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CONSTRUCTION OF A CRISPR VECTOR FOR INDUCING *TYR* GENE MUTATIONS IN ANEMONEFISH *Amphiprion ocellaris*

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ABSTRACT

The anemonefish *Amphiprion ocellaris* is highly popular in the ornamental fish trade due to its distinctive coloration, which features a combination of orange, black, and white. The formation of black pigmentation in teleost fish is controlled by the tyrosinase-producing gene (*tyr)*, which is responsible for melanin production. Studies have indicated that deletion mutations in the *tyr* gene family in fish and many other vertebrates lead to skin color abnormalities, such as albinism. The clustered regularly interspaced short palindromic repeat (CRISPR) system has emerged as a powerful and precise tool for gene editing with diverse applications. In this study, we construct a CRISPR/Cas9 system targeting the exon 2 region of the *tyr* gene in *A. ocellaris*. A guide RNA (gRNA) was designed and incorporated into the gRNA cloning vector and was subsequently introduced into *Escherichia coli* strain DH5-α. Afterward, the successfully incorporated gRNA sequence was validated via Sanger sequencing. The *E. coli* DH5-α strains carrying this recombinant plasmid were subsequently checked and screened for stability via generations. This gRNA vector together with the Cas9 plasmid will be used to generate knock-out mutations, enabling further investigation of the function of the *tyr* gene in melanin formation in anemonefish *A. ocellaris.*

Keywords: Anemonefish, CRISPR/Cas9, exon 2, gRNA, plasmid, tyrosinase-producing gene

INTRODUCTION

The anemonefish *Amphiprion ocellaris*, belonging to the family *Pomacentridae*, is one of the most recognized species in the ornamental fish trade worldwide (Wabnitz *et al*., 2003). Beyond its commercial appeal, *A. ocellaris* serves as an experimental model species in ecological, evolutionary, and developmental biology (Eco-Evo-Devo) thanks to larval development, and the ontogeny and evolution of its pigmentation patterns. Unlike many large marine fish commonly used in aquaculture, *A. ocellaris*

is a small species that can be easily bred in captivity (Roux *et al.*, 2020), making it an ideal research subject in marine biotechnology. Additionally, with the availability of assembled genomes for various anemonefishes (Lehmann *et al*., 2019; Marcionetti *et al*., 2019; Tan *et al*., 2018), *A. ocellaris* has become more approachable for the application of gene editing techniques to study the functions of genomic genes (Mitchell *et al*., 2021).

Tyrosinase is a multi-functional copper-containing enzyme found in melanocytes of nearly all animals and plants, playing as a physiological/biochemical marker for melanocyte maturation. This enzyme plays a critical role in converting the amino acid tyrosine into melanin, which is the primary contributor to skin, hair, and eye pigmentation across numerous species. In fish and many other vertebrates, black pigmentation is regulated by the tyrosinase-producing gene (*tyr*), which is directly involved in melanin production. Mutations in the *tyr* gene can result in pigment abnormalities. Most albinism in fish is associated with mutations regarding the tyrosinase gene family, resulting in a deficiency of melanin in the skin of albino fish and impaired eye development (Wang *et al*., 2007). Therefore, the expression of the tyrosinase gene is a valuable marker for studying the development of melanin-producing cells during embryogenesis (Camp & Lardelli, 2001).

The clustered regularly interspaced short palindromic repeat (CRISPR) system is one of the most advanced and widely utilized gene-editing techniques, enabling precise targeting and modification of genome sequences. These modifications might induce functional or dysfunctional changes in the targeted gene segment, thereby enabling a more thorough analysis of gene function. Guide RNA (gRNA), which can be laboratory synthesized, guides the Cas9 nuclease to create a double-strand break at a specific gene location. Consequently, DNA repair via non-homologous end joining (NHEJ) often introduces insertions or deletions (indels), which can lead to frameshifts and disrupt gene function (Hsu *et al*., 2014; Mitchell *et al*., 2021). In the CRISPR/Cas9 system, gRNA and the Cas9 nuclease are the key components. GRNA can exist in the form of plasmid DNA (pDNA) or mRNA. GRNA in the form of pDNA has the advantage of being resistant to degradation by exonuclease. Additionally, pDNA has a high efficiency of cellular entry thanks to its supercoiled structure, which facilitates the gRNA's penetration into the cell nucleus. Furthermore, pDNA can be easily stored and generated via the cultivation of the bacteria carrying the modified plasmid (Kobelt *et al*., 2013; Levacic *et al*., 2017). Although producing gRNA using pDNA requires several time-consuming steps, this cloning process is low cost and utilizes materials derived from recombinant DNA technology. In this study, we describe in detail the design and construction procedure of a gRNA vector containing the target sequence for exon 2 of the *tyr* gene.

