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T5 exonuclease-dependent assembly offers a low-cost method for efficient cloning and site-directed mutagenesis

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ABSTRACT

The assembly of DNA fragments with homologous arms is becoming popular in routine cloning. For an *in vitro* assembly reaction, a DNA polymerase is often used either alone for its 3′-5′ exonuclease activity or together with a 5′-3′ exonuclease for its DNA polymerase activity. Here, we present a 'T5 exonuclease DNA assembly' (TEDA) method that only uses a 5′-3′ exonuclease. DNA fragments with short homologous ends were treated by T5 exonuclease and then transformed into *Escherichia coli* to produce clone colonies. The cloning efficiency was similar to that of the commercial In-Fusion method employing a proprietary DNA polymerase, but higher than that of the Gibson method utilizing T5 exonuclease, Phusion DNA polymerase, and DNA ligase. It also assembled multiple DNA fragments and did simultaneous site-directed mutagenesis at multiple sites. The reaction mixture was simple, and each reaction used 0.04 U of T5 exonuclease that cost 0.25 US cents. The simplicity, cost effectiveness, and cloning efficiency should promote its routine use, especially for labs with a budget constraint. TEDA may trigger further development of DNA assembly methods that employ single exonucleases.

INTRODUCTION

Gene cloning is a major milestone for molecular biology (1). With the rapid development of molecular biology, metabolic engineering, and synthetic biology, the construction and modification of cloned genes become more routine than before, and the desire for reliable, simple, and cost-efficient methods also grows (2). Although the traditional restriction-ligation method is still widely in use, its low efficiency, site-dependence, and non-modularity do not meet the growing need (3). Recently, both *in vivo* and *in vitro* assemblies for DNA fragments with short homologous ends have been developed for cloning.

Recombineering is an *in vivo* method that clones desired DNA fragments introduced with a linear vector or directly captured from the host genome (4,5). The method requires that the host possesses a strong ability to join short homologous ends, such as *Saccharomyces cerevisiae* or *Escherichia coli* overexpressing the bacteriophage recombination system RecETγ (4,6–9). In general, these methods are reliable and easy to perform. The limitation is the requirement of relatively long homologous ends of about 50 bp or longer, which may require extra effort to generate (9).

In vitro assembly methods are still commonly used due to the use of shorter homologous ends of 15 bp or longer (10–12) (Table 1). The assembly can use cell extracts, the mix of several enzymes, or single enzymes. SLiCE (Seamless Ligation Cloning Extract) uses the cell extracts of *E. coli* that overexpresses the RecETγ system for DNA assembly *in vitro* (12,13); the method is sufficient for routine cloning, although slightly less efficient than commercial methods like the In-Fusion method (14). The enzyme-based assemblies apply one to three enzymes in the *in vitro* reaction, in which a DNA polymerase is used. The Gibson method uses three commercial enzymes for the assembly of DNA fragments with short homologous ends *in vitro*, and this method is especially useful for the assembly of large DNA fragments (15). First, T5 exonuclease degrades the homologous ends to generate 3′-overhangs, which anneal to each other; then, Phusion DNA polymerase fills the gaps; finally, Taq DNA ligase covalently links the fragments. It also could be used for assembly of large fragments extending to several hundreds of kilobases (16). For assembly of large DNA fragments, homologous ends longer than 50 bp are recommended; for short DNA fragments, homologous ends can be as short as 20 bp (15). The Hot Fusion method is a simplification of the Gibson assembly, in which the reaction system without Taq DNA ligase works well for routine assembly (17). Single DNA polymerases are also directly used for DNA assembly, but only their proof-reading activ-

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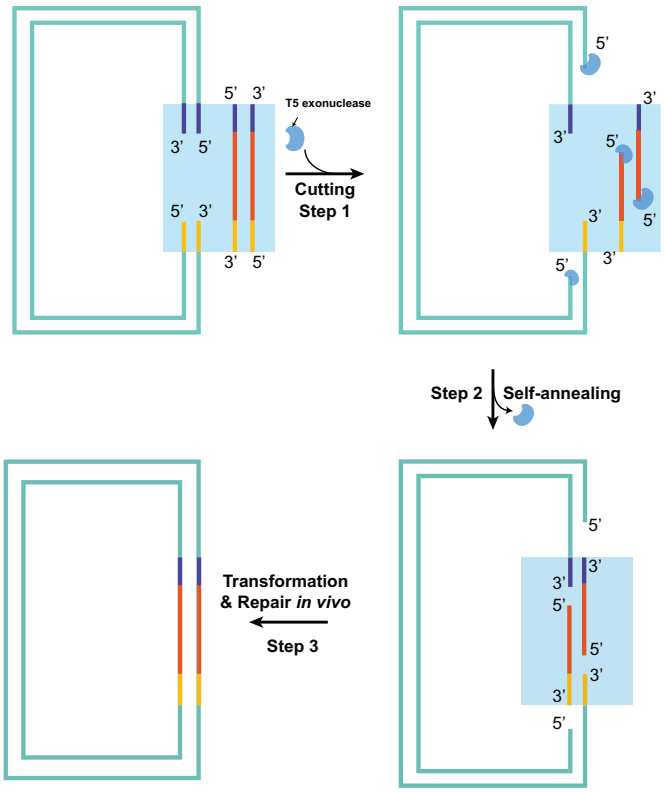

Figure 1. The schematic of the TEDA method. The blue half-moon represents T5 exonuclease. The double lined rectangle with a gap represents a linearized plasmid. The double vertical lines represent the insert DNA. Lines with same color indicate the homologous region. Step 1: T5 exonuclease cuts from the 5′ ends of linearized plasmid and insert DNA to generate 5′-overhangs. Step 2: the 5′-overhangs anneal to each other. Step 3: The cyclized DNA with DNA gaps is transformed into cells and the gaps are repaired *in vivo*.

ity is applied to generate 5′-overhangs that anneal to each other. The In-Fusion method applies a DNA polymerase of Human pox virus that is efficient for the assembly and has been successfully commercialized (11). Subsequently, the commercially available T4 DNA polymerase was shown to adequately assemble DNA fragments (10). Here we report a DNA assembly method that uses T5 exonuclease alone, in which homologous ends are digested by T5 exonuclease and annealed, with the rest of the repair being done inside *E. coli* host cells after transformation (Figure 1). The T5 exonuclease-dependent assembly (TEDA) can also be used for simultaneous site-directed mutagenesis (SDM) at multiple sites.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Strains and plasmids were listed in Supplementary Table S1. Primers were listed in Supplementary Table S2. Cells were cultured in lysogeny broth (LB) medium (Oxoid) containing the appropriate antibiotics (Sangon Biotech, Shanghai) at 37°C. Kanamycin, spectinomycin, ampicillin, and gentamycin were used at 50, 20, 100 and 15 μg/ml, respectively.

