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Validation of a high-performance liquid chromatographic assay for the quantification of adenovirus type 5 particles

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Abstract

An anion-exchange–high-performance liquid chromatography (AE–HPLC) method for the quantification of adenovirus type 5 (Ad5) total particles was validated according to performance criteria of precision, specificity, linearity of calibration and range, limit of detection, limit of quantification, accuracy and recovery. The viral particles were detected by absorbance at 260 nm using photodiode array detector (PDA). Cesium chloride (CsCl) purified Ad5 and lysate samples were used for the validation of the method. Relative standard deviations (RSDs) for the inter-day, intra-day precision and reproducibility for both the lysate and the Ad5 standard were less than 10 and 2% for the peak area and retention time, respectively. The method was specific for Ad5 which was eluted at 8.0 min. The presence of DNA does not affect the recovery of Ad5 particles for accurate quantification. Based on the error in prediction to be less than 10%, the working range was established between 2×10^{10} and 7×10^{11} VP/ml with correlation coefficient of 0.99975, standard deviation of 6.14×10^{9} VP/ml and a slope of 3.04×10^{5} VP/ml. The recovery of the method varied between 88 and 106% in all of the lysate samples investigated which is statistically similar to 100% recovery at 95% confidence interval.

Keywords: Adenovirus type 5 particles

1. Introduction

The demand for replication-defective adenovirus viral vectors for gene therapy applications has increased over the years. In order to meet these demands, robust production processes must be developed. One of the most important factors in the development of processes is to have fast, accurate and reliable quantification methods for the product of interest. Currently, physical methods for the quantification of adenovirus type 5 (Ad5) total particles include spectral measurement at 260 nm after cell lysis [1], anion-exchange–HPLC [2–6] and electron microscopy [1]. However, to the best of our knowledge a report on the validation of these methods is not published elsewhere.

The emphasis on the validation of analytical methods has become increasingly important especially if such a method is used in the quantification of the drug product or drug substance intended for human use. The purpose of method validation is to

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demonstrate by generation of data if it meets the requirements for its intended application. The characteristics associated with an analytical method are expressed in terms of analytical performance parameters such as precision, reproducibility, specificity, limit of detection (LOD), limit of quantification (LOQ), linearity of calibration, working range, accuracy and recovery [7–9].

We have developed in our laboratory an anion-exchange–HPLC method using a UNO Q polishing column for the quantification of Ad5 in the lysate samples [2]. The present paper focuses on the results obtained for the validation of this method according to the earlier mentioned parameters.

2. Experimental

2.1. Materials and methods

An HPLC System (Waters, Milford, MA) equipped with a 996 photodiode array detector (PDA), 717 Plus autosampler, Waters 600 controller, and Waters on-line degasser was used in this study. A Millennium32 software was used for data acquisition and peak integration. A UNO Q polishing column (4.6×10 mm, 0.16 ml) from Bio-Rad was used to isolate the Ad5 peak from the rest of the components in the sample. The column was equilibrated with buffer A (0.25 *M* HEPES, pH 7.5)–buffer B (2 *M* NaCl)–Milli-Q H_2O mixture (20:15:65, v/v) for 10 min. All buffers were filtered through a 0.45-μm filter membrane (Acrodisc syringe filter from Pall Gelman Sciences). Injector purge for 6.5 min and needle wash for 30 s were performed after column equilibration. After equilibration of the column, a blank run with 50 m*M* HEPES, pH 7.5 was always performed prior to sample injection(s) to ensure a flat baseline. Sample injection volume was always 25 μl unless otherwise mentioned. Samples were diluted in buffer (50 m*M* HEPES, pH 7.5) whenever necessary. A linear gradient was applied from 300 to 600 m*M* NaCl at a flow-rate of 1 ml/min for the elution of Ad5. The virus peak was eluted at 450 m*M* NaCl in about 8 min. After the virus elution, a 1.2 *M* NaCl wash was performed for 4 min and the column was re-equilibrated for 10 min before the next injection.

