgC site, and a spectinomycin resistance gene and I-SceI recognition sites (IsceI-spcR-IsceI cassette) was inserted into galP site, by successive electroporation steps for strain A10. yqgC and galP are nonessential genes next to the essential metK gene.

A 2.5 kb PCR fragment containing part of the speA gene, full-length yqgB-yqgC-metK genes and part of the galP gene was cloned into pKSI-1 for pKSI-metK. Site-directed mutagenesis of metK followed the protocol in QuikChange Site-Directed Mutagenesis kits (Agilent) to obtain the donor plasmid pKSI-metKmut (28). A 1 kb fragment with the gene for the SAM transporter (29, 30) was synthesized and replaced the metK coding region in pKSI-metK, yielding pKSImetKSamT.

Mutagenesis or replacement of metK was the same as for the nonessential genes, with 1 mmol/L SAM added to LB medium if required. For replacing metK gene with the Rickettsia SAM transporter gene, clones were also analyzed for SAM auxotrophy.
RESULTS
Scarless modification method strategy

This method included a conventional initial step and a final recombination step (Figure 1AB). In the initial step, a resistance gene flanked by I-SceI recognition sites was inserted into the chromosome by conventional λ-Red PCR targeting (Figure 1A); meanwhile, a fragment carrying the desired modification was inserted into donor plasmid with I-SceI recognition sites. In the final recombination step, expression of I-SceI endonuclease induces DSBs within both the genome and the plasmid. The fragment with modification is excised from a donor plasmid, and then incorporated into the genome at the DSB locus via λ-Red recombination. (Figure 1B)

Three groups of plasmids (bifunctional helper plasmid, templates plasmid and modular donor plasmid) were constructed (Table 1, Figure 2) for performing this method. The plasmids have been distributed to Addgene (http://www.addgene.org/) with IDs 51625~51628, 51652~51655 and 51725.

The method could be initiated by transformation of a host strain with a bifunctional helper plasmid (Figure 1A), which were constructed from the λ-Red helper plasmid pKD46 (3). The λ-Red genes gam, bet and exo were driven by the araBAD promoter and inducible with L-arabinose (Figure 2A). These genes mediated recombination between target regions and homologous arms. Plasmids with the I-SceI gene under the control of the trc or rhaB promoter were inducible with IPTG or L-rhamnose. The temperature sensitivity of the pSC101 replication origin maintained plasmids at low copy number and for easy curing by growth at
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42°C. Two versions of the helper plasmids were differentiated by use of either the kanamycin or ampicillin resistance markers. This design made the plasmids compatible with different donor plasmids (Figure 2A).

Plasmids pMDIAI and pMDISI were used as PCR templates to amplify apramycin-resistance or spectinomycin-resistance genes flanked by I-SceI recognition sites. (Figure 2C). The small construct sizes allowed simple and reliable integration of resistance marker genes into any location using established λ-Red PCR-targeting recombination methods. (3) (Figure 1A).

To facilitate donor plasmid construction, several modular vectors were constructed based on the high-copy vector pBluescript II KS (-) (23). Plasmid pKSI-1 consisted of a pBluescript II KS (-) backbone, an MCS, and two I-SceI recognition sites (Figure 2B). The MCS could be used for further subcloning steps. Plasmids pKSKI-1 and pKSKI-2 were similar, with a kanamycin-resistance gene kanMX in the MCS. Alternatively, donor plasmids could be constructed with common high-copy T-vectors such as pMD19-T simple and pBackZero-T (Takara). In this case, I-SceI recognition sites were introduced by a PCR step using longer primers. Donor plasmids based on pKSI-1 or common T-vectors showed high recombination frequencies (Table 2).

Markerless knockout and knock-in of selected genes

Knock-out of cadA

An apramycin-resistance gene cassette flanked by I-SceI recognition sites was integrated into the cadA locus by λ-Red PCR targeting to obtain an intermediate strain. Positive clones
were screened by PCR (Figure 3A). PCR products were digested with I-SceI, to check for functionality of the inserted I-SceI restriction sites (data not shown).

To construct a donor plasmid, truncated cadA segments with I-SceI recognition sites was cloned into a pBackZero-T vector. The donor plasmid was transformed into the intermediate strain for the final recombination step. In the recombination step, λ-Red enzymes, then I-SceI were induced, leading to cleavage of both the donor plasmid and the chromosome. Once DSBs were generated by I-SceI, recombination between the resulting donor fragment and the DSB chromosomal locus was mediated by λ-Red enzymes (Figure 1B).

