Protocol

Adeno-Associated Virus–Mediated Delivery of CRISPR–Cas Systems for Genome Engineering in Mammalian Cells

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The CRISPR–Cas9 system has emerged as a highly versatile platform for introducing targeted genome modifications into mammalian cells and model organisms. However, fully capitalizing on the therapeutic potential for this system requires its safe and efficient delivery into relevant cell types. Adeno-associated virus (AAV) vectors are a clinically promising class of engineered gene-delivery vehicles capable of safely infecting a broad range of dividing and nondividing cell types, while also serving as a highly effective donor template for homology-directed repair. Together, CRISPR–Cas9 and AAV technologies have the potential to accelerate both basic research and clinical applications of genome engineering. Here, we present a step-by-step protocol for AAV-mediated delivery of CRISPR–Cas systems into mammalian cells. Procedures are given for the preparation of high-titer virus capable of achieving a diverse range of genetic modifications, including gene knockout and integration.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

AAV (adeno-associated virus) lysis buffer <R>
AflII, BsmBI (New England Biolabs R0580S), and KpnI restriction endonucleases
Agarose gels
Ampicillin
Antibiotic-antimycotic (Thermo Fisher Scientific 15240096)
Benzonase nuclease (Sigma-Aldrich E8263)
DMSO
DNase I (Roche 04716728001)
DNase dilution buffer (10×) <R>
Dry-ice–ethanol bath

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Dulbecco's modified Eagle's medium (DMEM)
Escherichia coli TOP10 cells (Thermo Fisher Scientific C4040-03)
Expand High Fidelity PCR System (Roche 11759078001)
Fetal bovine serum (FBS)
Gel extraction kit
Gel-loading dye (10×) <R>
Human embryonic kidney (HEK) 293 T cells (ATCC CRL-1573)
iCycler mix (2×) <R>

This is needed to make the qPCR master mix.

Iodixanol solution (54%) <R>
Iodixanol solutions (15%, 24%, and 40%) <R>
LB solid or liquid medium <R>
Linear polyethylenimine (PEI) (MW 25,000; Polysciences)
$MgCl_2$ (0.15 M)
Oligonucleotides (see Steps 4, 14, 76, and 78)
pAAV–Cas9–sgRNA plasmid

This is available on request to the authors.

PBS (10×) +/− Tween 20 <R>

Include the appropriate concentration of Tween 20 and dilute if necessary. This protocol requires 1× PBS, 1× PBS containing 0.001% Tween 20, and 1× PBS containing 5% Tween 20.

PBS-MK (10×) <R>

Include the appropriate concentration of NaCl and dilute if necessary. This protocol requires 10× PBS-MK containing 1.37 M NaCl, 1× PBS-MK containing 0.137 M NaCl, and 1× PBS-MK containing 2 M NaCl.

pHelper plasmid (available upon request from the authors) (or plasmid that contains adenovirus helper genes)
Polyacrylamide gels
Proteinase K (New England Biolabs P8107S)
Proteinase K incubation buffer (2×) <R>
pXX2 (Cell Biolabs VPK-422) (or plasmid that contains the desired AAV *rep* and *cap* genes)
qPCR master mix <R>
QuickExtract DNA Extraction Solution (Epicentre QE09050)
SURVEYOR Mutation Detection Kit, containing Enhancer, Nuclease, and Stop Solution (Integrated DNA Technologies 706021)
SYBR Safe (Thermo Fisher Scientific S33102)
T4 DNA ligase with buffer (New England Biolabs M0202L)
T4 polynucleotide kinase (New England Biolabs M0201L)
Terrific broth (TB) medium <R>
TBE electrophoresis buffer (10×) <R>

Use diluted in distilled water at 1× strength.

Trypsin-EDTA (Thermo-Fisher Scientific 25300054)

Equipment

96-well flat-bottom tissue-culture plate
Access to DNA sequencing facilities
Benchtop centrifuge
Cell-culture plates (15-cm)
Cell scraper
Conical tubes (sterile, 15- and 50-mL)
Freezer set at −20°C

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Gel imaging system
Heat block with adjustable temperature
Incubator at 37°C with 5% CO_2
Long blunt-ended cannulas
Microcentrifuge tubes (sterile, 1.7-mL)
Online DNA sequence and analysis tools (see Steps 1–3)
OptiSeal polyallomer centrifuge tubes (4.9-mL capacity; Beckman Coulter 362185)
Plasmid midi or maxiprep kit

An alternative is to use polyethylene glycol (PEG) precipitation (see Step 18).

