10.1071/RD18485_AC

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Supplementary Material

Optimisation of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 : single-guide RNA (sgRNA) delivery system in a goat model

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Supplementary methods

Animal ethics statement

18 rams (2–3 years old, bodyweight: 30–50 kg) and 50 ewes (44 donors and 6 for rams training, 2–3 years old, bodyweight: 30–40 kg) of the Shaanbei Cashmere goat breed were used for this study. All protocols involving the use of animals were in accordance with approved guidelines of the Animal Care and Use Committee of the Northwest A&F University (Approval ID: 2014ZX08008-002).

Production of Cas9 and sgRNA plasmid

To test the efficiency and specificity of diverse sgRNA in goat fetal fibroblasts, we synthesized DNA oligos; then, annealed the double-stranded and sub-cloned into the pGL3-U6-sgRNA (U6-sgRNA) vector. Next, we transferred the pGL3-U6-sgRNA vector and pST1374-Cas9-ZFNLS (Cas9)

plasmid into competent cells (DH5α). Cells were cultured for eight hours for rapid amplification. Finally, we extracted the U6-sgRNA and Cas9 plasmid using the Endo-free Plasmid Purification Kit (TaKaRa, Dalian, China).

Optimization of the CRISPR system in goat fetal fibroblasts

We established a fibroblast cell line from 45-day-old goat fetus trunk tissue and collected cells at the fourth generation for transfection. Cell-culture medium A consists of 10% FBS (ThermoFisher, Gibco, Australia), 100 μg/mL penicillin, 100 μg/mL streptomycin, and DMEM medium (ThermoFisher, Gibco, USA). 10 sgRNA plasmids and Cas9 plasmid were transfected into a 12-well plate to verify the efficiency in goat fetal fibroblasts. The transfection procedure was conducted using Lipofectamine 3000 Reagent (ThermoFisher, Gibco, USA) according to the manufacturer's instructions.

After transfection, we used 1.2 μg/mL purinomycin and 28 μg/mL blasticidin to screen the cells for 2 days, followed by culture in cell-culture medium (DMEM-F12; Gibco, Rockville, MD). Next, cells were changed to cell-culture medium A for 24 h until they covered the entire culture dish. Genomic DNA was extracted via the Universal Genomic DNA Kit (CWBIO, China).

T7E1 cleavage and sequencing

To quantitatively measure the quality of the obtained genomic DNA, we used high-fidelity Taq polymerase (TOYOBO, Japan) to amplify the target gene fragment and for the subsequent Sanger sequencing (Table S6). Furthermore, the products were purified with a PCR Cleanup Kit (Omega, USA Norcross, GA), then immediately digested via T7E1 assay (NEB, Beijing, China) for 30 min at 37°C after denaturation and re-annealing with NEBuffer 2. The digestive results were separated via 2.5% agarose gel electrophoresis. We defined the grey value of unedited bands as label a, and labeled the edited bands as b and c, the formula to calculate the targeting efficiency of each sgRNA is as follows:

$$\text{Targeting efficiency (\%)} = \left(1-\sqrt{1-\frac{b+c}{a+b+c}}\right)\times 100$$

Off-target assay

To determine off-target effects of each sgRNA, we determined the specificity of sgRNAs, using sgRNAcas9 (Xie *et al.* 2014) to predict possible off-target loci and sequences. Next, we designed specific primers for each off-target site (Table S6), using PCR analysis and T7E1 enzyme digestion

to detect the knock-out in off-target sites, according to our previous study (Wang *et al.* 2015).

Preparation of the zygote injection mixture

We used DNA oligo sg8 to anneal the double-strand and sub-cloned it into the pUC57kan-T7-sgRNA (T7-sg8) vector. Next, a fragment containing T7 promoter and sg8 sequence was obtained via amplification with PrimerSTAR HS DNA polymerase (Takara, Beijing, China). Then, sgRNA was produced via the MEGA shortscript kit (Life Technologies, Ambion, USA) and was purified via the MEGA clear kit (Life Technologies, Ambion, USA). The pST1374-Cas9-ZFNLS (Cas9) plasmid was the *in vitro* transcription template and we linearized the Cas9 plasmid by Age I endonuclease (NEB, Beijing China), then transcript it *in vitro* via the mMESSAGE mMACHINE T7 Ultra Kit (Life Technologies, Ambion, USA) and used the RNeasy Mini Kit (Qiagen, Germany) to purified the Cas9 mRNA. The sgRNA and Cas9 mRNA were mixed at varied ratios according Table S5, and were stored at -80°C for further study.

Microinjection of Cas9/sgRNA system in goat zygotes

Healthy ewes (2 - 3-year-old) with regular estrus were selected as donors for zygote collection. These donors were treated with the CIDR Sheep and Goat Device (Eazi- Breed™, Pfizer, NY, USA) via insertion into the vagina for 14 to 16 days. The superovulation was performed with intramuscular injection of exogenous FSH (Folltropin-V, Bioniche Animal Health, Canada) 60 h before removing the CIDR. This treatment was administered via intramuscular injection with FSH for seven times (75, 50, 50, 37.5, 37.5, 25, and 25 mg each). Treatment with 1 mL prostaglandin was conducted at the time of CIDR removal. The estrous status of the donor was monitored 12 h after CIDR withdrawal for mating.

Zygotes were collected from oviduct via surgery 14 h after the last fertilization, and were transferred into TCM199 medium (ThermoFisher, Gibco, NY, USA). The Cas9 mRNA and sgRNA mixture with varied concentrations were injected into the cytoplasm of zygotes via the FemtoJet system (Eppendorf, Hamburg, Germany). Microinjection was conducted in the manipulation medium TCM199 on a heated platform with the Olympus micromanipulation system ON3. The conditions of injection pressure, injection time, and compensatory pressure were conducted according to (Wang *et al.* 2016). After injection, the zygotes were transferred to Quinn's Advantage Blastocyst Medium (Sage Biopharma, NJ, USA) at 38.5°C, 5% concentration of CO_2, and saturated humidity conditions.

Target efficacy detection in zygotes

The cleavage rate of embryos was observed after 24 h of incubation. We calculated the zygote cleavage rate and then collected every single zygote into a clean tube with approximately 2 μL PBS. Genomic DNA was extracted from zygotes using the REPLI-g Single Cell Kit (Qiagen, Germany). Next, the KOD Plus Neo (TOYOBO, Code No. KOD-201) was used for PCR amplification (Table S5) and Sanger sequencing was used to screen the knock-out rate and off-target effects of each group.

References

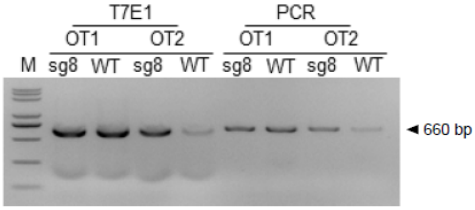
Wang, X., Yu, H., Lei, A., Zhou, J., Zeng, W., Zhu, H., Dong, Z., Niu, Y., Shi, B., and Cai, B. (2015) Generation of gene-modified goats targeting MSTN and FGF5 via zygote injection of CRISPR/Cas9 system. *Sci Rep* **5**, 13878.

Wang, X., Niu, Y., Zhou, J., Yu, H., Kou, Q., Lei, A., Zhao, X., Yan, H., Bei, C., and Shen, Q. (2016) Multiplex gene editing via CRISPR/Cas9 exhibits desirable muscle hypertrophy without detectable off-target effects in sheep. *Sci Rep* **6**, 32271.

