Article

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# Quantification of Autoantibodies to Annexin V in Plasma by an "In House" Sandwich ELISA

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Foi desenvolvido e validado um ELISA tipo sanduíche "*in house*" para a quantificação de anticorpos (Ac) anti-anexina V em plasma. Os parâmetros de validação estudados foram: (*i*) precisão, expressado como coeficiente de variação (CV) inter- e intra-ensaio, (*ii*) exatidão, expressado como porcentagem de desvio entre o valor obtido e o valor real, (*iii*) limite de detecção (LOD), avaliado a partir do branco de reagentes e (*iv*) robusteza, obtida através da introdução propositada de pequenas variações em diferentes parâmetros. Além disso, a técnica "*in house*" foi comparada com um método comercial. Encontrou-se que ambos CV foram < 20%, a exatidão foi de 100 ± 20%, o limite de detecção foi menor que 1 U $mL^{-1}$ e as pequenas variações na técnica não produziram variações significativas nos resultados. A comparação com o método comercial mostrou uma correlação aceitável. Concluiu-se que o método desenvolvido cumpre satisfatoriamente com os parâmetros de padronização e validação para imuno-análise.

An "in house" sandwich ELISA for the quantification of plasma anti-annexin V antibodies was developed and validated. The validation parameters studied were: (*i*) precision, expressed either as the intra- or the inter-assay coefficient of variation (CV), (*ii*) exactitude, expressed as the percentage deviation between the obtained value and the real value, (*iii*) limit of detection (LOD), evaluated from the reagents blank and (*iv*) robustness, obtained by deliberately introducing slight variations in different parameters. Also, a comparison between the "in house" technique and a commercial method was performed. The research revealed that both CV were < 20%, exactitude was within the 100 ± 20% range, limit of detection was below 1 U $mL^{-1}$ and that slight variations in the technique did not produce any significant variations in the results. Comparison with the commercial method showed an acceptable correlation. It was concluded that the method developed here satisfactorily accomplishes the parameters of standardization and validation for an immunoassay.

**Keywords:** ELISA, annexin V, antibodies, anti-phospholipid syndrome, foetal losses

## Introduction

Annexins belong to a family of proteins that are able to bind to negatively charged phospholipids and membrane bilayers through calcium dependent interactions. Though their fine structure has been well described, their functions have not been clearly identified yet.[1] Like others, annexins constitute a group of ubiquitous cytoplasmic proteins involved in signal transduction.[2]

Annexin V is a 320-amino acid-residue, 36-kDa-protein that is folded into a planar cyclic arrangement of four repeats with each repeat composed of five alpha-helical segments.[3,4] It is expressed in various cell types, including placental trophoblasts and vascular endothelial cells. This protein is highly expressed in an apparently constitutive manner by placental trophoblasts and is displayed

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on the apical membrane of the trophoblast cell line.[5] Moreover, annexin V has been shown to be required for the maintenance of placental integrity in mice. Infusion of anti-annexin V IgG antibodies (Ab) into pregnant animals decreased annexin V availability to bind to the trophoblast surface and caused placental thrombosis, necrosis and foetal loss.[6] There is also evidence that this protein plays a role in the maintenance of placental function in humans. Annexin V polymorphisms have been implicated as a risk factor for pregnancy losses.[7,8] Reduction of annexin V in preeclampsia was associated with intrauterine growth restriction and with elevations of plasma level of fibrin degradation products and thrombin-antithrombin III complexes.[9,10] These data support the concept that this protein may have a thrombomodulatory function at the maternal-foetal interface within the placental blood circulation by shielding apical membrane phospholipids from the critical phospholipid-dependent enzymatic reactions leading to blood clotting.

Anti-annexin V Ab were initially detected in patients with systemic lupus erythematosus (SLE).[11] These Ab have been associated with thrombotic events and/or recurrent abortions in patients with SLE and antiphospholipid syndrome (APLS), an autoimmune condition in which venous or arterial thrombosis and recurrent pregnancy losses occur in patients having serologic evidence of Ab against anionic phospholipid-protein complexes. Although the mechanisms leading to the occurrence of anti-annexin V Ab have not been completely elucidated yet,[12] it has been proposed that, in the context of increased apoptosis, extracellular/membrane annexin V might constitute an antigenic stimulus for specific Ab production. Moreover, it is suspected that anti-annexin V Ab may interfere with annexin V functions and exerts a detrimental role leading to thrombosis and/or vascular occlusion.[13] Nevertheless, there has been conflicting evidence regarding the correlation of Ab against annexin V with disease, with some studies indicating an association with clinical manifestations such as increased risk of pregnancy losses or thrombosis[14-19] whereas others have found this not to be the case.[20-22]

The scarce information available and the controversy in the literature prompted us to design and implement an "in house" sandwich enzyme-linked immunosorbent assay to quantify plasma levels of anti-annexin V Ab, thus providing for the first time a standarized method only commercially available at present. This assay will allow us to clarify whether these Ab constitute a risk factor for thromboembolism and for miscarriages in a rapid, simple way of processing a large number of samples.

