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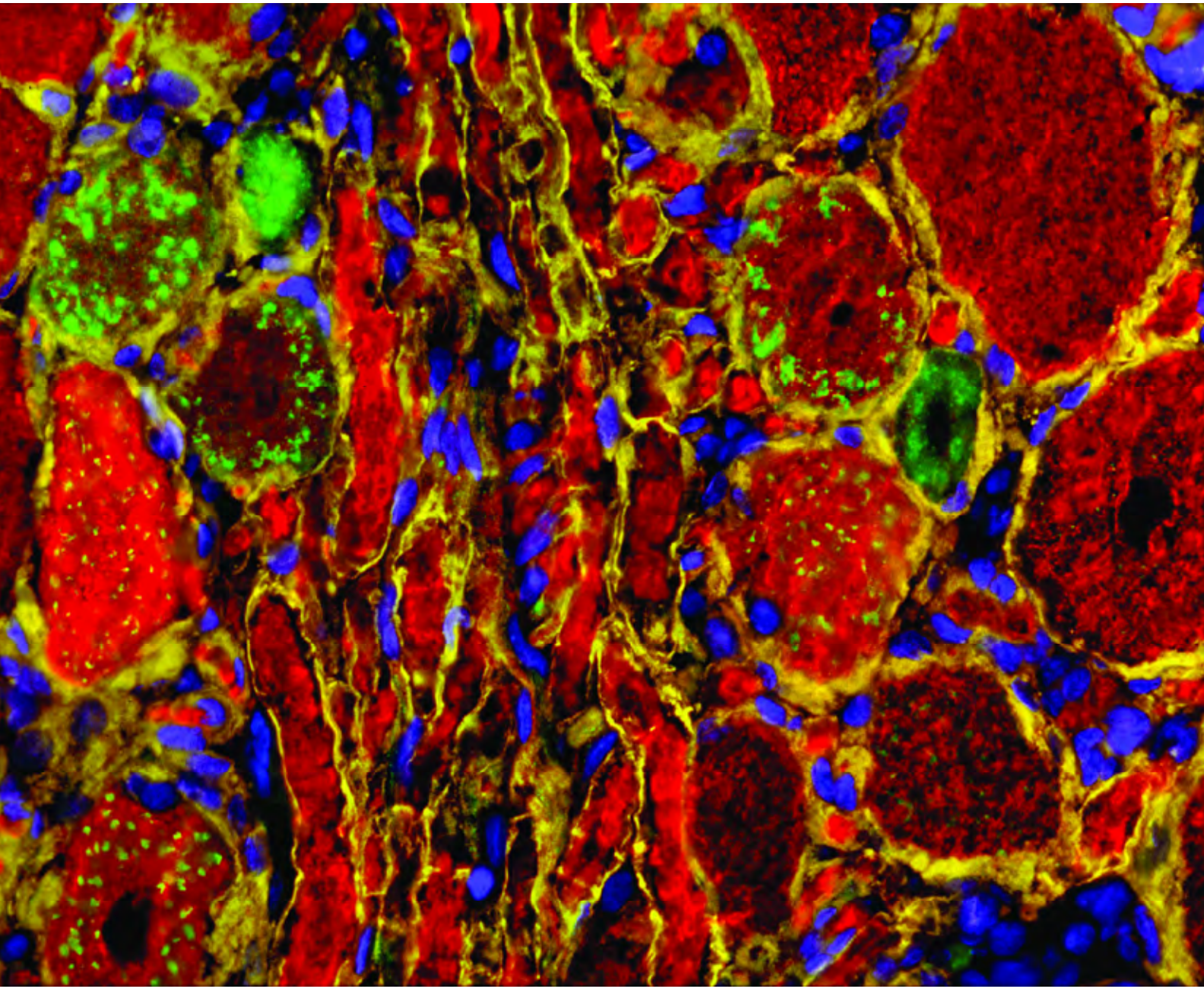

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Adenovirus Reference Material:
Determination of Particle Concentrations Obtained by Orthogonal, Physical-Chemical Methods