(a)

Position	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	N	G	G	Location			
FGF5 sg8	G	A	G	C	C	A	T	T	G	A	C	T	T	T	G	C	C	A	T	C	C	G	G	Chr.	Start	End	Strand
OT1	C	A	G	G	C	T	C	T	G	A	C	T	T	T	G	C	C	A	T	C	C	A	G	18	8045804	8045826	+
OT2	A	A	C	C	T	G	T	T	G	A	C	T	T	T	G	C	C	A	T	C	C	A	G	6	40127127	40127149	-

(b)



(c)

*FGF5*_sg8 OT1

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*FGF5*_sg8 OT2

```
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```
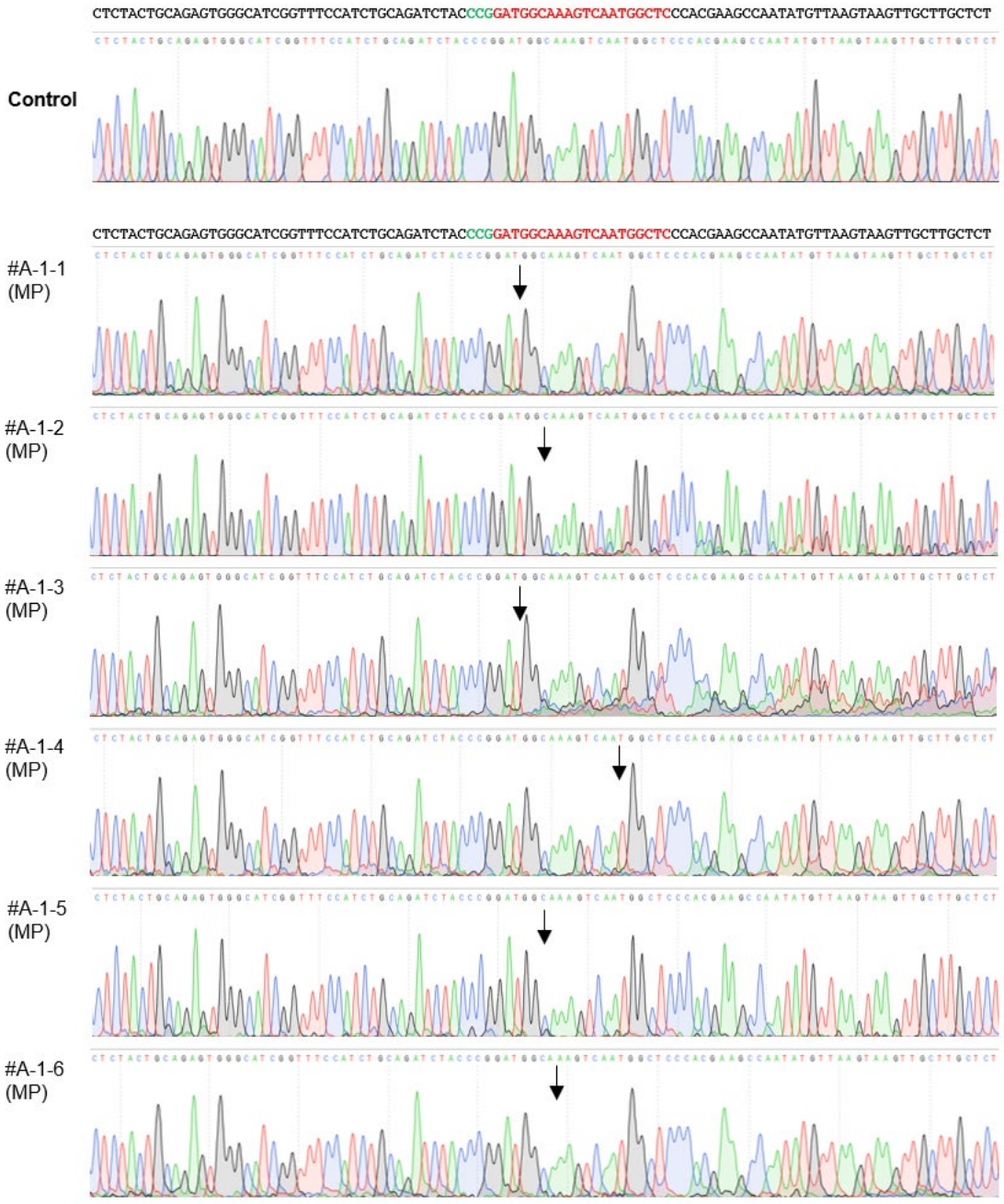

Fig. S1. (a) List of predicted off-target sites of sg8. (b) Left, detection of sgRNA:Cas9-mediated on-target cleavage of off-target sequence via T7E1 cleavage assay. Right, PCR products of the off-target sequence of sg8 from goat fibroblasts transfected with Cas9 and sg8. PCR products from the left panel were subjected to T7E1 cleavage assay. M, marker; WT, wild type PCR product from fibroblasts that have not been treated with CRISPR/Cas9. (c) Sequences of the FGF5 off-target site in fibroblasts and injected zygotes. Target sequences complementary to the FGF5 off-target sequence are shown red text, while SNP are shown in blue.