2.2. Ad5 standard preparation

Recombinant adenovirus serotype 5 (rAd5) was prepared by a two-step cesium chloride density gradient ultracentrifugation using a 100× concentrated cell suspension frozen at −80°C. The cells were lysed by two freeze–thaw cycles at 37°C for 10 min. Benzonase® was added to the lysate with final concentration of 100 U/ml. The lysate was incubated at room temperature for 1 h with slow shaking and centrifuged at 3000 rpm (2620 *g*) for 15 min (Sorvall RC-3B). The pellet was washed with 10 m*M* Tris–HCl, pH 7.9 and centrifuged at 3000 rpm (2620 *g*) for 15 min (Sorvall RC-3B). The supernatants were collected and the pellets were discarded. The collected supernatant was layered on tubes containing a step CsCl gradient of density 1.4/1.2 and centrifuged at 23 000 rpm (100 000 *g*) at 4°C for 90 min (Beckman L8-55M). The virus band was collected and centrifuged at 3000 rpm (2620 *g*) for 15 min. This virus solution was again layered on tubes containing a continuous CsCl gradient of density 1.4/1.2 and centrifuged at 23 000 rpm (100 000 *g*) at 4°C for 24 h. The band specific for Ad5 was collected and dialyzed overnight against 10 m*M* Tris–HCl, 1 m*M* $MgCl_2$, pH 7.9. The virus stock solution was stored in 10 m*M* Tris–HCl, 1 m*M* $MgCl_2$ and 5% sucrose, pH 7.9 in aliquots at −80°C.

2.3. Lysate preparation

The Ad5 was produced in a 20-l bioreactor [10] with the permissive human embryonic kidney cell line 293S in a liquid media containing serum. After 48 h post infection, the cells were collected by centrifugation and resuspended in 10 m*M* HEPES, 2 m*M* $MgCl_2$, pH 7.4, total volume 2 l. This solution was aliquoted in volumes of 5 and 10 ml and stored at −80°C. Before the analysis, thawing at 39°C for 6 or 7 min for the 5- or 10-ml aliquots, respectively, lysed the cells. The samples were then centrifuged at 7500 rpm (4610 *g*) for 5 min (Eppendorf 5415C

centrifuge). The supernatant was collected and filtered through a 0.45-μm Acrodisc syringe filter (Pall Gelman Sciences) prior to the injection.

2.4. Total viral particle determination

The total particle concentration of the CsCl-purified Ad5 standard was determined spectrophotometrically according to the method of Maizel et al. [1]. The pure virus was diluted appropriately with 0.5% SDS in 0.02 *M* sodium phosphate buffer, pH 7.2. The sample as well as the SDS buffer blanks were then heated at 55°C for 5 min and centrifuged at 12 000 rpm for 1 min (Eppendorf 5415C centrifuge). The virus sample was taken out of the tube carefully not disturbing the bottom portion and read at 260 nm using a Bausch and Lomb Spectronic 2000 spectrophotometer. The system was blanked first with the treated SDS buffer without virus prior to the reading of the sample. The total Ad5 particle concentration (VP/ml) was determined by multiplying the absorbance at 260 nm by the dilution factor and by 1.1×10^{12} (since 1.0 $OD_{260\ nm}=1.1\times10^{12}$ VP/ml) [1]. The total VP/ml thus obtained was used as the starting reference concentration for the generation of a standard curve for the HPLC method.

3. Results and discussions

3.1. Precision

The two most common precision measures are repeatability and reproducibility.

3.1.1. Repeatability

The repeatability of the method was performed by five repeated (intra-day) injections of the CsCl-purified Ad5 standard diluted to 4.75×10^{10} VP/ml, and for five consecutive days (inter-day). The means of the peak area and retention time of the five injections were determined and the overall mean for the five consecutive days was calculated along with the standard deviation. The percent relative standard deviation (% RSD), a measure of precision, was determined by dividing the standard deviation by the mean multiplied by 100 to evaluate the precision of the system (Table 1A). A good repeatability with an RSD of 4.41% for the determination of the concentration (peak area) and 0.75% for the retention time was demonstrated. The virus concentration determined was in the range of 4.34×10^{10} and 5.16×10^{10} at 95% confidence level and the retention time was in the range of 7.88 and 8.11 min with the corresponding mean values of 4.75×10^{10} VP/ml and

Table 1
Inter-day and intra-day assays of the (A) CsCl-purified standard and (B) infected 293 cell lysate

