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Simple and efficient custom transcription activator-like effector gene synthesis via twin primer assembly

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ABSTRACT

Transcription activator-like effector (TALE) nucleases (TALENs) efficiently recognize and cleave DNA in a sequence-dependent manner. However, current TALE custom synthesis methods are either complicated or expensive. Here we report a simple and low-cost method for TALE construct assembly. This method utilizes the denaturation/reannealing nature of double-stranded DNA to create a unique single-stranded DNA overhang for proper ordering of TALE monomers in an engineered multimer. We successfully synthesized two TALEN pairs targeting the endogenous *TET1* locus in human embryonic kidney cells and demonstrated their editing efficiency. Our method provides an alternative simple, low-cost method for effective TALEN assembly, which may improve the application of TALE-based technology.

METHOD SUMMARY

We report a simple and cost-effective transcription activator-like effector construct assembly method. This novel method is also suitable for the construction of other transcription activator-like effector fusion genes.

KEYWORDS:

genome editing ● TALE ● TALEN ● *TET1* ● twin primer

Transcription activator-like effector nuclease (TALEN) and CRISPR/Cas9 represent two popular technologies widely used in the genome editing field [1]. Owing to its simplicity, the CRISPR/Cas9 system has been widely used worldwide [2]. However, Cas9 recognizes DNA sequences via the protospacer adjacent motif (PAM), and the seed sequence adjacent to the PAM determines Cas9 specificity [3]. In addition, mismatches distal to the PAM are tolerated. Therefore potential off-target cleavage activity still occurs both *in vitro* and *in vivo* [4,5]. Recently, Cas9-based gene editing in human embryos demonstrates frequent loss and reshuffling of the targeted chromosome [6,7]. In addition, Cas9 is much larger than the TALEN monomer, making it difficult to deliver into cells [8]. Furthermore, TALEN can be used for the selective elimination of mitochondrial mutations [9]. These advantages favor the complementary usage of these two technologies.

The functional TALEN protein comprises a series of 34 amino acid residue repeats arranged in tandem, where the 12th and 13th residues (repeat variable diresidue) determine the DNA base recognition specificity [10]. Typically, a functional TALEN construct contains 15–20 repeats assembled in a defined order. However, achieving this goal is technically challenging due to the repetitive nature of transcription activator-like effector (TALE) array genes. To address this issue, several modular assembly methods utilizing single or multiple repeat libraries have been developed. The most widely used cloning strategy is Golden Gate assembly [11–15]. This strategy enables the simultaneous ligation of multiple repeat units with high specificity and efficiency [16]. It utilizes type IIS restriction enzymes to generate distinct cohesive ends. These enzymes target asymmetric DNA sequences and cut outside their target sequences; thus the restriction site is eliminated after ligation. Using this strategy, multiple DNA fragments can be ligated in tandem in a defined order. Alternatively, TALEN can be assembled via ligation-independent cloning. In one system, T4 DNA polymerase was used to create a unique single-stranded DNA overhang for complementary annealing [17]. In another system, the uracil-specific excision reagent (USER) cloning method was employed for this purpose [18]. In addition, TALEN can also be synthesized via streptavidin-biotin system-based iterative assembly or restriction enzymes-based unit assembly [19–21]. However, these methods are either highly complicated or expensive, so a low-cost TALEN assembly method is highly desirable.

Here we present a simple and low-cost method for the custom synthesis of TALE constructs that utilizes the denaturation/reannealing nature of double-stranded DNA to create unique cohesive ends by using two sets of primers. This twin primer-based assembly strategy enables the creation of complementary cohesive ends on the TALE monomer by a post-PCR denaturation/reannealing process. This

simple procedure does not require additional post-PCR enzymatic treatment and does not involve restriction enzyme sites or modified primers [22]. Thus this twin primer-based assembly strategy could be a useful supplement to custom TALEN synthesis.

Materials & methods

Reagents

TALE toolbox was obtained from Addgene (http://www.addgene.org/tale_toolbox/). Q5® Hot Start High-Fidelity 2X Master Mix, *Xba*I, *Bsa*I-HF, Nt.*Bbv*CI, T7 DNA ligase and T7 Endonuclease I were purchased from New England Biolabs (MA, USA). Lipofectamine 2000 was obtained from Life Technologies (CA, USA). pGEM®-T Easy Vector System I was purchased from Promega (WI, USA). TIANprep Mini Plasmid Kit was purchased from TIANGEN Biotech (Beijing, China).

