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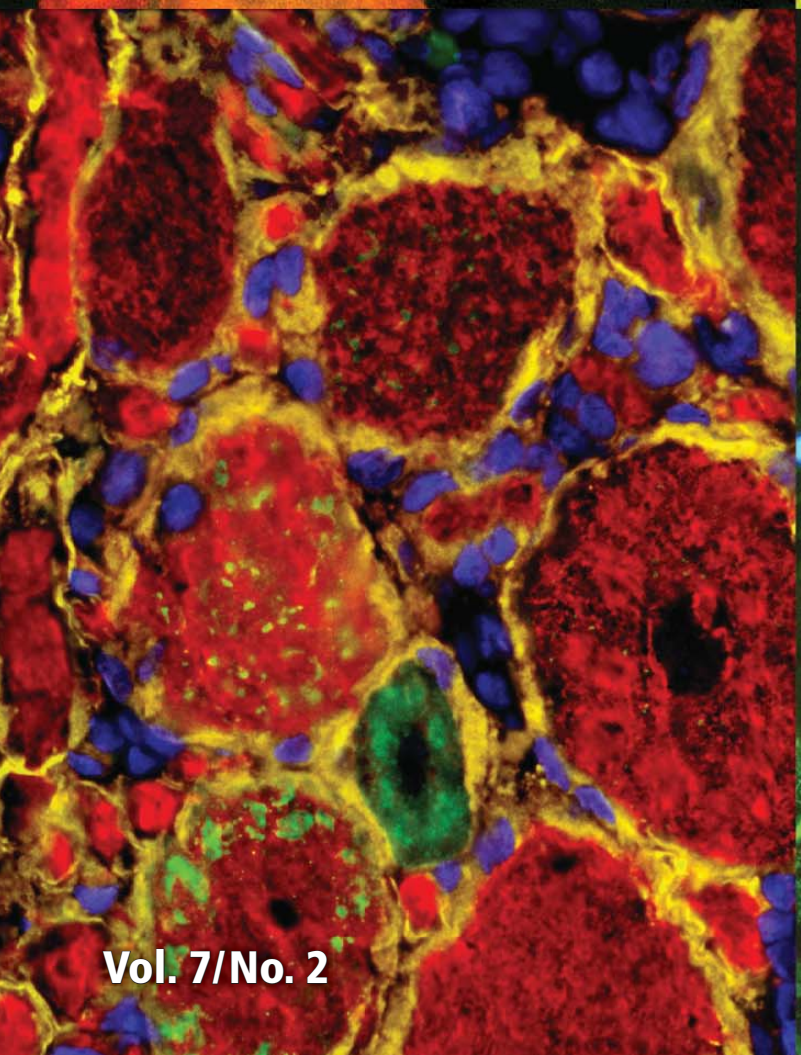

