LOUISVILLE APL DIAGNOSTICS, INC.

2622 NASA Pkwy Ste G2, Seabrook TX 77586 USA

LAPL ELISA™ aβ$_2$GPI IgG HRP Kit

REF	LAPL-K-HRP-aβ2GPI-1G

An Enzyme Immunoassay
For the Detection of IgG Anti-β$_2$ Glycoprotein I Antibodies

IVD **For *In Vitro* Diagnostic Use**

Technical Assistance
Tel: 770-455-7129
Fax: 770-455-6499
Customer Support: 800-624-3192
USA & Canada only
Email: support@louisvilleapl.com
Suggestions: feedback@louisvilleapl.com
Website: www.louisvilleapl.com

Corgenix, Inc.
11575 Main Street, Suite 400
Broomfield, Colorado 80020, USA

EC REP
MT Promedt Consulting GmbH
Altenhofstrasse 80
D-66386 St. Ingbert/Germany

EN

INSTRUCTIONS BOOKLET
LAPL ELISA™ aβ$_2$GPI IgA HRP Kit

TABLE OF CONTENTS **Page**

1 – INTENDED USE

The *LAPL ELISA™ aβ_2GPI IgG HRP Kit* is an enzyme-linked immunosorbent assay (ELISA) for the semi-quantitative determination of IgG anti-Beta2 Glycoprotein l (β_2GPI) antibodies in human serum or citrated plasma (3.2% sodium citrate).

The *LAPL ELISA™ aβ_2GPI IgG HRP Kit* is intended for the detection and semi-quantitation of IgG anti-β_2GPI antibodies in individuals with systemic lupus erythematosus (SLE) and lupus-like disorders (anti-phospholipid syndrome).

2 – EXPLANATION OF THE TEST

Anti-phospholipid antibodies are a heterogeneous group of immunoglobulins that bind to several anionic phospholipids, including cardiolipin and phosphatidylserine.[1,2] High serum levels of anti-phospholipid antibodies are frequently detected in patients with autoimmune (e.g., SLE) and non-autoimmune diseases, as well as in apparently healthy individuals.[3,4] These antibodies have been associated with an increased risk for recurrent arterial and venous thrombotic events, thrombocytopenia, and fetal loss.These manifestations are the main features of the anti-phospholipid syndrome (APS).[5,6] Most autoimmune anti-phospholipid antibodies require a serum cofactor (β_2GPI) for optimal binding.[7-10] It has been shown that many anti-phospholipid antibodies may react to a neoepitope formed on the β_2GPI molecule by the interaction between the phospholipid and β_2GPI.[11,12] Most assays for anti-phospholipid antibodies contain bovine serum as the source of cofactor. More recently, it has been shown that the binding of β_2GPI to the microwell surface may produce a neoepitope similar to that when combined with a phospholipid and the results with this system showed a good correlation with the anti-phospholipid syndrome.[13-16] The serologic detection of anti-β_2GPI antibodies provides enhanced clinical sensitivity for thrombosis. The *LAPL ELISA™ aβ_2GPI IgG HRP Kit* uses the well known ELISA format to detect anti-β_2GPI antibodies in human serum.

Patients with positive reactions to both anti-phospholipid and anti-β_2GPI assays were more likely to have clinical complications than those positive for only one. Higher prevalence and mean serum levels of IgGanti-β_2GPI antibodies have been reported in autoimmune patients. In addition, anti-β_2GPI antibodies in SLE patients correlated with clinical manifestations of anti-phospholipid syndrome.[17]

3 – PRINCIPLES OF THE PROCEDURE

The *LAPL ELISA™ aβ_2GPI IgG HRP Kit* is performed as an indirect ELISA. Diluted serum or plasma samples, calibrator sera, and controls are incubated in microwells coated with purified human - β_2GPI. Incubation allows the anti-β_2GPI antibodies present in the samples to react with the immobilized antigen. After the removal of unbound serum or plasma proteins by washing, antibodies specific for human IgG, labeled with horseradish peroxidase (HRP), are added forming complexes with the β_2GPI bound antibodies. Following another washing step, the bound enzyme-antibody conjugate is assayed by the addition of a single solution containing tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) as the chromogenic substrate .Color develops in the wells at an intensity proportional to the serum concentration of anti-β_2GPI antibodies.

