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Abstract
Gene splicing by fusion PCR is a versatile and widely used methodology, especially in synthetic biology. We here describe a rapid method for splicing two fragments by one-round fusion PCR with a dual-asymmetric primers and two-step annealing (ODT) method. During the process, the asymmetric intermediate fragments were generated in the early stage. Thereafter, they were hybridized in the subsequent cycles to serve as template for the target full-length product. The process parameters such as primer ratio, elongation temperature and cycle numbers were optimized. In addition, the fusion products produced with this method were successfully applied in seamless genome editing. The fusion of two fragments by this method takes less than 0.5 day. The method is expected to facilitate various kinds of complex genetic engineering projects with enhanced efficiency.

Keywords: Fusion PCR, Dual-asymmetric primers, Two-step annealing

Introduction

Splicing DNA fragments are enormously useful in genetic engineering, as they can be used to investigate element functions, edit genomes and generate new genes [1, 2]. The construction of such recombinant products usually involves using conventional restriction enzyme cloning end adapters, which sometimes cannot be done without availability of restriction sites, or requires introduction of unwanted sequences into the

junction. Fusion PCR was developed to solve these problems by gene fusion using the polymerase chain reaction (PCR) at any chosen location without introducing any extra base pairs [3]. This technology has been improved and developed for multiple applications thereafter [4–6]. However, the use of traditional fusion PCR can be tedious and time-consuming [7]. It not only calls for three PCRs to facilitate fusion of two fragments, but also requires complicated operational conditions such as adjustment of PCR conditions and cleanliness of the initial templates [8].

It has been reported that dual-asymmetric PCR could facilitate construction of synthetic genes [9]. In this system, the asymmetric primers will lead to asymmetric amplification of intermediate products. In the following PCR cycles, the dual asymmetrically amplified fragments can overlap and serve as templates for the full-length product. Small-sized products (200–350 bp) were produced with fixed protocols in the published research. Besides, a GC-rich adapter was reported to enhance fusion PCR efficiency as it could increase the intermediate fragments annealing efficiency [10]. Based on the two reports above, we developed and optimized a simple and efficient fusion method based on dual-asymmetric primers and two-step annealing (ODT) PCR without introducing adapter sequences.

The design principle is shown in Fig. 1. Dual-asymmetric primers and two templates were used in one PCR system. There will be three reaction types in one PCR system. At the preliminary stage, the first reaction is the main reaction, which will produce asymmetrically amplified fragments. In the second reaction, the dual-asymmetric amplified fragments overlap each other in both annealing step and elongation step, which will supply templates for the full-length product. In the third reaction, intact product will be produced with primers P1 and P4 and used as template. As the reaction progresses through more cycles, the concentration of inner primers decreases, and the third reaction becomes dominant. Over time, the intact fragment will be the major product in the PCR system. The final intact fragments can be cloned into vector via Gibson assembly [11], Slic [12] or Blunt cloning [13] for conservation and subsequent application.

Materials and Methods

Primers, Plasmids and E. coli Strains

Primers for fusion PCR used in this study were designed with Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Table 1. Two flanking primers P1 and P4 were designed as normally. The inner primers P2 or P3 should have a tail containing a complementary overlap fragment with the 3′-terminal of the other fragment (Fig. 1 and Table 1). The high GC content of the overlap is beneficial for the fusion of two fragments A and B. Therefore, primer P2 would be designed as normal if fragment A has a relative higher GC content in its 3′-terminal, while primer P3 has an overlap tail and vice versa. The primers used for conservation and application of fusion products are listed in Table S1.

In this study, three different gene fusions were constructed based on plasmids and strains used as templates listed in Table 2. For the first fusion, eutC tag cassette of genomic DNA of *E. coli* strain K-12 MG1655 and GFP cassette of plasmid DNA of pLei-sfGFP were used as temples and the fusion fragment named as EG. For the second fusion, *kan* cassette and *sacB* cassette from the same genomic DNA of MGL1 M were used, named as KS [14]. For the third fusion, the temples for FAR (Fatty acyl- CoA reductase) and *cat–sacB* are from genomic DNA of MGFARINS and MGKA, respectively, named as FS [7]. Plasmids pMD™18-T Vector, pZS13S, pEASY-Blunt were used to conserve the three fusion fragments EG, KS and FS. Plasmid templates (1 pg–1 ng) or genomic templates (1 ng–1 ug) were used for fusion PCR. Overnight cultured colonies were directly used as template for the robust test of the ODT.

Fusion PCR Conditions

For all PCR producers, the system included: 1 µl KOD Hot Start DNA polymerase (Novagen, 71086-3), 5 µl 10 × Buffer, 3 µl 25 mM $MgSO_4$, 5 µl dNTPs (2 mM each), 2 µl primer mix, equal molar amounts of templates (around 4×10^{-6} pmol), add water to 50 µl. The PCR cycles comprised an initial denaturation step at 94 °C for 2 min, followed by 30–46 cycles at 94 °C for 20 s. Annealing was conducted at 58, 55 and 55 °C for EG, KS and FS, respectively, at 20-s duration. Finally, elongation was done at 64–72 °C, for EG, KS and FS at 1-, 3- and 4-min duration, respectively, due to their sequence lengths. Thermo Fisher Scientific T_m Calculator was used for T_m calculation.

Primer ratio is a critical factor for the successful ODT. The prepared 10 µM solution of these four primers were mixed at a ratio and used in the PCR system. The primer ratios of P1:P2:P3:P4 of 1:1:1:1, 5:1:1:5, 10:1:1:10, 20:1:1:20, 40:1:1:40, and 60:1:1:60 were investigated to optimize the fusion products. Elongation temperature is another key factor for the ODT, because the fragment A and B annealing at this step will benefit the accumulation of the intact fragment AB (Fig. 1). Therefore, the elongation temperatures of 72, 70, 68, 66 and 64 °C were tested for all three fusions. In addition, cycles' number of 30, 34, 38, 42 and 46 were examined to optimize the fusion PCR.

Analysis of PCR Products

The fragment size distribution was determined using 1-kb ladder (Promega, Madison, WI) or 100-bp ladder as molecular weight markers. The images were acquired by Bio-Rad Gel Doc XR + Imaging System and quantitated using Bio-Rad Image Lab Software. For quantification analysis, the marker band, which is clear and totally separated from other bands, was chosen as reference. The band of 500 bp was used as reference for EG and 2000 bp for KS and FS. The products were confirmed by sequencing.

Genome Editing

The fusion fragments were cloned to vectors for conservation. Slic cloning (sequence and ligase independent cloning) was used to clone EG fragment into vector pZS13S resulted in pZS–EG [12]. Gibson assembly kit (NEB, E2611S) was used to clone KS into the pMD18-T vector resulted in pMD–KS [11]. Gibson assembly method is analogous to Slic cloning, except that it uses a dedicated exonuclease and a ligase to seal the single-stranded nicks. Blunt cloning kit (TransGen, CB101) was employed to clone FS into pEASYBlunt vector resulted in pEASY–FS [13]. The constructed pMD–KS was used for seamless deletion of *poxB* in *E. coli* strain MGL1 (Fig. 4) according to our developed method [7]. The *kan–sacB* cassette was amplified with M13 primers (Table S1), and then, the products were digested with Dpn I and purified. First, fragments containing *kan–sacB* flanked by tandem repeats were constructed with primers (Table S1). It was introduced into the target site via intermolecular homologous recombination assisted by lambda red enzymes (pKD46). Then, recombinants were selected from Luria–Bertani (LB) agar plate containing kanamycin (30 mg/L). Seamless excision of the selectable marker was achieved using sucrose [7].