DNA polymerases and reagents

Phusion DNA polymerase (referred as 'Phusion' in text, Thermo Fisher, Beijing) was used to amply DNA fragments. T5 exonuclease (New England Biolab, Beijing) was used in Gibson, Hot Fusion, and TEDA. Premix PrimeSTAR HS polymerase (referred as 'PrimeSTAR' in text, Takara, Japan) and TransStart FastPfu Fly DNA Polymerase (referred as 'FastPfu' in text, Transgen, Beijing) were used in testing the Gibson and Hot Fusion methods. Taq DNA ligase (New England Biolab) was used to prepare the self-produced Gibson reaction mixture. 2X HotStart Taq plus Master Mix (Novoprotein, Beijing) was used for colony PCR test. The 5- and 1-kb DNA markers of Transgen (Beijing) were used to estimate DNA fragments. DNA and PCR product purification kits (Omega, US) were used to purify DNA fragment. All other chemicals were purchased from Sangon Biotech except PEG8000 that was purchased from Sigma-Aldrich (Shanghai). All restriction enzymes were purchased from Thermo Fisher. All oligos were synthesized by Beijing Genomics Institute.

The preparation of vector and insert for TEDA optimization

The plasmids used in this study are listed in Supplementary Table S1. All the primers and the PCR amplified fragments used for plasmids construction are given in Supplementary Table S2.

The promoter Pkat that constitutively expresses the kanamycin resistant gene from pKD4 and the *egfp* gene from pCL1920-eGFP were cloned into pBluescript SK- at the SmaI site to obtain pSK::eGFP, in which *egfp* is under the control of Pkat. The Pkat-eGFP fragment was used as the template for PCR to introduce 30-, 20-, 15-, 9- and 6-bp homologous ends to the SmaI-digested pBluescript SK- (SmaI-pSK). These fragments were cloned into SmaI-pSK with the TEDA method.

The *phbCAB* operon from pBHR68 was cut by NisI and XhoI and ligated into p5TG to form p5TG::phbCAB (18,19). The *phbCAB* under the control of five *tac* promoters (5Ptac-phbCAB) from p5TG::phbCAB was amplified via PCR as one fragment (4.3 kb), two fragments (2.8 kb/1.7 kb and 1.4 kb/3.1 kb), or three fragments (1.4, 1.4 and 1.7 kb) with 20-bp homologous ends to adjacent fragments or to SmaI-pSK. These fragments were assembled with SmaI-pSK to produce pSK::5Ptac-phbCAB by using TEDA.

5Ptac-phbCAB was also cloned into the PCR-amplified pBBR1MCS-2 to produce pBBR1MCS2::5Ptac-phbCAB by using TEDA. A TAA stop codon was inserted into *phbC* gene to obtain pBBR1MCS::5Ptac-phbCAB_TAA encoding a truncated and inactive PhbC. Similarly, pBBR1MCS2::5Ptac-phbCAB_2TAA contained a TAA insertion in both *phbC* and *phbB*, and pBBR1MCS2::5Ptac-phbCAB_3TAA contained a TAA insertion in *phbC*, *phbB*, and *phbA*. The TAA stop codons from these three plasmids

were removed by using TEDA to test for SDM at single or multiple sites.

The *trc* promoter (Ptrc) from pTrc99a and the *lacZ* gene from MG1655 genome were cloned into PCR-amplified pBBR1MCS-5 and pCL1920 to obtain pMCS5::Ptrc-lacZ and pCL1920::Ptrc-lacZ, respectively, with the lac promoter in the original plasmids being removed. The *lacZ* gene was also cloned into PCR-amplified pBluescript SK- to get pSK::Plac-lacZ. The middle region of *lacZ* was removed from these three plasmids to obtain the truncated *lacZ*. Then, the middle region of *lacZ* was cloned back into these three PCR-amplified plasmids to produce the functional *lacZ* by using TEDA.

The kanamycin-resistant gene with its promoter sequence from pKD4 was amplified and treated with SmaI to generate the Kan-SmaI fragment. Kan-SmaI was ligated into pBluescript SK- at the SmaI site to obtain pSK-Kan. Then, the pSK-Kan plasmid was cut with SmaI, KpnI-SacI or HindIII-XbaI to produce three linearized vectors with blunt ends, 5′-overhangs (5′Oh) or 3′-overhangs (3′Oh), named as pSK-blunt, pSK-5′Oh, and pSK-3′Oh, respectively. The Pkat-eGFP fragment from pSK::eGFP was amplified with different primers to generate Pkat-eGFP-blunt, Pkat-eGFP-5′Oh and Pkat-eGFP-3′Oh, containing ends that were homologous to the ends of pSK-blunt, pSK-5′Oh, and pSK-3′Oh, respectively. To generate 20-bp homologous arms of Pkat-eGFP-5′Oh and Pkat-eGFP-3′Oh, the 4-bp overhangs were either added into the homologous arms or not to generate Pkat-eGFP-5′Oh-4bp-plus and Pkat-eGFP-3′Oh-4bp-plus or Pkat-eGFP-3′Oh-4bp-minus and Pkat-eGFP-3′Oh -4bp-minus. Further, 9-bp homologous arms of Pkat-eGFP-5′Oh-plus and Pkat-eGFP-3′Oh-plus were also generated. These inserts were assembled with pSK-5′Oh and pSK-3′Oh, respectively.

5X TEDA stock solution

One milliliter of 5X TEDA solution contained 0.5 M Tris–HCl pH 7.5, 50 mM $MgCl_2$, 50 mM dithiothreitol, 0.25 g of PEG 8000, and 1 μl of 10 U/μl T5 exonuclease (New England Biolab, Beijing). Aliquots of 100 μl could be stored at −80°C for long-term storage. For cloning, aliquots of 4 μl were separated into 200-μl PCR tubes and frozen at −80 or −20°C. The 5X solution was also diluted to 4/3X and 2X stock solutions.

The recommended TEDA procedure

A 4-μl aliquot of 5X TEDA reaction solution was thawed on ice, and 16 μl of DNA solution including the linear vector and insert was added. The linearized vector was used between 100 and 200 ng for a 3-kb plasmid and the molar ratio of vector to insert was 1:1–1:4. The linearized vectors were obtained by either enzyme-digestion or PCR amplification. The insert was obtained by PCR amplification, and homologous ends were introduced via primers. Both vectors and inserts were purified either via gel purification (Omega, US) or a commercial PCR Cycle-Pure Kit (Omega, US). After mixing, the reaction was carried out at 30°C for 40 min and terminated by placing on ice, and the reaction solution was then used for transformation.

The Gibson and SLIC methods were done as reported (10,15), and the In-Fusion method was done according to the instruction of the commercial kit (Takara). For comparison, 100 ng of the vector was used for all reactions.

