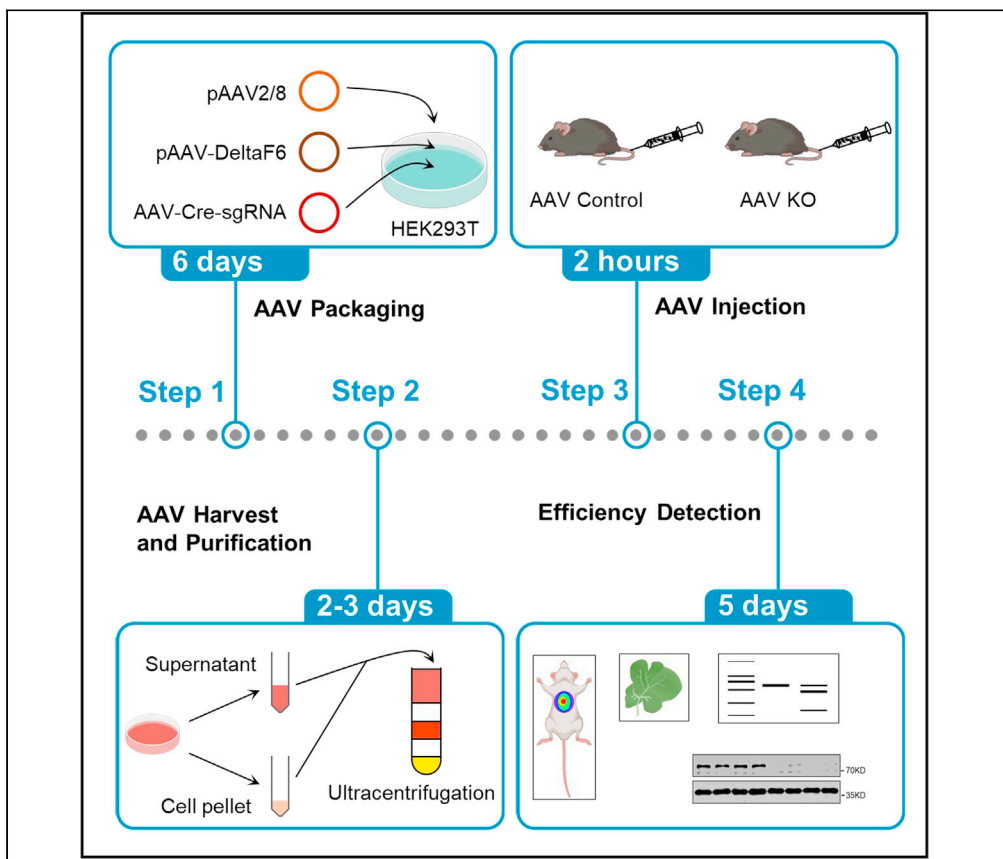


Protocol

Optimized protocols for efficient gene editing in mouse hepatocytes *in vivo* using CRISPR-Cas9 technology



We provide a protocol for gene editing in mouse hepatocytes *in vivo* using the CRISPR-Cas9 technology via AAV delivery. This protocol describes the construction of AAV plasmids, AAV packaging, injection, and the detection of *in vivo* knockout efficiency. Using this protocol, we can get up to 10^{14} AAV and knock out genes in hepatocytes efficiently within 15 days. Moreover, we describe an optimized protocol to simultaneously target two genes via AAV delivery of CRISPR-Cas9 materials in the liver.

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Highlights

Gene editing in hepatocytes *in vivo* using CRISPR-Cas9 technology

Dual gene editing *in vivo* using optimized protocol

High titer of AAV can be produced using this protocol

Multiple techniques to detect AAV infection efficiency

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Protocol

Optimized protocols for efficient gene editing in mouse hepatocytes *in vivo* using CRISPR-Cas9 technologyYanhao Chen^{1,3,*} and Qiurong Ding^{1,2,4,*}

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SUMMARY

We provide a protocol for gene editing in mouse hepatocytes *in vivo* using the CRISPR-Cas9 technology via AAV delivery. This protocol describes the construction of AAV plasmids, AAV packaging, injection, and the detection of *in vivo* knockout efficiency. Using this protocol, we can get up to 10^{14} AAV and knock out genes in hepatocytes efficiently within 15 days. Moreover, we describe an optimized protocol to simultaneously target two genes via AAV delivery of CRISPR-Cas9 materials in the liver.

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

BEFORE YOU BEGIN

Preparation of Rosa26-LSL-Cas9 knock-in mice

⌚ Timing: n/a

1. The Rosa26-LSL (loxP-stop-loxP)-Cas9 knock-in mice harbor a Cas9 transgene expression cassette in the Rosa26 locus (Platt et al., 2014), with an EGFP cDNA also linked via P2A to Cas9. Thus, the expression of Cas9 and EGFP is induced after exposing to the CRE recombinase. With this, introducing a liver cell specific-Cre and sgRNA into the mouse can specifically knock out the target gene in hepatocytes.

Preparation of AAV constructs

⌚ Timing: ~7 days

2. sgRNA design and synthesis.
 - a. sgRNA design. sgRNAs can be designed using the online CRISPR design tools. For example, Broad Institute CRISPick (Doench et al., 2016; Sanson et al., 2018). Online calculation of sgRNA obtained through algorithm optimization may have a higher probability of obtaining sgRNA with higher editing efficiency and lower off-target efficiency. At the same time, online calculation will also give potential off-target sites and probability, so we can choose suitable sgRNAs. You can also design sgRNAs manually as previously described (Qiu and Ding, 2021).

