AAV Production Protocol

Packaging Concentration and Purification

GENEMEDI
Innovation In Gene Therapy

Production protocol

Adeno-associated virus (AAV)

Introduction of AAV

Adeno-associated virus (AAV) is a small single-strand DNA virus infecting human and some other primate species. Currently, AAV has not known to cause disease and only induces very mild immune responses. As a member of the family Parvoviridae, wild type AAV requires the assistance of adenovirus or herpesvirus to complete the duplication, which is the reason why it's called adeno-associated virus [1,2]. The wild-type AAV2 genome consists of the viral rep and cap genes (encoding replication and capsid genes, respectively), flanked by inverted terminal repeats (ITRs) that contain all the cis-acting elements necessary for replication and packaging. The genome of typical AAV2 is about 4800bp, consisting of two upstream and downstream open read frames (ORFs) which are between two inverted terminal repeats (ITR) comprising Rep and Cap. ITR is required for synthesis of complementary DNA strand, while Rep and Cap can be translated into various proteins, including AAV virus cycle essential protein Rep78, Rep68, Rep52, Rep40 and enveloped protein VP1, VP2, VP3, etc. [3].

The present recombinant AAV (rAAV) vectors are generated by replacing all of the viral genome between the ITRs with a transcriptional cassette of less than 5 kilobases in length. The resulting construct is then co-expressed with two other plasmids: 1) an AAV-RC plasmid that provides the Rep and Cap genes in trans (separate from the ITR/Transgene cassette) and 2) an AAV helper plasmid that harbors the adenoviral helper genes. AAV-293 cells are used as the packaging cell line since they provide the E1a protein in trans as well. By modifying the Rep and Cap genes, scientists can control the serotypes to guide the recombinant AAV infection towards certain tissues. This 3-plasmid co-transfection system liberates the need for adenovirus during AAV production, which greatly simplifies the purification process.

To date, a total of 12 serotypes of AAV have been described with their unique traits and tropisms [4]. Concerning high safety, low immunogenicity, long-term expression of exogenous genes, AAV is thought to be the best gene delivery tool for gene function research in vivo.

Over the past decades, numerous AAV serotypes have been identified with variable tropism. To date, 12 AAV serotypes and over 100 AAV variants have been isolated from adenovirus stocks or human/nonhuman primate tissues. Different AAV serotypes exhibit different tropisms, infecting different cell types and tissue types. So, selecting the suitable AAV serotype is critical for gene delivery to target cells or tissues.

Due to the exhibition of natural tropism towards certain cell or tissue types, rAAV has garnered considerable attention. Highly prevalent in humans and other primates, several AAV serotypes have been isolated. AAV2, AAV3, AAV5, AAV6 were discovered in human cells, while AAV1, AAV4, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12 in nonhuman primate samples [5,6]. Genome divergence among different serotypes is most concentrated on hypervariable regions (HVRs) of virus capsid, which might determine their tissue tropisms. In addition to virus capsid, tissue tropisms of AAV vectors are also influenced by cell surface receptors, cellular uptake, intracellular processing, nuclear delivery of the vector genome, uncoating, and second-strand DNA conversion [7].

In order to better improve the infection efficiency and specificity of AAV to target tissues, scientists have genetically modified the viral capsid, and generated mosaic vectors to create chimeric AAV by swapping domain's or amino-acids between serotypes [8,9], which may allow researchers to specifically target cells with certain serotypes to effectively transduce and express genes in a localized area [10].

Protocol Overview

A schematic overview of recombinant AAV production is shown in Figure 1. The first step is to clone the gene of interest (GOI) into an appropriate plasmid vector. For most applications, the cDNA of interest is cloned into one of the ITR/MCS containing vectors. The inverted terminal repeat (ITR) sequences present in these vectors provide all of the cis-acting elements necessary for AAV replication and packaging.

The recombinant expression plasmid is co-transfected into the AAV-293 cells with pHelper (carrying adenovirus-derived genes) and pAAV-RC (carrying AAV2 replication and capsid genes), which together supply all of the trans-acting factors required for AAV replication and packaging in the AAV-293 cells. Recombinant AAV viral particles are prepared from infected AAV-293 cells and may then be used to infect a variety of mammalian cells.

