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An easy method to improve the recombinant efficiency of psi-CHECK2

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ABSTRACT: The vector psi-CHECK2 is widely used to validate the interactions of microRNA with mRNA. Typical primers for recombinant psi-CHECK2 are usually designed as protective bases at 5′ end of DNA (5 prime) followed by enzyme restriction sites with partial target sequences at 3′ end of DNA (3 prime). However, the recombinant efficiency of psi-CHECK2 is usually not high enough to fulfill our molecular experiments. Here, we demonstrated that using ∼15 bases of each dangling ends of linearized psi-CHECK2 instead of traditional protective bases could easily improve the recombinant efficiency of psi-CHECK2. In this study, the T4 ligation products of linearized psi-CHECK2 and digested PCR products were transformed into DH5α. The results showed that compared with the conventional primers, our improved primers have better performance in the number of bacterial colonies, the positive rate of PCR amplifications of bacterial solutions of randomly selected colonies, and the recombinant efficiency confirmed by Sanger sequencing. We also co-transformed the linearized psi-CHECK2 and digested PCR products without T4 ligase treatment to estimate the effects of endogenous homologous recombination of DH5α. Indeed, endogenous homologous recombination could promote the recombinant efficiency of psi-CHECK2 but was significantly lower than that of our method (combination of improved primers and T4 ligase treatment). This method may be an alternative to the constructions of other recombinant vectors.

KEYWORDS: vector construction, homologous recombination, double enzyme digestion, IGFBP3

INTRODUCTION

Vector construction is one of the basic technologies in gene engineering and molecular biology [1, 2] and plays a vital role in the whole molecular experiment, including transient overexpression, stable knock-off, and genome editing [3–5]. The vector psi-CHECK2 is widely used to validate the interactions of microRNA with mRNA [6]. The multiple cloning sites of psi-CHECK2 are immediately downstream of *Renilla* luciferase gene. If interactions existed, the fusion mRNA of *Renilla* luciferase will be decreased. Usually, the interested DNA or cDNA regions are amplified with primers designed as protective bases at 5′ end followed by enzyme restriction sites with partial target sequences at 3′ end. Then the cloned fragments are digested by 2 enzymes, and the products are ligated to psi-CHECK2 [7]. However, the recombinant efficiency of this method is not high enough to fulfill our molecular experiments, given one of the facts that the lengths of protective bases in PCR products can affect the digestion efficiency of restriction enzymes.

In our laboratory, a two-step procedure mediated by T vector is commonly used as an alternative of the construction of recombinant psi-CHECK2 when the above classic approach does not work. Firstly, the PCR fragments are easily cloned into pMD19-T, one kind of commercial T vector with high recombinant efficiency based on TA cloning. After PCR amplifications of bacterial solutions and Sanger sequencing, the positive bacterial colonies are subjected to plasmid extraction followed by digestions of 2 enzymes. Thirdly, the digested and recovered fragment will be ligated to linearized psi-CHECK2. This two-step procedure is time-consuming and laborious despite of its improved ligation efficiency and positive rate. In addition to the above 2 methods based on ligation, In-Fusion PCR Cloning Kit is increasingly popular because of high recombinant efficiency. It is based on homologous recombination, making recombination steps convenient and time-saving without the overnight ligation [8]. However, the kit is usually costly. We are interested in developing an easy method to improve the vector construction efficiency.

Insulin-like growth factor (IGF) binding protein 3 (IGFBP3) is a member of IGFBP family whose binding to IGFs could deter the access of IGF to IGFR and result in the inhibition of IGF function [9–11]. Besides, it has been reported that IGFBP3 has IGF-independent activities and regulates cell proliferation, differentiation, survival, migration, etc [12]. In our previous study, we had planned to validate the interactions between

IGFBP3 and the predicted microRNA, but we failed to clone the 3′-UTR (chr4: 76123453–76124382, ARS-UCD1.2) of IGFBP3 into the psi-CHECK2. Finally, we had to turn to In-Fusion PCR Cloning Kit to overcome this problem. In the meantime, lab members accidentally introduced the primers designed for In-fusion PCR into the traditional method but unexpectedly succeeded. Therefore, the present study is aimed to quantitatively determine the effectiveness of the improved primers used for traditional method.

