AAV Reference Material Working Group
Bid Submission Form

Method of vector production, RFP 1.0

Item for Submission

A proposal for production of one lot of AAV2CMVGFP vector with a yield of ≥ 2 x 10^{15} virus genomes in total. All methods of AAV vector production at a scale that is able to meet the yield requirement will be considered. This would include 293 cells/transient transfection, helper adneovirus infection of HeLa or other cell line derived AAV producer cells, use of recombinant herpes simplex virus (rHSV) or adenovirus-AAV hybrid and other scaleable processes such as baculovirus based production.

General Requirements for Bidding

A detailed description of the production process, reagents and equipment needed, such as, cell line, helper virus or plasmids, tissue culture reagents, tissue culture plastic ware and equipment, must be included. Conditions and procedures for storage and shipment of crude virus lysate should also be described. The QC tests and QA standard for cell lines, helper viruses, plasmids and crude vector product should be described. The bidder should include a description of the facility, plus the qualifications of the individuals who will do the work. There should also be information about similar work that has been accomplished in this facility by these personnel.

Documentation Requirements

Provide information as stated above. Production batch records or summary information of production lots generated by the proposed method should be included to demonstrate performance of the production method (e.g. virus genome yield per cell, sizes of lots, ratios of virus genome to infectious particles, etc).

Please complete the following fields:

Contact Information – RFP 1.0

Contact Individual:	***Richard O. Snyder***
Institution:	***University of Florida***
Address:	***PO Box 100266, Gainesville, Fl 32610-0266***
Phone Number:	***352-392-8459***
Email Address:	***rsnyder@gtc.ufl.edu***

Please indicate if your institution is also submitting proposals for the other activities:

AAV reference material purification
Down stream processing equipment and materials (filtration, concentration, etc)
Column chromatography materials (resins, columns, tubing, bags, containers and other accessories)
Chromatography work station
Formulation and storage condition for the AAV reference material
Donation of Cell Bank
Adenovirus Helper Virus or rHSV Helper Bank Production
Plasmids for transfection
Tissue culture reagents
Tissue culture plastic wares and equipment
Donation of other supplies/services
Vialing, storage and distribution services for the AAV reference material

Please attach:

Documentation mentioned above

Submit this completed form and all attached information for receipt **by October15, 2003** to the address below. Electronic submissions are encouraged. Final decisions will be communicated by or about November15, 2003. Please note that all information submitted will be publicly available. Please do not mark any information confidential, as we cannot honor that request. Please include an estimated cost and market value for goods and services donated.

Williamsburg BioProcessing Foundation
Attn: AAV Reference Material Working Group
1206 Laskin Road, Suite 201
Virginia Beach, VA 23451
(757) 423-8823
Fax: 423-2065

This proposal is for production of one batch of AAV2CMVGFP vector with a yield of $\geq 2X10^{15}$ virus genomes in total using transient transfection methodology.

Background

The Vector Core now transfects approximately 800 cell factories per year (each $1X10^9$ cells) including 500 individual cell factory preparations (average vector yield per preparation = ~1X10e13 vg, see Fig 1) and 300 cell factories used in large preparations (10 cell factories or more combined) of rAAV vectors.

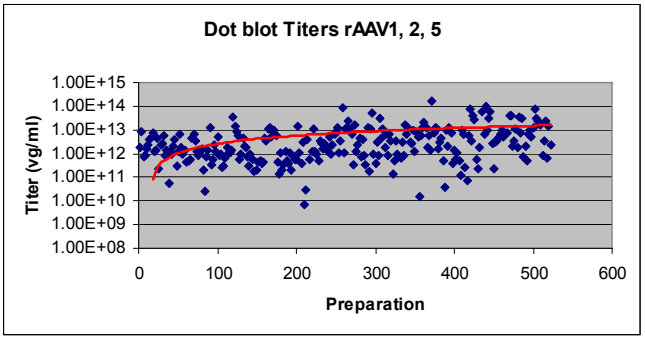


Figure 1. Small-scale rAAV preparations made during yr2002. 67% of all preps were AAV2. The remaining were AAV2 capsid mutants, or AAV1 or AAV5 vectors.