## Experimental

### Obtaining of "in house" calibrators

Different-titer anti-annexin V plasma samples collected from SLE and APLS patients (negative for hepatitis B surface antigen and human immunodeficiency virus by U. S. Food and Drug Administration-approved methods) were identified using a commercially available ELISA kit for quantification of plasma levels of annexin V Ab (Orgentec Diagnostika GmbH, Mainz, Germany). These samples were used as calibrators. The volume of 3 mL of venous blood was collected from every subject under complete aseptic conditions and added to EDTA-containing tubes (1:80). The tubes were gently rocked several times immediately after collection of blood to avoid clotting. Plasma was separated by centrifugation of the tubes at 3000 rpm for 10 min and stored frozen at −70 ºC until examination of anti-annexin V Ab. Plasma is preferred to serum for the determination of these Ab to avoid inhibition of the immunological reaction by annexin V released from blood cells during clotting.[23] Thus, samples showing slight hemolysis were readily discarded. No interference has been observed with lipemic or icteric plasma. The patients need not to be fasting, and no special preparations are necessary.

This study has been approved by the Bioethics Committee of our institution and patients have signed an informed consent.

### Controls

All assays were validated by the inclusion of a positive and a negative control, as internal quality controls. Low-, medium- and high-titer positive controls were prepared by using pooled plasma obtained as described in section 1. Aliquots of 100 μL were saved in eppendorf tubes and stored at −70 °C. Samples collected from healthy individuals were used as negative controls. The concentration of anti-annexin V Ab in these samples was below 6 U $mL^{-1}$, the inferior limit of normality suggested by the commercial method. The Ab concentrations in the pooled plasma used as control were determined using the commercial kit mentioned in the former section and the values obtained with our "in house" method were considered as acceptable when they did not differ by more than 20%.[24,25]

### "In-house" sandwich ELISA for detection of Ab to annexin V

The procedure is based on the sandwich principle of heterogeneous ELISA for detection of Ab, where the

antigen is immobilized on the solid phase, the sample contains the Ab to be determined, and the whole system is developed by means of a second Ab enzymatically labeled and directed to the first one.

Standarization of the technique was based on both the international literature available[26-28] and our own previous experience.[29] Plates were coated with different amounts (5, 7.5 or 10 µg *per* well) of native annexin V isolated from human placenta, (purity by sodium dodecyl sulfate electrophoresis: 93%, Sigma Chemical Co.; St. Louis, MO, USA) and incubated at 4 ºC. No significant difference was found among the different amounts of antigen ($p > 0.05$), so the lowest one (5 µg *per* well) was chosen in order to optimize resources (data not shown).

Microwell strips (Nunc, Copenhagen, Denmark) were coated with annexin V (5 µg *per* well, used as capture antigen) diluted in carbonate-bicarbonate buffer pH 9.6 (Sigma Chemical Co., St. Louis, MO, USA) and incubated overnight at 4 ºC. Wells were then washed five times with phosphate buffer saline (PBS, 10 mmol $L^{-1}$ sodium phosphate, 2.7 mmol $L^{-1}$ potassium chloride and 137 mmol $L^{-1}$ sodium chloride, pH 7.4) containing 0.05% Tween 20 (PBS-T) and blocked with 100 µL of 1% m/v bovine serum albumin (BSA, Sigma Chemical Co., St Louis, MO, USA) in PBS for 60 min at room temperature. After three washes with PBS-T, 100 µL of calibrators and positive and negative controls (diluted 1:100 in PBS-BSA) were added to each well. After 60 min of incubation at room temperature, wells were washed four times with PBS-T. The amount of 50 µL of peroxidase-conjugated goat Ab to either human IgG or IgM (Sigma Chemical Co., St Louis, MO, USA) diluted in PBS-BSA (1:15000 and 1:30000, respectively) were added to each well and incubated for 30 min at room temperature. After four short washes and one five-min-wash with PBS-T, 50 µL of chromogenic substrate (3,3',5,5'-tetramethylbenzidine, TMB, Sigma Chemical Co., St Louis, MO, USA) were added to each well and allowed to develop for 15 min. The reaction was stopped by addition of 50 µL of 1 mol $L^{-1}$ HCl and optical densities were recorded at 450 nm by means of a Sirio S ELISA microplate reader (Radim, Italy), using a 630 nm filter as a reference. A calibration curve was performed by plotting optical density *vs.* calibrators concentrations (5, 15, 30, 40 and 80 U $mL^{-1}$) on a semi logarithmic scale (Figure 1).

