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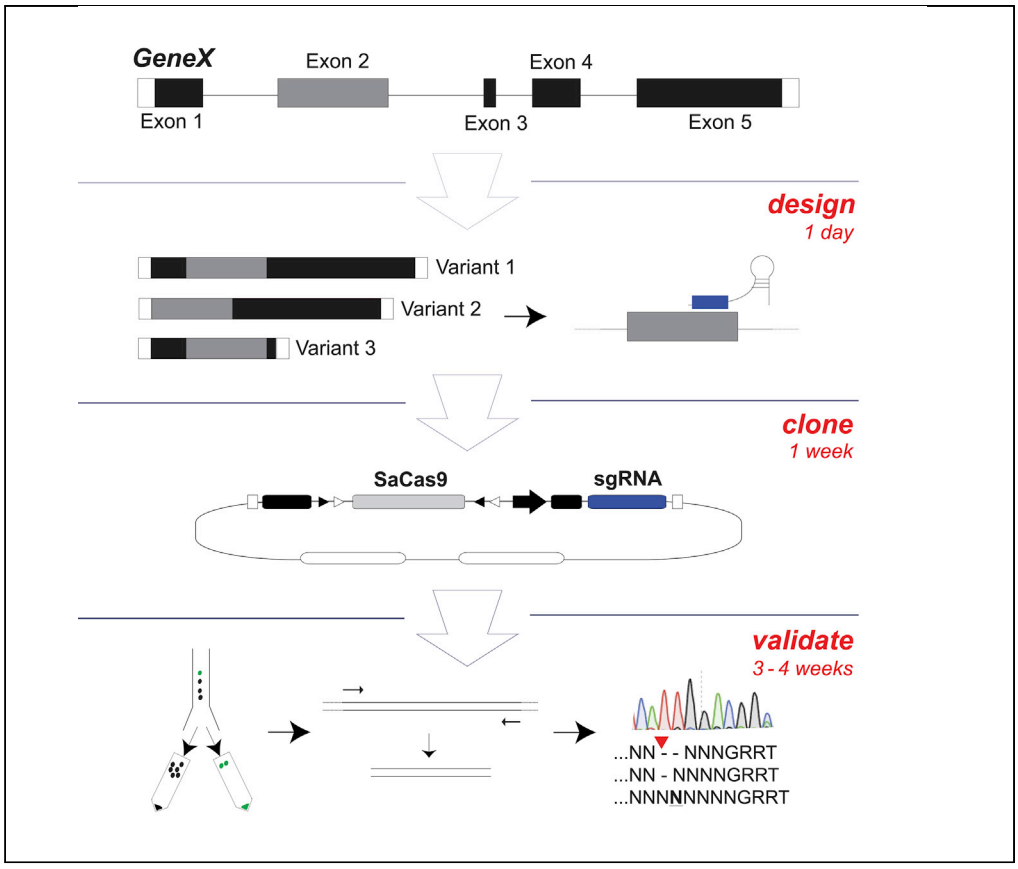
Protocol to Design, Clone, and Validate sgRNAs for *In Vivo* Reverse Genetic Studies



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HIGHLIGHTS

CRISPR/SaCas9 sgRNA design and cloning

Cre- and Flp-dependent viral gene mutagenesis

Validation of sgRNA by targeted deep sequencing

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Protocol to Design, Clone, and Validate sgRNAs for *In Vivo* Reverse Genetic Studies

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SUMMARY

AAV-CRISPR/Cas9 permits gene mutagenesis in the adult CNS. Current methods determining *in vivo* on-target mutagenesis have been limited by the ability to isolate virally transduced cells. This protocol optimizes a workflow for the design, cloning, and validation of sgRNAs delivered by AAVs *in vivo* that can be applied to any target gene in the CNS of rat or mouse model systems and can be adapted to Cre or Flp driver lines using AAV-FLEX-SaCas9-sgRNA or AAV-FLEXfrt-SaCas9-sgRNA, respectively.

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

BEFORE YOU BEGIN

⚠ CRITICAL: This protocol consists of two distinct sections. The first section "*Insertion of sgRNAs into Vector*" outlines the design and cloning of sgRNAs into AAV-FLEX-SaCas9-sgRNA or AAV-FLEXfrt-SaCas9-sgRNA. The second section "*Validation of sgRNA*" outlines the process for obtaining deep sequencing reads with SaCas9-mediated mutations. There will be *6–8 weeks* between completion of the first section and the ability to start of the second section. Please read the entire protocol carefully before beginning.

