Chapter 11

Delivering Base Editors In Vivo by Adeno-Associated Virus Vectors

Colin K. W. Lim, Angelo J. Miskalis, Pablo Perez-Pinera, and Thomas Gaj

Abstract

CRISPR base editors are genome-modifying proteins capable of creating single-base substitutions in DNA but without the requirement for a DNA double-strand break. Given their ability to precisely edit DNA, they hold tremendous therapeutic potential. Here, we describe procedures for delivering base editors in vivo via adeno-associated virus (AAV) vectors, a promising engineered gene delivery vehicle capable of transducing a range of cell types and tissues. We provide step by step protocols for (i) designing and validating base editing systems, (ii) packaging base editors into recombinant AAV vector particles, (iii) delivering AAV to the central nervous system via intrathecal injection, and (iv) quantifying base editing frequencies by next-generation sequencing.

Key words Base editing, AAV, CRISPR, Central nervous system, Genome editing, Intrathecal injections

1 Introduction

Genome editing technologies have revolutionized biomedical research by providing an efficient and flexible means for genetically modifying cells. Among the technologies used for DNA editing are CRISPR base editors, a class of genome-modifying proteins that are capable of inducing target base substitutions in cells but without the traditional requirement for a DNA double-strand break (DSB) [1–3]. Most broadly, base editors consist of fusions of a targetable Cas9 (Cas9) nickase (nCas9) protein [4–6] with a nucleobase deaminase enzyme that catalyzes the deamination of a specific base within a stretch of sequence specified by a single-guide RNA (sgRNA) [3, 7]. To date, the most widely used base editing systems are cytosine base editors (CBEs) [1], which can convert a cytosine base to a thymine, and adenosine base editors (ABEs) [2], which

The authors "Colin K. W. Lim" and "Angelo J. Miskalis" contributed equally to this work.

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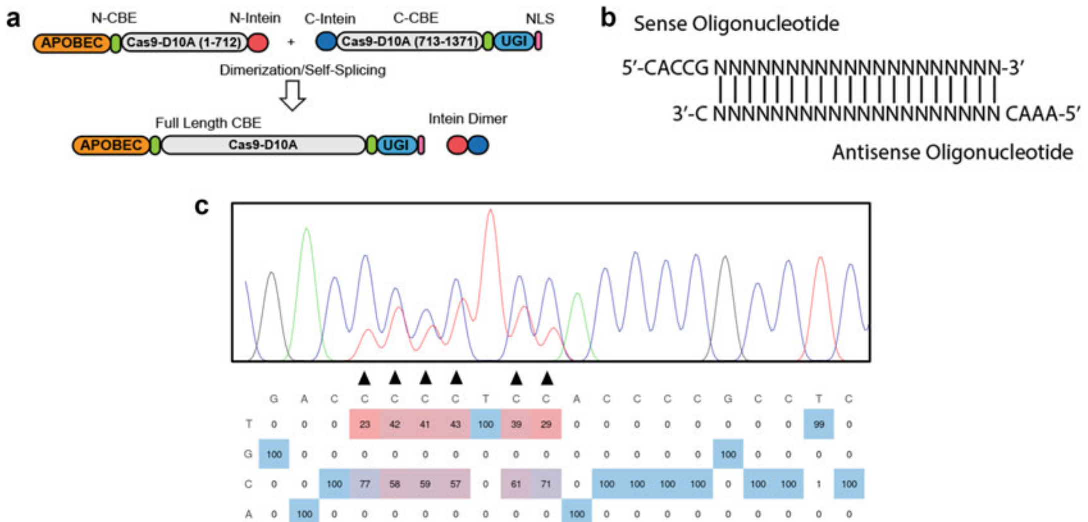
can transform an adenosine base to a guanosine. CBEs, specifically, rely on a cytidine deaminase catalytic domain to deaminate a target cytosine to create a uracil intermediate that is then converted to a thymine by the action of cellular replication and repair mechanisms [1, 8], while ABEs use an engineered TadA adenine deaminase domain to transform a target adenosine to inosine, a nucleobase that is ultimately resolved to guanosine through DNA repair [2, 3, 9].

Importantly, while traditional gene-editing modalities can also edit DNA with single-base resolution, they achieve this typically through homologous recombination, a DNA repair pathway that is largely available only in dividing cells [10–12]. Base editors, by contrast, employ DNA repair pathways that are sufficiently active in both dividing and non-dividing cells, thereby offering a means for introducing single-base edits in post-mitotic cells [13, 14]. Thus, due in part to the expanded range of precision editing outcomes available to them, base editors hold tremendous therapeutic potential [15]. However, capitalizing on these expanded therapeutic opportunities requires the availability of methods for safely and efficiently delivering base editors to cells. Chief among these are methods based on adeno-associated virus (AAV) vectors, a class of gene delivery vehicles with the ability to deliver transgene(s) to a range of cell types and tissues [16–19]. However, while promising, AAV vectors possess a limited capacity that restricts their ability to deliver a full-length base editor via a single vector particle. To overcome this limitation, our team [20, 21] and others [22, 23] have developed strategies for delivering base editors in vivo by two AAV vector particles using a process known as intein-mediated protein *trans*-splicing [24]. Our approach, in particular, relies on intein domains from the DnaB protein of *Rhodothermus marinus* [25], which we have shown can be inserted into a disordered linker connecting the α-helical recognition lobe and the nuclease lobe of the nCas9 protein to facilitate the reassembly of a full-length base editor protein from two expressed halves (Fig. 1a) [21]. This approach has enabled the delivery of an engineered CBE to the spinal cord, which we showed could be used to inactivate the expression of a mutant gene causative for amyotrophic lateral sclerosis [21]. Additionally, other teams have demonstrated the potential of this strategy by using it to deliver base editors to the liver [22, 23, 26, 27], retina [23, 28, 29], muscle [23, 30, 31], ear [32], and brain [23], which altogether highlights the potential of this approach for gene therapy.

Here, we describe procedures for delivering base editor proteins in vivo via dual AAV vector particles. We provide step-by-step protocols for (i) designing and validating base editing systems, (ii) packaging base editors into recombinant AAV vector particles, (iii) delivering AAV to the central nervous system via an intrathecal injection, and (iv) quantifying base editing frequencies by next-generation sequencing.



