# Two-step Red-mediated recombination for versatile high-efficiency markerless DNA manipulation in *Escherichia coli*

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*BioTechniques 40:191-197 (February 2006)*
doi 10.2144/000112096

*Red recombination using PCR-amplified selectable markers is a well-established technique for mutagenesis of large DNA molecules in* Escherichia coli. *The system has limited efficacy and versatility, however, for markerless modifications including point mutations, deletions, and particularly insertions of longer sequences. Here we describe a procedure that combines Red recombination and cleavage with the homing endonuclease I-*Sce*I to allow highly efficient, PCR-based DNA engineering without retention of unwanted foreign sequences. We applied the method to modification of bacterial artificial chromosome (BAC) constructs harboring an infectious herpesvirus clone to demonstrate the potential of the mutagenesis technique, which was used for the insertion of long sequences such as coding regions or promoters, introduction of point mutations, scarless deletions, and insertion of short sequences such as an epitope tag. The system proved to be highly reliable and efficient and can be adapted for a variety of different modifications of BAC clones, which are fundamental tools for applications as diverse as the generation of transgenic animals and the construction of gene therapy or vaccine vectors.*

## INTRODUCTION

Bacterial artificial chromosomes (BACs), mini-F plasmids into which DNA of up to 300 kb can be cloned (1), have made possible rapid and efficient mutagenesis of large sequences by techniques that were previously restricted to genetic manipulation of small plasmids in *Escherichia coli*. This advance has accelerated progress in genome sequencing projects, has revolutionized work with DNA viruses (2,3), and has facilitated the generation of transgenic animals through the use of modified clones of genomic BAC libraries (4).

Besides RecA-mediated mutagenesis, Red recombination or RecET cloning is one of the most commonly exploited techniques to engineer sequences in large DNA constructs (5,6). Substrates for Red mutagenesis are DNA double-strand breaks (DSB) wherein homologous recombination is focused close to the ends of the linear double-stranded DNA (dsDNA). Because replication of the substrate is not required, Red recombination allows utilization of PCR-amplified positive selection markers using primers bearing 40–50 bp extensions homologous to the target sequences (5,7–9).

To avoid long residual foreign sequences within modified constructs, *FRT* or *loxP* sites flanking selection markers have been used for excision of the positive selection markers with the corresponding recombinases Flp or Cre (6). The persistence of at least one copy of the *FRT* or *loxP* sites limits the repeatability of the procedure and may interfere with gene expression in some circumstances. Alternative systems developed for markerless mutagenesis of large DNA sequences in *E. coli* first insert a cassette consisting of both positive and negative selection markers or bifunctional markers, which allow both positive and negative selection by one gene product. Those cassettes are then replaced with the desired markerless (linear) DNA by a second recombination event and subsequent counterselection (6,10–13). One problem associated with negative selection markers is that they can be lost unspecifically such as by recombinations between repeat sequences, which are common in both mammalian and viral sequences (data not shown). Methods for markerless replacement without selection markers, such as the use of single oligonucleotides harboring modified sequences (14), the "gene gorging" technique (15), or the "hit-and-fix" protocol (16), lack a powerful procedure to screen for desired recombination events. To raise efficiencies, those methods employ PCR screening of pooled samples (17) or a preselection for recombination events with a marker targeting the *E. coli* chromosome as a second substrate (18).

The 18-bp recognition site of the homing endonuclease I-*Sce*I was used previously as the negative selection marker with an in vivo cleavage as the counterselection step (10). The technique presented here was refined such that I-*Sce*I is utilized not only for counterselection but also for generation of the substrate for a second Red recombination in *E. coli*. In this modified strategy, the positive selection markers that were used to introduce the desired target modification in a first step are removed in a second step by the combination of I-*Sce*I cleavage and intramolecular Red recombination utilizing a previously introduced sequence duplication. We show that this modification renders the method versatile and very efficient.

## MATERIALS AND METHODS

### Cells, Viruses, and BAC Clones

Equine herpesvirus type 1 (EHV-1) was propagated on rabbit kidney cells (RK13), maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Two parental BAC clones were used; both are derived from EHV-1 strain RacL11 in which gene 71 located in the unique-short region was replaced by the mini-F sequence pHA1 (resulting in pL11w) or pHA2 harboring an additional *gfp* gene within vector sequences (resulting in pL11g; also see Supplementary Figure S1 available online at www.BioTechniques.com) (19,20). Transfection of RK13 cells

with pL11w or pL11g DNA isolated from *E. coli* was used to reconstitute recombinant viruses vL11w or vL11 g (21).

