REPORT OF FIELD EMISSION SCANNING ELECTRON MICROSCOPY RESULTS FOR ARMWG CHARACTERIZATION OF VR-1516 REFERENCE MATERIAL.

FESEM Data and

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PURPOSE:

This report summarizes results using field emission scanning electron microscopy (FESEM) analysis to characterize the VR-1516 reference material. VR-1516 is a wild-type Ad5 virus proposed for use as a comparative adenovirus (Ad) standard.

SUMMARY:

VR-1516 was analyzed using FESEM image analysis to characterize mean particle diameter, perimeter, and multiplet order. The total estimated mean diameter of the single particles was 86.2 $\pm$ 5.37 nm across dilutions and surface variations. The total estimated mean perimeter of the single particles was 335.9 $\pm$ 25.6 nm. We observed a dilution buffer effect on both the diameter and perimeter measurement. As well, use of ARMWG buffer resulted in some inconsistencies of Ad particle distribution on the grid surfaces and idiosyncratic aberrant imaging, relative to our previous experiments using SCH 58500 in GFB buffer. Particle perimeter distribution analysis was applied to look at micro-aggregation with VR-1516. The analysis revealed that the material consisted predominantly (>70%) of icosahedral shaped single adenovirus particles, with the remaining virus population being doubles, triplets, or multiplets composed of more than three adenovirus particles.

PROCEDURE:

Sample preparation: VR-1516 was thawed at room temperature and an initial 1:2 dilution (v/v) was prepared by adding the appropriate amount of ARMWG buffer directly into the Ad sample. The formulation for ARMWG buffer was 20mM Tris, 25mM $NaCl_2$, 2.5% (w/v) glycerol, pH 8.0 (storage condition: 4°C). Sequential

dilutions (1:4 and 1:8) were then prepared in this buffer from the initial 1:2 dilution using ARMWG buffer. Parallel 1:2 dilutions were made in a cross buffer experiment using gel filtration buffer (GFB) from the SCH 58500 program. The formulation for GFB was 14mM Tris, 10.6mM NaH_2PO_4, 2mM $MgCl_2$, 2% sucrose, 10% glycerol (w/v), pH 7.8 (storage condition: -80°C). Each sample was prepared for FESEM image analysis. A 20 μL aliquot of VR-1516 (undiluted and diluted samples) was adsorbed onto the surface of a 200 mesh formvar stabilized carbon coated copper grid (Ted Pella, Redding, CA) for 2 minutes at room temperature, fixed with 1.5% gluteraldehyde in phosphate buffered saline (pH 7.6) for 5 minutes at room temperature, then quickly rinsed once with phosphate buffered saline (PBS) and twice (<30 seconds) with distilled water. Residual droplets from each step were removed by wicking with filter paper. Samples were immediately stained with 0.22 μm filtered 1% w/v aqueous uranyl acetate for 10 minutes at room temperature, rinsed 4 times with distilled water, and dried by wicking with filter paper. Aliquots of each sample were also fixed for the analysis using the same grids described above except each grid was pre-treated for 20 minutes with 1% poly-L-lysine. All grids were affixed onto aluminum metal support stubs using carbon tape, grounded further with a fine tip carbon pen, and stored in a dry container until imaging.

Image analysis: FESEM images were prepared with a JEOL model 6330F FESEM (JEOL USA, Inc., Peabody, MA) using the following parameters: 5.0 kV accelerating voltage, 12.5 μamp emission current, 7.0 probe current (spot size), secondary electrons at 20,000x magnification and slow capture scan. Magnification accuracy for the FESEM was tested using an ASTM-certified magnification reference standard (MRS-4, Geller Microanalytical Laboratories, Topsfield, MA).

