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AAV capsid library production

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We use this protocol and it's working

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Abstract

We recently developed adeno-associated virus (AAV) capsids to facilitate efficient and noninvasive gene transfer to the central and peripheral nervous systems. However, a detailed protocol for generating and systemically delivering novel AAV variants was not previously available. In this protocol, we describe how to produce and intravenously administer AAVs to adult mice to specifically label and/or genetically manipulate cells in the nervous system and organs, including the heart. The procedure comprises three separate stages: AAV production, intravenous delivery, and evaluation of transgene expression. The protocol spans 8 d, excluding the time required to assess gene expression, and can be readily adopted by researchers with basic molecular biology, cell culture, and animal work experience. We provide guidelines for experimental design and choice of the capsid, cargo, and viral dose appropriate for the experimental aims. The procedures outlined here are adaptable to diverse biomedical applications, from anatomical and functional mapping to gene expression, silencing, and editing.

Attachments

699-1488.pdf
5MB

Guidelines

Introduction

Recombinant AAVs (rAAVs) are commonly used vehicles for in vivo gene transfer and promising vectors for therapeutic applications[1]. However, AAVs that enable efficient and noninvasive gene delivery to defined cell populations are needed. Current gene delivery methods (e.g., intraparenchymal surgical injections) are invasive, and alternatives such as intravenous administration require high viral doses and provide relatively inefficient transduction of target cells. We previously developed Cre recombination-based AAV targeted evolution (CREATE) to engineer and screen for AAV capsids that are capable of more efficient gene transfer to specific cell types via the vasculature[2–4]. Compared to naturally occurring capsids (e.g., AAV9), the novel AAV-PHP capsids identified by CREATE exhibit markedly improved tropism for cells in the adult mouse central nervous system (CNS), peripheral nervous system (PNS), and visceral organs. In this protocol, we describe how to package genetic cargo into AAV-PHP capsids and intravenously administer AAVs for efficient, noninvasive, and targeted gene delivery at sites throughout the body (Fig. 1).

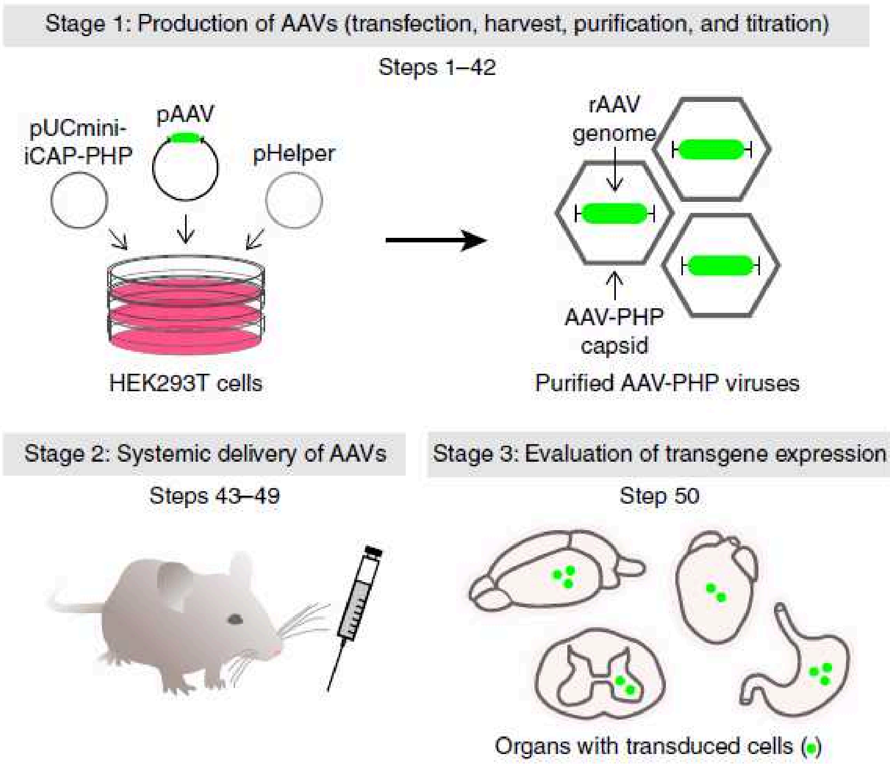

Fig. 1 | Overview of the protocol. The Procedure comprises three main stages: AAV production (Steps 1–42), intravenous delivery (Steps 43–49), and evaluation of transgene expression (Step 50). The pAAV plasmid contains the rAAV genome (e.g., containing a fluorescent reporter, shown in green) (Fig. 6 and Table 1), which is packaged into an AAV-PHP capsid via triple transient transfection. Systemic administration of AAV-PHP viruses is achieved by retro-orbital injection into wild-type or transgenic mice; transgene expression is evaluated after adequate time has passed for viral transduction and protein expression. AAV-PHP viruses target cells in the CNS (e.g., in the brain and spinal cord) or PNS and visceral organs (e.g., in the heart and gut). Filled green circles represent transduced cells. For illustrative purposes, we use fluorescent labeling as an example of how to assess transgene expression; however, assessment can take other forms (see 'Experimental design' section for details). See Fig. 7a for a time line of the Procedure.

Among our new capsid variants[2–4], AAV-PHP.B and the further evolved AAV-PHP.eB efficiently transduce neurons and glia throughout the CNS (Fig. 2); another variant, AAV-PHP.S, displays improved tropism for neurons within the PNS (Fig. 3) and organs, including the gut[2] and heart (Fig. 4). Importantly, these capsids target cell populations that are normally difficult to access because of their location (e.g., sympathetic, nodose, dorsal root, and cardiac ganglia) (Figs. 3a–c and 4d) or broad distribution (e.g., throughout the brain or enteric nervous system) (Figs. 2 and 3d) and can be utilized in several mouse and rat strains (Fig. 5). Together with the capsid, the genetic cargo (or rAAV genome) can be customized to control transgene expression (Fig. 6 and Table 1). The rAAV genome contains the components required for gene expression, including promoters, transgenes, proteintrafficking signals, and recombinase-dependent expression schemes. Hence, different capsid–cargo combinations create a versatile AAV toolbox for genetic manipulation of diverse cell populations in wild-type and transgenic animals. Here, we provide researchers, especially those new to working with AAVs or systemic delivery, with resources that will help them utilize AAV-PHP viruses in their own research.

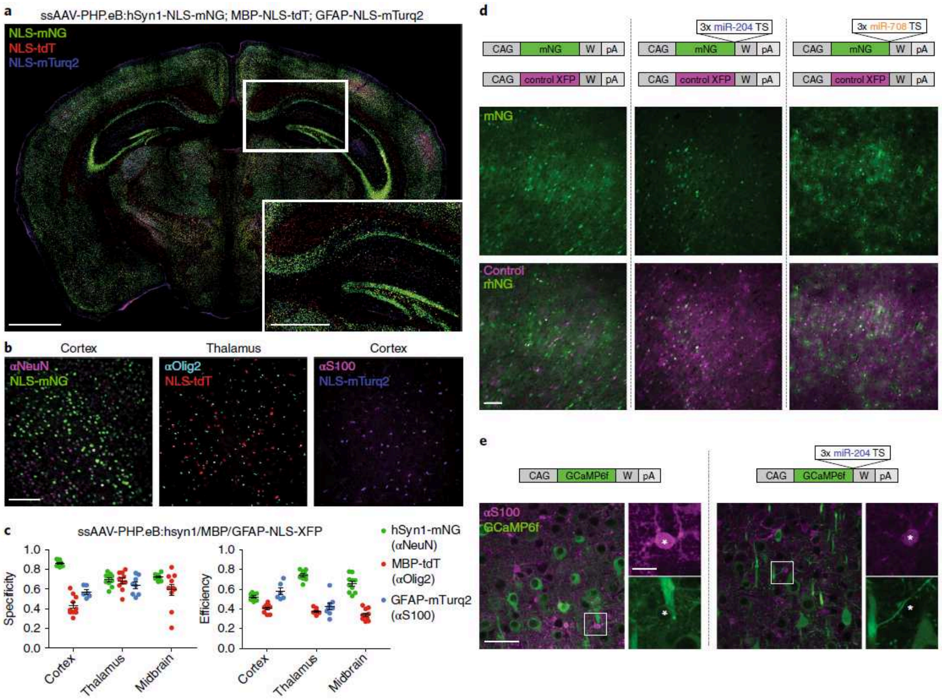

Fig. 2 | AAV-PHP.eB and gene regulatory elements enable cell type–specific gene expression in the brain. **a–c**, We used AAV-PHP.eB to package single-stranded (ss) rAAV genomes that express fluorescent reporters (XFPs), each with two nuclear localization signals (NLS), from cell type–specific promoters. Genomes containing the hSyn1, MBP, or GFAP ($GfABC_1D$) promoters were used to target neurons, oligodendrocytes, or astrocytes, respectively. Viruses were co-delivered by retro-orbital injection to 7-week-old C57BL/6N mice (*n* = 2) at 3×10^{11} vector genomes (vg)/virus (9×10^{11} vg total). Native fluorescence in coronal brain sections was evaluated 4 weeks later using confocal microscopy. All sections were mounted in Prolong Diamond Antifade before imaging. **a**, Cell type–specific, nuclear-localized XFPs label distinct cell types throughout the brain. Tile scan of a coronal brain slice, presented as a maximum-intensity projection; inset shows a zoomed-in view of the hippocampus. XFPs were mNeonGreen (mNG; green), tdTomato (tdT; red), and mTurquoise2 (mTurq2; blue). Scale bars, 1 mm and 500 µm (inset). **b,c**, Antibody staining can be used to determine the specificity and efficiency of cell type–specific promoters. **b**, Brain sections were stained with NeuN (purple), Olig2 (light blue), and S100 (purple) to mark neurons, oligodendrocyte lineage cells, and a population of glia that consists mainly of astrocytes, respectively. NLS-mNG (green), NLS-tdT (red), and NLS-mTurq2 (dark blue) indicate nuclear-localized XFPs. Images are from a single z plane. Scale bar, 100 µm. **c**, AAV-PHP.eB differentially transduces various regions and cell types throughout the brain. 'Specificity' or 'Efficiency' are defined as the ratio of double-labeled cells to the total number of XFP- or antibody-labeled cells, respectively. For image processing, median filtering and background subtraction using morphological opening were first applied to each image to reduce noise and correct imbalanced illumination. Each nucleus expressing XFPs and labeled with antibodies was then segmented by applying a Laplacian of Gaussian filter to the pre-processed images. We considered cells that were both expressing XFPs and labeled with antibodies if the nearest center-to-center distance between blobs (nuclei or cell bodies) in two channels was <7 µm (half of the cell body size). Five images per brain region were analyzed in each mouse; we excluded images with tissue edges because bright edges prevent accurate cell detection. Mean ± s.e.m. is shown. **d,e**, miRNA target sequences (TS) miR-204-5p or miR-708-5p[77] can be used to achieve expression that is more restricted to neurons or astrocytes, respectively. **d**, The indicated pairs of vectors were separately packaged into AAV-PHP.eB and co-administered via retro-orbital injection to 6- to 8-week-old C57BL/6J mice (*n* = 2) at 1×10^{11} vg/virus (2×10^{11} vg total); mNG and control XFP fluorescence were evaluated 3 weeks later using confocal microscopy. The CAG-mNG genome (green) contained no miRNA TS (left) or three tandem copies of miR-204 (middle) or miR-708 (right) TS; the CAG-XFP genome (magenta) contained no miRNA TS and was injected as an internal control. miR-204 reduced expression in cells with the morphology of astrocytes, and injected as an internal control. miR-204 reduced expression in cells with the morphology of astrocytes, and miR-708 reduced expression in cells with neuronal morphology. Scale bar, 100 µm. **e**, ssAAV-PHP.eB:CAG-GCaMP6f-3x-miR122-TS (left) or ssAAV-PHP.eB:CAG-GCaMP6f-3x-miR204-5p-3x-miR122-TS (right) was injected into 6- to 8-week-old C57BL/6J mice (n = 2) at 1×10^{11} vg/mouse; gene expression was evaluated 3 weeks later using confocal microscopy. The miR-204 TS reduced GCaMP6f expression (green) in $S100^{+}$ glia (magenta) in the cortex. Both vectors contained three tandem copies of miR-122 to reduce expression in hepatocytes[78]. Insets and asterisks highlight representative images of $S100^{+}$ glia. Scale bars, 50 µm and 10 µm (insets). Refer to Table 1 for details of rAAV genomes. Experiments on vertebrates conformed to all relevant governmental and institutional regulations and were approved by the Institutional Animal Care and Use Committee (IACUC) and the Office of Laboratory Animal Resources at the California Institute of Technology. In our primary publication[2], results were obtained using the C57BL/6J mouse line. pA, polyadenylation signal; W, WPRE.

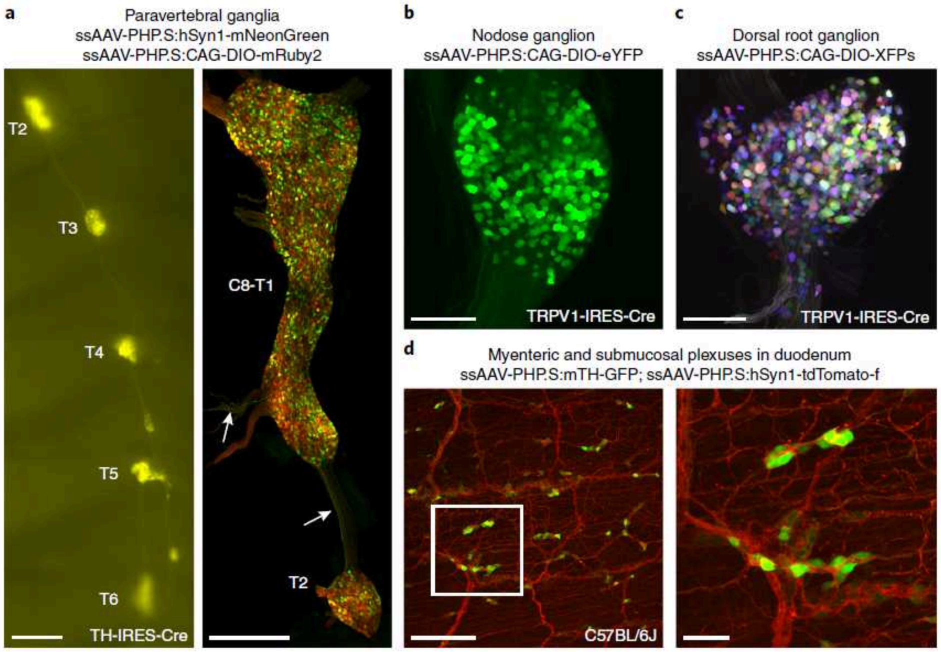

Fig. 3 | AAV-PHP.S transduces neurons throughout the PNS. We used AAV-PHP.S to package single-stranded (ss) rAAV genomes that express fluorescent reporters from either neuron-specific (e.g., hSyn1 and TH (tyrosine hydroxylase)) or ubiquitous promoters (e.g., CAG). Viruses were delivered by retro-orbital injection to 6- to 8-weekold C57BL/6J or Cre transgenic mice, and transgene expression was evaluated 2–3 weeks later. Whole-mount tissues were optically cleared using either ScaleSQ[64] (**a** (right), **c**, and **d**) or RIMS[57] (**b**) and imaged using wide-field or confocal microscopy; confocal images are presented as maximum-intensity projections. **a**, ssAAV-PHP.S:hSyn1- mNeonGreen and ssAAV-PHP.S:CAG-DIO-mRuby2 were co-injected into a TH-IRES-Cre mouse at 1×10^{12} vg/virus (2×10^{12} vg total). Native mNeonGreen (green) and mRuby2 (red) fluorescence were assessed 2 weeks later using wide-field (left) or confocal fluorescence microscopy (right). Images are from the second to sixth thoracic (T2–T6) (left) and eighth cervical to second thoracic (C8–T2) (right) paravertebral ganglia, which provide sympathetic innervation to thoracic organs, including the heart. Arrows denote mNeonGreen+ nerve fibers. Scale bars, 1 mm (left) and 500 µm (right). **b**, ssAAV-PHP.S:CAG-DIO-eYFP was injected into a TRPV1-IRES-Cre mouse at 1×10^{12} vg; gene expression in a nodose ganglion was evaluated 3 weeks later. Scale bar, 200 µm. **c**, A mixture of three separate viruses (ssAAV-PHP.S:CAG-DIO-XFPs) was injected into a TRPV1-IRES-Cre mouse at 1×10^{12} vg/virus (3×10^{12} vg total); gene expression in a dorsal root ganglion was evaluated 2 weeks later. XFPs were mTurquoise2 (blue), mNeonGreen (green), and mRuby2 (red). Scale bar, 200 µm. **d**, ssAAV-PHP.S:mTH-GFP and ssAAV-PHP.S:hSyn1- tdTomato-f (farnesylated) were co-injected into a C57BL/6J mouse at 5 × 1011 vg/virus (1×10^{12} vg total); gene expression in the duodenum was assessed 22 d later. The image stack includes both the myenteric and submucosal plexuses. Inset shows a zoomed-in view of ganglia containing TH+ cell bodies (green); tdTomato-f (red) labels both thick nerve bundles and individual fibers. Scale bars, 200 µm (left) and 50 µm (right). Refer to Table 1 for details of rAAV genomes. Experiments on vertebrates conformed to all relevant governmental and institutional regulations and were approved by the Institutional Animal Care and Use Committee (IACUC) and the Office of Laboratory Animal Resources at the California Institute of Technology. In our primary publication[2], results were obtained using the ChAT-IRES-Cre driver mouse line.