Results are obtained by reading the O.D. (optical density or absorbance) of each well in a spectrophotometer. Calibrator sera are provided, with the IgG anti-β_2GPI antibody concentrations expressed in G units. For multipoint calibration, perform a linear regression analysis with calibrator values against calibrator O.D.s. Controls and patient results are determined from the calibration curve. These units are traceable to available reference preparations.

4 – COMPONENTS

Store at 2 - 8°C. Do Not Freeze.

Each *LAPL ELISA™ aβ_2GPI IgG HRP Kit* contains the following reagents (**volumes may vary depending onthe kit size and configuration**):

- 12 x 8 stabilized β_2GPI (from human serum) coated microwells with frame
- 60 mL Sample Diluent IV (blue-green solution)
- 3 vials (0.250 mL) IgG β_2GPI Calibrator Serum* (1-high, 2-moderate, 3-low) (human); see vial label for antibody concentration in G units.
- 0.250 mL IgG β_2GPI Positive Control Serum* (human); see vial label for expected G unit range
- 0.250 mL Normal Control Serum* (human); see vial label for expected G unit range
- 15 mL anti-human IgG (goat) HRP-Conjugated Antibody Solution (blue solution)
- 15 mL One Component Substrate Solution (TMB and H_2O_2); ready to use
- 15 mL Stopping Solution (0.36 N sulfuric acid)
- 2 bottles (30 mL) Wash Concentrate (33X PBS/Tween)

***CAUTION**: Contains sodium azide.

WARNINGS AND PRECAUTIONS

1. For *in vitro* use only.
2. Human source material used to prepare the calibrators and controls included in this kit has been tested and shown to be negative to HBsAg, HCV, and HIV 1 & 2 by FDA required tests. However, all human blood derivatives, including patient samples, should be handled as potentially infectious material
3. Do not pipette by mouth.
4. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
5. Wear disposable gloves while handling kit reagents and wash hands thoroughly afterwards.
6. Certain components of this product contain sodium azide as a preservative. Sodium azide has been reported to form lead and copper azides when left in contact with these metals. These metal azides are explosive. Any solutions containing azide must be thoroughly flushed with copious amounts of water to prevent the build-up of explosive metal azides in the plumbing system
7. One Component Substrate Solution can cause irritation to the eyes and skin. Use gloves when handling substrate and wash thoroughly after handling. Keep reagent away from ignition sources. Avoid contact with oxidizing agents.
8. Certain components are labeled with the following:
   Irritating to eyes (R 36). Avoid contact with skin (S 24). Avoid contact with eyes (S 25). In case of contact with eyes, rinse immediately with plenty of water and seek medical advice (S 26). If swallowed, seek medical advice immediately and show this container or label (S 46).

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 - 30°C) for 30 minutes prior to use.
3. Avoid direct sunlight.

SPECIMEN COLLECTION AND HANDLING

No Serum or citrated plasma (3.2% sodium citrate) should be used as the sample matrix. Blood should be collected by venipuncture and the serum separated from the cells by centrifugation after clot formation. If not tested immediately, the specimens should be stored at 2 to 8°C. If specimens are to be stored for more than 72 hours, they should be frozen at -20°C or below. Avoid repeated freezing

and thawing. Do not use hemolyzed, icteric, or lipemic serum or plasma as these conditions may cause aberrant results. Specimens containing visible particulate matter should be clarified by centrifugation before testing.
If citrated plasma is to be used, blood should be collected by venipuncture and the plasma separated from the cells immediately by centrifugation at 1500g for 10 minutes. The supernatant must be carefully removed after centrifugation to avoid contamination with platelets. Repeating the centrifugation and separation steps may be advisable to minimize platelet contamination. Lysed or aged platelets can lead to aberrant results. If not tested immediately, plasma samples should be stored a described for serum.

