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Improved CRISPR-Cas12a-assisted one-pot DNA editing method enables seamless DNA editing

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Abstract

As the clustered regularly interspaced short palindromic repeats (CRISPR)–Cas12a (previously known as Cpf1) system cleaves double-stranded DNA and produces a sticky end, it could serve as a useful tool for DNA assembly/editing. To broaden its application, a variety of engineered FnCas12a proteins are generated with expanded protospacer adjacent motif (PAM) requirements. Two variants (FnCas12a-EP15 and EP16) increased the targeting range of FnCas12a by approximately fourfold. They can efficiently recognize a broad range of PAM sequences including YN (Y = C or T), TAC and CAA. Meanwhile, based on our demonstration that FnCas12a is active from 16 to 60°C, we developed an "improved CRISPR-Cas12a-assisted one-pot DNA editing" (iCOPE) method to facilitate DNA editing by combining the crRNA transcription, digestion, and ligation in one pot. By applying iCOPE, the editing efficiency reached 72–100% for two DNA fragment assemblies, and for the 21 kb large DNA construct modification, the editing efficiency can reach 100%. Thanks to the advantages of Cas12a, iCOPE with only one digestion enzyme could replace current a variety of restriction enzymes to perform the cloning in one pot with almost no sequence constraints. Taken together, this study offers an expanded DNA targeting scope of CRISPR systems and could serve as an efficient seamless one-pot DNA editing tool.

KEYWORDS
Cas12a variants, DNA editing, FnCas12a, one pot, synthetic biology

1 | INTRODUCTION

The discovery of Type II restriction endonucleases facilitated the wide application of DNA recombination technologies. Later, the rapid development in synthetic biology triggered the demand for versatile DNA assembly/editing strategies. There have been two main types of DNA assembly/editing methods: restriction-ligation assembly and homology-directed assembly. At present, the most frequently used restriction-ligation assembly methods are traditional cloning and the Golden Gate assembly method (Engler, Gruetzner, Kandzia, & Marillonnet, 2009; Engler, Kandzia, & Marillonnet, 2008). The Golden Gate assembly method is based on Type IIs restriction endonucleases and has been optimized and applied in multiple DNA fragment assemblies in a predetermined order (Werner, Engler, Weber, Gruetzner, & Marillonnet, 2012). However, the limited number of Type IIS restriction endonucleases and the high frequencies of their recognition sites in a given long DNA sequence sometimes limit their applications. For homology-directed assembly, such as the well-characterized and high-efficiency method Gibson Assembly (Gibson et al., 2008, 2009), sometimes the efficiency may drop when the number of fragments is increased or the target is GC rich. Also, if we want to add the same gene fragment, that is, a promoter, multiple times into the same vector, the sequence homology would restrict

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the use of homology-directed assembly methods. Therefore, these limitations highlight the emerging needs for more versatile and effective DNA editing tools.

The booming technology-clustered regularly interspaced short palindromic repeats (CRISPR) system has developed rapidly over the last decade (Bayat, Modarressi, & Rahimpour, 2018; Ferreira, David, & Nielsen, 2018; Hille et al., 2018; Sander & Joung,). The widely used CRISPR-Cas9 from *Streptococcus pyogenes* has been designed and optimized to facilitate molecular cloning (W. Jiang et al., 2015; Kang, Charlop-Powers, & Brady, 2016; Liu et al., 2015; H. Wang et al., 2018), such as CATCH (Cas9-Assisted Targeting of Chromosome) (W. Jiang et al., 2015), ICE (in vitro CRISPR/Cas9-mediated Editing; Liu et al., 2015) and exoCET (exonuclease Combined with RecET recombination; H. Wang et al., 2018). All these strategies are successfully used in large DNA construct cloning or modification. However, these tools are homology-directed ligation for ends produced by Cas9, which may limit their applications for repeated DNA sequences that are likely to be found in a long target DNA. In 2015, the discovery and characterization of the Class II Type V CRISPR-Cas12a (Zetsche et al., 2015) offered an alternative potential for functional genomics of various organisms (Y. Jiang et al., 2017; Tang et al., 2017). Cas12a has several advantages over Cas9 for in vitro DNA editing, thus it could serve as a great supplement to current methods. For instance, Cas12a cleaves double-stranded DNA to produce relatively consistent sticky ends rather than uncertainly 1–3 bp sticky ends (Shou, Li, Liu, & Wu, 2018), a trans-acting crRNA is not required thus the crRNA is shorter (~42 bp), and it can process its own crRNA array into mature crRNA to enable easy multiplex gene editing (Fonfara, Richter, Bratovič, Le Rhun, & Charpentier, 2016; P. Gao, Yang, Rajashankar, Huang, & Patel, 2016). These characteristics facilitate the utilization of Cas12a as a DNA editing tool in vitro.

Among the characterized proteins in the Cas12a family, FnCas12a from *Francisella novicida* shows advantages in short PAM sequence recognition and preference to cleave the 18th to 23rd base distal from the PAM site, thus generating a 5 bp user-defined sticky end (S. Y. Li, Zhao, & Wang, 2016). Therefore, the FnCas12a can sever as a Type II RE like digestion enzyme in molecular cloning, and it has already been demonstrated in the C-brick standard and CCTL method (Lei et al., 2017; S. Y. Li et al., 2016). However, digestion and ligation are performed as separate time-consuming steps in those methods, and fragment purification is required to eliminate repeated digestion and back ligation. Moreover, the requirement of a TTN PAM limits its application.

To address these issues, we have devised an iCOPE (improved Cas12a-assisted one-pot DNA Editing) method that can either edit or assemble DNA fragments in a user-defined one-pot reaction (Figure 1). The PAM specificity of both Cas9 and Cas12a was expanded via either structure-guided design or evolutionary strategies (L. Gao et al., 2017; Hu et al., 2018; Kleinstiver et al., 2015). In this study, we generated a variety of FnCas12a mutants with different PAM recognition capabilities, which can recognize 60 out of 64 PAMs, and is especially efficient with YN, TAC, and CAA PAMs. The targeting range was increased by nearly fourfold relative to WT FnCas12a. Therefore, crRNAs can be designed to almost any sequence to facilitate seamless cloning. In this approach, by taking advantages of the widespread recognition sites of Cas12a variants, it serves as the restriction endonuclease to cleave target DNA at the user-defined sites following the guidance of a pair of corresponding sgRNA transcribed in vitro. As individually operating the in vitro RNA preparation, DNA digestion and ligation would take one more day than traditional work, we further combined the three steps together. This helps to eliminate the additional time as well as DNA purification. Moreover, this approach is also promising in manipulating large plasmids, which is not feasible via current popular methods. Taken together, the iCOPE method eliminates many of the sequence-based constraints of current technologies and can serve as a versatile DNA editing tool in vitro.

2 | RESULTS

2.1 | Engineered Cas12a variants with expanded protospacer adjacent motif targeting range

To address the limitation involving the requirement of a YTN (Y = C or T) PAM, we engineered FnCas12a variants with altered PAM specificities using specifically structure-guided mutagenesis. Studies on both Cas9 and Cas12a proved that the selectivity of PAM is determined by the residues around the PAM DNA duplex (Anders, Bargsten, & Jinek, 2016; L. Gao et al., 2017; Hirano, Nishimasu, Ishitani, & Nureki, 2016; Hu et al., 2018; Kleinstiver et al., 2015). Recently, the targeting range of AsCas12a was expanded by randomizing the codons at the PAM-recognition region, generating variants RR (S542R/K607R) and RVR (S542R/K548V/N552R) which efficiently recognize TYCV (V = A, C or G) and TATV PAMs respectively (L. Gao et al., 2017; Nishimasu et al., 2017). Because the crystal structure of FnCas12a has been solved and demonstrated a similar PAM-binding pattern as that of AsCas12a (Anders et al., 2016), we carried out structure-based engineering on FnCas12a via comparing the PAM-recognition regions of FnCas12a and AsCas12a variants (Figure 2a,b). First, mutations were introduced at four sites (N607R, K613V, N617R, and K671R) that correspond to the optimized AsCas12a variants (S542R, K548V, N552R, and K607R). Furthermore, FnCas12a has two residues D616 and K180, which are involved in PAM recognition but are quite different from their corresponding residues (N551 and S170) in AsCas12a (Figure 2a). To generate a similar PAM-recognition environment as that of AsCas12a variants, mutations D616N and K180S were introduced as well. Additionally, a charge interaction (K660-E733 in FnCas12a, D596-K689 in AsCas12a, Figure 2b) caught our attention for its engagement in the domain movements of WED-II and LKL regions, where the different distances of D596-Oδ and K689-Nζ in RR (5 Å) and RVR (3.3 Å) might come along with the distinct PAM selectivity. Thus, an Arg substitution was made on K660 of FnCas12a (K660R), expecting to enforce the charge interaction between its WED-II and LKL domains. The above site-directed mutagenesis studies were

