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Efficient Seamless Cloning of Full-length and Truncated GFP-tagged FAM210B Plasmids and Comparison with Traditional Restriction Cloning

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Abstract

The aim was to construct a series of expressing plasmids containing full-length or truncated FAM210B cDNA fused with GFP by seamless cloning technique. According to the design principle of seamless cloning, primers were designed and synthesized to amplify corresponding cDNA. On the other hand, the GFP-vector plasmid was linearized by restriction enzyme digestion, then they were ligated with each other by homologous recombinant kit. Finally, the competent E. coli DH5 was used to transform the linked products. In this study, we successfully constructed the fusion plasimds of FAM210B or its truncates with GFP by seamless cloning technology and verified by sequencing. The expressions of various recombinant GFP-tagged fusion plasimds were confirmed by fluorescence microscopy after transfection into H1299 cells. The successful construction and expression of these fusion plasmids would provide tools for the functional study of FAM210B in the future.

Keywords

Seamless Cloning; Restriction Cloning; FAM210B; Structural Domain.

1. Introduction

DNA cloning is undoubtedly one of the most basic molecular biology techniques[1]. There are many methods of DNA cloning, such as traditional T4 ligase mediated DNA cloning, EASY cloning, seamless cloning and so on. T4 ligase-mediated cloning is to use restriction enzyme to cut the carrier and fragment to produce the matched end, and then use T4 ligase to connect or TA clone, which is a tedious and time-consuming process.

Seamless Cloning/In-Fusion Cloning is a new, rapid and simple cloning method, which can insert one or more fragments of target DNA at any site of plasmid. The vector and target DNA can be closely linked by the pairing of homologous sequence bases, without any restriction enzymes and ligases. Therefore, the recombinant plasimd can be cloned with high efficiency, which can bypass the laborious traditional double digestion and enzyme ligation, and only need one step recombination procedure.

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There are many ways to achieve seamless genetic fusion, including overlap PCR[2, 3], inverse PCR[4], site-directed mutagenesis[5, 6], type IIS restriction enzyme-mediated gene fusion[7, 8], ligation-independent cloning (LIC)[9], RecA-dependent/independent recombination[10], and gene synthesis[11]. Seamless cloning is a process that allows two or more DNA fragments to be precisely joined together. It plays an important role in promoter and exon studies[12-14], gene and protein markers[15, 16], domain studies, and gene mutation[17] experiments.

FAM210B is a protein encoded by 576 nucleotides with a molecular weight of about 20 kDa. It contains an unknown functional domain 1279 (DUF1279), which contains a transmembranes peptide. Researchers from HuaZhong University of Science and Technology found that FAM210B is located in the outer membrane of mitochondria, and its N-terminal 47 amino acids MTS (mitochondrial targeting sequence) are necessary for its mitochondrial localization[18]. With the completion of various genome sequencing projects, the functional analysis of gene products has entered the stage[15]. Functional proteins are usually composed of several domains. In order to elucidate the function of a particular domain in a defined protein, it is usually necessary to delete or replace some domain in the homologous protein, and seamless cloning will play an important role in the experiment of domain deletion or exchange [15, 19].

In this study, we constructed three expression plasmids of FAM210B or its truncated fragments fused with EGFP using seamless cloning technology. The aim is to pave the way for the follow-up study on the function, structure and localization of FAM210B protein. At the same time, two methods of molecular cloning were analyzed to highlight the advantages of seamless cloning.

2. Materials and Methods

2.1 Molecular Cloning

2.1.1 Primer Design

SnapGene software was used to design specific primers for the target genes, and the following corresponding primers were synthesized by the company (Tsingke Biotechnology, China):

EGFP-MTS-forward: TCGAGCTCAAGCTTCGAATTCATGGCCGGGTTGCTGGCGTTGC;

EGFP-MTS-reverse: ATGGTGGCGACCGGTGGATCCGTCTAGCCTGGGCGGGAG;

EGFP-△MTS-forward: TCGAGCTCAAGCTTCGAATTCGCCCGGCTGCTCCGC;

EGFP-△MTS-reverse: ATGGTGGCGACCGGTGGATCCAGGTTTTGCAGCTGG;

EGFP-FAM210B-forward:
TCGAGCTCAAGCTTCGAATTCATGGCCGGGTTGCTGGCGTTGC;

EGFP-FAM210B-reverse: ATGGTGGCGACCGGTGGATCCAGGTTTTGCGCTGG.

