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SGRNA FOR EDITING SHEEP FGF5 TO REALIZE ALTERNATIVE SPLICING, COMPLETE SET OF NUCLEIC ACIDS AND USE

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(56) Related Art
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ABSTRACT

The present disclosure provides a single guide RNA (sgRNA) for editing sheep fibroblast growth factor 5 gene (FGF5) to achieve alternative splicing, a complete set of nucleic acids and use, and belongs to the technical field of cell engineering and genetic engineering. sgRNA is obtained by annealing nucleotide sequences of SEQ ID Nos. 1-6; the nucleotide sequences of SEQ ID No. 1 and SEQ ID No. 2 are annealed pairwise; the nucleotide sequences of SEQ ID No. 3 and SEQ ID No. 4 are annealed pairwise; and the nucleotide sequences of SEQ ID No. 5 and SEQ ID No. 6 are annealed pairwise. The sgRNA provided by the present disclosure can achieve editing of the sheep FGF5 by alternative splicing.

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COMPLETE SPECIFICATION FOR THE INVENTION ENTITLED

SGRNA FOR EDITING SHEEP FGF5 TO ACHIEVE ALTERNATIVE SPLICING, COMPLETE SET OF NUCLEIC ACIDS AND USE

This invention is described in the following statement:-

SGRNA FOR EDITING SHEEP FGF5 TO ACHIEVE ALTERNATIVE SPLICING, COMPLETE SET OF NUCLEIC ACIDS AND USE

TECHNICAL FIELD

[0001] The present disclosure belongs to the technical field of cell engineering and genetic engineering, and in particular relates to a single guide RNA (sgRNA) for editing sheep fibroblast growth factor 5 gene (FGF5) to achieve alternative splicing, a complete set of nucleic acids and use.

BACKGROUND

[0002] CRISPR/Cas9 system is an adaptive immune defense system formed during the long-term evolution of bacteria and archaea and can be used to fight against invading viruses and foreign DNA. CRISPR/Cas9 gene editing technology is used for specific DNA modification of targeted genes. The gene editing technology system mainly consists of Cas9 nuclease and sgRNA. Cas9 nuclease has an active domain that cleaves a double-stranded DNA to make it fragmented; sgRNA is a chimeric RNA formed by combining tracrRNA as a scaffold function and specific crRNA. sgRNA achieves a targeted recruitment of a Cas9/sgRNA complex to a gene of interest through complementary pairing of RNA-DNA bases. Cas9 then cleaves a double-stranded DNA in a site-directed way by recognizing a PAM motif (5'-NGG-3') on a gene of interest, and plays an editing role for the gene of interest.

[0003] CRISPR/Cas9 lentiviral system (Lentiviral CRISPR/Cas) can infect a variety of mammalian cells and co-express an optimized Cas9 nuclease encoded by a mammalian and sgRNA to facilitate genome editing. The lentivirus vector is a gene therapy vector based on HIV-1 (Human Immunodeficiency Virus Type I). The lentivirus vector has the ability to infect both dividing cells and non-dividing cells, so as to distinguish it from a normal retroviral vector. The vector can effectively integrate a foreign gene into a host chromosome to achieve persistent expression. In terms of infection ability, the vector can effectively infect a various type of cells such as neuron cells, liver cells, cardiomyocytes, tumor cells, endothelial cells, stem cells and the like, so as to achieve favorable therapeutic effect.

[0004] The construction principle of lentiviral vectors is that the packaging system of the lentiviral vectors generally consists of two parts, namely a packaging component and a vector component. The packaging component is constructed by removing cis-acting sequences required for packaging,

reverse transcription and integration from HIV-1 genome, which can supply protein necessary for the production of virus particles in trans; the vector component is complementary to the packaging component, i.e., containing the cis-acting sequences required for packaging, reverse transcription and integration, and having a multiple cloning site under the control of a heterologous promoter and a gene of interest inserted at this site at the same time. Multiple plasmids of the packaging component and the vector component are co-transfected into packaging cells, and then replication-defective lentiviral vector particles carrying genes of interest can be harvested from the supernatant of the packaging cells. The collected supernatant of the packaging cells can be directly used for infecting host cells. The genes of interest are integrated into the genome of the host cells through reverse transcription after entering them to express effector molecules at a high level.

[0005] FGF5 belongs to one of the members of fibroblast growth factor FGFs (a class of multifunctional peptide growth factor for regulating cell growth) family and is considered to inhibit a growth period of hair during hair growth. This function can be regulated by truncated FGF5 (FGF5s) produced by an alternative spliced form of FGF5 to relieve the inhibitory effect of FGF5 on hair growth. The FGF5s is the product of alternative splicing of FGF5 mRNA during maturation. Alternative splicing refers to the process of producing different mRNA splicing isoforms from an mRNA precursor through different splicing manners (selecting different splicing site for combining), so that the final protein product will exhibit different or mutually antagonistic functional and structural properties, or result in different phenotypes due to different expression levels in a same cell. Two FGF5 subtypes have been found to be isolated from rat, mouse and human brain tissues. Among them, one of the subtypes is a full-length mRNA of FGF5 including 3 exons; the other subtype is a FGF5s, that is, exon 2 in the full-length mRNA sequence of FGF5 is detected by an alternative spliced form. However, there are no reports about alternative splicing of the sheep FGF5 using CRISPR/Cas9 genome editing technology in prior art.

SUMMARY

[0006] In view of this, an objective of the present disclosure is to provide a sgRNA for editing sheep FGF5 to achieve alternative splicing, a complete set of nucleic acids and use. The sgRNA provided by the present disclosure can achieve editing of the sheep FGF5 by alternative splicing.

[0007] To achieve the above objective, the present disclosure provides the following technical solutions:

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[0008] The present disclosure provides a sgRNA for editing sheep FGF5 to achieve alternative splicing, where the sgRNA is obtained by annealing nucleotide sequences of SEQ ID Nos. 1-6; where

the nucleotide sequences of SEQ ID No. 1 and SEQ ID No. 2 are annealed pairwise; and

the nucleotide sequences of SEQ ID No. 3 and SEQ ID No. 4 are annealed pairwise; and

the nucleotide sequences of SEQ ID No. 5 and SEQ ID No. 6 are annealed pairwise.

[0009] The present disclosure also provides a complete set of nucleic acids including the sgRNA in the above technical solution and a lentiviral vector lentiCRISPRv2.

[0010] The present disclosure also provides use of the sgRNA in the above technical solution in editing sheep FGF5.

[0011] Preferably, the use includes the following steps of:

1) digesting a lentiviral vector lentiCRISPRv2 with BsmB I to obtain a digested vector, and ligating the above-described sgRNA with the digested vector to obtain a recombinant plasmid;

2) co-transfecting the recombinant plasmid obtained in step 1) with plasmids psPAX2 and pMD2.G into 293T cells to obtain recombinant lentiviruses;

3) infecting sheep muscle cells with the recombinant lentiviruses obtained in step 2) to achieve editing of sheep FGF5 by the sgRNA.

[0012] Preferably, the mass of the recombinant plasmids, the mass of the plasmid psPAX2, the mass of the plasmid pMD2.G, and the quantity of the 293T cells at the time of plating in step 2) are in a ratio of 20 μg: 15 μg: 6 μg: 2-2.5×10^6.

[0013] Preferably, the recombinant plasmids and the plasmids psPAX2 and pMD2.G are co-transfected into the 293T cells using a calcium phosphate transfection method.

[0014] The present disclosure provides a sgRNA for editing sheep FGF5 to achieve alternative splicing. The sgRNA is obtained by annealing nucleotide sequences of SEQ ID Nos. 1-6; the nucleotide sequences of SEQ ID No. 1 and SEQ ID No. 2 are annealed pairwise; the nucleotide sequences of SEQ ID No. 3 and SEQ ID No. 4 are annealed pairwise; the nucleotide sequences of SEQ ID No. 5 and SEQ ID No. 6 are annealed pairwise. The sgRNA provided by the present disclosure can achieve editing of the sheep FGF5 by alternative splicing.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows an enzymatic cleavage map of lentiCRISPRv2-Puro, lane 1: lentiCRISPRv2-Puro original plasmids; lane 2: Mark 1kb; lanes 3-6: a cleavage map of lentiCRISPRv2-Puro;

FIG. 2 shows a verification map of the recovery of lentiCRISPRv2-Puro from the gel of the enzymatic cleavage, lanes 1-3: a verification map of the recovery of lentiCRISPRv2-Puro from the gel of the enzymatic cleavage; lane 4: Mark 1kb;

FIG. 3 shows a sequencing map of lentiCRISPRv2-sgRNA;

FIG. 4 shows the expression of green fluorescent protein observed in cells after infection with recombinant lentiviruses. The first row: cells infected with pLEX-EGFP-containing recombinant viruses; the second row: cells transfected with LentiCRISPRv2-GFP-containing recombinant viruses;

FIG. 5 shows the sequence analysis of editing target sites in cells after infection with recombinant viruses carrying FGF5-sgRNA2;

FIG. 6 shows the sequence analysis of editing target sites in cells after infection with recombinant viruses carrying FGF5-sgRNA4;

FIG. 7 shows the sequence analysis of editing target sites in cells after infection with recombinant viruses carrying FGF5-sgRNA7;

