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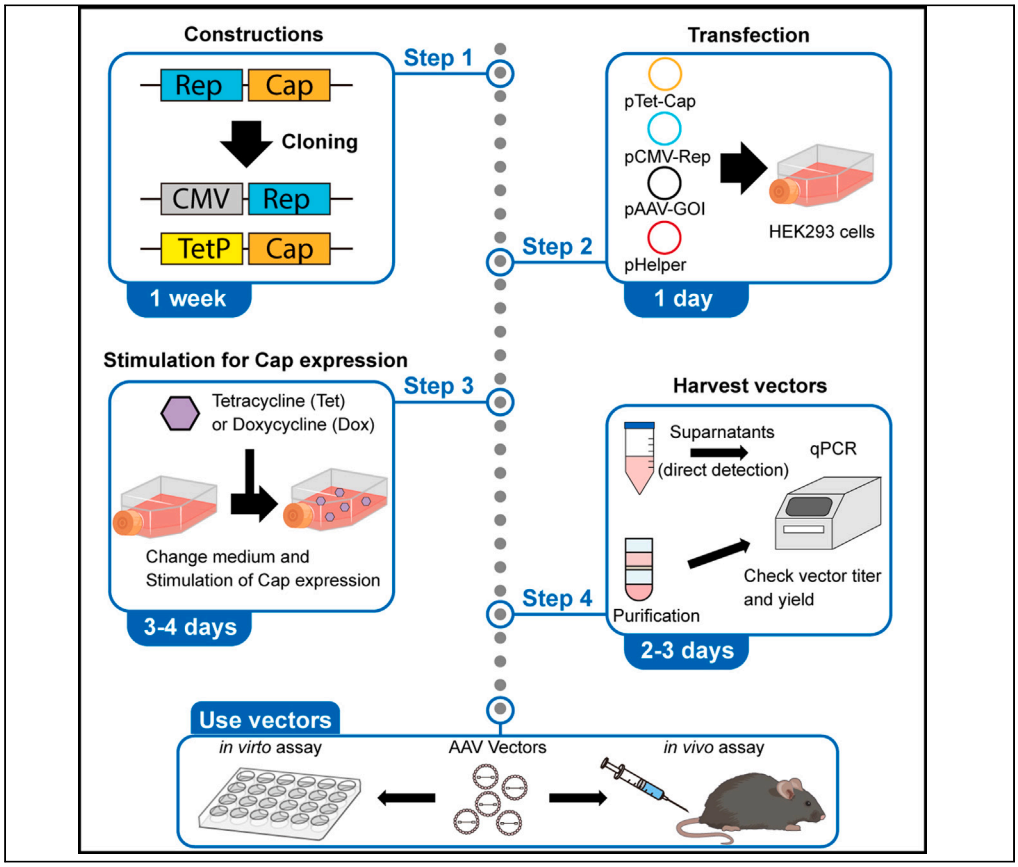
Protocol for producing an adeno-associated virus vector by controlling capsid expression timing



Conventional adeno-associated virus (AAV) production systems generate vast numbers of empty capsids, which should be eliminated before clinical use. Here, we present a protocol for efficient AAV vector production. We describe steps for separating replicase and capsid genes from the plasmid and controlling capsid expression until sufficient AAV vector genome replication is achieved. This protocol can produce AAV vectors in various serotypes.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

AAV vector production system controlling capsid expression timing

Protocol for AAV vector production by split expression of replicase and capsid

Increase of total AAV vector yield and improvement of empty capsid ratio

No characteristic change of AAV vector function by this protocol

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Protocol for producing an adeno-associated virus vector by controlling capsid expression timing

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SUMMARY

Conventional adeno-associated virus (AAV) production systems generate vast numbers of empty capsids, which should be eliminated before clinical use. Here, we present a protocol for efficient AAV vector production. We describe steps for separating replicase and capsid genes from the plasmid and controlling capsid expression until sufficient AAV vector genome replication is achieved. This protocol can produce AAV vectors in various serotypes.

For complete details on the use and execution of this protocol, please refer to Ohba et al.[1]

BEFORE YOU BEGIN

Adeno-associated virus (AAV) vectors are promising tools for gene therapy, and these vectors are globally used in clinical studies to treat various genetic disease.[2–7] However, these vector systems generate many empty capsids, which should be eliminated before clinical use. Moreover, the increasing cost of using conventional AAV vector systems for gene therapy is a significant issue. Herein, we provide an improved protocol for AAV vector production, which increases the vector yield and reduces empty capsids. This protocol requires conventional plasmids to construct replicase-expressing plasmids in the AAV vector production system and capsid-expressing plasmids regulated by tetracycline-dependent promoter. In addition, HEK293 cells are needed to produce AAV vectors using constructed plasmids.

Institutional permissions

Animal experiments were conducted humanely after receiving approval from the Institutional Animal Experiment Committee of the Jichi Medical University (Japan). Experiments followed the Institutional Regulation for Animal Experiments and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology (Japan).

Plasmid construction

Timing: > 1 week

1. Obtain PCR primer sets to amplify AAV2 replicase and capsid of various serotypes.

Note: To accomplish proper splicing of AAV genes, design PCR primer sets that target 15 nucleotides (nt) before the start codon and 92 nt after the stop codon for replicase gene and 350 nt before the start codon and 55–166 nt after the stop codon for capsid genes.

