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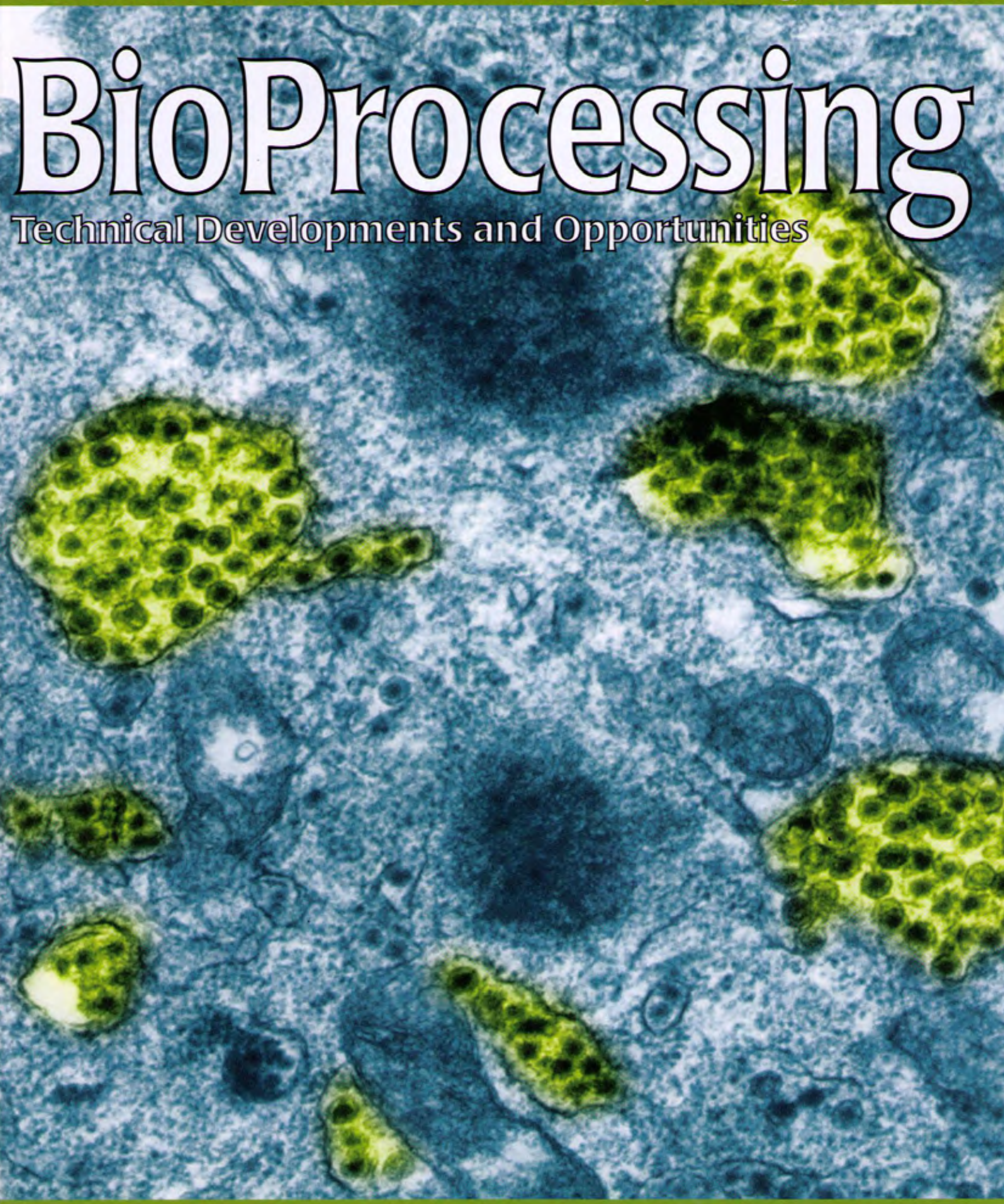

A publication of
The Williamsburg BioProcessing Foundation

Fall 2002

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A Short-Term Field Use and Shipping Stability Study of a Wild Type Ad5 Adenoviral Reference Material

BY KODJO ADADEVOH, MARIA CROYLE, DANIEL MALARME, EDWIGE BONFILS, MARK A. BOWE

Adenoviral vectors for gene delivery are being tested in the clinic for a number of indications and therapeutic uses. In order to facilitate the comparison of studies from different laboratories, the Adenovirus Reference Material Working Group (ARMWG) has developed a reference testing reagent, which will be referred to as the Wild Type Ad5 Adenoviral Reference Material (ARM).[1,2] This ARM will allow laboratories to standardize in-house controls employed in assays for the determination of particle concentration and infectious titer of their own adenoviral preparations. As part of this project, short-term field use and shipping studies were performed on the ARM. The virus was found to be stable under simulated shipping conditions, for one thaw after shipping, and at 4 °C for up to four hours after thawing. However, there was evidence of aggregation in some vials with repeated freeze-thaw cycles. Therefore, we recommend that each vial be treated as a single-use aliquot, and that it be used within four hours of thawing.