The constructed pEASY–FS was used for substitution of *araBAD* (arabinose degradation cluster) with *FAR* in *E. coli* strain MGL2 according to the "pop-in/pop-out" genome editing method [15]. The FS cassette was amplified with M13 primers (Table S1), and the products were digested with DpnI and purified. Subsequently, fragments containing FS and homologous ends flanking *araBAD* were constructed with primers FSSBADP1/FSSBADP2 (Table S1). The prepared fragment was introduced into the target site via intermolecular homologous recombination assisted by lambda red enzymes (pKD46). Then, recombinants were selected from Luria–Bertani (LB) agar plate containing kanamycin (30 mg/L). Seamless excision of the selectable marker was achieved using fusion products of upstream and downstream fragments of *araBAD*.

Results and Discussion

Optimization of Primer Ratio

According to the design principle, primer ratio is crucial for the feasibility of ODT. The primer ratios of P1:P2:P3:P4 of 1:1:1:1, 5:1:1:5, 10:1:1:10, 20:1:1:20, 40:1:1:40 and 60:1:1:60 were tested in triple fusion PCR for EG, KS and FS (Fig. 2a–c, respectively). As shown in Fig. 2a–c, it is obvious that the intact segments increased first and decreased as the primer ratio increased from 1:1:1:1 to 60:1:1:60. When the ratio of flank to inner primers was low, it is beneficial to the intermediate fragment formation, which was verified by the decreasing intermediate fragment concentrations combined with the increasing flank to inner primer ratio. As the amount of primers in the PCR system was fixed, the concentration of inner primers

was reduced when the ratio of flank to inner primers increased, which favored the amplification of intact fragment. However, the formation of intact fragment was based on fusion of the two intermediate fragments. When the ratio of flank to inner primers increased too much, the concentrations of inner primers were too low to produce enough intermediate fragments to form the intact fragment, which also resulted in a low yield of intact fragment.

In order to find out the proper ratio of flank to inner primers, we built the fitting functions based on each (y) relative intensity of resulted intact product and (x) its ratio of flank to inner primers. The fitting curves and expressions are shown in Fig. 2d. Similar trend lines were observed in three different fusions, and the best ratio of flank to inner primers for the maximum intensity of fusion products was calculated by the parabola vertex formula $(x, y) = (- b/2a, (4ac - b_2)/4a)$. For the three parameter fitting curves, similar ratios of flank to inner primers ($X_{EG} = 31.03$, $X_{KS} = 30.65$, $X_{FS} = 31.88$) were found for the maximum relative intensity of fusion products (Table S2). Therefore, the primer ratio 30:1:1:30 of P1:P2:P3:P4 is recommended for a higher intact fragment yield.

Optimization of Elongation Temperature

To further reduce the portion of intermediate fragments and enhance the formation of intact fusion products, the elongation temperature was optimized. Figure 3a–c shows the impacts of elongation temperature ranged from 72 to 64 °C on the intact fusion products of EG, KS and FS, respectively. According to the design principle, it is believed that there are two ways to enhance the production of the intact fusion products. Endogenous overlapping fragments with higher T_m were the first way, which is suitable for fusion of fragments containing an ideal end with high GC concentration. The second way was to reduce the elongation temperature, taking it as the second annealing step. This two-step annealing was used for fusion of fragments without a GC-rich ideal end.

As shown in Fig. 3a, for EG fusion, with overlap fragment having a relative high GC concentration (Table 1), the normal elongation temperature was acceptable for the fusion reactions. The decrease in the elongation temperature did not enhance the accumulation of the intact fragment. In fact, with the decrease in temperature, the concentration of the intact fragment decreased as a result of reduced polymerase activity [16]. For KS, the situation was different (Fig. 3b); with decrease in elongation temperature, the concentration of the intact fragment increased firstly and then decreased slightly. That is because the decrease in elongation temperature was beneficial for the annealing of intermediate fragments in the elongation step but was averse to the polymerase activity, which in addition happened in the FS fusion (Fig. 3c). The calculated T_m of the overlapping end for the three fusions were T_{mEG} 88.7 °C, T_{mFS} 82.5 °C and T_{mKS} 74.7 °C for EG, FS and KS, respectively. However, the best elongation temperature for each case was chosen as T_{EG} 72 °C, T_{FS} 70 °C and T_{KS} 68 °C, respectively. With these optimized elongation temperatures (T_{EG} 72 °C, T_{FS}

70 °C, T_{KS} 68 °C) and flank to inner primer ratio (30:1), the intact fusion products showed excellent amplifications, while the amounts of intermediate fragments dropped significantly compared with Fig. 2a–c.

Optimization of Cycles' Number

Figure 3d–f shows the effects of cycles' number from 30 to 46 on the formation of intact fusion products of EG, KS and FS, respectively. The overall trend for triple fusion PCR is that the increase in cycles' number increases the intact fragment production and reduces the intermediate fragment accumulation. The intact fragment concentration increased gradually as the cycles' number increased from 30 to 42. However, when the cycles' number increased from 42 to 46, the intact fragment concentration did not increase. Besides the triple fusion cases, the intermediate fragment concentration decreased gradually as the number of cycles increased. All the intermediate fragments nearly disappeared when the cycles' number increased to 42. Therefore, the recommended cycles' number is 42 for the developed method. As to the protocol of the commercial enzyme KOD, this is acceptable for the amplification accuracy [16]. In addition, overnight cultured colonies were directly used as templates to simplify the method, which was successfully carried out with this optimized ODT (Fig. S1).

Genome Editing with Fusion Fragments Generated by ODT

To conserve and enlarge the prepared fragments, three of the most applied cloning methods were tested. EG fragment was cloned into vector pZS13S resulting in pZS–EG. Colony PCR was conducted to test the efficiency showing that 18 out of 20 colonies had the right insertion, accounting for 90%. KS fragment was cloned into the pMD18-T vector. Among the 20 tested colonies, 90% contained the target insert. Blunt cloning was applied to clone the FS fragment prior cloned into pEASY-Blunt vector. Colony PCR test was conducted with 20 colonies too and only 5 colonies had the right insertion, accounting for 25% of the cloning efficiency. For both Slic and Gibson method, the amplified vector parts include overlap regions homologous to intact fragment ends, which can exclude the possibility of ligation between plasmid and intermediate fragment parts, while for blunt cloning, all double-stranded fragments in the products, including the intermediate fragments, can be ligated into the vector. Since trace amounts of intermediate fragments are unavoidable with ODT, Slic and Gibson assembly is better choices for the subcloning of the fusion fragments.