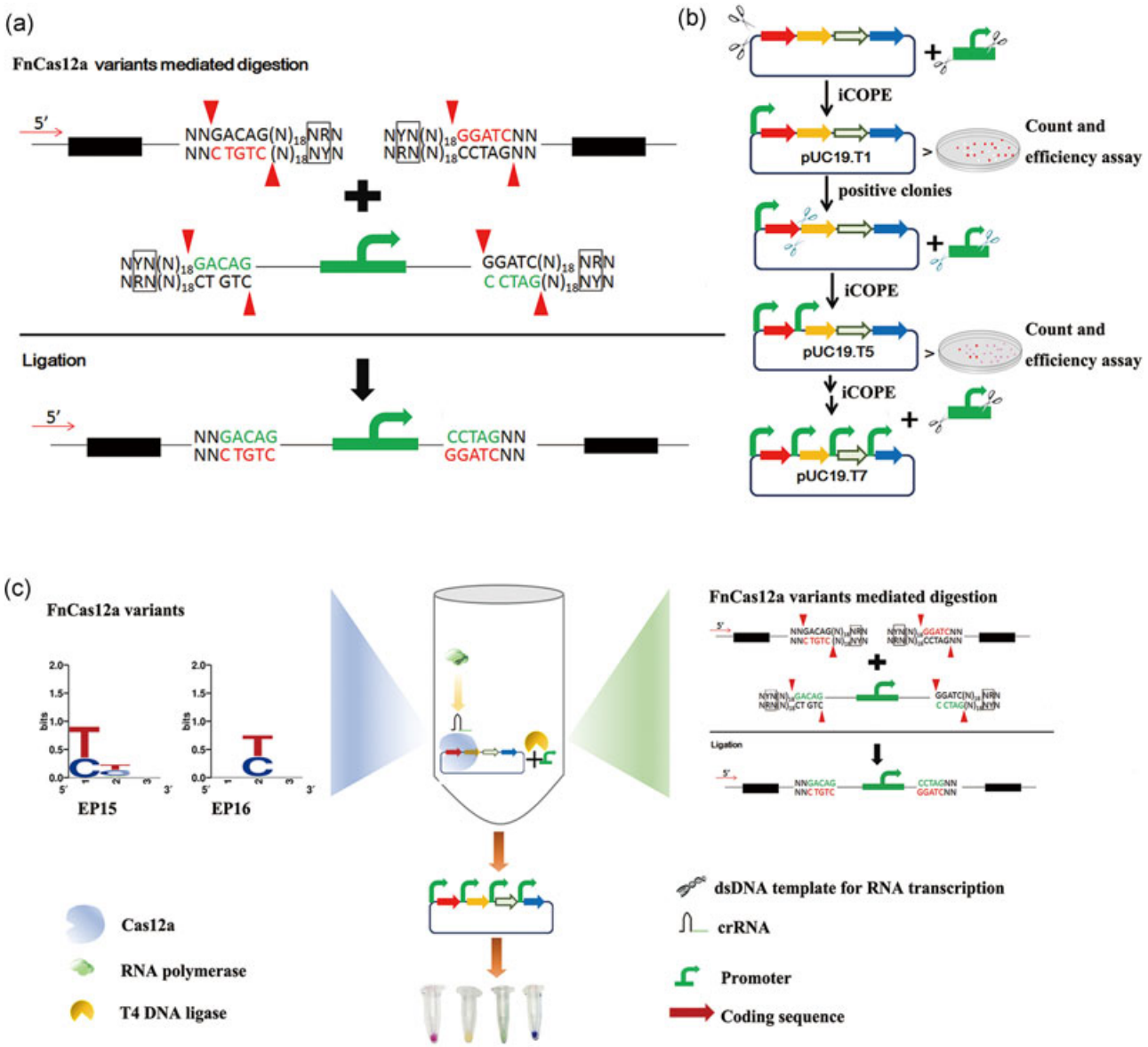


FIGURE 1 The design of iCOPE. (a) The double-digestion design of both the vector and insert. The PAMs are boxed. YN, TAC, TAA, and TGC PAMs can also be recognized in this study. The sticky ends of the vector and insert were colored. (b) Schematic chart for promoter insertion via iCOPE. The same promoter can be introduced into the vector iteratively. (c) the overall design of iCOPE. The iCOPE reaction is set up by adding the vector, the insert, the FnCas12a variant (EP15 or EP16), the corresponding crRNAs or the spacer template dsDNA with RNA transcription mix (NTP and RNA polymerase in the transcription buffer), and T4 DNA ligase all in the same reaction mixture. iCOPE: improved CRISPR-Cas12a-assisted one-pot DNA editing; NTP: nucleoside triphosphate; PAM: protospacer adjacent motif [Color figure can be viewed at wileyonlinelibrary.com]

grouped in series combinations and finally, 17 types of FnCas12a mutants were generated (Table S1).

To test the PAM targeting range of the FnCas12a mutants, in vitro cleavage assays were conducted with 0.625 pmol DNA substrate and the results were analyzed by gel electrophoresis. Before the PAM assay, we selected a pre-validated crRNA E-SP15 to construct the PAM assay library. Sixty-four designated sequences (5′-NNN TTGAGAGTGGTGAGTGACAGCGC-3′) were individually cloned into pRS426.Then, the DNA substrates (1234 bp), which contained the target sites, were amplified by primer Fn-PAM-temp-For and Fn-PAM-temp-Rev. If the mutants recognized the PAM sequences, a 707 and 527 bp products would be produced.

To evaluate the potential of FnCas12a variants, a 3-hr reaction was performed to ensure complete digestion. PAM profiling of FnCas12a variants is shown in Figure S1 and S2. As expected, the efficiency of FnCas12a-mediated DNA cleavage of TTV, TCV, CTV (V = A, C or G) PAMs was much higher than that of other recognizable PAMs like TTT and TCT, supporting the previously reported findings (L. Gao et al., 2017; Tu et al., 2017). Compared with WT FnCas12a, the FnCas12a variant N607R/K613V/N617R (hereafter referred to EP2), which is the analog to AsCas12a variant RVR, showed decreased activity and

