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[24] Streamlined Large-Scale Production of Recombinant Adeno-Associated Virus (rAAV) Vectors

By MARK POTTER, KYE CHESNUT, NICHOLAS MUZYCZKA, TERRY FLOTTE, and SERGEI ZOLOTUKHIN

Recent progress in development of rAAV production protocols in several laboratories has allowed a widespread testing of purified vectors of high titers in a variety of animal models. Many of these trials have resulted in successful therapies derived from long-term expression of transgenes delivered by rAAV. The positive outcome of most rAAV-mediated therapies appears to depend on the quality of the vector reagent, i.e., its purity, infectivity, and titer. The last parameter is a function of manufacturing capacity, specifically, the number of cells included into one production run. The requirements of preclinical *in vivo* studies in most cases reach beyond the regular production formats that rely on cells grown in flasks or dishes. In this report we describe a preindustrial scale-up protocol carried out in a cell factory format (about 10^9 cells per factory), allowing a modest facility to increase vector production at least 10- to 100-fold.

Earlier, we developed a new rAAV purification protocol resulting in higher yield and improved infectivity of viral particles.[1,2] This method utilizes a bulk purification of a crude lysate through an iodixanol step gradient followed by conventional heparin affinity or HPLC ion-exchange chromatography. Although quick and reproducible, this protocol is not readily amenable to large-scale production of a clinical-grade vector because of the limiting capacity of the iodixanol centrifugation step. To incorporate the requirements of an increased starting cell volume we describe further improvements in the production protocol introducing new chromatography purification steps that eliminate the need for any centrifugation methods. We also characterize the purified rAAV in terms of purity, infectivity,

[1] S. Zolotukhin, B. J. Byrne, E. Mason, I. Zolotukhin, M. Potter, K. Chesnut, C. Summerford, R. J. Samulski, and N. Muzyczka, *Gene Ther.* **6,** 973 (1999).

[2] W. W. Hauswirth, A. S. Lewin, S. Zolotukhin, and N. Muzyczka, *Methods Enzymol.* **316,** 743 (2000).

0076-6879/02 $35.00

and packaged particle composition. As a case study, we describe the production of a National Reference Standard (NRS) rAAV vector, sponsored by the National Gene Vector Laboratory.

The National Reference Standard effort stems from a multicenter effort to share preclinical data with regard to the long-term potential risks for insertional mutagenesis and/or germline transmission of rAAV. The types of preclinical studies which may be required to adequately address these issues may be very large and may require resources beyond those traditionally available to academic centers involved in the treatment of rare genetic diseases. In an effort to address this issue, a joint FDA/NIH Workshop was held on May 2nd and 3rd, 1999. At that workshop, members of the rAAV gene therapy community from academia, industry, and the federal government discussed the potential for developing a shared platform of preclinical data to address these vector-specific safety issues. It was generally recognized that in order to pool preclinical data in a meaningful way, it would be necessary for a wide range of groups to be able to discuss vector dosage, strength, and potency in equivalent titer units. It was further recommended that to facilitate this goal, a reference standard stock of rAAV with a precisely defined titer should be generated and made generally available to all members of the research community. All users of this reference stock would essentially be able to calibrate their titering assays against a common standard, thus allowing each group to state their titers in units that were precisely understood by all. The goal of the current application is to generate the rAAV reference standard stock, aliquot it into a large number of individual user vials, validate its utility as a reference standard among a handful of rAAV laboratories, and then transfer it to an appropriate distribution service. Within this application, we describe the production process to be used in the generation of this rAAV stock, the basic quality control assays that will be in place to confirm its purity and identity, and the standard protocols (SPs) to be used for physical and biological titering by the producing center, the validating centers, and the eventual individual users.

The rAAV construct chosen for the National Vector Standard was pTR-UF5.[3] It contains a humanized *gfp* gene[4] under the control of a CMV promoter and a *neo* gene under the control of a TK promoter.

Propagation of Cells

Protocol

Low-passage ($P < 40$) HEK 293 cells are used to propagate the rAAV vector. A working cell bank of 293 cells (P30), derived from a batch certified by

[3] R. L. Klein, E. M. Meyer, A. L. Peel, S. Zolotukhin, C. Meyers, N. Muzyczka, and M. A. King, *Exp. Neurol.* **150,** 183 (1998).

[4] S. Zolotukhin, M. Potter, W. W. Hauswirth, J. Guy, and N. Muzyczka, *J. Virol.* **70,** 4646 (1996).

Microbiology Associates for GMP-grade production, is being stored in vials (10^7 cells/aliquot) in liquid N_2.

Seeding Cell Factory with 293 Cells from T255 Flasks

1. Cells from one vial are thawed and seeded into a T225 flask. After cells reach confluence, they are split and seeded into three T225 flasks using regular cell culture techniques, followed by one more split into eight T225 flasks. The initial propagation cycle takes about 10 days.

2. Cells from eight confluent T225 flasks are then seeded into one cell factory (6320 cm^2 of a culture area, NUNC, Roskilde, Denmark). To split cells, 2 liters of medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1× antibiotic–antimycotic] is warmed to 37°. Five ml of 10 × trypsin-EDTA stock solution is diluted in 45 ml 1× phosphate-buffered saline (PBS) in a 50 ml conical tube. Medium from eight flasks is discarded and cells in each flask are washed with 10 ml PBS. Four ml of diluted trypsin–EDTA is added and flasks are rocked until cells start to peel, at which point flasks are knocked against a hard surface to lift cells off the surface. The trypsin–EDTA solution is then neutralized by addition of 16 ml per flask DMEM–5% FBS. The cells are resuspended by triturating with a pipette and collected in a 250 ml disposable conical tube. About 1090 ml of medium is mixed with the pooled cells in an aspirator bottle and the cell factory is then loaded following the manufacturer's instructions. The cell factory is incubated at 37° for about 20 hr until transfection.

