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A simple and efficient seamless DNA cloning method using SLiCE from *Escherichia coli* laboratory strains and its application to SLiP site-directed mutagenesis

Ken Motohashi

Abstract

Background: Seamless ligation cloning extract (SLiCE) is a simple and efficient method for DNA assembly that uses cell extracts from the *Escherichia coli* PPY strain, which expresses the components of the λ prophage Red/ET recombination system. This method facilitates restriction endonuclease cleavage site-free DNA cloning by performing recombination between short stretches of homologous DNA (≥15 base pairs).

Results: To extend the versatility of this system, I examined whether, in addition to bacterial extracts from the PPY strain, other *E. coli* laboratory strains were suitable for the SLiCE protocol. Indeed, carefully prepared cell extracts from several strains exhibited sufficient cloning activity for seamless gene incorporation into vectors with short homology lengths (approximately 15–20 bp). Furthermore, SLiCE was applied to the polymerase chain reaction (PCR)-based site-directed mutagenesis method, in a process termed "SLiCE-mediated PCR-based site-directed mutagenesis (SLiP site-directed mutagenesis)". SLiP site-directed mutagenesis simplifies the steps of PCR-based site-directed mutagenesis, as it exploits the capability of the SLiCE method to insert multiple fragments.

Conclusions: SLiCE can be performed in the laboratory with no requirement for a special *E. coli* strain, and the technique is easily established. This method increases the cloning efficiency, shortens the time for DNA manipulation, and greatly reduces the cost of seamless DNA cloning.

Keywords: Homologous recombination, Seamless DNA cloning, SLiCE, Site-directed mutagenesis, Plant redox-related gene

Background

The manipulation of recombinant DNA molecules is an indispensable step in current molecular biology research. Type IIP restriction endonucleases and DNA ligases were the original "workhorses" utilized to generate plasmids or other types of DNA vectors [1]. Recently, various restriction endonuclease cleavage site-independent cloning methods, which overcome the limitations associated with the lack of unique restriction enzyme sites, have been described [2–13]. These methods are based on homologous recombination, in which overlapping sequences present at the 5′ and 3′ ends of DNA fragments are combined.

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Seamless Ligation Cloning Extract (SLiCE), which was initially developed as a novel bacterial cell extract-based DNA cloning method, utilizes the *in vitro* homologous recombination activity of *Escherichia coli* cell extracts. Zhang *et al.* reported that cell extracts from the *E. coli* PPY strain, which contains the λ prophage Red/ET recombination system, could efficiently assemble DNA fragments with short end overlaps of at least 15 base pairs (bp) *in vitro* [14]. SLiCE is a simple and highly cost-effective approach for vector construction because, apart from adenosine triphosphate (ATP) and commonly used buffers, only an *E. coli* lysate was required for the seamless ligation of DNA fragments into vectors. However, cell extracts from DH10B (a common *E. coli* laboratory strain) did not exhibit efficient cloning activity

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with short end homology fragments (lengths with a range of 15–20 bp) [14].

Many methods for site-directed mutagenesis have been developed, including QuickChange site-directed mutagenesis [15], the overlap extension method [16, 17], and the megaprimer method [18, 19]. QuickChange site-directed mutagenesis is widely used as a simple polymerase chain reaction (PCR)-based method that does not require the purification of PCR fragments [20–23]. However, mutations may occur in the vector at non-desired sites during PCR amplification, which can compromise the fidelity of the approach. On the other hand, because the overlap extension and megaprimer methods utilize vectors that have been digested with restriction endonucleases, introduction of mutations in the vector region is avoided [24–26]. However, vector construction by these methods requires two sequential PCR reactions, and the purification of insert DNA fragments. To simplify these multiple-step methods, SLiCE has been applied to overlap extension PCR-based site-directed mutagenesis, because it can simultaneously integrate several DNA fragments into a vector [14]. In this study, I demonstrated that SLiCE-mediated PCR-based site-directed mutagenesis (SLiP site-directed mutagenesis) could be performed using extracts of a regular laboratory *E. coli* strain. This approach can be adopted in studies that require precise generation of mutants in the absence of unwanted alterations to the vector backbone.

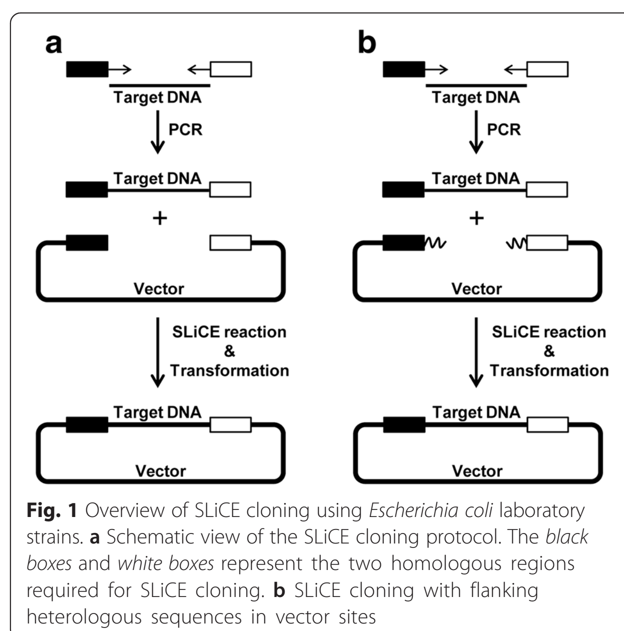

Fig. 1 Overview of SLiCE cloning using *Escherichia coli* laboratory strains. **a** Schematic view of the SLiCE cloning protocol. The *black boxes* and *white boxes* represent the two homologous regions required for SLiCE cloning. **b** SLiCE cloning with flanking heterologous sequences in vector sites