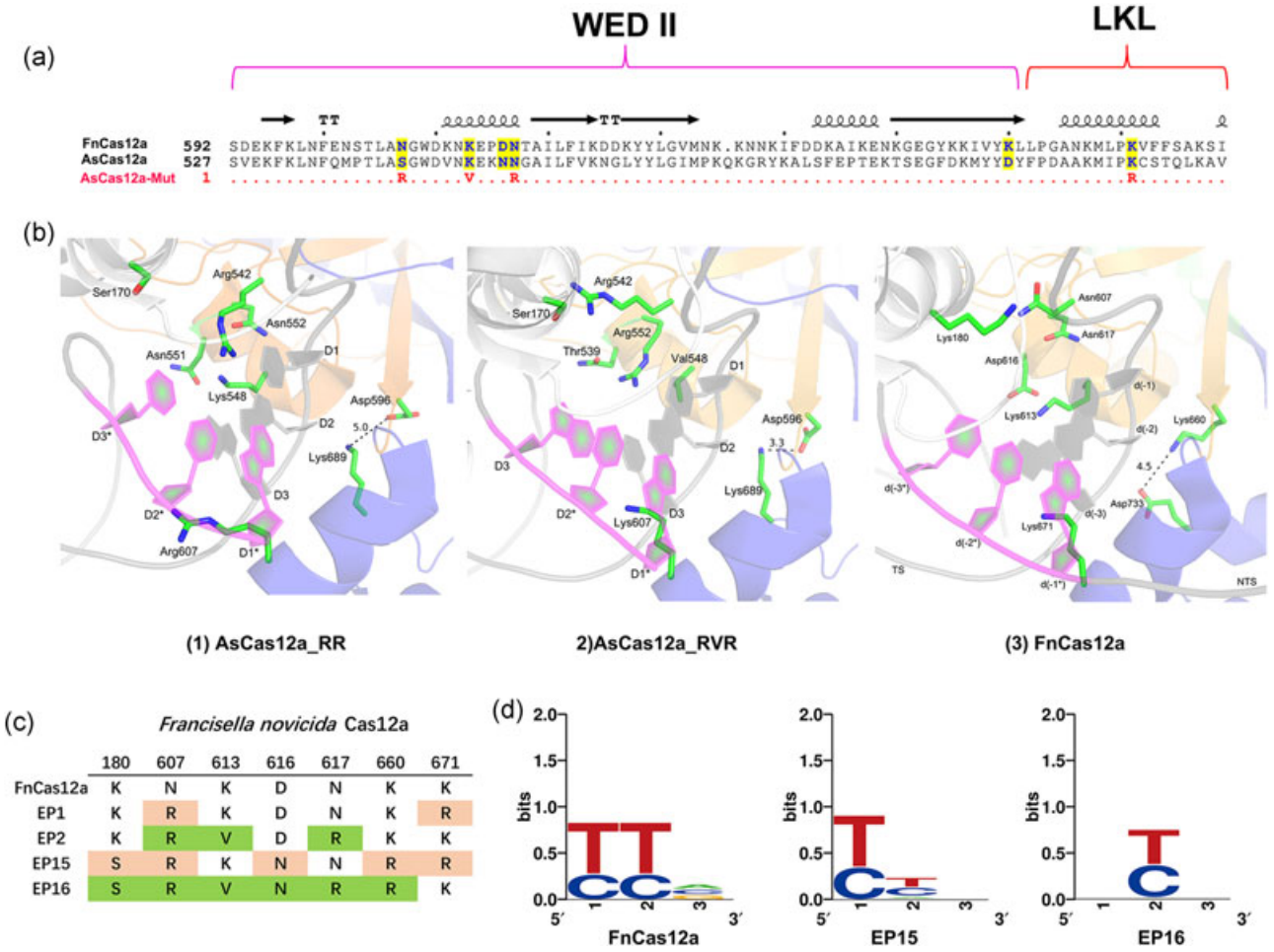


FIGURE 2 Structure-guided engineering of FnCas12a for broadened PAM compatibility. (a) Sequence alignment of FnCas12a, AsCas12a and AsCas12a mutants. The corresponding residues related to interactions with the nucleotides and PAMs are shadowed. (b) protospacer adjacent motif (PAM) recognition interaction of AsCas12a variants and the WT FnCas12a. The corresponding residues related to interactions with the nucleotides and PAMs are labeled. (c) Genotypes of FnCas12a and some variants, colored by different variants' types. EP1 is the FnCas12a mutant corresponding to AsCas12a-RR and EP2 is the FnCas12a mutant corresponding to AsCas12a-RVR (L. Gao et al., 2017). EP15 is the optimized version of EP1 and EP16 is the optimized version of EP2. (d) Sequence logos of the high-efficiency (>50%) cleavage PAMs for WT FnCas12a, FnCas12a-EP15, and FnCas12a-EP16 [Color figure can be viewed at wileyonlinelibrary.com]

decreased PAM recognition sites. However, if it was combined with K180S, K660R, or D616N (hereafter referred to as EP4, EP6, EP10, respectively), the variants showed similar cleavage activity at the same PAMs with WT FnCas12a. Meanwhile, new cleavage activity at PAMs VYV appeared (Figure S1). Surprisingly, after combining K180S, K660R and D616N to generate EP16 (N607R/K613V/N617R/K180S/K660R/D616N), we found that EP16 cleaved YN, TAC at considerable efficiency (>50%), and cleaved the other PAMs weakly excluding CGG, AGT, GGA, GGG (Figure S2c and S2d). This expanded the scope of EP16 to 60/64 PAMs (Figure S1b). Another type of variant N607R/K671R (EP1), analog to AsCas12a RR, showed similar PAM recognition sites with WT FnCas12a (Figure S2). After combination with K180S, K660R, and D616N, the variant EP15 produced a commendable cleavage efficiency at YYN PAMs (>95%) except for CCA PAM (Figure S1a, S2c, and S2d). Meanwhile, we combined the seven mutagenesis sites together and generated the variant EP17 (N607R/K613V/N617R/K180S/K660R/D616N/K671R). EP17 showed similar PAM recognition capacity with EP16, but decreased efficiency in TAV PAM sites. Taken together, EP16 has the broadest PAM recognition (60/64 sites) and prefers to target YN PAMs, and EP15 is more efficient than EP16 in TAA, TGC, and CTT PAM sites (Figure 2 and S2). In summary, EP15 and EP16 can effectively target almost any given sequence.

2.2 | Design and optimization of the iCOPE method

To develop an assembly method that can combine the digestion step with the ligation step, the digestion enzyme should have (a) site-specific target and cleavage sites, and (b) loss of digestion ability with the assembled constructs. The sticky ends produced by FnCas12a from *F. novicida* make it ideal as the digestion enzyme in the cloning system. FnCas12a cuts target DNA via crRNA guidance to the designated PAM area, so the PAM sites have to be altered or deleted to prevent repeated digestion. Here we designed the double-digestion on both the vector and insert (Figure 1a,b). In this case,

the loss of the PAM sequence after ligation prevents repetitive digestion. After digestion, the fragments are ligated in the designated order based on the user-defined 5-bp sticky ends. Different from restriction enzyme-based cloning/editing, paired crRNAs must be generated before the digestion process of CRISPR involved editing. However, crRNA in vitro transcription requires overnight incubation and RNA purification. To achieve the Cas12a-based one pot design, our system further combined RNA transcription, digestion and ligation in one reaction mixture (Figure 1c). Thus, we eliminated the additional steps for in vitro RNA preparation, required in Cas9 or Cas12a assisted in vitro editing. To test the efficiency of the one-pot reaction, the two fragment cloning was illustrated in the subsequent studies.

To optimize the iCOPE method, we checked the influence of the reaction buffer and its thermostability (Figure S3, S4a). The differences among CutSmart, NEB buffer 2, T4 ligase buffer and the NEB buffer 4 were not significant. Our experiment showed that FnCas12a is thermostable and active from 16 to 60°C (Figure S3). The flexibility in reaction buffers and the thermostability of FnCas12a make it perfect for us to apply FnCas12a as the digestion enzyme in a one-pot assembly method. As a result, T4 DNA ligase buffer was selected for subsequent experiments to accommodate the ligation reaction with T4 ligase. A final digestion temperature 50°C was chosen to eliminate back ligation (Figure S3) as T4 ligase is active from 16 to 37°C, but FnCas12a is active from 16 to 60 °C. To design an optimal final digestion step, we incubated the reaction at 50°C for different time lengths and we found that 10 min is enough (Figure S3).

Previous studies demonstrated that FnCas12a cleaved around YTV PAM more efficiently than around YTT PAM (Leenay et al., 2016; Tu et al., 2017). To explore the proper one-pot reaction condition suitable for YTN, we first selected the spacer E-SP15 to perform the enzyme cleavage activity assay. Linearized DNA $T_{PAM}3$ or $T_{PAM}2$ served as the template corresponding to the substrate with TTC PAM or TTT PAM, respectively. The influence of the concentrations of crRNA and FnCas12a were tested and we found that 1 μM crRNA and 500 nM FnCas12a works best for 3-hr digestion experiments (Figure S4b–d). Digestion time course assay was performed as well and 2-hr digestion time should be optimal (Figures S4e,f). Considering the interference among these parameters, we designed an orthogonal experiment to test both the FnCas12a concentration and digestion time. We found that 500 nM FnCas12a and 2 hr digestion time was optimal for subsequent experiments (Figure S5).

Stated thus, the recommended conditions for the initial digestion-ligation one-pot assay are: 1 μM crRNA and 500 nM FnCas12a in T4 DNA ligase buffer in a total volume of 10 μl, repeating the digestion step (37°C ,10 min) and ligation step (16°C ,10 min) 12 times with a final digestion incubated at 50°C for 10 min.

2.3 | TheiCOPE method is an efficient tool for in vitro DNA editing

In this study, most of the spacer used were designed *via*www.rgenome.net (Park & Bae, 2018; Table S2). To test the efficiency of the iCOPE method, a four-fluorescent protein or chromoprotein (mRFP, eYFP, amilCP, and cjBlue) cassette was introduced into pUC19 to generate pUC19.S4. Meanwhile, we selected the promoter pLac to activate the fluorescent proteins or chromoproteins in pUC19.S4, that is, when the promoter pLac with RBS was inserted in front of the chromoproteins in pUC19.S4, the colonies would show the corresponding colors (Figure 3a). Therefore, the editing efficiency can be calculated by counting the correct colored colonies (Figure 1b). Then the accuracy would be validated via Sanger sequence. First, the *pLac* was inserted in front of each chromoprotein individually by producing constructs pUC19.T1, pUC19.T2, pUC19.T3, and pUC19.T4 (Table S3). The efficiency of one fragment insertion ranged from 75 to 97.22% (Figure 3b and Table S4).