2. Clone the AAV2 replicase gene downstream of CMV promoter in a plasmid.
3. Clone each capsid gene from various serotypes downstream of Tetracycline (Tet) -regulating promoter (TetP) into plasmids.

Note: The optimal length of the additional sequence after the capsid gene depends on the AAV serotype. Both TetP systems (TetP and repressor are separated into two plasmid, or one plasmid containing TetP and repressor) can be used.

Plasmid preparation

Timing: 1 week

4. Transform plasmids (pAAV-GOI*, pHelper, pCMV-Rep, pAAV-RC, pTet-Cap, etc) in *E. coli*. *Gene of Interest.

Note: Any *E. coli* strain can be used for plasmid amplification. If some plasmids are not amplified efficiently, special competent cells such as NEB stable and Stbl3 can improve plasmid yield.

5. Culture *E. coli* in optimal medium volume at 37°C for 12–20 h.

Note: Amplifying some plasmids may be challenging. In this case, culture volume and time can be increased.

6. Harvest and purify DNA plasmids from *E. coli*, aliquot plasmid DNA in tubes, and store at −20°C before use.

Establishing stable cell lines (Optional)

Timing: > 2 weeks

7. Prepare HEK293 cells to 60%–70% confluency.
8. Transfect a plasmid containing the *Tet repressor* gene, such as pcDNA6/TR, and culture cells for 24–48 h.
9. Change the medium to fresh culture medium containing antibiotics (Zeocin for pcDNA6/TR) and culture the cells at 37°C in a CO_2 incubator for 24–72 h.

Note: Single colonies can be isolated at this time.

10. Repeat the passage and culture cells for 2–3 weeks, then store cells at −80°C before use.

Note: To obtain HEK293 cells with the *Tet repressor* gene in the genome, you must culture cells in an antibiotic-containing medium for at least 2 weeks.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
AAV8/9 intact particle antibody, ADK8/9 (I.P. = 1:50–200, W.B. = 1:200–500)	PROGEN	651161
AAV VP1/VP2/VP3 rabbit polyclonal, VP51 (W.B. = 1:1000–2000)	PROGEN	61084