FIG. 8 shows the sequence analysis of editing target sites in cells after infection with recombinant viruses carrying FGF5-sgRNA8;

FIG. 9 shows the sequence analysis of editing target sites in cells after infection with recombinant viruses carrying FGF5-sgRNA9;

FIG. 10 shows electrophoresis bands of amplification of related cDNA after editing of sheep FGF5 by sgRNA. Lanes 1-6 are electrophoresis bands of FGF5-sgRNA2/4/7/8/9 and FGF5 cDNA in the control group; Lane 7 is Marker 1kb; Lanes 8 -13 are electrophoresis bands of FGF5-sgRNA2/4/7/8/9 and reference gene (β-actin) in the control group;

FIG. 11 shows electrophoresis bands of amplification of related cDNA after editing of sheep FGF5 by sgRNA. Lanes 1-4 are electrophoresis bands of FGF5-sgRNA4/8/9 and FGF5 cDNA in the control group; Lane 5 is Marker 1kb; Lanes 6-9 are electrophoresis bands of FGF5-sgRNA4/8/9 and reference gene (β-actin) in the control group;

FIGS 12-14 show the probability statistics of different Indel gene editing types in FGF5-sgRNA4 (3 figures show three repeats); in which, FIG. 12 shows statistics of FGF5-sgRNA4-1 gene editing; FIG. 13 shows statistics of FGF5-sgRNA4-2 gene editing; FIG. 14 shows statistics of FGF5-sgRNA4-3 gene editing;

FIGS 15-17 show the probability statistics of different Indel gene editing types in FGF5-sgRNA8 (3 figures show three repeats); in which, FIG. 15 shows statistics of FGF5-sgRNA8-1 gene editing; FIG. 16 shows statistics of FGF5-sgRNA8-2 gene editing, FIG. 17 shows statistics of FGF5-sgRNA8-3 gene editing;

FIGS 18-20 show the probability statistics of different Indel gene editing types in FGF5-sgRNA9 (3 figures show three repeats); in which, FIG. 18 shows statistics of FGF5-sgRNA9-1 gene editing; FIG. 19 shows statistics of FGF5-sgRNA9-2 gene editing; FIG. 20 shows statistics of FGF5-sgRNA9-3 gene editing;

FIG. 21 shows the type of transcriptional expression products of FGF5 and its relative expression level affected by sgRNA.

DETAILED DESCRIPTION

[0015] The present disclosure provides a sgRNA for editing sheep FGF5 to achieve alternative

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splicing. The sgRNA is obtained by annealing nucleotide sequences of SEQ ID Nos. 1-6; the nucleotide sequences of SEQ ID No. 1 and SEQ ID No. 2 are annealed pairwise; the nucleotide sequences of SEQ ID No. 3 and SEQ ID No. 4 are annealed pairwise; the nucleotide sequences of SEQ ID No. 5 and SEQ ID No. 6 are annealed pairwise.

[0016] In the present disclosure, the accession number of the sheep FGF5 is GenBank: KJ647161.1.

[0017] In the present disclosure, the nucleotide sequence of SEQ ID No. 1 is specifically as follows:

CaccgGAAATATTTGCTGTGTCTCA

[0018] The nucleotide sequence of SEQ ID No. 2 is specifically as follows:

aaacTGAGACACAGCAAATATTTCc.

[0019] In the present disclosure, the nucleotide sequences of SEQ ID No. 1 and SEQ ID No. 2 are annealed by boiling the nucleotide sequences of SEQ ID No. 1 and SEQ ID No. 2 containing an annealing buffer in a water bath for 11 minutes, and cooling them to room temperature naturally.

[0020] In the present disclosure, the nucleotide sequence of SEQ ID No. 3 is specifically as follows:

CaccgTGGTTCTACTTACACTTGCA

[0021] The nucleotide sequence of SEQ ID No. 4 is specifically as follows:

aaacTGCAAGTGTAAGTAGAACCAc.

[0022] In the present disclosure, the nucleotide sequences of SEQ ID No. 3 and SEQ ID No. 4 are annealed by boiling the nucleotide sequences of SEQ ID No. 3 and SEQ ID No. 4 containing an annealing buffer in a water bath for 11 minutes, and cooling them to room temperature naturally.

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[0023] In the present disclosure, the nucleotide sequence of SEQ ID No. 5 is specifically as follows:

CaccgCAAATATTTCCAAAATACCT

[0024] The nucleotide sequence of SEQ ID No. 6 is specifically as follows:

aaacAGGTATTTTGGAAATATTTGc.

[0025] In the present disclosure, the nucleotide sequences of SEQ ID No. 5 and SEQ ID No. 6 are annealed by boiling the nucleotide sequences of SEQ ID No. 5 and SEQ ID No. 6 containing an annealing buffer in a water bath for 11 minutes, and cooling them to room temperature naturally.

[0026] The present disclosure also provides a complete set of nucleic acids including sgRNA in the above technical solutions and a lentiviral vector lentiCRISPRv2.

[0027] The present disclosure also provides use of sgRNA in the above technical solutions in editing the sheep FGF5.

[0028] In the present disclosure, the use preferably includes the following steps of:

1) digesting a lentiviral vector lentiCRISPRv2 with BsmB I to obtain a digested vector, and ligating the sgRNA in the above technical solutions with the digested vector to obtain a recombinant plasmid;

2) co-transfecting the recombinant plasmid obtained in step 1) with plasmids psPAX2 and pMD2.G into 293T cells to obtain recombinant lentiviruses;

3) infecting sheep muscle cells with the recombinant lentiviruses obtained in step 2) to achieve editing of the sheep FGF5 by the sgRNA.

[0029] The present disclosure has no particular limitations on the source of the lentiviral vector lentiCRISPRv2, and conventional commercial products can be used. The present disclosure has no particular limitations on a method for digesting the lentiviral vector lentiCRISPRv2 with the BsmB I, and a conventional method for digesting the lentiviral vector lentiCRISPRv2 with the BsmB I can

be used. The present disclosure has no particular limitations on a method for ligating the sgRNA with the digested vector, and a ligating method conventionally used by those skilled in the art can be used.

[0030] In the present disclosure, the obtained recombinant plasmids and plasmids psPAX2 and pMD2.G are co-transfected into 293T cells to obtain recombinant lentiviruses. In the present disclosure, the mass of the recombinant plasmids, the mass of the plasmid psPAX2, the mass of the plasmid pMD2.G, and the quantity of the 293T cells at the time of plating are in a ratio of 20 μg: 15 μg: 6 μg: 2-2.5×10^6. In the present disclosure, the recombinant plasmids and the plasmids psPAX2 and pMD2.G are co-transfected into the 293T cells using a calcium phosphate transfection method. The present disclosure has no particular limitations on the calcium phosphate transfection method, and the method conventionally used by those skilled in the art can be used.

[0031] In the present disclosure, sheep muscle cells are infected with the obtained recombinant lentiviruses to achieve editing of sheep FGF5 by the sgRNA. The present disclosure has no particular limitations on the method for infecting the sheep muscle cells with the recombinant lentiviruses, and a method conventionally used by those skilled in the art can be used. In the present disclosure, the recombinant lentiviruses preferably select the muscle cells with puromycin after infecting the sheep muscle cells to obtain resistant muscle cells, DNA and RNA of the resistant muscle cells are extracted, and editing of sheep FGF5 by the sgRNA is analyzed.

[0032] The technical solutions provided by the present disclosure will be described in detail below with reference to examples, but the examples should not be construed as limiting the claimed scope of the present disclosure. The experimental methods in the following examples are conventional methods unless specially stated. For details, see "Molecular Cloning (3rd Ed.)". The test materials used in the following examples are purchased from conventional biochemical reagent companies unless otherwise specially stated.

Example 1

[0033] The nucleotide sequences of SEQ ID No. 1 and SEQ ID No. 2 containing annealing buffer were boiled in a water bath for 11 minutes, and cooled to room temperature naturally to obtain sgRNA.

Example 2

[0034] The nucleotide sequences of SEQ ID No. 3 and SEQ ID No. 4 containing annealing buffer were boiled in a water bath for 11 minutes, and cooled to room temperature naturally to obtain sgRNA.

Example 3

[0035] The nucleotide sequences of SEQ ID No. 5 and SEQ ID No. 6 containing annealing buffer were boiled in a water bath for 11 minutes, and cooled to room temperature naturally to obtain sgRNA.

Comparative Example 1

[0036] The nucleotide sequences of SEQ ID No. 7 and SEQ ID No. 8 containing annealing buffer were boiled in a water bath for 11 minutes, and cooled to room temperature naturally to obtain sgRNA;

SEQ ID No.7: CaccgTTCTGTCATCCTAGGTATTT;

SEQ ID No.8: aaacAAATACCTAGGATGACAGAAc.

Comparative Example 2

[0037] The nucleotide sequences of SEQ ID No. 9 and SEQ ID No. 10 containing annealing buffer were boiled in a water bath for 11 minutes, and cooled to room temperature naturally to obtain sgRNA;

SEQ ID No.9: CaccgTTTTTAGCGATGTCAAAAAA;

SEQ ID No.10: aaacTTTTTTGACATCGCTAAAAAc.

