# UltramicroELISA for Measuring Tetanus Antitoxin in Human Sera[1]

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*This article describes a combination of methods—a solid-phase enzyme-linked immunosorbent assay (ELISA) combined with an ultramicroanalytical system (UMAS)—that can be used to measure tetanus antitoxin activity in human sera or plasma. The test, which is rapid and permits analysis of 78 samples of serum per reaction plate with a volume of 10 μL of diluted serum per sample, is proposed as an alternative to the traditional biologic assay in mice based on seroneutralization of a known dose of tetanus toxin.*

*The study reported here compared these two procedures, using them both to evaluate 100 sera from the Clinical Laboratory of the General Calixto García Hospital in Havana, Cuba. The two sets of results showed a high degree of correlation (r = 0.99) when subjected to linear regression analysis (95% CI = 0.985–0.993). These and other findings indicate that the cheap and rapid ultramicroELISA method can perform certain tasks for which the slower and costlier traditional assay is not well suited, such as field evaluation of tetanus toxoid vaccines and identification of hyperimmune plasmas appropriate for use in producing specific antitetanus immunoglobulin.*

The traditionally accepted method for determining tetanus antitoxin titers in the sera of humans or animals immunized with tetanus toxoid is *in vivo* seroneutralization (*1–3*), which quantifies the activity of the antibodies, reflecting their actual capacity for neutralizing the tetanus toxin in the organism. Despite this strength, the method has serious drawbacks when a large number of samples must be analyzed, because it takes five days, requires a minimum sample volume of 0.5 mL, and employs a large number of animals—which in turn require appropriate facilities and trained personnel to care for them. This makes *in vivo* seroneutralization so expensive as to render it impractical for large-scale use—in such work as epidemiologic study of antitetanus immunity, field evaluation of tetanus toxoid vaccines, or identification of hyperimmune plasmas for producing specific antitetanus immunoglobulin.

In recent decades, various authors have described *in vitro* methods—including hemagglutination (*4, 5*), radioimmunoassay (*6*), and more recently solid-phase enzyme-linked immunosorbent assay (ELISA) (*7–9*)—for determining levels of tetanus antitoxin present in sera or plasmas used in studies of tetanus immunity. The results obtained with ELISA correlate well with those of the traditional biologic assay. In addition, Cuba has the

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technology needed for an ultramicroanalytical system (UMAS) (*10–11*)[5] that permits quantification of the tetanus antitoxin in large numbers of serum samples rapidly at low cost. Utilizing this system, the ELISA method was combined with it to create the ultramicroELISA (UME) procedure described here.

## MATERIALS AND METHODS

### Sera

One hundred serum samples from the clinical laboratory of Havana's General Calixto García Hospital were evaluated. The samples, which were kept refrigerated at −20 °C until tested, were subjected to both the traditional biologic assay and the UME in order to assess tetanus antitoxin activity.

### Tetanus Antitoxin Standards

For the traditional biologic assay, a lyophilized equine antitoxin previously evaluated by comparison with the Second International Tetanus Antitoxin Standard established by WHO in 1969 was used as a working standard. The human antitoxin that served as a standard for the UME was prepared from a standard commercial immunoglobulin treated with diethylaminoethyl (DEAE) Sephadex A-50 (Pharmacia, Sweden), lyophilized, and evaluated using the traditional biologic assay for comparison with the equine antitoxin that served as the working standard. The estimated level of antitetanus activity of the standard human antitoxin, reconstituted with 1 mL of diluent, was 1.2 international units (IU) per mL.

[5]See also Fernández JL. Desarrollo de micrométodos de inmunoanálisis para el diagnóstico. [Doctoral thesis]. Havana: Centro Nacional de Investigaciones Científicas, Ministerio de Educación Superior; 1980.

### Solutions

A buffer solution of 0.05M sodium carbonate/bicarbonate (pH 9.6) was used to dilute the tetanus toxoid and sensitize the ELISA plates. To wash the reaction plates during each step of the assay, a buffer solution (PBS-T) containing phosphates and Tween 20 (8 g NaCl, 1.215 g $Na_2HPO_4 \cdot 2H_2O$, 0.2 g $KH_2PO_4$, 0.2 g KCl, 0.2 g $NaN_3$, 0.5 mL Tween 20, and distilled water to a volume of 1 L) with a pH of 7.3–7.5 was employed.