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-rabbit IgG, HRP-linked Antibody (W.B. = 1:2000–5000)	Cell Signaling Technology	7074S
Bacterial and virus strains		
AAV1-EGFP	Ohba et al.[1]	N/A
AAV2-EGFP	Ohba et al.[1]	N/A
AAV2-EGFP-P2A-Nluc	Ohba et al.[1]	N/A
AAV3B-EGFP	Ohba et al.[1]	N/A
AAV5-EGFP	Ohba et al.[1]	N/A
AAV6-EGFP	Ohba et al.[1]	N/A
AAV7-EGFP	Ohba et al.[1]	N/A
AAV8-EGFP	Ohba et al.[1]	N/A
AAV9-EGFP	Ohba et al.[1]	N/A
AAV9-EGFP-P2A-Nluc	Ohba et al.[1]	N/A
AAVrh10-EGFP	Ohba et al.[1]	N/A
AAVPHP. eB-EGFP	This paper	N/A
AAVPHP. S-EGFP	This paper	N/A
Competent high DH5 alpha	TOYOBO	DNA-903F
NEB stable competent *E. Coli*	New England Biolabs	C3040I
One Shot Stbl3 chemically competent *E.Coli*	Thermo Fisher	C737303
Chemicals, peptides, and recombinant proteins		
Eagle's minimum essential medium (E-MEM)	FUJIFILM Wako	051–07615
Fetal bovine serum (FBS) (HyClone)	Cytiva	SH30910.03
MEM non-essential amino acid (×100)	FUJIFILM Wako	139–15651
Penicillin-streptomycin Solution (×100)	FUJIFILM Wako	168–23191
HilyMax transfection reagent	DOJINDO	H357-10 (342–91103)
Doxycycline	Takara Bio	631311 (Z1311N)
PEI MAX	Polysciences	24765–1
Polyethylene glycol 8000	Santa Cruz Biotechnology	SC-281693
Pluronic F68	Thermo Fisher Scientific (GIBCO)	24040–32
Opti-Prep	Serumwerk	1893
TURBO DNase	Thermo Fisher	AM2238
TURBO DNA-*free* kit	Thermo Fisher	AN1907
4%–20% Mini-PROTEAN® TGX™ Precast Protein Gels	Bio-Rad Laboratories	4561096
Clarity Western ECL substrate	Bio-Rad Laboratories	1705061
Dynabeads™ Protein A for Immunoprecipitation	Thermo Fisher Scientific	10001D
Dynabeads™ Protein G for Immunoprecipitation	Thermo Fisher Scientific	10003D
Blasticidin	InvivoGen	ant-bl-05
Zeocin	InvivoGen	ant-zn-05
Benzonase	Merck	70746-3CN
Critical commercial assays		
TB Green® *Premix Ex Taq*™ II	Takara Bio	RR820A
Experimental models: Cell lines		
HEK293 cells (human, kidney epithelial cells)	RIKEN Cell Bank	RCB1637
2v6.11 cells (human, kidney epithelial cells)	Mohammadi et al.[8]	N/A
Oligonucleotides		
Inverted terminal repeat (ITR)-specific 5′ primer: 5′-GGAACCCCTAGTGATGGAGTT-3′	Aurnhammer et al.[9]	N/A
Inverted terminal repeat (ITR)-specific 3′ primer: 5′-CGGCCTCAGTGAGCGA-3′	Aurnhammer et al.[9]	N/A
Recombinant DNA		
pAAV-EGFP	Ohba et al.[1]	N/A
pAAV-EGFP-P2A-Nluc	Ohba et al.[1]	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pAAV-RC2 (pAAV-RC)	Agilent	240071
pAAV-RC1	Ohba et al.[1]	N/A
pAAV-RC3B	Ohba et al.[1]	N/A
pAAV-RC5 (pRC5)	Takara Bio	6650
pAAV-RC6	Ohba et al.[1]	N/A
pAAV-RC7	Ohba et al.[1]	N/A
pAAV-RC8	Ohba et al.[1]	N/A
pAAV-RC9	Ohba et al.[1]	N/A
pAAV-RCrh10	Ohba et al.[1]	N/A
pAAV-RC PHP.eB	This paper	N/A
pAAV-RC PHP. S	This paper	N/A
pTet-Cap1 (pCW-AAV Cap1-WPRE-hPGK-Hyg-2A-rTetR)	Ohba et al.[1]	N/A
pTet-Cap2 (pCW-AAV Cap2-WPRE-hPGK-Hyg-2A-rTetR)	Ohba et al.[1]	N/A
pTet-Cap3B (pCW-AAV Cap3B-WPRE-hPGK-Hyg-2A-rTetR)	Ohba et al.[1]	N/A
pTet-Cap5 (pCW-AAV Cap5-WPRE-hPGK-Hyg-2A-rTetR)	Ohba et al.[1]	N/A
pTet-Cap6 (pCW-AAV Cap6-WPRE-hPGK-Hyg-2A-rTetR)	Ohba et al.[1]	N/A
pTet-Cap7 (pCW-AAV Cap6-WPRE-hPGK-Hyg-2A-rTetR)	Ohba et al.[1]	N/A
pTet-Cap8 (pCW-AAV Cap6-WPRE-hPGK-Hyg-2A-rTetR)	Ohba et al.[1]	N/A
pTet-Cap9 (pCW-AAV Cap6-WPRE-hPGK-Hyg-2A-rTetR)	Ohba et al.[1]	N/A
pTet-Caprh10 (pCW-AAV Cap6-WPRE-hPGK-Hyg-2A-rTetR)	Ohba et al.[1]	N/A
pTet-CapPHP. eB (pCW-AAV CapPHP. eB-WPRE-hPGK-Hyg-2A-rTetR)	This paper	N/A
pTet-CapPHP. S (pCW-AAV CapPHP. S-WPRE-hPGK-Hyg-2A-rTetR)	This paper	N/A
pHelper	Takara Bio	6650
pcDNA3.1(+)-Rep2	Ohba et al.[1]	N/A
pcDNA4/TO-Cap2	Ohba et al.[1]	N/A
pcDNA6/TR	Thermo Fisher Scientific	V102520
Software and algorithms		
GraphPad Prism 8	GraphPad	https://www.graphpad.com/scientific-software/prism/
Other		
Ultracentrifuge	Beckman Coulter	Optima L-100 XP
Amicon Ultra -4 or -15, 100K Centrifugal Filter Device	Merck	UFC810024 or UFC910024
Ultra-clear centrifuge tubes (14 × 95 mm or 25 × 89 mm)	Beckman Coulter	344060 or 344058

MATERIALS AND EQUIPMENT

150 mM NaCl buffer

Reagent	Final concentration	Amount
NaCl	150 mM	8.767 g
MilliQ	-	Up to 1 L

Note: Sterilize the 150 mM NaCl buffer using an autoclave (121°C, 15 min), or bottle-top filter (0.22 μM), and store it at room temperature (RT; 20°C–25°C) before use (Maximum time for storage is one year at RT).

PEI-MAX solution		
Reagent	Final concentration	Amount
PEI-MAX	1 mg/mL	100 mg
MilliQ	–	Up to 100 mL

Note: Sterilize the PEI-MAX solution using a 0.22 μM syringe filter. After preparing aliquots (10 mL each), store them at −20°C before use (Maximum time for storage is 1–2 years at −20°C).

AAV Lysis buffer		
Reagent	Final concentration	Amount
NaCl	200 mM	11.689 g
10% Pluronic F68	0.001%	0.1 mL
PBS	–	Up to 1 L

Note: Sterilize the AAV lysis buffer using a bottle-top filter (0.22 μM) and store it at room temperature (20°C–25°C) before use (Maximum time for storage is one year at RT).

40% PEG solution		
Reagent	Final concentration	Amount
PEG-8000	40%	400 g
NaCl		24 g
MilliQ	–	Up to 1 L

Note: Adjust the pH to 7.4. Sterilize 40% PEG solution using an autoclave (121°C, 15 min). Store the solution at 4°C before use (Maximum time for storage is 3–6 months at 4°C).

10 × PBS-MK		
Reagent	Final concentration	Amount
1 M $MgCl_2$	10 mM	1 mL
3 M KCl	25 mM	0.833 mL
10 × PBS	–	Up to 100 mL

Note: Sterilize 10 × PBS-MK using a bottle-top filter (0.22 μM) and store it at room temperature (20°C–25°C) before use (Maximum time for storage is one year at RT).

