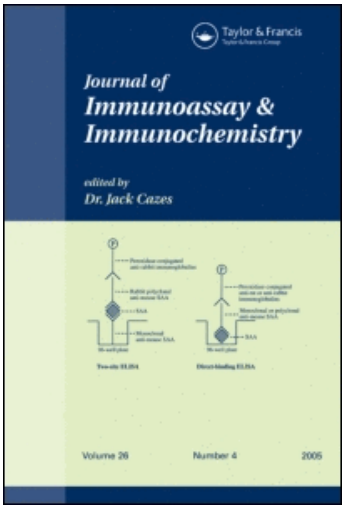

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A New End-Point for ELISA Titrations

José Vidal[a]
[a] Departamento de Personalidad, Facultad de Psicología, Universidad de Barcelona, Barcelona, Spain

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A New End-Point for ELISA Titrations

José Vidal*

Departamento de Personalidad, Facultad de Psicología, Universidad de Barcelona, Barcelona, Spain

ABSTRACT

This report describes a new ELISA procedure based on end-point titrations. This end-point ELISA takes advantage of the change of color intensity that occurs when peroxidase-containing wells of an ELISA plate are revealed with diaminobenzidine–nickel and further intensification with silver: as antibody concentration and, therefore, peroxidase concentration, decreased, the color became stronger in some wells and, afterwards (i.e., at lower antibody and peroxidase concentrations), the color faded toward clear background. It is proposed that the reciprocal of the sample dilution at which the color intensifies can be used as a measure of the sample antibody content. This report verifies the validity and precision of that procedure.

Key Words: ELISA; End-point; Silver enhancement; Diaminobenzidine; Poly(lys, phe); Immunoglobulin measurement.

*Correspondence: José Vidal, Departamento de Personalidad, Facultad de Psicología, Universidad de Barcelona, Passeig de la Vall d'Hebron 171, 08035 Barcelona, Spain; E-mail: jvidal@ub.edu.

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INTRODUCTION

Diffusion-in-gel enzyme-linked immunosorbent assay (DIG-ELISA)[1] is a simple, cheap, and sensitive procedure to measure the antibody concentration in a sample. In this technique, a layer of gel (agar or agarose) is deposited onto a polystyrene surface that has antigen adsorbed on it, then holes are bored in the gel and they are filled with antibody-containing solution; after several hours, the gel is removed and antibody-containing areas are visualized enzymatically. The original technique[1] was modified in order to increase its sensitivity and reliability.[2] Nevertheless, the technique still has a drawback, namely, performance takes a long time (24 hr to measure IgG concentration and 48 hr to measure IgM concentration). Those long times are due to the slow diffusion of IgG and IgM antibodies in the gel.

When antibody-containing areas, in DIG-ELISA, were revealed with an excess of peroxidase conjugate and the substrate diaminobenzidine–nickel–silver,[2] those areas had the aspect shown in Fig. 1, i.e., a circular spot of