Plasmid miniprep kit
Polyacrylamide gel electrophoresis (PAGE) apparatus
Preparative ultracentrifuge with fixed-angle rotor
Regular-bevel needle (21-gauge, 1½″)
Ring stand and clamp
Shaking incubator at 37°C
Syringes (sterile, 1- and 3-mL)
Thermocycler
Ultra-15 Centrifugal Filter Units (MWCO 100-kDa; Amicon UFC910024)
Vortex mixer
Water baths set to 37°C and 42°C

METHOD

Cloning

1. Retrieve the DNA sequence of the targeted gene using a reference genome database (e.g., http://www.ncbi.nlm.nih.gov/genome/).
2. Search for potential Cas9 cleavage sites using an online CRISPR design tool (Cradick et al. 2014; Montague et al. 2014) or DNA sequence viewing software.
3. For the *Streptococcus pyogenes* (SpCas9) protein, search the gene sequence for the motif 5′-$G(N)_{19}$-NGG-3′, where 5′-NGG-3′ is the protospacer-adjacent motif (PAM) recognized by SpCas9 (Jinek et al. 2012; Cong et al. 2013; Mali et al. 2013). Alternatively, for the *Staphylococcus aureus* (SaCas9) protein (Ran et al. 2015), search the gene sequence for the motif 5′-$G(N)_{21–24}$-NNGRRT-3′ (where R is A or G).

   A "G" nucleotide is recommended at the 5′ end of the single guide RNA (sgRNA) transcript for efficient expression from the human U6 promoter.

4. Design and order custom sense and antisense oligonucleotides encoding the selected sgRNA protospacer sequences, as shown in Figure 1A.
5. Phosphorylate 1 μM of each oligonucleotide with 5 units (U) of T4 polynucleotide kinase in recommended buffer in a total volume of 20 μL for 30 min at 37°C.
6. Anneal oligonucleotides by incubation for 5 min at 95°C, followed by fast cooling on ice for 5 min.
7. Digest pAAV–Cas9–sgRNA (empty) with BsmBI in recommended buffer for 3 h using 10 U of enzyme per microgram of DNA. Visualize DNA by agarose gel electrophoresis using a 1.2% agarose gel and fluorescent intercalating dye, such as SYBR Safe.

   All AAV vectors and sequences used here are available from the authors on request.

8. Purify the linearized pAAV–Cas9–sgRNA (empty) using a gel extraction kit, according to the manufacturer's instructions.