	G	A	C	C	C	C	C	T	C	C	A	C	C	C	C	G	C	C	T	C
T	0	0	0	23	42	41	43	100	39	29	0	0	0	0	0	0	0	0	99	0
G	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0
C	0	0	100	77	58	59	57	0	61	71	0	100	100	100	100	0	100	100	1	100
A	0	100	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0

Fig. 1 Designing and validating base editing systems. (**a**) Schematic of a split-intein base editor. When expressed, the two intein-containing halves participate in a *trans*-splicing reaction that reconstitutes a full-length base editor protein. (**b**) Design template for sense and antisense oligonucleotides for cloning sgRNAs into pAAV plasmids. 5′-CACC-3′ and 5′-AAAC-3′ overhangs are required for inserting the sgRNAs into the plasmids used in this protocol. (**c**) Sanger sequencing trace showing the editing frequencies of a CBE engineered to edit the *VEGF1A* gene in HEK293T cells, as determined by EditR. Arrowheads indicate the targeted cytosine bases

2 Materials

2.1 sgRNA Cloning

1. Custom synthesized oligonucleotides.
2. MAX Efficiency DH5α Competent Cells (ThermoFisher Scientific #18258012).
3. pAAV-CAG-N-BE-Int-U6-sgRNA and pAAV-CAG-C-BE-Int-U6-sgRNA plasmids (or equivalent pAAV plasmids encoding N- and C- split-intein base editors) [21].
4. BsaI restriction enzyme (New England Biolabs® (NEB) #R3733S).
5. SOC Outgrowth Medium.
6. 100 mg/mL ampicillin: Dissolve 1 g of ampicillin in 10 mL of deionized (DI) water, and filter sterilize using a 0.22 μm syringe filter.
7. LB medium: Dissolve 25 g of granulated LB broth in 1 L of deionized water. Sterilize by autoclaving.
8. 10 cm polystyrene petri dishes.
9. LB agar plates with 100 μg/mL ampicillin: Dissolve 40 g of LB Agar Dehydrated Culture Media in 1 L of deionized water using a magnetic stir bar. Sterilize by autoclaving. After autoclaving, and once the solution is warm to touch, add 1 mL of

100 mg/mL ampicillin, and mix for 1 min. Pour 25 mL of the resulting mixture per 10 cm polystyrene petri dish, and allow to cool. Flip the plates upside down after 2 h, and allow them to cool overnight at room temperature (RT). Store at 4 °C.

10. T4 Polynucleotide Kinase (NEB® #M0201S).
11. T4 DNA Ligase (NEB® #M0202S).
12. 10× T4 DNA Ligase Buffer (NEB® #B0202S).
13. Kapa 2G Robust PCR Kit (Roche #07960743001).
14. 20× Tris–acetate–EDTA (TAE) buffer: Dissolve 48.5 g Tris base in deionized water. Add 11.4 mL glacial acetic acid and 20 mL pH 8.0 0.5 M EDTA.
15. Agarose gel (2% w/v): Dissolve 2 g of agarose powder in 100 mL of 1× TAE buffer. Microwave until all the powder has dissolved and the solution is clear. Pour the solution into a gel cassette with a comb to cast the gel. Cover with saran wrap and store at 4 °C for up to a week. When running the gel, a 1× TAE solution can be used as the running buffer at a constant voltage of 180 V.
16. PureLink Quick Plasmid Miniprep Kit (ThermoFisher Scientific # K210010).
17. Thermocycler.
18. 37 °C shaking incubator.
19. Gel imaging system.
20. UV spectrophotometer.
21. Molecular Biology Grade Water (Corning #46-000-CM).
22. PCR microcentrifuge tubes.
23. U6-Fwd primer: 5′- GGGCCTATTTCCCATGATTCCT -3′. This primer will be used for Sanger sequencing.

2.2 Evaluating Base Editing in Mammalian Cells

1. Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate.
2. Opti-MEM I Reduced Serum Medium.
3. Lipofectamine 2000 Transfection Reagent (ThermoFisher Scientific #11668030).
4. PEI MAX (Polysciences #24765): Dissolve 100 mg of PEI MAX in 62.5 mL of sterile Molecular Biology Grade Water. Adjust pH to 7.0.
5. Fetal Bovine Serum (FBS).
6. Penicillin/Streptomycin.
7. Trypsin–EDTA (0.25%; ThermoFisher Scientific #25200056).
8. Human embryonic kidney (HEK) 293T cells.
9. 10 cm cell culture dishes.

10. 24-well flat-bottom cell culture plates.
11. Cell scrapers.
12. pAAV-GFP (Addgene Plasmid #32395).
13. Mammalian cell culture incubator.
14. Benchtop Centrifuge 5425 (Eppendorf #5405000107) or equivalent.
15. Fluorescence microscope.
16. DNeasy Blood and Tissue Kit (QIAGEN #69504).
17. Zymo DNA Clean & Concentrator (Zymo Research #D4003).

2.3 AAV Vector Packaging

1. pAdDeltaF6 plasmid (Addgene Plasmid #112867).
2. pAAV2/9 plasmid (Addgene Plasmid #112865) or an alternate rep/cap-expressing AAV packaging plasmid.
3. Beckman Coulter Optima LE-80K ultracentrifuge or equivalent.
4. VTi 65.2 Vertical-Tube Rotor with torque wrench (Beckman Coulter #362754) or equivalent.
5. VTi 65.2 Vertical-Tube Rotor Spacers (Beckman Coulter #362198) or appropriate spacers for an equivalent rotor.
6. VTi 65.2 Vertical-Tube Rotor Plugs (Beckman Coulter #368545) or appropriate plugs for an equivalent rotor.
7. Benchtop Centrifuge 5425 (Eppendorf #5405000107) or equivalent.
8. Retort stand and clamp.
9. Luer Slip Tip 1 mL syringe.
10. 4.6 mL OptiSeal Polypropylene Tubes (Beckman Coulter #362185).
11. Amicon Ultra-15 Centrifugal Filter Units (MWCO 100 kDa; Amicon #UFC910024).
12. Cell scrapers.
13. Benzonase Nuclease.
14. Tween-20.
15. 10× PBS (0.1 M): Dissolve 80 g of NaCl, 2 g of KCl, 14.4 g of Na_2HPO_4, and 2.4 g of KH_2PO_4 in 1 L of DI water. Adjust pH to 7.4 using HCl. Sterilize with a disposable 0.22 μm vacuum filtration system. Store at room temperature (RT) for up to a year.
16. 10× PBS-MK: Dissolve 2 g of $MgCl_2$–$6H_2O$ and 1.9 g KCl in 1 L of 10× PBS. Sterilize using a disposable 0.22 μm vacuum filtration system. Store at RT for up to a year.
17. 1× PBS-MK w/ NaCl: Dissolve 58.4 g of NaCl in 50 mL of 10× PBS-MK. Adjust volume to 500 mL with DI water.

