

Design, Construction, and Analysis of Specific Zinc Finger Nucleases for *microphthalmia* - associate transcription factor

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ABSTRACT

This work studied the design, construction, and cleavage analysis of zinc finger nucleases (ZFNs) that could cut the specific sequences within *microphthalmia* - associate transcription factor (*mitfa*) of zebra fish. The target site and ZFPs were selected and designed with zinc finger tools, while the ZFPs were synthesized using DNAWorks and two-step PCR. The ZFNs were constructed, expressed, purified, and analyzed *in vitro*. As expected, the designed ZFNs could create a double-strand break (DSB) at the target site *in vitro*. The DNAWorks, two-step PCR, and an optimized process of protein expression were firstly induced in the construction of ZFNs successfully, which was an effective and simplified protocol. These results could be useful for further application of ZFNs – mediated gene targeting.

Key words: Zinc finger nuclease, DNAWorks, gene synthesis, gene knockout

INTRODUCTION

Reverse genetics has been a focus in the fields of gene functions and diseases' gene therapy. However, the reverse genetics techniques of non-mammalian vertebrates and plants are limited to Morpholinos (MO), RNAi and TILLING. Performed in the early period of embryonic development, morpholinos have no effect on genome (Sumanas 2002). Heritable mutants can't be obtained by RNAi technology either. (Hannon 2002). Although heritable mutants can be obtained by TILLING, TILLING technology is non-effective and time-consuming for genes containing many introns (Sessions 2002).

Gene targeting using the homologous recombination has performed well in embryonic stem cell lines of mouse. However, targeted genomic manipulation has failed in other non-mammalian vertebrates (Deiters 2006).

Zinc finger nuclease (ZFN) has become a new tool for gene knockout in other metazoan, plants and

even human cell lines. ZFNs are engineering restriction endonucleases, which can be used to cut the DNA sequence and create double-stranded breaks (DSBs) at target points in the chromosomes. Most DSBs in genome may be repaired by the homologous recombination (HR). While some DSBs of the damaged chromosomes can be repaired by non-homologous ending joining (NHEJ), small deletions or insertions were followed by ligations. Hence, the mutants with site-specific manipulation could be obtained through NHEJ.

ZFNs are fusion proteins composed of DNA-binding domains and non-specific cleavage domains consisting of the cleavage domain of wild type restriction enzyme *Fok I* (WT *Fok*). The WT *Fok I* was discovered in *Flavobacterium okeanoikoites*, which was a member of type IIS restriction endonucleases. The WT *Fok I* is also made up of a cleavage domain and a DNA recognition domain. The recognition domain of

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WT *Fok I* binds to the sequence 5'-GGATG-3' (Sugisaki, 1981), while the cleavage domains form a dimer to cut DNA sequences. The cleavage domains of WT *Fok I* constitute the cleavage domains of ZFNs, which are more active in form of heterodimers. Thus, ZFNs form heterodimers to induce the DSB at target points in chromosomes (Bibikova, 2001).

Another domain of ZFNs is the DNA-binding domain, which is zinc finger protein consisting of several C₂H₂ zinc finger (ZF) motifs. Each ZF motif comprises 30 amino acids, and contains a α -helix and two β -sheets. A ZF motif binds to a triplet of DNA sequence by crucial amino acids at the positions -1, 2, 3 and 6 of α -helix (Erickson, 1999). Thus, different ZF motifs may be designed by changing these crucial amino acids to recognize different triplets of DNA sequence, while the other amino acids can be maintained as an unalterable backbone (Wolfe 1999). Each ZF motif binds to a triplet, while ZFP consists of consecutive ZF motifs recognizing consecutive triplets. ZFs recognizing 64 possible triplets have been detected and isolated with phage display (Segal 1999; Liu 2002). Thus, ZFPs and ZFNs can be designed to bind and cleave arbitrarily chosen sequences in principle. The interests of ZFNs application have been stimulated in the fields of gene knockout and gene replacement at target sites in the genome of many model organism, including *Xenopus laevis* (Bibikova 2001), plant cells (Lloyd 2005), *Drosophila* (Bibikova 2002), *Caenorhabditis elegans* (Morton 2006), *Danio rerio* (Doyon 2008) and even human cells (Porteus 2003).