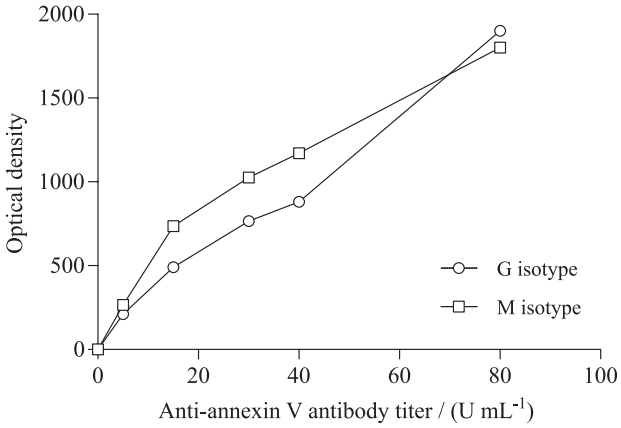


**Figure 1.** Calibration curves for IgG and IgM isotypes were obtained by plotting optical density *vs.* concentration of the calibrators used (5, 15, 30, 40 and 80 U $mL^{-1}$) in a semilogarithmic scale. Each point represents mean of four repeats.

## Validation parameters

### Precision

Precision is the concordance rate among the values obtained in a measurement system and it is expressed as the coefficient of variation (CV). The CV is the ratio between the standard deviation (SD) of the values obtained from the assays and the mean concentration of the analyte (%). Different types of precisions can be determined: (*i*) the intra-assay precision (or repeatability) is the CV of multiple determinations of only one sample processed in a unique series of assays; (*ii*) the inter-assay precision (also known as intermediate precision) is expressed as the CV of multiple determinations of only one sample, controls and reagents processed in various series of assays performed in the same laboratory; and (*iii*) the reproducibility, that is the precision among different laboratories, is worth determining in collaborative studies, but it has no meaning in the validation of assays within the same laboratory. In the present study, precision was estimated by titrating three plasma samples of high, medium and low titers of IgG and IgM anti-annexin V Ab. Four replicates were carried out for intra- and inter-assay studies. CV was calculated and a value below 20% was considered acceptable.[24,25]

### Exactitude

Exactitude is the concordance rate between the real value and the measured value for the assayed parameter. It is expressed as the percentage deviation or the percentage error between the observed value and the real value (experimental value/real value) × 100%.[24,25]

In this study, a sample of known titer of anti-annexin V IgG or IgM was assayed sevenfold in the same plate and a percentage deviation of 100 ± 20% was considered acceptable.[30]

### Limit of detection

The limit of detection (LOD) is the lowest amount of the analyte that can be detected but not necessarily quantified

as an exact concentration or amount. For the evaluation of this parameter, four repeats of the reagents blank were performed in three different plates for each isotype of anti-annexin V Ab evaluated in this study. The LOD was calculated by adding three times the standard deviation to the mean value.[31]

### Robustness

Robustness is the ability of a test not to be affected by slight modifications deliberately introduced in different parameters and constitutes an indication of the reliability of the method in normal conditions. Such variations can be introduced either in the temperature or humidity of the incubation room or incubation chamber, in the incubation periods of time or in the pH value of a reagent (inside a narrow range), among others. The exactitude and precision or any other valuation parameter must be assessed for each condition in order to determine which modifications are well tolerated under assay conditions.[25] Slight variations were introduced in the currently developed technique with the aim of determining their influence on the final results. Variations in BSA concentration used in the blocking solution (1, 2 or 3%), blocking periods of time (60 or 90 min), temperature applied to the blocking process (room temperature or 37 ºC) and periods of time for the recording of final color optical density (5, 15, 30 or 45 min) were performed. Also, it was tested the reproducibility of the assay of different plate lots (1 and 2) and antigen lots (1 and 2) from the same commercial supplier. These variations were carried out for each Ab isotype. Besides, results obtained using a polyclonal peroxidase-conjugated rabbit Ab to either human IgG or IgM (DakoCytomation, Glostrup, Denmark) diluted in PBS-BSA (1:6000 and 1:1000, respectively) were also analyzed.

### Comparison between the "in house" method and the commercial kit

The "in house" ELISA was compared to one of the two kits available in our country, provided by Orgentec Diagnostika GmbH, Mainz, Germany. Sixty-eight plasma samples, 38 from non-pregnant, childbearing age healthy women and 30 from SLE and APLS women patients, were analyzed through both techniques, as well as low, medium and high titer positive controls. This comparison was carried out for each Ab isotype.

