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Interpreting ELISA Data and Establishing the Positive-Negative Threshold

Ten years ago, Voller et al (6) and Clark and Adams (3) introduced enzyme-linked immunosorbent assay (ELISA) to plant pathology. Since then, ELISA has been used to assay for a great variety of plant viruses and several other plant pathogens. Few of the resulting publications, however, have dealt with the problems of interpreting ELISA data (2). Our own experiences in interpreting and analyzing ELISA data led to this article, in which we discuss the setting of positive-negative ELISA thresholds, some pitfalls that can lead to erroneous interpretation of ELISA data, and some recent developments in analyzing ELISA data.

The Problem of Test Thresholds

The setting of test thresholds is a crucial feature in the design, verification, and routine use of an assay. While this appears to be well recognized in the clinical (human) laboratory use of ELISA and similar assays, we believe it is not as well recognized in the plant sciences. The medical literature contains many examples of careful attention to the problem of threshold choice—an obvious necessity when dealing with life-threatening illnesses. In the plant sciences, however, false negatives and false positives do not have such serious consequences and ELISA data analysis has not received such careful attention. Granted, a false positive in a test of, for example, potato ring rot, with zero tolerance of disease, could mean a large, unnecessary financial loss to a grower. In most cases, however, a false negative or false positive simply yields erroneous data, which tend to be ignored or, worse yet, incorporated into the final results.

To obtain an idea of how other plant pathologists interpret their data, we surveyed PLANT DISEASE, *Plant Pathology*, *Annals of Applied Biology*, and *Journal of General Virology* for ELISA-related papers published during the 2 years prior to October 1985. We found 81 such articles and determined: 1) the method used to set threshold values, 2) the absorbance range and number of healthy standards used, 3) the replication of experiments and samples, and 4) comparison of ELISA with other assay methods.

The survey revealed many opportunities for improving test reporting practices. Over one-half of the authors did not state how they determined the positive-negative threshold (Table 1). Over three-fourths of the articles did not give the absorbance range and the number of healthy standards, about two-thirds did not state if experiments were replicated, and three-fourths did not state if samples were replicated (Table 2). Approximately one-half of the papers reported use of an additional method to evaluate the test plants for comparison with ELISA (Table 3).

Using a number of different individuals for the negative standards is important to ensure adequate coverage of the possible range of healthy values, and the cultivar, rootstock, age, growing conditions, and tissue type of the standards should be matched to the unknown test samples. The range of the healthy background absorbance interval should be known. Not all ELISA systems are equivalent when it comes to false positives and false negatives; systems with very low background levels (e.g., <0.1 absorbance unit) usually yield fewer questionable samples than systems with higher backgrounds. Replication of samples is also important.

Table 1. Results of 2-year (1984 and 1985) survey of four journals[a] for methods used to set ELISA threshold values

Method	Number of papers
Not stated[b]	49
Visual	7
$2\bar{x}$ Negative mean	10
$3\bar{x}$ Negative mean	5
Negative mean + $2s$	2
Negative mean + $3s$	5
Negative mean + $4s$	1
Other	2
Total	81

[a] PLANT DISEASE, *Plant Pathology*, *Annals of Applied Biology*, and *Journal of General Virology*.
[b] Some articles cited another article for materials and methods.

Use of Histograms

A useful way to look at ELISA data is in the form of a histogram (frequency distribution). The process of constructing a histogram from ELISA data is straightforward. One chooses a convenient interval (generally 0.02 or 0.05 absorbance unit), counts the number of data points falling within each interval, and plots the frequency distribution. For even easier construction of a histogram, some ELISA optical readers can be interfaced with a microcomputer via an RS-232 port, which allows direct input of data from the ELISA reader into a software package, e.g., Lotus 1-2-3. From there, a

Table 2. Absorbance range, number of healthy controls, and replication of experiments and samples reported in 81 ELISA-related articles[a]

Item	Stated (no.)	Not stated or not done (no.)
Absorbance range of healthy controls	18	63
Number of healthy controls	9[b]	72
Experiments replicated	32	49
Samples replicated within experiments	21	60

[a] Published in PLANT DISEASE, *Plant Pathology*, *Annals of Applied Biology*, or *Journal of General Virology* during 1984 and 1985.
[b] Five articles reported fewer than five controls; three, five to 10; and one, more than 10.

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simple program can produce a histogram automatically.

