Designing a Restriction Enzyme-free Method to Construct a MicroRNA Precursor gene for microRNA Cloning

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Research Article

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Abstract

Common cloning strategies depend on the enzymatic digestion of the insert. In addition, the enzymatic digestion of PCR product ends by restriction enzymes is of low efficiency. These limitations are related to the need for enzymatic digestion to produce sticky ends in the insert sequence. Hence, in the present study, we aimed to present a new generation of pre-microRNA cloning method without using restriction enzymes for constructing pre-microRNA gene. In this strategy, by engineering an expression vector's sequence and designing two intelligent primer sets for two consecutive PCR reactions, the pre-microRNA sequence with appropriate restriction sites related to the expression vector was produced, without restriction enzymes. The recombinant expression vector was transfected into HEK293 cells, and microRNA-21 expression was assayed in these cells by real-time PCR, confirming the high efficacy of the presented cloning method. The present method can be an inexpensive and reliable method for microRNA precursor cloning by providing a high-performance protocol.

1. Introduction

MicroRNAs are non-coding RNA molecules that act as endogenous inhibitors of gene expression. MicroRNAs are commonly transcribed by type II RNA polymerases as a long-acting precursor called primary-microRNA (pre-microRNA). They are then processed in the nucleus by an enzymatic complex, (RNase III) and Pasha / DGCR8, and converted to a liberated hairpin referred to precursor-microRNA (pre-microRNA) with approximately 60–80 nucleotides in length. Pre-microRNA is then transported from the nucleus to the cytoplasm by exportin-5 protein, cleaved by the RNase III enzyme called Dicer, and converted to a double-stranded 18–24 nucleotide RNA molecule. The RNA molecule is located in the RNA-induced silencing complex (RISC) and, depending on the relative thermodynamic stability of the 5'-end, one of the strands remains as the guide strand or mature microRNA and another strand is degraded. MicroRNA guides the RISC to its target mRNA, cleaving the mRNA or preventing translation [1–2]. MicroRNAs are widely used in biological processes, including cell proliferation and apoptosis. Aberrant expression of microRNAs can lead to disorders and malignancies in humans, including cancer. In addition, altered microRNA expression levels are associated with the type and stage of cancers [1]. These molecules regulate gene expression through specific interaction with 3'-untranslational region (3'-UTR) of mRNA after transcription. The discovery of microRNAs as the main regulators of genes opens up a new avenue to develop more powerful and effective ways to control and treatment of diseases [3]. Due to the potential of microRNAs in targeting a large number of mRNAs, these molecules are involved in many biological phenomena, and therefore, many of the researches have been conducted to use microRNAs in the diagnosis and treatment of diseases [4–6]. For example, the normal expression of microRNAs that have been removed from the genome or their expression has been reduced can be compensated by microRNA restoration based-therapy through microRNA-mimic or microRNA-mimetic [7]. Therefore, the expression of microRNAs in both in vivo and in vitro can be regulated by the synthesis of pre-microRNA molecules or antisense oligonucleotides, which is a promising prospect for the treatment of a variety of diseases. Pre-microRNA gene cloning is one of the strategies of this technology. In this strategy, the pre-

microRNA gene is cloned into an expression vector. This method has been widely used in many studies to detect microRNAs, detecting their target genes, investigating cell signaling pathways, and generating model cells expressing specific microRNAs [8]. Common cloning methods usually involve using restriction endonucleases to produce DNA fragments with complementary ends that ligate together by a DNA ligase. This process involves preparing the insert (microRNA precursor gene) and the expression vector. These DNAs are cleaved by two specific restriction enzymes that identify the restriction sites on either side of the insert sequence and the multiple cloning site of the vector. By using two different restriction enzymes, two different ends are produced in each of these DNAs, which leads to the directional binding of the insert sequence to the expression vector. Directional cloning is very important for maintaining an open reading frame as well as the activity of regulatory factors [9]. In addition, non-directional cloning can be performed using a restriction enzyme. In this case, a screening step is needed to determine the correct direction of the gene and to prevent the self-ligation, the vector should be dephosphorylated, which reduces the efficiency of the cloning reaction [10].