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With the continued progress of adenoviral vectors in gene therapy studies it is increasingly evident that a more formalized approach to the characterization and analysis of these viral vectors is urgently needed. Today, adenoviral vectors are beginning to be considered "well characterized biologics," as shown by numerous publications describing sophisticated analytical approaches for recombinant adenovirus product candidates.[1–7] Because the analytical definitions of adenoviral vectors currently lack comparison to a common standard, the problem for regulatory agencies is how to objectively evaluate safety in relation to the administered dose. This well-recognized need for an adenovirus standard has been addressed by a consortium of representatives from regulatory agencies, industry, and academic organizations — the Adenoviral Reference Material (ARM) Working Group. Its work has come to fruition in the recent public availability of the ARM,a purified wild type 5 adenovirus. Many aspects of the history, production, and characterization of the ARM have been published in detail.[8–11]

FDA's perspective on the need for the ARM and its intended use was discussed by Simek et al.[12] The authors point out that fundamentally different methods for the determination of viral particle concentration make it difficult to compare the dosing in various clinical trials. This is of particular concern because of the sharp threshold of the dose-toxicity curve. The $A260_{nm}$/SDS method has often been used to determine particle concentrations. The availability of the ARM,and its analysis by multiple investigators and methods, now provides regulatory agencies with a means of comparison. However, the authors clearly state that although FDA does not expect a duplication of the ARM particle concentration value,the results from the $A260_{nm}$/SDS method should fall within the published range,as this range is the outcome of a large number of measurements in many different laboratories.

This article summarizes the use of orthogonal methods other than the $A260_{nm}$/SDS method for the determination of viral particle concentration of the ARM. Values from orthogonal analyses are compared to the designated particle concentration as determined by the $A260_{nm}$/SDS method, and can benefit users of the ARM as they validate their own orthogonal methods and internal reference standards.

Methods and Materials

Adenoviruses are nonenveloped, icosahedral (20-sided) dsDNA viruses. The Adenovirus Reference Material (ARM) is a column-purified wild type 5 adenovirus (Ad5). It was produced and vialed by Int rogen Therapeutics, Inc. in

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Table 1. Forward and reverse primers and probe sequences for the hexon and E4 qPCR assays

Assay	*Reagent*	*Sequence (5' to 3')*
Hexon qPCR Assay	Forward Primer	ACT ATA TGG ACA ACG TCA ACC CAT T
	Reverse Primer	ACC TTC TGA GGC ACC TGG ATG T
	Probe	6-FAM-ACC ACC GCA ATG CTG GCC TGC-TAMRA
E4 qPCR Assay	Forward Primer	CAC CAC CTC CGC GTA CCA TA
	Reverse Primer	CCG CAC CTG GTT TTG CTT
	Probe	6-FAM-AAC CTG CCC GCC GGC TAT ACA CTG-TAMRA

Houston, TX under the guidance of the Adenovirus Reference Material Working Group (ARMWG),and is available from the American Type Culture Collection, Manassas, VA (catalog no. VR-1516).[8] *All information regarding the purification and characterization of the ARM is published on the Williamsburg BioProcessing Foundation's (WilBio) website at www.wilbio.com.*

Determination of Virus Particle Concentration

Typically, each method was carried out by the originating laboratory and at least one other laboratory. The following organizations participated: Berlex Biosciences (Berlex); Canji Inc (Canji); Schering-Plough Research Institute (SPRI); Transgene, S.A. (TRANS); and the University of Texas at Austin, College of Pharmacy (UT). Additional data was submitted by Cobra Therapeutics (Cobra), the Biotechnology Research Institute of the National Research Council Canada (BRI), and Cell Genesys, Inc. (CG), for physical methods that were performed only by these laboratories.