	Day 1*	Day 2*	Day 3*	Day 4*	Day 5*	Inter-day
(A) CsCl-purified standard						
Mean peak area	137807	138937	149582	146192	152253	144594
Standard deviation	6351	3649	4801	1857	5444	6394
% RSD	4.61	2.63	3.21	1.27	3.58	4.41
Retention time (min)	7.960	8.060	7.937	8.063	7.965	7.997
Standard deviation	0.034	0.019	0.026	0.019	0.029	0.06
% RSD	0.43	0.24	0.33	0.23	0.36	0.75
(B) Infected 293 cell lysate						
Mean peak area	222486	204245	193161	190366	202101	202472
Standard deviation	2805	2539	3176	8017	4956	12620
% RSD	1.26	1.24	1.64	4.21	2.45	6.23
Retention time (min)	7.831	8.004	7.989	8.101	7.931	7.971
Standard deviation	0.018	0.007	0.026	0.023	0.011	0.099
% RSD	0.23	0.08	0.32	0.29	0.14	1.25

* Intra-day (5 repeated injections on each day).

8.00 min, respectively. Table 1A also includes intra-day %RSD for the peak area and retention time for CsCl-purified Ad5 standard. The obtained RSDs also demonstrated good repeatabilities, 1.27–4.61 and 0.23–0.43% for the peak area and retention time, respectively.

The repeatability of the method was also determined by injecting a lysate five consecutive times for five consecutive days (Table 1B). This also demonstrated a good repeatability of lysate samples with an RSD of 6.23% for the determination of the concentration (peak area) and 1.25% for the retention time. With a 95% confidence level, the virus concentration in the lysate was estimated to be in the range of 5.26×10^{10} and 6.62×10^{10} and the retention time in the range of 7.78 and 8.16 min with the corresponding mean values of 5.94×10^{10} VP/ml and 7.97 min, respectively. Table 1B also includes intra-day RSDs for the peak area and retention time of the lysate. The obtained values also demonstrated good repeatabilities, 1.24–4.21 and 0.08–0.32% for the peak area and retention time, respectively.

The mean retention times for the Ad5 standard and lysate are statistically equal at 95% confidence level. In general, a relative standard deviation of $\leq$15% is a normally accepted value for the concentration (peak area) and $\leq$2% is a normally accepted value for the retention time. These values are mostly used as limits when setting acceptance criteria according to industrial guidelines [7,8,11,12]. However, the method described here generates RSDs below these values, therefore, this method can be considered repeatable.

3.1.2. Reproducibility

The reproducibility of the method was determined by compiling results obtained from five different days of sample analysis performed in duplicate injections by three different operators using both the CsCl-purified Ad5 standard and the lysate preparation. The RSD for each day was determined to evaluate the reproducibility of the method. Table 2A,B presents the results obtained for the reproducibility of the method. For the Ad5 standard and the lysate sample, the RSD values were below 10% for the peak area, while for the retention time, the values were less than 2%. Therefore, the method is considered reproducible for both the standard and the lysate samples.

Since, this method is within the normally accepted %RSD values for repeatability and reproducibility, therefore, it can be concluded that this method is precise.

Table 2
Mean, standard deviation and RSD values obtained by duplicate injections of (A) CsCl-purified Ad5 and (B) lysate sample by three different operators over a period of 5 days

Days	Peak area			Retention time (min)		
	Mean of duplicates by three operators	SD	RSD %	Mean	SD	RSD %
(A) CsCl-purified Ad5						
1	129390	4900	3.79	7.902	0.120	1.52
2	130027	2610	0.20	7.895	0.147	1.86
3	130502	3404	2.61	7.951	0.150	1.89
4	130485	8649	6.63	7.934	0.115	1.45
5	144892	12757	9.63	7.923	0.107	1.35
(B) Lysate sample						
1	194569	12991	6.68	7.852	0.100	1.28
2	203069	13614	6.70	7.933	0.056	0.70
3	184225	18192	9.87	8.064	0.084	1.05
4	172497	9247	5.36	8.017	0.156	1.95
5	197738	14141	7.15	8.069	0.083	1.03