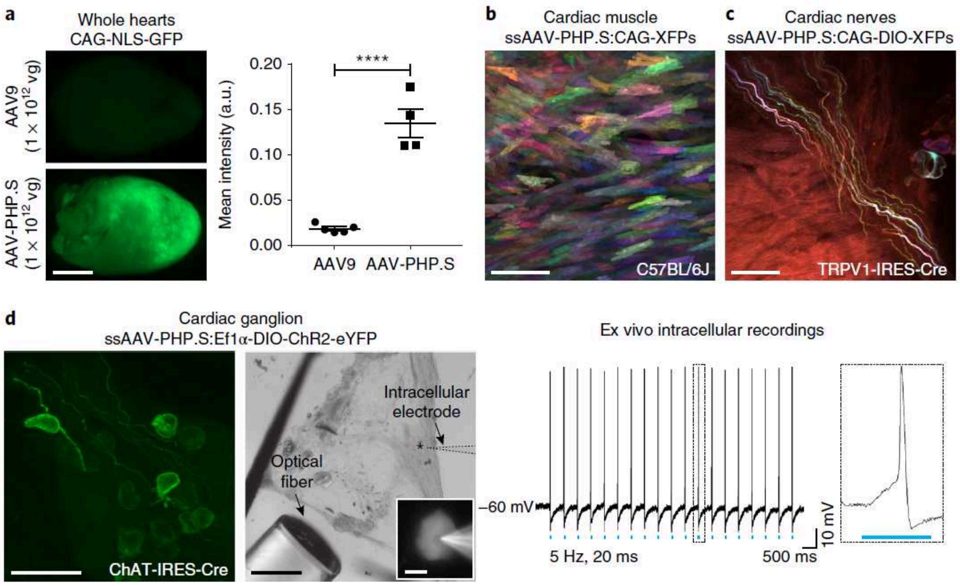

Fig. 4 | AAV-PHP.S for mapping the anatomy and physiology of the heart. AAV-PHP.S viruses were delivered by retro-orbital injection to 6- to 8-week-old C57BL/6J or Cre transgenic mice. **a**, AAV-PHP.S transduces the heart more efficiently than the current standard, AAV9. ssAAV9:CAG-NLS-GFP or ssAAV-PHP.S:CAG-NLS-GFP were injected into C57BL/6J mice at 1×10^{12} vg/mouse. Native GFP fluorescence was assessed in whole-mount hearts 4 weeks later using wide-field fluorescence microscopy (unpaired *t* test, $t_7 = 8.449$, *****P* <0.0001). For AAV9 and AAV-PHP. S, *n* = 5 and 4 mice, respectively. a.u., arbitrary units. Mean ± s.e.m. is shown. Scale bar, 3 mm. **b**, A mixture of three viruses (ssAAV-PHP.S:CAG-XFPs) was injected into a C57BL/6J mouse at 3.3×10^{11} vg/virus (1×10^{12} vg total); gene expression in cardiac muscle was evaluated 11 d later. Individual cardiomyocytes can be easily distinguished from one another. Scale bar, 200 µm. **c**, A mixture of three viruses (ssAAV-PHP.S:CAG-DIO-XFPs) was injected into a TRPV1-IRES-Cre mouse at 1×10^{12} vg/virus (3×10^{12} vg total); gene expression in cardiac nerves was evaluated 2 weeks later. Scale bar, 50 µm. **d**, ssAAV-PHP.S:Ef1α-DIO-ChR2-eYFP was injected into ChAT-IRES-Cre mice (*n* = 2) at 1×10^{12} vg; gene expression in a cardiac ganglion was evaluated 3 weeks later (left). Ex vivo intracellular recordings were performed after 5 weeks of expression. Differential interference contrast (DIC) image (middle) shows the optical fiber for light delivery and electrode for concurrent intracellular recordings; inset shows a higher magnification image of a selected cell (asterisk). Cholinergic neurons generated action potentials in response to 473-nm light pulses (5 Hz, 20 ms) (right). Scale bars, 50 µm (left), 300 µm (middle), and 10 µm (inset). Whole mount tissues in **b**, **c**, and **d** (left) were optically cleared using ScaleSQ[64] and imaged using confocal microscopy; confocal images are presented as maximum-intensity projections. XFPs in **b** and **c** were mTurquoise2 (blue), mNeonGreen (green), and mRuby2 (red). Refer to Table 1 for details of rAAV genomes. The pAAV-Ef1α-DIO-ChR2-eYFP plasmid was a gift from K. Deisseroth, Stanford University (Addgene, plasmid no. 20298). Experiments on vertebrates conformed to all relevant governmental and institutional regulations and were approved by the Institutional Animal Care and Use Committee (IACUC) and the Office of Laboratory Animal Resources at the California Institute of Technology. In our primary publication[2], results were obtained using the ChAT-IRES-Cre driver mouse line.

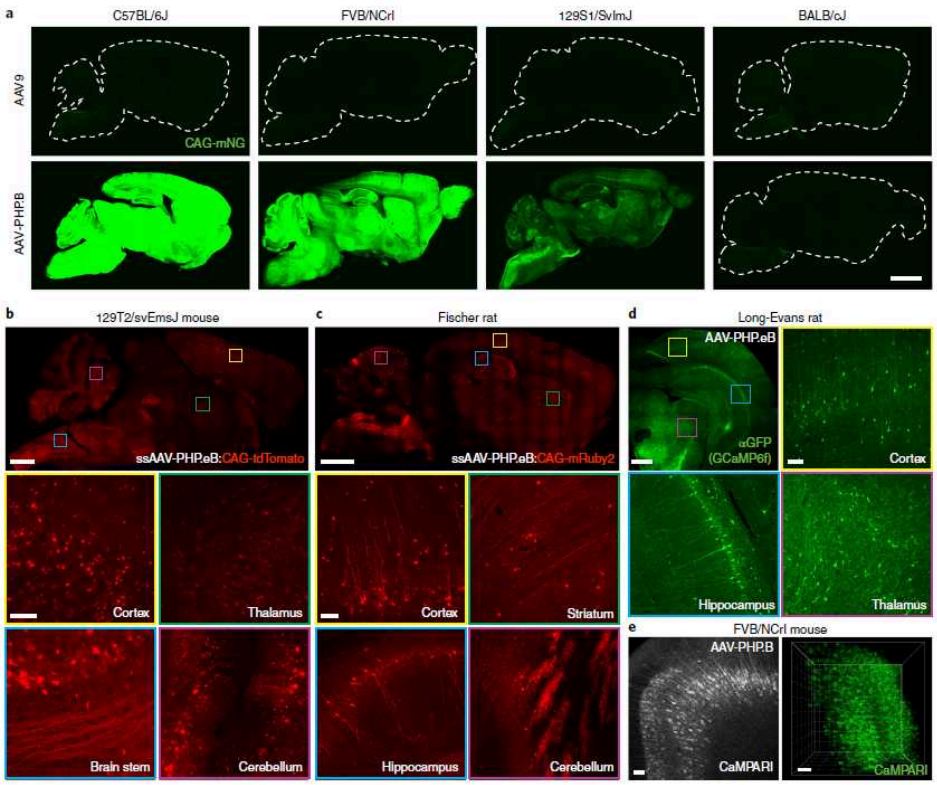

Fig. 5 | AAV-PHP.B and AAV-PHP.eB can be used in several mouse and rat strains. **a**, AAV-PHP.B transduces the brain more efficiently than AAV9 in C57BL/6J, FVB/NCrl, and 129S1/SvImJ mice, but not in BALB/cJ mice. ssAAV9:CAG-mNeonGreen or ssAAV-PHP.B:CAG-mNeonGreen were systemically delivered to 6- to 8-week-old C57BL/6J (*n* = 1–2 mice per group), FVB/NCrl (*n* = 2 mice per group), 129S1/SvImJ (*n* = 2 mice per group), and BALB/cJ mice (*n* = 2 mice per group) at 1×10^{12} vg/mouse. 3 weeks later, sagittal brain sections were mounted in Vectashield and imaged using confocal microscopy. Imaging and display parameters are matched across all panels. Scale bar, 2 mm. **b–e**, Examples of AAV-PHP.B- and AAV-PHP.eB-mediated brain transduction for fluorescent labeling (**b,c**) and calcium imaging (**d,e**) in different mouse and rat strains. Gene expression was evaluated using confocal microscopy. **b**, ssAAV-PHP.eB:CAG-tdTomato (Addgene) was delivered by retro-orbital injection to a 10-week-old 129T2/SvEmsJ mouse at 3×10^{11} vg; tdTomato fluorescence (red) was examined 2 weeks later. Scale bars, 1 mm (top) and 100 µm (insets). **c**, ssAAVPHP. eB:CAG-mRuby2 was administered by tail-vein injection to a 6-week-old female Fischer rat at 3×10^{12} vg; 3 weeks later, brain slices were mounted in Prolong Diamond Antifade for imaging. Scale bars, 2 mm (top) and 100 µm (insets). **d**, ssAAV-PHP.eB:CMV-hSyn1-GCaMP6f-3x-miR122- TS was delivered by tail-vein injection to a 4-week-old female Long-Evans rat at 1×10^{13} vg; 3 weeks later, brain slices were stained with a GFP antibody (green) for imaging. Scale bars, 1 mm (top left) and 100 µm (insets). The vector contained three tandem copies of miRNA target sequence (TS) miR-122 (CAAACACCATTGTCACACTCCA) to reduce expression in hepatocytes[78]. Images in d courtesy of M. Fabiszak/W. Freiwald lab, Rockefeller University. **e**, ssAAV-PHP.B:CaMKIIa-CaMPARI (calcium-modulated photoactivatable ratiometric integrator[79]) was administered by retro-orbital injection to a 8-week-old FVB/NCrl mouse at 3×10^{11} vg and cortical expression was assessed 2 weeks later. Images are a 50-µm maximum-intensity projection of the cortex (left) and 500-µm-thick ScaleSQ[64]-cleared 3D volume (right). Scale bars, 100 µm. Experiments on vertebrates conformed to all relevant governmental and institutional regulations and were approved by the Institutional Animal Care and Use Committee (IACUC) and the Office of Laboratory Animal Resources at the California Institute of Technology. In our primary publication[2], results were obtained using the C57BL/6J mouse line. CaMKIIa, calcium/calmodulin-dependent protein kinase type IIa; CMV, cytomegalovirus early enhancer element.

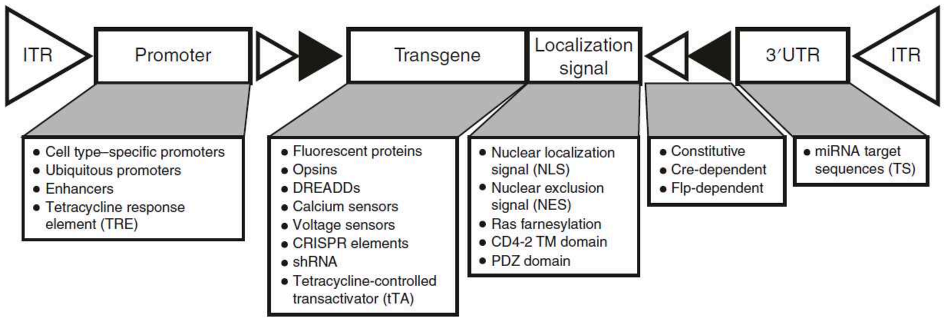

Fig. 6 | A modular AAV toolbox for cell type–specific gene expression. The rAAV genome, contained in a pAAV plasmid (not shown), consists of an expression cassette flanked by two 145-bp inverted terminal repeats (ITRs); the entire genome, including the ITRs, cannot exceed 4.7–5 kb. The promoters, transgenes, localization signals, and recombination schemes are interchangeable. Gene regulatory elements, such as promoters and microRNA (miRNA) target sequences (TS) (Fig. 2d,e), determine the strength and specificity of transgene expression54. Transgenes may be constitutively expressed or flanked by recombination sites for flippase (Flp)- or Cre recombinase (Cre)-dependent expression. In the latter approach, the transgene remains in the double-floxed inverted orientation (DIO); Cre-mediated inversion of the transgene enables cell type–specific expression in transgenic animals (Figs. 3a–c and 4c,d). Localization sequences further restrict gene expression to distinct cellular compartments such as the nucleus (via one or more nuclear localization signals (NLS)) (Fig. 2a,b), cytosol (via a nuclear exclusion signal (NES)[80]), or cell membrane (via farnesylation[76], the CD4-2[81] transmembrane (TM) targeting domain, or PDZ[82] protein–protein interaction domains) (Fig. 3d). Note that the 3' UTR contains the woodchuck hepatitis posttranscriptional regulatory element (WPRE) (609 bp) and a polyadenylation signal (e.g., the human growth hormone (hGH) polyA) (479 bp) (not shown), both of which enhance transgene expression[54].We recommend that foreign genes be codon-optimized to match the host species to increase expression from the rAAV genome. Use sequence-editing and annotation software to determine the unique attributes of each rAAV genome. In Table 1, we list genomes used here and in our previous work[2,3]; see also Addgene's plasmid repository for pAAVs that may be suitable for different applications. CRISPR, clustered regularly interspaced short palindromic repeats; DREADDs, designer receptors exclusively activated by designer drugs; shRNA, short hairpin RNA.

Table 1 | pAAV plasmids

	A	B	C	D
		Vector name pAAV-	**Expression class**	**Addgene no.**
	Tunable expression	TRE[a]-mTurquoise2	tTA-dependent	99113
		TRE-eYFP		104056
		TRE-mRuby2		99114
		TRE-DIO[b]-mTurquoise2	Cre- and tTAdependent	99115
		TRE-DIO-eYFP		117383

	A	B	C	D
		TRE-DIO-tdTomato		99116
		TRE-DIO-mRuby2		99117
		CAG[c]-tTA[d]	Inducer	99118
		hSyn1[e]-tTA		99119
		ihSyn1[f]-tTA		99120
		ihSyn1-DIO-tTA		99121
	Tissue-wide expression	CAG-mTurquoise2	Constitutive	99122
		CAG-eYFP		104055
		CAG-mRuby2		99123
		CAG-NLS[g]-GFP		104061
		CAG-DIO-mTurquoise2	Cre-dependent	104059
		CAG-DIO-eYFP		104052
		CAG-DIO-mRuby2		104058
	Cell type–specific expression	hSyn1-mTurquoise2	Cell type–specific	99125
		hSyn1-eYFP		117382
		hSyn1-mRuby2		99126
		GFAP[h]-2xNLS-mTurquoise2		104053
		hSyn1-2xNLS-mTurquoise2		118025
		MBP[i]-2xNLS-tdTomato		104054
		mTH[j]-GFP		99128
		hSyn1-tdTomato-f[k]		104060
		GFAP-mKate2.5-f		99129
		mDlx[l]-NLS-mRuby2		99130
		CAG-eYFP-3x-miR204-5p-TS[m]		117380
		CAG-eYFP-3x-miR708-5p-TS[n]		117381
		CAG-GCaMP6f-3x-miR204-5p-3xmiR122-TS[o]		117384

A comprehensive list of pAAV plasmids used in this and related work[2,3].

[a]TREpi, second-generation tetracycline-regulated promoter.
[b]DIO, double-floxed inverted orientation.
[c]CAG, synthetic promoter containing the cytomegalovirus early enhancer element, the promoter, first exon, and first intron of chicken beta-actin gene, and the splice acceptor from the rabbit beta-globin gene.
[d]tTA, tetracycline-controlled transactivator.
[e]hSyn1, human synapsin I promoter.
[f]ihSyn1, inducible intron human synapsin I promoter.
[g]NLS, nuclear localization signal.
[h]GFAP (GfABC1D), glial fibrillary acidic protein promoter.
[i]MBP, myelin basic protein promoter.
[j]mTH, mouse tyrosine hydroxylase promoter.
[k]f, farnesylation signal from c-Ha-Ras.
[l]mDlx, mouse distal-less homeobox promoter.
[m]miR-204-5p-TS: AGGCATAGGATGACA AAGGGAA.
[n]miR-708-5p-TS: CCCAGCTAGATTGTAAGCTCCTT.
[o]miR-122-TS: CAAACACCATTGTCACACTCCA.

Overview of the protocol

We provide an instruction manual for users of AAV-PHP variants. The procedure includes three main stages (Fig. 1): AAV production (Steps 1–42), intravenous delivery (Steps 43–49), and evaluation of transgene expression (Step 50).

The AAV production protocol is adapted from established methods. First, HEK293T cells are transfected with three plasmids[5–7] (Steps 1–3, Figs. 1 and 7): (i) pAAV, which contains the rAAV genome of interest (Fig. 6 and Table 1); (ii) pUCmini-iCAP-PHP, which encodes the viral replication and capsid proteins (Table 2); and (iii) pHelper, which encodes adenoviral proteins necessary for replication. Using this triple-transfection approach, a single-stranded rAAV genome is packaged into an AAV-PHP capsid in HEK293T cells. AAV-PHP viruses are then harvested[8] (Steps 4–14), purified[9,10] (Steps 15–31), and titered by quantitative PCR (qPCR)[11] (Steps 32–42) (Fig. 7). Purified viruses are intravenously delivered to mice via retro-orbital injection[12] (Steps 43–49), and gene expression is later assessed using molecular, histological, or functional methods relevant to the experimental aims (Step 50).

This protocol is optimized to produce AAVs at high titer (≥1 × 10^{13} vector genomes (vg)/ml and ≥1 × 10^{12} vg/dish) and with high transduction efficiency in vivo[2,3].

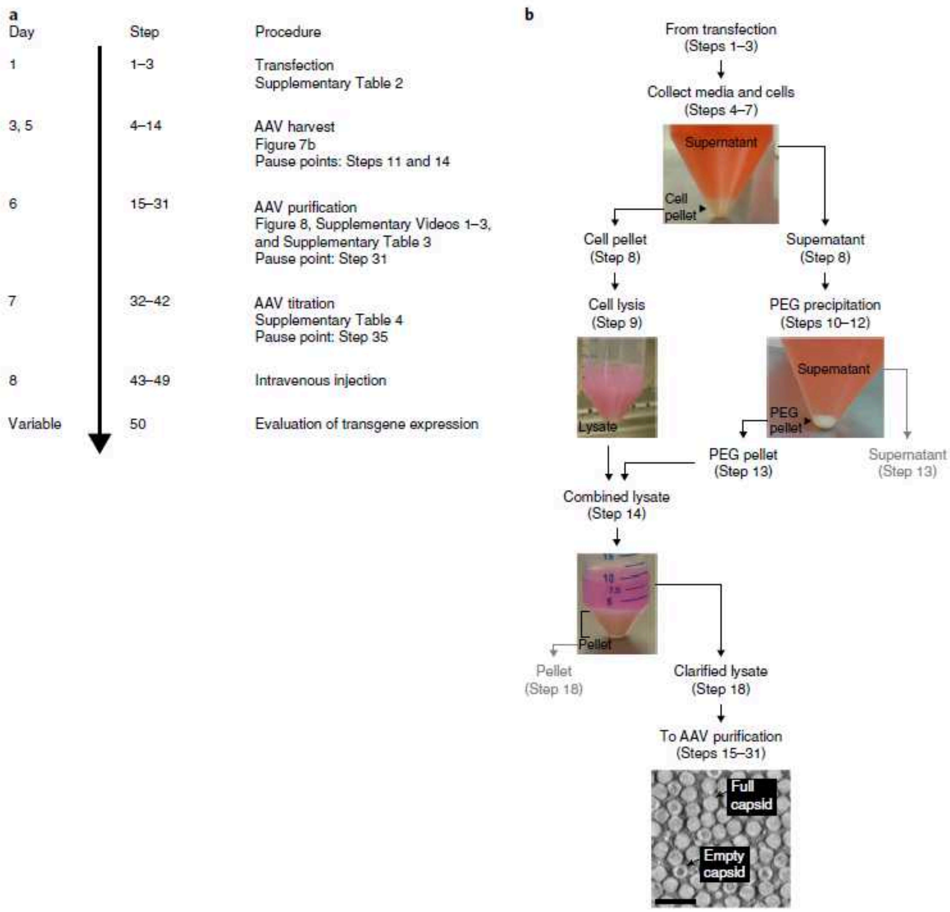

Fig. 7 | Time line and AAV harvest procedure. a, Time line of the procedure. The entire protocol spans 8 d, excluding pause points on days 5 (Steps 11 and 14), 6 (Step 31), and 7 (Step 35) and the time required to evaluate transgene expression (Step 50). Days 1–7 (Steps 1–42) constitute the AAV production stage (Fig. 1). **b**, Schematic of the AAV harvest procedure, with images corresponding to indicated steps. The iodixanol-based purification protocol does not eliminate empty capsids (i.e., capsids that fail to package an rAAV genome), as determined by negative-staining transmission electron microscopy; empty particles are characterized by an electron-dense core. Scale bar, 50 nm. Gray arrows and text denote steps at which the supernatant and pellet can be bleached and discarded (Steps 13 and 18).