MATERIALS AND METHODS

Primer designing

As mentioned above, the typical primer pairs (TPrimer) were designed as protective bases at 5′ end followed by enzyme restriction sites with partial target sequences at 3′ end. The improved primer pairs (IPrimer) were designed as partial vector sequences at 5′ end followed by enzyme restriction sites with partial target sequences at 3′ end. Three interested regions from *IGFBP3* (chr4: 76123453–76124382, ARS-UCD1.2), *HMGA2* (chr5: 47965479–47966447), and *BMP8b* (chr3: 106310114–106311105) were cloned into psi-CHECK2. TPrimer were designed by NCBI Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast), and IPrimer were created by In-Fusion Cloning primer design (www.takarabio.com/learning-centers/cloning/primer-design-and-other-tools). Primer information was listed in the Table S1.

Experimental group designing

Experimental group information was illustrated in Fig. 1. Briefly, Group-1: [IPrimer PCR product + double restrictions] + [linearized psi-CHECK2] + [T4 ligation] + [DH5α transformation]; Group-2: [IPrimer PCR product] + [linearized psi-CHECK2] + [DH5α transformation]; Group-3: [TPrimer PCR product + double restrictions] + [linearized psi-CHECK2] + [T4 ligation] + [DH5α transformation].

PCR amplification and ligation

The genomic DNA was extracted from whole-blood samples of cattle by phenol-chloroform according to standard procedures [13]. The details of genomic DNA extraction were not provided because the length restriction of the paper. Each PCR amplification was performed in 25 μl reaction mixture containing 50 ng of genomic DNA according to Taq PCR Mix (Sangon Biotech, China). The cycling protocol was as follows: 5 min at 95 °C, 35 cycles at 94 °C for 30 s, annealing at 60 °C (*IGFBP3*), 63 °C (*HMGA2*), or 61 °C (*BMP8b*) for 30 s, and 72 °C for 45 s. The sequences of PCR products were confirmed by Sanger sequencing. The PCR products were purified with SanPrep Column PCR Product Purification Kit (Sangon Biotech). The DNA concentrations of purified fragments and recovered psi-CHECK2 were quantitated using Microvolume UV-Vis Spectrophotometer (NanoDrop, US). The 2 μg of psi-CHECK2 plasmid and 0.5 μg of purified PCR products (Sangon Biotech Kit) were digested by 2 μl of QuickCut *Xho* I and 2 μl of QuickCut *Not* I (Takara, Japan) at 37 °C for 15 min. The digested PCR products were purified again, while the linearized psi-CHECK2 was separated on a 1% agarose gel and recovered by SanPrep Column DNA Gel Extraction Kit (Sangon Biotech). Finally, 10 ng of interested fragments and 10 ng of linearized vector were ligated with T4 DNA ligase (Takara) at 16 °C overnight.

DH5α transformation

The corresponding products were transformed into DH5α by the following procedure: 5 μl of ligated products and 30 μl of DH5α were incubated on ice for 30 min, followed by incubation at 42 °C for 90 s. For Group-3 (endogenous homologous recombination, HR), 10 ng of PCR products (undigested) and 10 ng of linearized vector were directly introduced into 30 μl of DH5α.

Construction efficiency

Three plates were coated with transformed DH5α for each group. The number of bacterial colonies (ColonyNumber) were counted automatically using ImageJ software; the number of positive amplifications of bacterial colony solutions by PCR (PositivePCR) were detected by agarose gel electrophoresis; the number of correct recombinant psi-CHECK2 (SequencingConfirmed) were confirmed by Sanger sequencing. Eight bacterial colonies of each plate (a total of 24 colonies for each group) were randomly selected to determine the PositivePCR and SequencingConfirmed.

Statistical analysis

SPSS one-way ANOVA was used to analyze the difference of ColonyNumber among the 3 groups. SPSS chi-square test was used for PositivePCR and SequencingConfirmed analyses.