Cell culture

Human embryonic kidney (HEK 293T) cells were grown in Dulbecco's modified Eagle medium (Corning, NY, USA), supplemented with 10% fetal bovine serum (Corning) in a 37°C incubator.

Monomer library construction

PCR amplification is depicted in Figure 1A. Each 100-μl reaction contained 50 ng template plasmid, 10 μM primers and 50 μl Q5® Hot Start High-Fidelity 2X Master Mix. The amplification was performed according to the manufacturer's instructions.

Linearization of pGEM-USER plasmid

pGEM-USER vector was constructed as previously described [18]. Briefly, 10 μg pGEM-USER vector was digested with 40 U of *Xba* I in 100 μl reaction volume for 18 h at 37°C, then incubated with 20 U of Nt.*Bbv*CI for 1 h at 37°C. The cleaved product was purified by phenol–chloroform extraction and ethanol precipitation, then dissolved in sterile distilled water and adjusted the concentration to 100 ng/μl.

Functional TALEN expression vector assembly

TALEN target site and expression backbone selection was carried out as previously described [13]. For pentamer assembly, 2.5 μl of each PCR product were mixed well for a defined position, and cross-hybridization performed as previously described [13]. After that, 3.6 μl of the cross-hybridization products were incubated with 100 ng linearized pGEM-USER vector in 20 μl reaction volume for 30 min at 37°C. Then the products were ready for transformation. Briefly, the 20 μl reaction products were added to 100 μl DH5α-competent cells. Then the mixture was incubated for 30 min on ice, for 45 s at 42°C and for 2 min on ice. The products were then plated onto LB plates for overnight incubation. Correct pentamer colonies were picked up by colony PCR and the plasmids were extracted. For TALEN assembly, 30 fmol each of TALEN expression backbone and three pentamer vectors incubated with 1 μl ATP (10 nM, New England Biolabs), 0.25 μl T7 DNA ligase (3000 U/μl) and 0.75 μl *Bsa*I-HF (20 U/μl). The reaction was carried out as follows: 20 cycles of 5 min at 37°C and 5 min at 20°C, then 1 cycle of 5 min at 37°C and 5 min at 80°C. Positive TALEN vectors were screened by colony PCR.

Cell transfection

Human HEK 293T cells were seeded in six-well plates and grown to 90% confluence. 2 μg left and 2 μg right TALEN plasmids were transfected per well according to the manufacturer's instructions.

TALEN activity verification

Cells were collected 48 h posttransfection and lysed in 500 μl lysis buffer (0.5% sarcosyl, 200 mM NaCl, 10 mM EDTA, pH 8.0, 10 mM Tris-HCl, pH 8.0, 1 mg/ml proteinase K; Sigma-Aldrich, MO, USA). Cells were incubated at 37°C overnight. Next day, the cell lysate was transferred to sterile 1.5-ml Eppendorf tubes containing 500 μl isopropanol. The DNA precipitate was transferred into 500 μl 70% ethanol using a sterile pipette tip, pelleted by centrifugation and air-dried before being dissolved in 50 μl sterile distilled water. The target region was amplified by PCR according to the manufacturer's recommendation. For the T7 endonuclease I assay, 200 ng PCR products were mixed with 1.1 μl of 10 × NEB buffer 2 in 10.5 μl reaction volume. Then the products were sent to cross-hybridization as previously described [13]. Next, 0.5 μl T7 endonuclease I (10 U/μl) was added into the products and incubated for 20 min at 37°C. The products were analyzed on 2% agarose gel. The density of the cleaved products density was determined using ImageJ (NIH, MD, USA) and editing efficiency of TALEN pairs was calculated as previously described [23].

Results & discussion

Strategy for TALE repeat assembly

We used a two-round assembly strategy to synthesize TALE-related constructs. To create unique cohesive ends, we initially amplified TALE monomer units with two sets of primers, both of which contained a short and a long primer (Figure 2A). After amplification, each of the PCR products included an additional linker region. Then the PCR products were mixed and denatured/reannealed. Half produced sticky ends, and only a quarter of the denaturation/reannealing products were usable in subsequent ligation. Next, five monomer denaturation/reannealing products were ligated into an intermediate vector (Figure 2B). Finally, three TALE pentamers were assembled into expression backbone vectors via an additional round of Golden Gate cloning (Figure 2C).

