1. INTRODUCTION

The first study on the anti-phospholipid antibodies began in 1906 when Wasserman introduced a serological test for syphilis. In 1942 it was found the active component that is a phospholipid indicated by the name of Cardiolipin. In the 50's it was observed that a large number of people appeared to be positive for syphilis tests but did not show any evidence of disease. At the beginning the phenomenon was classified as a series of false positive syphilis test, then a more accurate analysis revealed, for this group of patients, a high prevalence of autoimmune disorders including Systemic Lupus Eritematousus (SLE) and Sjögrens syndrome. The term lupus anticoagulant (LA), used for the first time in 1972, derives from experimental observations in which it was observed an increased risk of thrombosis, paradoxically, with the presence of some anticoagulants factors; the term LA is not totally correct, in fact the disease is present more frequently in patients without lupus and it is associated with thrombosis rather than to abnormal bleeding. Some years later the role of a cofactor has been investigated, the β2-glycoprotein I (apolipoprotein H) also said β2GPI, and its interactions with anionic phospholipids in human serum / plasma. This cofactor is a β-globulin with a molecular weight of 50 kDa that has the concentration of 200 g / mL in plasma. The β2GPI is involved in the regulation of blood coagulation, inhibiting the intrinsic way. β2GPI in vivo is associated with negatively charged substances such as anionic phospholipids, heparin and lipoproteins. The region that binds phospholipids is in its fifth domain. The acronym "aPL" (anti-phospholipid antibodies) indicates improperly antibodies directed against phospholipids negatively charged like Cardiolipin (CL), Phosphatidyl serine (PS) Phosphatidyl inositol (PI) and phosphatidic acid (PA); more correctly the term anti-phospholipid antibodies indicate those antibodies directed against the complex between β2GPI and anionic phospholipids that can bind to the fifth domain of β2GPI. Among these, the Cardiolipin is the most commonly used phospholipid as an antigen for determining the aPL with ELISA method. Diagnostic laboratories measure the antibodies directed against the complex between β2GPI and negatively charged phospholipids, as Phosphatidyl serine (PS) Phosphatidyl inositol (PI) and phosphatidic acid (PA). Some researchers suggest the use of PS instead of Cardiolipin in ELISA assays, for a more precise diagnosis. However, these antibodies against phospholipids are less commonly used, even if their use may increase the clinical sensitivity of patients samples with suspected Anti-phospholipid Syndrome (APS), but it can’t replace the determination of autoantibodies anti-Cardiolipin.

2. INTENDED USE

Anti beta 2 Glycoprotein 1 IgG kit is an indirect enzyme-linked immunosorbent assay (ELISA) designed for the quantitative measurement of IgG class antibodies directed against the β2-Glycoprotein 1 in human serum or plasma. Anti beta 2 Glycoprotein 1 IgG kit is intended for laboratory use only.

3. PRINCIPLE OF THE ASSAY

Anti-β2-GP1 test is based on the binding of antibodies present in calibrators, controls or pre-diluted patient samples to the β2-GP1 coated on the inner surface of the microplate wells. After a 60 minutes incubation the microplate is washed with wash buffer to remove the non-reactive serum components. An anti-human-IgG horseradish peroxidase conjugate solution recognizes IgG class antibodies bound to the immobilized antigens. After a 30 minutes incubation excess enzyme conjugate, which is not specifically bound is washed away with wash buffer. A chromogenic substrate solution containing TMB is dispensed into the wells. After 15 minutes of incubation colour development is stopped by adding the stop solution. The solution turns yellow at this point. The level of colour is directly proportional to the concentration of IgG antibodies present in the original sample.