After growth in IPTG or L-rhamnose to induce I-SceI expression with L-arabinose to induce expression of λ-Red enzymes, most surviving cells that were apramycin sensitive were confirmed as having the desired deletion. The deletion was confirmed by PCR, restriction endonuclease analysis and sequencing (Figure S2A).

Meanwhile, in the negative control experiments without donor plasmid, the frequency of apramycin sensitivity of the surviving cells was lower than 5% of the total, indicating the knock-out of cadA would occur by repair with the donor DNA, not by non-homologous end-joining of the double stranded breaks.

Knock-in of gdhA

Results for knock-in of the gdhA gene were similar to results for knocking out cadA. In the recombination step, I-SceI were induced, leading to cleavage in the chromosome at gntT gene locus and evict spectinomycin-resistance gene. Once DSBs were generated at gntT locus, recombination happened between the donor fragment with gdhA and the DSB chromosomal
locus, mediated by λ-Red enzymes. After recombination, the gdhA gene fragment replaced the spectinomycin-resistance gene. The resulting strain had the desired modification of gdhA integrated into the chromosome (Figure S2B).

*Multiple modifications*

We also inserted the gdhA gene into the chromosome of a cadA-disrupted E. coli strain. Similar modification efficiency was obtained (data not shown). The bifunctional helper plasmid for cadA disruption was retained, and directly used for the PCR targeting experiments to integrate spectinomycin-resistance gene into gntT locus. These results demonstrate the protocol could be used repeatedly, to make multiple modifications in a strain.

*Seamless deletion of pepD*

To determine if the new method could be used for seamless deletion of a selected gene, we deleted the pepD gene following a procedure similar to the process described above. An apramycin-resistance gene cassette flanked by I-SceI recognition sites was integrated into the pepD locus, replacing 600 bp in the pepD ORF (Figure 3A). To construct a donor plasmid, upstream and downstream fragments for the pepD gene were joined and subcloned into pKSI-I. The fusion fragment was suitable for seamless deletion of pepD (Figure 3B). The apramycin-resistance gene fragment and remaining 1.0 kb pepD fragments were replaced by “nothing” (Figure 3B). The 1.6 kb pepD ORF was seamlessly deleted from the ATG start codon to the TAA terminator codon (Figure 3C).
Modifications in the essential \textit{metK} gene

To use the new method to modify an essential gene locus, a novel strategy for an essential gene was designed. Two pairs of I-SceI sites with 2 different resistance genes were inserted into the loci next to the target gene by consecutive λ-Red PCR targeting. Meanwhile, donor plasmid with fragment carrying the desired modification was constructed as same process for the non essential genes. In the final recombination step, the wild-type target gene and the two resistance genes were removed from the chromosome. Then the fragment with modification is excised by I-SceI from a donor plasmid and incorporated into the genome at the gene locus via recombination. (Figure 4)

To determine if the new method could be used to modify an essential gene locus, we used the method to modify the \textit{metK} gene. \textit{E. coli} \textit{metK} is essential because it produces the only enzyme for synthesis of SAM, which is involved in many metabolic reactions, and \textit{E. coli} cannot take up SAM from medium(31). As an essential gene, \textit{metK} could not be directly deleted, but mutations can be made in its coding region. The \textit{metK} gene can be replaced by a \textit{Rickettsia} SAM transporter gene and the resulting cells take up extracellular SAM and survive with SAM in the medium (29, 30).

Site-directed mutations in \textit{metK}

The intermediate strain A10 was constructed with two resistance genes with 2 pairs of I-SceI sites inserted into the loci next to \textit{metK} (Figure 4A). A donor plasmid was constructed with mutagenesis fragments containing \textit{metK} sequences with three synonymous mutations.

In the recombination step, I-SceI cut at the 2 pairs of I-SceI sites next to \textit{metK}, and the
wild-type metK gene and the two resistance genes were removed from the chromosome. Subsequently, the mutation fragment from the donor plasmid replaced the wild-type fragment via homologous recombination, introducing the mutations into the metK ORF without scar fragments (Figure 4B). Mutations were confirmed by PCR, restriction endonuclease analysis and sequencing (Figure 4C).

*Replacement of metK with Rickettsia SAM transporter*

A donor plasmid containing the SAM transporter coding region, the metK promoter and the metK terminator was constructed. The plasmid was transformed into the A10 intermediate strain. Using the same procedure, the SAM transporter fragment was used to replace wild-type metK via homologous recombination (Figure 4B,C). The resulting strains had SAM auxotrophy and grew in medium supplemented with SAM (Figure S1).

