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Original Article

A Novel Prokaryotic Green Fluorescent Protein Expression System for Testing Gene Editing Tools Activity Like Zinc Finger Nuclease

Abstract

Background: Gene editing technology has created a revolution in the field of genome editing. The three of the most famous tools in gene editing technology are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases, clustered regularly interspaced short palindromic repeats (CRISPR), and CRISPR-associated systems. As their predictable nature, it is necessary to assess their efficiency. There are some methods for this purpose, but most of them are time labor and complicated. Here, we introduce a new prokaryotic reporter system, which makes it possible to evaluate the efficiency of gene editing tools faster, cheaper, and simpler than previous methods. **Materials and Methods:** At first, the target sites of a custom ZFN, which is designed against a segment of ampicillin resistance gene, were cloned on both sides of green fluorescent protein (GFP) gene to construct pPRO-GFP. Then pPRO-GFP was transformed into *Escherichia coli* TOP10F' that contains pZFN (contains expression cassette of a ZFN against ampicillin resistant gene), or p15A-KanaR as a negative control. The transformed bacteria were cultured on three separate media that contained ampicillin, kanamycin, and ampicillin + kanamycin; then the resulted colonies were assessed by flow cytometry. **Results:** The results of flow cytometry showed a significant difference between the case (bacteria contain pZFN) and control (bacteria contain p15A, KanaR) in MFI (Mean Fluorescence Intensity) ($P < 0.0001$). **Conclusion:** According to ZFN efficiency, it can bind and cut the target sites, the bilateral cutting can affect the intensity of GFP fluorescence. Our flow cytometry results showed that this ZFN could reduce the intensity of GFP color and colony count of bacteria in media containing amp + kana versus control sample.

Keywords: *Gene editing tools, green fluorescent protein expression system, zinc finger nuclease*

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Introduction

Gene editing technology has progressed in recent years; therefore, now there is exceptional situation for investigators to manipulate substantially any gene in a wide range of gene editing project by targeted custom-made nucleases that combined with sequence-specific DNA-binding domains. Three of common genome-editing tools in order to their advent in the gene editing technology world are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR), and CRISPR-associated (CAS) systems. ZFNs are DNA-cutting enzymes built by adding the endonuclease domain from Type IIs restriction endonuclease fokI to a 3 zinc finger protein (ZFP).[1,2] The wonder of ZFN technology is the individual arrays of the ZFPs, which are about 30 amino-acid-long protein domains and they can recognize specific nucleotide triplets by generating hydrogen bonds in the major groove of the DNA. The second component of ZFNs, the FokI endonuclease domain is a nonspecific endonuclease that fulfilling the action of DNA cleavage. FokI works as a dimer, so it is required to position two ZFNs on adjacent recognition sites until cleavage can be take place. In the most routinely used conformation, the ZFP domain of a ZFN (left and right arrays) is contained three or four ZFs.

TALEs are naturally exist in genus *Xanthomonas*, the plant pathogenic bacteria and contain DNA-binding domains consisted of 33–35 amino acid repeat domains that each one identifies a nucleotide. The specificity of TALE is due to two amino acids that they have a hyper variable composition and this position is known as the repeat-variable di-residues.[3,4]

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How to cite this article: Sabzehei F, Kouhpayeh S, Dastjerdeh MS, Khanahmad H, Salehi R, Naderi S, *et al.* A Novel Prokaryotic Green Fluorescent Protein Expression System for Testing Gene Editing Tools Activity Like Zinc Finger Nuclease. Adv Biomed Res 2017;6:155.

Received: March, 2015. **Accepted:** July, 2015.

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DOI: 10.4103/2277-9175.219420