The constructed plasmids were applied in the following experiments: The constructed plasmid pZS–EG was transformed in *E. coli* NEB 5-alpha resulting in successful GFP protein expression (Fig. S2). The constructed pMD–KS was successfully used for several seamless gene deletions, including *poxB* (Fig. 4), in *E. coli* strain MGL1 according to our developed seamless genome editing method [7]. The FS fragment was used to

substitute *araBAD* cluster with FAR gene in a recombinant strain MGL2 resulting in strain MGL4 (Table 2). The developed ODT method is helpful for many genetic engineering experiments.

Traditional fusion PCR for splicing DNA fragments was developed 3 decades ago, and then modified and used for variable applications [3–6]. However, the existing method needs three PCRs and sometimes a GC-rich adapter. The novelty of the ODT method resides in two aspects: Firstly, it allows fusing two fragments with only one PCR, and secondly, the fusion efficiency can be secured by two-step annealing. Thus, ODT is a fast and efficient method to generate recombinant DNA products.

We used the ODT method to fuse fragments with templates from sources, including genomic DNA of different origin and plasmid DNA. The ODT method proved feasible in all selected situations, and it is easy to perform on modern PCR machines. Moreover, the robustness of the ODT method was verified by colony PCR, which avoided DNA extraction, separate cloning steps and gel purification. The optimized ODT method saves more than 2/3 of time and costs less than half a day compared with existing fusion methods. However, there are two limits for the utilization of ODT method. Firstly, it cannot realize site-directed mutagenesis as the flanking primers will mainly produce the wild type fragment. Secondly, it needs a spacer (10 Kb) between the two fragments if they are on the same genome.

