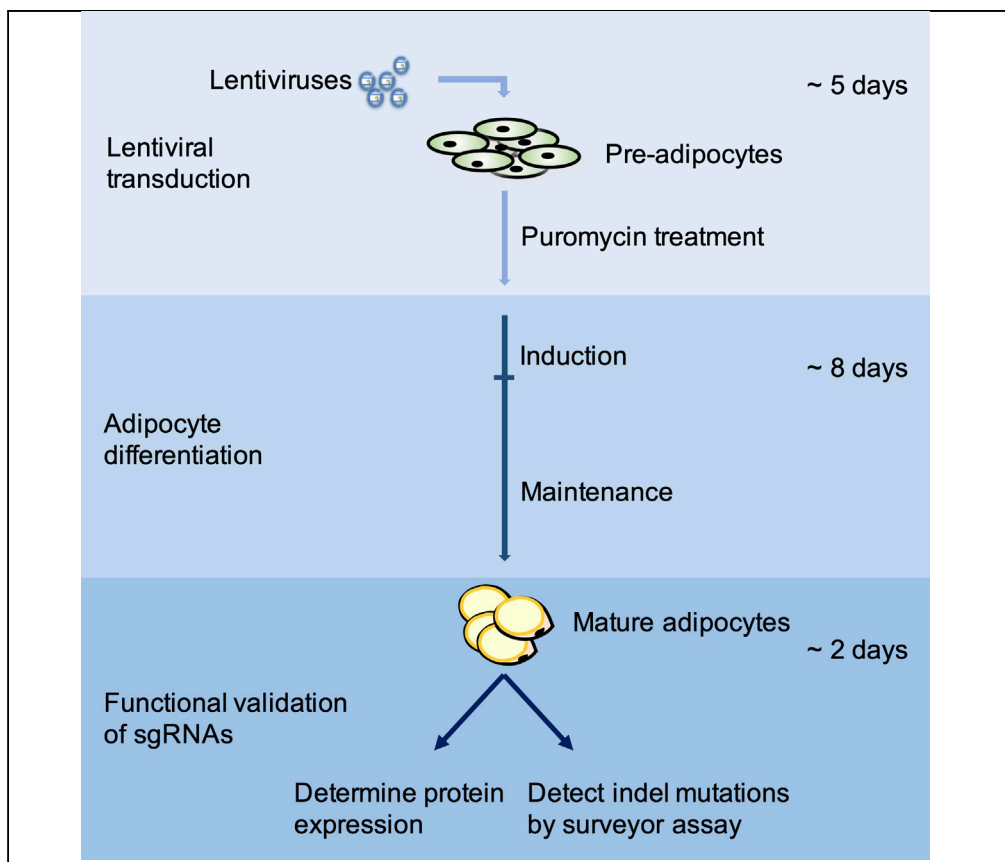


Protocol

Optimized protocol for gene editing in adipocytes using CRISPR-Cas9 technology



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HIGHLIGHTS

Gene editing protocol in adipocytes using CRISPR-Cas technology

Double gene editing in adipocytes using optimized protocol

High-efficiency sgRNA will produce cleavage bands on agarose gel

High-efficiency sgRNA will significantly decrease target protein expression

We present a detailed protocol for gene editing in adipocytes using the CRISPR-Cas technology. This protocol describes sgRNA design, preparation of lentiCRISPR-sgRNA vectors, functional validation of sgRNAs, preparation of lentiviruses, and lentiviruses transduction in adipocytes. Moreover, an optimized method of gene editing using the lentiCRISPRv2 vector expressing two sgRNAs targeting two different genes has also been described.

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Protocol

Optimized protocol for gene editing in adipocytes using CRISPR-Cas9 technology

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SUMMARY

We present a detailed protocol for gene editing in adipocytes using the CRISPR-Cas technology. This protocol describes sgRNA design, preparation of lenti-CRISPR-sgRNA vectors, functional validation of sgRNAs, preparation of lentiviruses, and lentiviruses transduction in adipocytes. Moreover, an optimized method of gene editing using the lentiCRISPRv2 vector expressing two sgRNAs targeting two different genes has also been described.

For complete details on the use and execution of this protocol, please refer to Qiu et al. (2020).

BEFORE YOU BEGIN

The RNA-guided CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9) technology has been widely used in gene editing. CRISPR/Cas9 system consists of the nuclease Cas9 and the guide RNA, which guides Cas9 to cleave DNA and produce DNA double-stranded breaks (DSBs) at a specific genomic locus. The generated DSBs can be repaired via the NHEJ (non-homologous end joining) or HDR (homology directed recombination) pathway, both of which can be adopted to mediate a desired editing outcome.

This protocol describes the use of lentiCRISPR mediated gene knockout in pre-adipocytes, which can be then differentiated into mature adipocytes for functional studies.

Note: This protocol can also be applied to adipocyte cell lines.

sgRNA design and synthesis

⌚ Timing: 30 min

1. sgRNA design.

Input target genomic DNA sequence. sgRNAs can be designed using the online CRISPR Design Tool. For example, <http://tools.genome-engineering.org> (Ran et al., 2013), Broad Institute GPP (Sanson et al., 2018, Doench et al., 2016), Synthego (<https://design.synthego.com/#/>), etc.

