


## TECHNOLOGICAL NOTE

# Cloning short DNA into plasmids by one-step PCR

Cheng-Cheng Tao<sup>1†</sup>, Ying Yang<sup>1†</sup>, Fang Li<sup>2†</sup>, Ling Qiao<sup>3</sup>, Yue Wu<sup>1</sup>, Xiao-Dong Sun<sup>1</sup>, Yuan-Yuan Zhang<sup>1,4</sup>  & Chang-Long Li<sup>1</sup>

1 West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu, China

2 Department of Medical Oncology, Sichuan Cancer Hospital and Institute, School of Medicine, University of Electronic Science and Technology of China, Chengdu, China

3 School of Basic Medicine, Chengdu University of Traditional Chinese Medicine, Chengdu, China

4 School of Biological Sciences and Technology, Chengdu Medical College, Chengdu, China

## Keywords

Homologous recombination; one-step PCR; plasmid construction; restriction enzyme-based cloning method.

## Correspondence

Yuan-Yuan Zhang and Chang-Long Li, West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu 610041, P.R. China.  
Tel: +86 28 8550 1278  
Fax: +86 28 8550 1237  
Email: sarahyyzhang@hotmail.com (Zhang); changlongli@scu.edu.cn (Li)

<sup>†</sup>These authors contributed equally

Received: 6 August 2020;

Accepted: 30 August 2020.

doi: 10.1111/1759-7714.13660

Thoracic Cancer **11** (2020) 3409–3415

## Introduction

Plasmid vectors with small or multiple-tags, and small RNAs are commonly used in biomedical studies. Current methods of plasmid construction require obtaining the gene of interest from the amplification of the DNA template or chemically synthesized oligonucleotides.<sup>1–3</sup> Obtaining a gene fragment of interest from the DNA library is the most commonly used method.<sup>1</sup> It relies on digestion of DNA by type II restriction enzymes, and cutting the plasmid DNA circles with the same restriction nuclease to create linear DNA molecules. Finally, the gene

## Abstract

**Background:** Plasmid construction of small fragments of interest (such as insertion of small fragment marker genes, expression of shRNA, siRNA, etc) is the basis of many biomolecular experiments. Here, we describe a method to clone short DNA into vectors by polymerase chain reaction (PCR), named one-step PCR cloning. Our method uses PCR to amplify the entire circular plasmid. The PCR was performed by the primers containing the gene of short DNA with overlapping sequences between 10–15 bp. The PCR products were then transformed into *E. coli* and cyclized by homologous recombination in vivo.

**Methods:** The pEGFP-N1-HA plasmid was constructed by one-step PCR and transformation. Cells were transfected with pEGFP-N1-HA and pEGFP-N1 plasmid using TurboFect transfection reagent. Protein expression was detected by western blotting and the HA-GFP fusion protein was detected by confocal microscopy.

**Results:** The pEGFP-N1-HA plasmid was successfully constructed and HA expression in cells.

**Conclusions:** Free from the limitations of restriction enzyme sites and omitting the ligation process, our method offers a flexible and economical option of plasmid construction.

## Key points

**Significant findings of the study** A method to clone short DNA into plasmids was found.

**What this study adds:** Our study provides a flexible and economical option to clone short DNA into plasmids.

of interest and the linearized vector are separately purified, mixed together at a certain molar ratio, and ligated with T4 DNA ligase to form recombinant DNA circles.<sup>4–7</sup> This process involves cumbersome subcloning procedures including designing primers, digestion, ligation and verification of recombinant plasmids.<sup>8, 9</sup> Therefore, its efficiency is dependent on both the restriction enzyme and the DNA ligase. In addition, unwanted nucleotide sequences may be added to the insert, and result in undesirable changes in the final products.<sup>10</sup> The chemical synthesis method is also called oligo overlap cloning, which can be used to add a

short stretch of DNA to a plasmid, such as adding short tags (HA-tag or Flag) and cloning shRNAs.<sup>3, 11</sup> The DNA fragment of interest with restriction site is obtained through chemical synthesis, and the linearized vector with sticky ends is obtained by digesting the vector with restriction endonuclease.<sup>11, 12</sup> Both methods share similar limitations.