To increase the efficiency of iCOPE, we tested the reaction parameters as follows: incubation for 10 min at 37°C and 10 min at 16°C or incubating for 5 min at 37°C and 5 min at 16°C, both steps repeated 3, 6, 9, 12 or 15 times. These conditions were similarly efficient, and the 2-hr reaction time is long enough for one promoter insertion (Figure 3c and Table S5). At the same time, we tested the influence of DNA fragments' forms. Circle vector–linearized promoter, circle vector–circle plasmids containing the corresponding promoter and linearized vector–linearized promoter were studied in two cases (the mRFP expression and the amilCP expression), and we found that the editing efficiency among varied types of DNA fragments showed no significant difference, but the number of colonies decreased when linearized DNA fragments were introduced (Figure 3d,e and Table S6). The same result was also observed in the Golden Gate assembly (Figure 3d,e and Table S6), indicating that it may be a common phenomenon which still needs further experiments to give explanations. To confirm its potential for sequence-independent ligation, the same promoter pLac was inserted iteratively via iCOPE to produce pUC19.T5, pUC19.T6, pUC19.T7 and pUC19.T8 and no significant differences of efficiency (the pLac has been inserted into pUC19.S4) or accuracy (the pLac has been inserted into pUC19.S4 as user-designed) were observed (Figure 3f). This result further indicated that iCOPE could serve as an alternative DNA editing tool in constructs containing repeated sequences.

To evaluate the influence of varied crRNA forms, we synthesized a series of crRNAs including two spacers in one RNA strand and four spacers in one RNA strand. Then we applied these crRNAs in iCOPE for the mRFP activation (insertion of the *pLac* in front of the *mRFP* in the pUC19.S4), the eYFP activation (insertion of the *pLac* in front of the *eYFP* in the pUC19.S4) and the amilCP activation (insertion of the *pLac* in front of the *amilCP* in the pUC19.S4). The results presented that the experiments added the four spacers on one array showed similar editing efficiency to the one added four spacers individually. However, the assembly efficiency decreased when adding two arrays each containing two spacers (Figure 3g and Table S7). Meanwhile, the enzymatic assay of different crRNA forms was also performed, and the result concurred with the DNA editing performance (Figure S6). This consequence may be due to the architecture of CRISPR arrays on genomes (Zetsche et al., 2015), where the CRISPR arrays are always in multiples of four. Therefore, based on our design, adding a

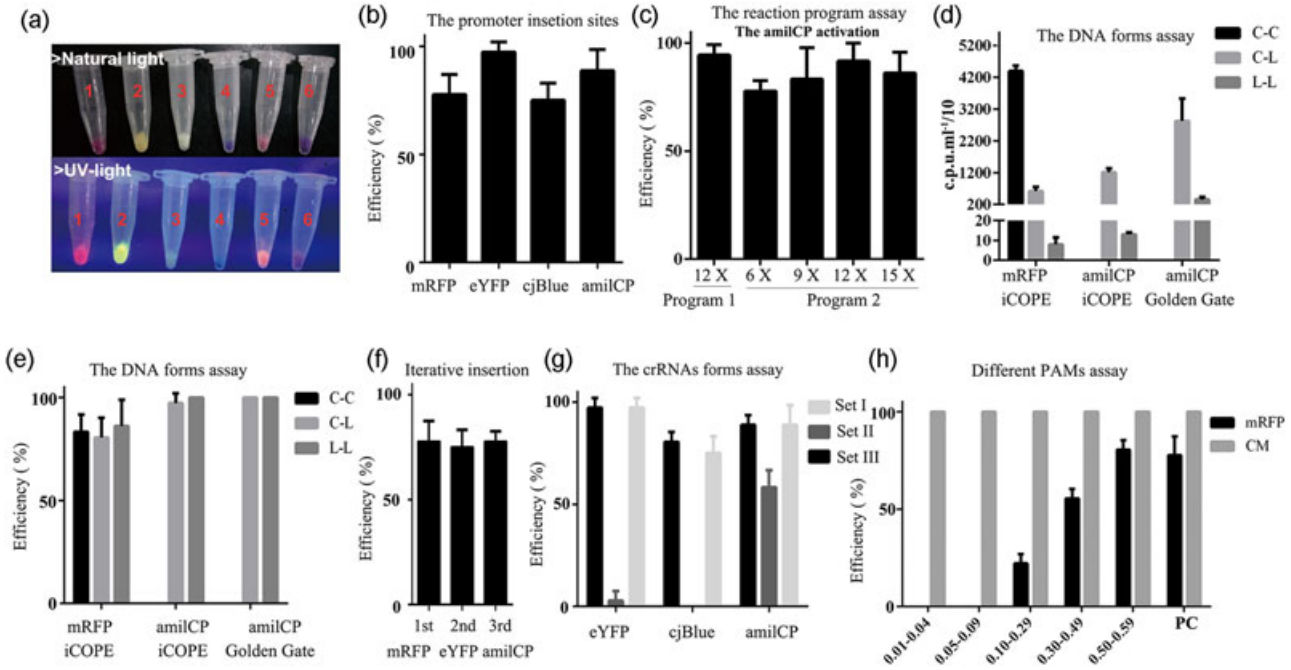


FIGURE 3 The efficiency assay of iCOPE. (a) The result from chromoprotein and fluorescent proteins expression. Four individual chromoproteins, assembled dual chromoproteins and triple chromoproteins were expressed in *E. coli* grown in liquid LB medium. From 1–6, constructs expressed mRFP (pUC19-T1), eYFP (pUC19-T2), cjBlue (pUC19-T4), amilCP (pUC19-T3), mRFP plus eYFP (pUC19-T5), and mRFP plus eYFP, and amilCP (pUC19-T6), respectively. (b) The application of iCOPE in DNA editing. The promoter *pLac* was inserted in front of each protein individually by producing constructs pUC19.T1 (mRFP expression), pUC19.T2 (eYFP expression), pUC19.T3 (amilCP expression) and pUC19.T4 (cjBlue expression). (c) The influence of reaction program. The promoter pLac was inserted in front of amilCP in pUC19.S4. The reaction program was set as following: the reaction mix was performed as Program 1(incubating the reaction mix at 37°C for 5 min and at 16°C for 5 min, and repeat the two steps for designed times) or Program 2 (incubating the reaction mix at 37°C for 10 min and at 16°C for 10 min, and repeat the two steps for designed times) first, and then at 75°C for 10 min (heat inactivation) before transformation to *E.coli*. (d,e) The influence of varied DNA forms. In this study, two examples were tested. The mRFP activation was performed by inserting *pLac* in front of pUC19.S4 via iCOPE, and the amilCP activation was performed by inserting the amilCP expression cassette (*pLac* + B0034 + *amilCP*) into the *BsaI* cloning sites in plasmid Fn.VU6 via iCOPE or Golden Gate assembly method. C–C, circle vector–circle plasmids containing the corresponding promoter; C–L, Circle vector–linearized promoter; L–L, linearized vector–linearized promoter. Notably, the C–C in amilCP activation tests have not been performed. (f) The influence of the iterative insertion of the same promoter. (g) The influence of varied crRNA forms. To investigate the influence of crRNA forms, inserting promoter pLac in front of the *eYFP*, *cjBlue* and *amilCP* in pUC19.S4 individually were performed. Set I containing four spacers on four crRNAs individually; Set II containing four spacers on two strands of crRNAs; Set III containing four spacers on one crRNA array. (h) The test of the cloning efficiency of different PAMs. Two examples were selected to exploring the cloning efficiency of PAMs in iCOPE, including the mRFP insertion and the CM insertion. The mRFP insertion was performed by inserting mRFP expression cassette (*pLac* + B0034 + *mRFP*) into pUC19. The efficiency was calculated by mRFP expression (red colonies/ total). The CM insertion was performed by inserting chloramphenicol resistance gene expression cassette into pUC19. In the CM insertion case, the transformation reaction mix was plate on LB media containing chloramphenicol (25 μg/ml). The PAMs used in this study including GAG (0.01), GGT (0.03), AAA (0.07), TAT (0.08), TGA (0.13), AGC (0.15), TGC (0.31), CAA (0.41), ACC (0.50), GCT (0.52), TCA (0.99), and ACG (0.99). In general, 12 colonies were picked for validation in this study. Error bars: mean ± SD. for $n = 3$ [Color figure can be viewed at wileyonlinelibrary.com]

crRNA array containing four spacers could replace the adding of four separated crRNAs. In summary, the crRNA array containing four spacers could be used in iCOPE to simplify the reaction components.

