



EL- β 2GPI™ (IgM-IgG-IgA)

*An enzyme immunoassay for the detection and measurement of
IgM, IgG, and IgA isotypes of
anti-Beta-2-Glycoprotein I antibodies*

For professional use only

IVD

**For In Vitro Diagnostic Use
Instruction Manual**

REF

Catalog No.: 401-404

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INTRODUCTION

Name: TheraTest *EL-β2GPI*[™] (*IgM-IgG-IgA*)

Intended Use

FOR IN VITRO DIAGNOSTIC USE

The TheraTest EL-β2GPI[™] (IgM-IgG-IgA) is a set of in vitro diagnostic tests for the measurement of IgM, IgG, and IgA autoantibodies in human serum directed against serum Beta-2-Glycoprotein I (β2GPI). This measurement aids in the diagnosis of antiphospholipid syndrome (APS) or certain autoimmune thrombotic disorders such as those secondary to systemic lupus erythematosus.

Summary and Explanation of Test

Beta-2-Glycoprotein I (β2GPI) is a single chain polypeptide with an apparent molecular weight of 50 kD (non-reduced) and 70 kD (reduced). It is present in plasma at a concentration of about 200 μg/mL. β2GPI has been shown to inhibit the intrinsic blood coagulation pathway, ADP-mediated platelet aggregation, and prothrombinase activity of activated platelets.¹

Autoantibodies against phospholipids in general and cardiolipin in particular, have been associated with thrombosis, fetal wastage, inflammation of the central nervous system, thrombocytopenia, as well as some other manifestations. These have been generally lumped together as anti-phospholipid antibody syndrome (APS).^{2,3,4} Evidence has been accumulated that some antiphospholipid antibody molecules recognize cardiolipin whereas others recognize a complex formed by cardiolipin and a carrier protein, β2GPI.⁵⁻⁸ Further studies have shown that, in large part, antibodies recognize molecules of β2GPI at high density⁹ attached to an x-irradiated solid phase.¹⁰

Recent studies have shown that elevated levels of IgM and IgG anti-β2GPI antibodies may be more specific for the diagnosis of patients with APS than the elevated levels of anticardiolipin antibodies.^{4,11} In addition, a large proportion of patients with SLE were shown to have IgA anti-β2GPI which was correlated with the presence of APS manifestations¹² (see also Expected Values, below).

The distribution of immunoglobulin (Ig) heavy chain isotypes in APS patients may vary with ethnic background and disease manifestation. IgG anticardiolipin and IgG anti-β2GPI have been shown to have relatively high specificity for APS.¹³ For anticardiolipin antibodies, IgG has been found to be the dominant isotype in Hispanic and African American populations, whereas IgA is dominant in the Afro-Caribbean population. IgM anticardiolipin was found to be associated with hemolytic anemia in Spanish¹⁴ and Mexican¹⁵ patients with SLE. The IgA isotype of anti-β2GPI was shown to be prevalent in SLE patients with APS manifestations.¹² The presence of all three isotypes of anti-β2GPI has a higher specificity for APS than any isotype alone (see Guide to Interpretation below).

Method Description

The TheraTest EL-β2GPI Test is a solid phase enzyme immunoassay with a 96-well plate format for the measurement of anti-β2GPI antibodies. Isotype determinations can be performed simultaneously or individually. Pairs of wells (i.e., blank and β2GPI-coated) are incubated in parallel with Calibrator(s), diluted serum Specimens, and Positive and Negative Controls. During the incubation, the anti-β2GPI antibodies present in the test sample are bound to the solid phase antigen. The wells are washed and isotype-specific horseradish peroxidase-labeled anti-human immunoglobulin antibody (the enzyme conjugate) is added. After incubation of the wells with the enzyme conjugate, unbound labeled antibody is removed by aspiration and washing. A chromogenic substrate solution is then added and the presence of anti-β2GPI antibodies is detected by a color change read by an ELISA reader. The absorbance value in the control well (blank) is subtracted from the value obtained from the well coated with β2GPI.

REAGENTS

β 2GPI Coated Wells in 96-well format. For single use only! All wells are coated with β 2GPI. Wells are printed with name of the antigen. The unused wells and the frame may be stored and used at a later date. They are returned to their desiccant-containing pouch, carefully sealed in the plastic resealable pouch and stored at 2° - 8°C until the expiration date.

Blank Wells for β 2GPI. For single use only! The unused wells and the frame may be stored and used at a later date. They are returned to their desiccant-containing pouch, carefully sealed in the plastic resealable pouch and stored at 2° - 8°C until the expiration date.

Wash Buffer (10X) - Concentrated buffer with Tween 20.

Anti-IgM Enzyme Conjugate - Anti-Human IgM (Fc5 μ specific) conjugated with horseradish peroxidase; with preservative and dye (colored blue).

Anti-IgG Enzyme Conjugate - Anti-Human IgG (Fc γ specific) conjugated with horseradish peroxidase; solution with preservative and dye (colored green).

Anti-IgA Enzyme Conjugate - Anti-Human IgA (α -chain specific) conjugated with horseradish peroxidase with preservative and dye (colored red).

Chromogen - 3,3',5,5' tetramethylbenzidine in buffer with hydrogen peroxide.

Stop Reagent - 2M phosphoric acid.

β 2GPI Positive Control* - Human serum containing IgM, IgG, and IgA antibodies to β 2GPI and preservative. See enclosed Data Sheet for performance characteristics.

β 2GPI Negative Control* - Human serum containing preservative. See enclosed Data Sheet for performance characteristics.

β 2GPI Calibrators*: a) IgM; b) IgG; c) IgA- (**Ready-to-use**) Human serum, diluted in stabilizing buffer, containing IgM, IgG, *and/or* IgA antibodies to β 2GPI with preservative. See enclosed Data Sheet for performance characteristics.

* Reagents containing sodium azide

WARNINGS AND PRECAUTIONS

FOR IN VITRO DIAGNOSTIC USE

Reagents containing Human Source Material

CAUTION: Controls and Calibrators contain Human Serum. Treat as potentially infectious.

The materials used to prepare the Calibrator and Controls were derived from human blood. When tested by FDA-cleared methods for the presence of antibody to HIV (Human Immunodeficiency Virus) and Hepatitis B

Surface Antigen (HBsAg), the materials were nonreactive. Inasmuch as no test method can offer complete assurance that HIV, hepatitis virus or other infectious agents are absent, these materials and all patient specimens should be handled as though capable of transmitting infectious diseases. Human material should be handled in accordance with good laboratory practice using appropriate precautions as described in the Centers for Disease

Control and Prevention, "*Biosafety in Microbiological and Biomedical Laboratories*" 5th edition, Dec 2009. For Web site: Search "CDC BMBL 5th edition".