18. 1× PBS w/ Tween-20 (5%). Combine 10 mL of 10× PBS with 85 mL of DI water and 5 mL of Tween-20. Mix well. Make the solution fresh before use.
19. 1× PBS w/ Tween-20 (0.001%). Combine 50 mL of 10× PBS with 450 mL of DI water and 5 μL of Tween-20. Mix well. Make the solution fresh before use.
20. AAV lysis buffer: Dissolve 3.03 g of Tris base and 4.38 g of NaCl in 500 mL of DI water. Adjust pH to 8.2. Sterilize using a disposable 0.22 μm vacuum filtration system. Store at RT for up to a year.
21. Liquid nitrogen.
22. OptiPrep Density Gradient Medium (60% w/v) (Sigma-Aldrich #D1556).
23. Phenol red solution (0.5% w/v): Dissolve 50 mg of phenol red sodium salt in 10 mL of DI water. Sterilize using a disposable 0.22 μm vacuum filtration system. Store at RT for up to a year.

2.4 AAV Vector Titering

1. Dry block incubator.
2. Real-time PCR system.
3. 10× DNase Buffer: Dissolve 3.03 g of Tris base and 2 g of $MgCl_2$–$6H_2O$ in 100 mL of DI water. Adjust pH to 7.4. Sterilize using a disposable 0.22 μm vacuum filtration system. Store at RT for up to a year.
4. 2× Proteinase K Buffer: Dissolve 0.81 g of Tris base, 0.74 g of Na_2-EDTA-$2H_2O$, and 0.12 g of NaCl in 100 mL of DI water. Adjust pH to 8. Sterilize using a disposable 0.22 μm vacuum filtration system. Store at RT for up to a year.
5. DNase I.
6. Proteinase K.
7. iTaq Universal SYBR Green Supermix (Bio-Rad #1725120).
8. BGH-Fwd primer: 5′-GCCTTCTAGTTGCCAGCCAT-3′.
9. BGH-Rev primer: 5′-GGCACCTTCCAGGGTCAAG-3′.
10. SilverXpress Silver Staining Kit (ThermoFisher Scientific #LC6100).

2.5 Intrathecally Delivering AAV Vector to Mice for In Vivo DNA Editing

1. Isoflurane.
2. Isoflurane vaporizer.
3. Airtight container with inlet and outlet.
4. 25 μL Hamilton gas tight syringe with 28 gauge, 1 inch, point style 2 needle.
5. Mouse Nosecone.
6. Mouse Dissecting Kit (World Precision Instruments #MOUSEKIT).

7. 1× PBS.
8. Luer-Lok sterile 30 mL syringe.
9. Paraformaldehyde (PFA; 4% w/v).
10. Ketamine (100 mg/mL).
11. Xylazine (20 mg/mL).

2.6 Measuring Base Editor Expression and DNA Editing

1. Cryostat microtome.
2. Fluorescence microscope.
3. Sucrose solution (30% w/v).
4. 2 mL cryogenic vials.
5. Scigen Tissue-Plus O.C.T. Compound (Fisher Scientific #23-730-571).
6. Peel-A-Way Disposable Embedding Molds (Epredia #1220).
7. Cryoprotectant solution: Combine 125 mL of ethylene glycol and 125 mL glycerol with 250 mL of 10× PBS. Mix well. Store at RT for up to a year.
8. 12-well cell culture plates.
9. Sable Brush.
10. Netwell inserts (74 μm Mesh Size).
11. Normal Donkey Serum.
12. Triton X-100.
13. Rabbit anti-HA (Cell Signaling Technology #3724S).
14. Chicken anti-GFAP (Abcam #ab4674).
15. Alexa Fluor 647 AffiniPure Donkey Anti-Chicken IgY (Jackson ImmunoResearch #703-605-155).
16. Alexa Fluor 488 AffiniPure Fab Fragment Donkey Anti-Rabbit IgG (Jackson ImmunoResearch #711-547-003).
17. Microscope slides.
18. Slide coverslips.
19. VectaShield Hardset Antifade Mounting Medium (Vector Laboratories #H-1400-10).
20. Pellet Pestles (FisherBrand #121-141-367) or equivalent.
21. KAPA HIFI PCR Kit (Roche #07958854001).
22. Nextera XT DNA Library Prep Kit (Illumina #FC-131-1096).
23. Illumina Next Generation Sequencing (NGS) instrument compatible with Nextera XT kit.
24. AMPure XP beads (Beckman #A63880).
25. 80% Ethanol: Mix 20 mL Molecular Biology Grade Water with 80 mL 200 proof ethanol.

26. 10 mM Tris pH 8.5 buffer: Add 26.87 mg Tris–HCl and 39.91 mg Tris–base to 50 mL Molecular Biology Grade Water. Adjust pH accordingly.
27. Magnetic stand.
28. Qubit Flex Fluorometer (ThermoFisher Scientific #Q33327).
29. Qubit Assay Kit (ThermoFisher Scientific #Q33230).

3 Methods

3.1 Constructing a Base Editing System

3.1.1 Designing and Cloning the sgRNA

1. Identify candidate target sites within a gene of interest (GOI) using Benchling or an equivalent CRISPR design resource [33, 34]. CBEs and ABEs typically convert bases within a 3–5 bp window 15 bps upstream the protospacer adjacent motif (PAM).
2. Design oligonucleotides encoding the sgRNA protospacer sequence with appropriate overhangs for cloning into the plasmids pAAV-CAG-N-BE-Int-U6-sgRNA and pAAV-CAG-C-BE-Int-U6-sgRNA (Fig. 1b).
3. Combine 1 μM of each oligonucleotide in a PCR microcentrifuge tube, and incubate in a thermocycler using the following cycle to anneal the oligonucleotides: 95 °C for 5 min; 10 °C for 5 min (*see* **Note 1**).
4. Mix the annealed oligonucleotides with the pAAV plasmid (pAAV-CAG-N-BE-Int-U6-sgRNA or pAAV-CAG-C-BE-Int-U6-sgRNA) in a PCR microcentrifuge tube with the following components (*see* **Note 2**):

pAAV plasmid	170 ng
Annealed oligonucleotide mixture	1 μL
T4 PNK	10 U
T4 DNA ligase	400 U
BsaI	20 U
10× T4 DNA ligase buffer	2 μL
Molecular biology grade water	To 20 μL