The DNA-binding domain of ZFNs contains several ZF motifs whose number can be changed. Three ZF motifs are believed to be the minimum to achieve the adequate specificity and affinity. Although adding more ZF motifs may enhance the binding specificity, it also increases the difficulty of ZFP gene synthesis and searching for an appropriate site. Three or four ZF motifs have been used widely and successfully for strictly cleavage in genome (Bibikova 2002; Porteus 2006).

The widely application of ZFNs can be imagined, due to the targeted gene manipulation in non-mammalian vertebrates. Microphthalmia-associate transcription factor (*mitf*), a member of basic helix-loop-helix zipper proteins, regulates the specific gene expression and signal transmission of melanocytes. *Mitf*, a kind of conservative gene in evolution, encodes five

protein isoforms, including MITF-A, MITF-B, MITF-C, MITF-H, and MITF-M. It encodes two isoform proteins, MITF-A and MITF-B, in zebra fish (Shibahara 2001). The *mitfa* was chosen as the target gene for constructing the specific ZFNs in this work to establish the basis for gene function research and human disease models' construction. This work studied the construction protocol of ZFNs for target site, in which DNAWorks was induced to design the coding sequences of ZFPs. Then a simplified method of gene synthesis, a two-step gene synthesis method, was carried out successfully using DNAWorks software.

MATERIALS AND METHODS

Search for ZFN target sites

The DNA sequence and cDNA sequence of *mitfa* were found at NCBI website. When these sequences are put into the website of zinc finger tools (<http://www.scripps.edu/mb/barbas/zfdesign/zfdesignhome.php>), several parameters should be chosen, such as "separated target sites", "the core sequence" and "triplets to search" (Mandell 2006). Then all the target sites on the DNA sequence are output. According to the scores of the website, the conserved sites of the DNA sequences and the difficulty of gene synthesis, the DNA segment "ttgactcttatacaagacctgat" between 2965bp and 2988bp of *mitfa* was chosen as a plausible target site.

Design of the coding sequences of ZFPs

While the target site was output, the amino acid sequences of ZFP1 and ZFP2 were also obtained with zinc finger tools. To design the coding sequence of ZFPs, their amino acid sequences were input into the website of DNAWorks (<http://helixweb.nih.gov/dna-works/>). While several parameters needed to be defined, including codon optimization, codon frequencies, melting temperature and hairpins formation (Hoover 2002). Firstly, the codon usage table of Zebra fish was found in the codon usage database and input into DNAWorks. Secondly, the melting temperature was limited to 60°C. Thirdly, the hairpin formation was avoided in the coding sequences of ZFPs. Finally, the website of DNAWorks output the coding sequences of ZFPs and fourteen oligonucleotides sequences for gene synthesis of each ZFP.

Construction of ZFPs' coding sequences

To construct the coding sequences of ZFPs, the two-step PCR method, overlap extension (OE) PCR and amplification PCR, was used for gene synthesis according to Dong et al (Dong, 2007). Fourteen oligonucleotides were mixed and diluted to 2 μ M with ddH₂O, which were used as the primer mixture in the PCR for synthesizing gene. The first-step PCR, OE PCR, was carried out in a 50 μ l reaction mixture including 4 μ l oligonucleotides mixture, 250 μ M dNTP, 1 \times PCR buffer, and 0.25 U of *TransStart Fastpfu* DNA polymerase (Beijing TransGen Biotech Co., lid.). The OE PCR process consisted of 30 cycles at 95 $^{\circ}$ C for 30s, 64 $^{\circ}$ C for 60s, and 72 $^{\circ}$ C for 60s. Then 1.5 μ l of the OE PCR product was added into the amplification PCR reaction mixture, including 0.4 μ M outer primers, 250 μ M dNTP, 1 \times PCR buffer, and 0.25 U of *TransStart Fastpfu* DNA polymerase in a volume of 50 μ l, and the process consisted of 30 cycles at 95 $^{\circ}$ C for 30s,