4. MATERIALS

4.1. Reagents supplied

- **Anti-β2-GP1 Standards Coated Wells:** 12 breakapart 8-well snap-off strips coated with Beta-2-Glycoprotein 1; in resealable aluminum foil.
- **Stop Solution:** 1 bottle containing 15 ml sulphuric acid, 0.15 mol/l (avoid any skin contact), ready to use
- **Conjugate:** 1 bottle containing 15 ml Anti h-IgG conjugate with horseradish peroxidase (HRP), BSA 0,1%, Proclin < 0,0015%
- **TMB Substrate Solution:** 1 bottle containing 15 ml 3,3’,5,5’-tetrachloro-3,3-diaminobenzidine 0,26 g/L, hydrogen peroxide 0,05%
- **Wash solution:** 1 bottle containing 50 ml (10x conc.) Phosphate buffer 0,2M, proclin < 0,0015%
- **Sample Diluent:** 1 bottle containing 100 ml Phosphate buffer 0,1M, NaN3 < 0,1%
- **Anti-Beta2-GP1 Standards:** 5 bottles, 1,2 ml each, ready to use
- **Standard:** 0: 0 AU/ml
Standard 1: 10 AU /ml
Standard 2: 20 AU /ml
Standard 3: 40 AU /ml
Standard 4: 160 AU /ml

**Positive Control:** 1 bottle containing 1.2 ml, Phosphate buffer 0.1M, NaN₃ < 0.1%, human serum, ready to use

**Negative Control:** 1 bottle containing 1.2 ml, Phosphate buffer 0.1M, NaN₃ < 0.1%, human serum, ready to use

### 4.2. Materials supplied

- 1 Strip holder
- 1 Cover foils
- 1 Test protocol
- 1 Distribution and identification plan

### 4.3. Materials and Equipment needed

- ELISA microwell plate reader, equipped for the measurement of absorbance at 450 nm
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 µl
- Vortex tube mixer
- Distilled water
- Disposable tubes
- Timer

### 5. STABILITY AND STORAGE

Store all the kit reagents at 2-8°C. Do not freeze. Reagents are stable until the expiration date when stored and handled as directed. Unused antigen coated microwell strips should be resealed securely in the foil pouch containing desiccants and stored at 2-8°C.

### 6. REAGENT PREPARATION

*It is very important to bring all reagents, samples and standards to room temperature (22…28°C) before starting the test run!*

#### 6.1. Coated snap-off Strips

The ready to use break apart snap-off strips are coated with Beta-2-Glycoprotein 1. Store at 2...8 °C. Open the bag only when it is at room temperature. Immediately after removal of strips, the remaining strips should be resealed in the aluminium foil along with the desiccant supplied and stored at 2...8 °C; stability until expiry date. Do not remove the adhesive sheets on the unused strips.

#### 6.2. Anti-Beta2 Glycoprotein 1 IgG Standards / control

Since no international reference preparation for Anti-β2-GP1 antibodies is available, the assay system is calibrated in relative arbitrary units. The standards are ready to use and have approximately the following concentrations:

<table>
<thead>
<tr>
<th></th>
<th>S0</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
</tr>
</thead>
<tbody>
<tr>
<td>AU/mL</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>40</td>
<td>160</td>
</tr>
</tbody>
</table>

**Positive Control:** 1 bottle containing 1.2 ml, Phosphate buffer 0.1M, NaN₃ < 0.1%, human serum, ready to use

**Negative Control:** 1 bottle containing 1.2 ml, Phosphate buffer 0.1M, NaN₃ < 0.1%, human serum, ready to use

#### 6.3. TMB Substrate Solution

The bottle contains 15 ml of 3,3′,5,5′-tetramethylbenzidine 0.26 g/L, hydrogen peroxide 0.05%. The reagent is ready to use and has to be stored at 2...8°C in the dark. *The solution should be colourless or could have a slight blue tinge. If the substrate turns into blue, it may have become contaminated and should be thrown away.*

#### 6.4. Stop Solution

The bottle contains 15 ml 0.15 M sulphuric acid solution (R 36/38, S 26). This ready to use solution has to be stored at 2...8°C.

#### 6.5. Wash Solution

Dilute the contents of each vial of the buffered wash solution concentrate (10x) with distilled water to a final volume of 500 ml prior to use. For smaller volumes respect the 1:10 dilution ratio. The diluted wash solution is stable for 30 days at 2-8°C. In concentrated wash solution it is possible to observe the presence of crystals. In this case mix at room temperature until complete dissolution of crystals is observed. For greater accuracy dilute the whole bottle of concentrated wash solution to 500 mL, taking care also to transfer the crystals completely, then mix until crystals are completely dissolved.
6.6. Sample Diluent
The bottle contains 100 ml Phosphate buffer 0.1M, NaN3 < 0.1%