Introduction

The use of reference testing reagents to standardize measurements can greatly facilitate the comparison of data between laboratories. Such reagents can be particularly useful when working with products whose properties are inherently complex, such as proteins or viral vectors. While the field of adenoviral vectors has progressed on many fronts, the lack of measurement unit standardization has nevertheless hampered the exchange of safety and efficacy data.

The Adenovirus Reference Material Working Group (ARMWG) was formed to oversee the development of a reference testing reagent for adenoviral vectors.[1,2] This volunteer effort, comprised of representatives from industry, academia, and government organizations, has led to the development and characterization of a Wild Type Ad5 Adenovirus Reference Material (ARM).

Characterization of the ARM included a multi-laboratory determination of the particle concentration and infectious titer. This information will be provided with the ARM when it is distributed, and is considered to represent the primary utility of the ARM.[3]

The ARM is intended to be used by laboratories to standardize in-house controls employed in assays to determine the particle concentration and infectious titer of adenoviral preparations. Although the ARMWG did provide protocols to participating laboratories for characterization of the ARM, the ARMWG does not advocate that specific assays be used when characterizing additional adenoviral preparations. However, FDA representatives have indicated that product sponsors will need to validate their analytical methods and in-house standards against the ARM, thus providing greater comparability.[4]

Additional characterization of the ARM has been organized by the ARMWG. Participating laboratories have analyzed the ARM for purity by a variety of methods, and have sequenced the viral genome for eventual deposit in GenBank®.[1] A five-year stability study has begun at the recommended storage temperature of less than -55 °C, and also at -20 °C. The results of these analyses will be made available through future publication and on the WilBio website (www.wilbio.com).[4]

The studies reported in this article are intended to define acceptable environmental and handling conditions, and they examine stability during shipping, and under conditions expected for typical lab use. To simulate shipping under adverse conditions, ARM vials were packed in shipping contain-

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ers approved by the distributor (ATCC®), and then exposed to temperatures exceeding those expected during routine shipments. ARM samples were also exposed to multiple cycles of freezing and thawing to determine whether a thawed vial could be re-frozen for later use. Finally, ARM vials were thawed and then maintained at conditions simulating either common refrigerator (4 °C) or bench-top (room temperature) storage. Following these treatments, the samples were analyzed for particle concentration, infectious titer, and aggregation. These studies indicate that the ARM is stable during shipping, and for one thaw after shipment. In some vials, re-freezing the ARM resulted in viral aggregation. It is therefore recommended that ARM vials not be refrozen, and that each vial should be considered as a single-use aliquot. The ARM was also found to be stable when held at 4 °C for up to four hours. However, storage at 25 °C was more problematic, with evidence of aggregation beginning as early as four hours after thawing.

Materials and Methods

1. Conduct of the Study

The experiments and analyses were conducted at three laboratories: Genetic Therapy, Inc. in Gaithersburg, MD; Laboratory of Maria Croyle, University of Texas in Austin, TX; and Transgene S.A. in Cedex, France. Each participating laboratory received ARM vials, and then performed the specified analyses (except Appearance, which was assessed at only two facilities).

2. Particle Concentration Methods

Optical Density (performed by Genetic Therapy, Inc.): Using ARM formulation buffer or "excipient" (20 mM Tris, 25 mM NaCl, 2.5% Glycerol, pH 8.0), four independent dilutions of each sample were prepared to a volume of 450 µl. Then, 50 µl of 1% SDS (in excipient) was added to achieve a final volume of 500 µl with 0.1% SDS. Each dilution was mixed and allowed to sit at room temperature for 15 minutes prior to determining absorbance readings. Samples were analyzed using a Beckman DU 640 spectrophotometer at 260 nm. Using excipient only, the absorbance reading was adjusted to zero at 260 nm, and then three readings of each dilution were obtained and averaged. Viral particle concentrations were determined using the following formula:[5]

Concentration (particles/mL) = (average A260 nm) x (1.1×10^{12} particles/mL) x (1/Dilution factor)

