Title:

"Quick and Affordable DNA Cloning by Reconstitution of Seamless Ligation Cloning Extract (SLiCE) using Defined Factors"

A Short title:

"Seamless Cloning using Defined Factors"

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Abstract

The cloning of DNA fragments to plasmid vectors is at the heart of molecular biology. Recent developments have led to various methods utilizing homologous recombination of homology arms. Among them, Seamless Ligation Cloning Extract (SLiCE) is an affordable alternative solution that uses simple *Escherichia coli* lysates. However, the underlying molecular mechanisms remain unclear and the reconstitution of the extract by defined factors has not yet been reported. We herein show that the key factor in SLiCE is Exonuclease III (ExoIII), a double-strand (ds) DNA-dependent 3'->5' exonuclease, encoded by *XthA*. SLiCE prepared from the *xthA*Δ strain is devoid of recombination activity, whereas purified ExoIII alone is sufficient to assemble two blunt-ended dsDNA fragments with homology arms. In contrast to SLiCE, ExoIII is unable to digest (or assemble) fragments with 3' protruding ends; however, the addition of single-strand DNA-targeting Exonuclease T overcomes this issue. Through the combination of commercially available enzymes under optimized conditions, we achieved the efficient, reproducible, and affordable cocktail, "XE cocktail", for seamless DNA cloning. By reducing the cost and time required for DNA cloning, researchers will devote more resources to advanced studies and the careful validation of their own findings.

Introduction

Plasmid construction is a necessary step in most molecular biology projects. The ligation of restriction enzyme-digested double-strand (ds) DNA fragments has long been used as the only option for this purpose. In recent years, seamless cloning using homologous recombination, such as Gibson Assembly(Gibson et al., 2009) and In-Fusion Cloning(Hamilton et al., 2006; Irwin et al., 2012), is replacing the classical method. However, the commercial products currently available for this technology are expensive, which strains the budgets of laboratories and places significant constraints on student research groups, such as iGEM (International Genetically Engineered Machine competition) teams.

The above two systems for seamless cloning both connect two dsDNA fragments by an *in vitro* homologous recombination reaction using a 15- to 20-bp sequence called a "homology arm" designed at both ends of the 2 fragments. In Gibson Assembly and a related protocol(Xia et al., 2019), T5 exonuclease initially creates 5'->3' resections at both DNA ends, producing a single-strand (ss) DNA region at each homology arm. The exposed mutually complementary ssDNAs spontaneously bind to form dsDNA connecting two DNA fragments (Fig. S1, left panel). After filling the gap by DNA polymerase, the nick is finally closed by DNA ligase. In-Fusion Cloning, which uses different enzymes to create the 3'->5' resection in the first step(Hamilton et al., 2006; Irwin et al., 2012), also works according to a similar principle (Fig. S1, right panel).

To avoid these expensive systems, several attempts to produce simple and affordable lab-made cloning systems have recently been reported. Among them, the Seamless Ligation Cloning Extract (SLiCE) method, which does not require any commercial enzymes, is one of the most accepted protocols(Zhang et al., 2012). A simple lysate of *Escherichia coli* (SLiCE) was shown to induce highly efficient homology recombination between dsDNA fragments with homology arms *in vitro*. A subsequent study demonstrated that this extract may be prepared from any *E. coli* strains derived from K12, including mutants devoid of classical homologous recombination enzymes,

such as RecA, RecB, and RecJ(Motohashi, 2015). Modifications and improvements to SLiCE preparation protocols have also been reported(Guo et al., 2022; Messerschmidt et al., 2016; Motohashi, 2015; Okegawa & Motohashi, 2015), leading to the establishment of systems for affordable DNA cloning. However, the molecular mechanisms underlying the assembly reaction in SLiCE remain unclear.

In recent years, a method called “direct cloning”, in which two DNA fragments with long homology arms are directly transformed into *E. coli* competent cells to obtain recombinant plasmids(Jones & Howard, 1991), has been attracting increasing attention. Several attempts have been made to elucidate the molecular mechanisms of this not very efficient, but simple method(Nozaki & Niki, 2019; Yang et al., 2021). The findings obtained revealed that Exonuclease III (ExoIII), which previously received limited attention in the context of homologous recombination, is essential for direct cloning. Therefore, we hypothesized that ExoIII is also crucial for the SLiCE reaction. In genetic and biochemical experiments, we demonstrated that ExoIII was not only necessary, but also sufficient for *in vitro* homologous recombination cloning. Furthermore, we identified Exonuclease T (ExoT) as an auxiliary factor to expand the substrate preference of ExoIII cloning. The present results provide a simple and affordable system for seamless DNA cloning through the reconstitution of SLiCE with defined factors.

Results

XthA **is the genetic requirement of an *E. coli* strain for efficient SLiCE preparation**

Since the first report of SLiCE, the factors responsible for recombination activity in this extract have been investigated. The original study showed that RecA was not required for SLiCE activity(Zhang et al., 2012). Furthermore, SLiCE from derivatives of the *E. coli* B strain, such as BL21(DE3), exhibited negligible or no recombination activity. Other strains were tested for SLiCE preparation(Motohashi, 2015); however, the factors involved in this recombination activity have yet to be identified.

We initially assumed that the most reasonable candidate was the RecE/RecT system because the expression of the λ RED system, an ortholog of the RecE/RecT system, was employed in the original study to enhance SLiCE activity(Zhang et al., 2012). The λ RED system facilitates strand exchange by forming a 5'->3' resection at the end of the substrate DNA, similar to Gibson Assembly(Muyrers et al., 2001) (Fig. S1, left panel). *In vivo* homologous recombination cloning by the RecE/RecT system was also previously reported(Oliner et al., 1993; Zhang et al., 1998). BL21(DE3), which produces inactive SLiCE, carries a 1.5-kb long deletion in the RecE gene in its genome(Jeong et al., 2015).

We also noted that RusA, a Holliday junction resolvase, was absent in BL21(DE3)(Jeong et al., 2015). Therefore, we added it to the list of the candidate factors essential for SLiCE activity, together with the well-studied Holliday junction resolvase, RuvC. XthA was also added to the list because it was reported to be required for direct cloning using *in vivo* homologous recombination(Nozaki & Niki, 2019; Yang et al., 2021). We collected and used strains with the disruption of each candidate gene to prepare SLiCE. The activity of each extract was examined using two blunt-ended PCR products, a linearized pUC118 plasmid, and a chloramphenicol acetyltransferase gene (CAT), with two 20-bp homology arms at each end of DNA (Fig. 1a). The colonies

harboring correct recombinant plasmids were grown on LB plates containing ampicillin and chloramphenicol. The results obtained are shown in Fig. 1b.

Among the strains examined, the pronounced loss of recombination activity was observed in SLiCE prepared from the *xthA*Δ strain. The number of recombinant colonies obtained with this extract was as few as that of the negative control. In contrast, only a moderate reduction in colony numbers was observed for *recE*Δ and *rusA*Δ. The strain overexpressing RecE/RecT(Zhang et al., 1998) (*sbcA*23^-, *recB*21^-, and *recC*22^-) did not enhance or prevent recombination activity. SLiCE from *recT*Δ or *ruvC*Δ often delivered more colonies than that from a wild-type *E. coli* strain. Based on these results, we concluded that XthA is required for recombination activity in SLiCE.

Homologous recombination activity in SLiCE may be replaced by commercially available ExoIII

XthA encodes the enzyme ExoIII, a 3'->5' exonuclease specific for dsDNA, which has been used to generate ordered deletion clones of DNA fragments for sequencing(Henikoff, 1984; Kitabatake & Inokuchi, 1993). A protocol for seamless DNA cloning by ExoIII was already reported in 1993(K. Hsiao, 1993). In this protocol, a 3'->5' resection is created and used for the connection between inserts and vectors through the spontaneous annealing of complementary single-strand homology arms. To avoid the over-digestion of fragments, the reaction was performed on ice for 30 sec and phenol/chloroform extraction was used to inactivate ExoIII after digestion. The careful manipulation of the reaction mixture and the use of organic solvents prevented this protocol from being accepted by a wide variety of users. Several groups subsequently reproduced and/or improved the use of ExoIII in the *in vitro* DNA cloning protocol(Dao et al., 2022; Gibson et al., 2009; Yang et al., 2021). The findings obtained suggested that ExoIII was not only required, but also sufficient for efficient seamless DNA cloning by SLiCE. We investigated whether ExoIII may replace SLiCE in the cloning assay using two blunt-ended dsDNA fragments. As shown in Fig. 2a, DNA cloning was

successful with high efficiency when ExoIII diluted in 1× SLiCE buffer(Zhang et al., 2012) was used.