66 $^{\circ}$ C for 30s, and 72 $^{\circ}$ C for 60s and finally extension at 72 $^{\circ}$ C for 5min. The final PCR products were detected using agarose gel electrophoresis and purified with Gel purification kit (Beijing TransGen Biotech Co., lid.). The whole sequences were cloned into pUC57 vectors, and subsequently sent to a commercial company (Shanghai Sangon Biotech Co., lid.) to sequence and detect its accuracy.

Assemblage of ZFNs

To assemble the coding sequences of ZFNs, *Fok I* (RV) (the two complementary *Fok I* cleavage domain variants named as *Fok I* (RV) and *Fok I* (DA)) was amplified from the plasmid pCMV-GZFN1-*Fok*-RV using PCR, while *Fok I* (DA) was obtained from the plasmid pGK-GZF3-*Fok*-DA. The purified DNA sequences of *Fok I* (RV) and *Fok I* (DA) were cloned into two pET-30a vectors to form the pET-*Fok I* (RV)-30a and pET-*Fok I* (DA)-30a, respectively (Fig.1).

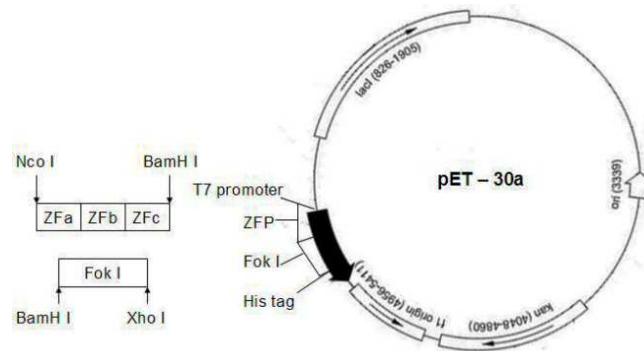


Figure 1 - The construction of ZFNs. †The sequence of *Fok I* and ZFP were linked into the plasmids pET-30a to form pET- ZFN-30a.

The expression vector pET-ZFN1-30a was constructed by linking the ZFP1 sequence into pET-*Fok I* (RV)-30a. The expression vector pET-ZFN2-30a was constructed by linking the ZFP2 sequence into pET-*Fok I* (DV)-30a. Both pET-ZFN1-30a and pET-ZFN2-30a were amplified in DH5 α *E. Coli*.

Expression and Purification of ZFNs

To obtain the ZFNs towards *mitfa* gene *in vitro*, three steps were carried out including expression, detection, and purification. To express the ZFNs, the vectors pET-ZFN1-30a and pET-ZFN2-30a were transformed into competent DE3 *E. coli* cells separately, and selected on LB plate containing kanamycin (10 μ g/ml) growing at 37 $^{\circ}$ C for 12h.

One positive clone of each vector was cultured at 37 $^{\circ}$ C overnight in LB medium containing kanamycin (10 μ g/ml) and ZnCl₂ (0.1mM). Then 50 ml culture was added into 1 L high concentration medium, including 32 g tryptone, 20 g yeast extract, 5 g NaCl, 2.7 g ZnCl₂, 5 mM NaOH, 12.54 g KHPO₄, and 2.31 g KH₂PO₄. After growing at 37 $^{\circ}$ C for 3 h, the culture mixture was added into 0.3 mM IPTG and shifted to 22 $^{\circ}$ C overnight to induce the expression of ZFNs.

To detect the expression of ZFNs, the expression strain cells were collected with centrifuging at 6000 g for 3 min from 400 μ l culture mixture at 4 $^{\circ}$ C. Then they were re-suspended in protein treatment solution, including 200 μ l 10% SDS, 200 μ l beta-mercaptoethanol, and 600 μ l protein

loading buffer, treated in water of 100°C and subsequently analyzed with SDS-PAGE.

