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# Standardization of an Immunoassay for the Detection of Antibodies to B2 Glycoprotein-I

Eric N. Erickson Jr.  
*Seton Hall University*

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STANDARDIZATION OF AN IMMUNOASSAY FOR THE  
DETECTION OF ANTIBODIES TO  $\beta_2$  GLYCOPROTEIN - I

BY

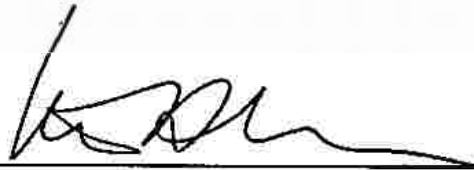
ERIC N. ERICKSON JR  
July, 1999

Submitted in partial fulfillment of the requirements for the degree of  
Master of Science from the Department of Biology of Seton Hall  
University

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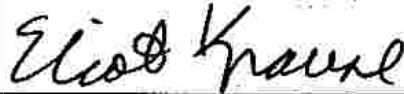
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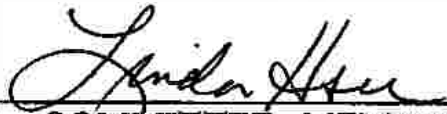
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## ACKNOWLEDGMENT

No acknowledgment could begin, nor come to fruition without the steadfast foundation and support provided by both a patriarch and matriarch. I am therefore always indebted to both my father and mother.

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## Abstract

Antiphospholipid Syndrome (APS) is an evolving autoimmune disease with numerous clinical manifestations. APS occurs in two forms: Primary Antiphospholipid Syndrome (PAPS) and secondarily in association with other autoimmune disorders such as Systemic Lupus Erythematosus (SLE). In the brief period since its discovery as a cofactor for anticardiolipin antibodies,  $\beta_2$ -glycoprotein I ( $\beta_2$ GPI; apolipoprotein H) has been recognized as the autoantigen in the absence of anionic phospholipid when appropriately presented to human autoantibodies. Recent studies suggest that this presentation requires the surface-dependent unmasking of a cryptic epitope. Immunoassays of autoantibodies directed against  $\beta_2$ GPI, developed through the application of suitable polymeric matrices, may be clinically relevant in that they may be more specific for (APS). Assays of Anti- $\beta_2$  Glycoprotein I ( $\alpha\beta_2$ GPI) have used a variety of materials in the antigen-specific presentation system. This situation renders this assay particularly susceptible to interlaboratory variation when raw absorbance data are reported. Presently, many assay systems for the detection of APS lack standardization and are plagued with technical problems. Reference materials for a uniform enzyme immunoassay of IgG  $\alpha\beta_2$ GPI have not, as yet, been available.

The following thesis focuses on the development and standardization of a clinically significant assay for the detection of APS. The assay centers on the detection of  $\alpha\beta_2$ GPI antibodies. Described herein, is a method utilizing commercially available, chemically activated polystyrene microtiter plates with a surface which binds proteins covalently. Additionally, a commercial source of human  $\beta_2$ GPI has been obtained. Reference calibrators have been developed consisting of a control group of 111 men and 93 women, yielding a range of 0-19 standard IgG anti- $\beta_2$ GPI units (SGU) for the ninety-fifth percentile of values. These are intended as interim calibrators that can be used to develop primary international standards for an immunoassay of IgG  $\alpha\beta_2$ GPI.

## Introduction

The study of anti-phospholipid antibodies (aPL) can be traced to the first clinical assay for syphilis, developed by Wasserman (1907). Thirty-four years later Pangborn (1941), demonstrated that the active antigenic component of the assay was a phospholipid, which was named cardiolipin (CL). All subsequent tests were based on the detection of antibodies to extracted CL. Thereafter, the venereal disease research laboratories (VDRL) antigen assay was created, consisting of a combination of cholesterol, phosphatidylcholine (PC) and CL. During the wide use of the assay it became apparent that there was a large percentage of the population with positive tests, who showed no clinical or epidemiological manifestations of infection.

This phenomenon was termed biological false positive serological test for syphilis (BFP-STs), and occurred under two distinct circumstances. The first BFP-STs were detected in a number of patients with infections other than syphilis. The BFP-STs disappeared with convalescence. Chronic BFP-STs, had an apparently idiopathic origin, persisting over many years (Moore and Mohr, 1952). Further studies showed an association of chronic BFP-STs and autoimmune diseases. Systemic Lupus Erythematosus (SLE) was the major autoimmune disorder associated with chronic BFP-STs (Hasserick and Long, 1951).

Conly and Hartman (1952), described two patients with SLE who had both BFP-STs and an unusual plasma constituent that inhibited *in vitro* coagulation. This factor was termed the lupus anticoagulant (LA) by Feinstein and Rapaport (1972). Detection of

LA activity was determined by measuring prolonged clotting times in phospholipid-associated coagulation tests; prolonged clotting was indicative of LA.

The LA is somewhat paradoxical. Lupus anticoagulant was not found in patients with syphilis and its association with BFP-STS became widely recognized (Johansson and Lassus, 1974). Contrary to the anticoagulant effect observed *in vitro* these patients did not exhibit prolonged bleeding. Bowie and coworkers (1963) reported that patients with LA were more prone to thrombosis than to hemorrhage.

LA consists of a heterogeneous group of antibodies which may require more than one clotting test to demonstrate anticoagulant activity (Triplett and Brandt, 1989). The best method for detecting LA is the subject of some controversy. Tests which are used include the following: partial thromboplastin time (PTT), prothrombin time (PT), activated partial thromboplastin time (aPTT), koalin clotting time (KCT), dilute Russel viper venom time (dRVVT) and tissue thromboplastin inhibition assay (TTI) (Schleider et al., 1976, Rauch et al., 1989, Triplett and Brandt, 1989, McNeil et al., 1991). Furthermore, the LA tests do not distinguish from the antibodies occurring in syphilis and those occurring in Primary Antiphospholipid Syndrome (PAPS), SLE and other autoimmune disorders.

This led Thiagarajan and coworkers (1980), to use monoclonal antibodies with lupus anticoagulant activity to test the possibility that LA was an antiphospholipid antibody. The monoclonal antibodies reacted with several negatively charged phospholipids, thus suggesting that the antibodies may be directed to phospholipid (Thiagarajan et al., 1980). This prompted Harris and colleagues (1983) to develop a solid phase immunoassay to directly detect circulating antibodies that bind CL, a negatively charged phospholipid.



This enzyme linked immunosorbent assay (ELISA) was developed to improve the sensitivity of detecting anti-cardiolipin (aCL) antibodies in patients with SLE.

The ELISA is performed by immobilization of a purified antigen. In ELISA the antibody reacts with the immobilized antigen to form an immune complex immobilized on the surface. Detection is achieved by reacting the immobilized complex with a second antibody, which has previously been tagged with a detectable molecule (Ekins, 1971). The small serum sample required for the ELISA system provides an advantage over the coagulation assay. Using ELISA for aCL Harris and coworkers (1983) demonstrated a majority of LA samples were aCL positive, however, the converse was not true.

The sensitive immunoassays based on purified cardiolipin stimulated a new era of investigation into Antiphospholipid Syndrome (APS), a syndrome characterized by arterial and venous thrombosis, strokes, migraines, livedo reticularis, recurrent fetal loss, abortion and thrombocytopenia (Harris et al., 1983). Initially, aCL antibodies and the LA assays were believed to detect antibodies specific for phospholipids or phospholipid-protein complexes (Harris et al., 1988), leading to the current nomenclature of the syndrome, APS. Antibodies detected in the aCL assay, however, appear to represent an immunologically distinct population. Those present in patients with infections such as syphilis, leprosy, tuberculosis, and endocarditis the antiphospholipid antibodies appear not to be associated with vascular spasm (Costello and Green, 1986, Santiago et al., 1989, Wang et al., 1995). Additionally aCL antibodies associated with autoimmune diseases were found to react with other anionic phospholipids (McNeil et al., 1991).

In 1990, it was reported that aCL reactivity by autoantibodies from patients with APS was dependent on the presence of a serum protein, apolipoprotein H or

$\beta_2$ glycoprotein I ( $\beta_2$  GPI) (Galli et al., 1990, McNeil et al., 1990). After years of controversy over the precise nature of the epitope(s) presented by the cardiolipin- $\beta_2$ GPI complex, it is apparent that the epitope for the relevant autoantibodies is encrypted in circulating  $\beta_2$ GPI. Several groups have determined that when  $\beta_2$ GPI is bound to an appropriately polar surface, IgG class autoantibodies from subjects with APS recognize and bind to the protein. (Matsuura et al., 1994, Keil et al., 1995, Roubey et al., 1995). Recently it has been established that IgG a $\beta_2$ GPI may be more specific than aCL for APS (Balestrieri et al., 1995, Cabiedes et al., 1995, Cabral et al., 1995, El-Kadi et al., 1995, Matsuura et al., 1994).

$\beta_2$ GPI is a 48kd phospholipid binding plasma protein with anticoagulant properties (Schousboe, 1985, Harris et al., 1994, Hunt and Krilis, 1994). The properties of  $\beta_2$ GPI that have been established are: binding to DNA and phosphocellulose, lipoproteins, heparin, negatively charged phospholipid vesicles, intralipid, platelets, mitochondria, and deoxycholate (Schousboe, 1985). Additionally,  $\beta_2$ GPI has been shown to inhibit contact activation of the intrinsic blood coagulation pathway, ADP-mediated platelet aggregation, and prothrombinase activated platelets (Schousboe, 1985). The inhibiting effect of  $\beta_2$ GPI on prothrombinase activity of resting platelets was not found to be inhibited by increasing the concentrations of Stuart-Prower Factor (Xa) and Platelet Factor V (Nimpf et al., 1986).  $\beta_2$ GPI is distributed among several plasma protein fractions. Approximately 17% is bound to high-density lipoprotein (HDL), 16% associated with chylomicrons and very-low density lipoproteins (VLDL), 2% with low density lipoprotein (LDL), and the remaining 65% is not associated with lipoproteins (Nimpf et al., 1986). The

concentration of  $\beta_2$ GPI in the blood (0-300 $\mu$ g/ml) is the function of two codominant autosomal alleles, BgN and BgD with a BgN frequency of 0.94 in Caucasians (Cleve and Ritter, 1969, Koppe et al., 1970, and Schousboe, 1985). The fifth domain of  $\beta_2$ GPI has been found to contain a phospholipid binding site and a region that is recognized by anticardiolipin antibodies (Hunt and Krilis, 1994).

Additional studies have shown that the clinical manifestations of APS in patients with SLE associate more strongly with a $\beta_2$ GPI than with antiphospholipid antibodies. Cabiedes and colleagues (1995), demonstrated a strong association between a $\beta_2$ GPI and clinical APS even in the absence of antiphospholipid antibodies (aPL). Furthermore, Blank and coworkers (1994) have shown that immunization of naive mice with  $\beta_2$ GPI induced experimental APS. High titers of a $\beta_2$ GPI antibodies were recorded in the sera of mice immunized with  $\beta_2$ GPI. The immunized mice expressed clinical manifestations of APS consisting of prolonged aPPT's, significant thrombocytopenia, reduced fecundity, increased fetal loss as well as a decrease in placental and embryo mean weights (Blank et al., 1994). These findings suggest a model in which antibodies from patients with APS bind to a cryptic epitope on  $\beta_2$ GPI that is exposed after binding to a phospholipid (Hunt and Krilis, 1994, Wang et al., 1995)

#### Current Evidence

Mounting evidence pinpointed a $\beta_2$ GPI antibodies as the primary link to APS. Conflicting hypotheses emerged suggesting that  $\beta_2$ GPI may be the antigen,  $\beta_2$ GPI might modify the PL to form an epitope, that PL may modify  $\beta_2$ GPI to form an epitope or that the epitope contains both moieties (Hunt and Krilis, 1994, Keil et al., 1995) In direct

conflict with the former reports, Gharavi and coworkers (1993) studied sera from 146 patients with rheumatic diseases, including 75 that were aCL positive using ELISA, and determined that there was no increase in the binding to  $\beta_2$ GPI coated wells compared to 40 normal human sera.