During experiments, we noted that the composition of the dilution buffer significantly affected the stability of ExoIII. When 1× SLiCE buffer was used for dilutions, the optimum condition was a 20,000-fold dilution of the commercially available enzyme in the final concentration (Fig. 2a). When the enzyme was further diluted with the same buffer, a marked reduction in activity was observed, reflecting the loss of enzyme activity beyond a reduction in the number of enzyme molecules. We reasoned that certain additive(s) are required for the stable dilution of this enzyme. A good candidate was glycerol used in the original SLiCE. ExoIII was more stable in the diluted condition when we used a new dilution buffer containing 50% glycerol (see Experimental Procedures). Therefore, the optimum dilution condition for the cloning reaction was corrected to a 320,000-fold dilution at the final concentration (Fig. 2b). Since the ExoIII enzyme purchased (2170A from Takara-Bio) contains 25 μL of 200 U/μL enzyme, theoretically, a 10-μL reaction may be performed ~800,000 times. The use of the commercial product of ExoIII (M0206L from NEB) in the seamless DNA cloning assay was previously reported(Dao et al., 2022). The findings obtained showed that a 30-fold dilution was optimal for the purchased enzyme. Using our dilution buffer, we also tested the same product (M0206S) that contains 100 U/μL enzyme and found that the best dilution was 80,000-fold in the final reaction mixture (data not shown), confirming that the composition of the dilution buffer significantly affects the stability of ExoIII.

Under these conditions, we examined the effects of the homology arm length using the blunt-ended dsDNA fragments prepared by PCR. As shown in Fig. 2c, a minimum 15-bp homology arm was required for reproducible DNA cloning. These results are consistent with the findings of previous studies that characterized SLiCE or ExoIII cloning(Dao et al., 2022; Messerschmidt et al., 2016; Motohashi, 2015; Zhang et al., 2012). We also investigated the effects of the GC content in the homology arm sequence on assembly efficiency. To achieve this, we prepared a series of DNA fragments with 20-bp homology arms containing different numbers of GC nucleotides

from 0 to 100% (Fig. 3a). Recombinant plasmids were successfully generated in all cases tested (Fig. 3b). We concluded that diluted ExoIII may be used for DNA cloning with a number of homology arms containing a high or low GC content bias.

The substrate preference of ExoIII cloning may be expanded by adding ExoT

The original study reported the principle and feasibility of seamless DNA cloning by ExoIII(K. Hsiao, 1993), and subsequent studies described re-examinations of and improvements to the protocol(Dao et al., 2022; Gibson et al., 2009; Yang et al., 2021). These modified protocols and that described in this study use the same enzyme, ExoIII, a dsDNA-dependent 3'->5' exonuclease. Since ExoIII cannot digest dsDNA fragments with 3' protruding ends, these methods share a common limitation. Our diluted ExoIII system did not produce any recombinant plasmids when the *Pst*I (producing 4-nucleotides 3' protruding end)-digested plasmid was used as a vector. A previous study showed that DNA fragments with 3' protruding ends generated by *Sac*I were successfully assembled by the SLiCE method, indicating that SLiCE contains an additional factor to treat 3' protruding ends(Messerschmidt et al., 2016).

To identify the enzyme that assists ExoIII activity by blunting 3' protruding ends, we selected and tested three candidate enzymes, including two from *E. coli*, namely, Klenow fragment and ExoT, and T4 DNA polymerase as a positive control. Klenow fragment is a large fragment of *E. coli* DNA polymerase I that harbors 3'->5' exonuclease activity. ExoT is a single strand-specific 3'->5' DNA/RNA exonuclease(Y.-Y. Hsiao et al., 2012). These *E. coli* enzymes were previously reported to be useful for the blunting reaction of 3' protruding ends(Zuo, 1999). T4 DNA polymerase is also a widely used enzyme in the blunting reaction of the staggered ends of dsDNA. As shown in Fig. 4a, these enzymes all restored the activity of ExoIII in DNA cloning with *Pst*I-digested fragments, whereas no colonies were produced in the absence of these enzymes. The reaction produced the largest number of colonies when ExoT was used at a certain amount, inferring that ExoT is a blunting enzyme that works in SLiCE (Fig. 4b). Since a large amount of ExoT was needed for the reaction, we also developed a

purification system for recombinant ExoT. We confirmed that a large amount of the pure enzyme may be prepared by single Ni-NTA column chromatography (Fig. 4c).

Optimization of Exo III cloning provides an efficient cloning system

In the present study, we demonstrated that a cocktail of two enzymes, ExoIII and ExoT, functionally reconstituted SLiCE. To further improve the protocol, we attempted a series of optimizations for the usability of the enzyme cocktail.

We initially aimed to develop a 2× enzyme mix that is stored at -30˚C and may be directly mixed with DNA for use. To achieve this, ExoIII diluted with the dilution buffer were directly mixed with equal amounts of DNA fragments and kept at 37˚C for various reaction times to assess the efficiency of the assembly. As shown in Fig. 5a, DNA assembly efficiently proceeded under this condition in the presence of 25% glycerol and other components for enzyme preservation. We further optimized the enzyme concentration with the aim of constructing a system in which the reaction is completed at 37˚C for 5 min to reduce the reaction time. As shown in Fig. 5b, large numbers of colonies were obtained in a 5-min reaction using a 2× mix containing the ExoIII enzyme diluted 20,000-fold.

Optimization of the concentration of ExoT was performed in a 5-min protocol using the 2× mix. The efficiency of the reaction was examined by inserting a PCR-generated insert fragment into the multi-cloning site (MCS) of *Pst*I-cleaved pUC118. As shown in Fig. 5c, a very large number of colonies was observed under conditions where a 100- to 200-fold dilution of ExoT was included in the final reaction mixture. Based on these results, we concluded that the optimal 2× mix needs to contain 1/100 of ExoT in addition to 1/20,000 of ExoIII. We named this mixture "XthA-ExoT cocktail" or "XE cocktail".

The parameters optimized for XE cocktail are summarized in Table 1. These results are consistent with data reported for SLiCE and ExoIII cloning by other groups(Dao et al.,

2022; Gibson et al., 2009; Guo et al., 2022; K. Hsiao, 1993; Messerschmidt et al., 2016; Motohashi, 2015; Okegawa & Motohashi, 2015; Zhang et al., 2012), except for the use of ExoT and the dilution buffer. We finally developed a 2× mix that assembles two DNA fragments with virtually any sequences in 5 minutes. The ultra-low cost 2× mix presented herein, XE cocktail, may be simply produced by diluting either commercially purchased or lab-made enzymes. This cocktail may be stored at -30˚C for at least three years without experiencing a noticeable loss of efficiency (data not shown), in contrast to the stability of SLiCE, which decreases within 2-3 months when stored under the same conditions.

Success rates of cloning and 5-fragment assembly

The colonies observed in the series of experiments described above formed on LB plates containing ampicillin and chloramphenicol. Since only correct clones were selected and counted in these experiments, the rate of the successful cloning of correct inserts still needed to be examined. Therefore, we performed replica-plate assays (Fig. S2a). After the assembly reaction of a linear plasmid fragment carrying the ampicillin resistance gene and an insert carrying the chloramphenicol resistance gene, DNAs were transformed into competent cells and plated on LB-ampicillin plates. One hundred colonies were selected from each plate and streaked onto another LB plate containing chloramphenicol. The number of resistant colonies is summarized in Fig. S2b. As indicated, 98-99% of colonies grown on LB-ampicillin plates carried the correct insert fragment with the chloramphenicol resistance gene. These results demonstrate that the XE cocktail protocol is highly accurate and reliable for DNA cloning. We assumed that chloramphenicol-sensitive colonies were derived from a trace amount of an undigested plasmid included in the PCR amplification mixture for the preparation of the linear plasmid fragment.