6.7. Conjugate IgG:
The bottle containing 15ml Anti h-IgG conjugate with horseradish peroxidise (HRP), BSA 0.1%, Proclin < 0.0015%

7. SPECIMEN COLLECTION AND PREPARATION
For determination of Anti-β2-GP1 human serum or plasma are the preferred sample matrixes. All serum and plasma samples have to be pre-diluted with sample diluent 1 : 100. Therefore 10 L of sample may be diluted with 990 L of sample diluent. The patients need not to be fasting, and no special sample preparation is necessary. Collect blood by venipuncture into vacutainers and separate serum (after clot formation) or plasma from the cells by centrifugation. Samples may be stored refrigerated at 2-8°C for at least 5 days. For longer storage of up to six months samples should be stored frozen at -20°C. To avoid repeated thawing and freezing the samples should be aliquoted. Neither Bilirubin nor Hemolysis have significant effect on the procedure. The controls are ready to use.

8. ASSAY PROCEDURE

8.1. Test Preparation
Please read the test protocol carefully before performing the assay. Result reliability depends on strict adherence to the test protocol as described. Prior to commencing the assay, the distribution and identification plan for all specimens and controls should be carefully established on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder. Please allocate at least:
1 well (e.g. A1) for the substrate blank
2 wells (e.g. B1+C1) for standard 0
2 wells (e.g. D1+E1) for standard 1
2 wells (e.g. F1+G1) for standard 2
2 wells (e.g. H1+A2) for standard 3
2 wells (e.g. B2+C2) for standard 4
2 wells (e.g. D2+E2) for positive control
2 wells (e.g. F2+G2) for negative control

It is recommended to determine standards and patient samples in duplicate. Perform all assay steps in the order given and without appreciable delays between the steps. A clean, disposable tip should be used for dispensing each standard and each patient sample.

8.2. Test Procedure

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Standard</th>
<th>Sample or Controls</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard S0-S4</td>
<td>100 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>100 µL</td>
<td></td>
</tr>
<tr>
<td>Diluted Sample</td>
<td></td>
<td>100 µL</td>
<td></td>
</tr>
</tbody>
</table>

Incubate for 60 minutes at room temperature (22-28°C). Remove the content from each well, wash the wells three times with 300 µL of diluted wash solution.

Conjugate | 100 µL | 100 µL |

Incubate for 60 minutes at room temperature (22-28°C). Remove the content from each well, wash the wells three times with 300 µL of diluted wash solution.

TMB substrate | 100 µL | 100 µL | 100 µL |

Incubate for 15 minutes in the dark at room temperature (22-28°C).

Stop solution | 100 µL | 100 µL | 100 µL |
Shake the microplate gently. Read the absorbance (E) at 450 nm against Blank

9. RESULTS

For Anti-β2-GP1 a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is the data reduction method of choice. Smoothed-Spline Approximation and log-log coordinates are also suitable. However a Lin-Log plot is recommended. First calculate the averaged optical densities for each calibrator well. Use lin-log graph paper and plot the averaged optical density of each calibrator versus the concentration. Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.

Typical results (for example only) The table below shows typical results for Anti-β2 glycoprotein 1 IgG. The data are for illustration only and should not be used to calculate the results.

<table>
<thead>
<tr>
<th>N</th>
<th>OD1</th>
<th>OD2</th>
<th>mean</th>
<th>C1</th>
<th>C2</th>
<th>mean</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>STD0</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>STD1</td>
<td>0.297</td>
<td>0.302</td>
<td>0.300</td>
<td>9.91</td>
<td>10.09</td>
<td>10.00</td>
<td>1.22</td>
</tr>
<tr>
<td>STD2</td>
<td>0.588</td>
<td>0.598</td>
<td>0.593</td>
<td>19.83</td>
<td>20.17</td>
<td>20.00</td>
<td>1.22</td>
</tr>
<tr>
<td>STD3</td>
<td>1.101</td>
<td>1.141</td>
<td>1.121</td>
<td>39.16</td>
<td>40.85</td>
<td>40.01</td>
<td>3.00</td>
</tr>
<tr>
<td>STD4</td>
<td>2.501</td>
<td>2.390</td>
<td>2.446</td>
<td>171.6</td>
<td>148.5</td>
<td>160.1</td>
<td>10.22</td>
</tr>
</tbody>
</table>

9.1. REFERENCE VALUES

In a normal range study with serum samples from healthy blood donors the following ranges have been established with Anti-Beta2Glycoprotein 1 IgG test:

Anti-Beta2 Glycoprotein 1 IgG [AU/ml] Negative: < 20 Positive: > 20

Positive results should be verified concerning the entire clinical status of the patient. Also every decision for therapy should be taken on an individual patient basis. It is recommended that each laboratory establishes its own normal and pathological ranges of seric Anti-β2-GP1 IgG.