### Statistical analysis

Statistical analysis was performed by using one-way analysis of variance followed by the Bonferroni's multiple range test, student's *t* test and Friedman test. Values of $p < 0.05$ were considered to be statistically significant.

## Results and Discussion

In this study, a diagnostic Ab-detection ELISA for anti-annexin V Ab was developed and evaluated and the optimal conditions for the determination of this Ab were established.

### Validation of the ELISA

### Precision

The CV values obtained in the intra-assay precision study of three plasma samples were lower than 20% (Table 1) for each Ab isotype. These results are below the limit accepted by the World Health Organization (WHO) for such techniques.[24,25] The same happened to the CV values obtained in the inter-assay study (Table 2).

These results are considered satisfactory for immunoassays as reported by Laureen *et al*.[32] It has to be born in mind that precision is limited by both accidental and technical, manual errors that cannot always be avoided.[33] This lets us assume that the slight imprecision found here does not abolish the reproducibility of our results.

### Exactitude

A sample of known titer was assayed sevenfold in the same plate and exactitude results are shown in Table 3.

**Table 1.** Coefficient of variation (CV) in the repeatability assay

| Sample | Anti-annexin V antibody | | | |
|---|---|---|---|---|
| | IgG | | IgM | |
| | Mean concentration / (U mL$^{-1}$) | CV / % | Mean concentration / (U mL$^{-1}$) | CV / % |
| High titer | 44 | 15 | 38 | 16 |
| Medium titer | 16 | 8 | 18 | 11 |
| Low titer | 8 | 9 | 8 | 13 |

The intra-assay precision (or repeatability) was estimated by titrating three plasma samples of high, medium and low titers of IgG and IgM anti-annexin V antibody processed in a unique series of assays. Each value represents the mean of four experiments.

**Table 2.** Coefficient of variation (CV) in the intermediate precision assay

| Sample | Anti-annexin V antibody | | | |
|---|---|---|---|---|
| | IgG | | IgM | |
| | Mean concentration / (U mL$^{-1}$) | CV / % | Mean concentration / (U mL$^{-1}$) | CV / % |
| High titer | 42 | 14 | 40 | 15 |
| Medium titer | 15 | 13 | 16 | 13 |
| Low titer | 8 | 12 | 7 | 16 |

The inter-assay precision (or intermediate precision) was estimated by titrating three plasma samples of high, medium and low titers of IgG and IgM anti-annexin V antibody processed in various series of assays performed in the same laboratory. Each value represents the mean of four experiments.

**Table 3.** Percentage deviation in the exactitude assay

| Anti-annexin V antibody isotype | Real value / (U mL$^{-1}$) | Mean measured value / (U mL$^{-1}$) | SD | CV / % | Percentage deviation / % |
|---|---|---|---|---|---|
| IgG | 47.0 | 43.29 | 5.21 | 12 | 92 |
| IgM | 40.0 | 39.07 | 5.02 | 13 | 98 |

The exactitude of the assay was estimated by titrating a sample of known titer for IgG and IgM anti-annexin V antibody in the same plate. Each value represents the mean of seven experiments; SD: standard deviation; CV: coefficient of variation.

Percentage deviation was inside the established interval ($100 \pm 20\%$)[30] for both isotypes of Ab. Considering that the exactitude depends on the immunoenzymatic reaction, the sample studied and the specificity of the method, it can be assumed that the current strategy of development of our "in house" assay was adequate.

### Limit of detection

The LODs obtained for our assay were 0.6 and 0.8 U mL$^{-1}$ for IgG and IgM isotypes, respectively (Table 4). This indicates that our assay would be capable of detecting minimum quantities of anti-annexin V Ab. It is worth noting that the coating process of the solid phase is essential to obtain good results for this parameter.[34]

### Robustness

Different concentrations of BSA in the blocking solution (Figure 2a), different incubation temperatures (Figure 2b) and different incubation periods (Figure 2c) were tested. Neither variations in the concentrations of BSA ($p > 0.05$) nor variations in the duration and temperature of the blocking period ($p > 0.05$) produced any significant changes. Final color intensity was assessed by recording absorbance at 450 nm using a reference filter (630 nm), at different periods of time (Figure 2d). No significant difference was obtained among the periods of time evaluated ($p > 0.05$) either for IgG and IgM anti-annexin V Ab.

When analyzing the effect of different plate lots (Figure 3a) and antigen lots (Figure 3b), no statistically significant difference was found ($p > 0.05$). Using another conjugate (Figure 3c) did not produce any significant change in the results either ($p > 0.05$).

Taken together, these results suggest that slight variations in the ELISA technique developed do not produce any significant variations in the assessment of Ab titers.

### Comparison between the "in house" method and the commercial kit

Figures 4a and 4b shows the results obtained with individual plasma samples in both ELISA techniques in which an acceptable correlation can be seen (r = 0.94 and r = 0.93 for G and M isotypes, respectively; $p < 0.05$).

