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MINIREVIEW

Modern and Simple Construction of Plasmid: Saving Time and Cost

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Construction of plasmids has been occupying a significant fraction of laboratory work in most fields of experimental biology. Tremendous effort was made to improve the traditional method for constructing plasmids, in which DNA fragments digested with restriction enzymes were ligated. However, the traditional method remained to be a standard protocol more than 40 years. At last, several recent inventions are rapidly and completely replacing the traditional method, because they are far quicker with less cost, and requiring less material. We here introduce three such methods that cover up most of the cases. Moreover, they are complementary with each other. Our lab protocols are provided for "no strain, no pain" construction of plasmids.

Keywords: plasmid, cloning, recombineering, PCR cloning, fusion PCR, Gibson assembly, SLiCE

Introduction

In any field of biology, cloning a gene as well as its controlling sites has been one of the time-consuming and intelligence-demanding tasks. Now, it recently became more than 10 times quicker without increased cost, no need for broad and deep knowledge, and even becomes easier in some sense than washing lab instruments, which requires a certain amount of care. Therefore, cloning is no longer the rate-limiting step of a project, and this revolution is now changing the schedules and strategies adopted not only in a bacteriology lab, but also in most experimental biology labs. In this review, at first, we briefly summarize the historical background of the recent major advances in the recombination technique. We then concentrate this review on introducing three new or revived techniques for the routine construction of plasmids of ordinary lengths, rather than the high technologies used in synthetic biology and systems biology, which deal with DNAs of genome sizes. In the last section, we provide our lab protocols for simplified methods. Because everything is evolving, and because we are far from perfect, we welcome reader suggestions of simpler and newer methods which save both time and cost.

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Restriction enzyme-free and ligase-free cloning

Since cloning and genome editing are composed of a replacement of the original DNA sequence with another sequence, it is a kind of site-specific recombination. Therefore, the restriction enzyme/ligase-free cloning requires a homologous recombination system and is called recombineering. The donor DNA fragment to be inserted should be flanked by two DNA segments homologous to the target DNA segments to be replaced. Such DNA fragments are generally linear and thus can be the products of the polymerase chain reaction (PCR).

The history of restriction enzyme/ligase-free cloning started by using yeast (Orr-Weaver *et al*., 1983; Moerschell *et al*., 1988), but the method has been developed in *E. coli* by the use of the recombining systems of Rac prophage (RecE/T) (Orr-Weaver *et al*., 1983; Zhang *et al*., 1998) and of lambda phage (Red αβγ) (Murphy, 1998; Muyrers *et al*., 1999; Yu, 2000). These systems are composed of 5′ to 3′ exonuclease (RecE or Red α) and single-stranded DNA binding protein (RecT or Red β). The Red γ (Gam) inhibits the recombination by host SbcCD and RecBCD, which otherwise prevents the invasion of foreign DNA in cells (Kulkarni and Stahl, 1989; Murphy, 1991).

This method is applied in mutating, inserting, replacing, deleting, and inverting chromosomal and plasmid DNA. Since PCR is a restriction enzyme-free construction of DNA, its product can be used as the exogenous DNA fragment to replace an intact gene (Datsenko and Wanner, 2000). By using this PCR-based method, genome-wide correction of a single-knock out strain of K-12 *E. coli*, KEIO clones, has been constructed (Baba, 2006). The donor DNA is not limited to double-stranded DNA. The recombination efficiency was improved by using single-stranded DNA (Ellis *et al*., 2001), enabling a selection without using antibiotic markers. In this way, the basic method for recombineering was established in early 2000.

This method has been developed by combination with the recombinase-mediated cassette exchange, and the artificially designed nucleases, TALEN (Hockemeyer *et al*., 2011; Miller *et al*., 2011) and CRISPR (Barrangou *et al*., 2007; Garneau *et al*., 2010; Gasiunas *et al*., 2012), yielding a powerful genome-editing tool (Esvelt and Wang, 2013; Thomason *et*

al., 2014). The method greatly facilitated the handling of long DNA such as BAC, which is extensively used for genomic libraries of a variety of organisms, and for BAC transgenic animals (Ting and Feng, 2014), as well as transgenic plants through agrobacterium (Hu *et al*., 2014). Since simultaneous parallel mutagenesis is possible, heterogeneous mutants can be prepared in a single procedure (Wang *et al*., 2009). In *E. coli*, an impressive parallel genome editing is the conversion of all 314 TAG termination codons to TAA, which is associated with the deletion of otherwise essential gene encoding RF1 (Isaacs *et al*., 2011). Variations of the host strains for recombineering are increasing (Ryu *et al*., 2014; Thomason *et al*., 2014).