Alternatives: sgRNAs can also be designed manually.

- Obtain the gene coding sequence (CDS) from the NCBI gene database (<https://www.ncbi.nlm.nih.gov>) by searching the gene name in the designated assembly.



- b. Copy the CDS and import the CDS into the UCSC genome browser (<http://genome.ucsc.edu>) to obtain the corresponding genomic sequence.
- c. Select the most common exon among all isoforms to obtain the DNA sequence.

Note: In this protocol, we do not consider the functional difference of different isoforms, thus recommend to select the most common exon among all isoforms for sgRNA design; in case researchers are interested in functional differences between different isoforms, targeting isoform specific exon would be a better choice.

- d. Single-guide RNA (sgRNA) sequences can be manually selected by identifying the 20-bp sequence directly upstream of any 5'-NGG (protospacer adjacent motif, PAM) or downstream of any 5'-CCN.

△ **CRITICAL:** It is recommended to select sgRNAs with high specificity, which can be assessed by searching in the UCSC genome browser (<http://genome.ucsc.edu>). Typically, design three or more sgRNAs for the first time according to the target gene coding region. Then select sgRNA with the highest editing activity for gene editing in pre-adipocytes.

2. sgRNA synthesis.

- a. Design the sgRNA oligonucleotide primers as follows:
 sgRNA Forward: 5'-CACCGNNNNNNNNNNNNNNNNNNNNNN-3',
 sgRNA Reverse: 5'-AAACNNNNNNNNNNNNNNNNNNNNNC-3'.

Note: The highlighted region represents the 20-nt sgRNA sequence; the forward primer is 20-nt upstream of 5'-NGG, and the reverse primer complements the 20 highlighted nucleotides of the forward primer.

- b. Order the designed sgRNA oligonucleotides.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
lentiCRISPRv2	Addgene	Cat# 52961
pMDLg/pRRE	Addgene	Cat# 12251
pRSV-Rev	Addgene	Cat# 12253
pMD2.G	Addgene	Cat# 12259
pUC57-MU6-gRNA	Genewiz	n/a
pCW-Cas9	Addgene	Cat# 50661
Chemicals, peptides, and recombinant proteins		
T7 Endonuclease I	NEB	Cat# M0302L
BsmBI	NEB	Cat# R0580
NEbuffer 3.1	NEB	n/a
NEbuffer 2	NEB	n/a
BbsI-HF	NEB	Cat# R3539S
CutSmart	NEB	n/a
EcoRI	NEB	Cat# R3101S
KOD buffer	TOYOBO	Cat# KOD-401
Ligation high	TOYOBO	Cat# LGK-101
2× Taq Master Mix (Dye)	TransGen Biotech	Cat# CW0682
Polyethylenimine (PEI)	Polysciences	Cat# 23966-1
Polybrene	Merck	Cat# TR-1003-50UL
Gelatin	Santa Cruz	Cat# sc-218567
Insulin	Santa Cruz	Cat# sc-29062

(Continued on next page)

<i>Continued</i>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
T3	Sigma	Cat# T-2877
Indomethacin	Sigma	Cat# I-7378
Dexamethasone	Sigma	Cat# D-4902
IBMX	Sigma	Cat# I-5879
Rosiglitazone	Santa Cruz	Cat# sc-20279
DMEM	Gibco	Cat# 8117254
FBS	Gibco	Cat# 16000044
Puromycin dihydrochloride	BioVision	Cat# 295160
RIPA buffer	Millipore	Cat# 20188
SDS-PAGE gel	Epizyme	Cat# PG112
Ampicillin	Beyotime Biotechnology	Cat# ST007
DTT	Sangon Biotech	Cat# A100281-0005
Tris-HCl	Sinopharm Chemical Reagent	Cat# 73509461
SDS	Amresco	Cat# 0227-1KG
Glycerol	Sinopharm Chemical Reagent	Cat# 10010618
Bromophenol blue	Sangon Biotech	Cat# A500922-0025
Agarose	Biowest	Cat# BY-R0100
Yeast extract	OXOID	Cat# LP0021
Tryptone	OXOID	Cat# LP0042
NaCl	Sangon Biotech	Cat# 7647-14-5
Agar powder	Solarbio	Cat# A8190
Calcium chloride dihydrate	Sigma	Cat# C7902
<i>Critical commercial assays</i>		
TIANprep Mini Plasmid Kit	TIANGEN	Cat# DP103
TIANamp Genomic DNA Kit	TIANGEN	Cat# DP304-03
Gel Extraction Kit	CWBIO	Cat# CW2302M
qPCR Lentivirus Titration Kit	Abm	Cat# LV900
<i>Experimental models: cell lines</i>		
HEK293T	Cell Bank, Type Culture Collection Committee, Chinese Academy of Sciences	n/a
NIH3T3	Cell Bank, Type Culture Collection Committee, Chinese Academy of Sciences	n/a
Brown adipocytes	n/a	n/a
White adipocytes	n/a	n/a
<i>Other</i>		
0.22- μ m vacuum filter	Millipore	Cat# SLGP033RB
0.45- μ m vacuum filter	Millipore	Cat# SLHV033RB
Beckman JA25.50 rotor	Beckman	n/a
<i>Bacterial and virus strains</i>		
Trans5 α Chemically Competent Cell	TransGen	Cat# 431675
NEB Stable competent cell	NEB	Cat# C3040I
One Shot Stb13 chemically competent cell	Invitrogen	Cat# C737303
<i>Oligonucleotides</i>		
sgRNA forward: 5'-CACCGNNN NNNNNNNNNNNNNNNNN-3'	n/a	n/a
sgRNA reverse: 5'-AAACNNNN NNNNNNNNNNNNNNNNC-3'	n/a	n/a