Manufacturing Process

Summary. The vector is produced in the Vector Core Laboratory at the University of Florida. rAAV vector is produced by expanding 293 cells into 10-layer Cell Factories (Nunc) and cells are co-transfected using a $CaPO_4$ precipitation method with helper and vector plasmids. After 60 hours, cells are harvested and frozen.

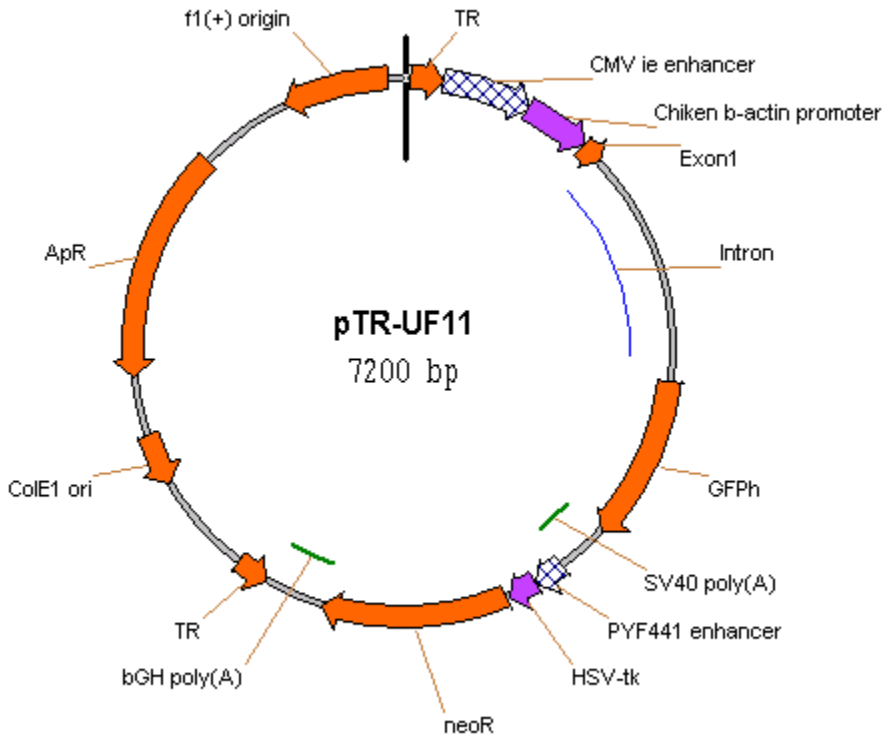
Methods. Cell and virus processing is performed exclusively in biosafety cabinets during open steps. Tissue culture cells. 293 cells (ATCC) are cultured in DMEM supplemented with 5% Fetal Bovine Serum and antibiotics (called DMEM-complete). PBS, and 0.05% Trypsin are used during cell passage. Briefly, 293 cells are split 1:3 the day prior to transfection, so at the time of transfection the cell confluency is ~75-80%. A production

run utilizes about $1X10^9$ cells seeded in a Cell Factory (Nunc) and purified plasmid DNA. The $CaPO_4$-precipitate is formed by mixing 1.8 mg of pDG and 0.6 mg of the rAAV vector plasmid (~1:1 molar ratio) in a total volume of 50 ml of 0.25 M $CaCl_2$ followed by the addition of 50 ml of 2XHBS pH 7.05 to the DNA/$CaCl_2$. The mixture is incubated for 1-2 minutes at room temperature, at which time the formation of precipitate is stopped by diluting the mixture into 1100 ml of pre-warmed DMEM-complete. The conditioned culture media is removed from the cells and the fresh precipitate-containing media is added immediately. Cells are incubated at 37°C, 5% CO_2 for 60 hrs and the $CaPO_4$ precipitate is allowed to remain on the cells during this incubation period. At the end of the incubation the culture media is discarded, cells are washed with PBS, and harvested using PBS containing 5mM EDTA. The collected cells are centrifuged at 1000g for 10 minutes, resuspended in 60 ml Lysis Solution (150 mM NaCl, 50 mM Tris pH 8.4) or other buffer that will be compatible with purification, combined, and stored at -20 °C until purified. Alternatively, cell pellets can be combined, centrifuged, supernatant removed, and cell pellets frozen at –20°C without buffer.