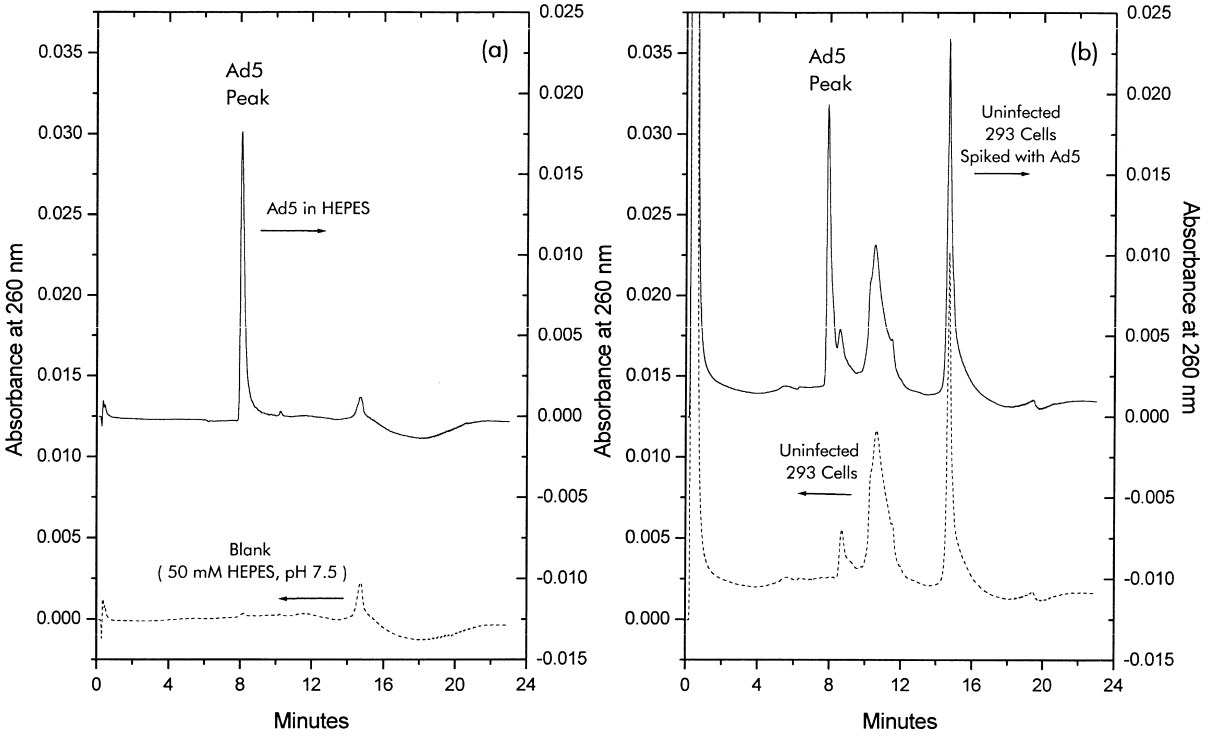

Fig. 1. Elution profiles. (a) A blank containing 50 m*M* HEPES at pH7.5 and Ad5 standard at a concentration of 1×10^{11} VP/ml prepared in HEPES buffer; (b) uninfected 293 cells and uninfected 293 cells spiked with 1×10^{11} CsCl-purified Ad5 VP/ml.

3.2. Specificity of Ad5 peak

A blank (50 m*M* HEPES, pH 7.5), the CsCl-purified Ad5 standard in HEPES at a concentration of 1×10^{11} VP/ml, uninfected 293 cells, and uninfected 293 cells spiked with 1×10^{11} Ad5 VP/ml (Fig. 1a and b) were visually inspected for the presence of substances which might have co-eluted with the Ad5 peak. No peak was found in HEPES buffer that could interfere with CsCl-purified Ad5 peak (Fig. 1a). Although, there is a peak in the 293 cell lysate chromatogram right after the Ad5 peak at about 8.6 min, the spiked peak was clearly distinguishable from this peak when compared with non-spiked to spiked Ad5 regions (Fig. 1b). The lysate chromatogram in Fig. 2 reveals that the above mentioned peak is far away from Ad5 peak. The Ad5 peak at about 8 min was an independent peak and no apparent interfering or co-eluting peaks with similar

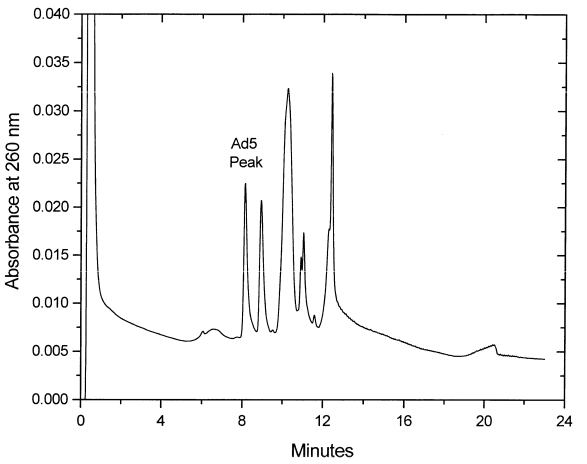

Fig. 2. Elution profile of a lysate sample (infected 293 cells).

retention times were found on chromatograms of blanks, the Ad5 standard, uninfected 293 cell lysates, uninfected 293 cell lysates spiked with Ad5 and lysate samples (infected 293 cells).

