Bid Submission Form
Short-term and Field Stability Studies
RFP 11.0

Contact Information – RFP 11.0

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***If laboratories are submitting a proposal as a group, a main contact should be provided along with contact information for each participating laboratory (attach additional copies of this form).**

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

- ☒ Determination of Particle Concentration
- ☒ Determination of Infectious Titer
- ☐ Long-term Stability Study
- ☐ Other Characterization
- ☐ Donation of Supplies/Other Services for Characterization Phase

General Capability Statement

Genetic Therapy, Inc. (GTI) proposes to participate in the short-term stability studies utilizing the assay protocols provided by the ARMWG in RFP 8.0 and RFP 9.0. Additionally, the following tests will be performed using GTI's protocols: Appearance, pH, Strength by anion exchange HPLC (AX-HPLC), and infectivity by Hexon-FACS. Laboratory personnel of GTI's Quality Control (QC) group will perform both the ARMWG's standard operating procedure and GTI in-house procedures. The data will be reviewed minimally by one QC group member who did not participate in the assay, and by a GTI Quality Assurance representative. GTI has been producing and analyzing adenoviral vectors for over ten years. A large body of data has been collected using each of the in-house methods proposed for the stability study.

Equipment

All instruments used are calibrated at a minimum of once every year. Equipment is maintained and used according to Standard Operating Procedures. Equipment logs and results of all assays are reviewed by a Quality Control supervisor.

Personnel

GTI's QC personnel are fully qualified to design and perform all necessary testing for the short-term field stability studies. All laboratory personnel have a minimum of a bachelor's degree in science. In addition, all laboratory personnel are trained in cGMP's, GLPs, Biosafety, and specific laboratory techniques. A training file is maintained for each employee.

Quality Assurance auditors have a minimum of a bachelor's degree in Biology (or equivalent field), a working knowledge of laboratory procedures, and are trained in auditing GLP and GMP documentation.

Experience

GTI has over two years of experience running the anion exchange HPLC method for adenovirus. Additionally, the QC staff has over ten years of experience running HPLC assays.

GTI's staff has been analyzing adenoviral vectors under GLP conditions for thee years. Personnel have familiarity with a variety of infectious titer assays, including plaque assay and $TCID_{50}$, and have qualified an infectious titer assay that is routinely used. They have also qualified an assay for particle concentrations by optical density at 260 nm.

GTI's staff has over ten years of experience analyzing Adenovirus by Flow Cytometry.

GTI In-house methods

1) Anion-exchange HPLC method for quantitation of adenoviral particles

GTI has developed a method for determining adenovirus particle concentrations by HPLC. The method employs an anion-exchange column and a gradient elution system with varying strengths of buffer. The

assay has been shown to reliably quantitate adenovirus particles in the concentration range from 3 X 10^9 to 3 X 10^{11} VP/ml .

The analysis is performed using a Hewlett Packard 1100 HPLC System with ChemStation software at 260 nm. See attachment 2 for details of the analytical procedure.

The reference standards used are purified in-house vector lots that have been quantitated by optical density at 260 nm. The standard has been shown to be stable at –65 º C for at least 24 months.

2) pH

   The pH of the sample will be recorded at each of the time points specified in the protocol following the procedure in the United States Pharmacopeia.

3) The appearance of the sample will be examined upon thawing at each of the pre-determined time points. The formation of particulates, and change of color will be observed.

4) Hexon-FACS assay

GTI has developed a HPLC method for rapidly detecting adenovirus infection utilizing a Fluorescein-directly conjugated anti-adenovirus antibody. The assay is performed by plating S8 cells at a density of 1x 10^6 per well for 24 hours. The cells are then infected with 1ml of thee-fold dilutions of the virus for a total of 12 dilutions. The infected cells are allowed to incubate at 37º C for 4 hours. 1ml of media is added and the infected cells are incubated overnight. Cells are trypsinized and trypsinization is stopped, cells are fixed, permeabilized and then stained. The cells are then analyzed by flow Cytometry.

GTI's method uses a Beckman Biomek 2000 laboratory automation workstation to perform all dilutions and a Beckton-Dickenson Facscalibur Flow Cytometer for analysis. GTI's method is capable of detecting 1 PFU/ml; the typical analytical range is $2.8 \times 10^7 – 5.0 \times 10^{12}$ particles/ml with a R^2 of 0.99. See attachment 1 for details of the analytical procedure.

Test plan

At each test point of 4h, 8h, 1 day, 3 days and 7 days the test article will be tested in duplicate using two samples for a total of two results per sample and four results per test, see table I.