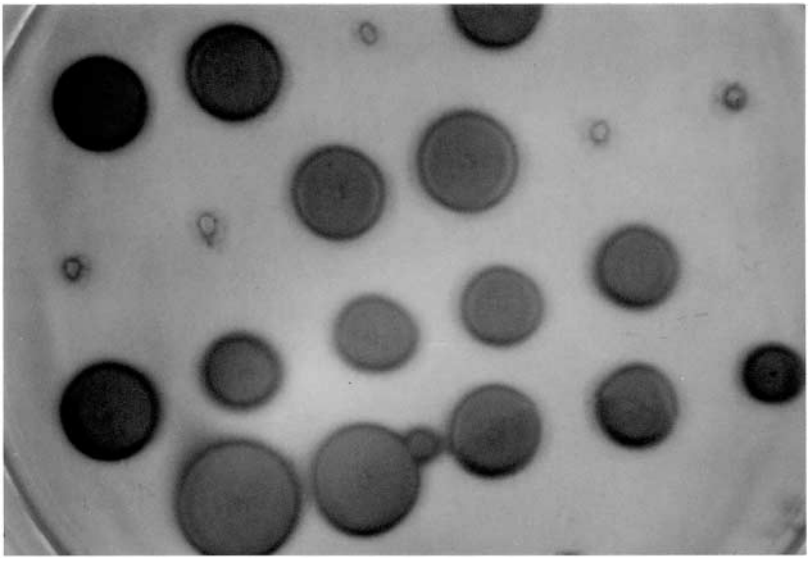

Figure 1. DIG-ELISA revealed with diaminobenzidine–nickel–silver. Murine serum, containing IgG antibodies to rat erythrocytes diffused, in an agar layer, over polystyrene with erythrocyte stromata bound to it. Antibody-containing areas were disclosed with (an excess of) peroxidase-conjugated anti-IgG antibodies and diaminobenzidine plus nickel, with color intensification with silver. A ring of stronger color can be seen in the periphery of the spots.

pale color was surrounded by a ring of stronger color. Because the antibody concentration decreased from the center of the spot toward the periphery, at a certain (low) antibody concentration, the color became more intense, and thereafter, i.e., at lower antibody concentrations, the color faded away. In principle, this increase in color intensity would take place also in the wells of an ELISA plate that contains a serial dilution of antibodies, and the intensification of color could be used as an end-point. Consequently, one could perform an enzyme-linked immunosorbent assay (ELISA) and quantitate the results by the increase in color intensity, since the dilution corresponding to the color increase would reflect the antibody content. In this manner, there would be no need to use a spectrophotometer, and ELISA would become simpler and cheaper; besides, this end-point ELISA would be faster than DIG-ELISA.

This report shows that the end-point ELISA provides an approximate estimate of the antibody content of a sample, and that the procedure is relatively precise.

EXPERIMENTAL

Reagents

3,3′-Diaminobenzidine tetrahydrochloride dihydrate (DAB) was from Fluka (Alcobendas, Madrid, Spain), and the following reagents were from Sigma (Alcobendas, Madrid, Spain): glutaraldehyde (25% aqueous solution), phosphate–buffered saline (PBS; 10 mM phosphate, 2.7 mM potassium chloride, 0.137 M sodium chloride, pH 7.4), poly (lysine, phenylalanine) hydrobromide (lysine : phenylalanine = 1 : 1, molecular weight 20,000–50,000), ethanolamine hydrochloride, human IgG (reagent grade), human IgM (reagent grade), affinity-purified goat antibodies to human IgG (γ chain-specific), affinity-purified goat antibodies to human IgM (μ chain-specific), peroxidase-conjugated affinity-purified goat antibodies to human κ light chain, peroxidase-conjugated affinity-purified goat antibodies to human λ light chain, peroxidase-conjugated affinity-purified goat antibodies to mouse IgG (γ chain-specific) and peroxidase-conjugated affinity-purified goat antibodies to mouse IgM (μ chain-specific).

Animals and Immunizations

Ten female mice of the RjOrl : Swiss strain, approximately 3 months of age, were purchased from Janvier España, Madrid, Spain; the mice were

housed 4 per cage and received food and water ad libitum; the temperature of the room was 21°C ± 1°C. The mice were injected intraperitoneally with varying numbers of erythrocytes from a rat of the Long Evans strain: two mice were injected with 1×10^7 erythrocytes, two mice with 5×10^7 erythrocytes, three mice with 1×10^8 erythrocytes, and three mice with 1.5×10^8 erythrocytes; the red cells were suspended in PBS and each mouse received 0.1 mL of the suspension. Each mouse was boosted, 16 days later, with the same dose of erythrocytes, and was bled 5 days after the booster. The mice were bled from the tail vein.