Selection and Design of sgRNAs for Insertion into AAV1-FLEX-SaCas9-sgRNA or AAV1-FLEXfrt-SaCas9-sgRNA

Timing: 1 h

1. Obtain genomic sequence from the UCSC genome browser (Waterston et al., 2002) by searching the gene name in the designated assembly, e.g., mouse or rat.
2. Import genomic sequence to DNA annotation software and label all coding exons (Figure 1A).

Note: The software Snapgene is excellent for displaying and annotating genomic information (Insightful Science; available at snapgene.com).

3. Align all known isoforms of the gene to the reference genomic sequence.

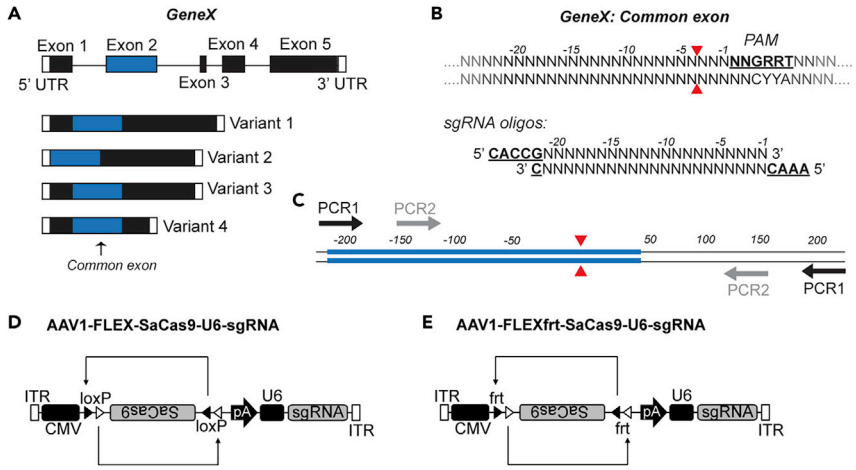

Figure 1. Designing sgRNAs for Cloning into AAV-FLEX-SaCas9-U6-sgRNA or AAV-FLEXfrt-SaCas9-U6-sgRNA

(A) Top: schematic of a hypothetical gene with the exons represented as boxes and introns as lines. Bottom: examples of hypothetical gene isoforms with the most 5′ common exon in blue.

(B) Top: schematic of gene sequence with protospacer adjacent motif (PAM) sequence underlined. Bottom: Guidelines for ordering the forward and reverse primers for synthesis of the sgRNA. Red arrows: SaCas9 cleavage site.

(C) Schematic of primer design for amplicon production. Red arrows: SaCas9 cleavage site. Blue line: exon 2. Black line: intron.

(D and E) Schematic of (D) AAV-FLEX-SaCas9-U6-sgRNA and (E) AAV-FLEXfrt-SaCas9-U6-sgRNA.

Note: Isoforms for mouse genes can be found at the Mouse Genomics Informatics (MGI) website (Bult et al., 2019; Smith et al., 2019).

4. Choose the most 5′ exon that is common to all isoforms.
5. Input this sequence into the CRISPOR website (http://crispor.tefor.net/) (Concordet and Haeussler, 2018; Haeussler et al., 2016) and choose the appropriate genome (e.g., mouse or rat) and PAM (21 bp-NNG(A/G)(A/G)T – Cas9 S. Aureus).
6. Choose a sgRNA that has both high specificity (fewest predicted off-target sites in exons) and high chance of a frameshift mutation.

Note: It is recommended that there are two or less predicted off-targets in exons.

7. Order the forward and reverse oligos such that the forward oligo (the one on the same strand as the PAM sequence) has "CACCG" on the 5′ end, and the reverse oligo has "AAAC" on the 5′ end, and "C" on the 3′ end (Figure 1B). Sequences with overhangs added can be directly copied from the CRISPOR website using the "U6 expression from an Addgene plasmid" and selecting pX601-AAV-CMV::NLS-SaCas9-NLS-3XHA-bGHpA;U6::BsaI-sgRNA (Zhang lab) (Ran et al., 2015).

Note: Failure to add the correct overhangs will hinder proper cloning into the vector.

Design PCR Primers for Amplification of Targeted Region following FACS of Virally Transduced Nuclei

Timing: 30 min

8. Design forward and reverse primers for PCR 1 that span the target region with the predicted SaCas9 cut site (located in the sgRNA) in the center and will result in a ~400–600 bp product (Figure 1C).