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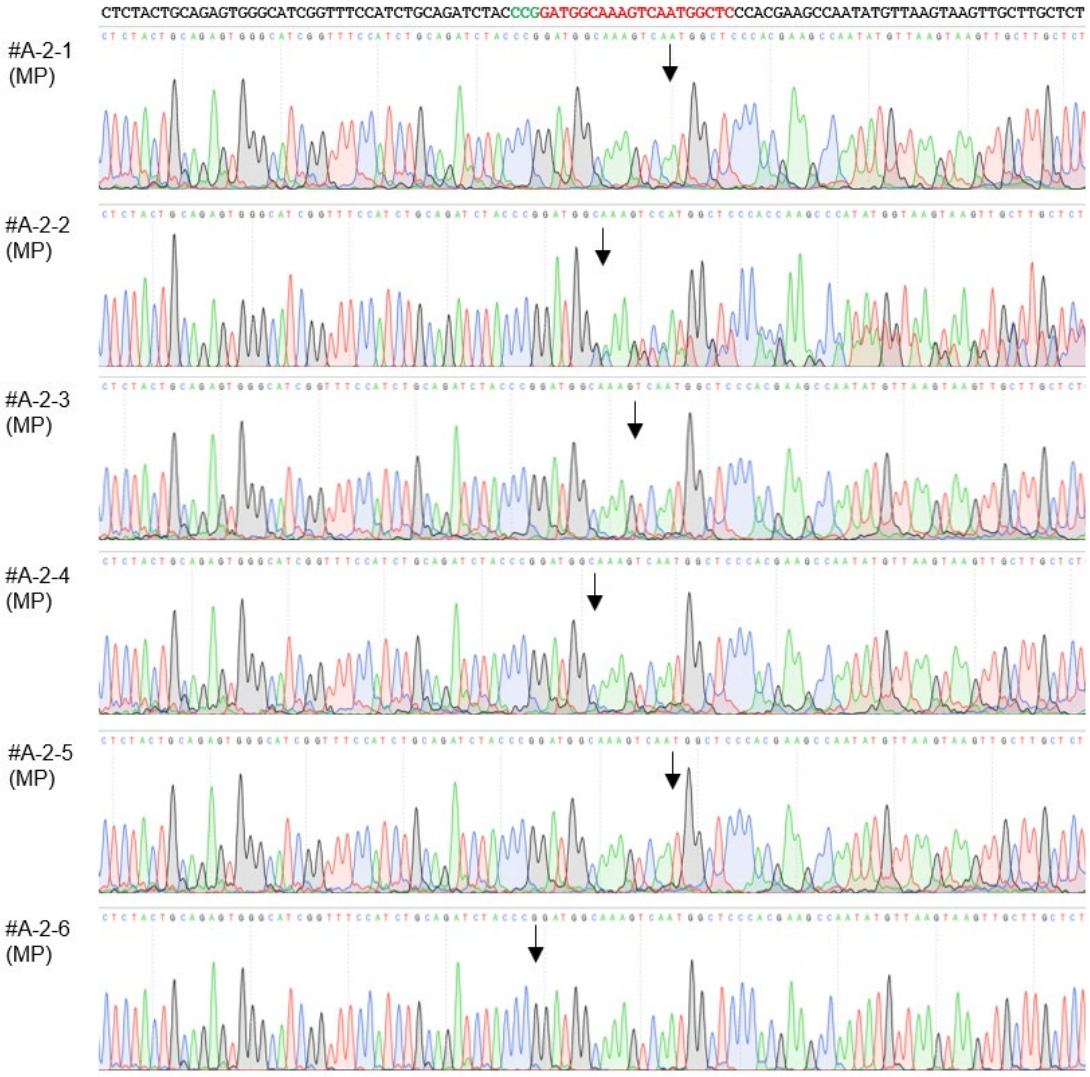
#A-1-6 (MP)

(A-1 group)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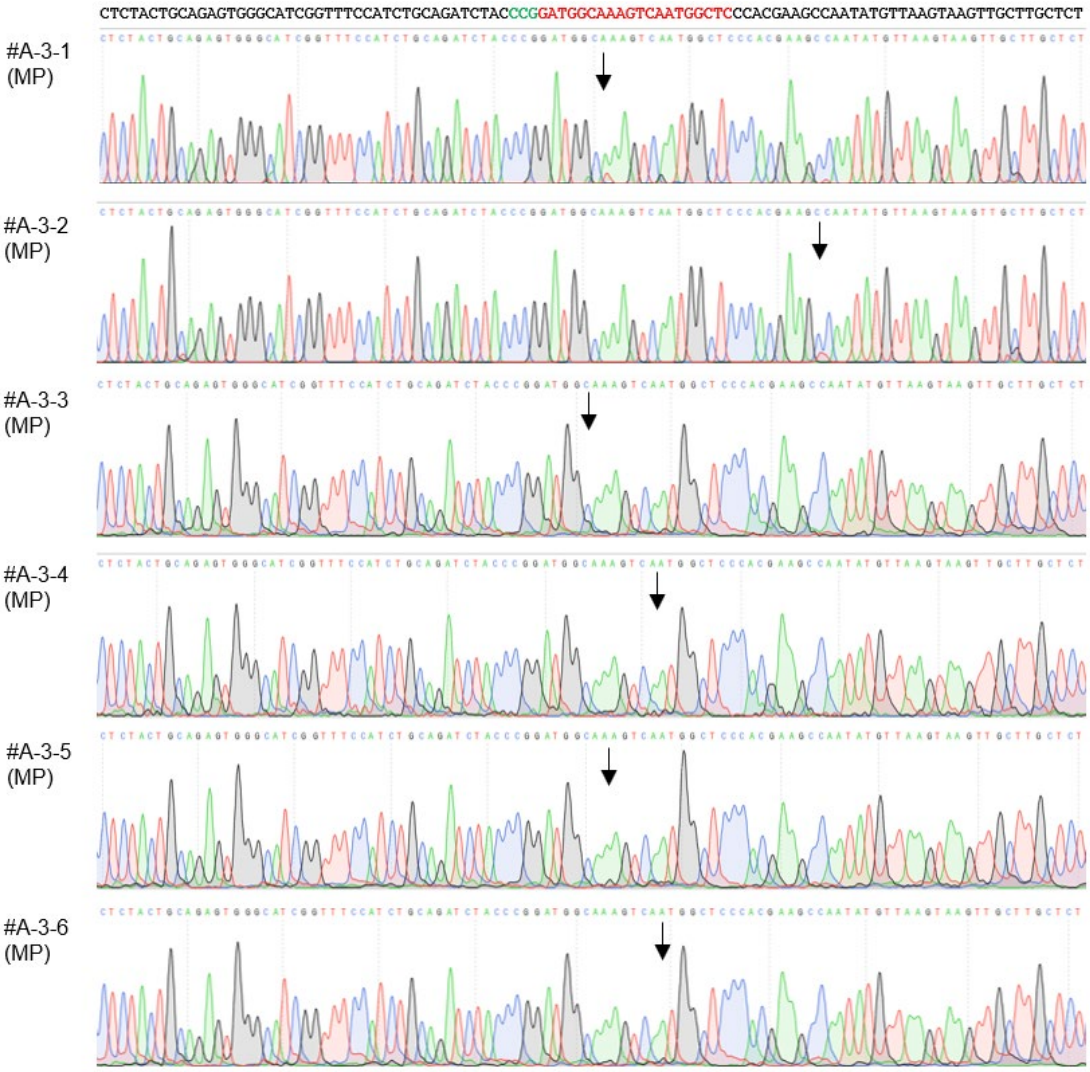
(MP)

(A-2 group)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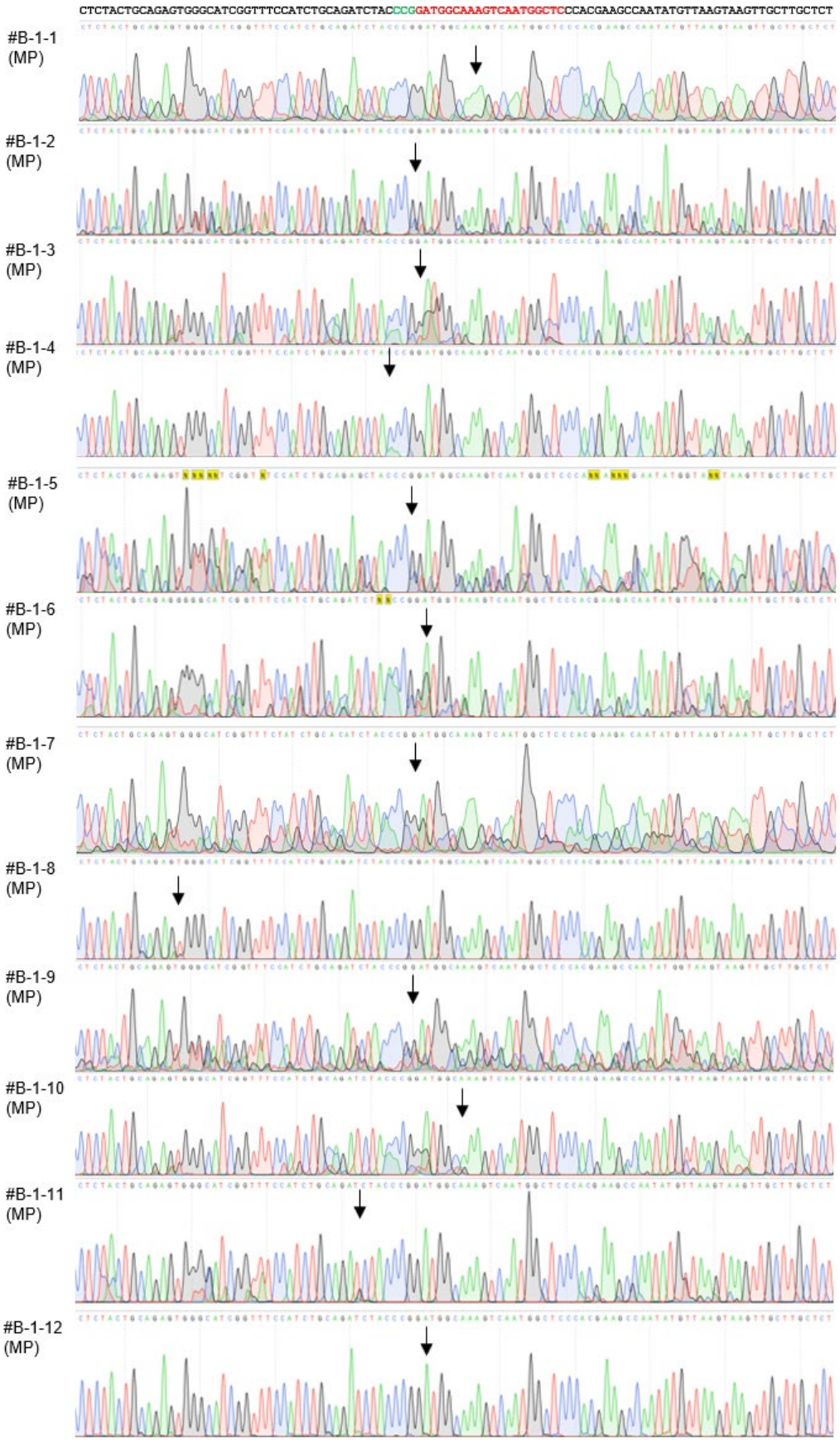
(MP)

(A-3 group)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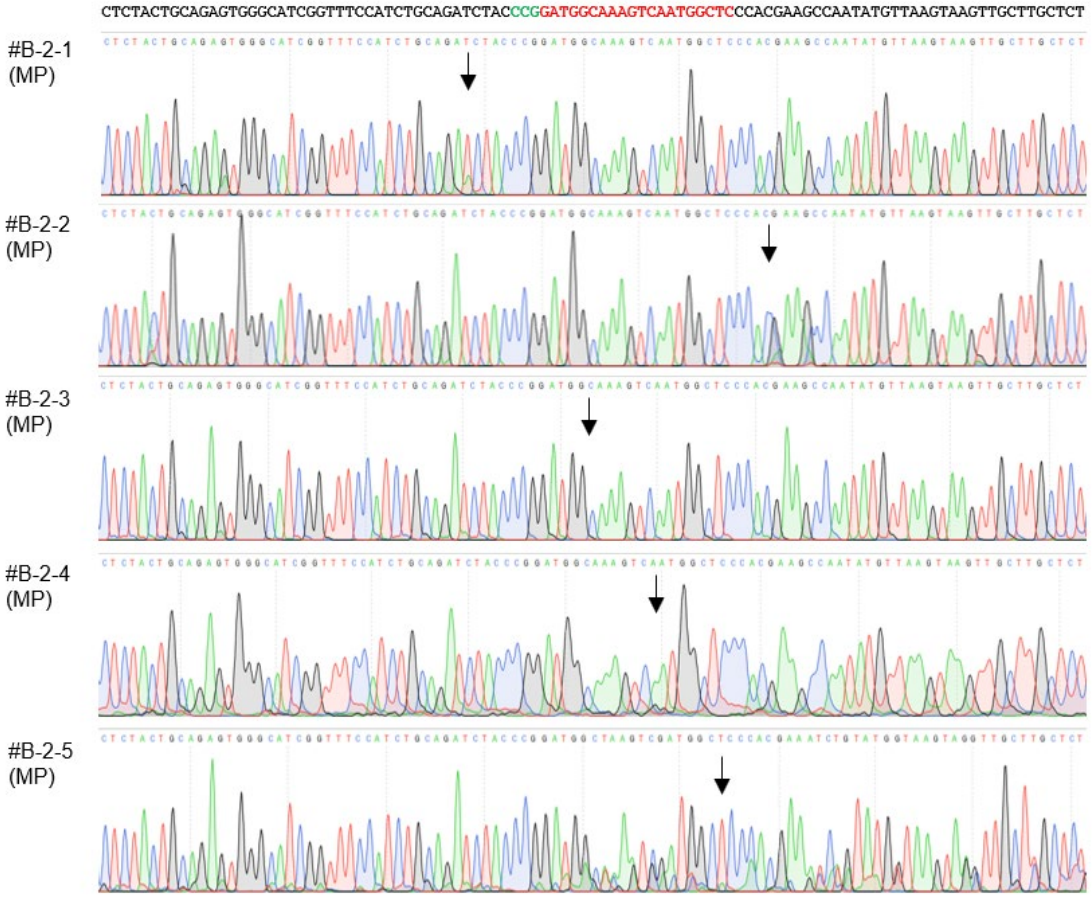
(B-1 group)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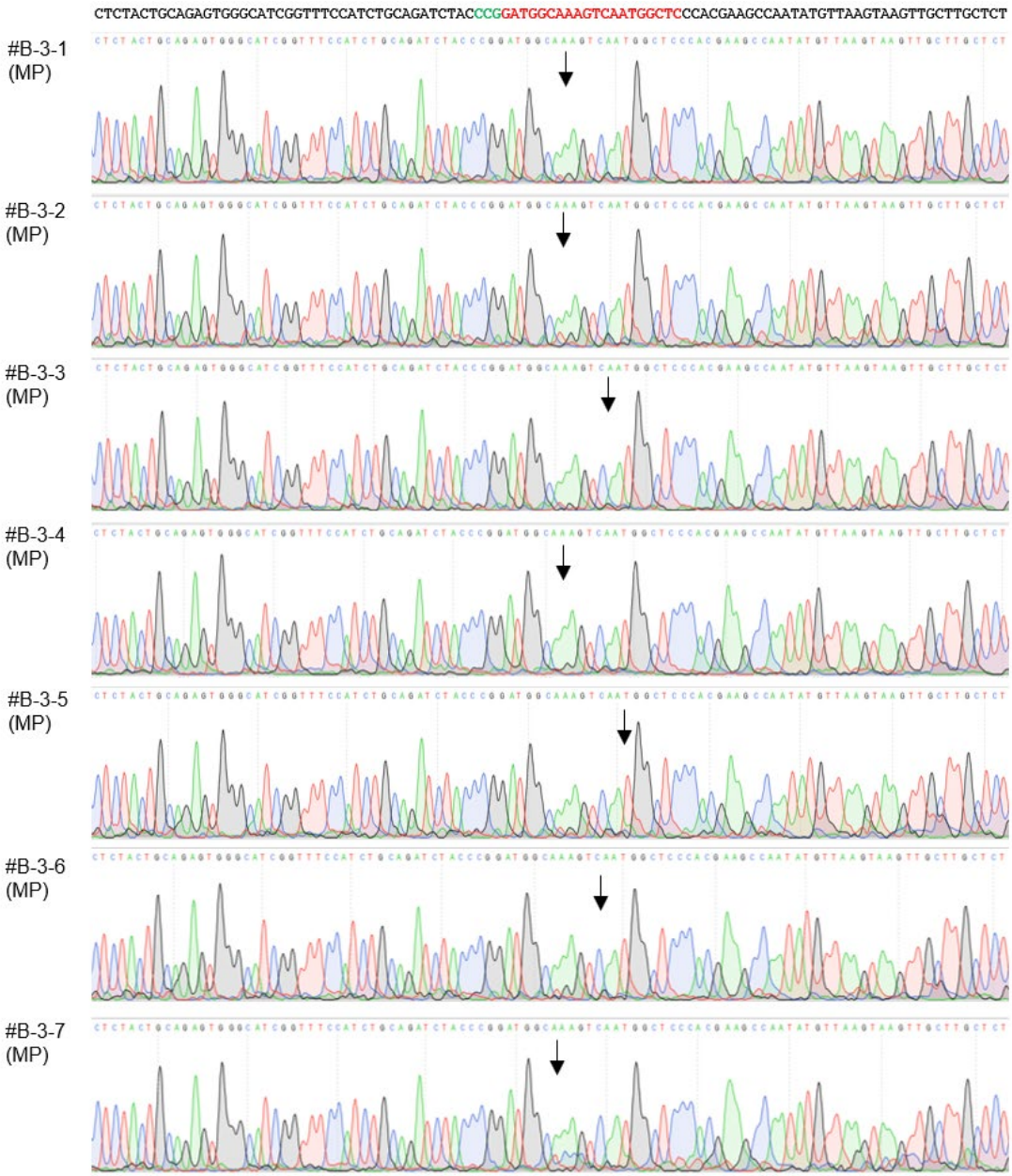
(B-2 group)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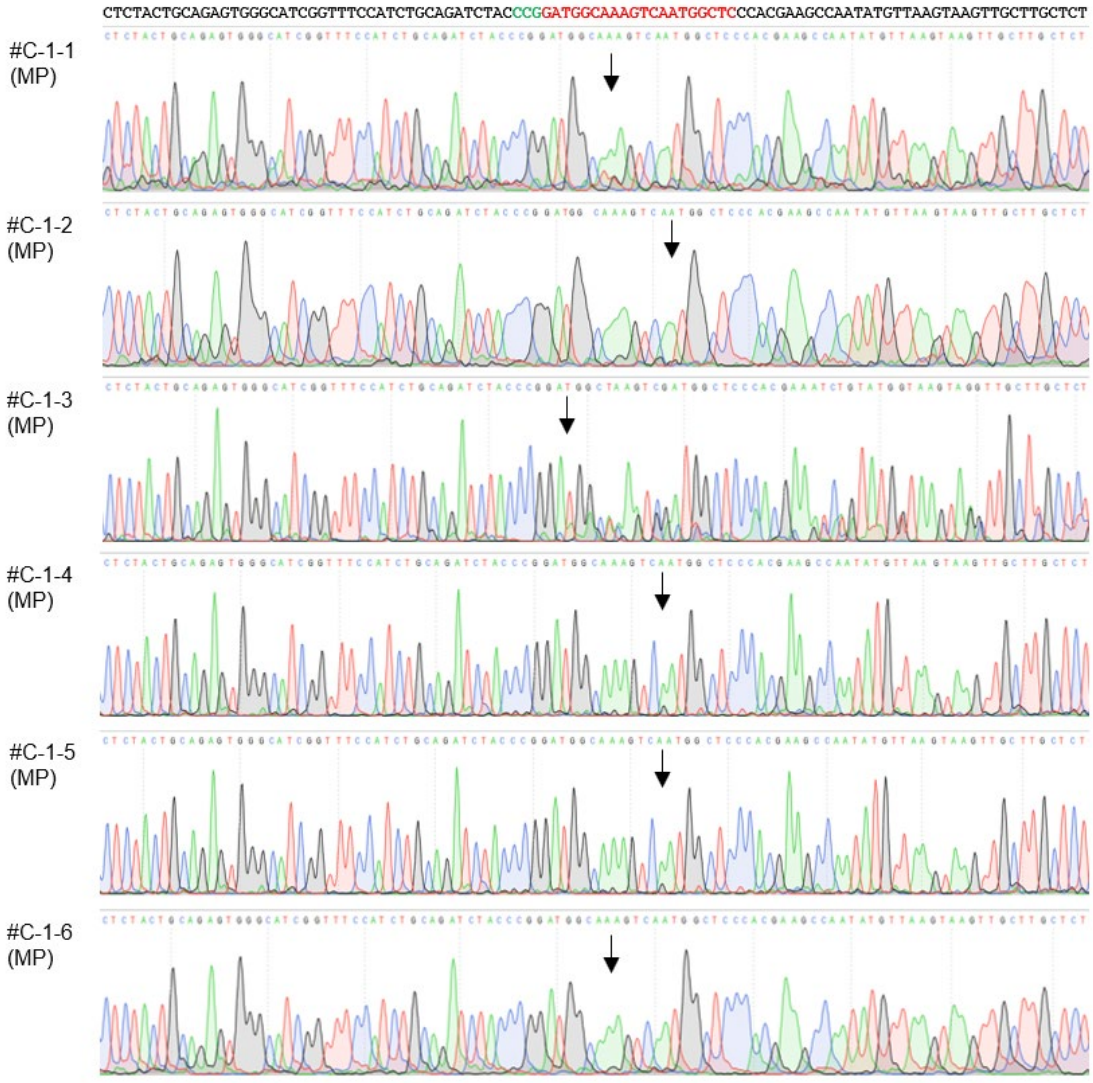
(B-3 group)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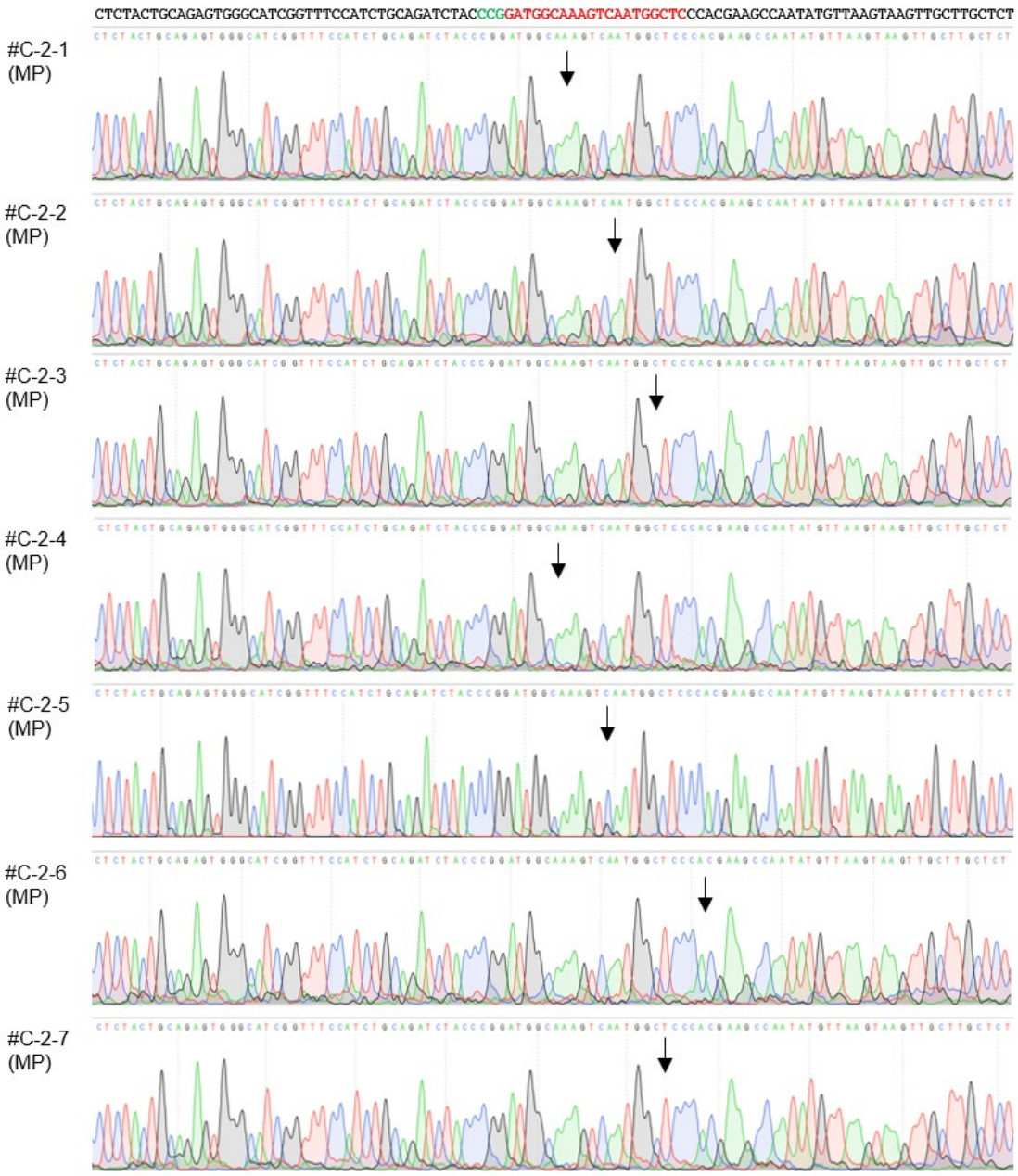
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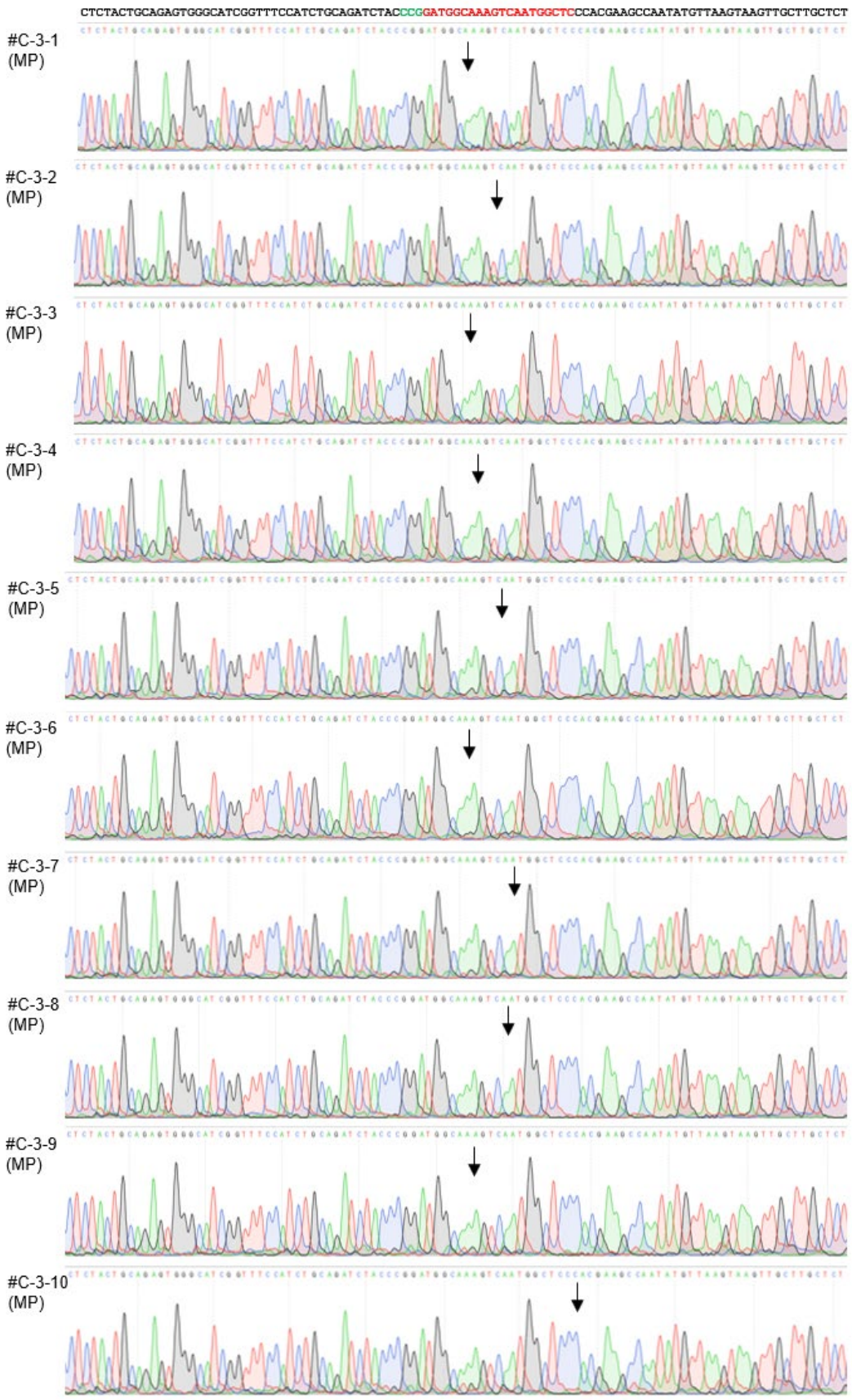