Preparation of competent cells

Competent cells were prepared via three different methods: the KCM, Inoue and electroporation methods (20–22). A modified Inoue method was shown to be the most effective. Briefly, a fresh colony of *E. coli* from LB plates was picked up and transformed into LB broth for culturing at 30°C with shaking. Then, 0.5 ml of the overnight culture was transferred into 50 ml of LB broth and incubated at 30°C until the OD_{600} reached 0.4–0.6. The cells were harvested by centrifugation at 2900 × *g* for 10 min. The cells were washed with 20 ml of ice-cold Inoue solution (55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl and 10 mM PIPES (pH 6.7)) and resuspended in 2 ml of the Inoue solution; 0.15 ml DMSO was added and mixed. The preparation was divided into 100-μl aliquots and stored at −80°C.

Transformation

A modified Inoue transformation procedure was adopted. An aliquot of 100-μl Inoue competent cells from −80°C was thawed on ice. Five microliter of a TEDA reaction solution was mixed with the Inoue competent cells. The tube was stored on ice for 30 min, heated at 42°C in a water bath for 90 s, and then transferred on ice for 2 min. 900 μl of LB medium was added into each tube and incubated with gentle shaking at 37°C for 1 h. 10 to 100 μl of the cell suspension was spread onto LB plates with indicated antibiotics for screen.

The screening of positive colonies

Phenotypes were used for initial screening to obtain the positive rates (positive colonies/total colonies). Cells that overexpress *phbCAB* formed white colonies on LB plates with 2% glucose due to the accumulation of polyhydroxybutyrate (PHB) (18). Colonies producing eGFP were checked for fluorescence by using FluorChem Q (ProteinSimple, USA). Blue-white screening was used to check for the expression of *lacZ* on LB plates with the appropriate antibiotic, 1 mM isopropyl β-D-1-Thiogalactoside (Sangon Biotech, China), and 0.5 mg/ml of X-gal (Sangon Biotech, China) (22). Ten to 20 positive colonies were then checked via colony PCR. Five or ten plasmids from the positive colonies were sent for sequencing at TsingKe Biological Technology Company (Beijing, China) to verify the inserts as indicated in the text. The PCR and sequencing primers for the plasmids used in TEDA optimization and testing were listed in Supplementary Table S2.

RESULTS

T5 exonuclease in a Tris buffer with PEG 8000 works well for cloning

The Gibson method applies three enzymes, and it can be simplified by removing Taq DNA ligase without reducing

the cloning efficiency (17,23). To test whether the method could be further simplified, we checked the requirement of the enzymes and other components in the Gibson system for cloning Pkat-eGFP into pBluescript SK- (Figure 2A). The simplified method with only T5 exonuclease worked well at a similar efficiency to the Gibson method and the Hot Fusion method (Figure 2B and C). All yielded >95% positive clones with green fluorescence. Twenty positive colonies in each group were tested via colony PCR and confirmed through isolation and restriction digestion. All were correct. Ten of the isolated plasmids were then sent for sequencing by using M13F and M13R primers, and no errors were found. Thus, T5 exonuclease alone is sufficient for DNA assembly with the gap filling by DNA polymerase and ligation being completed after transformation inside *E. coli* cells (Figure 1).

The optimization of the TEDA reaction

Fourteen factors that might affect TEDA efficiency were tested for optimization (Table 2). The optimal reaction was at 30°C for 40 min with 0.04– 0.08 U T5 exonuclease per reaction (Supplementary Figure S1A–C). PEG 8000, glycerol and bovine serum albumin (BSA) are known to increase the enzyme's stability or activity (24,25). 5% of PEG 8000 in the reaction solution was the best for the cloning efficiency (Supplementary Figure S2A). BSA decreased the efficiency (Supplementary Figure S2B). Glycerol from 0 to 20% in the reaction solution did not affect the cloning efficiency (Supplementary Figure S2C). Several low temperatures were also tested to facilitate annealing, but they did not improve the efficiency (Supplementary Figure S2D).

The amount of DNA was optimal between 100 and 200 ng in a 20-μl reaction for a 3-Kb vector (Supplementary Figure S3A). The vector to fragment molar ratio between 1:1 and 1:4 had the most cloning efficiency (Supplementary Figure S3B). The length of homologous ends could be decreased to 15 bp without affecting the cloning efficiency (Supplementary Figure S3C). Since TEDA was performed at 30°C instead of 50°C, the reduced temperature may allow short single stranded DNA to anneal. In all tests, the percentages of positive colonies were higher than 95%.

Different types of plasmid ori were tested to evaluate if they could affect the TEDA efficiency. We first cloned *lacZ* (3075 bp) as a reporter gene into pBBR1MCS-5, pCL1920 and pBluescript SK- with pBBR ori, pSC101 ori, and pUC ori, respectively. The middle part of *lacZ* (1937 bp in length, from 17 bp to 1953 bp of *lacZ*) was removed, to obtain pBBR1MCS-5::lacZ-truncated, pCL1920::lacZ-truncated, and pSK::lacZ-truncated. Then the middle part of *lacZ* (Middle-*lacZ*) was generated by PCR with the overlapping region between the vector and the fragment being 15- or 20-bp, and cloned back into the vectors by using TEDA (Supplementary Figure S4A–C). The assembly efficiency showed no significant difference between the 15-bp overlapping group and the 20-bp overlapping group or among different replication origins (Supplementary Figure S4D). The only exception was with pBBR1MCS-5, which generated 2-fold more colonies with the 20-bp homologous ends than that with 15-bp homologous ends. The positive rate (blue colonies/total colonies) in each group was higher than 95%.