Example 4

Construction of LentiCRISPRv2 recombinant plasmids

1. Design of sgRNA and syntheses of oligonucleotide chains

[0038] According to CRISPR/Cas9 targeting principle, sgRNA was designed for PAM sequence in exon 2 region of FGF5 using online CRISPR design tool, wherein CACC was added to the 5' end of sgRNA sense strand templates, and AAAC was added to the 5' end of sgRNA antisense strand templates, so that sgRNA was complementary to the sticky end sequence formed by BsmB I digestion. The sequence was as follows:

Table 1 FGF5-sgRNA sequence information

Name	Sequence	No.	Positions of PAM in FGF5 genome
FGF5-sgRNA-F2	CaccgTTCTGTCATCCTAGGTATTT	SEQ ID No.7	8512-8514
FGF5-sgRNA-R2	aaacAAATACCTAGGATGACAGAAc	SEQ ID No.8	
FGF5-sgRNA-F4	CaccgGAAATATTTGCTGTGTCTCA	SEQ ID No.1	8534-8536
FGF5-sgRNA-R4	aaacTGAGACACAGCAAATATTTCc	SEQ ID No.2	
FGF5-sgRNA-F7	CaccgTTTTTAGCGATGTCAAAAAA	SEQ ID No.9	8591-8593
FGF5-sgRNA-R7	aaacTTTTTTGACATCGCTAAAAAc	SEQ ID No.10	
FGF5-sgRNA-F8	CaccgTGGTTCTACTTACACTTGCA	SEQ ID No.3	8600-8602 (reverse complementary)
FGF5-sgRNA-R8	aaacTGCAAGTGTAAGTAGAACCAc	SEQ ID No.4	
FGF5-sgRNA-F9	CaccgCAAATATTTCCAAAATACCT	SEQ ID No.5	8501-8503 (reverse complementary)
FGF5-sgRNA-R9	aaacAGGTATTTTGGAAATATTTGc	SEQ ID No.6	

2. Construction of recombinant eukaryotic expression plasmid lentiCRISPRv2-sgRNA

(1) Digesting lentiCRISPRv2 plasmids with single restriction enzyme BsmB I, and then performing 1% agarose gel electrophoresis (see FIG. 1 for 1% agarose gel electrophoresis map of the digested product) to recover and purify linearized plasmids (FIG. 2).

(2) Annealing primers FGF5-sgRNA-F and FGF5-sgRNA-R to obtain double-stranded DNA molecules with sticky ends at both ends.

(3) Ligating the product of step (1) with the double-stranded DNA molecules obtained in step (2) to obtain a recombinant plasmid. Sequencing a fragment of interest in the recombinant plasmids found that a sequence of interest appeared in the sequencing result, which was consistent with the original sequence (FIG. 3), indicating that vectors were successfully constructed.

(4) For the recombinant vectors identified correctly by sequencing and without base mutation, extracting the plasmids according to the instruction of QIAGEN Midi kit, measuring the concentration and purity of the extracted plasmids by Nanodrop One nucleic acid quantifier, and storing at -20°C for later use.

Example 5

Packaging of LentiCRISPRv2 recombinant lentiviruses

[0039] According to the packaging instruction of LentiCRISPRv2 lentiviruses, recombinant lentiviral plasmids and packaging plasmids psPAX2 and pMD2.G were co-transfected into 293T cells using a calcium phosphate transfection method for packaging of the lentiviruses.

1. 2-2.5x10^6 293T cells were seeded in per 10 cm cell culture plate, and prepared for transfecting with lentiviral plasmids when the cells adhered to the wall and reached 70-80% growth confluence.

2. The recombinant lentiviral plasmids and the packaging plasmids psPAX2 and pMD2.G were co-transfected into 293T cells by the calcium phosphate transfection method for packaging of the lentiviruses. Transfection system (1 ml/10 cm plate): recombinant lentiviral plasmids 20 μg; packaging plasmids (psPAX2) 15 μg; enveloped plasmids (pMD2.G) 6 μg; diluted with sterile H_2O to 500 μl; 2 × HBS (Hepes buffered saline) 500 μl was added; the mixture was mixed thoroughly by rapid vortexing.

3. While vortexing, 50 μl 2.5 M $CaCl_2$ was slowly added dropwise to the above mixture. The mixture was added to the cell culture plate after incubating at room temperature for 20 minutes.

4. The medium was exchanged for fresh medium after 12-14 h, and the cells were transferred to an incubator at 32°C after 10 h.

5. The cell supernatant was collected after 14-16h, and the supernatant was filtered with a 0.45 μm filter (that is, lentivirus infection solution). The filtered supernatant could be directly used to infect target cells.

6. The packaging of recombinant lentiviruses was evaluated by observing the expression of GFP reporter gene in cells after infection with recombinant viruses containing pLEX-EGFP and LentiCRISPRv2-GFP respectively. Fluorescence detection results showed that the recombinant lentiviruses had high packaging efficiency and could continue to be used to infect the target cells (FIG. 4).

Example 6

Infection of target cells with recombinant lentiviruses carrying FGF5-sgRNA

1. Target cells (sheep muscle cells) were seeded in a 6-well cell plate, and infected with lentiviruses when the cells adhered to the wall and reached 60-70% growth confluence.

2. The cell supernatant containing lentivirus packaging plasmids and fresh medium (v/v, 1:1) and 10 μg/ml polybrene was added to the target cells. Then, the cells were placed in an incubator at 32°C.

3. The cells were transferred to an incubator at 37°C after 14-16 h, the medium was exchanged for fresh medium after 10 h to continue culturing.

4. The cells were transferred to a 10 cm cell culture plate after 48 h, and at the same time cell culture medium containing 1.5 μg/ml puromycin was added for cell selection for 15 days, and the cell culture medium containing 1.5 μg/ml puromycin was replaced once every 3-4 days.

FGF5 editing and identification of transcript types in resistant cells

[0040] After lentivirus infection and 15 days of puromycin selection, muscle cells infected with

FGF5-sgRNA-2, FGF5-sgRNA-4, FGF5-sgRNA-7, FGF5-sgRNA-8 and FGF5-sgRNA-9 recombinant lentiviruses all gained resistance. Each type of resistant cells was collected and used for DNA extraction and RNA extraction.

1. DNA detection

[0041] According to the instruction of TIANamp Genomic DNA Kit, DNA was extracted from muscle cells after virus infection and 15 days of puromycin selection, and DNA was used as template to perform PCR amplification on the sequence of the second exon region of FGF5 of the resistant cells (see Table 2 for PCR amplification primers) to obtain a 402 bp amplified product including a target sequence, and it was determined whether gene editing has occurred near the corresponding PAM sequence by sequencing. Sequencing results showed that a set of peaks appear at the sequence after PAM site in the muscle cells after infection with FGF5-SgRNA4 (FIG. 6), FGF5-SgRNA8 (FIG. 8) and FGF5-SgRNA9 (FIG. 9) recombinant viruses, which preliminarily showed that they played an editing role for FGF5. No significant changes occurred in a gene sequence of interest in the muscle cells after infection with FGF5-sgRNA2 (FIG. 5) and FGF5-sgRNA7 (FIG. 7) recombinant viruses, which preliminarily showed that they didn't play an editing role for FGF5.

Table 2 PCR amplification primers for the second exon of FGF5

Name of the primers	Sequence	No.	Fragment size
FGF5-8357-F	CATAATCAATGTCACAATAAACAAGG	SEQ ID No.11	402 bp
FGF5-8759-R	CTTATAAAAACACATACAATTCTCTTGG	SEQ ID No.12	

2. Hi-Tom DNA sequencing

[0042] To further clarify the types of FGF5-sgRNA gene editing and the rate of different gene editing types, with reference to Hi-TOM gene editing site detection kit, Hi-TOM PCR was performed using DNA of the obtained resistant cells as a template (see Table 3 for primer information). The PCR product was recovered and purified for targeted deep sequencing analysis.

Table 3 Hi-TOM PCR (DNA) amplification primers

Name of the primers	Sequence	No.	Fragment size
Hi-Tom-DNA-FGF5-F	GGAGTGAGTACGGTGTGCGATATATAGAGGAGTCTGTG	SEQ ID No.19	201 bp
Hi-Tom-DNA-FGF5-R	GAGTTGGATGCTGGATGGTGTAAAATCTCTGTAACACC	SEQ ID No.20	

[0043] Hi-Tom DNA sequencing results showed that: (1) compared with the Control group, 2 bases were deleted at positions 8529-8530 of FGF5 genome in main FGF5-sgRNA4, and this result was obtained in three repeated experiments (FIGS 12-14); (2) compared with the Control group, a base A was inserted after position 8605 of FGF5 genome in main FGF5-sgRNA8, and this result was obtained in three repeated experiments (FIGS 15-17); (3) compared with the Control group, 4 bases were deleted at positions 8505-8508 of FGF5 genome in main FGF5-sgRNA9, 2 bases were deleted at positions 8508-8509, a base was deleted at position 8507, 2 bases were deleted at positions 8505-8506, a base T was inserted after position 8507 and this result was obtained in three repeated experiments (FIGS 18-20); (4) compared with the Control group, FGF5-sgRNA2 and FGF5-SgRNA7 didn't play an gene editing role.

