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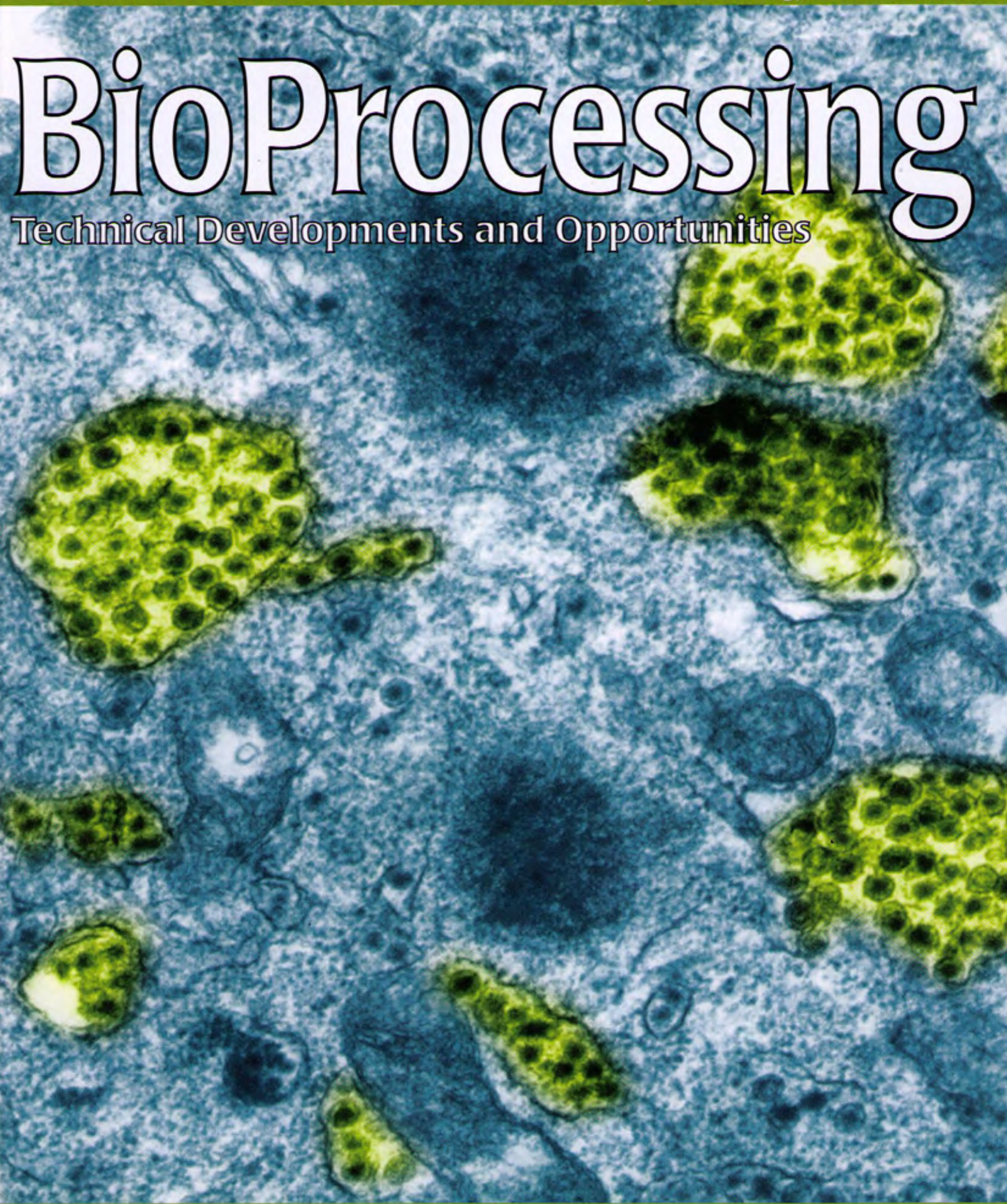