In the traditional restriction enzyme/ligase method, two most distressful results are a low efficiency of transformation and a high background of re-cloning of the vector DNA lacking insert DNA. Furthermore, designing a plasmid mostly requires a compromise due to the lack of a restriction site. To solve these difficulties, many clever inventions have been developed. However, some of them require a longer time, more steps, more amounts of DNA, high quality chemicals, or proficient researchers. As a consequence, the original traditional method has been preserved in most labs together with various restriction enzymes and T4 ligase. The newly developed methods also kept failing in replacing the traditional method, until recently. At last, three methods independent of restriction enzymes and ligases have recently become successful in resolving the long-lasting conservatism. They are simply quicker and less expensive. They show more efficient transformation with a lower background of uninserted plasmid. Namely, the first set of the methods superior to the traditional counterpart was established only 3–4 years ago, although some parts of the method had been developed long before. Therefore, there is no longer a compromise in the DNA sequence and thus an ideal sequence is the designed sequence itself.

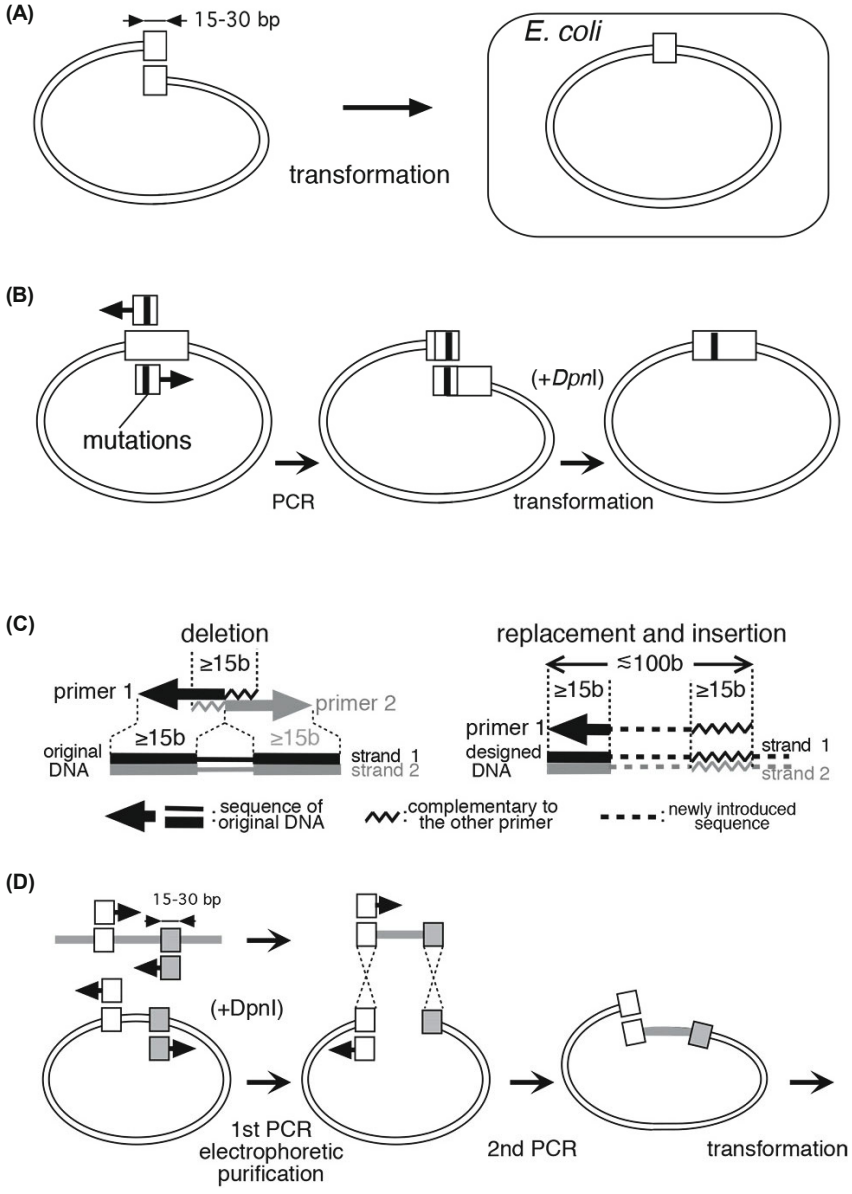

Fig. 1. PCR cloning. (A) A linear DNA with overlapping ends of 15–30 bp is converted to a closed circular DNA in *E. coli* cells. (B) Mutagenesis of a gene (white rectangular) with two primers, which contain 15 bp or longer complementary sequence with each other (white square box) as well as a mutated sequence (black bar). Treatment with *Dpn*I reduces the vector DNA background. (C) The design of the shortest primers for deletion, replacement, and insertion. The DNA strand 1 (black line) is shown with its 3′-end on the left and strand 2 (gray line) with its 3′-end on the right. In the left panel, the maintained sequence (thick solid line) is distinguished from the deleted segment (thin solid line). A primer for deletion is composed of 3′-end half with the maintained sequence longer than 15 b (arrow) and the 5′-end half with the complementary sequence to the other primer (zigzag line) to make an overlup longer than 15 bp for cyclization. The arrows in the primers point to their 3′-ends. In the right panel, only one primer is shown because the other primer is symmetrically designed. The designed DNA is shown in the same polarity as that in the left panel with the replacement or the insert (dotted line). The largest length