RESULTS AND DISCUSSION

To evaluate the feasibility of our developed method (Group-1), 3 different regions from 3′ UTR of *IGFBP3*, promoter of *HMGA2*, and exon of *BMP8b* were used as illustrations of recombinant psi-CHECK2 construction. The experimental scheme of this study was present in Fig. 1. Group-1 used IPrimer and T4 ligation, Group-2 adopted IPrimer and endogenous homologous recombination (HR) of DH5α, and Group-3 was carried out with TPrimer and T4 ligation. Double restriction (*Xho* I and *Not* I) of PCR products was performed for Group-1 and Group-3, but not for Group-2.

Three indicators were used to assess the performances of the 3 methods, including the ColonyNumber, the PositivePCR, and the SequencingConfirmed.

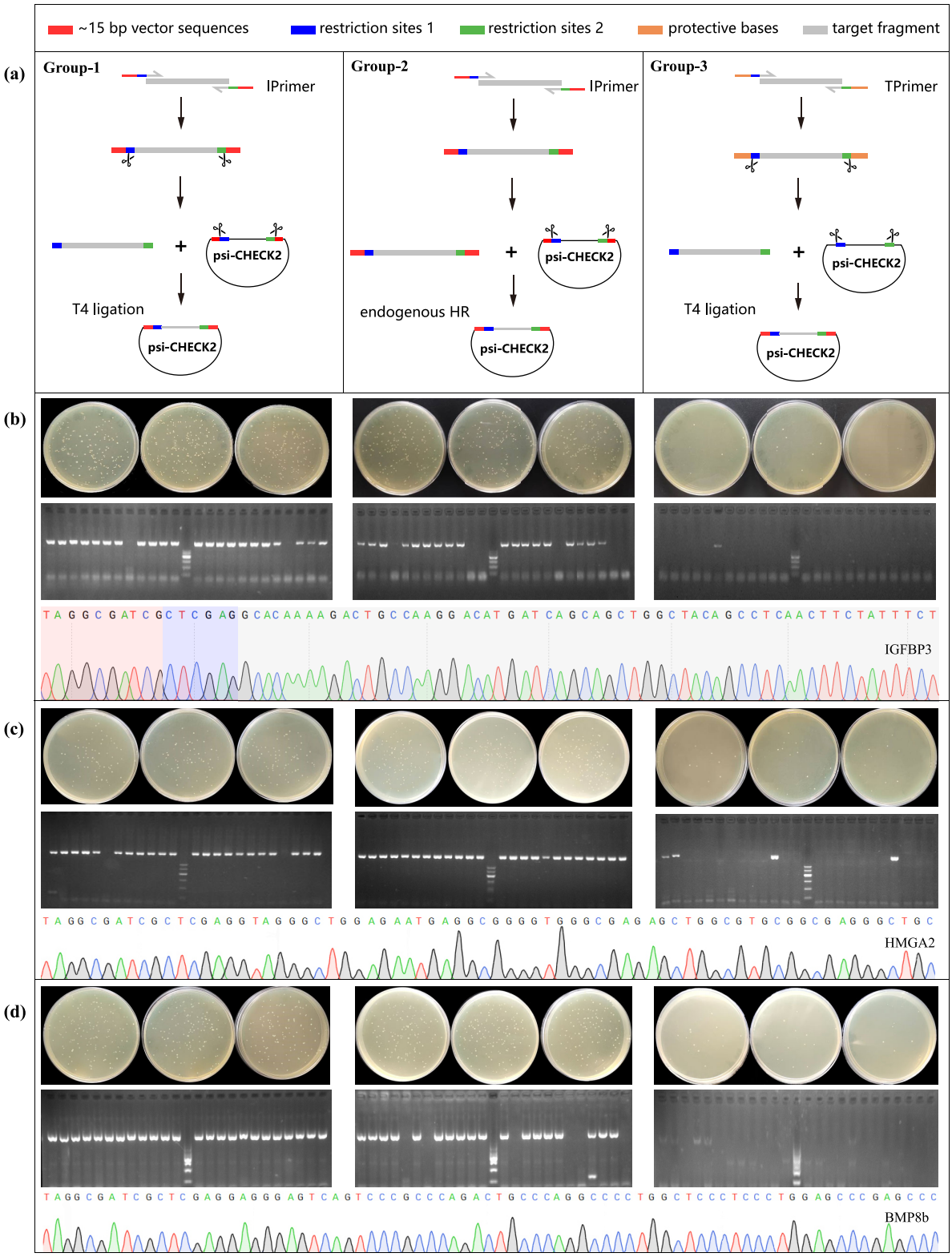

Fig. 1 Experimental scheme and construction efficiency. (a) Vector construction procedure for the three groups; (b–d) Overview of ColonyNumber, PositivePCR, and SequencingConfirmed for *IGFBP3* (b), *HMGA2* (c), and *BMP8b* (d). ColonyNumber and SequencingConfirmed were both detected from twenty-four colonies. Primer psi-check2-F (5′-ATGGGTAAGTACATCAAGAG-3′) was used for Sanger sequencing.

Table 1 Comparisons of three methods for recombinant psi-CHECK2 construction.

Gene	Indicator	Group-1	Group-2	Group-3	*p*-value
IGFBP3	ColonyNumber	167/191/177[a]	173/155/161[a]	10/9/6[b]	6.66E-07
	PositivePCR	7/8/7[a]	7/6/5[a]	1/0/0[b]	2.97E-09
	SequencingConfirmed	7/8/6[a]	4/6/3[b]	1/0/0[c]	4.57E-08
HMGA2	ColonyNumber	138/117/107[a]	166/169/180[b]	11/16/16[c]	4.00E-06
	PositivePCR	7/8/7[a]	8/8/8[a]	2/1/1[b]	4.49E-11
	SequencingConfirmed	5/7/7[a]	7/6/4[a]	0/1/1[b]	2.32E-07
BMP8b	ColonyNumber	76/74/89[a]	101/138/134[b]	8/15/12[c]	1.13E-06
	PositivePCR	8/8/8[a]	6/7/5[b]	4/0/1[c]	2.94E-08
	SequencingConfirmed	8/8/6[a]	6/5/4[b]	0/0//0[c]	7.06E-10