MATERIALS AND METHODS

Materials

The gRNA cloning vector was purchased from Addgene (USA) (Addgene plasmid #41824, 3914 bp in length; Figure 1). This vector is an empty gRNA expression plasmid, utilized to create gRNA targeting specific sequences. *A. ocellaris* embryos were provided by a local commercial

breeder (Nha Trang, Vietnam). The *E. coli* DH5-α strain was purchased from Invitrogen (USA) and adapted in the Nha Trang University laboratory. Specific primers were designed on the *tyr* gene of *A. ocellaris* (NCBI, ID: 111572659) and presented in Table 1.

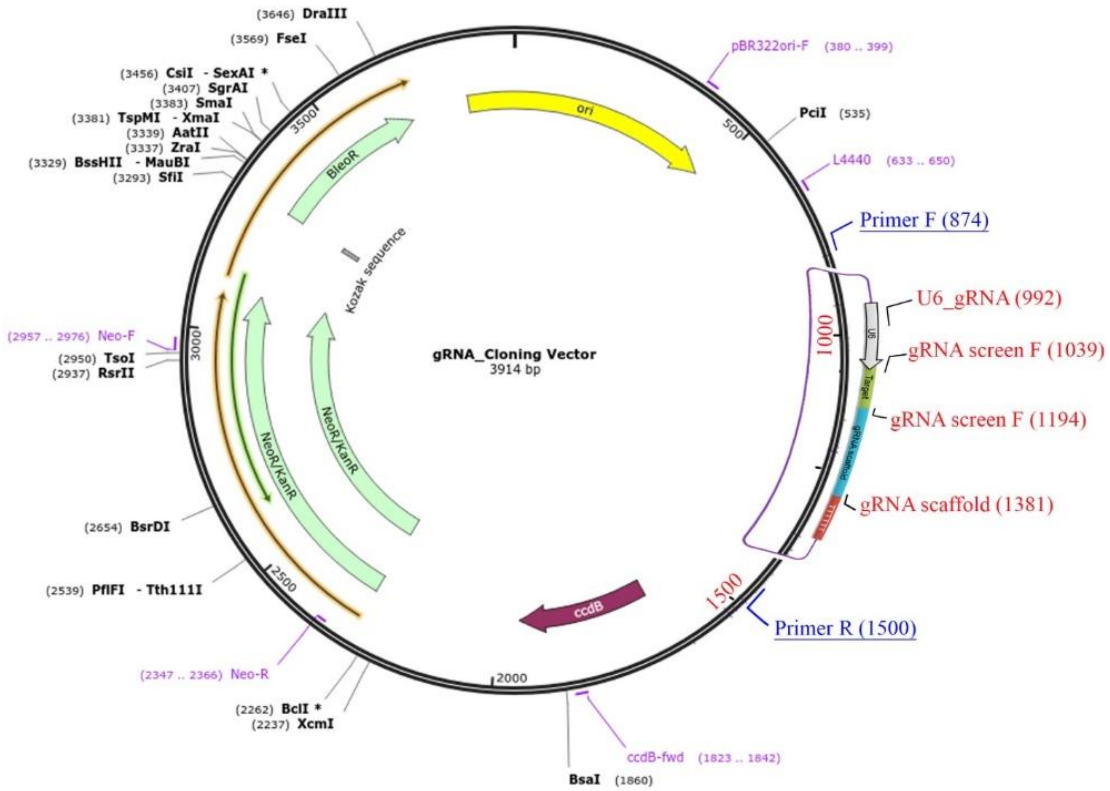

Figure 1. Schematic structure of gRNA_cloning vector

Table 1. List of primers in this study

Primers	Sequence (5'-3')	Length (bp)	Purpose
tyrF1229	CACATTCCAAGCAGTCACACCAC	23	Amplification of the exon 2 region of the *tyr* gene
tyrR1769	TCCATAGGTCCCGTCTCGTACTC	23	
gRNA screen F	TTTCTTGGGTAGTTTGCAGTTTT	23	Colony screening, amplification of the gRNA-spacer site
gRNA screen R	ACTCGGTGCCACTTTTTCAA	20	
F874	AACGACGGCCAGTGAATTGTAATACG	26	Amplification of the gRNA-spacer site in the plasmid, used for sequencing
R1500	GCTATGACCATGATTACGCCAAGCTA	26	