A publication of
The Williamsburg BioProcessing Foundation

Fall 2002

A Statistical Analysis of Adenovirus Reference Material Assay Results

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The Adenovirus Reference Material (ARM) was developed under the guidance of the Adenovirus Reference Material Working Group (ARMWG) and the U.S. Food and Drug Administration (FDA), and was made possible through the donation of services and supplies by a large number of laboratories and institutions from the United States, Canada, France, The Netherlands, Germany, and the United Kingdom.[1,2] All information regarding the development and characterization of the ARM can be found on The Williamsburg BioProcessing Foundation's (WilBio) website: http://www.wilbio.com.

The purpose of the ARM is to provide a reference material for use in validating assays and internal standards for adenoviral particle concentration and infectious titer. The NIH Recombinant DNA Advisory Committee recommended the development of such a reference-testing agent in their report issued January 2002.[3]

The ARM consists of purified wild type 5 Adenovirus as described by ATCC®'s catalog number VR-5. This material has been formulated as a sterile liquid in 20 mM TRIS, 25 mM NaCl, 2.5% glycerol, at pH 8.0 at room temperature, and has been stored at -70 °C. The configuration is 0.5 mL in a Type II glass vial with a Teflon-coated gray butyl stopper and an aluminum seal with crimp closure.

The ARMWG assigned the particle concentration and infectious titer based on a statistical analysis of data derived during the characterization phase of the project (presented in this report). Procedures for obtaining and analyzing these data were provided by the ARMWG and have been posted on the WilBio website (http://www.wilbio.com). The particle concentration is 5.8×10^{11} particles/mL, with a 95% certainty that the true particle concentration lies within a range of 5.6×10^{11} to 6.0×10^{11} particles/mL. The infectious titer on HEK 293 cells is 7×10^{10} NAS Infectious Units (NIU)/mL, with

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a 95% certainty that the infectious titer on HEK 293 cells lies within a range of $7x10^{10}$ to $8x10^{10}$ NIU/mL.

The purpose of this report is to document the statistical analyses performed in estimating the particle concentration and infectious titer of the reference material, and to create limits within which future results can be expected to fall.

METHODS

Particle concentration was estimated using the calculations contained in the template spreadsheet ARMWG Particle OD 102601 wk1.xlt.[4] The equations for this calculation can be found in Appendix A-1. Infectious titers were estimated using the following five methods:

1. Average Poisson (the default method in the template spreadsheet ARMWG Infect Titer v0110291.xlt)[5]
2. Spearman-Kärber
3. 20% Trimmed Spearman-Kärber (10% trimmed from each side)
4. 50% Trimmed Spearman-Kärber (25% trimmed from each side)
5. Maximum Likelihood

The equations for these calculations can be found in Appendix A-2, and the statistical properties of the five estimation methods are compared and discussed in Appendix A-3.

The primary focus of the statistical analysis is to characterize the particle number and infectious titer of the reference material. Ninety-five percent confidence intervals of the mean were calculated, which provide statements about the values of the reference material and how well those values are known.

A second calculation presents two and three standard deviation confidence bounds that serve as limits within which any individual observation can be expected to fall. The two standard deviation limits correspond to nominal 95% bounds on an individual value, and the three standard deviation limits correspond to nominal 99.7% bounds on an individual value. These confidence intervals will be useful in the future when laboratories perform assays on this reference material and derive results, which should fall within these limits. In particular, values outside the three standard deviation limits will occur with only a 0.3% probability, and should be considered as outliers and an indication of a problem.

In our analysis, observations outside the three standard deviation limits were identified as outliers and were excluded. Means and confidence intervals were then recalculated without these data points.

Confidence intervals for infectious titer were calculated with data that had been log-transformed, since the standard deviations were large relative to the mean (large CVs), and titer data are typically log-transformed for statistical analysis. The following procedure was used:

1. Log-transform the data (Log base 10).
2. Calculate the mean, standard deviation, and confidence limits of the transformed data.
3. Inverse-transform (antilog) the mean and confidence limits back to the original units (10 raised to the power of the result).