To analyze the expression of soluble ZFNs, DE3 *E. coli* cells were collected using centrifuging at 6000 g for 5 min in 2 ml culture mixture, and re-suspended with 2.0 ml PBS in an ice bath. After the cells in PBS were broken by sonicating in an ice bath, total soluble proteins in the supernatant were collected with centrifuging at 6000 g for 3 min at 4°C. The sediment consisted of total insoluble proteins of the cells. The soluble protein and insoluble protein were analyzed using SDS-PAGE respectively.

To purify the ZFNs, total soluble protein of DE3 *E. coli* cells were collected with ultrasonic wave in an ice bath and purified using Ni-NTA resin (Nanjing genscript Co., Lid.) according to its instruction.

The cleaving activity detection of ZFNs in vitro

To detect the cleaving activity of ZFNs, the targeted sequence was linked into a plasmid pEASY-blunt simple as the DNA substrate. If ZFNs cut the targeted sequence in the plasmid, the super-helical conformations of the plasmid would

be changed into linear conformation. The change could be detected with agarose gel electrophoresis. The first step was the construction of the DNA substrate (Fig. 2). The DNA substrate sequence (cgcggtacctttgactcttatcaaagacctgataagcttggg) which contained the targeted sequence of ZFN1 and ZFN2 (tttgactattatcaaagacctgat) was synthesized using PCR with primers Pt1 and Pt2. The sequence of Pt1 was “cgcggtacctttgactcttatcaaagacctg”, and that of Pt2 was “ccaagcttatcaggtctttgataagagctcaaagg”. The PCR was performed in 30 µl reaction mixture, including 0.3 µM Pt1, 0.3 µM Pt2, 1×PCR buffer, and 0.25 U of *TransStart Fastpfu* DNA polymerase, and the process consisted of 35 cycles at 95°C for 10 s, 54°C for 30 s, and 72°C for 30 s, and extension at 72 °C for 10 min. The PCR products of 42 bases were detected using 3% agarose gel electrophoresis for 10 min. The product bands were cut and purified with Gel purification kit. The targeted sequence was cloned into pEASY-blunt simple, and subsequently sequenced to detect its accuracy. The plasmid pEASY-target-blunt simple was the DNA substrate of the analysis of ZFNs activity *in vitro*.

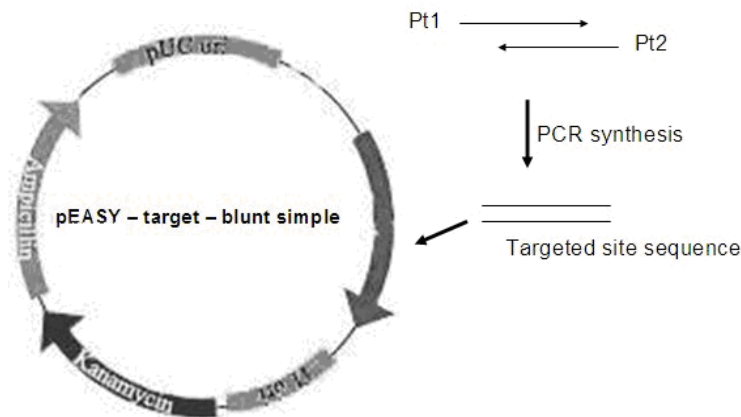


Figure 2 - The construction of pEASY-target-blunt simple.

According to Dana Carroll's report (Carroll, 2006), 20 µl reaction mixture consisted of 1×ZFN reaction buffer, 50 mM NaCl, 1mM DTT, 100 µg/ml yeast RNA, 50 ng DNA substrate, and the ZFNs. In the reaction mixture, ZFN1 and ZFN2 were equimolar as well as the DNA substrate. The reaction was started with 1.0 µl of 0.2 M MgCl₂ at 25°C for 1 h. The ZFN reaction buffer consisted of 0.1 M Tris (pH8.5), 0.5 mM ZnCl₂, 250 µg/ml

BSA. The reaction results were analyzed using agarose gel electrophoresis.