Applications of the method

We anticipate that AAV-PHP capsids (Table 2) can be used to package rAAV genomes (contained in pAAV plasmids that are available through Addgene and elsewhere) (Fig. 6 and Table 1) to enable a wide range of biomedical applications. Below, we highlight current and potential applications of this method.

Table 2 | AAV-PHP capsid plasmids

	A	B	C	D	E
	AAV-PHP capsid	**Plasmid name**	**In vivo characteristics**	**Production efficiency**	**Addgene no.**

	A	B	C	D	E
	AAV-PHP.B	pUCmini-iCAP-PHP.B	Broad CNS transduction	Good	103002
	AAV-PHP.B2	pUCmini-iCAP-PHP.B2	Broad CNS transduction	Good	103003
	AAV-PHP.B3	pUCmini-iCAP-PHP.B3	Broad CNS transduction	Good	103004
	AAV-PHP.eB	pUCmini-iCAP-PHP.eB	Broad CNS transduction	Good	103005
	AAV-PHP.S	pUCmini-iCAP-PHP.S	Broad transduction in PNS and visceral organs	Good	103006
	AAV-PHP.A[a]	piCAP-PHP.A	Broad astrocyte transduction in CNS	Poor	CLOVER

AAV-PHP capsid plasmids have a built-in tTA-TRE-based inducible amplification loop to increase virus production. If the rAAV genome has a tetracycline-regulated element (e.g., TRE), the tTA on the capsid plasmid will drive a high level of expression from the TRE-containing rAAV genome, which may reduce virus production. To increase viral yields, increase the number of dishes per viral prep.
[a]Given the poor production efficiency of AAV-PHP.A, and its tendency to aggregate after purification, we suggest using AAV-PHP.eB to target astrocytes. Use an astrocyte promoter, such as GFAP, to drive transgene expression (Fig. 2a–c). To request AAV-PHP.A (listed as CLOVER in the table), visit http://www.clover.caltech.edu/. iCAP, inducible cap expression; pUCmini, pUC origin of replication.

Anatomical mapping

Fluorescent reporters are commonly used for cell type–specific mapping and phenotyping[2,13,14] (Figs. 2–5). AAV-mediated multicolor labeling (e.g., Brainbow[15]) is especially advantageous for anatomical mapping approaches that require individual cells in the same population to be distinguished from one another. We and others have demonstrated the feasibility of this approach in the brain[2,15], retina[15], heart (Fig. 4b,c), and gut[2], as well as the peripheral ganglia (Fig. 3c). Spectrally distinct labeling is well-suited for studying the organization of cells (e.g., cardiomyocytes (Fig. 4b)) in healthy and diseased tissues and long-range tract tracing of individual fibers through extensive neural networks (e.g., the enteric[2] or cardiac nervous systems (Fig. 4c)).

Functional mapping

AAV-PHP capsids are also relevant for probing cell function. AAV-PHP.B was previously used to target distinct neural circuits throughout the brain for chemogenetic[16,17] and optical imaging applications[18,19]. We predict that AAV-PHP viruses will be beneficial for manipulating neural networks that are typically difficult to access, such as peripheral circuits controlling the heart (Fig. 4d), lungs[20], or gut[21]. AAV-PHP variants could also be utilized to interrogate the function of non-neuronal cell types, including cardiomyocytes[22], pancreatic beta cells[23,24], and hepatocytes[25]. Harnessing AAV-PHP viruses to modulate cell physiology may reveal novel roles for different cells in regulating organ function and/or animal behavior[26].

Gene expression, silencing, and editing

AAV-PHP viruses can be used to test potential therapeutic strategies that would benefit from organwide or systemic transgene expression[27]. Recently, AAV-PHP.B was used to treat[16] and model[28] neurodegenerative diseases with widespread pathology. Other potential applications include gene editing (e.g., via CRISPR[29–32]) or silencing (e.g., via shRNA[33]); importantly, these approaches could be utilized to broadly and noninvasively manipulate cells in both healthy and diseased states for either basic research or therapeutically motivated studies.

AAV capsid engineering

AAV-PHP capsids can be further evolved for more efficient transduction of specific organs and cell types throughout the body. This protocol can be used for AAV engineering applications (e.g., our in vivo capsid selection method CREATE[2,3]). Using a modified transfection protocol (Steps 1–3 and online methods in ref. [3]), DNA libraries (generated by diversification of the AAV cap gene) are packaged to produce AAV capsid libraries, which are then harvested (Steps 4–14 and online methods in ref. [3]), purified (Steps 15–31), and titered (Steps 32–42). Libraries are systemically administered to Cre transgenic animals (Steps 43–49) or wild-type animals in which Cre is introduced (e.g., by AAV delivery), and Cre-dependent cap recovery from tissues of interest facilitates further rounds of selection to isolate enriched variants. This protocol can also be used to characterize novel serotypes identified with CREATE or other engineering methods[34].

Limitations of the method

A major limitation of AAV capsids, including AAV-PHP variants, is their relatively small packaging capacity (<5 kb). Some elements of the rAAV genome, such as the woodchuck hepatitis posttranscriptional regulatory element (WPRE), can be truncated[35] or removed[36,37] to accommodate larger genetic components. The development of smaller promoters[38,39] and dual expression systems[40], in which genetic elements are split between two or more viruses (requiring efficient cotransduction), has also enabled the delivery of larger genomes. Continued development of these approaches will help bypass restrictions on rAAV genome size.

Intravenous administration of AAVs also presents unique challenges. For example, systemic transduction may be undesirable for applications in which highly restricted gene expression is vital to the experimental outcome. Possible off-target transduction, due to the broad tropism of AAV-PHP variants and/or lack of compatible cell type–specific promoters, can be reduced by miRNA-mediated gene silencing. Sequences complementary to miRNAs expressed in off-target cell populations can be introduced into the 3' UTR of the rAAV genome (Fig. 6); this has been shown to reduce off-target transgene expression and better restrict expression to cell types of interest[41,42] (Fig. 2d,e).

Another challenge of systemic delivery is that it requires a high viral load, which can elicit an immune response against the capsid and/or transgene and reduce transduction efficiency in vivo[43]. Immunogenicity of AAVs may be exacerbated by empty capsid contamination in viral preparations[44,45]. The viral purification protocol (Steps 15–31) reduces, but does not eliminate, empty capsids (Fig. 7b). If this poses a concern for specific applications, viruses can be purified using an alternative approach[8,9,46].

Last, generation of viruses for systemic administration may impose a financial burden on laboratories due to the doses of virus required. Nevertheless, viral-mediated gene delivery is inexpensive compared to creating and maintaining transgenic animals. Moreover, intravenous injection is faster, less invasive, and less technically demanding than other routes of AAV administration, such as stereotaxic injection, thereby eliminating the need for specialized equipment and survival surgery training.

Experimental design

Before proceeding with the protocol, a number of factors should be considered, namely the expertise and resources available in the lab; the animal model, capsid, and rAAV genome to be used; the dose for intravenous administration; and the method(s) available for assessing transgene expression. Each of these topics is discussed below to guide users in designing their experiments.

Required expertise and resources

This protocol requires that the scientists have basic molecular biology, cell culture, and animal work experience. Users should be approved to handle laboratory animals, human cell lines, and AAVs. A background in molecular cloning is advantageous, although not necessary if relying on available plasmids.

In addition to having the above expertise, the labs must be equipped for the molecular and cell culture work relevant to the procedure; we suggest that users read through the entire 'Materials' and 'Procedure' sections beforehand to ensure that the required reagents and equipment are available and appropriate safety practices and institutional approvals are in place.

Animal model

This protocol describes the production of AAVs for intravenous delivery to 6- to 8-week-old male and female mice. AAV-PHP viruses have been validated in C57BL/6J mice[2,3,16,47] (Figs. 2–5) and numerous Cre driver lines[2,16–18], including, but not limited to, TH-IRES-Cre (Fig. 3), TRPV1-IRESCre (Figs. 3 and 4), and ChAT-IRES-Cre mice[2] (Fig. 4). Intriguingly, AAV-PHP.B demonstrates low transduction throughout the brain when systemically administered to BALB/cJ mice[48] (Fig. 5a). However, the neurotropic properties of AAV-PHP.B are not limited to the C57BL/6J strain in which they were selected. AAV-PHP.B transduces the brain more efficiently than AAV9 in both FVB/NCrl and 129S1/SvImJ mice (Fig. 5a). We also show examples of AAV-PHP.eB transducing neurons in C57BL/6NCrl (Fig. 2a–c) and 129T2/SvEmsJ mice (Fig. 5b), as well as Fischer (Fig. 5c) and Long- Evans rats (Fig. 5d). Compared to AAV9 and AAV-PHP.B, AAV-PHP.eB results in more efficient neuronal transduction in Sprague–Dawley rats after either intravenous administration or intraparenchymal stereotaxic injections[28,49]. We predict that

AAV-PHP capsids can be used in multiple species and strains for diverse applications, such as those requiring fluorescent labeling (Fig. 5a–c) and calcium imaging (Fig. 5d,e). We have not compared the transduction efficiencies of AAV9 and AAV-PHP capsids across all rodent strains and species or determined the optimal dose for transducing specific organs and cell types in different animal models. Users should test these parameters to determine the utility of AAV-PHP variants in their research. See 'Reagents' for mouse and rat catalog numbers.

Selecting an AAV-PHP capsid

We recommend choosing an AAV-PHP capsid (Table 2) based on its tropism and production efficiency. Capsid properties are listed in Supplementary Table 1; we include species, organs, and cell populations examined to date and note typical viral yields. We anticipate that most researchers will use AAV-PHP.eB (Addgene, plasmid no. 103005) or AAV-PHP.S (Addgene, plasmid no. 103006) in their experiments. AAV-PHP.eB and AAV-PHP.S produce viral yields similar to those of other high-producing naturally occurring serotypes (e.g., AAV9) and enable efficient, noninvasive gene transfer to the CNS or PNS and visceral organs, respectively[2] (Figs. 2–5).

The earlier capsid variants, which provide widespread CNS transduction, either produce suboptimal yields (AAV-PHP.A)3 or have since been further evolved for enhanced transduction efficiency in vivo (AAV-PHP.B (Addgene, plasmid no. 103002))2. We therefore recommend using AAV-PHP.eB for CNS applications, especially when targeting neurons. Note, however, that the chosen capsid will ultimately depend on the experimental circumstances; multiple factors, including species[50], strain[48] (Fig. 5), age[51], gender[52], and health[53], can influence AAV tropism. Testing the AAV-PHP variants in a variety of experimental paradigms will continue to reveal the unique attributes of each capsid and identify those most suitable for different applications.

Selecting an rAAV genome

Users must select an rAAV genome, contained in a pAAV plasmid, to package into the capsid (Figs. 1 and 6; Table 1). In Table 1, we list the pAAVs used here (Figs. 2–4) and in our previous work[2,3]; we direct users to Addgene's plasmid repository for additional pAAVs developed for various applications.

Depending on the experimental aims, users can elect to design their own genomes[54] and clone from existing pAAVs. When customizing plasmids, it is imperative that the rAAV genome, the sequence between and including the two inverted terminal repeats (ITRs), does not exceed 4.7–5 kb (Fig. 6); larger genomes will not be fully packaged into AAV capsids, resulting in truncated genomes and low titers. The ITRs are 145-bp sequences that flank the expression cassette and are required for replication and encapsidation of the viral genome. ITRs are typically derived from the AAV2 genome and must match the serotype of the rep gene contained in the capsid plasmid; pUCmini-iCAP-PHP plasmids contain the AAV2 rep gene and are therefore capable of packaging genomes with AAV2 ITRs (i.e., almost any pAAV available from Addgene). Other genetic components (e.g., promoters, transgenes, localization signals, and recombination schemes) are interchangeable and can be customized for specific applications (Fig. 6).

Dosage for intravenous administration

The optimal dose for intravenous administration to target cell populations must be determined empirically. We encourage users to refer to Figs. 2–5 and related work for suggested AAV-PHP viral doses. AAV-PHP variants have been successfully administered to adult mice[2,3,16,47] (Figs. 2–5), neonatal mice16, and neonatal and adult rats[28,49] (Fig. 5c,d) for fluorescent labeling; they have also been used for calcium imaging[18,19] and optogenetic (Fig. 4d), chemogenetic[16,17], and therapeutic applications[16,28].

We typically administer between 1 × 10^{11} and 5 × 10^{11} vg of AAV-PHP.eB or between 3 × 10^{11} and 1 × 10^{12} vg of AAV-PHP.S to adult mice (6–8 weeks old). However, dosage will vary depending on the target cell population, desired fraction of transduced cells, and expression level per cell. AAVs independently and stochastically transduce cells, typically resulting in multiple genome copies per cell[2]. Therefore, higher doses generally result in strong expression (i.e., high copy number) in a large fraction of cells, whereas lower doses result in weaker expression (i.e., low copy number) in a smaller fraction of cells. To achieve high expression in a sparse subset of cells, users can employ a two-component system in which transgene expression is dependent on co-transduction of an inducer (e.g., a vector expressing Cre[55], Flp[2], or the tetracycline-controlled transactivator (tTA)[2]); inducers are injected at a lower dose (typically 1 × 10^{9} to 1 × 10^{11} vg) to limit the fraction of cells with transgene expression. Note that transgenes and gene regulatory elements (e.g., enhancers, promoters, and miRNA target sequences (Fig. 2d,e)) can influence gene expression levels. Therefore, users should assess transgene expression from a series of doses and at several time points after intravenous delivery to determine the optimal experimental conditions.

Evaluation of transgene expression

Following in vivo delivery, AAV transduction and transgene expression increase over the course of several weeks. Although expression is evident within days after transduction, it does not reach a steady-state level until at least 3–4 weeks after transduction. Therefore, we suggest waiting for a minimum of 2 weeks before evaluating fluorescent labeling[2,3,16,28] (Figs. 2–5) and at least 3–4 weeks before beginning optogenetic (Fig. 4d), chemogenetic[16,17], and calcium imaging[18,19] experiments. Note that, like other AAVs, AAV-PHP variants are capable of providing long-term transgene expression. AAV-PHP.B-mediated cortical expression of a genetically encoded calcium indicator, GCaMP6s, was reported to last at least 10 weeks post-injection without toxic side effects[19] (i.e., nuclear filling56), and we have observed GFP expression throughout the brain >1 year after viral administration (see Supplementary Fig. 4 in ref. [3]). However, the time points suggested here are only meant to serve as guidelines; gene expression is contingent on multiple factors, including the animal model, capsid, genome, and dose.

The appropriate method(s) for evaluating transgene expression will vary among users and may include functional (e.g., optical imaging[56]), histological[57] (e.g., using endogenous fluorescence, antibodies, or molecular probes), or molecular (e.g., Western blot[58] or qPCR3) approaches[59]. To assess transduction efficiency across different organs, users can perform a qPCR-based vector biodistribution assay, in which vector genomes are quantified and normalized to the mouse genome (e.g., a housekeeping gene)[3]. Other approaches typically involve examining fluorescent protein expression in thin or thick (≥100 µm) tissue samples. The CLARITY-based methods such as passive CLARITY technique (PACT) and perfusion-assisted agent release in situ (PARS)[60] render thick tissues optically transparent while preserving their three-dimensional molecular and cellular architecture, and facilitate deep imaging of large volumes (e.g., using confocal or light-sheet microscopy)[61–63]. Cleared tissues are compatible with endogenous fluorophores, including commonly used markers such as GFP[3,57,60], eYFP[60], and tdTomato[57]. However, some fluorescent signals, such as those from mTurquoise2, mNeonGreen, and mRuby2, can deteriorate in chemical clearing reagents. To visualize these reporters, we suggest using optical clearing reagents such as refractive index-matching solution (RIMS)[57] or ScaleSQ[64] (Figs. 3a,c,d, 4b–d, and 5e) or commercially available mounting media (Step 50) (Fig. 5a,c). Some fluorescent proteins are sensitive to photobleaching. For example, mRuby2 may bleach over long imaging sessions or at high magnification; tdTomato exhibits similar spectral properties and may be a more suitable alternative, given its photostability[65]. Also, note that autofluorescent lipofuscin accumulates in aging postmitotic tissues (e.g., the brain and heart)[66] and may interfere with examination of transduced cells; in this case, either reduce autofluorescence using histological methods[57,67] or, if possible, inject younger adults (≤8 weeks old) and determine the minimum time required for transgene expression.

Troubleshooting

Troubleshooting advice can be found in Table 3.