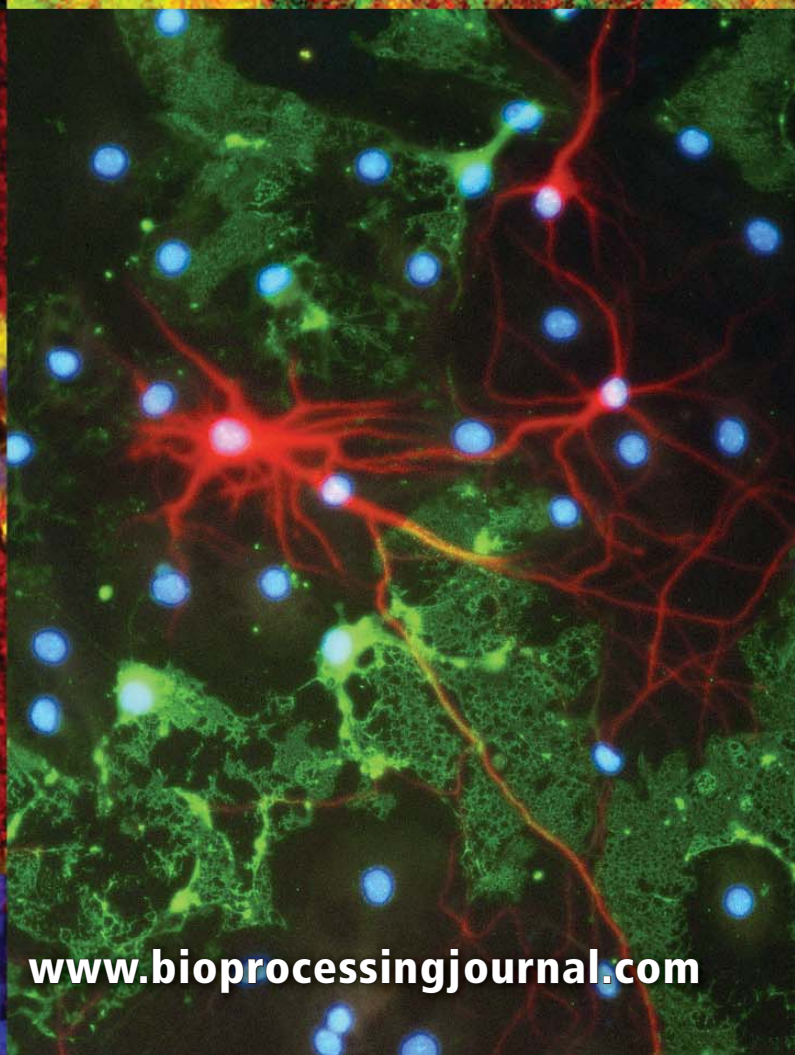

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Manufacture and Stability Study of the Recombinant Adeno-Associated Virus Serotype 2 Vector Reference Standard

By RICHARD O. SNYDER* et al.

Introduction

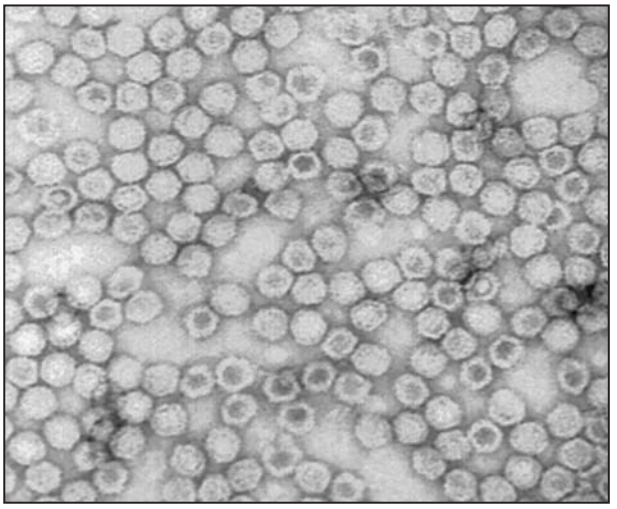
Recombinant adeno-associated viral (rAAV) vectors have proven to be efficient vehicles for gene transfer in animal models. The attractive features of this vector system are long-term gene expression with little or no associated toxicities following administration to a variety of tissues.[1-6] Previous and ongoing clinical trials in humans demonstrate a very good over-all safety profile.[7-11] However, one of the caveats of this work that has been carried out by several laboratories is the inability to normalize vector doses administered by different investigators to animals and humans. Most of the work to date has involved AAV serotype 2 vectors, but vector systems based on other AAV serotypes are being rapidly developed.[12-14]

Administered doses are usually based on vector genomes (using hybridization, real-time PCR, or spectrophotometry), but give no information as to the infectivity of the vector. Determining infectious titer is critical as the ratio of infectious virions to vector genome-containing virions is important in determining the dose, potency, and strength of the vector preparation.

Following the example set by the adenoviral reference material working group (ARMWG) for developing the ARM,[15] the FDA encouraged the AAV community to develop a high-quality rAAV serotype 2 reference standard stock (AAV2 RSS) and to use it to validate each laboratory's internal reference standard and test methods. The AAV community responded to facilitate comparisons among nonclinical or clinical studies, aid in the manufacture of more consistent and higher quality vectors, and ultimately help formulate regulatory policy.[16]

The AAV reference standard working group (AAV2RSWG), a volunteer organization comprised of members from the AAV community, includes six industry members and 23 universities from nine countries, FDA/CBER, NIH, and The Williamsburg BioProcessing Foundation (WilBio, www.wilbio.com). It was recognized that generating the AAV2 RSS would be a major undertaking because it must be supplied in sufficient quantity to each requestor for use in all necessary tests at each location, be of high quality, and remain stable for an extended period of time. A profile of the AAV2 RSS was established for filling and characterizing several thousand vials (Table 1).

A consensus was reached by the AAV2RSWG to manufacture the AAV2 RSS using helper virus-free transient transfection and chromatographic purification. In this article, we will describe the production and purification of the AAV2 RSS filtered formulated bulk. The bulk material has been vialed and is now being characterized prior to making it available for distribution to the community in the coming months.