As mentioned above, common cloning methods depend on the presence of suitable restriction sites in vectors and the insert sequences. Restriction enzymes used in these methods are often expensive [11]. In addition, the low efficiency of enzymatic digestion for PCR products leads to the failure of the common cloning protocol. T-A cloning technology can solve this problem, but this technique is time-consuming and expensive. In addition, using the TA enzyme recognition sites adds extra nucleotides to the insert sequence and may disturb the open reading frame [12]. Therefore, it is better to place the recognition sites of the restriction enzymes on the primers. These limitations are the result of the need for enzymatic digestion to produce sticky ends in the insert sequence. In order to overcome these limitations, Li et al. proposed a cloning method independent of the restriction sites, which is based on homologous recombination and single-stranded annealing under in vitro conditions [13]. In addition, there are several reports of restriction enzyme-independent cloning strategies, including CPEC (circular polymerase extension cloning), SLICE (seamless ligation cloning extract), RSFC (restriction site-free cloning), SLIC (sequence- and ligation-independent cloning), and Gibson assembly [13–15, 18]. Despite the effectiveness of these methods, they are complex and dependent on other special enzymes and laborious and time-consuming protocols. Therefore, designing a simple, fast and accurate method that eliminates the need to use restriction enzymes for producing PCR products with appropriate sticky ends paves the way for using pre-microRNA cloning strategy for the diagnostic and treatment.

In the present study, we decided to provide a new generation of precursor microRNA cloning method without need to restriction enzymes for producing PCR products with appropriate sticky ends (insert sequence). In the proposed strategy, by engineering an expression vector's sequence and designing two intelligent primer sets for two consecutive PCR reactions, the pre-microRNA sequence with appropriate sticky ends counterpart with the expression vector were produced, without restriction enzymes (Fig. 1). The present method can be a fast, convenient, inexpensive and reliable method for microRNA precursor cloning by providing a high-performance protocol.

2. Materials And Methods

2.1. Pre-microRNA gene amplification

In this study, microRNA-21 was used as a microRNA model. In order to amplify the pre-microRNA-21 gene, 2 µg template DNA (extracted from HEK293 human cell line), 0.5 µL of each of the forward and reverse primers (Table 1), 7 µL of water and 10 µL of PCR master mix were mixed. The PCR reaction program includes an initial denaturation at 95°C for 10 min, 38 cycles including denaturation at 95°C for 20 s and annealing at 61°C for 20 s and DNA extension at 72°C for 40 s. PCR products were loaded in 1% agarose gel.

Table 1
Primers used for pre-microRNA-21 gene amplification.

F-primer	ATAGAAGCGGCCGCGGGTTCGATCTTAACAGGCC
R-primer	GCATTCACTATCAAACCCACAATGCAGCT

2.2. Producing pre-microRNA-21 gene with appropriate sticky ends

To produce the sticky ends in the pre-microRNA gene, a primer set was used (Table 2). Two separate PCR reactions were performed that each of which performs with the forward primer and reverse primer, respectively. In these reactions, the product of the previous PCR reaction was used as the template DNA. The PCR reaction program for both reactions includes an initial denaturation at 95°C for 5 min, 60 cycles including denaturation at 95°C for 55 s, annealing at 58°C for 20 s and DNA extension at 72°C for 40 s. The first PCR reaction results in the production of a single forward strand (complement to the sense strand of microRNA-21 precursor gene), which has an overhanging end that is complementary with one of the vector's sticky end. The second PCR reaction results in the production of a single reverse strand which is complement with an anti-sense strand of the microRNA-21 precursor gene and has a complementary overhang that is complementary with another sticky end of the vector. In order to annealing two synthesized forward and reverse strands, 3 µL of the forward strand, 3 µL of the reverse strand, 2 µL of annealing buffer, 4 µL of NaCl (0.5 M) and 8 µL of water were mixed and the mixture was annealed by heating at 95°C for 5 min, gradually cooled to 25°C in the thermo-cycler. In this way, the pre-microRNA gene with appropriate sticky ends (BbsI restriction sites, CACC and AAAC) is produced.