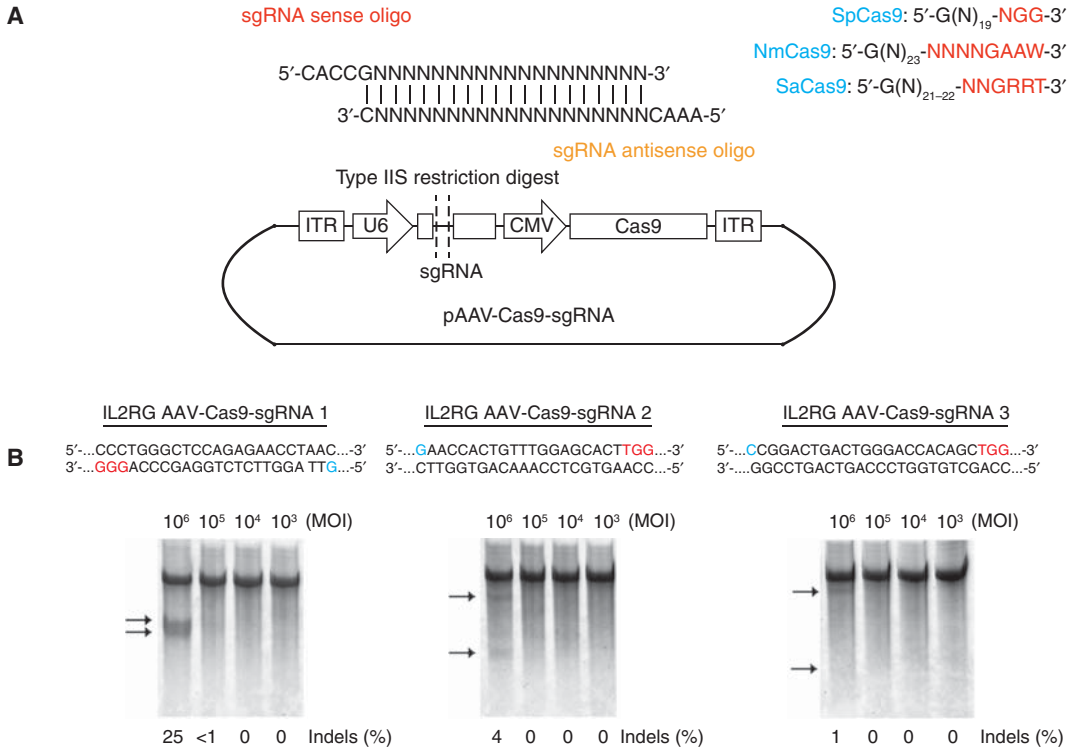

FIGURE 1. Adeno-associated virus (AAV)-mediated delivery of CRISPR–Cas9 for genome editing in mammalian cells. (*A*) Vector preparation. *Streptococcus pyogenes* (Sp), *Neisseria meningitidis* (Nm), and *Staphylococcus aureus* (Sa) Cas9 target sites and sense and antisense oligonucleotides for constructing sgRNA. Sense and antisense sgRNA oligonucleotides encode 5′-CACC-3′ and 5′-AAAC-3′ overhangs, respectively, for insertion into pAAV–Cas9–sgRNA. AAV vectors encoding SpCas9 and NmCas9 should be digested with BsbI, whereas AAV vectors encoding SaCas9 should be digested with BsaI. (*B*) Frequency of endogenous interleukin-2 receptor γ chain (*IL2RG*) gene modification in HEK293 T cells infected with AAV–Cas9–sgRNA of increasing MOI with three different sgRNAs (1–3), as determined by a SURVEYOR nuclease assay. Arrows indicate the position of the expected cleavage product. The protospacer-adjacent motif (PAM) and "G" initiation nucleotide are colored red and blue, respectively. Cas9, CRISPR-associated protein 9; CMV, cytomegalovirus promoter; CRISPR, clustered regularly interspaced short palindromic repeat; indel, insertion or deletion; ITR, inverted terminal repeat; MOI, multiplicity of infection; sgRNA, single guide RNA.

9. Ligate the sgRNA duplex DNA into 20–50 ng of linearized pAAV–Cas9–sgRNA (empty) using 1 U of T4 DNA ligase for 1 h at room temperature.

   A 6:1 molar insert:vector ratio is recommended for ligation.

10. Thaw 100 µL of chemically competent *E. coli* TOP10 cells on ice and mix gently with ligated pAAV–Cas9–sgRNA.

11. Keep the cells on ice for 30 min. Heat-shock the mixture for 35 sec at 42°C and recover the cells in 1 mL of lysogeny broth (LB) for at least 30 min at 37°C with shaking.

12. Spread 50–100 µL of bacterial cell culture on a LB agar plate with 100 µg/mL ampicillin and incubate overnight at 37°C.

13. The following day, inoculate 2–4 mL of terrific broth (TB) medium containing 100 µg/mL ampicillin with one colony from the LB agar plate and culture overnight at 37°C with shaking.

14. Purify pAAV–Cas9–sgRNA by plasmid miniprep and confirm plasmid identity by DNA sequencing using the primer U6 Seq (5′-GACTGTAAACACAAAGATATTAGTAC-3′).

15. Test the ability of Cas9 to induce modifications at the genomic target site in mammalian cell culture by transient transfection of pAAV–Cas9–sgRNA using the procedures described in Steps 60–75.

    It is strongly recommended that this control be performed.