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Results and Discussion

Production and Manufacture of the AAV2 RSS Purified Bulk

Requests for proposals were drafted and issued, and the AAV2RSWG Donations Subcommittee and the Manufacturing Subcommittee reviewed proposals for raw materials donations, production and purification, vialing, and repository. Recommendations were then made to the Executive Committee and winning bids were awarded (Table 2). The goal was to produce one lot of rAAV2-GFP vector with a yield of $1x10^{15}$ virus genomes. Eighteen batches (Table 3) were produced using transient transfection of 293 cells seeded into ten 10-layer Nunc cell factories (CF-10), and purified by column chromatography (see *Materials and Methods*). The 18 harvests were generated from February 2006 to December 2006, and the 18 purification runs overlapped from August 2006 to January 2007.

Cells from the ten CF-10 cell factories were harvested, thawed, lysed with 0.5% sodium deoxycholate, treated with Benzonase® (Merck KgaA), and then disrupted by microfluidization. Virions were then purified by STREAMLINE™ (GE Healthcare Life Sciences) heparin affinity chromatography (Figure 1A). Peak fractions were pooled and the NaCl concentration was adjusted to 1M. The pooled fractions were applied to a Phenyl Sepharose™ (GE Healthcare Life Sciences) chromatography column, and the rAAV was collected in the flow-through. This flow-through was diluted with water, purified, and concentrated by sulfopropyl cation exchange chromatography.

Vector was eluted in 5–10 ml of 135 mM NaCl in phosphate buffered saline (PBS) (equivalent to 285 mM ionic strength), and the purified bulk was stored frozen at –80°C. In-process testing was performed on each purified bulk for purity, infectious titer, and vector genome titer. The 18 batches produced a total of 150 ml of vector with

TABLE 1. AAV2 RSS Assays Utilized in Materials Characterization.

• Confirmation of the serotype and capsid titer by ELISA
• Evaluation of the purity, capsid subunit stoichiometry, and chemical integrity of the capsid by SDS-PAGE
• Vector genome titer by qPCR
• Infectious titer by TCID50 with qPCR read-out, and by transduction with GFP read-out
• Full sequencing of the vector
• Stability testing

TABLE 2. AAV RSS2 Committees in 2006.

Executive Committee:
Richard Snyder - *Univ. Florida* **Parris Burd** - *Bayer Corporation* **Olivier Danos** - *Genethon* **Keiya Ozawa** - *Jichi Medical School* **Maritza McIntyre** - *FDA* **Denise Gavin** - *FDA* **Keith L. Carson** - *WilBio* **Richard Knazek** - *NIH NCRR*
Donations Subcommittee:
Keith L. Carson - *WilBio* **Richard Snyder** - *Univ. Florida* **Denise Gavin** - *FDA CBER*
Manufacturing Subcommittee:
Guang Ping Gao - *Univ. Penn.* **Phil Cross** - *Harvard* **Anna Salvetti** - *Univ. Nantes* **Susan Washer** - *AGTC* **K. Reed Clark** - *OSU* **Guang Qu** - *Avigen*
Raw Materials/Filling/Repository Services:
HyClone, Aldevron, Nunc, Mediatech, Corning, Fisher Scientific, Introgen Therapeutics, and **ATCC**
Production and Purification Site:
The University of Florida's Powell Gene Therapy Center Vector Core