Note: If you do not have an sgRNA sequence that efficiently targets the gene of interest, you need to design at least three sgRNAs and screen for efficient ones as previously described



(Qiu and Ding, 2021). And it is recommended that at least two different sgRNAs are applied in all experimental procedures, with careful evaluation of the sgRNA off-target effects (Ding et al., 2014).

- b. sgRNA oligonucleotides synthesis. Synthesis of two sgRNA oligonucleotides as follows:
 sgRNA-F: 5'-ACCGNNNNNNNNNNNNNNNNNNNNNN-3'
 sgRNA-R: 3'-CNNNNNNNNNNNNNNNNNNNNNNCAA-5'

Note: The polyN sequence represents the 20 bp sgRNA sequence that targets the gene of interest.

3. Preparation of AAV-Cre-sgRNA.

- a. Construct the AAV-Cre-sgRNA plasmid (Figure 1).
- i. Delete the GFP expression cassette between two inverted terminal repeats (ITRs) of pAAV-GFP.
 - ii. The CRE expression cassette was inserted between the two ITRs. The *Cre* gene is driven by a liver-specific TBG promoter, which is also linked via a T2A sequence to a *Luciferase* gene to facilitate the *in vivo* imaging of CRE-expressing cells later.
 - iii. A U6 driven sgRNA expression cassette was inserted after the *Cre* expression cassette. The sequence containing two *Sap I* sites was added before the sgRNA scaffold sequence, where the sgRNA targeting sequence will be inserted for each gene. The sequence containing two *Sap I* sites is as follows:
 5'-ACCGAAGAGCAAGCTCTTCGGTT-3'
 3'-TGGCCTTCTCGTTCGAGAAGCCAA-5'
- b. Digest 1 µg AAV-Cre-sgRNA plasmid with *Sap I* restriction enzyme for 1 h at 37°C.

Reagent	Amount
AAV-Cre-sgRNA	1 µg
<i>Sap I</i>	1 µL
CutSmart Buffer	5 µL
ddH ₂ O	n/a
Total	50 µL

- c. Run the *Sap I* digestion products on a 1% agarose gel.
- d. Cut the 7399 bp DNA band. Extract DNA from the gel using a gel extraction kit.
- e. Denature the pair of oligos from step 2b at 95°C for 5 min, then slowly cool the solution 0.1°C/s to 25°C in a thermocycler.

Reagent	Amount
sgRNA-F (100 µM)	1 µL
sgRNA-R (100 µM)	1 µL
Annealing buffer (10×)	5 µL
ddH ₂ O	n/a
Total	50 µL

- f. Set up the ligation reaction and incubate at 16°C for 30 min.

Reagent	Amount
Digested plasmid from 3d	50 ng
Annealed oligos from 3e	2 µL
Ligation High (premixed T4 ligase)	3 µL
ddH ₂ O	n/a
Total	9 µL

AAV-Cre-sgRNA:

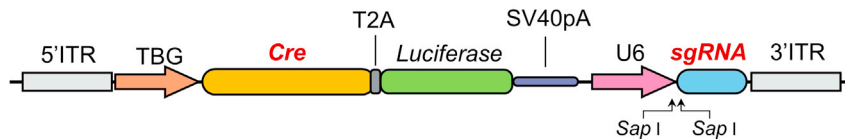


Figure 1. Schematic of the AAV-Cre-sgRNA vector

- g. Set up a ligation reaction without annealed oligos as a negative control and incubate at 16°C for 30 min.
- h. Transform all the ligation products from step f into Stbl3 chemically competent cells. Transform all the products from step g into Stbl3 competent cell as a negative control.

Note: It is recommended to transform the AAV plasmids into Stbl3 competent cells to reduce potential homologous recombination through the ITRs.

- i. Add 100 μ L LB medium to the competent cells, then incubate in a 37°C shaker for 1 h.
- j. Spread competent cells evenly on an LB plate containing 100 μ g/mL ampicillin.
- k. Incubate the LB plates at 37°C for 12–16 h.
- l. Assess the plate for colony growth. Typically, multiple colonies should grow on the plate, and the negative control plate should not have colony growth.
- m. Pick three single colonies with pipette tips and put them into different 15 mL centrifuge tubes.
- n. Add 2 mL of LB medium containing 100 μ g/mL ampicillin to each centrifuge tube and incubate in a shaker at 37°C for 6 h.
- o. Isolate plasmid DNA from the bacterial cultures using a plasmid miniprep kit.
- p. Use U6 sequencing primer U6-F to identify whether the 20 bp sgRNA oligonucleotides sequence is inserted correctly into the AAV-Cre-sgRNA vector.

Optional: Preparation of AAV-Cre and AAV-mCherry-sgRNA1-sgRNA2 plasmids.