9.2. Precision and reproducibility

Intra-Assay

Within run variation was determined by replicate 12 times three different sera with values in the range of standard curve. The within assay variability is ≤7.0%.

Inter-Assay

Between run variation was determined by replicate the measurements of two different control sera with different lots of kits and/or different mix of lots of reagents. The between assay variability is ≤10.4%.

9.3. Sensitivity

Test against two commercial reference kits, performed on 41 sera (including 15 positive sera and 26 negative sera) showed a sensitivity of 100% (the first one) and of 82.3% (the second one).

9.4. Specificity

Test against two commercial reference kits, performed on 41 sera (including 14 positive and 27 negative) showed a specificity of 90% (the first one) and of 95.8% (the second one).

9.5. Detection limit

The lowest concentration of anti-beta 2 glycoprotein 1, which can be distinguished from zero standard is about 0.47 AU/mL with confidence limit of 95%.
10. Warnings and Precautions

WARNINGS This kit is intended for research use by professional persons only. Use appropriate personal protective equipment while working with the reagents provided. All human source material used in the preparation of standards and controls for this product has been tested and found negative for antibody to HIV 1&2, HbsAg, and HCV. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, the Standard and the Controls should be handled in the same manner as potentially infectious material. Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy and the bovine protein has been obtained from countries not infected by BSE, but these materials should be handled as potentially infectious. Some reagents contain small amounts of Sodium Azide (NaN3) or Proclin 300R as preservatives. Avoid the contact with skin or mucosa. Sodium Azide may be toxic if ingested or absorbed through the skin or eyes; moreover it may react with lead or copper plumbing to form potentially explosive metal azides. If you use a sink to remove the reagents, allow scroll through large amounts of water to prevent azide build-up. The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes. The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes. Avoid the exposure of reagent TMB/H2O2 to directed sunlight, metals or oxidants.

PRECAUTIONS Please adhere strictly to the sequence of pipetting steps provided in this protocol. All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. Allow all kit components and specimens to reach room temperature (22-28°C) and mix well prior to use. Do not interchange kit components from different lots. The expiry dates printed on the labels of the box and of the vials must be observed. Do not use any kit component beyond its expiry date. WARNING: the conjugate reagent is designed to ensure maximum dose sensitivity and may be contaminated by external agents if not used properly; therefore, it is recommended to use disposable consumables (tips, bottles, trays, etc.). For divided doses, take the exact amount of conjugate needed and do not re-introduce any waste product into the original bottle. In addition, for doses dispensed with the aid of automatic and semi-automatic devices, before using the conjugate, it is advisable to clean the fluid handling system, ensuring that the procedures of washing, deproteinization and decontamination are effective in avoiding contamination of the conjugate; this procedure is highly recommended when the kit is processed using analyzers which are not equipped with disposable tips. If you use automated equipment it is your responsibility to make sure that the kit has been appropriately tested. The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background. It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate. Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction. Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. Maximum precision is required for reconstitution and dispensation of the reagents. Samples microbiologically contaminated should not be used in the assay. Highly lipemic or haemolysed specimens should similarly not be used. Plate readers measure vertically. Do not touch the bottom of the wells.

11. BIBLIOGRAPHY


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Pengo V, Biasiolo A, Fior MG. Autoimmune antiphospholipid antibodies are directed against a cryptic epitope expressed when β2-glycoprotein 1 is bound to a suitable surface. Thromb Haemost 1995;73:29-34


SCHEME OF THE ASSAY

Anti-Beta2 Glycoprotein 1 IgG

Test Preparation

Prepare reagents and samples as described. Establish the distribution and identification plan for all specimens and controls on the resultsheet supplied in the kit.

Select the required number of microtiter strips or wells and insert them into the holder.

Assay Procedure

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