LAL Update

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Letter From the President

Dear LAL User:

This Update will clarify some of the statistics used with turbidimetric and chromogenic LAL tests. Most LAL users are concerned with repeatability, i.e. checking replicate determinations for outliers and, reproducibility, checking whether two laboratories (or technicians) are obtaining significantly different results. Unfortunately, there is little LAL-specific guidance provided by either the FDA or the USP in this regard other than limit values which indicate a valid test, i.e. a correlation coefficient of = 0.98 and a spike recovery of 50 to 200%. The FDA in their "Guidance for Industry --- Validation of Analytical Procedures: Definition and Terminology" uses the terms Precision, Repeatability, Intermediate Precision, and Reproducibility. The latter three terms are all subsets of Precision which is defined as "...the variance, standard deviation or coefficient of variation of a series of measurements." Thus, the LAL user is mainly left to his own devices as to how to analyze results to determine whether they are in control. At least one LAL manufacturer recommends in a chromogenic product insert under "Performance Characteristics" that: "Replicate samples should be run to establish good technique and low coefficient of variation." This manufacturer further recommends: "The C.V. of [these] absorbance values should be less than 10%. With experience, values of 3-4% should be attainable." In a turbidimetric product insert, this same manufacturer indicates that the "...(C.V.) equals the "sample" standard deviation of the reactions times divided by the mean..." Although no examples are provided, it is obvious that this manufacturer recommends raw values, i.e. optical density values (for chromogenic endpoint) and time of onset values for kinetic turbidimetric to calculate C.V. It should be noted that these recommendations are ***not*** *general requirements for all LAL reagents, but are specific to the LAL product(s) covered by their respective product inserts and would be supported by data generated by this manufacturer. Associates of Cape Cod, Inc. (ACC) on the other hand does not include its expectations for C.V. in its product insert but does include this calculation in the software which accompanies the LAL-5000 and Pyros Kinetix machines as a convenience for our customers. ACC's C.V.s however are calculated from EU/ml values rather than time of onset. Although C.V.s should be a relatively simple and convenient check for precision, due to a lack of clarification on the part of LAL manufacturers and the FDA, there is definitely some confusion among LAL users. In addition, some LAL users may be using C.V.s incorrectly, leading to a false sense of security related to the reproducibility (or lack thereof) of their assays. Hopefully this UPDATE will clarify the use of C.V.s. For this UPDATE I have relied heavily on the experience of Mr. Keith Richardson, ACC's Instrumentation Manager. Keith has been with ACC for 15 years, actually beginning as a part-time horseshoe crab "bleeder" and working his way through our Technical Services Department. Although Keith is currently responsible for machine manufacture, maintenance, and calibration, he is still ACC's "kinetic assay expert" and continues to help with technical inquiries related to the LAL-5000, Pyros Kinetix machine, and plate readers, and participates in workshops and site visits. During his tenure at ACC he has accumulated more experience with kinetic data than anyone else in the company and possibly more than any other LAL user. This UPDATE concludes with an article on Simple Statistics for the LAL User - Standard Deviation, Repeatability, Reproducibility and a clarification of the Coefficient of Variation.*

Sincerely,

Thomas J. Novitsky, Ph.D.

Simple Statistics for the LAL User - Standard Deviation, Repeatability, Reproducibility and a Clarification of the Coefficient of Variation.

By Keith Richardson & Thomas J. Novitsky

Using the standard deviation and coefficient of variation

The standard deviation (actually the standard deviation of the mean as used in this context) is calculated according to the formula(s) in Figure 1. There are at least two ways to calculate standard deviation depending on whether the entire population (n) is measured or only a representative sample (n-1) is used. It can be argued that the values (e.g. times of onset, EU/ml) generated for each replicate in an LAL assay represent the entire population (the population in this case being all the replicates from a single concentration). On the other hand, the volume of endotoxin standard solution used in the actual LAL test usually represents only a small percentage of the original dilution. The test also employs only a single vial from large lots of CSE, LAL, LRW, etc., therefore it may be more appropriate to use equation 2. Note that for small numbers of replicates, equation 2 is significantly more conservative, i.e. results in higher values for standard deviation and subsequently coefficient of variation (C.V.). than equation 1. For these reasons, Associates of Cape Cod, Inc. (ACC) prefers to use equation 2. The standard deviation has a unit value that is the same as that attributed to the mean, e.g. if the mean of the times of onset is used to determine the standard deviation, seconds would be the unit; likewise, if endotoxin units were used, EU/ml would be the unit. The standard deviation is also sometimes referred to as the standard error. It is a direct measure of precision, i.e. the lower the standard deviation, the higher the precision. The standard deviation is also a measure of variation, i.e. the lower the standard deviation, the lower the variation (variability).