Variations in the assay systems used may have been, at least partially, responsible for the conflicting data reported. Additionally, there was a wide range in the percentage of positive patients reported by investigators using  $\beta_2$ GPI in their ELISA systems for the detection of APS. Conflicting conclusions may have arisen from the coexistence of a number of auto antibodies in the sera to be assayed, particularly with SLE and related autoimmune disorders. Significant proportions of these patients have ACA, LA activity, antiendothelial cell antibodies, antiplatelet antibodies, anti-DNA antibodies and immune complexes (Shi et al., 1993). As mentioned previously, further confusion stemmed from early assumptions that LA and aCL were identical. Moreover, the role of  $\beta_2$ GPI in the antigen-antibody system was unclear.

Monoclonal antibodies were used to confirm previous data regarding the functions of  $\beta_2$ GPI in APS. Three families of monoclonal antibodies to purified  $\beta_2$ GPI have been produced. All were found to react with  $\beta_2$ GPI in ELISA and none reacted with negatively charged phospholipids. It should be noted, that one monoclonal antibody failed to recognize  $\beta_2$ GPI bound to PL while the other two demonstrated enhanced binding when  $\beta_2$ GPI was added to PL (Wagenknecht and McIntyre, 1993). Monoclonal aCL antibodies have also been developed. These required  $\beta_2$ GPI for anionic PL binding (Ichikawa et al., 1994). Further, monoclonal aCL bound to solid phase  $\beta_2$ GPI on polystyrene plates that

had carboxyl groups on their surface, but did not react with solid phase  $\beta_2$ GPI on ordinary polystyrene plates. Ichikawa and colleagues (1994) showed that the degree of binding was dose dependent and reached a plateau at  $\beta_2$ GPI concentrations of 4-20  $\mu$ g/ml .

APS results in a serious derangement of hemostasis. Injury to the vessel wall causes platelets to adhere to the subendothelial structures, change their morphological structure and release the content of their granula and aggregate (Nimpf et al., 1986). The concurrent platelet aggregation and clot formation are the focal point of  $\alpha\beta_2$ GPI antibodies in APS. The antibodies are thought to predispose individuals to thrombosis through various mechanisms. Anticardiolipin antibodies (aCL) and LA have been shown to be a discreet group of antiphospholipid antibodies (Pangborn, 1941). These antibodies often coexist although they are separable from the plasma. At physiological levels  $\beta_2$ GPI naturally inhibits the generation of factor  $X_a$ , although aCL arrests this action leading to unopposed factor  $X_a$  generation and a prothrombotic state (Nimpf et al., 1986, Kandiah et al., 1996). LA immunoglobulins behave differently they have also been found to inhibit factor  $X_a$  generation in a similar manner (Nimpf et al., 1986, Kandiah et al., 1996). The elevated numbers of individuals with genetically-predetermined protein C and S deficiency has led some investigators to speculate on role of antibodies to these proteins in hemostasis. Malia and coworkers (1990), theorized that lupus like anticoagulants precipitate thrombosis through an effect on protein C. With activated protein C and S playing pivotal roles inhibiting blood coagulation through degradation of factors  $V_a$  and  $VIII_a$ .

Presently, there is a growing awareness that surfaces used to bind  $\beta_2$ GPI are critical in presenting the epitope in such a manner that is recognized by  $\alpha\beta_2$ GPI antibodies. Matsuura and colleagues (1994) as well as Cabiedes and coworkers (1995), found that human SLE sera and sera from mice with APS recognize  $\beta_2$ GPI only if polystyrene plates are previously oxygenated by irradiation. These studies were confirmed by Koike (1994). Experimental data showed that specificity for an epitope on  $\beta_2$ GPI is expressed by a conformational change occurring when  $\beta_2$ GPI interacts with an oxygen-substituted solid phase surface (Koike 1994). Galli and coworkers (1990) have demonstrated that the aPL cofactor was the target antigen for the aPL and that the antibody binding was proportional to the amount of cofactor coated on the microtiter wells in the absence of phospholipid. Collateral studies utilizing chemically activated polystyrene have been reported to bind proteins covalently, supporting the above findings (Keil et al., 1995). However, it should be noted that the latter did not observe a significant correlation between cofactor dependent aCL and  $\alpha\beta_2$ GPI antibodies, as was observed by Koike (1994).

The use of polystyrene in anticardiolipin and  $\beta_2$ GPI ELISA's has assumed a pivotal role in identifying patients prone to APS manifestations, albeit with varying degrees of success. Presently, few methodologies have been proposed for covalent binding of  $\beta_2$ GPI in the ELISA system. Although, irradiation has been the method of choice for activating the surface of polystyrene, results have been inconsistent. Enhancing the previous ELISA systems through covalent binding (through the utilization of Xenobind™), application of the proper analyte ( $\beta_2$ GPI), and performing consistent uniform technique have been critical to the ELISA system developed herein. Xenobind™ covalent microwell plates

provide a uniform surface of high binding capacity and low variability (Douglas and Monteith, 1994). Variation among previous assay systems employed may be due to several factors such as, effective blocking of the plate to prevent binding of the antigen or detector antibody to the plate surface, sample dilutions, consistent, thorough washing between steps to remove non-specifically bound material, and consistent, uniform immobilization of the capture antibody. Although the most notable and consequential variation presumably stems from a non-uniform binding surface for the analyte.

As above, various authors have observed a correlation between  $\beta_2$ GPI and APS. In the assay system herein,  $\beta_2$ GPI serves as the antigenic determinant whose cryptic epitope is revealed by its covalent binding to the solid surface of Xenobind™.

### Methods and Materials - Pilot Study

Prior to the characterization of the novel assay described herein, the analytical and clinical relationship between human IgG autoantibodies to  $\beta_2$ GPI and aCL antibodies was investigated. Sera were obtained from a bank of specimens submitted to the laboratory at St. Joseph's Hospital and Medical Center for routine aCL assay, and were maintained at  $-70^\circ\text{C}$  (range  $-68$  to  $-72^\circ\text{C}$ ). In addition to the patients on the Rheumatology Service the laboratory accepts diagnostic test referrals from all areas of the university-affiliated 780-bed urban medical center. This includes acute and long-term care in-patients and a number of out-patient satellite health centers, as well as from private physicians' offices in the region.

Routine clinical measurement of aCL was made with kits from Reaads Medical Products (Westminster, CO, U.S.A.). The HRP secondary (anti-human IgG) antibody was also obtained from Reaads as part of the kit and used without modification, as were the substrate (3,3',5,5'-tetramethylbenzidine and  $\text{H}_2\text{O}$ , solution) and a stopping solution (1.25 M sulfuric acid). Rabbit anti- $\beta_2$ GPI was obtained from the Biotechnology Research Institute (Organon Teknika Corp., Rockville, MD, U.S.A.), HRP-conjugated goat anti-rabbit IgG from Cappel Research Products (Organon Teknika Corp., Durham, NC, U.S.A.), and purified  $\beta_2$ GPI was from Crystal Chem. Inc. (Chicago, IL, U.S.A.). Chemically activated (Xenobind<sup>TM</sup>) and untreated polystyrene microtiter plates were purchased from Xenopore Corp. (Saddle Brook, NJ, U.S.A.) and were used in strip format containing 8 wells per strip. Gammabind G-Sephararose was obtained from



Pharmacia Corp. (Piscataway, NJ, U.S.A.). BSA (Fraction V) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Biotin-LC-hydrazide (Immunopore) and HRP-conjugated streptavidin were obtained from Pierce Chemical Co. (Rockford, IL, U.S.A.).

ELISA was used in several formats. The clinical aCL assay yielding values in GPL (IgG anti-phospholipid) units standardized to the Louisville values (Cassilius et al., 1991), was the commercial kit (described above) method. This was used without modification, and data obtained in this assay format are presented in GPL units. Wells for assay of  $\beta_2$ GPI binding were prepared by incubating (for 2 h at 37°C) 100 $\mu$ l volumes of 10 $\mu$ g/ml  $\beta_2$ GPI, or biotinylated  $\beta_2$ GPI dissolved in PBS to yield the final mass per well values indicated in the results. These plates were blocked with 3% BSA in PBS, which was also used as diluent and wash solution in the anti- $\beta_2$ GPI assay. The Reaads HRP-anti-human IgG and substrate were used as recommended by the manufacturer to detect bound human IgG. The rabbit IgG was detected using HRP-goat anti-rabbit IgG at a titer of 1:75000 followed by substrate reaction using the Reaads protocol. Biotinylated IgG was detected by HRP-streptavidin (30ng/ml) binding. For all binding studies, Reaads' substrate and stop solution were used, following the manufacturer's method. Absorbance was read at 450 nm in a BioTek Instruments, Inc. (Winooski, VT, U.S.A.) model EL307C microplate reader.

Precipitins between the rabbit antibody and  $\beta_2$ GPI were detected by double diffusion in agarose. Equivalence was optimally detected at an antigen range of 0.03-0.06mg/ml when antibody concentration was 1 mg/ml. A whole IgG fraction was purified from the

serum subject DB (see Table 1) by ligand affinity chromatography using recombinant protein G (GammaBind G-Sepharose). The eluted IgG was buffer-exchanged with PBS and concentrated to approximately the starting volume of serum as described previously (Cassulis et al., 1991). This IgG fraction was used to study precipitin formation by double diffusion against  $\beta_2$ GPI and in the elution experiments with high ionic strength, low pH citrate (HL citrate solution: sodium citrate/citric acid, pH 2.5, 1.4 M citrate).

#### Methods and Materials - Establishment of de novo method.

Commercially available products based on the Reaads kit were utilized to reveal a cryptic epitope for the binding of  $\beta_2$ GPI. The appropriate titer of horseradish peroxidase-conjugated goat anti-human IgG (affinity-purified antibody containing ~1g/L total protein, obtained from Cappel Research Products, Div. of Organon Teknika, Durham, NC; prod. no. 55252) was established. The absorbance of titers from 1:250 to 1:1250 of horseradish peroxidase-conjugated goat anti-human IgG to human IgG, were read at 450nm in a BioTek Instruments, Inc. (Winooski, VT, U.S.A.) model EL307C microplate reader. Xenobind™ microtiter wells (Xenopore, Hawthorne, NJ) were used to provide the anionic surface for the binding of  $\beta_2$ GPI. Utilizing two subjects, EE (low control) and DB (high control), volume fractions were plotted against the  $A_{450}$ . Succeeding the volume fraction analysis, the samples and volume fractions were assayed for viability in subsequent days after thawing and days after harvest.

#### Methods and Materials - Development of Standards

Phlebotomy was performed on two subjects. The first subject (HRM-01), was a 35-year-old man with APS and positive laboratory test results for lupus anticoagulant (by

dilute Russell's viper venom method) and IgG anticardiolipin antibodies. The second subject (LRM-01), was a 24-year-old male graduate student in excellent health with no clinical or laboratory evidence of immunological or other disease. The protocol for harvesting one unit (500mL) of blood from these subjects was approved by the Institutional Review Board (responsible for the ethical conduct of experiments involving human subjects) at St. Joseph's Hospital and Medical Center.

Blood was collected into standard citrate phosphate-dextrose-adenine (CPDA) donor collection bags (Miles, Elkhart, IN) containing 0.19g of anhydrous citric acid, 1.66g of sodium citrate (dihydrate), 0.14g of sodium phosphate, monobasic (monohydrate), 2.01g of dextrose (monohydrate), and 0.017g of adenine. The nominal capacity of these bags is 450mL. Plasma was aseptically transferred to the satellite bag attached to the main collection bag. This resulted in the collection of 286mL of plasma from HRM-01 and 249mL of plasma from LRM-01. To achieve a target absorbance signal of ~1.5 A at 450nm at a 1:100 dilution in our assay system, we further diluted the plasma preparation from HRM-01 with 52mL of outdated plasma from an anonymous blood donor and 10mL of CPDA prior to recalcification with 12mL of 0.9mol/L CaCl<sub>2</sub>. This plasma did not clot and was maintained as a plasma preparation. Plasma LRM-01 was recalcified with 7.5mL of CaCl<sub>2</sub> solution, allowed to clot, and maintained as a serum preparation. Each preparation was aseptically divided into 50-uL aliquots that have been stored at -70°C (range -68 to -72°C) in our laboratory. Both HRM-01 and LRM-01 were nonreactive for antibodies to HIV, HTLV-1, hepatitis C and hepatitis B core antigen, and for hepatitis B surface antigen.