Chromogen

Irritant! This product contains 3,3',5,5'-tetramethylbenzidine (TMB) ($\leq 0.05\%$), a chromogenic indicator of horseradish peroxidase activity. It has shown neither mutagenic nor carcinogenic effects in laboratory experiments.¹⁷

Hazardous Substance Risk & Safety Phrases:

- R36/37/38 – Irritating to eyes, respiratory system, and skin. Avoid inhalation and direct contact.
- S24/25 – Avoid contact with skin or eyes.
- S26 – In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
- S36 – Wear suitable protective clothing.
- S51 – Use only in well-ventilated areas.

Reagents Containing Sodium Azide

Calibrators and Controls contain sodium azide which can react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush drain with large quantities of water to prevent azide build-up.

Hazardous Substance Risk & Safety Phrases:

- R22 - Harmful if swallowed.
- R36/37/38 - Irritating to eyes, respiratory system, and skin. Avoid inhalation and direct contact.
- S26 - In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
- S28 - After contact with skin, wash immediately with plenty of water.
- S36/37/39 - Wear suitable protective clothing, gloves and eye/face protection
- S46 - If swallowed, seek medical advice immediately and show this container label.

Stop Reagent (2M Phosphoric Acid)

May cause severe burns upon contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes.

Hazardous Substance Risk and Safety Phrases

- R34 - Causes burns.
- S26 - In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
- S36/37/39 - Wear suitable protective clothing, gloves, and eye/face protection.
- S45 - In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).

General Precautions and Information

1. Wash Buffer, Chromogen, and Stop Reagent are interchangeable among the $\beta 2$ GPI kits. All other reagents are kit and lot specific and therefore not interchangeable.
2. The Stop Reagent can irritate eyes and mucous membranes.
3. Do not allow Chromogen to come in contact with metal or oxidizing agents.
4. Handle patient sera and kit reagents with appropriate precautions. Do not pipette by mouth.
5. Work in a well ventilated area when using kit reagents.
6. Do not use test components beyond expiration date.
7. Avoid microbial contamination of the reagents. If solutions become turbid, they should not be used.
8. Avoid exposure of reagents to excessive heat or light during storage.
9. Use disposable labware or wash all glassware and plasticware thoroughly according to standard laboratory practice.
10. Dispose of containers and unused kit reagents in accordance with all local, state and federal regulatory requirements.

STORAGE AND HANDLING



1. Store all reagents at 2° - 8°C when received. Avoid freezing reagents.
2. All reagents must be brought to room temperature (18 - 25°C) for 30 minutes prior to use.
3. Avoid subjecting reagents to direct sunlight.
4. When stored at 2° - 8°C, the diluted Wash Buffer is stable for 8 weeks.

SPECIMEN REQUIREMENTS

Collection and Storage of Serum

No special preparation of the patient is required. A whole blood specimen should be obtained (in a red top, serum separating tube) using accepted medical techniques to avoid hemolysis. The blood should be allowed to clot and the serum separated by centrifugation. Unseparated blood can be stored at 18 – 25°C for 24 hours before serum separation. The test serum should be clear and non-hemolyzed. Serum samples may be stored at 2 – 8°C for up to 14 days prior to testing. If testing cannot be completed within 14 days of collection, the separated serum must be stored at -20°C. Allow serum to equilibrate to room temperature (18 – 25°C) prior to testing. Do not use serum that has been thawed more than once or that has been heat inactivated.

TEST PROCEDURE

Before starting the assay, read the Instructional Manual carefully. Instructions should be followed exactly as they appear in this Instruction Manual to ensure valid results.

A. Materials provided

<u>Item</u>	<u>Component</u>	<u>No. / Vol.</u>
1	β2GPI Coated Wells	2 plates
2	Blank Wells for β2GPI	2 plates
3	Wash Buffer (10X)	2 x 100 mL
4	Anti-IgM Enzyme Conjugate	1 x 27 mL
5	Anti-IgG Enzyme Conjugate	1 x 27 mL
6	Anti-IgA Enzyme Conjugate	1 x 27 mL
7	Chromogen	2 x 27 mL
8	Stop Reagent	2 x 27 mL
9	β2GPI Positive Control (IgM, IgG, IgA)	1 x 0.35 mL
10	β2GPI Negative Control	1 x 0.35 mL
11	β2GPI IgM Calibrator	1 x 1.5 mL
12	β2GPI IgG Calibrator	1 x 1.5 mL
13	β2GPI IgA Calibrator	1 x 1.5 mL
14	Resealable plastic pouch	1

B. Materials required but not provided

1. Calibrated adjustable micropipettors:

- a. multichannel pipettor, 12 channels, 50-300 μL ($\pm 5\%$)
- b. single channel micropipettor, 5-50 μL ($\pm 5\%$)
- c. single-channel micropipettor, 200-1000 μL ($\pm 5\%$)
- d. 8-channel repeat pipettor, 50-200 μL (10%) - for plate washing (optional)
2. Pipette tips (100 – 1000 μL , 200 – 1000 μL , 1 – 200 μL , plugged)
3. Glass or plastic pipettes (1-mL, 5-mL, and 10-mL)
4. 1.2-mL minitubes (cluster tubes or dilution tubes)
5. Interval timer
6. Multichannel pipette reagent reservoirs
7. Single (450 nm) or dual (450 nm test, 620 – 690 nm reference) wavelength spectrophotometer (ELISA reader) for 96-well microtiter plates, capable of linear absorbance reading from 0.02 to 2.5.
8. Deionized water (resistivity $>1\text{M}\Omega$) or purified water for irrigation, USP
9. Clean wash bottle
10. Automated plate washer (optional)
11. 1-L graduated cylinder (Class B or better)
12. Absorbent paper towels for blotting washed plates