### Bacteria and Plasmids

All plasmids were constructed and maintained in *E. coli* DH10B cells grown at 37°C in Luria Bertani (LB) medium. BAC maintenance and Red recombinations were performed in *E. coli* strain EL250 [which contains a λ prophage encoding the recombination enzymes Exo, Beta, and Gam (8)] harboring pL11w or pL11g.

To construct pEPkan-S (see Supplementary Figure S2), the kanamycin resistance gene *aphAI* derived from plasmid pACYC177 (New England Biolabs, Ipswich, MA, USA) was amplified by PCR (Supplementary Table S1), adding I-*Sce*I recognition sites and a sequence encoding the FLAG® epitope (Sigma, St. Louis, MO, USA). The PCR product was cut with *Bam*HI and *Eco*RV and cloned into the *Bgl*II and *Nru*I sites of pcDNA3 (Invitrogen, La Jolla, CA, USA).

The 1.6-kb *Eco*RV/*Pst*I fragment of plasmid pACBSR (15), harboring the *I-sceI* gene and a part of *araC*, was cloned into the *Eco*RV and *Pst*I sites of pBAD24 (ATCC, Manassas, VA, USA), resulting in pBAD-*I-sceI* (see Supplementary Figure S3).

To delete a *Pst*I site present in the mRFP1 encoding plasmid (22), pmRFP1 was digested with *Bgl*II and *Bam*HI before religation. The resulting plasmid without the multiple cloning site (MCS) was named pmRFP1/-M.

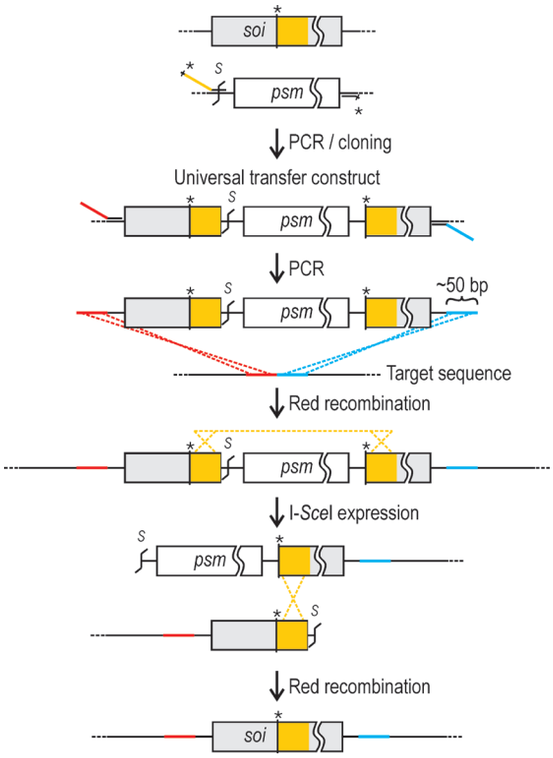


**Figure 1. Insertion of long sequence stretches using the developed mutagenesis protocol.** First, a universal transfer construct was generated. The transfer construct was amplified by PCR and integrated into the target site with a first Red recombination. The cleavage of the I-*Sce*I site in vivo and a subsequent second Red recombination resulted in the removal of the positive selection markers, leaving behind the sequences of interest. Lines or boxes of identical colors symbolize identical sequences. Colored dotted lines indicate single homologous recombination events. *soi*, sequence of interest; *psm*, positive selection marker; S, I-*Sce*I restriction site; asterisk, unique restriction site. For details on PCR constructs and primer design, see Supplementary Figure S4 and Supplementary Table S1.

### Primers

Primers that we used for the manipulations are listed in Supplementary Table S1. Oligonucleotides were custom-synthesized, and all primers longer than 50 bp were polyacrylamide gel electrophoresis (PAGE) purified (Integrated DNA Technologies, Coralville, IA, USA).