Methods

Determine the exon 2 sequence of tyr gene from A. ocellaris

The *tyr* sequence of *A. ocellaris* was obtained from the NCBI database (ID: 111572659). Sequence data of the *tyr* gene were collected and exon regions for the entire genome were identified by command lines (grep, awk, and egrep) on the Ubuntu system (https://ubuntu.com/). Gene annotations and protein predictions were accessed from the EMBL (https://asia.ensembl.org/index.html/) and the NCBI database (https://www.ncbi.nlm.nih.gov/genbank/).

The structure of the *tyr* gene with exon 2 and the primers for exon 2 amplification was illustrated in Figure 2.

Based on the sequence of the *tyr* gene and the exon 2 region referenced from the NCBI database, a pair of primers (tyrF1229, tyrR1769; Table 1) was designed to amplify the exon 2 region of the *tyr* gene of the fish from the local commercial farm. The 540 bp amplified product of the *tyr*'s exon 2 region of the local *A. ocellaris* was then sequenced using the Sanger sequencing method. The resulting sequence was then compared to the exon 2 sequence of *A. ocellaris* retrieved from GenBank (GB) to assess similarity using BioEdit.

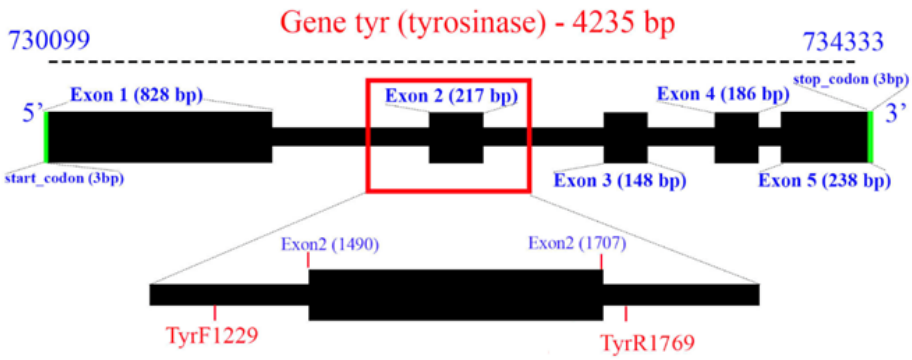

Figure 2. Structure of *tyr* gene with exon 2 and the primers for exon 2 amplification

Design specific gRNA for exon 2 region of tyr

The exon 2 sequence of the *tyr* gene obtained in this study was used as the input of the online tool CRISPR-P version 2.0 (CRISPR-P v2.0, http://crispr.hzau.edu.cn/CRISPR2/) to design specific gRNA sequences. A target sequence should have a length of 19 or 20-bp with an 'NGG' protospacer-adjacent-motif (PAM) site located on the 3' end of the sequence. The target sequence with the most suitable indexes (high on-target and low off-target scores) was selected for the synthesis. The gRNA sequence was subsequently inserted into the gRNA_cloning vector. The insertion site is located downstream of the U6 promoter, which has been efficiently utilized for sgRNA expression in teleost such as zebrafish (Halbig *et al.*, 2008), grouper (Yan *et al.*, 2023), and fugu (Zenke *et al.*, 2008), enabling the expression of the gRNA targeting the exon 2 region of the *tyr* gene in anemonefish.

Cloning selected gRNA into the gRNA cloning vector

The cloning of selected gRNA followed the protocol of Mali *et al*. (2013). Firstly, the selected gRNA sequence was incorporated into two 60-bp oligonucleotides (InsertF, InsertR; Table 2). The sequences are 5' to 3', and the N regions marked in green and red are targeted sequences (20 nucleotides of a protospacer) and reverse complements of each other.