RESULTS

Targeting site of ZFN

The DNA and cDNA sequences of *mitfa* in zebra fish were obtained in NCBI database and

subsequently input into zinc finger tools to search for the possible sites. According to conservative sites analysis and difficulty analysis of gene synthesis, the sequence between 2965 bp and 2988 bp of *mitfa* DNA sequence was chosen as targeting site, which was “tttgactcttatcaaacctgat”.

Amino acid sequence of ZFPs

The reorganization sequences of ZFP1 and ZFP2

are shown in Figure 3. The amino acid sequence of ZFP1 was “LEPGEKPYKCPECG KSFSTSGNLVRHQRTHTGEKPYKCPECGKSFSTKNSLTEHQRTHTGEKPYKCPECGKSFSQLAHLRAHQRTHTGKKTS”, and that of ZFP2 was “LEPGEKPYKCPECGKSFSDPGALVRHQRTHTGEKPYKCPECGKSFSQLAHLRAHQRTHTGKKTS”.

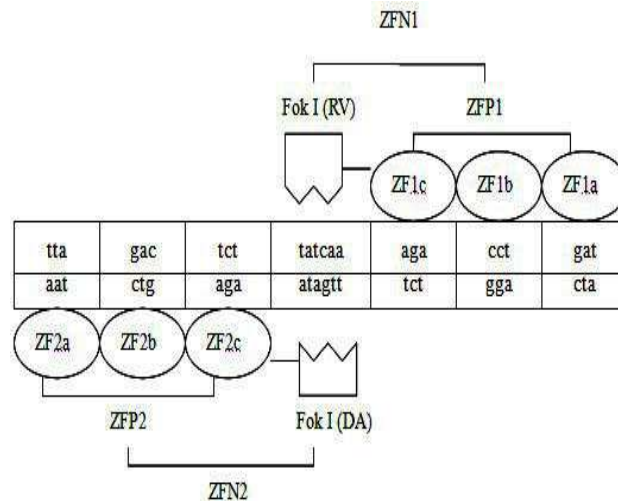


Figure 3 - The reorganization between targeted sequence and ZFNs.

Coding sequences of ZFPs

After the amino acid sequences of ZFPs were input into DNAWorks, the coding sequences of ZFPs were obtained after codon optimization in DNAWorks. The coding sequence of ZFP1 was “ctggaaccgggcgagaaaccgtacaagtgccagagtgcggcaa gagcttcagcacctctgtaatctcgtgcgccatcagcgtaccacac gggtgaaaaaccttacaatgtccggagtgtggcaaatctttccacc aaaaacagcctaccgaacaccagcgcaccatacgggcgaaaag ccgtataaatgcccggaatgcgtaagtctttctcagctggcgcac tgcgtgccaccacgtacgcacaccggtaaaaagacctct”. The coding sequence of ZFP2 was “ctggaaccgggcgaaaagccttacaatgcccgagtgcgtaag tctttctcccagcgcgcaaacctcgtgcgcacactcagcgcactcacac ggcgagaaccatataagtgcctgaatgtggcaaatcttttagcgac ccaggcgcgctcgttcgtaccagcgtaccatacgggtgagaagcc gtacaagtgtccagaatgcggcaagtctttctcagctggcacatctc cgcgctcaccacgtacgcataccggcaaaaagacctct”.

Oligonucleotides for ZFPs gene synthesis

The oligonucleotides for ZFPs gene synthesis were obtained using DNAWorks, and synthesized by a commercial company. The oligonucleotides for ZFP1 and ZFP2 are shown in Table 1 and Table 2, respectively.