Note: Primers should be 18–20 bp in length, 40%–60% G-C content, and have similar annealing temperatures.

9. Design forward and reverse primers for PCR 2 that anneal within the product from PCR 1 and will result in a ~200–400 bp product.

Note: The forward primer should be downstream of the forward primer from PCR 1, and the reverse primer should be upstream of the reverse primer from PCR 1 (Figure 1C).

Note: Again, primers should be 18–20 bp in length, 40%–60% G-C content, and have similar annealing temperatures.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
pAAV-FLEX-SaCas9-sgRNA[a]	Addgene	Cat# 124844
pAAV-FLEXfrt-SaCas9-sgRNA[b]	Addgene	Cat# 124845
pAAV-FLEX-EGFP-KASH[a]	Addgene	Cat# 154373
pAAV-FLEXfrt-EGFP-KASH[b]	Addgene	Cat# 154374
Chemicals, Peptides, and Recombinant Proteins		
D-Sucrose	Fisher	Cat# BP220-212
$CaCl_2$	Sigma	Cat# C4901-100G
$Mg(Ac)_2$	Sigma	Cat# M5661-50G
Tris Base	Fisher	Cat# BP152-5
EDTA	Sigma	Cat# ED-500G
β-mercaptoethanol	Bio-rad	Cat# 1610710
CutSmart buffer	NEB	Cat# B7204S
Ampicillin	Fisher	Cat# BP1760-25
Quick calf intestinal phosphatase (CIP)	NEB	Cat# M0525S
BsaI-HFv2	NEB	Cat# R3733S
HindIII-HF	NEB	Cat# R3104L
T4 polynucletide kinase (PNK)	NEB	Cat# M0201S
T4 ligase buffer	NEB	Cat# B0202S
T4 ligase	NEB	Cat# M0202S
Deoxynucleotide (dNTP) Solution Set	NEB	Cat# N0446S
OptiPrep density gradient medium	Sigma	Cat# D1556-250ML
Protease Inhibitor Cocktail[c]	Sigma	Cat# P8340
NP-40 Surfact-Amps™ Detergent Solution[c]	Thermo	Cat# 28324
Experimental Models: Organisms/Strains		
Mouse: Flp or Cre driver lines	n/a	n/a
Bacterial and Virus Strains		
DH10B electrocompetent *E. coli* cells	Thermo	Cat# 18290015
Adeno-associated virus serotypes 1-9	n/a	n/a
Critical Commercial Assays		
QiaQuick gel extraction kit	Qiagen	Cat# 28704

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
MinElute gel extraction kit	Qiagen	Cat# 28604
QiaPrep Spin Miniprep DNA kit	Qiagen	Cat# 27104
Maxiprep DNA kit[c]	Invitrogen	Cat# K210007
REPLI-g Advanced DNA Single Cell kit	Qiagen	Cat# 150363
Phusion High-Fidelity DNA Polymerase kit	Thermo	Cat# F530L
Software and Algorithms		
CRISPOR sgRNA prediction algorithm	Concordet and Haeussler, 2018	crispor.tefor.net
ImageJ	Schneider et al., 2012	https://fiji.sc/
Snapgene software	GSL biotech	Snapgene.com
Other		
MicroPulser Electroporation Apparatus[c]	Bio-Rad	n/a
Dounce tissue homogenizers (2 mL)[c]	Millipore Sigma	n/a
Precision mouse brain slicer[c]	Braintree Scientific, Inc.	n/a
Reusable Biopsy punch[c]	Produstrial	n/a
Ultracentrifuge[c]	Thermo or Beckman	n/a
BD AriaFACS III	BD Biosciences	n/a
Thermocycler[c]	Bio-Rad	n/a
High-Performance Conical Tubes (50 mL)[c]	VWR	Cat# 89039-656
High-Performance Concial Tubes (15 mL)[c]	VWR	Cat# 89039-666
Membrane filter, 0.025 um pore size	Millipore	Cat# VSWP01300
Beckman centrifuge tubes 1x31/2	Beckman	Cat# 344058

[a]Only necessary if gene mutagenesis is Cre-dependent
[b]Only necessary if gene mutagenesis is Flp-dependent
[c]Other sources than the ones noted may be used for these products

MATERIALS AND EQUIPMENT

Buffers for Validation of sgRNA Targeting *In Vivo*

– Homogenization buffer (individual stocks can be stored at 4°C for up to 1 year, but please make fresh Homogenization buffer day of)