Using a modification of the method previously described (EL-Kadi et al., 1995), 100 $\mu$ L to 10 $\mu$ g/mL  $\beta_2$ GPI (Crystal Chemical, Chicago, IL) in phosphate-buffered saline (PBS, pH 7.4) was added to chemically activated microtiter wells (Xenobind™, Xenopore, Hawthorne, NJ), incubating for 2 h at 37°C, washing three times with 30 g/L bovine serum albumin (Cohn Fraction V; Boehringer Mannheim Biochemicals, Indianapolis, IN) in PBS (BSA/PBS), and then blocking with BSA/PBS (2h at 37°C). These antigen-coated plates were used soon after coating as they exhibited pseudo-first-order decay with a  $t_{0.5}$  of ~23 days after storage at 2-8°C. All assays were performed within 24h of antigen-coated plate preparation.

The assay calibrators were diluted in 1:100 in BSA/PBS, then added (100 $\mu$ L) to antigen-coated wells and incubated for 15 min at room temperature (RT: 25  $\pm$  1°C). After washing the wells three times with BSA/PBS, we further incubated (15 min, RT) the samples with 100 $\mu$ L of horseradish peroxidase-conjugated goat anti-human IgG (1:500 dilution of stock affinity-purified antibody containing ~1g/L total protein, obtained from Cappel Research Products, Div. of Organon Teknika, Durham, NC; prod. no. 55252). We then washed the wells as before with BSA/PBS and incubated each well with 100 $\mu$ L of substrate solution, prepared by mixing a tetramethylbenzidine solution with an equal volume of H<sub>2</sub>O<sub>2</sub> solution (both from Kirkegaard & Perry Labs., Gaithersburg, MD; prod. no. 50-76-00). After 10 min at RT, the reaction was stopped by adding 100 $\mu$ L of 1.25 mol/L sulfuric acid (reagent grade). We also measured  $A_{450}$  with a Model EL307C microtiter plate reader (Bio-tek Instruments, Winooski, VT) vs. a reagent blank in which BSA/PBS was substituted for diluted specimen.

### Results - Pilot Study

Sera were grouped into three categories: a reference group of apparently normal sera with low IgG aCL values (<22GPL units), a group of moderate binding sera containing those with additional 23 incremental GPL units (23-45 GPL units), and a high binding group, with  $\geq 46$ GPL units. Thus the latter two groups were considered positive for aCL. This was done to compare clinical features of APS in patients with high  $\alpha\beta_2$ GPI activity with those having low activity within the same range of aCL.

The aCL values of 82 sera were distributed as follows: 44 had values within our laboratories reference (normal) interval of 0-22 GPL units; 38 sera had higher aCL values of 23-87 GPL units. The latter sera were considered positive and subdivided into two groups, a moderate binding group comprising sera ranging from 23-45 GPL units (n=18) and a high binding group with sera having  $\geq 46$  GPL units (n=20). The distribution of  $\alpha\beta_2$ GPI binding activity (in absorbance units) in these three groups are presented in Figure 1.

The low ( $\leq 22$ GPL units) and moderate (23-45 GPL units) aCL groups had comparable  $\alpha\beta_2$ GPI binding activities with the low group having  $0.19 \pm 0.15$  (SD) and the moderate aCL group having  $0.21 \pm 0.09$  absorbance units. The high aCL group ( $\geq 46$ GPL units) had significantly higher  $\alpha\beta_2$ GPI activity ( $0.56 \pm 0.59$ ). ANOVA indicated that the differences among the groups were significant at  $p < 0.0001$ , with a Newman-Keuls test showing statistical significance ( $p < 0.05$ ) between the high GPL group and each of the other groups.

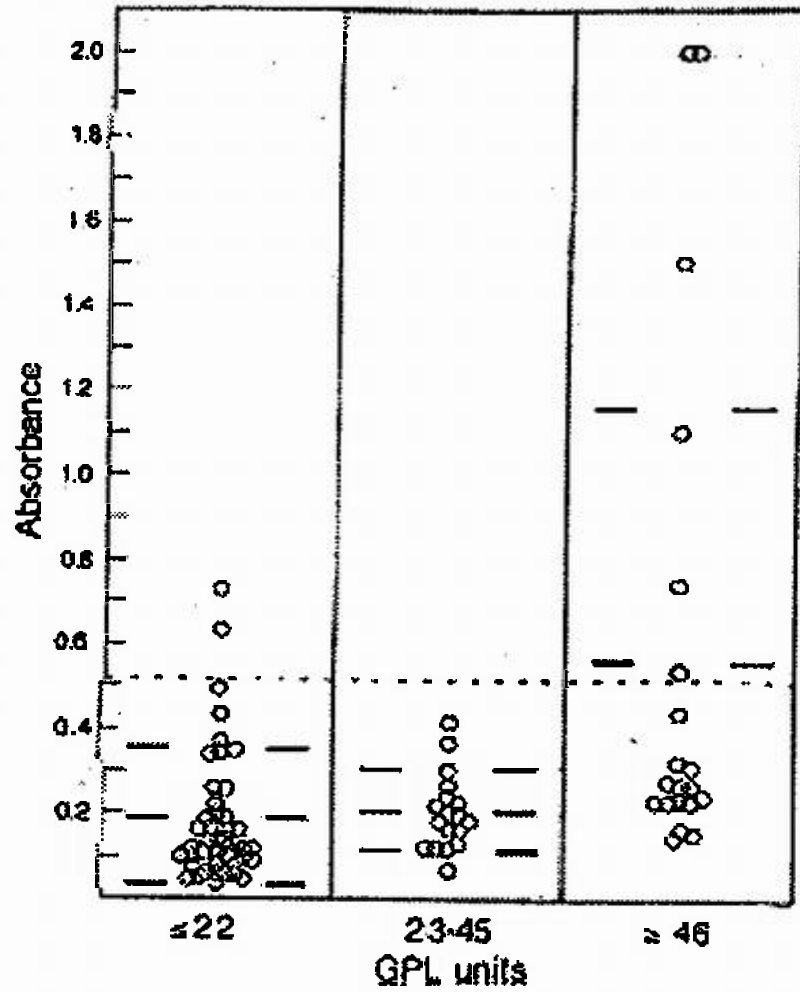


Figure 1

Anti- $\beta_2$ GPI (absorbance units) in 3 groups of sera with low ( $\leq 22$  GPL), moderate (23-45 GPL) and high ( $\geq 46$  GPL) aCL activity. Means  $\pm$  1 SD are shown by the bars (except for the  $\geq 46$  GPL group, in which case the mean minus 1 SD is below zero). The broken line is mean plus 2 SD for the reference group, taken as the upper limit for the reference interval (normal range).

A preliminary reference interval was established based on the mean  $\pm$  2SD (absorbance units) for the  $\alpha\beta_2$ GPI activity in the normal aCL group. Thus, values above 0.49 absorbance units were considered positive. By this criterion, the sera subjects in the normal aCL group represented outliers. No subject in the moderate aCL was in this category, but six patients in the high aCL group were positive. A 3 x 2 table contingency table analysis of outliers ( $>2SD$  above the mean of the low group) yielded  $X^2 = 12.61$  ( $p=0.002$ ) compared to  $X^2= 0.016$  ( $p = 0.90$ ) for the low and moderate groups alone in a 2 x 2 analysis.

A pilot series of 12 reference sera, six with high and six with low GPL values, was screened for reactivity with  $\beta_2$ GPI bound to activated wells. The values obtained are shown in Table I. Two sera (DA and DB), both from patients with APS secondary to systemic lupus erythematosus (SLE), exhibited binding substantially higher than the BSA background control. At a higher serum titer (1:100) than that used in the experiment in Table I (1:50) on wells containing  $1\mu\text{g}$  of  $\beta_2$ GPI, both sera again exhibited binding characteristics substantially different from other sera in the pilot group that were weakly or non-reactive (Fig. 2).

We chose one of these high-binding sera (DB) to use as the index serum to compare the binding of  $\beta_2$ GPI to activated wells with the binding to plain, unactivated polystyrene wells. These isotherms are shown in Figure 3a. In the unactivated wells, very little binding activity is observed over the entire range of  $\beta_2$ GPI mass per well. The activated wells showed a sharp increase in activity over the range of  $0.1\text{-}1.0\mu\text{g}$  per well, at which point maximum binding was observed.

**Table 1. Absorbance Values for Anti- $\beta_2$ GPI Activity in 12 Reference Sera.**

Serum	Diagnosis	aCL (GPLunits)	Anti- $\beta_2$ GPI <sup>o</sup> (absorbance units)	Control <sup>•</sup> (absorbance units)
VT	SLE	39	0.10	0.29
DA	SLE	47	≥ 2.0	0.35
MJ	FM	31	0.14	0.31
DB	SLE	91	≥ 2.0	0.24
SB	SLE	56	0.20	0.52
GM	n.d.	38	0.54	0.41
AB	n.d.	5	0.05	0.10
CR	n.d.	9	0.10	0.09
JI	n.d.	7	0.20	0.26
HB	n.d.	5	0.04	0.08
KT	n.d.	9	0.12	0.22
NE	n.d.	4	0.03	0.04

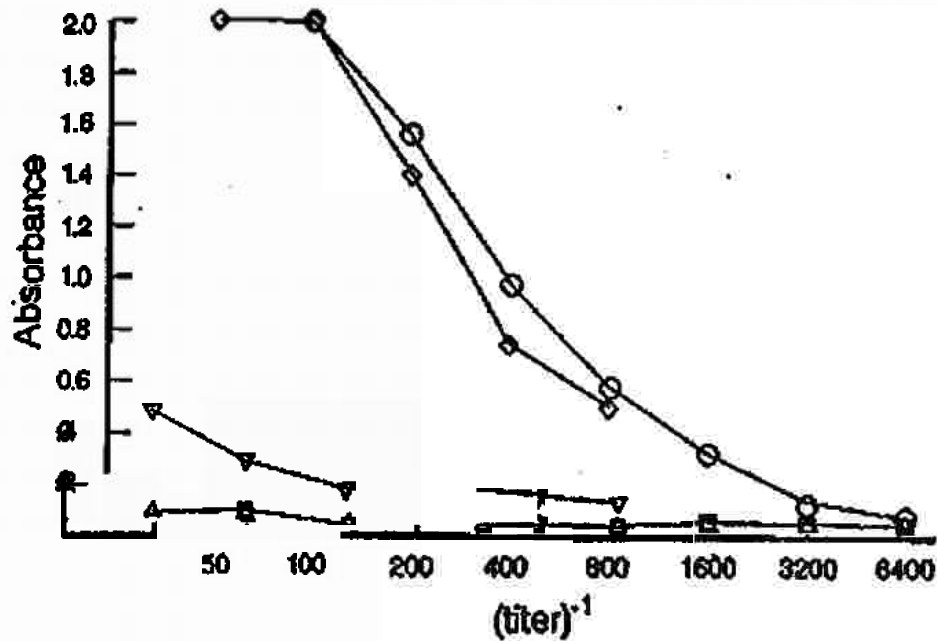
All values are duplicate measurements

\*Abbreviations: SLE, systemic lupus erythematosus; FM, fibromyalgia; n.d., not determined.

<sup>o</sup>Conditions:  $\beta_2$ GPI, 10 $\mu$ g/well with 3% BSA in PBS; washed only with PBS only (no BSA); serum diluted 1:50.

<sup>•</sup>Conditions: As above but without  $\beta_2$ GPI, i.e. BSA only.





**Figure 2**

Change in absorbance with titer of human sera in a binding assay for anti-b<sub>2</sub>GPI. b<sub>2</sub>GPI was added to activated polystyrene at 1 mg per well and incubated for 2 hr at 37EC. Wells were blocked with BSA under the same conditions. Sera studied: DB (circle), DA (diamond), KT (triangle), GM (inverted triangle) and VT (square).

