

Multiplex Gene Editing via CRISPR/Cas9 System in Sheep

Yiyuan Niu, Yi Ding, Xiaolong Wang and Yulin Chen*

College of Animal Science and Technology, Northwest A&F University, Yangling, China

*For correspondence: chenyulin@nwfau.edu.cn

[Abstract] Sheep is a major large animal model for studying development and disease in biomedical research. We utilized CRISPR/Cas9 system successfully to modify multiple genes in sheep. Here we provide a detailed protocol for one-cell-stage embryo manipulation by co-injecting Cas9 mRNA and RNA guides targeting three genes (*MSTN*, *ASIP*, and *BCO2*) to create genetic-modified sheep. Procedure described sgRNA design, construction of gRNA-Cas9 plasmid, efficient detection in fibroblast, embryos and sheep, and some manipulative technologies. Our findings suggested that the CRISPR/Cas9 method can be exploited as a powerful tool for livestock improvement by targeting multiple genes that are in charge of economically significant traits simultaneously.

Keywords: Cas9, Sheep, *MSTN*, *BCO2*, *ASIP*

[Background] Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have been used to modify many cell lines and organisms in the past. The recent CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) technology development provides an efficient tool for genome modifying of targeting loci. This system has an advantage of modifying multiple loci at the same time. Sheep with specific gene modifications may contribute to breeding. The results demonstrate the first detailed evidence of large animal modification in sheep.

Materials and Reagents

1. Pipette tips
2. Centrifuge tubes
3. 6-well culture plate
4. HiBindTM DNA Mini Column
5. Sheep (Healthy ewes [3-5 years old] were selected about 160)
6. PGL3-U6-gRNA vector (Addgene, catalog number: 51133)
7. pUC57-T7-gRNA vector (Addgene, catalog number: 51132)
8. *E. coli* DH5 α competent cells
9. Cas9 mRNA *in vitro* transcription vector (Addgene, catalog number: 44758)
10. T vector (Takara Bio, catalog number: D103A)
11. *Bsa*I enzyme (New England Biolabs, catalog number: R3535)
12. DNA Gel Extraction Kit (Corning, catalog number: D205-04)

13. T4 ligase (New England Biolabs)

14. Ampicillin

15. EndoFree Plasmid Maxi Kit (QIAGEN, catalog number: 12362)

16. Primers

a. M13R (-47) primer

5'-CGCCAGGGTTTTCCCAGTCACGAC-3'

b. Primers for amplifying the selected potential off-target loci, designed by Primer5 software.

