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SLiCE: a novel bacterial cell extract-based DNA cloning method

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ABSTRACT

We describe a novel cloning method termed SLiCE (Seamless Ligation Cloning Extract) that utilizes easy to generate bacterial cell extracts to assemble multiple DNA fragments into recombinant DNA molecules in a single *in vitro* recombination reaction. SLiCE overcomes the sequence limitations of traditional cloning methods, facilitates seamless cloning by recombining short end homologies (≥15 bp) with or without flanking heterologous sequences and provides an effective strategy for directional subcloning of DNA fragments from Bacteria Artificial Chromosomes (BACs) or other sources. SLiCE is highly cost effective as a number of standard laboratory bacterial strains can serve as sources for SLiCE extract. In addition, the cloning efficiencies and capabilities of these strains can be greatly improved by simple genetic modifications. As an example, we modified the DH10B *Escherichia coli* strain to express an optimized λ prophage Red recombination system. This strain, termed PPY, facilitates SLiCE with very high efficiencies and demonstrates the versatility of the method.

INTRODUCTION

The generation of recombinant DNA molecules is an essential tool in modern molecular biology. The conventional DNA cloning strategies that have been used for several decades typically involve the use of type II restriction enzymes to generate appropriate DNA fragments, the modification of DNA ends to generate blunt or sticky ends and the ligation of the DNA fragments to generate plasmid or other type DNA vectors (1–3). However, these procedures depend on the presence of appropriate restriction sites to generate both vector and insert molecules and often leave unwanted sequences at the junction sites. In addition, the restriction enzymes and modifying enzymes required for these manipulations are often expensive making these procedures costly especially in high throughput settings. To circumvent these limitations, we developed a new restriction site independent cloning method that does not leave any unwanted sequences at the junction sites (seamless) and is based on *in vitro* recombination between short regions of homologies (15–52 bp) in bacterial cell extracts termed SLiCE (Seamless Ligation Cloning Extract). SLiCE allows for efficient restriction site independent cloning of DNA fragments generated by restriction digestion or PCR amplification into linearized vectors. In addition, SLiCE does not require the use of enzymes for the modification of vector and insert end sequences (such as Klenow or T4 DNA polymerase) or ligases. The SLiCE method can be used for virtually any type of cloning approach including the simple subcloning of PCR or restriction fragments, the generation of tagged expression vectors, the construction of more complex vectors such as gene targeting vectors or the directional subcloning of larger DNA fragments from more complex vectors such as bacterial artificial chromosomes (BACs). In addition, SLiCE allows the assembly of multiple DNA fragments in one cloning step, which may make it an ideal method for the assembly of multiple DNA fragments during gene synthesis applications.

The SLiCE method is based on bacterial extracts that can be derived from a variety of common RecA$^-$ *Escherichia coli* laboratory strains such as DH10B and JM109. These strains can also be further optimized by simple genetic modifications to improve SLiCE cloning efficiencies and capabilities making SLiCE highly versatile. For example, we established a DH10B-derived strain, termed PPY that was engineered to contain an optimized λ prophage Red recombination system (4–6). We found that extracts derived from this strain provide the highest cloning efficiencies thus far and that it can be used for all cloning approaches that are routinely used in the laboratory. The SLiCE method overcomes many problems related to conventional cloning procedures and provides a highly cost-effective approach for the

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generation of recombinant DNA molecules in a seamless and restriction site independent manner. In this report, we describe the SLiCE method, its principal features and applications.

MATERIALS AND METHODS

Bacteria strains

The following laboratory *E. coli* strains were used: DH10B (Invitrogen), JM109 (Promega), BL21(DE3) (Invitrogen), BLR(DE3) (Novagen) and ER2566(NEB).

The DH10B derived *E. coli* strain PPY was constructed by Suicide Plasmid Based Genome Modification (7) using plasmid pGT1 (PPY genotype: F^- *end*A1 *rec*A1 *gal*E15 *gal*K16 *nup*G *rps*LΔ*lac*X74 *Φ80lac*ZΔM15 *ara*D139 Δ(*ara,leu*)7697 *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *cyn*X:: [*ara*C pBAD- *redα* EM7- *redβ* Tn5-*gam*] λ^-).

The competent cells used for transformation of SLiCE generated recombination products were ElectroMAX *DH10B*^{TM} cells and MAX Efficiency® *DH10B*^{TM} competent cells (Invitrogen).

Plasmids

The plasmid pBL was constructed by insertion of a 70-bp chemically synthesized multiple cloning site into the 2.5-kb PCR-generated plasmid backbone of pBluescript II KS(+) (Stratagene) and deletion of the *LacZα* ORF by conventional cloning. The plasmid pBL-DL was constructed by insertion of a 1-kb PCR fragment from pGEM®-luc (Promega) into the NotI/SalI sites of pBL by SLiCE. The suicide plasmid pGT1 was constructed by SLiCE-mediated insertion of a 830-bp PCR-amplified fragment spanning the 3′ region of the *E. coli* DH10B *cyn*X gene and an *ara*C-pBAD-*redα*/EM7-*redβ*/Tn5-*gam* expression cassette isolated from plasmid pBAD24 (8) and lambda phage DNA (NEB) into the *Sma*I site of plasmid pEL04 (9). pGT1 also contains a temperature-sensitive replicon and a chloramphenicol selection marker.

Preparation of SLiCE extract

Escherichia coli strains were grown at 37°C in 100 ml 2X YT medium until they reached $OD_{600} \approx 5.3$ (OD_{600} readings were calculated by diluting the sample to enable photometric measurement in the linear range between 0.1 and 0.5 OD_{600}). PPY was subsequently incubated for 2 h in 2X YT medium containing 0.2% L-arabinose to express λ prophage protein Redα. The cells were harvested by centrifugation at 5000g for 20 minutes at 4°C. The cells from 96 ml of original culture (wet weight ≈0.92 g) were washed 1 time with 200 ml ddH_2O and resuspended in 1.2 ml CelLytic^{TM} B Cell Lysis Reagent (Sigma). The resuspended cells were incubated at room temperature for 10 minutes to allow lysis to occur. The cell lysates were centrifuged at 20 000*g* for 2 min at room temperature to pellet the insoluble material. The resulting supernatants were carefully removed from the cell debris into a low binding 1.5 ml tube (Protein LoBind Tube 1.5 ml, Eppendorf). The cell extracts were mixed with equal volume of 100% glycerol, aliquoted into 40–60 ul portions in low binding 0.5 ml tubes (Protein LoBind Tube 0.5 ml, Eppendorf), and stored at −20°C for about 2 months without significant loss of activity. For long-term storage, the aliquoted cell extracts were stored at −80°C in 50% glycerol, which can be thawed on wet ice and refrozen up to 10 times without significant loss of activity.