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16. Thaw 100 µL of chemically competent *E. coli* TOP10 cells on ice and mix gently with 100 ng of pAAV–Cas9–sgRNA plasmid. Transform as above (Steps 10–12).
17. The following day, inoculate 50–100 mL of TB medium containing 100 µg/mL ampicillin with one colony and grow overnight at 37°C with shaking.
18. Purify plasmid DNA by plasmid midiprep or maxiprep, according to the manufacturer's instructions, or by PEG precipitation.
19. Store plasmid at −20°C until transfection.

Adeno-Associated Virus Production

20. Maintain HEK293 T cells in DMEM containing 10% (v/v) FBS and 1% antibiotic-antimycotic at 37°C in a fully humidified atmosphere with 5% CO_2.
21. Seed HEK293 T cells onto a 15-cm plate at a density of $2.5–3 \times 10^7$ cells/plate.
22. At 24 h after seeding, or once cells are ~90% confluent, add 15 µg pAAV–Cas9–sgRNA, 15 µg pXX2, and 15 µg pHelper plasmids to 4 mL of cell-culture medium in a sterile 15-mL conical tube.
23. Add 135 µL PEI (1 µg/µL) and mix immediately by vortexing for 10 sec.

    The volume of PEI is based on a 3 to 1 ratio of PEI (µg) to total DNA (µg).
24. Incubate transfection solution for 10 min at room temperature.
25. Add transfection solution dropwise to cells.
26. (Optional) Change media 8–12 h posttransfection to reduce transfection-reagent-associated toxicity.
27. Harvest virus from cells 48–72 h posttransfection by manually dissociating cells from plate using a cell scraper and pipetting media and cells into 50-mL conical tubes.
28. Pellet cells by centrifugation at 1500*g* for 5 min at room temperature.
29. Remove media and resuspend cells in 2 mL of lysis buffer for each 15-cm plate.
30. Freeze–thaw cells three times using a dry-ice–ethanol bath and a 37°C water bath.

    The cell lysate can be stored at −20°C after the third freeze.
31. Incubate the cells with 10 U of benzonase per milliliter of cell lysate. Incubate the samples for 30 min at 37°C.
32. Centrifuge cell lysate at 10,000*g* for 10 min at room temperature.
33. Transfer supernatant to new tubes and store at 4°C until purification.

Iodixanol Density Gradient Centrifugation

34. Pipette 1.2 mL of 15% iodixanol solution into an OptiSeal polyallomer centrifuge tube.

    Ensure that each iodixanol solution contains the appropriate amount of 10× PBS-MK containing 1.37 M NaCl, 1× PBS-MK containing 0.137 M NaCl, or 1× PBS-MK containing 2 M NaCl.
35. Underlay the 15% iodixanol solution with 0.7 mL of 24% iodixanol solution containing phenol red using a long blunt-ended cannula attached to a 3-mL syringe.
36. Underlay the 24% iodixanol solution with 0.6 mL of 40% iodixanol solution.
37. Underlay the 40% iodixanol solution with 0.6 mL of 54% iodixanol solution containing phenol red.
38. Gently pipette 1.8 mL of crude lysate onto each gradient.
39. Weigh tubes to ensure that they are properly balanced. Use AAV lysis buffer to balance tubes as necessary and seal tubes using the caps provided.

40. Set preparative ultracentrifuge to slow acceleration and deceleration settings and centrifuge gradients at 174,000*g* for 2 h at 18°C.
41. Carefully remove centrifuge tubes from the rotor and secure the centrifuge tube in a clamp attached to a ring stand. Remove the cap.
42. Carefully puncture the tube at the interface between the 40% and 50% iodixanol solutions using a 21-gauge 1½″ regular-bevel needle attached to a 1-mL syringe.
43. Collect the bottom four-fifths of the 40% iodixanol solution (bevel up) and the top one-fifth of the 54% iodixanol solution (bevel down) (Zolotukhin et al. 1999).

    Contaminating proteins from the cell lysate will be present in a band at the interface between the 24% and 40% iodixanol layers. Do not collect the protein band.

44. Store collected fractions in a sterile 1.7-mL microcentrifuge tube or a 15-mL conical tube at 4°C until further purification.