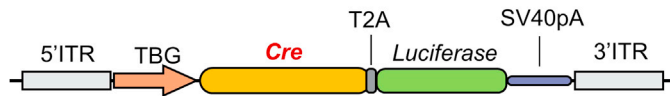
The AAV-Cre-sgRNA only targets one gene. If you want to simultaneously knockout two genes, you can construct the AAV-Cre and AAV-mCherry-sgRNA1-sgRNA2 plasmid (Figure 2). In the AAV-Cre plasmid, the sgRNA cassette was removed from the AAV-Cre-sgRNA construct via a PCR strategy. The AAV-mCherry-sgRNA1-sgRNA2 was inserted into an mCherry expressing cassette driven by the TBG promoter and two sgRNA cassettes with one driven by the mouse U6 promoter, the other sgRNA driven by the human U6 promoter. With these two AAV constructs, two different genes can be simultaneously and equally targeted *in vivo* via the CRISPR/Cas 9 technology. In addition, the co-expression of the two fluorescent proteins can indicate the targeting efficiency—the mCherry expression indicates the successful delivery and expression of sgRNAs, while the GFP expression demonstrates the delivery of the AAV-Cre virus and expression of Cas9.

Preparation of buffer and solution

⌚ Timing: ~1 day

4. Preparation of polyethylenimine solution.
 - a. Add 1 g polyethylenimine (PEI) to 1 L ddH₂O.
 - b. Stir to dissolve the PEI.
 - c. Add 1 M NaOH to adjust pH to 7.0.
 - d. Sterilize the solution using a 0.22 μ m sterile filter.
 - e. Store at –20°C after aliquoting.

AAV-Cre:



AAV-mCherry-sgRNA1-sgRNA2:



Figure 2. Schematic of the AAV-Cre and AAV-mCherry-sgRNA1-sgRNA2

5. Preparation of luciferin solution.
 - a. Prepare a 15 mg/mL fluorescein stock solution with sterile DPBS
 - b. Sterilize with 0.2 μ m filter membrane.
 - c. After aliquoting, store at -80°C and protect from light.
6. Preparation of 10 \times DNA annealing buffer.

Note: Refer to Materials and equipment.

7. Preparation of lysis buffer.

Note: Refer to Materials and equipment.

8. Preparation of iodixanol solutions of different concentrations.

Note: Refer to Materials and equipment.

Design qPCR primers for AAV titer determination

⌚ Timing: \sim 5 min

9. Choose one gene on the AAV vector to design qPCR primers for AAV titer determination. The primers used in this protocol are Cre-F and Cre-R.

Preparation of -80°C alcohol bath

⌚ Timing: \sim 5 min

10. Pour 300 mL ethanol into a 500 mL enamel cup and put it at -80°C .

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Sap I	New England Biolabs	Cat# R0569L
T7 Endonuclease I (T7EI)	New England Biolabs	Cat# M0302L
Ligation High	TOYOBO	Cat# LGK-101
Polyethylenimine (PEI)	Polysciences	Cat# 23966-1

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Benzonase Nuclease	Merck	Cat# 70746-3
OptiPrep Density Gradient Medium	Sigma-Aldrich	Cat# D1556
DNase I Amplification Grade	Sigma-Aldrich	Cat# AMPD1-D5307
10× Reaction Buffer	Sigma-Aldrich	Cat# AMPD1-R6273
Proteinase K	TIANGEN	Cat# RT403
D-Luciferin, Potassium Salt	YEASEN	Cat# 40902ES01

Critical commercial assays

PowerUp SYBR Green Master Mix	Applied Biosystems	Cat# A25742
TIANprep Mini Plasmid Kit	TIANGEN	Cat# DP103
EndoFree Maxi Plasmid Kit	TIANGEN	Cat# DP117
TIANamp Genomic DNA Kit	TIANGEN	Cat# DP304-03
Gel Extraction Kit	CWBIO	Cat# CW2302M

Deposited data

AAV-Cre-sgRNA	This paper	Mendeley Data: https://doi.org/10.17632/y5g3kffbg9.1
AAV-Cre	This paper	Mendeley Data: https://doi.org/10.17632/y5g3kffbg9.1
AAV-mCherry-sgRNA1-sgRNA2	This paper	Mendeley Data: https://doi.org/10.17632/y5g3kffbg9.1

Experimental models: Cell lines

HEK293T	Chinese National Collection of Authenticated Cell Cultures	Cat# GNHu17
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Experimental models: Organisms/strains

Rosa26-LSL-Cas9 knock-in mouse	The Jackson Laboratory	Stock No: 024857
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Oligonucleotides

sgRNA-F	CACCGNNNNNNNNNNNNNNNNNNNN	N/A
sgRNA-R	AAACNNNNNNNNNNNNNNNNNNNNC	N/A
U6-F	GACTATCATATGCTTACCGT	N/A
Cre-F	TGAAGACCATCCAACAGCAC	N/A
Cre-R	CAAAGTCAGTGCGTTCAAAGG	N/A

Recombinant DNA

pAAV2/8	Gift from James M. Wilson	Addgene plasmid # 112864
pAdDeltaF6	Gift from James M. Wilson	Addgene plasmid # 112867
pAAV-GFP	Cell Biolabs	Cat# AAV-400

Other

Centrifugal Filter	Merck Millipore	Cat# UFC910096
0.22 μm Sterile Filter	Merck Millipore	Cat# SLGPR33RB
Centrifuge Bottle	BECKMAN COULTER	Cat# 355618
Optima XPN Ultracentrifuge	BECKMAN COULTER	Model No: XPN-100 - IVD
QuantStudio 6 Flex Real-Time PCR System	Thermo Fisher Scientific	Cat# 4485697
IVIS Lumina II In Vivo Imaging System	PerkinElmer	Model No: Lumina II
Laser Scanning Microscopes	OLYMPUS	Model No: FV1200

