Cloning of intronic sequence within DsRed2 increased the number of cells expressing red fluorescent protein

Rishikaysh V. Pisal[a], Hana Hrebikova[a], Jana Chvatalova[a], Tomas Soukup[a], Stanislav Filip[b], Jaroslav Mokry[a]

Aim. Cloning of artificial intronic sequence within the open reading frame (ORF) of DsRed2 gene.
Method. Splice prediction software was used to analyze DsRed2 sequence to find an ideal site for cloning artificial intronic sequence. Intron was cloned within DsRed2 using cyclic ligation assembly. Flow cytometry was used to quantify the number of cells expressing red fluorescence.
Result. Sequencing data confirmed precise cloning of intron at the desired position using cyclic ligation assembly. Successful expression of red fluorescence after cloning of intron confirmed successful intron recognition and splicing by host cell line. Cloning of intron increased the number of cells expressing red fluorescent protein.
Conclusion. Cloning of intronic sequence within DsRed2 has helped to increase the number of cells expressing red fluorescence by approximately four percent.

Key words: DsRed2, Cyclic ligation assembly (CLA), intron cloned DsRed2

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[a]Department of Histology and Embryology, Faculty of Medicine in Hradec Kralove, Charles University, Simkova 870, 500 03 Hradec Kralove, Czech Republic
[b]Department of Oncology and Radiotherapy, Faculty of Medicine in Hradec Kralove, Charles University, Simkova 870, 500 03 Hradec Kralove, Czech Republic
Corresponding author: Jaroslav Mokry, e-mail: mokry@lfhk.cuni.cz

INTRODUCTION

DsRed is a red fluorescent protein (RFP) from coral *Discosoma sp.*[1] with excitation and emission maxima at 558 and 583 nm respectively[2]. Shi-Lung Lin et al used EcoO109I restriction site within hcRed1 for cloning an intron within its ORF (ref.[3]). Intron cloned HcRed1 construct was successfully utilized to generate transgenic miRNA-expressing cell lines and animals[4-5] and gene-knockout mice for human disease research[6]. The authors show successful splicing of pre-mRNA and further excision into small miRNA, which in turn are able to trigger targeted gene silencing making this type of construct a good platform for miRNA expression[3].

We extended this finding to DsRed2 and after analyzing the sequence using splice prediction software it was found that EcoO109I restriction site within ORF of DsRed2 encompasses a prominent intron splicing enhancer region, which can be used for cloning intronic sequences. In this study, we opted for cycled ligation based DNA assembly procedure to clone the intron within the ORF. This method employs a thermostable ligase and short scaffold oligonucleotides connectors (SOCs) that are homologous to the ends and beginnings of two adjacent DNA sequences[7].

A brief overview of cyclic ligation assembly is as follows: First, denaturation of the two fragments is carried out in the presence of suitable SOC, which allows the SOC access to the complementary strands of the two sequences when the reaction is returned to an annealing temperature[7]. Binding of the SOC to the complementary strands creates a temporary dsDNA structure with an absence of a single phosphodiester bond between the two complementary strands[7]. The thermostable Taq Ligase[8] specifically ligates the complementary strands together to produce a continuous ssDNA structure without any loss or addition of nucleotides at the joint. SOCs serve only as initial scaffolds for fragment assembly, with successfully assembled products becoming additional templates as they assemble[7]. The two ligated strands anneal to their complementary strands, again creating a single nick in a dsDNA structure. The amount of correctly assembled product increases exponentially as the reaction cycles between denaturing and annealing/ligation temperatures[7].

In the current study, we have successfully cloned intron within ORF of DsRed2 using cyclic ligation assembly. This cloning resulted an increase in number of cells expressing red fluorescent as compared to un-cloned DsRed2.

MATERIALS AND METHODS

Splicing prediction

DsRed2 sequence was analyzed for splice site prediction using NetGene2 World Wide Web Server software developed and maintained by Center for Biological Sequence Analysis, The Technical University of Denmark DK-2800 Lyngby, Denmark[9] Link to website is http://www.cbs.dtu.dk/services/NetGene2/

Table 1. Sequences of synthetic oligonucleotides used in the experiment.