Anion Exchange HPLC (performed by Genetic Therapy, Inc.): Duplicate preparations of each sample were made in excipient with one part sample to 24 parts excipient buffer. The calibration curve used for quantitation utilized a range of 5.5×10^{11} to 5.5×10^{9} particles/mL, and was based on a wild-type Ad5 standard used by Genetic Therapy, Inc. — TCA-349 (5.5×10^{12} particles/mL, assigned using the optical density method as described above). Samples were analyzed at a flow rate of 1 mL/min on an Agilent HP1100 HPLC (Hewlett Packard) and utilizing a 1 mL Resource™ Q column (Amersham Pharmacia Biotech). The 100 µl samples were loaded in 300 mM NaCl in 50 mM HEPES at pH 7.5, and then eluted with a linear gradient of 300-600 mM NaCl in 50 mM HEPES at pH 7.5.[6] Peaks were detected at a 260 nm wavelength.

3. Infectious Titer Methods

Hexon FACS (performed by Genetic Therapy, Inc.): The cells used for the hexon FACS assay are an E1/E2a complementing derivative of A549 (AE1-2a).[7,8] AE1-2a cells were maintained and cultured in Richter's medium supplemented with 10% FBS. Twenty-four hours before infection, the AE1-2a cells were seeded at a density of 5×10^{5} cells/well in six-well tissue culture plates. On the day of infection, three-fold serial dilutions (1:1 x 10^{3} to 1:5.65 x 10^{9}) of the sample were prepared in Richter's medium with 5% FBS. Viral dilutions (1 mL/well) were applied to the cells in duplicate, and the plates were incubated at 37 °C with 5% CO_2. At 24 hr post-infection, the cells were trypsinized and treated overnight with 1% paraformaldehyde in DPBS at 2-8 °C. The samples were then permeabilized and stained with a FITC-conjugated mouse anti-Hexon-antibody (Chemicon), and then analyzed on a BD Facscalibur Flow Cytometer (FACS).[7] The data (% hexon positive cells as a function of viral dilution) were fit to a four parameter logistic equation using analysis software (GraphPad Prism). The FACS infectious titer (F.I.T.), in FACS Infectious Units per mL (FIU/mL), was calculated as:

F.I.T. (FIU/mL) = [(0.5 FIU/cell) x (5×10^{5} cells/well)] / [(EC_{50}) x (1 mL/well)]

where EC_{50} is expressed as the viral dilution at which 50% of the cells were positive for hexon staining. The constant (0.5 FIU/cell) assumes one infectious unit per positively stained cell. However, when 50% of the cells stain positive for hexon, it could be expected that a significant number of the infected cells have been infected by more than one adenoviral particle.[9] Therefore, this method of calculation is expected to underestimate the infectious titer. Typically less variable than many other methods, this method is particularly useful for stability studies, and similar comparisons, where precision may be more critical than accuracy.

NAS (normalized adjusted standard) Titer (performed by Genetic Therapy, Inc.):[9,10] HEK 293 cells were maintained in DMEM with high glucose (4.5 g/L D-glucose) and supplemented with 10% bovine calf serum. Twenty-four hours before infection, HEK 293 cells were seeded at a density of 4×10^{4} cells/well in 96-well tissue culture plates containing DMEM with low glucose (1.0 g/L D-glucose) and supplemented with 10% bovine calf serum. Four 96-well plates were prepared for each sample.

On the day of infection, dilutions of the sample were prepared in DMEM with low glucose and no serum.[10] Duplicate dilution series were prepared for each sample, and fourteen viral dilutions (1:5 x 10^{7} through 1:1.28 x 10^{10}, with a 1.41-fold or 2-fold separation

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Table 1. Particle Concentration, Infectious Titer, pH, and Appearance Analysis

Treatment	OD_{260} (vp/ml)	AX-HPLC (vp/ml)	Hexon FACS (FIU/ml)	NAS Titer (IU/ml)	pH
Freeze-Thaw Cycles					
1	5.87×10^{11}	6.25×10^{11}	9.3×10^{9}	1.9×10^{11}	7.94
2	5.79×10^{11}	6.66×10^{11}	8.4×10^{9}	5.5×10^{10}	7.94
3	5.78×10^{11}	6.24×10^{11}	8.5×10^{9}	1.4×10^{11}	7.94
Shipping simulation	ND	6.56×10^{11}	9.0×10^{9}	1.1×10^{11}	8.00
2-8°C Stability					
4 hr	5.93×10^{11}	5.23×10^{11}	1.0×10^{10}	2.3×10^{11}	7.92
8 hr	5.75×10^{11}	5.85×10^{11}	ND	1.4×10^{11}	7.93
24 hr	5.57×10^{11}	5.47×10^{11}	5.6×10^{9}	1.3×10^{11}	7.95
72 hr	5.68×10^{11}	ND	6.4×10^{9}	6.8×10^{10}	8.01
168 hr	5.72×10^{11}	4.72×10^{11}	7.0×10^{9}	7.8×10^{10}	7.97
22-25°C Stability					
4 hr	5.81×10^{11}	5.46×10^{11}	1.0×10^{10}	2.1×10^{11}	7.91
8 hr	5.90×10^{11}	5.35×10^{11}	ND	6.1×10^{10}	7.94
24 hr	5.53×10^{11}	5.53×10^{11}	4.5×10^{9}	1.4×10^{11}	7.89
72 hr	5.56×10^{11}	ND	5.9×10^{9}	5.6×10^{10}	7.99
168 hr	5.41×10^{11}	4.47×10^{11}	5.9×10^{9}	1.2×10^{11}	7.98