DIG-ELISA

This technique was performed as previously described[2] with two modifications: (i) erythrocyte stromata were bound to poly(lysine, phenylalanine) by means of glutaraldehyde [instead of suberic acid *bis*(*N*-hydroxysuccinimide ester)], and (ii) the concentration of the silver reagent used to intensify the spots was one half of the concentration used before. In brief, the procedure was as follows: polystyrene petri dishes of 13.6 cm in internal diameter were coated with poly(lysine, phenylalanine), 40 μg/mL, and rat erythrocytes (20 mL/dish of a 1% suspension in saline) were allowed to settle; after three washes with saline, bound red cells were lysed, and the stromata were linked to poly (lysine, phenylalanine) with 0.1% glutaraldehyde in PBS (20 mL/dish) for 30 min; peroxidase activity of the stroma was quenched with 0.3% hydrogen peroxide, unreacted glutaraldehyde residues were quenched with a mixture of ethanolamine and gelatin, and loosely bound material was detached by a wash with PBS–Tween (0.05% Tween 20 in PBS). After addition of agar and serum samples, diffusion took place for 1 day (for IgG), or 2 days (for IgM); afterwards, peroxidase label (peroxidase–anti-IgG antibodies or peroxidase–anti-IgM antibodies) was added and areas of antibody content were revealed with diaminobenzidine–nickel reagent with further intensification with silver reagent.

End-Point ELISA

End-point ELISA was used to measure concentrations of human IgM, or human IgG, in samples thereof, and to estimate the content of antibodies to rat erythrocytes in mouse sera. To measure concentrations of human immunoglobulins, antibodies to human IgM, or IgG, were bound to 384-well MaxiSorp plates (Nunc, Roskilde, Denmark) by Hobbs' procedure;[3] the plates were incubated overnight with poly (lysine, phenylalanine), 40 μg/mL in

PBS, 25 μL/well, washed three times with water, and incubated with 0.5% glutaraldehyde in PBS (25 μL/well) for 30 min, washed twice with water and once with saline, incubated with human IgM (15 μg/mL in PBS), or IgG (15 μg/mL in PBS), 25 μL/well, for 60 min, washed twice with saline, incubated with ethanolamine hydrochloride (1 mg/mL in PBS) plus gelatin (5 mg/mL), 50 μL/well, for 30 min, washed twice with saline and once with PBS–Tween.

To measure concentrations of antibodies to rat erythrocytes, red-blood-cell-stromata were linked to 384-well plates as follows: plates were coated with poly (lysine, phenylalanine) as above, a 1% suspension of erythrocytes in saline was added, 25 μL/well, and allowed to settle for 30–40 min; thereafter, the unbound erythrocytes were washed away with saline and bound erythrocytes were lysed with PBS : water (1 : 10, v/v). After several washes with PBS : water 1 : 10 (until the washing fluid was no longer pink), 0.1% glutaraldehyde in PBS was added, 25 μL/well, and kept there for 30 min; after three washes with saline, the wells were incubated for 15 min with 0.3% hydrogen peroxide in PBS, 25 μL/well. After three washes with saline, the wells were incubated for 30 min with ethanolamine hydrochloride (1 mg/mL in PBS) plus gelatin (5 mg/mL), 50 μL/well; the plates were washed twice with saline and once with PBS–Tween.

Samples (containing human IgM, human IgG, or antibodies to rat erythrocytes) were serially diluted in the wells of the ELISA plate; dilutions, by a factor of 1.3, were done in PBS–Tween with 10 mg/mL gelatin. Each well received 25 μL of fluid. After 90 min, the plates were washed three times with PBS–Tween, and the appropriate peroxidase conjugate was added (for human immunoglobulins, anti-human kappa light chain + anti-human lambda light chain antibodies; for murine antibodies, anti-mouse IgM or anti-mouse IgG antibodies); after approximately 90 min, wells containing antibodies were revealed with diaminobenzidine–nickel and further treatment with a silver developer (this is the procedure described by Ludány et al.[4] with a minor modification: the concentration of the silver developer was one half of that used by Ludány et al.). The incubation time for diaminobenzidine–nickel was 20–30 min, whereas the time for silver enhancement was 10–15 min.

A reference sample (a solution of human immunoglobulins of known concentration or a murine serum rich in antibodies to rat erythrocytes) was run together with the test samples, and results are expressed as the quotient: antibody concentration in the test sample/antibody concentration in the reference sample (this quotient equals the quotient: reciprocal of test-sample dilution when color increases/reciprocal of reference-sample dilution when color increases).