$A260_{nm}$/SDS: This method for determination of total virus particle concentration was first described by Maizel et al.[13] Briefly, a virus sample is lysed in 0.1% SDS for 15 minutes at room temperature. The virus particles disintegrate under these conditions into proteins and DNA. The DNA content is subsequently measured by UV absorption at 260 nm. According to Maizel et al., one OD_{260} unit contains 1.1×10^{12} particles.[13] This method was also described in detail by Adadevoh et al.[10]

In some cases,particle determination by orthogonal methods required comparison of the ARM to certain standard materials. An Ad5 standard was provided by Canji and is referred to as the "SZCE rAd standard" (SCZE 92AAH, 1.16×10^{12} particles/ml as determined by A260 in SDS).[13]

Anion Exchange-HPLC (AE-HPLC): This method measures intact virus particles. It uses a 1 ml Resource Q column (Pharmacia Biotech, Piscataway, NJ) and is described in detail by Shabram et al.[1] Purified Ad5 samples can be analyzed by this assay without pre-treatment. Buffer B is 50 mM HEPES at pH 7.5, and buffer C is 1.5 M NaCl and 50 mM HEPES at pH 7.5. The column is equilibrated at 20% B (300 mM NaCl) for 2 minutes, followed by an increase of B to 40%, held at 40% for 2 minutes, ramped up to 100% B, then followed by an immediate decrease to 20% in 14 minutes,and a final equilibration step at 20% B for 4 minutes. The flow rate is 1 ml/min. The HPLC systems used were either a Waters 625 (Waters, Milford, MA) or an Agilent 1100 (Agilent Technologies, Palo Alto, CA), depending on the investigator. Elution profiles were monitored at 260 nm and 280 nm. Particle concentrations were calculated by comparing peak areas at 260 nm with the SZCE rAd5 standard curve in a range from 1.16×10^{10} to 1.16×10^{11} particles per injection. The Transgene and BRI laboratories used AE-HPLC standards that were not the SZCE rAd5 but did consist of purified adenovirus preparations whose particle concentrations had been established via the $OD260_{nm}$/SDS method of Maizel et al.[13] These laboratories also varied the elution procedure slightly. Transgene performed an equilibration for 2 minutes at 20% B (300 mM NaCl), followed by a linear gradient from 0 to 375 mM NaCl over 10 minutes, followed by 2 minutes at 375 mM NaCl, followed by a 2.6 minute wash at 1.5 M NaCl, and a final equilibration with 20% B (300 mM NaCl) for 9 minutes. BRI used a bovine serum albumin-conditioned 0.16-ml UNO Q polishing column (BioRad Laboratories). Its method uses slightly different buffers: Buffer A consists of 0.25 M HEPES at pH 7.5, buffer B is 2 M NaCl, and buffer C consists of Milli-Q water.[18] BRI equilibrates the column with 20% A (50 mM HEPES), 15% B (300 mM NaCl), and 65% C (water) at 1 ml/min, before running a gradient elution.

Reverse Phase HPLC (RP-HPLC): The viral particles disintegrate into their proteins under the RP conditions and elute as a characteristic protein fingerprint. This method was first described by Lehmberg et al.[2] Samples of intact virus particles containing 0.048% Tween 20 were injected onto a Jupiter C4 column (2 x 150 mm, Phenomenex, Torrance, CA) and eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid. The instruments used were an HP1090 or a Waters Alliance 2690 system, again depending on the

investigator. The flow rate was 200 µl/min, with detection at 214 nm, and 40° C column temperature. The ARM hexon peak areas were used to calculate the particle concentration by comparison to the hexon peak areas from the SCZE rAd standard. For this study, the SCZE rAd standard had to be diluted by a factor of ten to be in a suitable range for the assay. Standard curves were generated by injecting increasing amounts of the diluted SCZE rAd standard ranging from 1 x 10^9 to 1 x 10^{10} particles per injection. Potentially, the particle concentration also can be determined by comparison of the hexon peak area of an unknown Ad sample to a known protein standard peak, such as BSA, as first published by Lehmberg et al.[2]