Figure 1 is a histogram of ELISA results for potato virus S in plantlets derived from 90 characterized potato tissue cultures. Thirty-one assay points (vertical axis) were found in the absorbance interval 0.05–0.10 (horizontal axis), 10 assay points in the 0.10–0.15 interval, and so on. These results represent an apparently satisfactory assay. The distribution of data is bimodal, with the two populations separated by a large interval of absorbance units. The population on the left contains all the negative samples, and the population on the right contains all the positive samples. No false positives or false negatives were displayed in repeated ELISA tests with these samples. The interpretation of this data set is straightforward. These data would be difficult to interpret, however, if known negative or known positive samples were absent and if the positive-negative threshold for the test was not known.

We routinely use histograms to examine our ELISA data, and we find them to be useful devices for quickly recognizing many features of a data set. For example, during construction and testing of a new plant virus ELISA, a histogram of the test data often displays negative and positive distributions like those in Figure 1 when the test is challenged with approximately equal numbers of known positives and negatives. If the positive and negative populations are not well separated by a reasonable absorbance interval, we can expect interpretation problems in applying the ELISA test to such samples.

Table 3. Comparison of ELISA with other methods of detection reported in 81 articles[a]

Method	Number of articles
None	44
Symptomatology	2
Infectivity	18
Transmission (vectors)	3
Graft inoculation	1
Electron microscopy	3
Immunosorbent electron microscopy	6
cDNA hybridization	1
Indirect ELISA	2
Agar gel double diffusion	4
Sucrose density gradient centrifugation	1
Total	85[b]

[a] Published in PLANT DISEASE, *Plant Pathology*, *Annals of Applied Biology*, or *Journal of General Virology* during 1984 and 1985.

[b] Some articles reported comparisons with more than one method.

Samples from virus-tested programs, especially elite or nuclear stocks, give data that produce histograms like Figure 2, which shows data from a tobacco ringspot virus ELISA with nursery stock blueberry bud samples.

An Example

Histograms by themselves do not solve the problem of setting thresholds. Figure 3 shows the results of three successive years of ELISA testing for the presence of Prunus necrotic ringspot virus in a block of 194 sour cherry trees. All three components of Figure 3—A, B, and C—show compact, dense distributions of positives and negatives separated by about 1.2 absorbance units. Depending on the year, a considerable number of samples lie between these peaks. The negative population of individuals in an ELISA histogram is not always normally distributed, i.e., in a bell-shaped curve, but instead may be skewed to the right. This "tail" area has a high potential for containing both false negatives and false positives, depending on how the data are interpreted.

The ELISA values for three problematic trees falling to the right of the negative population in the 1983 assay (Fig. 3A) were similar (Table 4), with a range above twice the mean of the healthy controls ($2\bar{x} = 0.12$ absorbance unit, 1983). By the standards commonly used in plant ELISA ($2\bar{x}$ or $\bar{x} + 3$ standard deviations [s] of healthy control samples), these trees would be rated positive. Tree 8-3 had much higher readings in 1984 and 1985 than in 1983 (Table 4). Tree 8-4 also had a "positive" reading in 1983 but had readings lower than $2\bar{x}$—and even lower

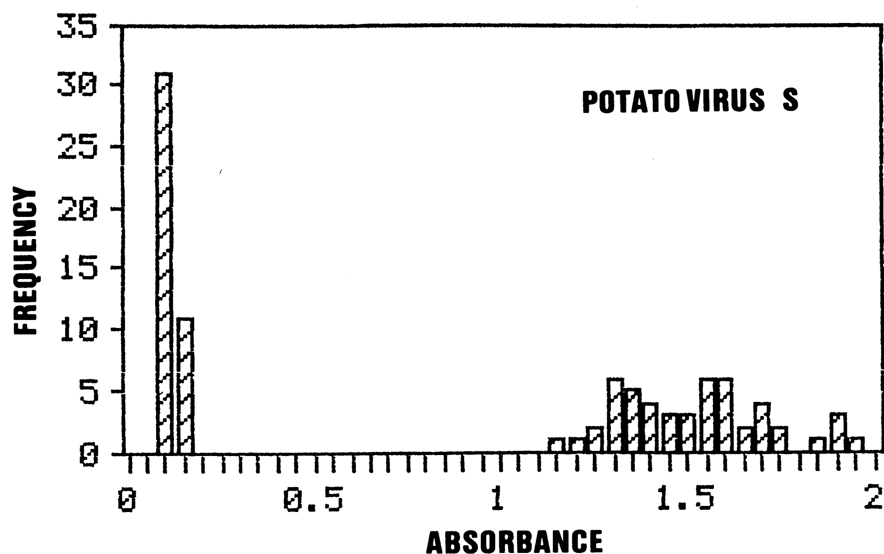


Fig. 1. Histogram of ELISA results for potato virus S in plantlets derived from 90 characterized potato tissue cultures. The bimodal distribution of data is ideal, with a large interval of absorbance separating healthy (negative) plants on the left and diseased (positive) plants on the right.