3. cDNA detection

[0044] According to the instruction of TransZol Up Plus RNA Kit, total RNA was extracted from the cells after virus infection and 15 days of drug selection, and the concentration and purity of total RNA were measured by Nanodrop One nucleic acid quantifier. The total RNA was reverse transcribed into cDNA according to SuperScript™ IV First-Strand Synthesis System reverse transcription kit, and cDNA was used as a template for RT-PCR detection (see Table 4 for RT-PCR amplification primers). The RT-PCR product was verified by 2% agarose gel electrophoresis and analyzed and photographed by UV gel imaging system. The results showed (FIGS 10, 11) that the relative brightness of bands of full-length FGF5 (including 3 exons of FGF5) as RT-PCR product was lower than that of the Control group, the relative brightness of bands of truncated FGF5 (FGF5s, including only exons 1 and 3) was higher than that of the Control group in FGF5-sgRNA4, FGF5-sgRNA8, FGF5-sgRNA9

electrophoresis bands, indicating that FGF5-sgRNA4, FGF5-sgRNA8, and FGF5-sgRNA9 played an gene editing role in the sheep muscle cells, and exon 2 in the full-length mRNA sequence of FGF5 was detected by an alternative splicing form under sgRNA editing. The brightness of the bands of the full-length FGF5 and the truncated FGF5 of FGF5-sgRNA2 and FGF5-sgRNA7 had no relatively significant changes in the selected resistant cells and the unselected control cells (FIG. 10).

Table 4 RT-PCR identification primers for different spliceosome types of FGF5

Name of the primers	Sequence	No.	Fragment size
cDNA-FGF5-F	CTACCCGGATGGCAAAGTCA	SEQ ID No.13	230 bp / 126bp
cDNA-FGF5-R	TGTGTATGGCGGAGGCATAG	SEQ ID No.14	
β-actin-F	TGACCCAGATCATGTTTGAGA	SEQ ID No.15	186 bp
β-actin-R	CAAGGTCCAGACGCAGGAT	SEQ ID No.16	

4. Hi-Tom cDNA sequencing

[0045] To further clarify the different types of transcriptional expression products of FGF5 and their relative ratios in cells with FGF5-sgRNA editing, with reference to Hi-TOM gene editing site detection kit, Hi-TOM PCR was performed using DNA of the obtained resistant cells as a template (see Table 5 for primer information). The PCR product was recovered and purified for targeted deep sequencing analysis.

Table 5 Hi-TOM PCR (cDNA) amplification primers

Name of the primers	Sequence	No.	Fragment size
Hi-Tom-cDNA-FGF5-F	GGAGTGAGTACGGTGTGCCTACCCGGATGGCAAAGTCA	SEQ ID No.17	230 bp /126bp
Hi-Tom-cDNA-FGF5-R	GAGTTGGATGCTGGATGGTGTGTATGGCGGAGGCATAG	SEQ ID No.18	

[0046] Hi-Tom cDNA sequencing results showed that: (1) compared with the Control group, FGF5-sgRNA4, FGF5-sgRNA8 and FGF5-sgRNA9 all played an gene editing role, thereby reducing the expression of mRNA including full-length FGF5 (that of cells with FGF5-sgRNA4, FGF5 -sgRNA8 and FGF5-sgRNA9 editing decreased from 65.52% of the control to 22.99%, 2.42% and 15.07% respectively), and the expression of mRNA including truncated FGF5 increased (that of cells with FGF5-sgRNA4, FGF5 -sgRNA8 and FGF5-sgRNA9 editing increased from 30.69% of the control to 43.25%, 52.45% and 69.11% respectively), and the amount of expression products with Indel mutation in FGF5 transcription products of edited cells increased (that of cells with FGF5-sgRNA4, FGF5-sgRNA8, FGF5-sgRNA9 editing increased from 3.79% of the control to 33.76%, 45.13% and 15.83% respectively) (FIG. 21). Therefore, the sequencing results showed that exon 2 in mRNA sequence of transcriptional expression products with FGF5 editing was detected by an alternative splicing form under sgRNA editing, and the changes of the expression of the full-length and truncated FGF5 in the sequencing results were consistent with the verification results of agarose gel electrophoresis (FIG. 10 and FIG. 11); (2) compared with the Control group, FGF5-sgRNA2 and FGF5-sgRNA7 didn't play an gene editing role. Therefore, the expression of mRNA of the full-length FGF5 didn't decrease, and the expression of mRNA of the truncated FGF5 didn't increase (FIG. 21).

[0047] The foregoing descriptions are only preferred implementation manners of the present disclosure. It should be noted that for those skilled in the art, several improvements and modifications may further be made without departing from the principle of the present disclosure. These improvements and modifications should also be deemed as falling within the protection scope of the present disclosure.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A single guide RNA (sgRNA) for editing sheep fibroblast growth factor 5 gene (FGF5) to achieve alternative splicing, wherein the sgRNA is obtained by annealing nucleotide sequences having SEQ ID Nos. 1-6; wherein:

the nucleotide sequences of SEQ ID No. 1 and SEQ ID No. 2 are annealed pairwise; and

the nucleotide sequences of SEQ ID No. 3 and SEQ ID No. 4 are annealed pairwise; and

the nucleotide sequences of SEQ ID No. 5 and SEQ ID No. 6 are annealed pairwise.

2. A complete set of nucleic acids, comprising the sgRNA according to claim 1 and a lentiviral vector lentiCRISPRv2.

3. Use of the sgRNA according to claim 1 in editing sheep FGF5.

4. The use according to claim 3, comprising the following steps of:

1) digesting a lentiviral vector lentiCRISPRv2 with BsmBI to obtain a digested vector, and ligating the sgRNA according to claim 1 with the digested vector to obtain a recombinant plasmid;

2) co-transfecting 293T cells with the recombinant plasmid obtained in step 1) with plasmids psPAX2 and pMD2.G to obtain recombinant lentiviruses;

3) infecting sheep muscle cells with the recombinant lentiviruses obtained in step 2) to achieve editing of sheep FGF5 by the sgRNA.

5. The use according to claim 4, wherein the mass of the recombinant plasmids, the mass of the plasmid psPAX2, the mass of the plasmid pMD2.G, and the quantity of the 293T cells at the time of plating in step 2) are in a ratio of 20 μg: 15 μg: 6 μg: 2-2.5×10^6.

6. The use according to claim 4, wherein the recombinant plasmids and the plasmids psPAX2 and pMD2.G are co-transfected into the 293T cells using a calcium phosphate transfection method.