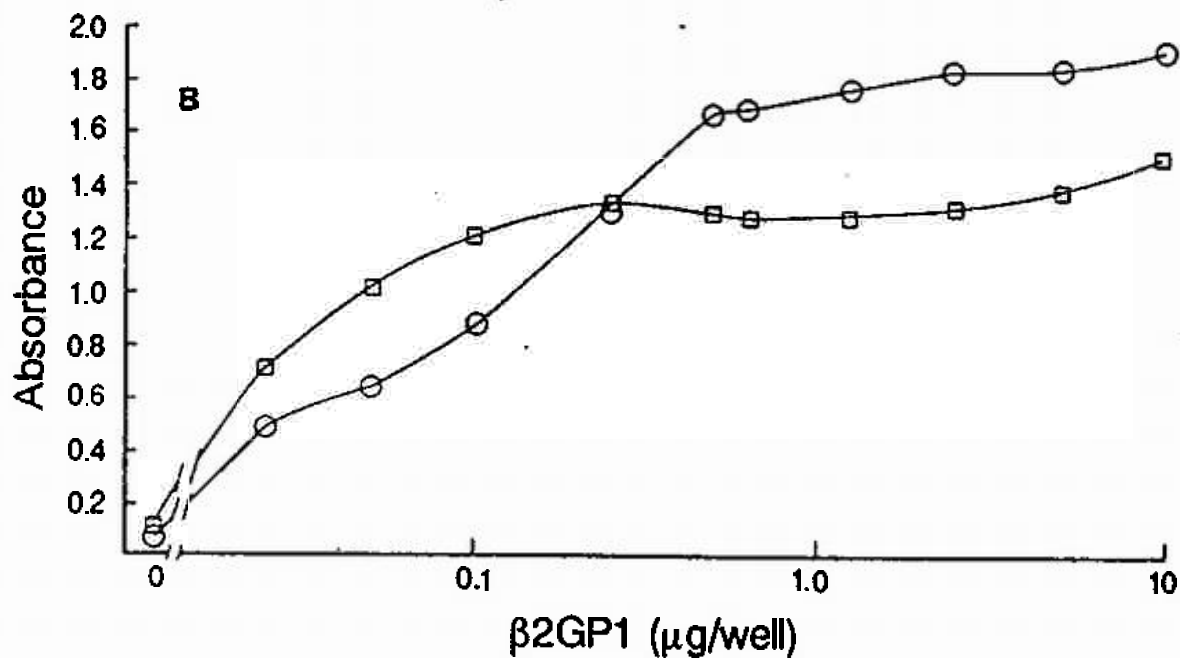
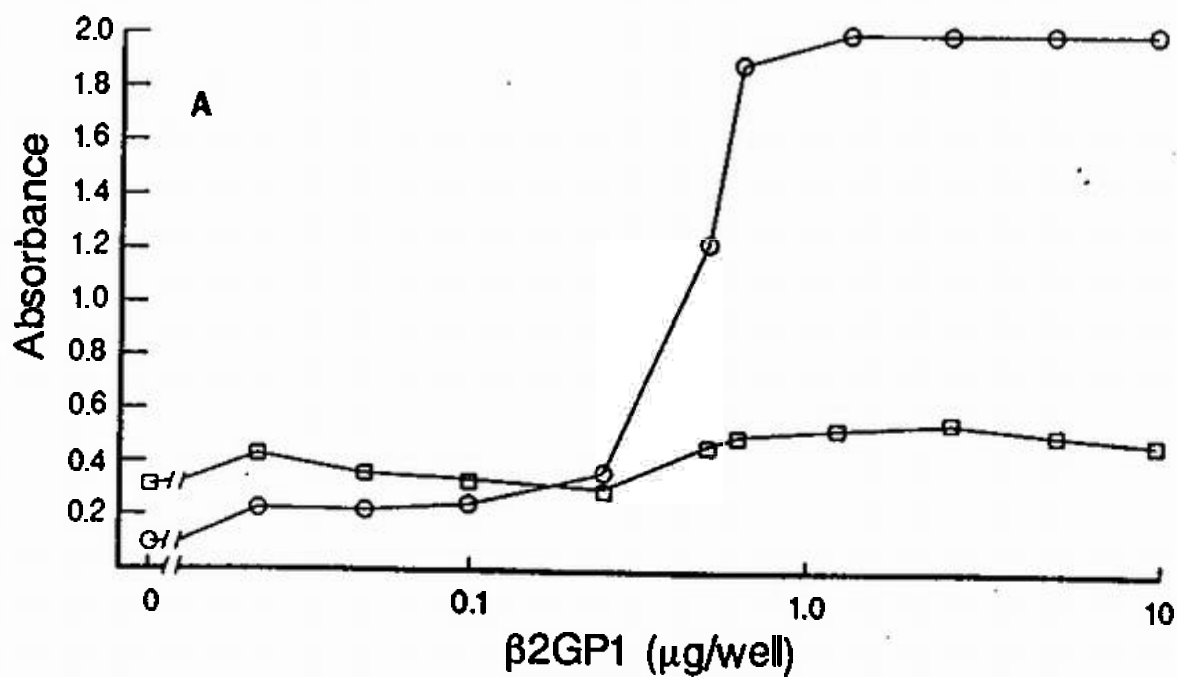


Figure 3

- A) Absorption isotherms for  $\beta_2$ GPI on activated (O) and unactivated ( $\square$ ) polystyrene. Activity detected with human serum (DB) and anti-human IgG.
- B) Absorption isotherms for  $\beta_2$ GPI on activated (O) and unactivated ( $\square$ ) polystyrene. Activity detected with rabbit and anti-rabbit IgG.

To investigate whether these observations were purely a demonstration of quantitative differences in binding, or whether epitope presentation was a surface-dependent phenomenon, we examined activity based on the recognition of bound antigen by a rabbit polyclonal IgG  $\alpha\beta_2$ GPI. This reagent exhibited liquid-phase reaction with  $\beta_2$ GPI as demonstrated by precipitin arcs in a double-diffusion system, whereas neither serum DB nor IgG purified from serum DB was reactive. These isotherms, shown in Figure 3b, again demonstrate near-maximal binding at 1 $\mu$ g per well on the activated polystyrene. However, the unactivated wells also show substantial binding activity over the range of 1-10 $\mu$ g per well.

The apparent ability of  $\beta_2$ GPI to bind to the unactivated polystyrene was confirmed by the use of a biotin-avidin detection system (Fig.4), at a concentration of HRP-streptavidin of 30ng/ml. Biotinylated  $\beta_2$ GPI bound to unactivated polystyrene at nearly 80% of its binding to activated polystyrene in the saturation region of the isotherm. Elution by HL citrate solution of either bound  $\beta_2$ GPI or IgG  $\alpha\beta_2$ GPI was studied by using a series of procedures as described in Table 2. After initial assay of  $\alpha\beta_2$ GPI activity of the IgG fraction of serum DB (absorbance= 1.86, 100% activity by definition), a parallel set of wells was incubated with HL citrate (30 min at 25°C). Of these, half (two wells) were washed with PBS/BSA and assayed for IgG remaining. This procedure resulted in an absorbance of 0.09, demonstrating that over 95% of the IgG had been removed. Re-incubation of the other two wells with the original IgG fraction, followed by re-assaying, resulted in an absorbance of 1.68, indicating a restoration of 90.3% of the original IgG  $\alpha\beta_2$ GPI activity.

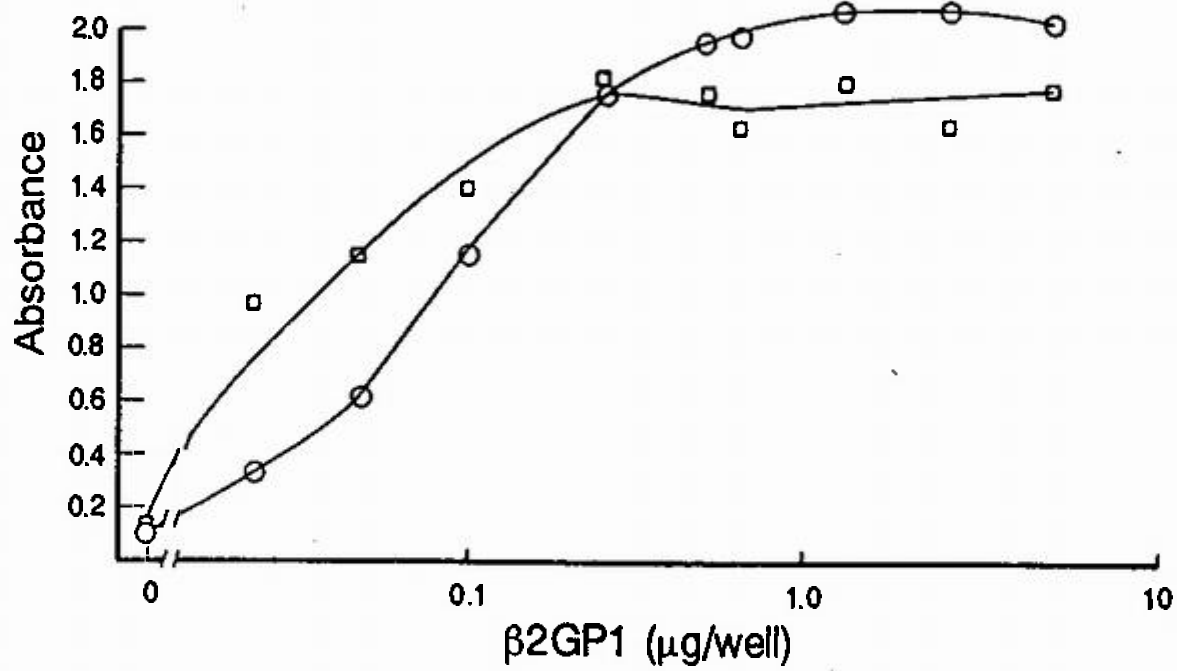


Figure 4

Adsorption isotherms for biotinylated  $\beta$ 2GPI on activated (O) and unactivated ( $\square$ ) polystyrene detected with HRP-streptavidin at 30ng/ml

**Table 2. Protein Elution Profiles with HL Citrate Solution of the System IgG Anti- $\beta_2$ GPI on Activated Polystyrene**

<b>Number of Wells</b>	<b>Experimental Procedure</b>	<b>Absorbance</b>	<b>Activity Yield</b>
6:2	Complete System (IgG + $\beta_2$ GPI) Assay (HRP-anti IgG)	1.86	100
6:4	Assay (HRP-anti-IgG)		
4:2	Incubate with HL citrate / Assay	0.09	4.8
6:2	Reincubate with IgG		
2:2	Assay	1.68	90.3

Absorbance results are means of duplicate measurements

To determine the prevalence of  $\alpha\beta_2$ GPI in a larger group of sera we screened 113 sera that had been submitted for aCl measurement. Binding of  $\beta_2$ GPI above the cut-off of 0.45 absorbance units (the mean  $\pm$  2S.D. for 61 sera with GPL values within our normal range of 0-22 was  $(0.15 \pm 0.30)$ ) was observed in only nine sera. Of these, six had aCl measurements of 47 GPL or higher.

### **Results - Establishment of de Novo Method**

Until this point standard kit reagents were used in the pilot study (Keil et al 1995). The kit reagents seemed the natural progression to the establishment of a *de novo* assay utilizing similar basic biochemical reactions. The novel assay utilized  $\beta_2$ GPI in place of cardiolipin. The titer of the horseradish peroxidase conjugated goat anti-human IgG (antibody containing 1mg/ml total protein, Cappel Research Products, Durham, NC) was established. Figure 5, shows the optimal antibody titer to be used in succeeding assays to be 1:500. In addition the volume fraction of EE and DB showed a linearity with a  $r = 0.97$  and  $S_{xy} = 0.09$ , shown in Figure 6. In addition, the two samples of DB and EE were utilized to establish sample longevity in days after harvest (Fig. 7), and in days after thawing (Fig. 8).