Primer name	Sequence
FP LF DsRed	5'-ATCTCGAGCTCAAGCTTCGAATTCTG-3'
RP LF DsRed	5'-CTTGTGGGTCTCGCCCTTCA-3'
FP SF DsRed	5'-GCCCTGAAGCTGAAGGAC-3'
RP SF DsRed	5'-CTCTACAAATGTGGTATGGCTG-3'
Intron amplification FP	5'-GTAAGAGTGGTCCGATCGTCGC-3'
Intron amplification RP	5'-CTGCAGGATATCAAAAAAGGGACAGG-3'
DsRed2N1 FP (Hind III RS)	5'-ATGCAAGCTTGCCACCATGGCCTCCTCCGAGAAC-3'
DsRed2N1 RP (XbaI RS)	5'-ATGCTCTAGACGGCCGCTACAGGAACAGGTG-3'
SOC1	5'-TGAAGGGCGAGACCCACAAGGTAAGAGTGGTCCGATCGTC-3'
SOC2	5'-CCTTTTTTGATATCCTGCAGGCCCTGAAGCTGAAGGACGG-3'
Sequence of artificial intron sense strand	5'-GTAAGAGTGGTCCGATCGTCGCGACGCGTCATTACTAACTATCAAT ACTTAATCCTGTCCCTTTTTTGATATCCTGCAG-3'

Table 2. CLA reaction setup and thermocycler program[7].

Cycled Ligation Reaction		
Component	Amount (μL)	Final concentration
20 nM Insert Mix	2	2 nM
200 nM SOC Mix	1	10nM
10X Taq Ligase Buffer	2	1X
40 U/μL Taq DNA Ligase	2	80 U
H_2O	12	
Total	20	
Thermocycler Program:		
1. 95 ˚C 2 min		
2. 95 ˚C 30 s		
3. 60 ˚C 2 min		
4. GOTO 2 30×		
5. 55 ˚C 1 0 min		
6. 4 ˚C hold		

Plasmids construction

Using DsRed2N1 plasmid as template entire DsRed2 gene was PCR amplified as two fragments; a 556 bp fragment (LF) and 233 bp fragment (SF) using LF DsRed and SF DsRed primer pairs respectively. Intronic sequence was amplified from 79 bp single stranded oligonucleotide sequence using primer pair Intron FP and Intron RP. All PCR amplifications were carried out using high fidelity polymerase (Q5 high fidelity polymerase NEB).

Three fragments were assembled into a single 763 bp fragment using cyclic ligation assembly (CLA) procedure. Reaction setup and thermocycler program is mentioned in Table 2. Before CLA the PCR amplified fragments were phosphorylated using T4 kinase. CLA reaction mix contained scaffold oligonucleotide connectors (SOC), PCR amplified fragments and Taq ligase a thermostable ligase. For joining three fragments two SOC connectors were used sequences of which are in Table 1.

2 μL of CLA product was PCR amplified using DsRed2N1 FP (Hind III RS) and DsRed2N1 RP (XbaI RS) primer pair respectively followed by restriction digestion and cloning into HindIII and XbaI sites of multiple cloning sites of pcDNA3.1 (+) mammalian expression plasmid.

Cell culture and transfection

HeLa cells were provided by Emil Rudolf (Charles University, Czech Republic) and were maintained in a humidified incubator at 37 °C with 5% CO_2 and grown in Dulbecco's modified eagle medium containing 10% fetal bovine serum (Sigma). Cells were plated into 12-well tissue culture plates at a density of 1.6×10^5 cells per well 24 hr prior to transfection. Cells were transfected using X-tremeGENE™ HP DNA Transfection Reagent (Roche). Transfection was carried out as per manufacturer's guidelines.

Fluorescent image

Fluorescent cells were observed using a Nikon Eclipse Ti-E inverted fluorescence microscope. The images were captured by a Nikon DS-Qi2 camera under a 10× objective and recorded using NIS-Elements for advanced research (NIS-Elements AR) software.

Flow cytometry

Flow cytometry was performed on transiently transfected HeLa cells using Beckman Coulter Cell Lab Quanta MPL flow cytometer. More than 15,000 events of each sample were counted for quantifying number of cells expressing red fluorescence and only live cells were gated. Data was analysed using Cell Lab Quanta analysis software. Cells were analysed by flow cytometry at various time points over a span of 72 hrs post transfection.