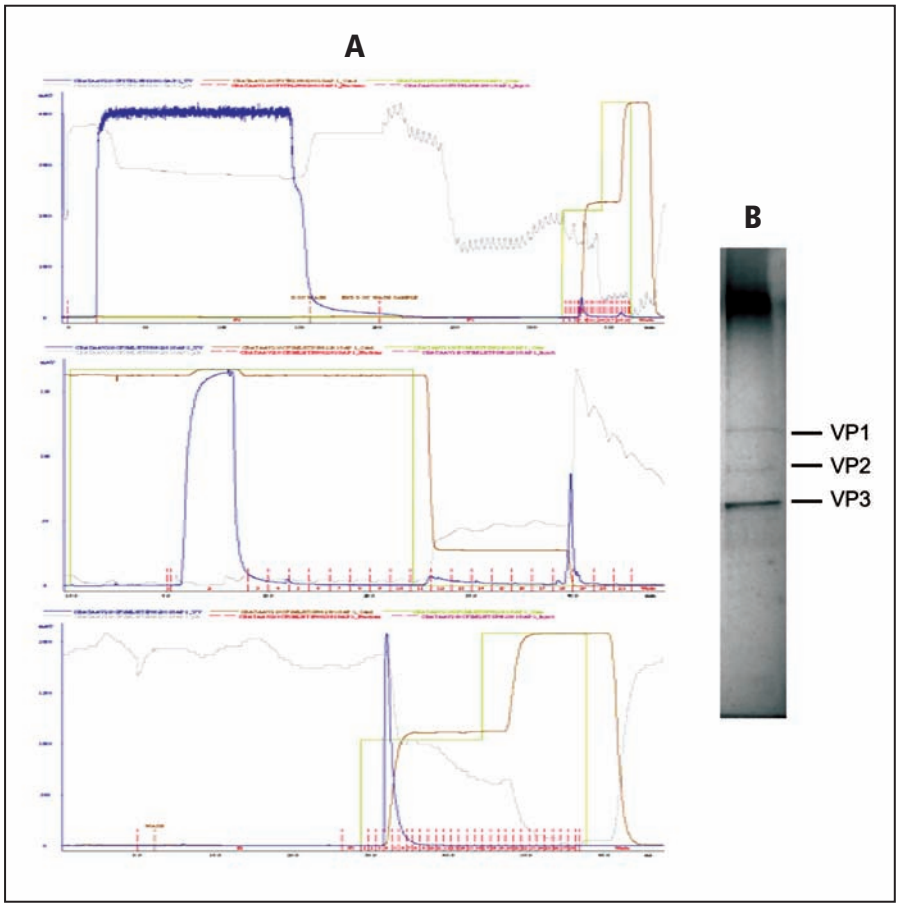

FIGURE 1. FPLC Purification of rAAV. A: Ten CF-10 of rAAV-UF11 producer cells were microfluidized in the presence of deoxycholate and immediately loaded on the first of three FPLC columns. The first column is a Streamline (Pharmacia) heparin column. The second column is phenyl sepharose, and the third column is SP sepharose. Shown are tracings for A280, conductivity, and fraction numbers. B: Silver stained SDS-PAGE gel.

TABLE 3. Yield and Material Theoretical Mass Balance.								
Batch #	HE Peak AUC	PS Peak AUC	SP Peak AUC	SP Run Date	SP Volume (ml)	SP Titer (vg/ml)	SP Titer (IU/ml)	Total (vg/batch)
NRS 08-29-06	179.65	914.75	434.63	08/29/2006	12.9	5.68E+12	4.50E+10	7.33E+13
NRS 09-06-06	20.04	71.77	42.03	09/06/2006	4.6	9.48E+11	6.50E+08	4.36E+12
NRS 09-11-06	14.59	50.30	27.87	09/12/2006	4.0	6.03E+11	8.10E+09	2.41E+12
NRS 09-20-06	137.71	493.81	350.67	09/20/2006	11.4	7.44E+12	3.70E+10	8.48E+13
NRS 09-27-06	108.14	363.02	272.68	09/28/2006	10.4	3.49E+12	3.60E+10	3.63E+13
NRS 10-03-06	91.63	297.68	229.07	10/03/2006	9.6	3.67E+12	3.50E+10	3.52E+13
NRS 10-10-06	67.24	244.05	176.40	10/10/2006	8.75	1.68E+12	3.00E+10	1.47E+13
NRS 10-17-06	52.41	173.13	130.89	10/17/2006	7.65	1.95E+12	1.37E+10	1.49E+13
NRS 10-24-06	48.74	168.70	123.50	10/24/2006	7.5	3.66E+12	9.10E+09	2.75E+13
NRS 10-30-06	67.21	229.02	172.34	10/31/2006	8.5	4.32E+12	8.90E+09	3.67E+13
NRS 11-06-06	27.80	55.06	57.58	11/07/2006	5.45	7.51E+11	1.80E+09	4.09E+12
NRS 11-15-06	54.66	197.89	150.22	11/15/2006	8.25	1.92E+12	5.30E+10	1.58E+13
NRS 11-21-06	43.56	157.60	116.30	11/21/2006	7.35	2.98E+12	4.90E+10	2.19E+13
NRS 11-28-06	66.75	245.35	183.34	11/28/2006	8.75	7.36E+12	3.20E+10	6.44E+13
NRS 12-06-06	88.89	337.04	239.61	12/06/2006	9.5	7.35E+12	2.80E+10	6.98E+13
NRS 12-12-06	117.17	421.37	296.14	12/12/2006	10.4	4.33E+12	4.30E+10	4.50E+13
NRS 12-20-06	74.64	272.88	199.05	12/20/2006	8.85	1.35E+12	2.30E+10	1.19E+13
NRS 01-04-07	40.57	126.58	85.85	01/04/2007	6.45	8.47E+11	1.40E+10	5.46E+12
							TOTAL vg	**5.69E+14**

The area under the peak curve (AUC) was obtained from the ÄKTA (GE Healthcare Life Sciences) for each column (Heparin Sepharose [HE]; Phenyl Sepharose [PS]; Sufopropyl Sepharose [SP]). Vector genomes as determined by dot-blot (vg); infectious units as determined by infectious titer assay (IU).

a total of 5.69×10^{14} vg. The purified bulks were combined, diluted to ~2×10^{11} vg/ml, and sterile filtered into two 1.3 L portions. This filtered formulated bulk was stored frozen (–80°C) in anticipation of vialing.