Results

Estimation of SLiCE from *E. coli* laboratory strains

Both the colony formation rate (number of colonies) and the ratio of correct clones (cloning efficiency) in transformation are important determinants for efficient cloning of PCR fragments. Cell lysates from *E. coli* $RecA^-$ strains such as DH10B contain endogenous *in vitro* homologous recombination activity, and can be used to clone PCR fragments into vectors with homology regions. However, cloning with lysates from this strain is not efficient, particularly in the case of inserts with short homology lengths (approximately 15–20 bp), because of a lower colony formation rate [14]. An *E. coli* PPY strain that expresses an optimized λ prophage Red/ET recombination system circumvents this problem by increasing the colony formation rate during PCR fragment cloning [14]. To extend the utility of this method, I prepared SLiCE extracts from several *E. coli* laboratory strains with some modifications, and estimated the efficiency with which redox-related genes from *Arabidopsis* could then be cloned into expression vectors [27–30] (Fig. 1a). The SLiCE extracts from common $RecA^-$ *E. coli* laboratory strains such as DH10B, JM109, DH5α, XL10-Gold and Mach1 T1 supported the cloning of PCR fragments containing a 19-bp overlap region into the vectors, and were associated with a high colony formation rate (Table 1). Additionally, SURE2, a $RecA^+$, *recB recJ* strain, also facilitated the cloning of PCR fragments with almost the same efficiency. No extract that contained only insert DNA fragments and linearized vector could also clone the PCR fragments into vectors using homologous recombination activity *in vivo* in host cells (Table 1) [31]. However, the transformation efficiencies ("Number of colonies" in Table 1) were less effective with 1/40 – 1/200 of bacterial SLiCE from several *E. coli* laboratory strains. Together, these data highlight the specificity of the new method. The improved SLiCE preparation could support the cloning of the PCR fragments into vector, with an efficiency of $2 – 10 \times 10^3$ colonies/ng vector; this yield was obtained with a 19-bp overlap region and using ~2×10^8 CFU/μg of pUC19 DNA chemically competent cells. In contrast, a previously reported SLiCE from the DH10B strain yielded a more modest efficiency of 75–80 colonies/ng vector; in this case, 15–20 bp overlap lengths and 1×10^{10} CFU/μg of pUC19 DNA high efficiency electrocompetent cells were used [14]. The SLiCEs from *E. coli* laboratory $RecA^-$ strains used in this study were sufficiently active to achieve seamless cloning with respect to both colony formation rate (number of colonies) and the ratio of the correct clones (cloning efficiency). These results indicate that *E. coli* laboratory strains other than *E. coli* PPY can be used as the SLiCE sources. Therefore, SLiCE from the JM109 strain was used in all subsequent experiments.

Next, the effect of short homology length on cloning efficiency was evaluated using PCR fragments containing overlaps of varied lengths (Table 2). To determine whether the cloning fidelity was maintained at all overlap lengths, DNA sequencing was performed. This revealed

Table 1 The cloning efficiencies using SLiCE from different *E. coli* laboratory strains

Strain	Number of colonies[a]		Cloning efficiency[b]	
	Prx IIE (AT3G52960)	G6PDH1 (AT5G35790)	Prx IIE (AT3G52960)	G6PDH1 (AT5G35790)
no extract	47.0 ± 4.6	63.7 ± 5.5	16/18	17/18
DH10B	4,630 ± 879	3,020 ± 63.5	17/18	18/18
JM109	9,960 ± 240	5,300 ± 820	18/18	18/18
DH5α	6,130 ± 348	4,340 ± 979	18/18	18/18
XL10-Gold	6,210 ± 652	3,610 ± 287	17/18	18/18
Mach1 T1	9,530 ± 411	2,310 ± 416	18/18	18/18
SURE2	8,490 ± 896	6,040 ± 1,380	16/18	17/18

[a]Number of colonies is represented as CFU per nanogram of vector. Each value of "number of colonies" is the mean ± standard deviation of three independent experiments. [b]Cloning efficiencies for the insert DNA are represented as "number of clones with the confirmed correct insert length by colony-PCR/number of colonies subjected to colony-PCR". The insert DNA fragments were amplified using 19-bp overlap primers. The linearized vector DNA was prepared by PCR. The SLiCE reaction was performed for 60 min at 37 °C with an insert:vector ratio of 1:1 and 3:1 for Prx IIE and G6PDH1, respectively

that a minimum overlap length of 15 bp was required for correct insertion of the DNA fragments into the vector. Although DNA assembly by homologous recombination with a SLiCE from the PPY strain required more than 15 bp of overlapping homology [14], I found that a 10-bp overlap was sufficient for the assembly of insert DNA into the vector. However, both the cloning efficiency and fidelity of a 10-bp overlap assembly were slightly reduced. PCR fragments with a 19-bp overlap region resulted in the maximum number of colonies upon transformation.