Harvest testing:

- Mycoplasma
- In vitro adventitious viruses
- Bioburden

Vector Plasmid



The TR-UF-11 vector is a GFP-expressing vector under the control of the Chicken β-actin promoter CMV IE enhancer. The vector cassette is flanked by AAV2 ITRs.

pTR-UF-11 AAV vector plasmid master cell bank specifications

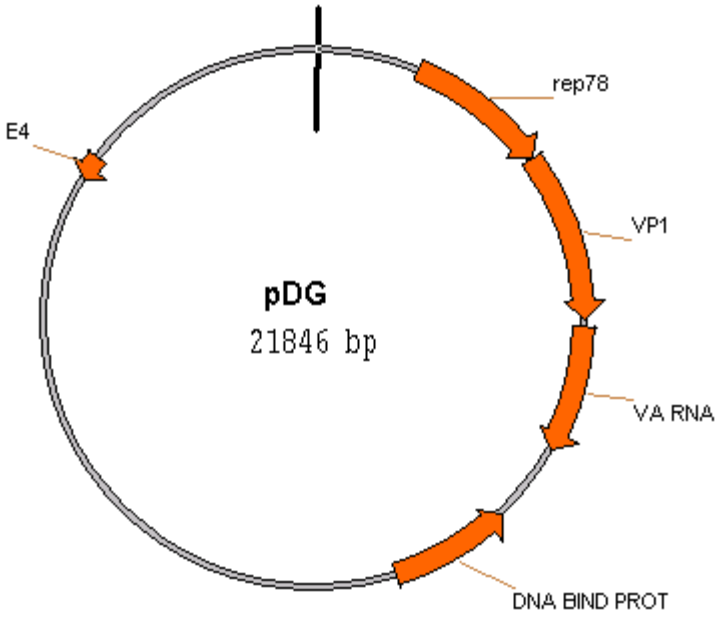
Test	Method	Specification
Antibiotic Resistance	LB Plate Culture	Ampicillin Resistant
Morphology	Phase Contrast Microscopy	E. coli Rods: Pass
Restriction Enzyme Digest with PvuII	Restriction Enzyme Pattern Analysis	According to Map
Restriction Enzyme Digest with PstI	Restriction Enzyme Pattern Analysis	According to Map
Restriction Enzyme Digest with SmaI	Restriction Enzyme Pattern Analysis	According to Map
Phage Contamination	LB Plate Culture	Negative
DNA Sequencing	Double-stranded Primer walking	According to Sequence

pTR-UF-11 plasmid DNA specification

Test	Method	Specification
Appearance	Visual inspection	Clear, no foreign particles
Sterility	USP	Sterile
Endotoxin	LAL	< 100 EU/mg
DNA Homology	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
Restriction Digests	Restriction endonuclease digestion (3 enzymes) Agarose gel electrophoresis	Matches expected restriction pattern
Concentration	Spectrophotometric	1 mg/ml

Helper plasmid.

The pDG helper plasmid reported by Grimm. et al., (Hum Gene Ther. 1998 Dec 10;9(18):2745-60) was obtained from Dr. Jurgen Kleinschmidt's laboratory in Heidelberg, Germany. This plasmid supplies the AAV and Adenoviral gene functions required for



amplification and packaging of AAV2 vectors, but does not generate infectious adenovirus. The pDG construct was transformed into E. coli strain DH5α.

pDG Plasmid Master Bank Release Specifications

Test	Method	Specification
Antibiotic Resistance	LB Agar Plate Culture	Ampicillin Resistant
Morphology	Phase Contrast Microscopy	Rod-shaped bacteria
Restriction Enzyme Digest with PvuII	Restriction Enzyme Pattern Analysis	According to Map
Restriction Enzyme Digest with PstI	Restriction Enzyme Pattern Analysis	According to Map
Restriction Enzyme Digest with SmaI	Restriction Enzyme Pattern Analysis	According to Map
Phage Contamination	LB Agar Plate Culture	Negative
DNA Sequencing	Double stranded Primer Walk	According to Sequence

pDG plasmid DNA specification

Test	Method	Specification
Appearance	Visual inspection	Clear and colorless solution
Sterility	USP	No microbial growth detected
Endotoxin	LAL	< 100 EU/mg
DNA Homology	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
Restriction Digests	Restriction endonuclease digestion (3 enzymes) Agarose gel electrophoresis	Matches expected restriction pattern
Concentration	Spectrophotometric	1 mg/ml

293 Master Cell Bank

A 293 Master Cell bank was prepared by BioReliance under cGMPs. All vials are stored at the University of Florida Human Applications Laboratory.