Bioburden Testing

Prior to freezing, the filtered formulated bulk was sampled (5 ml) and tested for bioburden by AppTec (now WuXi AppTec, Inc.). Aerobes, fungi, spores, and obligate anaerobes all tested negative with an assay sensitivity of < 5 CFU/sample.

Purity and Titer

Samples of each purified bulk were titered by dot-blot assay, infectious titer assay, and evaluated for purity. Table 3 summarizes the vector genome and infectious titers obtained for each of the 18 batches. Figure 1B is a representative silver gel of batch purity. The filtered formulated bulk was titered by infectious titer assay and had a titer of 2×10^9 IU/ml, with the assay control having known titers of 1.8×10^{12} vg/ml and 1.2×10^{10} IU/ml (p.i. = 150). Therefore, we estimated that the filtered formulated bulk had a titer of 3×10^{11} vg/ml.

Mycoplasma Testing

Mycoplasma testing was performed as part of the RSS2 characterization. Mycoplasma testing occurs on the cell harvest (i.e., when the transfected cells are collected from the cell factories), and includes cells mixed with the spent culture supernatant. 1×10^7 cells in 15 ml of spent culture supernatant were tested according to a Good Laboratory Practices (GLP) "Points to Consider" assay using direct and indirect tests (AppTec). Since 18 batches of ten cell factories were each transfected, the harvests were pooled prior to testing to reduce cost. The sample submitted (1×10^7 cells in 15 ml of spent media) was equal to 6.7×10^{-3}% of the total number of cells from the 18 batches (= 1.5×10^{11} cells). The harvest was positive for *Mycoplasma arginini* (bovine origin), and the test was valid (i.e., all controls performed). The test was repeated and the result was also positive.

HyClone, who donated the serum used in cell culturing, was contacted to determine if they had recalled any of the serum lots used. According to HyClone, none of the serum lots tested positive for mycoplasma. At this time, it is not known which of the batches were contaminated or when the contamination occurred during the 11 months of production because the harvests were pooled. However, other AAV vector batches made concurrently in the same laboratory during production of the RSS tested negative for mycoplasma.

Next, the filtered formulated bulk was tested for mycoplasma. A modified mycoplasma test was developed and used by AppTec. A 3 ml sample of the filtered formulated bulk (equal to 0.12%

of the total 2570 ml; thus, a sample 18x larger [on a percentage basis] than was tested for the harvest) was inoculated onto Vero cells. The Vero cells were harvested (by scraping) and used to inoculate a second set of Vero cells, followed by a third round of Vero inoculation. These cycles were designed to allow growth and amplify any mycoplasma that might be present in the filtered formulated bulk. The cells from the final inoculation cycle were tested by the same GLP "Points to Consider" assay using direct and indirect tests (AppTec). The filtered formulated bulk tested negative for mycoplasma. The test was valid, and spike-in controls performed as expected.

Lastly, the harvest and filtered formulated bulk were tested by PCR (AppTec) for mycoplasma DNA. The harvest was confirmed positive and the filtered formulated bulk tested negative, with all controls performing as expected. The PCR assay can detect a 16S rRNA gene region for the following mycoplasma species: *M. arginini*, *M. orale*, *M. hyorhinis*, *M. synoviae*, *M. gallisepticum*, *M. pnumoniae*, *S. citri*, *A. laidlawii*, and *M. fermentans*.

The purified bulk is likely negative for mycoplasma following purification because: 1) The cells were lysed using microfluidization — a highly effective method of disintegrating cells and bacteria in the presence of deoxycholate; 2) the three chromatographic steps may have separated mycoplasma and mycoplasma DNA from the AAV; and 3) the series of filtration steps may have removed mycoplasma. There isn't any data to support this other than the negative culture and PCR test results following these three process steps.

The AAV reference standard was made in a research vector core, not in a GMP facility. Making the AAV2 RSS under GMP would have been cost-prohibitive. If this lot of AAV had been intended for humans, it would not be acceptable to use a harvest that was mycoplasma-positive, and the entire lot would have been discarded. However, because this is a reference standard to be used in research and QC labs, the AAV2 RSS WG recommended that we continue forward and fill, bank, characterize, and distribute the AAV2 RSS.