5. Incubate the one-step digestion-and-ligation reaction in a thermocycler using the settings in Table 1.
6. Thaw MAX Efficiency DH5α Competent Cells on ice. Mix 1 μL of the reaction mixture from **step 5** with 10 μL of the thawed cells (*see* **Note 3**).
7. Incubate the transformation mixture on ice for 30 min, and then incubate at 42 °C for 45 s. Transfer the cells to a culture

Table 1
Thermocycler protocol for the one-step digestion-and-ligation reaction

Step	Temperature	Time	No. of cycles
Initial digestion	37 °C	20 min	1
Digestion Ligation	37 °C 16 °C	10 min 10 min	4
Final digestion	37 °C	30 min	1
Final ligation	16 °C	4 h	1

tube with 45 μL of pre-warmed SOC medium, and incubate the cells with shaking at 37 °C for 45 min.

8. Spread 10–50 μL of the mixture from **step 7** on an LB agar plate containing 100 μg/mL ampicillin. Incubate overnight at 37 °C.
9. The following day, inoculate 5 mL of LB media containing 100 μg/mL ampicillin with one colony from the LB agar plate from **step 8**. Culture overnight at 37 °C with shaking.
10. The following day, purify plasmid DNA from the inoculated culture from **step 9** using the PureLink Quick Plasmid Miniprep Kit according to the manufacturer's instructions.
11. Quantify plasmid concentration by a UV spectrophotometer (*see* **Note 4**). Plasmid can be stored indefinitely at −20 °C.
12. Confirm the insertion and sequence identity of the sgRNA by Sanger sequencing with the U6-Fwd primer.

3.2 Evaluating Base Editing in Mammalian Cells

3.2.1 Transfection

1. Seed 2×10^5 HEK293T cells per well onto a 24-well cell culture plate in 0.5 mL of DMEM with 10% (v/v) FBS and 1% penicillin/streptomycin. Incubate overnight at 37 °C with 5% CO_2.
2. The following day, mix 500 ng of the pAAV-CAG-N-BE-Int-U6-sgRNA plasmid with 500 ng of the pAAV-CAG-C-BE-Int-U6-sgRNA plasmid (both should carry the target sgRNA from Subheading 3.1.1, **step 12**) in 50 μL of Opti-MEM I Reduced Serum Medium. Incubate at RT for 5 min. Separately, add 1 μg of pAAV-GFP plasmid with 50 μL of Opti-MEM I Reduced Serum Medium for a separate transfection. GFP fluorescence can be used as a visual indicator to confirm transfection.
3. Add 4 μL of Lipofectamine 2000 Transfection Reagent to 96 μL of Opti-MEM I Reduced Serum Medium. Add 50 μL of the resulting mixture to the each of the Opti-MEM I Reduced Serum Medium mixtures from **step 2**.
4. Incubate at RT for 20 min.

5. Gently add the 100 μL transfection mixture from **step 4** drop-wise to a well of seeded HEK293T cells from **step 1.** Gently sway the plate to facilitate mixing.
6. Incubate the transfected cells in a cell culture incubator for 72–120 h. Transfection efficiency can be determined by measuring GFP fluorescence in cells transfected with pAAV-GFP (*see* **Note 5**).

3.2.2 Analyzing Base Editing Outcomes

1. Aspirate media and wash cells by adding 0.25 mL of 1× PBS to each well.
2. Aspirate 1× PBS and add 0.25 mL of trypsin-EDTA to each well to release cells. Incubate the cells in a cell culture incubator for 5 min.
3. Neutralize the trypsin–EDTA by adding 0.25 mL of DMEM with 10% (v/v) FBS and 1% penicillin/streptomycin to the well.
4. Using a micropipette, transfer the cell suspension to a 1.5 mL microcentrifuge tube. Centrifuge the cells at 500*g* for 5 min.
5. Gently aspirate the supernatant. If analysis needs to occur at a later time point, cell pellets can be frozen at −20 °C for DNA extraction.
6. Purify the genomic DNA from the cells harvested from **step 5** using the DNeasy Blood and Tissue Kit, per the manufacturer's instructions.
7. Amplify the region of genomic DNA containing the sgRNA target site by the PCR reaction conditions below and the thermocycler protocol from Table 2.

Genomic DNA	2 μL
5× KAPA enhancer 1	5 μL
KAPA 2G buffer A	5 μL
10 μM forward primer	1.25 μL
10 μM reverse primer	1.25 μL
10 mM dNTP mix	0.5 μL
KAPA 2G robust polymerase	0.1 μL
Molecular biology grade water:	To 25 μL

8. Purify the PCR product using a Zymo DNA Clean and Concentrator or an equivalent PCR purification kit.
9. Sequence the PCR product by Sanger sequencing. Use EditR or an equivalent software as shown in Fig. 1c to quantify the frequency of editing at the target base [35].

Table 2
Thermocycler protocol for amplifying genomic DNA

Step	Temperature	Length	No. of cycles
Initial denaturation	95 °C	3 min	1
Denaturation Annealing Extension	95 °C 65 °C 72 °C	20 s 15 s 15 s/kb	35
Final extension	72 °C	1 min/kb	1

3.3 AAV Packaging

3.3.1 Transfection

1. Seed 2.5 × 10^7 HEK293T cells onto a 15 cm cell culture dish in 25 mL of DMEM with 10% (v/v) FBS and 1% penicillin/streptomycin. Incubate overnight in a cell culture incubator.
2. At 16 h after seeding, or when cells are 80–90% confluent, add 15 μg of pAAV-CAG-N-BE-Int-U6-sgRNA or pAAV-CAG-C-BE-Int-U6-sgRNA with 15 μg of pAdDeltaF6 and 15 μg pAAV2/9 in a 1.5 mL microcentrifuge tube with 750 μL of serum- and antibiotic-free DMEM. These volumes are for one 15 cm cell culture dish. Scale as necessary and use a 15 mL conical tube for volumes exceeding 1.5 mL.
3. Mix 60 μL of PEI-MAX with 690 μL of serum- and antibiotic-free DMEM. Combine the resulting 750 μL mixture with the plasmid-containing solution from **step 2**. Vortex to mix. Incubate at RT for 10 min.
4. Gently add the solution dropwise across the entire 15 cm dish. Gently swirl to further distribute the mixture. Incubate the dish at 37 °C.