For the freeze thaw studies a minimum of 7.7 ml will be required for testing. The test sample will be prepared in duplicate at each time point.

For the shipping studies, samples will be tested in duplicate at the conclusion of the study (72 h).

Stability design

X represents stability testing at each of the time points listed below; Stability after specified time at 2-8ºC after thawing samples. Stability after specified time at room temperature after thawing samples, and Stability upon storage at -20º C.

TABLE I

Test	Method	**Sample amount	Test time point				
			4h	8h	1 day	3 days	7 days
Strength	AX– HPLC	66µl	X	X	X	X	X
pH	USP	1 ml	X	_	_	_	X
Appearance	Visual Inspection	*1ml	X	_	_	_	X
Infectivity	Hexon-FACS	100µl	X	_	X	X	X
Strength	RFP 8.0	*951µl	X	X	X	X	X
Infectivity	RFP 9.0	100µl	X	X	X	X	X
Sample amount			2.2ml×2×3 = 13.3ml	1.1ml ×2×3 = 6.6ml	1.2ml×2×3 = 7.2ml	1.2ml×2×3 = 7.2ml	2.2ml×2×3 = 13.3ml

* Same sample used in two assays.
** Sample amount for duplicates.

Freeze thaw studies

A minimum of 8 ml of sample will be required for the freeze thaw studies. The samples will be frozen thawed and analyzed once a day over a five day period for appearance, pH, strength and Infectivity. pH and Hexon-FACS will be performed on samples from day one and day five only. Samples will be thawed by warming them up quickly in 37º C water bath just until all of the ice in the vial has completely melted. See table II below for freeze thaw study design. Five sets of samples will be frozen on day 0, each subsequent day each sample will be thawed, one sample will be tested and the remaining samples will be returned to the freezer.

Freeze thaw study design

TABLE II

Test	Method	Sample amount	Time				
			Day 1	Day 2	Day 3	Day 4	Day 5
Strength	AX–HPLC	66µl	X	X	X	X	X
pH	USP	1 ml	X	_	_	_	X
Appearance	Visual Inspection	*1ml	X	X	X	X	X
Infectivity	Hexon-FACS	100µl	X	_	_	_	X
Strength	RFP 8.0	*951µl	X	X	X	X	X
Infectivity	RFP 9.0	100µl	X	X	X	X	X
Sample amount			2.2ml	1.1ml	1.1ml	1.1ml	2.2ml

* Same sample used in two assays.

Shipping studies

A shipping study will be simulated by packaging two samples of Ad5 with additional vials filled with buffer in the same configuration as will be used to ship the standard. The samples will be frozen on dry ice in a Styrofoam container with an outer box. The box will be exposed to temperatures 40º C (104º F) and 50º C (122º F) for up to 72 hours. The box will be stored in a 40º C environment for two days and in a 50º C environment for one day. The product in the box will be tested after 72 hours for strength, appearance, pH and infectivity. Throughout the study the temperature within the box will be monitored utilizing a temperature monitoring device (Datalogger) with thermocouples calibrated to a NIST standard. The temperatures recorded will represent the actual temperatures to which the product is exposed.

Sample amount

The total volume of virus required for the study will be 58ml.

Reporting

At the conclusion of each of the following studies a report will be written detailing the raw data and the analytical methods and results.

a) Effects of multiple freeze thaws
b) Stability at -20ºC
c) Stability at 2-8ºC after thawing at suggested times
d) Stability at room temperature after thaw at suggested times
e) Stability under shipping conditions used by ATCC

At the conclusion of all of the studies a summary report will be prepared and copies of the primary data will be attached for reference.

Timing

GTI is ready to accept Reference Material samples for testing anytime they are available in the third or fourth quarter of 2001. The total time expected to perform the assay and to review and report the results is 6-7 weeks.

Attachment 1

TITLE: Adenovirus Titer Determination by Hexon Flow Cytometric Assay using directly labeled FITC antibody and multi-well sampler

REFERENCES

Linda Weaver and Michael Kadan, "Evaluation of Adenoviral Vectors by Flow Cytometry", *Methods* **21**: 297-312 (2000), Academic Press

1.0 MATERIALS AND EQUIPMENT

1.1 Equipment

1.1.1 6-well plates (Falcon 3046 or equivalent)

1.1.2 96-well cell processing V-bottom assay blocks: Corning Costar No. 3960 or equivalent.

