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Analytical Biochemistry 360 (2007) 306–308
ANALYTICAL BIOCHEMISTRY
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Notes & Tips

One-step generation of recombineering constructs by asymmetric-end ligation and negative selection

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Received 28 March 2006
Available online 19 May 2006

Recombineering [1] or Red/ET recombination [2], a recently developed technique based on homologous recombination in *Escherichia coli*, is useful for the efficient production of a gene-targeting vector (TV)[1] by modification of bacterial artificial chromosome (BAC) clones. This method requires the preparation of a linear DNA recombineering fragment, containing a selectable gene flanked by homologous regions (HRs) for recombination. The PCR method using primers containing HR sequences is the simplest way to produce the recombineering fragment with HRs placed at both ends. This procedure is not advantageous, however, when complex components such as reporter genes are included in the amplified region due to the high degree of mutations possibly generated by PCR. To avoid this problem, Liu and coworkers [3] and Valenzuela and coworkers [4] employed a ligation-based cloning method to introduce HRs into a vector used for recombineering. They used HRs provided as PCR products and oligonucleotides, respectively. In this strategy, however, several restriction enzymes (REs), including six-base cutters, are employed for cloning of HRs. Suitable enzyme sites or HRs, therefore, should be chosen according to the construct introduced. These properties sometimes necessitate screening to identify and select the correct clones. In addition, background clones derived from uncut vector could appear after recombination [3], although most of them can be eliminated by *Dpn*I digestion when PCR-amplified recombineering fragments are used [2].

In this study, we developed five new DNA vectors (Fig. 1A) that enable the efficient generation of recombineering constructs using a one-step ligation and cloning method. The inclusion of a combination of restriction sites, selection markers, and reporter genes into our vectors as described below also circumvents some of the problems generated in the previous methods for constructing TVs.

The five vectors that we developed for the generation of recombineering constructs are shown in Fig. 1A and are described fully in the Supplementary materials and methods. First, pADY vector was developed for the generation of recombineering fragments. This vector has multicloning sites (including *Asc*I, *Xba*I, *Spe*I, and *Nhe*I) for the insertion of a DNA fragment that subsequently will be introduced into the target locus. The *GFPuv* gene was introduced into the downstream *lac* promoter of pADY (Figs. 1A and B) to easily distinguish the GFPuv-negative recombined clones from the GFPuv-positive nonrecombined clones under the UV lamp. The pADY vector with HRs, *GFPuv* gene, and target fragment can be digested with meganuclease I–*Sce*I to prepare a ready-to-use recombineering fragment (Fig. 1C). Second, pAEF was designed to construct a TV by subcloning the BAC insert with recombineering. pAEF has had a diphtheria toxin fragment A (DT-A) cassette inserted as a negative selection marker for gene targeting in ES cells. The GFPuv cassette was included in pAEF to help with the elimination of the GFPuv-positive nonrecombined clones after recombineering (Fig. 1B). After the *SacB* genes in pAEF are replaced by HRs, the resulting DNA with the ligated HRs can be subjected to BAC subcloning following digestion with *Asc*I. The I–*Sce*I site was included in pAEF for linearization of TVs. Third, pADW has the multiple cloning site of pADY and the DT-A cassette of pAEF but lacks the

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[1] *Abbreviations used:* TV, targeting vector; BAC, bacterial artificial chromosome; HR, homologous region; RE, restriction enzyme; DT-A, diphtheria toxin fragment A.