MATERIALS AND EQUIPMENT

10× DNA annealing buffer

Reagent	Final concentration	Amount
Tris-HCl (pH8.0)	400 mM	2.4227 g
NaCl	500 mM	1.4611 g
MgCl ₂	100 mM	0.4761 g
ddH ₂ O	n/a	n/a
Total	n/a	50 mL

Store at 4°C for up to 1 year

Lysis buffer

Reagent	Final concentration	Amount
Tris-HCl (pH8.0)	20 mM	0.1211 g
NaCl	150 mM	0.4383 g
ddH ₂ O	n/a	n/a
Total	n/a	50 mL

Store at 4°C for up to 1 year

17% iodixanol

Reagent	Final concentration	Amount
10× PBS	1 ×	5 mL
MgCl ₂ (1 M)	1 mM	0.05 mL
KCl (1 M)	2.5 mM	0.125 mL
NaCl (5 M)	1 M	10 mL
OptiPrep	n/a	12.5 mL
ddH ₂ O	n/a	22.325 mL
Total	n/a	50 mL

Store at 4°C for up to 1 year

25% iodixanol

Reagent	Final concentration	Amount
10× PBS	1 ×	5 mL
MgCl ₂ (1 M)	1 mM	0.05 mL
KCl (1 M)	2.5 mM	0.125 mL
OptiPrep	n/a	20.83 mL
ddH ₂ O	n/a	23.995 mL
Total	n/a	50 mL

Store at 4°C for up to 1 year

40% iodixanol

Reagent	Final concentration	Amount
10× PBS	1 ×	5 mL
MgCl ₂ (1 M)	1 mM	0.05 mL
KCl (1 M)	2.5 mM	0.125 mL
OptiPrep	n/a	33.33 mL
ddH ₂ O	n/a	11.495 mL
Total	n/a	50 mL

Store at 4°C for up to 1 year

60% iodixanol

Reagent	Final concentration	Amount
MgCl ₂ (1 M)	1 mM	0.05 mL
KCl (1 M)	2.5 mM	0.125 mL
OptiPrep	n/a	50 mL
Total	n/a	50.175 mL

Store at 4°C for up to 1 year

STEP-BY-STEP METHOD DETAILS

Production of AAV

⌚ Timing: 6 days

1. Preparation of HEK293T cells.
 - a. Seed HEK293T cells into a 10 cm dish. Cells were cultured in DMEM supplemented with penicillin-streptomycin and 10% FBS at 37°C in a 5% CO₂ incubator.
 - b. When the cells reach 80% confluence, split cell to 5 10 cm dishes.
 - c. Pass 5 dishes of HEK293T cells to 25 dishes, when the cells reach 80% confluency.

Note: 25 dishes of HEK293T cells can pack 10¹³–10¹⁴ viruses. You can use the appropriate amount of 293T cells for virus packaging according to your needs.

2. Plasmids transfection.
 - a. When the cell density reaches 80%–90%, prepare for transfection.
 - b. Preheat PEI solution and DMEM at 37°C.
 - c. Add the pAAV2/8, pAdDeltaF6, and AAV-Cre-sgRNA to DMEM, pipetting up and down a few times to mix.

Reagent	Amount
pAAV2/8	10 µg
pAdDeltaF6	10 µg
AAV-Cre-sgRNA	10 µg
PEI	90 µL
DMEM	n/a
Total	1 mL

- d. Add 90 µL PEI (the ratio of PEI volume (mL) to the amount of total DNA (mg) is 3:1) to the Plasmid/DMEM mixture.
 - e. Vortex the mixture. Place at 20°C–24°C for 10 min.
 - f. Gently add the mixture dropwise to the cell culture medium and shake gently to mix.

Note: The amount of reagent used in step 2 is for transfecting 1 dish of HEK293T cells.

Optional: The AAV-Cre-sgRNA plasmid can be replaced with the pAAV-GFP plasmid, and one dish of HEK293T cells can be transfected at the same time to test the transfection efficiency. 24 h after transfection, GFP-positive cells should be greater than 80%. [Troubleshooting 1](#)

3. After 10–12 h of plasmids transfection, replace the medium with fresh culture medium.

Harvest of AAV

⌚ Timing: 1–2 days

After 60 h of plasmid transfection, harvest the AAV from the culture medium and HEK293T cells.

4. Harvest the AAV from the culture medium.
 - a. Collect all the culture medium in several 50 mL centrifuge tubes.
 - b. Centrifuge at 3000 g for 10 min to remove cell debris.
 - c. Transfer all the supernatant to new centrifuge tubes.
 - d. Pipette 15 mL of supernatant into the centrifugal filter.

- e. Centrifuge at 3000 g for 30 min at 4°C.
- f. Remove the bottom fraction. Collect the concentrated supernatant in the filter into a new 50 mL centrifuge tube.
- g. Repeat steps d-f, until all the supernatant is concentrated.

△ **CRITICAL:** All supernatant should be concentrated to less than 7 mL, otherwise one centrifuge bottle will not be enough to hold all the concentrated supernatants.