Particle measurements for mean diameter, perimeter, and multiplet order: Objects in the images were sized and counted by importing FESEM-generated files into Image-Pro Plus v.4.1 (Media Cybernetics, Silver Spring, MD). The counting and

sizing functions were automated using a macro within Image-Pro Plus (IPP). The macro is composed of multiple subroutines to crop the entire area of each image to a standard size, filter the background, and identify the objects within an image that were to be sized and counted. Diameter and perimeter measurements were calculated for each object in the nanometer spatial scale and the objects were sorted by descending size, then exported into Microsoft Excel 97. Sorted objects were then analyzed to determine if they had 1 (single), 2 (double), 3 (triple), or >3 (multiplet) adenovirus particles. No determination of the exact number of virus particles in multiplets was made. This prevented the potential underestimate of virus number from viruses that may lie behind the plane of view. The IPP software was calibrated for magnification using an ASTM-certified magnification reference standard.

RESULTS:

Results for this study can be found in notebook number 46935. Electronic images are stored in the SEM intra-lab server, Union, NJ. The results presented in this report are for research purposes only.

Sample preparation: Preparations of VR-1516 on both substrates (carbon and poly-L-lysine treated carbon) demonstrated good preservation of typical adenovirus particle morphology. However, the adenoviral particles from this sample, whether undiluted or diluted in the ARMWG buffer, did not always evenly distribute over both surfaces. This observation was historically in contrast with results obtained from SCH 58500 preparations prepared with this method on these surfaces (data not shown). A cross buffer study was performed to investigate further. In this experiment both VR-1516 and a batch of rAd/p53 (batch 98W-585-1008) were evaluated undiluted and diluted 1:2 (v/v) in each buffer then prepared for FESEM analysis on untreated carbon substrates. Substrates were prepared in duplicate to evaluate for reproducibility of the sampling method. SCH 58500 particle distribution

was evenly distributed on the carbon substrates and a normal icosahedral geometery of the virus was evident. However, particle distribution for VR-1516 in ARMWG buffer was evenly distributed on the surface of only one grid out of two prepared; normal icosahedral geometry of the viral particles in this sample preparation was evident.

MRS evaluation: The error measurement calculated from the MRS using a 2.0 μm pitch pattern ranged from –0.4% to +0.2%, a value well within the manufacturer's specifications.

Quantitation of virus particles in FESEM images: Images were collected from different areas of grids and the diameter and perimeter for each object in the fields was calculated in the nanometer spatial scale. A total of 85 FESEM images were collected during the analysis, from which 48 were evaluated further using Image-Pro Plus and Microsoft excel 97. Representative FESEM images of VR-1516 on carbon or poly-L-lysine treated carbon are shown in Figures 1 and 2. We also observed aberrant images of VR-1516 that had not been observed either in previous SCH 58500 analyses, or in the cross buffer experiment performed in this study (Figure 3).

Estimated mean diameter and perimeter measurements were calculated for VR-1516 on two different substrates (carbon or poly-L-lysine treated carbon) using IPP and are summarized in Table 1. Ten images were prepared for each sample preparation. The estimated mean diameter and perimeter measurements of VR-1516 in the cross buffer study are summarized in Table 2. Preparations for this experiment were adsorbed onto untreated carbon substrates only. Two images were prepared and analyzed for each sample preparation. The overall mean diameter and perimeter measurements of single adenovirus particles from sample VR-1516, regardless of surface or dilution in ARMWG buffer, ranged from 83.5 ± 3.55nm to 93.1 ± 13.1nm, and 324.5 ±16.0nm to 384.9 ± 75.6nm, respectively

(Tables 1 and 2). The mean diameter and perimeter of undiluted VR-1516 on carbon ranged from 83.5 ± 3.55nm to 90.8 ± 8.20nm and 324.5 ± 16.0 to 358.9 ± 48.7nm, respectively. When diluted 1:2 in ARMWG buffer, diameter and perimeter measurements ranged from 88.8 ± 6.55nm to 93.1 ± 13.1nm and 345.7 ± 28.8nm to 384.9 ± 75.6nm, respectively. But when diluted in GFB buffer, diameter and perimeter measurements were 84.7 ± 8.25nm and 334.3 ± 46.16nm, respectively. Mean diameter and perimeter measurements of undiluted VR-1516 could not be assessed on poly-L-lysine treated carbon because few particles were present on the substrate. In addition, the particles that were present were unevenly distributed over the surface. Upon dilution however, the particles were more densely populated and evenly distributed over the substrate surface. The mean diameter and perimeter measurements at the 1:2 dilution was 87.3 ± 3.74 and 339.1 ± 15.2, respectively. Mean diameter and perimeter measurements at the 1:4 dilution were similar to the 1:2 dilution and was 88.8 ± 3.35nm and 345.7 ± 28.8nm, respectively.