MATERIALS AND EQUIPMENT

- Induction medium for differentiation of brown pre-adipocytes

Reagent	Stock concentration	Final concentration	Volume to add
DMEM	n/a	n/a	45 mL
10% FBS	n/a	n/a	5 mL
Insulin	5 mg/mL	1 µg/mL	10 µL
T3	10 µM	1 nM	5 µL
Indomethacin	0.125 M	0.125 mM	50 µL
Dexamethasone	1 mM	1 µM	50 µL
IBMX	0.5 M	0.5 mM	50 µL
Rosiglitazone	1 mM	1 µM	50 µL
Total	n/a	n/a	50 mL

Note: Always prepare freshly before use.

- Maintenance medium for differentiation of brown pre-adipocytes

Reagent	Stock concentration	Final concentration	Volume to add
DMEM	n/a	n/a	45 mL
10% FBS	n/a	n/a	5 mL
Insulin	5 mg/mL	1 µg/mL	10 µL
T3	10 µM	1 nM	5 µL
Total	n/a	n/a	50 mL

Note: Always prepare freshly before use.

- Preparation of PEI stock

Add linear PEI (50 mg) to 50 mL sterile water in a 50-mL conical tube. Place the tube at 55°C in a water bath and vortex every 10 min until PEI is completely dissolved. The PEI solution is cooled to room temperature (~25°C), filter sterilized using a 0.22-µm vacuum filter and stored at 4°C for up to 5 months or longer.

- Preparation of LB buffer

Reagent	Amount
Yeast extract	5 g
Tryptone	10 g
NaCl	10 g
ddH ₂ O	to 1 L
Total	1 L

LB buffer is autoclaved at 121°C for 15 min. Add antibiotics after the LB buffer is cooled adequately. The LB buffer can be stored at room temperature (~25°C) for up to 1 month or longer.

Note: The use of antibiotics depends on the vectors that used. When culture LentiCRISPRv2-based vector, add ampicillin to the LB buffer before use. The final concentration of ampicillin is 100 µg/mL.

- Preparation of LB agar plates

To prepare LB agar plates, add 15 g agar powder to 1 L LB buffer and autoclave for 15 min at 121°C. After adequate cooling, add antibiotics to the LB buffer, and pour into the petri dishes. Allow to solidify. The LB agar plates can be stored at 4°C for up to 45 days or longer.

- Preparation of 3× SDS-PAGE loading buffer

Reagent	Amount
1 M Tris-HCl (pH 6.8)	7.5 mL
DTT	2.313 g
SDS	3 g
Glycerol	15 mL
Bromophenol blue	0.015 g
ddH ₂ O	to 50 mL
Total	50 mL

The 3× SDS-PAGE loading buffer can be stored at room temperature (~25°C) for up to 1 month or longer.

STEP-BY-STEP METHOD DETAILS

Preparation of lentiCRISPR-sgRNA vector

⌚ Timing: 3 days

Alternatives: sgRNA can also be delivered by retrovirus or by electroporation. If you already have the established retroviral vector, directly jump to step 6 of the sgRNA screening part for instructions on how to screening sgRNAs with high editing activity. Skip the part on lentiviruses preparation and infection (steps 4–6), if you are preparing to deliver genes via electroporation.

1. Day 1. Part One-Preparation of sgRNA oligonucleotide inserts.
 - a. Dissolve the sgRNA oligonucleotides in ddH₂O and dilute to a final concentration of 10 μM. Set up the following reaction for each pair of sgRNA oligonucleotides:

Component	Amount (μL)
sgRNA-F (10 μM)	1
sgRNA-R (10 μM)	1
KOD buffer (10×)	10
ddH ₂ O	88
Total	100

Note: The KOD buffer is from the KOD-Plus-Neo kit (TOYOBO, # KOD-401).

- b. Anneal these sgRNA oligonucleotides at 95°C for 5 min, and cool the mixture to room temperature (~25°C).
2. Day 1. Part Two-Digestion of the lentiCRISPRv2 plasmid.

Note: This step can also be done before step 1.

- a. The lentiCRISPRv2 plasmid is obtained as a gift from Feng Zhang (Addgene plasmid #52961; RRID: Addgene_52961):
Digest the lentiCRISPRv2 plasmid (1 μg) with 0.5 μL BsmBI enzyme at 55°C for 1 h. Run samples on 1% (wt/vol) agarose gel to verify the appropriately sized fragment (12,994-nt DNA fragment). Successful digestion should yield 12,994-nt and 1,879-nt DNA fragment.