Gene synthesis of ZFPs

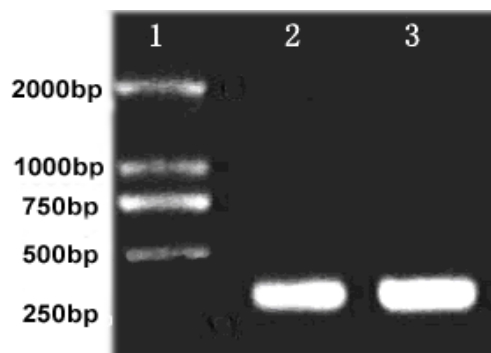
Two-step PCR was carried out to synthesize the coding sequences of ZFPs, which were detected subsequently using agarose gel electrophoresis (Fig.4). The length of successful PCR products was 276 bp. The coding sequences of ZFPs were sequenced analyzed accurately. The two-step PCR and DNAWorks were proved successfully applied in ZFPs gene synthesis.

Table 1 - The oligonucleotides for ZFP1.

No.	oligonucleotides
1	ctggaaccgggcgagaaaccgtacaagtgccca
2	tgaagctcttgcgcactctggcactgtacggt
3	gtcggcaagagcttcagcacctctgtaatctcgtg
4	tgggtacgctgatggcgcacgagattaccagaggtgc
5	cgcacatcagcgtaccacacgggtgaaaaccttaca
6	tgccacactccggacatttgaaggtttaccctg
7	atgtccggagtgtggcaatcctttccacaaaaacagc
8	cgtctgtgttcggtgaggctgttttgggtgaaaaggatt
9	ctcaccgaacaccagcgacccatacggcgaaaa
10	ttccggcatttatacggcttttcccgtatgggtg
11	gccgtataaatgcccgaatgcggtaagtcttctcag
12	cacgcagatgcgccagctgagagaaagacttaccgca
13	ctggcgcatctgctgcccaccaacgtacgcac
14	agaggctctttaccgggtgctgtacgttggtggg

Table 2 - The oligonucleotides for ZFP2.

No.	oligonucleotides
1	ctggaaccgggcgaaaagccttacaatgcccggagt
2	ctgggagaaagactaccgcactccggcatttgaagg
3	gcggtaaagtcttctccagcgcgcaaacctccgtg
4	gtgagtgcgctgatgacgcacggaggttgctgcg
5	cgcacatcagcgcactcacacgggcgagaacctataag
6	atttgcacattcaggcacttatatggttctcggccgt
7	tgccctgaatgtgcaaatccttagcgaccaggcg
8	ctggtgacgaacgagcgcctgggtcgttaaagg
9	cgtctgttcgtaccagcgtaccatacgggtgaga
10	tctggacactgtacggcttctaccgtaggggtacg
11	agccgtacaagtgtccagaatggcgaagtcctttct
12	cggagatgtccagctgagaaaaggacttccgcat
13	cagctggcacatctccgcgtcaccaacgtacgc
14	agaggctcttttggcgtatgctgtacgttggtgagcg

**Figure 4** - The PCR products of coding sequences of ZFPs. †Lane 1 contains size standards of DL2000 DNA marker (Beijing Transgene Co., Ltd.). Lane 2 is the coding sequence of ZFP1. Lane 3 is the coding sequence of ZFP2.

Expression vector of ZFNs

The coding sequence of ZFP1 and *FokI* (RV) were cloned into pET-30a to form ZFN1 about 900 bp,

while ZFP2 and *FokI* (DA) were used to form ZFN2. The results of ZFNs construction were detected using PCR and shown as Figure 5.

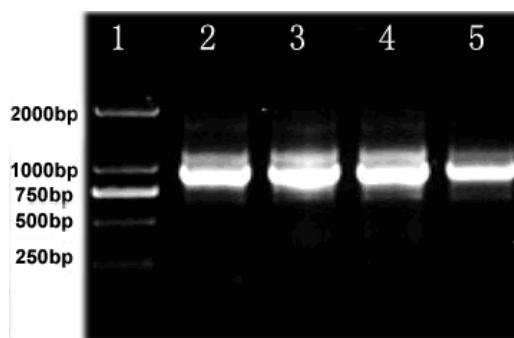


Figure 5 - The PCR results of ZFNs construction. †Lane 1 is the DL2000 DNA marker. Lane 2 and 3 are the coding sequences of ZFN1. Lane 4 and 5 are the coding sequences of ZFN2.