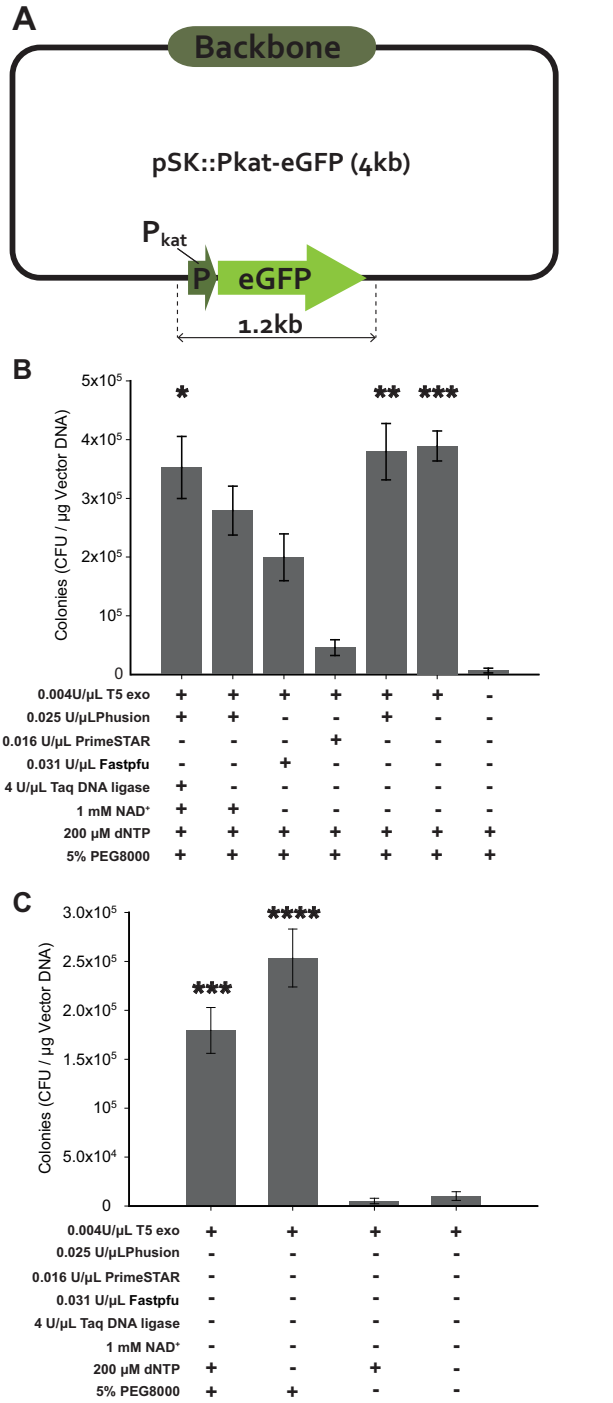

Figure 2. Enzymes and buffer components required for TEDA. (**A**) The pKat-eGFP fragment was cloned into SmaI-digested pBluescript SK–. The assembly of the two fragments was used as a model for the test. (**B**) Taq DNA ligase, Phusion DNA polymerase, T5 exonuclease (T5 exo), NAD^+ were tested for their necessity for the DNA assembly. In addition, PrimeSTAR or FastPfu was also used instead of Phusion for testing; (**C**) PEG 8000 and dNTPs were further tested for their necessity for the DNA assembly. The concentrations of relevant components mentioned above were indicated in the figure. The base solution contained 0.1 M Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 10 mM dithiothreitol. The reaction was processed at 50°C for 1 h, which was the same as the Gibson assembly. *, Gibson; **, Hot Fusion; **, TEDA with dNTPs and at 50°C; ****, TEDA without dNTPs at 50°C. The data are averages of three parallel experiments with STDEV.

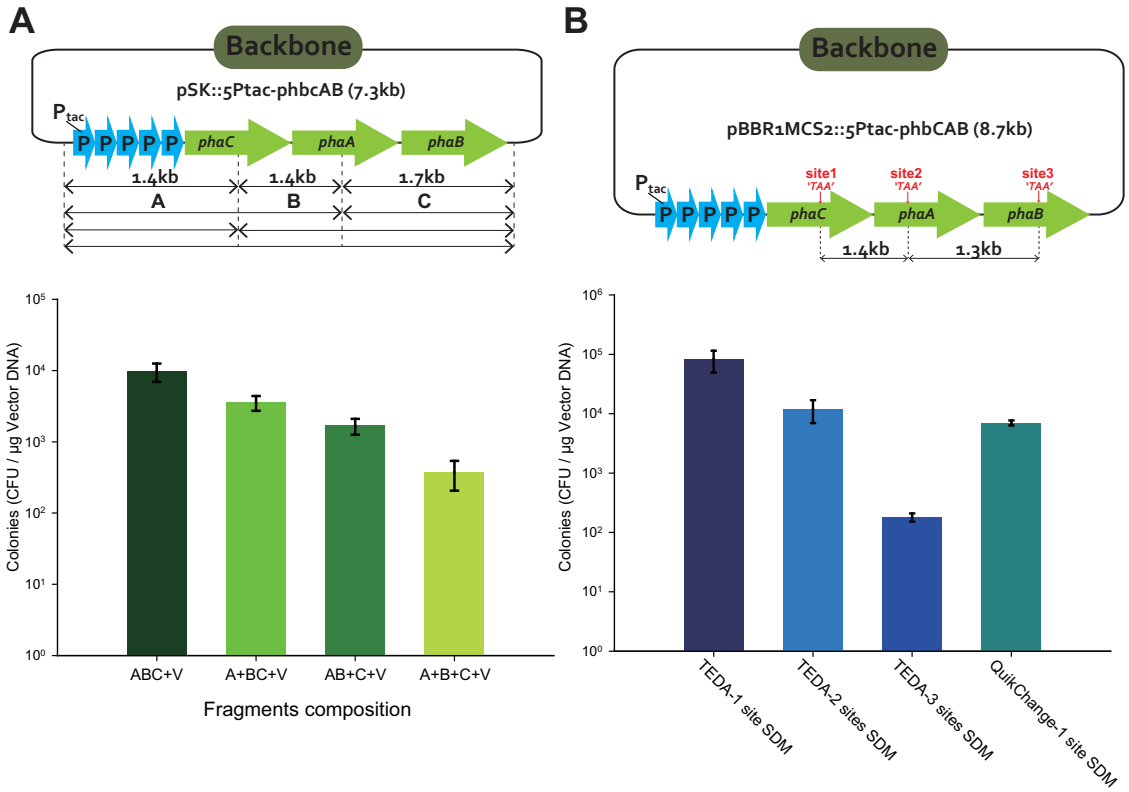

Figure 3. TEDA was used for multiple DNA fragment assembly and SDM at multiple sites. (**A**) The *phbCAB* gene cluster was used as ABC, or separated into three fragments (A, B and C), or two fragments (AB and C, or A and BC). These fragments were assembled with SmaI-digested pBluescript SK- (V) by using TEDA. (**B**) The *phbA*, *phbB* and *phbC* genes on pBBR1MCS2–5Ptac-phbCAB contained one, two, or three stop codons (TAA). The efficiency of TEDA to remove one (1 site SDM for site 1), two (2 sites SDM for sites 1 and 3) or three (3 sites SDM for all 3 sites) stop codons in one single TEDA reaction was tested. The modified QuikChange SDM method was applied to remove one 'TAA' codon at site 1 as a control. The data are averages of three parallel experiments with STDEV.

These results showed that the TEDA assembly works well with different cloning vectors.

The storage conditions and TEDA stock solution concentrations were also tested. The TEDA stock solutions were prepared as 4/3-, 2- and 5-fold concentrated, and were stored at −20 and −80°C. The efficiency of TEDA was tested after storage (Supplementary Figure S5A&B). The results indicated that the cloning efficiency was not affected with the tested stock solutions when stored at −20 or −80°C for one month. We also compared the cloning efficiency for a 5X TEDA solution stored at −80°C for 4 months and a newly prepared one, and no efficiency difference was found (Supplementary Figure S5C). Gibson assembly reaction solution has been reported to have no efficiency loss after one-year storage at −80°C (15). Since TEDA has only one enzyme and Gibson has three, longer storage time for 5X TEDA solution is also expected.