3.3.2 Harvesting

1. At 72 h after transfection, aspirate the culture media from the 15 cm dish, and add 5 mL of 1× PBS to each plate. Harvest the cells by manual dissociation using a cell scrapper. Transfer the dissociated cells to a 50 mL conical tube.
2. Centrifuge the suspension at 1500*g* for 5 min at RT.
3. Discard the supernatant and resuspend the resulting cell pellet in AAV lysis buffer (use 1.6 mL of lysis buffer per 15 cm dish).
4. Freeze-thaw the cell suspension by submerging the 50 mL conical tube in liquid nitrogen (recommended time, 10 min). Transfer the frozen samples to a 37 °C water bath until the contents are thawed. Repeat twice. Frozen samples can be stored at −80 °C for up to 1 month.
5. Add Benzonase enzyme to cells (10 Units per 1 mL of lysate), and incubate at 37 °C for 30 min.
6. Transfer the solution to 1.5 mL tubes and centrifuge at 18,500*g* for 30 min at RT. Following centrifugation, transfer the supernatant to a 50 mL tube. The solution can be stored at 4 °C for up to 1 day.

3.3.3 Iodixanol Gradient Purification

1. Prepare the iodixanol solutions as described in Table 3.
2. Pipette 1.2 mL of the 15% iodixanol solution into a 4.6 mL OptiSeal Polypropylene Tube.
3. Gently underlay the 15% iodixanol solution with 0.7 mL of the 25% iodixanol solution using a 1 mL syringe with a blunt-ended canula.
4. Gently underlay the 25% iodixanol solution with 0.6 mL of the 40% iodixanol solution.
5. Finally, gently underlay the 40% iodixanol solution with 0.6 mL of the 54% iodixanol solution.
6. Gently pipette the lysate from Subheading 3.3.2, **step 6** onto the top of the gradient. The lysate should reach the halfway point of the "neck" of the tube (~1.6 mL of lysate per OptiSeal Polypropylene Tube).
7. Weigh the tubes to ensure they are balanced (±0.1 g) for ultracentrifugation. Add additional AAV lysis buffer for balancing, if necessary. Seal the tubes securely (*see* **Note 6**).
8. Carefully place each tube into the VTi 65.2 Vertical-Tube Rotor, and cover each tube with VTi 65.2 Vertical-Tube Rotor Spacers. Then, place VTi 65.2 Vertical-Tube Rotor Plugs on top of the spacers, and screw each plug tightly by exerting at least 120 Newtons of torque using an appropriate torque wrench.
9. Place the filled VTi 65.2 Vertical-Tube Rotor into the Beckman Coulter Optima LE-80K ultracentrifuge, and centrifuge the gradients at 174,000*g* for 2 h at 18 °C using slow acceleration and slow deceleration settings.

Table 3
Iodixanol solutions

	Volume			
Buffers and reagents	**54% iodixanol**	**40% iodixanol**	**25% iodixanol**	**15% iodixanol**
60% iodixanol density gradient medium	21.60 mL	–	–	–
54% iodixanol without phenol red	–	14.82 mL	11.12 mL	9.44 mL
10× PBS-MK	2.40 mL	–	–	–
1× PBS-MK	–	5.18 mL	12.88 mL	7.56 mL
1× PBS-MK with NaCl	–	–	–	17.00 mL
0.5% phenol red solution	120 μL	–	120 μL	–

Note: The 54% iodixanol solution is prepared in the absence of phenol red, as it is used to create the 40%, 25%, and 15% iodixanol solutions

10. Gently remove the tubes from the ultracentrifuge rotor.
11. Firmly secure the OptiSeal Polypropylene Tube to a retort stand. Securely clamp and remove the cap.
12. Using a 38.1 mm, regular beveled, 21-gauge needle attached to a Luer Slip Tip 1 mL syringe, puncture the OptiSeal Polypropylene Tube between the 40% and 50% iodixanol interfaces, and collect only the 40% and 50% fractions (*see* **Notes 7** and **8**).
13. Eject the collected iodixanol solution into a 50 mL tube, and store at 4 °C for buffer exchange and concentration.
14. Repeat **steps 12** and **13** for additional OptiSeal Polypropylene Tubes.

3.3.4 AAV Buffer Exchange and Concentration

1. Incubate an Amicon Ultra-15 Centrifugal Filter Unit with 1× PBS w/ 5% Tween-20 for 20 min at RT. The Filter Unit accommodates ~15 mL of solution.
2. Discard the solution and wash the Filter Unit with 1× PBS w/ 0.001% Tween-20.
3. Pour the extracted iodixanol solution into the Amicon Centrifugal Filter Unit, and dilute with 1× PBS w/ 0.001% Tween-20.
4. Centrifuge the Amicon Ultra-15 Centrifugal Filter Unit at 3000*g* for 1 h at RT. Discard the flowthrough, and dilute the solution again with 1× PBS w/ 0.001% Tween.
5. Repeat **steps 3** and **4** until the iodixanol is cleared from the solution, as indicated by its appearance and viscosity. We recommend at least four centrifugation cycles.
6. Concentrate the purified AAV to the desired volume and store it at 4 °C (short term) or −80 °C (long term).

3.3.5 Titering by qPCR

1. Add 1 μL of AAV vector solution to 5 μL of 10× DNase Buffer, 1 μL of DNase, and 43 μL of Molecular Biology Grade Water in a 1.5 mL tube, and incubate at 37 °C for 30 min.
2. Incubate the solution at 75 °C for 10 min to inactivate the DNase enzyme.
3. Add to the microcentrifuge tube from **step 2** 60 μL of 2× Proteinase K Buffer and 10 μL of Proteinase K, and mix by gentle vortexing. Incubate the solution at 37 °C for 1 h.
4. Incubate the solution at 95 °C for 20 min to inactivate the Proteinase K.
5. Dilute by combining 10 μL of the solution to 90 μL of water. The solution can be stored at 4 °C until further use.
6. Create tenfold serial dilutions from 0.2 ng/μL to 0.02 pg/μL of pAAV-CAG-N-BE-Int-U6-sgRNA or another appropriate plasmid for the standard curve.