Buffer Exchange and Concentration

45. Incubate Ultra-15 Centrifugal Filter Unit in 1× PBS containing 5% Tween 20 for 30 min at room temperature. After incubation, wash filter once with 1× PBS containing 0.001% Tween 20.
46. Dilute collected iodixanol fraction to 15 mL in 1× PBS containing 0.001% Tween 20 and add to Ultra-15 Centrifugal Filter Unit.
47. Centrifuge at 4000*g* for 30 min or until solution has been concentrated to <2 mL.
48. Add 15 mL of 1× PBS containing 0.001% Tween-20 and mix well.
49. Repeat Steps 47 and 48 three times or until all iodixanol has been eliminated and the viscosity of the solution is similar to that of 1× PBS containing 0.001% Tween 20.
50. Concentrate the virus to the desired volume and store at 4°C.

Viral Titering

51. Combine 1 µL of virus with 5 µL of 10× DNase dilution buffer, 0.5 µL of DNase I, and 43.5 µL of water. Incubate virus sample for 30 min at 37°C.
52. Incubate the sample for 10 min at 75°C to inactivate DNase I.
53. Add 60 µL of 2× proteinase K incubation buffer and 10 µL of proteinase K to virus sample and incubate for at least 1 h at 37°C.
54. Incubate for 20 min at 95°C to inactivate the proteinase K.
55. Create 10-fold serial dilutions of pAAV–SpCas9–sgRNA plasmid between 0.2 ng/µL and 0.02 pg/µL for generating a standard curve.
56. Prepare qPCR master mix containing iCycler mix.
57. Dilute virus sample 10-fold for qPCR.
58. Combine 15 µL of qPCR master mix with 5 µL of virus sample or linear plasmid for the standard curve and run qPCR using the following protocol.

1 cycle	5 min	95°C
40 cycles	30 sec	95°C
	30 sec	60°C
	20 sec	72°C

59. Plot threshold cycle (C_t) values for standards against the $\log_{10}$ of the starting plasmid copy number. Correlate the C_t value of the virus sample to the copy number of the standard from a corresponding C_t value.

    See Troubleshooting.

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Genome Modification

60. Seed HEK293 T cells (or the most relevant cell type) onto a 96-well flat-bottom tissue-culture plate at a density of 4×10^4 cells per well.
61. At 24 h after seeding, add AAV–SpCas9–sgRNA vector to cells at a genomic multiplicity of infection (MOI) of ~10^6.

    Vector can be diluted in serum-containing medium.
62. (Recommended) Vary the MOI five- to 10-fold from 10^6 to 10^2 to further assess vector activity.
63. At 72 h after infection, wash cells once with 1× PBS and isolate infected cells by trypsin–EDTA digestion.

    See Troubleshooting.
64. Use benchtop centrifuge to collect cells (1100*g* for 3 min).
65. Remove supernatant and resuspend cells by vigorous pipetting with 50 µL of QuickExtract DNA Extraction Solution.
66. Incubate samples for 15 min at 65°C, followed for 15 min at 98°C. Hold samples at 4°C or store indefinitely at −80°C.
67. Amplify the targeted genomic region by polymerase chain reaction (PCR) using the Expand High Fidelity PCR System. Perform a 50-µL PCR.

Template DNA	3 µL
Expand high fidelity buffer (10×) with $MgCl_2$	5 µL
Each primer	0.4 µM
High fidelity *Taq* DNA polymerase	0.5 µL
DMSO	5%
Water	to 50 µL

68. Verify amplification by agarose gel electrophoresis.
69. Denature and re-anneal the PCR amplicon to generate mismatched duplex DNA for the SURVEYOR nuclease assay using the following profile.

95°C	10 min
95°C–85°C	−2°C/sec
85°C–25°C	−0.1°C/sec
4°C	Hold

70. Mix 10 µL of heteroduplex DNA with 1 µL of 0.15 M $MgCl_2$, 1 µL of SURVEYOR Enhancer S, and 1 µL of SURVEYOR Nuclease S. Incubate the reaction for 1 h at 42°C.
71. Quench reaction with 1 µL of Stop solution and add 2 µL of 10× DNA gel loading dye to each sample.
72. Load the samples in a PAGE apparatus on a 10%–14% TBE acrylamide gel and run the gel at 140–180 V until the xylene cyanol band from the gel loading dye is located in the middle or bottom third of the gel.
73. Remove the gel and stain with 10 µL of SYBR Safe in 30 mL of 1× TBE electrophoresis buffer for 10 min. Wash the gel at least once with water.
74. Visualize the gel using a gel imaging system and measure the density or intensity of each band.
75. Determine the percentage gene modification by measuring the fraction of parental band cleaved at the anticipated location, as described previously (Guschin et al. 2010).