Seeding Four Cell Factories from One Confluent Factory. A fresh stock of trypsin–EDTA solution is made by diluting 25 ml of 10 × stock with PBS in a 250 ml conical tube while medium is being warmed at 37°. Medium from the confluent cell factory is poured off and cells are rinsed with ~250 ml PBS. Trypsin/EDTA solution is added to the factory and cells are dislodged after rocking the factory at room temperature for 2 min. About 850 ml of media is added to the aspirator bottle, and the cells are rinsed off with the media and transferred into an empty media bottle (total volume is about 1.1 liter). Cell clumps are dispersed by vigorously shaking the capped bottle and cells are aliquoted into the four factories. The cell factory that was used for seeding is reused and receives 200 ml of cell suspension (along with 1 liter of fresh medium). This factory is then used to seed new factories on subsequent days. One cell factory can be recycled for seeding up to five times. The remaining three factories receive 300 ml of cell suspension and 1 liter of fresh medium each and are transfected the next day.

Transfection

The basic strategy for producing rAAV via cotransfection of two plasmid DNAs has been extensively described.[1,2] The scale-up protocol adapted for one cell factory is described below.

Protocol

Cell confluency is determined by focusing the microscope on the plane of the bottom layer of the cell factory. Transfection is carried out when cells are about 75% confluent.

2× Hepes-buffered Saline (HBS) buffer is thawed and kept at 37° until ready to use. One liter of media and 50 ml of FBS are prewarmed and mixed in an aspirator bottle. The helper plasmid pDG and the rAAV plasmid (1867.5 μg and 622.5 μg, respectively) are mixed in a 250 ml conical tube and the mixture is diluted with H_2O up to 46.7 ml final volume. $CaCl_2$ is added to the same tube (5.2 ml of 2.5 *M* stock solution) and mixed with the DNA. Old media from the cell factory is discarded and formation of the $CaPO_4$ precipitate is initiated by adding 52 ml of prewarmed 2× HBS buffer. The mixture is swirled for 1 min and transferred into an aspirator bottle containing medium. Medium is then loaded into the cell factory, and the cells are incubated at 37° for 48–60 hr without additional media change.

Harvesting Transfected Cells

Protocol

In a dedicated rAAV hood the cell factory is rocked to dislodge nonadherent cells and medium is discarded. Cells are washed with 500 ml of PBS using an aspirator bottle. EDTA is added to 500 ml PBS to a 5 m*M* final concentration, and the solution is transferred into the factory and spread evenly over all layers. The cells are lifted off the plastic by vigorously shaking the factory, and the cells are poured into two 250 ml conical tubes. The remaining cells are rinsed out with an additional 500 ml PBS and transferred into two more conical tubes. Cells are harvested by low-speed centrifugation at 300*g* for 10 min at 4°. The supernatant is discarded and cell pellet is stored at −20° until processing.

Purification of rAAV Vector

Generation of Crude Lysate

As an alternative to repeated freeze–thaw cycles, which produce a crude cell lysate requiring further purification either by filtration or centrifugation prior to any subsequent processing, we sought a method that would generate a lysate that would be immediately suitable for chromatography. One such method utilizes a microfluidizer processor (model M-110, Microfluidics International Corporation). The processor works by introducing low-pressure air (or gas) through a series of sequentially smaller cylinder piston pumps into a product stream generating high-pressure force (1 psi of supply air generates 230 psi liquid pressure). The high-pressure stream enters the interaction chamber into precisely defined

microchannels where three primary forces, shear, impact, and cavitation, act on the sample as it passes through the chamber. We have found one pass of a stock material through the microfluidizer to be sufficient for our needs. However, recycling the product through the interaction chamber is possible. To process the cell suspension through the chamber, we routinely pool cell pellets from factories transfected with the same vector. In the case of the National Reference Standard, cell pellets from 10 individual cell factories were pooled.

Protocol

Cells are allowed to thaw at room temperature for approximately 15 min and placed on ice. To each cell pellet 15 ml deoxycholate lysis buffer (0.5% sodium deoxycholate, 20 m*M* Tris-HCl pH 8.0, 150 m*M* NaCl) is added and mixed well by triturating, and mixtures are pooled into one 250 ml conical tube. To reduce the viscosity the lysate is treated with benzonase (100 U/ml) for 30 min at 37° on the addition of $MgCl_2$ (1 m*M* final concentration). The sample is then split into two equal parts, 125 ml each, and the volumes in each tube are adjusted to 150 ml by the addition of an equal volume of fresh deoxycholate lysis buffer. Both aliquots of diluted sample are then homogenized by one pass through a microfluidizer and collected in a single vessel.

Streamline Heparin Affinity Chromatography. All chromatography steps are performed using AKTA FPLC Pharmacia's system. A Pharmacia FPLC XK/26 column containing the Streamline Heparin chromatography matrix (fixed bed format, column bed volume of 55 ml) is equilibrated with deoxycholate lysis buffer (see above). The entire crude sample (~300 ml) is applied to column at a flow rate of 3 ml/min. After loading, the column is washed with 330 ml (about 6 column volumes, CV) of the same buffer at a flow rate of 5 ml/min, and then with 18 CV (about 1 liter) of PBS at a flow rate of 10 ml/min. The sample is then eluted from the column by a single step gradient using PBS containing 0.5 *M* NaCl at a flow rate of 3 ml/min. Ten ml fractions are collected (Fig. 1A) and positive fractions are determined by fluorescent cell assay (FCA).[1] In the example shown in Fig. 1A fractions 6 through 8 are pooled.