The C.V. is the standard deviation expressed as a percentage of the mean. It is useful when comparing means that differ substantially. Although it is expressed as a percentage and is thus unitless, **units are implied**. Therefore C.V.s calculated from times of onset cannot be compared to C.V.s calculated from optical densities or EU/ml. While it is easy to use the standard deviation when comparing one set of LAL measurements to another, if the two measurements differ substantially in their means, a direct comparison of the standard deviation is not possible. This is because values obtained at the extremes of a measurement spectrum could be expected to have more variation. For example, measurement of a number of replicates of 5 EU/ml (very short time of onset) resulted in a standard deviation of 0.13 and a C.V. of 2.9% (calculated from endotoxin values as EU/ml) while replicates at 0.05 EU/ml (moderate time of onset) had a standard deviation and C.V. of 0.01 and 22% respectively. Thus, in this example, if only standard deviations are compared, the variability of the standard for 5 EU/ml looks worse than that at 0.05 EU/ml when in fact the C.V. indicates more variability for the lower concentration. Therefore if a technician is interested in assessing their LAL assay skills, they should use standard deviations as a measurement of precision **only** when comparing a single concentration from one assay with the same concentration in a separate assay. To compare precision **between** concentrations, the analyst would need to use C.V.s. It should be pointed out at this point (so as not to discourage our readers) that excellent (i.e. low) C.V. values can be obtained across the spectrum of LAL concentrations. Thus, although higher values **tend** to be obtained at the ends of the test range, a technician with good laboratory skills can expect low values at all points on the standard curve. There is however no **absolute** C.V. that is indicative of a "good" or a "bad" test. As a rule of thumb, a "< 10%" value is considered "good". However, as I will illustrate in the next example, not all C.V.s are created equal.

As mentioned above, although C.V.s are unitless, units are implied. We have also learned that we cannot compare C.V.s that were calculated using different units. But what units should be used for this calculation (or does it even matter)? The answer to this question is not as straightforward as one might think since the choice of units affects the size of the C.V. value. In Vol. 9 of the Endosafe Times, C.V.s were used to compare standards performed by an analyst vs. those obtained by a robot. To perform the comparison, time of onset values were used. The resulting C.V.s for all assays were less than 10% although the analyst was generally better than the robot (8 out of 12 paired C.V.s were lower for the analyst although this observation was not highlighted by the Endosafe Times author). Interestingly, one of the conclusions drawn from this study was that since all C.V.s were less than 10%, the robot method could be interchanged with the analyst method. But is this true? What if the statistician had decided

to use calculated EU/ml values instead of times of onset? Since EU/ml are calculated from time of onsets, what difference could it make? Table 1. uses the data from the "Comparison: Reaction Times of Robotic vs. Analyst Curves" as published in Vol. 9, No. 1 of the Endosafe Times converted to EU/ml. C.V.s were then calculated from the standard deviations of the means and compared to the C.V.s calculated from the times of onset. The results are dramatic. Although there is definitely a correlation between the two sets of C.V.s, i.e. high C.V.s calculated from time of onset data correspond to high C.V.s calculated from EU/ml values, the EU/ml C.V.s are substantially greater than those from times of onset (range of C.V.s from time of onset = 0.16 to 5.15% while those calculated from EU/ml range from 0.98 to 32.31%). Thus if one used EU/ml values to calculate C.V., the "general rule" of <10% would have failed some of the assays. It is interesting to note that only 2 out of 12 assays for the "analyst" resulted in C.V.s >10% (one was close at 10.4%) while 4 out of 12 assays for the "robot" would have failed. Should the statistician therefore choose the data set that gives the lowest values when calculating the C.V.? There is no solid answer to this although if one is comparing an assay or standard whose intention is to measure endotoxin concentration, then it seems reasonable to **use** endotoxin concentrations for the statistical analysis. This is the position taken by ACC. The calculation for C.V. included in Pyros (and Pyrosoft 11) employs the standard deviation of the means in EU/ml units. Table 2. illustrates an actual kinetic turbidimetric assay that compares C.V.s calculated from the time of onset vs. those calculated from endotoxin values. Note that although there is a correlation between C.V.s calculated from the different data sets, the actual values for those calculated from time of onset are always of a lower magnitude. Since the EU/ml values are obtained from a standard curve that employs the time of onset, ACC believes using these transformed values provides a more rigorous test of precision. In order to be useful, C.V. values need to be compared with those determined after performing the test on a large number of samples. At ACC, C.V. values (calculated from EU/ml) under 10% are routinely obtained for all standard concentrations, however, values between 10 and 20% are common for the low concentrations, e.g. 0.001 EU/ml. Users of our reagent and software should expect similar results after sufficient practice. The real value of C.V.s, however, is as a continual check for precision and with the new Pyrosoft 11 software users will be able to trend their own C.V.s.

Repeatability and Reproducibility Calculations

The FDA in their "Guidance for Industry-Validation of Analytical Procedures defines Repeatability as measurement that "…expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision." In other words, repeatability is the precision within a laboratory. Reproducibility on the other hand is defined as a measurement that "…expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology)." The FDA provides an additional definition of "Intermediate precision" defined as a measurement that "…expresses within-laboratories variations: different days, different analysts, different equipment, etc." C.V.s of course with the cautions noted above could be compared to get an idea of the precision for all these groups.

