Statistical Analysis

of

Adenovirus Reference Material Assay Results:

Determination of Particle Concentration and Infectious Titer

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1 INTRODUCTION

The Adenovirus Reference Material (ARM) consists of purified Adenovirus, Type 5 (wild type adenovirus, see ATCC VR-5) formulated as a sterile liquid in 20 mM TRIS, 25 mM NaCl, 2.5% glycerol, pH 8.0 at room temperature, and stored frozen at –70°C. The configuration is 0.5-mL in a Type II glass vial with a Teflon-coated gray butyl stopper and aluminum seal and crimp closure.

The ARM was developed under the guidance of the Adenovirus Reference Material Working Group (ARMWG) and the U.S. Food and Drug Administration (FDA) 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 at The Williamsburg BioProcessing Foundation's website http://www.wilbio.com. The purpose of the ARM is to define the particle unit and infectious unit for adenovirus gene vectors and establish a reference point for comparisons. The NIH Recombinant DNA Advisory Committee recommended the development of such a reference-testing agent in their report issued January 2002 [3].

The ARMWG assigned the particle concentration and infectious titer based on statistical analysis of data derived during the characterization phase of the project and presented in this report. Procedures for obtaining and analyzing these data were provided by the ARMWG (http://www.wilbio.com). The particle concentration is 5.8×10^{11} particles/mL, with 95% certainty that the true particle concentration lies within the 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 95% certainty that the infectious titer on HEK 293 cells lies within the range of 7×10^{10} to 8×10^{10} NIU/mL.

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

2 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 5 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. The statistical properties of the 5 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. 95% Confidence intervals of the mean were calculated for this purpose. The 95% confidence intervals on the mean make statements on the values of the reference material and how well those values are known.

A second calculation presents 2 and 3 standard deviation confidence bounds as limits within which any individual observation can be expected to fall. The 2 standard deviation limits correspond to nominal 95% bounds on an individual value and the 3 standard deviation limits correspond to nominal 99.7% bounds on an individual value. These confidence intervals will be useful in the future when laboratories are performing assays on this reference material as they give limits within which the results should fall. In particular, values outside the 3 standard deviation limits will occur with only 0.3% probability. Thus, results outside the 3 standard deviation limits can be considered as true outliers and an indication of a problem.

Observations outside 3 standard deviation limits were identified as outliers and were excluded. Means and confidence intervals were recalculated without the outliers.

Confidence intervals for infectious titer were calculated on log transformed results. The transformation was performed because the standard deviations were large relative to the mean (large CVs), and, typically, titer data are log transformed for statistical analysis. The procedure used was the following:

1. Log transform the data. Log base 10 was used.
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 antilogged mean of log transformed data is the geometric mean. The geometric mean can also be calculated as the Nth root of the product of N numbers. The geometric mean is usually less affected than the average by large outliers. Thus, the geometric mean is smaller than the average. The antilogged confidence limits will always be greater than zero and will not be symmetric around the geometric mean. The distance from the geometric mean to the lower limits will always be less than the distance from the geometric mean to the upper limit.

The effects of the number of cells plated, passage number, and confluency were investigated with 3 separate one-way Analyses of Variance (ANOVAs). Linear regressions were also performed to test for non-zero slopes with passage number.

Replicates by laboratories were considered as independent observations for the above calculations.

Repeatability was estimated as a pooled standard deviation. The root mean squared error (RMSE) from a one-way ANOVA with Laboratory as the classification variable was used to estimate repeatability. This method is equivalent to the following procedure:

1. Calculate the mean infectious titer for each laboratory.
2. For each laboratory, subtract its mean from each of its data values. These are deviations.
3. Square all the deviations.
4. Sum all the squared deviations.
5. For each laboratory, calculate the number of replicates minus one, and sum these over all laboratories.
6. Divide the sum of 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. The repeatability standard deviation is a measure of how close replicates from the same facility will be.