    Representative results are shown in Figure 1B. See Troubleshooting.

Gene Targeting

Nuclease-induced double-strand breaks can stimulate integration of donor DNA into an endogenous locus through homology-directed repair (HDR) (Rouet et al. 1994; Bibikova et al. 2001). AAV, in particular, can enhance gene targeting by >1000-fold compared with plasmid DNA (Russell and Hirata 1998; Jang et al. 2011; Asuri et al. 2012; Gaj et al. 2015).

76. To construct AAV vectors for gene targeting, design primers to PCR-amplify "left" and "right" homology arms that flank the intended modified sequence. (The 5′ [sense] primer for the "left" homology arm should encode an AflII restriction site, and the 3′ [antisense] primer for the "right" homology arm should encode a KpnI restriction site.) Situate the Cas9 cleavage site within 50 bp of each homology arm.

    Optimal homology arm length ranges from 0.5 to 1.5 kb.

77. Encode single-base modifications on the 3′ and 5′ ends of the antisense and sense primers of the "left" and "right" homology arms, respectively. Encode a silent restriction site on the donor template for downstream analysis.

78. For gene integration, design a second set of primers to amplify the gene of interest (GOI), with 20–30 nucleotides of sequence complementary to the 3′ and 5′ ends of the "left" and "right" homology arms, respectively. Ensure that the Cas9 cleavage site is not present in the donor construct.

    Because of the carrying capacity of AAV, the donor template should not exceed 4.7 kb.

79. PCR-amplify the homology arms from genomic DNA and the GOI from cDNA or plasmid DNA using the primers designed in Steps 76 and 78.

80. Gel-purify the homology-arm-encoding and GOI-encoding amplicons using a gel extraction kit, according to the manufacturer's instructions.

81. Fuse the amplicons together by overlap PCR or Gibson assembly (Gibson et al. 2009) to generate the donor template.

82. Gel-purify the donor template by gel extraction and digest both it and the AAV plasmid (e.g., AAV–Cas9–sgRNA) with AflII and KpnI restriction enzymes.

83. Ligate the donor template into 20–50 ng of digested AAV plasmid and transform into cells, as described in Steps 21–26.

84. Purify the AAV donor plasmid by miniprep and confirm plasmid identity by DNA sequencing.

85. Construct the accompanying AAV–Cas9–sgRNA vector, and package and purify both it and the AAV donor, as described in Steps 20–59.

86. Infect cells with purified AAV vectors, as described in Steps 60–62.

    Cells infected with AAV donor vector containing a selectable marker, such as a puromycin-resistance gene or enhanced green fluorescent protein (EGFP), can be subjected to antibiotic selection or harvested for fluorescence-activated cell sorting (FACS) at 72 h after infection. Limiting dilution is recommended for isolation and expansion of clonal cell lines.

87. Isolate genomic DNA, as described in Steps 63–66, and PCR-amplify the genomic target across the integration junctions using the Expand High Fidelity PCR System. If the donor template contained a silent restriction site, evaluate the integration frequency by restriction digestion analysis. Determine gene modification by measuring the fraction of the parental band cleaved at the anticipated location. Finally, use DNA sequencing to confirm the presence of gene modifications.

    See Troubleshooting.

TROUBLESHOOTING

Problem (Step 59): The calculated titer value is too low.

Solution: A low titer can arise for a number of reasons, including using an impure plasmid preparation, mutations within the AAV plasmid from modification of inverted terminal repeats, toxicity from PEI transfection, off-target cleavage within the AAV vector genome by Cas9, or the vector being released from cells. Possible solutions include using phenol–chloroform extraction of the

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AAV plasmid to improve vector purity, diagnostic restriction digestion of the AAV vector to establish vector integrity, transfection by calcium phosphate to eliminate the possibility of PEI-induced toxicity, and harvesting the cells 48 h after transfection.