C. Reagent preparation:

1. **1X Wash Buffer** - The 10X Wash Buffer must be diluted 1:10 prior to use. Prepare 1X Wash Buffer by pouring the contents of the 10X wash Buffer into a clean one liter volumetric container. Rinse the bottle with deionized or distilled water to remove residual buffer. Add the rinse to the one liter container. Add deionized or distilled water until a total volume of 1.0 liter is reached; mix thoroughly. When stored at 2 – 8°C, the diluted Wash Buffer is stable for 8 weeks. The 1X Wash Buffer is used to dilute samples (i.e., as Specimen Diluent) and to wash microwells. **Note: Prior to use, check the 10X Wash Buffer for crystals formed during storage in the cold. If crystals are present, warm the bottle until the crystals dissolve. Failure to take this precaution will cause test failure.**
2. **Chromogen** - Disposable glassware or plasticware should be used to dispense the Chromogen. Alternatively, all labware employed must be washed thoroughly according to standard laboratory practice. The Chromogen should be pipetted into the reagent reservoir (an excess of 10% of the necessary amount should be measured) no sooner than 5 minutes prior to use. Discard any unused Chromogen after completion of the test procedure. Do not pour excess Chromogen back into the stock bottle.
3. **Specimens, Positive Control, and Negative Control** - The Specimens and Controls must be diluted 1:101 prior to use. Pipette 10 μL of the appropriate Specimen or Control into 1 mL of Specimen Diluent (i.e., 1X Wash Buffer). Unused diluted Controls and Specimens should be discarded after assay completion. The acceptable range for each Control is reported on the enclosed Data Sheet.
4. **$\beta 2\text{GPI}$ Calibrators** – All Calibrators are pre-diluted and ready for use. Do not dilute further.
5. **$\beta 2\text{GPI}$ Coated Wells and Blank Wells for $\beta 2\text{GPI}$** – The entire strip of break-apart wells may be employed or individual wells may be used. An example for distribution of the antigen-coated wells and blank wells is shown on the Data Sheet. For each specimen tested, place one antigen-coated well and one blank well in the frame for each isotype determination.

D. Assay procedure

1. Allow all reagents and patient sera to come to room temperature prior to use (18 – 25°C).
2. Calibrator(s), Positive Control, and Negative Control must be included with all assays.

3. Select the appropriate plate format; mark the position of the samples (i.e., Calibrator(s), Positive Control, Negative Control, and Specimens) on a worksheet. See **suggested plate layouts in the Data Sheet and Fig. 1.**

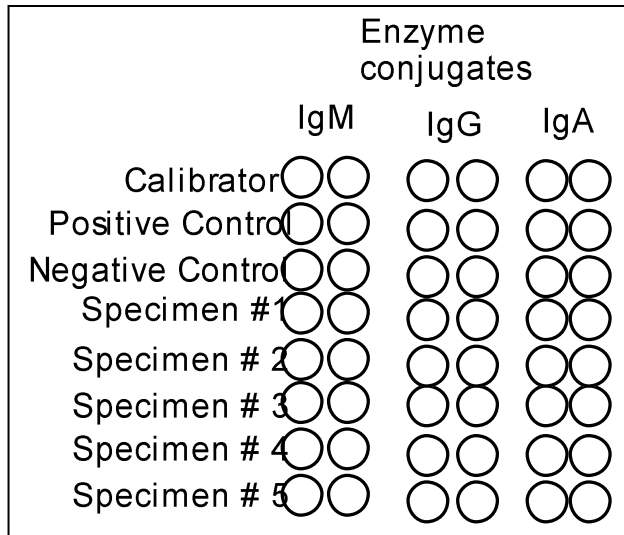


Fig. 1. Arrangement of Calibrator(s), Positive Control, Negative Control, and Specimens

4. Determine the number of antigen-coated and blank wells needed. Open the metallized pouches and remove the appropriate number of wells. Return any remaining wells to the pouches with the desiccant; carefully reseal in the resealable plastic pouches and refrigerate. Antigen-coated wells are labeled (B2) and specimen blank wells are labeled (B2BL). Assemble the plate with one column of antigen (B2) wells and next to it a column of blank (B2BL) wells. See suggested plate layout diagram in the Data Sheet.
5. Dilute all Specimens, and Controls (but not the Calibrator(s)) 1:101 (e.g., 10 μ L into 1 mL) in 1X Wash Buffer and mix well. Pipette 100 μ L of Calibrator(s) (as supplied), 100 μ L of each of the diluted controls and samples into each well of a pair (antigen-coated and Specimen Blank). For best results pipette all samples within 5 minutes of the start of the assay. This step is facilitated by the use of multichannel or repeat pipettes.
6. Incubate the plate for 30 – 35 minutes at room temperature (18 - 25°C).
7. For manual washing of the plate, remove the sample fluid from the wells by aspiration or by shaking the liquid from the plate into a designated disposal container or sink. Fill all wells with 1X Wash Buffer (250-350 μ L per well) but do not overflow wells. Remove liquid from wells and repeat the washing procedure 2-3 more times for a total of 3-4 washes. For washing the plate(s) with automated or semi-automated instruments, follow their accompanying instructions. Whether washing is done manually or with an automated washer, remove all residual fluid from the wells after the last wash by blotting the inverted plate on absorbent paper.
8. Pipette 100 μ L of the isotype-appropriate Enzyme Conjugate into each well of matched isotype designation. Complete this step within 5 minutes for each plate.
9. Incubate plate(s) for 30 – 35 minutes at room temperature (18 - 25°C).
10. Aspirate or decant the Enzyme Conjugates from all wells and wash the plate as in Step 7 above.
11. Dispense 100 μ L of Chromogen into each well. Incubate the plate for 15 (\pm 1) minutes at room temperature (18 - 25°C). The β 2GPI-coated wells, incubated with the Positive Control or the

Calibrator(s), will develop a blue color.

12. Pipette 100 μ L of Stop Reagent into each well and mix by gently tapping the side of the plate. The blue color changes to yellow.
13. Firmly seat the microtiter plate in the reader and measure absorbance of each well at 450 nm. Absorbance values should be read within 30 minutes of completing the assay. If a dual wavelength ELISA reader is used, set the test wavelength at 450 nm and the reference at 620 – 690 nm. Follow the instructions provided with your instrument.