Table 3 | Troubleshooting table

A	B	C	D
Step	**Problem**	**Possible reason**	**Solution**
24 (Transfection)	Transfection solution is not cloudy	DNA–PEI complexes have not formed	Thoroughly vortex the transfection solution for 10 s and incubate at RT for 2–10 min before use; always use PEI at RT
		Transfection miscalculation	Carefully follow the instructions in the Reagent setup and Supplementary Table 2 (‘Transfection calculator’ sheet) to prepare the PEI + DPBS master mix and DNA + DPBS solutions
25 (Transfection)	Low or no fluorescent protein expression post transfection	Low DNA purity	Use an endotoxin-free plasmid purification kit to isolate plasmids; assess DNA purity (i.e., 260/ 280 and 260/230 ratios) before transfection
		Mutations in plasmids	Verify the integrity of pAAV plasmids by sequencing and restriction digestion before transfection
		Poor cell health	Maintain cells in an actively dividing state at recommended

	A	B	C	D
				ratios (Reagent setup). Ensure cells are not over-confluent at the time of transfection, and change media no more than 24 h post transfection
			Weak fluorescent reporter and/or promoter, or promoter cannot initiate gene expression in HEK293T cells	Include a positive transfection control (e.g., pAAV-CAG-eYFP). Note that some promoters may take 2–3 d to show expression
			Transgene expression depends on Flp or Cre recombinase	Include a positive transfection control (see above)
			Transfection miscalculation	Carefully follow the instructions in the Reagent setup and Supplementary Table 2 (‘Transfection calculator’ sheet) to prepare the PEI + DPBS master mix and DNA + DPBS solutions
	31 (AAV harvest)	Cell lysate is not pink	pH of the lysate is too low	Check the pH of the lysate by pipetting 30 µl onto a pH strip; adjust the pH to 8.5 with NaOH suitable for cell culture. In subsequent viral preps, ensure that the pH of SAN digestion buffer is ~10.0; during cell lysis, the pH should drop to 8.5–9.0, which is optimal for SAN digestion
			Fluorescent protein expression from a strong promoter (e.g., CAG)	Expression of blue/green or red fluorescent proteins from strong promoters can cause the lysate to turn yellow or red, respectively; proceed with AAV production
	38 (AAV purification)	Density gradients have no clear delineation between iodixanol layers	Layers are mixed	Repour the gradients (Supplementary Video 1, 0:00–1:45, or Supplementary Video 2, 0:00–1:13); gradients should be poured fresh and not allowed to sit for too long
	46(AAV purification)	Tube collapsed during ultracentrifugation	An air bubble was trapped underneath the black cap	Carefully remove the tube from the rotor and wipe it with fresh 10% (vol/vol) bleach before proceeding with AAV purification. In future viral preps, remove air bubbles with a P200 pipette before ultracentrifugation

	A	B	C	D
			The rotor and/or OptiSeal tubes were not in proper working order	Carefully remove the tube from the rotor and wipe it with fresh 10% (vol/vol) bleach before proceeding with AAV purification. In future viral preps, check that the rotor and tubes are completely dry; moisture between tubes and the tube cavity can cause tubes to collapse. Also check tubes for dents before pouring the density gradients
	48 (AAV purification)	Cannot puncture the OptiSeal tube with the needle	Not enough force is used	Use a forward-twisting motion to insert the needle into the tube (Supplementary Video 3, 0:06–0:21); practice on an OptiSeal tube filled with water
		Two holes were punctured through the OptiSeal tube	Too much force was used	See above. Do not remove the needle; carefully insert a new needle and proceed to collect virus
		Cannot collect virus with the needle	Black cap was not removed	Use the tube removal tool to remove the black cap from the tube after inserting the needle but before collecting virus (Fig. 8g and Supplementary Video 3, 0:22–0:30); practice on an OptiSeal tube filled with water
			Plastic from the tube is lodged inside the needle	Firmly replace the black cap and remove the needle from the tube; insert a new needle into the same hole, remove the black cap, and try collecting virus again
		Density gradient flows out of the needle hole in the tube after removal of the needle	Black cap was not firmly replaced	Act quickly; use the beaker of bleach to catch the liquid and firmly replace the black cap to stop the flow. In subsequent viral preps, ensure that the black cap is replaced before removing the needle from the tube (Supplementary Video 3, 1:19–1:30); practice on an OptiSeal tube filled with water
	53 (AAV purification)	Purified virus is cloudy	The virus/DPBS mixture was not mixed and contains iodixanol	Repeat the buffer exchanges in Steps 28–30. In future viral preps, thoroughly mix the virus/DPBS mixture using a P1000 pipette in Steps 27, 29, and 30

	A	B	C	D
		Unknown material is suspended in purified virus	Salt, DNA, or viral precipitation	Before titering or injecting the virus, spin down the precipitate at 3,000g for 5 min at RT and transfer the supernatant (i.e., the virus) to a new screwcap vial. We have not noticed a decrease in titer after removing precipitate from our preps; however, it is a good practice to re-titer a virus if precipitate has formed
			Bacterial contamination	Bleach the virus. In future viral preps, filtersterilize viruses after purification, and only open tubes containing viruses in a biosafety cabinet. During intravenous injections, never use the original virus stock; bring only an aliquot of what is needed for injection
			Carry-over from the filtration membrane of the Amicon filter device	Filter-sterilize the virus
	64 (AAV titration)	No SYBR signal detected for DNA standards or virus samples	Missing reagents (e.g., primers) in qPCR reaction	Check that all qPCR reagents were added to the master mix and that the DNA standards and virus samples were added to their respective wells
			Degraded reagents	Use fresh, properly stored qPCR reagents
		No SYBR signal detected for virus samples	DNase was not inactivated, resulting in degradation of the viral genome during proteinase K treatment	Repeat the titration procedure; be sure to inactivate DNase with EDTA at 70 °C (Step 34)
			Proteinase K was not inactivated, resulting in degradation of DNA polymerase during qPCR	Repeat the titration procedure; be sure to inactivate Proteinase K at 95 °C (Step 37)
		Triplicates do not have similar Ct values	Inaccurate pipetting and/or inadequate mixing of reagents	Repeat the qPCR; pipette accurately and thoroughly mix all reagents before use
		Standard curve is not linear	Inaccurate pipetting and/or inadequate mixing of reagents while preparing the DNA standard dilutions	Repeat the qPCR; pipette accurately and thoroughly mix all reagents while preparing the DNA standard dilutions. Note that at low concentrations (high Ct values), standard nos. 1 and 2

	A	B	C	D
				will deviate from linearity (Supplementary Table 4, ‘Example’ sheet). This is normal; the qPCR does not need to be repeated
			DNA standards degraded and/or stuck to the walls of 1.5-ml tubes	Repeat the qPCR; prepare the DNA standard dilutions fresh, immediately before use, and use only DNA/RNA LoBind microcentrifuge tubes. Note that at low concentrations (high Ct values), standard nos. 1 and 2 will deviate from linearity (Supplementary Table 4, ‘Example’ sheet). This is normal; the qPCR does not need to be repeated
		Viral yield is lower than expected (Supplementary Table 4, cell K27)	Transfection, AAV harvest, AAV purification, and/or AAV titration were not successful	Include a positive transfection/virus production control (e.g., pAAV-CAG-eYFP) and a positive titration control. To determine at which stage the virus may have been lost, collect a 30-µl sample from the cell lysate (Step 9), the media before PEG precipitation (Step 10), the PEG pellet resuspension (Step 14), and the lysate before (Step 18) and after iodixanol purification (Step 26). Store samples at 4 °C for up to 1 week for titering (Steps 32–42)
			AAV capsid and/or genome results in poor production efficiency	Scale up viral preps to ensure enough virus is produced for downstream applications
			ITRs underwent recombination during bacterial growth	After plasmid purification, but before transfection, digest pAAVs with SmaI to confirm the presence of ITRs, which are required for replication and encapsidation of the viral genome; always propagate pAAVs in recombination-deficient bacterial strains
	65 (intravenous injection)	A large volume (e.g., more than 100 µl, depending on user preference) of virus needs to be injected	Virus concentration is too low	Reconcentrate the virus using an Amicon filter device. Add 13 ml of DPBS and the virus to the top chamber of the Amicon filter device and use a P1000 pipette to mix. Centrifuge at 3,000g at RT until the desired volume of solution remains in the top chamber

	A	B	C	D
	70 (Intravenous injection)	Virus spills out of the eye during injection	Incorrect needle placement	Absorb the spilled virus using a paper towel; disinfect AAV-contaminated surfaces and materials with fresh 10% (vol/vol) bleach or an equivalent disinfectant. Load the same insulin syringe with more virus, position the needle behind the globe of the eye in the retro-orbital sinus, and try the injection again. Practice injections using DPBS or saline until comfortable with the procedure
		Bleeding before, during, or after injection	Incorrect needle placement	Position the needle behind the globe of the eye in the retro-orbital sinus; never puncture the eye itself. Inject the virus slowly; following injection, carefully remove the needle at the same angle at which it was inserted. Practice injections using DPBS or saline until comfortable with the procedure
			Needle is left in the injection site for too long	Once the needle is correctly placed in the eye, immediately inject the virus
	72 (Evaluation of transgene expression)	Weak or no transgene expression in the tissue of interest	Sufficient time has not passed for protein expression	Wait longer for optimal protein expression
			Dose is too low, or dose is too high, causing cell toxicity	Inject multiple animals with a series of doses and sacrifice them at different time points (e.g., weekly) to determine the optimal dose
			Titer is inaccurate	Re-titer viruses before injection if more than 1 month has passed since titration; this will ensure that the animals are administered the most accurate dose possible
			Virus degraded	See above. Store AAV-PHP viruses at 4 °C for up to 3 months, during which time we have not noticed a decrease in titers or transduction efficiency in vivo. Do not store diluted viruses; dilute only what is needed immediately before retro-orbital injection

	A	B	C	D
			Weak or no expression from the AAV genome	Verify the integrity of pAAV plasmids by sequencing and restriction digestion before transfection. If possible, verify viral transduction and transgene expression in vitro before systemic administration
			Poor viral injection	Inject multiple animals to increase the chance of success
			Fluorescent protein and/or signal deteriorates in chemical-clearing reagents	Ensure that the chosen clearing protocol is compatible with the fluorescent protein(s) under investigation (see 'Experimental design' and 'Anticipated results' sections for details)
		Fluorescent signal photobleaches during imaging	Fluorescent protein is sensitive to photobleaching (e.g., during long imaging sessions or at high magnification)	Use a different fluorescent protein with similar spectral properties but higher photostability (e.g., tdTomato rather than mRuby2, or eGFP rather than Emerald)
		Lipofuscin accumulation	Aging tissue	Reduce autofluorescence using histological methods (e.g., Sudan black) or, if possible, inject younger adults (≤8 weeks old) and determine the minimum time required for transgene expression

Timing

Refer to Fig. 7a for a time line of the Procedure.
Steps 23–25, triple transient transfection of HEK293T cells: 1–2 h
Steps 26–36, AAV harvest: 5 d
Steps 37–53, AAV purification: 1 d
Steps 54–64, AAV titration: 1 d
Steps 65–71, intravenous (retro-orbital) injection: <5 min per mouse, excluding setup and cleanup time
Step 72, evaluation of transgene expression: variable

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Note

Competing interests

The California Institute of Technology has filed patent applications related to (but not on) this work: Recombinant AAV Capsid Protein (US patent no. 9,585,971); Selective Recovery (US patent application no. 15/422,259); Targeting Peptides for Directing Adeno-Associated Viruses (AAVs) (US patent application no. 15/374,596). The authors declare no other competing interests.

Materials

Biological materials

Note

! **CAUTION** To address the issue of cell line misidentification and cross-contamination, it is recommended that cell lines be regularly checked to ensure they are authentic and are not infected with mycoplasma.

- Plasmids, supplied as bacterial stabs (Addgene; see Tables 1 and 2 for plasmids used in this and related work)

Note

CRITICAL Three plasmids (pAAV, pUCmini-iCAP-PHP, and pHelper) are required for transfection (Fig. 1). The pHelper plasmid is available in Agilent's AAV helper-free kit
AAV Helper-Free System **Agilent Technologies Catalog #**240071

- Human embryonic kidney (HEK) cells (293 or 293T;
Human Embryonic Kidney (HEK293) Cells **ATCC Catalog #**CRL-1573 or
293T **ATCC Catalog #**CRL-3216™ , respectively)

Note

! **CAUTION**
HEK cells pose a moderate risk to laboratory workers and the surrounding environment and must be handled according to governmental and institutional regulations. Experiments involving HEK cells were performed using biosafety level 2 practices as required by the California Institute of Technology and the US Centers for Disease Control and Prevention.

CRITICAL
HEK293 and HEK293T cells constitutively express two adenoviral genes, E1a and E1b, which are required for AAV production in these cells[7]; we do not recommend using an alternative producer cell line with this protocol.

- Plasmid DNA containing the target sequence to be amplified during AAV titration; used for preparing the DNA standard stock.

Note

CRITICAL The plasmid used to make the DNA standard must contain the same target sequence as the pAAV plasmid used to generate virus. The target sequence must be within the rAAV genome; we typically amplify a portion of the WPRE or hGH polyA (see Fig. 6 caption for abbreviations and 'Reagents' for primer sequences).

- Animals to be injected. Wild-type mouse strains used in this work include C57BL/6J (Jackson Laboratory, stock no. 000664), C57BL/6NCrl (Charles River Laboratories, strain code 027), FVB/NCrl (Charles River Laboratories, strain code 207), 129S1/SvImJ (Jackson Laboratory, stock no. 002448), and 129T2/SvEmsJ (Jackson Laboratory, stock no. 002065). Cre driver lines include ChAT-IRES-Cre (Jackson Laboratory, stock no. 028861, heterozygous), TH-IRES-Cre (European Mutant Mouse Archive, stock no. EM00254, heterozygous), and TRPV1-IRES-Cre mice (Jackson Laboratory, stock no. 017769, homozygous). Fischer rats (Charles River Laboratories, strain code 002) and Long-Evans rats (Charles River Laboratories, strain code 006) were used in Fig. 5. All rats were 4–6 weeks old at the time of AAV administration; mice were 6–10 weeks old. Refer to the 'Experimental design' section, Fig. 5, and Supplementary Table 1 for species and strain considerations.

Note

! CAUTION Experiments on vertebrates must conform to all relevant governmental and institutional regulations. Animal husbandry and experimental procedures involving mice and rats were approved by the Institutional Animal Care and Use Committee (IACUC) and the Office of Laboratory Animal Resources at the California Institute of Technology.

- For molecular cloning: Recombination-deficient *Escherichia coli* strains such as

NEB Stable Competent E.coli (High Efficiency) - 20×0.05 ml **New England Biolabs Catalog** #C3040H ,

Invitrogen™ One Shot™ Stbl3™ Chemically Competent E. coli **Fisher Scientific Catalog** #C737303 , or

SURE 2 Supercompetent Cells **Agilent Technologies Catalog** #200152 .

Reagents

Plasmid DNA preparation

- Agarose **VWR International (Avantor) Catalog** #N605-250G
- Antibiotics (e.g., Carbenicillin disodium salt **Alfa Aesar Catalog** #J61949 ; all plasmids used in this work carry antibiotic resistance genes to ampicillin/carbenicillin)

- Quick-Load Purple 1 kb Plus DNA Ladder - 250 gel lanes **New England Biolabs Catalog #**N0550S
- Lysogeny broth (LB; Amresco, cat. no. J106-1KG)

Note

CRITICAL For large-scale plasmid preparations, such as maxi and giga preps, we typically use Plasmid+ media (Thomson Instrument, cat. no. 446300), an enriched medium formulated to support higher cell densities and plasmid yields, as compared to those of LB.

- LB Broth with agar (Miller) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**L3147-1KG
- NucleoBond Xtra Maxi EF Maxi kit for endotoxin-free plasmid DNA **Macherey-Nagal Catalog #**740424.50

Note

CRITICAL Triple transient transfection requires large amounts of pUCmini-iCAP-PHP (22.8 µg/dish) and pHelper plasmid DNA (11.4 µg/dish) (Supplementary Table 2, 'Detailed calculations' sheet); isolating these plasmids may be more convenient with a giga-scale purification kit (NucleoBond PC 10000 EF; Macherey-Nagel, cat. no. 740548). All plasmids should be purified under endotoxin-free conditions. Endotoxin contamination in plasmid preparations can reduce transfection efficiency, and contaminating endotoxins in viral preparations could elicit immune reactions in mammals in vivo.

- Restriction enzymes, including SmaI - 2,000 units **New England Biolabs Catalog #**R0141S ; used for verifying plasmid and ITR integrity
- Sequencing primers (Integrated DNA Technologies); used for verifying plasmid sequence integrity
- SYBR SAFE DNA stain **Invitrogen - Thermo Fisher Catalog #**S33102
- TAE Buffer (Tris-acetate-EDTA) (50X) **Thermo Fisher Catalog #**B49

Cell culture

- Gibco™ DMEM high glucose GlutaMAX™ Supplement pyruvate **Fisher Scientific Catalog #**10-569-044
- Ethanol, absolute **J.T. Baker Catalog #**8025

Note

! **CAUTION** Ethanol is flammable.

- FBS (GE Healthcare, cat. no. SH30070.03)

Note

CRITICAL
Divide into aliquots and store at 20 °C for up to 1 year. Avoid freeze–thaw cycles.

- Gibco™ MEM Non-Essential Amino Acids Solution (100X) **Fisher Scientific Catalog** #11-140-050
- Gibco™ Penicillin-Streptomycin (5000 U/mL) **Fisher Scientific Catalog** #15-070-063

Note

CRITICAL Divide into aliquots and store at −20°C for up to 1 year. Avoid freeze–thaw cycles.

- Gibco™ TrypLE™ Express Enzyme (1X) phenol red **Fisher Scientific Catalog** #12-605-036

Transfection

- Polyethylenimine Linear MW 25000 Transfection Grade (PEI 25K™) **Polysciences, Inc. Catalog** #23966-1

Note

CRITICAL Compared to other commonly used transfection reagents (e.g., Lipofectamine or calcium phosphate), PEI is less expensive, given the scale of transfection, and produces high viral yields (≥1 × 10^{12} vg/dish), which are needed for systemic administration.

- Water For Injection (WFI) for Cell Culture **Thermo Fisher Catalog** #A1287304
- Gibco™ DPBS no calcium no magnesium **Fisher Scientific Catalog** #14-190-250
- Hydrochloric acid solution 1.0 N **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #H9892

Note

! **CAUTION** HCl is corrosive. Use personal protective equipment.