Table 2
Primers used to perform the PCR reaction for producing forward and reverse strands, as well as colony PCR.

F-primer	CACCTATAGAAGCGGCC
R-primer	AAACTGCATTCACTATC

Table 3
Oligonucleotides used for engineering the expression vector.

Oligonucleotide-1	GGCCGCCACCGGGTCTTCGAGAAGACCTGTTTTG
Oligonucleotide-2	TCGACAAAACAGGTCTTCTCGAAGACCCGGTGGC

2.3. Construction of a pre-microRNA expressing vector with BbsI recognition sites and GTTT and GGTG restriction sites

In this study, the pJEBB vector was used for microRNA precursor gene cloning and microRNA overexpression. This vector was gifted by Dr. Karim Rahimi (Department of Molecular Biology and Genetics - Gene Expression and Gene Medicine, Aarhus University, Denmark). This vector has restriction sites for Not I and Sal I enzymes. To create appropriate sticky ends in this vector that be proportional to the synthesized pre-microRNA gene sequence, the BbsI enzyme recognition site, and GTTT and GGTG sequences as restriction sites were located in the vector (Fig. 2). In order to produce pJEBB vector containing BbsI recognition site and GTTT and GGTG restriction sites, two complementary oligonucleotides were used, which have two BbsI recognition sites, two different desired sequences (GTTT and GGTG) and the restriction sites of NotI and Sall enzymes at the overhanging 3'- and 5'- ends of their double-stranded hybrids, respectively (Fig. 2). In order to annealing two oligonucleotides, 3 µL of oligonucleotide – 1, 3 µL of oligonucleotide – 2, 14 µL of annealing buffer (10 mM HCl, 10 mM DTT, 10 mM PEG, 100 mM NaCl and 10 mM MgCl₂) were mixed and the mixture was annealed by heating at 95°C for 5 min, gradually cooled to 25°C in the thermo-cycler. This double-stranded product is called annealing product. In order to ligation, the annealing product to the pJEBB linear vector, which has sticky ends of restriction sites of Not I and Sal I enzymes, 5 µL of linear vector, 2.5 µL of ligase buffer, 1 µL of T4 DNA ligase, 1 µL of annealing product, 0.5 µL of NaCl (0.5 M) and 10 µL of water were mixed and the mixture was incubated at 22°C for 1 h. Then, the transformation and screening steps were performed and to confirm the ligation process, the colony PCR technique was performed using primers in Table 4. The engineered expression vector was digested and linearized by the BbsI restriction enzyme. For this purpose, 5 µL of the circular vector, 5 µL of universal buffer and 0.2 µL of BbsI enzyme in a final volume of 50 µL were mixed and the mixture was incubated at 37°C for 45 min. To deactivate the enzyme, the reaction mixture was incubated at 65°C for 10 minutes.

Table 4
Primers used to perform colony PCR related to the expression vector.

Oligonucleotide-1	GGCCGCCACCGGGTCTTCGAGAAGACCTGTTTTG
RVpJEBB	GTCGAGGACCTGGAGGG
FVpJEBB	CTGCCCCGACAACCACTACC
Oligonucleotide-2	TCGACAAAACAGGTCTTCTCGAAGACCCGGTGGC

