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AAV production and purification

Forked from Individual AAV production and purification

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Gerard Michael Coughlin[1]

[1]California Institute of Technology

Transcriptional crosstalk

Gerard Michael Coughlin
California Institute of Technology

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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.

IMPORTANT
This protocol is based on Challis et al., *Nat Protoc 14*: 379, published in 2019. Since that publication, we have made a number of modifications to the protocol to increase throughput or yield. With these modifications, we routinely achieve yields of >5e12 vg per 150 cm dish, with a well packaging capsid (e.g. AAV9, AAV-PHP.eB, and AAV.CAP-B10).

In Challis et al., *Nat Protoc 14*: 379, a Type 70 Ti rotor is used for ultracentrifugation of the AAV through a density gradient (steps 32-36). This rotor can hold 8 tubes, allowing for simultaneous purification of 8 separate AAV variants, at 1-10 plates (150 mm plates) per variant. To increase throughput, we also provide a smaller scale purification workflow with a Type 70.1 Ti rotor, which can allow for purification of 12 separate AAV variants, at 1-3 plates (150 mm plates) per variant.

Significant modifications to the referenced protocol are indicated by ***MODIFICATION***. Read and understand the original protocol, before following a modified protocol. Following the original protocol should also yield expected results.

Attachments

PDF

699-1488.pdf
5MB

Guidelines

This protocol is based on Challis et al., *Nat Protoc 14*: 379, published in 2019. Since that publication, we have made a number of modifications to the protocol to increase throughput or yield. With these modifications, we routinely achieve yields of >5e12 vg per 150 cm dish, with a well packaging capsid (e.g. AAV9, AAV-PHP.eB, and AAV.CAP-B10).

In Challis et al., *Nat Protoc 14*: 379, a Type 70 Ti rotor is used for ultracentrifugation of the AAV through a density gradient (steps 32-36). This rotor can hold 8 tubes, allowing for simultaneous purification of 8 separate AAV variants, at 1-10 plates (150 mm plates) per variant. To increase throughput, we also provide a smaller scale purification workflow with a Type 70.1 Ti rotor, which can allow for purification of 12 separate AAV variants, at about 1-3 plates (150 mm plates) per variant.

Significant modifications to the referenced protocol are indicated by ***MODIFICATION***. Read and understand the original protocol, before following a modified protocol. Following the original protocol should also yield expected results.

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

Note

CRITICAL Three plasmids (pAAV, pUCmini-iCAP or rep-cap plasmid, and pHelper) are required for AAV production.

1. The pAAV genome contains the cargo to deliver, flanked by AAV inverted terminal repeats.
2. The iCAP or rep-cap plasmids contain AAV rep and cap genes, encoding machinery for replication and capsid production. The sequence of the cap gene will determine the capsid that the pAAV genome is packaged into.
3. pHelper plasmid contains adenovirus genes necessary for AAV production and 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 cells7; we do not recommend using an alternative producer cell line with this protocol.

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

NEB Stable Competent E.coli (High Efficiency) - 20x0.05 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 Catalog #**N605-250G
- Antibiotics (e.g., 100 mg/mL carbenicillin solution **Teknova Catalog #**C8001)
- Quick-Load Purple 1 kb Plus DNA Ladder - 250 gel lanes **New England Biolabs Catalog #**N0550S
- Lysogeny broth (LB; Amresco, cat. no. J106-1KG) or alternative

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 Agar plates with 100 mg/mL carbenicillin, 100mm **Teknova Catalog #**L1010
- ZymoPURE II plasmid maxiprep kit **Zymo Research Catalog #**D4202

Note

CRITICAL Triple transient transfection requires large amounts of pUCmini-iCAP-PHP (22.8 µg/dish) and pHelper plasmid DNA (11.4 µg/dish). Preparing these plasmids may be more convenient with a giga-scale purification kit. 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™ TrypLE™ Express Enzyme (1X) phenol red **Fisher Scientific Catalog #**12-605-036

Transfection

- PEI MAX® - Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40000) **Polysciences, Inc. Catalog #**24765-1

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.