AAV production

- 10% (vol/vol) Bleach (prepare fresh from concentrated liquid bleach (e.g., Clorox))

Note

CRITICAL AAV-contaminated equipment, surfaces, and labware must be disinfected for 10 min with fresh 10% (vol/vol) bleach; ethanol is not an effective disinfectant against non-enveloped viruses. AAV waste disposal should be conducted according to federal, state, and local regulations.

- Dry ice; optional
- Potassium chloride 99.0-100.5% granular AR® ACS Macron Fine Chemicals™ **Avantor Sciences Catalog** #6858-06
- Magnesium chloride hexahydrate 99.0-102.0% crystals AR® ACS Macron Fine Chemicals™ **VWR International (Avantor) Catalog** #MACR5958-04
- Sodium Chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #SX0420
- OptiPrep - Density Gradient Media (Iodixanol) **Cosmo Bio Catalog** #AXS-1114542
- Phenol red solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #1072420100
- Pluronic F-68 **Gibco - Thermo Fisher Scientific Catalog** #24040-032 (optional)
- Polyethylenglycol (MW=8000) **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #89510-1KG-F
- Salt Active Nuclease High Quality (Bioprocessing grade) **Arcticzymes Catalog** #70910-202 (SAN; 25 U/µl
- Tris ultra pure >99.9% **MP Biomedicals Catalog** #819620
- Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water **Fisher Scientific Catalog** #10-977-023
- Water For Injection (WFI) for Cell Culture **Thermo Fisher Catalog** #A1287304
- Gibco™ DPBS no calcium no magnesium **Thermo Fisher Scientific Catalog** #14190250

AAV titration

- Calcium chloride anhydrous ≥96.0% (by argentometric titration) BAKER ANALYZED® ACS J.T.Baker® **JT Baker Catalog** #1311-01
- DNAse I **Roche Catalog** #04716728001
- Magnesium chloride hexahydrate 99.0-102.0% crystals AR® ACS Macron Fine Chemicals™ **VWR International (Avantor) Catalog** #MACR5958-04
- NaCl (Millipore, cat. no. SX0420-3)
- N-Lauroylsarcosine sodium salt **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #L9150-50G
- Primers corresponding to the target sequence to be amplified during qPCR (Integrated DNA Technologies)
  WPRE-forward: GGCTGTTGGGCACTGACAAT
  WPRE-reverse: CCGAAGGGACGTAGCAGAAG
  hGH polyA-forward: GTGCCCACCAGCCTTGTC

hGH polyA-reverse: TGTCTTCCCAACTTGCCCCTT

Note

CRITICAL The proximity of the primer binding sites to the ITRs can affect titering results; do not use primers corresponding to the ITRs. Note that titers measured with different primers or across laboratories may not be directly comparable.

- Proteinase K (recombinant, PCR grade; 50 U/ml (2.5 U/mg); Proteinase K rec. PCR Grade lyo. **Roche Catalog** #03115828001)
- Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog** #Q32854
- ScaI-HF - 1,000 units **New England Biolabs Catalog** #R3122S or other enzyme that cuts outside of the rAAV genome and within the pAAV backbone
- FastStart Universal SYBR Green Master (Rox) **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #4913850001
- Tris ultra pure >99.9% **MP Biomedicals Catalog** #819620
- Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog** #10977023
- UltrapPure 0.5M EDTA pH 8.0 **Invitrogen - Thermo Fisher Catalog** #15575020
- 1M Tris-HCl pH=7.5 **Invitrogen - Thermo Fisher Catalog** #15567-027

Intravenous (retro-orbital) injection

- 10% (vol/vol) Bleach, prepared fresh, or equivalent disinfectant (e.g., Accel TB surface cleaner; Health Care Logistics, cat. no. 18692)
- Isoflurane USP Liquid for Inhalation. **Piramal Heathcare Catalog** #NDC 66794-017-25

Note

! CAUTION Isoflurane is a halogenated anesthetic gas associated with adverse health outcomes in humans and must be handled according to governmental and institutional regulations. To reduce the risk of occupational exposure during rodent anesthesia, waste gas was collected in a biosafety cabinet using a charcoal scavenging system as approved by the California Institute of Technology.

- Proparacaine hydrochloride ophthalmic solution, USP (0.5% (wt/vol); Akorn Pharmaceuticals, cat. no. 17478-263-12)
- Gibco™ DPBS no calcium no magnesium **Fisher Scientific Catalog** #14-190-250

Equipment

Plasmid DNA preparation equipment

- Centrifuge (Beckman Coulter, model no. Allegra X-15R)
- Gel electrophoresis system (Bio-Rad horizontal electrophoresis system)
- Gel-imaging system (Bio-Rad, Gel Doc EZ system)
- Incubating shaker (Eppendorf, model no. I24)
- Incubator (Thermo Fisher Scientific, Heratherm model) or 37°C warm room
- Sequence-editing and annotation software (e.g., Lasergene by DNASTAR (https://www.dnastar.com/software/lasergene/), SnapGene by GSL Biotech (http://www.snapgene.com/), or Vector NTI by Thermo Fisher Scientific (https://www.thermofisher.com/us/en/home/life-science/cloning/vector-ntisoftware.html))
- Spectrophotometer (Thermo Fisher Scientific, NanoDrop model)

Plasmid DNA preparation supplies

Falcon® 100 mm x 15 mm Not TC-treated Bacteriological Petri Dish 20/Pack 500/Case Sterile **Corning Catalog #**351029

- Falcon® 14 mL Round Bottom High Clarity PP Test Tube Graduated with Snap Cap Sterile 25/Pack 50 **Corning Catalog #**352059
- Ultra Yield flasks and AirOtop seals (250 ml; Thomson Instrument Company, cat. nos. 931144 and 899423, respectively); use with Plasmid+ media. Alternatively, use LB and standard Erlenmeyer flasks.

AAV production equipment

- Biological safety cabinet.

Note

! **CAUTION** HEK293T cells and AAVs are biohazardous materials and must be handled according to governmental and institutional regulations. All experiments involving the aforementioned materials were performed in a Class II biosafety cabinet with annual certification as required by the California Institute of Technology and the US Centers for Disease Control and Prevention.

- Centrifuge that can reach speeds up to 4,000g, refrigerate to 4°C, and accommodate 250-ml conical centrifuge tubes (Beckman Coulter, model no. Allegra X-15R).
- Fluorescence microscope for cell culture (Zeiss, model no. Axio Vert A1)
- Incubator for cell culture (humidified at 37 °C with 5% CO_2;

Equipment	
Heracell™ 240i CO2 Incubator, 240L	NAME
CO2 Incubator	TYPE
Thermo Scientific™	BRAND
51032875	SKU
https://www.thermofisher.com/order/catalog/product/51032875	LINK

)

- Laboratory balance (with a readability of 5–10 mg)
- Support stand with rod and clamp (VWR International, cat. nos. 12985-070, 60079-534, and 89202-624, respectively) (Fig. 8f)

Equipment	
Talon® Support Stands, Cast Iron, Rectangular Base	NAME
Support Stands	TYPE
VWR®	BRAND
12985-070	SKU
https://us.vwr.com/store/product/4617761/vwr-talon-support-stands-cast-iron-rectangular-base	LINK

Equipment	
Talon® Rods, Stainless Steel	NAME
Rods	TYPE
VWR®	BRAND
60079-534	SKU
https://us.vwr.com/store/product/4617540/vwr-talon-rods-stainless-steel	LINK

Equipment	
Talon® Two- and Three-Prong Swivel Clamps	NAME
Swivel Clamps	TYPE
VWR®	BRAND
89202-624	SKU
https://us.vwr.com/store/product?keyword=89202-624	LINK

- Ultracentrifuge (preparative ultracentrifuge for in vitro diagnostic use;

Equipment

Optima XE-90	NAME
Ultracentrifuge	TYPE
Beckman Coulter	BRAND
A94471	SKU
https://www.beckman.com/centrifuges/ultracentrifuges/optima-xe/a94471	LINK

, with a Type 70Ti fixed-angle rotor).

Note

! **CAUTION** During ultracentrifugation, rotors are subjected to enormous forces (350,000g in this protocol). Rotor failure can have catastrophic consequences, including irreparable damage to the centrifuge and laboratory and fatal injuries to personnel. Inspect the rotors for signs of damage or weakness before each use, and always follow the manufacturer's instructions while operating an ultracentrifuge.

- Water bath (Fisher Scientific, Isotemp model)

AAV production supplies

- Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-100 membrane **Merck Millipore (EMD Millipore) Catalog #**UFC910024
- Olympus Plastics 23-430 1000µl Reach Olympus Premium Barrier Tips Low Binding Racked Sterile 8 R **Genesee Scientific Catalog #**23-430
- Falcon® Cell Scrapers with 25 cm Handle and 3.0cm Blade Sterile Individually Packaged 100/Case **Corning Catalog #**353089
- Centrifuge Bottle Rack for 250mL Tubes (6-Well) **Universal Medical Catalog #**HS23224 or empty beakers
- Conical centrifuge tubes (50 ml, 250 ml, and 500 ml (optional);
  Falcon® 50 mL High Clarity PP Centrifuge Tube Conical Bottom Sterile 25/Rack 500/Case **Corning Catalog #**352098, 430776, 431123
  respectively)
- Costar Spin-X centrifuge tube filters (Corning, cat. no. 07-200-385); optional
- Empty, sterile media bottles

- 32.4 mL OptiSeal Polypropylene Tube 26 × 77mm - 50Pk **Beckman Coulter Catalog** #361625 ; includes black caps
- 32.4 mL Tube Kit Polypropylene OptiSeal Tubes 26 × 77mm **Beckman Coulter Catalog** #361662 ; includes a tube rack, spacers, and spacer- and tube-removal tools

Equipment

Portable Pipet-Aid® XL Pipette Controller	NAME
Pipette Controller	TYPE
Drummond Scientific	BRAND
4-000-105	SKU
https://www.drummondsci.com/product/pipet-aid/portable-pipet-aid-xl/	LINK

Note

CRITICAL Use a pipetting device with precise control to pour the density gradients (Step 38) and load the virus (Step 40).

- pH-indicator strips pH 7.5 - 14 **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #109532
- pH-indicator strips pH 2.0 - 9.0 **Merck Millipore (EMD Millipore) Catalog** #109584
- Screw-cap vials 1.6ml **National Scientific Catalog** #BS16NA-PS
- Serological pipettes (2 ml, 5 ml, 10 ml, 25 ml, and 50 ml; Falcon® 2 mL Serological Pipet Polystyrene 0.01 Increments Individually Packed Sterile 100/Box **Corning Catalog** #356507 , and Genesee Scientific, cat. nos. 12-102, 12-104, 12-106, and 12-107, respectively)

Note

CRITICAL Corning brand 2-ml serological pipettes consistently fit into OptiSeal tubes while pouring the density gradients (Step 38.1) and loading the virus (Step 40); other brands should be tested before use.

- Stericup sterile vacuum filtration system (0.22 µm; 1 liter; Stericup Quick Release-GP Sterile Vacuum Filtration System **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #S2GPU11RE)
- Sterile bottles (500 ml; VWR® Round Media Bottles **VWR International (Avantor) Catalog** #89166-106)
- Syringes (5 ml and 10 ml; 5 mL BD Luer-Lok™ Syringe sterile single use **Becton Dickinson (BD) Catalog** #309646 and 309604, respectively)
- Syringe filter units (0.22 µm; Millex-GP Syringe Filter Unit, 0.22 µm **Merck Millipore (EMD Millipore) Catalog** #SLGP033RS)
- Tissue culture dishes (150 mm × 25 mm; Corning® 150 mm TC-treated Culture Dish **Corning Catalog** #430599)
- Tubing, e.g., polytetrafluoroethylene (PTFE) standard tubing (2 mm i.d. × 3 mm o.d.; Fluorostore) and Tygon tubing (2 mm i.d. × 4 mm o.d.; United States Plastics, cat. no. 57658); optional

Note

CRITICAL Ensure that the PTFE tubing fits on the tip of a 5-ml serological pipette and into an Optiseal tube before pouring the density gradients (Step 38.2). Use the Tygon tubing to secure the PTFE tubing at the pipette tip.

- 16-gauge × 1 1/2 inch needles (BD safety needles **Becton Dickinson (BD) Catalog** #305198)

AAV titration equipment

- Centrifuge (

Equipment	
Centrifuge 5418 R - Microcentrifuge	NAME
Microcentrifuge	TYPE
Eppendorf	BRAND
5418	SKU
https://www.eppendorf.com/us-en/eShop-Products/Centrifugation/Microcentrifuges/Centrifuge-5418R-p-PF-240995	LINK

)

- Dry bath and heating blocks (Fisher Scientific, Isotemp models)
-

Equipment	
Microplate Centrifuge, PCR Plate Spinner	NAME
PCR Plate Spinner	TYPE
VWR®	BRAND
VWRU89184-610	SKU
https://in.vwr.com/store/product/8081983/microplate-centrifuge-pcr-plate-spinner	LINK

or centrifuge equipped with plate adapters

- Quantitative PCR machine (Analytik Jena, model no. qTOWER 2.2)

Equipment

Invitrogen™ Qubit™ 3 Fluorometer	NAME
Accurately measures DNA, RNA, and protein using the highly sensitive fluorescence-based Qubit quantitation assays	TYPE
Invitrogen™ Q33216	BRAND
Q33216	SKU
https://www.fishersci.co.uk/shop/products/qubit-3-0-fluorometer/15387293	LINK

AAV titration supplies

- Barrier pipette tips (low binding; 10 µl, 20 µl, 200 µl, and 1,000 µl; Olympus Plastics 23-401 10µl Reach Olympus Premium Barrier Tips Low Binding Racked Sterile 10 Ra **Genesee Scientific Catalog** #23-401 , 23-404, 23-412, and 23-430, respectively)
- DNA Clean & Concentrator kit (DNA Clean & Concentrator-25 **Zymo Research Catalog** #D4033 (DCC-25)), for purification of up to 25 µg of the DNA standard
- Microcentrifuge tubes (1.5-ml DNA/RNA LoBind; Eppendorf, cat. no. 86-923)
- Qubit™ Assay Tubes **Invitrogen - Thermo Fisher Catalog** #Q32856
- Excel Scientific TS-RT2-100 ThermalSeal RT Sealing Film Non-Sterile 50µm Polyester 100 Films/Uni **Genesee Scientific Catalog** #12-529
- Stericup-GP Sterile Vacuum Filtration System **Fisher Scientific Catalog** #SCGPU02RE
- VWR® Round Media Bottles **VWR International (Avantor) Catalog** #89166-104
- Olympus Plastics 24-310W Olympus 96-Well PCR Plate FAST-type Low Profile White 10 Plates/Unit **Genesee Scientific Catalog** #24-310W

Intravenous (retro-orbital) injection equipment

- Animal anesthesia system (

Equipment

Tabletop Laboratory Animal Anesthesia System	NAME
Animal Anesthesia System	TYPE
V-1	BRAND
901806	SKU
http://www.vetequip.com/item.asp?cat=0&catalogID=901806	LINK
1 h 1"w x 8"d x 14" 19"w x 18"d x 50" 22"w x 19"d x 50"h Base Footprint: 19"w x 18"d	SPECIFICATIONS

, 901807, or 901810)

Note

CRITICAL Most animal facilities provide anesthesia systems equipped with an induction chamber, isoflurane vaporizer, nose cone, and waste gas scavenging system.

Intravenous (retro-orbital) injection supplies

- Activated charcoal adsorption filters **VetEquip Catalog** #931401
- Insulin syringes with permanently attached needles (31 gauge × 5/16 inches; BD, cat. no. 328438)
- Oxygen gas supply (Airgas)
- Screw-cap vials (1.6 ml; National Scientific Supply, cat. no. BC16NA-PS)

Safety warnings

CAUTION

- AAVs are biohazardous materials and must be handled according to governmental and institutional regulations. Experiments involving AAVs were performed using biosafety level 2 practices as required by the California Institute of Technology and the US Centers for Disease Control and Prevention.

- rAAVs, although replication-incompetent, are potent gene-delivery vehicles and must be handled according to governmental and institutional regulations. The safety of packaged transgenes (e.g., oncogenic genes) should be carefully considered. Perform all procedures in a certified biosafety cabinet and clean AAV-contaminated equipment, surfaces, and labware with fresh 10% (vol/vol) bleach.

- HEK293T cells and AAVs are biohazardous materials and must be handled according to governmental and institutional regulations. All experiments involving the aforementioned materials were performed in a Class II biosafety cabinet with annual certification as required by the California Institute of Technology and the US Centers for Disease Control and Prevention.

- Ethanol is flammable.

- HCl is corrosive. Use personal protective equipment.

- Isoflurane is a halogenated anesthetic gas associated with adverse health outcomes in humans and must be handled according to governmental and institutional regulations. To reduce the risk of occupational exposure during rodent anesthesia, waste gas was collected in a biosafety cabinet using a charcoal scavenging system as approved by the California Institute of Technology.

- During ultracentrifugation, rotors are subjected to enormous forces (350,000g in this protocol). Rotor failure can have catastrophic consequences, including irreparable damage to the centrifuge and laboratory and fatal injuries to personnel. Inspect the rotors for signs of damage or weakness before each use, and always follow the manufacturer’s instructions while operating an ultracentrifuge.

Reagent setup: Plasmid DNA

1

Note

CRITICAL
All reagents used for viral production and administration should be prepared using endotoxin-free materials. Glassware is not endotoxin-free, and autoclaving does not eliminate endotoxins. To minimize contamination, we dissolve chemicals in sterile bottles by shaking and/or heating to mix, use demarcations on bottles to bring solutions to the final volume, and use pH strips rather than a pH meter. When filter-sterilizing solutions, do so in a biosafety cabinet.

Grow bacterial stocks in LB or Plasmid+ media containing the appropriate selective antibiotic; refer
to the Addgene catalog for suggested growth conditions. Use a large-scale endotoxin-free plasmid purification kit to isolate plasmids; elute plasmid DNA with the supplied Tris-EDTA (TE) buffer. Measure the DNA purity and concentration using a spectrophotometer and freeze at -20 °C or
-80 °C for up to several years.

Note

CRITICAL

- Always verify the integrity of purified plasmids by Sanger sequencing (using a DNA sequencing facility) and restriction digestion (https://www.neb.com/tools-and-resources) before proceeding with downstream applications. pAAV plasmids contain ITRs (Fig. 6) that are prone to recombination in *E. coli*. pAAVs should be propagated in recombination-deficient strains such as NEB Stable, Stbl3, or SURE 2 competent cells to prevent unwanted recombination. After purification, pAAVs should be digested with SmaI to confirm the presence of ITRs, which are required for replication and encapsidation of the viral genome; use sequence-editing and annotation software to determine the expected band sizes. Note that it is difficult to sequence through the secondary structure of ITRs[68]; avoid ITRs when designing sequencing primers.
- Create bacterial glycerol stocks and store at -80 °C for up to several years.