Phenyl-Sepharose Hydrophobic Interaction Chromatography. The pooled heparin column fractions (30 ml) are adjusted to 1 *M* NaCl by the addition of 5 *M* stock solution. The sample is then loaded onto a Pharmacia FPLC XK/16 column (column bed of 18 ml) containing hydrophobic interaction chromatography (HIC) medium phenyl Sepharose at a flow rate of 5 ml/min. Prior to loading sample the column is preequilibrated with PBS–1 *M* NaCl. The column is monitored by UV absorption and the virus elutes in the flow-through (~100 ml) (Fig. 1B).

Heparin Affinity Chromatography. The virus is concentrated by chromatography on heparin. The phenyl Sepharose fraction is diluted to ~150 m*M* NaCl by the addition of dH_2O (usually a 6-fold dilution). The diluted sample (~700 ml)

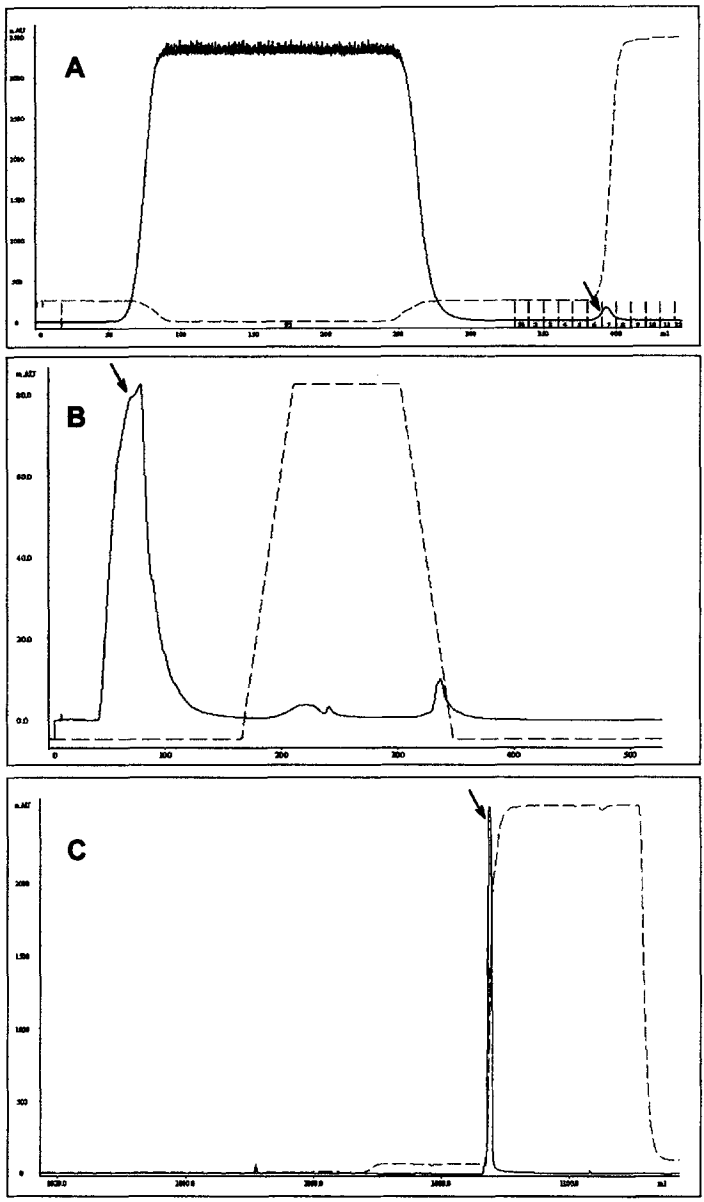

FIG. 1. Chromatography profiles of NRS rAAV purification steps. (A) Streamline heparin affinity chromatography. (B) Phenyl Sepharose hydrophobic interaction chromatography (C). Heparin affinity chromatography. Broken lines show conductivity profiles of the respective processes. Solid lines indicate OD_{280}. Arrows mark the pooled peak fractions.

TABLE I
SUMMARY OF rAAV-UF5 PURIFICATION

	Crude lysate		Streamline heparin affinity chromatography		HIC phenyl sepharose chromatography		Heparin affinity chromatography	
Run	Total inf. particles	% yield	Total inf. particles	% yield	Total inf. particles	% yield	Total inf. particles	% yield
1	4.8×10^{12}	100	2.7×10^{12}	56	2.3×10^{12}	48	1.3×10^{12}	27
2	9.6×10^{12}	100	1.8×10^{13}	186	1×10^{13}	104	4.2×10^{12}	43
3	3.6×10^{12}	100	1.2×10^{12}	33	1.3×10^{12}	36	1.2×10^{12}	33

is then loaded onto a heparin affinity column, BioPerceptive's Poros HE/20 column (bed volume 1.7 ml) at a flow rate of 5 ml/min. After loading, the column is washed with 100 ml PBS at the same flow rate and virus is eluted with PBS containing 0.5 *M* NaCl using a single step gradient. One ml fractions are collected and aliquots analyzed by fluorescence cell assay (FCA). The major peak of UV absorbance contains rAAV-GFP vector (Fig. 1C).

To estimate the yield, the vector obtained after each purification stage is titered by FCA. The yields of three representative vector runs, each consisting of 10 pooled cell factories, are shown in Table I.