- Gibco™ DPBS no calcium no magnesium **Fisher Scientific Catalog #**14-190-250

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 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 Fischer Catalog #**24040-032
- 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

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® 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% CO2;

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

Equipment

Type 70 Ti Fixed-angle titanium rotor	NAME
Ultracentrifuge Rotor	TYPE
Beckman Coulter	BRAND
337922	SKU
https://www.beckman.com/centrifuges/rotors/fixed-angle/337922	LINK

, or a Type 70.1 Ti fixed-angle rotor

Equipment

Type 70.1 Ti Fixed-angle titanium rotor	NAME
Ultracentrifuge rotor	TYPE
Beckman Coulter	BRAND
342184	SKU
https://www.beckman.com/centrifuges/rotors/fixed-angle/342184	LINK

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 #**910024
- 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

For Type 70 Ti rotor:

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

For Type 70.1 Ti rotor:

- 8.9 mL OptiSeal Polypropylene Tube, 16 x 60 mm **Beckman Coulter Catalog #**361623 ; includes black caps
- 8.9 mL Tube Kit Polypropylene OptiSeal Tubes 16 x 60 mm **Beckman Coulter Catalog #**361660

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).

Equipment

Repeater® E3/Repeater® E3x - Electronic Multi-Dispenser Pipette	NAME
Repeater pipette	TYPE
Eppendorf	BRAND
4987000410	SKU
https://www.eppendorf.com/us-en/Products/Liquid-Handling/Manual-Pipettes-Dispensers/RepeaterE3-RepeaterE3x-p-PF-135444	LINK

- Screw-cap vials 1.6ml **National Scientific Catalog #**BS16NA-PS
- Serological pipettes (2 ml, 5 ml, 10 ml, 25 ml, and 50 ml)
- Repeater pipette tips (10 ml, 25 ml)
- Stericup sterile vacuum filtration system (0.22 µm; 1 liter;
  Stericup Quick Release-GP Sterile Vacuum Filtration System **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S2GPU11RE
)
- 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)

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

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

- Verify the integrity of purified plasmids by Sanger sequencing and restriction enzyme digest, or through whole plasmid sequencing before proceeding with downstream applications. pAAV plasmids contain ITRs (Fig. 6) that are necessary for replication and encapsidation of the viral genome. Because ITRs 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. Presence of ITRs in purified pAAV genomes can be assessed through restriction enzyme digest with SmaI (use sequence-editing and annotation software to determine the expected band sizes). Note that it is difficult to Sanger sequence through the secondary structure of ITRs[68]; avoid ITRs when designing sequencing primers. Alternatively, whole plasmid sequencing with Oxford Nanopore Technology (e.g. as offered through Plasmidsaurus) is able to fully sequence the ITRs.
- Create bacterial glycerol stocks and store at -80 °C for up to several years.

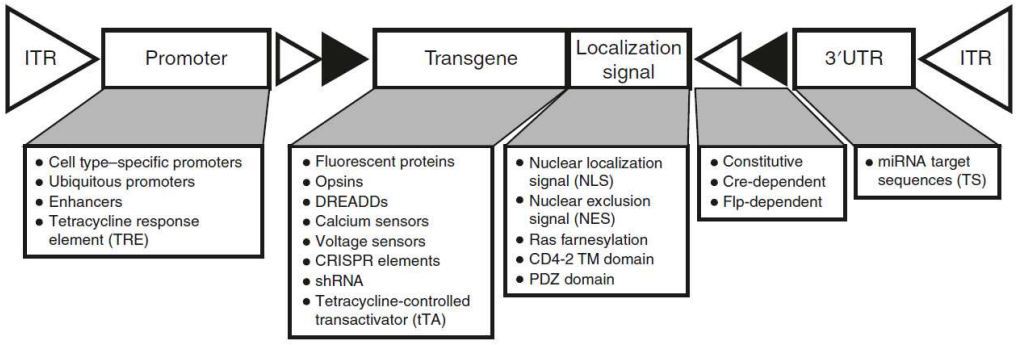


Fig. 6 | A modular AAV toolbox for cell type–specific gene expression (from Challis et al., *Nat Protoc 14*: 379)

Reagent setup: Cell culture media

2 Add

	A	B
	FBS	55 ml or 27.5 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 10% or 5% (vol/vol) FBS.

Note

CRITICAL
To quickly expand cells for large viral preps, we use DMEM with 10% FBS. After triple transfection with AAV packaging plasmids, we switch to DMEM with 5% FBS.

To enable detection of contaminated cultures, we avoid using antibiotics in culture media. Furthermore, because transfection increases membrane permeability, antibiotics in the media may lead to cytotoxicity and lower transfection efficiency. Because we do not use antibiotics in the culture media, proper sterile technique is necessary.

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 25 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 day or 1:6 every other day when expanding cells for viral production; split cells at a 1:2.5 ratio 24 h before transfection. Always use sterile technique.
- Before starting tissue culture, plan the number plates and ideal transfection day and work backwards to determine the number of plates to start with. We recommend passaging cells at least 3 times before transfecting, to ensure high cell viability and to allow for potential contamination to become detectable.
- Follow the manufacturer's recommendations to create frozen stocks of HEK cells.