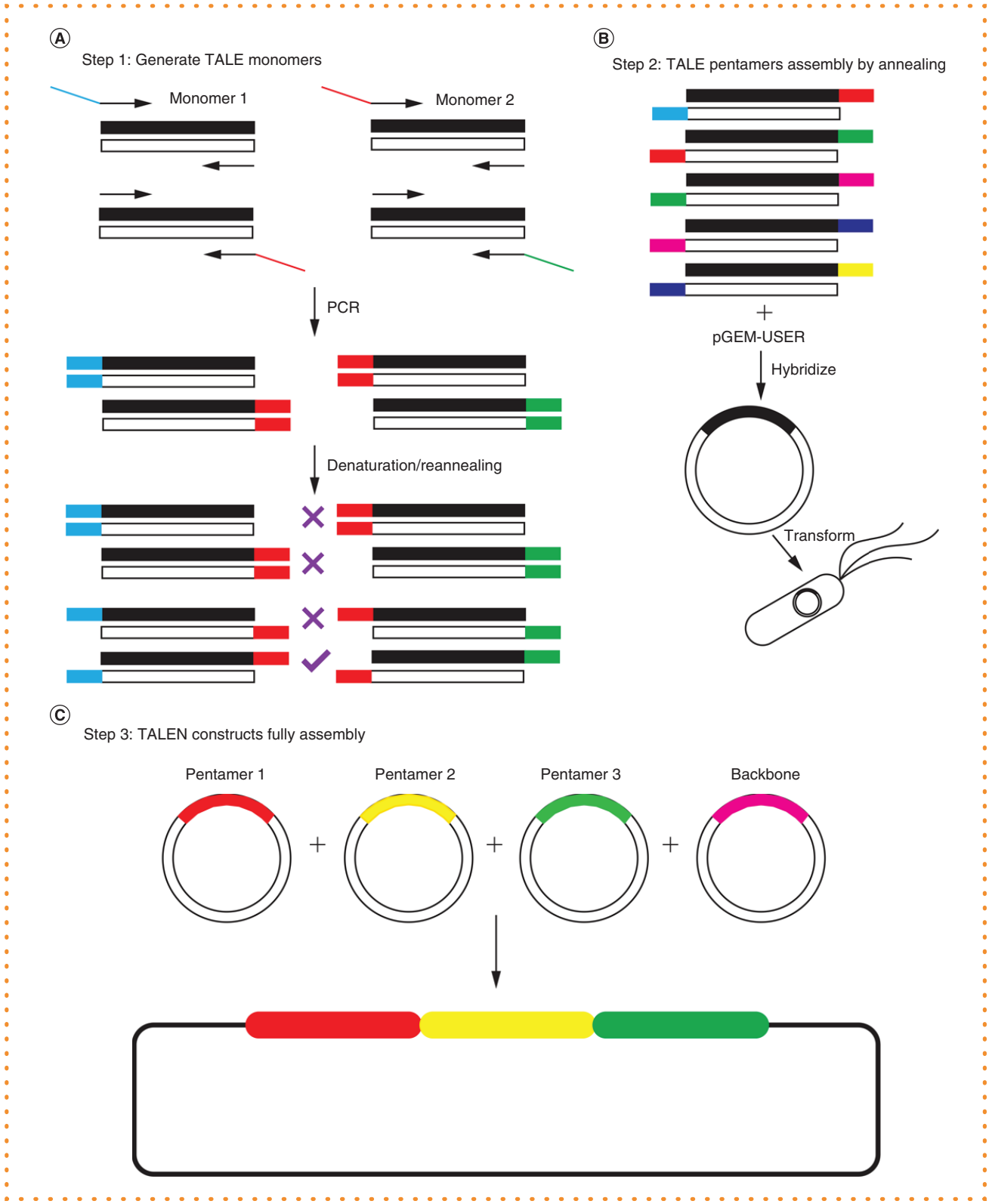

Figure 1. Custom transcription activator-like effector nuclease assembly via a twin primer-based strategy. **(A)** PCR amplification setup for the TALE monomer combination. 100 μl of PCR products can be used to assemble dozens of TALEN constructs. No purification procedure is necessary. **(B)** Samples were analyzed by colony PCR to verify positive TALE pentamer clones which were approximately 613 bp long. Lanes 1–5 indicate correctly assembled TALE pentamers. **(C)** Samples were analyzed by colony PCR to verify fully assembled TALEN constructs. Lane 1 shows the left TALEN and lane 2 shows the right TALEN. Note the repeat pattern of the PCR products due to incomplete amplification. TALE: Transcription activator-like effector; TALEN: Transcription activator-like effector nuclease.