TEDA could assemble multiple DNA fragments and simultaneously mutate at multiple sites

A gene cluster for PHB synthesis under the control of five *tac* promoters (5Ptac-phbCAB, 4.3 kb) was cloned into pBluescript SK– at the SmaI site by using TEDA as a single fragment, two fragments, or three fragments (Figure 3A). When PHB is overproduced in *E. coli* cells, the colonies will turn white (18,19). In all tests, the positive colonies accounted for more than 90% of the total colonies. The number of positive colonies exponentially decreased with the increase in fragment numbers (Figure 3A). Ten white colonies from the four fragments assembly (noted as 'A+B+C+V') were confirmed by using colony PCR and restriction enzyme digestion of the isolated plasmids. Five of the isolated plasmids were also sequenced. All results showed the tested white colonies contained the right clones.

The plasmids with functional PHB synthesis genes or with a stop codon TAA insertion into one, two, or three genes were constructed. We then tested to use TEDA to remove these stop codons in a single step. The plasmids pBBR1MCS2::5Ptac-phbCAB-1TAA, pBBR1MCS2::5Ptac-phbCAB-2TAA and pBBR1MCS2::5Ptac-phbCAB-3TAA were divided into one, two and three fragments, respectively. The break points were at the sites where the TAA stop codons were inserted. The TAA stop codons at these sites were removed via PCR primers, and 20-bp homologous ends were generated via PCR (Figure 3B). The TEDA method could efficiently edit three sites of pBBR1MCS2::5Ptac-phbCAB-3TAA in a single step (Figure 3B). The efficiency increased more than 10 folds for single SDM than our previously modified QuikChange SDM method (22) (Figure 3B). White colonies were used for initial screening. The positive

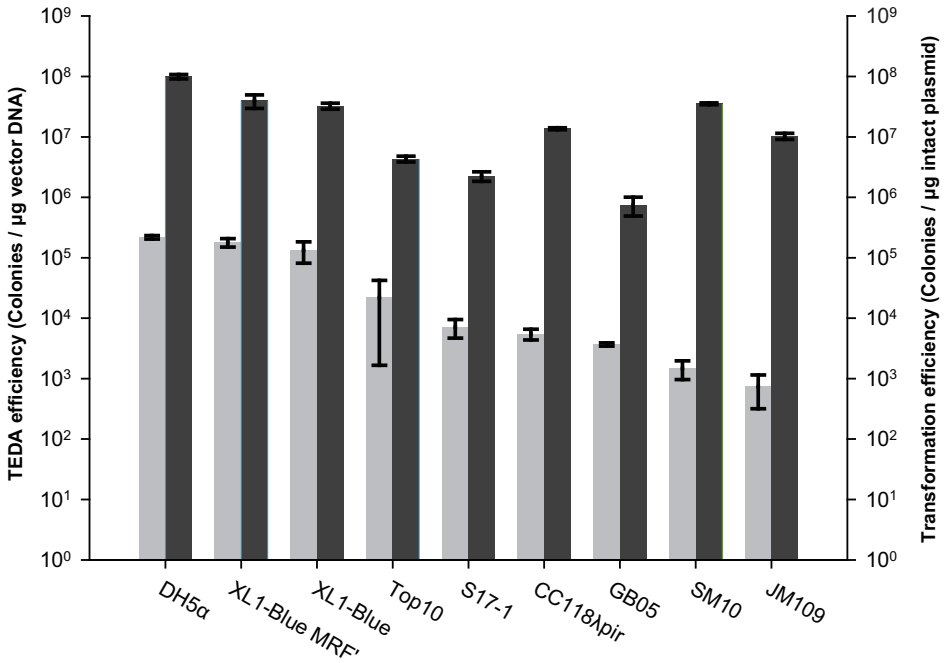

Figure 4. The effect of host strains on TEDA efficiency. The Inoue method was used to prepare the competent cells. Middle-*lacZ* and pBBR1MCS5::lacZ-truncated with 15 bp homologous arms were assembled by using TEDA (Supplementary Figure S4B). The TEDA reaction mixture was transformed into competent cells of different *E. coli* strains (gray columns). As a control, the intact pBluescript SK- plasmid was transformed into the competent cells to test for the transformation efficiency (dark columns). The data are averages of three parallel experiments with STDEV.

rates for all tests were all higher than 90%. The negative colonies were mainly from the undigested vector, which was used as the PCR template and then digested by DpnI. Ten white colonies from the mutations at three sites (noted as 'TEDA-3 sites SDM') were confirmed by using colony PCR and restriction enzyme digestion check. Then five plasmids of these 10 colonies were sequenced, and they all had the correct mutation.

The effects of competent-cell preparation and *E. coli* strains on the TEDA efficiency

Three different competent cell preparation methods were investigated (Table 3). The Inoue method, electroporation method, and KCM method all have similar efficiency when tested with an intact vector pBluescript SK-, but the electroporation method had the lowest efficiency with significantly less positive colonies when used together by using TEDA to assemble pBBR1MCS-5::lacZ-truncated and Middle-lacZ with 15 bp homologous arms, suggesting that electroporation was not ideal for TEDA. The Inoue method was recommended since it showed the highest efficiency for generating positive colonies.

Nine *E. coli* strains were used to test their compatibility with TEDA (Figure 4). DH5α, JM109, Top10, XL1-Blue, CC118λpir, SM10 and XL1-Blue MRF' had higher than 10^7 CFU/μg of intact plasmid by using the Inoue method to prepare competent cells. However, DH5α, XL1-Blue and XL1-Blue MRF' showed higher efficiency for cloning than others with higher than 10^5 CFU/μg of linearized plasmid. Hence, the competent cells of these three strains prepared by the Inoue method were recommended for TEDA.

The effects of linearized vectors with blunt ends, 3′-overhangs, or 5′-overhangs

The linearized vector pSK-5′Oh was assembled with Pkat-eGFP-5′Oh-4bp-plus or Pkat-eGFP-5′Oh-4bp-minus by using TEDA to test the effect of 5′-overhangs on TEDA efficiency (Figure 5A). The insert Pkat-eGFP-5′Oh-4bp-plus contained the 4-bp overhangs of the linear vector in the 20-bp homologous arms, and Pkat-eGFP-5′Oh-4bp-minus did not. In a similar way, pSK-3′Oh was assembled with Pkat-eGFP-3′Oh-4bp-plus or Pkat-eGFP-3′Oh-4bp-minus (Figure 5A). For the control, pSK-blunt was assembled with Pkat-eGFP-blunt (Figure 5A). Positive colonies displayed fluorescence. For all groups, the positive colonies were higher than 95%. Ten positive colonies were further checked with colony PCR and restriction enzyme digestion. Five of them were sequenced with M13F and M13R primers. The results were all correct. The cloning efficiencies as measured by the number of positive colonies were similar when using the linearized vectors with blunt ends or 3′-overhangs, but the efficiency was clearly lower with the linearized vector containing 5′-overhangs (Figure 5B). The averaged positive colonies from the Pkat-eGFP-5′Oh-4bp-plus and -minus groups were only 11% and 8% of that of the Blunt-blunt group (Figure 5B). The 3′- and 5′-overhangs from the linearized vector that did not share homology to the insert were removed during assembly (Figure 5A); the removal of these 4-bp overhangs during the assembly did not affect the cloning efficiency (Figure 5B).