**Table 4.** Limit of detection for the immunoassay

| Anti-annexin V antibody isotype | Mean optical density for the reagents blank | SD | Limit of detection (OD measurement)) | Limit of detection / (U mL$^{-1}$) |
|---|---|---|---|---|
| IgG | 31 | 9 | 58 | 0.6 |
| IgM | 26 | 9 | 53 | 0.8 |

The limit of detection was determined by four repeats of the reagents blank in three different plates for each isotype of the anti-annexin V antibody. Each value represents the mean of three series of experiments. OD: optical density.

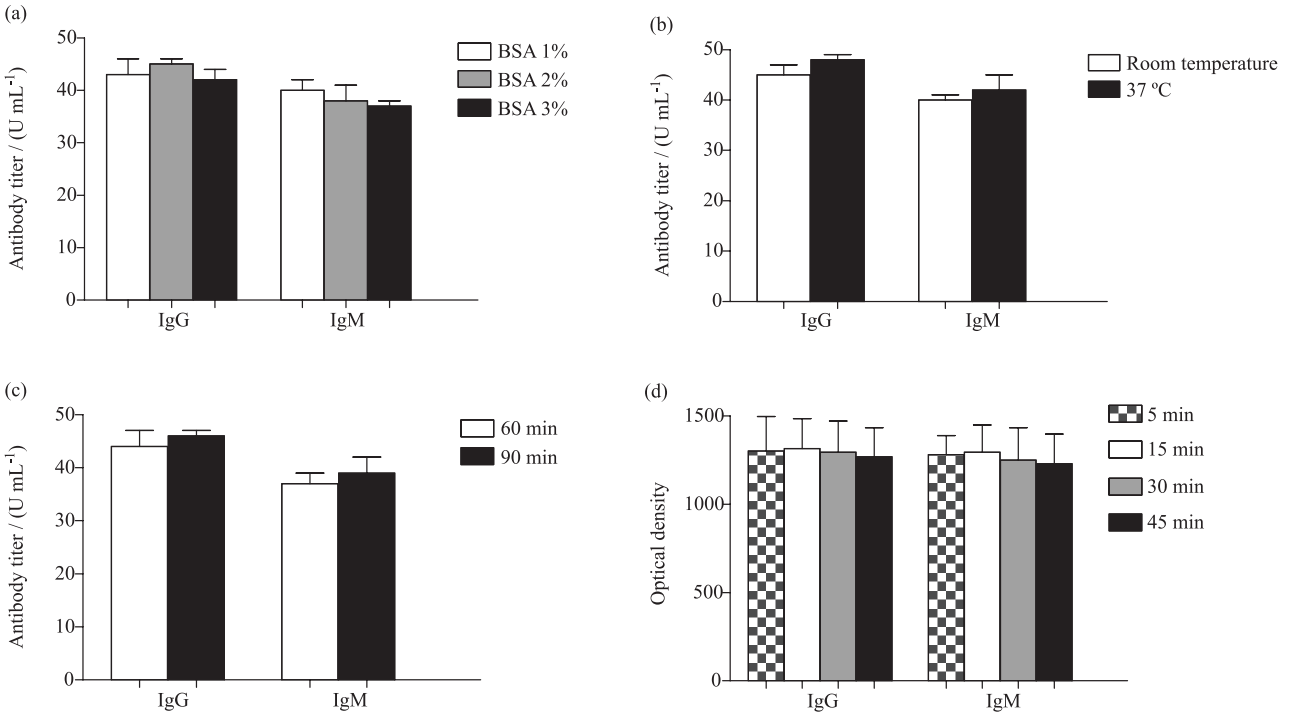


**Figure 2.** (a) Variations in the concentration of BSA in the blocking solution. IgG and IgM anti-annexin V Ab titers were determined using 1, 2 or 3% BSA in the blocking solution. No significant difference was found among the different concentrations either for IgG and IgM ($p > 0.05$). Each value represents the mean ± SD (n = 3). (b) Variations in the temperature of the blocking period. IgG and IgM anti-annexin V Ab titers were determined after blocking either at room temperature or 37 ºC. No significant difference was observed between the temperatures tested for both isotypes ($p > 0.05$). Each value represents the mean ± SD (n = 3). (c) Variations in the duration of the blocking period. IgG and IgM anti-annexin V Ab titers were determined after blocking for 60 and 90 min. No significant difference was found between the periods of time assayed for either isotype ($p > 0.05$). Each value represents the mean ± SD (n = 3). (d) Variations in the final color recording time. Final color intensity was assessed by recording absorbance at 450 nm using a reference filter (630 nm) at 5, 15, 30 and 45 min. No significant difference was obtained among the periods of time evaluated either for IgG and IgM ($p > 0.05$). Each value represents the mean ± SD (n = 3).