Diameter and perimeter measurements serve a different purpose in the analysis. The diameter measurement assesses particle size by assuming icosahedral symmetry exists. Perimeter is a measure of an object outline and thus is sensitive to morphology changes in the particle. Diameter measurements calculated from samples diluted in ARMWG buffer were more variable and appeared to be slightly larger in size than samples prepared in undiluted or diluted GFB buffer. This trend was not statistically significant in either study however. The expected mean diameter range of adenovirus particles was approximately 60-100 nm. They fall within the expected range for an adenovirus (1,2).

Perimeter measurements of all adenovirus objects in the chosen fields were also analyzed and are summarized in Figure 4. Distribution of the various adenovirus objects was calculated as the percentage total perimeter by object size. All adenovirus particles were sized by perimeter analysis and the total percentage of

perimeter was calculated for singles, doubles, triples and for multiplets with greater than three adenovirus particles. Most of the virus particles from VR-1516 distributed over the grid surfaces were singles. The icosahedral geometry of the virus particles was evident. The number of singles calculated in the sample on carbon was approximately 70-73%, while on poly-L-lysine treated carbon the percentage was approximately 80-90%. The distributions of other adenovirus objects from this sample were significantly less and of those most particles were doubles comprised of two adenovirus particles. During the cross buffer study we compared an expired batch of SCH 58500 to VR-1516 to assess for similarities and differences of particle distributions from both samples. Both samples were prepared undiluted and diluted 1:2 in ARMWG and GFB buffer on untreated carbon. The results for this experiment are shown in Figure 5. Two images were prepared for each sample preparation. SCH 58500 particle distribution was evenly distributed on the carbon surfaces and a normal icosahedral geometry of the virus was evident. Particle distribution for the SCH 58500, in both buffer preparations, was typical for this sample. The number of singles present in the sample ranged from 60-73%, and an apparent increase in multiplet size was noted, but the result is not unexpected since the batch was expired. The particle distribution for VR-1516 was approximately 73%.

Interestingly VR-1516 samples prepared on either of the carbon surfaces using ARMWG buffer did not always distribute the viral particles evenly. This observation was in contrast with results obtained from SCH 58500 samples prepared using this method on either surface but using GFB buffer. In addition to unevenly distributed particles from VR-1516 there appeared to be a very small and random population of aberrant shaped adenovirus particles prepared in the ARMWG buffer (Figure 3). It should be noted that this observation was idiosyncratic. Thus it remains to be seen if this population is inherent in the sample or if it is an artefact of the method. Since the volume of sample supplied for the analysis was small, we could not further

investigate this observation. However, the methodology described in this report can be adapted to further reduce inconsistency with this type of adenovirus sample.

REFERENCES:

1. Bondoc, L.L., et al. (1998). Size distritution analysis of recombinant adenovirus using disc centrifugation. J. Industrial Microbiology and Biotechnology. 20, 317-322.
2. Harrison, S.C. (1991). Principles of virus structure (chapter 3). In Fundamental Virology, Second Edition, edited by B. N. Fields, etl al. Raven Press, Ltd., New York.37-61.

Table 1 Adenovirus particle number, diameter, and perimeter of sample VR-1516 on carbon and poly-L-lysine treated carbon surfaces. Sample dilutions were performed in ARMWG buffer. FESEM mean diameter and perimeter for all singlet adenovirus particles captured were calculated using the Image-Pro Plus automation macro.