Component	Amount
lentiCRISPRv2	1 μg
BsmBI	0.5 μL
NEbuffer3.1 (10×)	2 μL
ddH ₂ O	to 20 μL
Total	20 μL

3. Day 1. Part Three—Cloning of sgRNA into the lentiCRISPRv2 vector.

- a. Set up the ligation reaction for each sgRNA as following:

Component	Amount (μL)
BsmBI-digested lentiCRISPRv2 from step 2	0.3
sgRNA from step 1	5.7
Ligation high	3
Total	9 μL

- b. Incubate the ligation reaction at room temperature (~25°C) for 2 h.
 c. Transform the ligation reaction into a Trans5α chemically competent cell. Incubate the mixture on ice for 30 min, followed by heat shock at 42°C for 30 s, and immediately re-incubate on ice for 2 min.

Note: It is recommended to transform the lentiviral plasmids in NEB Stable competent cell or One Shot Stbl3 chemically competent cell (Invitrogen) to reduce potential homologous recombination.

- d. Add 100 μL LB buffer to the ligation mixture from step 3c, then incubate the mixture at 37°C for 1 h in a shaker.
 e. Add the mixture from step 3d onto an LB plate with 100 μg/mL ampicillin. Incubate the LB plate at 37°C for 12–16 h.

△ **CRITICAL:** Prepare a negative control plate (ligation of BsmBI-digested lentiCRISPRv2 alone without annealed sgRNA oligo insert).

- f. Day 2: Assess the plate for colony growth. Typically, multiple colonies should grow on the lentiCRISPR-sgRNA (sgRNA inserted into lentiCRISPRv2 plasmid) plate, and the negative control plate should not have colony growth.
 g. Select two or three colonies from the lentiCRISPR-sgRNA plate to confirm the insertion of sgRNA into lentiCRISPRv2 vector. Grow each single colony in 3 mL LB buffer with 100 μg/mL ampicillin and shake the LB buffer at 37°C for 6 h.

Note: The time for incubation is flexible.

- h. Isolate plasmid DNA from the bacterial cultures using a TIANprep Mini Plasmid Kit, according to the manufacturer's instructions (<https://www.tiangen.com/asset/imsupload/up0062501001604555834.pdf>).
 i. Day 3. Verify the CRISPR vector, designated as lentiCRISPR-sgRNA1, by sequencing. Sequence each colony from the U6 promoter using the U6 primer. The 20-bp sgRNA sequence should be inserted between the U6 promoter and the remainder of the sgRNA scaffold. Further details and sequence of the lentiCRISPRv2 vector can be found at <http://n2t.net/addgene:52961>.

Screening sgRNAs with high editing activity

⌚ **Timing:** 11 days

Note: It is recommended to screen for sgRNAs with high editing activity in HEK293T cells (for gene editing in human cells) and NIH3T3 cells (for gene editing in mouse cells).

4. Preparation of lentiCRISPR-sgRNA virus

⌚ **Timing:** 5 days

- a. HEK293T cells culture. Culture cells in DMEM supplemented with 10% FBS at 37°C and 5% CO₂.

- b. Day 1. Prepare cells for transfection. Plate HEK293T cells onto 10-cm plates in DMEM supplemented with 10% FBS 16–24 h prior to transfection. Typically, plate $\sim 4 \times 10^6$ cells in a 10-cm plate ($\sim 5 \times 10^4$ cells/cm²).

Note: It is recommended to transfect the plasmid mix when cells are 80%–90% confluent.

- c. Day 2. On the day of transfection, replace the growth medium with 6–8 mL DMEM 4–6 h before transfection. Prepare polyethylenimine (PEI) transfection reagents for each 10-cm plate as following:

⚠ **CRITICAL:** It is important to perform this step gently and carefully because HEK293T cells can be easily detached. It is also recommended to pre-coat cell culture plates with poly-L-lysine or collagen to prevent cell detachment.

Alternatives: PEI-based transfection can be replaced with calcium phosphate or lipofection-based approaches.

- i. Mix the lentiviral plasmid with the packaging vectors (total ~ 25 μ g plasmid DNA), and subsequently dilute the mixture in 300 μ L DMEM by pipetting up and down for a few times.

Component	Amount (μ g)
pMDLg/pRRE	7.35
pMD2.G	3.95
pRSV-Rev	2.85
lentiCRISPR-sgRNA	11.25
Total	25.4

- ii. Mix the PEI stock gently before use. Thereafter, add 75 μ L PEI (the ratio of PEI volume (μ L) to the amount of total DNA (μ g) is 1:3) to 300 μ L DMEM and mix gently by pipetting up and down for a few times.
- iii. Add the PEI/DMEM solution (375 μ L) to the diluted DNA/DMEM mixture and mix immediately by pipetting up and down for a few times or vortexing.
- iv. Incubate the mixture at room temperature ($\sim 25^\circ\text{C}$) for 15 min, and add the mixture to each plate with gentle rocking for 2 or 3 times.
- v. After 4–6 h, replace DMEM with growth medium.
- vi. Days 4 and 5. Collect the virus-containing medium 48 h and 72 h after transfection. Thereafter, filter the medium with a 0.45 μ m vacuum filter, and store the virus-containing medium at 4°C for immediate use. For longer term storage at -80°C , the virus-containing medium can be concentrated by centrifugation with a Beckman JA25.50 rotor (capacity for eight tubes) at $48,384 \times g$ for 2 h at 4°C.