### Red Recombination

Red recombination was performed as described earlier (8). PCRs were performed with *Taq* DNA polymerase (New England Biolabs), *Dpn*I was added to digest the template, and the amplification product was purified from an agarose gel with the Perfectprep® Gel Cleanup Kit (Eppendorf, Westbury, NY, USA). *E. coli* strain EL250 harboring pL11w or pL11g was grown at 32°C in LB with 30 µg/mL chloramphenicol to an $A_{600}$ of 0.5 to 0.7. The EL250 culture was then transferred into a 42°C water bath shaker for 15 min at 220 rpm to induce the expression of the Red recombination system. Finally, bacteria were cooled on ice for 20 min, washed 3 times with ice-cold 10% glycerol in water, and resuspended in 1/100 of the original culture volume of 10% glycerol in water. Recombination-competent EL250 cells were either frozen in aliquots (50 µL) at -70°C or used fresh for electroporation using approximately 100 ng of PCR product (0.1 cm, 1.5 kV, 25 µF, 200 Ω). After electroporation, bacteria were shaken in 1 mL LB medium at 32°C for 1.5 to 2 h and finally spread on appropriate selective agar plates (see Supplementary Protocol).

## RESULTS AND DISCUSSION

### Markerless Introduction of Long Sequences

The major problem of markerless DNA modifications utilizing Red mutagenesis has been the absence of efficient protocols for the insertion of longer sequences. We began to optimize the developed mutagenesis procedure to allow for the efficient insertion of sequences of interest such as sequences encoding fluorescent proteins or promoters. Universal transfer constructs were generated that contained the sequences of interest with an inserted positive selection marker (*aphAI*, which confers kanamycin resistance), an adjoining I-*Sce*I site, and a duplication of a short sequence of the sequences of interest (Figure 1, Supplementary Video S1). The transfer constructs generated by PCR amplification of the I-*Sce*I-*aphAI* cassette with a forward primer contained an approximately 50 bp DNA duplication of nucleotides present in the sequences of interest. In addition, both the forward and reverse primers contained restriction sites to allow cloning of the PCR product in the correct orientation into a unique restriction site within the sequences of interest. The unique restriction site was chosen such that it is located immediately upstream of the approximately 50 bp sequence duplicated within the forward primer. Supplementary Video S1 summarizes the steps involved in