Characterization of Purified rAAV

An important index of virus quality is the ratio of physical particles to infectious particles in a given preparation. To characterize the quality of the virus, we used several independent assays to titer both physical and infectious rAAV particles. A conventional dot-blot assay (not shown) and real-time polymerase chain reaction (PCR) (Fig. 2) were used for physical particle titers. Infectious titers were determined by infectious center assay (ICA) and a fluorescence cell assay (FCA), which scored for expression of GFP (not shown). In order to avoid adventitious contamination of rAAV stocks with wtAAV, the use of wtAAV was eliminated in the ICA (as well as all other protocols in the laboratory). This was made possible by the use of the C12 cell line,[5] which contains integrated wtAAV *rep* and *cap* genes, for both the infectious center assay and the fluorescent cell assay. Adenovirus serotype 5 (Ad5), which was used to coinfect C12 along with rAAV, was titered using the same C12 cell line in a serial dilution cytopathic effect (CPE) assay. The amount of Ad producing well-developed CPE in 48 hr on C12 cells was used to provide helper function for both the ICA and FCA assays. Both physical particle

[5] K. R. Clark, F. Voulgaropoulou, D. M. Fraley, and P. R. Johnson, *Hum. Gene Ther.* **6,** 1329 (1995).

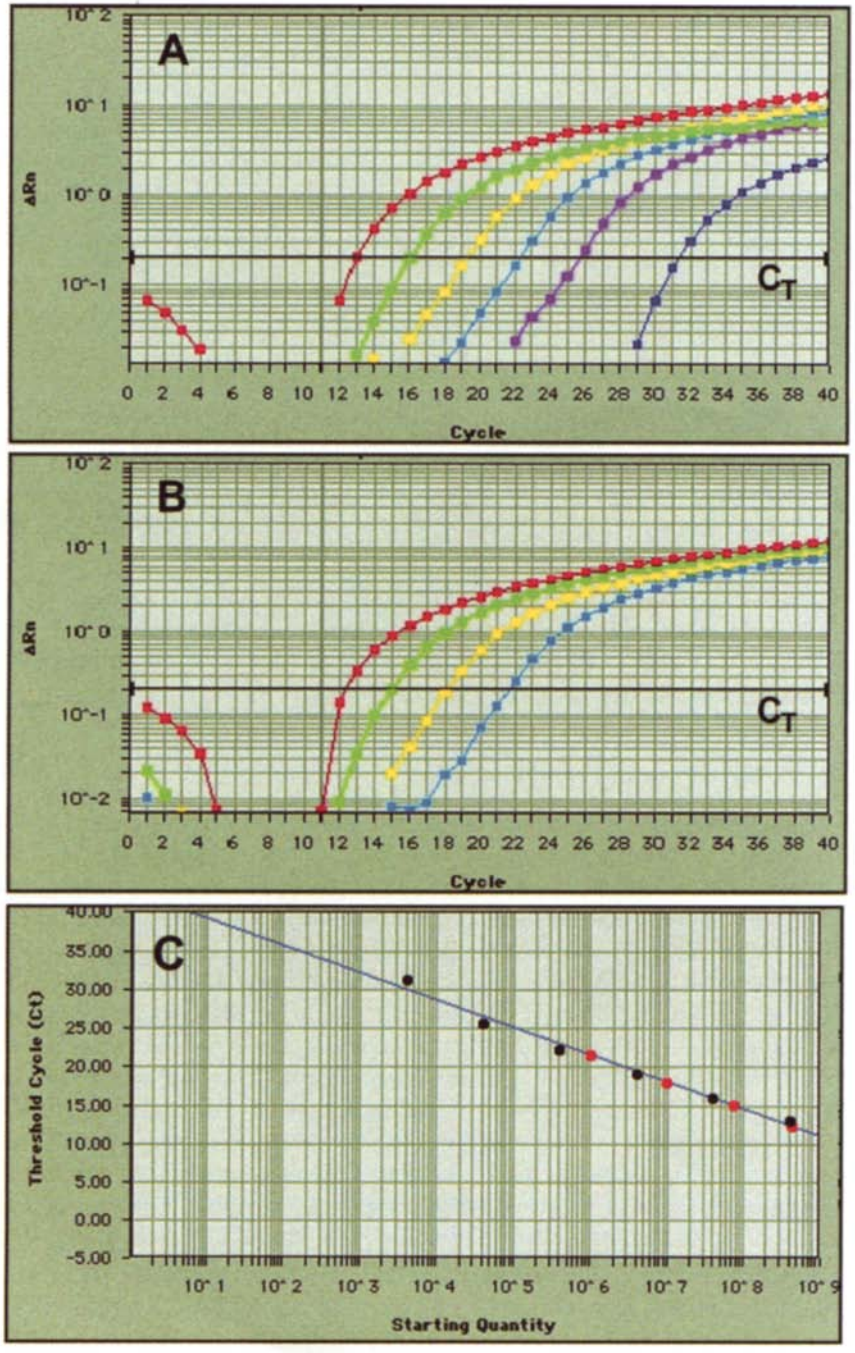
A
ΔRn
Cycle
C_T
B
ΔRn
Cycle
C_T
C
Threshold Cycle (Ct)
Starting Quantity

TABLE II
PHYSICAL AND INFECTIOUS TITERS OF NATIONAL REFERENCE STANDARD rAAV AS DETERMINED BY FOUR ASSAYS

	Dot blot	Real-time PCR	ICA	FCA
NRS rAAV titer	1.12×10^{13} part/ml	1.46×10^{13} part/ml	2.0×10^{12} infect.part/ml	2.16×10^{12} infect.part/ml

titers and infectious titers, each obtained by two independent methods, were generally in agreement, differing in most cases by a factor of 2 or less (Table II). The particle to infectivity ratio (p/i) was approximately 6.

Dot-Blot Assay (DBA)

Protocol

1. Eppendorf tubes are marked 1 through 12 (tubes for 2× dilution series) and 1* through 12* (tubes to denature the diluted DNAs). Two additional tubes to denature viral DNA samples are marked 10^0 and 10^{-1}.

2. To make a standard DNA dilution curve, 50 μl of H_2O is aliquotted into each of the tubes marked 1 through 12. The first dilution is made by adding 50 μl of standard plasmid DNA (5 ng/μl) containing the gene of interest to tube 1. Normally, the same transfer vector plasmid that was originally packaged is being used. Twofold series dilutions are made by vortexing the tube and transferring 50 μl from tube 1 into tube 2 and so on until all dilutions are done. *Caution:* Make sure to change tips after each dilution.