All vials were clear and flawless by appearance.

between each dilution) were used for infection. Twelve replicates of each dilution were plated, while leaving one row of uninfected cells on each assay plate as a negative control. The viral dilutions were applied to the cells for one hour, and then replaced with DMEM with low glucose and supplemented with 10% bovine calf serum. The plates were incubated for 10 days at 37 °C ± 2 °C and 5% CO_2. On the tenth day following infection, the samples were scored for the presence of CPE. The infectious titer (Infectious Units/mL or IU/mL) was determined utilizing the following equation:

$$\text{NAS Titer (IU/mL)} = -\{[\ln(1 - (p_w / n))] \times D\} / (A_w \times C_w \times I \times t^{1/2})$$

where p_w is the number of positive wells per dilution, n is the total number of wells per dilution, D is the dilution factor, A_w is the area of the bottom of the well in cm^2 (i.e.: 0.32 cm^2), C_w is the confluence (estimated by visual examination) of the well at the time of infection, I is a constant (2.38×10^{-4} cm per particles $sec^{1/2}$) that incorporates the diffusion coefficient, and t is the exposure time in seconds.

4. Physical Characterization Methods

Appearance (performed by Genetic Therapy, Inc. and University of Texas at Austin). The appearance of each sample was evaluated using white light. Using both black and a white backgrounds, the vials were visually inspected for any particulate matter, as well as for color and overall clarity.[11] The following definitions were applied:

·**clear and flawless** - preparation had no visible color, particulate matter, or other visual interference when observed against a white or a black background.

·**hazy** - preparation had a faint white color that was only detectable against a black background, but was not apparent in bright light. It was possible to see objects placed behind the preparation when using either a white or black background.

·**cloudy** - preparation looked white against a black background. When looking through the vial against either a white or black background, it was difficult to distinguish the detail of an object, even though its outline could be detected.

·**opaque** - preparation had an opalescent color (blue-white) against a black background and in bright light. It was not possible to visualize any object placed behind the sample, regardless of background color.

pH (performed by Genetic Therapy, Inc.). pH readings were obtained utilizing a standard pH meter (Corning) with a combination electrode. The meter had been calibrated with pH 7 and pH 10 calibration standards (Fisher).

Particle size distribution by Dynamic Light Scattering (performed by University of Texas at Austin). As a test for aggregation, particle size was assessed with a DynaPro LSR laser light scattering device and detection system (Protein Solutions, Lakewood, NJ). Forty-five microliters of sample were slowly added to a quartz cuvette, which

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was then sealed with a plastic stopper. The cuvette was placed in the detection system, and particle size readings were collected every ten seconds over a one-minute period. Using either Dynamics or DynaLS software (Protein Solutions, Lakewood, NJ), regularization histograms were developed and hydrodynamic radii values were assigned to various subpopulations within the sample. Each analysis was qualified by comparing the sample cuvette to a standard solution of polystyrene beads with a hydrodynamic radius approximately the size of the adenovirus (35 + 1.5 nm, Duke Scientific, Palo Alto, CA), and to a cuvette containing the excipient (blank control). The absence of any detectable particles in the excipient, and readings within 2 nm of the labeled value of the bead standard, were the criteria for qualification of each analysis.