Picogreen: This quantitation assay for DNA is based on the Picogreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR). The assay and its application for adenovirus particle quantitation were previously described by Murakami et al.[3] For this study, some modifications of the published method were made, therefore, a description of the experimental details that differ from the publication are outlined here: ARM samples were diluted 1:5 and 1:10 in phosphate-buffered saline (PBS), containing 2 mM $MgCl_2$ and 2% sucrose. The SZCE rAd standard was diluted 1:10 and 1:20 with the same buffer. Ten µl of these diluted samples were mixed with an equal volume of 15 µl of 10 U/µl DNase I and 15 µL RNase A (at 10 mg/ml) in 80 mM Tris at pH 7.5, with 20 mM NaCl and 12 mM $MgCl_2$. The samples were then incubated for 15 minutes at 37° C. For the SDS lysis, 20 µL of 0.2% SDS and 50 mM EDTA were added to the enzyme sample mix. Diluted ARM samples without enzyme treatment were analyzed in parallel. Fifteen µL of these samples were mixed with equal volumes of 0.2% SDS and 50 mM EDTA. Incubation was again for 15 minutes at 37° C. The reaction with Picogreen and data evaluation was performed according to the insert from the Molecular Probes assay kit and the description.[3]

An alternate Picogreen method was performed by Cobra Therapeutics at the 1-ml cuvette scale and did not include SZCE in the analysis. Cobra prepared samples and standards without SDS but using the Tris-HCl, EDTA buffer, and Proteinase K provided in the kit. Samples and standards were prepared in duplicate at each dilution, heating them at 56° C for 90 minutes. Cobra added 1.5 ml of each standard or sample to a plastic cuvette. 1.5 ml of the working solution of Picogreen was added, then the solutions were mixed and left at room temperature for at least 30 minutes prior to fluorescent measurement. Sample DNA concentration was calculated as:

p/ml = (sample fluorescence – intercept of calibration line)/(slope of calibration line) * (dilution factor)/1000 * 0.02 * 1.1 x 10^{12}

Hexon quantitative polymerase chain reaction (qPCR) assay: The particle concentration of a purified adenovirus sample can be determined based on the qPCR assay for hexon DNA. This technique requires template DNA from the sample source to be purified before analysis. The purification of the ARM and the SZCE rAd standard was accomplished by following the DNA Blood Mini Kit protocol (Qiagen). One microliter of the purified DNA was used to determine its concentration utilizing the Perkin-Elmer Taqman 5' nuclease real-time PCR methodology (programmable thermocycler, Applied Biosystems, ABI Prism 7700 Sequence Detector). PCR primers were hexon forward and reverse oligonucleotide (Applied Biosystems, both custom orders) and a fluorescently tagged hexon Taqman probe (Applied Biosystems, custom order). Probe and primer sequences for the hexon qPCR assay can be found in Table 1. The hexon DNA present in a sample is detected, quantified, and converted to viral particles based on a viral DNA standard curve in a range from 5 x 10^4 to 5 x 10^8 starting particles.

E4 qPCR assay: Cell Genesys performed a qPCR assay similar to the hexon qPCR assay. In this case, the target adenoviral gene was the E4 gene. E4 forward and reverse oligonucleotide (Applied Biosystems, both custom orders) and a fluorescently tagged hexon Taqman probe (Applied Biosystems, custom order) were used as in the hexon qPCR assay. Table 1 shows the probe and primer sequences for the E4 qPCR assay.

Results and Discussion

Total particle concentration derived by orthogonal methods

The $A260_{nm}$/SDS method was chosen for the total particle concentration determination because it is easy to perform, widely accepted, does not rely on a purified standard, and thus can be executed in any laboratory working with adenovirus. The results from 13 laboratories were used to determine the "official" particle concentration of the ARM as published in the product information sheet.[15] A detailed discussion of the statistical analysis of these results was reported by Callahan.[9] The relative standard deviations of the absorbance readings at 260 nm varied, depending on the testing laboratory, from 0.05 to 15%, with most of the values being <2%. The range of the particle numbers was from 5.34 x 10^{11} to 6.73 x 10^{11} p/ml, with the average being 5.82 x 10^{11} ± 3.41 x 10^{10} p/ml. *The ARMWG-assigned particle concentration is 5.8 x 10^{11} p/ml* — with 95% certainty that the true particle concentration lies within the range of 5.6 x 10^{11} to 6.0 x 10^{11} p/ml. The range that includes three standard deviations of the mean is 4.8 x 10^{11} to 6.9 x 10^{11} p/ml.