E. Procedural Comments

1. **Storage and Handling of Microwells** - The wells that are not used during the assay should be promptly placed in the metallized pouch with desiccant, sealed in the resealable pouch, and stored at 2° - 8°C. Wells stored in a previously opened pouch should be used within 3 months. To avoid false positive readings, the bottom of the microwells should be kept clean at all times.
2. **Crystallization of Reagents** - The Wash Buffer (10X), may crystallize at 2° - 8°C. **Important:** *Inspect the bottle for crystals prior to dilution. If crystals are present, warm the reagent at 37°C until crystals dissolve.*
3. **Washing** - Each column of wells may be washed using a multichannel pipettor or a repetitive pipettor. The wells may be aspirated or decanted into an appropriate disposal container or sink. Alternatively, commercial semi-automated washing systems may be used by following the manufacturer's instructions. Irrespective of the method, blot the wells on absorbent paper after the final wash. **Note:** *Insufficient washing may elevate background giving imprecise test results.*
4. **Pipetting** - To avoid cross-contamination and sample carry-over, Calibrator(s), Positive Control, Negative Control, and patient Specimens **must** be pipetted with separate pipette tips. When testing multiple Specimens, a multichannel pipettor should be used to pipette the Specimens, Enzyme Conjugates, 1X Wash Buffer, Chromogen, and Stop Reagent. Avoid cross-contamination of these reagents by using separate tips also.
5. **Plate setup** – To minimize confusion that can result from detachment of the strips from the frame, write a symbol that identifies the nature of the strip on its textured tab before starting the assay.
6. **Measurement of Absorbance Values** - Absorbance values should be measured within 30 minutes after completion of an assay. If the absorbance value exceeds the limit of detection for the instrument, an approximate value may be obtained by one of two methods:
 - a. Remove 100 μ L from the well and its corresponding Specimen Blank and add 100 μ L of deionized or distilled water to each; determine the net absorbance value and then multiply that value by 2. This dilution procedure may be repeated. **LIMITATION:** Patient antibody levels may exceed the available antigenic sites of the well.
 - b. Repeat the assay testing the Specimen at a 1:1010 dilution (or greater dilution) in Specimen Diluent. Multiply the measured Unit value by the additional dilution factor beyond 1:101 (i.e., Unit value x 10 for a 1:1010 dilution). **LIMITATION:** Approximation of measurement is subject to the intrinsic nonlinearity of (diluted) serum antibodies. The absorbance values obtained from this dilution procedure are only approximations of antibody bound to the well.

QUALITY CONTROL

1. **Specimen Blanks:** If the Specimen Blank absorbance values for Calibrator(s), Positive Control, Negative Control, and for multiple test Specimens exceed 0.4, the assay should be repeated. If the IgG Specimen Blank absorbance value obtained for any given patient Specimen exceeds 0.4, the given Specimen should be retested. If upon repeat testing, the IgG Specimen Blank absorbance value is again above 0.4, the patient Specimen may be abnormal with respect to IgG (e.g.,

hypergammaglobulinemia). **If a more definitive explanation is needed, please contact the manufacturer.** Inappropriately stored Specimens may have relatively high blanks.

2. **Calibrators:** The Calibrator(s) should have net absorbance values that are within the ranges specified on the **Data Sheet**. If the values obtained fall outside the ranges provided, the entire run should be discarded and the test repeated.

2. Positive and Negative Controls: Positive and Negative Controls **must** be run each time the assay is performed. In order to validate the assay, the Positive and Negative Control Unit values must be within the ranges stated on the enclosed **Data Sheet**. When the results for Positive and Negative Controls are not within the specified ranges, the run is repeated.

Previous runs of the same lot should be reviewed to determine if a shift or a sustained change (e.g., downward trend) in values has occurred. (See Troubleshooting.)

4. **Patient Specimens:** If the net absorbance value for a Patient Specimen exceeds the linear range of the ELISA reader (usually 2.0 – 2.5 OD), retest by further diluting the Specimen 1:10 (final dilution = 1:1010) and repeat the test procedure. To obtain Units/mL, use the following formula: (*See below for an explanation of the Conversion Factor.*)

Conversion Factor x Net Abs (10-fold diluted Specimen) x 10 = Units/mL in Specimen

RESULTS

Most ELISA readers are computer compatible and data may be calculated with the help of computer programs. Check periodically that the program chosen yields the same results as obtained by manual calculations.

Calculation of Results

1. Determination of Net Absorbance Values:

The net absorbance for Calibrators, Positive Controls, Negative Controls, and Specimens is calculated by subtracting the absorbance value of the blank well (R) from the absorbance value of the antigen-coated well (L). Each test well (β 2GPI IgM, IgG, IgA) has a separate Specimen Blank well. The absorbance value of the Specimen Blank well is subtracted from the absorbance value of the test well to obtain the net absorbance.

Example:

Absorbance of β 2GPI antigen-coated well (on Left) = 1.150

Absorbance of corresponding Blank well (no antigen coating, on Right) = 0.150

Net Absorbance of anti- β 2GPI = 1.150 – 0.150 = 1.000

Note: If the absorbance in the Blank well is higher than in the β 2GPI-coated well, the net absorbance should be considered zero.

2. Calculation of anti- β 2GPI antibody activity:

- a) The calculation of **X** Units of antibody in a Specimen is based on the following principle:

$$\frac{\text{Units/mL in Calibrator}}{\text{Net Absorbance of Calibrator}} = \frac{\text{X Units/mL in Specimen}}{\text{Net Absorbance of Specimen}}$$

- b) From this formula a Conversion Factor (CF) is calculated.

Note: *The CF is Ig class specific. Calculate separate CF(s) for IgM, IgG, and IgA anti- β 2GPI antibody activities.*

$$\text{CF} = \frac{\text{anti-}\beta\text{2GPI Units/mL in Calibrator}}{\text{Net Absorbance of Calibrator}}$$

(Net Absorbance value of Calibrator)

- c) The CF can then be used to calculate the Units/mL in each Specimen, as follows:

$$\text{CF} \times \text{Net Abs. of Specimen} = \text{Units/mL in Specimen.**}$$

- d) The Conversion Factors for the IgM, IgG, and IgA autoantibody Units/mL must be calculated each time the Calibrator is used for testing.

**If the net absorbance value of a Specimen is equal or higher than 2.0, it should be diluted at least an additional 1/10 and retested. Otherwise the result should be reported as >n Units/mL where n has been calculated with a Net Absorbance of 2.0.

Limitations of the Procedure

1. Diagnosis should not be made solely on the basis of a positive test result. The results must be interpreted in conjunction with all clinical information available to the physician (i.e., history, physical exam, and other diagnostic procedures).
2. Treatment of a patient should not be initiated solely on the basis of a positive EL-β2GPI test. Supportive clinical information should be available.
3. Patients with a positive syphilis serology including the fluorescence test may also show a false positive test result for anti-β2GPI antibodies. Syphilis serology should be known if a Specimen is abnormal.
4. As for any indirect solid phase immunoassay, rheumatoid factor of any isotype may react with IgG attached to the solid phase in the form of anti-β2GPI antibody. However, if IgG anti-β2GPI is not present, rheumatoid factor does not react with the antigen-coated wells. Rheumatoid factors IgM and IgA may mask the presence of anti-β2GPI IgG antibody and may cause it to appear as IgM and/or IgA antibody instead of IgG.
5. The individual laboratory should verify normal values for different isotypes, since the normal values may vary with age and ethnic groups. However, abnormal values in elderly should not be attributed only to age.¹⁶

EXPECTED VALUES

Sera from 100 random blood bank donors were tested. The following upper limits of anti-β2GPI Units/mL, for normals, were established for each Ig isotype. A cut-off at 98-99 percentile was used for the “normal” population.