Note: Lentivirus can be dissolved in cold PBS or DMEM. Usually, for one 50 mL tube, it is recommended to dissolve the virus with 200 μ L cold PBS or DMEM. For more detailed information about the production and purification of lentiviruses, please refer to (Tiscornia et al., 2006, Dull et al., 1998).

Alternatives: Lentivirus can also be concentrated via polyethylene glycol (PEG)-based system (Larcombe et al., 2019). Troubleshooting 1 (Low titers of lentiviruses)

⌚ **Timing:** 2–3 h

5. Lentivirus titration (: Titer the concentrated lentivirus with a commercial qPCR Lentivirus Titration Kit, according to the manufacturer's instructions (https://www.abmgood.com/pub/media/productdocument/document//l/v/lv900_datasheet_v18.pdf). Typically, the lentivirus titer of a 10-cm plate is $\sim 1 \times 10^7$ pfu.

6. Lentiviruses transduction in NIH3T3 cells

⌚ **Timing: 6 days**

- a. Day 1. Plate NIH3T3 cells in a 12-well plate 1 day before transduction.
- b. Day 2. Infect cells with lentiviruses at a MOI of ~30 and allow lentivirus-infected cells to grow for another 2 days.
- c. Day 4. Treat cells with 3.5 µg/µL puromycin for 2 days to remove non-infected cells.
- d. Day 6. Collect cells for genomic DNA extraction.

Note: Polybrene can be added to increase transduction efficiency.

⚠ **CRITICAL:** Do not forget to set up control groups (cells infected with lentiviruses that carry no sgRNA or express only GFP). At least include one of them as a control, and it is recommended to include both as controls.

7. Surveyor assay to validate sgRNA function

⌚ **Timing: 1 day**

Note: The Cas9 and sgRNA in cells can cause genomic insertion or deletion (indel) mutations, which can be detected via either the surveyor assay or sequencing. The surveyor primers can be designed via the online Primer Design Tool. For example, Primer 3.0 Web (<http://primer3.ut.ee>).

- a. Extract genomic DNA from different group cells using the TIANamp Genomic DNA Kit, according to the manufacturers' instruction (<https://www.tiangen.com/asset/imsupload/up0484377001604554551.pdf>).
- b. Set up the PCR reaction as follows:

Component	Amount
Surveyor-F (10 µM)	1 µL
Surveyor-R (10 µM)	1 µL
2× Taq	50 µL
Genomic DNA	500 ng
ddH ₂ O	to 100 µL
Total	100 µL

Note: Design PCR primers for amplification (Surveyor-F/R) of the target region with the predicted SpCas9 cut site in the center. It is recommended to design a primer pair that will produce a ~600 bp band.

Then perform PCR using the following cycling conditions:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial denaturation	94°C	2 min	1
Denaturation	94°C	30 s	25–35 cycles
Annealing	58°C	30 s	
Extension	72°C	15 s	
Final extension	72°C	5 min	1
Hold	4°C	forever	

Note: The time for extension is mainly determined by the length of DNA product and the DNA polymerase used.

- c. Run the PCR products on a 1% (wt/vol) agarose gel. Purify the PCR product using a Gel Extraction Kit, according to the manufacturers' instruction (<https://www.cwbiotech.com/uploads/websitpdf/216c4037-3eae-4ac2-b86a-762277a7adc1.pdf>).
- d. Anneal the PCR products as follows:

Component	Amount
DNA product from step 7c	250 ng
NEbuffer 2 (10×)	2 μL
ddH ₂ O	to 20 μL
Total	20 μL

Note: Put the mixture at 95°C for 5 min.

Note: Take equal amounts of DNA product from different lentiviruses-treated cells (lentiviruses carrying sgRNA or no sgRNA) for analysis.

- e. Digest the annealed DNA fragments with 0.5 μL T7 Endonuclease I (T7EI) enzyme at 37°C for 1 h.
- f. Run samples on a 1% (wt/vol) agarose gel.
- g. Determine the cleavage efficiency by quantifying gel bands without overexposing them. A high-efficiency sgRNA will result in obvious cleavage bands, whereas the negative controls should have no cleavage bands. Troubleshooting 4 (No obvious cleavage bands after T7 endonuclease I digestion).

Optional steps

⌚ **Timing: 5 days**

8. Two sgRNAs can be cloned into one lentiCRISPRv2 vector. The lentiCRISPRv2 vector can be modified to target two different genes simultaneously.
 - a. Day 1. Prepare the following reaction mixture to digest 1 μg of pUC57-MU6-gRNA plasmid DNA:

Component	Amount
pUC57-MU6-gRNA	1 μg
BbsI-HF	0.5 μL
CutSmart (10×)	2 μL
ddH ₂ O	to 20 μL
Total	20 μL

Digest the pUC57-MU6-gRNA plasmid with 0.5 μL BbsI enzyme at 37°C for 2.5 h and run the product on a 1% (wt/vol) agarose gel to verify successful fragment.

Note: The pUC57-MU6-gRNA plasmid can also be digested with BbsI enzyme at 37°C for 8–12 h.