Reagent setup: PEI stock solution

4 Add 323 mg of PEI to a 1 litre bottle of WFI water. PEI 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.

Note

CRITICAL
PEI MAX (MW 40,000) should readily dissolve in water without any pH adjustment needed. We do not adjust the pH of the PEI solution as it will be mixed with buffered DPBS during subsequent steps.

Reagent setup: PEI + DPBS master mix

5 Thaw PEI in a 37 °C water bath. Bring the PEI to Room temperature and vortex to mix. Add PEI and DPBS to a 50-ml conical centrifuge tube and vortex again to mix. Use Supplementary Table 2 from Challis et al., *Nat Protoc 14*: 379 ('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. Use Supplementary Table 2 from Challis et al., *Nat Protoc 14*: 379 ('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.

Reagent setup: SAN digestion buffer

7 Add

	A	B
	NaCl	29.22 g

	A	B
	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 U of SAN (4 µL of 25 U/µL SAN) per milliliter of SAN digestion buffer; pipette to mix.

> **Note**
>
> **CRITICAL**
> Prepare the day of 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 Molarity (M) 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 Molarity (M) 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 from Challis et al., *Nat Protoc 14*: 379 to determine the volumes of each reagent needed (see note below). 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
- Do not pour the density gradients until Step 30.
- We prepare stocks of gradient steps in advance and store at room temperature for months
- OptiPrep is 60% iodixanol in water, and therefore does not need to be diluted further (except with the addition of a small amount of phenol red) for the 60% step.

MODIFICATION
To ensure complete digestion of the producer cell nucleic acid and complete solubilization of the AAV capsids, a higher volume of SAN buffer with SAN may be used to resuspend and digest the cell pellet (Step 23).

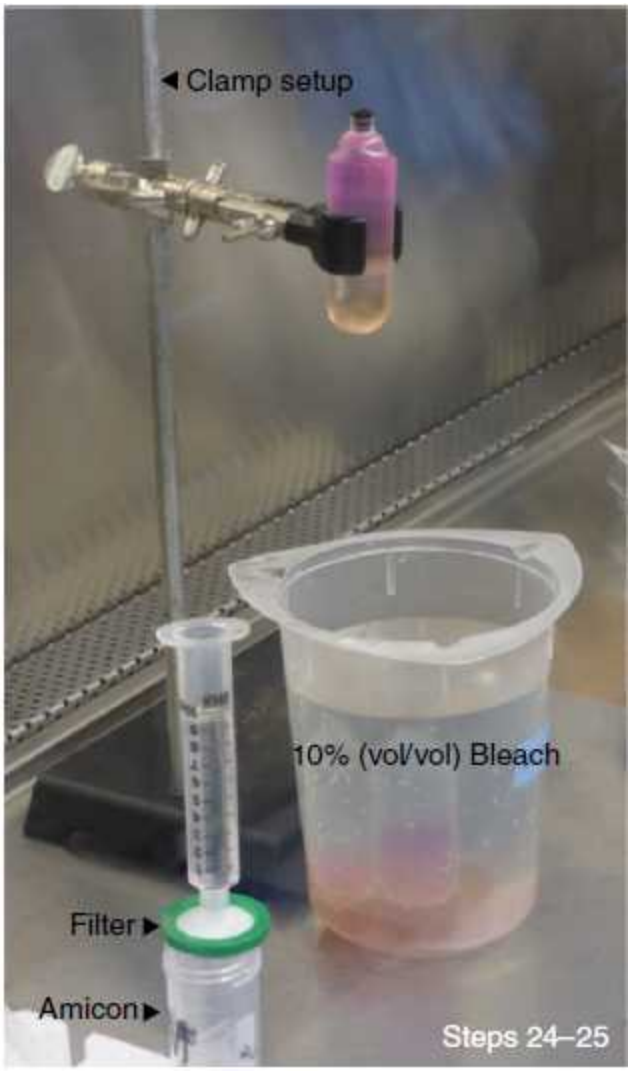
To enable loading of a higher volume of SAN buffer with SAN, a modified gradient can be used.

For a Type 70 Ti rotor:
- 6 mL of 15% step
- 4 mL of 25% step
- 5 mL of 40% step
- 3 mL of 60% step

For a Type 70.1 Ti rotor
- 1.8 mL of 15% step
- 1.2 mL of 25% step
- 1.5 mL of 40% step
- 0.8 mL of 60% step

Equipment setup: Clamp setup for AAV purification

13 Attach the rod to the support stand. Secure the clamp 25–30 cm above the stand (Fig. 8f from Challis et al., *Nat Protoc 14*: 379).