In a previous study, ExoIII was also used to assemble more than two DNA fragments (Dao et al., 2022). Therefore, we investigated whether our XE cocktail assembled five fragments in a single reaction (Fig.6). To achieve this, we used one linear vector

fragment to clone four insert fragments containing partial fragments of the chloramphenicol and kanamycin resistance genes, as shown in Fig. 6a. Assembled DNA was transformed into *E. coli* and plated on LB-chloramphenicol kanamycin plates. Three independent assays produced 78, 416, and 48 colonies, respectively, on each plate. Eight colonies were selected from each plate, and the insert length of each clone was examined by colony PCR. All tested clones had the correct insert length (Fig.6b). These results demonstrate the applicability of our XE cocktail to multiple fragment assembly.

Discussion

We identified ExoIII as an essential factor for seamless DNA cloning in SLiCE by preparing a lysate from the *xthA*Δ mutant strain. We demonstrated that SLiCE in the recombination reaction may be replaced by a trace amount of ExoIII prepared using an improved dilution buffer (summarized in Fig. 7). We also showed that ExoIII cannot assemble DNA fragments with 3' protruding ends and that the addition of ExoT compensates for this limitation. By optimizing conditions, we succeeded in making a very simple and cost-effective cocktail for seamless DNA cloning using only commercially available enzymes.

The present results revealed that the SLiCE method is based on the 3'->5' exonuclease reaction. Therefore, the assumption that the original study premised for their modification of SLiCE by installing the λ RED system (PPY strain)(Zhang et al., 2012), which relies on a 5'-3' exonuclease, was incorrect. By overproducing the λ RED system, natural 3'-5' resection activity by ExoIII, which competes with the λ RED system, is overwritten in their modified system. Since the modified PPY extract generally produces more colonies than normal SLiCE, this type of extract is still useful, but requires a special strain and week-long biochemical procedures for its preparation. The present results predict that the PPY method will be more efficient when the *xthA*Δ strain is used as a host.

In our XE cocktail, ExoT is added for the blunting reaction of 3' protruding ends. Since a previous study reported that ExoT stops digestion when a contiguous CC is present at the 3' end(Y.-Y. Hsiao et al., 2012), restriction enzyme-digested fragments with these ends may influence the efficiency of the recombination with our cocktail. Nevertheless, we anticipate that such a case rarely exists; among the restriction sites present in the MCS of two representative plasmids, pUC118 and pBluescript SK II, the only site generating such a cutting end was ApaI (GGGCC/C). According to the datasheet from the vendor (Takara-Bio), ExoIII may exceptionally digest the four-base protruding GGCC-3' end generated by ApaI. We also confirmed that XE cocktail may efficiently

assemble an insert and ApaI-digested plasmid (data not shown). Therefore, XE cocktail used herein may be effective for all plasmid vectors linearized by restriction enzymes.

ExoT is used in relatively large quantities and represents a major cost in the preparation of XE cocktail. We created an expression plasmid for ExoT, performed simple purification (Fig. 5a), and confirmed that it was as effective as the commercially available enzyme (data not shown). Lab-made ExoT may be an option when a large amount of this enzyme is needed. Another option is the storage and use of the ExoIII-only solution (X cocktail) and XE cocktail separately to save ExoT when using blunt-ended fragments or those with 5'-protruding ends. The protocol for the preparation of recombinant ExoIII was previously reported(Dao et al., 2022). The preparation of these enzymes from *E. coli* is simple because the contamination of any protein(s) from the *E. coli*-soluble fraction to the enzymes does not interfere with recombination unless they do not exceed the concentration in SLiCE. Seamless DNA cloning may be more affordable if these enzymes are prepared in each laboratory.

Table 1 Optimization of the XE cocktail reaction

Terms	Unit	Optimal Condition	Tested Conditions
Reaction temperature	˚C	37	30, 37, 42
Inactivation temperature	˚C	65	60, 65, 70, 75, 80
Inactivation time	min	5	0, 5, 10, 15 (at 65˚C)
pH	-	7.5	7.0-9.5
KCl	mM	40	0-300
$MgCl_2$	mM	10	5-10
DNA concentration	ng/µL	0.1-3	0.02-10
homology arm length	bp	> 15	5-40
ExoIII concentration	U/mL	10	1.25-200
ExoT concentration	U/mL	50-100	1-250

Experimental procedures

Bacterial strains and SLiCE preparation

DH5α was purchased from Toyobo. The mutant strains shown in Fig. 1 were provided by NBRP (National Bioresource Project) for *E. coli* at the National Institute of Genetics, Japan. Among these strains, gene-disrupted mutants (*xthA*Δ, *recE*Δ, *recT*Δ *rusA*Δ, and *ruvC*Δ) were from the KEIO collection (JW1738-KC, JW1344-KC, JW1343-KC, JW0538-KC, and JW1852-KC, respectively). RecE/T overexpression strains were AQ3625 [*thr*-1 *leuB*6 *thi*-1 *lacY*1 *galK*2 *ara*-4 *xyl*-5 *mtl*-1 *proA*2 *his*-60 *argE*3 *rpsL*31 *tsx*-33 *sup*E44 *recB*21 *recC*22 *sbcA*23] and AQ3626 [*thr*-1 *leuB*6 *thi*-1 *lacY*1 *galK*2 *ara*-4 *xyl*-5 *mtl*-1 *proA*2 *his*-60 *argE*3 *rpsL*31 *tsx*-33 *sup*E44 *recB*21 *recC*22 *sbcA*23 *recE*159]. SLiCE was prepared from each strain according to the protocol(Okegawa & Motohashi, 2015) using Triton X-100 as a detergent.

The seamless DNA cloning assay

Unless indicated, the seamless DNA cloning assay was performed in 10 μL of 1× SLiCE buffer (50 mM Tris-HCl (pH 7.5 at 25˚C), 10 mM $MgCl_2$, 1 mM ATP, and 1 mM DTT) using 10 ng of each DNA fragment of the insert and vector. After a 15-minute incubation at 37˚C, the reaction mixtures were heat treated at 65˚C for 5 minutes. Materials were placed and mixed on ice, and incubation steps were immediately performed in a pre-warmed thermal cycler. Regarding transformation, 1 μL of the reaction mixture was removed and added to 5 μL of DH5α competent cells (DNA-903F, Toyobo). Cells were incubated on ice for 15 minutes followed by 45 seconds of heat shock at 42˚C. After a 1-minute incubation on ice, 50 μL of pre-warmed SOC medium was added to cells and incubated at 37˚C for 60 minutes. Cells were inoculated on LB plates containing ampicillin (final 50 μg/mL) and chloramphenicol (final 34 μg/mL). Transformation efficiency by this protocol was $\sim 3\times 10^7$ CFU/μg of pBR322 DNA. All assays were performed at least in triplicate; 3 reaction mixtures were independently prepared and analyzed.

In the 2-fragment assembly with 2× mix (the X cocktail or XE cocktail), 2 μL of 2× mix and 1 μL of each DNA fragment (10 ng/μL) were used for a 4-μL reaction. Two microliters of the reaction mixture was then transformed into 10 μL of competent cells. In the 5-fragment assembly, 5 μL of 2× mix and 1 μL of each DNA fragment (50 ng/μL) were used in a 10-μL reaction. After assembly, the entire reaction mixture was transformed into 100 μL of competent cells. In both cases, each DNA fragment was amplified via PCR in a 20-μL reaction and treated with *Dpn*I to remove the plasmid DNAs used as PCR templates.