The antilog mean of the log-transformed data is the geometric mean, which can also be calculated as the Nth root of the product of N numbers. The geometric mean is usually less affected by large outliers than the arithmetic average would be, and also results in a smaller number. The antilog confidence limits will always be greater than zero and will not be symmetrical around the geometric mean, with the result that the distance from the geometric mean to the lower limit will always be less than the distance from the geometric mean to the upper limit.

For the infectious titer assay analysis, three separate, one-way Analyses of Variance (ANOVAs) were used to investigate the effects on the data by variations in: the number of cells plated, the passage number used for the starting cells, and the confluency of the cells when the virus was introduced. Linear regressions were also performed to test for non-zero slopes with passage number.

Laboratory replicates were considered as independent observations for the above calculations.

Repeatability was estimated as a pooled standard deviation by using Laboratory as the classification variable and calculating the root mean squared error (RMSE) from a one-way ANOVA. This method is described by the following procedure:

1. Calculate the mean infectious titer for each laboratory.
2. Subtract the mean from each of the data values to derive the deviations.

Table 1. Estimated particle concentration and its standard error (p/mL 10^{10})

Laboratory – replicate	Estimate	Standard error	N
1	59.54	2.68	4
4	60.30	0.34	4
5 – A	53.36	1.85	4
5 – B	54.82	1.97	4
6	67.28	7.08	4
8	55.88	7.85	4
9	57.82	2.98	4
10	57.37	1.08	4
18	57.57	0.73	4
11	54.59	1.63	4
14	59.17	0.59	4
15	60.96	0.87	4
16	58.44	0.70	4
17	58.39	4.46	4

3. Square all of the deviations.
4. Sum all the squared deviations.
5. Subtract one from the number of replicates that has been summed across all laboratories.
6. Divide the sum of the squared deviations (Step 4) by the sum of the number of replicates minus one (Step 5).
7. Take the square root.

Repeatability was estimated on untransformed data, and its standard deviation serves as a measure of how close replicates will be within the same facility.

RESULTS

Raw data used in statistical analyses are listed in Appendix B.

Particle Concentration

Particle concentration results were received from 15 laboratories; however, data from two laboratories were excluded because the required SOP was not followed. Therefore, the following analysis involves data from the remaining 13 laboratories. Each laboratory performed two replicates resulting in 14 observations, and each observation consists of triplicates at 4 dilutions (one at 80% Ad5 and three at 30% Ad5). Table 1 (page 44) shows the calculation results of the particle concentration for each laboratory and replicate. Values ranged from 53.36×10^{10} p/mL to 67.28×10^{10} p/mL.

Table 2 provides information on how well the particle concentration is known, and shows the particle concentration mean, standard deviation, and 95% confidence bounds about the mean. The mean was 58.25×10^{10} p/mL with 95% limits from 56.28×10^{10} p/mL to 60.22×10^{10} p/mL. This result indicates with 95% certainty that the true particle concentration mean of the reference material lies within 56.28×10^{10} to 60.22×10^{10} p/mL.

Table 3 presents 2 and 3 standard deviation limits for the particle concentration. The two standard deviation limits are from 51.41×10^{10} p/mL to 65.09×10^{10} p/mL. The three standard deviation limits are from 48.00×10^{10} p/mL to 68.59×10^{10} p/mL.

Table 2. 95% confidence on the interval on the mean (p/mL 10^{10})

Lower	Upper	Mean	Standard deviation	N
56.28	60.22	58.25	3.42	14

Table 3. Two and three standard deviation limits (p/mL 10^{10})

Number of standard deviations	Lower bound	Upper bound	Mean	Standard deviation	N
2	51.41	65.09	58.25	3.42	14
3	48.00	68.50	58.25	3.42	14

No observations fell outside the three standard deviation limits, so no outliers were identified or excluded from the analysis.