2.1.2 Amplification and Purification of Inserted cDNA

The volume of reaction system was 20 μL, containing 2× Taq PCR MasterMix (AG, China)10 μL, 10 μmol/L upstream and downstream primers 1 μL each, and template cDNA 1 μL. The amplification conditions were pre-denaturation for 5 min at 98℃, denaturation at 98℃ for 30 s, annealing at 58℃ for 15 s, extension at 72℃ for 45 s, 35 cycles, full extension at 72℃ for 10 min, and preservation at 4℃. PCR products were purified and recovered using FitAmp Gel DNA Isolation Kit (Magen, China).

2.1.3 Linearization of the Vector Plasmid

The vector was linearized using restriction endonuclease (Takara, China) at 37℃ for 4 h, and then purified and recovered with the FitAmp Gel DNA Isolation Kit.

2.1.4 Ligation

The amplified PCR products were separately linked to linearized pEGFP-N1 vectors using One Step Cloning Kit (Vazyme, China) at 37℃ for 30 min. And the amount of PCR products and vector plasmid is calculated as follows:

Insert PCR products (X) = [0.04 x Insert base pairs] ng (0.06pmol);

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Vector plasmid (Y) = [0.02 x base pairs of cloned carrier] ng (0.03pmol).

2.1.5 Transformation and Colony Screening

The ligands were directly transformed into E. coli DH5α and screened on LB culture plates containing 100 μg/mL ampicillin to obtain the transformed bacteria. Then the monoclonal colonies were selected for PCR vertifying and sequencing to confirm the correct ligation. Plasmid with correct sequencing result was extracted with kit (Magen, China) and reserved at -20°C.

2.2 Cell Culture and Plasmid Transfection

Human non-small cell lung adenocarcinoma cell line H1299 (Cell Resource Center, Institute of life science Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM(Gibco BRL,USA) and supported with 10% FBS in humidified chambers at 37°C in an atmosphere with 5% CO2. When the cells reach to 70%-80% confluence rate, they were used to transfect with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) and the plasmid. After 48 hours at 5% CO2 and 37°C, the cells were observed under an inverted microscope.