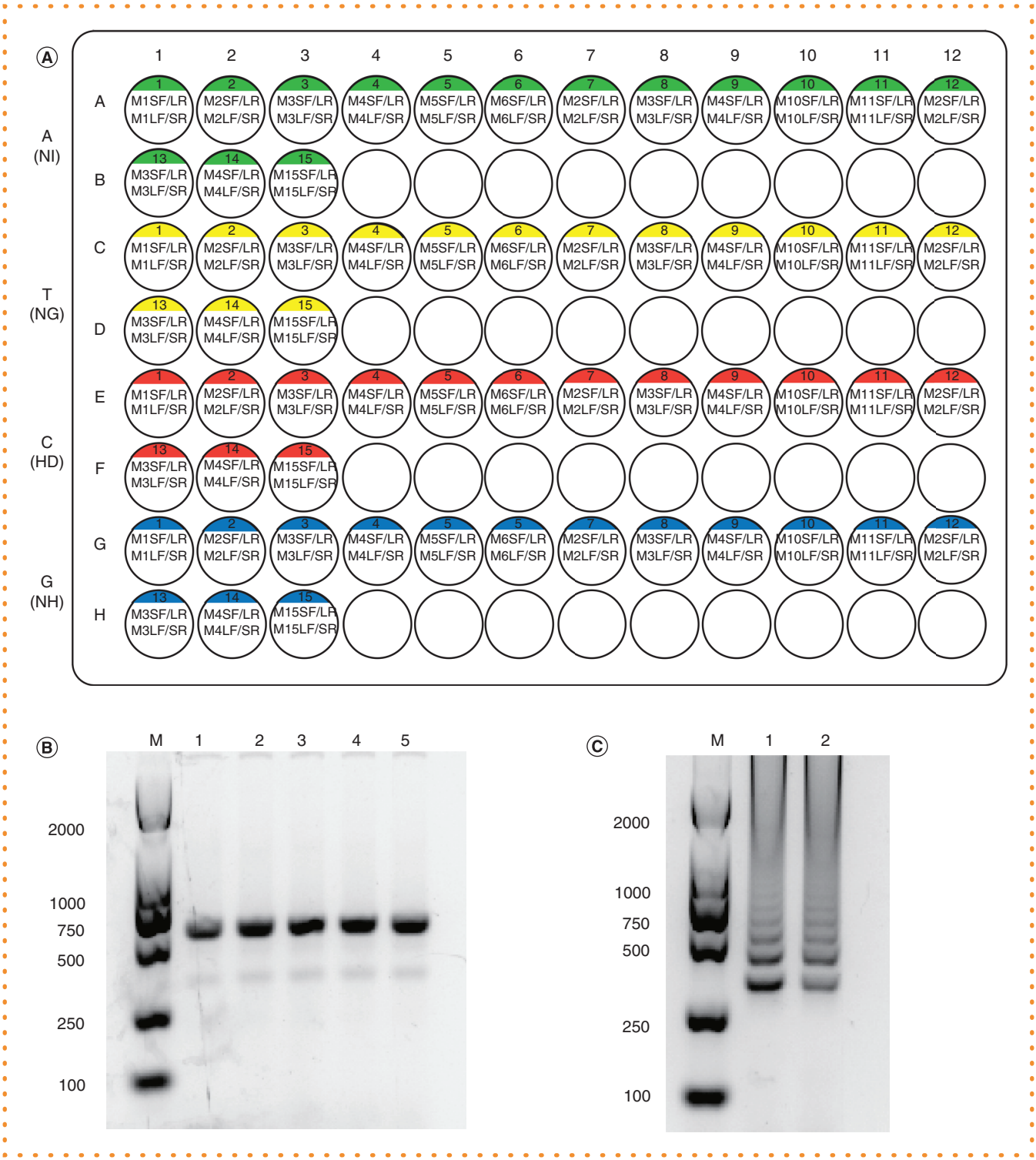

Figure 2. Twin primer-based transcription activator-like effector array gene assembly workflow. (A) Twin primer assembly workflow illustrating an assembly of five monomers. For step 1, only a portion of the five monomers are illustrated. After denaturation/reannealing, half of the products are expected to have compatible sticky ends, while the other half of the products are blunt ends. However, only a quarter of the products are compatible with pGEM-USER vectors. **(B)** Five reannealed monomer PCR products were mixed with pGEM-USER vectors and subsequently subjected to transformation. **(C)** Positive TALE pentamer colonies were selected via colony PCR and subsequently ligated into final expression backbone vectors. TALE: Transcription activator-like effector; USER: Uracil-specific excision reagent.

Table 1. Oligonucleotide sequences used in this study.

Primers	Sequences (5′–3′)
M1SF	GGTCTCCTGACCCCTGAGCAGGTCGT
M1LR	ATGCGCTTGGCACAGCACAGG
M1LF	GGGAAAGTGGTCTCCTGACCCCTGAGCAGGTCGT
M1SR	GGCACAGCACAGGCAGCAACC
M2SF	GGTCTGACCCCTGAGCAGGTC
M2LR	AGGCCGTGTGCTTGGCACAG
M2LF	AAGCGCATGGTCTGACCCCTGAGCAGGTC