SLiCE reaction and transformation

The vectors used for SLiCE were linearized by restriction digestion or PCR amplification. The cloning inserts were PCR amplified using primers containing 5′-end homologies to the vector or to other inserts for coassembly. Vector or insert DNAs that were generated by PCR amplification using plasmid DNA as templates were treated with DpnI prior to purification to remove residual plasmid template DNA. The linearized vectors and PCR inserts were subjected to gel electrophoresis and purified using the QIAEX II gel extraction kit. For SLiCE cloning of BAC DNA, the restriction digested BAC DNA was purified by phenol/chloroform extraction.

The standard SLiCE reaction mix contained the following ingredients: 50–200 ng linear vector, appropriate amount of insert DNA in a 1 : 1 to 10 : 1 molar ratio of insert to vector, 1 µl 10X SLiCE buffer (500 mM Tris–HCl (pH 7.5 at 25°C), 100 mM $MgCl_2$, 10 mM ATP, 10 mM DTT), 1µl SLiCE extract and ddH_2O to a total volume of 10 µl. The SLiCE reaction was incubated at 37°C for 1 h and subsequently 1 µl of the SLiCE reaction was electroporated into 20 µl ElectroMAX *DH10B*^{TM} cells (Invitrogen) or chemically transformed into 100 µl MAX Efficiency® *DH10B*^{TM} competent cells (Invitrogen) following the manufacturer's instructions. The transformation efficiencies of ElectroMAX *DH10B*^{TM} cells (Invitrogen) and MAX Efficiency® *DH10B*^{TM} competent cells (Invitrogen) were $\sim 1 \times 10^{10}$ and 1×10^9 transformants/µg of pUC19 DNA, respectively. The transformed cells were plated on ampicillin/Xgal agar plates or agar plates containing appropriate antibiotics.

RESULTS

Comparison of *E. coli* K12 strains for SLiCE

SLiCE is a cloning method that is based on *in vitro* recombination in bacterial extract. SLiCE is a simple and efficient procedure with the entire process consisting of three steps (Figure 1a): (i) The preparation of linear vector and insert fragments containing short end homologies introduced by PCR with primers having appropriate 5′ extension sequences; (ii) the SLiCE *in vitro* reaction and (iii) the standard transformation (electroporation or chemical transformation) of recombination products into suitable host bacteria. In this article, all bacterial transformations were performed by electroporation using ElectroMAX DH10B^{TM} cells (Invitrogen) unless otherwise noted.

To determine the most efficient bacterial strains for SLiCE, five standard laboratory *E. coli* K12 strains were tested including DH10B, JM109, ER2566, BL21 (DE3) and BLR (DE3). The main criteria for their selection

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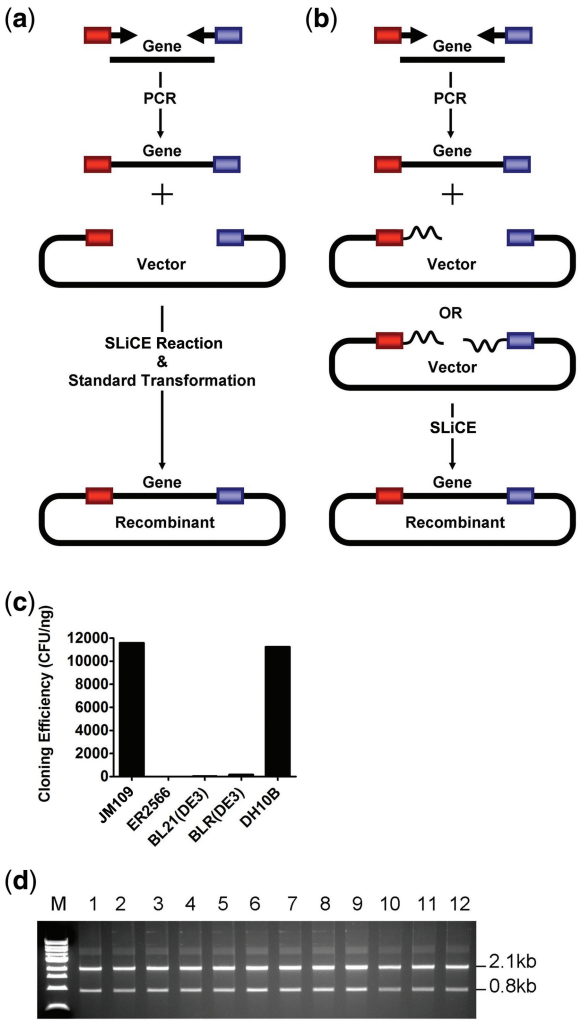

Figure 1. SliCE cloning. (**a**) Outline of SLiCE Cloning. (**b**) Schematic illustrating seamless SLiCE cloning with flanking heterologous sequences. (**c**) Comparison of SLiCE efficiency of *E. coli* K 12 strains. (**d**) BsaAI/SapI restriction analysis of the recombinants derived from SLiCE cloning. Plasmid DNAs from 12 independent ampicillin-resistant blue colonies (lanes 1–12) were digested with BsaAI/SapI. The digestion products were separated on a 1% agarose gel and visualized after ethidium bromide staining. Recombinant plasmids contain one BsaAI site and one SapI site yielding diagnostic 2.1- and 0.8-kb restriction fragments.

included their genetic status with regard to the RecA homologous recombination protein and the presence of restriction systems that could interfere with the stability of exogenous DNA (Supplementary Table S1).