Plasmids and PCR products

The majority of assays (Figs. 1b, 2a, 2b, 5a, 5b) were performed with the two DNA fragments amplified by PCR with PrimeStar GXL (R050A, Takara-Bio). The insert was a chloramphenicol acetyltransferase gene of the pHSG399 plasmid amplified by the primers m911 and m912 (details for all primer sequences are available in Supplementary Table S1). The vector was linearized pUC118 amplified by m835 and m834. The assay with the 3'-protruding end (Figs. 4a and 5c) used a *Pst*I-digested pUC118 plasmid and the PCR product of the chloramphenicol acetyltransferase gene amplified by k095 and k096, where *Pst*I recognition sites are capitalized. Other primer sequences, including those for Figs. 3b and 4a, are also listed in Table S1. Before use in the cloning assays, all PCR fragments were purified by spin columns with a silica membrane (EP-11201, Ajinomoto Bio-Pharma).

Enzymes

Unless indicated, ExoIII was purchased from Takara-Bio (2170A). Exonuclease T was purchased from NEB (M0265S) or prepared from *E. coli* cells overproducing the enzyme (see below). Enzymes were diluted by the dilution buffer (50 mM Tris-HCl pH 7.5, 40 mM KCl, 10 mM $MgCl_2$, 5 mg/mL BSA, 50% glycerol, and 1 mM DTT). In the

optimized 2× mix, 10 U/mL of ExoIII (Takara-Bio) and 50 U/mL of ExoT were included in the dilution buffer (Table 1).

Preparation of the recombinant ExoT protein

E. coli ExoT was amplified by PCR with k089 and k090 from genomic DNA. The fragment was cloned to the *Nde*I-*Bam*HI-digested pET-11a vector by XE cocktail described in this study. Regarding protein expression, the plasmid pMK682 was transformed into the BL21(DE3) strain. Colonies were directly resuspended in a 500-mL LB supplemented with ampicillin (50 μg/mL) and cells were grown at 30˚C with 180 rpm shaking for ~5 h until OD600 reached 0.4. IPTG was added to the culture (final 0.5 mM) to induce protein expression. Cells were harvested after 3 h and 30 min of induction. Cells (~300 mg) were disrupted by sonication in a lysis buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, and 20 mM imidazole) and the supernatant was applied to Ni-sepharose beads (GE, 300 μL slurry). After washing 4 times, ExoT was eluted by the lysis buffer containing 250 mM imidazole. The recovery of 1.5 mL of 22.2 μM protein from a 500-mL culture was performed. To prepare 2× mix, 110 nM of the enzyme was used.

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Conflict of interest

All authors have no conflicts of interest to declare.

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Figure Legends

Figure 1

SLiCE cloning methods require XthA in the genetic background of an *E. coli* strain used for lysate preparation

(a) Outline of the experiment. The insert containing chloramphenicol acetyltransferase (Chl^R) was amplified from a plasmid by PCR and used in the seamless DNA cloning assay. An ampicillin-resistant plasmid amplified and linearized by PCR was used as a vector. The colonies observed on LB plates with ampicillin and chloramphenicol double selection represent the successful assembly of transformants carrying the correct recombinant plasmids. Two 20-bp homology arms were designed and attached at each end of the insert by PCR.

(b) The activity of seamless DNA cloning using SLiCE prepared from various *E. coli* strains was examined. Each of the genotypes ("xthA", "recE", "recT", "rusA", "ruvC", "recB/sbcA", and "recBC/sbcA/recE") indicates the disruption or mutation of the gene(s) in the strain from which SLiCE was prepared. "50% Glycerol" represents a negative control where SLiCE was substituted by 50% Glycerol solution. "WT" is a wild-type *E. coli* strain for the positive control. Regarding "WT", we used DH5α, which carries intact alleles of the genes disrupted or mutated in other strains in the figure. Three independent reactions were performed, and the colony numbers observed were plotted. Box bars represent the average of data and error bars represent the standard deviation.

Figure 2

ExoIII may substitute for SLiCE in the seamless DNA cloning of blunt-ended dsDNA fragments

(a) Seamless DNA cloning was performed using a diluted ExoIII enzyme. The enzyme was diluted by 1× SLiCE buffer to various concentrations and used in the assay. The indicated number (0, 2,500-, 5,000-, 10,000-, 20,000-, or 40,000-fold) is the final dilution of the purchased enzyme in the reaction mixture. Except for the diluted enzyme in the place of SLiCE, experiments were performed under the same conditions as those described in the SLiCE method. Two DNA fragments used in the experiments in Fig. 2 were employed and the colonies that formed on LB containing both ampicillin and chloramphenicol were counted and presented as shown in Fig. 2(b).

(b) The effects of the buffer for ExoIII dilution on cloning efficiency were examined. The same cloning experiments were performed as in (a), but using a different dilution buffer containing 50% glycerol (see Experimental Procedures). The indicated number (40,000-, 80,000-, 160,000-, 320,000-, or 640,000-fold) is the final dilution of the enzyme in the reaction mixture.

(c) The lengths of the homology arms were changed and the effects on DNA cloning efficiency were examined. In all assays, a common DNA fragment was used as an insert. Regarding vectors, different linearized plasmids were generated by PCR with different primer sets. Two homology arms with the indicated lengths were designed and attached at both ends of the vector. ExoIII was diluted with the improved dilution buffer at a 32,000-fold concentration and was used as 10× enzyme. Reactions were performed according to the SLiCE protocol.

Figure 3

DNA cloning with diluted ExoIII works with homology arms containing a wide range of GC contents

(a) A schematic representation of a designed plasmid and insert is indicated. One of the two homology arms is fixed (20 bp at GC 45%) and common in all pairs of the inserts

and vectors used here. The other side of the homology arm is changed by the pairs of fragments.

(b) Twenty-two pairs of inserts and vectors were created and tested. Each pair of fragments contains the homology arm with the indicated GC% at the end to be tested. Regarding each GC%, two different sequences were generated and separately used for the cloning assay. Three independent assembly reactions were performed and analyzed for each set of fragments.

Figure 4

The addition of ExoT enables ExoIII to clone fragments with 3'-protruding ends

(a) The insert containing the chloramphenicol resistance gene was amplified by PCR and cloned into a pUC118 plasmid linearized by *Pst*I. The reaction mixture was supplemented with KF; Klenow fragment of *E. coli* DNA polymerase I, ExoT; Exonuclease T (Ribonuclease T) of *E. coli*, T4pol; T4 DNA polymerase. Each enzyme was purchased and diluted using 1× SLiCE buffer. Regarding each enzyme, the three different dilutions (10, 100, and 1,000-fold) indicated were prepared and used as a 10× enzyme mix. Each 10× enzyme mix contained ExoIII diluted to 1/2,000 by 1× SLiCE buffer.

(b) Outline of the process involving ExoT and ExoIII in the cloning system. ExoT may digest the four nucleotides at the 3' protruding ends generated by a restriction enzyme, such as *Pst*I or *Sac*I. After the ends are blunted by ExoT, ExoIII starts creating a 3'->5' resection to proceed to the connection of the two complementary strands of the homology arms.

(c) Purified ExoT. SDS-PAGE and CBB staining was used to analyze the amount and purity of recombinant ExoT. The single visible band shown corresponds to ExoT, a 27-kDa protein.

Figure 5

Optimization of the ExoIII-ExoT cocktail provides an efficient 2× mix for the DNA cloning reaction

(a) The assembly between two blunt-ended DNA fragments with the 2× enzyme mix was performed. ExoIII was diluted in the dilution buffer at 1/80,000 and used as a 2× enzyme mix. Reactions were performed at 37˚C and terminated at the indicated time points. The number of ampicillin-chloramphenicol double-resistant colonies was plotted.

(b) To optimize the enzyme dilution factor for the 5-minute reaction, ExoIII was diluted to different concentrations (5,000-, 10,000-, 20,000-, 40,000-, and 80,000-fold) as indicated. Each enzyme was used as a 2× mix to assemble the two blunt-ended DNA fragments, as in (a).

(c) The concentration of the ExoT enzyme in a 2× mix was optimized. A series of dilutions（20-, 40-, 100-, and 200-fold) of ExoT were prepared in the 2× mix containing ExoIII at a 20,000-fold dilution. The efficiency of the DNA assembly was monitored using a PCR-amplified insert fragment and PstI-digested plasmid in the 5-minute reaction. (-); negative control experiments performed without ExoT.