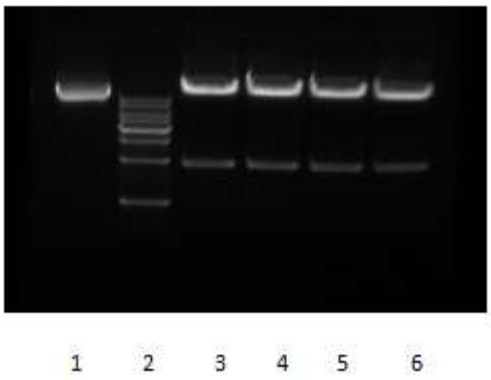

FIG. 1

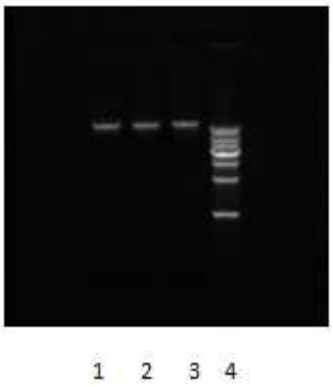

FIG. 2

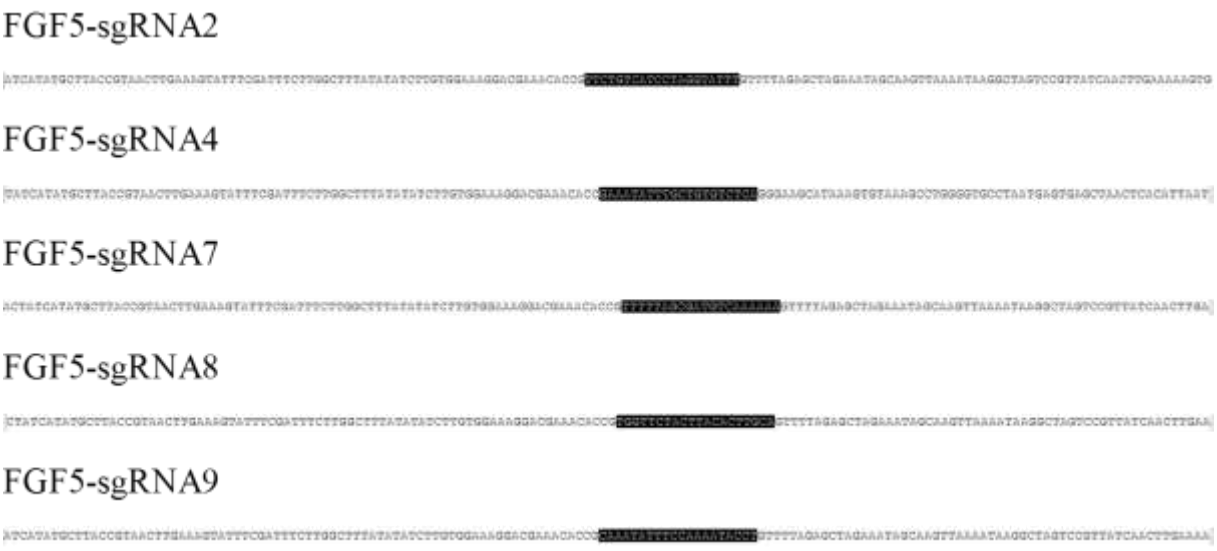

FIG. 3

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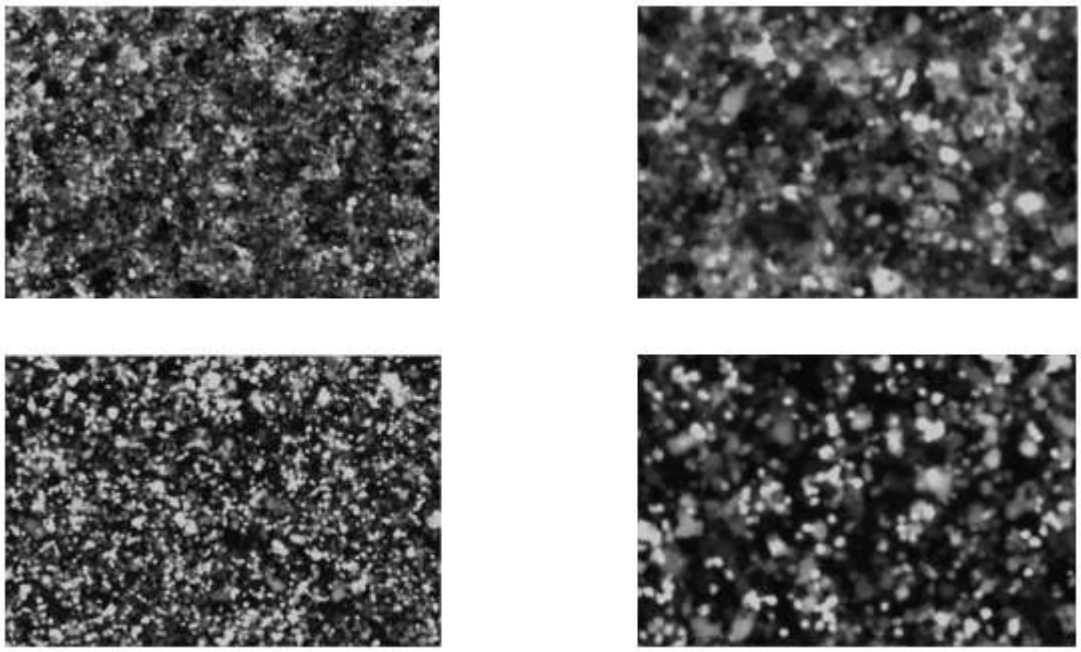

FIG. 4

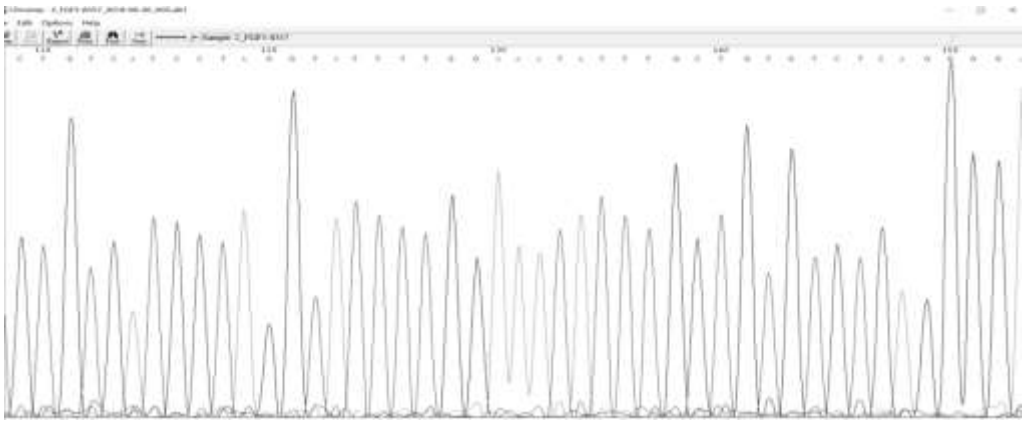

FIG. 5

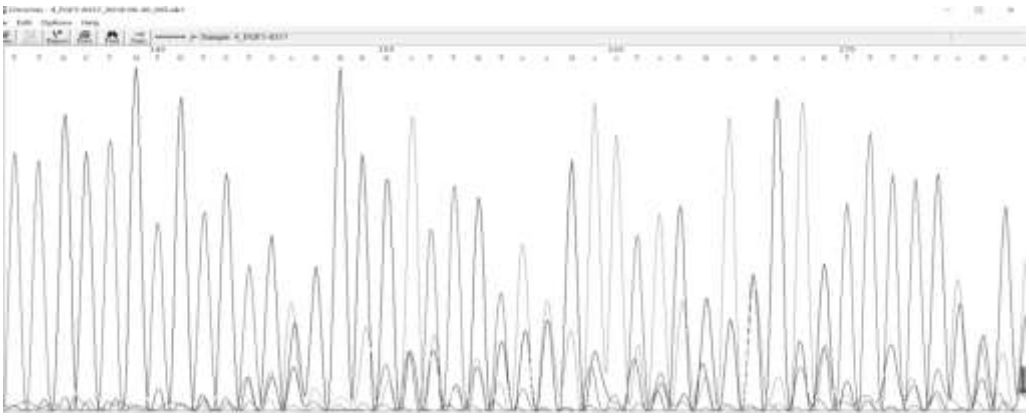

FIG. 6

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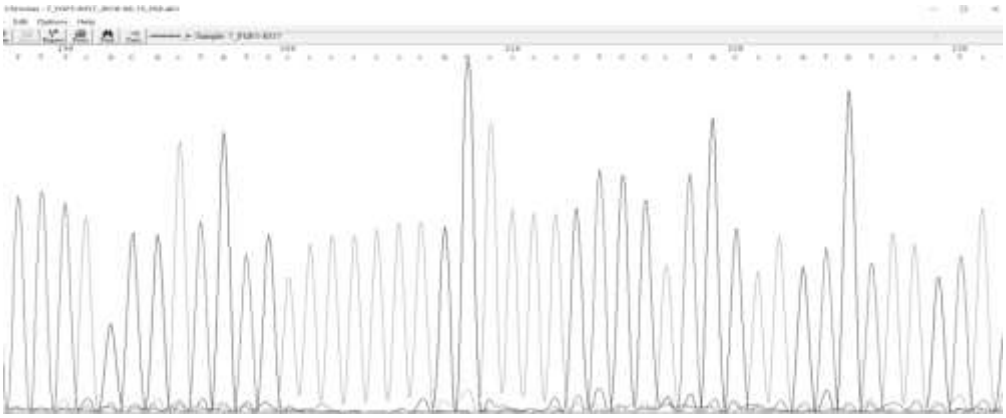