3. To denature DNA, 200 μl of alkaline buffer (0.4 *M* NaOH–10 m*M* EDTA) is aliquotted into each tube marked 1* through 12*, including the tubes 10^0 and 10^{-1}. Diluted DNA samples, 10 μl each, are transferred from tubes 1 through 12 into tubes 1* through 12*, respectively, and mixed by vortexing. *Note:* The transfer is usually done in reverse order (starting from tube 12) using one tip.

4. To prepare the viral DNA sample, the purified viral stock is first treated with DNase I to digest any contaminating unpackaged DNA. Four μl of a purified

FIG. 2. Real-time PCR assay for NRS rAAV. (A) Amplification profiles of standard plasmid pTR-UF5 dilution series. Red graph, 1 ng of plasmid DNA per reaction; green graph, 100 pg; yellow, 10 pg; light blue, 1 pg; purple, 100 fg; dark blue, 10 fg. (B) Amplification profiles of NRS viral DNA dilution series. Red graph, 5 μl out of 10^2 dilution (including 10-fold original dilution after DNAse I and Proteinase K treatment) per reaction; green graph, 5 μl out of 10^3 dilution; yellow, 5 μl out of 10^4 dilution; light blue, 5 μl out of 10^5 dilution. (C) Calibration graph of threshold cycle values plotted against starting genome equivalents of the standard plasmid. Black dots represent plasmid DNA values generated at different dilutions (A); red dots represent viral DNA dilutions (B).

virus stock is incubated with 10 U of DNase I (Boehringer) in a 200 μl reaction mixture, containing 50 m*M* Tris-HCl, pH 7.5, 10 m*M* $MgCl_2$ for 1 hr at 37°. At the end of the reaction, 20 μl of 10 × proteinase K buffer (10 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, 1% SDS final concentration) is added, followed by the addition of 2 μl of proteinase K (18.6 mg/ml, Boehringer). The mixture is incubated at 37° for 1 hr. Viral DNA is purified by phenol/chloroform extraction (twice), followed by chloroform extraction and ethanol precipitation using 10 μg of glycogen as a carrier. The DNA pellet is dissolved in 40 μl of water, resulting in a 10-fold dilution of the original sample. To denature viral DNA, 1 μl and 10 μl are transferred into tubes containing prealiquotted alkaline buffer, marked 10^{-1} and 10^0, respectively.

5. To apply DNA to the filter, an 11.5 × 4 cm rectangular piece of nylon filter (0.45 μm, MagnaGraph Nylon Transfer Membrane, Osmonics Inc.) is wetted in H_2O. The filter is then placed on top of two layers of prewetted Whatman 3 MM filter papers (8 × 11 cm) and the whole stack is assembled in a dot-blot manifold apparatus (Schleicher and Schull Inc.) following the manufacturer's instructions. To equilibrate the membrane alkaline buffer (0.5 *M* NaOH–1.5 *M* NaCl) is pipetted into each well (400 μl per well) and house vacuum is applied for slow (about 100 μl/min) suction until wells are empty. Denatured DNA is then transferred into the wells in a reverse order, i.e., starting with tube 12*. Viral DNA samples are then applied, skipping one full row in the minifold. *Caution:* Eliminate inadvertent bubbles by slowly pipetting the solution in a well up and down. After wells are dry, the filter is washed one time with alkaline buffer (400 μl/well). The apparatus is disassembled and the nylon membrane is placed on dry Whatman 3 MM paper for 5 min. The filter is then rinsed in 4× SSC for 10-15 sec; air dried on Whatman 3 MM filter; and DNA is fixed to the nylon membrane in a microwave oven (high setting for 4 min). *Caution:* Be sure to have about 500 ml of water in a separate beaker in the microwave or the membrane may catch on fire.

6. The membrane is prehybridized in 7% (w/v) SDS–0.25 *M* $NaHPO_4$ (pH 7.2)–1 m*M* EDTA (pH 8.0) at 65° for at least 1 hr before adding the denatured probe. The probe is a ^{32}P-labeled DNA (any fragment of the DNA in the rAAV cassette being titered). About 20 ng of a random-primed labeled probe (specific activity of 10^9 cpm/μg) is denatured and added to 5 ml of hybridization buffer (see above). The hybridization is carried in a glass cylinder in a rotational oven at 65° for 12 hr. The membrane is then washed three times (20 min each) in 2× SSC at room temperature, air dried, and exposed in a Phospho-Imager (Storm 860, Molecular Dynamics) cassette.

7. The image is processed using ImageQuant software and subsequently plotted to derive the standard curve using software GraphPhad Prism (GraphPad Software, Inc). The viral physical particles titer is calculated taking into account the dilution factor using the average values of two dilutions (see Table II for the titer of NRS rAAV).

Real-Time PCR Assay (RTPA)

Earlier we described two independent assays for determining rAAV physical titer: dot-blot assay and QC-PCR.[1] Below we describe another PCR-based assay using the PE-Applied Biosystems Prism 7700 sequence detector system similar to the method described by Clark *et al.*[6] To perform the assay, viral DNA is isolated by sequential treatment with DNase I and Proteinase K as described in the preceding section.