1 × PBS-MK		
Reagent	Final concentration	Amount
1 M $MgCl_2$	1 mM	1 mL
3 M KCl	2.5 mM	0.833 mL
10 × PBS	1× PBS	100 mL
MilliQ	–	Up to 1 L

Note: Sterilize 1 × PBS-MK using a bottle-top filter (0.22 μM) and store it at room temperature (20°C–25°C) before use (Maximum time for storage is one year at RT).

2 M NaCl / 1× PBS-MK		
Reagent	Final concentration	Amount
5 M NaCl	2 M NaCl	100 mL
1 × PBS-MK	–	150 mL

Note: Sterilize 2 M NaCl/1× PBS-MK using a bottle-top filter (0.22 μM) and store it at room temperature (20°C–25°C) before use (Maximum time for storage is one year at RT).

54% (w/v) Opti-prep (Iodixanol) buffer		
Reagent	Final concentration	Amount
Opti-prep (60% Iodixanol)	54%	45 mL
10 × PBS-MK	1 × PBS-MK	5 mL

Note: Prepare the buffer day of use. Add 45 μL of phenol red/10 mL of 54% Opti-prep buffer for a 54% gradient.

40% (w/v) Opti-prep buffer		
Reagent	Final concentration	Amount
54% Opti-prep buffer	40%	20 mL
1 × PBS-MK	–	7 mL

Note: Prepare the buffer day of use.

25% (w/v) Opti-prep buffer		
Reagent	Final concentration	Amount
54% Opti-prep buffer	25%	12.5 mL
1 × PBS-MK	–	14.5 mL
Phenol red	–	0.0675 mL

Note: Prepare the buffer day of use. Add 30 μL of phenol red/12 mL of 25% Opti-prep buffer for 54% gradient.

15% (w/v) Opti-prep buffer		
Reagent	Final concentration	Amount
54% Opti-prep buffer	15%	7.5 mL
2 M NaCl / 1× PBS-MK	–	13.5 mL
1 × PBS-MK	–	6 mL

Note: Prepare the buffer day of use.

Alternatives: Different transfection reagents will likely not impact AAV production. Other preferred reagents can be used in this protocol. Cesium chloride (CsCl) gradients could be used for ultracentrifugation purification step of AAV instead of iodixanol.

STEP-BY-STEP METHOD DETAILS

Preparing HEK293 cells – Day 0

Timing: 12–24 h

1. Plate HEK293 cells in a Minimum Essential Medium (MEM)-containing plate containing 10% fetal bovine serum (FBS) and non-essential amino acid (NEAA) (If possible, it would be better not add antibiotics to the culture medium at this time).

   Note: The cell seeding number depends on the culture scale.

2. Check cell confluency before transfection.

   Note: Cells should reach 80%–90% confluency by observing under microscope at the time of transfection.

   Note: Cell condition is one of the crucial factors for AAV vector production. If the condition is poor, including the presence of strange cell shapes, use a new cell stock to properly produce AAV vectors.

Transfection of AAV plasmids – Day 1–5

Timing: 1 h (for step 3)

Timing: 1 h (for steps 4 to 8)

Four plasmids containing the AAV genome, adeno virus helper genes, Tet-Cap, and CMV-Rep2 are co-transfected into HEK293 cells for AAV vector production. Plasmids for conventional AAV vector transfection can be prepared as control. For comparison, the plasmid transfection volume must be adjusted similarly by adding an empty plasmid. Any transfection reagents can be used for this protocol.

3. Transfect plasmids to HEK293 cells.
   Small scale (such as 6-, 12-, or 24-well-plates).
   a. Commercialized transfection reagents can be used according to manufacturer's instructions. (Example; HilyMax https://www.dojindo.co.jp/manual/H357e.pdf, Lipofectamine 3000 https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Flipofectamine3000_protocol.pdf)
   b. Prepare Opti-MEM medium in a tube (the suitable medium volume depends on transfection reagent)
   c. Add plasmids to the tube in the following ratio:

	μg for 12 well	(Ratio)
pAAV-EGFP (or pAAV-GOI)	1 μg	(1)
pTet-Cap (or pAAV-RC)	1 μg	(1)
pHelper	1 μg	(1)
pcDNA3.1 (+)-Rep (or pcDNA3.1(+)-empty)	0.25 μg	(0.25)
Total	3.25 μg	

All plasmids are added in a tube containing Opti-MEM medium.

   d. Add transfection reagent to each tube according to manufacturer's instructions.
   e. Mix samples well by vortex (it depends on manufacturer's instructions) and incubate them for 10–20 min at room temperature (RT; 20°C–25°C).

f. Add samples dropwise to each well for plasmid transfection into cells.
g. Incubate cells at 37°C, 5% CO_2, 90%–95% humidity for 12 h in a CO_2 incubator.

Large scale (such as T225 flask, Cell Factory [2528 $cm^{2]}$]).

h. Prepare PEI-MAX solution and 150 mM NaCl solution.