Procedure

14

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; once cells have been transfected, AAVs are harvested on days 3 and 5. Plan accordingly during this time window.

MODIFICATION
Modifications (indicated by ***MODIFICATION***) to the referenced protocol (Challis et al., *Nat Protoc. 14:* 379-414) can help to increase yield and/or throughput. This increased yield can be especially helpful in cases where large doses of multiple AAVs are necessary. Using these modifications, we routinely obtain yields >5e12 vg per plate with well packaging capsids.

In the referenced protocol, a Type 70 Ti rotor is used for ultracentrifugation of the AAV through a density gradient (Steps 32-37). This rotor can hold 8 tubes, allowing for simultaneous purification of 8 separate AAV variants, at 1-8 plates (150 mm plates) per variant. To increase throughput, we also provide a smaller scale purification workflow with a Type 70.1 Ti rotor, which can allow for purification of 12 separate AAV variants, at about 1-3 plates (150 mm plates) per variant.

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

10s

15

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.

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

Note

CRITICAL
Cell health is critical for efficient transfection and capsid production. We do not include antibiotics in our culture media and we passage cells at least 3 times before transfecting. This is to ensure that they are healthy and free of contamination. Transfecting cells while there are antibiotics in the media may lead to cytotoxicity and lower transfection efficiency.

Deviations in doubling time may indicate cell health issues or contamination. In our hands, healthy HEK 293T cells should double every 16 hours when cultured in 10% FBS (triple every 24 hours).

CRITICAL
When passaging cells the day before transfection, care should be taken to ensure uniform cell density across the culture dish. Using a 250 mL or 500 mL tube and resuspending cells to the full volume, then plating can help to achieve uniform density (i.e. if plating 8 plates, bring total volume of cell suspension to 200 mL and plate 25 mL in each plate). Dropwise addition of high concentration cell suspensions to pre-plated media can lead to non-uniformity in cell density and poor transfection.

CRITICAL
While expanding the number of plates, save used media bottles. These bottles are sterile and can be used during AAV harvest (Steps 18-28).

16 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 from Challis et al., *Nat Protoc. 14:* 379-414, '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. Mix by gently pushing plate forward and backward, then side-to-side, and finally in a 'Figure 8' pattern, before returning the dishes to the cell culture incubator.

10s

Note

CRITICAL STEP

- We use a pAAV:pUCmini-iCAP-PHP:pHelper plasmid ratio of 1:4:2 based on micrograms of DNA. We use 40 µg of total DNA per 150-mm dish (5.7 µg of pAAV, 22.8 µg of pUCmini-iCAP-PHP, and 11.4 µg of pHelper) (Supplementary Table 2, 'Detailed calculations' sheet). The plasmid ratio was optimized during the initial development of the AAV production protocol; 1:4:2 gave the best viral yields.
- 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.
- Mixing the PEI-DNA complexes into the media by swirling will lead to the complexes clustering in the middle of the dish and non-uniform transfection across the plate surface.
- Users can opt to run a positive transfection/virus production control (e.g., pAAV-CAG-eYFP); this is especially important if using an untested rAAV genome.

17 Change the media 12–24 h post transfection by aspirating the old media in 10% (vol/vol) bleach and replacing it with 25 mL of fresh, pre-warmed DMEM with 5% FBS (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.
- Do not put a contaminated serological pipette back into your stock media bottle. To reduce cost and plastic waste, a 250 mL (1-4 plates) or 500 mL conical tube (5-8 plates) can be used. You will need 1 such conical tube per virus. Decant the required amount of pre-warmed media into the conical tube, and then use the same serological pipette to replace media for all plates. Keep this conical tube, as it can be used for AAV harvest (Steps 18-28).
- 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.
- At this point, we switch from 10% FBS to 5% FBS. This helps to reduce cost, and reduces overgrowth of the producer cells on the plate.
- While changing media, save used media bottles. These bottles are sterile and can be used during AAV harvest (Steps 18-28).

Procedure: AAV harvest ● Timing 5 d

2h 15m

18

Safety information

! CAUTION
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.

Note

CRITICAL
Carefully label all tubes and replace gloves, pipettes, and cell scrapers between viral preps to avoid cross-contamination. Refer to Fig. 7b for a schematic of the AAV harvest procedure.