PCR primers, designed by PerkinElmer to amplify GFP have the following sequences:

Forward:	5′-TTTCAAAGATGACGGGAACTACAA-3′
Reverse:	5′-TCAATGCCCTTCAGCTCGAT-3′
Probe:	5′-6FAM-CCCGCGCTGAAGTCAAGTTCGAAG-TAMRA-3′

The reaction components used in setting up the reactions and their corresponding volumes are listed below:

TaqMan Universal PCR Master Mix (2×)	25 μl
Forward primer (9 μ*M*, 10× stock solution)	5 μl
Reverse primer (9 μ*M*, 10× stock solution)	5 μl
TaqMan probe (2 μ*M*, 10× stock solution)	5 μl
DNA sample	5 μl
Water	5 μl
Total volume	50 μl

The reactions are initiated by incubating samples at 50° for 2 min to activate uracil-*N*-glycosylase (UNG), followed by incubation at 95° for 10 min (AmpliTaq Gold activation step). The cycling parameters are as follows: 15 sec at 95°, 1 min at 60°, 40 cycles total.

Protocol

To quantify the titer, semilog plots of the increase in reporter fluorescence (ΔRn) vs PCR cycle number are derived for dilutions of the standard and test sample DNAs.

1. To derive a standard curve, plasmid DNA pTR-UF5 is serially diluted in H_2O to derive 6 separate dilutions covering a range of 10 fg to 1 ng (4×10^3–4×10^8 genome equivalents of single-stranded viral DNA, respectively) in a volume of

[6] K. R. Clark, X. Liu, J. P. McGrath, and P. R. Johnson, *Hum. Gene Ther.* **10,** 1031 (1999).

5 μl. This is added into the PCR reaction and amplified as described above. The amplification profiles of six different dilutions are shown in Fig. 2A.

2. To derive a viral DNA sample amplification curve, four 10-fold serial dilutions are made using 10 μl of purified DNA derived from a viral stock, which had been digested with DNase I and treated with proteinase K (see above). After all dilutions are made, 5 μl of each dilution is added to 45 μl of preassembled mix and the reactions are performed as described above. The amplification profiles of serial dilutions are shown in Fig. 2B.

3. To generate a copy number for the standard curve, the C_T value (an arbitrary set threshold cycle) is plotted against the input molecule copy number (Fig. 2C, black dots, coefficient of linearity, 0.985). The viral titer of 7.3×10^7 is derived using the system's software on plotting viral DNA amplification values (Fig. 2C, red dots). This value is then corrected for the dilution factor to derive a final value of 1.46×10^{13} particles/ml (Table II).

Infectious Center Assay/Fluorescent Cell Assay

Protocol

A modification of the previously published protocol[7] was used to measure the ability of the virus to infect C12 cells, unpackage, and replicate. The same cells are used for both ICA and FCA if rAAV encodes the GFP reporter gene.

1. C12 cells are plated in DMEM in a 96-well dish at about 75% confluence. Serial dilutions of the rAAV to be titered are set up as follows: add 250 μl of medium to the first well and add 225 μl of medium to the adjacent wells. Add 2.5 μl of virus to be titered to the first well and serially dilute (10× steps) by transferring 25 μl per dilution to the adjacent well, being certain to change tips after each dilution. Add adenovirus (Ad5) to the cells at a multiplicity of infection (MOI) of 20. Leave a few wells as "adenovirus only" controls.

2. At 40 hr postinfection, cells infected with rAAV-UF5 are visually scored using a fluorescence microscope. Green fluorescence is monitored using high sensitivity CHROMA filter 41012 High Q FITC.

3. To perform ICA, set up a 12-port filter manifold built to hold 4-mm diameter filters as follows: first, wet two pieces of Whatman (Clifton, NJ) paper with PBS and apply it to the entire manifold. Next, apply a nylon DNA transfer membrane (MagnaGraph; Micron Separations, Westboro, MA) wetted with PBS to the Whatman paper. Tighten the assembled manifold and fill each well of the manifold with 5 ml of PBS. (Alternatively, individual glass-fritted 4-mm diameter disk filter holders fitted into a vacuum manifold can be used.)

[7] S. K. McLaughlin, P. Collis, P. L. Hermonat, and N. Muzyczka, *J. Virol.* **62,** 1963 (1988).

4. Detach cells from the 96-well dish by pipeting vigorously eight times and apply each infected cell sample to one well of the manifold. Wash the well with 200 μl of PBS and apply this also to the appropriate well. After transferring all of the cells to the filter manifold, allow 5 min to pass before gentle vacuum (about 1 cm of water) is applied.

5. Allow the nylon membrane to air dry for 5 min on Whatman 3MM paper. Denature the DNA on the filter for 5 min in 0.5 *M* NaOH, 1.5 *M* NaCl and then blot on Whatman paper. Neutralize the membrane for 5 min in 1.5 *M* NaCl, 0.5 *M* Tris-HCl, pH 7.8, and blot on Whatman paper. Rinse in 4× SSC (1× SSC is 0.15 *M* NaCl plus 0.015 *M* sodium citrate) for 30 sec and air dry for 10 min. Microwave the nylon membrane on a high setting for 4 min to fix the DNA as described above. *Caution:* Be sure to have about 300 ml of water in a separate beaker in the microwave or the membrane may catch on fire.

6. Prehybridize the membrane in 7% (w/v) SDS–0.25 *M* $NaHPO_4$ (pH 7.2)–1 m*M* EDTA (pH 8.0) at 65° for at least 1 hr before adding the probe. The probe is a ^{32}P-labeled DNA (any fragment of the DNA in the rAAV cassette being titered). Denature about 50 ng of a random-primed labeled probe (specific activity of 10^9 cpm/μg), add to 15 ml of hybridization buffer (see above) in a glass hybridization cylinder, and hybridize at 65° for 12 hr. Wash the membrane twice for 1 hr each in the same buffer at 60°, dry, and expose the filter to standard X-ray film. An example of such autoradiographs was published earlier.[1] To calculate the infectious titer of the rAAV preparation, the number of positive dots on the filters (optimally 20–200 positive dots per filter) is counted and corrected for the dilution of the stock used on that filter.