A summary will be included on the product information sheet supplied with each shipment of the AAV2 RSS stating that the harvest was mycoplasma positive but the purified bulk was negative. "The AAV2 RSS has been exposed to mycoplasma, but is mycoplasma-free." Thus, laboratories requesting the RSS2 will be fully informed and can decide if they want to bring it into their QC laboratories.

Stability and Absorption Study

In anticipation of filling the filtered formulated bulk into vials, a study was conducted to compare the stability and absorption of an AAV2-GFP vector in polypropylene and glass vials. Data was generated using an AAV2-GFP vector filled into cryovials (a standard container for UF) and glass vials (Figure 2) with a volume of 0.5 ml at a vector concentration of 2×10^{11} vg/ml in the same formulation as the AAV2 RSS (PBS + 135 mM NaCl). This study was conducted to evaluate the short-term stability (1 hour, 1 day, 3 days, 7 days) of the vector at room-temperature (the temperature for the vial filling), and long-term –80°C storage temperature (1 hour, 1 day, 14 days, 35 days, 124 days) in these containers. The

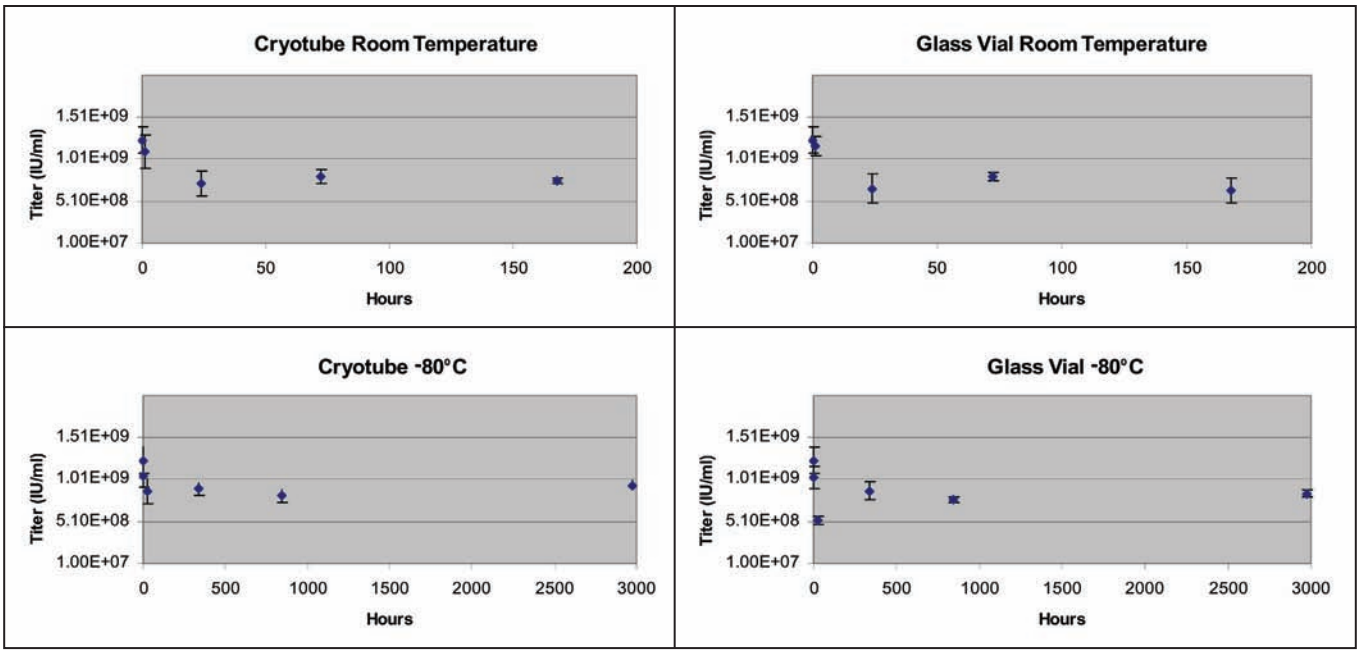

FIGURE 2. Stability Study. An AAV2-GFP vector (2×10^{11} vg/ml) was stored in glass or polypropylene cryovials at room temperature (21°C) or frozen at –80°C for up to 7 days or 124 days, respectively. The vector was titered using the infectious titer assay (described in *Materials and Methods*). Titers (IU/ml) are reported ± STDEV.

AAV vector was filled into vials, stored, and then titered by the infectious titer assay according to the schedule. In all scenarios, there was a 30–40% drop between the initial titer and the average of samples taken during the time course, which most likely indicates absorption to the container surfaces at this low vector concentration.