SLiCE can assemble insert DNA fragments into restriction enzyme-digested vectors with flanking heterologous sequences

PCR-amplified insert DNAs have often been cloned into vectors digested at restriction enzyme cleavage sites as a standard protocol [32–37]. To evaluate the cloning efficiency using such an approach, pET23 vectors were digested with restriction enzymes to yield flanking heterologous sequences (Fig. 1b). The cloning efficiency for the digested vector was determined using multiple pairwise combinations of cloning sites (Table 3). I found that inserts could be incorporated into vectors with heterologous flanking regions, although the cloning efficiency varied according to the precise combination of restriction enzymes used (Table 3 and Additional file 1: Figure S1). The presence of the heterologous flanking region at the 5′ or 3′ end of the vector DNA did not inhibit the correct ligation of the insert DNA to the vector (Table 3; *Nde*I[or *Nco*I]-*Bam*HI and *Bam*HI-*Xho*I). However, the presence of heterologous flanking regions at both the 5′ and 3′ ends of the vector DNA markedly reduced the cloning efficiency (Table 3; *Bam*HI). The fidelity of colony-PCR positive clones was minimally affected by the presence of the heterologous flanking sequences.

Optimization of the SLiCE reaction

Next, the conditions for the SLiCE reaction were optimized for extracts from *E. coli* JM109. Time-course experiments indicated that transformation efficiencies were more than 3×10^3 colonies/ng vector over incubation periods between 5 and 60 min, and the reaction reached saturation rapidly (Fig. 2a). These transformation efficiencies were sufficient for usual DNA cloning, using conventional chemically competent cells. Incubations lasting more than 90 min reduced the colony formation rate. Longer incubation times might induce degradation of the insert and vector DNA, since the SLiCE contains

Table 2 Effect of end homology length on SLiCE cloning

Homology length (bp)	Number of colonies[a]		Cloning efficiency		Cloning accuracy (%)[b]	
	Prx IIE	G6PDH1	Prx IIE	G6PDH1	Prx IIE	G6PDH1
10	558 ± 74.5	585 ± 121	11/16	16/16	100.0	93.8
15	875 ± 43.9	777 ± 186	16/16	15/16	100.0	100.0
19	972 ± 162	1,070 ± 289	15/16	16/16	100.0	100.0
24	926 ± 28.6	519 ± 74.8	16/16	16/16	100.0	100.0
29	483 ± 34.8	520 ± 51.4	16/16	16/16	100.0	100.0
34	105 ± 37.2	150 ± 47.1	14/16	16/16	100.0	87.5

[a]Each value of "number of colonies" is the mean ± standard deviation of three independent experiments. [b]Cloning accuracies are given as the percentage of correctly cloned expression vectors in colony-PCR positive clones. The insert DNA fragments and linearized vector DNA were prepared by PCR. The SLiCE (JM109) reaction was performed for 10 min at 37 °C with 1:1 and 3:1 molar ratios of insert to vector for Prx IIE and G6PDH1, respectively

Table 3 The SLiCE cloning efficiencies of linearized vectors prepared by digestion with different restriction enzymes

Restriction enzymes	Flanking heterologous length (bp)	Number of colonies[a]		Cloning efficiency		Cloning accuracy (%)	
		Prx IIE	G6PDH1	Prx IIE	G6PDH1	Prx IIE	G6PDH1
*Nde*I - *Xho*I	0 + 0	9,680 ± 651	-	18/18	-	100	-
*Nco*I - *Xho*I	0 + 0	-	8,470 ± 2,180	-	17/18	-	94.1
*Bam*HI	40 + 40	926 ± 62.0	576 ± 17.6	1/18	5/18	100	100
*Nde*I - *Bam*HI	0 + 40	164 ± 20.8	-	12/18	-	75.0	-
*Nco*I - *Bam*HI	0 + 40	-	195 ± 83.3	-	4/18	-	100
*Bam*HI - *Xho*I	40 + 0	857 ± 298	960 ± 71.3	9/18	12/18	88.9	83.3

The insert DNA fragment was prepared by PCR. The linearized pET23a or pET23d vectors were prepared by digestion with restriction enzymes. The multiple cloning site of pET23a (or pET23d) are displayed in Additional file 1: Figure S1. The SLiCE (JM109) reaction was performed for 60 min at 37 °C with 1:1 and 3:1 molar ratios of insert to vector for Prx IIE and G6PDH1, respectively. [a]Each value of "number of colonies" is the mean ± standard deviation of three independent experiments

bacterial nucleases. A 1:1 to 3:1 molar ratio of insert:vector yielded highly efficient cloning, with more than 2×10^3 colonies/ng vector being formed (Fig. 2b). The enhancement of transformation efficiency in *E. coli* by modification of the SLiCE preparation protocol allows the simplification of several steps, including purification of insert and vector DNA fragments. Therefore, the influence of the DNA fragment purification steps on cloning efficiency was also evaluated (Table 4). Unpurified PCR fragments could be inserted to the vector, although with a lower transformation efficiency. By contrast, ethanol precipitation or ExoSAP-IT (Affymetrix, Santa Clara, CA) treatment of insert DNA fragments improved the transformation efficiency between 5- and 7-fold. PCR purification columns or purification via agarose gel electrophoresis improved the efficiency between 37- and 138-fold. We also observed a high cloning efficiency (at the optimal insert:vector ratio) regardless of the purification steps used.