3 RESULTS

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

3.1 Particle Concentration

Particle concentration results were received from 15 laboratories. Data from 2 of these laboratories were excluded because the SOP protocol was not followed. Data from 13 laboratories were analyzed. One laboratory performed two replicates. There were 14 observations. Each observation consists of triplicates at 4 dilutions (one at 80% Ad5 and 3 at 30% Ad5). Table 1 presents the results of the calculations of the particle concentration for each laboratory and replicate. Values ranged from 53.36E10 p/mL to 67.28E10 p/mL.

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

Table 2 shows the mean, standard deviation and 95% confidence bounds on the mean for particle concentration. The mean was 58.25E10 p/mL with 95% limits from 56.28E10 p/mL to 60.22E10 p/mL. This indicates with 95% certainty that the true mean particle concentration of the reference material lies within 56.28E10 to 60.22E10 p/mL. This is a statement on how well the mean particle concentration is known.

Table 2. 95 % confidence 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 presents 2 and 3 standard deviation limits on particle concentration. The 2 standard deviation limits are from 51.41E10 p/mL to 65.09E10 p/mL. The 3 standard deviation limits are from 48.00E10 p/mL to 68.59E10 p/mL.

Table 3. Two (2) and 3 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 3 standard deviation limits, so no outliers were identified or dropped.

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, in the future, only means of 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 58.25E10 p/mL ± 1.97E10 p/mL. With time, as more observations are collected, this interval will become narrower and the mean particle concentration will become very well known.

However, any laboratory performing one replicate assay on the reference material can expect to get a result quite different from the mean particle concentration. Table 3 presents limits within which these future observations should fall. Nominally, 99.7% of all future results should fall within the 3 standard deviation limits. These limits will not narrow as more information is collected. Rather, the mean and standard deviation will become better known and may shift somewhat, but the 3 standard deviation limits will remain wide. These are the limits to be used when deciding if a given assay performed as expected.

3.2 Infectious Titer

Infectious titer results were received from 18 laboratories. Data from one laboratory were excluded from these statistical analyses because the SOP was not followed; a different dilution series and a different number of wells were used in the assay. Thus, results from 17 laboratories were analyzed. Additionally, 1 replicate from each of two laboratories was not included because the assays did not meet specifications. Thirteen laboratories sent results on 2 replicates, 2 laboratories had 1 replicate and 2 laboratories had 4 replicates, for a total of 36 observations. Replicates were considered as independent observations, meaning the laboratories with 4 replicates "counted" more than the others and the laboratories with 1 replicate "counted" less. Table 4 presents results for all laboratories and replicates. Values varied widely across methods and laboratories, from a low of 3.968E10 IU/mL to a high of 38.500E10 IU/mL.

Table 4. Estimated infectious titers ((IU/mL)*10**10)

Laboratory - Replicate	Average Poisson	Spearman-Kärber untrimmed	Spearman-Kärber 20% trim	Spearman-Kärber 50% trim	Maximum Likelihood
1 - A	7.811	10.960	9.351	7.584	8.867
1 - B	13.500	12.160	12.130	12.760	10.380
2 - A	36.340	11.630	38.500	36.080	35.720
2 - B	29.040	24.780	24.430	29.470	21.250
3 - A	8.761	8.763	7.750	8.271	7.522
3 - B	4.538	6.757	5.848	5.062	5.605
3 - C	5.834	5.136	4.659	4.778	4.444
3 - D	10.320	9.765	8.559	7.263	8.015
4 - B	7.772	6.610	5.552	3.968	5.434
5 - A	10.710	10.120	9.836	10.420	8.709
5 - B	10.460	8.391	7.722	6.378	7.018
6 - A	19.410	11.430	9.765	7.368	8.915
6 - B	9.383	7.695	6.470	4.381	6.210
7 - A	5.121	7.263	5.955	4.778	6.081
7 - B	4.894	5.933	5.305	4.778	5.024
8 - A	12.820	16.300	14.010	12.760	15.120
8 - B	10.560	9.836	9.907	10.120	8.279
9 - A	10.950	12.210	10.120	8.035	10.130
9 - B	13.600	11.360	10.800	9.019	9.586
10 - B	18.720	24.270	20.260	17.660	20.310
10 - C	21.080	24.620	21.010	21.010	20.400
11 - A	7.365	6.378	6.020	6.565	5.520
11 - B	6.201	7.476	6.684	5.682	6.274
11 - C	5.365	7.159	6.517	5.210	5.966
11 – D	9.045	9.979	9.217	8.513	8.520
12 – A	4.255	5.600	5.723	5.363	4.616
12 – B	6.265	5.441	5.580	6.020	4.561
13 – A	10.440	9.019	8.890	8.763	7.422
14 – A	7.727	8.637	8.006	8.035	7.418
14 – B	9.227	9.625	9.085	7.584	8.024
15 – A	9.002	9.907	8.778	8.890	8.485
15 - B	10.630	10.570	9.521	8.513	8.711
16 - A	7.666	10.350	9.385	10.120	8.801
16 - B	8.828	9.019	7.949	7.584	7.433
17 - A	15.590	15.390	15.390	14.320	12.090
17 - B	15.160	14.320	14.270	15.170	10.940