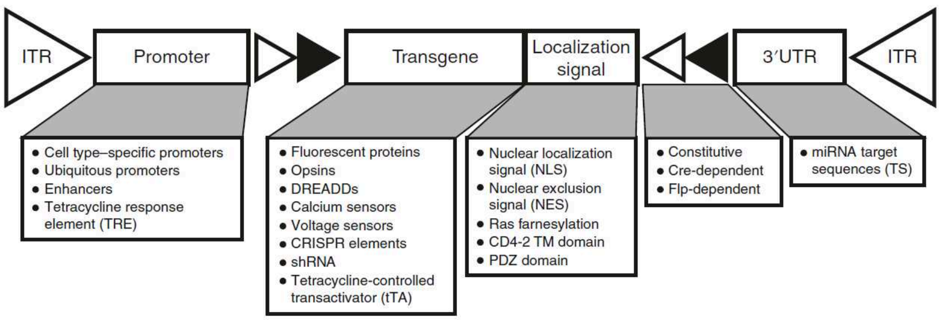


Fig. 6 | A modular AAV toolbox for cell type–specific gene expression.

Reagent setup: Cell culture media

2 Add

	A	B
	FBS	25 ml
	NEAA	5 ml
	pen–strep	5 ml
	DMEM	500-ml bottle

Invert to mix and store at 4 °C for up to several months; warm to 37 °C before use. The resulting cell culture media should have a final concentration of 5% (vol/vol) FBS, 1× NEAA, and 50 µL pen–strep.

Note

CRITICAL
To quickly expand cells for large viral preps, consider using a final concentration of 10% (vol/vol) FBS in the cell culture media; see guidelines on cell culture below.

Reagent setup: Cell culture

3 Thaw HEK293T cells according to the manufacturer's recommendations. Passage cells using either
TrypLE Express enzyme or a standard trypsinization protocol for adherent cultures[69].
Seed cells in 150-mm tissue culture dishes with a final volume of 20 mL of media per dish. Maintain in a cell culture incubator at 37 °C with 5% CO_2.

Note

CRITICAL
- We suggest passaging cells at a ratio of 1:3 (i.e., divide one dish of cells into three new dishes of cells) every other day when expanding cells for viral production; split cells at a 1:2 ratio (or 6×10^4 cells/cm^2) 24 h before transfection. Use higher split ratios if using 10% (vol/vol) FBS. Always use sterile technique.
- Follow the manufacterer's recommendations to create frozen stocks of HEK cells.

Reagent setup: PEI stock solution

4 Pipette 50 mL of WFI water into a 50-ml conical centrifuge tube for later use. Add 323 mg of PEI to the remaining 950 mL bottle of WFI water and adjust the pH to 2–3 by adding [M] 1 Mass Percent HCl suitable for cell culture, keeping track of the volume of HCl added. Heat in a 37 °C water bath for several hours (or Overnight) and occasionally shake to mix. Once dissolved, add reserved WFI water to a total volume of 1 L . Filter-sterilize, make aliquots in 50-ml conical centrifuge tubes, and store at -20 °C for up to 1 year. We routinely freeze–thaw our PEI aliquots.

1d

Note

CRITICAL
Both our PEI stock solution recipe and PEI calculations (Supplementary Table 2, 'Detailed calculations' sheet) are based on ref. 5. We adjust the pH to 2–3 so that PEI dissolves in water. The designated pH range does not appear to adversely affect cell viability, transfection efficiency, or viral titers. The transfection solution, created by mixing the PEI + DPBS master mix and DNA + DPBS solution (Step 24 and Supplementary Table 2), has a final pH of 6.5–7.0. To transfect one dish, 2 mL of transfection solution is added to 20 mL of media (Step 24), which further dilutes the PEI.

Reagent setup: PEI + DPBS master mix

5 Thaw PEI in a 37 °C water bath. Bring the PEI to Room temperature (23 °C) and vortex to mix. Add PEI and DPBS to a 50-ml conical centrifuge tube and vortex again to mix. Use Supplementary Table 2 ('Transfection calculator' sheet) to calculate the volumes of PEI (cell I9) and DPBS (cell J9) needed.

Note

CRITICAL
Prepare fresh master mix before use.

Reagent setup: DNA + DPBS

6 Bring plasmid DNA to Room temperature and briefly vortex to mix. For each viral prep, add DNA and DPBS to a 50-ml conical centrifuge tube; the solution is vortexed in Step 24. Use Supplementary Table 2 ('Transfection calculator' sheet) to calculate the quantities of DNA (e.g., cells E9, E11, and E13) and DPBS (e.g., cell F9) needed.

Note

CRITICAL
Prepare fresh DNA + DPBS solution before use. Re-measure plasmid DNA concentrations immediately before use; multiple freeze–thaw cycles may cause DNA degradation.

Reagent setup: SAN digestion buffer

7 Add

	A	B
	NaCl	29.22 g
	Tris base	4.85 g
	$MgCl_2 \cdot 6H_2O$	2.03 g
	WFI water	1-liter bottle

and shake to mix. Filter-sterilize and store at Room temperature for up to several months. The resulting SAN digestion buffer should have a final pH of ~ pH 10.0 and a final concentration of [M] 500 millimolar (mM) NaCl, [M] 40 millimolar (mM) Tris base, and [M] 10 millimolar (mM) $MgCl_2$.

Reagent setup: SAN + SAN digestion buffer

8 Add 100 µL of SAN (4 µL of 25 µL SAN) per milliliter of SAN digestion buffer; pipette to mix.

Note

CRITICAL
Prepare fresh solution before use.

Reagent setup: 40% (wt/vol) PEG stock solution

9 Decant ~ 500 mL of WFI water into a 500-ml sterile bottle for later use. Add 146.1 g of NaCl to the remaining 500 mL (in the 1-liter bottle of WFI water) and shake/heat until dissolved. 1d

Once completely dissolved, add 400 g of PEG and heat at 37 °C Overnight for up to 2 nights. Add reserved WFI water to a total volume of 1 L . Filter-sterilize and store at Room temperature for up to several months. The resulting stock solution should have a final concentration of [M] 2.5 Mass Percent NaCl and 40% (wt/vol) PEG.

Note

CRITICAL

- Prepare in advance. To expedite the procedure, heat the solution at 65 °C until the PEG is dissolved. The solution will appear turbid, but no flecks of PEG should remain; the mixture will become clear upon cooling.
- Pre-wet the entire filter surface with a minimal volume of water before adding the solution. This solution is extremely viscous and will take 1–2 h to filter.

Reagent setup: DPBS + high salt

10 Add

	A	B
	NaCl	29.22 g
	KCl	93.2 mg
	$MgCl_2 \cdot 6H_2O$	101.7 mg
	DPBS	500-ml bottle

and shake to mix. Filter-sterilize and store at Room temperature for up to several months. The resulting buffer should have a final concentration of [M] 1 Mass Percent NaCl, [M] 2.5 millimolar (mM) KCl, and [M] 1 millimolar (mM) $MgCl_2$ (in addition to the salts in the DPBS).

Reagent setup: DPBS + low salt

11 Add

	A	B
	NaCl	2.92 g
	KCl	93.2 mg
	$MgCl_2 \cdot 6H_2O$	101.7 mg
	DPBS	500-ml bottle

and shake to mix. Filter-sterilize and store at Room temperature for up to several months. The resulting buffer should have a final concentration of

[M] 100 millimolar (mM) NaCl, [M] 2.5 millimolar (mM) KCl, and [M] 1 millimolar (mM) $MgCl_2$ (in addition to the salts in the DPBS).

Reagent setup: Iodixanol density gradient solutions (15%, 25%, 40%, and 60% (wt/vol) iodixanol)

12 For each layer, add iodixanol (OptiPrep), DPBS + high salt or DPBS + low salt, and phenol red (if applicable) to a 50-ml conical centrifuge tube. Invert or briefly vortex to mix. Use Supplementary Table 3 to determine the volumes of each reagent needed. The 25% and 60% layers contain phenol red, which turns the solutions red and yellow, respectively, and facilitates clear demarcation of the gradient boundaries (Fig. 8).

Note

CRITICAL

- Prepare fresh solutions on the day of AAV purification. Alternatively, prepare up to 1 d in advance; store at Room temperature and protect from light. Do not pour the density gradients until Step 38.
- In Step 38.2, prepare more iodixanol solutions than are needed. For six or fewer gradients, prepare enough of each solution to pour an extra gradient. For eight gradients, prepare enough of each solution to pour two extra gradients. The extra solution is needed to fill the 5-ml pipette and prevent an air bubble from disturbing the gradient when releasing the last of the required volume.

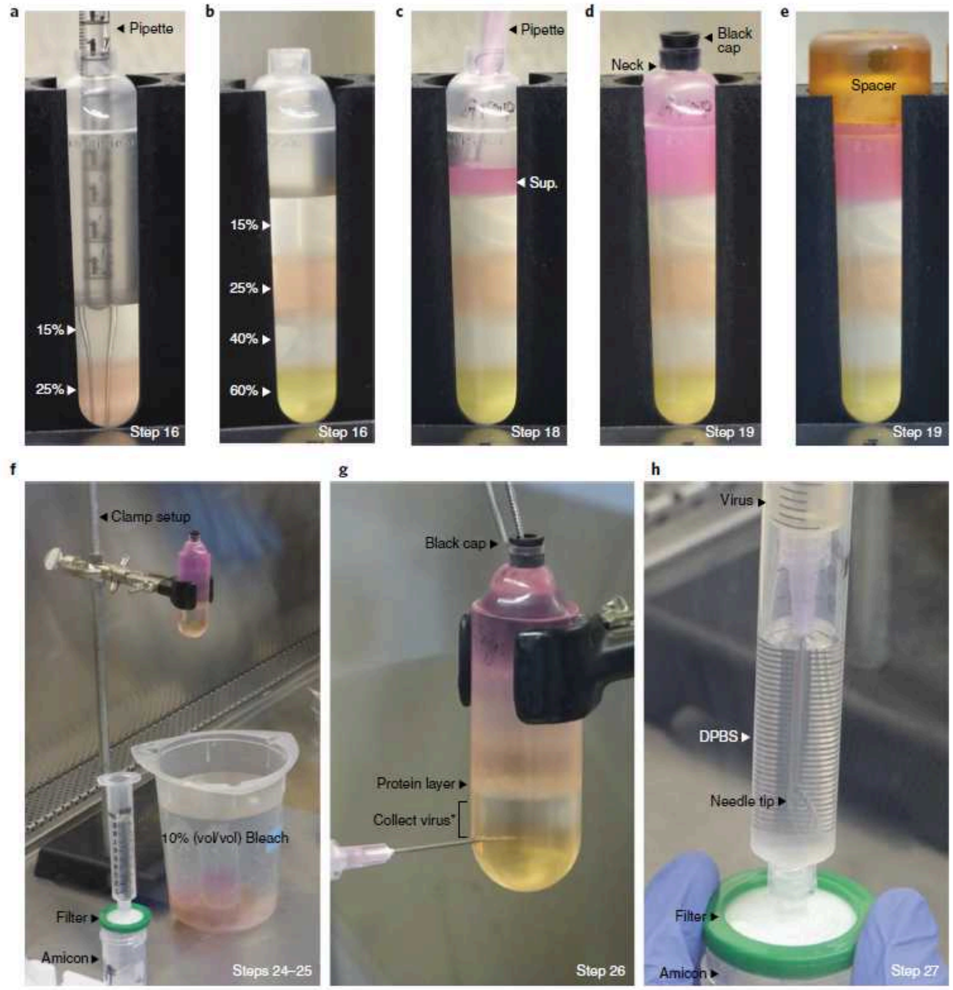


Fig. 8 | AAV purification procedure. **a,b**, In Step 38, pipette the iodixanol density gradients (Supplementary Video 1, 0:00–1:45, or Supplementary Video 2, 0:00–1:13). **a**, Layer the 25% (wt/vol) iodixanol underneath the 15% layer. **b**, Add layers of increasing density under the previous layer; the gradients should have a sharp delineation between layers. **c**, In Step 40 the supernatant (Sup.) from Step 39 (Fig. 7b) above the 15% layer (Supplementary Video 1, 1:46–2:22; the same step is also shown in Supplementary Video 2, 1:14–1:55). **d,e**, In Step 41, fill each tube up to the neck with SAN digestion buffer and insert a black cap (**d**); place a spacer on top before weighing the tubes (**e**). **f**, After ultracentrifugation (Step 44), secure the tube into the clamp setup above a container of fresh 10% (vol/vol) bleach (Step 46). Allow 10 ml of DPBS to begin dripping through the syringe filter unit into an Amicon filter device (Step 47). **g**, In Step 48, collect the virus (Supplementary Video 3, 0:00–1:30). Insert the needle ~4 mm below the 40/60% interface (i.e., where the tube just starts to curve). Do not collect virus (asterisk) until the black cap is removed; do not collect from the white protein layer at the 25/40% interface. **h**, In Step 49, filter the virus/iodixanol (Supplementary Video 3, 1:31–2:32). Inject the virus below the DPBS in the filter-attached syringe barrel before pushing the virus/DPBS through the syringe filter unit and into the Amicon filter device.

Reagent setup: DNase digestion buffer

13 Use a 50-ml serological pipette to measure 247.5 mL of UltraPure water into a 250-ml sterile bottle. Add

	A	B
	$CaCl_2$	55.5 mg
	1 M Tris-HCl	2.5 ml
	$MgCl_2 \cdot 6H_2O$	508 mg

and shake to mix. Filter sterilize and store at Room temperature for up to several months. The resulting buffer should have a final concentration of [M] 2 Molarity (M) $CaCl_2$, [M] 10 Molarity (M) Tris-HCl, and [M] 10 Molarity (M) $MgCl_2$.

Reagent setup: DNase I + DNase digestion buffer

14 Add 50 µL of DNase I per milliliter of digestion buffer (a 1:200 dilution of 10 µL DNase); pipette to mix.

Note

CRITICAL
Prepare fresh solution before use.

Reagent setup: Proteinase K solution

15 Use a 50-ml serological pipette to measure 250 mL of UltraPure water into a 250-ml sterile bottle. Add 14.61 g of NaCl and shake to mix. Add 2.5 g of N-lauroylsarcosine sodium salt to the mixture and gently swirl to mix; N-lauroylsarcosine sodium salt is a surfactant and will generate bubbles during vigorous mixing. Filter-sterilize and store at Room temperature for up to several months. The resulting solution should have a final concentration of [M] 1 Mass Percent NaCl and 1% (wt/vol) *N*-lauroylsarcosine sodium salt.

Reagent setup: Proteinase K + proteinase K solution

16 Add 100 µg of proteinase K per milliliter of solution (a 1:200 dilution of 50 µL (2.5 U/mg) proteinase K); pipette to mix.

Note

CRITICAL
Prepare fresh solution before use.

Reagent setup: DNA standard stock

17 Set up a single 50-µl restriction digest reaction; use 60-80 µL (3–4 µl) of ScaI (or another suitable enzyme) to linearize 20 µg of the plasmid DNA containing the target sequence. Run a small amount of the reaction on an agarose gel to ensure complete digestion. Purify the reaction using two DNA clean-up columns. Measure the DNA concentration (ng/µl) using a spectrophotometer. Dilute to ~5–10 × 10^9 single-stranded (ss) DNA molecules/µl and use the Qubit assay to verify the concentration (ng/µl). Divide into 20 µL aliquots in DNA/RNA LoBind microcentrifuge tubes and freeze at -20 °C for up to 1 year.

Note

CRITICAL
- Before preparing the standard, use sequence-editing and annotation software to confirm that the plasmid contains a single ScaI site in the ampicillin resistance gene.
- Refer to ref. 11 and use Supplementary Table 4 (cells B7–10) to calculate the number of ssDNA molecules in a given plasmid (cell B13). We typically use linearized pAAV-CAG-eYFP diluted to 10 µL, which corresponds to 6.6 × 10^9 ssDNA molecules/µl (Supplementary Table 4, 'Example' sheet).

Reagent setup: DNA standard dilutions

3s

18 Prepare three sets of eight (1:10) serial dilutions of the DNA standard stock. For each set, begin by pipetting 5 µL of the standard into 45 µL of UltraPure water (standard no. 8). Mix by vortexing for 00:00:03 and proceed with the seven remaining dilutions (standard no. 7 to standard no. 1). The final concentrations of the standard

3s

dilutions should range from 5–10 × 10^8 (standard no. 8) to 5–10 × 10^1 (standard no. 1) ssDNA molecules/µl.

Note

CRITICAL
Prepare fresh solutions in DNA/RNA LoBind microcentrifuge tubes immediately prior to use; at low concentrations, the linearized DNA is prone to degradation and/or sticking to the walls of the tube[11]. One 20 µL aliquot of the DNA standard stock will provide enough DNA for preparing the dilutions and verifying the concentration via the Qubit assay before qPCR.