Application of SLiCE to PCR-based site-directed mutagenesis

Previously, the SLiCE method, using the *in vitro* homologous recombination activity of lysates from the *E. coli* PPY strain, was employed to integrate multiple DNA fragments into vectors in a single cloning reaction [14]. The overlap extension method, which is used for PCR-based site-directed mutagenesis (Fig. 3a), has an advantage in that it can use restriction enzyme-digested vectors, which excludes the possibility of acquiring a mutation in the vector region [16, 17]. SLiCE was applied to generate cysteine-to-serine mutants at critical residues in redox-related proteins [26, 38–40]. To generate the cysteine mutants, pairs of insert DNA fragments containing a mutation site and short end homologies at both sides were amplified by PCR, and two fragments were simultaneously integrated into pET vectors using SLiCE (Fig. 3b). Both unpurified and column-purified DNA fragments could be inserted into the vector. The use of column-purified insert DNA increased the number of colonies following transformation by approximately 4- to 16-fold, although the cloning efficiencies were not improved (Table 5). Thus, this new SLiCE-mediated PCR-based site-directed mutagenesis (SLiP site-directed mutagenesis) technique simplifies the original overlap extension method by removing several steps. Although QuickChange site-directed mutagenesis also minimizes the number of steps, SLiP site-directed mutagenesis has the added advantage that only the insert region (and not the whole plasmid) is amplified by PCR.

Discussion

In this study, I demonstrated that a wide variety of laboratory $RecA^-$ strain extracts can be used to clone PCR fragments, in the absence of any special modifications to the strains. In the case of short homology lengths (approximately 15–20 bp), SLiCEs from several *E. coli* laboratory strains yielded good colony formation rates, which were at least three orders of magnitude greater than those obtained in a previous study [14]. Unexpectedly, SURE2, which is a $RecA^+$, *recB recJ* strain, also retained SLiCE activity. Although the mechanisms associated with the *in vitro* homologous recombination activity of *E. coli* extracts are not fully characterized, routine cloning using several *E. coli* extracts works well even with cells that are not highly electrocompetent. The SLiCEs from *E. coli* laboratory strains in this study have such high transformation efficiencies because several steps of the original SLiCE preparation protocol [14] have been modified. Firstly, *E. coli* cells were harvested at OD_{600} = 2.0–3.0 (late log phase). Secondly, extraction of *E. coli* lysates was performed under 4 °C, and ice-cold solutions were used during the preparation. These modifications might improve the transformation efficiency following the use of SLiCEs from *E. coli* laboratory strains.

In addition, the SLiCE method was simplified by optimizing the reaction conditions (Fig. 2 and Table 4). Although purification of insert DNA fragments was not essential for the assembly of fragments into the vectors, selecting a purification step improved cloning efficiency (Table 4). A 19-bp overlap sequence is recommended as