- b. Days 1–3. Clone another sgRNA into the pUC57-MU6-gRNA plasmid, which is called pUC57-sgRNA2. The protocol is identical to that in step 3.
- c. Day 3. Part One. Digest the pUC57-sgRNA2 vector with EcoRI enzyme at 37°C for 1 h. Run samples on a 1% (wt/vol) agarose gel to verify successful fragment (the 430-bp-long product), which is designated as Mouse U6-sgRNA2. Successful reactions should yield a 430-bp fragment and a 2,713-bp fragment.
- d. Day 3. Part Two. Digest the lentiCRISPR-sgRNA1 vector (from step 3) with EcoRI enzyme at 37°C for 1 h. Run samples on a 1% (wt/vol) agarose gel to verify successful fragment.

- e. Day 3. Part Three. Ligate the Mouse U6-sgRNA2 fragment from step 8c into the digested lentiCRISPR-sgRNA1 from step 8d. Set up the ligation reaction as follows:

Component	Amount (μL)
lentiCRISPR-sgRNA1 from step 8d	0.3
Mouse U6-sgRNA2 fragment from step 8c	5.7
Ligation high	3
Total	9 μL

Days 3–5. All other steps are identical to step 3.

Note: It can also be achieved by delivering two sgRNAs from two separate viruses when using higher MOI for infection to simultaneously knockout two genes in one cell.

Lentiviral transfection of adipocytes

⌚ Timing: 5 days + differentiation

9. Pre-adipocytes culture and transduction

⌚ Timing: 5 days

Note: This protocol does not consider the effect of a specific gene on adipocytes differentiation. To exclude possible effect of a gene during adipocyte differentiation, we recommend the use of inducible CRISPR/Cas9 system, for example, the pCW-Cas9 (Addgene, #50661), to start gene editing after adipocyte differentiation in mature adipocytes. Infect pre-adipocytes with the inducible CRISPR/Cas9 system to introduce the Cas9 and sgRNA cassettes to cells, and induce the Cas9 expression with doxycycline after differentiation to knockout certain genes of interest.

- a. Maintain brown pre-adipocytes and white pre-adipocytes in DMEM supplemented with 20% FBS at 37°C and 5% CO₂. Passage the cells at 1:6 or more every 2 days.

Note: Plates can be pre-coated with 0.1% gelatin to prevent cell detachment. The volume of 0.1% gelatin added is as follows: 12-well plate, 1 mL/well, 6-well plate, 1.5 mL/well, and 10-cm culture dish, 6 mL/dish.

- b. Plate pre-adipocytes in 6-well plates for lentiviral infection. After approximately 8 h, add lentiviruses (carry sgRNA) to cells (day 1). Add $\sim 6 \times 10^5$ viral particles to 2×10^5 cells, which typically resulted in 20%–30% of cells infected (Figure 1A).

⚠ **CRITICAL:** It is recommended to set up control groups (cells infected with lentiviruses that carry no sgRNA or express GFP).

Note: Polybrene (8 μg/mL) can be added to increase transduction efficiency.

- c. After 48 h of transduction, treat cells with 1.5 μg/μL puromycin for 2 days to remove non-infected cells (Figure 1B).
- d. After 2 days of puromycin treatment, collect cells and plate cells in a new 12-well plate for differentiation. Troubleshooting 2 (The number of infected pre-adipocytes might be variable, thereby resulting in experimental variability)

Note: It is recommended to allow these infected cells to grow in growth medium for one more day before differentiation.