Upon infection of the host cell, the single-stranded virus must be converted into double-stranded for gene expression. The AAV virus relies on cellular replication factors for the synthesis of the complementary strand. This conversion is a limiting step in recombinant gene expression and can be accelerated using adenovirus superinfection or etoposides, such as camptothecin or sodium butyrate. Whereas these agents are toxic to the target cells and can kill target cells if left on the cells, so the use of etoposides is only recommended for short-term use or in obtaining viral titers. Typically, the AAV genome will form high molecular weight concatemers which are responsible for stable gene expression in cells.

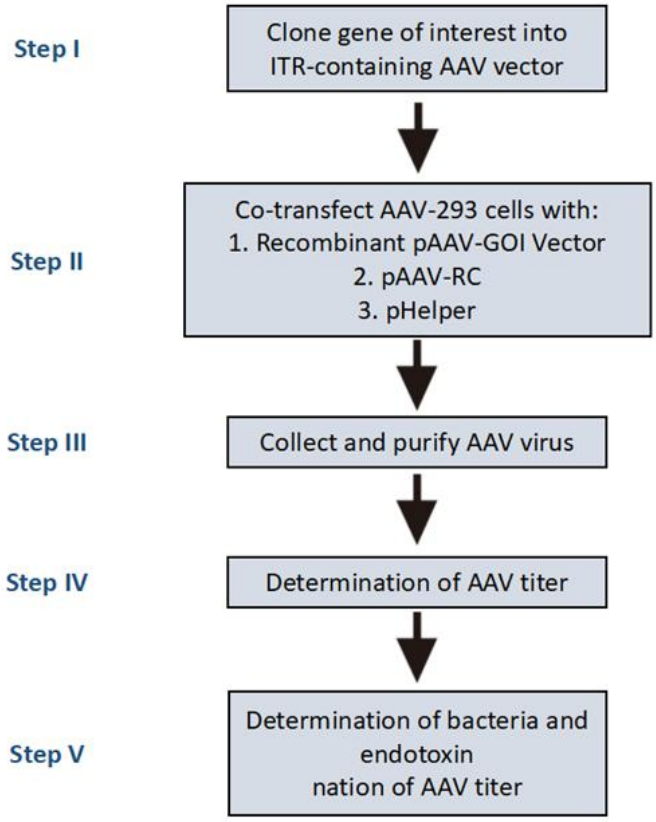

Figure 1. AAV packaging experiment flow chart.

Experimental Materials

GeneMedi's AAV Vectors System

GeneMedi's AAV Vector System, also named AAV expression system or AAV packaging plasmid system, is a powerful tool for in-vivo gene delivery, gene editing, and gene therapy. You can easily produce a recombinant AAV (rAAV) particle in 293T cell line in high titer using GeneMedi's AAV Vector System. The Genemedi AAV vector system including multiple AAV expression vector plasmids, AAV helper plasmid and the serotypes-specific AAV Rep-Cap plasmids (AAV-RC).

GeneMedi's AAV expression vectors have been inserted with different expression cassettes, containing kinds of verified protomers and reporters including GFP, zsgreen, RFP, mcherry and luciferase. The GeneMedi's AAV expression vectors have been proved very suitable for unique gene overexpression or shRNA-mediated knock-down (also called RNAi (RNA interference). You can also achieve gene knock-out(KO) or gene editing using our Crispr-cas9-gRNA AAV expression vector.