Previous in vitro DNA cleavage experiments demonstrated the wide PAM recognition scope of FnCpf1 variants EP15 and EP16. To test the minimal depletion score of the PAM for an efficient DNA cloning, we divided the PAM scores lower than 0.6 into five groups (a) 0.50–0.59, (b) 0.30–0.49, (c) 0.10–0.29, (d) 0.05–0.09, (e) 0.01–0.04) and tested their cloning efficiency via the mRFP gene cloning and the chloramphenicol (CM) resistant gene cloning (Figure 3h and Table S8). In each group, we selected two PAMs to test. Therefore, spacers targeting PAMs ACC (0.50) and GCT (0.52) from group a; TGC (0.31) and CAA (0.41) from group b; TGA (0.13) and AGC (0.15) from group c; AAA (0.07) and TAT (0.08) from group d; GAG (0.01) and GGT (0.03) from group e; as well as TCA (0.99) and ACG (0.99) as positive controls were synthesized to target pUC19. After collection of the corresponding inserts, iCOPE reactions were performed in an RNase-free reaction system of 10 μl volume, employing 60 ng pUC19 plasmid, 40 ng prepared inserts and 1 μM corresponding crRNAs. As shown in Figure 3h and Table S8, the editing efficiency of the PAMs ACC and GCT (the recognition efficiency range 0.5–0.6) is comparable to that of the PAMs TCA and ACG (the positive controls). This

finding supported our definition of the PAM recognition efficiency-the PAM depletion scores larger than 0.50 would be regarded as high-efficiency PAMs. With selection pressure, as illustrated in the chloramphenicol (CM) resistant gene cloning case, the efficiency of PAMs GAG and GGT (the recognition efficiency range 0.01–0.04) for cloning achieved 100%. This result proved that FnCas12a variant EP16 can cleave the PAMs with a score of 0.01.

To test the efficiency of iCOPE, we used this method to introduce promoter pLac to activate amilCP expression. The new reaction system was performed by simply adding an RNA transcription mix (ATP/CTP/GTP/UTP/T7 pol mix/ transcription buffer) and the dsDNA template for spacer transcription into the previous reaction mix. Several parameters were investigated (Table S9), and we concluded that when the transcription mix is 6 μl and the reaction program including a 37°C incubation for 2 hr before the standard program, the iCOPE method showed nearly the same efficiency as before.

Theoretically iCOPE could be applied in multiple DNA fragment assembly. To test its performance, the three and four fragment assembly experiments via iCOPE were performed. The efficiency of the 3 fragment assembly is around 33.33 ± 14.38% and that of the 4 fragment assembly is only 1.85 ± 0.81% (Table S10). We speculated that with the increased number of crRNAs, the complexity of the reaction components affected the assembly efficiency. Therefore, further optimization of iCOPE for multiple fragment assembly would be considered.

2.4 | The application of iCOPE in seamless DNA editing

Previous work demonstrated that FnCas12a can perform DNA editing in vitro (Lei et al., 2017; S. Y. Li et al., 2016). However, limited PAM recognition restricts its application. For example, to replace the promoter in actII-orf4 cluster, a short sequence was ligated to the target promoter *ermE* in advance to create the desired recognition site as in the reported CCTL method (Lei et al., 2017). As iCOPE employs FnCas12a variants with expanded PAM targeting range, it could perform more versatile DNA editing. Here, we designed two parallel assays. One substituted the *lacZ* gene in pUC19 with the *mRFP* gene, and the other replaced the ampicillin resistance gene with a chloramphenicol (hereafter called CM) resistance gene in pUC19 (Figure S7). By applying iCOPE with EP16, we have fewer constraints in cutting site selection; therefore, the replacement was designed with no scar left (Figure S7). In *lacZ* replacement, two spacers with PAMs ACA, TTG were produced. With the help of EP16 in the iCOPE system, about 92% of the colonies were correctly assembled (Table 1). For substitution of chloramphenicol resistance gene, two spacers with PAM TCA, ACG were generated and the efficiency reached 100% (Table 1) as the selection marker helps to eliminate false positives. These results confirmed that with the FnCas12a variants, the iCOPE method could serve as a seamless DNA editing tool in vitro with almost any given DNA sequences.

2.5 | The application of iCOPE in large DNA modification

Numerous DNA assembly technologies have been developed for large DNA construction, among which the in vitro Gibson assembly, Golden Gate Assembly, and the in vivo DNA assembler technology are widely used for one-step assembly of multiple DNA fragments (Engler et al., 2009; Shao, Zhao, & Zhao, 2009). However, in pathway engineering, for example, to study the functional genes of the natural products producing pathways, we need to generate a variety of deletion constructs based on the full-length gene clusters. Applying these methods to modify a designated part within a large DNA construct is difficult and time-consuming, if it is not designed well in advances.

To investigate the capacity of iCOPE to edit large gene clusters and specifically to construct in-frame deletion recombinants, we used plasmid pATRTH (~21 kb, containing the salidroside biosynthetic gene cluster) as a target to test (Figure 4). To study the function of *Aro7* (encoding chorismate mutase) in the salidroside biosynthetic gene cluster, we need to knock out the *Aro7* gene expression cassette (1.8 kb) for comparative analysis. However, via traditional cloning methods, without pre-designed editing sites, we cannot modify such size plasmid easily. First, we designed two spacers pATRTH-P1(PAM: GTT) and pATRTH-P2 (PAM: ACA) and then the iCOPE with EP16 was conducted. To verify cloning efficiency, 33 colonies from three parallel experiments were validated by PCR, demonstrating that 100% colonies with the expected seamless deletion were obtained (Figure 4b). Then, the accuracy was confirmed by Sanger sequencing (Figure 4c,d). The result showed that the *Aro7* expression cassette was successfully knocked out from the pATRTH plasmid as designated.

3 | DISCUSSION

With our finding of the thermostability and broad PAM recognition of mutant FnCas12a, we developed a one-pot seamless in vitro DNA

TABLE 1 The results of DNA editing via iCOPE

Application	Efficiency (%)	Accuracy (%)
mRFP replacement	92 ± 4	100
Chloramphenicol resistance gene replacement	100	100
pATRTH modification	100	100

To determine the cloning efficiency of the mRFP replacement, colonies were counted and calculated by color (mRFP expression). Accuracy (the *mRFP* was inserted into pUC19 as designed) was confirmed by Sanger sequencing of the positive colonies, in which eight colonies from three parallel experiments were sequenced (Figure S8a); To determine the cloning efficiency (the chloramphenicol resistance gene was inserted into pUC19) and accuracy (the chloramphenicol resistance gene was inserted into pUC19 to replace Ampicillin resistance gene as designed) of the CM replacement, 18 colonies from three parallel experiments (six samples for each) were sequenced (Figure S8b); the efficiency and accuracy were shown in the form of mean ± *SD*, $n = 3$.