Surface	Sample Dilution	N	Mean diameter, nm	Perimeter, nm
Carbon	Undiluted	2196	83.5 ± 3.55	324.5 ± 16.0
	1:2	765	88.8 ± 6.55	345.7 ± 28.8
Poly-L-Lysine treated carbon	Undiluted	ND	ND	ND
	1:2	1171	87.3 ± 3.74	339.1 ± 15.2
	1:4	594	88.8 ± 3.35	345.4 ± 18.4
	Grand Total	4979	86.2 ± 5.37	335.9 ± 25.6

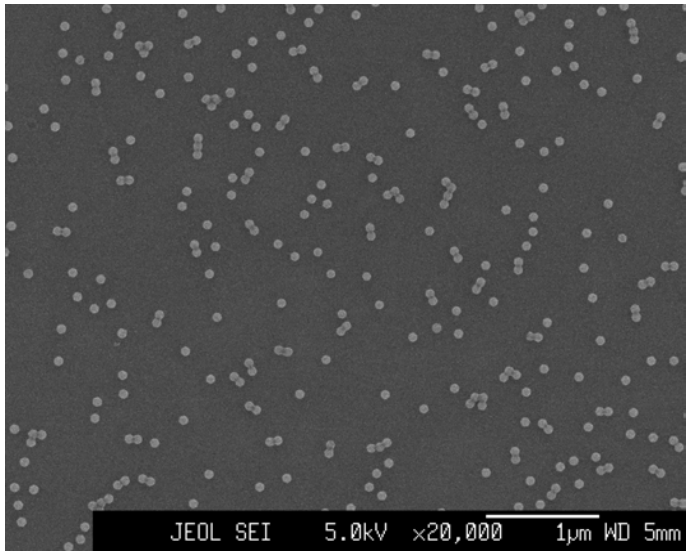
SD standard deviation of the mean
N total number of singlet adenovirus particles
ND not done

Table 2 Cross buffer study: Adenovirus particle number, diameter, and perimeter of sample VR-1516. Sample dilutions were prepared in ARMWG and GFB buffer then prepared in duplicate on carbon surfaces. A total of 2 images were captured and analyzed for each sample. FESEM mean diameter and perimeter for all singlet adenovirus particles captured were calculated using the Image-Pro Plus automation macro.

Buffer	DIlution	N	Mean diameter, nm	Perimeter, nm
---	Undiluted	168	90.8 ± 8.2	358.9 ± 48.66
GFB	1:2	85	84.7 ± 8.25	334.3 ± 46.16
ARMWG	1:2	85	93.1 ± 13.1	384.9 ± 75.59

Figure 1 Representative FESEM image (a) and sorted macro image (b) of VR-1516, diluted 1:2 in ARMWG buffer, and prepared on an untreated carbon grid surface. Images were prepared at magnification of 20,000x and a 5 kV accelerating voltage.

(a) Original FESEM Image



(b) Sorted Image

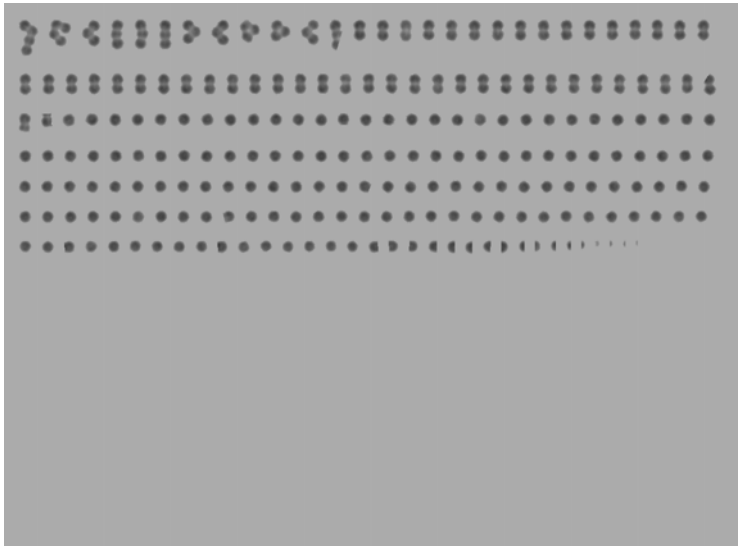
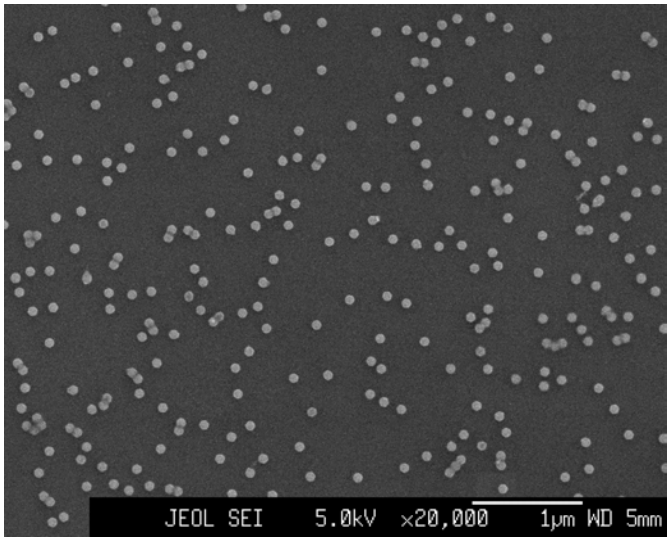