Anti-β2GPI IgM: 4 β2GPI Units/mL

Anti-β2GPI IgG: 25 β2GPI Units/mL

Anti-β2GPI IgA: 4 β2GPI Units/mL

Because no national or international Standard(s) exist for anti-β2GPI antibodies, TheraTest based its Units on an anticardiolipin standard provided by the Anti-Phospholipid Standardization Laboratory (Atlanta, Georgia). The Units were set such that the net absorbance for a Unit of anti-β2GPI (on an Ig-isotype-specific basis) approximates the net absorbance for a Unit of anti-cardiolipin antibody (on an Ig-isotype-specific basis) with use of the anticardiolipin Standard.

Specimens should be considered in the “gray” area of positive range as follows: IgM, 5 - 10 Units/mL, IgG, 25 - 40 Units/mL, and IgA, 5 - 6 Units/mL). These results should be reported as borderline positive

and the clinician is advised to consider repeating the test in 4-8 weeks. Abnormal levels are considered: > 10 IgM anti-β2GPI Units/mL, > 40 of IgG anti-β2GPI Units/mL or > 6 of IgA anti-β2GPI Units/mL. See **Table 1, Reference Ranges**.

Table 1. Reference Ranges for Serum anti-β2GPI

	IgM	IgG	IgA
Normal	≤ 4	≤ 25	≤ 4
Borderline Positive	5 - 10	26 - 40	5 - 6
Abnormal	≥ 11	≥ 41	≥ 7

GUIDE TO INTERPRETATION

The expected clinical sensitivity of the TheraTest EL-β2GPI for APS is shown in **Tables 2 and 3**. Previous studies have shown sensitivities of IgM anti-β2GPI and IgG anti-β2GPI within the same ranges as reported here, with specificities of >80% for APS.¹³ The sensitivity for APS in SLE patients with severe manifestations of the syndrome is >90%, when IgA-anti-β2GPI is also considered along with IgM- and IgG- anti-β2GPI.¹² On 100 specimens from patients expected to have anti-phospholipid syndrome there was a highly significant correlation and a large overlap between the levels of anticardiolipin and anti-β2GPI (**Figs. 2 and 3**) (p<0.001 for all three isotypes).

The values for sensitivity, specificity and agreement expressed in **Table 2** were calculated as follows. For calculation of disease sensitivity of the EL-anti-β2GPI test for APS, all patients in the study that met the usual definition of antiphospholipid syndrome (N=87) (i.e., clinical manifestation(s) plus anti-cardiolipin antibodies¹¹) were considered as truly diseased (APS). Specificity of the test for APS was calculated with two sets of controls: a) blood bank donors (N=100) and b) disease controls (N=114) (i.e., patients with collagen vascular diseases: [50 with rheumatoid arthritis] + [44 with scleroderma] + [20 with SLE without manifestations of APS]). The agreement was calculated by considering the truly diseased (i.e., APS) as 100% of all patients that had clinical manifestations of antiphospholipid syndrome and at least one of the two antibody specificities, either anticardiolipin or anti-β2GPI (N=105). The best sensitivity (88%) and agreement (85%) values were obtained when the specimen was positive for at least one isotype (any isotype). The best specificity was observed when the specimen was positive for all three isotypes (M+G+A together) and the patients were compared to normal donors (100% specificity) or disease controls (98% specificity). A high level of antibody (vs borderline) was also important in providing a relatively high specificity (**Table 2, col.7**).

Table 2. Sensitivity, specificity and agreement for APS of anti-β2GPI test

	2	3	4	5	6	7	8	9
Parameter	IgM	IgG	IgA	Any iso-type	Any two iso-types	Abnormal level any isotype*	col. 7 and/or col. 9	M+G +A together**
Sensitivity	58	72	45	88	55	67	75	37
Specificity (BBD)	98	99	97	96	99	99	99	100
Specificity (Disease Controls)	85	95	92	76	96	94	93	98
Agreement	75	84	51	85	N/A	N/A	N/A	N/A

* Abnormal level is considered: ≥ 10 IgM anti-β2GPI U/mL, ≥ 40 IgG anti-β2GPI U/mL or ≥ 6 IgA anti-β2GPI U/mL

Table 3. Percentages of positive samples for IgM, IgG, and IgA anti-β2GPI antibodies
(See Table 2 for disease sensitivity and specificity.)

Diagnosis	Number Tested	Anti-β2GPI % Positive			
		IgM	IgG	IgA	IgM or IgG or IgA (any isotype)
Blood bank donors	100	2	1	1	4
Syphilis	86	9	12	9	18
Rheumatoid Arthritis	50	14	6	12	20
Scleroderma	44	11	4	0	14
APS clinical	100	48	59	36	72
SLE	48	27	25	58	73
SLE with record of APS	28	25	25	75	82
SLE with record of severe APS*	17**	29	35	81	94

*APS= antiphospholipid syndrome; the sensitivity of anticardiolipin antibody test for the same group was 78% (for details see also **Fig. 3** below).

**The 17 patients were a subset of the 28 patients in the line above with SLE and record of APS.

Antibodies against β2GPI may be found in patients with collagen vascular diseases other than APS (**Table 3**). They are found, although with less frequency than anticardiolipin, in patients with syphilis. Thus, syphilis serology should also be known in patients positive for anti-β2GPI. Anti-β2GPI may be found as one Ig isotype alone or as combinations of two or three isotypes (**Table 4**). The majority of APS patients tested had both anticardiolipin and anti-β2GPI antibodies (**Table 5**).

Table 4. Distribution of single isotypes and combination of

isotypes in 148 patient specimens: 100 with antiphospholipid syndrome and 48 with SLE.

Isotype of antibodies identified	Number positive for anti- β 2GPI	Number positive for anti- β 2GPI with >2 x normal limit
IgM only	15	5
IgG only	24	13
IgA only	17	11
IgM+IgG	12	N/A
IgM+IgA	2	N/A
IgG+IgA	6	N/A
IgM+IgG+IgA	32	N/A

N/A: not applicable

Table 5. Relationship between the positive and negative test results with anticardiolipin and anti- β 2GPI (TheraTest EL-aCL and TheraTest EL- β 2GPI) for IgM, IgG, and IgA isotypes separately and for any isotype (IgM, IgG, and /or IgA) in 140 specimens (patients with APS and normals).