Table 5 presents the 95% confidence bounds and the 2 and 3 standard deviation confidence limits for all estimation methods. A comparison of this table with Table 4 shows that the values for laboratory # 2 are statistical outliers for the Spearman-Kärber 20% trim and maximum likelihood methods. The laboratory # 2 values for the other methods are outside the 2 standard deviation bounds (except the Spearman-Kärber untrimmed, replicate A). These two observations were subsequently dropped from the analysis.

Table 5. Confidence bounds for infectious titers ((IU/mL)*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 presents confidence bounds for infectious titers without the outlier observations. All of the means and limits shifted lower. This is expected as an upper outlier was dropped. Also, the sample size dropped from 36 to 34 reflecting the loss of the two replicates from the one laboratory dropped.

Table 6. Confidence bounds for infectious titers ((IU/mL)*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

The ANOVA and regression results on passage number found no significant differences. Table 7 presents the p-values for these analyses and all are greater than 0.05.

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

Significant differences were found between cell numbers plated. Table 8 shows that the p-values were less than 0.05 for all estimation methods. The infectious titer geometric means for 10,000 cells plated (13.77E10 IU/mL to 16.80E10 IU/mL) are nearly twice those for 40,000 cells plated (7.34E10 IU/mL to 8.83E10 IU/mL).

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

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

Similarly, there were significant differences between confluency values. The infectious titer geometric means for confluency = 0.25 (19.26E10 IU/mL to 24.44E10 IU/mL) were nearly triple those for confluency = 0.8 (7.48E10 IU/mL to 8.97E10 IU/mL).

Table 9. ANOVA results for confluency

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

Table 10 shows that only one laboratory, laboratory # 10, had a confluency different from 0.8 and two laboratories, laboratory # 10 and laboratory # 1 plated 10,000 cells rather than 40,000. These two laboratories each had two replicates. Note that laboratory # 2, although not included in these ANOVA analyses, also had 10,000 cells plated and 0.2 confluency.

Dropping these 4 observations, confidence limits were calculated on laboratories that used the same cell number plated (40,000) and the same confluency (0.8). These 30 observations were taken from a distribution with the same mean and with variances due only to inter-laboratory variability and replicate variability.

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

Table 11 presents descriptive statistics and confidence bounds for all estimation methods. The geometric means have again declined as upper outliers have been dropped and the N is down to 30 with 4 more observations dropped. The variabilities have also declined as seen in the smaller widths of the confidence bounds. For the maximum likelihood method, mean infectious titers of the reference material is 7.37E10 IU/mL with 95% confidence limits from 6.60E10 IU/mL to 8.23E10 IU/mL. The 3 standard deviation limits are 3.04E10 IU/mL to 17.86E10 IU/mL.

Table 11. Confidence bounds for infectious titers ((IU/mL)*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 displays repeatability estimates for all estimation methods. Repeatability standard deviations vary from 1.47E10 IU/mL for 20% trimmed Spearman-Kärber to 2.46E10 IU/mL for the average Poisson. Repeatability measures how close replicates can be expected to be. Thus, smaller repeatability is better. For the maximum likelihood estimator, replicate observations should be within ± 3 x 1.60E10 IU/mL which equals ± 4.80E10 IU/mL.