0003-2697/$ - see front matter
doi:10.1016/j.ab.2006.05.001

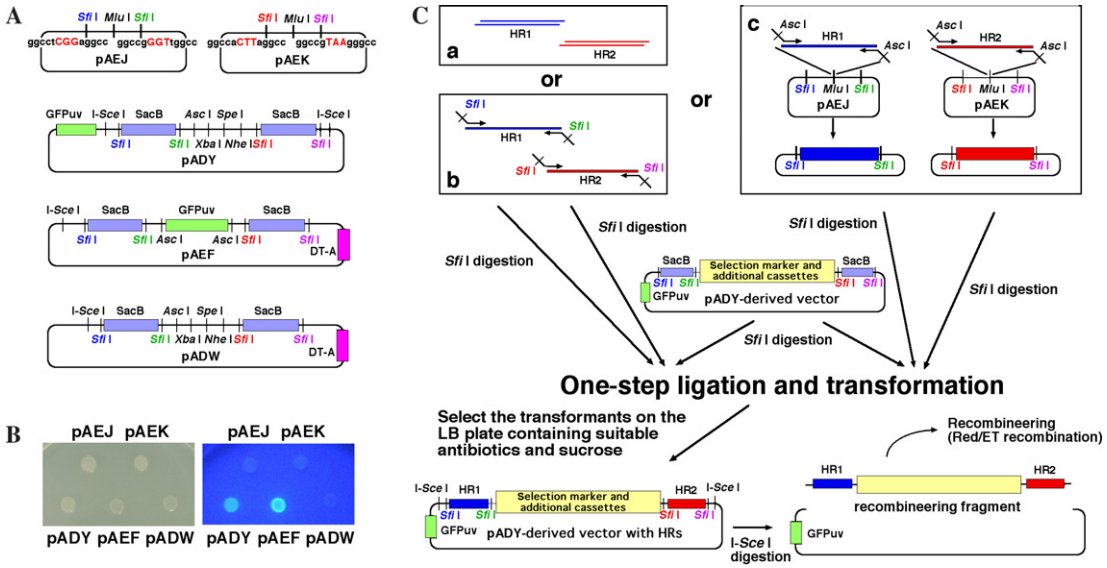

Fig. 1. Cloning vectors and one-step cloning of HRs. (For detailed explanation, see also Supplementary figure legand.) (A) The cloning vectors developed in this study. (B) *GFPuv*-derived fluorescence of *E. coli* containing pADY and pAEF. (C) Cloning of HRs in a single ligation step.

GFPuv cassettes of pADY and pAEF. This vector was generated for one-step TV construction using PCR amplification to add the homology arms. In this case, the homology arms should be much longer (~10 kb) because they facilitate efficient homologous recombination in ES cells. Fourth, pAEJ and pAEK are "supplemental" plasmids for providing *Sfi*I sites at both ends of the HRs. PCR-amplified HRs with *Asc*I sites at both ends can be cloned into the *Mlu*I site of these vectors after *Asc*I digestion. Because *Asc*I is a rare-cutter RE, longer DNA fragments can be cloned into these vectors and used as HRs. These vectors may also be helpful when confirmation of the DNA sequence is required prior to the one-step cloning.

We introduced *Sfi*I sites (5′-GGCCNNNNNGGCC-3′) into these vectors for the following two reasons. First, digestion of the vectors with *Sfi*I, an enzyme capable of recognizing an interrupted palindrome, results in the generation of nonidentical, asymmetric, and noncomplementary 3′ overhangs with three nucleotides. Because four distinct *Sfi*I sites were introduced into the vector (as shown in Fig. 1A), self-ligation of a vector alone or of inserts does not occur. If inserts are designed to possess complementary sequences at both ends, we are able to clone them easily in the correct position and direction (Fig. 1C). This property of *Sfi*I facilitates the cloning of two HRs by a one-step ligation reaction, and no additional enzymes are required for this step. Second, because *Sfi*I is a rare-cutter RE, it is possible to digest relatively long DNA fragments that can serve as the HRs. This property of *Sfi*I is advantageous because the frequency of recombination increases significantly as the size of the HRs increases [2,3]. A similar cloning strategy employing the *Sfi*I site was reported by Scott and Smith [5]. They introduced two *Sfi*I sites into the bacteriophage vector fUSE5 for the efficient cloning of a DNA fragment. We expanded this strategy in our current study by introducing four *Sfi*I sites into a pBluescript-based vector, and we established the efficient four-fragment ligation method using *Sfi*I sites as mentioned below.

The vectors pADY, pAEF, and pADW each contained two *SacB* genes flanked by two *Sfi*I sites (Fig. 1A). The *SacB* gene product, levansucrase, converts sucrose to levan, which is highly toxic to the host cells [6]. The *SacB* genes, therefore, are beneficial as a counterselection marker and can easily be replaced by HRs on cloning (Fig. 1C).

Our one-step cloning strategy is depicted in Fig. 1C. HRs can be supplied as double-stranded oligos (Fig. 1C-a) or PCR-amplified fragments with cohesive ends complementary to the *Sfi*I sites of a vector (Figs. 1C-b and C-c). The *Sfi*I-digested vector (pADY-derived vector) can be directly ligated with both HR1 and HR2 in a single reaction without gel purification and dephosphorylation of the vector. Theoretically, correctly ligated clones such as "pADY-derived vector with HRs" should be obtained through ligation using nonidentical and asymmetric cohesive ends generated by *Sfi*I digestion.