In the UME, inactivated normal sheep serum (5% in PBS-T) was used to dilute the samples, control sera, conjugate, and standard tetanus antitoxin used to establish the calibration curve. A 10% v/v diethanolamine buffer (pH 9.8) was used as the diluent for the fluorogenic substrate, which was 4-methyl umbelliferyl phosphate in a solution containing 0.13 mg/mL (Koch Light, England). (The hydrolysis produced by the alkaline phosphatase releases 4-methyl umbelliferone, which emits light with wavelengths in the range of 420–529 nm when excited by light with a wavelength in the range of 356–365 nm.)

### Antigens

For the traditional biologic assay, a lyophilized reference tetanus toxin (Empresa de Producción de Biológicos Carlos J. Finlay, Cuba) was used as antigen. The doses for indirect tests (L+/10, L+/100, L+/1 000 and L+/10 000) were determined by conducting the seroneutralization assay *in vivo* with the standard tetanus antitoxin of equine origin a minimum of six times. The doses selected for evaluating the sera were determined in accordance with the levels of antitetanus activity detected earlier with the UME procedure, which employed a concentrated and purified tetanus toxoid (Empresa de Producción de Biológicos Carlos J. Finlay) whose flocculation units per mL

(Lf/mL) had been determined previously by means of a flocculation assay (*12*). This toxoid was appropriately diluted and lyophilized so that when reconstituted with 1 mL of sodium carbonate/bicarbonate buffer it would have 10 Lf/mL.

## Control Sera

A positive control serum was prepared from a mixture of sera from a Havana blood bank. The mixture tested negative for both hepatitis B virus surface antigen (HBsAg) and antibodies to the human immunodeficiency virus (HIV). Its estimated tetanus antitoxin activity—3.25 ± 0.75 IU/mL—was determined by means of the traditional biologic assay.

It was not feasible to find serum mixtures in Cuban blood banks from which tetanus antitoxin was absent. For this reason, a negative control serum was prepared by extracting the antitoxin from the positive control serum using immunoaffinity chromatography in a column of AH-Sepharose 4-B (Pharmacia, Sweden) with immobilized tetanus toxoid.[6] Elimination of antitetanus activity was confirmed by means of a biologic assay in which tetanus toxin at a dose of L+/10 000 was used. The total protein concentration (as determined by the Biuret method) following this process was 6.2 g/dL, a level that is within the range of concentrations found in normal sera (6.0–7.5 g/dL).

## Human Anti-IgG

A heavy-chain human anti-IgG produced in sheep was obtained and conjugated with alkaline phosphatase using the method described by Voller et al. (*13*). BALB/c mice weighing 16–18 g, all of the same sex, were used in each of the traditional biologic assays.

## Ultramicroanalytical System

For the ultramicroanalytical system (UMAS) testing designed to examine many samples quickly and cheaply, three items of equipment are needed. These are (1) a 96-tip multipipette with the ability to dilute samples and to add them or other components to the reaction plate simultaneously, (2) an automatic washer to wash the plates during the various phases of the assay, and (3) a computerized reader to read the plates and analyze and present the results.

For the UME (*10–11*),[7] polystyrene plates having 96 microwells with an individual capacity of 20 μL each were employed. The reaction volume in all cases was 10 μL. Readings were expressed in percent fluorescence; and, through computer programs specific for each assay, the corresponding values were transformed into units of measure appropriate for the analysis in question.

## Biologic Assay

The *in vivo* seroneutralization method used was that established by the Empresa de Producción de Biológicos Carlos J. Finlay (*3*). The doses of tetanus toxin varied in accordance with the neutralizing activity obtained with the UME when the serum samples were analyzed. The standard antitoxin for the UME, the control sera, and the study sera were evaluated using this method, and a reading of the antitetanus activity, expressed in IU/mL, was obtained for each. The estimated level of antitetanus activity of the standard antitoxins and the control sera was determined using a minimum of six assays conducted under identical condi-

[6]Fajardo EM, Silva C. Portuondo B, Leyva JL, Noroña M. Obtención de suero negativo de antitoxina tetánica mediante cromatografía de afinidad. Paper presented at the Pharmaceutical Sciences 87 meeting held in Havana, Cuba, on 29–31 October 1987.