Harvest the cell culture media 72 h (3 d) post transfection. Tilt each dish at a 30° angle and use a 25-ml serological pipette to collect the media. Store in an empty, sterile media bottle (saved from earlier expansion or media changes) 4 °C until Step 19. Replace the media with 25 mL of fresh, pre-warmed DMEM with 5% FBS (Reagent setup). While expanding the number of plates, save used media bottles. These bottles are sterile and can be used during AAV harvest (Steps 18-28).

Note

! CAUTION
Tilt dishes away from the front grill of the biosafety cabinet to prevent media from spilling out of the biosafety cabinet.

CRITICAL STEP
- To avoid cross-contamination, harvest the media from one viral prep at a time.
- To reduce cost and plastic waste, reuse the 250 mL (1-4 plates) or 500 mL conical tube (5-8 plates) from Step 17. Decant the required amount of pre-warmed media into the conical tube, and then use the same serological pipette to replace media for all plates producing the same virus. Keep this conical tube, as it can be used again during AAV harvest (Steps 18-28).
- For AAV-PHP production in HEK293T cells, the media at 72 h post transfection contains ~2×10^{11} vg per dish, or 10–20% of the expected viral yield. Failure to collect and change media at this time point will decrease yields.
- If time is limited, media and cells can be harvested together at 72 h or 96 h rather than 120 h, but total yields will be reduced.

19 Harvest the media and cells 120 h (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 the 250 mL or 500 mL conical tube used during earlier media changes. Use some of the media collected at 72 h post-transfection to wash remaining cells off each plate and combine with the scraped cell suspension.

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.

20 Combine the remaining media collected at 72 h post transfection with the media and cells collected at 120 h post transfection.

Note

CRITICAL
Save the empty media bottle

21 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

Note

CRITICAL
When pelleting cells from large volumes in 500 mL conical tubes, longer centrifugation times may be necessary to completely pellet cells from the media. Visually inspect the cell pellet and media to ensure that the media is clear and free of cell clumps.