Figure 6

The XE cocktail may be used for the 5-fragment assembly

(a) Outline of the experiment. This experiment consisted of dividing the chloramphenicol and kanamycin resistance genes into two fragments, which were separately amplified by PCR. Linear plasmid vectors were also prepared via PCR. Homology arms were designed for assembly and are indicated by the colored boxes.

(b) Colonies were grown on LB plates containing chloramphenicol and kanamycin antibiotics. Eight clones were randomly selected from each plate and examined via colony PCR. The expected length for the correct insert (1875-bp) is shown.

Figure 7

Schematic diagram of DNA assembly with ExoIII

Mix a linear vector created by PCR or restriction enzyme digestion with an insert fragment containing homology arms designed at both ends, as shown. The addition of ExoIII results in resection from the 3' end of both fragments, and one strand of the homology arm is removed. Exposed ssDNA is expected to spontaneously hybridize in the reaction solution. Assembled DNA fragments, with gaps in both strands, are transformed into *E. coli*. These gaps are expected to be repaired by the DNA repair system in *E. coli*, resulting in the formation of a replicative plasmid *in vivo*.

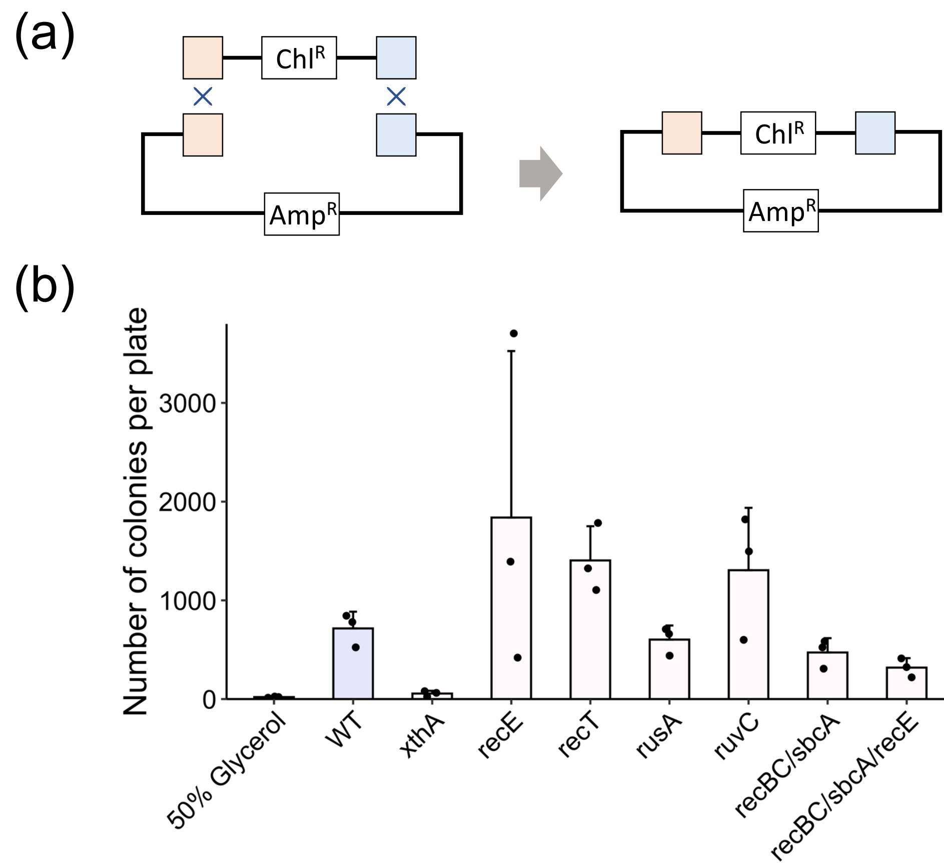
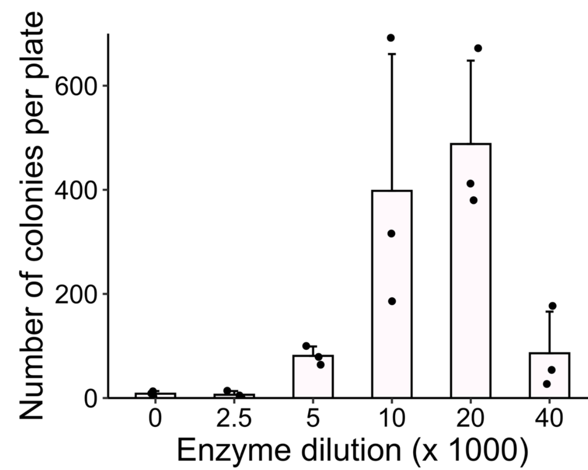
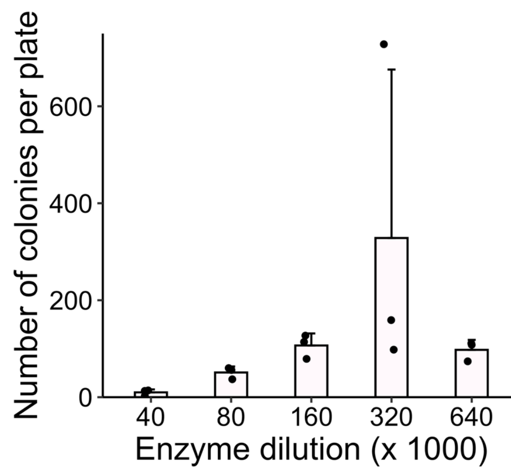


FIGURE 1





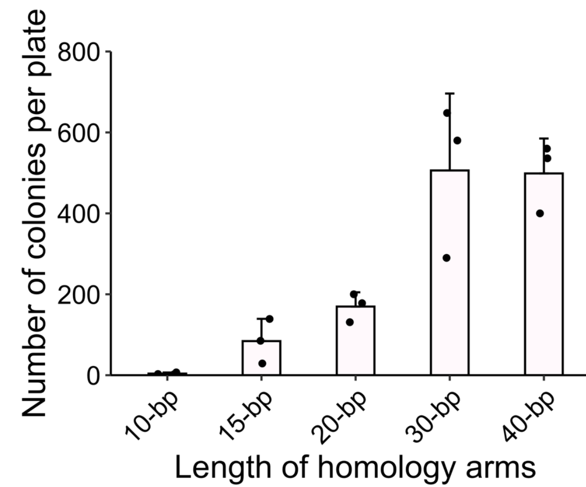
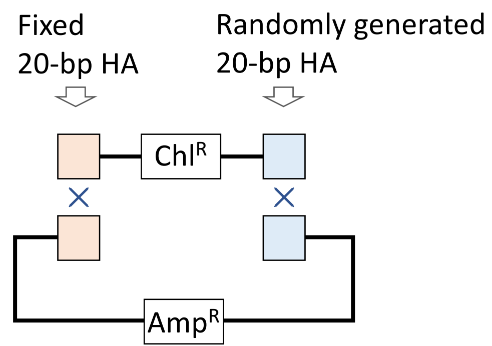


FIGURE 2

(a)



(b)