ColonyNumber, number of bacterial colonies; PositivePCR, positive amplifications of bacterial solutions of colony by PCR; SequencingConfirmed, the number of correctly recombinant psi-CHECK2 confirmed by Sanger sequencing. ColonyNumber and SequencingConfirmed were both detected from twenty-four colonies. One-Way ANOVA was used for ColonyNumber and chi-square test was used for PositivePCR and SequencingConfirmed. Different letter means significant difference at the 0.05 level.

As shown in Table 1 and Fig. 1, Group-1 and Group-2 had similar ColonyNumber and were significantly more than that of Group-3. For *IGFBP3* gene, the mean ColonyNumber were 178, 163, and 8; those for *HMGA2* gene were 121, 171, and 14; those for *BMP8b* gene were 80, 124, and 12, for Group-1, Group-2, and Group-3, respectively.

Eight bacterial colonies of each plate were randomly selected to determine the PositivePCR and SequencingConfirmed. No significant differences in PositivePCR were observed between Group-1 and Group-2. The total PositivePCR of *IGFBP3* gene were 22 and 18; those for *HMGA2* gene were 22 and 24; those for *BMP8b* gene were 22 and 15, for Group-1 and Group-2, respectively. However, much less PositivePCR (no more than 5, $p < 0.05$) were identified for Group-3 of each gene, suggesting the low recombinant efficiency of the traditional method. We further confirmed the results of PositivePCR using Sanger sequencing for the 24 colonies. Expectedly, the SequencingConfirmed of Group-3 was the least (no more than 2, $p < 0.05$) among the 3 groups. In contrast, total SequencingConfirmed of Group-1 were 21, 19, and 22 for *IGFBP3*, *HMGA2*, and *BMP8b*, respectively, and those of Group-2 were 13, 17, and 15. On the whole, the recombinant efficiencies were 86.11% (62/72, Group-1), 62.50% (45/72, Group-2), and 12.50% (3/24, Group-3). These results demonstrated that partial vector sequences instead of protective bases in the typical primer pairs could improve psi-CHECK2 recombinant efficiency.