[7]See also footnote 5.

tions. The results obtained using this traditional biologic assay served as a reference for comparing the results obtained with the UME.

## UltramicroELISA Procedure

The indirect UME method devised by Voller et al. (*13, 14*) was used to detect antitetanus antibodies after determining the optimum dilutions of antigen, standard antitoxin, conjugate, control sera, and samples; the intra-assay (2%) and interassay (5%) inaccuracy for the samples, taking the positive control as the assay sample; and the intra-assay (3%) and interassay (5%) inaccuracy for the calibration curve.

The reaction plates were sensitized with tetanus toxoid diluted in carbonate/bicarbonate buffer until a final concentration of 0.3 Lf/mL was obtained. They were then left overnight at a temperature of 2–8 °C and were washed three times consecutively with PBS-T for 30 seconds. After that the plates were shaken dry against nonfluorescent absorbent paper, and a 1% solution of bovine seroalbumin in PBS-T was added as a blocking agent. The plates were then kept at a temperature of 20–25 °C for one hour. The contents of the microwells were subsequently removed by aspiration, and any residual blocking solution was removed. The serum samples, together with the control sera, were then added; and a calibration curve was applied that was based on the standard antitoxin for the UME, represented by five successive double dilutions (from 0.16 to 0.01 IU/mL) and a 0 value (sheep serum with no antitoxin added). The samples and control sera were diluted 1:100 for the analysis.

The control sera and those used to establish the points on the calibration curve were tested twice. This was not done with the experimental serum samples, however, in order to permit a larger number to be tested (although if greater precision were desired, each sample could have been tested twice, a procedure that would have increased the cost of the determination). As a result, it was possible to examine 78 samples on each reaction plate. The plates were incubated for one hour at 37 °C and were washed as previously described. The conjugate was then added, at a ratio of 1:3 000, and the plates were incubated for another hour at 37 °C. Following the required wash, the fluorogenous substrate was added, after which the plates were maintained at a temperature of 20–25 °C for 30 minutes. A reading was then taken to determine the tetanus antitoxin activity, expressed in IU/mL, of the samples and control sera, using the calibration curve for reference.

Samples with an antitoxin activity <1 IU/mL were not detected by this system when dilutions of 1:100 were prepared. To study these samples it was necessary to use a dilution of 1:10, which yielded results in an analytical spectrum of 0.1–1.6 IU/mL.

## Computer Analysis

The computer systems used to analyze the data and interpret the results were written in UCSD Pascal computer language (*15, 16*). The data for each sample were introduced into the system using a code that made it possible later to identify each result.

Employing the algorithm used to perform the calculations and interpret the results (*17*), levels of fluorescence, expressed in percentages, were analyzed relative to different points on the calibration curve with known levels of antitoxin activity (IU/mL) and the positive and negative control sera. The readings obtained were accepted or rejected in accordance with the following criteria, which were established through validation assays for the method:

- Calibration curve: A maximum variation of 10% between the fluorescence values (%) of two duplicates was accepted. If this condition was not met at only one point on the curve, the anomalous duplicate was eliminated and a correction was made in order to permit use of the curve. If such variation was observed at more than one point on the curve, the assay was considered invalid. Also, to meet the minimum sensitivity requirement, the point showing the least antibody activity had to exhibit a percentage fluorescence greater than the median value of the sheep serum without antitoxin plus two standard deviations (SD). It was also required that the overall chart of analytical values have a linear distribution with a slope approaching 1, and that the fluorescence of the point on the calibration curve with the greatest activity (0.16 IU/mL) have a value between 100% and 150%.
- Control sera: A maximum variation of 10% between the fluorescence values (%) of two duplicates was accepted. In the case of the negative serum (SERON), the average fluorescence (%) had to be less than the average fluorescence (%) of the sheep serum without antitoxin plus two SD. In the case of the positive serum (SEROP), the average fluorescence (%) had to fall between the second and third of the five points on the calibration curve, and its tetanus antitoxin activity had to be between 2.5 and 4.0 IU/mL, as calculated using the traditional biologic assay.