The serotypes-specific AAV Rep-Cap plasmid (AAV-RC plasmid, or called AAV-RC plasmid) contain the AAV2-Rep gene with different serotypes of AAV's Cap gene(also called AAV capsids gene).
GeneMedi's AAV Rep-Cap plasmids is including AAV2, AAV5, AAV6, AAV8, AAV9, AAV-PHP.B, AAV-PHP.eB, AAV PHP.s, AAV-Retro (Retrograde), AAV-Anc80 (L65), AAV-DJ, AAV-DJ8. GeneMedi also supplies capsid optimized AAV variant including AAV2 variant(Y444F), AAV2 variant (Y272F, Y444F, Y500F, Y730F), AAV2 variant (Y444F, Y730F, Y500F, Y272F, Y704F, Y252F), AAV2.7m8, AAV8 variant (Y733F), AAV8 variant(Y733F, Y447F), AAV8 variant(Y733F,Y447F,Y275F) and some other engineering AAV serotypes not mentioned.

Visit https://www.genemedi.net/i/aav-vector-system here for more information about GeneMedi's AAV vector system and multiple serotypes of AAV Rep-Cap plasmids.

Bacterium Strain

E. coli strain DH5α is used for amplification of shuttle and backbone vectors.

Packaging Cell Line

AAV-293 is the virus packaging cell line that can facilitate initial production, amplification and titration of rAAV. Originated from the 293 cell line and established for plaque assays, this cell line was identified to be an easy-to-handle transfection host.

The complete growth medium of AAV-293 is Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep). For a continuous culture, cells should not exceed 70% confluence to maintain proper characteristics. Usually, starting from cell passage number one, optimal results can be obtained within 30 passages. Once reached, it is best to start a new culture from another frozen stock in case of any unexpected mutations and unhealthy growth. Therefore, banking your own AAV-293 frozen stocks is very important to ensure experimental integrity and continuity. Freezing cells at the logarithmic phase will improve post-thaw viability.

Notices:

To maintain cells in a healthier condition and improve production efficiency of AAV, it is recommended to use our Genemedi anti-mycoplasma reagent, CurePlasma™.

Other Materials and Reagents

Gene of interest
LB broth
Agar and Agarose
Kanamycin
Ampicillin
70 and 100% ethanol
Sterile PBS
Iodixanol
Pluronic-F68
Chlorine bleach
DNA gel apparatus and power supplies
Class II Biosafety Cabinet
37℃ orbital shaker
37℃ bacteria incubator
37℃, 5% CO_2 incubator
15- and 50-ml conical tubes
25- and 75-cm^2 tissue culture flasks
Cell scrapers
Dry-ice/methanol bath
Liquid nitrogen tank
Low-speed swinging-bucket centrifuge
Microcentrifuge
Centrifuge tube (thick-wall polycarbonate tube with cap)

Packaging and Concentration of rAAV

Construction and Amplification of Plasmids

Constructed rAAV vector plasmids and the backbone vector should be amplified in DH5α and purified by Maxi-Prep kit to remove endotoxins (note: QIAGEN Plasmid Maxi Kit is recommended). A concentration over 1 μg/μl and the A260/A280 ratio range between 1.7-1.8 is required for virus packaging. Please be cautious that plasmid quality would affect the transfection efficiency and titer of product viruses.

Note:

In order to construct vectors quickly and efficiently, it is strongly recommended to use Genemedi - ClonEasy™ One Step Cloning Kit（Cat. GM-GC-01/02/03）.

Click here to find more about Genemedi - ClonEasy™ One Step Cloning Kit

Transfection of Virus Plasmids into AAV-293 Packaging Cells

a. AAV-293 cells should be prepared at least a day ahead to reach a confluence of 50%-70% monolayer morphology before transfection.

b. On the day of transfection, DMEM needs to be pre-warmed at 37℃ water bath and LipoGene™ transfection reagent should be equilibrated to room temperature and tapped to mix before use.

c. To prepare viral plasmids for each reaction using a 10-cm dish according to the following table 1:

Table 1. Plasmid and transfection reagent required for transfection.

Component	Amount
pAAV-RC	10 μg
pHelper	20 μg
pAAV-GOI	10 μg
LipoGene™	100 μl

d. Mix plasmids with transfection reagent in DMEM and add drop-wise to pre-seeded AAV-293 cells. Incubate in 37℃, 5% CO2 and refresh with complete culture medium in 6 hours.