Up to date, the traditional method (as described for Group-3) is still widely used to construct various recombinant vectors such as psi-CHECK2 because of the low cost and easy access of the materials [6, 14, 15]. To improve the recombinant efficiency of the traditional method, innovation has been developed. An example of commercial success is In-fusion Kit which is based on homologous recombination enzyme [8]. Despite of the high cost, this method is becoming popular because it is convenient, fast, and multipurpose [16, 17]. A method for gel extraction of mixed target DNA fragments before ligation was developed for recombination between a vector and different DNA fragments at a time, which is required for the consideration of sequence features [18]. Additionally, this method may be unable to improve the recombinant efficiency of a given fragment. Ligation-independent cloning of PCR products (LIC-PCR) has been developed for the efficient cloning of complex PCR mixtures, resulting in libraries exclusively consisting of recombinant clones, where 5′ ends of the primers used to generate the cloneable PCR fragments contain an additional 12 nucleotide (nt) sequence lacking dCMP [19]. Compared with the above methods and traditional method, our improvement does not need additional materials. The addition of ∼15 bp vector sequences instead of protective bases in primer pairs could significantly improve the recombinant efficiency of psi-CHECK2. Higher digestion efficiency of IPrimer PCR products may make contributions. But we could not exclude the possibility of homologous recombination between linearized vector and incompletely digested IPrimer PCR products. These speculations need further validation experiments. Additionally, although we do not use this method for other vectors, our lab members have given positive feedback when they constructed pGL3-Basic and pCDNA3.1. Detailed comparative study on other vectors, however, is still needed.

CONCLUSION

The addition of ∼15 bp vector sequences instead of protective bases in primer pairs could significantly improve the recombinant efficiency of psi-CHECK2, which may provide an alternative method to construct other recombinant vectors.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2023.007.

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Appendix A. Supplementary data

Table S1 Primer sequence information.

Gene	Primer	Primer sequence	For group	Product	Enzyme
IGFBP3	IPrimer-F	TAGGCGATCGCTCGAGGCACAAAAGACTGCCAAGGACA	Group-1/2	963 bp	*Xho* I
	IPrimer-R	TTGCGGCCAGCGGCCGCCACCAAGCAAGGGCGATT			*Not* I
	TPrimer-F	CCGCTCGAGGCACAAAAGACTGCCAAGGA	Group-3	955 bp	*Xho* I
	TPrime-R	ATAAGAATGCGGCCGCCACCAAGCAAGGGCGATTTT			*Not* I
HMGA2	IPrimer-F	TAGGCGATCGCTCGAGGTAGGGCTGGAGAATGAGGCG	Group-1/2	1002 bp	*Xho* I
	IPrimer-R	TTGCGGCCAGCGGCCGCCGGGATTCCCGGCCTGTCCCTT			*Not* I
	TPrimer-F	CCGCTCGAGGTAGGGCTGGAGAATGAGGCG	Group-3	994 bp	*Xho* I
	TPrime-R	ATAAGAATGCGGCCGCCGGGATTCCCGGCCTGTCCCTT			*Not* I
BMP8b	IPrimer-F	TAGGCGATCGCTCGAGGAGGGAGTCAGTCCCGCCCAGA	Group-1/2	1025 bp	*Xho* I
	IPrimer-R	TTGCGGCCAGCGGCCGCAGTCCCCTCCACTGAGAAAGGC			*Not* I
	TPrimer-F	CCGCTCGAGGAGGGAGTCAGTCCCGCCCAGA	Group-3	1017 bp	*Xho* I
	TPrime-R	ATAAGAATGCGGCCGCAGTCCCCTCCACTGAGAAAGGC			*Not* I

IPrimer, improved primer pairs used for Group-1 (improved method developed by this study) and Group-2 (endogenous homologous recombination); TPrimer, typical primer pairs used for Group-3 (traditional method).