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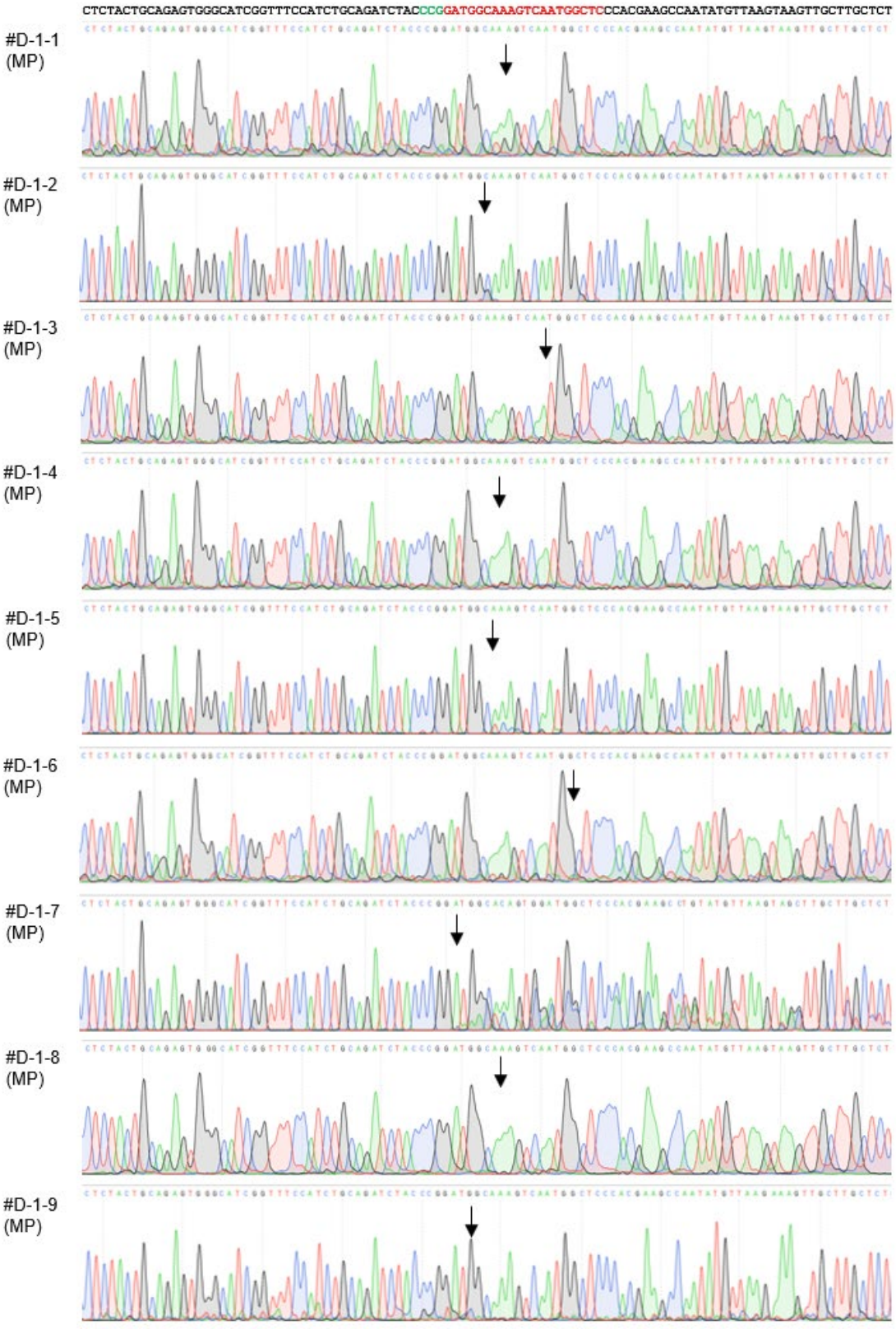
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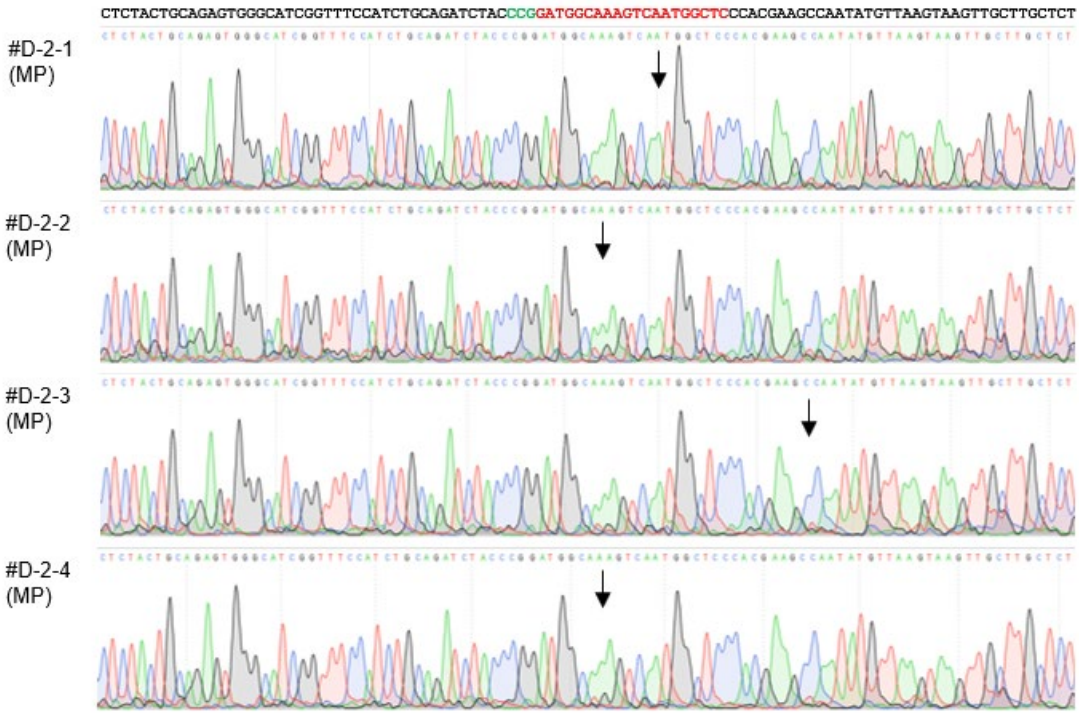
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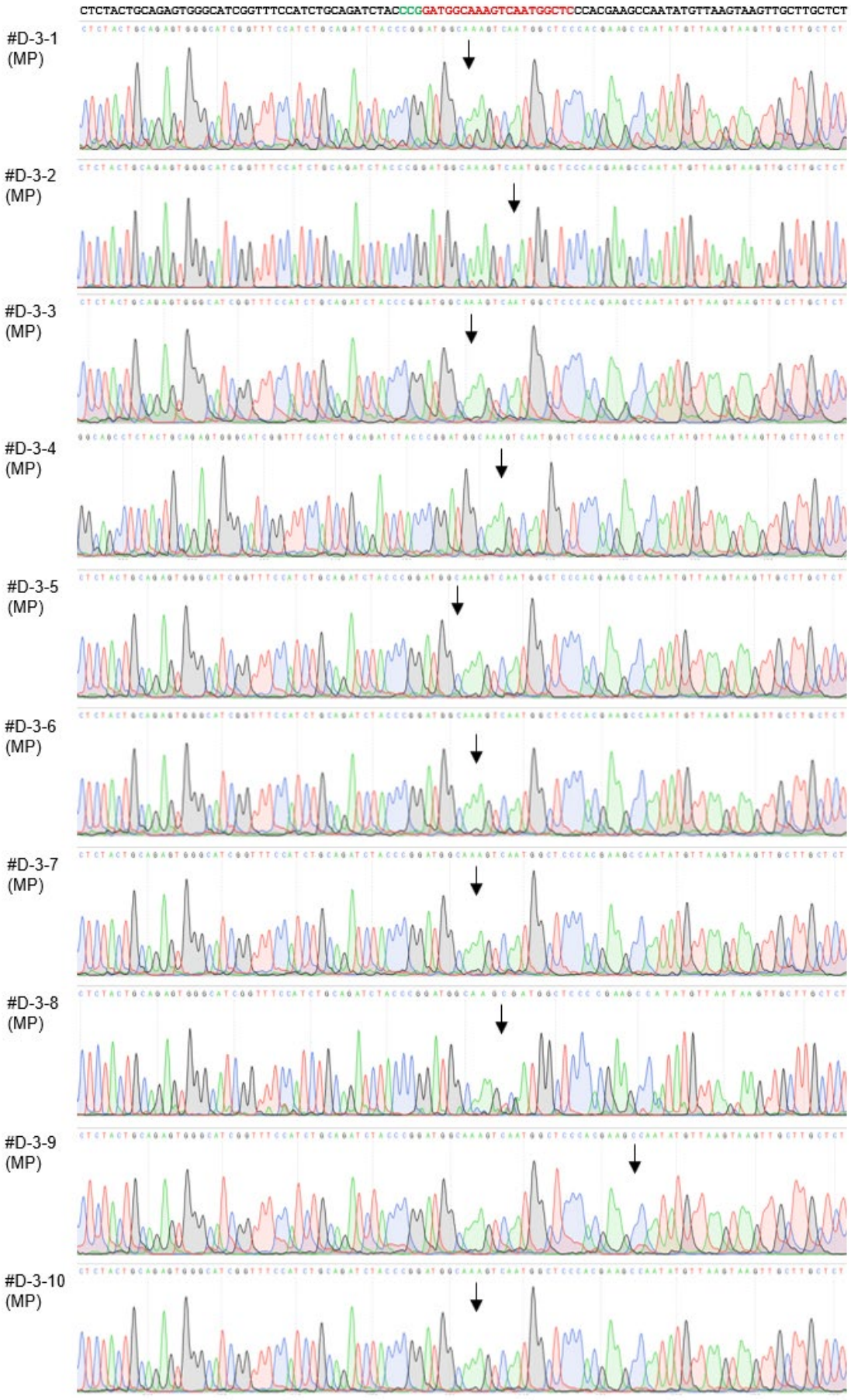
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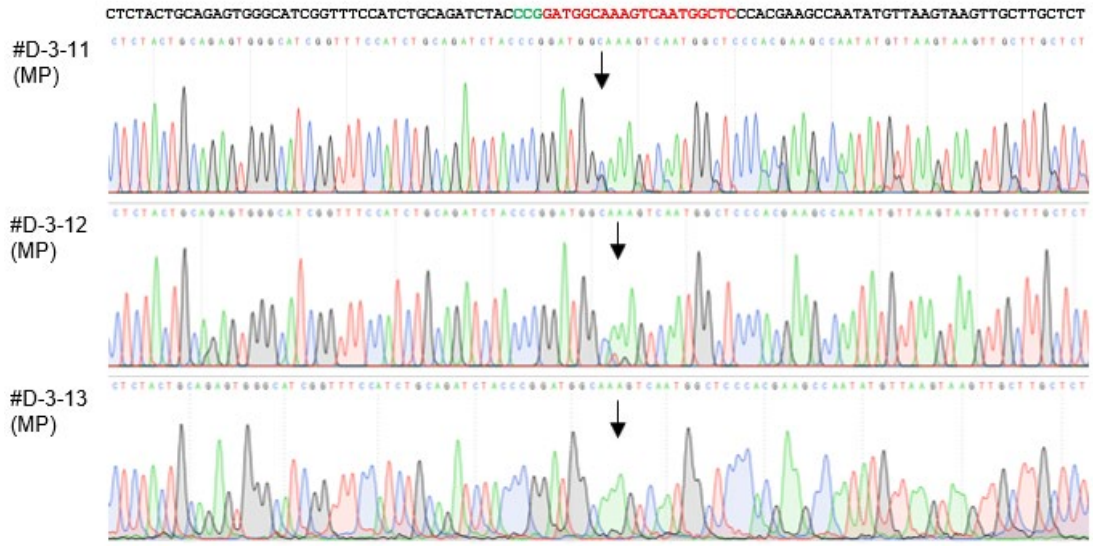

(D-3 group)

Fig. S2. Evaluation of genetic modifications in the injected embryos based on different Cas9:sgRNA concentration combinations. #A-1-10 indicates the group id (A), subgroup id (1), and the embryo id (10). MP, multiple peaks. Arrows indicate the position of modification.

Table S1. Comparison of different computational programs

Screen indexes	sgRNAcas9	CasOT	Cas-OFFinder	SSFinder
Type	off-line	off-line	online	off-line
PAM type contains "NGG"	√	√	√	√
Containing the goat genome	√	√	√	√
GC% content	√	×	×	×
Maximum number of mismatches	5	-	10	-
Seed sequence detection	√	√	×	√
Export target site detail	√	√	√	×
Export off-target site detail	√	√	√	×