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CRISPR and CAS systems are originated from adaptive immune systems of bacteria. It uses short RNAs for targeting specific DNA sequences and it also can cleave DNA by protein component. The efficiency of these gene editing tools need to specific binding and cutting, so it is necessary to assay their efficiency before use of them in a project. Some methods have been designed to assay efficiency of binding and cutting of ZFN including cell-based luciferase reporter system,[5] surrogate reporter system,[6] cell-I assay,[7] and green fluorescent protein (GFP) gene targeting reporter system in eukaryotic cells.[8] Most of these methods have been set up in the eukaryotic cells and in the more cases need to time and cost labored process. For example in the eukaryotic system, there is an essential need to stable cell line that express reporter gene like GFP. Therefore, developing a cheaper and faster prokaryotic assay for evaluation of gene editing tools can address this issue. In this project, we inserted a prokaryotic GFP gene in an expression cassette that has embedded two multiple cloning sites on both sides of it for subcloning two target sites of each one of the GE tools. Hence, if we transfer the expression cassette of each one of the GE tools to the *Escherichia coli* TOP10F', then this ZFN expressing bacteria must be transformed with pPRO-GFP, if this GE tools can bind and cut the target sites that has embedded on the two sides of GFP gene, the intensity of GFP color in the bacteria must be changed and this method can evaluate the efficiency of this GE tools.

Materials and Methods

Construction of vector containing green fluorescent protein expression cassette

In the first step a gene construct containing lacUV5 promoter, ribosome binding site, prokaryotic codon optimized GFP coding sequence, and terminator rrnB-T1 flanked by two 32 bp of ampicillin resistance gene as target sites, was designed. The gene construct was ordered to synthesize and cloned in pUC57 to Genecust company (GeneCust, Dudelange, Luxembourg). The constructed plasmid was named as pPRO-GFP [Figure 1a]. The pPRO-GFP was transformed into chemically competented *E. coli*. TOP10F' according to Higa and Mendel protocol and selected on LB (Luria Bertani) agar medium contains 100 µg/ml ampicillin.

Flow cytometric analysis

In order to evaluate the GFP expression intensity, flow cytometric analysis was run. The confirmed bacterial clone was inoculated and cultured in LB broth (Merck, NJ, USA) and induced by 1 mM isopropylthio-β-D-galactoside (IPTG) (Sigma-Aldrich, MI, USA) for overnight at 37°C and 250 rpm in a shaking incubator. Cultures were precipitated and subsequently washed twice and resuspended in phosphate buffered saline (PBS) (pH = 7.4). GFP expression intensity was analyzed with an FACSCalibur flow cytometer (Becton Dickinson, NJ, USA) by accumulating up to 100,000 events per tube. The pUC57 transformed *E. coli* Top10F' was used as negative control to enable correct compensation.

pP15A-KanaR plasmid

The pP15A-KanaR plasmid was constructed by Dr. Khanahmad (in press). This plasmid has the p15A origin of replication and the kanamycin resistance gene (KanaR) of M13KO7 helper phage DNA [Figure 1b].

pZFN construct description

The pZFN expression vector containing the left and right ZFP arrays fused to ELD/KKR FOKI variants under the two lacUV5 promoters, cloned in p15A, KanaR vector had been constructed in our lab. The described pZFN was designed to target a specific 25 bp sequence of pTZ57R ampicillin resistance gene. The ZFN target site is provided in [Figure 1c]. The pZFN expression vector length is 4800 bp. The required nucleotide sequences coding left and right ZFP arrays were provided from http://zifit.partners.org/ZiFiT website. The FokI endonuclease domain contained an obligate heterodimeric variant, ELD/KKR, was downloaded from add gene plasmids pCLR2070 and pCLR2068, respectively. The ZFN arrays and linker were codon optimized for *E. coli* and linked to the FOKI KKR and ELD. This ZFN was synthesized by Genecust Company and cloned in the pP15A-Kana between BamHI and XbaI restriction sites (GeneCust, Dudelange, Luxembourg) and named as pZFN [Figure 1d].

The synthesized pZFN was subsequently transformed into competent *E. coli* strain Top10' and selected on LB plate containing 25 µg/ml kanamycin (Merck, NJ, USA) at 37°C. One positive clone was inoculated into LB broth medium supplemented with 25 µg/ml of kanamycin, $ZnCl_2$ 0. 1 mM (Merck, NJ, USA) and incubated overnight at 37°C. To optimize ZFN expression, 1 mM IPTG was added into medium and incubated at 30°C for 4 h.

Evaluation of zinc finger nuclease expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

To detect the expression of ZFN, *E. coli* strain TOP10' cells were collected and precipitated at 12,000 rpm for 3 min. The cells then were resuspended in a lysis solution containing 200 µl 10% sodium dodecyl sulfate (SDS) solution, 200 µl beta-mercaptoethanol, and 600 µl protein loading buffer heated at 96°C for 5 min and run on 12% SDS-polyacrylamide gel electrophoresis (PAGE).