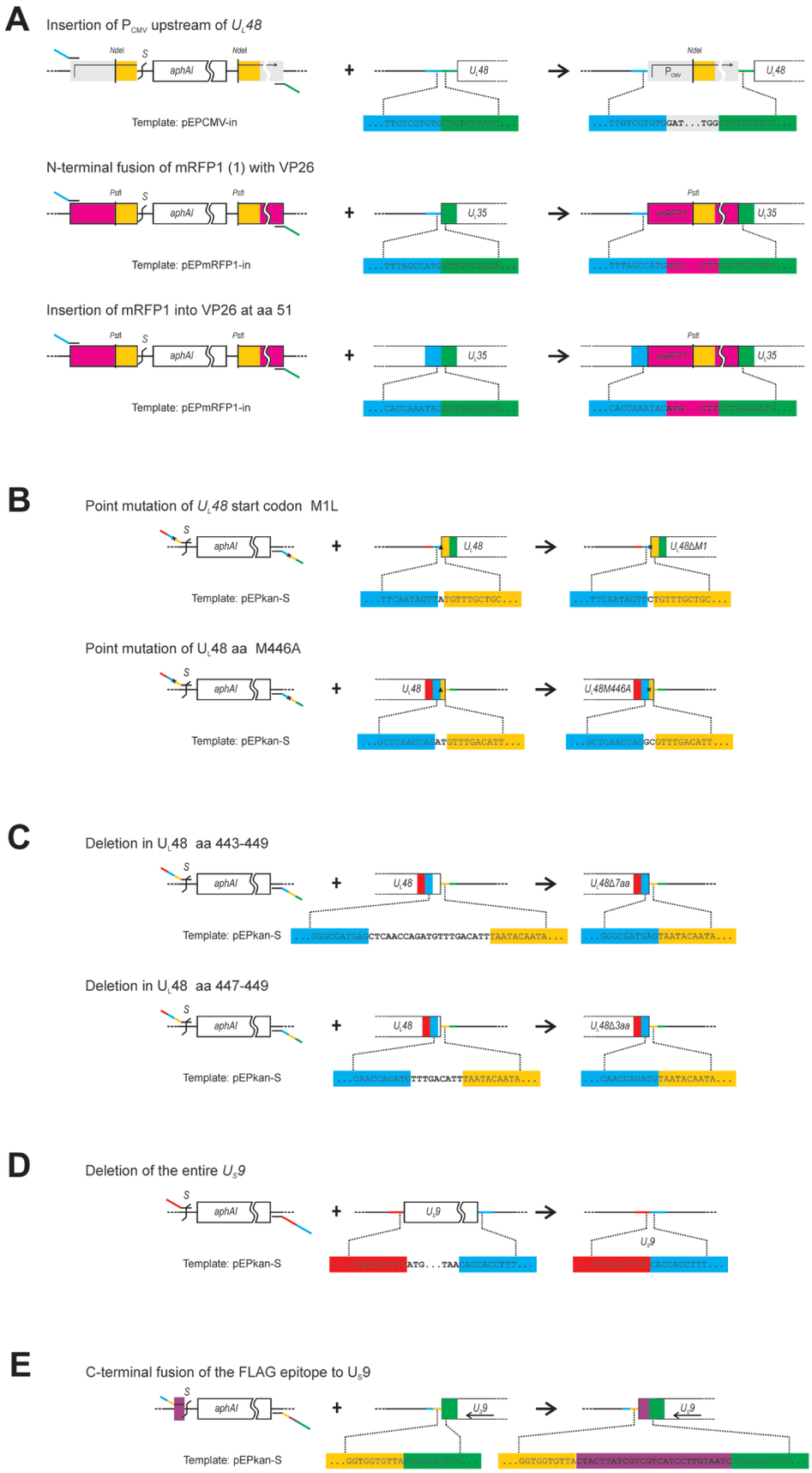


**Figure 2. Diagram of PCR constructs, targets, and derived mutants.** Given are presentations of (A and E) generated sequence insertions, (B) point mutants, and (C and D) deletion mutants using the developed method. The left column displays blueprints of the generated PCR products; middle column, target sequences; and right column, final mutants. Identical colors represent identical sequences. X, new sequence; triangle, old sequence, *S*, I-*Sce*I restriction site; aa, amino acid.

**Table 1. Efficiency of Two-Step Red-Mediated Recombination for Mutagenesis**

| | Specific Mutation in BAC Clones pL11w or pL11g | | Positive Colonies/Tested Colonies (%) First Recombination[a] | Positive Selection Marker Excision[b] | Second Recombination[c] | Overall Efficiency |
|---|---|---|---|---|---|---|
| Long Insertion | Insertion of $P_{CMV}$ upstream of *$U_L$48* | | 5/5 (100%) | 23/27 (85.2%) | 3/5 (60%) | 51% |
| | N-terminal fusion of the mRFP1 (22) with VP26 | | 1/1 (100%) | 16/16 (100%) | 6/6 (100%) | 100% |
| | Insertion of mRFP1 into VP26 at amino acid 51 | | 3/4 (75%) | 27/28 (96.4%) | 13/13 (100%) | 72% |
| Point Mutation | Point mutation of $U_L$48, M1L | without arabinose | 2/2 (100%) | 0/24 (0%) | N.A. | N.A. |
| | | with arabinose | | 21/24 (87.5%) | 7/7 (100%) | 87% |
| | Point mutation of $U_L$48, M446A | without arabinose | 10/10 (100%) | 0/24 (0%) | N.A. | N.A. |
| | | with arabinose | | 25/28 (89.3%) | 11/11 (100%) | 89% |
| Short Deletion | Deletion in $U_L$48, amino acids 443–449 | | 2/2 (100%) | 28/30 (93.3%) | 4/4 (100%) | 93% |
| | Deletion in $U_L$48, amino acids 447–449 | without arabinose | 18/18 (100%) | 0/24 (0%) | N.A. | N.A. |
| | | with arabinose | | 39/45 (86.7%) | 4/4 (100%) | 87% |
| Long Deletion | Deletion of the entire *$U_S$9* | | 3/5 (60%) | 24/60 (40%) | 4/4 (100%) | 24% |
| Short Insertion | C-terminal fusion of the FLAG epitope to *$U_S$9*-encoded protein | | 3/5 (60%) | 44/87 (50.6%) | 4/5 (80%) | 24% |

BAC, bacterial artificial chromosome; N.A., not applicable.
[a]Colony PCR or restriction fragment length polymorphism (RFLP) screening of $Amp^r$ and $Cm^r$ resistant colonies.
[b]Kanamycin-sensitive/total colonies.
[c]As determined by sequencing.

generating and using universal transfer constructs, and Supplementary Figure S4 provides details about the sequences of interest employed in this study.