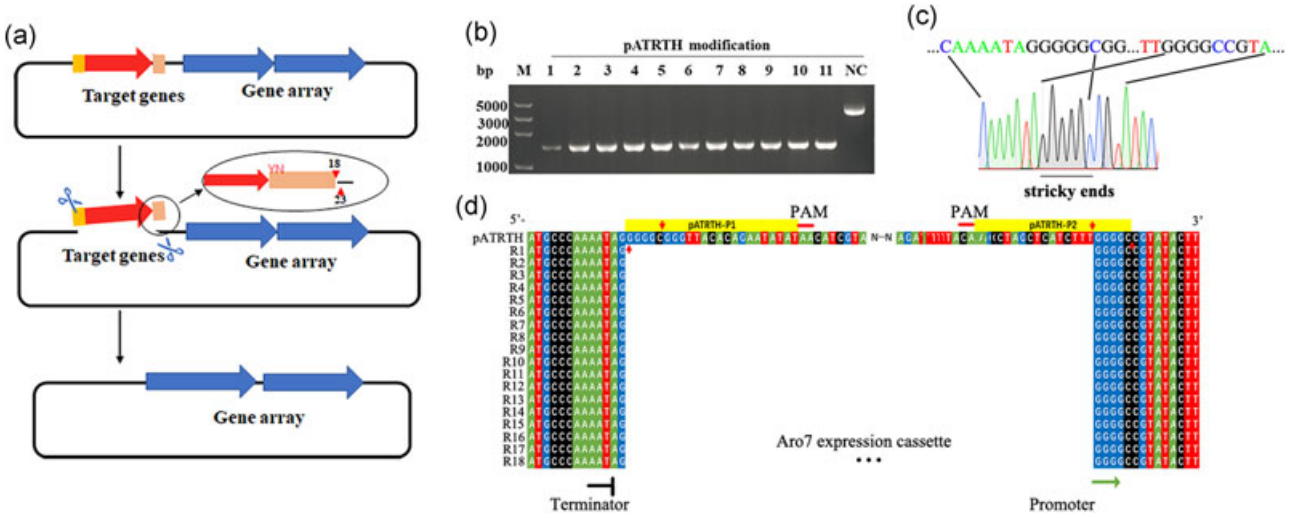


FIGURE 4 The Cas12a assisted target gene deletion in large DNA construct. (a) Schematic chart for deleting target gene *Aro7* expression cassette in pATRTH via iCOPE. A pair of 23-nt spacer sequences were chosen nearby the target gene cassette. The pATRTH was cleaved by EP16 in vitro. (b) Positive rates of iCOPE, which was analyzed by PCR using primers of pATRTH-CKF and pATRTH-CKR. PCR bands with the size of 1.3 kb indicated the acquisition of the positive clones. NC is negative control, and the PCR band should be 3.12 kb. (c,d) Verification of the editing accuracy by Sanger sequencing. In d, spacers sequences were indicated, while the PAM sites were shown by lines, and the cut sites were labeled with arrows. The R1 to R18 represented different samples from tree parallel experiments corresponding to b (six clones from each trail), and the gap indicates the deleted 1.8 kb *Aro7* expression cassette [Color figure can be viewed at wileyonlinelibrary.com]

cloning/editing method we call "iCOPE." By applying the mutants of FnCas12a instead of Type IIs RE in the one-pot reaction for non-artificial constructs editing, iCOPE provides the advantages of the Golden Gate assembly method with higher programmability, versatility, and specificity because of the extended PAM recognition ability and the long crRNA recognition sites. Moreover, iCOPE program combines the RNA transcription with digestion and ligation, that is, this is simply performed by incubating the spacer template dsDNA, Cas12a protein, and dsDNA templates all in the same reaction mixture. The iCOPE allows obtaining nearly 100% correct recombinants in one tube and one step after just a 4–6 hr incubation time, while traditional RE or Cas9/Cas12a-based digestion-ligation tools should take a day or more to perform the cloning. Comparing with the newly developed C-brick standard and CCTL method, iCOPE is more efficient in DNA editing, even in large DNA construct modification.

More important, the engineered FnCas12a mutants EP15 and EP16 expanded the scope of the iCOPE method. In this study, the engineered FnCas12a mutant EP16 (N607R/K613V/N617R/K180S/K660R/D616N) can cleave 60/64 PAMs and around 33/60 PAMs with relatively high activity, while EP15 is more efficient than EP16 in TAA, TGC, CTT PAM sites (Figure S2). By combing EP15 and EP16, 35/64 sites could be effectively targeted and those could be potentially applied in the cloning processes. With a selection pressure, the PAM with the efficiency of 0.01 can work (Figure 3h). This feature improved the iCOPE method to be an in vitro DNA editing tool with almost no sequence constraints. That iCOPE leaves no unwanted base pairs in the target vector makes it a perfect tool for DNA editing and cloning, especially in large gene constructs modification. Proof of concept, we succeed in deleting a designated 1.8 kb from a 21 kb DNA construct with 100% efficiency.

Among the various in vitro DNA editing methods, such as SLIC (M. Z. Li & Elledge, 2012), USER (Salomonsen, Mortensen, & Halkier, 2014), SSRTA (Zhang, Zhao, & Ding, 2011) and SIRA (Colloms et al., 2014), the low efficiency at certain conditions or the special requirements for certain applications limit their wide utilization. Therefore, more versatile DNA editing/cloning methods were developed, such as Type IIs RE based Golden Gate assembly and PfAgo mediated PfAgo/AREs platform (Enghiad & Zhao, 2017; Engler et al., 2009). Comparing with the Golden Gate assembly method, iCOPE takes advantages of the CRISPR-Cas12a system, using crRNA to design the recognition site and generating a 5-bp user-defined sticky end. Thus, it possesses the merits of the Golden Gate method. While the crRNA is long, which ensures a low repeated emergence rate on a given DNA fragment, the iCOPE gets rid of the limitations of the Golden Gate method. However, the Golden Gate assembly method has a competitive advantage in multiple fragment assembly in one pot. The PfAgo/AREs are capable of recognizing and cleaving DNA sequences at any arbitrary site and generating defined sticky ends of varying length. Compared with the PfAgo/AREs, (a) both Cas12a variants and PfAgo/AREs can be used to replace REs in general molecular cloning, but PfAgo/ARE may be more reprogrammable as the AREs cleave DNA sequences at virtually any arbitrary site and generate defined sticky ends of varying length, (b) PfAgo/AREs cleave target DNA at high temperatures (>87°C), thus it cannot work together with the ligase to perform one-pot cloning, and (c) 5′-phosphorylated ssDNA is required in PfAgo/ARE platform, whereas dsDNA or RNA is required in iCOPE. Taken all together, iCOPE is promising to sever as a complementary tool for editing or cloning large DNA segments, especially those containing large natural products gene clusters. Because the editing and optimization of

those large gene clusters are challenging, iCOPE offers an alternative to current Cas9-based methods as the crRNA can be designed to target near-arbitrary sequences for seamless editing with high efficiency.

As the colonies decreased in contrast to Golden Gate assembly and the efficiency reduced in multiple DNA fragment assembly, the limitations of iCOPE attracted our attentions. One is that the reaction composition is more complicated than that of the Golden Gate assembly, which may affect the transformation efficiency and result in the reduction of colonies. Another aspect is that the varied cleavage efficiency among different crRNAs may affect the editing efficiency. Especially, with the increased number of assembled fragments, the number of crRNAs increases. Then the editing preference of the crRNAs may reduce the efficiency of iCOPE. Therefore, future optimization of iCOPE including improvements of the purification process of Cas12a of changing the forms or structures of the crRNAs may help to improve its efficiency in multi-fragment assembly. On the other hand, several crRNA design tools have been developed for CRISPR systems to deal with the off-target or low-efficiency issues (Labun, Montague, Gagnon, Thyme, & Valen, 2016; Moreno-Mateos et al., 2015; Xie, Shen, Zhang, Huang, & Zhang, 2014). Recently, a design tool was reported that can enable accurate prediction of AsCas12a crRNA activities in 125 cells (Kim et al., 2018). With further understanding of the guide RNA activities in vitro, the selection of crRNAs for iCOPE could be standardized and optimized. Then, it would also be promising to apply iCOPE in multi-fragments assembly with high efficiency. Also, with the future evolution of Cas12a and exploration of new enzymes, there would be more choices for sequence-independent tools development. Moreover, the reaction components, such as the RNA transcription mix or T4 ligase, are easy to get from biological reagent companies, and the Cas12a protein is easy to be purified in biolabs. In summary, our work provides an efficient one-pot DNA editing tool in vitro and it is potential to be used in a broad range of applications.

4 | MATERIALS AND METHODS

4.1 | Cloning, expression, and purification of wild type and mutant FnCas12a proteins

FnCas12a (WP003040289) expression plasmid pET28-FnCas12a-TEV was a gift from Professor Jin Wang from the Shanghai Institute of Plant Physiology & Ecology, CAS, China(S. Y. Li et al., 2016). Expression plasmids of FnCas12a variants were generated by the standard QuikChange mutagenesis method (W. Wang & Malcolm, 1999). All above FnCas12a and mutants' expression plasmids were transformed into *E. coli* BL21 (DE3) to produce protein. Expression of FnCas12a and its mutants (FnCas12a-EP1~EP17) were induced with 0.5 mM IPTG at OD600 of 0.6-0.8. Purification was performed via immobilized affinity chromatography with Ni^{2+} charged HiTrap metal chelating sepharose resin (GE, Boston, MA) as described before (Zetsche et al., 2015). The protein purity was analyzed by SDS-PAGE and fractions containing homogeneous FnCas12a proteins were concentrated by ultrafiltration. Protein concentration was determined by the Bradford method (Hammond & Kruger, 1988). Relevant primers and plasmids were shown in Table S11 & S1.