5. Isolation of the AAV from HEK293T cells.
 - a. Add 1–2 mL DMEM into one dish and resuspend the HEK293T cells with a pipette by pipetting up and down several times.
 - b. Transfer the suspension to a 50 mL centrifuge tube.
 - c. In the same way, transfer the remaining 24 dishes of HEK293T cells to the 50 mL centrifuge tube.
 - d. Centrifuge at 3000 g for 15 min at 4°C
 - e. Discard the supernatant and resuspend the cell pellet in 3 mL lysis buffer.
 - f. Freeze the resuspended cells in an alcohol bath at –80°C for 30 min
 - g. Quickly transfer the frozen cells to a 37°C water bath for 20 min.
 - h. Repeat steps f to g.
 - i. Put the thawed cell suspension in the –80°C alcohol bath.

Purification of AAV

⌚ **Timing:** 1 day

6. Quickly transfer the frozen cells from step 5 to a 37°C water bath for 20 min.
7. Mix the concentrated supernatant from step 4 and the freeze-thaw cell suspension, add 1 M MgCl₂ to a final MgCl₂ concentration of 1 mM.
8. Add Benzonase to the mixture to a final Benzonase concentration of 25 U/mL, mix well and incubate at 37°C for 30 min.
9. Centrifuge at 3000 g for 20 min at 4°C.
10. Preparation of discontinuous iodixanol gradients ([Figure 3](#)). Slowly add 3.5 mL 60%, 3.5 mL 40%, 4 mL 25%, 4 mL 17% iodixanol to the centrifuge bottle from bottom to top.
11. Add the supernatant from step 9 onto the top of the 17% layer of iodixanol gradients very slowly and gently.
12. Use Beckman Optima XPN-100-IVD ultracentrifuge, 70Ti rotor – centrifugation at 60000 rpm (max rcf ≈ 370000 g) for 2 h at 4°C, acceleration 6, deceleration 9.
13. Using a 5 mL syringe and a 20-gauge needle to collect the 40% layer of iodixanol gradients.
14. Transfer the 40% iodixanol to a centrifugal filter.
15. Fill up the centrifugal filter with PBS. The volume after filling is about 15 mL.
16. After mixing upside down, centrifuge at 3000 g for 30 min at 4°C. [Troubleshooting 2](#)
17. Remove the bottom fraction.
18. Repeat step 15 to 17 twice.
19. Collect the remaining virus suspension in the filter into a 1.5 mL centrifuge tube, add PBS to dilute to about 1 mL. [Troubleshooting 3](#)
20. After pipetting and mixing, aliquot 100 µL virus into each tube and freeze at –80°C. Aspirate 5 µL and prepare for AAV titer measurement later.

⏸ **Pause point:** Store at –80°C until titer measurement or AAV injection.

qPCR for AAV genome titer measurement

⌚ **Timing:** 4 h

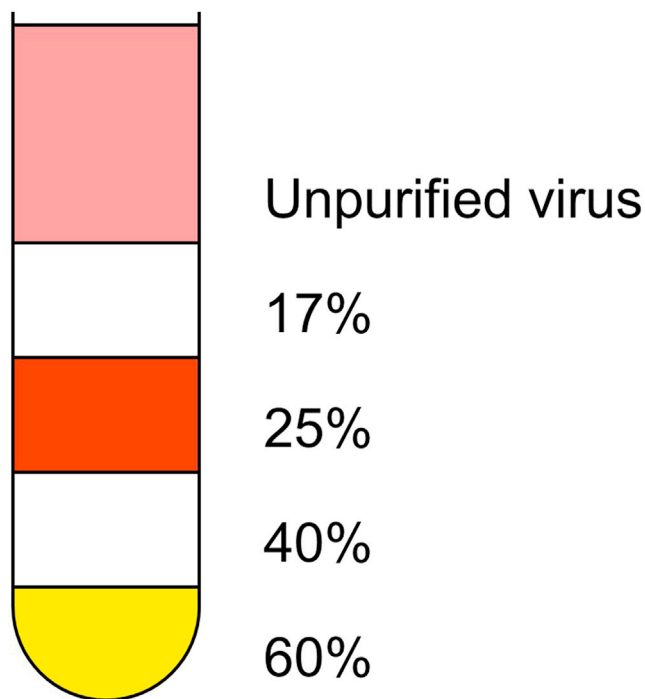


Figure 3. Illustration of iodixanol gradients before centrifugation

The liquid in the centrifuge bottle from bottom to top is 60%, 40%, 25%, 17% iodixanol, and unpurified AAV.

21. Prepare standards. Dilute the AAV-Cre-sgRNA plasmid at a ratio of 1:10 into 7 gradients. The highest starting concentration is recommended to be around 10 $\mu\text{g}/\text{mL}$.
22. Preparation of viral genomic DNA
 - a. Incubate 5 μL virus with DNase I at 37°C for 10 min to remove residual DNA in the solution.

Reagent	Amount
Virus	5 μL
DNase I	5 μL
10 \times Reaction Buffer	5 μL
ddH ₂ O	35 μL
Total	50 μL

- b. Incubate the mixture at 95°C for 10 min to denature DNase I
- c. Add 1 μL proteinase K and incubate at 37°C for 10 min to digest the virus protein coat.

Reagent	Amount
From step b	50 μL
Proteinase K	1 μL
10 \times Reaction Buffer	5 μL
ddH ₂ O	44 μL
Total	50 μL

- d. Inactivate proteinase K at 95°C for 10 min.