To evaluate whether the presence of *Sfi*I sites and *SacB* genes works efficiently for the cloning of HRs, we examined the cloning efficiency of this method in the following experiments (Supplementary Table 1). Several concentrations of HRs, which had been supplied as 50-bp double-stranded oligos, were subjected to ligation with a vector containing the 4.3 kb target fragment, including the kanamycin-resistant gene after digestion with *Sfi*I. Comparisons of cloning efficiencies of HRs were made between the gel-fractionated and non-gel-fractionated vectors and between the presence and absence of sucrose in the Luria–Bertani (LB) plates (Supplementary Table 1). When higher concentrations of oligos were used as HRs for cloning, the resulting clones were mostly the correct ones with HRs even without undertaking sucrose selection. A number of

background clones were generated, however, when lower concentrations of oligos were ligated to non-gel-fractionated vectors and transformants were cultured in the absence of sucrose. This unwanted result was improved greatly when transformants were cultured in the presence of sucrose. Notably, we confirmed that all of the selected recombinant clones contained both HRs in the correct position and direction. These results confirmed that it was possible to replace the *SacB* genes with two HRs with a high efficiency by a single-step cloning. Negative selection of *SacB*-containing clones and the use of *Sfi*I sites were also beneficial for eliminating time-consuming steps such as gel purification and dephosphorylation, all of which may result in considerable sample loss. We also obtained good results when we used 400–600 bp of PCR-amplified fragments as a source of HR (Figs. 1C-b and C-c) (data not shown). The strategy of sucrose selection and *Sfi*I asymmetric ligation worked perfectly, and no unwanted clones were identified in more than 12 independent experiments.

We also successfully applied the current method to clone 6.5 and 1.4 kb of PCR-amplified HRs in a single ligation step for direct construction of TV using the pADW-derived vector (data not shown). This indicates the suitability of our vector for the introduction of long HRs that can be used directly for homologous recombination in ES cells. With this approach, TV can be constructed within 1 week, although there is a possibility of introducing mutations by PCR into the homology arms.

Because linear fragments isolated from RE-digested vectors are used in our recombineering procedure, the occasional contaminating background colonies derived from uncut plasmid need to be eliminated. Therefore, the *GFPuv* gene included in pADY and pAEF (Figs. 1A and B) was useful in circumventing this problem. We found that small numbers (1–10%) of clones exhibited green fluorescence after the recombinogenic procedure when the *E. coli* DY380 strain [3] was used (data not shown). Although the rate of background clones derived from uncut plasmids appears to be relatively low, our data indicate the usefulness of the *GFPuv* gene for detection of background clones.

Although pADY and pADW are useful plasmids for construction of TVs, they need to be modified for the successful and efficient selection in the *E. coli* and ES cells. For this purpose, we constructed universal vectors (pAGE, pAGF, pAGI, pAGJ, and pAHV [shown in Supplementary Table 2]) derived from the pADY. These vectors were used for the construction of standard or conditional TV. By using these vectors in combination with the 129/Ola BAC clones [7], we generated several kinds of TVs that already have been used successfully to generate several lines of knockout ES cells and mice (data not shown).

In summary, we have produced vectors useful for improving the generation of recombineering DNA for TV construction. These vectors contain *Sfi*I sites, dual *SacB* genes, and the *GFPuv* gene, all of which simplify the cloning of HRs and improve the efficient screening of the correct clones after recombination. The use of these new vectors does not shorten the time required to perform recombinogenic engineering; rather, it simplifies the procedure itself. Because our vectors are applicable to many recombinogenic engineering-based methods, including TV construction, they can be used as tools for high-throughput functional analyses based on DNA modifications.

Acknowledgments

We thank Neal G. Copeland for providing the recombinogenic bacterial strain, DY380. This work was supported in part by a Grant-in-Aid for Scientific Research (16651102) from the Japan Society for the Promotion of Science (JSPS) and by the Research and Study Program of the Tokai University Educational System General Research Organization.

Appendix A. Supplementary data

Supplementary material for this article is available in the online version at doi:10.1016/j.ab.2006.05.001.

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