5 – TEST PROCEDURE

A. Material provided

LAPL ELISA™ aβ_2GPI IgG HRP Kit, see "Reagents" section for a complete list

B. Material required but not provided

- Reagent grade water to prepare PBS wash solution (2L)
- Graduated cylinders
- Precision pipettors capable of delivering between 10 µL and 1000 µL, with appropriate tips
- Miscellaneous glassware appropriate for handling small volumes
- Flasks or bottles, 1 liter
- Wash bottles, preferably with the tip partially cut back to provide a wide stream, or an automated or semi-automated washing system
- Disposable gloves
- Plate-reading spectrophotometer capable of reading absorbance at 450 nm (650 nm reference if dual beam)
- Multichannel pipettors capable of delivering to 8 wells simultaneously
- Microdilution tubes and a 96-well microdilution tube holder for sample dilutions and rapid delivery to microwell plate Precision micropipettes with disposable plastic tips which deliver 10 µl, 100 µl, and 1 ml (±2%)

C. Procedural Notes

1. Bring serum or plasma samples and kit reagents to room temperature and mix well before using; avoid foaming. Return all unused samples and reagents to refrigerated storage as soon as possible.
2. All dilutions of calibrators, controls, and test sera or plasma must be made just prior to use in the assay.
3. A single water blank well can be included in each plate with each run. No sample or kit reagents are to be added to this well. Instead, add 200 µL of

reagent grade water to the well immediately prior to reading the plate in the spectrophotometer. The plate reader should be programmed to "zero" or "blank" against an air or a water well.

4. Good washing technique is critical for optimal performance of the assay. Adequate washing is best accomplished by directing a forceful stream of wash solution into the bottom of the microwells from a plastic squeeze bottle with a wide tip. Wash solution in the water blank well will not interfere with the procedure. An automated plate washing system can also be used.
5. **IMPORTANT**: Failure to adequately remove residual wash solution can cause inconsistent color development of the Substrate Solution.
6. Use a multichannel pipettor capable of delivering to 8 wells simultaneously when possible. This speeds the process and provides more uniform incubation and reaction times for all wells.
7. Carefully controlled timing of all steps is critical. All calibrators, controls, and samples must be added within a five minute period. Batch size of samples should not be larger than the amount that can be added within this time period.
8. For all incubations, the start of the incubation period begins with the completion of reagent or sample addition. Incubation periods of 30 minutes are recommended, however incubation periods of up to 40 minutes may be used with this format.
9. Addition of all samples and reagents should be performed at the same rate and in the same sequence.
10. Incubation temperatures above or below normal room temperature (18 to 26°C) may contribute to inaccurate results.
11. Avoid microbial and cross-contamination of reagents when opening and removing aliquots from the primary vials.
12. Do not use kit components beyond expiration date.
13. Do not use kit components from different kit lot numbers.

D. Reagent Preparation

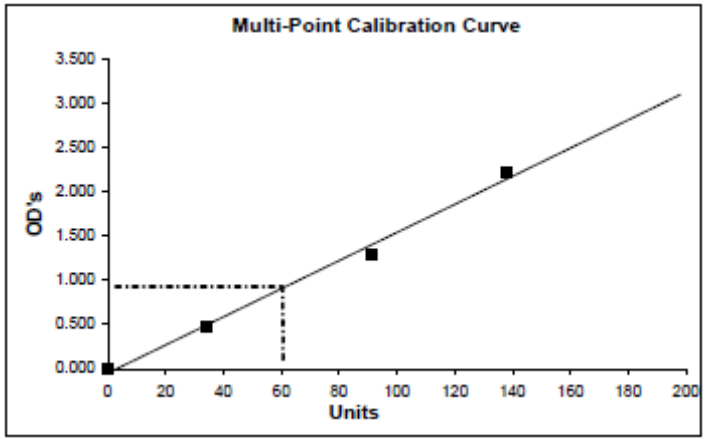
Wash Solution (PBS/Tween):
Measure 30 mL of Wash Concentrate and dilute to 1 liter with reagent grade water. The pH of the final solution should be 7.35 ± 0.1. Store unused wash solution in the refrigerator at 2-8°C. Discard if the solution shows signs of microbial or cross-contamination.