23. Set up the qPCR reactions.

Reagent	Amount
PowerUp SYBR Green Master Mix (2×)	5 μL
Cre-F	0.4 μL
Cre-R	0.4 μL
DNA template	1 μL
Nuclease-free water	3.2 μL
Total	10 μL

24. Set up and run the real-time PCR instrument.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Activation	50°C	2 min	1
	95°C	10 min	
Denaturation	95°C	15 s	40
Annealing/ Extension	60°C	1 min	

25. Calculate the titer of the virus

- The number of copies N of single-stranded DNA per microliter in each standard can be approximated using the following formula from the concentration C ($\mu\text{g}/\mu\text{L}$) and the length L (bp) of the standard of the plasmid.

$$N \approx 2 \times \frac{6.02 \times 10^{23}}{2 \times 330 \times L} \times C \times 10^{-6}$$

Note: The average relative molecular mass of each deoxyribonucleoside is approximately 330. The length of the plasmid standard is L . Therefore, the relative molecular mass of the standard product is $2 \times 330 \times L$. 6.02×10^{23} is Avogadro constant. So, the copy number of $2 \times 330 \times L$ g standard plasmid is about 6.02×10^{23} .

- Calculate the number of copies of single-stranded DNA in each standard
- The C_t value of each standard product as the independent variable x , and the logarithm of the copy number with base 10 as the dependent variable y , to fit the linear regression equation (Figure 4). [Troubleshooting 4](#)
- The C_t value of the viral genome is substituted into the linear regression equation, and the value y is calculated. [Troubleshooting 5](#)
- 10 to the power of y is the number of copies of the viral genome.
- Because the virus is diluted 20 times in step 22, the concentration of the virus stock solution needs to be multiplied by 20.

Injection of AAV

⌚ Timing: 2 h

- Remove the AAV from -80°C and dissolve it at 37°C .
- Dilute the virus titer with sterile PBS to $1 \times 10^9/\mu\text{L}$.
- Inject 250 μL of virus per mouse via tail vein injection.

⚠ CRITICAL: When mice in the experimental group were injected with AAVs carrying sgRNA (AAV-sgRNA), the control group should be injected with the same titer of AAVs without sgRNA (AAV-control).

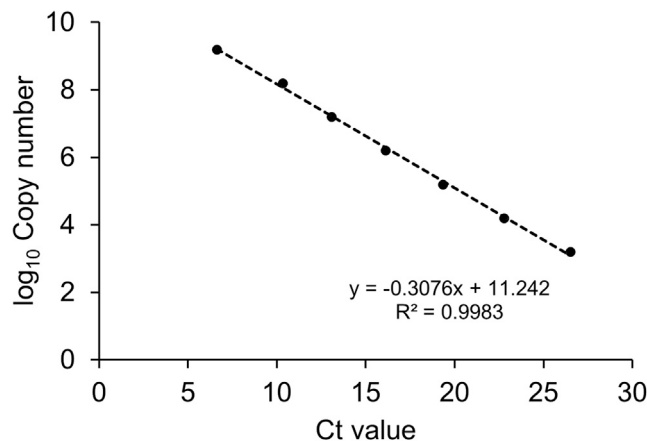


Figure 4. An example of linear regression equation curve for standard products

The abscissa is the Ct value of the standard product. The ordinate is the logarithm of the standard DNA copy number with the base ten. The linear regression equation is $y = -0.3076x + 11.242$. The coefficient of determination (R^2) is 0.9983.

Efficiency detection

⌚ Timing: 5 days

29. *In vivo* bioluminescence imaging to detect whether the virus successfully infects the animal livers.

Note: The AAV-Cre-sgRNA vector contains the luciferase gene, so *in vivo* bioluminescence imaging can be used to detect whether the virus successfully infects liver cells and expresses the viral genes.

- a. 1–2 weeks after virus injection, the virus infection is detected by *in vivo* imaging.
- b. Each mouse was injected intraperitoneally with a luciferin/body weight concentration of 150 mg/kg.
- c. After 10–15 min, when the light signal reaches the strongest stable plateau, perform the imaging analysis.

30. Detect the green fluorescence of mouse liver tissue to determine whether the mouse successfully expresses the Cas9 protein.

Note: In the Rosa26-LSL-Cas9 animals, the EGFP protein is co-expressed with Cas9 via a P2A linker sequence. Therefore, by observing the green fluorescence of the mouse liver, we can judge whether expression of the Cas9 protein in liver cells is successfully induced.

- a. Detect the green fluorescence of the whole liver.
 - i. The mice were anesthetized by intraperitoneal injection of 4% chloral hydrate at a dose of 10 mL/kg.
 - ii. Open the abdominal cavity of the mouse and remove the liver.
 - iii. The liver was washed to remove residual blood in PBS.
 - iv. Put the liver in a 6 cm culture dish.
 - v. Observe the green fluorescence of the liver with a fluorescence microscope.
- b. Detect the green fluorescence of frozen sections of the liver.
 - i. Cut the liver tissue block with a thickness of less than 5 mm.

- ii. Place the tissue block onto a cryomold.
- iii. Cover the entire tissue block with the OCT compound.
- iv. Slowly put the bottom of the mold into liquid nitrogen until the entire tissue block is completely frozen.
- v. Transfer the frozen tissue block to a cryotome cryostat. Cut sections at a thickness of 4 μm .
- vi. Observe the green fluorescence of liver slices with a fluorescence microscope.