To overcome these limitations, several PCR-based cloning protocols have been proposed to skip the use of DNA ligases and restriction endonucleases. Similar to our method, these methods are based on homologous recombination. The PCR products are flanked by 15 to 60 bp sequences, which exactly match the ends of the linear vector.<sup>13</sup> These include in vitro Gateway recombination cloning technology,<sup>14</sup> seamless ligation cloning extract (SLiCE)<sup>15</sup> and RecET,<sup>16</sup> etc. The SLiCE method from a commonly used laboratory *E. coli* strain, JM109 and DH5 $\alpha$ , can efficiently function as an effective alternative to commercially available seamless DNA cloning kits.<sup>15</sup> It also includes in vivo recombination methods, such as relying on RecA recombination enzymes, Red/ET recombination systems,<sup>17, 18</sup> and DH5 $\alpha$  competent cells.<sup>19</sup> These methods require preparing the target gene and linearized vector, respectively. The target gene can also be inserted by the QuikChange site-directed mutagenesis method.<sup>20</sup> Similar to our one-step PCR method, the target fragment can be inserted at any amplifiable site of the vector. The difference is that when we designed the primers, we divided the target sequence into three parts, the target sequence at the 5' end of the forward primer, the target sequence at the 5' end of the reverse primer, and a 15 bp homology arm with overlapped region for target gene. In the QuikChange site-directed mutagenesis method, the sequence of the target fragment was designed at the 5' end of the forward primer, while the 5' end of the reverse primer contained only a 15 bp homologous arm. Compared with the QuikChange site-directed mutagenesis method, our one-step PCR method reduces both the cost for primer synthesis and the formation of primer dimers.

Although DNA inserts below 90 bp can be inserted using the SLiCE method,<sup>21</sup> it is still difficult in most plasmid construction methods to insert short DNA fragments less than 150 bp.<sup>22</sup> DNA inserts below 150 bp can be directly synthesized, but it is cost prohibitive with limited productivity. Also, the effects of synthesizing two short oligonucleotides below 150 bp still depend on the efficiency of annealing, endonuclease, and ligase, etc. The fidelity also significantly decreases when synthesizing oligonucleotides between 100 bp and 150 bp. Therefore, with these traditional methods it is especially inappropriate to construct plasmids with DNA fragments below 150 bp. In this study, we amplified a linear vector including the short target gene below 150 bp by one-step PCR.

A DNA repair system in *E. coli*, homologous recombination, which can cyclize linearized vectors with homology

arms, has been previously reported.<sup>10, 22</sup> Employing the characteristics of *E. coli*, we developed a method for constructing plasmids based on one-step PCR and homologous recombination. Previously reported methods of constructing plasmid using homologous recombination of *E. coli* are all for genes longer than 150 bp.<sup>22</sup>

Here, we describe an efficient and flexible method based on one-step PCR and homologous recombination for constructing a plasmid containing the fragment of interest below 150 bp. We constructed the pEGFP-N1-HA plasmid by inserting the target fragment HA-tag into the vector pEGFP-N1 for proof-of-principle validation.

## Methods

### Cell culture

Human 293T cells and HeLa cells were purchased from American Tissue Culture Collection (ATCC) and maintained in our laboratory. Cells were maintained in Dulbecco's modified Eagle's medium (HyClone, Logan City, Utah, USA) containing 10% fetal bovine serum (Biological Industries, Cromwell, USA) and 1% penicillin-streptomycin in a humidified incubator with 5% CO<sub>2</sub> and 37°C.

### Construction of the pEGFP-N1-HA plasmid

The pEGFP-N1 vector (4733 bp) used in this study was provided by TaKaRa. The sequence of HA-tag was 5'-TAC-CCATACGACGTCCCAGACTACGCT-3' (27 bp). The linearized vector pEGFP-N1 carrying the HA-tag was obtained by PCR. The PCR products were verified by gel electrophoresis. The original template plasmids were digested by *DpnI* enzyme (purified PCR products). Linearized vectors were transformed into *E. coli* and self-recombinant plasmids based on homologous recombination.