To confirm whether the 5′-overhangs of the vector were removed, pSK-5′Oh and pSK-3′Oh were assembled with Pkat-eGFP-9bp-5′Oh-4bp-plus and Pkat-eGFP-9bp-3′Oh-

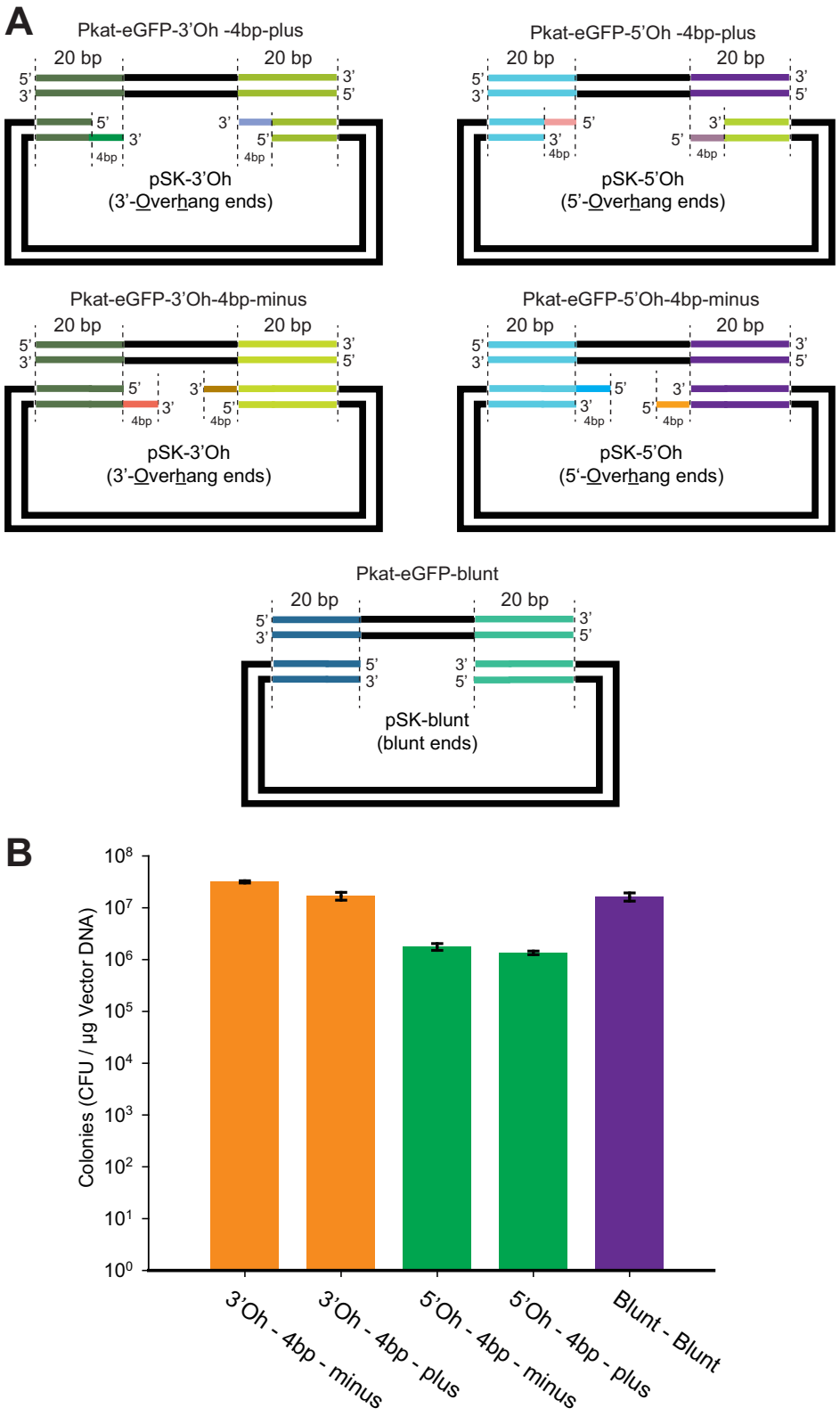

Figure 5. The effect of the linearized vector with different ends on TEDA. (**A**) The schematic presentation of the linearized vector with different ends and insert containing 20-pb homologous arms. Pkat-eGFP-3′Oh-4bp-plus and Pkat-eGFP-5′Oh-4bp-plus, the 4 bp of the overhangs were homologous to the 20-bp arm of the insert; Pkat-eGFP-3′Oh-4 bp-minus and Pkat-eGFP-5′Oh-4bp-minus, the 4 bp of the overhangs was not homologous to the insert arm. (**B**) The cloning efficiency with different ends and inserts containing 20-bp arms by using TEDA. The data are averages of three parallel experiments with STDEV.