Photon Correlation Spectroscopy (performed by Transgene): A N4 Plus Submicron Particle Size Analyzer (Beckman Coulter, Miami, FL) was set to 90° and used to determine aggregation by photon correlation spectroscopy. N4 L100 and L500 Latex Control Particles (Beckman Coulter) were used during each run to confirm correct operation of the instrument. Samples of a Transgene adenoviral standard were diluted with excipient in disposable micro-cuvettes (2 dilutions per sample), and then analyzed in both Unimodal and Size Distribution Processor (SDP) analysis modes. The unimodal size and polydispersity indexes of the ARM samples were compared to those of the in-house standard. The SDP intensity bar charts were also examined for evidence of particles larger than those of the in-house standard. A sample is considered aggregated when any of the following conditions are met: the unimodal size of the sample is at least 10% larger than the expected value; the polydispersity index is >0.12; or particles, whose sizes are distinct and superior to the reference size in the same buffer, are detected on the SDP intensity bar chart.

Table 2. Dynamic Light Scattering Analysis

Treatment	Replicate #	Radius ± S.D. (nm)	Proportion (%)	Aggregated?	Appearance
Freeze-Thaw Cycles					
1	1	55.7 ± 6.2	100%	No	Clear and flawless
	2	54.3 ± 8.2	100%	No	Clear and flawless
	3	56.7 ± 6.2	100%	No	Hazy
	4	56.6 ± 4.5	100%	No	Hazy
2	1	57.3 ± 4.7	100%	No	Clear and flawless
	2	58.7 ± 7.6	100%	No	Clear and flawless
3	1	56.1 ± 4.7	100%	No	Hazy
	2	55.1 ± 10.0	100%	No	Hazy
Shipping simulation	1	58.9 ± 12.4	100%	No	Clear and flawless
	2	54.8 ± 11.4	100%	No	Clear and flawless
2-8°C Stability					
4 hr	1	60.0 ± 2.8	100%	No	Clear and flawless
	2	54.5 ± 6.4	100%	No	Clear and flawless
8 hr	1	77.0 ± 12.2	3.0%	Yes	Opaque
		721 ± 139	70%		
		3960 ± 460	27%		
	2	58.5 ± 11.7	100%	No	Clear and flawless
24 hr	1	424 ± 64	34%	Yes	White precipitate
		3650 ± 440	66%		
	2	407 ± 31	29%	Yes	Cloudy
		2790 ± 930	71%		
72 hr	1	321 ± 92	20%	Yes	White precipitate
		3890 ± 360	80%		
	2	56.3 ± 5.7	59%	Yes	Clear and flawless
		320 ± 23	12%		
		4030 ± 580	29.1%		
168 hr	1	189 ± 24	8.9%	Yes	Extremely opaque
		3870 ± 710	91%		
	2	56.0 ± 11.8	100%	No	Clear and flawless
22-25°C Stability					
4 hr	1	63.8 ± 17.5	100%	No	Clear and flawless
	2	56.1 ± 5.3	100%	No	Clear and flawless
8 hr	1	180 ± 1	2.7%	Yes	Opaque
		612 ± 84	31%		
		3980 ± 460	66%		
	2	55.9 ± 6.4	100%	No	Clear and flawless
24 hr	1	617 ± 87	31%	Yes	Opaque
		3930 ± 560	69%		
	2	58.8 ± 12.6	100%	No	Clear and flawless
72 hr	1	694 ± 82	30%	Yes	Opaque
		4030 ± 220	70%		
	2	59.4 ± 2.3	2.0%	Yes	Clear and flawless
		895 ± 119	42%		
		4050 ± 630	56%		
168 hr	1	54.2 ± 8.9	1.9%	Yes	Extremely opaque
		895 ± 96	45%		
		3860 ± 470	53%		
	2	58.7 ± 6.1	100%	No	Clear and flawless

Results

Stability during repeated freeze-thaw cycles. The Adenoviral Reference Material is shipped frozen and must be thawed for use. The initial thaw of a vial is defined as one freeze-thaw. In order to determine whether a partially used vial of the ARM might be re-frozen for later use, stability of the ARM was assessed over a series of one, two, or three freeze-thaw cycles. Duplicate vials were sampled for each freeze-thaw cycle, requiring six vials per experiment. To eliminate day-to-day analytical variability, the freeze-thaw cycles were scheduled so that all analyses were performed on the same day.