3. Results

3.1 Comparison of Traditional Restriction Cloning with Seamless Cloning

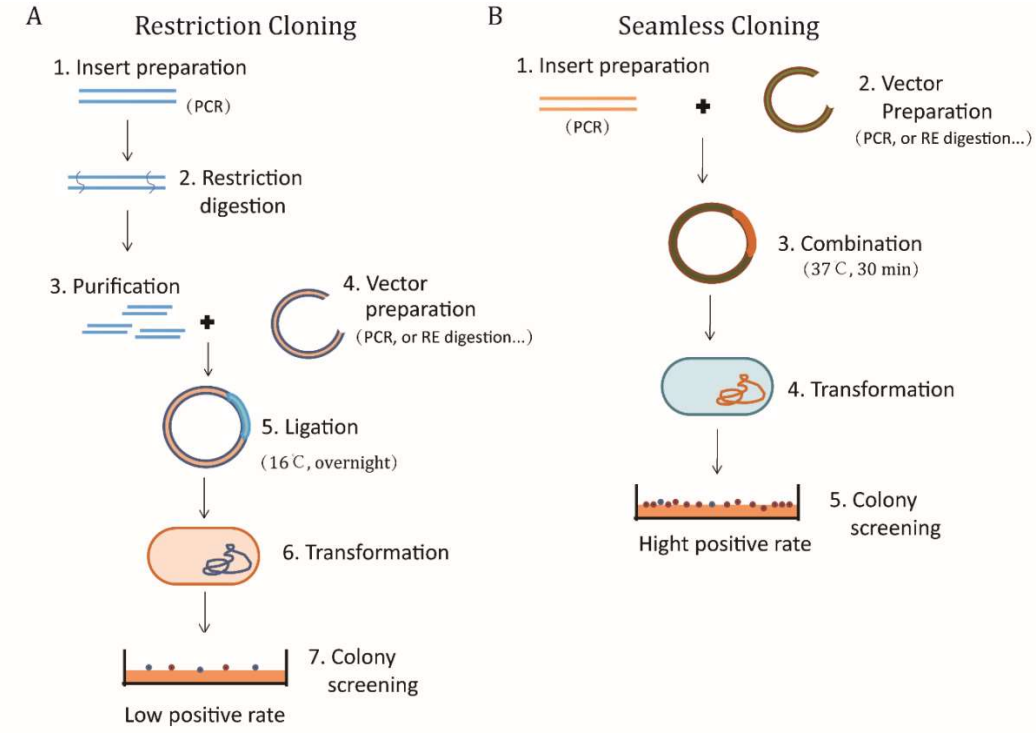

Fig. 1 The model schemes of plasmid construction. (A) Restriction Cloning. (B) Seamless Cloning.

In order to choose a efficient method for plasmid constructing, we compared the characteristics of Restriction Cloning and Seamless Cloning, as shown in Fig. 1. The amplification of the target gene, linearization of the vector, and subsequent transformation steps are the same in both methods. However, Restriction Cloning has the disadvantages of taking longer time and displaying low positive rate. Moreover, in Restriction Cloning, the design of PCR primers needs to find suitable restriction endonuclease, which must exist on the vector plasmid but not on the gene itself, so the introduced restriction sites are relatively limited. However, Seamless Cloning has no requirement on the restriction site, and the cDNA of interest can be inserted into any site of any vector. In addition, the PCR products in Restriction Cloning need to go through a series of processes such as enzyme digestion, glue recovery and ligation before they are directed to the target vector, multiple cDNAs can only be spliced several times. Seamless Cloning only needs one reaction to complete directional

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cloning, omiting the process of enzyme digestion and enzyme ligation, and can simultaneously clone multiple cDNA fragments.