Table 2. 60-bp oligonucleotides incorporated with gRNA sequence

Name	Sequence (5'-3')	Length (bp)
InsertF_ *Tyr*	TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACC*GNNNNN NNNNNNNNNNNNNN*	60
InsertR_ *Tyr*	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC*NNNNNNN NNNNNNNNNNNNC*	60

Subsequently, the two oligonucleotides were annealed to create a DNA fragment containing 20 bp double-stranded regions flanked by 40 bp 5' overhangs at each end. The resulting annealed fragment was then extended to create a 100-bp-length double-stranded gRNA fragment. The annealing reaction was conducted with the addition of NaCl (50mM) at 95ºC for 5 minutes and cooled down to room temperature for 1 to 2 hours. The extension of the gRNA fragment was performed using Herculase (72ºC for 15 minutes). The resulting double-stranded gRNA fragment was checked by electrophoresis and then purified using the MinElute Clean Up Kit (QIAGEN).

The gRNA_cloning vector was linearized using AflII (New England Biolabs) at 37ºC for 3 hours, then 65ºC for 20 minutes. Subsequently, the linearized vector was separated by electrophoresis and the desired band was extracted from the gel using the QIAquick Gel Extraction Kit (QIAGEN). The extracted vector was then further purified using the QIAquick PCR Purification Kit (QIAGEN).

To generate recombinant vectors, the 100-bp-length double-stranded gRNA fragment was ligated to the purified linearized gRNA_cloning vector at a 2:1 volume ratio, using the Gibson Assembly Master Mix (New England Biolabs) at 50ºC for 1 hour to create a recombinant plasmid with a 60-bp inserted gRNA sequence. To ensure the ligated plasmids carry the correct insertion, a PCR reaction to amplify the insert site was performed using gRNA screen primers (Table 1), and the PCR products were then separated by electrophoresis.

Transform recombinant plasmids into E. coli

To generate the recombinant vector containing the desired gRNA fragment in *E. coli* DH5-α, the ligated product was mixed with competent cells at a 1:10 volume ratio. After incubating on ice for 30 minutes, the cells were subjected to a thermal shock at 42ºC for 30 seconds, followed by immediate cooling on ice for 5 minutes. After that, cells were recovered in S.O.C. medium, and incubated at 37ºC for 1 hour with shaking. Finally, the bacteria were plated on LB medium containing kanamycin (125 µg/ml)

and incubated overnight at 37ºC. Colonies with the gRNA-inserted plasmids had PCR amplification products of 210 bp, while colonies that did not receive the gRNA insert ad amplification products of 150 bp.

Determine the stability of recombinant strains of E. coli carrying the gRNA plasmid

Colonies with gRNA-injected plasmids were cultured through F1 and F2 generations to evaluate the stability of the recombinant plasmid in the transformed *E. coli* strains. Concurrently, the colonies were cultured in liquid LB medium containing 125 µg/ml kanamycin for the extraction of plasmid using the QIAprep Spin Miniprep Kit (QIAGEN). Subsequently, the miniprep products underwent PCR amplification using gRNA screen F/R primers to confirm the presence of the inserted plasmids. All the amplicons were analyzed by electrophoresis. Finally, the gRNA-spacer site of the recombinant plasmid was amplified via PCR reaction with the specific primers (F2415 and R3041; Table 1). The expected 650-bp-length amplicon was then sequenced by the Sanger sequencing method to confirm the presence of the entire gRNA structure within the recombinant gRNA_cloning vector.

RESULTS AND DISCUSSION

Designing target gRNA of the exon 2 of the *tyr* gene

The sequencing results of the 540 bp amplicon, amplified from exon 2 region of the *tyr* gene in *A. ocellaris* from Nha Trang, were compared for similarity with the sequences in the NCBI database using the BLAST program. The results indicated that the exon 2 of the *tyr* gene of *A. ocellaris* from Nha Trang has a length of 217 bp, which is 100% matched to that of the GB sequence. Exon 2 of the *tyr* gene is sufficiently upstream where reading frame shifts produced by indel mutations would effectively disrupt gene function, while being far enough downstream to minimize the chances of alternative transcription start sites being utilized (Mitchell *et al*., 2021). The deletion of exon 2 mediated by CRISPR has been demonstrated to significantly reduce melanin pigmentation in clownfish (Mitchell *et al*., 2021). Therefore, this exon 2 sequence was also used to design the sequence of gRNA for the CRISPR/Cas9 system targeting the exon 2 region of the *tyr* gene in the local *A. ocellaris* in this study.