Table 12. Repeatability estimates ((IU/mL)*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

4 CONCLUSIONS

The mean particle concentration was 5.825E11 p/mL with 95% limits from 5.628E11 p/mL to 6.022E11 p/mL. This means with 95% certainty the true mean particle concentration of the reference material lies within 5.628E11 p/mL to 6.022E10 p/mL.

The WG decided to use the maximum likelihood results on data from laboratories that followed the stipulated protocol. The infectious titer was 7.37E10 IU/mL with 95% confidence limits from 6.60E10 IU/mL to 8.23E10 IU/mL. This means with 95% certainty the true mean infectious titer of the reference material lies within 6.60E10 IU/mL to 8.23E10 IU/mL.

The WG also decided to keep 2 digits for particle concentration and one digit for infections titer. Thus, the particle concentration is 5.8×10^{11} particles/mL, with 95% certainty that the true particle concentration lies within the 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 95% certainty that the infectious titer on HEK 293 cells lies within the range of 7×10^{10} to 8×10^{10} NIU/mL.

5 REFERENCES

1. Hutchins, B. (2002). Development of a Reference Material for Characterizing Adenovirus Vectors. *BioProcessing* **1(1)**: 25-28.
2. Hutchins, B., Sajjadi, N., Seaver, S., Shepherd, A., Bauer, S.R., Simek, S., Carson, K., and Aguilar-Cordova, E. (2000). Working Toward an Adenoviral Standard. *Molecular Therapy* **2(6)**: 532-534.
3. NIH Recombinant DNA Advisory Committee (January 2002) "NIH Report: Assessment of Adenoviral Vector Safety and Toxicity: Report of the National Institutes of Health Recombinant DNA Advisory Committee," *Human Gene Therapy 13 (1):* 3-13.
4. Adenovirus Reference Material Standard Operating Procedure for Determination of Particle Concentration via Spectrophotometric Analysis, Version 4.0, Nov. 7, 2001.
5. Adenovirus Reference Material Standard Operating Procedure for Determination of Infectious Titer in 293 Cells in a 96-Well Format, Version 3.0, dated Nov. 7, 2001.

APPENDIX A: ESTIMATION TECHNIQUES

A-1 FORMULA FOR ESTIMATING PARTICLE CONCENTRATION

Viral particle concentration was determined using the following formula:

Concentration ($_{\text{particles/mL}}$) = [$A260_{\text{nm corrected for blank}}$] x [1.1E12 ($_{\text{particles/mL}}$)] x [1/Dilution],

where the dilutions were:

0.30 for the 30% Ad5 runs, and
0.80 for the 80% Ad5 runs,

$A260_{\text{nm corrected for blank}}$ was the average of the triplicate readings corrected for blank, and the value of 1.1E12 particles/mL is from published data [1].

A-2 FORMULAE FOR ESTIMATING INFECTOUS TITER UNITS

Infectious titers were estimated with the following 5 methods: Average Poisson [2] (the default method in the Excel template), Spearman-Kärber [3], 20% trimmed Spearman-Kärber [4], 50% trimmed Spearman-Kärber [4], and maximum likelihood [5]. This section presents the formulae for the estimation. Section A-3 compares the methods and discusses pros and cons.

Average Poisson

The differential corrected infectious titer value was computed for each dilution for which the number of positive wells (out of 12) were greater than 2 and less than 10. This value was then averaged over the dilutions. The differential corrected infectious titer value was computed as follows:

$$dcitv_d = \frac{-D_d \ln\left(1 - \frac{p_d}{12}\right)}{(0.32)C_F(0.000238)\sqrt{T_I}}$$

$$dcitv = \frac{\sum_{(2<p_d<10)} dcitv_d}{\sum_{(2<p_d<10)} 1}$$

Here, D_d is the dilution value for case d, p_d is the number of positive wells at that dilution, C_F is the confluency, and T_I is the infection time.