ZFNs protein

The soluble ZFNs were expressed successfully with 0.3 mM IPTG at 22°C in DH5α *E.coli*, and purified using Ni resin (Fig. 6). The targeted proteins of 30KD were expressed in DH5α *E.coli*

with IPTG inducement. The soluble proteins were obtained with sonication and centrifugation. The results proved that the protein expression protocol and the high concentration medium were effective to express the soluble ZFNs.

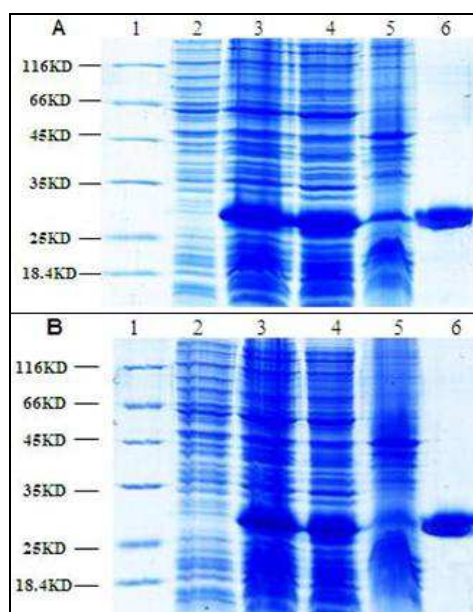


Figure 6 - ZFNs expression detected with SDS-PAGE. † (A) The analysis of ZFN1 expression. (B) The analysis of ZFN2 expression. Lane 1 is the Protein Marker (Fementas Co., Lid.). Lane 2, the total protein of DH5α *E.coli* without IPTG inducement; Lane 3, the total protein with IPTG inducement; Lane 4, the total soluble proteins with IPTG inducement; Lane5, the total insoluble proteins with IPTG inducement; Lane6, purified ZFNs.

Cleaving activity of ZFNs

The targeted DNA was linked into pEASY-blunt simple, which were super-helix plasmids. The cleaving activity of ZFNs was detected *in vitro* and analyzed using agarose gel electrophoresis.

Results in Figure 7 showed that ZFNs could cut the super-helix conformation plasmids into linear molecules at 25°C in a reaction mixture with approximately equimolar ZFNs of the DNA substrate.

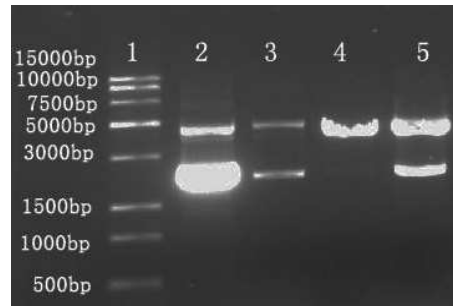


Figure 7 - The cleaving activity of ZFN. †Lane 1 is the DNA marker; lane 2, the control DNA substrate; lane 3 and lane 5, the incomplete cleavage of DNA substrate; lane 4, the complete cleavage of the DNA substrate.

DISCUSSION

ZFNs are known as effective tools for “directed gene knockout” of the plants and vertebrates as well as human cell lines. Here, the protocol of ZFNs design, engineering and cleaving activity detection has been shown with an example of *mitfa* (Fig. 8). Several critical factors are involved in the protocols of ZFNs design and engineering.

The first factor is the selection of target site in an interested gene. Although many possible sites and relevant ZFPs could be obtained with zinc finger tools, choosing a target site one should pay attention to the following points: (1) To make mutagenesis occur at target site and a unique site should be chosen. (2) Since ZFPs binding all ANN and GNN triplets have been reported detailedly (Liu 2002), target sites consisting of GNN triplets or ANN have priority to be chosen. ZFPs of high binding ability was obtained in this study by choosing a target site, including two GNN and two ANN triplets. (3) The distance between two reorganization sequences in target sites ranged from 4 bp to 6 bp was successful and effective to be cut by ZFNs *in vitro* (Bibikova 2001). (4) The target sites could be selected considering the critical conservative sites of the gene. The target site chosen was near the critical sites encoding the DNA binding domain of transcription factor *mitfa*. The second involving factor was that the amino acid sequences of ZFPs which should be converted

into coding sequences automatically using the DNA Works software.

