Determination of the Free Hexon Concentration
of the Adenovirus Reference Material (ARM)

Sundari Ravindran, Gary Vellekamp, (SPRI)

Summary: Using the immunoaffinity /gel filtration assay, the free hexon concentration of the ARM was determined to be 1.16 µg/ml. If the virus concentration of the ARM is assumed to be 5.9 x 10^{11} particles/ml (from the A_{260}SDS assay at SPRI), this gives a relative concentration of 2.0 µg free hexon / 10^{12} particles, or 1.5% of the total hexons are free hexons.

Procedure: The determination of the free hexon concentration of the ARM was performed according to the general research procedure for the immunoaffinity/ gel filtration free hexon determination (Attachment 1).
More specifically, free hexon standards were run before and after the test samples. ARM samples were run at 5X and 10X dilutions in duplicate. rAd internal standards (5 x 10^{11} particles/ml) were also run in duplicate. Blank runs were interspersed between the free hexon standards, the different dilutions of the ARM, and the rAd internal standards, to confirm that there was no run-to-run carry over. 20 µl of fluorescent-labeled antibody was used per 100 µl reaction.

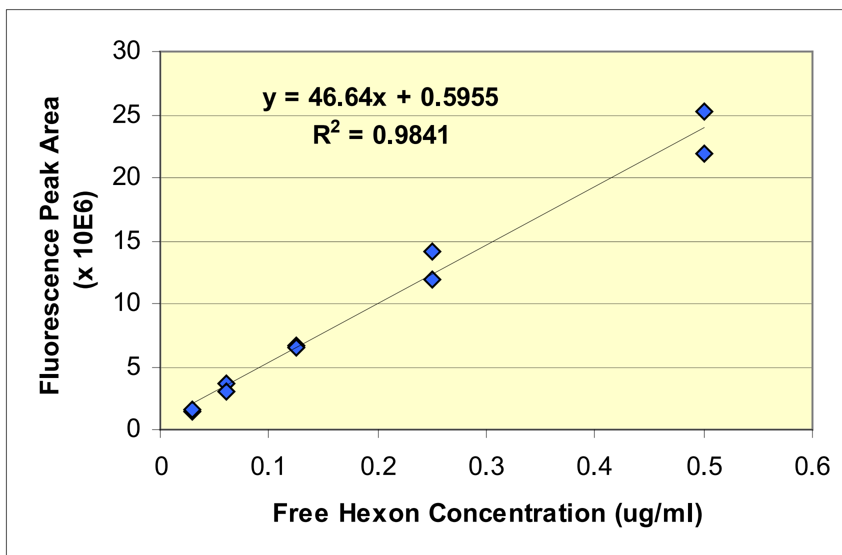
Results: Table 1 below records the results and calculations to determine the free hexon concentration of the ARM. The samples are listed in the order of analysis.

Table 1. Immunoaffinity/ gel filtration determination of the free hexon concentration for ARM: fluorescent peak areas and calculations.

Sample	Free hexon standard in the vial (ug/ml)	Dilution Factor	Free Hexon Fluorescence Peak Area (10E6)	Free Hexon Fluorescence Peak Area minus blank (10E6)	Free Hexon in the vial from std. curve (ug/ml)	Free Hexon in the sample (ug/ml)
Blank	0		0.72			
free hexon standard	0.030		2.21	1.49		
free hexon standard	0.060		4.39	3.67		
free hexon standard	0.125		7.33	6.61		
free hexon standard	0.250		14.90	14.18		
free hexon standard	0.500		25.89	25.17		
blank			0.54			
rAd internal std. (1008)		5.0	3.91	3.19	0.056	0.28
rAd internal std. (CCGV)		12.5	8.93	8.21	0.163	2.04
blank			0.60			
ARM		10.0	6.82	6.10	0.118	**1.18**
ARM		10.0	7.49	6.77	0.132	**1.32**
blank			0.61			
ARM		5.0	11.40	10.68	0.216	**1.08**
ARM		5.0	11.01	10.29	0.208	**1.04**
blank			0.64			
rAd internal std. (1008)		5.0	4.46	3.74	0.067	0.34
rAd internal std. (CCGV)		12.5	8.57	7.85	0.156	1.94
blank	0		0.66			
free hexon standard	0.03		2.29	1.57		
free hexon standard	0.06		3.69	2.97		
free hexon standard	0.125		7.26	6.54		
free hexon standard	0.25		12.61	11.89		
free hexon standard	0.5		22.60	21.88		

The fluorescence peak areas for the free hexon standards were used to generate the standard curve shown in Fig. 1.