Name	[Stock]	[Final]	Volume to Add
D-Sucrose	500 mM	320 mM	3.2 mL
$CaCl_2$	200 mM	5 mM	125 µL
$Mg(Ac)_2$	60 mM	3 mM	250 µL
Tris (pH=7.8)	200 mM	10 mM	250 µL
EDTA (pH=8)	10 mM	0.1 mM	50 µL
NP40	–	0.1%	5 µL
Protease inhibitor cocktail	10 mM	0.1 mM	50 µL
β-mercaptoethanol	14.2 M	1 mM	35.2 µL
Water	–	–	1.035 mL
Total Volume			**5 mL**

– Upper gradient centrifugation buffer (make day of)

Name	[Stock]	[Final]	Volume to Add
$CaCl_2$	200 mM	5 mM	125 µL
$Mg(Ac)_2$	60 mM	3 mM	250 µL
Tris (pH=7.8)	200 mM	10 mM	250 µL
Protease inhibitor cocktail	10 mM	0.1 mM	50 µL
β-mercaptoethanol	14.2 M	1 mM	35.2 µL
60% OptiPrep Gradient	60%	50%	4.17 mL
Water	-	-	120 µL
Total volume			**5 mL**

– Lower gradient centrifugation buffer (make day of)

Name	[Stock]	[Final]	Volume to Add
60% OptiPrep Gradient	60%	29%	4.83 mL
Water	-	-	5.17 mL
Total volume			**10 mL**

STEP-BY-STEP METHOD DETAILS

Insertion of sgRNAs into Vector

Timing: 5 days

1. Day 1. Vector Digestion using BsaI
   a. Set up the following reaction using vector of choice (Figures 1D and 1E):

Reagents	Amount
pAAV-FLEX-SaCas9-sgRNA **or** pAAV-FLEXfrt-SaCas9-sgRNA	(6–7 µg)
BsaI-HFv2	1 µL
NEB CutSmart Buffer	4 µL
ddH_2O	Bring up to 40 µL
Total	**40 µL**

   b. Digest 16 h (overnight) at 37°C.
2. Day 2. Part One – Purification of cut vector
   a. Add 2 µL of Quick CIP and allow reaction to go for 30 min at 37°C.
   b. Run the cut vector on a 1% agarose gel and perform a gel extraction using the Qiagen gel extraction kit following manufacturer instructions.

   Note: The linear vector should run at ~7.8 kb.

3. Day 2. Part Two – Anneal and phosphorylate each pair of oligos

   Note: Part Two can be completed simultaneously with Part One.

a. Combine the following reagents:

Reagent	Volume (μL)
100 μM sgRNA primer for	1
100 μM sgRNA primer rev	1
ddH_2O	6.5
T4 ligase buffer	1
T4 polynucleotide kinase (PNK)	0.5
Total	**10**

b. Incubate for 30 min at 37°C.
c. Boil the reaction at 100°C for 5 min.
d. Turn off the heat and allow the reaction to *slowly* reach 20°C–22°C (room temperature).

Alternatives: This can also be completed using a ramp down protocol in a thermocycler.

4. Day 2. Part Three – Ligate the phosphorylated and annealed oligos into the cut vector
   a. Add 1 μL T4 ligase and ~50 ng of cut vector directly to the reaction from Part Two.
   b. Allow the ligation to sit at 20°C–22°C (room temperature) for 3 h.
   c. Remove excess salts via dialysis.
   d. Transform 1.5 μL of the ligation using 25 μL DH10B electrocompetent *E. coli* cells.
   e. After transformation, quickly resuspend in 500 μL LB.
   f. Allow 1-h recovery in 37°C with shaking and then plate 100 μL on LB + 100 μg/mL AMP antibiotic plates.
   g. Allow the colonies to grow 12–16 h at 37°C.

Pause Point: Plate may be stored at 4°C for multiple days.

5. Day 3. Colony selection and miniprep setup
   a. Set up eight minipreps using 3 mL LB + 100 μg/mL AMP in 14 mL tubes that allow aeration.
   b. Place in 37°C with 250 rpm shaking for 12–16 h.
6. Day 4. DNA purification and validation of sgRNA insertion
   a. Perform a miniprep of each of the colonies using the Qiagen miniprep kit to extract the plasmid DNA according to manufacturer instructions.
   b. Prepare the following reaction for each purified colony DNA:

Reagent	Volume per Reaction (μL)
DNA [100–300 ng/μL]	5
Cutsmart buffer	2
Water	12.4
BsaI-HFv2	0.3
HindIII-HF	0.3
Total	**20**

   c. Let each reaction go 3–4 h at 37°C.
   d. Run a 1% gel to determine if the oligos inserted.