FIG. 7

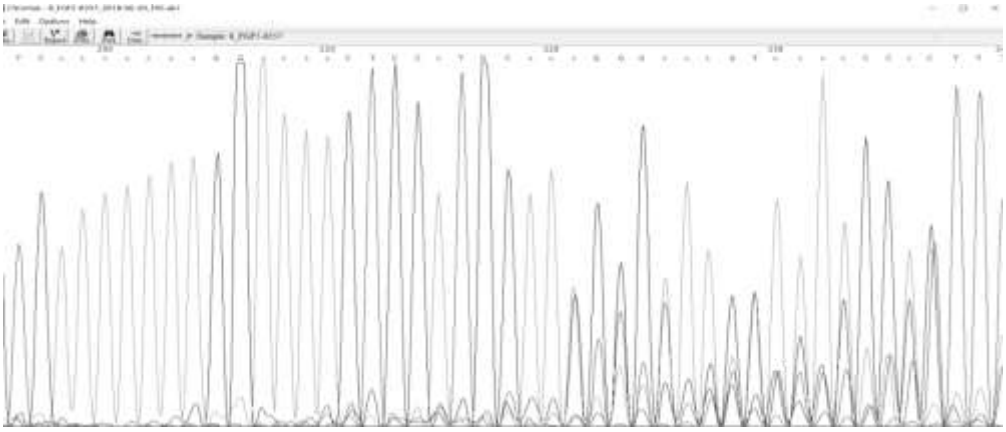

FIG. 8

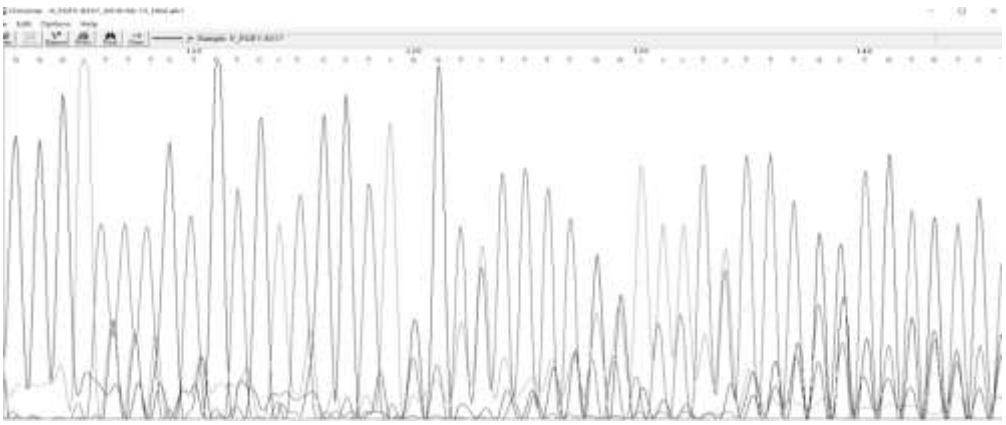

FIG. 9

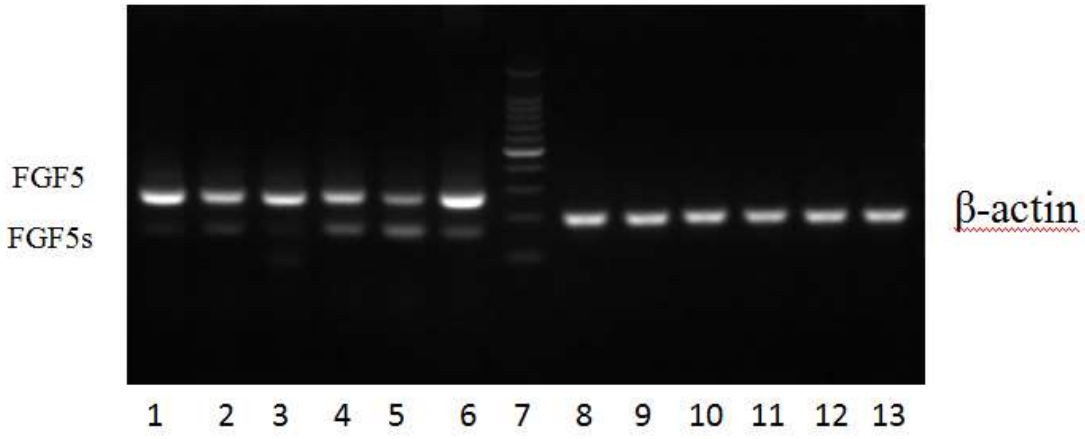

FIG. 10

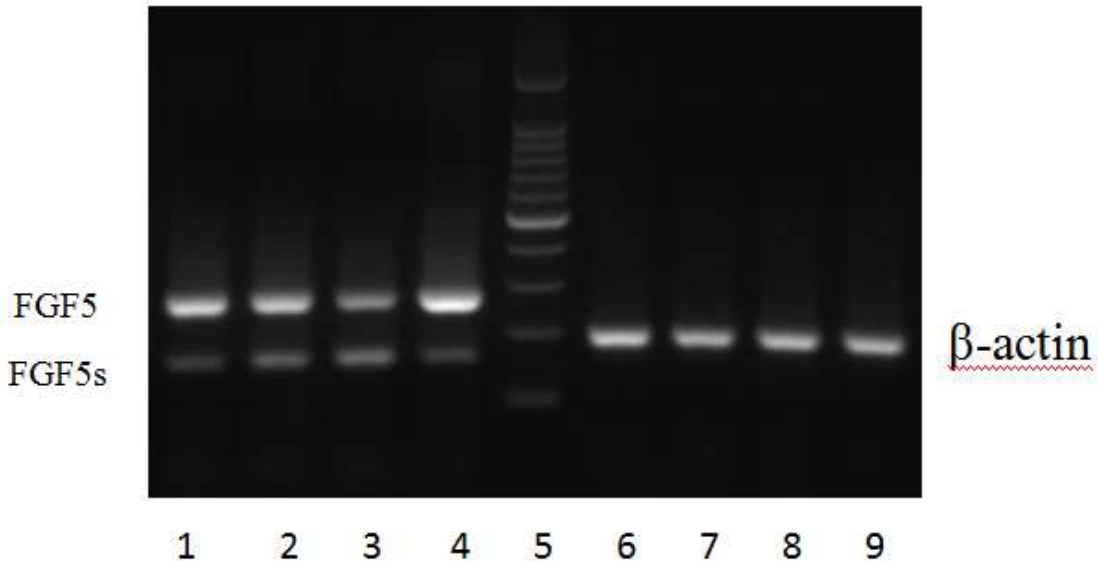

FIG. 11

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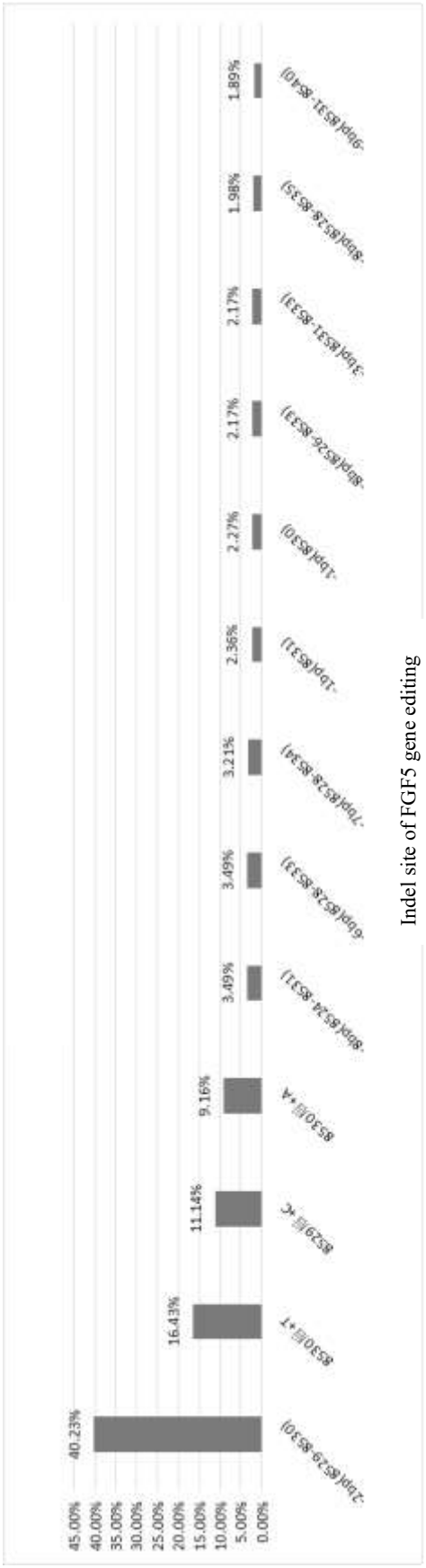

FIG. 12

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FIG. 13

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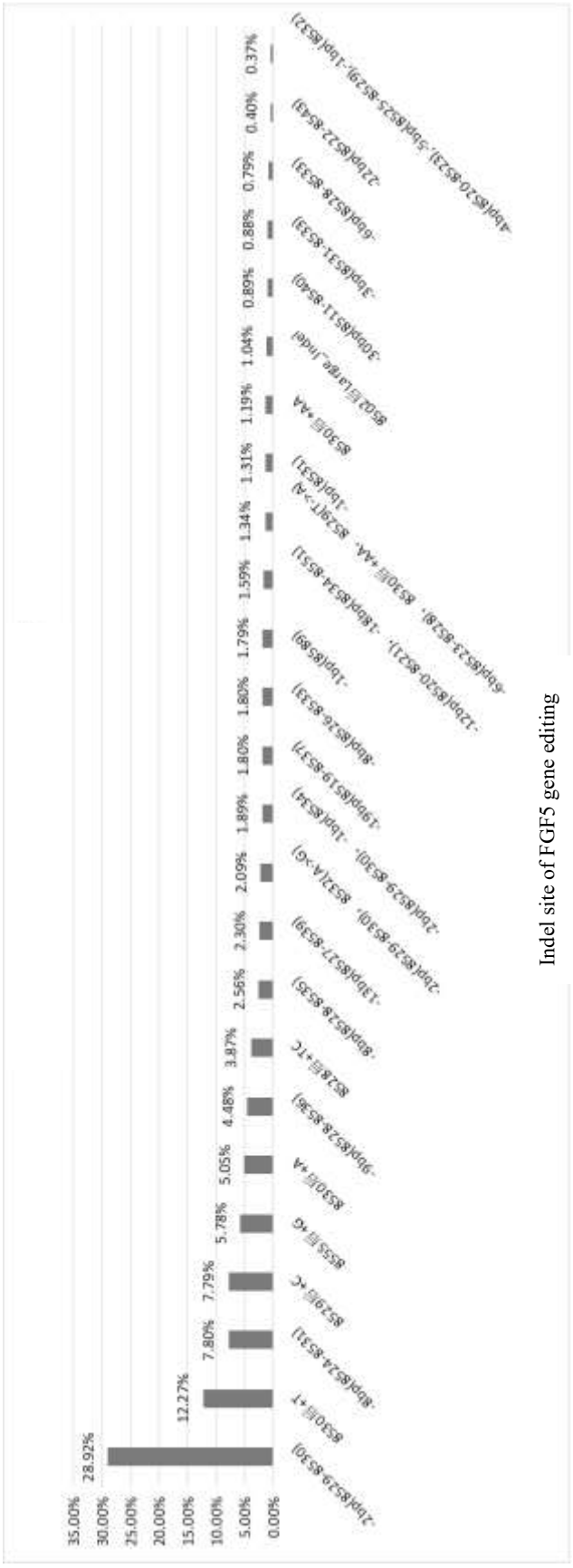