Spearman–Kärber

The Spearman–Kärber method has four parts. The first part makes the data monotonic, the second part trims the edges, the third part performs the estimate of the 50% filled dilution, and the last part converts that result to the differential corrected infectious titer value.

The non-trimmed case is calculated by setting the trimming parameter equal to zero.

Monotonic transformation

The monotonic transformation adjusts the data such that the positive well counts increase with a decrease in dilution. This is done by averaging the number of positive wells over neighboring dilutions. As a result the well counts will no longer be integers.

Some experimentation was done to arrive at an algorithm that would successfully perform the monotonic transformation.

a) The method mentioned in Hamilton *et.al.* was not efficient to implement as a computer algorithm. In this method, successive well counts are averaged if the second (less diluted) is smaller than the first (more diluted). This averaging is repeated until the sequence of well counts are non-decreasing. It was found that this procedure took over 10,000 iterations for one of the data sets. Most data sets converged in just a few iterations. A few took over a hundred iterations. (The convergence problems would be easily overcome if the adjustments were done manually.)
b) The method used converged in two iterations for all data sets. In this method dilution intervals are formed and averaged, and the average replaces the original values over the interval. A dilution interval extends from a beginning sample to the sample just before one that is larger than any sample in the interval. That larger sample is then the beginning sample in the next dilution interval.

Trimming

Let y_d be the monotonic transformed proportion of well occupancy (0 to 1.0) for a dilution index, d. Let α be the trim factor. (2α is the fraction trimmed.) Then the occupancies are adjusted as follows:

$$y_d = \begin{cases} 1 & \frac{y_d - \alpha}{1-2\alpha} > 1 \\ 0 & \frac{y_d - \alpha}{1-2\alpha} < 0 \\ \frac{y_d - \alpha}{1-2\alpha} & \text{otherwise} \end{cases}$$

All proportions of well occupancies get changed by this algorithm. The proportions for the lowest, trimmed dilutions get set to zero. The proportions for the highest trimmed dilutions get set to one. The proportions in between get adjusted away from one-half. That is, some get adjusted higher and some get adjusted lower. The dilutions at the trimmed values may also get modified (See reference 3).

Spearman-Kärber calculation

The concentration at which 50% of the wells are positive is estimated to be

$$LC_{50} = \exp\left\{-0.5\sum_d \left[-\ln(D_{d+1}) - \ln(D_d)\right]\left(y_{d+1} - y_d\right)\right\}$$

where the y_d is the adjusted proportion at dilution D_d.

This is related to concentration by

$$C = LC_{50} \ln 2$$

Titer corrections

To scale the $LC_{50}\ln 2$ to the differential corrected infectious titer value, the following equation was used:

$$dcitv = \frac{LC_{50} \ln 2}{(0.32)C_F(0.000238)\sqrt{T_I}}$$

where D_d is the dilution value for case d, p_d is the number of positive wells at that dilution, C_F is the confluency, and T_I is the infection time.

Maximum Likelihood

The samples are K replicates at each of N concentrations. The number of replicates with no entities is k_i where $0 \leq i < N-1$. Each concentration has a dilution factor, d_i. The concentration at each dilution is represented by c/d_i where c is the unknown quantity to be estimated.

The probability that no entities are detected in a sample is assumed to be Poisson and equal to

$$p_i = e^{-c/d_i}$$

The dilution at which 50% of the samples would on average have zero entities is given by

$$\frac{1}{2} = e^{-c/d_{50}}$$

$$d_{50} = \frac{c}{\ln 2}$$

Then, the probability of obtaining the k_i is given by a product of binomials as follows:

$$P = \prod_{i=0}^{N-1} \binom{K}{k_i} p_i^{k_i} (1-p_i)^{K-k_i}$$

$$= \prod_{i=0}^{N-1} \binom{K}{k_i} e^{-k_i c/d_i} \left(1-e^{-c/d_i}\right)^{K-k_i}$$