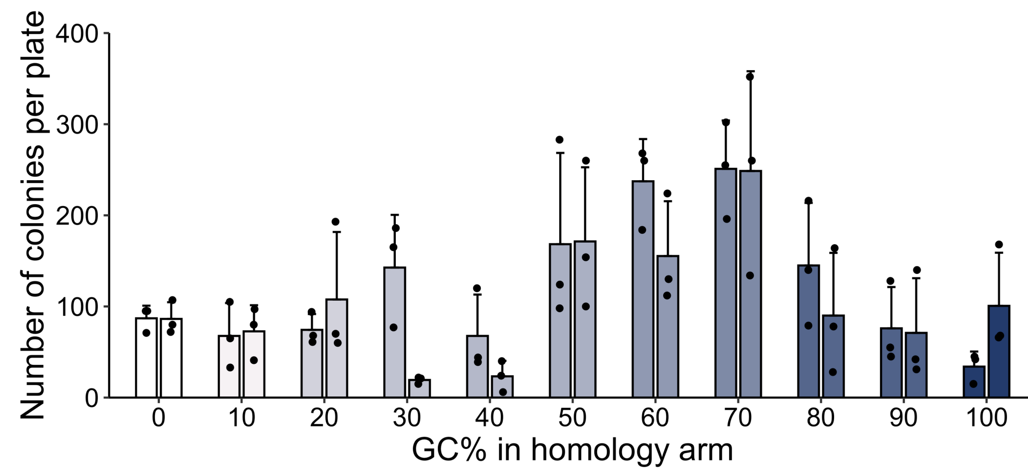
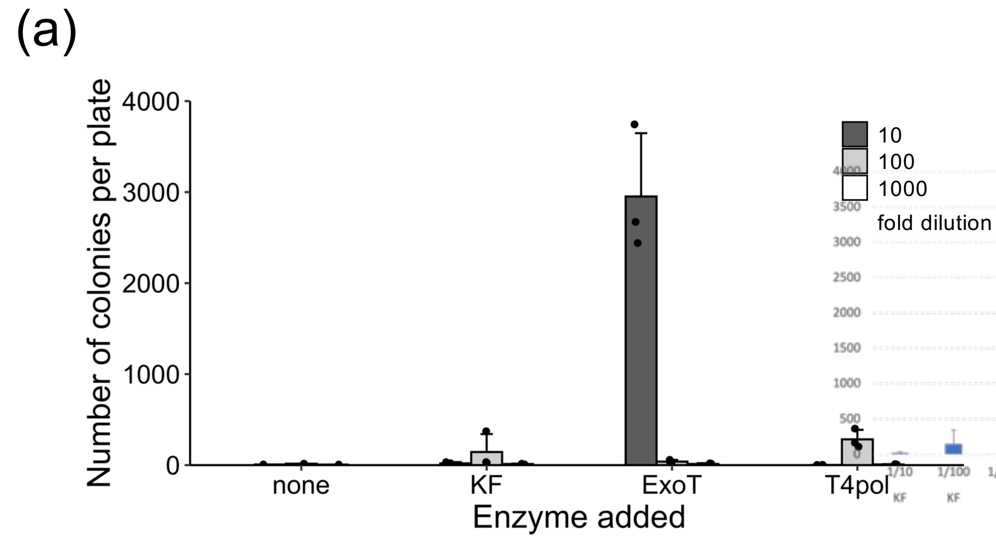


FIGURE 3

(a)



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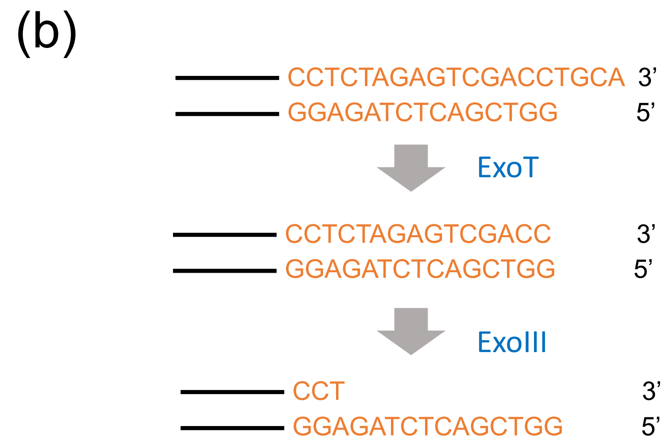


(c)

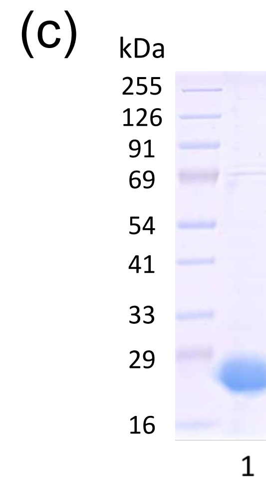
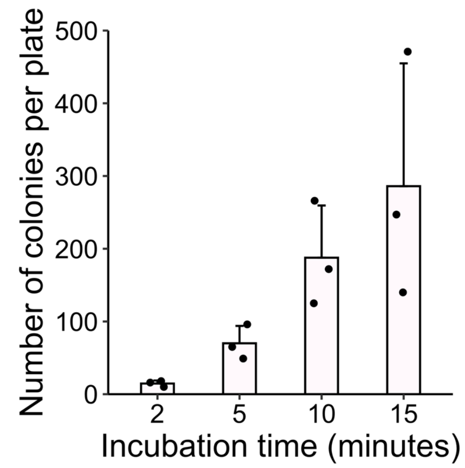
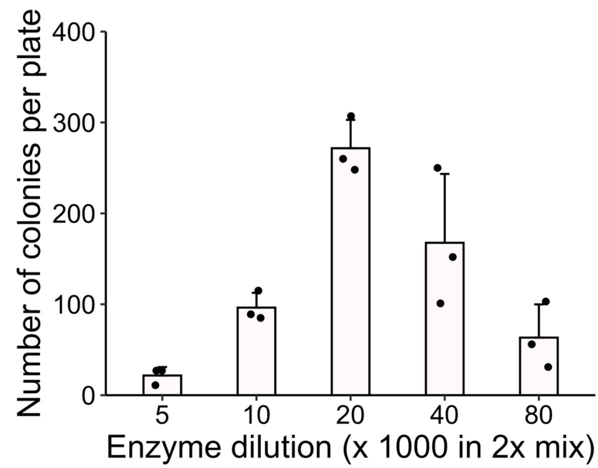


FIGURE 4

(a)



(b)



(c)

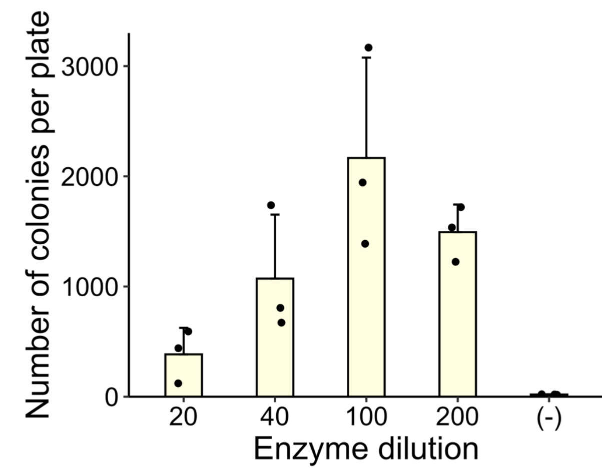
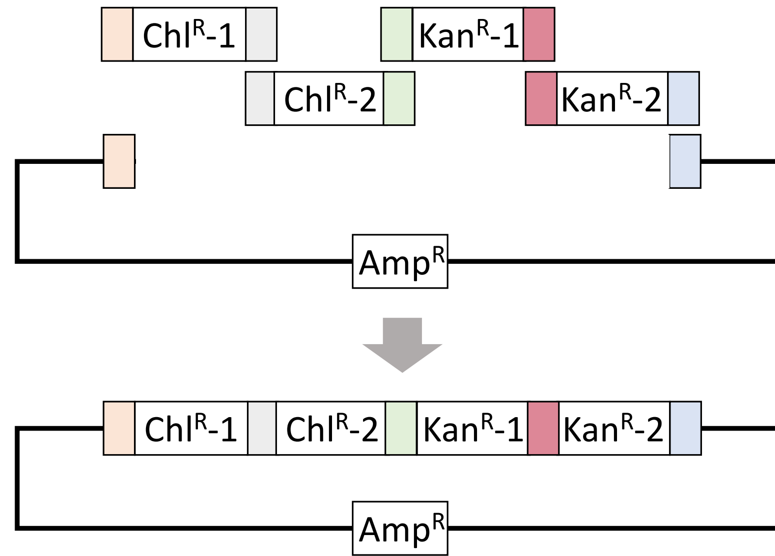


FIGURE 5

(a)



(b)