Note:

1. *A detailed protocol of the transfection reagent can be referred to Genemedi LipoGene™ Transfection Reagent User Manual.*
   Click here to find more about Genemedi - LipoGene™ Transfection Reagent

2. *Cells should be in a healthy growth state for use before transfection.*

Collection of AAV

a. Around 72 hours after transfection, harvest the cells from the 10 cm plate with a cell scraper.

b. Spin the cells at 1,500g for 5 minutes to collect the cell pellet. Resuspend the cell pellet in 0.5ml lysis buffer (10 mM Tris-HCl (pH8.5), 150 mM NaCl). Freeze/thaw the cell pellet 3 times through a dry ice/ethanol bath and a 37°C water bath to obtain the crude lysate.

c. Spin down the crude lysate at 3,000g for 10 minutes. Collect the supernatant fraction, which contains harvested rAAV. Keep the virus at -80℃.

Purification of AAV

a. Reagent Preparation:

1× PBS-MK buffer: Dissolve 52.6 mg of MgCl2, and 29.82 mg of KCl in 1× PBS in a final volume of 200 mL.

1 M NaCl/PBS-MK buffer: Dissolve 5.84 g of NaCl in 1× PBS-MK buffer in a final volume of 100 mL.

Note: The buffer should be sterilized by passing through a 0.22- μ m filter and store at 4 ℃.

15% iodixanol: mix 4.5 mL of 60% iodixanol and 13.5 mL of 1 M NaCl/PBS-MK buffer;

25% iodixanol: mix 5 mL of 60% iodixanol and 7 mL of 1x PBS-MK buffer and 30 μL of phenol red;

40% iodixanol: mix 6.7 mL of 60% iodixanol and 3.3 mL of 1x PBS-MK buffer;

60% iodixanol: mix 10 mL of 60% iodixanol and 45 μL of phenol red.

b. Overlay each solution into a QuickSeal tube in the order (5 mL of 60% iodixanol; 5 mL of 40% iodixanol; 6 mL of 25% iodixanol; 8 mL of 15% iodixanol) using a 10 mL syringe and an 18 g needle. Carefully add up to 5 mL of clarified virus supernatant on top of the gradient. Use 1× PBS (or cell lysis buffer) to top off the tube. Seal the QuickSeal tubes. Centrifuge at 350,000 g for 90 min in a T70i rotor at 10 ℃.

c. Carefully take the QuickSeal tubes out of the rotor and place them in a stable rack.

Note:
Take care to avoid bubbles during purification.

If more centrifugation time is needed, you can alternatively centrifuge for 2 h at 200,000 g at 18 ℃.
Make sure not to disturb the gradient before virus collection.

d. Pierce the QuickSeal tube slightly below the 60-40% interface with an 18 g needle attached to a 10 ml syringe. The bevel of the needle should be up, facing the 40% iodixanol step. Collect up to 5 ml per tube, and avoid collecting the proteinaceous material at the 40-25% interface.

Note:

Unless otherwise specified, all AAV viruses are purified with iodixanol gradient ultracentrifugation at 350,000g for 90 min in a T70i rotor at 10 ℃ to separate contaminants from the impure AAV preparations. The 15% iodixanol step helps destabilize ionic interactions between macromolecules with the addition of 1M NaCl. The 40% and 25% steps are used to remove contaminants with lower densities, including empty capsids, while the 60% step acts as a cushion for genome-containing virions. After several steps of gradient ultracentrifugation, the virus particles will be enriched.

Concentration of AAV (Ultrafiltration method)

It may be necessary to remove iodixanol (molecular weight: 1,550 Dalton) from the gradient fractions with filtration or dialysis method either to concentrate the viral particles or avoid interfering with some add-on processes.

a. Pluronic-F68 Preparation:

0.1% Pluronic-F68: 49.5 ml PBS + 500 μl 10% Pluronic F68

0.01% Pluronic-F68: 45 ml PBS + 5 ml 0.1% Pluronic-F68

0.001% Pluronic-F68 with 200mM NaCl: 45 ml PBS + 5 ml 0.01% Pluronic-F68 + 200 mM NaCl
0.001% Pluronic-F68 (formulation buffer): 45 ml PBS + 5 ml 0.01% Pluronic-F68.