Gene	Name of primer	Sequence
MSTN	1-sMSTNsg1OT-1F	GAGTCCTTCCAGTGAAGCTCATC
	1-sMSTNsg1OT-1R	TCTTAGCCAGCACACCATCCTA
	2-sMSTNsg2OT-1F	GATGATGGCAGATGAGATGACT
	2-sMSTNsg2OT-1R	TTCAGTATGAGTGGAACAGGTT
	3-sMSTNsg2OT-1F	CCGAAGACTTGTTCAGGAAGAT
	3-sMSTNsg2OT-1R	CGAATAGGAGCAGATCATTGACA
	4-sMSTNsg2OT-1F	CTCAAGGCAAGAATACTGAAGTG
	4-sMSTNsg2OT-1R	CTTGGAGGAGGTCGCTATTAAC
BCO2	5-sBCO2sg2OT-1F	GTGCTCTGGCTTCAATAATGTA
	5-sBCO2sg2OT-1R	TTGGACGAGTGATGATGTAAGA
ASIP	6-sASIPsg1OT-1F	AGTGACAATGACAGATGAGTGG
	6-sASIPsg1OT-1R	CAGACATGACTGAGTGACTAACA
	7-sASIPsg1OT-1F	CTTCAGCAGACAAGGAACACTT
	7-sASIPsg1OT-1R	AATGGAGCATGGAGATCCTGAT
	8-sASIPsg1OT-1F	CTGCTGAGTCATAGCTGAATTAG
	8-sASIPsg1OT-1R	TAGATGGAATACTTGAGGAGGAG
	9-sASIPsg1OT-1F	AATATTGAGTCATGCCAGTTCC
	9-sASIPsg1OT-1R	GTAAGTCATACCTGTTGCCTTAG
	10-sASIPsg1OT-1F	ATTACTCAGGCTCACACCAACC
	10-sASIPsg1OT-1R	CCTCTGCTCAGTCTCTGTCAATC
	11-sASIPsg1OT-1F	ATTACTCAGGCTCACACCAACC
	11-sASIPsg1OT-1R	CCTCTGCTCAGTCTCTGTCAATC
	12-sASIPsg2OT-1F	AATGAGTCCATGAGGTCACAGA
	12-sASIPsg2OT-1R	GCTACTGAACTGAACTGAACTGA
	13-sASIPsg2OT-1F	GAGAGTCAGTCTATGGTTCAACT
	13-sASIPsg2OT-1R	CTTCCTAGTAAGCCTCCACAAG
	14-sASIPsg2OT-1F	ACCAATAGCAGGTCTGACCACAT
	14-sASIPsg2OT-1R	GCTTACTCACAGCCAGACTTGC
	15-sASIPsg2OT-1F	AGGCAAGTCTTCATACCAAGTG
	15-sASIPsg2OT-1R	TGAGGAGGCAAGTGATACAATG
	16-sASIPsg2OT-1F	GTAAGTCAAAGAGCTGGCAGAT
	16-sASIPsg2OT-1R	CTGTAAGCGAGATTAGAAGTTGG
	17-sASIPsg2OT-1F	GACAGGTGCATCTTTGAGACTGC
	17-sASIPsg2OT-1R	CAGATGGTATCATGCTCCACTGC

17. Ethanol

18. Ice acetic acid (Guangzhou Chemical Reagent Factory, catalog number: CB39-GR-0.5L)

19. Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, Gibco™)

20. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™)

21. Penicillin-streptomycin (Thermo Fisher Scientific, Gibco™)

22. Lipofectamine3000 reagent (Thermo Fisher Scientific, Invitrogen™)
23. Opti-MEM medium (Thermo Fisher Scientific, Gibco™)
24. Blastidene S hydrochloride
25. Universal Genomic DNA Kit (CWBIO, catalog number: CW2298M)
26. KOD-Plus-Neo
27. PCR cleanup kit (AppliChem, catalog number: A7089,1000)
28. T7EI
29. Agarose
30. TCM199 medium (Thermo Fisher Scientific, Gibco™)
31. Quinn's Advantage Cleavage Medium and Blastocyst Medium (Sage Biopharma)
32. Phosphate-buffered saline (PBS) (Thermo Fisher Scientific)
33. REPLI-g Single Cell Kit (QIAGEN, catalog number: 150343)
34. D2000 DNA marker
35. Tris
36. Ethylenediaminetetraacetate acid (EDTA)
37. Agar powder
38. Tryptone
39. Yeast extract
40. Sodium chloride (NaCl)
41. 50x TAE (see Recipes)
42. LB medium (see Recipes)

Equipment

1. Pipettes
2. 37 °C water bath
3. Thermal cycler (Bio-Rad thermocycler)
4. DNA electrophoresis apparatus
5. Microcentrifuge
6. Eppendorf FemtoJect system
7. Olympus micromanipulation system ON3 (Olympus, model: ON3 SERIES)
8. Microinjection device
9. Biopsy forceps (Olympus, model: FB-11K-1)

Software

1. Primer5 software

Procedure

A. sgRNA design

1. Select specific target sites in sheep genome and then design sgRNA-oligo sequence.
2. After design of sgRNA-oligo sequence, appropriate adaptor sequences should be added for cloning. The upstream primer of both pUC57-T7-gRNA vector and PGL3-U6-gRNA should include 5'-TAGG-3' adaptor sequence, while the downstream primer for both should contain an adaptor with the sequence of 5'-AAAC-3'.