To induce mutations in the *tyr* gene using the CRISPR/Cas9 system, it is essential to design a gRNA sequence specific to exon 2. In this study, we obtained 10 optimal protospacer sequences for the *tyr* gene (see the Table S1 for a list of protospacer DNA sequences). Those sequences that have a high on-target efficiency (0M = 1), low off-target (total number of off-target - OT = 0; total number of potentials off-target – POT = 0), non-appearance of mismatches with the coding sequences (CDS) of other genes in the *A. ocellaris* genome (M1, M2, M3, M4 and M5 = 0), and specificity to the *tyr* gene within the *A. ocellaris* genome. The sequence *tyr*_exon2_S_3 (Table 3) was chosen as one of the two gRNA candidates with the highest IDT scoring (Table S1).

Table 3. Protospacer that used to derive gRNA for this study

Name	Sequence (5'-3')	% GC	Length (bp)
*tyr*_exon2_S_3	AGGCGTTGTGTAACGCAACAGGG	50.00	23

Ligation of gRNAs into the gRNA_cloning vector

The gRNA sequence was incorporated into two 60-bp oligonucleotides. After annealing and extension, a 100-bp double-stranded gRNA fragment was produced and confirmed by electrophoresis on a 2% agarose gel. The result in Figure 3 shows that the annealed fragment had been successfully extended to create a 100-bp-length double-stranded DNA fragment containing the gRNA sequence and can be used for subsequent experiments.

After ligating the gRNA fragment into the linearized vector, to ensure the ligation had been successfully performed, a pair of primers (gRNA screen F/R; Table 1) was used to amplify the gRNA segment of the inserted plasmid, and the amplicon was checked by electrophoresis. Figure 4 indicated that the gRNA fragment was successfully inserted into the gRNA cloning vector, which was shown by the 210 bp amplicon compared to the 150 bp amplicon of the non-inserted vector. This result confirms that the gRNA had been successfully incorporated into the gRNA cloning vector and can be used for downstream experiments.

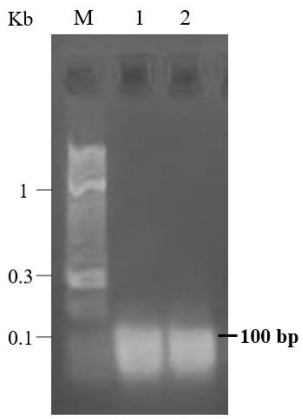

Figure 3. Extended gRNA fragment; M: 50 bp DNA Marker; 1 and 2: ds DNA

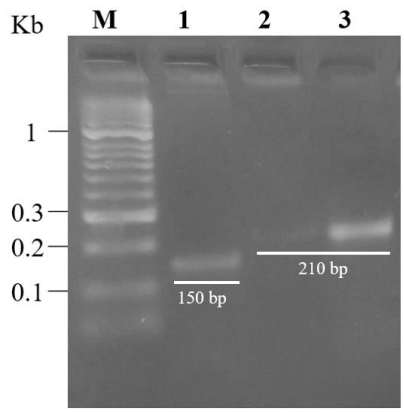

Figure 4. gRNA segment amplified results; M: 50 bp DNA Marker; 1: PCR product of non-inserted vector; 2 and 3: PCR products of inserted vector

Transformation of gRNA-CRISPR vector into *E. coli* competent cells

After incorporating the gRNA and the gRNA_cloning vector, the recombinant vector was subsequently transformed into *E. coli* DH5-α competent cells. After recovering in S.O.C. medium, the transformed cells were then cultured on LB agar plates containing kanamycin. After 16

hours, 5 small, round-shaped colonies were observed.

Colony screening

PCR screening was conducted to identify the successfully transformed colonies carrying the recombinant vectors. The insertion of the gRNA segment into the gRNA_cloning vector was verified via PCR reaction using the gRNA screen F/R primers (Table 1) and the resulting amplicons were then analyzed by electrophoresis on a 2% agarose gel. Figure 5 shows that 5 colonies had the anticipated length of approximately 210 bp. From these results, the selected colonies were then sequenced to confirm the presence of the targeted gRNA. Furthermore, cultures were used to determine the stability of the transformed vector over generations.