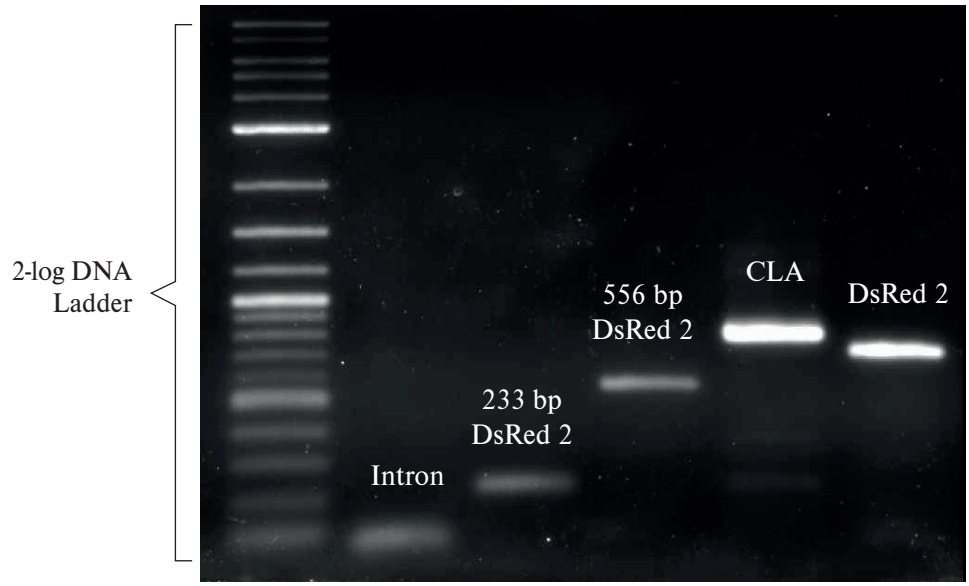

Fig. 1. Directed assembly of intron between two fragments of DsRed2 gene. Lane 1 2-log DNA ladder; Lane 2 PCR amplification of 79 bp intron; Lane 3 PCR amplification of 233 bp fragment of DsRed2 gene; Lane 4 PCR amplification of 556 bp fragment of DsRed2 gene; Lane 5 PCR amplification of 763 bp CLA product; Lane 6 PCR amplification of DsRed2 gene.

>16_Pisalek_1_20161130_3_A06_3130
CATGKGAAYWTATAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACSAYTYACTATA
GGGAGACCCAAGCTGGCTAGCGTTTAAACTTAAGCTTGCCACCATGGCCTCCTCCGAGAACGTCATCACCGAGTTCATGCGCT
TCAAGGTGCGCATGGAGGGCACCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCCAC
AACACCGTGAAGCTGAAGGTGACCAAGGGCGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCCCAGTTCCAGTACGGCTC
CAAGGTGTACGTGAAGCACCCCGCCGACATCCCCGACTACAAGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGA
TGAACTTCGAGGACGGCGGCGTGGCGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCTGCTTCATCTACAAGGTGAAGTTC
ATCGGCGTGAACTTCCCCTCCGACGGCCCCGTGATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCC
CCGCGACGGCGTGCTGAAGGGCGAGACCCACAAGGTAAGAGTGGTCCGATCGTCGCGACGCGTCATTACTAACTATCAATACT
TAATCCTGTCCCTTTTTTGATATCCTGCAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGTCCATCTA
CATGGCCAAGAAGCCCGTGCAGCTGCCCGGCTACTACTACGTGGACGCCAAGCTGGACATCACCTCCCACAACGAGGACTACA
CCATCGTGGAGCAGTACGAGCGCACCGAGGGCCGCCACCACCTGTTCCTGTAGCGGCCGTCTAGAGGGCCCGTTTAAACCCGC
TGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGC
CACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAATAGGTGTCATTCTATTCTTGGGGGGTGGG
GTGGGGCAGGAAAACCAAGGGG

Fig. 2. Result of sequencing reaction shows precise cloning of intron within DsRed2 gene. Font in light grey depicts DsRed2 gene, font which is in black and underlined depicts intronic sequence and font in plain black is the sequence outside the DsRed2 gene.