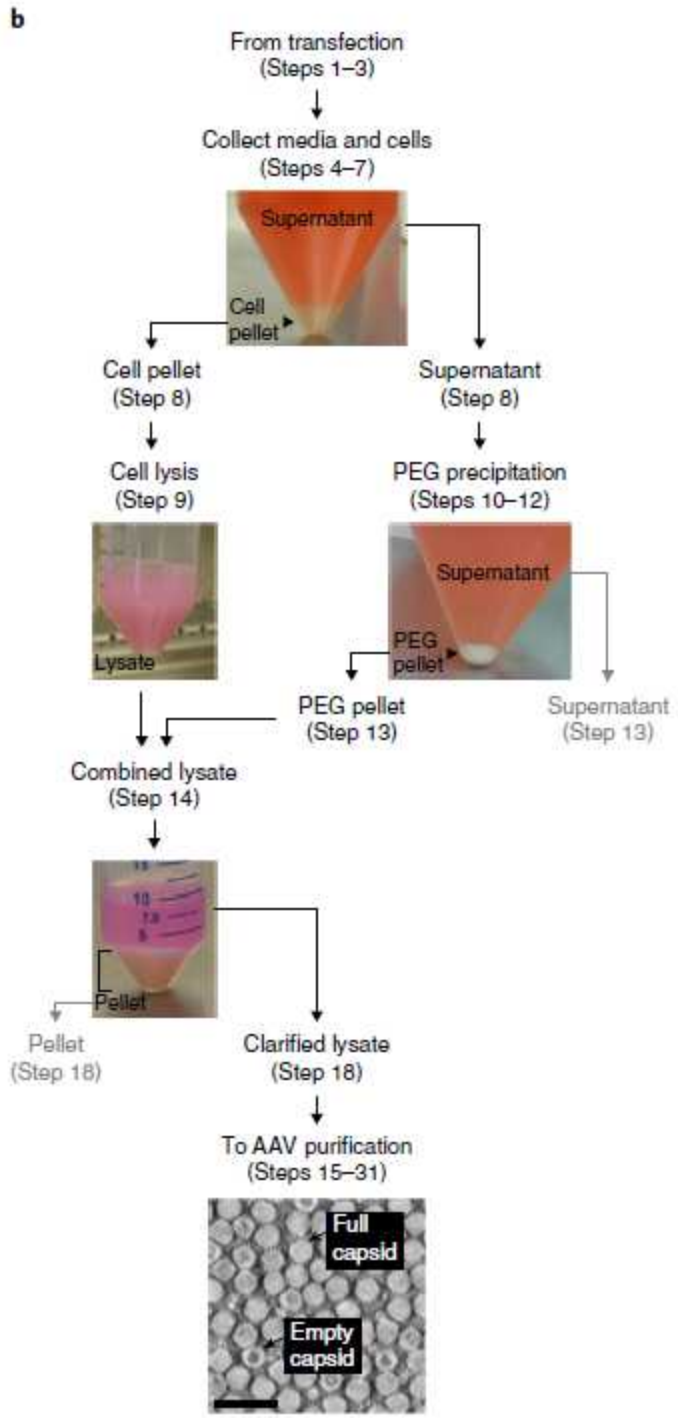


Fig 7b

22 Pour off the supernatant (i.e., the clarified media) into the reserved media bottle. Centrifuge again at 2000 x g, Room temperature, 00:01:00 and remove media using a P1000 pipette and add to the supernatant. 1m

Note

Incomplete removal of supernatant will cause several milliliters of media to dilute the SAN digestion buffer in Step 23

23 Prepare 9.5 mL (if using a Type 70 Ti rotor) or 3.0 mL (if using a Type 70.1 Ti rotor) of SAN + SAN digestion buffer (Reagent setup) per viral prep. Use a 10-ml serological pipette to gently resuspend the cell pellet in 8.0 mL (Type 70 Ti) or 2.4 mL (Type 70.1 Ti) of SAN + SAN digestion buffer; pipette into a 50-ml tube to finish resuspending the pellet (Fig. 7b). Incubate in a 37 °C water bath until Step 27. Remaining SAN + SAN digestion buffer can be stored at 4 °C until Step 27.

Note

CRITICAL STEP
- Save the conical tube for Step 24
- 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.

MODIFICATION
In Challis et al., *Nat Protoc. 14:* 379-414, 5 mL of SAN + SAN digestion buffer was used to resuspend the cell pellet. Resuspending and digesting the cell pellet in larger volumes of SAN + SAN digestion buffer can help to ensure more complete digest of nucleic acid and thus more complete solubilization of AAV particles from the cell lysate.

24 Retrieve the supernatant collected in Step 22, and transfer into the corresponding saved conical tube from Step 23. Using markings on the side of the conical tube, decant 1/4 of the supernatant volume of 40% (wt/vol) PEG stock solution into the supernatant (i.e. the PEG solution is formulated as 5X working concentration and the final solution should contain 8% (wt/vol) PEG). Tighten the cap and invert ten times to mix. Incubate On ice for at least 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].

Save the used media bottle for Step 26.

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

25 Centrifuge the PEG–media mixture at 4000 x g, 4°C, 00:45:00 . Centrifugation will result in the formation of a PEG pellet (Fig. 7b). 45m

26 Pour off the supernatant (i.e., the PEG-clarified media) into used media collection bottle for bleaching. Centrifuge the conical tube again at 4000 x g, 4°C, 00:05:00 and remove remaining supernatant with a P1000 pipette and add to the already collected supernatant. 5m

Note

CRITICAL
During the first centrifugation of PEG solution (Step 25), the precipitate can come to rest along the walls of the conical tube, especially if using a 500 mL tube. This second centrifugation step collects the precipitate in the bottom of the tube and ensures complete removal of clarified media.

27 PEG pellet resuspension. Resuspend the PEG pellet in 1 mL (Type 70 Ti) or 0.3 mL (Type 70.1 Ti) of SAN + SAN digestion buffer (Reagent setup) per viral prep. Using a P1000 pipette, pipette up and down to resuspend the PEG pellet until smooth and free of clumps. Combine the resuspended PEG pellet with the corresponding lysate from Step 23. 30m

Incubate in a 37 °C water bath for an additional 00:30:00 .

Note

Resuspending the PEG pellet is difficult and can take ~ 00:05:00 per pellet. Do not use a serological pipette to resuspend the pellet, which can become lodged within the barrel of the pipette.

28 Store the lysate at 4 °C Overnight . 5m

Note

Previously, we have accidentally left lysates in the 37 °C water bath overnight. We did not observe a drastic decrease in yields.

Procedure: AAV purification ● Timing 1 d 2h 15m

29

Note

CRITICAL
The AAV purification steps are most easily learned by visualization; refer to Fig. 8 and Supplementary Videos 1–3 of Challis et al., *Nat Protoc. 14:* 379-414 for details.

For a Type 70 Ti rotor, use 32.4 mL OptiSeal tubes (maximum capacity of 29.9 mL). For a Type 70.1 Ti rotor, use 8.9 mL OptiSeal tubes.