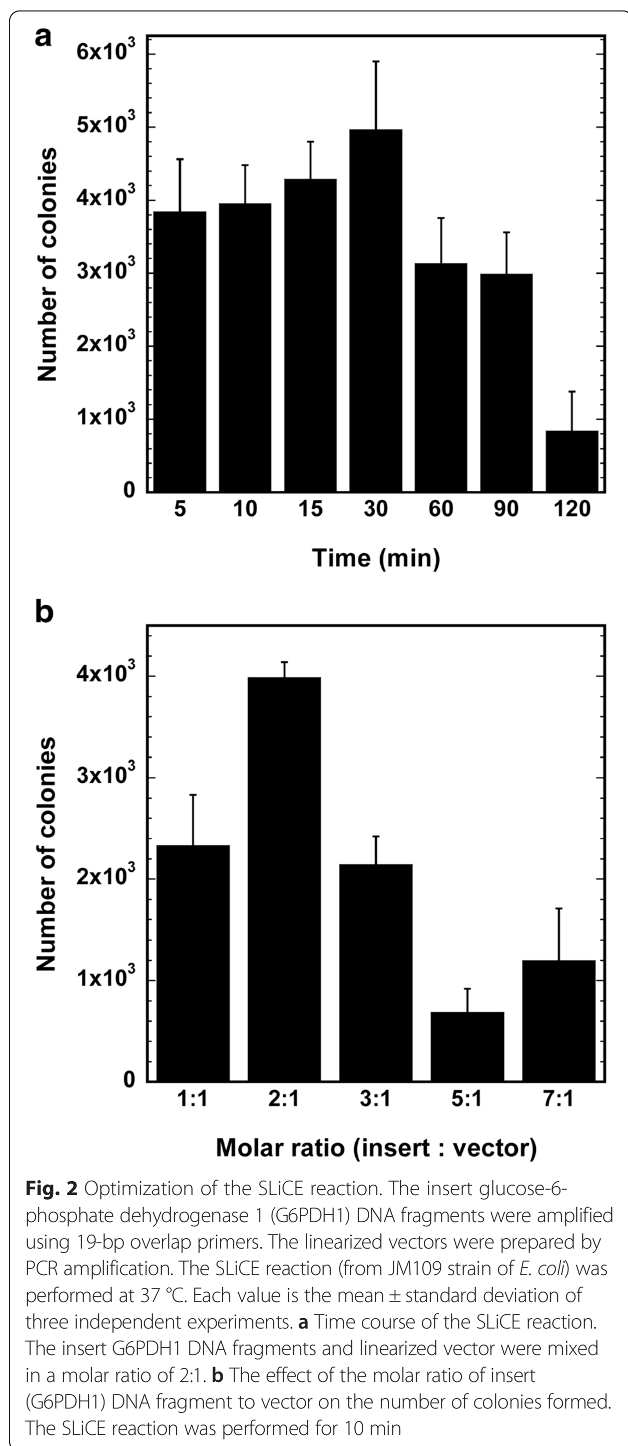

Fig. 2 Optimization of the SLiCE reaction. The insert glucose-6-phosphate dehydrogenase 1 (G6PDH1) DNA fragments were amplified using 19-bp overlap primers. The linearized vectors were prepared by PCR amplification. The SLiCE reaction (from JM109 strain of *E. coli*) was performed at 37 °C. Each value is the mean ± standard deviation of three independent experiments. **a** Time course of the SLiCE reaction. The insert G6PDH1 DNA fragments and linearized vector were mixed in a molar ratio of 2:1. **b** The effect of the molar ratio of insert (G6PDH1) DNA fragment to vector on the number of colonies formed. The SLiCE reaction was performed for 10 min

Table 4 Effect of PCR fragment purification method on SLiCE efficiency

Purification method	Number of colonies[f]		Cloning efficiency	
	Prx IIE	G6PDH1	Prx IIE	G6PDH1
None[a]	86.3 ± 2.3	76.0 ± 4.0	15/18	17/18
EtOH ppt[b]	546 ± 102	514 ± 68.7	18/18	17/18
ExoSAP-IT[c]	481 ± 117	507 ± 105	18/18	18/18
Column[d]	7,170 ± 806	4,660 ± 1,400	18/18	18/18
Agarose gel[e]	11,900 ± 1,750	2,830 ± 100	18/18	18/18

The insert DNA fragments were amplified using 19-bp overlap primers and KOD DNA polymerase [51] (for Prx IIE gene) and PrimeSTAR Max DNA polymerase (for G6PDH1). The linearized vector DNA was prepared by PCR. The PCR solutions were treated with *Dpn*I. The SLiCE (JM109) reaction was performed for 10 min at 37 °C. [a]Unpurified insert DNA was directly used for the SLiCE reaction. [b]Insert DNA was precipitated by ethanol. [c]PCR solution was treated with ExoSAP-IT (Affymetrix). [d]Insert DNA was purified from the PCR solution using a Gel/PCR Extraction Kit (FastGene). [e]Insert DNA was purified by agarose gel electrophoresis and using a Gel/PCR Extraction Kit (FastGene). An equivalent volume to 1/20 of the PCR solution was used for the SLiCE reaction. [f]Each value of "number of colonies" is the mean ± standard deviation of three independent experiments

the short end homology region, since this yielded the maximum number of transformants (Table 2). However, a 15-bp overlap sequence was also sufficient to provide colonies harboring the correct clone. The SLiCE method also has the advantage that linearized vectors (generated by either PCR amplification or restriction enzyme digestion) may be used, as its efficiency and accuracy were not affected by the presence of blunt ends or 5′ sequence overhangs at the ends of the vector (Table 2 and Table 3). I summarize the SLiCE protocol from *E. coli* laboratory strains in Fig. 4. For the standard protocol, various insert DNA fragments, including small amounts of PCR-products amplified as multiple bands can be cloned into vectors with high efficiency. In contrast, a rapid protocol is available for high abundance PCR-products that have been amplified as a single band.