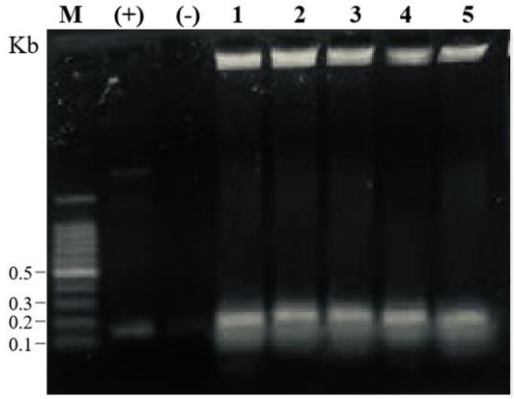

Figure 5. Electrophoresis of PCR products of colonies containing gRNA inserted plasmid. M: 100 bp DNA ladder; (+) positive control (with non-inserted gRNA_cloning vector), (-) negative control (without DNA template); 1-5: PCR products from transformed colonies 1-5.

Stability of recombinant strains

To assess the stability of the recombinant strains, PCR reactions were conducted using similar primers (gRNA screen F/R primers; Table 1). These reactions were done to confirm the presence of the gRNA vector in the F1 and F2 generations of the recombinant strain. Figures 6 (6A and 6B) demonstrated that the amplicons obtained from PCR reactions, using the miniprep products of the F1 and F2 generations of the recombinant strain, had the expected length of 210 bp. This result indicated that the gRNA_cloning vector containing the gRNA structure within the recombinant strain is stable. Furthermore, this recombinant strain is suitable for the production of gRNA plasmid and can be preserved for future research.

Although the recombinant strains' stability is confirmed through PCR for F1 and F2 generations, the long-term *in vivo* stability of the CRISPR vector has not been confirmed yet.

Further studies may explore these constructs' performance over time and under varying environmental conditions.

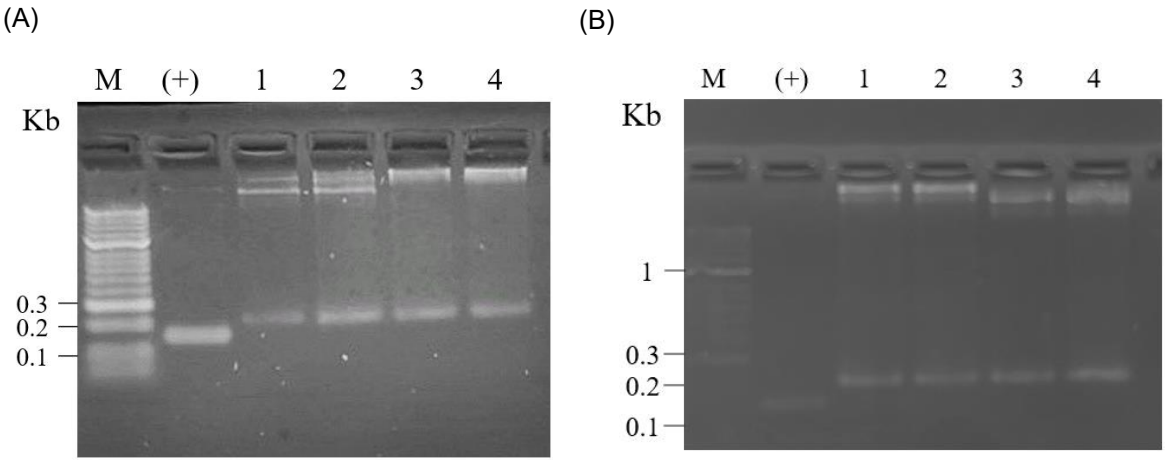

Figure 6. Electrophoresis of PCR products of: A) F1 colonies containing gRNA inserted plasmid; B) F2 colonies containing gRNA inserted plasmid; M: 50 bp DNA ladder; (+) positive control (with non-inserted gRNA_cloning vector); 1-4: F1/F2 recombinant strain miniprep products

Recombinant CRISPR vector sequencing

The sequences derived from the 650 bp amplicon containing the gRNA sequence on the recombinant vector were compared for similarity with the sequence of the original gRNA_cloning vector on GenBank, the InsertF and InsertR of *tyr*_exon2_S_3 (Table 3), the gRNA scaffold sequence on the plasmid and the protospacer site (Figure 7). The fasta sequences derived from 650 bp amplicons for the recombinant vector, the gRNA_cloning vector and its components were listed in Supplementary material S1.