### **Results of the Development of Standard Material.**

From absorbance measurements obtained over the first 3 months of storage ( $n=21$ ),  $A_{450}$  was  $1.45 \pm 0.24$  (mean  $\pm$  SD, 95% confidence interval = 1.34-1.56) for the HRM-01 plasma calibrator and  $0.11 \pm 0.07$  (95% confidence interval = 0.07-0.14) for the LRM-01 serum calibrator. These values served as the basis for the assignment of arbitrary units to the materials, the units being a simple function of absorbance ( $A_{450}$ ) and dilution. Thus, a

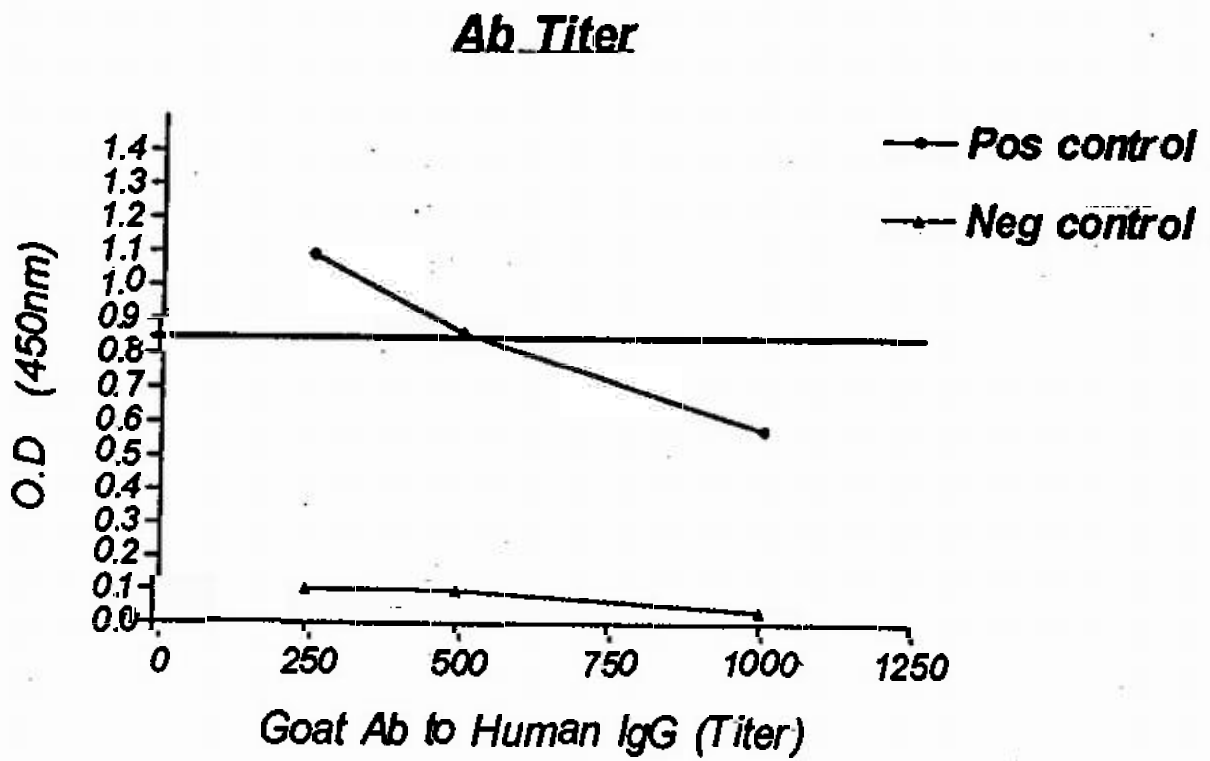


Figure 5  
Optimal Antibody Titer

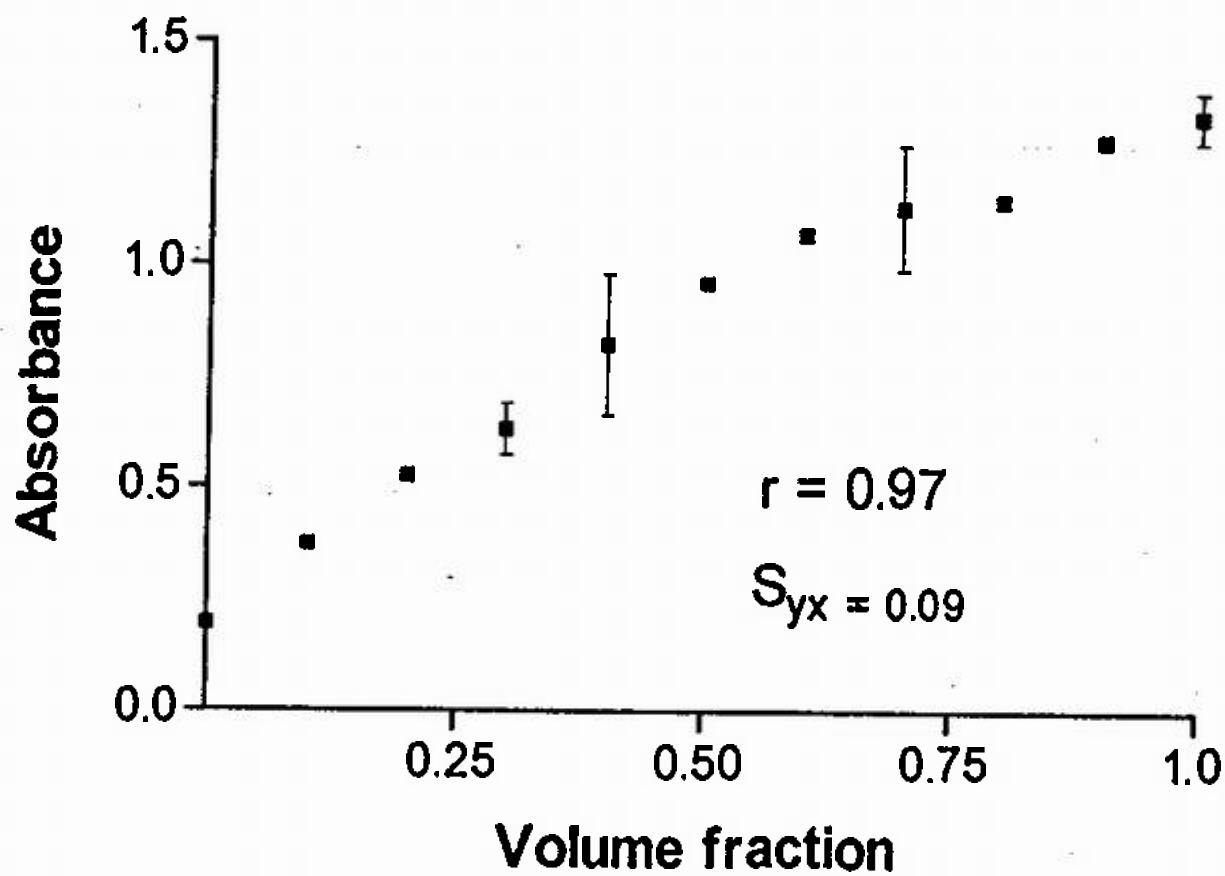


Figure 6. Volume Fraction

Absorbance at 450nm plotted against volume fractions. Vertical lines through the box (■) plots signify 1 (small vertical line) and 2 (larger vertical line) standard deviations (SD).



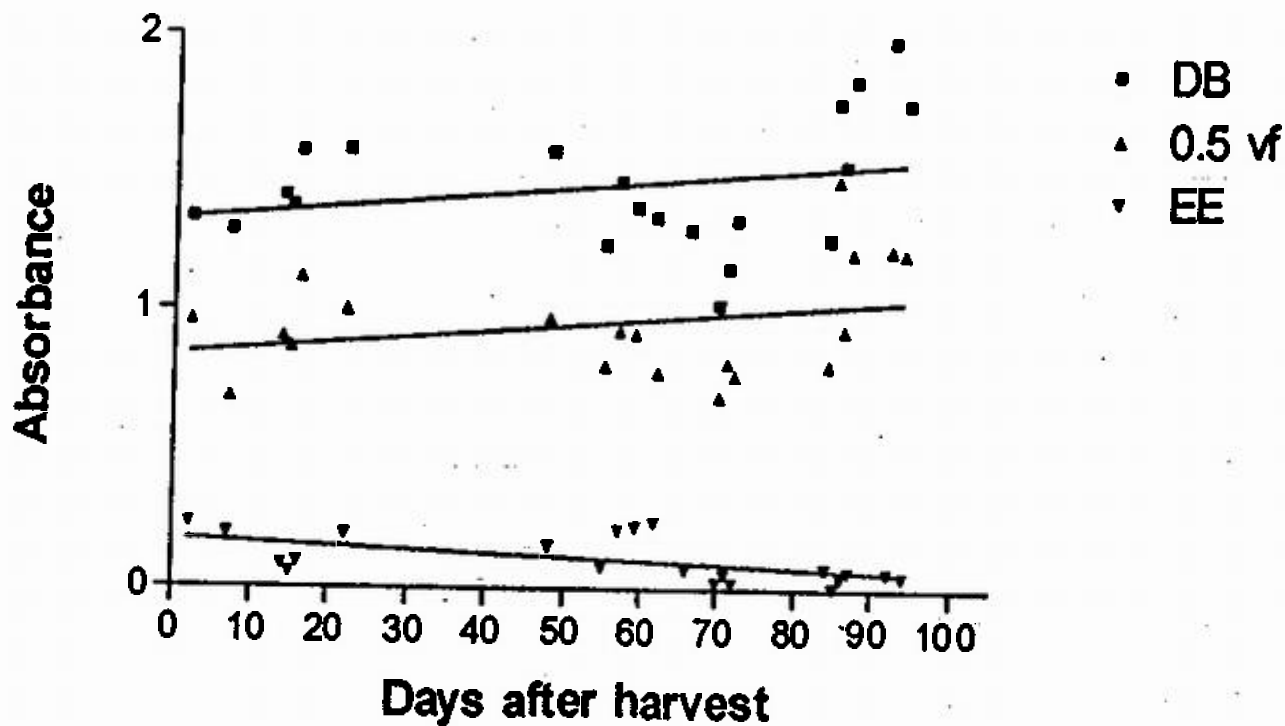


Figure 7. Days after Harvest.

Absorbance at 450nm plotted against days after harvest. Samples assayed; DB (■), EE (▼) and 0.5 volume fraction of DB and EE (▲).

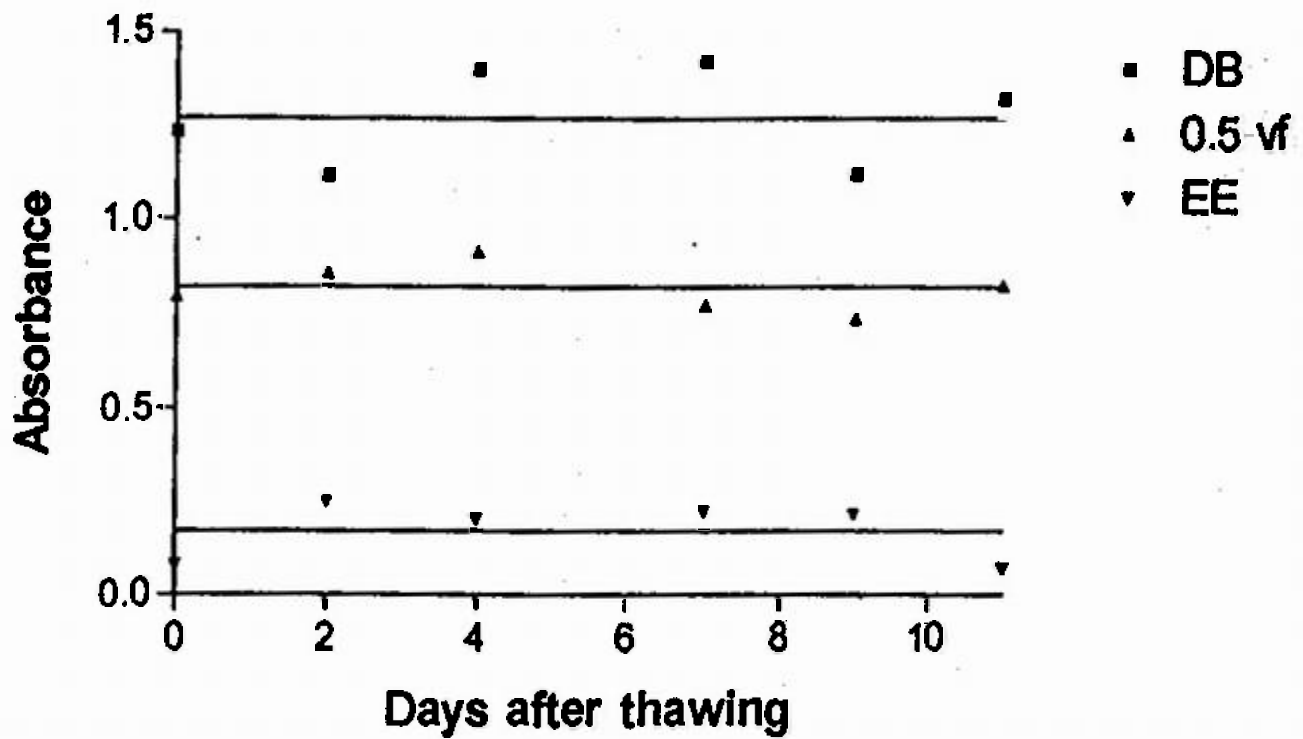


Figure 8. Days after thawing.