To determine the suitability of extracts derived from these strains for SLiCE, a simple cloning strategy was devised. A plasmid vector termed pBL was linearized by restriction digestion and incubated together with a 500-bp PCR-amplified LacZα in the different bacterial extracts. To facilitate recombination, the LacZα fragment contained 42-bp end sequences that were homologous to the end sequences of pBL. According to the experimental design positive recombinant clones could be identified by blue/white selection after transformation and growth of bacteria on ampicillin/Xgal agar plates.

This analysis showed that extracts from the two RecA$^-$ strains DH10B and JM109 yielded the highest cloning efficiencies, indicating that SLiCE is facilitated by a RecA independent mechanism (Figure 1c).

Due to the high cloning efficiency, cell extract from the DH10B strain was further used for the analysis of SLiCE. Using the pBL–LacZα cloning strategy described above, the influence of several parameters on SLiCE cloning efficiency was tested including the lengths of end homologies, the vector/insert ratio, the overall DNA concentration and the transformation methods. To determine the effect of end homology length on SLiCE, homologies ranging from 0 to 100 bp (as counted from the 3′-ends of the vector) were tested (Table 1). This analysis showed that inserts without end homology or 10-bp end homology did not yield any recombinant colonies. In contrast, 15 bp of end homology already yielded an appreciable number of recombinant colonies at a cloning efficiency of 75 colony forming unit (CFU)/ng of vector, while 30 bp of end homology provided very robust cloning efficiencies (920 CFU/ng of vector). A further increase in end homology length resulted in even higher cloning efficiencies with an end homology length of 52 bp providing the highest efficiency (21 965 CFU/ng of vector). However, in contrast to *in vivo* homologous recombination cloning, the cloning efficiency dropped significantly when the end homology length was further increased (Table 1) indicating that SLiCE promotes *in vitro* recombination by a different pathway. Control reactions that contained the same vector/insert combinations with increasing end homologies but without SLiCE extract did not yield any recombinant colonies. Next, SLiCE was performed with varying vector/insert molar ratios at a vector concentration of 10 ng/μl and 42 bp of end homology. These studies showed that vector/insert ratios of 1 : 1, 1 : 2, 1 : 6 and 1 : 10 yielded 1335, 2330, 11 120 and 12 120 CFU/ng of vector, respectively, demonstrating that increased insert ratios could yield higher cloning efficiencies. Compared to the standard vector concentration of 10 ng/μl, SLiCE using low concentrations of vector (1 ng/μl) and a vector/insert at ratio of 1 : 1 led to a 200-fold decrease in cloning efficiency which is likely due to the instability of vector and insert DNA at these low concentration in the SLiCE reaction mix. All the data above were derived by electroporation of the SLiCE reaction products. We also performed chemical transformation of SLiCE reaction products with 42 bp of end homology using MAX Efficiency® *DH10B*^{TM} competent cells (Invitrogen), which yielded about a 10-fold lower cloning efficiency than electroporation (1063 CFU versus 10 480 CFU/ng of vector, chemical transformation versus electroporation).

Besides promoting recombination between homologous sequences at the ends of vector and inserts, SLiCE is also capable of facilitating recombination between DNA fragments that contain flanking heterologous sequences and of deleting the extra flanking sequences to generate precise junctions at the recombination sites. This feature of SLiCE provides a highly useful cloning tool, especially in those cases where the absence of suitable restriction sites in a vector prevent the seamless cloning of an insert

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Table 1. Influence of End Homology Length on SLiCE Cloning

Homology length (bp)	Cloning efficiency		Cloning accuracy	
	DH10B SLiCE	PPY SLiCE	DH10B SLiCE	PPY SLiCE
Vector only	0	0	–	–
0	0	0	–	–
10	0	0	–	–
15	75	4 640	90%	99%
20	80	34 500	88%	99%
30	920	124 000	98%	99%
42	10 480	632 000	99%	99%
52	21 965	766 000	99%	99%
68	1795	162 000	99%	99%
78	3325	119 250	99%	99%
88	1890	68 000	99%	99%
100	3320	32 500	99%	99%

Cloning efficiencies using different lengths of end homologies are given as CFUs of blue colonies per nanogram of vector. Cloning accuracies are given as the percentage of blue colonies among the total number of all amp^r colonies (blue and white). The 2.5-kb vector pBL was linearized by NotI/SalI digestion and the 500–700-bp LacZα fragments were prepared by PCR. Experiments were performed using 10 ng/μl of vector and the corresponding amount of insert DNA at a 1 : 6 molar ratio of vector:insert. The blue colonies contain recombinant plasmid and the white colonies contain non-recombinant vector background.

fragment into a desired vector region (Figure 1b). To test this feature, DH10B SLiCE reactions were performed with vector pBL-DL that was designed to provide heterologous flanking sequences at the cloning sites. pBL-DL was linearized with appropriate restriction enzymes to generate flanking heterologous sequences on one side (319, 738 and 998 bp) or on both sides (45 bp plus 23 bp and 319 bp plus 738 bp) and was together with a LacZα fragment of 500 bp with 42 bp of end homologies (Figure 1b) subjected to SLiCE followed by blue/white selection. Our results showed that DH10B SLiCE can efficiently remove 45 bp plus 23 bp on both sides of flanking heterologous sequences but it cannot facilitate DNA cloning with longer flanking heterologous sequences of 319, 738, 998 bp on one side or 319 bp plus 738 bp on both sides (Supplementary Table S2).

Generation of a modified DH10B strain for the optimization of SLiCE

In vivo homologous recombination in *E. coli* can be facilitated by three different recombination pathways: the RecA dependent pathway, a RecA independent pathway of unknown nature and a RecA independent pathway that utilizes prophage Red/ET recombination systems (4–6,10–14). The studies above indicate that a RecA independent pathway catalyzes SLiCE. To optimize SLiCE and acquire even more efficient strains as a source for cell extracts, we modified the DH10B genome using a suicide plasmid based strategy to insert an optimized λ prophage Red recombination system into the bacterial genome. Specifically, the genome of DH10B bacteria were modified to constitutively express the λ phage *red*β and *gam* genes under the control of the EM7 and Tn5 promoters, respectively, and also the *red*α gene under the control of an arabinose-inducible pBAD promoter (*araC*-pBAD) (8). The modified DH10B strain was termed PPY and tested for SLiCE. Extracts derived from PPY bacteria yielded significantly higher cloning efficiencies and a more robust seamless cloning activity in the presence of heterologous flanking sequences than the DH10B extracts (see below) and were used in the following series of experiments for the analysis of optimized SLiCE capabilities.