RESULTS

NetGene2 software predicted that the intron would be recognised and spliced by splicing machinery

Even though the region around EcoO109I restriction site was reported rich in splicing enhancer sequences it was essential to find out if it was also true in the case of DsRed2 since the previous experiment was carried out in hcRed1 (far red fluorescent protein). In order to find the precise site we had to repeatedly perform the analysis by re-entering the engineered sequence close to the EcoO109I restriction site until the highest confidence level for donor splice site and acceptor splice site was obtained.

After a couple of trials, finally NetGene2 software analysis revealed 0.99 confidence for donor splice site on direct strand and 1.0 confidence for acceptor splice site on direct strand. This gave us an assurance that the site around EcoO109I restriction site was rich in splicing enhancer sequence.

Successful cloning of intron within ORF of DsRed2 gene

Cyclic ligation assembly was used to rope the three fragments together. Elegance of CLA lies in the fact that no optimization of reaction condition is essential for new assembly. Hence we used the pre-standardised conditions mentioned by Roth et al.[7] and obtained the site specific cloning with precision and in the first attempt (Fig. 1 and 2). Exclusion of nuclease from the reaction makes this procedure precise, time-saving and less complicated. Precision of cloning was confirmed by DNA sequencing and intron splicing was confirmed by checking expression of red fluorescence (Fig. 3).

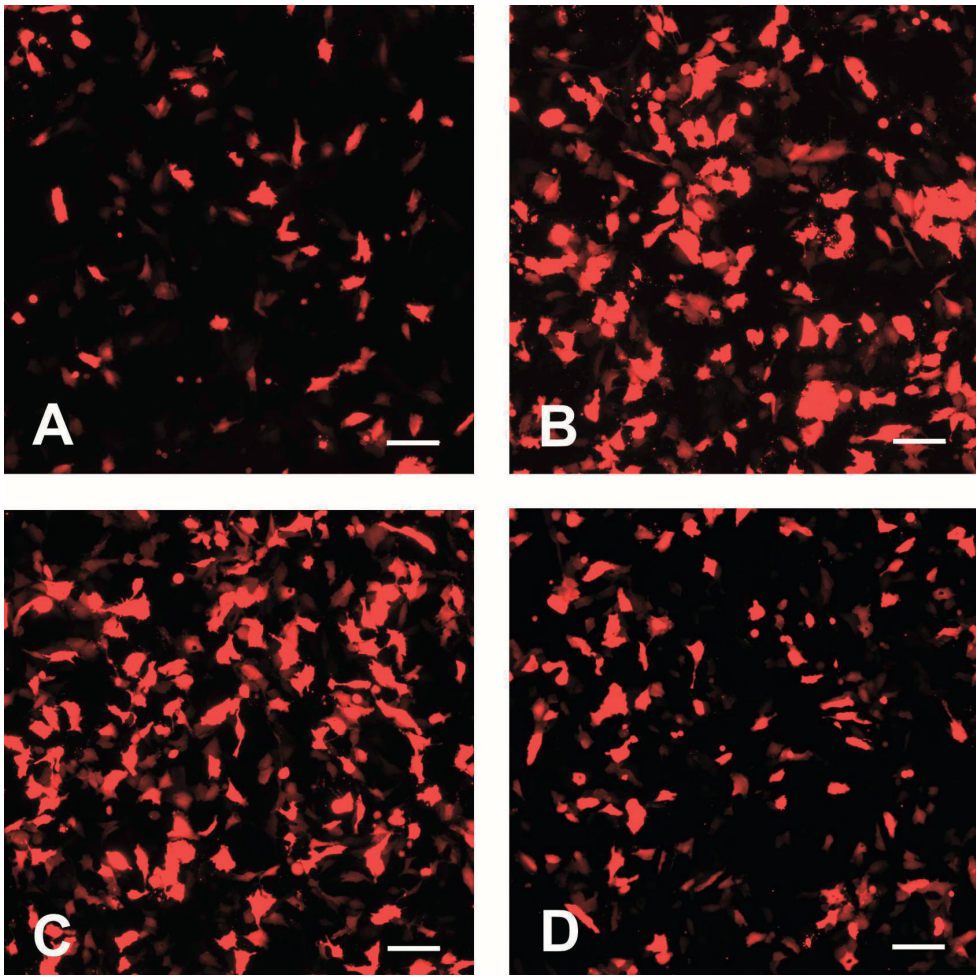

Fig. 3. Successful splicing of the intron. HeLa cells were transiently transfected with pcDNA3.1 (+) plasmid containing intron cloned DsRed2 gene. Expression of red fluorescence at (a) 24 h; (b) 48 h; (c) 60 h and (d) 72 h post transfection was recorded.