of primer is limited to about 100 b because of the fidelity and cost. We succeeded in inserting a 130 bp fragment with the arrow length of 20 b. The replacement or insertion of the shortest segment, a single base, requires the shortest primers of 23 b in this method. They are composed of the arrow part of 15 b, new one base, and the zigzag part of 7 b, because the 7 b downstream half of the arrow part can also be the upstream part of the zigzag one. (D) Cloning with fusion PCR. The first PCR amplifies the insert DNA and the vector DNA with a set of primers sharing two 15–30 bp sequences (white and gray boxes). The amplified fragments are electrophoresed, cut out of the gel, and extracted. The second PCR uses either set of the primers.

PCR cloning

The simplest among the three is a PCR-based method belonging to the technique sometimes called "fusion PCR" or "overlapping PCR". To transform *E. coli*, one need not necessarily prepare a closed circular plasmid DNA. It is enough to make a linear DNA with more than 15 bp overlapping segments at both its ends (Fig. 1A). Therefore, plasmids of moderate sizes can be amplified by PCR as a linear DNA. When the mutation is a replacement, insertion, or deletion localizing within 130 bp or so on the final designed plasmid, it can be introduced by two oligo DNA primers as similarly proposed in ref (Zheng, 2004) (Fig. 1B). Note that a long insertion requires a high quality oligo DNA. The best cost-performance design is to make the shortest primers. In this method, the best design is obviously made by positioning the center of the mutation at the center of the 15 b complementary region (Fig. 1C), yielding the shortest primer set. As has been used in a commercially available kit, a set of perfectly complementary primers is also allowed in this method. Although designing such primers is prohibited in classic textbooks of PCR because of the unwanted production of primer dimers, setting a suitable time for elongation enables productive PCR using modern DNA polymerases which produce blunt ends.

The second application of this method deserving the name of "fusion" is the ligation of two DNA segments by PCR (Fig. 1D). Since the early period of PCR, an artifact has been observed: two DNA fragments are eventually ligated (Mullis, 1991). This ligation was applied in early developments of PCR (Yon and Fried, 1989; Rashtchian, 1995). Therefore, the method described here is a revival of the old method, but the reason for revival is based on the recent improvements of PCR enzymes and the buffers which show high fidelity and