The working dilution of each peroxidase conjugate has to be found by cross titration, since the working concentration of the enzyme conjugate is

usually higher than the one suggested by the manufacturer for ELISA work (a concentration that is too low does not produce the color increase mentioned in the Introduction).

Statistics

Straight lines were fitted to scatter-plots by a least-squares method. Correlation coefficients are Pearson product-moment coefficients. The statistical software used was Statistica 6.0 (Tulsa, OK).

RESULTS

The Basic Phenomenon

A solution of human IgG (10 mg/mL), and a solution of human IgM (1 mg/mL), were titrated by ELISA as described above, dilutions being from 1 : 10,000 to 1 : 20,480,000 for IgG (wells B1–B12 in Fig. 2), and from 1 : 25 to 1 : 51,200 for IgM (wells E1–E12). Figure 2 shows the plate after development: an increase in color intensity is observed at dilution 1 : 80,000 for IgG (well B4) and at dilution 1 : 1600 for IgM (well E7). This assay was performed on a 96-well plates because the wells of a 384-well plates were too small and too close together to yield a good-quality picture.

Rows C and F represent assays run on wells coated with goat albumin: no color is observed in any well, which shows the absence of nonspecific adsorption by IgM, IgG, or the peroxidase conjugate (at the dilutions used). Wells D1, D2, and D3 were coated with antibodies to human IgG, but did not receive IgG: no color is perceived in those wells. Similarly, wells G1, G2, G3 were coated with antibodies to human IgM did not receive IgM, and did not develop color. Wells D4, D5, D6 were coated with IgG, and wells G4, G5, G6 were coated with IgM, but did not receive the peroxidase conjugate: no color is observed in those wells.

Validity of End-Point ELISA as a Procedure to Measure Antibody Concentration

The abrupt change in color that takes place with dilution could be used as an end-point for the measurement of the antibody content in a sample.

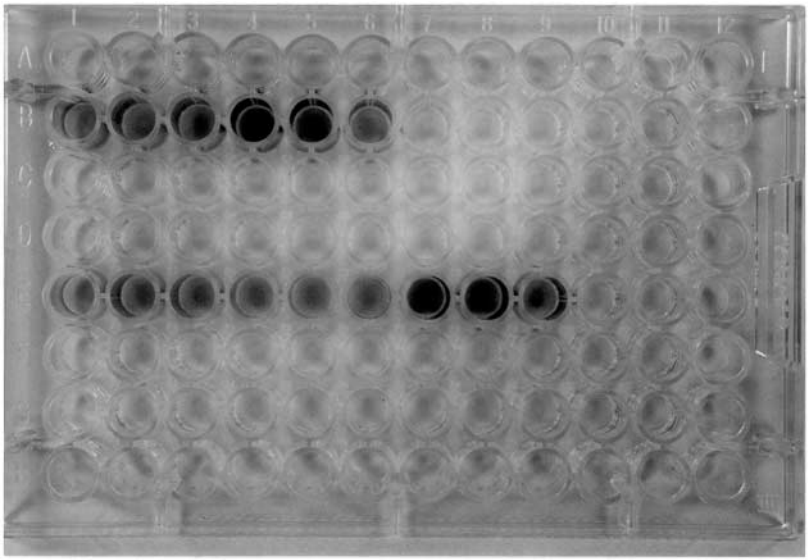

Figure 2. The basic phenomenon. Row B: serial dilution of a human IgG solution (10 mg/mL), from 1 : 10,000 (well B1) to 1 : 20,480,000 (well B12); the wells had anti-IgG antibodies bound to them. Row C: serial dilution as for row B, but the wells had goat albumin bound to them. Wells D1–D3: wells with anti-IgG antibodies bound to them, but did not receive IgG. Row E: serial dilution of a human IgM sample (1 mg/mL), from 1 : 25 (well E1) to 1 : 51,200 (well E12); the wells had anti-IgM antibodies bound to them. Row F: serial dilution of the IgM sample on wells coated with goat albumin. G1–G3: wells coated with anti-IgM antibodies and not incubated with IgM. Wells D4, D5, D6 were coated with IgG, and wells G4, G5, G6 were coated with IgM, but did not receive the peroxidase conjugate. The plate was developed with peroxidase and diaminobenzidine–nickel, with further intensification of color with silver. Color intensification is observed at dilution 1 : 80,000 for IgG (well B4), and at dilution 1 : 1600 for IgM (well E7).