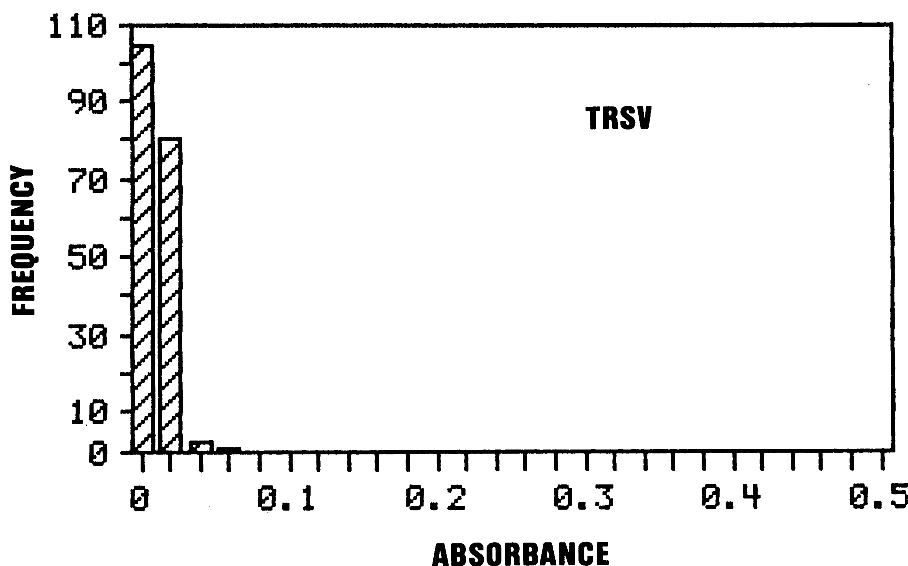


Fig. 2. Histogram of ELISA results for tobacco ringspot virus in elite blueberry stocks, representing the best source of healthy plants. All absorbance values are clustered near zero.