When the above conditions were not met, the assay was considered invalid and the computer program rejected the reaction plate. If during this initial step of the analysis no rejections occurred, the fluorescence values of the 78 samples were interpolated on the calibration curve and their tetanus antitoxin activity was determined, in IU/mL, by multiplying the activity value obtained on the curve times the dilution of the sample. Since activity levels of less than 1 IU/mL could not be detected with samples having a dilution of 1:100, the program interpreted these samples as though they were negative and attributed to them an activity of 0.0 IU/mL. In order to determine their true antitoxin activity, it was necessary to repeat the assay using a dilution of 1:10 and entering appropriate data for the new dilution. Regarding control sera, the program treated these as though they were study samples and accordingly provided results for a total of 82 samples.

With regard to further processing, the serum samples' tetanus antitoxin activity levels, as indicated by both *in vivo* seroneutralization and UME, were subjected to linear regression analysis.

## RESULTS

The antitetanus activity (IU/mL) detected with the traditional biologic assay was consistent, in most cases, with the activity indicated by the UME. This was true both for the samples diluted 1:100 ($n$ = 93) and for those diluted 1:10 ($n$ = 7). This consistency is shown clearly in Figure 1, which presents the results of the linear regression analysis. The equation of the line and the regression coefficient (r) show a linear correlation between the values obtained in both assays, as a result of which it was concluded that the UME did satisfactorily reproduce the results obtained by subjecting the 100 sera in the study sample to the traditional biologic assay.

Figure 2 shows a computer printout of the UME results for a single test plate. As one of the initial aims in developing this technique was to use it in investigating plasmas with an antitetanus activity of at least 5 IU/mL for production of specific immunoglobulin, the processing

**Figure 1.** Correlation between the tetanus antitoxin titers of the study sera (expressed in IU/mL) obtained by the ultramicroELISA (UME) method and by the traditional biologic assay, as determined by linear regression analysis.

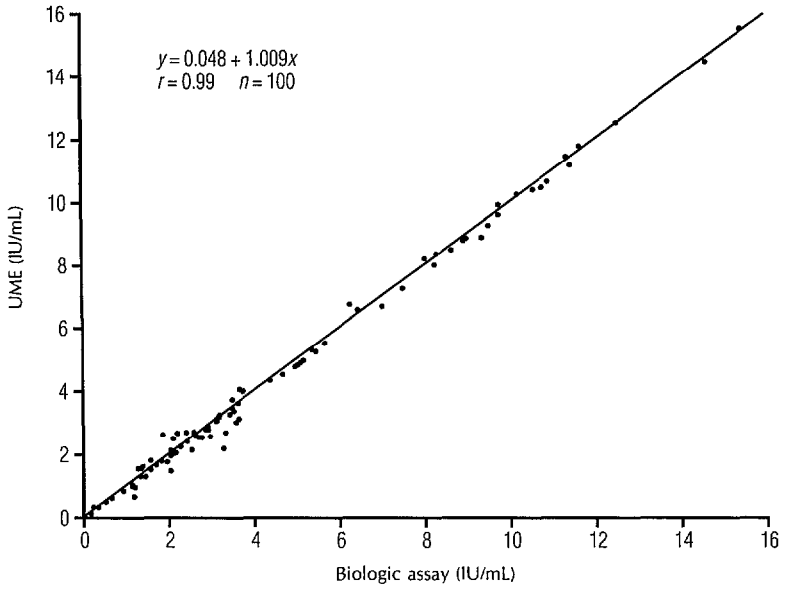


**Figure 2.** The format in which the study's computer program printed out the tetanus antitoxin titers (in IU/mL) obtained by the ultramicroELISA (UME) method. The meanings of abbreviations used in the printout are as follows: STAND = Values of antitetanus activity (IU/mL) on the calibration curve. FMIN-FMAX = Minimum and maximum fluorescence (%) of the serum pairs at each of six points on the curve. DIF (%) = The percentage difference between the fluorescence values of the two members of each serum pair. CORREC = Correction (if necessary) of the median value of the pairs (in the event of an aberrant value). EST = Average value for each point used in constructing the assay calibration curve. SEROP = Positive control serum. SERON = Negative control serum. APT = Designation of a sample exhibiting tetanus antitoxin activity ≥5 IU/mL.