E. Assay procedure

1. The assay is performed using a four-point calibration curve. (Calibrators 1, 2, and 3 plus sample diluents/reagent blank as Calibrator 4 equal to 0 A units.) A reagent blank control (Calibrator 4) must be run with each assay in which Sample Diluent without serum is added to the well. This well is then treated the same as sample wells in subsequent assay steps.
2. Remove any microwell strips that will not be used from the frame and store them in the bag provided.
3. Prepare a 1:50 dilution of the calibrators, controls, and patient samples in sample diluent (blue-green solution); e.g., 10 µL of sample added to 490 µL of Sample Diluent equals a 1:50 sample dilution.
4. Add 100 µL of diluted calibrators (including the reagent blank/Calibrator 4), controls, and patient samples to the appropriate microwells.
5. Incubate 30 minutes at room temperature. After the incubation is complete, carefully invert the microwells and empty the sample fluid. Do not allow samples to contaminate other microwells.
6. Wash 4 times with wash solution. Each well should be filled with wash solution per wash. Invert microwells between each wash to empty fluid. Use a snapping motion of the wrist to shake the liquid from the wells. To retain microwell modules during washing, the frame must be squeezed at the top and bottom of the longest sides. Blot on absorbent paper to remove residual wash fluid. Do not allow wells to dry out between steps.
7. Add 100 µL anti-human IgG HRP-Conjugated Antibody Solution (blue) to the wells.
8. Incubate for 30 minutes at room temperature. After the incubation is complete, carefully invert the microwells and empty the conjugate solution.
9. Wash 4 times with wash solution, as in step 6. Use a snapping motion to drain the liquid, and blot on absorbent towels after the final wash. Do not allow the wells to dry out.
10. Add 100 µL One Component Substrate to each well and incubate for 30 minutes at room temperature. Add substrate to the wells at a steady rate. Blue color will develop in wells with positive samples.
11. Add 100 µL Stopping Solution (0.36 N sulfuric acid) to each well to stop the enzyme reaction. Be sure to add the acid to the wells in the same order and at the same rate as the Substrate was added. Blue Substrate will turn yellow and colorless solution will remain colorless. Blank or zero the plate reader against an air or a water blank well. Read the O.D. of each well at 450 nm (and 650 nm reference if dual beam). The O.D. values should be measured within 5 minutes of the addition of the Stopping Solution.

6 – CALCULATION OF RESULTS

1. Calculate the mean O.D. values if duplicates of the calibrators, controls and patient samples were performed.
2. Perform linear regression or quadratic/2nd order polynomial regression analysis with the four calibrator values against the mean O.D.s for each calibrator (see vial labels for G units; Calibrator 4 [sample diluent] is equal to 0 G units).
3. The calibrator curve can be plotted either automatically using a validated software program or manually with graph paper. It is recommended to use a zero intercept when generating the regression line to avoid negative values. If this option is not available, any negative values should be reported as zero units. When generating the curve manually, draw a best fit line through the plotted points using a zero incercept.
4. Determine the control and patient sample values from the calibrator curve.
5. Example of a multi-point curve calibration.



Using the example calibration curve provided, a specimen O.D. of 0.860 at 450 nm would correspond to a calculated value of 60 units. The calibration curve provided is an example only and should not be used to calculate patient results. A new calibration curve should be performed with every test run

7 – QUALITY CONTROL

1. The O.D. value of Calibrator 2 should be at least 0.600 to assure that the kit is functioning properly. Calibrator 2 O.D. readings of less than 0.600 may indicate that the kit is no longer suitable for use.

2. The O.D. of Calibrator 4 or reagent blank should be less than 0.050 when the spectrophotometer has been blanked against air or a water well. Readings greater than 0.050 may indicate possible reagent contamination or inadequate plate washing.
3. The anti-β_2GPI values obtained for the control sera should be within the ranges indicated on the vial labels. Occasional small deviations outside these ranges are acceptable.
4. O.D. values for duplicates of the controls or patient samples should be within 20% of the mean O.D. value for samples with absorbance readings greater than 0.200.
5. Each laboratory should periodically determine its own normal cut-off values for the appropriate population of patients.
6. Samples with anti-β_2GPI values greater than 200 G units may be reported as "greater than 200 G units."
7. Assure that all quality control parameters have been met before reporting test results.