*Curing plasmids*

Donor plasmids are efficiently cured by I-SceI cleavage (14). After recombination, at least half of colonies with the desired genotype were sensitive to ampicillin or kanamycin (Table 2), showing efficient curing of donor plasmids. For the colonies with the desired genotype and the donor plasmid remaining, prolonged culture in IPTG or L-rhamnose medium to induce I-SceI expression resulted in donor plasmid curing (data not shown). Helper plasmids can be cured by 42°C growth (3).
Method efficiency

Preparatory steps followed an established λ-Red PCR targeting method(3). Integration frequencies (colonies with expected PCR bands per tested spectinomycin/apramycin resistant colonies) were greater than 50%, depending on the size of the homologous regions and the targeted genomic locus (data not shown).

For recombination for modification and dominant marker recycling, IPTG induction of the trc promoter to stimulate I-SceI expression gave higher recombination frequencies and donor plasmid curing frequencies than rhaB promoter. (Table 2).
The scarless genetic modification strategy described here was based on a simple two-plasmid procedure using λ-Red recombination and endonuclease I-SceI cleavage. A fragment with I-SceI recognition sites was inserted into the target site using λ-Red recombination and positively selected by conventional λ-Red PCR targeting (2, 3). I-SceI cleaved both the donor plasmid and the chromosome. Incorporation of the insertion fragment was enhanced by the expression of λ-Red enzymes and led to integration of the donor fragment and scarless genetic modification (Figure 1). Generally, the strategy took about 1.5-2 weeks, with donor plasmid construction and λ-Red PCR targeting procedure performed simultaneously. We used this strategy in both *E. coli* K-12 strain MG1655 and the B strain BL21(DE3). This strategy should be applicable in any bacterial strains that can be modified with λ-Red or other PCR targeting recombination systems. As endonuclease I-SceI directly cleavage at the chromosome, this method would be applicable for host strains with any genotypes, including those with genotypes not suitable for published counterselectable markers.

Our method use two separated rounds of recombination, and this approach is also used by some similar previously reported methods (14, 15, 20). This approach avoids the integration of a duplicated sequence in a first recombination to be a substrate for the second recombination (16, 17) and gives flexibility in designing and performing experiments. As shown by our *pepD* seamless deletion, in the first PCR-targeting step, short arms homologous to
pepD ORF were used for the first recombination, deleting a pepD coding region of approximately 600 bp by replacement with a resistance marker gene and leaving pepD coding region of approximately 1 kb. Subsequently, fragments homologous to upstream and downstream regions of pepD were used for the second recombination, resulting in complete deletion of the truncated pepD segments and the resistance genes from the start codon to the terminator codon (Figure 3).

Our method shows some similarity to a method described previously by Kuhlman and Cox for integrating large fragments into the E. coli genome (14, 15). Kuhlman and Cox’s method requires integration of 25bp exogenous sequences into the genome to generate a “landing pad”, so it is not a seamless method. Our method uses the endogenous fragment for recombination in both initial and the final recombination steps. No redundant sequences are introduced into the genome, so seamless modification is possible.

Our method could be used for both nonessential and essential genes. Of the total 4288 E. coli genes, 3985 are nonessential and 303 genes are essential candidates including 37 with unknown function (1). To modify nonessential genes, I-SceI recognition sites and resistance gene could be inserted directly into the gene (Figure 1). For modifying essential genes, the I-SceI recognition site and resistance genes could be inserted upstream and downstream of the essential gene (Figure 4). In both cases, subsequent recombination was accurate as expected.

As previously reported, in vivo cleavage of donor plasmids by I-SceI expression has advantages over introduction of linear DNA fragments by direct transformation(14). Repair and editing mechanisms during chromosomal and plasmid replication result in high fidelity. The
higher transformation efficiencies of supercoiled plasmids facilitates the experiments. High plasmid copy numbers are important for maintaining a high concentration of intracellular fragments (14). The gene gorging method is reported to result in chromosomal modification frequencies as high as 1–15%, even without cutting chromosomes (21).

I-SceI expression must be considered. In our experiments, using the rhaB promoter for I-SceI expression resulted in about a half of the colonies with the resistant phenotype. PCR confirmed that no mutations occurred within or next to the resistance genes and the integration site (data not shown). The trc promoter gave a higher efficiency for fragment replacement and donor plasmid curing, probably because of differences in expression levels from the different promoters (32).