IgM		EL-aCL	
		Positive	Negative
EL- β 2GPI	Positive	45	7
	Negative	7	81
IgG		EL-aCL	
		Positive	Negative
EL- β 2GPI	Positive	58	1
	Negative	10	71
IgA		EL-aCL	
		Positive	Negative
EL- β 2GPI	Positive	21	17
	Negative	6	96
Any isotype		EL-aCL	
		Positive	Negative
EL- β 2GPI	Positive	67	8
	Negative	12	53

Table 6. Relative sensitivity, specificity, and agreement between anti- β 2GPI

and anti-cardiolipin (TheraTest EL- β 2GPI™ and EL-aCL™) calculated on 140 specimens (from patients with APS and normals) tested in parallel for the two antibodies. The results obtained with the anticardiolipin test were used to categorize patients as truly diseased (APS). (Data calculated from Table 5 above.)

Parameter	IgM (%)	IgG (%)	IgA (%)	Any isotype (%)
Sensitivity	86	85	78	85
Specificity	91	99	85	87
Agreement	90	92	84	87

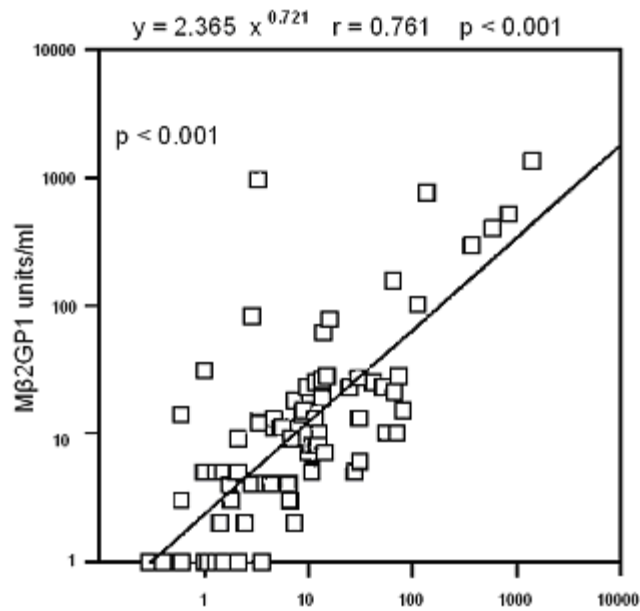


Fig 2a IgM aCL Units/mL

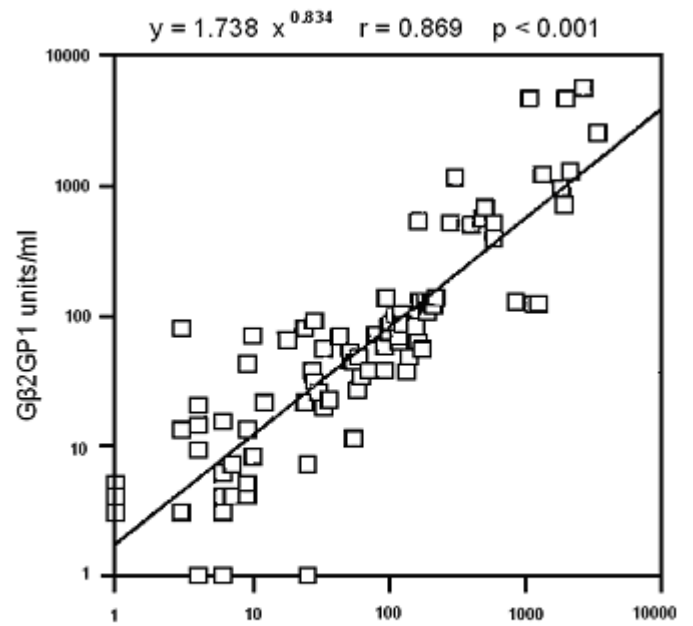


Fig 2b IgG aCL Units/mL

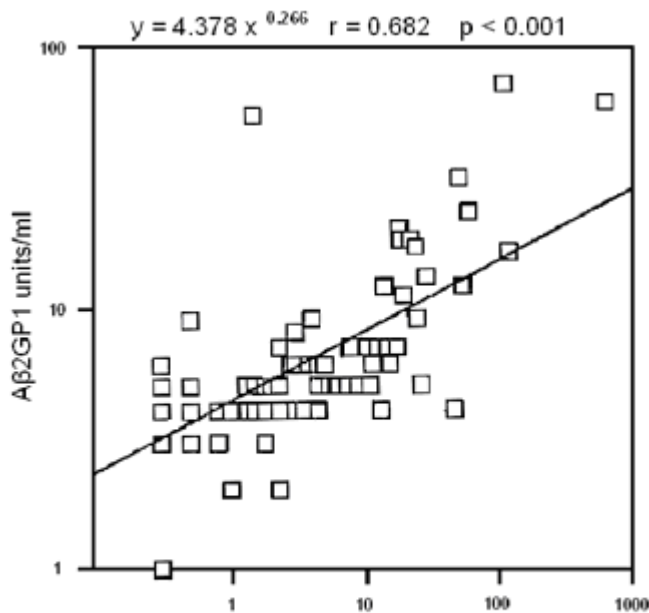


Fig 2c IgA aCL Units/mL

Fig. 2 a,b,c: The correlation between the levels of anti-β2GPI (EL-β2GPI, single point calibration) and anticardiolipin [aCL] antibodies (TheraTest EL-aCL, standard curve) in specimens from 100 patients with manifestations of antiphospholipid syndrome (1 aCL Unit \approx 1 β2GPI Unit).

Note: Mβ2GPI = IgM anti-β2GPI, Gβ2GPI = IgG anti-β2GPI, and Aβ2GPI = IgA anti-β2GPI.