Conclusion

This rapid fusion method ODT leaves out the intermediate steps and maximizes the efficiency of fusion PCR. This new strategy was proved to be rapid and an efficient fusion PCR method. Its efficiency and simplicity might make it a valuable strategy for generating chimeric fragments in genetic engineering projects.
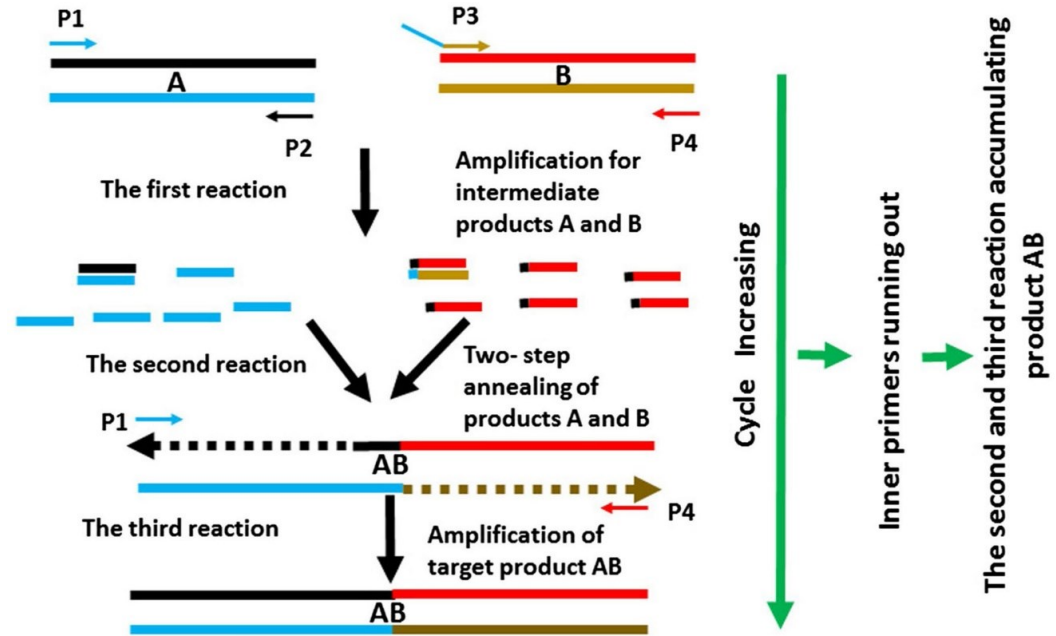
Fig. 1. Schematic diagram of one-step fusion PCR based on dual asymmetric primers and two-step annealing (ODT). The first reaction: primers P1 and P2 produced intermediate fragment A, and primers P3 and P4 produced intermediate fragment B. The second reaction: fragments A and B annealed by their overlap region. The third reaction: primers P1 and P4 produced the intact fusion product AB. The three reactions almost happen at the same time in the PCR system. However, at the preliminary stage, the first reaction was the major reaction due to the sufficient inner primers P2 and P3. As the PCR cycle increasing, concentrations of P2 and P3 primers decreased and annealed AB product increased, the third reaction became the major reaction and formed more intact fragment AB
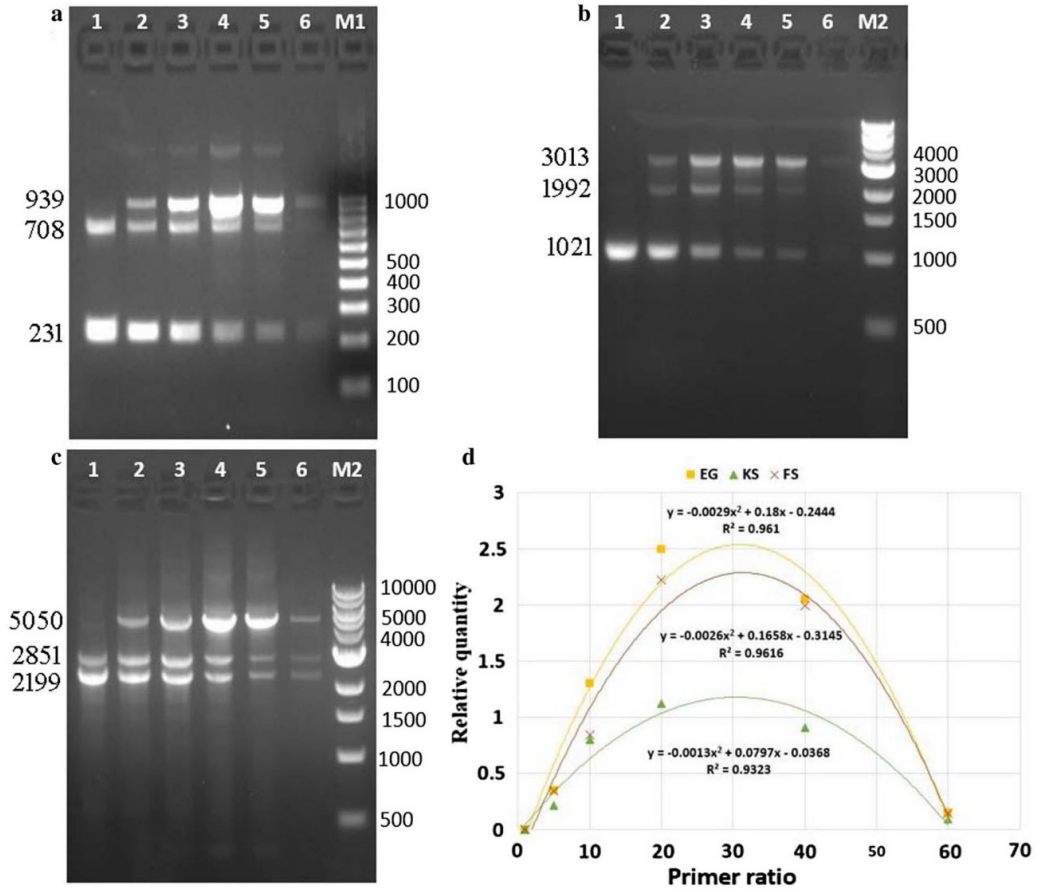
Fig. 2. Amplification results of primer ratio optimization for ODT method. **a–c** Optimization of primer ratio for EG, KS and FS, respectively. Lane 1 to lane 6, P1:P2:P3:P4, 1:1:1:1, 5:1:1:5, 10:1:1:10, 20:1:1:20, 40:1:1:40, 60:1:1:60. **d** Fitting curves and expressions of (y) relative intensity of intact product and (x) ratio of flank to inner primers. The parabola vertex formula $(x, y) = (-b/2a, (4ac - b^2)/4a)$ was used to calculate the best ratio (x) and maximum intensity (y).
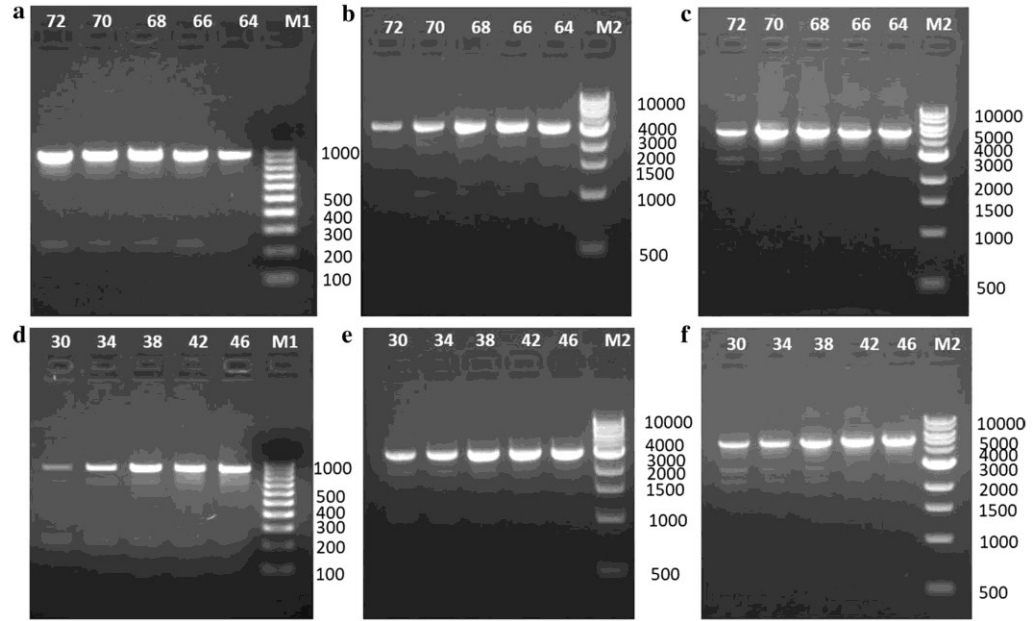
Fig. 3. Amplification results of elongation temperatures (**a**–**c**) and total cycles' number (**d**–**f**) optimization for ODT method. **a**, **d** EG; **b**, **e** KS; **c**, **f** FS. The elongation temperatures and cycles' number were marked on the top of each lane.
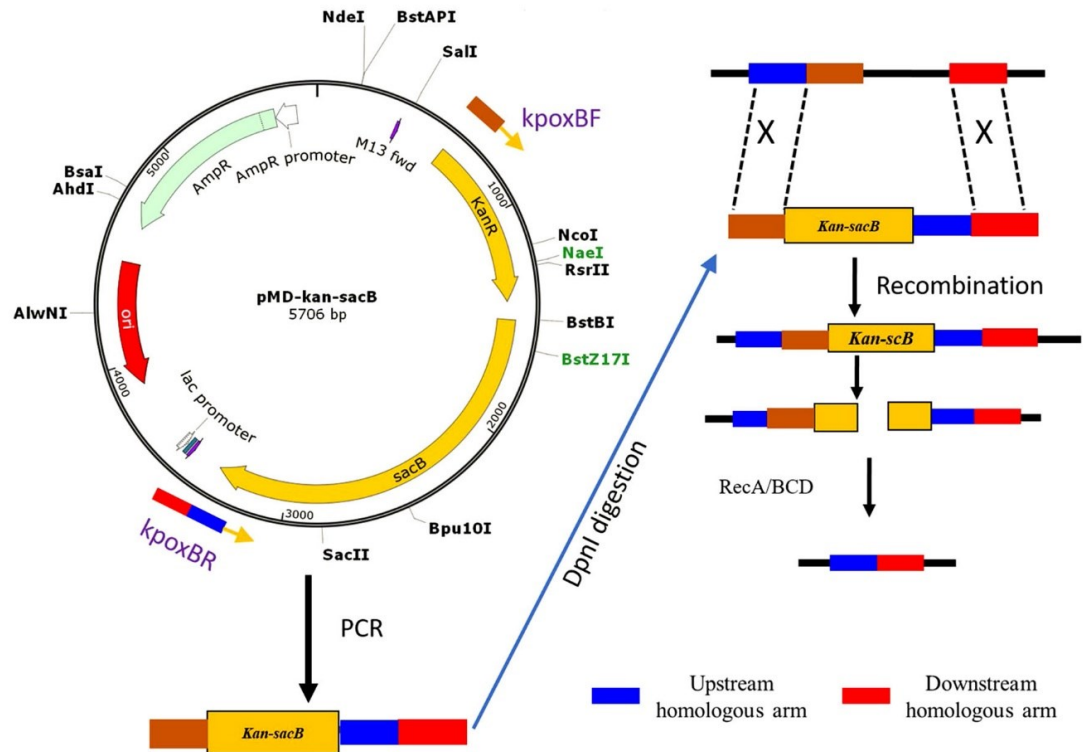

Fig. 4. Schematic process of seamless deletion of *poxB* from *E. coli* strain MGL1 assisted by the fusion fragment KS.

Conflict of interest — The authors declare that they have no conflicts of interest.