In the first experiment (Table 1, page 64), samples were analyzed for pH, appearance, particle concentration

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Table 3. Analysis of aggregation by Photo Correlation Spectroscopy

Treatment	Replicate #	Unimodal size (nm)	Polydispersity Index	Aggregated?
Freeze-Thaw Cycles				
1	1	113	0.04	No
	2	108	0.08	No
2	1	753	1.37	Yes
	2	609	1.34	Yes
3	1	364	1.04	Yes
	2	317	1.27	Yes
Shipping simulation	1	105	-0.02	No
	2	106	-0.03	No
2-8°C Stability				
4 hr	1	106	0.02	No
	2	106	-0.02	No
8 hr	1	106	-0.04	No
	2	106	-0.01	No
24 hr	1	106	0.01	No
	2	107	0.01	No
72 hr	1	105	-0.01	No
	2	105	-0.02	No
168 hr	1	104	-0.03	No
	2	104	0.01	No
22-25°C Stability				
4 hr	1	106	-0.01	No
	2	106	0.01	No
8 hr	1	106	-0.01	No
	2	106	0.00	No
24 hr	1	107	-0.03	No
	2	107	0.00	No
72 hr	1	106	-0.02	No
	2	105	-0.02	No
168 hr	1	104	-0.03	No
	2	103	0.01	No

by OD_{260} and Anion Exchange Chromatography, as well as infectivity by NAS Infectious Titer and Hexon-FACS. Using either OD_{260} or Anion Exchange Chromatography (ANOVA, $p > 0.05$), no significant loss of particle titer was observed after two or three freeze-thaw cycles, when compared to the control condition of one freeze-thaw. While the NAS infectious titer appears lower after the second freeze-thaw, this apparent decrease is within the expected variability for the assay (see Discussion). Analysis of the replicates by ANOVA indicated no significant change in the values with freeze-thaw cycle ($p > 0.05$). Likewise, Hexon FACS did not indicate a significant drop in infectious titer. Appearance and pH of the samples were also unchanged.

Using Dynamic Light Scattering, samples of the ARM were screened for aggregation phenomenon during repeated freeze-thaw cycles. The vials tested in this experiment were also examined for appearance (Table 2, page 65). When compared to one freeze-thaw, there was no observable change in the average hydrodynamic radius of the ARM after two or three freeze-thaw cycles. However, significant clouding of the formulation was observed sporadically. This effect appeared to be vial specific since it was detected in two of four vials after one freeze-thaw, and two of two vials after three freeze-thaws, but not in either of the samples receiving two freeze-thaws.

Aggregation during repeated freeze-thaw cycles was also explored using Photon Correlation Spectroscopy (Table 3). This experiment was performed using separate vials from those used in the experiments detailed in Table 1 and Table 2. The measured unimodal size of the ARM and the polydispersity index increased after 2 or 3 freeze-thaw cycles, suggesting that aggregation of the ARM occurred. Size Distribution Processor analysis of the same data (an alternate analysis mode designed to detect discrete populations) also indicated multiple particle sizes in all samples that had gone through 2 or 3 freeze-thaw cycles (data not shown).

Shipping simulation. For each shipping simulation, two vials of the ARM and eight vials containing 0.5 mL ARM formulation buffer were wrapped in brown paper, or bubble wrap, and placed in an aluminum screw-top container. The aluminum container was placed in a 14 kg ATCC®-approved shipper having 8 cm thick foam walls. The shipper was filled with 12-13 kg of dry ice, and then sealed with a 10 cm thick foam lid and standard packing tape. The shipper was exposed to 40 °C for two days and then 50 °C for one additional day. At the end of the simulation, the package was opened and the contents were examined and analyzed. Approximately one-third of the original dry ice remained at the end of the simulations.

Samples from one simulation were analyzed for pH, appearance, particle concentration by Anion Exchange Chromatography, and infectivity by NAS Infectious Titer and Hexon FACS (Table 1). Aggregation was evaluated by Dynamic Light Scattering in a second simulation (Table 2), and by Photon Correlation Spectroscopy in a third simulation (Table 3). Each simulation was performed in a different facility. In all analyses, the results were within an acceptable range of the control values (see Freeze-Thaw #1), indicating that the ARM was stable during

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the simulated shipping conditions.

Short-term stability. Finally, the Adenoviral Reference Material was tested for stability during prolonged exposure to common laboratory conditions. Vials of the ARM were removed from storage in an ultra-low freezer (-80 °C) and thawed at room temperature. The samples were then maintained at conditions simulating either refrigerator (2-8 °C) or countertop (22-25 °C) storage. Samples were removed for analysis at five time points: 4, 8, 24, 72, and 168 hours.

Samples were analyzed for pH, appearance, particle concentration by OD_{260} and Anion Exchange Chromatography, and infectivity by NAS Infectious Titer and Hexon FACS (Table 1). Appearance and pH of these vials did not change with exposure to either storage condition. At both 4 °C and 25 °C, particle concentrations by AX-HPLC decreased approximately 17% between 24 and 168 hours (ANOVA with Tukey post-hoc test, $p < 0.05$). When exposed to bench-top storage conditions, a drop of 6% was observed between eight and 24 hours for the OD determination of particle concentration (ANOVA with Tukey post-hoc test, $p < 0.05$). At either temperature, there was a trend, although not statistically measurable, toward a loss of infectious titer after four hours.