1.1.3 Sealing film for 96-well assay blocks (Fisher Scientific Catalog # 07-200-375 or equivalent)

1.1.4 12-channel pipettor

1.1.5 Becton-Dickenson™ Facscalibur Flow Cytometer or equivalent

1.1.6 Cytek Automated Multisampler

1.1.7 Beckman GS-KR Centrifuge or equivalent, with 96-well adaptors

1.1.8 37^{o}C water bath

1.1.9 37^{o}C CO_2 incubator

1.2 Cells and tissue culture reagents

1.2.1 S8 cells, GTI working cell bank

1.2.2 10% Richter's, stored at 2-8 ^{o}C

1.2.3 5% Richter's, stored at 2-8 ^{o}C

1.2.4 Versene (EDTA) solution, 0.2 mg/ml (BioWhittaker # 12-711A or equivalent), stored at 2-8 ^{o}C

1.2.5 Trypsin-Versene Solution, 0.5 g/L Trypsin and 0.2 g/L Versene, sterile (BioWhittaker # 17-161E or equivalent), store unopened at –18 to -20 ^{o}C. store at 2-8 ^{o}C after thawing

1.3 Immunostaining Reagents

1.3.1 Buffered formaldehyde (Polyscientific Catalog # S2334) or equivalent. Dilute all formaldehyde preparations in Dulbecco's PBS immediately prior to use and discard excess after use.

1.3.2 BD Facsflow Buffer (Catalog # 342003), sheath buffer for flow cytometers and for sample preparation. No substitutions accepted.

1.3.3 Dulbecco's PBS (DPBS)

1.3.4 Tween-20 (Polyoxyethylene Sorbitan Monolaurate, Sigma, catalog # P-7949)

1.3.5 Permeabilization Buffer: Dulbecco's PBS + 0.2% Tween-20. Prepare sufficient buffer for the number of samples being stained.

1.3.6 Antibody wash buffer: Dulbecco's PBS + 2% FBS, chilled to 4^{o}C.

1.3.7 Chemicon Fluorescein-directly conjugated anti-adenovirus antibody, catalog # MAB-8052 F-K.

2.0 PROCEDURE

2.1 Adenovirus Infection of S8 Cells

2.1.1 Seed S8 cells in 6-well plates using a cell concentration of 2.5 x 10^5 cells/ml. Deliver 2 ml of cell suspension, or 5 x 10^5 cells per well.

2.1.2 Prepare virus 24 hours after seeding cells for infection by performing thee-fold serial dilutions to generate nine concentration levels.

2.1.3 Count viable cells from 3 representative wells and record the data.

2.1.4 Infect cells with virus:

2.1.4.1 Gently aspirate media, one 6-well plate at a time, to prevent cells from drying out.

2.1.4.2 Deliver 1 ml of 5% Richter's, or diluted virus to each well, beginning with the most dilute virus and ending with the most concentrated virus.

2.1.4.3 After infecting all plates, incubate cells for 4 hours on a rocker platform in a 37^{o}C, 5% CO_2 humidified incubator.

2.1.4.4 Aspirate infection media (mock infected, then low to high virus concentration) and replace with 2 ml of 10%Richter's. Return cells to incubator.

2.1.4.5 Incubate cells in a 37^{o}C, 5% CO_2 humidified incubator for a total of 24 hours from the start of virus infection.

2.1.5 Harvesting Infected S8 Cells (Performed 24 hours post-infection):

2.1.5.1 Aliquot 1 ml of cold 10% Richter's to each well of an Assay Block Place the block on ice, awaiting trypsinized cells.

2.1.5.2 Wash cells with 2 ml/well Dulbecco's PBS. Add 0.5 ml Versene and incubate plate on a rocker platform in a 37^{o}C, 5% CO_2 humidified incubator. An entire run can be treated in this manner, awaiting the trypsin-versene step.

2.1.5.3 Add 0.5 ml Trypsin-Versene solution to each of 6 wells. Pipet cells about five times to suspend. Transfer the cells to the appropriate wells in the assay block. Pipetting and transferring should be performed one well at a time. Keep a record of the location of each sample, using the 96-well grid provided.

2.1.5.4 Centrifuge cells using the appropriate adaptors, at approximately 1000 RPM for 5-10 minutes.

2.1.5.5 Decant medium and re-suspend the cell pellets in DPBS with 1% formaldehyde and store for 15-24 hours at 2-5^{o}C before staining.