Sequences of primers of HA-tag were as following: forward, 5'-TAC GAC GTC CCA GAC TAC GCT AAG CTT CGA ATT CTG CAG TCG AC-3'; reverse, 5'-CTA CCG GAC TCA GAT CTC GAG CTC ATG TAC CCA TAC GAC GTC CCA-3'. As for PCR reactions, each 20  $\mu$ L sample in the PCR thermocycler (Gene-Explorer Touch, Zhengzhou, China) contained 1  $\mu$ L DNA template (30 ng/ $\mu$ L), 1  $\mu$ L forward primer (10 nM), 1  $\mu$ L reverse primer (10 nM), 7  $\mu$ L double distilled H<sub>2</sub>O, and 10  $\mu$ L I-5 2 $\times$  high fidelity master mix (MCLAB, San Francisco, USA). A negative control replacing master mix with double distilled H<sub>2</sub>O was employed. The PCR cycling profile for all reactions was as follows: initial denaturation at 98°C for 2 minutes, denaturation of template at 98°C for 10 seconds, annealing at 64°C for 15 seconds, and

extension at 72°C for 75 seconds, amplification for 18 cycles with the final extension at 72°C for 1 minute.

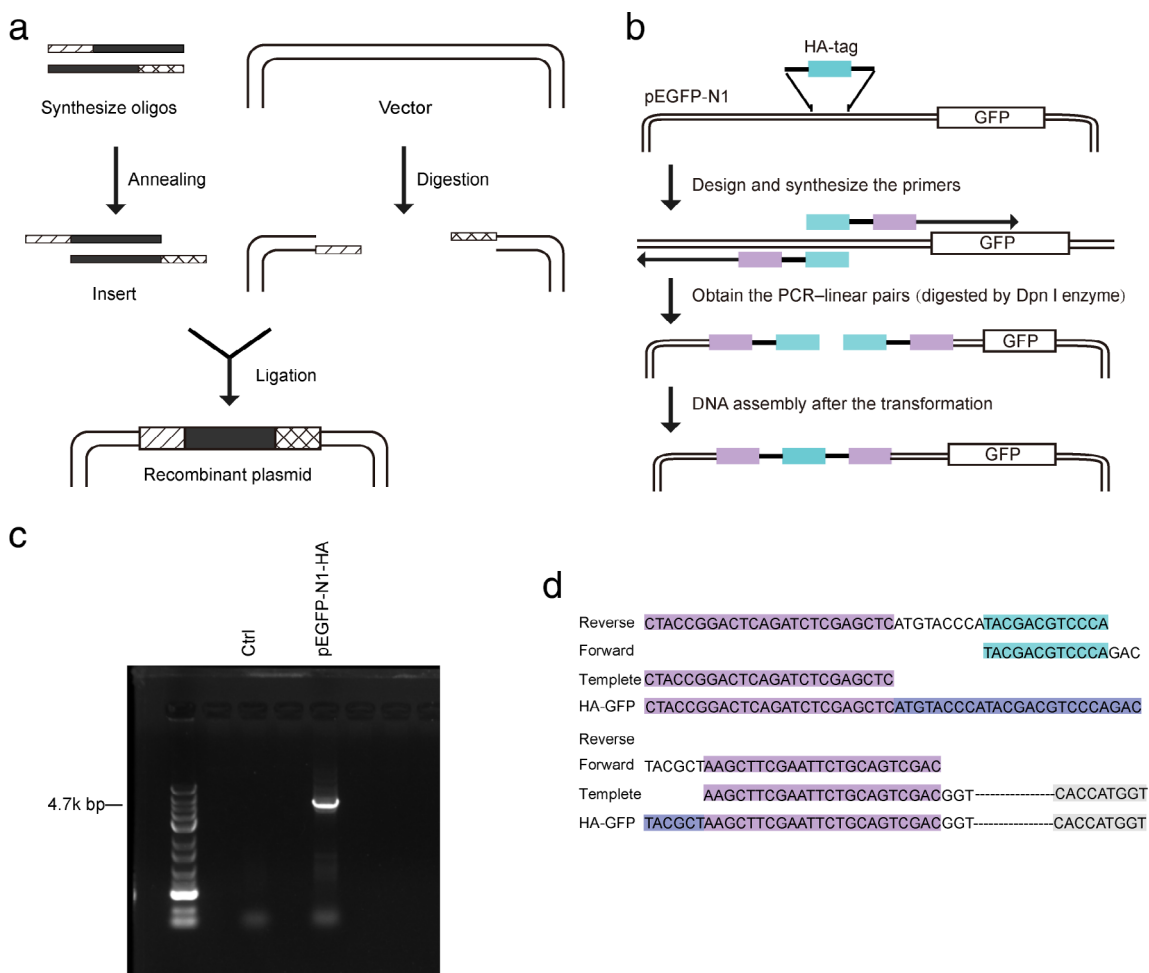
The PCR products (4774 bp, containing homologous sequences) were verified by electrophoresis on 0.9% agarose gel. The remaining 17 µL PCR products were mixed with 0.5 µL *DpnI* enzyme (BioLabs, Ipswich, USA), centrifuged, and incubated at 37°C for 3 hours.