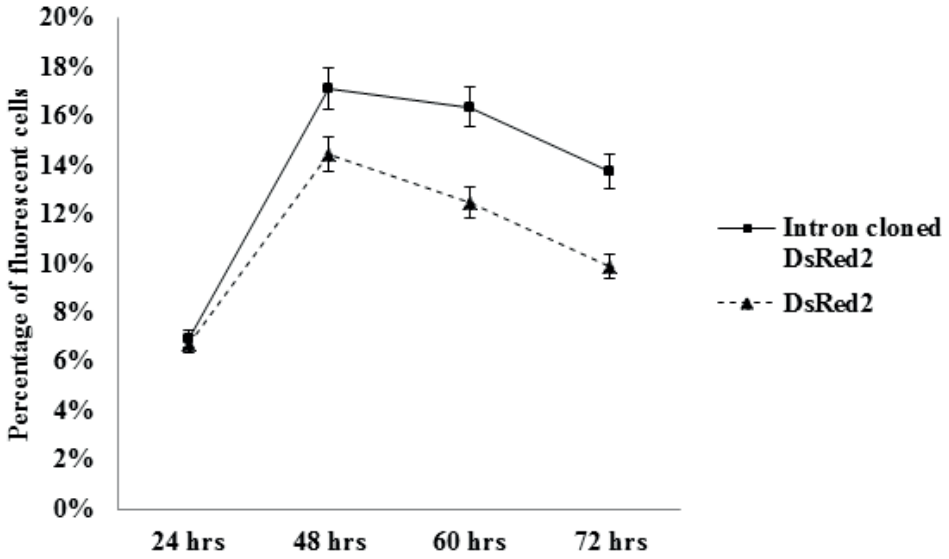

Fig. 4. Comparative analysis of number of cells expressing red fluorescence when transfected with native DsRed2 gene and intron cloned DsRed2 gene.

Effect of intron cloning on dsRed2

Flow cytometry results of intron cloned DsRed2 and native DsRed2 showed four percent increase in number of cells expressing red fluorescence in intron cloned DsRed2 over native DsRed2 (Fig. 4). The expression was measured at different time point over a period of 72 h post transfection. In both variants i.e. intron cloned DsRed2 and native DsRed2 number of cells expressing red fluorescence increased exponentially, peaked at 48 h post transfection and then declined. At 24 hour there was marginal difference between number of cells expressing red fluorescence in the variants but at later time points, difference started to increase gradually; at the peak of expression i.e. 48 h post transfection intron cloned DsRed2 showed 3 percent increase over native DsRed2 while at 60 h and 72 h there was a 4 percent increase.

DISCUSSION

A non-classical approach to cloning made the daunting task easier and with utmost precision. In the beginning, we tried the classical way of using restriction endonuclease, single strand exonuclease and T4 polymerase to clone the intron at the precise position within EcoO109I restriction site but without any success. Recent advances in synthetic biology have provided more powerful and efficient in vitro genetic assembly strategies than restriction digestions[10].

Apart from cyclic ligation assembly there are other approaches like Gibson assembly, Successive hybridization assemblies, Golden gate assemblies etc. for assembling DNA fragments. Each of these approaches has its own advantages and disadvantages. Gibson assembly[11] and successive hybridization assembly[12] are not suitable for cloning small fragments and reaction set up for Gibson assembly is expensive and the reaction parameters need to be standardized for each new assembly. A major disadvantage of golden gate assemble is that it has complex preparatory work, it is not 100% sequence independent and it can only be applied for cloning if type IIS restriction site essential for generating flanking ends is not present within the fragments to be assembled[13-14].

Cyclic ligation assembly[7] is an ideal cloning technique for our experiment because the fragments to be assembled are relatively small and insertional precision is obligatory as insertion or deletion of a single base pair would cause frame shift leading to formation of nonsense protein; also reaction setup is comparatively inexpensive and the ligation of fragments is scarless. Another important feature of CLA is that standardization of reaction parameters is not essential and same parameters can be extended to any new assembly reaction.