Efficiency and fidelity of PPY SLiCE

In the first series of experiments we investigated the efficiency and fidelity of the improved PPY SLiCE extract. First, we examined the influence of end homology length on PPY SLiCE mediated cloning in more detail. Using the pBL–LacZα cloning strategy end homologies varying from 0 to 100 bp were examined (Table 1). Vector and insert fragments with no end homology or a short homology of 10 bp did not yield any recombinant colonies. Similar to DH10B SLiCE without the Red system, the minimum length of homology required for efficient cloning was 15 bp, however, the PPY SLiCE extract yielded a dramatic increase in the number of blue recombinant colonies (4 640 CFU versus 75 CFU per ng of vector, PPY SLiCE versus DH10B SLiCE) with a high cloning accuracy. Similar to DH10B SLiCE cloning, the cloning efficiency for PPY SLiCE increased with homology length in a range up to 52 bp but dropped significantly when the end homologies were further increased (Table 1).

PPY SLiCE-mediated cloning was also performed using another vector/insert combination (p3XFLAG-CMV-7.1 vector (Invitrogen) and a 800-bp PCR insert with end homologies ranging from 0 to 42 bp). These studies yielded similar results (data not shown).

The recombinant colonies derived from PPY SLiCE cloning were further analyzed by colony PCR, restriction digestion and DNA sequencing analyses. More than 300 blue colonies were screened using colony PCR (data not shown) and some of the colonies were verified by restriction digestion (Figure 1d). All of the analyzed clones contained the correct insert. The vector/insert junctions of

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30 recombinant clones were sequenced and all of the clones contained the correct cloning junctions indicating that SLiCE fuses vector and insert in a precise manner. The small number of white background colonies that were observed in these test experiments could be traced back to spurious amounts of undigested pBL vector during linearization.

We next examined the fidelity of PPY SLiCE without prior selection of positive recombinant clones. For these studies we used a NotI/XbaI linearized 5.2-kb plasmid vector and a 1.4-kb PCR-amplified insert with 30 bp of end homologies. The entire insert and the junction regions of 20 positive recombinants were sequenced. Eighteen recombinants contained completely correct sequences and 2 recombinants presented one mutant nucleotide located in the PCR insert, which is consistent with the error rate of the DNA polymerase that was used for PCR amplification (Fastart Fidelity PCR system, Roche) at 2.4×10^{-6}/bp/cycle and 30 cycle amplification. The error rate and location of mutations indicate that these mutations were caused by PCR amplification of inserts and that SLiCE did not introduce further mutations.

We next examined the effect of various molar ratios of vector and insert and the overall DNA concentration on PPY SLiCE. The results were again similar to DH10B SLiCE mediated cloning. PPY SLiCE with pBL–LacZα vector/inserts at molar ratios of 1 : 1, 1 : 2, 1 : 6 and 1 : 10 with 20-bp end homologies yielded 19 240, 27 320, 34 500 and 65 350 CFU/ng of vector, respectively, showing that increasing the amount of insert yields slightly higher cloning efficiencies for PPY SLiCE. We also observed that PPY SLiCE at a low concentration of vector and insert (1 ng/μl) at a vector/insert ratio of 1 : 1 also resulted in a 10-fold reduced cloning efficiency.

We also determined the sequence dependence of PPY SLiCE in more detail. For this, we first generated four LacZα fragment and vector combinations containing 20-bp end homologies that each differed in GC content (ranging from 40% to 80%) and sequence. These vector/insert combinations were subjected to PPY SLiCE. We did not find a significant difference in the cloning efficiencies indicating that SLiCE is indeed sequence independent within the range of sequence diversity tested (Supplementary Table S3). Next, we generated four fragments with 20-bp end homologies containing a mismatch at different positions. We found that a single mismatched nucleotide at the end of the 20-bp homology produced one type of recombination product and the heterologous nucleotide was removed during SLiCE. A single mismatched nucleotide within the middle of the 20-bp homology produced two types of recombination products in which either one of the mismatched nucleotides was retained. In addition, we observed that the presence of a single mismatch only led to a slight reduction in cloning efficiency (Supplementary Table S4).

To determine the effect of insert length on PPY SLiCE cloning efficiencies we attempted to assemble vectors ranging in size from 2 to 15 kb and containing inserts ranging from 80 bp to 21 kb in size. We found that the cloning of larger fragments by SLiCE occurred at robust but somewhat reduced efficiencies. For example, the assembly of an 11-kb recombinant plasmid containing an 8-kb insert was achieved at a cloning efficiency of 140 CFU/ng of vector. The restriction analysis of 24 clones revealed that 22 contained the expected recombinant plasmid.

At present, we have successfully used SLiCE-mediated cloning to generate more than 100 recombinant plasmids employing various cloning strategies and many different vector/insert combinations. Our results indicate that SLiCE can be considered a universal cloning method for the generation of recombinant DNA at high fidelity. Furthermore, the nature of vector and insert ends such as blunt ends or 3′ or 5′ sequence overhangs did not influence SLiCE efficiency or accuracy. However, the use of vectors with complementary 5′ or 3′ overhanging ends for SLiCE increased the formation of empty vector background colonies, which is probably due to annealing of the single-stranded ends in the bacterial extracts or in the transformed host cells.

PPY SLiCE with flanking heterologous sequences

The seamless cloning activity of PPY SLiCE was examined using the same pBL-DL–LacZα cloning strategy as for DH10B SLiCE with flanking heterologous sequences at one side (2, 319, 738 and 998 bp) or on both sides (45 bp plus 23 bp and 319 bp plus 738 bp) and with various end homologies.