2.1.6 Staining Fixed Cells

2.1.6.1 Centrifuge fixed cell suspensions at 1000 RPM for 5-10 minutes.

2.1.6.2 Decant fixative into an appropriate waste container.

2.1.6.3 Tap tubes or assay blocks to dislodge pellets and add 2 ml of DPBS per tube or well.

2.1.6.4 Centrifuge cell suspensions as in Step 5.3.2

2.1.6.5 Decant supernatants and re-suspend cell pellets in 0.5 ml Permeabilization buffer using either a multichannel pipet or repeat pipettor.

2.1.6.6 Incubate cell suspensions in a 37^{o}C water bath for 15-20 minutes.

2.1.6.7 Immediately place samples on wet ice. Dispense 1.5 ml Wash Buffer per sample

2.1.6.8 Centrifuge cells as in step 5.3.2.

2.1.6.9 Decant supernatants. Place samples on wet ice.

Suspend pellets in 100µl of Fluorescein-labeled primary antibody, diluted 1:200.

2.1.6.10 Incubate on wet ice or in the refrigerator, from 30-45 minutes. Overnight incubation can also be performed, with flow Cytometric analysis done on the following day.

2.1.6.11 Dilute all samples with 0.6 ml BD Facsflow buffer. It is not necessary to wash samples.

2.1.6.12 Transfer 0.2 ml cell suspensions to a flat-bottomed 96-well plate.

2.1.6.13 If multiwell sampler is not available, samples can be transferred to polystyrene tubes and analyzed one at a time.

2.1.6.14 Analyze for FITC fluorescence on flow Cytometer. Use Mock Infected cells as negative controls.

Attachment 2

TITLE: Anion Exchange HPLC Method for Quantitation of Adnoviral Vectors

REFERENCES

Paul Shabram, *et al*., "Analytical anion-exchange HPLC of recombinant type-5 adenoviral particles", *Human Gene Therapy* **8**: 453-465 (1997).

1.0 MATERIALS AND EQUIPMENT

Note: Equivalent reagents and supplies may be used.

1.1. Equipment
- 1.1.1. Hewlett Packard 1100 HPLC System, ChemStation Rev A06.03 software
- 1.1.2. Pipetman
- 1.1.3. Biological safety carbinet (NUARE, NU-425-400)
- 1.1.4. Ultra low freezer (Revco, -80°C freezer)
- 1.1.5. Refrigerator
- 1.1.6. Analytical balance (Mettler Toledo, PB 1502)
- 1.1.7. Stir plate (Barnstead/Thermolyne, model S 46725)
- 1.1.8. Vortexer (VWR)
- 1.1.9. pH meter (Beckman, Φ40 pH meter)

1.2. Materials
- 1.2.1. Adenoviral Vector Standards: OV1 (3.3x1012 vp/ml, GTI lot: TCA cc061113, prepared by Vector Process Development in GTI), stored at -80°C, expiration date Jan-11-03.
- 1.2.2. 5X THP Dialysis buffer, BioWhittaker Cat. # 04-881Q
- 1.2.3. NaCl, Fisher Cat. # S271-1
- 1.2.4. NaOH, Fisher Cat. # SS255-1
- 1.2.5. HPLC grade H_2O, Fisher Cat. # 010856
- 1.2.6. HEPES (base form), Sigma Cat. # H-3784
- 1.2.7. HEPES (acid form), Sigma Cat. # H-4034
- 1.2.8. HCL, LabChem Cat. # 0188-24
- 1.2.9. Ethanol (200 Proof), Spectrum Cat. # 64-17-5
- 1.2.10. 1 ml Pharmacia Biotech Resource Q anion exchange HPLC column (6.4mm x 30mm, part number: 17-1177-01)
- 1.2.11. Disposable filter unit with pore sizes 0.2µm (NALGENE Cat. 0001660020)
- 1.2.12. 1.5 ml microcentrifuge tube (Eppendorf, Cat. 2236320-4)
- 1.2.13. HPLC amber injection vial with glass insert and snap cap (Agilent)
- 1.2.14. Aerosol Resistant pipette tips (20, 100, 200, and 1000µL), Molecular BioProducts
- 1.2.15. Biohazard sharp container (B-D Guardian)

1.3. Prepared Reagents
- 1.3.1. HPLC buffer A (300 mM NaCl, 50 mM HEPES, pH 7.50 ± 0.01): Weigh 6.196g of HEPES (acid form), 6.247g of HEPES (base form), and 17.532g NaCl dissolve into approximate 800 ml of HPLC grade H_2O, adjust pH to 7.50 ± 0.01 with 1N NaOH or 1N HCl as needed. Dilute this buffer to exactly 1000 ml in a class A volumetric flask and mix well. Filter with 0.2µm filter and store at 4°C until use. Expiration date is 6 days later at room temperature due to microorganism growth.