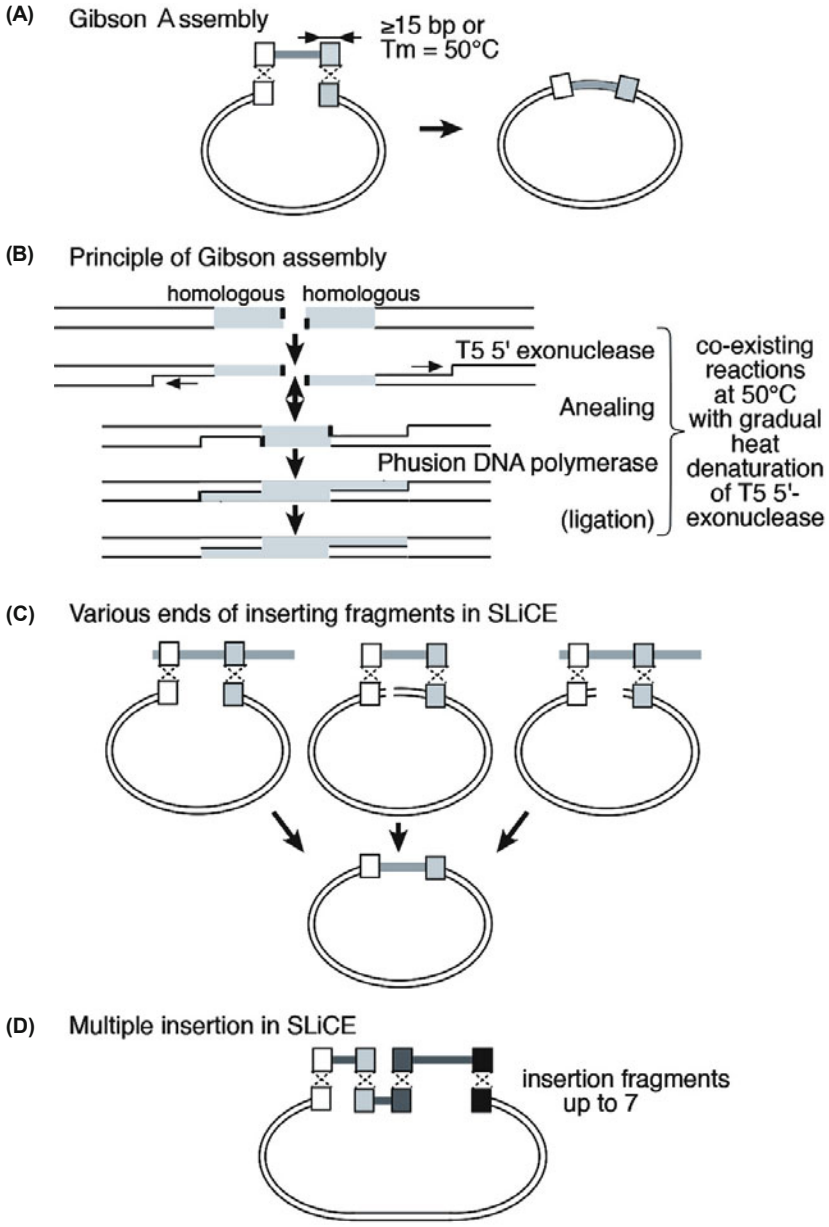

Fig. 2. Gibson Assembly and SLiCE. (A) The vector DNA (partial circle) and the insert DNA (gray thick line) produce the product (completed circle) with two homologous sequences (white and gray boxes). In the case of Gibson Assembly, their length is determined so that the melting temperature is 50°C, while, in the case of SLiCE, the length is 15 bp or longer independent of the melting temperature. (B) The reactions of Gibson Assembly. The two 3′-ends of the joining fragments (thick vertical lines) disappear in the final product after ligation. (C) In SliCE, a common sequence does not have to be at a DNA end, and can be an interior of the insert DNA or vector DNA fragment. The most interior position we ever successfully used is 100 bp from an end. This freedom allows more selections of PCR primer sequences. (D) SLiCE can insert multiple DNA fragments up to 7 in the designed order, at once.

high processivity. When different joint sequences of 15 bp or longer are designed, the two or more segments are ligated by only a single PCR, depending on the PCR enzymes and buffers. The number of inserts is theoretically unlimited and a repetitive PCR ligation is possible to ligate many fragments in a stepwise process. However, the ligation is not always successful, and excessive time and effort should not be expended because a high-cost performance method for multi-insertion is available, as introduced in a later section.

We very often prepare linear vector DNAs by colony PCR, eliminating the need for liquid cultures and plasmid preparations. The colony PCR is sometimes avoided because of a poor success rate. The failure is mostly due to excessive collection of cells. Note that 10^2 or 10^3 cells are sufficient for amplifying plasmid DNA or genomic DNA, respectively. A single colony of 1 mm diameter contains 10^8–10^9 colonies. Thus, the sufficient amount is less than 10^{-5} or less portion of a colony, which is far from being visible. If one collects a visible quantity of cells, probably 10^6–10^8 cells, it is excessive for allowing PCR, which requires a 1,000–10,000-fold dilution. We collect cells with a linear platinum wire and wipe most of the attached cells off by inserting the wire into a new agar plate before inserting it in a PCR mixture. This is convenient because replica plating is often required in a colony PCR. Using a toothpick to collect cells is not recommended.

There is anxiety that PCR will introduce mutations in your plasmids, which is true for *Taq* DNA polymerase. However, modern high fidelity PCR enzyme can elongate 10 kb without any errors. In fact, we have so far sequenced the final plasmids more than several million bp in total, but found no mutations by our protocols shown in the last section. If the linear DNA of the designed length is the dominant product, there is no need to purify the PCR product before transformation. If extra bands are shorter than the length between the replication origin and the antibiotic marker, a purification is not needed, either. Only when there are longer extra bands, the correct band must be extracted and purified from the gel. During the extraction process, the vector DNA and insert DNA fragments are recovered with a single tube of the extraction kit, halving the cost and enhancing the yield.