b. Balance the filter membrane with 15 ml of 0.1% Pluronic F68 and incubate for 10 min at room temperature.
c. Remove the 0.1% Pluronic F68 and add 15 ml of 0.01% Pluronic F68.
d. Centrifuge at 3000 rpm for 5 min at 4 ℃.
e. Discard the filtrates and add 15 ml of 0.001% Pluronic F68 with 200mM NaCl PBS.
f. Centrifuge at 3000 rpm for 5 min at 4 ℃.
g. Discard the filtrates and add virus purifications.
h. Centrifuge at 3500 rpm for 8 min at 4 ℃, discard the flow-through.
i. Replenish more virus purifications and spin 3500 rpm for 4 min at 4°C, discard the filtrates. Repeat this step as needed.
j. Utilize a P1000 to pipette up and down to wash off the filter wall to recover as much virus as possible.
k. Keep the concentrates at 4 ℃ for short term (less than one week), or in small aliquots and keep at -80 ℃ for long term usage.

Note:

1. *It is difficult to remove Iodixanol. Add more formulation buffer and virus purifications and pipet back and forth a few times in order to mix the iodixanol settled at the bottom or wall of the column into the solution after each centrifugation.*

2. *Concentrating on a minimum of 500 µl is recommended. If the concentrate volume is less than 500 µl, bring up the volume with formulation buffer.*

Quality Assurance of rAAV

AAV capsid contains VR1 82kDa, VR2 72kDa and VR3 62kDa, which can be detected using the method of polyacrylamide gel electrophoresis (PAGE) followed by silver staining or Coomassie blue staining. Pure AAV should display only three major protein bands, such as the following virus purified in Genemedi shown in Figure 2. We guarantee the purity of the AAV virus based on the specification that we give for different services.

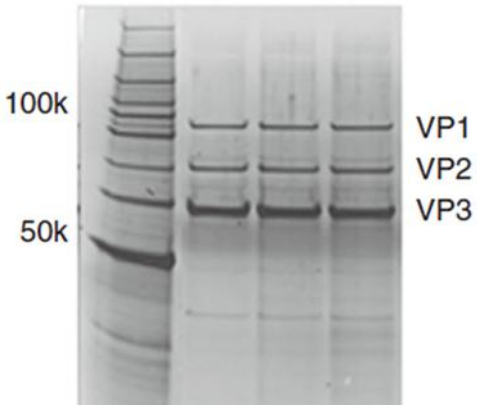

Figure 2. Purity of purified AAV virus.

Titer Detection of rAAV

AAV titers are determined by real-time quantitative PCR using primers targeted the ITR. The amplicons are detected using SYBR green technology. Titer values are then determined by comparison to a standard curve of a plasmid sample of known concentration. An example of the Ct value of the standard sample and sample to be tested is shown in table 2, while the standard curve is displayed in figure 3.

Table 2. Ct value of standard and sample in a typical absolute quantification process.

Standard	Copy Number (vg/ml)	Ct value 1	Ct value 2	Ct value 3
Standard_1	10^{10}	4.51	4.32	4.42
Standard_2	10^{9}	7.21	7.61	7.25
Standard_3	10^{8}	10.97	10.55	10.67
Standard_4	10^{7}	14.19	14.38	14.35
Standard_5	10^{6}	17.75	17.58	17.68
AAV Sample		12.78	12.65	12.81

Set the average Ct value of each group as Y-axis, the logarithm of corresponding group as X-axis. Substitute the average Ct value of samples to be tested into formula, obtaining X = 7.45. Substitute the AAV virus titer calculation formula: $10^{x} \times 40000$ vg/ml= $10^{7.45} \times 40000 = 1.1 \times 10^{12}$ vg/ml.