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Table 1. Primers used for fusion PCR

Primers	Sequences	Application
EGP1	ATG GAT CAA AAA CAG ATT GAA GAA A	EG construction
EGP2	GCG GGC CAC GGT GCT	EG construction
EGP3	**GCG CCG CAG CAC CGT GGC CCGC** ATG TCC AAG GGC GAA GAG C	EG construction
EGP4	TTA TTC GTC CAT ACC GTG AGT GAT	EG construction
KSP1	TTG CAG TGG GCT TAC ATG G	KS construction
KSP2	**AGG CGT TTA GGC GCA CCA ATA ACT GCC TTA** TCA GAA GAA CTC GTC AAG AAGGC	KS construction
KSP3	TAA GGC AGT TAT TGG TGC CCT TAA AC	KS construction
KSP4	ATA GAT ACA TCA GAG CTT TTA CGA G	KS construction
FSAP1	ATG GCA ATA CAG CAG GTA CAT CAC G	FS construction
FSAP2	**GGG CTT CCC GGT ATC AAC AGG GAC ACC AGG A**TCA GGC AGC TTT TTT GCG CTG	FS construction
FSAP3	TCC TGG TGT CCC TGT TGA TA	FS construction
FSAP4	ATA GAT ACA TCA GAG CTT TTA CGA G	FS construction

The bold part in the primers is the overlapping part for fusion; the melting temperatures for the overlapping region: T_{mEG} (88.7 °C), T_{mKS} (74.7 °C), T_{mFS} (82.5 °C)

Table 2. Plasmids and strains used and constructed in this study

Plasmids/strains	Relevant characteristics	Reference/source
Plasmids		
pLei-sfGFP	sfGFP expression vector	Lab collection
pMD™18-T vector	T-easy vector	Takara, code: D10A
pZS13S	Expression vector	Lab collection
pEASY-Blunt	Blunt T-easy vector	TransGen Biotech CB101
pZS–EG	pZS13S containing EG fragment	This study
pMD–KS	T-easy vector with *kan–sacB* cassette	This study
pEASY–FS	T-easy vector with FS fragment	This study
Strains		
E. coli K-12 MG1655	Wild type *E. coli*	Lab collection
MGFARINS	MG ldhA::FAR	[7]
MGKA	MGK ΔtesA:: tesA 3'50-Chi-cat-sacB	[7]
MGL1 M	*E. coli* K-12 MG1655 Δ*tesC* Δ*tesB* Δ*ldhA::kan* Δ*pta* Δ*ackA::cat-sacB*	[14]
MGL1	*E. coli* K-12 MG1655 Δ*tesC* Δ*tesB* Δ*ldhA::kan* Δ*pta* Δ*ackA*	[14]
MGL2	*E. coli* K-12 MG1655 Δ*tesC* Δ*tesB* Δ*ldhA::FRT* Δ*pta* Δ*ackA* Δ*poxB*	This study
MGL3	*E. coli* K-12 MG1655 Δ*tesC* Δ*tesB* Δ*ldhA::FRT* Δ*pta* Δ*ackA* Δ*poxB* Δ*araBAD::FS*	This study
MGL4	*E. coli* K-12 MG1655 Δ*tesC* Δ*tesB* Δ*ldhA::FRT* Δ*pta* Δ*ackA* Δ*poxB* Δ*araBAD::FAR*	This study

Table S1 Primers used for conservation and application of fusion products.

Primers	Sequence	Application
pZSEGP1	CTGTTTTTGATCCATGTCGACTTCTCCTCTTGCTAGC	Slic cloning for EG
pZSEGP2	GGTATGGACGAATAAGGTCGACTCTAGAAAGCTTCC	Slic cloning for EG
pMDKSP1	GTAAGCCCACTGCAAATCGTCGACCTGCAGGCA	Gibbson assembly for KS
pMDKSP2	CTCTGATGTATCTATTATCTCTAGAGGATCCCCGGGT	Gibson assembly for KS
M13fwd	GTAAAACGACGGCCAGT	Template preparation
M13rev	CAGGAAACAGCTATGAC	Template preparation
kpoxBF:	**GGTAAAGAACATGTCGAATACGATAATCCGTATGATGTTGGAATGACCGG**TTGCAGTGGGCTTACATGG	poxB deletion
kpoxBR	CGTAAATCAATCATGGCATGTCCTTATTATGACGGGAAATGCCACCCTTTGGTTCTCCATCTCCTGAATGTGATAACGGTAACAAGTTTAGTTCATCTGAATAGATACATCAGAGCTTTTACGAG	poxB deletion
FSSBADP1	**GCAACTCTCTACTGTTTCTCCATACCCGTTTTTTTGGATGGAGTGAAACG**ATGGCAATACAGCAGGTACATCACG	Ara BAD substitution
FSSBADP2	**GCTTGAGTATAGCCTGGTTTCGTTTGATTGGCTGTGGTTTTATACAGTCA**ATAGATACATCAGAGCTTTTACGAG	Ara BAD substitution
FSSBADP3	ATTGCCGTCACTGCGTCTT	Up and down stream fusion
FSSBADP4	CGTTTCACTCCATCCAAAAAAAC	Up and down stream fusion
FSSBADP5	CTCCATACCCGTTTTTTTGGATGGAGTGAAACGTGACTGTATAAAACCACAGCCAATC	Up and down stream fusion
FSSBADP6	GATCGGCGGGAATAAACG	Up and down stream fusion

Note: the yellow-highlightened parts of pZSEGP1and pZSEGP2 are overlapping ends for Slic cloning; the green-highlightened parts are of pMDKSP1 and pMDKSP2 overlapping ends for Gibson assembly cloning.The bold parts of the primer kpoxBF are homologous fragment in the target gene; the green-shaded part of kpoxBR to is homologous to the 3' end of *poxB*; The underlined parts of the primer kpoxBR is homologous to the 5' end of poxB; the yellow-shaded parts of kpoxBF and kpoxBR are homologous to *kan-sacB* cassette; the bold part of the primer FSSBADP1 is homologous to the 5' end of araBAD; the green-shaded part of kpoxBR is homologous to the 3' end of *poxB;* the yellow-shaded parts of FSSBADP1 and FSSBADP2 are homologous to the constructed FS cassette. The red part of primer FSSBADP5 is the overlap sequence for fusion PCR

Table S2 Calculations of the vertexes for the ratio of flank to inner primers and relative intensity value of resulted intact product.

Fusion fragments	Fitting curves	a, b, c values in the fitting curves	Vertex values $X=-b/2a$, $Y=(4ac-b^2)/4a$
EG	$Y=-0.0029X^2+0.18X-0.2444$	-0.0029, 0.18, -0.2444	**X=31.03**; Y=2.55
KS	$Y=-0.0013X^2+0.0797X-0.0368$	-0.0013, 0.0797, -0.0368	**X=30.65**; Y=1.18
FS	$Y=-0.0026X^2+0.1658X-0.3145$	-0.0026, 0.1658, -0.3145	**X=31.88**; Y=2.33

Note: X is the ratio of flank primer: inner primer; Y is the relative intensity value of resulted intact fusion product.

Table S3 Sequence information for the fusion fragments of EG, KS and FS.