Analysis of Vector Purity by SDS–Gel Electrophoresis

To investigate the purity of the final vector stock further, virus was analyzed using a 12% SDS polyacrylamide gel for 5 hr at 200 V under standard buffer conditions and visualized by silver staining (Fig. 3A). Tenfold serial dilutions were loaded into three separate wells. In addition to three major bands representing rAAV capsid proteins V1, VP2 and VP3, some low molecular weight bands are visible in lane 1. These proteins appeared to be products of proteolysis of viral capsid proteins, since the Western blotting analysis performed on a separate segment of the gel revealed that all low-molecular peptides hybridized to anti-capsid antibodies (Fig. 3B). The serial dilution experiment over a 100-fold range illustrates that the vector is 99.9% pure.

Analysis of Vector Composition by Continuous Iodixanol Density Gradient

To examine the density of viral particles a new analytical procedure has been introduced. The virus-containing fractions from the POROS column were pooled

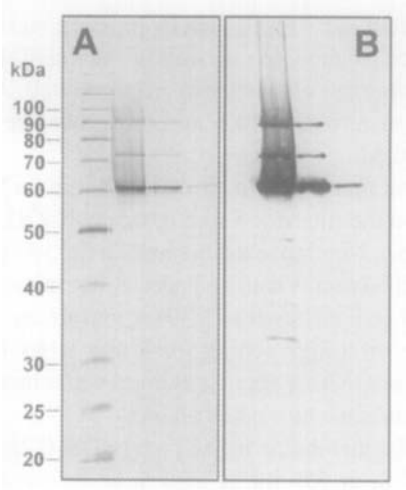

FIG. 3. SDS-protein gel analysis of purified NRS rAAV. Tenfold serial dilutions were loaded into three separate lanes in duplicate. The duplicate portions were stained with either silver to visualize proteins (Panel A) or Western blotted with the monoclonal antibody B1[9] against all three AAV capsid proteins (Panel B). Lane 1 in panel A contains protein standards where molecular masses are shown on the left in kDa.

and analyzed in a continuous gradient of iodixanol. The gradient is formed by mixing virus with 30% iodixanol prepared in PBS containing 0.9 *M* $MgCl_2$. The sample is transferred into a quick-sealing tube, placed in a Ti-70 rotor, and spun at 70,000 rpm for 8 hr at 15°. After centrifugation the sample is fractionated by puncturing the tube at the bottom and collecting 1 ml fractions.

Fractions are analyzed by FCA (Fig. 4A) and by polyacrylamide gel electrophoresis (Fig. 4B). Two distinct peaks of rAAV are seen upon silver stain analysis: fractions 6 through 8 and fractions 13 through 15. The virus peak that bands in the gradient at a refractive index of about 1.425 η contains most of the infectious virus (Fig. 4A, filled diamonds), whereas the more prominent peak in the middle of the gradient contains mostly empty, noninfectious particles. To compare two viral stocks processed by the current method and the method described earlier[1,2] we subjected an rAAV-GFP vector preparation that had been purified by an iodixanol step gradient and conventional heparin affinity chromatography to the iodixanol continuous gradient described above. As expected, the empty particle peak is no longer seen in the viral stock (Fig. 4C) since it was separated away during the preceding iodixanol density step gradient purification. The only peak that is seen, fractions 6 through 9, consists of fully infectious particles, as judged by FCA (Fig. 4A, open circles). The additional bands seen in fraction 22 are contaminating cellular proteins.