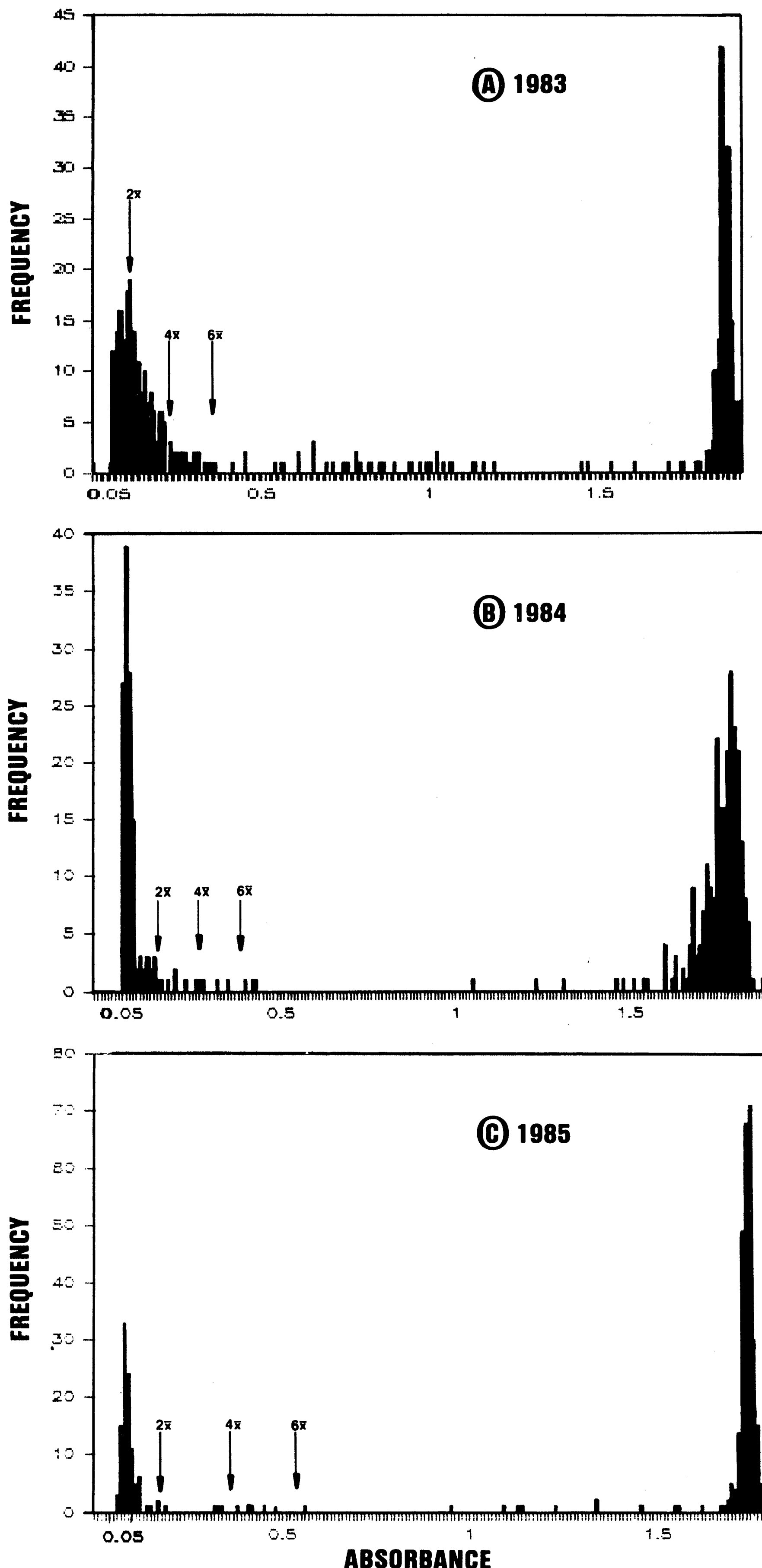


Fig. 3. Histograms of ELISA results for Prunus necrotic ringspot virus in 194 sour cherry trees tested in (A) 1983, (B) 1984, and (C) 1985; each histogram contains 388 values because ELISA plate wells were run in duplicate. Arrows indicate the $2\bar{x}$, $4\bar{x}$, and $6\bar{x}$ of the 10 negative control samples.

than $\bar{x} + 3s$—in 1984 and 1985. Was the virus missed when tree 8-4 was sampled in 1984 and 1985, or was the 1983 reading interpreted incorrectly? The ELISA results for tree 6-7 are similarly puzzling—positive in 1983 and again in 1984, seemingly corroborating the 1983 interpretation, but definitely negative in 1985.

These three trees were not the only ones with data that moved in and out of positive and negative populations over the 3-year period. In assays of the 194 trees, the ELISA values were grouped into four categories: A) $<2\bar{x}$ of healthy controls, B) $2\bar{x}$ to $<4\bar{x}$, C) $4\bar{x}$ to $<6\bar{x}$, and D) $\geqslant 6\bar{x}$. Category A represents those values below the commonly used negative threshold. The $\bar{x} + 3s$ (Chebychev's equivalent) is also becoming popular for use as a threshold. In this data set, however, the two values ($2\bar{x}$ and $\bar{x} + 3s$) are very similar. Category D represents those values that are strong positives; category C, those values that can be considered low positives; and category B, those values that may be very low positives or unusually high negatives. Category B may contain a higher percentage of false positives or false negatives, depending on how the data are interpreted, and is often in the tail region of the negative population.

The ELISA readings of more than 100 trees behaved in a very straightforward manner, remaining for all 3 years $<2\bar{x}$ for 24 trees and $\geqslant 6\bar{x}$ for 84 trees. The values for 52 other trees also fit an understandable pattern, going from negative to positive categories over the 3-year period. Results were less certain in some instances, however. For example, eight trees had category D values ($\geqslant 6\bar{x}$) in 1983 but category A values ($<2\bar{x}$) in both 1984 and 1985. Were the 1983 readings a mistake, due perhaps to contamination during the ELISA process, or was the virus simply missed in 1984 and 1985 because of sampling errors? The latter is probably true, since the 1983 absorbance readings were so high. Similarly, another eight trees tested as very low positives (category B) in 1983,

Table 4. ELISA values for three sour cherry trees tested three successive years for Prunus necrotic ringspot virus

Tree	Absorbance values[a] 1983	1984	1985
6-7	0.23	1.05	0.08
	0.21	1.23	0.08
8-3	0.27	1.82	1.75
	0.30	1.82	1.75
8-4	0.29	0.10	0.08
	0.20	0.10	0.08

[a] 1983 and 1984, $2\bar{x} = 0.12$; 1985, $2\bar{x} = 0.18$.

then very negative (category A) in 1984 and 1985. Were these trees negatives or positives? They would be considered positives if just the 1983 data were used, but when they failed to yield positive values in successive years, they began to look like negatives.

