Determination of Adenovirus pVIII (31K) Concentration for Estimation of the Empty Capsid Concentration of the Adenovirus Reference Material (ARM)

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Summary: RP-HPLC of the ARM detected no adenovirus pVIII protein**.** This indicates that the empty capsid concentration of the ARM based on this assay is less than ~1 x 10^9 empty capsid particles /ml.

Procedure: Samples of the ARM were evaluated for presence of empty capsids using the RP-HPLC research procedure "Determination of Adenovirus pVIII (31K) Concentration for Estimation of the Empty Capsid Concentration of the Adenovirus Preparations" attached below (Attachment 1). More specifically the ARM was run with two different injection volumes in duplicate, and compared to a purified empty capsid standard also run with two different injection volumes in duplicate. Samples of rAd internal standards, with and without empty capsids were also run. Two blank injections were run between each pair of samples. All samples had added to them the nonionic detergent octyl-B-d-glucopyranoside (10%; Sigma) to make a final concentration of 0.5%, to avoid losses to surfaces during handling.

Results: The results from the four injections of the purified empty capsid standard (1.0 x 10^{11} particles / ml) are shown in Table 1. The pVIII peak area was divided by the injection volume to give the pVIII peak area /ml. The average value of these four injections was used to estimate the empty capsid concentrations of the other samples. The ARM had no detectable pVIII peak area even with the 20 ul injection. This indicates that the ARM has less than ~1 x 10^9 empty capsid particles /ml. Our two rAd internal standards, with and without empty capsids, both gave their expected values.

Table 1. Results of empty capsid estimation by RP-HPLC evaluation of pVIII.

Sample*	injection volume (ul)	pVIII peak area (10E6)	pVIII peak area / ml (10E6)	Empty capsid conc. (particle/ ml 10E11)
empty capsid standard	5	0.112	22.3	
empty capsid standard	20	0.565	28.3	
empty capsid standard	5	0.117	23.5	
empty capsid standard	20	0.525	26.3	
			25.1	
ARM	5	Not detected		
ARM	20	Not detected		
ARM	5	Not detected		
ARM	20	Not detected		
rAd internal std. (CCGV)	20	Not detected		
rAd internal std. (CCGV)	20	Not detected		
rAd internal std. (1008)	20	0.295	14.8	0.59
rAd internal std. (1008)	20	0.267	13.3	0.53

* Empty capsid standard was 1 x 10^{11} particles/ ml as determined by $A_{260scattering}$ and confirmed by hexon determination on RP-HPLC. Both rAd standards were at 5 x 10^{11} particles/ ml determined by $A_{260\ SDS}$. The rAd internal standard (1008) was purified by the two-column procedure, and the purified empty capsid and highly-purified rAd internal standard (CCGV) were prepared from the column-purified rAd, by both CsCl and glycerol density gradient centrifugations as described in Vellekamp et el. (2001) "Empty capsids in column-purified recombinant adenovirus preparations" Human Gene Therapy 12:1923-1936.

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Performed Feb. 12, 2002

Attachment 1.

Research Procedure

Title: Determination of Adenovirus pVIII (31K) Concentration for Estimation of the Empty Capsid Concentration of the Adenovirus Preparations

Purpose:

This reverse phase HPLC assay indirectly determines the amount of empty capsid present in an adenovirus sample by measuring the pVIII (31K) protein present in the sample.

Theory of Operation:

In this RP-HPLC assay, the adenovirus sample is disrupted into its protein components giving a characteristic protein elution profile. The identities of all the major peaks have been determined. The pVIII (31K) protein has been found to be present in empty capsids at a fixed concentration. The concentration of pVIII in the test adenovirus sample is compared to the pVIII concentration of either a purified empty capsid reference standard or to a recombinant adenovirus preparation reference standard containing empty capsids that has had its empty capsid concentration previously determined by comparison to a purified empty capsid reference standard. The result is reported in particles/ml of empty capsid.

Reference:

This procedure is based on a modification "Reverse Phase HPLC Assay for the Analysis of SCH 58500 Development and Validation Report" (Mei Lin, Bioanalytical/ Biotechnology Development, SPRI; Oct. 1999) of the RP-HPLC procedure reported in Vellekamp et el. (2001) "Empty capsids in column-purified recombinant adenovirus preparations" Human Gene Therapy 12:1923-1936.