7. Prepare a qPCR master mix using 2× iTaq Universal SYBR Green Supermix as follows:

2× iTaq Universal SYBR Green Supermix	10 μL
10 μM BGH-Fwd primer	0.4 μL
10 μM BGH-Rev primer	0.4 μL
Molecular Biology Grade Water	4.2 μL

8. Combine 15 μL of the master mix with 5 μL of the extracted vector genome from **step 5** or the standards from **step 6** in a 96-well plate, and initiate qPCR using the settings in Table 4 (*see* **Note 9**).
9. Plot the cycle threshold (C_t) values for the standards against the respective $\log_{10}$ plasmid copy number (*see* **Note 10**). A sample curve is shown in Fig. 2a, b.
10. Construct a line of best fit for the curve in **step 9**, and fit the C_t value(s) of the AAV sample(s) to the best fit equation to obtain unscaled copy numbers for the AAV vector genomes.

Table 4
qPCR thermocycler settings

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	5 min	1
Denaturation Annealing Extension	95 °C 60 °C 72 °C	30 s 30 s 20 s	40

Note: A melt curve is recommended to assess product purity

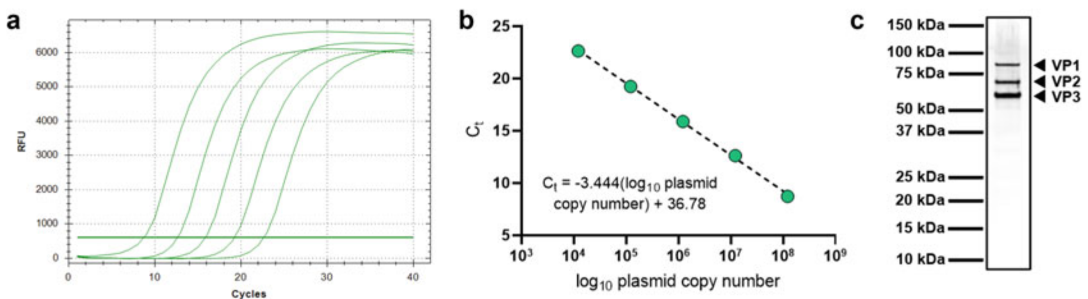


Fig. 2 Measuring the titer and purity of AAV. (**a**) qPCR amplification curves of representative plasmid standards. The green horizontal line indicates the cycle threshold (C_t). (**b**) Measured C_t values versus the $\log_{10}$ plasmid copy number of each standard. (**c**) SDS-PAGE of purified AAV visualized by a SilverXpress Silver Staining Kit. VP1 (82 kDa), VP2 (67 kDa), and VP3 (60 kDa) are the major structural proteins that comprise the AAV capsid

11. Multiply this value by two, as AAV consists of a single-stranded DNA genome.
12. Divide this value by five to obtain the amount of AAV vector genomes (vg) per μL (this step accounts for the 5 μL of sample initially loaded into each well in the qPCR plate).
13. Finally, multiply by 1200 to account for the dilutions in **steps 1–5** to obtain the titer of the AAV vector in vg/μL.
14. Conduct SDS-PAGE analysis on the purified AAV vector to determine its purity. Protein bands can be visualized using the SilverXpress Silver Staining Kit. A representative SDS-PAGE with silver-staining is shown in Fig. 2c.

3.4 Intrathecally Delivering AAV Vector to Mice for In Vivo DNA Editing

3.4.1 Intrathecal Injection

1. Mix 1×10^{11} vg each of the N- and C-terminal split-intein-CBE AAV vectors in 10 μL of 1× PBS w/ 0.001% Tween-20 (*see* **Note 11**). Keep the solution on ice prior to injections.
2. Anesthetize the mouse by placing it in an airtight container connected to an isoflurane vaporizer configured to emit 3% isoflurane at a rate of 1 L/min.
3. Transfer the mouse to a nose cone connected to the isoflurane vaporizer, and measure its paw pinch reflex by firmly pinching its hind paw.
4. When no pinch reflex is observed, shave ~1.5 cm^2 of fur from its lower back to facilitate visualization of the needle during the injection.
5. Fill a 25 μL gas tight Hamilton syringe connected to a 1 inch, 28-gauge, point style 2 needle with 10 μL of the AAV solution from **step 1** (*see* **Note 11**).
6. Feel along the spine of the animal to locate the most prominent spinous process, which corresponds to the L6 vertebra.
7. Insert the needle from **step 5** in the gap between the spinous processes of the L6 and L5 vertebra until a tail flick is observed, which indicates that the needle has penetrated the intradural space.
8. Slowly inject the AAV solution into the mouse (rate, 0.2 μL/s). After injection, leave the needle in place for 30 s, and then gently remove the needle.
9. Place the mouse back into a clean cage, and monitor the mouse until it regains consciousness and can freely move (~20 min). A heating pad can be placed below the cage to keep the mouse warm.
10. Repeat **steps 2** through **9** for additional mice.

3.4.2 Tissue Collection

1. Four weeks after injection, but shortly before tissue harvesting, aliquot 1 mL of chilled PFA (4% w/v) into 1.5 mL microcentrifuge tubes. Keep the tubes on ice. Additionally, pre-chill 2 mL cryogenic vials (*see* **Notes 12** and **13**).
2. Anesthetize a mouse with 100 mg/kg of ketamine and 10 mg/kg of xylazine via intraperitoneal injection. Check for the absence of a toe-pinch reflex indicating that the mouse is fully sedated before proceeding to the next step.
3. Transcardially perfuse the mouse by inserting a 25-gauge needle attached to a Luer-Lok sterile 30 mL syringe with 25 mL of 1× PBS into the apex of the left ventricle of the heart, cutting the right atrium. Steadily expunge the 1× PBS into the left ventricle at a rate of 5 mL/min. Successful perfusion is indicated by blanching of the liver, which, in turn, indicates that the liver is cleared of blood.
4. Isolate the spinal column using a mouse dissecting kit, and separate the cervical, thoracic, and lumbar spinal cord by cutting between the C7 and T1 and the T10 and T11 vertebrae using surgical scissors.
5. Gently remove the vertebrae to expose the spinal cord.
6. Place tissues in either chilled PFA (4% w/v) for immunohistochemistry or a cryogenic vial for nucleic acid isolation. Repeat for each spinal cord segment.
7. Repeat **steps 2–6** for additional mice.
8. Submerge cryogenic vials in liquid nitrogen for 1 min to snap freeze the tissues. Store at −80 °C (*see* **Note 14)**.
9. For tissues in PFA (4% w/v), incubate overnight at 4 °C for fixation. Then, transfer to a tube with 5 mL of sucrose solution (30% w/v), and incubate at 4 °C until the tissues sink to the bottom of the tube (~2 days).