Fragment	Length (bp)	full sequence
eutC tag for EG	231	ATGGATCAAAAACAGATTGAAGAAATTGTACGCAGCGTGATGGCGTCAATGGGACAAGCGGCCCCCGCGCCGTCAGAAGCAAAGTGCGCCACCACCAACTGTGCGGCACCGGTGACCTCGGAAAGCTGTGCGCTGGATTTAGGTTCCGCTGAAGCAAAAGCGTGGATTGGTGTTGAAAATCCGCATCGCGCAGACGTATTAACAGAACTGCGCCGCAGCACCGTGGCCCGC
GFP for EG	708	ATGTCCAAGGGCGAAGAGCTCTTCACCGGAGTTGTTCCAATCCTCGTAGAACTGGACGGTGATGTTAACGGCCACAAATTTTCTGTGCGTGGTGAGGGCGAAGGTGACGCTACCAACGGCAAACTGACTCTGAAATTTATCTGCACCACCGGCAAACTGCCGGTTCCGTGGCCGACCCTGGTAACTACCCTCACTTATGGTGTCCAGTGCTTCTCTCGTTACCCCGACCACATGAAACGTCACGACTTCTTCAAAAGCGCTATGCCGGAGGGTTACGTTCAGGAACGTACGATTAGCTTCAAGGATGACGGTACCTACAAAACTCGTGCGGAAGTTAAATTCGAGGGAGACACCCTGGTGAACCGTATCGAACTGAAAGGTATTGACTTCAAGGAGGATGGCAATATCCTGGGTCACAAGCTGGAGTATAACTTCAATAGCCACAACGTGTACATCACTGCTGATAAACAGAAAAACGGTATCAAGGCTAACTTCAAAATTCGCCACAACGTAGAAGACGGCTCCGTTCAGCTGGCTGACCACTACCAGCAGAACACCCCGATCGGCGACGGTCCAGTACTGCTGCCTGACAACCACTATCTGTCCACGCAGTCCGTTCTGTCTAAAGACCCGAACGAGAAACGCGATCACATGGTTCTGCTGGAATTCGTTACTGCTGCTGGCATCACTCACGGTATGGACGAATAA
kan for KS	1021	TTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCAAGCGAACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGATAAGGCAGTTATTGGTGCCCTTAAACGCCT
sacB for KS	1992	GGTGCTACGCCTGAATAAGTGATAATAAGCGGATGAATGGCAGAAATTCGAAAGCAAATTCGACCCGGTCGTCGGTTCAGGGCAGGGTCGTTAAATAGCCGCTAGATCTAAGTAAATCGCGCGGGTTTGTTACTGATAAAGCAGGCAAGACCTAAAATGTGTAAAGGGCAAAGTGTATACTTTGGCGTCACCCCTTACATATTTTAGGTCTTTTTTTATTGTGCGTAACTAACTTGCCATCTTCAAACAGGAGGGCTGGAAGAAGCAGACCGCTAACACAGTACATAAAAAAGGAGACATGAACGATGAACATCAAAAAGTTTGCAAAACAAGCAACAGTATTAACCTTTACTACCGCACTGCTGGCAGGAGGCGCAACTCAAGCGTTTGCGAAAGAAACGAACCAAAAGCCATATAAGGAAACATACGGCATTTCCCATATTACACGCCATGATATGCTGCAAATCCCTGAACAGCAAAAAAATGAAAAATATCAAGTTCCTGAATTCGATTCGTCCACAATTAAAAATATCTCTTCTGC

		AAAAGGCCTGGACGTTTGGGACAGCTGGCCATTACAAAACGCTGACGGCACTGTCGCAAACTATCACGGCTACCACATCGTCTTTGCATTAGCCGGAGATCCTAAAAATGCGGATGACACATCGATTTACATGTTCTATCAAAAAGTCGGCGAAACTTCTATTGACAGCTGGAAAAACGCTGGCCGCGTCTTTAAAGACAGCGACAAATTCGATGCAAATGATTCTATCCTAAAAGACCCAAACACAAGAATGGTCAGGTTCAGCCACATTTACATCTGACGGAAAAATCCGTTTATTCTACACTGATTTCTCCGGTAAACATTACGGCAAACAAACACTGACAACTGCACAAGTTAACGTATCAGCATCAGACAGCTCTTTGAACATCAACGGTGTAGAGGATTATAAATCAATCTTTGACGGTGACGGAAAAACGTATCAAAATGTACAGCAGTTCATCGATGAAGGCAACTACAGCTCAGGCGACAACCATACGCTGAGAGATCCTCACTACGTAGAAGATAAAGGCCACAAATACTTAGTATTTGAAGCAAACACTGGAACTGAAGATGGCTACCAAGGCGAAGAATCTTTATTTAACAAAGCATACTATGGCAAAAGCACATCATTCTTCCGTCAAGAAAGTCAAAAACTTCTGCAAAGCGATAAAAAACGCACGGCTGAGTTAGCAAACGGCGCTCTCGGTATGATTGAGCTAAACGATGATTACACACTGAAAAAAGTGATGAAACCGCTGATTGCATCTAACACAGTAACAGATGAAATTGAACGCGCGAACGTCTTTAAAATGAACGGCAAATGGTACCTGTTCACTGACTCCCGCGGATCAAAAATGACGATTGACGGCATTACGTCTAACGATATTTACATGCTTGGTTATGTTTCTAATTCTTTAACTGGCCCATACAAGCCGCTGAACAAAACTGGCCTTGTGTTAAAAATGGATCTTGATCCTAACGATGTAACCTTTACTTACTCACACTTCGCTGTACCTCAAGCGAAAGGAAACAATGTCGTGATTACAAGCTATATGACAAACAGAGGATTCTACGCAGACAAACAATCAACGTTTGCGCCAAGCTTCCTGCTGAACATCAAAGGCAAGAAAACATCTGTTGTCAAAGACAGCATCCTTGAACAAGGACAATTAACAGTTAACAAATAAAAACGCAAAGAAAATGCCGATATTGACTACCGGAAGCAGTGTGACCGTGTGCTTCTCAAATGCCTGATTCAGGCTGTCTATGTGTGACTGTTGAGCTGTAACAAGTTGTCTCAGGTGTTCAATTTCATGTTCTAGTTGCTTTGTTTTACTGGTTTCACCTGTTCTATTAGGTGTTACATGCTGTTCATCTGTTACATTGTCGATCTGTTCATGGTGAACAGCTTTAAATGCACCAAAAACTCGTAAAAGCTCTGATGTATCTAT
FAR for FS	2199	ATGAGCTGTTGACAATTAATCATCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCGCCGCTGAGAAAAAGCGAAGCGGCACTGCTCTTTAACAATTTATCAGACAATCTGTGTGGGCACTCGACCGGAATTATCGATTAACTTTATTATTAAAAATTAAAGAGGTATATATTAATGTATCGATTAAATAAGGAGGAATAAACCATGGGGGGTTCTCATCATCATCATCATCATGGTATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATCTGTACGACGATGACGATAAGGATCGATGGGGATCCATGGCAATACAGCAGGTACATCACGCTGACACTTCATCATCAAAGGTGCTCGGACAGCTCCGTGGCAAGCGGGTTCTGATCACCGGTACCACTGGCTTTCTGGGCAAGGTGGTCCTCGAAAGGCTGATTCGGGCGGTGCCTGATATCGGCGCAATTTACCTGCTGATCCGGGGCAATAAACGGCATCCGGATGCTCGTTCCCGTTTCCTGGAAGAAATTGCCACCTCCTCGGTGTTTGACCGTCTTCGCGAGGCCGATTCAGAGGGATTTGACGCCTTTCTGGAAGAGCGCATTCACTGCGTGACCGGTGAGGTGACCGAAGCGGGTTTCGGGATAGGGCAGGAAGACTATCGCAAACTCGCCACCGAACTGGATGCGGTGATCAACTCCGCTGCAAGCGTGAATTTCCGTGAAGAGCTCGACAAGGCGCTGGCCATCAACACCCTGTGCCTTCGGAATATTGCCGGCATGGTGGATTTGAATCCGAAGCTTGCGGTCCTGCAGGTCTCCACCTGCTATGTCAATG
		GCATGAACTCGGGGCAGGTAACCGAATCGGTGATCAAGCCGGCAGGCGAGGCCGTGCCGCGTTCCCCGGACGGCTTCTATGAGATAGAAGAGCTTGTTCGCCTGCTTCAGGATAAAATTGAAGACGTTCAGGCCCGTTATTCCGGCAAAGTGCTGGAGAGGAAGCTGGTGGACCTGGGGATTCGGGAAGCCAACCGCTATGGCTGGAGCGATACCTACACCTTTACCAAGTGGCTGGGCGAACAGTTGCTGATGAAGGCGTTAAACGGGCGCACGCTGACCATTCTGCGTCCTTCGATTATCGAAAGTGCCCTGGAGGAACCAGCGCCCGGCTGGATTGAGGGGGTGAAGGTGGCAGATGCCATCATCCTGGCTTACGCACGGGAAAAGTCACCCTCTTCCCGGGCAAACGCTCCGGTATCATCGATGTGATTCCAGTGGACCTGGTGGCCAACTCCATCATCCTTTCCCTGGCGGAAGCTCTTGGAGAACCCGGTCGACGTCGCATCTATCAATGTTGCAGCGGGGGCGGCAATCCAATCTC