31. Use T7E1 assay to detect genome editing efficiency.

- a. Design primers to amplify mouse genome sites targeted by the sgRNA.

Note: We recommend that the length of the PCR product should be between 600 bp–1000 bp. Long PCR products will increase the difficulty of amplification. The distance from the cut site should be greater than 150 bp. Otherwise, it will be difficult to separate the undigested DNA band and the longer digested fragment through DNA gel, and the shorter digested fragment will also be blurry on the DNA gel.

- b. Extract genomic DNA from mouse liver.
- c. Use the primers to amplify the genomic sequence targeted by sgRNA.
- d. Run the PCR products on a 1% agarose gel. Cut the target DNA band and extract the DNA using a gel extraction kit.
- e. Denature the DNA at 95°C for 5 min, then slowly cool the solution 0.1°C/s to 25°C in a thermocycler.

Reagent	Amount
DNA product from step 31b	500 ng
NEBuffer 2 (10 \times)	2 μL
ddH ₂ O	n/a
Total	20 μL

- f. Digest the annealed DNA fragments with 0.5 μL T7 Endonuclease I (T7EI) enzyme at 37°C for 30 min.
- g. Run samples on a 1% agarose gel.
- h. 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 band.

32. Use Western blot to detect whether the target protein in mouse liver has been successfully knocked out.

EXPECTED OUTCOMES

After the qPCR for AAV genome titer measurement, the total titer of the viral genome you can get is about 10^{14} . The coefficient of determination (R^2) of the standard linear regression equation should be greater than 0.98 and the amplification efficiency of primers should be between 90%–110%. This will ensure that your calculated titer is more accurate. One week after AAV injection, you can observe significant fluorescence intensity in mouse liver through *in vivo* imaging (Figure 5A). Three weeks after AAV injection, the liver DNA sequence targeted by sgRNA will generate obvious cleavage bands after T7E1 enzyme digestion (Figure 5B). If cutting bands are observed in the control group, it may indicate that there are natural heterozygous mutations (e.g., single nucleotide polymorphism, SNP) in the amplified DNA sequence, which can also be recognized by the T7E1 enzyme. Also, in some occasional situations, the inactivation of the T7E1 enzyme can also lead to the failure to detect digested fragments. Sanger sequencing can be performed on the amplified DNAs to directly determine whether the site has been edited. We can observe the green fluorescence of the liver with a fluorescence microscope (Figure 5C). We can also observe the green fluorescence of hepatocytes

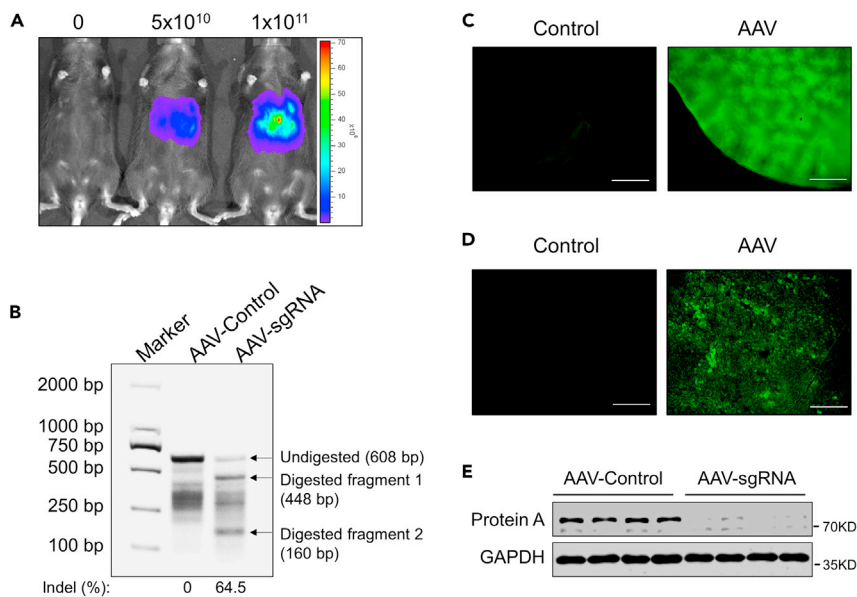


Figure 5. The anticipated outcomes of the protocol

(A) *In vivo* bioluminescence imaging of mice one week after administration with 0, 5×10^{10} , 1×10^{11} AAV-*Cre-sgRNA* AAVs.

(B) Gel image of liver DNA T7E1 assay after 3 weeks of infection with AAVs that does not carry (AAV-Control) and carries sgRNA (AAV-sgRNA).

(C) The images of GFP expression in fresh liver tissues from mice upon two weeks' infection with 0 or 1×10^{11} AAV-*Cre-sgRNA* AAVs. Scale bars represent 1 mm. (D) The images of GFP expression in frozen liver sections from mice upon two weeks' infection with 0 or 1×10^{11} AAV-*Cre-sgRNA* AAVs. Scale bars represent 250 μ m.

(E) Western analysis of protein A expression in liver tissues from animals after 3 weeks' infection with AAVs not carrying (AAV-Control) or carrying sgRNA (AAV-sgRNA).

in frozen sections of the liver with a fluorescence microscope (Figure 5D). The target protein expression level will be significantly reduced, 3 weeks after injection of AAV carrying CRE and sgRNA (Figure 5E).