Canji and Berlex performed the same AE-HPLC assay, while Transgene and BRI performed AE-HPLC assays that were slightly different. In all cases a standard was used whose particle concentration was assigned using the $A260_{nm}$/SDS method. The results from Canji and Berlex were within a very narrow range (each laboratory n=6) and the relative standard deviations were 1.3 and 2.2% (Table 2). The average particle concentration from both labs was 6.46 x 10^{11} p/ml. Although Transgene and BRI (each laboratory n=6) did not use the exact same AE-HPLC assay conditions or standard, these two laboratories produced results very similar to those of Canji and Berlex. When all

four laboratories' results are examined, the average particle concentration is still 6.46 x 10^{11} p/ml with a standard deviation of 0.96 x 10^{11}, or 14.9%. This value falls within the broader three standard deviation range for particle concentration established by the ARMWG.

The RP-HPLC assay was performed by SPRI and Berlex (each laboratory n=4). The results obtained with this method (5.30 x 10^{11} average of both laboratories) were significantly lower than those obtained with the AE-HPLC method, even though the same reference standard was used in both assays ($p<0.0001$, two-tailed, unequal variance). The SZCE rAd standard was found to contain roughly 6% free hexon and the ARM contains 1.5% free hexon (data not shown). Because the accuracy of the RP-HPLC assay as used in this study depends on the accuracy of the standard assignment, the results shown in Table 2 were adjusted for the free hexon content, which slightly increased the calculated concentration compared to the original values published on the WilBio.com website.

The Picogreen method and the qPCR assays measure DNA content by comparison to a DNA standard and then convert those data into adenovirus particle concentration. The results for both methods are shown in Table 2. The particle concentration derived by the qPCR assays were slightly greater than for the other methods, but were very similar to each other, regardless of the adenovirus gene target. The average qPCR-derived particle concentration for the ARM based on the data from Canji, Berlex, and Cell Genesys was 8.08 x 10^{11} p/ml, with a standard deviation of 0.27 x 10^{11} p/ml, or 3.4%. This value is significantly outside the broader three standard deviation range for particle concentration established for the ARM.

There was more variability among Picogreen-derived results for particle concentration. For the three laboratories performing the Berlex method, the particle concentration was determined as 3.56 x 10^{11} p/ml with a standard deviation of 0.90 x 10^{11} p/ml, or 25.3%. When the results from Cobra Therapeutics are included, the mean particle concentration via Picogreen was 3.44 x 10^{11} p/ml, with a standard deviation of 0.15 x 10^{11} p/ml, or 22.5%. The 3.44 x 10^{11} p/ml concentration is outside of the broader three standard deviation range for the ARM.

The ARMWG determined that the precision of the $A260_{nm}$/SDS method was such that only two significant figures could be reported. When the values derived from the different methods were expressed similarly and compared with the assigned ARM particle concentration of 5.8 x 10^{11} p/ml, only the two HPLC methods resulted in values within the three standard deviation range of the assigned ARM concentration (Table 3). In fact, these methods resulted in values within two standard deviation range. This is not surprising as the HPLC assays depend upon a standard that in each case was assigned its particle concentration based on the $A260_{nm}$/SDS method. As long as the standard is qualified against the ARM, or the $A260_{nm}$/SDS method is qualified against the ARM,then both HPLC assays provide an alternative means of determining adenovirus particle concentration.