The use of vectors prepared from *E. coli* has one benefit. One can dramatically reduce the appearance probability of the colonies containing only circulized vector DNA, which is inevitable in the traditional method due to incomplete digestion with restriction enzymes. The DNA prepared from *E. coli* is methylated and thus able to be digested with *Dpn*I, a four-cutter restriction enzyme working at various salt concentrations. A 30 min treatment with *Dpn*I just before the transformation is sufficient, and the transformation is not affected by the presence of trace amounts of *Dpn*I (Fig. 1B). Because the storage buffer for *Dpn*I usually contains salt, adding too much volume of *Dpn*I prevents electroporation. Actually, it is the only restriction enzyme kept in our freezers.

The largest merit of this method based on PCR, as well as the ones introduced in the following sections, is the simplicity of the design of a plasmid. There is no compromise due to the lack of restriction sites, no need to select the time and the temperature for ligation, and no need to stock the intermediates of the reaction. Because of the high efficiency, the shortest time, and the lowest cost, this method is the first choice among the three. However, because it relies on PCR, there are cases where DNAs were not amplified because of unclear reasons. When a linear fragment is the designed product, the existence of a sequence homologous to the 15 bp overlapping region sometimes produces unwanted longer products. The success depends on the PCR enzymes and their specific buffers. We usually try this method with two enzyme systems, and, if both fail, we set those enzymes aside and start with another method described below.

Gibson Assembly

This clever method is an *in vitro* homologous recombination, which inserts a DNA fragment into a vector DNA according to two homologous regions existing at the ends of the insert DNA and the linearized vector DNA (Fig. 2A). This method first made it possible to synthesize a whole genome of a creature from synthesized DNA fragments (Gibson *et al.*, 2008). As mentioned above, a recombination system is composed of 5′ exonuclease and a factor annealing the produced single-stranded part of the DNA fragments, which are single-stranded DNA binding proteins in the above-mentioned cases. In the presence of T5 5′ exonuclease, Phusion DNA polymerase, and *Taq* ligase, three sequential reactions proceed at 50°C: the 5′→3′ exonuclease reaction, annealing of the generated protruding 3′-ends, and the gap-filling DNA polymerase reaction expanding the homologous region (Fig. 2B). In the presence of the optional ligase, the final product is a closed circular plasmid molecule, which inhibits the exonuclease reaction as the nicked circular molecule generated in the absence of ligase. Furthermore, the exonuclease is gradually denatured at 50°C. The expanded homologous region makes strand separation less probable at the specified temperature. As a consequence, the reactants are unidirectionally driven to produce the designed product.

Individual enzymes are commercially available, as well as a kit, which are not inexpensive. However, the standard protocol is for long DNA, say several hundred kb, and is an over specification for the construction of ordinal sizes. Thus, one several tenths amount of the enzymes is enough. Furthermore, the expensive *Taq* ligase can be omitted in the transformation of *E. coli*. Therefore, it is possible to use such small amounts of the enzymes that the cost becomes close to the price of a synthetic oligo DNA per base.

This method is essentially an end-end joining, and thus the product is independent of the existence of repetitive sequences. Since it is using the purified enzymes, it is highly reproducible. The DNA required is a stoichiometric mixture of vector DNA and insert DNA of the amounts as small as 2–10 fmol each, much less than that required in the traditional method. A high concentration is avoided because the final product is formed by self-circularization rather than bimolecular reaction. Therefore, this method is recommended as the second method when PCR cloning does not work. However, if any sets of the prepared primers cannot make the DNA fragments to be joined exactly, which sometimes occurs, the following third method is recommended.

SLiCE

Even in such a case, one can use Seamless Ligation Cloning Extract (SLiCE) method (Zhang *et al.*, 2012). This is an *in vitro* version of recombineering using the homogenate of *E. coli* cells with lambda Red α-γ overexpressed. Therefore, the total reaction is the same as Gibson Assembly as depicted in Fig. 2A. When the cell homogenate is prepared as exactly indicated, the efficiency of ligation with ends that overlap more than 15 bp is as efficient as is Gibson Assembly, but much less expensive, unless labor costs are an issue. The largest benefit of this system is that the homologous region does not necessarily locate at the very end as does the Gibson Assembly. It can be in interior positions, which are shown in Fig. 2C. We confirmed that one end can be as far as 100 bp from the homologous region. This protruding end gives enough choices of PCR primers so that the fragment could be amplified. A drawback associated with this benefit is that the unique product cannot be obtained if there are repetitive sequences.