2.4. Generation and transformation of the recombinant vector containing pre-microRNA gene

To ligation of the generated pre-microRNA gene to the expression vector, 7 μL of deionized water, 2 μL of pre-microRNA, 4 μL of the vector (with a concentration of 20–30 ng/ μL), 2 μL of T4 DNA ligase buffer and 0.2 μL of T4 DNA ligase enzyme were mixed and the mixture was incubated at 22°C for 1 h. To transform the ligation product to DH5 α , 7 μL of the ligation product was added to 100 μL of competent cells. After mixing, the tube containing the mixture was incubated on ice for 30 min. The tube was placed into a 42°C water bath for 60 s and then put back on the ice for 2 min. 800 μL of LB media was added to the bacteria and they were grown in 37°C shaking incubator for 120 min. For screening, all of the transformations were placed onto an LB agar plate containing the ampicillin antibiotic. The PUC19 vector was used as a positive control. A number of colonies were observed on the culture medium after 16–24 hours.

2.5. Colony PCR technique

To confirm the ligation of the insert sequence (microRNA precursor) with the expression vector, colony PCR was performed. One colony was inoculated at 10 μL of LB medium. In order to perform colony PCR, 10 μL of PCR master mix, 0.5 μL of the primers in Table 2 (30 μM) and 8 μL of water were mixed. 2 μL of the inoculated bacteria resuspended to the reaction. The PCR program for the reaction includes an initial denaturation at 95°C for 10 min, 38 cycles including denaturation at 95°C for 20 s, annealing at 58°C for 20 s and DNA extension at 72°C for 20 s.

2.6. Bacterial culture and recombinant plasmid extraction

After confirmation by colony PCR, a colony of recombinant bacteria was dissolved in 10 mL LB medium containing ampicillin (1 $\mu\text{g}/\text{mL}$). After 16–24 h, the bacteria were precipitated and the plasmids were isolated according to the instruction of GeneAll plasmid prep kit (Gene All Biotechnology, South Korea).

2.7. Cell culture and transfection

To evaluate microRNA overexpression, before transfection, HEK293 cells were seeded in a 12-well cell-culture plate at a concentration of 80×10^3 cells per well in 900 μL of DMEM high glucose culture medium containing 1% penicillin-streptomycin antibiotic. After a day, at a confluence of 70–80%, the cells were transfected with the recombinant expression vector. Transfection was done using Turbofect (Invitrogen) according to the manufacturer's instructions and the plate was placed in a 37°C incubator for 48 hours. We used 3 μL of Turbofect for 2 μg vector and the final volume for 12-well plates was 1000 μL .

2.8. RNA extraction and cDNA synthesis

After 24 h of incubation following transfection, total cellular RNA was collected and extracted. The extraction of total RNA from the control and transfected cells was performed using the TRIzol reagent (Cinnagene, Iran) according to the manufacture's instruction. The quality of the extracted RNAs was evaluated by spectrophotometry. MicroRNA-21 and RNA U48 were reverse transcribed for cDNA synthesis by cDNA synthesis kit (Parstoos company, Iran) and specific primers for microRNA-21 and RNA U48 (Table 5), respectively. RNA U48 was used as the endogenous reference gene.

Table 5
RT-PCR reaction primers.

miR-21 RT Primer	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTTTGA
U48 RT Primer	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTTTGG

2.9. Real-time PCR

Expression levels of microRNA-21 and the endogenous control (RNA U48) were determined by real-time quantitative PCR. The primer sequences were listed in Table 6. The reaction was performed using 1 μ L of cDNA samples, 1 μ L of each primer and 10 μ L of SYBR Green PCR Master Mix (Parstoos company, Iran) in a total volume of 20 μ L. The Analytik Jena system was used to perform real-time PCR. The PCR program for the reaction includes an initial denaturation at 95°C for 3 min, 38 cycles including denaturation at 95°C for 8 s, annealing at 61°C for 20 s and DNA extension at 72°C for 15 s.

Table 6
Primers used to perform real-time PCR.