Our method could be a simple and easy way to move mutations from one strain into another. For example, generating a strain with a desired phenotype generated by random mutagenesis or evolution require introducing every mutation into a wild-type strain to analyze the effect on function (33). Such mutations can be identified by high-throughput genome sequencing and confirmed by PCR and dideoxy chain-termination sequencing. The pKSKI series could be made into T-vectors by simple digestion (34–36), so donor plasmids could be constructed by direct T-A cloning. A resistance gene cassette and I-SceI recognition sites could be integrated into the gene locus of the recipient strain by λ-Red PCR targeting. The fragment from the donor plasmid harboring the mutation could be integrated into the genome of recipient strains to replace the wild type allele.

Another advantage of our new method is that different modifications could be performed on a single intermediate strain. By using different donor plasmid, a target gene of interest could
be modified differently. Any methods could be used to construct the donor plasmid, even by artificial whole gene synthesis. The only requirement is inclusion of upstream and downstream homologous regions and I-SceI recognition sites. According to our experiments, homologous regions in 300–500bp length could benefit both plasmid construction and recombination efficiency. The designed sequence on the donor plasmid will be the exact sequence introduced in the final strains.

The system could potentially be improved. For making multiple modifications, a different set of resistance marker genes flanked by I-SceI recognition sites could be integrated successively into target sites. Donor fragments for each modification could be assembled together with I-SceI sites between the fragments. The assembled fragments could be cloned into a single donor plasmid. Upon I-SceI cleavage of the chromosome and donor plasmid, the linear dsDNA fragments from the donor plasmid could introduce a series of genetic modifications into the chromosome. Recent studies demonstrated that Cas/CRISPR RNA-guided targeting methods can be used to edit bacterial genomes (37). These methods appear to be more efficient than previously reported TALENs method (38). Two-plasmid recombination methods could be further modified by replacing I-SceI cleavage with Cas/CRISPR and combining two-step recombination into one step.

Our data demonstrated that the new method presented here are a useful tool for unmarked genetic modification of *E. coli*. This effective method can be used with both essential and nonessential genes modification, and will benefit basic and applied genetic research.
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Conflict of interest statement: None declared.

SUPPLEMENTAL MATERIAL

Supplementary Table S1, Supplementary Figures S1–S2, Supplementary Methods, Protocol.

REFERENCES


Figure Legends

Figure 1. Diagram of this two-plasmid method.

A: Antibiotic cassette fragment with I-SceI recognition sites integrated at a target via λ Red-mediated recombination creating an intermediate strain. B: Intermediate strain with mutation(s) and I-SceI recognition sites was transformed with donor plasmid. Expression of I-SceI was induced by L-rhamnose or IPTG (isopropyl β-D-1-thiogalactopyranoside). I-SceI recognition sites in donor plasmid and chromosome were cleaved. Integration of donor fragment at the cleaved site of chromosome was mediated by λ Red recombination.

Figure 2. Plasmids used for this method.

A: pREDTKI and pREDTAI (helper plasmids) with arabinose-inducible (araB promoter) λ-Red recombinase functions, IPTG-inducible (trc promoter) I-SceI expression. B: pKSI-1 (modular plasmids for donor) based on the high-copy vector pBluescript II KS (-), with multiple cloning site (MCS) and two I-SceI recognition sites. C: Part of plasmid pMDIAI (template plasmid) with an apramycin-resistance gene flanked by FRT (Flippase Recognition Target) sites and I-SceI recognition sites. For pMDISI, apramycin-resistance was replaced with spectinomycin-resistance genes. Pink arrow: binding sites for primers MDF and MDR.

Figure 3. Seamless pepD deletion and PCR analysis

A: 40 bp short arms homologous to pepD ORF; 600 bp pepD segment replaced by marker. B: Arms with upstream (U) and downstream (D) homology to pepD, with truncated 1.0 kb pepD segments and apramycin (apr) resistance genes seamlessly deleted from ATG to TAA. C: PCR of pepD. BL21(DE3) (wild-type pepD), 2.5 kb; intermediate strain (pepD::apr), 3.3 kb; final strain (ΔpepD), 0.9 kb.
Figure 4. Modifications to metK gene and PCR analysis.