The maximum likelihood estimate of the concentration, c, is found by differentiating this expression with respect to c, setting that expression equal to zero, and solving for c as follows:

$$\frac{\partial P}{\partial c} = P\sum_{j=0}^{N-1}\left(-\frac{k_j}{d_j}\right) + P\sum_{j=0}^{N-1}\frac{(K-k_j)e^{-c/d_j}}{d_j\left(1-e^{-c/d_j}\right)} = PA(c) = 0$$

where

$$A(c) = \sum_{j=0}^{N-1}\frac{1}{d_j}\left\{-k_j + \frac{\left(K-k_j\right)}{\left(e^{c/d_j}-1\right)}\right\} = 0$$

A(*c)* must be zero since P is always positive and the product of A(*c)* and P is zero.

Note that

$$A(0) = +\infty$$

$$A\left(+\infty\right) = -\sum_{j=0}^{N-1}\frac{k_j}{d_j}$$

In addition

$$\frac{\partial A(c)}{\partial c} = -\sum_{j=0}^{N-1}\frac{\left(K-k_j\right)}{d_j^2}\frac{e^{c/d_j}}{\left(e^{c/d_j}-1\right)^2} < 0 \quad \left(0 < c < +\infty\right)$$

As *A* moves from positive infinity to a negative value as *c* goes from zero to infinity and the slope of *A* is negative throughout this region, *A(c)* only crosses zero once. Consequently there is a single solution for *c*. Also since *A(c)* is monotonic, it is relatively easy to iterate to a solution.

To search for a solution, following steps are taken:

1 Pick an initial value for concentration, *c*, at the low end of the range.

2 Compute *A(c)*. If *A(c)* is negative then divide *c* by two and repeat this step. If *A(c)* is positive, go to the next step.

3 Iterate updating *c* with

$$c = c - \frac{A(c)}{\left(\frac{\partial A(c)}{\partial c}\right)}$$

This seems to converge very well in a few iterations. The convergence is from the low side of the zero crossing.

The differential corrected infectious titer value is given by

$$dcitv = \frac{C}{(0.32)C_F(0.000238)\sqrt{T_I}}$$

where C_F is the confluency at the time of infection, and T_I is the infection time.

A-3 MONTE CARLO ANALYSES AND COMPARISONS

Monte Carlo analysis is a technique for investigating statistical properties of estimators that cannot be found through mathematical manipulation. For this Monte Carlo analysis, a set of data was simulated by assuming the following:

1. The concentration, C, equals 1
2. The dilutions, D, went from $(\sqrt{2})^K/16$, K = 1 to 14.
3. P = the probability of a negative well = $e^{-C/D}$ for a given dilution.
4. The binomial distribution with parameter (1-P) was used to randomly assign the total number, N, of wells positive out of 12 wells.
5. The number of negative wells = 12-N.

The concentration (known to be one) was then estimated using the 5 estimation methods. This was repeated 1000 times.

Table A-1 summarizes the results of the Monte Carlo simulations. In the first row, the arithmetic mean was 1.17, or 17% higher than the concentration of 1 used to generate the data. This is a positive 17% bias. The geometric mean is 1.15, or a 15% bias. Thus, the average Poisson, the default method in the template spreadsheet has a large bias.

The Spearman-Kärber method has a negative bias of 9% from the geometric mean. The trimmed Spearman-Kärber methods have slight positive bias of 2% for 20% trimming and 4% for 50% trimming. The maximum likelihood method is unbiased.

Table A-1. Summary of 1000 Monte Carlo simulated data sets

	Arithmetic		Geometric	Confidence intervals					
				2 Standard Deviation		3 Standard Deviation		95%	
Analysis Method	Mean	StD	Mean	Lower	Upper	Lower	Upper	Lower	Upper
Average Poisson	1.17	0.22	1.15	0.79	1.68	0.66	2.02	1.14	1.17
Spearman-Kärber	0.92	0.16	0.91	0.64	1.28	0.54	1.52	0.90	0.92
Spearman-Kärber 20% trim	1.04	0.17	1.02	0.73	1.43	0.62	1.69	1.01	1.03
Spearman-Kärber 50% trim	1.06	0.19	1.04	0.73	1.50	0.61	1.80	1.03	1.06
Maximum likelihood	1.01	0.14	1.00	0.77	1.31	0.67	1.49	0.99	1.01

A biased estimation technique is not necessarily bad as long as everyone uses the same technique and the technique has small variability. In this case, however, the average Poisson has the largest standard deviation of all techniques and the maximum likelihood has the smallest standard deviation.