This idea was tested thus: several samples were prepared by diluting a human IgM solution (1 mg/mL), and the IgM concentration of the ensuing samples was determined by end-point ELISA (the undiluted solution was run in the plate as the reference sample). The upper part of Fig. 3 shows a plot of the estimated concentration vs. the actual concentration: the equation of the line was (measured IgM) $= -0.004 + 1.190 *$ (actual IgM); the standard errors of the intercept and the slope were 0.005 and 0.050, the correlation coefficient was 0.98, and the number of points was 17. The difference between the intercept (-0.004) and 0 did not reach statistical significance

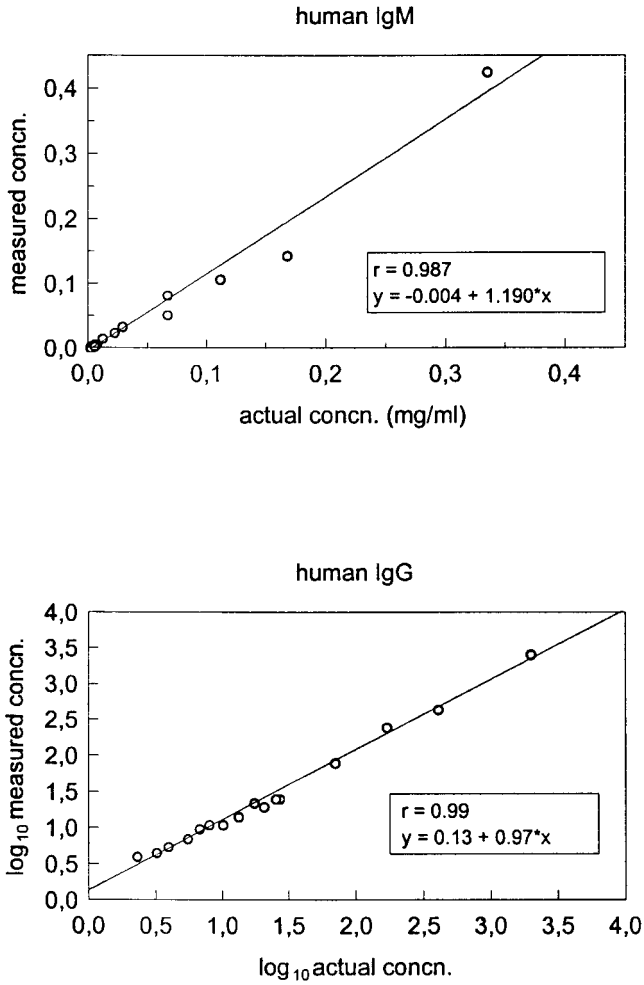

Figure 3. Accuracy of end-point ELISA. Upper graph: Plot of measured IgM concentration vs. actual concentration [obtained by diluting a sample of known concentration (1 mg/mL)]. Lower graph: plot of measured IgG content (expressed as log 10 of concentration) vs. actual content (as log 10 of concentration); the actual concentrations were obtained by diluting a sample whose concentration was 10 mg/mL.

(95% confidence limits: −0.014 to 0.006), and the slope (1.190) was slightly higher than 1 (95% confidence limits: 1.083–1.298).

The same validity test was performed with the IgG sample: the equation of the line was (measured IgG) = − 5.79 + 1.35* (actual IgG). The lower part

of Fig. 3 shows the plot of the estimated concentrations (as log 10) vs. the actual concentrations (as log 10): the equation of the line was (log measured IgG) = 0.13 + 0.97 * (log actual IgG); the standard errors of the intercept and the slope were 0.04 and 0.02, the correlation coefficient was 0.99, and the number of points was 16. The intercept (0.13) was slightly above 0 (95% confidence limits: 0.05–0.22) and the slope (0.97) was not different from 1 (95% confidence limits: 0.92–1.03).