Determine the number of gradients needed and prepare the iodixanol density gradient solutions, if necessary (Reagent setup and Supplementary Table 3 from Challis et al., *Nat Protoc. 14:* 379-414). 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.

30 Pour the density gradients (Fig. 8a,b and Supplementary Video 1, 0:00–1:45, or Supplementary Video 2, 0:00–1:13 of Challis et al., *Nat Protoc. 14:* 379-414). Each gradient is composed of the following layers, from top-down:

- 15% (wt/vol) iodixanol in high salt buffer
- 25% (wt/vol) iodixanol in low salt buffer, with phenol red
- 40% (wt/vol) iodixanol in low salt buffer
- 60% (wt/vol) iodixanol in low salt buffer, with phenol red.

Pour the layers with a 2-mL serological pipette. Begin by pipetting the necessary volume of 15% (wt/vol) iodixanol to each tube. Next, add the required volume 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).

For a Type 70 Ti rotor:
- 6 mL of 15% step
- 4 mL of 25% step
- 5 mL of 40% step
- 3 mL of 60% step

For a Type 70.1 Ti rotor
- 1.8 mL of 15% step
- 1.2 mL of 25% step
- 1.5 mL of 40% step
- 0.8 mL of 60% step

Note

CRITICAL STEP

- When loading the 25%, 40%, and 60% layers, 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.
- Visually inspect the gradients before loading SAN-digested samples. Gradients without sharp delineations between steps should not be used.

MODIFICATION

Pouring density gradients can also be achieved using an electronic repeater pipette with a 25 mL (Type 70 Ti) or 10 mL (Type 70.1 Ti) tip. To create a tip that will fit into the OptiSeal tube, score a 1 mL serological pipette with a razor and break it into a 3 inch fragment. Do not use the tapered end of the serological pipette; rather, create a new end by scoring and breaking the tapered end off. Slide this 3 inch piece over the end of the repeater pipette tip and turn to ensure that it is firmly attached. Gradients can be poured as described above. When pouring the 25%, 40%, and 60% steps, set the electronic controller to dispense at the lowest possible rate to minimize disruption to the gradient. Pour a practice gradient first, to ensure that your electronic repeater pipette dispenses slowly enough.

31 Centrifuge the lysate from Step 36 at 2000 x g, Room temperature, 00:10:00 .

10m

Centrifugation will result in the formation of a pellet (Fig. 7b).

Note

To save time, start centrifugation once you begin pouring the 40% steps

32 Use a 2-ml serological pipette to load the supernatant (i.e., the clarified lysate) from Step 31 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 from Challis et al., *Nat Protoc. 14:* 379-414). Touch the pipette tip to the surface of the 15% layer and slowly release the lysate such that a layer forms on top.

For a Type 70 Ti rotor, you should be able to load about 9 mL of supernatant. For a Type 70.1 Ti rotor, you should be able to load about 2.7 mL of supernatant.

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.
- Pipette slowly to avoid drawing up the pellet
- 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.

33 Re-extract AAV from the pellet, by adding 3 mL (for Type 70 Ti) or 1 mL (for Type 70.1 Ti) SAN buffer to the pellet, briefly vortexing, and centrifuging at 3000 x g, Room temperature, 00:10:00 10m

Note

MODIFICATION
In our original protocol (Challis et al., *Nat Protoc. 14:* 379-414), OptiSeal tubes were topped up with SAN buffer to reach the necessary volume. Re-extracting the pellet with the necessary volume of SAN buffer can help to recover AAV particles still present in the pellet. In our experience, about 10-20% of the yield from the first supernatant can be extracted in this second step.

34 Using a 2-ml serological pipette, load the remaining supernatant into the OptiSeal tube or until the volume reaches the neck of the tube. If you have loaded all the supernatant and there is still remaining space, add SAN buffer to reach the neck of the tube.

Firmly insert a black cap (Fig. 8d from Challis et al., *Nat Protoc. 14:* 379-414) 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. Air bubbles can be removed using a small pipette tip or by gently tapping the bottom of the OptiSeal tube against the workspace.

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.
- Pipette slowly to avoid drawing up the pellet
- The black cap should fit right above or touch the lysate.

35 Weigh the tubes with the caps and spacers on. Balance the tubes to within 5-10 mg of each other by adding lysate or SAN digestion buffer, or by removing lysate. 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 adjust the volume with a P20 or P200 pipette. The density of the SAN digestion buffer and of the lysate are close to that of water (about 1000 mg/mL)

Note

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

36 Load balanced tubes into the ultracentrifuge rotor and fasten the lid.

Note

! CAUTION
Ensure that the rotor is in proper working order. This includes checking that the o-rings are intact and lubricated with vacuum grease. 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.