B. Construct sgRNA-Cas9 plasmid

1. Digest the PGL3-U6-gRNA vector and pUC57-T7-gRNA vector by *BsaI*.

pUC57-T7-sgRNA	3 µg
CutSmart buffer	5 µl
<i>BsaI</i> (NEB)	1.5 µl
Add H ₂ O to	50 µl

2. Incubate at 37 °C for 3-4 h.
3. Purify the digested vector using DNA Gel Extraction Kit.
4. Double-strand sequences and prepare double-strand oligo DNA reaction system:

MSTN: sgRNA1: 5' CCAACTGTGGATATATCTGAGAC 3'

5' GTCTCAGATATATCCACAGTTGG 3'

sgRNA2: 5' GGATTTTGAAGCTTTTGGATGG 3'

5' CCATCCAAAAGCTTCAAATCC 3'

ASIP: sgRNA1: 5' CCAGAGATGAAAGGAACCTGAAG 3'

5' CTTCAAGTTCTTTTCATCTCTGG 3'

sgRNA2: 5' CAATTCTTCCATGAACCTGTTGG 3'

5' CCAACAGGTTTCATGGAAGAATTG 3'

BCO2: sgRNA1: 5' CCATGCATTGCACCGCTTCTAAC 3'

5' GTTAGAAGCGGTGCAATGCATGG 3'

sgRNA2: 5' GGTCGTCTCAGCTCGAGTCCAGG 3'

5' CCTGGACTCGAGCTGAGACGACC 3'

Top strand (100 µM)	4.5 µl
Bottom strand (100 µM)	4.5 µl
NEB buffer 2	1 µl

5. Perform PCR using the following cycling conditions:

95 °C	5 min	
95-85 °C at -2 °C/cyc	5 sec	4 cycles
85-25 °C at 0.1 °C/cyc	5 sec	400 cycles
hold at 4 °C	forever	

6. Ligate the diluted double-strand oligo DNA with the PGL3-U6-gRNA vector digested by *Bsa*I:

<i>Bsa</i> I digested vector (25 ng/μl)	1 μl
Oligo-duplex	2 μl
10x T4 DNA ligase buffer	0.5 μl
T4 ligase (NEB)	1 μl
Add ddH ₂ O to	50 μl

7. Incubate at 16 °C overnight.
8. Transform 1 μl of ligation product into 50 μl *E. coli* DH5α competent cells.
9. Pick up one colony and inoculate in a 15 ml tube containing 5 ml LB medium with 50 μg/ml ampicillin (4 colonies for each transformation). Incubate overnight in a 37 °C shaker.
10. Extract plasmids from transformed DH5α cells using Endo-Free Plasmid Mini Kit following the manufacturer's instructions (see [Supplement 1](#)).
11. Confirm the positive clones containing PGL3-U6-gRNA and pUC57-T7-gRNA by Sanger sequencing using M13R (-47) primer.

C. Cas9/sgRNA efficacy test in sheep fibroblast

1. Sheep fibroblasts were obtained from a 40-day-old fetus and then transported to the laboratory.
2. The fibroblasts are cultured for five passages in DMEM medium (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco) until 80-90% confluence.
3. Cell transfection.
 - a. The transfection procedure is performed using Lipofectamine3000 reagent (Invitrogen) according to the specification (Table 1).

Table 1. The specification about transfection using Lipofectamine3000 Reagent (Invitrogen)

Steps	Component	6-well
Seed cells to be 70-90% confluent for transfection	Adherent cells	0.25-1 x 10 ⁶
Dilute Lipofectamine3000 reagent in Opti-MEM medium-mix well	Opti-MEM medium	125 µl
	Lipofectamine3000 reagent	5 µl
Prepare master mix of DNA by diluting DNA in Opti-MEM medium, then add P3000 reagent	Opti-MEM medium	125 µl
	DNA (0.5-5 µg/µl)	5 µg
	P3000 reagent (2 µl/µg DNA)	5 µl
Add diluted DNA to each tube of diluted Lipofectamine3000 reagent (1:1 ratio)	Diluted DNA (with P3000 reagent)	125 µl
	Dilute Lipofectamine3000 reagent	125 µl
Incubate	Incubate for 15 min at room temperature	
Add DNA-lipid complex to cells	DNA-lipid complex	250 µl
Visualize/analyze transfected cells	Incubate cells for 2-4 days at 37 °C. Then, analyze transfected cells.	