The above results showed a good linear correlation between the actual immunoglobulin concentration and the concentration measured with the ELISA described here. Yet, all the samples had antibodies of the same affinity, because the samples were obtained by diluting the same original solution. Therefore, it was necessary to verify that the same linear association between measured concentrations and actual concentrations of immunoglobulins held for different sera. To do this, the anti-rat-erythrocytes antibody content of 10 mouse sera was measured with an established method (DIG-ELISA) and with the end-point ELISA described here. Figure 4 shows linear plots for both IgM and IgG. For IgM, the equation of the line was (IgM by end-point ELISA) = 0.71 + 0.54 * (IgM by DIG-ELISA) and the correlation coefficient was 0.87. For IgG, the equation was (IgG by end-point ELISA) = 0.03 + 0.63 * (IgG by DIG-ELISA); the correlation coefficient was 0.91.

Reliability of End-Point ELISA

To assess the intra-assay reliability, the IgM, or IgG, concentration of four replicates of a human serum was measured, in the same plate, by end-point ELISA as described above; the measurement was again repeated with two more plates. The root mean square coefficient of variation, a pooled coefficient of variation from several assays,[5] was 12% for IgM and $<1\%$ for IgG.

To assess the inter-assay reliability, the IgM, or the IgG, concentration of a human serum was measured, by end-point ELISA, in four different plates. The root mean square coefficient of variation was 31% for IgM and 12% for IgG.

The sensitivity of the procedure (the antibody concentration at which color intensification took place) could be estimated from the human serum used for reliability assessment: for IgM, the mean ($\pm$standard error of the mean) of four determinations was 1.5 ($\pm$0.6) μg/mL, whereas, for IgG, the mean ($\pm$standard error of the mean) of four determinations was 0.10 ($\pm$0.02) μg/mL.