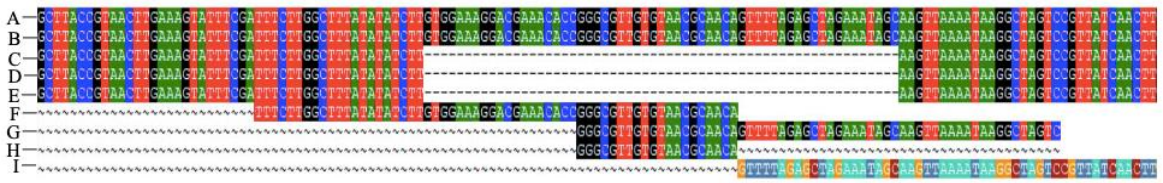

Figure 7. Sequences of the recombinant gRNA vectors, gRNA cloning vector and its components. (A, B) The gRNA_cloning vector sequence with the protospacer inserted from recombinant strains T1 and T2; (C) The gRNA_cloning vector sequence from GenBank; (D) U6 promoter sequence; (E) The gRNA_cloning vector used in the study; (F) InsertF_tyr_exon2_S_3 (5'-3'); (G) InsertR_tyr_exon2_S_3 (3'-5'); (H) Tyr_exon2_S_3 sequence; (I) gRNA scaffold sequence.

Detailed sequences of the recombinant vector isolated from the recombinant strains (T1 and T2) are presented and compared with the non-inserted gRNA_cloning vector sequence and the component of the CRISPR vector (U6 promoter, protospacer). The recombinant vectors are 60 nucleotides longer than the original gRNA_cloning vector, and these additional sequences match the sequences of the initial InsertF_*Tyr* and InsertR_*Tyr* constructs. Furthermore, the inserted gene sequence in the recombinant

vector completely matches the gRNA-spacer sequence targeting exon 2 of the *tyr* gene in *A. ocellaris*. This gRNA-spacer is 20 bp in length and is the matching region of the two primers, InsertF_*Tyr* and InsertR_*Tyr*, which were synthesized to form the dsDNA segment. The final 20 nucleotides of the inserted sequence were followed by the gRNA scaffold sequence of the plasmid. This complete match confirms that the gRNA-spacer has been inserted in the correct position within the recombinant vector.

CONCLUSION

This study designed gRNA sequences targeting the *tyr* gene and created a recombinant gRNA vector for CRISPR technology in the anemonefish *A. ocellaris*. The gRNA was designed based on the exon 2 sequence in *A. ocellaris* obtained from Nha Trang. The gRNA sequence was then successfully inserted into the gRNA cloning vector. Sequencing confirmed that the gRNA was successfully inserted into the recombinant vector at the position following the U6 promoter and before the gRNA scaffold sequence. This insertion site plays an important role in ensuring that the gRNA is efficiently transcribed and performs its gene editing function. The recombinant plasmid will be used in combination with the Cas9 plasmid to generate mutants in the anemonefish *A. ocellaris*. The construct procedure is also utilized to generate recombinant plasmids for CRISPR technology in other marine fish species.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY MATERIALS

Table S1. The list of 10 optimal protospacer sequences of exon 2 of the *tyr* gene from the anemonefish *Amphiprion ocellaris*

Name	Start base	End base	Protospacer + PAM(5'-3') sequences	Length (bp)	CG ration (%)	Scoring (IDT)	0M (on-/off-)	1M/2M/3M/4M/5M	Total_No.of_OT	1M/2M/3M/4M/5M	Total_No.of_POT	Risk evaluation
tyr_exon2_S_1	18	40	GGAGGAGTACAACAGCCGAGAGG	23	60,00%	37	1	0	0	0	0	Best
tyr_exon2_S_2	37	59	GAGGCGTTGTGTAACGCAACAGG	23	55,00%	48	1	0	0	0	0	Best
tyr_exon2_S_3	**38**	**60**	**AGGCGTTGTGTAACGCAACAGGG**	**23**	**50,00%**	**57**	**1**	**0**	**0**	**0**	**0**	**Best**
tyr_exon2_S_4	39	61	GGCGTTGTGTAACGCAACAGGGG	23	55,00%	37	1	0	0	0	0	Best
tyr_exon2_S_5	42	64	GTTGTGTAACGCAACAGGGGAGG	23	55,00%	45	1	0	0	0	0	Best
tyr_exon2_S_6	43	65	TTGTGTAACGCAACAGGGGAGGG	23	50,00%	53	1	0	0	0	0	Best
tyr_exon2_S_7	64	86	GGTCCACTGTTGCGTAACCCTGG	23	60,00%	57	1	0	0	0	0	Best
tyr_exon2_S_8	114	136	ACTCCCCACAACAGCTGATGTGG	23	55,00%	56	1	0	0	0	0	Best
tyr_exon2_S_11	147	169	GGGCCTCCCCGAGTACGAGACGG	23	70,00%	32	1	0	0	0	0	Best
tyr_exon2_S_12	148	170	GGCCTCCCCGAGTACGAGACGGG	23	70,00%	32	1	0	0	0	0	Best