Western blot analysis was then performed with SDS-PAGE kit (Cyto Matin Gene, Iran).

Briefly, the protein bands were transferred into nitrocellulose membrane. The membrane was blocked with the PBS buffer containing 5% nonfat milk overnight, followed by being incubated with horseradish peroxidase-conjugated anti-his tag antibody (Thermo scientific, USA) with

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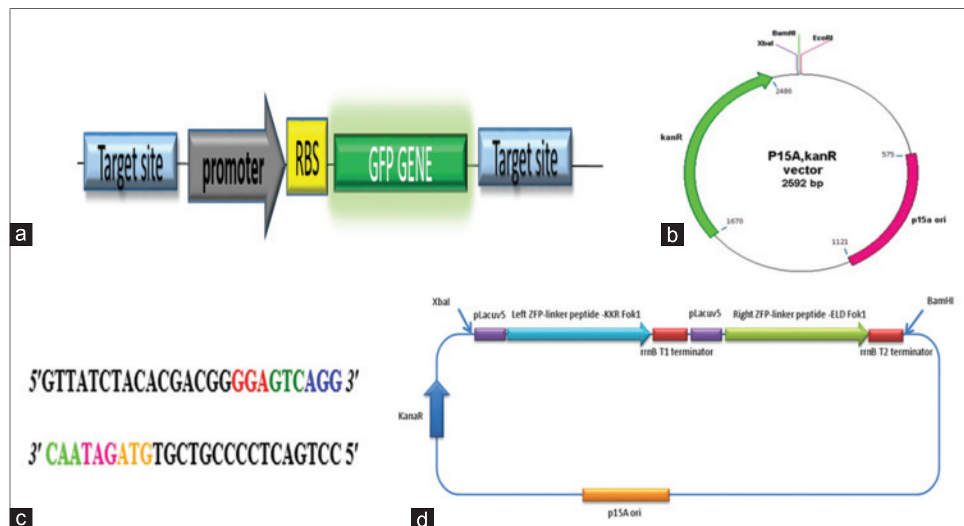

Figure 1: The green fluorescent protein expression cassette (a). The p15A, KanaR vector constructed for zinc finger nucleases cloning and expression (b). The zinc finger nucleases 25-bp target site identified in ampicillin resistance gene of pTZ57R (c). This figure illustrates the pZFN containing zinc finger nuclease expression cassette cloned into p15A, KanaR vector, which is 4800 bp in length (d)

dilution (1:2000) and incubated at 4°C overnight and subsequently washed for 3 times (each time for 10 min). Finally, detection was done by DAB.

Transformation of pPRO-green fluorescent protein into the *Escherichia coli* TOP10F' containing pZFN or pP15-Kana

The extracted of pUC57-GFP plasmids were transformed into chemically competent Top10' cells that already contains pZFN or pP15-Kana vector separately. The transformed bacteria were cultured on 100 µg/ml ampicillin, 30 µg/ml kanamycin, and ampicillin/kanamycin containing LB agar medium. Moreover, all media contain 1 mM IPTG. The plates incubated overnight at 37°C and 30°C. The bacteria containing pP15-kana vector were cultured on LB agar plate supplemented with 30 µg/ml kanamycin as a control.

Evaluation of zinc finger nuclease activity

To evaluate the efficiency of ZFN, the rate of colony growth on amp-kana LB plates was calculated manually. Moreover, optical density at 600 nm (OD600) was also measured for washed colonies in the same volume of LB broth of plates containing amp-kana media in case and control groups.

Flow cytometric analysis

Flow cytometric analysis using FACSCalibur flow cytometer (BD bioscience, NJ, USA) was performed to assess ZFN activity on ampicillin resistance gene and consequently GFP expression.

Bacterial colonies grown on amp-kana and amp containing media in the case (pZFN) and control (pP15A-Kana) groups were washed with LB broth, centrifuged at 9000 rpm for 3 min, and subsequently resuspended in 1 ml sterile PBS. *E. coli* strain Top10' containing pUC57-GFP vector and Top10' alone was used as positive and negative controls, respectively. One hundred thousand events were counted for each sample.