Table S2. List of sgRNA information designed by sgRNAcas9

sgRID	Protospacer_sequence+PAM (5'-3')	GC%	Mismatch						No. of POT	Risk evaluation
			0	1	2	3	4	5		
sg1	AGAAGCGCCTCGCACCCAAAGGG	60	1	0	0	0	0	0	0	Very low
sg2	CATCTGCAGATCTACCCGGATGG	55	1	0	0	0	0	0	0	Very low
sg3	CATCCGGGTAGATCTGCAGATGG	55	1	0	0	0	0	0	0	Very low
sg4	GGTAGCCTCTACTGCAGAGTGGG	55	1	0	0	0	0	1	1	Low
sg5	TTTCCATCTGCAGATCTACCCGG	45	1	0	0	0	0	1	1	Low
sg6	TGCAGTAGAGGCTACCGGTCCGG	60	1	0	0	0	1	0	1	Low
sg7	TACCCGGATGGCAAAGTCAATGG	50	1	0	0	0	2	0	2	Low
sg8	GAGCCATTGACTTTGCCATCCGG	50	1	0	0	0	2	0	2	Low
sg9_1	AGCCATTGACTTTGCCATCCGGG	50	1	0	0	0	3	0	3	Low
sg10_1	TCTGCTCCAAGCCGCTTCCTGGG	60	1	0	0	0	3	0	3	Low
sg11	CTCTACTGCAGAGTGGGCATCGG	55	1	0	0	1	3	0	4	Low
sg12	CGGTAGCCTCTACTGCAGAGTGG	60	1	0	0	2	2	0	4	Low
sg13	CGATGCCCACTCTGCAGTAGAGG	60	1	0	0	0	4	1	5	Low
sg14	CACTCTGCAGTAGAGGCTACCGG	55	1	0	0	1	5	0	6	Moderate
sg15	TCAGGTGGCTAAGGAAGAGGAGG	55	1	0	0	1	8	6	15	Moderate
sg16	TGGAGCAGAGCAGCTTCCAGTGG	60	1	0	1	2	13	4	20	Moderate