293 Master Cell Bank Specifications

Test	Method	Specification
Inapparent Viruses	Suckling Mice	No evidence of contamination
	Adult Mice	No evidence of contamination
	Embryonated Hen's Eggs - Yolk Sac Route	No evidence of contamination
	Embryonated Hen's Eggs - Alantoic Route	No evidence of contamination
	Guinea Pigs	No evidence of contamination
In Vitro Viruses	Indicator Cells – Cytopathic Effect	No evidence of contamination
	Indicator Cells – Hemadsorption	No evidence of contamination
	Indicator Cells – Hemagglutination	No evidence of contamination

Test	Method	Specification
In Vitro Assay for Presence of Porcine Viruses	Indicator Cells – Cytopathic Effects	Not detected
	Indicator Cells Hemadsorption	Not detected
	Indicator Cells, fixed Immunofluorescence	No fluorescence
In Vitro Assay for Presence of Bovine Viruses	Indicator Cells – Cytopathic Effects	Not detected
	Indicator Cells Hemadsorption	Not detected
	Indicator Cells, fixed Immunofluorescence	No fluorescence
CMV DNA	PCR	Negative
AAV DNA	PCR	Negative
EBV DNA	PCR	Negative
HTLV I/II DNA	PCR	Negative
HIV 1 and 2 DNA	PCR	Negative
Hepatitis B Virus DNA	PCR	Negative
Hepatits C Virus RNA	RT-PCR	Negative
Retrovirus-like Particles	Transmission Electron Microscopy	No identifiable virus-like particles
Human Parvovirus B19 DNA	PCR	Negative
Mycoplasma	Agar/broth cultivation	Negative
	Hoechst Stain	Negative
Cell Identity	Isoenzyme Analysis	Human
Sterility Test	Direct Inoculation	Satisfactory

293 working Cell bank.
A WCB of HEK 293 cells was established at University of Florida under cGMPs. All vials are stored at the University of Florida Human Applications Laboratory.

293 Working Cell Bank Specifications

Test	Method	Specification
In Vitro Viruses	Indicator Cells – Cytopathic Effect (Contract Lab)	Viral contaminants not detected
	Indicator Cells – Hemadsorption (Contract Lab)	Viral contaminants not detected
Mycoplasma	Agar/broth cultivation (Contract Lab)	Mycoplasma not detected
	Hoechst Stain (Contract Lab)	Mycoplasma not detected
Viability	Trypan Blue Stain	> 80%
Sterility Test	Direct Inoculation (Contract Lab)	Negative
Total Viable Cells/Vial	Trypan Blue Stain	$5 \times 10^6 – 2 \times 10^7$

Cell culture reagents

a. DMEM supplemented with 5% Characterized Fetal Bovine Serum and antibiotics

i. Characterized Fetal Bovine serum

ii. Antibiotics

b. Porcine trypsin

c. Dulbecco's Phosphate Buffered Saline

d. DPBS with 5mM EDTA

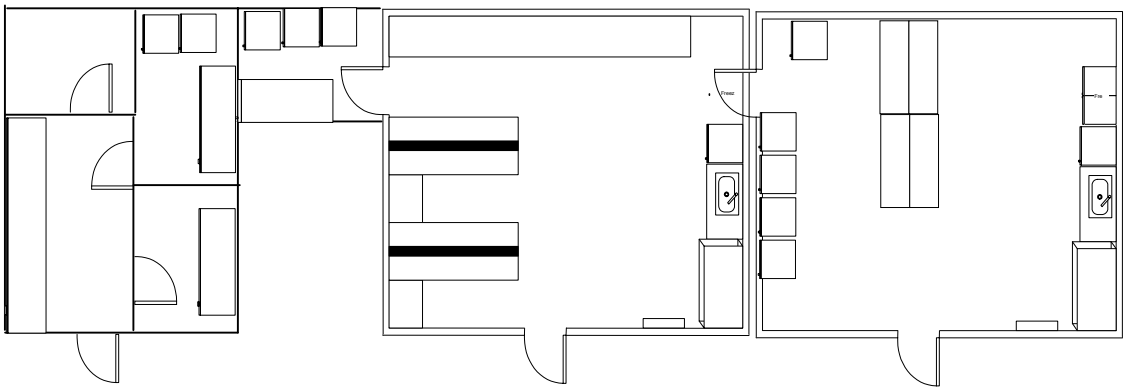
e. Sterile plasticware.