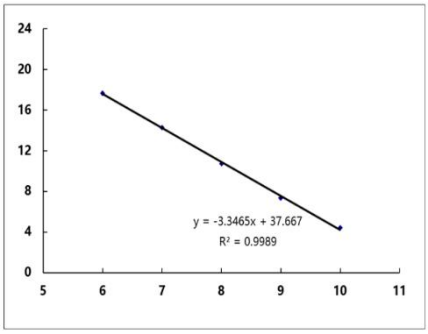

Figure 3. Standard curve in absolute quantification.

Cell Infection Test of AAV

After AAV titer detection, the infection activity needs to be evaluated before animal experiments. Test the expression of target gene by infecting cells, such as 293T, CHO. MOI will be controlled ranging from 10^{4} to 10^{5} (MOI is multiplicity of infection, namely the number of virus particles needed for infecting a cell). The detailed AAV infection protocol can be found in the AAV User Manual.

Safe Use of AAV

1. AAV related experiments should be conducted in biosafety level 2 facilities (BL-2 level).

2. Please equip with lab coat, mask, gloves completely, and try your best to avoid exposing hand and arm.

3. Be careful of splashing virus suspension. If biosafety cabinet is contaminated with virus during operation, scrub the table-board with solution comprising 70% alcohol and 1% SDS immediately. All tips, tubes, culture plates, medium contacting virus must be soaked in chlorine-containing disinfectant before disposal.

4. If centrifuging is required, a centrifuge tube should be tightly sealed. Seal the tube with parafilm before centrifuging if condition allowed.

5. AAV related animal experiments should also be conducted in BL-2 level.

6. AAV associated waste materials need to be specially collected and autoclaved before disposal.

7. Wash hands with sanitizer after experiment.

Storage and Dilution of AAV

Storage of AAV

Virus can be stored at 4°C for a short time (less than a week) before using after reception. Since AAV viruses are sensitive to freeze-thawing and the titer drops with repeated freeze-thawing, aliquot viral stock should be stored at -80°C freezer immediately upon arrival for long-term usage. While virus titer redetection is suggested before using if the AAV viruses have been stored for more than 12 months.

Dilution of AAV

Dissolve virus in ice water if virus dilution is required. After dissolving, mix the virus with medium, sterile PBS or normal saline solution, keeping at 4°C (using within a week).

Precautions

- Avoid AAV exposure to environmental extremes (pH, chelating agents like EDTA, temperature, organic solvents, protein denaturants, strong detergents, etc.)

- Avoid introducing air into the AAV samples during vortex, blowing bubbles or similar operations, which may result in protein denaturation.

- Avoid repeated freezing and thawing.

- Avoid exposing to "regular" plastics (especially polystyrene or hydrophobic plastics) for prolonged periods in liquid phase. Most AAV viruses are very sticky and loss can occur if exposed to regular plastics, including tubes, cell culture plates, pipette tips, if not frozen. It is best to store AAV in siliconized or low protein binding tubes. Pluronic F-68 used at 0.01%-0.1% in the formulation buffer will minimize sticking if regular plastics are used.

- Avoid diluting AAV into low salt solution. Some AAV serotypes, such as AAV2, aggregates in low salt solution, which will be non-infectious.

References

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Related products

1. AAV packaging system

AAV1 vector system	AAV6 vector system	AAV-DJ vector system
AAV2 vector system	AAV8 vector system	AAV-Dj8 vector system
AAV2 variant(Y444F) vector system	AAV8 variant (Y733F) vector system	AAV2-Retro (Retrograde) vector system
AAV2 variant (Y272F,Y444F,Y500F,Y730F) vector system	AAV8 variant(Y733F, Y447F) vector system	AAV9-PHP.B vector system
AAV2 variant(Y444F,Y730F,Y500F,Y272F,Y704F,Y252F) vector system	AAV8 variant(Y733F,Y447F,Y275F) vector system	AAV9-PHP.eB vector system
AAV2 variant(AAV2.7m8) vector system	AAV9 vector system	AAV9-PHP.S vector system
AAV5 vector system	AAV-Rh10 vector system	

More details please visit: https://www.genemedi.net/i/aav-vector-system

2. **AAV Promise-ORF™** :

Sequence-verified CDNA clones in AAV and mammalian expression vectors.

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3. AAV custom production

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