   Note: Insertion of the sgRNA into the vector will produce a single band at 7.8 kb. Failure to insert the sgRNA will result in two bands (2.5 kb and 5.3 kb).

   e. Select a colony and add 200 μL from miniprep culture in 200 mL LB + 100 μg/mL AMP in a 1L Erlenmeyer flask. Incubate 16 h (overnight) at 37°C with 250 rpm.

7. Day 5. Purification of plasmid DNA
   a. Perform a maxiprep using the Invitrogen maxiprep kit.
   b. Send the DNA to Genewiz for sequencing using the U6 Genewiz Universal primer (sequence: GACTATCATATGCTTACCGT).

Pause Point: The product can be stored at −20°C for years.

Viral Package, Injection, and Expression

Timing: >7 weeks

8. Package plasmids into AAVs.

Note: For packaging DNA into AAVs, please refer to (Gore et al., 2013) for step by step instructions or use a commercial AAV packaging facility.

⚠ CRITICAL: AAV serotypes and titer for AAV-FLEX-SaCas9-sgRNA and AAV-FLEX-EGFP-KASH or AAV-FLEXfrt-SaCas9-sgRNA and AAV-FLEXfrt-EGFP-KASH must be the same to maximize co-transduction efficiencies.

9. Co-inject Cre- or Flp-expressing animals with newly synthesized AAV-FLEX-SaCas9-sgRNA or AAV-FLEXfrt-SaCas9-sgRNA (targeting your gene of choice) and AAV-FLEX-EGFP-KASH or AAV-FLEXfrt-EGFP-KASH, respectively (Figure 2A).

Note: KASH is a protein domain that is inserted into the nuclear envelope. EGFP-KASH integrates EGFP into the nuclear envelope to appropriately identify virally transduced nuclei by EGFP fluorescence (Swiech et al., 2015).

⚠ CRITICAL: Viral concentrations should be between 1 and 3 × 10^{12} particles per mL and injected at a volume of 0.5 mL and rate of 0.25 mL/min. AAV-FLEX-SaCas9-sgRNA and AAV-FLEX-EGFP-KASH should be mixed at equal ratios. Optimal injection volumes, ratios, and viral titers should be tested to ensure optimal expression for each targeted cell type and brain region.

⚠ CRITICAL: Allow *4 weeks* minimum post-surgery for viral expression, and SaCas9 targeting and genomic DNA cleavage before beginning the next section.

Validation of sgRNA

Timing: 2 days

10. Day 1. Part One – Homogenize tissue and isolate nuclei

Timing: 1–1.5 h

   a. At 4 weeks (or longer) following viral injection, harvest tissues from the injected animals. If harvesting from a localized brain region, slice brain into relatively thin (approx. 500 μm) coronal slices using a precision mouse brain slicer and biopsy punch. 3–6 tissue punches weighing <2 mg each can be combined into a single reaction.
   b. Place 2 mL of cold homogenization buffer in a 2 mL Dounce homogenizer. Keep everything on ice.
   c. Extract the tissue containing the virus injection using a small Biopsy punch.