Seamless DNA assembly kits based on *in vitro* homologous recombination activity have recently become

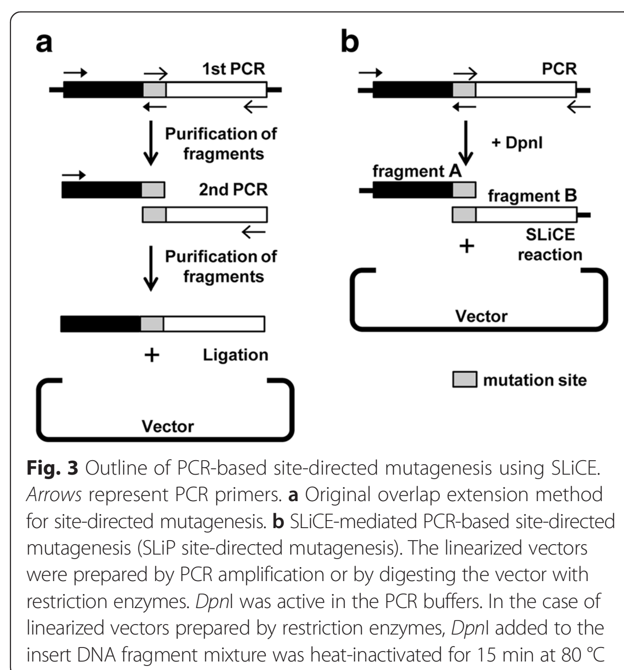

Fig. 3 Outline of PCR-based site-directed mutagenesis using SLiCE. *Arrows* represent PCR primers. **a** Original overlap extension method for site-directed mutagenesis. **b** SLiCE-mediated PCR-based site-directed mutagenesis (SLiP site-directed mutagenesis). The linearized vectors were prepared by PCR amplification or by digesting the vector with restriction enzymes. *Dpn*I was active in the PCR buffers. In the case of linearized vectors prepared by restriction enzymes, *Dpn*I added to the insert DNA fragment mixture was heat-inactivated for 15 min at 80 °C

Table 5 SLiCE-mediated PCR-based site-directed mutagenesis

Mutation site	Number of colonies[c]	Cloning efficiency	Mutation(%)[d]
Prx IIE C51S[a]	22.0 ± 14.0	10/16	100
Prx IIE C76S[a]	18.0 ± 7.5	15/16	100
G6PDH1 C97S[a]	32.0 ± 4.6	9/16	93.3
G6PDH1 C105S[a]	54.0 ± 13.5	9/16	88.9
Prx IIE C51S[b]	197 ± 49.2	14/16	100
Prx IIE C76S[b]	283 ± 21.2	15/16	100
G6PDH1 C97S[b]	189 ± 39.0	6/16	100
G6PDH1 C105S[b]	220 ± 30.0	9/16	100

Target cysteine residues that are reduced by chloroplast thioredoxins were substituted with serine residues [26, 52]. The insert DNA fragments were prepared by PCR using PrimeSTAR Max DNA polymerase. pET23a and pET23d vectors were linearized using restriction enzymes *Nde*I and *Xho*I (for pET23a) and *Nco*I and *Xho*I (for pET23d). The SLiCE (JM109) reaction was performed for 60 min at 37 °C. [a]One microliter of unpurified insert DNA fragments in 20 μL PCR solution was directly used for the SLiCE reaction. [b]PCR solution (20 μL) was purified using a Gel/PCR Extraction Kit (FastGene) and an equivalent volume to 1/20 of the PCR solution was used for the SLiCE reaction. [c]Each value of "number of colonies" is the mean ± standard deviation of three independent experiments. [d]The ratio of mutants generated is given as the percentage of mutated clones (determined by DNA sequencing) among colony PCR-positive clones

commercially available. These include the In-Fusion HD Cloning Kit [41, 42] (Clontech), GeneArt Seamless Cloning and Assembly Kit (Life Technologies), and Gibson Assembly Cloning Kit [2, 43] (New England BioLabs). However, many of the commercially available kits are associated with a high cost per reaction. The SLiCE method uses extracts from common *E. coli* laboratory strains. These extracts can be prepared easily in the laboratory and the use of this method greatly reduces the costs associated with DNA manipulation. SLiCE is highly efficient and maintains high fidelity for gene cloning. These results also allow the use of either commercial or laboratory "homemade" chemically competent cells. As an approximate guide, our laboratory has successfully used SLiCE with chemically competent *E. coli* cells that yield >10^7 CFU/μg of pUC19 DNA [44].

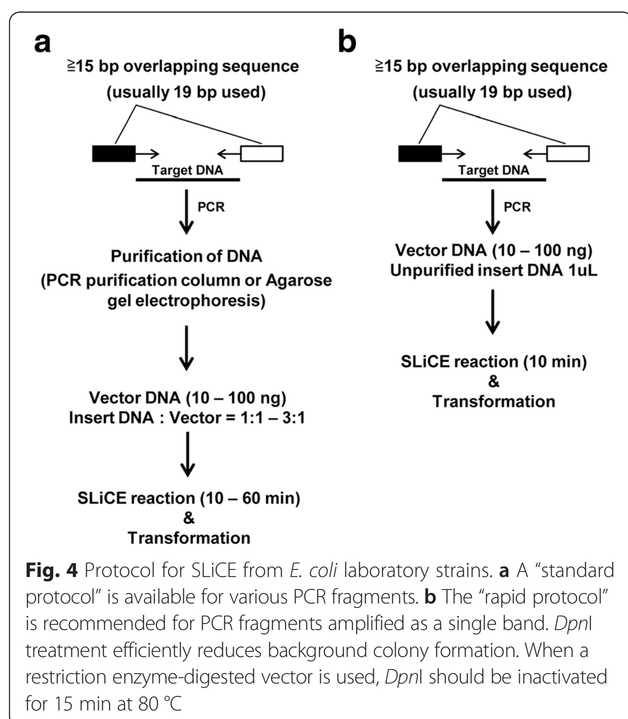

Fig. 4 Protocol for SLiCE from *E. coli* laboratory strains. **a** A "standard protocol" is available for various PCR fragments. **b** The "rapid protocol" is recommended for PCR fragments amplified as a single band. *Dpn*I treatment efficiently reduces background colony formation. When a restriction enzyme-digested vector is used, *Dpn*I should be inactivated for 15 min at 80 °C