The transfer plasmids were then used to insert the sequences of interest into desired target sequences present in the BAC. First, the universal transfer constructs were amplified by PCR with primers that each possessed approximately 50 bp of homology to the respective target sequence. The resulting linear DNA fragment was inserted into the target BAC clones by classical Red recombination using *E. coli* strain EL250 (8). Specifically, 100 ng of plasmid pBAD-*I-sceI* (for arabinose-inducible expression of I-*Sce*I) were electroporated together with the PCR product into the BAC-containing bacterial cells. Of the kanamycin (selection for *aphAI*)- and ampicillin (selection for pBAD-*I-sceI*)-resistant colonies, those in which successful integration of the PCR product into the BAC DNA had occurred were detected by colony PCR or restriction fragment length polymorphism (RFLP) analysis (Table 1, first column).

The next step of the protocol involved removal of the positive selection markers. Colonies were grown at 32°C for 2–4 h in 2 mL of LB medium with ampicillin and chloramphenicol to maintain the BAC. Two milliliters of LB containing 1% arabinose and antibiotics were added to the culture to induce the expression of I-*Sce*I from plasmid pBAD-*I-sceI*. Further incubation for 2 h at 32°C allowed cleavage at the I-*Sce*I site, thereby generating a DSB. Cultures were then transferred into a 42°C water bath shaker for 30 min to initiate a second Red recombination event between duplicated sequences (Figure 1, shown in yellow) now present within the modified BAC. Subsequently, bacteria were grown for another 2–4 h at 32°C, and serial dilutions were spread on agar plates containing chloramphenicol, ampicillin, and 1% arabinose. Replica plating was used to detect kanamycin-sensitive colonies (i.e., cells in which *aphAI* excision had occurred), which were examined by RFLP or colony PCR screening. Frequencies of positive clones are listed in the second column of Table 1;

diagrams of the constructs are shown in Figure 2; sample raw RFLP results are presented in Supplementary Figure S5. Finally, the affected loci of candidate positive mutants were PCR-amplified and sequenced to verify successful sequence modification in the BACs.

As summarized in the final column of Table 1, overall efficiencies for generating the desired long-insertion recombinants ranged from 72%–100%. Furthermore, when tested in RK13 cells, recombinant virus containing RFP inserted at amino acid 51 of VP16 displayed the expected phenotype and localization pattern as assessed by fluorescence microscopy (Supplementary Figure S6A).

### Markerless Generation of Point Mutants

To generate point mutations in BAC clones, cloning of transfer constructs was unnecessary to achieve the sequence duplications required for scarless mutagenesis. The I-*Sce*I-*aphAI* cassette from recombinant plasmid pEPkan-S (Supplementary Figure S2) was PCR-amplified with primers adding extensions of 60–80 bp to the cassette (Figure 3; Supplementary Video S2). The outer approximately 40 bp of primers and PCR products were homologous to the target locus in the BAC. Both primers also contained approximately 40 bp of modified target sequences, which were reverse complementary to each other and—depending on the type of modification—could also be a part of the outer approximately 40 bp (Figure 3). In the first Red recombination, the PCR product containing the positive selection marker flanked by appropriate sequence duplications and modifications was inserted into the locus of interest. In a second step, following the induction of cleavage at the I-*Sce*I site as described above, Red recombination between the duplicated sequences resulted in markerless point mutations (see Figure 3 and Supplementary Video S3 for a general overview and Figure 2 for sequence specifics). A negative control in which arabinose was not added revealed the absence of detectable Red recombination (Table 1), as expected. Western blot analysis of cells infected with recombinant virus was performed for two point mutants: $U_L48$ M446A and a mutant in which the first start codon was disrupted ($U_L48$ M1L). The blot showed the presence or absence, respectively, of bands corresponding to a protein of wild-type size (Supplementary Figure S6B).

### Markerless Deletion or Insertion of Short Sequences

If the duplications contained within the primers represent a noncontiguous sequence of the target DNA, the point mutations strategy can be adapted to allow markerless deletions. Deletion of shorter sequences appeared to be more efficient than long deletions; however, both procedures required minimal screening to derive positive clones (Table 1; see Figure 2 for explanatory diagrams). As before, the arabinose-negative control confirmed the dependence of the second recombination event on I-*Sce*I expression (Table 1). Western blot analysis of both small deletion mutants of $U_L48$ revealed proteins of sizes consistent with the expected mutations (Supplementary Figure S6B).

Inclusion of short sequences in the primers (e.g., a sequence encoding a FLAG tag) resulted in the insertion of these short sequences of up to 30 bp into the target sites (Table 1 and Figure 2).