| ELISA OF TETANUS ANTITOXIN: | | | | | PLATE: M6C-8689 | 8/8/89 |
|---|---|---|---|---|---|---|
| STAND | FMIN | FMAX | DIF (%) | MEAN | CORREC | EST |
| 0.00 | 6.98 | 7.30 | 4.48 | 7.14 | 7.14 | |
| 0.01 | 27.59 | 28.26 | 2.40 | 27.92 | 27.92 | 27.92 |
| 0.02 | 43.16 | 43.20 | 0.09 | 43.18 | 43.18 | 43.18 |
| 0.04 | 60.27 | 62.32 | 3.34 | 61.30 | 61.30 | 61.30 |
| 0.08 | 83.15 | 88.21 | 5.91 | 85.68 | 85.68 | 85.67 |
| 0.16 | 103.97 | 110.16 | 5.78 | 107.06 | 107.06 | 107.06 |

(Continued)

**Figure 2.** (Continued)

QUANTIFICATION OF TETANUS ANTITOXIN:

| NUMBER | CODE | | IU/mL | NUMBER | CODE | | IU/mL |
|---|---|---|---|---|---|---|---|
| 1 | SEROP | 52.34 | 2.9 | 2 | SEROP | 52.17 | 2.9 |
| 3 | SERON | 9.57 | 0.0 | 4 | SERON | 9.37 | 0.0 |
| 5 | 00G-159 | 72.01 | 5.5-->> APT | 6 | 00G-24 | 80.10 | 6.8-->> APT |
| 7 | 00G-85 | 111.58 | >16.0-->> APT | 8 | 00G-126 | 62.89 | 4.2 |
| 9 | 00G-45 | 39.58 | 1.7 | 10 | 00G-33 | 54.05 | 3.1 |
| 11 | 00G-44 | 98.41 | 11.9-->> APT | 12 | 00G-7 | 81.77 | 7.2-->> APT |
| 13 | 00G-94 | 31.82 | 1.2 | 14 | 00G-108 | 88.43 | 8.7-->> APT |
| 15 | 00G-57 | 70.54 | 5.3-->> APT | 16 | 00G-83 | 33.55 | 1.3 |
| 17 | 00G-89 | 51.01 | 2.8 | 18 | 00G-91 | 112.52 | >16.0-->> APT |
| 19 | 00G-121 | 104.19 | 14.4-->> APT | 20 | 00G-81 | 91.61 | 9.6-->> APT |
| 21 | 00G-17 | 90.47 | 9.2-->> APT | 22 | 00G-142 | 95.55 | 10.8-->> APT |
| 23 | 00G-51 | 86.03 | 8.1-->> APT | 24 | 00G-55 | 68.12 | 4.9 |
| 25 | 00G-140 | 89.31 | 8.9-->> APT | 26 | 00G-152 | 72.39 | 5.5-->> APT |
| 27 | 00G-80 | 48.14 | 2.5 | 28 | 00G-111 | 69.91 | 5.2-->> APT |
| 29 | 00G-84 | 54.20 | 3.1 | 30 | 00G-131 | 51.24 | 2.8 |
| 31 | 00G-99 | 70.42 | 5.2-->> APT | 32 | 00G-107 | 100.85 | 12.9-->> APT |
| 33 | 00G-130 | 71.05 | 5.3-->> APT | 34 | 00G-72 | 70.52 | 5.3-->> APT |
| 35 | 00G-25 | 46.24 | 2.3 | 36 | 00G-14 | 82.18 | 7.2-->> APT |
| 37 | 00G-8 | 120.28 | >16.0-->> APT | 38 | 00G-22 | 81.15 | 7.0-->> APT |
| 39 | 00G-78 | 83.47 | 7.5-->> APT | 40 | 00G-82 | 78.91 | 6.6-->> APT |
| 41 | 00G-42 | 49.68 | 2.6 | 42 | 00G-64 | 62.39 | 4.1 |
| 43 | 00G-1 | 104.43 | 14.6-->> APT | 44 | 00G-116 | 72.81 | 5.6-->> APT |
| 45 | 00G-29 | 50.24 | 2.7 | 46 | 00G-133 | 71.80 | 5.4-->> APT |
| 47 | 00G-38 | 13.66 | 0.0 | 48 | 00G-158 | 54.46 | 3.2 |
| 49 | 00G-48 | 59.92 | 3.8 | 50 | 00G-56 | 68.42 | 5.0-->> APT |
| 51 | 00G-86 | 68.95 | 5.0-->> APT | 52 | 00G-73 | 110.42 | >16.0-->> APT |
| 53 | 00G-62 | 24.29 | 0.0 | 54 | 00G-109 | 61.25 | 4.0 |
| 55 | 00G-79 | 63.12 | 4.2 | 56 | 00G-134 | 81.08 | 7.0-->> APT |
| 57 | 00G-50 | 92.13 | 9.7-->> APT | 58 | 00G-132 | 11.11 | 0.0 |
| 59 | 00G-115 | 50.62 | 2.7 | 60 | 00G-32 | 71.35 | 5.4-->> APT |
| 61 | 00G-120 | 31.31 | 1.2 | 62 | 00G-90 | 64.97 | 4.5 |
| 63 | 00G-95 | 38.59 | 1.6 | 64 | 00G-138 | 44.16 | 2.1 |
| 65 | 00G-87 | 78.92 | 6.6-->> APT | 66 | 00G-112 | 67.59 | 4.8 |
| 67 | 00G-52 | 64.65 | 4.4 | 68 | 00G-35 | 132.75 | >16.0-->> APT |
| 69 | 00G-71 | 40.29 | 1.8 | 70 | 00G-54 | 95.16 | 10.7-->> APT |
| 71 | 00G-61 | 82.95 | 7.4-->> APT | 72 | 00G-110 | 30.30 | 1.1 |
| 73 | 00G-46 | 54.50 | 3.2 | 74 | 00G-143 | 50.95 | 2.8 |
| 75 | 00G-149 | 110.83 | >16.0-->> APT | 76 | 00G-136 | 92.32 | 9.8-->> APT |
| 77 | 00G-39 | 44.87 | 2.1 | 78 | 00G-67 | 95.68 | 10.9-->> APT |
| 79 | 00G-67 | 44.66 | 2.1 | 80 | 00G-76 | 67.55 | 4.8 |
| 81 | 00G-47 | 82.20 | 7.2-->> APT | 82 | 00G-38 | 41.65 | 1.9 |