The following protocol is for transfection using Cell Factory (Culture medium; 400–500 mL.

i. Prepare 50 mL (1/10 volume of culture medium) of 150 mM NaCl solution in a 225 mL tube.
j. Add plasmids to the tube in the following ratio:

	μg for 2528 cm^2	(Ratio)
pAAV-EGFP (or pAAV-GOI)	600 μg	(1)
pTet-Cap (or pAAV-RC)	600 μg	(1)
pHelper	600 μg	(1)
pcDNA3.1 (+)-Rep (or pcDNA3.1(+)-empty)	150 μg	(0.25)
total	1950 μg	

All plasmids are added in a tube containing 150 mM NaCl solution.

k. Add 6825 μg of PEI-MAX (3.5-fold to total DNA amount (μg)) to each tube.

Note: The volume of PEI-MAX needs to be optimized to obtain best results by your cell condition such as transfection efficiency and cytotoxicity against DNA and reagents.

l. Mix samples well by up-side-down and incubate them for 20 min at RT.
m. Take approximately 100 mL of culture medium from culture flask and add it to a new tube.
n. Add the plasmids mixture to 100 mL of culture medium and mix well (the total volume is approximately 150 mL).
o. Return the mixture to the flask for plasmid transfection into cells and mix the medium gently.
p. Incubate the cells at 37°C for 12 h in a CO_2 incubator.

Note: Cells must be adherent in this transfection step to obtain maximum AAV vector production. If some cells float during transfection, the efficacy of AAV vector production decreases.

4. At 12 h post-transfection, prepare fresh, warmed complete culture medium.
5. Add doxycycline (Dox) with a final 2 μg/mL concentration to fresh medium.
6. Discard culture medium from the flask.
7. Add fresh complete medium containing Dox in the flask.
8. Incubate cells at 37°C in a CO_2 incubator for 3 days (small scale) or 4 days (large scale).

Note: Generally, adding Dox at 10–15 h post transfection is advantageous for efficient AAV vector production. However, vector production efficiency may depend on the cell condition and experiments.

Harvest cells and supernatants – Day 5

Timing: 1–2 h

9. Prepare the AAV lysis buffer, 1 × phosphate-buffered saline (PBS), chilled 5 mM EDTA/PBS solution on ice, and 40% PEG solution.
10. Harvest cells and supernatants.

Small scale.

a. Prepare the tubes for the samples.
b. Pipette the culture medium to detach all cells from plate.

c. Collect the cells and supernatant in a tube.
d. Store samples in −80°C before use.

Large scale.

e. Prepare the tubes for the samples.
f. Collect the culture supernatants in tubes and add 1/4 volume of 40% PEG solution, and store the mixture at 4°C for at least 4 h after mixing well until AAV vector collection in supernatants in subsequent steps.

Note: Only supernatant is needed to harvest AAV vector in culture meidum at this moment. Detached cells may exist in supernatant at this step. These cells can be separated from the supernatant by centrifugation, and the cell pellet can be mixed with sample at step b-5.

g. Carefully wash the cells using 1 × PBS while avoiding detaching cells, and discard PBS.

Note: If many detached cells are present in this step, collect the samples in a tube and mix them according to step b-5.

h. After the PBS wash, add 100 mL of ice-chilled 5 mM EDTA/PBS solution to the cell flask, and incubate the cells at RT for 10–15 min to detach the cells.
i. Collect solutions containing cells in a tube, and centrifuge at 2000–3000 × *g* for 10 min, and then discard the supernatant.
j. Wash the flask with 100 mL PBS and collect the cells in tubes.
k. Repeat step b-5 twice to collect the cells from the flask completely.
l. Centrifuge samples at 2000–3000 × *g* for 10 min, and then discard the supernatant.

Note: If the entire volume of supernatant cannot be collected in a tube, centrifugation can be performed separately for each wash sample.

m. Discard the supernatant and keep the pellets on ice or in −80°C before use.
n. Centrifuge the supernatants-PEG mix (after the 4-h 4°C incubation) from step b-2, at 2000–3000 × *g* for 20 min.
o. Discard the supernatant, and dissolve the supernatant-pellet in 8–10 mL of AAV lysis buffer.
p. Add dissolved samples (step b-11) to each tube containing cell pellet at step b-9, and store samples at −80°C before use.

Pause point: You can stop experiment after freezing samples.

AAV vector extraction and purification – Day 5–6

Timing: 1–2 days (for steps 11 to 34)

For AAV vector extraction and purification, you can use global standard methods.[10,11] Ultracentrifuge steps to purify AAV vectors require items described in Figure 1A and key resources table.

Extract AAV vectors

11. Prepare a 37°C water bath and liquid nitrogen (or cold ethanol using dry ice).
12. Incubate the frozen samples in a 37°C water bath for 10–15 min until they have thawed entirely.
13. Mix the samples well by vortexing for 10–20 s.
14. Then place samples in liquid nitrogen (or cold ethanol using dry ice) for 20 min until completely frozen.
15. Repeat steps 12–14 thrice (four times in total).