Up to 7 DNA fragments can be simultaneously inserted in a vector DNA in the order designed by the overlapping sequences (Fig. 2D). Although SLiCE relies on the Red system, one can use the *E. coli* recombination system if a homogenate can be prepared from a RecA$^-$ strain such as JM109 and DH10B (Zhang *et al.*, 2012). The efficiency will be reduced by about 100 fold but still orders of magnitude higher than the traditional methods. In conclusion, three tools, PCR cloning, Gibson Assembly, and SliCE can save time and cost in the construction of plasmid in labs. Almost all constructions of plasmids can be significantly improved by these three methods, and one can forget a brute-force construction with one exception. Since all three methods rely on PCR, a DNA fragment containing more than several repeats of sequences, such as TALEN, may still require a brute-force construction.

Our lab protocol for PCR cloning

We have found no available documents written in English so far, while there is one in Japanese (TaKaRa Bio, 2007) using a PCR polymerase with one of the fastest elongation rates. The manual describes the mutagenesis shown in Fig. 1B, but is applicable to the all fusion PCR as shown below.

1. Prepare 100 µl of PCR mixture containing 50 µl of PrimeSTAR® Max Premix (TaKaRa, Japan), the forward and reverse primers of final 0.2 µM each, and the template. In the case of Tks Gflex® DNA polymerase, 2.5 U of the DNA polymerase and 50 µl of 2×Gflex® PCR Buffer are added in place of 50 µl of PrimeSTAR® Max Premix.
2. The amount of a template DNA is10–100 pg of plasmid or 100 pg–10 ng of genomic DNA. In the case of colony PCR, a platinum wire is preferred for picking up the cells. If a Pipetman tip is used, cells must be suspended in 0.1–1 ml of water with sucking and blowing, and 1 µl of the suspension is added as a template DNA. The colony should not be scraped with a tip, but just touch the colony with its apex.
3. Set the PCR machine for the temperature gradient from 50°C to 67°C and PCR is made at various temperatures with 3–4°C increments. We distribute 16 µl of the mixture to each of the positions 1, 4, 6, 7, 9, and 12 in a 12-connected PCR tube array (This somewhat odd selection was determined to give the most regular increments.)
4. PCR is composed of 30–34 cycles of the denaturation at 98°C for 10 sec, the annealing at graded temperatures typically for 5 sec, and elongation at 72°C for 5 sec/kb, which is followed by further elongation for 5 min. In the case of colony PCR, a preheating at 94°C for 6 min is required. In the case of Gflex® DNA polymerase, the annealing is for 15 sec and the elongation is at 68°C for 30 sec/kb.
5. Analyze 2–5 µl mixture taken from each tube with agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer containing 1 µg/ml ethidium bromide.
6. Select a PCR mixture giving a clear single band of the designed length. Avoid one giving a smear even if it shows the highest yield of the designed band. If both PrimeSTAR® Max and Gflex® DNA polymerases did not yield any productive bands, this method should be abandoned, and use the Gibson Assembly or SLiCE.
7. If a template DNA is prepared from *E. coli*, add 2 U of *Dpn*I to 10 µl of the selected mixture, and then incubate for 30 min to remove the intact vector DNA. This treatment is significant especially when the template DNA was added from a stock solution. If a DNA prepared by colony PCR is used as the template DNA, this treatment is not needed.
8. Electroporate the linear DNA of at most 1 µl of the selected PCR mixture at 1.8 kV in a curette of 1mm gapped electrodes. Apply the pulse twice. The competent cells have been prepared as described in ref. 18 and stored at -80°C.
9. Suspend the cells in 1 ml LB Lenox. Make a recovery culture for 1 h if the antibiotic marker is not ampicillin.
10. The cell suspension is concentrated to about 50 µl by centrifugation at 3,000 rpm for 5 min and spotted at the center of an agar LB plate containing suitable antibiotics. Put the plate on a rotating table, and streak the agar surface with a platinum wire (but not a toothpick) from the center to the periphery to draw a spiral. By this spiral method, only one plate is used for single-colony selection without discarding any of the cell suspension.
11. Incubate the plate at a suitable temperature for 8 h or longer, and then a formed colony is subjected to PCR for sequence analysis.

Our lab protocol for Gibson Assembly and SLiCE

Our Gibson Assembly protocol is essentially the same as the one provided by New England BioLab (USA) with the following modifications. At first, the total volume of the reaction is reduced to 4 µl, because only 1 µl is required for electroporation. Secondly, the final concentrations of T5 exonuclease and Phusion DNA polymerase are reduced to 4 mU/µl and 5 mU/µl, respectively, and *Taq* ligase is omitted to transform an *E. coli* strain. Thirdly, the concentration of dNTP is reduced to 20 µM for better results. We use

a PCR machine to make the reaction for a small volume.