Even though DsRed has wide application it has some major shortcomings like cells expressing high levels of DsRed show cytotoxic effect due to oligomerization and aggregation[15]. Correlation between Bcl-xL protein and DsRed mediated cytotoxicity has been established and it has been shown that DsRed supresses translation of Bcl-xL protein and over-expression of Bcl-xL alleviates the cytotoxicity mediated by DsRed protein[16].

Here we report an increase of four percent in number of cells expressing red fluorescence upon transfection with intron cloned DsRed2 when compared to cells transfected with native DsRed2. Excluding the initial reading of 24 h, intron cloned DsRed2 showed consistent increase in number of cells expressing red fluorescence over native DsRed2. The exact mechanism underlying the increase in number of cells expressing red fluorescence after intron cloning is still unclear. Our research has confirmed that without meddling with Bcl-xL protein it is possible to increase the number of cells expressing red fluorescent protein by cloning an intron within ORF of DsRed2 gene. Increasing number of cells expressing red fluorescence might translate to reduced cytotoxicity and this was achieved without any interference from exogenous protein. This improvement will help in widening the application scope of the DsRed2 protein.

It has been reported that overexpression of Bcl-xL increased percentage of DsRed-Express2 expressing cells by 2 to 3% (ref.[16]). On a comparative note, we have obtained slightly better increase in percentage of cells expressing red fluorescence and without any exogenous interference. From an application point of view, the increase is significant because the percentage of cells expressing native DsRed2 is comparatively low and secondly if we increase the total count of cells taken into consideration then the number depicting the increase would be significantly large.

Even though we have demonstrated an increase in number of cells expressing red fluorescent protein, further research needs to be done in order to confirm the mechanism behind the increase and to check whether further increase is possible. In theory, it is possible to achieve larger number of cells expressing red fluorescence than reported, by increasing the length of intronic sequence. This increase in length will prolong the time required for transcription, translation[17] and in turn protein aggregation; which might reduce the aggregation-mediated cytotoxicity, resulting in an increase in the number of cells expressing red fluorescent protein.

Apart from increasing the percentage of cells expressing red fluorescence, intron cloned DsRed2 can also be used in acquiring advanced details of splicing mechanism as well as for cloning intronic microRNA (miRNA); in our case we have introduced cloning sites within its sequence. Intronic miRNA is a class of miRNAs derived from the processing of non-protein-coding regions of gene transcripts. The intronic miRNAs differ uniquely from intergenic miRNAs in the requirement of RNA polymerase (Pol)-II and spliceosomal components for its biogenesis[18-19]. Cloning of intronic miRNA within the intron of DsRed2 will firstly serve as an indicator for miRNA expression and secondly it will help to increase miRNA expression by readily promoting spliceosome assembly and splicing as the adjoining sequences contain prominent splicing sites.

One prominent use of this construct would be to en-

hance expression of genes by cloning any recombinant gene downstream of intron cloned DsRed2 (ref.[20]); the underlying mechanism is unknown but might be due to increase in stability of the fusion protein formed after splicing. Another application includes studying RNAi by targeting the cloned small interfering RNAs (siRNA) against the red fluorescent protein[21]. For RNAi study dual marker system like DsRed2 and eGFP is essential as the second marker i.e. eGFP will confirm expression of the construct since absence of red fluorescence gives rise to two possibilities, the construct is not expressed or successful targeting of siRNA.

CONCLUSION

We have demonstrated that cloning of intron within the DsRed2 gene helps to increase the number of cells expressing red fluorescent protein.

To the best of our knowledge, this is the first time an artificial intron has been cloned within the ORF of the DsRed2 gene using cyclic ligation assembly. The successful cloning increased the number of cells expressing red fluorescence by approximately four percent compared to native DsRed2 protein.

ABBREVIATIONS

DsRed, *Discosoma sp* Red Fluorescent protein; CLA, Cyclic ligation assembly; ORF, Open reading frame; SOC, Scaffold oligonucleotide connector; miRNA, MicroRNA; siRNA, Small interfering RNAs; RNAi, RNA interference; eGFP, Enhanced green fluorescent protein.