M2SR	GCTTGGCACAGCACAGGCAG
M3SF	GACCCCTGAGCAGGTCGTGGC
M3LR	AGTTAACCCATGCGCTTGGCACAGCAC
M3LF	ACACGGCCTGACCCCTGAGCAGGTCGTGGC
M3SR	GCGCTTGGCACAGCACAGGCA
M4SF	CCTGAGCAGGTCGTGGCAATT
M4LR	AGGCGTGAGTCCATGTGCTTGGCACAGCAC
M4LF	ATGGGTTAACTCCTGAGCAGGTCGTG
M4SR	CCATGTGCTTGGCACAGCACA
M5SF	GAGCAGGTCGTGGCAATTGCG
M5LR	GGAGACATGGTCTCCAGGCCATGCGCTTGGCACAG
M5LF	ACTCACGCCTGAGCAGGTCGTG
M5SR	GGTCTCCAGGCCATGCGCTTGGCACAG
M6SF	GGTCTCCGCCTGACCCCTGAGCAGGT
M6LR	Identical to M1LR
M6LF	GGGAAAGTGGTCTCCGCCTGACCCCTGAGCAGGT
M6SR	Identical to M1SR
M10SF	Identical to M5SF
M10LR	GGAGACATGGTCTCGTTAGTCCATGCGCTTGGCAC
M10LF	Identical to M5LF
M10SR	GGTCTCGTTAGTCCATGCGCTTGGCAC
M11SF	GGTCTCCCTAACCCCTGAGCAGGTCGTGGC
M11LR	Identical to M1LR
M11LF	GGGAAAGTGGTCTCCCTAACCCCTGAGCAGGTCGTGG
M11SR	Identical to M1SR
M15SF	Identical to M5SF
M15LR	GGAGACATGGTCTCTGAGTCCGTGCGCTTGGCACAGCAC
M15LF	Identical to M5LF
M15SR	GGTCTCTGAGTCCGTGCGCTTGGCACAGCAC
pGEM-F	CGGCCGCCATGGCGGCCGCG
pGEM-R	CCTGCAGGCGGCCGCGAATTC
TALE-seq-F	CCAGTTGCTGAAGATCGCGAAGC
TALE-seq-R	TGCCACTCGATGTGATGTCCTC-
guide RNA1 target sequence	TAAGTTATCTGATTCTTACCTGG
guide RNA2 target sequence	CAATCATGTTCACTTTTAATGGG