3.2 Primer Design for Seamless Cloning

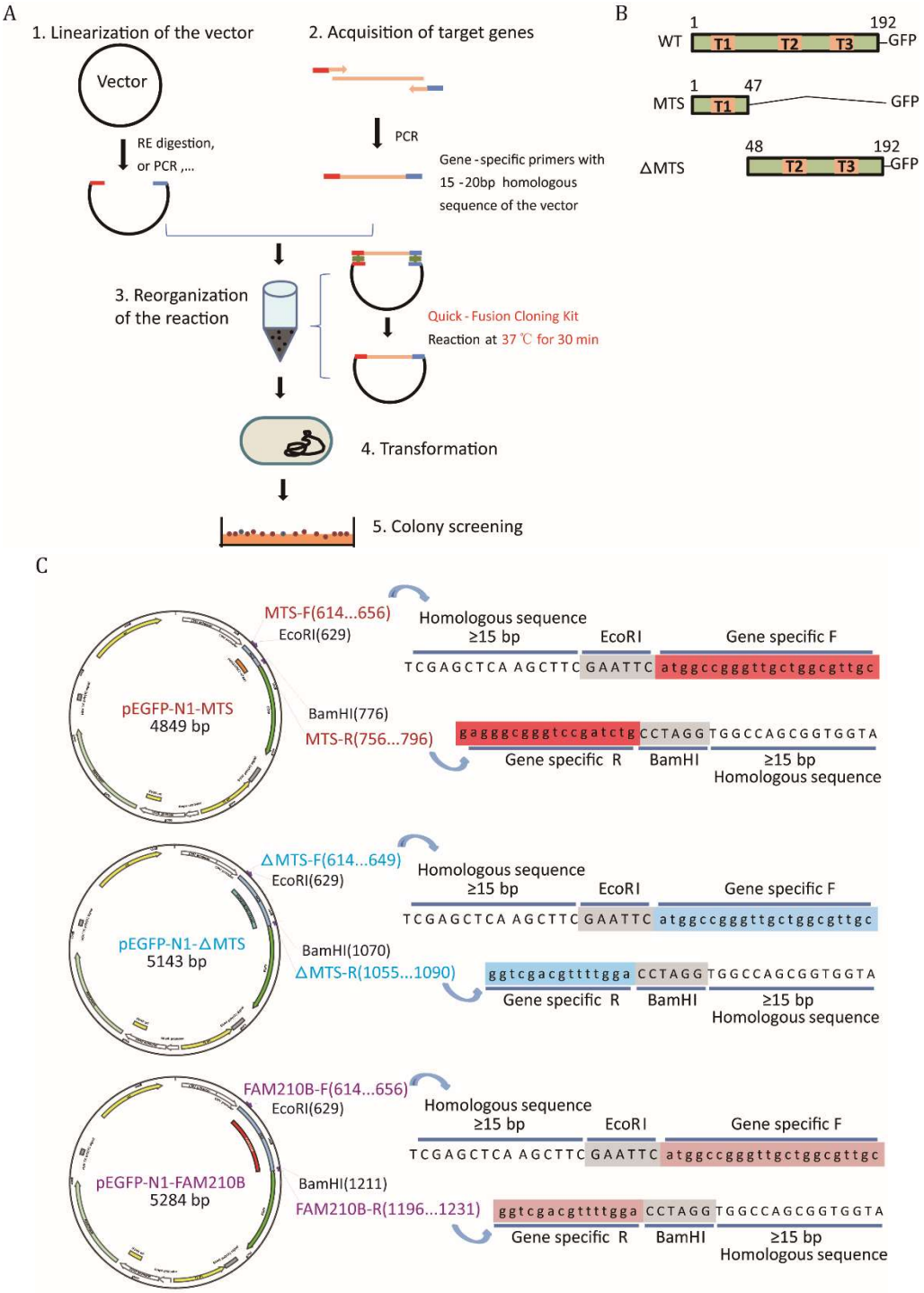

Fig. 2 Primers design for seamless cloning. (A) The specific process of seamless cloning. (B) The schematic diagram of the primary structure of FAM210B protein and its domain. (C) The design of primers for FAM210B and its truncated domains.

The two main advantages of seamless cloning are time saving and high positive rate. Fig. 2A shows the detailed processes of seamless cloning, including linearization of the vector by enzyme digestion or reverse PCR, amplification of the inserted cDNA fragment with homologous sequence, recombination reaction of the linearized vector and the inserted fragment using the Quick-Fusion

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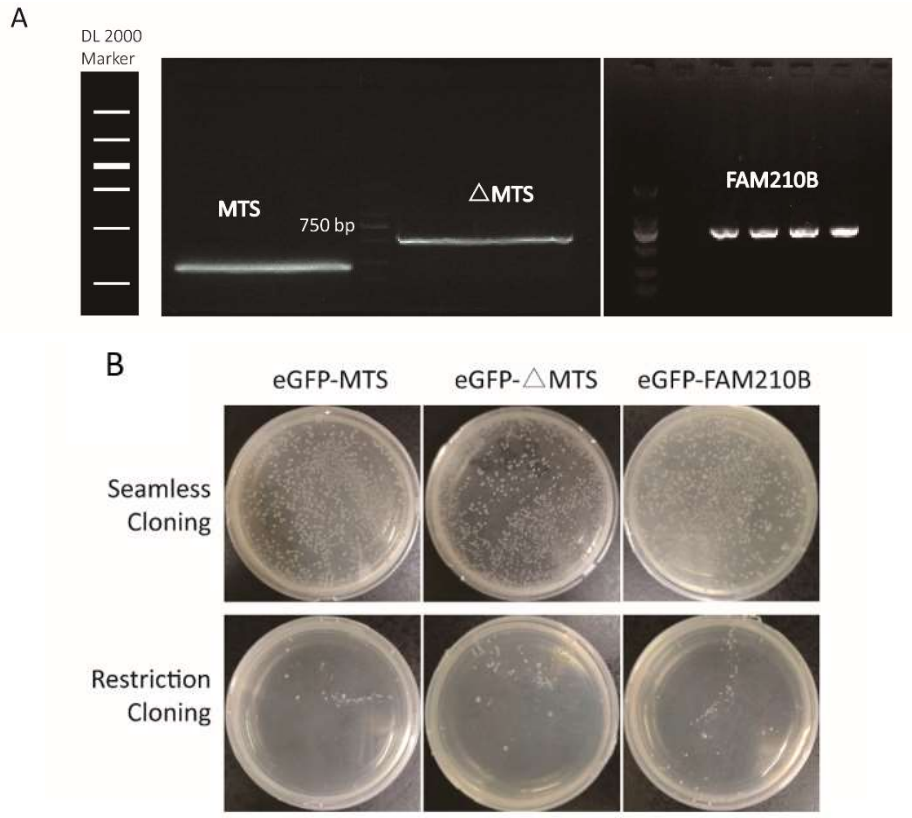
Cloning Kit at 37℃ for 30 min, transformation of the recombinant product and positive screening. Human protein sequence analysis predicted that the N-terminus of FAM210B might have a cleavage sequence as a mitochondrial targeting sequence (MTS)[18]. According to the prediction analysis, FAM210B was truncated into MTS and △MTS as shown in Fig. 2B, and GFP were added to their C- terminus. As shown in Fig. 2C, primers for FAM210B and its truncated domains were designed according to the design principle of seamless clone primers. The homologous sequences of the two ends of the linearized vector were introduced into the 5 'end of the forward and reverse amplification primer of the inserted fragment, so that the ends of the amplified inserted fragment had the same homologous sequences corresponding to the two ends of the linearized clone vector, which had 15-20 bp, but did not include the restriction site.