3.5 Measuring Base Editor Expression and DNA Editing

3.5.1 Measuring Base Editor Expression by Immunohistochemistry

1. Gently remove the spinal cord tissue from the sucrose solution using tweezers. Fully submerge the tissue in Scigen Tissue-Plus O.C.T. Compound in a Peel-A-Way Disposable Embedding Mold. Depending on the size of the tissue, up to 3 mL of O. C.T Compound may be required.
2. Remove the frozen O.C.T. Compound from the mold, and mount it in a cryostat-microtome configured at −20 °C.
3. Slice the frozen compound coronally to obtain 40 μm spinal cord sections. Transfer sections to a 48-well plate containing 1 mL of cryoprotectant solution per well (*see* **Note 15**). Sections can be maintained in cryoprotectant for 1 year at −20 °C.

4. Gently remove the tissue sections from cryoprotectant using a Sable Brush (#0 Round), and transfer them to a 12-well plate with 1× PBS and Netwell inserts (74 μm Mesh Size). Place the 12-well plate on a platform shaker for 5 min at RT.
5. Transfer the Netwell inserts with the sections into a well with fresh 1× PBS, and place the 12-well plate on a platform shaker for 5 min. Repeat.
6. Transfer the Netwell inserts with the sections into a well containing Blocking Solution [1× PBS with donkey serum (5% v/v) and Triton X-100 (0.5% v/v)]. Incubate the 12-well plate on a platform shaker for 2 h at RT.
7. Transfer the Netwell inserts with the tissue sections into a new well with Blocking Solution containing Rabbit anti-HA (1: 250; Cell Signaling Technology #3724S) and Chicken anti-GFAP (1:1000: Abcam #ab4674). Incubate on a platform shaker for 48–72 h at 4 °C (*see* **Note 16**).
8. Wash the tissues as described in **steps 5** and **6**.
9. Transfer the Netwell inserts with the tissue sections into a new well with Blocking Solution containing Alexa Fluor 647 AffiniPure Donkey Anti-Chicken IgY (1:150; Jackson ImmunoResearch #703-605-155) and Alexa Fluor 488 AffiniPure Fab Fragment Donkey Anti-Rabbit IgG (1:150; Jackson ImmunoResearch #711-547-003). Incubate the 12-well plate at RT for 2 h. Wrap the plate with aluminum foil to prevent photobleaching of the fluorophores.
10. Repeat **step 8**.
11. Remove the tissues, and carefully place them on a microscope slide using the Sable Brush (#0 Round). Add two drops of VectaShield Hardset Antifade Mounting Medium onto the slide, and gently cover the tissues and slide with a coverslip, ensuring that each section is fully immersed in mounting medium.
12. Wipe excess mounting medium from the microscope slide using a Kimwipe. Dry in a microscope slide box at 4 °C.
13. Analyze the sections using a fluorescent light microscope (Fig. 3).

3.5.2 Verification of In Vivo Base Editing by NGS

1. Design primers to amplify by PCR the target site with index-containing overhangs for next-generation sequencing (NGS). The overhangs will encode index primers for a second PCR. The amplicon length should be compatible with the maximum read length provided by an NGS instrument (*see* **Note 17**).
2. Add 170 μL of Buffer ATL from the DNeasy Blood and Tissue Kit to the tissue from Subheading 3.5.1, **step 8**, and grind the

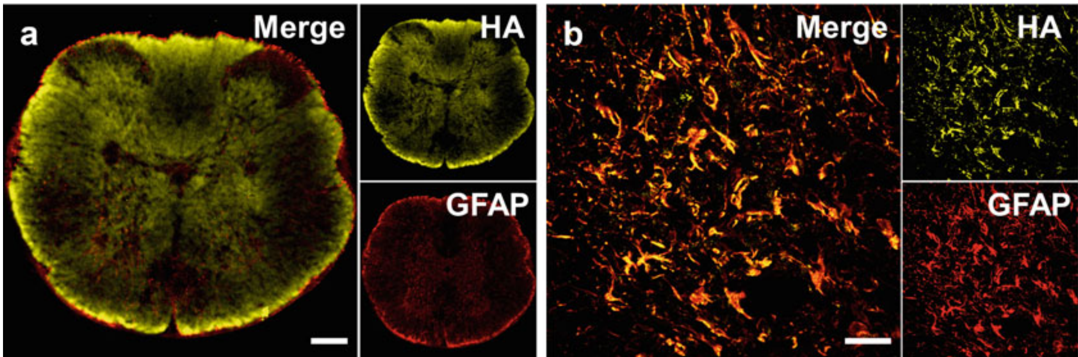


Fig. 3 Delivering a base editor to the spinal cord by AAV. (**a, b**) Representative immunofluorescence staining of the thoracic spinal cord 4 weeks after SOD1-ALS mice were injected with two AAV9 vectors encoding N- and C-terminal split-intein CBE. Scale bar, (**a**) 200 μm and (**b**) 30 μm. CBE was detected by its C-terminus human influenza hemagglutinin (HA) epitope tag. GFAP indicates reactive astrocytes, the targeted cell type

tissue with a pestle until fully homogenized. Tissues should be allowed to warm to room temperature before homogenizing.

3. Purify genomic DNA from the homogenized tissue using the DNeasy Blood and Tissue Kit per the manufacturer's instructions. Determine DNA concentration by a UV spectrophotometer.
4. Amplify the target site by PCR with the following conditions (Table 5):

Genomic DNA	50 ng
5× KAPA HIFI Buffer	5 μL
10 μM Fwd primer	0.75 μL
10 μM Rev primer	0.75 μL
10 mM dNTP mix	0.75 μL
KAPA HIFI DNA Polymerase	0.5 μL
Molecular Biology Grade Water	To 25 μL

5. Use AMPure XP beads to purify the PCR amplicons. Add 20 μL of the XP beads to each PCR reaction and mix well. Incubate at RT for 5 min.
6. Place the PCR microcentrifuge tube on a magnetic stand for 5 min. Discard the supernatant.
7. With the samples still on the magnetic stand, add 200 μL of ethanol (80%) to the sample. Incubate on the magnetic stand for an additional 1 min, and discard the supernatant. Repeat. Use a micropipette to remove any excess ethanol.