Construction of TALENs targeting the *TET1* locus

To verify the feasibility of this strategy, we synthesized two TALEN pairs targeting the human *TET1* locus. We first created a PCR library by using two sets of primers (Figure 1A, Table 1). The TALE monomer templates and expression backbone vectors were obtained from Addgene (https://www.addgene.org/kits/zhang-tale-toolbox/). A standard 100-μl PCR system was then set up using Q5 DNA polymerase (New England Biolabs). For a specific position in the TALEN target sequence, 2.5 μl of each PCR product were mixed, then 5-μl aliquots of these PCR products were used to perform cross-hybridization. The denaturation and reannealing process was performed as previously described [13]. Then 3.6 μl of the denaturation/reannealing products of five monomers were mixed with 100 ng of pGEM-USER vector at 37–50°C for 30 min [18]. After that, the reaction products were added into competent cells and subsequently transformed.

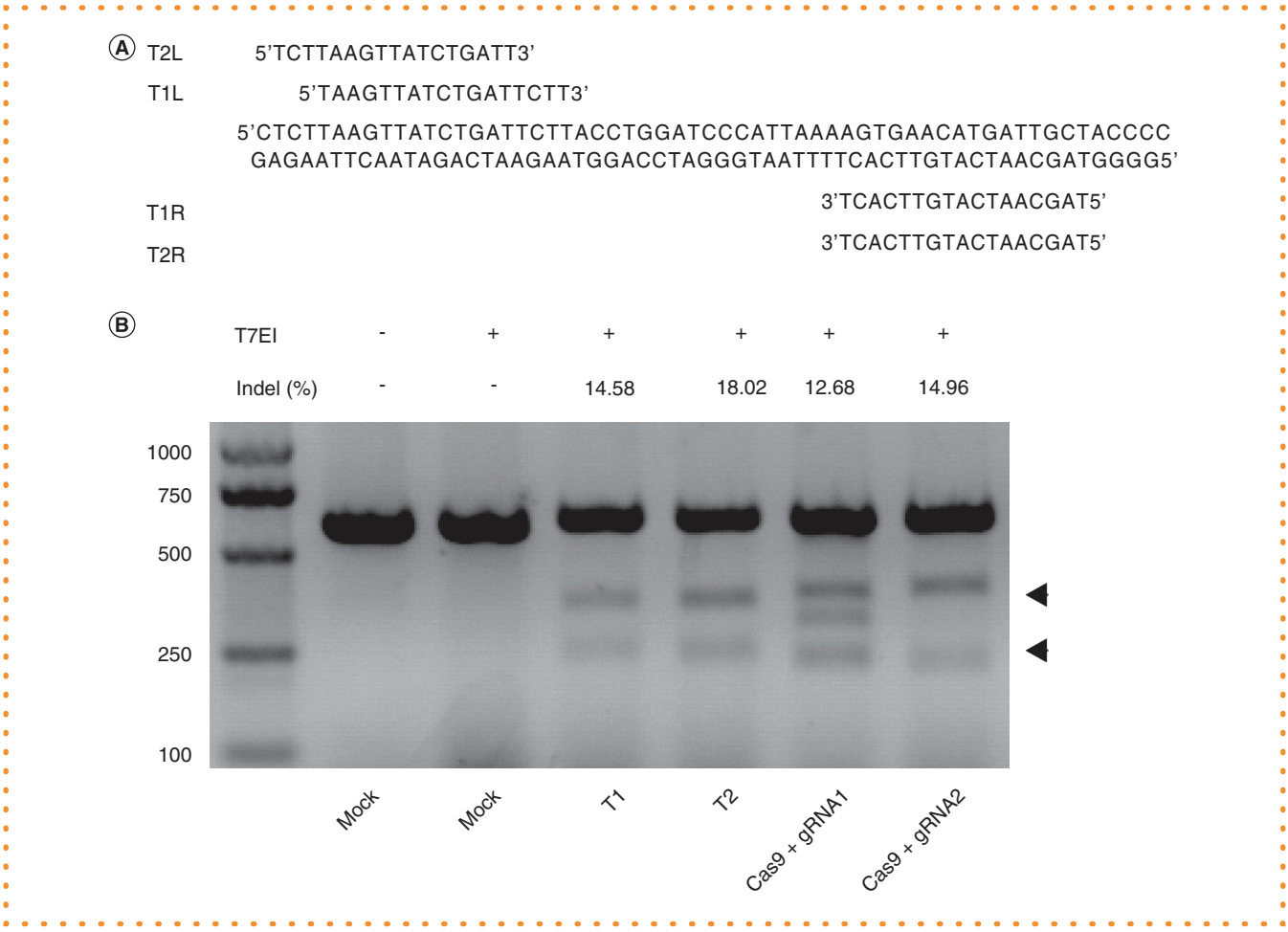

Figure 3. Transcription activator-like effector nuclease *in vivo* activity test using T7 endonuclease I assay. (A) Illustration of the human *TET1* locus and TALEN target sites. **(B)** T7 endonuclease I assay results of HEK 293T cells transfected with TALEN and Cas9/gRNA constructs. Arrows indicate T7 endonuclease I cleaved DNA fragments. HEK: Human embryonic kidney; TALEN: Transcription activator-like effector nuclease.

Positive pentamer colonies were identified by colony PCR using primers pGEM-F/R (Figure 1B & Supplementary Figure 1A & Table 1 & Supplementary material). Then plasmids were extracted and three pentamers were incubated with 30 fmol of the corresponding expression backbone vectors for subsequent Golden Gate cloning. Correctly assembled TALEN construct colonies were verified by colony PCR using primers TALE-seq-F/R (Figure 1C & Supplementary Figure 1B & Table 1). As shown in Figure 1C, positive colonies exhibited DNA ladder-like smear bands due to the repetitive nature of the TALE array genes. These data demonstrate that the twin primer-based TALEN assembly strategy is simple and feasible.

Verification of gene targeting efficiency