New Methods for Setting Positive-Negative Thresholds

Recently, Kramer et al (5) suggested that discriminate analysis could be a better method than the commonly used ones that set arbitrary limits (e.g., $2\bar{x}$ and $\bar{x} + 3s$) for determining positive-negative thresholds in ELISA tests. They reported on the use of ELISA to test human sera for levels of antimeasles antibody. They used the SPSS statistical package (4) to do discriminate analysis of the ELISA data. After establishing correlation coefficients among ELISA, indirect immunofluorescence, and hemagglutinin inhibition values, they calculated a value called the discriminate function to use as the positive-negative threshold for ELISA. They then compared the discriminate function value with the usual cutoff values, e.g., $2\bar{x}$ of healthy, $3\bar{x}$, and $\bar{x} + 2s$, $3s$, and $4s$.

We applied this technique to ELISA for potato leafroll virus (PLRV) in potato sprouts, with visual plant symptoms as the independent assay. This data set (supplied by Robert Goth, USDA, Beltsville, MD) consisted of 297 individual tubers from which the sprouts had been tested twice by ELISA (Fig. 4). The tubers were grown in a controlled greenhouse and observed twice for symptoms. We used the SPSS package for discriminate analysis (Wilk's interactive method). We converted the ELISA values to corrected absorbance interval values (CORROD, or A_{405nm} reading minus the $\bar{x}$ of healthy control plants, minus a correction factor, which we arbitrarily set at unity). We also calculated the discriminate function LnCORROD. This transformation was done following Kramer et al (5), in case the data did not conform to normal distribution. After calculating the two discriminate function values for the PLRV data set, we constructed a table of comparative ELISA positive-negative thresholds and also calculated the percentage of false-positive and false-negative values for ELISA as determined by the independent visual test for symptoms (Table 5).

Table 5 summarizes the positive-negative threshold in terms of its value, the method used to set the threshold, and the percentage of false positives and false negatives determined by visual symptoms. As expected, the threshold for positives set at $>2\bar{x}$ gave the lowest number of false negatives (0%) and a relatively high number of false positives (9.09%). The number of false negatives increased and that of false positives decreased as thresholds were selected at higher absorbance levels. The discriminate analysis resulted in thresholds of 0.12–0.13, giving about 9–11% false negatives and about 2% false positives.

The use of discriminate analysis for this data set appears to have been of no benefit. The attempt was not cost-effective because of the expense of computer time. Also, the results were not substantially better than those produced by hand methods with a calculator.

The PLRV-infected sprout data set was collected and interpreted before we knew about discriminate analysis as a possible method for setting the positive-negative threshold. The use of the data is what determined the method for setting the threshold. Picking sample tubers negative for PLRV was important, and a low absorbance interval threshold was chosen that apparently gave no false negatives.

Burrows and Barnett (1) have proposed another way to deal with interpretation of ELISA data: converting the dose response curve (absorbance unit antigen concentration) to a curve of detection rate vs. antigen concentration. This suggestion is based on an elegant but complicated analysis of detection rules by Burrows and merits further study and testing with plant systems.

Conclusions and Guidelines

Our examples and discussion suggest that there are no easy answers for setting positive-negative thresholds in plant ELISA. The most widely used method in clinical science is totally empirical. We expect that no test, and certainly no ELISA, can be 100% correct. ELISA must be directly compared with some other, independent method of defining pathogen presence. Then, using a population of negative and positive samples, the results from the reference and ELISA methods are compared and a threshold is chosen that yields the most "correct" results, i.e., the fewest false negatives and/or false positives. During