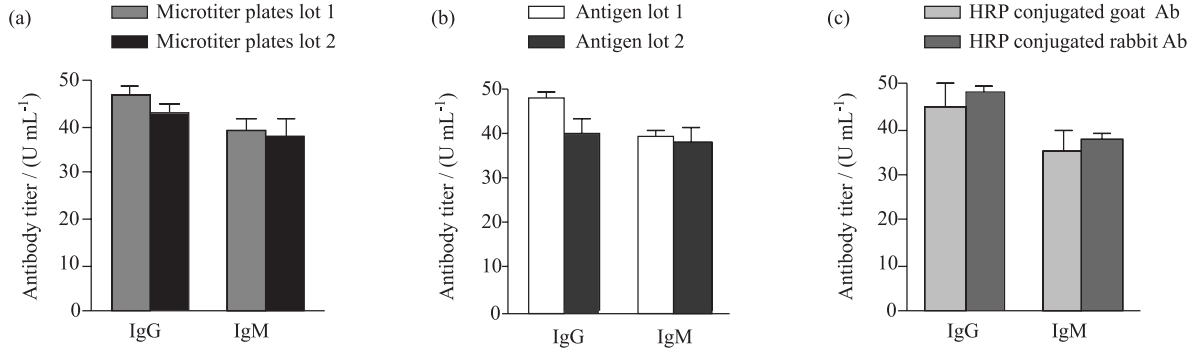


**Figure 3.** (a) Variation in plate lots. Both anti annexin V antibody isotypes were determined using different plate lots. No statistically significant difference was found between lots for both immunoglobulin isotypes ($p > 0.05$). (b) Variation in antigen lot. Both anti annexin V antibody isotypes were determined using different antigen lots. No statistically significant difference was found between lots for both immunoglobulin isotypes ($p > 0.05$). (c) Variation in the source and commercial supplier of the conjugate. Both anti annexin V antibody isotypes were determined using different conjugates, one developed in goat and the other in rabbit. No statistically significant difference was found between conjugates tested for both immunoglobulin isotypes ($p > 0.05$).

CV and LOD values obtained for both techniques are shown in Table 5. While no differences were observed in LOD values, intra- and inter-assay CV were higher for the "in house" technique than for the commercial method, nevertheless, values were within the acceptable range for validation.[25] Performance of the "in house" ELISA was comparable to the commercial kit.

## Conclusions

The laboratory methodology used for the detection of anti-annexin V Ab is crucial. Enzyme-linked immunoassays are currently the most commonly used methods because they are easily performed, potentially may be automated, and allow the screening of large numbers of samples.

**Table 5.** Comparison between the "in house" method and the commercial kit

| Anti-annexin V antibody isotype | Intra-assay CV / % | | Inter-assay CV / % | | Limit of detection / (U mL$^{-1}$) | |
|---|---|---|---|---|---|---|
| | "In house" EIA | Commercial EIA | "In house" EIA | Commercial EIA | "In house" EIA | Commercial EIA |
| IgG | 11 | 3.8-6.7 | 13 | 4.1-5.7 | 1.0 | 0.6 |
| IgM | 10 | 3.8-6.7 | 15 | 4.1-5.7 | 1.0 | 0.8 |

Validation parameters for the "in house" method were compared to those of the commercial kit provided by the supplier. No difference was observed for LOD values and, although intra- and inter-assay CV were higher for the "in house" technique, they were within the aceptable range for validation.