Statistical analysis

Independent *t*-test was used for the comparison of colony count in amp and amp-kana media and the measurement of OD600 between these media. Flow cytometric results were statistically analyzed using Mann–Whitney test. The level of significance was set at $P < 0.05$. All statistical analysis was done using software package SPSS version 20 (SPSS Inc., Chicago, IL, USA). The results were presented as mean ± standard deviation.

Results

Evaluation of green fluorescent protein expression level

The appearance of green colonies on amp containing LB plate demonstrated the pPRO-GFP transformation and expression of GFP [Figure 2].

Flow cytometric results for GFP expression level indicated stronger intensity for GFP expression in pPRO-GFP transformed bacteria when compared with pUC-57 transformed ones as negative autofluorescent control [Figure 3].

Evaluation of zinc finger nuclease protein expression

The expression of ZFNs proteins was induced with IPTG (0.25, 0.5.0.75, 1, 2, 3 mM) at 25°C in *E. coli* TOP10' and then colonies were screened on LB agar contained 25 µg/ml kanamycine. Because of weak ZFN expression in the early stage of membrane development there was a weak band. It should be noted that the specific band was detected, but it was so weak that it was not possible to capture and it merged with the back ground immediately, so we have not any informative picture.

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Evaluation of zinc finger nuclease biological activity in transformed *E. coli* with pPRO-green fluorescent protein

Flow cytometric analysis of ZFN cleavage activity on amp resistance gene was measured by MFI as a consequent of GFP expression level upon the transformation of pZFN containing *E. coli* with pPRO-GFP. The results indicated a significant difference of MFI for GFP among colonies grown on amp-kana (bacteria containing pZFN and pPRO-GFP versus bacteria containing pP15A-Kana and pPRO-GFP) ($P < 0.0001$) [Figures 4 and 5].

In addition, the manually counted number of colonies in the control group was significantly more than case group [Table 1].

Moreover, OD600 was also measured for washed colonies in the same volume of LB broth of plates containing the amp and amp-kana media in case and control groups. There was a significant difference between OD of case and control groups. ($P < 0.0001$) [Figure 6].

Discussion

As mentioned, GE tools have a predictable nature, so we can use them in a wide range of studies that need to manipulate a gene. This gene can be either human or prokaryotic gene and it is considerable that the efficiency of these tools must be evaluated with some methods. For example in cell-based luciferase reporter system,[5] a stable cell line, which expresses a single copy of luciferase interrupted with a target site for ZFN in the middle region of the functional luciferase gene. This interrupted luciferase can be restituted by co-transfection of ZFN expression cassette and a truncated luciferase fragment as a donor into the stable cell line. When ZFN create double strand break (DSB) in the target site, this break can stimulate cell repair system. Because of existing, a donor homologous recombination can be occurred and functional luciferase gene will be recovered. Hence, this correction reflects the ZFN efficiency. In the case of cel-I assay,[7] we must transfect a stable cell line with ZFN mRNA or protein, therefore, if ZFN binds and cleaves the target site, a DSB will occur. Under repair process of nonhomologous end joining (NHEJ), cell repairs the DSBs. In some cases, improper NHEJ leads to deletion, insertion, or substitution of nucleotides. After polymerase chain reaction (PCR) on genomic DNA of manipulated cells, denaturation-reannealing causes the formation of heteroduplex between wild type and modified allele. After the introducing the Cel-I enzyme to these resulted heteroduplex, this enzyme can cleave each heteroduplex molecules and these samples will be analyzed by PAGE to assess ZFN cleavage efficiency. In the surrogate reporter system, the reporter contains the mRFP gene, the target sequence, and eGFP gene. The mRFP is constitutively expressed from cytomegalovirus promoter, but functional eGFP only expressed when the programmable nuclease has activity and cleave the target