The Future

The filtered formulated bulk has been filled by ATCC into polypropylene cryovials (0.5 ml at a concentration of 2×10^{11} vg/ml). Twenty-three testing sites have committed to performing various assays (Table 1) to characterize the RSS2 with the number of replicates needed for statistical significance. In addition, there will be a stability study which will be comprised of: 1) the infectivity and identity/purity assays performed at three separate timepoints (initial characterization, six months and one year) with a set number of replicates at each timepoint; 2) the number of testing sites; 3) sterility testing; and 4) freeze/thaw studies. The method of shipping and laboratory onsite storage requirements have also been evaluated. The characterization of the AAV2 RSS will be published once the data is collected and statistically analyzed.

Materials and Methods

Plasmid DNA

The AAV vector plasmid pTR-UF-11[17] and the pDG-KanR helperplasmid, a kanamycin-resistant version of pDG[18] were produced by fermentation, purified by alkaline lysis and chromatography, formulated (1mg/ml) in TE, sterile filtered, aliquoted into 7 ml and 20 ml portions, tested, and released by Aldevron.[17,18]

Reagents and Supplies

Corning provided seropipets (1–100 ml sizes), T-225 tissue culture flasks, 1 L filter units, centrifuge tubes (15–500 ml sizes), and storage bottles. Fisher Scientific provided chemicals, latex gloves, and disinfectant concentrates. HyClone provided water for injection (WFI), DPBS, PBS + 5 mM EDTA, FBS, and DMEM. Mediatech provided pen/strep, 10x trypsin/EDTA, and trypan blue. Nunc provided 10-layer cell factories, funnels, and roller bottles.

TABLE 4. Plasmid Release Specifications.

Tests	Methods	Specifications
Appearance	Visual inspection	Clear and colorless solution
Sterility	USP	No microbial growth detected
Endotoxin	LAL	< 100 EU/mg
Homogeneity	Agarose gel electrophoresis	Predominantly supercoiled
Purity: A 260/280 ratio	Spectrophotometric	1.8-2.0
Identity	Agarose gel electrophoresis	Co-migrates with reference DNA and/or size confirmed versus supercoiled marker
Genomic DNA	Agarose gel electrophoresis	< 5%
Residual RNA	Agarose gel electrophoresis	Not visible on agarose gel
Identity	Restriction endonuclease digestion (3 enzymes) Agarose gel electrophoresis	Matches expected restriction pattern
Concentration	Spectrophotometric	1 mg/ml

Production

Cell and virus processing was performed exclusively in biosafety cabinets during open steps. 293 cells were cultured in DMEM, supplemented with 5% FBS and antibiotics (DMEM-complete), in culture flasks. PBS and 0.05% trypsin were used during cell passage. Briefly, 293 cells were split 1:3 the day prior to transfection, so at the time of transfection the cell confluency was ~75–80%. A production run utilized 5×10^9 cells seeded in five 10-layer cell factories and purified plasmid DNA Aldevron. The $CaPO_4$ precipitate was formed by mixing 9 mg of pDG and 3 mg of the rAAV vector plasmid pTR-UF-11 (~1:1 molar ratio) in 250 ml (total volume) of 0.25 M $CaCl_2$, followed by the addition of 250 ml of 2x HBS, pH 7.05, to the DNA/$CaCl_2$. The mixture was incubated for 1–2 minutes at room temperature, at which time the formation of precipitate was stopped by diluting 100 ml of the mixture into each of five bottles of 1100 ml pre-warmed DMEM-complete. The conditioned culture media was removed from the cells, and the fresh precipitate-containing media was added immediately. Cells were incubated at 37°C, 5% CO_2 for 60 hours. The $CaPO_4$ precipitate was allowed to remain on the cells during this incubation period. At the end of the incubation, the culture media was discarded, cells were washed with PBS, and harvested using PBS containing 5 mM EDTA. Samples of cells were combined with spent tissue culture media for mycoplasma and *in vitro* adventitious agent testing. The collected cells from each harvest of five CF-10 were centrifuged at 2000 g for 15 minutes, combined, and stored at –20°C in 500 ml conical centrifuge bottles until purified.

Cell Lysis

The harvests from ten CF-10 (~1×10^{10} cells) were thawed and resuspended in 600 ml lysis solution (150 mM NaCl, 20 mM Tris, 1% deoxycholate, pH 8.0). Benzonase (50 U/mL final concentration) in the presence of 2 mM $MgCl_2$ and Tris/NaCl buffer was added to the harvest BPC. After incubation with intermittent mixing for 60 minutes at 37±2°C, the Benzonase-treated harvest was further disrupted by passing it once through a microfluidizer (Microfluidics M-110S). The crude lysate was collected in a 1 L PETG bottle and fed into an ÄKTA FPLC to immediately begin chromatographic purification.