Table A-2 presents the pros and cons for the estimation techniques. All techniques are biased except the maximum likelihood. The maximum likelihood also has the smallest variability. The average Poisson requires no iteration. Although the Spearman-Kärber method used in this report (the 0% trimmed Spearman-Kärber) required iteration, the traditional Spearman-Kärber does not. All Spearman-Kärber methods assume data have come from a symmetrical distribution so that the mean, which is actually estimated, can be set equal to the LC_{50}, a median. See Finney [5] for further discussion of the Spearman-Kärber method.

The maximum likelihood method has the most desirable properties among the estimation method investigated. Its biggest disadvantage is that it requires iterating. An Excel spreadsheet has been developed for evaluation. This spreadsheet requires a minimum amount of information to perform the iterating.

Table A-2. Pros and Con of the estimation methods

Analysis Method	Biased	Variability	Iteration Required	Assumptions
Average Poisson	Yes	Largest	No	Restricted dilutions to 20% to 80% positive
Spearman-Kärber	Yes	2^{nd} smallest	Yes	0% & 100% readings, symmetry, monotonicity
Spearman-Kärber 20% trim	Yes	middle	Yes	0%, 100% readings, symmetry, monotonicity
Spearman-Kärber 50% trim	Yes	2^{nd} largest	Yes	0%, 100% readings, symmetry, monotonicity
Maximum likelihood	No	Smallest	Yes	None

A-4 APPENDIX REFERENCES

1. Maizel, JR., J., White, D., and Scharff, M. (1968). The Polypeptides of Adenovirus 1: Evidence for Multiple Protein Components in the Virion and a Comparison of Types 2, 7A, and 12. *Virology* **36**: 115-125.
2. Nyberg-Hoffman, C., Shabram, P., Li, Wei, Giroux, D. and Aguilar-Cordova, E. 1997. Sensitivity and reproducibility in adenoviral infectious titer determination. *Nature Medicine* **3(7)**: 808-811.
3. Finney, D.J. 1971. *Statistical Method in Biological Assay*. Second edition. P524-530.
4. Hamilton, M.A., Russo, R.C., and Thurston, R.V. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environmental Science and Technology* **11(7)**: 714–719.
5. Finney, D.J. 1971. *Statistical Method in Biological Assay*. Second edition. P520-522.

APPENDIX B: DATA LISTING

Table B-1. Listing of particle concentration data used in statistical analyses: A_{260nm} blank corrected spectrophotometer readings

Laboratory	80% Ad5						30% Ad5					
#	Replicate 1			Replicate 1			Replicate 2			Replicate 3		
	OD Reading			OD Reading			OD Reading			OD Reading		
	1	2	3	1	2	3	1	2	3	1	2	3
1	0.406	0.401	0.405	0.165	0.168	0.168	0.166	0.166	0.166	0.164	0.166	0.165
4	0.442	0.442	0.442	0.164	0.164	0.164	0.164	0.164	0.164	0.164	0.164	0.164
5 – A	0.401	0.401	0.401	0.138	0.139	0.140	0.148	0.149	0.150	0.141	0.145	0.146
5 – B	0.420	0.419	0.419	0.147	0.148	0.149	0.143	0.145	0.146	0.147	0.148	0.148
6	0.444	0.446	0.446	0.210	0.210	0.211	0.188	0.183	0.183	0.174	0.172	0.171
8	0.407	0.410	0.409	0.162	0.164	0.167	0.168	0.171	0.172	0.122	0.122	0.122
9	0.415	0.412	0.414	0.170	0.170	0.169	0.157	0.153	0.153	0.151	0.152	0.152
10	0.428	0.429	0.429	0.153	0.155	0.155	0.155	0.155	0.155	0.155	0.156	0.156
18	0.420	0.420	0.420	0.159	0.158	0.158	0.158	0.158	0.159	0.154	0.154	0.154
11	0.403	0.418	0.415	0.140	0.150	0.155	0.139	0.146	0.146	0.141	0.156	0.150
14	0.429	0.430	0.430	0.160	0.159	0.159	0.162	0.163	0.164	0.161	0.163	0.163
15	0.437	0.437	0.437	0.169	0.170	0.169	0.165	0.165	0.165	0.167	0.167	0.167
16	0.432	0.433	0.433	0.158	0.158	0.158	0.159	0.159	0.159	0.159	0.159	0.159
17	0.427	0.431	0.430	0.142	0.145	0.145	0.173	0.174	0.174	0.157	0.159	0.159