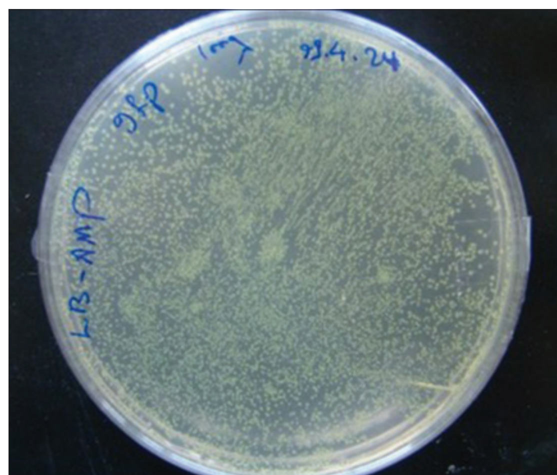

Figure 2: Transformed colonies of *Escherichia coli* with pPRO-green fluorescent protein plasmid: The growth of green colonies confirmed transformation of pPRO-green fluorescent protein and expression of green fluorescent protein

Table 1: The result of zinc finger nuclease biological activity counting of colonies was done manually and its result showed: In amp-kana media the count of colonies in both groups (case and control) was significantly meaning full ($P < 0.017$)

Group / Count	pZFN + pPRO + GFP	pP15kana + PPRO-GFP	*P*
AMP + KANA	130.67±30.31	302.67±13.42	0.017

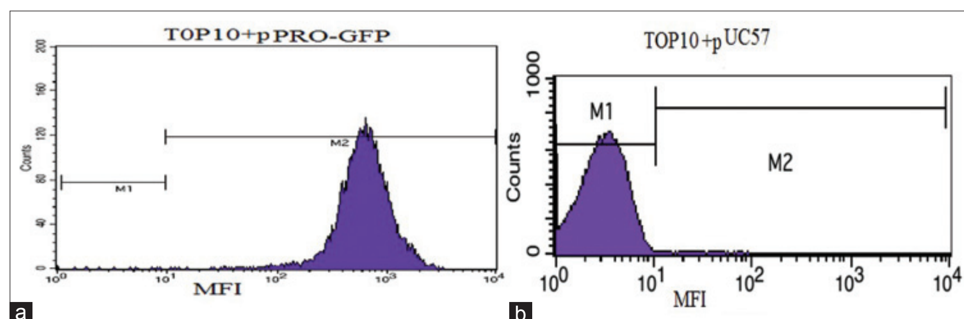

Figure 3: Analysis of green fluorescent protein expression by flow cytometry. The results showed that the MFI in transformed bacteria with pPRO-green fluorescent protein (a) was significantly higher in comparison with transformed bacteria with pUC57 (b)

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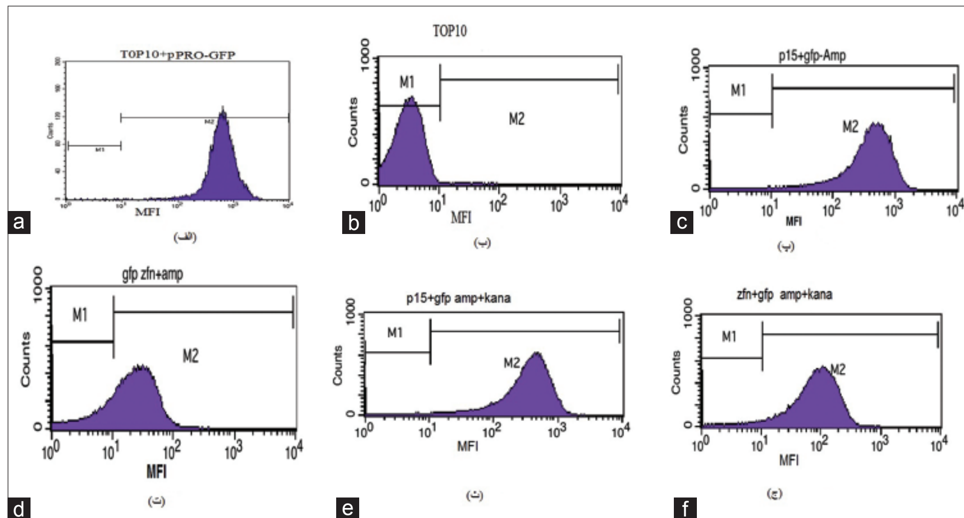