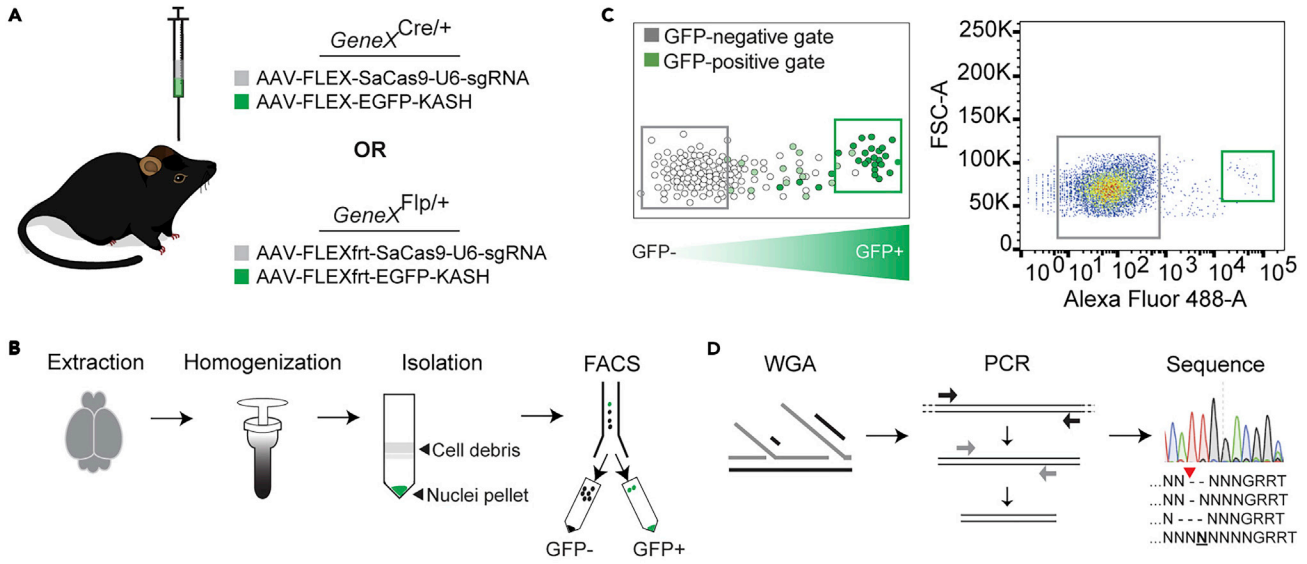

Figure 2. Process for the Validation of sgRNAs *In Vivo*

(A) Schematic of AAV co-injections into the mouse brain.

(B) Schematic of workflow necessary to prepare nuclei for FACS.

(C) Left: Schematic of proper gating to isolate GFP-negative and GFP-positive nuclei. Right: Example FACS with GFP-negative and GFP-positive gates labeled.

(D) Schematic of workflow for the generation and sequencing of amplicons following FACS. Red arrow: SaCas9 cleavage site.

Note: If there is a large amount of tissue, simply divide the tissue into multiple reactions. Too much tissue inhibits efficient homogenization.

Note: Tissue from the same region with no viral manipulations is a good negative control for setting gates for FACS. See step 11c for additional instruction.

Pause Point: Tissue may be flash frozen in liquid nitrogen and stored at −80°C.

d. Place the tissue directly into 2 mL of homogenization buffer (Figure 2B).
e. Homogenize the brain slices first by using Dounce A 25x. Then switch to Dounce B 25x.
f. Pipette this into a 15 mL conical tube on ice.
g. Add 3 mL of homogenization buffer to bring the volume to 5 mL. Keep on ice for 5 min.
h. Add 5 mL of Upper gradient centrifugation buffer and mix by inversion.
i. Gently load on top of 10 mL of the lower gradient centrifugation buffer into 1 × $3^1/_2$ Beckman tubes.
j. Centrifuge at 7,150 × *g* for 30 min at 4°C.
k. Remove the suspended debris using a KimWipe and then gently decant the supernatant.
l. Resuspend in 500–1,000 μL sterile 1× PBS.

11. Day 1. Part Two – FACS using a BD AriaFACS III

Timing: 1 h

a. Place 3 μL of REPLI-g Advanced Single Cell Storage buffer (Qiagen) into each well of an 8-well PCR strip. Keep everything on ice.
b. Sort using the 70 μm nozzle in single cell mode on a BD AriaFACS III. Sort 500 nuclei per well, which results in 1 μL of sorted cell suspension per well.
c. Design gates by comparing the negative control (no viral manipulations) to the experimental group. The GFP-positive gate should contain no GFP-negative nuclei as determined by the

negative control (Figure 2C). Load the negative control and position the GFP-positive gate such that it contains zero GFP-negative nuclei.

d. Sort both GFP-positive and GFP-negative nuclei.

e. After the sort, keep the samples on ice.

Pause Point: Samples may be frozen at −80°C or used directly for whole genome amplification (WGA).

12. Day 1. Part Three – Whole genome amplification

Timing: 2.5 h

a. Perform whole genome amplification (WGA) using the REPLI-g Advanced Single Cell kit (Qiagen) according to manufacturer's instructions (Figure 2D).

Note: WGA is used to limit bias during PCR amplification.

b. Dilute 2 µL of WGA FACS DNA into 100 µL of H_2O (1:50) and store at 4°C.

Pause Point: 1:50 WGA FACS DNA can be stored at 4°C, and the remaining WGA FACS DNA at −20°C for long-term storage.