### Transformation of the pEGFP-N1-HA plasmid into *E. coli*

*E. coli* competent DH5α (Transgene Biotech, Beijing, China) (50 µL) was thawed on ice and mixed with 5 µL PCR products. The mixture was then incubated on ice for 30 minutes and transferred into a water bath at 42°C for

45 seconds followed by incubation on ice for 2 minutes. The mixture was added to 1 mL prewarmed Luria Broth (LB) and gently shaken at 37°C and 150 rpm for 70 minutes. Finally, cells were plated on fresh LB-agar plates supplemented with 50 µg/mL Kanamycin (BBI, Shanghai China), and incubated overnight at 37°C. The monoclonal colonies were picked up and sequenced. The length of the pEGFP-N1-HA plasmid was 4.76 kb.

A colony of transformed *E. coli* cells was picked and grown in 1 mL LB at 37°C for 4 hours. Then, 200 µL were taken for DNA sequencing. The remaining cells were added into 250 mL LB and incubated overnight at 37°C. Plasmid DNA was extracted with the GoldHi Endofree Plasmid Miniprep Kit (Cwbio, Beijing, China) according to the manufacturer's instructions.



**Figure 1** The construction of pEGFP-N1-HA plasmid. **(a)** The schematic diagram of traditional plasmid construction method based on overlapping oligonucleotides and restriction enzymes. **(b)** Schematic diagram of our method based on one-step PCR and homologous recombination (■) Overlapping sequences of the templates (■) Homogenous arms. First, primers were designed and synthesized. The PCR-linear pairs were then obtained. Finally, the PCR products were transformed into chemically competent *E. coli*. **(c)** Electrophoresis on SDS-SAGE gel of the PCR products. The size of the pEGFP-N1-HA plasmid was about 4.7 kb. **(d)** Diagram of the PCR-linear pairs cyclization. Both the forward and reverse primer have a homologous region, which can be circularized in competent *E. coli* (■) Overlapping sequences of the templates (■) Homogenous arms (■) HA-tag (■) GFP.