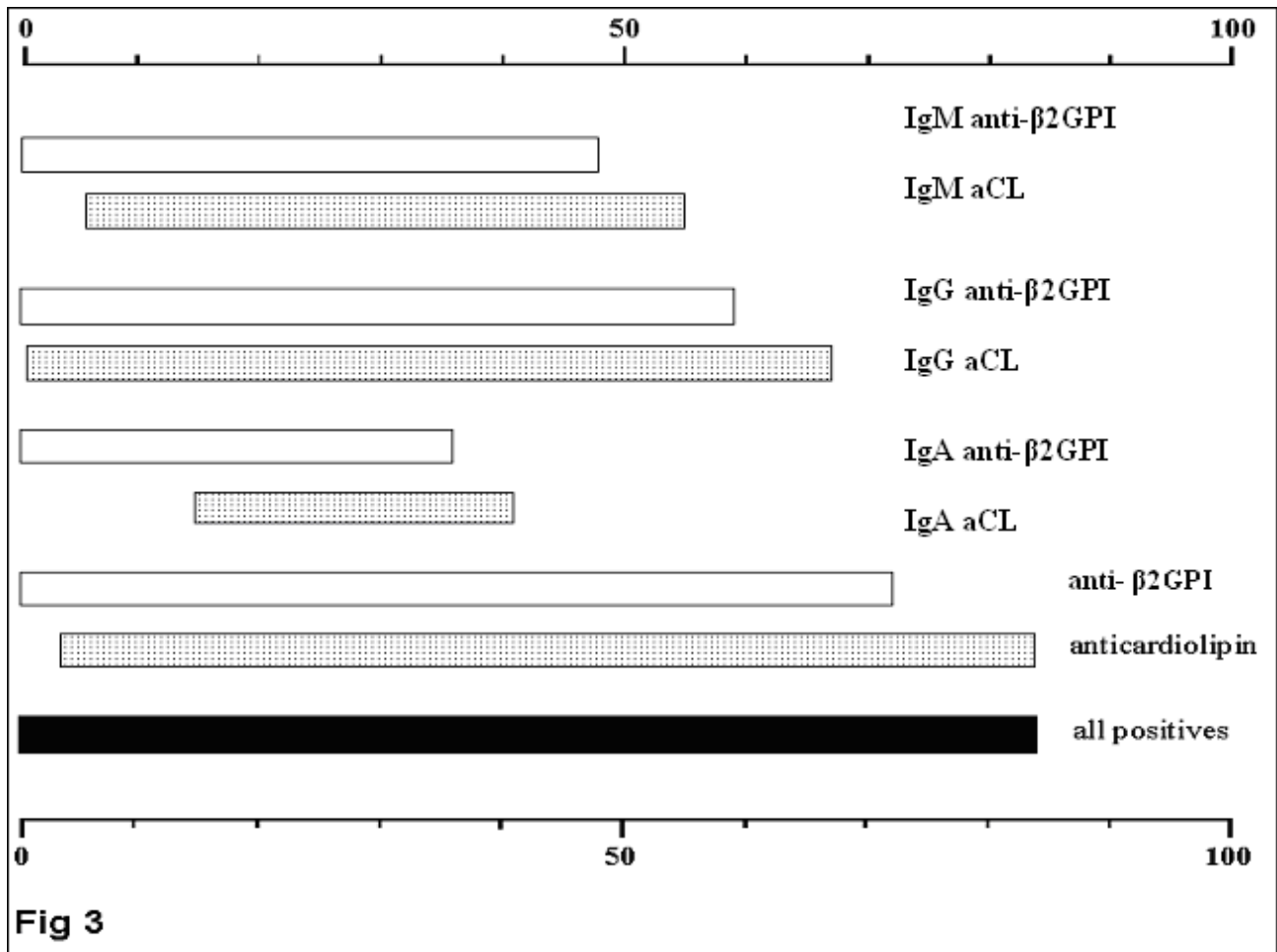


Fig. 3: The relationship between specimens with abnormal levels of anticardiolipin antibodies and/or anti-β2GPI in 100 specimens. Note the large overlap between the results obtained with the two assays as well as the existence of specimens deemed to be normal by only one of the two assays.

PERFORMANCE CHARACTERISTICS

Within-Run Precision:

Within-run precision was determined by assaying a specimen containing high levels of autoantibodies in 42 pairs of wells for each antibody class. The coefficient of variation for IgM, IgG, and IgA anti-β2GPI was found to be 6%, 11% and 11%, respectively.

Between-Run Precision:

Between-run precision was determined by assaying specimens containing high and low levels of autoantibodies in 20 separate runs. The coefficient of variation was found to be: IgM 5%; IgG <9% and IgA <11%.

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TROUBLESHOOTING

Problem	Possible Causes	Solution(s)
OD values of Specimen Blanks are >0.4.	<ol style="list-style-type: none"> 1. Chromogen may be contaminated. 2. Improper storage or aging of Specimen. 	<ol style="list-style-type: none"> 1. Take OD of Chromogen + Stop Reagent; if OD >0.1, discard Chromogen, use new bottle & repeat test. 2. Check storage conditions for reagents.
Single Specimen has high Specimen Blank for IgG.	<ol style="list-style-type: none"> 1. Specimen may have very high level of IgG. 	<ol style="list-style-type: none"> 1. Check IgG levels
Calibrator OD values out or range.	<ol style="list-style-type: none"> 1. Incorrect incubation temperature or timing. 2. Wrong Calibrator was used or placed in the wrong well. 3. Calibrator was diluted. 4. Wavelength of filter incorrect. 5. Contamination of Calibrator in well. 6. Cal Blank OD out of range. 	<ol style="list-style-type: none"> 1. Check that temperature was correct. Check that time was correct. See "Poor Precision" below. No. 2-4. Repeat test. 2. Repeat test. Pay close attention to instructions. 3. Repeat test. Calibrator is pre-diluted and ready-to-use. 4. Change filter to 450 ± 5 nm. 5. Repeat test; pipette carefully. 6. a) an excess volume of Conjugate was added to the Calibrator wells. b) the incubation time was too long. c) Insufficient washing. d) a damaged/dirty well; clean optical surface; reread. e) Chromogen is contaminated; replace.
OD value for Calibrator &/or Unit value for Positive Control (PC) show a sudden downward shift on their longitudinal graphs.*	<p>A sudden downward shift suggests:</p> <ol style="list-style-type: none"> 1. Improper filter selection for ELISA reader 2. A reduced volume or improper dilution of sample or one of the reagents. 3. A lower than usual incubation temperature 4. A shortened incubation time. 5. Poor quality water for Wash Buffer 	<ol style="list-style-type: none"> 1. Change filter to 450 nm for the ELISA reader; reread plate if time elapsed since development is <30 min. 2. Review sample & reagent volumes used for the test; make corrections, if needed, and rerun test. 3. Check that room temperature (RT) is between 20° & 25°C, and that reagents are warmed to RT. 4. Assure that the timer functions properly and that timed interval settings are correct. 5. Use bottled sterile water for irrigation, USP. <p>Note: For errors other than wrong filter, repeat the test.</p>
OD values for Calibrators and Unit values for PC show downward trends on their longitudinal graphs.*	<p>A downward trend of either or both sets of values suggests possible reagent decay (antigen-coated wells, Calibrator, PC, Conjugate and/or Substrate)</p>	<p>Assure that microwells and reagents are properly stored at 2° - 8°C. Check empty microwells for condensate and if present, contact manufacturer. Consider decay of Calibrator or PC if only one reagent shows downward trend. If OD <u>and</u> Unit values are within acceptable ranges for Calibrators and PCs, accept test results. Use new reagent(s) and repeat the test if a reagent error is suspected.</p>
OD values for Calibrators and/or Unit values for PCs show sudden upward shifts on their longitudinal graphs.	<p>A sudden upward shift suggests:</p> <ol style="list-style-type: none"> 1. An increased volume of the sample or one of the reagents. 2. A greater than usual incubation temperature 3. A lengthened incubation time. 4. Improper mixing of reagents. 5. Contamination of the well with 	<ol style="list-style-type: none"> 1. Review sample and reagent volumes used for the test; make corrections, if needed, and rerun test. 2. Check that Room Temperature (RT) is in 18° - 25°C range; adjust if necessary. 3. Assure that the timer functions properly and that timed interval settings are correct. 4. Mix all reagents before use.