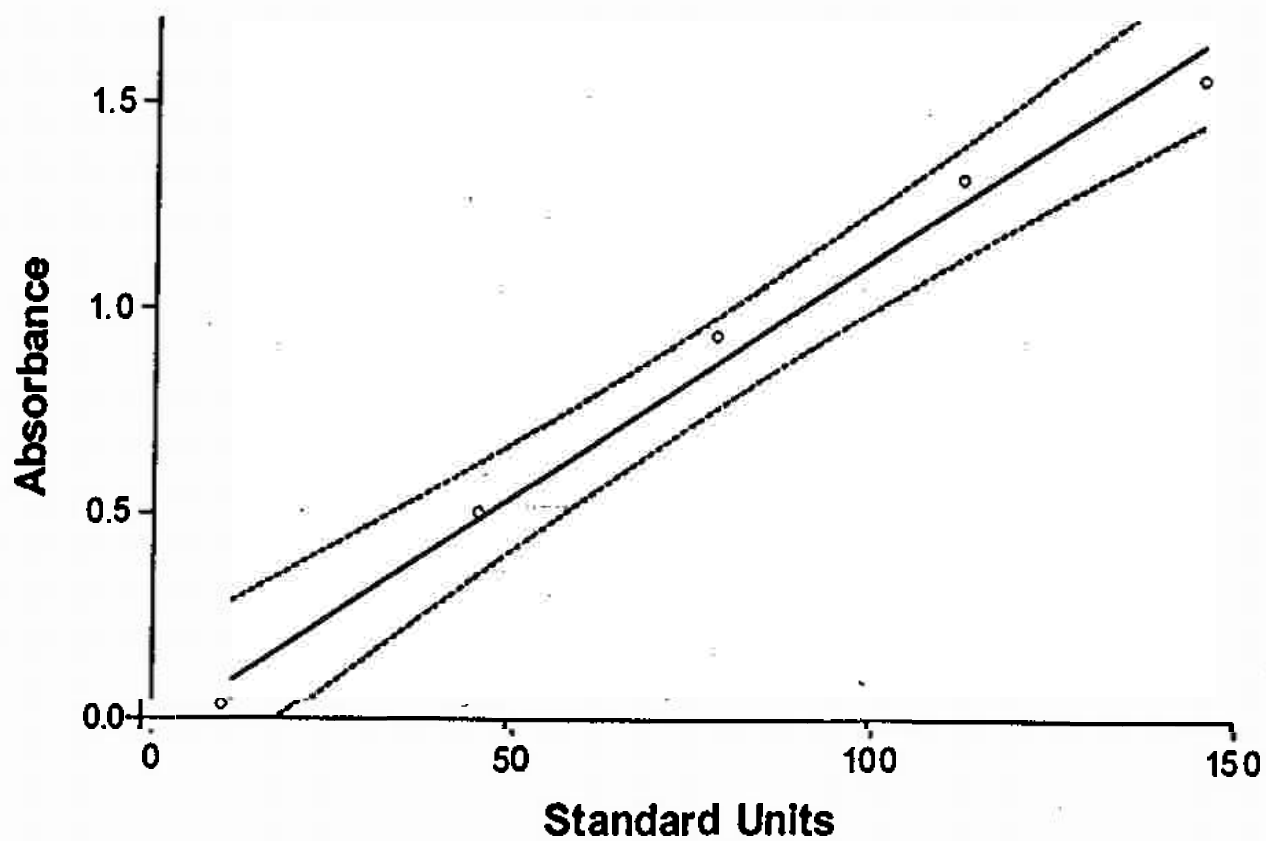
Absorbance at 450nm plotted against days after thawing. Samples assayed; DB (■), EE (▼) and 0.5 volume fraction of DB and EE (▲).

standardized unit is defined as  $A_{450}$  times the reciprocal titer [titer<sup>-1</sup>]. The resultant mean value for reference material HRM-01 was 145 SGU (standard IgG anti- $\beta_2$ GPI units), and LRM-01 was 11 SGU.

The application of these materials in an immunoassay is presented as a typical five-point calibration curve (Fig. 9). The values of the three intermediate calibrators are calculated from the assigned values of mixtures of HRM-01 and LRM-01. Thus undiluted LRM-01 and HRM-01 are equivalent to SGU = 11 and 145 respectively. Additional calibrators are as follows: 3 parts of LRM-01 and 1 part of HRM-01 yielding 44 SGU, equal parts of LRM-01 and HRM-01 yielding 78 SGU, and 1 part LRM-01 and 3 parts HRM-01 yielding 112 SGU. The curve is linear ( $r = 0.99$ ) over the applied range. The nonspecific binding of HRM-01, assessed by the signal from BSA-coated wells, is minimal (mean = 0.06  $A_{450}$ ,  $n = 20$ ).

#### **Results of the Application of Reference Standards.**

To study the stability characteristics of  $\beta_2$ GPI-coated microtiter plates stored and refrigerated at 2-8°C, the antigen coated wells were probed with four sera from subjects with APS. These were then subjected to the assay procedure at intervals of up to 125 days. The data obtained from this experiment were normalized to percentage of maximum absorbance. These data, shown in Figure 10, demonstrate excellent fit to a first order decomposition ( $r > 0.99$  for all cases). The means  $\pm$  1 SD were plotted against the natural logarithm of time (Fig. 10; inset); and the half-life ( $t_{1/2}$ ) was calculated from the rate constant ( $k' = -21.2d^{-1}$ ), given by the slope of the line ( $r = 0.95$ ,  $p < 0.05$ ), yielding  $t$



**Figure 9**  
Standard Units Calibration curve generated from reference materials HROM-1 and LRM-01. Results from a single run after the assignment of arbitrary units for the standards. Points are the mean absorbance of duplicate microtiter wells. Dashed lines represent the upper and lower 95% confidence intervals of the regression line.

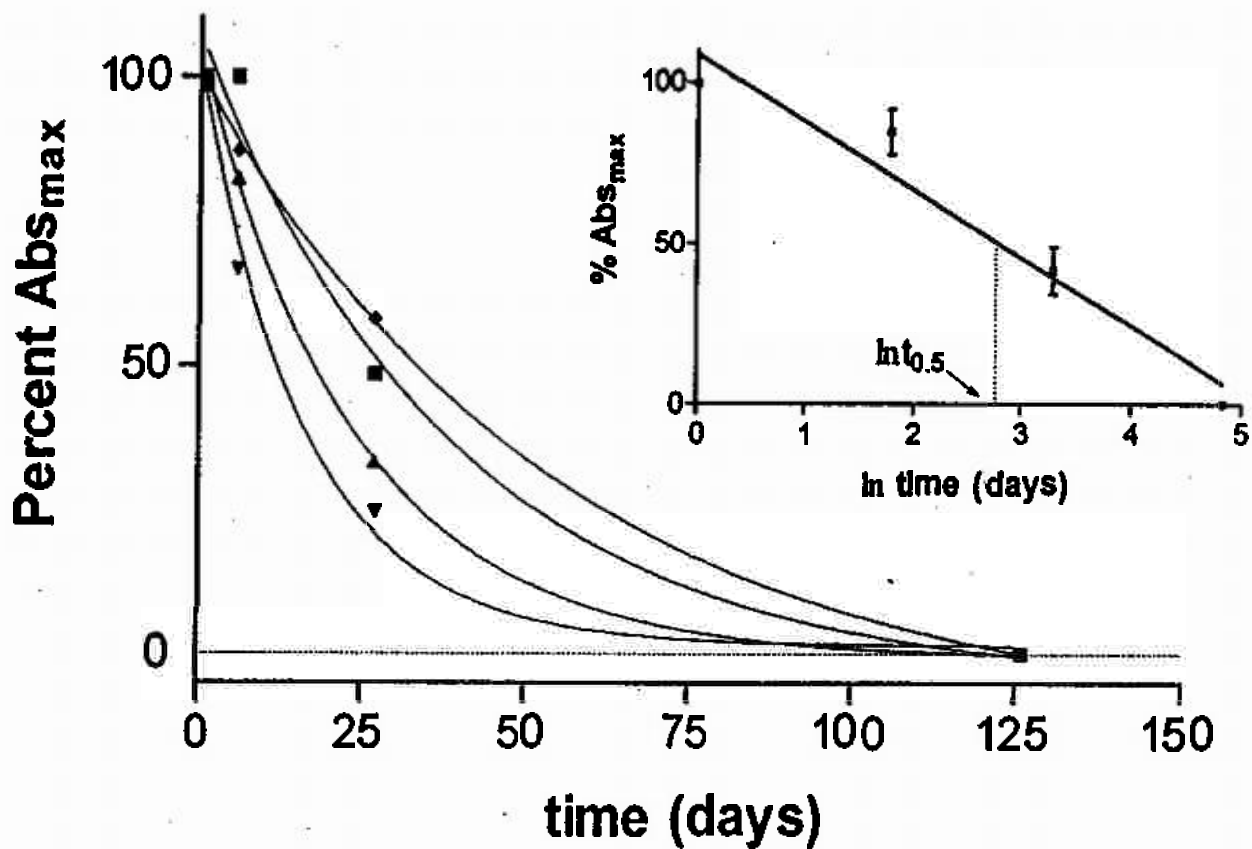


Figure 10

Normalized plots of absorbance versus time for stability study of antigen-coated plates after storage at 2-8°C. Inset shows regression line of Abs<sub>max</sub> versus ln time (ln t<sub>0.5</sub> = 2.76d or t<sub>0.5</sub> = 15.8 d).

$t_{1/2} = 15.8$  days. Thus in all studies that followed, the coated plates were used within 24hrs of the coating process, assuring at least 90% of the original activity.

Two reference calibrators, (HRM-01 = 145 SGU and LRM-01 11SGU) were utilized as assay standards (Erickson et al., 1996). Three point standard curves with standards in duplicate were generated for each run. Acceptance criterion for a run was based on a value of  $r^2 \geq .950$ . In Figure 11, the means and standard errors for 36 consecutive, accepted runs are depicted.

Sera at three levels of activity were used to assess the precision (coefficient of variation [CV] ) of the assay both between (interassay) and within (intraassay) runs. The data is presented in Table 3. To determine the effect of dilution on a specimen which lies beyond the high standard (at the screening titer of 1:100), a patient sample meeting the criterion was diluted serially. As can be seen from the results of the experiment, shown in Figure 12a, dilutions greater than 1:200 brought the value within range. The absorbance at each dilution was used to calculate SGU, which are shown as a function of titer in Figure 12b. The slope of this plot indicated no significant deviation from zero ( $p = 0.29$ ); the mean value obtained was 426 SGU, with a CV of 10.9%.