It is important to understand the difference between Tables 2 and 3. The confidence interval in Table 2 is for a mean of 14 observations. Thus, only means resulting from 14 observations can be expected to fall within these limits. At this point, the particle concentration of the Ad5 reference material can be estimated as 58.25×10^{10} p/mL $\pm$ 1.97×10^{10} p/mL. As more observations are collected, this interval will become narrower and the mean particle concentration will become far better known.

However, any laboratory performing a one-replicate assay on the reference material can expect to get a result that is quite different from the mean particle concentration that is reported in this article, and the limits within which these future observations should fall is shown in Table 3. Nominally, 99.7% of all future results should fall within the 3 standard deviation limits. As more information is collected, the mean and standard deviation will become better known and may shift. However, the three standard deviation limits will remain wide and can be relied upon to indicate if a future assay results could have been expected.

Infectious Titer

Infectious titer results were received from 18 laboratories. Data from one laboratory were excluded because the SOP was not followed (a different dilution series and a different number of wells were used). Additionally, one replicate from each of two laboratories was not included because the assays did not meet specifications. Thirteen laboratories sent results on two replicates, two laboratories had one replicate, and two laboratories had four replicates, resulting in a total of 36 observations. Replicates were considered as independent observations, meaning that the laboratories with four replicates "counted" more and the laboratories with one replicate "counted" less. Table 4 (see www.bioprocessingjournal.com/callahan) presents results for all laboratories and replicates. Values varied widely across methods and laboratories, and resulted in a range of 3.968×10^{10} IU/mL to 38.500×10^{10} IU/mL.

Table 5 (page 46) presents the 95% confidence bounds, as well as the two and three standard deviation confidence limits for all estimation methods. A comparison with Table 4 shows that the laboratory #2 values are statistical outliers for the Spearman-Kärber 20% trim and maximum likelihood methods. Except for the Spearman-Kärber untrimmed result (replicate A), the laboratory #2 values for the other methods are outside the two standard deviation bounds. These two observations were subsequently dropped from the analysis.

Table 6 (page 46) presents confidence

bounds for infectious titers without the outlier observations. All of the means and limits shifted downward, as could be expected since an upper outlier was dropped. Also, the sample size dropped from 36 to 34, reflecting the loss of two replicates from the excluded laboratory.

The ANOVA and regression analyses conducted on passage number resulted in no significant differences. Table 7 presents the p-values for these analyses, all of which were greater than 0.05.

However, the same analyses did result in significant differences being identified between the sets of data in which different cell numbers were plated. Table 8 shows that the p-values were less than 0.05 for all estimation methods. The infectious titer geometric mean values when 10,000 cells were plated ($13.77x10^{10}$ IU/mL to $16.80x10^{10}$ IU/mL) are nearly twice those calculated when 40,000 cells were plated ($7.34x10^{10}$ IU/mL to $8.83x10^{10}$ IU/mL).

Table 5. Confidence bounds for infectious titers (IU/mL x 10^{10}). All laboratories and all replicates

Estimation method	Arithmetic		Geometric mean	2 StD bounds		3 StD bounds		95% Bounds on the mean		
	Mean	StD		Lower	Upper	Lower	Upper	Lower	Upper	N
Average Poisson	11.23	6.75	9.84	3.64	26.65	2.21	43.85	8.32	11.65	36
Spearman-Kärber	10.69	5.00	9.83	4.44	21.76	2.99	32.37	8.59	11.24	36
Spearman-Kärber 20% trim	10.53	6.60	9.29	3.66	23.59	2.00	37.58	7.94	10.88	36
Spearman-Kärber 50% trim	9.95	6.82	8.56	3.05	23.97	1.82	40.13	7.19	10.18	36
Maximum likelihood	9.66	6.15	8.52	3.36	21.63	2.11	34.46	7.28	9.97	36