Table 5
Thermocycler protocol for NGS amplification PCR

Step	Temperature	Time	No. of cycles
Initial denaturation	95 °C	3 min	1
Denaturation Annealing Extension	98 °C 65 °C 72 °C	20 s 15 s 15 s/kb	28
Final extension	72 °C	1 min/kb	1

8. Air-dry the XP beads on the magnetic stand for 10 min.
9. Remove the microcentrifuge tube from the magnetic stand, and add 52 μL of 10 mM Tris pH 8.5 buffer (*see* Subheading 2.6, **step 26**), mixing samples with the beads. Incubate at RT for 5 min and then incubate on the magnetic stand for 3 min.
10. After the solution clears, collect 50 μL of the supernatant for the second (indexing) PCR. Samples can be analyzed by agarose gel electrophoresis to determine purity (*see* **Note 17**).
11. Determine DNA concentration with a UV spectrophotometer (*see* **Note 17**).
12. Reamplify the amplicon by PCR using Nextera XT Indexing Primers. Conduct the PCR using the following conditions (Table 6):

PCR amplicon from **step 5**	50 ng
5× KAPA HIFI Buffer	5 μL
10 μM index primer 1	5 μL
10 μM index primer 2	5 μL
10 mM dNTP mix	0.75 μL
KAPA HIFI DNA Polymerase	0.5 μL
Molecular Biology Grade Water	To 25 μL

13. Repeat **steps 5–10** to purify the index-containing amplicon (s) using AMPure XP Beads. Use 25 μL of Tris pH 8.5 buffer (*see* Subheading 2.6, **step 26**) when repeating **step 9**, and collect 20 μL of the supernatant when repeating **step 10**. Samples can be analyzed by agarose gel electrophoresis to determine purity (*see* **Note 18).**
14. Quantify the amount of amplicon DNA (in nM) by a Qubit Assay and Qubit Flex Fluorometer (*see* **Note 19**).

Table 6
Thermocycler protocol for NGS indexing PCR

Step	Temperature	Time	No. of cycles
Initial denaturation	95 °C	3 min	1
Denaturation Annealing Extension	95 °C 55 °C 72 °C	30 s 30 s 30 s	8
Final extension	72 °C	5 min	1

15. Dilute each sample to 10 nM using Molecular Biology Grade Water, and then pool 5 μL of each sample into a final library for sequencing.
16. Sequence the samples with an Illumina NGS sequencer instrument compatible with the Nextera XT kit, and quantify the frequency of base editing via CRISPResso2 or equivalent software [36].

4 Notes

1. For oligonucleotides with higher melting temperatures (>65 °C), the following annealing protocol is recommended: heat to 95 °C for 5 min; ramp down at 0.1 °C/s, and hold for 2 min at 5 °C intervals. Continue until 20 °C and then hold for 5 min. Hold at 10 °C indefinitely.
2. The Type IIS restriction enzyme used to digest a pAAV plasmid can vary. While the plasmids in our protocol rely on BsaI, depending on the plasmid, BbsI and BsmBI may also be used. When ligating the sgRNA into the pAAV plasmid, we recommended using a 20:1 molar ratio of sgRNA:pAAV.
3. As a negative control, a negative ligation reaction should be done, which can consist of all components for the one-pot reaction except the T4 DNA ligase to determine if the digestion occurred. We routinely observe no colonies with this control.
4. Purified plasmids should ideally possess 260/280 absorbance ratios of 1.7–1.9. Appreciably lower or higher absorbance ratios may indicate contaminants.
5. In our experience, the transfection efficiency of HEK293T cells exceeds 90%. If observing sub-optimal transfection efficiencies, we recommend varying the density of the seeded cells, transfecting less or more plasmid DNA, or using an alternate transfection reagent, such as Lipofectamine P3000 or PEI.

6. Because lysates from transfected HEK293T do not undergo inactivation, all steps should be performed in a certified Biosafety Cabinet using appropriate biosafety containment procedures.
7. Ensure that the OptiSeal Polypropylene Tubes are clamped securely before puncturing, and do not place extremities in the path of the needle.
8. A cloudy white line should be visible between the 24% and 40% iodixanol interfaces. This line contains proteins from the cell lysate. Care must be taken to avoid extracting this proteinaceous material.
9. Samples should be measured in at least technical duplicates to minimize error.
10. The copy number of a plasmid can be calculated from its mass using the formula below:

$$\text{Plasmid copy number} = \frac{(m \times \text{NA})}{L \times 660\ \frac{\text{g}}{\text{mol}} \times 10^{9}}$$

where m = mass of plasmid in ng; NA = Avogadro's number; L = length of plasmid/amplicon; and 660 g/mol = average molecular weight of a DNA base pair

11. Injection volumes can range from 5 μL to 12 μL per mouse. Lower volumes may result in inconsistent transduction, while higher volumes can create pressure, resulting in backflow.
12. Three tubes of PFA (4% v/v) are needed per animal for the cervical, thoracic, and lumbar spinal cord segments.
13. Tissues must be kept cold (<4 °C) to minimize degradation.
14. Snap freezing can prevent the formation of ice crystals that can damage the tissue and proteins.
15. One well with 1 mL of cryoprotectant solution can accommodate ~8 tissue sections.
16. Tissue sections can be stained with antibodies in a 12- or 24-well plate format. 12-well plates enable more efficient transfer of tissues via Netwell inserts but will require additional staining solution per well.
17. A majority of NGS sequencing techniques will be able to read 150–300 base pairs in both the forward and reverse directions beginning at the first base of the primer that binds to the amplicon. When designing and optimizing primers, it is important that the amplicon size does not exceed the combined length of the forward and reverse reads recommended by the NGS instrument. A longer length will result in incomplete reads for the amplicon.

18. If PCR yields are low, increase the amount of template DNA or the number of cycles. Additionally, if non-specific amplification is observed, increased annealing temperatures are recommended. Otherwise, new primers may be required.
19. DNA concentration in units of nanomolar for each sample in the pooled library can be calculated with the formula below:

$$\text{DNA Concentration (nM)} = \left(\frac{D}{\left(\frac{660\,\text{g}}{\text{mol}}\tilde{n}a\right)}\right)\tilde{n}10^6$$

where D = the concentration of the amplicon in ng/μL; and a = amplicon size in base pairs.

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