3.3. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD, limit of detection, which may be more precisely referred to as ''minimum detectable value'', is determined by the following formula for validation purposes [13,14]:

Sample blank value + 3*s*

where *s* is the sample standard deviation of the six injections of sample blanks.

The LOQ is the lowest concentration of analyte that can be quantified with an acceptable level of precision [8,14,15]. For validation purposes, it is defined as:

Sample blank value + 10*s*

The mean values of peak area and the standard deviations for six blanks (50 m*M* HEPES, pH 7.5) and six 5.0×10^8 VP/ml CsCl-purified Ad5 standard injections are shown in Table 3. The sample blank that was not supposed to have any viral particles had a mean peak area (noise) of 91 and the mean peak area of the Ad5 standard was 1395. The corresponding standard deviations were 86 and 206, respectively. Therefore, LOD was calculated as: $0 + (3\times86$ Area Units$)(5.00\times10^8$ VP/ml/1395 Area Units$) = 0.9\times10^8$ VP/ml. Similarly, LOQ was calculated as: $0 + (10\times86$ Area Units$)(5.00\times10^8$ VP/ml/1395 Area Units$) = 3.1\times10^8$ VP/ml.

3.4. Linearity of calibration and working range

The linearity of data was determined by plotting the Ad5 concentration as a function of the peak area response by injecting the CsCl-purified Ad5 in concentration ranges of 1.0×10^9–1.0×10^{12} VP/ml in triplicates. The correlation coefficient (r^2) was used to estimate the linearity of the calibration. A good linearity was obtained with a correlation coefficient of 0.99878, a standard deviation of 1.64×10^{10} VP/ml, and a slope of 2.90×10^5 (VP/ml/peak area). All triplicate injections in this concentration range gave an RSD of less than 5%. The percent difference (predicted minus expected value) was plotted against the expected virus concentration in Fig. 3. To accept ±10% difference, the operating range to determine the adenovirus particle concentration was limited between 2×10^{10} and 1×10^{12} VP/ml.

A linear plot of concentration range between 2×10^{10} and 1×10^{12} VP/ml against peak area revealed that 1×10^{12} VP/ml is an outliner from the linearity (Fig. 4). Therefore, the true working range for our analytical purpose was set between 2×10^{10} and 7×10^{11} VP/ml with a correlation coefficient of 0.99975, a standard deviation of 6.14×10^9 VP/ml of Ad5, and a slope of 3.04×10^5 (VP/ml/peak area). Working in this range would generate an error in prediction not more than ±10% from the expected value. Since this linear fit passes through zero, then a one point calibration at 7×10^{11} VP/ml is sufficient.

Table 3
Limit of detection and limit of quantitation determination

Injection #	Sample blank 50 m*M* HEPES, pH 7.5 (Peak area around 8 min)	Ad5 standard 5.00×10^8 VP/ml (Peak area around 8 min)
1	0	1548
2	152	1344
3	124	1092
4	0	1369
5	180	1623
6	107	1092
Mean	91	1395
s	86	206

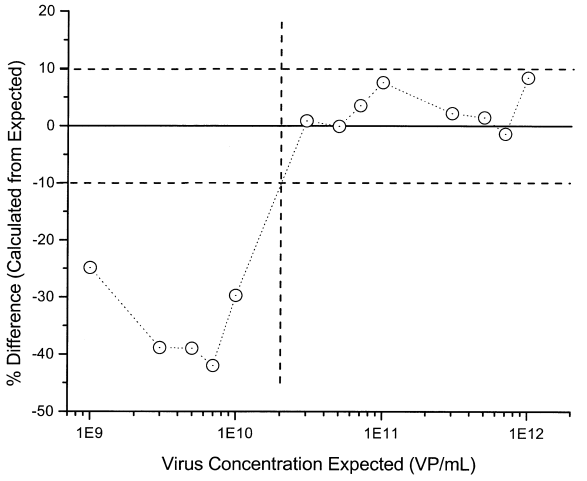

Fig. 3. Percent difference (calculated from expected) vs. virus concentration expected.