Mir21F primer	GGCGTAGCTTATCAGACTGATG
U48F primer	TGATGACCCCAGGTAAGTCTG
Ri (Revers primer for miR-21 and U48)	CCAGTGAGCAGAGTGACG

2.10. Statistical analysis

Statistical analysis was performed by SPSS statistics version 22. Data were represented as means $\pm$ Standard deviation and a P-value of < 0.05 was regarded as significant for the results.

3. Results

3.1. Primer design and generation of microRNA precursor with intended sticky ends without using restriction enzyme

In this study, microRNA-21 was used as a microRNA model. The primers for amplification of the pre-microRNA gene were designed so that the ATAGAAGGCGGCC sequence be at the 5'-end of the forward primer and the GCATTCACTATC sequence be at the 5'-end of the reverse primer (Fig. 1). These sequences are targeted at the second PCR, which is performed to create sticky ends for the pre-microRNA gene. Other parts of these primers are complementary to the pre-microRNA-21 gene (Table 1). The generation of the pre-microRNA-21 gene was performed by PCR and PCR products were loaded on 1% agarose gel. As expected, a product with a length

Of 430 nucleotides has been produced (Fig. 3).

To create sticky ends in the microRNA precursor, two primers were used. The forward primer is designed such that its 3'-end be complementary with the 12-nucleotides overhang sequence of the forward primer

in the previous PCR and its 5'-end has the CACCT sequence. The reverse primer is also designed so that its 3'-end be complementary with the 12-nucleotides overhang sequence of reverse primer in the previous PCR and its 5'-end has the AAAC sequence (Table 2). In this step, two separate PCRs are performed, in one of which only the forward primer is used and in the other only the reverse primer is used. In these reactions, the products that were produced in the previous PCR reaction are used as the template. The first PCR reaction results in the production of a single forward strand (complement to the sense strand of microRNA-21 precursor gene), which has an overhanging end that is complementary with one of the vector's sticky end. The second PCR reaction results in the production of a single reverse strand which is complement with an anti-sense strand of the microRNA-21 precursor gene and has a complementary overhang that is complementary with another sticky end of the vector. The results of these reactions have been shown in (Fig. 4). Annealing two synthesized forward and reverse strands was performed that the result has been shown in Fig. 4, fourth lane.

3.2. Construction of the pre-microRNA-21 gene expression vector

The pJEBB vector is a type of expression vector for microRNA expression in eukaryotic cells. The vector consists of a CMV promoter, an EGFP open reading frame, an intronic pre-microRNA expression cassette that has been flanked by the SD (splice-donor) and SA (splice-acceptor) sequences, and a beta-globin poly-A termination sequence. The vector is about 4517 nucleotides in length and has an ampicillin-resistant gene. This vector also has two restriction sites for Not I and Sal I enzymes. To construct a linear vector with suitable sticky ends, we used the BbsI recognition site. For creating appropriate sticky ends in this vector, two complementary oligonucleotides were used, which have two BbsI enzyme recognition sites that two different desired sequences (GTTT and GGTG) are located in their upstream. These upstream sequences are cleaved by the BbsI enzyme. In addition, these oligonucleotides are designed such that the restriction sites of NotI and Sall enzymes are located at the overhanging 3'- and 5'- ends of their double-stranded hybrid, respectively (Fig. 2). The ligation of the engineered and linear vector with the double-stranded hybrid was performed by T4 DNA ligase. To confirm the ligation process, two colony PCR was performed (Fig. 5). The vector was then cleaved by the BbsI restriction enzyme. Therefore, the linear vector with GTTT and GGTG ends was generated that are counterpart with the pre-microRNA gene ends. The reaction condition is optimized such that 0.2 μ L of the BbsI enzyme can cleave 1 μ g/ μ L of the vector.

3.3. Construction of the pre-microRNA-21 gene expressing vector

Ligation of pre-microRNA gene sequence with CACC and AAAC ends into the engineered linear expression vector carried out using the T4 DNA ligase enzyme. After transformation of competent bacteria and the screening step, the colony PCR performed using primers in Table 2. The presence of a band with 600 nucleotides in length demonstrated the ligation of the pre-microRNA gene into the expression vector (Fig. 6). Sequencing the constructed vector also confirmed the results of colony PCR (Fig. 7).