13. Day 2 (Figure 2D). Amplification of target region

Timing: 4 h

a. Set up the following reaction on ice (PCR 1) using the Phusion high-fidelity DNA polymerase kit and first set of PCR primers:

Reagent	Volume (µL)
100 µM For primer 1	1
100 µM Rev primer 1	1
1:50 diluted WGA FACS DNA	1
10x dNTPs	10
5x HF buffer	20
Water	62
DMSO	3
Phusion	2
Total Volume	**100 µL**

b. Mix well, then divide the 100 µL reaction into four 25 µL reactions.

c. Use the following thermocycler protocol:

Step	Cycle	Temperature (°C)	Time	Repeat
1	Initial denaturation	98	30 s	
2	Denaturation	98	10 s	×34
3	Anneal	66	20 s	
4	Elongation	72	10 s	
5	Final Extension	72	5 min	
6	Hold	12	Forever	

Note: Please refer to potential problems 3–5 in the Troubleshooting section to alter thermocycler protocol accordingly.

d. Combine the four reactions from PCR 1 into a single tube and mix well.
e. Set up the following reaction (PCR 2):

Reagent	Volume (μL)
100 μM For primer 2	1
100 μM Rev primer 2	1
PCR 1 reaction	1
10x dNTPs	10
5x HF buffer	20
Water	62
DMSO	3
Phusion	2
Total Volume	**100 μL**

f. Mix well, then divide the 100 μL reaction into four 25 μL reactions.
g. Use the exact same thermocycler protocol from step 13c.
h. Combine the four reactions from PCR 2 into a single tube and mix well.
i. Run the product on a 2% agarose gel.
j. Gel extract the final product (single band between 200–400 bp) using the MinElute gel extraction kit (Qiagen). Final product may be sent directly to sequencing or may be kept at 20°C for long-term storage.

14. Amplicon-EZ submission
    a. Please refer to Genewiz: Amplicon-EZ service for exact submission guidelines.
    b. Necessary items:
        i. Reference sequence of amplicon
        ii. PCR 2 forward and reverse primers sequence
        iii. 500 ng (20 ng/μL) of amplicons

EXPECTED OUTCOMES

Genewiz will upload the Amplicon-EZ data onto their server and will provide the login credentials necessary to access this data. The software FileZilla (https://filezilla-project.org/) can be used to access and transfer these files off their server. Once the data is transferred, there will be an Excel file labeled "Plate_abundance" that contains a trimmed sequence and number of reads obtained for every unique amplicon (along with other information). There will be anywhere from 1,000–10,000 unique reads. To remove possible errors due to amplification during PCR, the reads must be trimmed to ***just contain the sgRNA and PAM sequence***. Example data set is below. Reference sequence in A2, sgRNA and PAM bolded, deletion indicated by " – ", insertions are bolded and strike-through. Column A: Unique sequences. Column B: number of reads associated with each unique sequence. A6 contains a mutation that is due to PCR.

	A	B
1	Unique sequence	Reads
2	NNNNNNNNNNNNNNN**NNNNNNNNNNNNNNNNNNNNNNNNNNNNGRRT**NNNNN	
3	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN - - NNGRRTNNNNN	946
4	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN - NNNGRRTNNNNN	434
5	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGRRTNNNNN	111
6	NNNNNN - NNNNNNNNNNNNNNNNNNNNNNNNNNN - - NNGRRTNNNNN	58

Trimming can be achieved by:

1. Copy the amplicon sequences and corresponding read count and move to separate page in Excel.
2. Use the following function in C3 to trim the reads from the left:
   =RIGHT((unique read), LEN(unique read) – (# of bases))
   EXAMPLE: =RIGHT((A3), LEN(A3)-14)
   RESULT in column C: NNNNNNNNNNNNNNNNNNN - - NNGRRTNNNNN
3. Use the following function in D3 to trim the reads from the right:
   =LEFT((unique read), LEN(unique read) – (# of bases))
   EXAMPLE: =LEFT((C3), LEN(C3)-5)
   RESULT in column D: NNNNNNNNNNNNNNNNNNN - - NNGRRT

This results in only the sgRNA and PAM sequence remaining in column D.

Note: Sequences containing insertions will result in additional bases.

4. Recalculate the number of reads for each unique sequence using the SUMIF command. Complete this for all rows:
   EXAMPLE: =SUMIF(D3:D6,D3,B3:B6)
   RESULT: 1,004 reads (rows 3 and 6 have the same Cas9-mediated mutation)

This will result in the number of reads associated with each type of mutation.