Figure 1. Standard Curve: free hexon fluorescence peak area versus free hexon concentration



The linear regression equation was used to calculate the free hexon concentration of the virus samples in the reaction vial, using the fluorescence peak area. (The fluorescence peak area of the free hexon-antibody complex for the ARM showed a small amount of early-eluting fluorescence as is routinely observed for recombinant Ad samples; this was included for the determination.) This was multiplied by the dilution factor to give the free hexon concentration of the sample. The values determined for the rAd internal standards were consistent with previous analyses.

The values for the ARM samples were averaged to give a value of 1.16 µg/ml of free hexon (std. dev. = 0.13).

If the virus concentration of the ARM is assumed to be 5.9 x 10^{11} particle/ml (from the A_{260}SDS determination at SPRI), this gives a relative concentration of 2.0 µg free hexon / 10^{12} particles.

Using values of 720 hexon monomers per adenovirus particle and 108,000 molecular weight per monomer gives 129 µg of hexon per 10^{12} particles. Therefore the ARM is determined to have 1.5 % of the total hexon as free hexon (2.0 µg free hexon / 10^{12} particles / (129 µg of hexon per 10^{12} particles + 2.0 µg free hexon / 10^{12} particles).

S. Ravindran
G. Vellekamp, Ph.D.
Process Research / Biotechnology Development
Schering Plough Research Institute

Performed Feb. 12, 2002

Attachment 1.

Research Procedure **Jan. 21, 2002**

Title: Immunoaffinity/gel filtration assay for determination of free hexon concentration in the absence or presence of adenovirus.

Purpose:

This immunoaffinity/gel filtration assay, performed with an HPLC system with fluorescence detection, determines the concentration (μg/ml) of free hexons in absence or presence of intact adenovirus. If the particle concentration of the virus is known, the free hexon amount can be reported as μg of free hexon/10^{12} viral particles.

Theory of Operation:

The free hexons in the virus sample, bound to a fluorescein-conjugated monoclonal antibody (Chemicon, catalog #: 5016C-K) specific to the adenovirus hexon trimer is injected onto a size exclusion Superdex 200 HR column. The free hexon-antibody complex elutes near the void volume, well separated from the free antibody. Detection is with an in-line fluorescence detector with the excitation and emission wavelengths were 490nm and 520nm, respectively. The free hexon concentration is calculated from a standard curve of varying concentrations of purified free hexon versus the fluorescent peak area of the free hexon-antibody complex.

Equipment:

HPLC System: The Waters system includes a WISP autosampler with thermal control and an in-line fluorescence detector and absorbance detector. Data acquisition and integration were performed by Millennium software.

Column: Superdex 200 HR 10/30 (Amersham Biotech cat. # 17-1088-01)

1000 ml, 100 ml and 10 ml graduated cylinders

10-, 20-, 100- μl Pipetman or equivalent

Microsoft Excel software

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.

Materials:

Sodium Phosphate Monobasic Dihydrate (Fisher Cat. # S381-500)

Sodium Chloride (Fisher Cat. # S271-3)

Tris Hydroxymethyl Aminomethane (Fisher Cat. # T395-500)

Triton X-100 (Sigma Cat. # T-9284)

Magnesium Chloride (Fisher Cat. # M33-500)

Sodium Hydroxide (50%w/w Fisher Cat. # SS 254-1)

Glycerol (Fisher Cat. # G33-1)

Sucrose (EM Industries Cat. #0076535 B)

Urea (Fisher Cat. # BP 169-10)

Water (Milli-Q purified water)

Purified free hexon (Hexons were purified from a rAd–infected 293 cell lysate clarified by tangential flow filtration through a 0.65 µm filter (Millipore, Bedford,MA). The benzonase-treated concentrated lysate was adjusted to 280 mM NaCl and passed through a DEAE-Fractogel column to remove virus and DNA. This flow through was then diluted to 150 mM NaCl, reapplied to the DEAE-Fractogel, and eluted with a 150-600 mM NaCl gradient. The major A_{280} peak which contains the hexon was pooled and chromatographed on gel filtration (Superdex 200 pg, Amersham Biosciences, Piscataway, NJ,) with 14 mM Tris, 11 mM sodium phosphate, pH 8.1-8.2 at 4°C, 2 mM $MgCl_2$, 2% sucrose, and 10% (w/v) glycerol. The concentration of the gel filtration pool was 2.3 mg/ml as determined by Bio-Rad DC protein assay and the A_{280} was 3.2. Aliquots were stored at –80°C. Dilute solutions of the purified hexon included 0.1% Triton X-100 to avoid losses to surfaces.)