The particle concentration derived from the Picogreen methods was low and outside the three standard deviation range. In fact, it resulted in concentrations that were approximately 60%

Table 2. ARM virus particle concentration (p/ml) determined by orthogonal methods

Assay	Laboratory	Canji	Berlex	SPRI	UT	*Average Sub-group*	TRANS	BRI	CG	Cobra	*Combined Average*
AE-HPLC	p/ml	6.37 x 10^{11}	6.55 x 10^{11}	NA	NA	*6.46 x 10^{11}*	5.29 x 10^{11}	7.64 x 10^{11}	NA	NA	*6.46 x 10^{11}*
	SD	8.52 x 10^{9}	1.41 x 10^{10}			*1.27 x 10^{10}*	4.60 x 10^{9}	6.93 x 10^{9}			*9.62 x 10^{10}*
	%CV	1.3	2.2			*2.0*	0.9	0.9			*14.9*
RP-HPLC	p/ml	NA	5.11 x 10^{11}	5.49 x 10^{11}	NA	*5.30 x 10^{11}*	NA	NA	NA	NA	*5.30 x 10^{11}*
	SD		1.75 x 10^{10}	2.23 x 10^{10}		*2.69 x 10^{10}*					*2.69 x 10^{10}*
	%CV		3.4	4.1		*5.1*					*5.1*
Picogreen	p/ml	3.78 x 10^{11}	4.33 x 10^{11}	NA	2.57 x 10^{11}	*3.56 x 10^{11}*	NA	NA	NA	3.08 x 10^{11}	*3.44 x 10^{11}*
	SD	4.07 x 10^{10}	5.76 x 10^{9}		3.13 x 10^{10}	*9.00 x 10^{10}*				2.96 x 10^{10}	*7.73 x 10^{10}*
	%CV	10.8	1.3		12.2	*25.3*				9.6	*22.5*
qPCR	p/ml	8.03 x 10^{11}	7.84 x 10^{11}	NA	NA	*7.94 x 10^{11}*	NA	NA	8.38 x 10^{11}	NA	*8.08 x 10^{11}*
	SD	1.39 x 10^{10}	4.81 x 10^{10}			*1.34 x 10^{10}*			3.99 x 10^{10}		*2.74 x 10^{10}*
	%CV	1.7	6.1			*1.7*			4.8		*3.4*

Table 3. Assigned ARM virus particle concentration and standard deviation ranges

Assigned Particle Concentration (p/ml)	**5.8×10^{11}**	
	Lower Limit	*Upper Limit*
+1 S.D. Range	5.5×10^{11}	6.1×10^{11}
+2 S.D. Range	5.1×10^{11}	6.5×10^{11}
+3 S.D. Range	4.8×10^{11}	6.9×10^{11}

compared to the $A260_{nm}$/SDS method. Although it is beyond the scope of this paper to fully explore the reasons for this discrepancy, there are several possible causes. One contributing factor may be that the DNA standard is lambda DNA and not purified adenoviral DNA. Another is that the Picogreen dye may not completely access the adenovirus DNA during the assay or may do so somewhat differently compared to the lambda DNA standard. In any case, the resulting signal is lower than one might expect.

Berlex scientists commented that the Picogreen assay is performed frequently in their process development laboratories and that the concentrations are usually in good agreement with the RP-HPLC derived concentrations. The ratio of RP-HPLC to Picogreen assay results is usually about 1.2 ± 0.2. For the data presented here, the ratio was 1.18 when taking only the Berlex data into account, but was 1.54 for the data combined from all laboratories. This may indicate laboratory-to-laboratory variation and suggests that an individual laboratory would be able to qualify a Picogreen assay against the ARM or an ARM-qualified internal adenovirus standard.

In the case of the qPCR methods,the particle concentration derived in each case was high and outside the three standard deviation range. In the case of either the hexon qPCR or E4 qPCR Assays, the resulting concentration was approximately 140% of the 5.8×10^{11} p/ml assigned concentration. Again, it is not within the scope of this paper to determine the reasons for this discrepancy. However, given that the qPCR method detects DNA fragments, it is possible for the method to overestimate intact particle concentration depending on viral particle purity from residual adenovirus DNA and host cell DNA.