3.5. Accuracy and recovery

Accuracy is defined as the closeness of the results to the true values and when expressed in terms of bias as the difference between the mean of the results produced by the method and the true value. Therefore, the mean of triplicates was compared with the expected value of a spiked (fortified) CsCl-purified Ad5 standard in the corresponding test samples.

The accuracy and recovery of the assay was determined by spiking with a 10^{11} VP/ml of the CsCl-purified Ad5 standard into four different samples: (1) lysate (infected 293 cells); (2) lysate (infected 293 cells) with Benzonase® treatment; (3) lysate (non-infected 293 cells), and (4) lysate (non-infected 293 cells) with Benzonase® treatment. The use of Benzonase® at concentrations of 100 U/ml of lysate was carried out to determine whether there is an interference due to the presence of DNA in the accurate quantification of Ad5 particles. The percent recovery was calculated as:

$$\%\ \text{Recovery of spiked Ad5} = \frac{(C1 - C2)}{C3}\ 100\%$$

where $C1$ is the concentration of Ad5 in the spiked sample (spiked concentration + original concentration in the non-spiked sample), $C2$ is the concentration of Ad5 in the non-spiked lysate, and $C3$ is the concentration of Ad5 used for spiking (i.e. 1×10^{11} VP/ml).

For all of the samples investigated the % recoveries obtained range from 88 to 106%, a bias of less than 15% which is an inherited error associated with the method (Table 4). The obtained recoveries are not statistically significant from each other at 95% confidence interval. With the expected concentration of 1.0×10^{11} VP/ml of Ad5, the range is between 8.1×10^{10} and 1.2 $x10^{11}$ VP/ml of Ad5. Therefore, the accuracy of the method is considered to be good with or without Benzonase® treatment.

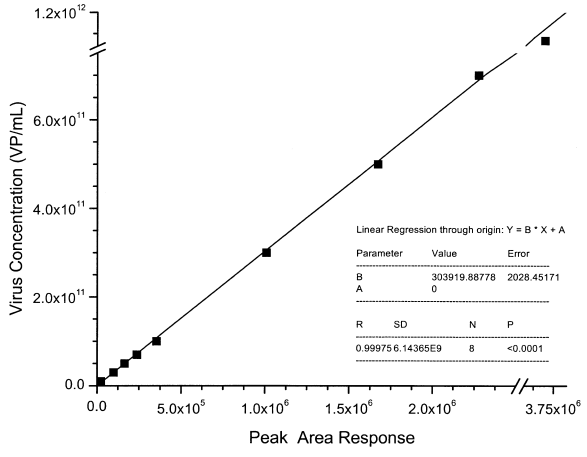

Fig. 4. Linearity of calibration curve using CsCl-purified Ad5.

4. Conclusions

The AE–HPLC using the UNO Q polishing column for the quantification of total Ad5 particles was shown to be precise, specific, accurate, and linear in the range of 2.0×10^{10}–7.0×10^{11} VP/ml of Ad5.

This method is now being routinely used in our laboratory to determine the Ad5 concentration in cell lysates, semi-purified and purified Ad5, to monitor the kinetics during the production phase and for the assessment of peak purity and calculation of yield during the purification process. Benzonase® treatment of the lysate samples did not show a significant increase in the recovery of Ad5 particles, and it is not used prior to analysis.

Table 4
Accuracy and recovery of Ad5 spike (1×10^{11} VP/ml)

Sample	Non-spiked peak area	Spiked peak area	Recovered peak area	Ad5 spike recovered (VP/ml)	% Recovery of spiked Ad5
Lysate	195914	508113	312199	9.5×10^{10}	95
Lysate with Benzonase®	252975	547885	294190	9.0×10^{10}	90
293S Cell lysate (non-infected)	NA	290077	NA	8.8×10^{10}	88
293S Cell lysate (non-infected) with Benzonase®	NA	349852	NA	10.6×10^{10}	106

All values presented under spiked, non-spiked and recovered peak areas are means of triplicate injections. NA, not applicable, since there were no viral particles in the non-infected 293 cell lysate.

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