Reagent setup: qPCR master mix

19 Prepare a qPCR master mix for the total number of reactions (i.e., wells) needed. One reaction requires

	A	B
	SYBR Green master mix	12.5 µl
	UltraPure water	9.5 µl
	each primer (from a 2.5-µM stock concentration)	0.5 µl
	Total	23 µl/well

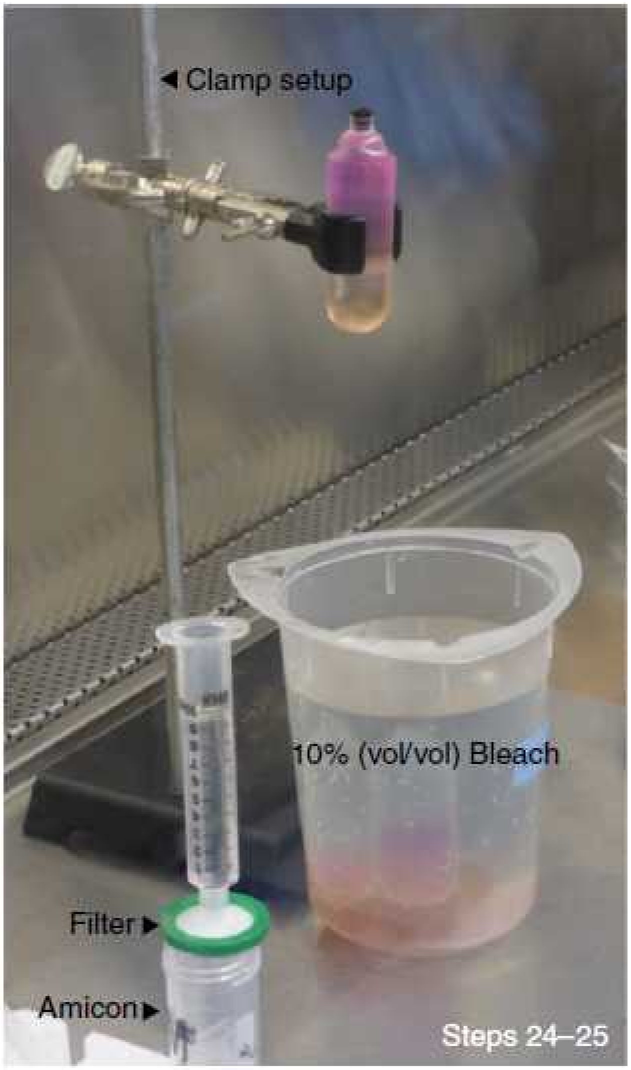
Pipette or vortex for 1–2 s to mix.

Note

CRITICAL
Prepare fresh solution before use.

Equipment setup: Clamp setup for AAV purification

20 Attach the rod to the support stand. Secure the clamp 25–30 cm above the stand (Fig. 8f).



Procedure

21

Safety information

! CAUTION
AAVs are biohazardous materials and must be handled according to governmental and institutional regulations. Experiments involving AAVs were performed using biosafety level 2 practices as required by the California Institute of Technology and the US Centers for Disease Control and Prevention.

Note

CRITICAL
The entire procedure spans 8 d, excluding pause points and the time required to evaluate transgene expression (Fig. 7a). There are no pause points between days 1 and 5, until Step 33; once cells have been transfected, AAVs are harvested on days 3 and 5. Plan accordingly during this time window.

Procedure: Triple transient transfection of HEK293T cells ● Timing 1–2 h

10s

22

Note

CRITICAL
For capsids that package well (i.e., AAV9, AAV-PHP.B, AAV-PHP.eB, and AAV-PHP.S), the PEI transfection protocol typically yields ≥1 × 10^{12} vg per 150-mm dish, as measured post purification[2,3]. Before starting the protocol, determine the number of dishes needed per viral prep and use Supplementary Table 2 (‘Transfection calculator’ sheet) to calculate the quantities of PEI, DPBS, and plasmid DNA required for transfection. Skip to Step 65 if custom AAVs were obtained elsewhere.

24 h before transfection, seed HEK293T cells in 150-mm dishes to attain 80–90% confluency the next day[69]. Incubate the cells in a cell culture incubator at 37 °C with 5% CO_2.

23 At the time of transfection, make the PEI + DPBS master mix and the DNA + DPBS solution for each viral prep (Reagent setup and Supplementary Table 2, ‘Transfection calculator’ sheet). Using a 5- or 10-ml serological pipette, add the required volume of the PEI + DPBS master mix (e.g., ‘Transfection calculator’ cell G9) dropwise to the DNA + DPBS solution (e.g., ‘Transfection calculator’ cells E9 + E11 + E13 + F9) while gently vortexing to mix. Cap the tube and thoroughly vortex for 00:00:10 to mix. Allow the mixture to sit at Room temperature for 2–10 min. Add 2 mL of the transfection solution dropwise to each dish and swirl to mix before returning the dishes to the cell culture incubator.

10s

Note

CRITICAL STEP

- We use a AAV2/9 REP-AAP-ΔCAP:pUC18:pHelper plasmid ratio of 1:1:2 based on micrograms of DNA. We use 40 µg of total DNA per 150-mm dish (9.98 µg of AAV2/9 REP-AAP-ΔCAP, 9.98 µg of pUC18, and 19.97 µg of pHelper). Additionally, we add 10 ng of t he plasmid-safe DNase treated library per 150-mm dish.
- The transfection solution will appear slightly cloudy because of the formation of DNA–PEI complexes[5,6]. Allowing the mixture to sit for >10 min may reduce transfection efficiency.

? TROUBLESHOOTING

24 Change the media 12–24 h post transfection by aspirating the old media in 10% (vol/vol) bleach and replacing it with 20 mL of fresh, warmed media (Reagent setup).

Note

CRITICAL STEP

- Do not allow the cells to remain without media for more than a few minutes. To protect the cells from unwanted stress, aspirate the media from five plates at a time and promptly replace it with new media. PEI is moderately cytotoxic[6] and cell death of up to 20% is common[71]. Do not allow the media to remain unchanged for more than 24 h post transfection. Failure to change the media in a timely manner will result in poor cell health and low titers.
- Beginning 72 h post transfection, examine the cells under a fluorescence microscope to assess fluorescent protein expression, if applicable. Note that expression of the rAAV genome does not necessarily correlate with final viral yield and will depend on the reporter and/or promoter under investigation.

? TROUBLESHOOTING

Procedure: AAV harvest ● Timing 3 d

2h 15m

25 Harvest the media and cells 60 h (2.5 d) post-transfection. Use a cell scraper to gently scrape the cells in the media. After scraping the first dish, prop it at a 30° angle, using an empty 1.5-ml microcentrifuge tube rack for support. Scrape down the residual cells and media such that they are pooled together. Return the dish lid and scrape the next plate; prop dishes up against one another along the length of the biosafety cabinet until scraping is complete. Use a 25-ml serological pipette to collect the media and cells from

each dish; transfer to a 250-ml conical centrifuge tube. Pool the media and cells from up to 10 dishes in a single tube.

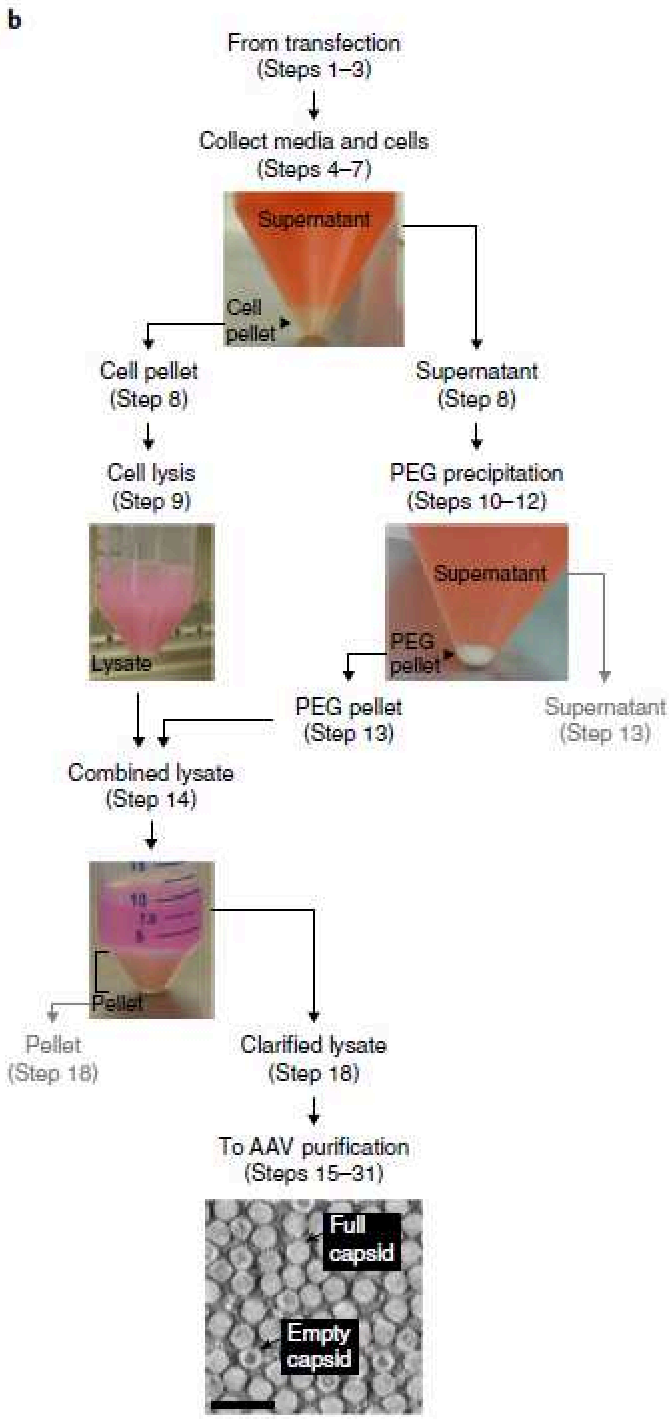
Note

! CAUTION
Scrape the cells with a forward motion (i.e., away from the front grill of the biosafety cabinet) to prevent media and cells from splashing out of the biosafety cabinet. If a spill does occur at this or any other step, immediately cover with paper towels and carefully saturate the towels with fresh 10% (vol/vol) bleach.

CRITICAL STEP

- To avoid cross-contamination, harvest the media and cells from one viral prep at a time.
- For larger viral preps (6–10 dishes), a 250- or 500-ml conical centrifuge tube can be used to harvest the media and cells (Steps 27–31). However, we recommend using two 250-ml tubes in Step 32.2 because the PEG pellet (Step 34) is difficult to remove from the walls and edges of 500-ml tubes (Step 36).

26 Centrifuge the media and cells at 2000 x g, Room temperature, 00:15:00 . Ensure that the tube caps are tightly secured. Centrifugation will result in the formation of a cell pellet (Fig. 7b). 15m



27 Pour off the supernatant (i.e., the clarified media) into the corresponding bottle from Step 26. Allow excess media to drip back down onto the beveled edge of the 250-ml tube; remove using a P1000 pipette and add to the supernatant. Store the supernatant at 4 °C until Step 32.

Note

CRITICAL STEP
Failure to remove excess media from the pellet will cause several milliliters of media to dilute the SAN digestion buffer in Step 31.

28 Cell pellet resuspension. Prepare 5 mL of SAN + SAN digestion buffer (Reagent setup) per viral prep. For smaller viral preps (1–5 dishes), use option 31.1. For larger preps (6–10 dishes), use option 31.2.

28.1 **Harvest from 1–5 dishes**

1h

1. Use a 5-ml serological pipette to gently resuspend the cell pellet in 5 mL of SAN + SAN digestion buffer; pipette into a 50-ml tube to finish resuspending the pellet (Fig. 7b).
2. Incubate in a 37 °C water bath for 01:00:00 and store at 4 °C until Step 36 (up to 1 d).

Note

CRITICAL STEP

- Be sure to collect the entire pellet, which will stick to the walls and beveled edges of 250-ml tubes. Save the 250-ml tubes for Step 32.
- The high salt content of SAN digestion buffer lyses the cells, which release the viral particles and nucleic acids into the solution. Initially, the cell lysate may be viscous and difficult to pipette; SAN will degrade nucleic acids and reduce the viscosity after incubation at 37 °C. The pH of the lysate will decrease to 8–9 or lower during cell lysis, but the lysate should appear pink rather than yellow/orange because of residual phenol red (Fig. 7b). Note that the expression of fluorescent proteins from strong promoters (e.g., CAG) can alter the color of the lysate.
- (**Optional**) Collect a 30 µL sample from the cell lysate for troubleshooting; store at 4 °C for up to 1 week. If the viral yield is lower than expected, the sample can be titered (Steps 54–64) to determine at which stage the virus may have been lost.

? TROUBLESHOOTING

28.2 **Harvest from 6–10 dishes**

1h

- Use a 10-ml serological pipette to partially resuspend the smaller cell pellet in 5 mL of SAN + SAN digestion buffer. Pipette into the second 250-ml tube containing the larger pellet and resuspend together; pipette into a 50-ml tube to finish resuspending the pellet (Fig. 7b).

- Incubate in a 37 °C water bath for 01:00:00 and store at 4 °C until Step 36 (up to 1 d).

Note

CRITICAL STEP

- Be sure to collect the entire pellet, which will stick to the walls and beveled edges of 250-ml tubes. Save the 250-ml tubes for Step 32.
- The high salt content of SAN digestion buffer lyses the cells, which release viral particles and nucleic acids into solution. Initially, the cell lysate may be viscous and difficult to pipette; SAN will degrade nucleic acids and reduce the viscosity after incubation at 37 °C . The pH of the lysate will decrease to 8–9 or lower during cell lysis, but the lysate should appear pink rather than yellow/orange because of residual phenol red (Fig. 7b). Note that expression of fluorescent proteins from strong promoters (e.g., CAG) can alter the color of the lysate.
- (**Optional**) Collect a 30 µL sample from the cell lysate for troubleshooting; store at 4 °C for up to 1 week. If the viral yield is lower than expected, the sample can be titered (Steps 54–64) to determine at which stage the virus may have been lost.

? TROUBLESHOOTING

29 Retrieve the supernatant collected in Step 30. For smaller viral preps (1–5 dishes), use option 32.1. For larger preps (6–10 dishes), use option 32.2.

29.1 **Harvest from 1–5 dishes**

Pour the supernatant from Step 30 into the corresponding 250-ml tube from Step 31.

Note

CRITICAL STEP

(**Optional**) Collect a 30 µL sample from the media for troubleshooting; store at 4 °C for up to 1 week. If the viral yield is lower than expected, the sample can be titered (Steps 54–64) to determine at which stage the virus may have been lost.

29.2 **Harvest from 6–10 dishes**

Equally divide the supernatant from Step 30 between the two corresponding 250-ml tubes from Step 31.

Note

CRITICAL STEP
(**Optional**) Collect a 30 µL sample from the media for troubleshooting; store at 4 °C for up to 1 week. If the viral yield is lower than expected, the sample can be titered (Steps 54–64) to determine at which stage the virus may have been lost.

30 Use a 25-ml or 50-ml serological pipette to add a 1/5 final volume of 40% (wt/vol) PEG stock solution to the supernatant (i.e., the supernatant should contain a final concentration of 8% (wt/vol) PEG solution). Tighten the cap and thoroughly invert ten times to mix. Incubate On ice for 02:00:00 . 2h

Note

CRITICAL STEP
For AAV production in HEK293T cells, the cell culture media contains a large fraction of the expected yield[72]. Failure to PEG-precipitate AAV particles in the media will result in lower viral yields[8].

PAUSE POINT
The PEG–media mixture can be incubated at 4 °C Overnight .

31 Centrifuge the PEG–media mixture at 4000 x g, 4°C, 00:30:00 . Centrifugation will result in the formation of a PEG pellet (Fig. 7b). 30m

32 Pour off the supernatant (i.e., the PEG-clarified media) into a used media collection bottle for bleaching. Allow excess media to drip back down onto the beveled edge of the 250-ml tube; aspirate or pipette to remove.

33 PEG pellet resuspension. Prepare 1 mL of SAN + SAN digestion buffer (Reagent setup) per viral prep. For smaller viral preps (1–5 dishes), use option 36.1. For larger preps (6–10 dishes), use option 36.2.

33.1 **Harvest from 1–5 dishes** 30m

1. Use a P1000 pipette to carefully resuspend the PEG pellet in 1 mL of SAN + SAN digestion buffer; pipette into the corresponding lysate from Step 31 (Fig. 7b).
2. Incubate in a 37 °C water bath for an additional 00:30:00 .

Note

CRITICAL STEP

- Resuspending the PEG pellet is difficult and will take ~ 00:05:00 per pellet. Be sure to collect the entire pellet, some of which will stick to the walls and beveled edges of 250-ml tubes. During resuspension, avoid air bubbles, which can be difficult to collect with a pipette and may disrupt capsid structure. Do not use a serological pipette to resuspend the pellet, which can become lodged within the barrel of the pipette.
- (**Optional**) Collect a 30 µL sample from the PEG pellet resuspension, before adding it to the corresponding lysate, for troubleshooting; store at 4 °C for up to 1 week. If the viral yield is lower than expected, the sample can be titered (Steps 54–64) to determine at which stage the virus may have been lost.

PAUSE POINT

Store the lysate at 4 °C Overnight . Alternatively, use a dry ice–ethanol bath to freeze the lysate; store at -20 °C for up to 1 week.

33.2 **Harvest from 6–10 dishes** 30m

1. Use a P1000 pipette to partially resuspend one of the PEG pellets in 1 mL of SAN + SAN digestion buffer. Pipette into the second 250-ml tube containing the second pellet and carefully resuspend together; pipette into the corresponding lysate from Step 31 (Fig. 7b).
2. Incubate in a 37 °C water bath for an additional 00:30:00 .

Note

CRITICAL STEP
Resuspending the PEG pellet is difficult and will take ~ 00:05:00 per pellet. Be sure to collect the entire pellet, some of which will stick to the walls and beveled edges of 250-ml tubes. During resuspension, avoid air bubbles, which can be difficult to collect with a pipette and may disrupt capsid structure. Do not use a serological pipette to resuspend the pellet, which can become lodged within the barrel of the pipette.

- (**Optional**) Collect a 30 µL sample from the PEG pellet resuspension, before adding it to the corresponding lysate, for troubleshooting; store at 4 °C for up to 1 week. If the viral yield is lower than expected, the sample can be titered (Steps 54–64) to determine at which stage the virus may have been lost.

PAUSE POINT
Store the lysate at 4 °C 00:00:00 . Alternatively, use a dry ice–ethanol bath to freeze the lysate; store at -20 °C for up to 1 week.

Procedure: AAV purification ● Timing 1 d

2h 15m

34

Note

CRITICAL
One iodixanol density gradient is sufficient to purify virus from up to ten 150-mm dishes. If more than ten dishes per prep are used, divide the lysate into more than one gradient. The AAV purification steps are most easily learned by visualization; refer to Fig. 8 and Supplementary Videos 1–3 for details.

Determine the number of gradients needed and prepare the iodixanol density gradient solutions (Reagent setup and Supplementary Table 3). Set the OptiSeal tubes in the rack provided in the OptiSeal tube kit; alternatively, use the long edge of a 50-ml tube Styrofoam rack.

Note

CAUTION
Check the OptiSeal tubes for defects; tubes with dents may collapse during ultracentrifugation.