of the data was programmed so as to tag with the word "apt" any sample showing that degree of activity, in order to facilitate rapid and error-free selection.

## DISCUSSION AND CONCLUSIONS

The UME procedure described above affords a way of producing results quickly—without any need for making subsequent calculations that could delay analysis and be a source of error. The sample used in this study was sufficiently large to permit reproduction of the results obtained, providing similar samples are used. However, possible exception to this statement should be taken in those cases where little antitetanus activity (0.1 to 1.6 IU/mL) is detected and where it is necessary to subject the samples to dilutions of 1:10 in order to perform the assay.

It should be noted that the UME method described here has been used successfully to identify plasmas[8] with antitetanus activity and to conduct field evaluations of diphtheria-tetanus vaccine (*18, 19*). Overall, the UME's speed, low cost, and ability to produce results consistent with those of the traditional biologic assay have shown that it is suitable for field-testing tetanus toxoid vaccines and for identifying hyperimmune plasmas with a view toward producing the specific immunoglobulins involved.

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## *1995 Soper Award Recipient*

The Board of Trustees of the Pan American Health and Education Foundation (PAHEF) bestowed the 1995 Fred L. Soper Award on Dr. John Gay and collaborators for their article "Factores dietéticos de la neuropatía epidémica en la Isla de la Juventud, Cuba" ["Dietary Factors in Epidemic Neuropathy on the Isle of Youth, Cuba"], which was published in Spanish in the *Boletín de la OSP* 116(5), May 1994, and in English in the *Bulletin of PAHO* 29(1), March 1995.

The award is given annually for an outstanding original scientific contribution to public health, with special relevance to Latin America and/or the Caribbean, that was published in the preceding year in a scientific journal listed in the *Index Medicus*. Eligibility is limited to works by authors whose principal affiliation is with a teaching, research, or service institution located in a Latin American or Caribbean country. Nominations for the 1996 award are currently being accepted by the Executive Secretary, PAHEF, at 525 Twenty-third Street, N.W., Washington, D.C. 20037, U.S.A.