sg17	AGGGGAAGAAGAGGAAGACGCGG	55	1	2	2	14	9	0	27	High
sg18	CGCTGAGGATCAGGTGGCTAAGG	60	1	0	0	14	476	10	500	Low
sg19	GGATCAGGTGGCTAAGGAAGAGG	55	1	0	0	8	56	22	86	Low
sg20	GGTGGCTAAGGAAGAGGAGGAGG	60	1	0	6	48	196	158	408	High
sg21	GGCTAAGGAAGAGGAGGAGGAGG	60	1	3	32	153	330	141	659	High
sg22	AAGGAAGAGGAGGAGGAGGAAGG	55	6	29	185	263	347	82	911	High

Note: POT, potential off-target sites.

Table S3. List of sgRNA sequence and efficiency in goat fibroblasts

sgRNA	Targeting site (PAM sequence)	Location on Chr6	Strand	Target efficiency (%)
sg1	AGAAGCGCCTCGCACCCAAA*GGG*	95419312-95419334	-	15.4
sg2	CATCTGCAGATCTACCCGGA*TGG*	95419548-95419570	-	4.5
sg3	CATCCGGGTAGATCTGCAGA*TGG*	95419547-95419569	+	10.2
sg4	GGTAGCCTCTACTGCAGAGT*GGG*	95419515-95419537	-	27.3
sg5	TTTCCATCTGCAGATCTACC*CGG*	95419544-95419566	-	2.1
sg6	TGCAGTAGAGGCTACCGGTC*CGG*	95419508-95419530	+	12.8
sg7	TACCCGGATGGCAAAGTCAA*TGG*	95419560-95419582	-	18.3
sg8	GAGCCATTGACTTTGCCATC*CGG*	95419563-95419582	+	42.9
sg9	GGCTGCCACGGAGAGGAACC*CGG*	95419346-95419368	-	10.5