37 Carefully transfer the rotor to the ultracentrifuge and centrifuge: 2h 45m

For a Type 70 Ti rotor: 350000 x g, 18°C, 01:30:00 (58,400 r.p.m.)

For a Type 70.1 Ti rotor: 350000 x g, 18°C, 01:15:00 (61,700 r.p.m.)

Use 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 reaches 500 r.p.m., then take 6 min to stop) profiles.

Note

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

CRITICAL
The lower centrifugation time for a Type 70.1 rotor reflects the lower path length. If adapting the protocol for another rotor or centrifuge, use a rotor calculator (e.g. https://www.beckman.com/centrifuges/rotors/calculator) to ensure sufficient force and time.

MODIFICATION
Challis et al., *Nat Protoc. 14:* 379-414 uses a longer centrifugation time (02:25:00). We have not noticed any systematic difference in yield between AAVs purified with a 1.5 hour centrifugation vs. the original longer spin. This longer time is not necessary, but can be helpful in situations where a longer break in the purification protocol is desired.

38 During ultracentrifugation, gather the supplies and equipment for Steps 39-42. Assemble the clamp setup (Equipment setup) and collect one of each of the following per gradient: PES Ultra-15 centrifugal filter device, 5-ml syringe, 10-ml syringe, 0.22-µm syringe filter unit, and a 16-gauge needle.

39 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 of Challis et al., *Nat Protoc. 14:* 379-414). 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.

40 Prepare the supplies for Steps 41 and 42. 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 PES filter device. Next, add 10 mL of DPBS supplemented with 0.001% of Pluronic F-68 (DPBS + 0.001% Pluornic F-68) 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

- (**Optional**) Rinse the filtration membrane of the PES filter device by adding 15 mL of DPBS + 0.001% Pluornic F-68 to the top chamber and centrifuging at 3000 x g, Room temperature, 00:01:00 ; discard the flow-through. The manufacturer recommends using the device immediately after rinsing.

41 From the tube clamped in Step 46, collect the virus, starting just below the 40/60% interface, through the 40% layer[9,10] (Fig. 8g and Supplementary Video 3, 0:00–1:30 from Challis et al., *Nat Protoc. 14:* 379-414). 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 60% cushion.
- Before attempting to collect virus from the density gradient, practice on an OptiSeal tube filled with water.

42 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 from Challis et al., *Nat Protoc. 14:* 379-414). 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 PES 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 PES 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.
- Use DPBS with 0.001% Pluronic-F68 for all subsequent buffer exchanges, and for the for the final storage bufffer

43 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 PES filter device is 500-1500 µL (>10× concentrated).

Note

CRITICAL STEP
This step, and subsequent buffer exchange steps, may take longer than the indicated time. In this case, mixing the solution in the upper chamber by pipetting with a P1000 pipette before centrifuging again, can help to expedite the process.

44 Discard the flow-through for bleaching. Add 13 mL of DPBS + 0.001% Pluornic F-68 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.

45 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 150-200 µL of solution in the top chamber.

Note

CRITICAL STEP
- The minimum volume for the PES filter is around 150 to 200 µL. Once the solution has reached this volume, it will not concentrate any further.
- 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

46 Use a P200 pipette to transfer the virus from the top chamber of the PES filter device directly to a 1.6-ml screw-cap vial. Then wash the PES filter off with another 150-200 µL of DPBS + 0.001% Pluronic F-68, pipetting the wash buffer on the walls 10 times to recover any residual AAV. A second wash may help to increase yield further, but will also decrease the

concentration. Combine wash(es) with the rest of the virus in the 1.6-ml screw-cap vial and store at 4 °C .

Note

CRITICAL STEP

- PES 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 PES 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 .
- Avoid pipetting the wash buffer on the filter itself, as this may cause the filter to break apart and result in pieces of the filter in the final product. If this does occur, the above sterile filtration step can help to remove these.
- 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.
- The final volume will affect the AAV concentration (i.e. titer). We routinely keep virus suspensions in 4 °C at titers ranging from 1e12 to 1e14 vg/mL, for at least 3 months without any issue. Note though that the stability will depend upon the capsid itself and the concentration.

MODIFICATION

We have found that washing the PES filter can enable recovery of a significant amount of AAV particles; separately titering the original concentrate and the wash shows that the yield from the wash was 50-100% of that in the concentrate. A second wash allowed for an additional 10% recovery.

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