b. Fibroblasts are separately transfected as followed types:

- (1) MSTN-sg1 (0.8 µg), MSTN-sg2 (0.8 µg), and MSTN-sg1 (0.4 µg) + MSTN-sg2 (0.4 µg)
 - (2) ASIP-sg1 (0.8 µg), ASIP-sg2 (0.8 µg), and ASIP-sg1 (0.4 µg) + ASIP-sg2 (0.4 µg)
 - (3) BCO2-sg1 (0.8 µg), BCO2-sg2 (0.8 µg), and BCO2-sg1 (0.4 µg) + BCO2-sg2 (0.4 µg)
- Along with 0.8 µg of Cas9 plasmid by Lipofectamine3000 in a 6-well culture plate.

4. Dilute by adding 10 mg/ml of blasticidin S hydrochloride into the medium (1:1,000 dilution) 24 h after transfection.
5. Then incubate for 24 h at 37 °C.
6. Genomic DNA is extracted from fibroblasts at 72 h after transfection using a Universal Genomic DNA Kit.

7. Amplify targeted fragments using KOD polymerase, then purify with a PCR cleanup kit.
a. Primers used for amplifying Cas9/sgRNA targeted *MSTN*, *ASIP* and *BCO2* fragment.

Gene	Name of primer	Sequence	Amplicon (bp)
<i>MSTN</i>	MSTN E2 forward	GACATGGAGGCGTTCGTTTCATT	422
	MSTN E2 reverse	CTGGGAAGGTTACAGCAAGATCA	
	MSTN E3 forward	TAGAAGTCAAGGTAACAGACAC	509
	MSTN E3 reverse	GTTTCATATACTGTAGCTTGTGC	
<i>ASIP</i>	sASIP CKE1 1F	GTTCTCCTTCCATGTCCTAAGC	541
	sASIP CKE1 1R	ACTAGATTGGTGAGAGGCAGAG	
<i>BCO2</i>	sBCO2 CK 1F	ATTGCGGTTTACAAGTGGAGAA	500
	sBCO2 CK 1R	AGGGATTGTGTAGCAATGTTAGC	

- b. Prepare the reaction mixtures as follows:

10x PCR buffer for KOD-Plus-Neo	5 μ l
dNTPs (2 mM)	5 μ l
MgSO ₄ (25 mM)	3 μ l
Forward primer (10 μ M)	1 μ l
Reverse primer (10 μ M)	1 μ l
KOD-Plus-Neo (1.0 U/ μ l)	1 μ l
DNA sample (Genomic DNA extracted from transgenic sheep fibroblast or wild type)	100 ng
Add H ₂ O to 50 μ l	

- c. Perform PCR using the following conditions [annealing temperature (T) and extension time (L)].

94 °C	2 min	1 cycle
98 °C	10 sec	 34 cycle
Annealing temperature	30 sec	
68 °C	Extension time	
68 °C	5 min	1 cycle
12 °C	Forever	1 cycle

8. The purified PCR products are denatured and re-annealed in NEBuffer 2 (NEB) using a Bio-Rad thermocycler.
9. The PCR products are digested with T7EI for 30 min at 37 °C and separated by 2.5% agarose gel electrophoresis.
10. PCR products of mutations detected by T7EI cleavage assay are sub-cloned into T vector.
11. For each sample, the colonies are randomly picked and sequenced using an M13F (-47) to confirm the positive colony.

D. Off-target detection in sheep fibroblasts

1. Predict the potential off-target sites using SeqMap.
2. Extract sheep fibroblasts DNA for PCR amplification using specific primers (see Materials and Reagents).

- a. Mix the reaction components according to the follows:

10x PCR buffer for KOD-Plus-Neo	5 µl
dNTPs (2 mM)	5 µl
MgSO ₄ (25 mM)	3 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
DNA sample (Genomic DNA extracted from transgenic sheep fibroblast or wild type)	100 ng
KOD-Plus-Neo (1.0 U/µl)	1 µl
Add H ₂ O to 50 µl	33 µl

- b. Perform PCR using the following conditions [annealing temperature (T) and extension time (L)].