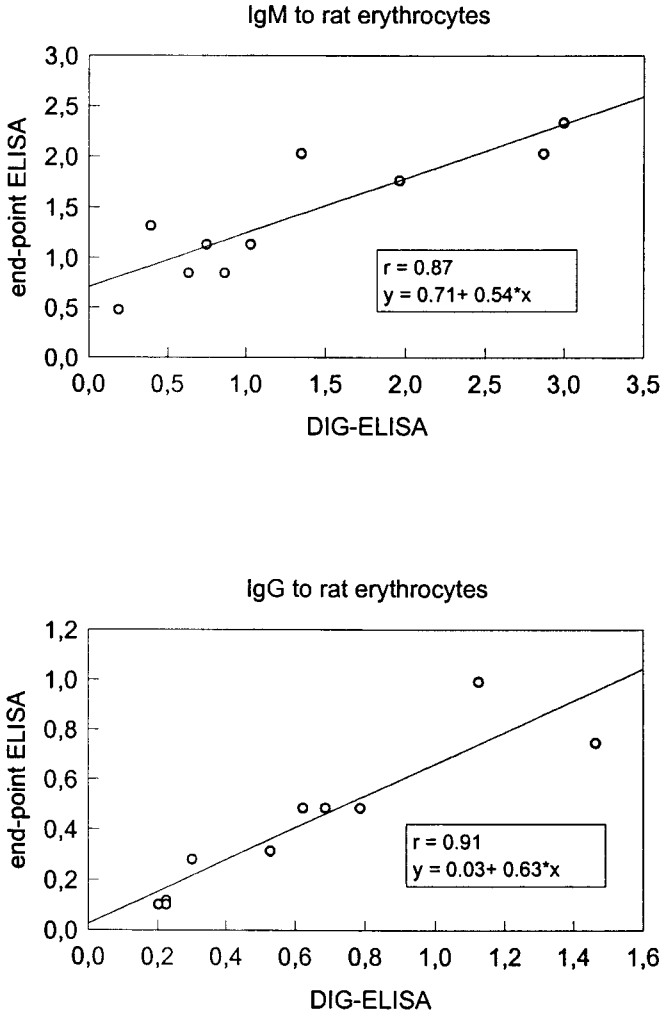

Figure 4. Comparison of end-point ELISA with DIG-ELISA. Ten mice were immunized and boosted with varying doses of rat erythrocytes (range: 1×10^7 erythrocytes/mouse–1.5×10^8 erythrocytes/mouse). Upper graph: plot of IgM content measured by end-point ELISA vs. content measured by DIG-ELISA. Lower graph: plot of IgG content measured by end-point ELISA vs. content measured by DIG-ELISA. For both end-point ELISA and DIG-ELISA, antibody content in a serum is expressed as the fraction: concentration in the serum/concentration in a reference serum.

DISCUSSION

In this report, it is proposed that the intensification of color that took place at certain antibody dilution, in plates developed with diaminobenzidine–nickel–silver, could be used as an end-point for antibody titration. This implies that the method should be accurate (i.e., that the estimated antibody concentration is close to the actual concentration) and precise (i.e., that its coefficient of variation is not too high). This report intends to verify both conditions.

Accuracy was tested by dilution experiments: several samples were created by diluting a solution of human immunoglobulin (IgM or IgG) of known concentration, their immunoglobulin concentration was estimated by end-point ELISA as described in this report, and the estimated values were plotted against the actual concentrations (Fig. 3). Ideally, the intercept and the slope of the ensuing straight line should be, respectively, 0 and 1. For the IgM line, the intercept (-0.004) was not different from 0 (Results) and the slope (1.19) was close to 1 (95% confidence interval: 1.08–1.29). For the IgG line, estimated and actual concentrations were transformed into logarithms (to the base 10) in order to dampen the influence of the higher concentrations in the regression (the concentration interval was from 0 to 3000 μg/mL). After the transformation, the intercept (0.13) was close to 0 (95% confidence interval: 0.05–0.22) and the slope (0.97) was not different from 1 (95% confidence interval: 0.92–1.03).

Besides testing the accuracy of a new method, a comparison with other current methods is necessary. Consequently, the end-point method reported here was compared with DIG-ELISA (an established method);[1,2] for the measurement of the level of anti-rat erythrocytes antibodies (Fig. 4). The correlation coefficients for IgM and IgG were, respectively, 0.87 and 0.91. These coefficients are comparable with the ones between DIG-ELISA and ELISA [$r = 0.84$, [6]], DIG-ELISA and hemagglutination [$r \approx 0.90$,[7]; $r = 0.89$,[8]], DIG-ELISA and immunofluorescence [$r \approx 0.80$,[7]; $r = 0.90$,[8]], DIG-ELISA and complement fixation [$r = 0.83$,[8]], ELISA and Farr assay and immunofluorescence [$0.53 < r < 0.85$].[9]

The use of 384-well plates has some advantages over the use of 96-well plates: (i) more samples can be processed in a plate, and (ii) the dilution factor may be lowered from 2 to 1.3, thus achieving a more continuous distribution of antibodies in a row (if dilutions were done by a 1.3 factor in a 96-well plates, each sample would take up a lot of wells and only a few samples could be processed in a plate).

The end-point procedure reported here has two advantages over ELISA: (i) it is simpler and cheaper (because it does not require a plate reader), and (ii) it may work when the test sample and the reference sample yield non-parallel

lines in a plot of absorbance vs. dilution (because the end-point does not depend on the color content of the wells). Therefore, the end-point method described here is appropriate for field studies, laboratories with a low budget, and situations where antibody content is to be correlated with a less precise variable (e.g., some behaviors).

ABBREVIATIONS

ELISA	Enzyme-linked immunosorbent assay
DAB	3,3′-Diaminobenzidine tetrahydrochloride dihydrate
PBS	Phosphate-buffered saline
DIG-ELISA	Diffusion-in-gel enzyme-linked immunosorbent assay

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