Purification

The lysate was applied to a 160 ml STREAMLINE heparin column using automated programs which captured the AAV type 2 vector particles. The column was washed with PBS and the vector was then eluted with a 350 mM salt bump using PBS/NaCl. The vector-containing fractions (the HE peak) were collected in a 250 ml polypropylene conical bottle and stored at 2–8°C. The salt concentration of the HE peak was increased from 350 mM to 1.1 M by the addition of 4M NaCl. The salt-adjusted HE peak was then passed through a 5 ml Phenyl Sepharose column. The column was monitored by UV absorbance, and the vector was eluted in the flowthrough. Vector was collected in a 250 ml conical bottle. WFI was added to the Phenyl Sepharose flowthrough to decrease the salt concentration to approximately 140 mM NaCl. This material was then loaded onto a 5 ml SP HP (sulfopropyl cation exchange) column, and the purified bulk was eluted with a 285 mM PBS/NaCl salt bump. All process equipment underwent cleaning and sanitization using NaOH, according to standard operating procedures.

In-Process Testing

Each purified bulk was sampled for vector genome titer, infectious titer, and vector identity and purity testing to ensure that each purified bulk met at least the minimum specifications.

Formulation

The purified bulks were thawed and mixed, diluted with PBS/NaCl (285 mM) in a 5 L BioProccess Container™ ([BPC]HyClone). The formulated bulk was then filtered using a Sterivex-GV 0.22 µm filter, and filled during filtration at 118 ml/min into two 1.3 L portions each in a 2 L BPC. Samples for bioburden testing were taken post-filtration.

Stability Study

A rAAV2-GFP vector was dispensed into Type I borosilicate glass vials with gray butyl stoppers or 1.2 ml polypropylene cryovials (Corning), and stored frozen at –80°C or at room temperature for the indicated time. The infectious titer (green cells) was tested at each timepoint in triplicate. Forty-eight vials (cryo and glass) were filled with 0.5 ml at 2×10^{11} vg/ml each (= 24 ml at 2×10^{11} vg/ml = 4.8×10^{12} vg total used), as specified for the actual reference standard.

Assay for Identity and Protein Purity of rAAV Stocks

Vector stocks were analyzed by silver staining (Bio-Rad) following electrophoresis of the fully-reduced capsid proteins on 10% SDS polyacrylamide gels (Bio-Rad).

Infectious Titer Assay

In this assay, 96-well plates seeded with 2×10^4 C12 cells, a gift of P. Johnson,[19] were infected 16 hours after seeding with ten-fold dilutions of rAAV and superinfected with wild-type adenovirus type 5 (Ad5) at a multiplicity of infection (MOI) of ten. Ad5-infected cells complement AAV DNA replication and amplify the rAAV genomes. Thirty hours later, individual cells infected with rAAV-GFP were visually scored using a fluorescence microscope, and the titer was calculated according to the dilution factor.

Dot Blot Assay for rAAV Vector Genome Titer and the Particle-to-Infectivity Ratio

The dot blot assay was used to determine the titer of rAAV virions that contain vector genomes. For each sample, plasmid and unpackaged vector DNA was digested for one hour at 37°C in a final volume of 200 µl containing 20 U of DNase I (Roche), 10 mM Tris-Cl, pH 7.5, and 1 mM $MgCl_2$. Encapsidated rAAV vector genomes were liberated by adding an equal volume of 2 x proteinase K buffer (20 mM Tris-Cl, pH 8.0, 20 mM EDTA, pH 8.0, 1% SDS), followed by the addition of proteinase K (30 µg), and incubated at 37°C for one hour. The liberated vector DNA was phenol-extracted and ethanol precipitated using glycogen as a carrier. Precipitated DNA was dissolved in 40 µl of TE, and up to 10 µl was diluted into 400 µl 0.4 N NaOH/10 mM EDTA immediately prior to immobilization. A two-fold dilution series of the plasmid DNA that was packaged and then prepared in water and diluted into 0.4 N NaOH/10 mM EDTA immediately prior to immobilization. Denatured vector DNA was immobilized onto a nylon membrane along with the plasmid standard curve using a dot-blot apparatus (S&S). The blots were probed for the transgene and exposed to film or Phosphoimager screen (Molecular Dynamics). The vector DNA signal was compared to the signal generated from the plasmid DNA standard curve and extrapolated to determine a vector genome titer. A comparison of the vector genome titer to the infectious titer produced the particle-to-infectious (p.i.) ratio.

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