Indel site of FGF5 gene editing

FIG. 14

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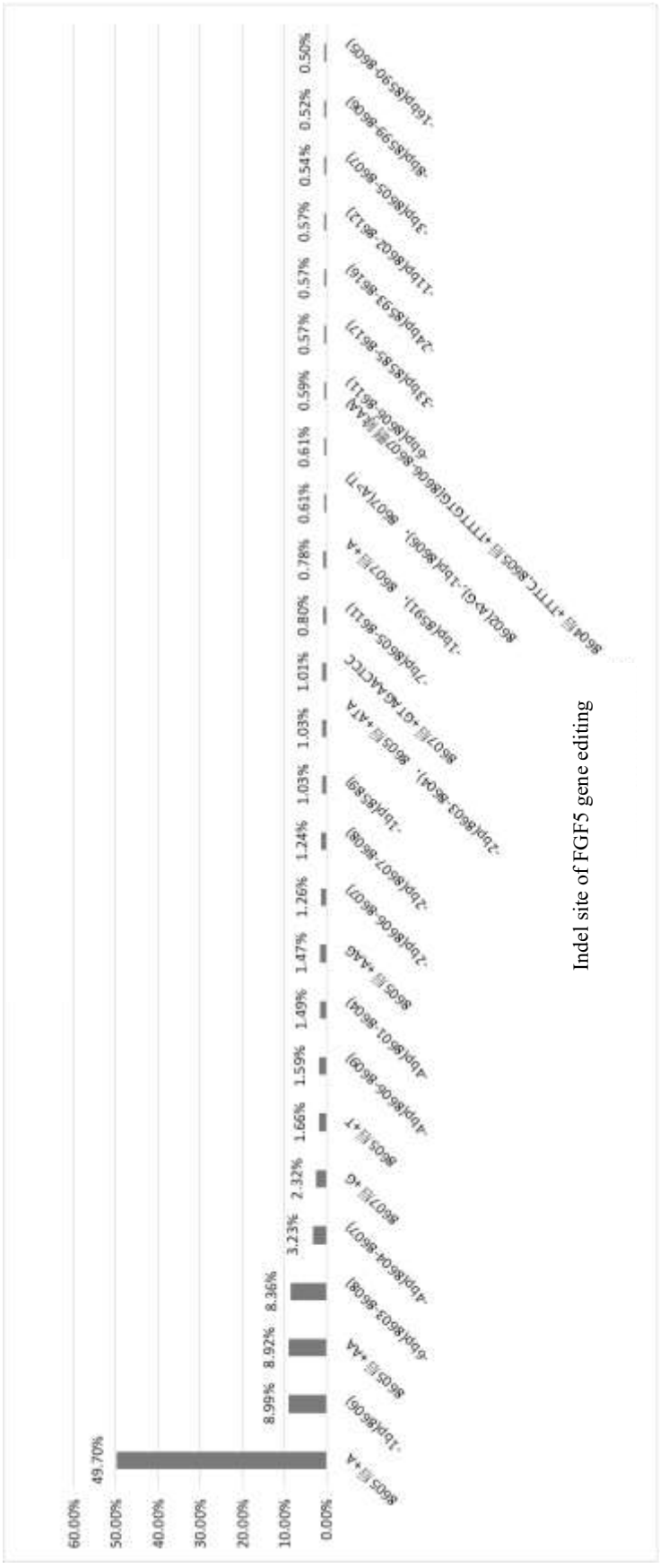

FIG. 15

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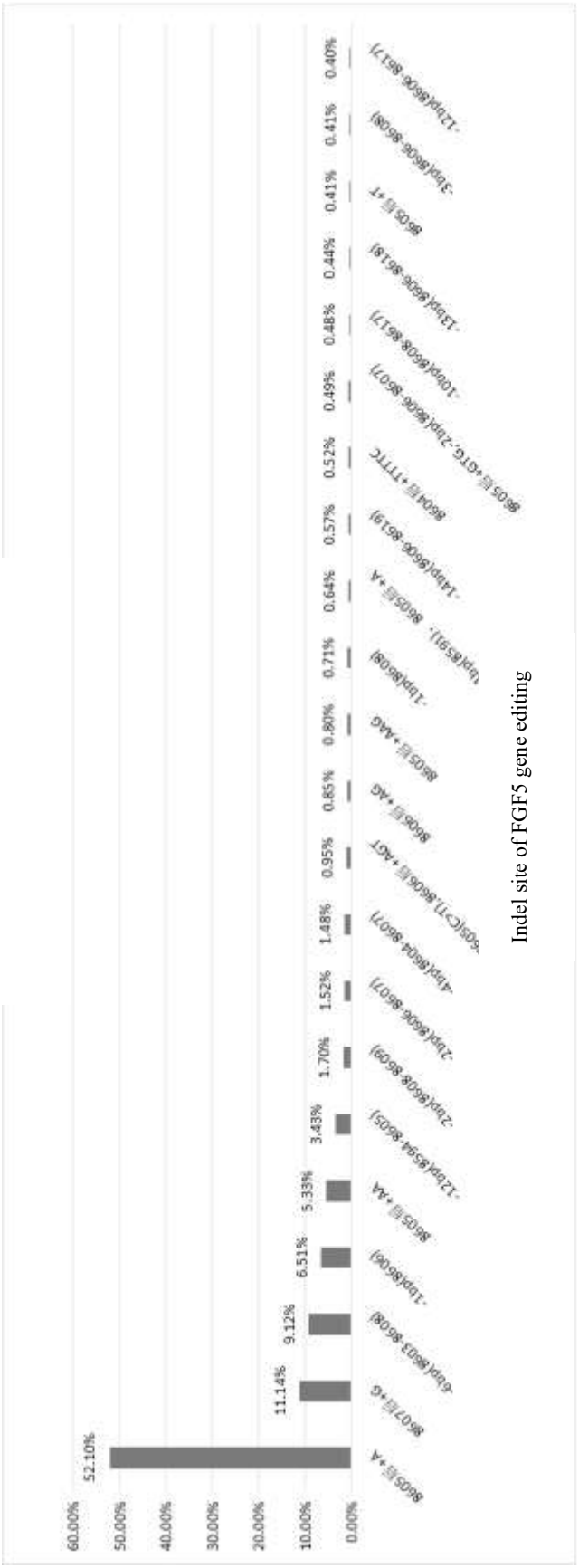

FIG. 16

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FIG. 17

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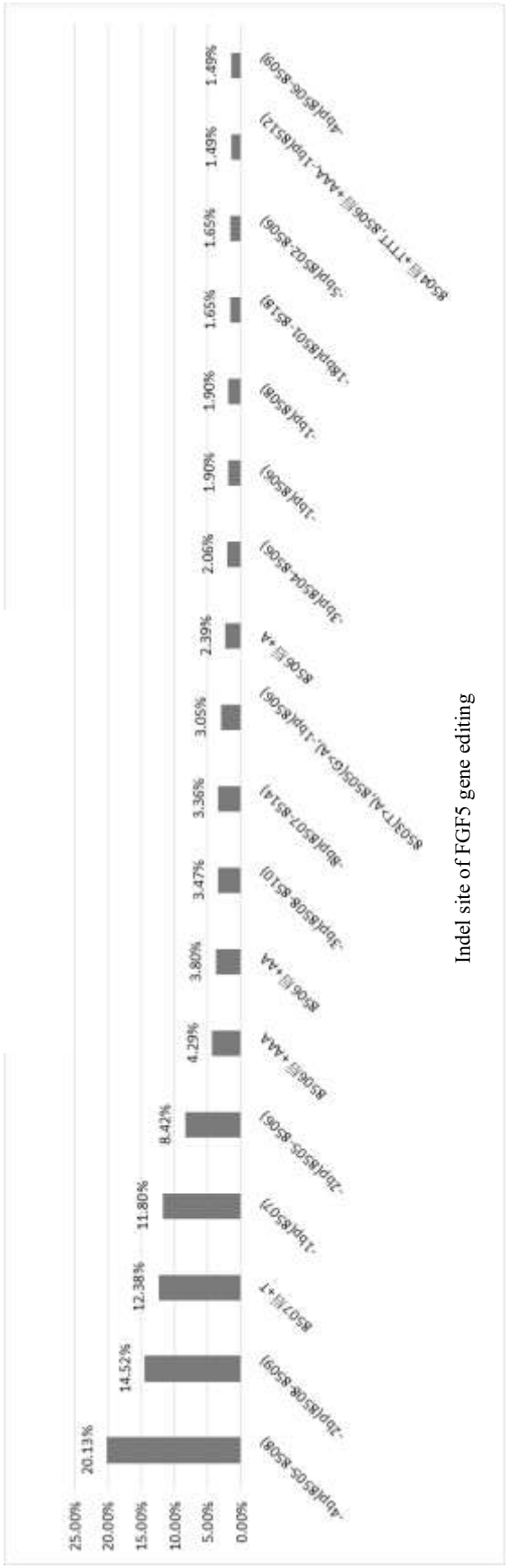