10. Adipocyte differentiation.

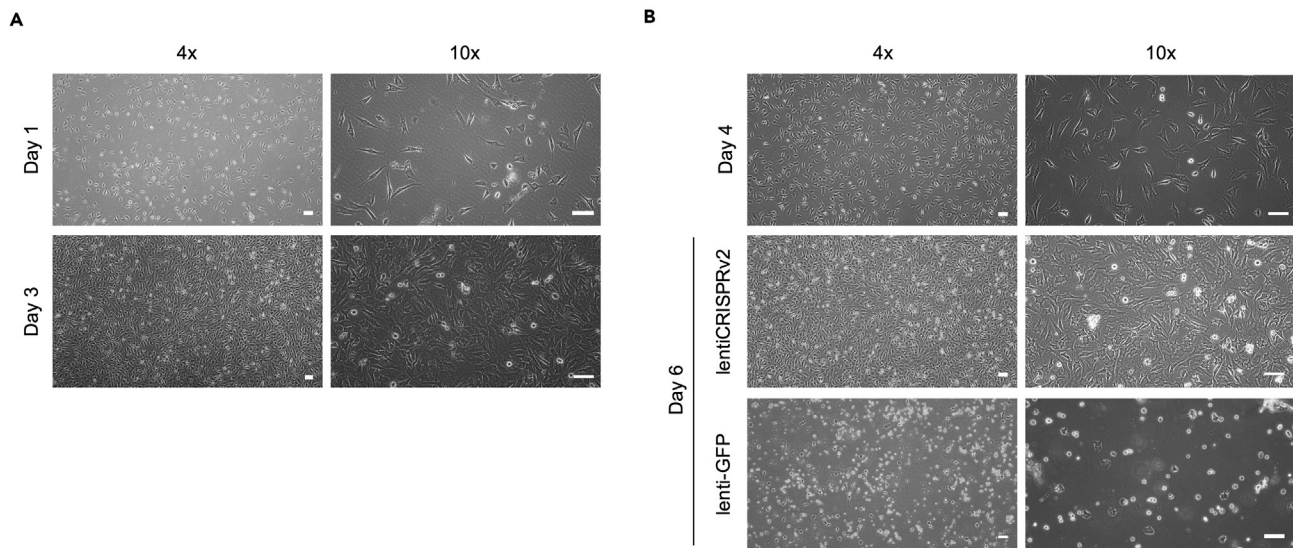


Figure 1. Lentiviral infection and puromycin treatment

Representative images show the density variation of brown pre-adipocytes after lentiviral infection from day 1 to day 3 (A); or after puromycin treatment from day 4 to day 6 (B). The lentiCRISPRv2 vector contains the puromycin cassette while the lenti-GFP vector does not. Thus, cells infected with lentiCRISPRv2 viruses are resistant to puromycin treatment, and cells infected with lenti-GFP viruses are puromycin-irresistant. Scale bar, 200 μm .

Note: The protocol for the differentiation of white pre-adipocytes is similar to that for the differentiation of brown pre-adipocytes, with the exception that no T3 and 5 $\mu\text{g}/\text{mL}$ insulin is used during the whole differentiation process (Qiu et al., 2018, Feng et al., 2019). Thus, this protocol mainly describes the brown pre-adipocytes differentiation.

△ **CRITICAL:** Prepare all reagents for adipocyte differentiation before use. Aliquot stock solutions into small volumes. Avoid repeatedly freezing and thawing.

- a. When brown pre-adipocytes grow confluent (termed “day 0”), remove the growth medium and add induction medium to induce adipogenesis.

Note: The induction medium should be freshly prepared before use.

- b. On day 3, remove the induction medium and add the maintenance medium.

Note: Do not wash cells with PBS. The maintenance medium should be freshly prepared before use.

- c. Change the maintenance medium every 2 days. On day 6 or 7, Typically more than 90% of cells are differentiated. Troubleshooting 3 (Low differentiation potential of pre-adipocytes)

Note: Change the maintenance medium daily if it appears yellow.

Functional validation of sgRNAs

⌚ Timing: 1–2 days

11. Functional testing for the detection of indel mutations by surveyor assay (Timing: 1 day):
Confirm the function of sgRNA in adipocytes by detecting the indel mutations generated by specific sgRNAs. The protocol is identical to that in step 7. Troubleshooting 4 (No obvious cleavage bands after T7 endonuclease I digestion)
12. Functional testing to determine protein expression

⌚ **Timing: 2 days**

- a. Collect the well-differentiated adipocytes in 1.5 mL tube. For one well of 12-well plate, add 60 μ L RIPA buffer into the tube, mix gently by pipetting up and down for a few times, and put the mixture on ice for 15 min.

Note: The volume of RIPA buffer can be flexible. For one well of 12-well plate, it is recommended to add 60 μ L RIPA buffer to lysis cells.

- b. Centrifuge 15 min at 15,294 $\times g$, and transfer the lysis buffer (middle layer) to a new 1.5 mL tube (Figure 2).

Note: This step can be repeated to remove lipids. Troubleshooting 5 (Lipid droplets in mature adipocytes reduce protein extraction efficiency)

- c. Add 3 \times SDS-PAGE loading buffer to each tube, and incubate the mixture at 95°C for 5 min.

Note: The volume of 3 \times SDS-PAGE loading buffer depends on the volume of RIPA buffer in step 12a. For 60 μ L RIPA buffer, add 30 μ L 3 \times SDS-PAGE loading buffer.

- d. Place the solution on ice for immediate use or store the solution at -20°C or -80°C for future use.
- e. Run the protein samples on a SDS-PAGE gel, and detect the target protein expression using corresponding antibodies.

EXPECTED OUTCOMES

When running the DNA samples on 1% (wt/vol) agarose gel, high-efficiency sgRNA will generate obvious cleavage bands, whereas the negative controls will have no cleavage bands (Figure 3). When running protein samples on SDS-PAGE gel, high-efficiency sgRNA will result in significantly decreased target protein expression.

LIMITATIONS

In this protocol, as gene editing is performed in a group of cells, it usually results in a knockdown effect, as in some cells with a certain percentage, CRISPR editing results in indels with multiples of 3 nt, thus resulting in deletion or insertion of several amino acids, but not frameshift or early protein truncation. In order to ensure a knockout effect, single clones must be isolated with sequencing confirmed with a frameshift indel mutation.

Another limitation of this protocol is that genes are edited in pre-adipocytes but not directly in mature adipocytes. It is possible that knockout of certain gene may have functional consequences to adipocyte differentiation. If researchers want to exclude possible effect of a gene during adipocyte differentiation, we recommend using inducible CRISPR/Cas9 system, for example, the pCW-Cas9 (Addgene, #50661), to start gene editing after adipocyte differentiation in mature adipocytes. This works by infecting pre-adipocytes with the inducible CRISPR/Cas9 system to introduce the Cas9

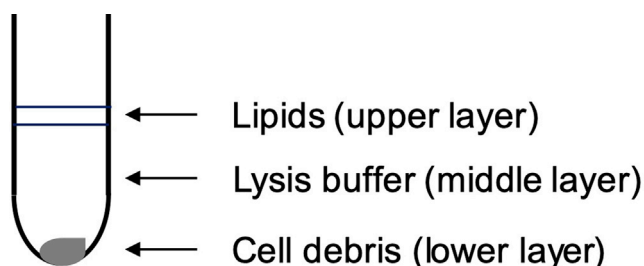


Figure 2. Schematic view of the layers after centrifugation (step 12b)

The upper layer is lipid, the middle layer is the lysis buffer, and the lower layer is the cell debris.