Transfection reagents

a. 2.5M CaCl2 will be prepared at the University of Florida
b. 2X Hepes Buffered Saline will be prepared at the University of Florida
c. Water for injection

Facility

The University of Florida Vector Core Laboratory facilities include two laboratories of 660 sq. ft each, and a laboratory of 330 sq. ft (floorplan shown below). In addition, the Vector Core shares an equipped darkroom, cold room, equipment room, phosphohimager, dishwashing facilities, and a microscope room with three other faculty members.



Major equipment located in the laboratory includes: Beckman centrifuges: (2) J6-HC, (2) J2-HS, (3) LE-80 ultras, LE-90 ultra with appropriate rotors; Microfluidics Microfluidizer; Biosafety cabinets: Forma 4-foot A2/B3, (2) Baker 6-foot, Baker 4-foot, (3) NuAire 4-foot, NuAire 6-foot; (2) Biometra OV-5 hybridization ovens; (2) Forma -86°C Ultra low freezers; (3) Fisher Isotemp refridgerators; (3) Sears -20°C Freezers; (16) Forma CO_2 Tissue Culture Incubators; Barnstead Water purification system; PE PCR machine; Spectrophotometer; UVP UV Transilluminator; FPLC systems: (3) Pharmacia AKTA FPLC with dedicated computers and BioRad FPLC system all with fraction collectors; Bacterial shakers: New Brunswick and Amerex; Microscopes: Nikon light microscope, Meiji light microscope, Zeiss Axiovert 25 with Fluorescence, Zeiss Axiovert 135 with Fluorescence, and Nikon Optiphot-2; (6) Eppendorf microfuges; (3) electronic balances; Conductivity meter; pH meter.

Computer: Three Dell 8100 (Pentium 4) computers with two laser printers located in offices. In the laboratory space: a Dell Dimension computer (Pentium II) with laser printer, a Gateway computer (Pentium I), a Compaq Computer (Pentium III), a flat bed scanner.

Other: The Department of Molecular Genetics and Microbiology and the Powell Gene Therapy Center provide all secretarial and fiscal services that are needed for projects. A machine shop and electronic shop are located in the Health Center and are available on a fee-for-service basis. Photographic services are also available on a fee basis. The University of Florida has core facilities (ICBR) for oligonucleotide synthesis, peptide synthesis, and monoclonal antibody generation. Protein microsequencing and electron microscopy are available on a fee-for-service basis.

Personnel

Richard O. Snyder, Ph.D. is the Director of the Vector Core and Human Applications Laboratory. Mark Potter, the Assistant Director of the Vector Core, heads a group of 13 technicians involved in producing and purifying research-grade vector. In addition, a The Human Applications Laboratory (cGMP manufacturing facility) operates in a separate building with a separate staff of 15 people. A Quality Control group (5 people) and a Quality Assurance Unit (3 people) work closely with the Vector Core and Human Applications Laboratory.

For the Vector Core, General training sessions are held on a periodic basis for personnel working in Manufacturing, QC, and QA. Individuals are presented with protocols covering a particular procedure before the training session. The individual receives hands-on training under guidance of a supervisor. When individuals can complete the task under supervision, they are allowed to complete the tasks on their own and in teams. Personnel have been trained in aseptic technique and specific procedures, and undergo yearly refresher training. Additionally, dedicated facilities maintenance operators have been trained and oversee the regular cleaning and maintenance of the facility and equipment. The Assistant Director of Quality Control coordinates environmental and personnel monitoring and testing of raw materials, in-process and product samples. The Director of Quality Assurance has independent oversight of manufacturing and oversees Quality Control. The Director of QA is responsible for release of raw materials and final product, validation support, document development and review, training, change control, auditing, document control, and conducting deviation and failure investigations.

Approximate value of the donation: $125,000 (this includes vials of the 293 working cell bank, labor, and facilities costs, and excludes all other reagent, E.coli banks, and testing costs).