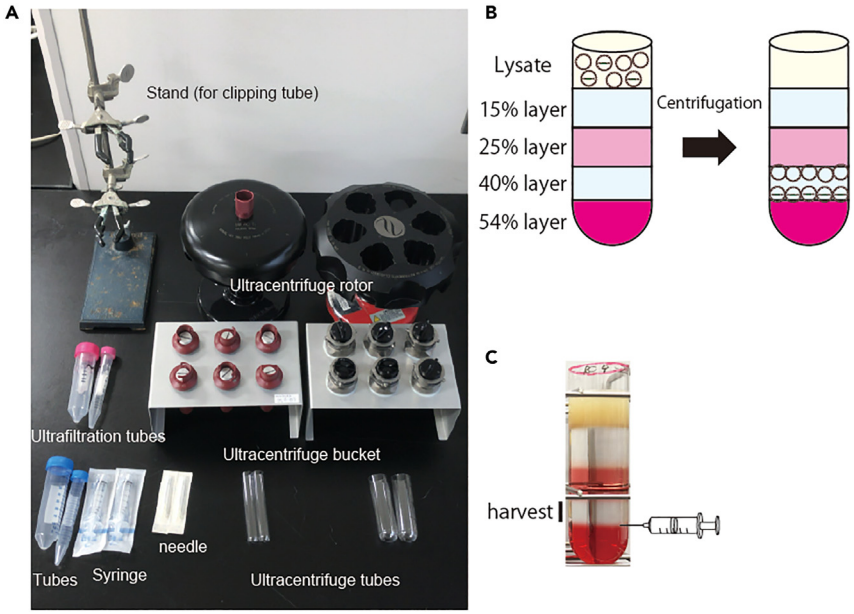

Figure 1. Ultracentrifuge for AAV vector purification

(A) Items used for ultracentrifugation to purify AAV vectors.

(B) Diagram of layers containing AAV vectors before and after ultracentrifugation.

(C) The position of the needle to collect AAV vectors after centrifugation.

16. Centrifuge the samples at 5000–8000 × *g* for 10 min to pellet the cell debris.
17. Transfer the supernatants to new tubes (The supernatants comprise the crude AAV vector solutions).

Note: For small scale preparations, you can filter the supernatants using 0.22 μm syringe-top filter to eliminate cell debris after centrifugation (step b-16).

18. Digest free DNAs in solution with a DNase treatment.
    [Small scale]
    a. Prepare DNase solution, such as Turbo DNase, according to manufacturer's instructions.
    b. Take 4 μL of sample and digest free DNAs in a 20 μL reaction volume. Example: TURBO DNase or TURBO DNA-*free* kit (ThermoFisher)

Sample	4 μL
10× reaction buffer	2 μL
TURBO DNase	1 μL
Pure water	13 μL
total	20 μL

    c. Incubate the samples at 37°C for at least 4 h.
    d. After the reaction, inactivate the DNase according to the manufacturer's instructions. (For example, inactivate DNase by adding 1 μL of inactivation reagent from the Turbo DNA free kit and mixing samples well. Centrifuge samples at 8,000–10,000 × *g* for 5 min and then collect supernatants into a new tube for elimination of DNase.)
    e. Add 1 volume of water to dilute DNase-treat samples.

Note: Samples are diluted with 1/10 from the initial concentration.

Note: Because AAV vector yield by small scale is small, samples before DNase treatment (step 17) can be used for various assay. However, the step of DNase treatment (step 18) is required to check AAV vector titer in solution (Quantification; step 34–40).

f. Proceed to the quantification step.

[Large scale]

g. Add benzonase with 50 units/mL as the final concentration to each tube.

h. Incubate the samples for at least 4 h at 37°C.

i. Proceed to the purification step.

Purify AAV vectors

19. Prepare ultracentrifugation tubes and 54, 40, 25, 15% Opti-prep (Iodixanol) buffers.

Note: Opti-prep buffers should be prepared ahead of time.

20. Layer 54, 40, 25, and 15% Opti-prep gradients (from the bottom to the top) in ultracentrifugation tubes (Figure 1B) slowly to not mix layers.

	Tube for SW32 Ti	Tube for SW40 Ti
54% layer	6.0 mL	2.0 mL
40% layer	6.0 mL	2.0 mL
25% layer	7.0 mL	2.5 mL
15% layer	8.5 mL	3.0 mL
Sample	8.5–9.5 mL	2.5–3.0 mL

21. Apply the sample (step 18 b-3) on top of the 15% layer.
22. If making multiple samples can add AAV lysis buffer to the top for balance.
23. Perform ultracentrifugation at 30,000–40,000 rpm (approximately 170,000–280,000 × *g*) for 6–15 h at 4°C–10°C.

Note: Normally, centrifugation is performed for 12–15 h (overnight).

24. Check the layers after ultracentrifugation and collect the entire second layer from the bottom (Figures 1B and 1C), which contains full and empty AAV vectors, with syringe.

Note: The second layer contains full (including AAV genome) and empty AAV particles. To select the portion containing full AAV vectors, the fractionation for second layer can be performed.

Note: If further purification is needed, re-ultracentrifugation can be performed using the samples in this step.

25. Add 1 volume of AAV lysis buffer to dilute the sample.
26. Prepare Amicon Ultra 100 kDa MWCO column (4 mL or 15 mL, Merk) by adding 4–15 mL of AAV lysis buffer to pre-wash the membrane, and then centrifuging the column at 3000 × *g* for 5 min.
27. Discard the flow-through, and immediately add the sample (at step 25) to the pre-washed column.
28. Centrifuge the sample at 3,500–4,000 × *g* for 40–60 min.