A second series of vials was stored in a temperature-controlled environment at each storage condition, and then analyzed for appearance and for aggregation by Dynamic Light Scattering (Table 2). Under refrigerated storage, the hydrodynamic radius of the ARM did not change for the first four hours of storage. Both vials analyzed at that time point also remained clear and flawless. However, by eight hours the observed hydrodynamic radii increased in one of two vials, suggesting aggregation. This result was also observed in the vials analyzed after 24, 72, and 168 hours. At bench-top storage conditions, there was some indication that aggregation began as early as four hours, because the hydrodynamic radius of the ARM in one of two vials increased to 63.8 ± 17.5 nm from a control value of 55.72 ± 6.2 nm — the other vial remained unchanged. After eight hours at bench-top storage, multiple populations of particles, with hydrodynamic radii much greater than the control value were detected in at least one of two vials. Some vials, which appeared to contain aggregated ARM as determined by the hydrodynamic radii, no longer appeared clear and flawless. However, there were vials with severely altered hydrodynamic radii that did not change in appearance.

Finally, fresh vials of the ARM were analyzed for aggregation by Photon Correlation Spectroscopy (Table 3). Neither the unimodal size nor the polydispersity index changed after exposure to either storage condition for any time point from four to 168 hours. Analysis of the data by Size Distribution Processor yielded the same result (data not shown).

Discussion

The wild type Ad5 Adenoviral Reference Material is intended for use in standardizing measurements of particle concentration and infectious titer. Such standardization will increase the comparability of safety and efficacy data for this maturing class of gene therapy products. The studies described in this report were designed to evaluate handling and storage conditions of the ARM in a typical laboratory.

The ARM is being distributed by ATCC® as a frozen material in its approved containers containing dry ice. No evidence of vector or formulation breakdown was observed in the shipping simulations. Appearance, pH, particle concentration, and infectious titer remained unchanged by the shipping conditions described. Likewise, two independent assays did not detect aggregation of the ARM following shipping simulations. These studies indicate that the shipping configuration approved and used by ATCC® is suitable for this material.

Analysis of particle concentrations and infectious titers did not indicate any deleterious effect from up to three sequential freeze-thaw cycles of the ARM. Likewise, analysis of samples by Dynamic Light Scattering gave no indication that repeated freeze-thaw cycles caused aggregation. This information is in agreement with preliminary data submitted to the ARMWG about the specific formulation chosen for the ARM.[12] However, data obtained by Photon Correlation Spectroscopy suggested that some aggregation began after the second freeze-thaw. Given this information, we recommend that the ARM should not be re-frozen for later use after its initial thaw.

From the short-term stability studies, the particle concentrations and infectious titers fluctuated throughout the 168-hour time period, whether the material was stored at refrigerator (2-8 °C) or benchtop (22-25 °C) conditions. Using HPLC, we were able to detect a small loss of particle concentration. While the infectious titers did appear to trend downward with time, resolving those trends statistically was not possible. It should be noted that the design of the study only allowed two vials to be used for each condition, and thus limited the ability to determine the significance of the small losses in titer. As is typical with biological assays, the intra- and inter-assay variability of the NAS Titer was relatively large. During characterization of the ARM, the repeatability standard deviation for this method was 27%.[13] Given an observed average NAS Titer (Table 1) of 1.2×10^{11} IU/mL, replicate observations would be predicted to fall within +/- 3 x 27% or 9.8×10^{10} IU/mL. The assessment of infectious titer by hexon FACS, and the particle concentration by either method used, provided a greater degree of precision. However, the study design did not allow robust statistical analysis of a sufficient number of independent samples. While tentative conclusions may be reached from examination of the data trends, a larger sample size would be required to substantiate that the observed changes were statistically significant.

In the studies performed at bench-top and refrigerator storage conditions, the two methods employed to assess aggregation phenomenon provided conflicting results. Photon Correlation