Table 6. Confidence bounds for infectious titers (IU/mL x 10^{10}), Without Laboratory #2

Estimation method	Arithmetic		Geometric mean	2 StD bounds		3 StD bounds		95% Bounds on the mean		
	Mean	StD		Lower	Upper	Lower	Upper	Lower	Upper	N
Average Poisson	9.97	4.24	9.18	4.01	20.97	2.66	31.70	7.94	10.60	34
Spearman-Kärber	10.25	4.50	9.52	4.51	20.10	3.10	29.20	8.36	10.84	34
Spearman-Kärber 20% trim	9.29	3.88	8.66	4.15	18.09	2.87	26.13	7.62	9.85	34
Spearman-Kärber 50% trim	8.61	3.91	7.91	3.50	17.86	2.33	26.85	6.86	9.12	34
Maximum likelihood	8.55	3.76	7.95	3.78	16.70	2.61	24.20	6.98	9.05	34

Table 7. P-values from ANOVAs

Classification Variable	Average Poisson	Spearman-Kärber untrimmed	Spearman-Kärber 20% trim	Spearman-Kärber 50% trim	Maximum likelihood
Passage Number Category	0.8636	0.4678	0.2811	0.1455	0.4836
Passage Number	0.6292	0.4350	0.2403	0.0783	0.3606

Similarly, there were significant differences observed between the sets of data when different confluency values were used. The infectious titer geometric mean values when the cell confluency was 0.25 ($19.26x10^{10}$ IU/mL to $24.44x10^{10}$ IU/mL) were nearly triple those calculated when the confluency was 0.8 ($7.48x10^{10}$ IU/mL to $8.97x10^{10}$ IU/mL).

Table 10 (inserted following pg. 47) shows that only laboratory #10 used a cell confluency other than 0.8, and both laboratory #10 and laboratory #1 plated the test wells at 10,000 cells versus 40,000. These two laboratories each had two replicates. Although not included in these ANOVA analyses, note that laboratory #2 also plated with 10,000 cells and used a 0.2 cell confluency.

Dropping these four observations, confidence limits were calculated for laboratories that plated 40,000 cells and used an 80% cell confluency. These 30 observations were taken from a distribution with the same mean, and with variances due only to inter-laboratory and replicate variability.

Table 11 (page 47) presents descriptive statistics and confidence bounds for all of the estimation methods. The geometric means have again declined since upper outliers have been dropped, and the N is down to 30 after deleting four more observations. The variabilities have also declined, as shown in the smaller widths of the confidence bounds. For the maximum likelihood method, the mean infectious titer is $7.37x10^{10}$ IU/mL with 95% confidence limits from $6.60x10^{10}$ IU/mL to $8.23x10^{10}$ IU/mL. The three standard deviation limits are $3.04x10^{10}$ IU/mL to $17.86x10^{10}$ IU/mL.

Table 12 (page 47) displays repeatability estimates for all analysis methods. Repeatability standard deviations vary from $1.47x10^{10}$ IU/mL for 20% trimmed Spearman-Kärber, to $2.46x10^{10}$ IU/mL for the average Poisson. Repeatability measures how close replicates can be expected to be, and thus smaller repeatability is better. For the maximum likelihood estimator, replicate observations should be within $\pm 3x1.60x10^{10}$ IU/mL, which equals $\pm 4.80x10^{10}$ IU/mL.

Table 8. ANOVA results for cell number plated: P-values and geometric means ((IU/mL)10^{10}

Estimation method	P value	Cell number plated	
		10,000	40,000
		Geometric Mean ((IU/mL)*10**10)	
Average Poisson	0.0005	13.97	7.37
Spearman-Kärber untrimmed	0.0202	14.28	8.65
Spearman-Kärber 20% trim	0.0005	16.80	8.83
Spearman-Kärber 50% trim	0.0010	14.82	8.06
Maximum likelihood	0.0024	13.77	7.34

CONCLUSIONS

For the Adenoviral Reference Material, the mean particle concentration was determined to be $5.825x10^{11}$ p/mL. With 95% certainty, the true mean particle concentration of the ref-

erence material lies between 5.628×10^{11} p/mL and 6.022×10^{10} p/mL.