Figure 2 Representative FESEM image (a) and sorted macro image (b) of VR-1516, diluted 1:2 in ARMWG buffer, and prepared on a poly-L-lysine treated carbon grid surface. Images were prepared at magnification of 20,000x and a 5 kV accelerating voltage.

(a) Original FESEM Image



(b) Sorted Image

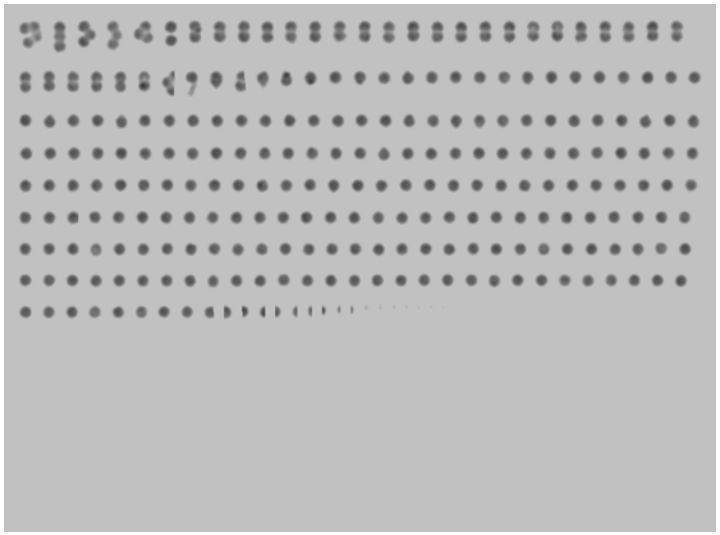
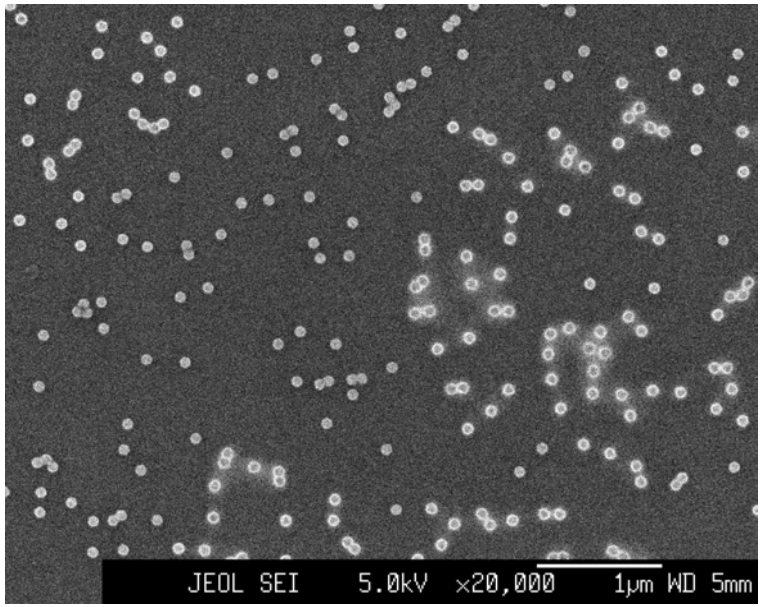
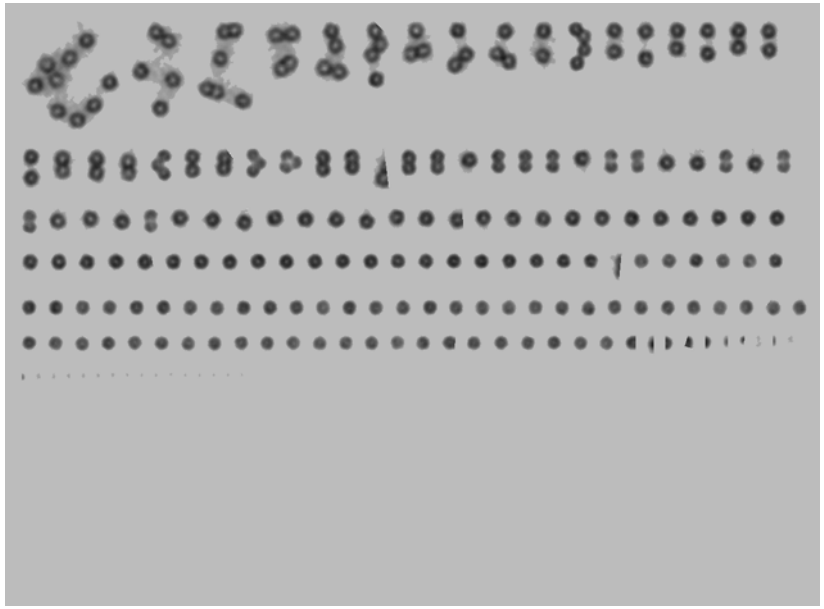