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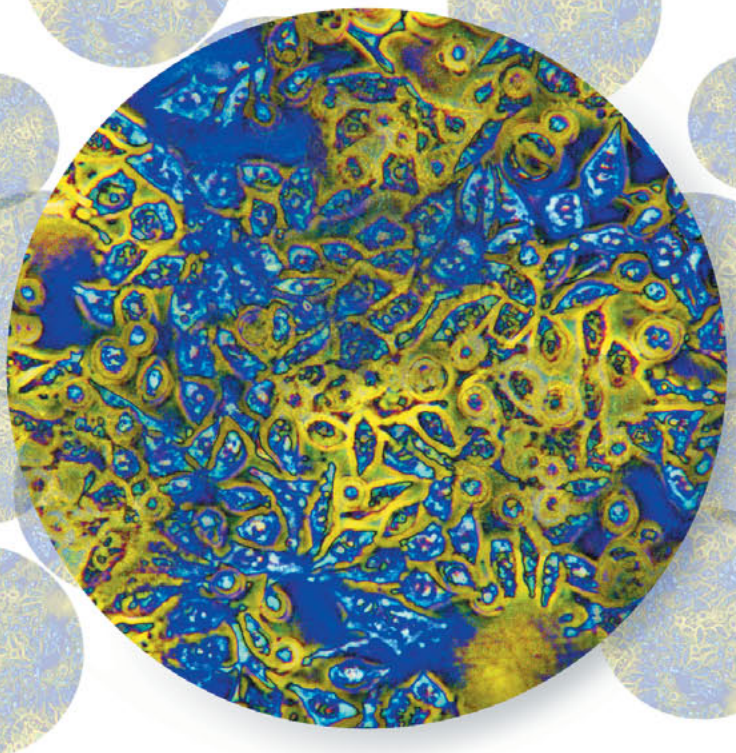

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spectroscopy indicated that no significant aggregation occurred at either temperature for up to 168 hours. However, Dynamic Light Scattering indicated that particles of increased size began appearing after four hours at either temperature. Still, this effect was vial specific, since half the time (five out of the ten time points assessed for both studies) one vial demonstrated significant aggregation formation while the other did not. A larger sample number would be required to determine the significance of this observation, and this study further illustrates the importance of incorporating sufficient sample numbers in any testing plan for product stability.[14]

The vials that were analyzed by Dynamic Light Scattering were visually examined. Many of the vials that exhibited particles with increased hydrodynamic radii also appeared hazy, cloudy, opaque, or to contain a white precipitate. Throughout the experiments, some vials (Table 2) appeared hazy even though no change was observed in the hydrodynamic radius of the ARM. Still other vials continued to appear clear and flawless even though particles were detected with increased size. It is therefore unclear what the relationship is between the measured hydrodynamic particle radii and the sample appearance. The cause of the hazy or opaque appearance may not be aggregated viral particles, but may instead be precipitation of a formulation component or impurity. A highly chromogenic impurity, that is not associated with the virus, has been detected in the ARM.[15] The samples labeled as cloudy and opaque in which aggregates were found, as well as some labeled as clear, were tested for the presence of the contaminant by RP-HPLC. In all samples tested, there were very low levels of the highly chromogenic contaminant (personal communication with Gary Vellekamp of Schering Plough Research Institute). Thus, there is not a good correlation between any two of the phenomena: cloudy vials, increased hydrodynamic radii observed by Dynamic Light Scattering, or the presence of the chromogenic impurity, and it remains uncertain whether there are any causal links.

There were considerable discrepancies between the results obtained with Photon Correlation Spectroscopy and Dynamic Light Scattering for both the short-term stability studies and the freeze-thaw experiments. Moreover, in cases where either method indicated aggregation, the infectious titer did not decrease to the extent that might be expected for a highly aggregated virus preparation (i.e.: samples from 72 hours at 22-25 ºC). It is important to note that separate samples were used for the aggregation tests and the titer assays. In addition, the two methods used for aggregation produced similar measurements for unaggregated particles, even though Dynamic Light Scattering data is reported as the radius, and Photon Correlation Spectroscopy data is reported as the diameter. Discrepancies in detecting apparent aggregation may be due to differences in the software packages used to assess particle size data for assignment to specific populations within a single sample. It is also theoretically possible that some small difference in sample handling, by the different laboratories performing those analyses, led to different amounts of aggregation. Given the vial-specific nature of some observed phenomena, plus the intra-laboratory and intra-assay differences in observed stability, it might have been useful for at least one assay, such as a determination of infectious titer, to be performed by all participating laboratories on all samples.

Based upon the information currently available, we have decided to make the most conservative recommendations for handling the ARM. The final recommendation for laboratory use is to thaw the vial once and use the material within four hours after thawing. Longer storage after thawing, or repeated freezing and thawing cycles, may cause the viral particles to aggregate.

Acknowledgements
We thank Shellie Callahan (UT Austin), David Dolinger Few (GTI), Catherine Fahrner (Transgene), Tracey German (GTI), and Mano Venkatesan (GTI) for their expert technical assistance with this study, and Mike Kadan (GTI) and Russette Lyons (GTI) for critical review of this manuscript.

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