Table B-2. Listing of infectious titer data used in statistical analyses: Number of positive wells.

	Dilution (*10^7)													
Laboratory # - Replicate	5	10	20	28.3	40	56.6	80	113	160	226	620	453	640	1280
1 - A	12	11	12	8	4	5	4	3	2	2	1	2	1	0
1 - B	12	11	10	10	9	7	5	2	2	2	0	1	1	0
2 - A	12	11	12	9	7	4	3	4	0	1	0	0	2	1
2 - B	12	9	8	6	6	7	2	1	0	0	0	1	0	0
3 - A	12	12	8	7	7	4	6	1	1	0	2	1	0	0
3 - B	12	12	12	5	3	1	3	1	0	1	0	0	0	0
3 - C	12	7	7	6	5	6	2	1	1	0	1	0	0	0
3 - D	12	12	5	10	6	2	5	5	3	2	1	2	0	0
4 - B	12	12	7	4	2	4	2	4	3	1	0	0	1	0
5 - A	12	12	8	8	7	5	6	5	2	1	0	0	0	0
5 - B	12	12	9	4	8	3	2	2	4	3	0	0	0	0
6 - A	12	12	8	6	7	4	4	4	2	2	1	3	4	0
6 - B	12	11	5	7	4	3	2	2	3	4	2	2	1	0
7 - A	12	12	7	5	5	3	3	2	1	2	2	1	0	0
7 - B	12	12	7	6	3	4	2	0	2	0	0	0	0	0
8 - A	12	12	12	11	12	8	4	2	3	0	1	2	1	0
8 - B	12	11	10	7	6	6	6	4	1	1	0	1	0	0
9 - A	12	12	12	8	5	5	4	3	2	2	3	0	2	0
9 - B	12	12	10	7	8	5	4	3	1	4	3	0	0	0
10 - B	12	12	8	5	4	5	3	2	1	2	1	1	0	0
10 - C	12	10	8	7	6	4	3	2	1	1	1	2	1	0
11 - A	12	12	8	8	6	1	2	0	0	1	0	0	0	0
11 - B	12	12	9	5	5	3	2	3	2	2	0	0	0	0
11 - C	12	11	8	5	5	3	3	2	2	2	2	0	0	0
11 – D	12	12	11	7	5	5	6	2	2	1	1	0	0	0
12 – A	12	12	11	6	3	0	0	0	0	0	0	0	0	0
12 – B	12	12	9	7	4	0	0	0	0	0	0	0	0	0
13 – A	12	10	8	6	7	6	4	3	5	2	0	1	0	0
14 – A	12	11	9	9	7	3	3	1	2	2	1	1	0	0
14 – B	12	11	10	8	6	4	3	3	2	3	1	0	1	0
15 – A	12	12	8	9	7	4	4	4	1	1	2	1	0	0
15 - B	12	12	9	6	6	5	3	6	2	3	2	1	0	0
16 - A	12	12	7	10	10	3	4	4	1	1	1	0	1	0
16 - B	12	11	7	7	7	4	4	5	1	1	2	1	1	0
17 - A	12	11	10	9	8	7	6	4	4	3	3	2	1	0
17 - B	12	10	9	9	8	7	6	5	4	3	3	2	1	0