1. Prepare the stoichiometric mixture of the vector DNA and the insert DNA of 2–10 fmol each in less than 1.9 or 2.0 µl, depending on the optional addition of ca. 0.1 µl of *Dpn*I (0.1 µl is symbolic and essentially means less than 0.2 µl).
2. Add 2.0 µl of 2× Gibson Premix (Note that the premix in the original protocol is 1.33×.).
3. Adjust the volume to 4.0 µl by adding water.
4. When *Dpn*I is added, incubate the mixture at 37°C for 30 min.
5. Then incubate the mixture typically at 50°C for 30 min. Since the reaction is unidirectional, this time is not critical and 15–60 min is allowed in the original protocol. The reaction mixture can be stored at 4°C overnight.

The original protocol of SLiCE (Zhang *et al*., 2012) is modified as follows. The *Dpn*I treatment before the electroporation is optionally added. Because ATP was hydrolyzed in the presence of Mg, ATP was removed from 10× SLiCE buffer to increase its storage life.

1. Prepare the stoichiometric mixture of the vector DNA and the insert DNA of 15–70 fmol each in less than 6.9 or 7.0 µl, depending on the optional addition of ca. 0.1 µl of *Dpn*I.
2. Add 1.0 µl of 10× SLiCE buffer (-ATP), 1.0 µl of 10 mM ATP, 1.0 µl of SLiCE Extract, and optionally ca. 0.1 µl of *Dpn*I.
3. Adjust the volume to 10 µl by adding water.
4. Incubate the mixture at 37°C for 1 h.
5. Transformation must be made in less than 1 h, even when stored at 4°C, otherwise store the mixture at -20°C.

Transformation of *E. coli*

We routinely transform competent cells, which have been stored at -80°C, at high efficiency by electroporation (Seidman *et al*., 2001) and a heat-shock method, called Inoue method (Sambrook and Russell, 2006). The former has higher transformation efficiency, but the latter accepts more volume of a DNA solution, yielding a similar total number of colonies when a DNA solution of 10 µl or more is provided. When stored competent cells are not available, one-step transformation is convenient (Chung *et al*., 1989; Seidman *et al*., 2001), although the efficiency is orders of magnitude lower.

Acknowledgements

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References

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., and Mori, H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**, 2006.0008.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., and Horvath, P. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**, 1709–1712.

Chung, C.T., Niemela, S.L., and Miller, R.H. 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. USA* **86**, 2172–2175.

Datsenko, K.A. and Wanner, B.L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**, 6640–6645.

Ellis, H.M., Yu, D., DiTizio, T., and Court, D.L. 2001. High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides. *Proc. Natl. Acad. Sci. USA* **98**, 6742–6746.

Esvelt, K.M. and Wang, H.H. 2013. Genome-scale engineering for systems and synthetic biology. *Mol. Syst. Biol.* **9**, 641.

Garneau, J.E., Dupuis, M.È., Villion, M., Romero, D.A., Barrangou, R., Boyaval, P., Fremaux, C., Horvath, P., Magadán, A.H., and Moineau, S. 2010. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**, 67–71.

Gasiunas, G., Barrangou, R., Horvath, P., and Siksnys, V. 2012. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. USA* **109**, E2579–86.

Gibson, D.G., Benders, G.A., Andrews-Pfannkoch, C., Denisova, E.A., Baden-Tillson, H., Zaveri, J., Stockwell, T.B., Brownley, A., Thomas, D.W., Algire, M.A., and *et al.* 2008. Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. *Science* **319**, 1215–1220.

Hockemeyer, D., Wang, H., Kiani, S., Lai, C.S., Gao, Q., Cassady, J.P., Cost, G.J., Zhang, L., Santiago, Y., Miller, J.C., and *et al.* 2011. Genetic engineering of human pluripotent cells using TALE nucleases. *Nat. Biotechnol.* **29**, 731–734.

Hu, S., Fu, J., Huang, F., Ding, X., Stewart, A.F., Xia, L., and Zhang, Y. 2014. Genome engineering of *Agrobacterium tumefaciens* using the lambda Red recombination system. *Appl. Microbiol. Biotechnol.* **98**, 2165–2172.