M: the number of nucleotide mismatches (1M, 2M, 3M, 4M or 5M); 0M: perfect match to the on-target site, and if the number of 0M sites >1, maybe off-target sites are contained; bp: base pair; OT: off-target; POT: potential off-target.

Supplementary material S1: Sequences of the recombinant CRISPR vectors, gRNA cloning vector and its components

>Tyr_exon2_NT

GTGATCTGCACCCAGCCGGAGGAGTACAACAGCCGAGAGGCGTTGTGTAACGC
AACAGGGGAGGGTCCACTGTTGCGTAACCCTGGCAACCATGATCCAAATCGCG
TGACGCGACTCCCCACAACAGCTGATGTGGATTTCACTGTGGGCCTCCCCGAGT
ACGAGACGGGACCTATGGACCGATTTGCCAACATGAGCTTTAGAAACGTCCTA
GAGG

>T1

TCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCC
CTTTGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGG
TACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCA
TATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACA
CAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAG
TTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTT
GAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG
GGCGTTGTGTAACGCAACAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG
CTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTAGA
CCCAGCTTTCTTGTACAAAGTTGGCATTAAAGGGCGAATTCCAGCACACTGGCG
GCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGATGCATAGCTT

>T2

TCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCC
CTTTGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGG
TACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCA
TATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACA
CAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAG
TTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTT
GAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG
GGCGTTGTGTAACGCAACAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG
CTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTAGA
CCCAGCTTTCTTGTACAAAGTTGGCATTAAAGGGCGAATTCCAGCACACTGGCG
GCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGATGCATAGCTT

> gRNA cloning vector (GB)

TCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCC
CTTTGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGG
TACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCA

TATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACA
CAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAG
TTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTT
GAAAGTATTTCGATTTCTTGGCTTTATATATCTTAAGTTAAAATAAGGCTAGTC
CGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTAGACCCAGC
TTTCTTGTACAAAGTTGGCATTAAAGGGCGAATTCCAGCACACTGGCGGCCGTT
ACTAGTGGATCCGAGCTCGGTACCAAGCTTGATGCATAGCTT

>U6_gRNA (GB)

TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTAC
CAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATAT
ACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAA
AGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTG
CAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAA
AGTATTTCGATTTCTTGGCTTTATATATCTTAAGTTAAAATAAGGCTAGTCCGTT
ATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTAGACCCAGCTTTC
TTGTACAAAGTTGGCATTA

> gRNA cloning vector in this study

TCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCC
CTTTGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGG
TACCAAGGTCGGGCAGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCAT
ATACGATACAAGGCTGTTAGAGAGTAATTAGAATTAATTTGACTGTAAACACA
AAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTT
GCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGA
AAGTATTTCGATTTCTTGGCTTTATATATCTTAAGTTAAAATAAGGCTAGTCCGT
TATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTAGACCCAGCTTT
CTTGTACAAAGTTGGCATTAAAGGGCGAATTCCAGCACACTGGCGGCCGTTACT
AGTGGATCCGAGCTCGGTACCAAGCTTGATGCATAGCTT

>InsertTyrF(5'-3')

TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGGGCGTTGTGTAAC
GCAACA

>InsertTyrR(3'-5')

GGGCGTTGTGTAACGCAACAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
GCTAGTC

> Tyr_exon2_S_3 sequence

AGGCGTTGTGTAACGCAACAGGG

>gRNA scaffold

GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA
AAAAGTGGCACCGAGTCGGTGCTTTTTTT