LIMITATIONS

Due to the upper limit of cargo size in AAV packaging, it is difficult to express the spCas9 using AAV vectors. Therefore, our *in vivo* knockout system must use the Cas9 transgenic mice.

An sgRNA that can efficiently target the gene of interest will need to be screened out and inserted into the AAV vector. Usually we will need to first verify the activity of different sgRNAs on the cell line *in vitro*, and select the most efficient sgRNA for AAV packaging. However, due to the significant differences in cell types infected, as well as the delivery method adopted between the *in vivo* and *in vitro* experiments, some sgRNAs which work *in vitro* in cell lines may not work as well *in vivo* in liver cells.

Knocking out of the target gene directly in liver *in vivo* through the CRISPR system may also have potential off-target effects. Different sgRNAs targeting the same gene can be applied to exclude potential phenotypic changes due to off-target effects. Direct PCR and sequencing the potential off-target sites or even whole genome sequencing can be applied to evaluate the off-target effects.

We use qPCR to quantify the number of viral genomic DNA to determine the titer of AAV. However, this may overestimate the titer of active AAVs, as some AAVs may not have infectious activity despite having the AAV genome. In addition, freezing and thawing can also affect the AAV activity.

Therefore, the number of active AAV viruses we obtain should be lower than the titer we determined by qPCR.

TROUBLESHOOTING

Problem 1

Few GFP-positive cells. Plasmid transfection efficiency is low.

Potential solution

Plasmids transfection should be performed when the HEK293T cells reach 80%–90% confluency, otherwise the transfection efficiency will be greatly reduced.

After the frozen PEI solution is thawed, there will be some precipitate. Ensure that your PEI solution has been fully dissolved.

HEK293T cells are easily contaminated by mycoplasma. Mycoplasma contamination will reduce the transfection efficiency of cells. Make sure your cells are clean by testing cell supernatant with a mycoplasma detection kit.

Plasmids extraction with an endotoxin-free plasmid maxiprep kit can improve the transfection efficiency.

Problem 2

The liquid in the centrifugal filter is difficult to be filtered by centrifugation.

Potential solution

The 40% iodixanol and PBS in the centrifugal filter should be thoroughly mixed. Otherwise, the density of the 40% iodixanol at the bottom is too high to be centrifuged.

Problem 3

The virus solution is too turbid.

Potential solution

Centrifuge the virus solution at 3000 g for several minutes. Collect the virus supernatant and discard the pellet.

Problem 4

The coefficient of determination (R^2) of the standard product linear regression equation is less than 0.95. A point deviates from the linear regression curve.

Potential solution

The standard was not sufficiently mixed when diluted. Ensure that each standard is thoroughly mixed when diluted.

The highest concentration of standard is recommended to be around 10 $\mu\text{g}/\text{mL}$. A too high concentration of DNA will not be able to obtain accurate Ct value through qPCR.

Problem 5

qPCR amplification curve of virus sample is not smooth. Did not detect the Ct value of virus samples.

Potential solution

The concentration of the viral genome was too high. Dilute the viral genome 10 times or 100 times before qPCR.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Qiorong 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

Original data have been deposited to Mendeley Data: <https://doi.org/10.17632/y5g3kffbg9.1>.

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AUTHOR CONTRIBUTIONS

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

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Ding, Q., Strong, A., Patel, K.M., Ng, S.L., Gosis, B.S., Regan, S.N., Cowan, C.A., Rader, D.J., and Musunuru, K. (2014). Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing. *Circ. Res.* 115, 488–492. <https://doi.org/10.1161/CIRCRESAHA.115.304351>.
- Doench, J.G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E.W., Donovan, K.F., Smith, I., Tothova, Z., Wilen, C., Orchard, R., et al. (2016). Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat. Biotechnol.* 34, 184. <https://doi.org/10.1038/nbt.3437>.
- Platt, R.J., Chen, S.D., Zhou, Y., Yim, M.J., Swiech, L., Kempton, H.R., Dahlman, J.E., Parnas, O., Eisenhaure, T.M., Jovanovic, M., et al. (2014). CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell* 159, 440–455. <https://doi.org/10.1016/j.cell.2014.09.014>.
- Qiu, Y., and Ding, Q. (2021). Optimized protocol for gene editing in adipocytes using CRISPR-Cas9 technology. *STAR Protoc.* 2, 100307. <https://doi.org/10.1016/j.xpro.2021.100307>.
- Sanson, K.R., Hanna, R.E., Hegde, M., Donovan, K.F., Strand, C., Sullender, M.E., Vaimberg, E.W., Goodale, A., Root, D.E., Piccioni, F., and Doench, J.G. (2018). Optimized libraries for CRISPR-Cas9 genetic screens with multiple modalities. *Nat. Commun.* 9, ARTN 5416. <https://doi.org/10.1038/s41467-018-07901-8>.
- Wei, Y.D., Tian, C., Zhao, Y.X., Liu, X.J., Liu, F., Li, S., Chen, Y.H., Qiu, Y., Feng, Z.H., Chen, L.L., et al. (2020). MRG15 orchestrates rhythmic epigenomic remodelling and controls hepatic lipid metabolism. *Nat. Metab.* 2, 447. <https://doi.org/10.1038/s42255-020-0203-z>.