3.3 Construction and Validation of GFP-MTS, GFP-△MTS or GFP-FAM210B Plasmids

The primers designed and synthesized above were used for PCR amplification, and agarose gel electrophoresis was used to verify the PCR products, as shown in Fig. 3A. The actual size of PCR product was consistent with the predicted one, indicating that the designed primers were correct and the PCR reaction procedure was appropriate. The purified PCR product was then linked to the linearized GFP plasmid after digest, and the recombinant product was transformed into competent cell DH5a. As shown in Fig. 3B, hundreds of monoclone were formed on the recombination reaction transformation plate after overnight culture, while the number of clones from transformation plate cultured with the restriction cloning product is significantly less than the former. A number of clones on the recombinant reaction transformation plate were selected for colony PCR identification. The clones were correct, and bands with length consistent with the size of inserted cDNA fragments appeared (Fig. 3C). The colonies identified as positive by PCR were inoculated into liquid LB medium containing appropriate antibiotics for overnight culture, and plasmids were extracted and sequenced correctly as shown in Fig. 3D.



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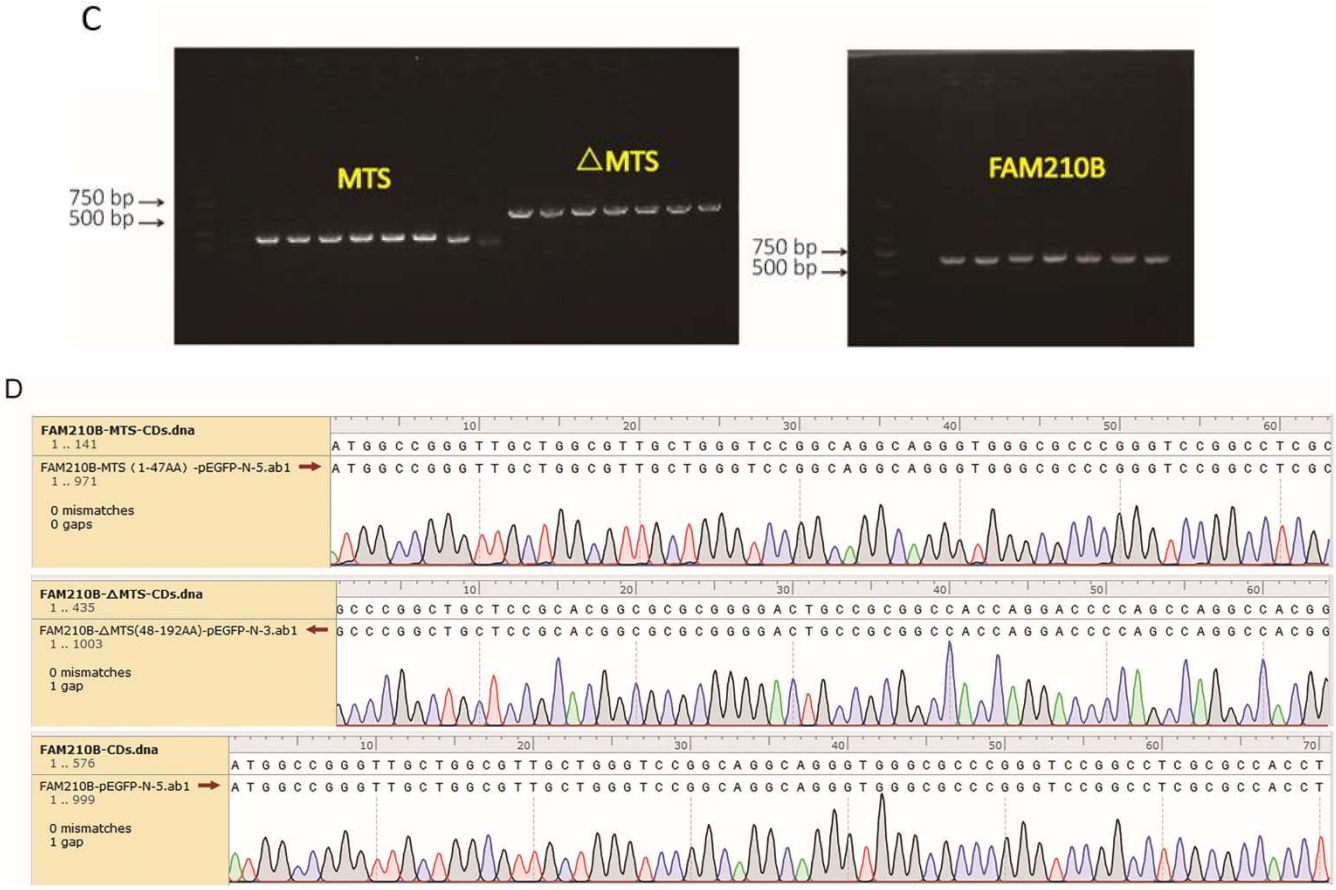