		CCTGGGTGAGTTCATCGATCATCTCATGGCGGAATCAAAAGCCAATTACGCTGCCTACGATCACCTGTTCTACCGGCA GCCCAGCAAGCCGTTTCTGGCGGTTAACCGGGCGCTGTTTGATTGGTGATCAGTGGTGTTCGCTTACCGCTCTCCCTG ACGGACCGTGTGCTCAAATTACTGGGAAATTCCCGGGACCTGAAAATGCTCAGGAATCTGGATACCACCCAGTCGCTG GCAACCATTTTTGGTTTCTACACCGCGCCGGATTATATCTTCCGGAACGATGAGCTGATGGCGCTGGCGAACCGGATG GGTGAGGTCGATAAAGGGCTGTTCCCGGTGGATGCCCGCCTGATTGACTGGGAGCTCTACCTGCGCAAGATTCACCTG GCCGGGCTCAATCGCTATGCCCTGAAAGAACGAAAGTGTACAGTCTGAAAACCGCGCGCCAGCGCAAAAAAGCTGC CTGAGAATTCGAAGCTTGGCTGTTTTGGCGGATGAGAGAAGATTTTCAGCCTGATACAGATTAAATCAGAACGCAGAA GCGGTCTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAAGTG AAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAA AGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCC GGGAGCGGATTTGA
*cat-sac*B for FS	2851	TCCTGGTGTCCCTGTTGATACCGGGAAGCCCTGGGCCAACTTTTGGCGAAAATGAGACGTTGATCGGCACGTAAGAGG TTCCAACTTTCACCATAATGAAATAAGATCACTACCGGGCGTATTTTTTGAGTTATCGAGATTTTCAGGAGCTAAGGA AGCTAAAATGGAGAAAAAATCACTGGATATACCACCGTTGATATATCCCAATGGCATCGTAAAGAACATTTTGAGG CATTTCAGTCAGTTGCTCAATGTACCTATAACCAGACCGTTCAGCTGGATATTACGGCCTTTTTAAAGACCGTAAAGA AAAATAAGCACAAGTTTTATCCGGCCTTTATTCACATTCTTGCCCGCCTGATGAATGCTCATCCGGAATTCCGTATGGC AATGAAAGACGGTGAGCTGGTGATATGGGATAGTGTTCACCCTTGTTACACCGTTTTCCATGAGCAAACTGAAACGTT TTCATCGCTCTGGAGTGAATACCACGACGATTTCCGGCAGTTTCTACACATATATTCGCAAGATGTGGCGTGTTACGGT GAAAACCTGGCCTATTTCCCTAAAGGGTTTATTGAGAATATGTTTTTCGTCTCAGCCAATCCCTGGGTGAGTTTCACCA GTTTTGATTTAAACGTGGCCAATATGGACAACTTCTTCGCCCCCGTTTTCACCATGGGCAAATATTATACGCAAGGCGA CAAGGTGCTGATGCCGCTGGCGATTCAGGTTCATCATGCCGTTTGTGATGGCTTCCATGTCGGCAGAATGCTTAATGA ATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGTAATTTTTTTAAGGCAGTTATTGGTGCCCTTAAACGCCTGG TGCTACGCCTGAATAAGTGATAATAAGCGGATGAATGGCAGAAATTCGAAAGCAAATTCGACCCGGTCGTCGGTTCA GGGCAGGGTCGTTAAATAGCCGCTAGATCTAAGT*AAATCGCGCGGGTTTGTTACTG*ATAAAGCAGGCAAGACCTAAAA TGTGTAAAGGGCAAAGTGTATACTTTGGCGTCACCCCTTACATATTTTAGGTCTTTTTTTATTGTGCGTAACTAACTTGC CATCTTCAAACAGGAGGGCTGGAAGAAGCAGACCGCTAACACAGTACATAAAAAAGGAGACATGAACGATGAACAT CAAAAAGTTTGCAAAACAAGCAACAGTATTAACCTTTACTACCGCACTGCTGGCAGGAGGCGCAACTCAAGCGTTTGC GAAAGAAACGAACCAAAAGCCATATAAGGAAACATACGGCATTTCCCATATTACACGCCATGATATGCTGCAAATCC CTGAACAGCAAAAAATGAAAAATATCAAGTTCCTGAATTCGATTCGTCCACAATTAAAAATATCTTCTGCAAAAG GCCTGGACGTTTGGGACAGCTGGCCATTACAAAACGCTGACGGCACTGTCGCAAACTATCACGGCTACCACATCGTCT TTGCATTAGCCGGAGATCCTAAAAATGCGGATGACACATCGATTTACATGTTCTATCAAAAGTCGGCGAAACTTCTA TTGACAGCTGGAAAAACGCTGGCCGCGTCTTTAAAGACAGCGACAAATTCGATGCAAATGATTCTATCCTAAAAGACC AAACACAAGAATGGTCAGGTTCAGCCACATTTACATCTGACGGAAAAATCCGTTTATTCTACACTGATTTCTCCGGTA AACATTACGGCAAACAAACACTGACAACTGCACAAGTTAACGTATCAGCATCAGACAGCTCTTTGAACATCAACGGT GTAGAGGATTATAAATCAATCTTTGACGGTGACGGAAAAACGTATCAAAATGTACAGCAGTTCATCGATGAAGGCAA CTACAGCTCAGGCGACAACCATACGCTGAGAGATCCTCACTACGTAGAAGATAAAGGCCACAAATACTTAGTATTTG AAGCAAACACTGGAACTGAAGATGGCTACCAAGGCGAAGAATCTTTATTTAACAAAGCATACTATGGCAAAAGCACA TCATTCTTCCGTCAAGAAAGTCAAAAACTTCTGCAAAGCGATAAAAAACGCACGGCTGAGTTAGCAAACGGCGCTCTC