94 °C	2 min	1 cycle
98 °C	10 sec	34 cycle
T	30 sec	
68 °C	L	
68 °C	5 min	1 cycle
12 °C	Forever	1 cycle

3. The PCR products are then subjected to a T7EI cleavage assay as described above and results below (Figure 1).

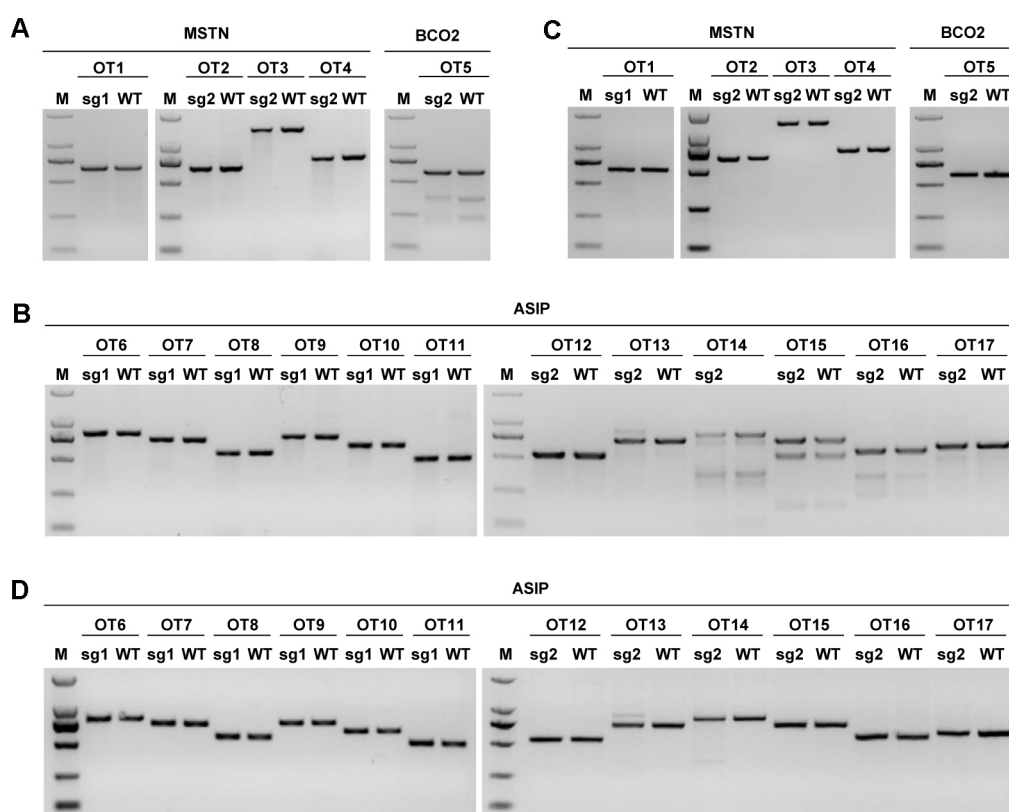


Figure 1. Detection of the sgRNA:Cas9-mediated off-target cleavage of *MSTN*, *ASIP*, and *BCO2* by using the T7EI cleavage assay. The PCR products of the potential off-target sites in *MSTN*, *ASIP*, and *BCO2* sgRNA:Cas9 loci from sheep fibroblasts (A and B). 17 predicted off-target sites that were most homologous to *MSTN*, *ASIP*, and *BCO2* sgRNAs were named OT1 to OT17. C and D. Detection of the sgRNA:Cas9-mediated off-target cleavage of *MSTN*, *ASIP*, and *BCO2* by using the T7EI cleavage assay. More messages see [Wang et al. \(2016\)](#).

E. Zygote collection

Note: Healthy ewes (3-5 years old) were selected to be desired donors for zygote collection which include regular estrus cycles.