	Conjugate.	5. Repeat test.
		Note: Rerun assay after corrections are made.
Positive and/or Negative Control Unit values out of range.	<ol style="list-style-type: none"> 1. Incorrect temperature or timing of incubations. 2. Incorrect volumes of reagents pipetted. 3. Reagents not mixed. 4. Cross-contamination of Controls 5. Optical pathway not clean. 	<ol style="list-style-type: none"> 1. Check that incubation temperature & timing were correct. Rerun assay. 2. Assess accuracy/precision of pipettes and assure correct volumes were delivered. Rerun assay. 3. Mix all reagents before use. 4. Pipette carefully, changing tips for each reagent. 5. Check for moisture, dirt, or air bubbles in or on wells. Tap plate, wipe bottom and reread.
No color development	<ol style="list-style-type: none"> 1. One or more reagents not added, or added in wrong sequence. 2. Antigen-coated wells inactive. 3. Improper dilution of Wash Buffer concentrate. 	<ol style="list-style-type: none"> 1. Recheck procedure. Be sure stop reagent is added last. Check for unused reagent(s). 2. Check for obvious moisture in unused wells – an indicator of condensate from opening a cold pouch. Use microwells from a new pouch and repeat test. 3. Prepare new 1X Wash Buffer and repeat test. Do not confuse Stop Reagent with Wash Buffer Concentrate (similar bottles) when preparing 1X Wash Buffer. Use of diluted Stop Reagent in place of 1X Wash Buffer will destroy color development. <p>Note: Repeat tests on Calibrator and Controls first, for a check on good performance after changes. Contact manufacturer if trouble persists.</p>
All test results are yellow.	<ol style="list-style-type: none"> 1. One or more reagents may be contaminated; focus first on Chromogen. 2. Contaminated buffers and reagents. 3. Wash Buffer (1X) contaminated or improper washing due to crystals formation in the Wash concentrate 4. Improper dilution of serum/Controls. 	<ol style="list-style-type: none"> 1. Check absorbance of unused Chromogen + Stop Reagent; discard if absorbance is high. 2. Check all solutions for turbidity. Discard turbid reagents and repeat with new reagents. 3. Use clean container. Check quality of water used to prepare buffer; check for crystal formation in the Wash concentrate (<i>dissolve crystals before preparing 1X Wash Buffer.</i>) 4. Check by measuring residual volume of diluted Specimen. Make corrections and repeat test.
Poor precision.	<ol style="list-style-type: none"> 1. Pipettor delivery CV greater than 5% or samples not added slowly. 2. Serum or reagents not mixed sufficiently; reagents not at room temperature prior to addition. 3. Reagent addition taking too long; inconsistency in timing intervals, air bubbles. 4. Air currents blowing over plate during incubations. 5. Optical pathway not clean. 6. Instrument not equilibrated before readings were taken. 	<ol style="list-style-type: none"> 1. Check calibration of pipettor. Use reproducible technique. 2. Mix all reagents gently but thoroughly and equilibrate to room temperature. 3. Develop consistent uniform technique and avoid splashing; use multi-tip device or autodispenser to decrease reagent delivery time. 4. Cover plate or place in chamber. 5. Wipe bottom of plate with soft tissue. Check instrument light source and detector for dirt. 6. Check instrument manual for warm-up procedure.

Poor precision (cont'd)	7. Washing not consistent; trapped bubbles; liquid left in wells at end of wash cycle.	7. Use only acceptable washing devices. Lengthen timing delay on automated washing devices. Check that all wells are filled and aspirated uniformly. Dispense 1X Wash Buffer above level of reagents previously added to wells.
	8. Improper pipetting.	8. Avoid air bubbles in pipette tips. Develop consistent technique.

* **Longitudinal Graphs:** For each anti- β 2GPI assay performed with a given kit lot number, the OD values of the Calibrators (IgM, IgG, IgA) and the Unit values of the PCs and NCs (IgM, IgG, IgA) are recorded on longitudinal graphs which are maintained in the *Bench Manual*. These graphs can be used to see an upward or downward shift or trend over time for Calibrator OD values and /or for PC/NC Unit values.

Abbreviated Test Procedure

- 1. Dilute Controls and Specimens 1:101 with Specimen Diluent (1X Wash Buffer).**
- 2. Pipette 100 μ L of pre-diluted Calibrator(s) into each of the designated paired wells (antigen-coated and Blank wells).**
- 3. Pipette 100 μ L of diluted Controls and Specimens into the designated paired wells (antigen-coated and Blank wells).**
- 4. Incubate plate for 30 – 35 minutes at room temperature (18 - 25°C).**
- 5. Wash the wells three times with 1X Wash Buffer.**
- 6. Add 100 μ L of the appropriate Enzyme Conjugate to each well.**
- 7. Incubate plate for 30 – 35 minutes at room temperature (18 - 25°C).**
- 8. Wash the wells three times with 1X Wash Buffer.**
- 9. Add 100 μ L Chromogen to each well.**
- 10. Incubate plate for 15 \pm 1minutes at room temperature (18 - 25°C).**
- 11. Add 100 μ L Stop Reagent to each well.**
- 12. Read the absorbance at 450 nm (reference 620 – 690 nm) within 30 minutes.**

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