4.2 | Preparation of plasmids

pUC19 and pSB1C3 were selected as cloning vectors. Reporter sequences, that is, *amiCP* (K592015), *mRFP* (E1010), *eYFP* (E0030), and *cjBlue* (BBa_K592011) were amplified from corresponding iGEM parts with appropriate primers (Table S12) and then cloned into pUC19 to generate a series of plasmids such as pUC19.S1, pUC19.S2, pUC19.S3, and pUC19.S4 (Table S3). The promoter pLac with varied spacers, terminator and RBS were amplified by PCR (Fast pfu DNA polymerase, Novoprotein, Shanghai, China) and assembled into pSB1C3 to produce plasmids pSB1C3.P1, pSB1C3.P2, pSB1C3.P3, and pSB1C3.P4 (Table S3) using Gibson Assembly Master Mix (New England Biolabs, MA).

PAM plasmids library construction was based on Golden Gate assembly method (Engler et al., 2009) with some modifications. Briefly, paired oligonucleotides (Table S13) flanked with *BsaI* sites were annealed in prior of assembling into pRS426 to produce PAM assay plasmids library (Table S14).

4.3 | Synthesis of crRNAs

The templates for in vitro transcription were prepared via PCR amplification with primers of T7-crRNA-F and corresponding oligonucleotides (Table S15). Notably, DNA templates for 4 crRNAs one array were amplified from plasmid pUC57. PAM (Sequence S1, GENEWIZ, Suzhou, China). The synthesis of crRNAs was performed using the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, MA). T7 in vitro transcription was performed overnight (about 14 hr) with 0.8 μg DNA template, and then RNA was purified using the RNA Clean & Concentrator-5 kit (Zymo Research, CA) and quantified with NanoDrop2000C (Thermo Fisher Scientific, MA).

4.4 | Cas12a in vitro cleavage assay

Linearized DNA T_{PAM}1-64 and T_{Red}1 were chosen as templates. T_{PAM}1 (1234 bp) was amplified with primers of Fn-PAM-temp-For and Fn-PAM-temp-Rev (Table S13), using plasmid pRS426.P1-P64 (Table S14) as the template. T_{Red}1 (3036 bp) was amplified with primers eforRed-For and pUC19-B-Rev, using plasmid pUC19. eforRed (Table S3) as the template. The FnCas12a in vitro cleavage assay was performed at 37°C in cleavage buffer (NEB Buffer 3.1 or T4 DNA ligase buffer) with 500 ng substrate DNA, 500 nM Cas12a, 1 μM crRNAs and 10 units RNase inhibitor (Solarbio, Beijing, China) in an RNase-free reaction system of 10 μl volume for 3 hr. Reactions were quenched by heating at 75°C for 10 min in before gel electrophoresis. If needed, gel electrophoresis result was quantified

by Gel Doc XR + with Image Lab Software (BIO-RAD, CA) and obtained the depletion score.

4.5 | The iCOPE method

The iCOPE program is set up by adding 0.03 pmol circle vector, 0.09 pmol linearized insertion, 6 μl in vitro transcription mix (New England Biolabs, MA), 1 μl Cas12a (5 μM), 100 ng short dsDNA for each corresponding crRNA, eight units of RNase inhibitor and 10 units of T4 DNA ligase in a total volume of 20 μl. Then the reaction program was running as following: after incubating for 2 hr at 37°C (in vitro transcription), incubation for 10 min at 37°C and 10 min at 16°C for six times, followed by incubation for 10 min at 50°C (final digestion) and then 10 min at 75°C (heat inactivation). Notably, the addition of Cas12a varies slightly depending on the batch and purification conditions.

4.6 | Apply iCOPE for gene replacement

The mRFP coding sequence was PCR amplified from pUC19.S4 with primers mRFP-For and mRFP-Rev, and the chloramphenicol resistance gene was PCR amplified from pSB1C3 with primers CM-For and CM-Rev. For the replacement of *LacZ* with *mRFP*, FnCas12a-EP16 were applied as cleavage enzyme in iCOPE and dsDNA for preparing crRNA (E-SP20, E-SP21, E-SP22, and E-SP23) were added. To determine the cloning efficiency, colonies were first counted by color (positive in red while the rest should be blue). Accuracy (the *mRFP* was introduced to pUC19 as designed) was confirmed by Sanger sequencing of the positive colonies, in which 8 colonies from 3 parallel experiments were sequenced (Figure S8a). For the substitution of the ampicillin resistance gene with the chloramphenicol resistance gene, FnCas12a-EP16 and dsDNA for preparing crRNA (E-SP24, E-SP25, E-SP26, and E-SP27) were introduced. As the change of resistance, Sanger sequencing was directly performed using the primer of Amp-seq-For to check the efficiency (the CM was introduced to pUC19) and accuracy (the CM was introduced to pUC19 as designed) and 18 colonies from 3 parallel experiments (six samples of each) were sequenced (Figure S8b).

4.7 | Apply iCOPE in large DNA construct pATRTH modification

For deleting the Aro7 expression cassette, two spacers were designed as pATRTH-P1 (GTTATATATTCTGTGTAACCCGCCCC) and pATRTH-P2 (ACAAAAGCCTAGCTCATCTTTGGGGC). Here the iCOPE program was set up as following: 100 ng plasmid pATRTH, 6 μl in vitro transcription mix (New England Biolabs, MA), 100 ng short dsDNA (containing pATRTH-P1 and pATRTH-P2 for crRNA preparation), eight units of RNase inhibitor and 10 units of T4 DNA ligase in a total volume of 20 μl. After running of the standard incubation program, 10 μl of the reaction mix was transformed into DH10B, and 125 ± 19 colonies were obtained. To verify the cloning efficiency, 33 colonies from three parallel experiments (11 colonies for each) were validated by PCR with primer pATRTH-CKF and pATRTH-CKR. Then, 18 positive samples from PCR validation (six colonies of each) were sequenced for the accuracy confirmation.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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REFERENCES

Anders, C., Bargsten, K., & Jinek, M. (2016). Structural plasticity of PAM recognition by engineered variants of the RNA-guided endonuclease Cas9. *Molecular Cell*, *61*(6), 895–902. https://doi.org/10.1016/j.molcel.2016.02.020

Bayat, H., Modarressi, M. H., & Rahimpour, A. (2018). The conspicuity of CRISPR-Cpf1 system as a significant breakthrough in genome editing. *Current Microbiology*, *75*(1), 107–115. https://doi.org/10.1007/s00284-017-1406-8

Colloms, S. D., Merrick, C. A., Olorunniji, F. J., Stark, W. M., Smith, M. C. M., Osbourn, A., … Rosser, S. J. (2014). Rapid metabolic pathway assembly and modification using serine integrase site-specific recombination. *Nucleic Acids Research*, *42*(4), e23. https://doi.org/10.1093/nar/gkt1101

Enghiad, B., & Zhao, H. (2017). Programmable DNA-guided artificial restriction enzymes. *ACS Synthetic Biology*, *6*(5), 752–757. https://doi.org/10.1021/acssynbio.6b00324

Engler, C., Gruetzner, R., Kandzia, R., & Marillonnet, S. (2009). Golden gate shuffling: A one-pot DNA shuffling method based on type IIs restriction enzymes. *PLoS One*, *4*(5), e5553. https://doi.org/10.1371/journal.pone.0005553

Engler, C., Kandzia, R., & Marillonnet, S. (2008). A one pot, one step, precision cloning method with high throughput capability. *PLoS One*, *3*(11), e3647. https://doi.org/10.1371/journal.pone.0003647

Ferreira, R., David, F., & Nielsen, J. (2018). Advancing biotechnology with CRISPR/Cas9: Recent applications and patent landscape. *Journal of Industrial Microbiology and Biotechnology*, *45*(7), 467–480. https://doi.org/10.1007/s10295-017-2000-6

Fonfara, I., Richter, H., Bratovič, M., Le Rhun, A., & Charpentier, E. (2016). The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes

precursor CRISPR RNA. *Nature*, *532*(7600), 517–521. https://doi.org/10.1038/nature17945

Gao, L., Cox, D. B. T., Yan, W. X., Manteiga, J. C., Schneider, M. W., Yamano, T., … Zhang, F. (2017). Engineered Cpf1 variants with altered PAM specificities. *Nature Biotechnology*, *35*(8), 789–792. https://doi.org/10.1038/nbt.3900

Gao, P., Yang, H., Rajashankar, K. R., Huang, Z., & Patel, D. J. (2016). Type V CRISPR-Cas Cpf1 endonuclease employs a unique mechanism for crRNA-mediated target DNA recognition. *Cell Research*, *26*(8), 901–913. https://doi.org/10.1038/cr.2016.88

Gibson, D. G., Benders, G. A., Andrews-Pfannkoch, C., Denisova, E. A., Baden-Tillson, H., Zaveri, J., … Smith, H. O. (2008). Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. *Science*, *319*(5867), 1215–1220. https://doi.org/10.1126/science.1151721

Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., 3rd, & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, *6*(5), 343–345. https://doi.org/10.1038/nmeth.1318

Hammond, J. B., & Kruger, N. J. (1988). The bradford method for protein quantitation. *Methods in Molecular Biology*, *3*, 25–32. https://doi.org/10.1385/0-89603-126-8:25

Hille, F., Richter, H., Wong, S. P., Bratovič, M., Ressel, S., & Charpentier, E. (2018). The biology of CRISPR-Cas: Backward and forward. *Cell*, *172*(6), 1239–1259. https://doi.org/10.1016/j.cell.2017.11.032

Hirano, S., Nishimasu, H., Ishitani, R., & Nureki, O. (2016). Structural basis for the altered PAM specificities of engineered CRISPR-Cas9. *Molecular Cell*, *61*(6), 886–894. https://doi.org/10.1016/j.molcel.2016.02.018

Hu, J. H., Miller, S. M., Geurts, M. H., Tang, W., Chen, L., Sun, N., … Liu, D. R. (2018). Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature*, *556*(7699), 57–63. https://doi.org/10.1038/nature26155

Jiang, W., Zhao, X., Gabrieli, T., Lou, C., Ebenstein, Y., & Zhu, T. F. (2015). Cas9-Assisted Targeting of CHromosome segments CATCH enables one-step targeted cloning of large gene clusters. *Nature Communications*, *6*, 8101. https://doi.org/10.1038/ncomms9101

Jiang, Y., Qian, F., Yang, J., Liu, Y., Dong, F., Xu, C., … Yang, S. (2017). CRISPR-Cpf1 assisted genome editing of *Corynebacterium glutamicum*. *Nature Communications*, *8*, 15179. https://doi.org/10.1038/ncomms15179

Kang, H. S., Charlop-Powers, Z., & Brady, S. F. (2016). Multiplexed CRISPR/Cas9- and TAR-mediated promoter engineering of natural product biosynthetic gene clusters in yeast. *ACS Synthetic Biology*, *5*(9), 1002–1010. https://doi.org/10.1021/acssynbio.6b00080

Kim, H. K., Min, S., Song, M., Jung, S., Choi, J. W., Kim, Y., … Kim, H. (2018). Deep learning improves prediction of CRISPR-Cpf1 guide RNA activity. *Nature Biotechnology*, *36*(3), 239–241. https://doi.org/10.1038/nbt.4061

Kleinstiver, B. P., Prew, M. S., Tsai, S. Q., Topkar, V. V., Nguyen, N. T., Zheng, Z., … Joung, J. K. (2015). Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature*, *523*(7561), 481–485. https://doi.org/10.1038/nature14592

Labun, K., Montague, T. G., Gagnon, J. A., Thyme, S. B., & Valen, E. (2016). CHOPCHOP v2: A web tool for the next generation of CRISPR genome engineering. *Nucleic Acids Research*, *44*(W1), W272–W276. https://doi.org/10.1093/nar/gkw398

Leenay, R. T., Maksimchuk, K. R., Slotkowski, R. A., Agrawal, R. N., Gomaa, A. A., Briner, A. E., … Beisel, C. L. (2016). Identifying and visualizing functional PAM diversity across CRISPR-Cas systems. *Molecular Cell*, *62*(1), 137–147. https://doi.org/10.1016/j.molcel.2016.02.031

Lei, C., Li, S. Y., Liu, J. K., Zheng, X., Zhao, G. P., & Wang, J. (2017). The CCTL (Cpf1-assisted Cutting and Taq DNA ligase-assisted Ligation) method for efficient editing of large DNA constructs in vitro. *Nucleic Acids Research*, *45*(9), e74. https://doi.org/10.1093/nar/gkx018

Li, M. Z., & Elledge, S. J. (2012). SLIC: A method for sequence and ligation-independent cloning. *Methods in Molecular Biology*, *852*, 51–59. https://doi.org/10.1007/978-1-61779-564-0_5

Li, S. Y., Zhao, G. P., & Wang, J. (2016). C-Brick: A new standard for assembly of biological parts using Cpf1. *ACS Synthetic Biology*, *5*(12), 1383–1388. https://doi.org/10.1021/acssynbio.6b00114

Liu, Y., Tao, W., Wen, S., Li, Z., Yang, A., Deng, Z., & Sun, Y. (2015). In vitro CRISPR/Cas9 system for efficient targeted DNA editing. *mBio*, *6*(6), e01714–e01715. https://doi.org/10.1128/mBio.01714-15

Moreno-Mateos, M. A., Vejnar, C. E., Beaudoin, J. D., Fernandez, J. P., Mis, E. K., Khokha, M. K., & Giraldez, A. J. (2015). CRISPRscan: Designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. *Nature Methods*, *12*(10), 982–988. https://doi.org/10.1038/nmeth.3543

Nishimasu, H., Yamano, T., Gao, L., Zhang, F., Ishitani, R., & Nureki, O. (2017). Structural basis for the altered PAM recognition by engineered CRISPR-Cpf1. *Molecular Cell*, *67*(1), 139–147. e132. https://doi.org/10.1016/j.molcel.2017.04.019

Park, J., & Bae, S. (2018). Cpf1-Database: Web-based genome-wide guide RNA library design for gene knockout screens using CRISPR-Cpf1. *Bioinformatics*, *34*(6), 1077–1079. https://doi.org/10.1093/bioinformatics/btx695

Salomonsen, B., Mortensen, U. H., & Halkier, B. A. (2014). USER-derived cloning methods and their primer design. *Methods in Molecular Biology*, *1116*, 59–72. https://doi.org/10.1007/978-1-62703-764-8_5

Sander, J. D., Joung, J. K. (2014). CRISPR-Cas systems for genome editing, regulation and targeting. *Nature Biotechnology*, 32(4), 347-355.

Shao, Z., Zhao, H., & Zhao, H. (2009). DNA assembler, an in vivo genetic method for rapid construction of biochemical pathways. *Nucleic Acids Research*, *37*(2), e16. https://doi.org/10.1093/nar/gkn991

Shou, J., Li, J., Liu, Y., & Wu, Q. (2018). Precise and Predictable CRISPR Chromosomal Rearrangements Reveal Principles of Cas9-Mediated Nucleotide Insertion. *Molecular Cell*, *71*(4), 498–509. e494. https://doi.org/10.1016/j.molcel.2018.06.021

Tang, X., Lowder, L. G., Zhang, T., Malzahn, A. A., Zheng, X., Voytas, D. F., … Qi, Y. (2017). A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. *Nat Plants*, *3*, 17018. https://doi.org/10.1038/nplants.2017.18

Tu, M., Lin, L., Cheng, Y., He, X., Sun, H., Xie, H., … Gu, F. (2017). A 'new lease of life': FnCpf1 possesses DNA cleavage activity for genome editing in human cells. *Nucleic Acids Research*, *45*(19), 11295–11304. https://doi.org/10.1093/nar/gkx783

Wang, H., Li, Z., Jia, R., Yin, J., Li, A., Xia, L., … Zhang, Y. (2018). ExoCET: Exonuclease in vitro assembly combined with RecET recombination for highly efficient direct DNA cloning from complex genomes. *Nucleic Acids Research*, *46*(5), 2697–2697. https://doi.org/10.1093/nar/gkx1296

Wang, W., & Malcolm, B. A. (1999). Two-stage PCR protocol allowing introduction of multiple mutations, deletions, and insertions using QuikChange site-directed mutagenesis. *Biotechniques*, *26*(4), 680–682. https://doi.org/10.2144/99264st03

Werner, S., Engler, C., Weber, E., Gruetzner, R., & Marillonnet, S. (2012). Fast track assembly of multigene constructs using Golden Gate

cloning and the MoClo system. *Bioengineered Bugs*, *3*(1), 38–43. https://doi.org/10.1371/journal.pone.0016765

Xie, S., Shen, B., Zhang, C., Huang, X., & Zhang, Y. (2014). sgRNAcas9: A software package for designing CRISPR sgRNA and evaluating potential off-target cleavage sites. *PLoS One*, *9*(6), e100448. https://doi.org/10.1371/journal.pone.0100448

Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S., Essletzbichler, P., … Zhang, F. (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell*, *163*(3), 759–771. https://doi.org/10.1016/j.cell.2015.09.038

Zhang, L., Zhao, G., & Ding, X. (2011). Tandem assembly of the epothilone biosynthetic gene cluster by in vitro site-specific recombination. *Scientific Reports*, *1*, 141. https://doi.org/10.1038/srep00141

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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