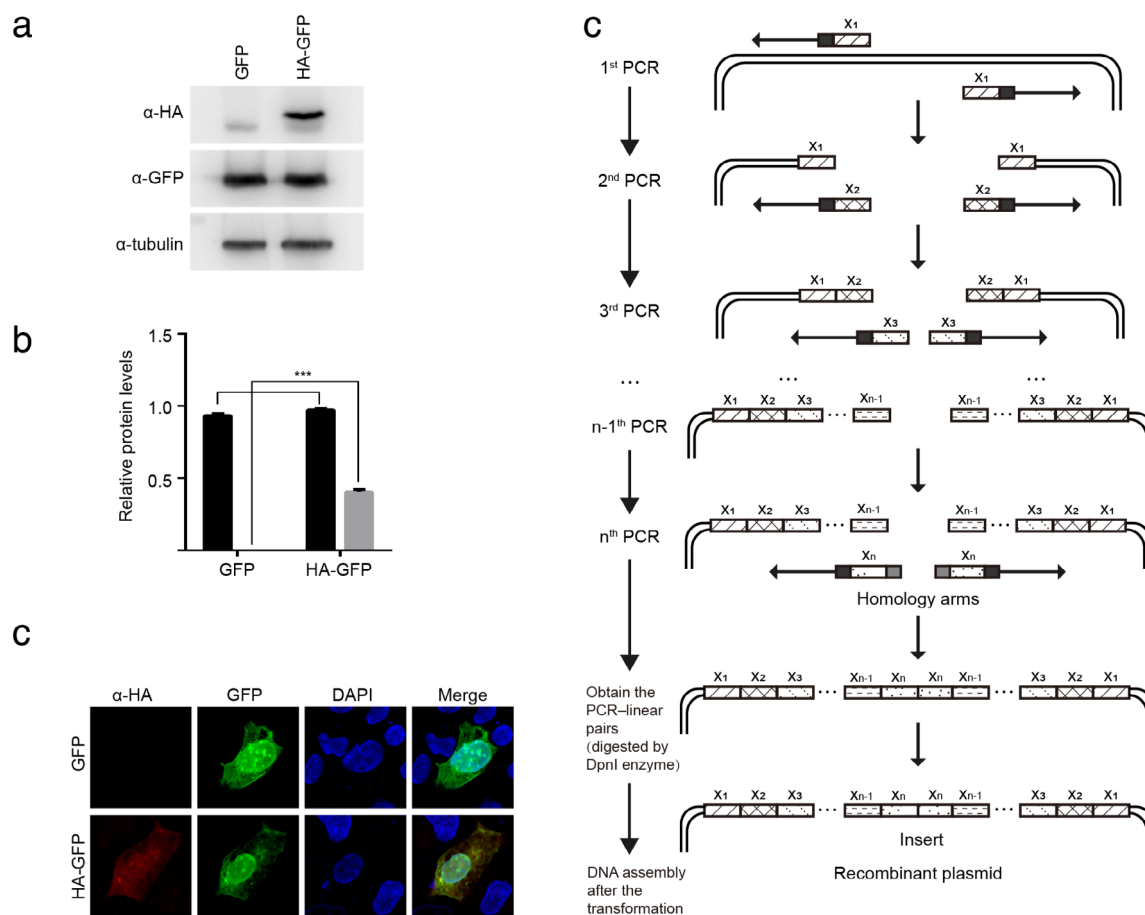
## Western blotting

Cells were transfected using TurboFect Transfection reagent (Thermo, Waltham American) according to the manufacturer's instructions. Cells were lysed in ice-cold lysis buffer 48 hours later, and lysates were centrifuged at 12000 rpm for 20 minutes at 4°C. After measuring the protein content using a bicin-choninic acid (BCA) assay, equal amounts of protein (1 g/mL) were electrophoresed by SDS-PAGE on 12% acrylamide gels and transferred onto a PVDF membrane. The membranes were blocked by 5% skim milk in Tris-buffered saline with 0.1% Tween (TBST) for 1 hour, and then incubated with anti-HA antibody (Santa Cruz Biotechnology, Dallas, USA) and anti-GFP antibody (Santa Cruz Biotechnology, Dallas, USA) at room temperature for 2 hours. After three washes with TBST,

membranes were incubated with a secondary polyclonal antibody to rabbit IgG conjugated with HRP at room temperature for 1 hour. Labeled proteins were detected using an ECL substrate (Mei5bio, Beijing, China).