In comparison to DH10B extracts, PPY extracts presented a much stronger seamless activity, which can remarkably increase the efficiency of DNA cloning especially with shorter flanking heterologies (45 bp plus 23 bp on both sides; 7600 CFU versus 1265 CFU per ng of vector, PPY extract versus DH10B extract). In addition, PPY SLiCE can efficiently remove longer flanking heterologous sequences of up to 998 bp on one side or up to 319 bp plus 738 bp on both sides (Table 2). In general, vectors with shorter flanking sequences or flanking sequences on only one side yielded higher cloning efficiencies compared to vectors with longer and/or double-sided flanking heterologous sequences (Table 2). Similar to SLiCE cloning without flanking heterologous sequences, longer end homologies between vector and insert resulted in higher cloning efficiencies (Table 2).

SLiCE cloning with multiple fragments

The high cloning efficiency and fidelity of PPY SLiCE suggested it might be possible to generate more complex recombinant plasmids using multiple inserts in a single cloning reaction. To test this idea we designed two different SLiCE strategies for the cloning of multiple insert fragments. In the first strategy, we attempted to clone multiple inserts into one vector in one SLiCE reaction to generate a single recombinant DNA molecule derived from multiple fragments that was termed multiple-way SLiCE cloning (Figure 2a). The second strategy was designed to clone several different inserts carrying the same end homology into a vector in one SLiCE reaction in parallel. This strategy creates multiple different recombinant DNA molecules and was termed SLiCE batch cloning (Figure 2b).

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Table 2. PPY SLICE with flanking heterologous sequences

Homology length (bp)	Flanking heterology length (bp)		Vector length (bp)	Cloning efficency	Cloning accuracy (%)
	Side 1	Side 2			
20	2	0	2500	10 000	99
42	319	0	2803	2270	99
30	319	0	2803	1250	98
42	738	0	3222	1232	87
30	738	0	3222	432	77
42	998	0	3482	570	94
42	45	23	2552	7600	81
30	45	23	2552	1288	63
20	45	23	2552	710	59
42	319	738	3541	5	98

Cloning efficiencies are given as CFUs of blue colonies per nanogram of vector. Cloning accuracies are given as the percentage of blue colonies among the total number of all amp^r colonies (blue and white). Vectors containing different end heterologies were derived from plasmid pBL-DL by digesting with various restriction enzymes. LacZ inserts of 500-bp size containing the indicated end homologies were generated by PCR. The experiments were performed using 10–40 ng/μl vector DNA and the corresponding amount of insert DNA at a 1 : 6 molar ratio of vector:insert in a 10 μl reaction volume. The blue colonies contain recombinant plasmid and the white colonies contain non-recombinant vector background.

To examine the potential of SLiCE for multiple-way cloning, three-, four- and seven-way SLiCE cloning was performed using the pBL vector and three different sets of PCR amplified inserts with 42 bp of end homology that can assemble into a single 1.9-kb DNA fragment expressing LacZα activity (Figure 2c and Table 3). Our studies showed that PPY SLiCE mediated three-, four- and seven-way cloning occurred at significant efficiencies and high accuracies (Table 3). DNA sequencing analysis showed that all of the multiple fragments were precisely joined by SLiCE-mediated multiple-way cloning.

We next examined multiple-way SLiCE cloning using other vectors and inserts. A three-way SLiCE using a 4.7-kb vector (p3XFLAG-CMV-7.1, Invitrogen) and two 250-bp inserts with 24 bp of end homologies produced about 500 CFU/ng of vector with a 10-fold stimulation over non-recombinant background colonies. In another three-way experiment we successfully assembled a 3-kb vector and two 2.6- and 2.5-kb inserts using 42 bp of end homology with 80% accuracy and a cloning efficiency of 60 CFU/ng of vector. To determine the ability of multiple-way SLiCE cloning to assemble highly complex vector/insert combinations, a four-way cloning strategy (42-bp end homology, 2.5 kb of vector, inserts of 500 bp, 1.4 and 2.5 kb) and a seven-way strategy with shorter end homology (24-bp homology, 2.5 kb of vector and six inserts totaling 2 kb) were performed. For both multiple-way cloning strategies the cloning efficiencies were reduced but still provided at least 20 CFU/10 ng of vector.

For SLiCE batch cloning, two sets of experiments were performed. Six PCR inserts varying from 300 bp to 1 kb with 30 bp of end homology were mixed together with a linearized 6.7-kb prokaryotic expression vector (PTXB1, NEB) and incubated in PPY SLiCE extract (Figure 2b). About 340 CFU/ng of vector were obtained. The analysis of 32 colonies showed that all six possible recombinant vector/insert combinations were obtained (Figure 2d and e). Another experiment using a 4.7-kb mammalian expression vector (p3XFLAG-CMV-7.1, Invitrogen) and three inserts of 1, 1.5 and 2.5 kb with 24-bp homology yielded similar results (data not shown).

SLiCE cloning of genomic fragments from BAC clones

It is often challenging to subclone a genomic DNA fragment from larger DNA vectors such as BACs into a plasmid vector. Due to the high cloning efficiency of PPY SLiCE, we tested whether SLiCE could also facilitate this type of cloning. A SLiCE cloning strategy was designed to subclone individual genomic DNA fragments from BAC vectors (Figure 3a). Specifically, BAC DNA isolated from clone *RP23-303G13* (CHORI), 165 kb in size and containing 66 *Bgl*II and 19 *Eco*RV restriction sites, was digested with either *Bgl*II or *Eco*RV to generate a complex pool of DNA fragments. The digested BAC DNA was phenol/chloroform purified and subjected to PPY SLiCE cloning with PCR-generated pBluescript II KS(+) (Stratagene) derived vectors that contained end homologies to different *Bgl*II or *Eco*RV BAC restriction fragments. We attempted SLiCE cloning of several *Bgl*II BAC fragments of different sizes (830 bp, 3.7, 6.7, 8.7 and 14 kb) with 42 or 52 bp of end homology. In addition, SLiCE cloning was also performed for several *Eco*RV BAC fragments of larger sizes (5.3, 6.3, 12.2 and 21 kb) (Table 4). In all cases we were able to obtain recombinant clones carrying the different BAC fragments with high or acceptable cloning efficiencies (Table 4 and Figure 3b and c), indicating that SLiCE cloning is an effective strategy for the directional subcloning of small or large BAC genomic fragments.