PCR-based site-directed mutagenesis is a common technique used in molecular biology experiments. The QuickChange mutagenesis kit is frequently used for site-directed mutagenesis because it is a simple process that does not require the purification of PCR fragments. However, this method relies on PCR amplification of the entire plasmid, which is accompanied by the risk of introducing a mutation in the vector region. In contrast, in the overlap extension method only the insert DNA is amplified. Furthermore, the possibility of introducing a mutation in the vector region is excluded by the use of a restriction enzyme-digested vector. A limitation of the overlap extension method is that it follows an elaborate protocol and requires multiple steps (Fig. 3a). SLiP site-directed mutagenesis removes one PCR cycle and two DNA purification steps from the overlap extension method, and thus provides an efficient and accurate method for the generation of site-directed mutants.

Conclusions

SLiCEs prepared from a wide variety of laboratory $RecA^-$ *E. coli* strains could be used to clone PCR fragments, in the absence of any special modifications to the strains. SLiP site-directed mutagenesis is a simple and efficient method that removes any possibility of mutation in the vector region. The combination of SLiCE using *E. coli* laboratory strains and SLiP site-directed mutagenesis will facilitate the generation of recombinant plasmids and mutants, and shorten the time for DNA manipulation in the laboratory. The elucidation of mechanisms that control *in vitro* homologous recombination activity in *E. coli* lysates will lead to further enhancement of SLiCE efficiency.

Methods

Reagents

CelLytic B Cell Lysis Reagent (SIGMA B7435) was used for *E. coli* cells lysis buffer. Adenosine-5′-triphosphate disodium salt hydrate from yeast (Nacalai Tesque, Inc. 01072–24) was used for ATP contained in SLiCE buffer (10×).

E. coli strains

The following *E. coli* laboratory strains were used to prepare SLiCE extracts (Additional file 1: Table S1): DH10B [45], JM109 [46], DH5α [47], XL10-Gold (Agilent Technologies, Santa Clara, CA), Mach1 T1 (Life Technologies, Carlsbad, CA) and SURE2 (Agilent Technologies, Santa Clara, CA). *ECOS* X Competent *E. coli* DH5α (Nippon gene, Tokyo, Japan) chemically competent cells were used to transform the recombinant DNA generated using the SLiCE method, in order to obtain the constant transformation efficiency.

Preparation of the SLiCE extract from *E. coli* laboratory strains

E. coli strains precultured in LB Miller medium (1 mL) at 37 °C were transferred to 2× YT medium (50 mL) in a 100-mL round-bottom, long-neck Sakaguchi shake flask. The cells were grown at 37 °C in a reciprocal shaker (160 rpm) until OD_{600} reached a value of 2.0–3.0 (late log phase). The cultures were generally incubated for 3.5–6.5 h. The cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4 °C. The cells were then washed with 50 mL sterilized water (ice-cold), and centrifuged at 5,000 × *g* for 5 min at 4 °C. The wet cells were recovered with a yield of 0.25–0.40 g, and gently resuspended in 1.2 mL CelLytic B Cell Lysis Reagent and incubated for 10 min at room temperature. The cell lysates were then centrifuged at 20,000 × *g* for 2 min at 4 °C. All subsequent procedures were performed on ice. The supernatants were carefully transferred into 1.5-mL microtubes to remove the insoluble materials, and an equal volume of ice-cold 80 % (v/v) glycerol was added and mixed gently. Forty microliters of each SLiCE extract was aliquoted into a 0.2-mL 8-strip PCR tube. The SLiCE extracts were snap-frozen in a bath of liquid nitrogen, and stored at −80 °C in 40 % (v/v, final concentration) glycerol. For short-term storage, the aliquoted SLiCE extracts could be stored at −20 °C in 40 % (v/v, final concentration) glycerol for about 3 months without significant loss of activity.

SLiCE reaction and transformation

SLiCE buffer (10×; 500 mM Tris–HCl, pH 7.5, 100 mM $MgCl_2$, 10 mM ATP, and 10 mM dithiothreitol) was passed through a 0.2-μm filter, and dispensed in 40-μL aliquots into 0.2-mL 8-strip PCR tubes and stored at −20 °C. The *Arabidopsis* type II peroxiredoxin E (Prx IIE, 0.6 k base pairs (kbp), AT3G52960) [48, 49] and chloroplastic glucose-6-phosphate dehydrogenase 1 (G6PDH1, 1.6 kbp, AT5G35790) [50] genes were used as insert DNA molecules. The linearized pET23a DNA and insert DNA fragments were amplified using the primers listed in Additional file 1: Table S2 and Tks Gflex DNA polymerase (Takara-Bio, Otsu, Japan) by PCR. To compare the reaction efficiency of SLiCE with that of restriction enzyme-digested vectors, the pET23a and pET23d vectors were linearized by digestion with restriction enzyme and purified using a Gel/PCR Extraction Kit (FastGene) following agarose gel electrophoresis.