Figure 3 Representative FESEM image (a) and sorted macro image (b) of aberrant samples observed in sample VR-1516. The sample was diluted 1:2 in ARMWG buffer, prepared on a formvar stabilized carbon copper grid surface, and imaged on the FESEM at the parameters indicated. Images were prepared at magnification of 20,000x and a 5 kV accelerating voltage.

(a) Original FESEM Image



(b) Sorted Image



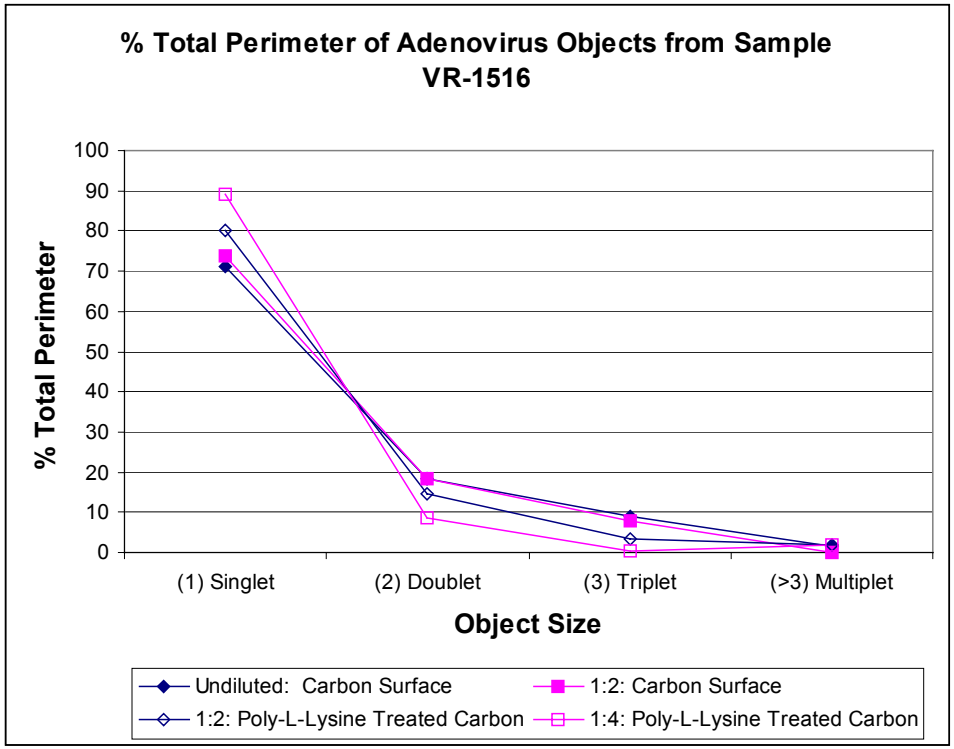


Figure 4 Adenovirus particle distribution of sample VR-1516. Perimeter measurements were calculated for all single, double, triple, and multiplet adenovirus particles at the various dilutions indicated. Distribution of the various adenovirus objects