Antibody (Chemicon, catalog #: 5016C-K)

Autosampler vials, inserts and caps (Analytical Sales and Services cat. #s 31531, 30502 and 53127 respectively.

Genie vortex

0.22 µm Millipore Stericup filters (Cat. # SCGPU05RE)

Procedure:

Record and label all buffers.

1. Preparation of buffers:

1.1 Gel filtration buffer: (For 1000 ml)

Fill a graduated cylinder about 900 ml with Milli-Q water. Weigh and add 3.12 grams sodium phosphate monohydrate, 5.84 grams sodium chloride, 0.41 gram magnesium chloride. Add 1 ml Triton X-100. Mix. Calibrate a pH meter with two standard solutions: pH 7 and pH 10. Measure the pH of the solution. Add concentrated sodium hydroxide solution until the pH meter reads 8.0. Add Milli-Q water to a volume of 1000ml. Mix. Read the pH again and make necessary adjustment with sodium hydroxide or hydrochloric acid to give pH 8.0 +/- 0.5. Filter the buffer.

1.2 Reaction buffer: (for 100 ml)

Weigh 10 gram of glycerol into a graduated cylinder; fill with Milli-Q water up to 80 ml. While stirring, add 0.17 gram of sodium phosphate, 0.1695 gram of Tris, 2 grams of sucrose. After those components are in solution add 0.04 gram of magnesium chloride and adjust to 100 ml with Milli-Q water. Measure the pH. It should be 7.8 +/-0.5 at room temperature. Filter and store the buffer.

1.3 Triton X-100 solution (2%): (for 10 ml)

Add 200 µl of Triton X-100 to 10 ml of Milli-Q water. Mix well. Filter the solution.

2. Preparation of samples:

Add to the HPLC vial the following solutions in the order: reaction buffer, Triton X-100 solution (5µl), fluorescent-labeled antibody (15-20 µl), and the hexon or virus sample to be tested. Determine the volumes of the reaction buffer and the sample so that the total volume in the HPLC vial is 100 µl. Mix the reaction mixture by trituration with a pipetman. Cap and briefly vortex the vial.

3. HPLC setup:

Place the HPLC vials were in the WISP autosampler that was pre-chilled at 4°C. Pump the gel filtration buffer through the column, the fluorescence detector and the UV detector at a flow rate of 0.5 ml/min. The backpressure generated with the column in-line should be approximately 180 psi. The excitation and emission wavelengths for the fluorescence detector are set at 490 nm and 520 nm, respectively. Autozero the fluorescence detector manually prior to the first injection. The injection volume is constant at 50 µl and the run time for each assay is 65 minutes.

4. Assay setup:

Two blank controls are run with no free hexon or virus sample in the reaction mixtures. Then a set of standards are run with purified free hexon ranging from 30 ng/ml to 500 ng/ml in the HPLC vial followed by the test samples. Test samples are generally run in duplicate or triplicate. A second set of free hexon standards should be run if total assay time exceeds approximately 30 hours.

5. Integration and calculation of free hexon concentration:

The peak areas of the free hexon-antibody complex region, free antibody, and free fluorescence are integrated with a fixed baseline using the Millennium software. The areas are recorded in an Excel table. The above fluorescent peak areas are totaled for each chromatograph to check for fluorescence recovery. The fluorescent peak area of free hexon–antibody complex of the second blank is subtracted from all the standards and unknown samples. The concentration of purified free hexon in the HPLC vial is plotted versus the corrected fluorescent peak area of free hexon-antibody complex to generate a standard curve. Linear regression analysis is used to determine the slope and intercept of the standard curve. The free hexon-antibody complex fluorescent areas of unknown samples that are within the linear range of the standard curve are evaluated; other unknown samples are retested at other dilutions. Using the standard curve linear regression, the free hexon concentrations in the HPLC vial of in the unknown samples are determined as micrograms per milliliter. Multiplication by the dilution factor of the unknown sample in the HPLC vial gives the free hexon concentration of the

sample. Additionally, if the viral particle concentration of the unknown sample is known, then the free hexon amount is also reported as micrograms of free hexon/10^{12} viral particles.

6. Cleaning and disinfecting the column:

When the column was extensively used or when the backpressure of the system exceeds 250 psi with the column in-line, the following cleaning procedure is implemented. The column is washed with two column volumes of Milli-Q water, 0.5 column volume of 4M urea/0.5 N sodium hydroxide solution, followed by two column volumes of Milli-Q water. Then the column is equilibrated with two column volumes of gel filtration buffer.

For long term storage, instead of equilibrating with gel filtration buffer, the column and the system are equilibrated with 0.01N sodium hydroxide.