After the SLiCE reaction solution had been prepared, the SLiCE extract was thawed on ice and mixed gently. The standard SLiCE reaction solution comprised the following components: 10 ng linear vector (PCR amplified or restriction enzyme-digested), an appropriate amount of insert DNA (1:1 to 3:1 molar ratio of insert to vector), 1 μL 10× SLiCE buffer, 1 μL SLiCE extract, and sterilized distilled water to a total volume of 10 μL. The SLiCE reaction mixture was incubated at 37 °C for the indicated time (5–120 min). One microliter of the SLiCE reaction solution was chemically transformed into 20 μL *ECOS* X Competent *E. coli* DH5α (Nippon gene, Tokyo, Japan) according to the instruction manual. The transformation efficiency of the competent cells (20 μL) was approximately 2×10^8 CFU/μg of pUC19 DNA. Transformed *E. coli* cells were plated on LB agar plates containing ampicillin and incubated at 37 °C for 12–16 h.

Evaluation of the SLiCE reaction

Various parameters were used to measure the efficiency of the SLiCE reactions under each experimental condition. Number of colonies was represented as CFU per nanogram of vector. Cloning efficiencies for the insert DNA were given as the ratio of colonies with an insert of the confirmed correct length as estimated by colony-PCR. In particular, cloning efficiencies were represented as "the number of clones with the confirmed correct length of insert DNA by colony-PCR/number of colonies subjected to colony-PCR". The cloning accuracy was expressed as the percentage of correctly cloned expression vectors in colony-PCR positive clones. The insert cloning sites were determined by DNA sequencing.

Insert-check by colony PCR in transformed *E. coli*

Each colony was picked with a sterile toothpick and put into the bottom of a 0.2-mL 8-strip PCR tube or a 96-well PCR plate. After the toothpicks were removed from the PCR-tube, 10 μL of KAPATaq EXtra DNA polymerase (KAPA Biosystems, Wilmington, MA) PCR mix was added to each sample; this mixture included the T7P and T7T primers corresponding to the T7 promoter and T7 terminator sequences of the pET vectors, respectively (Additional file 1: Table S3). The sample solutions were

reacted according to the KAPATaq EXtra standard protocol. When the target DNA was >1.5 kbp in length, the Tks Gflex DNA polymerase (Takara-Bio, Otsu, Japan) was used instead of KAPATaq EXtra.

SLiCE-mediated PCR-based site-directed mutagenesis (SLiP site-directed mutagenesis)

Primers for site-directed mutagenesis were designed using the PrimerX program (http://www.bioinformatics.org/primerx/) under the following conditions: melting temperature >78 °C and primer termination with guanine (G) or cytidine (C) for QuickChange site-directed mutagenesis kit (Additional file 1: Table S3). Pairs of insert DNA fragments for a cysteine mutant of Prx IIE and G6PDH1 were amplified in a 20-μL reaction volume using PrimeSTAR Max DNA polymerase (Takara-Bio, Otsu, Japan) along with the T7P primer/Mutant reverse primers and Mutant forward primers/T7T primer (Additional file 1: Table S3). The two PCR fragments for mutant generation were incubated with *Dpn*I for 60 min at 37 °C to digest the template plasmid DNA. Subsequently, *Dpn*I was inactivated by incubating the mixture at 80 °C for 15 min. For the unpurified insert DNA fragments, 1 μL of each unpurified insert DNA fragment in a 20-μL PCR reaction solution was mixed with the linearized pET23a vector (10 ng; digested using *Nde*I and *Xho*I [*Nco*I and *Xho*I for pET23d]), and then added to the SLiCE reaction. For the purified insert DNA fragments, an equivalent volume to 1/20 of the PCR solution was added to the SLiCE reaction.

Additional file

Additional file 1: Figure S1. The multiple cloning site of pET23a (or pET23d) vector. **Table S1.** Escherichia coli strains used for SLiCE preparation. **Table S2.** Oligonucleotides for PCR amplification. **Table S3.** Oligonucleotides for site-directed mutagenesis.

Abbreviations

ATP: Adenosine triphosphate; CFU: Colony-forming units; G6PDH1: Glucose-6-phosphate dehydrogenase 1; PCR: Polymerase chain reaction; Prx IIE: Type II peroxiredoxin E; SLiCE: Seamless ligation cloning extract; SLiP site-directed mutagenesis: SLiCE-mediated PCR-based site-directed mutagenesis.

Competing interests

The author declares that he has no competing interests.

Authors' contributions

KM designed and performed the experiments, analyzed the data and wrote the manuscript. The author read and approved the final manuscript.

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