was calculated as the percentage total perimeter by object size. A comparison of undiluted preparations of VR-1516 could not be done because particles did not absorb evenly on the poly-L-lysine treated carbon surface.

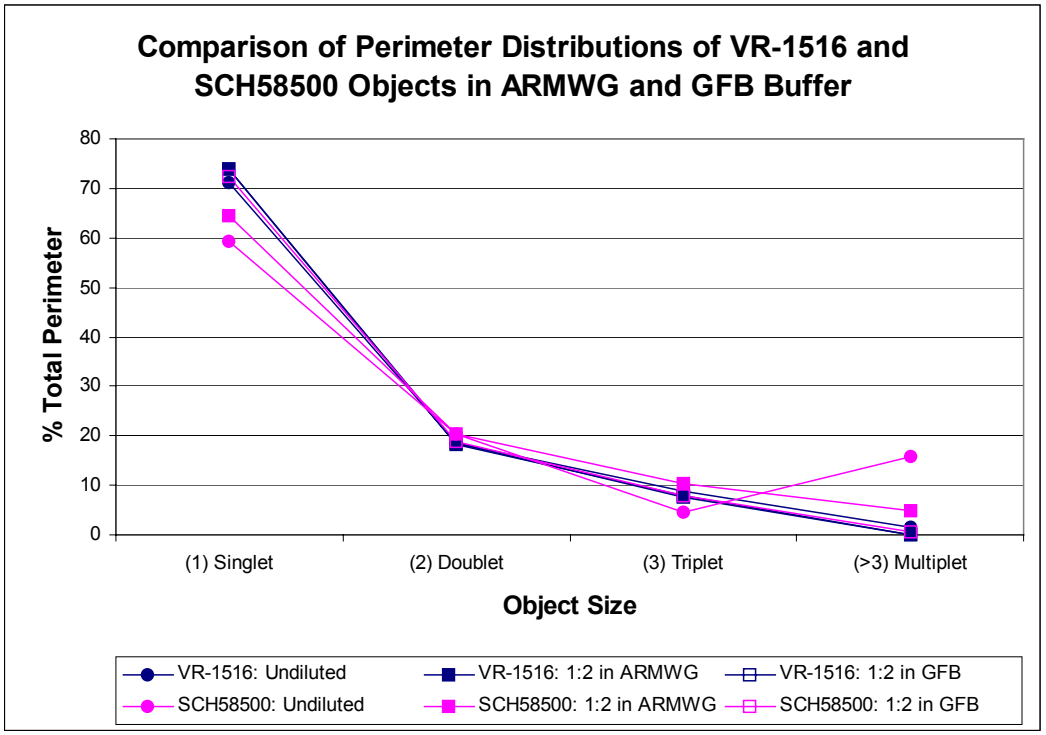


Figure 5 Adenovirus particle distribution of sample VR-1516 and SCH58500 prepared in ARMWG and GFB buffer. Perimeter measurements were calculated for all single, double, triple, and multiplet adenovirus particles at the dilutions indicated. Distribution of the various adenovirus objects were calculated as the percentage total perimeter by object size. All samples were prepared on untreated formvar stabilized carbon copper grids.