sg10	AGCCTCTACTGCAGAGT*GGG*	95419518-95419537	-	27.4

Coordinates of sgRNA target sites are based on the goat genome assembly CHR2.0.

Table S4. Cleavage rate and mutant efficiency of zygotes for each group

Group	Cas9 mRNA (ng/μL)	Cas9:sgRNA concentration	Cleavage rate at 24 h (%)	Targeting efficiency (%)
A_1	10	20:1	16/25 (64.0)	6/25 (24.0)
A_2	10	4:1	25/31 (80.7)	6/31 (19.4)
A_3	10	2:1	13/25 (52.0)	6/25 (24.0)
B_1	20	20:1	20/22 (90.9)	12/22 (54.5)
B_2	20	4:1	18/27 (66.7)	5/27 (18.5)
B_3	20	2:1	22/22 (100.0)	7/22 (31.8)
C_1	50	20:1	27/28 (96.4)	6/28 (21.4)
C_2	50	4:1	22/24 (91.7)	7/24 (29.2)
C_3	50	2:1	19/24 (79.2)	16/24 (66.7)
D_1	100	20:1	19/22 (86.4)	9/22 (40.9)
D_2	100	4:1	4/17 (23.5)	4/17 (23.5)
D_3	100	2:1	22/27 (81.5)	13/27 (48.1)
Control	0	0	15/24 (62.5)	0/24 (0.0)

Table S5. List of primers used in this study

Primer	Sequence (5'-3')	Purpose
sg1_F	ACCGAGAAGCGCCTCGCACCCAAA	Production of sgRNA plasmid
sg1_R	AAACTTTGGGTGCGAGGCGCTTCT	
sg2_F	ACCGCATCTGCAGATCTACCCGGA	