We successfully assembled two TALEN pairs that theoretically target the human *TET1* genome locus (Figure 3A). To test the DNA cleavage ability of custom synthesized TALENs, we transfected these TALEN pairs separately into HEK 293T cells with Lipofectamine 2000. We also transfected two Cas9/gRNA groups as positive controls. After 48 h of incubation, 619-bp DNA fragments containing the targeting region were amplified by PCR, then the products were denatured/reannealed. After that, the T7 endonuclease I assay was used to assess the cutting efficiency of the corresponding TALEN pairs [24]. As expected, cleaved fragments of approximately 261 bp and 358 bp were observed on a 2% agarose gel in the TALEN and Cas9/gRNA transfection groups, whereas the control groups contained almost intact 619-bp fragments (Figure 3B). We calculated 14.58 and 18.02% cutting efficiencies of the two TALEN pairs at the *TET1* locus, comparable with those of the Cas9/gRNA groups. This result suggested that the assembled TALEN pairs were effective *in vivo*.

In summary, we provide a simple and low-cost method to custom synthesize TALE-related constructs. This method needs only to perform denaturation/reannealing after PCR amplification. In addition, only 2.5 μl of PCR products were used for a specific monomer position. Thus, one round of amplification can assemble dozens of TALE array genes. Our twin-primer assembly strategy may facilitate and expand the application of TALE-related technology.

Future perspective

The simplicity, versatility and easy accessibility of CRISPR/Cas9 make it popular in the world. However, several recent studies conducted in human embryos have raised safety concerns about heritable genetic changes [6,7]. These studies revealed frequent loss and reshuffling of the targeted chromosome and heightened concerns surrounding the uncertain risk of CRISPR/Cas9 editing. They also raise the possibility that the phenotype after gene editing is due to chromosome loss, rather than homologous recombination-based repair [6,7]. Thus several improvements should be made in the near future. Firstly, accurate methods for off-target detection should be developed. Secondly, multiple high-fidelity versions of Cas9 nuclease should be developed to minimize the off-target effect. Thirdly, zinc finger nuclease and TALEN should also be considered since they are function as dimers and rely on protein–DNA recognition. Last but not least, the factors that decide chromosome end rejoining or loss and their mechanisms should be elucidated [6].

Author contributions

S Wang and Y Zhu conceived the idea, designed the study and wrote the manuscript. Y Pan, H Wang, Y Zhao and M Qu synthesized TALE monomer libraries. S Wang and Y Yu constructed TALEN vectors and collected the data.

Financial & competing interests disclosure

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