### Conclusions

The mutagenesis strategy described here is based on a simple two-step procedure and utilizes various previously established methods such as Red recombination and counterselection with the homing endonuclease I-*Sce*I. In the first step, a linear marker construct is inserted via Red recombination into the target site using positive selection. In the second step, I-*Sce*I cleaves at its recognition site and creates a DSB. The adjoining duplicated sequence is then used as the new substrate for a second intramolecular Red recombination, which results in the loss of the previously introduced marker (Figures 1 and 3). This novel combination and adaptation of various techniques results in an accurate and highly efficient recombination method that enables a wide variety of different manipulations of large DNA molecules in *E. coli*. Besides the examples shown here, we have prepared a gene expression unit consisting of promoter, multiple cloning site, and poly(A) signal as a universal transfer construct. Sequences encoding various viral antigens were introduced into the generic transfer plasmid, inserted into viral BAC sequences by the described method, and recombinant vaccine viruses were generated (data not shown). The combination of flexible transfer constructs and well-established or novel vector constructs has great potential to allow timely generation of vector-based vaccines or gene transfer constructs for therapeutic purposes.

Probably the biggest advantage of the methodology described here is the efficiency of the protocol, which mainly rests on the fact that Red recombination focuses the recombination event close to a DSB (5) that is generated at an I-*Sce*I site adjoining the duplicated sequence. Because the second Red recombination takes place within this short duplication of sequences, false-positive kanamycin-sensitive colonies resulting from spurious recombinations at other sites were extremely rare (Table 1). A second reason for the efficiency of the method is that both the substrate and the target for the second recombination step are present in all cells of the *E. coli* culture. Since loss of the positive selection marker and not only negative selection are used to select clones for further screening, error-prone repair at the DSB or lack of the I-*Sce*I cleavage were not of concern. In our experience with EHV-1 and Marek's disease virus (MDV) BAC clones, this strategy has in a number of cases been the only means to generate certain recombinants that could otherwise not be obtained by established methods such as RecA-mediated mutagenesis because these methods resulted in the introduction of illegitimate recombination that mainly occur between tandem repeat sequences (data not shown).

Because the two limiting screening steps have efficiencies of 60% to 100%, an adaptation of the method even for high-throughput generation of libraries of mutant BACs should be possible, and

the method may increase the capacities for the production of transgenic animals (7) without the introduction of unnecessary scars, such as *loxP* sites, within modified sequences (23).

## ACKNOWLEDGMENTS

*We thank the members of the Osterrieder Laboratory, Joel Baines, and Tom Forest for inspiring discussions and help. We are indebted to Neal Copeland, Roger Tsien, and Joseph Peters for providing recombinant plasmids and bacteria. This study was supported by grant awards from the National Institutes of Health (AI060843-02), the United States Department of Agriculture NRI program (CSREES 2003-02234), the Morris Animal Foundation (D03EQ-74), and the Harry M. Zweig Memorial Fund to N.O.*

## COMPETING INTERESTS STATEMENT

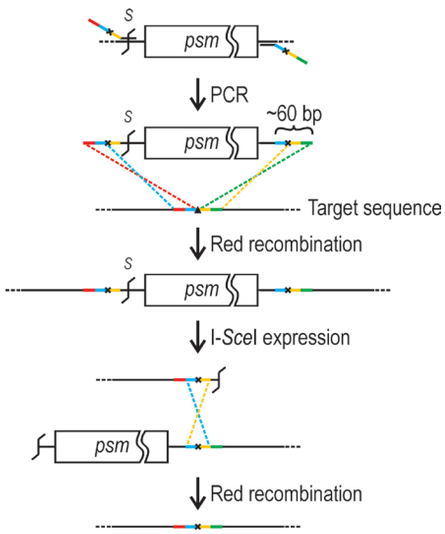


**Figure 3. Diagram of the generation of point mutants by the developed protocol.** The I-*Sce*I-*aphAI* cassette was PCR-amplified to add approximately 40 bp of target sequences (red and cyan or yellow and green) and overlapping modified target sequences (cyan and yellow with an X). The desired point mutation using the linear DNA fragment was obtained. Colored lines symbolize identical sequences, whereas dotted lines indicate single homologous recombination events. *psm*, positive selelction marker; X, new sequence; triangle, old sequence, *S*, I-*Sce*I restriction site. For additional details, see Supplementary Video S3 and Supplementary Table S1.

*The authors declare no competing interests.*

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Received 19 August 2005; accepted 3 November 2005.

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