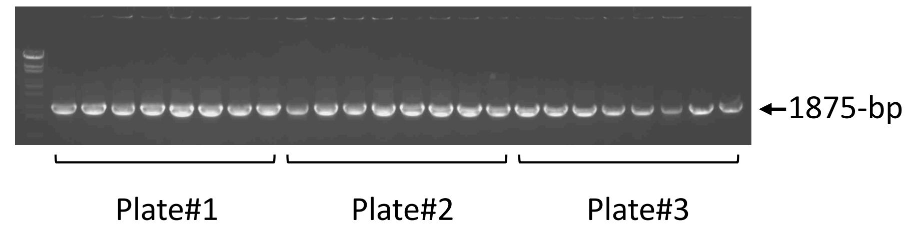
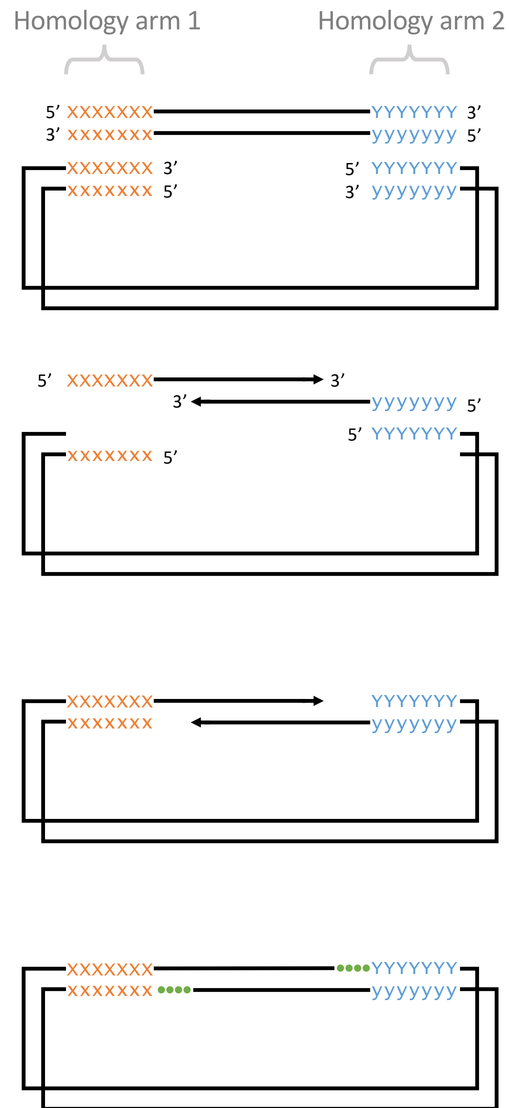


FIGURE 6



Insert

Linearized Vector

3’ resection by ExoIII

Spontaneous hybridization

(Transformation)

DNA repair in E. coli

FIGURE 7

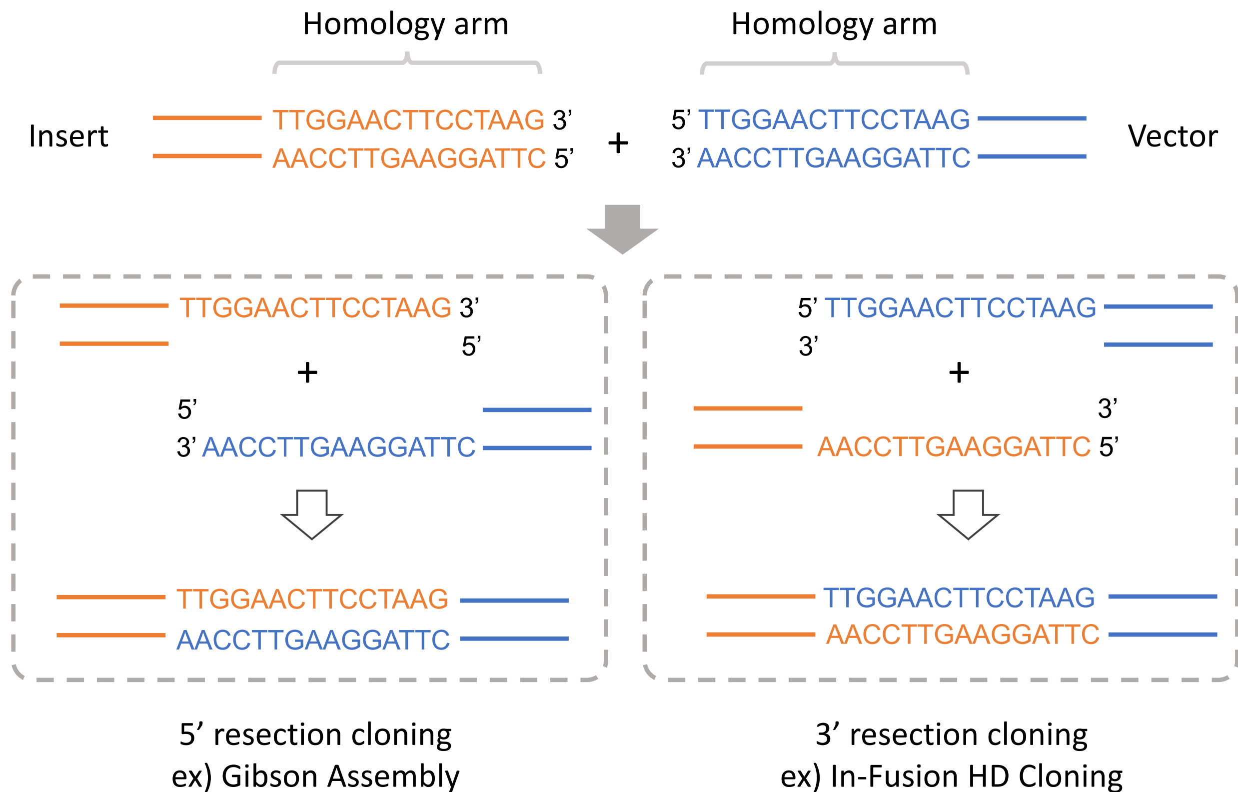


Figure S1

Schematic diagram of two types of seamless DNA cloning

In seamless DNA cloning, such as Gibson Assembly and In-Fusion HD Cloning, 15- to 20-bp homology arm sequences are designed and generated by PCR at both ends of the insert fragment. The homology arm region may essentially be any sequence as long as the common sequence appears at the end of the insert and vector. One pair of an insert and vector is shown. Depending on the enzyme used in the kit, a 3'->5' (left panel) or 5'->3' (right panel) resection is generated. The ssDNA region generated in the homology arm of the insert is complementary to that in the vector. These strands spontaneously form dsDNA, which is repaired by other enzymes in the reaction or in *E. coli* cells after transformation.

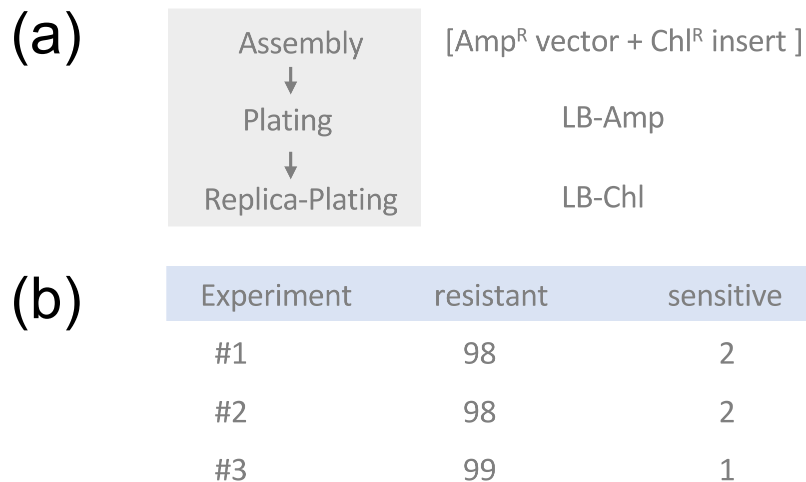


Figure S2

Evaluation of the Chl^R insert cloned by the XE cocktail.

(a) Schematic diagram of cloning followed by the replica plating experiment. The Chl^R insert was amplified and cloned into a linearized ampicillin-resistant plasmid. The resulting transformants were then plated onto LB-ampicillin plates. One hundred colonies were then randomly selected and inoculated on LB-chloramphenicol plates.

(b) The numbers of chloramphenicol-resistant and -sensitive colonies were counted and indicated. Three independent assembly reactions were performed and examined separately.