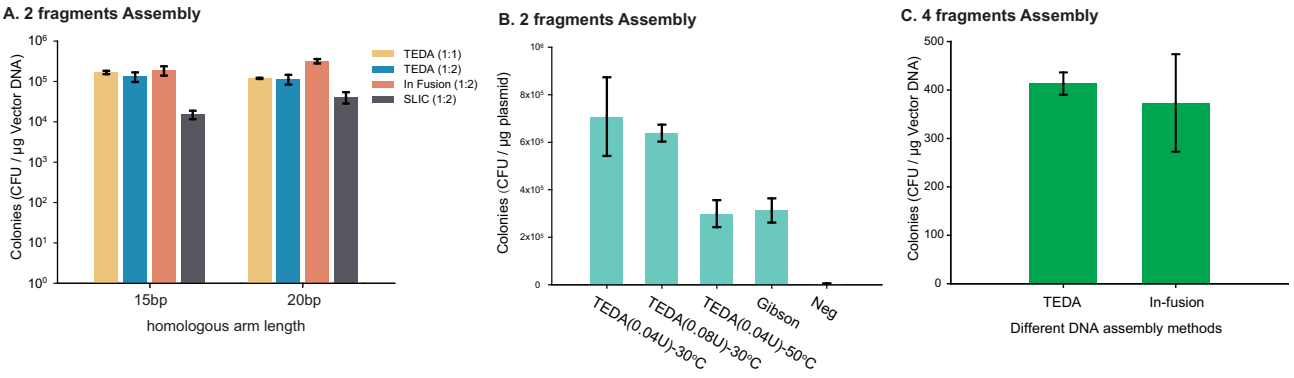

Figure 6. Comparison of different assembly methods. (**A**) TEDA was compared with In-fusion and SLIC for the assembly of two fragments. Middle-*lacZ* and pBBR1MCS5::lacZ-truncated with 15-bp or 20-bp overlaps were used. 1:1, the same molar ratio of the insert to vector was used for DNA assembly; 1:2, double molar amount of the insert to vector was used for DNA assembly. (**B**) TEDA was compared with Gibson and non-optimized TEDA methods. The Pkat-eGFP and SmaI-pSK was used for cloning. TEDA(0.04U)−30°C, 0.04 U T5 exonuclease at 30°C for 40 min; TEDA(0.08 U)−30°C, 0.08 U T5 exonuclease at 30°C for 40 min; TEDA(0.04 U)−50°C, 0.04 U T5 exonuclease at 50°C for 40 min; Gibson, 0.08 U T5 exonuclease with Phusion and Taq DNA ligase at 50°C for 60 min. Neg, DNA fragments were transformed without TEDA treatment. (**C**) TEDA was compared with In-fusion for 4 fragments assembly. The 5Ptac-phbCAB operon was separated into three fragments (Figure 2A), and they were assembled with linearized pBBR1MCS-2 to generate pBBR1MCS2::5Ptac-phbCAB. The data are averages of three parallel experiments with STDEV.

4bp-plus by using TEDA, respectively (Supplementary Figure S6A). The assembly of pSK-3′Oh and Pkat-eGFP-9bp-3′Oh-4 bp-plus formed positive colonies, but the assembly of pSK-5′Oh and Pkat-eGFP-9bp-5′Oh-4bp-plus did not (Supplementary Figure S6B). The results indicated that the TEDA worked via removing the 5′-overhang, and the resulting homologous region was only 5 bp, which was too short for *in vitro* assembly.

The comparison of TEDA with other DNA assembly methods

Different DNA assembly methods were compared for their efficiency with 2-fragment or 4-fragment assembly (Figure 6A–C). The efficiency of TEDA was similar to the commercial In-Fusion kit and higher than SLIC (Figure 6A) and Gibson (Figure 6B). TEDA method also had a similar efficiency for 4-fragment assembly as In-Fusion (Figure 6C). For all the methods, the positive colonies that produced blue color from isopropyl β-D-1-Thiogalactoside were all higher than 95%. Ten blue colonies were further confirmed by colony PCR and enzyme digestion. Five of these 10 colonies were also sequenced with no errors being found. The results suggest that the optimized TEDA could match or exceed the widely used DNA assembly methods for routine cloning.

DISCUSSION

TEDA is a simple method derived from the Gibson assembly and the Hot Fusion method (15,17). For the Gibson method, both filling the gaps by DNA polymerase and covalent linking by DNA ligase are done outside of the cells, which may be necessary for the assembly of large plasmids (15). For the Hot Fusion method, the filling is done outside of the cells but the covalent linkage is done inside the cells (17). For routine cloning as tested, removing Phusion DNA polymerase and Taq DNA ligase from the Gibson system did not affect the cloning efficiency (Figure 2B). The result is different from a recent report, showing a 3.7-fold lower efficiency when DNA polymerase was also removed; however, they used a vector-to-insert ratio of 1:8 instead of 1:1 or 1:2 (23). Since we found that the vector-to-insert ratio over 4 reduced the TEDA's efficiency (Supplementary Figure S1), the difference is likely caused by the ratio used. TEDA was 2- to 3-fold more efficient than the Gibson and Hot Fusion methods. Thus, for routine cloning, TEDA is preferred over Gibson and Hot Fusion. TEDA uses only T5 exonuclease to create 3′-overhangs of linear DNA fragments, which anneal to form circle DNA with gaps. After transformation, the gap repair and covalent linkage occur inside *E. coli* cells (Figure 1).

The DNA polymerase-based DNA assemblies, including In-Fusion and SLIC, also use a similar strategy to that of TEDA with the repair done after transformation (10,11). The DNA polymerases have the 3′-5′ exonuclease activity for proof-reading and generate 5′- overhangs that anneal to each other. TEDA uses T5 exonuclease that generates 3′-overhangs. Little is known about the DNA polymerase used in the commercial In-Fusion kit. However, both T5 exonuclease and T4 DNA polymerase are semi-processive exonucleases (26,27). For TEDA, we recommend using 0.04 U T5 exonuclease per reaction, incubating at 30°C for 40 min; whereas, SLIC uses 1.5 U of T4 DNA polymerase per reaction with a incubation of 2.5 min at room temperature, followed by incubating on ice for annealing (10). Considering the differences in incubation time and temperature, the total digestion by both is in a similar range. TEDA has a similar assembly efficiency as that of In-Fusion, but higher than that of SLIC (Figure 6A).

The simplicity of the TEDA reaction makes it easier to optimize. After optimization, the method is simple, fast, efficient, and cost-effective. For optimization, PEG 8000 and the proper dilution of T5 exonuclease were two key factors for TEDA (Figure 2A&B). PEG 8000 greatly enhanced the TEDA efficiency (Figure 2A), probably due to its ability to enhance the annealing of complementary DNA strands (28). TEDA requires 0.04–0.08 U T5 exonuclease per reaction (Supplementary Figure S2B). The low unit of T5 exonuclease ensures partial digestion. Further, without a

Table 1. The collections of the current popular DNA assembly methods

DNA assembly methods[a]	Price/reaction ($)[b]	Valuable components in one reaction	Origins
Enzyme methods			
Self-prepared Gibson	3.03	0.08 U T5 exonuclease (NEB), 0.5 U Phusion DNA polymerase (NEB) and 80 U Taq DNA ligase (NEB)	(15)
Commercial Gibson	12.6	Commercial kit (NEB)	NEB
In-Fusion	24.4[c]	Commercial kit (CloneTech)	CloneTech
GeneArt	36.3[c]	Commercial kit (Thermo)	Thermo
NEBuilder	18.5	Commercial kit (NEB)	NEB
SLIC, one step SLIC	0.264	0.6 U T4 DNA polymerase (NEB)	(10,33)
Hot Fusion	0.535	0.08 U T5 exonuclease (NEB), 0.5 U Phusion DNA polymerase (NEB)	(17)
TEDA	0.0025	0.04 U T5 exonuclease (NEB)	This study
DATEL	1.66	40 U Taq ligase (NEB), 1 U Taq DNA polymerase (Tiangen) and 1 U pfu DNA polymerase (Tiangen)	(34)
PCR			
CPEC	1.06	1 U Phusion DNA polymerase	(35)
TPA	2.1	2 U Q5 DNA polymerase for a 2 fragments assembly	(36)
Recombination *in vitro*			
SLiCE	0.29	25 μl of CelLytic B (Sigma) and one 0.5-mL protein-low-binding tube (Eppendorf) were required	(12)

[a]Only the DNA assembly methods that have competitive efficiency were considered.
[b]The cost only applies to the valuable components in the reaction.
[c]The price was transferred by rate calculation of RMB to USD.