sg2_R	AAACTCCGGGTAGATCTGCAGATG	
sg3_F	ACCGCATCCGGGTAGATCTGCAGA	
sg3_R	AAACTCTGCAGATCTACCCGGATG	
sg4_F	ACCGGTAGCCTCTACTGCAGAGT	
sg4_R	AAACACTCTGCAGTAGAGGCTAC	
sg5_F	ACCGTTTCCATCTGCAGATCTACC	
sg5_R	AAACGGTAGATCTGCAGATGGAAA	
sg6_F	ACCGTGCAGTAGAGGCTACCGGTC	
sg6_R	AAACGACCGGTAGCCTCTACTGCA	
sg7_F	ACCGTACCCGGATGGCAAAGTCAA	
sg7_R	AAACTTGACTTTGCCATCCGGGTA	
sg8_F	ACCGAGCCATTGACTTTGCCATC	
sg8_R	AAACGATGGCAAAGTCAATGGCT	
sg9_F	ACCGGCTGCCACGGAGAGGAACC	
sg9_R	AAACGGTTCCTCTCCGTGGCAGC	
sg10_F	ACCGAGCCTCTACTGCAGAGT	
sg10_R	AAACACTCTGCAGTAGAGGCT	
primer 1_F	TTCCCCTCCCATTCGCCCTCTCCC	Efficiency test in goat fetal fibroblasts
primer 1_R	ACGCACCTCCAACCCAACCCTCCG	
primer 2_F	GAGTACACAAAGCGGCGGGTGAG	Efficiency test in goat zygotes
primer 2_R	AGGTTCTGGAGGAGAGCAAGCAA	
OT1_F	GGAGGCAGAGGTTGGGGTT	Off-target assay
OT1_R	CAGAAAGTTGGAGGGGGCA	
OT2_F	TGGTTCTTTTCTGCTTCTATTTCACT	
OT2_R	GATCCCTTTTAGCTCTTTTAGGTTTG	
Primer_F	TCTCGCGCGTTTCGGTGATGACGG	*In vitro* transcription of sgRNA
Primer_R	AAAAAAAGCACCGACTCGGTGCCACTTTTTC	