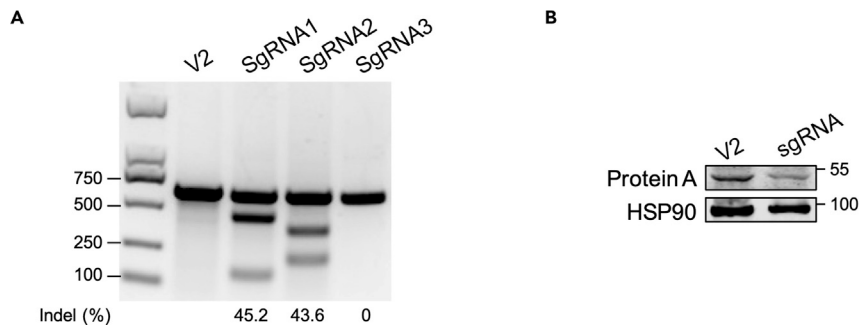


Figure 3. Surveyor assay and western blot results illustrating the editing activity of selected sgRNAs

(A) Assessment of sgRNA cleavage efficiency by surveyor assay. sgRNA that results in obvious cleavage bands indicates high-efficiency sgRNA, such as sgRNA1 and 2 showed here. sgRNA that hardly generates any cleavage bands indicate low-efficiency sgRNA, such as sgRNA3 showed here. The sgRNA efficiency is quantified by ImageJ. (B) Western blot confirming the reduction in protein levels resulted by selected sgRNA. Figure reprinted with permission from Qiu et al. (2020).

and sgRNA cassettes to cells, and induce the Cas9 expression with doxycycline after differentiation to knockout certain genes of interest.

TROUBLESHOOTING

Problem 1

Low titers of lentiviruses (step 4).

Potential solution

This might occur due to multiple reasons: first, poor HEK293T cell health. It is recommended to use low-passage cells. We usually discard cells that have been passaged for more than 15 times.

Second, low transfection efficiency of plasmids. Check the quality of transfection reagents or change to other more-efficient transfection reagents. Improper cell density (too low or too high) also results in low transfection efficiency.

Third, the time for transfection. According to our experience, transfect plasmids 16–24 h after seeding will improve lentiviruses titers.

Problem 2

The number of infected pre-adipocytes might be variable (step 9d), thereby resulting in experimental variability.

Potential solution

This might occur due to multiple reasons. The viral titer might not be accurate. Therefore, titer the lentiviral samples with a commercial qPCR Lentivirus Titration Kit before use.

If the target gene affects cell growth, the density of pre-adipocytes for different groups may be different. To determine this, seed the same number of cells for different groups in a 12-well plate to induce differentiation.

The concentration of puromycin might be different in each sample. To avoid this, prepare a puromycin stock solution and then make diluted samples to ensure homogeneity. Avoid addition of puromycin individually.

Problem 3

Low differentiation potential of pre-adipocytes (step 10).

Potential solution

This might occur due to multiple reasons. First, poor cell health can alter growth rate or morphology. Cells that have more than 15 passages should not be used. A higher number of cell divisions might decrease the differentiation capacity.

Second, the activity of compounds used for differentiation is low. Aliquot to avoid repeated freezing and thawing.

Third, improper cell density. Cell density in a too low or too high level may affect differentiation.

Problem 4

No obvious cleavage bands after T7 endonuclease I digestion (step 7g and step 11).

Potential solution

This might occur due to low editing activity of sgRNA. Therefore, it is recommended to design at least three sgRNAs and screen for sgRNAs with high editing activity. The sgRNA with the highest editing activity should be selected to transfect cells.

Second, low activity of T7EI can cause incomplete digestion. The editing activity of sgRNA can also be determined by sequencing the PCR product generated using surveyor primers.

Third, the time required for T7EI digestion might be short. Therefore, the PCR products can be incubated with T7EI enzyme for a longer duration to achieve complete digestion.

Problem 5

Lipid droplets in mature adipocytes reduce protein extraction efficiency (step 12b).

Potential solution

The lipid droplet content is positively correlated with the maturity of adipocytes. Remove the lipid droplets after addition of RIPA buffer to cells. Moreover, it is recommended to repeat this step until no obvious lipid droplets are observed.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Dr. Qirong Ding (qrding@sibs.ac.cn).

Materials availability

All unique materials generated from this study are available from the Lead Contact with a complete Materials Transfer Agreement.

Data and code availability

We did not generate any unique datasets or data.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.Q. and Q.D.; Investigation, Y.Q.; Writing – Original Draft, Y.Q.; Writing – Review & Editing, Y.Q. and Q.D.; Funding Acquisition, Q.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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