#### **Results - Determination of a Reference Interval:**

In Table 4, the data on the healthy control cohort, from whom sera were collected for the determination of the reference interval, are categorized by racial and ethnic background of the subjects. The distribution of SGU values obtained from the 204 subjects in the reference sample are shown in Figure 13. Forty-nine of the subjects (24%) had 0 SGU values as the distribution tailed to 54 SGU. As is apparent from the

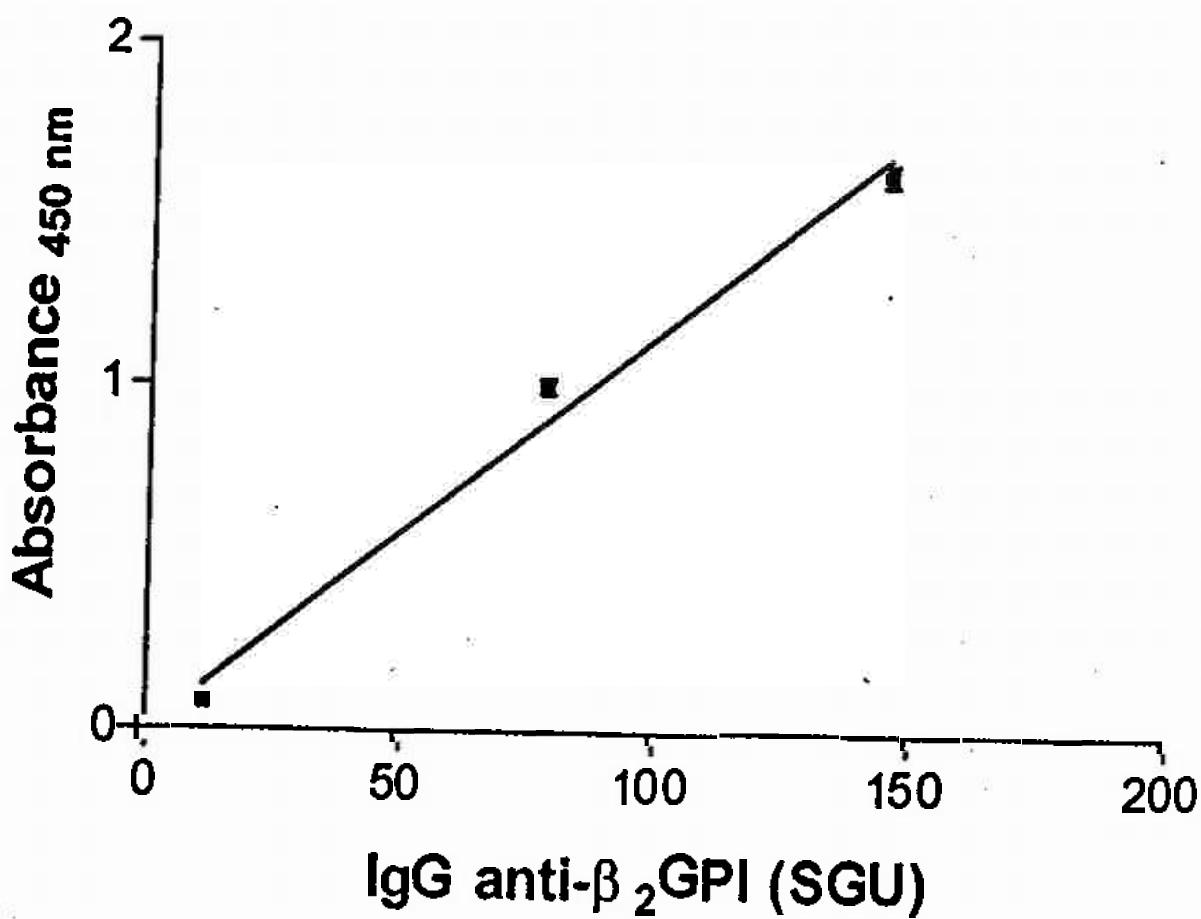


Figure 11

Summary plot standard curves of 36 runs. Means and standard errors of the mean (SEM) for absorbance of the three standards routinely used in this assay are plotted as a function of the value of the standards in SGU.

**Table 3. Precision of Immunoassay of IgG Anti- $\beta_2$ GPI at Three Levels.**

**Intra-assay Precision**

Level	n	means	SD	CV(%)
low	28	7.3	0.9	12.3
mid	23	42	2.9	6.9
high	22	113.7	9.4	8.3

Values of means and standard deviations (SD) are in standard IgG and Anti- $\beta_2$ GPI units (SGU)

**Inter-assay Precision**

Level	n	means	SD	CV(%)
low	19	7.9	2.8	35.4
mid	19	40.3	6.4	15.9
high	19	105.3	8.1	7.7

Values of means and standard deviations (SD) are in standard IgG and Anti- $\beta_2$ GPI units (SGU)



Figure A

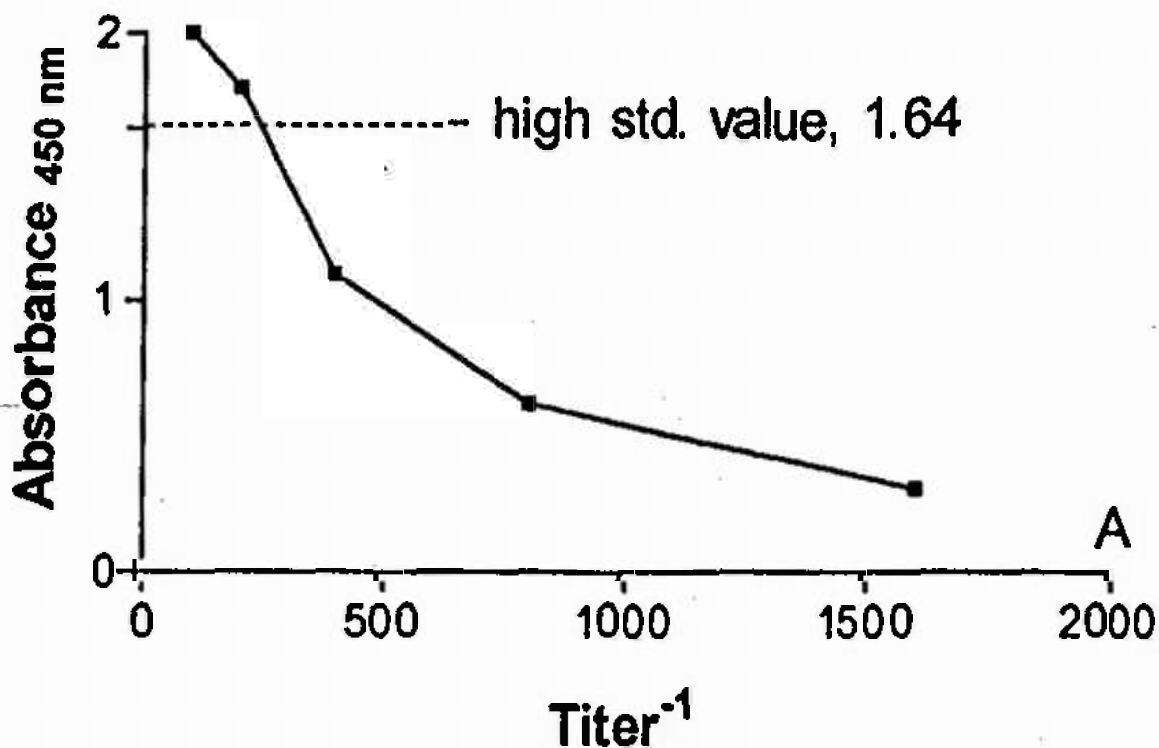


Figure B

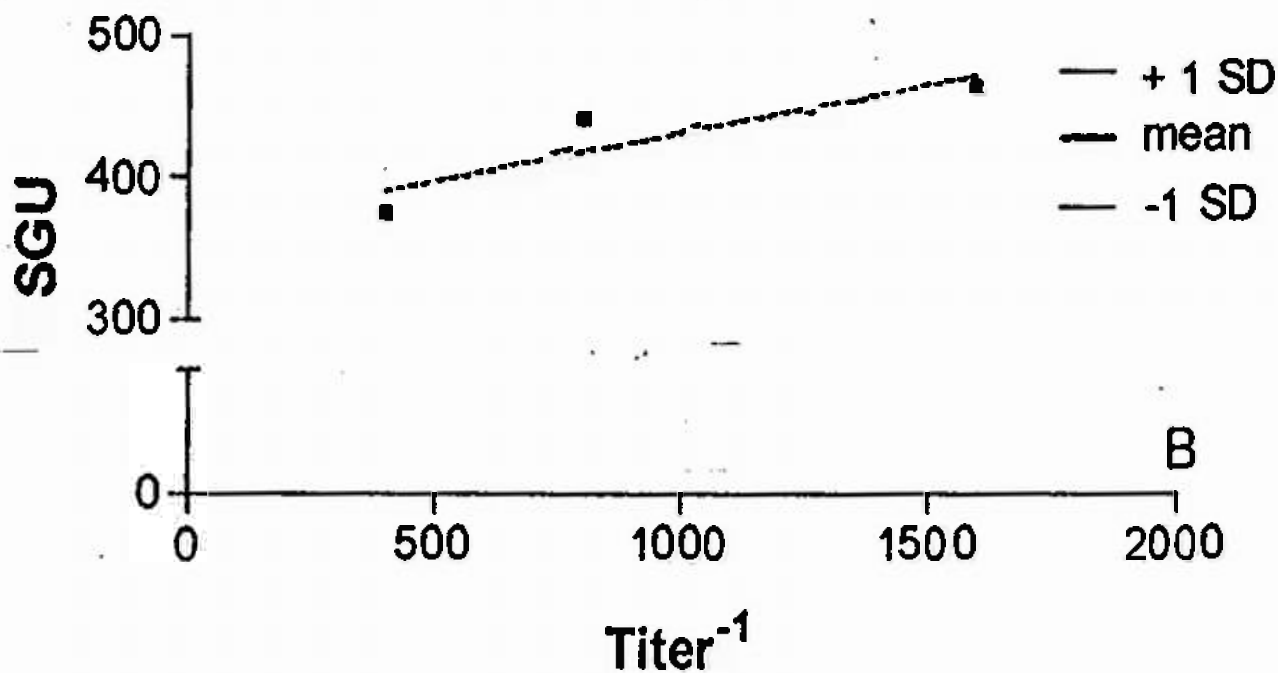
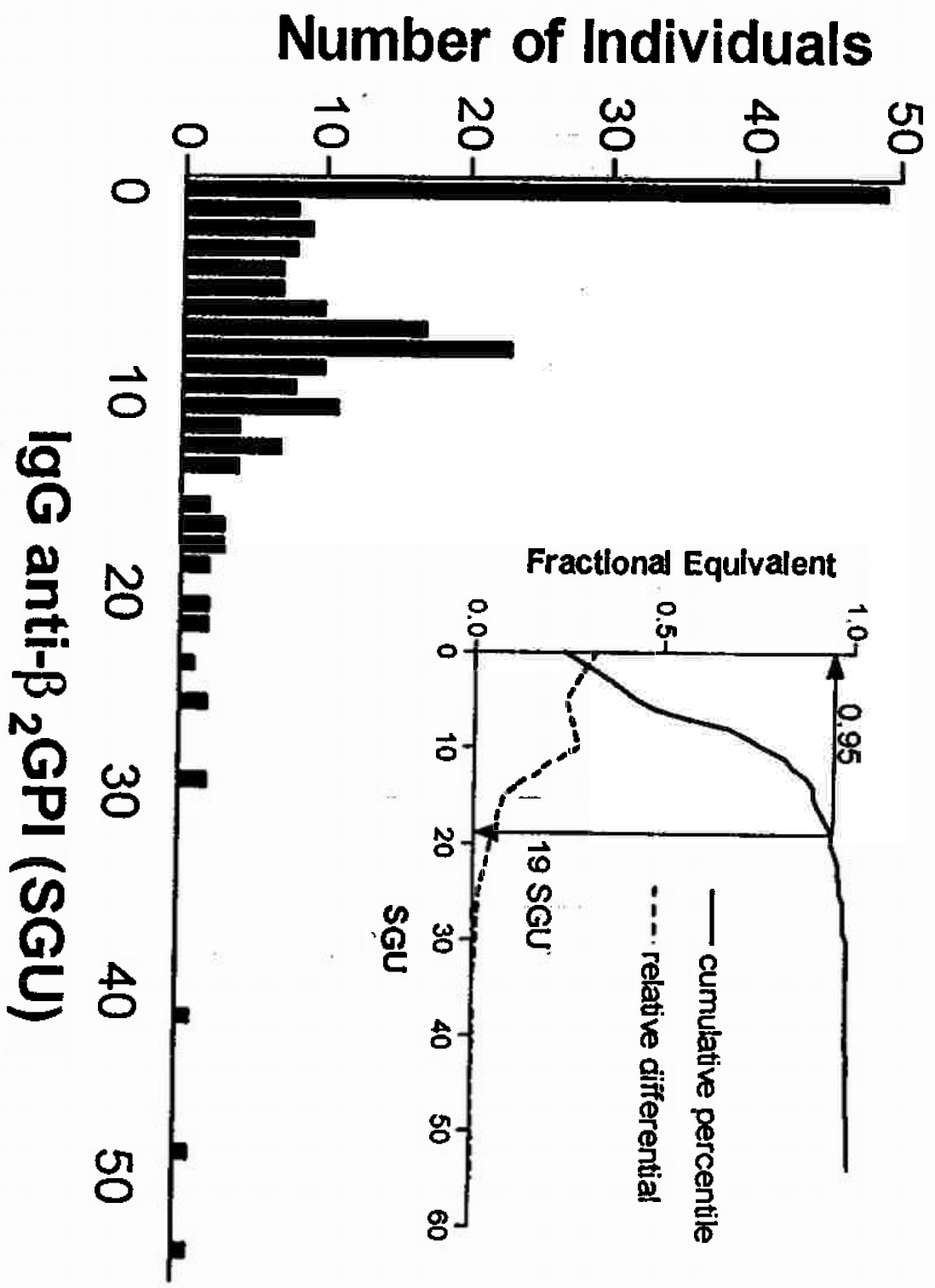