LIMITATIONS

This protocol will confirm mutagenesis by SaCas9 but will not provide the absolute number of edited cells in total. The number of edited reads can vary greatly depending on the positioning of the GFP-positive gate during FACS (Hunker et al., 2020). A more right-shifted gate will result in a higher number of edited reads. This is either due to 1) a higher concentration of virus in the brightest GFP population, resulting in more molecules of SaCas9 and a higher percentage of edited cells or 2) presence of GFP-negative, auto-fluorescing cells in GFP-positive gate.

TROUBLESHOOTING

Problem 1

The test digestion used to determine sgRNA insertion resulted in all negative colonies (for steps 6b–6d).

Potential Solution

There are two possible causes. The first possible cause is the vector digestion did not go to completion (steps 1a–1b). To solve this, digest the vector for a longer amount of time. Run 5 μL of the reaction on a 1% agarose gel to confirm a single band before moving forward with the gel extraction.

The second possible cause could be that the sgRNA sequence chosen contains a BsaI site. The additional BsaI site would cause the vector to be cut regardless of the insertion of the sgRNA, resulting in false-negative colonies in step 6d. To circumvent this, use PCR to determine sgRNA insertion (see **Alternative Protocol 1** below) and skip steps 6b–6d.

Alternative Protocol 1: PCR can be used to determine sgRNA insertion in place of the digestion. The U6 primer sequence is: GACTATCATATGCTTACCGT. The sgRNA primer rev is the reverse primer designed in step 7 of "Before You Begin". Use the following reaction:

Reagent	Volume (μL)
100 μM U6 primer for	1
100 μM sgRNA primer rev	1
10x dNTPs	10
5x HF buffer	20
Water	53
DMSO	3
Phusion	2
Total Volume	**90 μL**

1. Mix well, then add 9 μL to 8 PCR tubes.
2. Add 1 μL of miniprep DNA to each tube.
3. Use the following thermocycler protocol:

Step	Cycle	Temperature (°C)	Time	Repeat
1	Initial denaturation	98	30 s	
2	Denaturation	98	10 s	×34
3	Anneal	66	20 s	
4	Elongation	72	10 s	
5	Final Extension	72	5 min	
6	Hold	12	Forever	

4. Run on a 2% agarose gel. Positive colonies will have 100 bp product. Negative colonies will have no product.

Problem 2

Low numbers (<500 GFP+) of nuclei during FACS (steps 11a–11e).

Potential Solution

This was most likely due to insufficient homogenization (steps 10a–10e) caused by too much tissue per sample. To avoid this, after punching the tissue simply split the tissue into multiple reactions (step 10d) and proceed as detailed above. Samples may be recombined following resuspension in step 10i.

Problem 3

PCR 2 resulted in multiple bands (steps 13i–13j).

Potential Solution

This result could occur for multiple reasons. One, the primers used for PCR amplification could bind to genomic repeat regions. To determine this, display repeat as lowercase letters when obtaining the genomic sequence from the UCSC genome browser. If possible, design the primers upstream or downstream of these regions to avoid them.

If the primers are not in repeat regions, non-specific off-target amplification is occurring. To diminish non-specific amplification, increase the annealing temperature 1°C–2°C.

Problem 4
No bands in PCR 2 (steps 13i–13j).

Potential Solution
The PCR conditions are too stringent. Lower the annealing temperature 1°C–2°C during both PCR 1 (step 13c) and PCR 2 (step 13g) until the proper band is visible.

Problem 5
The band in PCR 2 is the wrong size (steps 13i–13j).

Potential Solution
This is most likely due to a G-C rich region in the amplicon, which can form hairpins during PCR. These hairpins get skipped over by the polymerase, thereby effectively deleting portions of DNA from the amplicon. To avoid this, first determine the location of the G-C rich regions in the genome (>85% G-Cs) and try to design primers to exclude the region (either downstream or upstream of the G-Cs). If the G-C rich region is too close to the sgRNA cut site (<100 bp), design primers that result in a shorter amplicon (~200 bp; steps 8 and 9 in Part Two of "Before You Begin"), increase the annealing temperature (up to 69°C) and use the 5x GC buffer (supplied in the Phusion high-fidelity DNA polymerase kit) in place of the 5x HF buffer during PCR 1 (step 13a) and PCR 2 (step 13e).

RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Larry Zweifel (larryz@uw.edu).

Materials Availability
Plasmids generated in this study have been deposited to Addgene, [124844, 124845, 154373, and 154374].

Data and Code Availability
This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS
A.C.H. and L.S.Z. conceptualized the study, designed experiments, analyzed data, and wrote the paper. A.C.H. performed all plasmid and AAV synthesis, FACS, and sequencing analysis.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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