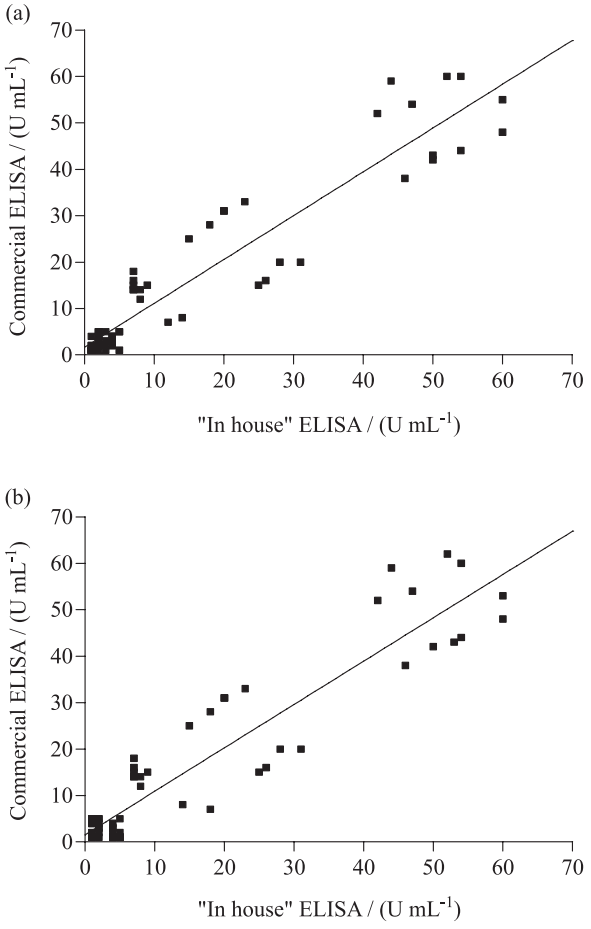


**Figure 4.** Correlation between "in house" ELISA and commercial ELISA in the determination of antibodies anti annexin V. Sixty-eight plasma samples were assayed by both techniques obtaining a correlation of 0.94 for IgG isotype [$p < 0.05$; panel (a)] and of 0.93 for IgM isotype [$p < 0.05$; panel (b)].

The method currently developed satisfactorily accomplishes the parameters of standardization and validation for an immunoassay.[26-28] Therefore, it is possible to rely on an "in house" ELISA technique for the evaluation of the content of anti-annexin V Ab in plasma with high reproducibility and reliability. Furthermore, because of its feasible execution, it can be recommended as a quantification assay for plasma levels of anti-annexin V Ab in patients with SLE and APLS. On the other hand, due to controversy towards the clinical implications of these Ab in the pregnancy losses, it is believed that a useful tool for the investigation of populations suffering from recurrent abortion is being provided.

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## References

1. Benz, J.; Hofmann, A.; *Biol. Chem.* **1997**, *378*, 177.
2. Gerke, V.; Moss, S.; *Physiol. Rev.* **2002**, *82*, 331.
3. Grundmann, U.; Abel, K. J.; Bohn, H.; Löbermann, H.; Lottspeich, F.; Küpper, H.; *Proc. Natl. Acad. Sci. U. S. A.* **1988**, *85*, 3708.
4. Huber, R.; Berendes, R.; Burger, A.; Luecke, H.; Karshikov, A.; *Behring Inst. Mitt.* **1992**, *91*, 107.
5. Vogt, E.; Ng, A. K.; Rote, N. S.; *Am. J. Obstet. Gynecol.* **1997**, *177*, 964.
6. Wang, X.; Campos, B.; Kaetzel, M. A.; Dedman, J. R.; *Am. J. Obstet. Gynecol.* **1999**, *180*, 1008.
7. de Laat, B.; Derksen, R. H.; Mackie, I. J.; Roest, M.; Schoormans, S.; Woodhams, B. J.; de Groot, P. G.; van Heerde, W. L.; *Ann. Rheum. Dis.* **2006**, *65*, 1468.
8. Rand, J. H.; Wu, X. X.; Guller, S.; Gil, J.; Guha, A.; Scher, J.; Lockwood, C. J.; *Am. J. Obstet. Gynecol.* **1994**, *171*, 1566.
9. Shu, F.; Sugimura, M.; Kanayama, N.; Kobayashi, H.; Kobayashi, T.; Terao, T.; *Gynecol. Obstet. Invest.* **2000**, *49*, 17.
10. Bogdanova, N.; Horst, J.; Chlystun, M.; Croucher, P. J. P.; Nebel, A.; Bohring, A.; Todorova, A.; Schreiber, S.; Gerke, V.; Krawczak, M.; Markoff, A.; *Hum. Mol. Genet.* **2007**, *16*, 573.
11. Gajtan, E.; Tambya, M. C.; Chanseauda, Y.; Servettaza, A.; Guillevinb, L.; Mouthona, L.; *Autoimmun. Rev.* **2005**, *4*, 55.
12. Rand, J. H.; *Curr. Rheumatol. Rep.* **2000**, *2*, 246.
13. Nakamura, N.; Shidara, Y.; Kawaguchi, N.; Azuma, C.; Mitsuda, N.; Onishi, S.; Yamaji, K.; Wada, Y.; *Biochem. Biophys. Res. Commun.* **1994**, *205*, 1488.