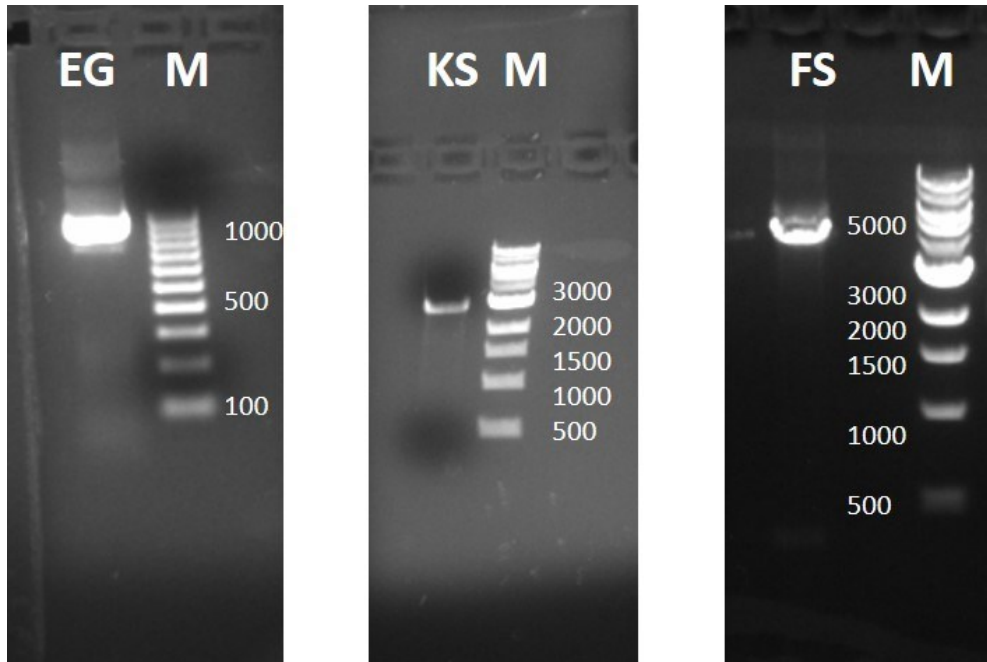| | | GGTATGATTGAGCTAAACGATGATTACACACTGAAAAAAGTGATGAAACCGCTGATTGCATCTAACACAGTAACAGA
TGAAATTGAACGCGCGAACGTCTTTAAAATGAACGGCAAATGGTACCTGTTCACTGACTCCCGCGGATCAAAAATGAC
GATTGACGGCATTACGTCTAACGATATTTACATGCTTGGTTATGTTTCTAATTCTTTAACTGGCCCATACAAGCCGCTG
AACAAAACTGGCCTTGTGTTAAAAATGGATCTTGATCCTAACGATGTAACCTTTACTTACTCACACTTCGCTGTACCTC
AAGCGAAAGGAAACAATGTCGTGATTACAAGCTATATGACAAACAGAGGATTCTACGCAGACAAACAATCAACGTTT
GCGCCAAGCTTCCTGCTGAACATCAAAGGCAAGAAACATCTGTTGTCAAAGACAGCATCCTTGAACAAGGACAATT
AACAGTTAACAAATAAAAACGCAAAAGAAAATGCCGATATTGACTACCGGAAGCAGTGTGACCGTGTGCTTCTCAAA
TGCCTGATTCAGGCTGTCTATGTGTGACTGTTGAGCTGTAACAAGTTGTCTCAGGTGTTCAATTTCATGTTCTAGTTGCT
TTGTTTTACTGGTTTCACCTGTTCTATTAGGTGTTACATGCTGTTCATCTGTTACATTGTCGATCTGTTCATGGTGAACA
GCTTTAAATGCACCAAAAACTCGTAAAAGCTCTGATGTATCTAT |

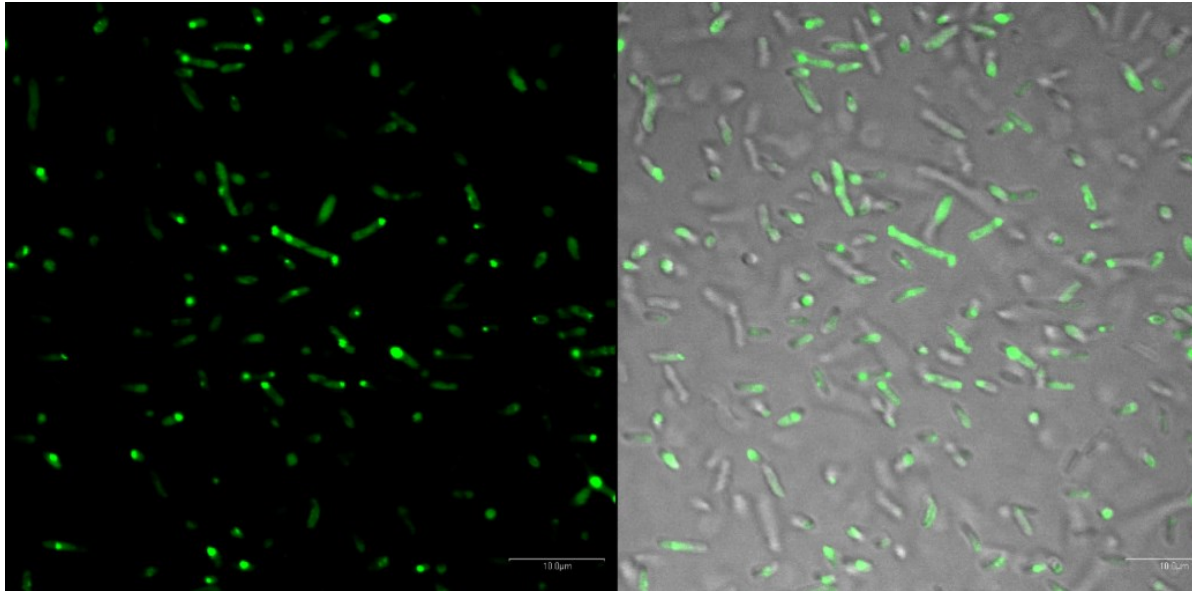Fig. S1 Amplification results of EG, KS and FS fusion using overnight cultured colonies directly as templates.

Fig. S2 GFP protein successfully expressed in *E. coli* NEB 5-alpha.

Protocol S1, Slic cloning method:

Hybridize reaction

Vector	150 ng if available, or as much as possible
Insert	1:1 molar ratio to vector
10×buffer 2 (NEB)	2 µL
20×BSA	1 µL
T4 DNA polymerase (1:6 dilution in buffer 2)	1 µL

H$_2$O   to 20 µL

Use following temperature sequence in a thermocycler,

22 ºC for 5 min; 75 ºC for 15 min; 37 ºC for 3 min; 4 ºC hold.

Then transform normally.