8 – NORMAL RANGE

Serum samples from 120 healthy blood donors were tested for IgG anti-β_2GPI antibodies. The following normal range was established:

- Less than 20 G units

9 – PERFORMANCE CHARACTERISTICS

Clinical Specificity

Normal Samples

Serum samples from 121 healthy blood donors were assayed for the presence of IgG anti-β_2GPI antibodies. Using the establish cutoff value of 20 G units, this normal population demonstrated 100% specificity (mean value = 2.1 G units). Serum samples from 41 infectious disease (syphilis), 42 progressive systemic sclerosis (PSS), and 42 rheumatoid arthritis (RA) patients were assayed for the presence of IgG anti-β_2GPI antibodies. These patient groups demonstrated similar results compared to the healthy blood donor population (mean values = 3.9, 2.1, and 2.0 G units respectively). Results of these groups along with the healthy blood donors are summarized in the table below..

	Healthy	Infectious (syphilis)	PSS	RA
# of Samples (n)	121	41	42	42
Mean (G units)	2.1	3.9	2.1	2.0
Standard Deviation	1.7	10.3	3.9	2.2
% Negative	100%	97.5%	97.6%	100%

Clinical Sensitivity

Systemic Lupus Erythematosus (SLE):

Serum samples from 40 unselected (consecutive) patients with SLE were tested with the kit. Nine of the samples (sensitivity of 22.5%) were positive for IgG anti-β_2GPI antibodies (mean value = 24.5 G units). A good correlation was found between IgG anti-β_2GPI with both IgG anti-phosphatidylserine (r = 0.928) and IgG anti-cardiolipin (r = 0.864) antibody levels in this group

Serum samples from 12 selected female patients with SLE who had a clinical history of thrombosis, thrombocytopenia, or recurrent fetal loss were evaluated for IgG anti-β_2GPI antibodies. Seven of the samples (sensitivity of 58%) were positive in this population (mean value = 69 G units).

SLE Controls:

Serum samples from six selected female patients with SLE who had a history of thrombocytopenia (no thrombosis) were tested for IgG anti-β_2GPI antibodies. Only one sample (17%) tested weak positive (36 G units), with a mean value of 12.7 G units for this group.

Serum samples from 10 selected female patients with SLE who were known not to have had thrombotic episodes, nor any other feature of the anti-phospholipid syndrome, were tested in the assay. Two of the samples (20%) were weak positive for IgG anti-β_2GPI antibodies, with a mean value of 8.5 G units.

Primary Anti-phospholipid Syndrome (APS):

Serum samples from nine patients with the diagnosis of primary anti-phospholipid syndrome (APS) were tested on the *LAPL ELISA™ aβ_2GPI IgG HRP Kit*. Most, if not all, specimens were expected to be positive (above 20 G units) in this population. Eight of the nine samples resulted positive (88.9% sensitivity) with a mean value of 111 G units. A summary of sensitivity testing is presented below

	Unselected SLE	Selected SLE		Primary APS
		No Thrombosis	Thrombosis	
# of Samples (n)	40	16	12	9
Mean (G units)	24.5	10.1	69.0	111.0
Standard Deviation	49.6	12.7	66.2	55.2
% Positive	22.5%	18.8%	58.0%	88.9%

Technical Performance Comparison

Two disease populations (unselected SLE and primary APS) were tested on the *LAPL ELISA™ aβ_2GPI IgG HRP Kit* and a predicate device to study the correlation between positive and negative results. The results are summarized in the table presented below

		LAPL ELISA™ aβ_2GPI IgG HRP Kit	
		Negative	Positive
Predicate Device IgG anti-β_2GPI	Negative	32	8
	Positive	0	9

Relative Sensitivity	100%
Relative Specificity	80%
Agreement	84%

Precision

Three samples with known G unit values (one low, one moderate, and one high) were assayed in 23 replicates on three different occasions. The mean intra-assay and inter-assay coefficients of variation (%CVs) are presented in the following table. The reported intra-assay coefficient of variation is the mean of the three separate intra-assay %CVs. Inter-assay %CV is the coefficient of variation obtained from three plates from one lot.