Isaacs, F.J., Carr, P.A., Wang, H.H., Lajoie, M.J., Sterling, B., Kraal, L., Tolonen, A.C., Gianoulis, T.A., Goodman, D.B., Reppas, N.B., and *et al.* 2011. Precise manipulation of chromosomes *in vivo* enables genome-wide codon replacement. *Science* **333**, 348–353.

Kulkarni, S.K.S. and Stahl, F.W.F. 1989. Interaction between the *sbcC* gene of *Escherichia coli* and the *gam* gene of phage lambda. *Genetics* **123**, 249–253.

Miller, J.C., Tan, S., Qiao, G., Barlow, K.A., Wang, J., Xia, D.F., Meng, X., Paschon, D.E., Leung, E., Hinkley, S.J., and *et al.* 2011. A TALE nuclease architecture for efficient genome editing. *Nat. Biotechnol.* **29**, 143–148.

Moerschell, R.P., Tsunasawa, S., and Sherman, F. 1988. Transformation of yeast with synthetic oligonucleotides. *Proc. Natl. Acad. Sci. USA* **85**, 524–528.

Mullis, K.B. 1991. The polymerase chain reaction in an anemic mode: how to avoid cold oligodeoxyribonuclear fusion. *PCR Methods Appl.* **1**, 1–4.

Murphy, K.C. 1991. Lambda Gam protein inhibits the helicase and chi-stimulated recombination activities of *Escherichia coli* RecBCD enzyme. *J. Bacteriol.* **173**, 5808–5821.

Murphy, K.C. 1998. Use of bacteriophage lambda recombination functions to promote gene replacement in *Escherichia coli*. *J.*

Bacteriol. **180**, 2063–2071.

Muyrers, J.P.J., Zhang, Y.Y., Testa, G.G., and Stewart, A.F.A. 1999. Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res.* **27**, 1555–1557.

New England BioLabs Gibson Assembly® Protocol (E5510). https://www.neb.com/protocols/2012/12/11/gibson-assembly-protocol-e5510?device=pdf.

Orr-Weaver, T.L., Szostak, J.W., and Rothstein, R.J. 1983. Genetic applications of yeast transformation with linear and gapped plasmids. *Methods Enzymol.* **101**, 228–245.

Rashtchian, A. 1995. Novel methods for cloning and engineering genes using the polymerase chain reaction. *Curr. Opin. Biotechnol.* **6**, 30–36.

Ryu, Y.S., Biswas, R.K., Shin, K., Parisutham, V., Kim, S.M., and Lee, S.K. 2014. A simple and effective method for construction of *Escherichia coli* strains proficient for genome engineering. *PLoS ONE* **9**, e94266.

Sambrook, J. and Russell, D.W. 2006. The Inoue method for preparation and transformation of competent *E. coli*: "ultra-competent" cells. *CSH Protoc.* **2006**, 1–6.

Seidman, C.E., Struhl, K., Sheen, J., and Jessen, T. 2001. Introduction of plasmid DNA into cells. Current Protocols in Molecular Biology, John Wiley & Sons, Inc., Hoboken, NJ, USA.

TAKARA Bio. 2007. PrimeStarMax mutagenesis kit 1–3.

Thomason, L.C., Sawitzke, J.A., Li, X., Costantino, N., and Court, D.L. 2014. Recombineering: genetic engineering in bacteria using homologous recombination. *Curr. Protoc. Mol. Biol.* **106**, 1.16.1–1.16.39.

Ting, J.T. and Feng, G. 2014. Recombineering strategies for developing next generation BAC transgenic tools for optogenetics and beyond. *Front Behav. Neurosci.* **8**, 111.

Wang, H.H., Isaacs, F.J., Carr, P.A., Sun, Z.Z., Xu, G., Forest, C.R., and Church, G.M. 2009. Programming cells by multiplex genome engineering and accelerated evolution. *Nature* **460**, 894–898.

Yon, J. and Fried, M. 1989. Precise gene fusion by PCR. *Nucleic Acids Res.* **17**, 4895.

Yu, D., Ellis, H.M., Lee, E.C., Jenkins, N.A., Copeland, N.G., and Court, D.L. 2000. An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **97**, 5978–5983.

Zhang, Y.Y., Buchholz, F.F., Muyrers, J.P.J., and Stewart, A.F.A. 1998. A new logic for DNA engineering using recombination in *Escherichia coli*. *Nat. Genet.* **20**, 123–128.

Zhang, Y., Werling, U., and Edelmann, W. 2012. SLiCE: a novel bacterial cell extract-based DNA cloning method. *Nucleic Acids Res.* **40**, e55–e55.

Zheng, L. 2004. An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res.* **32**, e115–e115.