3.4. Expression of microRNA-21 in the cells transfected with the pre-microRNA-21 expressing vector

Relative expression of microRNA-21 was measured in cells transfected with the pre-microRNA-21 expressing vector using the real-time PCR method. Un-transfected cells were considered as control and the expression of microRNA-21 in these cells was considered 1. Transfected cells showed a significant increase in microRNA-21 expression (Fig. 8). The relative fold change for microRNA-21 expression in the cells transfected with the pre-microRNA-21 expressing vector compared with control (untransfected cells) was calculated to be 12.5 ($p < 0.05$).

4. Discussion

In the present study, a new generation of precursor microRNA gene cloning methods without the need to restriction enzymes has been presented. In this strategy, by engineering an expression vector sequences and designing two intelligent primer sets for two consecutive PCRs, the pre-microRNA gene sequence with appropriate sticky ends counterpart with the expression vector was produced, without using restriction enzymes. The efficiency of the method was demonstrated using colony PCR and sequencing. In addition, the construct was transfected to HEK293 cells, and the expression of microRNA-21 in these cells was assayed. The results confirmed the high reliability of the cloning method. In the Construction of the pre-microRNA-21 expression vector, the reaction condition is optimized such that 0.2 μL of the BbsI enzyme can cut 1 $\mu\text{g}/\mu\text{L}$ of the vector. In other words, the least quantity of the restriction enzyme is used at this step. It is noteworthy that to provide all linear vectors in all commercial cloning kits, two restriction enzymes is required for producing the vector with different sticky ends. In this study, the selection of the BbsI restriction enzyme is intelligent. Due to the different positioning of the recognition and restriction sites of this enzyme, we can only use one restriction enzyme to create the different sticky ends and therefore prevent the re-ligation process. In addition, the proposed method can perform the cloning process in about 4 h with high quality. The protocol can utilize for any pre-microRNA or DNA fragment and any other expression vector. In general, the present method can be a fast, convenient, inexpensive and reliable method for microRNA precursor cloning by providing a high-performance protocol.

Declarations

Conflicts of interest/Competing interests

The authors declare no conflicts of interest

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Availability of data and material

All data are available

Code availability

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Consent for publication

Not applicable

Author contributions

Mojdeh Amandadi was involved in wrote the paper and performed the analysis.

Mohammad Hashemabadi was involved in conceived and designed the analysis and wrote the paper.

Hosseinali Sasan was involved in conceived and designed of the analysis.

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Figures

Figure 1

schematic protocol for the proposed pre-microRNA cloning method.

Figure 2

schematic representation for the construction of expression vector with sticky ends (GTTT and GGTG).

Figure 3

Products of the first PCR reaction (production of microRNA-21 precursor gene). First lane: DNA marker, second lane: microRNA-21 precursor gene.

Figure 4

First and second lanes show products of the second PCR reactions using the forward and the reverse primer, respectively. The third lane shows the product of annealing reaction. The fourth lane shows the DNA marker.

Figure 5

Colony PCR products of the engineered pJEBB vector containing the BbSI recognition and restriction sites. Lane1: Colony PCR using reverse primer of the vector and the oligonucleotide-1 primer. Lane 2: Colony PCR using forward primer of the vector and the oligonucleotide-2 primer. Lane 3: DNA marker.

Figure 6

Results of colony PCR to confirm the pre-microRNA-21 gene cloning into the pJEBB vector. Lane 1: DNA marker. Lane 2: Colony PCR products.

Figure 7

The results of sequencing the constructed expression vector.

Figure 8

The expression of microRNA-21 expression in the cells transfected with the pre-microRNA-21 expressing vector (test) and un-transfected cells (control) (P-Value <0.05).