The third factor is the gene synthesis of ZFPs. The gene synthesis method used was a reproducible simple method of less error based on DNAWorks (Dong 2007). The genes could be synthesized using two-step PCR, including the overlap extension (OE) PCR and amplification PCR. DNAWorks software was used instead of manual design to obtain the oligonucleotides for gene synthesis, which could keep similar melting temperature of overlapped regions and make sure the specificity of the primers. Several synthesis strategies for target gene could be obtained in DNAWorks. Each synthesis strategy is scored by DNAWorks, and these scores are important parameters for the selection of synthesis strategy. The most critical parameter, overall score, would be zero in an ideal synthesis strategy (Hoover 2002). The two-step PCR is proved a successful method to be used to synthesize ZFP1 and ZFP2.

The fourth factor is the expression of soluble ZFNs. Soluble protein is easier to be extracted, purified, and keep active than the insoluble protein. Protein expression is affected by several factors, including the temperature, concentration of IPTG and medium. Amino acid sequences fold too quickly to form accurate conformation at high temperature and most proteins with inaccurate conformation are insoluble. The expression level of such protein is very low at low temperature.

The expression temperature used and detected in this work was from 16 to 37°C, while more soluble ZFNs were expressed at 22°C, which was induced with IPTG of 0.3 mM. The soluble ZFNs were expressed in high concentration medium than Luria-Bertani (LB) medium.

ZFN-mediated gene manipulation becomes an effective tool for gene function studies. Facile constructions and rapid cleavage detections of ZFNs *in vitro* are essential processes for gene manipulation. This work reported a simple, rapid and reproducible method of less error to design

and construct the ZFN. The optimization of ZFPs' coding sequences was simplified with DNAWorks software according to the codon usage frequencies of different organisms. Since the gene synthesis primers with similar melting temperatures were simply obtained using DNAWorks, the coding sequences of ZFPs were successfully carried out using two-step PCR. The facile protocol consisting of design, construction and testing of ZFNs could be widely applied to knock out target gene in the plants, vertebrates and even human cell lines.

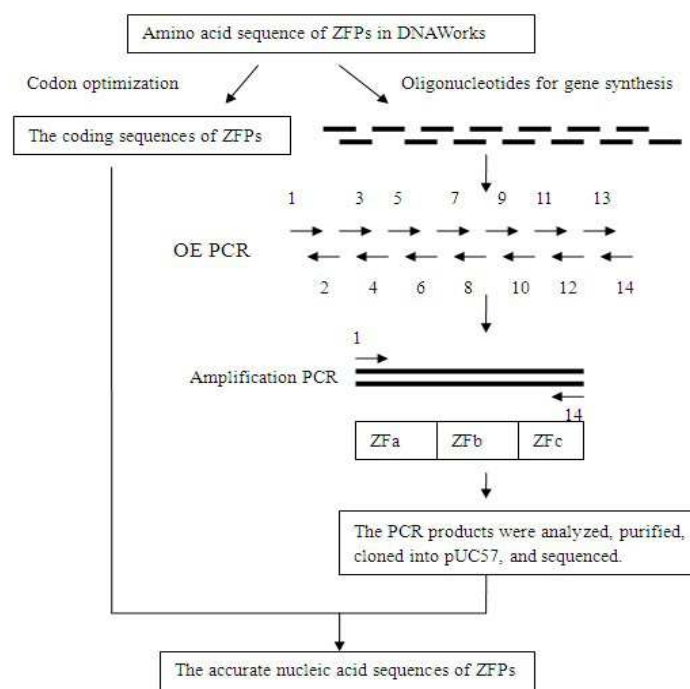


Figure 8 - ZFPs gene synthesis process.

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