The Working Group decided to use the maximum likelihood analysis results and only include data from the laboratories that followed the specified protocol. The infectious titer was determined to be 7.37×10^{10} IU/mL. With 95% certainty, the true mean infectious titer of the reference material lies between 6.60×10^{10} IU/mL and 8.23×10^{10} IU/mL.

The WG also agreed that the data should be presented with two significant digits for particle concentration and one significant digit for infectious titer. Thus, the particle concentration is henceforth reported as 5.8×10^{11} particles/mL, with a 95% certainty that the true particle concentration lies within the range of 5.6×10^{11} to 6.0×10^{11} particles/mL. Using the same rationale, the infectious titer on HEK 293 cells is now reported as 7×10^{10} NAS Infectious Units (NIU)/mL, with 95% certainty that the infectious titer on HEK 293 cells lies within the range of 7×10^{10} to 8×10^{10} NIU/mL.

Table 9. ANOVA results for confluency

Estimation method	P value	Confluency	
		0.25	0.8
		Geometric Mean ((IU/mL)*10**10)	
Average Poisson	<.0001	20.36	7.50
Spearman-Kärber untrimmed	0.0046	19.87	8.74
Spearman-Kärber 20% trim	<.0001	24.44	8.97
Spearman-Kärber 50% trim	0.0002	20.63	8.21
Maximum likelihood	0.0007	19.26	7.48

Table 11. Confidence bounds for infectious titers (IU/mL x 10^{10}), Laboratories 1, 2, and 10 not included

Estimation method	Arithmetic		Geometric mean	2 StD bounds		3 StD bounds		95% Bounds on the mean		N
	Mean	StD		Lower	Upper	Lower	Upper	Lower	Upper	
Average Poisson	9.26	3.53	8.65	4.06	18.45	2.78	26.94	7.51	9.96	30
Spearman-Kärber	9.21	2.81	8.83	4.86	16.02	3.61	21.57	7.90	9.86	30
Spearman-Kärber 20% trim	8.44	2.69	8.06	4.39	14.83	3.23	20.11	7.20	9.04	30
Spearman-Kärber 50% trim	7.79	2.81	7.34	3.67	14.69	2.60	20.78	6.45	8.36	30
Maximum likelihood	7.70	2.37	7.37	4.09	13.30	3.04	17.86	6.60	8.23	30

Table 12. Repeatability estimates (IU/mL x 10^{10}

Estimation method	Standard Deviation
Average Poisson	2.46
Spearman-Kärber	1.73
Spearman-Kärber 20% trim	1.47
Spearman-Kärber 50% trim	1.74
Maximum likelihood	1.60

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Table 10. Passage number, cell number plated and confluency for each laboratory

Laboratory	Passage number	Passage number category	Cell number plated	Confluency
1	4	3-4	10,000	0.8
2	5	5-7	10,000	0.2
3 – A, B	11	>7	40,000	0.8
3 – C, D	14	>7	40,000	0.8
4	10	>7	40,000	0.8
5	4	3-4	40,000	0.8
6 – A	23	>7	40,000	0.8
6 – B	24	>7	40,000	0.8
7 – A	6	5-7	40,000	0.8
7 – B	8	>7	40,000	0.8
8	8	>7	40,000	0.8
9	4	3-4	40,000	0.8
10 – B	4	3-4	10,000	0.25
10 - C	7	5-7	10,000	0.25
11	4	3-4	40,000	0.8
12	5	5-7	40,000	0.8
13	6	5-7	40,000	0.8
14	3	3-4	40,000	0.8
15			40,000	0.8
16	6	5-7	40,000	0.8
17	5	5-7	40,000	0.8