DISCUSSION

The observation that bacterial cell extracts can efficiently recombine DNA molecules using short-end homologies was a serendipitous discovery in our laboratory. After initial characterization and further optimization, we were able to establish a novel restriction site

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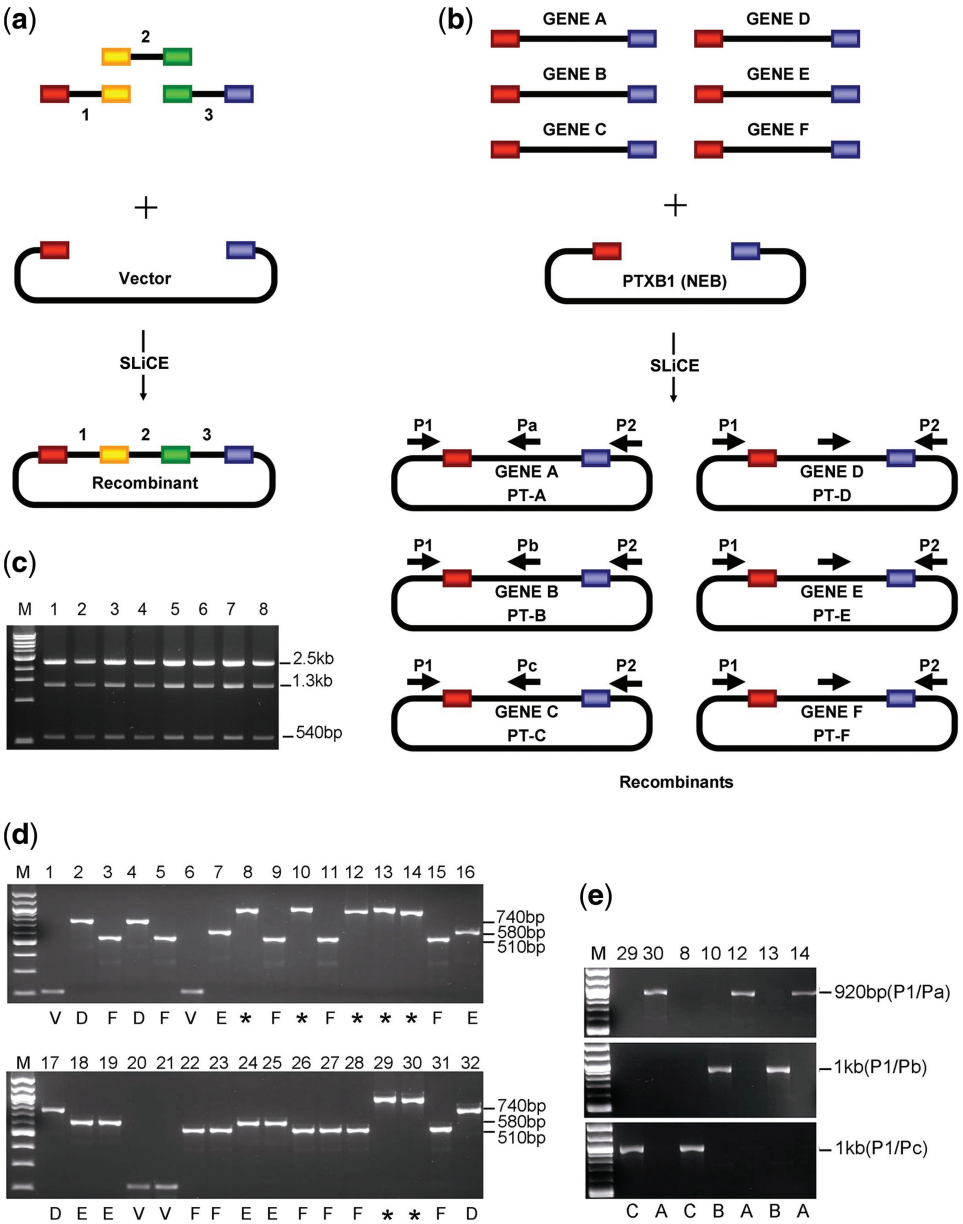

Figure 2. SLiCE cloning with multiple fragments. (**a**) Schematic illustrating multiple-way SLiCE cloning. A three-way cloning approach is shown. (**b**) Schematic illustrating SLiCE batch cloning. Six PCR inserts with 30-bp homology and plasmid vector PTXB1 (NdeI/SapI) were subjected to SLiCE batch cloning. P1, P2, Pa, Pb and Pc refer to the primers for colony PCR screening. (**c**) BsaAI/XmnI restriction analysis of the recombinants derived from seven-way SLiCE cloning. Plasmid DNAs from eight independent ampicillin-resistant colonies (lanes 1–8) were digested with BsaAI/XmnI. Recombinants contain one BsaAI site and one XmnI site located within the vector and one BsaAI site located in the insert yielding diagnostic 2.5-, 1.3-kb and 540-bp restriction fragments. (**d**) PCR screening of recombinants derived from SLiCE batch cloning of six different inserts. Thirty-two independent colonies (lanes 1–32) were subjected to PCR analysis with primer pair P1/P2. Recombinant plasmids PT-A, PT-B and PT-C all yielded PCR products of ~1 kb (lanes labeled with asteriscs). Recombinant plasmids PT-D, PT-E and PT-F yielded PCR products of 740, 586 and 510 bp, respectively (lanes labeled D, E and F). Non-recombinant vector pTXB1 yielded a PCR product of 210 bp (lanes labeled V). (**e**) To identify PT-A, PT-B and PT-C recombinants were further analyzed using primer pairs P1/Pa, P1/Pb and P1/Pc. The PCR products of PT-A using primer pair P1/Pa, PT-B using primer pair P1/Pb and PT-C using primer pair P1/Pc were 920 bp, 1 and 1 kb, respectively (lanes labeled A, B and C).

independent and seamless cloning method that we termed SLiCE. We found that simple cell extracts from two RecA deficient laboratory strains, JM109 and DH10B, were able to efficiently recombine vector and insert DNA containing short end homologies. In addition, we found that these strains can be further optimized for SLiCE by simple genetic modification. In this study we introduced the genes of the λ prophage Red recombination system and the *gam* gene into the DH10B genome to generate a new bacterial strain termed PPY. This strain currently provides the highest cloning efficiencies and facilitates SLiCE in a wide variety of cloning applications.