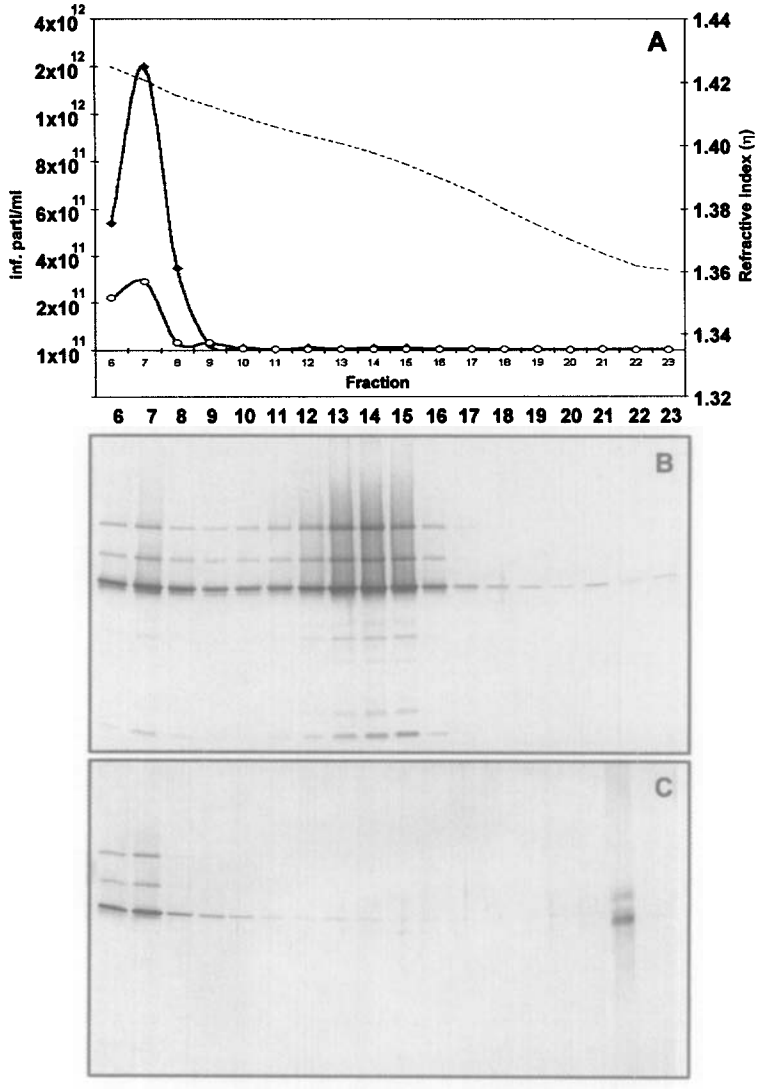

FIG. 4. Continuous iodixanol gradient analysis of purified rAAV. (A) Fluorescent cell assay analysis of gradient fractions. Filled diamonds; infectious profile of NRS rAAV fractions (separated as shown in Panel B); open circles, rAAV-UF5, prepurified as described earlier by iodixanol step gradient/heparin affinity chromatography[1] and subsequently separated as shown in Panel C. A plot of refractive index of gradient fractions is shown by a dotted line. (B) Silver-stained SDS/protein gel analysis of iodixanol fractions of the NRS rAAV separated by continuous gradient. (C) Similar analysis of iodixanol fractions of the prepurified rAAV-UF5 rAAV.

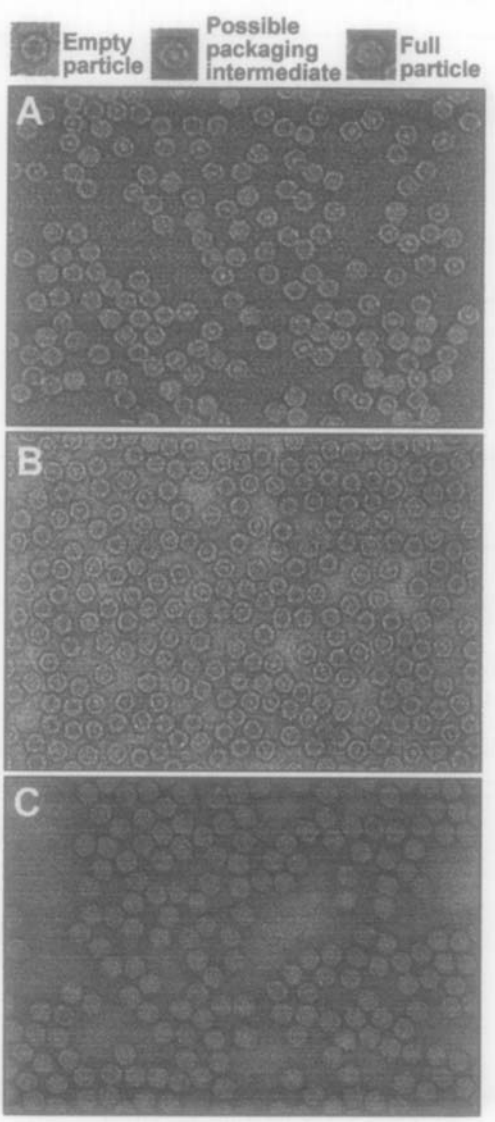

FIG. 5. Electron microscopy analysis of NRS rAAV particles. Electron micrographs were obtained using a Zeiss EM-10A transmission electron microscope operating at 80 kV accelerating voltage. The magnification factor is 49,500. (A) Electron micrograph of rAAV purified by three successive chromatography steps shown in Fig. 1. The preparation consists of a mixture of full particles (uniformly stained), empty particles (filled circle inside a particle), and a possible packaging intermediate (open circle inside a particle). (B) Electron micrograph of virus pooled in fractions 13–15 of iodixanol gradient (Fig. 4B). The preparation essentially consists of empty particles and packaging intermediates. (C) Electron micrograph of virus pooled in fractions 6–8 of iodixanol gradient (Fig. 4B). The preparation consists predominantly of full particles.

It is worth noting that separation of full and empty particles does not improve the physical-to-infectious particle ratio of a given stock, since both titering assays used in this protocol (DBA and RTPA) are based on quantification of packaged genomes, rather than on the assay of assembled particles. Removal of empty particles, however, improves the overall quality of a viral preparation by decreasing the capsid antigen burden of the stock and eliminating a competitor for cell surface receptors.

Analysis of Virus Structure by Electron Microscopy

To confirm the structure of infectious and noninfectious particles, two peaks from the continuous iodixanol gradient are concentrated by POROS HPLC chromatography as described earlier.[1] Following chromatography, EM analysis of concentrated samples is performed as described below and shown in Fig. 5. The sample is prepared by placing 5 μl of purified virus stock on support films of Formvar/Carbon 400 mesh copper grids (Ted Pella, Inc.) for 1 min. Excess sample is removed by blotting with a filter paper. The sample is then stained with 5 μl of 2% uranyl acetate for 10 sec and excess stain is removed as described above.

rAAV purified by three successive chromatography steps consists of a mixture of full (uniformly stained) and empty (filled circle inside a particle) particles, as well as a possible packaging intermediate (open circle inside a particle) (see Fig. 5A and captions above). This particle structure is consistent with the data documented by Grimm *et al.*[8] After separation in an iodixanol gradient as shown in Fig. 4, the virus pooled in fractions 13–15 consists essentially of empty particles and packaging intermediates (Fig. 5B). The virus pooled in fractions 6–8 of the iodixanol gradient consists predominantly of full particles (Fig. 5C).

Conclusions

Here we describe an improved protocol adapted for large-scale production of a preclinical grade rAAV. This protocol consists of three sequential chromatography purification steps resulting in highly purified (99.9% pure) and infectious (particle-to-infectivity ratios less than 10) vector preparations. In addition, we describe a new centrifugation procedure that allows the separation of full and empty particles. The described protocol was successfully implemented for the production the National Reference Standard rAAV vector, sponsored by the National Gene Vector Laboratory.

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[9] C. E. Wobus, B. Hugle-Dorr, A. Girod, G. Petersen, M. Hallek, and J. A. Kleinschmidt, *J. Virol.* **74,** 9281 (2000).

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