1. Zygote collection and treatment of donors are conducted as earlier described in goats (Wang et al., 2015).
2. Collect one cell stage zygotes (around 10-16 h after the last insemination) using a surgical way and immediately transfer the collected zygotes into a TCM199 medium.

F. Efficient detection of sheep zygotes

1. Microinject the sgRNA & Cas9 mRNA into sheep zygotes using an Olympus micromanipulation system ON3 in a manipulation medium TCM199 on the heated platform.

*Note: Mix Cas9 mRNA (20 ng/μl) and sgRNAs (5 ng/μl for each sgRNA) targeting *MSTN*, *ASIP* and *BCO2*.*

2. Inject the sgRNA & Cas9 mRNA into the cytoplasm of fertilized oocytes using an Eppendorf FemtoJect system with the following set-up.

The injection pressure	45 kpa
Injection time	0.1 sec
Compensatory pressure	7 kpa

3. The embryos are cultured for 168 h at 38.5 °C in Quinn's Advantage Cleavage Medium and Blastocyst Medium (Sage Biopharma).
4. Blastocyst embryos are used for efficient detection preserved in PBS.
5. Genomes of embryos are amplified using REPLI-g Single Cell Kit.
6. Targeted fragments are amplified as described above including primers, reactions and procedures.
7. Purification of PCR products, T7EI cleavage assay, and Sanger sequencing are performed according to Wang *et al.* (2015).

G. Production of gene-modified sheep

Note: Gene-modified sheep and control animals were fed the same diet, and managed at the same conditions throughout the experiment.

1. Select the surrogate ewes with their natural estrus cycles as gist.
2. Zygote collection and treatment of donors are conducted as described above.
3. The zygotes are cultured for 24 h at 37 °C using Quinn's Advantage Cleavage Medium (Sage Biopharma).
4. Then culture the zygotes in Quinn's Advantage Blastocyst Medium (Sage Biopharma) at 37 °C, 5% CO₂ and saturated humidity.
5. About 3-4 divisive embryos are transplanted into the ampullary-isthmic junction of the oviduct of the recipient ewes.
6. Pregnancy normally takes about 150 days in Tan sheep, and is determined based on the observation of estrus behaviors in every ovulation cycle.

H. Efficiency detection in Genetic-modified sheep

1. Jugular venous blood samples and tissues are collected from randomly selected genetic-modified sheep (n = 10) and randomly selected control animals (n = 10) at 240 days of age for blood and tissues chemistry analyses.
2. Genomic DNA is extracted from blood and tissues using corresponding assay from the University Genomic DNA Kit.
3. Targeted fragments are amplified as described above in off-target detection in sheep fibroblast (including primers, reactions and procedures) (Procedure D).
4. T7EI cleavage assay and sequencing are performed as previously described (step C9).
5. The results may confirm the mutation in gene-modified sheep. (Figure 2)

sgRNAs of targeted genes are in red text, while the PAM sequences are marked in green. The mutations are marked in blue, dashlines indicates deletions, and lowercase indicates insertions or replacements. Insertions (+), deletions (-), mutations (m) is shown to the right of each allele. The genotypes are shown to the right with the rates of total clones for TA-sequencing. For more information see [Wang et al. \(2016\)](#).

I. Germline transmission detection

1. Germ cells from the testis of the three founders (#28, #33, and #37) were obtained by using biopsy forceps.
2. The genomes of germ cells are amplified using REPLI-g Single Cell Kit.
3. PCR products with mutations are detected using the T7EI cleavage assay, followed by Sanger sequencing.

Recipes

1. 50x TAE

Dissolve 242 g of Tris, 37.2 g of EDTA in 700 ml dH₂O

Note: Heat solution to 60 °C to assist in dissolution.

Add 57.1 ml of ice acetic acid

Add dH₂O to 1,000 ml

Then 200 ml solution was diluted to 1x TAE working solution by adding 10 L dH₂O

2. LB medium

Dissolve 2 g agar powder, 2 g tryptone, 1 g yeast extract, 1 g NaCl in 200 ml dH₂O

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