14. Kaburaki, J.; Kuwana, M.; Yamamoto, M.; Kawai, S.; Ikeda, Y.; *Am. J. Hematol.* **1997**, *54*, 209.
15. Satoh, A.; Suzuki, K.; Takayama, E.; Kojima, K.; Hidaka, T.; Kawakami, M.; Matsumoto, I.; Ohsuzu, F.; *J. Rheumatol.* **1999**, *26*, 1715.
16. Sugiura, K.; Muro, Y.; *J. Rheumatol.* **1999**, *26*, 2168.
17. Zammiti, W.; Mtiraoui, N.; Hidar, S.; Fekih, M.; Almawi, W. Y.; Mahjoub, T.; *Arch. Gynecol. Obstet.* **2006**, *274*, 261.
18. Zammiti, W.; Mtiraoui, N.; Kallel, C.; Mercier, E.; Almawi, W. Y.; Mahjoub, T.; *Reproduction* **2006**, *131*, 817.
19. Rand, J. H.; Arslan, A. A.; Wu, X. X.; Wein, R.; Mulholland, J.; Shah, M.; van Heerde, W. L.; Reutelingsperger, C. P.; Lockwood, C. J.; Kuczynski, E.; *Am. J. Obstet. Gynecol.* **2006**, *194*, 182.
20. Siaka, C.; Lambert, M.; Caron, C.; Amiral, J.; Hachulla, E.; Hatron, P. Y.; Goudemand, J.; *Rev. Med. Interne* **1999**, *20*, 762.
21. Ogawa, H.; Zhao, D.; Dlott, J. S.; Cameron, G. S.; Yamazaki, M.; Hata, T.; Triplett, D. A.; *Am. J. Clin. Pathol.* **2000**, *114*, 619.
22. Arai, T.; Matsubayashi, H.; Sugi, T.; Kondo, A.; Shida, M.; Suzuki, T.; Izumi, S. I.; McIntyre, J. A.; Makino, T.; *Am. J. Reprod. Immunol.* **2003**, *50*, 202.
23. Kuragaki, C.; Kidoguchi, K.; Nakamura, N.; Wada, Y.; *Am. J. Hematol.* **1995**, *50*, 68.
24. Aboul-Enein, H. Y.; Emafo, P. O.; Gupta, P. K.; Layloff, T.; Lik, N.; Paal, T.; Rabouhans, M. L.; Zhong-Yuan, Y.; *Comité de Expertos de la OMS en Especificaciones para las Preparaciones Farmacéuticas, Prácticas Adecuadas para la Fabricación de Productos Farmacéuticos*, Ginebra, 1992, Serie de Informes Técnicos No. 823, Anexo 1. http://whqlibdoc.who.int/trs/WHO_TRS_823_spa.pdf
25. Chaloner-Larsson, G.; Anderson, R.; Egan, A.; *Guía de la OMS sobre los Requisitos de las Prácticas Adecuadas de Fabricación (PAF), Segunda parte: Validación* (WHO/VSQ/97.02), Ginebra, 1998, 70. http://whqlibdoc.who.int/hq/1997/WHO_VSQ_97.02_spa.pdf
26. Betts Carpenter, A. In *Manual of Clinical Laboratory Immunology*, 6th ed.; Rose, N.; de Macario, E.; Fahey, J.; Friedman, H.; Penn, G., eds.; America Society for Microbiology: Washington D. C., 1992, ch. 2.
27. Stenman, U. H. In *Principles and Practices of Immunoassay*, 2nd ed.; Price, C. P.; Newman, D. J., eds.; Macmillan: London, 1997, ch. 11.
28. Tijsen, P. In *Practice and Theory of Enzyme Immunoassay*; Burdon, R. H.; Van Knippenberg, P. H., eds.; Processing Elsevier Sci. Pub. B. V.: Amsterdam, Netherlands, 1985, ch. 15.
29. Basiglio, C.; Arriaga, S.; Pelusa, H.; Almará, A.; Roma, M.; Mottino, A.; *Biochem. Biophys. Acta* **2007**, *1770*, 1003.
30. Fajardo, E.; Delgado, I.; Riverón, L.; Izquierdo, L.; Iglesias, N.; Álvarez, E.; Perojo, A.; Costa, N.; Tamayo, Y.; Jorge, E.; Hernández, B.; Díaz, Y.; Cruces, A.; Gutiérrez, N.; Puig, A.; Mandiarote, A.; Martínez, R.; Cardoso, D.; *VacciMonitor.* **2006**, *15*, 5.
31. Broughton, P. M. G.; Bergonzi, C.; Lindstedt, G.; Loeber, I. G.; Malan, P. G.; Mathieu, M.; Pozet, S.; *Guidelines for a User Laboratory to Evaluate and Select a Kit for its Own Use, in Part 1: Quantitative Tests*; European Committee for Clinical Laboratory Standards: London 1986, 3.
32. Laureen, E. L.; *BioPharmaceutical* **1995**, 36.
33. Sokoll, L. J.; Chan, D. W.; *Anal. Chem.* **1999**, 71, 356.
34. Ochoa, R.; Martínez, J. C.; Estrada, E.; García, A. M.; Ferriol, X.; Blanco, R.; *VacciMonitor.* **2000**, *9*, 17.

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