Problem (Step 63): Infectivity is low.

Solution: Because of differences in primary receptor usage and capsid composition, many naturally occurring AAV vectors display differential infection abilities both in vitro and in vivo. Therefore, the use of AAV vectors with the intended cell or tissue tropism is essential for efficient gene delivery. Infection by AAV serotypes can be measured by using a fluorescent reporter gene, such as that encoding EGFP. Engineered or evolved AAV vectors with improved or altered tropism can also be used to enhance infection (Kotterman and Schaffer 2014).

Problem (Step 75): The efficiency of genome modification is low.

Solution: Poor genome modification could be due to a number of factors, including low levels of Cas9-mediated cleavage at the genomic target site, terminal truncations within the AAV vector genome, and low levels of Cas9 expression. Test the ability of the Cas9–sgRNA complex to induce modifications at the genomic target by transient transfection. Because of the limited carrying capacity of AAV, packaging a single vector containing both a large Cas9 variant (such as SpCas9) and sgRNA could lead to vectors with truncations at the 5′ end of the vector genome (Senis et al. 2014). Use Southern blot analysis to establish whether truncations are present. To minimize vector genome heterogeneity, SpCas9 and sgRNA can be delivered using two separate particles (Swiech et al. 2015). Smaller Cas9 orthologs, such as *Neisseria meningitidis* (NmCas9) (Hou et al. 2013) and SaCas9 (Ran et al. 2015), can also be used to induce genome modifications from a single AAV particle despite their more restrictive PAM requirements. Finally, confirm that the promoter is providing high levels of expression in the desired cell type by western blotting or by the use of a fluorescent reporter gene, such as one encoding EGFP.

Problem (Step 87): The efficiency of integration is low.

Solution: No integration could be the result of insufficient homology arm length, low levels of Cas9 activity, or poor infectivity. Test the ability of the Cas9–sgRNA complex to induce modifications at the genomic target by transient transfection. Use an alternative sgRNA if activity is low. In addition, test the ability of the AAV donor vector in combination with Cas9 to mediate HDR by transient transfection. Modify homology arm length in cases where the existing donor template does not trigger integration. Use of small molecules that inhibit nonhomologous end joining (NHEJ) can also enhance HDR (Chu et al. 2015; Wurst et al. 2015; Yu et al. 2015).

RECIPES

AAV Lysis Buffer

Reagent	Final concentration
Tris-HCl (pH 8.0)	50 mM
NaCl	150 mM

Sterilize using a disposable 0.22-µm vacuum filtration system in a tissue-culture hood and store at room temperature.

DNase Dilution Buffer (10×)

Reagent	Final concentration
Tris-HCl (pH 7.4)	250 mM
$MgCl_2$	100 mM

Sterilize using a disposable 0.22-µm vacuum filtration system in a tissue-culture hood and store at room temperature.

Gel-Loading Dye (10×)

Reagent	Final concentration
Glycerol	60%
EDTA	0.2 M
Bromophenol blue	0.5%
Xylene cyanol	0.5%

Adjust to pH 8.0. Store at room temperature.

iCycler Mix (2×)

Reagent	Volume/final concentration (for 1 mL)
PCR buffer (10×; 200 mM Tris-HCl, 500 mM KCl, pH 8.4)	200 µL
$MgCl_2$	25 mM
dNTPs	10 mM

Sterilize using a disposable 0.22-µm vacuum filtration system in a tissue-culture hood and store at room temperature.

Iodixanol Solution (54%)

Reagent	Volume to add
OptiPrep Density Gradient Medium (60% iodixanol; Sigma-Aldrich D1556)	40 mL
PBS-MK (10×, containing 1.37 M NaCl) <R>	4.44 mL

Sterilize the 54% iodixanol solution using a disposable 0.22-µm vacuum filtration system in a tissue-culture hood and store at room temperature. Add 60 µL of 0.5% phenol red to a 12-mL aliquot of the 54% iodixanol solution.