A: IsceI-apr-IsceI cassette inserted into yqgC and IsceI-spc-IsceI cassette inserted into galP. YqgC and galP are nonessential genes next to metK. B: Wild-type metK and resistant genes replaced by mutated metK or SAM transporter. galP fragment and speA-yqgB-yqgC fragment were homologous arms. C: PCR analysis of metK. MG1655 (wild-type metK), 2.8 kb; intermediate strain A10 (yqgC::apr, galP::spc), 5.9 kb; final strain with mutated metK, 2.8 kb, the same length as wild-type metK; final strain with metK replaced by SAM transporter, 2.6 kb.
Marker pepD pepD::apr ΔpepD

A

B

C

Marker pepD pepD::apr ΔpepD

3.2 k
2.5 k
0.9 k
3.0 k
0.5 k
1.0 k
### Table 1: Strains and plasmids used in this study

<table>
<thead>
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<th>Strain or plasmid</th>
<th>Characteristics</th>
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<td><strong>Strains</strong></td>
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<td>pREDIA</td>
<td>ori SC101(ts), Amp&lt;sup&gt;R&lt;/sup&gt;, araBAD promoter for λ-Red, rha promoter for I-SceI</td>
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<tr>
<td>pREDKI</td>
<td>ori SC101(ts), Kan&lt;sup&gt;R&lt;/sup&gt;, araBAD promoter for λ-Red, rha promoter for I-SceI</td>
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<td>pREDTAI</td>
<td>ori SC101(ts), Amp&lt;sup&gt;R&lt;/sup&gt;, araBAD promoter for λ-Red, trc promoter for I-SceI</td>
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<td>pREDTKI</td>
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<td><strong>Template plasmids</strong></td>
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<tr>
<td>pMDIAI</td>
<td>Apr&lt;sup&gt;R&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;, Apramycin resistance gene flanked by FRT and I-SceI sites. pMD18-T simple backbone</td>
<td>This work</td>
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<td>pMDISI</td>
<td>Spc&lt;sup&gt;R&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;, Spectinomycin resistance gene flanked by FRT and I-SceI sites. pMD18-T simple backbone</td>
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<td><strong>Modular plasmids for donor</strong></td>
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<tr>
<td>pKSKI-1</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;, pBluescript II KS(-) backbone with This work I-SceI site - kanMX - I-SceI site, differed in insert direction with pKSKI-2</td>
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<td>Amp&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;, pBluescript II KS(-) backbone with This work I-SceI site - kanMX - I-SceI site, differed in insert direction with pKSKI-1</td>
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<tr>
<td>pKSI-1</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, pBluescript II KS(-) backbone with I-SceI This work site - multiple cloning sites - I-SceI site</td>
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<td><strong>Other plasmid</strong></td>
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<tr>
<td>pMD18-T simple</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, cloning vector</td>
<td>Takara</td>
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<td>pMD19-T simple</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, cloning vector</td>
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<td>pBackzero-T</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;, cloning vector</td>
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<td>pUG6</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt; vector</td>
<td>EUROSCARF, Guldener, et al., 1996</td>
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<td>Plasmid Name</td>
<td>Description</td>
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<td>pPIC3.5K</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, <em>P. pastoris</em> expression plasmid</td>
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<td>pBluecriptII KS(-)</td>
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<td>pKD46</td>
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<td>Datsenko and Wanner, 2000,</td>
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<td>pKD46K</td>
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<td>pMDcadA</td>
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<td>pMDcadAL</td>
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<td>pBackZero-ISceI-cadA</td>
<td>Donor plasmid for cadA deletion, pBackzero-T backbone</td>
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<td>pKS1pepD0.7k</td>
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<td>pMD19-gntT</td>
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<td>pMD19-gntT-spc</td>
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<td>pMD19-gntT-gdhA</td>
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<td>pKSImetK</td>
<td>metK fragment cloned into pKSI-1</td>
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<td>pKSImetKmut</td>
<td>Donor plasmid for site-directed mutagenesis of the metK</td>
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<td>pKSImetKSamT</td>
<td>Donor plasmid for replacing metK with SAM transporter</td>
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Amp<sup>R</sup>: Ampicillin resistance; Kan<sup>R</sup>: Kanamycin resistance, Apr<sup>R</sup>: Apramycin resistance; Spc<sup>R</sup>: Spectinomycin resistance
<table>
<thead>
<tr>
<th>Modification</th>
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<td>cadA deletion</td>
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<td>PepD seamless deletion</td>
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<td>PepD seamless deletion</td>
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<td>MetK mutation</td>
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Amp: Ampicillin; Apr: Apramycin; Spc: Spectinomycin.