Note: Samples are sticky and may not easily pass through the column. In this case, increase the centrifugation time until most samples pass through the column.

29. Add 4 mL of fresh AAV lysis buffer to wash the column (If you use a 15 mL column, add 15 mL of AAV lysis buffer)
30. Centrifuge the sample at 3,500–4,000 × *g* for 20–40 min.

Note: Adjust the optimal remaining volume of solution in the column by centrifugation time.

31. (Optional) Wash column by repeating step 29–30.
32. Collect the remaining solution above the column, which contains AAV vectors.
33. Filter sample further using a 0.45 μm syringe.
34. Aliquot AAV vector solutions into tubes and keep them in −20°C or −80°C before use.

Note: Because AAV vectors are usually stable, they can be stored at 4°C for short periods.

Pause point: You can stop experiment after finishing this step.

Quantification of AAV vectors – Day 7

Timing: 3–4 h

To use the produced AAV vector for various assays, you need to determine the AAV vector titer using quantitative polymerase chain reaction (qPCR) or other methods.

35. Prepare the qPCR reagent, primer sets targeting the inverted terminal repeat (ITR), standard (quantified AAV vector, plasmid containing AAV genome, fragment of ITR), and samples.
36. Prepare 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 vg/μL of standard samples and a negative control.
37. Place the standard and samples in separate wells in a PCR plate in duplicate or triplicate, and seal the plate.
38. Incubate the plate at 95°C for 10 min to denature AAV vectors.

Note: Instead of DNA plasmids or fragments, we recommend using viral particles for the standard and denaturing them together with samples in the same plate because the efficacy of AAV genome release from particles and capsid proteins in the reaction may affect the qPCR result.

39. Prepare the qPCR master mix, including primers, according to manufacturer's instructions.

(As example of a qPCR reaction with a reaction volume of 20 μL): 1 μL of sample, 12.5 μL of 2 × qPCR buffer, 0.1 μL of primer 1 (10 μM), 0.1 μL of primer 2 (10 μM), 11.3 μL of pure water).

(Example; TB Green Premix Ex Taq II https://www.takarabio.com/documents/User%20Manual/RR82LR/RR82LR_UM.pdf)

Note: The concentration of the samples in this protocol are 1/10 dilution from the original samples.

40. Perform the qPCR reaction according to manufacturer's instructions.

Note: The reaction conditions depend on the reagents and instrument. Fast and conventional PCR cycles are applicable.

41. Analyze AAV vector titer in samples.

Note: After determining the AAV vector titer, these vectors can be used for various assays such as enzyme-linked immunosorbent assay (ELISA), western blots, immunoprecipitation, infection studies on cells and injection into mouse models.

Table 1. The comparison summary between the present protocol and conventional methods

	summary of results[a]
Increase in total AAV vector yield[1]	2–10-fold[b]
Full (incorporation of AAV genome)/empty vector ratio	10%–50% improvement[a]
Applicable serotypes	Various
AAV vector cell infectivity	No difference
AAV vector distribution in mice	No difference[b]

[a]The differences depend on serotypes.
[b]AAV9 distribution in mice.

Note: After aliquoting, the AAV vectors can be kept at −20 or −80°C for long-term storage, at 4°C for short-term storage, for 1–2 weeks, before use.

EXPECTED OUTCOMES

We show a detailed step-by-step protocol to efficiently produce AAV vectors by capsid (cap) expression timing control. Compared with conventional system, we have successfully produced high quantity and quality of AAV vectors using the tetracycline promoter controlling Cap expression (Tet-Cap) system. Furthermore, our protocol applies to most of AAV serotypes. AAV vectors produced by the Tet-Cap system have similar cell infectivity and tissue distribution in mice than the conventional system,[1] indicating that the change of protocol does not affect AAV vector function (Table 1).

Many studies have attempted to generate Cap-mutated AAV vector to modify tissue tropism. However, introducing mutant viral components, including capsid genes, sometimes reduces viral fitness, including a low viral yield. In contrast, our protocol can increase vector quantity (Figures 2A and 2B) and quality (Figure 2C), even for Cap mutants such as PHP.eB and PHP.s,[12,13] indicating that this method is possibly applicable for various AAV mutants to improve AAV vector production.

However, using AAV vectors for gene therapy has a high cost due to generating large numbers of empty capsids during vector production, requiring multi-purification step before clinical use.[14,15]