Fig. 3 Construction and validation of GFP-MTS,GFP-△MTS and GFP-FAM210B plasmids. (A) PCR products were detected by agarose gel electrophoresis. (B) Tablets for overnight culture. (C) Colony PCR assay by agarose gel electrophoresis. (D) Plasmids sequencing data.

3.4 Validation of the Expression of the Recombinant Plasmids

The recombinant plasmid was separately transfected into H1299 cells, and the fusion expression of GFP-MTS, GFP-△MTS and GFP-FAM210B were observed by inverted fluorescence microscope at 48 h after transfection. The green fluorescence of H1299 cells after transfection of the three recombinant plasmids GFP-MTS, GFP-△MTS and GFP-FAM210B, separately, was detected, and the transfection efficiency reached more than 70%.

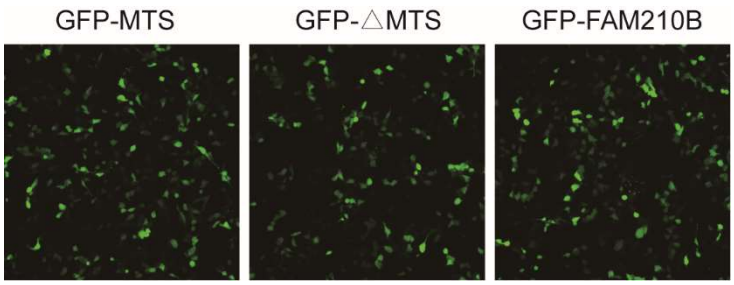

Fig. 4 H1299 cells were transfected with GFP-MTS, GFP-△MTS or GFP-FAM210B plasmid and observed under fluorescence microscope(x 100).

4. Discussion

Compared with traditional cloning methods, seamless cloning has several obvious advantages. Firstly, the selection of loci is flexible and the gene is cloned at any position of the vector. The second is that it is fast and simple. the laborious processes of enzyme cutting, gel cutting recovery and enzyme linking necessary for traditional restriction cloning are unnecessary in this method, as well as the

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operation of phosphorylation and terminal replenishment. Third, it can be precisely oriented, so the cloning efficiency and positive rate are high. The seamless cloning also can carry out multiple target gene recombinations at once. Thus, seamless cloning is an excellent choice in terms of time saving and accuracy.

Classical gene fusion techniques including type II restriction enzyme digestion and DNA linking reactions (the so-called cut-and-paste reaction) have been used as standard procedures for producing hybrid genes. However, such processes often leave restriction sites at the junction or introduce additional amino acid residues. These additional sequences change the spacing between elements and may adversely affect the structure and activity of the fusion protein, thus potentially interfering with the precise study of the fusion gene. Seamless cloning avoids these problems because it can connect any fragment to any vector and can connect multiple fragments simultaneously. Therefore, it has been widely used in molecular cloning.

Acknowledgments

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