FIG. 18

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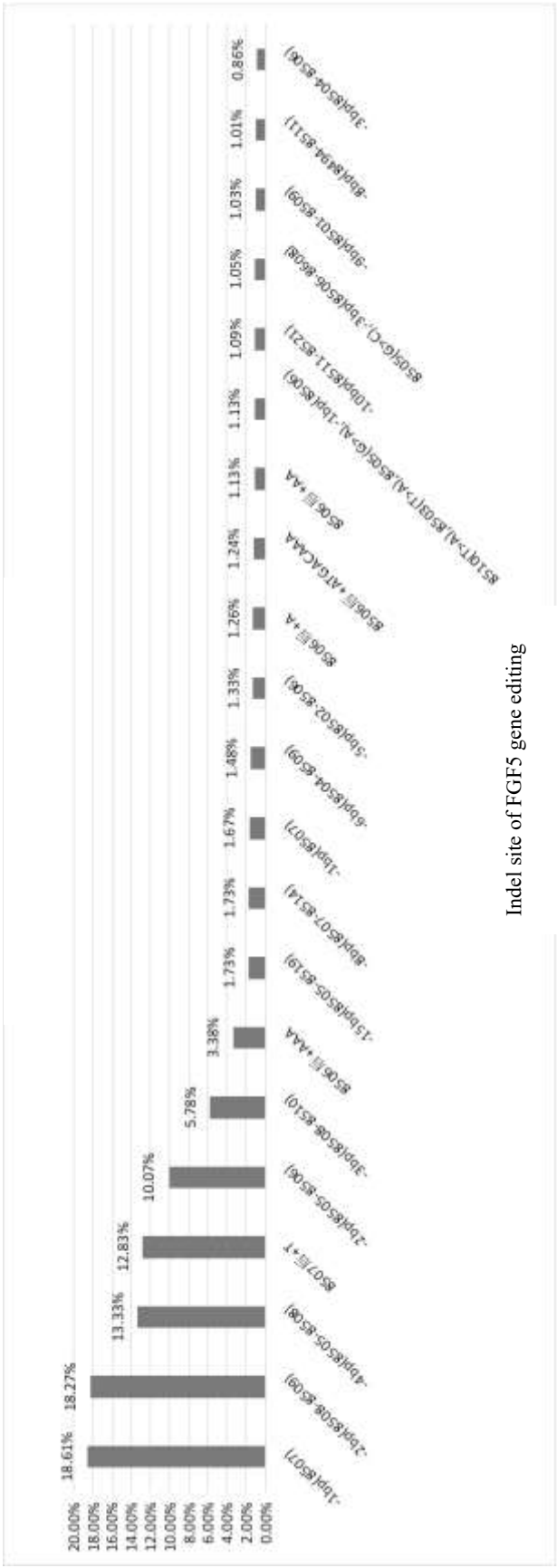

FIG. 19

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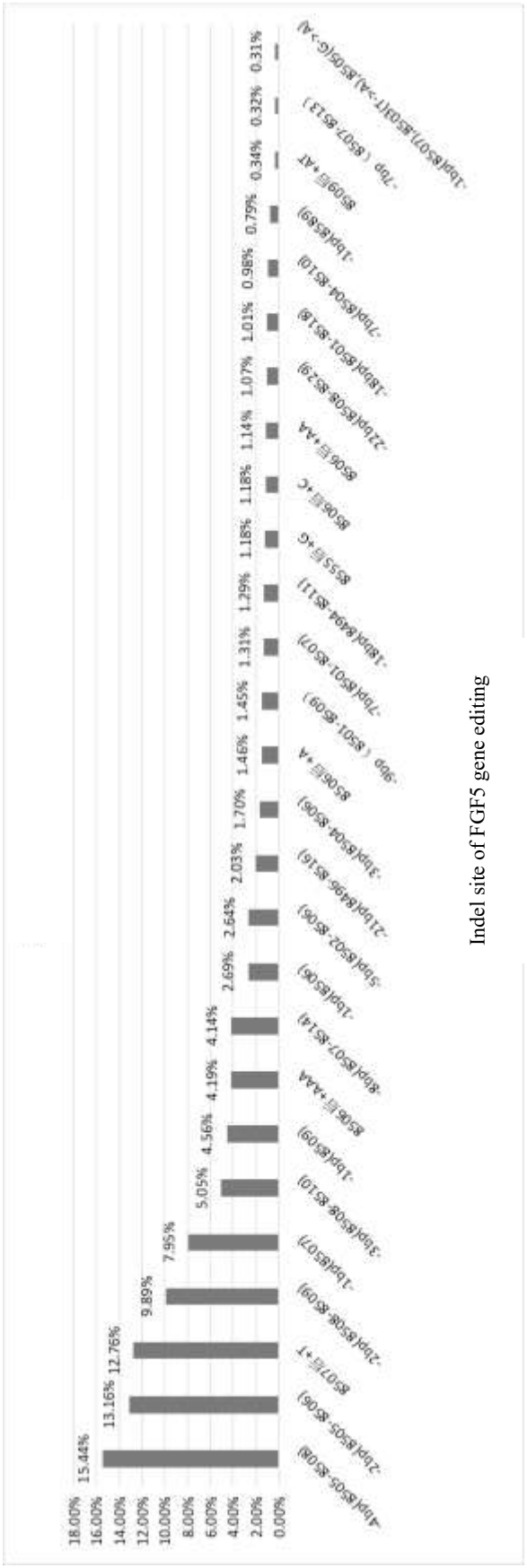

FIG. 20

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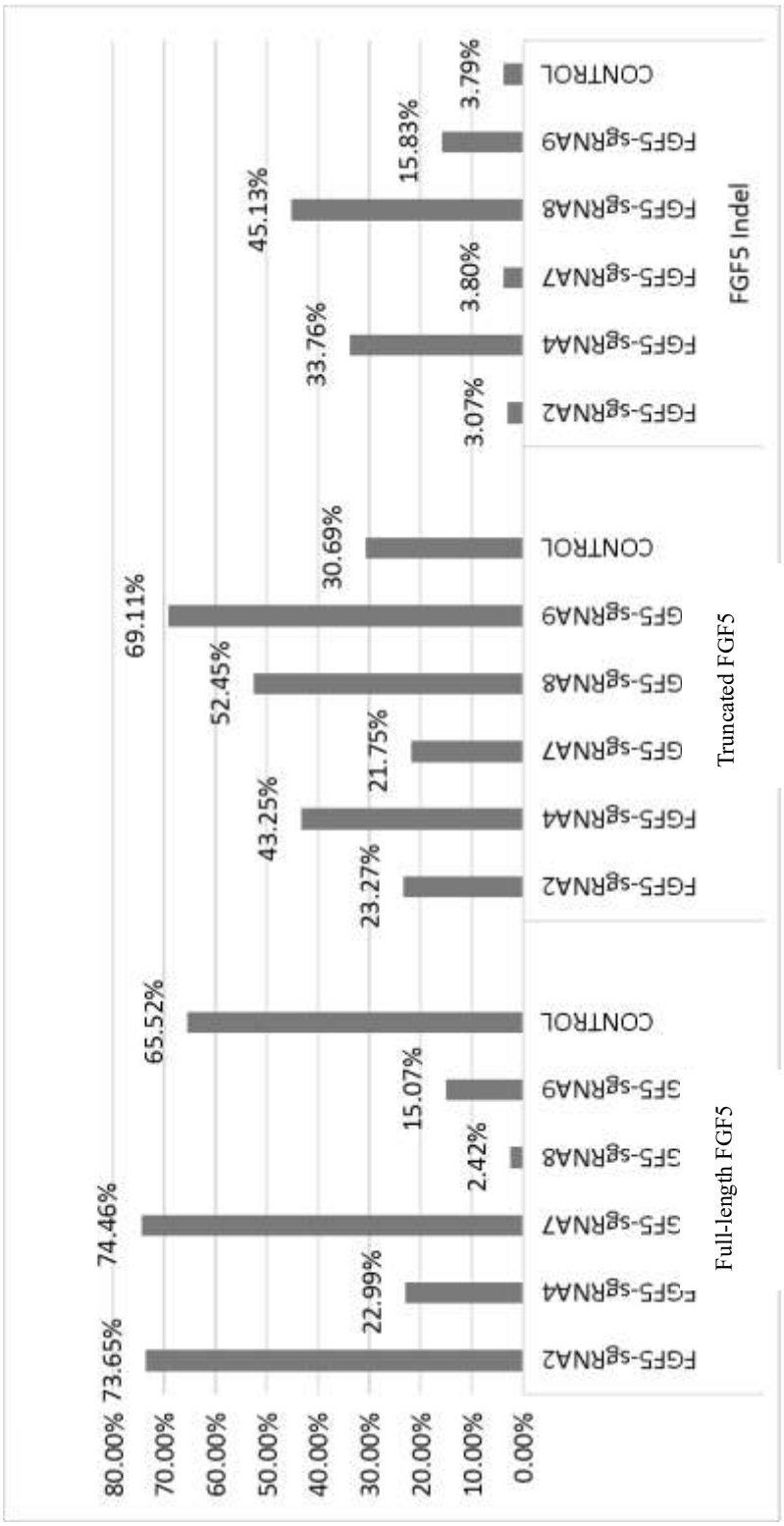

FIG. 21

Sequence List

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