## Fluorescence imaging

Cells were fixed with 4% paraformaldehyde (Sangon, Shanghai, China) for 20 minutes, permeabilized with 0.5% PBS-Triton X-100 (BBI, Shanghai, China) for 18 minutes, and blocked with 4% bovine serum albumin (BioFroxx, Einhausen, Germany) in PBS at room temperature for 90 minutes. The slides were then incubated with anti-HA antibody (Santa Cruz, Dallas, USA) for 2 hours at room temperature, and subsequently incubated with secondary antibody at room temperature for 2 hours. Nuclei were



**Figure 2** Validation of our method. (a & b) The expression of GFP and HA-tag proteins in 293T cells transfected with pEGFP-N1 or pEGFP-N1-HA was detected by western blotting, and quantified using ImageJ (■)  $\alpha$ -GFP (▨)  $\alpha$ -HA. The values were normalized by the intensities of the corresponding  $\alpha$ -tubulin band. (c) The expression of GFP and HA proteins in 293A cells transfected with pEGFP-N1 or pEGFP-N1-HA was detected by confocal microscopy. (d) Schematic diagram of our one-step PCR method for inserting large fragments. The large fragment insertion is segmented into a plurality of fragments with a length of less than 150 bp according to a one-step PCR method. PCR-linear pairs with large fragment insertion are obtained by multiple PCR amplification, digested by *DpnI* to eliminate the parent DNA, and directly transformed into competent *E. coli*. \* indicates  $P < 0.05$ , \*\* indicates  $P < 0.01$ , and \*\*\* indicates  $P < 0.001$ .

stained with Hoechst (Sigma, Saint Louis, USA). Images were captured using a confocal laser scanning microscopy (Zeiss, Oberkochen, Germany).

## Statistical analysis

All the data were normalized to the control. Data are shown as mean  $\pm$  SD and analyzed with *t*-test using the GraphPad Prism (GraphPad, USA).  $P < 0.05$  was considered statistically significant.

## Results

### pEGFP-N1-HA plasmid successfully constructed using our method

Compared with the traditional construction method in Fig 1a, the schematic map of our method is illustrated in Fig 1b. The linearized vector was obtained by one-step PCR. After annealing, the fragment of HA-tag was directly cloned into the pEGFP-N1 vector. The new constructed plasmid, pEGFP-N1-HA, was subjected to agarose-gel electrophoresis. As shown in Fig 1c, PCR products were absent in the negative control, while the pEGFP-N1-HA reaction system showed a 4.7 kb PCR product. The sequencing result of the pEGFP-N1-HA plasmid construction was identical to the GenBank sequence (see Fig 1d), which confirmed the electrophoresis results.

### HA-tag was expressed in 293T cells

The HA-tag expression in 293T cells was detected by western blotting. As shown in Fig 2a, HA-tag was successfully expressed in cells. The HA-GFP fusion protein was clearly detected by confocal microscopy (Fig 2b).

## Discussion

To efficiently and rapidly construct plasmids with inserts below 150 bp has prominent practical applications, such as small peptide fusion proteins, small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs). Traditional plasmid construction depends on restriction enzymes and ligases which are time-consuming and labor-intensive. There have been many developments in constructing recombinant vectors in vitro using recombinases and the endogenous recombinases of *E. coli*. Seamless ligation cloning extract (SLiCE) method is a commonly used method, which utilizes bacterial cell extracts to assemble multiple DNA fragments into recombinant DNA molecules in a single in vitro recombination reaction.<sup>23</sup> Compared with our method, the advantage of SLiCE is that SLiCE can simultaneously assemble multiple DNA fragments without using