In either the case of a Picogreen assay or a qPCR assay, it is important to realize that if the method can be adjusted slightly, or if the method utilizes an ARM-qualified standard, that the resulting particle concentrations would likely be within the ARM's three standard deviation range.

Assay characteristics

The $A260_{nm}$/SDS method is an absolute method (meaning it does not require a viral standard for calibration).[13] The two HPLC methods were calibrated against a purified adenovirus standard. For these studies, the SZCE rAd standard was used. This standard was assigned its particle concentration by the $A260_{nm}$/SDS method in a manner similar to the ARM. So, the HPLC assay results should fall within a relatively narrow range. The RP-HPLC method, however, can be used with an absolute calibration based on a highly purified protein, as described by Lehmberg et al.[4] Using a highly purified in-house, viral working standard from Berlex in the RP-HPLC assay, which was calibrated against BSA, the particle number derived for the ARM was $5.15 \pm 0.4 \times 10^{11}$ p/ml. This is in excellent agreement with the number obtained with the SZCE rAd standard reference calibration ($5.30 \pm 0.26 \times 10^{11}$ p/ml), and within the two standard deviation range.

The RP-HPLC method could overestimate virus concentration if free hexon is present in the sample, or on the other hand, might underestimate adenovirus particle concentration if the calibration standard contains a significant amount of free hexon. Therefore, it is crucial to characterize HPLC assay standards by methods that quantify the presence of free hexon.

An additional complication for both HPLC methods is the possible presence of empty capsids in the samples or in the comparative standards. The ARM was determined to have essentially no detectable empty capsid concentration as estimated from the pVIII concentration.[4] The SZCE rAd standard was estimated to have ~3% empty capsid (data not shown). Empty capsids would have a negligible effect on the $A260_{nm}$/SDS particle determination, but they would be detected by RP-HPLC (due to hexon content) and partially detected by AE-HPLC (since the majority of the signal at A260 is due to virus particle scatter). Again, characterization of standards for other impurities or viral forms is important in establishing a particle concentration assay.

The $A260_{nm}$/SDS method heavily depends on the purity of the virus samples because contaminating nucleic acids and some formulants will contribute to the reading at 260 nm, as might potentially viral contaminants as described by Vellekamp et al.[11] Also, the conversion factor, as described by Maizel et al., should be verified.[13] That is, it is possible to establish an extinction coefficient for a laboratory's own adenovirus product or internal standard. The factor of 1.1×10^{12} p/ml per one $A260_{nm}$ OD unit in 0.1% SDS was based on three assumptions from experimental data using adenovirus type 2. The first assumption was that the molecular mass of the DNA is 2.30×10^7 Da per particle. This was determined from the sedimentation rate of the purified virus DNA by Green et al.[16] Second,the protein constitutes 87% and the DNA 13% of the total viral particle, as determined by Green and Pina.[17] Third, there is 280 µg protein per $A260_{nm}$ in 0.5% SDS of purified virus, as determined by Lowry.[13] The first assumption has been confirmed by the complete DNA sequencing of the ARM. It might be useful to experimentally

reconfirm the other two assumptions using the ARM as well.

All particle concentration data presented here are in a range from 3.4 to 8.1 x 10^{11} p/ml, a difference of approximately a factor of two. It is impossible to say which number is the most accurate. Each method has its advantages and limitations and it is crucial that the investigator be aware of any potential shortcomings of the employed methods. More importantly, each method should be tied to and qualified using the ARM so that similar units are reported.

The impact of a two-fold variation in the stated administered particle dose is not tolerable given the sharp relationship between dose level and toxicities seen after administration of adenovirus via certain routes (intravenous or intrahepatic artery, for example) above 5 x 10^{12} particles. As long as the assay reports particle concentration using a scale that is tied to the ARM,then the method will provide the relative data.

This is the first time that a single virus preparation has been tested by different laboratories and by an array of orthogonal methods. These results clearly illustrate the importance of the ARM. It provides a much needed tool for regulatory agencies and for investigators to compare the concentrations of adenovirus preparations and the resulting preclinical and clinical data sets from different studies, laboratories, or sponsors.

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