Bacterial extracts have been shown to efficiently catalyze RecA-dependent homologous recombination (15). However, SLiCE-mediated cloning is working most efficiently in RecA-deficient extracts indicating that it

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Table 3. Multiple-way PPY SLICE cloning

Multiple-way SLiCE	Inserts length (bp)	Cloning efficiency	Cloning accuracy (%)
Three-way	520 and 1400	6610	88
Four-way	520, 762 and 760	4080	96
Seven-way	420, 400, 330, 220, 280 and 550	3260	90

Cloning efficiencies are given as CFUs of blue colonies per nanogram of vector. Cloning accuracies are given as the percentage of blue colonies among the total number of all amp^r colonies (blue and white). The 2.5-kb vector pBL was linearized by NotI/SalI digestion and the inserts were prepared by PCR. Experiments were performed using 10 ng/µl of vector and the corresponding amount of insert DNA at a 1 : 6 molar ratio of vector:insert. The blue colonies contain recombinant plasmid and the white colonies contain non-recombinant vector background.

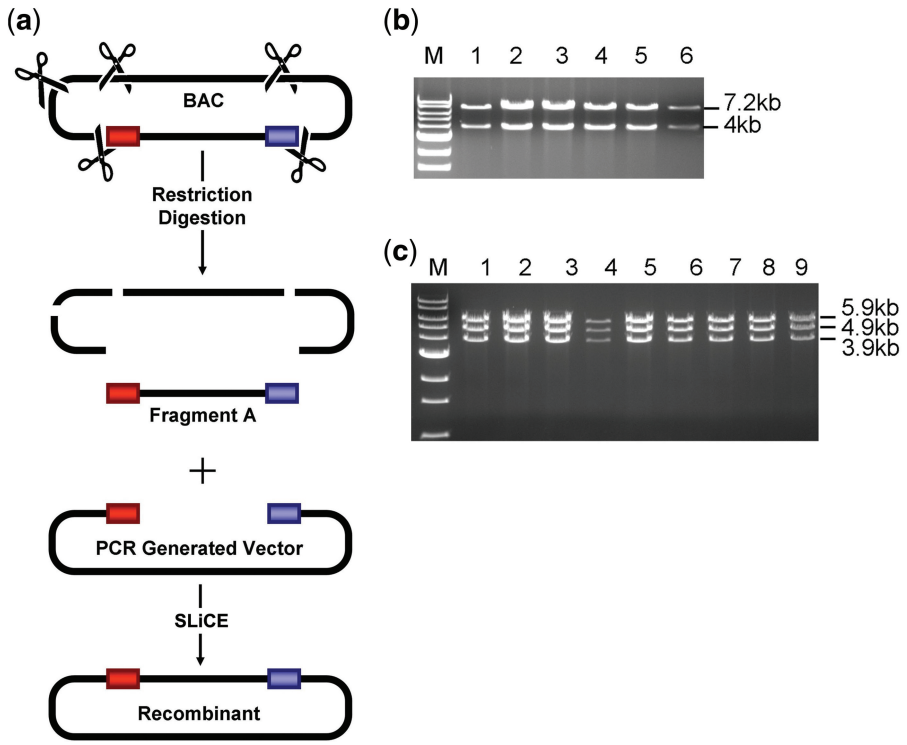

Figure 3. BAC SLiCE Cloning. (**a**) Schematic illustrating BAC SLiCE cloning. (**b**) XmnI/XhoI restriction analysis of recombinants derived from SLiCE cloning of an 8.7-kb BglII BAC fragment. Plasmid DNAs from six independent ampicillin-resistant colonies (lanes 1–6) were digested with XmnI and XhoI. Recombinants contain one XmnI site within the vector and one XhoI site within the insert yielding diagnostic 7.2- and 4-kb restriction fragments. (**c**) BamHI/BglII restriction analysis of recombinants derived from SLiCE cloning of a 12.2-kb EcoRV BAC fragment. Plasmid DNAs from nine independent ampicillin-resistant colonies (lanes 1–9) were digested with BamHI and BglII. Recombinants contain one BglII site within the vector and two BamHI sites in the insert yielding diagnostic 5.9-, 4.9- and 3.9-kb restriction fragments.

Table 4. BAC SLICE cloning

End homology length (bp)	Restriction enzyme	Insert length (kb)	Cloning efficiency	Cloning accuracy (%)
42	BglII	0.83	97	75
42	BglII	3.7	37	44
42	BglII	6.7	171	47
52	BglII	8.7	52	12
52	BglII	14	42	14
42	EcoRV	5.3	197	62
42	EcoRV	6.3	277	76
52	EcoRV	12.2	130	66
52	EcoRV	21	19	53

Cloning efficiencies are given as CFUs per nanogram of vector. Cloning accuracies are given as the percentage of correct clones among the total number of amp^r clones. pBluescript II KS+ (Stratagene) was used as template to PCR amplify linear vectors containing end homologies corresponding to various BglII or EcoRV restriction fragments in BAC clone RP23-303G13. Vector DNA (10–20 ng/µl) and total BglII or EcoRV digested BAC DNA (1 ug/µl) were subjected to PPY SLiCE cloning.