recombinase, restriction enzyme and DNA ligase. However, SLiCE involves the extraction of bacterial solution and the preparation of linearized vectors and target fragments. The recombination efficiency of different bacterial cell extracts is also different. There have been studies using the recombination system of *E. coli* competent DH5 $\alpha$ . This method required neither engineering bacteria, nor recombinase and bacterial cell extracts. However, the linearized vector and the target fragment have to be prepared, respectively. Therefore, these have to be digested by *DpnI* enzyme and transformed.<sup>19, 24</sup> The biggest difference between this method and our method is that our method only requires one step of PCR to obtain the linearized vector with the target fragment. In addition, our method is also more flexible in position selection than the existing one-step PCR method for constructing shRNA plasmid.<sup>20</sup> Our method is based on homologous recombination, which is different from the method for constructing site-directed mutagenesis<sup>25</sup> and generation of shRNA expression vectors<sup>20</sup> in previous reports. Compared to these previously reported methods, our method is more flexible in selecting insertion sites. The DNA fragment of interest can be inserted at any position other than the promoter. Compared with direct synthesis of short oligonucleotides, our method is more economic. We also compared the cloning efficiency and accuracy of constructing the pEGFP-N1-HA plasmid by our one-step PCR method and the In-Fusion cloning method. Our one-step PCR method synthesized 89 bp DNA, about 47% less than 131 bp of the In-Fusion cloning. There were more procedures in the In-Fusion cloning, such as DNA fragment purification from PCR enzymatic reactions, in vitro recombination, etc. According to the sequencing results, there was the same accuracy between our one-step PCR method and the In-Fusion cloning method (data not shown).

Although our method was specifically designed for plasmids with small inserts below 150 bp, it can also be applied to construct plasmids with large fragments (Fig 2c). Small fragments can be obtained by PCR one by one, and ligated to form a large DNA fragment. Our method has several limitations. The efficiency may not be constant because the plasmid template used in PCR amplification has some transformation potential.<sup>25</sup> In order to eliminate the influence of the plasmid template, it is necessary to digest the template by *DpnI* endonuclease. The *DpnI* digestion time of the PCR products plays key roles in the success of our method. DNA polymerase may also induce errors. The error rate may be high when PCR is used to amplify genomic sequences. The amplified genomic sequences are longer, and the DNA polymerase mismatch rate is higher.<sup>26, 27</sup> The use of high-fidelity *Taq* enzyme, such as *Pfu* DNA polymerase,<sup>28</sup> can reduce the mismatch rate in PCR, which will also increase the cost.

Overall, our method combines the advantages of both PCR-based strategies and homologous recombination (Figure S1). Due to its simplicity and versatility, our method makes it possible to rapidly and cost-effectively construct plasmids with inserts below 150 bp, which will greatly contribute to the functional study of small peptides and RNA interference.

## Acknowledgments

Funding was provided by the National Natural Science Foundation of China (Grant No. 81770580) and the Sichuan Science and Technology Program (Grant No. 2019YJ0050).

## Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## References

- Fenderson BA. Molecular biology of the cell, 5th edition. *Shock* 2008; **30**: 100.
- May M. Engineering a new business. *Nat Biotechnol* 2009; **27**: 1112–20.
- Liang J, Luo Y, Zhao H. Synthetic biology: Putting synthesis into biology. *Wiley Interdiscip Rev Syst Biol Med* 2011; **3**: 7–20.
- Berg P, Mertz JE. Personal reflections on the origins and emergence of recombinant DNA technology. *Genetics* 2011; **184**: 9–17.
- Kaufman RI, Nixon BT. Use of PCR to isolate genes encoding sigma54-dependent activators from diverse bacteria. *J Bacteriol* 1996; **178**: 3967–70.
- Weiss B, Richardson CC. Enzymatic breakage and joining of deoxyribonucleic acid, I. repair of single-strand breaks in DNA by an enzyme system from *Escherichia coli* infected with T4 bacteriophage. *Proc Natl Acad Sci U S A* 1997; **57**: 1021–8.
- Ahluwalia JK, Khan SZ, Soni K et al. Human cellular microRNA hsa-miR-29a interferes with viral nef protein expression and HIV-1 replication. *Retrovirology* 2008; **5**: 117.
- Xu R, Li QQ. Protocol: Streamline cloning of genes into binary vectors in agrobacterium via the gateway<sup>®</sup> TOPO vector system. *Plant Methods* 2008; **4**: 4–4.
- Kiesel B. Book review: An introduction to genetic engineering. By Desmond S T Nicholl. *Eng Life Sci* 2002; **22**: 374–4.
- Liu CJ, Jiang H, Wu L, Zhu LY, Meng E, Zhang DY. OEPR cloning: An efficient and seamless cloning strategy for large- and multi-fragments. *Sci Rep* 2017; **7**: 44648.
- Miyagishi M, Sumimoto H, Miyoshi H, Kawakami Y, Taira K. Optimization of an siRNA-expression system with an improved hairpin and its significant suppressive effects in mammalian cells. *J Gene Med* 2004; **6**: 715–23.
- Arber W. DNA modification and restriction. *Prog Nucleic Acid Res Mol Biol* 1974; **14**: 1–37.
- Jacobus AP, Gross J. Optimal cloning of PCR fragments by homologous recombination in *Escherichia coli*. *PLOS One* 2015; **10**: e0119221.
- Hartley JL, Temple GF, Brasch MA. DNA cloning using in vitro site-specific recombination. *Genome Res* 2000; **10**: 1788–95.
- Okegawa Y, Motohashi K. A simple and ultra-low cost homemade seamless ligation cloning extract (SLiCE) as an alternative to a commercially available seamless DNA cloning kit. *Biochem Biophys Res Commun* 2015; **4**: 148–51.
- Zhang Y, Buchholz F, Muyrers JP, Stewart AF. A new logic for DNA engineering using recombination in *Escherichia coli*. *Nat Genet* 1998; **20**: 123–8.
- Lovett ST, Hurley RL, Sutura VA Jr, Aubuchon RH, Lebedeva MA. Crossing over between regions of limited homology in *Escherichia coli*. RecA-dependent and RecA-independent pathways. *Genetics* 2002; **160**: 851–9.
- Muyrers JP, Zhang Y, Buchholz F, Stewart AF. RecE/RecT and Redalpha/Redbeta initiate double-stranded break repair by specifically interacting with their respective partners. *Genes Dev* 2000; **14**: 1971–82.
- Motohashi K. Evaluation of the efficiency and utility of recombinant enzyme-free seamless DNA cloning methods. *Biochem Biophys Res Commun* 2017; **9**: 310–5.
- Li J, Li C, Xiao W, Yuan D, Wan G, Ma L. Site-directed mutagenesis by combination of homologous recombination and DpnI digestion of the plasmid template in *Escherichia coli*. *Anal Biochem* 2008; **373**: 389–91.
- Messerschmidt K, Hochrein L, Dehm D, Schulz K, Mueller-Roerber B. Characterizing seamless ligation cloning extract for synthetic biological applications. *Anal Biochem* 2016; **509**: 24–32.
- Kostylev M, Otwell AE, Richardson RE, Suzuki Y. Cloning should be simple: *Escherichia coli* DH5 $\alpha$ -mediated assembly of multiple DNA fragments with short end homologies. *PLOS One* 2015; **10**: e0137466.
- Zhang Y, Werling U, Edelman W. SLiCE: A novel bacterial cell extract-based DNA cloning method. *Nucleic Acids Res* 2012; **40**: e55.
- Huang F, Spangler JR, Huang AY. In vivo cloning of up to 16 kb plasmids in *E. coli* is as simple as PCR. *PLOS One* 2017; **12**: e0183974.
- Xu J, Zeng JQ, Wan G, Hu GB, Yan H, Ma LX. Construction of siRNA/miRNA expression vectors based on a one-step PCR process. *BMC Biotechnol* 2009; **9**: 1–10.
- Farina M, Berenguer J, Pons S, da Rocha JBT, Aschner M. Introducing cloned genes into cultured neurons providing novel in vitro models for neuropathology and neurotoxicity studies. *NeuroMethods* 2011; **56**: 185–222.

- 27 Tindall KR, Kunkel TA. Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry* 1988; **27**: 6008–13.
- 28 Cline J, Braman JC, Hogrefe HH. PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Res* 1996; **24**: 3546–51.

## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Figure S1.** Supplementary Figure.