Name	sequence (5' to 3')	references
m834	tcatggtcatagctgtttcctg	pUC118 amplification, forward
m835	aacgtcgtgactgggaaaac	pUC118 amplification, reverse
m911	GGAAACAGCTATGACCATGAtcgaacggaagatcacttcgcag	CAT insert, forward
m912	GTTTTCCCAGTCACGACGTTgggcaccaataactgcctta	CAT insert, reverse
m915	ggcaatgaaagacggtgagc	CAT insert partial fragment 2, forward
k071	aacactatcccatatcaccagctcaccg	CAT insert partial fragment 1, reverse
k072	taagcagacagttttattgtgggcaccaataactgcctta	CAT insert partial fragment 2, reverse
k073	taaggcagttattggtgcccacaataaaactgtctgctta	Kan resistance insert partial fragment 1, forward
k074	caaccaaaccgttattcattcgtgattgcgcctgagcgag	Kan resistance insert partial fragment 1, reverse
k075	ctcgctcaggcgcaatcacgaatgaataacggtttggttg	Kan resistance insert partial fragment 2, forward
k076	ttaagttgggtaacgccagggttttcccagtcacgacgttcaggtggcacttttcggggaaa	Kan resistance insert partial fragment 2, reverse
k089	gtttaactttaagaaggagatatacatatgCATCACCATCATCATCATtccgata acgctcaacttaccggtc	ExoT cloning, forward
k090	ctttcgggctttgttagcagccggatccttacacctcttcggcggcagatag	ExoT cloning, reverse
k095	acccggggatcctctagagtcgacCTGCAGacggaagatcacttcgcag	CAT insert forward for PstI-site cloning, forward
k096	acggccagtgccaagcttgcatgcCTGCAGgggcaccaataactgcctta	CAT insert forward for PstI-site cloning, reverse
k261	ATTATAAAAATTAAAAAAATcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k262	ATTTTTTTAATTTTTATAATgggcaccaataactgcctta	Fig.3, supplemental Table S2
k263	AAATTTTTATATATTTTAAAcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k264	TTTAAAATATATAAAAATTTgggcaccaataactgcctta	Fig.3, supplemental Table S2
k265	TAATATTAGAAACTTATTATcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k266	ATAATAAGTTTCTAATATTAgggcaccaataactgcctta	Fig.3, supplemental Table S2
k267	TATAATAAGATGTAAATTAAcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k268	TTAATTTACATCTTATTATAgggcaccaataactgcctta	Fig.3, supplemental Table S2
k269	AATTTCAGATTCTTCTTATAcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k270	TATAAGAAGAATCTGAAATTgggcaccaataactgcctta	Fig.3, supplemental Table S2
k271	TATAACGTTCATTAAATGATcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k272	ATCATTTAATGAACGTTATAgggcaccaataactgcctta	Fig.3, supplemental Table S2
k273	GATTAAGTGAATTGACATGAcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k274	TCATGTCAATTCACTTAATCgggcaccaataactgcctta	Fig.3, supplemental Table S2
k275	CTGTTAAACAGACATACTAAcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k276	TTAGTATGTCTGTTTAACAGgggcaccaataactgcctta	Fig.3, supplemental Table S2
k277	CTCTAGTATACAGACGAGATcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k278	ATCTCGTCTGTATACTAGAGgggcaccaataactgcctta	Fig.3, supplemental Table S2
k279	TGCCTAATACCTCTCAAATGcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k280	CATTTGAGAGGTATTAGGCAgggcaccaataactgcctta	Fig.3, supplemental Table S2
k281	TCCCCTTTACCACATGCTAGcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k282	CTAGCATGTGGTAAGGGGAgggcaccaataactgcctta	Fig.3, supplemental Table S2
k283	ACGAGTCTCGATTTGCACTTcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k284	AAGTGCAAATCGAGACTCGTgggcaccaataactgcctta	Fig.3, supplemental Table S2
k285	CCCGACAAGTTAGCAGACCGcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k286	CGGTCTGCTAACTTGTCGGGgggcaccaataactgcctta	Fig.3, supplemental Table S2
k287	AACACACGCAGTCTCCGGTGcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k288	CACCGGAGACTGCGTGTGTTgggcaccaataactgcctta	Fig.3, supplemental Table S2
k289	GTCCGATCAGTGCCGGAGCCcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k290	GGCTCCGGCACTGATCGGACgggcaccaataactgcctta	Fig.3, supplemental Table S2
k291	GCCCTGAACCGCCCAACCCGcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k292	CGGGTTGGGCGGTTCAGGGCgggcaccaataactgcctta	Fig.3, supplemental Table S2
k293	TGTGGGGAGGGGCTGCGCTCcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k294	GAGCGCAGCCCCTCCCCACAgggcaccaataactgcctta	Fig.3, supplemental Table S2
k295	GCTACGTGGCGCCCACGGCCcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k296	GGCCGTGGGCGCCACGTAGCgggcaccaataactgcctta	Fig.3, supplemental Table S2
k297	GGGGTCGCCGGTCCCGCCCCcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k298	GGGGCGGGACCGGCGACCCCgggcaccaataactgcctta	Fig.3, supplemental Table S2
k299	GCCCCCGGCGGCGTGGGGGAcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k300	TCCCCCACGCCGCCGGGGGCgggcaccaataactgcctta	Fig.3, supplemental Table S2
k301	CGGCGCCGGCCGCGCGCGCGcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k302	CGCGCGCGCGGCCGGCGCCGgggcaccaataactgcctta	Fig.3, supplemental Table S2
k303	GCCGGCGCGCCCCCCGCCGGcctggcgttacccaacttaa	Fig.3, supplemental Table S2
k304	CCGGCGGGGGGCGCGCCGGCgggcaccaataactgcctta	Fig.3, supplemental Table S2

Supplemental Table S1

Primers used in the present study. All primers were synthesized by Eurofins on a scale of 10 nmoles and purified using the OPC protocol.

experiment	GC%	vector number	vector primer	insert number	insert primer
1	0	V1	m834,k261	I1	m911,k262
2	0	V2	m834,k263	I2	m911,k264
3	10	V3	m834,k265	I3	m911,k266
4	10	V4	m834,k267	I4	m911,k268
5	20	V5	m834,k269	I5	m911,k270
6	20	V6	m834,k271	I6	m911,k272
7	30	V7	m834,k273	I7	m911,k274
8	30	V8	m834,k275	I8	m911,k276
9	40	V9	m834,k277	I9	m911,k278
10	40	V10	m834,k279	I10	m911,k280
11	50	V11	m834,k281	I11	m911,k282
12	50	V12	m834,k283	I12	m911,k284
13	60	V13	m834,k285	I13	m911,k286
14	60	V14	m834,k287	I14	m911,k288
15	70	V15	m834,k289	I15	m911,k290
16	70	V16	m834,k291	I16	m911,k292
17	80	V17	m834,k293	I17	m911,k294
18	80	V18	m834,k295	I18	m911,k296
19	90	V19	m834,k297	I19	m911,k298
20	90	V20	m834,k299	I20	m911,k300
21	100	V21	m834,k301	I21	m911,k302
22	100	V22	m834,k303	I22	m911,k304

Supplemental Table S2

Design for the amplification of DNA fragments used in Fig. 3. Twenty-two pairs of vectors and insert fragments were generated and used in experiments with 22 different homology arm sequences. Each experiment was repeated at least three times, and the data obtained are shown in Fig. 3.

Supplemental Figures and Tables, legends

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In seamless DNA cloning, such as Gibson Assembly and In-Fusion HD Cloning, 15- to 20-bp homology arm sequences are designed and generated by PCR at both ends of the insert fragment. The homology arm region may essentially be any sequence as long as the common sequence appears at the end of the insert and vector. One pair of an insert and vector is shown. Depending on the enzyme used in the kit, a 3’->5’ (left panel) or 5’->3’ (right panel) resection is generated. The ssDNA region generated in the homology arm of the insert is complementary to that in the vector. These strands spontaneously form dsDNA, which is repaired by other enzymes in the reaction or in *E. coli* cells after transformation.

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