Table 2. Summary of optimized TEDA conditions

Terms	Unit	Tested conditions	Optimal condition
Reaction temperature	°C	15, 20, 25, 30, 35, 40, 45, 50, 55, 60	30
Enzyme concentration	U/reaction	0.02, 0.04, 0.08, 0.16, 0.32, 0.64	0.04–0.08 U
Reaction time	min	10, 20, 30, 40, 50, 60	40
PEG 8000	% (w/v)	0, 1, 3, 5, 10, 15, 20, 25	3%–10%
BSA	mg/ml	0, 0.05, 0.1, 0.2, 0.4, 0.8	0
Glycerol	% (v/v)	0, 5, 10, 15, 20	No difference
Annealing temperature step	°C	0, 10, 20	No difference
Vector (3Kb size) amount	ng/reaction	50, 100, 200, 300, 400	100–200
Molar ratio	Vector:Insert	8:1, 4:1, 2:1, 1:1, 1:2, 1:4, 1:8	1:1–1:4
Homologous arm	bp	6, 9, 15, 20, 30	≥15 bp
Vector ori	–	pUC ori, pBBR1MCS ori, pSC101 ori	No difference
Storage temperature	°C	−20°C, −80°C	No difference
Strorage time	day	3, 10, 30	No difference
Stock solution	fold	4/3×, 2×, 5×	No difference

thermophilic DNA polymerase that requires high temperature for activity, TEDA allows the reaction at 30°C rather than 50°C as in the Gibson and Hot Fusion methods (Supplementary Figure S1A). As reported, 50°C keeps Phusion DNA polymerase and Taq DNA ligase active as well as inactivating T5 exonuclease after its has generated the 5′-single strand ends (15). However, the supplier claims that T5 exonuclease is not inactivated by heating (https://www.neb.com). Our results showed that inactivation of T5 exonuclease is not required as long as the concentration of the enzyme is low, consistent with the heat-resistant property of the enzyme. The low temperature may also facilitate annealing of short overhangs. On the basis of our results we recommend 9- to 15-bp overhangs for one-fragment cloning (Supplementary Figure S3C) and 20-bp overhangs for multiple-fragment assembly (Figure 3A). The short overlaps also reduce the cost for primer synthesis.

For TEDA, the vector can be prepared by PCR or by restriction enzyme digestion. When prepared by the digestion, the TEDA method works equally well for the linearized vector with blunt ends or 3′-overhangs, but the efficiency was only about 10% with the linearized vector containing 5′-overhangs (Figure 5). T5 exonuclease is not only a very efficient exonuclease (29), but also a flap endonuclease (30), capable of threading 5′-overhangs and cleaving them (31). When it treats 4-bp 5′-overhangs, it uses its flap endonuclease activity to cut off the 5′-overhangs, and then uses its exonuclease activity for further digestion. For the 3′-overhangs and blunt ends, the enzyme directly uses its exonuclease activity (32). Thus, the vectors can be linearized with any restriction enzymes, but the enzymes that create blunt ends and 3′-overhangs are preferred.

Besides efficiency and simplicity, the cost is an important factor for cloning (Table 1). Several reports have tried to reduce the cost (Table 1). Jae-Yeon Jeong *et al*. optimized the SLIC method, removing RecA and ATP, without sacrifice of cloning efficiency (10). Hot Fusion was modified from the Gibson method without the addition of Taq DNA ligase and NAD^+ (17). The SLiCE method used a cell extract for DNA assembly, but it required a commercial cell lysis buffer CelLytic B for cell lysis and a special 0.5-mL low protein-binding tube, both of which increase the cost

Table 3. The effects of competent cell preparation methods on TEDA efficiency

Competent cells	Transformation Eff.[a]	TEDA Eff.[b]	Positive Perc.[c]
Electroporation	$3.10 \times 10^7 \pm 3.61 \times 10^6$	$1.69 \times 10^3 \pm 9.26 \times 10^2$	69.9% ± 6.4%
Inoue	$1.63 \times 10^7 \pm 1.51 \times 10^6$	$9.43 \times 10^4 \pm 1.65 \times 10^4$	98.8% ± 0.3%
KCM	$7.00 \times 10^6 \pm 1.51E \times 10^6$	$1.61 \times 10^4 \pm 2.61 \times 10^3$	96.1% ± 3.1%

[a]Transformation Efficiency was estimated with the number of formed colonies after transformation with intact pBluescript SK- normalized to 1 μg.
[b]TEDA efficiency was estimated with the number of formed colonies after transformation of the TEDA product of the linearized plasmid pBBR1MCS-5::lacZ-truncated and the insert Middle-lacZ with 15 bp homologous arms at molar ratio 1:1, normalized to 1 μg of the plasmid.
[c]Positive percentage was the percentage of positive clones.

(12). Recently, DNA assembly methods that only depend on PCR techniques were developed (Table 1) (35,36), offering a different approach to DNA assembly. The PCR method requires two sets of primers and two PCR reactions for each fragment and the vector. We believe that TEDA has the lowest cost for cloning among all reported methods that have the features with the sequence-independent and ligase-independent manner (Table 1). Diluted T5 exonuclease (0.25 cents/reaction) in the Tris-buffer containing PEG 8000, Mg^{2+}, and dithiothreitol is effective for the cloning. With the low cost, scientists from developing countries and students in high schools and colleges will be more likely to engage in cloning and SDM.

We recently revised the QuikChange™ SDM method (22). The PCR reaction proceeds with an exponential amplification of the plasmid to produce linear DNA with homologous ends that are joined to generate the mutated plasmid after transformation inside the host *E. coli* cell (22). The ability of *E. coli* to assemble DNA fragments has been known for a long time and been used to develop DNA assembly methods (23,37–42). Most recently, Huang and colleagues further optimized this method and offered a low-cost solution for DNA assembly (39). However, the efficiency is low. When the QuikChange™ PCR product was treated with T5 exonuclease according to TEDA, the efficiency of forming positive colonies was 10-fold higher than without T5 exonuclease treatment (Figure 3B). Further, the number of colonies formed by using TEDA for two-fragment assembly were 50 to 500 fold higher than co-transformation of the same amount of linearized vector and insert directly into competent cells, which were used as negative control as reported in several figures (Figure 6B, Supplementary Figure S1A, B–S3A, B and S5A, B). Therefore, instead of generating several site-directed mutations in a cloned gene one by one, several DNA fragments with mutations in the homologous arms can be generated through PCR and linked by TEDA in one reaction (Figure 3B). The results imply that the exonuclease digestion is likely a limiting step inside *E. coli* when blunt-end DNA fragments are directly transformed into the cell. The use of TEDA can overcome this limitation. This finding resonates with previous use of DNA assembly methods for SDM (43–48).

In summary, TEDA is efficient for routine cloning and simultaneous SDM at multiple sites. The latter employs the ability of TEDA to assemble several DNA fragments in one reaction. The low cost, simplicity, and efficiency should promote its adaption, which may trigger further improvement and the use of exonucleases for DNA assembly and SDM.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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