1.3.2. HPLC buffer B (600 mM NaCl, 50 mM HEPES, pH 7.50 ± 0.01): Weigh out 6.196g of HEPES (acid form), 6.247g of HEPES (base form), and 35.064g NaCl dissolve into approximate 800 ml of HPLC grade H_2O, adjust pH to 7.50 ± 0.01 with 1N NaOH or 1N HCl as needed. Dilute this buffer to exactly 1000 ml in a class A volumetric flask and mix well. Filter with 0.2µm filter and store at 4°C until use. Expiration date is 6 days later at room temperature due to microorganism growth.

1.3.3. HPLC buffer C (1.5M NaCl, 50 mM HEPES, pH 7.50 ± 0.01): Weigh out 6.196g of HEPES (acid form), 6.247g of HEPES (base form), and 87.66g NaCl dissolve into approximate 800 ml of HPLC grade H_2O, adjust pH to 7.50 ± 0.01 with 1N NaOH or 1N HCl as needed. Dilute this buffer to exactly 1000 ml in a class A volumetric flask and mix well. Filter with 0.2µm filter and store at 4°C until use. Expiration date is 14 days later at room temperature due to microorganism growth.

1.3.4. 1X THP buffer: Dilute 10ml of 5X THP to 50ml with HPLC grade H_2O, a final composition of 200 mM Tris, pH 8.0, 50 mM HEPES. Store at 4°C, and expire in 2 months.

1.3.5. 1N HCl: Dilute 20ml of 5N HCl to 100ml with HPLC grade H_2O, store at room temperature. Expiration date is 2 months later.

1.3.6. 1N NaOH: Dilute 10ml of 10N NaOH to 100ml with HPLC grade H_2O, store at room temperature. Expiration date is 2 months later.

2.0 PROCEDURE

2.1 Virus standard samples in buffer: Adenoviral vector standard (OV1, 3.3×10^{12} vp/ml) is diluted with 1X THP to make the following set of standards.

Standard #	Stock Origin	Stock Volume (µL)	1X THP Buffer Volume (µL)	Particles/ml
1	$3.3x10^{12}$	20	200	3×10^{11}
2	Standard #1	75	150	1×10^{11}
3	Standard #2	75	175	3×10^{10}
4	Standard #3	100	200	1×10^{10}
5	Standard #4	100	233	3×10^{9}

The standard samples can be used freshly or stored at –80°C freezer, and thawed before daily use. 150µL of standard samples are transferred to HPLC injection vial for HPLC analysis.

2.2 Test sample preparation

Sample in similar buffer as 1X THP: no sample preparation is needed, sample can be injected directly.

Sample's concentration higher than upper limit quantitation: dilute with buffer in which the sample was originally dissolved, and lower the concentration to the quantitation range.

Sample in high salt buffer: sample needs to be diluted with 1X THP to reduce salt concentration before HPLC analysis. Subsequent dilutions may be made if necessary.

2.3 HPLC conditions

2.3.1 Gradient schedule for HP Quaternary Pump

	Time (min)	%A	%B	%C	%D	Flow	Pressure
1	0.00	100	0	0	0	1.00	80
2	2.00	100	0	0	0	1.00	80
3	2.01	100	0	0	0	1.00	80
4	12.00	0	100	0	0	1.00	80
5	15.00	0	100	0	0	1.00	80
6	15.01	0	0	100	0	1.00	80
7	20.00	0	0	100	0	1.00	80
8	20.01	100	0	0	0	1.00	80
9	30.00	100	0	0	0	1.00	80

2.3.2 Autosampler parameter

- Injection volume: 100µL
- Store temperature: 4°C
- Syringe size: 100 µL
- Sample loop: 100µL

2.3.3 Multiple wavelength detector parameters:

- Signal quantitation: 260nm with BW 4nm
- Reference wavelength: 450nm with BW 4nm
- Attenuation output: 1000mAU

2.3.4 Integration Event:

Time	Type	Value
Initial	Slope sensitivity	5
Initial	Peak width	0.04
Initial	Area reject	1.00
Initial	Height reject	0.5
Initial	Shoulders	Tan

2.3.5 Column cleaning program:

The column should be cleaned when the pressure for the whole system is higher than 40 bar. The cleaning procedure is: Inject 100 µL 1N NaOH followed by the gradient described below:

Time (min)	% 1N NaOH	% 1N HCl	% H2O	% Buffer A	Flow (ml/min)	Pressure
0.0	100	0.0	0.0	0.0	1	80
5.0	100	0.0	0.0	0.0	1	80
5.01	0.0	100	0.0	0.0	1	80
10.0	0.0	100	0.0	0.0	1	80
10.1	0.0	0.0	100	0.0	1	80
20.0	0.0	0.0	100	0.0	1	80
20.1	0.0	0.0	0.0	100	1	80
60.0	0.0	0.0	0.0	100	1	80

2.3.6 Column storage:

When the column is not used for a short periods(24 hrs), turn pump flow rate down to 0.1 or 0.2 ml/min all the time. When column is not be used for long period(>24hrs), pump 20% ethanol at flow rate 1.0 ml/min for 30 min and stop the pump. Store the column at room temperature.

2.3.7 Calculations:

Adenoviral vectors elute at a retention time of approximately 10 ± 0.2 min. A typical chomatogram of a purified OV1 sample is shown in Figure 1.

Peak areas are used to calculate final viral concentration using linear standard curve generated by standard samples prepared as in 2.1 in material and method section.

Linear standard curve (equation show below) can be obtained by performing regression analysis on the standards using Microsoft Excel or ChemStation or equivalent software. The acceptable linear curve should have R^2 value ≥0.98

$Y = AX + B$

Where Y = peak area, X = viral concentration, A is slope and B is y-intercept.

EXAMPLE FIGURES

Figure 1. Typical chomatogram for OV1 in 1X THP ($3x10^{11}$ vp/ml) injection

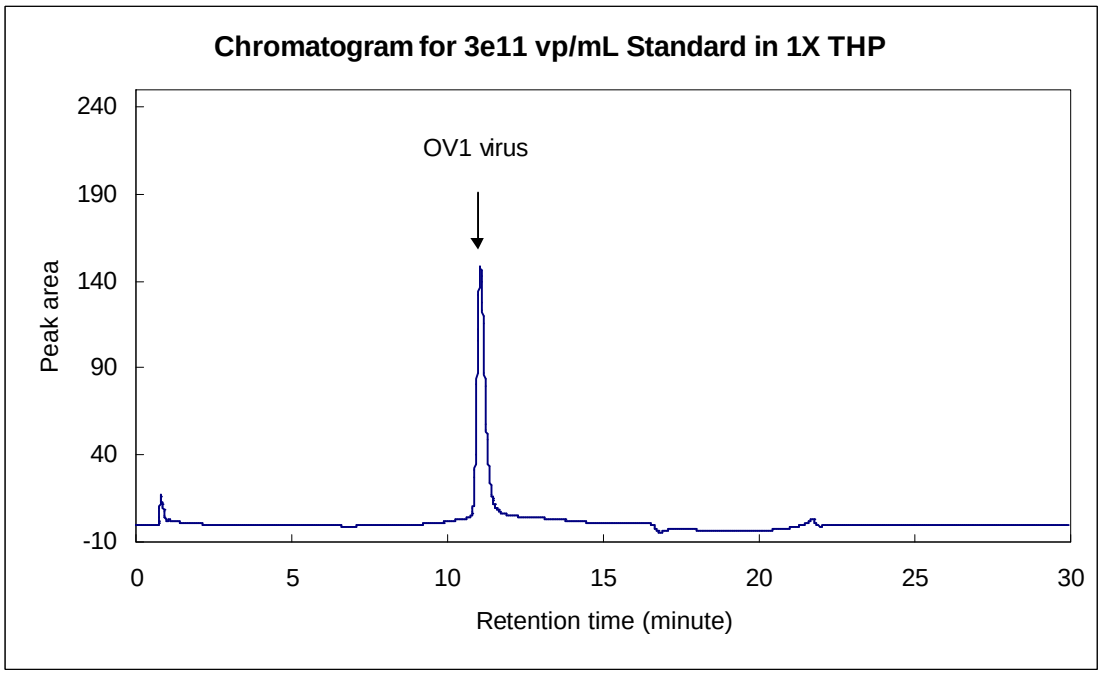

Figure description: Anion Exchange HPLC chomatogram of purified Adenoviral Vector Standards (GTI lot: TCA cc061113) in 1X THP. The chomatogram was obtained in the analysis of 100µL of solution containing $3x10^{11}$ vp/ml.

Figure 2. Typical Standard curve for OV1 in 1X THP