	Value Range	Intra-assay Mean %CV	Inter-assay Mean %CV
Low	30 -50 G units	4.3%	4.0%
Moderate	60 -70 G units	4.7%	3.5%
High	>110 G units	3.4%	1.7%

10 – LIMITATIONS OF THE TEST

The anti-β_2GPI antibody concentration values obtained from this assay are an aid to diagnosis only. Each physician must interpret these results in light of the patient's history, physical findings, and other diagnostic procedures. If clinical findings suggest the presence of anti-phospholipid antibodies and the patient is negative for anti-β_2GPI antibodies, some investigators recommend testing for anti-cardiolipin antibodies, anti-phosphatidylserine antibodies, and the lupus anticoagulant to confirm the

negative result. A patient may be considered positive for anti-phospholipid antibodies if one or all of the tests give positive results.

Warranty

This product is warranted to perform as described in this package insert. The Manufacturer disclaims any implied warranty of merchantability or fitness for a particular use, and in no event shall The Manufacturer be liable for consequential damage.

11 – REFERENCES

1. McNiel HP, Chesterman CN, Krilis SA. Immunology and clinical importance of antiphospholipid antibodies. Adv Immunol 1991; 49:193-280.
2. Harris EN. Annotation. Antiphospholipid antibodies. Br J Haematol 1990; 74:1-9.
3. Love PE, Santoro SA. Antiphospholipid antibodies: Anticardiolipin and the lupus anticoagulant in systemic lupus erythematosus (SLE) and in non-SLE disorders. Prevalence and clinical significance. Ann Intern Med 1990; 112:682-698.
4. Sammaritano LR, Gharavi AE, Lockshin MD. Antiphospholipid antibody syndrome: immunologic and clinical aspects. Seminars in Arthritis and Rheumatism 1990; 20:81-96.
5. Hughes GRV, Harris EN, Gharavi AE. The anticardiolipin syndrome (Editorial). J Rheumatol 1986; 13:486-489.
6. Hughes GRV. The antiphospholipid syndrome: ten years on. Lancet 1993; 342:341-344.
7. McNeil HP, Simpson RJ, Chesterman CN. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: β2-Glycoprotein I (apolipoprotein H). Proc Natl Acad Sci USA 1991; 87:4120-4124.
8. Galli M, Comfurius P, Maassen C. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. The Lancet 1990; 335:1544-1547.
9. Matsuura E, Igarashi Y, Fujimoto M. Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease. The Lancet 1990; 336:177-178.
10. Roubey R. Immunology of the antiphospholipid antibody syndrome. Arthritis & Rheumatism 1996; 39:1444-1454.
11. Wagenknecht D, McIntyre J. Changes in β2-Glycoprotein I antigenicity induced by phospholipid binding. Thrombosis and Haemostasis 1993; 64:361-365.
12. Pengo V, Biasiolo A, Grazia Fior M. Autoimmune antiphospholipid antibodies are directed against a cryptic epitope expressed when β2-glycoprotein I is bound to a suitable surface. Thrombosis and Haemostasis 1995; 73:29-34.
13. Matsuura E, Igarashi Y, Tasuda T. Anticardiolipin antibodies recognize β2-glycoprotein I structure altered by interacting with an oxygen modified solid phase surface. J Exp Med 1994; 179:457-462.
14. Keil L, Galazka H, El-Kakdi HS. Binding of β2-glycoprotein I to activated polystyrene and its recognition by human IgG autoantibodies. Biotechnol Appl Biochem 1995; 22:305-313.

15. Amengual O, Atsui T, Khamasgta MA. Specificity of ELISA for antibody to beta 2-glycoprotein I in patients with antiphospholipid syndrome. Br J Rheumatol 1996: 35:1239-1243.
16. Tsutsumi A, Matsuura E, Ichikawa K. Antibodies to β2-glycoprotein I and clinical manifestations in patients with systemic lupus erythematosus. Arthritis & Rheumatism 1996; 39:1466-1474.
17. Lopez L, Dier K, Lopez D, Merrill J, Fink C. Anti-β2-Glycoprotein I and Antiphosphatidylserine Antibodies Are Predictors of Arterial Thrombosis in Patients With Antiphospholipid Syndrome. Am J Clin Pathol 2004;121:142-149

Louisville APL Diagnostics, Inc.

Revision #5 –March 31st, 2013

REF	LAPL-K-HRP-aβ2GPI-1G