References

1) Biometry, Sokal, Robert R., and F. James Rohlf, second ed. W.H. Freeman and Company, New York. 1981.

2) 63 Guidance for Industry Validation of Analytical Procedures: Definition and Terminology. Final Guidance. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Veterinary Medicine, July 1999.

3) Endosafe Times Volume 9, Number 1. March 2002.

Figure 1. Formulas for the Calculation of Standard Deviation

1)* Standard Deviation = $\sqrt{\frac{n\sum x^2 - (\sum x)^2}{n^2}}$

2) Standard Deviation = $\sqrt{\frac{n\sum x^2 - (\sum x)^2}{n(n-1)}}$

where: n = sample size, e.g. number of replicates
x = mean (of time of onset, EU/ml, etc.)

** Formula used to calculate Standard Deviations and subsequent C.V.s in Reference 3.*

Table 1. "Comparison: Reaction Times of Robotic vs. Analyst Curves" Using C.V.s From EU/ml.*

Day 1.

	Etxn. Hand	Std. Dev.	C.V.	Etxn. Robot	Std. Dev.	C.V.	Robot Etxn. Using Hand Regression
1	4.924	0.31	6.49	5.253	0.31	6.09	6.381
2	4.492			4.819			5.853
3	0.580	0.02	3.56	0.531	0.05	10.19	0.640
4	0.551			0.459			0.554
5	0.049	0.00	4.66	0.054	0.01	11.06	0.065
6	0.046			0.047			0.056
Slope Y - intercept r	-0.205 2.977 -0.999			-0.206 2.960 -0.999			

Day 2.

	Etxn. Hand	Std. Dev.	C.V.	Etxn. Robot	Std. Dev.	C.V.	Robot Etxn. Using Hand Regression
1	4.753	0.13	2.90	4.923	0.41	7.85	4.109
2	4.562			5.502			4.533
3	0.621	0.06	10.40	0.442	0.03	5.95	0.489
4	0.536			0.481			0.527
5	0.055	0.01	22.07	0.047	0.01	12.97	0.068
6	0.040			0.057			0.080
Slope Y - intercept r	-0.219 2.999 -0.997			-0.194 2.999 -0.999			

Day 3.

	Etxn. Hand	Std. Dev.	C.V.	Etxn. Robot	Std. Dev.	C.V.	Robot Etxn. Using Hand Regression
1	5.010	0.26	5.47	4.173	0.67	14.50	3.176
2	4.637			5.126			3.838
3	0.552	0.02	3.59	0.562	0.03	5.49	0.502
4	0.524			0.607			0.539
5	0.050	0.00	6.01	0.046	0.00	0.98	0.050
6	0.046			0.047			0.051
Slope Y - intercept r	-0.253 2.949 -0.999			-0.233 2.967 -0.998			

Day 4.

	Etxn. Hand	Std. Dev.	C.V.	Etxn. Robot	Std. Dev.	C.V.	Robot Etxn. Using Hand Regression
1	4.725	0.09	1.89	4.150	0.97	19.98	2.174
2	4.600			5.515			3.565
3	0.570	0.01	1.19	0.504	0.06	11.33	0.360
4	0.580			0.592			0.420
5	0.045	0.00	5.04	0.038	0.02	32.31	0.030
6	0.048			0.060			0.047
Slope Y - intercept r	-0.231 2.932 -0.999			-0.222 2.969 -0.996			

* *Raw data from Ref. 3*

Table 2.
Comparison of C.V.s Calculated From Onset Time and Endotoxin Concentration

File: Test .lv3

Well	Description	Standard Conc. Units	Raw Onset	Adjusted Onset	CV Onset	Calc. Endotoxin	CV Endotoxin
1	Neg. Ctl.						
2	Neg. Ctl.						
3	Standard 1	0.001 EU/ml	3760	4093.4	3.44	0.000676	15.75
4	Standard 1	0.001 EU/ml	3670	3898.8		0.000845	
5	Standard 1	0.01 EU/ml	2100	2142.5	0.19	0.0132	0.86
6	Standard 1	0.01 EU/ml	2080	2148.2		0.0131	
7	Standard 1	0.1 EU/ml	1260	1287.3	0.61	0.137	2.79
8	Standard 1	0.1 EU/ml	1280	1298.4		0.132	
9	Standard 1	1 EU/ml	880	881.3	1.26	0.781	5.77
10	Standard 1	1 EU/ml	890	897.1		0.720	

Slope:	0.217753
Y Intercept:	2.9218
Correlation Coefficient:	-0.994

CALENDAR OF EVENTS

DECEMBER 2002

December 9-13
PDA Annual Meeting
New Orleans Marriott Hotel
New Orleans, LA
Booth # 200

MARCH 2003

March 17-19
PDA Spring Conference
Paradise Point Hotel
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