Equipment and Reagents:

HPLC System: Waters system with W2690 pump, 996 PDA or 486 UV detector with wavelength set at 214 nm, W2690 autosampler with the temperature set at 10°C.

Column: Jupiter, 2 x 150 mm, 5µ, 300 A, C4 column. Cat. no. 00F-4167-B0. The column is heated with Waters Column Heater set at 50°C.

Materials: Acetonitrile, Optima grade, purchased from Fisher, Cat. No. A996-4.

Trifluoroacetic acid, HPLC/Spectro Grade, purchased from Pierce, Cat. No. 28901

Recombinant Adenovirus Standard (~1 x 10^{12} particles /ml) containing empty capsids, or purified adenovirus empty capsid standard.

47 mm glass vacuum filtration system purchased from Rainin Cat. No. 419380 or equivalent.

47 mm diameter (0.45 µm pore size) Nylon filtration membrane purchased Fisher (P/N N04SP04700) or equivalent.

Solutions: Mobile phase A: 0.1% (v/v) trifluoroacetic acid aqueous solution.

1. Add 4.0 ml of trifluoroacetic acid to a 4-liter graduated cylinder containing 4 L deionized water and mix well.
2. Vacuum filter through a 0.45 μm Nylon filter.

Mobile Phase B: Acetonitrile/Aqueous (90:10, v/v) 0.1% Trifluoroacetic acid.

1. Add 4.0 ml trifluoroacetic acid, 400 ml deionized water to a 4L graduated cylinder containing 3600 ml acetonitrile.
2. Vacuum filter through a 0.45 μm hydrophobic filter (or equivalent).

Recombinant Adenovirus Standard Solution:
150 μl of the standard solution is transferred to HPLC small volume vial with 300 μl inserts, stoppered and stored at -80°C

Gradient Conditions

Step#	Time (min)	Flow (ml/min)	% A	% B	Curve
1	0.00	0.2	75	25	0
2	10	0.2	60	40	6
3	25	0.2	56	44	6
4	40	0.2	40	60	6
5	41	0.2	0	100	6
6	43	0.2	0	100	6
7	43.5	0.2	75	25	6
8	50	0.2	75	25	6
9	65	0.01	75	25	11

Safety Precautions:

Handle all adenovirus samples with caution. Work in a bio-safety hood. Dispose all the used vials and pipette tips into a bio-safety container. Wear lab coat, gloves and safety glasses.

Blank and Standard sample analysis:

1. Withdraw a fresh vial of the standard from the -80°C freezer.
2. Thaw the standard completely at room temperature, vortex gently to ensure sample homogeneity prior to use.
3. Transfer an appropriate amount of sample into a HPLC sample vial (sample volume must be at least 50 μl).
4. Inject 10 μl standard onto the HPLC column and the run time is 55 minute gradient.
5. Run 2-3 blanks at the beginning of sample set
6. Run the standard in triplicate at the beginning of each sample set for system suitability. The % relative standard deviations for retention time and peak area should be less than 5%.
7. Run a blank and standard every 10^{th} sample. The % relative standard deviations for retention time and peak area for the entire sample set should be less than 10%.

System Suitability:

1. Use Waters HPLC system suitability software or calculate manually.
2. The % relative standard deviation for retention time and area of the VII and 31K peak for standard should be less than 5% for the system suitability and less than 10% with entire sample set.

Column Installation and Documentation:

1. Columns are monitored with system suitability and the resolution between 31K and 7K peak. Replace the column when the resolution is lesser than 1. Under normal sample conditions, columns are replaced after about approximately 500 injections.
2. When a new column is installed, condition the column with mobile phase B for at least one half hour. Make ten injections of standard to condition the column.
3. Record the HPLC column serial number and the date the column is installed in system log book. Record the total injection number in the log book when the column is replaced.

Calculations:

1. Calculate the concentration of empty capsid in the unknown sample from the area of pVIII (31K) protein as follows:

$$\text{Concentration of empty capsid}_{\text{unknown}} = \frac{\text{pVIII peak area/ml}_{(\text{unknown})} \times \text{concentration of empty capsid}_{(\text{standard})}}{\text{pVIII peak area/ml}_{(\text{standard})}}$$