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Author contributions: RP: experiments design and manuscript writing; RP, HH, JC: performed the experiments; RP, HH, JC, SF, JM: data analysis; TS: contributed analysis tools.

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REFERENCES

1. Mizuno H, Sawano A, Eli P, Hama H, Miyawaki A. Red fluorescent protein from Discosoma as a fusion tag and a partner for fluorescence resonance energy transfer. Biochemistry 2001;40:2502-10.
2. Matz MV, Fradkov AF, Labas YA, Savitsky AP, Zaraisky AG, Markelov ML, Lukyanov SA. Fluorescent proteins from nonbioluminescent Anthozoa species. Nat Biotechnol 1999;17:969-73.
3. Lin SL, Chang DC, Chang-Lin S, Lin CH, Wu DT, Chen DT, Ying SY. Mir-302 reprograms human skin cancer cells into a pluripotent ES-cell-like state. *RNA* 2008;14:2115-24.
4. Lin SL, Ying SY. Gene silencing in vitro and in vivo using intronic microRNAs. Methods Mol Biol 2006;342:295-312.
5. Lin SL, Chang SJ, Ying SY. Transgene-like animal models using intronic microRNAs. Methods Mol Biol 2006;342:321-34.
6. Xia XG, Zhou H, Samper E, Melov S, Xu Z. Pol II-expressed shRNA knocks down Sod2 gene expression and causes phenotypes of the gene knockout in mice. PLoS Genet 2006;2:e10.
7. Roth TL, Milenkovic L, Scott MP. A rapid and simple method for DNA engineering using cycled ligation assembly. PLoS One 2014;9:e107329.
8. Takahashi M, Yamaguchi E, Uchida T. Thermophilic DNA ligase. Purification and properties of the enzyme from Thermus thermophilus HB8. J Biol Chem 1984;259:10041-7.
9. Hebsgaard SM, Korning PG, Tolstrup N, Engelbrecht J, Rouzé P, Brunak S. Splice site prediction in Arabidopsis thaliana pre-mRNA by combining local and global sequence information. Nucleic Acids Res 1996;24:3439-52.
10. Khalil AS, Collins JJ. Synthetic biology: applications come of age. Nat Rev Genet 2010;11:367-79.
11. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA,3rd, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 2009;6:343-5.
12. Jiang X, Yang J, Zhang H, Zou H, Wang C, Xian M. In vitro assembly of multiple DNA fragments using successive hybridization. PLoS One 2012;7:e30267.
13. Engler C, Kandzia R, Marillonnet S. A one pot, one step, precision cloning method with high throughput capability. PLoS One 2008;3:e3647.
14. Engler C, Gruetzner R, Kandzia R, Marillonnet S. Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. PLoS One 2009;4:e5553.
15. Strongin DE, Bevis B, Khuong N, Downing ME, Strack RL, Sundaram K, Glick BS, Keenan RJ. Structural rearrangements near the chromophore influence the maturation speed and brightness of DsRed variants. Protein Eng Des Sel 2007;20:525-34.
16. Zhou J, Lin J, Zhou C, Deng X, Xia B. Cytotoxicity of red fluorescent protein DsRed is associated with the suppression of Bcl-xL translation. FEBS Lett 2011;585:821-7.
17. Swinburne IA, Miguez DG, Landgraf D, Silver PA. Intron length increases oscillatory periods of gene expression in animal cells. Genes Dev 2008;22:2342-6.
18. Danin-Kreiselman M, Lee CY, Chanfreau G. RNAse III-mediated degradation of unspliced pre-mRNAs and lariat introns. Mol Cell 2003;11:1279-89.
19. Lin SL, Chang D, Wu DY, Ying SY. A novel RNA splicing-mediated gene silencing mechanism potential for genome evolution. Biochem Biophys Res Commun 2003;310:754-60.
20. Lacy-Hulbert A, Thomas R, Li XP, Lilley CE, Coffin RS, Roes J. Interruption of coding sequences by heterologous introns can enhance the functional expression of recombinant genes. Gene Ther 2001;8:649-53.
21. Seyhan AA. A multiplexed miRNA and transgene expression platform for simultaneous repression and expression of protein coding sequences. Mol Biosyst 2016;12:295-312.