Figure 4: The result of zinc finger nuclease biological activity. Zinc finger nuclease repressed green fluorescent protein expression through cleavage of pPRO-green fluorescent protein targeted site which results in amp resistance and green fluorescent protein expression destruction and reduction. (a) MFI for green fluorescent protein expression level related to pPRO-green fluorescent protein containing *Escherichia coli* colonies as positive control, (b) MFI for green fluorescent protein expression level for *Escherichia coli* strain TOP10' alone as negative control, (c-f) MFI for green fluorescent protein expression level in *Escherichia coli* colonies containing PZFN and pP15A-Kana transformed by pPRO-green fluorescent protein remained on amp and amp-kana containing plates, respectively

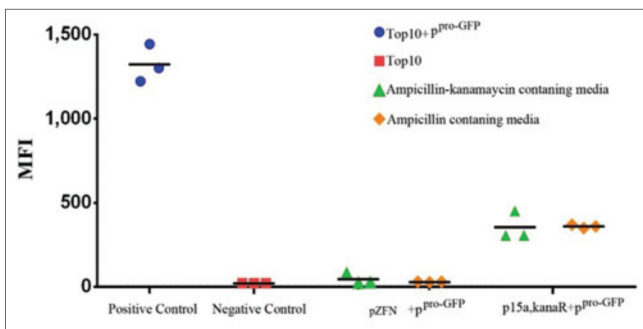

Figure 5: Significant differences between four groups: Comparison of case and control (pZFN + pPRO-green fluorescent protein and pP15A-KanaR + pPRO-green fluorescent protein) in two amp and amp-kana containing media ($P < 0.001$)

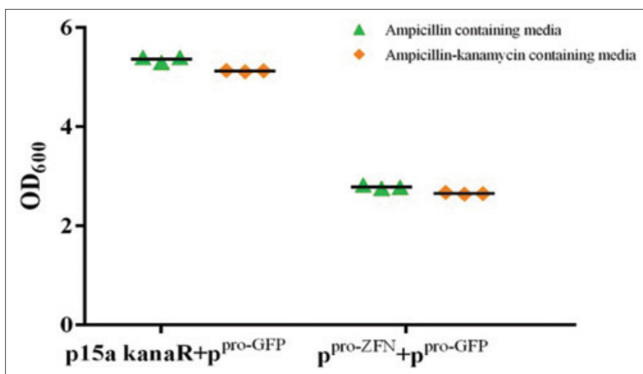

Figure 6: The result of optical density measurement in case and control group. The optical density measurement at 600 nm indicates significant differences of optical density measurement at 600 nm in two groups (pP15A-KanaR + pPRO-GFP and pZFN + pPRO-GFP) in two amp and amp + kana containing media

sequence. When a custom designing nucleases is offered to the target sequence, the resulted break is repaired by NHEJ. The created mutations can cause eGFP to be in frame with mRFP and induce the expression of the mRFP-eGFP fusion protein.[6]

In this study, we have introduced a new method for evaluating of GE tools. As mentioned above, for example about the methods that have been used GFP or luciferase reporter gene in a cell line; first, a stable cell line have to be produced that expresses mutant version of those genes, and in second step, this stable cell line must be transfected with ZFN expression cassette. One of the obvious difficulties of these methods is the production of the stable cell line. However, in our new prokaryotic system that has embedded the target site of GE tools on both sides of GFP gene, not need to produce a stable cell line, and this method could be simpler, cheaper, and faster than above methods. In addition this simple prokaryotic system also, let investigators evaluate the efficiency of every GE tool that has been designed to target each gene. In recent study, CRISPR-CAS9 was used to target antimicrobial resistance gene,[9] the efficiency of these tools could be evaluated with this simple prokaryotic system. In our study, we evaluated the efficiency of ZFN that designed for targeting of ampicillin resistant gene. The result showed that MFI in case group was decreased and this item indicates the level of GFP expression indirectly. Nowadays high resolution melting (HRM) PCR is a new facility to evaluate indel effect of GE tools. HRM usually uses as a screening method to assess single nucleotide polymorphism or mutation and also can be used for indel.[10] If there is any deviation in HRM, it could be confirmed by sequencing. Sequencing could be used to confirm the effect of GE tools on prokaryotic, plasmid, or even eukaryotic genome. Indeed with these suggested methods the indel effect of

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NHEJ resulted from treating with a custom GE tools can be detected.

Financial support and sponsorship

This study was supported by vice chancellery of Isfahan University of medical science.

Conflicts of interest

I have no conflicts of interest with no body.

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