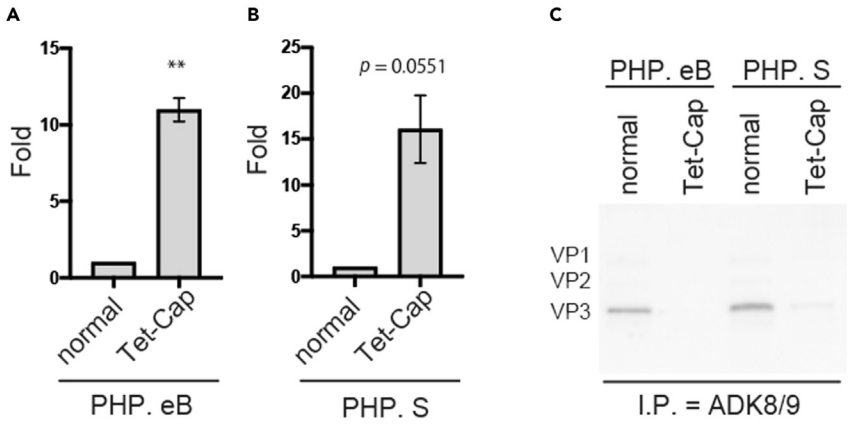

Figure 2. The present protocol applies to capsid mutants

(A and B) AAV vector yield in PHP. eB and PHP. S capsid mutants using Tet-Cap system. The fold difference of AAV vector yield on AAV PHP. eB (A), and AAV PHP. S (B) with the change in medium and doxycycline (Dox) stimulation 12 h post-transfection. The data was normalized to the qPCR value of normal samples (RC; conventional system). Graphs and statistical analyses were performed using GraphPad v 8 software from three independent experiments. The asterisk in panel indicates as follow: ** = $p < 0.01$, which presents statistical significance of the t-test with Welch's correction. Error bars indicate the standard error of the mean (SEM).

(C) Western blot of Cap proteins after immunoprecipitation of AAV PHP.eB and PHP.S. The same titer of AAV vector (5×10^8 vg/sample for PHP.eB and 2×10^8 vg/sample for PHP. S) calculated using qPCR was subjected to immunoprecipitation using ADK8/9 antibodies, and protein A/G magnetic beads before western blotting. The panel shows representative western blotting image.

Our protocol can efficiently produce AAV vectors, increase the total yield by approximately 2–10-fold, and reduce the empty capsid ratio. Our system could be used as a bioreactor system to increase the production scale.[16] Thus, we expect that our system can help reduce the cost of gene therapy in the future.

LIMITATIONS

Our protocol can increase total AAV vector yield and improve its quality compared to conventional AAV production system. However, because improving the AAV vector production is based on the comparing the Tet-Cap and commercialized AAV vector production systems, it is unclear whether Tet-Cap system has an advantage over the modified AAV vector production system. Additionally, the cell condition in each laboratory may differ, such as the number of passages and cell culture medium for example. Because cell condition is a crucial factor for viral production, improving AAV vector production varies between laboratories. Moreover, the different techniques used to purify AAV vectors in each laboratory may affect the AAV vector yield results.

TROUBLESHOOTING

Problem 1

Cells are detached from the plate after transfection to produce AAV vectors (refer to step 3 to 8).

Potential solution

Plasmid DNA and proteins expressed in plasmids are sometimes toxic for cells. Therefore, optimizing the total plasmid number for transfection, suitable for your laboratories' cells, could reduce detached cells.

In addition, the character of the HEK293 cells may differ from the original cells after long-term culturing or changing culture conditions such as using Dulbecco's Modified Eagle Medium (DMEM) medium. Therefore, this protocol recommends using appropriate culture conditions like MEM supplemented with 10% FBS and NEAA, the original culture medium for HEK293 cells. In some cases, you may obtain similar results to our paper[1] if cells used for AAV vector production are cultured with a different medium, such as DMEM supplemented with 10% FBS. However, if you cannot obtain a similar result, using original culture condition for HEK293 cells is recommended for this protocol. Additionally, long-term culturing using different media results in different cell characteristics, which may not be recovered to the original characteristics. In this case, using original cells and proper culture conditions as far as possible are strongly recommended for this protocol.

Problem 2

Improving the total yield and full/empty particles (E/F) ratio of AAV vectors cannot be observed (refer to step 40 and expected outcomes).

Potential solution

Usually, HEK293 cells are adherent and must be active state for AAV vector production. Like the solution for problem 1, transfection conditions need to be optimized for efficient AAV vector production. Additionally, because viruses are generated in cells, the cellular machinery for viral production must be intact. Therefore, the cell condition is crucial to produce AAV vectors efficiently. In addition of culture conditions, cells must be healthy and adherent during AAV vector production as long as possible. If cell condition is poor, preparing healthy cells using original cell stocks or obtaining cells from companies or cell banks would improve the results.

HEK293 grown in suspension can be used for this protocol. However, because AAV vector production system using suspension HEK293 cells is already more efficient than the conventional system, the improvement of the total yield and E/F ratio using this protocol may be small.

Problem 3

Many empty AAV vectors remain (refer to step 19–33 and expected outcomes).

Potential solution

In this protocol and in our previous paper,[1] we compared the total yield and E/F ratio of AAV vectors between the Tet-Cap and conventional system. Therefore, we aimed to collect all AAV vectors, including full and empty particles. If no empty vectors are needed, you can perform an additional ultracentrifuge or fractionation to obtain more purified AAV vectors at step 24, as previously reports.[10,11]

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kenji Ohba (ohbak@jichi.ac.jp).

Materials availability

Antibodies were obtained from commercial sources described in the STAR Methods key resources table. All unique materials generated in this study will be available from the lead contact after completing a Material Transfer Agreement.

Data and code availability

- The data reported in this paper will be shared by the lead contact (ohbak@jichi.ac.jp) upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

K.O. conceived and designed the research, performed all experiments and data analysis, interpreted the results of all experiments, and prepared the manuscript. K.O. and H.M edited the manuscript. All authors reviewed and approved the manuscript.

DECLARATION OF INTERESTS

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Based on the present study, K.O. and TAKARA Bio Inc. have applied for an international patent.

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