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utilizes a different recombination pathway. The existence of a RecA-independent recombination pathway in *E. coli* that mediates exchange at short homologies has been proposed (10). It was suggested that this RecA-independent recombination mechanism involves the generation of short 5′ and 3′ tailed strands that anneal to homologous molecules (11) and that *E. coli* single-strand exonucleases such as RecJ, ExoVII, ExoI and ExoX could degrade these tails and abort the exchange reaction. Consistent with this notion, the RecA-independent recombination was stimulated in the absence of these single-strand exonucleases. However, the mechanism of RecA-independent recombination is unknown and it remains to be seen whether this recombination pathway is also responsible for SLiCE-mediated recombination. It is likely that SLiCE-mediated cloning involves the activities of exonucleases or helicases for the generation of single-strand tails at the ends of vectors and inserts, single-strand binding proteins for the stabilization of these single-strand overhangs and possibly other factors that protect 5′ and 3′ tails from degradation and facilitate their annealing. Consistent with this idea we found, that although SLiCE mediated recombination is efficient in the absence of the prophage Red/ET recombination systems, the introduction of the *red*α, *red*β and *gam* genes, which facilitate similar transactions at single stranded DNA ends, into DH10B bacteria greatly enhanced SLiCE mediated cloning.

Although SLiCE shares some features with other recently developed *in vivo* recombineering methods (16–19), it is different in several aspects. Recombineering methods provide useful tools for DNA modification and depend on homologous recombination that is mediated by the λ-prophage encoded Red recombination system *in vivo* in bacterial cells. In contrast, SLiCE is an *in vitro* recombination method facilitated by bacterial cell extracts. The Red recombination system is not required for SLiCE but can be used to further increase the cloning efficiencies of SLiCE. In addition, the main application of recombineering is the modification of large DNA molecules such as BACs or bacterial genomes, while SLiCE can be used as a general cloning method for the generation of recombinant plasmids.

Compared to conventional ligation dependent cloning methods including the cloning of DNA fragments with sticky or blunt ends generated by restriction digestion or the TA cloning of PCR fragments, SLiCE has several important advantages: (i) It is a time and labor saving method that consists of a one-hour/one-tube reaction followed by standard transformation of host bacteria. (ii) It does not require any prior treatment of end sequences. (iii) It can be used to directionally clone one or more fragments into any vector with high efficiency and fidelity. (iv) It promotes seamless cloning without leaving any unwanted sequences at the cloning junctions.

In addition to SLiCE, several other *in vitro* homologous recombination based cloning systems have recently been described, such as LIC-PCR and SLIC (20–23), Exonuclease III induced ligase-free directional subcloning of PCR products (24), Ribocloning (25), Enzyme-free cloning (26) and six commercially available cloning systems including In-Fusion™ PCR Cloning (27–29) (Clontech), Cold Fusion Cloning Kit (SBI), Fast Seamless Cloning Kit (Dogene), CloneEZ® Kit(Genescript), and GENEART® Seamless Cloning and Assembly Kit (invitrogen). SLIC uses the 3′–5′ exonuclease activity of T4 DNA polymerase to generate ssDNA overhangs in insert and vector which are required for the fusion of vector and insert fragments by single strand annealing with or without the addition of RecA. Exonuclease III induced ligase-free directional subcloning uses 3′–5′ exonuclease activity of Exonuclease III to generate ssDNA overhangs which facilitate cloning (24). Ribocloning uses Rnase A to cleave at single rC or rU bases that were introduced by PCR into vector and insert and subsequent heating to generate ssDNA overhangs for cloning. Enzyme-free cloning creates complementary ssDNA overhangs by PCR with tailed primer sets and post-PCR denaturation-hybridization reactions. In-Fusion™ PCR Cloning promotes PCR cloning by the In-Fusion enzyme, a poxvirus DNA polymerase with 3′–5′ exonuclease activity. The mechanisms or enzymatic activities involved in the other commercial cloning systems have not been disclosed by the suppliers, but it is likely that they utilize processes that are similar to that of SLIC and In-Fusion™ PCR Cloning.

Exonuclease III induced ligase-free directional subcloning is only useful for cloning fragments with blunt or 5′ protruding ssDNA ends but not compatible for cloning of fragments with 3′ protruding ssDNA ends. Furthermore, the method probably depends partially on the helical structure of the DNA fragments and displays sequence dependence (C > A = T > G) (24). Ribocloning and Enzyme-free cloning require PCR amplification to generate both vector and insert and cannot facilitate the cloning of DNA fragments generated by restriction digestion. In addition, Ribocloning requires special PCR primers containing ribonucleotides (rC or rU) at the 3′-ends and enzyme-free cloning requires four pairs of primers for one reaction and is not suitable for multiple-way cloning (25).

In comparison to SLiCE, SLIC requires optimization. Vector and insert fragments for SLIC need to be treated with T4 DNA polymerase and the treatment duration is not always constant but depends on the homology length (23).

The efficient seamless cloning activity is one of the most important features of SLiCE as it allows the recombining of vector and inserts *in vitro* even in the presence of flanking heterologous sequences of up to 998 bp on one side or 319 bp plus 738 bp on both sides. This property decreases the sequence dependence of end cloning by SLiCE and greatly extends its usefulness for many applications such as replacing unwanted sequences adjacent to the cloning sites without any prior treatment even in the absence of appropriate restriction sites. For example, in a single SLiCE reaction, we subcloned the open reading frame of the Pms2 gene into the mammalian expression vector p3XFLAG-CMV-7.1 (Invitrogen) and deleted 72 bp of an unwanted sequence tag flanking the cloning site within the vector, which could not be deleted by restriction digestion. SLIC also has seamless cloning

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activity; however, it is limited to flanking heterologies of only up to 20 bp. There are no reports that In-Fusion™ PCR Cloning (Clontech) or any of the other *in vitro* cloning systems have such activity. Furthermore, SLiCE is the only known *in vitro* recombination based method for the directional subcloning of genomic BAC fragments into plasmid vectors. At present it is not clear if the other *in vitro* cloning systems can be used for this application.

In summary, SLiCE is an easy, efficient and inexpensive cloning method that allows the generation of recombinant plasmid vectors in a seamless and precise fashion. It requires the generation of simple bacterial cell extracts from readily available lab strains and does not require the use of restriction enzymes or DNA end modification enzymes such as Klenow or T4 DNA polymerase. In addition, the joining of vector and insert fragments by DNA ligase is not required. SLiCE is also a highly versatile method, and its capabilities can be expanded by the generation of additional optimized bacterial strains in the future.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–4.

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The PPY strain can be obtained upon request (yongwei.zhang@einstein.yu.edu).

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