

# A Protocol for AAV vector production and purification

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## 1. Introduction

The protocol describe at below is typical of methods that are used to propagate and purify AAV vectors for experiments both *in vitro* and *in vivo*.

## 2. Principles of the Triple Plasmid Transfection System

The minimum regions in helper adenovirus that mediate the replication of the AAV vector are E1, E2A, E4, and VA<sup>1</sup>. The 293 line of human embryonic kidney cells encodes the E1 region of the Ad5 genome<sup>2</sup>. When a helper plasmid that encodes the E2A, E4, and VA regions (Ad-helper plasmid) is used to transfect 293 cells, together with plasmids that encode the genome of the AAV vector (vector plasmid), as well as the *rep* and *cap* genes (AAV-helper plasmid), the AAV vector is produced as efficiently as when infection by adenovirus is used as a source of helper virus. Elimination of the heat-inactivation step directed against contaminating adenovirus can improve the yield of the virus. Furthermore, contamination of the AAV vector stock by most adenoviral proteins can be avoided when this helper virus-free method is used.

## 3. Reagents

Helper plasmid DNA (pHLP, pAdeno)

Vector plasmid harboring the gene of interest flanked by inverted terminal repeats (ITRs )

293 cells (human embryonic kidney cells)

DMEM/F12 culture medium

Fetal bovine serum

2 x HBS buffer, containing 290 mM NaCl, 50 mM HEPES buffer and 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.1

300 mM CaCl<sub>2</sub>

Phosphate-buffered saline (PBS)

1 M HEPES buffer, pH 7.4

100 mM Tris-HCl (pH 8.0) plus 150 mM NaCl (TBS)

0.5 M EDTA (pH 8.0)

40% sucrose plus 0.01% BSA in TBS

DNase buffer, containing 50 mM Hepes (pH 7.6), 0.15 M NaCl and 10 mM MgCl<sub>2</sub>

HNE buffer, containing 50 mM Hepes (pH 7.4), 0.15 M NaCl and 25 mM EDTA

A solution of CsCl in HNE (1.25 g/ml) in HNE

A solution of CsCl in HNE (1.50 g/ml) in HNE

#### **4. Plasmids**

The AAV vector plasmid, pAAVlacZ, harbors a beta-galactosidase expression cassette flanked by inverted terminal repeats (ITRs). The AAV-helper plasmid pHLP, harboring *rep* and *cap*, has been described previously as pHLP19. The Ad-helper plasmid pAdeno is identical to pVAE2AE4-5 and encodes the entire E2A and E4 regions plus the VA RNA I and II genes<sup>1</sup>.

#### **5. Transfection and Extraction of Virus**

This protocol is for the transfection of cells in one 225-cm<sup>2</sup> flask. For cultures of other sizes, multiply volumes on a linear basis.

Plate trypsinized 293 cells at 5 x 10<sup>6</sup> cells per 225-cm<sup>2</sup> flask to generate a monolayer of 20% to 40% confluence when cells attach initially to the surface of the flask. Use 40 ml of medium per flask and try to avoid plating clumps of cells and to make sure that cells are distributed evenly on the substratum. An even density of cells over the entire substratum is essential for high yield and it can be achieved by moving the flask of newly plated cells gently in a crosswise pattern before cells become attached. Place the plate in an incubator in 5% CO<sub>2</sub> in air and allow cells to grow to 80% confluence (24 to 48 h.).

One hour before transfection, replace half the medium in the flask with fresh medium. Add 23 µg of vector and of each helper plasmid to 4 ml of 300 mM CaCl<sub>2</sub>. Gently add this solution to 4 ml of 2x HBS and mix immediately by gentle inversion three times. Immediately pipette this mixture into the 225-cm<sup>2</sup> flask of 293 cells in 40 ml of DMEM/F12 medium

plus 10% FCS and swirl to produce a homogeneous solution. Immediately return the plate to the incubator and incubate at 37° for 4 to 6 hr. Do not disturb the plate during this period. At the end of the incubation, replace the medium with pre-warmed DMEM/F12 culture that medium contains 2% FCS. Three days after transfection, add 1 ml of 0.5 M EDTA to the flask and incubate for 3 min at room temperature. Collect the suspension of cells and centrifuge it at 300xg for 10 min. Remove the supernatant and resuspend the cells in the pellet in 2 ml of TBS.

Freeze and thaw the cells that have been suspended in TBS three times by placing them alternately in a dry ice/ethanol bath until the suspension is completely frozen and in a water bath of 37°C until it is completely thawed. Return the sample to the ice bath immediately when it is completely thawed. Remove tissue debris by centrifugation at 10,000xg for 10 min and collect the supernatant.

#### ***6. Purification of the AAV vector***

Prepare supernatants, as described above from 24 flasks. Place 11 ml of a solution of 40% sucrose plus 0.01% BSA in TBS in a sterile ultracentrifuge tube (Ultrabottle #3430-3870; Nalge Nunc, Rochester, NY). Carefully overlay the 48 ml of pooled supernatants on this solution. Pellet the crude viral particles by centrifugation at 100,000xg for 16 hr at 4°C. Resuspend the pellet by vigorous agitation in 5 ml of DNase buffer. Add 1,000 units of DNase I and incubate for 1 hr at 37°C. Add 250 µL of 0.5 M EDTA and then remove debris by centrifugation at 10,000xg for 2 min. Then filter the supernatant through a low-protein-binding 5-µm syringe filter (Sterile Acrodisc Syringe Filter; Pall Gelman Laboratory, Ann Arbor, MI). Load the filtered material onto a two-tier CsCl gradient (1.25 g/ml and 1.50 g/ml) prepared in HNE buffer. Spin the gradient at 35,000 rpm for 2 h at 16°C in an SW40 rotor (Beckman Instruments, Palo Alto, CA). Collect the band of viral particles and load it on a second two-tier CsCl gradient (1.25 g/ml and 1.50 g/ml) prepared in HNE buffer. Spin the gradient at 65,000 rpm for 2 h at 16°C in a VTi65.2 rotor (Beckman Instruments). Collect fractions of 0.5 ml each and select the virus-rich fraction by semi-quantitative PCR analysis, Western blotting with antibodies against Cap, or quantitative DNA dot-blot hybridization. Use a dialysis cassette (Slide-A-Lyzer; Pierce, Rockford, IL)

to desalt the virus-rich fraction by three cycles of dilution with 300 ml of HNE buffer. Concentrate the material to 50  $\mu$ L with Ultrafree-4 (Millipore, Bedford, MA) according to the manufacturer's instruction. The final titer usually ranges between  $1 \times 10^{13}$  and  $5 \times 10^{13}$  particles from  $5 \times 10^8$  293 cells, as determined by quantitative DNA dot-blot hybridization or Southern blotting. In the case of an AAV vector that expresses lacZ, production of the vector can be assessed functionally by titration with 293 cells in the presence of a wild-type adenovirus type 2 at an MOI of 50. The transduced cells are incubated for 24 h at 37°C before fixation and X-gal staining. Stained cells are counted under a light microscope. The ratio of genomes to functional viral particles is determined from the total number of genomes divided by the total number of functional units (as determined by X-gal staining) for verification of infectivity.

## ***7. References***

1. T. Matsushita, S. Elliger, C. Elliger, G. Podsakoff, L. Villarreal, G. J. Kurtzman, Y. Iwaki, and P. Colosi, *Gene Ther.* 5, 938 (1998).
2. F. L. Graham, J. Smiley, W. C. Russell, and R. Nairn, *J. Gen. Virol.* 36, 59 (1977).