35 Pour the density gradients (Fig. 8a,b and Supplementary Video 1, 0:00–1:45, or Supplementary Video 2, 0:00–1:13). Each gradient is composed of the following layers: 6 mL of 15% (wt/vol) iodixanol, 6 mL of 25% (wt/vol) iodixanol, 5 mL of 40% (wt/vol) iodixanol, and 5 mL of 60% (wt/vol) iodixanol (Supplementary Table 3). Pour the layers with a 2- or 5-ml serological pipette. We typically use a 2-ml pipette; using a 5-ml pipette is faster but requires the use of PTFE and Tygon tubing and extra reagents. To load the layers with a 2-ml pipette, choose option 38.1. To load the layers with a 5-ml pipette, choose option 38.2.

35.1 **Loading with a 2-ml pipette**

Begin by pipetting 6 mL (measure to the 3 ml mark twice) of 15% (wt/vol) iodixanol to each tube. Next, add 6 mL of 25% (wt/vol) iodixanol under the 15% layer by lightly touching the pipette tip to the bottom of the tube and slowly releasing the solution (Fig. 8a and Supplementary Video 1, 0:13–1:29). Continue adding layers of increasing density under the previous layer. The gradients should have a sharp delineation between layers (Fig. 8b).

Note

CRITICAL STEP

- When loading the 25%, 40%, and 60% layers with a 2-ml pipette, stop releasing the solution and slowly remove the pipette once the iodixanol is ~5 mm from the tip of the pipette (Supplementary Video 1, 0:42–0:58 and 1:20–1:25). This will prevent an air bubble from disturbing the gradient. The remaining iodixanol will be released when the pipette is removed from the tube.
- Corning brand 2-ml serological pipettes consistently fit into OptiSeal tubes; other brands should be tested before use.

? TROUBLESHOOTING

35.2 **Loading with a 5-ml pipette**

Attach a piece of tubing (see Equipment) to a 5-ml pipette. Begin by pipetting 6 mL of 15% (wt/vol) iodixanol into each tube. Next, add 6 mL of 25% (wt/vol) iodixanol under the 15% layer by lightly touching the tubing to the bottom of the tube and slowly releasing the solution (Supplementary Video 2, 0:17–1:13). Continue adding layers of increasing density under the previous layer. The gradients should have a sharp delineation between layers (Fig. 8b).

Note

CRITICAL STEP
Fill the 5-ml pipette with more layer solution than is needed (e.g., an extra 1 ml per layer); this will prevent an air bubble from disturbing the gradient when releasing the last of the required volume (Supplementary Video 2, 1:09–1:11). Remember to prepare extra solution (Reagent setup).

? TROUBLESHOOTING

36 Centrifuge the lysate from Step 36 at 2000 x g, Room temperature, 00:10:00 . Centrifugation will result in the formation of a pellet (Fig. 7b). 10m

37 Use a 2-ml serological pipette to load the supernatant (i.e., the clarified lysate) (~6–7 ml total) from Step 39 above the 15% (wt/vol) iodixanol layer (Fig. 8c and Supplementary Video 1, 1:46–2:22 or Supplementary Video 2, 1:14–1:55). Touch the pipette tip to the surface of the 15% layer and slowly release the lysate such that a layer forms on top.

Note

CRITICAL STEP
- Use a pipetting device with precise control. Do not allow the lysate to drip from the pipette tip onto the 15% layer; this will cause the lysate to mix with the gradient. Note that Corning brand 2-ml serological pipettes consistently fit into OptiSeal tubes; other brands should be tested before use.
- The pellet may be soft, making it difficult to retrieve all of the supernatant. After loading 6-7 mL of lysate above the 15% layer, spin the lysate for an additional 3000 x g, Room temperature, 00:15:00 ; use a P200 or P1000 pipette to slowly load the remaining supernatant onto the lysate layer. Discard the pellet in 10% (vol/vol) bleach or a biohazard waste bin.
- (**Optional**) Collect a 30 µL sample from the lysate for troubleshooting; store at 4 °C for up to 1 week. If the viral yield is lower than expected, the sample can be titered (Steps 54–64) to determine at which stage the virus may have been lost.

38 Using a 2-ml serological pipette, fill each tube up to the neck with SAN digestion buffer. Firmly insert a black cap (Fig. 8d) and place a spacer on top (Fig. 8e). Caps and spacers are provided with the OptiSeal tubes and in the OptiSeal tube kit, respectively.

Note

! CAUTION

- Overfilling the tube can cause a spill when inserting the black cap. Handling the tubes without caps, or with loosely fitted caps, can also cause spills.
- Avoid air bubbles, which can cause the OptiSeal tubes to collapse during ultracentrifugation.

CRITICAL STEP
The black cap should fit right above or touch the lysate.

39 Weigh the tubes with the caps and spacers on. Balance the tubes to within 5-10 mg of each other using SAN digestion buffer. Be sure to adjust the tube weight in the biosafety cabinet; use the tube removal tool provided with the OptiSeal tube kit to remove the black cap and add the appropriate volume of SAN digestion buffer with a P20 or P200 pipette.

Note

! CAUTION
Failure to balance the tubes before ultracentrifugation could result in damaged equipment.

40 Place the ultracentrifuge rotor in the biosafety cabinet. Load the tubes and fasten the lid.

Note

! CAUTION
Ensure that the rotor is in proper working order. This includes checking that the o-rings are intact, as cracked o-rings can cause virus to spill during ultracentrifugation. Also, check that the rotor and tubes are completely dry; moisture between tubes and the tube cavity can cause tubes to collapse. To prevent damage to the rotor, set it on a paper towel so that the overspeed disk at the bottom is not scratched.

41 Carefully transfer the rotor to the ultracentrifuge. Spin the Type 70 Ti rotor at 350000 x g, 18°C, 02:25:00 (58,400 r.p.m.) with slow acceleration (no. 3; the instrument will take 3 min to accelerate to 500 r.p.m., followed by maximum acceleration) and deceleration (no. 9; the instrument will deccelerate at maximum speed until it 2h 25m

reaches 500 r.p.m., then take 6 min to stop) profiles. Alternatively, a Type 60 Ti rotor can be used at 358000 x g (59,000 r.p.m.).

Note

! CAUTION
Always follow the manufacturer's instructions while operating an ultracentrifuge.

42 During ultracentrifugation, gather the supplies and equipment for Steps 46–49. Assemble the clamp setup (Equipment setup) and collect one of each of the following per gradient: Amicon Ultra-15 centrifugal filter device, 5-ml syringe, 10-ml syringe, 0.22-µm syringe filter unit, and a 16-gauge needle.

43 After ultracentrifugation, bring the rotor inside the biosafety cabinet and remove the lid. Use the spacer removal tool provided with the OptiSeal tube kit to remove the spacer from the first tube. Next, use the tube removal tool to grip the tube neck. Slowly remove the tube from the rotor and secure it into the clamp setup above a 500-ml or 1-liter beaker containing fresh 10% (vol/vol) bleach (Fig. 8f). Clean the side of the tube with a paper towel or a Kimwipe sprayed with 70% (vol/vol) ethanol.

Note

! CAUTION
- The black cap may become dislodged from the tube during removal, increasing the likelihood of a spill. Try replacing the cap before removing the tube from the rotor. Otherwise, replace the cap once the tube is secured in the clamp setup.
- If a tube collapses during ultracentrifugation, take extra care when removing the tube from the rotor. Use fresh 10% (vol/vol) bleach to wipe the tube before proceeding with AAV purification. Viruses purified from collapsed tubes may have lower yields.

? TROUBLESHOOTING

44 Prepare the supplies for Steps 48 and 49. First, remove and save the plunger from a 10-ml syringe. Attach a 0.22-µm syringe filter unit to the syringe barrel and place it on top of an Amicon filter device. Next, add 10 mL of DPBS to the barrel and allow the solution to begin dripping through the syringe filter unit and into the filter device (Fig. 8f). Last, attach a 16-gauge needle to a 5-ml syringe.

Note

CRITICAL STEP

- Amicon filter devices contain traces of glycerine. If this interferes with downstream applications, rinse the device with DPBS before use.
- (**Optional**) Rinse the filtration membrane of the Amicon filter device by adding 15 mL of DPBS to the top chamber and centrifuging at 3000 x g, Room temperature, 00:01:00 ; discard the flow-through. The manufacterer recommends using the device immediately after rinsing.

45 From the tube clamped in Step 46, collect the virus from the 40/60% interface and 40% layer[9,10] (Fig. 8g and Supplementary Video 3, 0:00–1:30). Hold the top of the OptiSeal tube with your nondominant hand; use your dominant hand to hold the needle/syringe. Use a forward-twisting motion to insert the needle ~4 mm below the 40/60% interface (i.e., where the tube just starts to curve). Use the tube removal tool in your non-dominant hand to remove the black cap from the tube to provide a hole for air entry. With the needle bevel up, use the needle/syringe to collect 4.0–4.5 ml of virus/ iodixanol from the 40/60% interface and 40% layer. Do not collect from the white protein layer at the 25/40% interface; as this interface is approached, rotate the needle bevel down and continue collecting from the 40% layer. Firmly replace the black cap before removing the needle from the tube.

Safety information

! CAUTION

Keep your hands out of the path of the needle to prevent accidental exposure to AAVs. Failure to firmly replace the black cap before removing the needle will cause the AAV contaminated solution to flow out of the needle hole in the tube and potentially onto and out of the biosafety cabinet. Perform this step over a large beaker of fresh 10% (vol/vol) bleach (Fig. 8f).

Note

CRITICAL STEP

- The virus should concentrate at the 40/60% interface and within the 40% layer[10]. There will not be a visible virus band, but the phenol red in the 25% and 60% layers helps to better define the 40% cushion.
- Before attempting to collect virus from the density gradient, practice on an OptiSeal tube filled with water.
- (**Optional**) Collect a 30 µL sample from the virus/iodixanol for troubleshooting; store at 4 °C for up to 1 week. If the viral yield is lower than expected, the sample can be titered (Steps 54–64) to determine at which stage the virus may have been lost.

? TROUBLESHOOTING

46 Add the 4.0-4.5 mL of virus/iodixanol to the syringe barrel containing 10 mL of DPBS (prepared in Step 47) (Fig. 8h and Supplementary Video 3, 1:31–2:06). Layer the virus below the DPBS by placing the needle near the bottom of the barrel and pressing on the plunger. Insert the 10-ml syringe plunger into the barrel and push the virus/DPBS mixture through the syringe filter unit and into the Amicon filter device (Supplementary Video 3, 2:07–2:32). Mix well using a P1000 pipette.

Note

CRITICAL STEP

- This filtration step reduces the likelihood of clogging the filtration membrane in the Amicon filter device. The virus/iodixanol mixture will be difficult to push through the syringe filter unit; DPBS will be easy to push through as it washes the filter.
- AAVs adhere to hydrophobic surfaces, including plastics; use low-binding pipette tips (Reagents). Pluronic F-68 is a nonionic surfactant that may reduce virus loss associated with sticking to plastics.
- (**Optional**) Include 0.001% (vol/vol) Pluronic F-68 in the DPBS for Steps 49–52.

47 Centrifuge the virus/DPBS mixture at 3000 x g, Room temperature for 5–8 min, or until the volume of the solution remaining in the top chamber of the Amicon filter device is 500-1500 µL (>10× concentrated).

Note

CRITICAL STEP
This step may take longer because iodixanol initially slows the passage of the solution through the filtration membrane.

48 Discard the flow-through for bleaching. Add 13 mL of DPBS to the virus in the top chamber and use a P1000 pipette to mix.

Note

CRITICAL STEP
Remove the filter device, which contains the virus, before discarding the flow-through.

49 Centrifuge the virus/DPBS mixture as in Step 50. Wash the virus two more times for a total of four buffer exchanges. During the last spin, retain 300-500 µL of solution in the top chamber.

Note

CRITICAL STEP

- The third and fourth washes may require only a 2–3-min spin until the desired volume remains in the top chamber.
- The volume retained in the top chamber will affect the final virus concentration (vg/ml) (i.e., the lower the volume, the higher the concentration). A final volume of 300-500 µL should work for most applications, assuming a production efficiency of at least 1×10^{12} vg/dish and a dose and injection volume of no more than 1×10^{12} vg and 100 µL, respectively (see 'Experimental design' section and Step 65 for dose and injection volume recommendations, respectively). For direct injections, a final volume of 200 µL may be optimal. Note that retaining too low a volume may cause the virus to aggregate during storage at 4 °C (see Step 64 for details).

50 Use a P200 pipette to transfer the virus from the top chamber of the Amicon filter device directly to a 1.6-ml screw-cap vial; store at 4 °C.

Note

CRITICAL STEP

- Amicon filter devices are not sterile. If this is a concern for specific applications, the virus can be filter-sterilized before storage. (**Optional**) Filter-sterilize the virus. Use a P200 pipette to transfer the virus from the top chamber of the Amicon filter device directly to a Costar Spin-X filter unit within a centrifuge tube. Centrifuge the virus at 3000 x g, Room temperature, 00:01:00 . Discard the filter unit and transfer the purified virus from the centrifuge tube to a 1.6-ml screw-cap vial; store at 4 °C .
- The screw-cap vials are not low protein binding; however, they help prevent the formation of aerosols when opening and closing the tubes. We store AAVs in screw-cap vials at 4 °C and typically use them within 3 months, during which time we have not noticed a decrease in titers or transduction efficiency in vivo. We have not rigorously tested the effects of long-term storage at -20 °C or -80 °C for systemically delivered viruses.

? TROUBLESHOOTING

PAUSE POINT Store the purified virus at 4 °C for up to 3 months.

Procedure: AAV titration ● Timing 1 d

1h 0m 10s

51

Note

CRITICAL
The AAV titration procedure below is adapted from ref. 11. Each virus sample is prepared in triplicate in separate 1.5-ml DNA/RNA LoBind microcentrifuge tubes and later loaded into a 96-well plate for qPCR. All solutions must be accurately pipetted and thoroughly mixed; qPCR is highly sensitive to small changes in DNA concentration.

Prepare a plan for the PCR plate setup. Allocate the first 24 wells (A1–B12) for the DNA standards such that standard no. 1 occupies wells A1–A3, standard no. 2 occupies wells A4–A6, and so on. Use the remaining wells for the virus samples such that the first virus sample occupies wells C1–C3, the second sample occupies wells C4–C6, and so on.

Note

CRITICAL STEP
Include DPBS as a negative control and a virus sample with a known concentration as a positive control; prepare the controls with the virus samples in Steps 55–62.

52 Use DNase I to digest DNA that was not packaged into the viral capsid. Prepare DNase I + DNase digestion buffer (Reagent setup) and add 100 µL to each 1.5-ml tube. Vortex each virus for 1–2 s to mix; alternatively, use a P200 pipette to mix. Add 2 µL of the virus to each of three tubes. Vortex for 1–2 s to mix and spin down (2000 x g, Room temperature, 00:00:10); incubate in a 37 °C water bath for 01:00:00 .

1h 0m 10s

Note

CRITICAL STEP
- Do not vortex/pipette the virus vigorously or vortex longer than 1–2 s; exposure to force may disrupt capsid structure.
- When dipping the pipette tip into the virus stock, insert the tip just below the surface of the liquid rather than dipping it deep inside. Excess virus carried on the outside of the tip will carry over into the DNase digestion buffer and cause variations in the titer.
- Prepare each virus sample in triplicate.

53 Inactivate the DNase. Add 5 µL of EDTA to each tube; vortex for 1–2 s to mix, spin down (2000 x g, Room temperature, 00:00:10), and incubate in a 70 °C dry bath for 00:10:00 .

10m 10s

Note

CRITICAL STEP
DNase must be inactivated or else it will degrade the viral genome when it is released from the viral capsid in Step 57.

54 Use proteinase K to digest the viral capsid and release the viral genome. Prepare proteinase K + proteinase K solution (Reagent setup) and add 120 µL to each tube.

2h 0m 10s

Vortex for 1–2 s to mix and spin down (2000 x g, Room temperature, 00:00:10); incubate in a 50 °C dry bath for 02:00:00 .

Note

PAUSE POINT Samples can be incubated at 50 °C Overnight .

55 During the last 20 min of the proteinase K digestion, prepare the DNA standard dilutions (Reagent setup) and use the Qubit assay to measure the concentration (ng/µl) of the DNA standard stock.

Note

CRITICAL STEP
The concentration of the standard stock solution is used to generate the standard curve after qPCR (Supplementary Table 4, cell B9). To measure the concentration of the standard stock solution, use the Qubit fluorometer, which measures low DNA concentrations with high sensitivity and accuracy.

56 Inactivate the proteinase K. Incubate the tubes in a 95 °C dry bath for 00:10:00 . 10m

Safety information

! CAUTION
Tube caps may pop open unexpectedly; use safety glasses while removing the tubes from the 95 °C dry bath.

Note

CRITICAL STEP
Proteinase K must be inactivated or else it will digest the DNA polymerase during qPCR.

57 Allow the tubes to cool for 00:05:00 . Vortex each sample for 1–2 s to mix and add 3 µL to a new tube containing 897 µL of UltraPure water (a 1:300 dilution). 5m 3s

Vortex the diluted samples for 00:00:03 to mix.

58 Prepare the qPCR master mix (Reagent setup).

59 Load the PCR plate based on the experimental plan from Step 32. First, pipette 23 µL of qPCR master mix into each designated well. Next, pipette 2 µL of each standard into wells A1–B12. Last, pipette 2 µL of each diluted sample from Step 38 into wells C1 and onward. Seal the plate with sealing film and briefly spin down (500 x g, 00:00:10) in a plate spinner. 10s

60 Place the PCR plate into the qPCR machine. Use the following cycling parameters: 11m 15s

Step 63.1: 95 °C , 00:10:00
Step 63.2: 95 °C , 00:00:15
Step 63.3: 60 °C , 00:00:20
Step 63.4: 60 °C , 00:00:40
Repeat steps 63.2–63.4 40×.

61 When the qPCR run is complete, export the cycle threshold (Ct) values to an Excel file. Copy and paste the Ct values into Supplementary Table 4 ('AAV titration calculator' sheet) to generate a standard curve and calculate the titer (vg/ml) (cell G27) of each virus; calculate per-plate production (vg/dish) (cell K27) to assess production efficiency. Be sure to enter the appropriate values in cells B7–10 and B18; see 'Example' sheet.

Note

CRITICAL STEP
If the titer is ≥1 × 10^{14} vg/ml, the virus may aggregate during storage at 4 °C .
Dilute the virus to between 2 × 10^{13} and 5 × 10^{13} vg/ml with DPBS and re-titer the diluted stock.

? TROUBLESHOOTING

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