Iodixanol Solutions (15%, 24%, and 40%)

Reagent	Volume (for 15%)	Volume (for 24%)	Volume (for 40%)
Iodixanol solution (54%) <R>	4.72 mL	5.56 mL	7.41 mL
PBS-MK (1×, containing 2 M NaCl) <R>	8.50 mL	–	–
PBS-MK (1×, containing 0.137 M NaCl) <R>	3.78 mL	6.44 mL	2.59 mL
Phenol red (0.5%)	–	60 µL	–
Total	17 mL	~ 12 mL	10 mL

Sterilize each solution using a disposable 0.22-µm vacuum filtration system in a tissue-culture hood and store at room temperature.

LB Solid or Liquid Medium

10 g	Tryptone
5 g	Yeast extract
5 g	NaCl
20 g	Agar (for solid medium only; omit for liquid medium)

Combine the ingredients and bring to 1 L with ddH_2O. Autoclave and store at room temperature or 4°C.

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PBS (10×) +/− Tween 20

Reagent	Final concentration (10×)
Na_2HPO_4 (pH 7.4)	100 mM
KH_2PO_4	18 mM
NaCl	1.37 M
KCl	27 mM
Tween 20	as required

Sterilize 10× PBS without Tween 20 using a disposable 0.22-µm vacuum filtration system in a tissue-culture hood and store at room temperature. Dilute to 1× and/or add Tween 20 as needed: For 1× PBS containing 0.001% Tween 20, dilute 10× PBS 1:9 in distilled water to give a volume of 500 mL, and add 5 µL of Tween 20. For 1× PBS containing 5% Tween 20, dilute 10× PBS 1:9 in distilled water to give a volume of 475 mL, and then add 25 mL of Tween 20 for a final volume of 0.5 L.

PBS-MK (10×)

Reagent	Final concentration (10×)
Na_2HPO_4 (pH 7.4)	100 mM
KH_2PO_4	18 mM
NaCl	as appropriate
$MgCl_2$	10 mM
KCl	25 mM

Sterilize 10× PBS-MK containing the appropriate concentration of NaCl using a disposable 0.22-µm vacuum filtration system in a tissue-culture hood and store at room temperature. When required, dilute to 1× in distilled water.

Proteinase K Incubation Buffer (2×)

Reagent	Final concentration
Tris-HCl (pH 8.0)	10 mM
Na_2EDTA	20 mM
$NaCl_2$	20 mM

Sterilize using a disposable 0.22-µm vacuum filtration system in a tissue-culture hood and store at room temperature.

qPCR Master Mix

Reagent	Volume (for 150 µL)
iCycler mix (2×) <R>	100 µL
Sense primer (1 µM)	2 µL
Antisense primer (1 µM)	2 µL
Fluorescein (1 µM)	2 µL
SYBR Green (40×) (Thermo Fisher Scientific S-7563)	2 µL
Taq DNA polymerase:JumpStart *Taq* Antibody (1:1) (New England Biolabs M0267X)	2 µL
Distilled water	40 µL

Sterilize using a disposable 0.22-µm vacuum filtration system in a tissue-culture hood and store at room temperature.

TBE Electrophoresis Buffer (10×)

Reagent	Quantity (for 1 L)	Final concentration
Tris base	121.1 g	1 M
Boric acid	61.8 g	1 M
EDTA (disodium salt)	7.4 g	0.02 M

Prepare with RNase-free H_2O. Dilute 100 mL to 1 L to make gel running **buffer**. Store for up to 6 mo at room temperature.

Terrific Broth (TB) Medium

Reagent	Quantity	Final concentration
Yeast extract	24 g	24 g/L
Tryptone	20 g	20 g/L
Glycerol	4 mL	4 mL/L
Phosphate buffer	100 mL	0.017 M KH_2PO_4 and
(0.17 M KH_2PO_4, 0.72 M K_2HPO_4)		0.072 M K_2HPO_4

Add 900 mL of deionized water to 24 g of yeast extract, 20 g of tryptone, and 4 mL of glycerol. Shake or stir until the solutes have dissolved and sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2). Allow the solution to cool to ~60°C and add 100 mL of sterile phosphate buffer. Store TB at room temperature; it will keep for at least 1 yr.

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