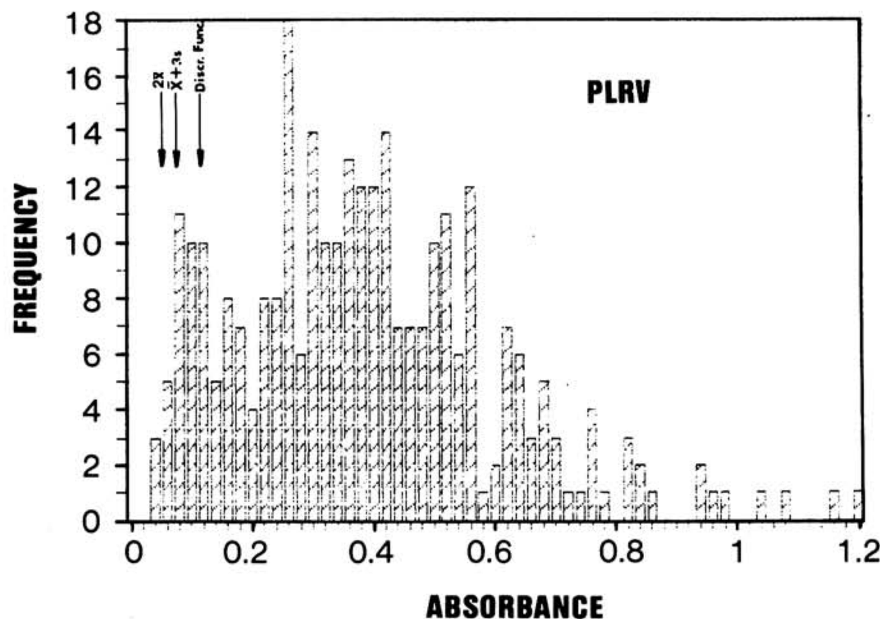
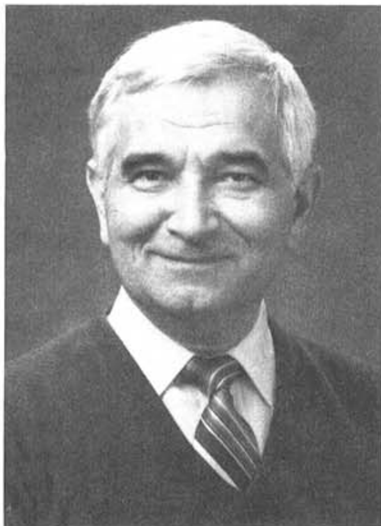


Fig. 4. Histogram of ELISA results (mean of two tests performed 1 month apart) for potato leafroll virus in 297 potato sprouts. Ten negative control plants were used to determine the healthy mean value. (ELISA data set supplied by Robert Goth, USDA, Beltsville, MD.)

Table 5. Summary of false-negative and false-positive results[a] of ELISA for potato leafroll virus infection in 297 potato sprouts, employing commonly used statistical methods vs. discriminate analysis for determining the positive-negative threshold

Threshold absorbance value	Method for setting threshold	Percentage of false negatives	Percentage of false positives
0.040	$2\bar{x}$	0.00	9.09
0.050	$\bar{x} + 3s$	0.34	8.42
0.060	$3\bar{x}$	1.01	7.07
0.065	$\bar{x} + 3s$	1.01	6.73
0.080	$\bar{x} + 4s$	2.02	4.71
0.118	CORROD[b]	9.09	2.36
0.131	LnCORROD[b]	11.11	2.04

[a] Determined by visual plant symptoms.
[b] Discriminate function.



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routine use, any ELISA must be controlled by maintaining the threshold in a constant range of absorbance values. Also, satisfactory assay performance should be proved by contrasting and using test standards or controls.

The common use of such thresholds as $2\bar{x}$, $3\bar{x}$, $\bar{x} + 3s$, etc., should be recognized as arbitrary at least and often misleading. A threshold should be adopted on the basis of an acceptable reference. These detection rules are not, by themselves, based on any fundamental property of ELISA or of the samples to justify their use.

For adequately reporting ELISA data at this time, we suggest the following guidelines:

1. Clearly state the positive-negative threshold used.
2. Test enough plants to become familiar with the range of negative (healthy) values involved.
3. Include enough known negative controls in each routine assay to ensure representation of the previously established range of negative background values.
4. Always include a positive control.
5. Match control samples and test samples with respect to host type, tissue type, age, and position.
6. Strongly consider replication of test samples.

We hope we have succeeded in pointing out some of the pitfalls associated with doing ELISA and reporting the resulting data. We hope also that this article will serve as a focus for further discussion and research toward developing more meaningful and accurate ELISAs and data presentation.

Acknowledgment

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