Figure 12 A and B

Dilutional linearity of an out of range specimen. (A) Absorbance at 450nm at serial dilutions between titers of 1:100 and 1:1600. The value of the high standard (HRM-01) for this run is indicated. (B) Plot of three values obtained by conversion to SGU reciprocal titer. Short solid lines to the right of the dotted regression line indicate the SD of SGU for these three points.

**Table 4. Distribution of  $\beta_2$ GPI values (SGU) from Reference Interval Cohort by Ethnicity**

<b>Ethnicity / Race</b>	<b>n (%)</b>	<b>Mean</b>	<b>Median</b>	<b>Range</b>
White	152 (74.5)	7	6	0-54
Hispanic	18 (8.8)	8	7	0-30
Asian/ Pacific Island	15 (7.4)	10	8	0-49
Black	10 (4.9)	12	13	0-26
Undetermined	9 (4.4)	6	8	0-13
Total	204 (100)	7	7	0-54



**Figure 13**  
 Distribution of values from 204 healthy subjects. Insert shows relative differential and cumulative percentiles plots.

bar graph, the distribution tested strongly non-Gaussian by the Kolmogorov-Smirnov statistic ( $P < 0.0001$ ). To include 95% of our values, as is normally done in the case of parametric reference intervals, the cumulative percentile plot (Fig.13, insert) was constructed. The value of SGU at the ninety-fifth percentile was determined and was taken to represent the upper limit of the reference interval, resulting in a range of 0-19 SGU.

## Discussion

The first test in which aPL were detectable was the complement fixation assay described by Wasserman in 1906. The antigen, bound by reagin was identified as cardiolipin by Pangborn in 1941. Subsequent worldwide distribution of anti-treponemal assays led to the observation of a phenomenon known as biological false positive serologic test for syphilis (BFP-STS). An acute and chronic BFP-STS were soon identified. Acute BFP-STS was usually infection induced, e.g. leprosy, tuberculosis, endocarditis ect. Chronic BFP-STS was observed to persist for greater than six months, and was associated with autoimmune, or lymphoproliferative disease (Moore and Mohr, 1952, Hasserick and Long, 1951). Additionally, pharmacologic agents such as procainamide, hydralazine, and chlorpromazine were observed to cause BFP-STS.

Subsequently an unusual plasma constituent that inhibited *in vitro* coagulation, and paradoxically demonstrated an hypercoaguable state *in vivo* was detected. First termed the lupus anticoagulant (LA), the plasma constituent was later identified as an anticardiolipin antibody (aCL) (Feinstein and Rapaport, 1972 and Thiagarajan et al., 1980). The advent of molecular biology led to the development of an anticardiolipin radioimmunoassay and standardization by Harris and colleagues in 1983.

Antiphospholipid antibodies encompass at least three reactivities: BFP-STS, LA, and aCL. The latter two are associated with symptomatology although the significance continues to be debated (Gharavi et al., 1993, Alarcon-Segovia and Cabral, 1994, Blank et al., 1994, Hunt and Krilis, 1994, El-Kadi et al., 1995). The complications resulting from the autoimmune hypercoaguable state of APS has been associated with antibodies to

cardiolipin, and other anionic phospholipids (Hughes, 1983, Galli et al., 1990, Cabiedes et al., 1995).

Recent evidence has identified  $\beta_2$ GPI, once thought to be the cofactor for aCL as the phospholipid binding protein which is the true epitope for APS antibodies (Blank et al., 1994, Najmey et al., 1996). Current data demonstrate that the recognition of  $\beta_2$  GPI in the absence of phospholipid by human aCL is dependent on the surface to which  $\beta_2$ GPI is bound. When  $\beta_2$ GPI is not bound to a solid phase, there appears to be a lack of recognition by human autoantibodies (Matsuura et al., 1994, Keil et al., 1995). Results have demonstrated that irradiated as well chemically activated surfaces provide recognition of this epitope(s) (Matsuura et al., 1994, Keil et al., 1995). These surfaces appear to mimic the *in vivo* binding of  $\beta_2$ GPI to presumably anionic physiologic surfaces which reveal the proteins cryptic epitope, providing recognition of human autoantibodies.

A pilot study was conducted to establish the foundation for a novel assay using  $\beta_2$ GPI as the antigen. This preliminary study made use of conventional methods to obtain aCL measurements from of 82 sera. Subsequently, the sera were assayed for  $\alpha\beta_2$ GPI activity through the use of chemically activated polystyrene, with BSA as a blocking agent. The reagent blanks demonstrated minimal binding; typically in the range of 0.02 - 0.04 absorbance units. Further, the CV for the low, midlevel, and high intra-assay precision was 8.3%, 6.9% and 12.3% respectively. Additionally, the CV for the inter-assay precision was 35.4%, 15.9% and 7.7% for the low, midlevel and high respectively.

The data from the pilot study suggest that substantial binding of  $\beta_2$ GPI does not occur in sera with aCL activity of less than 50 GPL units. Elevated  $\alpha\beta_2$ GPI activity was

found in 2 of the 62 sera with an aCL greater than 46 GPL units (Fig. 1). Therefore, there was no significant correlation between cofactor dependent aCL and  $\beta_2$ GPI. Subsequently 12 reference sera, six with high and six with low GPL values, were screened for reactivity with  $\beta_2$ GPI bound to activated wells. Two of the subjects, DA and DB (Table 1), had a high GPL, as well high a $\beta_2$ GPI absorbance units. Sera from DA and DB were chosen as the index sera to demonstrate absorption isotherms for  $\beta_2$ GPI on activated and non-activated polystyrene. Clearly, Figure 3, demonstrates the dependence of an "activated" surface for the binding of  $\beta_2$ GPI. These results demonstrate binding to  $\beta_2$ GPI in untreated, hydrophobic polystyrene as well as activated polystyrene. Thus, the recognition of  $\beta_2$ GPI on either chemically activated or irradiated polystyrene is a qualitative phenomenon, dependent not on the surface of the polystyrene but rather how the protein is bound to the surface.

The chemically activated polystyrene works by covalently binding  $\beta_2$ GPI. Covalent binding of the assay system was demonstrated utilizing elution experiments with HL citrate.  $\beta_2$ GPI dependent on ionic interaction would have been removed. Additionally 90% of the original IgG a $\beta_2$ GPI activity can be reconstituted when a $\beta_2$ GPI reactive IgG is reincubated with bound  $\beta_2$ GPI (Table 2).

Prior to the work described herein, there were no standardized immunassays for the detection of antibodies to  $\beta_2$  GPI. In this study, a standard serial antibody titer (Fig. 5), and standard curves were established (Fig. 6). Also, the variability of standards over time (Fig. 7), and after thawing (Fig. 8), were investigated.

The application of these materials for the *de novo* assay is presented in a 5 point calibration curve (Fig. 9). The values of the three intermediate calibrators were calculated from the assigned values of the mixtures of HRM-01 and LRM-01. The curve is linear ( $r = 0.99$ ) over the applied aforementioned ranges. The non-specific binding of HRM-01 assessed by the signal from the BSA-coated wells is minimal (mean = 0.06  $A_{450}$  and  $n = 20$ ). Standard curves consistently demonstrated excellent linearity. The coefficient of determination ( $r^2$ ) was minimal (0.95 limit of acceptance) and more typically in the range of 0.97 - 0.99 (Fig. 11).

These data demonstrate the standardization of an assay for IgG antibodies to human  $\beta_2$ GPI. Generally, the precision of the assay (Table 1) is fairly good. The confidence intervals between arising from these measurements clearly suggest that discrimination between levels of  $\alpha\beta_2$ GPI is satisfactory in this assay. The first standardized  $\alpha\beta_2$ GPI immunoassay and calibrators developed herein, can be used to develop international standards. Following the standardized detection of  $\alpha\beta_2$ GPI antibodies, correlation's may be drawn between disease states with similar pathology.

The pathologic, and complete *in vivo* physiological and molecular presentation  $\beta_2$ GPI- $\alpha\beta_2$ GPI has not yet been elucidated. However, during the high mitotic and apoptotic rate of the embryo, apoptosis has been observed to be a source of immunogens (Vaishnav et al.,1997). Several intervillous changes and fibrin changes have been reported in the placenta (Rosa et al., 1994). The mechanism of injury to the fetus by  $\alpha\beta_2$ GPI antibodies is variable, occurring on the trophoblast and villi surfaces of the placenta. Thrombotic and infarcted areas are not uniform (Rosa et al., 1994)



Lockwood (1986), observed an association between IgG-aCL subtype and fetal death, while intrauterine growth restriction was associated with IgM-aCL subtypes. Additionally, a high prevalence of antiphospholipid antibodies has been described in children with idiopathic cerebral ischemia (Schoenberg et al., 1978, Angelini et al., 1994). It would be of interest to characterize the  $\alpha\beta_2$ GPI antibodies with the standardized ELISA developed herein to determine a correlation between apoptosis and the generation of  $\alpha\beta_2$ GPI antibodies in these hypercoaguable states.

## Conclusion

Antiphospholipid Syndrome (APS) is a hypercoaguable state in which the following clinical manifestations may be present: arterial and venous thrombosis, livedo reticularis, strokes, migraines, recurrent fetal loss, and thrombocytopenia. The underlying pathophysiologic process appear to be thrombotic in nature, although the mechanism responsible for the production of  $\alpha\beta_2$ GPI antibodies is unclear. However, some authors have theorized the externalization of phosphatidylserine during apoptosis plays a role. (Vaishnaw et al., 1994, Quasim et al., 1998).

Laboratory characterization and detection of APS continues to be debated. Assays of  $\beta_2$ GPI have used a variety of materials in the assay presentation system (Arvieux et al., 1991, Koike 1994, Roubey et al., 1995, Keil et al., 1995). Lack of standardization has been well documented (Ekins 1991). The data presented herein describe the performance characteristics and reference range of a standard ELISA for the detection of  $\alpha\beta_2$ GPI antibodies. Therefore, standardized  $\alpha\beta_2$ GPI units detected in hypercoaguable or pathological states, may begin to be correlated with aCL and LA assays previously standardized.

During the development of the ELISA, the plates were used within 24hrs of preparation. Viability and decomposition after 24hrs was not evaluated. Moreover, it would be of interest to determine the response of these calibrators in assays that utilize irradiated plates and (or) noncommercial sources of  $\beta_2$ GPI.

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